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Purine Nucleoside Phosphorylase: A New Marker for Free Oxygen Radical Injury to the Endothelial Cell

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The effect of ischemia and reperfusion on purine nucleoside phosphorylase was studied in an isolated perfused rat liver model. This enzyme is localized primarily in the cytoplasm of the endothelial and Kupffer cells; some activity is associated with the parenchymal cells. Levels of this enzyme accurately predicted the extent of ischemia and reperfusion damage to the microvascular endothelial cell of the liver. Livers from Lewis rats were subjected to 30, 45 and 60 min of warm (37° C) no flow ischemia that was followed by a standard reperfusion period lasting 45 min. Purine nucleoside phosphorylase was measured at the end of the no flow ischemia and reperfusion periods as was superoxide generation (O_2^-). Bile production was monitored throughout the no flow ischemia and reperfusion periods. Control perfusions were carried out for 120 min. A significant rise in purine nucleoside phosphorylase levels as compared with controls was observed at the end of ischemia in all the three groups. The highest level, 203.5 ± 29.2 mU/ml, was observed after 60 min of ischemia. After the reperfusion period, levels of purine nucleoside phosphorylase decreased in the 30- and 45-min groups 58.17 ± 9.66 mU/ml and 67.5 ± 17.1 mU/ml, respectively. These levels were equal to control perfusions. In contrast, after 60 min of ischemia, levels of purine nucleoside phosphorylase decreased early in the reperfusion period and then rose to 127.8 ± 14.8 mU/ml by the end of reperfusion ($p < 0.0001$). Superoxide generation at the beginning of reperfusion was higher than in controls with similar values observed at the end of 30, 45 and 60 min of ischemia. During reperfusion, production of superoxide continued. Bile production was significantly lower at the end of 30 min (0.044 ± 0.026 μl/min/gm), 45 min (0.029 ± 0.022 μl/min/gm) and 60 min of ischemia (0.022 ± 0.008 μl/min/gm) when compared with bile production by control livers during the corresponding time (0.680 ± 0.195, 0.562 ± 0.133 and 0.480 ± 0.100 μl/min/gm respectively; $p < 0.001$). During reperfusion, rates of bile production were normal after 30 and 45 min of ischemia. In contrast, significantly lower rates of bile production, 0.046 ± 0.36 μl/min/gm ($p < 0.001$) occurred during reperfusion after 60 min of ischemia. Control livers during the same period produced 0.330 ± 0.056 μl/min/gm of bile. The results indicate that purine nucleoside phosphorylase levels may be a good index of oxidative injury to the liver in ischemia reperfusion and reliably predict the functional state of the organ after reperfusion. (HEPATOLOGY 1990;11:193-198.)

Liver transplantation is now the therapy of choice for end-stage liver disease (1). Procurement, preservation and subsequent transplantation of solid organs involve a period of ischemia followed by reperfusion. Ischemia results in the breakdown of ATP to hypoxanthine, which is the substrate for xanthine oxidase–mediated conversion to xanthine (2). During the reperfusion, this reaction produces superoxide and other reactive oxygen species, which have been implicated in the reperfusion injury observed after prolonged ischemia (3-5). Organ viability has been correlated with duration of ischemia and the breakdown of energy metabolism (6-9). Based on this, a number of methods have been developed to measure the levels of purine catabolites such as adenosine, inosine, hypoxanthine and xanthine to function as indices of ATP breakdown and markers of organ viability after transplantation. An accumulation of hypoxanthine and xanthine in the perfusate with concomitant low levels of inosine is associated with poor liver function after transplantation (10). Although these methods are accurate, they all require sophisticated instrumentation such as HPLC and cannot be performed routinely and sufficiently rapidly enough to be helpful or predictive clinically. Since ischemia and reperfusion injury involve the microvascular endothelial cell (11, 12) and the generation of hypoxanthine from inosine occurs in the endothelial cell (13), we investigated whether the time course of changes in the levels of the enzyme responsible for this

Received December 14, 1988; accepted August 24, 1989.

