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^{31}P-NMR SPECTROSCOPY OF RAT LIVER DURING SIMPLE STORAGE OR CONTINUOUS HYPOTHERMIC PERFUSION[1]

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SUMMARY

The ATP content and intracellular pH (pH_i)[3] of isolated rat liver before, during, and after cold preservation in either UW-lactobionate (UW, n=10) or Euro-Collins (EC, n=8) solutions were monitored using phosphorus-31 nuclear magnetic resonance (^{31}P-NMR) spectroscopy. The ^{31}P-NMR spectra were obtained on a 4.7-Tesla system operating at 81 MHz. Fructose metabolism, liver enzyme release, O_2 consumption, and rat survival after liver transplantation were also evaluated. During simple cold storage (SCS), the ATP level declined to undetectable levels with both preservation solutions while the pH_i declined to approximately 7.0. In contrast, during continuous hypothermic perfusion (CHP), hepatic ATP levels remained measurable during the 24-hour EC preservation and actually increased significantly ($p>0.01$) during UW preservation. After reperfusion at 37°C with Krebs-lactate, the SCS livers treated with EC differed significantly from the UW livers in terms of their ATP and pH_i as well as their response to a fructose challenge. In contrast, livers undergoing CHP demonstrated similar behaviors with both solutions. These results demonstrate an increase in the hepatic ATP content during CHP which occurs with UW but is not seen with EC. On the other hand, only livers that were simply stored with UW achieved significant survival after transplant, while CHP livers were affected by vascular damage as demonstrated by fatal thrombosis after transplant. These data suggest that ATP content is not the only determinant of good liver function although a system of

hypothermic perfusion might further improve liver preservation efficacy should injury to vascular endothelium be avoided.

INTRODUCTION

Clinical liver transplantation has changed markedly since the introduction of the University of Wisconsin (UW) preservation solution (1). With this solution, the cold preservation time for a liver has been extended to 24 hours (2-4), considerably longer than that which can be achieved with the Euro-Collins (EC) solution (5). The impact of this extension in cold preservation time on the logistics and costs of liver transplantation has been substantial: almost immediately, both a wider procurement and better distribution of donor organs became possible. Moreover, fewer donor organs are wasted, and the transplant procedure itself has become a semi-elective operation rather than an urgent procedure at most institutions. Most importantly, graft survival after preservation with the UW solution has increased and a reduced frequency of primary graft non-function and hepatic artery thrombosis has been observed compared to that which had been achievable with the EC solution (4, 6-8).

The precise mechanisms responsible for the improvement in transplant success achieved with the UW cold preservation solution is not yet clear. In designing the solution, Belzer and associates (9) combined the available knowledge relative to the principles of anaerobic cold ischemia with known principles of liver metabolism. The constituents of the UW solution were chosen in an effort to create a solution that would prevent: hypothermic-induced cell swelling, intracellular acidosis, expansion of the interstitial space,

injury from oxygen-free radicals, and depletion of the tissue adenosine phosphate content (ATP, ADP, etc.) (9, 10).

Several studies have attempted to relate the hepatic content of ATP in a donor liver with the degree of post-operative graft function (11-13). Each of these reports has suggested that the ability of the liver to recover from the many insults experienced between the donor and recipient operations can be predicted from the ATP content of the donor organ at the time of transplant and/or the rapidity of its restoration after reperfusion. Hepatic ATP levels also correlate highly with the rate of subsequent bile flow, a parameter currently used as an early post-operative sign of good graft function (11).

Phosphorus-31 nuclear magnetic resonance (^{31}P-NMR) spectroscopy allows non-invasive monitoring of tissue phosphorus compound levels (ATP, ADP, etc.), of fructose metabolism and of the intracellular pH (pH_i) both in vivo and ex vivo (14-18).

The purpose of this study was to evaluate the metabolic function, ATP content and pH_i of isolated rat liver using two models of liver preservation: simple cold storage (SCS) and continuous hypothermic perfusion (CHP) with both UW and EC solutions. The questions whether the ATP content and the pH_i at any time during the preservation and recovery period are reliable indices of organ viability and whether ^{31}P-NMR spectroscopy can provide a valuable non-invasive tool in assessing the efficacy of preservation have been addressed.

METHODS

Study Design

The present study was designed to allow a baseline evaluation by ^{31}P-NMR spectroscopy of isolated and perfused rat liver followed by the sequential events similar to those occurring during organ procurement, liver preservation, and transplantation. For this reason the following phases were performed: (i) liver isolation; (ii) Perfusion with Krebs-lactate (KL) at 37 °C and fructose challenge; (iii) cooling; (iv) preservation at 6-10 °C (by two methods) with UW or EC; (v) reperfusion; and (vi) re-evaluation with perfusion with Krebs-lactate (KL) at 37 °C and fructose challenge.

Finally, in separate experiments, rat liver transplantation was performed in a representative number of livers after 24 hr of SCS or CHP.

Rat livers were surgically isolated (phase 1, see: Animals and Surgical Procedures) and connected to a perfusion apparatus. Each liver was perfused with KL at 37°C for 20 min to achieve a stable baseline before ^{31}P-NMR spectra were collected. In separate control experiments, a stable base-line for O_2 consumption, lactate dehydrogenase (LDH) leakage, glucose production, and ^{31}P-NMR spectra were obtained within 15-20 min from the isolation-perfusion of the liver. These parameters were stable for more than 6 hr under normothermic perfusion with KL. No livers were discarded in the present data set for technical reasons.

A fructose load was administered and its metabolism followed for an additional 40 min (phase 2, fructose before preservation)

after which the temperature was reduced to 6-10°C while simoultaneously switching the preservation solution, stopping the oxygenation, reducing the flow rate and recycling the perfusate (phase 3, cooling). Livers were then randomly assigned to either be stored (SCS) or continuously perfused at a low flow (CHP) with either UW or EC at 6-10°C (phase 4, preservation). Before closing the perfusion cycle, enough perfusate (UW or EC, approximately 100 ml) was used to flush the liver and the NMR cell, in order to avoid significant contamination of the preservation solution with KL. After 24 hr of cold preservation, the livers were reperfused at 37°C with oxygenated KL (phase 5, reperfusion) and organ function was reassessed by examining the response to a second fructose load (phase 6, fructose after preservation).

Throughout the experimental period, ^{31}P-NMR spectra were collected to monitor the intrahepatic content of phosphate metabolites (ATP, PM, Pi, F-1-P) and pH_i. Perfusate samples were collected periodically to determine oxygen consumption, pH, LDH, and glucose content.

