Histol Histopathol (1995) 10: 111-116

Histology and Histopathology

# The role of xanthine oxidase in ischemia/reperfusion damage of rat liver

**W.M. Frederiks and K.S. Bosch**
Department of Cell Biology and Histology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

**Summary.** Oxygen radicals have been proposed to be involved in the induction of liver cell damage during reperfusion after ischemia. The role of xanthine oxidase in this process and the potential of the antioxidant system have been studied in a model of *in vivo* ischemia of rat liver followed by 1 h reperfusion by the use of enzyme histochemistry. Based on decreased lactate dehydrogenase activity in certain areas of liver parenchyma, cell damage could already be detected at 1 h reperfusion after ischemia. Incubations performed on serial sections showed that the same areas contained decreased activities of xanthine oxidoreductase, xanthine oxidase, catalase and glucose-6-phosphate dehydrogenase. Some individual cells in the undamaged liver parenchyma expressed a very high glucose-6-phosphate dehydrogenase, which suggests that these cells have a good defence against oxidative stress. It is concluded that oxygen radicals derived from xanthine oxidase do not play a decisive role in the induction of cell damage immediately at reperfusion after ischemia. However, it cannot be excluded that xanthine oxidase present in the blood stream can give rise to the development of additional damage later on.

**Key words:** Enzyme histochemistry, Ischemia, Reperfusion, Oxygen radicals, Rat liver

## Introduction

Ischemia is a widely occurring event which can take place generally in an organ during surgery, under conditions of shock and with transplantation. Knowledge of the processes which play a role in the appearance of ischemic damage is necessary in order to try to prevent injury. Ischemia induces diminished production of ATP due to anaerobic glycolysis and this is accompanied by acidosis. As a consequence of reduced amounts of ATP ion balances over membranes are disturbed and swelling occurs (Trump et al., 1982). Increasing periods of ischemia give rise to enhanced damage, firstly reversibly but when passing the point of no return, irreversibly. Irreversible damage has been morphologically characterized by the appearance of flocculent densities in mitochondria (Jennings et al., 1975; Glaumann et al., 1977; Trump et al., 1982; Myagkaya et al., 1985). Reperfusion following ischemia induces additional damage to the ischemically injured cells (Bulkley, 1983). Then cytoplasmic enzymes derived from the damaged cells appear into the blood.

In previous studies we have found that high levels of lactate dehydrogenase are already found at 1 h reperfusion after ischemia of rat liver (Frederiks et al., 1983, 1984a). Moreover, areas with decreased lactate dehydrogenase activity were observed at the same time in rat liver tissue (Frederiks et al., 1983; Kamiike et al., 1989). Leakage of cytoplasmic enzymes was followed by leakage of enzymes from organelles such as mitochondria (Nishimura et al., 1986; Frederiks and Marx, 1987), lysosomes (Frederiks and Marx, 1989), and peroxisomes (Frederiks et al., 1992).

It has been proposed that oxygen-derived free radicals may be responsible for the ischemia-reperfusion phenomenon (Bulkley, 1983; Parks et al., 1983; Granger et al., 1986). The enzyme which has been thought to be one of the most likely candidates as producer of oxygen radicals is xanthine oxidase (Nordstrom et al., 1985; Granger, 1988; Lamesch et al., 1989). Granger et al. (1981) developed a theory on the working mechanism of this enzyme in ischemia, but until now a definite proof for this hypothesis has not been given. The theory was based on data that the NAD-using (D-)form of xanthine oxidoreductase can be transformed into the $O_2$-using (O-)form of the enzyme by a protease activated by elevated intracellular calcium concentrations. During ischemia one of the substrates for the enzyme, hypoxanthine, would be generated by breakdown of ATP, whereas during reperfusion after ischemia the other substrate, oxigen, becomes available to produce oxygen radicals. However, recent studies have made clear that