Supported by research grants from the Veterans Administration and Project Grant No. DK 29961 from the National Institutes of Health, Bethesda, Maryland.

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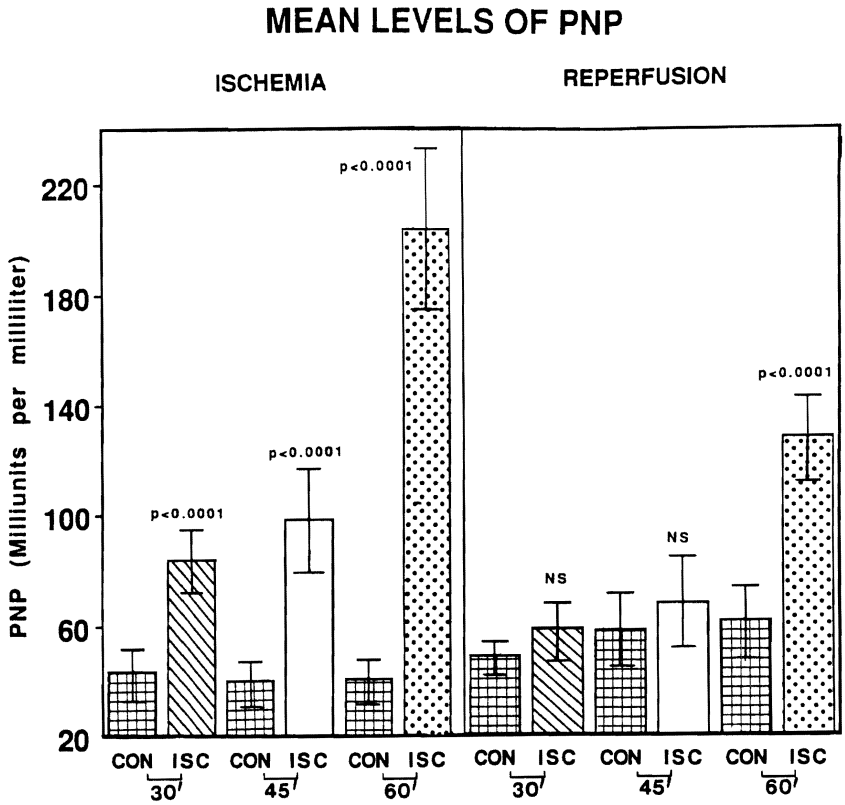

FIG. 1. Effluent levels of PNP in control *(CON)* livers and livers subjected to 30, 45 and 60 min of warm ischemia (ISC) (mean ± S.D., n = 6 for each group). PNP levels are significantly higher than corresponding controls ($p < 0.0001$) at end of ischemic period in all three groups. During reperfusion period of 45 min, PNP levels drop to control levels in 30 and 45 min group. In 60 min group PNP levels remain significantly higher ($p < 0.0001$) than control levels and levels in 30 and 45 min groups. NS = not significant.

conversion, namely purine nucleoside phosphorylase (PNP), reflected the extent of ischemic damage to the endothelial cell and differentiated between reversible and irreversible injury to the liver.

MATERIALS AND METHODS

Chemicals. PNP from calf spleen and xanthine oxidase from cow's milk were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN) and were >99% pure. Inosine, horse heart cytochrome *c* (grade III) and bovine superoxide dismutase were from Sigma Chemical Co. (St. Louis, MO).

Animals. Inbred male Lewis strain rats weighing 250 to 350 gm were purchased from Charles River Breeding Laboratories, Inc. (Wilmington, MA). All rats were acclimatized for at least 3 days before the experiment and were allowed food and water *ad libitum*. Animals were anesthetized with inhalational methoxyflurane (Metofane; Pitman-Moore, Inc., Washington Crossing, NJ) for both induction before and maintenance during surgery. The liver was exposed and immobilized through a transverse abdominal incision. The common bile duct, portal vein and inferior vena cava were cannulated. While the liver was being excised, it was gently perfused with Krebs buffer containing 2% bovine serum albumin (Krebs-Henselert buffer–bovine serum albumin) equilibrated with 95% O_2/5% CO_2 to clear it of all red blood cells and to minimize the trauma of procurement. Immediately after the liver was harvested, it was placed within the perfusion circuit.

