REPERFUSION INJURY FOLLOWING HYPOTHERMIC RENAL PRESERVATION: EVIDENCE OF EARLY OXIDATIVE DAMAGE IN THE RABBIT KIDNEY

by

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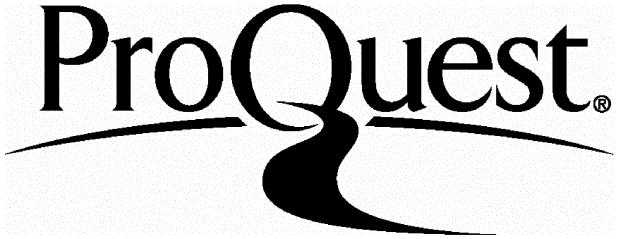

This Thesis is dedicated to my parents Sam and Rosa, and to my sister Susan, for their support and encouragement throughout my studies.

REPERFUSION INJURY FOLLOWING HYPOTHERMIC RENAL PRESERVATION: EVIDENCE OF EARLY OXIDATIVE DAMAGE IN THE RABBIT KIDNEY

Rabbit kidney preservation for 72h by methods used clinically followed by *ex vivo* normothermic reperfusion produced functional changes associated with injury to vascular and urinary systems. Protein leaked into these spaces and solute reabsorption was disrupted, although energy substrate production persisted. Experiments tested the hypothesis that reactive oxygen species produced following kidney reoxygenation caused reperfusion damage, and that injury was dependent upon biochemical changes which provided a transition metal catalyst (iron) during ischaemic preservation.

Fresh and preserved kidneys were reperfused normoxically or hypoxically in the presence of salicylate which produced characteristic hydroxyl radical (•OH) dependent metabolites during early reperfusion, particularly in preserved kidneys reperfused normoxically. In the absence of •OH scavenging by salicylate, LDH release into the renal vasculature was elevated by normoxic reperfusion and there was evidence of oxygen-dependent injury to proximal tubules, however lipid peroxidation was unaltered.

Groups of kidneys were given a cold re-flush (CRF) following preservation to remove putative pathological metabolites before normothermic reperfusion. Urinary and vascular spaces were cleared, and kidneys showed less tissue damage upon reperfusion than non-CRF controls, characterized

by decreased enzyme release from cytosolic (LDH) compartments and tubule cell membranes (γ-GT). After storage and CRF kidneys were reperfused either hypoxically or normoxically to determine whether tissue damage upon reperfusion was oxygen-dependent. LDH release into the vasculature at the onset of reperfusion was elevated by normoxia.

The anti-malarial drug chloroquine was added to the preservation solution to prevent intracellular acidification during kidney storage. Chloroquine improved some functional parameters in 48h preserved kidneys compared with untreated controls. For example release of the intralysosomal enzyme N-acetyl-ß-D-glucosaminidase into vascular and urinary spaces was decreased and urinary tubule and glomerular integrity was better maintained.

Studies of the distribution of an iron chelator, desferrioxamine (DFX) showed urinary clearance rates equal to glomerular filtration rate, and no accumulation in kidney tissues. DFX administered in storage and reperfusion solutions decreased •OH production during early reperfusion, suggesting a role for iron in the synthesis of these species. DFX did not appear to prevent tissue damage.

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ABBREVIATIONS

ACE	:	angiotensin converting enzyme
ADP	:	adenosine diphosphate
AMP	:	adenosine monophosphate
ATP	:	adenosine triphosphate
ATPase	:	adenosine triphosphatase
BSA	:	bovine serum albumen
CAPD	:	continuous ambulatory peritoneal dialysis
CoA	:	coenzyme A
CRF	:	cold re-flush
CQ-HCA	:	hypretonic citrate containing chloroquine
DFX	:	desferrioxamine
2,3-DHB	:	2,3-dihydroxybenzoate
2,5-DHB	:	2,5-dihydroxybenzoate (Gentisic acid)
1,25-DHCC	:	1,25-dihydroxycholecalciferol
DNA	:	deoxyribonucleic acid
EC	:	energy charge
EDRF	:	endothelium-derived relaxing factor
EDTA	:	ethylenediaminetetraacetic acid
EM	:	electron microscopy
ESR	:	electron spin resonance spectrometry
GFR	:	glomerular filtration rate
GSH	:	glutathione (reduced form)
GSSG	:	glutathione (oxidized form)
τ-GT	:	gamma glutamyl transpeptidase (transferase)
H_2O_2	:	hydrogen peroxide

HCA : hypertonic citrate

HCl : hydrochloric acid

HES : hydroxyethyl starch (Hespan)

HPLC : high performance liquid chromatography

hSOD : human recombinant superoxide dismutase

IM : intramuscular

IV : intravenous

JG : juxtaglomerular cells

LDH : lactate dehydrogenase

MUADG : 4-methyl-umbelliferyl-2-acetamido-2-deoxy-ß-D-glucopyramoside

NAD : nicotinamide adenine dinucleotide

NAG : N-acetyl-ß-D-glucosaminidase

NTA : nitrilotriacetic acid

NZW : NewZealand White (rabbits)

PAGE : polyacrylamide gel electrophoresis

PEG : polyethylene glycol

PBN : n-phenyl-t-butyl nitrone

PBS : phosphate-buffered saline

PCA : perchloric acid

P_i : inorganic phoshate

PUFA : polyunsaturated fatty acid

RAFT : Restoration of Appearance and Function Trust

RNA : ribonucleic acid

ROS : reactive oxygen species

SDS : sodium dodecyl sulphate

SLS : sodium-lactobionate-sucrose (solution)

SOD : superoxide dismutase

TBA : thiobarbituric acid

U/W : University of Wisconsin (solution)

XD : xanthine dehydrogenase

XO : xanthine oxidase

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LIST OF ELECTRON MICROGRAPHS

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Plate 4.2 : Glomerulus from 48h preserved kidney reperfused normoxically for 5min. Magnification = x5,000

Plate 4.3 : Proximal tubules from kidneys stored for 48h and then reperfused normoxically for 5min. Magnification = x4,000

Plate 4.4 : Proximal tubule from kidney stored for 48h and then hypoxically reperfused for 5min. Magnification = x4,000

Plate 4.5 : Thick ascending tubule from kidney stored for 48h and then reperfused hypoxically. Magnification = x4,000

INTRODUCTION

RENAL PHYSIOLOGY - PRESERVATION/REPERFUSION EFFECTS

The kidney has a number of physiological functions which must resume following transplantation for the grafted organ to be of benefit to the recipient. It is desirable that full renal function is immediate, but delayed graft function can be accommodated by using appropriate support techniques such as haemodialysis and careful dietary management. However, this increases the overall cost of the procedure and also increases the risk of infection and morbidity.

Renal structure and functions

The major role of the kidneys (structure shown in Fig.1.1) is to form urine and thereby facilitate the excretion of metabolic waste products from the body, and to maintain fluid and electrolyte homeostasis. Although kidneys exist in pairs in the normal healthy individual, they have a great deal of reserve function and it is possible for individuals to live in good health with only one functional organ. Blood flow to the organs is relatively large; although the kidneys represent only approximately 0.5% of total body weight they receive 25% of the cardiac output. It is therefore important that following transplantation an adequate supply of blood is restored to the kidney and that the renal vasculature is maintained in a physiologically functional state to allow normal flow.

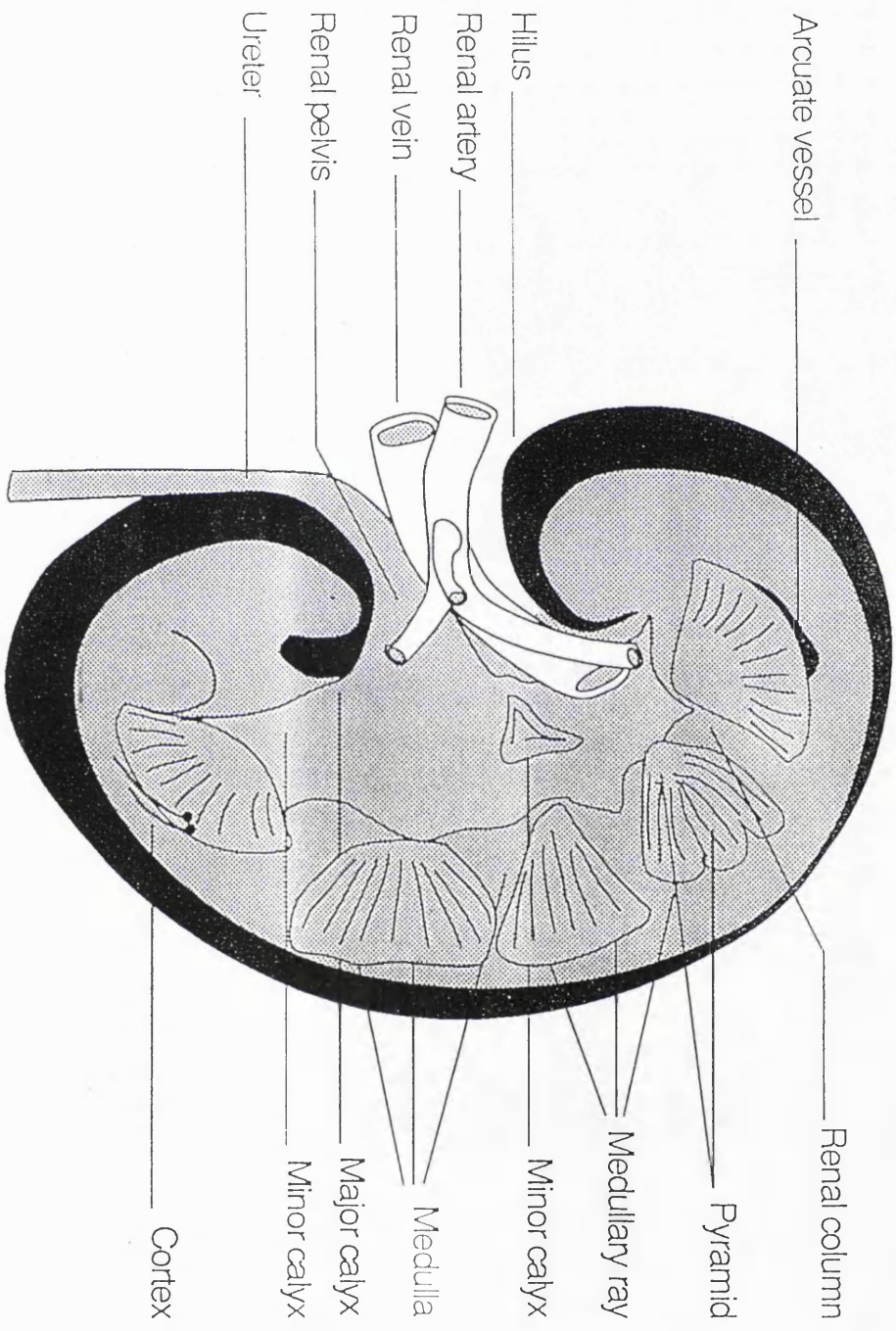


Fig.1.1: Gross kidney structure

Urine is formed by the ultrafiltration (filtration under pressure) of plasma; the hydrostatic pressure required for this is generated from the circulatory pressure plus the additional effect of a narrowing of the efferent arterioles from the glomeruli - the bundles of capillaries which function as filtration units (Fig.1.2). Damage to the afferent and efferent arterioles, or inappropriate release of vasoactive agents, due to transplant procedures, may effect the ultrafiltration process.

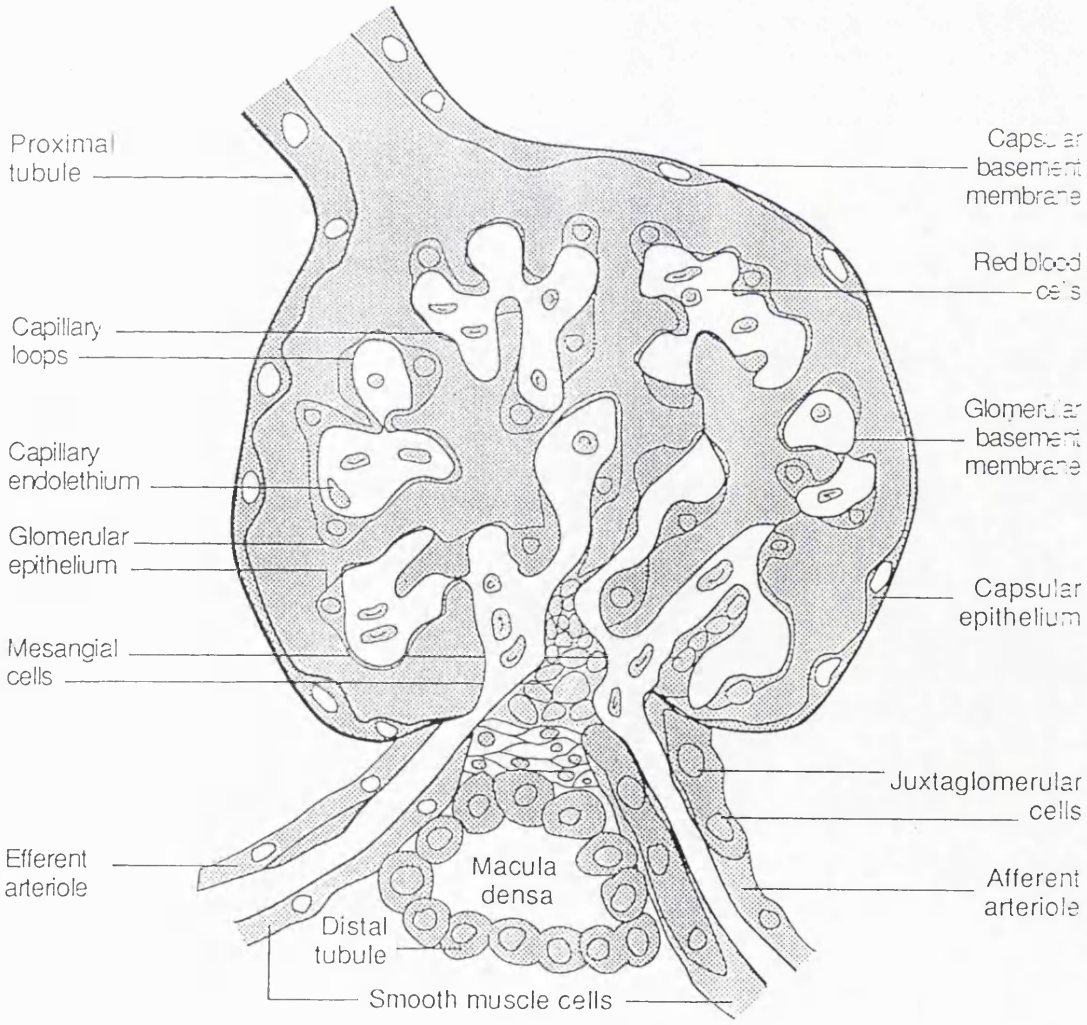


Fig.1.2: Structure of the glomerulus

Some solutes and water are reabsorbed in various parts of the renal tubules and other solutes are secreted by the tubular epithelial cells into the urinary spaces. The composition of the plasma ultrafiltrate, the glomerular filtrate, is dependent upon the permeability of the membranes between the plasma and glomerular capsule space. The glomerular capillaries are fenestrated by the presence of gaps at the junctions of their endothelial cells. The basement membrane is a continuous layer of mucopolysaccharide and glycoprotein. On the capsular side of the basement membrane are epithelial cells, podocytes, which are in contact with the membrane via interdigitating processes. The above structures constitute a selective permeability barrier between vascular and glomerular compartments, and therefore dictate the composition of the glomerular filtrate. It is therefore a fundamental prerequisite for normal renal function that this barrier does not lose its selectivity as a result of preservation of the organ or due to damage occurring following transplantation. The glomerular membrane is freely permeable to water, electrolytes, and low molecular weight plasma constituents (up to a molecular weight of approximately 69,000 a.m.u.) but does not allow passage of cells and most proteins. Preservation of glomerular structure and viability of podocytes and endothelial cells is needed to prevent proteinuria and the possibility of passage of cells into the renal tubules.

The volume of urine produced and its composition are

greatly different to the glomerular filtrate due to reabsorption and excretion mechanisms of the epithelial cells of the tubules. Solutes in the fluid passing through the tubules are reabsorbed by both active and passive mechanisms. Solutes such as glucose are usually fully reabsorbed unless the blood glucose concentration, and therefore the glomerular filtrate glucose concentration, rises above a level which exceeds the capacity of tubular reabsorption. This phenomenon is called the renal threshold for glucose. Glucose is fully reabsorbed from the proximal tubule (Fig.1.3). Damage to the tubular epithelium by the ischaemic insult of flush preservation and/or reperfusion injury may decrease the ability of the cells to reabsorb glucose, thereby artificially lowering the renal threshold for glucose. Since reabsorption is an active process, depletion of intracellular adenine nucleotide pools during cold ischaemic preservation, and in particular diminished ATP levels, may lead to inefficient glucose reabsorption, and the appearance of elevated glucose levels in the urine early after transplantation.

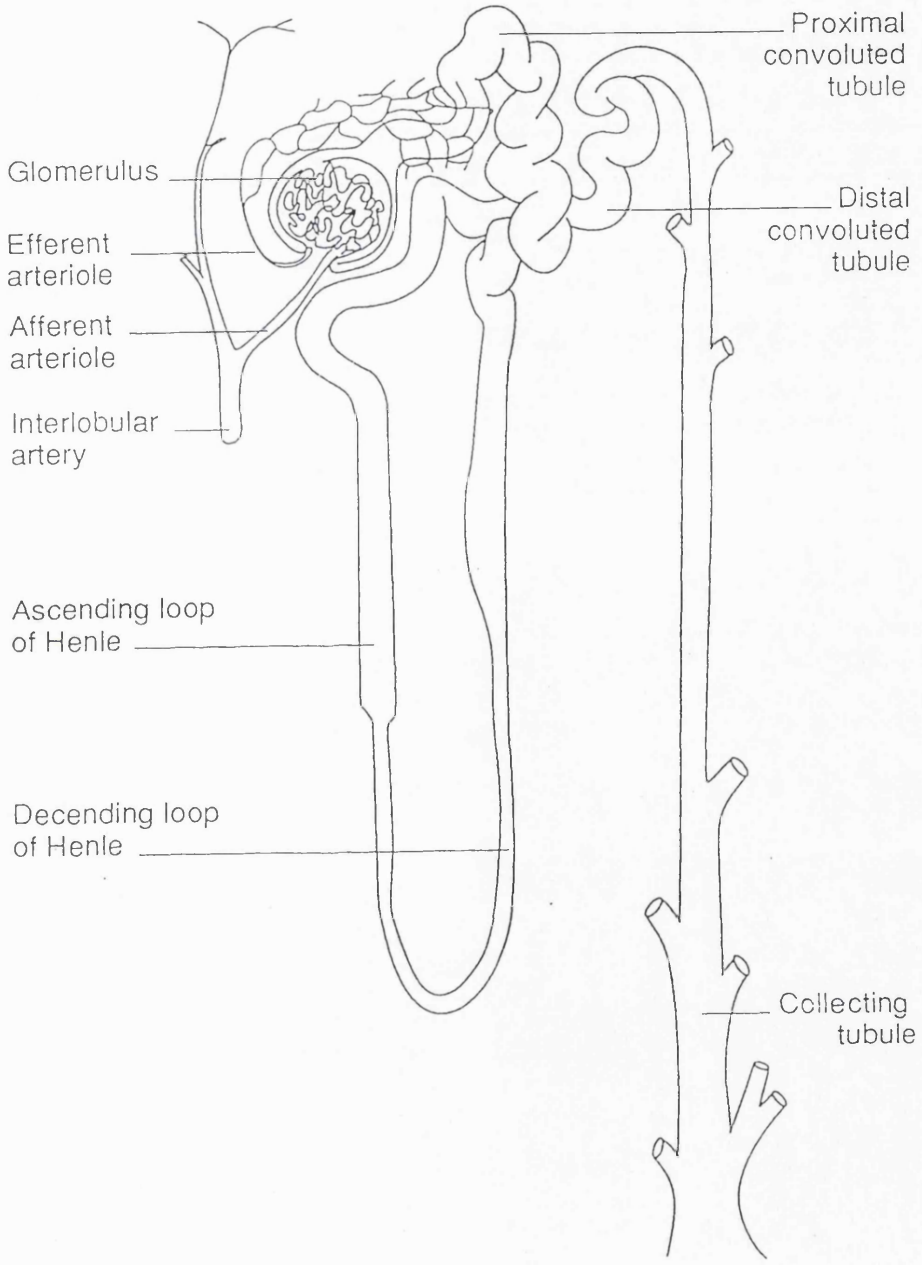


Fig.1.3: Tubule structure

Amino acids pass freely into the glomerular filtrate but are present only in trace amounts in the urine. A transport mechanism proposed by Meister (1973) accounts for the transport of a large proportion of most amino acids. The mechanism involves the enzyme τ-glutamyl transpeptidase (τ-GT) and glutathione (τ-glutamyl-cysteinyl-glycine).

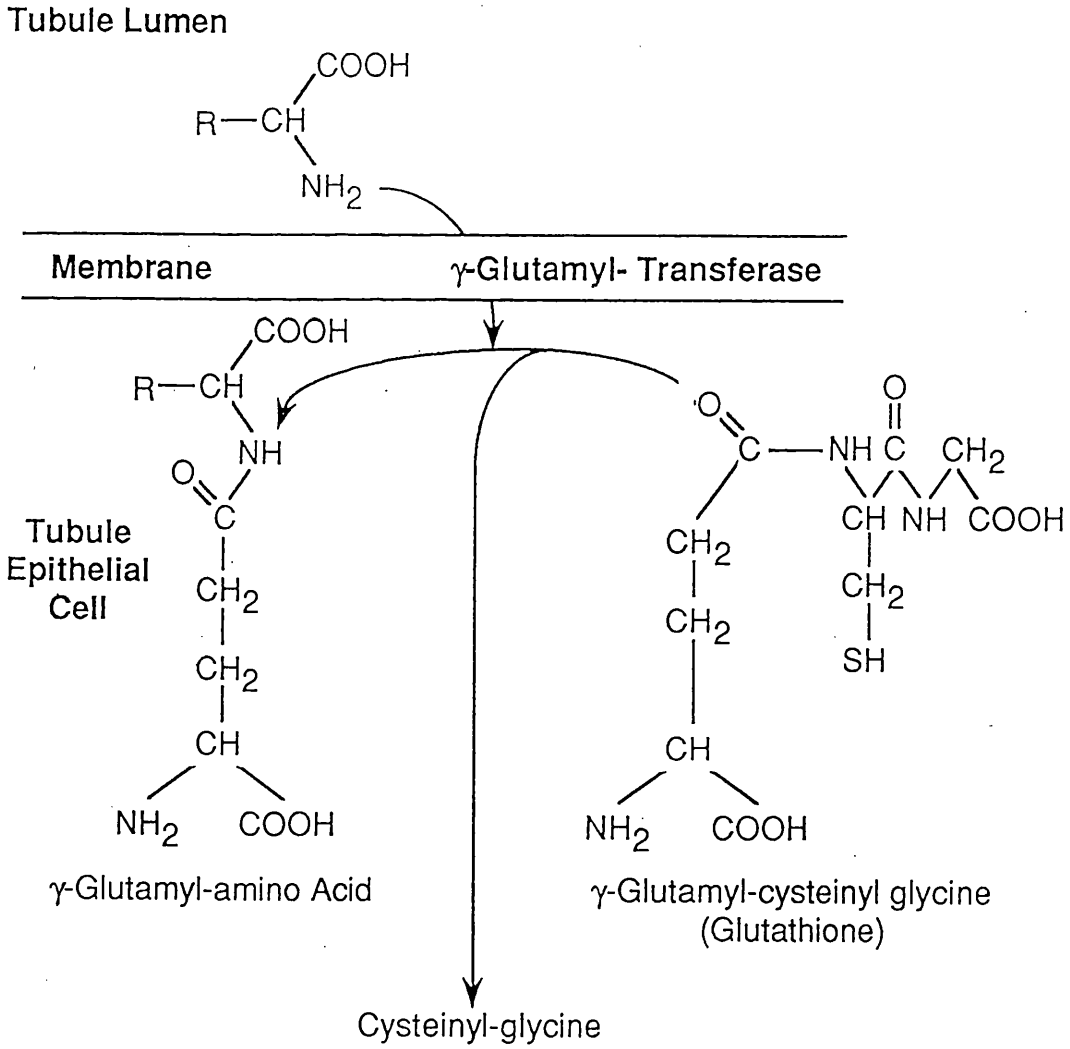


Fig.1.4: Site of τ-GT in proximal tubule membranes

γ-GT is a membrane-bound enzyme located in proximal tubule brush border membranes (Fig.1.4). Analysis of γ-GT in urine, either during flush preservation procedures or following reperfusion, gives an indication of the integrity of the proximal tubule cells and therefore reabsorptive capability.

Small quantities of protein, mainly albumin, are filtered by the glomeruli and most is reabsorbed in the proximal tubule, probably by pinocytosis (Lathem *et al.*, 1960). Proteinuria is therefore not only evidence of glomerular damage, but also may indicate suppressed pinocytotic mechanisms.

Sodium ions are reabsorbed actively by a specific Na^+/K^+ -dependent ATPase enzyme which transports Na^+ ions into the peritubular space, thereby causing a concentration gradient which favours passive transport of Na^+ ions from the tubule lumen into tubule cells. Analysis of sodium ion concentration in the urine compared with the filtered sodium load in the glomerular filtrate, gives an indication of the activity of the Na^+/K^+ ATPase following transplantation. Sodium reabsorption also occurs via a sodium-glucose symport mechanism. Both surfaces of the proximal tubule cells actively transport potassium (K^+) ions ; there is a net movement of K^+ ions into the peritubular space by passive diffusion. The other major ions reabsorbed in the proximal tubule are phosphate and bicarbonate.

Further reabsorption of solutes, mainly sodium and chloride (Cl^-) ions, takes place in the loop of Henle and in

the distal tubules. In the distal tubules sodium reabsorption is dependent upon the adrenocortical hormone aldosterone, whose release is stimulated by circulating angiotensin II. Water reabsorption is passive and occurs in the loop of Henle, distal tubules and collecting ducts. Water reabsorption occurs in the loop of Henle via a process known as the counter-current multiplier mechanism. The basis for this mechanism is the fact that the descending limb of the loop of Henle is freely permeable to water while the ascending limb is relatively impermeable to water. Cl^- ions are actively transported from ascending limb epithelial cells into the interstitial fluid. The movement of these negatively charged ions into the interstitium produces an electrochemical gradient which causes cations (mainly Na^+) to pass out of the epithelial cells into the interstitial fluid which then becomes hypertonic. Water moves out of the descending limb under an osmotic gradient, which thereby causes the fluid in the descending limb to become hypertonic. Water reabsorption from the descending limb facilitates active Cl^- reabsorption from the ascending limb by diluting the interstitial fluid, thereby lowering the concentration gradient against which Cl^- is transported.

Permeability of the distal tubules is increased by antidiuretic hormone (ADH) released by the posterior pituitary. Ischaemic injury or reperfusion injury following transplantation, may not in isolation significantly influence water reabsorption but, since the response to ADH is mediated

by adenylate cyclase, depletion of adenine nucleotides may influence the response to the hormone.

Endocrine functions

The enzyme renin is synthesized in the juxtaglomerular (JG) cells and cleaves the circulating plasma tetradecapeptide angiotensinogen to angiotensin I, which is subsequently converted to the vasoactive peptide angiotensin II by angiotensin converting enzyme (ACE) in the lungs. Renin secretion is stimulated by a decrease in mean renal arterial pressure or by decreased sodium ion concentration in the macula densa, a group of specialized cells in the distal tubule. It is feasible that vascular damage, or damage to the macula densa, caused by ischaemia and/or reperfusion could lead to inappropriate synthesis or release of renin by JG cells. Erythropoetin is a hormone of renal origin which stimulates bone marrow to increase red blood cell production, and is known to be released during hypoxia. An active metabolite of vitamin D, 1,25-dihydroxycholecalciferol (1,25-DHCC), necessary for calcium transport across membranes, is also produced and released in kidneys.

Thus by detailed analysis of urine composition and by comparison with the composition of solutes entering the kidney via the renal artery, it is possible to identify sites of dysfunction within the kidney. Once those cells and tissues which are most susceptable to ischaemic injury and reperfusion

injury have been defined, it might be possible to institute therapy to prevent or lessen this damage.

RENAL DISEASES

Renal diseases are diagnosed by measurement of kidney functions and comparison of these data with standard values obtained from normal subjects. Renal functions commonly measured include: 1)tubular transport maximum (T_m) -maximum rate of solute reabsorption, such as glucose; 2)effective renal plasma flow; and 3)glomerular filtration rate (GFR). The pathophysiological manifestations of renal disease will be dictated by the site of injury within the kidney, (e.g. glomerular injury, tubular injury etc). Renal dysfunction is often secondary to other disorders such as hypertension, diabetes, infection, or accute hypotension.

Major renal diseases

1) Glomerulonephritis: This is an inflammatory disease which may follow infection by a member of the ß-haemolytic streptococci family and in which an antigen-antibody reaction damages the glomerulus so that filtration is decreased. Most patients recover, but a few progress to the chronic stages of the disease which may then require surgical removal of the kidneys (nephrectomy).

2) Nephrotic syndrome: This is a disease characterized by proteinuria and oedema, and most commonly arises secondary to glomerulonephritis, resulting in an increase in the pore size

in the glomerular basement membrane. Most of the protein lost in the urine is plasma albumin which leads to reduced colloid osmotic pressure in the plasma allowing fluid to diffuse into the interstitial spaces of body tissues, thereby causing oedema.

3) Tubular necrosis: In this condition the tubular epithelium may become necrotic as a result of prolonged ischaemia or the action of toxins, leading to decreased urine production (oliguria) or complete cessation of urine production (anuria). Support therapy such as haemodialysis may allow time for regeneration of the damaged epithelium over a number of weeks if necrosis is not too far advanced.

The kidney is particularly susceptible to drug-induced damage because concentration of the glomerular filtrate in the renal tubules also causes concentration of drugs which are able to pass across the glomeruli. These high concentrations of drugs may then damage tubule cells. Drugs in a number of therapeutic groups can be nephrotoxic, including antibiotics (e.g.streptomycin), anti-inflammatories (e.g.aspirin), and anaesthetics (e.g.methoxyflurane). In some instances, however, there is no obvious cause of renal failure.

Consequences of renal failure

Renal failure leading to anuria and oliguria results in uraemia, which is an accumulation in the blood of metabolic waste products which are usually excreted in urine. These have toxic effects in a number of organs if the patients are not

treated by haemodialysis, dietary control, or renal transplantation, uremia progresses to cause lethargy, mental deterioration, coma and ultimately death. Although elevated plasma urea is indicative of ureamia it is not the only cause of symptoms observed in uraemic patients. These effects are due to elevated blood potassium, phoshate and sulphate levels, and decreased levels of calcium and sodium. There is an accumulation of protein deamination products and metabolic acidosis occurs.

The symptoms which are manifested as a consequence of renal failure are dependent upon the specific sites of damage in the kidney. These symptoms will also be dependent upon the number of nephrons affected since the kidney has a substantial reserve capacity which may obscure changes in kidney performance until a specific number of nephrons have been damaged. Any kind of renal dysfunction affects all parts of the nephron to some extent, although either tubular or glomerular dysfunction may predominate. For instance, a decline in blood flow to the kidney for any reason will result in a diminished glomerular filtration rate (GFR). Glomerular damage results in increased proteinuria, causing a decrease in the amount of plasma albumin in the systemic circulation; tissue oedema ensues due to oncotic imbalances between intracellular and extracellular environments. Damage to kidney tubules will cause decreased reabsorption of solutes, resulting in their appearance in the urine at levels in excess of normal limits.

If GFR is reduced but tubular function is normal a large proportion of the reduced filtered volume will be reabsorbed and urine volume will therefore be greatly diminished. Since the rate of excretion of urea and creatinine is dependent upon the GFR, their concentration in the systemic plasma will increase if the diminished rate of excretion does not balance the rate of their production. Plasma levels of phosphate and urate also increase due to decreased filtration of these solutes. The reduction in the amount of filtered sodium allows much of that which is filtered to be reabsorbed. This results in decreased potassium secretion in the distal tubule and overall potassium retention since this can still be reabsorbed proximally. Less sodium ions are available for exchange with hydrogen ions. Since bicarbonate ions are reabsorbed by a process which requires loss of hydrogen ions the plasma bicarbonate concentration will decrease.

If tubule function is reduced but glomerular function is normal the adjustment of urine composition will be affected. Reduced solute reabsorption in the proximal tubule is accompanied by reduced water reabsorption at this site, resulting in the production of a large volume of dilute urine. Secretion of hydrogen ions is diminished and therefore bicarbonate ion reabsorption is inhibited. The response to the adrenocortical hormone aldosterone, which stimulates the reabsorption of sodium ions from the tubular fluid in distal tubules, may be inhibited and therefore the excretion of sodium may be inappropriately high for the rate of renal blood

flow. Potassium reabsorption in the proximal tubule is impaired and this may lead to low potassium concentrations in the plasma. In addition the reabsorption of glucose, amino acids, phosphate ions and magnesium ions will be impaired.

TRANSPLANTATION

Kidney transplantation is the most ethical and economical treatment for end-stage renal failure and is the most frequently performed of all solid-organ transplants. Haemodialysis, however, is the most frequently used treatment for renal failure even though long term costs are greater than for transplantation. Dialysis may also lead to problems such as brittle bones in some patients. Continuous ambulatory peritoneal dialysis (CAPD) is less costly although the dialysis fluid itself is expensive, and problems with infections are frequent. Therefore it would be desirable to offer kidney transplants to all suitable patients with end-stage renal failure as soon as this condition manifests, thereby avoiding the problems associated with renal failure and long term dialysis.

Two of the most important considerations in renal transplantation (apart from immunological graft rejection), and in organ transplantation generally, are preservation of the donor organ prior to transplantation, and immediate or early graft function. These components are intimately associated. Effective preservation will increase the probability of immediate graft function or promote early graft

function. Immediate function is absolutely essential in organs such as the heart and liver for which there are no long term alternative methods of duplicating organ function. In kidney transplantation any improvement in early graft function is also desirable and is beneficial in a number of ways. Primary nonfunction necessitates a longer stay in hospital for patients and requires continued haemodialysis until the transplanted kidney resumes life-sustaining function. There is evidence that the 1-year graft survival rate decreases by up to 20% in kidneys which do not have good primary function (Albrecht *et al.*, 1988). It is also important to prevent primary nonfunction because this condition may be difficult to differentiate from acute rejection (Parrott *et al.*, 1990).

There are a number of reasons why it is desirable to promote early function and to preserve organs for longer periods while still maintaining viability:

1) Better cross matching: The ability to spend more time carrying out immunological tissue type analyses and thereby match the organ to the most similar donor, would decrease the incidence of organ rejection and therefore prevent organ wastage and extended hospitalisation. In the future there may also be time for the possibility of specific donor or recipient treatment to reduce immune rejection.

2) Transport over greater distances - this allows kidneys to be obtained from hospitals which may not have their own transplantation programme, and also allows better cross matching of donor and recipient. The ability to share organs

across national boundries, for example within the European Community would be an advantage because the potential recipient pool, and thus the likelihood of a good match, would increase. Such international cooperation would result in decreased recipient waiting times. This is important since it is not uncommon for patients with end-stage renal failure to wait for months or even years for a kidney transplant in the U.K.

3) Less coordination of surgical teams would be needed - heart transplantation and to a lesser extent liver transplantation still require that donor and recipient surgical teams work to closely timed schedules, because currently used preservation solutions can only maintain organ viability for a few hours.

By achieving the above aims the available pool of usable kidneys would increase, even if the rate of organ donation remained constant. A better understanding of ischaemic disease may allow the prediction of organ viability prior to transplantation thus allowing the use of non-ideal donors such as non-heart-beating cadavers to increase the supply of kidneys. Improved early post-transplant function would allow recipients to return to their communities earlier and resume normal activity, thereby decreasing the demand on health service resources. Decreased ischaemic/reperfusion injury has also been shown to be important in kidney transplantation in preventing the combined damaging effects of ischaemia and nephrotoxicity of immunosuppressant drugs such as cyclosporin

which must be given to patients to prevent graft rejection (Kanazi *et al.*, 1986).

RENAL TRANSPLANT HISTORY

The Russian surgeon Voronoy performed the first human kidney allograft in 1933 (Voronoy, 1936), but the patient died within two days. He performed six similar operations in the next sixteen years with no success. The first successful human renal transplant was performed in Boston in 1954 when a kidney was transplanted between identical twins (Murray *et al.*, 1955). In the mid 1940's Medawar discovered that the immune system could be modulated to prevent infection and promote the survival of skin grafts in animals (Medawar, 1944). The advent of immunosuppression, and in particular the discovery of Cyclosporin in 1970, has probably been one of the most significant advances in the field of kidney transplantation, and has made liver (Calne *et al.*, 1981), heart, and heart-lung (McGregor *et al.*, 1986) transplantation possible. The routine use of this drug in renal transplantation was introduced following studies by Calne and collegues in Cambridge in 1978.

Clinical intervention to promote kidney function

During organ procurement and transplantation there are several opportunities for therapeutic intervention to limit organ damage and thereby promote viability. These are:

1) by donor treatment;
2) during the harvest procedure using modified flush solutions;
3) during the storage period;
4) immediately prior to organ grafting -reflush solutions;
5) by recipient treatment before transplantation;
6) by post-transplant recipient therapy.

Option (2) offers the best opportunity because it is the least complicated and requires little change to current methods of organ harvesting. For this reason the improvement of organ preservation solutions has been the most intensely studied method for promoting viability after longer periods of storage.

SOURCES OF ORGANS

Brain-dead, heart-beating cadaveric donors are the preferred source of kidneys, although many clinicians and researchers now also advocate the use of non-heart-beating donors to increase the number of organs available (Rigotti *et al.*, 1991). The main problem with this latter procedure is the long period of warm ischaemia experienced by these organs, and the resulting delayed graft function, although acute rejection episodes are reported not to be higher than in recipients of organs from heart-beating cadaveric donors (Castelao *et al.*, 1991).

Satisfactory results have been reported using kidneys from paediatric cadavers of less than two years of age

transplanted into adult recipients (Abouna *et al.*, 1991). These kidneys were not considered suitable by centres in Europe and the United States and were transported to Kuwait for transplantation. The group found that paediatric kidneys showed compensatory hypertrophy, enabling them to cope with the increased demands on the organs due to the body mass of the adult recipients. Segoloni and colleagues (1991) advocated the use of older donors (50+ years) to increase the supply of kidneys. They found that donor age did not affect recipient survival, but increasing recipient age had a negative influence on survival. Sumrani and colleagues (1991) reported that transplants using kidneys of living distant relatives (grandparents, cousins etc.) were as equally successful as transplants from immediate family members.

Transplantation ethics are primarily determined by cultures, but economics also play a role because the source of kidneys for transplantation is influenced by the availability of, and entitlement to, healthcare. Some countries have no organized dialysis facilities or cadaveric transplantation program, e.g. India, and therefore patients who can afford treatment are dependent upon a kidney from a relative or one purchased from a living unrelated donor (Dossetor and Manickavel, 1991). Recently there has been controversy over the use of kidneys from executed prisoners in China for transplantation in Hong Kong.

Future organ sources

a) Animals : The use of xenografts, organ transplants in which the donor is of a different species to the recipient, has been an attractive prospect since the early attempts at transplantation (Reemstra, 1969). When these first transplants were performed little was known about the extensive immunological problems which arise as a result of tissue incompatibility. Although this major obstacle still exists even with modern immunosuppression techniques, research into renal xenografting continues because the success of such a procedure would mark an immense advance in the treatment of renal failure. With continuing advances in genetic engineering problems with tissue incompatibility may not be insurmountable. Ethical considerations aside, xenografts could theoretically provide an almost inexhaustible source of kidneys, altough this idea is still far from becoming reality.

b) Permanent artificial implants : Current research efforts are still a long way from developing an artificial implant capable of performing life-sustaining haemodialysis. There is little incentive for such research because at present the technical difficulties of producing a small filtration system with a very large surface area and the capability of selective reabsorption of solutes, to meet the requirements of the body, prohibit the idea from becoming reality. Currently there is much research into a permanently implantable artificial heart which, although complex, would probably not require the capability to perform functions as intricate as

those needed in the kidney.

c) Cryopreserved kidneys : Cryopreservation, the method by which cells and tissues are stored at very low temperatures, usually in liquid nitrogen (-196°C), is an attractive concept for long-term preservation and "banking" of organs. However, such procedures are currently only possible with either cell monolayers and free cell cultures, discrete cells such as sperm (Polge *et al.*, 1949) and bone marrow (Pegg, 1964) or certain tissues (Hanna and Sherman, 1971). The major problems are considered to be the formation of ice crystals throughout the tissue, which damage cells, and the difficulty in achieving the desired cooling rate in larger tissues (Lehr *et al.*, 1964). There is also the need for a method of delivering cryoprotective agents (Pegg and Robinson, 1978) to all cells in an organ as large as the kidney with equal efficiency. There is also the need to select an effective and relatively harmless cryoprotectant (Armitage and Pegg, 1979). Until these major problems have been overcome it is unlikely that there will be a suitably effective method of cryopreservation of kidneys with life-sustaining function.

CONSEQUENCES OF ISCHAEMIA AND REPERFUSION

The passage of blood or any other liquid through the vasculature of an organ such as the kidney is termed *perfusion*. This is the process by which metabolic substrates are delivered to tissues and waste products are removed. Interruption of this flow for any length of time is known as

ischaemia, although this term is sometimes also used to describe situations involving decreased, but not ceased, blood flow to tissues. *Reperfusion* is the process by which the flow of blood or other fluids is restored to tissues following a period of ischaemia.

The consequences of ischaemia and reperfusion have been the focus of much research for a number of years. In addition to the importance of these phenomena in organ transplantation, they are also implicated in the aetiology of many other pathological conditions such as ischaemic heart disease (Hess *et al.*, 1982) and stroke (Rhencrona *et al.*, 1979). Ischeamia and reperfusion are also inevitable consequences of some surgical procedures. It is therefore important that we understand the biochemical events which occur during ischaemia and during reperfusion in order to prevent cell injury. It is generally accepted that reperfusion injury and ischaemic injury are intimately associated events, the extent of damage occurring during reperfusion being dependent upon the length of the ischaemic period and the biochemical changes which occur during ischaemia. Reperfusion injury has been described in a number of tissues; kidney (Summers and Jamison, 1975), heart (Hearse, 1977), liver (Buckland *et al.*, 1965), and brain (Jenkins *et al.*, 1981). It is important to establish whether such injury is physiologically significant or is just an inevitable consequence of transplantation with limited significance. For reperfusion injury to be considered of long-term significance to normal organ function damage must occur

at a greater rate, or to such an extent, that cellular repair mechanisms are exceeded.

Adenine nucleotides in ischaemia

The examination of changes to tissue adenine nucleotide concentration following ischaemia and reperfusion is important because of the fundamental role for these compounds in almost all biochemical processes. Adenosine triphoshpate (ATP), an adenine nucleotide, is used in all biological systems as an energy transduction molecule, and is therefore a necessity for normal cell function. In addition adenine nucleotides have a role in control of cell metabolism via altered enzyme activity, and by controlling cell responses to external stimuli via cyclic AMP (cAMP). They are precursors of nucleic acids, and components of coenzymes such as coenzyme A (CoA), and nicotinamide adenine dinucleotide (NAD).

The purine ring of adenine is sythesized from glycine, aspartate, glutamine, tetrahydrofolate and carbon dioxide (CO_2) by ATP-dependent processes. This has implications for *de novo* synthesis of adenine nucleotides during reperfusion following ischaemia when the level of many of these precursors may be diminished.

During ischaemia there is a rapid decay of ATP to ADP and then to AMP; degradation to hypoxanthine occurs at a slower rate (Buhl *et al.*, 1975). Guanine and uridine nucleotides, which represent only a fraction of the total nucleotide pool, are also degraded giving uracil and guanine respectively as

the end products. There appears to be a correlation between depletion of adenine nucleotides and extent of ischaemic injury, but a more accurate index of tissue metabolic status is the ability to restore the balance of the components of the nucleotide pool following reperfusion (Vogt and Farber, 1968). Recovery of adenine nucleotides following reperfusion probably occurs via two distinct mechanisms : 1) salvage pathways utilizing purine and nucleoside degradation products accumulated in ischaemic tissues (Murray, 1971), and 2) *de novo* synthesis utilizing substrates supplied by blood. Wilkinson and Robinson (1974) noted that depletion of adenine nucleotides led to increased permeability of lysosomal and cell membranes which are normally stabilized by ATP.

Under normal physiological conditions, adenine nucleotides are in dynamic equilibrium with their smaller precursor molecules. Intracellular enzymes such as the 5'-nucleotidases rapidly degrade them to nucleosides and ultimately bases. These bases may be reconverted to nucleotides. Such interconversions may give a false picture of metabolic events in the kidney if they are allowed to continue during extraction and analytical procedures.

Free Radicals

Free radicals are atoms or molecules which contain one or more unpaired electrons. Unpaired electrons are those which are alone in an atomic orbital. The presence of an unpaired electron (•) is the reason for the extreme reactivity of these

species. If a radical donates an electron to a nonradical or takes an electron from a nonradical, this second species will itself become a radical, unless it also has an unpaired electron.

The process of reperfusion of the kidney after transplantation following ischaemic preservation re-introduces oxygen to the organ, and there is strong evidence for a specific oxygen radical-mediated component of reperfusion injury. It is unlikely that free radical mediated cell damage is the only cause of organ dysfunction upon reperfusion but it is plausible that radical activity makes a significant contribution to the outcome of the graft. There is evidence for free radical damage at both intracellular (Otani *et al.*, 1984) and extracellular (Drugas *et al.*, 1991) sites, following ischaemia and reperfusion. Such injury has been shown to occur in a number of organs and tissues including kidney (Baker *et al.*, 1985; Green *et al.*, 1986), heart (Bernier *et al.*, 1986; Zweier, 1988), brain (Aust *et al.*, 1985), intestine (Granger *et al.*, 1981), skin (Manson *et al.*, 1983), pancreas (Sanfey *et al.*, 1984) and liver (Marubayashi *et al.*, 1983; Thurman *et al.*, 1988), often by indirect methods such as the demonstration of protective effects of the inhibition of radical production or the scavenging of radicals after production.

The depletion of intracellular ATP during cold ischaemic preservation not only removes the source of energy to maintain ionic gradients, but may also lead to the accumulation of

products which can initiate free radical generation (Granger *et al.*, 1981). During ischaemic kidney preservation ATP degrades to AMP, then to adenosine, then to inosine, and onwards to hypoxanthine. At the same time the cytosolic enzyme xanthine dehydrogenase (XD) undergoes proteolysis (Engerson *et al.*, 1987), converting it to the enzyme xanthine oxidase (XO). When oxygen is reintroduced during reperfusion, xanthine oxidase catalyses the oxidation of hypoxanthine to xanthine also resulting in the reduction of oxygen to superoxide radicals. Xanthine oxidase also catalyses the oxidation of xanthine to uric acid, again resulting in oxygen reduction to superoxide. The superoxide radicals may then directly cause damage to cell constituents, or may undergo further reactions to form more damaging species. This free radical production can be inhibited in a number of cells by treatment with XO inhibitors such as allopurinol (Terada *et al.*, 1992).

Some free radical production occurs under normal physiological conditions *in vivo*, but the sites, rate, and significance of this production are not entirely known although it is believed that the mitochondria are a major site of radical generation (see below). The superoxide radical ($\bullet O_2^-$) is considered to be one of the most ubiquitous radical species in biological environments. Superoxide production occurs *in vivo* by either enzyme-directed processes such as those which occur in activated phagocytes (Baboir, 1978), or by uncontrolled mechanisms such as leakage of electrons from mitochondrial transport chains to oxygen (Fridovich, 1989).

These chains function to oxidise reduced substrates, namely reduced nicotinamide adeninedinucleotide (NADH) and reduced flavin adeninedinucleotide ($FADH_2$), produced during the Krebs cycle, thereby releasing energy to drive ATP sythesis. The electron transport chain is located in the inner mitochondrial membrane and consists of a number of flavoproteins, non-haem-iron proteins and cytochromes, which pass electrons to oxygen, thereby reducing it to water. Some components of the chain may "leak" electrons, so that not all of the electrons which reach them are conveyed to the next member of the transport chain but instead are passed to oxygen causing its reduction to superoxide. The main sites of electron leakage are thought to be the NADH-coenzyme Q reductase complex and the reduced forms of coenzyme Q (Halliwell and Gutteridge, 1985).

Production of radicals under controlled conditions as part of normal metabolism may not always cause damage to tissues and may be beneficial. Damage may occur if radicals are produced in excessive amounts (i.e. a rate which exceeds control by cellular defence mechanisms) or at inappropriate sites. A number of biological molecules react with superoxide radicals leading to loss of normal function; one example is nitric oxide which is itself a radical (NO•), and is converted to peroxynitrite (Saran *et al.*, 1989). The loss of the vasorelaxant effect of nitric oxide by reaction with superoxide has been shown to cause vasospasm (Laurindo *et al.*, 1991). It has also been suggested that this mechanism may be involved in the control of vascular tone *in vivo* (Halliwell,

1989). Although superoxide toxicity may be due to a direct effect of the radical, it may also be mediated via the generation of more reactive hydroxyl radicals in a transition metal-catalysed reaction. Iron is considered to be one of the most important transition metal catalysts involved in free radical production *in vivo*. Intracellular iron is stored as ferritin, a non-catalytic complex with apoferritin which is a protein with a molecular weight of 450,000. Release of this iron may result in catalysis of undesirable biochemical reactions both intracellularly and extracellularly (see below).

Hydroxyl radicals are believed to react with almost all physiological molecules. The iron-catalysed synthesis of hydroxyl radicals is thought to proceed as follows:
Ferric iron (Fe^{3+}) is reduced to ferrous iron (Fe^{2+}) by superoxide ($\bullet O_2^-$). Fe^{2+} is then reoxidised by hydrogen peroxide (H_2O_2) which is formed by the dismutation of $\bullet O_2^-$ (either spontaneously or by the action of the enzyme superoxide dismutase SOD (Fridovich, 1975)), resulting in the production of the hydroxyl radical ($\bullet OH$).

$$\bullet O_2^- + Fe^{3+} \text{ ----------> } Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \text{ ----------> } Fe^{3+} + OH^- + \bullet OH$$

(Fenton Reaction)

The catalyst-free net reaction was first described by Haber

and Weiss (1934). When iron is present, as in the Fenton reaction, this is often called an iron-catalysed Haber-Weiss reaction :

$$\bullet O_2^- + H_2O_2 \text{ ----i-r-o-n----> } O_2 + \bullet OH + OH^-$$

Hydrogen peroxide, which itself is not a free radical, may also be cytotoxic. Inhibition of any such cytotoxicity is achieved by the conversion of H_2O_2 to water by the enzymes catalase and glutathione peroxidase. The possible sources of free iron to catalyse these reactions is currently unknown. Superoxide itself can mobilize iron from ferritin, a protein which is the major intracellular iron-binding molecule, (Biemond *et al.*, 1984) and hydrogen peroxide can degrade heme proteins thereby releasing iron (Gutteridge, 1986).

Hydroxyl radical attack can lead to the propagation of a chain reaction, resulting in damage to a number of adjacent molecules such as membrane lipid peroxidation of polyunsaturated fatty acids (Halliwell and Gutteridge, 1986).

$$\text{Lipid-H} + \bullet OH \text{ ---------> } H_2O + \text{Lipid}\bullet$$

The lipid carbon-centred radical combines with oxygen to form a peroxy radical.

$$\text{Lipid}\bullet + O_2 \text{ ----------> Lipid-OO}\bullet$$

Peroxy radicals can then attack adjacent membrane lipids, causing the chain reaction.

$$\text{Lipid-OO}\bullet + \text{lipid-H} \longrightarrow \text{lipid}\bullet + \text{Lipid-OOH}$$

$$\text{Lipid}\bullet + O_2 \longrightarrow \text{lipid-OO}\bullet$$

.....and so on.....

Lipid hydroperoxides (Lipid-O_2H) can also break-down by an iron catalysed reaction to form peroxy radicals and alkoxy radicals (LO•) as follows:

$$2\ \text{Lipid-OOH} \xrightarrow{\text{-I-R-O-N-}} \text{LOO}\bullet + \text{LO}\bullet + H_2O$$

Thus radical generation at one particular site can lead to damage to parts of the cell which are some distance from this site.

Free radicals are produced during normal cell metabolism but are usually short-lived because of inactivation by protective antioxidant mechanisms. Normal cells have sufficient capacity to protect themselves from basal free radical production by non-enzymic (ascorbate and vitamin E) and enzymic (superoxide dismutase, glutathione peroxidase and catalase) defence mechanisms. However as can be seen from the above reaction mechanisms, SOD activity in excess of that of enzymes responsible for the removal of hydrogen peroxide (glutathione peroxidase and catalase) could be deleterious

because this would produce relatively higher concentrations of hydrogen peroxide. Free radicals have been implicated in the pathology of reperfusion injury, and in a number of clinical conditions including atherosclerosis, arthritis and cancer (Halliwell, 1987). Pathological manifestations are likely to be due to either increased free radical production in excess of cellular antioxidant capabilities, or decrease in antioxidant efficiency resulting in an inability to cope with normal radical production. The cause of oxidative injury is likely to be a combination of these phenomena (Farrari *et al.*, 1986). The sources of these free radicals include degradation products of ATP, infiltrating leukocytes and damage to the mitochondrial electron transport chain. There is also evidence for a role for decompartmentalized iron in the generation of free radicals in tissues (Paller and Hedlund, 1988).

Uncontrolled radical generation damages cells in a number of ways. The propagation of free radical chain reactions results in damage to large numbers of molecules by each radical molecule formed. One of the most common pieces of evidence for this is lipid peroxidation of polyunsaturated fatty acids (PUFA's) in cell membranes. Damage to cell membranes and alterations in their permeability have obvious implications for normal function in all aspects of cell metabolism. The lipid peroxides produced can also fragment to produce a number of cytotoxic derivatives such as aldehydes, and this has been the basis of a number of assays of free radical damage to physiological systems (Yagi,K. 1982).

Proteins can be denatured by oxidants resulting in increased susceptability to proteolysis (Huang *et al.*, 1992) with potentially disastrous effects on enzyme-mediated metabolic processes. Damage to nucleic acids such as DNA by free radicals may have serious consequences if not repaired, and has been implicated in the pathogenisis of certain cancers (Sausville *et al.*, 1978; Robertson, 1983). Pathological events such as lipid peroxidation and protein denaturation may lead to the tissue dysfunction seen to result from radical generation, for example increased microvascular permeability (Dauber *et al.*, 1991). The major problems associated with tissue incompatibility leading to graft rejection are not primarily due to oxidative injury. However, tissue infiltration by phagocytic cells which produce reactive oxygen species, such as polymorphonuclear leukocytes (PMN's), may lead to a significant component of rejection-dependent renal damage being due to oxidative injury. Phagocyte infiltration into the vascular endothelium has been demonstrated, concurrent with increased superoxide production and increased vascular permeability to albumin (Terada *et al.*, 1992). There is also evidence that free radical injury to the vascular endothelium is a cause of the loss of post-reperfusion endothelium-dependent vasorelaxation (Lefer and Ma, 1991). Infusion of recombinant human superoxide dismutase (hSOD) was found to prevent the inhibition of endothelium-derived relaxing factor (EDRF) activity following reperfusion in the cat spleen. Similar but reversible effects have been

demonstrated in canine coronary arteries (Kim *et al.*, 1992).

It is important to determine whether free radical production upon reperfusion is an initiating factor in the production of tissue damage, or whether radicals are produced secondary to initiating pathological events, since in many human diseases oxidative stress is a secondary phenomenon (Halliwell and Gutteridge, 1984).

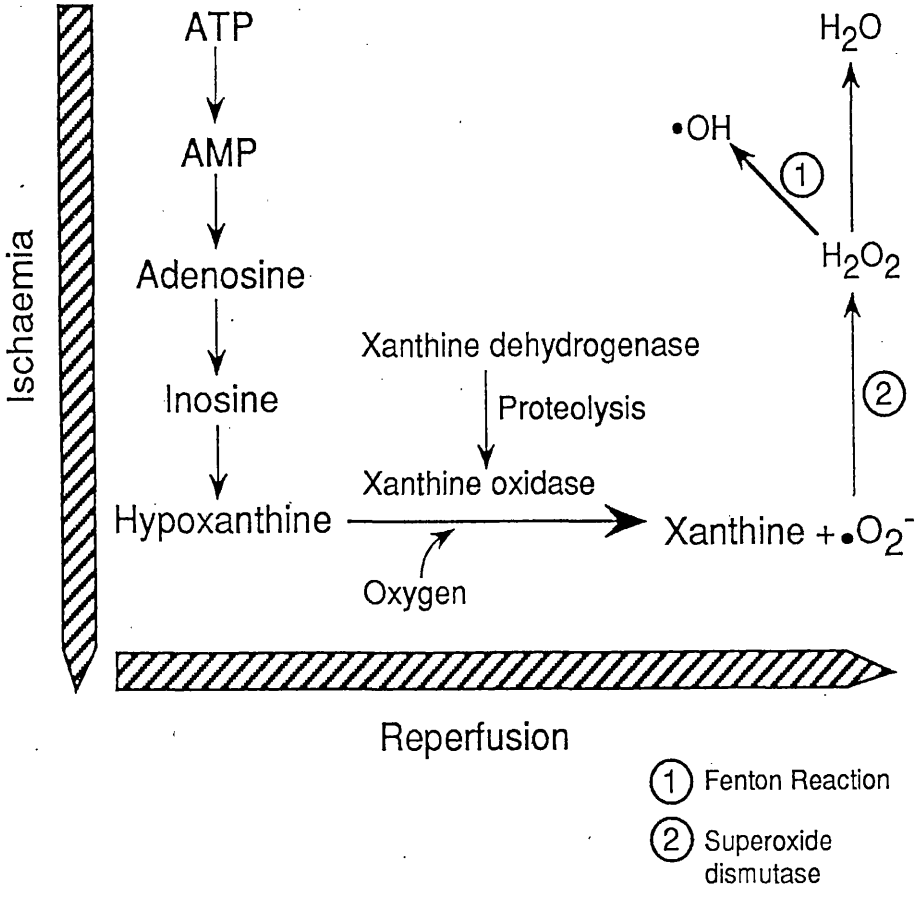


Fig.1.5: Mechanism by which free radicals may be produced as a result of ischaemia and reperfusion.

OTHER DAMAGING EFFECTS OF ISCHAEMIA

Cell Swelling

The regulation of cell volume is dependent upon active extrusion of Na^+ ions, under conditions where the extracellular Na^+ concentration is high, and the maintenance of a negative intracellular potential with respect to the extracellular environment to inhibit the entry of Cl^- ions. These processes are dependent upon the activity of the Na^+/K^+ ATPase and, via ATP production, on aerobic metabolism. The membrane-bound ATPase transports three Na^+ ions out of cells for every two K^+ ions which it transports in. This causes the above-mentioned electrical gradient across the membrane. Intracellular macromolecules which may be negatively charged, such as some proteins, in addition to directly causing an osmotic gradient across cell membranes also attract positively charged K^+ ions, thereby adding to this effect. During ischaemia the activity of the membrane pumps is greatly decreased due to the lack of ATP as a substrate for the process, and the use of hypothermia in preservation does not prevent ionic exchanges accross membranes. As a result of this, if the extracellular Na^+ concentration is higher than the intracellular concentration, Na^+ and Cl^- ions enter cells causing an osmotic gradient which favours the entry of water into cells, resulting in cell swelling (Frega *et al.*, 1976). In addition to this tissue oedema being directly detrimental to those cells which have swollen, it also has implications

for surrounding tissues which themselves may not have been significantly injured. Swelling of the endothelial cells lining the renal vasculature has been implicated in processes which lead to erythrocyte clustering in the kidney microvasculature upon reperfusion with blood (Summers and Jamison, 1971). This occurrence, known as the "no reflow phenomenon" is not a true coagulation process in that it is not thought to be mediated by blood coagulation factors. The occlusion contributes to further secondary ischaemic damage which may be more severe than any injury which the organ may have sustained during cold preservation because the kidney will now have returned to normothermia, thereby accelerating the pathologiacal processes of ischaemia.

Calcium redistribution

The role of calcium in the pathology of ischaemic injury and reperfusion injury has been investigated in a number of studies (Chien *et al.*, 1977; Watts *et al.*, 1980; Opie, 1989). Cytosolic calcium concentrations in most cells are kept low (≈0.1μM) with respect to the extracellular concentration (≈1mM) by active extrusion and sequestration mechanisms. Ca^{2+}ATPases are situated in the endoplasmic reticulum and plasma membrane (Carafoli, 1987). The reduction in intracellular ATP which occurs during ischaemia therefore prevents normal calcium extrusion and the concentration of free Ca^{2+} ions in the cytosol increases. In addition, numerous animal models of ischaemia and reperfusion have shown an

increase of cellular free calcium levels upon reperfusion as a common biochemical feature (Nayler *et al.*, 1988). This may then lead to the activation of intracellular enzymes such as phospholipases (Otamiri *et al.*, 1987) with the resulting degradation of phospholipids in cell membranes, and possibly increased production of prostaglandins and leukotrienes (Craven and DeRubertis, 1983), and concurrent superoxide production. Accelerated phospholipid catabolism is likely to affect the activity of membrane-bound enzymes such as the Na^+/K^+ATPase, and may have secondary effects on all cell functions since membrane permeability may be affected. The action of phospholipase A_2 upon cell membranes produces arachidonic acid which then acts as a substrate for cyclooxygenase resulting in prostaglandin synthesis and concurrent superoxide production. The inappropriate production of prostaglandins, which are vasoactive, in the renal vasculature may also be a cause of the "no-reflow" phenomenon referred to above. The lysophosphatides which result from increased phospholipase activity during ischaemia have also been shown to cause injury (Shaikh and Das, 1981).