*Offprint requests to:* Dr. W.M. Frederiks, Department of Cell Biology and Histology, Academic Medical Center, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands

conversion of the D- into the O-form does not occur in rat liver after ischemia (McKelvey et al., 1988; Marubayashi et al., 1991; Frederiks et al., 1993a; Kooij et al., 1994). Until now, the effects of reperfusion following ischemia on the localization of these enzymes have not been investigated. Therefore, in the present study we investigated the localization of xanthine oxidoreductase and xanthine oxidase activities at 1 h reperfusion after 60 min of ischemia of rat liver. The characteristics of some enzymes involved in the defence against oxygen radicals, catalase and glucose-6-phosophate de-hydrogenase, were also studied.

## Materials and methods

Male Wistar rats weighing 200-250 g were used. The animals were fasted for 24 h before operation, but water was allowed *ad libitum*.

### *Experimental procedures*

*In vivo* ischemia of rat liver was induced by clamping the afferent vessels to median and left lateral liver lobes with a neuroclip (Frederiks et al., 1982). After 60 min ischemia the blood flow to the liver was restored by removing the clamp. At 1 h reperfusion livers were removed. Liver fragments, maximally 5 $mm^3$, were frozen in liquid nitrogen and stored at -80 °C.

### *Enzyme histochemical methods*

Cryostat sections (8 μm thick) were cut on a motor-driven Bright cryotome fitted with a retraction microtome at a cabinet temperature of -25 °C (Van Noorden and Frederiks, 1992). Sections were picked up onto clean glass slides and stored in the cryotome cabinet until used. Only in the case of demonstrating xanthine oxidoreductase activity sections were incubated immediately after cutting (Kooij et al., 1991; Frederiks et al., 1993b). In all other cases cryostat sections were allowed to dry for 5 min at room temperature before incubation.

The incubation for lactate dehydrogenase activity was performed in a medium containing 18% (w/v) polyvinyl alcohol (PVA, hot water soluble, Sigma, St. Louis, USA), 100 mM phosphate buffer, pH 7.4, 150 mM lactic acid, 3 mM $NAD^+$, 0.47 mM methoxyphenazine methosulphate (mPMS), 5 mM sodium azide and 5 mM tetranitro BT (Frederiks et al., 1983). The incubation lasted for 5 min at room temperature.

Xanthine oxidoreductase activity was demonstrated according to Kooij et al. (1991) with some minor modifications using an incubation medium containing 18% (w/v) PVA, 100 mM phosphate buffer, pH 8.0, 0.5 mM hypoxanthine, 0.45 mM mPMS and 5 mM tetranitro BT. The incubation lasted for 15 min at 37 °C.

Incubations for the demonstration of xanthine oxidase activity were performed with a medium consisting of 10% (w/v) PVA, 100 mM Tris maleate buffer, pH 8.0, 10 mM cerium chloride, 100 mM sodium azide and 0.5 mM hypoxanthine (Ferederiks and Marx, 1993; Frederiks et al., 1994). Incubations lasted for 60 min at 37 °C. After incubation, the reaction product was visualized by incubating for 30 min at 37 °C in a medium containing 50 mM sodium acetate buffer, pH 5.3, 100 mM sodium azide, 1.4 mM diaminobenzidine, 42 mM cobalt chloride and 0.6 mM hydrogen peroxide.

The reaction for glucose-6-phosphate dehydrogenase activity was performed in a medium containing 18% (w/v) PVA, 100 mM phosphate buffer, pH 7.4, 5 mM glucose-6-phosphate, 0.8 mM $NADP^+$, 5mM magnesium chloride, 0,45 mM mPMS, 5mM sodium azide and 5 mM tetranitro BT (Van Noorden, 1984). The incubation lasted for 10 min at 37 °C.

The procedure for the demonstration of catalase activity was based on the method of Angermüller and Fahimi (1981) with some minor modifications (Ankum et al., in preparation). The sections were fixed for 5 min at room temperature in 0.3% (v/v) glutaraldehyde in distilled water. The sections were incubated for 30 min at 37 °C in a medium containing 2% (w/v) PVA, 100 mM glycine-NaOH buffer, pH 10, 15 mM hydrogen peroxide and 5 mM diaminobenzidine.

