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THE MODULATION OF LIPID PEROXIDATION IN LIVER ISCHAEMIA

Graham Stuart Wynward Whiteley MB BS FRCS

Submitted for the Degree of Master of Surgery

of the University of London

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ABSTRACT

The Modulation of Lipid Peroxidation in Liver Ischaemia

Iatrogenic and pathological organ ischaemia and subsequent reperfusion are common occurences in surgical practice.

The inevitable ischaemia which follows removal of an organ for transplantation and its reperfusion in the recipient, the planned occlusion of blood vessels to control haemorrhage during surgery on the organs supplied by them, or to repair or replace the vessels themselves are examples.

Understanding the sequelae of these unphysiological situations, with a view to reducing irreversible damage, is essential.

Oxygen derived free radicals have been implicated as agents of damage during both ischaemia and reperfusion.

To investigate this hypothesis, the effect of normothermic ischaemia on rat liver has been investigated.

Lipid peroxidation, which has been shown to be an indicator of free radical production was measured over three hours normothermic ischaemia. This was assessed by measuring the peroxidation products malondialdehyde and conjugated dienes and the ratio of oxidised to reduced glutathione.

Glutathione is an endogenous scavenger of oxygen derived free radicals and exists in both oxidised and reduced forms. Lipid peroxidation results in the oxidation of reduced glutathione. The total glutathione level remains constant as it is not used up in the conversion to the oxidised from the reduced form. Thus both oxidised and reduced forms of glutathione were measured and a ratio of oxidised to reduced glutathione was calculated and used as an estimation of the extent of lipid peroxidation.

Any changes in liver metabolism after three hours warm ischaemia in vitro was determined by measuring one element of synthetic ability, urea production. A repeatable pattern of deterioration was established.

The production of lipid peroxidation products, malondialdehyde and conjugated dienes increased significantly after periods of 60 minutes ischaemia. The oxidised/reduced glutathione ratio showed highly

significant changes after the same period. Taken together these results suggest significant free radical production during this time.

Having established that lipid peroxidation due to free radical production increased and rat liver synthetic function decreased, the modulation of this peroxidation by free radical scavenging agents was then studied.

The free radical scavenging agents superoxide dismutase, allopurinol, desferrioxamine and mannitol were given to the animals before experimental ischaemia was induced.

When these were administered, with the exception of mannitol, significant reduction in the parameters of lipid peroxidation were observed. Different scavengers were found to have effects on different aspects of lipid peroxidation, with superoxide dismutase being effective on all parameters.

When the effect of these scavengers on synthetic function was measured, superoxide dismutase partially preserved the synthetic function. The other scavengers had no significant effect.

The conclusion of the study is that significant lipid peroxidation, an index of free radical damage, occurs with periods of ischaemia of 60 minutes and above in the intact rat liver and that this peroxidation can, by the administration of superoxide dismutase, allopurinol and desferrioxamine, be reduced. The deterioration of liver function cannot totally be explained by free radical activity since only partial preservation of function was possible with superoxide dismutase.

It remains to be shown if the administration of free radical scavenging agents prior to any clinical procedure which leads to hepatic ischaemia, would be beneficial in reducing hepatocellular damage.

Statement of Originality

All experimentation contained in this Thesis was performed by the author whilst holding the post of Stanley Thomas Johnson Research Fellow in the Academic Department of Surgery at the Royal Free Hospital and School of Medicine, London.

The literary survey and review was performed and written by the author.

The bibliography was compiled by the author.

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CHAPTER 1.

AN INTRODUCTION TO ISCHAEMIA, REPERFUSION AND FREE RADICALS.

The Clinical Background to Ischaemia

Tissues within the body require a supply of oxygen for many of their metabolic processes. Without oxygen these processes and functions would not continue.

In general, oxygen is supplied to tissues in combination with haemoglobin in the erythrocyte in a reversible oxidation-reduction process.

Most tissues can withstand a minor reduction in oxygen supply without a severe effect on function.

The rapidity of onset of changes in oxygen supply is important. Changes in circulatory dynamics and increase in haemoglobin content of the red blood cell result from lower atmospheric oxygen

concentration over several days or weeks, which occurs at high altitudes. The tissues, to some extent, suffer some degree of ischaemia before these circulatory adjustments take place. If the transition to high altitude is too rapid, the result is mountain sickness which can be avoided by acclimatisation.

This type of oxygen deprivation is usually well tolerated, hovever, if the blood supply to an organ or tissue suddenly and completely ceases, the result tends to be more extensive. An example being hepatic necrosis following hepatic artery transection due to trauma (Lucas C.E. & Ledgerwood A.M., 1978).

The duration of ischaemia plays a part. No lasting damage occurs following occlusion of the circulation in the arm during blood pressure measurement with a sphygmomanometer but a compartment syndrome lasting for three hours produces permanent changes in muscle (Heppenstal R.B.,1986).

Factors Influencing The Extent of Ischaemic Damage

Complete ischaemia exists when the blood supply to

an organ or tissue ceases. Partial ischaemia occurs when the blood supply is pathologically low.
The extent of ischaemic damage within a tissue may depend upon certain basic factors. These include the organ or type of tissue involved in the process and the species of animal studied.

Ischaemic tolerance of the liver of the baboon is over 30 minutes (Heaney J.P. et al.,1965). That of the dog was reported to be less than 20 minutes when the afferent blood supply to the liver is clamped (Raffucci F.L.,1953) although more recent work shows survival after 1 hours ischaemia (Hasselgren P-O. et al., 1979).The human liver can tolerate between 60 and 120 minutes of ischaemia (Huguet C. et al.,1978) but the pig appears to be the most tolerant to liver ischaemia with survivors after 3 hours (Khan D. et al., 1986).

The tissues subjected to ischaemia show different degrees of tolerance. Kidneys of dogs can survive ischaemic periods of 1 hour with no apparent damage (Pegg D.E. et al., 1984), whereas the above study by Raffucci shows death of some dogs after 20 minutes liver ischaemia.

The duration of ischaemia and its rapidity of onset together with the severity of the ischaemia influence the extent of the damaging process.

The presence of a collateral circulation is an important factor in protecting an organ from ischaemic damage, as it prevents ischaemia when the major blood supply is occluded. Collateral circulation can develop however as a result of ischaemia. Hollenberg identifies a neovascular stimulating agent in the lymph draining ischaemic kidneys (Hollenberg N.K. et al.,1985).

The temperature at which the ischaemia occurs determines the extent of damage to the tissue. In the ischaemic rat liver which is perfused with a given storage solution, the reduction in adenine nucleotides is affected by temperature (Attenburrow V.D. et al., 1981). A temperature of 10C. was beneficial, whereas 2-4C. and normothermic ischaemia showed equivalent adenine nucleotide content.

The concept of cold conditions failing to reduce ischaemic damage as they would a chemical reaction may appear difficult to grasp. Some reactions in tissue fractions occur faster in ice than in liquid water (Grant N.H. & Alburn H.E., 1965), for example the copper catalysed splitting of hydrogen peroxide.

The severity of damage related to body temperature in ischaemic kidneys is shown by Zager (Zager R.A. & Altschuld R. 1986).

The Variation in Tissue Response to Ischaemia

The organ or tissue involved in the ischaemic process has great bearing on the resultant damage. The brain is notably sensitive to periods of ischaemia lasting a few minutes. Complete recovery seldom occurs following ischaemic periods of this magnitude (Siesjo B.K. 1985). The neural tissue of the spinal cord is also sensitive to ischaemic damage (Anderson D.K. et al.,1985. Lim K.H. et al., 1986. Coles J.C. et al., 1986).

The kidney (Pegg D.E. et al., 1984) and liver have intermediate sensitivity and tolerance to normothermic ischaemia (Harris et al.,1982. Nordlinger B. et al. 1980). These organs may recover following ischaemic periods of one to two hours duration (Myers B.D. et al. 1984). There is speculation at present that the liver can recover from normothermic ischaemia after a greater time than previously thought (Kahn D. et al. 1986).

Muscle is relatively resistant to ischaemic damage as highlighted by the frequency of recovery after arterial occlusion in the legs by emboli or surgical cross clamping for bypass surgery. Four hours ischaemia is often tolerated in such circumstances.

In skeletal muscle of the rat hind limb , a tourniquet may be applied for 3 hours with full recovery (Heppenstal B. et al., 1986) although this recovery may take over 3 hours (Newman R.J. 1984). The severity of ischaemic damage in rat skeletal muscle is also dependent upon the activity of the muscle. Resting muscle will tolerate 3 hours ischaemia whereas glycogen depleted muscle will not (Jennische E. 1985).

The tolerance of the gut to ischaemia is variable, depending on whether the vascular occlusion is acute or chronic (Marston A. 1985). McCord suggests that "intestinal mucosa is the tissue most exquisitely sensitive to ischaemic injury" (McCord J.1985).

Species Variation in Ischaemia

The extent of ischaemic damage in tissues is

thought to be species dependent. Examples of tolerance to liver and kidney ischaemia between species have been cited above.

In those animals in which the effects of liver ischaemia has been studied, the rat is said to have a liver which is very sensitive to ischaemia. The liver of the pig has been noted to survive 6 hours normothermic ischaemia (Khan D. et al., 1986). This observation is not supported by most authors when death occurs after 3 hours ischaemia (Nordlinger B. et al. 1980).

The longer the ischaemic period, the greater is the damage. This is borne out in a clinical setting by the transient arterial occlusion during blood pressure measurement with a sphygmomanometer resulting in complete recovery. The intermittent release of a tourniquet will reduce the recovery time of a limb (Newman R.J. 1984). Embolic occlusion for instance lasting over 12 hours often has disasterous consequences resulting in the loss of the limb and may lead to death due to tissue metabolites affecting the heart on reflow (Mathieson M.A.et al.,1983).

The Effect of Rapidity of Onset of Ischaemia

A rapid onset of ischaemia is usually more damaging than a gradual occlusion. Adaptation to a gradual onset of ischaemia can take place. Collateral circulation develops in a partially ischaemic tissue in an attempt to counteract the decrease in blood flow (Hollenberg N.K. et al. 1985). Progressive lower limb ischaemia demonstrates this phenomenon where arteriograms are studied prior to bypass procedures being undertaken (Abbott W.M. & Meier G.H. 1987). Many collaterals are seen even though major vessels are totally occluded. Rapid embolic occlusion of the same vessels in the absence of collaterals results in severe ischaemic damage if embolectomy is not performed in good time.

PROBLEMS SPECIFIC TO LIVER ISCHAEMIA

The Clinical Background To Liver Ischaemia

Current trends in Surgery favour a more radical approach to the resection of liver tumours which would previously have been regarded as inoperable (Fortner J.G. et al.,1974).

Starzl describes the operation of hepatic trisegmentectomy. This extensive liver resection involves resecting all of the right lobe together with the medial part of the left lobe (Starzl T.E. et al. 1975).

Due to the better prognosis after resection, particularly in the fibrolamellar type of primary liver cell carcinoma (Soreide O. et al. 1986), extensive resection is now thought to be indicated. Second resections for recurrent hepatocellular carcinoma are now advocated (Nagasue N. et al. 1986).

The liver is an extremely vascular organ from which large volumes of blood may be lost if it is traumatised.

During major liver resections, haemostasis is therefore essential. This was recognised at the turn of the century and was achieved by the temporary occlusion of the hepatic artery by clamping the vascular triad at the porta hepatis (Pringle J.H., 1908). This manouvre is effective in reducing haemorrhage from the cut surface of the liver.

For major resection of the liver amounting to lobectomy, the Pringle manouvre is said to be

useless in preventing major venous bleeding (Huguet et al., 1978). Huguet suggested a much more complex vascular exclusion when extensive resection is undertaken. His method involved portal triad clamping, supra and infrahepatic clamping of the vena cava and clamping of the upper aorta.

The point is also made that there is a significant blood supply from vessels within the hepatic ligaments and these must be divided in the dissection prior to applying the clamps.

A similar exclusion technique was described by Fortner with the addition of liver cooling to 4C. to prolong ischaemic tolerance up to 2 hours and 14 minutes (Fortner J.G. et al. 1974). Huguet thought that the cooling was not as necessary as first imagined provided the vascular exclusion did not exceed 30 minutes (Huguet C., 1976).

In liver trauma similar manouvres are employed to control haemorrhage whilst definitive procedures are undertaken (Lim R.C. et al. 1976, Pachter H.L. et al. 1983).

In Pachter's study the Pringle manouvre was used together with crude cooling which involved pouring

iced Ringer lactate over the liver to produce hypothermia.

A study by Aaron S. in 1975 described the effects of selective hepatic artery ligation to control haemorrhage in liver trauma at normothermic temperatures.

The Tolerance of the Liver to Ischaemia

The above manouvres, although effective in controlling haemorrhage, necessarily lead to tissue ischaemia due to the occlusion of the afferent blood supply at normal body temperature. This may result in lasting damage if the period of vascular occlusion exceeds approximately 1 hour (Huguet C.et al. 1978).

Total vascular occlusion including subdiaphragmatic aortic clamping is possible (Huguet C. 1976). It is said to be harmless for periods of up to 30 minutes normothermic ischaemia in man.

Pachter points out the difference between clamping for trauma and tumour resection in which normothermic clamping of 1 hour was reported as being safe (Huguet & Nordlinger 1978). It is implied

however that 30 minutes is still considered to be the safe working time for hepatic resection.
He gives two reasons for a lower tolerance to normothermic ischaemia in hepatic trauma. One is that hepatic tumours develop spontaneous collaterals which may increase the liver's tolerance to ischaemia. The other is that the general condition of the severely injured patient may reduce tolerance to ischaemia.

The Outcome of Ischaemia in the Liver

From the above studies, the implication is that the Surgeon has a safe ischaemic time in which to complete a hepatic resection. This time can be increased by cooling (Fortner J.G., 1974. Pachter H.L. 1983) or by administration of steroids (Figueroa I. 1975).

What changes can be expected in the liver if the safe ischaemic time is exceeded ?

The changes can be divided into structural and metabolic.

The Histological Changes in Liver Ischaemia

What is the microscopic rather than the biochemical result of ischaemia?
Detectable histological changes are only noted after two hours ischaemia or greater. There appears to be little microscopic change after shorter periods of ischaemia (Nordlinger B. et al. 1980).

The changes seen occur in the centrilobular region and follow a relatively well defined sequence of events. (Adkison et al. 1986).

a) Clumping of nuclear chromatin
b) Dilation of the endoplasmic reticulum
c) Swelling of mitochondria
d) Distortion of the plasma membrane of the cell
e) Vacuolation at the periphery of the cell within the cytoplasm.

Distension of the sinusoids with blood cells, cytoplasmic condensation and nuclear shrinkage is seen, together with some cell necrosis in pig liver at 180 minutes normothermic ischaemia (Nordlinger B. et al. 1980).

Extension of these changes away from the centrilobular region of the liver occur after three hours ischaemia or longer accompanied by fatty change leading to hepatic necrosis.

The integrity of cellular membranes is affected during ischaemia by a process affecting the fatty acid components. This process is called lipid peroxidation (Slater T.F. 1982, Kappus H., 1985) and it affects both cellular and subcellular membranes (Bridges J.W. et al., 1983). Lipid peroxidation is mediated by free radicals and is discussed later in the Chapter.

Mitochondrial membranes appear to be sensitive to lipid peroxidation (Marubayashi S., et al. 1982). This results in impaired transport of ions especially calcium. (Bellomo G. & Orrenius S. 1985).

Permeability of the cell membrane to enzymes increases, leading to increased blood levels of hepatic enzymes especially aspartate transaminase (AST, SGOT) and glutamate pyruvate transaminase (SGPT). (Adkison D et al 1986).

The membrane of the cell and of subcellular components has been shown to become more viscous as a result of lipid peroxidation (Eichenberger et al., 1982) and may be due to cross linking of membrane phospholipids by malondialdehyde (Nair V. et al. 1986).

Cellular damage during ischaemia may occur due to the release of lysosomal enzymes. During ischaemia there is a release of enzymes which progresses within the period of ischaemia. A high concentration of lysosomes occurs within the liver cell and damage due to hydrolytic enzyme release may be significant. (Chaudry T.H., 1983).

The Biochemical Changes After Liver Ischaemia

Several biochemical changes which occur during normothermic ischaemia have been described. Battersby (Battersby C. et al. 1974) showed that a metabolic alkalosis developed during ischaemia which returned to the pre ischaemic level at 60 minutes.

ATP concentration within cells has been shown to decrease rapidly during ischaemia (Hems D.A. & Brosnan J.T. 1970, Takenaka M. et al. 1981, Marubayashi S. et al. 1986). The regeneration of ATP following ischaemia is taken to be a good indicator of cellular recovery.

Radical scavenging agents including a-tocopherol have been shown to improve regeneration of ATP over control animals (Marubayashi S. et al. 1986).

The level of ATP during ischaemia falls dramatically after 60 minutes but increases after 120 and 180 minutes ischaemia (Nordstrom G., 1985). A similar pattern is seen for ADP level. The changes in ATP level are seen seconds after interruption of liver blood supply in rats (Hems D.A. & Brosnan J.T. 1974).

Nordstrom also observes that the tissue water content increases with ischaemia. Both intra and extracellular water increase but the increase in intracellular water, due to altered cell permeability, contributes to cell injury (Granger D.N., Hollwarth M.E. 1986). The cell swelling is thought to cause microvascular compression.

Synthetic function of the liver during ischaemia has been measured by several authors.
The protein synthesis falls rapidly during 60 minutes ischaemia (Hasselgren P.O., 1979 & 1982, Nordstrom G., 1985). The rate of fall then decreases at 120 and 180 minutes.

This is assessed by the incorporation of the amino acid leucine into protein. Similar results are seen by both authors, with amino acid incorporation falling to between 30%(rat) and 50%(dog) of the pre ischaemic value between 120 and 180 minutes.

This may be due to a decrease in tissue energy level, tissue acidosis or deranged intracellular ion composition Nordstrom G. et al. 1984).

Changes in liver enzymes, the so called liver function tests, are noted to be insignificant during 60 minutes ischaemia by Hasselgren in the dog. Battersby shows a significant increase in SGOT which returns to normal 30 minutes after cessation of ischaemia. There is also an increase in alkaline phosphatase after 60 minutes ischaemia but this is within the normal range. Elevation of aspartate amino transferase occurs with acute hepatic ischaemia (Wustrow T. et al. 1981).

The above histological and biochemical changes are observed after normothermic ischaemia in the liver. They occur when the safe ischaemic time is exceeded.

What might cause these changes ?

Fridovich (1983) stated that " temporary interruption of blood flow to a tissue results in damage to that tissue. It has usually been assumed that this damage occurs during the period of hypoxia

and is due to depletion of ATP. Another possibility is that the deleterious effects actually occur during reperfusion and are due to free radical generation."

INTRODUCTION TO FREE RADICALS

What is a Free Radical?

In the normal molecule, chemical stability and reactivity depend upon a balance of electrical charge within their electron shells or orbitals. If the molecule is balanced there will be a tendency to stability. A free radical is a highly reactive chemical species which has electrons with unpaired spins in its orbitals or single electrons in the orbitals. The inequality of charge accounts for the reactivity of free radicals.

Free radicals in organic chemical reactions are documented in the 1930's (Hey D.H. & Waters W.A. 1937). Some industrial significance of free radicals is shown in research from the rubber industry. Free radicals cause certain rubbers to become brittle (Farmer E.H. et al. 1943).

Free radicals are implicated in pathological processes for example pulmonary oedema caused by high altitude or hyperoxia (Taylor A.E. et al 1983). They are thought to be responsible for the cellular damage induced by radiation (Proctor P.H. and Reynolds E.S., 1984) and ischaemia. Free radicals

are also thought to be involved in the processes of chemical toxicity (Trush M.A. et al. 1982, Ryle P.R. 1984, Slater T.F. 1984, Eklow-Lastbom L. et al. 1986). Some side effects of drug therapy are thought to be caused by free radical activity (Docampo R. et al. 1981).

Free radicals occur in normal physiological body processes which are not damaging to the animal. These include the oxidation of alcohol, phagocytosis (Chance B. et al. 1979) and inflammation (Michelson A.M. & Puget K. 1986). Natural scavengers of free radicals are identified. These include the enzyme catalase, superoxide dismutase (Weisiger R.A. 1986) and glutathione peroxidase (Chance B. et al., 1979).

A compound may become a free radical by either losing or gaining an electron in a chemical oxidation-reduction reaction. These reactions will generate two radicals at the same time because one substance will gain an electron whilst another will by necessity lose an electron for the reaction to proceed. If the free radicals which are produced are very reactive, the concept of a chain reaction has to be considered.

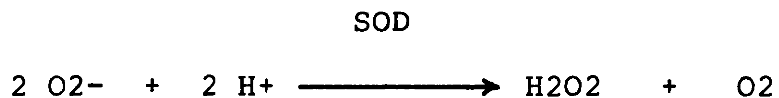
Chain reactions are often involved in the pathological processes which are thought to be

mediated by free radicals (Kappus H. 1985).

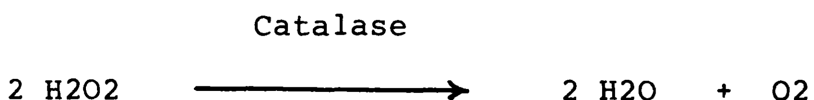
Cellular Defence Against Free Radicals

Natural cellular defence against the damaging effects of free radicals has been identified. The mechanisms which have been identified at present, fall into those which rely on an enzymatic process and those which rely on the formation of a stable end product of a free radical reaction.

Examples of the former group are SUPEROXIDE DISMUTASE which inactivates the potentially harmful superoxide radical (Fridovich I. 1983) to molecular oxygen or hydrogen peroxide (McCord J.M. 1983).



CATALASE converts hydrogen peroxide, which is itself a reactive species, to water and molecular oxygen, both of which are chemically more stable.



A multi-enzyme system for the disposal of free radicals involves oxidised and reduced glutathione and is controlled by GLUTATHIONE OXIDO-REDUCTASE (Bellomo G. & Orrenius S. 1985, Reed D.J. 1985) . Reduced glutathione will accept electrons from peroxides to reduce them to water.

$$ROOH + 2\ GSH \longrightarrow ROH + H2O + GSSG$$

Reduced glutathione is then oxidised in turn in a process using NADPH to yield oxidised glutathione (Ross D. et al. 1985). Subsequent reduction of oxidised glutathione replaces the pool of reduced glutathione (Brigelius R. 1985).

In addition to the enzymatic disposal of active free radical species, several chemical substances can accept electrons to form new free radicals which are relatively stable and non damaging. These substances are generally of high molecular weight although this is not always the case. Tocopherol (Tsen C.C. & Collier 1960, Marubayashi S. et al. 1986, Scott G. 1988) and some carotenes are recognised free radical scavengers together with coenzyme Q10 (Takenaka M. et al. 1981, Marubayashi S. et al. 1982). Respiratory substrates such as succinate and B-

hydroxy-butyrate may also act as free radical scavengers (Meszaros L. et al. 1982).

The Detection and Estimation of Free Radicals

The detection and direct measurement of free radicals remains difficult. Due to the high reactivity of some free radicals e.g. the hydroxyl radical, their existance can be defined in milli or even micro seconds (Dormandy T.L. 1983). This is because such a highly reactive entity reacts with a second substance before it can be measured and steady state conditions are hardly ever achieved.

The possibility of highly reactive species ever reaching high concentrations for measurement is therefore remote. Despite these considerations methods for the estimation of free radical species exist (Smith C.V. et al.,1987).

Direct Electron Spin Resonance Spectroscopy

The major direct estimation of free radicals is electron spin resonance. This is a method of measurement specific for molecules which have unpaired electron spins. The charge set up by such

an unpaired electron will be affected by a magnetic field applied to it (Knowles P.F. et al.,1976).

This phemomenon can be detected and measured to yield a particular line on an electromagnetic spectrum (Blum H. et al. 1986). Different radical species will yield different spectral lines allowing some identification (Swartz H.M. 1970, Makino K. et al. 1986). The superoxide radical may be identified in this manner (Chance B. et al. 1979). ESR methods are used by some authors to elucidate electron transfer reactions involving metal ions (Gilbert B.C. & Jeff M. 1988) and the reactions involved in lipid peroxidation (Makino K. et al. 1986).

The technique relies on a sufficient concentration of free radicals. This would require either a high rate of production or a low rate of elimination for the radical system.

Spin Trapping

Spin trapping is an adaptation of the electron spin resonance system. A free radical can be made to bind to a non reactive substance to form a stable product. This is known as a spin trap. An example of

this is the reaction of the hydroxyl radical with 5,5 -dimethyl-1-pyrroline-1-oxide (DMPO) (Weiss S.J. 1986, Makino K. et al. 1986). The resonance spectra for the common traps are known and any change in the spectrum as a result of binding of the radical can be detected. The production of free radical species may therefore be estimated using the electron spin resonance method. The most significant advantage of this indirect spectroscopic method is that the free radicals generated in the system will progressively accumulate as they bind to the trap. Measurement of short lived species therefore becomes possible.

Chemiluminescence

The generation of free radicals can be measured using chemiluminescence. As free radicals combine with certain chemical substances, they cause the emmision of light. If this emission is amplified , a fairly accurate measure of free radical production would be possible (Iwaoka T. et al., 1987). Chemiluminescence is used successfully in the detection of free radicals in the catalyzed oxidation of polyunsaturated fatty acids (Schulte-Herbruggen T. & Cadenas E. 1985) and in the initiation of lipid peroxidation (Cadenas E. 1985).

Singlet oxygen can be measured by a chemiluminescent method (Sies H. 1985).

A possible limitation is once again seen in the case of highly reactive and hence short lived species. The free radical must combine with the chemiluminescent compound and the detector must be sensitive to the low intensity and short duration of the chemiluminescence if the method is to work.

The Measurement of Relatively Stable Intermediates

During the free radical reactions relatively stable intermediates are often produced. In the case of the lipid peroxidation pathway for example these stable products are identified in detail (Esterbauer H., 1985). Estimations of these substances can therefore be made. Conjugated dienes (Dormandy T.L. 1985) and malondialdehyde have been assayed (Plaa G.L. & Witschi H. 1976, Yagi K., 1982, Suematsu T. & Abe H. 1982). The limitation of these estimations of reaction intermediates is in the fact that the measurements are indirect. The nature of the original radical is still not identified. These intermediates are however a good indicator that a

free radical reaction has taken place and may be useful in determining the extent of the reaction.

Depletion of Endogenous Scavenging Agents

Free radical scavengers exist as a protective mechanism within cells.

Superoxide dismutase for example exists in the cells of many species. It is specific for the dismutation of the superoxide radical to a stable entity. It would appear that the superoxide radical is produced in the normal cell. If free radicals are produced in a cell system, a feasible method for their detection would be the rate or extent of depletion of endogenous scavengers.

Similarly the conversion of substances involved in redox systems from the reduced to the oxidised form could be measured as in the case of glutathione.

Gas Chromatography-Mass Spectrometry

This technique for the measurement of lipid peroxidation products is proposed as being an

accurate method for both in vitro and in vivo use (Hughes H. et al. 1986). Previously described in vitro methods for the estimation of peroxidation products are thought by Hughes to be subject to interference in vivo.

THE ROLE OF OXYGEN DERIVED FREE RADICALS IN ISCHAEMIC AND REPERFUSION DAMAGE IN CELLS AND TISSUES

Introduction

Once an initiating mechanism for production of free radicals is instituted within a cell, the reaction with available oxygen may progress. An activated oxygen species or oxy radical is produced (Halliwell B. & Gutteridge J.M.C. 1985).

These radicals have been implicated in damaging reactions to cells and subcellular components (Eichenberger K. et al. 1982). Bulkley lists some clinical conditions in which free radicals may play a significant role. They may be involved in myocardial infarction, intestinal ischaemia, cerebral ischaemia and skin graft survival (Bulkley G.B. 1983).

Biochemical disruption in terms of enzyme activity, fluid and ionic imbalance (Moore G.A. et al. 1983. Bellomo G. et al. 1985) together with vascular permeability (Granger D.N. et al. 1986) have also been attributed to free radical activity.

Oxygen derived free radicals have been shown to react with any class of macromolecule within the cell including proteins (Gutteridge J.M.C. 1981. Fornander J. et al. 1984) carbohydrates (Gutteridge J.M.C. 1984) and DNA (Brawn K. & Fridovich I. 1981, Ueda K. et al. 1985).

Reaction with the lipids contained within cell membranes leads to peroxidation reactions. The results range from an alteration in fluidity of the membrane (Eichenberger K. et al. 1982) to changes in permeability to both intracellular and extracellular substances. Complete disruption of cell membranes or the membranes of intracellular components including mitochondria and lysosomes (Peters T.J. et al. 1985) is thought to occur as a result of peroxidation. This may lead to cell death.

Protein components of cells are also susceptible to attack by free radicals. Suggested consequences of such attack includes the alteration of enzyme and transport functions within the cell (Marubayashi S. et al. 1982). Damage to nucleic acids (Ueda K. et al. 1985) as a result of free radical attack may lead to mutation. Several cytotoxic agents are thought to act in this way, emphasising that free radical activity may be beneficial as well as harmful (Plaa G.L. & Witschi H. 1976).

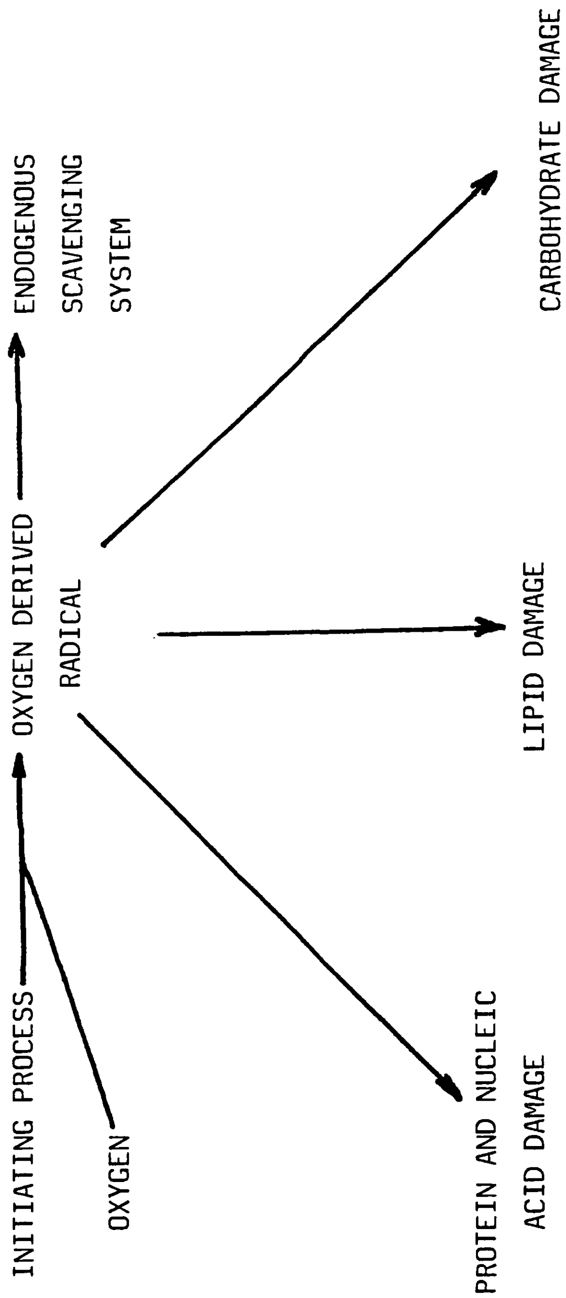


Figure 1.i The Effects of Oxygen Derived Free Radicals on Cellular Macromolecules.

Lipid Peroxidation in Ischaemia and Reperfusion Damage

Lipid peroxidation is the subject of much interest (Clark I.A. et al. 1985, Tribble D.L. et al. 1987). The mechanism of lipid peroxidation (Wills E.D. 1965) in various organ systems such as heart (Roth E. et al. 1985), lung (Torok B. et al. 1986) and artificially produced subcellular fractions (Wills E.D. 1969) has been studied .

Identification of the products of lipid peroxidation is summarised in an article by Esterbauer (Esterbauer H. 1985).

Experimental models have been established to drive lipid peroxidation to a measurable end point to elucidate the peroxidation process (Mak I.T. et al. 1983, Ungemach F.R. 1985).

Interest has been shown in the study of several tissues. Heart (Gardner T.J. et al.1983), neural tissue (Korthals J.K. et al.1985), intestine (Parks D.A. et al. 1983, Koningsberger J.C. et al. 1988), kidney and skeletal muscle have been studied. Recently attention has been given to free radical activity in the liver, not only from the pharmacological or toxicological view (Sugino K. et

al. 1987) but from the standpoint of ischaemia and reperfusion (Adkison D. et al. 1986, Hasselgren P-O. 1987). This could have been spurred on by the advances in liver transplantation and the ability to perform extended liver resections.

Myocardial ischaemia has become a topic of interest in recent years from a free radical viewpoint. Several groups are now researching into the reduction of myocardial damage using free radical scavenging agents [Hearse et. al. 1983, Gardner T.J. et.al. 1983].

Several authors stress the importance of low oxygen tension on lipid peroxidation. De Groot shows a doubling of the amount of MDA* production at an oxygen partial pressure of 10 mm.Hg. over that at 70 mm.Hg. in rat liver microsomes (DeGroot H. & Noll T. 1986). The same Author demonstrates a peak of MDA production at a partial pressure of oxygen of 1 mm.Hg falling as the partial pressure increases in isolated hepatocytes.

The optimum partial pressure of oxygen to promote lipid peroxidation in liver microsomes and hepatocytes is thought to be between 1 and 40 mm.Hg. (DeGroot H. & Noll T. 1985).

* MALONDIALDEHYDE

The Structure of Cellular and Intracellular Membranes

A brief account of the structure and composition of cellular membranes draws attention to the fundamental importance of lipids, especially phospholipids in the maintainance of cellular integrity and membrane fluidity.

Due to the high lipid content of cells, the effect of lipid peroxidation would be devastating if the production of oxygen derived radicals was allowed to continue unchecked by naturally occuring scavengers or was overwhelmed by potent initiator of peroxidation such as ischaemia or damage by toxins.

Biological membranes consist essentially of phospholipid and protein. The structure of cellular membranes was proposed by Darson and Danielli in 1935. It was later refined to form the unit membrane hypothesis by Robertson.

The structure is now taken to be a phospholipid bilayer which measures 50-70 nanometres thick. Hydrophobic hydrocarbon chains are orientated towards the inside of the membrane and the polar groups are orientated towards the outside of the

membrane. The outer layer of the cell is hydrophilic.

The phospholipids in membranes have been shown to move laterally but not from one side of the membrane to the other. This led Singer and Nicholson to propose the fluid mosaic model . The individual movement of lipid molecules gives the cell membrane the essential fluidity required for biological function.

Singer and Nicholson showed that the membrane proteins are arranged in the bilayer in one of two ways. Some proteins appear to reach across both sides of the membrane. Others appear to be embedded in the membrane from either side but do not seem to penetrate.

This penetration difference is thought to be explained by the distribution of polar groups of the amino acids on the outside of the protein. Thus the proteins form a mosaic within the lipid bilayer. The proteins are free to move laterally as are the lipid components.

The arrangement of the proteins is said to determine the permeability of the membrane.

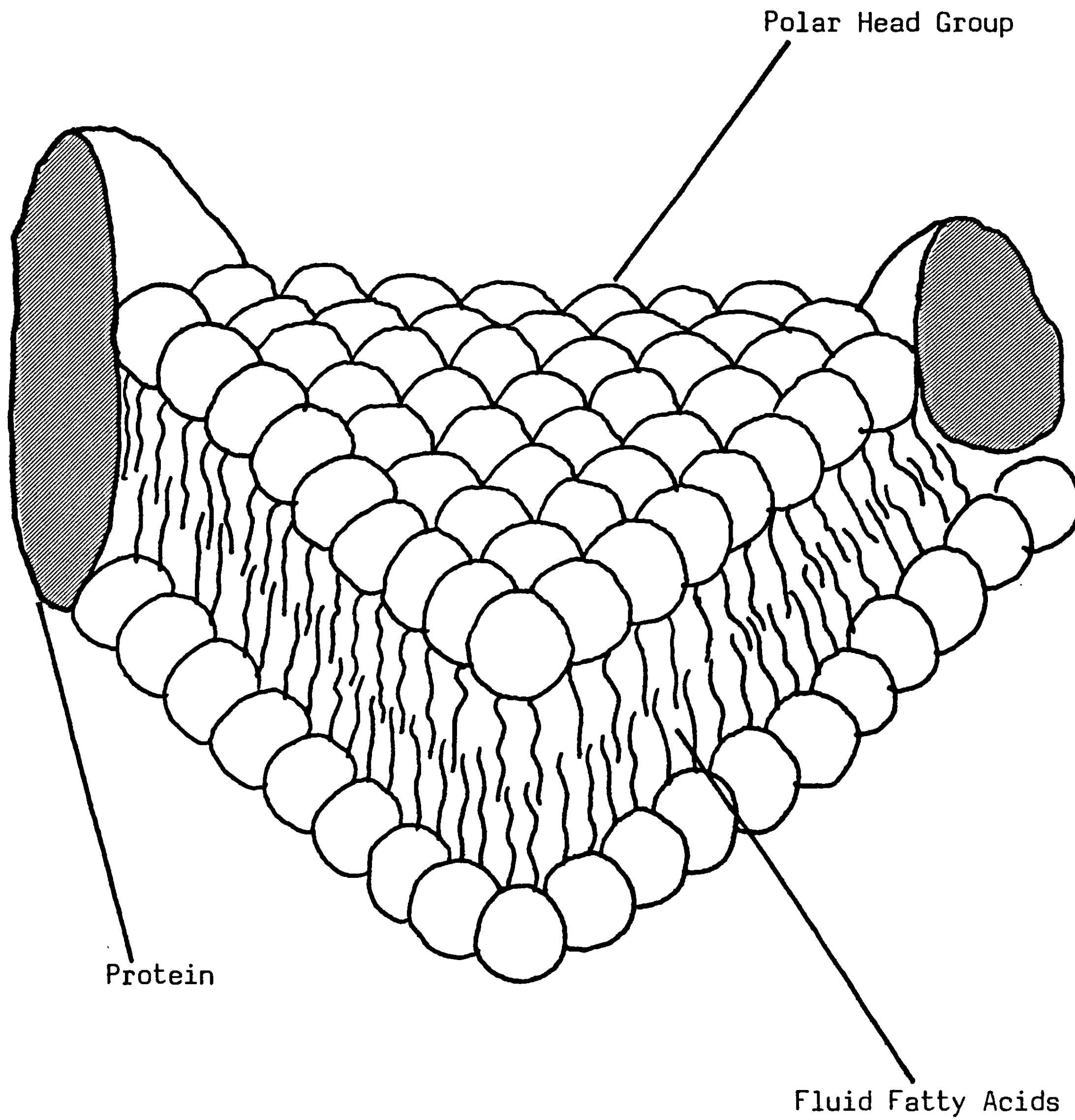


Figure 1.ii The Fluid Mosaic Model of the Structure of Cell Membranes (after Singer & Nicholson 1972, modified)

The Lipid Component of the Plasma Membrane

Most membranes contain between 40% and 50% lipid. However some membranes contain as little as 20% lipid, for example the inner mitochondrial membrane. At the other end of the scale a high lipid content of 75% is found in myelin sheaths around nerves (In Biochemistry Lehninger, Worth Publications 1982). Various unsaturated fatty acids are found in the lipid moiety, usually with an even number of carbon atoms, especially C18, C20 and C22 fatty acids. Slater (Slater T.F. 1984) reported that the C20 and C22 fatty acids are more susceptible to lipid peroxidation.

Free Radical Involvement in Lipid Peroxidation

A free radical mechanism is generally accepted for peroxidation of unsaturated fatty acids (Halliwell B. & Gutteridge J.M.C., 1985. Slater T.F.,1984.).

This has been ascribed to oxygen derived free radicals such as the superoxide radical (Halliwell B., 1981). Several other radicals have been implicated including the hydroxyl, hydroperoxyl and singlet oxygen species.

There is evidence that the superoxide radical may not be as important as first thought (Bors W. et al. 1980). This must be weighed against the majority of Authors work involving beneficial effects of superoxide dismutase (Hansson R. et al. 1983, Atalla S.L. et al. 1985, Im M.J. et al. 1985, Ouriel K et al. 1985, Jadot G. et al. 1986).

Singlet oxygen has been suggested as the initiating radical (King M.M. et al. 1975) and that singlet oxygen could well be derived from the superoxide anion. This in turn could be derived from the auto oxidation of thiols (Misra H.P. 1974), as the reaction of oxygen with iron and sulphur containing proteins (Orme-Johnson W.H. & Beinert H., 1969).

There is conflicting evidence as to the part played by the hydroxyl radical in lipid peroxidation. Koster and Slee (Koster J.F. & Slee R.G. 1980) offered evidence for its involvement, although the hydroxyl radical has been rejected as a component in the initiation of the lipid peroxidation reaction (Morehouse L.A. 1983). It has also been suggested that the peroxy radical is important in initiating peroxidation (Bielski B.H.J. et al., 1983).

Although these radicals are implicated in lipid

peroxidation, it is unclear which species has the major role. It appears certain that the transition metal catalysts especially iron and copper, as described by Wills in the mid 1960s (Wills E.D. 1965), which are involved in chemical functions in most cells are also involved in the generation of free radicals.

Cobalt and manganese have also been implicated as catalysts. These metal ions are able to catalyse reactions which can generate any of the above free radical species especially when in the reduced state. Ascorbic acid is probobly involved in maintaining iron in the divalent state (Wills E.D. 1965).

Many peroxidation products have been identified following free radical activity on lipids. These include aldehydes, ketones, alkenes, alkanes and carboxylic acids. These are produced either alone or in combination. The toxicity produced by lipid peroxidation is thought to be due to either the build up of these products or to disruption of the fatty acid component of the cell membrane (Tseu, 1960), endoplasmic reticulum (Wills E.D. 1971) and lysosomes (Wills E.D. & Wilkinson A.E. 1966). It is shown that lipid peroxidation occurs in dehydrated

systems and the level of tissue water is important (Mouradian R. et al. 1985).

The substrates for lipid peroxidation include unsaturated fatty acids in any form but especially free fatty acids, triglycerides and phospholipids, which are important components of cellular and subcellular membranes. The degree of lipid peroxidation is shown to be proportional to the number of double bonds in the substrate (Schulte-Herbruggen T. & Cadenas E. 1985).

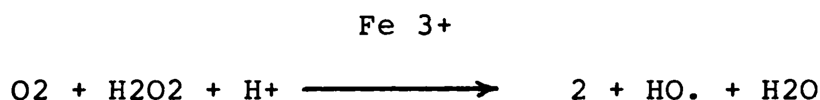
The degree of lipid peroxidation shows species differences. Jordan and Schenkman demonstrate the different rates of malondialdehyde production in liver microsomes in small mammals (Jordan R.A. & Schenkman J.B. 1982).

Experimental work (Esterbauer H. 1982), has shown that the type of lipid peroxidation product depends upon the fatty acid substrate. Membrane phospholipid peroxidation is held to be a rapid process (Wills E.D. 1969), which can be readily shown in vivo and in experimentally produced liposomes (Konings A.W.T. et al. 1979).

A Mechanism For Lipid Peroxidation

Kappus suggested (Kappus H. 1985) that although the superoxide radical is the most predominant in aqueous solution at physiological pH, it does not have the reactivity to abstract a hydrogen atom from an unsaturated fatty acid. It is thought not to be an initiator of the peroxidation cascade. For this reason it is suggested that the perhydroxy radical HO2., which is most active at lower pH but is active enough at physiological pH to initiate lipid peroxidation (Bielski B.H.J. et al., 1983. Gebicki J.M. & Bielski B.H.J. 1981). This work was however carried out in a chemical model and not at physiological pH.

There has been great emphasis on the Haber-Weiss reaction in the peroxidation chain (Gutteridge J.M.C. 1981). This reaction involves transition metal catalysts in which superoxide reacts with hydrogen peroxide and hydrogen ions to yield the highly reactive hydroxyl radical, molecular oxygen and water.