Liver Perfusion. The perfusion apparatus used was recirculating, and the perfusate was continually oxygenated. Perfusate levels of O_2, CO_2, pH and bicarbonate were checked every 15 min by a Radiometer ABL-2 blood gas analyzer (Radiometer, Copenhagen, Denmark). The perfusate and liver were maintained at 37° C throughout the preischemic, ischemic and reperfusion periods. A standard experimental protocol was used for all the experimental groups in which the livers were equilibrated on the perfusion apparatus for 15 min. During this equilibration period, flow was gradually increased to a level of 2.5 to 3.5 ml/min/gm of tissue while maintaining the perfusion pressure below 15 cm H_2O. After the equilibration period, no flow ischemia was induced by bypassing flow around the liver for 30, 45 or 60 min. Six rats were studied per ischemic group. Reperfusion lasted a standard 45 min because pilot studies with our apparatus (unpublished data) showed that this length of perfusion would demonstrate whether a liver was able to recover normal function as measured by bile production and tissue levels of adenine nucleotides. Samples of effluents were collected at 15-min intervals for superoxide (O_2^-) (14) and PNP estimations (15). Samples were stored at −70° C until they were assayed. Bile production was monitored throughout the perfusion. Bile production rate during a particular period was calculated by dividing the difference in volume of bile produced in this period by the wet weight of the liver and expressed as microliters per minute per gram wet weight of liver.

Light Microscopy. Samples of each liver from the end of reperfusion were saved in formalin, stained with hematoxylin and eosin and analyzed by an independent pathologist. Control perfusions for 120 min were carried out in six rats.