After incubation with PVA-containing media, the sections were rinsed with distilled water (60 °C) in order to stop the reaction immediately and to rinse off the viscous PVA-containing media. The sections were mounted in glycerol jelly.

## Results

In control liver, high activity of the cytoplasmic enzyme lactate dehydrogenase was found in liver parenchymal cells, with a somewhat higher activity in periportal than in pericentral hepatocytes. Certain areas of liver parenchyma showed a decreased activity at 1 h reperfusion after ischemia (Fig. 1). These areas seem to be randomly distributed within the parenchyma.

Xanthine oxidoreductase activity was found in endothelial cells of sinusoids, Kupffer cells and liver parenchymal cells, with a higher activity in pericentral than in periportal hepatocytes. Xanthine oxidoreductase activity in both parenchymal and non-parenchymal cells decreased in those areas which also showed decreased lactate dehydrogenase activity (Fig. 2).

The localization of xanthine oxidase activity was largely similar to xanthine oxidoreductase activity in control liver, but the activity was much lower. Again, areas with decreased lactate dehydrogenase activity showed decreased xanthine oxidase activity at 1 h reperfusion after ischemia (Fig. 3).

The peroxisomal enzyme catalase was localized in a granular form homogeneously distributed within the liver lobules. At 1 h reperfusion after ischemia the activity of this peroxisomal enzyme also decreased in the areas which showed decreased lactate dehydrogenase activity (Fig. 4).

A rather low activity of glucose-6-phosphate de-

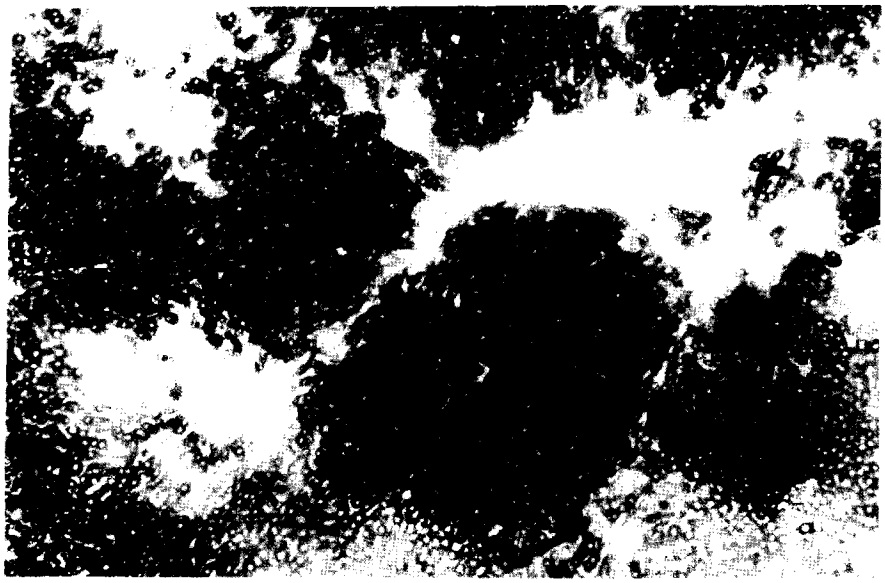

**Fig. 1.** Micrograph of unfixed cryostat section of rat liver after incubation for lactate dehydrogenase activity. Liver tissue was obtained at 1 h reperfusion after ischemia. Areas with decreased amounts of final reaction product are found (*). x 75