This reaction will not take place without the presence of metal ions. It has been suggested that other transition metals such as copper are able to catalyse reactions (Gutteridge J.M.C. & Wilkins S. 1982).

$$H_2O_2 + Cu^{+} \longrightarrow 2HO. + Cu^{2+}$$

Further production of hydroxyl radicals may result from another iron dependent reaction, the Fenton reaction. This involves the reaction of hydrogen peroxide with ferrous iron and hydrogen ions, to yield the hydroxyl radical, ferric iron ions and water.

$$H_2O_2 + Fe^{2+} + H^{+} \longrightarrow HO. + Fe^{3+} + H_2O$$

Speculation exists as to the reaction between superoxide and hydroxyl radical being involved in the peroxidation process. If this reaction was to proceed, singlet oxygen would be produced.

$$O_2 + OH. + H^{+} \longrightarrow O_2 + H_2O$$

Although singlet oxygen is reactive enough to initiate the reaction, it is not as reactive as the

hydroxyl radical. It is thought that this difference in reactivity would preclude the initiation by singlet oxygen.

The Chain Reaction of Lipid Peroxidation

After the initiation step, a complex chain reaction occurs. This is shown diagramatically in the figures below.

The first stage is thought to be a hydrogen abstraction from an unsaturated fatty acid to yield a lipid radical. This in turn will react with any molecular oxygen present within the system to form the lipid peroxyl radical [LOO.].

This reactive species leads to further chain reaction with lipids and subsequent molecular rearrangement to form the measurable product malondialdehyde (Yagi K. 1982. Kappus H. 1985). This is taken to be an indicator of lipid peroxidation and has been used frequently as an experimental assay (Reiter R. & Burk R.F. 1987).

Other measurable products include dienes, alkanes and other lipid aldehydes such as 4 Hydroxynonenal (Esterbauer H. 1982).

In summary, the peroxidation of lipids is a many stage chain reaction which has commanded much speculation as to its exact course.

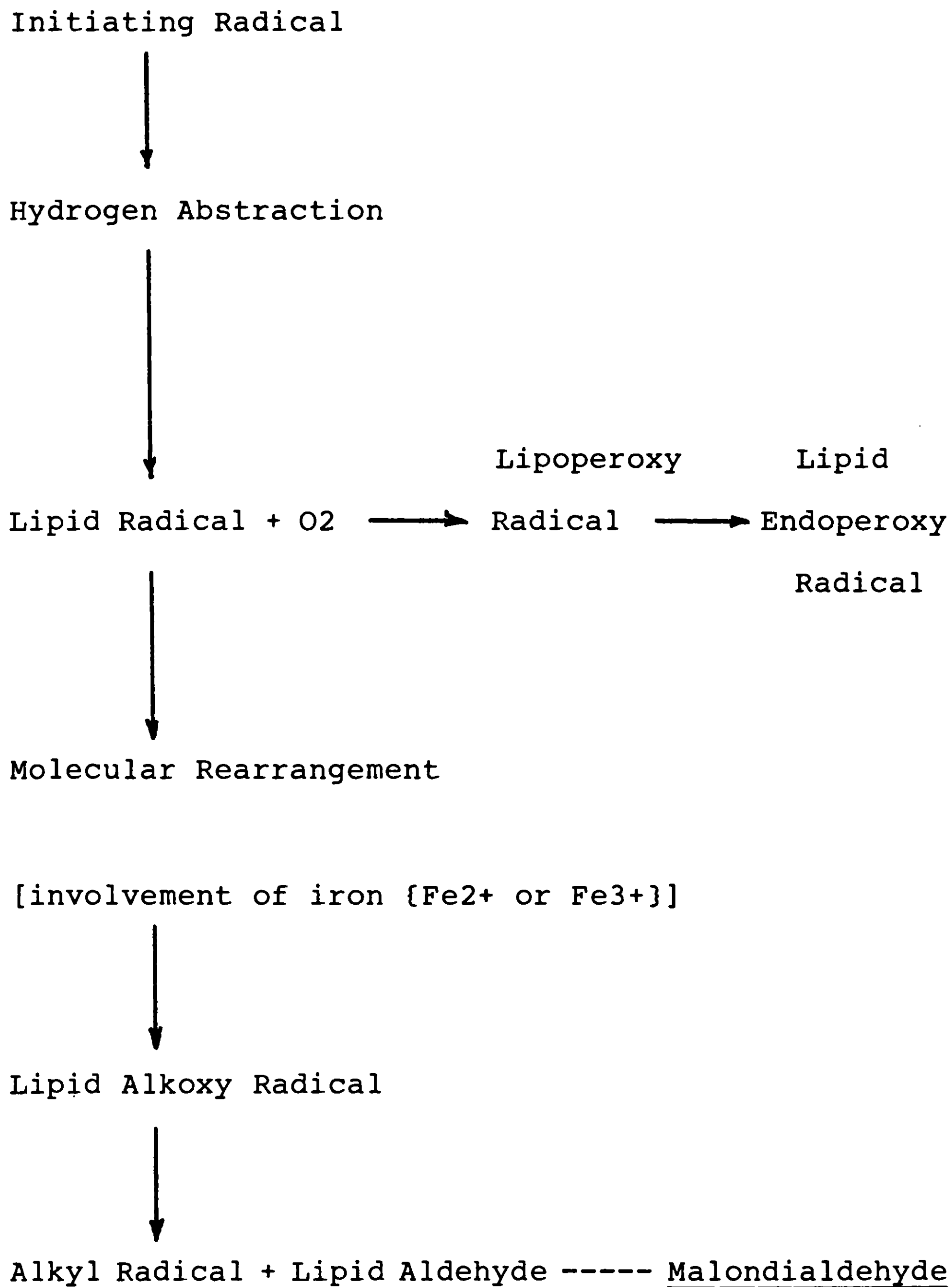
The chain can only be terminated when two radicals react together to form a stable product. If this did not occur, destruction of lipid material within cells would progress, especially in those lipids which are components of the cell membranes. This is because they contain an abundance of unsaturated fatty acids.

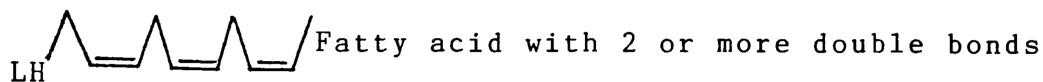
Further Chain Reactions in Lipid Peroxidation

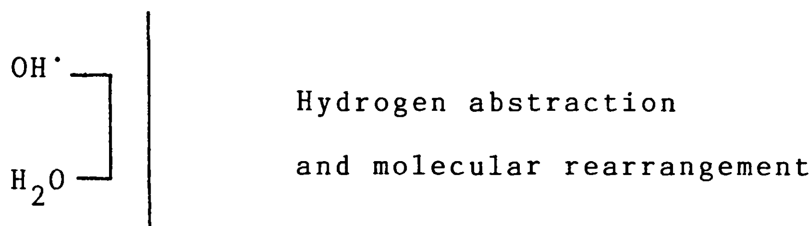
The stages in the progression of lipid peroxidation from the initiation of free radical production to malondialdehyde formation are now considered . It must be emphasised that this is to a great extent speculation drawn from a study of the substances which have been observed in peroxidation models and systems (Halliwell B. & Gutteridge J.M.C. 1984).

A schematic representation of the reactions thought to be involved in lipid peroxidation is shown below.

It shows the sequence of events following the production of the initiating radical from whatever source. The sequence ends with the production of relatively stable intermediates, which are commonly used to demonstrate free radical activity.







Diene
conjugate
LOOH
$O_2^{\cdot -}$
LOO·
LH
(peroxyl
radical)
LO· + OH·
LH
L·
LH
LOO·
LOOH
LH
further
hydrogen
abstraction
LO· + OH^- + Fe^{3+}
LOOH
L·
LOO· + H^+ + Fe^{2+}
pentyl
radical
LOO·
$O_2^{\cdot -}$

$R_1N{=}CH{-}CH{=}CH{-}NHR_2 \leftarrow O{=}CH{-}CH_2{-}CHO$

(Schiff base) (malondialdehyde)

Figure 1.iii Schematic Representation of Lipid Peroxidation.

This reaction can progress in cells in their normal metabolic situation. Usually it is limited by endogenous defence systems.

There are defence systems which interact with the initiation phase. Internal sequestration of transition metals occurs. Ferritin and transferrin sequester iron, thus preventing the Haber-Weiss reaction. Caeruloplasmin may well be a factor in endogenous defence by maintaining iron in the divalent state (Yamashoji S. & Kajimoto G. 1983).

If initiating reactions in the cell occur, endogenous superoxide dismutase will deactivate superoxide (Fridovich I. 1983). Catalase will convert hydrogen peroxide to oxygen and water thus preventing the Fenton reaction producing hydroxyl radicals.

Endogenous substances which can break the chain reaction of lipid peroxidation include vitamin E and coenzyme Q.

The Effect of Lipid Peroxidation on Cells and Tissues

In the previous sections, the chemical events of lipid peroxidation have been described. Evidence regarding the effect on whole cells and tissues suggests that the lipids within membranes are destroyed by lipid peroxidation. This process affects both cellular and subcellular membranes alike. The effect of lipid peroxidation is to alter the fluidity of the membrane and to alter their permeability to solutes (Bridges J.W. et al. 1983. Kunimoto M. 1981. Slater T.F. 1982).

Membrane damage by lipid peroxidation in lysosomes has resulted in the auto digestion of cells due to release of digestive enzymes (Mak I.T.et al. 1983). Damage to endoplasmic reticulum (Plaa G.L. & Witschi H. 1976) and the membranes of mitochondria (Chance B. et al. 1979. Meszaros L. et al. 1982) as a result of lipid peroxidation has been demonstrated.

Cell lysis has been observed when the plasma membrane is damaged during lipid peroxidation. The effects of peroxidation include the inactivation of enzymes within membranes and the inactivation of protein receptors on membranes. The inactivation of

enzymes involved in the respiratory chain within mitochondria would be an example.

The effect of highly reactive oxygen derived free radical species appears to take place locally. The effect of the less reactive radicals however may occur at a distant site. An example quoted is that of malondialdehyde reacting with proteins (Eichenberger K. et al.,1982). There has been suggestion that the damage caused at a site distant from that of production is more likely to be due to metabolic interference than further lipid peroxidation (Moore G.A.et al. 1983). Free radical activity also decreases the pool of free radical scavengers (Kappus H. 1985). This involves cellular anti-oxidants and glutathione, thus making cells and tissues more prone to further free radical damage.

Free Radical Activity Involved in Liver Ischaemia and Reperfusion.

Much work has been performed to elucidate the action of free radicals in diseases of neural tissue, muscle, kidney and heart. Oxygen radicals contribute to ischaemic liver injury (Parks D.A.,

Bulkley G.B., Granger D.N.,1983, McEnroe C.S. et al. 1986).

A microvascular effect of ischaemia has been suggested (Granger D.N., Hollwarth M.E. and Parks D.A., 1986). Capillary filtration increases in the small gut after ischaemia leading to interstitial oedema and accumulation of fluid in the gut lumen. It has been suggested that free radical activity is implicated because superoxide dismutase reduces this ischaemically induced permeability (Granger D.N., Rutili G. and McCord J.M.,1981). Free radicals also increase vascular permeability in hamster cheek pouch. Though no reference is applicable to the ischaemic liver model this vascular permeability may account for early damage on reperfusion by oedema formation.

Could this response be mediated by Histamine, or Prostaglandins? Granger (Granger D.N., Rutili G. & McCord J.M., 1981) concluded that the permeability was not abolished by prior administration of antihistamines, indomethacin or methyl prednisolone. It seems that neither histamine nor the prostaglandins are specifically involved suggesting that free radicals are likely to be involved.

Radical Production in Ischaemia and Reperfusion

Several authorities agree that a pathway involving xanthine oxidase is implicated as the source of oxygen derived free radicals (Granger, Rutili, McCord 1981, Baker G.L. et al. 1985, Adkison D. et al. 1986).

Since:-

1. Xanthine oxidase is experimentally useful for free radical production.

2. Allopurinol offers protection against ischaemic reperfusion injury in myocardial infarction and renal ischaemia (Granger D.N.1981) and is shown to be a hydroxyl radical scavenger (Moorhouse P.C. et al. 1987).

Mechanism

(i) There is catabolism of ATP to hypoxanthine via AMP and Inosine (after Granger D.N. et al, 1981).

(ii) Calcium ingress to cells as a result of decreased activity of the calcium pump (via

ATPase). This calcium ion influx activates the conversion of Xanthine dehydrogenase to Xanthine oxidase via a reduction of NAD+ and a protease enzyme. In the presence of oxygen the reaction between hypoxanthine and xanthine oxidase produces superoxide anions and xanthine. The superoxide anion may then be responsible for production of H_2O_2 and hydroxyl radicals. Calcium ions are shown to increase lipid peroxidation in both normothermic and hypothermic experimental systems (Cotterill L.A. et al. 1988).

Production of Free Radicals in the Liver

The NADPH Cytochrome P450 System

This system is a major electron transfer chain. It occurs on the endoplasmic reticulum and Cytochrome P450 is situated mainly in the liver although small amounts occur elsewhere (Benedetto C. et al. 1981).

The system involves both a cytochrome P450 and NADPH p450 reductase flavoprotein. The phospholipid membrane components would appear to be important to the system. The significance of this

system is in the direct donation of an electron to another molecule from the flavoprotein.

A redox cycling reaction which is known to produce superoxide anion radicals, (Trush M.A.,et al. 1982), is possible.

Lipid peroxidation is shown in vitro to deplete cytochrome P-450, as is chronic iron overload (Cheeseman K.H. et al. 1985). This suggests a role of the cytochrome P-450 system in iron mediated lipid peroxidation.

An important aspect of this reaction is in the activation of toxic substances. Halothane has been implicated in this toxic activation and could well compound damage in a surgical setting of hepatic ischaemia and reflow whilst using halothane inhalation anaesthesia. Xanthine oxidase is present in relatively high concentrations and this mechanism is feasible.

Conversion of Xanthine dehydrogenase is said to require 1 hours ischaemia in the rat liver (Ray & McCord 1983 Conference Summaries).

Suggested Mechanisms of Increased Vascular Permeability

a) This may be due to lipid peroxidation of capillary endothelial cells.

b) This may be due to the degradation of collagen and hyaluronic acid in basement membranes of capillary. (Brawn K. and Fridovich I., 1980.).

c) This may be due to leucocyte chemotaxis and the subsequent release of agents which increase permeability.

Protection Against the Effects of Oxygen Derived Free Radicals and the Prevention of Damage in Ischaemia.

It is suggested that free radical damage will only occur if the radicals are produced in excess of the capacity of the natural scavenging systems.

Once these systems are overwhelmed, free radical damage proceeds unhindered. The consequences of the free radical activities have been explained in previous sections.

Prevention of free radical damage has been attempted in many studies. A multiplicity of agents have been employed in a number of organ systems.

Only a handful of agents however have attained credibility in the literature.

Protective agents against free radical attack and damage may be endogenous i.e. those which occur naturally within cells and tissues. They may also be exogenous, affecting chemical reactions in the free radical cascade but not produced within the cell or tissue.

The major endogenous scavengers take part in enzyme mediated reactions. They comprise various cellular peroxidases, including the glutathione system (Chance B. et al. 1979). Catalase and superoxide dismutase are also involved and have been studied in previous experimental work. The alpha tocopherol component of Vitamin E and coenzyme Q10 (Marubayashi S. et al. 1986) have been administered experimentally as have certain prostaglandins (Sikujara O. et al. 1983).

Exogenous scavenging agents used in experimental studies within the literature include mannitol, desferrioxamine, allopurinol and ATP with magnesium chloride. Each agent has been assigned a beneficial effect in certain organ systems.

Natural Protection Against Free Radical Damage

In order to protect the cell against damage from the excessive activity of free radicals, cells possess a number of chemical systems. There are species differences between these systems although general considerations apply.

Enzymic systems exist in animals, plants and micro-organisms including bacteria and yeasts.

Peroxidase catalysed reactions such as those shown ~~in~~ appear to be almost universally involved in cellular defence.

In animal systems emphasis has been placed on the glutathione peroxidase catalysed reactions which will be described in more detail below.

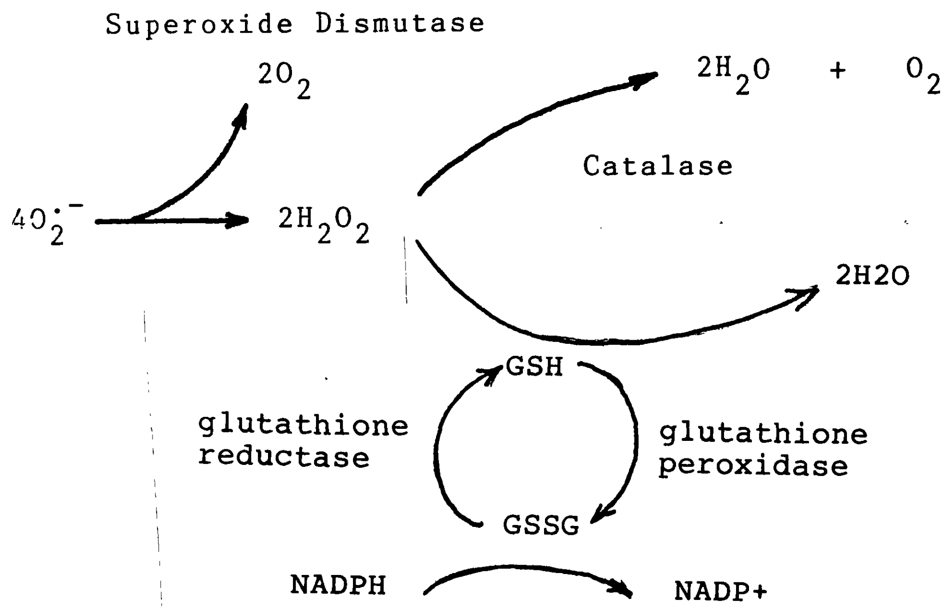


Figure 1.iv The Cellular Enzyme System For Oxygen Radical Elimination

Peroxidase catalysed reactions involve simultaneous oxidation and reduction of the reacting components. Free radicals, with unpaired electrons can be either oxidised or reduced at the expense of substances involved in peroxidation reactions.
In this way free radical damage can be diverted from essential cellular components.

The Importance of the Peroxidase Systems

The peroxidases are involved in oxidation and reduction reactions within animal plant and bacterial cells. They appear to be species specific.
In animal cells the major peroxidase system involves glutathione (Chance B. et al. 1979).

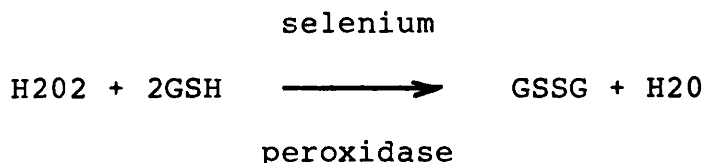
The Glutathione Peroxidase System

The glutathione system was introduced by the discovery of glutathione peroxidase in 1957 by G.C. Mills. Glutathione exists in both oxidised and reduced forms in all animal cells together with the essential enzymes glutathione peroxidase and

glutathione reductase. Glutathione is found in millimolar concentrations but there is a tissue variation. The rat liver has a concentration of 7-8 mmolar glutathione (Halliwell B & Gutteridge J.M.C. 1985).

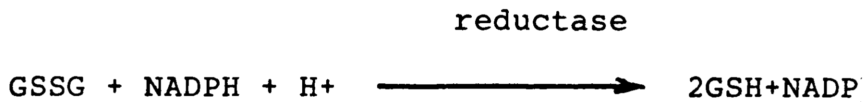
Most of the cellular glutathione exists in the reduced form with a ratio of reduced to oxidised glutathione of 10:1. To maintain this ratio the activity of both glutathione perioxidase and glutathione reductase is high in the rat liver.

The structure of glutathione is of a simple tripeptide. The constituent amino acids are Glycine, Cysteine and glutamic acid. The conversion of reduced glutathione to oxidised glutathione involves a selenium catalysed reaction involving glutathione peroxidase. There is a linkage between two sulphydryl (SH) groups of the amino acid cysteine of reduced glutathione under the influence of the activated oxygen species hydrogen peroxide.



Thus hydrogen peroxide is inactivated by the glutathione system.

Restoration of reduced glutathione to the normal ratio following this reaction is via glutathione reductase involving cellular NADPH.



The rate limiting factor in this reaction is the restoration of NADPH by the pentose phosphate pathway. This is an oxidative reaction which would not be favoured in ischaemia. This leads to an increased production of oxidised glutathione (GSSG) but decreased restoration of reduced glutathione (GSH) unless there is enough available oxygen.

Glutathione peroxidase also acts on lipid peroxides as well as hydrogen peroxide. It is difficult to assess which of these substances is responsible for the major changes in glutathione status in lipid proxidation.

Oxidised glutathione is released from cells during oxidative stress such as ischaemia. A closed experimental system in which there is no redistribution of oxidised glutathione to other tissues is therefore desirable in experimental

studies. In this way an accurate ratio of oxidised to reduced glutathione may be obtained.

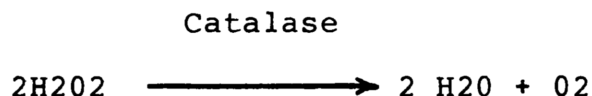
In the rat liver a reduction of total glutathione by 18.3% was recorded after 90 minutes ischaemia (Marubayashi S. et al., 1986) and glutathione peroxidase activity remained unchanged.

Pretreatment of livers with reduced glutathione has not been extensively studied. There is evidence that myocardium can be protected by GSH after ischaemia.

It has been demonstrated (Jennische E., 1984) that liver necrosis is less extensive when high levels of reduced glutathione are present during ischaemia.

Catalase

A protective agent which appears to be important in man is the enzyme catalase (Chance B. et al. 1979). This enzyme occurs within most aerobic cells. It catalyses the reaction to convert hydrogen peroxide to water and ground state oxygen.



$$2H_2O_2 \xrightarrow{\text{Catalase}} 2\,H_2O + O_2$$

This occurs via an intermediate compound. The reaction also requires a ferric iron catalyst. A number of experimental studies have been recorded using catalase either alone or in combination. Indeed catalase is one of the more popular scavenging agents to be used in various systems which lead to free radical production.

Evidence that catalase is an important protector of cells and tissues subjected to free radical damage has been produced in heart (Hearse D.J. & Tosaki A. 1987) kidney and liver (Kupcsulik P. & Kokas P.,1979) under certain conditions. Its probable role in preventing free radical damage is twofold.

Firstly the conversion of hydrogen peroxide. Although not a particularly reactive free radical,it can induce damage to cells. More importantly it can be converted into the much more reactive hydroxyl or peroxy radicals which are thought to be responsible for the initiation of lipid peroxidation (Kappus H., 1985).

Superoxide Dismutase as a Free Radical Scavenging Agent

The first observation of superoxide dismutase occurred in 1930 in England by Mann and Keilin when they discovered a copper containing protein in bovine erythrocytes. They called this protein erythrocuprein.

In 1953 a similar protein was isolated in horse liver which was given the name hepatocuprein. This protein was found to contain zinc as well as copper.

McCord and Fridovich in 1968 showed that the bovine erythrocyte protein removed the superoxide free radical. It was known from then on as Superoxide Dismutase.

Copper-Zinc superoxide dismutase (Cu-Zn SOD) is a highly stable compound which is found in animals, plants and yeasts. It has a molecular weight of 32,000 and is comprised of two protein subunits. It is resistant to chemical degeneration by urea or sodium lauryl sulphate (SDS), and is resistant to heating.
The reaction which is catalysed by superoxide dismutase involves two superoxide radicals.

The zinc is thought to play no part in this reaction but exists to stabilise the enzyme.

Species variation in the efficacy and physical characteristics of superoxide dismutase exist (Jadot G. et al. 1986). In a chemically induced inflammatory reaction involving superoxide radical the superoxide dismutase activity of nine mammalian species is tested (Marklund S.L. 1984). Great variation is identified. Similar differences in superoxide dismutase activity are noted when plant as well as animal enzymes are studied .

Optimum pH for the reaction of various superoxide dismutases also shows species variation. The pH range at which the superoxide dismutase from bovine erythrocytes is effective is wide 5.3 - 9.5 and hence can probably function in many adverse cellular conditions.

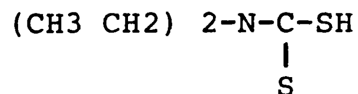
Superoxide is distributed both intra and extracellularly. Studies exist to compare

extracellular with intracellular SOD (Marklund S.L. 1984). They indicate that the cellular form is the more effective.

Apart from species variation and variation dependent on site of action there are isoenzymes in existence.

Together with the major form of Cu-Zn both a manganese superoxide dismutase (Mn-SOD) and iron superoxide dismutase (Fe-SOD) exist in animal, plant and bacterial cells. Mn-SOD constitutes 10% of the superoxide dismutase in rat liver and interestingly is not inactivated by diethyldithiocarbamate, one of the most frequently used inhibitors of Cu-Zn SOD (Heikkila R.E. et al. 1976, Misra H.P. 1979). The FeSOD exists in bacterial cells and plant cells only and hence cannot be implicated in free radical defence involving animal cells.

Denaturation of Cu-Zn superoxide dismutase has been noted to be difficult. Cyanide is effective but for in vivo experimentation is a nonsense. An effective in vivo inactivator of this enzyme is diethyldithiocarbamate (Heikilla R.E. et al. 1976).



Diethyldithiocarbamate binds to the copper at the active sites of the enzymes and actively removes copper from it. In mice, doses of up to 1.5 g/kg have been used to inactivate SOD to reduce the liver activity by 71% in three hours (Heikkila R.E. et al. 1976). It must be noted that high doses can affect other enzymes too.

Non enzymic reactions which protect against free radical damage also take place within cells and tissues. Although several substances have been suggested as endogenous scavengers of oxygen derived free radicals, their significance is far from certain.

Ascorbic acid however, is thought to be an important agent in extracellular protection. Uric acid and some sugars, glucose and fructose (Sagone A.L. et al., 1983) have been implicated as agents in extracellular protection. No specific site of action has been implicated. Hydroxyl radical and singlet oxygen are thought to be disposed of by these extracellular scavengers.

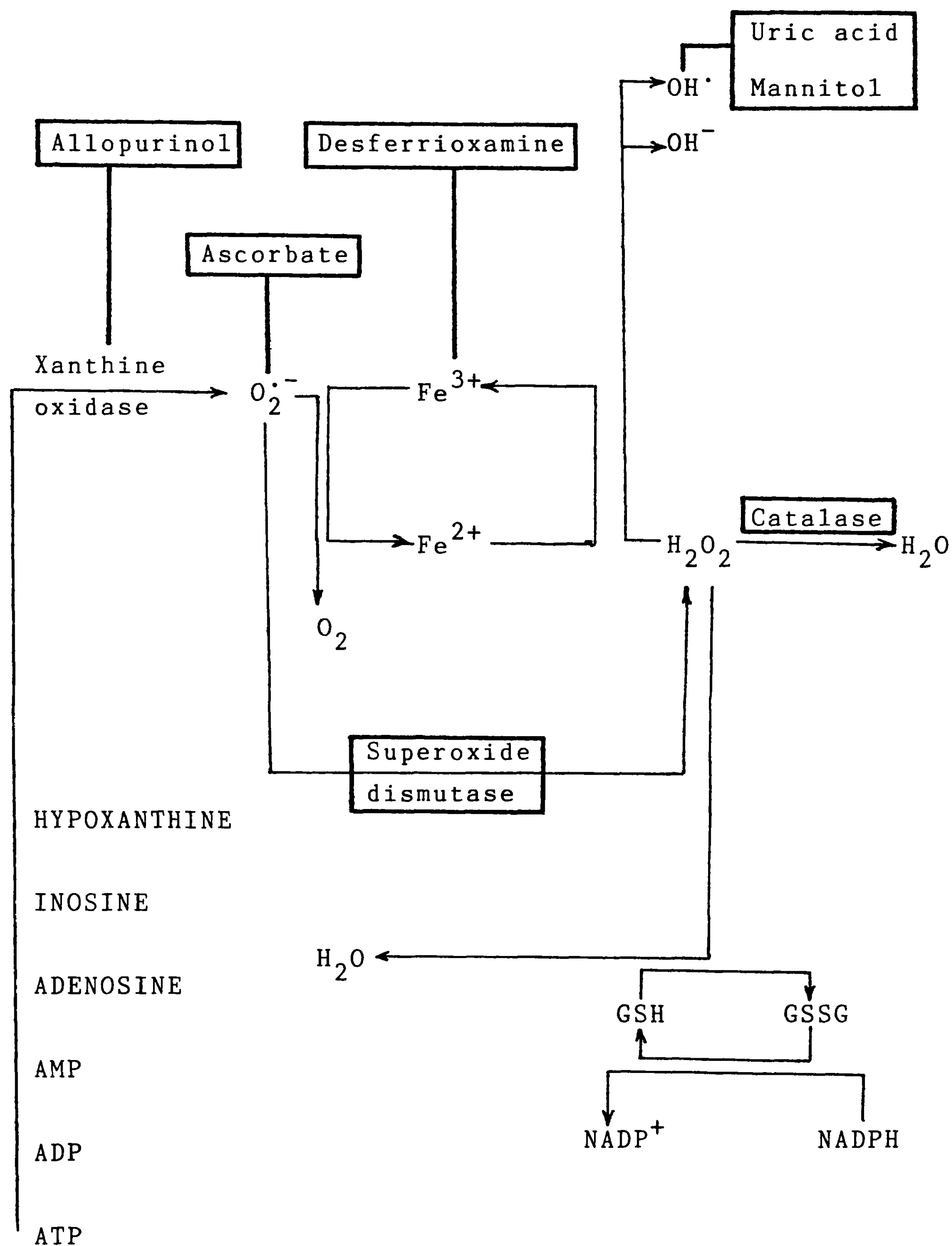


Figure 1.v The Formation of Superoxide and Hydroxyl Radicals: Site of Action of Scavengers (after Granger et al.)

Ascorbic Acid

Can be synthesised in plants and most animals with the exception of man, primates, guinea pig and fruit bat, which rely on a dietary source. Ascorbic acid has a role as cofactor in several enzymic processes. It is also involved in the synthesis of collagen. Ascorbic acid acts as an electron donor (reducing agent) promoting the reaction $Fe3+ \rightarrow Fe2+$.It promotes iron uptake in the gut by maintaining the iron in the reduced form. Ascorbate reacts rapidly with the superoxide, hydroperoxy and hydroxyl radicals (02., H02. and OH.). Ascorbate will also scavenge oxygen.

Ascorbate appears to have its main role as an extracellular antioxidant (Weiss S.J. 1986). The ascorbate must be restored to its original form and the mechanism for conversion back again to ascorbate proceeds via a semidehydroascorbate stage and involves the conversion of $NADH \rightarrow NAD+$.

Uric Acid

In normal plasma concentrations uric acid is a scavenger of hydroxyl radicals and singlet oxygen. Urate can inhibit lipid peroxidation. It is

thought to play a part in the extracellular scavenging system to form the less active peroxyradical.

Sugars

Glucose in vivo and sucrose in vitro scavenges the hydroxyl radical (Sagone A.L. et al. 1983).

Vitamin E

The alpha tocopherol form of this vitamin is thought to be important in antioxidant defence (Marubayashi S. et al. 1986). This substance is fat soluble and hence hydrophobic. Because of this it has a tendency to concentrate in the inner parts of the cellular membrane and this may be important in its defensive role.

The reactions of alpha tocopherol are said to include those with the superoxide anion, singlet oxygen and various lipid peroxy radicals (Marubayashi S et al. 1984). The reaction with lipid peroxy radicals has importance in the interruption of the lipid peroxidation chain. Subsequent reaction between a free radical and alpha

tocopherol yields a vitamin E radical which is relatively unreactive. The chain reaction cannot continue as a result. The Vitamin E radical is probably then converted to Vitamin E by ascorbate (Slater T.F. et al.1985).

In recent years, prostaglandins have been investigated protective agents against free radical damage. Protection of harvested organs for transplantation has been attempted and a synopsis of prostaglandin action is offered by Shaw, (Shaw J.F.L. 1986). Direct experimental findings of the usefulness of certain prostaglandins noteably prostaglandin I2 (PGI2) or prostacyclin on liver ischaemia has been offered by Sikujara (Sikujara O. et al. 1983). Survival of rats subjected to up to 90 minutes of hepatic ischaemia was improved after prolonged infusion of prostaglandin I2. The action is thought to be on platelet aggregation which has led to its use in large animal and human veno veno bypass systems during experimental liver ischaemia.

Exogenous Free Radical Scavenging Agents

Introduction

Many agents have been tested as potential scavenging agents for oxygen derived free radicals. The administration of substances already present in the body has been reviewed in the previous section.

Various chemicals not normally present in the body have been studied in relation to free radical damage in a number of tissues including muscle, neural tissue, kidney and liver. Some agents have a specific site of action for example the xanthine oxidase inhibitor Allopurinol. Others are said to have an effect by acting as a general target for attack. An example of this latter group is mannitol which is used frequently in kidney systems (Zager R.A. et al. 1985, Green C.J. et al. 1986). Mannitol is a substance thought to be acted upon by the hydroxyl radical to effectively reduce free radical action on more essential cellular components. Gelatin polypeptides (Haemaccel) is also effective as a scavenger in liver perfusion experiments (Fuller B.J. et al. 1978).

Specific agents used as scavengers will be described below.

In studies of ischaemia - reperfusion injury to various tissues only a small group of potential scavenging agents have been given serious consideration. The iron chelating agent desferrioxamine, xanthine oxidase inhibitor allopurinol and the osmotic diuretic mannitol have been studied most frequently. The efficacy of sugars, for example glucose (Sagone A.L. et al. 1983), has been investigated when administered in the pre-ischaemic phase.

Replacement of ATP has been studied in combination with magnesium chloride. Chaudry (Chaudry I.H. 1983), and his co-workers have investigated this agent (ATP-MgCl2) extensively in tissue ischaemia with recent application to liver ischaemia-reperfusion-damage (Toledo-Peyrera L.H. 1974).

ATP Magnesium chloride and liver ischaemia/reperfusion

In the early 1980's, a relatively large number of publications appear devoted to the effect of ATP-MgCl2 and liver ischaemia.

There would seem to be a beneficial effect of this agent in ischaemia - reperfusion injury according to

most authors although Hasselgren (Hasselgren P-O. et al. 1982) has offered evidence that there is no beneficial effect. The microcirculation is said to be improved by some authors (Clemens MG et al. 1985) whereas maintenance of calcium gradients by the magnesium concentration has been shown (Ohkawa M. et al. 1983) as a possible beneficial effect.

ATP magnesium chloride pretreatment of livers before ischaemia has been shown to increase the bile production of ischaemic liver tissue in vitro and increase survival time of animals following liver ischaemia in vivo (Kupusculik P. & Kokas P. 1979).

Not only does the ATP-MgCl2 pretreatment improve the above parameters but regeneration of liver tissue following ischaemia is said to be improved.

Increases in hepatic blood flow or reflow following ischaemia of 1 hour are noted over control animals when ATP-MgCl2 is administered (Ohkawa M., Clemens M.G. & Chaudry I.H., 1983).

Mitochondrial functions are impaired when calcium accumulates within this organelle. A fourfold increase in Ca2+ concentration is seen in liver ischaemia of 1 hour (Ohkawa M. et al. 1983 Chaudry I.H. 1983). The restoration of normal calcium

concentration is shown to be improved by the administration of ATP-MGCl2.

The role of Allopurinol in Liver Ischaemia and Reperfusion

It has been mentioned in the recent literature (Hasselgren P.O. et al. 1982) that there are few studies on the beneficial effect of allopurinol in liver ischaemia and reperfusion. The results are conflicting in the three studies identified by him. A further study not mentioned by Hasselgren is noted (Kupsculik P. & Kokas P. 1979). Although the range of identified studies on liver is small, the weight of results indicates a beneficial effect in both phases.

Ischaemia itself is neglected in the studies with most attention being focussed on the reperfusion phase. In studies involving other organs the ischaemic phase does not appear to contribute much to free radical production whereas the reperfusion phase leads to more gross and hence measureable change. Studies in liver ischaemia/reperfusion studies follow suit with the ischaemic phase being apparently neglected.

The ischaemic period, however, represents the initiation of the xanthine oxidase pathway with the degradation of ATP to inosine and hypoxanthine. If enough available oxygen is available in a solid organ, the progression via xanthine oxidase may occur.

Xanthine oxidase is inhibited by allopurinol. Because of this, allopurinol is used for the treatment of hyperuricaemia and gout. The inhibition of xanthine oxidase is theoretically of importance in the reduction of free radical damage to cells and tissues. This is borne out by the few existing studies mainly in the rat model.

Dosage of allopurinol in ischaemia reperfusion studies appears to be far in excess of the therapeutic dose for hyperuricaemia. Doses as high as 50 gm/kg in a single bolus are used in some experiments. To be of use in a clinical situation this fact must be considered. Further points in the use of allopurinol must include the poor solubility of this drug at physiological pH. Alkaline pH is necessary for allopurinol to dissolve before administration. Therefore metabolic disorders may be encountered with large doses administered parenterally.

Desferrioxamine as an antioxidant

Desferrioxamine is a specific chelator of iron in the trivalent ferric form. It will only be effective if iron is unbound. Haemoglobin and ferritin are unaffected by this drug (Halliwell B. 1984). The drug is used primarily for the treatment of iron overload (Ciba Pharmaceuticals 1969). Clinically this is of use when multiple blood transfusion is necessary as this represents a massive infusion of iron. This high iron stress becomes damaging if untreated leading to haemosiderosis.

In the setting of free radical damage caused by ischaemia and reperfusion injury an iron chelator has been shown to have a beneficial effect. Iron has been shown to be a catalyst in both the Haber-Weiss and the Fenton type reactions which generate oxygen derived free radicals (Sies H., Halliwell B. 1985). The available iron is believed to occur in a free iron pool. Desferrioxamine would have a role in the prevention or reduction of these reaction by chelating the iron catalysts (Aust S.D. & White B.C. 1986).

Aust proposes a mechanism for the initiation of lipid peroxidation involving a type of iron complex

which is independent of superoxide, hydrogen peroxide or hydroxyl radicals. Aust also goes so far as to suggest that superoxide dismutase or catalase may initiate lipid peroxidation by altering the redox state of iron.

A specific iron chelator may be more desireable for free radical scavenging therapy. This chelator is desferrioxamine. Further supportive evidence for the usefulness of an iron chelator in liver systems is found in studies of iron overload (Movassaghi N. et al. 1969, Bacon B.R. et al. 1985, Masini A. et al. 1985).

Desferrioxamine acts primarily as a specific iron chelator but it has been suggested that this agent can react directly with both the superoxide and hydroxyl radical will occur not only with desferrioxamine but with the desferrioxamine-iron III complex.

Evidence that Desferrioxamine is an effective scavenging agent in both in vitro and in vivo systems is available (Fuller B.J. & Green C.J. 1986). Its role initially appeared to be straight forward in the chelation of iron III. The action of desferrioxamine may be more complex than this however.

The Aim of the Thesis

Thus there appears to be confusion in relation to the relative damage produced by free radicals during the ischaemic and reperfusion phases. Some authors say that most damage occurs on reperfusion, because this is when oxygen is supplied to the ischaemic tissue.
However some say that free radical damage can occur at low oxygen tensions (Noll T. & DeGroot H. 1984). Also that the products of this damage might act as chemotactants for neutrophil accumulation thus leading to further damage.

This thesis describes an attempt to study the effect of ischaemia in rat livers.

To test the hypothesis that lipid peroxidation due to free radical activity occurs in the ischaemic phase of the normothermic ischaemia/reperfusion mechanism, rat livers will be subjected to periods of normothermic ischaemia.

The changes in lipid peroxidation products malondialdehyde and conjugated dienes during ischaemia will be measured together with the changes in oxidised and reduced glutathione.

The changes in metabolic synthetic function during normothermic ischaemia will also be studied by measuring urea production.

The effect of free radical scavenging agents on both aspects will be assessed.

CHAPTER 2

EXPERIMENTAL STUDIES

2.1 Experimental methods used in the study

a. Methods used in lipid peroxidation studies

b. Method of preparation and administration of free radical scavenging agents

c. Methods used in metabolic studies

d. Statistical Analysis of Results

EXPERIMENTAL METHODS

Introduction

The experimental work in this thesis is divided into several parts.

The first consists of studies to determine free radical activity in the normothermic ischaemic rat liver by the measurment of two lipid peroxidation products, malondialdehyde and conjugated dienes over ischaemic periods of 180 minutes.

The levels of both oxidised and reduced glutathione are measured, together with the ratio of oxidised/reduced glutathione, during ischaemia.

The second section determines the effect of specific free radical scavenging agents on these parameters over the same ischaemic periods.

In the final section, the effect of normothermic ischaemia on a synthetic function in rat liver, urea production, is studied for ischaemic periods up to 180 minutes. The effect of the same free radical scavengers on urea production is determined.

The following chapter details the experimental procedures used in this study.

2.1a Lipid peroxidation in the isolated, warm, ischaemic rat liver

This section deals with the production and measurement of lipid peroxidation products in the rat liver during normothermic ischaemia, which in turn may reflect damage due to free radical activity. The experimental model used in this study is described below, together with a description of the indices of lipid peroxidation measured.

The experimental model

Male outbred Sprague-Dawley rats weighing between 200-250 grammes were used for the study. They were allowed normal diet and free access to water.

In these studies, all drugs, including an isotonic saline control were administered intravenously via the lateral tail vein. The rats were lightly anaesthetised with ether inhalation. This prevented rat movement before and during the injection and made restraint unnecessary.

Vein puncture was performed using a 25g needle and confirmation of entry was made by withdrawal of blood.

The volume of injection was standardised at 1ml per kilogramme given 15 minutes before the induction of anaesthesia for excision of the liver.

The rats were re-anaesthetised by ether inhalation and a total hepatectomy was performed by dividing all the hepatic ligaments, then sectioning the portal vein, bile duct and hepatic artery at the porta hepatis. The superior and inferior venae cavae were then sectioned and the liver was then withdrawn dividing any posterior attachments.

A specimen of freshly excised liver weighing approximately 0.5 grammes was taken from a liver lobe immediately after hepatectomy. This was placed in an iced petri dish prior to processing for the initial assay.

The remainder of the excised liver was then maintained at 37 degrees celsius in a closed petri dish which was kept moist by gauze soaked in isotonic saline.

Livers were maintained for up to 180 minutes, with 0.5 gramme tissue specimens being taken from the liver at time 15, 30, 45, 60, 120 and 180 minutes from the onset of ischaemia.

The liver specimens were immediately cooled on ice, weighed and homogenised.

Homogenisation

Weighed samples of rat liver were placed in a 15 mm. internal diameter homogenisation tube which had been pre cooled. Iced Dulbecco A phosphate buffered saline, was then added to reach a volume of 10ml. The samples were homogenised in the tube using a teflon homogenisation plunger attached to a commercial laboratory motor.

Lipid peroxidation in the control animal

Lipid peroxidation products were determined in the samples of homogenised liver taken from saline treated animals. The values of lipid peroxidation products and their variation with the time of ischaemia was established as a control group.

Two products were measured, **malondialdehyde** and **conjugated dienes.** The values of **oxidised and reduced glutathione** were also determined. The ratio of oxidised to reduced glutathione was calculated and the value termed the **glutathione ratio** was obtained.

2.1b Studies to determine the effect of free radical scavenging agents on lipid peroxidation

Introduction

In this series of experiments the effect of free radical scavenging agents on the products of lipid peroxidation was studied.

The scavenging agents used were:-

a) **MANNITOL-** This agent is traditionally used as a scavenger for the **hydroxyl anion radical.** The subsequent mannitol radical is far less reactive than the hydroxyl radical originally scavenged.

b) **DESFERRIOXAMINE-** This agent is an **iron chelator** used in the therapy of iron overload. It is thought

to act by chelating the iron catalyst in the Haber Weiss reaction (Haber & Weiss).

c) **ALLOPURINOL**-Is an agent inhibiting **xanthine oxidase** which may be responsible for promotion of the reaction which forms the superoxide radical.

d) **SUPEROXIDE DISMUTASE**- This enzyme is specific for the inactivation of the **superoxide radical.** It exists naturally and is probably responsible for cellular protection in the endogenous production of superoxide.

Studies to determine the effect of different doses of scavengers were also carried out for Desferrioxamine in doses of 5 to 15mg/kg., doses of Allopurinol 5 to 10mg/kg. Superoxide dismutase 500, 1000 and 10000 units/kg.

Theoretically superoxide dismutase, being an enzyme, could be denatured. This in practice is a difficult task. Heating does not completely inactivate the enzyme (own work). In vitro cyanide will abolish the action of the superoxide dismutase but in vivo this is not a feasible proposition.

