Thiol compounds and their effects on ionic homeostasis in the isolated rat heart

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THIOL COMPOUNDS AND THEIR EFFECTS ON IONIC HOMEOSTASIS IN THE ISOLATED RAT HEART.

Submitted by Peter S. Haddock

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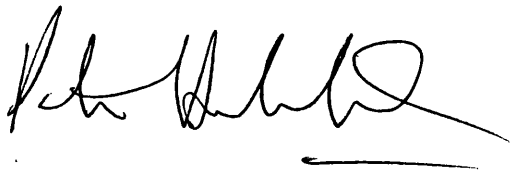

Peter S. Haddock.

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To Mom and Norman

for their endless love and support,

and Karen who put up with everything

and expected nothing.

A self-motivated person is, I suggest, someone who has no need of another person constantly pushing him or her on. The self-motivated have self-respect and goals which impel them to perform to the best of their capability without external pressure. Self-motivation is, in my view, a valuable and desirable asset. It could be argued that we cannot motivate others, but we can only help them motivate themselves by alerting them to their potential and encouraging them. (Author unknown).

ACKNOWLEDGEMENTS.

For his enthusiasm and belief in my work, I am very grateful to my supervisor, Dr. Brian Woodward, who has been both a friend and colleague over the past three years.

I also consider myself very lucky to have worked with Dr. Michael Shattock, Dr. Hiroshi Matsuura, Dr. William Coetzee and Prof. David Hearse who encouraged me and taught me a great deal during my short stay at The Rayne Institute, St. Thomas', London. I also thank them for their expert instruction and technical assistance in the experimental measurement of sodium pump current in isolated myocytes.

I would also like to thank Dr. Sue Peers and Amanda Taylor, at the William Harvey Research Institute, who performed the thromboxane B_2 assays and Dr. Robert Dodds, at Bath Institute for Rheumatic Disease, for his assistance with the histochemical assays of rat myocardial Na/K ATPase activity.

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SUMMARY.

In this study, the cardiovascular effects of reduced glutathione (GSH) and related compounds have been investigated using coronary and mesenteric vascular beds and the thoracic aorta of the rat. In addition, the effects of thiol compounds on an isolated bovine ventricular Na/K ATPase preparation and Na-pump current and free cytosolic calcium ($[Ca]_i$) in isolated rat ventricular myocytes and rabbit platelets were also studied. These investigations were performed in order to determine how reduced glutathione may partly exert its antiarrhythmic action in the ischaemic/reperfused rat heart.

Ischaemically-induced and potentially arrhythmogenic noradrenaline release from the myocardium was not affected by GSH suggesting an alternative mode of action by which GSH is antiarrhythmic. Myocardial ischaemia also resulted in contracture, the onset of which was delayed by GSH. This serendipitous finding led to investigations into how GSH and other sulphydryl compounds exert their protective, antiarrhythmic effect in the ischaemic/reperfused myocardium. Reduced glutathione significantly attenuated reperfusion-induced ^{86}Rb efflux, suggesting a potassium sparing action which is considered to be antiarrhythmic. The activity of an isolated bovine Na/K ATPase preparation was significantly potentiated and reduced, in a concentration-dependent manner, by reduced and oxidised glutathione (GSSG), respectively. GSH also lessened an ischaemically-induced loss of Na/K ATPase activity in isolated rat hearts. A stimulatory action of GSH on the Na/K pump may partly explain its effect on potassium loss during ischaemia. A concomitant reduction of intracellular sodium and Na/Ca exchange, as a result of Na/K pump stimulation, may also explain how GSH delays ischaemic contracture which is due to calcium overload. In isolated rat myocytes, the intracellular dialysis and extracellular superfusion of GSH and GSSG potentiated and decreased Na-pump current at all voltages, respectively, which correlates with the data obtained from the experiments with an isolated bovine Na/K ATPase preparation. Capacitance normalised Na-pump current, at 0mV, was also reduced in isolated myocytes prepared from glutathione-depleted hearts.

GSH lowered $[Ca]_i$ levels in isolated myocytes and platelets, while GSSG increased $[Ca]_i$ in a concentration-dependent manner. In both myocytes and

platelets, a GSSG-induced increase in $[Ca]_i$ was partly reversed by GSH. In addition, GSH reversed the increase in $[Ca]_i$ caused by the sulphydryl-reactive agents, *p*-hydroxymercuriphenylsulphonic acid and diamide, and by a thromboxane A_2 mimetic, U44619.

In experiments designed to study the effects of thiol compounds on the Na/H exchange mechanism, the physiological response of the heart in response to acidosis, in the presence and absence of reduced glutathione and related compounds, was investigated. In the Langendorff isolated rat heart, myocardial acidosis resulted in a pH-dependent rise in coronary perfusion pressure, indictative of coronary constriction. This effect was potentiated and attenuated by GSH and cysteine, respectively, in a concentration-dependent manner. Acidosis relaxed pre-contracted thoracic aorta but had no effect on the tone of pre-contracted mesenteric vascular beds. GSH caused an increase in tone only in pre-contracted mesenteric beds. Cyclooxygenase products are implicated in the pH-dependent constriction of the coronary bed, and its potentiation by GSH, as seen by the ability of flurbiprofen, indomethacin and a selective thromboxane A_2 receptor antagonist, GR 32191B, to attenuate these responses. Phentolamine significantly attenuated the effect of acidosis on coronary tone both in the presence and absence of GSH. In addition, the pH-dependent constriction was attenuated in hearts from animals previously depleted of catecholamines using 6-hydroxydopamine. This data suggests the some involvement of catecholamines and alpha-adrenoceptors in this coronary constriction, although no direct measurement of catecholamine release under acidotic conditions, in the presence and absence of reduced glutathione, was made. The sulphydryl-containing compounds, cysteine, reduced dithiothreitol, penicillamine and captopril all significantly attenuated the pH-dependent coronary constriction in a concentration-dependent manner, cysteine being the most potent. The involvement of endothelium-dependent relaxant factor in this effect was eliminated by the use of the reversible inhibitor of EDRF-dependent vasodilation, L-N^G-nitro-arginine. Finally, some data was obtained to suggest that cysteine may attenuate thromboxane A_2 release and increase the release of cGMP during acidosis which may explain how it attenuates a pH-dependent constriction in the coronary vasculature.

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LIST OF ABBREVIATIONS.

ATP	adenosine 5' triphosphate
b.p.m	beats per minute
BSA	bovine serum albumin
$[Ca]_i$	free intracellular calcium
Ca-ATPase	calcium-dependent adenosine 5' triphosphatase
CAT	catalase
DEM	diethylmaleate
DNTB	dinitrobenzoic acid
DTNB	5,5'-dithiobis-(nitrobenzoic acid)
DTT_{oxid}	oxidised dithiothreitol
DTT_{red}	reduced dithiothreitol
EDRF	endothelium-dependent relaxant factor
EDTA	ethylene diamine tetraaceetic acid
EGTA	ethylene glycol-bis(β-aminoethylether)*N,N,N',N'*-tetraacetic acid.
erc/minute	efflux rate coefficient
EIA	enzyme linked immunoassay
cGMP	cyclic guanosine monophosphate
g	gram
Gohm	giga ohm
Gs	GTP-binding stimulatory protein
GSH	reduced glutathione
GSSG	oxidised glutathione
GTP	guanosine 5' triphosphate
HEPES	*N*-2-hydroxy-ethylpiperazine-*N'*-2-ethanesulphonic acid
HPLC	high performance liquid chromatography
I-V	current-voltage
i.v	intravenous
i.p	intraperitoneal
ip	Na/K pump current
$[K]_o$	extracellular potassium concentration
MAN	mannitol
Mohm	mega ohm
MIE x 100	mean integrated extinction x 100
mmHg	millimetres of mercury
mM	millimolar
μM	micromolar
mV	millivolts
NAC	*N*-acetylcysteine
Na/Ca	sodium/calcium exchange
NADH	nicotinamide adenine dinucleotide (reduced form)

NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
Na/H	sodium/hydrogen exchange
Na/K ATPase	sodium/potassium adenosine 5' triphosphatase
NEFA	non-esterified fatty acids
NEM	N-ethylmalemide
L-NOARG	L-N^G-nitroarginine
NPY	neuropeptide Y
$O_2^{\cdot -}$	superoxide radical
pA	picoamperes
phent	phentolamine
DL-PSH	DL-penicillamine
pmoles	picomoles
PRP	platelet rich plasma
PSS	physiological salt solution
^{86}Rb	^{86}rubidium
S-A	sino-arterial
SDS	sodium dodecylsulphate
$\pm$s.e.mean	$\pm$ standard error of the mean
-SH	free sulphydryl group
SOD	superoxide dismutase
SR	sarcoplasic reticulum
TEA	tetraethyl ammonium chloride
X	xanthine
XO	xanthine oxidase

LIST OF CHEMICALS, DRUGS AND SUPPLIERS.

All analytical reagents for the preparation of standard Krebs-Henseleit buffer were obtained from BDH/Merck Chemical Company, Poole, Dorset, U.K.
^{86}Rb was obtained from Amersham Radiochemicals, Amersham, U.K.
GR 32191B was a gift from Glaxo Pharmaceuticals, Ware, Herts, U.K.
Captopril was kindly supplied by Squibb Pharmaceuticals, U.K.
BW 755C was a gift from Wellcome Pharmceuticals, U.K.
U44619 was purchased from Sigma, Poole, Dorset.
All gas mixtures were supplied by BOC.
Reduced and oxidised glutathione and other sulphydryl compounds were obtained from Sigma, Poole, Dorset.
All animals were bred in the on-site animal house.

CHAPTER 1.

INTRODUCTION.

1.1 METABOLIC AND IONIC CHANGES DURING MYOCARDIAL ISCHAEMIA.

Myocardial ischaemia is described as a deficit in the supply and demand of oxygen and metabolic substrates. During relatively severe periods of myocardial ischaemia, but prior to the onset of cell necrosis (infarction), there are a series of physiological and biochemical changes that occur. These are of a highly heterogeneous nature with respect to time and regional differences and many of them are potentially arrhythmogenic (*Hearse and Dennis, 1982*).

One of the first biochemical changes to occur following the onset of ischaemia is a rapid reduction in myocardial oxidative metabolism, due to a rapid depletion of available oxygen, which in turn results in a reduction in intracellular ATP. The most obvious mechanical change at this time is a depression of myocardial contractility and an increase in diastolic tension that occurs as a result of calcium overload (*Hearse et al., 1977*). Recovery from these changes upon reperfusion is dependent on the length of the preceding ischaemic period. Anaerobic glycolysis rapidly becomes the predominant metabolic pathway following the onset of ischaemia, causing an accumulation of lactate and H^+ (*Hearse and Dennis, 1982*). There is also a concomitant increase in the extracellular potassium concentration (*Knopf et al., 1990*) which may be the consequence of a parallel reduction in Na/K ATPase activity (*Kim and Akera, 1987*). There is also catecholamine release elevating tissue cAMP levels (*Podzuweit, 1982*). Although many of these changes have been implicated in arrhythmogenesis, the accumulation of extracellular potassium ($[K^+]_o$) is an important factor in the development of re-entry type dysrhythmia. Such an increase in $[K^+]_o$ reduces resting membrane potential and inactivates the fast sodium current, resulting in slow-response action potentials (*Corr and Sobel, 1979*). An enhanced arachidonic acid metabolism and other changes, such as the conversion of xanthine dehydrogenase to xanthine oxidase and the accumulation of hypoxanthine, may pre-dispose the ischaemic myocardium to the production of highly reactive and potentially damaging oxygen-derived free radicals upon reperfusion and the simultaneous re-introduction of molecular oxygen (*Murphy et al., 1987*). Free radicals have been indirectly implicated in reperfusion-induced arrhythmogenesis

from data showing the efficacy of selected free radical scavengers and antioxidants, such as reduced glutathione, in reducing their incidence (*Woodward and Zakaria, 1985*). However, the exact source and nature of these reactive and potentially damaging species is unknown and is the subject of considerable controversy.

1.2 FREE RADICALS AND OXIDATIVE STRESS.

1.2.1 Background.

Free radicals, and their effects in many organs and tissues, are recognised as an important factor in the aetiology of many disease states (*Shlafer et al., 1982; Sies, 1985; Simpson et al., 1987*; *Halliwell and Gutteridge, 1989*). Despite considerable evidence for a burst of oxygen-derived free radical production during the reperfusion of a previously ischaemic myocardium and the relatively low antioxidant capacity of the heart, the mode of action by which oxidant stress causes myocardial injury is unclear. Oxidative stress contributes to myocardial stunning (*Bolli, 1990*), the rate of myocardial infarction (*Engler and Gilpin, 1989*), impared endothelial function and vascular responsiveness (*Jackson et al., 1986; Hearse and Bolli, 1991)* and electrophysiological disturbances leading to ionic imbalance and arrhythmogenesis *(Bernier et al., 1986*; *Hearse, 1988*). In support of this, antioxidants or agents that inhibit radical production exert a protective effect against free radical-induced injury (*Bernier et al., 1986; Miura et al., 1988*; *Lucchesi et al., 1989)*. However, the identity, the sites of production and the mechanism by which these reactive species cause cell damage are still to be elucidated.

1.2.2 Oxidant stress and protein redox state.

Despite the fact that most radical-induced myocardial injury involves an imbalance of intracellular ionic homeostasis, that is controlled by both intracellular and intrinsic membrane-bound proteins, the vast majority of publications regarding this type of injury cite lipid peroxidation as the most likely mechanism by which this effect is mediated. Reeves *et al.* (1986) and Abramson

and Salama (1989) have shown, however, that several crucial ion-translocating proteins (such as the sodium/potassium pump, sarcolemmal Na/Ca exchange and the sarcoplasmic reticulum Ca-release protein) may be modulated by changes in redox state (thiol-disulphide interchange or mixed thiol formation) of free sulphydryl group(s) (-SH) on protein molecules. With this in mind, there is the possibility that either oxidative or reductive stress during reperfusion (radical burst or the accumulation of reducing equivalents, respectively) could alter protein redox state with great rapidity, resulting in the activation or inactivation of ion translocating processes, which may manifest as a loss of ionic homeostasis, myocardial stunning, abnormal vascular reactivity, contractile failure and arrhythmias.

1.2.3 Thiol groups and the control of protein and enzyme activity.

The activity of some proteins and enzymes depends on the regulation of the redox state of one or more thiol groups within the protein structure (*Rothstein, 1970; Brigelius, 1985; Zeigler, 1985*). In the tertiary structure of these proteins and enzymes the sulphydryl (-SH) groups, arising from cysteine residues, are often in close proximity to each other and may contribute, together with disulphide bonds, to the conformation of the active site. The formation of intramolecular disulphide bonds between adjacent -SH groups or their oxidation to intermolecular mixed thiols by interaction with low molecular weight sulphydryl compounds, such as reduced glutathione (GSH), can determine the conformation of proteins and thereby regulate enzyme activity (*Brigelius, 1985*). These intermolecular disulphide bridges are readily reversible and provide a switching mechanism that is sensitive to cellular redox state. Intramolecular bridges are not reversed so easily and may lead to the permanent inactivation of proteins and/or enzymes. Therefore, the extent and type of disulphide bridge formation may be an index of the degree and duration of oxidative stress. Several enzyme systems, including phosphofructokinase, hexokinase, glutathione reductase, glucose-6-phosphatase, adenylate cyclase, guanylate cyclase, cAMP-dependent protein kinase and cellular systems such as the cytoskeleton are regulated by this type of thiol-disulphide interchange mechanism (*Brigelius, 1985; Zeigler, 1985; Miller et al., 1990*). Glutathione reductase provides a good illustration of the rapidity with which

redox state can control enzyme activity since it can go through a cycle of activation and inactivation within a few milliseconds by switching pairs of protein thiol groups between the dithiol and disulphide state (*Trimm et al., 1986*). Therefore, cellular redox state (e.g intracellular reduced and oxidised glutathione content) may exert a regulatory role in cellular metabolism in a comparable manner to the phosphorylation and dephosphorylation of proteins and enzymes.

Under pathological conditions, the oxidation of protein thiols results in the impairment of transmembrane metabolite and ion transport, a loss of enzyme activity and the disturbance of contractile function (*Jones, 1985*). Cardiac thiol content can be reduced by sulphydryl oxidising agents (such as GSSG, cystamine and diamide) which correlates closely with a concomitant increase in heart rate and coronary flow (*Kosower, 1970; Gailis and Nguyen, 1975; Caparrotta et al., 1983*). In addition, diamide has a profound effect on inotropic state which is antagonised by ruthenium red, an inhibitor of sarcoplasmic reticulum (SR) calcium transport. If thiol-disulphide interchange is involved in the rapid modulation of ion translocating protein activity in cardiac membranes, then this may help to explain the rapid molecular events which initiate reperfusion arrhythmias, contractile failure and vascular injury.

1.2.4 Cellular thiol status.

Under normal conditions, intracellular concentrations of reduced glutathione are in the millimolar range whereas levels of oxidised glutathione are relatively low and maintained in the micromolar range (*Meister and Anderson, 1983*). The maintainance of a high GSH/GSSG ratio would be expected to keep the majority of protein-SH groups in the reduced state which may be critical for normal cellular function as most enzymes or proteins require free -SH groups for activity whereas the formation of disulphides is usually inhibitory.

1.2.5 Adrenergic receptors.

The binding of a beta-adrenoceptor agonist to an appropriate receptor stimulates the accumulation of cAMP by increasing the activity of adenylate cyclase via the

activation of a GTP-binding stimulatory protein, G_s (*Gilman, 1989*). It has been suggested that adrenergic receptor activity may be controlled by the formation of disulphide bridges between several cysteinyl residues that are known to be present in the transmembrane-spanning regions of the G_s protein (*Malbon et al., 1987*). Evidence in support of this comes from the work of Pendersen and Ross (1985) who have shown that thiol compounds, such as reduced dithiothreitol, can activate beta-adrenoceptors reconstituted in phospholipid vesicles in the absence of an agonist. The treatment of the G_s protein alone with dithiothreitol had no effect. This suggests that thiol activation is targetted at the receptor protein itself and not further downstream in the receptor-effector cascade. The recent work of Cotecchia *et al.* (1988) and Lefkowitz *et al.* (1989) has demonstrated major homology between the amino acid sequences of $alpha_1$, $beta_1$ and $beta_2$-adrenergic receptors with strict conservation of the cysteinyl residues that are thought to be located in the transmembrane spanning regions. Myocardial ischaemia leads to a significant increase in both alpha and beta adrenergic receptor numbers (*Corr et al. 1981; Strasser et al. 1988*) and sensitivity to catecholamines, in addition to noradrenaline release and accumulation in the synaptic cleft (*Schömig and Richardt, 1990*). During ischaemia, reductive stress, due to the accumulation of reducing equivalents, may further potentiate the adrenergic receptor activation via the reduction of intramolecular disulphide bonds in receptor proteins. Reperfusion-induced arrhythmias have been associated with both increased alpha- and beta-adrenergic stimulation and increased cAMP levels (*Sheriden et al., 1980; Corr et al., 1981*). Therefore, an increase in adrenergic receptor activity due to ischaemically-induced reductive stress may be deleterious in the face of increased receptor density and a net increase in noradrenaline release. Alternatively, it has been proposed that oxidant stress may desensitize adrenergic receptors thereby providing an explanation for the loss of contractile function during the early phase of myocardial reperfusion (*Pendersen and Ross, 1985*). In support of this, Schultz *et al.* (1990) have shown that myocardial contractile responses in the anaesthetized dog to exogenous noradrenaline are depressed during reperfusion following a 15 minute ischaemic period; full recovery of responsiveness was achieved after 24 hours. Both alpha and beta-adrenoceptors may, therefore, have a reduced responsiveness during reperfusion and the oxidation of free -SH groups in

the receptor protein may be responsible for this effect.

1.2.6 The sarcoplasmic reticulum.

The redox regulation of ion channel activity in the sarcoplasmic reticulum (SR) may be important in view of its role in the control of free cytosolic calcium. In a series of studies, Abramson *et al.* (1988a, 1988b) have shown that the opening and closing of calcium-release channels in the terminal cisternae in rabbit skeletal SR vesicles can be mediated via the reversible oxidation and reduction of -SH groups. Calcium release from the SR can be induced using micromolar concentrations of oxidising agents to promote disulphide formation. Reducing agents, such as reduced glutathione and reduced dithiothreitol, reverse this activation and promote calcium re-accumulation within the SR. From this work, Abramson *et al.* (1988a, 1988b) suggested a model to explain how oxidant stress could alter the gating of the SR calcium release channel leading to calcium overload and electrophysiological abnormalities (*Abramson and Salama, 1989*). With respect to this, the effect of oxidant stress on the ability of the SR to control cytosolic calcium has been demonstrated (*Matsuura and Shattock, 1989*). Oxidant stress, induced by the photoactivation of rose bengal, resulted in arrhythmias associated with calcium redistribution. A strong correlation between the SR content of a particular species and the degree of oxidant stress-induced damage was also shown. For example, the frog has no SR and is very resistant to the effects of rose bengal photoactivation (*Nakata and Hearse, 1990; Shattock et al., 1991*). Additional studies using isolated myocytes have shown that oxidant stress can induce oscillatory SR calcium release leading to arrhythmogenic transient inward currents (*Shattock et al., 1991; Matsuura and Shattock, 1991*a). This data is supported by the work of Scherer and Deamer (1986) and Yoshida and Tonomura (1976) who have shown that the activity of the SR Ca-ATPase can be reduced by the oxidation of -SH groups.

1.2.7 The sarcolemma.

Sodium/calcium exchange has been studied in sarcolemmal vesicles under oxidative stress (*Reeves et al., 1986*). Under these conditions Na/Ca exchange was stimulated up to 10 fold and this stimulation was inhibited by anaerobis and

catalase. Reduced and oxidised glutathione were both found to activate exchange and it has been suggested that the conformation of the Na/Ca exchange protein can be altered by the oxidation of three adjacent sulphydryl groups resulting in a modulation of channel activity. This system would be expected to be extremely vunerable to oxidative stress as at this time there is a net increase in the synthesis of superoxide, GSSG and hydrogen peroxide and the cell may be sodium-loaded and therefore susceptible to calcium gain. It is important to note that Reeves *et al.* (1986) observed these changes in Na/Ca exchange in the absence of detectable lipid peroxidation. This adds support to the hypothesis that changes in protein conformation as opposed to gross changes in membrane phospholipid content are important with regard to the early phase of oxidant stress in the myocardium.

In support of the theory that oxidative stress can selectively and adversely affect sarcolemmal function is the work of Bhatnager *et al.* (1990), Kim and Akera (1987) and Kramer *et al.* (1987). These groups have reported on the deleterious effect of oxygen-derived free radicals on ouabain binding to guinea-pig, frog and dog myocardial Na/K ATPase. Neutrophil-derived free radicals have also been shown to inhibit this ion translocating system (*Kukreja et al., 1988, 1989).* Kaneko *et al.* (1989a, 1989b) have shown that superoxide radical generating systems can inhibit rat myocardial sarcolemmal ATPase activity which can be reversed by superoxide dismutase, catalase or mannitol. Nitrendipine binding studies have shown that calcium channel activity is also reduced in the sarcolemmal membrane under conditions of oxidant stress (*Kaneko et al., 1989*a, 1989b). In studies involving exogenous oxidant stress, Dixon *et al.* (1990) also showed that oxidant stress results in alterations in Na/Ca exchange and Ca-ATPase activity that are matched by changes resulting from 30 minutes myocardial ischaemia and 5 minutes of subsequent reperfusion. This inhibition of Ca-ATPase activity could be attenuated by reducing agents such as dithiothreitol and cysteine and therefore it was speculated that Ca-pump activity may be modified by the oxidation of sulphydryl groups (*Kaneko et al., 1989*a, 1989b).

1.3 GLUTATHIONE.

The thiol compound, reduced glutathione, constitutes a major part of the cell's defence system against oxidative stress and is known to be protective against reperfusion-induced arrhythmias (*Woodward and Zakaria, 1985*). For these reasons, the maintainance of a fully functional glutathione pathway and related enzymic systems is critical if a cell and/or organ is to survive an ischaemic insult.

1.3.1 Metabolism.

Reduced glutathione (L-γ-glutamyl-L-cysteinylglycine) is a commonly found tripeptide that functions directly or indirectly in protein synthesis, enzyme activity, cellular metabolism, muscle contraction and the protection of cells against potentially damaging oxidising species (*Kosower, 1970; Meister and Anderson, 1983*). It is the predominant intracellular thiol and its structure is shown in figure 1. The intracellular synthesis of glutathione occurs by the consecutive actions of γ-glutamylcysteine synthetase and glutathione synthetase with feedback inhibition by glutathione itself. The availability of the precursor amino acids from the outside of cells is regulatory in GSH synthesis. The breakdown of glutathione is under the control of γ-glutamyl transpeptidase which catalyses the transfer of the γ-glutamyl moiety to acceptors such as cystine, methionine, water and GSH itself. This transpeptidase is unusual in the respect that its substrate, GSH, is located intracellularly whereas the enzyme is on the cell surface. There is, therefore, a GSH transporter to allow the interaction of substrate and enzyme (*Griffith et al., 1978*). γ-Glutamyl amino acids formed by this process are then transported into cells. The γ-glutamyl cycle, which accounts for the synthesis and degradation of glutathione, is shown in figure 2. Blaustein *et al.* (1989) have shown that glutathione-depleted hearts can have their GSH content restored to control levels by the use of glutathione methyl esters. In addition, Tsan and colleagues (1989) have also shown that exogenous GSH can increase GSH levels in pulmonary artery endothelial cells as a result of GSH breakdown at the cell surface and internal resynthesis. It appears that the time of exposure as well as the GSH concentration will have a bearing on the extent of intracellular GSH enhancement. In the experiments described in this thesis, it is likely that intracellular levels of GSH may be modulated by the perfusion of extracellular GSH, but the scale of GSH enhancement will depend on the basal GSH status of

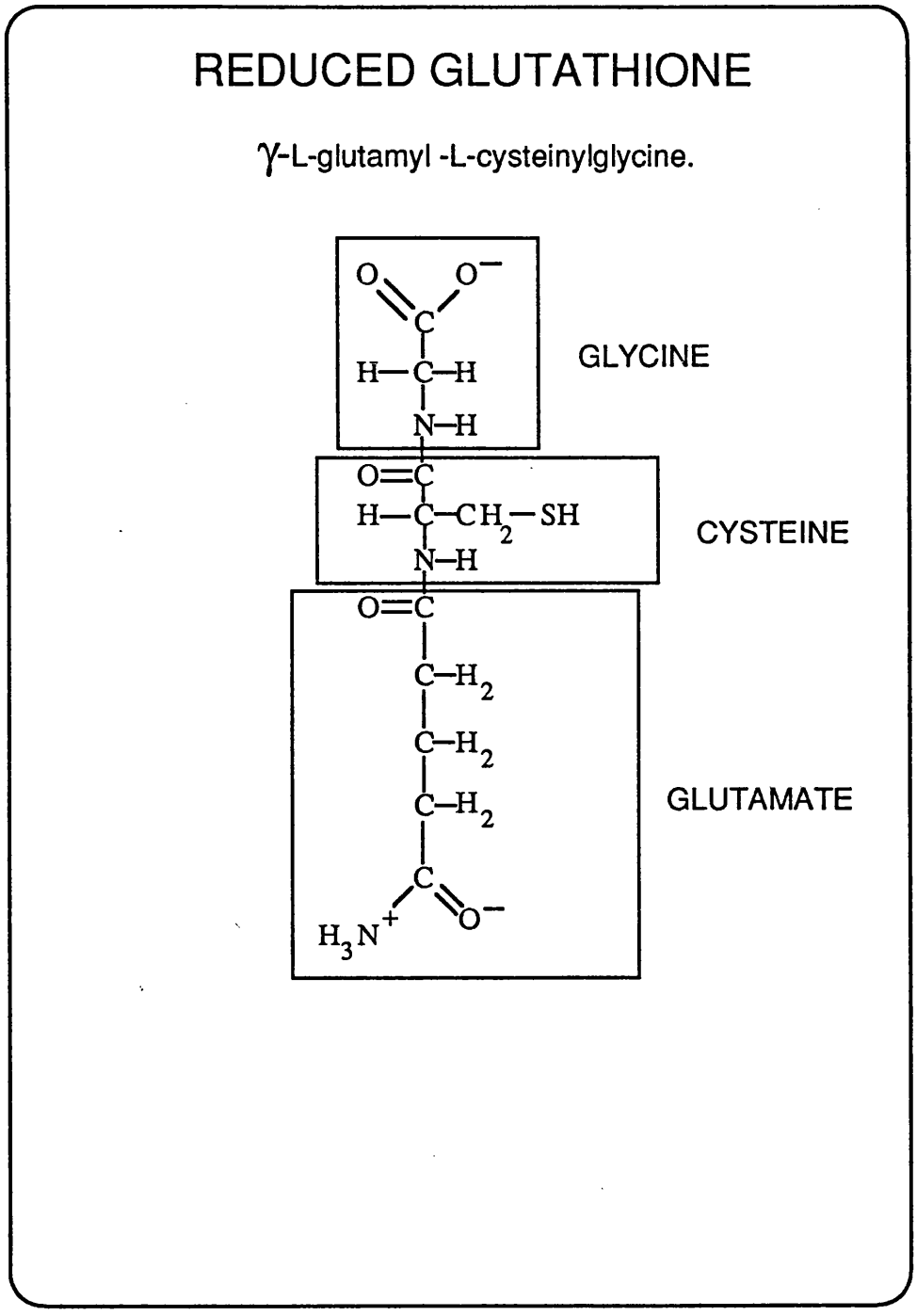


Figure 1. Structural formula of the tripeptide, reduced glutathione (GSH) (γ-L-glutamyl-L-cysteinylglycine) highlighting its component amino acid residues, glycine, cysteine and glutamate.

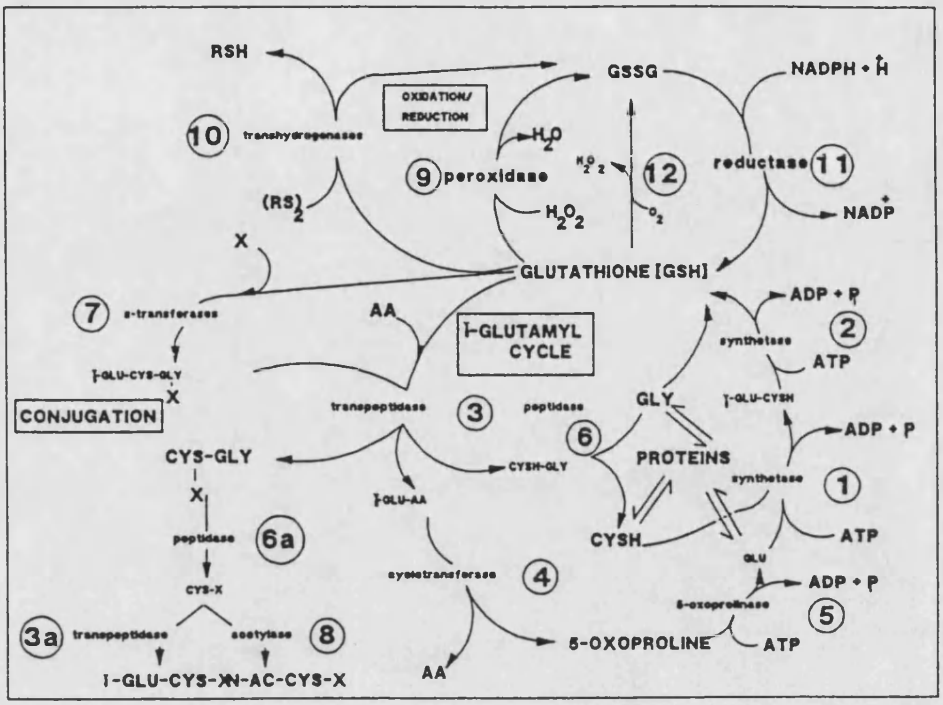


Figure 2. Overall summary of glutathione metabolism: Reaction 1. γ-GLU-CYSH synthetase; Reaction 2: GSH synthetase; Reaction 3 and 3a: glutamyl transpeptidase; Reaction 4: γ-glutamyl cyclotransferase; Reaction 5: 5-oxoprolinase; Reaction 6 and 6a: dipeptidase; Reaction 7: GSH S-transferases; Reaction 8: *N*-acetylase; Reaction 9: GSH peroxidase; Reaction 10: transhydrogenases; Reaction 11: GSSG reductase; Reaction 12: oxidation of GSH by $O_2^{\cdot-}$; conversion of GSH to GSSG is also mediated by free radicals; AA-amino acids.

the heart or single cell.

1.3.2 Glutathione transport.

The intracellular concentrations of GSH and GSSG are approximately 0.5-10mM and 1-2μM, respectively. Intracellular glutathione is, under normal conditions 99% GSH; levels of GSSG, being very low, only constitute 0.1 to 2% of the total glutathione pool. Under conditions of significant oxidative stress or toxicity, GSSG increases substantially and there may be a mechanism for its active transport out of the cell (*Meister and Anderson, 1983*). The export of glutathione from erythrocytes involves GSSG transport *(Beutler, 1983)*. When the GSSG level in such cells is artificially raised by the use of a GSH-oxidising agent, GSSG is actively exported. This process requires ATP, and Beutler (1983) suggested that this active transport mechanism may represent an emergency mechanism to protect cells from the toxic effects of GSSG. In addition, intracellular GSSG is reduced to GSH by glutathione reductase and the concomitant use of NADPH. The transport of GSH from the intracellular to the extracellular space is found in a wide variety of cells (*Meister et al., 1980; Meister, 1981*) and may function as a means of sulphur transport, in the form of cysteine, between cells. The function of GSH as a storage and transport form of cysteine has a distinct advantage in that the sulphydryl group of GSH is more stable than that of cysteine and GSH is far less toxic than cysteine, even at relatively high intracellular concentrations. The transport of GSH may also maintain essential -SH groups of proteins in the cell membrane during oxidative stress. There is good evidence that extracellular GSH functions as a reductant that may prevent the formation of intra- and intermolecular disulphides. It is also important in the removal of reactive oxygen intermediates and free radicals formed under normal and pathological conditions.

1.3.3 Functions.

Reduced glutathione is well known to toxicologists for its efficacy in the conjugation and detoxification of a wide range of xenobiotics and their metabolites and this function accounts for approximately 5-10% of GSH consumption. Glutathione is also a component of one of the cells main

endogenous defence systems against ischaemia/reperfusion and free radical-mediated oxidative damage. Studies of oxidant stress in a range of cell types have indicated that glutathione depletion may be one of the steps in the progression of irreversible cell injury. Human lymphoid cells and tumor cells depleted of GSH have been shown to be more sensitive to irradiation and cytolysis by reactive oxygen intermediates (*Griffith et al., 1978; Arrick et al., 1982*). A reduction of the glutathione pool has been related to the progression of lipid peroxidation and the alteration of protein-bound thiols and other changes that may alter cell membrane integrity (*Burton, 1988*). GSH is also a co-factor for enzymes such as glyoxylase, formaldehyde dehydrogenase and maleyl acetoacetate isomerase. There is evidence for its involvement in intracellular calcium homeostasis from its ability to maintain thiol groups in a reduced state at the active sites of Ca-ATPases.

1.3.4 Glutathione transhydrogenases.

Thiol-disulphide interchange is involved in several metabolic and physiological functions including protein synthesis and degredation, enzyme activation and inactivation, the synthesis of deoxyribose intermediates for DNA synthesis, and the reduction of cystine. Reduced glutathione has been thought to participate in these reactions in view of the fact that it is the major intracellular thiol. There is evidence for the existence of enzymes that catalyse thiol-disulphide interchange between GSH and low molecular weight disulphides, as well as between GSH and protein disulphides (*Mannervik and Eriksson, 1974*). GSSG reductase does not catalyse these reductions; they appear to be mediated by separate transhydrogenases. Although glutathione transhydrogenases have important functions with regard to the synthesis, structure, degredation, and function of proteins and enzymes, there are other systems that affect the thiol-disulphide status of cells that are also of considerable importance. These include the thio- and glutaredoxin systems which exhibit a high reducing potential.

1.3.5 Protection against myocardial ischaemia and infarction.

A range of pharmacological agents have been shown to be protective in animal models of myocardial ischaemia and infarction either by decreasing myocardial oxygen demand or by increasing oxygen supply (*Maroko et al., 1971*). However, their clinical significance is limited and as a result the limitation of infarct size has

been approached by other methods of myocardial cytoprotection.

The ischaemically-induced production of oxygen-derived free radicals is accompanied by a loss of protective enzyme and antioxidant mechanisms that are normally present in the myocardium (*Burton et al., 1984*). Among the more important protective systems is the glutathione redox pathway, whose role in the attenuation of free radical-induced myocardial necrosis has been investigated and demonstrated (*Forman et al., 1988*). In addition, glutathione peroxidase is significantly more active than other antioxidant enzyme systems (*Fantome and Ward, 1982*). Therefore, the maintainance of the glutathione pathway and related enzymic defence systems against oxidative stress is vital to prevent myocardial cell death under such conditions.

N-Acetylcysteine (NAC) is a glutathione precursor which has a longer plasma half-life than reduced glutathione. It also undergoes auto-oxidation at a slower rate than other sulphydryl compounds (*Estrela et al., 1983*) and has been used effectively to replenish intracellular glutathione stores in several disease processes (*Olson et al., 1980; Bernard et al., 1984*). Alberola *et al.* (1991) have shown NAC limits the extent of irreversible injury in the ischaemic canine heart but does not decrease myocardial oxygen demand or influence the regional distribution of myocardial blood flow. Therefore, it appears that NAC is protective by other mechanisms. The studies of Alberola *et al.* (1991) showed NAC to attenuate an ischaemically-induced reduction in intracellular reduced glutathione (GSH). It is unclear, however, whether this represents the protection of existing GSH stores from depletion or the replenishment of depleted GSH. NAC is also a free radical scavenger and inhibits neutrophil-mediated free radical production (*Bernard, et al., 1984; Tribble et al., 1987*). More recent studies have highlighted the beneficial effects of sulphydryl compounds against myocardial infarction which supports the theory that these compounds, and related enzymatic systems, are essential for myocardial protection against oxidative stress. Although the protective mechanism of NAC is unknown, the maintainance of intracellular GSH levels may be critical.

1.4 SODIUM/POTASSIUM ADENOSINE 5' TRIPHOSPHATASE (Na/K ATPase).

The Na/K ATPase is considered to be the biochemical representation of the Na/K pump that is critical for the maintainance of cellular ionic homeostasis. The modification of its structure, function and activity during periods of ischaemia and oxidative stress (*Kim and Akera, 1987*) may represent a mechanism by which free radicals are arrhythmogenic. It may also represent a site where radical scavengers and antioxidants, such as reduced glutathione, act, thereby attenuating ischaemically-induced potassium loss to the extracellular space and the incidence of reperfusion-induced arrhythmias.

1.4.1 The nature of the enzyme.

Na/K ATPase (EC 3.6.1.3) has been extensively purified and characterized, especially from cardiac tissue, with little species to species variation. The enzyme consists of a catalytic alpha subunit that is a polypeptide of about 95 000 daltons to which cardiac glycosides bind, and a glycoprotein, beta subunit with a molecular weight of approximately 45 000 daltons. It is thought that the enzyme exists as a dimer *in vivo* with each monomer consisting of a catalytic and glycoprotein subunit (figure 3). Hiatt *et al.* (1984) have suggested that the beta subunit may be involved in the insertion of the alpha subunit into the membrane. It has been shown that Na/K ATPase is present in the membranes of all animal tissues in which a sodium gradient is present. In addition, enzyme activity is highest in those tissues, such as the myocardium, which extrude sodium at a relatively high rate. In intact cells, the Na/K ATPase is the biochemical representation of the sodium pump and is one of the best-studied electrogenic transporters. It transports three intracellular sodium ions for two extracellular potassium ions resulting in a net outward membrane current with a K_m for potassium and sodium of 1.2mM and 18mM, respectively (*Gadsby et al., 1989; Shattock and Matsuura, 1990*). Inhibition of Na/K ATPase results in a decrease in sodium extrusion and potassium uptake. Although both sodium and potassium are both determinants of sodium pump activity, intracellular sodium has a greater influence than extracellular potassium.

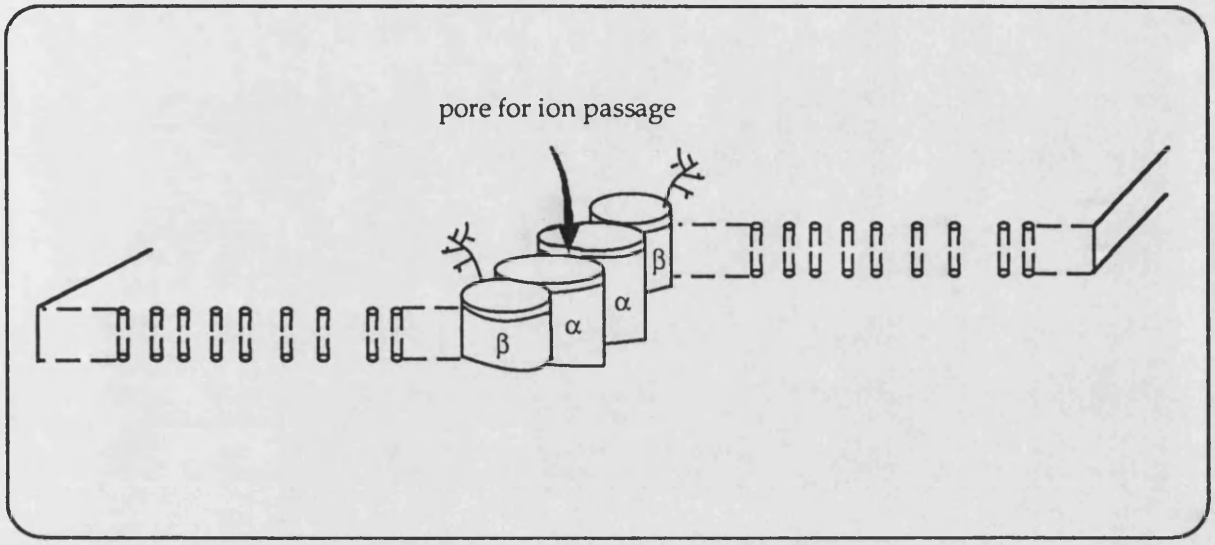


Figure 3. Diagramatic representation of an oligometric (α–β) structure for Na/K ATPase, highlighting the pore for ion passage between the two α-subunits. (*Redrawn from Jorgensen, 1982*).

1.4.2 Na/K ATPase activation and inhibition.

1.4.2.1 Sulphydryl groups.

In addition to the well known inhibition of the Na/K pump by cardiac glycosides, Na/K ATPase activity is significantly reduced by several other agents. Both thallium and rubidium have been reported to have an inhibitory action (*Ku et al., 1975, 1976*) which may have bearing on the use widespread use of ^{86}rubidium as a marker for potassium movement via sarcolemmal ion transporters. The literature also describes the inhibitory action of several sulphydryl blocking agents that produce dose-related, positive inotropic effects in isolated heart preparations (*Temma et al., 1978*). This type of compound, such as *N*-ethylmaleimide and *p*-chloromercuribenzoate, is thought to act at a different site to the cardiac glycosides and monovalent cations. Kawamura and Nagano (1984) have shown that there may be a single disulphide bridge in the beta subunit that may lead to loss of enzyme activity upon its cleavage with beta-mercaptoethanol. There is also thought to be one free sulphydryl group in the beta subunit. There are also several classes of sulphydryl groups are present in the alpha subunit that have been classified with respect to their rate of reaction with different probes. However, reactivity does not correlate with those groups essential for ATPase activity (*Askari et al., 1979; Schoot et al., 1979; Harris and Stahl, 1980*).

Kirley (1990) has shown that ATPase activity and ouabain binding is partly decreased by the reduction of a single, specific disulphide bond in the beta subunit. There are, however, three disulphides in the beta subunit that can be protected from reduction by relatively high concentrations of Na^+ and K^+. The presence of these cations also attenuates ouabain binding and increases ATPase activity. If there is a relationship between disulphide reduction in the beta subunit and the loss of ATPase activity and ouabain binding, it is much more complex than the simple one to one relationship postulated previously (*Kawamura and Nagano, 1984; Kawamura et al., 1985*). Kirley (1990) has suggested that the reduction of multiple disulphides may cause a change in the folding of the beta subunit, resulting in steric interference with extracellular cation binding sites on the alpha subunit or a change in the interaction between the subunits which is

critical for ATPase function. Therefore, the function of the alpha subunit may be affected in the absence of any direct effect on the alpha subunit itself. However, Kirley (1990) proposed that in the absence of high temperature and organic solvents used in the experimental protocol in his investigations, the degree of disulphide cleavage in the beta subunit would be insufficient to cause domain inactivation. Therefore, this indicates a delocalized protein denaturation mechanism rather than a specific bond cleavage is responsible for the reduction in Na/K ATPase activity by beta-mercaptoethanol. This work, however, has provided useful information on the presence and location of sulphydryl groups and disulphides in the alpha and beta subunits. Some workers have reported 12-17 free sulphydryl groups per alpha subunit (*Esmann, 1962; Gupte and Lane, 1979)* whilst Kirley (1990) has published data suggesting the presence of three disulphide bonds in the alpha subunit that has implications concerning the structure and stability of Na/K ATPase. These disulphides are present in the hydrophobic, membrane spanning regions of the protein where they contribute to the stability of the structural elements involved in sodium and potassium transport and their manipulation may prove critical in the regulation of cellular ion translocation.