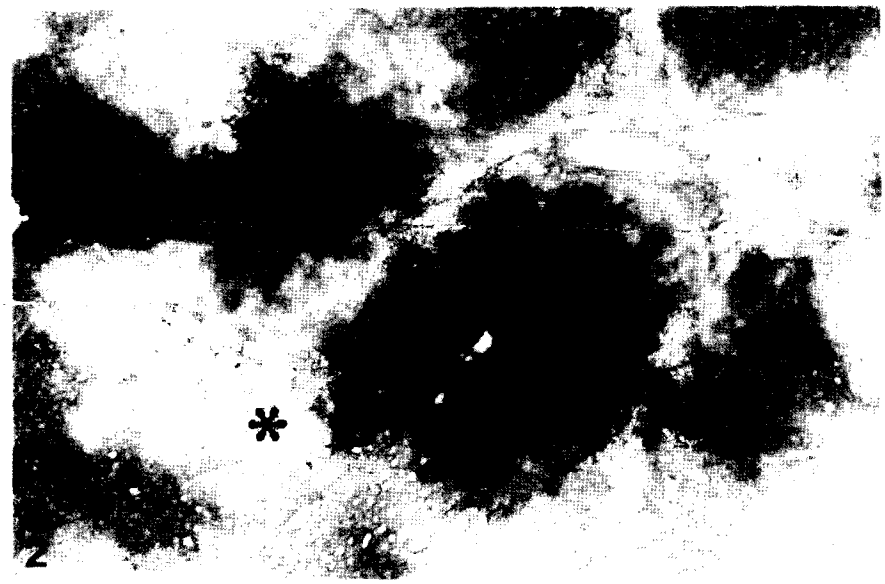

**Fig. 2.** Micrograph of unfixed cryostat section of rat liver after incubation for xanthine oxidoreductase activity. Liver tissue was obtained at 1 h reperfusion after ischemia. Areas with decreased amounts of final reaction product are found (*). In undamaged tissue activity is observed in parenchymal and sinusoidal cells. x 75

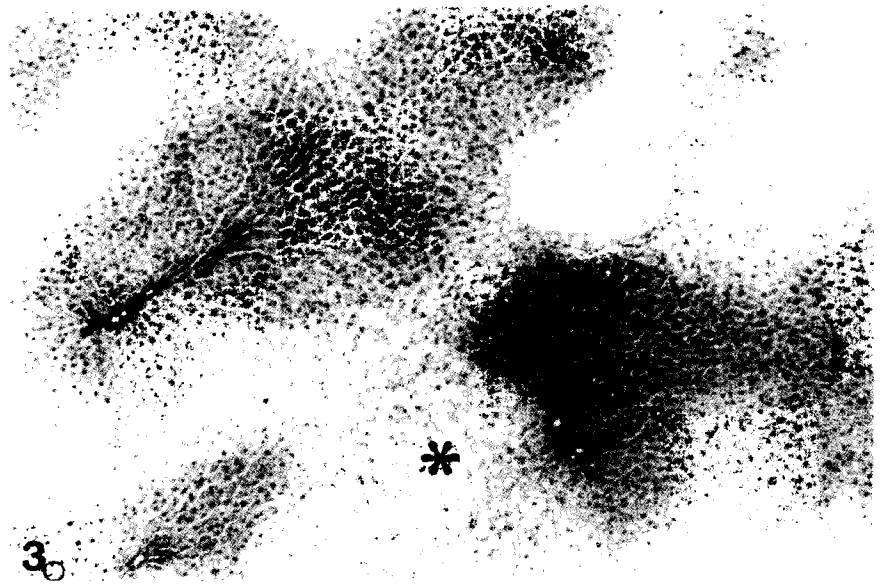

**Fig. 3.** Micrograph of unfixed cryostat section of rat liver after incubation for xanthine oxidase activity. Liver tissue was obtained at 1 h reperfusion after ischemia. Areas with decreased amounts of final reaction product are found (*). x 75

hydrogenase was found in liver parenchymal cells and a high activity was present in Kupffer cells. Again, during reperfusion after ischemia enzyme activity decreased in the same areas which showed a lowered lactate dehydrogenase (Fig. 5). Moreover, the number of cells with an extremely high glucose-6-phosphate dehydrogenase activity increased in the undamaged areas (Fig. 5). Some of these cells are Kupffer cells and/or monocytes, but others seem to be liver parenchymal cells.