PRESERVATION METHODS

1:Cold ischaemic flush storage

In the early days of kidney transplantation from cadaver donors it was necessary to graft the organ into a recipient as a matter of urgency following harvest. Using current techniques and preservation solutions, transplantation can be

scheduled within approximately three days of kidney procurement. Extended preservation allows transport of organs over greater distances, opportunities for improved tissue-typing and possibly *ex vivo* functional assessment and bench surgery. The single most important principle in preservation is the use of hypothermia, since this greatly reduces cellular metabolism (Levy *et al.*, 1959; Buhl and Jorgensen, 1975), although metabolic processes still continue at a decreased rate in the cold-stored kidney at temperatures close to 0°C (Pettersson *et al.*, 1974). This conserves intracellular metabolic substrates and slows the accumulation of toxic metabolic products.

The aim of flushing organs with preservation solutions is to remove blood, clear extracellular spaces (Pegg, 1978) and to achieve rapid cooling. The vascular and extracellular spaces are thereby filled with a solution designed to minimize injury during the preservation period and at reperfusion. The initial solutions used for renal preservation were designed to mimic extracellular fluids but it is unclear whether they actually protected kidneys from preservation-related injury since their use experimentally showed no significant improvement over simple surface cooling procedures (Calne *et al.*, 1963; Pegg *et al.*, 1964) in which kidneys were immersed in an ice-cold solution, usually saline, without vascular flushing (Dempster *et al.*, 1964). It was later realized that such procedures allowed loss of intracellular ions, particularly potassium, into extracellular spaces. Flush

solutions were later developed to resemble intracellular fluids in their ionic composition. These were hyperkalemic and also contained increased concentrations of magnesium ions. One such solution was described by Collins *et al.* (1969), who had developed what was arguably the first organ preservation solution with intracellular ionic characteristics. This solution also contained glucose as an additional solute in an effort to control cell swelling. However, Fuller and Pegg (1976) reported that potassium concentrations much higher than normal plasma levels led to poor kidney function in some biochemical assays. A number of more recent studies have shown solutions containing high concentrations of sodium ions, rather than potassium ions, to be equally or more effective (Moen *et al.*, 1989; Sumimoto *et al.*, 1989; Marshall *et al.*, 1991).

The osmolarity of the preservation solutions was increased by the inclusion of glucose, sucrose, or mannitol to oppose the net movement of water into cells in order to prevent cell swelling. Other additions include the use of vasorelaxants to prevent vasospasm and heparin to inhibit coagulation of any remaining blood. Preservation solutions currently used in clinical transplantation procedures, for example hypertonic citrate solution (Ross *et al.*, 1976) or University of Wisconsin (UW) solution (Belzer and Southard, 1988), are generally hyperkalemic and contain antioxidants and other pharmacological agents (see Chapter 2). In addition Marshalls solution is hypertonic. These solutions were

designed to protect the organ in a number of ways including: a)prevention of tissue oedema; b)maintenance of intracellular ionic composition; c)prevention of oxidative damage; and d)conservation of intracellular energy pools. University of Wisconsin solution (including a number of variants) is now the gold standard against which other preservation solutions are compared. Much of the efficacy of UW solution has been attributed to the presence of two specific impermeant solutes, lactobionate and raffinose. However, UW solution is not yet widely accepted for renal transplantation. Hypertonic citrate solution is still widely used since it offers satisfactory results at a fraction of the cost of UW solution. For this reason hypertonic citrate solution was the chosen preservation solution for my studies in order to be consistent with current clinical practices.

Other pharmacological agents used in preservation solutions have included diuretics such as frusemide and mannitol, although their mechanisms of protection during kidney preservation are not believed to be mediated by their diuretic effects. Mannitol is osmotically active and functions by decreasing swelling of cells during preservation, preventing compression of the renal vasculature and thus preventing the "no reflow" phenomenon upon reperfusion (Flores *et al.*, 1972; Jamison, 1974). Mannitol has also been shown to be a directly-acting hydroxyl radical scavenger (Magovern *et al.*, 1984). Frusemide is thought to act beneficially by protecting the kidney from vasospasm which may result from

handling the organ during harvest (Panijayanond *et al.*, 1973; VanHerweghem *et al.*, 1974). Membrane stabilizers such as chlorpromazine and methylprednisolone have been used to prevent structural damage, via mechanisms including phospholipase inhibition and increase of intralysosomal pH. However, it is worth noting that a solution which produced some of the best functional results in kidneys following simple cold flush preservation for 72h was a phosphate-buffered sucrose solution (Lam *et al.*, 1989). It is therefore clear that there is a need for further investigations into the development of an ideal organ preservation solution; either one solution for all organs or a variety of solutions individually tailored for each organ.

2:Continuous perfusion

An alternative to flush preservation, the method of continuous hypothermic perfusion *ex vivo* has also been used to preserve kidneys for transplantation. This has the advantage of continuous delivery of metabolic substrates to the kidney and removal of waste products with the possibility resuscitation of organ function prior to transplantation. In addition, continuous perfusion offers the prospect of *ex vivo* organ function assessment (Foreman *et al.*, 1981). Selection of the appropriate perfusion buffer, with the optimum ionic composition, suitable oncotic agents and optimum pH value, is important since continuous perfusion has been shown to decrease organ viability if attempts are made to maintain kidneys for extended periods (Johnson, 1982). Selection of an

appropriate temperature is also important to achieve an optimum for slowing unwanted metabolic processes while still allowing desired enzyme systems to function, for example those responsible for the maintenance of cell integrity. Therefore temperatures of 6°C to 10°C have often been used (Pegg *et al.*, 1981; Johnson, 1982). However, very few centres routinely practice continuous perfusion at present due to cost considerations and requirements for trained staff and complex equipment.

In my studies, therefore, I have chosen to examine cold flush preservation (method 1) as this is the most frequently used technique in clinical transplantation. The specific aims of my thesis are listed overleaf.

AIMS OF THESIS

1) To investigate early reperfusion events in the kidney.

2) To determine the consequences of ischaemia and reperfusion in the kidney.

3) To identify sites of free radical generation and targets of radical attack.

4) To determine whether free radicals are produced during early reperfusion.

5) To investigate ways of improving kidney function immediately following reperfusion.

MATERIALS & METHODS

MATERIALS

All chemicals were purchased from Sigma or BDH unless otherwise stated and were Analar grade for most applications. HPLC grade reagents were used for chromatographic analyses.

H=HPLC grade reagent

Acetic acid (glacial)
Acetonitrile H
Acrylamide
Adenosine diphosphate
Adenosine monophosphate
Adenosine triphosphate
Ammonium acetate H
Ammonium persulphate
Bis-acrylamide
Bovine serum albumin
Bromophenol blue
Calcium chloride
Conalbumin
Coomassie blue
Creatinine
Desferrioxamine (Desferal, Ciba-Geigy Ltd)

2,3-Dihydroxybenzoate

2,5-Dihydroxybenzoate

Dipotassium hydrogen orthophosphate

Eosin

Ethanol

Ethylenediaminetetraacetic acid (EDTA)

Formalin

Glucose

Gluteraldehyde

Glycine

Haematoxylin

Hydrochlroic acid

Hydroxyethyl starch (Hespan, Dupont pharmaceuticals)

Isopropanol

Marshalls solution

Mercaptoethanol

4-methyl-umbelliferone

4-methyl-umbelliferyl-2-acetamido-2-deoxy-ß-D-glucopyramoside

Nitrilotriacetic acid (NTA) H

Paraffin wax

Perchloric acid

n-Phenyl-t-butyl-nitrone

Phosphate-buffered saline

Potassium chloride

Potassium citrate

Potassium hydrogen carbonate

Potassium hydrogen orthophosphate

Potassium hydroxide

Sodium chloride

Sodium citrate

Sodium dodecyl sulphate

NNN'N'-Tetramethylethylenediamine (TEMED)

Toluene

Tris base

Tris-HCl

Xylene

Assay kits

LDH (Boehringer Mannheim)

γ-GT (Boehringer Mannheim)

Creatinine (Sigma)

Glucose (Boehringer Mannheim)

Molecular weight markers for polyacrylamide gel electrophoresis (Sigma): a mixture of carbonic anhydrase (bovine erythrocytes, 29KD); albumin (egg, 45KD); albumin (bovine plasma, 66KD); phosphorylase B (rabbit muscle, 97.4KD) ß-galactosidase (E.coli, 116KD) and Myosin (rabbit muscle, 205KD)

METHODS

SURGICAL PROCEDURES AND ANAESTHESIA

Kidney Isolation

Male New Zealand White rabbits (2-3Kg) were used in all studies. General anaesthesia was induced by intramuscular (i.m.) injection of 0.2ml/kg fentanyl citrate 0.315mg/ml + fluanisone 10mg/ml (Hypnorm, Janssen Pharmaceuticals Ltd) followed by diazepam (5mg/ml, Phoenix Pharmaceuticals Ltd) at 1mg/kg intravenously (i.v.), and Hypnorm by slow i.v. infusion as required. Animals were supplied with 100% oxygen (1l/min), via an open face-mask, throughout the surgical procedure. Heparin (Monoparin 1000 units, CP Pharmaceuticals Ltd) and frusemide (Lasix 10mg/ml, Hoechst Pharmaceuticals) at 3mg/kg were administered i.v. prior to laparotomy. This was done to optimise renal function and to standardise the procedure with other studies by our group using *in vivo* transplantation. There is also evidence that frusemide prevents the vasospasm which may occur due to handling of kidneys during removal from the donor (Panijayanond *et al.*, 1973; VanHerweghem *et al.*, 1974).

Both kidneys were removed from the rabbits using the following method which allowed one organ to be removed and flushed without interruption of blood flow to the second kidney until it was also removed. Once deep anaesthesia had been achieved, as indicated by the absence of foot- and eye-touch reflexes, a midline abdominal incision was made and the

left kidney exposed and fully mobilized by dissection from the surrounding connective tissue. The ureter was severed, leaving a length of approximately 3cm attached to the kidney. The left renal artery and vein were ligated and divided proximal to the kidney to allow removal of the kidney, which was immediately plunged into sterile ice-cold hypertonic citrate (HCA) solution. HCA kidney perfusion solution was obtained from Baxter Healthcare Ltd (see below for composition). The renal artery and the ureter were cannulated using nylon cannulae (1.65mmO/D, Portex Ltd.) constructed from a 5FG i.v. cannula cut to length 5cm, and the vasculature of the kidney was flushed with 100ml of sterile HCA solution at 4-7°C, delivered from a Viaflex container at a height of 100cm above the cannula. Effluent venous blood was allowed to run to waste from the open end of the renal vein. The right kidney was removed during this flushing procedure. Kidney weights were in the range 10-15g. Samples of arterial blood and urine were taken from the aorta and bladder respectively when required for selected experiments. The animals were then killed by thoracotomy, cardiac puncture and exsanguination under anaesthesia.

Perfusion Apparatus

Kidneys were subjected to normothermic bloodless perfusion on an *ex vivo* perfusion circuit for periods of up to 30 minutes. The circuit was constructed from nylon tubing (3mm O/D, Portex) based on a system described for the *ex vivo*

assessment of kidney function (Fuller and Pegg, 1976; Fuller *et al.*, 1977) and had a recirculating volume of 500ml. The perfusion circuit was assembled in a room maintained at a temperature of ≈37°C. Perfusate was aspirated, using a Watson-Marlow peristaltic pump, from a reservoir in a 37°C water bath through a series of filters (coarse pre-filter followed by 200μm and then 5μm pore size, Millipore) then through a bubble trap to the renal arterial cannula. The arterial pressure was monitored using an electronic isometric pressure transducer (Elcomatic EM750) attached to a side-arm close to the cannula, connected to an oscilloscope (DM722, Rigel Research Ltd), and was thereby maintained at 150/100mmHg (systolic/diastolic) by adjustment of the pulsatile flow rate of the perfusate delivered to the kidney. The buffer in the reservoir was constantly vigorously gassed with $95\%O_2/5\%CO_2$ or other appropriate gases (e.g. in one group of experiments a mixture of $95\%N_2/5\%CO_2$ was used) via a nylon sinter. Samples of perfusate were collected from the reservoir at 5 minute intervals, and samples of urine were collected from the ureteric cannula into a graduated tube simultaneously. For some experiments perfusate effluent samples were also collected from the renal venous outflow. Perfusion flow rate and urine flow rate were calculated by collecting and measuring the effluent from the renal vein and ureter respectively in unit time.

The apparatus had two modes of operation, a 'closed' (recirculating perfusate) circuit and an 'open' (non-

recirculating perfusate) circuit. In either case care was taken to ensure that air did not enter the renal vasculature at the time of attachment of the cannula to the circuit. This was achieved by keeping the cannula completely filled with flushing solution and placing it in the stream of perfusate flow whilst it was being positioned on the circuit.

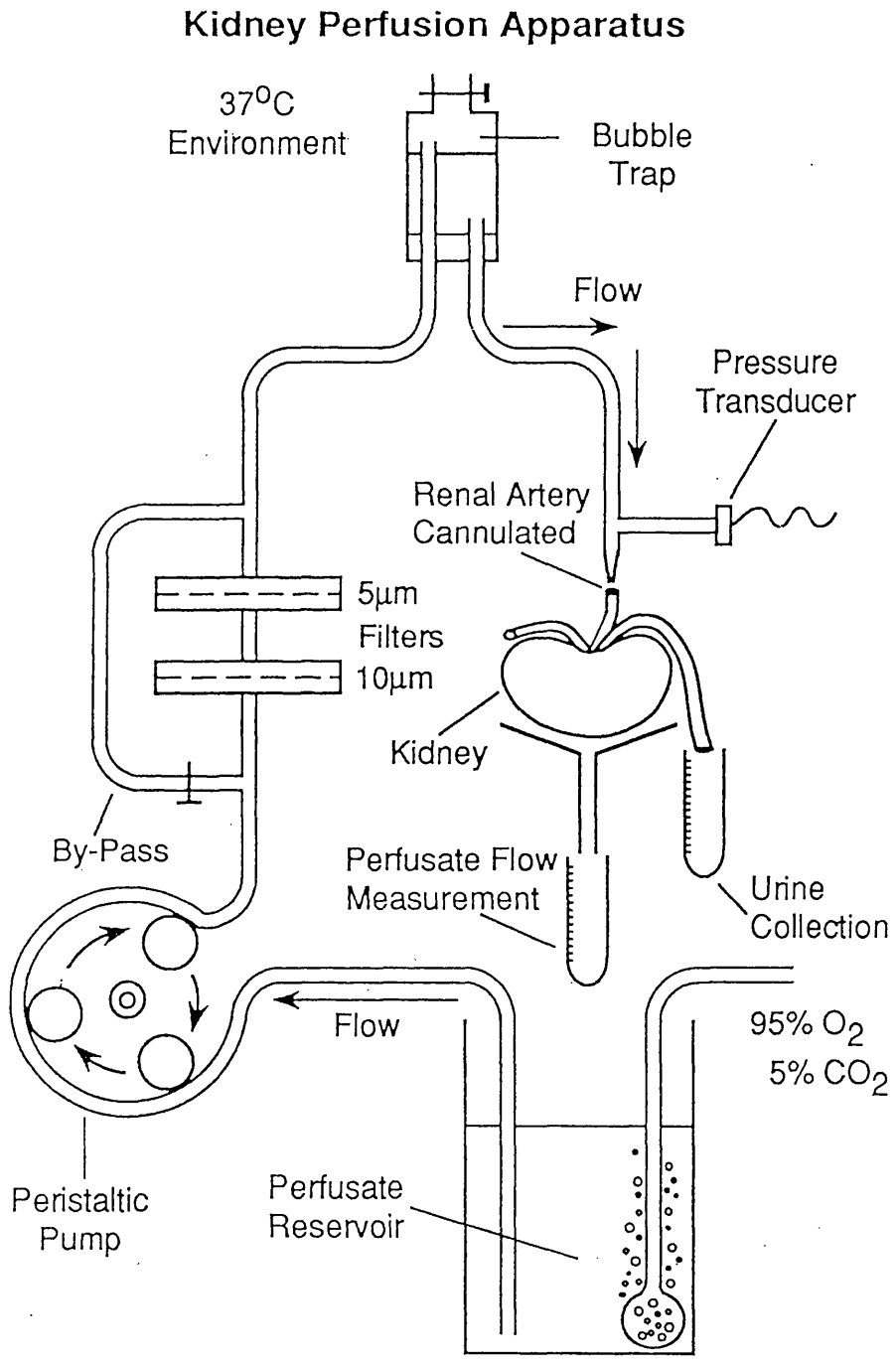


Fig. 2.1: PERFUSION CIRCUIT

Uninterrupted flow model

This model was designed to allow kidney perfusion with a perfusate solution *in situ*, without cessation of flow through the vasculature and therefore causing no tissue ischaemia. This method was developed for specific experiments to assess whether free radicals were produced as a result of the very short period of cold ischaemia inevitably experienced by fresh kidneys during normal harvesting and immediately prior to reperfusion.

Anaesthesia, laparotomy and removal of the right kidney were performed as previously described in this chapter. The left kidney was used for this procedure and was maintained intact in its position in the abdomen. The left kidney was chosen because the longer length of renal artery and vein allowed easier access for cannulation and sample collection (≈2cm left vessels vs. ≈1cm right vessels). Loose cotton ligatures were placed around the aorta both proximal and distal to the origin of the renal artery. The ureter was cannulated with a length of nylon tubing (1.65mm O/D), tied in position approximately 4cm from the hilus using cotton sutures. The lower abdominal aorta was then clamped in two places behind the distal ligature leaving a gap of 2cm between the clamps, so that blood flow to the kidney was not interupted by this procedure. A nylon cannula (5FG i.v. cannula, 1.65mm O/D), prefilled with saline and clamped at its open end, was introduced into the aorta between two clamps. The cannula was held in position by a cotton ligature which

was tight enough to prevent blood from leaking out of the incision, but not too tight to prevent the cannula from being advanced further into the aorta after removal of the clamp closest to the kidney. The clamp was then removed, and the cannula was advanced until its tip was close to the origin of the renal artery. The outflow from the perfusion circuit was attached to the cannula, whilst ensuring that no air entered the tubing, and the perfusion pump was switched on. Simultaneously, the ligature on the proximal side of the bifurcation was tightened to stop the blood supply to the kidney. This allowed the perfusate flow to be pumped through the renal artery to the kidney. A butterfly needle (21G, Venisystems) was inserted into the renal vein and tied in position for sample collection. These kidneys were not removed from animals but were perfused *in situ*.

PERFUSION / PRESERVATION SOLUTIONS

Perfusate for Warm Perfusion

The ionic composition of the perfusate was similar to that of rabbit serum, whilst hydroxylethyl starch (HES) was used as the major colloid. The composition of HES (Hespan, Dupont Pharmaceuticals) was as follows: Hetastarch (average mol. wt. 450,000)- 60g/l; NaCl- 9g/l; pH 5.5. This starch solution was diluted twofold in the final perfusate solution since 500ml of Hespan was used per litre of perfusate. The perfusate composition was (mMole/l): Na^+-155; K^+-6.8; Ca^{2+}-2; Cl^--139; HCO_3^--25; $H_2PO_4^{2-}$-1.5; HPO_4^{2-}-0.3; glucose-5;

creatinine-0.1; HES-3%. Bovine serum albumin (BSA, Sigma) was included (1g/l) as a tracer for protein excretion assessment. The pH was adjusted to 7.4 with 1M HCl at room temperature (≈20°C), and major buffering during reperfusion at 37°C was regulated by the HCO_3^-/CO_2 system acheived by gassing with $95\%O_2/5\%CO_2$. Double distilled water was used in the preparation of this solution.

Kidney preservation solution

Hypertonic citrate solution (HCA) was the standard kidney preservation solution used experimentally since this was the solution used clinically. HCA was obtained from Baxter Healthcare Ltd and has the following composition (mMole/l): potassium citrate- 28.0; sodium citrate- 28.0; mannitol-33.8g/l; magnesium sulphate- 40.6; pH≈7.

FUNCTIONAL ASSESSMENT

Glomerular Filtration Rate

The GFR was calculated from urine and perfusate creatinine concentrations (measured using the assay method of Bonsnes and Taussky, 1945) by the relationship described below.

$$GFR = \frac{\text{Urine [creatinine](mM)}}{\text{Perfusate [creatinine](mM)}} \times \text{Urine flow (ml/min)}$$

Creatinine clearance is an accurate gauge of GFR in the rabbit kidney as shown by comparable results to inulin clearance over a range of plasma concentrations of both compounds (Pitts, 1974). Secretion of creatinine by the kidney tubules, as occurs markedly in man, guinea pigs and rats, is not significant in rabbits.

Reabsorption of glucose and sodium

Tubular solute reabsorption was calculated as follows:

$$\% \text{ Reabsorption} = \frac{\text{Filtered load - Excreted load}}{\text{Filtered load}} \times 100$$

Filtered load = perfusate [solute](mM) x GFR(ml/min)

Excreted load = urine [solute](mM) x urine flow(ml/min)

Glucose concentrations in samples of perfusate and urine were measured spectrophotometrically (Micro-sample spectrophotometer 300N -Gilford instruments) by the glucose oxidase-peroxidase method (Werner *et al.* 1970). Sodium concentrations were measured by flame photometry (Richterich and Colombo, 1981) using a Corning clinical flame photometer 410C.

Proteinuria

Proteinuria was measured by comparison of the known protein concentration entering the renal vasculature (1g/l) with that of any protein appearing in the urine. Protein

concentration was measured spectrophotometrically using the method of Lowry *et al* (1951) with bovine serum albumin (BSA) as a standard, and expressed as a percentage of the perfusate protein concentration.

Enzyme activity in urine / vascular effluents

The enzyme τ-glutamyl transpeptidase (τ-GT) is an 80KD protein which, in the kidney, is located mainly in brush border membranes of proximal tubules, catalyzing the transfer of the τ-glutamyl group of glutathione (GSH, τ-glutamyl-cysteinyl-glycine) to amino acids and peptides (Tate and Meister, 1974). It was initially identified as an "antiglyoxylase" factor (Dakin and Dudley, 1913) because it was found to inhibit the conversion of methylglyoxal to D-lactate, but it was later shown to catalyse amino acid-stimulated breakdown of glutathione (Hanes *et al.*, 1950). The enzyme is important in the kidney for uptake of glutathione produced by the liver. τ-GT is a mixture of isoenzymes, each consisting of a large and a small subunit with proteolytic activity in the small subunits of these enzymes (Gardell and Tate, 1979).

Loss of τ-GT from proximal tubule brush border membranes may be indicative of a general τ-GT loss from other parts of the cell membrane and from the membranes of other cells where it is usually present in lower concentrations. Intracellular GSH is exported to membrane-bound τ-GT to be used for τ-glutamyl amino acid formation. The amino acid complexes are

then readily transported into certain cells (Allison and Meister, 1981). Loss of τ-GT from mebranes may therefore interfere with amino acid uptake into cells thereby slowing the synthesis of structural proteins and enzymes needed to replace those damaged by ischaemia and reperfusion. The enzyme τ-GT is also required for transport of GSH into cells, as demonstrated by the induction of a marked loss of GSH into the urine of experimental animals treated with τ-GT inhibitors (Griffith and Meister, 1979). Therefore loss of τ-GT may effect the antioxidant status of cells and render them less able to tolerate oxidative stress. Since GSH is required by the enzyme glutathione peroxidase to catalyse the degradation of hydrogen peroxide, decreased intracellular GSH levels may result in increased availability of hyrogen peroxide for hydroxyl radical production via the Fenton reaction (see Chapter 1). Glutathione also acts to maintain the thiol-disulphide status of cells by preventing oxidation of protein sulphydryl groups (Kosower and Kosower, 1978); hence lack of GSH may lead to changes in the secondary and tertiary structure of proteins. Plasma GSH is utilized by a number of tissues but particularly by the kidney (Bartoli *et al.*, 1978), in which damage to GSH-uptake mechanisms may play a significant role in post-transplant renal function. Thus preservation techniques which reduce ischaemia and reperfusion-induced loss of τ-GT from the kidney are likely to be beneficial to kidney function following transplantation. The measurement of τ-GT loss from rabbit kidneys perfused *ex*

vivo may therefore be a useful indicator of the capability for post-transplant tissue repair and protection against free radical damage, and ultimately may relate to organ viability.

The concentrations of the enzymes were measured in urinary and vascular effluents using commercially available kits. Assay was by spectrophotometry (Philips PU8700) according to the method of Persijn and Van der Slik (1976) for γ-GT, which used the following test principle:

l-γ-glutamyl-3-carboxy-4-nitroanilide + glycylglycine -----γ-GT-----> l-γ-glutamyl glyclglycine + 5-amino-2-nitrobenzoate

Lactate dehydrogenase was chosen as a marker of intracellular enzyme leakage and was assayed spectrophotometrically. This procedure used the following test principle:

$$\text{pyruvate} + \text{NADH} + H^+ \text{ <===LDH===> l-lactate} + NAD^+$$

Sample preparation

Samples of urine and vascular effluent perfusate for both LDH and γ-GT measurement were stored in liquid nitrogen (-196°C) until analysed. They were thawed at 4°C, and aliquots of appropriate volume were pipetted into plastic cuvettes (Starstedt) with a 1mm path length, and 1ml of reagent solution was added to each cuvette and stirred. The absorbance

change with time was measured over a period of 4min to give rates of reaction from which enzyme concentration could be calculated.

FREEZE CLAMPING

1) *In situ* : To measure certain unstable metabolites, e.g. adenine nucleotides, in kidney tissues *in vivo* it was necessary to freeze the organs as quickly as possible *in situ* without prior cessation of blood flow. Kidneys were mobilized in rabbits under anaesthesia as described earlier in this chapter by blunt dissection from surrounding tissue. A ligature was loosely placed around the renal artery and vein using a silk suture. The kidney was gently lifted from its position so that blood flow to the organ was not interrupted. Freeze-clamping was performed using a pair of stainless steel tongs with a jaw surface area of $16cm^2$. The clamping tongs were cooled to -196°C by immersion in liquid nitrogen. The largest possible accessable mass of kidney was compressed to a thickness of approximately 0.5cm between the jaws of the tongs. The intrarenal vessels were frozen instantaneously and therefore there was no bleeding from the crushed kidney. The ligatures around the renal artery and vein were tied and the vessels were severed distal to the ligatures. Areas of tissue which were not clamped were excised and discarded. Kidneys held in the cold clamping tongs were plunged into liquid nitrogen and stored in this at a temperature of -196°C until further preparation and the animal was killed under

anaesthesia.

2) *Ex vivo* : Kidneys undergoing isolated *ex vivo* perfusion were freeze-clamped without interruption of perfusate flow using a method similar to that described for *in situ* clamping. The perfusion pump was not switched off until immediately after freeze clamping so that metabolic delivery to tissues continued until the organs were frozen and to ensure that an unwanted period of ischaemia was not added to the procedure. The clamped kidney tissues were again plunged into liquid nitrogen and stored for further preparation.

Preparation of frozen tissue

The frozen kidneys were ground to a fine powder under a bath of liquid nitrogen using a pre-cooled mortar and pestle. Powdered tissues were stored (in 2ml Nunc Cryotubes) in liquid nitrogen until analysis. Samples prepared in this way were used for assays of adenine nucleotide concentration and lipid peroxidation (TBA-reactive material).

Estimation of tissue adenine nucleotide levels

The concentrations of adenine nucleotides were measured from kidneys which were freeze-clamped, either *in situ* in the anaesthetized animal (*in vivo* controls), or on the perfusion circuit at the end of the normothermic perfusion period. Adenine nucleotides were extracted by homogenisation (teflon pestle in 100ml glass tube, Potter-Elvehjem type, Camlab) of the powdered tissue (1g) in perchloric acid (10ml of 10%). The

acid extract was neutralized with a mixture of 1M potassium hydroxide (KOH) and saturated potassium hydrogen carbonate ($KHCO_3$) in an ice slurry. The resulting potassium perchlorate precipitate and remaining cell debris were removed by centrifugation (3000G) at 4°C for 10min (Mistral 3000i). Neutralized extracts were stored frozen (-196°C) in 2ml aliquots in Nunc cryotubes in liquid nitrogen until analysis. Adenine nucleotides in neutralized PCA extracts are stable for at least 6 months at -20°C (Perret, 1986). Samples were assayed against standards using reverse-phase anion-exchange HPLC. Relative concentrations of these three nucleotides were expressed as the tissue Energy Charge (EC) (Atkinson, 1968), calculated as follows:

$$EC = \frac{[ATP] + 0.5[ADP]}{[ATP] + [ADP] + [AMP]}$$

Assay for lipid peroxidation : TBA-reactive material

This assay procedure was based on the method described by Yagi (1982) for the fluorescent measurement (Perkin-Elmer LS5 luminescence spectrometer) of thiobarbituric acid (TBA) - reactive material (TBARs). Tissue samples which had been obtained from freeze-clamped kidneys and stored frozen (-196°C) in liquid nitrogen were used in this study. Tissues were ground to a powder under liquid nitrogen as previously described in this chapter. Powdered samples were homogenized (0.1g tissue/ 1ml) in ice-cold phosphate-buffered saline

solution (PBS) using a teflon pestle in a 100ml glass tube (Potter-Elvehjem type, Camlab). A volume of 2ml of crude homogenate was used for the assay. The protein content of this homogenate was also analysed using the method of Lowry (1951) in order to quantitate lipid peroxidation with reference to tissue proteins. This comparison was made because it was likely to be more accurate than expressing TBARs with reference to tissue wet weight.

DETERMINATION OF DESFERRIOXAMINE-AVAILABLE IRON IN KIDNEY TISSUES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

This method used desferrioxamine (DFX) to chelate available iron (i.e. "free" or loosely-bound iron) thereby forming ferrioxamine (FX) which could be measured by HPLC. To ensure that no contamination would arise from traces of iron in the buffers used in the assay, specific precautions were taken as described in the following section.

Removal of trace iron contamination from solutions

Double distilled water and all solutions used for kidney reperfusion and tissue homogenate preparation were dialysed against conalbumin (4%) for 48h in a cold room at 4°C, while being constantly stirred using magnetic stirrers. All glassware and disposables such as pipette tips were washed with 4mM EDTA followed by double distilled water which had been dialysed against conalbumin, to remove trace iron contamination.

Tissue sampling for desferrioxamine assay

Tissue samples were taken from kidneys immediately after reperfusion. Kidneys were divided longitudinaly in a petri dish on ice using a skin-graft knife (Sabre), then each half was divided into cortical and medullary tissue. Tissue homogenates (25% w/vol) were prepared in 0.1M Tris-HCl buffer (pH 7.4), which had been cooled in an ice slurry (≈0°C), using a Potter-Elvehjem homogenizer. Homogenates were centrifuged (Mistral 3000i) at 10,000G for 15min at 4°C. Supernatant aliquots (1ml triplicates) were mixed with 100μl of 22mM DFX prepared in dialysed distilled water. A further 1ml of each supernatant was immediately frozen and stored in liquid nitrogen (-196°C) for protein determination. Supernatant aliqots containing DFX at a final concentration of 2mM were incubated for 60min at 37°C to allow chelation of available iron. Standard iron samples were prepared using $FeCl_3$ to give concentrations of 10nM and 25nM Fe^{3+}. Aliquots of 1ml of these, along with an iron-free distilled water blank, were also incubated for 60min at 37°C with desferrioxamine (2mM). After incubation, DFX and FX were extracted from the supernatants using Bond-Elut C_{18} cartridges (6ml, Anachem). A vacuum elution system (Vacelute, Anachem) was used to draw all liquids through these columns. The columns, one for each triplicate aliquot, were wetted with 5ml of methanol followed by conditioning with 5ml of dialysed distilled water. Samples (1ml) were applied to the columns followed by washing with 1ml of dialysed distilled water. DFX and FX were eluted into EDTA-

washed eppendorf tubes (2ml) using 1.8ml of methanol. Methanol extracts were frozen in liquid nitrogen for up to 2 days before drying under vacuum in a rotary evaporator (Uniscience univap) at 50°C. Dried samples were stored at -70°C until HPLC analysis.

HPLC Analysis

This method was a modification of that described by Cramer *et al.* (1984). An Ultropac Spherisorb ODS2 5μm column (4.6mm i.d. x 80mm) was used for sample chromatography. A variable wavelength U/V detector set at 430nm and a fixed wavelength detector fitted with a 226nm filter were arranged in series for FX and DFX detection respectively. The mobile phase consisted of 0.02M sodium phosphate buffer (pH 6.6) containing 10% (v/v) acetonitrile, 4mM nitrilotriacetic acid (NTA), and 1M ammonium acetate. Samples were dissolved in 100μl of mobile phase and injected onto the column using a 20μl sample loop. The flow rate through the column was 1ml/min.

The amount of chelatable iron in tissue samples was calculated by comparison of the ratio FX/DFX with a standard curve prepared from the FX/DFX ratio of the standard iron samples.

HISTOLOGY

Electron microscopy

Tissue sections (≈3mm^3) from the cortex and medulla were cut from kidneys using a skin-graft blade (Sabre) and were placed in a solution of gluteraldehyde (3%) in phosphate buffer (0.1M) at room temperature for 2h. They were then stored at +4°C until further processing (up to 2 weeks). Specimens were removed from the fixing solution and washed in 0.1M phosphate buffer 3 times allowing 10min for each wash. Tissues were then placed in a 1% solution of osmium tetroxide in 0.1M phosphate buffer for 60min at room temperature. A further wash (0.1M phosphate buffer) was given prior to dehydration.

The fixed tissue sections were placed in acetone increasing in concentrations; from 25% to 50% then to 75% and finally to 100%. Twenty minutes were allowed for each stage until specimens reached the 100% acetone stage where they were given 4 changes of the liquid, each lasting 20min.

Dehydrated specimens were placed in a 1:1 mixture of Araldite / acetone in glass tubes overnight on a rotating mixer, without lids on the tubes to allow evaporation of the acetone. The specimens were placed in Araldite on a rotating mixer for 2h then the process was repeated with fresh Araldite.

Specimens were placed in fresh Araldite and placed in an oven at 60°C over-night. Sections were cut from the specimens embedded in araldite using an ultratome and were then stained

with osmium tetroxide before mounting on standard copper grids for histological examination using transmission electron microscopy.

GEL ELECTROPHORESIS

Serial samples of urine and vascular effluent collected from perfused kidneys were immediately frozen and stored by submerging 2ml aliquots in Nunc tubes (Intermed) in liquid nitrogen. Samples were thawed in an ice slurry, 200μl were removed and mixed vigorously with an equal volume of solubilizing buffer and allowed to stand at room temperature for 2 hours before applying them to the polyacrylamide gels for electrophoresis.

The buffer used to denature and solubilize proteins in urinary effluents had the following composition:
sodium dodecylsulphate (SDS)- 4% wt/v, mercaptoethanol- 0.05% v/v, Tris buffer (pH6.8) 0.094mM, bromophenol blue- 0.2% wt/v. A volume of 20ml of this solution was prepared using double distilled water, and was stored in a refridgerator at 4°C until used.

A separating gel containing 12.5% v/v acrylamide was used in all experiments. This had the following composition: acrylamide- 12.5% v/v; bis-acrylamide- 0.34% v/v; lower gel buffer- 0.375M (10ml of 1.5M Tris pH 8.8); SDS- 0.4% wt/v. This solution was made up to a final volume of 40ml with double distilled water. Ammonium persulphate- 100ul (0.2g/2ml) and TEMED- 20ul were added and thoughroughly mixed with the

above solution immediately prior to pouring gel.

The stacking gel onto which solubilized samples were loaded had the following composition: acrylamide- 6% v/v; methylene bis-acrylamide- 0.16% v/v; upper gel buffer- 0.125M (2.5ml of 0.5M TrisHCl pH6.8); SDS- 0.4% wt/v (0.4ml of 10%). This solution was made up to final volume of 10ml with double distilled water. Ammonium persulphate- 100ul (0.2g/2ml) and TEMED- 20ul were added and thouroughly mixed with the above mixture immediately prior to pouring the gel.

Molecular weight markers (Sigma) were run in the outside lanes of the 15 lane gels to provide a means by which the molecular weights of the unknown proteins in the urine samples could be estimated. A commercially available kit (Sigma) with a mixture of the following proteins was used: carbonic anhydrase (bovine erythrocytes, 29KD); albumin (egg, 45KD); albumin (bovine plasma, 66KD); phosphorylase B (rabbit muscle, 97.4KD) ß-galactosidase (E.coli, 116KD); Myosin (rabbit muscle, 205KD).

Electrophoresis was carried out with a constant current of 8mA per gel for approximately 5h, until the bromophenol blue dye front was 10mm from the end of the gel, with the positive electrode in the lower chamber. This chamber and the upper chamber contained a running buffer with the following composition: 18.12g Tris base; 86.4g glycine; 6g SDS; made up to 3l with double distilled water. After electrophoresis, gels were removed from the glass plates and washed with double distilled water. Gels were then placed in a fixing solution

(25% v/v isopropanol / 10% v/v acetic acid) and were left in this overnight. On the next morning gels were fully submerged in a stain solution (0.04% wt/v Coomassie blue; 10% v/v acetic acid; 25% v/v isopropanol) and left in this for approximately 8h. The gels were then destained (10% v/v acetic acid; 10% v/v isopropanol) overnight.

MEASUREMENT OF TISSUE DFX UPTAKE

The tissue concentration of desferrioxamine was determined according to the method of Paller and Hedlund (1988) by spectrophotometric assay of ferrioxamine before and after addition of saturating quantities of iron to supernatants of kidney tissue homogenates or to samples of urine and perfusate vascular effluents. These tissue homogenates (25% w/vol) were prepared in 0.1M Tris-HCl buffer (pH 7.4), which had been cooled in an ice slurry (≈0°C), using a Potter-Elvehjem homogenizer. Homogenates were centrifuged (Mistral 3000i) at 10,000G for 15min at 4°C. Supernatant aliquots (400μl triplicates) were mixed with 600μl of 10mM $FeSO_4$ and incubated at 25°C for 60min. Aliquots (500μl) of 20% trichloroacetic acid (TCA) were added and the precipitate was removed by centrifugation (10,000G for 15min at 4°C). The supernatant was mixed with an equal volume of 1M sodium acetate (pH 5.5). Absorbance at 429nm was measured before and after iron addition. DFX standards were in the range 0-500μM.

NAG ASSAY

Measurement of the lysosomal enzyme N-acetyl-ß-D-Glucosaminidase (NAG) in samples of urine and vascular effluent was carried out according to the method of Whiting and colleagues (1979). Aliquots (100µl) of urine and vascular effluents were decanted into glass tubes and 900µl of a reaction mixture containing 20mM citrate-phosphate buffer (pH 4.3) and 0.5mM 4-methyl-umbelliferyl-2-acetamido-2-deoxy-ß-D-glucopyranoside (MUADG) was added to each tube and thoroughly mixed. Samples were incubated at 37°C for 20min after which the reaction was terminated by addition of 2.0ml of 0.5M glycine-sodium hydroxide buffer (pH 10.4). The reaction during the incubation period produced 4-methylumbeliferone which was measured spectrofluorimetrically (excitation wavelength, 360nm; emission wavelength, 440nm) against 4-methylumbeliferone standards.

STATISTICAL METHODS

The results of the studies of post-reperfusion kidney function were analysed using either paired or unpaired Student's t-tests. Paired tests were performed when comparing kidneys from the same animal which had received different treatments. Unpaired tests were performed when comparing kidneys from different animals.

THE EFFECT ON ORGAN FUNCTION OF PRESERVATION OF RABBIT KIDNEYS FOR UP TO 72 HOURS

INTRODUCTION

It has been well established by numerous clinical and experimental studies that kidneys can be stored in current organ preservation solutions for extended periods with life-sustaining function following transplantation, although this function may be delayed for days or even weeks. The extrapolation of improvements made in maintaining individual biochemical pathways to improvements in expected organ funtion is tenuous. Therefore, in order to assess the function of stored kidneys more accurately it was necessary to examine a number of such biochemical processes simultaneously during reperfusion after preservation, as a better indication of integrated renal function.

The study in this chapter was performed to determine the biochemical changes in renal function during prolonged cold ischaemic preservation of rabbit kidneys in hypertonic citrate solution (HCA). It was necessary to define a set of suitable baseline functional parameters which could then be used to gauge the effects of any method of intervention to promote viability. Comparisons were made between freshly harvested kidneys, and kidneys stored for 24h and 72h. The longest

storage time of 72h was chosen because *in vivo* transplantation studies by our group suggested that this was the maximum possible preservation period for survival of recipient rabbits (33%), whilst 48h gave approximately 70% survival for 30 days (Gower *et al.*,1989).

METHODS

Kidneys from male NewZealand White rabbits were harvested and stored by flush preservation with the HCA solution (see Chapter 2). Fresh kidneys were harvested and immediately subjected to *ex vivo* normothermic bloodless perfusion on the circuit. Stored kidneys were perfused after flush preservation for the appropriate length of time. The perfusate composition was as previously described (Chapter 2); a volume of 500ml was used in a recirculating system. The perfusion buffer was prewarmed to 37°C, gassed with $95\%O_2/5\%CO_2$, and all procedures were performed in an environmentally-controlled room at 37°C.

Assessment of organ function was made by comparing markers of renal biochemistry and physiology in stored kidneys with those of freshly harvested kidneys. The indices of organ function used were as follows:

1) Perfusion flow rate (dependent upon vascular resistance in this constant perfusion pressure system, and equivalent to renal arterial flow rate)
2) Urine flow rate
3) Glomerular filtration rate (GFR)
4) Tubular glucose reabsorption

5) Tubular sodium reabsorption

6) Proteinuria

7) Tissue energy charge (adenine nucleotide ratio)

The GFR was calculated from urine and perfusate creatinine concentrations and flow rates, using the assay method of Bonsnes and Taussky (1945). Tubular glucose and sodium reabsorption were calculated as previously described (see Chapter 2).

Statistical analyses were performed using either paired or unpaired Students t-tests as appropriate.

RESULTS

(1) Perfusate flow rate:

The flow rate was proportional to the vascular resistance since the perfusion pressure was kept constant and perfusate viscosity did not alter. The perfusion flow rate tended to increase during the first 15min on the circuit in all three groups (fresh, 24h stored and 72h stored), and then stabilised. Although kidneys stored for 72h exhibited perfusion flow rates which were significantly lower than those of fresh kidneys ($p<0.05$ vs. fresh controls) at the onset of normothermic reperfusion, there were no significant differences between the groups after 15min and 30min of reperfusion (Table 3.1).

Perfusion Time (min)	Storage time (h)		
	Fresh (n=8)	24h (n=8)	72h (n=15)
0	66.13 ±7.97	59.85 ±11.23	52.56* ±12.68
15	75.13 ±17.60	77.71 ±17.81	74.81 ±17.93
30	80.25 ±22.76	77.71 ±24.47	79.56 ±17.72

Table 3.1: Flow rate (ml/min) through the renal vasculature in kidneys reperfused after cold ischaemic preservation for up to 72h. Values are means±SD.

(2) Urine production:

There were no significant differences in urine flow rate between fresh controls and cold ischaemic kidneys during reperfusion. Values ranged between 0.8ml/min and 3.5ml/min.

(3) Glomerular filtration rate (GFR):

Table 3.2: GFR (ml/min) in kidneys reperfused after storage for up to 72h. Values are means±SD.

Perfusion Time (min)	Storage time (h)		
	Fresh (n=8)	24h (n=8)	72h (n=15)
0	3.61 ±1.18	5.13 ±1.78	2.10*# ±1.28
15	3.67 ±1.07	2.85 ±1.39	3.07 ±1.83
30	4.50 ±1.18	3.80* ±1.62	3.07 ±1.88

*P<0.05 (vs. control)

#P<0.001 (vs. 24h stored)

In a similar way to perfusion flow rates, GFR values increased slightly during reperfusion of the kidneys in all groups. There was a trend for stored kidneys to exhibit lower GFR values than controls but only the initial GFR of 72h stored kidneys was significantly ($p<0.05$) depressed when compared to the other two groups. Kidneys subjected to 24 hours of cold ischaemia also had GFRs which were significantly ($p<0.05$) lower than control fresh kidneys after 30min of normothermic perfusion on the circuit (Table 3.2).

(4) Glucose reabsorption and sodium reabsorption:

Table 3.3: The effect of cold ischaemic kidney preservation for up to 72h on post-reperfusion tubule glucose reabsorption (% of filtered load). Values are means±SD.

Perfusion Time (min)	Storage time (h)		
	Fresh (n=8)	24h (n=8)	72h (n=15)
0	41.48 ±40.17	22.28 ±21.88	33.61 ±19.76
15	72.91 ±12.86	59.46 ±23.02	62.09 ±13.59
30	76.46 ±11.78	62.79 ±23.15	60.54* ±11.34

*P<0.01 (vs. control)

Tubular glucose reabsorption, shown in Table 3.3, measured after 30min of normothermic reperfusion on the *ex vivo* circuit, was significantly lower in kidneys subjected to 72h of cold ischaemic storage than in freshly harvested control organs.

Table 3.4: The effect of cold ischaemic kidney preservation for up to 72h on post-reperfusion tubule sodium reabsorption (% of filtered load). Values are means±SD.

Perfusion Time (min)	Storage Time (h)		
	Fresh (n=8)	24h (n=8)	72h (n=15)
0	-2.86 ±9.48	19.76 ±41.52	4.90 ±11.64
15	28.27 ±8.53	27.98 ±14.72	13.71* ±6.29
30	31.93 ±6.34	28.00 ±16.92	15.67* ±8.32

*P<0.05 (vs. controls and 24h stored)

For fresh control kidneys there was initially a very low rate of sodium reabsorption, but this increased and stabilised to about 30% of the filtered load during reperfusion. Kidneys stored for 24h showed a similar picture. However, this function was significantly depressed after both 15min and 30min of normothermic reperfusion in kidneys subjected to 72h of hypothermic ischaemia, compared to fresh kidneys.

(5) Protein leakage:

Table 3.5: The effect of cold ischaemic kidney preservation for up to 72h on post-reperfusion proteinuria (% of perfusate concentration). Values are means±SD.

Perfusion Time (min)	Storage Time (h)		
	Fresh (n=8)	24h (n=8)	72h (n=15)
0	44.83 ±35.48	78.76 ±25.21	179.26* ±79.20
15	13.28 ±6.64	31.65 ±19.83	71.36* ±26.74
30	11.50 ±4.98	42.47 ±37.04	59.94# ±20.19

*P<0.05 (vs. control & 24h stored)

#P<0.05 (vs. control)

When glomerular protein leakage was investigated in control kidneys, initially there was an apparently high leakage (44.8%), but this fell to a mean value of 11.5% of the perfusate protein concentration during reperfusion for 30min. As Figure 3.2 shows, increasing periods of hypothermic storage of the kidneys accentuated proteinuria. Kidneys stored for 24h showed an initial proteinuria which was markedly higher than control values for the first 10min of the reperfusion period.

The leakage of protein tended to be higher at all times during reperfusion of the 24h stored group, compared with fresh controls, but the differences were not statistically significant. Values for kidneys stored for 72h, however, were significantly higher ($p<0.005$) than those for fresh control kidneys at all sample time points. Interestingly, the initial value for the 72h stored group suggested a net loss of protein greater than the concentration in the perfusate, probably reflecting protein release following cellular damage during storage.

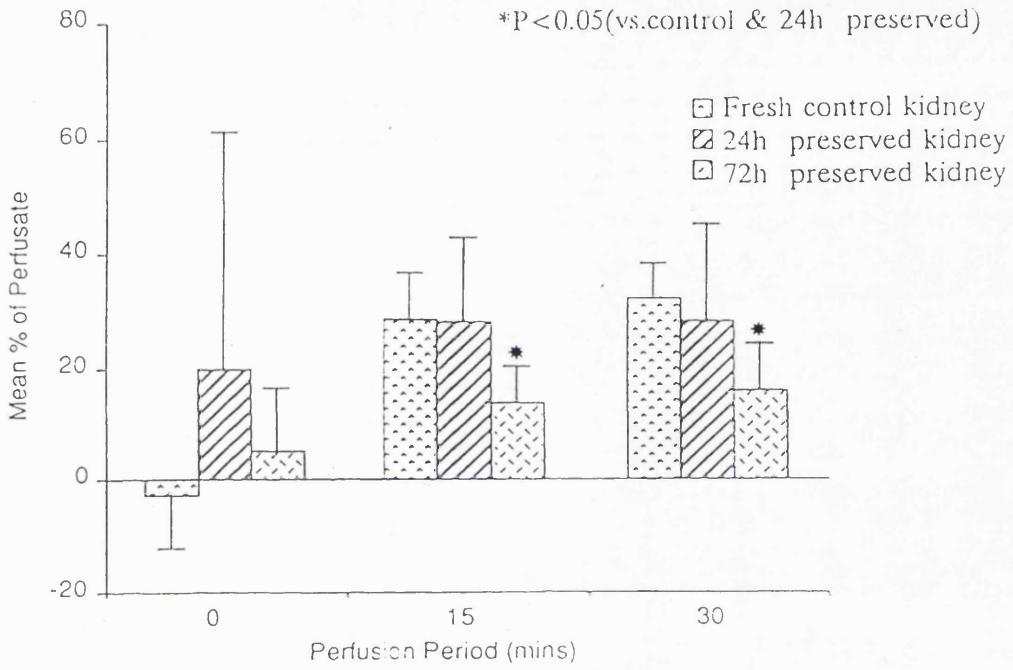


Fig. 3.1: Tubule sodium reabsorption in fresh, 24h preserved and 72h preserved kidneys.

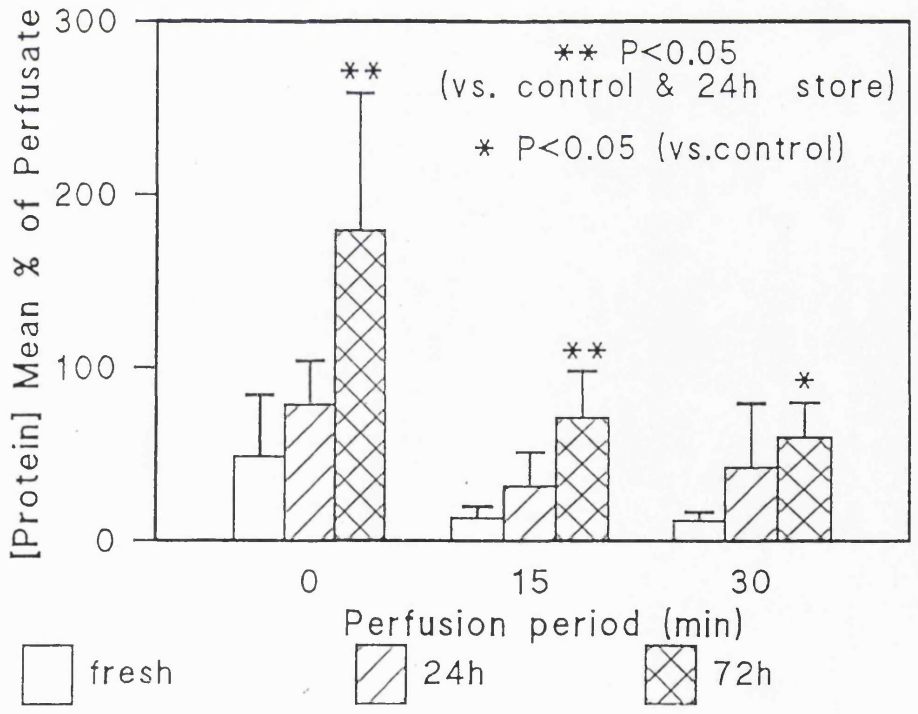


Fig. 3.2: Proteinuria in fresh, 24h preserved and 72h preserved kidneys.

(6) Energy charge:

Table 3.6: Tissue energy charge following 30min reperfusion of kidneys preserved for up to 72h. Values are means±SD.

Sample group				
24h no pfsn	*in vivo*	Fresh	24h	72h
0.21# ±0.02 (n=6)	0.85 ±0.08 (n=6)	0.79* ±0.05 (n=6)	0.70* ±0.10 (n=8)	0.70* ±0.09 (n=8)

#P<0.05 (vs. all other groups)

*P<0.05 (vs. *in vivo* controls)

The values for renal tissue energy charge, shown in Table 3.6, reflect the status of high-energy adenine nucleotides in the kidneys taken at the end of 30min reperfusion. There did not appear to be a marked difference in energy charge between any of the treatment groups compared to *in vivo* control values. In fresh, flushed kidneys, a value of 0.79±0.05 was found at the end of 30min of reperfusion. This was close to the value obtained by freeze-clamping control kidneys with uninterrupted blood flow *in vivo*, which produced values of 0.85±0.08. In both groups of stored kidneys a small decrease in energy charge (0.70±0.10 after 24h and 0.70±0.09 after 72h) was noted after reperfusion, but these were not statistically different

to control reperfused kidneys. In another group of kidneys stored for 24h but not reperfused, the energy charge was much lower, at 0.21±0.02. This was significantly lower than both controls ($P<0.05$) and 24h stored/reperfused kidneys ($P<0.01$).

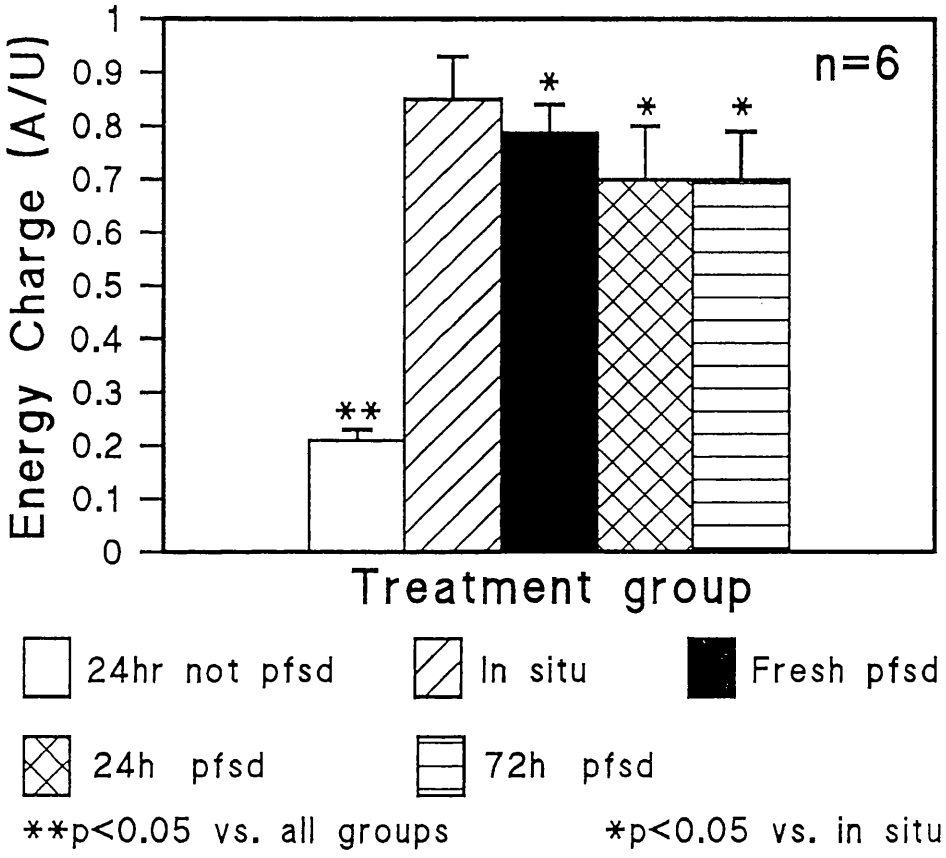


Fig. 3.3: Tissue energy charges in kidneys freeze-clamped *in situ*, after 24h cold preservation alone, and following 30min reperfusion after cold preservation for up to 72h.

DISCUSSION

Cold flush-preservation of rabbit kidneys for up to 72h followed by normothermic reperfusion produced characteristic functional changes. A significant reduction in the tissue content of energy substrates occurred within 24h of cold preservation, but these were restored to values similar to fresh controls upon normothermic, oxygenated reperfusion even after 72h preservation. These results suggest that metabolic pathways for ATP sythesis were conserved efficiently during cold ischaemic kidney storage. However, preservation-dependent damage to tubular reabsorptive mechanisms was apparent, and sodium reabsorption was particularly depressed. Since ATP seemed to be available for active metabolic processes, the reduced reabsorptive functions may have been due to specific damage to membrane ion pumps or transport proteins. Loss of endogenous protein into vascular and urinary spaces occurred upon reperfusion of preserved kidneys, and there was a marked glomerular proteinuria in 72h stored organs. These findings suggest that the permeability of vascular cell membranes, probably those of the vascular endothelium, and of tubular epithelial cells, was increased by ischaemic preservation and reperfusion, or that complete lysis of a number of these cells occurred. The proteinuria suggests damage to the glomeruli which resulted in increased permeability of the basement membrane. Perfusate flow rate through the vasculature and the rate of urine production were not affected by preservation for up to 72h, but the decreased viscosity of the perfusate

compared to that of blood and the absence of hormonal influences in this model may have made these parameters less useful for assessing renal function. However, as can be seen from this study, there were a number of biochemical mechanisms and kidney functions which showed characteristic changes dependent upon the length of ischaemic preservation, and these may be useful markers for the assessment of kidney function in experimental investigations of ischaemia and reperfusion.

Perfusate flow (vascular resistance)

These studies showed that renal arterial perfusate flow rate remained largely unaltered by the increase in cold storage time. The high arterial perfusate flows observed may simply reflect the fact that use of an acellular reperfusion solution may mask changes in blood flow which would be more pronounced when whole blood, containing erythrocytes, platelets and white cells encounters damaged endothelium of kidneys after prolonged ischaemia (Moldow and Jacob, 1984). The observation that the only significant difference in flow rates was between fresh and 72h preserved kidneys immediately at the onset of reperfusion may be explained in a number of ways. Under normal physiological conditions the renal vasculature is kept distended by the pressure of the blood passing through it. During preservation the intravascular pressure equilibrates with the atmospheric pressure, the vessels become flaccid and may collapse. Upon re-establishment of normothermic reperfusion, a filling pressure may have been

required to re-open the collapsed vascular bed before normal flow can be attained. Another possibility is that disruption of normal mechanisms for control of vascular tone occurred during the preservation period, including for example disruption of normal prostaglandin ratios (Lefer *et al.*, 1985). There is evidence for an accumulation of metabolites in the vascular space during cold ischaemia (Bayati *et al.*, 1990). The build-up of vasoconstrictors such as endothelin (Dinton & Anderson, 1990) and angiotensin (Linder *et al.*, 1982) may have been responsible for contracture of vascular smooth muscle, thereby increasing the renal vascular resistance. These may have been released as a result of cell damage and changes in cell metabolism during the 72h preservation period. The absence of elevated vascular resistance in 24h stored kidneys would then indicate that cells in this group of kidneys were better preserved and therefore the release of such vasoconstrictors would have been reduced. The flushing out of these vasoconstrictors during early reperfusion would then cause a decrease in vascular resistance and an increase in perfusion flow rate. There is a possibility that elevated cytosolic calcium during preservation may have caused vascular smooth muscle contracture which may have persisted into early reperfusion but this is unlikely since citrate in the HCA storage solution may effectively chelate free calcium in kidneys stored this way. However, if intracellular calcium levels did increase during ischaemia this is likely to have led to increased

prostaglandin and leukotriene production with concurrent increased superoxide production, due to activation of phospholipase A_2 (see Chapter 1) and the resulting increase in concentration of arachidonic acid which is a substrate for cyclooxygenase. Some vascular disturbances may have been due to delayed restoration of normal Na^+/K^+ membrane pump activity in vascular smooth muscle cells following storage in the hyperkalaemic preservation solution. Consequently, restoration of this ionic equilibrium following reperfusion would have returned the vascular tone to its normal physiological level. The simplest explanation for the observed initial depressed flow rate is that cell debris, accumulated in the vascular space during 72h preservation, impeded normal perfusion until such debris were flushed out of the kidney.

The fact that changes in vascular resistance were evident in this cell-free system has important implications for expected results with blood reperfusion following transplantation. Since the viscosity of blood is much greater than the cell-free perfusate, disruption of flow may be more pronounced following the grafting of a kidney into a recipient. It has also been demonstrated that renal vascular tone is dependent upon the release of endothelium derived relaxant factor (EDRF) into blood passing through the renal artery by the endothelial cells which line this vessel (Kon *et al.*, 1990). In my model very little of the renal artery endothelium came into contact with the perfusion buffer because the artery was cannulated close to the kidney and

therefore the release of EDRF into the perfusate entering the kidney would have been minimal. Ischaemic injury and reperfusion injury to the renal artery endothelium may therefore affect vascular resistance in a transplanted kidney in a way which could not be determined in these experiments.

Urine Production

Preservation for up to 72h did not appear to alter the ability of the kidneys to produce urine. Although there were no significant differences in urine production rate between the different groups of kidneys subjected to *ex vivo* perfusion, values exceeded expected *in vivo* values (see Tables B.1 and B.2, Appendix B). These expected values would be in the range 0.018-0.304ml/min from each kidney of a 2.5kg rabbit (Mitruka *et al.*, 1977). Some *ex vivo* values were as high as 3.5ml/min per kidney. This is probably due to a number of factors, in particular the composition of the acellular reperfusion buffer. The lack of neural and particularly humoral influences on tubule reabsorption is also likely to have been significant. The production of copious amounts of urine would have been expected if the promotion of water reabsorption in the collecting tubules by vasopressin was absent, as in this model. There may also have been some residual diuretic effects of the frusemide administered during the kidney removal procedure. Altered tubule sodium reabsorption (see below) may also have affected urine production since a large proportion of water reabsorption

occurs in the loop of Henle, by the counter-current multiplier mechanism.

Glomerular filtration rate (GFR)

The only obvious depression of GFR, as estimated by creatinine clearance, occurred at the onset of reperfusion in 72h preserved kidneys. Since creatinine clearance is an entirely passive process, with very little active reabsorption (Pitts, 1974), this difference cannot be explained by delayed or disturbed cell metabolism following prolonged organ storage. It may have been the result of occlusion of glomerular basement membrane fenestrations by particulate debris on either the vascular or tubular side of the glomeruli. This obstruction may then have been removed as the reperfusion solution cleared vascular and urinary spaces during early reperfusion. It is known that the tone of the mesangium, a cellular frame within the glomerulus, modulates the capillary filter surface area and therefore affects GFR. Changes in the tone of the mesangium during cold ischaemic preservation or delayed restoration of normal function may have reduced the filtration surface area and thereby caused initial depression of GFR. The mesangium is sensitive to vasoactive agents such as EDRF (Schultz *et al.*, 1990), and therefore inappropriate release (whether hypersecretion or hyposecretion) of this and other endogenous vasoactive compounds at the onset of reperfusion may have decreased initial GFR.