1.4.2.2 Oxygen metabolites.

It is accepted that oxygen-derived free radicals are produced in the reperfused myocardium following a period of ischaemia (*Zweier, 1988*). There are a number of avenues whereby these toxic metabolites may be produced under these conditions. There may be a role for xanthine oxidase in superoxide production (*McCord, 1985*). Hypoxanthine and xanthine are formed and xanthine dehydrogenase is converted to xanthine oxidase during ischaemia. On the re-introduction of molecular oxygen a burst of superoxide production can occur. In addition, leukocytes, macrophages and monocytes which migrate to the ischaemic area can contribute to the production of free radicals (*Burton, 1988*). Mitochondrial superoxide production is also increased which, along with the autooxidation of catecholamines, may represent sources of free radicals during ischaemia/reperfusion (*Burton, 1988*). It is now thought that these reactive metabolites are not only involved in the gross degradation of membrane lipids but also in more subtle alterations in intrinsic membrane-bound protein structure and

function. With regard to this, Kramer *et al.* (1984) demonstrated the *in vitro* inhibition of sarcolemmal Na/K ATPase in response to oxygen radicals. It is, however, difficult to establish if this pump inhibition is the result of relatively selective interactions of oxygen radicals with intrinsic proteins, such as the sodium pump (Na/K ATPase), or whether it represents more general oxidant-induced damage to a multitude of cellular constituents. Undoubtedly, there is a certain degree of non-specific damage on exposure to oxidising species and these non-specific effects may be dependent on the severity of the insult (eg. the length of the ischaemic insult prior to reperfusion). Data from the work of Xie *et al.* (1990) suggests that an oxidant stress induced by the xanthine/xanthine oxidase system results in damage to several other sarcolemmal proteins as well as the Na/K ATPase, although it seems that damage to the sodium pump is an early event in the development of the toxic effects of extracellularly generated oxidants in the myocardium. One major problem with such experiments and the interpretation of the resulting data is the unknown nature and local concentrations of the reactive species. Kim and Akera (1987) indirectly investigated the effects of oxygen-derived free radical on sarcolemmal Na/K ATPase using an ischaemia/reperfusion model in isolated guinea-pig hearts. A reduction in enzyme activity was observed during ischaemia and reperfusion which could be attenuated using selected radical scavengers and antioxidants, such as superoxide dismutase, catalase, mannitol, vitamin E and dimethylsulphoxide. However, no determination of the sites of action of free radical-induced damage to Na/K ATPase was made.

1.4.3.3 Fatty acid intermediates.

In addition to the production of various free radical species and changes in cellular redox state, long chain acyl carnitines are known to accumulate in millimolar concentrations in the ischaemic myocardium (*Liedtke et al., 1978*). These fatty acid intermediates have damaging biochemical, electrophysiological and mechanical effects in the myocardium. Despite their well known detrimental effects, little is known about the local concentrations of this class of compound or the degree of protein binding that occurs *in vivo*. A reduction in Na/K ATPase activity during periods of myocardial ischaemia has been documented, and as a

result the effect of these compounds on sodium pump activity has been investigated using sarcolemmal vesicles. In the majority of these studies relatively high concentrations of palmitoyl carnitine have been used which cause myoglobin loss and a fall in contracture in the whole heart (*Hulsmann et al., 1985*). For this reason, the data from such investigations should be viewed with caution especially when ascribing specific effects of this type of compound.

The therapeutic manipulation of acyl carnitine levels has been approached by the inhibition of carnitine acyl transferase 1 which is protective in the ischaemic myocardium. At the present time there are two major problems with the currently available inhibitors, namely their irreversibility and the fact that they cause cardiomyopathy at high doses (*Spedding, 1990*).

1.5 ACIDOSIS AND MYOCARDIAL FUNCTION.

As previously described, intracellular acidosis, as a result of H^+ and lactate accumulation, is a hallmark of myocardial ischaemia that results in a depression of contractile function. In addition to an increase in intracellular $[H^+]$, there is a concomitant increase in intracellular sodium and calcium as a result of Na/H and Na/Ca exchange. This may affect vascular tone and the degree of ischaemic damage following reperfusion.

1.5.1 Background.

There are few reports in the literature on the effects of acidosis on the coronary vasculature using the isolated perfused heart model. Some groups have used smooth muscle preparations whilst others have concentrated on the mechanical response of the heart to an acidotic insult. In vascular smooth muscle preparations, such as aortic and coronary artery strips, respiratory acidosis results in a decrease in tone whilst alkalosis results in an increase (*Rinaldi et al., 1987; Loutzenhiser et al., 1990).* Although the mechanisms responsible for these effects are, as yet, unknown, several proposals have been made. These include the inhibition of calcium influx at low pH and the stimulation of this process at high pH. There are

reports in the literature of the effects of acidosis on both relatively large and fine vessels. Steenburgen *et al.* (1977) reported on the effects of acidosis in the constant pressure, isolated rat heart model. Acidosis, which was then synonymous with ischaemia, was induced by the use of a perfusate buffered at pH 6.7. A fall in coronary flow was noted that is indicative of increased vascular resistance in this model. This is in contrast to the vasodilator action of acidosis on the pre-contracted rat aorta reported by Loutzenhiser *et al.* (1990).

Hydrogen ions are not passively distributed, and in order to maintain intracellular pH within narrow limits both a proton extrusion mechanism and an intracellular buffering system are essential. Three systems have been suggested, namely the $NaHCO_3$-Cl, the Cl-HCO_3 and the Na-H exchange mechanisms (*Poole-Wilson et al., 1984*), the latter of which appears to play a major role in the recovery from an acid load. In the short term, a significant proportion of acid can be neutralized by reversible and rapidly responding mechanisms that serve to buffer acid loads. In the long term, intracellular pH homeostasis relies on the cells ability to extrude protons or accumulate HCO^-_3 or OH^-. In the steady state, the intracellular accumulation of H^+ is balanced by the energy-requiring extrusion of acid. Siesjö and Messeter (1971) first investigated the cells response to an acid load, which caused a fall in pH_i of rat brain cells and subsequent recovery following washout with buffer at pH 7.4. The authors suggested that the initial CO_2-induced fall in pH_i was minimised by biochemical buffering but that the recovery phase was due to the active transport of protons or HCO^-_3 across the cell membrane. Following this work there has been increasing evidence for such a H^+ extrusion mechanism (*Roos and Boron, 1981*) which is enhanced in rat cardiac muscle by cAMP (*Fenton et al., 1978*). Previous studies in adult guinea-pig, rat, rabbit and ferret hearts have also shown that $[Ca]_i$ increases in acidosis (*Allen and Orchard, 1983; Orchard, 1987; Lee et al., 1988; Solaro et al., 1988).* The mechanism of this accumulation of free cytosolic calcium is unclear although it may be a result of a decrease in calcium sequestration by intracellular organelles such as the sarcoplasmic reticulum *(Orchard, 1987).* Also, at low pH values, calcium uptake and release from the SR may be decreased (*Fabiato and Fabiato, 1978*). An increased diastolic $[Ca]_i$ as a result of acidosis may also cause an increased

calcium-induced calcium release from the SR (*Orchard, 1987*). In addition, an acidosis-induced increase in intracellular [H^+] may result in an increase in intracellular sodium, via Na/H exchange. Sodium can exchange for calcium via the Na/Ca exchange mechanism that may manifest as an increase in free cytosolic calcium.

Severe acidosis is often accompanied by a decreased vasoconstrictor response to catecholamines (*Campbell et al., 1958; Dusting and Rand, 1975*). Several consequences of a reduction in pH contribute to this effect in smooth muscle, such as reduced in Ca^{2+} influx (*van Breemen et al., 1972; Kohlhardt et al., 1976*), inhibition of myofilament contractility (*Portzehl et al., 1969; Fabiato and Fabiato, 1978*) and alteration of cell surface receptors (*Flavahan and McGrath, 1981*). Loutzenhiser *et al.* (1990) suggested that a small degree of acidification stimulates calcium sequestration into intracellular stores that are sensitive to noradrenaline.

The effects of acidosis may be partly mediated by enzyme inhibition due to the pH optima of many enzyme systems. The synthesis and degradation of many vasoactive compounds are under the control of such systems and therefore may be susceptible and sensitive, to varying degrees to changes in extracellular and/or intracellular pH depending on the locality of the enzyme. For example, the ability of noradrenaline to constrict smooth muscle at lowered intracellular calcium concentrations is thought to be due to the activation of protein kinase C (*Rasmussen and Barrett, 1984; Jiang and Morgan, 1987*). Protein kinase C has a pH optimum of 7.5-8.0 and could theoretically be inhibited by reduced pH. This is in contrast to myosin light chain kinase which is relatively insensitive to pH changes between 6.5 and 9.0 (*Blumenthal and Stull, 1982*).

In addition to changes in vascular tone mediated via ion exchange mechanisms such as the Na/Ca, Na/H and Na/K exchange mechanisms, vasoactive substances released from the myocardium may be responsible for the response of the coronary vasculature to acidosis. There are some reports on the effect of acidosis on the release of catecholamines from the isolated rat heart (*Dart and Riemersma, 1989*) although it is not clear whether they are released in sufficient concentrations to cause a significant modulation of vascular tone. Neuropeptide Y (NPY) is a co-transmitter with noradrenaline and potentiates the action of other vasoactive

substances such as phenylephrine, histamine, angiotensin II and noradrenaline, at lower concentrations than are required for a direct response. Exercise results in extracellular acidosis and the release of NPY from the human heart and may contribute to an acidosis-induced change in coronary tone either directly or by potentiating the pressor effect of noradrenaline (*Walker et al.*, 1991).

CHAPTER 2.

MATERIALS AND METHODS.

2.1 ISOLATED LANGENDORFF PERFUSED RAT HEART PREPARATION.

This preparation was used to investigate the effects of selected thiol compounds on ischaemia/reperfusion-induced myocardial noradrenaline release and ^{86}Rb efflux as well as the physiological response of the heart to acidosis.

Hearts from male Wistar rats (University of Bath strain, 250-350g), were perfused using the Langendorff method at a constant flow rate of 10ml/minute at 37°C with a modified Krebs-Henseleit solution of the following composition (mM): lactate 5.0; NaCl 118.0; $NaHCO_3$ 25.0; KCl 4.7; KH_2PO_4 1.2; $CaCl_2$ 1.2; $MgSO_4$ 1.2, which was continuously gassed with 95% O_2, 5% CO_2. In all experiments, hearts were allowed to stabilize for 15 minutes prior to any interventions. During the final 5 minutes of the stabilization period hearts were paced at 300 b.p.m (pulses of 7 volts, rate 5 Hz and a duration of 2.5ms) using a Grass S88 stimulator). Hearts were paced in all experiments. Heart rate prior to pacing was 225±55 b.p.m (n=55). Perfusion pressure changes were monitored using a Bell and Howell pressure transducer connected to a side arm on the perfusion line. Developed tension, under a resting tension of 2g, was recorded with a Devices isometric transducer attached to the apex of the heart with a length of cotton and a barbless hook; the signal from this transducer was used to trigger an instantaneous rate meter. All recordings were made on an 8 channel Devices thermal recorder. Any drug injections were made in volumes of less than 100µl via a side-arm situated close to the aortic valves. Figure 4 illustrates the perfusion apparatus used in these experiments.

2.2 THE INITIATION OF GLOBAL, LOW-FLOW MYOCARDIAL ISCHAEMIA AND SUBSEQUENT REPERFUSION.

This protocol was used to investigate the effect of selected agents on ischaemia/reperfusion-induced noradrenaline overflow and contracture.

Following a 15 minute equilibration period, global, low-flow ischaemia was induced by reducing the flow rate of the perfusate by 90% to 1ml/min and superfusing the heart with anoxic perfusate (gassed with 95% N_2 / 5% CO_2) at 9ml/min. In these experiments the ischaemic period was either 20, 30 or 60

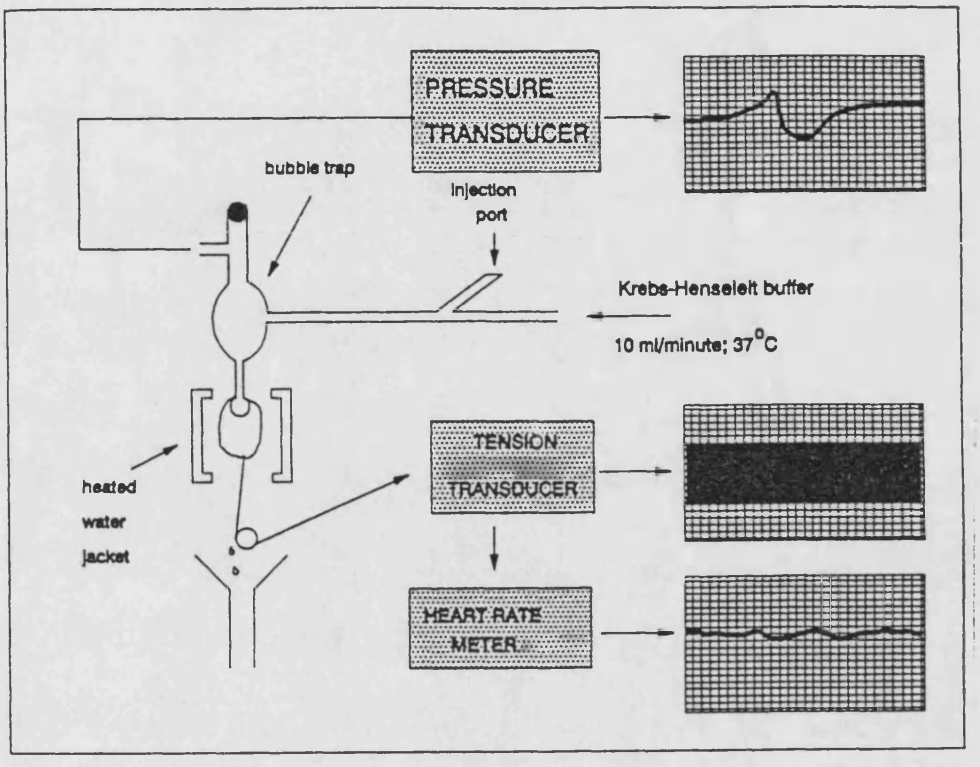


Figure 4. Diagram of the apparatus used for the isolated Langendorff perfused heart preparation.

minutes. Reperfusion was initiated, after the appropriate period of ischaemia, by restoring the flow to its pre-ischaemic level with oxygenated perfusate. In all experiments reperfusion was for 5 minutes after which the experiment was terminated and the heart removed from the apparatus, blotted dry and weighed.

2.3 MEASUREMENT OF CORONARY PERFUSATE NORADRENALINE LEVELS DURING MYOCARDIAL ISCHAEMIA AND SUBSEQUENT REPERFUSION.

This technique was utilized, in conjunction with the isolated perfused rat heart preparation, to determine both perfusate and tissue levels of noradrenaline during myocardial ischaemia and reperfusion

2.3.1 Collection of perfusate samples.

Hearts were perfused with Krebs-Henseleit solution (containing 5.9mM K^+) for a control period of 15 minutes. At this point hearts were paced at 300 b.p.m (pulses of 7 volts, rate 5 Hz and a duration of 2.5ms) using a Grass S88 stimulator. The successful initiation of pacing was confirmed by observation of the heart rate meter. Five minutes after the onset of pacing, global low-flow ischaemia was induced, as described in section 2.2, for the required period followed by 5 minutes reperfusion. Perfusate samples were collected in 1 minute aliquots at 5 minutes intervals during the equilibration period. During the ischaemic phase samples were collected in 1 minute aliquots at 10 minute intervals whilst during the 5 minutes of reperfusion samples were continously collected in 30 second aliquots. Sample collection was performed using a Gilson Model 203 automated fraction collector. The samples were kept on ice until the end of the experiment when they were all immediately assayed for noradrenaline content using HPLC with electrochemical detection.

2.3.2 Determination of perfusate noradrenaline levels by HPLC.

2.3.2.1 Preparation of HPLC mobile phase.

The composition of the mobile phase was (mM): 1-octanesulphonic acid (sodium

salt, HPLC grade) 0.5; sodium acetate (HPLC grade) 100; sodium hydroxide (HPLC grade) 60; citric acid (HPLC grade) 40, dissolved in 10% HPLC grade methanol: 90% Milli-Q grade water. The mobile phase was filtered through a 2µm Millipore™ filter using a Millpore™ vacuum filtration system, gassed to saturation with helium and finally degassed by sonication for 20 minutes prior to use.

2.3.2.2 Preparation of activated alumina (aluminium oxide).

Aluminium oxide was activated to obtain the grade recommended by Anton and Sayre (1962). 100 grams of aluminium oxide (neutral, Brockman grade 1, BDH) was added to 500 ml of 2M HCl in a Pyrex beaker, covered and heated to 90-100°C on a hot plate. The aluminium oxide was stirred for 45 minutes under these conditions. At the end of this period the aluminium oxide was allowed to settle and the yellow supernatant carefully decanted off. The aluminium oxide was washed twice with 250 ml aliquots of fresh 2M HCl at 70°C for 10 minutes, discarding the supernatant each time. Subsequently, the precipitate was stirred with 500 ml of 2M HCl for 10 minutes at 50°C. The aluminium oxide was then repeatedly washed (20-25 times) with 200 ml aliquots of distilled water until the supernatant had a pH of 3.4. Finally, the aluminium oxide was transferred to an evaporating dish and heated at 120°C for 60 minutes and at 200°C for a further 120 minutes in an oven. The activated alumina was then stored in a capped glass bottle in a dessicator at room temperature until required.

2.3.2.3 Extraction procedure for the assay of perfusate noradrenaline levels.

To a 10ml tube the reagents were added in the following order: 0.3ml perfusate, 0.05ml 300mM disodium EDTA, 0.05ml 50mM reduced glutathione, 0.05ml 3µM 3,4-dihydroxybenzylamine (DHBA) as an internal standard and 30mg of activated alumina. The reactants were mixed briefly by hand and then 0.15ml 1M Tris (pH 8.6) was added. The tubes were then stoppered and mixed on a rotary mixer for 20 minutes at room temperature. The tubes were centrifuged briefly, the supernatant decanted off and the alumina, containing absorbed noradrenaline, washed 3 times with 5ml of 3mM disodium EDTA pH 7.0. Finally the noradrenaline was eluted off the alumina by adding 0.3ml of 0.2M perchloric acid. The elutant was stored at

-25°C until its noradrenaline content was determined by HPLC.

2.3.2.4 Assay of perfusate extracts for catecholamines by high performance liquid chromatography.

The noradrenaline (NA) content of the perchloric acid extracts was determined using reversed phase, ion-pair HPLC with electrochemical detection. The liquid chromatograph consisted of an LDC Model III Constametric pump, a Rheodyne 7 125 injection valve with a 100µl loop, a 25 cm x 4.6 mm (i.d) stainless steel analytical column packed with 5µM diameter Hypersil-ODS (Shandon, U.K) particles and a Bioanalytical systems LC-4A amperometric detector. The detector was operated at +0.65V with an Ag/AgCl reference electrode (BAS RE-1) and a glassy carbon working electrode. The flow rate of the mobile phase (for composition, see above) was maintained at 1.0 ml/minute. Chromatograms, of which a typical example is shown in figure 5, were recorded on a JJ Instruments flat-top recorder. Peak heights and peak height ratios (NA/DHBA) were measured and calculated manually.

2.3.2.5 Calibration.

Aliquots of perfusate were spiked with known amounts of noradrenaline. After the addition of the internal standard (DHBA), the samples were extracted and assayed as previously described. In addition, blank samples of perfusate were assayed to allow the correction of the true noradrenaline content of experimental samples. The peak height ratios (NA/DHBA) were plotted against perfusate concentration to obtain a calibration curve. This curve was used to convert peak height ratios into concentration values when analysing perfusate samples.

2.3.2.6 Calculation of recovery.

The recovery of noradrenaline was determined by comparing the peak height ratios of spiked samples assayed after alumina extraction and of standards injected directly into the chromatograph. The recovery of DHBA was calculated by comparing the peak height ratios obtained during the construction of the calibration curve with those obtained when the DHBA was added during the final elution. The

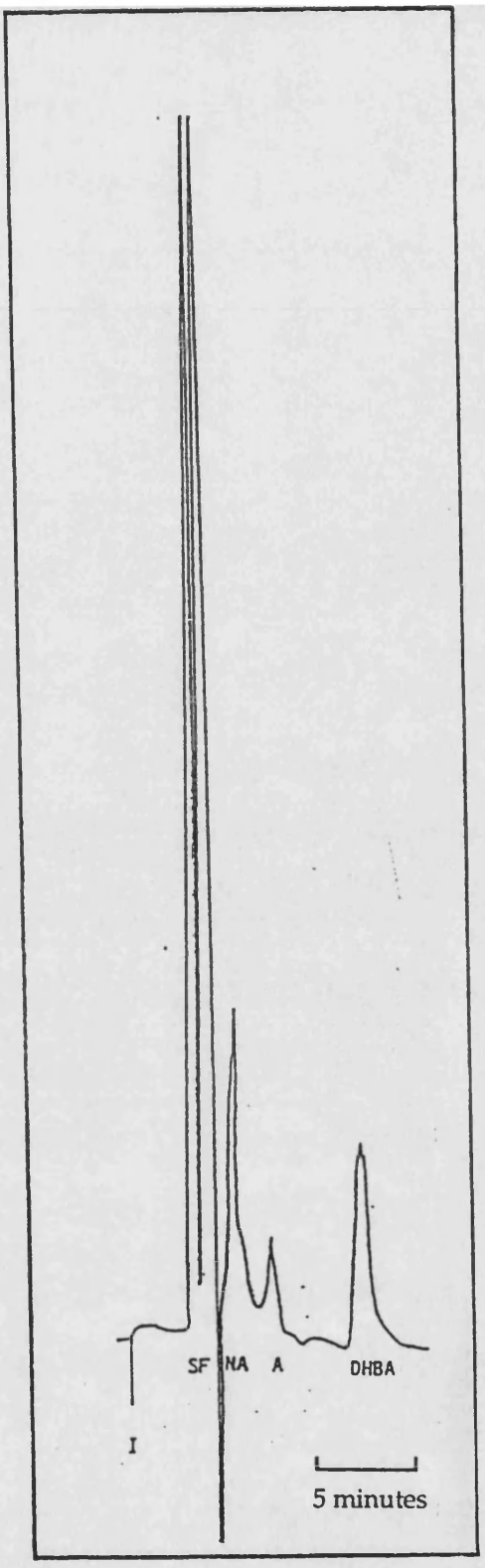


Figure 5. Typical high performance liquid chromatogram of the quantitative analysis of coronary perfusate for selected catecholamines. (Peak identification: I-sample injection, SF-solvent front, A-adrenaline, NA-noradrenaline, DHBA-3,4-dihydroxybenzylamine.

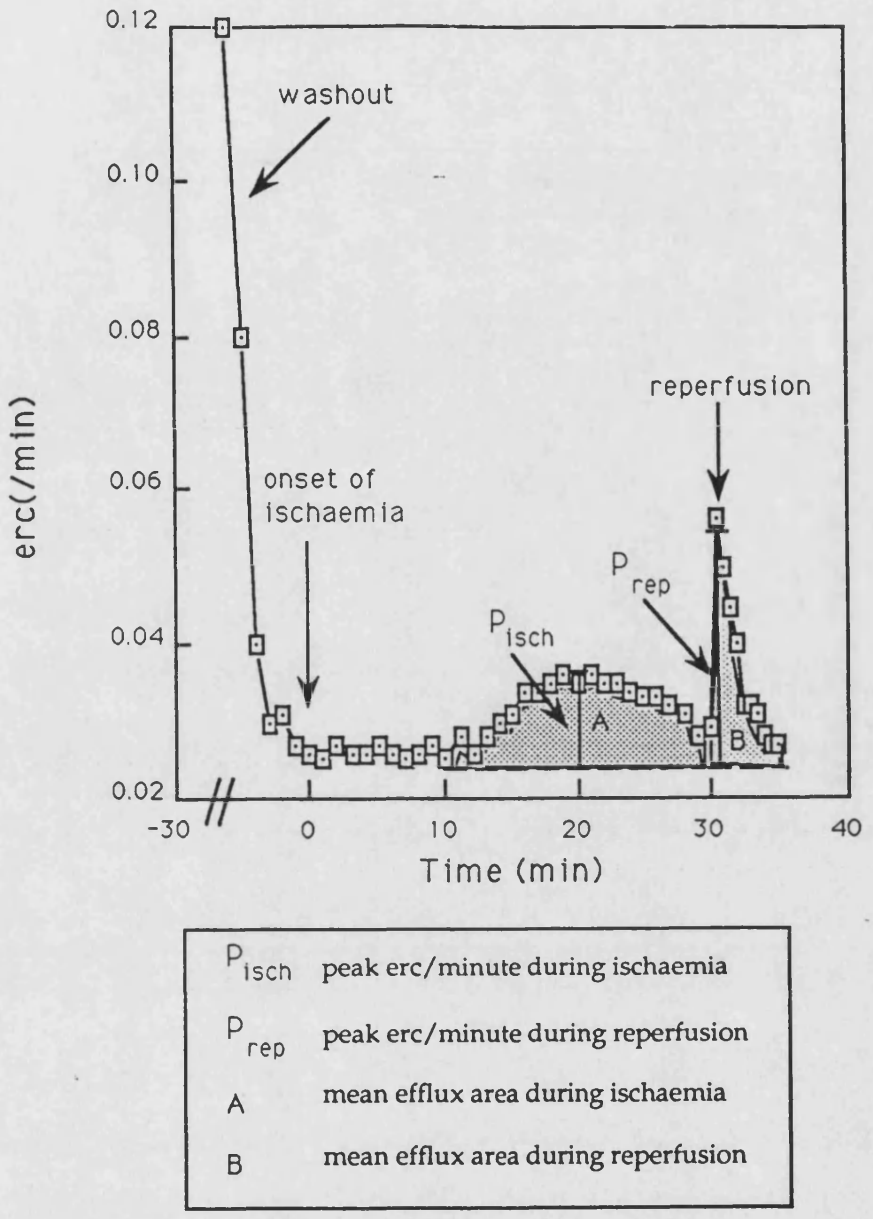


Figure 5a. Typical trace of ^{86}rubidium efflux rate coefficient (erc/min) during 30 minutes ^{86}rubidium washout, 30 minutes global, low flow ischaemia and 5 minutes subsequent reperfusion. Peak erc/min during ischaemia and reperfusion are represented as P_{isch} and P_{rep}, respectively. Mean efflux area during ischaemia and reperfusion are represented as A and B, respectively. The onset of ischaemia and reperfusion are indicated by the arrows.

recovery of noradrenaline and DHBA were 60% and 65% respectively.

2.3.2.7 Linearity of the extraction procedure and detector response.

The linearity of the extraction procedure was tested by spiking samples of perfusate with increasing amounts of noradrenaline and putting them through the normal extraction procedure. DHBA was only added with the 0.2M perchloric acid used for the final elution. The extracts were analysed and peak height ratios determined as before. Results from spiked samples were corrected for endogenous noradrenaline using results from blank perfusate samples. In addition, the linearity of the detection system was determined by the direct injection of standard solutions into the chromatogram. The concentration of DHBA was fixed. Both the extraction procedure and detection system were linear within the range corresponding to perfusate noradrenaline concentrations of 2.5-80 pmoles/ml.

2.4 DEPLETION OF ENDOGENOUS NORADRENALINE USING 6-HYDROXYDOPAMINE.

The effect of endogenous noradrenaline depletion on the onset of an ischaemic contracture was determined by reducing myocardial noradrenaline content with a single intravenous dose of 6-hyddroxydopamine.

An animal's endogenous noradrenaline may be depleted over a 24 hour period using a single intravenous dose of 50mg/Kg, 6-hydoxydopamine (HCl salt) (*Kostrzewa and Jacobowitz, 1974*) dissolved in 0.1ml of 0.8% ($^{w}/_{v}$) saline immediately prior to use. The animals' hair standing on end approximately 1 minute after dosing was used as an indication that the drug had been successfully delivered. These animals were sacrificed 17-24 hours later. In all experiments involving the use of noradrenaline depleted hearts, two hearts from the same group were assayed for tissue noradrenaline levels as were hearts from two untreated animals. This allowed calculation of the degree of tissue noradrenaline depletion expressed as a percentage of the control untreated hearts.

2.5 DETERMINATION OF TISSUE LEVELS OF NORADRENALINE.

Hearts were quick-frozen using aluminium tongs pre-cooled in liquid nitrogen. For the assay, hearts were ground in an aluminium grinder (pre-cooled in liquid nitrogen) to form a fine powder that was then further homogenised in 10ml of 0.1M perchloric acid containing 4.3mM disodium EDTA and 4.2mM $Na_2S_2O_5$, using a Polytron homogeniser. The homogenate was then centrifuged for 10 minutes at 400 rpm and 1ml of the resulting supernatant used for the assay. 50µl of 3µM DHBA, 500µl of 3M Tris (pH8.6) and 30mg of activated alumina were added to the supernatant and mixed for 10 minutes. After washing the alumina three times with distilled water, the amines were eluted from the alumina with 600µl of 0.2M perchloric acid (HPLC grade). 0.1 ml of the elutant was run on the column as previously described and its noradrenaline content determined.

2.6 ^{86}RUBIDIUM EFFLUX EXPERIMENTS.

^{86}Rubidium was used as a marker to monitor the net loss of potassium during myocardial ischaemia/reperfusion and the effect of selected compounds on this physiological response.

Hearts were perfused with Krebs-Henseleit solution for a control period of 5 minutes after which ^{86}Rb (0.2µCi/ml; Amersham, U.K) was added and perfused for a further 10 minutes. A 30 minute washout period then followed during which the heart was paced (same criteria as section 2.3.1). Any drugs were perfused for 10 minutes immediately after the washout period and during the subsequent period of ischaemia and reperfusion. 30 minutes global ischaemia was initiated during which the flow was reduced by either 90, 95 or 97.25%. Reperfusion was carried out by returning the flow to its pre-ischaemic level of 10ml/minute. Perfusate samples were collected in 1 minute aliquots during the 30 minute washout period, during the addition of drugs prior to the onset of ischaemia and throughout the ischaemic phase. During the reperfusion phase, perfusate was continuously collected in 30 second aliquots. 1ml of each sample was mixed with 8ml of scintillation fluid (Optiphase 'safe') and counted in an LKB 1215 scintillation counter using an external channels ratio method for 10,000 counts or for 10 minutes (*Durbin and*

Jenkinson, 1961).

At the end of the experimental period the heart was blotted with tissue paper, chopped into small pieces and left to digest in 5mls of 1M NaOH. After 48 hours the digested myocardial tissue was neutralized with 5mls of 1M HCl to prevent chemiluminesence (*Durbin and Jenkinson, 1961*), and the decay during this period allowed for. Finally, 1ml of the neutralized material was added to 8mls of scintillation fluid and counted as above. ^{86}Rubidium efflux is expressed as efflux rate coefficient at time *'t'* (erc/minute) calculated according to equation 1. To enable rapid data processing, computer software was utilized that was originally written by Dr D.M Coldwell (Smith-Kline Beecham Pharmaceuticals, Harlow, Essex) (Appendix 1). In these experiments, peak erc (min^{-1}) and the mean efflux area during ischaemia and reperfusion was determined which are diagramatically represented in figure 5a.

$$\text{erc } (min^{-1}) = \frac{\text{perfusate count}}{\text{total count x collection time (min)}} \qquad \textit{equation 1.}$$

2.7 RAT ISOLATED PERFUSED MESENTERIC VASCULAR BED PREPARATION.

The effect of acidosis on the tone of the pre-contracted mesenteric vascular bed was investigated using this preparation.

The superior mesenteric artery was cannulated, the middle colic artery ligated and the mesenteric vascular bed removed by careful dissection from male Wistar rats (University of Bath strain) weighing between 250-350g. Perfusion of the mesenteric vascular bed was performed at a constant flow rate of 4ml/minute with standard Krebs-Henseleit solution (see section 2.1) saturated with 95% O_2, 5% CO_2. Changes in perfusion pressure were monitored in a similar way to that described for the isolated perfused heart preparation (see section 2.1). The tissue was allowed to equilibrate for 30-40 minutes following dissection before any interventions were carried out. In some experiments the vascular tone was artificially elevated by the inclusion of pharmacologically active agents in the perfusion fluid.

2.8 ISOLATED RAT THORACIC AORTA PREPARATION.

The effect of acidosis on the tone of pre-contracted aortic rings was determined using this isolated tissue preparation.

Segments of thoracic aorta, from male Wistar rats (University of Bath, 250-300g), of about 0.5cm length were prepared as rings and mounted for isometric tension recording, under a resting tension of 2g, in a 10ml tissue bath containing Krebs-Henseleit solution saturated with 95% O_2, 5% CO_2 at 37°C. Tissues were left to equilibrate for 30-45 minutes before starting the experiment. In experiments where tissues were pre-contracted with 10μM noradrenaline the tissue was allowed to equilibrate for a further 10 minutes after maximal contraction was achieved. In all experiments the integrity of the endothelium was tested by the addition of acetylcholine (1μM). Endothelial cell damage was assumed to have occured if less than 80% relaxation of the aortic strip was observed in response to the prefusion of 1μM acetylcholine and these preparations were rejected. The tissues were then washed and, after a period of recovery, re-contracted with 10μM noradrenaline.

In addition, in some experiments an alternative physiological salt solution was used. The composition of this solution was (mM): NaCl 140; KCl 4.6; $CaCl_2$ 1.5; $MgCl_2$ 1.0; D-glucose 10.0; and HEPES 5.0. The perfusate was saturated with oxygen and the desired perfusate pH obtained by the addition of 1M HCl.

2.9 GUINEA-PIG TAENIA-COLI PREPARATION.

The guinea-pig taenia-coli preparation was used to determine any calcium-antagonist activity of cysteine.

Taenia preparations from the caecum of male guinea-pigs (University of Bath strain, 350-450g) were suspended in 10ml organ baths containing Krebs-Henseleit solution saturated with 95% O_2, 5% CO_2 at 37°C. Contractions were measured using a Devices isometric tension transducer connected to a Lectromed Multitrace 2 chart recorder. All preparations were mounted under a resting tension of 1g and initially left to equilibrate for 30 minutes before changing the bath solution to a calcium-free Krebs-Henseleit solution for a further 30 minutes. Following this, calcium chloride (0-2700μM) was added cumulatively to the perfusion solution, the

preparation allowed to recover in solution free of added calcium for 40 minutes and the addition of calcium chloride repeated. When assessing the effects of a drug on the contractile response to calcium, the drug was included in the perfusion solution for 20 minutes prior to the further addition of calcium.

2.10 ISOLATED RAT MYOCYTE PREPARATION.

Isolated ventricular myocytes were used to investigate the effect of selected agents on Na/K-activated pump current and free cytosolic calcium levels using appropriate electrophysiological and fluorometric techniques described in sections .

Isolated rat ventricular myocytes were prepared using an enzymatic procedure similar to that described by Powell and Twist (1976). Briefly, hearts from male Wistar rats (Bath University strain) weighing 250-300g were perfused at 37°C with a HEPES-Tyrode solution of the following composition (mM): NaCl 140; KCl 5.4; $CaCl_2$ 1.8; $MgCl_2$ 0.5; glucose 5.5; HEPES 5.0, adjusted to pH 7.4 with the required volume of 1M NaOH and saturated with O_2. After 5 minutes the perfusate was switched to a HEPES-Tyrode solution free from added calcium ("Ca^{2+}-free" Tyrode) for a further 5 minutes. This Tyrode solution contained an undetermined level of contaminant Ca^{2+} from the water source and the analytical reagents used. At this point, 50mls of "Ca^{2+}-free" Tyrode containing 20mg collagenase (EC 3.4.24.3, Type 1 from *Clostridium histolyticum*, Sigma, U.K) and 10mg protease (Pronase, Type XIV from *Streptomyces griseus*, Sigma, U.K) was perfused through the coronary circulation in a recycling mode for 15 minutes. A 3 minute washout period with "Ca^{2+}-free" Tyrode was then performed and the heart carefully removed from the cannula and placed in a petri-dish containing approximately 20mls "Ca^{2+}-free" Tyrode. Both atria were removed and the remaining ventricular tissue cut with scissors into about 10 equal pieces. These were then individually teased apart using small tweezers and gently agitated to free individual myocytes. Excess tissue fragments were removed from the petri dish and discarded. 0.5ml of calcium-containing Tyrode was then added to the cell suspension and the cells viewed under low power using a Nikon inverted microscope. Cells remained viable for at least 6-8 hours, confirmed by trypan-blue exclusion. The proportion of rod-shaped:round cells was within

50-60:1 immediately prior to and after the addition of calcium. Over the experimental time period (2-4 hours) the ratio of rods/round-shaped cells was maitained at 40-45:1.

2.11 ISOLATION AND PURIFICATION OF BOVINE VENTRICULAR Na/K ATPase.

An isolated Na/K ATPase preparation was utilized to investigate the effect of selected compounds on the activity of this intrinsic sarcolemmal protein.

To obtain purified bovine Na/K ATPase, ventricular tissue was processed by the method of Pitts and Schwartz (1975) which is described below.

2.11.1 Preparation of fragmented membranes.

Fresh bovine hearts (2.0-2.5 kg) were collected from the local abbatoir and transported back to laboratory on ice within 30 minutes. 200g of ventricular tissue, trimmed of excess fat, was roughly chopped and blended in a pre-chilled Waring blender for 30 seconds at low speed with 300 mls 0.25M sucrose, 1mM Tris-EDTA (pH 7.0) [Sucrose-EDTA]. A further 300 mls of sucrose-EDTA was then added and the suspension homogenised for 45 seconds at high speed. 600 mls of sucrose-EDTA was then added, mixed briefly, and the homogenate filtered through two layers of household muslin. The filtrate was then centrifuged at 12 000 x g for 10 minutes (MSE High Speed 18 centrifuge). The resulting pellet was resuspended in 400 mls of sucrose-EDTA and blended for an additional 60 seconds at low speed. 50 mls of sucrose-EDTA was used to washout the blender and the washings combined. 40 mls of 5% ($^{w}/_{v}$) sodium deoxycholate was added dropwise to the homogenate which was then stirred for 20 minutes and subsequently centrifuged at 96 000 x g for 25 minutes (Beckman L8-M ultracentrifuge, Ti-60 rotor). The supernatant was removed carefully, diluted to 840 mls with 1mM EDTA (pH 7.0) and centrifuged again at 96 000 x g for 90 minutes (Beckman L8-M ultracentrifuge, Ti-60 rotor). The resulting pellet was then resuspended in 80 mls of 1mM EDTA and stored overnight at -20°C. Poor sedimentation results in subsequent stages if the enzyme is not frozen at this

stage.

2.11.2 Sodium iodide treatment.

Fragmented membrane suspensions from 400g of ventricular tissue obtained from stage (i) of the enzyme preparation were pooled and washed with an equal volume of 1mM EDTA and centrifuged at 40 000 x g for 30 minutes (Beckman L8-M ultracentrifuge, Ti-45 rotor). The resulting pellet was resuspended in a final volume of 120 mls in 1mM EDTA. 30 mls of 6M NaI (containing 15mM EDTA and 150mM Tris base, pH 8.4) was added dropwise, stirred for 5 minutes, diluted with 240 mls 1mM EDTA and centrifuged at 96 000 x g for 25 minutes (Beckman L8-M ultracentrifuge, Ti-45 rotor). The relatively soft pellet was retained and resuspended in 390 mls of 25mM imidazole-HCl, 1mM EDTA (pH 7.0) [imidazole-EDTA] and centrifuged at 96 000 x g for 20 minutes. The pellet (NaI-treated enzyme) was resuspended in 40 mls of imidazole-EDTA and after determination of protein content, by the method of Bradford (1976), adjusted to 10mg protein/ml.

2.11.3 Deoxycholate-citrate treatment.

To achieve a high yield of pure enzyme the ratio of detergent:protein at this preparative stage is critical. From the data of Pitts and Schwartz (1975) the optima of this ratio is approximately 0.67 mg deoxycholate/mg protein which was subsequently used in all preparations. 30 mls of NaI-treated enzyme (10mg/ml) was added to a suitable tube and imidazole-EDTA added to give a total volume of 34 mls. 22 mls of 1.5M sodium citrate was then added followed by 4 mls of 5% ($^{w}/_{v}$) deoxycholate, mixing briefly after each addition. The suspension was incubated at 0°C for 10 minutes and centrifuged at 150 000 x g for 15 minutes (Beckman L8-M ultracentrifuge, Ti-45 rotor). 40 mls of the supernatant was transferred to a tube containing 28 mls of imidazole-EDTA and centrifuged at 150 000 x g for 20 minutes (Beckman L8-M ultracentrifuge, Ti-45 rotor). The supernatant from this stage was then transferred to a tube containing 46 mls of imidazole-EDTA and centrifuged at 150 000 x g for 60 minutes (Beckman L8-M ultracentrifuge, Ti-45 rotor). The pellet was resuspended in 40 mls of

imidazole-EDTA and stored at 4°C. The specific activity of the preparation was determined as described in section 2.12.

2.12 ASSAY FOR Na/K ATPase ACTIVITY.

Na/K ATPase activities of enzyme preparations, prepared as previously described, were indirectly determined by continuously monitoring the oxidation of NADH at at 340nM and 37°C in a Cecil instruments dual-beam recording spectrophotometer using a linked-enzyme system (*Schwartz et al., 1969*). Each cuvette contained (mM): $MgCl_2$ 5; NaCl 100; KCl 10; Tris-HCl (pH 7.4) 25; Tris-ATP 2.5; NADH 0.5; phosphoenolpyruvic acid 2.5 and 0.02 ml of a combined pyruvate kinase-lactate dehydrogenase suspension (Sigma, U.K) in a final volume of 1.9 mls. Ouabain octahydrate (Sigma, U.K) was added in aqueous solution in a volume of 0.02 ml to give a final concentration of 0-100μM. After appropriate temperature equilibration the reaction was started by the addition of the Na/K ATPase preparation in a volume of 10μl, equivalent to 100μg of protein. Reaction rate was determined by drawing a tangent along the linear portion of the data and expressed as change in absorbance units/minute. This data was then converted to determine enzyme activity as units/mg protein. When assessing the ouabain-sensitivity of an enzyme preparation, a control rate in the absence of ouabain was inititally determined. This represents total ATPase activity. Ouabain (0-100μM) was then added and the % inhibition determined. Under control conditions (100mM Na^+, 10mM K^+), total, ouabain-sensitive and ouabain-insensitive ATPase activity was constant for at least 20 minutes. From the data of Schwartz *et al.* (1969) it is clear that substrate levels are not rate-limiting.

To examine the effect of a selected compound on total ATPase activity of a sarcolemmal Na/K ATPase preparation, the compound was added directly to the cuvette and any change in reaction rate monitored. In a separate group of experiments, the effect of the compound on the ouabain-insensitive fraction was determined by adding ouabain (final concentration 30μM) followed 3 minutes later by the compound in question and any change in reaction rate monitored. In two further group of experiments, to determine the effect of a compound on the ouabain-sensitive ATPase fraction, the total activity in the presence of the compound was determined. The ouabain-insensitive activity, following the application of 30μM ouabain, was calculated using a further sample of isolated

enzyme. The data from each group of experiments (i.e. to determine the effect of a compound on total, ouabain-sensitive and ouabain-insensitive ATPase activity) was pooled and expressed as a mean value (±s.e.mean).

In all experiments where the effects of reduced glutathione were examined in the presence of GSSG, GSH was added following the establishment of a stable rate in the presence of GSSG.

Additional experiments were carried out prior to the collection of experimental data to validate the specifity of the assay with respect to sensitivity to ouabain, temperature, sodium concentration, pH and substrate availability. In view of the fact that the assay utilises a linked enzyme system the effect, if any, of any compound under investigation was studied on the activity of pyruvate kinase and lactate dehydrogenase using appropriate assay systems.

2.13 DEPLETION OF REDUCED GLUTATHIONE *in vivo*.

The reduction in myocardial reduced glutathione level by this method was utilized to investigate the role of cellular glutathione status on the onset of an ischaemically-induced contracture.

Diethylmaleate (DEM) is an alpha,beta-unsaturated carbonyl compound and a weak electrophile that reacts with reduced glutathione (GSH) in the presence of glutathione transferases (*Younes and Siegers, 1980*). Introduced by Boyland and Chausseaud in 1967, DEM is the most widely used depleting agent in this class and has been shown to reduce the hepatic GSH of rats by 80-94% at a dose of 0.8ml/Kg (i.p) within 30 minutes of administration. In this set of experiments male Wistar rats (University of Bath strain, 250-350g) were dosed with DEM, (0.8ml/Kg, i.p), and sacrificed 30 minutes later. Hearts from these animals were then used for experimental purposes. Tissue levels of reduced and oxidised glutathione in control and treated hearts were determined spectrophotometrically as described in section 2.14.

2.14 QUANTITATIVE DETERMINATION OF MYOCARDIAL REDUCED (GSH) AND OXIDISED (GSSG) GLUTATHIONE CONTENT.

Myocardial glutathione levels were determined using this method to enable the correlation of tissue glutathione status with the onset of ischaemic contracture.

Hearts were removed from the perfusion apparatus, blotted dry and weighed. The heart was finely chopped in a beaker containing 5 volumes 1% ($^{w}/_{v}$) picric acid and homogenised in a 10 ml Teflon hand-held homogeniser. The homogenate was transferred to a 15 ml centrifuge tube and centrifuged at high speed for 5 minutes (IEC centra-7 centrifuge). 5µl of the supernatant was assayed for GSH and GSSG content. In these experiments it was assumed that total glutathione=[GSH] + 2 x [GSSG] (*Ferrari et al., 1989*).

For the assay of total tissue glutathione three working solutions were made up in a stock buffer of 125mM sodium phosphate and 6.3mM Na-EDTA adjusted to pH 7.5. These 3 stock solutions were (i) 0.3mM NADPH/buffer, (ii) 6mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and (iii) 50 Units/ml glutathione reductase (EC 1.6.4.2; Type III from Bakers yeast, Sigma, U.K). To assay for total glutathione, 700µl of solution (i), 100µl of solution (ii) and glutathione, sample or water to give a final volume of 1.0 ml were mixed in a 1 cm light path cuvette and equilibrated to 30°C. To the warmed solution 10µl of solution (iii) was then added and the absorbance at 412nM monitored continuously, in a Cecil Instruments dual-beam recording spectrophotometer, until it reached a value approaching 2. The rate of DNTB reduction was then evaluated from the linear portion of the data. The glutathione content of the aliquot was determined by comparison of the rate observed to a standard curve generated with known amounts of glutathione (0.1-3.0 nmoles). The GSSG content of samples was determined by derivatisation of the GSH present with 2-vinylpyridine. 2µl of 2-vinylpyridine per 100µl of protein-free supernatant was mixed vigorously for 1 minute and allowed to stand at room temperature for 60 minutes. 5µl of this solution was then assayed as above. The GSH was then determined by inference using the equation shown above.