## Discussion

The present study has shown that at 1 h reperfusion following ischemia all the enzymes studied already leak from certain areas into the blood (Figs. 1-5). Previous studies made clear that the activities of these enzymes did not change after only ischemia, which means that reperfusion induced the leakage. The only enzymes which changed in rat liver after ischemia were 5'-nucleotidase and glycogen phosphorylase (Frederiks et al., 1988; Frederiks and Marx, 1990). The first enzyme is localized in normal liver in bile canalicular and sinusoidal plasma membranes of liver parenchymal cells and this localization changed into a diffuse way after ischemia (Frederiks et al., 1988). The ultrastructure of bile canalicular plasma membranes showed a multilamellar appearance after ischemia, whereas the plasma membranes at the sinusoidal surfaces were present as blebs (Frederiks et al., 1984b). It can be assumed that these blebs, formed during ischemia, are removed during reperfusion after ischemia. Reduced amounts of ATP present in the liver parenchymal cells

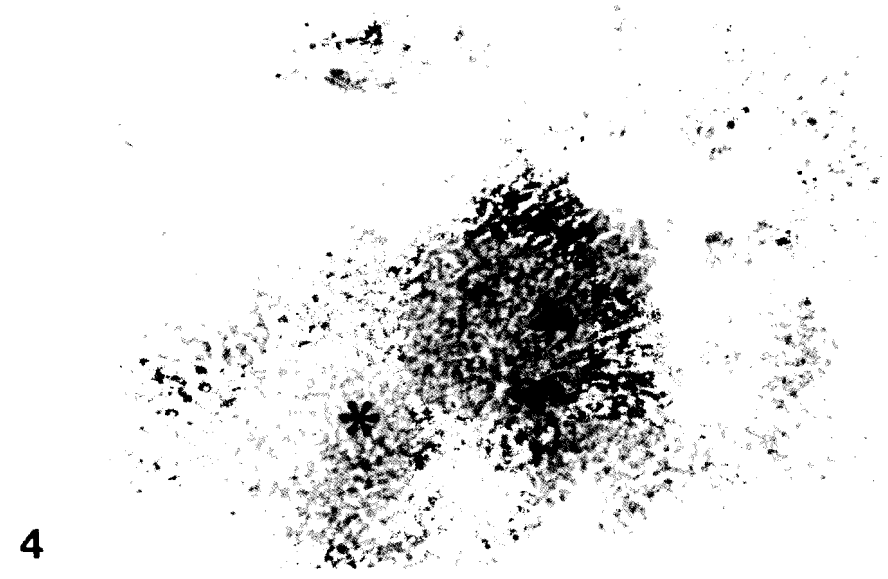


**Fig. 4.** Micrograph of cryostat section of rat liver after incubation for catalase activity. Liver tissue was obtained at 1 h reperfusion after ischemia. Areas with decreased amounts of granular reaction product are found (*). x 75


**Fig. 5.** Micrographs of unfixed cryostat section of rat liver after incubation for glucose-6-phosphate dehydrogenase activity. Liver tissue was obtained at 1 h reperfusion after ischemia. Areas with decreased amounts of final reaction product are found (*). High activity is present in individual cells in undamaged areas. x 75

may give rise to a disturbed balance of ions over the plasma membrane resulting in enlargement of the cell surface. Alterations in the cytoskeleton as a consequence of ischemia may also affect the appearance of the plasma membranes. The decreased glycogen phosphorylase activity in pericentral areas of rat liver after only short periods of ischemia also points to changes in the cytoskeleton (Frederiks and Marx, 1990). It has been assumed that the enzyme has strong connections with elements of the cytoskeleton which are necessary for expression of activity (Gustafson and Meijer, 1994).