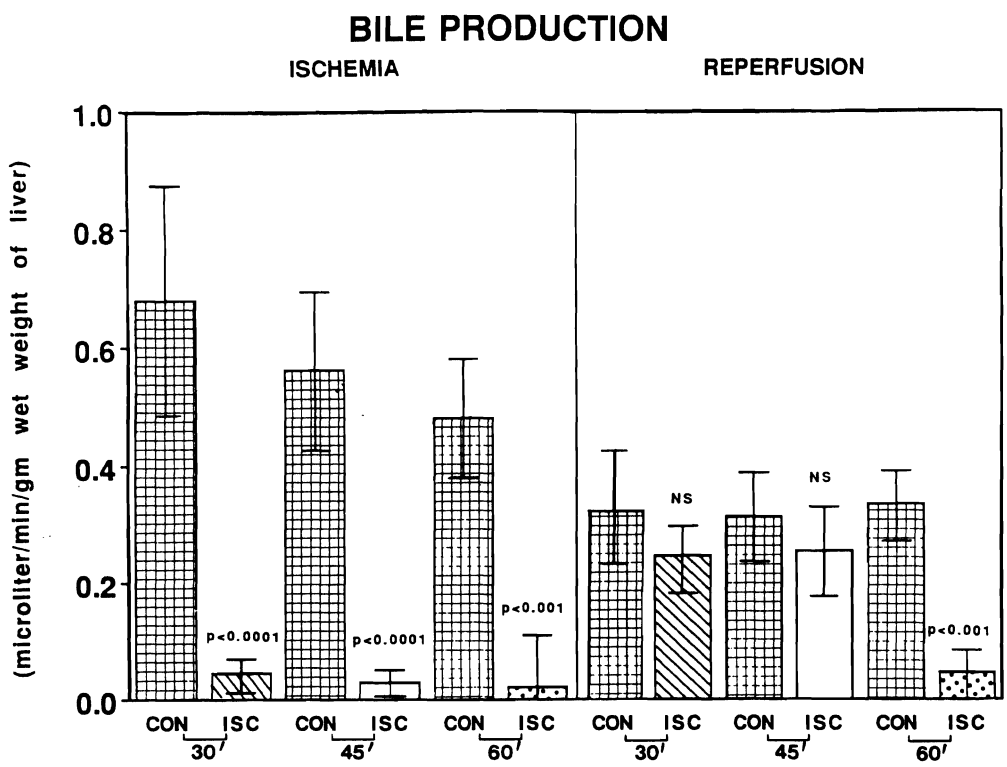

FIG. 2. Bile producton in control perfusions (CON) and at the end of 30, 45 and 60 min of warm ischemia (ISC) (mean ± S.D., n = 6 for each group). Bile production is significantly lower from control perfusions at end of 30, 45 and 60 min of warm ischemia ($p < 0.0001$). During reperfusion period, bile production returns to control levels in 30 and 45 min groups but remains significantly lower in 60 min group ($p < 0.001$).

Methods: Superoxide Generation. Superoxide levels in the effluent were measured by following the reduction of cytochrome *c* at 500 nm. Briefly, samples of effluent were diluted with phosphate buffer (50 mmol/L, pH 7.5) and placed in two cuvettes—a reference and a sample cuvette—at 37° C. The reference cuvette contained 300 μl of superoxide dismutase (1 mg/ml). The difference in optical density of cytochrome *c* between the reference and sample cuvette was used to calibrate the concentration of superoxide by using an extinction coefficient of 21.1 $mmol/L^{-1}$. All assays were carried out in triplicate according to the method of McCord and Fridovich (14).

Purine Nucleoside Phosphorylase. Enzyme activity in the effluent was measured by the method of Hoffee, May and Robertson (16). The breakdown of inosine to uric acid was measured by the increase in absorbance at 293 nm in a coupled assay system with xanthine oxidase.

Statistical Analysis. Data are expressed as mean ± S.D. The statistical significance of differences between group means was analyzed by Student's *t* test. The alpha level has been adjusted for the multiple comparisons using Bonferroni's adjustment. The alpha level per comparison was $p < 0.008$ (16).

RESULTS

PNP. Levels of PNP increased significantly at the end of the ischemic period in each of the three groups studied. Mean values at the end of 30, 45 and 60 min of no flow ischemia, 84.3 ± 10.8 mU/ml, 98.5 ± 18.6 mU/ml and 203.5 ± 29.2 mU/ml, respectively, were significantly ($p < 0.0001$) higher than control levels. In the reperfusion period of 45 min, PNP levels decreased in the 30 and 45 min ischemia groups to 58.17 ± 9.66 mU/ml and 67.5 ± 17.1 mU/ml, respectively, and were similar to control levels. A decrease in PNP levels also occurred during the early reperfusion period in the 60 min ischemia group followed by a sustained rise to 127.8 ± 14.8 mU/ml by the end of 45 min of reperfusion. This level of PNP was significantly higher ($p < 0.0001$) than that observed in control perfusion during the same period. Figure 1 shows the mean levels of PNP at the end of ischemia and reperfusion in the three groups.

Bile production during 30, 45 and 60 min of ischemia was respectively 0.044 ± 0.026 μl/min/gm, 0.029 ± 0.022 μl/min/gm and 0.022 ± 0.008 μl/min/gm wet weight. These rates were significantly lower than control rates during the same period, 0.680 ± 0.195 μl/min/gm, 0.562 ± 0.133 μl/min/gm and 0.480 ± 0.100 μl/min/gm wet weight, respectively ($p < 0.0001$).