Animals and Surgical Procedure

Male Sprague Dowley rats (200-250 g) were used as liver donors. They were provided free access to water and food until sacrifice under methoxifluorene anesthesia. Three hundred units of heparin was administered intravenously via the penile vein to prevent vascular thrombosis during the hepatectomy. The abdomen was entered through a midline abdominal incision and the infrarenal aorta was cannulated with a 20G catheter. The liver was perfused in

situ with 20 ml of cold (4°C) Lactated Ringer's solution, after incising the inferior vena cava above and below the liver and clamping the aorta proximal to the celiac axis. Immediately thereafter the liver was cooled in situ; the portal vein was cannulated using a second 20G catheter and the liver was continuously perfused with cold Lactated Ringer's via the portal vein and the aorta while the liver was being excised. The total ischemic time from the point of aortic clamping and flushing of the liver to time of initiation of the hepatic perfusion was less than six min in all cases.

Isolated Liver Perfusion

A wooden platform was constructed using non-magnetic holders for the glass condenser, a temperature probe, a glass syringe serving as a bubble-trap, a 30-mm diameter NMR sample tube, and Tygon tubing and connectors. The perfusion pump, the water bath, and the thermostat regulating the perfusate flow and temperature were placed in a room adjacent to the magnet. Insulated tubing was used to deliver the perfusate to the liver. The isolated rat liver with a pre-weighed catheter inserted in the portal vein was weighed and transferred to the NMR sample tube pre-filled with cold KL solution connected to the perfusion apparatus. The liver was perfused within the NMR sample tube which was placed inside the horizontal 40-cm diameter bore of the magnet. Three-way stopcocks were positioned in the system to allow for sampling of the perfusate at the out- and the in-flow sites of the perfused liver as well as for switching from one perfusion solution to another.

Before and after liver preservation, during phases 2 and 6, the KL was placed in a water-jacketed 4-liter perfusate reservoir connected to a 37°C water bath and pumped through the liver but without recycling. The flow rate during this perfusion at 37°C was maintained at 3 ml/min/g of liver (wet weight) and a 95%/5% O_2/CO_2 gas mixture was bubbled continuously into the perfusate reservoir at a constant pressure through a filter-stone. A filter valve allowed for the release of pressure within the reservoir as the gas was bubbled through the perfusate.

During phase 4 (preservation at 6-10 °C), a 250-ml Erlenmeyer flask containing the cold preservation solution (UW or EC) was maintained inside a cold water bath and connected to the pump using a recycling system. The perfusion rate during this phase of the study was 0.5 ml/min/g of wet liver weight. No oxygen was bubbled into the perfusate solution during this period. When the temperature reached 6°C in the NMR sample tube, the perfusate flow was stopped for the SCS experiments.

After 24 hr of preservation (SCS or CHP), the livers were reperfused with oxygenated KL at 37°C at a rate of 3 ml/min/g (phase 5, reperfusion). After a 30-min wash-out period, a second fructose load was delivered (phase 6, fructose after preservation).

Continuous temperature calibration using an on-line thermo-couple adjacent to the NMR probe allowed for maintenance of the perfusate temperature throughout <u>every</u> phase of the experiment.

Organ Viability

Hepatic viability was assessed continuously by measuring oxygen consumption (Radiometer AB2, Copenhagen), calculated from: $[(pO_2^{inflow} - pO_2^{outflow}) - pO_2^{blank}]$ multiplied by flow rate; LDH release into the perfusate solution, and glucose production. The livers were weighed both before and after each experiment. Basal LDH release into the perfusate during oxygenation and perfusion ranged between 20-35 U/L in phases 2, 5 and 6; O_2 consumption was 1.95±0.2 μmol/g/min and glucose production was 5±0.15 mg%. The metabolic response of the liver to a physiologic fructose challenge was assessed before and after preservation by monitoring the appearance and disappearance of the fructose-1-phosphate (F-1-P) peak in the ^{31}P-NMR spectra and the initial reduction and subsequent recovery of hepatic ATP content induced by the fructose load (0.15 mmole/g wet liver weight in 150 ml oxygenated Krebs-lactate, as a 5-min bolus).

^{31}P-NMR Spectral Measurements

The ^{31}P-NMR spectra were obtained using a Bruker BIOSPEC II 4.7-Tesla system equipped with a 40-cm horizontal bore superconducting solenoid. The signal was acquired by means of a 3.5-cm solenoid coil placed around the 30-mm diameter NMR tube containing the liver and tuned to 81 MHz for ^{31}P. The ^{31}P pulse width and the interpulse delay were 70 μsec and 2 sec except during the fructose study when the above ^{31}P parameters were 35 μsec and

0.3 sec, respectively. In the latter acquisitions, partial saturation of phosphomonoesters (PM) and inorganic phosphate (Pi) were considered. Under similar conditions, as recently reported by Delmas-Beauvieux et al. (19), no significant differences in longitudinal relaxation time (T_1) and saturation parameters for the phosphate metabolites of interest were observed under the various experimental phases and temperatures.

The ^{31}P-NMR spectra were obtained before, during, and 24 hr after low-temperature preservation (SCS or CHP) using either UW or EC. During phases 2 and 6 (KL perfusion at 37°C), ^{31}P-NMR spectra were recorded as 10-min blocks of 300 scans to establish a baseline, followed by 4-min blocks of 600 scans during the fructose challenge, and finally additional 10-min blocks during the cooling period (phase 3) and the reperfusion period (phase 5). During phase 4 of the continuous hypothermic perfusion (CHP), 30-min blocks of 900 scans were recorded continuously for 24 hours. Finally, during phase 4 of the SCS livers, spectra were acquired until ATP was observed in ^{31}P-NMR spectra.

A 5-mm spheric bulb containing a 0.15 M solution of methylenediphosphonic acid (MDPA) in D_2O at pH 9 was placed in the center of the NMR sample tube among the liver lobes to serve as a ^{31}P chemical shift and signal intensity reference for the intracellular pH determination and relative concentration measurements.

A calibration plot for determining the intracellular pH from the ^{31}P chemical shifts of P_i and F-1-P was developed using liver homogenate at 37°C containing 20 mM EDTA. After the addition of

10 mM F-1-P and 10 mM PCr, a titration curve (from pH 4.24 to 9.45) was generated. For each pH studied, the solution was positioned in the magnet and 10-min acquisitions were accumulated using a 3.5-cm diameter solenoid coil, 90° pulses, and a 5-sec interpulse delay. The ^{31}P-NMR chemical shifts were referenced to the PCr, taken as 0 parts per million (ppm). The ^{31}P signal of MDPA occurs at 20 ppm downfield from PCr. Titration curves were obtained by plotting the ^{31}P chemical shifts of P_i and F-1-P as a function of pH. A least-square fit was performed to establish the titration parameters for each curve. The intracellular pH (pH_i) was determined using the following equations (20):

$$pH_i = pK_{P_i} + \log [(\sigma_{P_i} - 3.34)/(5.81 - \sigma_{P_i})]$$

for P_i , and

$$pH_i = pK_{F\text{-}1\text{-}P} + \log (\sigma_{F\text{-}1\text{-}P} - 3.68)/(7.48 - \sigma_{F\text{-}1\text{-}P})$$

for F-1-P, where pK_{P_i} and $pK_{F\text{-}1\text{-}P}$ are 6.76 and 6.03, σ_{P_i} and $\sigma_{F\text{-}1\text{-}P}$ are the observed values of the chemical shift in ppm of P_i and F-1-P, respectively. The difference between the two determinations, when both peaks are present in the ^{31}P-NMR spectra, is less than 0.05 pH unit. Calibration curves prepared in KL provided equivalent information.