The present study showed that enzymes leaked not only from liver parenchymal cells, but also from Kupffer cells and sinusoidal endothelial cells. Therefore, the mechanism of leakage as proposed for liver parenchymal cells, also seems to be true for non-parenchymal cells (Rao et al., 1990; Vaubourdolle et al., 1993).

The leakage of xanthine oxidoreductase and xanthine oxidase activity from liver parenchyma during reperfusion after ischemia, combined with the absence of any effect of ischemia itself on these enzymes make it unlikely that oxygen radicals produced by xanthine oxidase are a candidate for the reperfusion damage. However, based on the findings of Kooij et al. (1994) that fast conversion of xanthine dehydrogenase into xanthine oxidase takes place in the blood, it can be concluded that the enzyme is responsible for the induction of additional damage; for instance, to sublethally damaged cells.

Damage at longer periods of reperfusion after ischemia may also occur as a consequence of infiltrating polymorphonuclear leukocytes (PMNs; Jaeschke et al., 1990; Koo and Komatsu, 1991). However, this phenomenon may only play a role at longer periods of reperfusion after ischemia, because increased numbers of PMNs have been observed at 3 h reperfusion (Frederiks et al., 1992).

The question arises as to whether the damage which appears at 1 h reperfusion after ischemia is irreversible injury. Previous studies have shown that the ultrastructure of lactate dehydrogenase-depleted cells shows inhomogeneity (Frederiks et al., 1989). Some cells were damaged irreversibly based on the appearance of flocculent densities in their mitochondria and karyolysis, whereas others only showed dilation and vesiculation of the endoplasmic reticulum and swelling of the mitochondria, which points to reversible damage. Accumulation of erythrocytes in the sinusoids of damaged areas was another finding described in that study, indicating a poor blood circulation in these areas. The damage to the endothelial lining and the presence of erythrocytes in the sinusoids make it unlikely that the reversibly damaged hepatocytes may restore anymore.

The question remains as to whether the cells surrounding the damaged areas are able to withstand the attact by oxygen radicals produced by xanthine oxidase in the blood. The present study indicates that some cells show an increased glucose-6-phosphate dehydrogenase activity (Fig. 5), suggesting that these cells possess an increased capacity to produce NADPH. The reduced coenzyme is a compound needed to keep glutathione via glutathione reductase in a reduced form. Glutathione can act as a potent antioxidant and therefore, glucose-6-phosphate dehydrogenase can be seen as an antioxidant parameter. However, more studies are necessary to support this theory.

In conclusion, the present study has shown that at 1 h reperfusion after ischemia of rat liver irreversibly damaged areas can already be detected based on the histochemical demonstration of cytoplasmic and organelle-bound enzymes. A direct role of xanthine oxidase in the production of oxygen radicals and the induction of early reperfusion damage is not likely, although the enzyme may be involved in the development of damage later on.

*Acknowledgements.* The authors wish to thank Dr. C.J.F. Van Noorden for stimulating suggestions, Mr. A. Maas for biotechnical support, Mrs. T.M.S. Pierik for preparing the manuscript and Mr. J. Peeterse for photographic work.

## References

Angermüller S. and Fahimi H.D. (1981). Selective cytochemical localization of peroxidase, cytochrome oxidase and catalase in rat liver with 3,3'-diaminobenzidine. Histochemistry 71, 33-44.

Bulkley G.B. (1983). The role of oxygen free radicals in human disease processes. Surgery 94, 407-411.

Frederiks W.M. and Marx F. (1987). Changes in cytoplasmic and mitochondrial enzymes in rat liver after ischemia followed by reperfusion. Exp. Mol. Pathol. 47, 291-299.

Frederiks W.M. and Marx F. (1989). Changes in acid phosphatase activity in rat liver after ischemia. Histochemistry 93, 161-166.

Frederiks W.M. and Marx F. (1990). The effect of ischemia on glycogen phosphorylase activity in rat liver: a quantitative histochemical study. Anal. Cell. Pathol. 2, 347-355.