The following experimental techniques were all performed by the author under the supervision of Dr M. Shattock and Dr H. Matsuura at the Cardiovascular Research Department, The Rayne Institute, St. Thomas' Hospital, London, SE1 7EH.

2.15 MEASUREMENT OF Na/K PUMP CURRENT IN ISOLATED RAT MYOCYTES.

This technique was utilized to determine the effect of the intracellular and extracellular application of selected compounds on Na/K pump current in isolated rat ventricular myocytes.

2.15.1 cell isolation.

Ventricular cells were obtained from hearts of male Wistar rats (St.Thomas' Hospital strain, 250-350g) using an enzymatic procedure similar to that described by Powell and Twist (1976) which is described in detail in section 2.10.

2.15.2 Recording arrangements.

Na/K pump currents were recorded in isolated ventricular myocytes using the whole-cell voltage-clamp technique originally described by Hammill *et al.* (1981). The recording arrangement used was that already described by Matsuura and Shattock (1990a). Briefly, patch electrodes were made from glass capillaries (o.d 1.5mm, i.d 1.0mm; Clark Electromedical Instruments, Reading, U.K). The electrode-puller itself was assumed to fire polish the electrodes sufficiently to obtain a high resistance seal between the tip and cell. Tip reistances were typically within the range 1-2 Mohm. The formation of a high resistance seal was confirmed by the reduction in voltage passing between the tip and cell. This was also confirmed by a reduction in an audio signal from the voltage-clamp amplifier. The apparatus and technique used for the intracellular dialysis and measurement of Na/K pump current in isolated rat ventricular myocytes are shown in figure 6a and 6b. A hydraulic micromanipulator (MO-102, Narishige Scientific Instruments, Tokyo, Japan) was used to position the patch electrode near the cell and a tight seal (resistance 10 to 100 Gohm) was established between the electrode tip and the

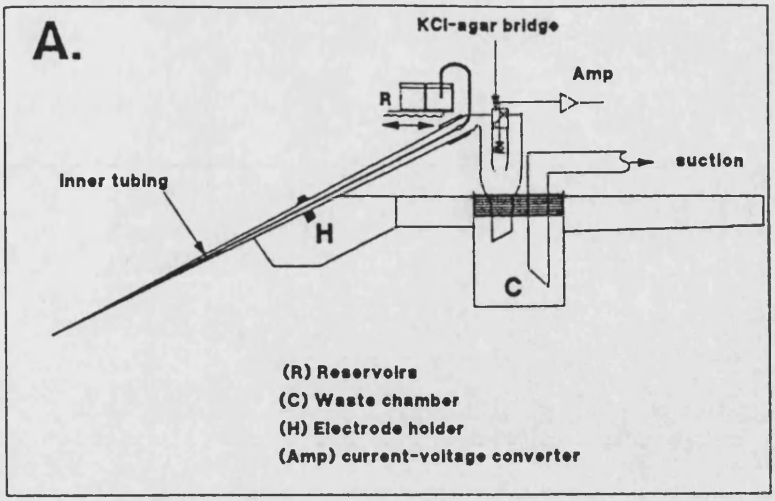
cell membrane by applying negative pressure (30 to 60 cmH_2O). The patch membrane was ruptured by a brief period of stronger suction. Current signals were recorded using an Axopatch 1-C single electrode voltage-clamp amplifier controlled by a microcomputer and pClamp software (Axon Instruments, Foster City, California, USA). All current records were filtered with a low-pass 500 Hz Bessel filter (cut-off -80 dB/decade) and were digitized at 1 KHz and stored on the computer hard disc. Membrane outward holding current was also recorded on slow-time base using a Gould two-channel pen recorder. All experiments were performed at room temperature (20-22°C).

2.15.3 Solutions used for measurements of Na/K pump currents.

Na/K pump currents were measured according to the method of Gadsby *et al.* (1985). Intracellular (pipette) sodium concentration was elevated to 30mM to maximally activate the Na/K pump and pipette solutions were formulated so as to block voltage-activated potassium channels and the Na/Ca exchange mechanism. The standard pipette soluton contained (mM): NaCl 30; CsOH 100; aspartic acid 100; tetraethyl ammonium chloride (TEA) 20, $MgCl_2$ 2; ethylene-glycol-bis-*N*,*N*,*N*',*N*'-tetraacetic acid (EGTA) 5; glucose 10, *N*-2-hydroxy-ethylpiperazine-*N*'-2-ethanesulphonic acid (HEPES) 10; adenosine triphosphate (Mg-salt) 5; creatine phosphate (Tris) 5, pH 7.2. The standard Tyrode solution used contained (mM): NaCl 140; CsCl 2; KCl 5; $MgCl_2$ 1; $NiCl_2$ 2; $BaCl_2$ 1; glucose 5.5; HEPES 5, pH 7.4. The Ca-inward current was prevented by using a nominally Ca-free Tyrode solution and the possibilty of a Ba current through the voltage-gated Ca channel was prevented by the inclusion of 2mM $NiCl_2$ (Shattock, M.J personnal communication). The Na current through voltage-gated channels was prevented by chosing an appropriate voltage protocol and potassium currents were blocked by replacing intracellular (pipette) potassium with Cs (100mM) and TEA (20mM). Na/Ca exchange current was inhibited by the inclusion of 2mM $NiCl_2$ in the Tyrode solution and 10mM EGTA in the pipette solution.

2.15.4 Voltage protocols.

In order to obtain the relationship between membrane potential and Na/K pump



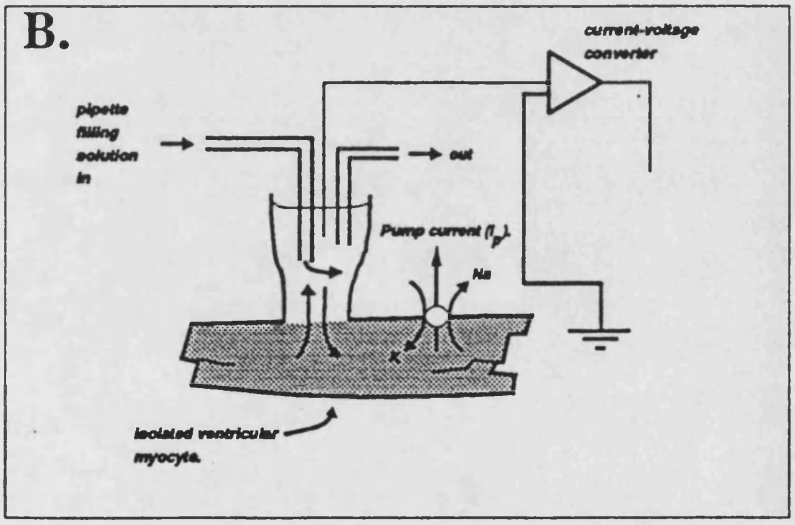
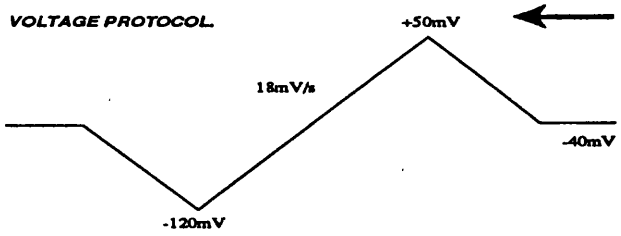


Figure 6. A: Digramatic representation of the microelectrode set-up used for the intracellular dialysis of selected pharmacological agents in an isolated rat ventricular myoicyte. B: Diagramatic representation of the internal dialysis and measurement of Na/K pump cursrent in an isolated rat ventricular myocyte.

current, a ramp voltage protocol was used which is shown diagramatically below. The protocol was sufficiently slow, at 18mV/second, to give essentially a quasi-steady state current-voltage (I-V) relationship (*Shattock and Matsuura, 1990b*). A negative-going ramp (from +50 mV to -120 mV) was used in order to prevent activating the voltage-gated Na-channel.



2.15.5 Validation of the technique and characterization of Na/K pump current.

Prior to the use of this method to investigate the effect of various agents on Na/K pump current, experiments were undertaken by Dr. M Shattock and Dr. H Matsuura to validate the experimental technique. They have demonstrated exact similarity between the passive currents recorded in the absence of external potassium and those recorded in the presence of 100μM ouabain suggesting that both interventions are eqi-potent in blocking the Na/K-pump current (i_p). The Na/K pump current is defined, therefore, in these experiments as the K_o-sensitive fraction of the total current recorded. This is more convienient than using the ouabain-sensitive fraction definition as the use of ouabain to block i_p is more difficult to reverse than exposure to K-free Tyrode (*Shattock and Matsuura, 1990b*).

2.15.6 Activation of Na/K pump current by intracellular sodium and extracellular potassium.

The relationship between $[Na]_i$, $[K]_i$ and i_p was established by Drs. Shattock and Matsuura prior to the initiation of this set of experiments. This was performed by dialysing different cells with different pipette solutions containing a range of Na^+ and K^+ concentrations between 0 and 50mM and 0 and 10mM respectively. The sensitivity of i_p to ouabain, extracellular K^+ and intracellular Na^+ and the similarity of these observations to previous studies (*Gadsby et al., 1985; Nakao and Gadsby,*

1989; Stimers et al., 1990) served to validate this experimental approach and, therefore, this technique was used to investigate the effect of selected sulphydryl-containing and disulphide compounds on Na/K pump current.

In experiments designed to investigate the effect of selected compounds on Na/K pump current the compound under investigation was either applied extracellularly or intracellularly by its inclusion in the perfusion fluid. Pump current recordings were made prior to and throughout the perfusion of the compound and during the following washout phase.

2.16 PREPARATION OF ISOLATED VENTRICULAR MYOCYTES FROM GSH-DEPLETED ANIMALS.

The effect of endogenous GSH on Na/K pump current was investigated by monitoring the effect of GSH-depletion on Na/K pump current in myocytes from control and DEM-treated animals.

In this set of experiments hearts were removed from male Wistar rats treated 30 minutes previously with diethylmaleate (0.8 ml/Kg, i.p). Isolated ventricular myocytes were subsequently prepared from these hearts, as previously described in the methods (section 2.10), and the Na/K pump current measured in single cells. This was compared to the control Na/K pump current in cells from untreated hearts, compensating for cell surface area and cell capacitance.

2.17 THE USE OF HIDDEN METAL-CAPTURE REAGENT FOR THE MEASUREMENT OF Na/K ATPase ACTIVITY IN RAT MYOCARDIAL TISSUE.

The effect of ischaemia/reperfusion and selected compounds on the activity of this cation exchanger, was determined using this cytochemicaltechnique.

In this set of experiments the effect of 30 minutes global, low-flow myocardial ischaemia and 5 minutes subsequent reperfusion on rat myocardial Na/K ATPase activity *in situ* in tissue slices, in the presence and absence of reduced glutathione, was investigated. In addition control data was obtained from hearts that were not subjected to an ischaemic insult. The methodology used for these experiments

was adapted from the technique described by Chayen *et al.* (1981).

Prior to the acquisition of experimental data, additional experiments were performed to validate the method with regard to sensitivity to ouabain, temperature, sodium concentration and substrate (ATP).

Hearts from male Wistar rats (University of Bath strain, 250-350g) were quick-frozen in 'dry-ice'/*n*-hexane (Fisons, free from aromatic hydrocarbons). The tissue was sectioned at 10μM in a Bright's cryostat with an automatic cutting device to ensure consistency of section thickness. The cabinet temperature was maintained between -25 and -30°C and the knife cooled with 'dry-ice'. Sections were picked off the knife and flash-dried onto glass slides. The method used to assay for Mg-activated Na/K ATPase activity was derived from Schwartz *et al.* (1969). The final reaction mixture contained the following reactants. A 40% solution ($^{w}/_{v}$) of Polypep 5115 (low viscosity, Sigma, U.K) was prepared in 0.2M Tris buffer (pH 7.4 at 37°C) containing 1mM sodium acetate. The following reactants were then added sequentially: NaCl (24 mg/ml; 410mM); $MgCl_2$ (4 mg/ml; 20mM); KCl (2.8 mg/ml; 37.5mM); disodium adenosine 5' triphosphate (10 mg/ml; 16.5mM). The lead ammonium citrate acetate complex (Sigma, U.K) was dissolved by constant shaking in the smallest volume of dilute ammonia (5 drops 0.88 ammonia per ml) to give a final concentration of 32 mg/ml. The final pH was adjusted to pH 7.5 by the addition of a 10:1 mixture (on a molar basis) of sodium and potassium hydroxides or 0.1M HCl. In order to remove free phosphate, that would give a strong background colouration, the sections were first immersed for 5 minutes in a 40% ($^{w}/_{v}$) solution of Polypep 5115 (Sigma, U.K) in Tris buffer (pH 7.5 at 37°C), containing 0.1M potassium acetate. After this time the medium was sucked off and replaced by fresh medium. This was then removed and replaced by the full reaction medium to a depth of 2-3 mm. To contain the medium over the sample a small Perspex ring was placed around each section. The medium, slides and sections were all pre-warmed to 37°C before these procedures were commenced. The reaction was then allowed to proceed for 15 minutes. After this time the sections were rinsed with several changes of 0.2M Tris buffer (pH 7.4 at 37°C) in Coplin jars, to remove any remaining Polypep media. They were then immersed for 1-2 minutes in water at room temperature that had been saturated with hydrogen sulphide. They were then rinsed several times with distilled water and allowed to dry. Sections were then mounted in

glycerol/water (2:1) medium. The density of the lead sulphide reaction product was measured using a Vicker M85 microdensitometer with a X40 objective, at 585nM and a flying spot of 0.5μM diameter in the plane of the section. Two succesive sections were fixed on one slide. In one set of sections, total ATPase activity was determined. At least 20 sections were cut from each heart. Ten readings from at least 10 cells per section were taken and a mean calculated. Data is expressed at mean integrated extinction x 100 (MIE x 100).

2.18 INDUCTION OF ACIDOSIS IN THE ISOLATED PERFUSED LANGENDORFF HEART MODEL.

The physiological respons of the coronary vasculature was investigated using the isolated perfused rat heart model.

Hearts from male Wistar rats (University of Bath strain) weighing 250-300g were perfused for a 15 minute equilibration period with a modified Krebs-Henseleit solution gassed with 95% O_2, 5% CO_2 at pH 7.4. and perfusion pressure, contractility and perfusion pressure continuously monitored as previously described in section 2.1. Acidosis was induced by perfusion with an acidotic Krebs- Henseleit solution. This had the same composition as the normal Krebs apart from the $NaHCO_3$ concentration which was reduced to give the desired pH within the range pH 7.2-6.8. Perfusion with acidotic Krebs-Henseleit solution was performed for 5 minutes and any changes in the criteria described above observed. A 10 minute washout period with normal Krebs-Henseleit buffer at pH 7.4 was then carried out. In experiments in which the effect of selected compounds on the physiological response of the heart to a reduction in pH were investigated, the compound under investigation was first pre-perfused for 5 minutes at pH 7.4. The compound was then perfused for a further 5 minutes at pH 6.8 and the same physiological responses monitored. As in control experiments, a 10 minute washout period with Krebs-Henseleit solution was then carried out.

2.19 DETERMINATION OF CORONARY PERFUSATE LEVELS OF CYCLIC GUANYLATE 5' MONOPHOSPHATE (cGMP) BY RADIOIMMUNOASSAY.

Perfusate levels of cGMP in response to acidosis, in the presence and absence of cysteine, was investigated using a specific radioimmunoassay technique.

For these experiments a commercially available cGMP radioimmunoassay kit (Amersham International Ltd., Amersham, UK; code TRK.500) was used with Amprep™ triethylaminopropyl SAX (strong anion exchange) minicolumns for sample preparation (Amersham International Ltd., Amersham, UK; code RPN.1918).

The assay is based on the competition between unlabelled cGMP and a fixed quantity of tritium-labelled compound for binding to an antiserum which has a high specificity and affinity for cGMP. The amount of labelled cGMP bound to the antiserum is inversely related to the amount of cGMP present in the assay sample. Measurement of the antibody-bound radioactivity enables the amount of unlabelled cGMP in the sample to be calculated. Separation of the antibody-bound cGMP from the unbound nucleotide is achieved by ammonium sulphate precipitaion, followed by centrifugation. The precipitate, which contains the antibody-complex, is dissolved in water and its activity determined by beta liquid scintillation counting. The concentration of unlabelled cGMP in the sample is then determined from a linear standard curve. The detection limit of the assay is 0.04 pmoles. Coronary perfusate levels of cGMP were determined under control conditions and in response to acidosis, in the presence and absence of selected compounds. After collection at selected time points, cGMP present in the perfusate samples was immediately absorbed onto individual pre-conditioned Amprep™ minicolumns which were refrigerated until all samples had been collected and the experiment terminated. At this point all samples were eluted off the columns and their cGMP content determined simultaneously. Krebs-Henseleit buffer was used as a negative control blank.

2.20 DETERMINATION OF CORONARY PERFUSATE LEVELS OF THROMBOXANE B_2 BY ENZYME-LINKED IMMUNOASSAY (EIA).

The effect of acidosis, in the presence and absence of reduced glutathione, on coronary perfusate levels of the satble thromboxane A_2 metabolite, thromboxane B_2, was determined using a specific enzyme-linked immunoassay.

For these experiments a commercially available thromboxane B_2 EIA kit was used (Cascade Biochem Ltd., Innovation Centre, Philip Lyle Building, University of Reading) by Ms. Amanda Taylor (The William Harvey Research Institute, Dept. of Biochemical Pharmacology, St.Bartholomew's Hospital College, Charterhouse Square, London, EC1 6BQ) who performed the thromboxane B_2 assays of coronary perfusate samples. Perfusate samples were prepared prior to assay. 2ml of coronary perfusate was mixed with 0.2 ml of 1N formic acid resulting in the acidification of the solution to pH 3. Four mls of ethyl acetate was then added and vortex mixed for 1 minute. The aqueous layer was then removed and the resulting organic fraction blown down to dryness with a stream of helium. The samples were then stored at -20°C prior to dispatch for thromboxane B_2 assay.

The assay is based on the competition between free thromboxane B_2 and acetylcholinesterase-linked thromboxane B_2 tracer for limited specific rabbit antiserum binding sites. The rabbit antiserum-thromboxane B_2 complex (free or tracer) then binds to a mouse monoclonal anti-rabbit antibody that is attached to an assay well. The plate is washed and Ellman's reagent (5,5'-Dithio-bis-(2-Nitrobenzoic acid) added to the well. The acetylcholinesterase tracer cleaves the Ellman's reagent to form a yellow compound (5-thio-2-nitrobenzoic acid) and the density of this chromophore is determined photometrically. The density of colour is proportional to the amount of tracer bound to the well, which is inversely proportional to the amount of free thromboxane B_2 present in the well during the incubation. Samples were collected prior to, during the perfusion and the washout of selected interventions and their thromboxane B_2 content determined. Data is expressed as pg thromboxane B_2 released/minute. The detection limit of the assay is 22.6 pg/ml of perfusate.

2.21 DETERMINATION OF CYTOSOLIC FREE CALCIUM CONCENTRATION IN ISOLATED RAT VENTRICULAR MYOCYTES AND RABBIT PLATELETS.

The cytosolic free calcium concentration of rat ventricular myocytes and washed platelets, in response to various pharmacological agents, was measured using a Photon Technology International Inc. Deltascan fluorimeter in conjunction with the calcium-sensitive dye, fura-2.

2.21.1 Cell preparation and loading with fura-2.

Isolated rat ventricular myocytes, prepared using an enzymic procedure previously described (section 2.10), or washed rabbit platelets (section 2.22) were suspended in 4 mls of HEPES Tyrode free from added calcium ("calcium-free" Tyrode) and incubated for 30 minutes at 37°C with 2.5μM fura-2 acetoxymethyl ester (Molecular Probes, Eugene, Oregon, U.S.A.). After incubation the cells were washed three times in 15 mls of "calcium-free" Tyrode and finally resuspended in 20 mls "calcium-free" HEPES-Tyrode.

2.21.2 Measurement of free intracellular calcium ($[Ca]_i$).

2 ml aliquots of cells were dispensed into quartz cuvettes and calcium chloride added to give final concentration of 1mM. Cuvettes were transferred to a Photon Technology Inc. Deltascan fluorimeter fitted with a thermostatted cuvette housing with stirring attachment for fluorescence reading at 37°C. Fura-2 is liberated from its methyl ester by cytosolic esterases inside the cell. Upon binding Ca^{2+}, fura-2 undergoes both a change in fluorescence intensity and spectral shift. Leakage of fura-2 from the loaded myocytes was shown to be insignificant as determined by the undetectable change in fluorescence signal produced by the addition of 1mM Ni^{+}. For our purposes, fura-2 fluorescence was continuously monitored using monochromator settings of 339nM and 380nM (excitation) and 410nM and 510nM (emission). The F_{max} was determined by lysing the cells with 40-50μM digitonin, thereby exposing the fura-2 to total intracellular Ca^{2+}. F_{min} was determined by adjusting the pH of the cell medium to 8.5 with 40μl of 2M NaOH followed by the addition of 120μl of 10mM EGTA. No correction was made for the background fluorescence of the myocytes. Fluorescence

readings were converted by an on-line microcomputer (NEC Powermate 286) with dedicated Deltascan software (PTI Instruments Inc., U.S.A) to display real-time $[Ca]_i$ by use of equation 2 (*Grynkiewicz et al., 1985*).

$$[Ca]_i = K_d \; X \; \frac{[R - V*R_{min}]}{[V*R_{max} - R]} \; X \; \frac{\text{no bound}\lambda 2}{\text{full bound}\lambda 2} \qquad \textit{equation 2}$$

where R = [signal λ1/signal λ2], K_d = dissociation constant for fura-2 = 224nM (*Roe et al., 1990*), V = viscosity coefficient = 1 (for aqueous solutions).

Cells were allowed to equilibrate at 37°C for 2 minutes before compounds or vehicle under investigation were added directly to the cuvette.

2.22 ISOLATED WASHED PLATELET PREPARATION FROM WHOLE RABBIT BLOOD.

Blood was obtained by venepuncture from male New Zealand white rabbits (body weight 1.0-1.5 Kg) and mixed with 3.2% ($^{w}/_{v}$) trisodium citrate (1ml/9ml of blood) as an anticoagulent. This blood was then centrifuged (1500 r.p.m, 10 minutes, IEC centra-7 centrifuge) and the supernatant (platelet-rich plasma, PRP) aspirated into plastic tubes. Plasma-free suspensions of platelets were then prepared. 150µl of 112µM prostacyclin was added to 20 ml aliquots of PRP and centrifuged (3000 r.p.m, 10 minutes, IEC centra-7 centrifuge). The purpose of the prostacyclin was to inhibit platelet aggregation during cell preparation. The supernatant was discarded and the pellet resuspended in 4 mls of HEPES-Tyrode of the following composition (mM): NaCl 0.14; HEPES 10; $MgCl_2$ 2; NaH_2PO_4 0.6; KCl 5. BSA (2.5g/L) and D-glucose (1g/L) was added immediately prior to use. It was at this stage that the cells were loaded with the calcium sensitive dye, fura-2 acetoxymethylester (2.5µM, 30 minutes), for the measurement of free cytosolic calcium levels (section 2.21.2). Following fura-2 loading the cell suspension was centrifuged at 3000 r.p.m and the pellet resuspended in 3mls of HEPES-Tyrode and 50µl of 112µM prostacyclin. Two further washes with 20ml aliquots of HEPES-Tyrode in the absence of added prostacyclin were subsequently performed.

2.23 STATISTICAL ANALYSIS.

Results are expressed as the mean ± standard error of the mean. Statistical differences between means were assessed using a one way analysis of varience followed subsequently by either Bonferroni's *t*-test or a paired or non-paired Student's *t*-test, where applicable.

CHAPTER 3.

ANTIOXIDANTS ON NORADRENALINE RELEASE AND CONTRACTURE IN THE ISCHAEMIC/REPERFUSED RAT HEART.

3.1 NORADRENALINE RELEASE FROM THE ISOLATED PERFUSED RAT HEART DURING 60 MINUTES, 30 MINUTES AND 20 MINUTES OF GLOBAL, LOW-FLOW ISCHAEMIA AND 5 MINUTES SUBSEQUENT REPERFUSION.

In view of the arrhythmogenic action of noradrenaline (*Daugherty et al., 1986*) and the corresponding antiarrhythmic action of reduced glutathione and other free radical scavengers and antioxidants (*Woodward and Zakaria, 1985*), experiments were undertaken to investigate whether these compounds exert their antiarrhythmic action by attenuating ischaemically-induced noradrenaline release.

There was no significant increase in noradrenaline release from the myocardium during the first 20 minutes of an ischaemic insult, whereas 30 and 60 minute periods of global, low-flow ischaemia (90% flow reduction from a control rate of 10ml/minute) resulted in a significant increase in noradrenaline release above control levels (figures 7,8,9). Pacing the hearts at 300 beats per minute had no significant effect on noradrenaline release during the pre-ischaemic phase or during ischaemia and reperfusion. Noradrenaline release upon subsequent reperfusion was dependent on the length of the preceding ischaemic period (figure 7,8,9). Typical responses of perfusion pressure, developed tension and heart rate during a 60 minute ischaemic period and 5 minutes subsequent reperfusion are illustrated in figure 10. An apparent rapid decline in heart rate is due to a concomitant decrease in contractility which fails to trigger the heart rate meter. Following the onset of ischaemia there was an increase in resting tension. The time to the onset of this ischaemically-induced contracture and the time for this contracture to plateau was recorded and this data is shown in table 1.

3.2 THE EFFECT OF REDUCED GLUTATHIONE, VERAPAMIL AND A SUPEROXIDE DISMUTASE/CATALASE/MANNITOL COCKTAIL ON NORADRENALINE OVERFLOW DURING ISCHAEMIA AND SUBSEQUENT REPERFUSION.

In this set of experiments, the concentration of radical scavengers and antioxidants used were those previously found to have antiarrhythmic activity in the isolated

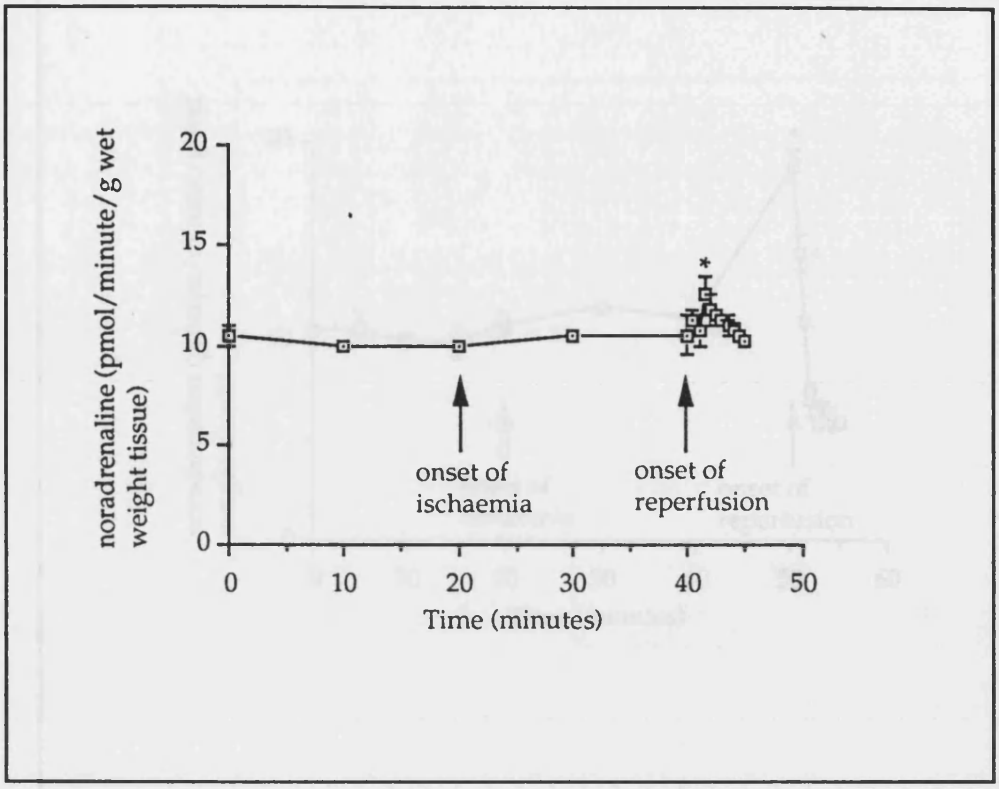


Figure 7. Effect of 20 minutes global, low-flow ischaemia and 5 minutes subsequent reperfusion on noradrenaline release from the isolated perfused rat heart. Hearts were paced at 300 b.p.m and the ischaemic and reperfusion periods initiated at the points indicated. Vertical bars represent ±s.e.mean. (n=6, *p<0.05 cw pre-ischaemic controls.)

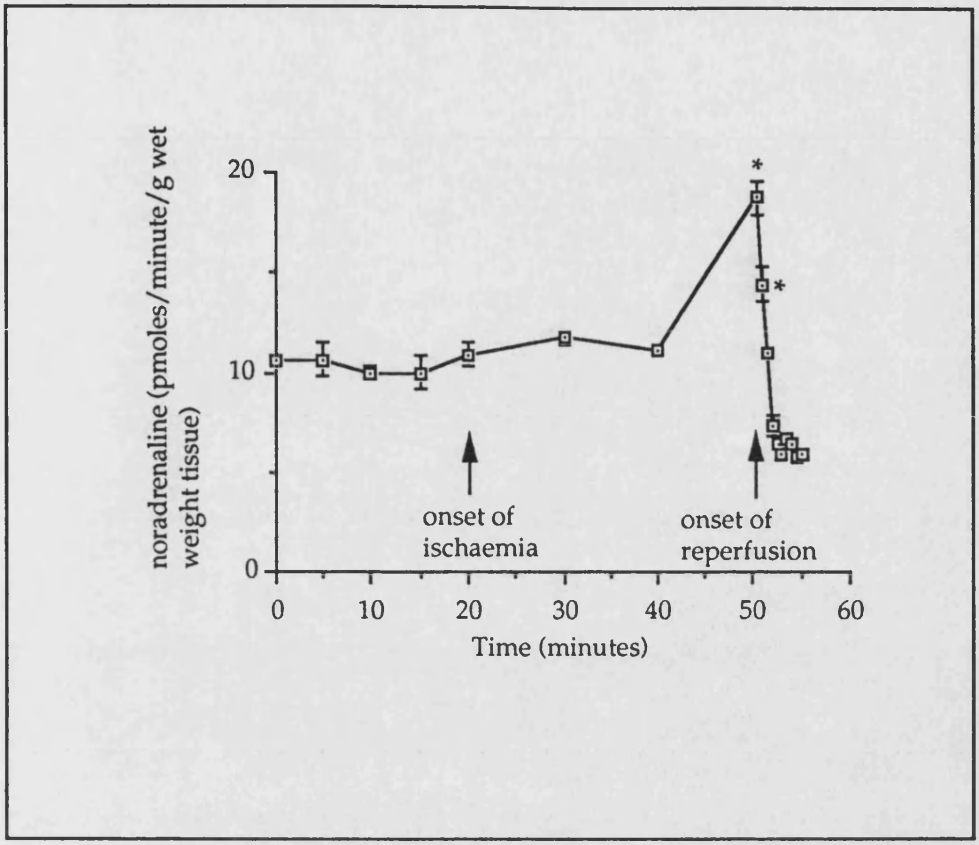


Figure 8. Effect of 30 minutes global, low-flow ischaemia and 5 minutes subsequent reperfuison on noradrenaline release from the isolated perfused rat heart. Hearts were paced at 300 b.p.m and the ischaemic and reperfusion periods initiated at the points indicated. Vertical bars represent $\pm$ s.e.mean. (n=6, *P<0.05 cw pre-ischaemic controls).

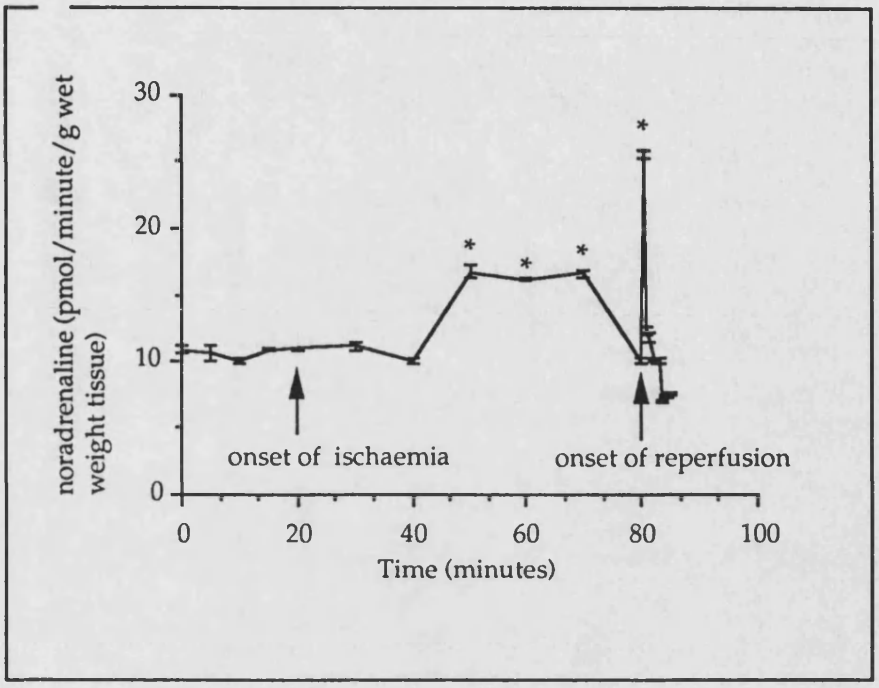


Figure 9. Effect of 60 minutes global, low-flow ischaemia and 5 minutes subsequent reperfusion on noradrenaline release from the isolated perfused rat heart. Hearts were paced at 300 b.p.m and the ischaemic and reperfusion periods initiated at the points indicated. Vertical bars represent $\pm$s.e.mean. (n=6, *$p<0.05$ cw pre-ischaemic controls).

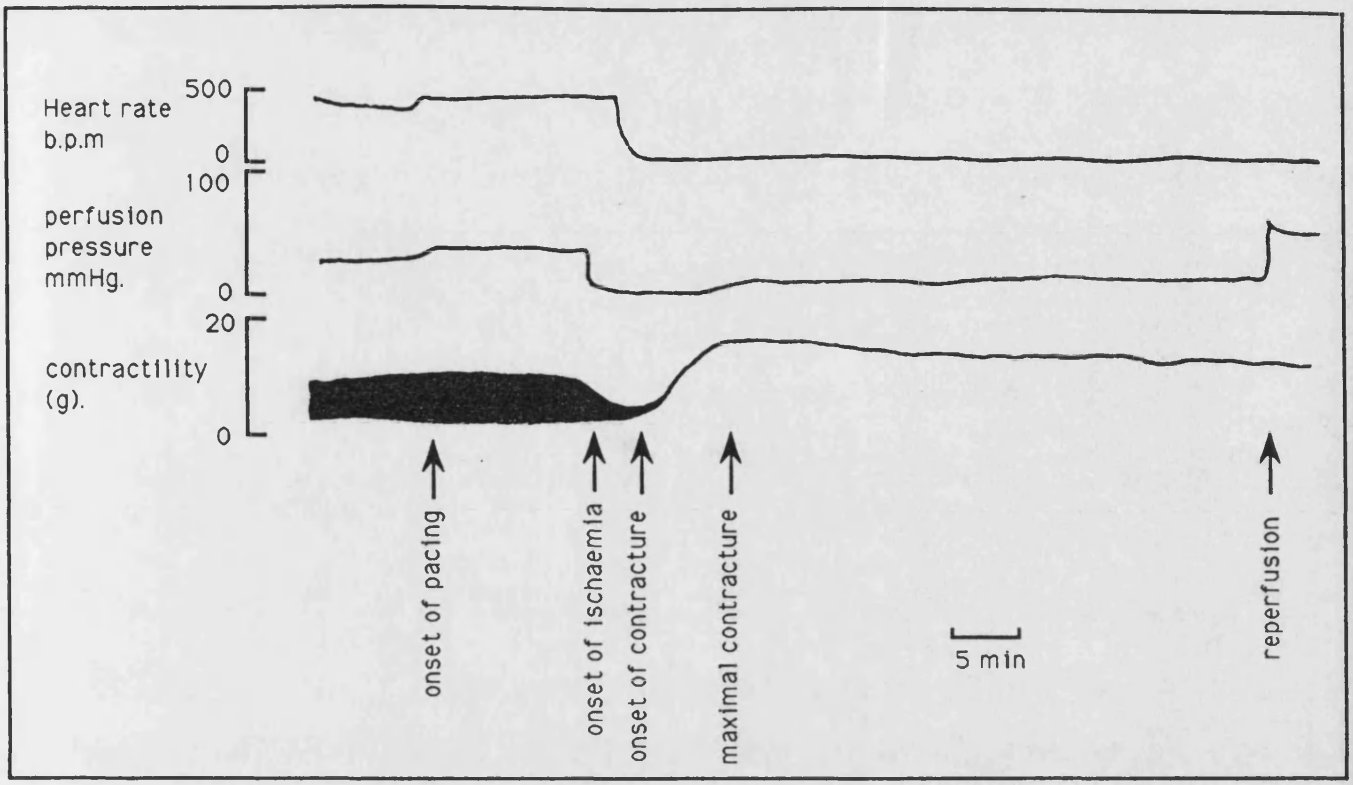


Figure 10. Typical trace showing the effect of 60 minutes global, low-flow ischaemia and 5 minutes subsequent reperfusion on coronary perfusion pressure, cardiac contractility and heart rate in the isolated perfused rat heart. The time to the onset and maximal contracture were calculated from the onset of ischaemia to the points marked 'onset' and 'maximal'.

DRUG	Concentration	n	ONSET (minutes)	MAXIMAL (minutes)
controls		6	7.6±0.2	15.3±0.2
captopril	1mM	4	9.6±0.28*	19.3±2.08*
	100μM	3	9.3±0.57*	18.6±1.54*
	75μM	3	8.2±0.28	17.6±1.15
	50μM	3	7.0±0.0	15.3±0.57
L-cysteine	1mM	4	10.1±0.2*	19.8±0.95*
	100μM	4	9.5±0.7*	20.5±0.7*
L-glycine	1mM	4	7.4±0.3	15.6±0.5
L-glutamate	1mM	4	7.3±0.2	15.5±0.4
L-cystine	1mM	3	6.1±0.3*	12.9±0.5*
L-methionine	1mM	3	7.1±0.2	15.0±0.2
reduced dithiothreitol	1mM	3	10.2±1.6*	18.6±1.5*
L-penicillamine	1mM	4	9.8±0.8*	18.6±2.3*
oxidised glutathione	1mM	4	6.0±0.2*	13.5±1.0*
reduced glutathione	1mM	8	10.1±0.3*	18.1±0.5*
verapamil	15nM	6	9.3±0.5*	16.2±0.3*
Superoxide dismutase,catalase, mannitol	10U/ml, 300U/ml, 50mM	5	10.1±0.2*	18.2±0.2*

Table 1. The effect of various pharmacological agents on the time to the onset and maximal contracture in the low-flow (90% flow reduction from a pre-ischaemic flow rate of 10ml/minute), globally ischaemic, isolated perfused rat heart. (n=3-8 hearts/group, * $p<0.05$ cw controls)

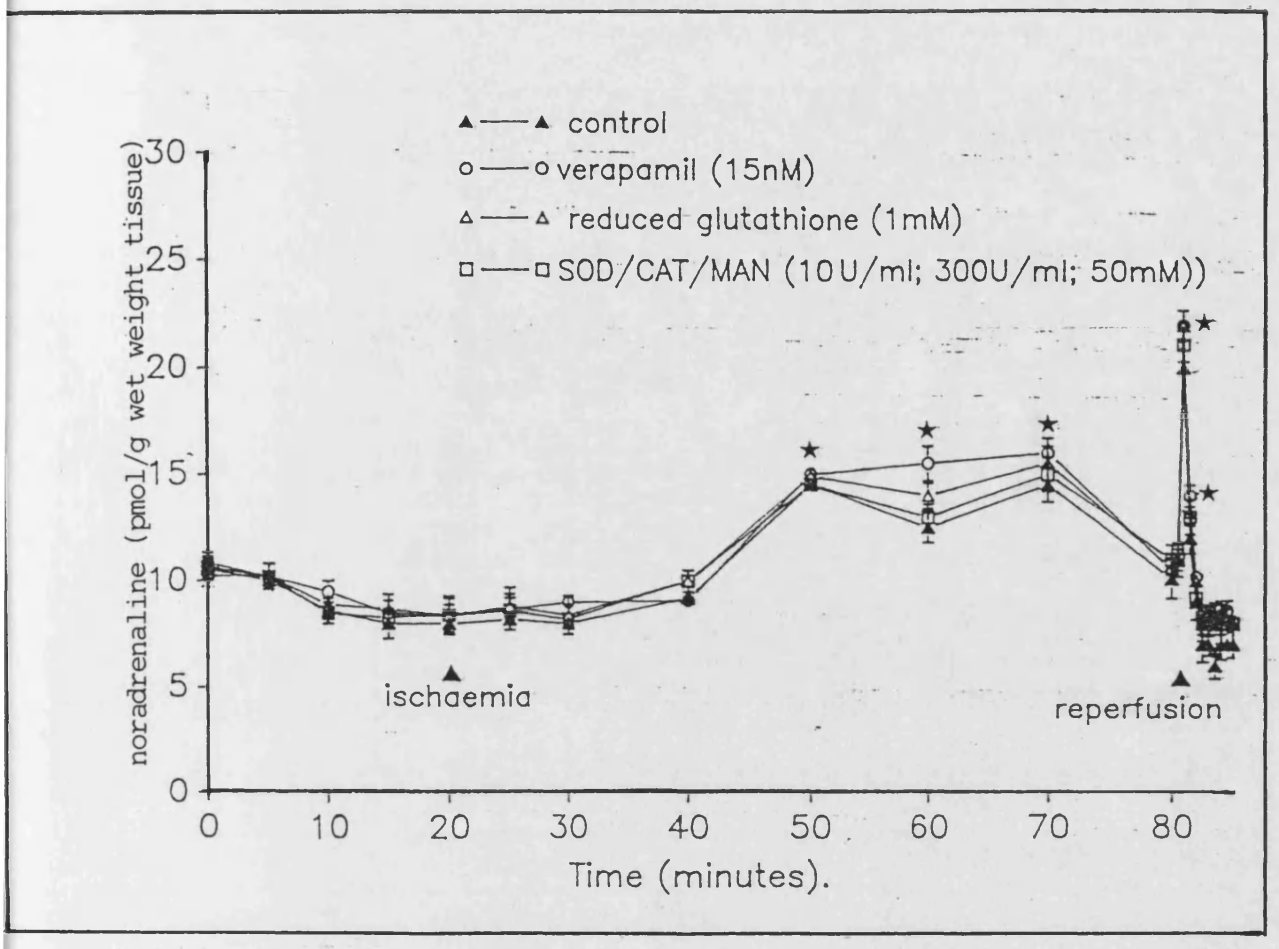
perfused rat heart (Woodward & Zakaria, 1985). 1mM reduced glutathione (GSH) had no significant effect on noradrenaline overflow from the isolated rat heart during both the control, ischaemic and reperfusion phase of the experiment. In addition, a cocktail of superoxide dismutase/catalase/mannitol (10 Units/ml, 300 Units/ml and 50mM respectively) had no significant effect on the profile of noradrenaline overflow during 60 minutes ischaemia and 5 minutes subsequent reperfusion (figure 11). The perfusion of 1mM reduced glutathione resulted in a significant coronary dilation equivalent to 30±2.5 mmHg. As a result, the effect of the L-type calcium channel antagonist verapamil (15nM), which causes a similar coronary vasodilator response to 1mM GSH, was investigated in this model. In common with 1mM GSH, 15nM verapamil had no significant effect on noradrenaline overflow during 60 minutes myocardial ischaemia and 5 minutes subsequent reperfusion.

3.3 THE EFFECT OF ENDOGENOUS NORADRENALINE DEPLETION ON AN ISCHAEMICALLY-INDUCED CONTRACTURE IN THE ISOLATED RAT HEART.

To investigate the possible involvement of noradrenaline release from the rat myocardium in the initiation and/or the progression of an ischaemic contracture observed in this model, the physiological responses of hearts previously chemically-depleted of noradrenaline (section 2.4) to an ischaemic insult were studied. The depletion protocol used resulted in a reduction of myocardial levels of noradrenaline by 91.4±0.1% (figure 12). Following a 20 minute ischaemic period and 5 minutes reperfusion, tissue levels of noradrenaline were reduced by a further 7% representing a loss of 800 pmoles of noradrenaline during the ischaemia/reperfusion insult (Table2a). Table 2b. shows that the time to the onset of contracture in noradrenaline-depleted hearts was significantly increased compared to the response of hearts from control, untreated animals.

3.4 THE EFFECTS OF REDUCED GLUTATHIONE, A SUPEROXIDE DISMUTASE/ CATALASE/ MANNITOL COCKTAIL, VERAPAMIL AND OTHER SELECTED COMPOUNDS ON AN ISCHAEMICALLY-INDUCED CONTRACTURE IN THE ISOLATED PERFUSED RAT HEART.