Proteinuria

At first inspection the fact that no significant differences in perfusate flow rate were observed might suggest that the vascular system of the kidneys was largely unaffected by prolonged hypothermic storage. However, the leakage of protein from the glomerular capillaries into the tubular lumens would indicate quite the opposite, highlighting an increase in capillary permeability, with a greater protein loss in the kidneys stored for 72h. A similar type of microvascular damage upon reperfusion following ischaemic storage has been shown in the kidney and in other organs (Smolens and Stein, 1981; McCord, 1985; Lambert *et al.*, 1986). Protein leakage therefore seemed to be a useful indicator of vascular damage. It is of interest that there was such a high protein leakage in the initial urine samples from kidneys after 72h preservation, which may reflect leakage of membrane-bound or intracellular enzymes. Similar leakage of enzymes during cold ischaemia/reperfusion injury in heart has been reported (Wickens *et al.*, 1987). The observation that the concentration of protein in urine at the onset of reperfusion in 72h stored kidneys was greater than the concentration of protein in the perfusate entering the kidney suggests that the excess protein was endogenous. This probably reflects increased cell membrane permeability, or cell lysis, of vascular endothelial cells and epithelial cells of the urinary tubules.

Energy Charge

Loss of the ability of mitochondria to resume ATP synthesis after re-establishment of blood flow has been described as the principal limiting factor in ischaemic / reperfusion injury (Trump *et al.*, 1976). Reactive oxygen species, in particular hydrogen peroxide (H_2O_2), have been shown to rapidly deplete ATP (Ward, 1991), although this does not necessarily correlate with cell injury. In my model the energetic status of the renal tissue after reperfusion showed little change with increasing cold preservation time, as indicated by the similarities in energy charge between fresh and preserved kidneys following 30min of warm reperfusion. It is clear that there was a substantial decrease in the energy charge during the storage period itself, dropping from 0.85±0.08 (the value *in vivo*) to 0.21±0.02 after 24h cold preservation. A decrease in the adenine nucleotide pool during ischaemia is a characteristic event and has been shown in a number of other organs (Asimakis *et al.*, 1992). The ability of stored kidneys, even those subjected to 72h of cold ischaemia, to increase their tissue energy charge to a level which, although not identical, was not significantly different to fresh reperfused kidneys, suggested that mitochondrial integrity was well preserved. It also suggests that the pool of substrate precursors for ATP synthesis were well maintained, or that *de novo* synthesis was highly efficient, since the levels af adenine nucleotides recorded were reached within 30min of reperfusion. A similarly rapid restoration of

tissue energy stores has been shown in transplanted human skeletal muscle subjected to reperfusion *in vivo* following ischaemia for up to 6h (Norman *et al.*, 1991). *De novo* synthesis is energetically unfavourable and studies of ischaemic acute renal failure *in vivo* (Stromski *et al.*, 1988) showed that ATP recovery following reoxygenation is predominantly from intracellular AMP. Regeneration from nucleosides and bases is limited by the availability of enzyme systems of the salvage pathways. This is less favourable since it requires ATP in the first instance to begin synthesis and the results of the energy charge measured after 24h preservation with no reperfusion suggested that this ATP may not be available. Nucleosides and bases are also more likely than AMP to leak out of cells during the ischaemic preservation period due to specific transport mechanisms (Plagemann *et al.*, 1988). Several studies have shown the ability to increase the intracellular ATP level by supplying exogenous nucleotides or adenosine to kidney tubules (Weinberg and Humes, 1986). In some cases these increases have been associated with improvements in renal function (Mandel *et al.*, 1988). However, few studies have addressed the importance of restoration of the normal ratios of nucleotides within cells; the energy charge. The restoration of this balance may be more important than simply trying to elevate the ATP content of cells by supplying exogenous substrates. Such manoeuvres may have inhibitory effects on the normal mechanisms of nucleotide synthesis due to negative-feedback mechanisms. It has also

been shown that intermittent reperfusion during organ storage, which removes some of the purine metabolites which may be utilised for nucleotide synthesis via salvage pathways, does not affect the recovery of ATP in the postischamic period (Thornton *et al.*, 1990).

A longer period of reperfusion, with the prospect of tissue repair and regeneration, and the continued supply of metabolic substrates, may have allowed further increases in energy charge in these studies. Mittnacht and Farber, (1981) suggested that provided that the integrity of the plasma membrane is well preserved, rapid and complete recovery of mitochondrial activity can be expected following ischaemia. Adequate levels of ATP would also have been needed to provide energy for cell repair at other sites.

The energy charge measured in kidneys which were freeze-clamped *in vivo* was significantly higher ($P<0.05$) than in all kidneys subjected to *ex vivo* reperfusion, even freshly harvested kidneys. This may represent differences in the ability of the two systems to deliver metabolic substrates or oxygen to the organ. It is unlikely that any refinements made in the design of a cell-free perfusion buffer solution could achieve the same capacity as blood to sustain normal cell function. This is because blood is a complex mixture of cellular and soluble components whose interactions with each-other and with the vasculature are an integral part of the maintenance of normal homeostasis. Although fresh kidneys reperfused *ex vivo* showed good maintenance of normal metabolic

equilibrium when expressed in this way, it is clear that energy production was not as efficient as *in vivo* which, in addition to the above considerations, may reflect the effects of the limited period of cold ischaemia to which these kidneys were inevitably subjected during harvesting (approximately 20min). Another reason for the observed high energy charge in 72h preserved kidneys may be due to decreased ATP utilization as a result of depressed active metabolic processes such as sodium and glucose reabsorption upon reperfusion.

The estimation of energy charge by the method described, using freeze-clamped whole kidneys, looks at the energy status in a number of renal compartments at the same time and is unable to identify the distribution and rate of energy recovery in individual cell types. It is possible that regeneration of ATP was more efficient in some regions of the kidney than in others, and this may have some bearing on the preferential recovery of certain aspects of renal metabolism. Cells which are highly dependent on ATP production, such as the cells of the proximal tubule are believed to be more susceptible to ischaemia-induced depletion of ATP (Guder and Ross, 1984).

Solute reabsorption

The reabsorptive processes which operate to recover sodium and glucose from the glomerular filtrate are well characterized. They rely upon both active and passive transport across cell membranes by mechanisms which are

essential for cell survival. The measurement of the activity of these transport mechanisms gives an indication of the potential ability of cells in the kidney to perform a number of essential functions: a)formation of ionic gradients; b)regulation of cell volume; c)regulation of cell pH; d)concentration of metabolic substrates; and e)extrusion of toxic metabolic waste products. Damage to transport sytems may also interfere with cell-cell comunication thereby preventing coherent operation resulting in decreased efficiency of organ function. Active transport of Na^+ and K^+ accounts for over 30% of ATP consumption in resting animals and may be even greater when normal ionic distribution needs to be restored following ischaemia. There is also evidence that the transport of glutathione (GSH) across renal brush border membranes is dependent upon the membrane potential generated by the distribution of these ions (Inoue and Marino, 1985). Thus functional recovery of Na^+/K^+ATPase activity is likely to influence GSH and amino acid transport into cells (see Chapter 1). Delayed recovery of the normal physiological membrane potential may therefore slow protein synthesis and the recovery of intracellular antioxidant defence mechanisms.

When tubular reabsorptive functions were examined, it was seen that the reabsorption of sodium was significantly depressed when the cold storage time was increased up to 72hr. Reabsorption of glucose was less affected, although this too was significantly depressed during the later stages of warm reperfusion after 72h storage. The decreased tubular

reabsorptions cannot be the result of a failure in ATP supply because of evidence from the energy charge studies, but may reflect more subtle damage, like a direct effect on the ATPase enzyme involved in sodium reabsorption. This type of selective damage has previously been suggested in ischaemia/reperfusion injury (Molitoris and Kinne, 1987), and free radicals have been shown to depress Na^+/K^+ATPase acivity in the kidney (Kako *et al.*, 1988) and in the heart (Kim and Akera, 1987). Huang and colleagues, (1992) demonstrated irreversible inhibition of purified Na^+/K^+ATPase upon exposure to hydrogen peroxide, superoxide radicals and hydroxyl radicals.It is possible that a loss of ATPase activity as a result of kidney storage, continued to cause suppressed ion transport even when ATP levels returned to normal. This was shown in studies by Varkarakis and colleagues, (1975). Funtional Na^+/K^+ATPase activity is required to control intracellular osmolarity otherwise oedema, and eventually cell lysis, may occur. Any altered activity in tubule cells is likely to reflect altered activity in other renal cells. Elevated free plasma concentrations of unsaturated fatty acids are known to modify Na^+/K^+ATPase activity (Kelly *et al.*, 1985). The concentration of these endogenous inhibitors may be locally elevated by phospholipase activity stimulated by ischaemia and reperfusion (Cheung *et al.*, 1986). Clerici and colleagues, (1992) reported that hydrogen peroxide (H_2O_2) decreased Na^+/K^+ATPase activity in rat alveolar epithelial cells, by a mechanism independent of H_2O_2-induced ATP depletion. Sodium reabsorption into the

epithelial cells of the kidney may be disrupted by a similar mechanism, thought to be mediated by changes in the cell membranes caused by lipid peroxidation. Hydrogen peroxide is known to be formed as a result of the dismutation of superoxide radicals (see Chapter 1) which may have been produced during reperfusion. Depletion of the antioxidant enzymes glutathione peroxidase and catalase, responsible for degrading H_2O_2, may thereby result in decreased sodium reabsorption even though the energy substrate to drive the reaction, ATP, is in adequate supply. This is in agreement with the study in rat alveolar cells, where it was demonstrated that d-α-tocoperol, a lipid-soluble antioxidant which prevents lipid peroxidation by scavenging peroxy radicals, prevented inhibition of Na^+/K^+ATPase by H_2O_2. Decrease in Na^+/K^+ATPase activity has been demonstrated in microsomal fractions prepared from canine kidneys following ischaemia and reperfusion (Kako *et al.*, 1988). They also found a concurrent decrease in sulfhydryl content, which suggests direct free radical-mediated damage to the enzyme. This may lead to disruption of the tertiary structure of the enzyme. The Na^+/K^+-ATPase is known to require an intact lipid domain for normal function, therefore damage to the basolateral membrane lipids in the immediate area of the enzyme, such as free-radical mediated lipid peroxidation (Halliwell and Gutteridge, 1986), may also influence sodium reabsorptive capabilities. It has also been demonstrated that direct radical mediated depression of enzyme function can be

separated from effects mediated by lipid peroxidation (Kako *et al.*, 1988).

It is also possible that during this early reperfusion period substrates for energy production were directed towards restoration of functions which were more critical to cells e.g. DNA / RNA synthesis and repair, enzyme synthesis, and the restoration of strucural integrity. As described above it is also possible that proximal tubule damage is evident because these cells are less able to maintain ATP concentrations during ischaemia than are the cells of the distal tubules (Bastin *et al.*, 1987). Although substantial sodium reabsorption does occur in the distal tubule *in vivo*, this process only occurs at this site when stimulated by circulating aldosterone. Since this hormone was not present in the reperfusion buffer, there was no such stimulation of sodium reabsorption in these segments in this model and therefore this would have allowed sodium excretion.

A secondary effect of reduced Na^+/K^+ATPase activity is reduced transmembrane Na^+-Ca^{2+} exchange (Hansson *et al.*, 1990), leading to an increased intracellular free calcium concentration. This may then have caused phospholipase activation leading to membrane damage or inapproriate prostaglandin / leukotriene production (Lelcuk *et al.*, 1985) accompanied by increased free radical production. Elevated calcium concentrations also activate the protease which is responsible for the limited proteolysis of xanthine dehydrogenase to xanthine oxidase (McCord, 1985). This in turn

may have lead to free radical generation as previously described (see Chapter 1).

Active sodium reabsorption occurs only across the basolateral membranes of tubule epithelial cells, releasing sodium ions into the peritubular space, thereby lowering the intracellular sodium ion concentration. This then allows sodium ions from the tubule lumen to diffuse passively into the cells. For these charged species (Na^+) to enter cells they must pass through the non-polar lipid environment of the cell membrane. This is achieved through protein channels (Devlin, 1992) which may be damaged during ischaemia and/or reperfusion, thereby preventing the ions from passing through. It must also be considered that the high potassium concentration in the HCA kidney storage solution would have resulted in a high intracellular potassium concentration in the cells of the kidney after 72h storage in this solution. Such high potassium concentrations in the proximal tubule cells may oppose potassium entry in early reperfusion, a process which is required for sodium to be pumped into the extracellular spaces by Na^+/K^+ATPase. High potassium concentrations can cause reversal of the enzyme pump, causing synthesis of ATP from ADP and inorganic phosphate (P_i), although this does not occur under normal physiological conditions. This may explain why ATP resynthesis was so efficient following organ preservation but sodium reabsorption was suppressed (Devlin,1992).

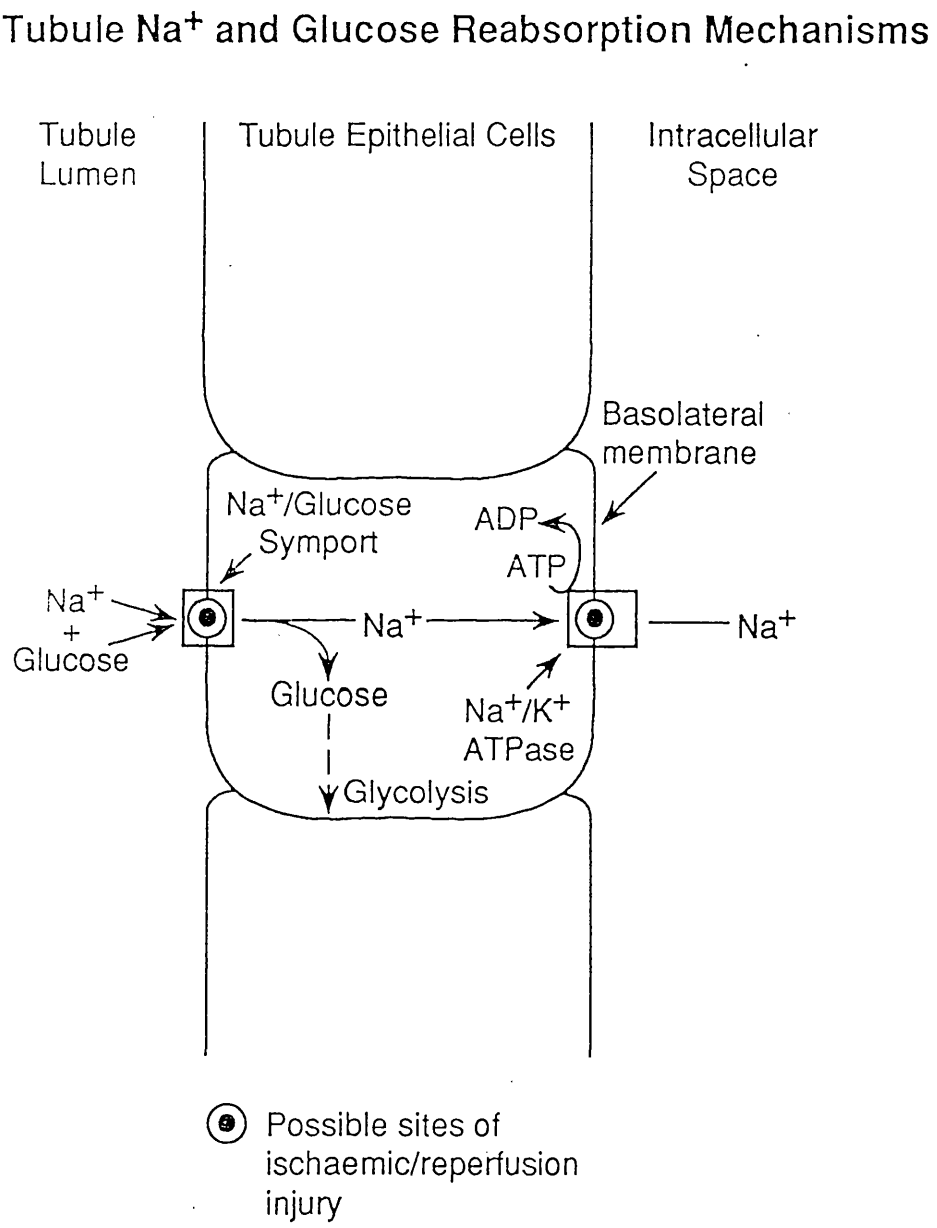


Fig 3.4: Possible sites of damage to solute transport mechanisms

The observation that glucose reabsorption was less affected by preservation-dependent injury in the early stages of *ex vivo* reperfusion than after 30min may reflect progressive reperfusion injury. Glucose translocation mechanisms may have been more susceptible than sodium transport mechanisms to those reperfusion events which characteristically occured later during reperfusion, such as lipid peroxidation and the production of particular free radical species. However, most glucose entry into cells is linked indirectly to active sodium extrusion. The pumping of Na^+ ions out of cells produces an ionic gradient which causes Na^+ ions to enter from the tubule lumen accompanied by glucose molecules which bind to the same specific transport protein. Damage to this symport protein may have been the cause of suppressed sodium and glucose reabsorption. It is possible that at the onset of reperfusion the majority of available ATP was utilized for active acquisition of glucose, the only available exogenous energy substrate, with suppression of active processes which were not of immediate benefit to cells. It was noticed that although solute reabsorption was more efficient in fresh kidneys than in stored organs, glucose reabsorption and sodium reabsorption did not reach the values which would have been expected *in vivo*.

SUMMARY

The results in this chapter demonstrated that during *ex vivo* perfusion after preservation, kidneys were able to restore their energetic status to near-normal values, even after 72h storage. However, solute reabsorption was depressed during reperfusion after 72h storage. Sodium reabsorption was significantly lower within the first 15min of the onset of reperfusion in these kidneys than in fresh controls or 24h stored organs, and glucose reabsorption was also significantly reduced in 72h stored organs after 30min reperfusion. Storage of kidneys for 72h also resulted in significantly higher proteinuria than either fresh or 24h stored organs. These findings suggest that prolonged kidney preservation produces functional changes associated with damage to the renal vasculature and urinary spaces, and that this loss of function is not due to a lack of high energy metabolic substrates.

OXYGEN-DEPENDENT REPERFUSION INJURY: HYDROXYLATION OF SALICYLATE AS A MARKER OF HYDROXYL RADICAL ACTIVITY

INTRODUCTION

The results of studies in the previous chapter suggested that specific sites in the kidney were affected by ischaemic preservation for up to 72h, and that biochemical changes such as ATP degradation occurred during ischaemia, which may have led to free radical production during reperfusion. The evidence presented showed that during warm reperfusion following cold ischaemic preservation changes in function such as increased proteinuria and decreased sodium reabsorption became evident, and it was suggested that oxygen-derived free radicals may have been a contributory factor. In order to test this, experiments were designed to investigate by direct measurement whether free radicals, and particularly the highly damaging hydroxyl radical (•OH), are formed during reperfusion. Comparisons were made between kidneys reperfused under normoxic conditions and those reperfused under hypoxic conditions in order to investigate the role of oxygen in reperfusion damage. A shorter cold preservation time of 48h was chosen for the following studies because it was thought that this time point may mark the transition between reversible and irreversible ischaemic injury in rabbit kidneys

using existing flush solutions.

The highly reactive and unstable nature of free radicals causes them to be extremely short-lived species and therefore their direct measurement is difficult. A number of methods for free radical detection have therefore been developed which rely on the reaction of radicals with other molecules (known as 'traps'), resulting in the production of more-stable characteristic adducts which can be analysed using appropriate techniques such as aromatic hydroxylation (see below) and electron spin resonance spectroscopy (see appendix A).

The use of aromatic hydroxylation as an assay for hydroxyl radical (•OH) activity is a valuable method since under physiological conditions these reactions occur at a diffusion-dependent rate with the production of hydroxylated end-products (Halliwell *et al.*, 1988). The reaction of salicylate with hydroxyl radicals produces characteristic adducts which can be identified and measured by high performance liquid chromatography (HPLC) with electrochemical detection (Grootveld and Halliwell, 1986). Catechol and 2,5-dihydroxybenzoate (2,5-DHB) are known to be formed *in vivo* by enzymatic hydroxylation of salicylate via mechanisms involving cytochrome P_{450} (Ingelman-Sundberg *et al.*, 1991), and may also be the result of the interaction of salicylate with normal basal metabolic •OH production and by reaction with singlet oxygen (Feix and Kalyanaraman, 1991). Another product, 2,3-dihydroxybenzoate (2,3-DHB), is believed to be specifically formed by •OH attack on the salicylate molecule, and no

enzymatic hydroxylation pathway for its production has been described. For this reason the formation of 2,3-DHB from salicylate, which was added to the kidney perfusion solution, was used as one indicator of •OH production in the isolated perfused rabbit kidney. However, as mentioned above the compound 2,5-DHB can also be formed by the reaction of hydroxyl radicals with salicylate, so the production of 2,5-DHB was also analyzed in this study. In isolation, the kidney experiences no influences from radical generation by other body tissues or the blood. A high concentration of salicylate (1mM) was chosen to increase trapping efficiency in the complex environment of kidney tissues which undoubtedly offered numerous competing endogenous biological targets for free radical attack. In addition there were a number of different compartments in the kidney which had to be accessed by the radical trap. For any compound to scavenge highly reactive free radicals such as the hydroxyl radical in biological systems it should ideally be present at a higher concentration than surrounding target molecules. Salicylate is particularly useful for this purpose because high concentrations can be produced *in vivo* (Aruoma and Halliwell, 1988) without harmful effects to the subject. However, it is not known whether salicylate is harmful in the isolated kidney at high concentrations. Salicylate has been used for the study of hydroxyl radical production in a number of organs both *ex vivo* and *in vivo* (e.g. brain, Cao *et al.*, 1988; intestine, Udassin *et al.*, 1991; and heart, Powell and Hall, 1990) but

there is little information about its use as a radical trap in the kidney.

METHODS

Reperfusion of stored kidneys

Standard perfusion buffer without bovine serum albumin (BSA) was used for these studies to prevent binding of salicylate to BSA and to prevent possible free radical quenching by reaction with BSA. One litre of this perfusate was gassed using a plastic sinter in the reservoir of the perfusion circuit, whilst being warmed in a water bath at 37°C for 1hr prior to the addition of salicylate. For normoxic reperfusion studies, the perfusate was gassed with $95\%O_2/5\%CO_2$, and for studies of hypoxic reperfusion it was gassed with $95\%N_2/5\%CO_2$. The respective oxygen concentrations measured in the reservoirs using an oxygen electrode (Strathkelvin instruments, model 781) were 95% for normoxic and 0% for hypoxic buffers, where readings were taken with reference to a solution of sodium borate (10mM) for 0% oxygen, and a perfusion buffer gassed with 100% oxygen. All measurements were taken from solutions at 37°C. Measurements of the actual oxygen concentration in the perfusion buffer delivered to the kidneys were taken at the tips of the cannulae prior to placing the organs on the perfusion circuit. These measurements were taken after an initial equilibration period of 15min during which the recirculating flow rate was kept at approximately 60ml/min whilst the perfusion buffer in

the reservoir (37°C) was gassed with the appropriate gas mixture (see Table 4.1). Following equilibration with the appropriate gas, 0.16g of salicylic acid (salicylate, Aldrich) was added to 1l of perfusate just before reperfusion and mixed thoroughly to give a concentration of 1mM. Salicylate was added at this stage to minimize the possibility of oxidative decomposition during the equilibration period.

Kidneys were either used immediately after harvesting from donor rabbits (fresh controls) or, prior to reperfusion, were flushed and preserved in Marshalls hypertonic citrate solution (HCA) for 48h on ice. One kidney of a pair was reperfused with oxygenated buffer and the other was reperfused with deoxygenated buffer. Samples of urine and vascular effluent were taken at the onset of reperfusion (time=0min) and then after 2min and 5min. Samples were taken at these times since the aim of the study was to investigate free radical production at the moment oxygen was reintroduced into kidney tissues in the very early stages of reperfusion. In addition, a non-recirculating perfusion system was used and due to the high perfusate flow rates achieved in this model, reperfusion for 30min, as used in other studies, would have required the use of very large volumes of perfusate. At time=0 the first 10ml of vascular effluent was collected and at each of the following two time intervals (2min and 5min) 2ml were collected. Samples of urine were collected for 1min at each sample interval. Perfusate flow rate (vascular resistance) and urine flow rate were recorded. Aliquots (500μl) of each sample

were acidified with 26μl of 1M HCl to give a final concentration of 50mM HCl. These acidified samples, along with the remainder of the urine and vascular effluents, were frozen and stored in liquid nitrogen until analysed.

Histology

Tissue samples for histological examination were immediately fixed in gluteraldehyde at the end of the reperfusion period and processed and examined as previously described (see Chapter 2).

Sample extraction

Acidified samples of urine and vascular effluent were thawed at room temperature. Aliquots (400μl) of each sample were placed in a 10ml glass tube (L.I.P. Ltd.) to which 8ml of HPLC grade ethylacetate (Rathburn chemicals Ltd.) was added. The samples and solvent were mixed thoroughly in a "vortex" mixer for 2min and then centrifuged for 10min at 3000rpm in a bench centrifuge (Mistral 2000). The solvent fraction containing the extracted salicylate metabolites and remaining salicylate was decanted into identical glass tubes and placed in a rotary evaporator (Uniscience Univap with refridgerated solvent trap) at 45°C to evaporate the ethylacetate. This step was repeated using a further 8ml of ethylacetate to extract the remaining metabolites from the samples. Dry sample extracts were stored at -20°C until HPLC analysis.

Chromatography

The dry sample extracts were dissolved in 200μl of 0.2M HCl immediately prior to injection (50μl sample loop volume) onto the HPLC column (Anachem S50DS2). An LKB HPLC system was used with a Trivector chromatography integrator. The method of sample detection was developed to require the use of a single detector to quantitate the various salicylate metabolites simultaneously. A Coulochem 5100A electrochemical detector was used with the following detection parameters: detector potential of +0.65 volts, guard cell potential of -0.08 Volts. The mobile phase used to elute the metabolites from the HPLC column under isocratic conditions (constant eluent composition) had the following composition: 30mM trisodium citrate (sigma); 4% vol/vol methanol (BDH); the pH was adjusted using glacial acetic acid (BDH) to give a value of 4.75. The mobile phase was passed through a 0.2μm nylon filter (Rainin Instrument Co.Inc.) prior to use in order to remove any contaminating particulate matter. The elution flow rate was 1ml/min.

Standards

A standard solution containing each of the metabolites of interest (2,3-DHB, 2,5-DHB; Sigma), in addition to salicylate itself, was prepared and analysed using the HPLC system to obtain characteristic retention times and peak areas (equal to concentration) for these compounds. The internal standard, resorcinol (Sigma), was also included in this solution. This compound was added to the perfusate effluent and urine samples

prior to extraction of metabolites in order to assess the extraction efficiency and thereby determine a correction factor for the calculation of sample concentrations from the peak areas obtained. The standard solution had the following composition: 2,3-DHB 0.1mM; 2,5-DHB 0.1mM; salicylate 1mM and resorcinol 0.0025mM.

Enzyme release

The release of the enzymes LDH and γ-GT into urine and vascular spaces were used as markers of tissue damage. The enzyme assay methods are described in Chapter 2.

RESULTS

The mean oxygen content of "normoxic" (gassed with 95% O_2/5%CO_2) and "hypoxic" (gassed with 95%N_2/5%CO_2) reperfusion solutions is shown below. Measurements were taken from fluid emmerging from the tip of the cannula. Values are means±SD, n=12 pairs of kidneys.

Perfusate oxygen content (% of a buffer gassed with 100% O_2)	
Normoxic	Hypoxic
94.59 ±0.37	1.01 ±0.35
t=726.89, p<0.001 ***	

Table 4.1: Mean oxygen content of normoxic and hypoxic reperfusion buffers.

Functional study results

Perfusate flow rate (ml/min)				
Time (min)	Fresh		48h	
	Normoxic	Hypoxic	Normoxic	Hypoxic
0	74.66 ±6.65	71.00 ±14.79	79.66 ±7.08	70.66 ±5.31
	t=0.87, p<0.50		t=4.88,p<0.001**	
2	73.00 ±7.87	77.33 ±11.84	92.33 ±8.98	85.00 ±13.25
	t=1.10, p<0.30		t=1.17, p<0.30	
5	63.00 ±12.69	82.66 ±17.32	101.00 ±11.08	98.00 ±15.64
	t=2.26, p<0.05*		t=0.49, p<0.70	

Table 4.2: Perfusion flow rate (ml/min) in fresh and 48h stored rabbit kidneys reperfused under normoxic or hypoxic conditions using perfusate containing 1mM salicylate. Values are means±SD, n=6.

Fresh kidneys showed no differences in perfusion flow rate between normoxically reperfused and hypoxically reperfused organs at the onset of reperfusion or at 2min of reperfusion. However, the flow rates were significantly higher ($p<0.05$) in hypoxically reperfused kidneys after 5min

compared to normoxic kidneys at this time. The flow rate in 48hr stored kidneys was significantly higher ($p<0.001$) in hypoxically reperfused kidneys at the onset of reperfusion, but there were no differences at 2min or at 5min of reperfusion.

Urine flow rate (ml/min)				
Time (min)	Fresh		48h	
	Normoxic	Hypoxic	Normoxic	Hypoxic
0	3.50 ±1.39	3.46 ±1.69	2.06 ±1.15	1.95 ±1.58
	t=0.08, p<0.90		t=0.30, p<0.80	
2	2.62 ±1.60	2.80 ±1.82	1.02 ±0.74	0.50 ±0.17
	t=0.54, p<0.70		t=1.65, p<0.20	
5	1.50 ±1.12	2.26 ±1.34	0.81 ±0.76	0.54 ±0.45
	t=2.45, p<0.05*		t=0.89, p<0.40	

Table 4.3: Urine flow rate (ml/min) in fresh and 48h stored rabbit kidneys reperfused under normoxic or hypoxic conditions using buffer containing 1mM salicylate. Values are means±SD, n=6.

There were no differences between urine flow rate at the onset of reperfusion or at 2min in either normoxically reperfused or hypoxically reperfused fresh kidneys. After 5min of reperfusion hypoxic fresh kidneys were seen to produce urine at a significantly higher rate ($p<0.05$) than normoxic fresh organs. Kidneys which had been stored for 48h showed no significant differences between urine flow rate in hypoxic and normoxic organs at any time during reperfusion.

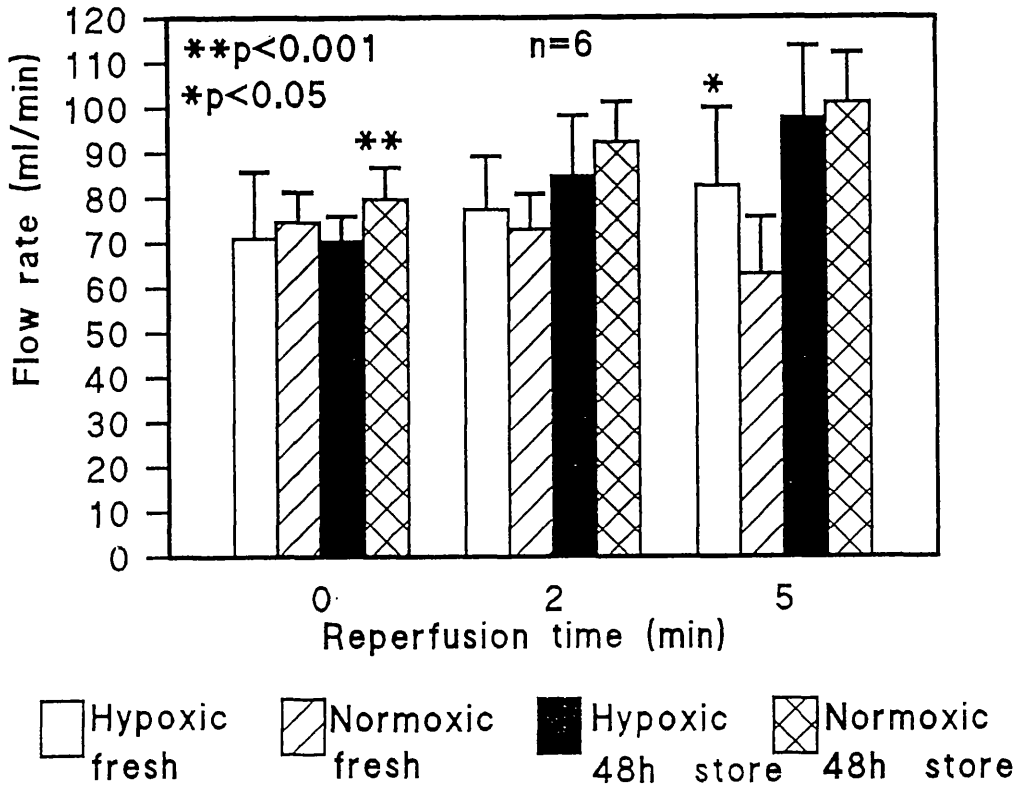


Fig.4.1: Perfusion flow rate in fresh and 48h stored kidneys reperfused under normoxic and hypoxic conditions in the presence of 1mM salicylate.

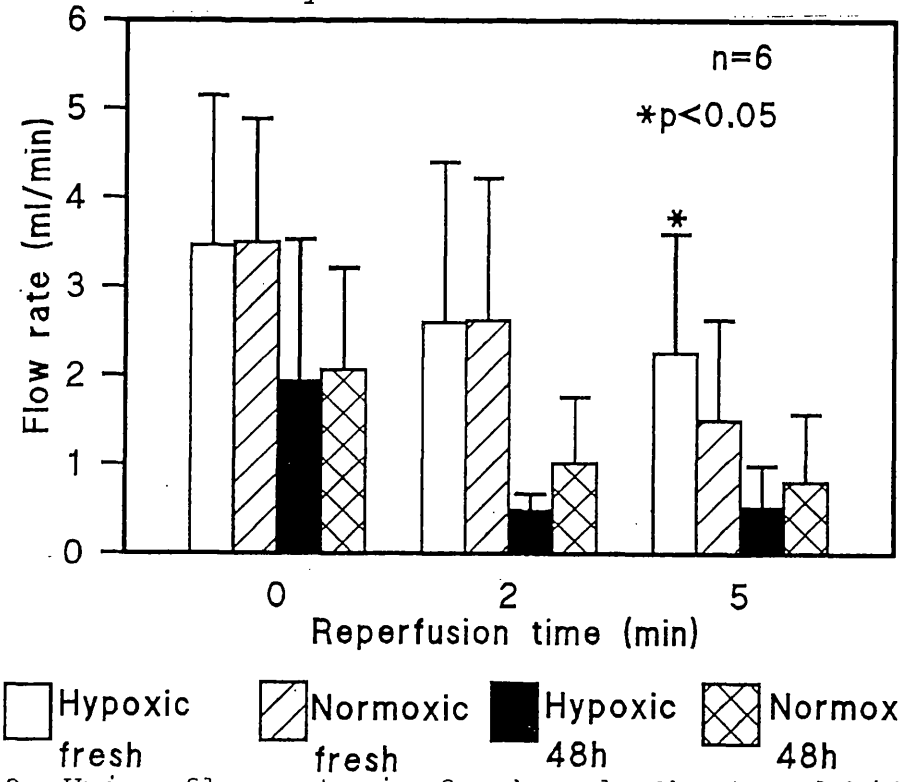


Fig.4.2: Urine flow rate in fresh and 48h stored kidneys reperfused under normoxic and hypoxic conditions in the presence of 1mM salicylate.

Production of salicylate metabolites

Salicylate metabolism in vascular spaces of fresh kidneys				
Time (min)	2,3-DHB (nMoles/ml)		2,5-DHB (nMoles/ml)	
	Hypoxic	Normoxic	Hypoxic	Normoxic
0	0.42 ±0.13	0.45 ±0.20	0.51 ±0.27	0.94 ±0.52
	t=0.56, p<0.60		t=2.09, p<0.10	
2	0.41 ±0.22	0.40 ±0.18	0.35 ±0.20	0.60 ±0.32
	t=0.07, p<1.0		t=3.89, p<0.01 **	
5	0.53 ±0.66	0.39 ±0.17	0.53 ±0.87	0.33 ±0.23
	t=0.44, p<0.7		t=0.50, p<0.70	

Table 4.4: Production of salicylate metabolites by radical-dependent (2,3-DHB) hydroxylation mechanisms, and by mechanisms which may be radical-dependent and -independent (2,5-DHB), in the vascular spaces of fresh reperfused rabbit kidneys. Values are means±SD, n=6.

There were no significant differences between the production of 2,3-DHB in the vascular space of hypoxic and

normoxic kidneys at any time during the reperfusion of fresh kidneys. The production of 2,5-DHB was, however, significantly higher ($p<0.01$) in normoxic kidneys than hypoxic kidneys after 2min of reperfusion (Table 4.4).

Salicylate metabolism in vascular spaces of 48h STORED kidneys				
Time (min)	2,3-DHB (nMoles/ml)		2,5-DHB (nMoles/ml)	
	Hypoxic	Normoxic	Hypoxic	Normoxic
0	3.18 ±3.63	6.14 ±7.56	60.56 ±58.45	59.77 ±56.57
	t=1.31, p<0.20		t=1.13, p<0.30	
2	0.58 ±0.16	0.90 ±0.39	3.84 ±2.75	4.03 ±2.39
	t=2.02, p<0.10		t=0.28, p<0.80	
5	0.28 ±0.08	0.67 ±0.38	1.27 ±0.76	2.01 ±1.20
	t=2.0, p<0.10		t=1.19, p<0.30	

Table 4.5: Production of salicylate metabolites by radical-dependent (2,3-DHB) hydroxylation mechanisms, and by mechanisms which may be radical-dependent and -independent (2,5-DHB), in the vascular spaces of rabbit kidneys reperfused after 48h cold ischaemic storage. Values are means±SD, n=6.

Kidneys which had been preserved for 48h showed no significant differences in either 2,3-DHB production or 2,5-DHB production at any time during reperfusion, when normoxically reperfused kidneys were compared with hypoxically reperfused organs (Table 4.5). However, values were elevated when compared to those seen in fresh kidneys (see later).

Salicylate metabolism in urinary spaces of fresh kidneys				
Time (min)	2,3-DHB (nMoles/ml)		2,5-DHB (nMoles/ml)	
	Hypoxic	Normoxic	Hypoxic	Normoxic
0	0.020 ±0.007	0.085 ±0.139	0.431 ±0.485	0.555 ±0.480
	t=1.05, p<0.40		t=0.39, p<0.80	
2-5	0.760 ±1.330	0.780 ±1.180	0.400 ±0.230	0.650 ±0.350
	t=0.27, p<0.80		t=1.96, p<0.10	

Table 4.6: Production of salicylate metabolites by radical-dependent (2,3-DHB) hydroxylation mechanisms, and by mechanisms which may be radical-dependent and -independent (2,5-DHB), in the urinary spaces of fresh reperfused rabbit kidneys. Values are means±SD, n=6.

There was no evidence of increased 2,3-DHB production or increased 2,5-DHB production in the urinary spaces of fresh kidneys reperfused normoxically when compared to those reperfused hypoxically.

Salicylate metabolism in urinary spaces of 48h stored kidneys				
Time (min)	2,3-DHB (nMoles/ml)		2,5-DHB (nMoles/ml)	
	Hypoxic	Normoxic	Hypoxic	Normoxic
0	6.18 ±7.64	9.22 ±12.43	72.05 ±81.15	94.06 ±102.67
	t=1.34, p<0.30		t=1.73, p<0.20	
2-5	1.56 ±2.12	1.93 ±2.89	26.42 ±27.72	29.44 ±25.85
	t=0.80, p<0.50		t=1.82, p<0.60	

Table 4.7: Production of salicylate metabolites by radical-dependent (2,3-DHB) hydroxylation mechanisms, and by mechanisms which may be radical-dependent and -independent (2,5-DHB), in the urinary spaces of rabbit kidneys reperfused after 48h cold ischaemic storage. Values are means±SD, n=6.

There were no significant differences in the production

of either 2,3-DHB or 2,5-DHB in the urinary spaces of normoxic kidneys compared with hypoxic kidneys at any time during reperfusion of either fresh or 48h stored organs (see Tables 4.6 and 4.7). Again, however, after storage the yields of both metabolites were greatly increased (see later).

PRESERVATION-DEPENDENT RADICAL PRODUCTION

Perfusate 2,3-DHB concentration (nMoles/ml)				
Time (min)	Normoxic		Hypoxic	
	Fresh	48h	Fresh	48h
0	0.45 ±0.20	6.14 ±7.56	0.42 ±0.13	3.18 ±3.63
	t=1.83, p<0.10		t=2.47, p<0.05*	
2	0.40 ±0.18	0.90 ±0.39	0.41 ±0.22	0.58 ±0.16
	t=2.69, p<0.05*		t=1.45, p<0.20	
5	0.39 ±0.17	0.67 ±0.38	0.28 ±0.08	0.53 ±0.66
	t=0.33, p<0.50		t=0.83, p<0.50	

Table 4.8: Vascular space 2,3-DHB production during hypoxic and normoxic reperfusion of fresh kidneys compared with 48h stored kidneys. Values are means±SD, n=6.

The production of 2,3-DHB in the vascular spaces of normoxically reperfused stored kidneys was greater ($p<0.05$) than that in fresh kidneys after 2min of reperfusion but not at the onset of reperfusion (t=0) or after 5min. The production of 2,3-DHB in the vascular spaces of hypoxically reperfused 48h stored kidneys was significantly higher ($p<0.05$) at the onset of reperfusion than in fresh kidneys. No differences in storage-dependent production of 2,3-DHB were detected at any other time during reperfusion (Table 4.8).

Perfusate 2,5-DHB concentration (nMoles/ml)				
Time (min)	Normoxic		Hypoxic	
	Fresh	48h	Fresh	48h
0	0.94 ±0.52	59.77 ±56.57	0.51 ±0.27	60.56 ±58.45
	t=2.54, p<0.05*		t=2.51, p<0.05*	
2	0.60 ±0.32	4.03 ±2.39	0.35 ±0.20	3.84 ±2.75
	t=3.47, p<0.01**		t=3.09, p<0.02*	
5	0.33 ±0.23	2.01 ±1.20	0.53 ±0.87	1.27 ±0.76
	t=3.34, p<0.01**		t=1.55, p<0.20	

Table 4.9: Vascular space 2,5-DHB production during hypoxic and normoxic reperfusion of fresh kidneys compared with 48h stored kidneys. Values are means±SD, n=6.

The production of 2,5-DHB in renal vascular spaces was significantly higher in stored organs than in fresh organs at the onset of normoxic reperfusion ($p<0.05$) and at the onset of hypoxic reperfusion ($p<0.05$). The production of this metabolite was similarly greater in stored kidneys than fresh organs after 2min of reperfusion under both normoxic ($p<0.01$) and hypoxic ($p<0.02$) conditions. Normoxic reperfusion continued to cause increased 2,5-DHB production

in stored kidneys ($p<0.01$) after 5min of reperfusion but hypoxic reperfusion did not (Table 4.9).

Urine 2,3-DHB concentration (nMoles/ml)				
Time (min)	Normoxic		Hypoxic	
	Fresh	48h	Fresh	48h
0	0.08 ±0.14	9.22 ±12.43	0.20 ±0.01	6.18 ±7.64
	t=1.79, p<0.20		t=1.78, p<0.20	
2-5	0.78 ±1.18	1.93 ±2.89	0.76 ±1.33	1.56 ±2.12
	t=0.83, p<0.50		t=0.75, p<0.50	

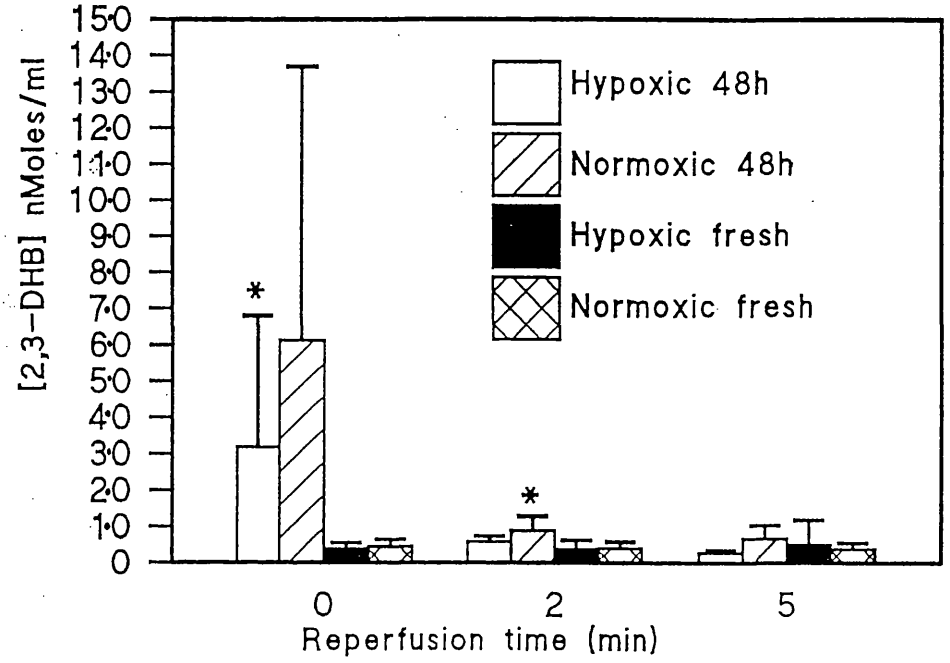
Table 4.10: Urinary space 2,3-DHB production during hypoxic and normoxic reperfusion of fresh kidneys compared with 48h stored kidneys. Values are means±SD, n=6.

There was no evidence of a difference in free radical production in the urinary spaces between fresh and stored kidneys when reperfused under either normoxic or hypoxic conditions (Table 4.10). Values in stored organs tended to be higher, but there was a wide range of results and the difference was not statistically significant.

Urine 2,5-DHB concentration (nMoles/ml)				
Time (min)	Normoxic		Hypoxic	
	Fresh	48h	Fresh	48h
0	0.56 ±0.48	94.06 ±102.67	0.43 ±0.49	72.05 ±81.15
	t=2.23, p<0.05*		t=2.16, p<0.10	
2-5	0.65 ±0.35	29.44 ±25.85	0.40 ±0.23	26.42 ±27.72
	t=2.41, p<0.05*		t=2.07, p<0.10	

Table 4.11: Urinary space 2,5-DHB production during hypoxic and normoxic reperfusion of fresh kidneys compared with 48h stored kidneys. Values are means±SD, n=6.

Stored kidneys produced significantly more 2,5-DHB in the urinary spaces at the onset of normothermic reperfusion than did fresh kidneys ($p<0.05$). However there were no differences between fresh and stored organs at this time (t=0) when reperfused under hypoxic conditions. This trend continued after 2min of reperfusion, and 2,5-DHB production remained higher in stored kidneys than fresh kidneys under normoxic conditions but not under hypoxic conditions. In hypoxic reperfusion the trend was for greater production of 2,5-DHB in stored kidney urinary spaces, but again there was a large spread of data and statistical significance was not achieved (Table 4.11).



*p<0.05 vs. fresh kidneys n=6

Fig.4.3: 2,3-DHB concentrations in vascular effluents of fresh and 48h stored kidneys reperfused for 5min under normoxic and hypoxic conditions.

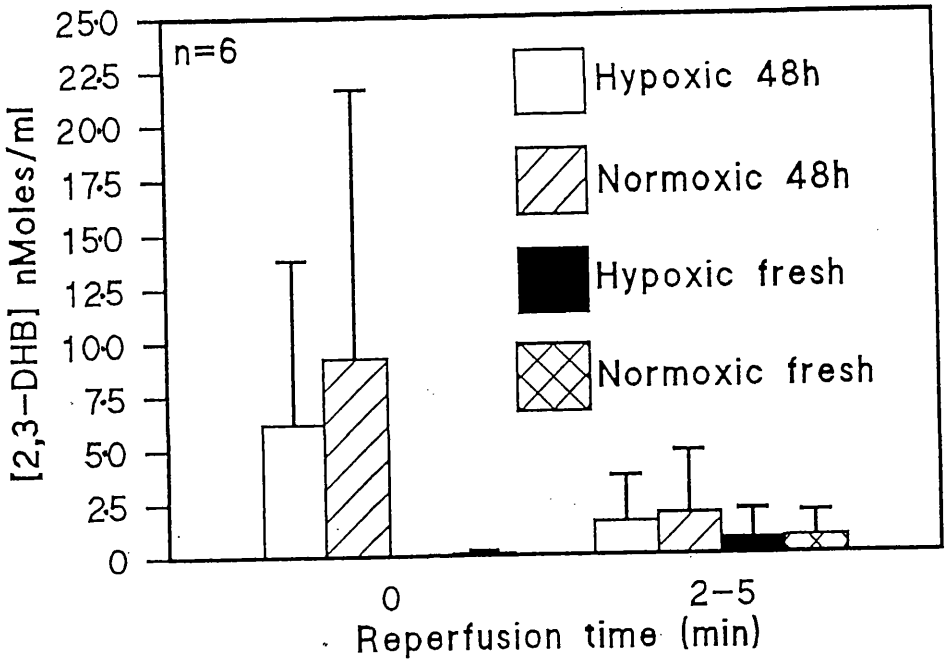


Fig.4.4: 2,3-DHB concentrations in urinary effluents of fresh and 48h stored kidneys reperfused for 5min under normoxic and hypoxic conditions.

Release of LDH into vascular and urinary spaces

Perfusate LDH activity (units/l)				
Time (min)	Fresh		48h stored	
	Normoxic	Hypoxic	Normoxic	Hypoxic
0	63.15 ±37.40	42.65 ±9.67	82.83 ±48.55	77.91 ±24.39
	t=1.25, p<0.30		t=0.29, p<0.80	
2	9.02 ±2.00	11.48 ±4.01	12.79 ±5.60	145.66 ±80.30
	t=1.16, p<0.30		t=3.63, p<0.01 **	
5	6.56 ±2.54	168.13 ±76.51	14.76 ±10.78	163.21 ±64.04
	t=5.07, p<0.001 ***		t=5.20, p<0.001 ***	

Table 4.12: Release of lactate dehydrogenase into the vascular space of fresh and 48h stored kidneys during normoxic and hypoxic reperfusion. Values are means±SD, n=6.

There were no significant differences in the release of LDH into the vascular effluent during normoxic or hypoxic reperfusion of fresh kidneys at the onset of reperfusion and at 2min into the procedure. Samples of vascular effluent collected at 5min from the onset of hypoxic reperfusion did however have increased LDH activity ($p<0.001$) when compared

to samples taken at this time from normoxic fresh kidneys. Kidneys which were stored for 48h prior to hypoxic reperfusion showed increased LDH release into the vasculature at 2min ($p<0.01$) and at 5min ($p<0.001$) from the onset of reperfusion when compared with normoxic organs. There were no significant differences at time=0min, the onset of reperfusion (Table 4.12).

Urine LDH activity (units/l)				
Time (min)	Fresh		48h stored	
	Normoxic	Hypoxic	Normoxic	Hypoxic
0	264.91 ±185.73	263.27 ±86.73	704.67 ±388.43	583.14 246.32
	t=0.02, p<0.90		t=1.14, p<0.30	
2	4.92 ±3.47	14.76 ±3.47	65.20 ±55.29	167.31 ±97.43
	t=3.16, p<0.05 *		t=1.72, p<0.20	
5	6.15 ±2.46	49.20 ±54.02	23.37 ±23.89	142.70 ±92.19
	t=1.57, p<0.20		t=2.55, p<0.10	

Table 4.13: Release of lactate dehydrogenase into the urine of fresh and 48h stored kidneys during normoxic and hypoxic reperfusion. Values are means±SD, n=6.

There was a significantly higher release of LDH ($p<0.05$) into the urinary spaces of hypoxic fresh kidney compared with normoxic fresh kidneys at 2min from the onset of reperfusion. There were no oxygen-dependent differences in the release of LDH into the urine at any other time during reperfusion of either fresh or 48h stored organs (Table 4.13).

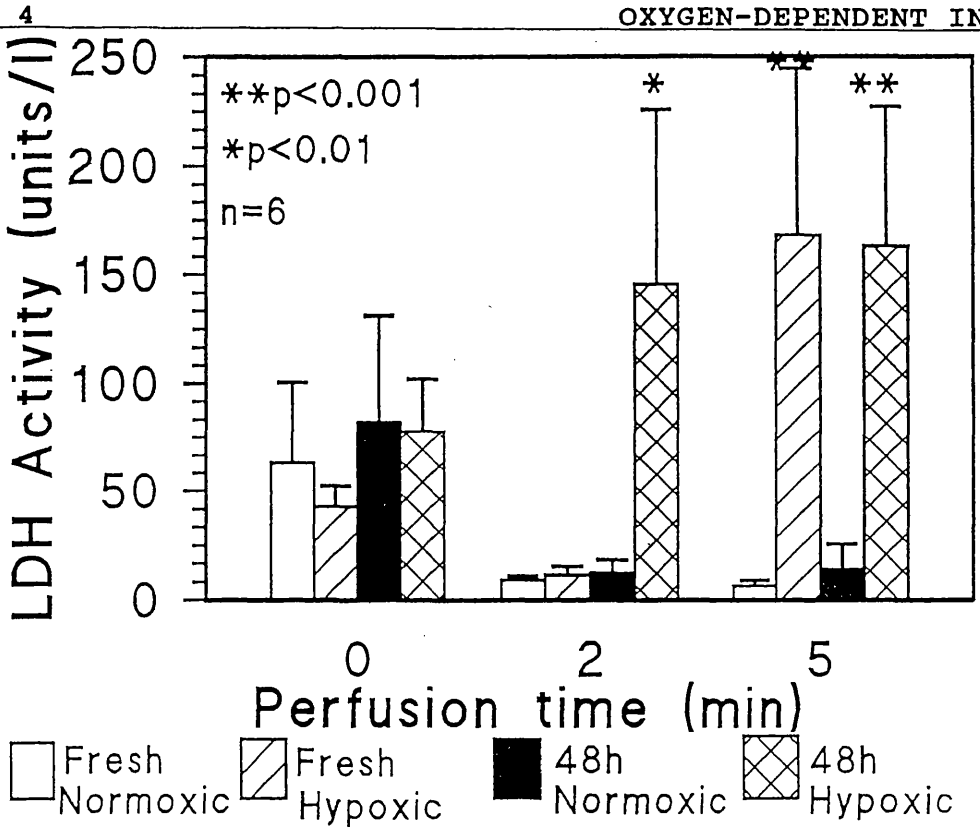


Fig.4.5: LDH activity in vascular effluents from fresh and 48h preserved kidneys reperfused under normoxic and hypoxic conditions.

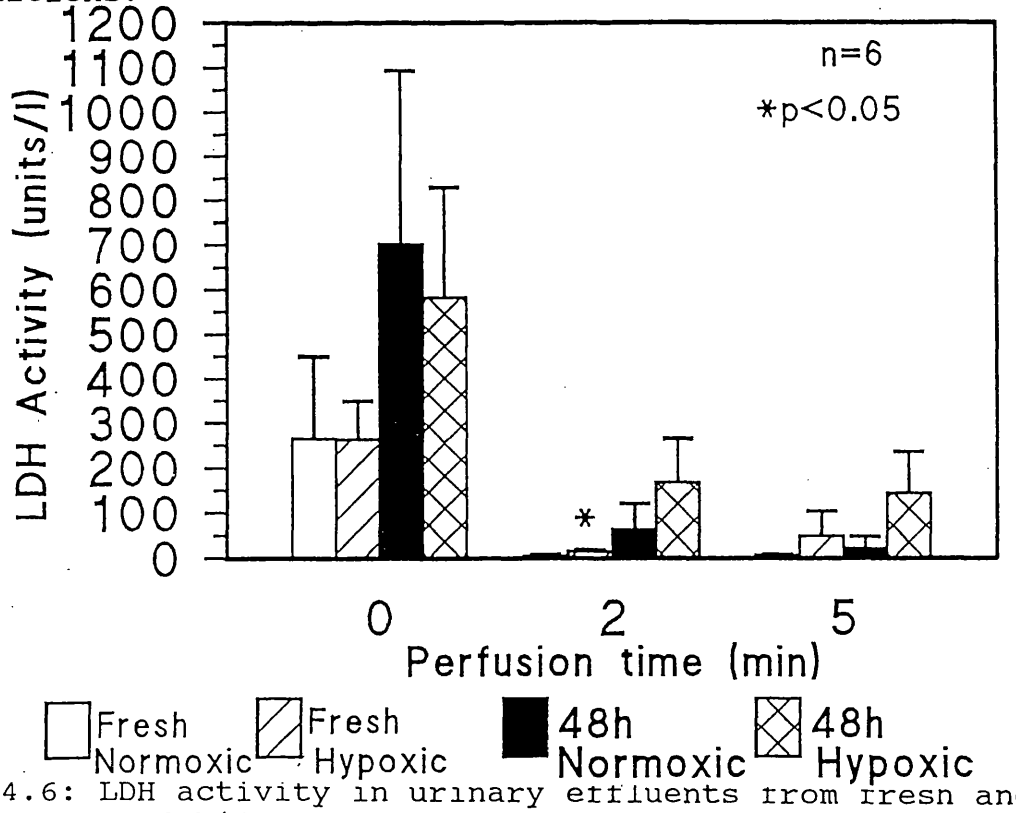


Fig.4.6: LDH activity in urinary effluents from fresh and 48h preserved kidneys reperfused under normoxic and hypoxic conditions.

Release of τ-GT into urinary spaces

Urine τ-GT activity (units/l)				
Time (min)	Fresh		48h stored	
	Normoxic	Hypoxic	Normoxic	Hypoxic
0	59.63 ±68.62	43.23 ±12.29	1472.97 ±1008.78	1391.10 ±731.28
	t=0.62, p<0.60		t=0.49, p<0.70	
2	23.54 ±23.25	24.89 ±11.52	175.78 ±124.84	246.65 ±99.18
	t=0.18, p<0.90		t=1.48, p<0.20	
5	10.42 ±7.75	120.04 ±177.78	91.48 ±39.16	345.54 ±344.22
	t=1.52, p<0.20		t=1.63, p<0.20	

Table 4.14: Release of τ-glutamyl transpeptidase into the urine of fresh and 48h stored kidneys during normoxic and hypoxic reperfusion.

There were no oxygen-dependent differences in the release of τ-GT into the urine of either fresh or 48h stored kidneys during 5min of normothermic, *ex vivo* reperfusion. However τ-GT was much higher in stored kidneys than fresh organs.

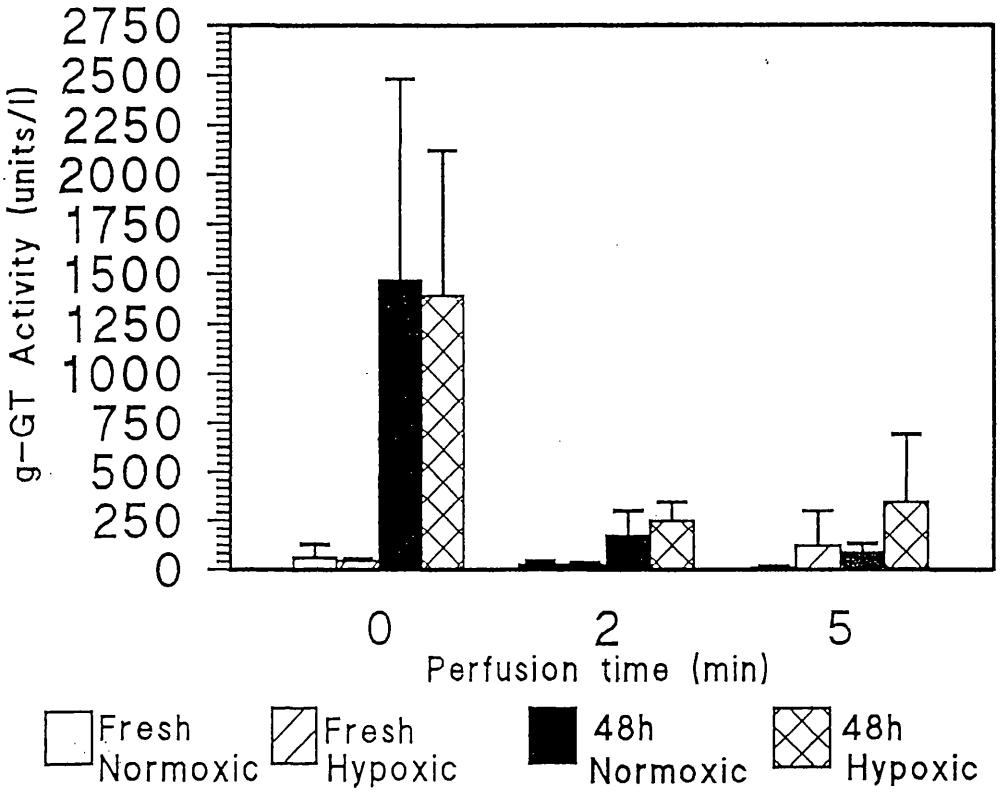


Fig.4.7: τ-GT activity in urinary effluents from fresh and 48h preserved kidneys reperfused under normoxic and hypoxic conditions.