During the reperfusion period, bile production returned to normal control levels in the 30 and 45 min ischemia groups. This was in contrast to the 60 min ischemia group, where the rate of bile production (0.046 ± 0.036 μl/min/gm) was significantly ($p < 0.001$) lower than the bile production in control liver, 0.330 ± 0.056 μl/min/gm. Figure 2 shows the

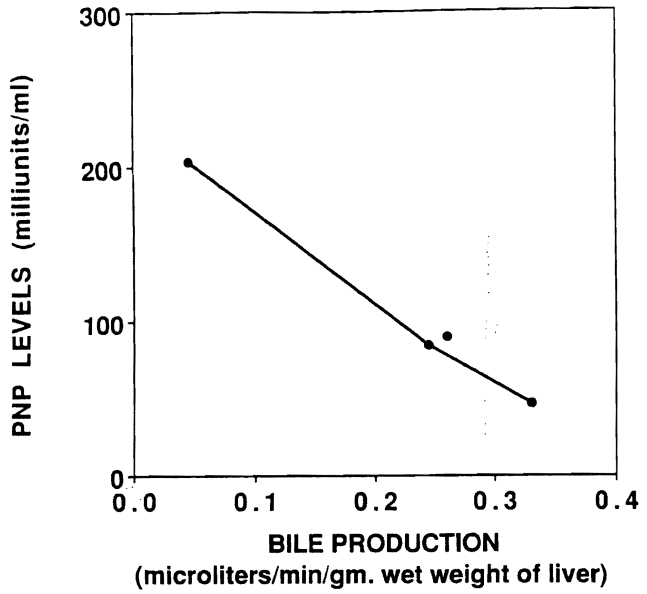

FIG. 3. Correlation between PNP levels at end of ischemia and bile production in reperfusion period (mean of six experiments in each group). Ischemia varied between 30 and 60 min followed by standard 45-min reperfusion period. High levels of PNP at end of ischemia were associated with significant reduction in bile production in reperfusion period. Conversely, normal levels of PNP were associated with normal rates of bile production during reperfusion. Mean PNP level of 46 mU/ml corresponded to mean bile production rate of 0.33 μl/min/gm.

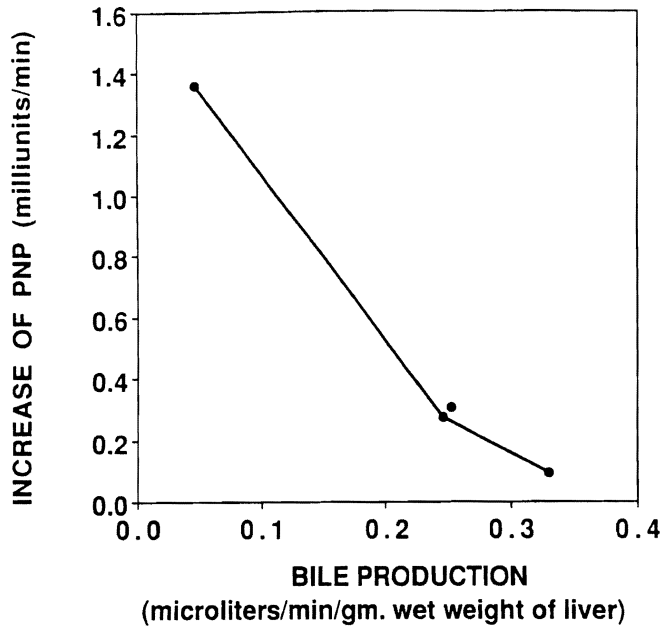

FIG. 4. Correlation between mean rate of increase in PNP levels and decrease in bile production during reperfusion (mean of six experiments). Ischemia varied between 30 and 60 min followed by reperfusion period of 45 min. Increase in PNP levels in 30 and 45 min groups are not significantly different from each other. In 60 min group PNP levels increase at a significantly greater rate than control perfusions and 30 and 45 min groups ($p < 0.0001$). Mean rate of increase in PNP levels in this group was 0.0092 μl/min/gm of liver. In normal livers mean rate of increase in PNP levels was 0.094 ± 0.05 mU/min and was associated with normal bile production rate of 0.33 μl/min/gm.

mean rates of bile production at the end of ischemia and reperfusion in the three groups.