The effect of the compounds used in the noradrenaline overflow experiments on



Figurell. Effect of reduced glutathione (1mM), verapamil (15nM) and a superoxide dismutase (SOD) (10 units/ml)/catalase (CAT) (300 units/ml)/mannitol (MAN) (50mM) cocktail on noradrenaline release from the isolated perfused rat heart during 60 minutes global, low-flow ischaemia and 5 minutes subsequent reperfusion. Vertical bars represent ±s.e.mean. (n=6, *$p<0.05$)

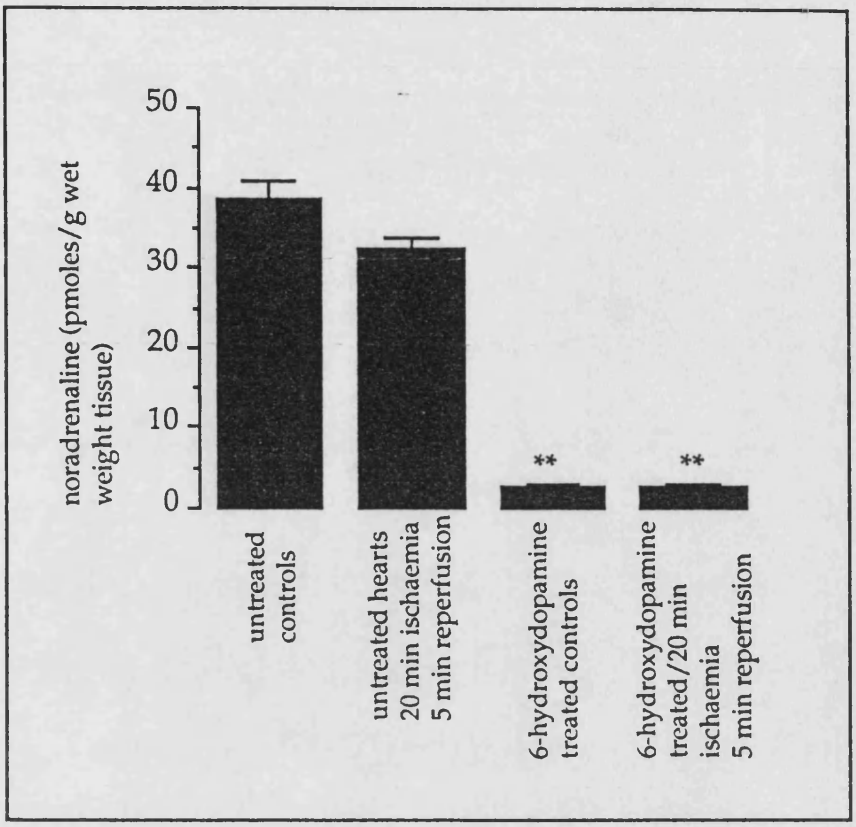


Figure 12. Comparison of the effect of 20 minutes global, low-flow ischaemia and 5 minutes subsequent reperfusion on the noradrenaline content of hearts from control, untreated animals and from 6-hydroxydopamine (50mg/kg, i.v, 17-24 hours pre-sacrifice) treated animals. Vertical bars represent ±s.e.mean. (n=8, **p<0.01 cw controls).

A.

GROUP	n	non-ischaemic	20 minutes ischaemia/5 minutes reperfusion
		Tissue noradrenaline (nmoles/g wet weight tissue).	
control, untreated hearts	6	11.4±0.2	10.45±0.3*
6-hydroxydopamine treated hearts	5	1.1±0.2	1.02±0.1*

B.

DRUG	n	ONSET (minutes)	MAXIMAL (minutes)
control untreated hearts	6	7.6±0.2	15.3±0.2
6-hydroxydopamine treated hearts	5	9.0±0.1*	16.2±0.3*

Table 2. A: Tissue noradrenaline levels in control and 6-hydroxydopamine treated animals under control conditions and following 20 minutes global, low-flow ischaemia and 5 minutes subsequent reperfusion. (n=5-6, *$p<0.05$ cw respective controls). B: Effect of myocardial noradrenaline depletion with 6-hydroxydopamine (50mg/kg, i.v 17-24 hours pre-sacrifice) on the time to the onset and maximal ischaemically-induced contracture in the isolated perfused rat heart. (n=5-6, *$p<0.05$ cw respective controls).

an ischaemically-induced contracture are shown in table 1. Reduced glutathione (1mM), a superoxide dismutase/catalase/mannitol cocktail (10Units/ml, 300Units/ml and 50mM respectively) and verapamil (15nM) all significantly delayed the onset and time to maximal contracture in this model. The concentrations of free radical scavengers and antioxidants used were those previously shown to attenuate the incidence of reperfusion-induced arrhythmias in the isolated rat heart model (*Woodward and Zakaria, 1985*). The effect of compounds related to GSH (L-cysteine, L-glycine, L-glutamate, L-cystine, reduced dithiothreitol, L-penicillamine, captopril, L-methionine and oxidised glutathione (GSSG)) on an ischaemic contracture were also investigated and the data from these experiments are shown in table 1. The sulphur-containing, non-sulphydryl amino acid, L-methionine, had no significant effect on the time to onset or maximal contracture. However, all the compounds containing a free sulphydryl group significantly delayed the onset and the time to maximal contracture. This is in contrast to the effects of oxidised glutathione and cystine which both significantly reduced the time to the onset of the contracture.

3.5 THE EFFECT OF ENDOGENOUS GLUTATHIONE DEPLETION ON AN ISCHAEMICALLY-INDUCED CONTRACTURE IN THE ISOLATED RAT HEART.

In view of the ability of exogenous reduced glutathione to significantly delay the onset and time to maximal contracture in the ischaemic rat heart, the role of endogenous glutathione in the protection against ischaemic contracture was investigated. This was approached by the chemical depletion of endogenous glutathione using diethylmaleate (section 2.13) and comparison of the physiological response of these hearts to ischaemia with the response of hearts from untreated animals. Time-matched controls to assess changes in basal myocardial glutathione levels in response to perfusion in the presence and absence of an ischaemic insult were also used. Data from these experiments are shown in table 3a The protocol used for the depletion of endogenous glutathione (section 2.13) resulted in reduction of total and reduced glutathione with no recordable change in tissue content of oxidised glutathione (table 3b). The inability to determine a change in GSSG levels may be due to the insensitivity of the assay system. This resulted in a 4-5 fold increase in the ratio of GSH/GSSG that is in common with the response

GROUP	TOTAL GLUTATHIONE	GSSG	GSH	GSH/GSSG
		(μmol/g wet weight tissue)		
untreated controls	1.6±0.09	0.013±0.009	1.55±0.07	125.6
30 mins post DEM	0.36±0.06*	0.014±0.007	0.34±0.05*	27
60 mins post DEM	0.32±0.05*	0.015±0.005	0.29±0.04*	23
20 min non ischaemic perfusion	0.52±0.02+	0.014±0.002	0.4±0.03+	28
20 min ischaemic/5 min reperfusion	0.40±0.03+	0.010±0.01	0.23±0.02+	10

Table 3a,b. Effect of diethylmaleate (0.864 g/kg i.p 30 or 60 minutes pre-sacrifice) or 20 minutes global low-flow ischaemia and 5 minutes subsequent reperfusion on myocardial glutathione levels. (n=4, +,*$p<0.05$ cw respective controls).

GROUP	n	ONSET (minutes)	MAXIMAL (minutes)
control untreated hearts	6	7.6±0.3	15.5±0.4
glutathione-depleted hearts.	5	6.2±0.2*	13.1±0.2*

Table 4. Effect of endogenous myocardial glutathione depletion, using diethylmaleate (0.8ml/kg i.p, 30 minutes pre-sacrifice), on the time to the onset and maximal contracture in the isolated perfused rat heart. (n=5-6 hearts/group, *$p<0.05$ cw controls).

seen following an ischaemic insult (*Ferrari et al., 1989*). The depletion of endogenous glutathione using this protocol also resulted in a significant shortening of the time to the onset and maximal contracture following the onset of ischaemia (table 4).

3.6 THE EFFECT OF SUPEROXIDE PRODUCTION USING THE XANTHINE/ XANTHINE OXIDASE SYSTEM ON AN ISCHAEMICALLY-INDUCED CONTRACTURE IN THE ISOLATED RAT HEART.

The effect of free radicals on the ischaemically-induced contracture seen in this model was investigated using the xanthine/xanthine oxidase system that results primarily in the production of the superoxide radical ($O_2^{\cdot-}$). Superoxide production in this set of experiments was confirmed by the detection of uric acid throughout the entire length of the experimental time course (*Xie et al., 1990*). In addition, the effect of exogenous superoxide dismutase or GSH on the effects of $O_2^{\cdot-}$ in this model was investigated. Neither xanthine, xanthine oxidase nor the ammonium sulphate vehicle in which the xanthine oxidase was suspended had any significant effect on the time to the onset of the ischaemic contracture. However, a bolus injection of 0.65 units of xanthine oxidase in the presence of 1mM xanthine, 5 minutes prior to the onset of the ischaemic insult, resulted in a significant shortening of the time to the onset of contracture. The perfusion of either 50 units/ml of superoxide dismutase or 1mM reduced glutathione throughout the experimental time course resulted in a significant attenuation of the combined

3.7 EFFECT OF REDUCED GLUTATHIONE ON ^{86}RUBIDIUM EFFLUX DURING MYOCARDIAL ISCHAEMIA AND SUBSEQUENT REPERFUSION.

To investigate a possible mechanism by which reduced glutathione is antiarrhythmic (*Woodward and Zakaria, 1985*) and delays the onset of an ischaemically-induced contracture, its effect on the efflux of radiolabelled rubidium (^{86}Rb), as a marker for potassium, from pre-labelled hearts during 30 minutes ischaemia and 5 minutes subsequent reperfusion was examined. Data is expressed as efflux rate coefficient against time (erc/minute) (see section 2.6). During the ischaemic period the perfusate flow rate was reduced by either 90, 95 or 97.5%

DRUG	n	ONSET (minutes)	MAXIMAL (minutes)
controls	4	7.5±0.3	15.2±0.2
0.65 units xanthine oxidase controls	5	7.3±0.4	15.3±0.5
1mM xanthine controls	4	8.0±0.3	15.9±0.4
35μM ammonium sulphate controls	5	7.6±0.4	15.4±0.3
1mM xanthine/0.65 units xanthine oxidase	4	4.1±0.2*	12.1±0.4*
1mM xanthine/0.65 units xanthine oxidase/1mM GSH	5	8.0±0.3	15.6±0.3
1mM xanthine/0.65 units xanthine oxidase/50 units/ml SOD	5	7.6±0.4	15.3±0.4

Table 5. Effect of xanthine/xanthine oxidase (1mM and 0.65 units, respectively), in the presence and absence of reduced glutathione (GSH) (1mM) or superoxide dismutase (SOD) (50 units/ml), on the time to the onset and maximal contracture in the low-flow, globally ischaemic isolated rat heart. (n=4-5 hearts/group, *$p<0.05$ cw controls).

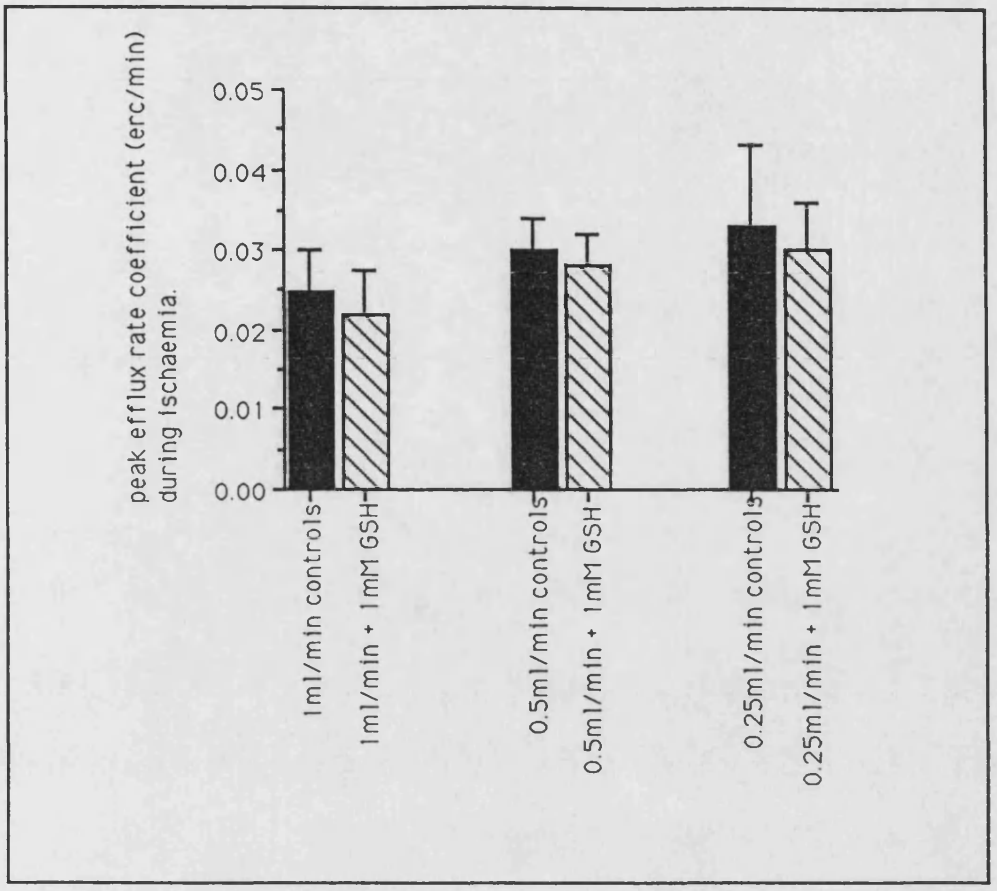


Figure 13. Effect of reduced glutathione (GSH) (1mM) and flow rate on the peak ^{86}rubidium efflux coefficient during 30 minutes global, low-flow ischaemia in the isolated perfused rat heart. Vertical bars represent ±s.e.mean. (n=4. *$p<0.05$ cw controls).

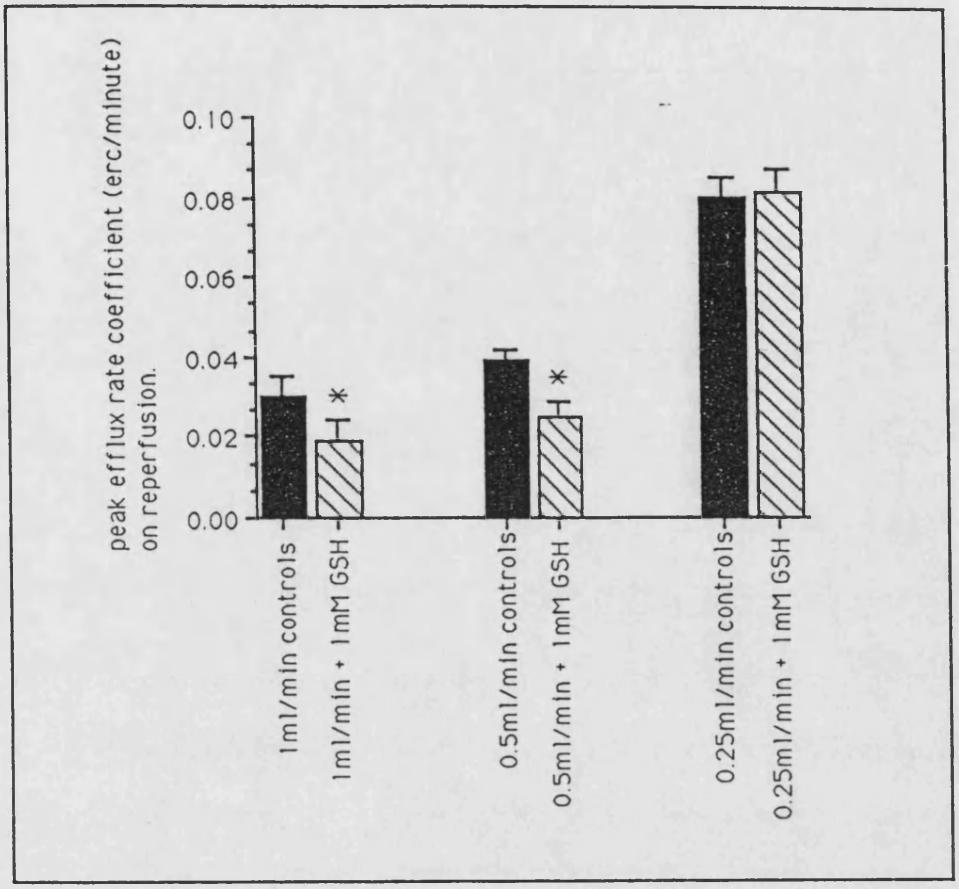


Figure 14. Effect of reduced glutathione (GSH) (1mM) and flow rate during 30 minutes global, low-flow myocardial ischaemia on the peak ^{86}rubidium efflux rate coefficient (erc/minute) upon subsequent reperfusion in the isolated perfused rat heart. Vertical bars represent ±s.e.mean. (n=4, *$p<0.05$ cw controls).

from a pre-ischaemic value of 10 ml/minute. There was no correlation between the degree of flow reduction and the maximum efflux coefficient during the ischaemic period (figure 13). However, the maximum erc/minute value achieved upon reperfusion was dependent on the degree of flow reduction during the preceding ischaemic phase (figure 14). The addition of 1mM reduced glutathione to the perfusate during ischaemia had no effect on the maximum erc/minute achieved (figure 13) whereas it reduced the peak erc/minute value upon reperfusion when the flow rate in the preceding ischaemic insult was reduced by 90 or 95% (figure 14). The effects of reduced glutathione on the mean efflux area during ischaemia and subsequent reperfusion were also assessed and this data is shown in figures 15 and 16. 1mM GSH reduced the mean efflux area only when the flow rate was reduced by 95% during the ischaemic period and during reperfusion when the flow was reduced by either 90 or 95% during the preceding ischaemic period. This data suggests a potassium sparing action of reduced glutathione in this model. (N.B peak erc/minute and mean efflux area were determined as outlined digrammically in figure 5a. Mean efflux area is expressed in arbitary units).

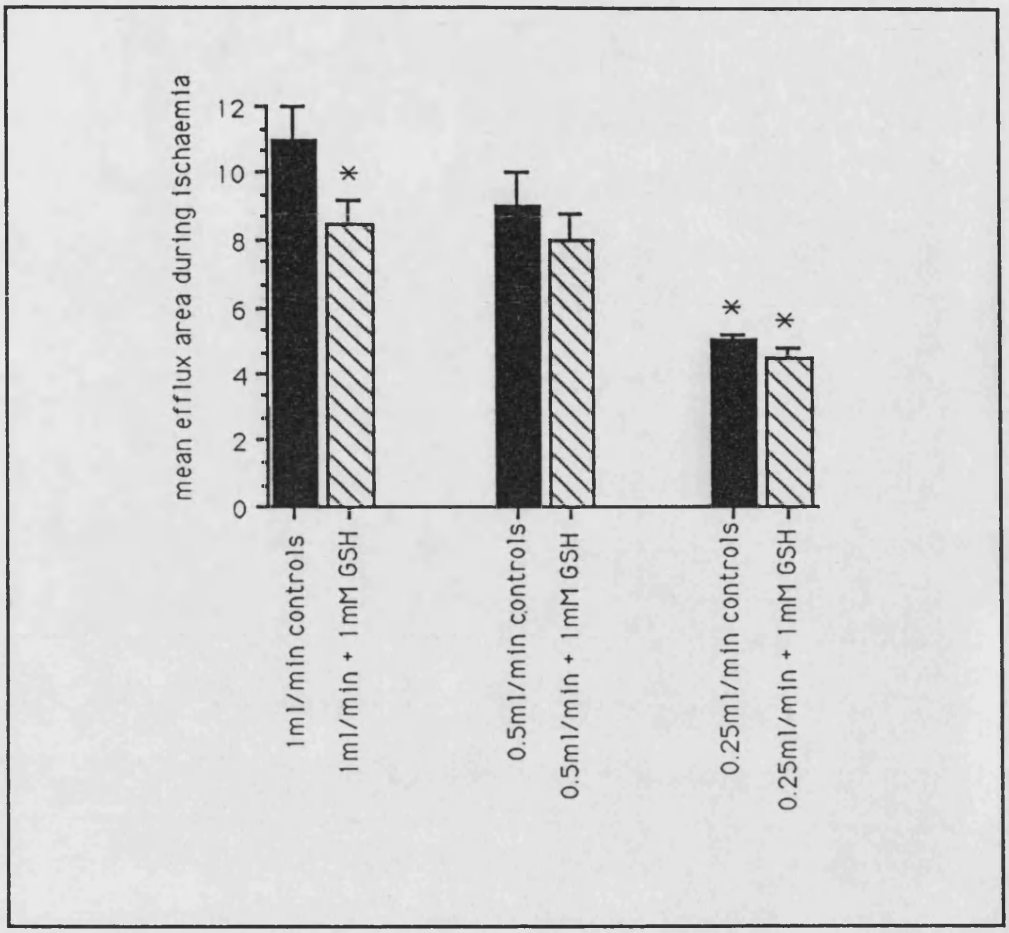


Figure 15. Effect of reduced glutathione (GSH) (1mM) and flow rate on the mean ^{86}rubidium efflux area during 30 minutes global, low-flow ischaemia in the isolated perfused rat heart. Vertical bars repesent ±s.e.mean. (n=4, *$p<0.05$ cw controls). Mean efflux area corresponds to the mean area under the plots of erc/minute during the ischaemic period (see figure 5b).

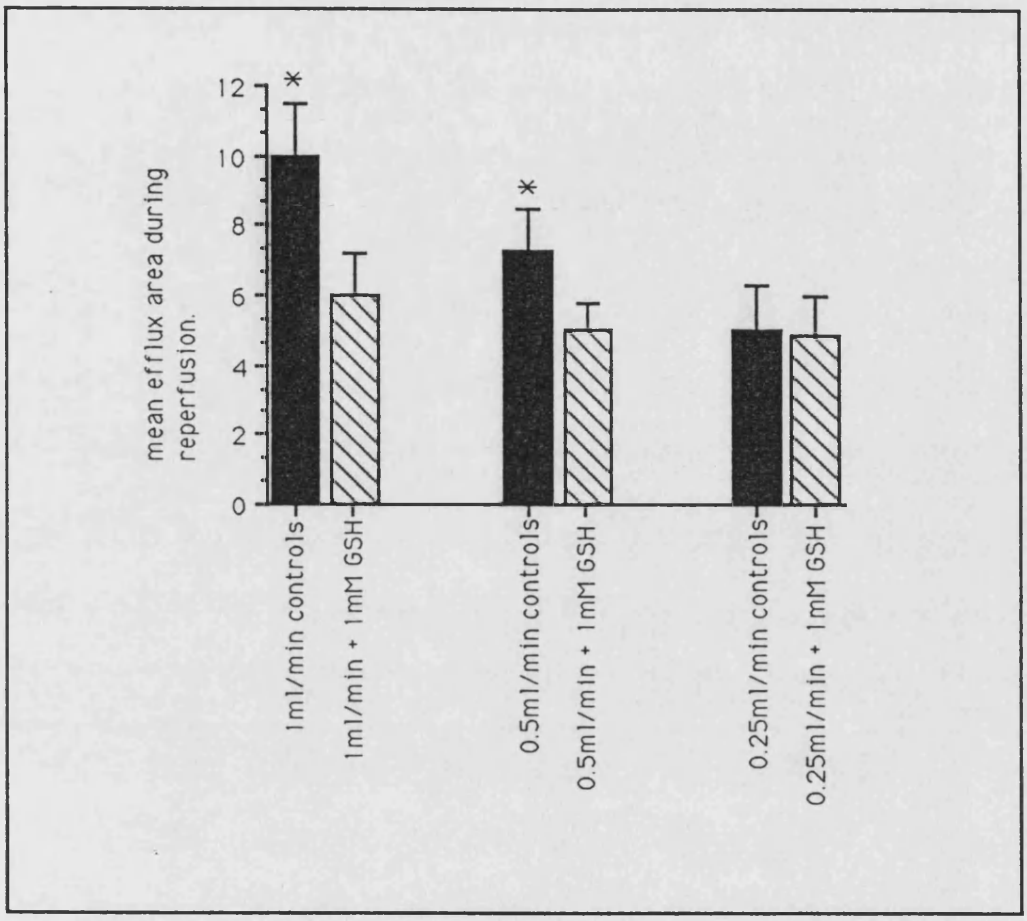


Figure 16. Effect of reduced glutathione (GSH) (1mM) and flow rate during 30 minutes global, low-flow ischaemia on the mean ^{86}rubidium efflux area during 5 minutes subsequent reperfusion in the isolated perfused rat heart. Vertical bars represent ±s.e.mean. (n=4, *$p<0.05$ cw controls).

CHAPTER 4.

DISCUSSION.

With a view to studying how free radical scavengers attenuate the incidence of reperfusion-induced arrhythmias (*Woodward and Zakaria, 1985*), the effect of reduced glutathione, selected free radical scavengers and the L-type calcium antagonist, verapamil, on noradrenaline overflow from the ischaemic myocardium was examined. None of the free radical scavengers or verapamil significantly reduced noradrenaline release during ischaemia or reperfusion at previously determined antiarrhythmic concentrations (*Woodward and Zakaria, 1985*). However, the contracture which occurred in response to an ischaemic insult was attenuated by several of the compounds studied. In light of these results, further experiments were undertaken to investigate how these compounds delay the onset of this ischaemic contracture, while ^{86}Rubidium efflux experiments were used to investigate whether GSH exerts an antiarrhythmic, potassium-sparing action during ischaemia and reperfusion. Reduced glutathione significantly attenuated ^{86}Rb efflux during ischaemia and reperfusion raising the possibility that the Na/K pump or other sarcolemmal potassium channels (eg ATP-dependent K^+ channels) are modulated by reduced glutathione and related compounds that may contribute to its potassium-sparing, antiarrhythmic action.

During the early phases of an ischaemic period, there is a reduction in the ability of the myocardium to maintain ionic homeostasis. Intracellular [Na^+], [H^+] and [Ca^{2+}] rise simultaneously within the first 5 minutes of ischaemia, whilst K^+ is lost from the cell in a biphasic manner (*Weishaar et al., 1979; Cobbe and Poole-Wilson, 1980; Hirche et al., 1980*). In addition, there is a reduction in cellular ATP (*Jennings et al., 1981*) and an accumulation of cAMP (*Lubbe et al., 1981; Opie, 1982*) which correlate with an increase in $[Ca]_i$ and the onset of contracture. There is also evidence for the mobilization of endogenous noradrenaline during ischaemia (*Mathes and Gudbjarnason, 1971; Holmgren et al., 1981; Muntz et al., 1984),* while on reperfusion and re-oxygenation of a previously ischaemic myocardium, cardiac reserves of noradrenaline are further depleted *(Riemersma and Forfar, 1982; Abrahamsson et al., 1983*) and the noradrenaline content of the coronary circulation is raised (*Abrahamsson et al., 1983; Nayler and Sturrock, 1983*). Noradrenaline has several effects on the myocardium including stimulation of oxygen consumption (*Rolett, 1974*), constriction of the coronary vasculature (*Pace,*

1977; Borda et al., 1980), production of oxygen free radicals via autoxidation (*Del Maestro, 1980*) and the generation of arrhythmias (*Opie and Thandroyen, 1983*). The mechanisms implicated in the genesis of reperfusion-induced arrhythmias are all mediated by the establishment of chemical and electrical gradients caused by the accumulation of metabolites in the ischaemic myocardium (*Surawicz, 1971*). Arrhythmogenic mechanisms, which are outlined in figure 17, include an increase in $[cAMP]_i$ (*Podzuweit et al., 1978*), lysophosphatide formation (*Corr and Sobel, 1982*), free radical production (*Manning and Hearse, 1984; Woodward and Zakaria, 1985; Bernier et al., 1986*), alpha- and beta-adrenoceptor stimulation (*Sheridan et al., 1980; Bralet et al., 1985; Bartel et al., 1986*) and disturbances in sodium (*Kleber and Wilde, 1986*), calcium (*Clusin et al., 1982*) and potassium homeostasis (*Curtis et al., 1986*; *Lubbe et al., 1986*). There is also evidence to suggest that adrenergic activation increases the size of the ischaemic area (*Waldenstrom et al., 1978*). The arrhythmogenic action of catecholamines during myocardial reperfusion suggests that adrenergic receptor stimulation may be involved in this action. Catecholamine autoxidation and the resulting production of free radicals and adrenochrome, a metabolite of noradrenaline, may also contribute to the damaging effects of noradrenaline release during reperfusion (*Yates et al., 1981*). It is, therefore, undesirable if noradrenaline reserves are mobilized, particularly if this occurs during ischaemic acidosis (*Cobbe and Poole-Wilson, 1980*) and reduced coronary flow which will reduce its rate of removal from the myocardium.

The protective role of reduced glutathione, a superoxide dismutase/catalase/ mannitol cocktail and verapamil during ischaemia was investigated with respect to their effect on myocardial noradrenaline release and uptake. Noradrenaline release during 60 minutes of global, low-flow ischaemia and 5 minutes subsequent reperfusion was unaffected by GSH and, therefore, it seems unlikely that GSH exerts its antiarrhythmic effect by directly attenuating ischaemically-induced noradrenaline overflow. In the light of these investigations, it would also seem that GSH has no effect on the neuronal uptake mechanism for noradrenaline that is inhibited during ischaemia (*Schömig et al., 1982*). Stimulation of this process would be expected to reduce the net accumulation and release of

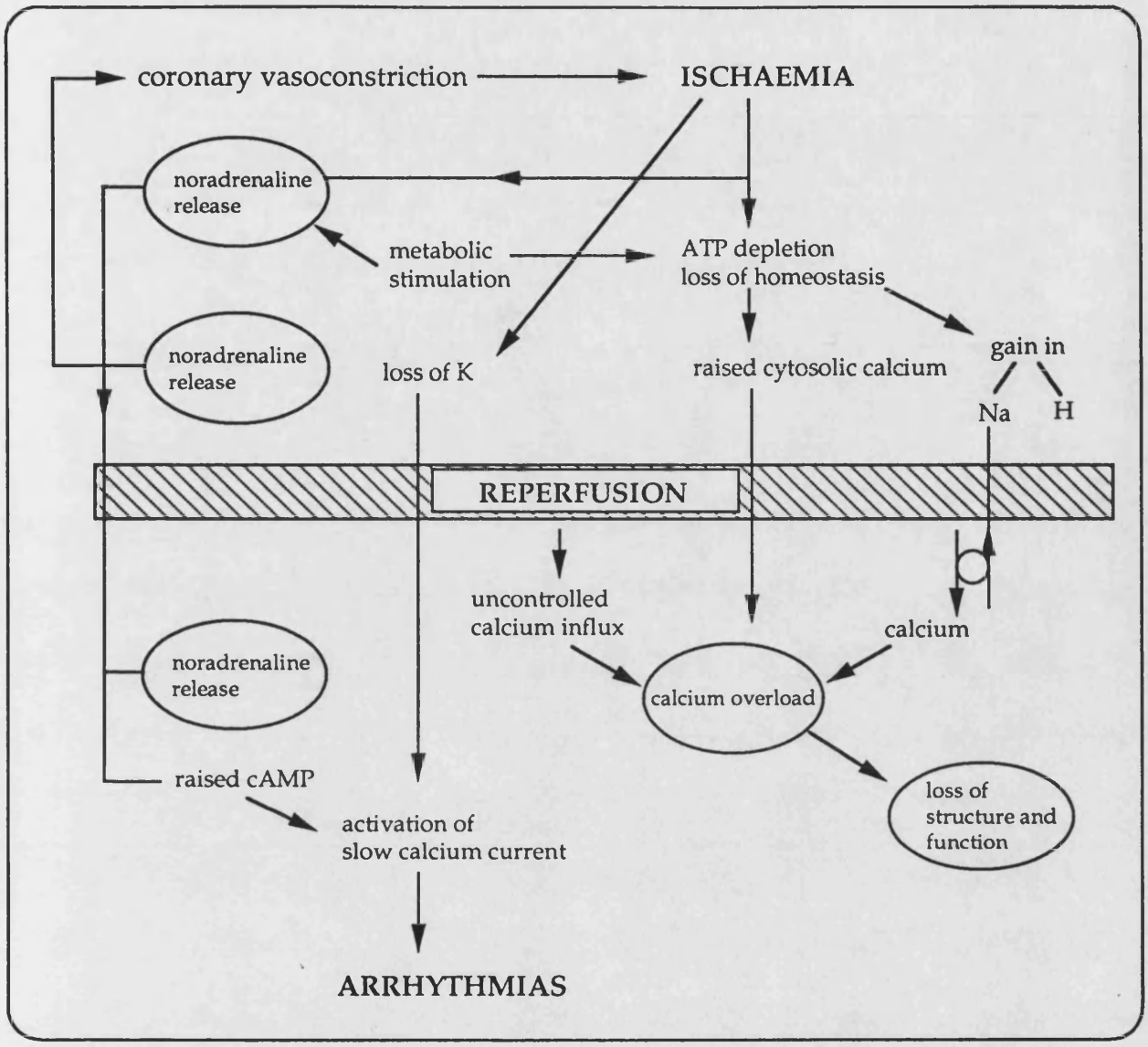


Figure 17. Diagramatic representation of factors involved in myocardial ischaemia/reperfusion-induced arrhythmogenesis, intracellular calcium overload and loss of cellular function.

noradrenaline during ischaemia. There is the further possibility that GSH and the other compounds studied may scavenge oxygen free radicals derived from catecholamines released under these conditions in the absence of any direct effect on noradrenaline release itself. If this is true then the production of adrenochrome would be reduced in the presence of free radical scavengers. At the present time there is no evidence to support this hypothesis. In addition, the contribution of this source of free radicals to the initiation of reperfusion-induced arrhythmias is unclear. In agreement with other groups (*Gauduel et al., 1979; Nayler and Scott, 1982*), cellular catecholamine depletion with 6-hydroxydopamine was found to be protective in that it significantly delayed the onset of an ischaemically-induced contracture. However, the amount of noradrenaline released from depleted hearts was not significantly different from that of the control, untreated hearts. In view of the fact that the protocol used for noradrenaline depletion resulted in only 91.4±0.1% depletion, the remaining 10% must be sufficient to account for ischaemically-induced noradrenaline overflow.

In view of the negative data from this set of experiments, it is likely that GSH exerts its antiarrhythmic action via another mechanism. The present investigations suggest a potassium-sparing action of GSH, which may be antiarrhythmic in view of the pro-arrhythmic effect of an elevated potassium concentration upon reperfusion of a previously ischaemic myocardium. ^{86}Rubidium efflux was dependent on the length of the ischaemic insult and the degree of flow reduction during this phase which may represent a correlation between the severity of an ischaemic insult and the degree of specific and non-specific cell damage to the cell membrane, intracellular organelles and membrane-bound ion pumps. However, this experimental data does not indicate whether the ^{86}Rb loss during reperfusion represents the washout of ^{86}Rb accumulated during the ischaemic phase or that released as a result of the restoration of flow. It is also impossible to determine whether the net ^{86}Rb loss represents a reduced uptake or an increase in actual efflux. There is no indication as to whether GSH attenuates ^{86}Rb loss due to the reperfusion phase itself or if it simply attenuates ^{86}Rb efflux during ischaemia. One experimental approach that may help resolve this anomaly would be to monitor myocardial lactate release during ischaemia and reperfusion. Lactate and

potassium are both released during myocardial ischaemia. Lactate is not released as a direct result of reperfusion and, therefore, lactate detected in the perfusate during reperfusion will represent washout of lactate accumulated during the preceding ischaemic phase. Differences in the profile of lactate and ^{86}Rb efflux may help to determine if ^{86}Rb, and therefore potassium, is released as a result of reperfusion or whether it is simply washout of ^{86}Rb released during ischaemia. Although the Na/K pump was targetted as a potential site for the action of GSH and related compounds, there is the possibility that potassium loss from the ischaemic myocardium may also occur as a result of changes in the activity of one or more of the various potassium channels in the heart, such as the ATP-sensitive K^+ channels (*Noma et al., 1983*). The sensitivity of the ischaemic/reperfusion-induced ^{86}Rb efflux to ouabain and TEA, for example, would be an experimental approach by which potassium loss via alternative potassium channels could be investigated.

The initiation of global, low-flow myocardial ischaemia resulted in a reduction in cardiac contractility and an ischaemically-induced contracture of the myocardium. It is clear from the results that reduced glutathione significantly delayed the onset of this contracture and so investigations were extended to include other compounds containing a free sulphydryl group. Cysteine, captopril, reduced dithiothreitol (DTT_{red},) and penicillamine (PSH) all significantly delayed the onset of contracture in response to ischaemia. This is in contrast to the effect of the sulphur-containing, non-sulphydryl amino acid, L-methionine, which had no significant effect. The pre-prefusion of 1mM reduced glutathione, cysteine, DTT_{red} or PSH resulted in a significant reduction in perfusion pressure of 30±2.5, 25.5±2.2, 28±3.5 and 20±2.2 mmHg, respectively, that is indicative of coronary vasodilation in this model. The concentration of verapamil that caused the same degree of coronary dilation (15nM) was, therefore, used as a non-sulphydryl control. Verapamil was also found to have no significant effect on noradrenaline overflow during ischaemia or reperfusion, yet it also significantly delayed the onset of myocardial contracture during the ischaemic phase. The presence of a free sulphydryl group is, therefore, not a requirement for a compound to attenuate an ischaemic contracture. Nayler and Sturrock (1985) showed that dihydropyridine-type calcium antagonists

attenuated the loss of cardiac noradrenaline during reperfusion following relatively short periods of ischaemia. However, Nayler and Sturrock (1985) and other groups (*Bourdillon and Poole-Wilson, 1982*; *Higgins and Blackburn, 1984*) failed to show an attenuation of ischaemia-reperfusion induced loss of noradrenaline with verapamil and diltiazem which is in agreement with the data presented here. In fact, when added just at the time of reperfusion, verapamil and diltiazem potentiated noradrenaline loss (*Nayler and Sturrock, 1985*). Recently, Richardt *et al.*, (1991) have reported on the ability of the L-type calcium channel antagonists verapamil, gallopamil, nifedipine and felodipine to attenuate the loss of noradrenaline from rat myocardium during reperfusion following a 20 minute period of global, no-flow ischaemia. The methodology used by this group differs from that used in the experiments described here with respect to the relatively low perfusate flow rate and that no-flow ischaemia was used. Because of this, coronary effluent was not collected or analysed during the ischaemic phase. In addition, the concentration of verapamil used by this group was 0.01-100μM, with an IC_{50} of 1μM, in contrast to 15nM used in the experiments described here. In view of this data, a verapamil concentration of 15nM would not be expected to attenuate ischaemically-induced noradrenaline loss from the myocardium.

Ischaemic contracture has also been reported to be dependent on cellular ATP content such that contracture is initiated at concentrations below approximately 12 μmoles/g dry weight (*Hearse et al., 1977*). GSH may attenuate a fall in tissue ATP content preventing the activation of ATP-dependent K^+ channels that would attenuate potassium loss during ischaemia. Talesnik and Tsoporis (1984) have shown that GSH protects against oxidant-induced damage to ATP-dependent microsomal Ca^{2+} sequestration in the guinea-pig and rat heart, which may represent an alternative method by which GSH delays the onset of ischaemic contracture. From the present investigations, the site of action of GSH is uncertain.

These experiments were negative in the respect that all of the interventions chosen failed to significantly attenuate noradrenaline release from the isolated rat heart during ischaemia and subsequent reperfusion. However, they provided evidence for the protective role of reduced glutathione and other sulphydryl compounds against ischaemically-induced myocardial injury. In light of the results, further work was

initiated to investigate potential mechanisms by which these compounds attenuate an ischaemically-induced contracture that is assumed to be ultimately due to calcium overload or an altered sensitivity of intracellular proteins and organelles to calcium. Examples of ion translocating systems involved either directly or indirectly in intracellular calcium homeostasis include the Na-pump (driven by the Na/K ATPase), the Na/Ca exchange mechanism, the sarcoplasmic reticulum Ca-ATPase and the Na/H exchange mechanism. As a starting point, the Na/K ATPase was chosen as a target for investigation as the activity of this enzyme has been shown to be significantly reduced during myocardial ischaemia (*Kim and Akera, 1987*) which would result in a rise in $[Na]_i$ favouring calcium influx via the Na/Ca exchange mechanism. This may represent a mechanism by which $[Ca]_i$ rises during ischaemia. The prophylactic modulation of the activity of this enzyme, resulting in its activation or the prevention of ischaemically-induced reduction in Na/K ATPase activity, would elicit a reduction in intracellular sodium, an increase in intracellular potassium and could contribute to the delaying effect of GSH on the onset of ischaemic contracture.

The data from this group of experiments has shown that although GSH has no effect on noradrenaline release during myocardial ischaemia and reperfusion, it delays the onset of ischaemic contracture and attenuates ischaemia/reperfusion-induced ^{86}Rb efflux which may explain its antiarrhythmic action in the heart. In view of the data available regarding an ischaemically-induced reduction in Na/K ATPase activity that can be attenuated by selected free radical scavengers (*Kim and Akera, 1987*), the effect of GSH on this ion translocating system was investigated as this could also affect ^{86}Rb efflux.

CHAPTER 5.

SULPHYDRYL COMPOUNDS AND SELECTED AGENTS ON THE ACTIVITY OF ISOLATED BOVINE VENTRICULAR AND RAT MYOCARDIAL SODIUM/ POTASSIUM ATPase AND SODIUM PUMP CURRENT IN ISOLATED RAT VENTRICULAR MYOCYTES.

5.1 THE EFFECT OF SULPHYDRYL COMPOUNDS AND SELECTED AGENTS ON ISOLATED BOVINE Na/K ATPase ACTIVITY.

The previously described effects of reduced glutathione (GSH) on an ischaemic contracture and ischaemia/reperfusion-induced ^{86}rubidium could be due to a stimulatory effect of the Na/K ATPase, that is the biochemical representation of the Na/K pump. For this reason, the effect of GSH, and related compounds, on the activity of Na/K ATPase was investigated using an isolated bovine ventricular preparation.

5.1.1 Validation and characterization of the Na/K ATPase preparation.

Before the examination of the effects of glutathione and other selected compounds on the activity of purified Na/K ATPase, preliminary studies were performed to characterize its activity with respect to stimulation by sodium and potassium, and sensitivity to cardiac glycosides, temperature and pH. The assay of Na/K ATPase activity relies on a linked-enzyme system and, therefore, it was also important to determine any stimulatory or inhibitory effect of the selected pharmacological interventions on the pyruvate kinase and lactate dehydrogenase preparations used in the assay, in addition to any regulatory effects on the Na/K ATPase preparation itself.

5.1.1.1 Ouabain inhibition of isolated bovine Na/K ATPase.

Schwartz *et al.* (1969) reported a dependence of the time of exposure and temperature of incubation on the affinity of ouabain for a bovine Na/K ATPase preparation. For this reason the time of exposure and temperature were kept constant at 10 minutes and 37°C, respectively. Because the inhibition of bovine Na/K ATPase by ouabain is curvilinear, enzyme activity at varying ouabain concentrations was determined during the initial linear phase (figure 18). At 37°C the Na/K ATPase preparation was ouabain-sensitive, within the range 0-100μM, with an IC_{50} value of approximately 25μM, which is in agreement with Schwartz *et al.* (1969). At ouabain concentrations greater than 30μM no further enzyme inhibition was observed (figure 19). As a result, a ouabain concentration of 30μM was used in all subsequent investigations to reveal the activity of the

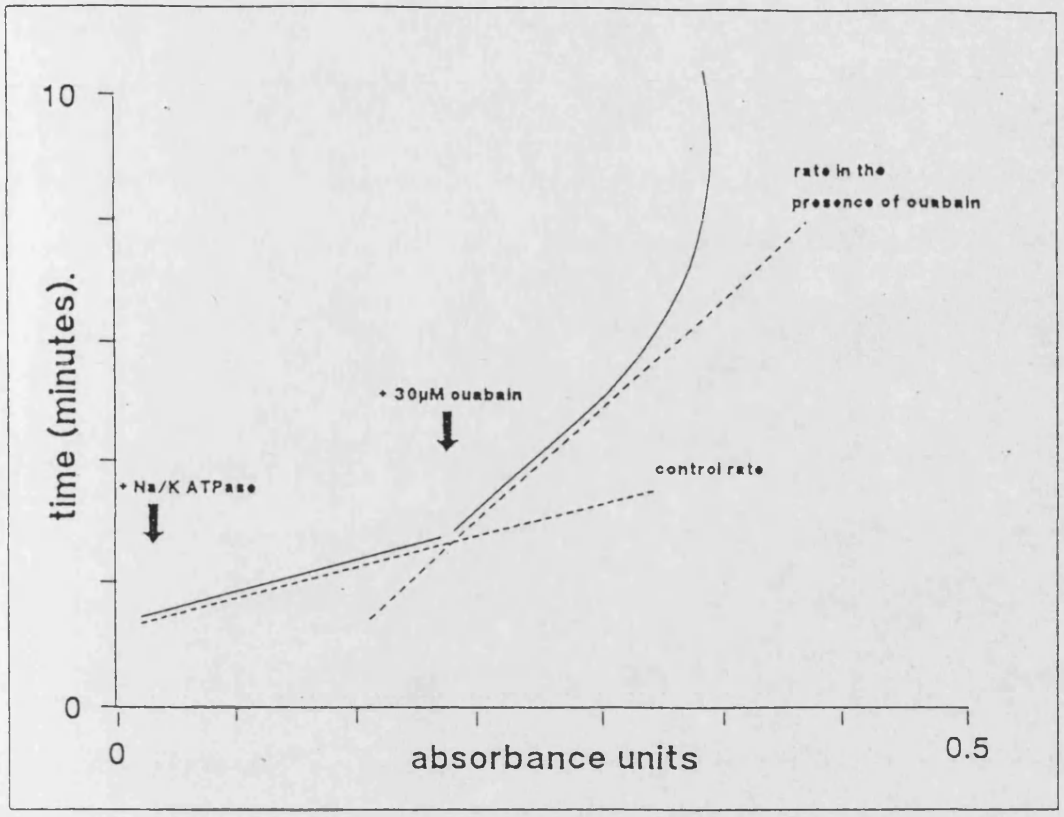


Figure 18. Diagramatic representation of the determination of ouabain sensitivity (0-100μM) of an isolated bovine Na/K ATPase activity. Enzyme activity was determined during the initial linear phase of the response.