HISTOLOGY

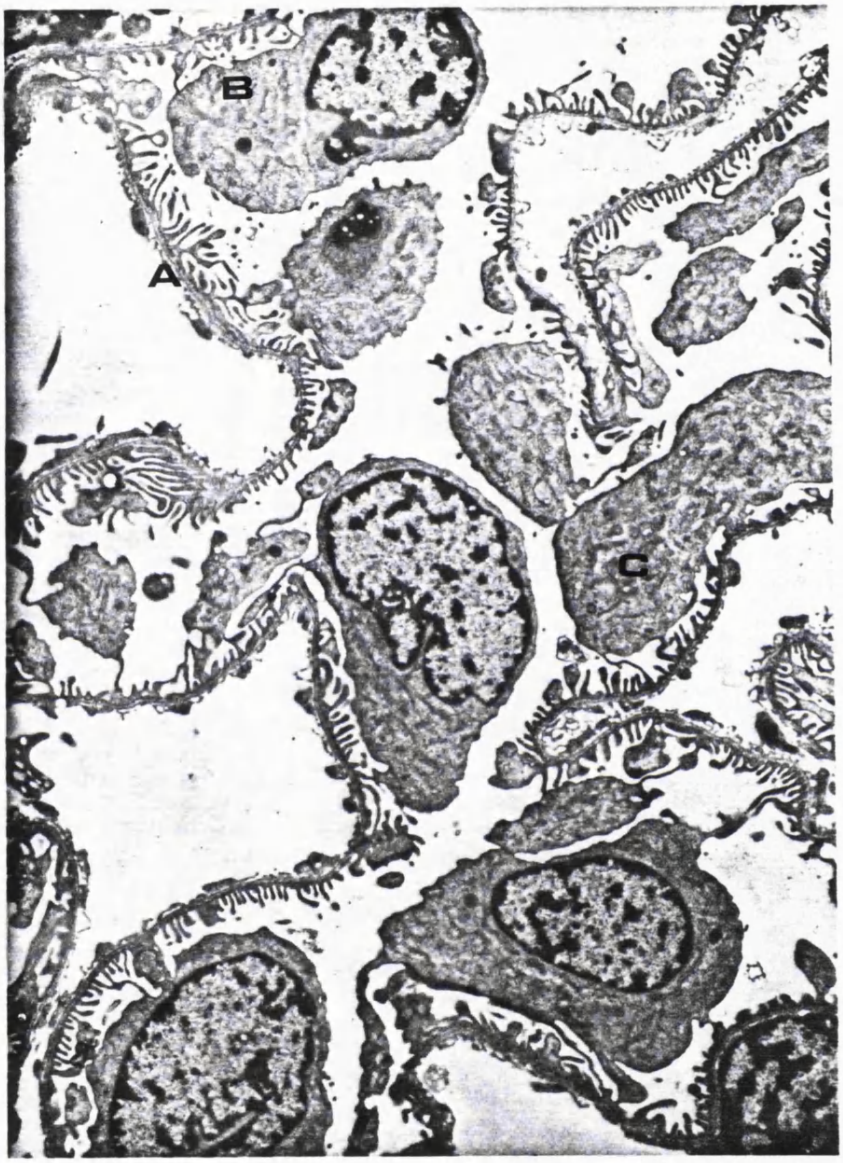


Plate 4.1 : Glomerulus from fresh kidney reperfused normoxically for 5min. Magnification = x5,000

The glomerular structure of this group of kidneys was essentially intact. The vascular endothelium appeared to be undamaged, as did the basal laminae (**A**). The structures of the podocytes (**B**) and mesangial cells (**C**) were well maintained, there was no evidence of cell swelling, nuclear swelling, or loss of nuclear material.

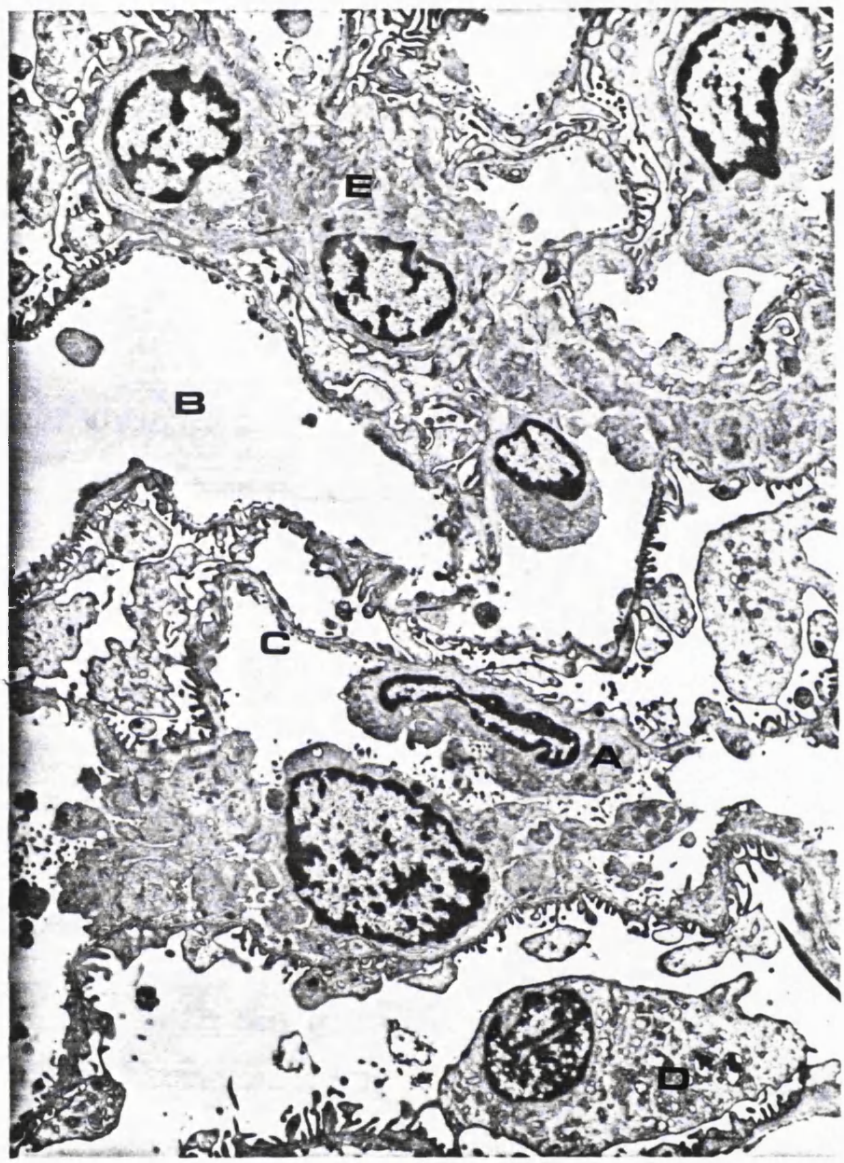


Plate 4.2 : Glomerulus from 48h preserved kidney reperfused normoxically for 5min. Magnification = x5,000

In contrast to fresh normoxic kidneys, stored organs showed damage to vascular endothelial cells (**A**), with some sloughing-off and loss of cell debris into urinary spaces (**B**). The basal lamina appeared to be generally undamaged (**C**). There was vacuolation of some podocytes (**D**), but on the whole the structure of these cells, and that of the mesangial cells (**E**), was well maintained. There was no evidence of nuclear damage in these cells.

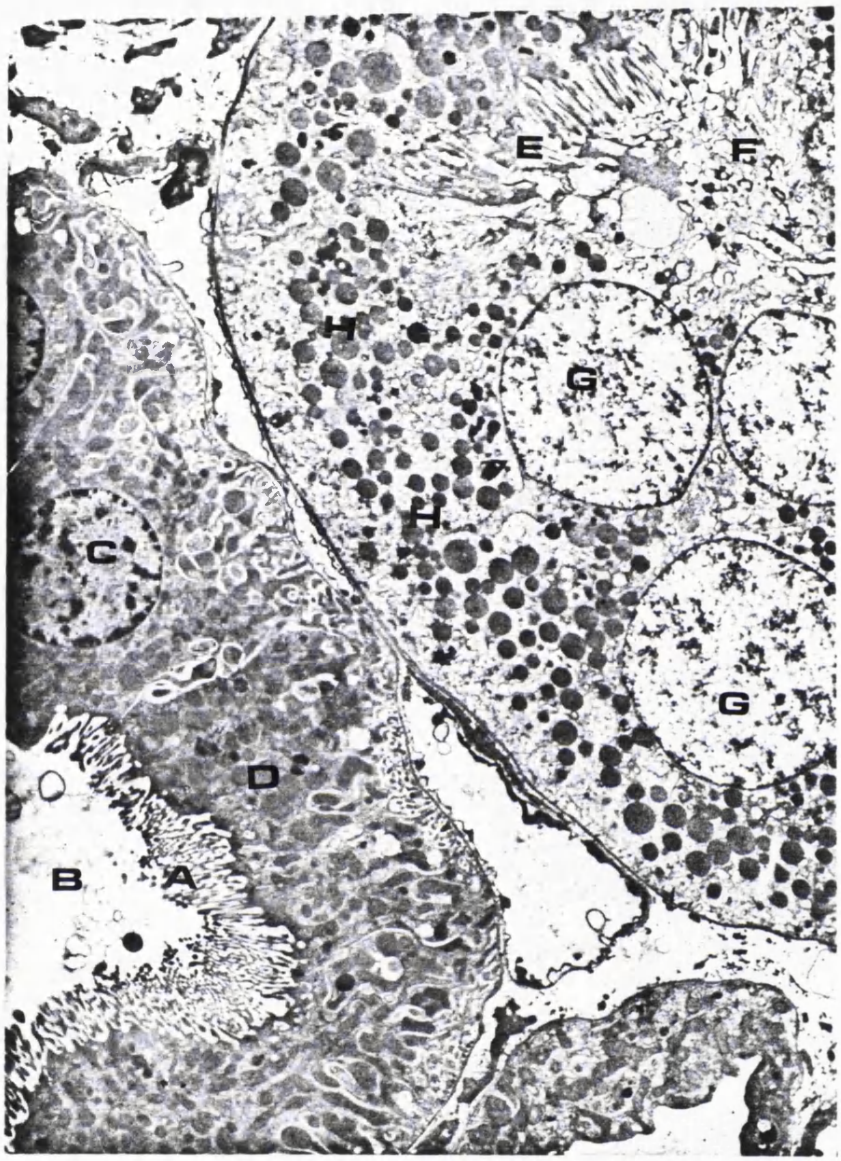


Plate 4.3 : Proximal tubules from kidneys stored for 48h and then reperfused normoxically for 5min. Magnification = x4,000

This electronmicrograph shows the frequently seen occurrence in which tubules in the same kidney, in this case adjacent proximal tubules, had sustained greatly contrasting degrees of injury. The tubule on the left showed an intact brush border (**A**) surrounding a relatively clear lumen (**B**). There did not appear to be any damage to cell nuclei (**C**)and gross mitochondrial structure (**D**) seemed to be intact. The tubule on the right, however, had extensive blebbing of the brush border microvilli (**E**) with loss of cellular material into the lumen (**F**) causing its complete occlusion. The nuclei were swollen (**G**), there was loss of nuclear material and the mitochondria were swollen (**H**).

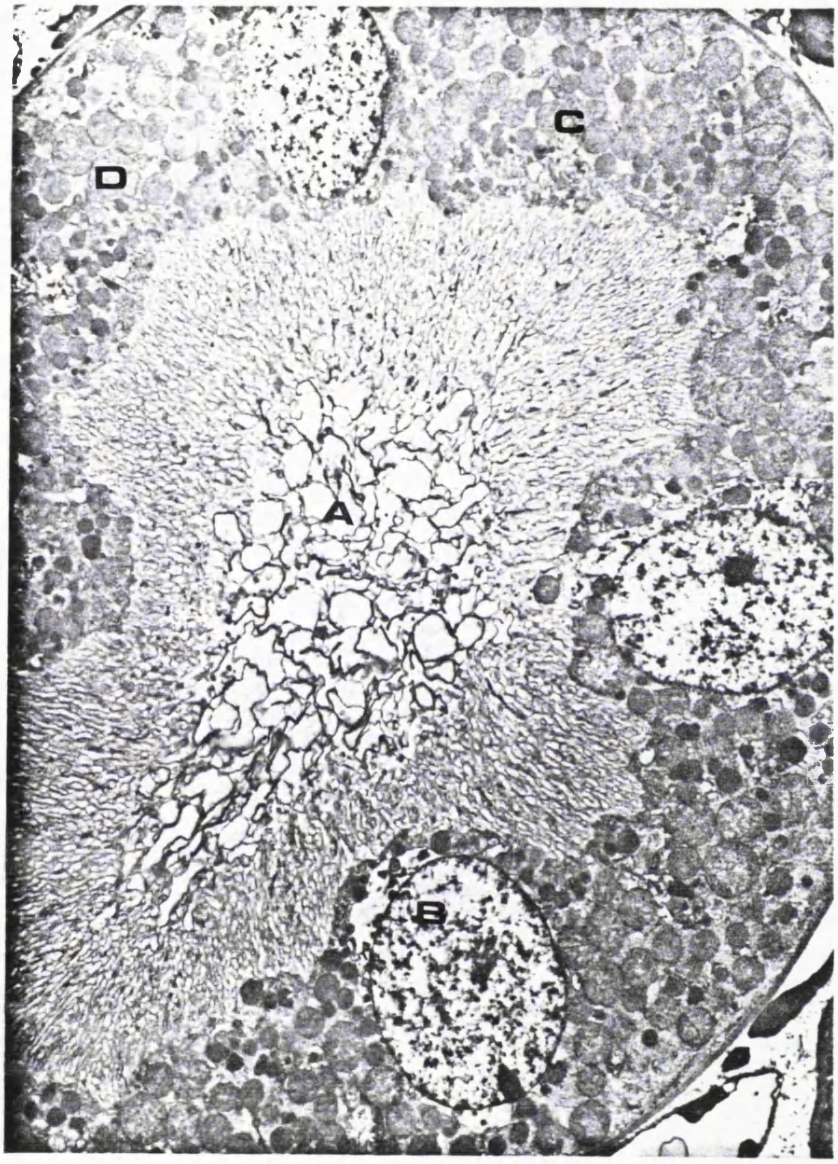


Plate 4.4 : Proximal tubule from kidney stored for 48h and then hypoxically reperfused for 5min. Magnification = x4,000

This kidney showed severe blebbing (**A**) in approximately 50% of the proximal tubules studied. In this section the nuclei (**B**) and mitochondria (**C**) were swollen and there was loss of mitochondrial cristae structure (**D**). This was probably hypoxic injury. However, in other parts of the same section (not shown) some tubules were completely intact, and the mitochondrial structure of distal tubules was well maintained.

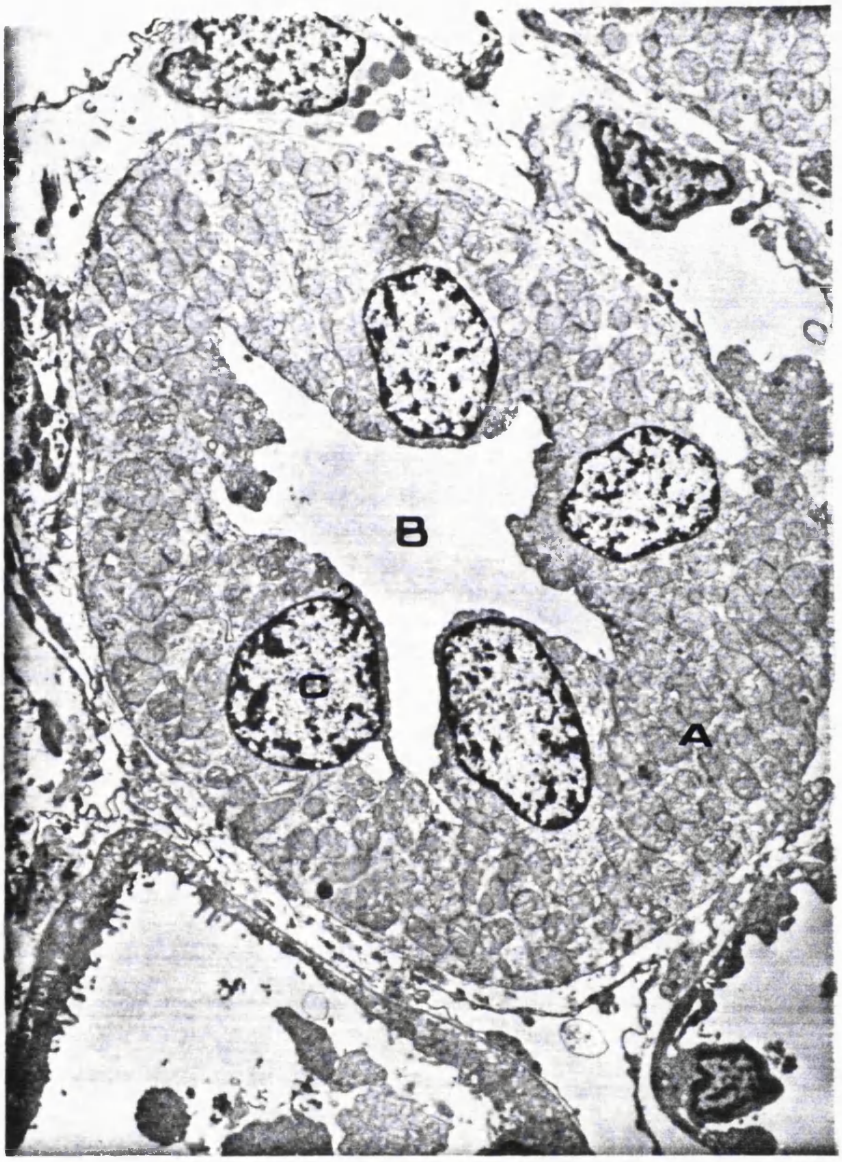


Plate 4.5 : Thick ascending tubule from kidney stored for 48h and then reperfused hypoxically. Magnification = x4,000

The mitochondrial structure appeared to be well maintained (**A**), there was no debris in the tubule lumen (**B**), and no loss of nuclear material (**C**).

DISCUSSION

Evaluation of hypoxic reperfusion as a model for the investigation of oxygen-dependent damage

The use of the isolated perfused kidney as a model in this series of experiments had many advantages over *in vivo* models. It was possible to perfuse kidneys with 1mM salicylate at a defined rate, free of the fluctuations in concentration that may arise over time due to *en route* metabolism of salicylate by other tissues *in vivo*. This allowed a high degree of confidence in the reproducibility of this aspect of the study. In addition, salicylate binds to circulating proteins such as albumin and therefore inaccuracies in the estimated distribution of the free compound in blood and tissues may arise *in vivo* due to fluctuations of the plasma protein content. The use of a protein-free reperfusion buffer in the isolated kidney should have largely avoided this source of error. Although superoxide and hydrogen peroxide have been shown to be produced in cultured kidney tubular epithelial cells (Rovin *et al.*, 1990), there is little information about free radical activity in the urinary spaces of the kidney. Due to the small size of the salicylate molecule it would be expected to be freely filtered across the glomeruli, and therefore may be a useful probe for radical activity in these compartments.

The *ex vivo* isolated perfused kidney model was used to

provide a method by which oxygen-mediated injury could be assessed without the use of exogenous antioxidants. The interpretation of results from experiments using antioxidants has a number of drawbacks including: a)the choice and dose of antioxidant used; b)elucidation of the precise site of antioxidant action including whether this is intra- or extracellular; c)the possibility of unwanted side reactions (whether beneficial or deleterious); and d)the need to understand the mechanism of action of the antioxidant (i.e. does it prevent radical formation, scavenge radicals or repair radical damage?). For instance, although ascorbate (vitamin C) is believed by some researchers to be the most important antioxidant in human plasma (Frei *et al.*, 1986), it also demonstrates paradoxical pro-oxidant characteristics under certain circumstances (Halliwell, 1990).

The model allowed hypoxic reperfusion of kidneys under defined conditions which could not be achieved *in vivo*. This method is probably only useful for appraisal of immediate organ function (up to 2-3min) upon reperfusion, since tissue enzyme studies and histology suggest that hypoxic injury after longer periods is likely to obscure the criteria used for assessment. The method is limited by glycolysis being the only mechanism by which ATP can be generated anaerobically, producing less ATP than oxidative phosphorylation.

Perfusate / urine flow rates

The increased urine flow rate measured at 5min of hypoxic reperfusion of fresh kidneys compared with normoxically reperfused organs may have been due to decreased sodium reabsorption in the absence of normal oxidative metabolism. This would have caused increased sodium delivery to the loop of Henlé and distal tubules thereby promoting the retention of water in these spaces, resulting in increased urine flow rates. This effect may not have been evident in 48h-stored kidneys due to the storage-dependent suppression of sodium reabsorption (see Chapter 3) in both normoxic and hypoxic organs. The increased rate of urine production may also have been due to the fact that perfusate flow through the kidney was greater at this time, and therefore it is likely that the amount of fluid filtered was greater than in normoxic organs. A similar effect may not have been noticed in the earlier sample measurements (0min and 2min) due to the higher flow rates at these times, which were possibly caused by naturesis as a result of a lack of energy substrates to drive sodium reabsorption during early reperfusion. The lack of differences between urine production after 48h storage in normoxic and hypoxic kidneys may indicate that the effects of cold ischaemia alone were more significant in causing injury than the amount of oxygen in the reperfusion solution; even very low concentrations of oxygen are capable of causing oxidative damage to stored organs.

The observation that perfusion flow rate was higher (vascular resistance was lower) at t=0min in normoxically reperfused stored kidneys than those which were reperfused hypoxically suggests that vascular tone at the onset of reperfusion is controlled by oxygen-dependent mechanisms. It is likely that active processes involved in the control of vascular smooth muscle, such as calcium extrusion and the synthesis of endogenous vasorelaxants, would have occurred more rapidly under normoxic conditions than in the absence of oxygen. The fact that this difference was not seen at any other time during reperfusion (2min or 5min) may indicate that even under hypoxia, kidneys were able to resume "normal" vascular tone, although this was achieved at a slower rate than occurred in normoxia. This would explain the similar flow rates between the two groups of kidneys at 2min and 5min of reperfusion. This similarity of vascular tone may, however, be achieved by different mechanisms in normoxic kidneys than in hypoxic kidneys. In normoxic organs the higher mean flow rate through the vasculature after 5min of reperfusion compared with the onset of reperfusion may be due to the resumption of physiological mechanisms for the control of vascular tone. In hypoxic organs this relatively lower vascular resistance may have been due to the effects of inadequate energy production for normal vascular smooth muscle function.

It was seen that the perfusion flow rate through the vasculature of hypoxically reperfused fresh kidneys was

greater than the flow rate through normoxic organs following 5min of reperfusion but at no other time. As described above, it is possible that 5min of hypoxic reperfusion caused depletion of substrates needed for active smooth muscle contraction thereby leading to vasorelaxation, and that anaerobic metabolism was not sufficient to support this function.

Some of the differences in vascular tone noticed in this study may have resulted from the effects of free radical production upon the activity of endogenous vasoactive compounds. It is known for instance that superoxide radicals react with endothelium-derived relaxant factor (EDRF) which is itself a radical (NO•), producing peroxynitrite (Saren *et al.*, 1989) which does not cause vasorelaxation. This may lead to vasospasm (Laurindo *et al.*, 1991) thereby decreasing flow through the renal vasculature. If superoxide radicals were produced in the fresh kidneys at t=5min during normoxic reperfusion, these may have interfered with normal EDRF activity thereby causing the decreased flow which was measured. The storage of organs for 48h however may have disturbed EDRF production to such an extent that its interaction with superoxide radicals was no longer detectable. It has also been shown that the renal vasoconstrictor effects of endothelin are potentiated by acetylsalicylic acid (Miura *et al.*, 1989), the precursor of salicylate. The presence of salicylate in the reperfusion buffer may therefore have interfered with normal control of

vascular tone.

Oxygen-dependent hydroxyl radical production

Some hydroxyl radical production in control fresh kidneys was to be expected since such production occurs *in vivo* within cells under normal physiological conditions (Halliwell and Gutteridge, 1985; Fridovich, 1989; Feix and Kalyanaramen, 1991). There were no differences between 2,3-DHB production in the renal vasculature during hypoxic and normoxic reperfusion, and the production of 2,5-DHB differed only at t=2min. This suggests that hydroxyl radical production was not affected by the oxygen concentration in the reperfusion buffer of fresh kidneys. However, although 2,3-DHB is the adduct which is believed to be produced solely by •OH reacting with salicylate, 2,5-DHB is also produced by this reaction. Studies by Onodera and Ashraf, (1991) showed that 30min of warm ischaemia in the rat heart containing 1mM salicylate in the vascular spaces did not cause production of 2,5-DHB until reperfusion was initiated. Upon reperfusion the production of 2,5-DHB was found to be greatest during the first 5min, and this could be inhibited by the presence of mannitol, a hydroxyl radical scavenger. However 2,3-DHB production was almost undetectable in their study. Therefore the production of 2,5-DHB should also be considered a useful indicator of hydroxyl radical production. In my studies the increased production of 2,5-DHB under normoxic conditions after 2min of reperfusion may

have been due to increased radical production at that time. This difference could also reflect a greater rate of normal physiological metabolism in normoxic fresh kidneys, but a decrease in mean rate of production of 2,5-DHB occurs from 0min to 5min of reperfusion. Normal physiological salicylate metabolism via enzyme-linked processes would have been expected to increase as the kidney regains physiological function during reperfusion. This suggests that some hydroxyl radical production may occur in the early stages of normoxic reperfusion of fresh kidneys which have been exposed to a minimal period of ischaemia (≈15min).

From the results in Table 4.5 there appears to be no evidence that normoxic reperfusion increased •OH production in the vasculature of kidneys compared with hypoxic reperfusion after 48h storage. This was also found to be the case when comparing the urinary spaces of fresh (Table 4.6) and 48h stored (Table 4.7) kidneys. There is therefore little evidence from this study to support the hypothesis that hydroxyl radical production during normothermic reperfusion occurred via a mechanism mediated by the concentration of oxygen in the reperfusion buffer. It is possible that 5min of reperfusion was not sufficient to produce differences in radical production between the groups of kidneys. However, this time limit was chosen in order to minimise hypoxic injury to the kidneys so that histological studies would not be compromised. It is possible that the small quantity of oxygen in the hypoxic reperfusion buffer

was sufficient to cause as much free radical production as the higher oxygen concentration in the normoxic solution.

It must also be remembered that by the use of this model fresh kidneys were subjected to short periods of warm and cold ischaemia whilst they were being harvested and cannulated. One of the questions that must be addressed is whether increased radical activity as a result of this short period of ischaemia actually reached a rate of production which was pathological. This will depend on the site of radical production and the effectiveness of defence mechanisms. If the antioxidant mechanisms which inhibit radical production were exceeded it would remain for scavenger defence mechanisms to prevent damage to cell components by radicals. Both such defences would be expected to operate with decreased effectiveness following ischaemic organ preservation due to loss through cell membranes with increased permeability, decreased synthesis, and diminished uptake of these compounds from the blood.

Preservation time-dependent radical production

A marked hydroxyl radical production, as shown by the formation of the 2,3-DHB adduct in the vascular spaces, was evident at the onset of reperfusion in kidneys which had been preserved for 48h and then hypoxically reperfused compared with similarly treated fresh kidneys. This suggested that the small quantity of oxygen present in the reperfusion buffer (<1.0%) was sufficient to allow radical

production. It is also possible that radicals were derived from trace amounts of oxygen present in the ischaemic kidney (from atmospheric oxygen which had equilibrated with the organs during preservation). Such hydroxyl radical production has been suggested to occur in rat kidney slices reoxygenated *in vitro* (Steiner and Babbs, 1990) and low oxygen concentrations have been shown to cause tissue damage in postischaemic tissues (Salaris and Babbs, 1989). Normoxically reperfused kidneys did not show a storage time-dependent increase in 2,3-DHB production at the onset of reperfusion, but did show a significant difference at t=2min, suggesting increased radical production in the vasculature of stored kidneys at this time.

The production of 2,5-DHB in the renal vasculature was seen to be significantly higher in stored organs at all times during reperfusion under normoxic conditions. Since this increased production is not likely to have been caused by increased metabolism via the cytochrome P_{450} mechanism in organs whose physiological functions are known to be depressed by storage, it is likely that this represents increased hydroxyl radical production. A significantly higher production of 2,5-DHB was also shown in hypoxic stored kidneys at the onset of reperfusion and after 2min, but was no longer significant after 5min. This decrease in hydroxyl radical production as hypoxic reperfusion progressed may indicate that a significant proportion of the radicals produced under hypoxic conditions were derived from

oxygen which had equilibrated with the kidneys during storage, as mentioned previously. As hypoxic reperfusion progressed this oxygen would have been utilized for normal metabolism and for free radical production until there was no longer sufficient oxygen to produce significant quantities of radicals. This hypothesis can be summarized as follows; there may have been more oxygen present in kidneys at the begining of hypoxic reperfusion than after 5min, and that stored kidneys are more susceptible to radical production from this oxygen than are fresh organs. The above results also raise the question that if the elevated production of 2,5-DHB detected in the vascular effluent of 48h stored organs is due to increased hydroxyl radical production and not due to cytochrome P_{450}-mediated metabolism, why is a corresponding increase in the production of 2,3-DHB not seen consistently? This phenomenon may reflect differing stabilities of the salicylate adducts in the kidney effluents, either during sample collection, storage or extraction for chromatography. It is unlikely to have been a problem with HPLC detection since samples were calibrated against standard solutions of 2,3-DHB and 2,5-DHB. It is not possible from the present work to determine whether radical adducts appearing in the vascular effluent were produced at intracellular sites (e.g. within endothelial cells) or within the vascular spaces (i.e. extracellular production). However, the fact that radicals were measured *immediately* upon reperfusion might suggest

that there would not have been time for significant intracellular uptake of salicylate, and therefore the radicals may have been derived from extracellular sources.

No significant differences in the production of 2,3-DHB in the urinary spaces were detected in either hypoxic or normoxic organs when fresh kidneys were compared with those which had been stored for 48h. Mean values of hydroxyl radical production tended to be higher in 48h preserved kidneys but these differences failed to reach statistical significance. This would suggest that hydroxyl radical production in the urinary compartments was not elevated by kidney preservation. However, again the production of 2,5-DHB in urinary spaces was higher in stored kidneys than fresh organs during normoxic reperfusion. Hypoxic reperfusion did not produce any significant differences. The complex nature of salicylate excretion and reabsorption in the kidney and variation in its distribution due to binding to proteins (see below) makes it difficult to identify the site of production of the adducts detected in urine. It is unclear whether radicals are produced in the epithelial cells of the urinary tubules, in the urinary spaces, or whether salicylate adducts enter this compartment after production at some other site (i.e the vascular spaces). The increased levels of 2,5-DHB in the urine of 48h stored, normoxically reperfused kidneys *did* correspond to increased levels in the vascular space. Oxygen concentrations are likely to have been higher in the vasculature than in

urinary tubules early in reperfusion, and therefore radical production may have been greatest in the vasculature, and salicylate adducts may have then filtered across the glomeruli into the urinary spaces. However, increased production of 2,5-DHB in the vasculature of hypoxically reperfused stored kidneys was not accompanied by a similar increase in the urinary spaces.

Salicylate distribution in the kidney

In blood 50-80% of salicylate binds to proteins, mainly albumin, and this is in equilibrium with the "free" salicylate pool. It may therefore bind to kidney proteins and then be released at a later time; proteinuria and protein release into the vascular space may thereby effect the amount of salicylate in the free pool. It has been shown that binding of salicylate to albumin inhibits the clearance of salicylate into the urine (Koren *et al.*, 1988). The isolated perfused kidney model has an advantage over *in vivo* studies because albumin-free perfusate can be used which should not have the same salicylate-binding capacity as blood.

Salicylate metabolites pass freely into the urine whereas free salicylate diffuses only slowly across the glomerulus under normal conditions, and is also secreted by proximal tubule cells into the tubule lumen by a carrier-mediated transport mechanism (Nickander *et al.*, 1979). The concentration of salicylate in the urine will therefore

depend to some extent upon the activity of this carrier. Another factor is that salicylate excretion is dependent upon the pH of the urine in the distal tubules. Under the acidic conditions to be expected during tissue ischaemia, salicylate remains non-ionized and may be reabsorbed into the vacular perfusate. Urine flow rate also affects salicylate reabsorption; slower flow results in greater reabsorption.

From the above information it is clear that there are a number of factors which may affect the way in which salicylate and its metabolites are distributed in the vascular and urinary spaces. It is also clear that due to reabsorptive mechanisms salicylate may come into contact with a number of tissues before emerging from the ureter, and metabolites in the vascular space may also have originated in other kidney localities. Never-the-less, salicylate adducts, particularly 2,5-DHB, were elevated upon reperfusion of stored kidneys, suggesting that radical formation during reperfusion is a real event.

Salicylate as an iron chelator

The ability of salicylate to chelate ferric (Fe^{3+}) iron (Miller *et al.*, 1990) may have been an important in these experiments. The high concentration of salicylate used (1mM) may make such chelation an inhibitory factor in the production of hydroxyl radicals in the proximity of biological molecules. Chelation of ferric iron in this way

may prevent it from being reduced to ferrous iron (Fe^{2+}) by superoxide, thereby ultimately inhibiting the formation of hydroxyl radicals:

$$\bullet O_2^- + Fe^{3+} \text{ ----------> } Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \text{ ----------> } Fe^{3+} + OH^- + \bullet OH$$

However since salicylate was only introduced at the time of reperfusion it may not have chelated intracellular iron released during the storage period. Therefore it may not be a significant factor in intracellular hydroxyl radical production at the immediate onset of reperfusion. It is also possible that the iron-salicylate complex retains the catalytic capability of iron and thereby ensures that any free radical production occurs directly in the vicinity of the salicylate molecules. This would give erroneously high estimations of hydroxyl radical activity. Under certain circumstances, iron chelation may favour hydroxyl radical production by stabilizing intermediates of the Fenton reaction (see Chapter 1). If salicylate is able to have a similar stabilizing effect on these intermediates it could be expected to promote radical production, once again giving falsely high estimations of radical production in the experimental system. For this reason the investigation of the role of iron chelation and the role of site-directed free radical generation will be important for the evaluation

of aromatic hydroxylation as a marker of hydroxyl radical production in biological sytems.

Hydroxyl radicals have also been shown to be produced by autoreduction of complexes of citrate with iron (Gutteridge, 1991). The composition of the kidney preservation solution may therefore have promoted radical generation, due to the high concentration of citrate ions present in this solution, if free iron was available to form complexes with these citrate ions.

Histology: Effects of reperfusion on kidney structure

Fresh kidneys which were examined after 5min of either normoxic or hypoxic reperfusion generally showed well maintained structural integrity. There was very little evidence of hypoxic injury in kidneys reperfused with hypoxic perfusates compared with those reperfused with normoxic perfusates. The structure of both the vasculature and the urinary tubules appeared undamaged in these groups of organs. This correlates well with functional study results which showed little evidence of differences in kidney performance during 5min of reperfusion. The structural results also concur with the results of the salicylate hydroxylation studies which showed no major differences in hydroxyl radical production between the two groups, other than increased 2,5-DHB production in the vascular spaces of normoxic kidneys after 2min of reperfusion.

Although fresh hypoxically reperfused kidneys showed significantly higher release of LDH into the vascular spaces at the end of reperfusion than did normoxic kidneys, there was little evidence of complete endothelial cell lysis. It is therefore likely that LDH leaked into the vascular spaces through membranes which were damaged sufficiently to allow passage of the enzyme, but not sufficiently for significant structural structural changes to be seen using the histological techniques in this study.

After 5min of reperfusion following 48h cold ischaemic preservation, injury to the renal vasculature was evident in both normoxic and hypoxic kidneys. Endothelial cells were sloughed off into the vascular spaces and there was evidence of cell debris in these spaces. The extent of damage varied considerably within each group, ranging from mild to severe in both normoxic and hypoxic organs, and there was no evidence of increased injury in either group. However reperfusion of these stored kidneys for 5min may have resulted in substantial hypoxic injury to the unoxygenated group of kidneys, as shown by the results of LDH release into the vascular spaces (Table 4.12), and this may have obscured differences which may have been due to oxidative damage. The glomerular structure seemed to be only moderately damaged in both normoxically and hypoxically reperfused 48h stored kidneys. There was no loss of nuclear material or evidence of mitochondrial damage in either mesangial cells or podocytes, although there was evidence of

vacuolation of some podocytes. The basal laminae were well maintained in both groups of kidneys, suggesting that the effectiveness of the the glomeruli as selectively permeable barriers would not have been greatly affected.

As plate 4.3 shows, there was a marked variation in the extent of damage to urinary tubules not only between kidneys within each of the groups , but also between tubules in the same kidney. Ultimately, the viability of kidneys will be determined by the ratio of irreparably damaged structures to those which can be repaired or are undamaged. It is unclear why adjacent tubules, as in Plate 4.3, should suffer such varying degrees of damage. This could have been caused by the uneven distribution of the protective preservation solution during initial flushing and preseravtion, or differences in the diffusion of oxygen through kidney tissues during reperfusion. ATP depletion has been shown to induce changes in cell morphology manifested as blebbing, in addition to changes to cytoskeletal protein structure, ultimately resulting in cell death (Gabai *et al.*, 1992).

It was not possible to determine precisely how much structural damage was due solely to oxidative reperfusion. However, histological examination by electron microscopy was able to demonstrate that reperfusion, whether normoxic or hypoxic, after 48h of cold ischaemic preservation caused noticeably more structural damage to the renal vasculature and urinary tubules than did reperfusion of fresh kidneys.

SUMMARY

The method of hypoxic reperfusion used in the studies described in this chapter was shown to produce oxygen concentrations which were approximately 1% of control normoxic values at the tip of the renal artery cannula. Both fresh and 48h stored kidneys were shown to produce metabolites of salicylate in urinary and vascular spaces, during both hypoxic and normoxic reperfusion, by mechanisms which are believed to be dependent upon the reaction of salicylate with hydroxyl radicals. Hydroxyl radical production appeared to be greatest at the onset of reperfusion in both vascular and urinary spaces. Although the mean rates of hydroxyl radical production tended to be higher in normoxically reperfused kidneys, in most cases there were no significant differences between hypoxic and normoxic reperfusion. The effects of cold storage for 48h were more noticable. Hydroxyl radical production in vascular and urinary spaces was greater in organs which had been stored than in fresh organs, particularly when both kidneys were reperfused normoxically. The results in this chapter therefore suggest that reperfusion induced hydroxyl radical production is dependent upon the length of the preceding period of cold ischaemia and may also be dependent upon the concentration of oxygen delivered to the kidneys in the perfusate.

NORMOXIC AND HYPOXIC REPERFUSION OF STORED KIDNEYS WITH SALICYLATE-FREE PERFUSATE

INTRODUCTION

This group of experiments was designed to determine whether similar effects upon renal biochemistry as those seen in Chapter 4 would be seen following reperfusion in the presence or absence of salicylate. To determine this, groups of fresh and 48h stored kidneys were subjected to either normoxic or hypoxic reperfusion; one kidney of each pair received one or the other treatment. Since salicylate molecules were shown to react with hyroxyl radicals, thereby preventing the radicals from reacting with cell constituents, it was possible that the scavenging activity of salicylate traps reduced injury to the kidney. In this way, it has been shown that salicylate reduces creatine kinase release and decreases the incidence of tachycardia and fibrillation in isolated reperfused rat hearts (Liu *et al.*, 1992). The levels of TBA-reactive material (lipid peroxidation products) were also decreased in these studies, suggesting that salicylate does indeed decrease free radical-mediated tissue injury. Studies of enzyme release into vascular and urinary spaces were therefore performed to investigate whether oxygenated reperfusion caused greater tissue injury than hypoxic

reperfusion. Urine and perfusate flow rates and tissue malondialdehyde concentrations were also measured.

It is also possible that salicylate, a compound which is known to be nephrotoxic when administered chronically (Black, 1986), may have adverse effects on renal biochemistry which may obscure functional assessment. Hence the above biochemical and physiological analyses were also used to assess possible salicylate toxicity.

METHODS

The methods used in this study were identical to those used in the previous study (Chapter 4) with the exception that a salicylate-free reperfusion solution was used.

RESULTS

Table 5.1: Tissue malondialdehyde (MDA) concentration in fresh kidneys and kidneys stored for 48h followed by 5min of reperfusion under either normoxic or hypoxic conditions (n=6, mean±SD μMoles/g protein).

	NORMOXIC	HYPOXIC
FRESH	1.31±0.34	1.38±0.44
48h PRESERVED	1.38±0.15	1.32±0.46

There was no significant difference between MDA concentration in hypoxically reperfused vs. control normoxically reperfused kidneys in either fresh ($p<0.70$) or 48h preserved ($p<0.90$)

organs. In addition there were no significant differences between fresh and stored kidneys in any treatment group. ($p<0.90$ for hypoxic fresh vs. hypoxic stored and $p<0.90$ for normoxic fresh vs. normoxic stored).

LDH release

Vascular effluent LDH activity (units/l)				
Time (min)	Fresh		48h	
	Normoxic	Hypoxic	Normoxic	Hypoxic
0	36.90 ±19.86	31.16 ±12.70	82.83 ±10.96	75.45 ±12.70
	t=0.93, p<0.40		t=2.66, p<0.05 *	
2	8.20 ±5.08	9.02 ±9.55	13.94 ±3.70	113.18 ±70.14
	t=0.34, p<0.80		t=3.60, p<0.01 **	
5	4.10 ±3.70	152.55 ±106.95	18.04 ±10.63	190.28 ±49.96
	t=3.45, p<0.01 **		t=9.22, p<0.001 ***	

Table 5.2: Release of LDH into the vascular space of fresh and 48h stored kidneys during normoxic and hypoxic reperfusion. Values are means±SD, n=6.

At the onset of reperfusion in 48h stored kidneys there was a significantly higher detectable LDH activity ($p<0.05$) in

the vascular effluent of normoxically reperfused kidneys when compared to hypoxically reperfused kidneys. After 2min and 5min of reperfusion this situation was reversed, vascular LDH activity being greatest in hypoxic kidneys ($p<0.01$ at 2min and $p<0.001$ at 5min). There was no significant difference between enzyme activity in the vascular effluents of fresh kidneys during the first 2min of reperfusion, but after 5min, LDH activity was greatest in hypoxic kidneys ($p<0.05$).

Urine LDH activity (units/l)				
Time (min)	Fresh		48h	
	Normoxic	Hypoxic	Normoxic	Hypoxic
0	398.60 ±204.34	278.87 ±161.75	1204.00 ±201.91	910.38 ±201.91
	t=1.82, p<0.10		t=1.43, p<0.20	
2	42.65 ±42.75	59.87 ±45.46	293.62 ±147.27	421.56 ±309.69
	t=1.84, p<0.01		t=1.06, p<0.40	
5	14.76 ±9.84	50.85 ±36.11	84.64 ±49.38	416.64 ±241.94
	t=2.43, p<0.05 *		t=3.12, p<0.05 *	

Table 5.3: Loss of LDH into the urine in fresh kidneys and 48h stored kidneys subjected to normoxic and hypoxic reperfusion. Values are means±SD, n=6.

LDH activity in the urine showed no differences between normoxic and hypoxic kidneys during the first 2min of reperfusion in either stored or fresh kidneys. After 5min of reperfusion enzyme activity was highest ($p<0.05$) in hypoxic kidneys in both groups.

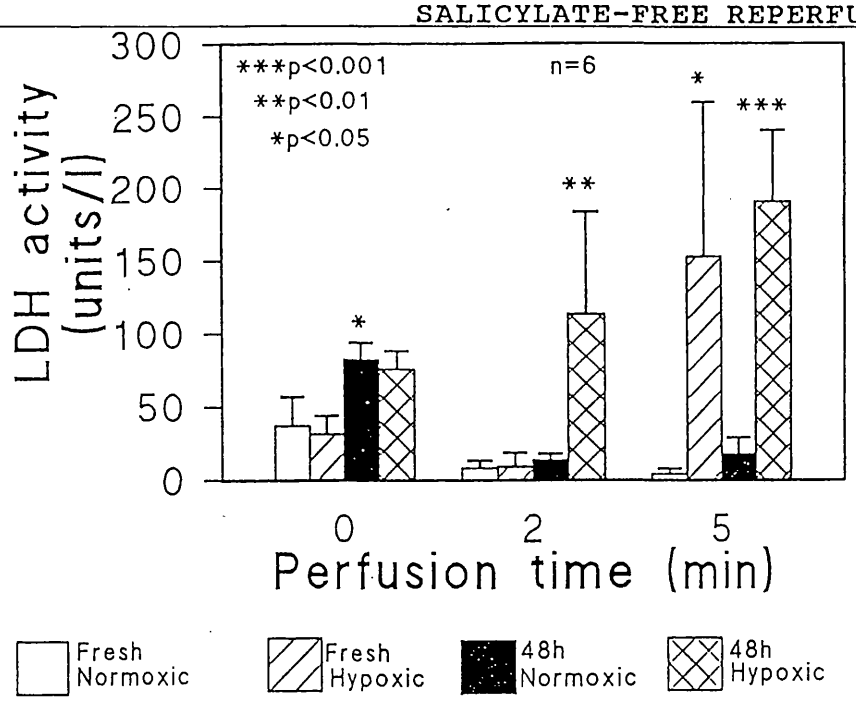


Fig.5.1: LDH activity in vascular effluents from fresh and 48h preserved kidneys reperfused normoxically and hypoxically for 5min.

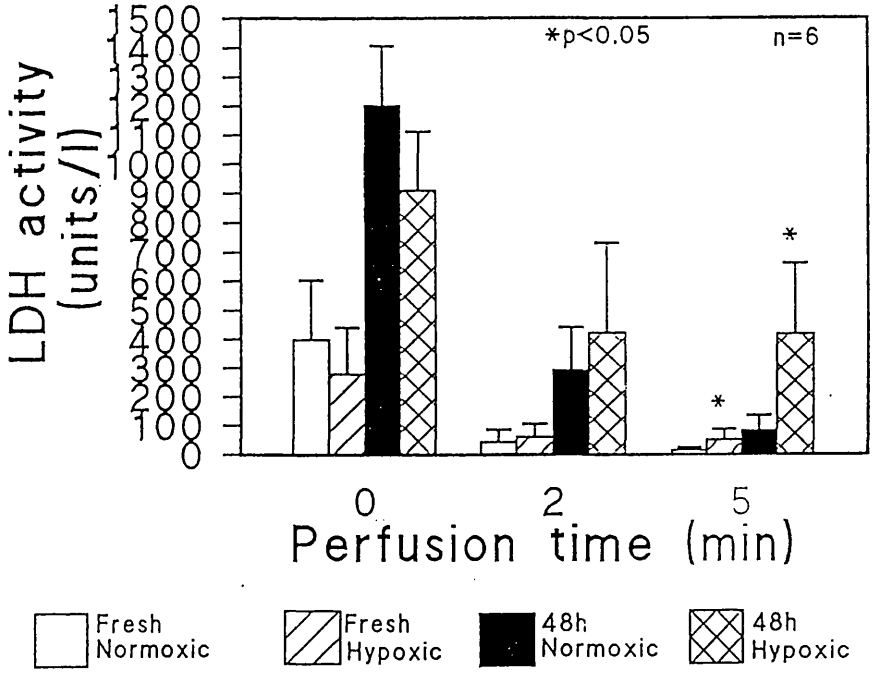


Fig.5.2: LDH activity in urinary effluents from fresh and 48h preserved kidneys reperfused normoxically and hypoxically for 5min.

Release of τ-GT into urine

Urine τ-GT activity (units/l)				
Time (min)	Fresh		48h	
	Normoxic	Hypoxic	Normoxic	Hypoxic
0	79.70 ±32.51	53.84 ±20.61	1203.12 ±703.48	1406.71 ±1177.11
	t=3.54, p<0.01 **		t=0.56, p<0.60	
2	53.26 ±45.05	41.88 ±47.83	555.84 ±408.27	390.20 ±86.35
	t=2.21, p<0.10		t=0.88, p<0.40	
5	44.97 ±44.43	31.45 ±27.66	276.99 ±120.74	878.53 ±267.14
	t=0.54, p<0.60		t=4.49, p<0.01 **	

Table 5.4: Loss of τ-GT into the urine in fresh kidneys and 48h stored kidneys subjected to normoxic reperfusion and hypoxic reperfusion. Values are means±SD, n=6.

Preserved kidneys showed no differences in urine τ-GT activity in hypoxic vs. normoxic organs during the early stages of reperfusion. However, after 5min hypoxic kidneys released greater quantities of the enzyme ($p<0.01$). In fresh kidneys normoxia produced significantly greater τ-GT release during the first two minutes of reperfusion ($p<0.01$ at t=0min, $p<0.05$

at t=2min), and at 5min of reperfusion there was no difference between release in normoxic and hypoxic kidneys.

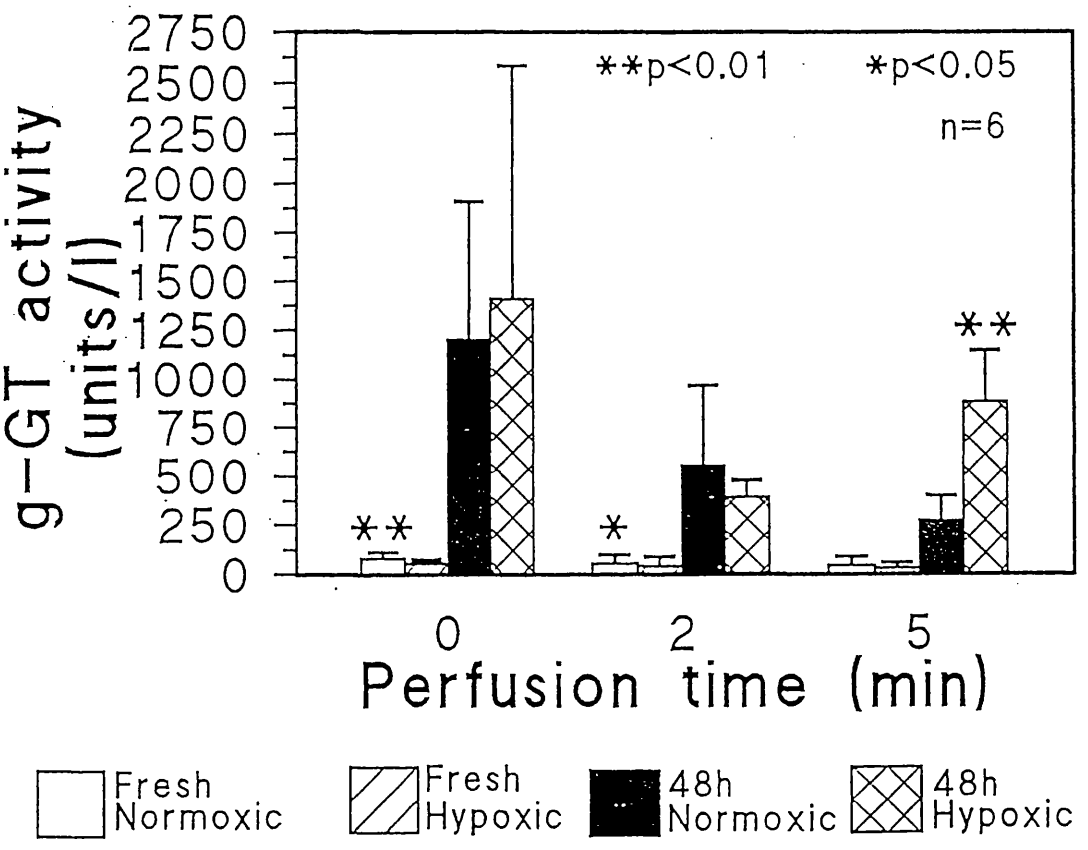


Fig.5.3: γ-GT activity in urinary effluents from fresh and 48h preserved kidneys reperfused normoxically and hypoxically for 5min.

Perfusion Time (min)	URINE FLOW RATE (ml/min)			
	Fresh		48h Preserved	
	normoxic	hypoxic	normoxic	hypoxic
0	1.6 ±0.61	1.58 ±0.59	0.50 ±0.33	0.53 ±0.43
2	0.98 ±0.63	0.95 ±0.78	0.46 ±0.32	0.19 ±0.17
5	0.98 ±0.50	1.08 ±0.94	0.36 ±0.35	0.15 ±0.13

Table 5.5: Urine flow rate in fresh and 48h preserved kidneys during normoxic and hypoxic reperfusion (mean±SD, n=6).

There were no statistically significant differences between the rate of urine production during reperfusion of normoxic and hypoxic kidneys. Neither fresh kidneys nor 48h stored organs showed differences in the rate of urine production under normoxia compared with hypoxia.

Perfusion Time (min)	PERFUSION FLOW RATE (ml/min)			
	Fresh		48h Preserved	
	normoxic	hypoxic	normoxic	hypoxic
0	65.66 ±8.04	59.00 ±7.23	68.00 ±11.04	62.16 ±9.51
2	70.66** ±8.45	56.83 ±13.06	88.16* ±17.91	73.33 ±9.43
5	72.16 ±11.63	60.33 ±15.14	90.16 ±15.44	82.66 ±15.73

Table 5.6: Perfusion flow rate (vascular resistance) in fresh and 48h preserved kidneys during normoxic and hypoxic reperfusion (mean±SD, n=6).

* $p<0.01$ vs. hypoxic kidneys

** $p<0.001$ vs. hypoxic kidneys

There were no significant differences between the perfusion flow rate in normoxic kidneys compared with hypoxic kidneys at the onset of reperfusion (t=0min) or after 5min in either fresh or 48h stored organs. However, in both fresh and stored kidneys flow was significantly higher ($p<0.01$ and $p<0.001$ respectively) under normoxic conditions when samples were taken at 2min from the onset of reperfusion.

Summary of enzyme release results from kidneys reperfused in the presence of salicylate and in its absence.

Repfn. Time (min)	Vascular space LDH release			
	Fresh + Sal	Fresh - Sal	48h + Sal	48h - Sal
0	---	---	---	Normoxia +++
2	---	---	Hypoxia +++	Hypoxia +++
5	Hypoxia +++	Hypoxia +++	Hypoxia +++	Hypoxia +++

Table 5.7: Summary of measurements of the release of LDH into vascular spaces of kidneys reperfused under normoxic or hypoxic conditions in the presence and in the absence of 1mM salicylate.

--- No significant increase

+++ Significant increase

Repfn. Time (min)	Urinary space LDH release			
	Fresh + Sal	Fresh - Sal	48h + Sal	48h - Sal
0	---	---	---	---
2	Hypoxia +++	---	---	---
5	---	Hypoxia +++	---	Hypoxia +++

Table 5.8: Summary of measurements of the release of LDH into urinary spaces of kidneys reperfused under normoxic or hypoxic conditions in the presence and in the absence of 1mM salicylate.

--- No significant increase

+++ Significant increase

Repfn. Time (min)	Urinary space γ-GT release			
	Fresh + Sal	Fresh - Sal	48h + Sal	48h - Sal
0	---	Normoxia +++	---	---
2	---	Normoxia +++	---	---
5	---	---	---	Hypoxia +++

Table 5.9: Summary of measurements of the release of γ-GT into urinary spaces of kidneys reperfused under normoxic or hypoxic conditions in the presence and in the absence of 1mM salicylate.

--- No significant increase

+++ Significant increase

DISCUSSION

The MDA analysis was performed on tissues which had been freeze-clamped at the end of reperfusion, but had not had any other treatment prior to the assay. Other studies have shown that following a further incubation period lipid peroxidation can be identified using this assay method (Gower *et al.*, 1987). Such procedures therefore represent the susceptibility of the tissue to lipid peroxidation (i.e. the tissue antioxidant status), not peroxidation during reperfusion. A second tissue incubation period following reperfusion was not used because the aim of this study was to identify any lipid peroxidation caused by reperfusion itself.

As can be seen from Table 5.1 there was no evidence of increased lipid peroxidation in kidneys reperfused with a well oxygenated perfusate compared with kidneys reperfused with a hypoxic perfusion solution. There was also no evidence of increased lipid peroxidation as a result of reperfusion after cold ischaemic preservation for 48h. This is in contrast with the results of the salicylate hydroxylation experiments which demonstrated increased hydroxyl radical activity in preserved kidneys. Such increased hydroxyl radical activity might be expected to result in increased lipid peroxidation (Gutteridge, 1984). However, the results of the salicylate hydroxylation studies do not give any information about the site of free radical production, so it is possible that the majority of radical activity occurred some distance away from the high density of lipids in cell membranes. It is also

possible that the hydroxyl radical activity detected was not sufficient to produce significant lipid peroxidation which could be detected by this assay procedure. MDA may have been formed to a greater extent in particular groups of kidneys during ischaemia or at the onset of reperfusion, but this may then have been washed out of the kidney or metabolized during later perfusion. Such loss of MDA formed during ischaemia has been shown following reperfusion of the kidney (Konya *et al.*, 1990) and of the heart (Park and Kehrer, 1991). MDA has been shown to be a substrate for peroxidase enzymes in reactions which result in the production of free radicals (Mottley *et al.*, 1991). It is also possible that the period of reperfusion (5min) was too short to produce significant lipid peroxidation; a number of studies have suggested that lipid peroxidation is a late event following free radical production (Comporti, 1987; Kappus, 1987), and therefore this phenomenon may accompany cell death rather than being one of the initiating factors. However it has also been shown that tissue acidosis, a process which increases lipid peroxidation in the rat liver, was actually *accompanied* by increased cell viability (Bronk and Gores, 1991). Therefore it is important to evaluate the significance of lipid peroxidation studies by simultaneous comparison with other indices of tissue injury.

Release of LDH into the vasculature

The significantly higher release of LDH into the vasculature of normoxically perfused stored kidneys suggests

that the presence of oxygen causes increased damage upon reperfusion after cold ischaemia. Although enzyme release in the analogous treatment group in 1mM salicylate-perfused organs was similar, it was not significantly different to the hypoxic group (+1mM salicylate). This suggests that the presence of salicylate in some way influenced the release of LDH in these organs.

Fresh kidneys seemed to be less susceptible to hypoxic reperfusion injury than 48h stored kidneys as evidenced by LDH release into the vasculature not being markedly different to normoxic kidneys over the first 2min of reperfusion. Increasing LDH release was expected during later stages of perfusion due to cell damage caused by suppression of normal oxidative metabolism, and has been shown in hypoxic perfusion of the heart (Park and Kehrer, 1991).

Release of τ-GT into urine

The observation that in fresh kidneys normoxia causes significant damage to proximal tubule brush border membranes, as demonstrated by increased τ-GT release in the first 2min of reperfusion, seems to conflict with the results of 48h preserved kidneys treated similarly. This may be due to high background levels of enzyme activity due to accumulation of τ-GT in tubules during cold ischaemic preservation. This may conceal any release which may occur as a result of oxygenated reperfusion. This apparent oxidative injury to the proximal tubule membrane may be mediated by the short period of cold

ischaemia which fresh kidneys inevitably experience (≈30min), thereby priming the kidney for reperfusion injury. The use of the "uninterrupted flow" *ex vivo* perfusion model (see Chapter 2 and Appendix A) may have abolished this phenomenon by removing the ischaemic period. The observation that this oxygen-dependent damage continued for the first 2min of reperfusion demonstrated that this injury was more pronounced than the effects of 2min of hypoxia. However after 5min the effects of hypoxia exceeded oxidative injury.

Urine flow rate

As can be seen from Table 5.5 the rate of urine production did not vary markedly between normoxic and hypoxic reperfusion in either fresh or 48h preserved kidneys. This demonstrates that normal oxidative metabolism was not required for the production of urine in the kidney in this model, although it would be expected that such metabolism is needed to control the urine composition. Active solute reabsorption in the tubules is likely to be depressed by hypoxia. Decreased sodium reabsorption would be expected to result in decreased water reabsorption, causing the production of increased volumes of dilute urine. This was not found to be the case as shown by the absence of a significant difference between hypoxic and normoxic kidneys at any time during reperfusion.

There was no evidence that oxygen-dependent damage alters the ability of the kidney to produce urine.

Perfusate flow rate

Table 5.6 shows that there were no significant differences in perfusion flow rate between normoxic or hypoxic reperfused fresh or 48h stored kidneys at either the onset of reperfusion (t=0min) or at the end (t=5min). However, measurements taken at 2min of reperfusion demonstrate that perfusion flow was significantly depressed in hypoxic fresh kidneys ($p<0.001$) and hypoxic 48h stored kidneys ($p<0.01$). This is evidence that renal vascular resistance was increased at this time since perfusion flow rate is entirely regulated by vascular resistance in this model. It appears that in hypoxic perfused kidneys there is a delay in reaching the maximum flow rate plateau, which is reached within 2min in normoxic kidneys. It is unclear why there should be a difference at this point in reperfusion, but the response is likely to be mediated either directly by changes in the metabolism of vascular smooth muscle cells or by changes in the secretion of endogenous vasoactive substances. As can be seen from the table of urine flow rate (Table 5.5), the difference in vascular resistance between hypoxic and normoxic kidneys at 2min of reperfusion did not result in a similar significant difference in urine production. This would suggest that pre-glomerular vessels, probably the afferent arterioles, are the vessels most affected by hypoxia. Increased post-glomerular vascular resistance would be expected to increase GFR resulting in increased urine production.

Histology

Transmission electron microscopic examination revealed blebbing of the proximal tubule brush border mebranes following reperfusion in a number of kidneys in each of the treatment groups (hypoxic or normoxic reperfusion). This may have been secondary to Na^+/K^+ATPase failure leading to loss of control of intracellular osmolarity (West, 1983). Excessive entry of water into cells may have forced the cell membrane away from the cytoskeleton causing the formation of blebs.

Evaluation of hypoxic reperfusion as a model for the investigation of oxygen-dependent damage

The *ex vivo* isolated perfused kidney model was used to provide a method by which oxygen mediated injury could be assessed without the use of exogenous antioxidants. The interpretation of results from experiments using antioxidants have a number of drawbacks including: a)the choice and dose of antioxidant used; b)elucidation of the precise site of antioxidant action including whether this is intra- or extracellular; c)the possibility of unwanted side reactions (whether beneficial or deleterious); and d)the need to understand the mechanism of action of the antioxidant (i.e. does it prevent radical formation, scavenge radicals or repair radical damage?). For instance although ascorbate (vitamin C) is believed by some researchers to be the most important antioxidant in human plasma (Frei *et al.*, 1986), it also demonstrates paradoxical pro-oxidant characteristics under

certain circumstances (Halliwell, 1990).

The model allowed hypoxic reperfusion of kidneys under defined conditions which could not be achieved *in vivo*. This method is probably only useful for appraisal of immediate organ function (up to 2-3min) upon reperfusion, since tissue enzyme studies and histology suggest that hypoxic injury after longer periods is likely to obscure the criteria used for assessment. The method is limited by glycolysis being the only mechanism by which ATP can be generated anaerobically, producing less ATP than oxidative phosphorylation.