A similar calibration plot at 10°C was developed in order to measure pH from the ^{31}P chemical shifts of P_i and PM during liver preservation.

The levels of phosphate metabolites (ATP, F-1-P, P_i) were determined by integrating the area under the curve for the appropriate peak in the ^{31}P-NMR spectra normalized to the MDPA area which served as a reference. When sequential changes were followed, peaks were normalized to the initial ^{31}P-NMR spectrum obtained after the equilibration period (100%).

Liver Transplantation

Rat liver transplantation of organs preserved either by SCS or CHP for 24 hr was performed according to the method of Kamada and Calne (21). Function of the transplanted liver was assessed for up to 3 days or determined by gross appearance of the organ, the portal vein, and the bile flow.

Materials

Krebs solution (22) with 1 mM L(+)lactic acid (Sigma) neutralized with NaOH (to pH 7.4 at 37°C) was used before and after cold preservation to perfuse the livers at 37°C. It was bubbled continuously with a 95%/5% O_2/CO_2 gas mixture and infused at a rate of 3 ml/min/g liver wet weight. The Euro-Collins (23) was purchased from Fresenius AG (Germany) and the UW (24) solution was a gift from the Dupont. All other chemicals used were reagent grade and were purchased from either Fisher, Sigma, or Abbott.

Statistical Analysis

The statistical analyses were performed using the Student's t test for paired or unpaired data. A $p<0.05$ was considered to be significant. All results are expressed as mean values ± SD .

RESULTS

Figure 1a shows a typical 81-MHz ^{31}P-NMR spectrum obtained for a perfused rat liver under normoxic conditions with Krebs-lactate (1 mM at 37°C, bubbled with 95% O_2/5% CO_2, pH 7.40, 3 ml/min/g liver wet weight) after a 20-min equilibration period and before cold preservation. The PM peak represents the sum of signals obtained from various sugar phosphates, glycolytic intermediates, and AMP. The ^{31}P chemical shift of the P_i peak relative to the external standard (MDPA) enabled a determination of the pH_i which was 7.28 ± 0.05 (n=18) in all rat livers perfused under these conditions. This finding agrees well with pH_i determined in mouse liver with PCr peak as an internal standard (25). The phosphodiester (PD) peak consists of the sum of the signals from glycero-3-phosphocholine, glycerolphosphoethanol-amine, phosphoenolpyruvate and other related compounds. The γ-ATP-phosphate and ß-ADP-phosphate signals occur about -2.4 ppm downfield from PCr; the α-phosphates of both ATP and ADP occur at -7.5 ppm; and the ß-ATP signal is a single peak at -16 ppm.

Following the administration of a fructose challenge (Figure 1b), major changes were observed in the ^{31}P-NMR spectra. An increase in the PM area was seen immediately due to an accumulation of F-1-P with an associated reduction in the P_i and ATP content of the liver.

The time course of the phosphate metabolites after fructose loading is shown in Figure 2, where data from 18 different perfused rat livers are shown. The F-1-P signal appeared in the first 4-min

spectrum, providing a 17 ± 16% increase ($p<0.01$) in the PM area, and reached a maximum in the following 4-min (83 ± 34%, $p<0.001$). It subsequently decreased rapidly as the fructose was metabolized. This process was complete within 20 min at which time the PM area in the ^{31}P-NMR spectrum returned to its pre-fructose level (2 ± 9%, n.s.). The intrahepatic ATP level declined to about 50% of its initial value 8 min after the fructose challenge (48 ± 11%, $p<0.001$). It then gradually recovered reaching a level about 80% of the pre-fructose value at 40 min (22 ± 5%, $p<0.001$). Two hours after the fructose challenge the hepatic ATP level had returned to approximately 90% of the initial basal value (results not shown). The intrahepatic P_i level decreased after the fructose load (8 min; 28 ± 10%, $p<0.001$), then rebounded to a level greater than the pre-fructose value (16 min; 47 ± 18%, $p<0.001$), and was still elevated at 40 min (16 ± 14%, $p<0.01$). Following the fructose load, the pH_i fell from a value of 7.28 ± 0.05 to 7.14 ± 0.05 ($p<0.001$) at 12 min and then returned to a value close to the original baseline value at 20 min (7.26 ± 0.06, n.s.) as measured by either the P_i or the F-1-P chemical shift.

After an initial 20-min equilibration period and the first fructose challenge, during which oxygen consumption and LDH release were constant, the perfusion solution was changed to one of the cold preservation solutions (UW: n=10, or EC: n=8). The flow rate was gradually decreased to 0.5 ml/min/g of wet liver weight. When the temperature within the NMR sample tube reached 6°C (approximately 30 min after switching), the perfusion was either stopped (SCS) or continued (CHP) for an additional 24 hr. During this

period (phase 3, "cooling"), ^{31}P-NMR spectra were obtained periodically and demonstrated a decline in intrahepatic ATP to a level approximately 30% of the initial value (UW: 35 ± 8%, n=10; EC: 25 ± 9%, n=8, n.s.) and the pH_i from 7.25 ± 0.05 to 7.20 ± 0.05, with no significant difference being evident among the various groups.

When the livers were simply stored (SCS) for 24 hr, hepatic ATP decreased within a few hours to undetectable levels (results not shown), confirming the findings of Busza et al. (26). The pH_i declined also to a value of 6.9 ± 0.1 (n=9, $p<0.01$). Although the time course of intrahepatic ATP decline was slower in the UW stored livers (n=5) as compared with those stored in EC (n=4), the difference was not statistically significant.

In Figure 3, sequential ^{31}P-NMR spectra are shown from livers continuously perfused (CHP) with UW solution at low temperature for 24 hr. The α-ATP and β-ATP peaks (right) have been measured to demonstrate the increase in area with time. The results are summarized in Figure 4 where the relative intrahepatic ATP concentrations are plotted for both the UW and EC experiments. The difference in ATP levels achieved with the two solutions was significant (UW: 55 ± 6%, n=5; EC: 26 ± 10, n=4, $p<0.01$) at 24 hr. The pH_i fell from a value of 7.20 ± 0.05 to a value of 7.10 ± 0.05 ($p<0.01$) for both solutions.