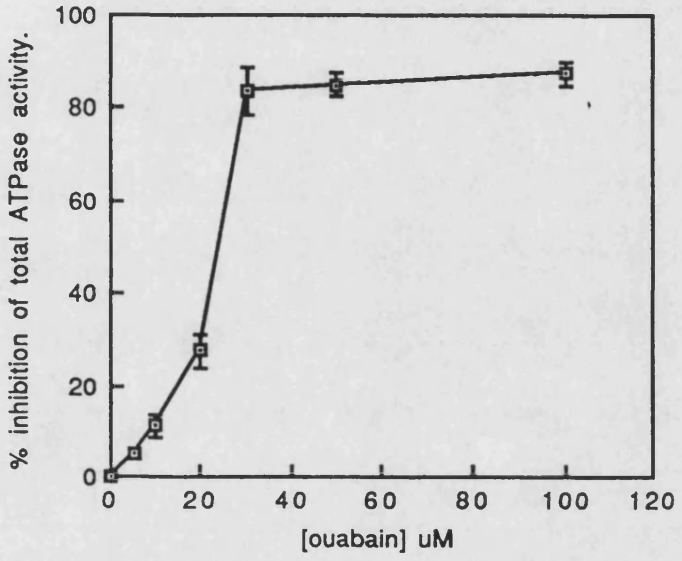


Figure 19. Effect of ouabain (0-50μM) on the activity of isolated bovine Na/K ATPase, expressed as % maximum activity. (Highest total activity measured in a single enzyme preparation = 32.2±2.2 units/mg protein).

ouabain-sensitive and insensitive fractions of the enzyme preparation (Total Na/K ATPase activity = activity of ouabain sensitive fraction + activity of ouabain-insensitive fraction).

To examine the effect of a selected compound on total activity, the compound was added directly to the cuvette and any change in reaction rate monitored. In a separate group of experiments, the effect of the compound on the ouabain-insensitive fraction was determined by adding ouabain (final concentration 30μM) followed 3 minutes later by the compound in question and any change in reaction rate monitored. In two further groups of experiments, to determine the effect of a compound on the ouabain-sensitive fraction the total activity in the presence of the compound was determined, as was the ouabain-insensitive activity following the application of ouabain. The data from each group was pooled and expressed as a mean value (±s.e.mean). The ouabain-insensitive fraction accounted for 18±5% of the total activity (n=5 preparations). The relative ouabain-sensitivity (and therefore the purity of the enzyme preparation) is probably a reflection of variability in the efficiency of the enzyme preparation and different sensitivities of the preparations to detergent solublization. The ouabain-insensitive ATPase activity is assumed to be due to calcium and magnesium-dependent ATPase.

5.1.1.2. Temperature sensitivity of bovine Na/K ATPase activity.

The effect of temperature on the activity of the total, ouabain-sensitive and insensitive fractions of bovine Na/K ATPase activity was determined at 25 and 45°C and the data are shown in figure 20. In response to a 20°C rise in incubation temperature (from 25 to 45°C), total enzyme activity rose significantly by 22.1±3.2 units/mg (basal activity at 25°C = 15.1±2.5 units/mg). The activity of the ouabain-sensitive fraction was also temperature dependent over the same range. The same temperature rise resulted in a concomitant increase in activity of 18±2.0 units/mg (basal activity at 25°C = 3.5±0.3 units/mg). Finally, the temperature sensitivity of the ouabain insensitive fraction was also determined. In this fraction, a 20°C rise in temperature significantly increased activity by 18±2.2 units/mg (basal activity at 25°C = 11.5±1.5 units/mg).

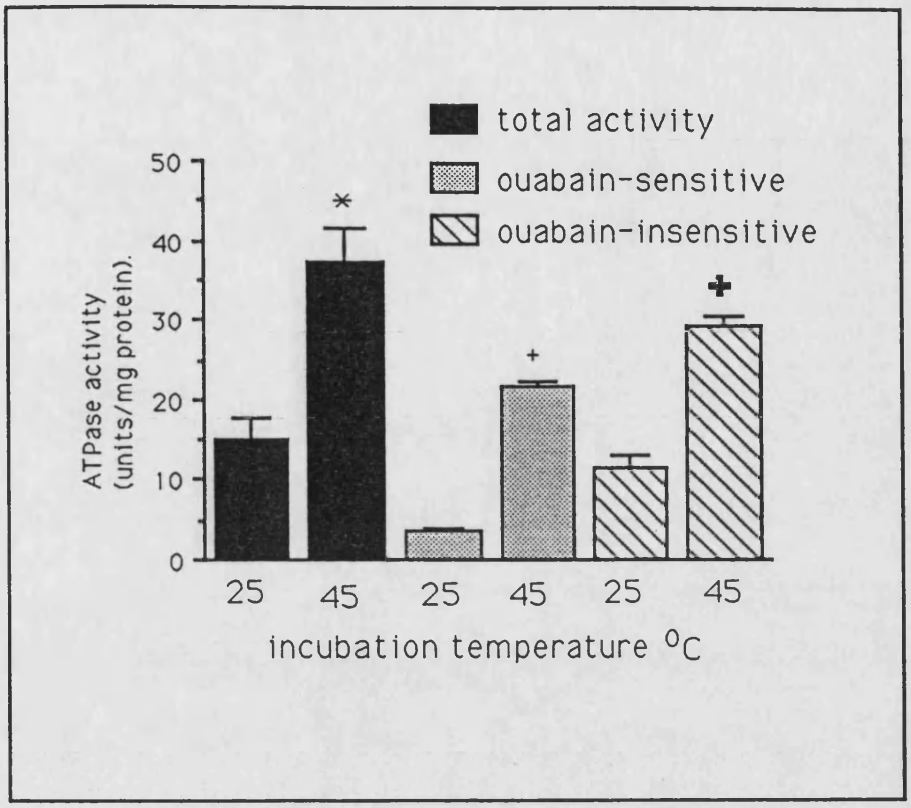


Figure 20. Effect of incubation temperature on the total, ouabain-sensitive and ouabain-insensitive ATPase activity of an isolated bovine sarcolemmal preparation. Vertical bars represent +s.e.mean. (n=6,*, +, ✚ $p<0.05$ cw respective control group). Highest total activity measured in a single enzyme preparation = 32.2+2.3 units/mg protein.

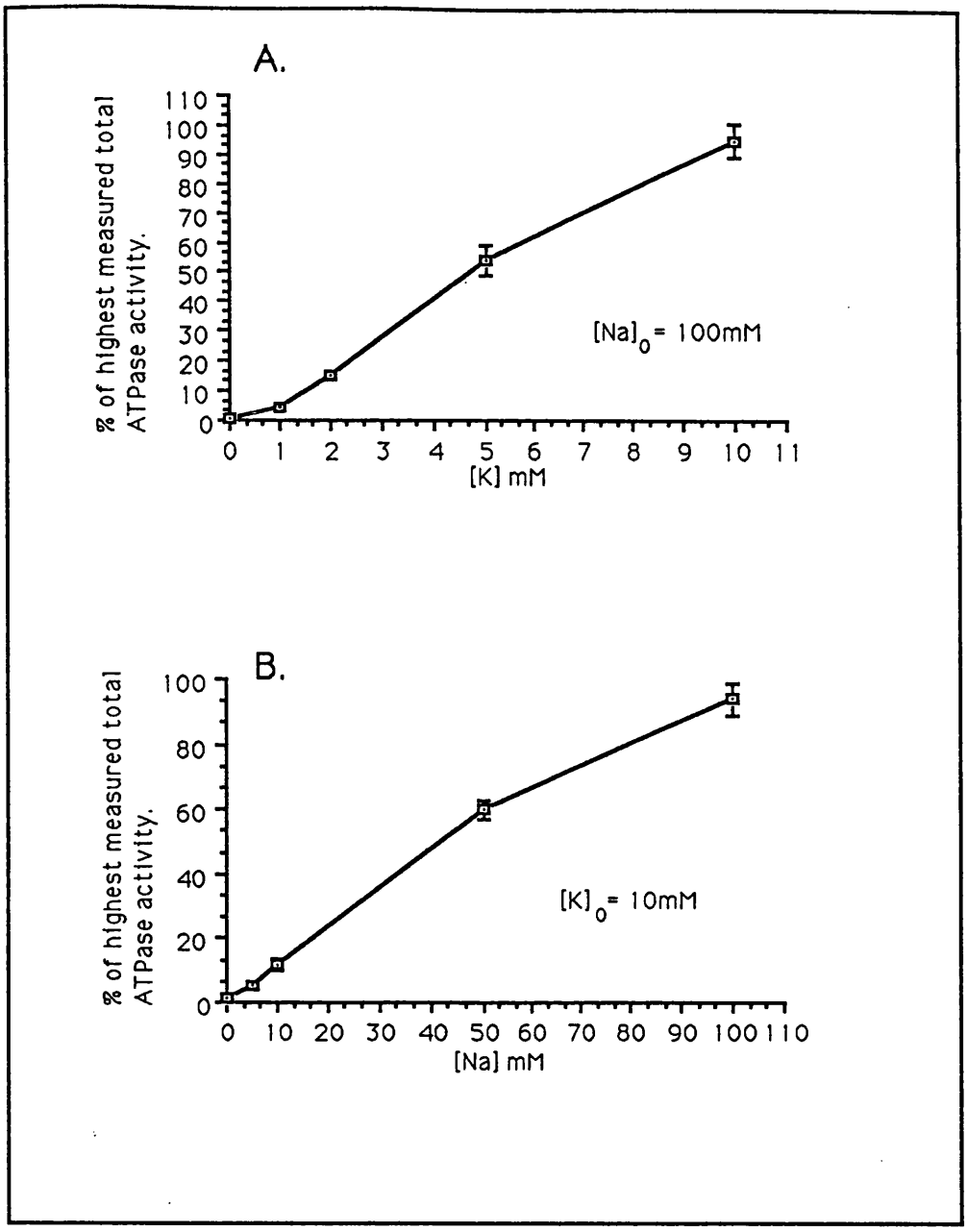


Figure 21. A: Effect of potassium concentration (0-10mM) on the total activity of an isolated bovine Na/K ATPase preparation expressed as % of highest measured total ATPase activity. B: Effect of sodium concentration (0-100mM) on the total activity of an isolated bovine Na/K ATPase preparation expressed as % of highest measured total ATPase activity. In both figures, vertical bars represent ±s.e.mean. (Highest total activity measured in a single enzyme preparation = 30.2±2.3 units/mg protein. [Na]=10mM, [K]=10mM, [ATP]=2.5mM). (n=4, *$p<0.05$ cw controls).

5.1.1.3 Activation of Na/K ATPase by potassium and sodium.

Figure 21a shows the potassium sensitivity (in the presence of a constant sodium concentration and temperature of 100mM and 37°C, respectively) of the total ATPase activity of an isolated bovine Na/K ATPase preparation. The total activity of the Na/K ATPase preparation was also dependent on the sodium concentration of the buffer (in the presence of a constant potassium concentration of 10mM) (figure 21b).

5.1.1.4 Effect of pH on Na/K ATPase activity.

The effect of acidosis, with respect to the optimal pH of 7.4 suggested (*Schwartz et al., 1969*), on the activity of the Na/K ATPase preparation was also investigated. Data from these experiments are shown in figure 22 and reveal the pH-dependency of the activity of the total, and ouabain-sensitive fractions of the enzyme within the pH range 6.8-7.4. The activity of the ouabain-insensitive fraction was unaffected by changes in pH within the same pH range.

The experiments described thus far were designed to validate and characterize the isolated enzyme preparation and the experimental technique used to quantify enzyme activity. The ouabain, temperature and pH sensitivity as well as its dependence on sodium and potassium concentration are similar to the data described by Pitts and Schwartz (1975) for an isolated bovine Na/K ATPase preparation and were assumed to validate the suitability of the enzyme preparation. This technique was, therefore, used to investigate the effect of glutathione and other selected compounds on the activity of the total, ouabain-sensitive and ouabain-insensitive fractions of bovine ventricular Na/K ATPase.

5.1.2 Effect of reduced glutathione on Na/K ATPase activity.

Figure 23 shows that the addition of reduced glutathione significantly increased the total activity and the activity of the ouabain-sensitive and insensitive fractions of the Na/K ATPase preparation in a concentration-dependent manner (0.25-1.0 mM). This activation was maintained for the duration of the experiment and did not significantly decay with time (data not shown).

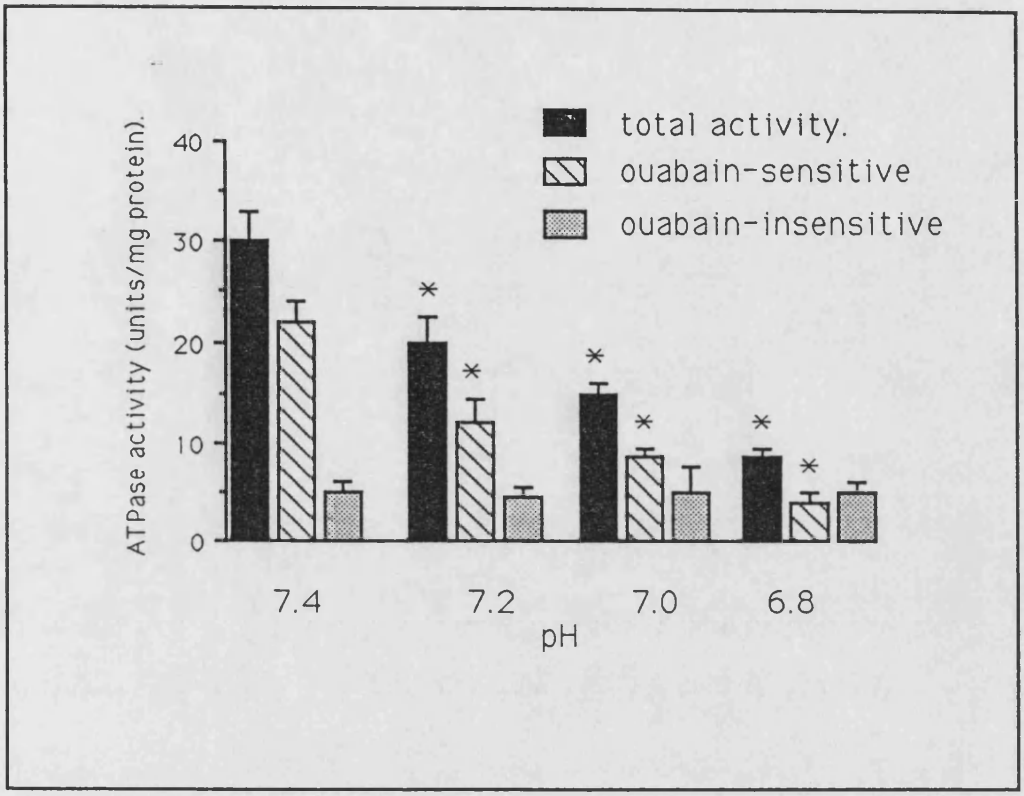


Figure 22. Effect of pH (7.4-6.8) on the activity of the total, ouabain-sensitive and ouabain-insensitive fractions of an isolated bovine Na/K ATPase preparation. Highest total activity measured in a single enzyme preparation = 32.2±2.3 units/mg protein. Vertical bars represent ±s.e.mean. (n=4, *p<0.05 cw controls).

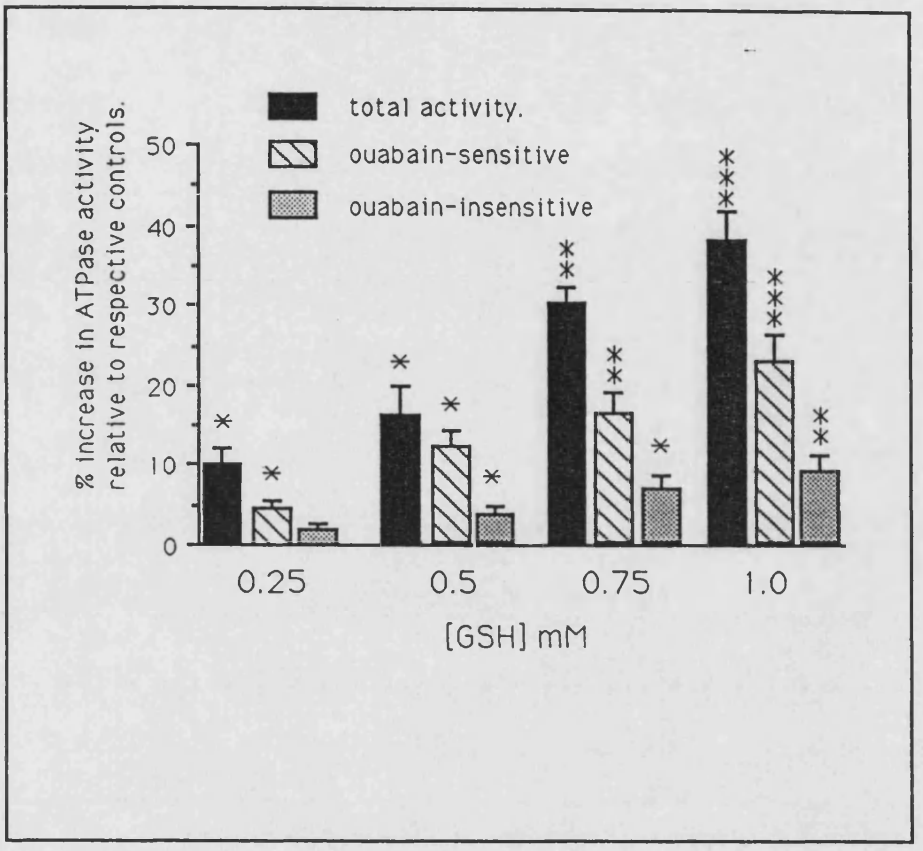


Figure 23. Effect of reduced glutathione (GSH) (0.25-1.0mM) on the activity of the total, ouabain-sensitive and ouabain-insensitive fractions of an isolated bovine Na/K ATPase preparation. Highest total activity measured in a single enzyme preparation = 32.2±2.3 units/mg protein. Vertical bars represent ±s.e.mean. (n=6, *p<0.05, **p<0.01, ***p<0.001).

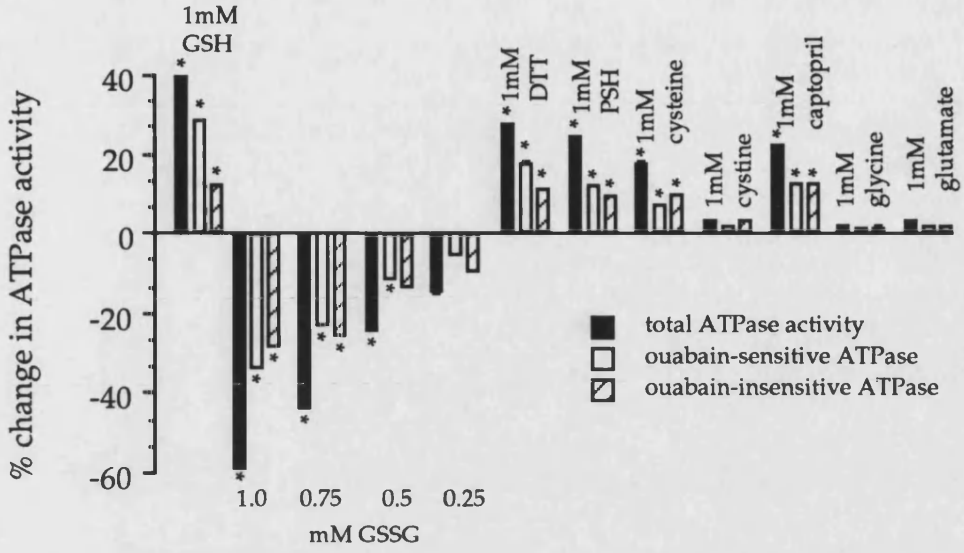


Figure 24. Effect of reduced glutathione (GSH) (1mM), oxidised glutathione (GSSG) (0.25-1.0mM), reduced dithiothreitol (DTT) (1mM), DL-penicillamine (PSH)s (1mM), cysteine (1mM), cystine (1mM), captopril (1mM), glycine (1mM) and glutamate (1mM) on the total, ouabain-sensitive and ouabain-insensitive ATPase activity of an isolated bovine Na/K ATPase preparation. Vertical bars represent $\pm$s.e.mean. (100% total ATPase activity=30.2$\pm$2.3 units/mg; 100% ouabain-sensitive ATPase activity=24.2$\pm$2.1 units/mg; 100% ouabain-insensitive ATPase=6$\pm$1.2units). n=6, *$p<0.05$ cw untreated control group.

5.1.3 Effect of glutamate, glycine and cysteine on Na/K ATPase activity.

In view of the fact that glutathione is a tripeptide composed of glutamate, glycine and cysteine, the effect of these individual amino acids on the activity of the isolated Na/K ATPase preparation was investigated. At a concentration of 1mM, neither glutamate nor glycine had a significant effect on the total activity or the activity of the ouabain-sensitive and ouabain-insensitive fractions of the Na/K ATPase preparation. This is in contrast to the effect of cysteine (1mM final concentration) which significantly increased the activity of all three enzyme fractions (figure 24).

5.1.4 Effect of cystine and oxidised glutathione on Na/K ATPase activity.

Oxidised glutathione and cystine are the disulphide forms of reduced glutathione and cysteine respectively and, therefore, the effect of their exogenous application on the activity of bovine Na/K ATPase activity was investigated. Total Na/K ATPase activity, in addition to the ouabain-sensitive and insensitive fractions of the enzyme preparation were inhibited in a concentration-dependent manner (0.25-1.0mM) by oxidised glutathione (figure 24). Cystine (1mM final concentration) had no significant effect on the activity of either the total, ouabain-sensitive or ouabain-insensitive fractions of the enzyme preparation (figure 24).

5.1.5 Effect of other selected sulphydryl compounds on Na/K ATPase activity.

In addition to reduced glutathione, the effects of the sulphydryl-containing compounds DL-penicillamine, reduced dithiothreitol and the angiotensin-converting-enzyme inhibitor, captopril on the activity of bovine ventricular Na/K ATPase were investigated. In common with reduced glutathione, all of these sulphydryl-containing compounds, at a concentration of 1mM, significantly increased the activity of the total, ouabain-sensitive and insensitive fractions of the isolated enzyme preparation (figure 24).

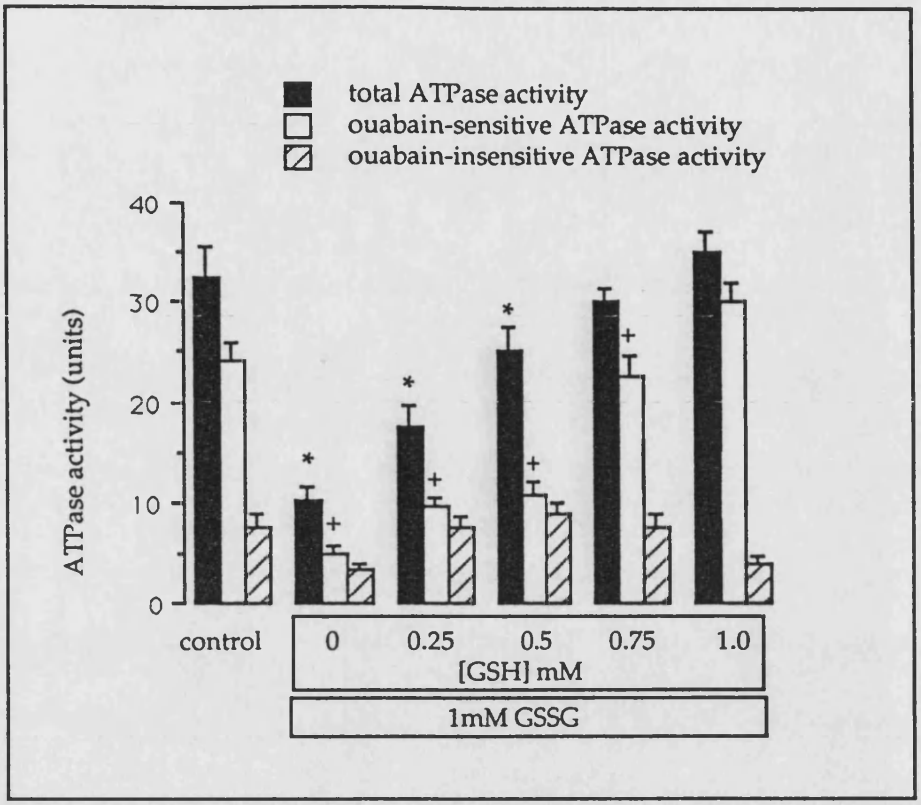


Figure 25. Effect of oxidised glutathione (GSSG) (1mM), in the presence of reduced glutathione (GSH) (0.25-1.0mM), on the total, ouabain-sensitive and ouabain-insensitive ATPase activity of an isolated bovine sarcolemmal preparation. Vertical bars represent ±s.e.mean. (n=6 hearts/group, *, +$p<0.05$ cw respective controls).

5.1.6 Effect of reduced glutathione on the oxidised glutathione-induced inhibition of Na/K ATPase.

When applied alone, reduced glutathione (GSH) and oxidised glutathione (GGSG) had opposing effects on the activity of the total, ouabain-sensitive and insensitive fractions of the isolated Na/K ATPase preparation. Therefore, the effect of reduced glutathione (0.25-1.0mM) on the inhibition of Na/K ATPase activity caused by 1mM oxidised glutathione was examined.

Figure 25 shows that reduced glutathione can overcome, in a concentration-dependent manner, the GSSG-induced inhibition of the activity of the total and ouabain-sensitive fractions of the Na/K ATPase preparation. A GSSG-induced inhibition of ouabain-insensitive activity was not significantly affected by the addition of GSH (0.25-1.0mM). 1mM GSSG caused a mean inhibition of total activity of 63.1±1.3%. This was reduced to 40%, 19.4% and 4.7% by 0.25, 0.5 and 0.75mM GSH, respectively. In the presence of 1mM reduced glutathione and 1mM oxidised glutathione there was a 7.3% increase in total enzyme activity which was not statistically significant.

5.1.7 Effect of selected pharmacological interventions on pyruvate kinase and lactate dehydrogenase.

As already mentioned, the assay of Na/K ATPase activity is based on a linked-enzyme system and it was, therefore, important to determine any effects of the compounds used on the activity of the pyruvate kinase and lactate dehydrogenase preparations. None of the interventions at the concentrations used had a significant effect on the activity of the enzyme preparations, confirming that the effects on enzyme activity of the exogenous application of these agents is due to an interaction with the Na/K ATPase preparation alone (figure 25b).

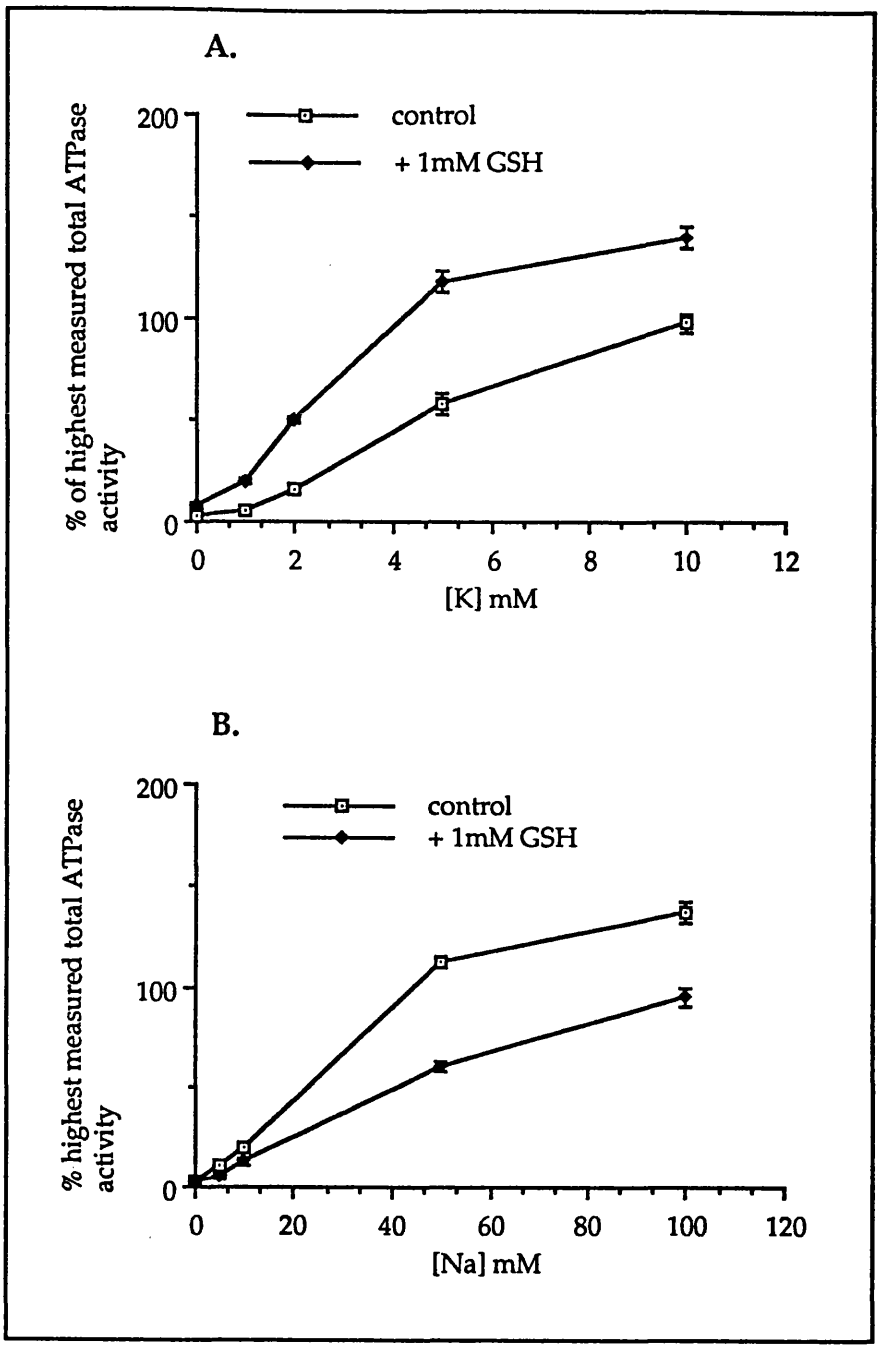


Figure 26. A: Effect of potassium concentration (0-10mM), in the presence of reduced glutathione (GSH) (1mM) on the total activity of an isolated bovine Na/K ATPase preparation expressed as % of highest measured total ATPase activity (n=4, *p<0.05 cw controls). B: Effect of sodium concentration (1-100mM), in the presence of reduced glutathione (GSH) (1mM) on the total activity of an isolated bovine Na/K ATPase preparation expressed as % of highest measured total ATPase activity. (Highest total activity measured in a single enzyme preparation= 30.2±2.3 units/mg protein. [Na]=100mM, [k]=10mM, [ATP]=2.5mM). (n=4, *p<0.05 cw controls). In both figures, vertical bars represent ±s.e.mean.

5.1.8 The effect of sodium and potassium concentration on the effects of reduced glutathione on bovine Na/K ATPase activity.

Figures 26a and 26b show that a [GSH]-dependent increase in the total activity of an isolated bovine Na/K ATPase preparation is [K^+] and [Na^+]-dependent (0.1-10mM and 1-100mM, respectively).

5.2 HISTOCHEMICAL ANALYSIS OF Na/K ATPase ACTIVITY IN RAT HEART SLICES.

5.2.1 Validation and characterization of the technique.

As with the assay of bovine Na/K ATPase activity, a clarification of the specificity of the histochemical Na/K ATPase assay was carried out with respect to ouabain and temperature sensitivity, in addition to ATP and sodium dependence.

5.2.1.1 Ouabain inhibition of rat myocardial Na/K ATPase.

Rat myocardial Na/K ATPase was relatively less sensitive to ouabain-induced inhibition than the isolated bovine Na/K ATPase preparation, with an IC_{50} of total activity of 0.75mM (figure 27). This is as expected in view of previous reports of the relative insensitivity of rat myocardial Na/K ATPase to inhibition by cardiac glycosides. At ouabain concentrations greater than 1mM no further enzyme inhibition was seen. A ouabain concentration of 1mM was used in all subsequent investigations to determine the activity of the ouabain-sensitive and insensitive fractions of the Na/K ATPase present in rat myocardial sections. In common with the methodology used in the isolated enzyme studies, the effect of selected compounds on cellular ATPase activity was approached by investigating the effect of each agent on each enzyme fragment individually. In one set of ventricular sections total ATPase activity was monitored. Ouabain-insensitive activity was monitored by prior exposure of sections to 1mM ouabain followed by the addition of the compound in question. Activity was measured pre- and post compound addition. Ouabain-sensitive activity was determined by the use of a separate group of sections that were pre-exposed to 1mM ouabain followed by the compound

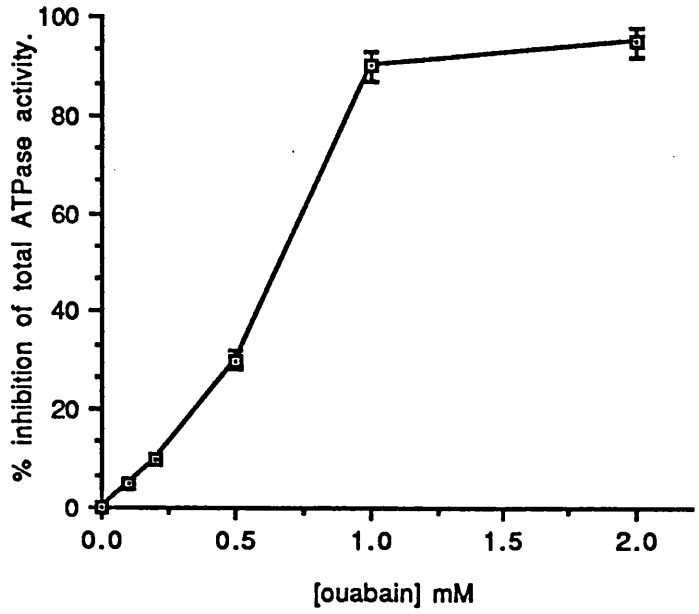


Figure 27. Effect of ouabain (0.0-2.0mM) on rat myocardial Na/K ATPase activity, expressed as % highest total activity. (Highest total activity = 25.2±2.1 mean integrated extinction x 100). Total activity was determined in a set of sections from each heart. Ouabain-sensitivity was determined in sequential fractions from the same hearts. The reason for this is that the assay does not allow measurement of total and ouabain-insensitive ATPase activity in the same section.

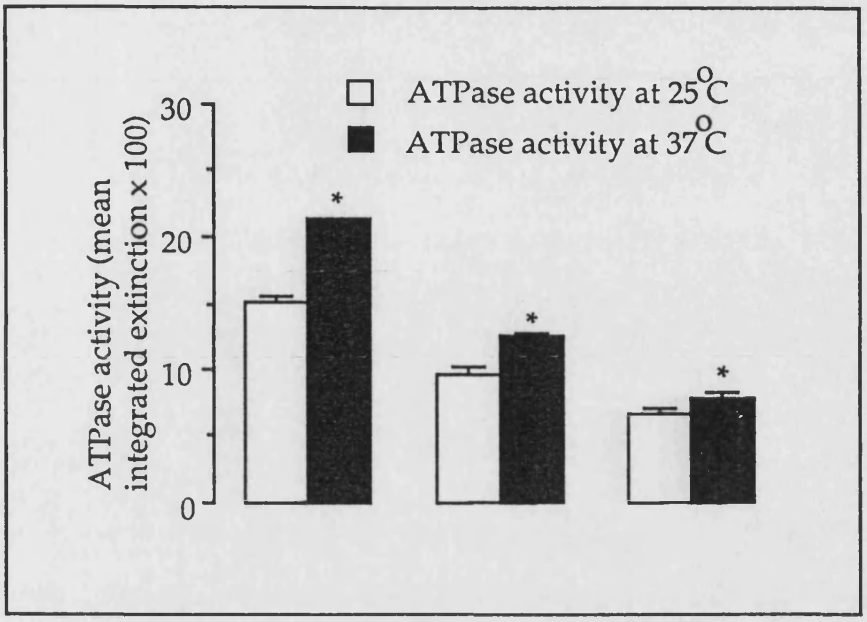


Figure 28. Effect of incubation temperature on the total, ouabain sensitive and ouabain-insensitive ATPase activity of rat myocardial slices. Vertical bars represent ±s.e.mean. (n=4, *p<0.05 cw activity at 25 C).

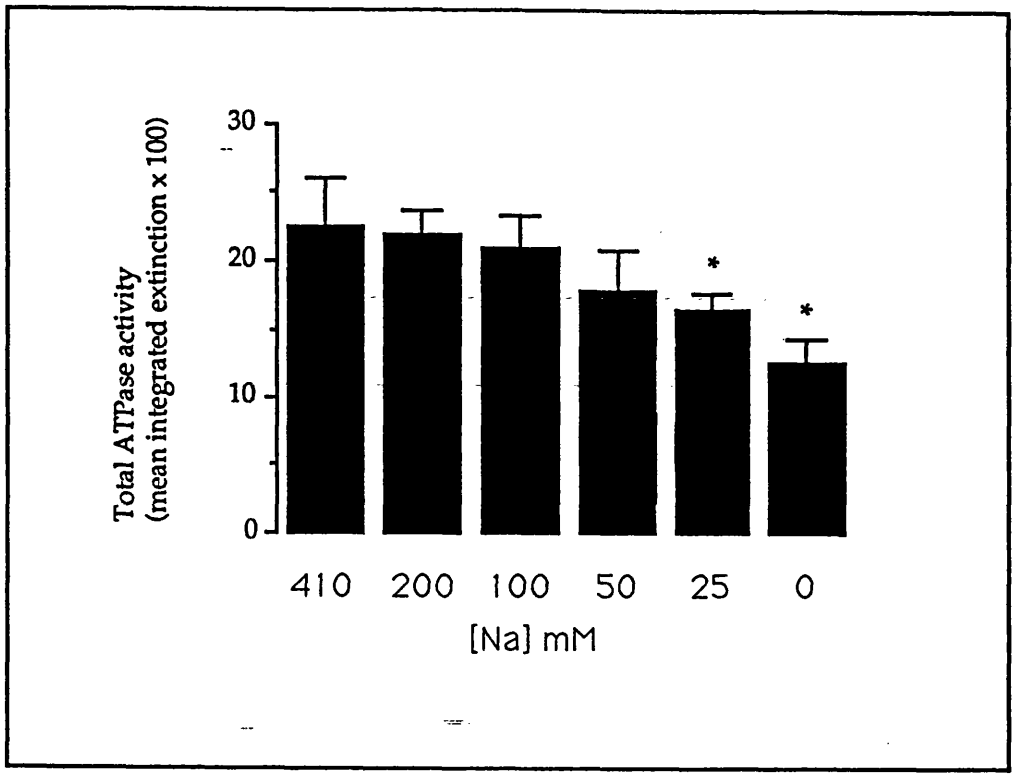


Figure 29. Effect of sodium concentration (0-410mM) on the total ATpase activity of rat myocardial slices. Vertical bars represent ±s.e.mean. (n=4 hearts/group, *p<0.05 cw 410mM Na controls.

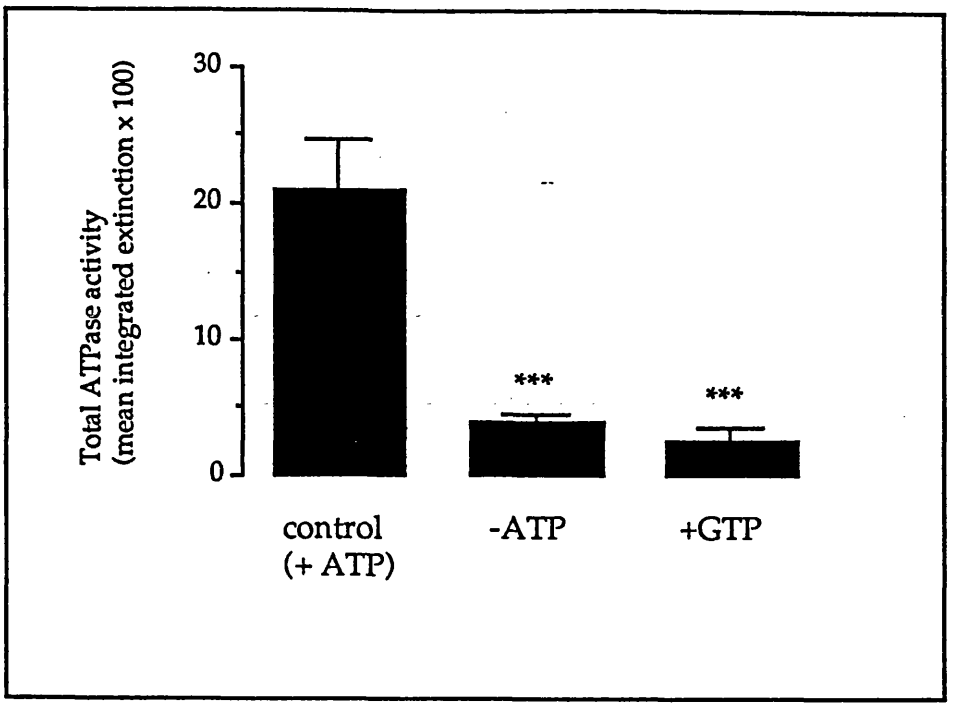


Figure 30. Effect of adenosine 5' triphosphate (ATP) (10 mg/ml) and guanosine 5' triphosphate (GTP) (10 mg/ml) on the total ATPase activity of rat myocardial slices. Vertical bars represent $\pm$s.e.mean. (n=4 hearts/group, ***$p<0.001$ cw control group).

under investigation. Ouabain-sensitive activiity was then determined by subtraction. The data from each group (ie total, ouabain-sensitive and ouabain-insensitive activity) was pooled and expressed as a mean value (±s.e.mean).

5.2.1.2 Temperature sensitivity of rat myocardial Na/K ATPase.

Rat myocardial Na/K ATPase activity was temperature dependent within the range 25-37°C (figure 28). At 37°C, total activity was 20.9±0.1 MIE x 100; ouabain-sensitive and insensitive activity was 12.5±0.2 and 7.9±0.3 MIE x 100, respectively. At 25°C, the activity of the total, ouabain-sensitive and insensitive fractions were significantly reduced to 15.2±0.2, 9.5±0.6 and 6.5±0.5, respectively.

5.2.1.3 Effect of sodium concentration on rat myocardial Na/K ATPase activity.

Figure 29 shows that the total activity of rat myocardial Na/K ATPase activity was sodium-dependent within the range 0-410mM. The large residual activity observed in the absence of added sodium may be due to the use of sodium/potassium hydroxide used to establish the final pH of the media, sodium present in the Polypep media itself or endogenous sodium within the section.

5.2.1.4 Assay dependence and specificity for adenosine 5' triphosphate (ATP) as a substrate.

The total ATPase activity of the enzyme preparation was determined in serial sections of rat myocardium in the presence of either adenosine 5' triphosphate (ATP) (10mg/ml) or guanosine 5' triphosphate (GTP) (10mg/ml). Figure 30 shows that relatively low specific Na/K ATPase activity was found when ATP was replaced by GTP in the reaction media and highlights its substrate specificity.

The experiments described thus far were designed to clarify the specificity of the histochemical assay. In common with the assay of isolated bovine Na/K ATPase activity, the ouabain and temperature sensitivity, as well the dependence on sodium and ATP were assumed to validate the suitability of the histochemical assay. This technique was, therefore, used to investigate the effects of global, low-flow myocardial ischaemia and subsequent reperfusion, in the presence and absence of

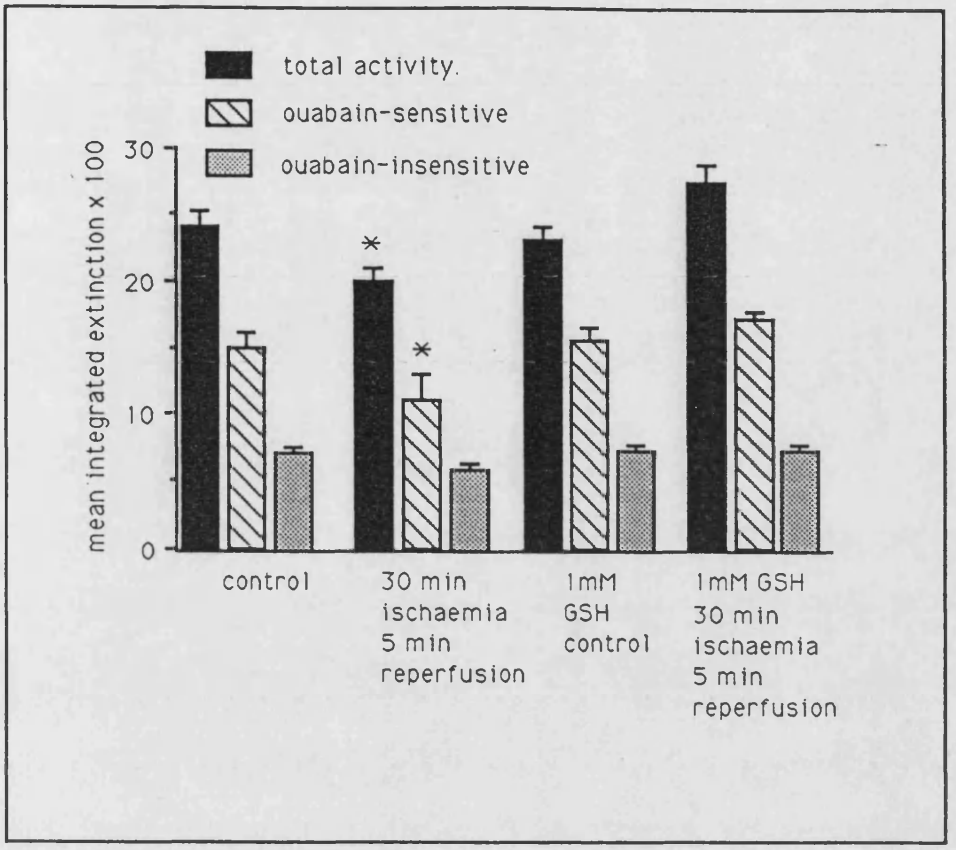


Figure 31. Effect of 30 minutes global, low-flow ischaemia and 5 minutes subsequent reperfusion, in the presence and absence of reduced glutathione (GSH) (1mM), on the activity of the total, ouabain-sensitive and ouabain-insensitive fractions of rat myocardial Na/K ATPase. Vertical bars represent ±s.e.mean. (n=4, *p<0.05 cw controls).

reduced glutathione, on the activity of rat myocardial Na/K ATPase.