SUMMARY

The studies described in this chapter were designed to determine whether there was a difference in the extent of reperfusion injury suffered by kidneys reperfused under normoxic and hypoxic conditions. The methods used in this study were similar to those used in Chapter 4, but salicylate was not added to the perfusate since this had been shown to react with hydroxyl radicals and may thereby have obscured any indication of oxygen dependent injury.

There was no evidence of increased lipid peroxidation as a result of either reperfusion using a perfusate with a high oxygen concentration, or as a result of organ preservation for 48h. Some degree of increased lipid peroxidation was expected in stored kidneys since increased hydroxyl radical production had previously been shown in these organs but the 5min reperfusion period may have been too short to cause

significant injury. The release of LDH into the renal vasculature was shown to be elevated by normoxic reperfusion, a response not seen previously in the presence of salicylate. There was also evidence of oxygen dependent injury to proximal tubule brush border membranes. There was no evidence of an oxygen dependent effect upon the rate of urine production or upon vascular resistance during reperfusion. From the results in this and the previous chapter it can be inferred that hydroxyl radical scavenging by salicylate attenuates reperfusion injury in the kidney to some degree.

COLD REFLUSH OF POSTISCHAEMIC KIDNEYS

INTRODUCTION

There has been recent interest in employing cold reflush (CRF) of stored kidneys just prior to transplantation, which may improve graft function following transplantation (Parrott *et al.*, 1990). Such CRF may be beneficial by removing: (i)debris which could otherwise obstruct vascular and urinary spaces (Bayati *et al.*, 1990); (ii)nephrotoxic substances which may cause tubular necrosis; or (iii)vasoactive substances such as prostaglandins (Lefer, 1985), endothelin (Perico *et al.*, 1992) and angiotensin (Linder *et al.*, 1982) which may accumulate in these spaces during cold ischaemia. The CRF may thereby facilitate better perfusion of the renal microvasculature following reperfusion, attenuating the "no-reflow" phenomenon and preventing areas of tissue ischaemia which result from this (Anaise *et al.*, 1987). It has been shown that cold perfusion with storage solutions washes out the products of nucleotide catabolism from tissues. This would be expected to have a detrimental effect on organ recovery following normothermic reperfusion since these products are utilized for resynthesis of ATP via salvage pathways (Stromski *et al.*, 1988). However, this has not been found to be the case (Thornton *et al.*, 1990). CRF also represents a method by which fresh supplies of antioxidants, osmotic agents, calcium antagonists, and other compounds can be supplied to the kidney

immediately prior to reperfusion, thus offering a further opportunity for therapeutic intervention to promote kidney function. The method also allows the possibility of employing a CRF solution of different composition to the organ preservation solution for specific purposes. One solution designed for such a purpose is the "Carolina Rinse" solution which contains adenosine, believed to be a key component (Gao *et al.*, 1991). The use of a second cold flush is analogous to the use of potassium-free flush solutions to remove hyperkalaemic preservation solutions from large organs such as the liver immediately prior to transplantation. This is done to prevent possible cardioplegic effects which may occur if hyperkalaemic solutions enter the systemic vasculature upon reperfusion. Plasma collected after passing through previously ischaemic rabbit kidneys has been shown to contain substances which have a negative inotropic effect on the heart (Hansson *et al.*, 1990). These substances may be removed by the CRF procedure and thereby augment normal cardiovascular function in the kidney transplant recipient.

In this series of experiments kidneys were subjected to CRF after storage for 48h using the same solution as that used for flush preservation. The aim was to allow the removal of tissue degradation products accumulated during the cold ischaemic preservation period, thereby allowing the analysis of products which were produced by storage alone or additionally after reperfusion.

A further study was performed to determine whether any

damage seen after reperfusion was caused by oxygen-dependent mechanisms.

METHODS

Rabbit kidneys were harvested and stored for 72h as previously described (see Chapter 2). The preservation time of 72h was chosen in order to determine whether CRF was beneficial to kidneys which had been shown to be significantly functionally damaged (see Chapter 3). One group (n=6) were subjected to blood-free isolated reperfusion at 37°C on the *ex vivo* circuit, whilst the second group (n=6) were perfused with 100ml of ice-cold hypertonic citrate solution (HCA) before normothermic reperfusion. Cold reflushing was performed via the renal artery using a bag (Viaflex) of HCA solution at a height of 100cm above the arterial cannula as in the method of initial kidney harvest. Serial samples of urinary and vascular effluents were collected both during CRF and during normothermic reperfusion. The standard reperfusion solution containing BSA (1g/l) was used in these experiments. Renal function tests were performed as described in Chapter 2. Indices of organ function measured were GFR (creatinine clearance), proteinuria, tubule sodium rebsorption and glucose reabsorption. In addition tissue damage was assessed by studying enzyme release from the proximal tubule brush border membrane (γ-GT) or cytosolic (LDH) compartments.

In the second study, kidney pairs were stored for 48h after which both organs were given a cold reflush as described

above. One kidney of the pair was reperfused normoxically and the other was reperfused hypoxically as described in Chapter 4. The preservation time of 48h was chosen to be consistent with the studies in Chapters 4 and 5, which suggested that free radicals were produced during reperfusion after 48h preservation, and that tissue dammage, demonstrated by increased enzyme release, also occurred at this time.

Paired and unpaired Student's t-tests were used for the statistical analysis of the data from these studies.

RESULTS

STUDY 1

Vascular resistance (perfusate flow rate)

The mean flow rate through the renal vasculature during the cold reflush procedure was 16.04ml/min, which was approximately 20% of the flow rate of normothermic reperfusion. There was no significant difference between the flow rate in CRF kidneys compared with non-CRF kidneys at the onset of normothermic reperfusion, with values in the range 50-74ml/min (see Table 6.1). Mean flow rates increased by approximately 20ml/min over the next 15min in both groups of kidneys. These rates reached a plateau in the later stages of reperfusion (15-30min). Perfusion flow rate through the renal vasculature was significantly greater in CRF kidneys ($p<0.01$) after 15min of reperfusion, demonstrating that the renal

vascular resistance was lower at this time. At the end of normothermic reperfusion there was no significant difference between flow rates in CRF and non-CRF kidneys.

Reperfusion Time (min)	**Perfusate flow (ml/min)**	
	Reflushed	Non-Reflushed
CRF	16.04±5.69	---
0	61.71±9.34	54.85±9.71
15	81.28±13.19	71.00±14.88
30	84.71±12.71	77.42±17.30

Table 6.1: Perfusion flow rate through the renal vasculature (ml/min) in kidneys which recieved CRF after 72h preservation prior to reperfusion, and control non-CRF organs. Values are means±SD, n=6.

Urine Flow Rate

The rate of urine production during the cold reflushing procedure was on average 50% of the rate of urine production during normothermic reperfusion. Mean values during the cold flush were 0.73±0.33ml/min compared with mean values in the range 1.14 to 1.78ml/min during normothermic reperfusion. There were no significant differences in the rate of urine production at 37°C in CRF kidneys compared with non-CRF kidneys.

Reperfusion Time (min)	Urine flow (ml/min)	
	Reflushed	Non-Reflushed
CRF	0.73±0.33	---
0	1.78±1.03	1.44±1.02
15	1.14±0.95	1.44±1.05
30	1.23±0.83	1.24±1.01

Table 6.2: Urine flow rate (ml/min) in kidneys which recieved CRF after 72h preservation prior to reperfusion, and control non-CRF organs. Values are means±SD, n=6.

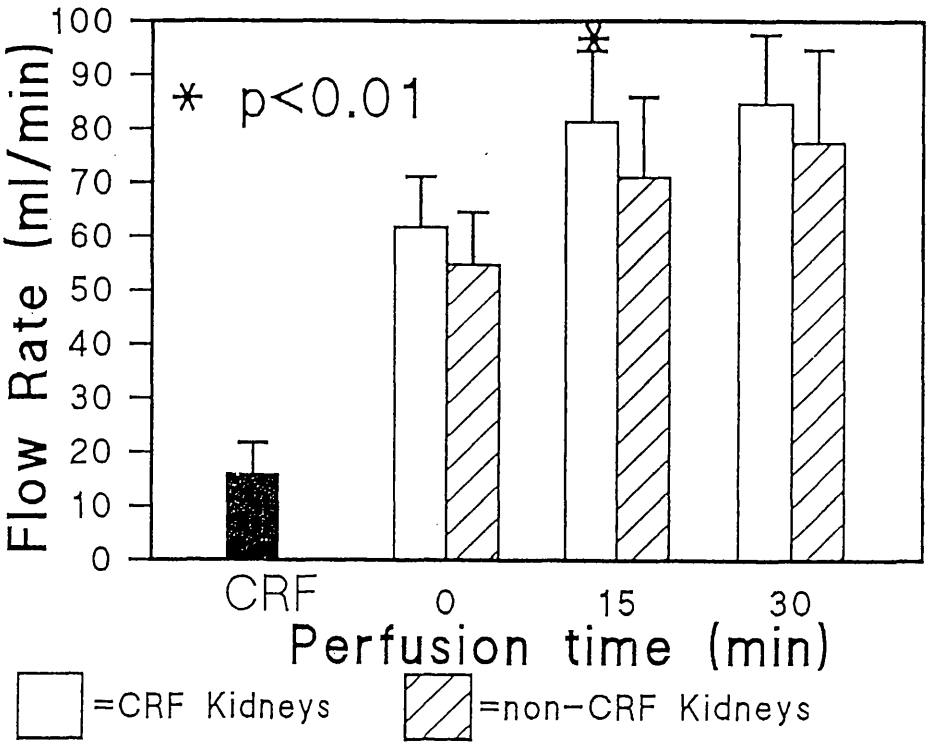


Fig. 6.1: Perfusion flow rate through the renal vasculature (ml/min) in kidneys which received CRF after 72h preservation prior to reperfusion, and control non-CRF organs.

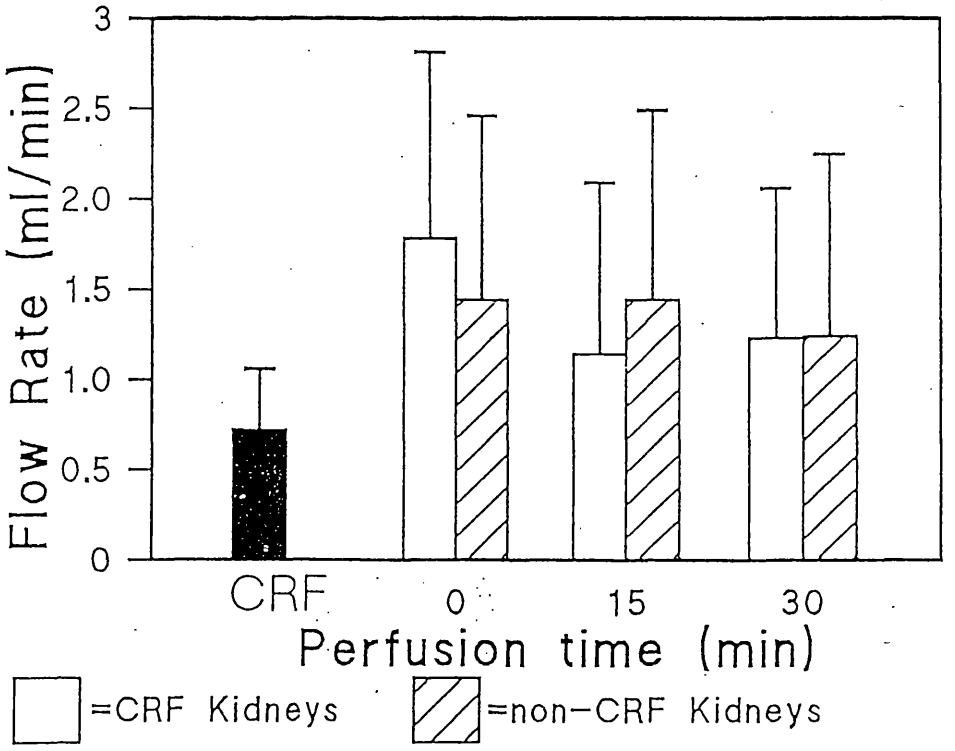


Fig. 6.2: Urine flow rate (ml/min) in kidneys which recieved CRF after 72h preservation prior to reperfusion, and control non-CRF organs.

Glomerular Filtration Rate (GFR)

GFR, as measured by creatinine clearance, was not significantly different between CRF and non-CRF treated kidneys at any time-point during normothermic perfusion. Mean values were in the range 1.74-2.29ml/min.

Reperfusion Time (min)	GFR (ml/min)	
	Reflushed	Non-Reflushed
0	1.98±0.81	2.29±1.18
15	1.74±1.02	2.03±1.37
30	2.06±1.44	1.91±1.20

Table 6.3: Glomerular filtration rate (ml/min) in kidneys which received CRF after 72h preservation prior to reperfusion, and control non-CRF organs. Values are means±SD, n=6.

Proximal tubule membrane damage: urine γ-GT level

The graph of urine γ-GT activity vs. perfusion time (Fig.6.4) shows that during the 10min cold reflush procedure there was a tenfold decrease in the amount of the enzyme detectable in the urine. The activity of the enzyme detectable in the urine at the end of the CRF period (10min) was significantly ($p<0.01$) lower than at the begining of the CRF. At the onset of normothermic reperfusion there was a burst of γ-GT release, comparable to that seen at the beginning of the

CRF procedure, but significantly lower ($p<0.01$) than the enzyme release at the same time-point in control kidneys which had not received a CRF. Enzyme release into the urine decreased over the next 15min in both kidneys, reaching a stable baseline value after approximately 30min of reperfusion. At this time τ-GT activity was seen to be significantly lower ($p<0.01$) in kidneys which received a CRF.

Reperfusion Time (min)	**Urine τ-GT activity (units/l)**	
	Reflushed	Non-Reflushed
CRF (0)	317.34±242.65	---
CRF (10)	9.73±6.80	---
0	306.66±207.12	1879.93±1115.48
15	239.58±227.75	320.86±237.73
30	131.40±95.36	228.93±156.31

Table 6.4: τ-GT release (u/l) into the urine of kidneys which received CRF after 72h preservation prior to reperfusion, and control non-CRF organs. Values are means±SD, n=6.

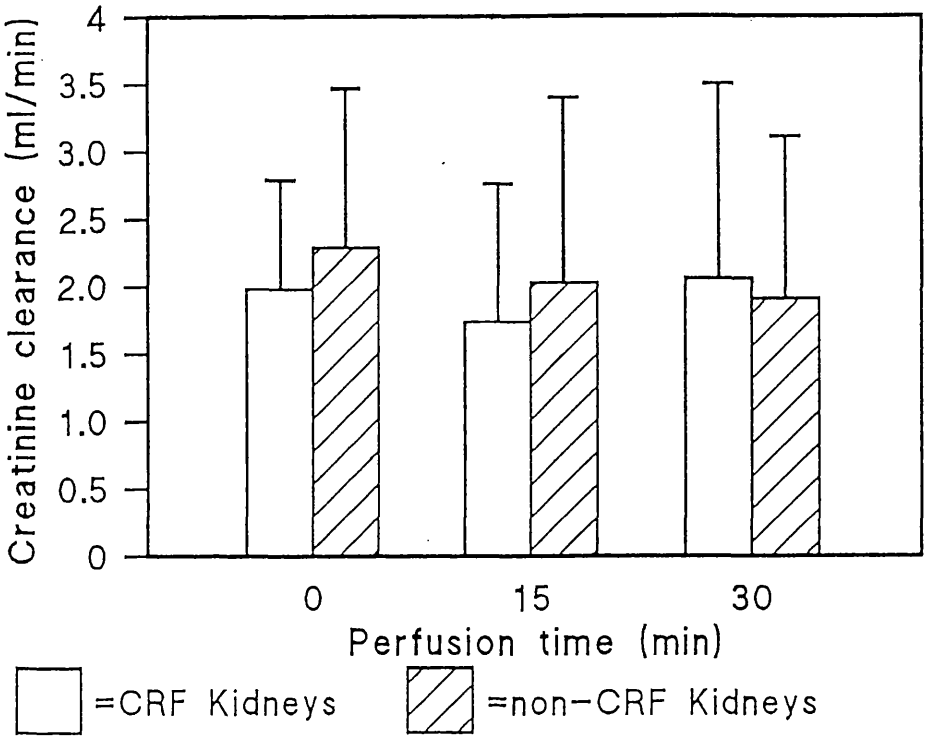


Fig. 6.3: Glomerular filtration rate (ml/min) in kidneys which received CRF after 72h preservation prior to reperfusion, and control non-CRF organs.

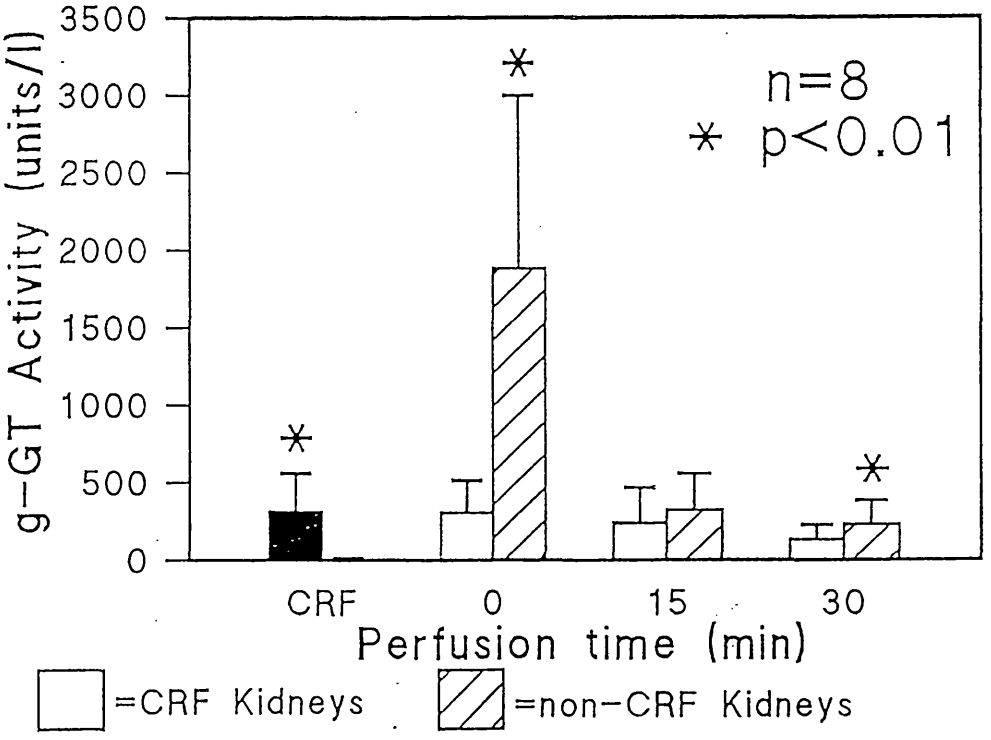


Fig. 6.4: γ-GT release (u/l) into the urine of kidneys which received CRF after 72h preservation prior to reperfusion, and control non-CRF organs.

Urine LDH level: gross tubule/collecting-duct damage

The graph of urine LDH activity vs. perfusion time (Fig. 6.5) shows similar trends to the release of γ-GT. Changes in the level of γ-GT release during CRF are shown by the dark bars on the graph. At the onset of CRF there is an initial high level of LDH activity detectable, decreasing to approximately 50% of this value at the end of the 10min cold flush. There was a significant decrease ($p<0.05$) in detectable LDH activity in the urine by the end of the CRF. At the onset of normothermic reperfusion there was a second burst of LDH release, exceeding the level in the initial CRF urine sample. This value was lower than the activity detectable in the initial urine sample of the control non-CRF kidney. The release of LDH diminished over the next 15min in both test and control kidneys, reaching a steady baseline after approximately 30min of reperfusion. During the reperfusion period, LDH release tended to be lower in CRF kidneys than in controls.

Reperfusion Time (min)	Urine LDH activity (units/l)	
	Reflushed	Non-Reflushed
CRF (0)	366.40±300.95	---
CRF (10)	64.76±20.70	---
0	275.40±140.90	694.00±246.52
15	89.96±83.41	169.86±229.96
30	78.31±73.67	115.80±159.62

Table 6.5: LDH release (u/l) into the urine of kidneys which received CRF after 72h preservation prior to reperfusion, and control non-CRF organs. Values are means±SD, n=6.

Proteinuria

The extent of proteinuria at the onset of normothermic reperfusion was significantly lower ($p<0.001$) in CRF kidneys than in non-CRF controls. However there was no difference between the two groups at any other time during reperfusion. Proteinuria was greatest at the onset of reperfusion and decreased during the first 10min of reperfusion in both CRF and non-CRF groups, reaching a steady rate between 15min and 30min.

Reperfusion Time (min)	**Proteinuria (% of [perfusate])**	
	Reflushed	Non-Reflushed
CRF (0)	140.00±12.00	---
CRF (10)	17.00±8.00	---
0	95.44±22.29	152.00±76.90
15	78.65±30.36	75.85±20.95
30	67.06±36.24	67.76±28.93

Table 6.6: Protein release (% of perfusate concentration) into the urine of kidneys which received CRF after 72h preservation prior to reperfusion, and control non-CRF organs. Values are means±SD, n=6.

This data is shown in Fig.6.6 . The dark shaded bars show proteinuria at the onset (t=0min) and at the end (t=10min) of the CRF procedure.

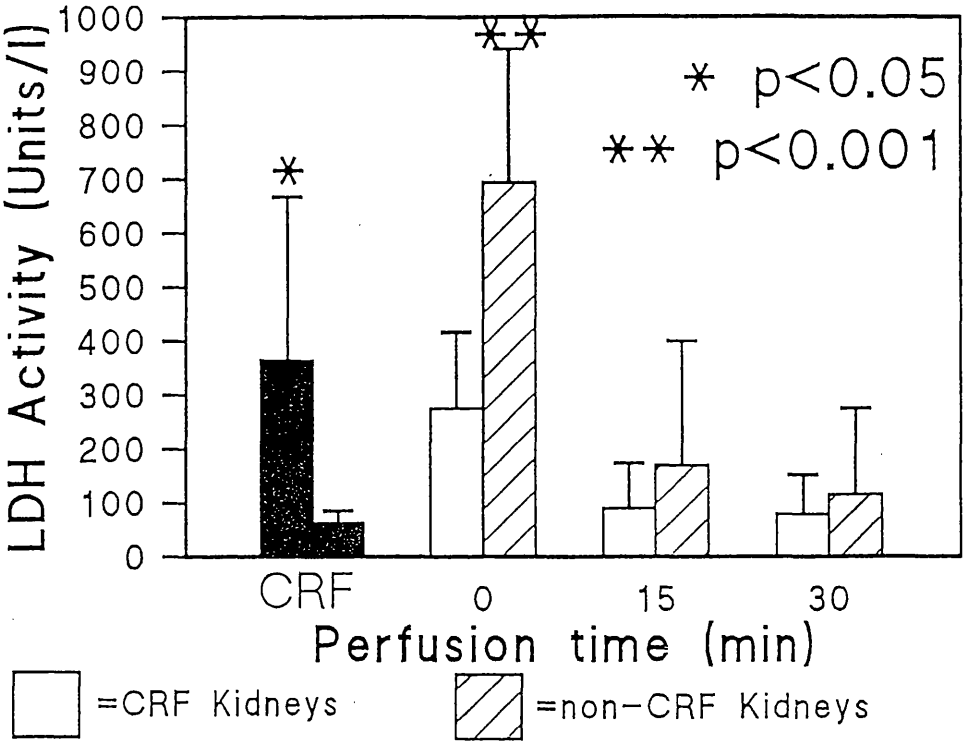


Fig. 6.5: LDH release (u/l) into the urine of kidneys which received CRF after 72h preservation prior to reperfusion, and control non-CRF organs.

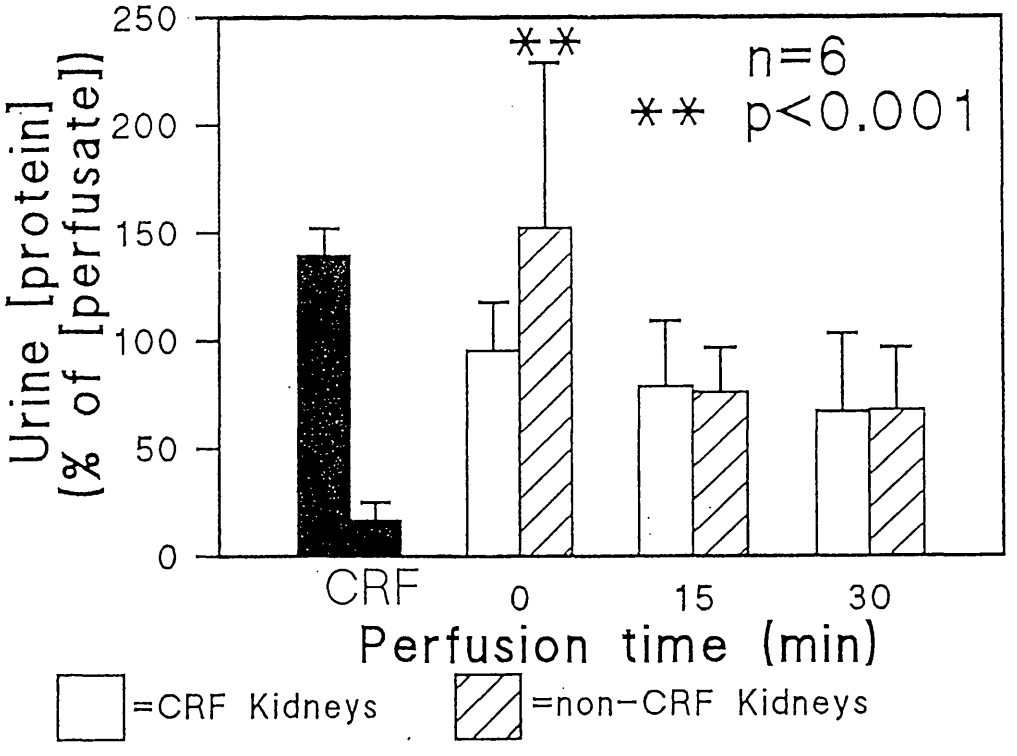


Fig. 6.6: Protein release (% of perfusate concentration) into the urine of kidneys which received CRF after 72h preservation prior to reperfusion, and control non-CRF organs.

Glucose Reabsorption

There was no significant difference in tubule glucose reabsorption between CRF and non-CRF kidneys at any time-point during the reperfusion period. Values ranged from 39.25±9.96% to 51±15.73% of the filtered glucose load.

Reperfusion Time (min)	**Tubule glucose reabsorption (% of filtered load)**	
	Reflushed	Non-Reflushed
0	43.45±19.22	39.25±9.96
15	45.38±14.95	51.38±15.73
30	46.08±21.27	46.56±28.87

Table 6.7: Tubule glucose reabsorption (% of filtered load) in kidneys which recieved CRF after 72h preservation prior to reperfusion, and control non-CRF organs. Values are means±SD, n=6.

Sodium reabsorption

There were no significant differences in tubule sodium reabsorption between CRF and non-CRF kidneys at any point during normothermic reperfusion. Reabsorption values ranged from -4.91% of the filtered load (net sodium excretion) to 35.87% of the filtered load (net sodium reabsorption).

Reperfusion Time (min)	**Tubule sodium reabsorption (% of filtered load)**	
	Reflushed	Non-Reflushed
0	13.17±7.08	10.84±12.79
15	9.36±2.65	12.77±6.27
30	16.94±9.97	15.18±9.57

Table 6.8: Tubule sodium reabsorption (% of filtered load) in kidneys which recieved CRF after 72h preservation prior to reperfusion, and control non-CRF organs. Values are means±SD, n=6.

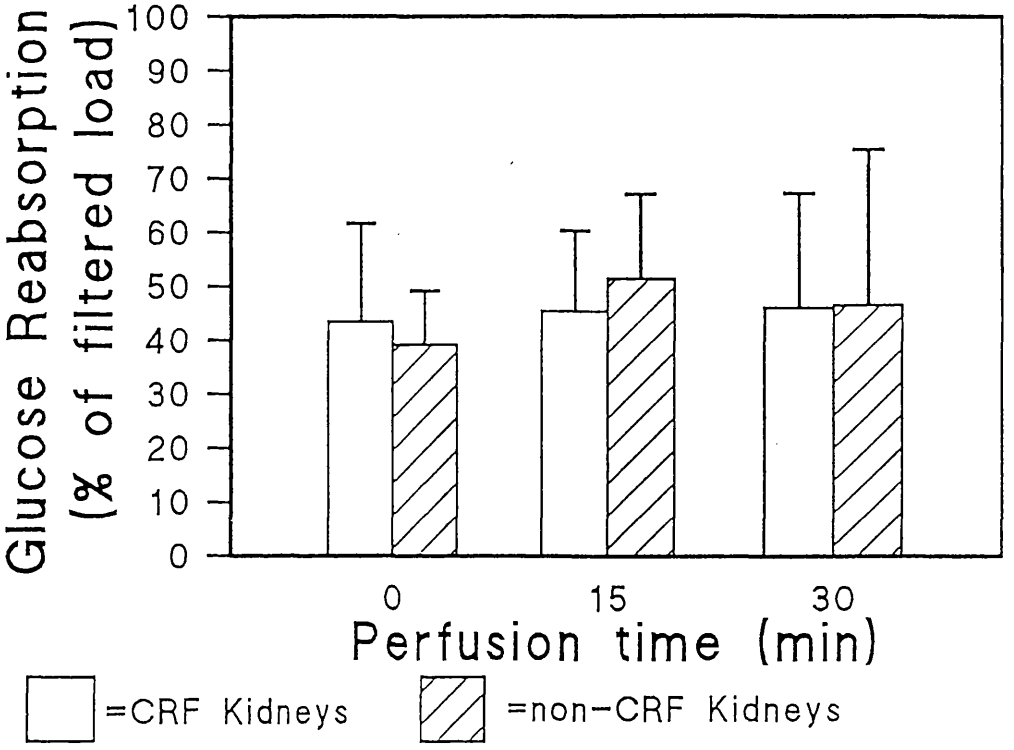


Fig. 6.7: Tubule glucose reabsorption (% of filtered load) in kidneys which received CRF after 72h preservation prior to reperfusion, and control non-CRF organs.

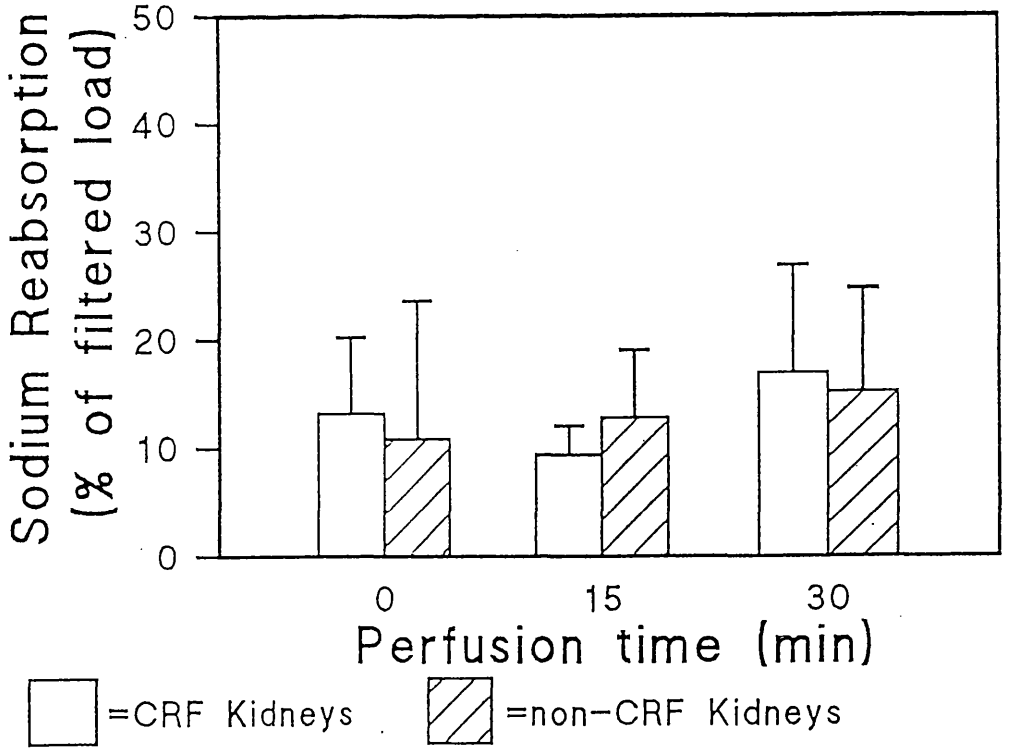


Fig. 6.8: Tubule sodium reabsorption (% of filtered load) in kidneys which received CRF after 72h preservation prior to reperfusion, and control non-CRF organs.

STUDY 2

THE ROLE OF OXYGEN IN REPERFUSION INJURY TO CRF KIDNEYS

Reperfusion time (min)	**Perfusate flow rate (ml/min)**	
	Normoxic	Hypoxic
0	79.66 ±7.08	72.33 ±4.27
	t=3.12, *p<0.02	
2	93.00 ±9.09	88.66 ±11.14
	t=0.78, p<0.50	
5	103.00 ±10.17	103.33 ±7.55
	t=0.06, p<0.90	

Table 6.9: Perfusate flow rate in 48h stored kidneys during normoxic and hypoxic reperfusion following cold re-flush. Values are means±SD, n=6.

As can be seen from Table 6.9, at the onset of reperfusion after CRF perfusate flow rate was significantly higher in normoxically reperfused kidneys ($p<0.02$) compared to hypoxically reperfused organs, although the difference in mean flow rate was small. Flow rate through the renal vasculature continued to increase in both groups of kidneys over the next

5min, but there were no significant differences between normoxic and hypoxic organs.

Reperfusion Time (min)	Urine flow rate (ml/min)	
	Normoxic	Hypoxic
0	1.51 ±0.77	1.28 ±0.55
	t=0.71, p<0.50	
2	0.91 ±0.71	0.50 0.15
	t=1.50, p<0.20	
5	0.74 ±0.67	0.41 ±0.18
	t=1.17, p<0.30	

Table 6.10: Urine flow rate in 48h stored kidneys during normoxic and hypoxic reperfusion following cold re-flush. Values are means±SD, n=6.

From Table 6.10 it can be seen that there were no significant differences in the rate of urine production between normoxic and hypoxic kidneys at any time during reperfusion after CRF.

Perfusate LDH activity (units/l) at t=0min	
Normoxic	Hypoxic
69.87 ±20.70	53.14 ±20.10
t=3.02, p<0.05 *	

Table 6.11: Release of lactate dehydrogenase into the vascular spaces of 48h stored kidneys during normoxic and hypoxic reperfusion following cold re-flush. Values are means±SD, n=6.

At the onset of reperfusion, normoxic reperfusion produced the release of the intracellular enzyme LDH into the renal vasculature in significantly higher concentrations than did normoxic reperfusion.

Reperfusion Time (min)	Urine LDH activity (units\l)	
	Normoxic	Hypoxic
0	476.51±290.78	344.47±303.34
5	61.51±66.66	186.17±135.06

Table 6.12: Release of LDH into the urinary spaces of 48h stored kidneys during normoxic and hypoxic reperfusion following cold re-flush. Values are means±SD, n=6.

There were no significant differences between LDH release into the urine upon normoxic or hypoxic reperfusion following 48h storage and CRF.

Reperfusion Time (min)	Urine τ-GT activity (units/l)	
	Normoxic	Hypoxic
0	352.99±370.11	381.75±396.41
5	63.30±97.61	179.24±311.12

Table 6.13: Release of τ-GT into the urinary spaces of 48h stored kidneys during normoxic and hypoxic reperfusion following cold re-flush. Values are means±SD, n=6.

There were no significant differences between τ-GT release into the urine upon normoxic or hypoxic reperfusion following 48h storage and CRF.

Urinary protein composition

Percentage of total urine proteins in 66-97KD range	
Normoxic	Hypoxic
43.00 ±22.22	30.16 ±20.54
t=3.73, p<0.01 **	

Table 6.14: Urinary proteins in molecular weight range 66-97KD (% of total urine protein concentration). Values are means±SD.

There was an increased release of proteins in the molecular weight range 66-97KD in kidneys which were reperfused under normoxic conditions after 48h preservation and CRF when compared with hypoxically reperfused kidneys.

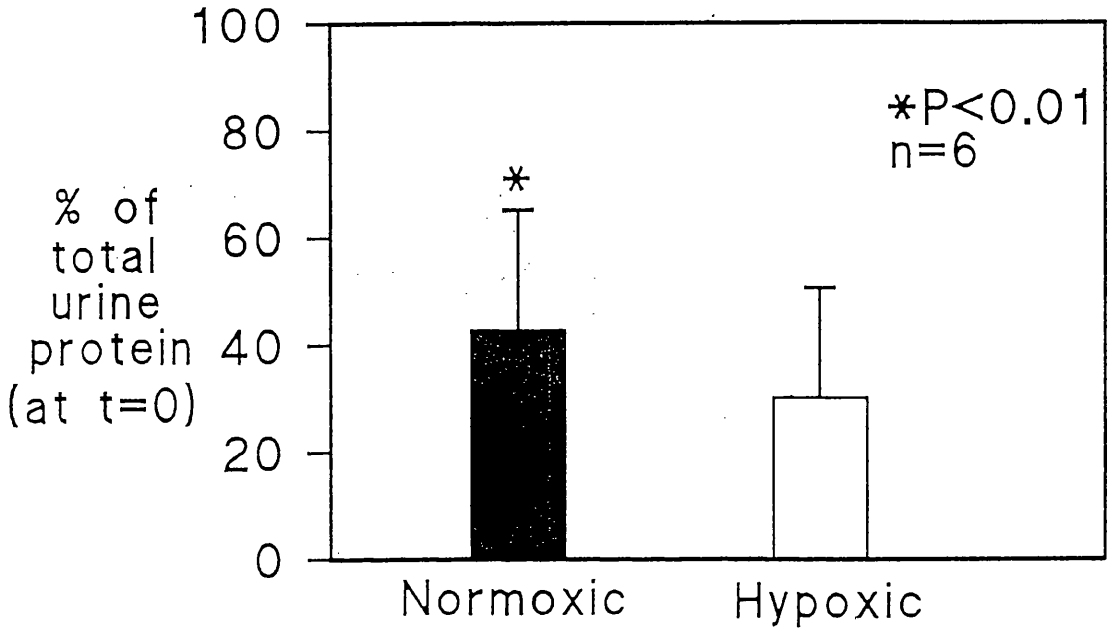


Fig. 6.9: Urinary proteins in molecular weight range 66-97KD (% of total urine protein concentration).

DISCUSSION

STUDY 1

Perfusate Flow

The low flow rate through the renal vasculature during CRF of 72h stored kidneys with HCA solution was due to a number of factors. It is likely that increased vascular resistance at the onset of this procedure was due to collapse of smaller renal blood vessels such as arterioles and capillaries. This collapse was chiefly due to the effects of relaxation of vascular smooth muscle due to the cold temperature (≈2°C) and the lack of any intravascular pressure during ischaemia. It may also have been partly due to accumulation of vasoconstrictors during the preservation period or the lack of production of vasorelaxant compounds (Lelcuk *et al.*, 1985). The fact that the renal vasculature collapsed during cold preservation meant that this had to be refilled by the CRF procedure, and therefore flow was slower than under conditions of normothermic reperfusion. Differences between the viscosity of the CRF solution and that of the perfusate used for normothermic reperfusion are also likely to have caused differences in the vascular flow rates. Flow through the vasculature increased slightly during the CRF; this was probably due to dilation of resistance vessels (arterioles) as the intravascular pressure was increased by the pressure of the fluid entering the kidney. This pressure could also have been sufficient to open smaller vessels which

may not have been perfused at the onset of CRF. The observation that CRF flow was approximately 20% of the mean flow during normothermic reperfusion demonstrated that normal vascular tone was not achieved under these conditions. It is also possible that cellular debris accumulated in the vasculature during the preservation period initially obstructed flow, and once this was cleared the CRF solution could flow more freely. A mechanism by which mannitol infused into kidneys following ischaemia could promote flow through the vasculature was described by Flores and colleagues, (1972). Since the CRF solution (HCA) contained mannitol (33g/l), it is possible that this exerted an osmotic effect causing the reduction of endothelial cell swelling, thereby facilitating better flow through the renal vasculature. However the CRF solution was identical in composition to the solution in which the kidney had been stored, and therefore this preservation solution should have opposed cell swelling to the same extent as the CRF solution. In spite of this mannitol is taken-up by cells during storage, hence these cells *do* swell. Accumulation within the kidney of endogenous vasoconstictors or the depletion of endogenous vasorelaxants during organ preservation may have influenced the rate of flow through the vasculature during CRF. The use of CRF may have washed some of these vasoconstrictors out of the kidney, thereby increasing the flow through the vasculature. These same substances may have also accumulated in the recirculating perfusate during normothermic reperfusion, thus leading to

varience in vascular resistance.

At the onset of normothermic reperfusion vascular resistance was similar in the two groups of kidneys, as shown by similar perfusion flow rates. This resistance decreased further in both groups of kidneys over the next 15min of perfusion, but was significantly lower ($p<0.01$) in CRF treated kidneys. Flow had increased further in both groups of kidneys by the end of the 30min perfusion period, the change in mean flow rate being greatest in non-CRF kidneys. These findings demonstrate that the CRF procedure allowed the perfusion flow rate to reach its maximum stable level sooner than maximum flow was achieved in non-CRF kidneys. This has important implications if it can be extrapolated to *in vivo* transplantation because it suggests that blood flow, and therefore metabolite delivery, to the kidney in early reperfusion may be increased by CRF. It is likely that such increased delivery would bring about a more rapid restoration of biochemical equilibria within tissues.

Urine Flow

Urine production was considerable during CRF of 72h stored kidneys, falling within the range of some of the rates which were produced by normothermic perfusion. This is in contrast to the markedly lower CRF flow through the renal vasculature compared with this flow during normothermic reperfusion. It is therefore possible that elevated postglomerular vascular resistance maintained a high

glomerular filtration pressure in the cold kidney resulting in the observed rate of urine production. It is also likely that the composition of HCA solution influenced the rate of urine production. Since this solution contained a high concentration of mannitol (33.8g/l), an osmotic diuretic, it is likely to have prevented water reabsorption and therefore urine production would have increased. Active transport of solutes in the kidney, particularly sodium, would have been greatly diminished in the cold kidney after depletion of metabolic substrates during the preservation period. There would therefore have been decreased mechanisms for urine concentration, such as the countercurrent multiplier system (see Chapter 1). Efficient urine production during the CRF may be of long-term benefit to the kidney if it allows the removal of cytotoxic substances prior to transplantation. For instance, catalytically-available iron may have entered the kidney extracellular spaces due to cell lysis or leakage through their membranes during ischaemia. If the CRF procedure is able to remove this "free" iron from the kidney, then oxidative damage to cells from the extracellular environment would be decreased. Oxidative damage would also be attenuated if the substrate for xanthine oxidase, hypoxanthine (see Chapter 1), was removed by this procedure. This is a possibility since the removal of adenine nucleosides and bases from postischaemic hearts has been achieved using similar methods (Schutz *et al.*, 1981; Jennings *et al.*, 1985).

There were no significant differences in urine flow rate

between the two groups of kidneys at any time during normothermic reperfusion suggesting that CRF did not influence early postreperfusion urine production. There was no evidence of increased production at the onset of reperfusion due to residual effects of mannitol in the CRF solution. This may be explained by the fact that all kidneys were preserved in a solution identical to the CRF solution. However, the observation that an increase in mean urine production did not accompany the increases in mean perfusion flow rate noted after 15min of normothermic reperfusion demonstrates that net glomerular filtration pressure did not change in this period. This may have occurred if the increased perfusion flow rate was due to dilation of the efferent arterioles during the reperfusion period. The glomerular filtration pressure would therefore not have increased and urine production would have remained constant.

The absence of humoral influences from substances such as vasopressin is also likely to have played a role in the high mean yield of urine seen in both groups of kidneys. Such humoral and neural influences would normally help regulate water and solute reabsorption, and therefore urine production. In the absence of vasopressin there would not have been any stimulus for water reabsorption in the distal tubules and collecting ducts, and therefore differences in the functional capability of the cells in these tissues in CRF and non-CRF kidneys may not have been apparent.

Glomerular Filtration Rate

Glomerular filtration rate seemed to closely resemble the rate of urine production in both CRF and non-CRF treated 72h stored kidneys, and there were no significant differences between the two groups at any time during normothermic reperfusion. This suggests that there were no noticeable urine concentrating effects in either group of kidneys. The fact that GFR was similar in CRF kidneys to non-CRF kidneys suggests that the accumulation of debris in the urinary and vascular spaces during ischaemia did not affect glomerular filtration (i.e. debris did not block the glomerular basement membrane fenestrations).

γ-Glutamyl Transpeptidase release

The burst of γ-GT release seen at the onset of normothermic reperfusion of 72h stored CRF organs when most of the enzyme had been removed by the cold flushing process, suggests that some injury to the proximal tubules was due to normothermic, oxygenated reperfusion. The lower γ-GT release in CRF kidneys at the onset of normothermic reperfusion compared with non-CRF kidneys suggests that either proximal tubule damage was significantly decreased by CRF or that the enzyme accumulated in the urinary spaces during storage and was released throughout reperfusion. However, the observation that γ-GT release was significantly lower in CRF kidneys at 30min of normothermic reperfusion, after which any enzymes accumulated during storage would be expected to have been

washed from the urinary spaces, suggests that the integrity of the proximal tubule brush border membranes was better maintained in CRF treated kidneys. The kidney is an organ which is particularly dependent upon the liver as a source of glutathione (GSH) which is carried to the kidney via the systemic blood circulation (Bartoli *et al.*, 1978). Uptake of this hepatically-derived GSH helps maintain renal antioxidant status. Therefore loss of γ-GT activity from cell membranes will decrease the ability of these cells to degrade hydrogen peroxide using the enzyme glutathione peroxidase and glutathione. Consequently non-CRF kidneys may be subjected to greater oxidative stress upon normothermic, normoxic reperfusion than kidneys which are given a CRF. Inability to take-up this systemic GSH may also prevent amino acid uptake into cells (Allison and Meister, 1981). Loss of γ-GT may indicate complete membrane disruption or may be due to the loss of the enzyme from an otherwise intact membrane. Either occurrence may decrease amino acid reabsorption resulting in amino aciduria and possible delayed tissue repair in the kidney.

Urine LDH activity

The significantly lower LDH release ($p<0.05$) into the urine at the end of the CRF compared with the beginning of the CRF of 72h stored kidneys demonstrated that the procedure removed cell contents which had accumulated in the urinary spaces during cold ischaemic kidney preservation. The bulk of

the enzyme detected is likely to have originated in the epithelial cells lining the urinary tubules and collecting ducts. Some may have been vascular in origin, passing across a potentially diminished glomerular barrier (see Chapter 3). It is unclear whether the enzyme passed across cell membranes whose permeability may have increased during storage, or whether LDH in the urinary spaces was due to cell lysis. At the onset of normothermic reperfusion there was a second discharge of LDH into the urine of CRF kidneys, in excess of the activity detectable at the end of CRF. This represented damage due to normothermic reperfusion, and goes some way towards separating reperfusion effects from effects which were solely due to ischaemia. However, urinary LDH release in CRF treated kidneys was significantly lower ($p<0.001$) at the onset of normothermic reperfusion than control non-CRF kidneys. This showed that CRF cleared the urinary tubules and collecting ducts of intracellular components which had built up in these spaces during preservation. Although mean enzyme release was lower in CRF kidneys until the end of the reperfusion period, the difference was not significant and probably represented continued reperfusion damage, possibly mechanical, to urinary epithelial cells. The observation that an intracellular enzyme such as LDH was lost into the urine both during CRF and during normothermic reperfusion suggests that other intracellular constituents which were not analysed in these studies are likely to have been lost into the urine. This loss of components of normal metabolism, including enzymes and their

substrates, may cause delayed graft function following transplantation until repair mechanisms prevent their continued leakage.

Proteinuria

The citrate solution used for cold reflush did not contain protein; hence, protein detected in the urine during this procedure originated from within the kidney. The most likely source of this protein was the epithelial cells of the urinary tubules, but some may have crossed the glomeruli from the vascular space where it may have accumulated during organ storage. It is unclear whether CRF proteinuria was the result of leakage of proteins through damaged cell membranes, the result of complete cell lysis, or loss of cell surface proteins. The results of urinary enzyme analysis suggest that intracellular proteins (LDH) and membrane-bound proteins (γ-GT) were lost. The observation that proteinuria was still evident at the end of the cold reflush procedure (0.17±0.08mg/ml) suggests that either some urinary spaces were cleared at a slower rate than others, or that leakage of proteins from cells and cell lysis continued during CRF. Mechanical damage of CRF may have been responsible for some of these occurrences.

Elevated proteinuria in non-CRF kidneys compared with CRF kidneys at the onset of normothermic reperfusion ($p<0.001$) is probably due to the flushing of protein accumulated during preservation from the urinary spaces by the CRF procedure.

This elevated proteinuria was not maintained during normothermic reperfusion, decreasing within 15min and reaching a plateau within 15-30min. The removal of this protein may produce more distinct benefits *in vivo* where tubule blockage at the onset of reperfusion often increases intratubular hydrostatic pressure with resulting decreased GFR. These proteins have been implicated in the long-term production of Tamm-Horsfall protein cylinders (Bayati *et al.*, 1990) which cause tubular obstruction. Towards the end of the reperfusion period proteinuria continued at a rate of approximately 70% of the perfusate protein concentration. This demonstrates that both groups of kidneys had suffered substantial glomerular injury, allowing perfusate albumin to cross, and that the CRF procedure was of no significant benefit to maintenance of glomerular integrity. This may indicate that in the case of early kidney glomerular function the extent of ischaemic injury (i.e. the preservation period) is more significant than reperfusion injury. From these experiments it was not possible to determine what proportion of protein in the urine originated in the perfusion buffer and how much was indigenous to the kidney.

Glucose / sodium reabsorption

CRF seems to be neither beneficial nor detrimental to tubule solute reabsorption as shown by the absence of significant changes in sodium and glucose reabsorption when kidneys were treated in this way. CRF may wash purine

metabolites out of the kidney tissues, thereby limiting their availability for resynthesis of ATP, and this may initially depress active solute reabsorptive processes. This could obscure any beneficial effects upon the maintenance of the integrity of reabsorption mechanisms such as the Na^+/K^+ATPase and Na^+-coupled glucose uptake. Since CRF seemed to prevent normothermic reperfusion injury to proximal tubule membranes, as shown by decreased γ-GT release after 30min of reperfusion, it may also help to conserve the integrity of the membrane-bound enzymes responsible for solute reabsorption in other parts of the tubules.

STUDY 2

THE ROLE OF OXYGEN IN REPERFUSION INJURY

Release of LDH into vascular spaces

The release of the intracellular enzyme LDH into vascular spaces at the onset of normothermic reperfusion following CRF was found to be significantly greater ($p<0.05$) in kidneys reperfused with a normoxic solution than those reperfused with a hypoxic solution. Since vascular spaces had been cleared of cell debris accumulated during storage by the CRF procedure, this release may have been due to reperfusion-induced oxidative damage to the vascular endothelium.

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Enzyme release into urinary spaces

There was no evidence of oxygen dependent release of LDH or γ-GT into the urinary spaces of 48h stored CRF kidneys following normothermic reperfusion. This is consistant with the results of an earlier study using identical reperfusion conditions for kidneys which had been stored for 48h but not given CRF (see Chapter 5). It would appear that the damage done to the epithelial cells of the urinary tubules during ischaemia was more conspicuous than oxidative reperfusion injury within the first 5min of reperfusion.

Urinary protein composition

The results of polyacrylamide gel electrophoresis of urinary proteins showed that there was an increased loss of proteins in the molecular weight range 66-97KD into the urine of kidneys reperfused normoxically compared to those reperfused hypoxically. However, it was not possible to determine the precise nature of these proteins or the anatomical site of their origin. It is likely that they were a mixture of intracellular and membrane proteins from the epithelial cells lining the urinary tubules, but it is also possible that some proteins from the vascular spaces may have leaked across the glomeruli into the urine.

It is important that any changes in protein structure or distribution detected should be correlated with functional changes in the cells from which they originate, since these protein changes cannot be assumed to be the cause of all

functional impairment. Huang and colleagues, (1992) showed loss of functional Na^+/K^+ATPase activity concomittant with fragmentation and cross-linking of enzyme subunits, demonstrated by SDS-PAGE, but this also occurred when there was no such evidence of structural changes. However, the oxidant-modified ATPase was shown to be more susceptible to degradation by a number of proteolytic enzymes.

SUMMARY

The results of Study 1 show that CRF washed out products of cell damage from the urinary and vascular spaces, and this was reflected in corresponding lower enzyme release during subsequent normothermic reperfusion. Accumulation of tubular debris has been noted previously following storage, and CRF may be one way of attenuating this, thereby improving early renal function. In 72h preserved kidneys CRF was found to allow flow through the renal vasculature to reach its maximum earlier than in non-CRF kidneys. This may be important for early restoration of normal organ function following *in vivo* transplantation. Urine production and GFR during reperfusion appeared to be unaffected by prior CRF in 72h stored organs. The observation that γ-GT release was significantly lower at the onset of reperfusion and after 30min of reperfusion in CRF kidneys suggests that reflushing afforded some degree of protection to proximal tubules from reperfusion injury. CRF did not significantly effect glomerular proteinuria, which may suggest that the majority of glomerular injury occurs during

ischaemic preservation rather than during normothermic reperfusion.

The results of these studies suggest that a short period of CRF has no adverse effects on immediate organ function, and may in fact be beneficial in promoting vascular flow and decreasing damage to the proximal tubule brush border membrane. It must be remembered, however, that cold re-flush with solutions not specifically designed for this process may cause further damage to the stored kidney. It has been shown that CRF with sodium-lactobionate-sucrose solution (SLS) actually increases damage to 48h stored canine kidneys, while the inclusion of 5% polyethylene glycol (PEG) significantly improves post-transplant function (Collins and Wicomb, 1992). The design of therapeutically useful cold reflush solutions may therefore be of significant benefit to kidney transplantation, and to the transplantation of those organs such as the heart, lungs, and liver, which must function immediately after grafting to allow recipient survival. The production of such a solution may also allow the clinical use of organs which have been stored for longer periods of time than currently permit life-sustaining function.

The results of the second study, the role of oxygen in reperfusion injury after CRF, suggest that the renal vasculature is susceptible to oxygen-dependent reperfusion injury and that this damage results in leakage of cytoplasmic contents into the vascular space. This leakage may be due to increased endothelial cell membrane permeability or may be the

result of total cell lysis. There does not appear to be an accompanying oxygen-dependent release of enzymes into the urinary spaces. Since the CRF procedure was shown to clear these spaces of enzymes accumulated during kidney preservation (see above), it is unlikely that this cell debris would have obscured the measurement of enzyme release which resulted from reperfusion alone. Modification of the CRF solution would be necessary to achieve maximum benefit from this procedure. The inclusion of substances such as metabolic energy substrates may help promote early restoration of normal kidney function.

THE ROLE OF IRON IN ISCHAEMIC / REPERFUSION INJURY TO THE KIDNEY AND THE INVOLVEMENT OF INTRACELLULAR pH AND LYSOSOMAL pH IN THIS INJURY

INTRODUCTION

The source of catalytic iron, involved in free radical generation, whether "free" iron or loosely-bound to other molecules, remains to be determined. Redistribution of intracellular iron to more accessible pools may play an important role in catalysing oxygen-derived free radical production (Healing *et al.*, 1989). The site and size of this intracellular pool is likely to play an important role in determining the extent of oxidative stress to tissues (Halliwell and Gutteridge, 1990). The intracellular iron pool is principally bound to the protein ferritin, but there is also the possibility of low molecular weight chelates with molecules such as ADP, ATP and citrate. Ferric ions (Fe^{3+}) have been shown to be released from transferrin, ferritin, and haemoglobin under acidic conditions (McCord, 1985), and in the presence of reducing agents such as O_2^- (Halliwell and Gutteridge, 1984; Thomas *et al.*, 1985; Biemond *et al.*, 1986). Iron has been shown to be released from ferritin by the

activity of xanthine oxidase by a process which can be inhibited by allopurinol or superoxide dismutase, suggesting that it is a process mediated by superoxide radicals (Powers *et al.*, 1992). Although the main purpose of flushing organs with preservation solutions was to remove blood, complete washout was rarely achieved. Small quantities of blood were often seen in the vascular effluent at the onset of reperfusion from rabbit kidneys flushed with HCA. This occurred despite the observation that after approximately 20ml of HCA had passed through the vasculature, there was no evidence of further blood washing out into the flush effluent at the time of harvest. Some vessels may trap blood as the result of a decrease in the volume of the vasculature due to vasoconstriction initiated by the high potassium content (28mM) of the flush solution. These sites of residual blood may be sources of localized elevated free iron if release from haemoglobin or transferrin can take place during ischaemic preservation. This release may even be caused by free radical species (Gutteridge, 1986). Tubule damage and increased lipid peroxidation, believed to be iron-mediated, have been reported to result from exposure of kidney tubules to haemoglobin and myoglobin (Paller, 1988), and both of these iron-containing proteins may react with hydrogen peroxide to form oxidizing substances capable of causing lipid peroxidation (Davies, 1990).

Transferrin is the major iron-binding protein in the plasma and forms a complex with ferric iron and bicarbonate.

Two molecules of Fe^{3+} are bound to each molecule of protein, but the average iron loading of transferrin in the blood is 20-30% of total, leaving a significant residual binding capability. Iron may be released if transferrin-bound ferric iron (stability constant $\approx 10^{20}$) is reduced to the weakly-binding ferrous iron (Fe^{2+}). Endothelial cells have also been shown to cause the release of iron from transferrin upon contact and this may be an important source of iron to catalyse free-radical mediated endothelial cell injury (Brieland *et al.*, 1992). It is unclear what contribution residual blood in the vasculature would make to increasing the size of the free iron pool in cold-preserved kidneys.

In addition to free iron, low molecular weight chelates of iron, such as citrate in a 1:1 complex with ferric ions, are often able to redox cycle and generate oxygen radicals (Gutteridge, 1991). This may have important implications for kidney preservation using the HCA solution, since the major anion in this solution is citrate. If there is a pH decrease, as occurs during cold ischaemic preservation, electron transfer can take place in ferric-citrate complexes to form ferrous ions. These then auto-oxidise if the pH becomes neutral, as occurs following reperfusion, resulting in •OH generation. However, for this reaction to occur the citrate and iron must be in the same compartment; hence citrate must enter the cells or iron must leak out or be present in the extracellular spaces during storage (e.g. in residual blood). From this information it is clear that any compound chosen to

chelate iron to prevent it from catalysing the Fenton reaction (see Chapter 1) must form a non-catalytic complex.

Lysosomes are the major organelles involved in the degradation of intracellular and extracellular material. They contain acid hydrolase enzymes capable of acting upon biological molecules such as proteins, lipids and carbohydrates. Lysosomal enzymes may be ultimately responsible for cell destruction following initial cell injury during ischaemia and reperfusion. Degradation of lysosomal membranes occurs during ischaemic preservation due to the action of phospholipases and the loss of stabilization of the membrane by ATP (Wilkinson and Robinson, 1974) which itself degrades during ischaemia. A number of studies have implicated lysosomal enzymes in a direct mechanism responsible for ischaemic cell injury (Lotke, 1966; Vogt and Faber, 1968), but less is known about the involvement of lysosomes in reperfusion injury. Inhibition of the degradation of tissue components by these enzymes may therefore prevent damage, initiated by some other mechanism, from proceding to cell death. The liberation and activation of enzymes from these membrane-limited compartments is enhanced by tissue acidification (Von Ardenne and Kruger, 1979). Liberation of lysosomal enzymes may cause damage to neighbouring cells such that these cells may in turn release the contents of their lysosomes, possibly including catalytically available iron, thus initiating a chain of damaging events in adjacent cells leading to areas of tissue necrosis. Tissue hyperacidification

occurs during cold ischaemic kidney preservation. The causes of this phenomenon include: 1) continued anaerobic metabolism leading to lactate accumulation (a decrease in ATP concentration stimulates glycolysis); and 2) loss of hydrogen ion (H^+,proton) active transport out of the cytoplasm due to lack of ATP. However, it has been claimed that acidosis protected rat livers from lethal oxidative stress (Bronk and Gores, 1991) even though lipid peroxidation was increased twofold at pH6.4 compared with 7.4. Lysosomal swelling, which may occur as a result of osmotic changes during kidney preservation (see Chapter 1), can also cause activation of lysosomal enzymes (Dingle *et al.*, 1979).

The accumulation of excess intracellular iron within lysosomes has been shown in a number of studies (Pechet, 1969; Theron and Mekel, 1971; Arborgh *et al.*, 1974; Seymour *et al.*, 1974). During ischaemia and/or reperfusion, iron which is liberated from the intracellular sites of its normal physiological activity, and possibly iron which may enter cells through a diminished plasma membrane barrier from extracellular compartments, may be sequestered by lysosomes to prevent cell damage. The release of iron from these lysosomes by pathological events during ischaemia and/or reperfusion could then provide a catalyst for free radical production as described in Chapter 1. It has also been suggested that hydroxyl radicals may be produced within lysosomes containing trace amounts of reactive iron, thereby damaging lysosomal membranes and releasing the acid hydrolases therein (Zhang *et*

al., 1992). The acidic medium of lysosomes is required for the liberation of iron from its major carrier in the blood, transferrin, following receptor-mediated endocytosis of the complex. Ferric ions (Fe^{3+}) have also been shown to be released from the storage protein ferritin and from haemoglobin ander acidic conditions (McCord, 1985). A similar pH-dependent mechanism (either in the cytoplasm or within lysosomes) may be responsible for the liberation of catalytic iron in the kidney during ischaemia and reperfusion. In the present chapter, studies were undertaken to test this hypothesis.