Superoxide Generation. Reperfusion of the livers was associated with significant generation of superoxide in each of the three ischemic groups. Superoxide generation at the end of 30, 45 and 60 min of ischemia was 0.852 ± 0.42 nmol cytochrome *c*–reduced/ml, 1.012 ± 0.31 nmol cytochrome *c*–reduced/ml and 1.013 ± 0.46 nmol cytochrome *c*–reduced/ml, respectively. All these values were significantly greater than those observed in control perfusions during the same interval ($p < 0.0001$). Superoxide levels at the end of the reperfusion period were significantly higher than the corresponding levels at the beginning of the reperfusion period. At the end of reperfusion superoxide levels in the 30-, 45- and 60-min groups were 1.64 ± 0.26 nmol cytochrome *c*–reduced/ml, 1.42 ± 0.15 nmol cytochrome *c*–reduced/ml and 1.67 ± 0.23 nmol cytochrome *c*–reduced/ml, respectively. These values were again significantly greater than the corresponding values in the control perfusion ($p < 0.0001$) (Table 1). Figure 3 represents the correlation between levels of PNP at the end of ischemia to rate of bile production during reperfusion in the three groups. Figure 4 shows the correlation between mean rate of increase in PNP levels and decrease in bile production during reperfusion in the three groups. Histopathological studies showed increasing injury to the sinusoidal endothelial cell with relative sparing of the hepatocytes (Fig. 5).

DISCUSSION

Ischemia resulted in significant elevation of PNP in the effluents of all livers. However a definite distinction was observed in the PNP profile during the reperfusion period. In the 30 min and 45 min ischemia groups, we observed a normalization of PNP levels, indicating a reversible endothelial cell injury. This was in contrast to the situation when livers were subjected to 60 min of warm ischemia, where a slight lowering of PNP levels in the early reperfusion period was followed by a sustained rise indicating an irreversible injury to the endothelial cell. LeMasters et al. (11) have demonstrated that hypoxic injury to the liver is associated with the formation of cell surface blebs on hepatocytes and enlargement of endothelial cell fenestrations with disruption of both cell types. Reoxygenation injury results in the shedding of these blebs and release of the cytosolic enzymes contained within (11, 12). Reversible injury was associated with cessation of the shedding and subsequent leakage of cytosolic enzymes, whereas irreversible injury was associated with a sustained release of endothelial enzymes. Interestingly, 45 min of warm ischemia represents the transition between reversible and irreversible ischemia in both studies (11, 17). In mammalian cells PNP catalyzes the breakdown of inosine to hypoxanthine (18), the substrate for xanthine oxidase–mediated generation of free oxygen

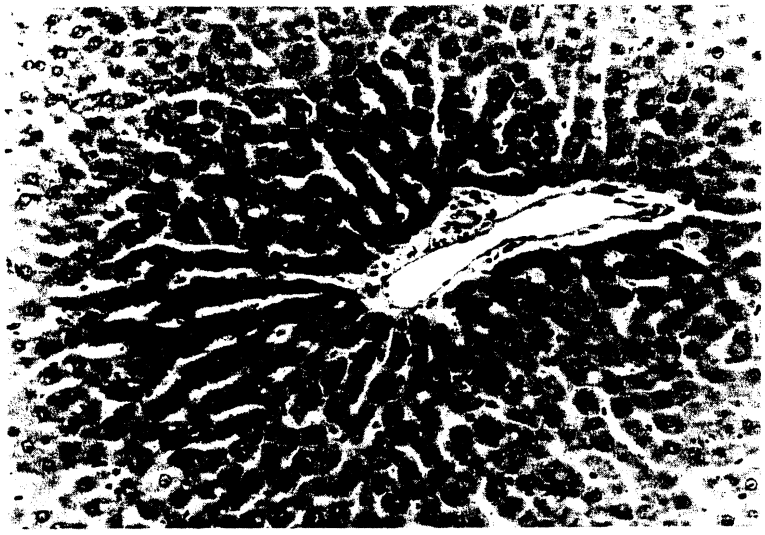

FIG. 5. Photomicrograph of rat liver during reperfusion after 60 min ischemia. Hepatocytes are only mildly injured with evidence of vacuolization, and portal vein endothelium is intact and appears normal. Sinusoidal endothelium has been severely damaged immediately adjacent to portal triad. (H & E stain, ×200.)