5.2.2 Effect of global, low-flow myocardial ischaemia/reperfusion in the presence and absence of reduced glutathione.

5.2.2.1 Effect of global, low-flow ischaemia and subsequent reperfusion on rat myocardial ATPase activity.

A 30 minute ischaemic period and 5 minutes reperfusion resulted in a significant loss of activity of the total, ouabain-sensitive and insensitive fractions of rat myocardial Na/K ATPase activity (figure 31).

5.2.2.2 Effect of reduced glutathione on basal Na/K ATPase activity.

The perfusion of 1mM reduced glutathione for 35 minutes under control conditions had no significant effect on activity of the total, ouabain-sensitive or insensitive fractions of rat myocardial Na/K ATPase (figure 31).

5.2.2.3 Effect of reduced glutathione on a loss of Na/K ATPase activity associated with global, low-flow myocardial ischaemia and reperfusion.

Figure 31 shows that 1mM reduced glutathione significantly attenuated the loss of total and ouabain-sensitive Na/K ATPase activity associated with a 30 minute ischaemic insult and 5 minutes reperfusion. Analaysis of variance and Bonferroni's *t*-test showed that there was no significant effect of GSH during ischaemia on total ATPase activity compared to the contol, non-ischaemic group.

The following electrophysiological studies were all performed by the author under the supervision of Dr M. Shattock and Dr H. Matsuura at the Cardiovascular Research Department, The Rayne Institute, St. Thomas' Hospital, London, SE1 7EH.

5.3 EFFECT OF EXOGENOUS THIOL COMPOUNDS ON SODIUM PUMP CURRENT IN ISOLATED RAT VENTRICULAR MYOCYTES.

Figure 32 shows the voltage protocol used in these studies and current traces associated with the descending phase of the ramp protocol. With standard pipette

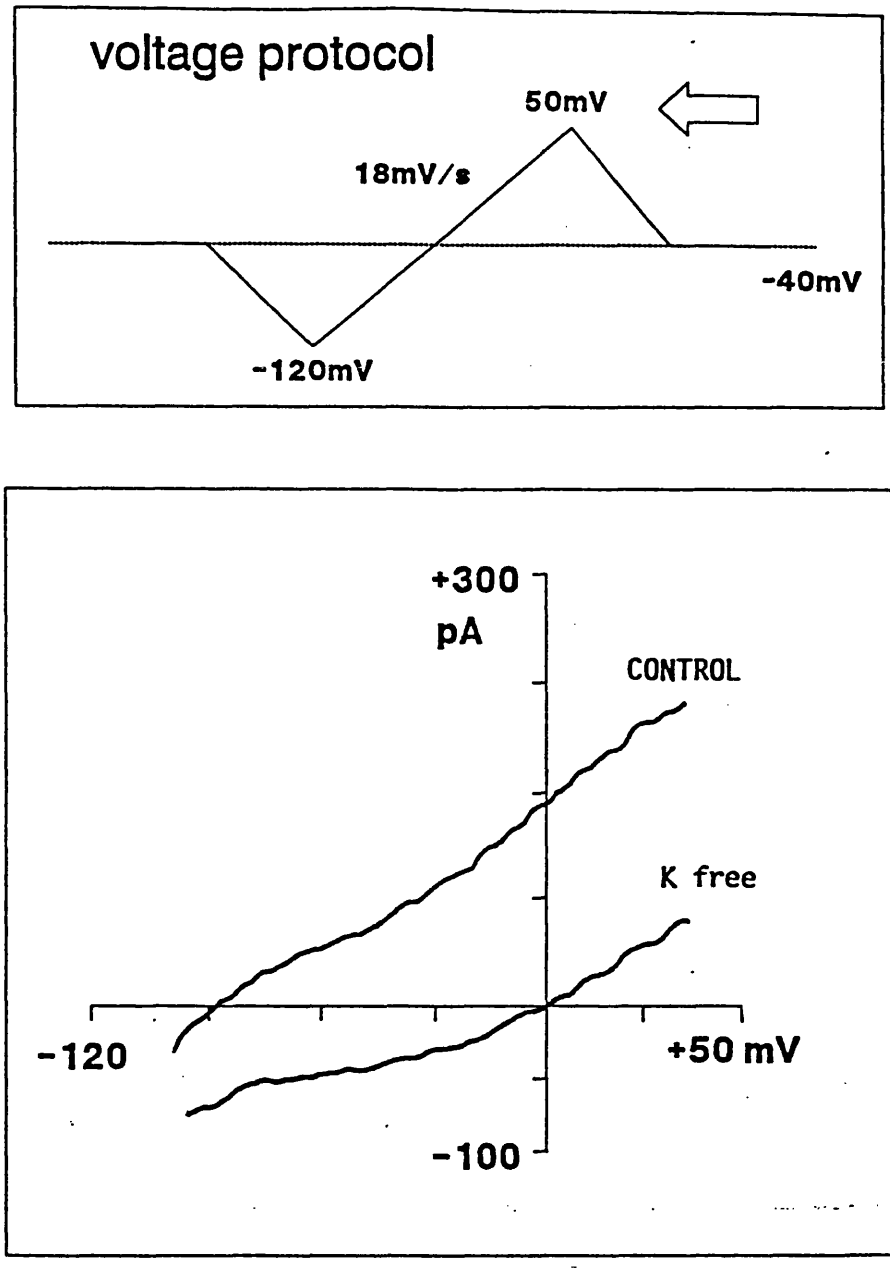


Figure 32. The ramp-voltage protocol used in this set of experiments (upper panel) and examples of currents recorded durin the ramp from +50 to -120mV under control conditions or in a K-free Tyrode solution (lower panel). The voltage protocol was imposed in the direction of the arrow from a holding potential of -40mV. Details of pipette and Tyrode solutions are given in the methods (section 2.15.3).

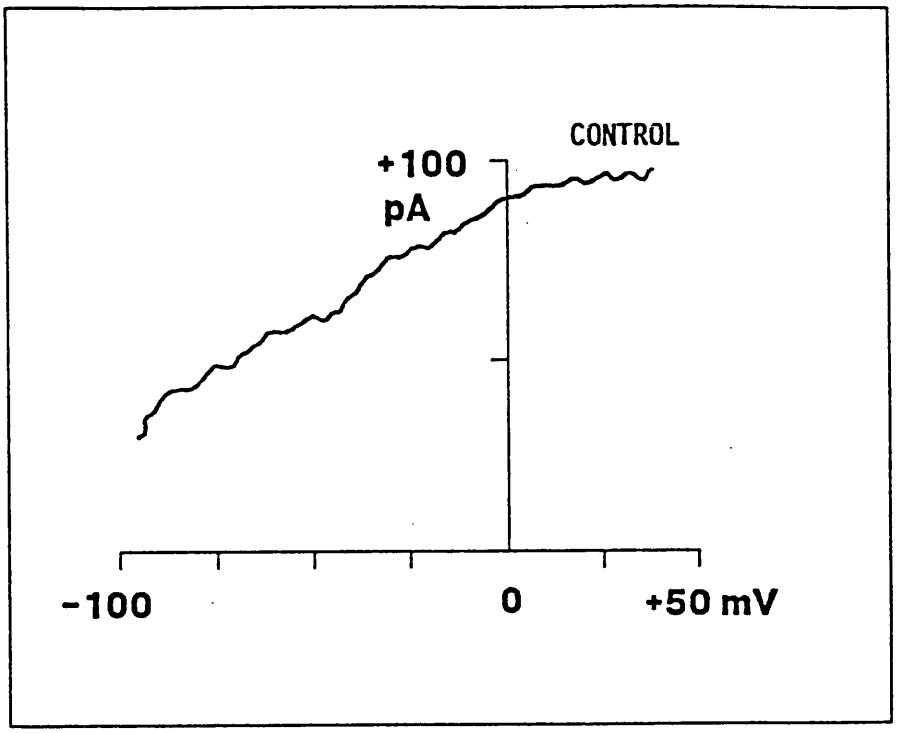


Figure 33. Ko-sensitive fractions of the currents recorded in figure 32. The current records shown here were obtained by subtracting the digitised potassium-free current trace from those recorded under control conditions. This reflects i_p uncontaminated by the currents through the passive membrane resistance. (*Reproduced by kind permission of Shattock and Matsuura, 1990*).

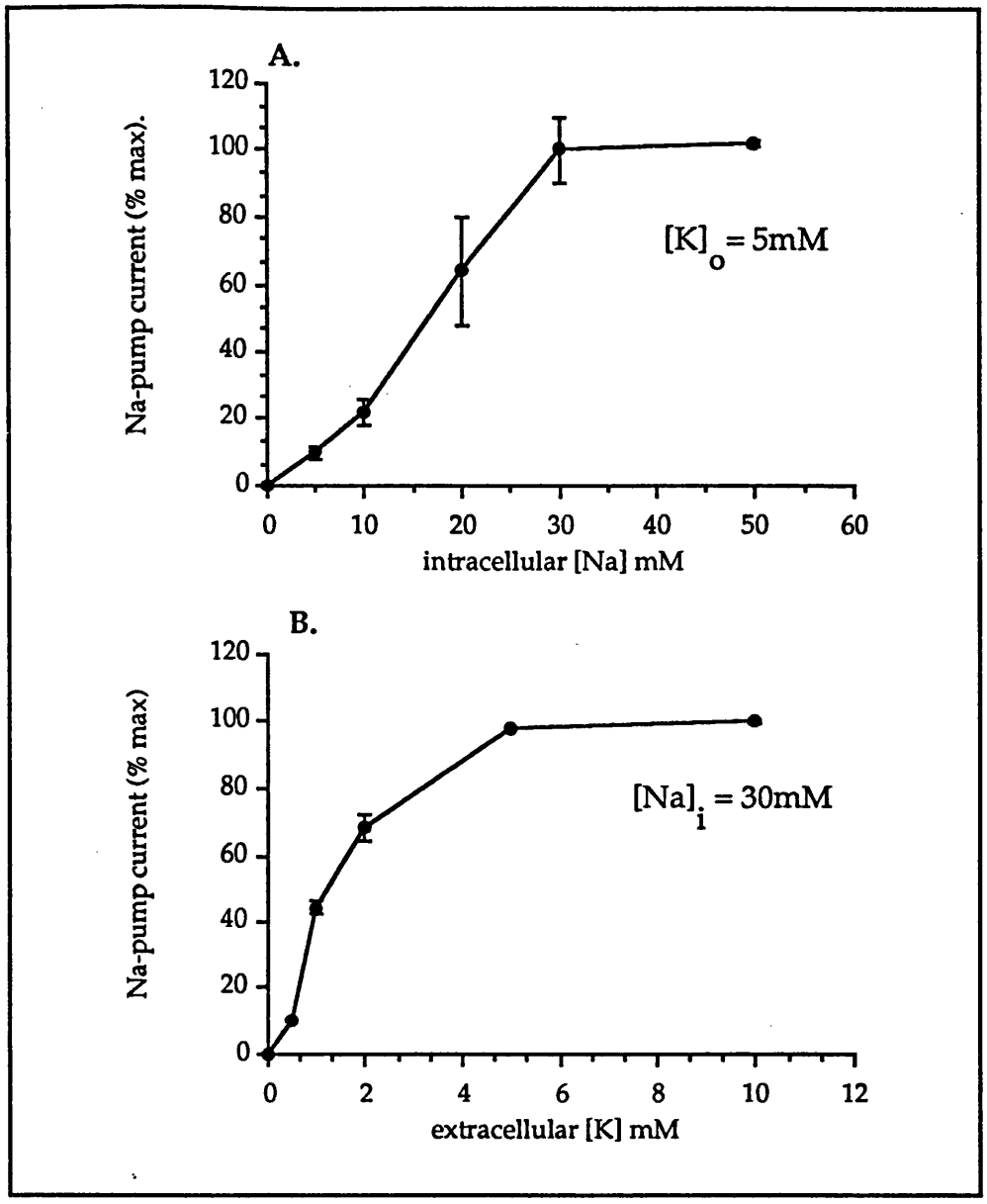


Figure 34. Activation of Na/K pump current by intracellular sodium and extracellular potassium. A: Relationship between intracellular sodium and Ko-sensitive Na/K pump current. This relationship was obtained by dialysing different cells with different pipette solustions containing a range of sodium concentrations between 0 and 50mM. The Ko-sensitive pump current was measured at 0mV and was expressed as a percentage of the maximally activated current. The Na/K pump current was half-maximally activated at a [Na] of 18.4mM. The values shown represent the mean±s.e.mean for at least 6 different cells for each Na concentration. B: Relationship between intracellular potassium and Ko-sensitive Na/K pump current. This relationship was obtained by dialysing different cells with different pipette solustions containing a range of potassium concentrations between 0 and 10mM. The Ko-sensitive pump current was measured at 0mV and was expressed as a percentage of the maximally activated current. The Na/K pump current was half-maximally activated at a [K] of 1.24mM. The values shown represent the mean±s.e.mean for at least 6 different cells for each Na concentration. (*Reproduced by kind permission of Shattock and Matsuura, 1990*).

and Tyrode solutions (previously described in 2.15.2 - 2.15.3), the current-voltage (I-V) relationship obtained consists of a Na-pump current component (i_p) and the current through the passive membrane resistance (R_{cell}). This component is revealed when the Na-pump current is inhibited by the removal of extracellular potassium.

The record in figure 33 was determined by the subtraction of the digitized K-free current trace from those recorded in the presence of extracellular potassium and represent i_p uncontaminated by the current through the passive membrane resistance.

5.3.1 Validation and characterisation of technique.

Due to the constraints of available time, the sodium and potassium dependence of Na-pump current (i_p) were not determined during the course of this set of experiments. However, data for this was obtained by Dr M. Shattock and Dr H. Matsuura prior to the initiation of this work using the same protocol and equipment. The relationship between Na_i and i_p was determined by dialysing different cells with a range of sodium concentrations. The K_o-sensitive pump current was measured at 0mV and expressed as a percentage of the maximally activated current. The relationship between i_p and sodium concentration was sigmoidal and i_p was half maximally activated at a sodium concentration of approximately 18mM (figure 34a). A similar protocol was used to investigate potassium dependence by superfusing cells with a range of potassium concentrations. I_p was half maximally activated at a potassium concentration of approximately 1.2 mM (figure 34b).

The prior characterization of this model using an identical experimental set-up in the laboratories of Dr M. Shattock and Dr H. Matsuura was assumed to be adequate to demonstrate the validity and suitability of this experimental approach to investigate the effects of exogenous thiol compounds on Na-pump current (i_p).

5.3.2 Effect of reduced glutathione on Na-pump current.

5.3.2.1 Extracellular superfusion.

Figure 35(i) shows that the extracellular superfusion of 1mM reduced glutathione

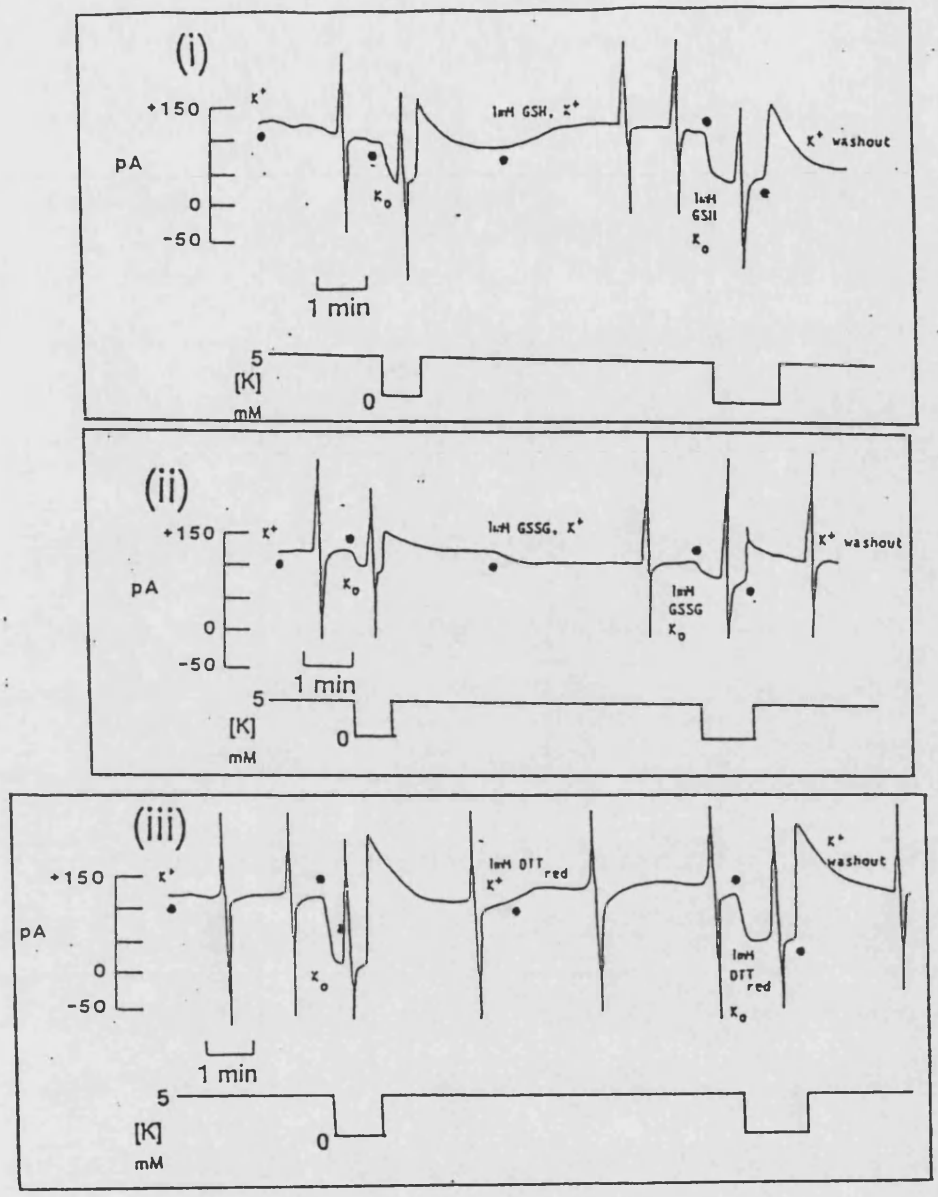


Figure 35. Chart recordings showing changes in holding current, recorded on a slow time-base, in the presence and absence of (i) extracellular reduced glutathione (GSH) (1mM), (ii) oxidised glutathione (GSSG) (1mM) or (iii) reduced dithiothreitol (DTTred). The timing of the changes in Tyrode potassium concentration are indicated and marked '●'. Ramp voltage protocols (as shown in figure 31) were imposed at the start of the trace and during potassium-free exposure and potassium-repletion (in the presence and absence of the compound under investigation). (Abbreviations: K+ -potassium-containing, Ko - potassium free.

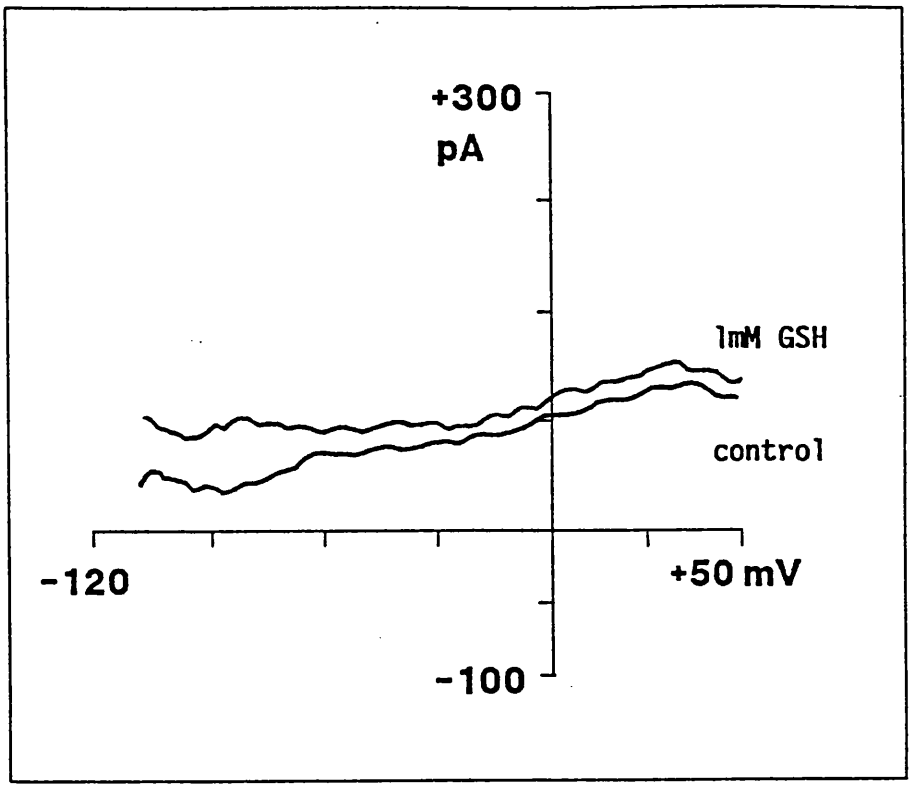


Figure 36. Influence of the extracellular superfusion of reduced glutathione (GSH) (1mM) on the current-voltage relationships for the Na/K pump current in a an isolated rat ventricular myocyte, using a ramp-pulse protocol stepping at 18mV/s between +50 and-120mV.

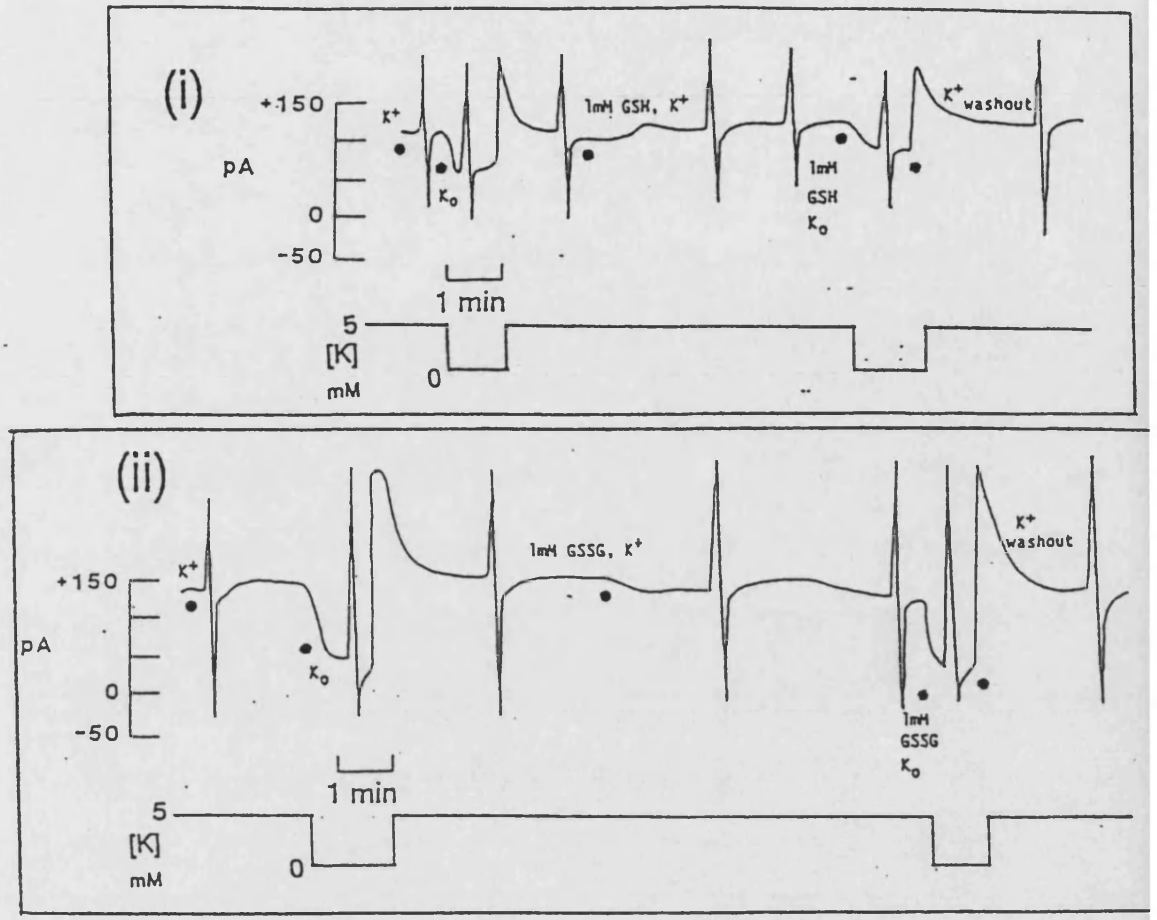


Figure 37. Chardt recordings showing changes in holding current, recorded on a slow time-base, in the presence and absence of (i) intracellular reduced glutathione (GSH) (1mM) or (ii) oxidised glutathione (GSSG) (1mM). The timing of the changes in Tyrode potassium concentration are indicated and marked '●'. Ramp voltage protocols (as shown in figure 31) were imposed at the start of the trace and during potassium-free exposure and potassium-repletion (in the presence and absence of the compound under investigation). (Abbreviations: K+ - potassium-containing, Ko - potassium free).

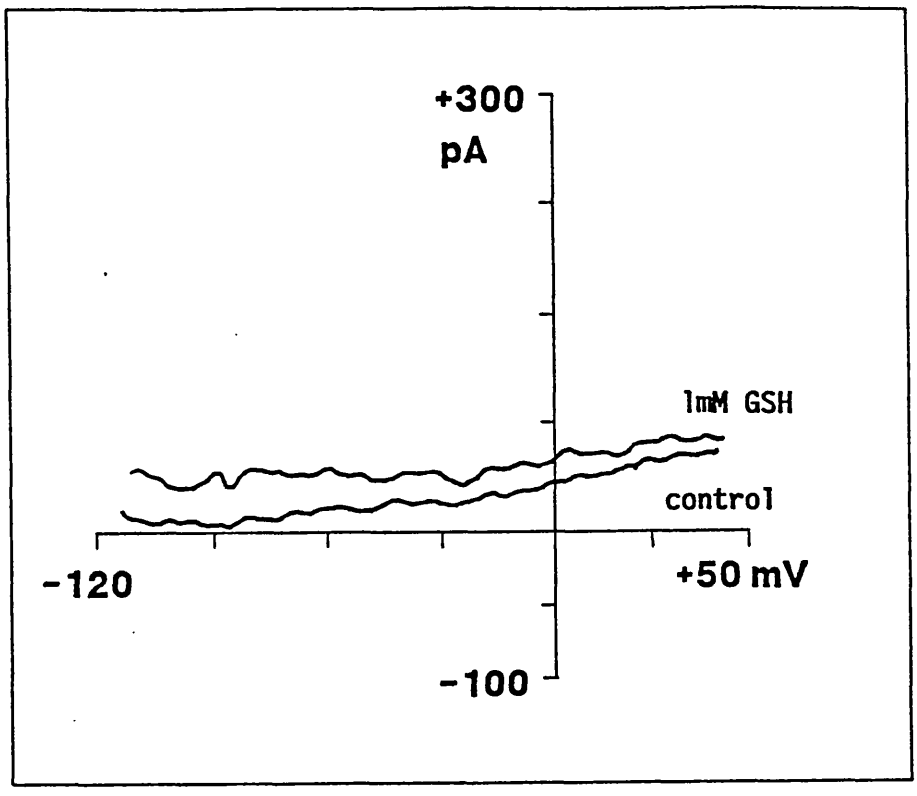


Figure 38. Influence of intracellular dialysis of reduced glutathione (GSH) (1mM) on the current-voltage relationships for the Na/K pump current in an isolated rat ventricular myocyte, using a ramp-pulse protocol stepping at 18mV/s between +50 and -120mV.

significantly increased the outward membrane holding-current recorded in an isolated rat ventricular myocyte. The extracellular superfusion of 1mM reduced glutathione (GSH) resulted in a significant increase in Na-pump current at all voltages (figure 36). Na-pump current returned to control values, at all voltages, on the removal of extracellular GSH. The re-admission of potassium after the potassium-free period (marked 'o') resulted in an overshoot of pump current that presumably reflects a reactivation of the Na/K pump in the presence of an elevated sodium concentration. This protocol of GSH addition and removal could be repeated three times on the same cell and similar results obtained.

5.3.2.2 Intracellular dialysis.

Current-voltage relationships were obtained prior, during and following the intracellular dialysis of 1mM reduced glutathione in isolated rat ventricular myocytes. The intracellular dialysis of 1mM GSH significantly increased the outward holding current (figure 37(i)) and Na-pump current at all voltages (figure 38). This effect was not significantly different to the effect of the extracellular superfusion of GSH.

Therefore, 1mM reduced glutathione significantly increased Na-pump current in isolated rat ventricular myocytes when applied either intra- or extracellularly.

5.3.3. Effect of oxidised glutathione on Na-pump current.

5.3.3.1 Extracellular superfusion.

The extracellular superfusion of 1mM oxidised glutathione (GSSG) significantly decreased the outward membrane holding-current (figure 35(ii)) and significantly attenuated Na-pump current at all voltages, in isolated rat ventricular myocytes (figure 39). As with reduced glutathione, this effect was readily reversible and could be repeated three times in the same cell and in cells prepared from different donor hearts (n=3).

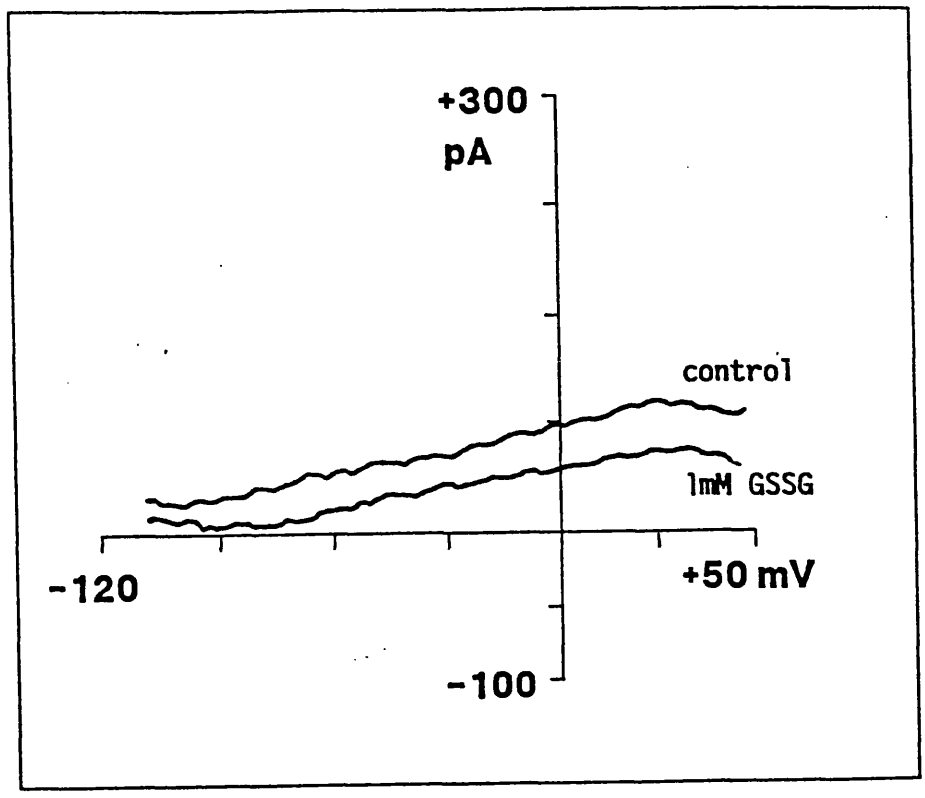


Figure 39. Influence of extracellular superfusion of oxidised glutathione (GSSG) (1mM) on the current-voltage relationships for the Na/K pump current in an isolated rat ventricular myocyte, using a ramp-pulse protocol stepping at 18mV/s between +50 and -120mV.

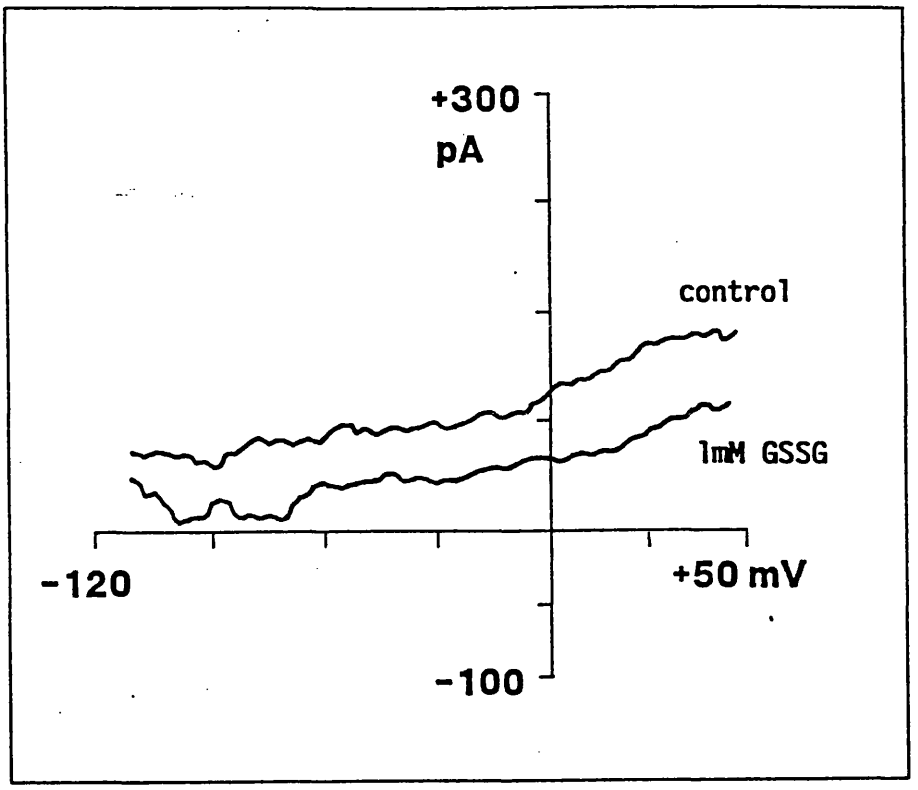


Figure 40. Influence of intracellular dialysis of oxidised glutathione (GSSG) (1mM) on the current -voltage relationships for the Na/K pump current in an isolated ventricular myocyte, using a ramp-pulse protocol stepping at 18mV/s between +50 and -120mV.

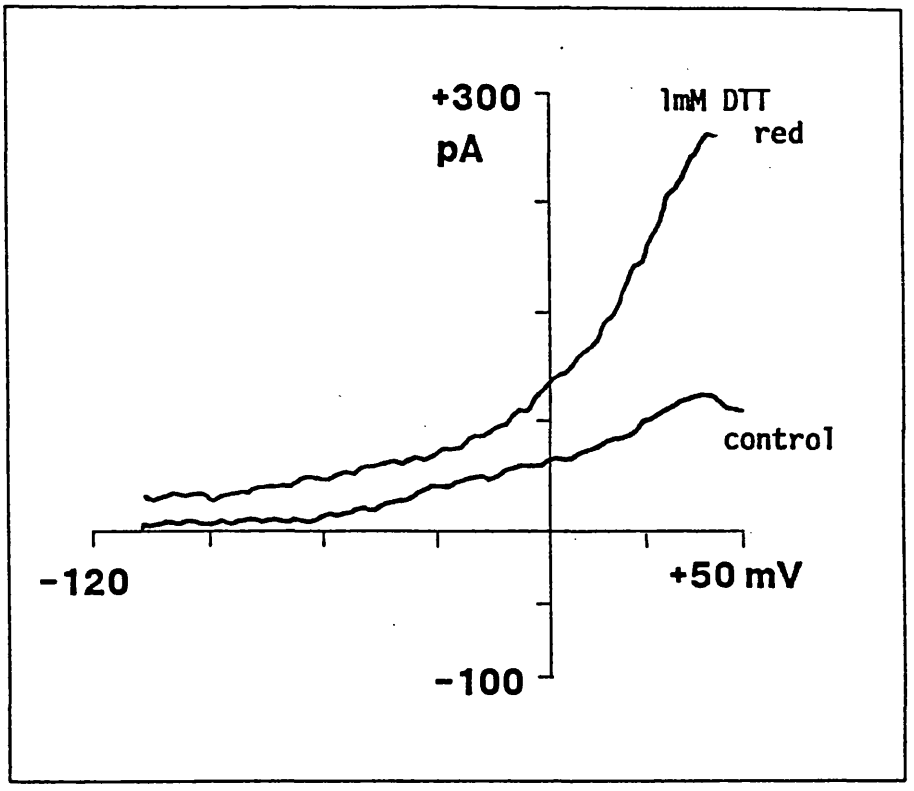


Figure 41. Influence of extracellular superfusion of reduced dithiothreitol (DTTred) (1mM) on the current-voltage relationships for the Na/K pump current in an isolated rat ventricular myocyte, using a ramp-pulse protocol stepping at 18mV/s between +50 and -120mV.

5.3.3.2 Intracellular dialysis.

In common with its extracellular superfusion, the intracellular dialysis of 1mM GSSG resulted in a reduction in outward membrane holding current (figure 37(ii)). In addition, Na-pump current was significantly attenuated at all voltages following the intracellular dialysis of 1mM GSSG (figure 40).

1mM oxidised glutathione, therefore, significantly attenuated Na-pump current at all voltages, in a reversible manner, whether applied intra- or extracellularly.

5.3.4 Influence of reduced dithiothreitol on Na-pump current.

5.3.4.1 Extracellular superfusion.

The superfusion of 1mM reduced dithiothreitol resulted in an increase in outward membrane holding current (figure 35(iii)). Na-pump current was also significantly increased at all voltages (figure 41).

No determination of the effects of the intracellular dialysis of DTT_{red} was performed due to problems with the formation of a brown precipitate upon the addition of 1mM DTT_{red} to the intracellular buffer.

5.3.5 Influence of intracellular glutathione depletion on Na-pump current.

Isolated rat ventricular myocytes were prepared from animals previously depleted of endogenous glutathione (as decribed in section 2.16) which was confirmed by enzymatic analysis. Na-pump current was compared in myocytes prepared from untreated and GSH-depleted animals. Whole cell capacitance (C_{cell}) was estimated using a ramp voltage protocol according to the method described by Shattock and Matsuura (1990). Na-pump current was normalised to cell capacitance by the method described by Isenberg *et al.* (1987). The rapid current deflection (ΔI) observed on switching from a negative-going to a positive-going ramp was measured and $C_{cell} = \Delta I/(2 \times dV/dt)$. Cell surface area was estimated assuming a specific membrane capacitance of 1.0 $\mu F/cm^2$ (*Hille, 1984*). At 0mV, in control myocytes from untreated animals, capacitance-normalised Na-pump current was

CONTROL CELLS.
AT 0mV, CAPACITANCE NORMALISED Na/K PUMP CURRENT = 0.015 ± 0.002 pA/μm^2.
GSH-DEPLETED CELLS.
AT 0mV, CAPACITANCE NORMALISED Na/K PUMP CURRENT = $0.007 \pm 0.002^*$ pA/μm^2.

Table 6. Comaprison of the effect of reduced glutathione (GSH) on capacitance normalised Na/K pump current, at 0mV, in isolated rat ventricular myocytes prepared from hearts from control, untreated animals with isolated rat ventricular myocytes prepared from hearts from glutathione-depleted animals using diethylmaleate (0.864 g/kg, i.p, 30 minutes pre-sacrifice). (n=3, *$p<0.05$ cw control group).

0.015±0.002 pA/μm^2 (n=3). This was significantly decreased in myocytes prepared from GSH-depleted hearts. In these cells, capacitance normalised Na-pump current was significantly reduced to 0.007±0.002 pA/μm^2 (n=3, *p<0.05). Therefore, endogenous glutathione depletion significantly attenuated Na-pump current in isolated rat ventricular myocytes (table 6).

CHAPTER 6.

DISCUSSION.

The investigations described above have shown that the activity of both a bovine ventricular and rat myocardial Na/K ATPase preparation can be modulated by the exogenous application of selected reductants and oxidants, such as reduced (GSH) and oxidised (GSSG) glutathione. These experiments were undertaken in an attempt to explain how reduced glutathione exerts its antiarrhythmic effect (*Woodward and Zakaria, 1985*), delays the onset of an ischaemically-induced contracture, as described in the present investigations, and attenuates ^{86}rubidium efflux from the rat myocardium (*Blackwell et al., 1986*). Modulation of Na-pump activity was also investigated in isolated rat myocytes. In this set of experiments the ability of GSH to increase and GSSG to decrease Na-pump activity was independent of whether they were applied intra- or extracellularly. A reduction in Na-pump activity was also observed in cells depleted of endogenous glutathione, using diethylmaleate, which may point to GSH being a component in the regulation of ion translocating systems such as Na/K ATPase.

The breakage and formation of intra- and intermolecular disulphide bridges has been shown to be crucial in the regulation of insulin-mediated hexose transport, phosphofructokinase, glutathione reductase, guanylate cyclase and fatty acid synthase (*Brigelius, 1985; Zeigler, 1985; Miller et al., 1990*) and may have a role to play in the modulation of Na/K ATPase activity observed in the investigations described above. The involvement of GSH and/or GSSG in such interactions would result in a change in cellular redox state, having a knock-on effect on the structure and activity of other enzymes and ion translocating proteins that may be susceptible to varying degrees of oxidant/reductant stress. Hence, the oxidation of protein thiols may be responsible for alterations in the activity and function of key enzyme systems, such as the Na/K pump, that may result in a reduction in the ability of the ischaemic myocardium to maintain ionic homeostasis, contractile function and rhythm. Ischaemia and subsequent reperfusion results in a significant loss of Na/K ATPase activity in guinea-pig hearts, an effect which can be attenuated by selected free radical scavengers and antioxidants (such as superoxide dismutase, catalase, dimethylsulphoxide, vitamin E and allopurinol) (*Kim and Akera, 1987*). In view of these results obtained by Kim and Akera (1987), the effect of reduced glutathione on the activity of Na/K ATPase was investigated as another example of a

compound involved in cellular defence against oxidative stress. The data from these experiments, described previously, is in agreement with Kim and Akera (1987) with respect to the ability of GSH as an antioxidant to attenuate an ischaemically-induced loss of Na/K ATPase activity. Kim and Akera, however, did not distinguish between the free radical scavenging and antioxidant activity of these compounds and the possibility of other direct or indirect effects on the Na/K pump itself.

In the past many authors have stressed the importance of free radical-induced lipid peroxidation as the central event in oxidative damage to the myocardium during ischaemia and reperfusion. This damage is likely to be dependent on many factors such as the severity of the ischaemic insult, the location of cell subtypes within a tissue and the efficiency of endogenous defence systems against oxidative stress. However, it is highly likely that ischaemia/reperfusion-induced oxidative stress may also affect the primary, secondary and tertiary structure of many membrane-bound proteins, such as the enzymes and ion translocating proteins involved in intracellular ionic homeostasis. Kim *et al.* (1989) investigated the effects of myocardial ischaemia on sarcolemmal Na/K ATPase activity with respect to the degree of lipid peroxidation and interactions with non-esterified fatty acids (NEFA) and their ester derivatives (acyl carnitines) that accumulate in the ischaemic myocardium (*Liedtke et al., 1978*). This group failed to show a correlation between the level of ischaemically-induced lipid peroxidation and an associated reduction in Na/K ATPase activity which lessens the significance of lipid peroxidation in the early stages of ischaemic damage to the myocardium and implies that there are other mechanisms by which the activity of membrane-bound proteins and enzymes are affected during this phase. It would be of interest to investigate the effect of reduced and oxidised glutathione on the accumulation of NEFA and acyl carnitines with a view to investigating the mode of action by which reduced and oxidised glutathione modulate Na/K ATPase activity during myocardial ischaemia and reperfusion.

From the work described here, it is unclear as to whether the ability of selected thiol compounds, such as reduced glutathione, to modulate the activity of Na/K ATPase results from direct interactions with the alpha and/or beta subunits of the

enzyme or by the involvement of these compounds in the removal of reactive oxygen species that cause oxidant/reductant stress. There is little doubt that glutathione, glutathione reductase and glutathione peroxidase are key components in the cells defence against highly reactive and potentially damaging reactive oxygen species (*Halliwell and Gutteridge, 1989*). During oxidative stress there is a reduction of total cellular glutathione and GSH during the removal of lipid peroxides and hydrogen peroxide, which also curtails a rise in intracellular GSSG levels. Ferrari *et al.* (1985) have documented such a change in the ratio of GSH/GSSG in response to an ischaemic insult. Under normal conditions, free sulphydryl (-SH) groups on proteins are maintained in the reduced state by a high intracellular GSH/GSSG ratio with only low concentrations of mixed thiols (*Brigelius, 1985*). However, in pathological conditions an associated fall in the GSH/GSSG ratio may result in a decrease in free -SH groups. Their oxidation and the formation of mixed thiols may be responsible for a loss of enzyme and ion channel activity. Therefore, the free radical scavenging action of GSH and related systems, that help protect against oxidative stress, may have an indirect effect on cellular redox state and enzyme activity. In view of this, it is reasonable to postulate that under conditions of oxidative stress, a change in the GSH/GSSG status of the heart could be an important factor in the regulation of Na/K ATPase, such that a fall in the GSH/GSSG ratio would lead to a net loss of potassium from the cell and a concomitant accumulation of intracellular sodium, both of which occur during ischaemia. These conditions would in turn result in a shift in sodium/calcium exchange favouring calcium influx, manifesting itself as contracture in this experimental model.