Chloroquine is an amphiphilic cationic compound which has been used as an antimalarial and antirheumatic drug, and has been shown to interfere with intralysosomal degradation of proteins, mucopolysaccharides and lipids, and to cause generalized lipidosis in man and in animals as a result (Lhermitte *et al.*, 1976). Chloroquine has also been shown to cause lipidosis in the kidney (Hruban *et al.*, 1972; Datsis, 1972). It has also been demonstrated (Yagil *et al.*, 1988) that chloroquine prevents the degradation of peptides such as insulin in kidney cells by inhibiting endosomal and lysosomal activity. Most compounds which cause lipidosis, characterized by accumulation of polar lipids including phospholipids and glycolipids, are amphiphilic bases; they contain a hydrophobic region (usually an aromatic ring) and a hyrophilic region (usually a side-chain which carries a primary, secondary or tertiary amine).

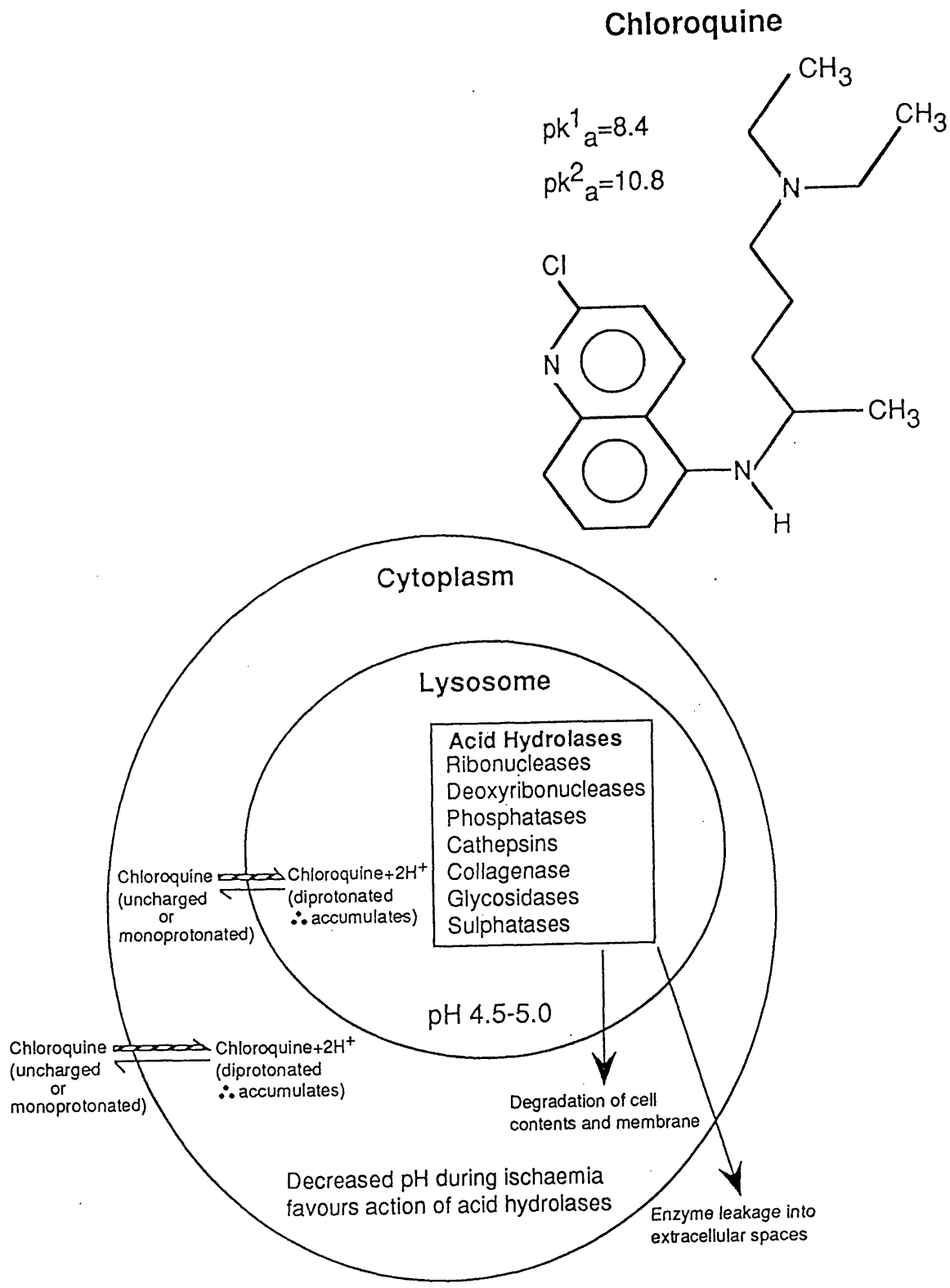

Fig. 7.1 Structure of the Chloroquine molecule and its hypothesized mechanism of action in the inhibition of intracellular acidification.

Chapter 7 ROLE OF IRON IN ISCHAEMIC/REPERFUSION INJURY

Intralysosomal accumulation of chloroquine has been shown to occur within minutes of intravenous administration, one of the fastest rates seen among this class of compound (Allison and Young, 1964; De Duve *et al.*, 1974). Chloroquine has two ionization constants (pK_a), 10.8 and 8.4. This means that at pH values below 10.8 the first cationic group is formed and at values below 8.4 the second cationic group is formed within the molecule. Chloroquine is able to cross biological membranes when it is in the unprotonised or monoprotonised form, but once a second proton binds the molecule can no longer traverse these membranes. As a result, under normal physiological conditions chloroquine crosses cell plasma membranes and lysosomal membranes, and once inside the lysosome the acidic environment causes protonation of the compound. Chloroquine can no longer cross the lysosomal membrane and therefore accumulates in this compartment. The binding of the hydrogen ions decreases their concentration, thereby increasing intralysosomal pH. Hydrogen ion "trapping" by chloroquine was proposed by Homewood and colleagues, (1972) who studied malarial parasites *in vitro*, and by Reijngoud and Tager, (1976) who investigated chloroquine uptake into isolated rat liver lysosomes. The second hypothesis investigated in this study was that a similar mechanism of hydrogen ion trapping can operate across the plasma membranes of cells experiencing cytoplasmic acidification during cold ischaemic flush-preservation.

Chapter 7 ROLE OF IRON IN ISCHAEMIC/REPERFUSION INJURY

METHODS

The standard methods of organ removal and flush preservation were used in this investigation (see Chapter 2). The only modification made to the procedure was the addition of 516mg of chloroquine to one of the 1L bags of HCA storage solution used for flush-preservation to give a final concentration of 1mM. Fresh chloroquine preservation solution (CQ-HCA) was prepared as follows for each experiment: the plunger of a sterile 50ml syringe was removed and the chloroquine, 516mg was placed inside the empty barrel. The plunger was carefully replaced, whilst ensuring that no chloroquine was expelled during the procedure. An 18G needle was fitted to the syringe and inserted into the injection port of a 1L bag of HCA preservation solution. Approximately 30ml of the solution was withdrawn, the syringe was capped and shaken vigorously to dissolve the compound. The CQ-HCA was then injected back into the bag of HCA to give a final concentration of 1mM. Gentle agitation of the HCA bag ensured that the chloroquine was fully dissolved and uniformly distributed. Care was taken throughout the procedure to maintain sterility. One kidney of each pair was flushed (2°C) with the CQ-HCA solution and stored on ice in this solution for 48h, the other kidney of each pair was preserved in the same way using standard HCA solution. Standard perfusate containing 1g/l BSA was used to reperfuse all kidneys on a non-recirculating (open) circuit, and reperfusion was maintained for 5min during which serial samples of urine and

vascular effluents were collected. Kidneys were freeze-clamped at the end of reperfusion and tissue, urine, and vascular effluent samples were stored under liquid nitrogen until analysis.

Free Iron analysis

Desferrioxamine-available iron (catalytically-available "free" iron) was measured by HPLC as described in Chapter 2.

RESULTS

Table 7.1: Effect of chloroquine (1mM) on the concentration of desferrioxamine-available iron in rabbit kidneys reperfused after storage for 48h. Values are means ±SD, n=6, statistical analysis by Mann-Witney test.

DFX-available Iron (μMol/g protein)			
Cortex		Medulla	
Control	Chloroquine	Control	Chloroquine
1.18 ±1.37	0.99 ±1.37	1.04 ±0.65	1.10 ±1.08
u=10, not sig. at p<0.05		u=17, not sig. at p<0.05	

The results in Table 7.1 show that there was a small decrease in DFX-available iron in the cortex of kidneys reperfused after 48h preservation in CQ-HCA, compared with reperfused untreated preserved organs, but this was not statistically significant. This trend was not shown in samples

of tissue from the medulla, where there was also no significant difference between the treated group and untreated controls. In addition, there were no statistically significant differences between the amount of free iron in the cortex and that in the medulla in either treated kidneys ($p<0.80$) or controls ($p<0.90$).

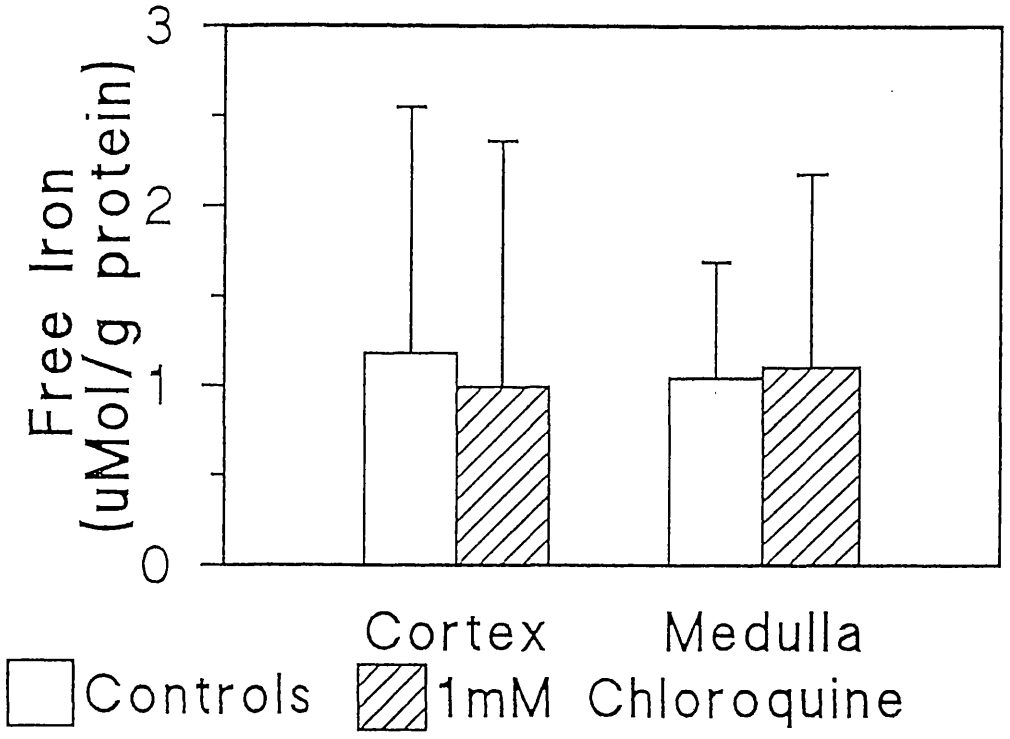

Fig. 7.2: Effect of chloroquine (1mM) on the concentration of Desferrioxamine-available iron in rabbit kidneys reperfused after storage for 48h.

Table 7.2: Perfusate flow rate in kidneys preserved for 48h in HCA containing 1mM chloroquine vs. standard HCA preservation. Values are means±SD.

Perfusate flow rate (ml/min)					
0min		2min		5min	
Control	+Chlrqn	Control	+Chlrqn	Control	+Chlrqn
51.33 ±7.86	57.66 ±5.85	61.66 ±6.53	68.00 ±9.79	66.16 ±3.92	74.00 ±8.00
t=1.76, p<0.20		t=4.42, p<0.01*		t=3.51, p<0.01*	

Table 7.2 shows the perfusion flow rate, which was proportional to vascular resistance in this model, through the vasculature of kidneys preserved for 48h in the presence and in the absence of 1mM chloroquine in the flush preservation solution. At the onset of reperfusion (t=0) the mean flow rate was lower in both groups of kidneys than it was at the subsequent sample points, but there was no significant difference between kidneys stored in CQ-HCA and those stored in standard HCA. Flow through the vasculature was significantly greater in the chloroquine-treated group following 2min of reperfusion, and in both groups the mean flow rate was greater than at the onset of reperfusion. Perfusion flow rate continued to increase in both groups

during the following 5min, and continued to be markedly higher in the kidneys which had been flush-preserved with the solution containing chloroquine.

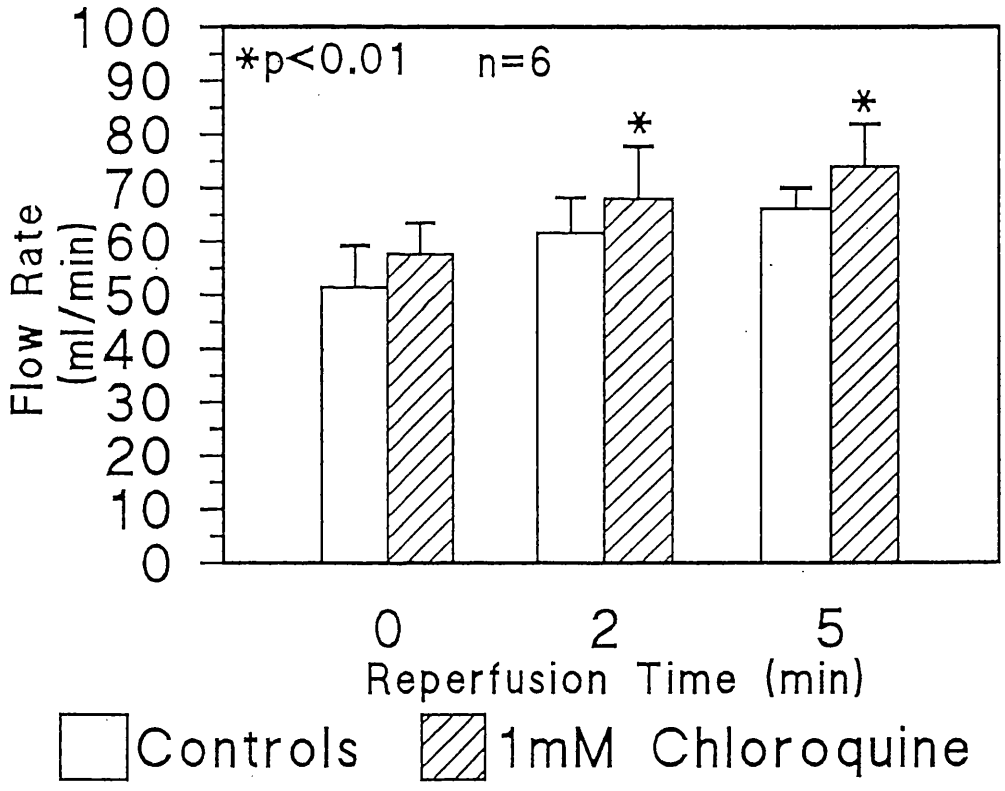

Fig. 7.3: Perfusate flow rate in kidneys preserved for 48h in CQ-HCA vs. standard HCA preservation.

Table 7.3: Urine production in kidneys preserved for 48h in CQ-HCA vs. standard HCA preservation. Values are means±SD.

Urine flow rate (ml/min)					
0min		2min		5min	
Control	+Chlrqn	Control	+Chlrqn	Control	+Chlrqn
1.93 ±0.51	2.50 ±0.86	1.73 ±0.58	2.23 ±0.90	1.70 ±0.83	1.80 ±0.60
t=1.31, p<0.30		t=1.03, p<0.40		t=0.22, p<0.90	

As shown in Table 7.3 there were no significant differences in the rate of urine production between the two treatment groups at any time during the 5min reperfusion period, although the mean urine flow rates tended to be higher in chloroquine treated kidneys.

Table 7.4: Glomerular filtration rate (creatinine clearance) in kidneys preserved for 48h in standard HCA solution and in CQ-HCA. Values are means ± SD.

Glomerular filtration rate (ml/min)					
0min		2min		5min	
Control	+Chlrqn	Control	+Chlrqn	Control	+Chlrqn
2.89 ±0.86	3.09 ±0.95	1.88 ±0.52	2.73 ±1.25	1.81 ±0.91	2.10 ±0.67
t=0.4, p<0.70		t=1.18, p<0.30		t=0.48, p<0.70	

The results in Table 7.4 show that there were no significant differences between GFR in kidneys preserved for 48h in standard HCA and those stored in CQ-HCA. Mean GFR was higher in both groups at the onset of reperfusion than at subsequent sample collection intervals.

Table 7.5: Tubule glucose (Glucs.) reabsorption and sodium reabsorption in kidneys stored for 48h in standard HCA solution and in CQ-HCA. Values are means ± SD, n=6. NB negative values (-) indicate the net excretion of a solute.

	Tubule solute reabsorption (% filtered load)					
	0min		2min		5min	
	Control	Chlrqn	Control	Chlrqn	Control	Chlrqn
Gluc. reab.	56.70 ±10.41	47.91 ±14.40	50.41 ±8.12	62.25 ±11.48	57.03 ±18.38	64.40 ±11.07
	t=0.95, p<0.40		t=2.90, p<0.02*		t=1.35, p<0.30	
Na^+ reab.	28.25 ±16.79	15.73 ±16.95	2.32 ±22.32	14.12 ±19.26	-0.01 ±30.32	11.82 ±17.87
	t=1.44, p<0.20		t=1.47, p<0.20		t=0.96, p<0.40	

As can be seen from Table 7.5, with the exception of increased tubular glucose reabsorption 2min into the reperfusion period, there were no statistically significant differences in either sodium or glucose reabsorption at any of the sample intervals during reperfusion. However, glucose and sodium reabsorption appeared to be higher after 2min and 5min of reperfusion but due to the large standard deviations only glucose reabsorption was significantly different at 2min.

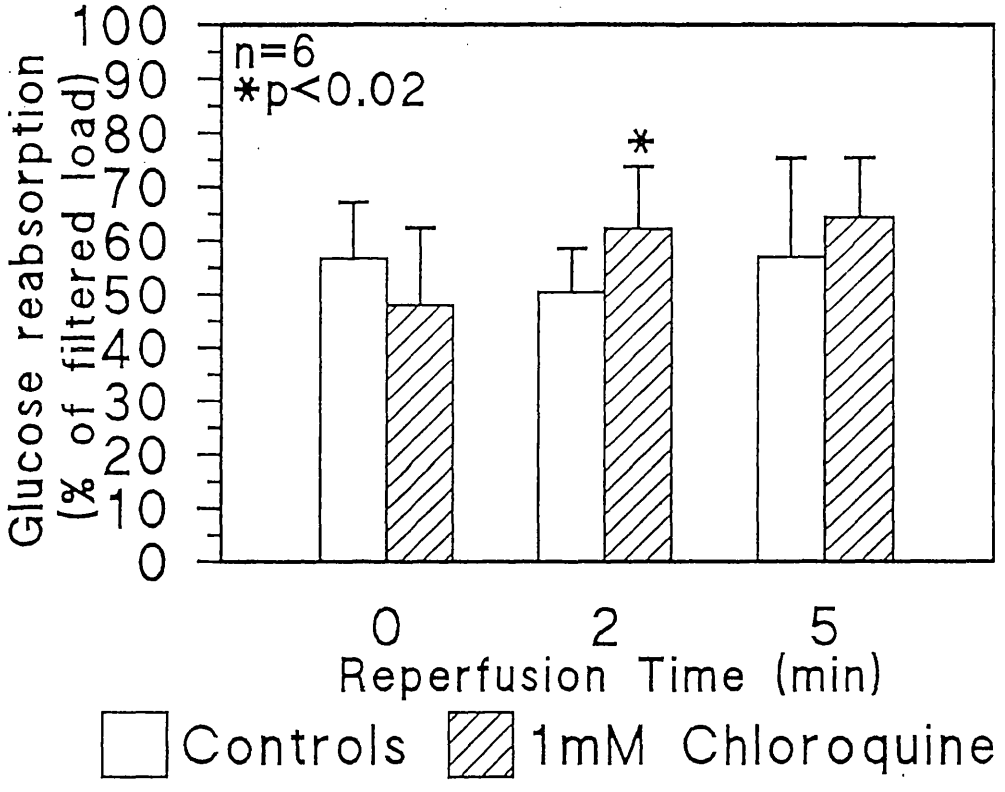

Fig. 7.4: Tubule glucose reabsorption in kidneys stored for 48h in standard HCA solution and in CQ-HCA.

Table 7.6: Release upon reperfusion of the enzyme N-acetyl-ß-D-glucosaminidase (NAG) into urine and vascular effluent of kidneys preserved for 48h in standard HCA and in CQ-HCA. Values are means±SD, n=6.

	Sample NAG activity					
	0min		2min		5min	
	Control	Chlrqn	Control	Chlrqn	Control	Chlrqn
Urine NAG	14357.5 ±1067.2	13427.8 ±1393.9	11267.5 ±345.1	10972.5 ±365.3	11024.2 ±516.3	10885 ±205.8
	t=1.14, p<0.30		t=3.57, p<0.01**		t=0.87, p<0.50	
Perft NAG	11407.7 ±244.41	11510.5 ±610.02	11163.3 ±369.23	10816.2 ±167.05	11111.2 ±517.19	11284 1057.7
	t=0.42, p<0.70		t=2.29, p<0.05*		t=0.71, p<0.50	

The results in Table 7.6 show that mean NAG release from chloroquine-treated kidneys at the onset of reperfusion was similar to untreated control kidneys in both urine and vascular effluents. Samples of these effluents taken at 2min of reperfusion showed small but significant decreases in enzyme activity in both urine and perfusate from treated kidneys. Following 5min of reperfusion, enzyme activity was found to be similar in treated and untreated kidneys in urine samples and in vascular effluent samples.

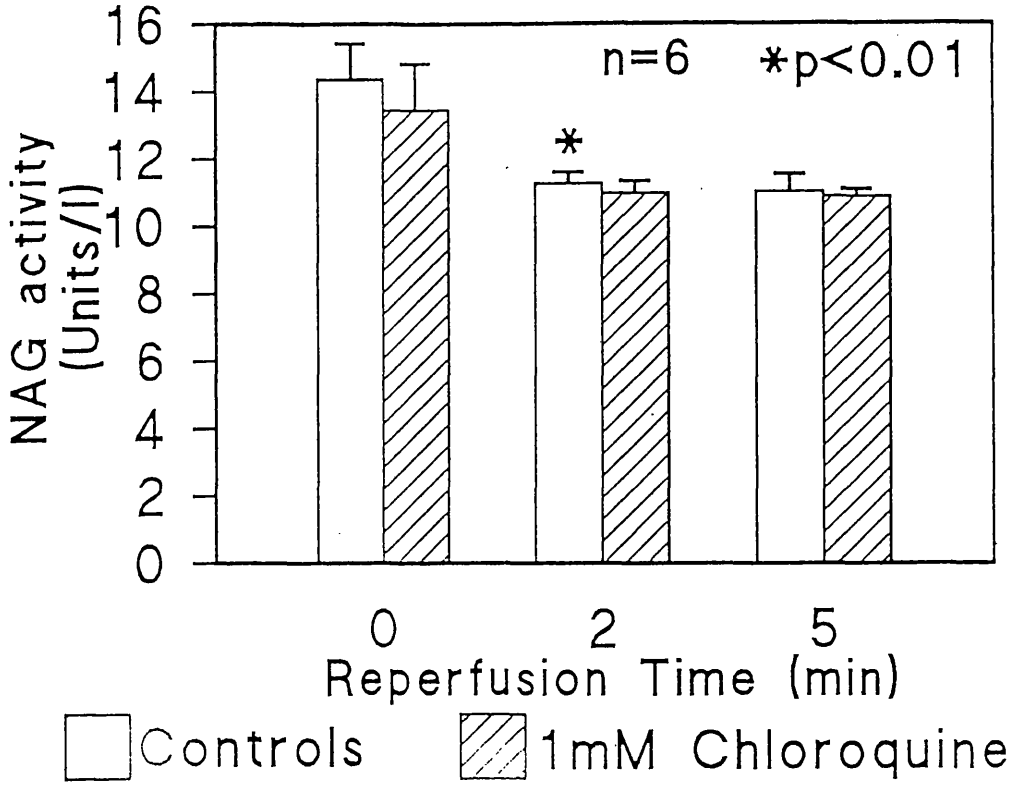

Fig. 7.5: Release upon reperfusion of the enzyme N-acetyl-ß-D-glucosaminidase (NAG) into urine of kidneys preserved for 48h in standard HCA and in CQ-HCA.

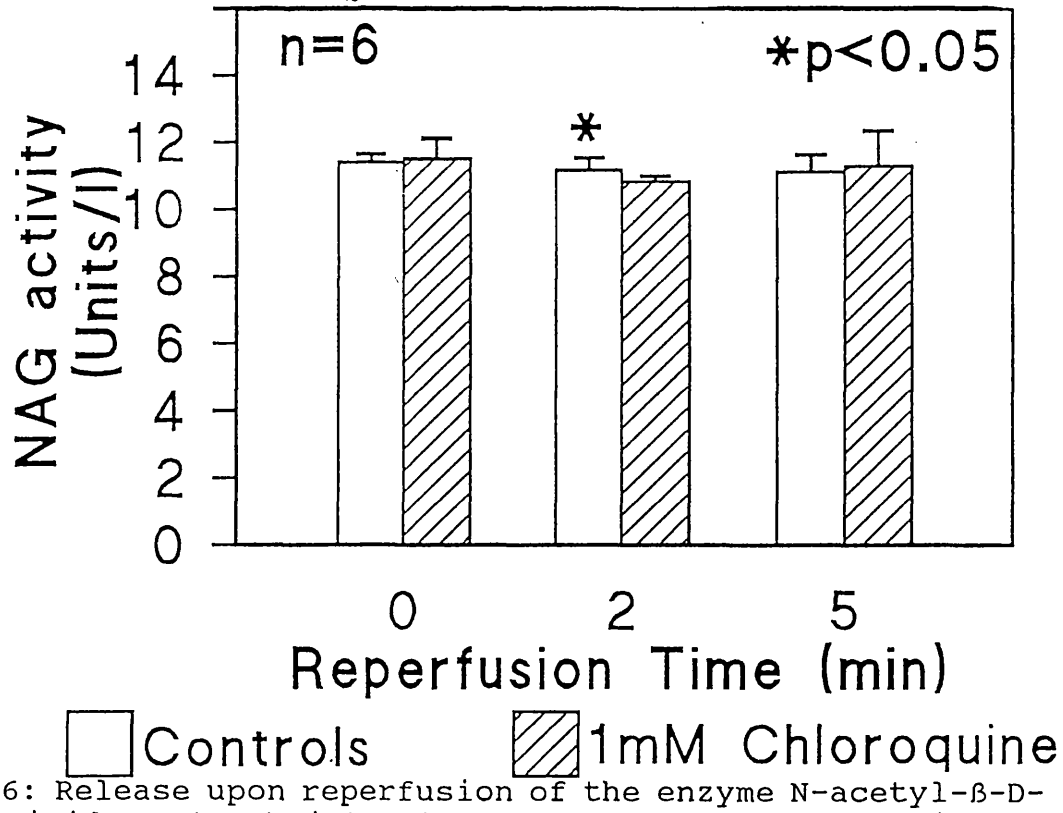

Fig. 7.6: Release upon reperfusion of the enzyme N-acetyl-ß-D-glucosaminidase (NAG) into the vascular effluent of kidneys preserved for 48h in standard HCA and in CQ-HCA.

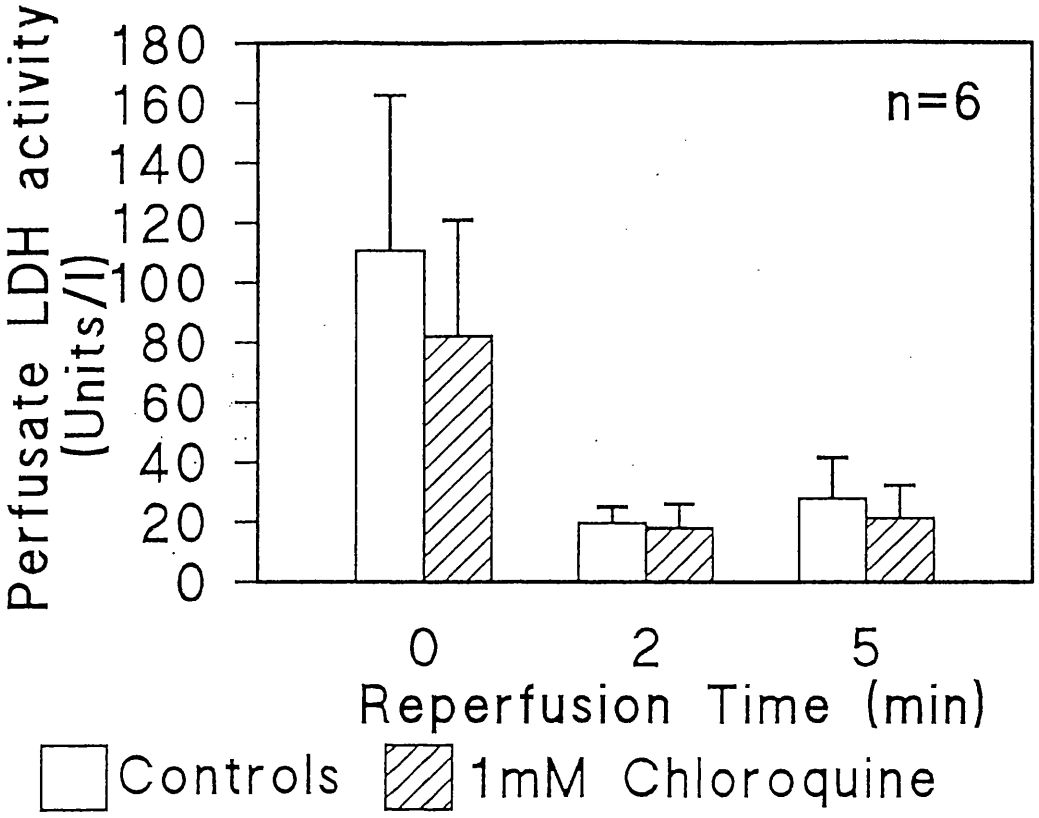

Fig. 7.7: Release of the intracellular enzyme LDH into the kidney vascular space during 5min of reperfusion following 48h preservation in CQ-HCA and in standard HCA.

As shown in Fig.7.7 at the onset of reperfusion (t=0min) mean values for activity of the enzyme in the vascular effluent were lower in kidneys preserved for 48h in the solution containing chloroquine than in control kidneys preserved in standard HCA (means±SD; 82.0±38.8u/l vs. 110.7±52.0u/l). However, this difference was not statistically significant ($p<0.20$). There was a marked decrease ($p<0.01$ for controls, $p<0.001$ for chloroquine-treated kidneys) in the release of LDH within 2min of reperfusion in both groups

(18.0±8.0u/l for chloroquine, 19.7±5.4u/l for controls), and this rate of release stabilized for the remainder of the reperfusion period as indicated by similar values at t=5min (21.3±11.1u/l for chloroquine, 27.9±13.8u/l for controls). There were no significant differences between treated kidneys and untreated controls at either 2min ($p<0.70$) or 5min ($p<0.50$) of reperfusion.

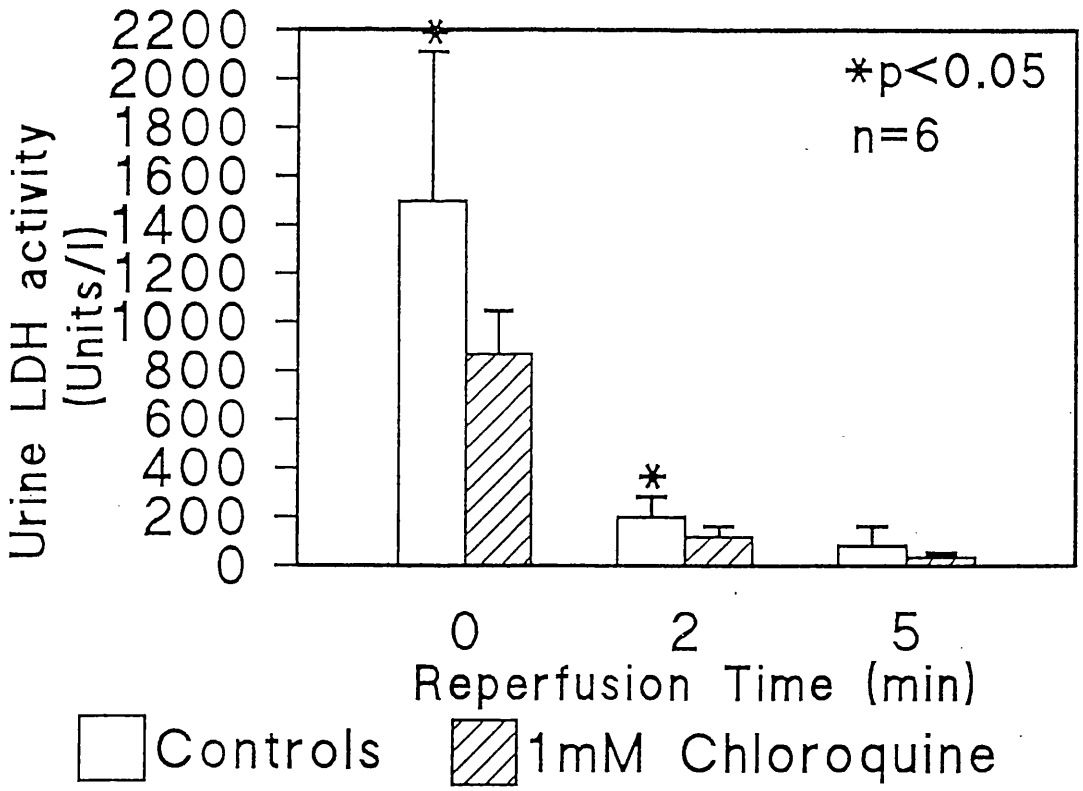

Fig. 7.8: Release of LDH into kidney urinary spaces during 5min of reperfusion following 48h preservation in CQ-HCA and in standard HCA.

As shown in Fig.7.8 there was a significantly lower release of LDH ($p<0.05$) at the onset of reperfusion in kidneys preserved for 48h in CQ-HCA than in control kidneys (means±SD; 868.6±177.8u/l for treated kidneys, 1498±610.6u/l for untreated controls). Enzyme release decreased significantly ($p<0.001$) within the next 2min in both groups of kidneys and continued to be significantly lower ($p<0.05$) in treated kidneys (117.2±44.4u/l) than in untreated controls (198.5±84.8u/l). The mean LDH release was further decreased when the final sample was taken (t=5min); values were 36.1±17.8u/l for treated kidneys and 82.0±79.2u/l for untreated controls. At t=5min there was no significant difference between the two groups ($p<0.20$), although the mean values of LDH release were lower for chloroquine-treated kidneys in all pairs of organs sampled (n=6).

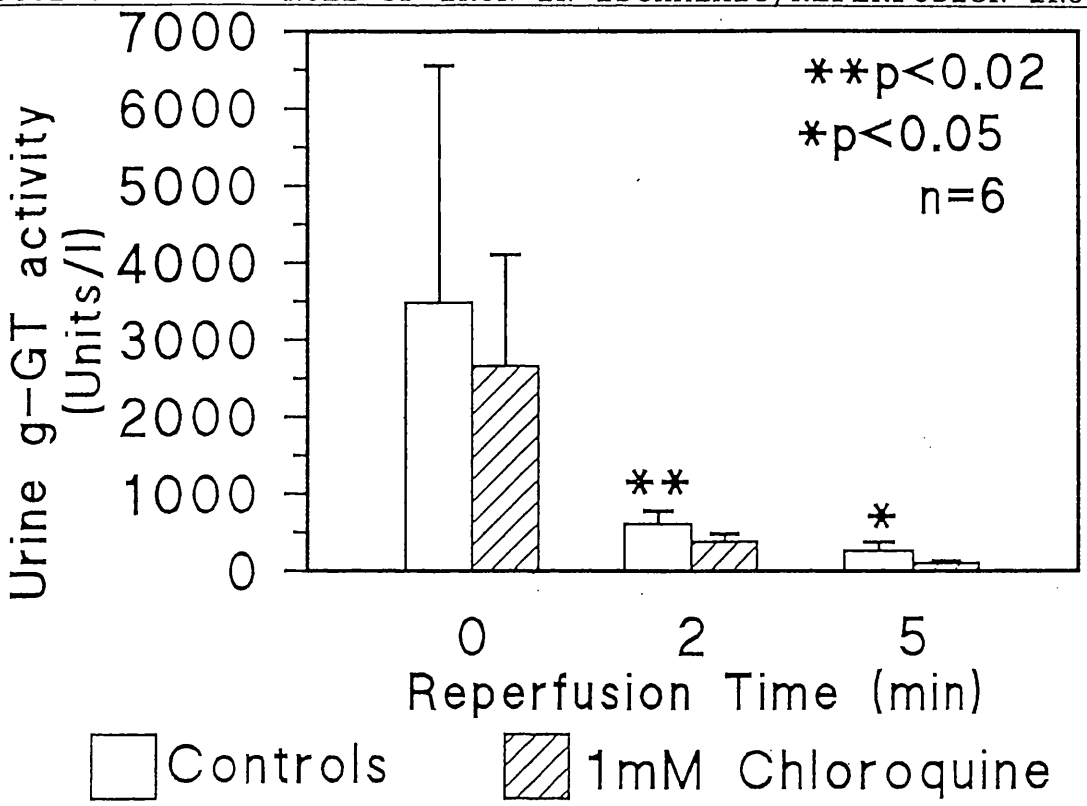

Fig. 7.9: The release into the urine of the proximal tubule brush border enzyme τ-glutamul transferase (τ-GT) in kidneys reperfused for 5min after 48h preservation in CQ-HCA and in standard HCA.

Fig.7.9 shows that mean values of τ-GT activity tended to be lower in the chloroquine-treated group at the onset of reperfusion (2657.6±1448.9u/l in treated kidneys vs. 3483.1±3072.2u/l in controls), but this difference was not statistically significant ($p<0.30$). There was a significant decrease ($p<0.05$ for controls vs. $p<0.01$ for treated kidneys) in τ-GT activity in both groups by t=2min (381.6±103.8u/l in treated kidneys vs. 609.9±170.5u/l in controls). Release was significantly lower ($p<0.02$) in chloroquine-treated organs

than in controls at this time. Enzyme release had further decreased by t=5min in both groups of kidneys and continued to be significantly lower ($p<0.05$) in treated organs. Values at t=5min were 99.6±33.2u/l for treated kidneys and 264.3±115.2u/l for untreated controls.

Table 7.7: Proteinuria (expressed as % of the perfusate protein concentration [g/l]) in kidneys preserved for 48h in either CQ-HCA or standard HCA (controls). Values are means ± SD, n=6.

Proteinuria (% of perfusate [protein])					
0min		2min		5min	
Control	Chlrqn	Control	Chlrqn	Control	Chlrqn
159.7 ±34.1	130.8 ±35.3	93.9 ±20.5	72.0 ±5.1	59.7 ±12.4	37.5 ±19.4
t=1.78, p<0.2		t=2.96, p<0.02*		t=3.16, p<0.01*	

As can be seen from Table 7.7 there was a marked proteinuria, in excess of the perfusate protein concentration, at the onset of reperfusion (t=0min) in both treated kidneys and in untreated controls. This then decreased within 2min to mean levels which were lower than the perfusate protein concentration in both groups of organs. Proteinuria was significantly lower in chloroquine-treated kidneys at the 2min sample point than in untreated controls. Protein loss into the

urine continued to decrease in both groups until the 5min sample point, at which time it was found that proteinuria was still lower in treated kidneys than in untreated controls. Proteinuria was significantly lower after 2min of reperfusion than at the onset of reperfusion (t=0) in both treated kidneys ($p<0.01$) and in untreated controls ($p<0.001$).

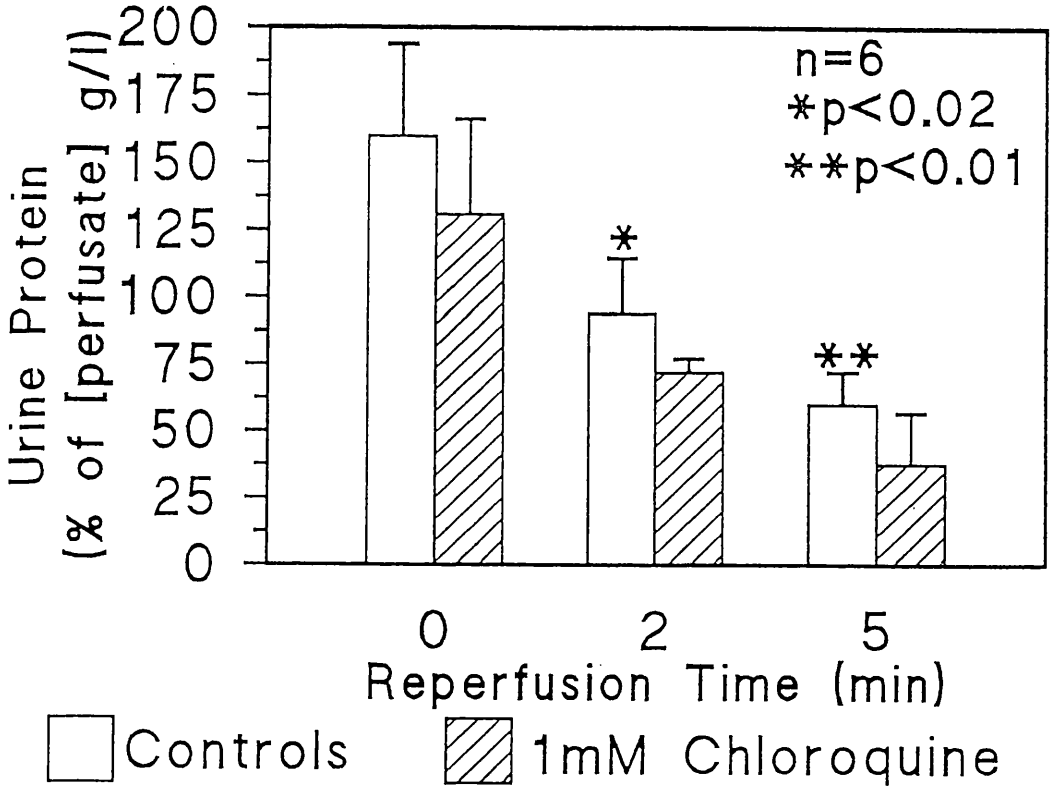

Fig 7.10: Proteinuria (expressed as % of the perfusate protein concentration [g/l]) in kidneys preserved for 48h in either CQ-HCA or standard HCA (controls).

Table 7.8: Protein release into the vascular space at the onset of reperfusion (t=0min) in kidneys preserved for 48h in either HCA containing 1mM chloroquine or in standard HCA (controls). Values are means ± SD of [total protein concentration in vascular effluent (g/l) - perfusate protein concentration (g/l)]. n=6 pairs.

Protein release (% of perfusate [protein]) into vascular effluent at t=0min	
Control	Chloroquine
47.6 ±13.9	40.3 ±22.9
t=0.59, p<0.60	

There was no significant difference in the release of kidney-derived proteins into the vascular space at the onset of reperfusion in kidneys preserved in HCA containing chloroquine compared with control preserved kidneys. The release of protein into the vascular space during reperfusion was undetectable by the assay method used (see Chapter 2) at the sample intervals which followed (2min and 5min).

Table 7.9: Tissue malondialdehyde concentration (μMol/g protein) in kidneys preserved for 48h in CQ-HCA and in standard HCA. Values are means ± SD, n=5 pairs of kidneys.

Kidney pair number	**Tissue MDA concentration (μMol/g protein)**	
	Control	Chloroquine
Mean	0.97	0.89
±SD	0.21	0.17
	t=1.77, p<0.20	

There was no evidence of any change in the extent of lipid peroxidation following reperfusion as indicated by tissue MDA concentration (Table 7.9) in kidneys stored in standard preservation solution compared with those stored in the solution containing chloroquine.

DISCUSSION

Availability of catalytic iron

The finding that the concentration of catalytic iron was decreased to some extent in the renal cortex by flush-preservation with a solution containing chloroquine suggests that a component of the mechanism of iron release during cold ischaemic preservation and reperfusion may be dependent upon the pH of the cytoplasm and/or lysosomes, or some other pathological event mediated by the pH in these compartments. This study did not allow the determination of the precise source of the iron liberated during ischaemia and reperfusion, and therefore particular sites in the kidney may be preferentially protected by the administration of chloroquine.

Perfusate / Urine flow and GFR

Perfusion flow rate through the renal vasculature was similar between the two groups of kidneys at the onset of reperfusion suggesting that vascular resistance was similar at this time. At 2min of reperfusion, perfusate flow rate was significantly higher in chloroquine-treated kidneys ($p<0.01$) and this trend was still evident at the end of the perfusion period. It is possible that chloroquine had a vasorelaxant effect on the renal vasculature either directly or via the release of an endogenous vasorelaxant. However, no such effect has been reported for chloroquine although other compounds

which have been used to prevent lysosomal disruption in the kidney, such as chlorpromazine (Bilde *et al.*, 1977), have been shown to have a vasorelaxant effect on the renal vasculature (Hanson *et al.*, 1971). Chloroquine may have prevented damage to cells involved in the control of vascular tone, such as endothelial cells which release endothelium-derived relaxant factor (Beierwaltes *et al.*, 1992; Poux *et al.*, 1992) and prostanoids (Stier *et al.*, 1992), and which are thought to be involved in the tissue renin-angiotensin system (Hofbauer *et al.*, 1976; Jin, 1988). Treatment of kidneys with chloroquine may have allowed the early restoration of normal control of vascular tone thereby preventing vasospasm which may occur following reperfusion (Anaise *et al.*, 1987). Prevention of damage to endothelial cells, possibly by the inhibition of damage by "free" lysosomal enzymes during preservation and reperfusion, would have been expected to result in improved total vascular integrity, which may explain the above observations.

The finding that the rate of urine production was not altered by preservation of kidneys in HCA containing chloroquine demonstrated that water reabsorption was not affected by this treatment. As in earlier studies, it is likely that the absence of hormonal influences such as promotion of water reabsorption by vasopressin in the distal convoluted tubules and collecting ducts resulted in urine production which was not controlled as precisely as *in vivo*, causing the excretion of large volumes of dilute urine. In the

absence of hormonal influences it was not possible to determine whether chloroquine prevented injury to the selective membrane permeability of the cells of the distal tubules and collecting ducts. In addition, since *in vivo* as much as 80% of the glomerular filtrate water is reabsorbed in the proximal tubules in response to osmotic imbalances caused by solute reabsorption, it is likely that similar rates of urine production between treatment groups reflect similar rates of solute reabsorption (see below).

Creatinine clearance, which was used to measure GFR, is a passive process and is therefore not dependent upon normal metabolic function of the kidney. Decreased injury to either treated or untreated kidneys would not have been obvious from the comparison of GFR values, but the similar values for the two groups suggest that neither experienced increased obstruction of glomerular basement mebrane fenestrations.

Glucose / Sodium reabsorption

Glucose reabsorption, which was unaltered by preservation in a solution containing chloroquine at the onset of reperfusion but improved at 2min of reperfusion, may have been depressed at time 0min due to a lag phase required for the restoration of 'normal' renal metabolism. During this period immediately after the onset of reperfusion, ATP levels, which are likely to have been largely depleted by 48h cold ischaemic preservation, would have started to be restored. The resynthesis of ATP may have been more efficient in

chloroquine-treated kidneys, possibly due to improved preservation of components of the synthetic pathway. Increased enzyme proteolysis by displaced lysosomal enzymes in untreated kidneys may have slowed the rate of recovery of the normal adenine nucleotide pool thereby decreasing the rate of recovery of active glucose reabsorption.

The Na^+/K^+ ATPase has been shown to be inhibited by free lysosomal enzymes in the preserved kidney even if the concentration of these enzymes is low (Varkarakis *et al.*, 1975). This finding would not have been supported by sodium reabsorption measurements in this study if chloroquine actually did effectively prevent cell disruption by inhibiting lysosomal enzyme liberation and activation. As described earlier, the dependence of sodium reabsorption upon hormonal influences may be the overwhelming factor when examining this criterion, and may obscure events in the *ex vivo* kidney model. Another possibility is that restoration of physiological energy substrate (e.g. ATP) concentrations in the cells of the proximal tubules and particularly the loop of Henlé where most sodium reabsorption occurs, required a longer period of reperfusion than was allowed in these experiments (5min). Upon reperfusion following ischaemic preservation there is a lack of these energy substrates within cells and therefore those which are available may be preferentialy directed to metabolic pathways which are most important for cell survival. For example glucose reabsorption may be promoted in order to provide cells with metabolic substrates, or ATP may have been

preferentially used for nucleic acid synthesis, enzyme synthesis or even structural repair. This may be one of the factors which causes delayed renal graft function following transplantation, as frequently occurs clinically.

Release of NAG into vascular and urinary spaces

The observation that the release of the lysosomal enzyme N-acetyl-ß-D-glucosaminidase (NAG) was significantly lower in urine and perfusate effluents from chloroquine-treated kidneys at the 2min sample interval alone could be explained by an obscuring of reperfusion-induced losses at the onset (t=0min) due to the accumulation of the enzyme in urinary and vascular spaces during preservation. Accumulation of intracellular enzymes in these compartments had been shown previously (see Chapter 6). At 2min, after this background enzyme activity had been flushed from the kidneys the effects of chloroquine could be seen. Since the perfusion buffer did not contain chloroquine, any of the compound remaining may have been washed-out into the urinary/vascular effluents, thereby removing any possible lysosomal-stabilizing effects.

Release of LDH into vascular spaces

Much of the LDH released into vascular spaces is likely to have come from damaged endothelial cells. Chloroquine did not appear to prevent damage to the vascular endothelium as shown by similar release of LDH into the vascular spaces of treated and control kidneys at all times during reperfusion.

However, LDH is not specific for endothelial cells and may have originated in other vascular cells such as smooth muscle cells. Release of LDH into the vasculature decreased significantly during the first 2min of reperfusion within each treatment group, suggesting that there was either a burst of enzyme release at the onset of reperfusion or that LDH had accumulated in the vasculature during ischaemic preservation as previously shown (see Chapter 6).

Release of LDH into urinary spaces

Treatment with chloroquine appeared to prevent damage to the urinary tubule cells during the preservation period as shown by decreased release of LDH into the urine at the onset of reperfusion. This lower enzyme release may also have been due to inhibition of reperfusion injury in the treated group. The decrease in enzyme release during the first 2min of reperfusion was probably due to flushing-out of enzymes accumulated during storage. The lower release of LDH into the urine of treated kidneys after 2min of reperfusion indicates that protection of tubule epithelial cells was sustained for at least 2min. This protection may have been the result of inhibition of damage to the cell plasma membrane or by inhibition of complete cell lysis. The permeability of the plasma membranes of cells in untreated kidneys may have increased due to the action of lysosomal lipases during ischaemia and early reperfusion. The release of these enzymes from lysosomes, or the prevention of their activation, may

have been inhibited by the treatment with chloroquine, thereby preventing damage to the cell membranes. The observation that after 5min of reperfusion there were no differences between LDH release from chloroquine-treated kidneys and from controls suggests that chloroquine was flushed-out during early normothermic reperfusion, thereby allowing release and activation of lysosomal enzymes, resulting in cell damage.

γ-GT release into urine

Mean release of γ-GT into urine at the onset of reperfusion was lower in chloroquine-treated kidneys than in controls but this difference was not statistically significant. This high release of γ-GT at the onset of reperfusion was probably due to flushing-out of enzymes accumulated in the urinary tubules during ischaemic organ preservation. Urine samples from control kidneys taken after 2min of reperfusion showed continued damage to proximal tubule brush border membranes, as shown by γ-GT release which was higher than chloroquine-treated kidneys. This continued until the end of the reperfusion period (5min) suggesting that chloroquine treatment had a lasting effect upon the maintenance of the integrity of brush border membranes. The γ-GT enzyme is maintained in the cell membrane by interactions with surrounding membrane components, and its activity is dependent upon these interactions. Destruction of membrane phospholipids in close proximity to the membrane-bound enzyme by phospholipases may have disrupted localised molecular

interactions in the cell membrane, allowing enzyme release into the fluid in the tubule lumens. Inhibition of the release and/or activation of such phospholipases by chloroquine would therefore have prevented loss of γ-GT into the urine.

Proteinuria

Protein appearing in the urine during reperfusion could have originated from two sources, either perfusate protein (BSA) or renal tissue proteins (enzymes or structural proteins). The observation that at the onset of reperfusion proteinuria was in excess of the perfusate BSA concentration shows that some of this protein must have come from kidney tissues. Some of this protein is likely to be the result of accumulation of cell debris, due to autolysis or leakage from cells, during the 48h cold ischaemic preservation period. This is confirmed by the finding that the burst of protein release at the onset of reperfusion was significantly higher than that measured after 2min, at which time much of the cellular debris which had accumulated during storage would have been washed out of the urinary spaces. The decreased proteinuria from chloroquine-treated kidneys compared to controls after 2min of reperfusion probably resulted from decreased loss of proteins from the epithelial cells lining the renal tubules, and also decreased loss of albumin across the glomeruli. The continued lower proteinuria in chloroquine-treated organs after 5min of reperfusion demonstrates that the integrity of the glomerular ultrafiltration mechanism (the structure of the glomerular

vasculature) was well maintained by including chloroquine in the flush-preservation solution. The glomerular basement membrane is likely to have been particularly susceptible to damage by activated lysosomal hydrolases released into the extracellular matrix, due to its close association with the glomerular capillary cells. The action of such hydrolases would have decreased the capability of the basement membrane to act as a selectively permeable barrier during filtration of the fluid passing through the glomeruli. Inhibition of the action of the lysosomal enzymes either within the lysosomes themselves or after liberation from these compartments, may have prevented degradation of the basement membrane.

Protein release into vascular spaces

The release of relatively large amounts of protein into the renal vascular effluent at the onset of reperfusion was probably due to the accumulation of cell degradation products in the vascular space during 48h cold ischaemic preservation, and the presence of residual blood from vessels which were incompletely flushed at the time of nephrectomy. However, the amount of blood remaining in the vasculature is likely to have been the same in all groups, including fresh kidneys which did not show such a large release of protein. Most of this protein probably originated from damaged endothelial cells, either leaking from the cytoplasm across damaged membranes or from the membranes themselves. It would appear therefore that 1mM chloroquine administered via the flush solution was unable to

significantly prevent vascular endothelial cell damage during ischaemic preservation by the suggested mechanism of inhibition of lysosomal enzyme activity, and the inhibition of tissue acidification. It is possible that neither the decreased cell pH nor the liberation and activation of lysosomal enzymes play a significant role in the pathology of ischaemic injury and early reperfusion injury to vascular endothelial cells. In addition the protein estimation assay may not have been sensitive enough to detect small changes in protein release into the vascular space in the presence of the relatively high protein concentration in the reperfusion buffer (1g/L of BSA). The observation that the release of enzymes (a specific group of proteins) into the vascular space was detectable at 2min and 5min after the onset of reperfusion, yet total protein release was undetectable, indicates that the assay procedure for total protein estimation was less sensitive than the enzyme assays employed for the estimation of NAG and LDH (see Chapter 2), and therefore the amount of protein released at t=0min was very large.

Tissue MDA content

From the results of the kidney tissue malondialdehyde (MDA) analysis there is no evidence of an inhibitory effect of chloroquine treatment on lipid peroxidation during reperfusion. However, a number of studies have suggested that lipid peroxidation is a late event in reperfusion injury

(Comporti,1987; Kappus,1987) and therefore the period of reperfusion used for these experiments (5min) may not have been sufficient to induce MDA formation. In this study the concentration of MDA was measured in powdered tissue samples which contained cells from a number of regions in the kidney. It was not possible to determine whether MDA levels were elevated in particular areas of the kidney following reperfusion. Results from the free iron study suggest that lipid peroxidation may be inhibited by chloroquine in particular parts of the kidney due to decreased availability of catalytic free iron in these regions. A further study may be able to determine whether increased lipid peroxidation occurs in tissues with higher levels of free iron.

Chloroquine was not included in the reperfusion solution because this may have resulted in the release of newly synthesized lysosomal enzymes directly into the extracellular space following the restoration of normal metabolism; chloroquine increases the pH of sorting vesicles (normal pH 5) leading to the export of enzymes from the cell rather than directing them to lysosomes. Under conditions of decreased metabolism during hypothermic storage the rate of enzyme synthesis would be expected to be greatly diminished and therefore release into the extracellular environment by this route would be limited.

The method used in this group of experiments to improve post-reperfusion renal function required only a minor modification to be made to the organ flush solution. Therefore

if found to be beneficial to the promotion of post-reperfusion organ function, the addition of chloroquine to flush solutions would be a simple modification to current clinical kidney harvest procedures.

A number of the criteria used to assess kidney function in this study, including perfusion flow rate, tubule glucose reabsorption, NAG release into the vascular spaces and urinary spaces and proteinuria only showed differences between treated and untreated organs after 2min of reperfusion. In most of these cases the reason why differences were not detected at the onset of reperfusion was because of the effects of accumulation of cell degradation products during the cold ischaemic preservation period. It would appear that chloroquine was unable to significantly prevent cell degradation during preservation but was able to inhibit a mechanism which acted to predispose the kidney to reperfusion injury. This would be consistent with the finding that inclusion of chloroquine in flush solutions decreased to some extent the concentration of catalytically-available iron detected in some tissues following 5min of reperfusion. The inhibition of the pathological events during ischaemia which lead to iron-catalysed free radical production (see Chapter 1) may therefore be an important component of the protective effect of chloroquine. Further studies need to be performed to determine whether chloroquine inhibits the release of catalytic iron during cold preservation alone, or whether it inhibits reperfusion-induced release of catalytic iron.

Chapter 7 ROLE OF IRON IN ISCHAEMIC/REPERFUSION INJURY

Some of the beneficial effects of chloroquine seen may be due to a secondary lysosomal membrane-stabilizing effect due to the inhibition of phospholipase activity. Such protection of kidneys from cold ischaemic injury and warm ischaemic injury has been shown by the use of a number of pharmacological agents (Chatterjee, 1977). The anti-inflammatory corticosteroid drug methylprednisolone and the neuroleptic drug chlorpromazine have been used as "membrane stabilizing agents" for this purpose (Starling *et al.*, 1973; Bilde *et al.*, 1977; Chien *et al.*, 1978) and it is possible that a similar mechanism may have been involved in the mechanism of the protective effects of chloroquine. Chlorpomazine has been found to be most effective when administered prior to renal ischaemia and less effective if administered via the preservation flush solution (Moses *et al.*, 1974). It may be necessary to administer chloroquine in a similar way to get optimum benefit from the compound although, as previously mentioned, the procedure chosen for this series of experiments provided the most simple method of drug administration.

SUMMARY

The inclusion of chloroquine in the HCA flush preservation solution of 48h stored kidneys was shown to significantly influence some biological and physiological indices of organ function. It was proposed that the effects seen were due to inhibition of excessive acidification of the

cytoplasm in cells throughout the kidney, and the preservation of lysosomal integrity. The concentration of "free" catalytically-available iron in the kidney may be decreased by flush preservation with CQ-HCA, which may be beneficial and desirable for the prevention of hydroxyl radical production as described in Chapter 1. However there was no evidence of decreased lipid peroxidation in treated kidneys. Perfusate flow rate (proportional to vascular resistance) was shown to be increased to some extent by preservation in CQ-HCA, although urine production and GFR were unaffected. There was evidence that preservation in CQ-HCA promoted tubule glucose reabsorption during reperfusion, but this was short-lived possibly due to the absence of a continued CQ-HCA infusion. There was no evidence of improved sodium reabsorption in treated kidneys. The release of the intralysosomal enzyme NAG into vascular and urinary spaces was found to be decreased during early reperfusion in kidneys which had been reperfused after 48h preservation in CQ-HCA when compared with standard preservation in HCA. Since lysosomal disruption is usually followed by intracellular degradation leading to cell death, the preservation of lysosomal integrity may be of considerable benefit to organ function following transplantation. There was evidence of improved maintenance of integrity in urinary tubule epithelial cells as indicated by decreased LDH and γ-GT release during reperfusion of CQ-HCA treated kidneys. In addition, proteinuria was significantly decreased in CQ-HCA treated kidneys, suggesting that structures vital to the

primary function of kidneys, the glomeruli, were less damaged by ischaemia and reperfusion when the organs were preserved in the solution containing chloroquine.

THE EFFECT OF IRON CHELATION ON REPERFUSION INJURY TO THE RABBIT KIDNEY

INTRODUCTION

In the previous chapter it was shown that the amount of catalytically-available iron in particular areas of kidneys following reperfusion may be decreased by inhibition of tissue acidification during organ preservation. The aim of the studies in this chapter was to determine whether chelation of iron, either intracellularly or extracellularly, decreased free radical production and tissue injury. Chelating agents which inhibit iron-catalyzed hydroxyl radical production are those which, like desferrioxamine, destabilize or prevent the formation of the intermediate iron-H_2O_2 complex. Agents which promote radical production are those which like iron-ethylenediaminetetraacetic acid (EDTA) stabilize the intermediate (Halliwell and Gutteridge, 1990).

Desferrioxamine (DFX) is a chelating agent for trivalent iron (Fe^{3+}) ions, resulting in the stable, non-toxic chelate, ferrioxamine. The chelator takes up iron which is either free or bound to ferritin or haemosiderin, and has a high affinity constant ($K_d=10^{-31}$) for ferric iron. It does not remove iron from haem-containing substances such as haemoglobin and transferrin. Desferrioxamine is routinely used clinically to

promote iron excretion in urine and faeces in cases of iron-overload, thus reducing pathological iron deposits in organs and tissues. DFX also directly inhibits lipid peroxidation and scavenges oxygen radicals (Hoe *et al.*, 1982; Sinoceur *et al.*, 1984). The fact that DFX is approved for clinical use would make it attractive for potential use in the prevention of ischaemia/reperfusion injury in clinical transplantation.