Denaturation of SOD is possible with **diethyl dithio carbamate** in vivo (Heikkila R. E. 1976, Misra H. P. 1979).

Denaturation was therefore carried out using a dose of 10000u/Kg superoxide dismutase to which was added 1mg diethyldithiocarbamate/1000 units mixed 5 minutes before administration. The results were compared with the control and the 10000 units SOD/kg series.

Materials and methods

The method of excision and preservation of the liver followed that described in 2.1a. The ischaemic periods studied were identical with those of the untreated animal.

The free radical scavenging agents were substituted for the control injection of 0.9% sodium chloride. The solutions were freshly prepared and formulated so as to give the final injection volume of 1ml/Kg. This was given as a slow injection over one minute.

Administration of the agents took place 15 minutes before induction of anaesthesia for the hepatectomy.

The experimental groups all consisted of ten liver specimens.

Table of free radical scavenging agents used in the study together with the dosage regimes employed

AGENT	DOSE(S)	ADMINISTRATION
CONTROL 0.9% Sodium Chloride		1ml into tail vein 15 minutes prior to ischaemia.
MANNITOL	1g/kg	Made up to 1ml in sterile water injected into tail vein 15 minutes prior to ischaemia.
DESFERRIOXAMINE	5mg/kg 15mg/kg	Made up to 1ml in sterile water injectedintotail vein 15 minutes prior to ischaemia.

AGENT	DOSE(S)	ADMINISTRATION
ALLOPURINOL	5mg/kg 10mg/kg	Made up to 1ml in sterile water injectedintotail vein 15 minutes prior to ischaemia.
SUPEROXIDE DISMUTASE	10000u/kg 1000u/kg 500u/kg	Made up to 1ml in sterile water injected into tail vein 15 minutes prior to ischaemia.
SUPEROXIDE DISMUTASE	Denatured	Denaturation by adding 10mg DEDTC to 10000u/kg and mixing for 10 minutes prior to administration.

2.1c Experimental method for metabolic studies involving synthesis of urea in rat liver

The experimental model followed that described in section 2.1, with the following modification.

Samples of liver were then taken 60, 120 and 180 minutes after hepatectomy. These time periods corresponded to the maximum changes in lipid peroxidation products and changes in glutathione seen in the previous section.

Preparation of specimens

Liver samples were lightly blotted and slices of thickness 0.4 millimetres were produced using a McIlwain automated tissue chopper. Samples of 0.2 grammes of the slices were weighed. They were then separated in chilled Krebs Ringer Bicarbonate solution at pH 7.4 to 7.6.

The slices were then tested for metabolic activity as follows:-

Metabolic Studies

Weighed slices were transferred to chilled glass incubation vessels containing 4 millilitres of a medium consisting of Krebs Ringer Bicarbonate containing ammonium chloride (4 millimoles/litre), ornithine hydrochloride (10 millimoles/litre) and d-l lactic acid (20 millimoles/litre).

The medium was equilibrated with a gas mixture of 95% oxygen and 5% carbon dioxide by bubbling through a glass pasteur pipette for one minute prior to addition of the slices.

The reaction vessels were then regassed for a further minute, stoppered and incubated at 37 degrees celsius for two hours in an agitator at 60 agitations per minute.

At the end of the incubation period the vessels were plunged into ice to halt the reaction. The medium in the vessel was decanted and stored at -20 degrees celsius until the biochemical estimation of urea was performed.

Urea estimation was performed using the colorimetric assay method of Berthelot as described in Appendix 1.

0.2g wet weight sample of slices was obtained after pre-blotting. Wet to dry weight ratios were calculated by a known wet weight of liver being heated to constant weight in a pre-weighed container at 100 degrees celsius.

This allowed the urea production to be calculated per gramme dry weight of liver.

2.1d Statistical Analysis

All data was subjected to analysis by Students unpaired "t" test.

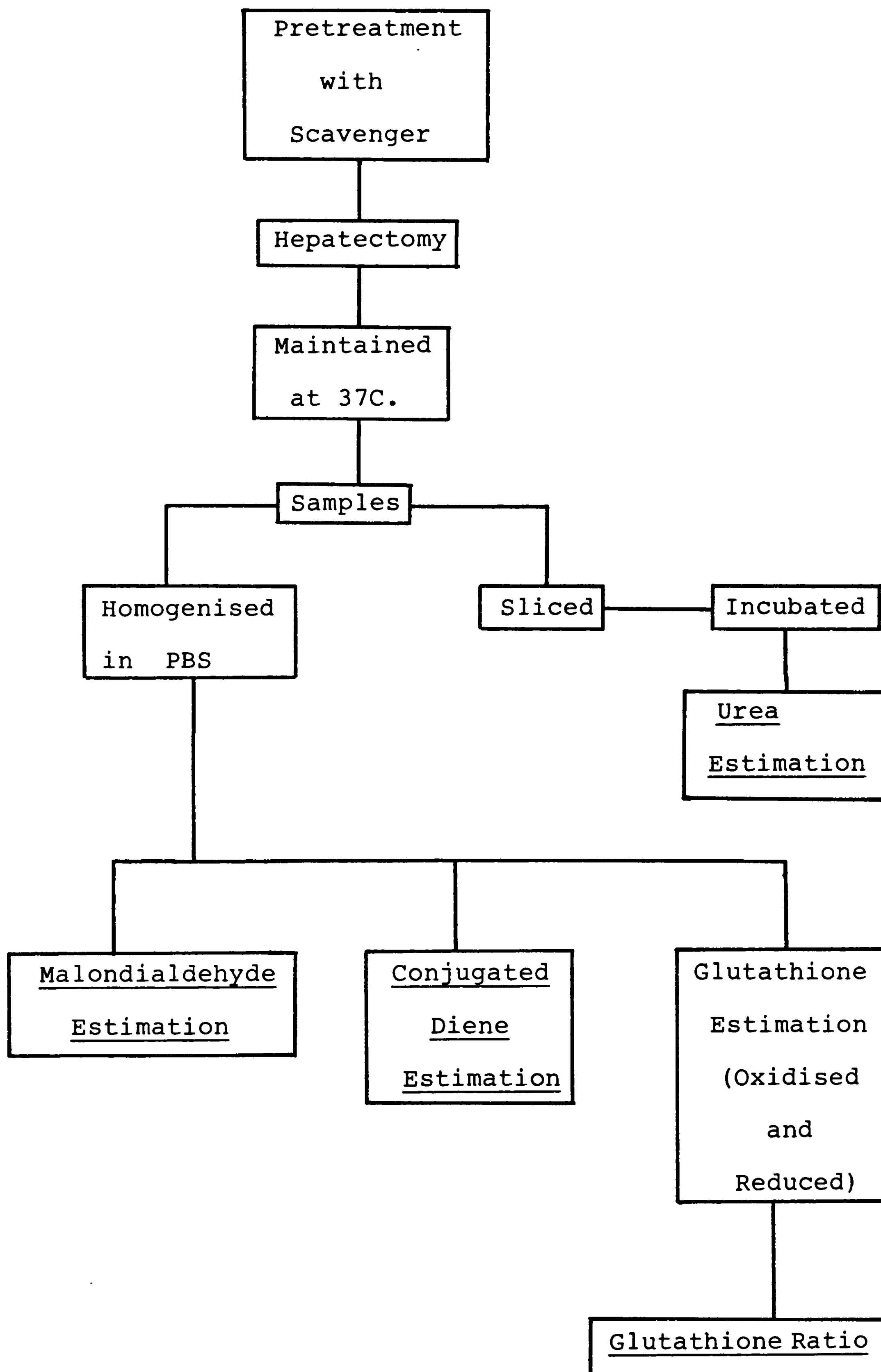


Figure 2.i Flow Diagram of Method for Ischaemia Studies in Rat Liver.

Timing of Samples

Time (min)	Saline Control	Superoxide dismutase (u/Kg) 500	1000	10000	Denatured
0	+	+	+	+	+
15	+	+	+	+	+
30	+	+	+	+	+
45	+	+	+	+	+
60	+	+	+	+	+
120	+	+	+	+	+
180	+	+	+	+	+

Time (min)	Allopurinol (mg/Kg) 5	10	Desferrioxamine (mg/Kg) 5	15
0	+	+	+	+
15	+	+	+	+
30	+	+	+	+
45	+	+	+	+
60	+	+	+	+
120	+	+	+	+
180	+	+	+	+

Figure 2.ii. Table showing the sampling regime for the rat liver during normothermic ischaemia.

Timing of Samples

Time (min)	Mannitol 1g/Kg	Urea estimation
0	+	+
15	+	-
30	+	-
45	+	-
60	+	+
120	+	+
180	+	+

Figure 2.iii Table to show the sampling regime for studies on the rat liver during normothermic ischaemia.

CHAPTER 3

RESULTS

THE EFFECT OF NORMOTHERMIC ISCHAEMIA ON THE ISOLATED RAT LIVER

3.1 Effect of ischaemia on lipid peroxidation

3.2 Effect of ischaemia on metabolism

3.1 THE EFFECT OF NORMOTHERMIC ISCHAEMIA ON THE ISOLATED RAT LIVER

Introduction

In this chapter the effect of normothermic ischaemia on the rat liver was demonstrated. The experimental results were divided into two sections. The first demonstrated the effect of ischaemia on lipid peroxidation.
Measurement of malondialdehyde and conjugated diene level was perfomed over an ischaemic period of 180 minutes. Sampling times were at 15, 30, 45, 60, 120 and 180 minutes together with a sample of freshly excised liver.
Reduced and oxidised glutathione levels were measured at the same time points and the ratio of oxidised to reduced glutathione was calculated as an overall indicator of glutathione status.

In the second part of the chapter the effect of ischaemia on the synthesis of urea was studied. Samples were taken over an ischaemic period of 180 minutes.

Method

The experimental method used in the studies and the assay methods are shown in Chapter 2. and Appendix 1. respectively.

Experimental results

a. Changes in malondialdehyde production during normothermic ischaemia

Freshly excised rat liver tissue gave a baseline value for malondialdehyde of 118.35 $\pm$ 21.49 nmoles/g (n=10). When rat liver was subjected to an ischaemic period of 15 minutes the value for malondialdehyde increased to 123.97 $\pm$ 18.01 nmoles/g (n=10). For progressively longer ischaemic periods malondialdehyde increased to 128.99 $\pm$ 17.86 nmoles/g (n=10) at 30 minutes, 138.46 $\pm$ 25.59 nmoles/g (n=10) at 45 minutes and 143.01 $\pm$ 22.18 nmoles/g (n=10) at 60 minutes. The increase in value of malondialdehyde continued reaching 154.09 $\pm$ 24.57 nmoles/g (n=10) at 120 minutes and 168.82 $\pm$ 24.95 nmoles/g (n=10) at 180 minutes.

The mean ratio

The variation in the values for malondialdehyde between rats at any one time period was

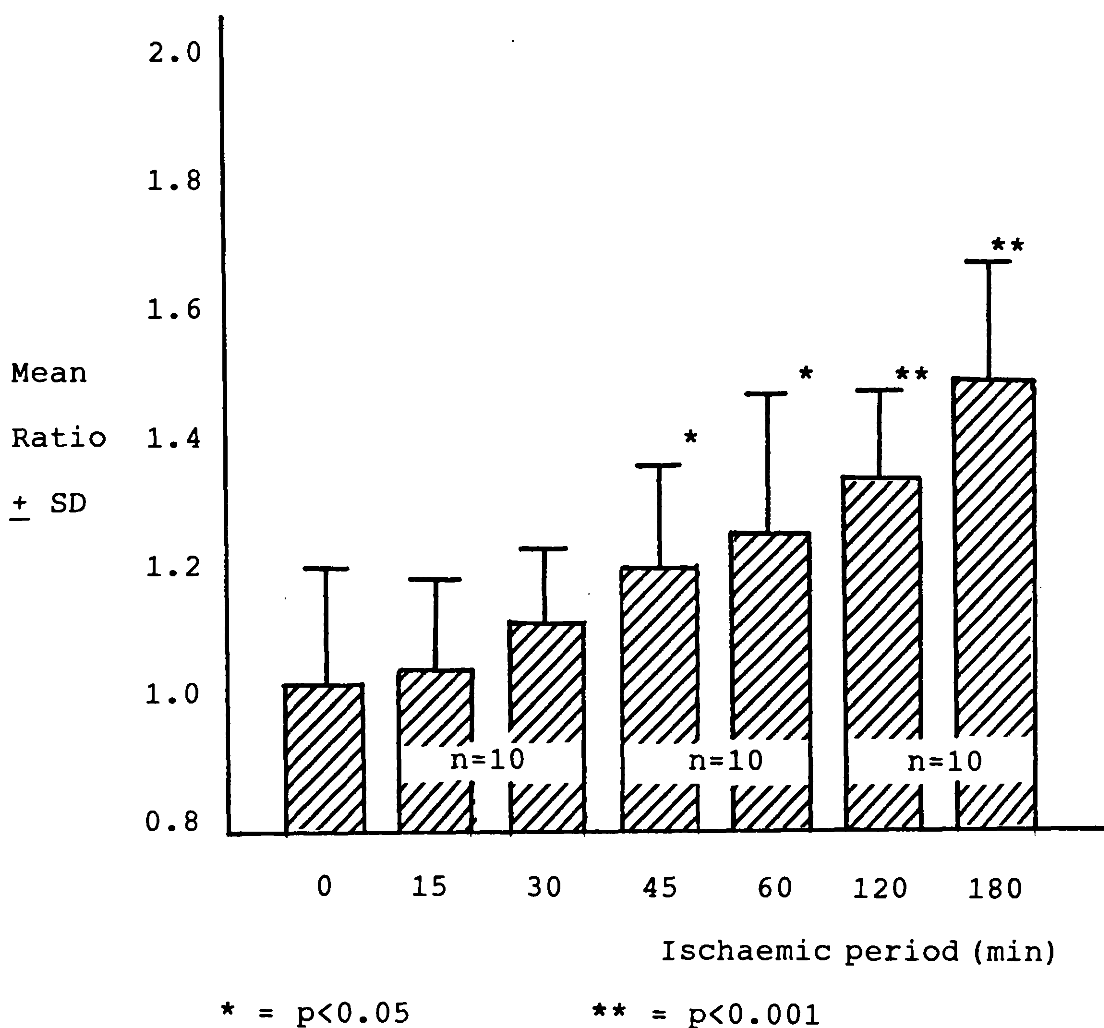


FIGURE 3.1i Graph to show the effect of normothermic ischaemia on malondialdehyde production in rat liver. The level of malondialdehyde increased significantly during progressive ischaemia. At 45 and 60 minutes a significance of $p<0.05$ was reached whilst that at 120 and 180 minutes was $p<0.001$.

considerable, leading to the relatively high standard deviations shown above. To compensate for this variation, each rat was considered separately.

A ratio of the value at a particular time period i.e. 60, 120 or 180 minutes, with that of the freshly excised liver was calculated. The mean value obtained from all rats in the group was termed the MEAN RATIO.

Taking the time zero value for the mean ratio as 1 the following results were obtained. The freshly excised liver had a value of 1.00 ± 0.17 (n=10). After 15 minutes of normothermic ischaemia the mean ratio was 1.01 ± 0.14 (n=10) which was not significant. At 30 and 45 minutes the ratios were 1.11 ± 0.11 (n=10) and 1.19 ± 0.15 (n=10). After 60 minutes of ischaemia the ratio had increased to 1.24 ± 0.21 (n=10) and to 1.32 ± 0.16 (n=10) after 120 minutes. The mean ratio at 180 minutes reached 1.45 ± 0.19 (n=10).

Significant increases in mean ratio of malondialdehyde production were seen after 45, 60 ($p<0.05$), 120 and 180 minutes ischaemia ($p<0.001$).

b. Changes in conjugated diene level during normothermic ischaemia

Freshly excised rat liver gave a baseline value for conjugated dienes of 8.61 ± 1.02 fluorescence units (fu)/g (n=10). At 15 minutes the value was 8.23 ± 1.02 fu/g (n=10), at 30 minutes 8.32 ± 1.52 fu/g (n=10) and at 45 minutes 7.90 ± 1.10 fu/g (n=10). After 60 minutes the value was 7.51 ± 1.37 fu/g (n=10), after 120 minutes 7.25 ± 1.44 fu/g (n=10) and after 180 minutes 6.84 ± 1.51 fu/g (n=10).

Again the mean ratios were calculated to compensate for the variation between rats. The baseline mean ratio was 1.01 ± 0.11 (n=10). Those at 15 and 30 minutes were 0.96 ± 0.07 (n=10) and 0.97 ± 0.09 (n=10) respectively. At 45 minutes the ratio decreased to 0.92 ± 0.10 (n=10) at 60 minutes it was 0.88 ± 0.14 (n=10) and at 120 minutes was 0.85 ± 0.12 (n=10). At 180 minutes of ischaemia the diene mean ratio was 0.73 ± 0.25 (n=10).

The trend was a decrease in the level of conjugated dienes with progressive normothermic ischaemia. The decrease achieved statistical significance at 60 minutes ($p<0.05$), 120 and 180 minutes ($p<0.01$).

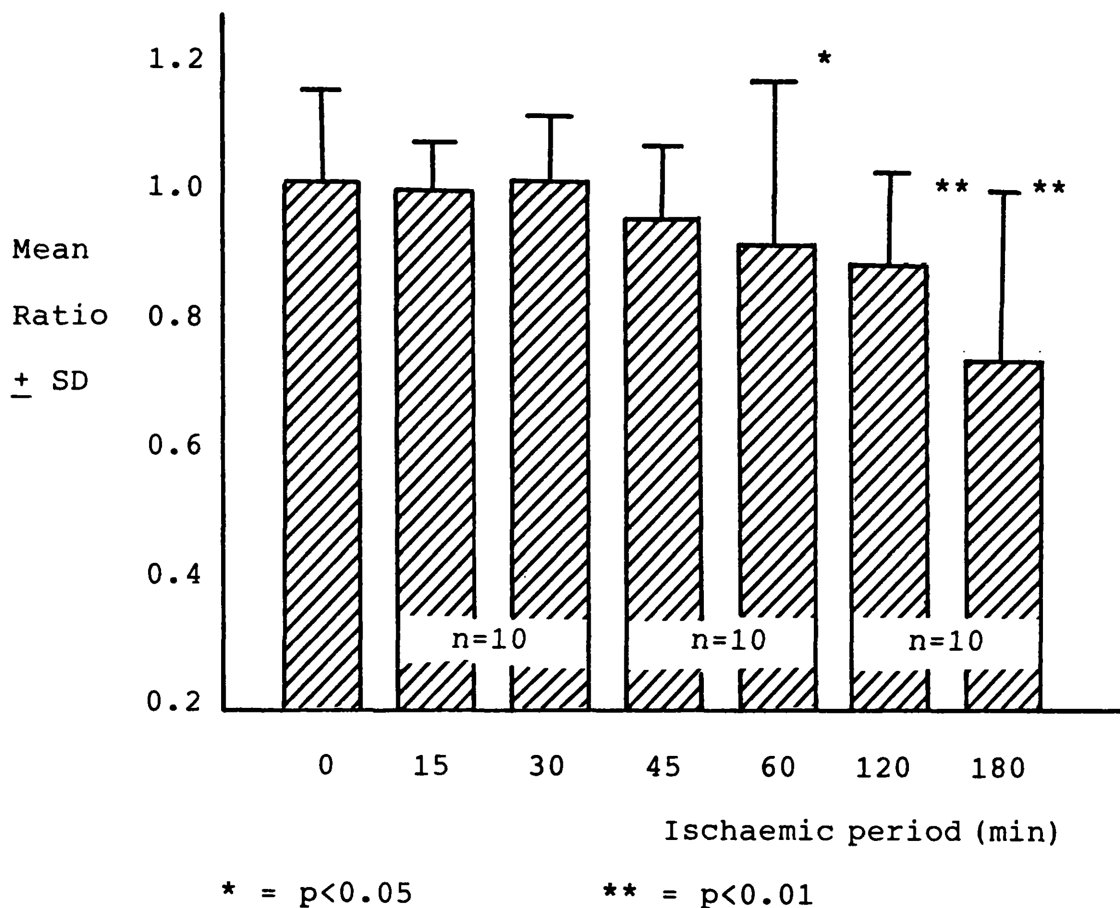


FIGURE 3.1ii Graph to show the effect of normothermic ischaemia on conjugated diene production in rat liver. The level of conjugated dienes decreased significantly during progressive ischaemia. At 60 minutes a significance of $p<0.05$ was reached whilst that at 120 and 180 minutes was $p<0.01$.

c. Changes in reduced glutathione during normothermic ischaemia

The freshly excised rat liver had a reduced glutathione level of 6.02 ± 0.87 nmoles/g (n=10). At 15 minutes ischaemia the level reached 6.03 ± 0.80 nmoles/g (n=10). With progressive ischaemia the level decreased. At 30 and 45 minutes the levels were 5.33 ± 1.18 nmoles/g (n=10) and 5.38 ± 1.11 nmoles/g (n=10) respectively. The level at 60 minutes was 5.14 ± 1.00 nmoles/g (n=10), that at 120 minutes was 4.25 ± 1.14 nmoles/g (n=10) and at 180 minutes reached 3.90 ± 1.13 nmoles/g (n=10).

The mean ratio of the freshly excised rat liver was 1.00 ± 0.14. At 15 minutes it was 1.03 ± 0.20 and at 30 minutes was 0.89 ± 0.15. At 45 minutes the ratio reached 0.91 ± 0.22. By 60 minutes the ratio was 0.86 ± 0.16. At 120 and 180 minuts the ratio had fallen to 0.71 ± 0.13 and 0.65 ± 0.12 respectively.

There was an overall decrease in reduced glutathione level which achieved significance at 120 and 180 minutes of normothermic ischaemia ($p<0.001$).

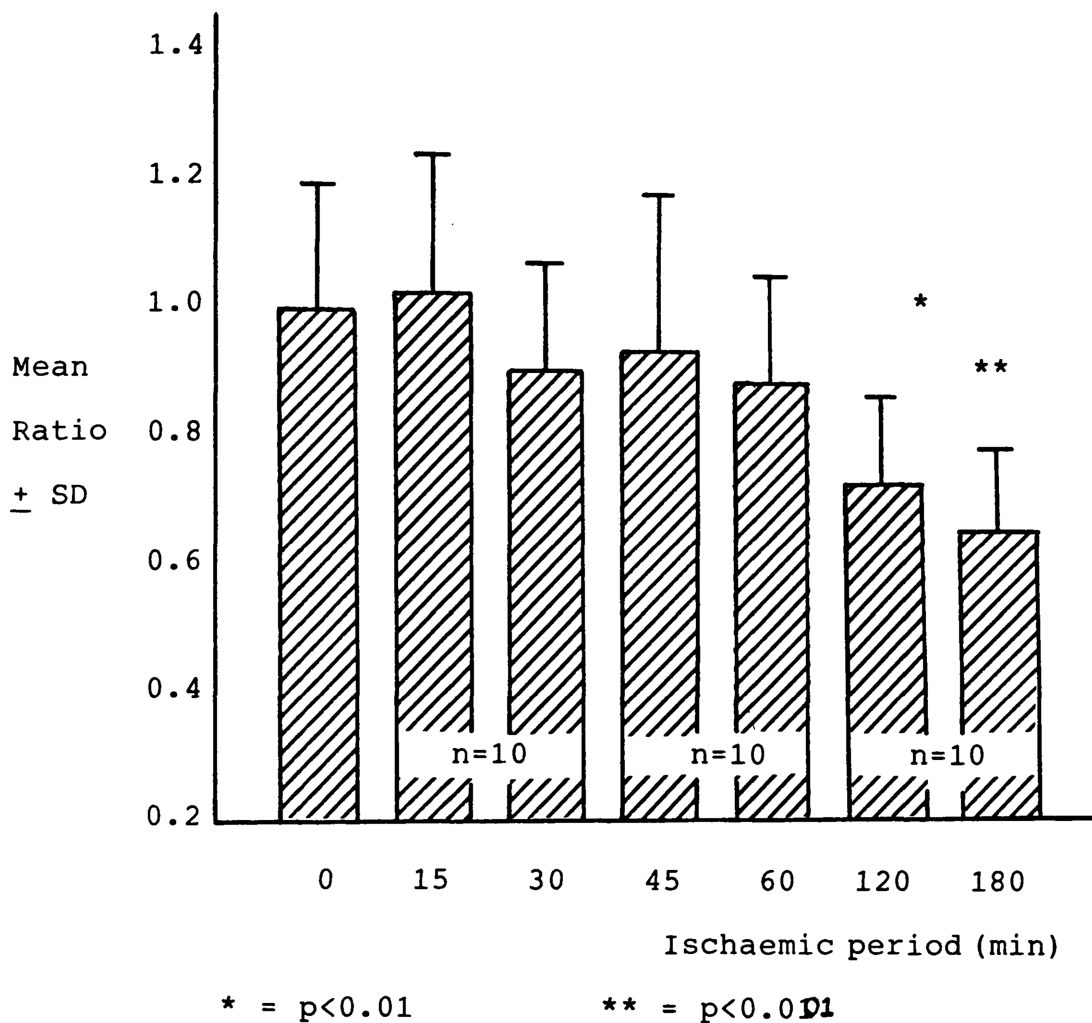


FIGURE 3.1iii Graph to show the effect of normothermic ischaemia on reduced glutathione level in rat liver.

The level of reduced glutathione decreased progressively with ischaemia achieving significance at 120 minutes ($p<0.01$) and 180 minutes ($p<0.001$).

d. Changes in oxidised glutathione during normothermic ischaemia

The oxidised glutathione level progressively rose with ischaemia. The time zero value was 2.15 ± 0.28 nmoles/g (n=10), whilst that at 15 minutes was 1.88 ± 0.57 nmoles/g (n=10). At 30 minutes the level was 2.21 ± 0.48 nmoles/g (n=10), rising to 2.46 ± 0.35 nmoles/g (n=10) at 45 minutes. At 60 minutes the level reached 2.54 ± 0.48 nmoles/g (n=10). At 120 and 180 minutes the levels had risen to 3.22 ± 0.72 nmoles/g (n=10) and 3.58 ± 0.66 nmoles/g (n=10) respectively.

The mean ratio indicated an overall rise. The time zero value was 1.00 ± 0.12 (n=10). The ratios at 15, 30 and 45 minutes were 0.94 ± 0.07 (n=10), 1.03 ± 0.12 (n=10) and 1.15 ± 0.12 (n=10). At 60 minutes the ratio was 1.18 ± 0.14 (n=10) whilst that at 120 minutes was 1.50 ± 0.25 (n=10). At 180 minutes the mean ratio had risen to 1.67 ± 0.21 (n=10).

Oxidised glutathione rose with progressive ischaemia. A significant increase over the time zero value was achieved at 60 minutes ($p<0.01$), 120 and 180 minutes ($p<0.001$).

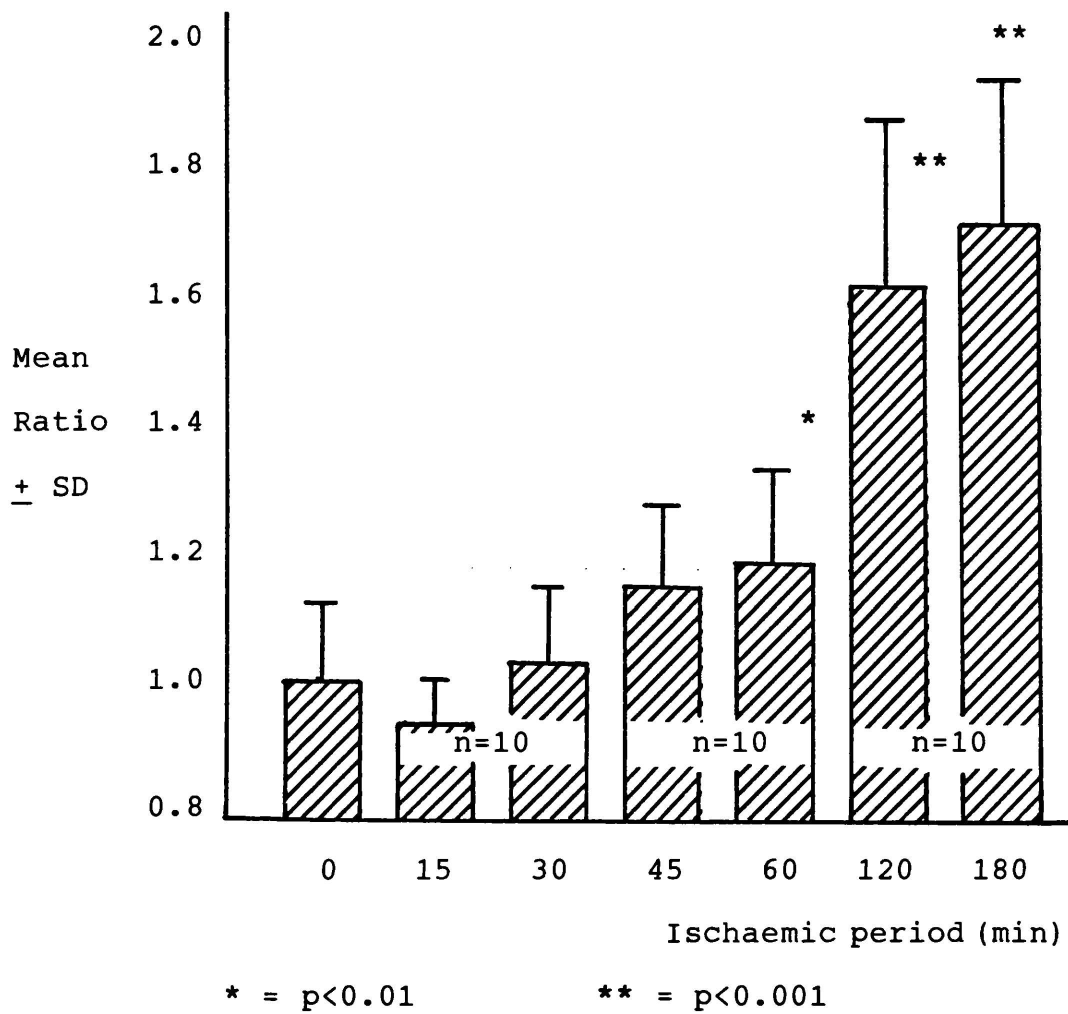


FIGURE 3.1iv Graph to show the effect of normothermic ischaemia on oxidised glutathione level in rat liver.

The level of oxidised glutathione increased progressively with ischaemia achieving significance at 60 minutes ($p<0.01$), 120 minutes ($p<0.001$) and 180 minutes ($p<0.001$).

e. Changes in glutathione ratio during normothermic ischaemia

The ratio of oxidised to reduced glutathione was calculated as an indicator of the conversion of glutathione during ischaemia.

The glutathione ratio of the freshly excised rat liver was 0.37 ± 0.07 (n=10). At 15 minutes the glutathione ratio was 0.34 ± 0.05 (n=10), whilst at 30 minutes it had risen to 0.43 ± 0.07 (n=10) and at 45 minutes it was 0.49 ± 0.14 (n=10). At 60 minutes a further rise to 0.50 ± 0.10 (n=10) was seen. Values of 0.79 ± 0.19 (n=10) at 120 minutes and 0.96 ± 0.19 (n=10) were observed.

The glutathione ratio in the normothermic rat liver rose with ischaemia. It became significantly greater than that of the freshly excised liver at 60 ($p<0.01$), 120 and 180 ($p<0.001$) minutes ischaemia.

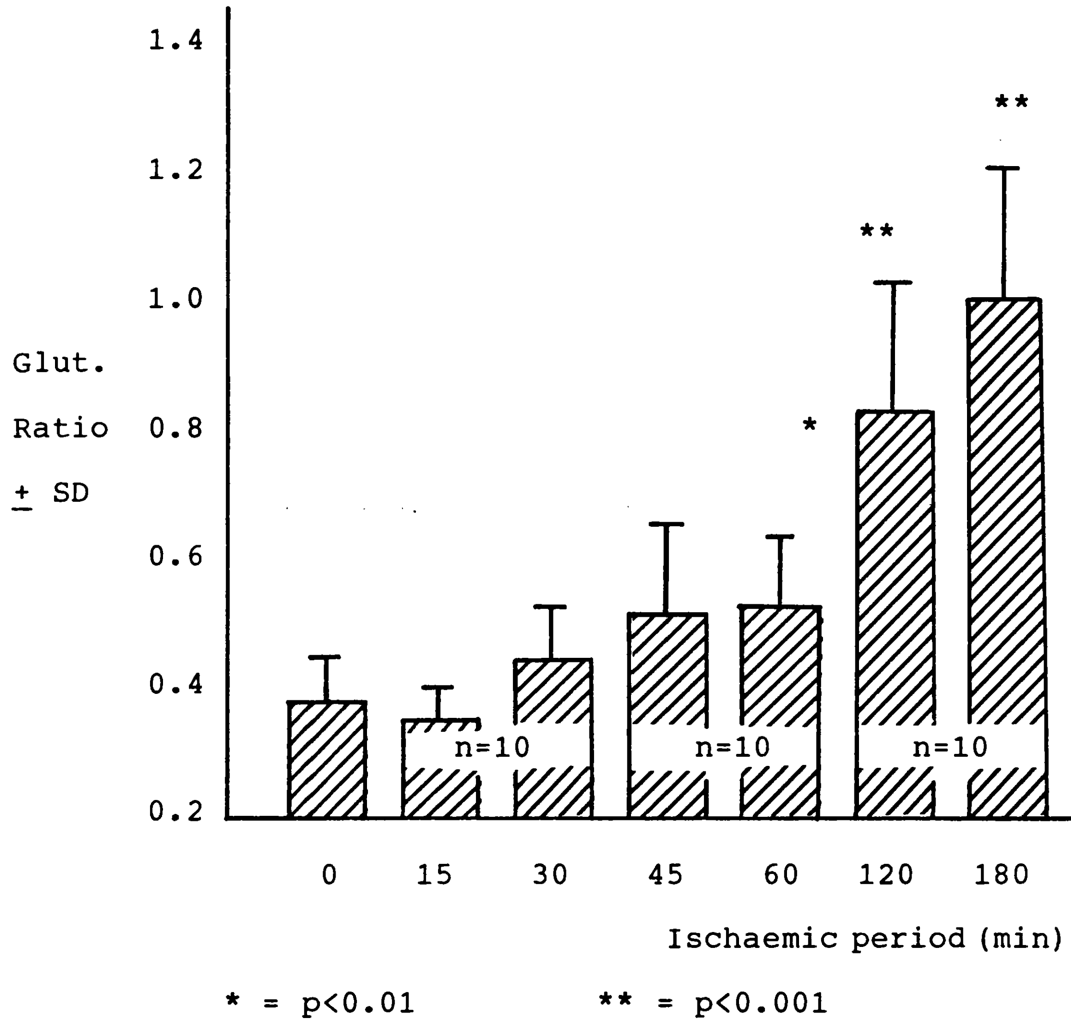


FIGURE 3.1v Graph to show the effect of normothermic ischaemia on glutathione ratio in rat liver.

The glutathione ratio increased progressively with ischaemia achieving significance at 60 minutes ($p<0.01$), 120 minutes ($p<0.001$) and 180 minutes ($p<0.001$).

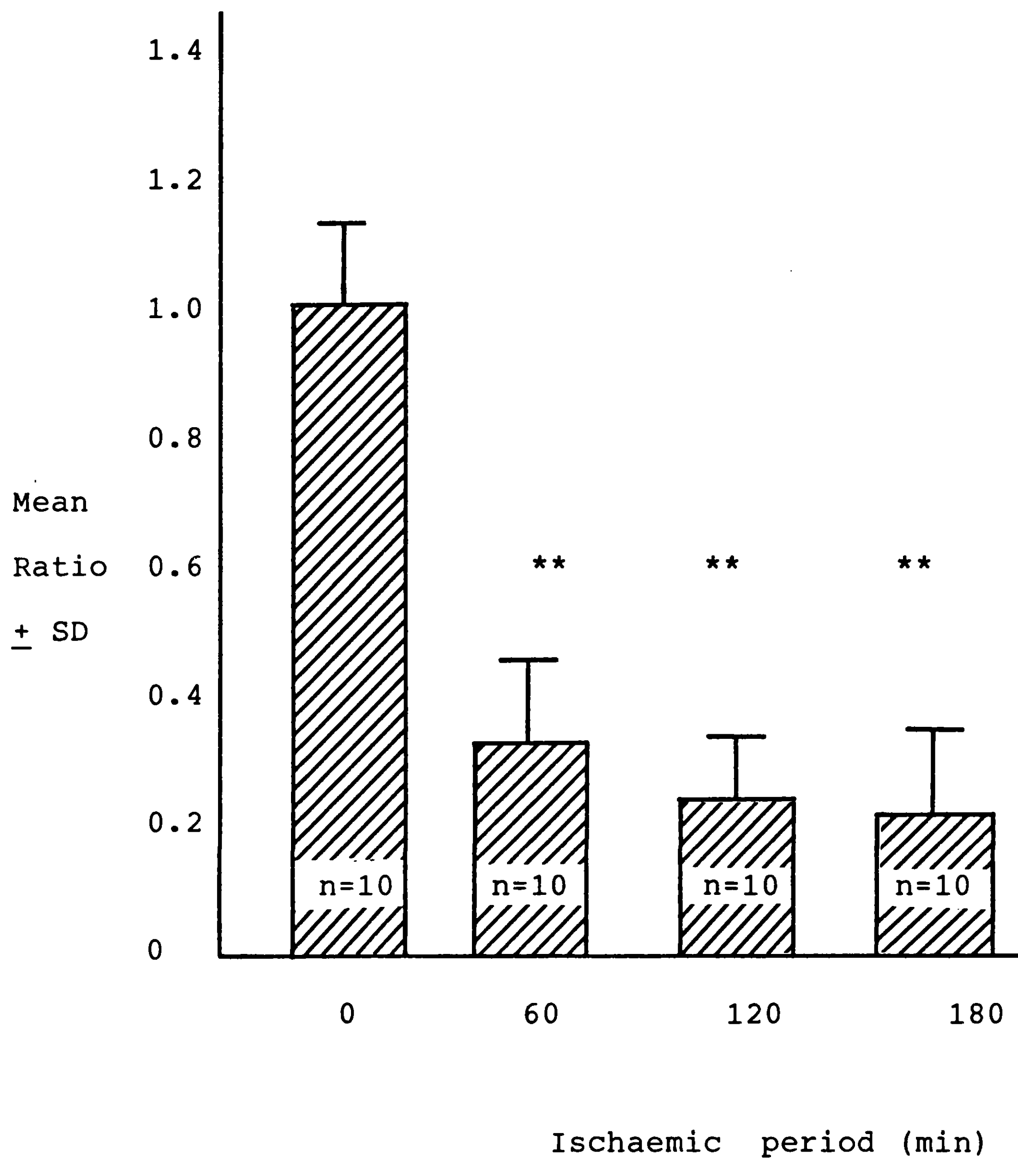
3.2 THE EFFECT OF ISCHAEMIA ON LIVER METABOLISM

Changes in urea production during normothermic ischaemia

The urea production in the freshly excised liver was 212.59 ± 43.98 mmoles/g dry weight (n=10). The urea production had fallen to 73.11 ± 31.20 mmoles/g dry weight (n=10) after 60 minutes ischaemia and 51.07 ± 18.85 mmoles/g dry weight (n=10) after 120 minutes. By 180 minutes the urea production had fallen to 49.67 ± 34.00 mmoles/g dry weight (n=10).

When the mean ratios were considered, the time zero value was 1.00 ± 0.22 (n=10). This had fallen to 0.34 ± 0.14 (n=10) at 60 minutes and 0.25 ± 0.10 (n=10) at 120 minutes ischaemia. After 180 minutes ischaemia the value was 0.23 ± 0.14 (n=10).

Urea production in the normothermic rat liver subjected to ischaemia was significantly decreased after 60, 120 and 180 minutes (p<0.001).



** = $p<0.001$

FIGURE 3.2i Graph to show the effect of normothermic ischaemia on urea production in rat liver.

Urea production fell during normothermic ischaemia. The values for 60, 120 and 180 minutes were all significantly lower than the initial value ($p<0.001$).

Summary of effects of normothermic ischaemia on rat liver

Significant increases in mean ratio of malondialdehyde production were seen after 45, 60 ($p<0.05$), 120 and 180 minutes ischaemia ($p<0.001$).

The trend was a decrease in the level of conjugated dienes with progressive normothermic ischaemia. The decrease achieved statistical significance at 60 minutes ($p<0.05$), 120 and 180 minutes ($p<0.01$).

There was an overall decrease in reduced glutathione level which achieved significance at 120 and 180 minutes of normothermic ischaemia ($p<0.001$).

Oxidised glutathione rose with progressive ischaemia. A significant increase over the time zero value was achieved at 60 minutes ($p<0.01$), 120 and 180 minutes ($p<0.001$).

The glutathione ratio in the normothermic rat liver rose with ischaemia. It became significantly greater than that of the freshly excised liver at 60 ($p<0.01$), 120 and 180 ($p<0.001$) minutes ischaemia.

Urea production in the normothermic rat liver subjected to ischaemia was significantly decreased after 60, 120 and 180 minutes ($p<0.001$).

CHAPTER 4

RESULTS

THE EFFECT OF FREE RADICAL SCAVENGERS ON NORMOTHERMIC ISCHAEMIA IN THE ISOLATED RAT LIVER

4.1 Effect of free radical scavengers on lipid peroxidation

4.2 Effect of free radical scavengers on liver metabolism

4.1 THE EFFECT OF FREE RADICAL SCAVENGERS ON LIPID PEROXIDATION

Introduction

In the previous chapter, the experimental results of studies of the effect of ischaemia on both lipid peroxidation and metabolism in the rat liver were shown.

These showed that the most significant changes in the parameters measured occurred after ischaemic periods of 60, 120 and 180 minutes.

In the following chapter, interest will be focussed on these particular time periods, although levels were measured at all time points indicated previously.

Method

The experimental method used in the studies together with details of the free radical scavengers used is shown in chapter 2.

Experimental results

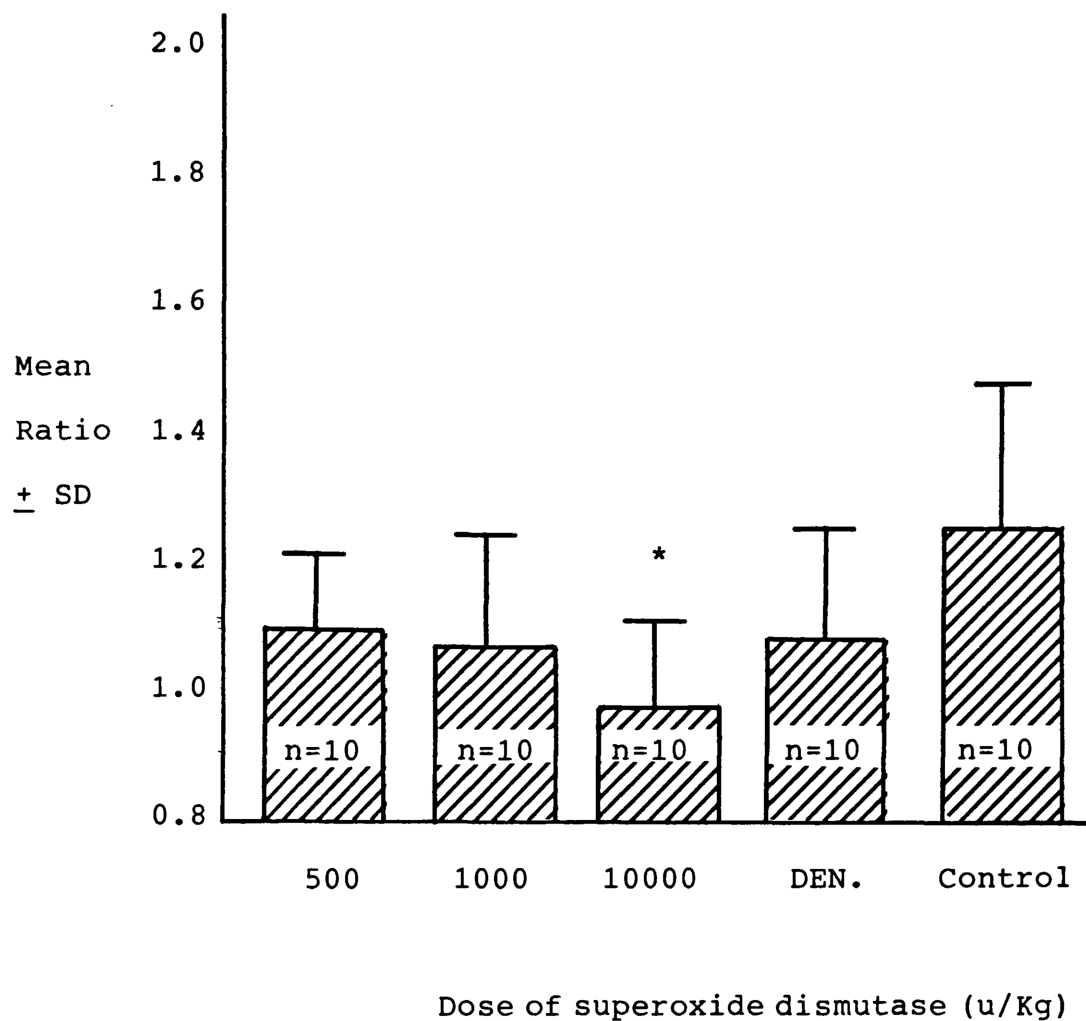
a. The effect of free radical scavengers on malondialdehyde production

SUPEROXIDE DISMUTASE

Three doses of superoxide dismutase were studied. These were 500, 1000 and 10000 units/Kg administered intravenously prior to ischaemia. Denaturation of superoxide dismutase was performed using diethyldithiocarbamate and the effect of this and the the three other doses were compared with the control of normal saline.

i. 500 u/Kg superoxide dismutase

The time zero value for MDA was 94.55 ± 12.94 nmoles/g (n=10) in the treated group compared with 118.35 ± 21.49 nmoles/g (n=10) in the control. The levels at 60, 120 and 180 minutes in the treated group were 101.39 ± 11.88 (n=10), 117.46 ± 12.06 (n=10) and 125.88 ± 14.69 nmoles/g (n=10) respectively. This compared with equivalent values in the control group of 143.01 ± 22.18 nmoles/g (n=10), 154.09 ± 24.57 nmoles/g (n=10) and 168.82 ± 24.95 nmoles/g (n=10) at 60, 120 and 180 minutes.