It is thought that the beta subunit of the Na/K ATPase dimer may be involved in the insertion of the alpha subunit into the lipid bilayer of the plasma membrane and, therefore, may be relatively more exposed and/or susceptible to oxidant/ reductant stress. Indeed, Kirley (1990) suggests that one of the three disulphide bonds found in the beta subunit may be more susceptible to cleavage by a reductant such as dithiothreitol. Subsequent cleavage of this bond renders the remaining two disulphide bonds liable to similar attack resulting in a loss of Na/K ATPase activity. Since GSH and dithiothreitol increased Na/K ATPase activity in the

present investigations, it would seem that either these compounds do not act at this site or that they cause other conformational changes that mask these disulphides from attack. An alteration in the structure of the beta unit may have a "knock-on" effect on the alpha unit resulting in an altered insertion of the alpha subunit into the membrane. This may modulate the activity of the enzyme in the absence of a direct effect on the alpha subunit. No correlation between enzyme inactivation and reduction of disulphide bonds exists, suggesting a requirement for multiple or sustained reductant attack to inactivate the enzyme. The ability of GSH to activate Na/K ATPase in the present investigations may be representative of a species or tissue difference between the two sources of enzyme. Using isolated Na/K ATPase from lamb kidney, Kirley (1990) reported an inhibitory effect of 50-100mM dithiothreitol which is in contrast to the stimulatory effect of 1mM dithiothreitol observed in experiments with bovine ventricular Na/K ATPase described here. It is not uncommon for drugs to have varying effects at relatively high and/or low concentrations and, therefore, it would be unwise to directly compare the two sets of data.

Reports on the number of cysteine residues in the alpha subunit of Na/K ATPase range between 9 and 14, whilst the concensus is that the beta subunit contains two *(Perrone et al., 1975; Peters et al., 1981).* Schoot *et al.* (1981) classified the sulphydryl groups of Na/K ATPase in terms of reactivity with *N*-ethylmaleimide (NEM) and dinitrobenzoic acid (DNTB). There are 12 Class A -SH groups per dimer that react with both NEM and DTNB; at least one of these -SH groups is essential for enzyme activity. There are 14 groups in Class B that only react with NEM. The remainder are in Class C and can only be alkylated after unfolding of the protein with a detergent, such as SDS. There is thought to be at least one thiol group in or near the catalytic centre. It is not possible to determine which groups, if any, are affected by GSH and GSSG from the experiments described in this thesis. Even though the extracellular application of GSH may enhance the intracellular [GSH], in the time course of these experiments it is more likely that GSH and/or GSSG may act in the short term at the thiol groups located on the extracellular side of the cell membrane in the histochemical studies of Na/K ATPase activity previously described. In ischaemically-induced oxidative stress in isolated hearts,

thiol groups located both intra- and extracellularly, are just as likely to be affected by free radical-induced oxidant stress. In the isolated enzyme preparation, the conformation of the enzyme is unknown and should not be assumed to be identical to that found *in vivo*. In this situation, GSH and/or GSSG may have access to a greater proportion of thiol groups required for enzyme activity. This may explain the ability of GSH to increase the activity of the isolated enzyme whereas it has no effect on basal Na/K ATPase activity in isolated heart preparations. Therefore, the location and relative susceptibility of free -SH groups and disulphides in an enzyme or protein to oxidative and reductive attack, respectively, may be important. With regard to Na/K ATPase, the location and relative vunerability to reductant attack of disulphides on the beta subunit may be critical.

Temma *et al.* (1978) investigated the relationship between the inotropic action of sulphydryl blocking agents and membrane sodium pump activity. These agents (of which *N*-ethylmaleimide, *p*-chloro-mercuribenzoate, *p*-chloromercuribenzenesulphonic acid and digitoxin are examples) are known Na/K ATPase inhibitors (*Skou and Hilberg, 1965*) but may affect membrane functions and properties other than Na-pump activity (*Rothstein, 1970*). The inotropic action of *N*-ethylmaleimide was partially reduced in the presence of the beta-adrenoceptor antagonist, propranolol or by reserpine pretreatment which suggests a role for catecholamine release in its mode of action. The functionally-related sulphydryl groups on the Na-pump are located at the inner surface of the cell membrane (*Rothstein, 1970*) and, therefore, these essential groups for Na-pump activity should be affected first by blocking agents that penetrate the cell membrane. This group, however, was unable to determine the site of action of these agents. Such blocking agents produce a biphasic effect with a relatively brief positive inotropic action followed by a negative inotropic effect. This is in common with the opposing effects of relatively high and low concentrations of dithiothreitol reported earlier. The latter effect of sulphydryl blockers may be due to a longer access time to subcellular organelles that may be responsible for this observation. The data from these experiments underlines, however, the potential involvement of -SH groups in the modulation of Na-pump activity. It would be of interest to study Na/K ATPase activity in response to ischaemia in hearts and isolated myocytes from animals

previously depleted of catecholamines, using reserpine or 6-hydroxydopamine, and the effect of the subsequent exogenous application of reduced and oxidised glutathione. As described earlier, the time to the onset of an ischaemically-induced contracture was significantly increased in hearts from animals previously depleted of endogenous catecholamines. However, in a separate set of experiments, reduced glutathione was shown to have no significant effect on noradrenaline release. Therefore, the role of GSH and catecholamines in ischaemic contracture and the modulation of Na/K pump activity remains unclear.

The exposure of myocytes to extracellularly generated oxidants results in alterations in membrane function and in cell viability (*Barrington et al., 1985; Sato et al., 1989; Shattock amd Matsuura, 1990).* Hydrogen peroxide and the superoxide radical may pass readily through the plasma membrane where they may react with metal ions bound to the plasma membrane or the membranes of intracellular organelles to produce the highly reactive hydroxyl radical (*Fridovich, 1989*). This may result in self propagating reactions and the amplification of the effects of the superoxide anion. Xie *et al.* (1990) reported on the effects of such oxidants on the function of selected membrane bound ion translocating proteins and suggested that, although their experimental approach resulted in some non-specific damage to the cell, there was some specificity in the interactions of superoxide and hydrogen peroxide-derived free radicals with sarcolemmal transport proteins, such as the Na/K ATPase. Both *in vivo* and *in vitro*, the long-term presence of such species may result in a time-dependent depression in activity and function of the cells' defence systems against oxidative stress, such as the glutathione/glutathione reductase/glutathione peroxidase system and a concomitant change in cellular redox state. Therefore, the direct effects of reactive oxygen species on membrane-bound proteins may represent irreversible damage whereas the modulation of enzyme activity via a change in cellular redox state (eg. the ratio of GSH/GSSG), due to a depression in the activity of cellular defence systems against oxidative stress, may be reversible. The effect of the exogenous application of oxidised glutathione on the activity of an isolated bovine Na/K ATPase preparation, described in the present investigations, may be synonymous with this situation. The depression of Na/K ATPase activity in response to GSSG was

readily reversible by the addition of GSH. The activity of this Na/K ATPase preparation may, therefore, be controlled by cellular redox state and may represent a mode of enzyme regulation that is downstream from the effects of free radicals themselves. *In vivo*, there is the possibility that oxidant-induced damage of this enzyme may render it more susceptible to subsequent redox regulation following the free radical-induced oxidation and/or reduction of free -SH groups and disulphides. In the experiments described earlier, using both isolated Na/K ATPase and rat Na/K ATPase in tissue slices, oxidant and reductant stress was mimicked using GSH and GSSG as opposed to the use of a free radical generating system, such as the xanthine/xanthine oxidase system that results, primarily, in the production of superoxide radicals. These experiments show that the activity of this enzyme can be modulated by changes in redox state as opposed to more permanant damage to membranes and proteins that results from the direct action of free radicals.

In the present investigations, reduced and oxidised glutathione were also used to study reductant and oxidant stress in isolated myocytes and their effect on Na-pump current investigated. These experiments were performed to confirm the data obtained using an isolated Na/K ATPase preparation and histochemical studies in isolated hearts. In rat ventricular myocytes, the intracellular dialysis and extracellular superfusion of reduced and oxidised glutathione resulted in a significant potentiation and attenuation of Na-pump current at all voltages, respectively. This suggests that there are free thiols and disulphides located both intra- and extracellularly that may be oxidised and reduced resulting in the modulation of Na-pump activity. The observation that the intracellular depletion of glutathione resulted in a depression of Na-pump activity suggests the involvement of cellular redox state in the modulation of this ion translocating system. However, other non-specific effects of diethylmaleate, used to deplete cellular glutathione, may be responsible for this effect. Therefore, it is important to repeat these experiments using alternative methods of intracellular glutathione depletion, such as buthionine sulphoximine or phorone *(Meister and Anderson, 1983)*. The re-addition of glutathione by intracellular dialysis to cells previously depleted of glutathione would be of interest to investigate any reversibility of the effects of diethylmaleate.

The preliminary results obtained with isolated ventricular myocytes reflect the experimental observations using isolated Na/K ATPase and whole hearts with regard to the effect of the exogenous application of reduced and oxidised glutathione and may explain how an ischaemically-induced reduction in the ratio of GSH/GSSG results in contracture.

In globally ischaemic hearts extracellular potassium concentration increases in a biphasic manner with a brief plateau phase 5 minutes after the onset of ischaemia and rising further to appoximately 20-30mM within 30 minutes (*Knopf et al., 1990*). Inhibition of Na/K ATPase with cardiac glycosides abolishes this plateau phase (*Weiss and Shine, 1982*). It has been proposed that this phase is due to the stimulation of Na/K ATPase by catecholamines that are released from the ischaemic myocardium in concentrations that may maximally activate the Na-pump (*Schömig et al., 1987; Knopf et al., 1990*). Na/K ATPase is also stimulated by a decrease in resting membrane potential (*Gadsby et al., 1985*) and by an increase in intracellular sodium (*Glitsch et al., 1981*). Therefore, there are several mechanisms that may be responsible for this brief activation of Na/K ATPase activity during ischaemia: (i). noradrenaline release, (ii). increase in intracellular [Na^+] and/or [H^+] and (iii). membrane depolarisation due to an increase in extracellular [K^+]. In view of this, reduced glutathione may increase Na/K ATPase activity by stimulating catecholamine release under resting and/or ischaemic conditions. However, in view of the potentially pro-arrhythmic effect of catecholamines under ischaemic conditions (*Pallandi et al., 1987; Daugherty et al., 1986*) and the antiarrhythmic action of GSH (*Woodward and Zakaria, 1985*), this seems unlikely. In experiments described earlier it was also shown that GSH attenuated ^{86}Rb efflux and, by inference, K^+ efflux during ischaemia and reperfusion which suggests an alternative mode of action in this model by which GSH influences Na/K ATPase activity. The second phase of an ischaemically-induced rise in extracellular [K^+] is thought to represent irreversible damage to ischaemic myocytes (*Kleber, 1984*). Fiolet *et al.* (1984) suggested that as this second phase of the increase in extracellular potassium coincides with a decline in cellular ATP and that Na/K ATPase activity is markedly reduced after 30 minutes of ischaemia (*Bersohn et al., 1982*), a fall in ATP availability may contribute to this rise in extracellular potassium. The data obtained

from the investigations described here have shown that GSH stimulates isolated Na/K ATPase and prevents an ischaemically-induced loss of myocardial Na/K ATPase activity. However, no studies of cellular ATP levels, in the presence and absence of GSH, during an ischaemic insult in isolated hearts were undertaken. It remains unclear whether cellular ATP levels and/or changes in specific ATP pools are important in the loss of Na/K ATPase associated with myocardial ischaemia and reperfusion. Reduced glutathione may attenuate this rise in extracellular potassium during ischaemia via an ATP-sparing action, or by stimulating ATP synthesis, in addition to a stimulatory action on Na/K ATPase. Xie *et al.* (1990) found that free radical-induced oxidative stress reduced Na/K ATPase activity prior to any significant reduction in cellular ATP content and loss of cell viability. In addition, Jennings *et al.* (1985) showed that myocardial reperfusion, following an ischaemic period, resulted in a recovery of tissue ATP but also an enhanced decrease in Na/K ATPase activity. Therefore, it seems unlikely that a fall in cellular ATP content is responsible for any significant loss of Na/K ATPase activity during ischaemia and reperfusion.

Cysteine, dithiothreitol, penicillamine and captopril all have a free sulphydryl group and, in common with reduced glutathione, they all significantly increased the activity of isolated bovine Na/K ATPase in the present investigations. Relatively high concentrations of captopril (0.37mM) have been shown to attenuate reperfusion-induced arrhythmias (*Van Gilst et al., 1986a*) and this may be related to its ability to potentiate and attenuate reperfusion-induced prostacyclin and catecholamine release, respectively (*Van Gilst et al., 1986*b). A stimulatory action on Na/K ATPase reported in the investigations described here, however, may also contribute to the antiarrhythmic action of captopril during reperfusion. However, at plasma concentrations well above those found therapeutically (approximately 100μM), captopril has no effect on the action potential in guinea-pig ventricular or S-A node cells (*Helmsworth, 1989*). Stimulation of an electrogenic Na/K pump would be expected to cause hyperpolarization and therefore it appears unlikely that captopril stimulates the Na/K ATPase at concentrations of approximately 100μM. However, there is no data on the electrophysiologcal effects of captopril during reperfusion and yet it is during this phase when oxidant stress is at its greatest, indicated by a fall in the GSH/GSSG ratio (*Ferrari et al., 1989*). If the fall in this ratio of reductants and oxidants inhibits Na/K ATPase during reperfusion then the

possibility remains that captopril may stimulate the inhibited enzyme during reperfusion but have no effect on the unstressed enzyme.

The role of cellular redox state in the control of systems involved in ion translocation *in vivo* is unclear. It would seem likely that if changes in redox state have a potentially profound effect on intracellular ion homeostasis that the processes controlling redox state would in turn be subject to tight regulation. In some respects this is true. Oxidised glutathione produced under oxidative stress is transported out of the cell where it is metabolized. In addition glutathione reductase re-synthesises reduced gluathione intracellularly from GSSG and NADPH, to maintain a low intracellular GSSG concentration. It would be logical to assume that the enzymes involved in glutathione homeostasis are liable to activation/ inactivation by reductant and/or oxidant stress. Both intra- and intermolecular disulphide bridges are critical in the regulation of glutathione reductase (*Brigelius, 1985*; *Zeigler, 1985; Miller et al., 1990*). Therefore, it is possible that the activity of this enzyme may be modulated during oxidative stress to cope with an increased intracellular GSSG concentration. The GSSG transporter mechanism may also be activated under severe or prolonged oxidative stress during which time glutathione reductase may be inactivated.

The relationship between myocardial ischaemia and a reduction in the ability of the heart to maintain ionic homeostasis is a complex situation. There are several alterations in the status of the myocardium that may have both a direct and indirect effect on the function of ion translocating systems, such as the Na/K ATPase. It is fairly certain that free radical-induced oxidative stress has a direct effect on such proteins. However, the potential to modulate the activity of these systems via pharmacological interventions that alter the cellular redox state is unclear. The selectivity of such an approach is a problem in view of the contrasting effects of sulphydryl oxidation and reduction on the activity of varying enzymes and ion translocating systems, as outlined above. Potential approaches may be to prophylactically enhance the hearts antioxidant capacity or inhibit major sources of oxidant stress.

CHAPTER 7.

REDUCED GLUTATHIONE AND RELATED COMPOUNDS ON FREE CYTOSOLIC CALCIUM IN ISOLATED RAT VENTRICULAR MYOCYTES AND RABBIT WASHED PLATELETS.

7.1 FREE CYTOSOLIC CALCIUM LEVELS IN ISOLATED RAT MYOCYTES AND PLATELETS IN RESPONSE TO SELECTED PHARMACOLOGICAL AGENTS.

7.1.1 Myocytes.

7.1.1.1 Control responses.

Under extracellular calcium-free conditions, basal free cytosolic calcium in isolated rat myocytes was 147.5±13.1nM (n=4). Upon adjustment of the external calcium concentration to 1mM, free cytosolic calcium rose slightly and plateaued at 196.2±16.2nM (n=4) (figure 43). Following equilibration for 2 minutes, in the presence of 1mM extracellular calcium, the effect of various pharmacological agents on $[Ca]_i$ was investigated.

7.1.1.2 Effect of reduced glutathione on extracellular calcium ($[Ca]_o$)-induced increase in free intracellular calcium ($[Ca]_i$) in isolated rat myocytes.

In the absence of added extracellular calcium, the addition of reduced glutathione (final concentration of1.0mM) had no significant effect on free cytosolic calcium levels (figure 42). The subsequent addition of calcium (final concentration 1mM) resulted in no significant change in $[Ca]_i$. In a separate group of experiments, the addition of extracellular calcium (final concentration 1mM) resulted in a rise in $[Ca]_i$. The subsequent addition of reduced glutathione (final concentration1.0mM) resulted in a rapid and significant fall in $[Ca]_i$ from 150±23nM (n=3) to 55±21nM (n=4) (figure 43).

7.1.1.3 Effect of oxidised glutathione (GSSG) on free cytosolic calcium in rat myocytes.

The effect of the extracellular addition of oxidised glutathione (GSSG) on $[Ca]_i$ in rat myocytes is shown in figure 44. GSSG significantly increased $[Ca]_i$ at a final concentration of 0.75-1.0mM. This effect was partly attenuated by the subsequent addition of reduced glutathione (final concentration1.0 mM).

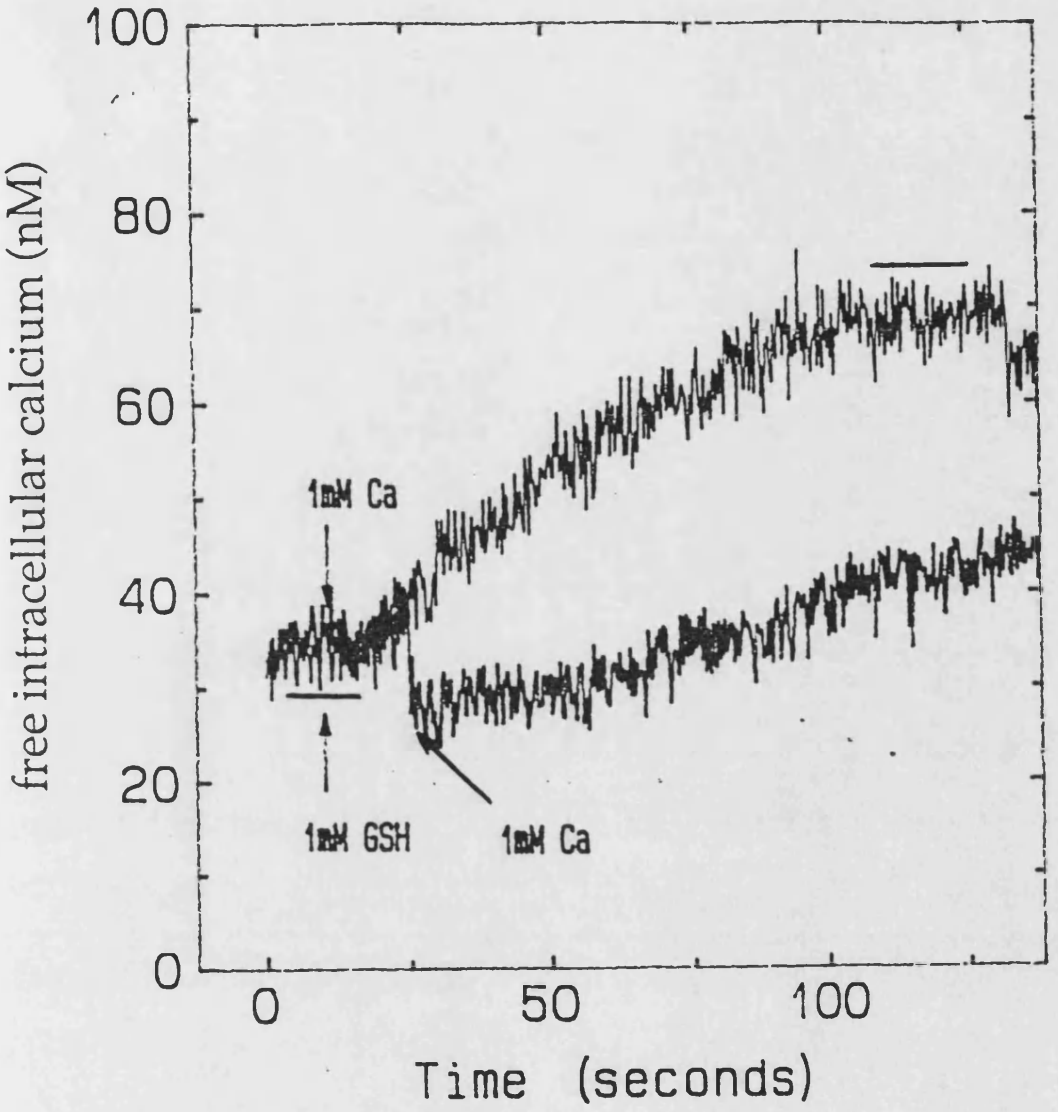


Figure 42. Typical trace of the effect of reduced glutathione (GSH) (1mM) on an increase in free intracellular calcium associated with adjusting extracellular calcium to 1mM (final concentration) in a suspension of isolated rat ventricular myocytes. The reduction in [Ca_i] following the addition of 1mM calcium in the cells pre-treated with 0.5mM GSH may represent inadequate stirring or an increase in intracellular calcium sequestration rendering it fura-2 insensitive. The traces overlap and cross over as indicated.

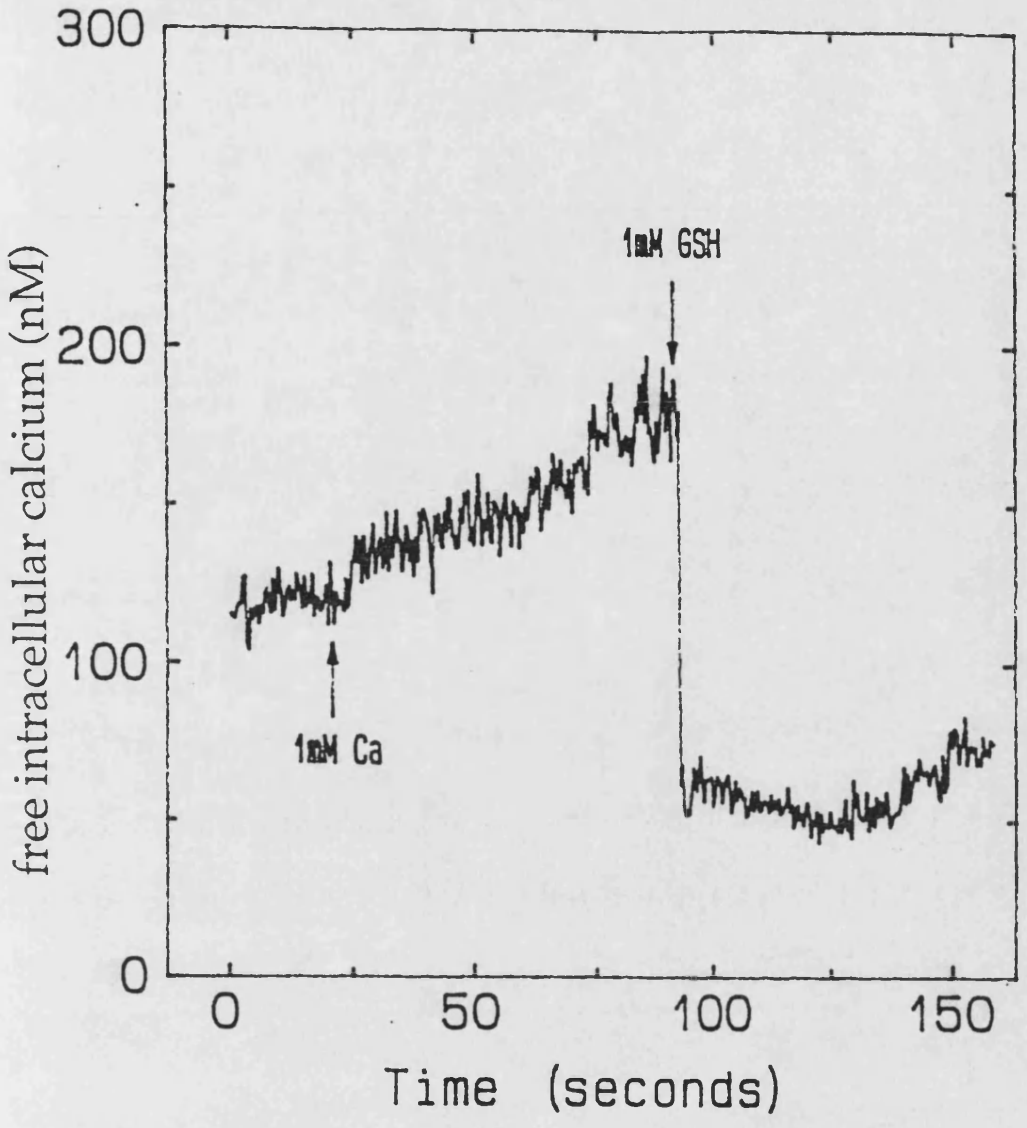


Figure 43. Typical trace of the effect of reduced glutathione (GSH) (0.5mM) on free intracellular calcium in a suspension of isolated rat ventricular myocytes. Extracellular calcium was increased to 1mM at the point indicated.

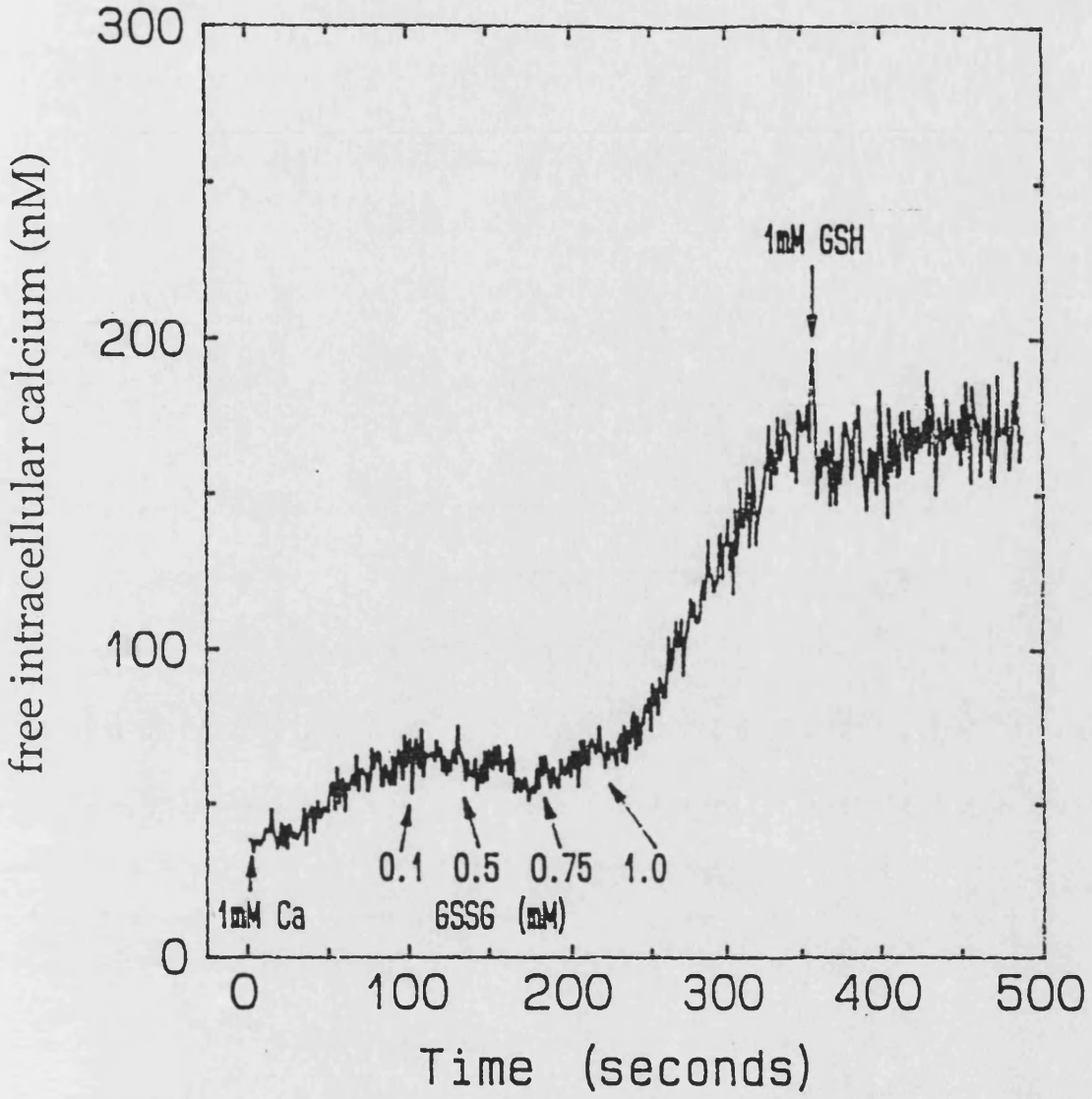


Figure 44. Typical trace of the effect of oxidised glutathione (GSSG) (0.1-1.0mM) and the subsequent addition of reduced glutathione (GSH) (0.5mM) on free intracellular calcium in a suspension of isolated rat ventricular myocytes.

7.1.1.4 Effect of U44619, GR 32191B and reduced glutathione on free cytosolic calcium in rat myocytes.

The potential role of thromboxane A_2 in changes in $[Ca]_i$, that may be modulated by reduced glutathione during ischaemia/oxidative stress, was investigated by the use of a thromboxane A_2 mimetic, U44619.

The addition U44619 (1μM final concentration), to rat myocytes in the presence of 1mM extracellular calcium, resulted in a sustained rise in $[Ca]_i$ of 115±55nM (n=3) from a resting value of 140±30nM (n=3) (figure 45). In a separate group of cells, the addition of a selective thromboxane A_2 receptor antagonist, GR 32191B (10μM final concentration), resulted in no significant change in $[Ca]_i$. There was a small, significant rise in $[Ca]_i$ of 15±6nM (n=3) in response to the subsequent addition of U44619 (1μM final concentration); this rise was significantly less than the rise in $[Ca]_i$ in response to 1μM U44619 alone confirming its effect is thromboxane A_2-receptor mediated. The increase in $[Ca]_i$ in the myocytes exposed to 1μM U44619 alone was reversed and sustained by the addition of reduced glutathione (final concentration 0.5mM). From a resting concentration of 255±30nM (n=5), $[Ca]_i$ fell to 92.1±14nM (n=5) following the addition of GSH (final concentration 0.5mM); 0.5mM GSH slightly but significantly reduced $[Ca]_i$ in the cells exposed to both 10μM GR 32191B and 1μM U44619 (figure 45).

7.1.2 Platelets.

In addition to the experiments with isolated rat myocytes, a similar group of experiments were performed using isolated rabbit platelets.

7.1.2.1 Effect of oxidised and reduced glutathione.

The addition of exogenous calcium to the cell suspension resulted in a sustained rise in intracellular calcium, reaching a plateau of 115±25 nM (n=3) within 2 minutes (figure 48). The subsequent addition of reduced glutathione (1mM) resulted in a rapid, significant and sustained fall in $[Ca]_i$ plateauing at 60±18 nM (figure 48). In a separate group of cells, the addition of oxidised glutathione (0.1-1.0mM) resulted in a rise in $[Ca]_i$ plateauing at 190±20 nM (figure 49).

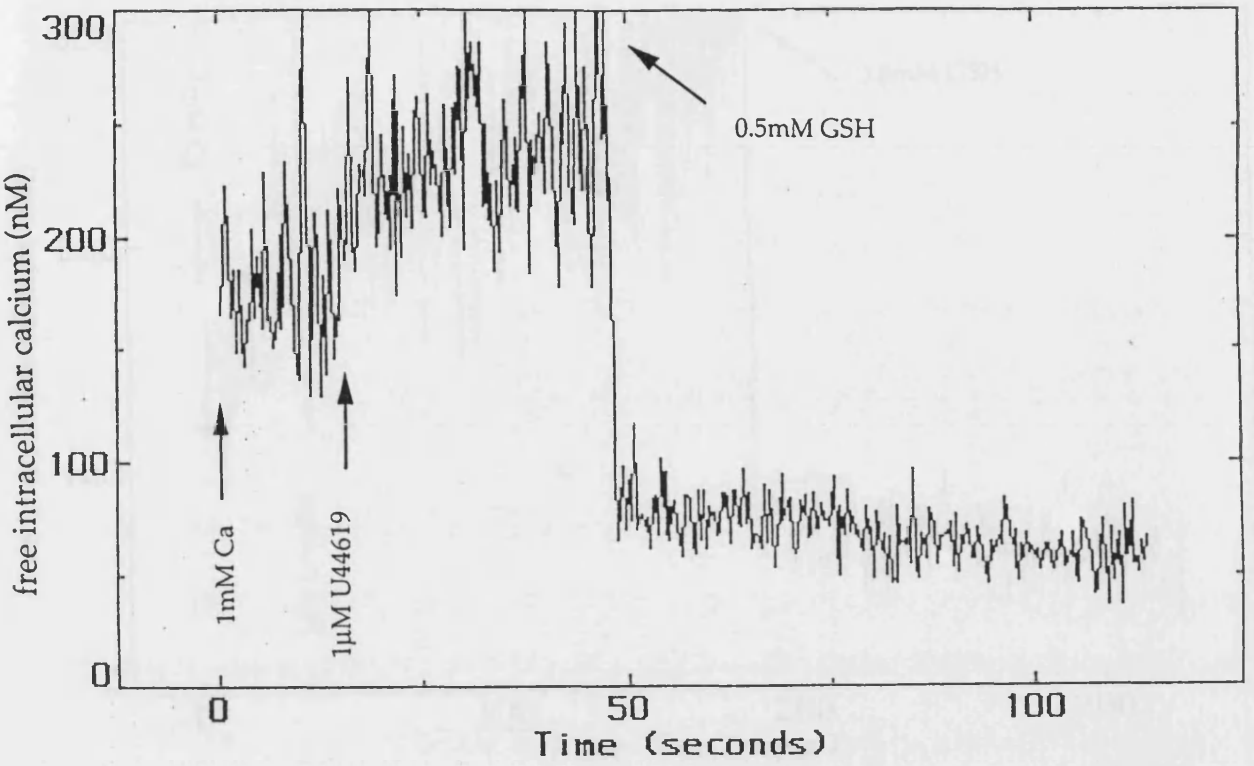


Figure 45. Typical traces of the effect of reduced glutathione (GSH) (0.5mM) on a rise in free intracellular calcium associated with the addition of the thromboxane A_2 mimetic, U44619 (1µM) to a suspension of isolated rat ventricular myocytes.

(Multiple copies of typical traces of this set of experiments are shown at the request of the examiners).

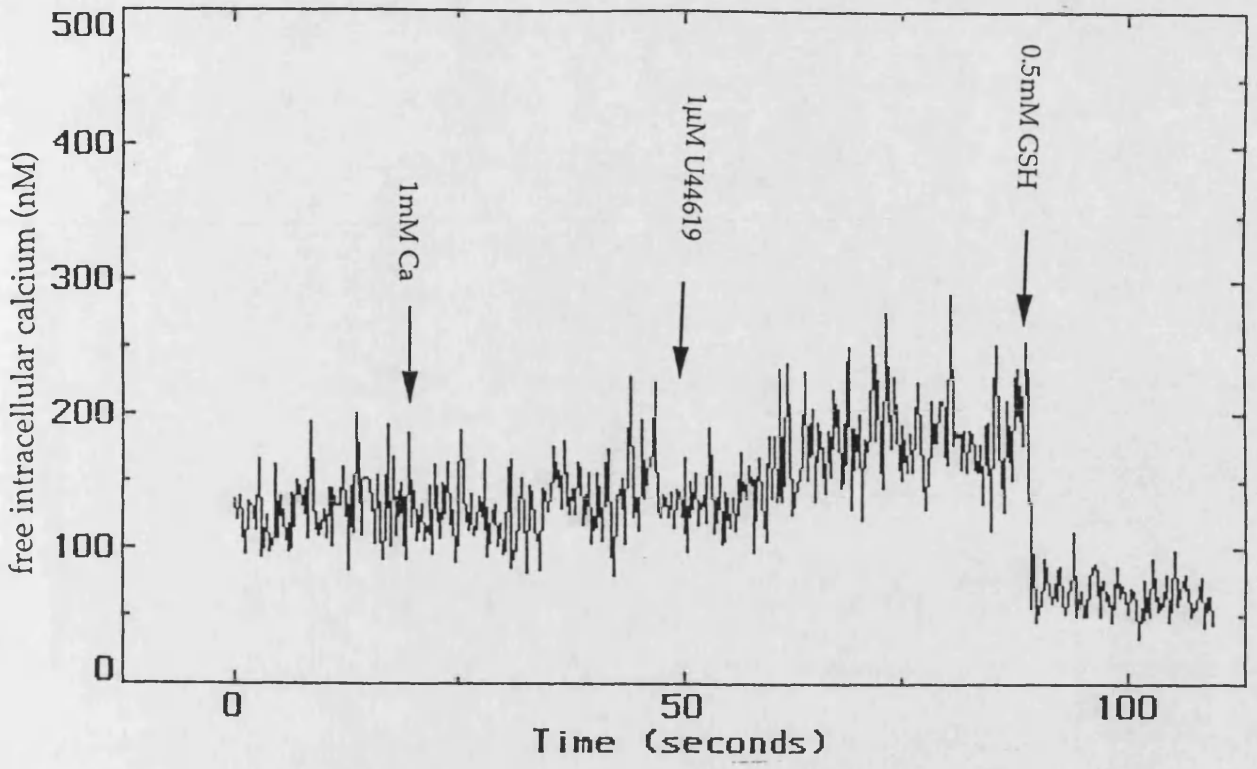


Figure 45. Typical traces of the effect of reduced glutathione (GSH) (0.5mM) on a rise in free intracellular calcium associated with the addition of the thromboxane A_2 mimetic, U44619 (1µM) to a suspension of isolated rat ventricular myocytes.

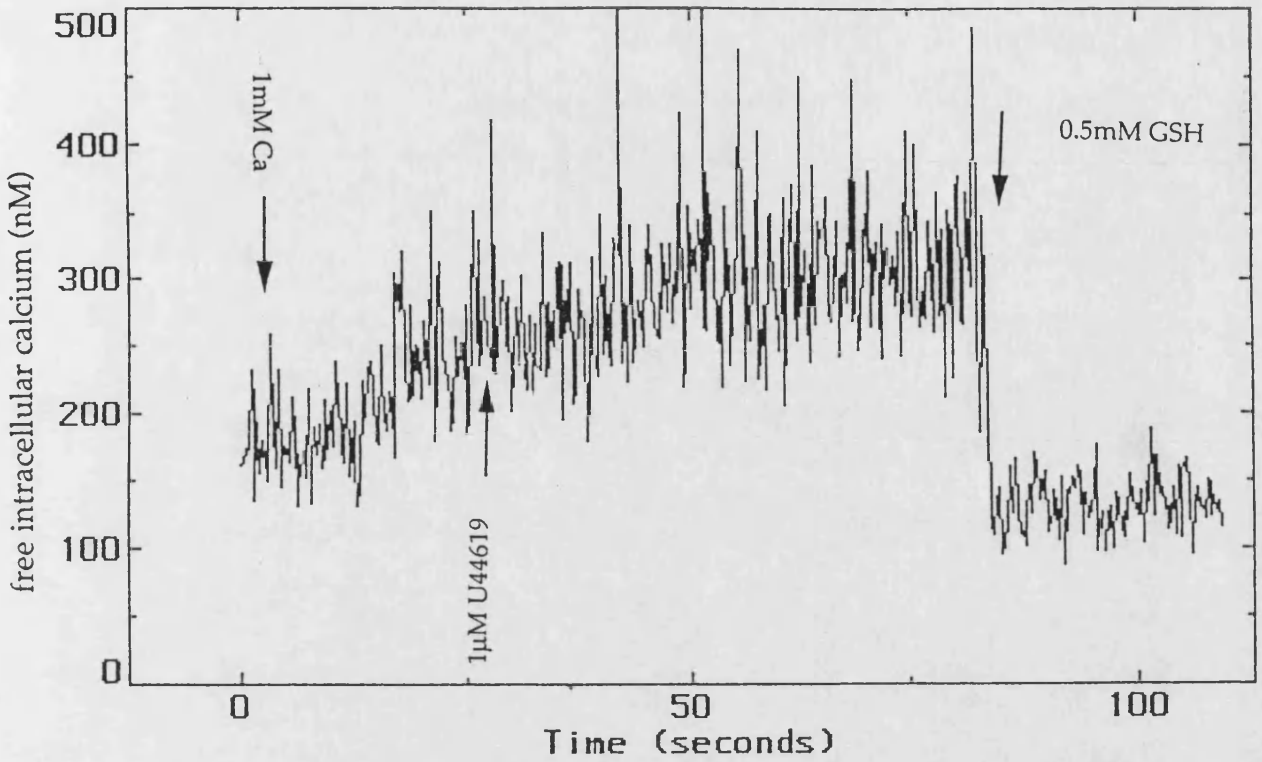


Figure 45. Typical traces of the effect of reduced glutathione (GSH) (0.5mM) on a rise in free intracellular calcium associated with the addition of the thromboxane A_2 mimetic, U44619 (1µM) to a suspension of isolated rat ventricular myocytes.

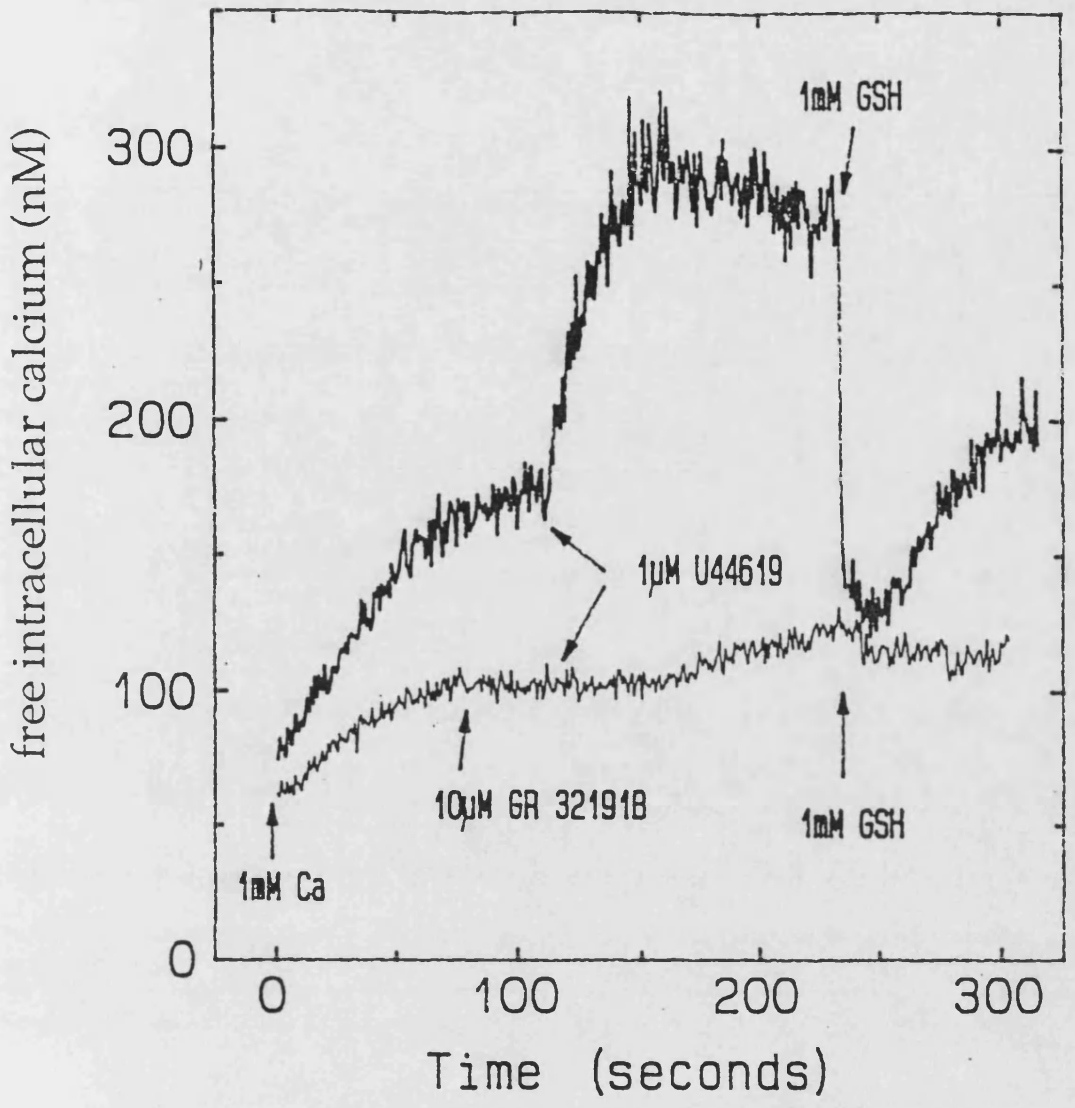


Figure 45. Typical traces of the effect of reduced glutathione (GSH) (0.5mM) on a rise in free intracellular calcium associated with the addition of the thromboxane A_2 mimetic, U44619 (1μM) and on the effect of GR 32191B (10μM) to a suspension of isolated rat ventricular myocytes.

7.1.2.2 Effect of U44619 and GR 32191B.

Following equilibration of washed platelets with extracellular calcium (final concentration 1mM), the addition of U44619 (final concentation 1μM) resulted in a sustained rise in $[Ca]_i$ of 575±190M (n=4) from a resting value of 37.5±4.7nM (figure 50). The subsequent addition of reduced glutathione (final concentration 0.5mM) resulted in a rapid and significant fall in $[Ca]_i$ to 35±7.9nM (n=5). In another group of cells left to equilibrate with extracellular calcium (final concentration 1mM), the addition of GR 32191B (final concentration 10μM) resulted in no significant change in $[Ca]_i$. The subsequent addition of U44619 (final concentration 1μM) resulted in a significantly depressed increase in $[Ca]_i$ compared to the cells exposed to the thromboxane A_2 mimetic alone. The subsequent addition of GSH (final concentration 0.5mM) resulted in a small but insignificant change of $[Ca]_i$ (figure 50).

The results obtained with both these cell types in response to selected pharmacological agents are both quantitatively and qualitatively similar, possibly indicating common methods by which free intracellular calcium is regulated and modulated by thiol compounds.