At reperfusion after 24 hr of SCS, the livers preserved with EC demonstrated less recovery of their ATP levels as compared to the UW solution (EC: 35 ± 10%, n=4; UW 65 ± 9%, $p<0.01$) and a very poor response to the fructose load (PM area increase at 8 min: EC 51 ± 20%, UW 93 ± 28%, $p<0.01$). The intracellular pH rapidly returned to

normal in UW preserved livers (7.30 ± 0.05, n=5), whereas EC livers continued to have lower pH_i (7.20 ± 0.05, n=4, $p<0.05$).

In the CHP experiments, following reperfusion, the hepatic ATP recovery averaged about 80% in both groups (UW: 84 ± 10%, n=5; EC: 79.5 ± 10, n=4, n.s.) (Figure 4), and a similar increase in the PM area after the fructose load was observed (at 8 min; UW: 112 ± 42%, EC: 91 ± 56%, n.s.). The recovery of hepatic ATP 40 min after fructose was greater in UW livers as compared to EC (UW: 60 ± 7%, n=5; EC: 44 ± 10, n=4, $p<0.05$). There was a long lag period between first and second fructose challenge (24 hr), ATP, P_i and pH_i were constant before each fructose challenge. Intracellular pH returned to normal with both solutions (7.30 ± 0.05, n=9).

The results of the transplant experiments performed using the 24-hr cold stored and perfused liver are shown in Table 1. Cold storage with the UW solution allowed for prolonged function of the transplanted liver. After 24 hr of cold perfusion with EC solution, the transplanted livers all ceased to function within 15 minutes. In contrast, the livers perfused with UW functioned for variable lenghts of time over 30 min to 3 hr when the experiments were discontinued. No experiments were performed with organs simply stored in EC as previous work by our group has shown such organs not to be viable.

DISCUSSION

The definitive assessment of hepatic viability after preservation is the capacity of the graft to survive after transplantation and to have all of its function restored (27). As an alternative model, an isolated perfused rat liver can be utilized to evaluate hepatic function during preservation, including measurement of the biochemical, metabolic, and morphologic integrity (28).

NMR spectroscopy is a unique tool which allows repeated, non-invasive measurement of metabolic changes in preserved organs that might be predictors of viability (29). The hepatic ATP level and the pH_i can be readily assessed using ^{31}P-NMR spectroscopy and are thought to be important indicators of organ function (14, 15). Although no significant differences in longitudinal relaxation time (T_1) and saturation parameters for the observed phosphate metabolites were shown during various experimental phases and temperatures by other investigators (19), we cannot exclude the possibility that saturation may have occurred affecting the peak height of Pi, PM and ATP. For this reason, we included, after proper control experiments, an external reference (MDPA) for relative measures of phosphate metabolites. In addition, the same ^{31}P-NMR recording schedule (2-sec delay, 70-msec pulse width) except during fructose challenge was always used, in order to minimize any errors in the relative intensity comparisons.

Previous studies using ^{31}P-NMR (30-35) as well as other biochemical methods (36-38) have demonstrated that the ATP levels

decline rapidly after flushing the liver with non-oxygenated cold preservation solutions so that within a few hours no ^{31}P-NMR visible ATP signal can be observed. The time course of its disappearance varies depending on the type of solution and cooling modalities being used. The present results with simple cold storage of the liver confirm these findings. Moreover, no significant difference in the ATP decay during the cooling period was seen between UW and EC. Following reperfusion, the capacity of the liver to regenerate ATP has been touted as a reliable index of graft viability either in perfusion experiments (35, 39) or in the transplant recipient (11). In recent clinical studies (12, 13), ATP levels within the donor liver have been shown to correlate positively with transplant outcome.

In the present study, we monitored the ATP levels and the pH_i in two experimental models of liver preservation: SCS and CHP. The first method has gained general acceptance in liver preservation, especially after the extension of the preservation time provided by the UW solution (4-8). The latter currently appears too complex to be clinically useful in liver transplantation (40) and the available human trials with this preservation technique have been performed only in kidney preservation (41). Recently, prolonged preservation time (72 hr) and subsequent successful liver transplantation have been obtained experimentally using CHP and a modified UW solution (42, 43). To our knowledge no data is available on the hepatic ATP levels and pH_i during continuos hypothermic perfusion.

The important components of the UW solution which make it a better preservation solution for the liver as compared with solutions used previously is still uncertain (9, 44). Many

constituents appear to be important either individually or in combination. Several studies have demonstrated that not all of the ingredients of the UW solution are essential (45-47). However, both adenosine and phosphates, which enable ATP synthesis during hypothermia (48-50), are thought to be essential for the recovery of ATP levels upon reperfusion (10). While the important role of adenosine in the solution has been demonstrated using isolated liver cells (51), not all studies using other models have found it to be necessary (38, 47). The current findings of an improved ATP recovery after 24 hr SCS with UW and upon normothermic reperfusion are in agreement with most other reports (19, 35, 52, 53) and suggest that the use of UW solution results in better liver viability as opposed to that achieved with the EC solution. On the other hand, when livers are continuously perfused for 24 hr at 8°C hepatic ATP levels are maintained at a low level with EC, and actually increase with time with UW perfusion. Indeed, on-line measurement of oxygen reveals some O_2 consumption during CHP (results not shown). It should be noted that after reperfusion both EC and UW-preserved livers recover similar levels of hepatic ATP in the hypothermically perfused for rat liver for 24 hr. Thus, the present data do not support the assumption that ATP precursors help the liver to recover during reperfusion (51). However, it is possible that longer periods of CHP may allow a difference to be detected (42).

The prevention of intracellular acidosis during cold storage is also an important function of the UW solution (9, 54). Cold ischemia activates anaerobic metabolism and a decline in the hepatic intracellular pH due to an increase in lactate and other factors (i.e.

ATP hydrolysis) (55). Thus, a high buffer capacity, provided mainly by inorganic phosphates, is one of the crucial requirements for an efficacious preservation solution (9).