* = p<0.01

FIGURE 4.1i Graph to show the effect of superoxide dismutase on malondialdehyde production in the rat liver at 60 minutes normothermic ischaemia.

A significant reduction in malondialdehyde production over control was seen at a dose of 10000u/Kg ($p<0.01$).

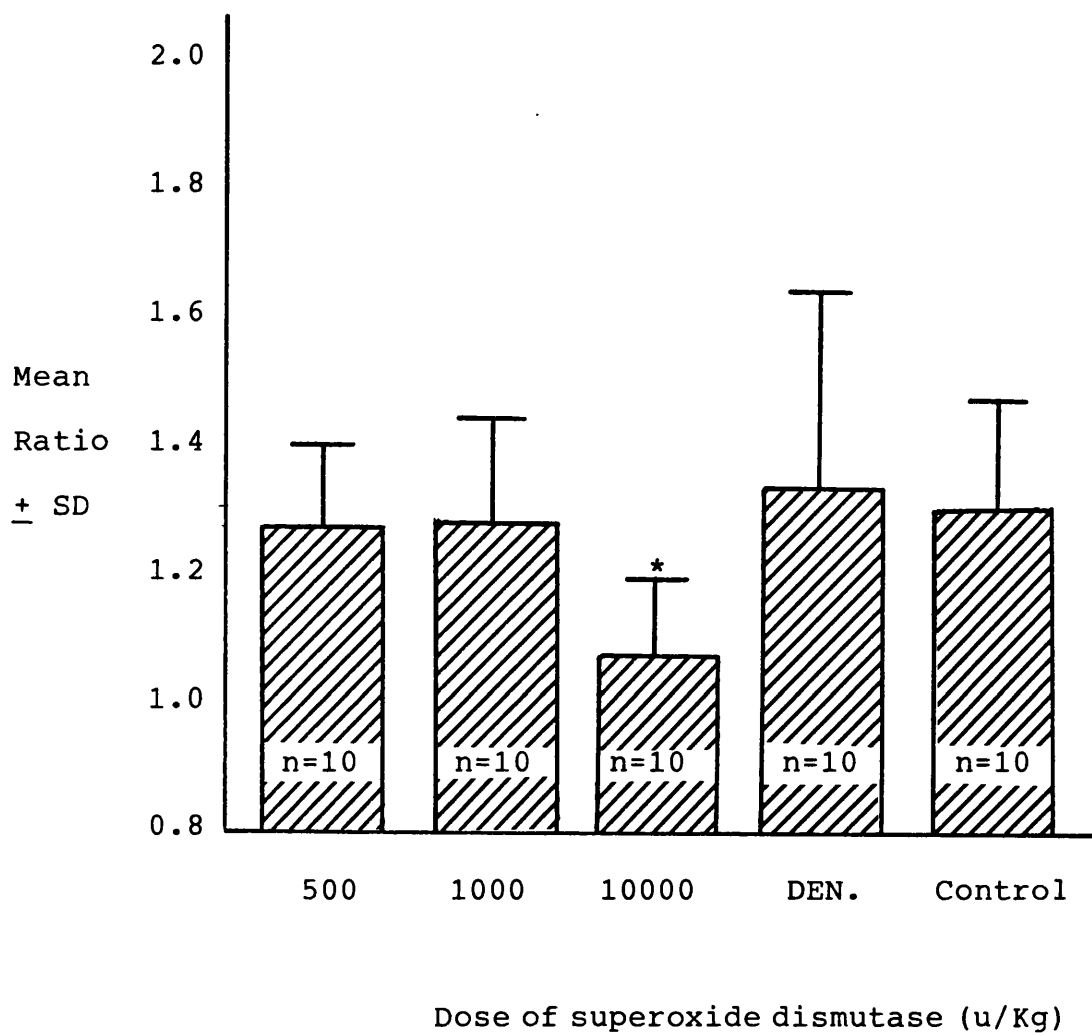
When the mean ratios were considered, a similar increase was observed in both groups. From an initial ratio of 1.00 ± 0.13 (n=10) in the treated group, there was a rise to 1.09 ± 0.11 (n=10) at 60 minutes. The increase continued, to reach 1.26 ± 0.12 (n=10) at 120 and 1.35 ± 0.16 (n=10) at 180 minutes.

The time zero value in the control group was 1.00 ± 0.17 (n=10). At 60, 120 and 180 minutes, the values rose to 1.24 ± 0.21 (n=10), 1.32 ± 0.16 (n=10) and 1.45 ± 0.19 (n=10) respectively.

Malondialdehyde production in both groups increased with time and was significantly greater than in the freshly excised liver ($p<0.001$) at 120 and 180 minutes. There was no significant difference between treated and untreated groups.

ii. 1000 u/Kg superoxide dismutase

The value for MDA in the freshly excised liver was 107.07 ± 5.70 nmoles/g (n=10) in the treated group and 118.35 ± 21.49 nmoles/g (n=10) in the control. The levels of MDA production in the treated group at 60, 120 and 180 minutes of ischaemia were 118.80 ± 15.14 nmoles/g (n=10), 134.44 ± 10.67 nmoles/g (n=10) and 140.42 ± 8.20 nmoles/g (n=10).



* = $p<0.01$

FIGURE 4.1ii Graph to show the effect of superoxide dismutase on malondialdehyde production in the rat liver at 120 minutes normothermic ischaemia.

A significant reduction in malondialdehyde production over control was seen at a dose of 10000u/Kg ($p<0.01$).

In the control group the equivalent levels of MDA production were 143.01 ± 22.18 nmoles/g (n=10), 154.09 ± 24.57 nmoles/g (n=10) and 168.82 ± 24.95 nmoles/g (n=10).

The mean ratio for the freshly excised liver in the treated group was 1.00 ± 0.05 (n=10) and that in the control group was 1.00 ± 0.17 (n=10). The values at 60, 120 and 180 minutes of 1.12 ± 0.17 (n=10), 1.27 ± 0,15 (n=10) and 1.32 ± 0.12 (n=10) showed a progressive increase in MDA production. The equivalent control values were 1.24 ± 0.21 (n=10), 1.32 ± 0.16 (n=10) and 1.45 ± 0.19 (n=10).

Malondialdehyde production progressively increased in both treated and untreated groups to reach statistical significance over the freshly excised liver at 120 and 180 minutes ($p<0.001$). There is no statistical difference in MDA production between treated and untreated groups.

iii. <u>10000 u/Kg superoxide dismutase</u>

MDA production in the freshly excised liver of the treated group was 109.99 ± 9.83 nmoles/g (n=10). That of the control group was 118.35 ± 21.49 nmoles/g (n=10). In the treated group the MDA

production rose slowly yielding 118.80 ± 15.14 nmoles/g (n=10) at 60 minutes. The values at 120 and 180 minutes were 134.44 ± 10.67 (n=10) and 140.42 ± 8.20 nmoles/g (n=10).
Equivalent values in the control group were 143.01 ± 22.18 nmoles/g (n=10) at 60 minutes, 154.09 ± 24.57 nmoles/g (n=10) at 120 minutes and 168.82 ± 24.95 nmoles/g (n=10) at 180 minutes.

When the mean ratios were calculated, it was noted that the production of MDA was far lower in the treated group than the control. Initial values were 1.00 ± 0.08 (n=10) in the treated group and 1.00 ± 0.17 (n=10) in the control. At 60, 120 and 180 minutes the ratio was 0.97 ± 0.12 (n=10), 1.06 ± 0.13 (n=10) and 1.16 ± 0.12 (n=10) respectively in the treated group but 1.24 ± 0.21 (n=10), 1.32 ± 0.16 (n=10) and 1.45 ± 0.19 (n=10) in the control.

Superoxide dismutase at a dose of 10000 u/Kg has a significant effect on the production of malondialdehyde. A statistically significant reduction ($p<0.01$) in malondialdehyde production was seen in the treated group at 60, 120 and 180 minutes. The rate of production of MDA was reduced <u>within</u> the treated group with a significant increase ($p<0.01$) over that of the freshly excised liver only occuring after 180 minutes.

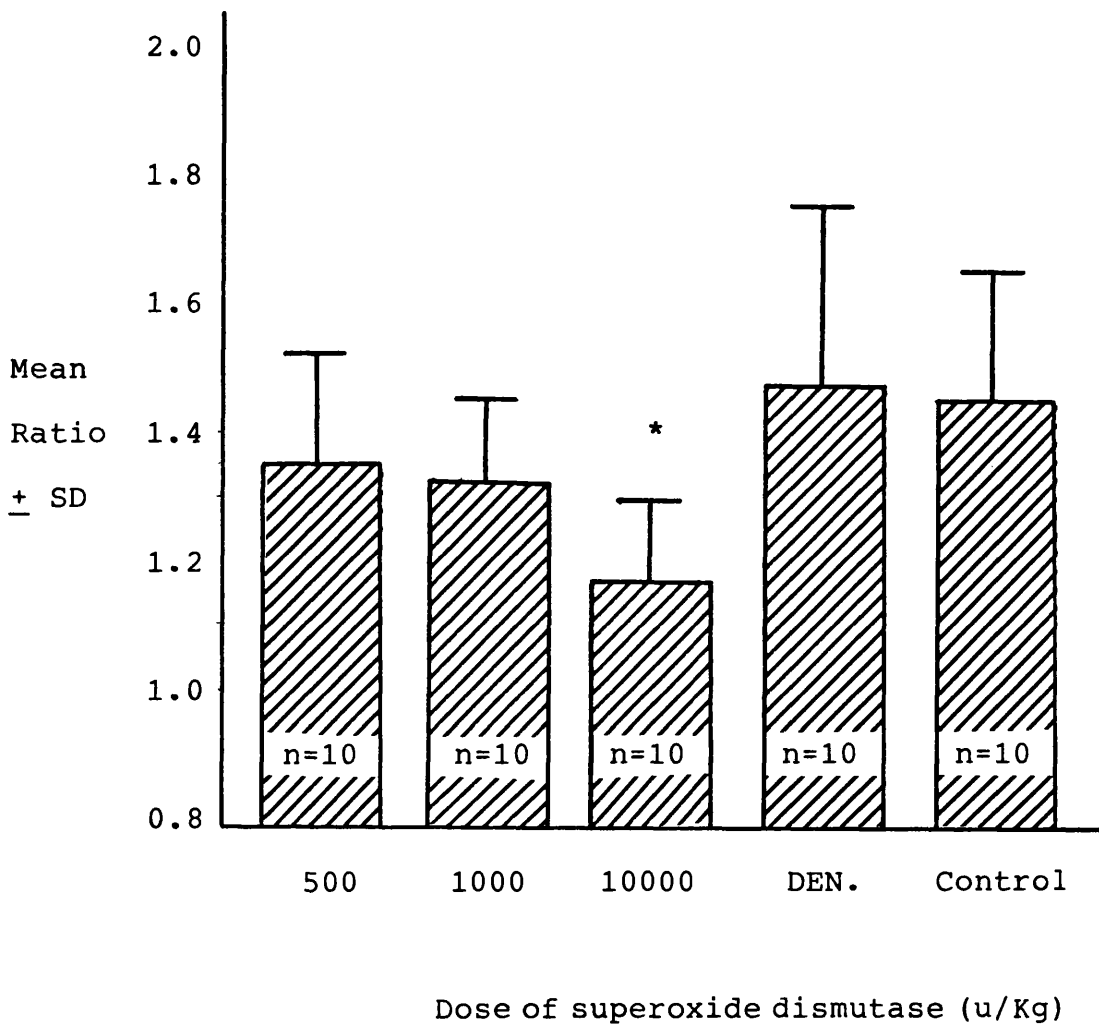


FIGURE 4.1iii Graph to show the effect of superoxide dismutase on malondialdehyde production in the rat liver at 180 minutes normothermic ischaemia.

A significant reduction in malondialdehyde production over control was seen at a dose of 10000u/Kg ($p<0.01$).

iv. Denatured superoxide dismutase

The value for malondialdehyde production in the freshly excised liver in the treated group was 141.51 ± 17.67 nmoles/g (n=10) and in the control group was 118.35 ± 21.49 nmoles/g (n=10). At 60, 120 and 180 minutes the values had risen to 150.39 ± 19.53 (n=10), 184.22 ± 31.41 (n=10) and 202.19 ± 30.85 nmoles/g (n=10). In the control group the values were 143.01 ± 22.18 nmoles/g (n=10) at 60 minutes, 154 09 ± 24.57 nmoles/g (n=10) at 120 minutes and 168.82 ± 24.95 nmoles/g (n=10) at 180 minutes.

The mean ratios showed a similar rise in MDA level with progressive ischaemia. The time zero value for the treated group was 1.00 ± 0.12 (n=10) and that of the control was 1.00 ± 0.17 (n=10). After ischaemic periods of 60, 120 and 180 minutes the values rose to 1.07 ± 0.17 (n=10), 1.33 ± 0.26 (n=10) and 1.46 ± 0.27 (n=10) respectively in the treated group. The values in the control group at 60, 120 and 180 minutes were 1.24 ± 0.21 (n=10), 1.32 ± 0.16 (n=10) and 1.45 ± 0.19 (n=10).

There is an increase in the production of MDA with ischaemia in rats treated with denatured superoxide dismutase which is significantly greater than the

freshly excised liver at 120 minutes ($p<0.01$) and180 minutes ($p<0.001$). There is no significant difference from the control group at any time point.

Summary

Superoxide dismutase can significantly reduce malondialdehyde production ($p<0.01$) over a control group. It appears to require a certain dose of superoxide dismutase (10000 u/Kg) to be effective. At lower doses there is no significant reduction of MDA. Denaturation of the 10000 u/Kg dose with diethyldithiocarbamate abolishes the effect of superoxide dismutase.

ALLOPURINOL

Two doses of allopurinol were used, 5mg/Kg and 10mg/Kg administered intravenously.

i. 5mg/Kg allopurinol

The malondialdehyde production of the freshly excised liver in the treated group was 172.68 ± 31.34 nmoles/g n=10) and in the control group was 118.35 ± 21.49 nmoles/g (n=10). There was an increase in malondialdehyde in both groups with progressive ischaemia. In the treated group at 60,120 and 180 minutes the values were 178.30 ± 25.83 nmoles/g (n=10), 183.00 ± 29.96 nmoles/g (n=10) and 203.54 ± 43.44 nmoles/g (n=10) respectively. In the control group the equivalent values were 143.01 ± 22.18 nmoles/g (n=10), 154.09 ± 24.57 nmoles/g and 168.82 ± 24.95 nmoles/g (n=10) at 60, 120 and 180 minutes.

The mean ratios at time zero were 1.01 ± 0.17 (n=10) for the treated group and 1.00 ± 0.17 (n=10). A progressive rise was seen in both groups. In the treated group at 60, 120 and 180 minutes the values were 1.05 ± 0.16 (n=10), 1.09 ± 0.26 (n=10) and 1.22 ± 0.34 (n=10). The control values were 1.24 ± 0.21

(n=10), 1.32 ± 0.16 (n=10) and 1.45 ± 0.19 (n=10) for 60, 120 and 180 minutes.

MDA production increased in the untreated group. In the group treated with 5mg/Kg allopurinol, there was no significant increase in MDA production. There was a significant difference ($p<0.05$) between the groups at 120 minutes.

ii. <u>10mg/Kg allopurinol</u>

In the allopurinol treated group the MDA production in the freshly excised liver was 108.16 ± 13.74 nmoles/g (n=10) and in the control 118.35 ± 21.49 nmoles/g (n=10). During ischaemia the production of MDA in the treated group rose to 113.89 ± 19.20 nmoles/g (n=10) at 60 minutes, 125.89 ± 22.50 nmoles/g (n=10) at 120 and 121.70 ± 19.47 nmoles/g (n=10) at 180 minutes. The control values were 143.01 ± 22.18 nmoles/g (n=10) at 60 minutes, 154.09 ± 24.57 nmoles/g (n=10) at 120 and 168.82 ± 24.95 nmoles/g (n=10) at 180 minutes.

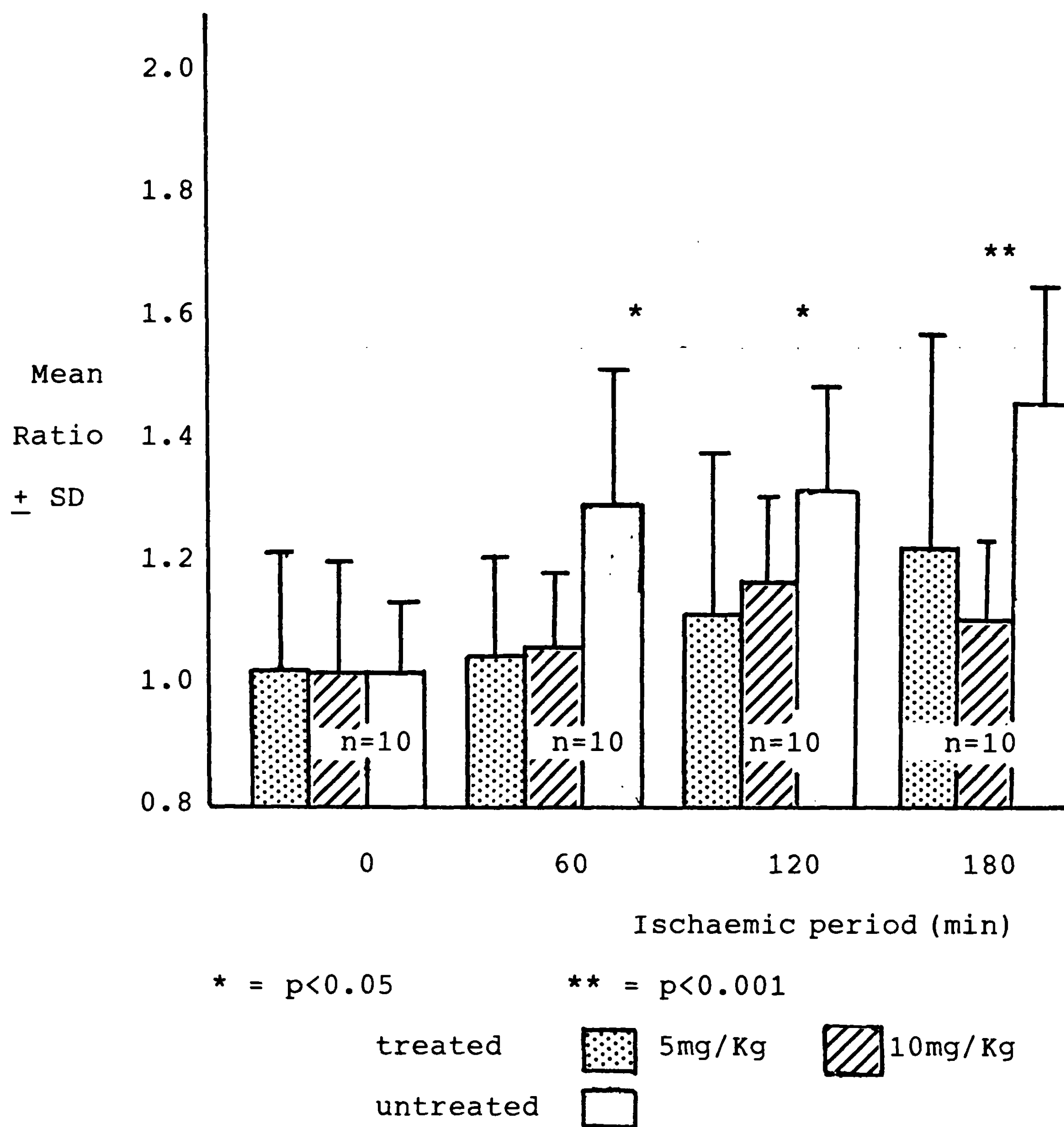


FIGURE 4.1iv Graph to show the effect of allopurinol on malondialdehyde level in rat liver during normothermic ischaemia.

The level of malondialdehyde increased significantly during ischaemia. 10mg/Kg allopurinol reduced this level over control at 60 minutes ($p<0.05$) and 180 minutes ($p<0.001$). 5mg/Kg was effective at 120 min.

The mean ratios at time zero were 1.00 + 0.12 (n=10) for the treated group and 1.00 ± 0.17 (n=10) for the control. For the allopurinol treated group, the values for 60, 120 and 180 minutes ischaemia were 1.06 ± 0.12 (n=10), 1.17 ± 0.15 (n=10) and 1.13 ± 0.11 (n=10). The equivalent values for the control were 1.24 ± 0.21 (n=10), 1.32 ± 0.16 (n=10) and 1.45 ± 0.19 (n=10).

Malondialdehyde production increased with ischaemia in both groups, achieving significance at 120 minutes ($p<0.02$) and 180 minutes ($p<0.05$). Allopurinol produced a significant reduction in malondialdehyde production ($p<0.05$) at 60 minutes and 180 minutes ($p<0.001$).

Desferrioxamine

Two doses of desferrioxamine were used, 5mg/Kg and 15 mg/Kg administered intravenously.

i. 5mg/Kg desferrioxamine

The malondialdehyde level in the freshly excised rat liver was 110.04 ± 6.55 nmoles/g (n=10) in the treated group and 118.35 ± 21.49 nmoles/g (n=10) in the control group. The level in the treated group slowly rose to 122.99 ± 10.58 nmoles/g (n=10) at 60 minutes, 135.44 ± 16.59 nmoles/g (n=10) at 120 minutes and reached 138.16 ± 12.86 nmoles/g (n=10) at 180 minutes ischaemia. The level in the control group reached 143.01 ± 22.18 nmoles/g (n=10) at 60 minutes, with 154.09 ± 24.57 nmoles/g (n=10) at 120 and 168.82 ± 24.95 nmoles/g (n=10) at 180 minutes ischaemia.

The mean ratios for the freshly excised livers were 1.00 ± 0.06 (n=10) in the treated group and 1.00 ± 0.17 (n=10) in the control. Mean ratios at 60, 120 and 180 minutes in the treated group were 1.13 ± 0.13 (n=10), 1.23 ± 0.11 (n=10) and 1.13 ± 0.11 (n=10) respectively. Those in the control group were

1.24 ± 0.21 (n=10), 1.32 ± 0.16 (n=10) and 1.45 ± 0.19 (n=10) for equivalent time periods.

There is a significant rise in MDA production at 60 minutes ($p<0.02$), 120 and 180 minutes $p<0.001$). A dose of 5mg/Kg desferrioxamine significantly reduced MDA production at 180 minutes of ischaemia ($p<0.001$) over the control group.

ii. <u>15mg/Kg desferrioxamine</u>

Malondialdehyde levels in the freshly excised liver were 122.89 ± 7.23 nmoles/g (n=10) in the treated group and 118.35 ± 21.49 nmoles/g (n=10) in the control group. The level rose in the treated group to 141.43 ± 11.90 nmoles/g (n=10) at 60 minutes, 151.82 ± 11.75 nmoles/g (n=10) at 120 minutes and 149.02 ± 12.39 nmoles/g (n=10) at 180 minutes ischaemia.

Levels in the control group rose to 143.01 ± 22.18 nmoles/g (n=10) at 60 minutes, 154.09 ± 24.57 nmoles/g (n=10) at 120 minutes and 168.82 ± 24.95 nmoles/g (n=10) after 180 minutes ischaemia.

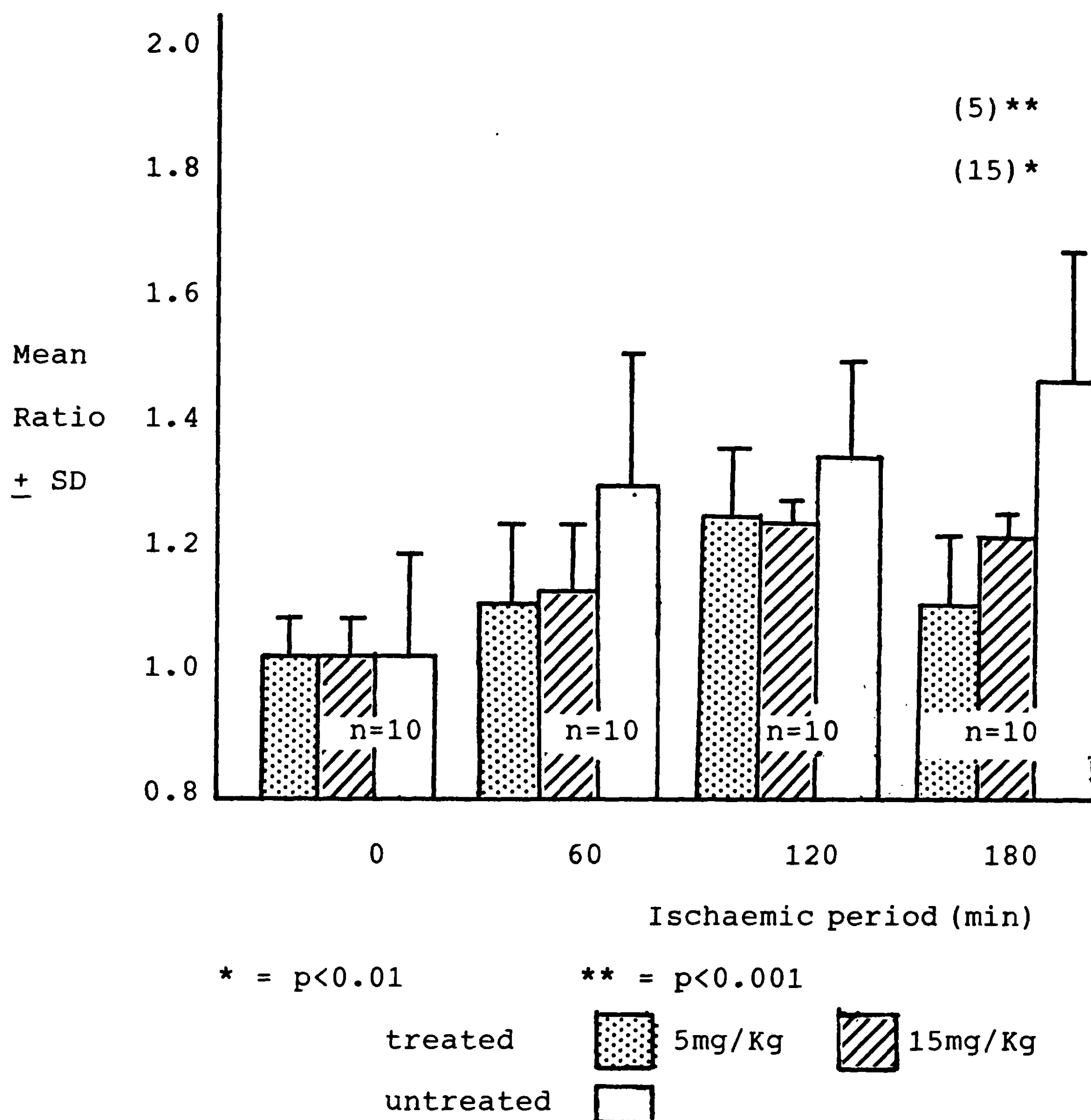


FIGURE 4.1v Graph to show the effect of desferrioxamine on malondialdehyde level in rat liver during normothermic ischaemia.

The level of malondialdehyde increased significantly during ischaemia. Desferrioxamine reduced this level over control at 180 minutes, 5mg/Kg ($p<0.001$) 15mg/Kg ($p<0.01$).

The mean ratios at time zero were 1.00 ± 0.06 (n=10) for the treated group and 1.00 ± 0.17 (n=10) for the control. The ratios rose with progressive ischaemia to 1.14 ± 0.11 (n=10) at 60 minutes, 1.22 ± 0.09 (n=10) at 120 minutes and 1.22 ± 0.05 (n=10) at 180 minutes ischaemia. The ratios in the control group were 1.24 ± 0.21 (n=10), 1.32 ± 0.16 (n=10) and 1.45 ± 0.19 (n=10) after 60, 120 and 180 minutes ischaemia respectively.

There was a significant increase in MDA production with ischaemia at 60 minutes ($p<0.01$), 120 and 180 minutes ($p<0.001$). 15mg/Kg of desferrioxamine reduced the production of MDA over control at 180 minutes ($p<0.01$).

Mannitol

One dose of mannitol was used. This was 1g/Kg and was administered intravenously.

A level of 94.72 ± 6.45 nmoles/g (n=10) MDA was found in the freshly excised liver of the treated group. The level in the control group was 118.35 ± 21.49 nmoles/g (n=10). At 60 minutes this had risen to 149.05 ± 10.08 nmoles/g (n=10) in the treated group, progressed to 164.64 ± 17.69 nmoles/g (n=10) at 120 minutes and 143.47 ± 20.79 nmoles/g (n=10) at 180 minutes.

The control values were 143.01 ± 22.18 nmoles/g (n=10), 154.09 ± 24.57 nmoles/g (n=10) and 168.82 ± 24.95 nmoles/g (n=10) at 60, 120 and 180 minutes respectively.

The mean ratio at time zero was 1.00 ± 0.07 (n=10) in the treated group and 1.00 ± 0.17 (n=10) in the control. At 60, 120 and 180 minutes the values for the treated group were markedly increased at 1.57 ± 0.13 (n=10), 1.74 ± 0.18 (n=10) and 1.52 ± 0.20 (n=10). The equivalent values for the control group were 1.24 ± 0.21 (n=10), 1.32 ± 0.16 (n=10) and 1.45 ± 0.19 (n=10).

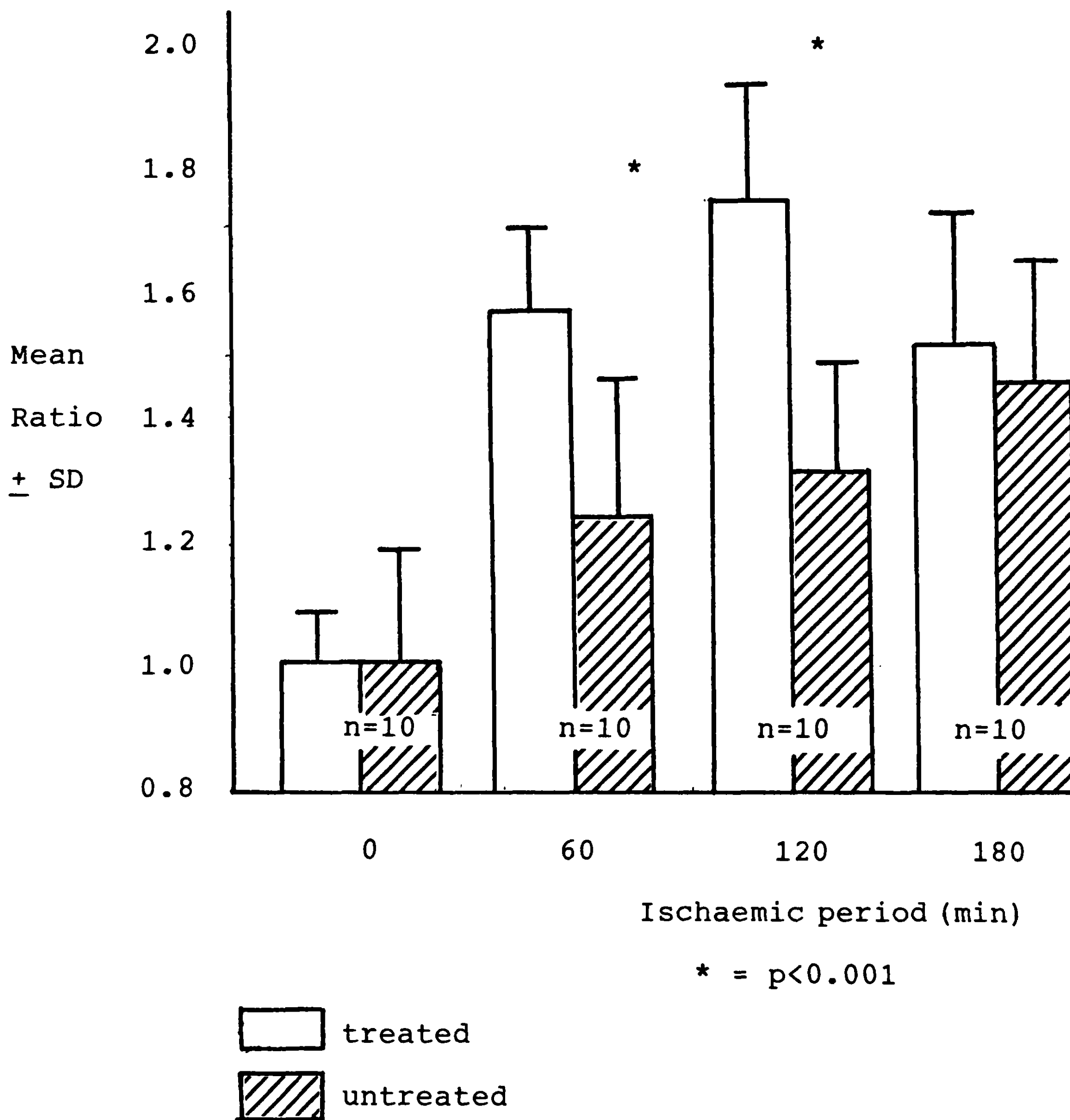


FIGURE 4.1vi Graph to show the effect of mannitol on malondialdehyde production in rat liver during normothermic ischaemia.

Malondialdehyde production was significantly <u>higher</u> than in the control group at 60 and 120 minutes ischaemia ($p<0.001$).

The level of malondialdehyde increased with ischaemia, to a highly significant level at 60, 120 and 180 minutes ($p<0.001$). The administration of mannitol resulted in a significant difference in MDA production over that of the control at 60 and 120 minutes ($p<0.001$). The mannitol resulted in an <u>increased</u> malondialdehyde production over that of the control.

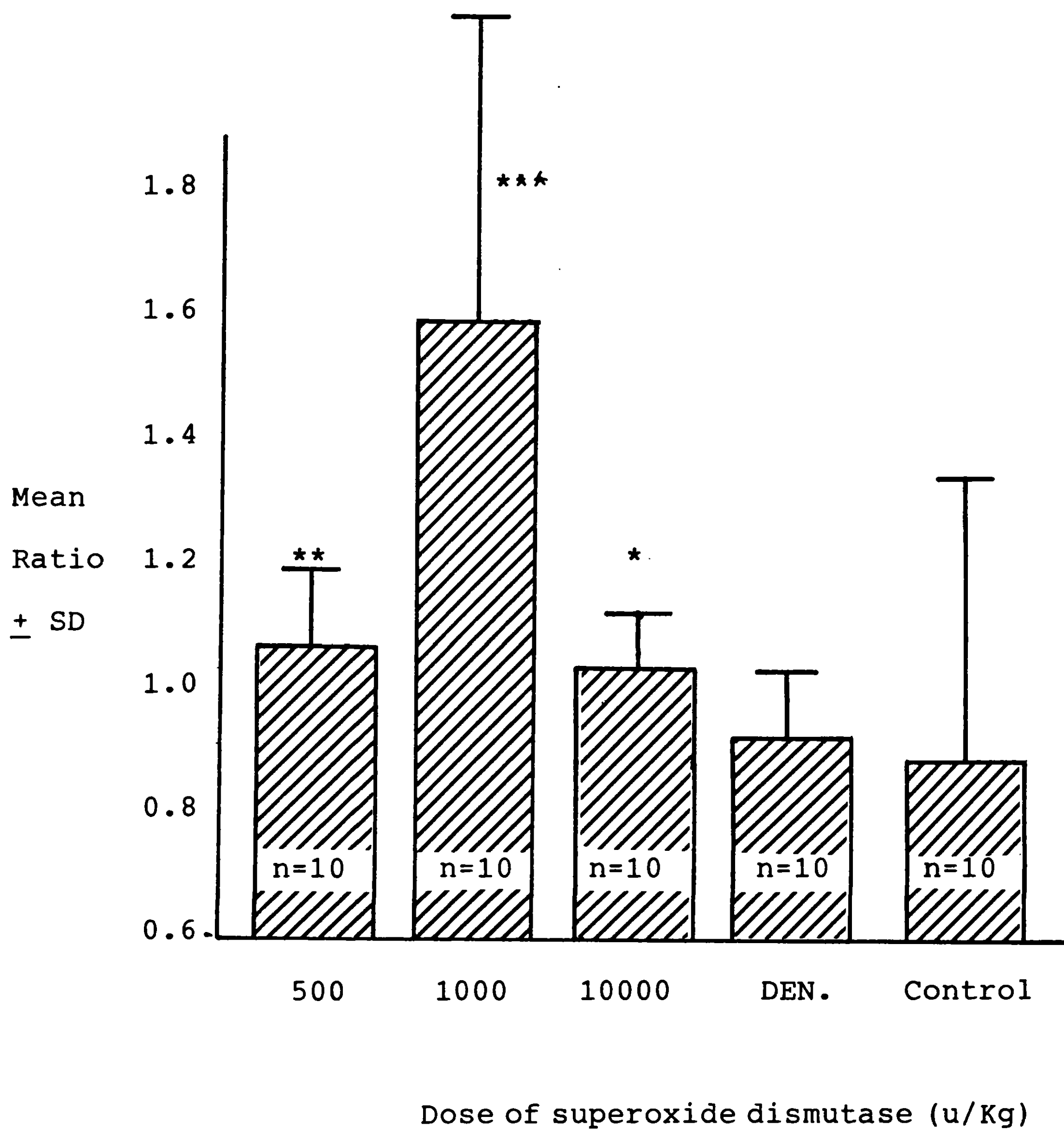
b. **The effect of free radical scavengers on conjugated diene production**

SUPEROXIDE DISMUTASE

i. 500u/Kg superoxide dismutase

The level of conjugated dienes in the freshly excised liver was 8.61 ± 1.02 fluorescence units(fu)/g (n=10) in the control group and 9.78 ± 1.20 fu/g (n=10) in the treated group.
In the treated group the level rose at 60 minutes to 10.27 ± 1.51 fu/g (n=10) then fell to 8.99 ± 1.43 fu/g (n=10) at 120 minutes and 8.68 ± 1.52 fu/g (n=10) at 180 minutes.
In the control group there was a steady fall in the level of dienes. Levels were 7.51 ± 1.37 fu/g (n=10), 7.25 ± 1.44 fu/g (n=10) and 6.84 ± 1.51 fu/g (n=10) at 60, 120 and 180 minutes respectively.

The mean ratio of the freshly excised liver was 1.00 ± 0.11 (n=10) in the control group and 1.00 ± 0.12 in the treated group.
In the treated group after 60, 120 and 180 minutes ischaemia the mean ratios were 1.06 ± 0.15 (n=10), 0.94 ± 0.20 (n=10) and 0.91 ± 0.21 (n=10) respectively.



* = $p<0.02$ ** = $p<0.01$ *** = $p<0.001$

FIGURE 4.1vii **Graph to show the effect of superoxide dismutase on conjugated diene production in the rat liver at 60 minutes normothermic ischaemia.**
The level of conjugated dienes was maintained at a significantly higher level than the control by superoxide dismutase.

In the control group the equivalent ratios were 0.88 ± 0.24 (n=10), 0.85 ± 0.12 (n=10) and 0.73 ± 0.25 (n=10).

The level of conjugated dienes in the liver of rats treated with 500u/Kg superoxide dismutase did not change sufficiently to achieve statistical significance when subjected to progressive ischaemia. When compared with the control group, the level of dienes was maintained at a higher value at 60 minutes ($p<0.02$) and 180 minutes ($p<0.05$).

ii. <u>1000u/Kg superoxide dismutase</u>

The level of conjugated dienes in freshly excised liver was 8.61 ± 1.02 fu/g (n=10) for the control and 6.86 ± 1.94 fu/g (n=10) for the treated group. In the treated group, the level again reached a higher value at 60 minutes of 10.11 ± 0.47 fu/g (n=10), which fell to 7.92 ± 1.46 fu/g (n=10) and 8.66 ± 1.13 fu/g (n=10) at 120 and 180 minutes.
The values for the control showed a steady fall, 7.51 ± 1.37 fu/g (n=10), 7.25 ± 1.44 fu/g (n=10) and 6.84 ± 1.51 fu/g (n=10) at 60, 120 and 180 minutes.

The mean ratios for freshly excised liver were 1.00 ± 0.11 (n=10) for the control and 1.00 ± 0.27 (n=10)

for the treated group.

In the treated group the values after 60, 120 and 180 minutes ischaemia were 1.60 ± 0.47 (n=10), 1.28 ± 0.52 (n=10) and 1.37 ± 0.38 (n=10).

In the control group the values were 0.88 ± 0.24 (n=10), 0.85 ± 0.12 (n=10) and 0.73 ± 0.38 (n=10) at 60, 120 and 180 minutes.

Once again the level of conjugated dienes in the treated group was seen to peak at 60 minutes. It was significantly higher at this dose of scavenger ($p<0.01$) than the fresh value.

The level of conjugated dienes was maintained at a significantly higher value than the control group at 60 and 180 minutes ($p<0.001$).

iii. <u>10000u/Kg superoxide dismutase</u>

The level of conjugated dienes in the freshly excised liver was 8.61 ± 1.02 fu/g (n=10) in the control group and 9.43 ± 1.12 fu/g (n=10) in the treated group.

In the treated group the level rose to 9.67 ± 0.91 fu/g (n=10) at 60 minutes, then fell to 8.16 ± 1.00 fu/g (n=10) at 120 and 8.30 ± 1.37 fu/g (n=10) at 180 minutes.