Frederiks W.M. and Marx F. (1993). A histochemical procedure for light microscopic demonstration of xanthine oxidase activity in unfixed cryostat sections using cerium ions and a semipermeable membrane technique. J. Histochem. Cytochem. 41, 667-670.

Frederiks W.M., James J., Bosch K.S., Schröder M.J.R. and Schuyt H.C. (1982). A model for provoking ischemic necrosis in rat liver parenchyma and its quantitative analysis. Exp. Pathol. 22, 245-252.

Frederiks W.M, Myagkaya G.L., Fronik G.M., Van Veen H.A., Vogels I.M.C. and James J. (1983). The value of enzyme leakage for the prediction of necrosis in liver ischemia. Histochemistry 78, 459-472.

Frederiks W.M., Vogels I.M.C. and Fronik G.M. (1984a). Plasma ornithine carbamyl transferase level as an indicator of ischemic injury of rat liver. Cell Biochem. Funct. 2, 217-220.

Frederiks W.M., Myagkaya G.L., Van Veen H.A. and Vogels I.M.C. (1984b). Biochemical and ultrastructural changes in rat liver plasma membranes after temporary ischemia. Virchows Arch. (B) 46, 269-282.

Frederiks W.M., Marx F. and Myagkaya G.L. (1988). A quantitative histochemical study of 5'-nucleotidase activity in rat liver after ischemia. J. Pathol. 154, 277-286.

Frederiks W.M., Marx F. and Myagkaya G.L. (1989). The «nothing

dehydrogenase» reaction and the detection of ischemic damage. Histochem. J. 21, 565-573.

Frederiks W.M., Marx F. and James J. (1992). Molecular basis of cell injury. In: Recent advances in cellular and molecular biology. Wegmann R.J. and Wegmann M.A. (eds). Peeters Press. Leuven. pp 215-221.

Frederiks W.M., Marx F. and Kooij A. (1993a). The effect of ischemia on xanthine oxidase activity in rat intestine and liver. Int. J. Exp. Pathol. 74, 21-26.

Frederiks W.M., Ouwerkerk I.J.M., Bosch K.S., Marx F., Kooij A. and Van Noorden C.J.F. (1993b). The effects of storage on the retention of enzyme activity in cryostat sections. A quantitative histochemical study on rat liver. Histochem. J. 25, 119-122.

Frederiks W.M., Bosch K.S., Van den Munckhof R.J.M. and Van Noorden C.J.F. (1994). A quantitative histochemical study of xanthine oxidase activity in rat liver using the cerium capture method in the presence of polyvinyl alcohol. J. Histochem. Cytochem. 42, 1091-1097.

Glaumann B., Glaumann H., Berezesky I.K. and Trump B.F. (1977). Studies on cellular recovery from injury. II. Ultrastructural studies on the recovery of the pars convoluta of the proximal tubule of the rat kidney from temporary ischemia. Virchows Arch. (B) 24, 1-8.

Granger D.N. (1988). Role of xanthine oxidase and granulocytes in ischemia-reperfusion injury. Am. J. Physiol. 255, H1269-H1275.

Granger D.N., Rutili G. and McCord J.M. (1981). Role of superoxide radicals in intestinal ischemia. Gastroenterology 81, 22-29.

Granger D.N., Hollwarth M.E. and Parks D.A. (1986). Ischemia-reperfusion injury: role of oxigen-derived free radicals. Acta Physiol. Scand. Suppl. 548, 47-63.

Gustafson L.A. and Meijer A. (1994). Interaction of cell swelling and glucose-induced translocation of glycogen synthase and phosphorylase. Submitted.

Jaeschke H., Farhood A. and Smith C.W. (1990). Neutrophils contribute to ischemia/reperfusion injury in rat liver in vivo. FASEB J. 4, 3355-3359.

Jennings R.B., Ganote C.E. and Reimer K.A. (1975). Ischemic tissue injury. Am. J. Pathol. 81, 179-198.