The measurement of pH_i using ^{31}P-NMR spectroscopy is based on the ^{31}P chemical shifts of the P_i and PM which are sensitive to changes in pH near their pK_a (14, 15). The positions of these resonances can be used as a sensitive indicator of the intracellular pH. We used an external reference standard (MDPA) and two calibration plots for the observed ^{31}P chemical shifts of P_i and F-1-P at different pHs using liver homogenate at different temperatures (15, 29, 56). A contribution to the P_i signal by the extracellular P_i cannot be avoided when using perfusates containing phosphates. However, different T_1 and relative saturation of the resonances (extra- vs intra-cellular) can be estimated (56), and utilized for correction of the measured ^{31}P signal intensities and chemical shifts. When Krebs-lactate is used, the liver/perfusate volume ratio in the control experiments (i.e. circulation only of perfusate and MDPA), allows us to estimate that the contribution of extracellular P_i to the observed ^{31}P-NMR P_i intensity (about 10%) is in agreement with previously reported findings (17, 56). This is further supported by observations during the fructose load that after 8 min the P_i peak is split into two peaks. As previously described (17, 18), the intracellular P_i and pH decline promptly after giving fructose as seen by ^{31}P NMR. The upfield ^{31}P-NMR resonance is assumed to be the extracellular P_i component (Figure 1b, P_i extra), and the pH is 7.4, as expected. When UW or EC solutions are used, a larger contribution to the P_i peak intensity in the ^{31}P-NMR spectra is

observed as a result of the high phosphate content of these solutions (Figure 3), making the determination of pH_i from P_i less accurate. In the case of Fuller and Busza (29), we determined the pH_i by using the chemical shift of PM, whose pK is not ideal for the pH range studied. Therefore, pH_i measurements under such experimental conditions can be considered to only be an approximation of the real value.

The data herein presented relative to pH_i during SCS confirm previous ^{31}P-NMR findings (19, 26, 53) and demonstrate the superiority of the UW solution in maintaining pH_i near neutrality. The pH_i was similar with either EC or UW with CHP, demonstrating a greater degree of maintenance of cellular metabolism with CHP over SCS at least for 24 hr.

In the present work, a fructose challenge was used as an additional liver function test before and after preservation, because the biochemical events following its administration can be rapidly monitored by ^{31}P-NMR spectroscopy (16-18). The response of the liver to the fructose load can be assessed by ^{31}P-NMR in terms of: (i) ability to take up the fructose load and its subsequent phosphorylation yielding an increase in the PM area; (ii) capacity to metabolize fructose with the return of the PM intensity to the pre-challenge level; (iii) the changes of the P_i and the pH_i; and (iv) the decline and the recovery in ATP levels. A baseline response to fructose under normothermic conditions was evaluated before preservation. The second challenge after 24 hr of SCS or HCP was normalized to the baseline response. A recent study on the ability of fructose to protect the liver during hypoxia has been reported (57).

Under our conditions, the time elapsed between administration of the challenge and the start of preservation probably ensures the complete metabolism of the fructose. Thus, a direct protective role for this substance is most unlikely. In addition, pH_i returned to a value close to the original baseline value 20 min after the fructose challenge. This finding essentially rules out a significant effect of the fructose load after the 24 hr of preservation. Other standard biochemical liver function parameters such as basal LDH release during different phases of the experimental design, O_2 consumption and glucose production were stable within a single liver preparation during both fructose challenges. It is our experience with liver perfusion experiments with a perfusate solution like KL that a single fructose challenge produces alterations in the ATP spectra which can be restored to a maximal pre-fructose level (90%) even after several hours have elapsed (unpublished data). From the foregoing argument, even if a fructose effect were present before preservation, it would not be sufficient to explain the differences observed among the various groups studied, since all received fructose at identical times and at identical doses. In our study, the UW preserved livers demonstrated a better response to a fructose challenge than did EC preserved livers under both preservation techniques. These metabolic observations confirm an earlier report using glucose formation as observed by ^{13}C-NMR spectroscopy (52), and provide non-invasively acquired additional useful metabolic information relative to the viability of the liver after cold preservation.

Livers preserved for 24 hr with either EC or UW were transplanted orthotopically into other Sprague-Dawley rats and their short-term performance was monitored. Organs preserved by SCS technique with UW solution functioned for two or more days when the animals were sacrified. In contrast, those preserved by CHP technique functioned only for short periods of time. Those perfused with EC all experienced an increase in the portal venous perfusion pressure during the preservation period and functioned only for minutes after revascularization. Those perfused with UW functioned for 30 min to three hr when the experiments were discontinued because of portal venous thrombosis. These observations document clearly that hepatocyte preservation is obtained with the methods utilized in these experiments, but that portal vein (endothelial cells) injury occurs and limits the long-term performance of the transplanted liver.

Only livers that were simply stored with UW achieved prolonged survival after transplantation, while CHP livers were affected by vascular damage as demonstrated by fatal thrombosis after transplant, even when UW solution was used. These data suggest that preservation of the hepatic ATP content is not the only determinant of good liver function following transplantation. Finally, a different system of hypothermic perfusion which does not injure endothelial cells might further improve liver preservation efficacy.

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FOOTNOTES

[1] Preliminary reports of this work have been presented as a poster at The British Society of Gastroenterology at Bradford, U.K., April 12-14, 1989, and as an oral presentation at the Nineth Annual Meeting of the Society of Magnetic Resonance in Medicine, New York, August 19-24, 1990.

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[3]Abbreviations used: AMP, adenosine monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; CHP, continuous hypothermic perfusion; EC, Euro-Collins solution; F-1-P, fructose-1-phosphate; KL, Krebs-lactate; MDPA, methylenediphosphonic acid; n.s., not significant; PCr, phosphocreatine; PD, phosphodiester; pH_i, intracellular pH; P_i, inorganic phosphate; PM, phosphomonoester; ^{31}P-NMR, phosphorus-31 nuclear magnetic resonance; ppm, parts per million; T_1, longitudinal relaxation time; SCS, simple cold storage; SD, standard deviation of the mean; UW, University of Wisconsin solution.

TABLE 1

Liver Transplantation After 24 Hours of Cold Storage or Organ Perfusion

Preservation Solution	Method	N	Rat Number	% Liver Weight Change	Portal Vein Pressure Change*	Survival Duration
Euro-Collins	CHP	2	1	+28.9	+3 cm	< 15 min
			2	+51.8	+7 cm	< 15 min
UW	SCS	2	1	**	-	2 days
		2	**	-	> 3 days	
	CHP	4	1	+11.6	0	3 hr
			2†	+26.7	0	2 hr
			3†	+12.5	-0.5 cm	1 hr
			4†	+40.7	+3.0 cm	0.5 hr

* Change in portal vein (inflow) pressure during the 24 hours of perfusion preservation.
** Not measured, but usually decreases 6-10% of initial weight.
† Portal vein thrombosis occurred terminating the observations.

FIGURE LEGENDS

Figure 1

^{31}P-NMR spectra of isolated rat liver perfused with Krebs-lactate at 37°C, before and after a fructose load.