Protection against reperfusion damage has been achieved in the kidney (Paller and Hedlund, 1988), the heart (Ambrosio *et al.*, 1987; Badylak *et al.*, 1987) and in liver cells (Omar *et al.*, 1989) using DFX which binds iron in a non-catalytic complex. However, to prevent reperfusion damage DFX would need to be present at possible sites of renal damage, including the tubular lumens, at the moment of reperfusion. DFX is known to be cleared from the body in the urine and faeces (Summers *et al.*, 1979) and therefore has a short half-life in the circulation. DFX has previously been used in the form of bolus injections given both to the kidney donor prior to kidney harvest, and to the recipient 5 minutes prior to restoration of the blood supply in a kidney transplant model (Gower *et al.*, 1989).

DFX has been shown to inhibit reperfusion injury in a number of organs (see above). In the heart this has been shown to result in greater myocardial function and energy metabolism (Ambrosio *et al.*, 1987; Boli *et al.*, 1987; Reddy *et al.*, 1989). This supports the hypothesis that iron plays a major role in the pathogenesis of reperfusion injury to the heart.

Various sites of iron-dependent oxidative injury have been described. The location and severity of this damage will dictate the pathological manifestations and therefore the nature of any cell, and ultimately organ, dysfunction. The mitochondria have been identified as sites of iron-dependent injury during ischaemia and reperfusion following the observation of improved mitochondrial function upon reperfusion in isolated rat hearts pretreated and reperfused with iron chelators (Van Jaarsveld *et al.*, 1990). This would therefore affect all aspects of cell metabolism. An improved rate of aerobic metabolism has been thought to be responsible for the rapid restoration of normal pH following reperfusion in isolated rat hearts treated with DFX compared with control untreated hearts (Amrosio *et al.*, 1987).

METHODS

Initial studies were undertaken to clarify the pharmacology of DFX distribution in perfused rabbit kidneys. The isolated *ex vivo* perfused kidney preparation was ideal for this purpose, since it allowed study of renal physiology under closely defined conditions. The urine was collected over timed periods from the ureter, and perfusate samples were collected from the arterial supply. From these, the kidney function tests (glomerular filtration rate (GFR), tubular reabsorption of glucose and sodium, urinary protein leakage) were measured by standard assays. The kidneys were perfused for 30 minutes to measure baseline functions, and then DFX (1mM final

concentration) was added to the circuit. During the next 60 minutes serial samples of perfusate and urine were collected. DFX was measured spectrophotometrically (Paller and Hedlund, 1988) and standard kidney function tests were also performed. At the end of the 60 minutes, a second washout-perfusion was instituted using 500 ml of fresh perfusate (minus DFX). Effluent perfusate was collected, and the kidney was homogenised in phosphate-buffered saline for measurements of tissue desferrioxamine levels (see Chapter 2).

The effect of desferrioxamine on post-ischaemic hydroxyl radical production : salicylate hydroxylation studies

In a further study, the hydroxylation of salicylate (1mM) in the kidney reperfusion solution was used to measure hydroxyl radical production in vascular and urinary spaces as previously described (see Chapter 4). The production of the salicylate metabolite 2,3-dihydroxybenzoate (2,3-DHB) was measured as a specific marker of hydroxyl radical (•OH) production. The metabolite 2,5-DHB was measured as a marker of salicylate hydroxylation by normal physiological pathways and by reaction with •OH. The aim of this study was to investigate whether the hydroxyl radical production demonstrated in the previous chapter occured by an iron-dependent mechanism.

Kidneys were removed from male NewZealand White rabbits (2-3Kg) using the standard procedure (see Chapter 2). One kidney of a pair was flushed with 100ml of standard HCA preservation solution from a bag (Viaflex) at a height of 1m

above the organ, the other was flushed using the same method with the same volume of HCA solution containing 400μM DFX. Both kidneys were stored on ice for 48h before *ex vivo* reperfusion for 5min on the perfusion circuit. The control kidney was perfused with standard perfusion solution (not containing bovine serum albumin) and the DFX-stored kidney was reperfused with the same solution containing 400μM DFX. This method of continuous DFX administration was used because earlier experiments (as described above) indicated that this was necessary to ensure that the iron chelator was present at possible sites of free radical production in urinary and vascular spaces during reperfusion. Serial samples of urine and perfusate vascular effluent were collected during the 5min reperfusion period for analysis of salicylate metabolites by HPLC (see Chapter 4) and for enzyme analysis to indicate tissue damage. Samples for analysis of salicylate metabolites were immediately acidified following collection (500μl of perfusate or urine + 26μl 1M HCL), and stored in Nunc tubes in liquid nitrogen (-196°C) until extraction and analysis as previously described (see Chapter 4). Samples of perfusate and urine for enzyme analysis were stored unacidified in identical tubes in liquid nitrogen. They were thawed in a cold-room at 4°C and the enzymes LDH (a cytosolic marker) and γ-GT (a marker of damage to the brush-border membrane of proximal tubules) were measured spectrophotometrically using the methods described previously (see Chapter 2).

RESULTS

Perfusion Sample	Creatinine clearance (ml/min)	Desferrioxamine clearance (ml/min)
30 minute preperfused	3.9 ± 2.0	--
30 minute DFX-perfused	4.6 ± 1.2	4.4 ± 1.1
60 minute DFX- perfused	4.6 ± 1.1	4.7 ± 1.0
DFX-free perfusion	--	0
Tissue DFX level at end		ND

Table 8.1: Clearance of creatinine and desferrioxamine in perfused fresh rabbit kidneys. Values are means±SD, n=5, ND=not detectable.

The mean values for a group of 5 experiments are shown in Table 8.1. During the 30 minute pre-perfusion, GFR had a mean value of 3.9 ml/min. Five minutes after the administration of DFX, the chelator was measurable in the urine, and by 10 minutes a steady-state rate of filtration was achieved. The clearance of DFX was similar to that of creatinine (GFR), which is the rate for a substance freely-filtered across the glomerular network of the kidney, but neither further secreted or reabsorbed by the tubules. Following the second washout-perfusion at the end of 60 minutes, DFX was negligible in the venous effluent, and also not detectable in the homogenised tissue (detection levels of >0.02umol/ml).

Vascular effluent [2,3-DHB] (nMoles/ml)		
Time (min)	Control	DFX
0	1.316±0.764	0.421±0.574*
2	3.424±4.660	2.897±3.974
5	0.475±0.235	0.267±0.196

Table 8.2: Production of 2,3-DHB in the vascular spaces of kidneys reperfused in the presence, and in the absence, of DFX following 48h preservation. Values are means±SD, n=6. *p<0.01

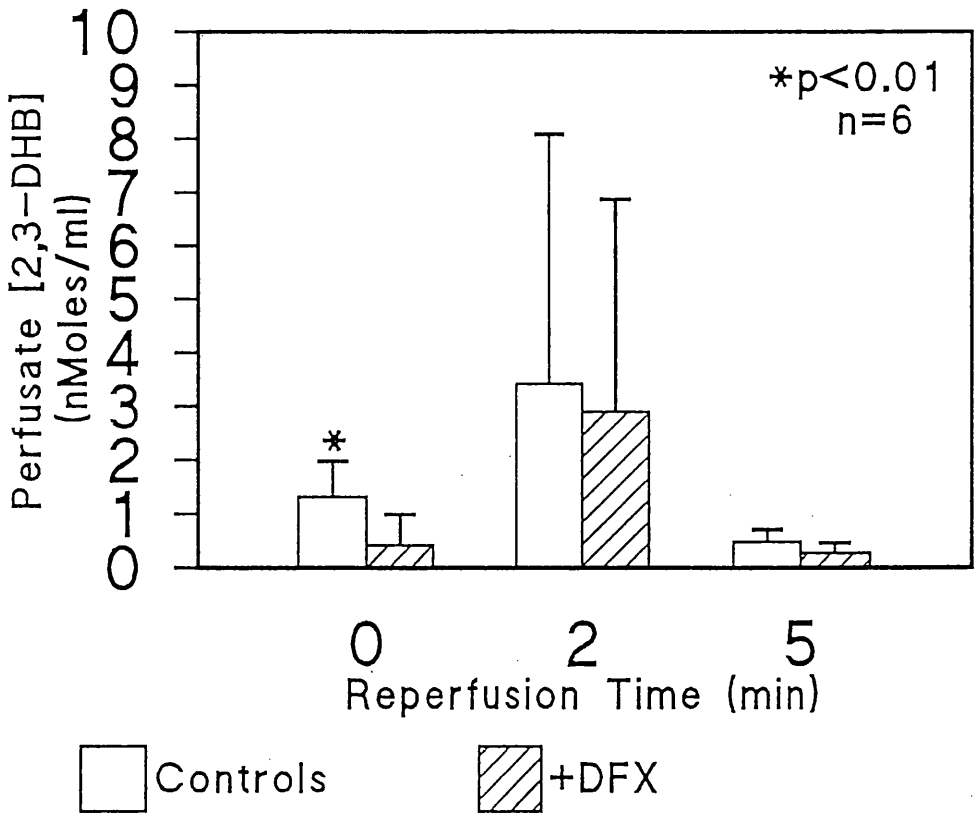

Fig. 8.1: Production of 2,3-DHB in the vascular spaces of kidneys reperfused in the presence, and in the absence, of DFX following 48h preservation.

Vascular effluent [2,5-DHB] (nMoles/ml)		
Time (min)	Control	DFX
0	0.857±1.51	0.294±0.333
2	0.972±0.956	0.756±0.891
5	6.089±3.019	5.210±2.205

Table 8.3: Production of 2,5-DHB in the vascular spaces of kidneys reperfused in the presence, and in the absence, of DFX following 48h preservation. Values are means±SD, n=6.

As shown in Table 8.2, the production of the hydroxyl radical-dependent metabolite of salicylate, 2,3-dihydroxybenzoate (2,3-DHB), at the onset of reperfusion, was significantly lower ($p<0.01$) in the vascular effluent of kidneys preserved and perfused in the presence of 400μM DFX compared with DFX-free control kidneys (0.421±0.574nM/ml vs. 1.316±0.764nM/ml). The production of the metabolite 2,5-DHB (Table 8.3), which is also produced by normal physiological mechanisms, was also elevated in control kidneys at the onset of reperfusion compared to DFX treated kidneys, although there was no statistically significant difference (mean±SD ; 8.57±15.16 vs. 2.94±3.33, $p<0.50$). Salicylate concentrations in the vascular effluent at this time followed similar trends.

Urine [2,3-DHB] (nMoles/ml)		
Time (min)	Control	DFX
0	28.796±19.305	9.523±6.152
2	47.706±35.096	23.370±13.124
5	5.806±1.997	3.699±2.774

Table 8.4: Production of 2,3-DHB in the urinary spaces of kidneys reperfused in the presence, and in the absence, of DFX following 48h preservation. Values are means±SD, n=6.

Urine [2,5-DHB] (nMoles/ml)		
Time (min)	Control	DFX
0	1.413±1.302	0.958±1.005
2	3.482±2.415	2.647±2.469
5	4.311±2.114	3.011±2.606

Table 8.5: Production of 2,5-DHB in the urinary spaces of kidneys reperfused in the presence, and in the absence, of DFX following 48h preservation. Values are means±SD, n=6.

The production of 2,3-DHB in the urinary space (Table 8.4) at the onset of reperfusion was significantly higher ($p<0.01$) in untreated control kidneys (27.49±17.56nM/ml) compared with DFX-treated kidneys (9.33±5.43nM/ml). The

production of 2,5-DHB (Table 8.5) was similarly elevated ($p<0.05$) in untreated kidneys (13.96±11.65nM/ml) compared with DFX-treated kidneys (9.44±9.00nM/ml). There were no significant differences in the amount of salycylate present in the urine between the two groups at the onset of reperfusion.

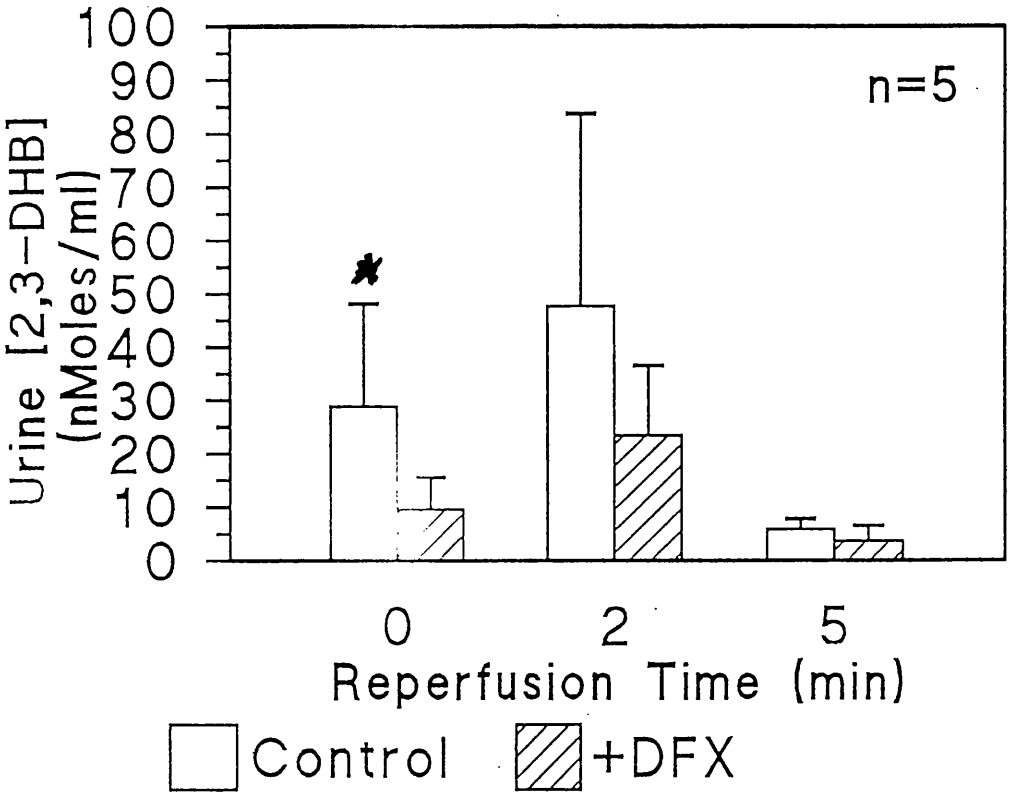

Fig. 8.2: Production of 2,3-DHB in the urinary spaces of kidneys reperfused in the presence, and in the absence, of DFX following 48h preservation.

Table 8.6: Perfusion flow rate and urine production during reperfusion of DFX-treated and control 48h stored kidneys, Mean±SD ml/min. n=6

Perfusate flow rate & Urine flow rate					
t=0		t=2		t=5	
Control	+DFX	Control	+DFX	Control	+DFX
P 70.66± 9.35	P 75.00± 10.93	P 78.00± 6.06	P *94.00± 18.15	P 82.50± 10.72	P 94.00± 18.15
U 1.85± 1.49	U 1.98± 2.00	U 1.71± 1.18	U 2.00± 1.70	U 1.48± 1.01	U 1.31± 1.24

P=Perfusion flow U=Urine flow *p<0.02

The graph of perfusate flow rate in kidneys perfused either in the presence or absence of DFX (Fig. 8.3) shows that mean flow rates in the DFX-treated group were higher than in the untreated control group. This difference was statistically significant ($p<0.02$) following 2min of reperfusion when values were 94.00±18.15ml/min for DFX-treated kidneys vs. 78.00±6.06ml/min for untreated kidneys. In both groups perfusion flow rate increased during the 5min reperfusion period but DFX-treated kidneys reached a maximum

flow rate plateau earlier (within 2min) than control kidneys. Urine flow rate was unaltered by the presence of DFX and remained similar in the two groups of kidneys throughout the reperfusion period.

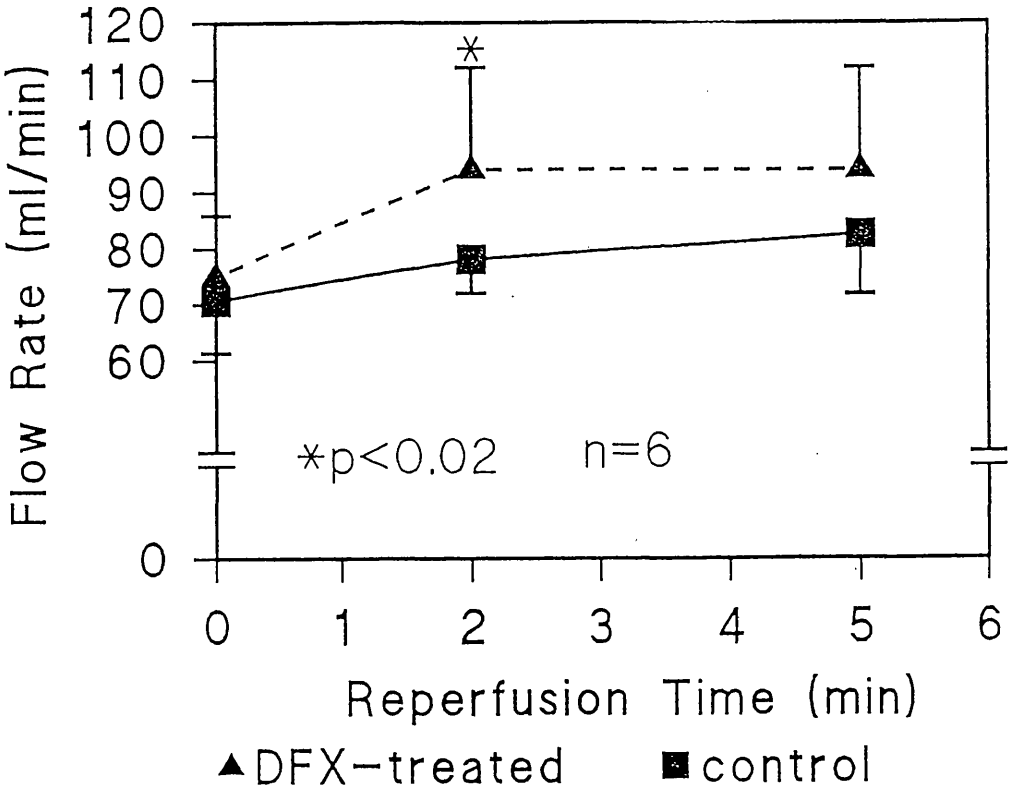

Fig. 8.3: Perfusate flow rate in kidneys reperfused in the presence, and in the absence, of DFX following 48h preservation.

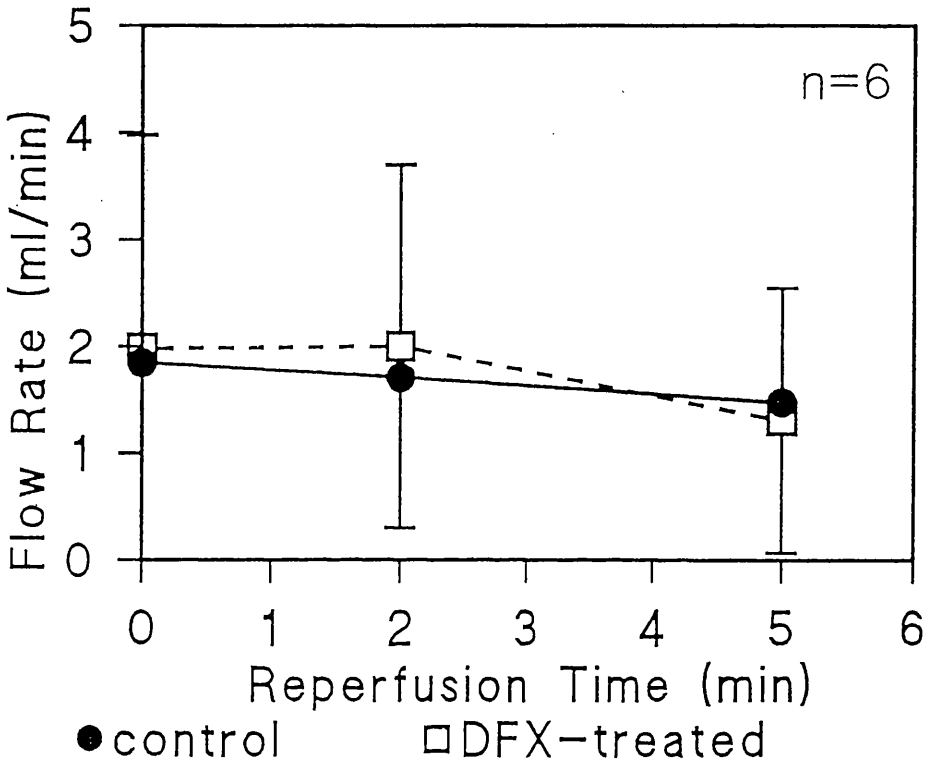

Fig 8.4: Urine flow rate in kidneys reperfused in the presence, and in the absence, of DFX following 48h preservation.

Table 8.7: Release of the intracellular enzyme lactate dehydrogenase (LDH) into vascular and urinary spaces, and proximal tubule membrane enzyme τ-glutamyl transpeptidase (τ-GT) into urinary space during 5min reperfusion (units/l). Values are mean±SD, n=6. Cntrl=control organs

Enzyme release into urinary / vascular spaces						
	0min		2min		5min	
	Cntrl	+DFX	Cntrl	+DFX	Cntrl	+DFX
Urine τ-GT (U/l) n=5	1882.82 ±805.18	1821.11 ±1236.8	199.39 ±85.90	335.32 ±449.11	71.04 ±26.14	82.86 ±48.31
	t=0.22, p<0.90		t=0.78, p<0.50		t=1.06, p<0.40	
Urine LDH (U/l) n=5	514.20 ±207.81	784.13 ±354.09	29.24 ±11.55	55.97 ±41.22	9.25 ±5.47	14.48 ±7.58
	t=1.34, p<0.30		t=1.51, p<0.20		t=1.32, p<0.30	
Pfst. LDH (U/l) n=6	88.17 ±67.62	87.22 ±70.55	10.23 ±3.25	9.41 ±3.92	11.49 ±7.41	11.35 ±9.63
	t=0.06, p<0.90		t=1.0, p<0.40		t=0.10, p<0.90	

As can be seen from Table 8.7 there were no significant differences in enzyme release into urinary and vascular spaces between DFX-treated kidneys and untreated control kidneys at any time during reperfusion. In all cases enzyme release was greatest at the onset of reperfusion and decreased during the following 5 minutes.

DISCUSSION

The results of the DFX distribution study demonstrated that DFX was rapidly filtered across the glomeruli of kidneys and passed into the tubular lumens. The clearance of DFX was similar to that of creatinine, and thus there was no evidence of active reabsorption from the tubular lumens as had been previously suggested (Summers *et al.*, 1979). After 60min perfusion of the kidneys with 1mM DFX, and subsequent short washout perfusion with fresh perfusate, no evidence of tissue accumulation of DFX was found. In relation to the use of DFX to chelate biologically-active iron and so prevent reperfusion damage in kidneys following transplantation, it was found that a bolus injection given just prior to restoration of blood supply would be expected to rapidly reach vascular and urinary extracellular spaces. However, to maintain DFX therapy for longer periods after reperfusion, or to maintain concentrations in order to attempt to chelate intracellular catalytic iron pools, a continuous infusion would be needed. This is confirmed by studies in which

discontinuation of DFX treatment showed loss of its protective effects (Van Jaarsveld *et al.*, 1992). The need for constant DFX infusion may be eliminated by current investigations into the use of DFX conjugated with a large molecule such as starch which will not easily pass into the urine (Drugas *et al.*, 1991; Rosenthal *et al.*, 1992), but still retains significant efficacy for iron chelation (Mousa *et al.*, 1992). Further modifications may continue to improve the effectiveness of DFX therapy.

The higher mean values for perfusion flow rate in DFX-treated kidneys demonstrates that vascular resistance was reduced in these organs since perfusion pressure (150/100mmHg) was kept constant during these experiments. This indicates either a direct vasorelaxant effect of DFX upon vascular smooth muscle or an indirect effect secondary to decreased availability of free iron in kidneys treated with the chelating agent. The vasorelaxant properties of DFX have been well described; DFX has been shown to cause severe hypotension when administered parenterally (Summers *et al.*, 1979) although the exact mechanism is unclear. This decreased vascular resistance did not appear to affect urine production which suggests that the transglomerular filtration pressure was similar in the two groups. Urine production was similar in the two groups of kidneys at the onset of reperfusion and throughout the 5min reperfusion period; hence, preservation of kidneys in a storage solution containing 400μM DFX, and perfusion with a buffer containing

400μM DFX did not alter primary function of the kidneys.

The graph of 2,3-DHB production in vascular spaces against reperfusion time in DFX-treated kidneys and in untreated controls (Fig. 8.1) shows a significant attenuation ($p<0.01$) of hydroxyl radical production at the onset of reperfusion in the presence of DFX. The decrease in initial production (time 0) of 2,3-DHB may have been due to decreased extracellular hydroxylation of salicylate, because of efficient chelation of catalytic extracellular iron by DFX. The greater production of 2,3-DHB seen after 2min of reperfusion may be due to the release of intracellular 2,3-DHB formed at sites which may be inaccessable to DFX administered by this method. This would exlain why DFX was not beneficial 2min into the reperfusion period. Production of 2,3-DHB at 5min of reperfusion marked a tailing-off of the initial bursts of hydroxyl radical production. Free radical production may have occurred at 2 different tissue sites; first extracellularly, the first site to receive oxygen upon reperfusion, and then intracellularly, possibly following diffusion of oxygen into cells. It is also possible that the increased hydroxyl radical production after 2min of reperfusion was due to DFX itself. High concentrations of DFX may exert a paradoxic pro-oxidant effect. It has been postulated (Borg and Schaich, 1986) that DFX has biphasic actions, increasing oxidative damage in a dose-dependent manner in the presence of reducing agents. However it is unlikely that the concentration of reducing

agents in the kidney would have been greater following 2min of oxygenated reperfusion than at the onset of reperfusion following 48h of ischaemia.

Although salicylate hydroxylation studies show that DFX significantly lowered hydroxyl radical activity in urinary and vascular spaces during the early reperfusion period, this was not reflected by correspondingly decreased tissue injury in these compartments as indicated by enzyme release. This suggests that although hydroxyl radicals are produced at the onset of reperfusion their role in independently causing marked direct tissue damage may be limited. It must also be borne in mind that the mechanism of inhibition of hydroxyl radical production may not be entirely due to iron chelation since there is evidence that DFX is also capable of acting as a direct scavenger of superoxide radicals (Menasch *et al.*, 1988). This would therefore decrease the amount of hydrogen peroxide produced by the dismutation of superoxide (both spontaneous and enzyme-catalysed) thereby removing any substrate available for the Fenton reaction.

SUMMARY

Preliminary studies showed that DFX in perfusate was rapidly cleared into the urine and did not accumulate in kidney tissues. In addition to inclusion of DFX in the HCA preservation solution, a continuous infusion of DFX was used during reperfusion to chelate iron which was in a form capable of catalysing hydroxyl radical production. This

produced a reduction in vascular resistance without affecting the rate of urine production. The presence of DFX caused a significant attenuation of hydroxyl radical production in vascular spaces during early reperfusion but this was not apparent in urinary spaces. This may have been due to biphasic radical production, first at extracellular sites, followed by production at intracellular sites. There was no evidence that inhibition of hydroxyl radical production by iron chelation prevented tissue damage as assessed by loss of cytosolic and membrane-bound enzymes into vascular and urinary spaces. The studies in this chapter thus suggest that iron chelation may have a significant role in the inhibition of post-reperfusion free radical production but DFX, administered as described, may not be capable of preventing tissue damage.

THESIS DISCUSSION

An isolated *ex vivo* perfused rabbit kidney model was used to investigate the role of oxidative stress in ischaemia / reperfusion injury to kidneys preserved by methods used in clinical transplantation. Early studies were aimed at determining which renal functional changes were associated with cold ischaemic preservation for up to 72h. Renal vascular resistance during 30min of reperfusion was largely unaffected by cold ischaemic preservation for up to 72h. Flow was decreased, however, in 72h preserved kidneys at the onset of reperfusion compared to fresh kidneys, possibly due to collapse of vessels during preservation or accumulation of cell degradation products in the vascular spaces at this time. Disruption of normal prostaglandin ratios (Lefer *et al.*, 1985) during ischaemia may also have affected control of vascular tone upon reperfusion. The high arterial flow rates measured reflected characteristics of perfusion with an acellular solution. Disruption of normal flow following extended preservation may be more pronounced when kidneys are grafted into a recipient *in vivo* because of the greater viscosity of blood. The differences referred to above may also explain why preservation for up to 72h did not significantly alter the rate of urine production during reperfusion. Even kidneys which were preserved for 72h produced urine at a rate which exceeded expected *in vivo* values (Mitruka *et al.*, 1977). In

addition, the absence of humoral and neural influences in the *ex vivo* model may have affected the ability of kidneys to reabsorb water. The administration of the diuretic frusemide during the nephrectomy procedure may also have caused elevated urine production. When fresh kidneys were compared with 72h stored organs glomerular filtration rate (GFR) was depressed only at the onset of reperfusion, possibly as a result of occlusion of glomerular basement membrane fenestrations by debris which may have accumulated during preservation. Changes in the tone of the mesangium during preservation, possibly due to distruption of the metabolism of endogenous vasoactive compounds (Schultz *et al.*, 1990), may also have reduced the filtration surface area until normal tone was resumed during later reperfusion. Proteinuria was greater in 72h stored kidneys than fresh organs, indicating increased permeability of glomerular capillaries. This was in aggreement with studies by Lambert and colleagues (1986). Urinary protein concentrations in excess of the perfusate concentration at the onset of reperfusion suggested that proteins from tubule epithelial cells passed into the urine. This loss may have represented reperfusion-induced cellular damage, or may have been due to washing out of proteins accumulated in urinary spaces after leakage into tubules during storage. These endogenous proteins probably included enzymes, which have been shown to leak from cells under similar conditions of ischaemia and reperfusion in the heart (Wickens *et al.*, 1987). In addition, measurement of the loss of kidney proteins has been

used clinically to monitor renal transplant rejection (Steinhoff *et al.*, 1991).

The ratios of adenine nucleotides in tissues (the energy charge) are known to be important for normal metabolism; possibly more so than the absolute concentration of ATP. The tissue energy charge decreased significantly within 24h of cold preservation. This decrease in energy charge is a characteristic event in ischaemia and has been shown in a number of other organs (e.g. heart, Asimakis *et al.*, 1992). Following 30min of reperfusion the energetic status of kidneys stored for 24h and 72h was restored to values similar to those of fresh perfused organs. This suggested that mitochondrial integrity was well preserved, and that the pool of substrates for ATP synthesis was well maintained (Stromski *et al.*, 1988). Loss of mitochondrial ability to resynthesise ATP has been described as the principal limiting factor in the recovery from ischaemic injury (Trump *et al.*, 1976). The energy charges of kidneys freeze-clamped *in vivo* were higher then those clamped after *ex vivo* reperfusion (including fresh kidneys). These differences were probably due to characteristics of *ex vivo* reperfusion such as the inability to deliver sufficient metabolic substrates to the kidney. A decrease in tubular sodium reabsorption was noticed within 15min of the onset of reperfusion of 72h stored kidneys. Since the supply of ATP was believed to have been sufficient, as described above, this decreased reabsorption may have been due to direct damage to the Na^+/K^+ATPase ion transporters in the basolateral membranes

(Molitoris and Kinne, 1987), or to the protein channels through which the solutes must pass to enter cells. Free radicals have been shown to depress the activity of the Na^+/K^+ATPase (Kako *et al.*, 1988; Huang *et al.*, 1992). Decreased glucose reabsorption was also seen after 30min of reperfusion of 72h stored kidneys, possibly as a result of direct damage to Na^+/glucose symport protein.

The subsequent experiments were designed to investigate whether free radical generation accompanied the functional disturbances described above. When salicylate was included in the reperfusion solution, two major metabolites were formed; 2,3-dihydroxybenzoate (2,3-DHB) believed to be formed solely by the action of hydroxyl radicals (•OH) upon salicylate (Halliwell *et al.*, 1991), and 2,5-dihydroxybenzoate (2,5-DHB), produced by the action of cytochrome P_{450} upon salicylate and also by reaction with •OH (Ingelman-Sundberg *et al.*, 1991; Onodera and Ashraf, 1991). When kidneys which were reperfused under hypoxic or normoxic conditions in the presence of salicylate were examined it was found that fresh hypoxic organs had a higher urine flow rate after 5min of reperfusion than did normoxic organs. Decreased tubular sodium reabsorption in the presence of low oxygen concentrations may have produced natriuresis with accompanying diuresis. This effect was not noticable in 48h preserved kidneys, possibly due to storage-dependent suppression of solute reabsorption, as described previously for 72h stored kidneys, being the overwhelming factor in these organs. Vascular resistance was

lower in normoxic 48h stored kidneys than in hypoxic kidneys at the onset of reperfusion, suggesting that metabolic processes involved in the control of vascular tone were resumed more rapidly in normoxic organs. No differences in perfusate flow rate were seen in the later stages of reperfusion, indicating that hypoxic organs eventually achieved a similar level of vascular resistance to normoxic organs. The production of the hydroxyl radical-dependent salicylate metabolite 2,3-DHB in the vascular and urinary spaces of fresh kidneys was not significantly different between normoxic and hypoxic kidneys. This suggested that the oxygen concentration in the reperfusion solution did not significantly affect the rate of free radical production in fresh kidneys. However, the metabolite 2,5-DHB, which is produced by the reaction of •OH with salicylate in addition to its production by the action of cytochrome P_{450}, was elevated at the 2min sample point. This may reflect some degree of radical production in these fresh kidneys.

There was no evidence of increased production of 2,3-DHB in vascular or urinary spaces of kidneys reperfused normoxically after 48h cold ischaemic preservation, when compared with hypoxically reperfused organs. The concentration of the metabolite 2,5-DHB was, however, elevated when a higher oxygen concentration was used. Since it was unlikely that normal metabolism via cytochrome P_{450} (Ingelman-Sundberg *et al.*, 1991) was stimulated at the onset of reperfusion after 48h of cold ischaemia, this elevated production of 2,5-DHB may

have been the result of a burst of free radical production. Hypoxically reperfused kidneys showed some evidence of hydroxyl radical production. Thus it is possible that the atmospheric oxygen which remained in the kidney tissues during storage was sufficient to produce oxygen-derived free radicals upon normothermic reperfusion, or that the small quantity of oxygen in the hypoxic perfusate caused this radical production. Low concentrations of oxygen have been shown to cause free radical-mediated damage (Salaris and Babbs, 1989). The finding that fresh kidneys also showed some evidence of free radical production may have been the result of metabolic changes during the short period of ischaemia to which fresh kidneys were inevitably subjected between harvest and reperfusion.

The production of 2,3-DHB in vascular spaces of 48h stored kidneys which were reperfused hypoxically was significantly higher than in fresh kidneys at the onset of reperfusion, demonstrating preservation-dependent radical production. It was believed that these radicals were produced by either the small quantity of oxygen present in the hypoxic reperfusion solution, or by oxygen which had equilibrated with the kidney tissues during storage. Similar results have been reported using rat kidney slices *in vitro* (Steiner and Babbs, 1989). Normoxic kidneys did not show storage-dependent free radical production in vascular spaces at the onset of reperfusion, but did show this after 2min of reperfusion.

The production of 2,5-DHB in the renal vasculature was

found to be significantly higher in stored organs than in fresh organs at all times during normoxic reperfusion. Since it is unlikely that cytochrome P_{450} mediated metabolism would be greater in stored organs, whose physiological functions were known to be suppressed, this probably represented increased •OH production. Significantly higher 2,5-DHB production was also seen in hypoxic stored kidneys compared with fresh organs at the onset of reperfusion and after 2min. Following 5min of reperfusion increased production was no longer apparent, suggesting that initial radical production was from oxygen within kidney tissues and that this was depleted as hypoxic reperfusion progressed. The exact anatomical sites of production of the salicylate metabolites detected in the vascular effluents are unclear. However, the rapid appearance of radical-dependent salicylate metabolites in the vascular spaces immediately at the onset of reperfusion would suggest that some •OH production occurred extracellularly.

No significant differences in the concentration of 2,3-DHB in the urinary effluents of fresh vs. 48h stored kidenys were seen during either normoxic or hypoxic reperfusion. However, normoxically reperfused kidneys showed elevated production of 2,5-DHB in the urinary spaces of 48h stored kidneys compared with fresh organs. This suggest that either there was elevated free radical production in the cells of the urinary tubules, previously demonstrated in tubule cultures by Rovin and colleagues (1990), or urinary spaces, or that the

products of the reaction of •OH with salicylate were filtered across the glomeruli into the urine. Radical production may have been greatest in areas with the highest oxygen concentration (i.e. the vasculature) and radical products may then have been filtered into the urine.

There was no evidence of increased lipid peroxidation in 48h preserved kidneys when reperfused normoxically, without the protective effects of salicylate (Liu *et al.*, 1992), compared with hypoxic reperfusion. In addition there was no evidence of increased lipid peroxidation when these stored organs were compared with similarly reperfused fresh kidneys. These results were observed despite the fact that the salicylate hydroxylation experiments suggested that hydroxyl radical production was higher in stored kidneys, and therefore some degree of hydroxyl radical induced lipid peroxidation was anticipated (Gutteridge, 1984). The level of radical production may not have been sufficient to cause significant lipid peroxidation or the majority of radical production may have occurred some distance from cellular polyunsaturated fatty acids. Peroxidation products may also have been washed out of the kidney or metabolized during later reperfusion (Konya *et al.*, 1990; Park and Kehrer, 1991). It is also possible that the 5min reperfusion period was too short to induce a noticable increase in peroxidation. Other studies have suggested that lipid peroxidation is a late event following free radical production (Comporti, 1987; Kappus, 1987). There was evidence that normoxic reperfusion after 48h

preservation caused more injury to vascular endothelial cells, measured by the release of LDH, than did hypoxic reperfusion. This is noteworthy because although suppression of normal oxidative metabolism was expected to cause increased cell damage (Park and Kehrer, 1991), the effects of oxidative injury in my experiments appeared to be more significant. This injury seemed less apparent when kidneys were reperfused in the presence of salicylate, possibly by scavenging •OH, thereby preventing them from damaging cell constituents. It is likely that the high "background" levels of γ-glutamyl transpeptidase (γ-GT) in the urinary spaces of 48h preserved kidneys, due to accumulation during storage, obscured the injurous effects of normoxic reperfusion that were evident in fresh kidneys. This reperfusion injury to proximal tubule brush border membranes of fresh organs may have been caused by the short period of ischaemia to which these kidneys were inevitably subjected. Although the rate of urine production did not appear to be different in hypoxic kidneys compared with normoxic organs, perfusion flow rate was significantly lower after 2min of normoxic reperfusion in both fresh and 48hr stored kidneys when compared with hypoxic organs. This increased vascular resistance did not continue until the end of the reperfusion period and was probably due to delayed restoration of normal vascular tone in pre-glomerular vessels since urine production was not affected. Transmission electron microscopy revealed some degree of vascular damage and blebbing of proximal tubule membranes in each of the treatment

groups (hypoxically and normoxically reperfused fresh and 48hr stored kidneys). This phenomenon may have been secondary to loss of cell membrane Na^+/K^+ATPase activity (West, 1983; Gabai *et al.*, 1992) resulting in loss of ability to control intracellular osmolarity.

The next study was carried out in order to investigate whether any of the reperfusion-induced changes in organ biochemistry and physiology seen in the earlier studies were caused by accumulation of metabolites within kidney tissues during cold ischaemic preservation. The method of cold reflushing (CRF), as described by Parrott and colleagues (1990), was employed. There were a number of factors which may have influenced the rate of flow through the renal vasculature during CRF following cold preservation of kidneys for 48h. These include collapse of the vasculature during storage, accumulation of cellular debris or endogenous vasoconstrictors, or depletion of endogenous vasorelaxants during storage (Linder *et al.*, 1982; Lefer *et al.*, 1985; Lelcuk *et al.*, 1985). CRF after preservation may have washed these components out of kidneys. Upon normothermic reperfusion, flow through the vasculature was found to reach a maximum earliest in CRF treated organs, possibly due to the removal of the impediments mentioned above or caused by mannitol, an osmotic diuretic, in the CRF solution (Flores *et al.*, 1972). Post-transplant blood flow may be improved in this way. Urine was produced at a considerable rate during CRF, possibly as a result of the presence of mannitol. Urine

production during normothermic reperfusion did not appear to be influenced by the preceding CRF and no differences in flow rate were seen between CRF and non-CRF kidneys. This may have been due to the absence of humoral influences in the *ex vivo* model. GFR was similar to urine flow rate and was not influenced by CRF. Injury to proximal tubule brush border membranes caused by normothermic reperfusion was shown by a burst of γ-GT release into the urinary spaces at the onset of reperfusion after CRF. This release was lower than in non-CRF kidneys, probably due to the removal of enzymes accumulated in urinary spaces during ischaemic preservation. The finding that γ-GT release continued to be lower in CRF kidneys after 30min of normothermic reperfusion, by which time enzymes accumulated during storage would have been washed out, suggests that the brush border membranes were less damaged in the CRF organs. This may have important implications for cell survival since γ-GT is involved in the uptake of certain amino acids (Allison and Meister, 1981) and the antioxidant cofactor glutathione into cells (Bartoli *et al.*, 1978). The enzyme lactate dehydrogenase (LDH), which had accumulated in urinary spaces during storage, was shown to be washed out by the CRF procedure, as shown by decreasing enzyme activity in the urine as CRF proceded. Most of this LDH is likely to have originated in tubular epithelial cells whose membrane permeability was increased by cold ischaemic storage. There was a burst of LDH release at the onset of normothermic reperfusion, and this was greatest in non-CRF kidneys. Such reperfusion induced loss of

cell constituents may have a significant role in causing delayed post-transplant organ function. Protein was not included in the CRF solution and therefore the proteinuria observed during CRF must have been caused by loss of protein from urinary tubule epithelial cells, and some may have been vascular in origin. Loss of protein into the urine continued until the end of the CRF procedure suggesting that some urinary spaces were cleared more slowly than others, or that cell damage was occurring during CRF. Kidneys which had been subjected to CRF showed lower proteinuria than non-CRF organs at the onset of normothermic reperfusion, probably because their urinary spaces had been cleared of most protein by the CRF. This may be important for the prevention of tubular obstruction by proteins (Bayati *et al.*, 1990) after *in vivo* transplantation. However, both groups of kidneys were shown to have sustained glomerular injury, as indicated by similar losses of perfusate protein into the urine throughout normothermic reperfusion. Tubular reabsorption of sodium and glucose during normothermic reperfusion was found to be unaltered by prior CRF. The lack of humoral influences in the *ex vivo* reperfusion model may have obscured injury to the reabsorptive mechanisms of either CRF or non-CRF kidneys. The above findings suggest that CRF after storage may be beneficial to the immediate post-transplant function of kidneys but modification of the composition of the CRF solution may be necessary to achieve maximum protection of all kidney functions (Collins and Wicomb, 1992).

In the next study experiments were performed to investigate the mechanisms of iron release from intracellular storage sites during ischaemia and reperfusion, shown to occur in the kidney (Gower *et al.*, 1989; Healing *et al.*, 1990) and the heart (Boucher *et al.*, 1992). The inclusion of chloroquine in the kidney flush preservation solution to inhibit tissue acidification during storage slightly decreased the concentration of catalytic iron in the renal cortex following reperfusion but not in the medulla when compared to untreated controls. This demonstrated that an element of the mechanism of iron release in the cortex may depend upon intracellular and intralysosomal pH. Chloroquine did not alter lipid peroxidation, which was measured after 5min of normothermic reperfusion. However, this study measured malondialdehyde concentration in tissue samples containing both cortical and medullary material, therefore it was not possible to determine whether increased iron release in the cortex corresponded with increased lipid peroxidation. Perfusate flow rate through kidneys which had been stored in the solution containing chloroquine was significantly greater than in untreated organs after 2min of reperfusion. Therefore treatment with cloroquine may have prevented the accumulation of cell degradation products in vascular spaces, or may have inhibited injury to the mechanisms which were involved in the control of vascular tone. This increased vascular flow was not accompanied by increased urine production. There were no differences in the rate of urine production or in GFR in treated kidneys compared

with untreated controls. Treatment with chloroquine did not appear to alter post-reperfusion glomerular function or the quantity of water reabsorbed. Tubule sodium reabsorption was unaffected by preservation of kidneys in the solution containing chloroquine, and there was no evidence that the inhibition of Na^+/K^+ATPase activity by free lysosomal enzymes (Varkarakis *et al.*, 1975) could be prevented by storage in this way. Glucose reabsorption was found to be increased in treated organs at the 2min sample interval. There may have been a lag phase during the early stages of reperfusion when ATP levels needed to be restored before these active reabsorptive processes could operate at an optimum level. This may therefore have obscured any differences in damage to the transport mechanisms of treated kidneys compared with untreated controls. Once cellular debris, which had accumulated in the vascular and urinary spaces during preservation, had been washed out of kidneys at the onset of normothermic reperfusion, the concentration of the lysosomal enzyme N-acetyl-ß-D-glucosaminidase (NAG) released into these spaces was found to be decreased in chloroquine-treated kidneys at 2min post reperfusion. By 5min the protective effect of chloroquine upon lysosomal integrity may have been lost due to washing out of chloroquine by the perfusate which did not contain the drug.

Damage to the vascular endothelium, as shown by LDH release into the vascular space during reperfusion, appeared to be unaffected by treatment with chloroquine. However,

damage to urinary tubules was significantly reduced at the onset of reperfusion in treated organs, and continued to be lower than controls for at least 2min. At the end of reperfusion (t=5min) this effect was not seen, possibly due to the loss of protection by chloroquine once it had been washed out of kidneys. Treatment with chloroquine was shown to have a lasting effect upon the maintenance of the integrity of proximal tubule brush border membranes, as shown by the lower release of the enzyme γ-GT into the urine compared with control kidneys. This protective effect may have been mediated by inhibition of the action of acidophilic lysosomal phospholipases upon the phospholipids in the immediate environment of the membrane-bound enzyme. A number of studies have implicated lysosomal enzymes in ischaemic cell injury (Lotke, 1966; Vogt and Faber, 1968). The integrity of glomerular basement membranes was well maintained in chloroquine treated kidneys as shown by the decreased proteinuria in these organs compared with controls at 2min and 5min after the onset of normothermic reperfusion. Inhibition of lysosomal enzymes, either within lysosomes or after release from these compartments, may have prevented glomerular damage. Although protein release into the vascular spaces at the onset of reperfusion was not found to be different in treated kidneys to untreated controls, the inability of the assay method to distinguish between various types of proteins may make it a less sensitive criterion than the measurement of specific proteins such as enzymes. The method used in this

study was a simple modification to present clinical kidney preservation techniques. However, to obtain maximum benefit from chloroquine the compound may need to be administered throughout reperfusion and not just in the preservation solution. The time and method of administration of other membrane-stabilizing agents such as chlorpromazine (Bilde *et al.*, 1977) have been shown to be decisive factors in determining the post-ischaemic renal function when such compounds were investigated (Moses *et al.*, 1974).

The next group of experiments were designed to further investigate the role of iron, seen in the previous study to be released from intracellular storage sites, in causing ischaemic / reperfusion injury. When a preliminary study was performed of the distrubution of the iron chelator desferrioxamine (DFX) in the isolated *ex vivo* perfused kidney it was found that the glomerular clearance of this compound was similar to that of creatinine (i.e. it was similar to GFR). There was no evidence of DFX reabsorption in the tubules, as suggested by Summers and colleagues (1979), or of tissue accumulation of the chelator. On this basis the following study, which aimed to investigate the effects of iron chelation on postischaemic reperfusion injury, included storage of kidneys for 48h in a solution containing DFX and reperfusion with perfusate containing DFX. Other studies (e.g. Van Jaarsveld *et al.*, 1992) have shown loss of effects if DFX administration is not maintained. Hydroxyl radical production at the onset of reperfusion, as shown by hydroxylation of

salicylate in the perfusate, was found to be reduced in vascular and urinary spaces of DFX treated kidneys. This showed that iron chelation (probably at extracellular sites) decreased •OH production. However, after 2min of reperfusion no differences were seen between DFX treated kidneys and untreated controls. This may have denoted radical production at intracellular sites which may have been inaccessable to DFX as suggested by the drug distribution study. It is possible that free radical production occurred at two distinct sites; initially extracellularly as oxygen first reached vascular and urinary spaces (DFX-sensitive mechanism), and then intracellularly as oxygen diffused into cells (DFX-insensitive mechanism).

The mean vascular resistance was reduced within the first 2min of reperfusion in both DFX treated kidneys and in untreated controls, but this occurred most rapidly in treated organs, possibly a direct effect of DFX upon the vasculature (Summers *et al.*, 1979). Therefore after 2min reperfusion flow was significantly higher in DFX treated kidneys than in controls. Similar results showing increased vascular flow in hearts preserved in a solution containing DFX have been reported (Ely *et al.*, 1992). This may have been a direct effect of DFX upon the vascular smooth muscle or may have occurred secondary to iron chelation. This decreased vascular resistance did not appear to affect the rate of urine production, suggesting that the transglomerular filtration pressure remained similar in the two groups of kidneys. When

the release of the intracellular enzyme LDH and the proximal tubule brush border membrane enzyme γ-GT were measured to determine the extent of tissue damage, no differences were noticed between DFX treated kidneys and untreated controls at any time during reperfusion. This suggested that although hydroxyl radicals were produced at the onset of reperfusion, their role in independently causing marked direct tissue damage may be limited.

AN APPRAISAL OF THE VALUE OF ISOLATED KIDNEY PERFUSION AS A METHOD FOR STUDYING REPERFUSION DAMAGE

This technique required the isolated perfusion of kidneys *ex vivo* with the maintenance of a supply of metabolic substrates and appropriate gas exchange between the vascular spaces and the renal tissues. The aim was to preserve the structural organisation of tissues, giving a closer representation of *in vivo* conditions than tissue homogenates and cell suspensions (systems commonly used in studies of renal biochemistry). Isolated tubule preparations (Burnier *et al.*, 1988), for instance, lack the intimate association with the renal vasculature that is essential for their normal physiological function. Kidney slices and homogenates are not usually subjected to "flow" in the form of blood or any other reperfusion medium and do not form a glomerular filtrate. They therefore have a number of shortcomings when investigating metabolic processes in the kidney. On the other hand information obtained from isolated perfusion studies relates to the interactions of all the renal cell types in these tissues and is therefore closer to renal function *in vivo*.

My model allowed the assessment of several different kidney functions to be made simultaneously on the intact kidney, thereby presenting a more accurate profile of kidney viability. The isolation of an organ *ex vivo* severs all neural influences and this must be considered when interpreting

results of functional analyses. Renal function *in vivo* is heavily dependent on endocrine control and this must also be considered when interpreting results. However, it may be advantageous that neural and humoral influences were removed by using this model, because processes such as sodium and glucose reabsorption were solely dependent upon the viability of the kidney under study and not on the response to the release of a hormone or a nervous stimulus. No attempt was made to mimic the influences of hormones such as antidiuretic hormone (ADH) and aldosterone because their role *in vivo* is highly dependent on the state of hydration of the animal and reproducing appropriate hormone release would not be possible. Another feature of isolated perfusion which may be viewed as either an advantage or a disadvantage is that organ-organ interactions were removed. The kidney itself has a number of endocrine roles, including renin and erythropoietin synthesis and release, which function *in vivo* as part of feedback mechanisms to ultimately regulate blood flow and sodium reabsorption in the kidney in order to maintain fluid and electrolyte homeostasis. It must also be remembered that isolation of the kidney severs the continuity of the lymphatic drainage from the organ to the systemic lymphatics.

The perfusate composition was accurately controlled and therefore the rate of delivery of solutes to the kidney was well defined. However, there are differences between the *ex vivo* and *in vivo* physiology (e.g. flow rate through the renal vasculature) which probably result largely from the fact that

in the isolated perfusion model an acellular solution is used (Fuller *et al.*, 1977). The cell-free perfusate used has the advantage of excluding free radical generation by blood cells and other blood-borne entities, such as platelets (Salvemini and Botting, 1990). Another fundamental difference between reperfusion with blood or with the chosen buffered solution was the fact that the perfusate did not contain any of the antioxidants which are usually present in blood, for example ascorbic acid or extracellular superoxide dismutase. Therefore the standard perfusate may not have provided the kidney with the same protection from oxidative damage as would blood. On the other hand this may have been advantageous in my study because it allowed the investigation of reperfusion injury without the influence of the variables in antioxidant status which can arise in recipients of experimental renal transplants. Since the perfusate did not come into contact with any other tissues prior to entering the renal vasculature, there was no interference from metabolism of perfusate constituents outside of the kidney tissue. It was also possible to deliver drugs to the kidney in defined concentrations, and to measure the concentration of their metabolites in urine and vascular effluents free from metabolism by other tissues and organs. The *ex vivo* system allows vascular and urinary effluents to be serially sampled at accurate time intervals as they emerged from the kidney. Such serial sampling could only be achieved *in vivo* by using invasive techniques which may compromise normal blood flow.

The perfusion method permitted perfusate flow-rate, pressure and temperature to be reliably controlled and monitored in a way which is not possible *in vivo*. A comparison of the advantages and disadvantages of isolated *ex vivo* kidney perfusion versus *in vivo* blood reperfusion are given in Table 9.1.

There is little doubt that isolated kidney perfusion with physiological buffers causes some damage to the organ, since it is not possible to maintain a functioning viable kidney in such a system indefinitely. However, the model is very useful for the precise study of organ function for a number of hours. The kidney has advantages over the other transplantable organs such as the heart, lungs, and liver, since it has an easily accessable second compartment for sample analysis (the urinary space), whose biochemistry has been well characterized, in addition to the vasculature. Therefore in my studies, isolated *ex vivo* perfusion was the method of choice for studying reperfusion events.

Components of Perfusate vs. Whole Blood	
HES Perfusate	**Whole Blood**
Cell-free	Contains erythrocytes, leukocytes & platelets
Temperature accurately controlled	Temperature accurately controlled
No hormones	Contains a complex mixture of hormones
Limited oxygen carrying / delivery capability	Highly effective oxygen-carrying / delivery mechanisms
pH controlled, buffering capacity good at pH 7.4	pH controlled & buffering capacity good *in vivo*
Contains major ionic compounds found in blood	Contains many ionic compounds, some it very low concentrations
Contains glucose as a metabolic substrate	Contains glucose as major metabolic substrate, plus fatty acids

Table 9.1: Comparison of the major characteristic components of HES perfusion solution with whole blood.

In conclusion,

1) *Ex vivo* normothermic bloodless reperfusion of rabbit kidneys is a useful method for the study of the effects upon specific organ functions of experimental techniques for the inhibition of ischaemic / reperfusion injury.

2) Cold reflushing of kidneys with organ preservation solution after storage prior to normothermic reperfusion cleared vascular and urinary spaces, and may remove some of the pathological entities which predispose the organ to reperfusion injury.

3) Free radicals are produced during early reperfusion, and some radical production can be inhibited by chelation of catalytic iron in extracellular spaces and also possibly at intracellular sites.

4) Inhibition of tissue acidification during cold ischaemic kidney preservation may inhibit the release and activation of lysosomal enzymes, and may prevent the release of catalytic iron from intracellular storage sites.

APPENDIX A

FREE RADICAL SPIN-TRAPPING IN THE ISOLATED PERFUSED RABBIT KIDNEY : ELECTRON SPIN RESONANCE SPECTROSCOPY STUDIES

Introduction

Electron spin resonance spectroscopy (ESR) is a technique which detects atoms and molecules containing unpaired electrons. When electromagnetic radiation in the form of microwaves is applied to radicals in a strong magnetic field it causes unpaired electrons to move from a low energy level (parallel to the field) to a higher energy level (antiparallel to the field). The rate of change of absorbance of the applied microwave radiation produces characteristic spectra which can be used to identify particular radical species.

Spin traps are molecules which react with short-lived free radicals to produce characteristic radical adducts which are stable for longer periods of time and are therefore more easily measured. By measurement of these adducts obtained from a system which is producing free radicals it is possible to determine the rate of radical production and which particular radicals are being produced.

PBN TOXICITY STUDIES

The spin trap α-phenyl-t-butyl nitrone (PBN) has been used extensively to study free radical activity *in vivo* and *in*

vitro (e.g. Bolli *et al.*, 1988; Pincemail *et al.*, 1990). PBN was chosen for these ESR studies because it was able to trap a large number of different types of free radicals and gave long-lived adducts. However, toxicity data about this compound was not available from the manufacturer and little information has been published about the secondary effects of PBN in experimental systems. This study was performed to investigate the acute effects of PBN on renal function.

Methods

In the first experiment a fresh kidney was subjected to *ex vivo* reperfusion on the circuit using the standard perfusate with the addition of 15mM PBN. The kidney was reperfused for 30min during which serial samples of vascular effluent perfusate and urine were collected. In the following two experiments 48h preserved kidneys were reperfused with PBN-free standard perfusate for 15min. Following this equilibration period, PBN was added in concentrations varying from 5mM to 25mM. Serial samples of urine and perfusate were collected for assessment of organ function as previously described.

Results

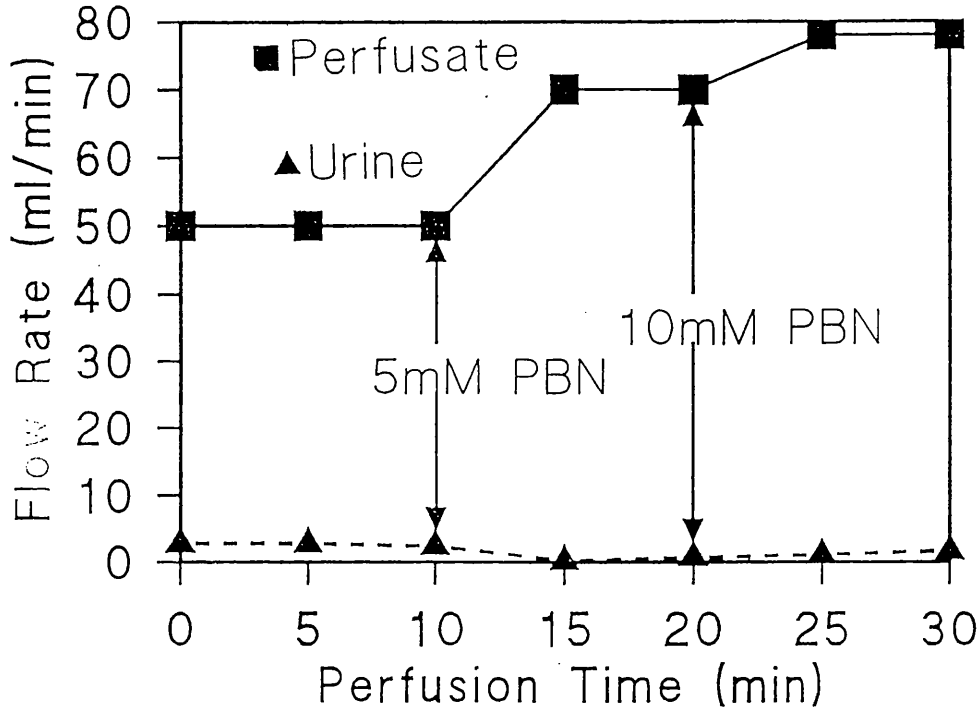

Fig. A.1: Effect of PBN (0-10mM) upon perfusion flow rate and urine flow rate in kidneys reperfused after 48h cold preservation.

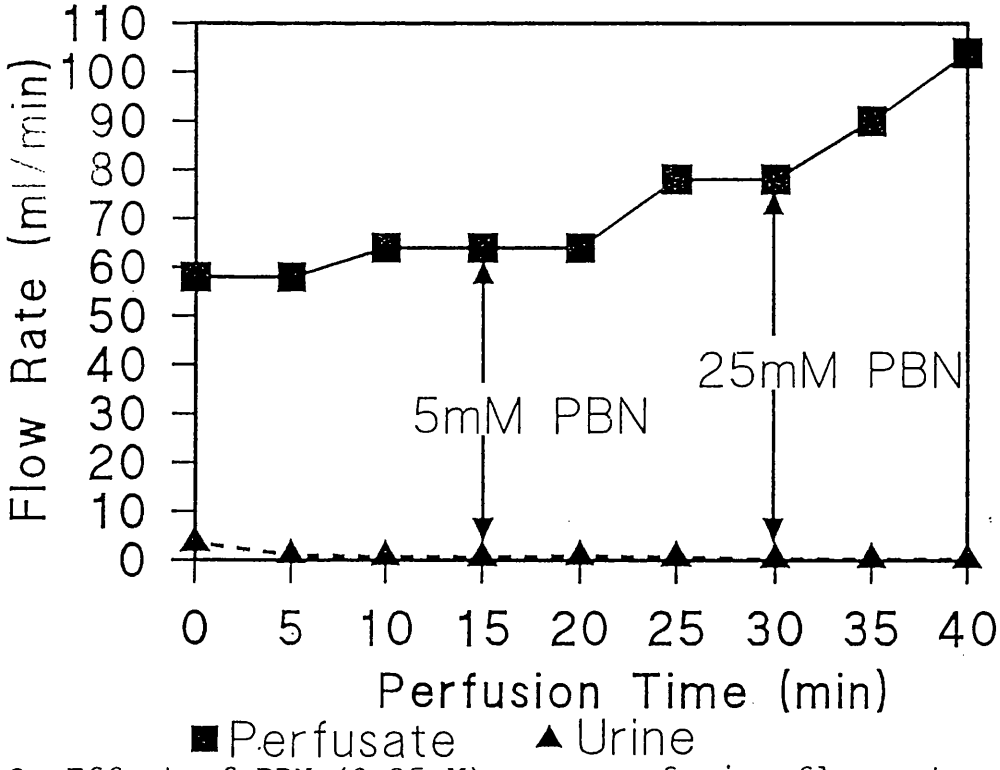

Fig. A.2: Effect of PBN (0-25mM) upon perfusion flow rate and urine flow rate in kidneys reperfused after 48h cold preservation.

Table A.1 : Effect of 15mM PBN on perfusion flow rate and urine flow rate in a fresh kidney.

Perfusate composition	Perfusion Time (min)	Perfusate Flow	Urine Flow (ml/min)
Standard Perfusate +15mM PBN	0	50	2.5
	5	78	0.1
	10	90	0.1
	15	102	0.1
	20	116	0.2
	25	118	0.3
	30	120	0.3

Table A.2 : Effect of PBN concentration (0-25mM) in the perfusate on perfusion flow rate and urine flow rate in a 48h stored kidney.