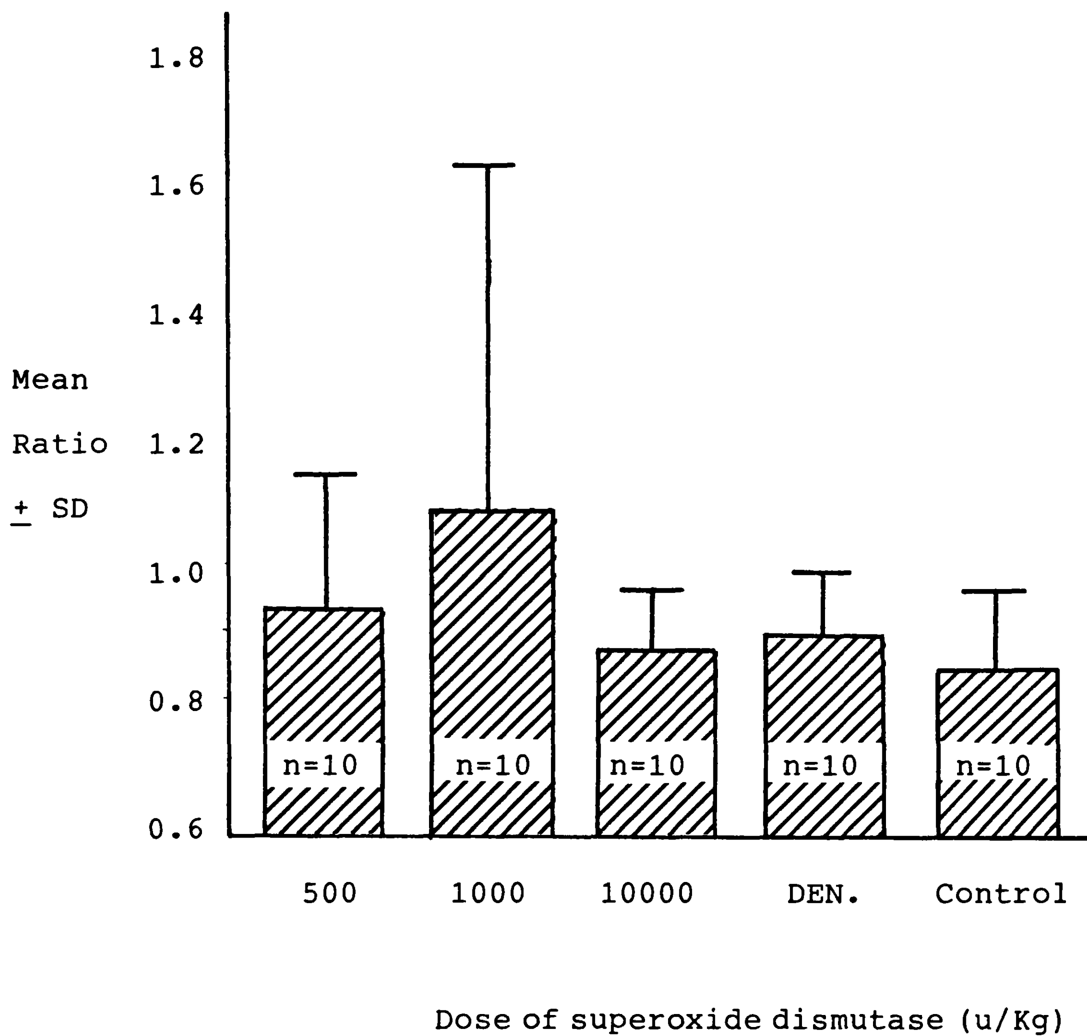


FIGURE 4.1viii Graph to show the effect of superoxide dismutase on conjugated diene production in the rat liver at 120 minutes normothermic ischaemia.
The level of conjugated dienes was not significantly higher than the control at 120 minutes after superoxide dismutase.

The equivalent control values showed a steady decrease at 7.51 ± 1.37 fu/g (n=10), 7.25 ± 1.44 fu/g (n=10) and 6.84 ± 1.51 fu/g (n=10) at 60, 120 and 180 minutes.

The mean ratios for freshly excised liver were 1.00 ± 0.11 (n=10) in both the control and treated groups. The values in the treated group were 1.03 ± 0.09 (n=10), 0.87 ± 0.09 (n=10) and 0.89 ± 0.12 (n=10) at 60, 120 and 180 minutes. The equivalent controls showed values of 0.88 ± 0.24 (n=10), 0.85 ± 0.12 (n=10) and 0.73 ± 0.25 (n=10) at 60, 120 and 180 minutes.

The level of conjugated dienes again reached a peak around 60 minutes and which fell significantly at 120 minutes ($p<0.02$). The level of dienes in the treated group was significantly higher than the control group at 60 minutes ($p<0.01$).

iv. Denatured superoxide dismutase

The level of conjugated dienes in freshly excised rat liver was 8.61 ± 1.02 fu/g (n=10) in the control and 8.51 ± 1.14 fu/g (n=10) in the treated group. In the treated group the level had fallen to 7.59 ± 0.66 fu/g (n=10) at 60 minutes. The fall continued

to 7.47 ± 1.00 fu/g (n=10) at 120 minutes and 7.19 ± 1.15 fu/g (n=10) at 180 minutes.
The values for the control group fell steadily at 60, 120 and 180 minutes to 7.51 ± 1.37 fu/g (n=10), 7.25 ± 1.44 fu/g (n=10) and 6.84 ± 1.51 fu/g (n=10) repectively.

The mean ratio for the freshly excised liver was 1.00 ± 0.11 (n=10) for the control and 1.00 ± 0.13 (n=10) for the treated group.
Values at 60, 120 and 180 minutes for the treated group were 0.91 ± 0.10 (n=10), 0.89 ± 0.09 (n=10) and 0.86 ± 0.12 (n=10).
The equivalent values for the control were 0.88 ± 0.24 (n=10), 0.85 ± 0.12 (n=10) and 0.73 ± 0.25 (n=10) at 60, 120 and 180 minutes respectively.

The administration of denatured superoxide dismutase returned the pattern of diene production to that of the control group, with no significant difference between the two. In the treated group the value for dienes fell with progressive ischaemia, achieving significance at 120 and 180 minutes ($p<0.05$).

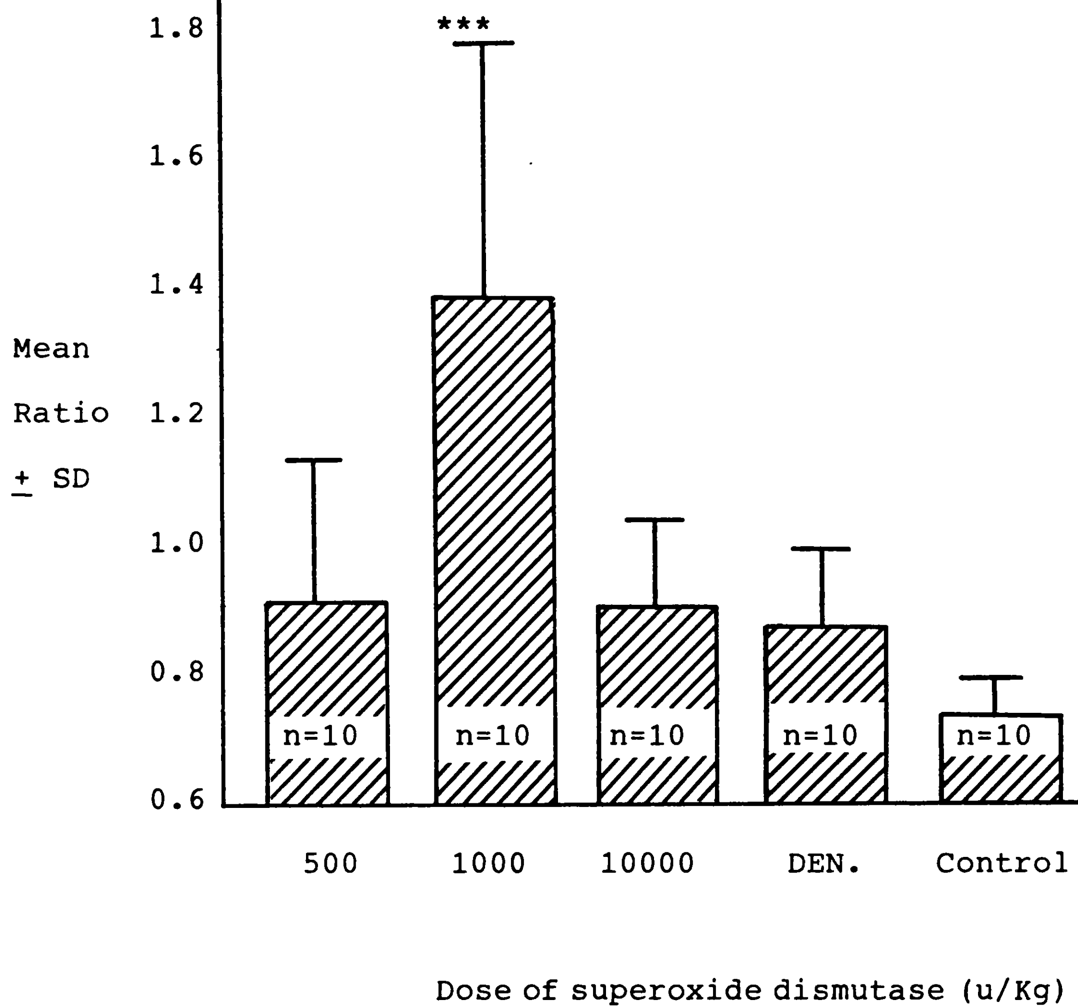


FIGURE 4.1ix **Graph to show the effect of superoxide dismutase on conjugated diene production in the rat liver at 180 minutes normothermic ischaemia.**
The level of conjugated dienes was maintained at a significantly higher level than the control after 1000u/Kg superoxide dismutase at 180 minutes ($p<0.001$).

Summary

Superoxide dismutase, when administered in doses of 500u/Kg, 1000u/Kg and 10000u/Kg maintains the level of conjugated dienes in the rat liver subjected to normothermic ischaemia. A peak value was seen at 60 minutes at all doses, with a subsequent fall in level with progressive ischaemia. The levels were significantly higher than the equivalent control group as ischaemia progressed.
Denaturation of superoxide dismutase with diethyldithiocarbamate abolished these findings and returned the pattern to that of the control.

Allopurinol

i. 5mg/Kg allopurinol

Conjugated diene levels in the freshly excised liver were 8.21 ± 2.18 fu/g (n=10) in the treated group and 8.61 ± 1.02 fu/g (n=10) in the control.
In the treated group the level fell to 6.66 ± 2.13 fu/g n=10), 7.24 ± 1.69 fu/g (n=10) and 6.71 ± 2.14 fu/g (n=10) at 60, 120 and 180 minutes of ischaemia.
The values for the control group were 7.51 ± 1.44 fu/g (n=10), 7.25 ± 1.44 fu/g (n=10) and 6.84 ± 1.51 fu/g (n=10) at 60, 120 and 180 minutes.

The mean ratio for freshly excised liver was 1.00 ± 0.25 (n=10) in the treated group and 1.00 ± 0.11 (n=10) in the control.
The values in the treated group were 0.85 ± 0.29 (n=10), 0.91 ± 0.18 (n=10) and 0.86 ± 0.31 (n=10) at 60, 120 and 180 minutes ischaemia.
The equivalent values for the control group were 0.88 ± 0.24 (n=10), 0.85 ± 0.12 (n=10) and 0.73 ± 0.25 (n=10) at 60, 120 and 180 minutes.

In both groups there was a fall in the level of dienes with ischaemia from that of the freshly excised liver. There was no significant difference between the treated and control group.

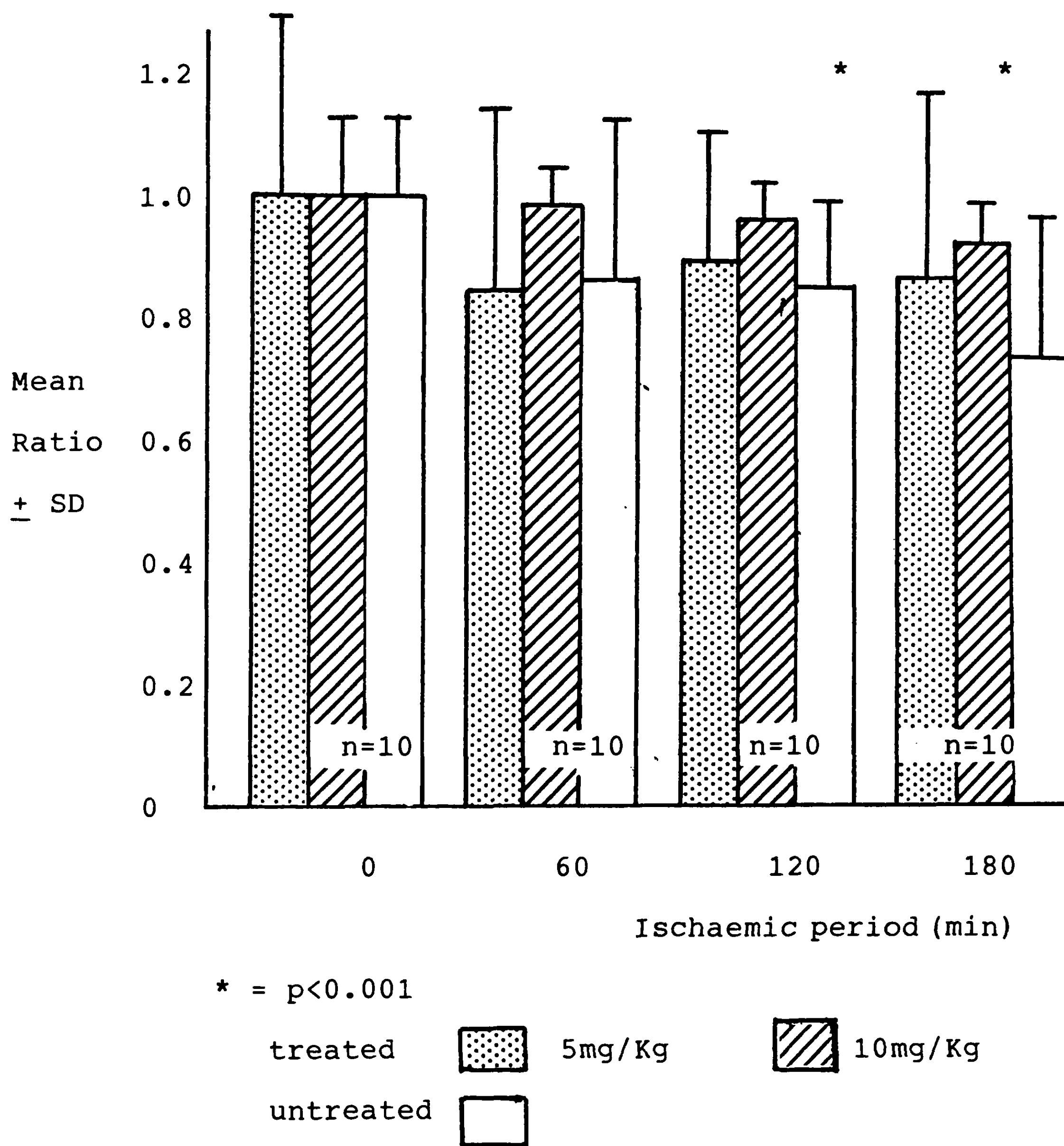


FIGURE 4.1x Graph to show the effect of allopurinol on conjugated dienes in rat liver during normothermic ischaemia.

The level of conjugated dienes fell withischaemia in all groups. There was a significantly higher level of dienes at 60 and 120 minutes in the 10mg/Kg group only ($p<0.05$).

ii. <u>10mg/Kg allopurinol</u>

For the higher dose of allopurinol the diene production in freshly excised liver was 9.01 ± 1.02 fu/g (n=10) in the treated group and 8.61 ± 1.02 fu/g (n=10) in the control.
The values during ischaemia in the treated group were 8.77 ± 0.76 fu/g (n=10), 8.48 ± 1.05 fu/g (n=10) and 8.11 ± 0.77 fu/g (n=10) at 60, 120 and 180 minutes.
In the control group the values were 7.51 ± 1.37 fu/g (n=10), 7.25 ± 1.44 fu/g (n=10) and 6.84 ± 1.51 fu/g (n=10) at 60, 120 and 180 minutes.

The mean ratio for freshly excised liver in both groups was 1.00 ± 0.11 (n=10).
In the treated group the mean ratio fell slowly to 0.98 ± 0.06 (n=10), 0.95 ± 0.05 (n=10) and 0.91 ± 0.05 (n=10) at 60, 120 and 180 minutes respectively.
The equivalent control values were 0.88 ± 0.24 (n=10), 0.85 ± 0.12 (n=10) and 0.73 ± 0.25 (n=10) at 60, 120 and 180 minutes.

There was no significant fall in diene level in the treated group until 180 minutes of ischaemia ($p<0.05$). The diene levels were higher in the treated group than the control at 60 and 120 minutes ($p<0.05$).

Desferrioxamine

i. 5mg/Kg desferrioxamine

The initial level of dienes in the treated group was 10.69 ± 0.98 fu/g (n=10) and that of the control group was 8.61 ± 1.02 fu/g (n=10).
During ischaemia in the treated group the level reached 9.78 ± 0.73 fu/g (n=10), 9.41 ± 0.63 fu/g (n=10) and 10.40 ± 0.97 fu/g (n=10) at 60, 120 and180 minutes.
The control values were 7.51 ± 1.37 fu/g (n=10), 7.25 ± 1.44 fu/g (n=10) and 6.84 ± 1.51 fu/g (n=10) at 60, 120 and 180 minutes.

The mean ratio for the feshly excised liver was 1.00 ± 0.09 (n=10) in the treated group and 1.00 ± 0.11 (n=10) in the control.
In the treated group the value of the mean ratio fell to 0.92 ± 0.07 (n=10), 0.89 ± 0.09 (n=10) and 0.99 ± 0.12 (n=10) at 60, 120 and 180 minutes of ischaemia.
The control values were 0.88 ± 0.24 (n=10), 0.85 ± 0.12 (n=10) and 0.73 ± 0.25 (n=10) at 60, 120 and 180 minutes.

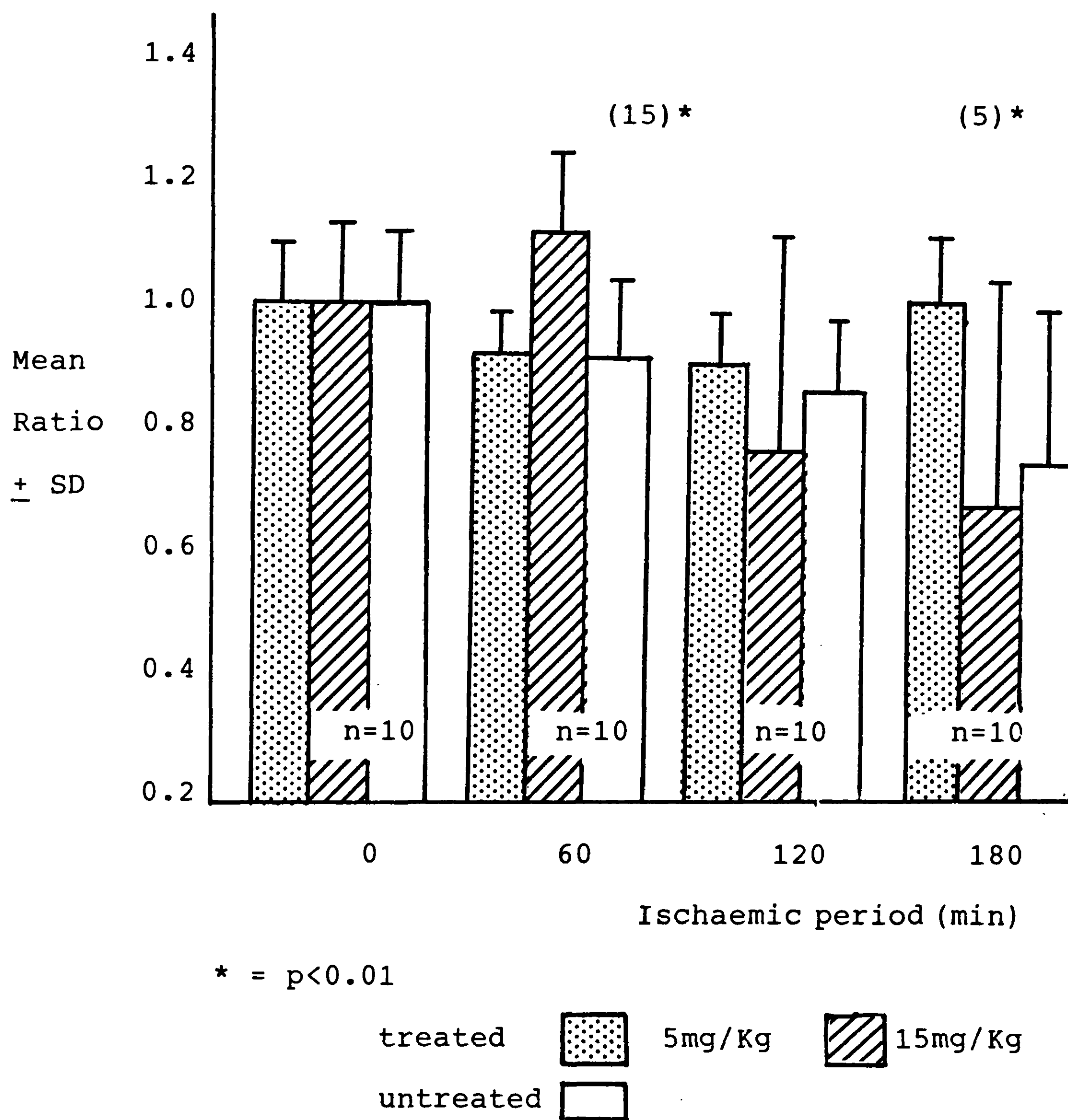


FIGURE 4.1xi Graph to show the effect of desferrioxamine on conjugated diene level in rat liver during normothermic ischaemia.

The level of conjugated dienes decreased significantly during ischaemia. Desferrioxamine maintained the level over control at 180 minutes, for 5mg/Kg ($p<0.01$) and 60 minutes for 15mg/Kg ($p<0.01$).

In the group treated with 5mg/Kg desferrioxamine, there is a significant fall in the level of dienes at 60 minutes ($p<0.05$) and 120 minutes ($p<0.02$). There is no significant difference between the treated and control groups until 180 minutes, when the level of dienes is higher in the treated group ($p<0.01$).

ii. <u>15mg/Kg desferrioxamine</u>

The level of conjugated dienes in the freshly excised liver was 9.30 $\pm$ 0.91 fu/g (n=10) in the treated group and 8.61 $\pm$ 1.02 fu/g (n=10) in the control.
During ischaemia the levels in the treated group were 10.25 $\pm$ 0.87 fu/g (n=10), 6.93 $\pm$ 3.00 fu/g (n=10) and 6.02 $\pm$ 3.36 fu/g (n=10) at 60, 120 and 180 minutes.
The control values were 7.51 $\pm$ 1.37 fu/g (n=10), 7.25 $\pm$ 1.44 fu/g (n=10) and 6.84 $\pm$ 1.51 fu/g (n=10) at 60, 120 and 180 minutes ischaemia.

The mean ratio for the freshly excised liver was 1.00 $\pm$ 0.12 (n=10) in the treated group and 1.00 $\pm$ 0.11 (n=10) in the control.

In the treated group the ratios during ischaemia were 1.12 ± 0.13 (n=10), 0.76 ± 0.32 (n=10) and 0.66 ± 0.35 (n=10) at 60, 120 and 180 minutes.
In the control group the equivalent values were 0.88 ± 0.24 (n=10), 0.85 ± 0.12 (n=10) and 0.73 ± 0.25 (n=10) at 60, 120 and 180 minutes ischaemia.

When the rats were treated with 15mg/Kg desferrioxamine, the trend over 3 hours ischaemia was an increase in conjugated dienes at 60 minutes followed by a fall at 120 ($p<0.05$) and 180 ($p<0.01$) minutes. There was a significant difference at 60 minutes between the treated and control group. The diene level was maintained at a higher value by desferrioxamine.

Mannitol

1g/Kg mannitol

The level of dienes in the freshly excised liver was 9.08 ± 0.49 fu/g (n=10) in the treated group and 8.61 ± 1.02 fu/g (n=10) in the control.
In the mannitol treated group the diene levels fell steadily to 7.98 ± 0.66 fu/g (n=10), 7.13 ± 0.53 fu/g (n=10) and 6.33 ± 0.91 fu/g (n=10) after 60, 120 and 180 minutes ischaemia.
The control levels also fell steadily to 7.51 ± 1.37 fu/g (n=10), 7.25 ± 1.44 fu/g (n=10) and 6.84 ± 1.51 fu/g (n=10) at 60, 120 and 180 minutes respectively.

This trend was shown in the mean ratios. Initial values were 1.00 ± 0.05 (n=10) in the treated group and 1.00 ± 0.11 (n=10) in the control.
In the treated group the values were 0.85 ± 0.05 (n=10), 0.79 ± 0.08 (n=10) and 0.71 ± 0.11 (n=10) after 60, 120 and 180 minutes ischaemia.
The control values were 0.88 ± 0.24 (n=10), 0.85 ± 0.12 (n=10) and 0.73 ± 0.25 (n=10) at 60, 120 and 180 minutes.

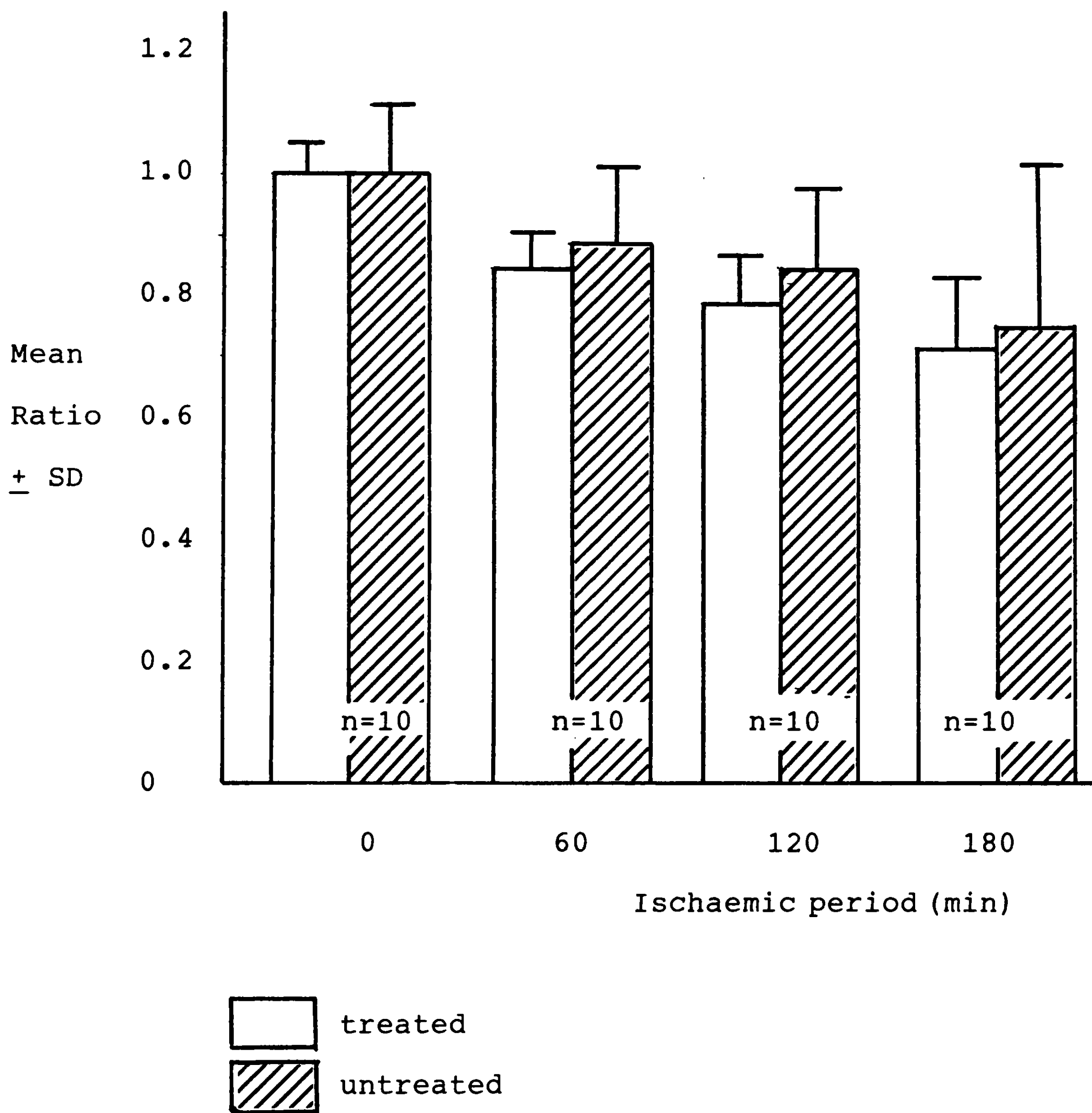


FIGURE 4.1xii **Graph to show the effect of mannitol on conjugated diene production in rat liver during normothermic ischaemia.**

Conjugated diene level fell in both treated and control groups during ischaemia. There was no significant difference between the two groups.

In the group treated with mannitol, there is a highly significant reduction in diene level over the initial value at 60, 120 and 180 minutes (all $p<0.001$).

There is no significant difference between the treated and untreated groups.

c. **The effect of free radical scavengers on the glutathione ratio**

SUPEROXIDE DISMUTASE

Again 3 doses of superoxide dismutase were used together with denatured superoxide dismutase. All were administered intravenously as shown in the methods chapter 2.

The glutathione ratio (oxidised glutathione/reduced glutathione) was studied in these experiments as an indicator of the changes in glutathione during ischaemia.

i. 500u/Kg superoxide dismutase

The glutathione ratio of the freshly excised control liver was 0.37 ± 0.07 (n=10) whereas that of the treated group was lower at 0.25 ± 0.05 (n=10). During ischaemia in the treated group the ratio increased to 0.31 ± 0.05 (n=10) at 60 minutes, 0.44 ± 0.09 (n=10) at 120 minutes and 0.57 ± 0.14 (n=10) at 180 minutes.

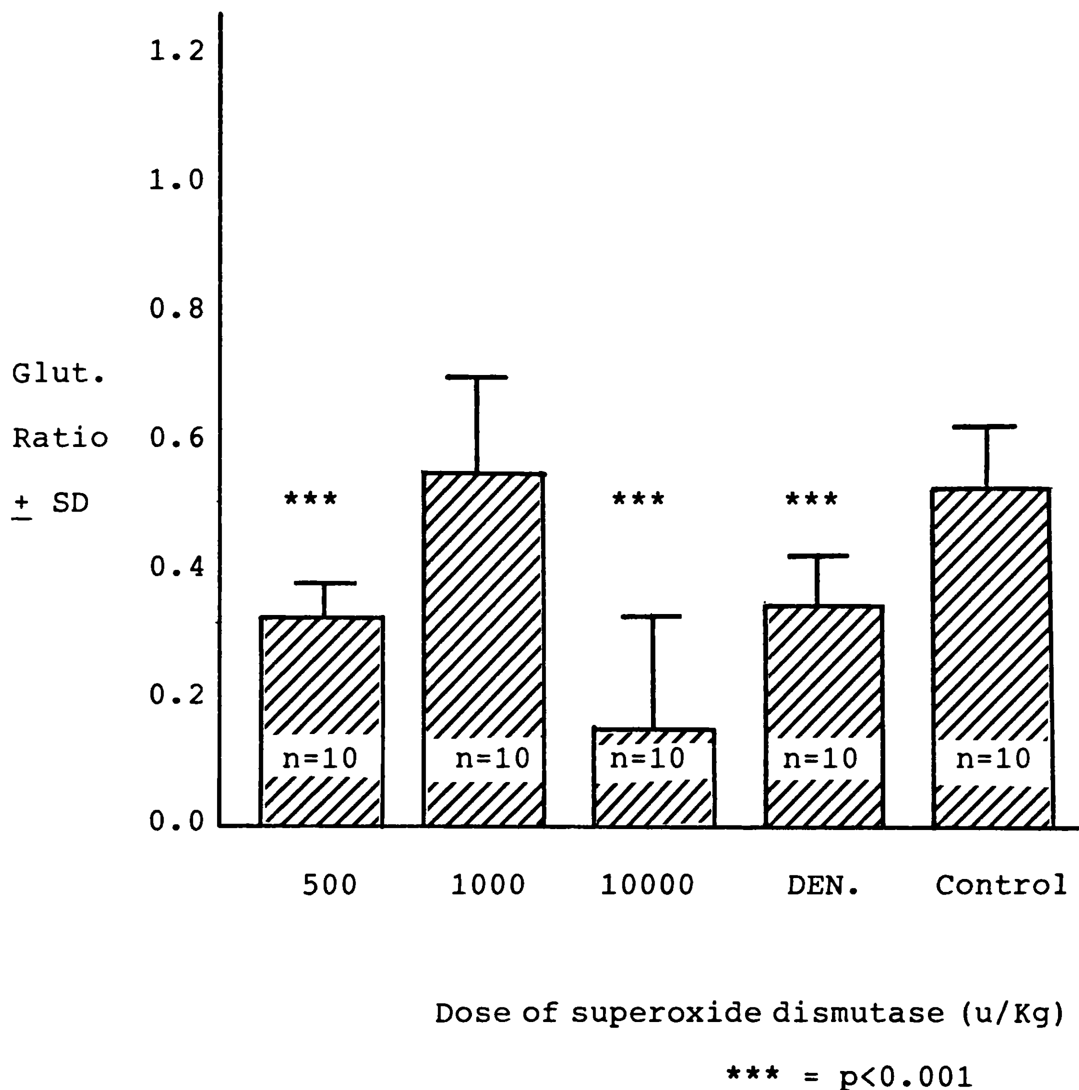


FIGURE 4.1xiii **Graph to show the effect of superoxide dismutase on glutathione ratio in the rat liver at 60 minutes normothermic ischaemia.**

The glutathione ratio was maintained at a significantly lower value than the control group with 500u/Kg and 10000u/Kg superoxide dismutase ($p<0.001$).

The ratio was much higher in the control group, yielding 0.50 ± 0.10 (n=10) at 60 minutes, 0.79 ± 0.19 (n=10) at 120 minutes and 0.96 ± 0.19 (n=10) at 180 minutes ischaemia.

The glutathione ratio in the control group progressively rose with ischaemia. Significant increases over those of the freshly excised liver were achieved at 60 minutes ($p<0.01$), 120 and 180 minutes ($p<0.001$).

In the group treated with superoxide dismutase the ratio was also seen to rise with increasing periods of ischaemia. The increase only became significant at 120 and 180 minutes ($p<0.001$).

The glutathione ratio was significantly reduced in the treated group at all time points ($p<0.001$) including the initial value.

ii. <u>1000u/Kg superoxide dismutase</u>

The initial value of the glutathione ratio in the treated group was 0.32 ± 0.02 (n=10) and that for the control 0.37 ± 0.07 (n=10).

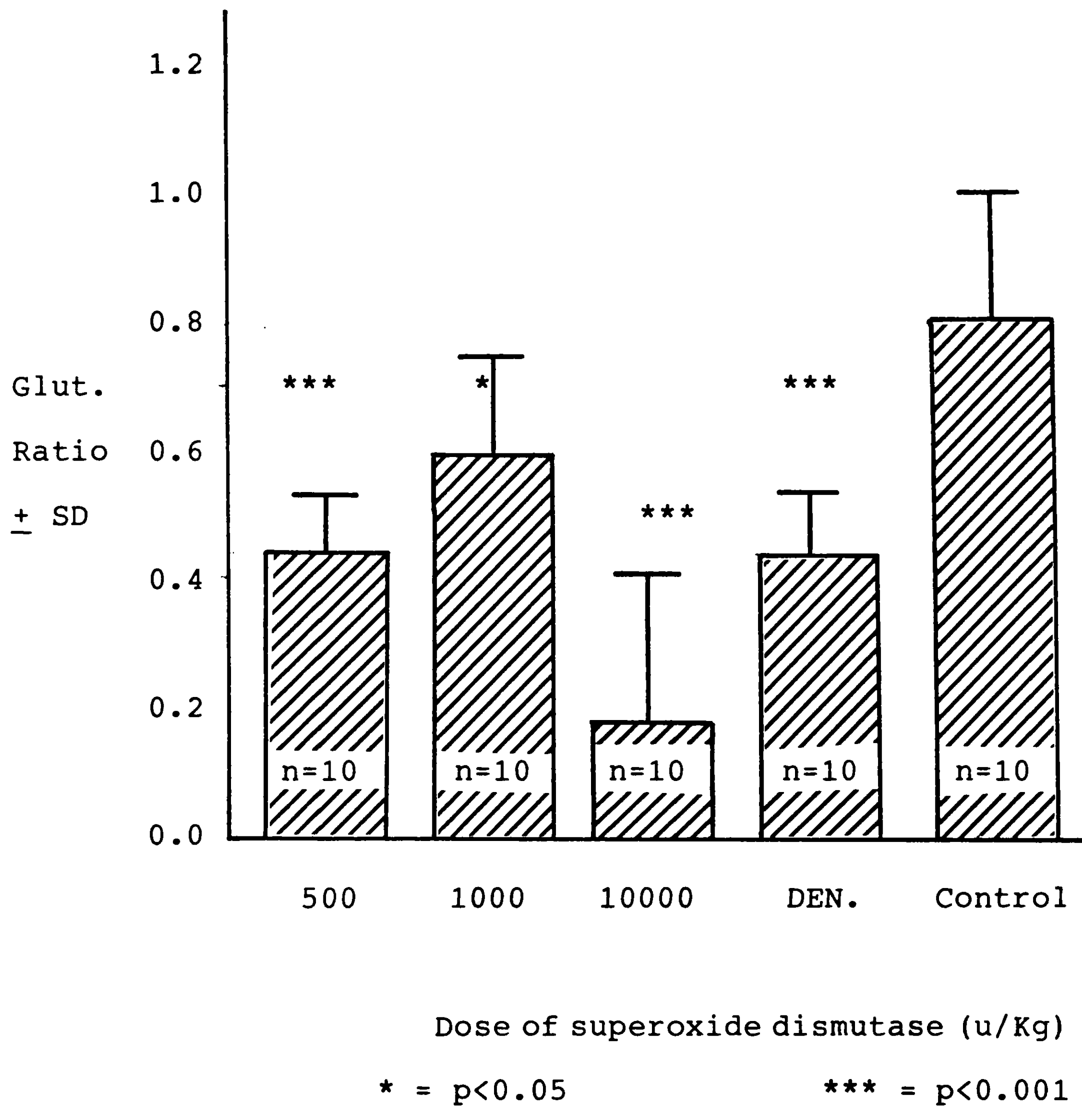


FIGURE 4.1xiv **Graph to show the effect of superoxide dismutase on glutathione ratio in the rat liver at 120 minutes normothermic ischaemia.**

The glutathione ratio was maintained at a significantly lower value than the control group with 500u/Kg and 10000u/Kg superoxide dismutase ($p<0.001$), and with 1000u/Kg ($p<0.05$).

In the treated group the values were 0.55 ± 0.14 (n=10), 0.59 ± 0.15 (n=10) and 0.96 ± 0.27 (n=10) at 60, 120 and 180 minutes ischaemia.
The equivalent control values were 0.50 ± 0.10 (n=10), 0.79 ± 0.19 (n=10) and 0.96 ± 0.19 (n=10) at 60, 120 and 180 minutes.

In the group treated with superoxide dismutase at a dose of 1000u/Kg there was a progressive rise in glutathione ratio with ischaemia, significantly higher than the initial value at 120 and 180 minutes ($p<0.001$.
There was no difference between the groups except at 120 minutes, when the treated group had a lower glutathione ratio ($p<0.05$).

iii. <u>10000u/Kg superoxide dismutase</u>

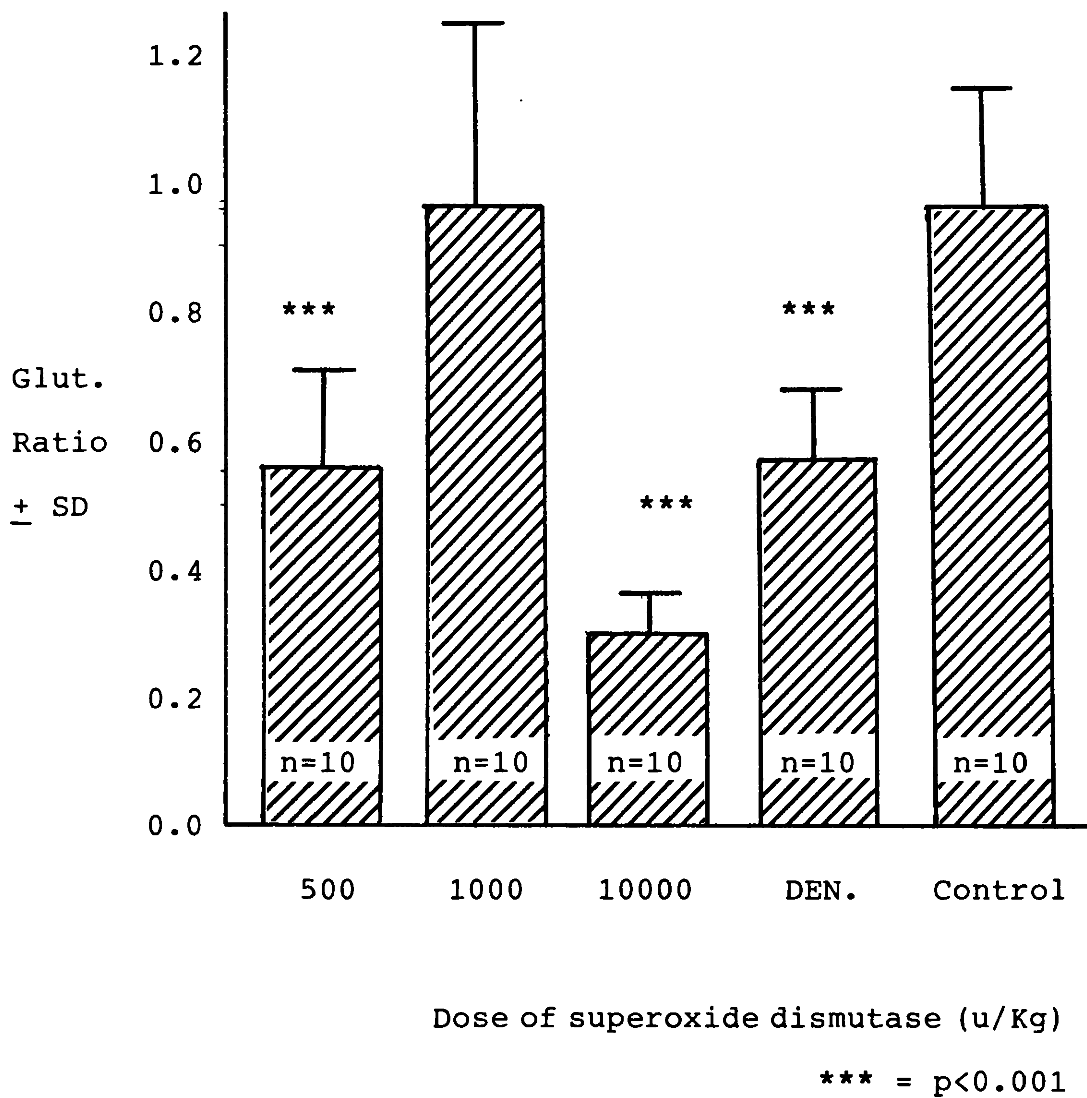
The glutathione ratio for the freshly excised liver in the treated group was 0.12 ± 0.13 (n=10), much lower than the control group 0.37 ± 0.07 (n=10).
In the treated group the ratios increased, reaching 0.15 ± 0.17 (n=10), 0.17 ± 0.22 (n=10) and 0.28 ± 0.06 (n=10) at 60, 120 and 180 minutes of ischaemia.
The control values were higher at 0.50 ± 0.10 (n=10), 0.79 ± 0.19 (n=10) and 0.96 ± 0.19 (n=10) at 60, 120 and 180 minutes.

Once again there was an increase in glutathione ratio with increasing ischaemia, becoming significant at 60, 120 and 180 minutes ($p<0.001$). The treatment of the rats with 10000u/Kg superoxide dismutase significantly reduced the glutathione ratio at all time points ($p<0.001$).

iv. Denatured superoxide dismutase

The initial ratio in the treateed group was 0.21 $\pm$ 0.05 (n=10) and that of the control was 0.37 $\pm$ 0.07 (n=10).
In the treated group there was a steady increase in glutathione ratio with ischaemia. The values were 0.33 $\pm$ 0.06 (n=10), 0.45 $\pm$ 0.09 (n=10) and 0.56 $\pm$ 0.10 (n=10) at 60, 120 and 180 minutes.
The control values were 0.50 $\pm$ 0.10 (n=10), 0.79 $\pm$ 0.19 (n=10) and 0.96 $\pm$ 0.19 (n=10) at 60, 120 and 180 minutes respectively.