Spectrum a): before fructose. The resonances were assigned as follows: 1) methylenediphosphonic acid (MDPA), which is the external reference standard used for the chemical shift measurements [-19.8 ppm downfield from that of hypothetical phosphocreatine (PCr)]; 2) phosphomonoesters (PM); 3) cytosolic inorganic phosphate (P_i); 4) phosphodiesters (PD); 5) γ-adenosine triphosphate (γ–ATP) + β-adenosine diphosphate (β–ADP); 6) α-adenosine triphosphate (α–ATP) + α–adenosine diphosphate (α–ADP); 7) β-adenosine triphosphate (β–ATP).

Spectrum b): 8 min after fructose. After fructose administration (1.5 mmole/10 g wet liver weight in Krebs-lactate, 5-min infusion), an increase in PM peak area, mainly due to phosphorylation of fructose to fructose-1-phosphate (F-1-P), and a decrease in P_i and ATP areas are shown. Also note the splitting of the P_i peak and shift to the right of one component (P_i intra), whose frequency corresponds to pH 7.1. The other P_i component (P_i extra) corresponds to pH 7.4. The ^{31}P-NMR spectra were acquired in 4-min blocks with 600 scans using a 0.3-sec interpulse delay and a 35-μsec pulse width.

Spectrum (b - a): computerized difference between the above ^{31}P-NMR spectra. The hashed area above the baseline (F-1-P) equal the ones underneath (P_i, ATP) in absolute numbers (in parenthesis).

Figure 2

Phosphate metabolites changes after a fructose challenge as seen by ^{31}P-NMR.

The time-course of fructose-1-phosphate (F-1-P), inorganic phosphate (P_i) and ATP relative changes after a fructose load (0.15 mmole/g wet liver weight in Krebs-lactate, 5-min infusion, given at 0 time). Values as relative changes from basal value (100%) at 0 time of phosphate metabolites areas (mean ± SD).

Figure 3

^{31}P-NMR spectra during 24 hours UW continuous hypothermic perfusion preservation (CHP).

Sequence from 30 min to 24 hr of ^{31}P-NMR spectra of rat liver during CHP with UW solution, showing a very intense resonance from inorganic phosphate (P_i, peak 4), due to the high phosphate content of UW solution and the progressive increase (from left to right, bottom to top) of α-ATP (peak 6) and β-ATP (peak 7) areas over time. The resonances were assigned as follows:1) methylenediphosphonic acid (MDPA), which is the external reference standard (-19.8 ppm downfield from that of hypothetical PCr); 2) phosphomonoesters (PM): including AMP and IMP; 3) phosphomonoesters (PM): including

sugar phosphates; 4) cytosolic inorganic phosphate (P_i); 5) γ-adenosine triphosphate (γ–ATP) + β-adenosine diphosphate (β–ADP); 6) α-adenosine triphosphate (α–ATP) + α–adenosine diphosphate (α–ADP); 7) β-adenosine triphosphate (β–ATP). ^{31}P-NMR spectra were acquired in 30-min block with 900 scans using a 2-sec interpulse delay and a 70-μsec pulse width. Each spectrum displays the same region between -30 ppm and +25 ppm. Following the first spectrum (30 min) all the others have been displaced laterally and only peaks 6 and 7 were shown for clarity.

Figure 4

ATP content by ^{31}P-NMR spectroscopy during 24 hours perfusion preservation with UW and EC solutions.

Relative changes of β–ATP area of ^{31}P-NMR spectra of rat livers at the end of each experimental phase of continuous hypothermic perfusion (CHP): i) 40 min after the first fructose load in Krebs-lactate at 37°C before preservation (F1); ii) 30 min after switching to the cold preservation solution (cool); iii) after 24 hr of CHP at 6-10°C (24 h); iv) 20 min after reperfusion with Krebs-lactate at 37°C (reper); v) 40 min after the second fructose load (F2). Relative concentrations were referred to an external standard (MDPA) to allow intra and intergroup statistical analysis. UW: University of Wisconsin, EC: EuroCollins. Values as mean ± SD from baseline value (100%) at 0 time. Student t test for unpaired data (* $p<0.05$, ** $p<0.01$).