Kamiike W., Fujikawa M., Koseki M., Siumimura J., Miyata M., Kawashina Y., Wada H. and Tagawa K. (1989). Different patterns of leakage of cytosolic and mitochondrial enzymes. Clin. Chim. Acta 185, 265-270.

Koo A. and Komatsu H. (1991). Leukocyte interactions with sinusoid endothelial cells in ischemic reperfused rat livers. In: Cells of the hepatic sinusoid. Vol. 3. Wisse E., Knook D.L. and McCuskey R.S. (eds). Kupffer Cell Foundation. Rijswijk. pp 364-366.

Kooij A., Frederiks W.M., Gossrau R. and Van Noorden C.J.F. (1991). Localization of xanthine oxidoreductase activity using the tissue protectant polyvinyl alcohol and final electron acceptor tetranitro BT. J. Histochem. Cytochem. 39, 87-93.

Kooij A., Schiller H.J., Schijns M., Van Noorden C.J.F. and Frederiks W.M. (1994). Conversion of xanthine dehydrogenase into xanthine oxidase in rat liver and plasma at the onset of reperfusion after ischemia. Hepatology 19, 1488-1495.

Lamesch P., Ringe B., Blumhardt G. and Pichlmayr R. (1989). Effect of xanthine oxidase inhibition on warm ischemic lesions of the liver. Transplant. P. 21, 1285-1286.

Marubayashi S., Dohi K., Yamada K. and Kawasaki T. (1991). Role of conversion of xanthine dehydrogenase to oxidase in ischemic rat liver cell injury. Surgery 110, 537-543.

McKelvey T.G., Höllwarth M.E., Granger D.N., Engerson T.D., Landler U. and Jones H.P. (1988). Mechanism of conversion of xanthine dehydrogenase to xanthine oxidase in ischemic rat liver and kidney. Am. J. Physiol. 254, G753-G760.

Myagkaya G.L., Van Veen H. and James J. (1985). Quantitative analysis of mitochondrial flocculent densities in rat hepatocytes during normothermic and hypothermic ischemia in vitro. Virchows Arch. (B) 49, 61-072.

Nishimura T., Yoshida Y., Watanabe F., Koseki M., Nishida T., Tagawa K. and Kawashima Y. (1986). Blood level of mitochondrial aspartate aminotransferase as an indicator of the extent of ischemic necrosis of the rat liver. Hepatology 6, 701-707.

Nordstrom G., Seeman T. and Hasselgren P.O. (1985). Beneficial effect of allopurinol in liver ischemia. Surgery 97, 679-684.

Parks D.A., Bulkley G.B. and Granger D.N. (1983). Role of oxygen free radicals in shock, ischemia and organ preservation. Surgery 94, 428-432.

Rao P.N., Walsh T.R., Makowka L., Rubin R.S., Weber T., Snyder J.T. and Starzl T.E. (1990). Purine nucleoside phosphorylase: a new marker for free oxygen radical injury to the endothelial cell. Hepatology 11, 193-198.

Trump B.F., Berezesky I.K. and Osornio-Vargas A.R. (1982). Cell death and the disease process. The role of calcium. In: Cell death in biology and pathology. Bowen I.D. and Lockshin R.A. (eds). Chapman & Hall. London. pp 209-242.

Van Noorden C.J.F. (1984). Histochemistry and cytochemistyr of glucose-6-phosphate dehydrogenase. Prog. Histochem. Cytochem. 15, 1-85.

Van Noorden C.J.F. and Frederiks W.M. (1992). Enzyme histochemistry: A laboratory manual of current methods. Microscopy Handbooks 26. Oxford University Press. Oxford.

Vaubourdolle M., Chzaouilleres O., Poupon R., Ballet F., Braunwald J., Legendre C., Baudin B., Kirn A. and Giboudeau J. (1993). Creatine kinase BB: a marker of liver sinusoidal damage in ischemia-reperfusion. Hepatology 17, 423-428.

Accepted October 5, 1994