**There is a similar progression in glutathione ratios to that of the control. Increases in the ratio over the fresh liver were seen at the three time points during ischaemia ($p<0.001$).
The ratios however were all significantly lower than those of the control ($p<0.001$).**



Dose of superoxide dismutase (u/Kg)

*** = p<0.001

FIGURE 4.1xv **Graph to show the effect of superoxide dismutase on glutathione ratio in the rat liver at 180 minutes normothermic ischaemia.**

The glutathione ratio was maintained at a significantly lower value than the control group with 500u/Kg and 10000u/Kg superoxide dismutase (p<0.001).

Summary

When rats are treated with superoxide dismutase, there is, in general, a reduction in the glutathione ratio during progressive ischaemia. This was seen well in the 500u/Kg and 10000u/Kg groups but was not as marked in the 1000 u/Kg group.

The denaturation of superoxide dismutase with diethyldithiocarbamate did not return the glutathione ratio to the control values in this case.

b. The effect of allopurinol on the glutathione ratio during liver ischaemia

i. 5mg/Kg allopurinol

The value of the glutathione ratio for freshly excised liver was 0.21 $\pm$ 0.03 (n=10) in the treated group and 0.37 $\pm$ 0.07 (n=10) in the control.
In the treated group the ratio rose to 0.26 $\pm$ 0.04 (n=10) at 60 minutes, 0.34 $\pm$ 0.06 (n=10) at 120 minutes and 0.32 $\pm$ 0.04 (n=10) at 180 minutes.
The ratios of the control group were higher at 0.50 $\pm$ 0.1 (n=10), 0.79 $\pm$ 0.19 (n=10) and 0.96 $\pm$ 0.19 (n=10) at 60, 120 and 180 minutes of ischaemia.

**Within the treated group there was a significant increase in glutathione ratio at 60 minutes ($p<0.01$), 120 and 180 minutes ($p<0.001$).
There was a significant reduction in the glutathione ratio in the allopurinol treated group at all time points ($p<0.001$), compared with the control group.
The freshly excised liver had a significantly lower ratio in the treated group ($p<0.001$).**

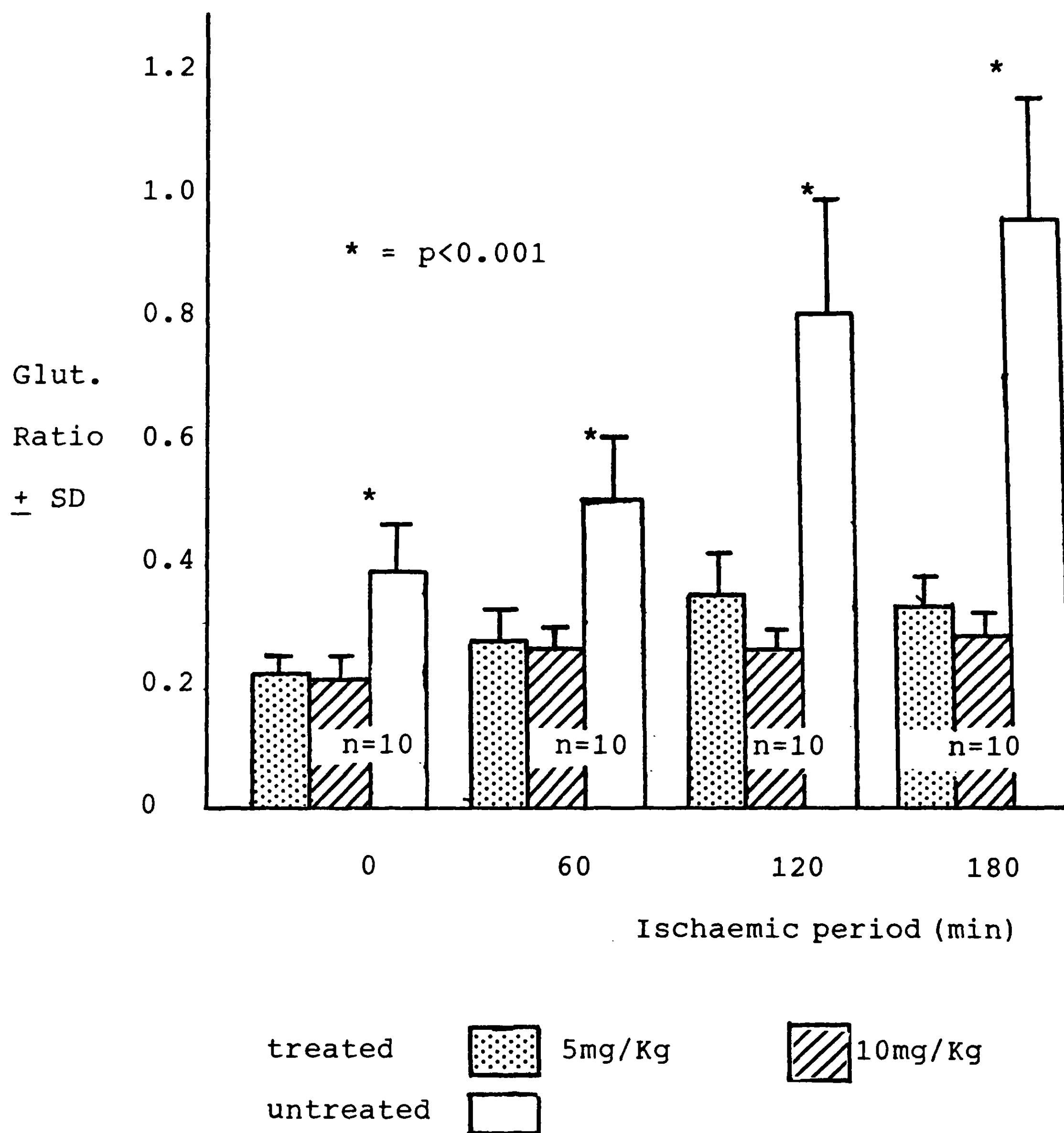


FIGURE 4.1xvi **Graph to show the effect of allopurinol on glutathione ratio in rat liver during normothermic ischaemia.**

The glutathione ratio rose with ischaemia in all groups. There was a highly significant reduction in the glutathione ratio over control at all time points at both doses.

ii. 10mg/Kg allopurinol

The values of glutathione ratio for freshly excised liver were 0.20 ± 0.02 (n=10) for the treated group and 0.37 ± 0.07 (n=10) for the control.

There was a gradual increase in the glutathione ratio within the treated group to 0.24 ± 0.03 (n=10) at 60 minutes, 0.24 ± 0.03 (n=10) at 120 and 0.28 ± 0.03 (n=10) at 180 minutes of ischaemia.

The control values were 0.50 ± 0.10 (n=10), 0.79 ± 0.19 (n=10) and 0.96 ± 0.19 (n=10) at 60, 120 and 180 minutes respectively.

There was a significant increase in glutathione ratio within the group treated with 10mg/Kg allopurinol, at 60, 120 ($p<0.01$) and 180 minutes ($p<0.001$).

There was a highly significant reduction in glutathione ratio in the allopurinol treated group compared with the control. This occurred at all time points including the initial value ($p<0.001$).

c. The effect of desferrioxamine on glutathione ratio in liver ischaemia

i. 5mg/Kg desferrioxamine

The glutathione ratio for freshly excised liver was 0.62 ± 0.12 (n=10) in the treated group and 0.37 ± 0.07 (n=10) in the control.
There was an increase in the ratio as ischaemia progressed. In the treated group the values were 0.87 ± 0.14 (n=10), 1.08 ± 0.14 (n=10) and 1.02 ± 0.15 (n=10) at 60, 120 and 180 minutes.
Control values were lower at 0.50 ± 0.10 (n=10), 0.79 ± 0.19 (n=10) and 0.96 ± 0.19 (n=10) at 60, 120 and 180 minutes ischaemia.

At the lower dose of desferrioxamine there is an increase in the glutathione ratio within the group at 60, 120 and 180 minutes ($p<0.001$) over the initial value.
Interestingly the glutathione ratio is substantially increased over the control group at 60 minutes ($p<0.001$) and 120 minutes ($p<0.01$). Although the ratio was higher at 180 minutes, statistical significance was not obtained.

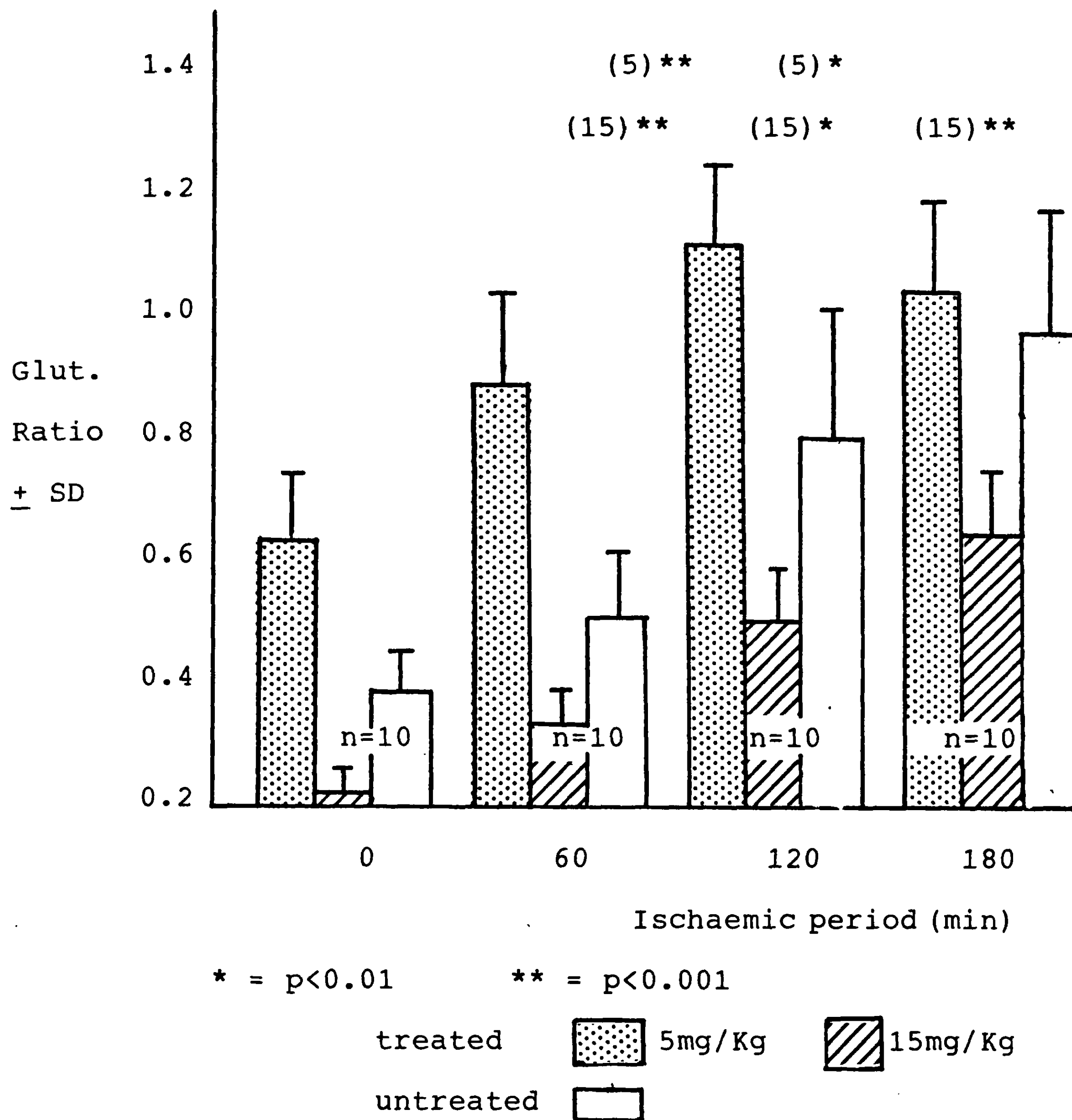


FIGURE 4.1xvii Graph to show the effect of desferrioxamine on glutathione ratio in rat liver during normothermic ischaemia.

The glutathione ratio increased with ischaemia in all groups. Desferrioxamine 5mg/Kg <u>increased</u> the level at 60 ($p<0.01$) and 180 ($p<0.001$) minutes. 15mg/Kg <u>reduced</u> the level at 60, 120 and 180 minutes.

ii. <u>15mg/Kg desferrioxamine</u>

The glutathione ratio of the freshly excised liver was 0.21 ± 0.05 (n=10) in the treated group and 0.37 ± 0.07 (n=10) in the control.
During ischaemia the ratio in the treated group rose to 0.30 ± 0.08 (n=10), 0.52 ± 0.09 (n=10) and 0.64 ± 0.11 (n=10) at 60, 120 and 180 minutes.
The values for the control group were 0.50 ± 0.10 (n=10), 0.79 ± 0.19 (n=10) and 0.96 ± 0.19 (n=10) at 60, 120 and 180 minutes.

The glutathione ratio in the group treated with the higher dose of desferrioxamine rose with ischaemia, to achieve significance at 60 ($p<0.01$), 120 and 180 ($p<0.001$) minutes over the fresh liver.
15mg/Kg desferrioxamine reduced the glutathione ratio when compared with the control group. Significant reduction occurred at 60 ($p<0.001$), 120 ($p<0.01$) and 180 ($p<0.001$) minutes.

d. **The effect of mannitol on the glutathione ratio in liver ischaemia**

A single dose of 1g/Kg mannitol was used as in previous experiments.

The glutathione ratio in the freshly excised rat liver was 0.47 ± 0.08 (n=10) in the treated group and 0.37 ± 0.07 (n=10) in the control.
The ratio increased in the treated group to 0.59 ± 0.12 (n=10), 0.78 ± 0.20 (n=10) and 0.97 ± 0.14 (n=10) at 60, 120 and 180 minutes of ischaemia respectively.
The values for the control were 0.50 ± 0.10 (n=10), 0.79 ± 0.19 (n=10) and 0.96 ± 0.19 (n=10) at 60, 120 and 180 minutes.

In the treated group the glutathione ratio of the freshly excised liver was significantly higher than control ($p<0.01$). The ratio rose significantly at 60 minutes ($p<0.02$), 120 and 180 minutes ($p<0.001$). There was no significant difference between the treated and untreated groups during 180 minutes ischaemia.

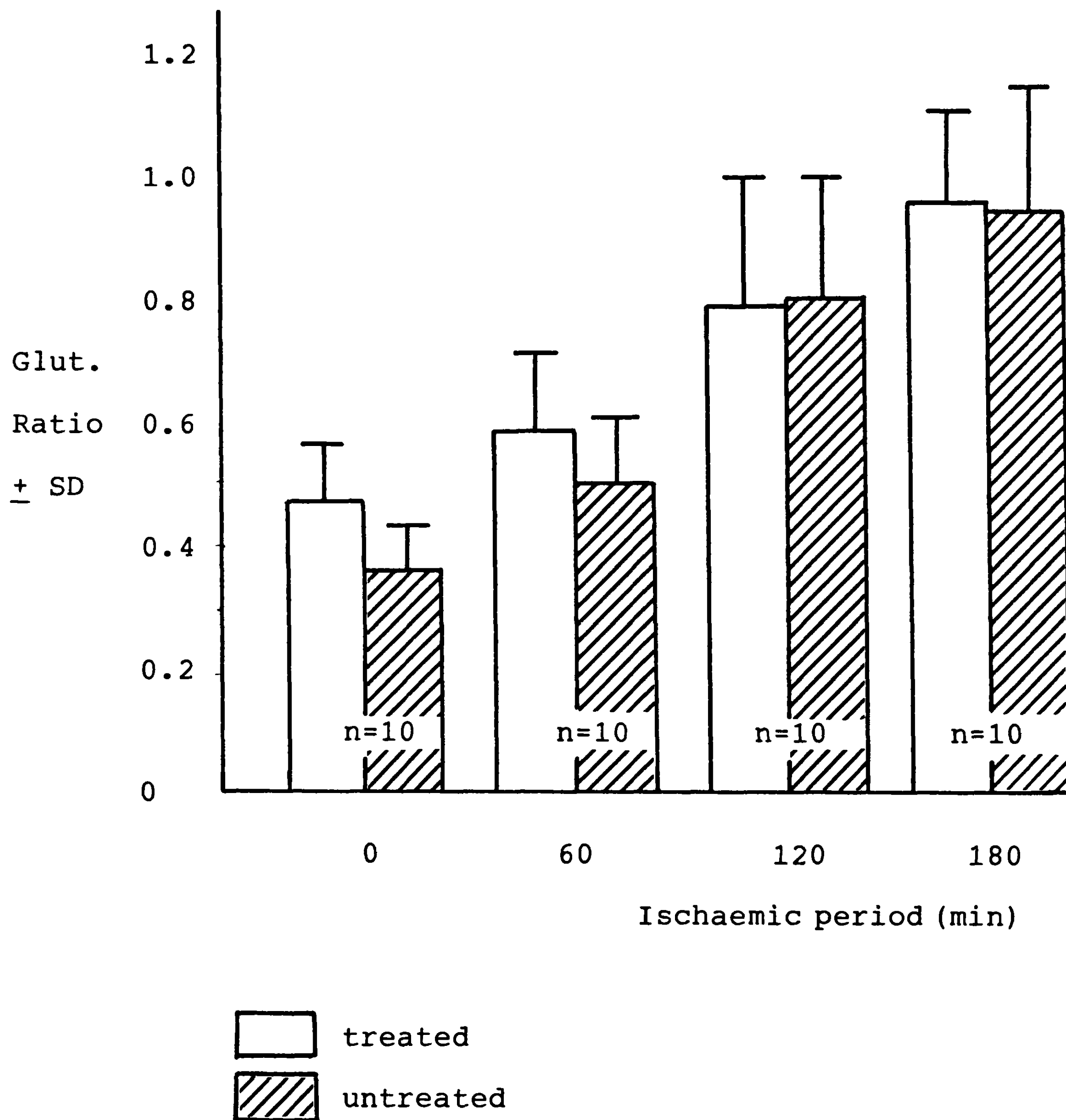


FIGURE 4.1xviii **Graph to show the effect of 1g/Kg mannitol on glutathione ratio in rat liver during normothermic ischaemia.**

The glutathione ratio rose with ischaemia in both groups. There was no difference between treated and untreated groups.

Summary of effects of free radical scavenging agents on normothermic ischaemia in rat liver

Effects of superoxide dismutase

Malondialdehyde production was reduced at 60, 120 and 180 minutes (p,0.001) by superoxide dismutase but only at the highest dose of 10,000 u/Kg.

Superoxide dismutase maintained the level of conjugated dienes at all doses used ($p<0.02$-$p<0.001$). The peak level of conjugated dienes occurred at 60 minutes ischaemia. The dose of 1000 u/Kg yielded the most significant values.

Superoxide dismutase reduced the value of the glutathione ratio over that of the control ($p<0.01$ - $p<0.001$) at all time points. 10,000 u/Kg appeared to be the most effective dose.

Superoxide dismutase was therefore an effective free radical scavenger in this system because it reduced the progression of the measured parameters of lipid peroxidation.

When the superoxide dismutase was denatured with diethyldithiocarbamate, the effect on malondialdehyde levels and conjugated diene levels was abolished. There was no significant difference over control values.

When the effect of denaturation of superoxide dismutase on glutathione ratio was studied, it was noted that the lowering of the ratio over control was still observed.

The Effect of Allopurinol

Allopurinol was successful in preventing the rise in malondialdehyde level observed in the control group during ischaemia.

This became significant with the higher dose of 10 mg/kg at 60 minutes of ischaemia ($p<0.05$) and reached a highly significant result at 180 minutes ($p<0.001$).

With the lower dose of 5mg/Kg the level of malondiaaldehyde was reduced significantly at 120 minutes ischaemia ($p<0.05$).

The conjugated diene level was maintained by the administration of 10mg/Kg allopurinol at 60 and 120 minutes ischaemia ($p<0.05$).

The lower dose of allopurinol was ineffective in maintaining the diene level and the results were not significantly different from the saline control.

Allopurinol was very effective in lowering the glutathione ratio during ischaemia.

The initial values of the ratio were significantly lower than those of the control group with both doses of allopurinol.

Significant reduction in the glutathione ratio by both doses of allopurinol was seen at all time points ($p<0.001$).

Allopurinol was shown to be an effective scavenger in this system. It had a very significant effect on the glutathione ratio. The higher dose used in the study was the more effective.

The Effect of Desferrioxamine

The beneficial effect of desferrioxamine on malondialdehyde production only became significant at 180 minutes ischaemia with both 5mg/Kg ($p<0.001$) and 15mg/Kg ($p<0.01$).
The effect of 5mg/Kg desferrioxamine on conjugated diene level became significant at 180 minutes ($p<0.01$), with the diene level maintained at a higher level than the control.

The effect of 15mg/Kg desferrioxamine had its effect on conjugated dienes at 60 minutes with a level significantly higher than control ($p<0.01$).

5mg/Kg desferrioxamine interestingly raised the glutathione ratio significantly at 60 minutes ($p<0.001$) and 120 minutes ($p<0.01$).

At the higher dose of 15mg/Kg desferrioxamine there was a beneficial effect on the glutathione ratio with a significant reduction in the ratio at all time points ($p<0.01$-$p<0.001$).

The effect of desferrioxamine as a free radical scavenger in this system was only seen at the higher dose of 15mg/Kg. There was an effect on conjugated diene level and on glutathione ratio.

Desferrioxamine was not a very effective scavenger when malondialdehyde was studied.

The Effect of Mannitol

Mannitol at a dose of 1g/Kg raised the level of malondialdehyde production over control at 60 and 120 minutes ($p<0.001$).

Mannitol did not maintain the level of conjugated dienes at any time point.

Mannitol did not significantly reduce the value of the glutathione ratio.

Mannitol at a dose of 1g/Kg did not appear to be a useful scavenger in this system.

4.2 THE EFFECT OF FREE RADICAL SCAVENGERS ON LIVER METABOLISM IN THE RAT DURING NORMOTHERMIC ISCHAEMIA

Introduction

In the previous chapter, the urea production of rat liver slices was measured during periods of normothermic ischaemia lasting up to 180 minutes. The results showed a statistically significant fall in urea production with ischaemia at 60, 120 and 180 minutes ($p<0.001$).

It was also shown that there were statistically significant changes in lipid peroxidation products (malondialdehyde and conjugated dienes) during equivalent periods of ischaemia. The changes in glutathione status which were demonstrated in the previous chapter also indicated that significant lipid peroxidation had taken place.

The aim of this experiment was to demonstrate that free radical activity had an effect on liver metabolism via lipid peroxidation.

In the earlier part of this chapter it was shown that certain scavenging agents specific for free radicals reduced the levels of lipid peroxidation

products during normothermic ischaemia. Certain agents significantly limited the increase in glutathione ratio, thus indicating a reduction in lipid peroxidation.

When these specific scavenging agents are administered prior to ischaemia they will reduce the lipid peroxidation due to free radical activity. Thus it will demonstrate how much of the decrease in urea production is due to free radical activity.

Method

The experimental method is shown in the methods chapter 2.

The scavenging agents chosen for this study were those which were shown to have a statistically significant effect on lipid peroxidation in previous experiments.

The agents were :-

10000u/Kg superoxide dismutase
10mg/Kg allopurinol
15mg/Kg desferrioxamine

a. <u>The effect of superoxide dismutase</u>

The urea production for the freshly excised liver in the treated group was 105.51 ± 13.10 mmoles/g (n=10) and that of the control 212.59 ± 43.98 mmoles/g (n=10).

In the treated group the urea production fell to 47.43 ± 15.73 mmoles/g (n=10) at 60 minutes, 49.36 ± 11.74 mmoles/g (n=10) at 120 minutes and 34.42 ± 8.52 mmoles/g (n=10) at 180 minutes.

In the control group the equivalent values were 73.11 ± 31.20 mmoles/g (n=10), 51.07 ± 18.85 mmoles/g (n=10) and 49.67 ± 34.00 mmoles/g (n=10) at 60, 120 and 180 minutes ischaemia.

The mean ratios were calculated and were shown to be 1.00 ± 0.13 (n=10) in both groups.

In the treated group the values were 0.47 ± 0.18 (n=10) at 60 minutes, 0.48 ± 0.13 (n=10) at 120 minutes and 0.34 ± 0.11 (n=10) at 180 minutes.

The control values were 0.34 ± 0.13 (n=10), 0.25 ± 0.10 (n=10) and 0.23 ± 0.14 (n=10) at 60, 120 and 180 minutes ischaemia.

The urea production decreased during ischaemia in both groups. At 60, 120 and 180 minutes there was asignificant fall over the freshly excised liver ($p<0.001$) in both groups.

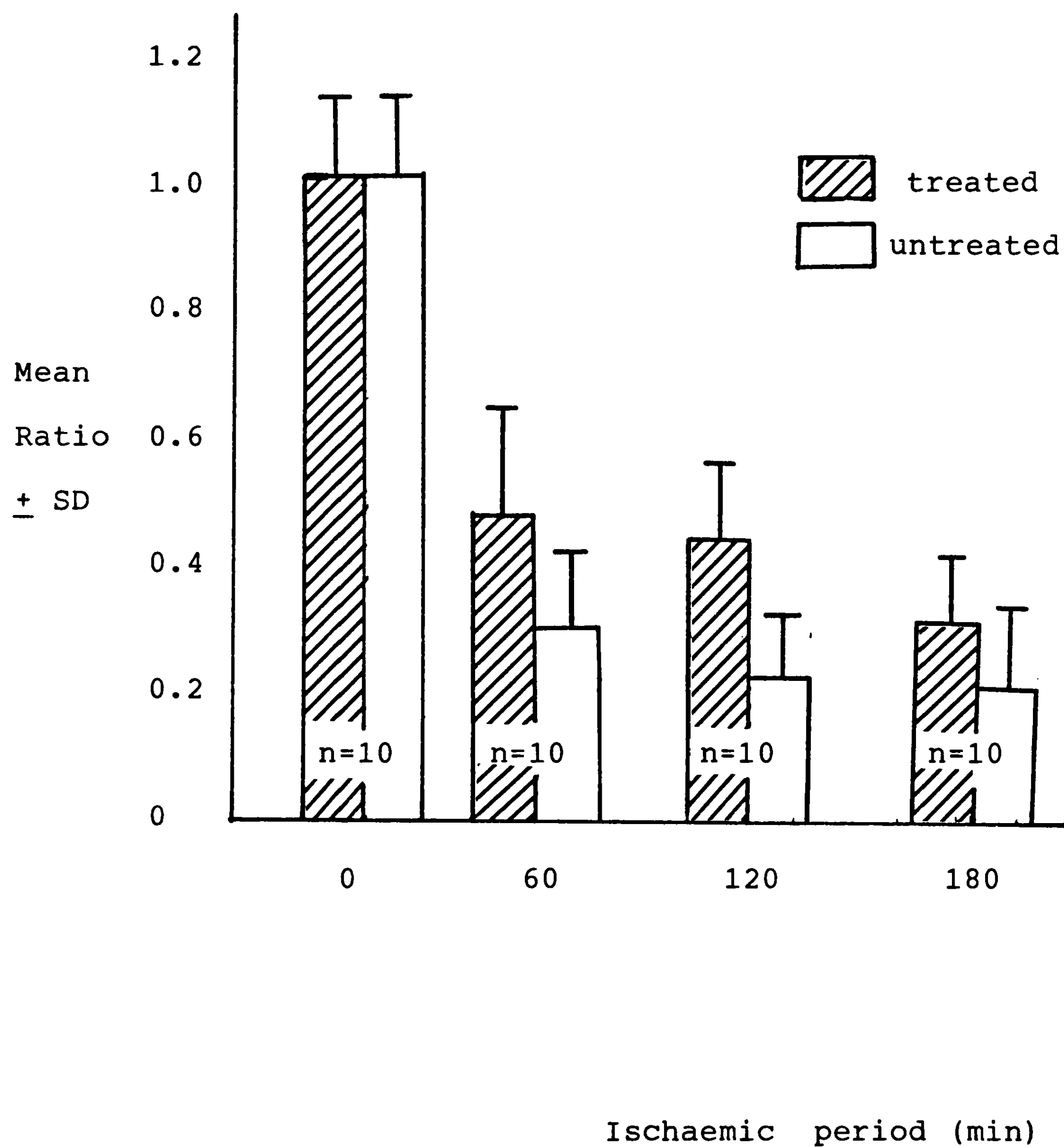


FIGURE 4.2i

Graph to show the effect of 10000u/Kg superoxide dismutase on urea production in rat liver during normothermic ischaemia.

Superoxide dismutase significantly maintains urea production at 120 minutes ischaemia.

The mean ratio urea production was higher in the group treated with superoxide dismutase and achieved significance at 120 minutes of ischaemia $p<0.001$).

b. <u>The effect of allopurinol</u>

The urea production in the freshly excised liver was 257.60 $\pm$ 81.15 mmoles/g (n=10) in the treated group and 212.59 $\pm$ 43.98 mmoles/g (n=10) in the control. In the treated group the urea production fell to 94.10 $\pm$ 58.33 mmoles/g (n=10) at 60 minutes, 81.72 $\pm$ 47.97 mmoles/g (n=10) at 120 minutes and 75.80 $\pm$ 43.92 mmoles/g (n=10) at 180 minutes.
In the control group the values were 73.11 $\pm$ 31.20 mmoles/g (n=10), 51.07 $\pm$ 18.85 mmoles/g (n=10) and 49.67 $\pm$ 34.00 mmoles/g (n=10) at 60, 120 and 180 minutes.

The mean ratio of urea production in the freshly excised liver was 1.00 $\pm$ 0.32 (n=10) in the treated group and 1.00 $\pm$ 0.13 (n=10) in the control.
In the treated group the ratio fell to 0.35 $\pm$ 0.13 (n=10) at 60 minutes, 0.31 $\pm$ 0.14 (n=10) at 120 minutes and 0.28 $\pm$ 0.14 (n=10) at 180 minutes.
In the control the equivalent values were 0.34 $\pm$ 0.13 (n=10), 0.25 $\pm$ 0.10 (n=10) and 0.23 $\pm$ 0.14 (n=10) at 60, 120 and 180 minutes.

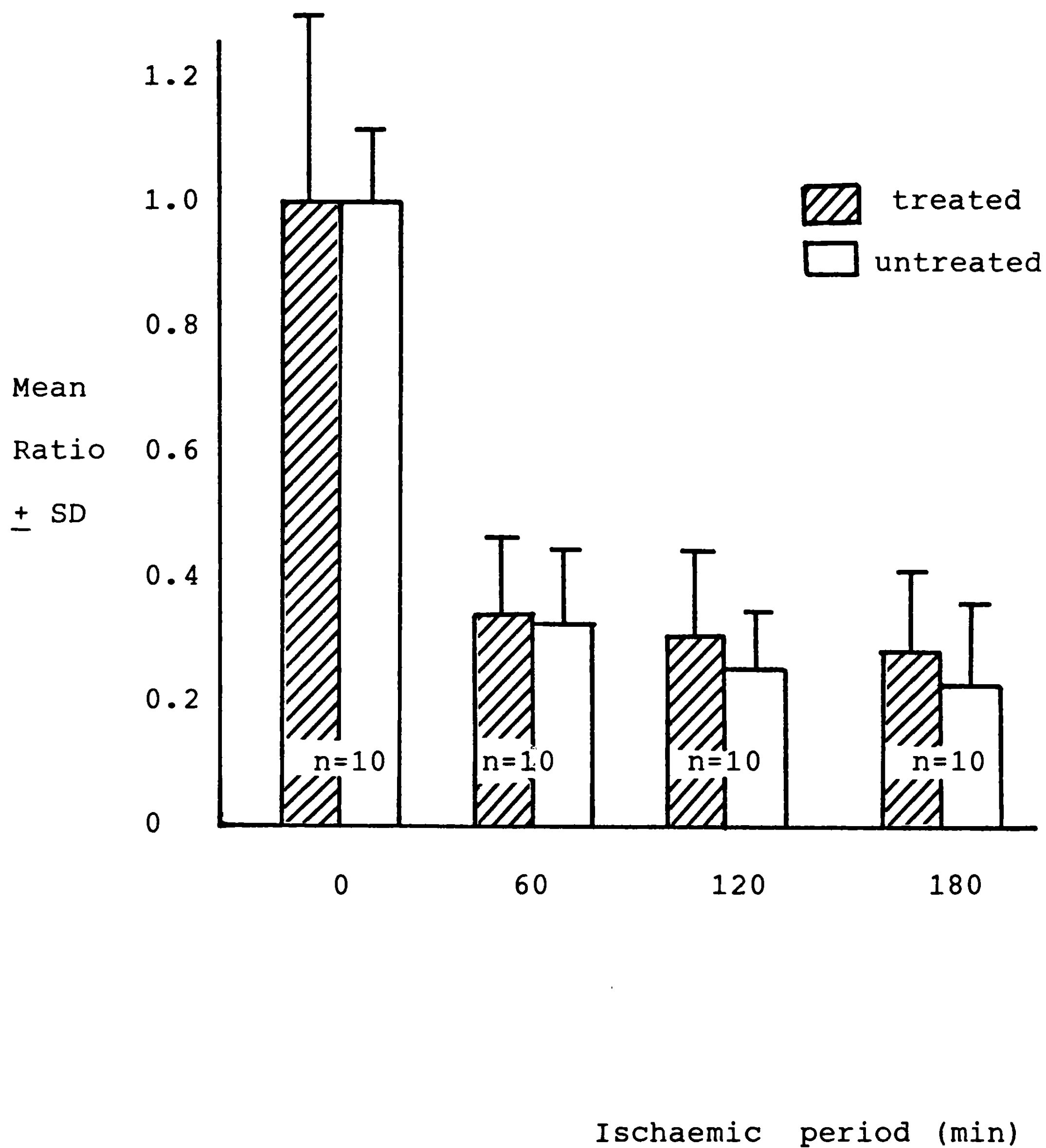


FIGURE 4.2ii

Graph to show the effect of 10mg/Kg allopurinol on urea production in rat liver during normothermic ischaemia.

Allopurinol does not significantly alter urea production in this system.

In both groups the urea production fell with ischaemia achieving significant decreases over the initial value at 60, 120 and 180 minutes ($p<0.001$). There was no significant effect of treatment with allopurinol over the control.

c. <u>The effect of desferrioxamine</u>

The urea production for the freshly excised liver was 239.05 ± 85.13 mmoles/g (n=10) in the treated group and 212.59 ± 43.98 mmoles/g (n=10) in the control.

In the treated group the urea production fell to 56.92 ± 30.55 mmoles/g (n=10) at 60 minutes, 47.40 ± 22.04 mmoles/g (n=10) at 120 minutes and 41.16 ± 14.94 mmoles/g at 180 minutes ischaemia.
In the control the equivalent values were 73.11 ± 31.20 mmoles/g (n=10) at 60 minutes, 51.07 ± 18.85 mmoles/g (n=10) at 120 minutes and 49.67 ± 34.00 mmoles/g (n=10) at 180 minutes ischaemia.

The mean ratio urea production for the freshly excised liver was 1.00 ± 0.36 (n=10) for the treated group and 1.00 ± 0.13 (n=10) for the control.
The mean ratio in the treated group fell to 0.24 ± 0.10 (n=10) at 60 minutes, 0.22 ± 0.10 (n=10) at 120

minutes and 0.19 $\pm$ 0.08 (n=10) at 180 minutes ischaemia.

In the control group the values were 0.34 $\pm$ 0.13 (n=10) at 60 minutes, 0.25 $\pm$ 0.10 (n=10) at 120 minutes and 0.23 $\pm$ 0.14 (n=10) at 180 minutes ischaemia.

In both groups the urea production fell significantly at 60, 120 and 180 minutes ($p<0.001$). There was no significant effect of treatment with desferrioxamine prior to ischaemia.

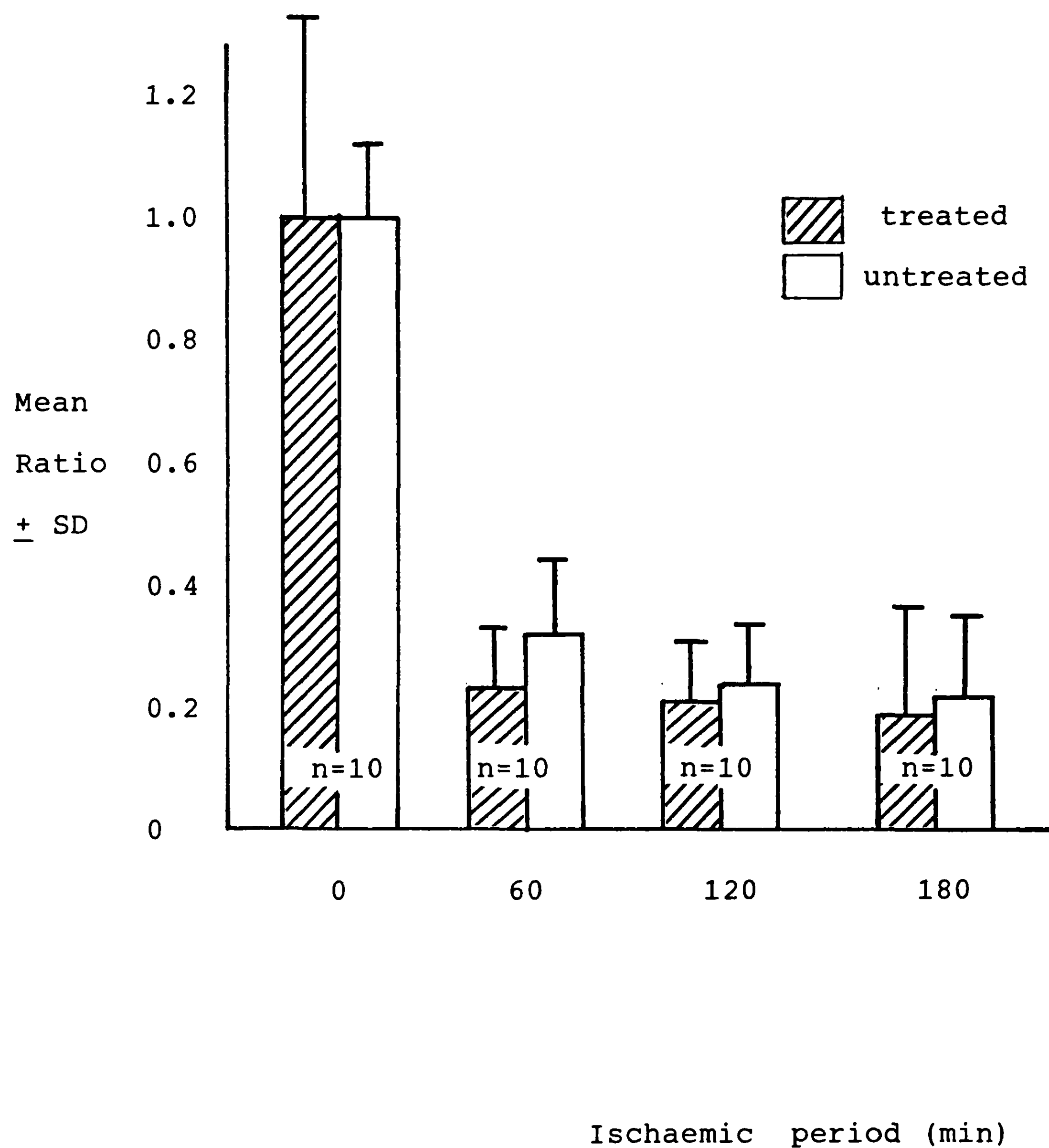


FIGURE 4.2iii

Graph to show the effect of 15mg/Kg desferrioxamine on urea production in rat liver during normothermic ischaemia.

Desferrioxamine does not significantly alter urea production in this system.

Summary

Urea production decreased significantly with ischaemic periods of 60 minutes and continued to do so up to 180 minutes.

Treatment of the rats with free radical scavenging agents failed to prevent a fall in urea production. The extent of the fall in urea production was not affected by allopurinol or desferrioxamine.

Superoxide dismutase maintained urea production at a significantly higher level than the control at 120 minutes ($p<0.001$).

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSION

Introduction

The results of this thesis reinforce those of other authors working with different organs and systems to investigate the result of ischaemic damage.

In a recent review by Hasselgren (Hasselgren P-O, 1988) the "time of no return" for hepatic ischaemia in rat was thought to be between 60 and 120 minutes. This ischaemic time appears from the results of this thesis to be the time after which free radical effects such as malondialdehyde level, conjugated diene level and changes in glutathione status become significant.

It has been shown that ischaemia of similar duration to those in the thesis has an effect on tissues at the microciculatory level (Parks D.A., 1983), cellular level (Chaudry I.H., 1981 & 1983) and subcellular level (Marubayashi S. et al., 1982).

The previous series of experiments demonstrate a repeatable sequence of events which occur during

ischaemia in the isolated rat liver which was maintained at 37 degrees C.

A measureable amount of lipid peroxidation was anticipated and this was shown to be the case in terms of Malondialdehyde production, conjugated Diene production and the changes in oxidised and reduced Glutathione.

In the following discussion, points have been arranged to follow the order of the experimental work. The first section is concerned with the changes in lipid peroxidation which are encountered in the **untreated** animals during ischaemia.

The reasons for the modulation of lipid peroxidation by the free radical scavenging agents are considered in the subsequent sections. Each free radical scavenging agent being discussed in turn.

The final section of the chapter takes the form of the conclusion drawn from the experimental work.

Ischaemia in the untreated animal

Conjugated Diene Level

Conjugated dienes have been used by other authors as a measure of free radical activity (Slater T.F., 1984. Dormandy T.L.,1985.). Fuller and Green (Fuller B.J. & Green C.J., 1986) used conjugated dienes as an indicator of peroxidation in renal ischaemia. Ungemach (Ungemach F.R.,1985) demonstrated a peak level of conjugated dienes in isolated hepatocytes at 30-60 minutes after exposure to a stimulator of lipid peroxidation, followed by a reduction in level up to 120 minutes.

In the experiments performed in Chapter 3 in the untreated animals, diene conjugates are shown to decrease with increasing normothermic ischaemia.

Diene conjugation is thought to occur at a relatively early stage in the lipid peroxidation chain reaction (Kappus H.,1985. Dormandy T.L., 1985) as seen in Chapter 1. As lipid peroxidation progresses, these early radicals would be expected to be converted to products occurring later in the lipid peroxidation chain. The concentration of the early components in the system should therefore decrease after their conversion.

This would appear to be the case in the rat liver system described in the previous experiments. This hypothesis is supported by the findings in experiments measuring malondialdehyde, a late product of peroxidation, which increased with ischaemia.

From this experimental finding the efficacy of a free radical scavenging agent should be reflected in either the maintenance of a higher concentration of conjugated dienes over a control group or of a particular concentration being maintained over a longer ischaemic period.

This theory does not, of course, take into account any specificity of a particular free radical scavenger and must assume that the effect can take place at any point in the stages of lipid peroxidation.

In the scheme for lipid peroxidation in Chapter 1, it is assumed that most of the scavengers used in the experiments have their site of action at the initiation stage of oxygen derived free radical production. Other scavengers, e.g. alpha tocopherol which has not been used in this series of experiments, may well have a site of action later in

the lipid peroxidation chain reaction.

Malondialdehyde Production

Malondialdehyde estimation by a thiobarbituric acid fluorescence method (Yagi K. 1982. Nair V. et al., 1986) has been used in renal ischaemia (Zager R.A., 1985), in systems using isolated hepatocytes (Reiter R. & Burk R.F. 1987) and in subcellular liver fractions (DeGroot H. & Noll T., 1986).

Malondialdehyde is a stable substance produced in the latter stages of lipid peroxidation. It is shown in the previous experiments that this product accumulates during peroxidation.

This has been shown experimentally in the above authors work using different systems and is supported by the results from this system.

Malondialdehyde increases progressively with periods of normothermic ischaemia. Accumulation of this product still occurs at three hours ischaemia.

In this system the malondialdehyde concentration increases as the diene concentration decreases which would substantiate the proposed reaction scheme.

The Glutathione System

The glutathione system is important in cellular defence against free radical damage . Oxygen derived species can become substrates for glutathiuone peroxidase with the resultant conversion of reduced glutathione (GSH) to oxidised glutathione disulphide (GSSG).

In the experimental system described previously, there is a reduction of reduced glutathione with progressive normothermic ischaemia. A corresponding increase in oxidised Glutathione disulphide is noted. This suggests the presence of oxygen derived free radicals in the system.

As an assessment of the conversion of reduced to oxidised glutathione the glutathione ratio was used. This is obtained by dividing the oxidised glutathione level by the reduced glutathione level in the same specimen. A progressive rise in the ratio with normothermic ischaemia is seen indicating free radical activity.