FIGURE 1

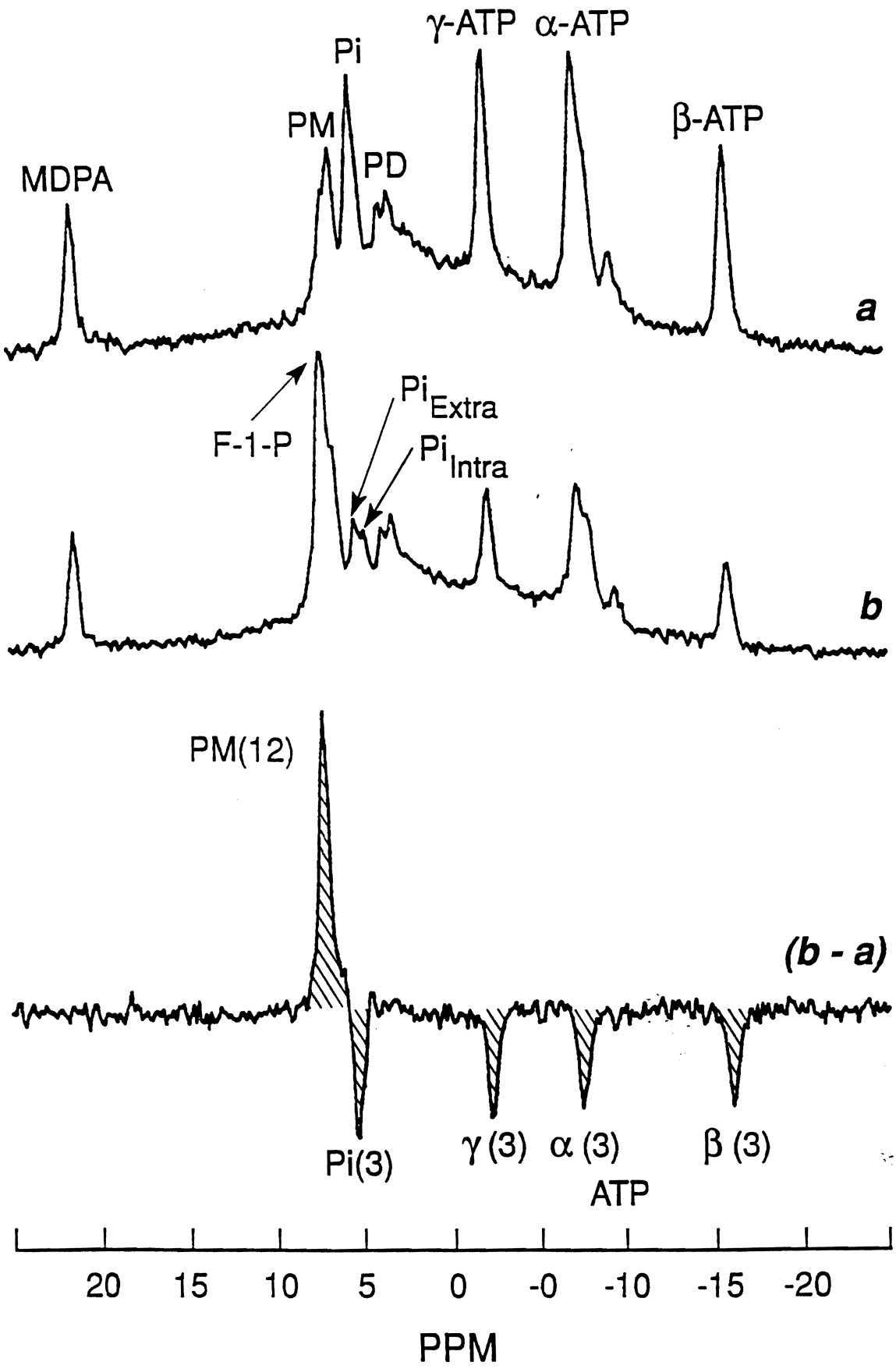

γ-ATP
α-ATP
Pi
PM
PD
β-ATP
MDPA
a
F-1-P
Pi Extra
Pi Intra
b
PM(12)
(b - a)
Pi(3)
γ (3)
α (3)
β (3)
ATP
20
15
10
5
0
-0
-10
-15
-20
PPM

FIGURE 2

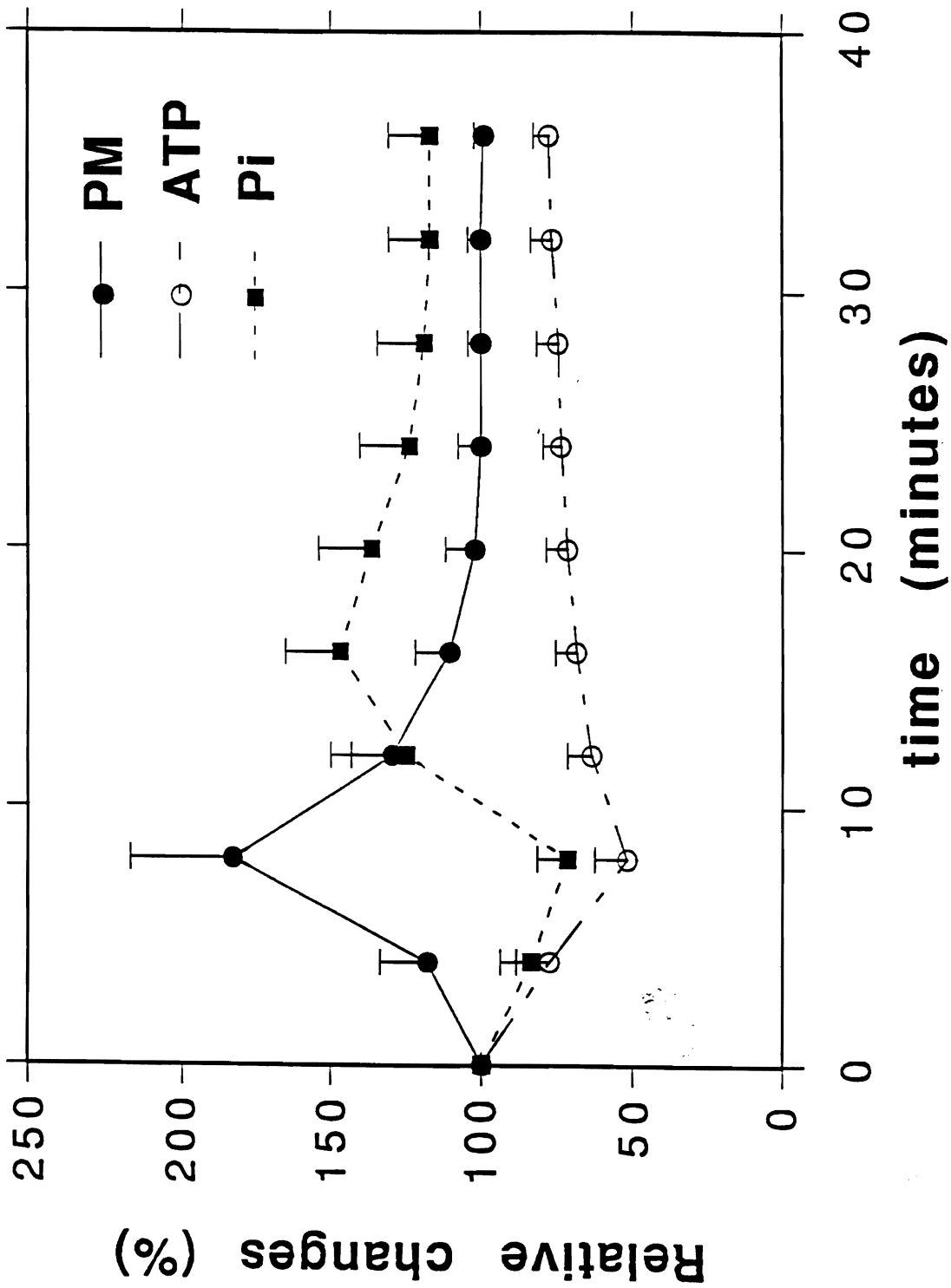

PM
ATP
Pi
Relative changes (%)
250
200
150
100
50
0
0
10
20
30
40
time (minutes)