Perfusate composition	Perfusion Time (min)	Perfusate flow (ml/min)	Urine Flow (ml/min)
Standard Perfusate	0	58	3.5
	5	58	1.1
	10	64	0.7
	15	64	0.6
+5mM PBN	20	64	0.9
	25	78	0.6
	30	78	0.3
+25mM PBN	35	90	0.3
	40	104	0.3

Table A.3 : Effect of PBN concentration (0-10mM) on perfusion flow rate and urine flow rate in a 48h-stored kidney.

Perfusate composition	Perfusion Time (min)	Perfusate Flow (ml/min)	Urine Flow (ml/min)
Standard Perfusate	0	50	2.8
	5	50	2.8
	10	50	2.4
+5mM PBN	15	70	0.2
	20	70	0.7
+10mM PBN	25	78	1.1
	30	78	1.7

ELECTRON SPIN RESONANCE STUDIES

Introduction

Although the results of the above study suggested that a low concentration of PBN (<5mM) might allow normal urine production, a higher concentration (25mM) was thought to be required to trap a sufficient quantity of free radicals for detection. A non-recirculating reperfusion system was used in order to prevent contamination of perfusate and urine samples by PBN adducts which may have accumulated in a recirculating system.

Methods

Study 1: Kidneys were reperfused under normoxic or anoxic conditions as previously described (see Chapter 2) on the *ex vivo* circuit for 10min during which serial samples of perfusate and urine were collected. Aliquot (10ml) of perfusate were collected into glass tube containing 2ml of toluene on ice. These samples were immediately shaken vigorously in a vortex mixer and then centrifuged at 5000G for 10min at 4°C. The lower toluene layer was then decanted into Nunc cryotubes and stored in liquid nitrogen until analysis (approximately 24h later). This procedure of PBN extraction into toluene was performed to concentrate the PBN radical adducts which are preferentially soluble in toluene rather than in the aqueous perfusion buffer. This produced a fivefold increase in the concentration of PBN. It was not

possible to use this method to concentrate urine samples because their volumes were too small. Samples, either in toluene or in aqueous solutions, were analysed in a Bruker ESP300 spectrometer using a standard aqueous sample cell at room temperature.

Study 2: a) A comparison was made of the PBN spin adducts from a kidney which was reperfused after storage under standard conditions with a kidney stored under nitrogen in order to investigate the role of atmospheric oxygen in the production of radicals following reperfusion; b) An uninterrupted flow model was used (a fresh kidney reperfused without interruption of flow from blood perfusion to buffer perfusion) in order to overcome any effects of the short period ischaemia inevitably suffered as a result of standard harvest procedures.

Results

Study 1

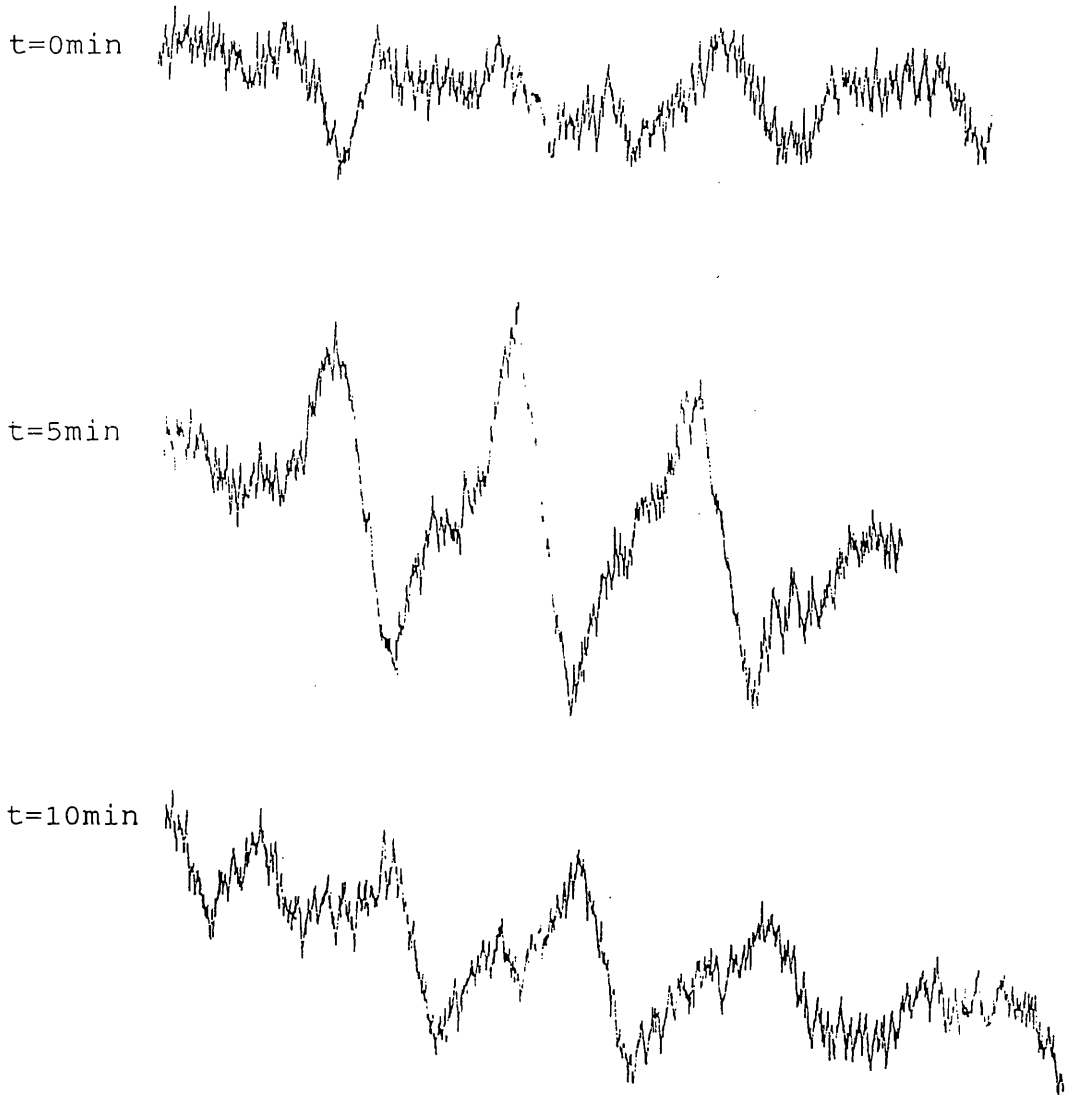

Fig. A.3: ESR spectra from fresh kidney reperfused hypoxically. Vertical axis represents signal intensity. Spectra shown from 0min-10min normothermic reperfusion.

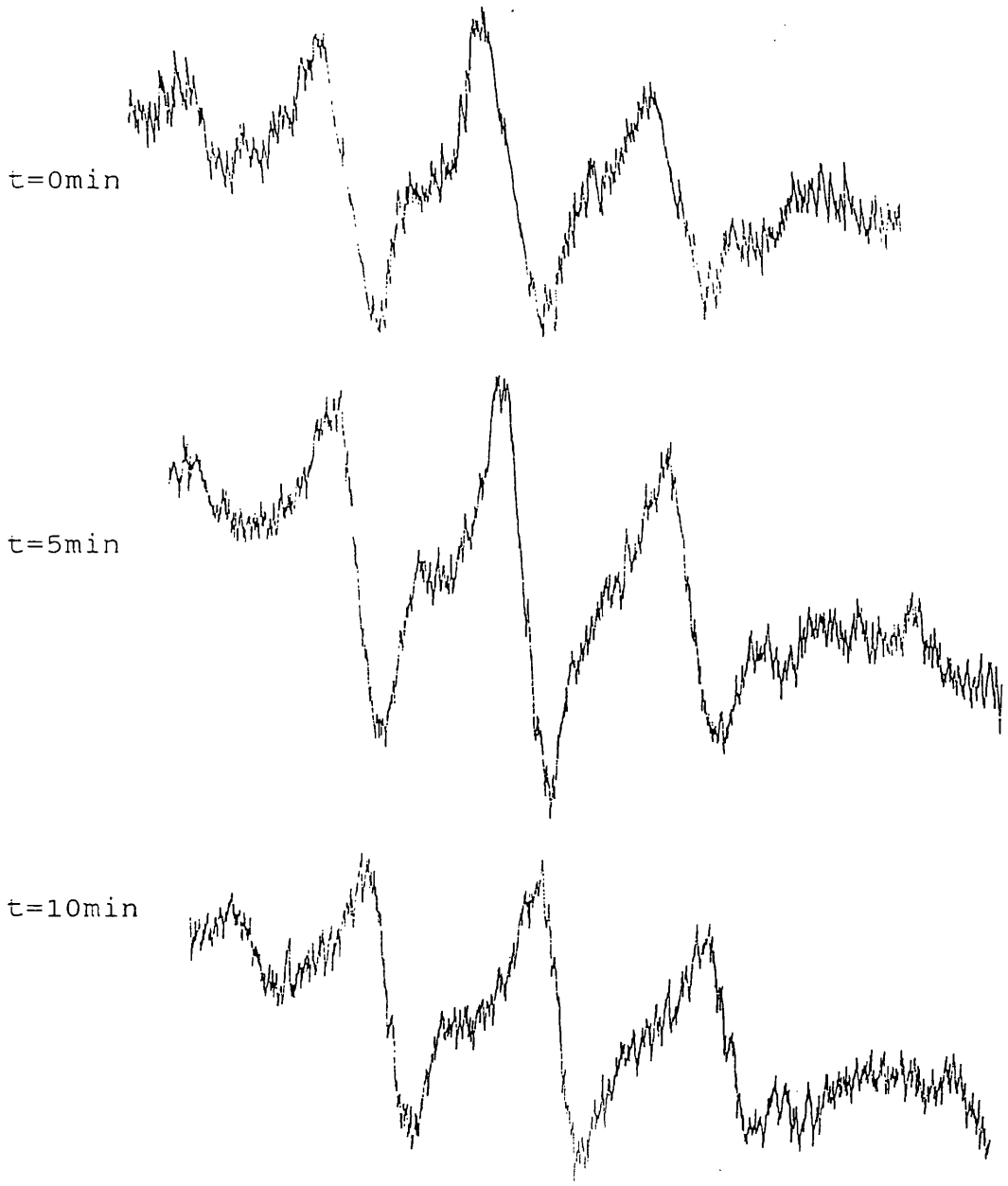

Fig. A.4: ESR spectra from vascular effluent of fresh kidney reperfused normoxically. Vertical axis represents signal intensity. Spectra shown from 0min-10min normothermic reperfusion.

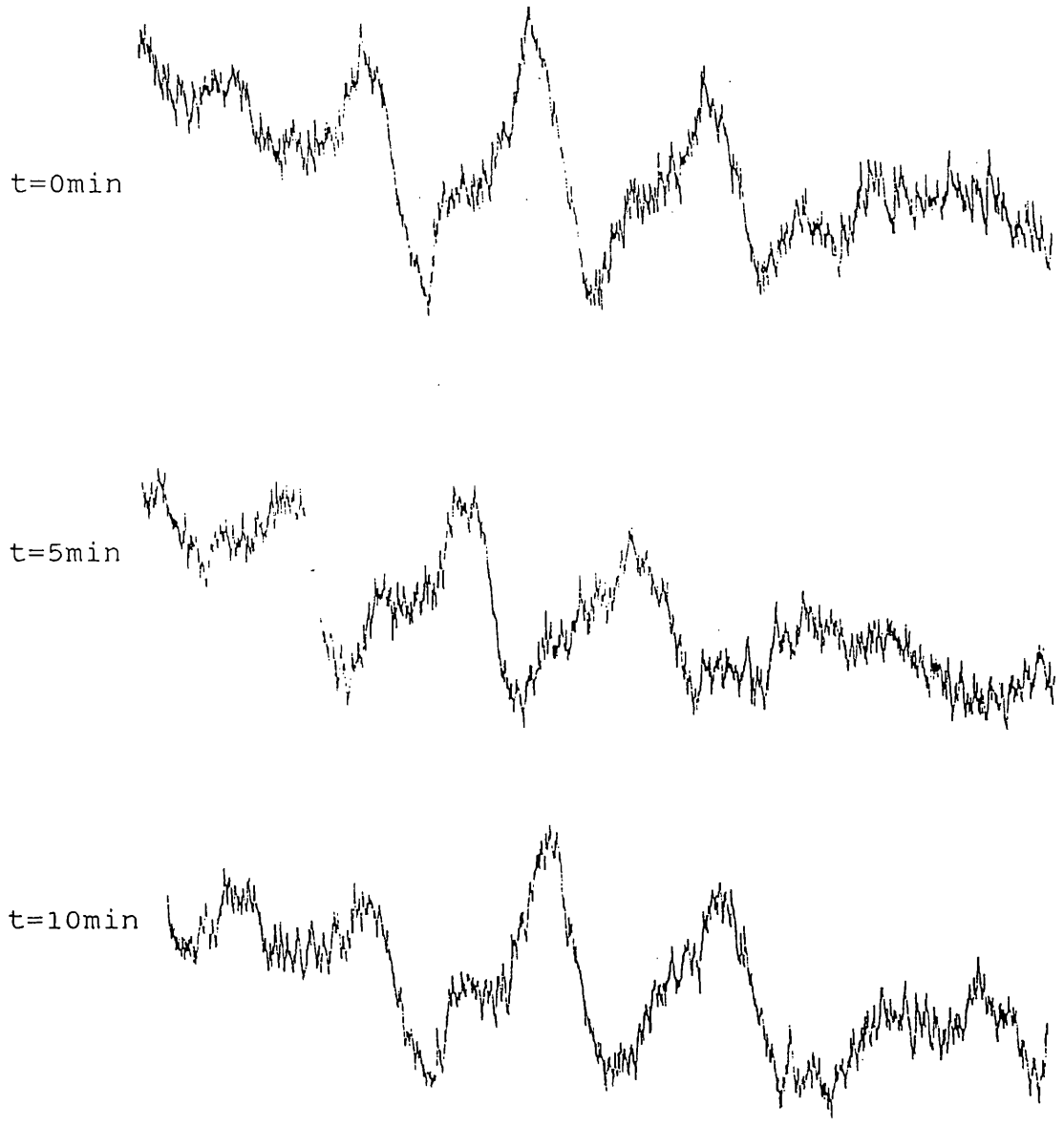

Fig. A.5: ESR spectra from vascular effluent of 48h stored kidney reperfused hypoxically. Vertical axis represents signal intensity. Spectra shown from 0min-10min normothermic reperfusion.

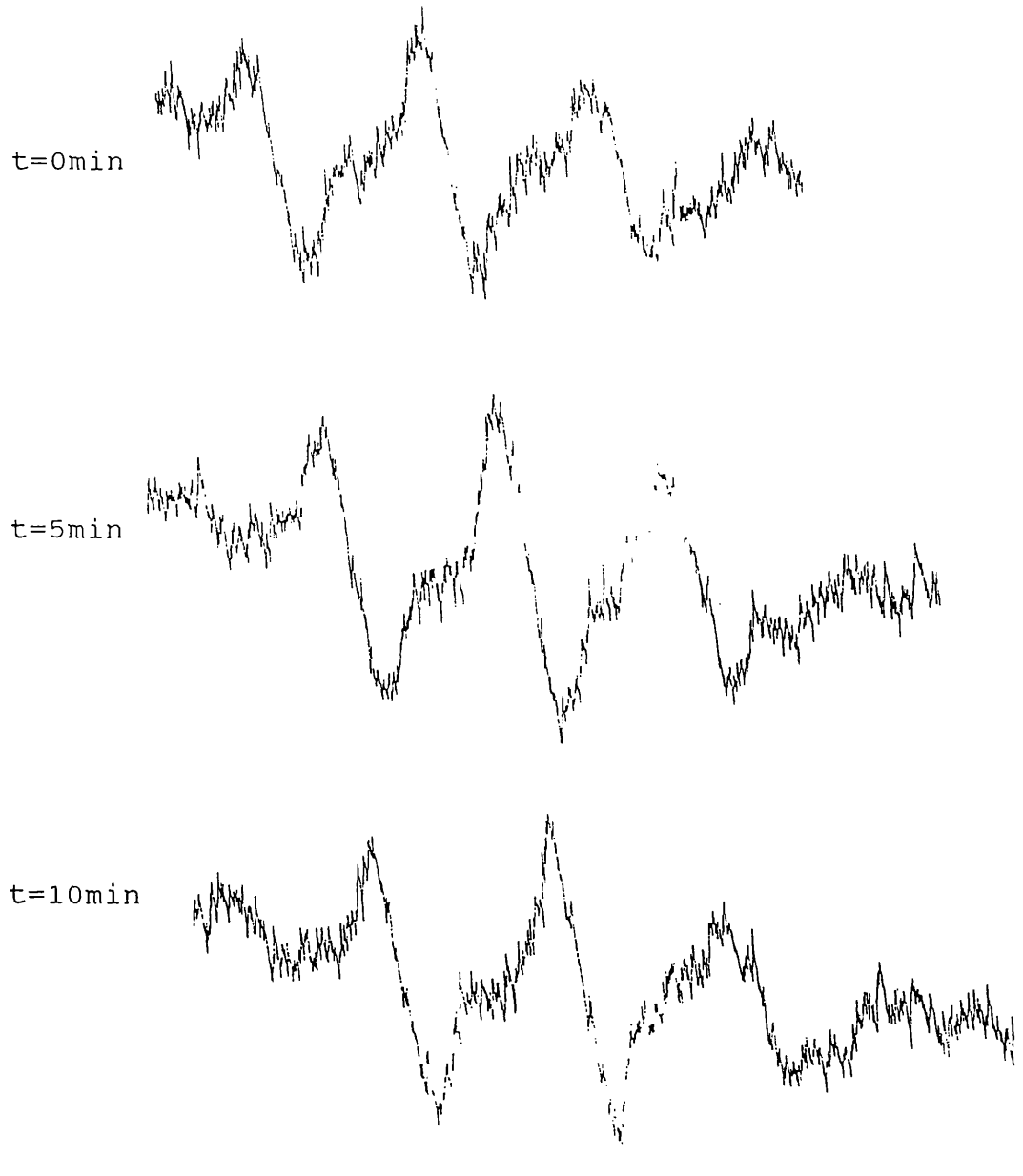

Fig. A.6: ESR spectra from vascular effluent of 48h stored kidney reperfused normoxically. Vertical axis represents signal intensity. Spectra shown from 0min-10min normothermic reperfusion.

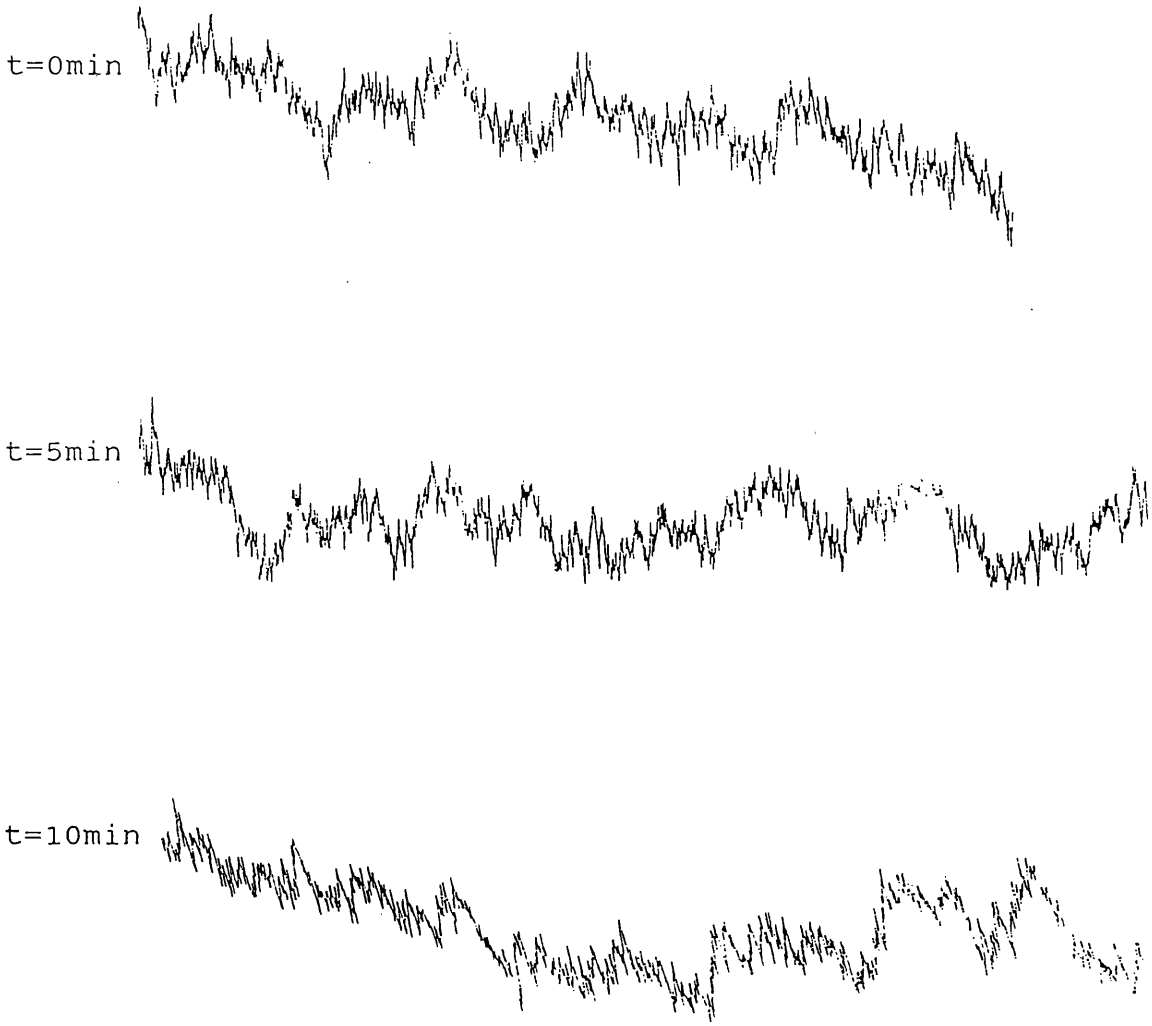

Fig. A.7: ESR spectra from urine of 48h stored kidney reperfused hypoxically. Vertical axis represents signal intensity. Spectra shown from 0min-10min normothermic reperfusion.

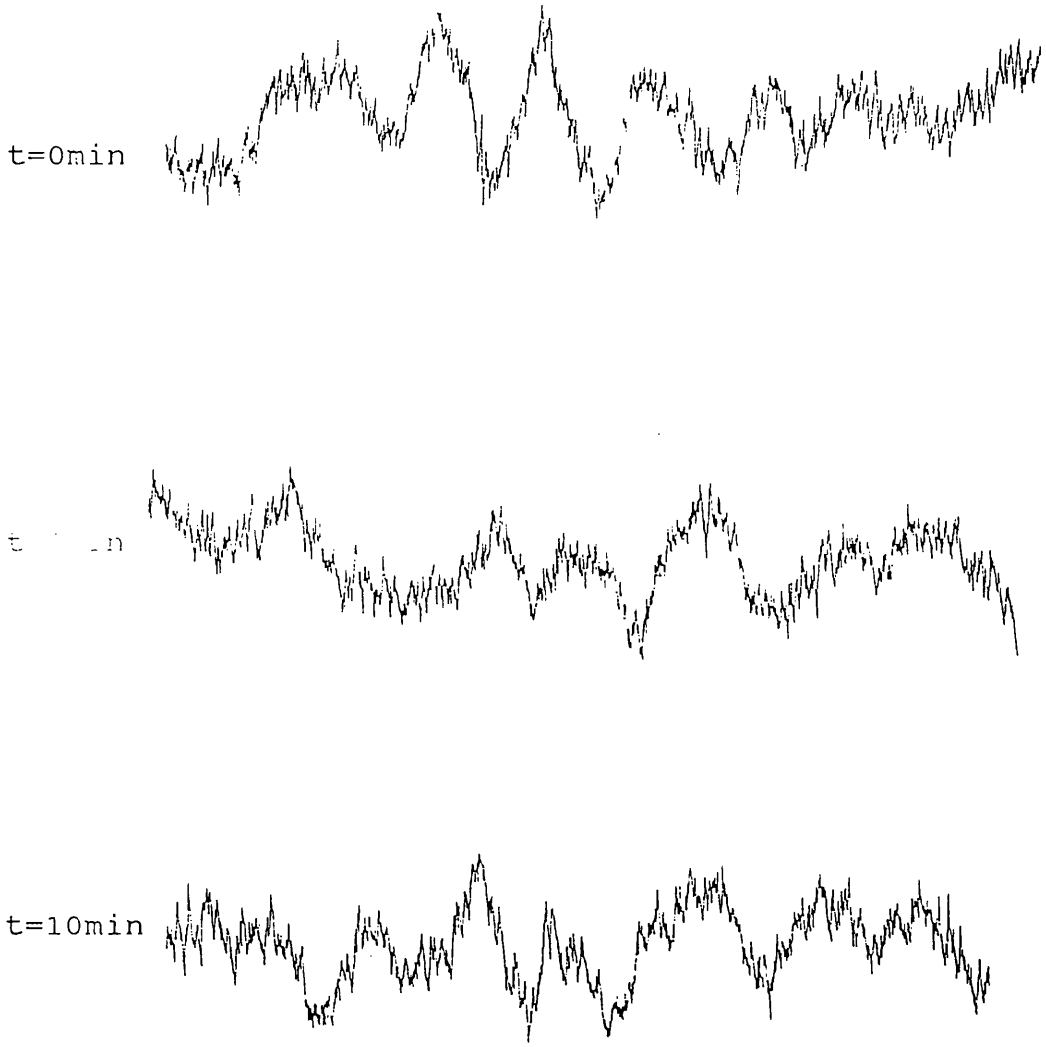

Fig. A.8: ESR spectra from urine of 48h stored kidney reperfused normoxically. Vertical axis represents signal intensity. Spectra shown from 0min-10min normothermic reperfusion.

a)Radical production in fresh kidneys: The signal obtained from a fresh hypoxic kidney was similar at the onset of reperfusion to 5min into reperfusion but decreased to approximately 50% of the initial signal intensity in the sample taken after 10min. In the normoxic kidney free radical generation increased during the first 5min of reperfusion and then decreased to give a signal at 10min which was similar to that at the onset of reperfusion. Free radical production did not diminish to the same extent as the decrease in the hypoxic kidney.

b)Radical production in 48h preserved kidneys: The signal obtained from vascular effluent samples from the kidney reperfused under hypoxic conditions was similar in intensity to the signal from control normoxic kidneys during the first 5min of reperfusion. Free radical production appeared to occur in both normoxic and hypoxic organs at this time. However, there was an increase in free radical production detected after 10min of reperfusion in normoxic kidneys, and a decrease in production in hypoxic kidneys. Urine samples gave a signal which was indistinct from background generation.

Study 2

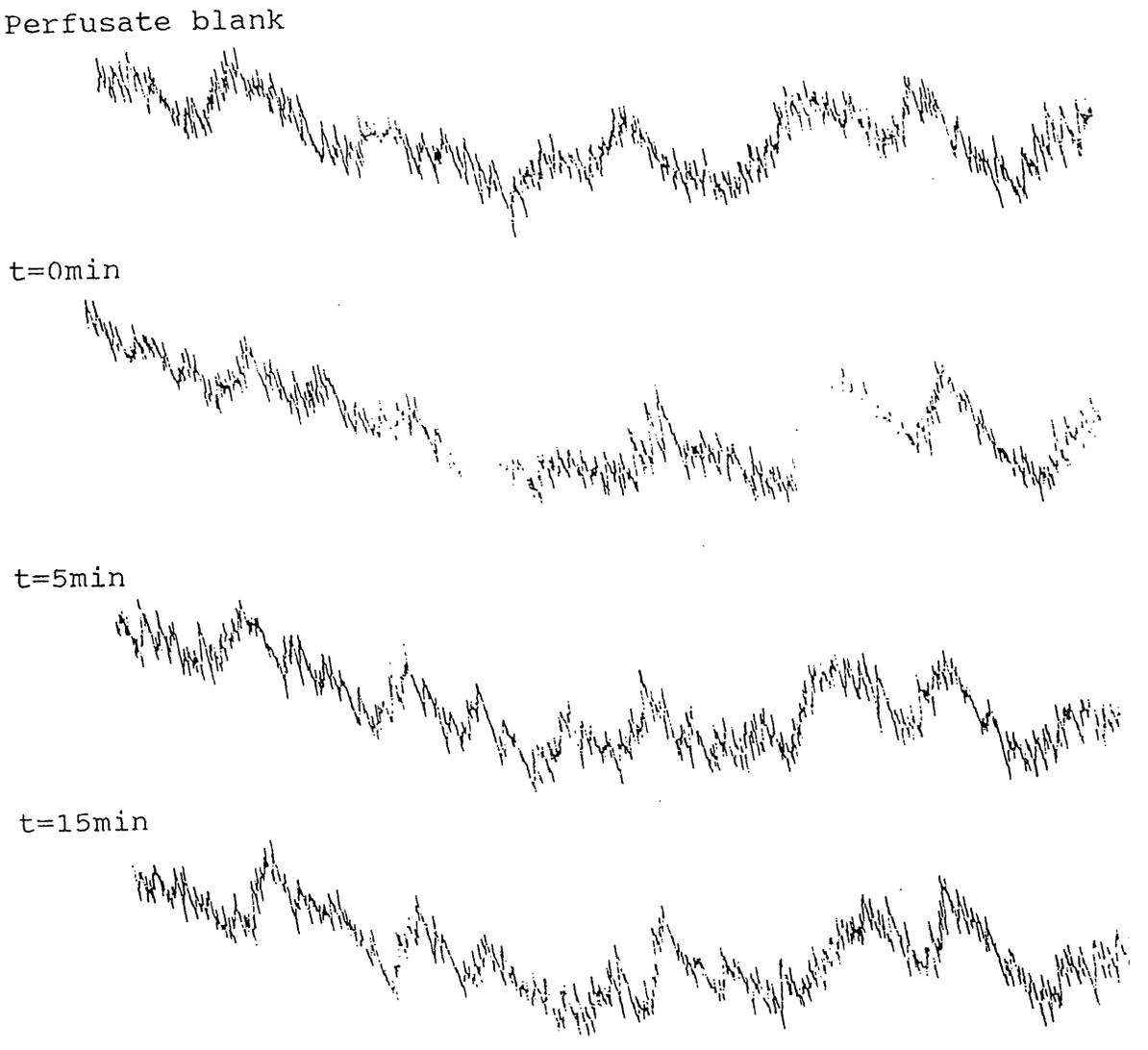

Fig. A.9: ESR spectra from vascular effluent of kidney stored for 48h under air and reperfused anoxically. Vertical axis represents signal intensity. Spectra shown from 0min-15min normothermic reperfusion.

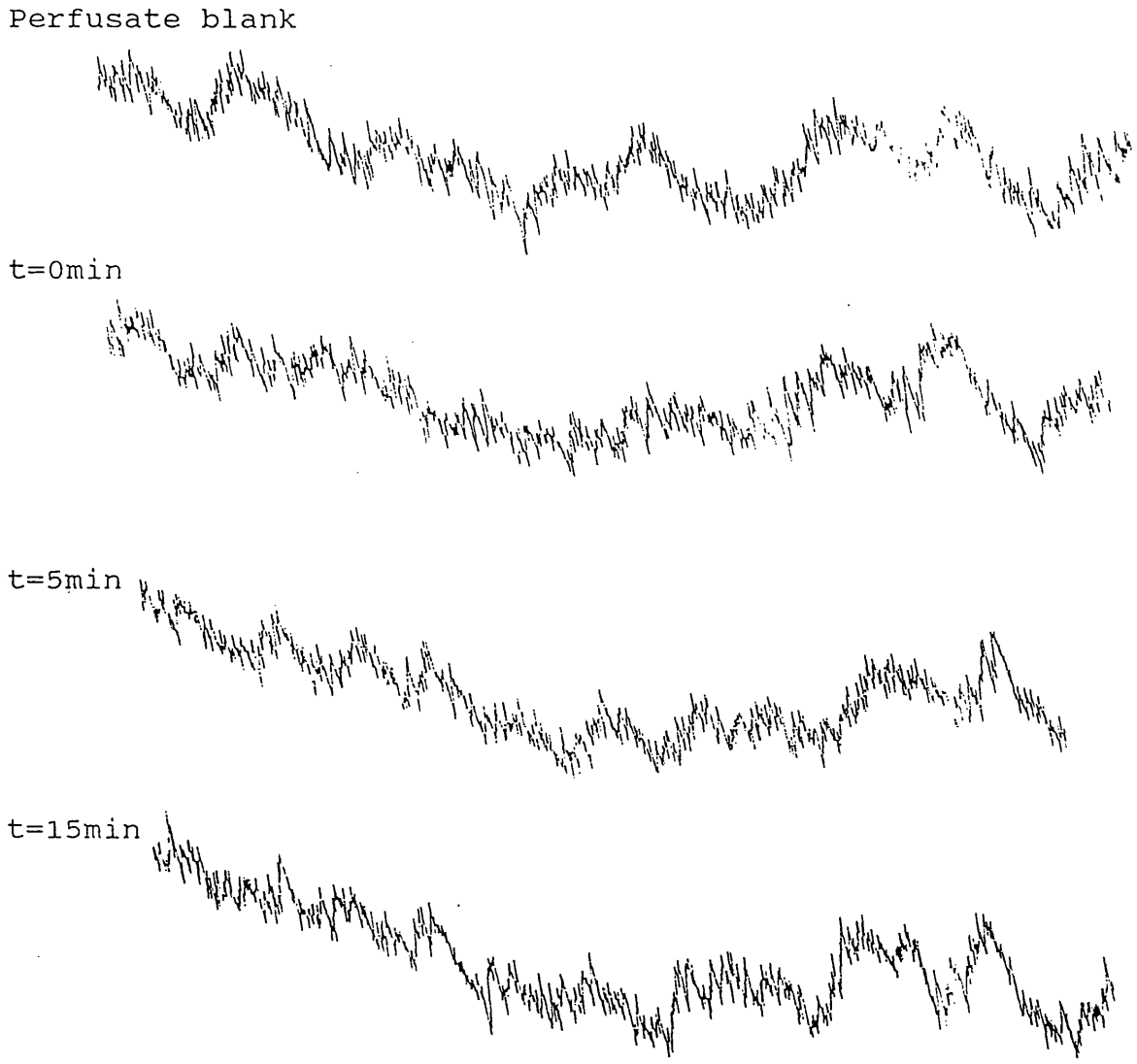

Fig. A.10: ESR spectra from urine of kidney stored for 48h under air and reperfused anoxically. Vertical axis represents signal intensity. Spectra shown from 0min-15min normothermic reperfusion.

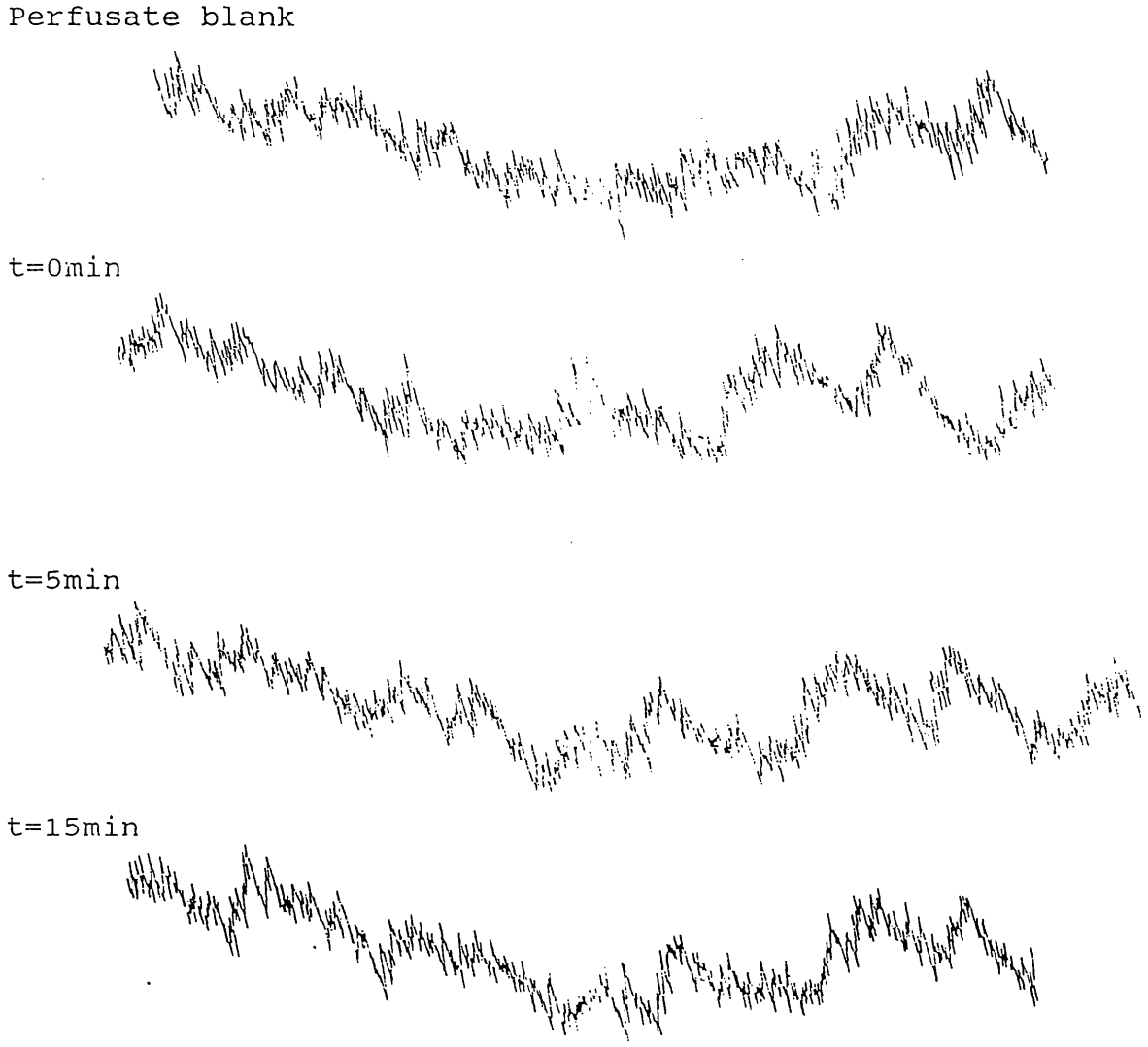

Fig. A.11: ESR spectra from vascular effluent of kidney stored for 48h under nitrogen and reperfused anoxically. Vertical axis represents signal intensity. Spectra shown from 0min-15min normothermic reperfusion.

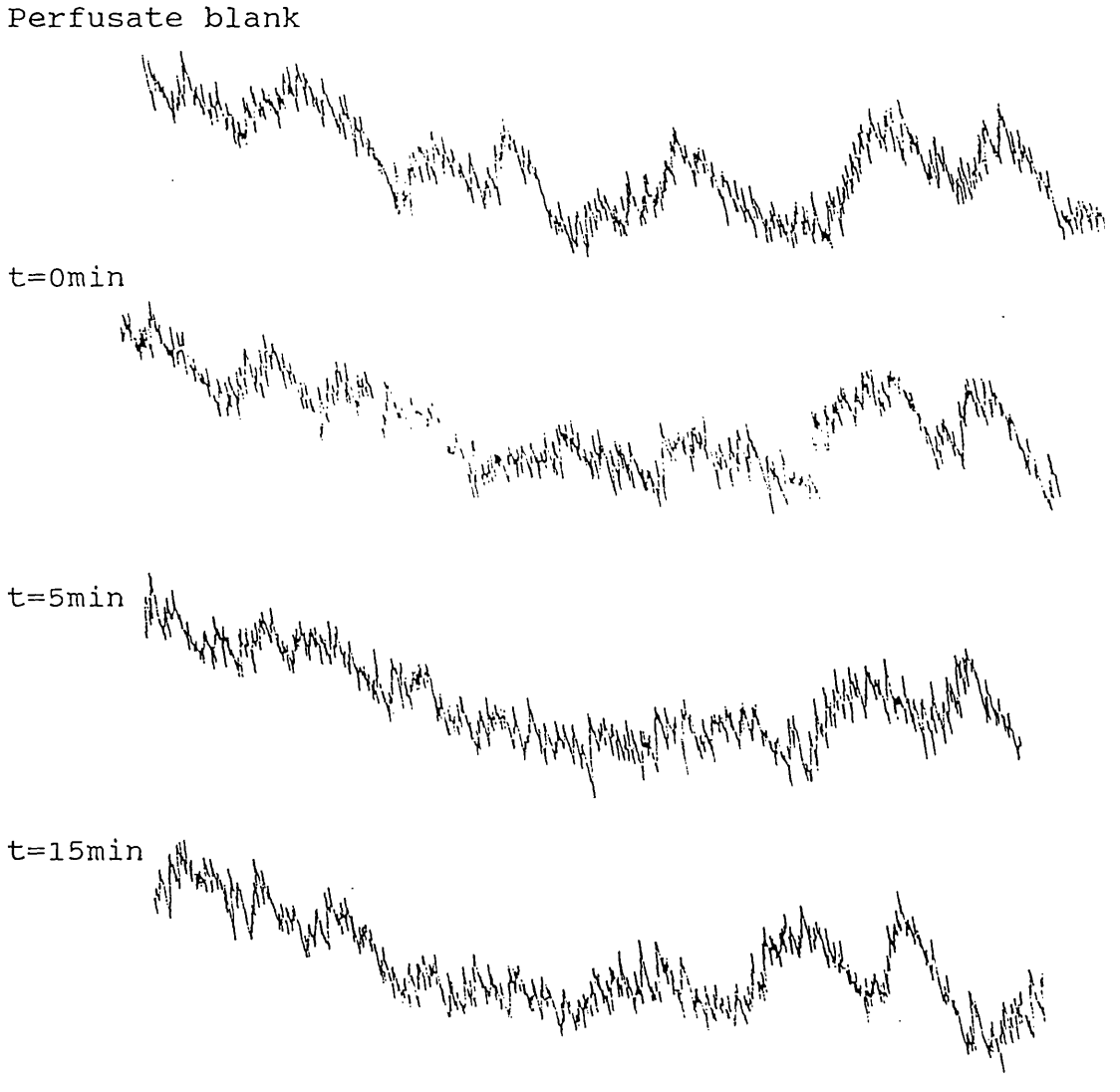

Fig. A.12: ESR spectra from urine of kidney stored for 48h under nitrogen and reperfused anoxically. Vertical axis represents signal intensity. Spectra shown from 0min-15min normothermic reperfusion.

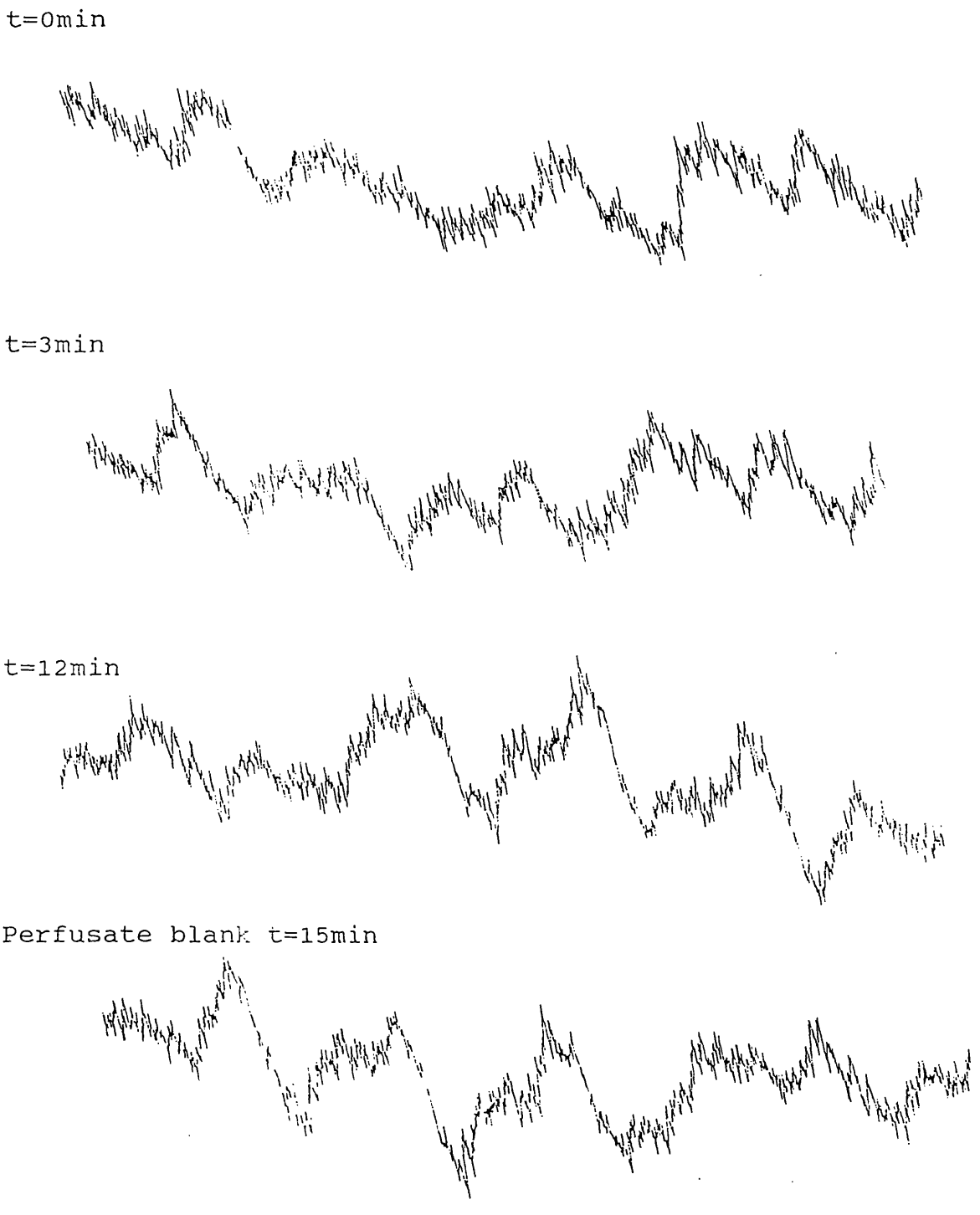

Fig. A.13: ESR spectra from vascular effluent of fresh kidney reperfused normoxically using the uninterrupted flow model. Vertical axis represents signal intensity. Spectra shown from 0min-10min normothermic reperfusion.

Discussion

The dose-response curves of vascular resistance (proportional to perfusate flow rate) against PBN concentration suggest that there was dose-dependent decrease in renal vascular resistance with increasing PBN concentration in the perfusion buffer. There also seemed to be a concommitant initial decrease in urine production when PBN was administered, possibly secondary to the decrease in renal vascular resistance. Diminished urine production may be have been produced by a lowering transglomerular vascular resistance causing decreased GFR. The mechanism by which PBN caused these acute changes in renal function is unknown and it is possible that normal function may have resumed if perfusion had been maintained for a longer period. For example, from Table A.3 it can be seen that the kidney perfused with concentrations of PBN not exceeding 10mM showed restoration of normal urine production after 25-30min of reperfusion.

The above results from study 1 suggest that even a short period of ischaemia, such as that experienced by "fresh" reperfused kidneys (approximately 15min of ischaemia), may have been long enough to initiate the priming mechanisms (see chapter 1) which predispose the kidney to elevated free radical production. It is also possible that the free radical production detected in fresh kidneys was the normal basal rate of physiological free radical production in this *ex vivo* system. There appeared to be an oxygen-dependent component of free radical production during the first 10min of reperfusion of 48h stored kidneys. The finding that radical production decreased during anoxic reperfusion suggests that radicals produced at the onset of reperfusion were generated independently of the oxygen concentration in the perfusion buffer. The increase in free radical production in normoxically reperfused kidneys after 10min of reperfusion is unlikely to have been due to increased oxygen concentrations in kidney tissues at this time, but may have been caused by mechanisms such as the liberation of catalytic iron from intracellular storage sites.

The finding that the spectra produced by all urine samples in this study were no greater than those produced by perfusate blanks may have been due to the inability to concentrate the samples by extraction into toluene because of low urine production.

In study 2 there was no evidence of free radical

production in either vascular or urinary spaces in kidneys reperfused anoxically for up to 15min after 48h storage under nitrogen or under atmospheric air. The signal intensity of the perfusate and urine samples was not noticably different to the perfusate blank. It is unclear why there was no evidence of free radical production in the kidney which was reperfused anoxically after storage under air, since a similarly treated organ did show evidence of radical production in study 1.

In the uninterrupted flow model with normoxic perfusion, there was no evidence of radical production in the vascular space at time 0min or 3min when these samples were compared to the perfusate blank. The perfusate blank taken from the buffer reservoir at the end of the experiment showed evidence of radical activity which may possibly be due to trace metal contamination. It would appear that radical production occurred between the onset of perfusion (when no signal was evident in samples) and the end of perfusion (15min; at which time a signal could be detected from samples). The evidence of free radical production in the perfusate sample taken at 12min post-perfusion is intermediate between these signals, and is probably due to an accumulation of radicals with continuous oxygenation of the buffer. Therefore there does not appear to be any evidence of free radical production in the kidney perfused in this way.

The exact identity of the radical species detected in

these experiments was not determined since the signals were relatively weak and resolution was low. However, the splitting between the peaks gave values of ≈13.97 Gauss for the larger peaks and ≈1.7 Gauss for the shoulders which can be seen on these peaks. These values are characteristic of an oxygen-centered adduct although the exact nature of this adduct, and that of the radical species which produced it, could not be determined.

APPENDIX B

IN VIVO RABBIT PLASMA AND URINE STANDARD VALUES

Plasma component	Plasma concentration in normal NZW rabbits
Protein (total)	53.00 - 79.00 g/l
Glucose	0.50 - 0.93 g/l
Creatinine	8.00 - 25.70 mg/l
Sodium	100.00 - 145.00 mEq/l
Potassium	3.60 - 6.90 mEq/l
Chloride	98.00 - 116.00 mEq/l
LDH	180.00 - 300.00 U/l

Table B1: Average concentrations of plasma components in blood from NZW rabbits. Data from Mitruka *et al.* (1977).

Urine	Rate of excretion (per kidney)
Urine volume	17.50 - 304.00 µl/min
Creatinine	8.75 - 69.50 µg/min
Protein	0.07 - 1.60 µg/min
Sodium	43.4 - 60.75 µg/min
Potassium	34.72 - 47.74 µg/min

Table B2: Estimated rate of excretion of metabolic waste products by each kidney in 2.5kg rabbits *in vivo*. Values calculated from data presented by Mitruka *et al.* (1977).

APPENDIX C

RAW DATA FROM SALICYLATE STUDIES IN CHAPTER 4

Perfusate oxygen content (%)	
Normoxic	Hypoxic
94.80	0.80
95.00	0.85
93.90	0.50
94.20	1.10
94.60	1.00
95.00	0.85
95.00	0.95
94.30	0.95
94.50	1.05
94.80	1.10
94.80	2.00
94.20	1.05
94.59 ±0.37	1.01 ±0.35
t=726.89, p<<0.001 ***	

Table C.1: Mean oxygen content of normoxic and hypoxic reperfusion buffers.

Sample Group	Perfusate 2,3-DHB concentration (nmoles/ml) at t=0min	
	Normoxic	Hypoxic
Fresh	0.2913 0.6389 0.2464 0.7647 0.3413 0.4697	0.3629 0.4693 0.2237 0.5504 0.3727 0.5869
48h	7.5142 20.1648 1.1066 6.8624 0.4829 0.7125	3.5336 8.7944 1.4959 6.0000 0.9008 1.1143

Table C.2: Concentration of 2,3-DHB (nmoles/ml) in vascular effluents from fresh and 48h stored kidneys at the onset of normoxic and hypoxic reperfusion.

Sample Group	Perfusate 2,5-DHB concentration (nmoles/ml) at t=0min	
	Normoxic	Hypoxic
Fresh	1.3254 1.6225 0.0981 0.7787 0.8437 1.0268	0.5138 0.5269 0.3554 0.2895 0.3683 1.0501
48h	39.3640 151.6518 104.0050 38.3254 0.5996 24.7312	32.0772 92.6367 163.0136 30.1973 0.7560 44.6951

Table C.3: Concentration of 2,5-DHB (nmoles/ml) in vascular effluents from fresh and 48h stored kidneys at the onset of normoxic and hypoxic reperfusion.

Sample Group	Perfusate 2,3-DHB concentration (nmoles/ml) at t=2min	
	Normoxic	Hypoxic
Fresh	0.3168 0.1504 0.4808 0.7221 0.3275 0.5270	0.3466 0.0908 0.8440 0.3408 0.4202 0.4276
48h	0.5172 0.8207 0.7915 1.6705 0.8057 0.8314	0.3614 0.6043 0.4848 0.5981 0.8527 0.6188

Table C.4: Concentration of 2,3-DHB (nmoles/ml) in vascular effluents from fresh and 48h stored kidneys after 2min of normoxic and hypoxic reperfusion.

Sample Group	Perfusate 2,5-DHB concentration (nmoles/ml) at t=2min	
	Normoxic	Hypoxic
Fresh	0.5766 0.1297 1.1641 0.6208 0.5948 0.5547	0.3259 0.0316 0.6556 0.3389 0.3249 0.4776
48h	3.4428 3.5632 3.5869 7.7635 0.5215 5.3025	6.2154 2.6727 2.4189 7.8293 0.2253 3.7291

Table C.5: Concentration of 2,5-DHB (nmoles/ml) in vascular effluents from fresh and 48h stored kidneys after 2min of normoxic and hypoxic reperfusion.

Sample Group	Perfusate 2,3-DHB concentration (nmoles/ml) at t=5min	
	Normoxic	Hypoxic
Fresh	0.3071 0.4657 0.1627 0.6834 0.4022 0.3382	0.1232 0.1089 1.8611 0.3759 0.3816 0.3539
48h	------ 1.1685 0.4813 0.1365 0.7872 0.8212	------ 0.1863 0.2075 0.3732 0.3436 0.3063

Table C.6: Concentration of 2,3-DHB (nmoles/ml) in vascular effluents from fresh and 48h stored kidneys after 5min of normoxic and hypoxic reperfusion.

Sample Group	Perfusate 2,5-DHB concentration (nmoles/ml) at t=5min	
	Normoxic	Hypoxic
Fresh	0.2856 0.3446 0.1771 0.7744 0.3367 0.0994	0.1303 0.0751 2.3173 0.3599 0.1957 0.1213
48h	1.5228 3.2143 3.0830 0.9622 0.4059 2.8759	0.6499 1.2718 1.2719 2.7360 0.7139 1.0042

Table C.7: Concentration of 2,5-DHB (nmoles/ml) in vascular effluents from fresh and 48h stored kidneys after 5min of normoxic and hypoxic reperfusion.

Sample Group	Urine 2,3-DHB concentration (nmoles/ml) at t=0min	
	Normoxic	Hypoxic
Fresh	0.0158 0.2879 0.0203 0.0420 0.0126 0.3330	0.0194 ------ 0.0192 0.0301 0.0093 0.0223
48h	30.6520 18.1429 1.0148 3.2862 1.5511 0.7062	20.8126 8.2617 2.4711 1.0824 0.9995 3.4945

Table C.8: Concentration of 2,3-DHB (nmoles/ml) in urine from fresh and 48h stored kidneys at the onset of normoxic and hypoxic reperfusion.

Sample Group	Urine 2,5-DHB concentration (nmoles/ml) at t=0min	
	Normoxic	Hypoxic
Fresh	0.3470 0.7520 1.4475 0.3575 0.2225 0.2060	0.2183 0.0389 0.2361 1.2350 0.8156 0.0544
48h	122.4460 178.0787 242.6742 9.2132 1.1439 10.8144	114.6000 101.2107 201.4278 3.4467 0.5048 11.1632

Table C.9: Concentration of 2,5-DHB (nmoles/ml) in urine from fresh and 48h stored kidneys at the onset of normoxic and hypoxic reperfusion.

Sample Group	Urine 2,3-DHB concentration (nmoles/ml) at t=2-5min	
	Normoxic	Hypoxic
Fresh	0.1037 0.4115 2.8936 ------ 0.2234 0.2870	0.0225 0.7409 2.7232 ------ 0.1012 0.2083
48h	7.7880 1.0374 0.4076 1.1895 0.7770 0.3841	5.5733 2.2697 0.5852 0.6206 0.0197 0.2685

Table C.10: Concentration of 2,3-DHB (nmoles/ml) in urine from fresh and 48h stored kidneys between 2min and 5min of normoxic and hypoxic reperfusion.

Sample Group	Urine 2,5-DHB concentration (nmoles/ml) at t=2-5min	
	Normoxic	Hypoxic
Fresh	0.4012 0.9312 0.8127 ------ 0.9735 0.1784	0.3020 0.2500 0.7478 ------ 0.5262 0.1805
48h	33.4928 48.2110 68.1853 20.2586 0.6383 5.8351	26.4419 67.8089 51.4297 6.6057 0.5127 5.6230

Table C.11: Concentration of 2,5-DHB (nmoles/ml) in urine from fresh and 48h stored kidneys between 2min and 5min of normoxic and hypoxic reperfusion.

Salicylate functional study results

	Perfusate flow rate (ml/min)					
	0min		2min		5min	
	Nmx	Hpx	Nmx	Hpx	Nmx	Hpx
1	84	100	84	100	64	100
2	66	64	66	72	46	96
3	80	70	64	74	58	72
4	76	70	70	70	58	54
5	72	60	80	68	84	82
6	70	62	74	80	68	92
mean	74.66	71.00	73.00	77.33	63.00	82.66
±SD	6.65	14.79	7.87	11.84	12.69	17.32
	t=0.87, p<0.50		t=1.10, p<0.40		t=2.26, p<0.10	

Table C.12: Perfusion flow rate (ml/min) in fresh rabbit kidneys perfused under normoxic or hypoxic conditions using perfusate containing 1mM salicylate. Nmx=normoxic, Hpx=hypoxic

	Perfusate flow rate (ml/min)					
	0min		2min		5min	
	Nmx	Hpx	Nmx	Hpx	Nmx	Hpx
1	70	70	84	70	84	100
2	80	68	96	82	114	98
3	84	72	84	98	106	98
4	90	80	90	100	102	104
5	80	70	108	90	108	118
6	74	64	92	70	92	70
mean	79.66	70.66	92.33	85.00	101.00	98.00
±SD	7.08	5.31	8.98	13.25	11.08	15.64
	t=4.88, p<0.01*		t=1.17, p<0.30		t=0.49, p<0.70	

Table C.13: Perfusion flow rate (ml/min) in 48h preserved rabbit kidneys perfused under normoxic or hypoxic kidneys using buffer containing 1mM salicylate. Nmx=normoxic, Hpx=hypoxic

	Urine flow rate (ml/min)					
	0min		2min		5min	
	Nmx	Hpx	Nmx	Hpx	Nmx	Hpx
1	4.2	5.5	4.0	3.5	2.5	2.5
2	5.0	5.5	4.4	5.5	2.9	3.8
3	2.3	1.5	1.0	1.0	0.3	0.7
4	5.0	3.5	3.4	2.6	1.9	2.6
5	2.0	2.3	0.5	0.6	0.2	0.6
6	2.5	2.5	2.4	3.6	1.2	3.4
mean ±SD	3.50 1.39	3.46 1.69	2.62 1.60	2.80 1.82	1.50 1.12	2.26 1.34
	t=0.08, p<0.90		t=0.54, p<0.70		t=2.45, p<0.05*	

Table C.14: Urine flow rate (ml/min) in fresh kidneys perfused under normoxic or hypoxic conditions using buffer containing 1mM salicylate. Nmx=normoxic, Hpx=hypoxic

	Urine flow rate (ml/min)					
	0min		2min		5min	
	Nmx	Hpx	Nmx	Hpx	Nmx	Hpx
1	1.5	2.2	1.5	0.6	-	-
2	2.6	1.4	2.0	0.5	1.7	0.3
3	0.6	1.0	0.3	0.6	0.2	0.5
4	2.1	1.5	1.0	0.6	0.3	0.5
5	1.6	0.6	0.3	0.2	0.3	0.1
6	4.0	5.0	-	-	1.6	1.3
mean ±SD	2.06 1.15	1.95 1.58	1.02 0.74	0.50 0.17	0.81 0.76	0.54 0.45
	t=0.30, p<0.80		t=1.65, p<0.20		t=0.89, p<0.50	

Table C.15: Urine flow rate (ml/min) in 48h preserved rabbit kidneys reperfused under normoxic and hypoxic conditions using buffer containing 1mM salicylate. Nmx=normoxic, Hpx=hypoxic

PROPOSALS FOR FUTURE STUDIES

1. Salicylate hydroxylation studies comparing citrate as a preservation solution with another impermeant to determine whether complexes of iron with the citrate in HCA cause free radical production.

2. Investigation of the role of atmospheric oxygen, equilibrated with kidneys during cold ischaemic preservation, in the post-reperfusion production of free radicals.

3. Determination of whether chloroquine inhibits delocalization of iron during ischaemic preservation in kidneys which have been flushed but not reperfused, or whether it inhibits reperfusion-induced iron delocalization.

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Date/place of examination:
8/12/93
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The following changes have been made to my PhD Thesis in accordance with the instructions of my examiners.

1)Suggested by Dr.D.Pegg:
page 97 Specific
page 97 distilled
page 181 has
page 185 of of (2nd deleted)
page 257 Fig.7.2 statistics re-analysed and corrected
page 266 membranes
page 332 electromagnetic
page 342 inverted page corrected

2)Suggested by Dr.T.Hallinan
page 65 hydroperoxides
page 66 Yaggi
page 108 Units inserted in table 3.1
page 109 Significance of results checked and confirmed
page 120 + 72 Superoxide production concurrent with prostaglandins and leukotrienes
page 275 Prostaglandins and other prostanoids
Page 308-309 Missing sentence replaced
Reference for Buhl et al. included

S. Toffa

Sam Toffa