Glutathione levels have been studied in liver systems (Marubayashi S. et al. 1984. Adkison D. et al., 1986) and the levels over ischaemic periods of 0-180 minutes show equivalent results in the 4-10

mmole/g range. Adkison also used the glutathione ratio as an indicator of glutathione status.

The findings of this and the other parameters measured suggests that the oxygen derived free radicals are present in amounts which are in excess of the capability of endogenous scavengers to eliminate them.

In this experimental system it is shown that there is sufficient free radical activity to cause lipid peroxidation which in turn may be sufficient to be toxic to liver tissue.

In the studies of Chapter 4, the effect of scavenging agents for oxygen derived free radicals is assessed. The parameters used are as described above. All treated animals are then compared with the untreated groups.

Theoretically an effective free radical scavenging agent would reduce the effects and hence the products of lipid peroxidation. Dependent upon its site of action the pathway could be broken. Figures in Chapter 1 show the proposed site of action of agents which have been used in these studies.

In the following sections the effect of individual free radical scavengers is discussed.

A consideration of individual free radical scavengers

Superoxide dismutase

Superoxide dismutase is a transition metal/protein complex which catalyses the conversion of superoxide anion radical to hydrogen peroxide. The superoxide anion radical is implicated by many authors in the initiation of the lipid peroxidation chain reaction.

The consequence of the effect of superoxide dismutase is to reduce diene conversion and reduce malondialdehyde production.

The glutathione status of the liver tissue in this system is preserved by superoxide dismutase.

In the previous series of experiments there appeared to be a dose relationship. 10,000 units of superoxide dismutase per kilogram lowered the production of malondialdehyde and maintained

glutathione status more effectively than lower doses of the order of 500 units per kilogram.

This effect was not as marked in the maintenance of conjugated diene levels where the dose of 1,000 units per kilogram appeared to be more effective than higher doses.

Superoxide anion radical is clearly involved in the lipid perioxidation reaction in this system.
This supports the xanthine oxidase pathway as the source of free radicals in this reaction during ischaemia. These effects also add weight to the theory that enough oxygen is contained within this experimental system to promote free radical formation.

Studies by other authors have shown that the administration of superoxide dismutase can be beneficial in protecting tissues against ischaemic damage.

Baker (Baker G.L. et al. 1985) showed that superoxide dismutase was beneficial in warm renal ischaemia of 45-60 minutes duration. Ouriel (Ouriel K. et al., 1985) also reported a beneficial effect of superoxide dismutase on kidneys after 60 minutes warm ischaemia. In a study by Adkison (Adkison D.

et al. 1986) a beneficial effect on hepatic function in terms of improvement of liver function tests and hepatic oxygen consumption was reported.

Denatured superoxide dismutase

Superoxide dismutase is relatively difficult to denature. It can be done by using diethyl dithiocarbamate. This substance was used in the experiments. In vivo denaturation of superoxide dismutase was reported to be effective in mice (Heikkila R.E. et al.,1976). Further elucidation of the denaturation process was offered by Misra (Misra H.P., 1979)

Denaturation of superoxide dismutase would appear from the experimental findings in Chapter 4 to have been very effective in the case of malondialdehyde production.

Diene conversion is marginally different from the untreated group indicating that denaturation is effective.

The levels of glutathione in both oxidised and reduced forms indicate that the levels correspond to those seen after free radical activity. The glutathione ratio however does not reach that of the

control group although it is higher than that of the group treated with 10,000u/Kg SOD at any time point.

Diethyldithiocarbamate is therefore effective in vivo in denaturing superoxide dismutase. Any impairment of scavenging ability beyond that of the control values would indicate that endogenous superoxide dismutase was being inactivated by diethyl dithiocarbamate.

Superoxide dismutase is difficult to denature effectively. Heat must be applied for prolonged periods and even after multiple boilings there is still demonstrable activity using a xanthine oxidase driven assay system (own work).

Cyanides are however, effective in denaturation of superoxide dismutase but their use in the living animal as a pretreatment is obviously inappropriate.

Allopurinol

Allopurinol is a drug which specifically inhibits the enzyme xanthine oxidase. In Chapter 1 it was

suggested that the xanthine oxidase was the major source of oxygen derived free radicals in the liver.

This free radical pathway has two phases. One occurs in ischaemia and involves the degradation of ATP to hypoxanthine via several steps, see Chapter 1.

The second phase is associated with a supply of oxygen which leads to the production of the superoxide anion radical.

Allopurinol is administered to rats in this series of experiments. At the two doses used an effect on free radical activity and lipid peroxidation is observed.

The findings regarding **malondialdehyde** production are of an overall decrease in the production which appears to be dose related. The higher scavenger dose decreases the production to a greater extent.

Dienes are converted at a slower rate after the administration of allopurinol.

The peroxidation chain reaction would therefore seem to be retarded by allopurinol.

Glutathione status is improved with allopurinol at both doses. A slower conversion of reduced glutathione (GSH) to oxidised glutathione disulphide (GSSG) is noted. This is reflected in a slow rising glutathione ratio.

All these findings indicate a beneficial effect of allopurinol in the ischaemic phase in rat liver in this system.

Allopurinol has previously been shown to be an effective scavenger in several systems including the liver (Nordstrom G. 1985), heart (Gardner T.J. et al., 1983), kidney (Hansson R. et al., 1983) and intestine (Parks D.A. et al., 1983).

Most studies involve reperfusion injury but in this thesis an effect is noted in the ischaemic phase. This would substantiate the theory that in whole liver studies, enough available oxygen is present in the early phase of ischaemia to drive the xanthane oxidase pathway to the second phase.

Adkison (Adkison D. et al., 1986) suggests that hepatocytes are resistant to ischaemic injury due perhaps to internal vascular regulation and the high oxygen abstraction capacity of the liver. He

demonstrated a beneficial effect of allopurinol on 60 minutes hepatic ischaemia when the drug was injected intraperitoneally. Adkison also demonstrates a reduction in glutathione ratio in rat liver during ischaemic periods between 0-4 hours.

Desferrioxamine

Desferrioxamine is a specific chelator of iron. Iron is involved in the metal catalysed Haber-Weiss reaction. The result of this reaction is the catalysed conversion of the superoxide anion radical to hydrogen peroxide.

Desferrioxamine should chelate any free iron present in the system, thus reducing oxygen radical conversion.

It should be noted that desferrioxamine does not chelate haem iron, this being the largest source of iron in the normal tissues.

Following the administration of desferrioxamine to the experimental animals there appears to be little difference between the untreated and treated groups up to 60 minutes ischaemia, although a late effect

is seen resulting in a decreased Malondialdehyde production at 120 and 180 minutes.

The diene levels are measured at a higher value than the control after 60 minutes at the higher dose of desferrioxamine.

At lower doses, it is interesting to note that desferrioxamine has the effect of **increasing** the glutathione ratio over that of a control. At the dose of 15 mg/Kg however, the glutathione ratio is reduced from that of the control values.

The precise level of iron within the system may be important in determining the rate of reaction in the peroxidation chain. The level of iron when the lower dose of desferrioxamine is administered may be sufficient to drive the reaction. At the higher dose, enough iron may be chelated to prevent the reaction.

In all, the higher dose of desferrioxamine has an effect on the glutathione ratio but does not appear to have great effect on either malondialdehyde or diene levels. Lower doses of desferrioxamine do not appear to be radical scavengers in this system. The beneficial effect is therefore variable, although Green (Green C.J. 1986) reports a

beneficial effect of desferrioxamine in rabbit kidney subjected to warm ischaemia and reflow.

Doses greater than 15 mg/kg tend to result in toxic effects to the rats used in this study. For this reason the maximum dose is limited to 15mg/Kg. Following the intravenous injection of desferrioxamine rats become drowsy and show reduced activity on recovery from initial anaesthesia.

The apparent lack of effect of desferrioxamine could be explained in several ways. Firstly, that the iron catalysed Haber-Weiss reaction is relatively unimportant in this system of lipid peroxidation. The peroxidation may well proceed by another route from the superoxide anion radical.

The level of free iron in the liver in the relevant oxidation state could be too low to promote the catalysed reaction. In ischaemic conditions most, if not all, the iron would be expected to be in the divalent ferrous state. As such, reactions involving the ferric iron would cease or be reduced.

Other considerations are the ability of desferrioxamine to penetrate to the site of the relevant radical. With the parenteral administration of the drug there is likely to be

little obstruction to desferrioxamine reaching the appropriate site.

Mannitol

Mannitol should act by "mopping up" the hydroxyl radical OH. This reactive radical if eliminated should lead to a decrease in peroxidation. There is evidence that mannitol is an effective scavenger in other systems. In the post ischaemic rat kidney, Aydin (Aydin G. et al., 1983) reports an effect after 60 minutes normothermic ischaemia. In a further study, Zager (Zager R.A.et al., 1985) reports a beneficial effect on glutathione and malondialdehyde levels after 25 minutes renal artery occlusion and reflow after a 2ml bolus of 20% mannitol.

The lack of beneficial effect in the isolated rat liver subjected to normothermic ischaemia is shown in the previous Chapter. Conjugated diene and malondialdehyde level is no different from that of the control group. The Glutathione status is unchanged by the administration of mannitol.

In this system therefore mannitol is ineffective as a free radical scavenger. Aydin shows no beneficial effect of mannitol in both dog and rat

kidney when administered as a pretreatment before normothermic ischaemia (Aydin G. et al. 1983). There may well be several explanations for this. One explanation is that the hydroxyl radical is not involved in this system. In other studies however, the hydroxyl radical is implicated in ischaemia / reperfusion injury (Kappus H.,1985).

Production of the hydroxyl radical is usually associated with an inflow of oxygen such as that occurring at reflow post ischaemia. Although there is evidence that oxygen is held in the relatively large mass of liver during ischaemia, it may well not reach a high enough concentration to produce the hydroxyl radical.

A second theory is that the mannitol is not reaching the site of radical production. Even a relatively large dose of mannitol does not penetrate the cell membrane easily. Therefore the only effective site for an effect of mannitol must be extra cellular. The sites of lipid peroxidation are mainly in cell membranes and the membranes of endoplasmic reticulum and mitochondria. Mannitol would not therefore have an effect on these sites.

On balance the second theory would appear most likely in this system.

CONCLUSION

A number of hypotheses are tested in the previous series of experiments.

Lipid peroxidation occurs in rat liver during normothermic ischaemia.

This hypothesis is proved in the results of experiments in chapter 3.1. The increase in malondialdehyde and changes in the level of conjugated dienes indicate that free radical activity occurs. This activity takes the form of lipid peroxidation.

The findings are corroberated by the conversion of reduced to oxidised glutathione during ischaemia and the subsequent increase in glutathione ratio. This indicates that endogenous defence against free radical activity is initiated in ischaemia.

Metabolic activity is affected by ischaemia.

The synthesis of urea by the liver is also shown to decrease during normothermic ischaemia. This is demonstrated in chapter 3.2.

It should be possible to reduce lipid peroxidation by the administration of specific free radical scavengers.

Free radical scavengers have an effect on lipid peroxidation during normothermic ischaemia as demonstrated in chapter 4.1.

Some scavengers such as 10,000u/Kg superoxide dismutase are very effective in reducing all measured effects of lipid peroxidation. This protective effect is abolished when the scavenger is denatured.

Other scavengers are ineffective in reducing the effects of lipid peroxidation. This is seen with 1g/Kg mannitol. None of the measured parameters differ from a control group.

Allopurinol and desferrioxamine appear to reduce some but not all of the effects of free radical activity during ischaemia.

Free radical activity affects a synthetic metabolic function of the rat liver during ischaemia.

This is studied in chapter 4.2, by administration of scavengers which are shown to effectively reduce

lipid peroxidation. The synthesis of urea is partially protected by superoxide dismutase but decreases with progressive ischaemia.

From the experimental results the overall conclusion is that free radical activity occurs in the rat liver during normothermic ischaemia. This activity, represented by lipid peroxidation, is reduced by the administration of specific free radical scavenging agents.

Some scavengers are more effective than others and different parameters of lipid peroxidation are affected by different scavengers.

This indicates that several pathways for the generation of free radicals exist in the isolated rat liver subjected to normothermic ischaemia.

Metabolic activity, represented by urea synthesis, falls during ischaemia in the isolated rat liver. Urea synthesis is improved by superoxide dismutase, the most effective scavenger in the lipid peroxidation experiments. Other scavengers are ineffective in improving urea synthesis.

Superoxide dismutase could not however prevent the fall in metabolic activity.

The conclusion is that free radical activity does play a part in ischaemic damage to the liver but it is not the major influence on metabolic activity during ischaemia.

CHAPTER 6

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CHAPTER 7

Suggested Further Research

In general the results of these studies would suggest that there is a supply of oxygen within the intact liver during the course of the assumed ischaemia.

There is experimental evidence that because the liver is a relatively massive organ oxygen diffuses from its surface for at least 30 minutes after induction of ischaemia.

The measurement of oxygen diffusion from the liver surface may well be possible using the surface oxygen electrode. Preliminary experiments in the pig support the theory that oxygen diffuses at a slow rate and may thus be available within the liver tissue for the promotion of a free radical lipid peroxidation chain. The continued progress of lipid peroxidation should not, on theoretical grounds, be possible in the absence of oxygen.

Potential problems encountered with oxygen electrode measurement could arise when the physical size of both electrode and rat liver are considered. The smallest practical oxygen electrode for surface

measurement is at present approximately 1 centimetre in diameter. It would be difficult to apply this electrode satisfactorily to several sites on the in situ rat liver.

Studies of Ischaemia/Reperfusion Injury in the Rat Liver

The results reported in this thesis show that production of free radicals occurred in the ischaemic phase. The production of these radicals could be modified by the administration of scavengers. It was observed that mannitol was ineffective and desferrioxamine was only partially effective.

Other scavengers have been tested in tissues other than liver and it may be informative to apply them to a rat liver system. Ascorbate, alpha tocopherol and calcium antagonists could be used.

A reliable model for the study of the reperfusion phase of hepatic injury is required.

Systems for the study of reperfusion injury in the rat require a veno-venous bypass procedure of the portal system during clamping of the vessels. Without this precaution the rat would die within

minutes. This is in common with the rabbit and the pig. Obviously this would preclude studies of long term ischaemia.

Whilst the technique of veno-venous bypass is accomplished with apparent ease by some authors, especially the Japanese, it is a technique which is not without problems.

The usual method of bypass uses a passive shunt from the femoral vein in the form of a small diameter plastic tube. Haemodynamic principles are against the success of such tubes primarily due to them clotting up. One solution would be to use heparin bonded tubing but this is not freely available in diameters less than 1mm.

An alternative would be to heparinise the rat. The liver however bleeds freely without heparinisation when its surface is cut. As multiple sequential biopsies are required for the experiment it would be anticipated that massive haemorrhage would occur with systemic anticoagulation.

An `in situ' model for the study of ischaemia / reperfusion damage in rat liver which does not require veno-venous bypass would be a technical advantage.

A model has been developed which allows ischaemia and reperfusion of 40% of the rat liver but which obviates the need for a veno-venous bypass (Author).

The rat liver has prominent lobes, separated by distinct and accessible vessels. It is possible to apply small arterial clamps such as a Scoville Lewis clamp to these vessels. The result is that approximately 40% of the liver is ischaemic and 60% remains non-ischaemic and allows the portal blood to circulate. The animal survives throughout the chosen ischaemic period.

The changes in reduced and oxidised glutathione, TBA reactive products and conjugated dienes have been established for ischaemic periods of 60 and 180 minutes followed by a reflow period of 15 minutes. Changes in the above parameters have also been measured in the non ischaemic lobes. The effect of free radical scavengers is planned for this model.

Studies of Ischaemia/Reperfusion in Other Species

The experimental model used in this thesis was the isolated normothermic rat liver. The effects of ischaemia/reperfusion on the pig liver has been investigated. The group size was small and further

numbers are needed. Preliminary experiments using superoxide dismutase as a free radical scavenger have been undertaken but again the numbers must be increased.

As a culmination of the animal work, the effect of clinical ischaemia in the human should be studied. The study would require operative liver biopsies before and after ischaemia which would be possible with ethical committee approval.

APPENDIX 1

A.1 Assay techniques used in the study

a. Urea assay

b. Glutathione assay

c. Malondialdehyde assay

d. Conjugated diene assay

APPENDIX 1

Methods used for the assay of specimens

A.1a Urea assay

The assay is an enzymatic colorimetric estimation using chemicals supplied by Boehringer Mannheim.

Urea is cleared by urease to form ammonium carbonate. The addition of phenol / sodium nitroprusside reagent and sodium hypochlorite result in a blue colouration following incubation at 37 deg. C.

$$CO(NH_2)_2 + 2H_2O \rightarrow (NH_4)_2CO_3$$

This is Berthelots reaction as applied by Fawcett and Scott in this assay. Although the usual test material is plasma, serum or urine, this reacton may be applied to media such as those produced in previous experiments with appropriate blank incubations.

In estimations of urea produced by liver slices the following method was used.

All tests were performed in duplicate.
Two samples, each containing 10 microlitres of liver incubate medium were diluted 1:10 with double distilled water and placed in bijou bottles. To one of these bottles 50 microlitres of urease reagent was added. A standard incubation, which consisted of 100 microlitres of urea standard (0.5 millimoles/litre) was used for comparison. In addition, a halved concentration was prepared.

The reagent blank consisted of 50 microlitres of urease reagent.

Each vessel was stoppered and incubated at 37 deg. C in a circulating water bath for 10 minutes. After this 2.5 millillitres of phenol (0.106 moles/litre and sodium nitroprusside (0.17 millimoles/litre) reagent was added to each vial using a repeating pipette. 2.5 millilitres of sodium hypochlorite (11 millimoles/litre) reagent was then added to the vials.

Samples were mixed using a rotary shaker, stoppered then incubated for a further fifteen minutes at 37 deg. C in the water bath.

Reading of urea samples

The absorbance of samples was measured using a Gilson spectrophotometer at a wavelength of 550 nanometres. The spectrophotometer was zeroed on the reagent blank prior to reading samples.

Readings of standards were performed at the beginning, middle and end of a batch of samples in order to assess assay variation.

Each pair of vessels (a) urease and (b) water for each sample was read together and recorded on printout paper in the automatic mode of the machine.

The basis of the calculation relies on a subtraction of the reading from the water cotaining sample from the urease containing sample.
This is divided by the reading from the standard before performing the calculation shown below:-

$$\frac{\text{Absorbance of sample - absorbance of blank}}{\text{Absorbance of the standard}}$$

Urea calculation

$$\frac{\text{U-C}}{\text{STD}} \times \text{STD CONCENTRATION} \rightarrow \text{Moles in sample } (10\mu l)$$

In 4 ml of incubate there is 0.2g of liver

Therefore

$$400 \times \frac{\text{U-C}}{\text{STD}} \times \text{STD CONCENTRATION}$$

In 1g wet weight of liver

$$\frac{1}{0.2} \times 400 \times \frac{\text{U-C}}{\text{STD}} \times \text{STD CONCENTRATION}$$

For 1g Dry weight of liver

$$\frac{1}{0.2} \times 400 \times \frac{\text{U-C}}{\text{STD}} \times \text{STD CONC.} \times \frac{1}{\text{Dry/wet weight}}$$

= micromoles of urea per gramme dry weight of liver

STD CONC. 0.50 m moles/litre = .0005 m moles/ml

= 0.05 micromoles per sample

Where U is the reading of the urease treated sample

C is the reading of the control sample

STD is the reading of the standard

Points in Criticism of Method and Discussion

1) It is necessary to determine over a dilution range, the standard concentration which is most suitable for this particular assay.

2) A warming up period of one hour is recommended for the Gilson spectrophotometer if repeatable results are to be obtained.

3) Specimens may be stored at - 20 deg. C for periods up to one year without deterioration.

4) Intra and inter assay variations of < 5% and<10% respectively are obtained in this assay.

5) The assay records all free ammonium irons. In the incubation experiment it was necessary to add both ammonium chloride and ornithine to the Krebs Ringer bicarbonate medium as substraits for urea production.
This is the reason for parallel reaction vials being studied.

The urease will cleave all urea present, with the subsequent release of ammonium ions into the medium. This will be incorporated into the coloured complex along with any free ammonium ions in the medium. An internal blank using double distilled water instead of urease will enable the non urea ammonium ions to be estimated and thence subtracted from the urease reaction reading. This will then allow the true urea production to be assayed.

6) Haemolysis will affect this assay. It is important therefore in the initial experiment to wash the specimen of liver in Krebs Ringer bicarbonate to remove obvious blood contamination.

A.1b Glutathione assay

Principle of glutathione reactions

Glutathione reacts with orthophthalaldehyde which is a fluorescent material. Orthophthalaldehyde will react with oxidised glutathione at an ambient pH of 12 and will react with reduced glutathione at an ambient pH of 8.

In this assay the pH of the solution is manipulated to give the optimum pH for each reaction by the addition of 0.1 molar sodium hydroxide for pH12 and EDTA phosphate buffer adjusted to pH8.

In assaying the oxidised glutathione it is possible that the relatively high concentration of reduced glutathione will interfere with the test and for this reason N ethyl maleimide is added to the mixture to combine with the reduced glutathione to form an inactive complex.

Reduced glutathione

A 50 microlitre sample of liver homogenate was diluted with 450 microlitres of phosphate EDTA buffer at pH8.

A 100 microlitre sample of this initial dilution was added to 3.7 millilitres of phosphate EDTA buffer in a 10 millilitre plain glass blood tube. To this mixture 200 microlitres of orthophthalaldehyde was added and the reactants mixed using a rotary shaker.

The reaction vessel was incubated at room temperature for 15 minutes. All samples were assayed in duplicate. A reduced glutathione standard and a reagent blank were treated in the same manner.

After incubation the contents of the tubes were fluoremetrically assayed using the Perkin Elmer LS5 at an excitation setting of 350 nanometres and a scanning emission range between 410 and 440 nanometres. The fluorescence peak occurred at 420 nanometres.

Oxidised glutathione

A 500 microlitre sample of liver homogenate was mixed with 200 microlitres of N ethyl maleimide which had been freshly prepared.

This mixture was incubated at room temperature for 30 minutes.
4.3 millilitres of 0.1 molar sodium hydroxide was added at the end of this period to quench the reaction and bring the pH to 12. An aliquot of 100 microlitres was taken and diluted with 3.7 millilitres of 0.1 molar sodium hydroxide. 200 microlitres of orthophthalaldehyde was then added and the whole mixture incubated at room temperature for a further 15 minutes. An oxidised glutathione standard and a reagent blank were prepared in the same manner. All samples were assayed in duplicate.

The specific fluorescence of the samples was measured using the Perkin Elmer LS5 luminescence spectrometer at an excitation wavelength of 350 nanometres and an emmision scan between 410 and 440 nanometres.

Preparation of glutathione standards

Reduced Glutathione

0.003g of reduced glutathione (BDH, Poole) was dissolved in 100ml. of ice cold phosphate EDTA buffer. This resulted in a nanomolar concentration for use in the assay.

Oxidised Glutathione

0.0061g of oxidised glutathione (BDH, Poole) was dissolved in 100ml. of ice cold phosphate EDTA buffer. This again gave a nanomolar concentration for use in the assay.

Reagents for the glutathione assay

Phosphate-EDTA Buffer (0.005m EDTA - 0.1m Sodium Phosphate)

To prepare one litre

17.6ml	0.5m NaH_2PO_4
60.8ml	0.5m Na_2HPO_4
1.635g	EDTA

This was made up in redistilled water with 1m sodium hydroxide slowly added whilst stirring the solution

until pH 8 was reached using a pH stick meter (Whatman).

Orthophthalaldehyde

0.02g of orthophthaldehyde was dissolved in 20ml of methanol.

N ethyl maleimide

To prepare a 0.04 molar solution, 0.05g N ethyl maleimide was dissolved in 10ml of phosphate buffer.

Glutathione standard curves

Standard curves for the glutathione assay were produced so that the accuracy and linearity of the assay could be determined over a range of concentrations including those used in the experimental work.

Tenth dilutions of a nanomole concentration solution of both reduced and oxidised glutathione were assayed by the respective glutathione techniques.

Their specific fluorescence was measured and a graph of peak height - blank height versus concentration of glutathione was plotted.

Reduced Glutathione

Concentration	Peak Height
1.0 nmole	98 mm
0.1 nmole	11.5 mm
0.01 nmole	2.5 mm

Oxidised Glutathione

Concentration	Peak Height
1.0 nmole	79 mm
0.1 nmole	9.5 mm
0.01 nmole	3.0 mm

In both cases the graphs were linear and accurate within the range of concentrations required.

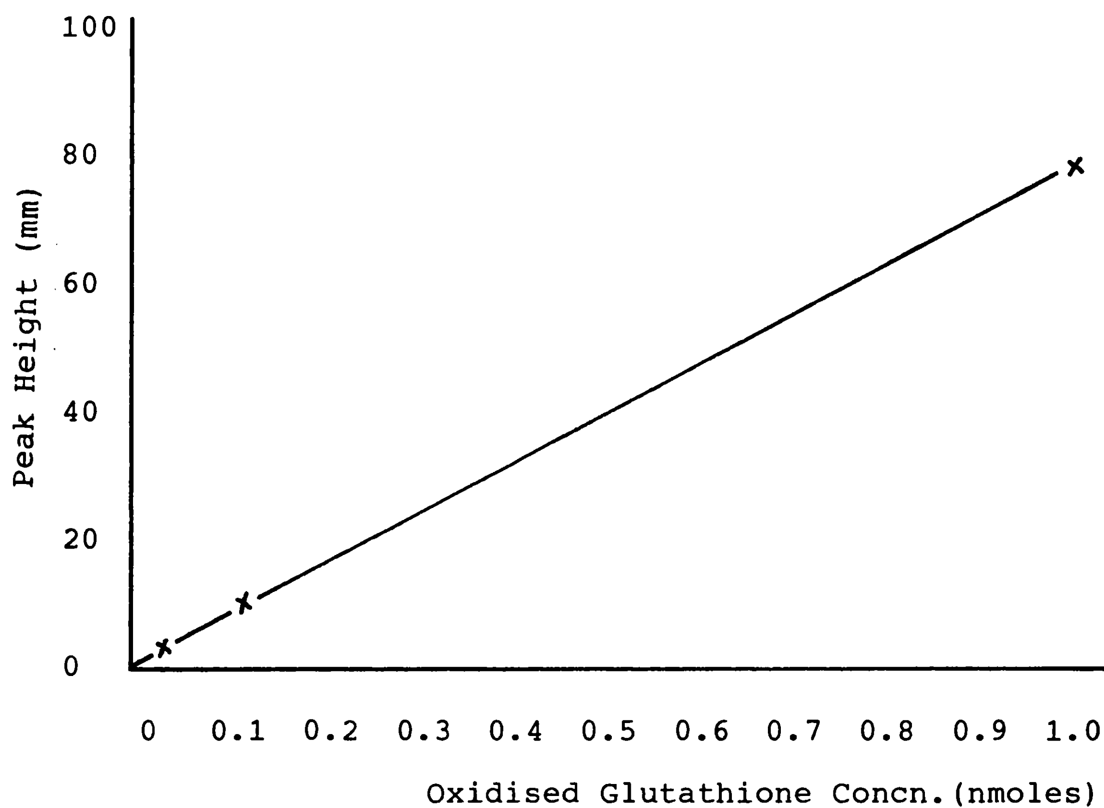


FIGURE 2.2i Graph to show the standard curve for oxidised glutathione. Concentrations of 0.01, 0.1 and 1.0 nanomoles of oxidised glutathione standard versus peak height of spectrophotometer trace (mm).

The standard curve was linear.

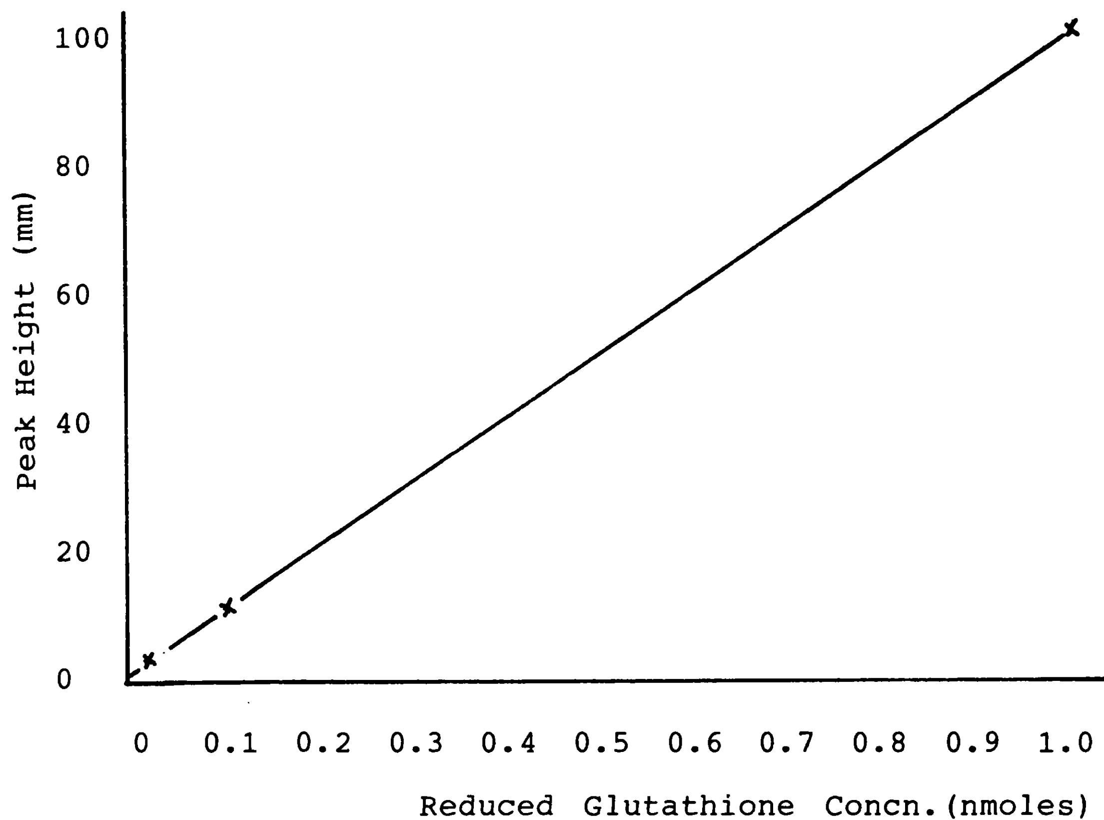


FIGURE 2.2ii Graph to show the standard curve for reduced glutathione. Concentrations of 0.01, 0.1 and 1.0 nanomoles of reduced glutathione standard versus peak height of spectrophotometer trace (mm).

The standard curve was linear.

Specific fluorescence traces were made of both oxidised and reduced glutathione standards to demonstrate the specific fluorescence pattern. In both cases the peak occured at an emission reading of 420 nm with excitation preset at 350 nm on the Perkin Elmer LS5.

A second peak was observed at 720 nm but this bore no relationship to the glutathione sample. If an empty cuvette was scanned, the 720 nm peak still occured although the 420 nm glutathione peak did not occur.

A.1c The assay of malondialdehyde

Principle of MDA assay

This is a fluorimetric assay of lipid breakdown products. The protein fraction of the whole liver homogenate is cleared from the reaction by the process of acid precipitation, effected by hydrochloric and phosphotungstic acids.

In the assay this protein forms a solid pellet in the reaction vial after incubation. The homogenate

is then treated with thiobarbituric acid which acts as the fluorescent material.

This binds on to the altered lipid fraction aided by the detergent effect of sodium lauryl sulphate. Fluorescent estimation of TBA reactive material is then possible.

Assay method for malondialdehyde

(Suematsu T, Abe H., 1982 , Yagi K., 1982)

The assay was carried out in 10ml plain glass blood tubes with screw caps (FBG Trident Ltd). The following solutions were added to each tube:

0.2ml	7%	Sodium Lauryl Sulphate
2.0ml	0.1 molar	Hydrochloric Acid
0.3ml	10%	Phosphotungstic Acid
1.0ml	0.67%	Thiobarbituric Acid

2mls of homogenate sample was added to each tube. All samples were assayed in duplicate.

A standard of 16 n moles MDA was set up by adding 2ml of the prepared standard tube containing the above solutions.

For the reagent blank, 2ml of Dulbecco A Phosphate buffered saline was substituted for the liver homogenate.

The screw tops were lightly applied and the contents of the tubes were mixed gently. The tubes were incubated at 95 degrees C in a boiling water bath (Grant Instruments, Cambridge Ltd.) for one hour.

At the end of the incubation the tubes were placed on ice for a period of 10 minutes. Following this the tops were removed and 5ml of ice cold Butanol was added using a repeating pipette (BCL). The screw tops were firmly applied and the tubes were well shaken using a rotary mixer (Clandon Maxi Mix 11). The tubes were then centrifuged at 3000 RPM for 10 minutes.

The upper, butanol extracted layer was removed and read on a fluorimeter (Perkin-Elmer Luminescence Spectrometer LS-5) using 10mm light path for UV cuvettes. The samples were read at an excitation of

515nm and a scanning emission of 540-560 nm.

The scanning traces were recorded on paper and the peak heights for each duplicated sample were compared with the standard.

To calculate MDA production

$$\frac{\text{Peak ht sample} - \text{Peak ht blank}}{\text{Peak height STD}} \times 16 = \text{nmoles MDA per sample}$$

(as the concentration of the standard in each tube was 16 n moles.)

For the liver homogenates in this study the concentration was 0.5 grammes liver in 10ml Dulbecco A Phosphate buffered saline, and 1ml of this was taken for each sample.

All values are calculated as n moles MDA per gramme of liver hence the calculation was as follows:

As the volume taken was 2ml the result must be multiplied by 5 to convert to a 10ml sample then by a further factor of 2 to convert the result to n moles MDA/g of liver wet weight.

$$\frac{\text{Sample ht.} - \text{Blank ht.}}{\text{STD ht.}} \times 16 \times 5 \times 2$$

Preparation of MDA standard

50 microlitre of MDA (Bis dimethyl acetal) was mixed with 2.45ml of 0.1 molar hydrochloric acid. This mixture was then incubated in a circulating water bath at 37 degrees C for 15 minutes.
This gave a concentration of acetal of 0.122 molar which is hydrolysed in the reaction to give 0.244 molar malondialdehyde.

The standard was then diluted 1:1000 by taking 10micro litres of the prepared solution and adding 9.99 (10) ml of Dulbecco A Phosphate buffered saline.

655 microlitres of the 1:1000 solution was then made up to 20ml with phosphate buffered saline to give an 8 n molar solution. This was the concentration of the working standard which compared well with levels of MDA produced in liver homogenates during the experimental work.

To establish a standard curve for the MDA assay

The sensitivity of the MDA assay needed to be established to relate to the nanomole quantities of MDA produced in the liver homogenates. The changes in TBA reactive products were relatively small in periods of 0 - 45 minutes hence the test had to be sensitive enough to detect these changes.

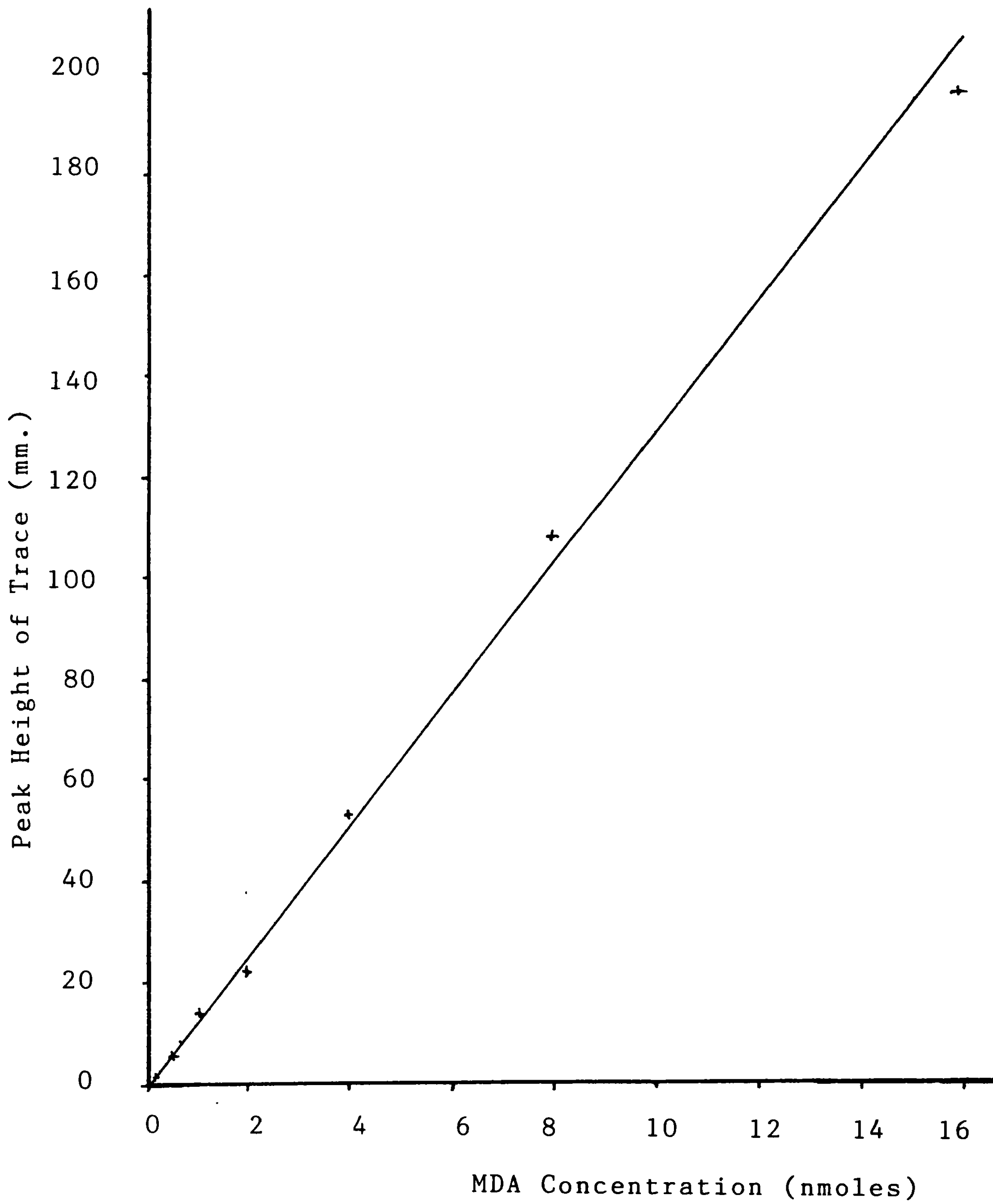
Method

Malondialdehyde standard solution of 16 n moles in 2ml phosphate buffered saline was progressively diluted to concentrations of 16, 8, 4, 2, 1, 0.5 and 0.25 n moles.

Using the Yagi assay (Yagi K., 1982) for MDA, a graph of peak height minus blank height versus MDA concentration was constructed.

The test was sensitive to 0.25 n moles of MDA and appeared to be most accurate in the range 0.5 to 8 n moles for this assay. The standard curve for this assay is linear as shown in the following figures.

Peak ht - Blank ht (mm)	MDA Concentration (n moles)
0.5	0.25
6.0	0.5
13.75	1.0
27.0	2.0
53.0	4.0
108.0	8.0
196.0	16.0



Standard Curve For The MDA Assay From 0.25 - 16 nmoles MDA.

Criticism and discussion of the MDA assay

Due to the toxic nature of malondialdehyde, care is required when handling the stock solution. The stock solution will also attack the plastic of the bijou bottles used for the assay.

For this reason the MDA standard should be added to the dilute acid and not vice versa.

A fresh standard should be made up for each assay although in practice the standard variation was minimal after 3-4 days storage at room temperature.

Heating of the homogenates during the incubation should be carried out between 90-95C. in the water bath. If solutions containing sucrose are used, the temperature must be reduced to 80C. to prevent charring of the sucrose and subsequent errors in the fluorimeter readings.

The incubation period is nominally one hour, although no further increase in activity appeared after 45 minutes in this series of experiments.

A graph of time versus activity is shown below. This should be compared with Yagi's original description of the estimation (Yagi, 1982). The sensitivity of the assay has been assessed using a graph of standard concentration versus activity on the fluorimeter. It can be seen that the assay is reliable for values of 0.5 nmoles to 32 nmoles of MDA. This is well within the desired limits of the experiments.

A.1d Estimation of conjugated dienes

Method

10 ml. plain glass tubes with screw caps were used. Into each of these was placed 8 ml. of a 2:1 mixture of chloroform and methanol.

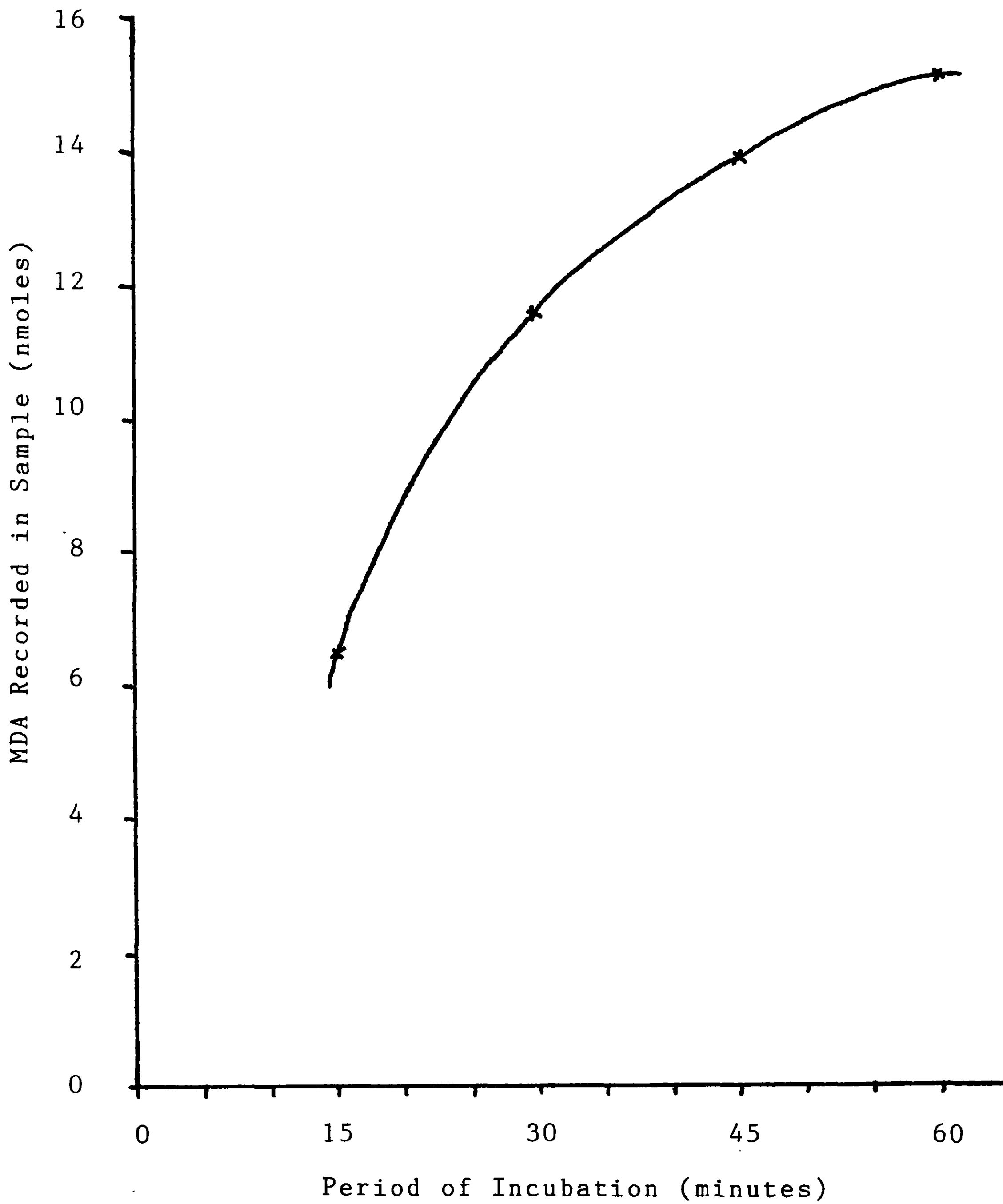
400 microlitres of liver homogenate was added and the tube was shaken well on a rotary mixer. The previously turbid fluid showed a definite clearing when mixing was complete.