FIGURE 3

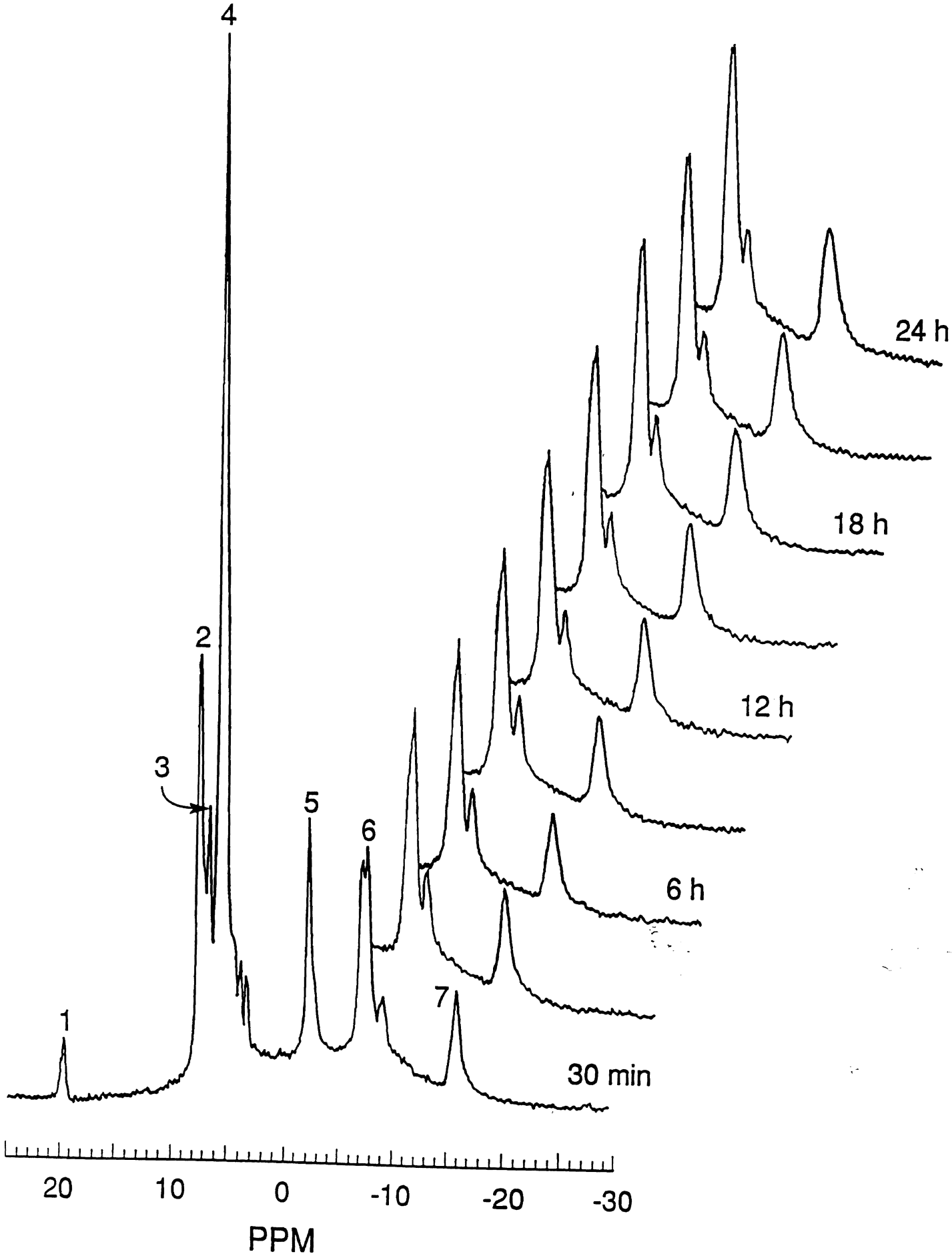
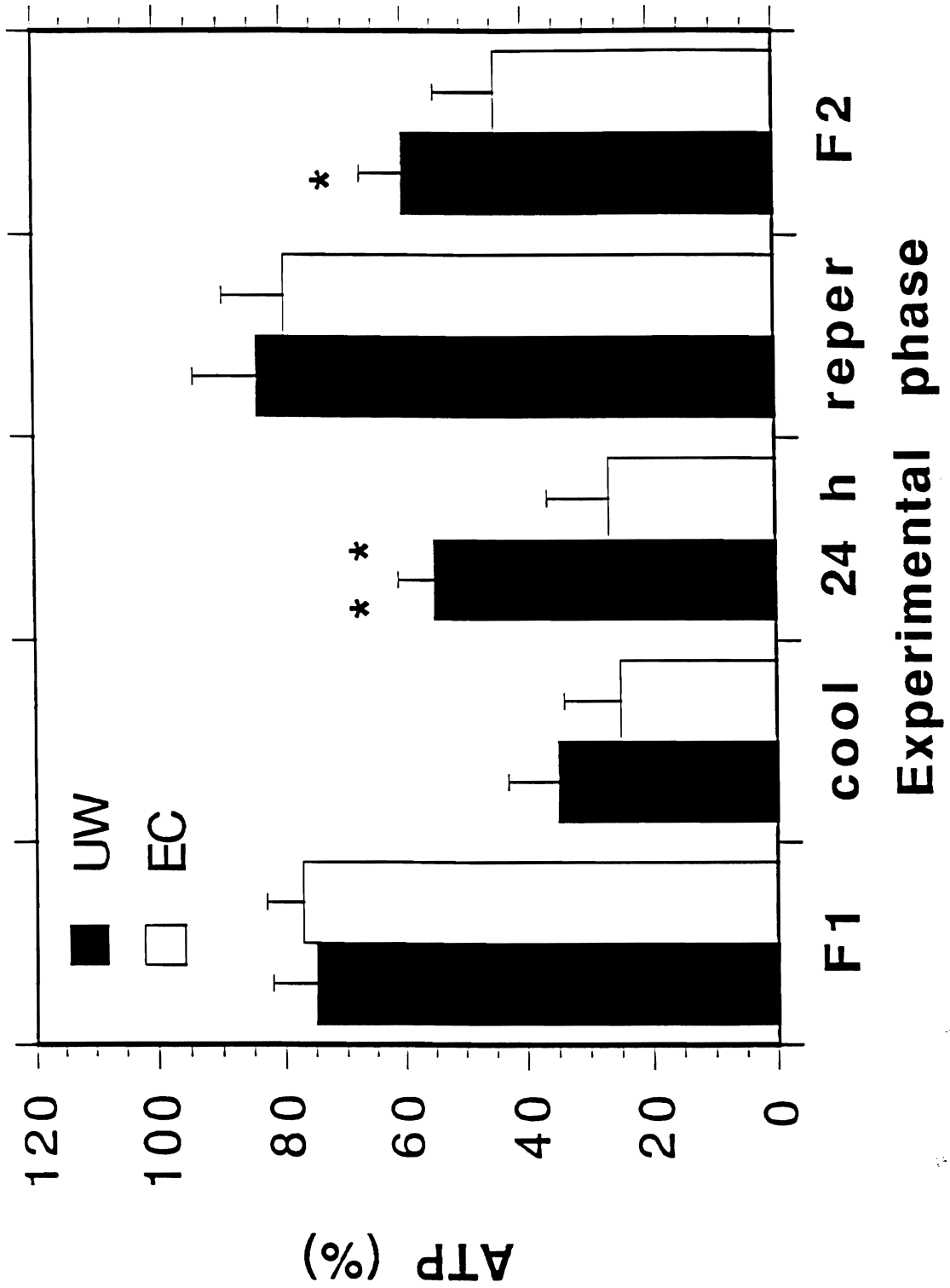


FIGURE 4


ATP (%)
120
100
80
60
40
20
0
UW
EC
F1
cool
24 h
reper
F2
Experimental phase
*
* *
*