TABLE 1. Superoxide generation at end of ischemia and reperfusion

Duration of ischemia (min)	End of ischemia	End of reperfusion	p (end of ischemia)	p (end of reperfusion)
30	0.85 ± 0.42	1.64 ± 0.26	$p < 0.0001$	$p < 0.0001$
	(0.13 ± 0.11)[a]	(0.10 ± 0.098)[a]		
45	1.012 ± 0.31	1.42 ± 0.15	$p < 0.0001$	$p < 0.0001$
	(0.16 ± 0.12)[a]	(0.19 ± 0.14)[a]		
60	1.013 ± 0.46	1.67 ± 0.23	$p < 0.001$	$p < 0.0001$
	(0.19 ± 0.18)[a]	(0.17 ± 0.12)[a]		

Mean ± S.D., 6 animals per group. Nanomoles cytochrome *c*–reduced per milliliter.

[a]Figures in parentheses indicate control levels at corresponding times.

Effluent samples obtained at mentioned time periods were centrifuged to remove any red blood cells. Superoxide dependent reduction of ferricytochrome *c* was monitored at 550 nm at 37° C using an extinction coefficient of 21.1 $mmol/L^{-1}$. Specificity of cytochrome *c* reduction was confirmed by including superoxide dismutase in the reaction mixture.

radicals. Although some of the adenosine produced is degraded in the parenchymal cells, most of it is converted to inosine and then to hypoxanthine in the sinusoidal endothelial cell. Elevated levels of PNP thus reflect enhanced breakdown of inosine to hypoxanthine and therefore an increased availability of substrate for reperfusion injury (Fig. 1) to the endothelial cell. Reperfusion was associated with a higher generation of the superoxide anion in all the three groups, with greater values observed at the end of the reperfusion period compared with those at the beginning of the reperfusion period. This is indicative of the oxidative injury associated with the reperfusion (Table 1).

If 30 and 45 min of ischemia indeed represent reversible injury to the liver, as is reflected in the PNP profiles, reperfusion should result in normal regeneration of ATP. Bile production has been used as a reliable and accurate measure (19-22) of cellular ATP in both donor liver preservation and in experimental liver perfusion (23-26). Using bile production as an index of ATP generation, we observed that the rate was indeed related to the length of ischemia and could be predicted accurately by the PNP profile (Fig. 3). In livers that were reversibly injured, we observed a normalization of the rates of bile production in the reperfusion period after a slight drop during ischemia (Fig. 2). Livers subjected to greater than 45 min of ischemia, however, demonstrated an inability to regenerate ATP as was reflected by a decrease in the rate of bile production (Fig. 3). Thus PNP profiles appeared to reflect the extent of ischemic damage to the liver and predict the extent of ATP generation in the reperfusion period. This is further demonstrated by the finding that the mean rate of increase in PNP appears to correlate with the mean decrease in bile production in the reperfusion period (Fig. 4).

In conclusion, this study demonstrates that PNP levels in the organ effluent is a quick, easy, reproducible

and relatively inexpensive assay and may be a reliable index of ischemic damage to the microvascular endothelial cell. Measurement of this enzyme can distinguish between reversible and irreversible injury to the liver and may prove useful as a reliable marker of organ function after transplantation.

Acknowledgments: We gratefully acknowledge the secretarial assistance of Donna Ross, Cynthia Meister, and Bernice Kula and the art work of Bob Karausky.

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