A fine precipitate was noted and this was consolidated by centrifugation in a MSE bench centrifuge at 2500 r.p.m. for 5 minutes.

A blank using 400 microlitres of Dulbecco A phosphate buffered saline was set up. The samples were duplicated and read against the blank on an ultraviolet spectrophotometer at a wavelength of 240 nanometres.

Discussion of the assay

The fluorescent material is composed of altered lipids. Whole phospholipid does not fluoresce. In this assay lipid components must be the only substances assayed as chloroform will only extract lipid material. The assay therefore measures altered lipid products which are taken to equate to the diene fraction which is produced early on in the lipid peroxidation chain. It does not by itself appear to represent an actual measure of lipid damage due to peroxidation.



Graph to Show The Effect of Incubation Time on MDA Recorded in The Sample

APPENDIX 2

DETAILS OF CHEMICALS AND EQUIPMENT USED IN THIS STUDY

CHEMICAL	SUPPLIER
Allopurinol (Zyloric injectable)	The Wellcome Foundation LondonEngland
Ammonium chloride	BDH Chemicals Ltd. Poole England
Butan-1-ol	BDH Chemicals Ltd. Poole England
Calcium chloride	BDH Chemicals Ltd. Poole England
Chloroform	BDH Chemicals Ltd. Poole England

Chemical	Supplier
Desferrioxamine	CIBA Laboratories HorshamEngland
Diethyldithiocarbamate	Sigma (London) Chemical Company Ltd. Poole England
Diethyl ether	May and Baker Dagenham England
Ethylene diamine tetra acetic acid (EDTA)	Sigma (London) Chemical Company Ltd. Poole England
Glutathione - oxidised	BDH Chemicals Ltd. Poole England
- reduced	
Hydrochloric acid	BDH Chemicals Ltd. Poole England

Lactic acid	BDH Chemicals Ltd. Poole England
Magnesium sulphate	BDH Chemicals Ltd. Poole England
Malondialdehyde bis dimethyl acetal	Aldrich Chemicals Gillingham England
Mannitol	Sigma (London) Chemical Company Ltd. Poole England
Methanol	BDH Chemicals Ltd. Poole England
N-ethyl maleimide	BDH Chemicals Ltd. Poole England

Ornithine (L-Ornithine monohydrochloride)	BDH Chemicals Ltd. Poole England
Orthophthaladehyde	BDH Chemicals Ltd. Poole England
Phosphate Buffered Saline (Dulbecco A Tablets)	Oxoid Ltd. England
Phosphotungstic acid	Sigma (London) Chemical Co. Ltd. Poole England
Potassium Chloride	Fisons Scientific Apparatus Loughborough England
Potassium dihydrogen orthophosphate	Hopkins and Williams Chadwell Heath England

Sodium bicarbonate	BDH Chemicals Ltd. Poole England
Sodium Chloride	BDH Chemicals Ltd. Poole England
Sodium hydrogen orthophosphate	Hopkins and Williams Chadwell Heath England
Sodium dihydrogen orthophosphate	Hopkins and Williams Chadwell Heath England
Sodium lauryl sulphate	BDH Chemicals Ltd. Poole England
Superoxide dismutase	Sigma (London) Chemical Co. Ltd. Poole England

Thiobarbituric acid	Sigma (London) Chemical Co. Ltd. Poole England
Urea Test Kit	Boehringer Mannheim Germany

APPENDIX 3

Data for malondialdehyde production in ischaemic rat liver (nmoles mda/g liver)

Isch. Period (min)	CONTROL		DENATURED SOD.	
	104.58	107.19	145.13	142.48
	120.26	135.95	143.72	110.48
0	126.01	156.85	131.60	144.00
	80.52	126.01	165.66	130.29
	98.30	128.10	171.33	130.29
	99.35	107.19	137.35	125.17
	113.46	123.40	130.27	145.52
15	128.63	158.93	147.27	166.86
	106.67	136.99	157.88	155.43
	127.06	138.04	149.38	140.19
	106.14	127.58	138.05	126.48
	118.69	144.31	134.51	142.48
30	128.63	165.75	123.19	166.86
	108.24	139.08	151.50	147.81
	118.69	132.81	132.39	157.71
	114.51	144.84	124.60	120.38
	109.28	140.65	133.10	137.14
45	132.81	191.90	136.64	160.76
	112.94	152.68	157.17	162.29
	124.44	160.62	138.05	137.90
	120.26	174.64	145.13	137.64
	123.40	145.36	125.31	118.86
60	124.97	152.68	155.04	158.48
	118.17	159.48	186.19	160.00
	134.38	176.73	159.29	158.48
	117.12	168.37	143.72	192.76
	147.97	169.41	164.96	147.81
120	152.16	176.73	229.38	226.29
	105.10	168.37	191.15	187.43
	161.05	174.64	151.50	207.24
	129.15	196.60	189.03	237.71
	164.71	177.78	172.74	172.19
180	189.80	197.65	206.85	210.29
	125.47	169.93	228.67	231.62
	166.27	170.98	146.55	227.05

Isch. Period (min.)	500u/Kg SOD		1000u/Kg SOD	
	91.53	102.65	102.35	115.46
	95.59	66.85	112.24	113.62
0	96.95	90.23	104.19	96.60
	109.15	93.52	106.72	104.42
	113.22	85.84	109.02	106.03
	90.00	82.19	117.30	112.47
	90.85	71.23	109.94	113.62
15	101.69	95.34	99.82	117.30
	86.10	81.83	108.33	102.12
	117.29	88.04	115.00	95.22
	98.98	75.98	111.78	132.48
	99.66	78.17	107.18	127.88
30	90.85	95.34	125.81	120.06
	92.88	72.69	126.96	93.84
	105.76	78.54	111.55	100.74
	101.02	93.52	121.44	112.01
	107.12	79.27	120.29	120.98
45	98.31	102.65	126.96	121.90
	109.15	88.40	126.27	104.42
	127.46	84.02	119.60	115.46
	113.90	104.84	110.40	103.50
	100.34	84.38	100.05	118.22
60	102.37	111.78	145.13	130.18
	105.08	92.79	140.53	109.94
	116.61	81.83	116.38	113.62
	127.46	115.07	143.29	115.92
	111.19	100.09	117.76	125.58
120	114.58	118.36	140.76	145.82
	137.63	105.21	138.00	138.92
	133.56	111.42	125.58	139.15
	130.17	113.97	144.21	120.52
	137.63	103.01	132.94	148.58
180	152.54	131.87	146.74	143.98
	128.81	117.99	144.67	140.76
	132.88	109.95	140.07	141.68

Isch. Period (min.)	10000u/Kg SOD		5mg/Kg ALLOPURINOL	
	112.18	102.35	212.70	237.09
	112.97	93.90	144.13	149.09
0	113.37	107.25	176.51	172.36
	117.17	103.35	160.00	136.00
	130.35	107.03	160.00	178.91
	96.78	71.20	189.21	195.63
	85.72	66.75	136.51	197.09
15	109.81	101.40	151.75	180.36
	103.10	113.75	157.46	162.91
	115.34	78.10	145.40	187.64
	94.80	82.77	142.22	155.64
	95.99	71.65	164.44	164.36
30	116.53	109.85	126.98	136.00
	120.87	98.15	156.83	211.64
	117.71	80.55	175.87	139.64
	92.83	73.43	193.02	160.73
	99.15	80.10	146.67	168.00
	99.15	118.30	175.87	148.36
45	108.23	107.90	172.70	202.91
	102.70	72.09	156.83	164.36
	96.38	89.89	206.98	213.09
	90.85	92.79	154.92	146.18
60	115.34	122.20	168.25	202.18
	116.13	120.90	176.51	194.91
	128.77	88.56	140.32	179.64
	114.16	101.02	173.33	211.64
	92.04	109.03	172.06	224.73
120	114.95	118.30	156.19	160.00
	119.29	130.00	139.41	214.55
	161.16	98.57	165.71	212.36
	135.49	114.37	165.08	247.27
	144.18	111.70	170.79	284.36
180	132.72	124.80	153.65	187.64
	137.46	137.15	177.78	237.82
	111.39	110.81	179.05	232.00

Isch. Period (min.)	ALLOPURINOL 10mg/Kg		DESFERRIOXAMINE 5mg/Kg	
	129.95	97.28	111.22	112.68
	123.20	95.68	115.37	118.07
	106.24	91.52	113.71	117.03
0	102.72	117.44	100.43	106.24
	96.00	121.60	100.43	105.21
	103.36	93.44	120.77	117.45
	115.20	97.92	112.26	114.13
15	108.80	102.72	107.47	111.64
	110.08	109.44	96.70	100.85
	91.20	114.56	88.40	90.68
	110.08	90.88	130.11	127.62
	113.28	82.24	108.94	112.88
30	103.04	93.12	128.24	132.18
	96.00	104.64	127.62	129.48
	87.36	103.68	93.79	94.00
	127.36	118.72	113.09	112.47
	122.88	99.84	109.77	113.92
45	122.64	96.32	114.75	117.45
	101.76	116.48	125.75	128.86
	98.24	103.04	121.39	123.26
	130.88	90.24	134.67	131.14
	142.40	90.88	101.47	107.07
	126.08	92.48	123.26	125.13
60	129.28	125.76	122.84	126.37
	104.96	105.92	129.07	128.86
	126.40	103.04	147.74	130.73
	156.80	113.92	132.60	133.22
120	144.64	91.52	156.25	167.04
	140.16	154.88	119.11	123.67
	108.16	119.36	128.86	115.17
	154.88	102.40	129.07	147.33
	139.84	112.96	126.37	130.31
180	138.24	90.56	152.31	155.01
	129.92	121.60	146.91	148.16
	106.88	119.68	121.60	124.50

Isch. Period (min.)	DESFERRIOXAMINE 15mg/Kg		MANNITOL 1g/Kg	
	124.82	116.13	96.33	94.01
	137.86	132.13	90.06	90.85
0	123.24	120.28	92.43	104.28
	121.66	119.89	102.70	98.75
	118.70	114.16	95.59	82.16
	119.69	111.59	88.14	81.37
	133.51	124.43	96.38	92.43
15	138.25	127.39	101.12	91.64
	127.19	121.27	99.54	91.64
	97.17	95.59	94.01	82.95
	118.50	108.63	86.58	104.28
	114.55	109.42	136.67	95.59
30	118.50	110.21	129.56	109.02
	110.41	104.48	127.98	112.18
	117.71	110.21	101.12	119.29
	143.78	131.74	101.40	123.24
	156.82	143.15	134.30	127.19
45	138.25	136.67	129.56	127.19
	119.69	112.38	142.99	150.10
	97.56	93.42	139.04	133.51
	162.35	152.08	139.62	155.63
	139.04	145.96	158.79	143.78
60	154.05	140.03	130.35	147.73
	133.91	128.38	164.32	156.42
	131.93	126.60	140.31	144.57
	164.32	148.92	183.28	181.70
	166.69	158.40	160.37	127.98
120	164.32	156.62	143.78	173.01
	144.97	136.67	180.91	169.85
	141.02	136.28	160.37	165.11
	160.37	144.38	153.26	180.91
	164.32	163.34	151.68	116.92
180	153.66	145.76	135.09	169.85
	152.87	145.76	141.41	119.29
	129.96	129.76	138.25	127.98

Data for Conjugated Diene Level in Rat Liver During Normothermic Ischaemia (fluorescence units/g liver)

Isch. Period (min.)	CONTROL		DENATURED SOD	
	7.95	7.98	7.25	9.80
	9.58	7.93	7.10	7.35
0	9.83	7.68	7.75	8.45
	10.05	8.40	8.80	8.65
	9.48	7.25	10.30	9.60
	9.03	7.63	6.70	8.35
	9.15	7.75	6.95	7.60
15	9.10	6.85	7.85	7.75
	9.70	7.50	9.45	9.35
	8.68	6.95	9.25	9.70
	8.78	6.70	6.85	8.05
	10.05	6.95	8.15	7.90
30	10.00	7.25	7.05	7.90
	10.83	7.43	7.90	9.55
	8.33	6.85	8.90	8.85
	9.43	7.15	8.05	7.15
	8.63	6.70	6.20	7.40
45	9.08	7.33	6.95	7.20
	9.35	7.23	7.90	8.10
	7.50	6.63	9.35	9.75
	9.68	7.58	6.85	6.85
	8.29	6.53	7.80	7.30
60	8.68	6.80	7.35	7.40
	9.08	6.25	7.50	7.40
	6.73	5.48	8.80	8.65
	8.78	6.73	7.60	7.60
	7.33	5.60	6.20	7.35
120	8.40	6.30	6.60	6.35
	10.08	6.18	8.10	6.95
	7.28	5.83	8.70	9.25
	7.48	5.40	7.30	7.50
	7.88	6.18	6.80	5.60
180	7.68	6.28	5.10	7.40
	10.18	5.68	7.00	8.95
	6.43	5.18	8.10	8.10

Isch. Period (min.)	500u/Kg SOD		1000u/Kg SOD	
	8.23	11.25	4.30	6.20
	8.10	10.43	3.70	9.15
0	8.73	10.05	7.15	9.30
	8.88	10.05	6.90	7.85
	11.05	10.98	5.50	8.50
	8.25	11.70	8.05	7.80
	6.90	10.05	8.25	8.25
15	8.23	10.98	9.40	8.45
	8.53	10.93	9.35	8.30
	12.80	10.83	7.85	8.25
	7.93	11.00	8.65	9.20
	7.80	9.40	8.35	9.90
30	8.58	10.65	9.50	10.35
	9.73	9.80	10.45	9.95
	10.25	9.70	9.05	9.70
	7.38	10.20	6.10	7.90
	10.95	8.95	5.90	8.35
45	10.15	9.33	8.25	9.35
	10.75	9.58	7.65	7.45
	10.98	10.48	7.05	7.70
	7.03	12.50	9.85	9.95
	10.53	8.68	9.45	10.80
60	11.40	10.23	9.80	10.80
	10.48	9.73	10.45	9.65
	11.63	10.85	10.35	10.00
	6.63	10.53	9.00	8.40
	11.13	9.20	8.45	9.00
120	10.28	7.85	5.15	8.85
	9.35	9.18	5.35	7.75
	8.15	7.55	8.65	8.10
	7.75	10.65	7.65	8.35
	10.78	8.05	7.85	8.20
180	10.65	9.20	11.25	8.20
	8.15	7.03	10.10	8.05
	7.43	7.08	8.30	8.60

Isch. Period (min.)	10000u/Kg SOD		ALLOPURINOL 5mg/Kg	
	7.85	9.80	5.80	6.90
	9.55	9.00	8.70	9.50
0	10.60	9.00	5.00	6.00
	11.40	7.73	8.20	10.40
	9.75	9.65	10.85	10.70
	7.70	9.00	6.70	5.80
	9.35	8.00	6.10	7.00
15	10.40	8.50	2.60	3.30
	10.20	10.00	3.30	8.70
	8.60	8.20	8.00	6.10
	10.05	10.50	4.70	4.60
	11.25	7.73	5.10	5.00
30	14.25	10.00	2.10	2.10
	12.20	11.50	5.20	7.20
	10.10	10.33	8.60	4.50
	8.90	12.00	6.40	5.10
	9.35	9.45	3.30	5.20
45	12.15	9.50	2.60	3.60
	9.35	9.38	4.50	3.30
	9.65	9.75	4.30	7.60
	8.30	9.90	7.50	8.60
	10.50	9.30	7.50	9.80
60	11.65	9.23	4.50	5.30
	9.90	9.50	2.40	6.50
	9.45	8.95	6.70	7.80
	6.75	9.00	6.90	8.60
	9.05	7.00	6.20	7.00
120	9.65	6.88	4.20	5.80
	8.50	8.00	7.00	7.70
	8.25	8.50	8.90	10.10
	6.50	8.00	8.90	8.40
	10.20	7.00	8.10	7.30
180	10.85	7.85	3.20	5.20
	8.20	8.23	4.20	4.80
	8.00	8.20	8.80	8.20

Isch. Period (min.)	ALLOPURINOL 10mg/Kg		DESFERRIOXAMINE 5mg/Kg	
	10.10	8.05	9.85	10.45
	10.70	7.30	9.85	10.50
0	8.60	8.30	9.90	10.70
	8.73	9.03	9.60	12.10
	9.60	9.68	11.80	12.15
	9.38	7.55	9.95	10.00
	10.13	7.70	9.70	10.65
15	8.85	8.00	11.40	10.45
	8.38	8.45	10.00	12.00
	9.90	10.00	10.10	12.20
	9.40	7.53	10.40	9.75
	9.30	8.20	10.20	10.40
30	9.38	7.63	10.05	10.00
	9.18	8.78	10.50	11.80
	9.85	9.33	10.00	12.20
	9.25	7.95	10.75	10.05
	9.65	8.38	10.30	10.70
45	8.30	7.78	10.60	10.45
	7.95	8.35	10.60	12.45
	8.58	8.98	10.05	12.05
	10.20	7.88	9.65	9.15
	9.23	7.98	10.70	9.70
60	8.60	8.18	9.55	9.75
	8.10	9.50	8.95	10.75
	9.05	9.00	8.85	10.75
	10.28	7.58	10.30	8.75
	10.35	7.60	10.30	9.10
120	7.98	7.55	9.10	8.70
	7.90	8.40	9.00	9.95
	8.88	8.23	8.97	9.90
	9.08	7.38	8.95	9.75
	9.35	7.58	9.95	12.45
180	7.68	7.48	11.10	9.85
	7.18	8.08	11.10	10.50
	8.55	8.78	9.85	10.45

Isch. Period (min.)	DESFERRIOXAMINE 15mg/Kg		MANNITOL 1g/Kg	
	8.60	8.80	7.93	9.50
	8.63	10.33	9.20	8.90
0	8.90	8.90	8.98	9.50
	10.50	10.20	9.50	9.35
	10.18	7.95	8.70	9.23
	9.65	9.83	8.20	9.35
	10.30	9.60	8.65	8.88
15	10.23	9.10	8.33	8.48
	11.43	10.08	8.63	8.63
	11.68	7.13	8.58	9.43
	10.83	9.65	7.83	8.35
	8.93	9.75	7.95	6.90
30	9.10	9.00	7.80	7.73
	9.50	9.23	8.55	8.13
	10.63	7.28	8.15	8.58
	10.95	9.10	7.88	7.98
	10.13	9.78	7.05	7.62
45	10.05	9.20	9.43	7.25
	10.73	9.10	8.45	8.20
	10.43	7.23	7.85	8.13
	11.83	9.45	7.58	7.70
	10.88	10.30	6.75	7.70
60	9.90	9.55	7.43	7.98
	11.00	9.05	8.25	7.98
	10.83	9.70	7.60	7.68
	9.10	4.03	7.85	7.35
	10.15	3.25	6.10	6.73
120	9.03	3.23	7.00	6.98
	10.00	3.83	7.65	7.75
	9.55	7.13	6.88	7.03
	8.35	2.85	6.90	6.15
	9.83	2.00	5.88	6.15
180	8.15	2.38	8.23	6.40
	9.38	2.33	6.98	5.50
	9.53	5.35	6.25	4.88

Data for reduced glutathione level in rat liver during normothermic ischaemia (mmoles/g liver)

Isch. Period (min.)	CONTROL (saline)		DENATURED SOD	
	6.96	4.52	6.43	6.64
	6.83	5.38	6.98	6.72
0	6.90	6.27	7.72	7.63
	6.54	4.79	7.09	7.75
	6.12	5.85	7.26	7.53
	6.31	7.17	7.04	5.69
	7.12	5.23	7.17	6.68
15	6.50	5.06	8.15	7.71
	5.94	4.88	8.06	6.76
	6.00	6.10	7.77	7.09
	5.50	4.56	6.70	6.14
	7.42	5.48	6.53	6.23
30	5.38	4.67	8.15	7.63
	7.15	4.17	7.60	7.05
	5.19	3.75	7.30	7.13
	5.65	4.25	6.55	8.98
	6.21	7.24	6.47	6.10
45	5.94	4.62	6.53	6.47
	5.77	6.12	6.89	6.93
	3.77	4.19	6.28	5.90
	5.83	5.71	6.38	5.81
	5.88	4.13	6.11	5.48
60	6.52	3.96	6.60	6.06
	5.87	3.52	6.72	6.23
	4.83	5.10	5.77	5.61
	5.75	3.81	5.49	5.36
	5.23	3.29	4.32	4.66
120	4.87	2.56	4.98	4.99
	5.81	3.02	5.79	5.57
	4.37	3.75	4.91	5.03
	5.06	2.83	4.47	4.25
	5.44	3.27	3.64	3.59
180	4.94	2.62	4.47	4.27
	5.31	2.96	5.30	5.20
	3.23	3.38	3.66	4.04

Isch. Period (min.)	500u/Kg SOD		1000u/Kg SOD	
	3.38	5.24	3.72	5.42
	4.22	5.85	4.26	5.20
0	4.38	4.65	4.68	5.78
	3.99	4.91	4.31	5.32
	4.24	5.78	4.41	4.98
	4.45	5.40	4.08	5.78
	3.74	5.64	4.19	5.42
15	4.04	4.28	4.83	5.50
	3.95	4.85	4.37	4.67
	4.21	5.36	4.59	4.98
	4.22	5.01	3.81	5.14
	3.57	5.45	4.01	4.98
30	4.02	4.23	4.25	5.40
	3.75	4.82	4.10	5.47
	3.72	4.82	4.16	4.90
	3.49	5.31	3.93	5.31
	3.70	5.03	3.98	5.14
45	3.61	4.22	4.10	5.63
	3.46	4.77	4.19	5.16
	3.87	5.01	4.35	4.88
	2.82	5.36	4.01	4.44
	3.49	5.31	4.17	4.99
60	3.53	4.17	3.96	5.52
	3.34	4.52	3.54	4.90
	3.50	5.24	3.54	5.08
	2.82	3.86	3.71	4.86
	2.73	3.81	3.99	4.92
120	2.89	3.17	3.86	4.95
	2.79	4.20	2.96	4.58
	2.55	5.24	3.35	4.82
	2.91	3.08	2.70	4.01
	2.73	4.08	2.48	3.04
180	2.95	2.67	2.36	4.20
	2.37	3.95	1.82	4.08
	2.04	4.92	2.34	4.44

Isch. Period (min.)	10000u/Kg SOD		DESFERRIOXAMINE 5mg/Kg	
	7.18	6.03	4.28	4.16
	7.50	6.75	3.77	3.50
0	6.80	6.05	4.41	4.01
	6.16	6.33	4.08	4.11
	6.36	7.41	4.22	4.16
	6.62	4.92	4.02	3.83
	7.28	6.23	4.41	4.50
15	5.92	5.69	6.50	5.06
	6.34	6.15	4.02	3.98
	5.62	7.89	3.95	4.01
	7.14	5.79	4.01	3.71
	6.44	5.49	3.36	3.50
30	6.76	5.76	3.30	3.17
	7.26	6.21	4.00	4.07
	7.12	8.37	3.72	3.68
	6.34	5.25	3.00	3.36
	6.86	6.03	3.57	3.35
45	5.96	5.84	3.30	3.17
	6.30	5.75	3.81	3.80
	6.48	8.55	3.84	3.81
	6.06	5.31	3.38	3.18
	6.54	5.58	2.76	2.73
60	5.98	5.78	3.35	3.20
	6.06	6.00	3.36	3.56
	6.14	6.90	4.02	3.98
	6.26	5.23	3.15	3.17
	5.20	5.39	2.49	2.87
120	6.02	5.75	3.38	3.05
	6.14	5.64	3.06	3.14
	4.56	6.99	2.73	2.70
	4.68	5.58	3.02	3.12
	3.94	5.51	2.73	2.75
180	3.70	5.94	2.96	3.02
	3.52	6.06	2.96	2.99
	3.66	6.95	2.45	2.54

Isch. Period (min.)	DESFERRIOXAMINE 15mg/Kg		ALLOPURINOL 5mg/Kg	
	6.90	6.45	5.04	5.81
	5.72	6.41	6.03	6.02
0	6.78	6.86	4.91	6.09
	5.88	5.75	6.39	7.59
	6.35	7.86	5.43	4.47
	7.20	6.26	4.71	5.39
	6.83	6.26	5.70	5.73
15	6.68	6.87	4.79	6.99
	5.97	6.78	6.23	6.00
	5.72	7.05	5.18	5.01
	6.80	7.08	5.68	6.48
	5.82	5.88	5.21	5.24
30	6.12	6.30	4.64	5.31
	6.03	5.94	5.75	5.19
	6.18	6.75	5.19	4.23
	6.00	6.90	4.54	6.14
	5.88	6.18	4.68	4.17
45	5.15	5.34	4.33	4.50
	6.03	5.19	6.69	6.54
	6.27	6.02	4.77	4.56
	6.00	6.72	4.51	5.26
	4.50	6.21	4.32	4.50
60	4.38	4.70	4.75	7.04
	5.85	5.72	5.57	7.26
	6.48	6.02	4.55	6.08
	4.52	5.25	4.24	5.37
	5.70	4.56	4.22	4.34
120	3.93	4.29	4.44	6.00
	3.96	4.47	5.51	6.57
	4.83	4.65	3.75	4.35
	3.87	4.80	4.64	4.82
	4.05	4.52	5.10	4.88
180	3.87	4.58	4.96	5.19
	4.35	4.26	4.91	6.60
	4.35	3.84	4.46	4.50

Isch. Period (min.)	ALLOPURINOL 10mg/Kg		MANNITOL 1g/Kg	
	5.10	5.60	6.98	8.40
	5.48	6.15	7.00	7.83
0	5.30	6.03	7.53	8.20
	4.85	5.67	7.40	7.85
	5.35	6.36	7.05	6.88
	5.53	6.20	6.40	7.68
	5.50	6.26	6.83	7.60
15	5.45	6.15	7.43	8.05
	5.35	6.23	6.50	6.70
	5.43	6.47	6.83	7.25
	5.30	5.97	7.05	8.18
	5.48	6.20	6.93	5.98
30	5.23	6.09	7.73	8.23
	5.00	5.94	6.55	6.83
	5.20	6.44	7.45	7.65
	5.20	5.73	5.43	6.25
	5.25	6.09	6.63	7.23
45	4.73	5.57	8.03	8.90
	4.48	5.64	7.18	7.43
	5.20	6.30	7.20	6.90
	4.90	5.43	5.05	6.00
	4.95	5.31	6.50	7.10
60	4.58	5.46	7.05	7.40
	4.58	5.76	5.85	6.08
	4.88	5.45	6.20	6.50
	4.95	5.55	4.80	5.63
	5.13	5.85	4.18	4.48
120	4.85	5.73	5.73	5.85
	4.73	5.64	5.50	5.65
	4.68	5.70	5.55	5.58
	4.45	4.98	4.10	4.78
	4.80	5.54	4.05	4.33
180	4.38	5.16	4.38	4.43
	4.60	5.45	4.05	4.05
	4.60	5.43	4.50	4.33

Data for oxidised glutathione level in rat liver during normothermic ischaemia (mmoles/g liver)

Isch. Period (min.)	CONTROL (Saline)		DENATURED SOD	
	2.28	2.42	1.60	1.43
	2.46	1.86	1.85	1.56
0	2.06	1.70	1.22	1.03
	2.32	2.03	1.26	1.09
	2.50	1.89	1.75	1.35
	2.00	1.90	1.78	1.63
	2.32	1.87	2.01	1.71
15	2.02	1.73	1.60	1.35
	2.20	1.86	1.27	1.06
	2.54	1.73	2.08	1.69
	2.54	1.94	1.66	1.39
	3.14	1.90	2.21	1.79
30	1.90	1.55	1.78	1.47
	2.46	2.15	1.27	1.07
	2.64	1.87	2.19	1.74
	2.72	2.30	2.03	1.81
	2.54	2.03	2.12	1.84
45	2.64	2.00	2.00	1.64
	2.66	2.06	1.38	1.12
	3.06	2.59	2.21	1.81
	2.90	2.23	2.19	1.91
	3.22	2.62	2.33	2.01
60	2.08	1.92	2.29	1.86
	3.04	2.41	1.55	1.24
	2.98	1.99	2.42	1.90
	3.50	2.66	2.64	2.31
	3.70	2.94	2.55	2.01
120	3.60	2.72	2.55	2.13
	3.86	3.23	1.96	1.55
	4.42	1.75	2.82	2.15
	3.62	3.06	2.60	2.20
	4.60	3.46	2.40	2.10
180	3.80	3.07	2.63	2.25
	4.36	3.00	2.33	1.89
	4.22	2.62	2.71	2.27

Isch. Period (min.)	500u/Kg SOD		1000u/Kg SOD	
	1.15	0.96	1.19	1.68
	1.24	1.05	1.46	1.76
0	1.05	1.24	1.38	1.68
	1.24	1.24	1.50	1.80
	0.96	1.15	1.34	1.61
	1.19	1.02	1.86	2.50
	1.05	1.05	1.85	2.13
15	1.02	1.05	1.53	2.00
	1.05	1.02	1.64	1.77
	1.02	1.19	1.55	1.79
	1.10	0.98	1.98	2.40
	1.19	1.05	2.31	2.74
30	1.05	1.05	1.79	1.97
	1.05	1.19	2.25	2.42
	0.98	1.10	1.58	1.68
	1.11	1.02	2.04	2.36
	1.16	1.01	2.13	2.34
45	1.01	1.10	1.79	1.91
	1.10	1.16	1.88	2.07
	1.02	1.11	1.79	1.92
	1.19	1.03	1.92	2.45
	1.31	1.22	2.09	2.39
60	1.22	1.39	1.82	2.04
	1.39	1.31	2.09	2.16
	1.03	1.19	1.86	1.96
	1.64	1.06	2.76	3.33
	1.57	1.30	2.78	3.00
120	1.30	1.45	1.65	1.89
	1.45	1.55	2.45	2.57
	1.06	1.65	1.79	1.80
	1.85	1.71	2.90	3.44
	1.64	1.53	2.96	3.20
180	1.53	1.72	2.45	2.63
	1.72	1.64	2.66	2.75
	1.71	1.85	2.48	2.46

Isch. Period (min.)	10000u/Kg SOD		DESFERRIOXAMINE 5mg/Kg	
	0.80	0.72	2.63	3.33
	0.81	0.83	2.05	2.53
0	0.80	0.88	1.78	1.85
	0.86	0.66	2.40	2.18
	0.72	0.72	2.65	2.98
	0.80	0.82	2.63	3.25
	0.80	0.80	2.00	2.28
15	0.99	0.81	1.88	2.05
	0.76	0.75	2.28	2.10
	0.81	0.78	2.65	2.75
	0.89	0.85	3.08	3.68
	0.80	0.82	2.30	2.78
30	0.82	0.81	2.15	2.18
	0.78	0.80	2.65	2.55
	0.88	0.82	3.20	3.13
	0.81	0.85	3.13	3.60
	0.84	0.77	2.30	2.55
45	0.82	0.82	2.15	2.23
	0.77	0.82	2.60	2.33
	0.90	0.85	3.30	3.35
	0.82	0.99	3.43	3.68
	0.82	0.85	2.53	2.65
60	0.81	0.82	2.25	2.25
	0.81	0.82	2.58	2.63
	0.86	0.84	3.55	3.43
	0.85	0.90	3.20	3.30
	0.99	0.99	3.23	3.23
120	0.90	0.97	2.68	2.78
	0.94	0.95	3.33	3.23
	0.94	0.94	3.30	3.30
	1.37	1.38	3.10	3.10
	1.34	1.25	2.73	2.95
180	1.29	1.24	2.45	2.50
	1.21	1.38	2.90	2.73
	1.30	1.37	3.10	3.23

Isch. Period (min.)	DESFERRIOXAMINE 15mg/Kg		ALLOPURINOL 5mg/Kg	
	1.10	1.25	1.01	1.12
	1.20	1.14	1.05	1.24
0	1.47	1.26	1.04	1.14
	1.59	1.08	1.36	1.34
	1.58	0.99	1.17	1.26
	1.10	1.40	0.93	0.92
	1.13	1.23	1.14	1.26
15	1.44	1.35	1.07	1.28
	1.56	1.11	1.25	1.44
	1.79	1.32	1.08	1.18
	1.51	1.54	1.22	1.46
	1.35	1.92	1.11	1.24
30	2.28	1.90	1.08	1.28
	1.57	1.32	1.40	1.34
	1.97	1.32	1.35	1.30
	1.43	1.65	1.14	1.24
	1.65	1.59	1.29	1.30
45	1.92	1.74	1.08	1.26
	2.06	1.58	1.52	1.32
	2.10	1.41	1.29	1.38
	1.29	1.75	1.16	1.32
	1.72	1.41	1.21	1.30
60	1.92	1.76	1.35	1.42
	1.67	1.71	1.55	1.28
	2.10	1.26	1.26	1.40
	2.25	2.31	1.44	2.00
	2.30	1.72	1.40	1.36
120	2.43	2.34	1.62	1.50
	2.52	2.28	1.83	1.48
	2.85	2.71	1.64	1.78
	2.71	3.15	1.56	1.78
	2.28	2.28	1.46	1.58
180	2.75	2.56	1.32	1.72
	2.72	2.27	1.64	1.86
	3.84	2.30	1.53	1.78

Isch. Period (min.)	ALLOPURINOL 10mg/Kg		MANNITOL 1g/Kg	
	0.99	1.07	3.84	3.18
	1.04	1.08	4.00	3.36
0	1.10	1.05	3.78	3.06
	1.11	1.04	3.58	2.73
	1.23	1.13	3.94	2.93
	1.11	1.28	3.82	3.23
	1.10	1.10	4.02	3.15
15	1.11	1.10	3.74	2.97
	1.13	1.11	3.38	2.54
	1.23	1.16	3.28	2.45
	1.10	1.19	3.78	3.18
	1.10	1.16	3.66	3.11
30	1.19	1.14	3.92	2.96
	1.10	1.05	3.40	2.55
	1.22	1.14	3.38	2.46
	1.13	1.19	4.08	3.44
	1.17	1.17	4.10	3.27
45	1.10	1.05	3.94	3.02
	1.16	1.10	3.60	2.64
	1.25	1.17	3.76	2.73
	1.35	1.37	4.16	3.47
	1.14	1.14	4.54	3.59
60	1.16	1.11	4.48	3.41
	1.17	1.17	3.98	2.97
	1.20	1.11	3.58	2.63
	1.37	1.38	4.30	3.63
	1.13	1.13	5.18	4.07
120	1.25	1.22	4.38	3.32
	1.19	1.16	4.62	3.48
	1.31	1.22	3.98	2.96
	1.40	1.40	4.44	3.81
	1.44	1.40	4.64	3.71
180	1.23	1.19	5.06	3.96
	1.32	1.31	4.64	3.48
	1.37	1.29	4.30	3.38

Data for glutathione ratio (oxidised/reduced) in rat liver during normothermic ischaemia

Isch. Period	CONTROL (saline)		DENATURED SOD	
	0.33	0.54	0.25	0.22
	0.36	0.35	0.27	0.24
0	0.30	0.28	0.16	0.14
	0.36	0.43	0.18	0.14
	0.41	0.33	0.25	0.18
	0.32	0.27	0.26	0.29
	0.33	0.36	0.28	0.26
15	0.31	0.35	0.20	0.18
	0.37	0.39	0.16	0.16
	0.43	0.29	0.27	0.24
	0.47	0.43	0.25	0.23
	0.43	0.35	0.34	0.29
30	0.36	0.34	0.22	0.20
	0.35	0.52	0.17	0.16
	0.51	0.50	0.30	0.25
	0.49	0.55	0.31	0.31
	0.41	0.28	0.33	0.31
45	0.45	0.44	0.31	0.37
	0.47	0.34	0.20	0.17
	0.82	0.62	0.36	0.31
	0.50	0.39	0.35	0.33
	0.55	0.64	0.39	0.37
60	0.32	0.49	0.35	0.31
	0.52	0.62	0.23	0.20
	0.62	0.39	0.42	0.34
	0.61	0.70	0.35	0.33
	0.71	0.90	0.59	0.44
120	0.74	1.07	0.52	0.43
	0.67	1.07	0.34	0.28
	0.97	0.47	0.56	0.43
	0.72	1.09	0.59	0.52
	0.85	1.06	0.66	0.59
180	0.77	1.18	0.59	0.53
	0.83	1.02	0.44	0.37
	1.31	0.78	0.74	0.56

Isch. Period (min.)	500u/Kg SOD		1000u/Kg SOD	
	0.34	0.19	0.32	0.31
	0.30	0.18	0.35	0.34
0	0.24	0.27	0.30	0.29
	0.31	0.22	0.35	0.34
	0.23	0.20	0.31	0.33
	0.27	0.19	0.46	0.44
	0.28	0.20	0.45	0.40
15	0.26	0.25	0.32	0.37
	0.27	0.22	0.38	0.38
	0.25	0.23	0.34	0.36
	0.26	0.20	0.52	0.45
	0.34	0.20	0.58	0.55
30	0.27	0.25	0.42	0.37
	0.28	0.25	0.55	0.45
	0.27	0.23	0.38	0.35
	0.32	0.20	0.52	0.45
	0.32	0.20	0.54	0.46
45	0.28	0.26	0.44	0.34
	0.32	0.25	0.45	0.41
	0.27	0.23	0.42	0.40
	0.43	0.20	0.48	0.56
	0.38	0.23	0.51	0.48
60	0.35	0.34	0.46	0.37
	0.42	0.25	0.59	0.44
	0.30	0.23	0.53	0.39
	0.52	0.28	0.75	0.69
	0.58	0.35	0.70	0.61
120	0.45	0.46	0.43	0.39
	0.52	0.38	0.83	0.57
	0.42	0.39	0.54	0.38
	0.64	0.56	1.08	0.86
	0.60	0.38	1.20	1.06
180	0.52	0.65	1.04	0.63
	0.73	0.42	1.47	0.68
	0.84	0.38	1.06	0.56

Isch. Period (min.)	10000u/Kg SOD		DESFERRIOXAMINE 5mg/Kg	
	0.12	0.12	0.62	0.80
	0.11	0.13	0.67	0.73
0	0.12	0.15	0.41	0.47
	0.14	0.11	0.59	0.53
	0.12	0.10	0.63	0.72
	0.12	0.17	0.66	0.85
	0.11	0.13	0.46	0.51
15	0.17	0.15	0.49	0.56
	0.12	0.13	0.57	0.53
	0.15	0.10	0.67	0.69
	0.13	0.15	0.77	1.00
	0.13	0.15	0.64	0.80
30	0.13	0.14	0.66	0.69
	0.11	0.13	0.66	0.63
	0.13	0.10	0.86	0.85
	0.13	0.17	1.05	1.08
	0.13	0.13	0.65	0.77
45	0.14	0.14	0.68	0.67
	0.13	0.15	0.69	0.62
	0.14	0.10	0.86	0.62
	0.14	0.19	1.02	1.16
	0.13	0.16	0.92	0.97
60	0.14	0.15	0.68	0.71
	0.14	0.14	0.77	0.74
	0.14	0.13	0.89	0.87
	0.14	0.18	1.02	1.05
	0.19	0.19	1.30	1.13
120	0.15	0.17	0.80	0.92
	0.16	0.17	1.09	1.03
	0.21	0.14	1.21	1.23
	0.30	0.25	1.03	1.00
	0.34	0.23	1.00	1.08
180	0.35	0.21	0.83	0.83
	0.35	0.23	0.98	0.92
	0.36	0.20	1.27	1.28

Isch. Period (min.)	DESFERRIOXAMINE 15mg/Kg		ALLOPURINOL 5mg/Kg	
	0.16	0.28	0.20	0.20
	0.21	0.18	0.18	0.20
0	0.22	0.19	0.22	0.19
	0.27	0.19	0.22	0.18
	0.25	0.13	0.22	0.29
	0.16	0.23	0.20	0.17
	0.17	0.20	0.20	0.22
15	0.22	0.20	0.23	0.19
	0.27	0.17	0.20	0.24
	0.32	0.19	0.21	0.24
	0.23	0.22	0.22	0.23
	0.24	0.33	0.22	0.24
30	0.38	0.31	0.24	0.25
	0.26	0.23	0.25	0.26
	0.32	0.20	0.26	0.31
	0.24	0.24	0.26	0.21
	0.32	0.26	0.28	0.28
45	0.38	0.33	0.25	0.28
	0.35	0.27	0.23	0.21
	0.34	0.24	0.27	0.31
	0.22	0.26	0.26	0.25
	0.39	0.23	0.28	0.29
60	0.44	0.38	0.29	0.21
	0.29	0.30	0.28	0.18
	0.33	0.21	0.28	0.23
	0.50	0.44	0.34	0.38
120	0.41	0.38	0.34	0.32
	0.62	0.55	0.37	0.25
	0.64	0.51	0.34	0.23
	0.59	0.59	0.44	0.41
	0.70	0.73	0.34	0.37
	0.57	0.51	0.29	0.33
180	0.71	0.56	0.27	0.34
	0.63	0.54	0.34	0.29
	0.89	0.60	0.35	0.40

Isch. Period (min.)	ALLOPURINOL 10mg/Kg		MANNITOL 1g/Kg	
	0.20	0.20	0.55	0.38
	0.19	0.18	0.58	0.43
0	0.21	0.18	0.51	0.38
	0.23	0.19	0.54	0.35
	0.23	0.18	0.56	0.43
	0.20	0.21	0.60	0.42
	0.20	0.18	0.59	0.42
15	0.21	0.18	0.51	0.37
	0.22	0.18	0.52	0.38
	0.23	0.18	0.48	0.34
	0.21	0.20	0.54	0.39
	0.20	0.19	0.53	0.52
30	0.23	0.19	0.51	0.36
	0.22	0.18	0.52	0.38
	0.24	0.18	0.46	0.33
	0.22	0.21	0.76	0.55
	0.23	0.20	0.62	0.46
45	0.24	0.19	0.49	0.34
	0.25	0.20	0.51	0.36
	0.24	0.19	0.53	0.40
	0.28	0.26	0.83	0.58
	0.23	0.22	0.70	0.51
60	0.26	0.21	0.64	0.46
	0.26	0.21	0.68	0.49
	0.25	0.21	0.58	0.43
	0.28	0.25	0.90	0.65
	0.22	0.20	1.24	0.91
120	0.26	0.22	0.77	0.57
	0.26	0.21	0.84	0.62
	0.28	0.22	0.72	0.53
	0.32	0.29	1.09	0.80
	0.30	0.26	1.15	0.86
180	0.28	0.23	1.16	0.90
	0.29	0.24	1.15	0.86
	0.30	0.24	0.96	0.78

Data for urea production in rat liver during normothermic ischaemia (micromoles/g liver)

Isch. Period (min.)	CONTROL (saline)		10000u/Kg SOD	
	184.75	276.97	127.17	89.06
	193.72	191.54	115.47	92.64
0	290.79	211.27	93.77	112.83
	256.29	151.06	87.74	119.06
	172.91	196.43	108.11	109.25
	64.52	129.78	19.25	61.32
	96.14	36.55	40.00	39.62
60	61.48	62.42	73.58	49.25
	99.67	18.02	38.49	45.66
	96.46	66.09	69.81	37.36
	71.85	64.89	36.04	70.94
	44.48	35.09	70.38	45.66
120	29.91	60.82	35.85	43.40
	54.92	15.24	45.66	53.02
	54.60	78.94	49.25	43.40
	93.84	107.62	31.13	36.23
	5.74	29.24	24.15	48.49
180	91.39	28.81	27.55	32.45
	48.82	12.60	44.53	30.00
	30.94	47.73	45.66	23.96

Isch. Period	ALLOPURINOL 10mg/Kg		DESFERRIOXAMINE 15mg/Kg	
	279.73	251.70	196.57	243.55
	191.81	366.70	192.34	277.24
0	168.46	425.17	147.66	434.59
	164.81	200.64	211.96	190.83
	287.38	239.58	155.12	340.67
	69.47	95.09	16.25	82.56
	55.60	97.66	81.74	58.48
60	37.66	240.22	43.25	87.36
	22.73	76.10	21.34	33.89
	134.77	111.69	33.24	111.05
	36.57	115.59	3.25	26.83
	53.81	61.75	62.15	54.14
120	15.86	180.70	53.69	41.49
	43.57	96.86	31.30	60.64
	134.77	77.70	51.71	88.46
	49.36	80.17	53.61	37.15
	19.73	65.77	62.51	23.82
180	65.40	114.70	34.30	56.78
	22.73	105.51	28.45	56.93
	142.70	46.94	16.62	41.41

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