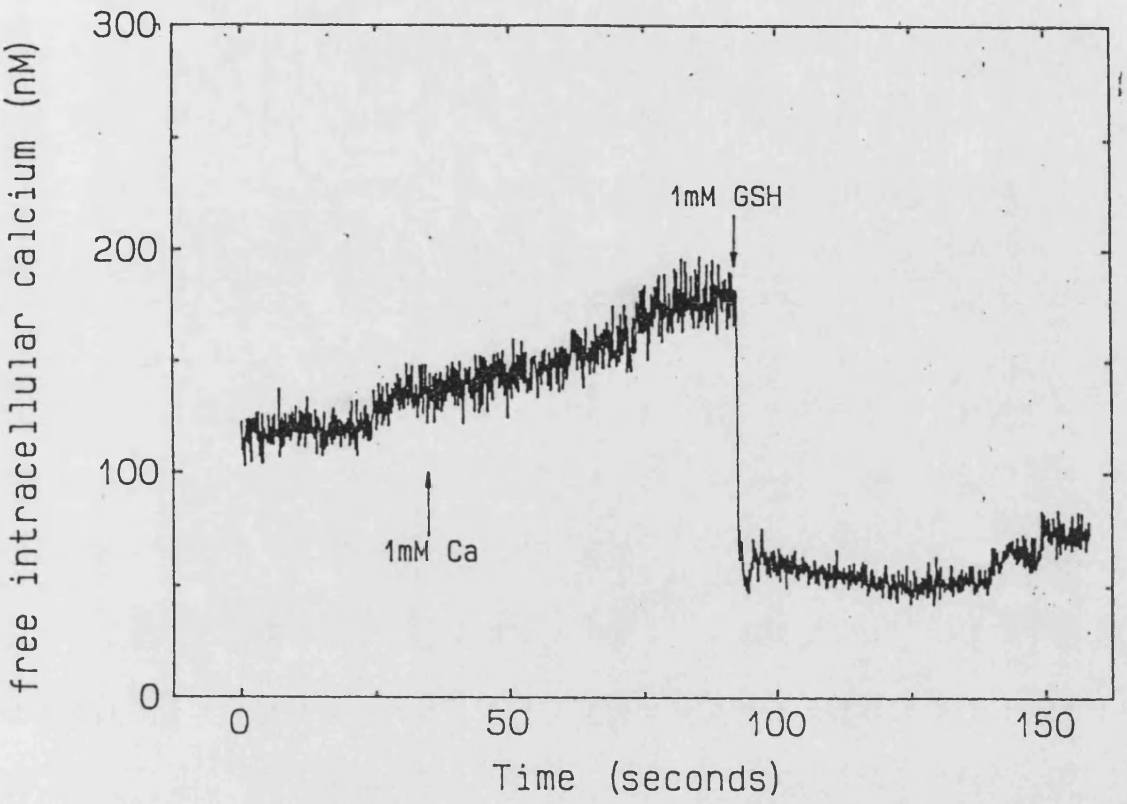


Figure 48. Effect of reduced glutathione (GSH) (1mM) on free intracellular calcium in isolated rabbit washed platelets. Extracellular calcium was increased to 1mM at the point indicated.

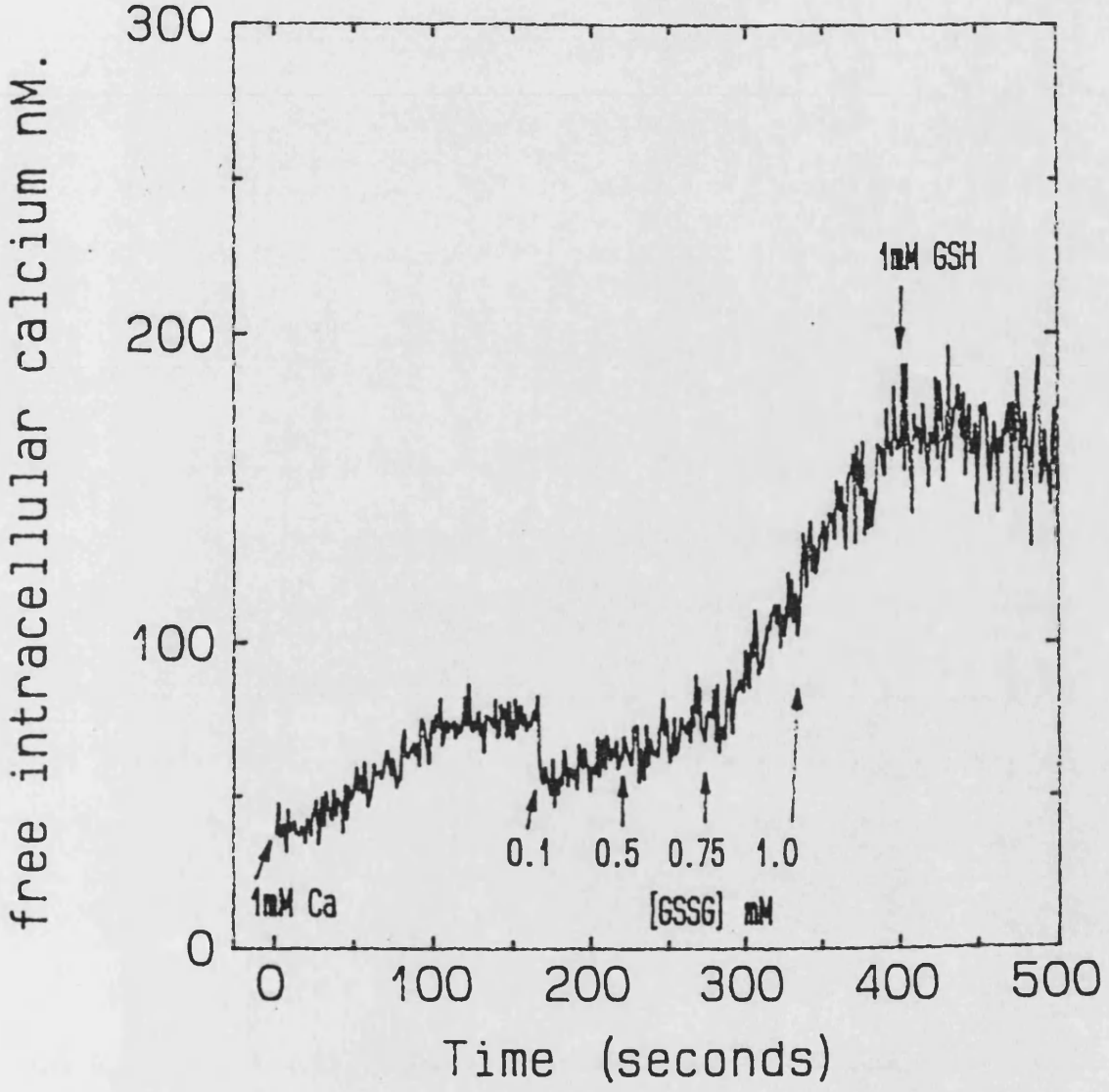


Figure 49. Effect of oxidised glutathione (GSSG) (0.1-1.0mM) on free intracellular calcium in isolated rabbit washed platelets. Extracellular calcium was increased to 1mM at the point indicated. Reduced glutathione (GSH) (1mM) was also added at the point indicated.

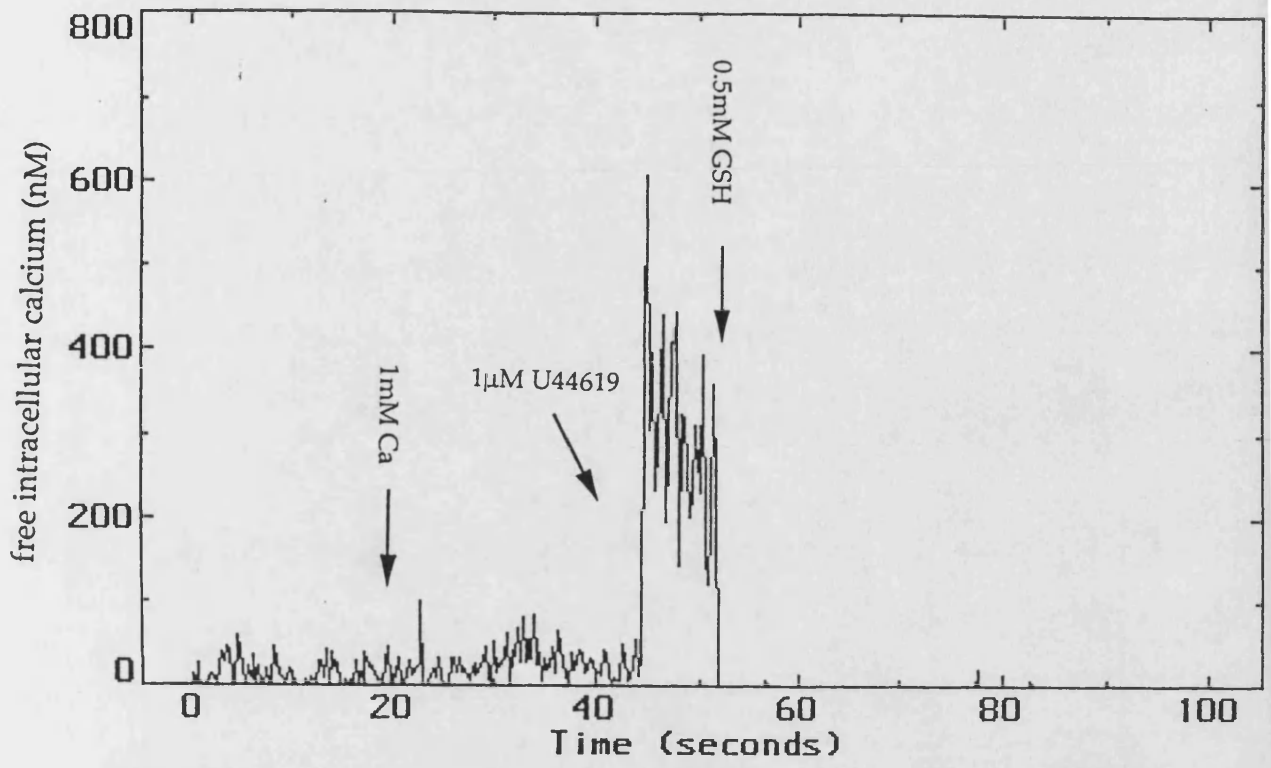


Figure 50. Typical trace of the effect of reduced glutathione (GSH) (0.5mM) on a rise in free intracellular calcium associated with the addition of the thromboxane A_2 mimetic, U44619 (1μM) to a suspension of isolated rabbit washed platelets.

(Multiple copies of typical traces of this set of experiments are shown at the request of the examiners).

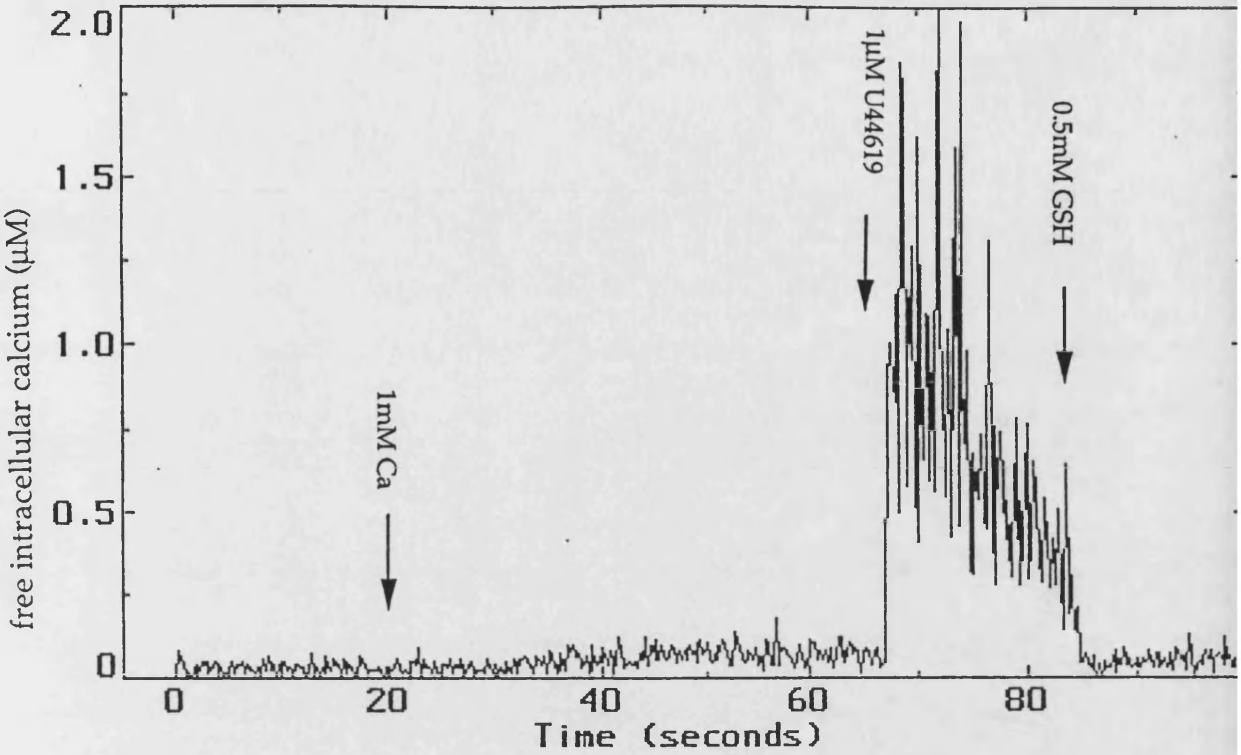


Figure 50. Typical trace of the effect of reduced glutathione (GSH) (0.5mM) on a rise in free intracellular calcium associated with the addition of the thromboxane A_2 mimetic, U44619 (1μM) to a suspension of isolated rabbit washed platelets.

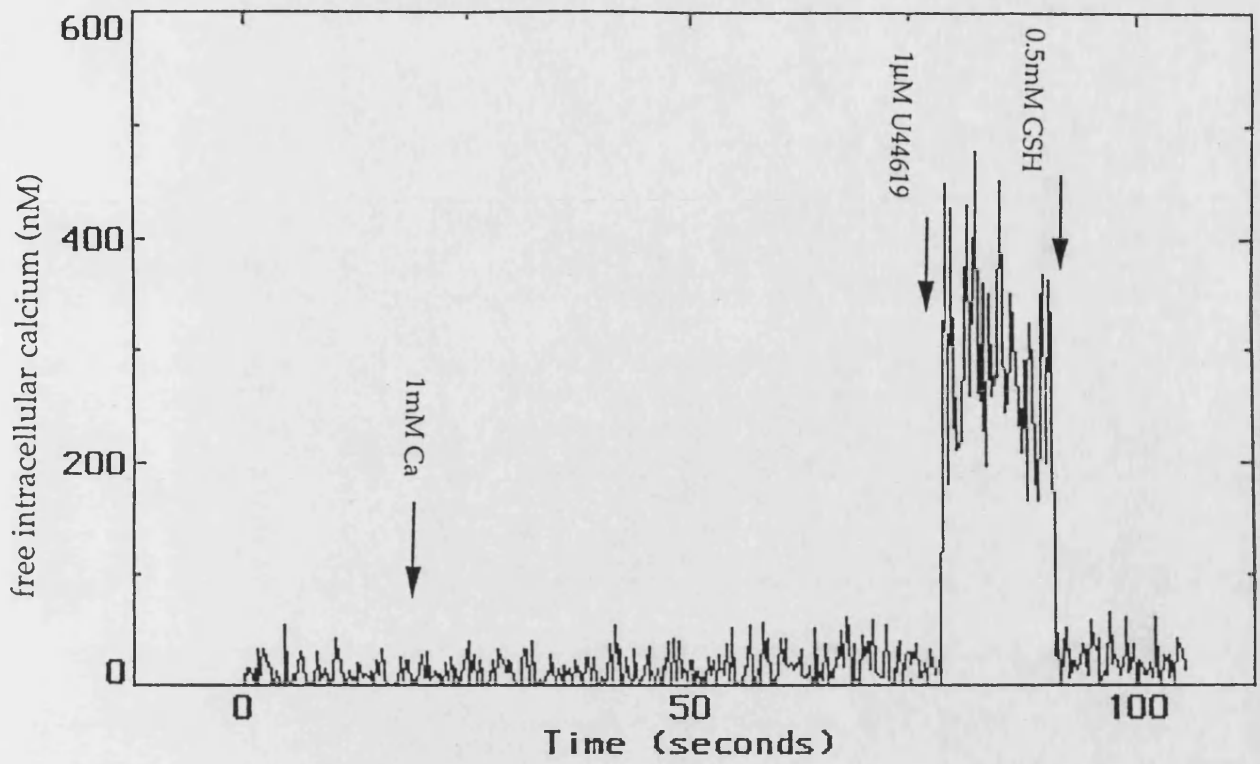
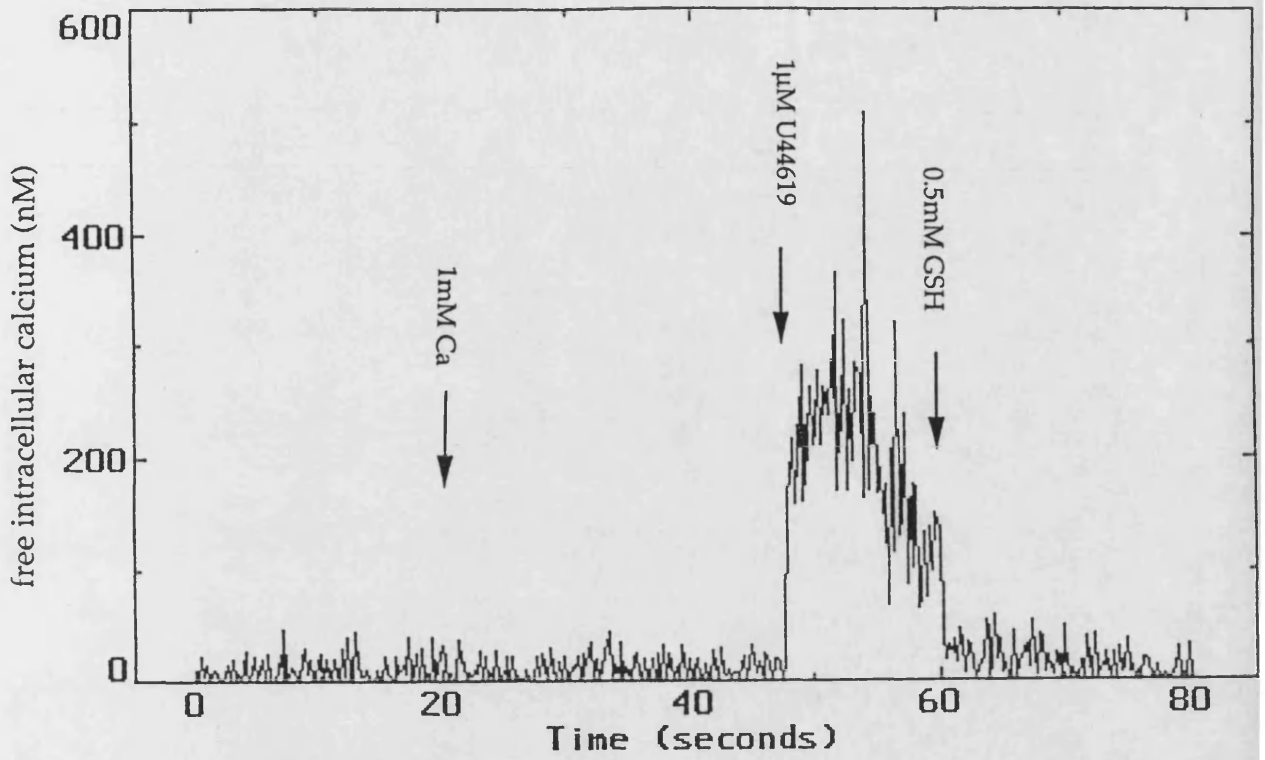


Figure 50. Typical trace of the effect of reduced glutathione (GSH) (0.5mM) on a rise in free intracellular calcium associated with the addition of the thromboxane A_2 mimetic, U44619 (1μM) to a suspension of isolated rabbit washed platelets.



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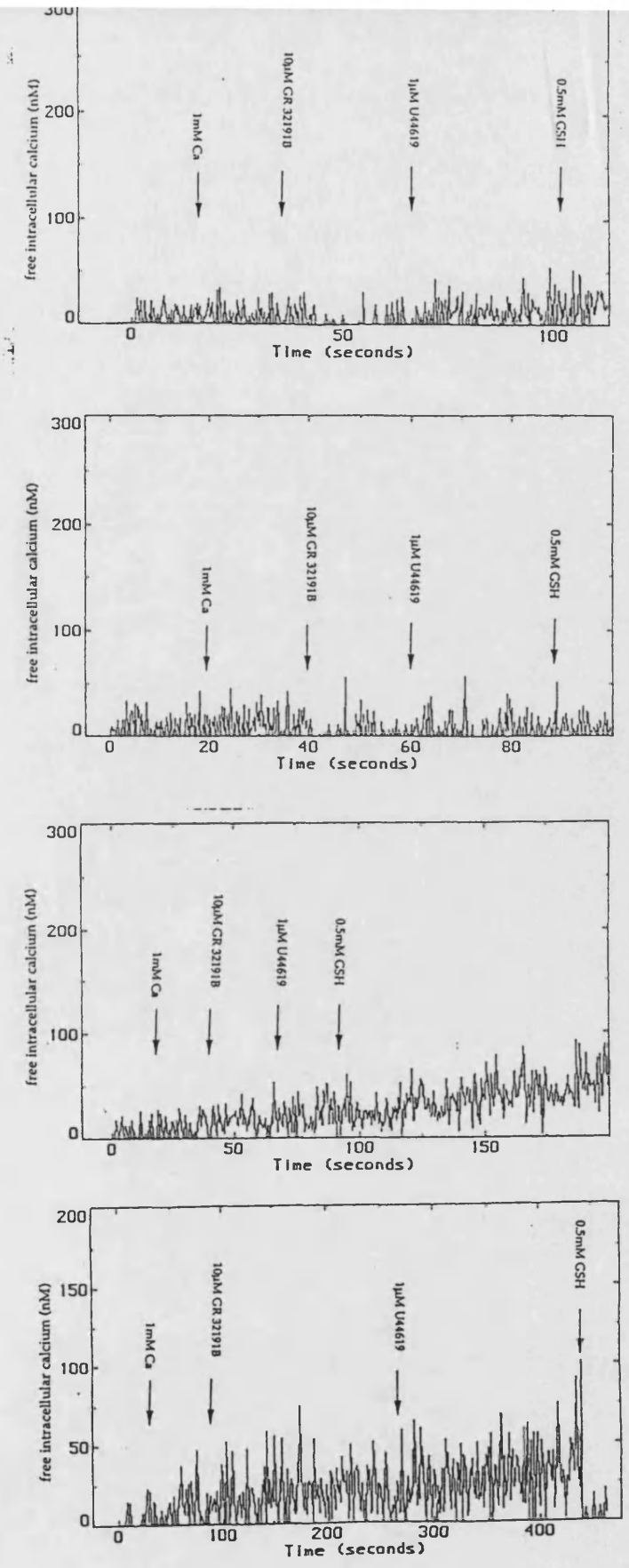

Figure 50a. Effect of GR 32191B (10μM), U44619 (1μM) and reduced glutathione (GSH) (0.5mM) on free intracellular calcium in isolated rabbit washed platelets.

CHAPTER 8.

DISCUSSION.

In the afore-mentioned experiments, reduced glutathione has been shown to delay the onset of an ischaemic contracture, reduce reperfusion-induced ^{86}rubidium efflux, attenuate the ischaemically-induced loss of Na/K ATPase activity in the rat heart, increase the activity of an isolated bovine Na/K ATPase preparation and increase Na-pump current in isolated rat ventricular myocytes. In this set of experiments, the change in free intracellular calcium concentration ($[Ca]_i$) in isolated rat ventricular myocytes was measured in response to reduced glutathione and related compounds using a fluorometric method and the calcium-sensitive probe, fura-2. The data from these experiments shows that GSH reduces and GSSG increases free intracellular calcium, respectively, adding weight to the hypothesis that the intracellular ratio of GSH/GSSG may be an important factor in the regulation of ion translocating systems involved in calcium homeostasis.

It is assumed that an ischaemically-induced contracture is a consequence of intracellular calcium overload (*Hearse et al., 1977*). This contracture is caused by an inadequate supply of ATP which cannot dissociate the actin-myosin cross bridges, or enable the restoration of basal cytosolic calcium levels to below the micromolar range (*Grossman and Barry, 1980*). An ischaemically-induced rise in intracellular calcium has been suggested to activate a variety of catabolic enzymes which may be partly responsible for cell injury under such conditions (*Katz and Reuter, 1979; Nayler et al., 1979; Poole-Wilson et al., 1984*). Several groups have measured free intracellular calcium in both isolated ischaemic tissues and cells using fluorescent calcium-sensitive dyes. A very rapid increase in systolic and diastolic $[Ca]_i$ following the onset of ischaemia has been shown in rabbit hearts loaded with the calcium-sensitive dye, indo-1 (*Lee et al., 1988*). In isolated myocytes, an increase in $[Ca]_i$ following metabolic inhibition preceded cell death (*Cobbold and Bourne, 1984*). However, there is evidence from this group that in an individual myocyte a rise in $[Ca]_i$ occurs after the cell exhibits extreme contracture.

In view of the stimulatory and inhibitory action of reduced and oxidised glutathione, respectively, on isolated bovine ventricular Na/K ATPase and the ability of reduced glutathione and other sulphydryl compounds to attenuate an ischaemic contracture in the isolated rat heart, the effect of these compounds on free cytosolic calcium in isolated rat myocytes was investigated. The effect of

sulphydryl blocking agents was also studied to assess the possible involvement of sulphydryl groups in intracellular calcium homeostasis. The exogenous application of reduced and oxidised glutathione, and other selected sulphydryl compounds, to a suspension of isolated myocytes may be analogous to the change in redox state observed during ischaemia (*Ferrari et al., 1989*) although other physiological parameters, such as intra- and/or extracellular [Na^+], [K^+] and [Ca^{2+}] are also altered in the pathological situation (*Knopf et al., 1990*). These experiments were undertaken in an attempt to extrapolate the effects of GSH and GSSG on an ischaemic contracture in the isolated rat heart and on the activity of bovine Na/K ATPase, to their effects on $[Ca]_i$ levels in rat myocytes. A stimulatory action of GSH on Na/K ATPase may indirectly reduce $[Ca]_i$ via an effect on Na/Ca exchange and this could be responsible for the ability of GSH to delay the onset of an ischaemically-induced contracture.

Under resting conditions, 1mM reduced glutathione significantly reduced $[Ca]_i$ in a suspension of isolated rat ventricular myocytes. In a separate group of cells in which the $[Ca]_i$ was artificially raised by the addition of a thromboxane A_2 mimetic U44619, (final concentration 30μM), 0.5mM GSH reduced $[Ca]_i$. GSH does not readily cross the cell membrane although its prolonged extracellular application may significantly increase intracellular GSH levels (Martensson et al, 1989) and in a suspension of isolated cells this effect may be potentiated. Therefore, during the time scale of these experiments, it would appear that GSH acts primarily at sites located on the extracellular surface but may ultimately influence the intracellular redox state/glutathione status by increasing intracellular GSH. There is no indication from these experiments as to whether GSH acts directly to sequest $[Ca]_i$ or whether it acts through a second messenger system. In contrast to the effects of GSH, GSSG increased $[Ca]_i$ in a concentration-dependent manner. GSSG would not be expected to enter the cell and, therefore, it would also appear to act primarily at extracellular sites. From the work of Ferrari *et al.* (1989), the concentration of GSSG in the coronary perfusate immediately after reperfusion is approximately 30nM. This is significantly lower than the concentrations of GSSG used in the experiments described here. However, there is the possibility that significantly higher concentrations of GSSG may occur in localized areas. The

concentration of GSSG in the perfusate is inevitably a mean of the concentration of GSSG in all localitites. 1mM reduced glutathione partially reversed the GSSG-induced increase in $[Ca]_i$. It is possible, therefore, that the reversible oxidation and reduction of disulphide bonds is responsible for the cells response to GSH and GSSG. If the Na/K pump is involved in calcium homeostasis in this experimental situation, GSH may reduce disulphides located extracellularly resulting in an increase in pump activity, leading to a net loss of calcium from the cell. The converse may be true for GSSG. However, the sulphydryl groups essential for Na-pump activity are located on the intracellular surface (*Rothstein, 1970*). This raises the possibility that GSH and GSSG affect the structure and function of the beta subunit of the Na-pump, thereby altering the insertion and activity of the catalytic alpha subunit. Alternatively, in the whole cell situation GSH and GSSG may affect the activity of other ion translocating systems involved in $[Ca]_i$ homeostasis such as the Na/Ca and Na/H exchange mechanisms. Although these experiments have only examined the extracellular application of GSH and GSSG, the data from the electrophysiological investigations of GSH and GSSG on Na-pump current described earlier in this thesis, show that this pump can be modulated by changes in intracellular redox state. Therefore, the intracellular presence of oxidants and reductants during ischaemia may modulate Na-pump activity and $[Ca]_i$. In a separate group of experiments, the effect of all the compounds used in this set of experiments, on the calcium binding of fura-2 was investigated. The free acid of fura-2 was used in these experiments. The fluorimeter was calibrated using 1mM calcium and excess EGTA. The fluorescence signal resulting from the binding of Ca^{2+} to fura-2 was monitored any any change following the addition of the compound in question determined. This data showed that neither GSH or GSSG, affected the calcium handling of fura-2, indicating that these compounds affect $[Ca]_i$ levels and not calcium/fura-2 binding.

The Na/Ca exchange mechanism may be involved in the rise of $[Ca]_i$ during ischaemia. After approximately 10 minutes of myocardial ischaemia there is a significant increase in $[Na]_i$ (*Pike et al., 1988*) which is secondary to an intracellular acidosis-stimulated increase in Na/H exchange. The time course of the

ischaemically-induced rise in $[Na]_i$ is similar to that of $[Ca]_i$ and therefore Na/Ca exchange may be responsible for an increase in $[Ca]_i$. If the Na/H exchange mechanism is blocked with amiloride or its derivatives, such as ethylisopropylamiloride (EIPA), the increase in $[Na]_i$ is prevented *(Cala et al., 1988)* as is the rise in $[Ca]_i$ *(Murphy et al., 1987)*. Interaction of GSH and GSSG with these proteins should be investigated via the use of amiloride and its derivatives to further elucidate the mechanism by which GSH and GSSG modulate $[Ca]_i$ under physiological conditions.

Unlike reduced glutathione, glutathione mono-isopropyl esters pass readily through the cell membrane where they are de-esterified. This causes an increase in intracellular glutathione concentration significantly above basal levels and this may represent an experimental approach by which the effect of $[GSH]_i$ on $[Ca]_i$ may be studied with more relevance to the *in vivo* situation (*Martensson et al., 1989*). There is the possibility that this type of compound and experimental strategy may be useful agents in the investigation of the relative contribution of cardioprotective systems against ischaemically-induced myocardial injury.

CHAPTER 9.

THE EFFECT OF ACIDOSIS ON ISOLATED VASCULAR PREPARATIONS

9.1 THE EFFECT OF ACIDOSIS IN ISOLATED VASCULAR PREPARATIONS.

9.1.1 THE CORONARY VASCULATURE.

In common with the Na/K pump, the Na/H exchange mechanisim is an intrinsic, membrane protein involved in intracellular ion homeostasis. In order to investigate whether GSH and other thiols could affect Na/H exchange, the physiological response of the heart to a change in pH and the modulation of the response by reduced glutathione and related compounds was investigated as this may be indicative of an effect on Na/H exchange. This was approached by perfusing the rat coronary vasculature with an acidotic perfusate and monitoring the physiological and mechanical responses.

9.1.1.1 Effects of acidosis in control hearts.

During myocardial ischaemia, intracellular pH is known to fall relatively rapidly to a minimum of approximately pH 6.8 (*Knopf et al., 1990*). For this reason a bicarbonate-buffered perfusate ($NaHCO_3$ final concentration 25.0mM) at pH 6.8 was used to induce acidosis in preliminary experiments. Figure 51 shows typical responses of cardiac contractility, perfusion pressure and heart rate in response to extracelluar acidosis. The reduction of extracellular pH to pH 6.8 resulted in a concomitant reduction in heart rate, developed tension and rise in perfusion pressure, that is indicative of coronary vasoconstriction in this model. In control hearts, perfusion pressure rose in response to acidosis at a rate of 23.6±0.7 mmHg/minute (n=6). The effects of acidosis on coronary tone and cardiac contractility were completely reversible by switching between perfusates buffered at pH 7.4 and 6.8, and this could be repeated at least 10 times in the same heart (data not shown).

9.1.1.2 Effect of a reduction in extracellular sodium concentration ($[Na]_o$) and the method of pH reduction of the Krebs-Henseleit solution on a pH-dependent vasoconstriction.

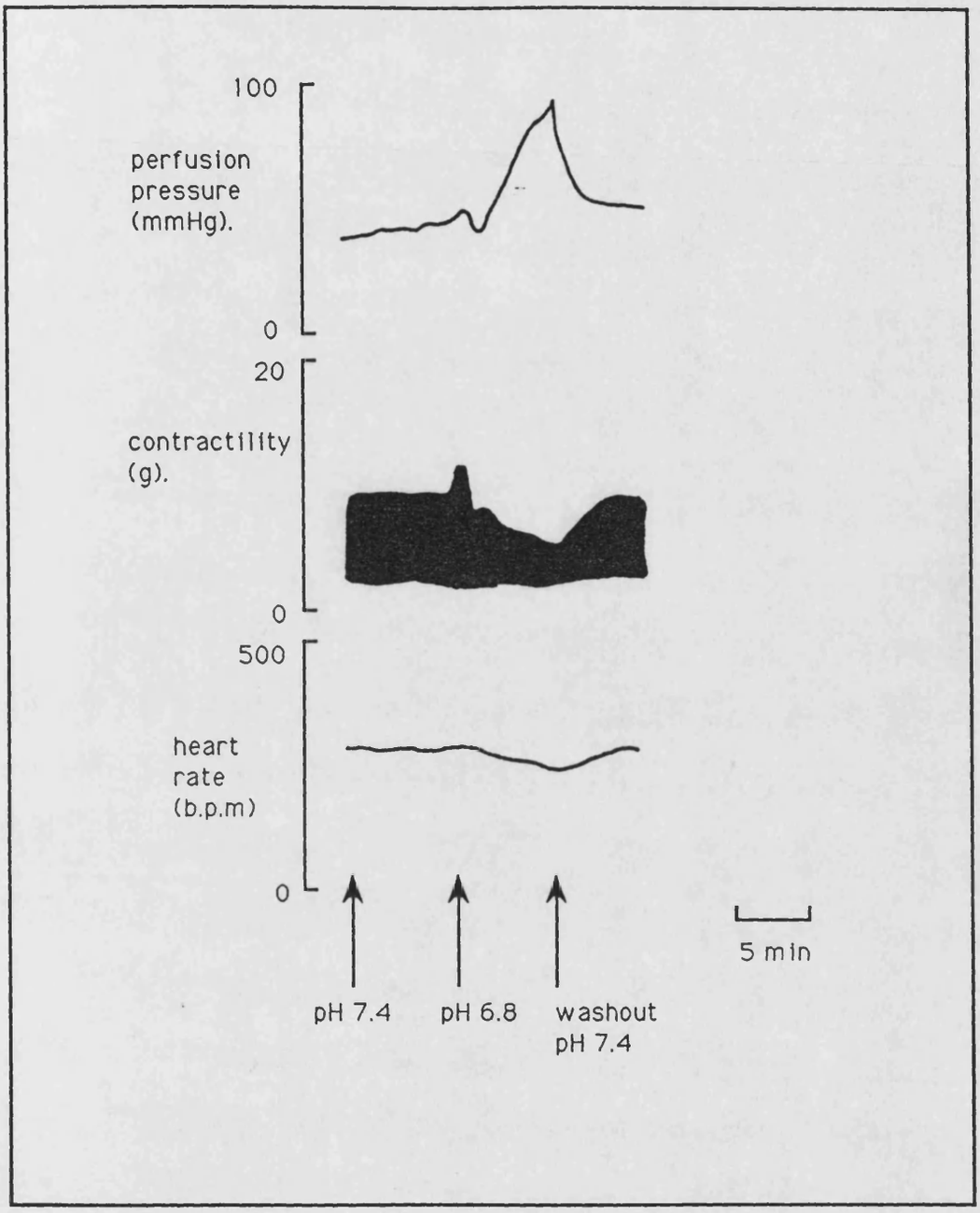

Figure 51. Typical trace of the effects of extracellular acidosis (pH 6.8) on coronary perfusion pressure, cardiac contractility and heart rate in the isolated perfused rat heart. The pH of the Krebs-Henseleit buffer is indicated by the arrows.

As described above, the pH of the Krebs-Henseleit solution was adjusted by lowering the $NaHCO_3$ concentration, which inevitably results in a reduction in sodium concentration (as well as bicarbonate concentration) which would be expected to alter Na/Ca exchange. Therefore, the effect of reducing the sodium concentration of the buffer on coronary tone, without affecting the HCO^-_3 concentration, was investigated. This was achieved by reducing the NaCl content to give the same reduction in sodium concentration ([Na]) as in the acidotic perfusate prepared by $NaHCO_3$ reduction. This represented a 5mM reduction in [Na]. The effect of reducing the [Na] was a slight but insignificant rise in cardiac contractility but no effect on coronary perfusion pressure (data not shown). It is unlikely that the decrease in bicarbonate concentration had any significant effect on ionised calcium concentration in the perfusate (*Schaer, 1974*).

To investigate the effect of the method of pH reduction of the Krebs-Henseleit buffer on the physiological response of the heart to acidosis, the pH of the solution was reduced to pH 6.8 by the addition of 1M HCl. There was no significant difference in the physiological response of hearts subjected to acidosis by perfusion with acidotic perfusate prepared by either of the two methods described, with respect to changes in perfusion pressure, cardiac contractility and heart rate (data not shown).

9.1.1.3 Effect of extracellular pH on coronary tone in the isolated perfused rat heart.

The relationship between coronary tone and extracellular pH was investigated by perfusion with Krebs-Henseleit solutions buffered at pH values betwen 7.4 and 6.8 and the rate of rise of perfusion pressure per minute recorded. Figure 52 shows that coronary tone was significantly increased, with respect to controls, at all pH values investigated. For the purposes of simplicity, cost and available time, all subsequent experiments were performed at pH 6.8.

9.1.1.4 Effect of adrenoceptor agonism.

To investigation any involvement of catecholamines, that are released in response

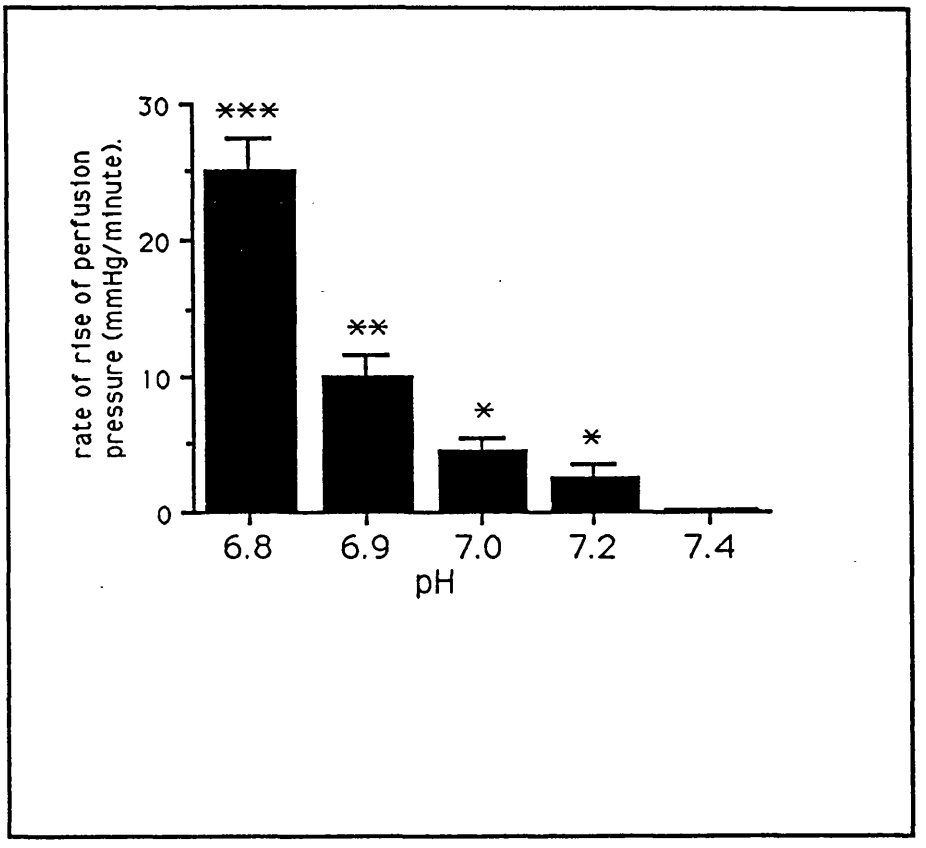

Figure 52. Effect of perfusate-pH (pH 6.8-7.4) on perfusion pressure in the isolated perfused rat heart. Vertical bars represent ±s.e.mean. (n=8-5 hearts/group, *p<0.05, **p<0.01, ***p<0.001 cw controls at pH 7.4.

to myocardial acidosis (*Dart and Riemersma, 1989*), in the progression of the pH dependent coronary vasoconstriction, alpha-adrenoceptors were blocked under acidotic conditions with 1μM phentolamine and the pressor response to acidosis monitored. Figure 53 shows the effect of the alpha-adrenoceptor antagonist, phentolamine, on the coronary vasoconstrictor response to perfusion with an acidotic perfusate at pH 6.8. At pH 7.4 and a concentration of 1μM, the perfusion of phentolamine resulted in a significant transient fall in perfusion pressure of 15.2±2.2 mmHg, that is indicative of coronary vasodilation. At pH 6.8, the same concentration of phentolamine significantly reduced the acidosis-induced coronary constriction which indicates the involvement of catecholamines or some other non-specific effect of phentolamine in the pressor response to acidosis (figure 53).

9.1.1.5 Effect of cyclooxygenase inhibitors.

The cyclooxygenase pathway is responsible for the synthesis of several vasoactive compounds (Figure 54) that may play a part in the pH-dependent vasoconstriction of the coronary vasculature in this model (thromboxane A_2, PGF_2 and several of the leukotrienes cause coronary constriction, while prostacyclin causes coronary dilation). To investigate this hypothesis, the effect of inhibiting this pathway with several selective cyclooxygenase inhibitors on the hearts physiological response to acidosis was studied. All compounds were perfused at previously determined concentrations that give complete cyclooxygenase inhibition (Flower, 1974).

10μM flurbiprofen perfused at pH 7.4 resulted in a transient and significant fall in perfusion pressure of 24.5±5.2 mmHg, from a resting pressure of 42.5±5.5 mmHg, that is indicative of coronary vasodilation in this model. The subsequent perfusion of the same concentration of flurbiprofen at pH 6.8 resulted in a significant reduction in the rate of rise of coronary perfusion pressure associated with acidosis. Under these conditions perfusion pressure rose at 12.3±2.8 mmHg/minute compared to the response in control hearts (in the absence of flurbiprofen), in which perfusion pressure rose at 26.2±2.1 mmHg/minute (figure 55).

A comparison was made of the effects of flurbiprofen with another cyclooxygenase inhibitor, indomethacin, under acidotic conditions. 10μg/ml indomethacin perfused

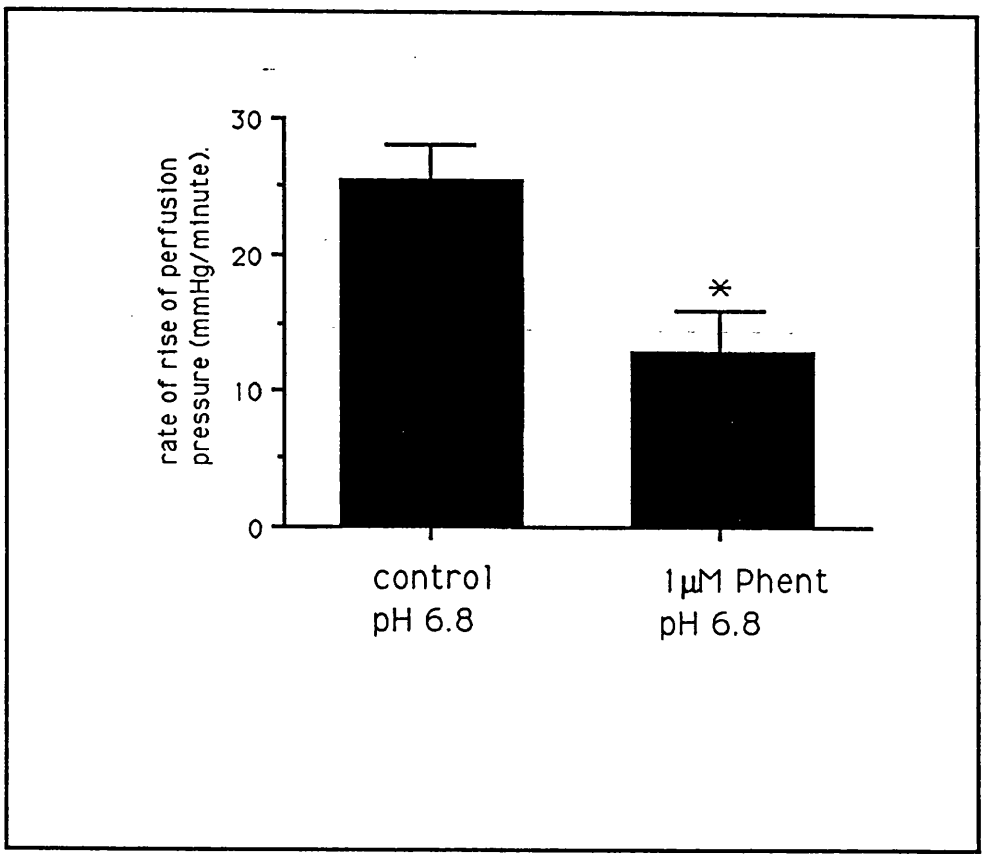

Figure 53. Effect of phentolamine (phent) (1μM) on a pH-dependent coronary constriction in the isolated, perfused rat heart. Vertical bars represent ±s.e.mean. (n=4, *p<0.05)cw controls.

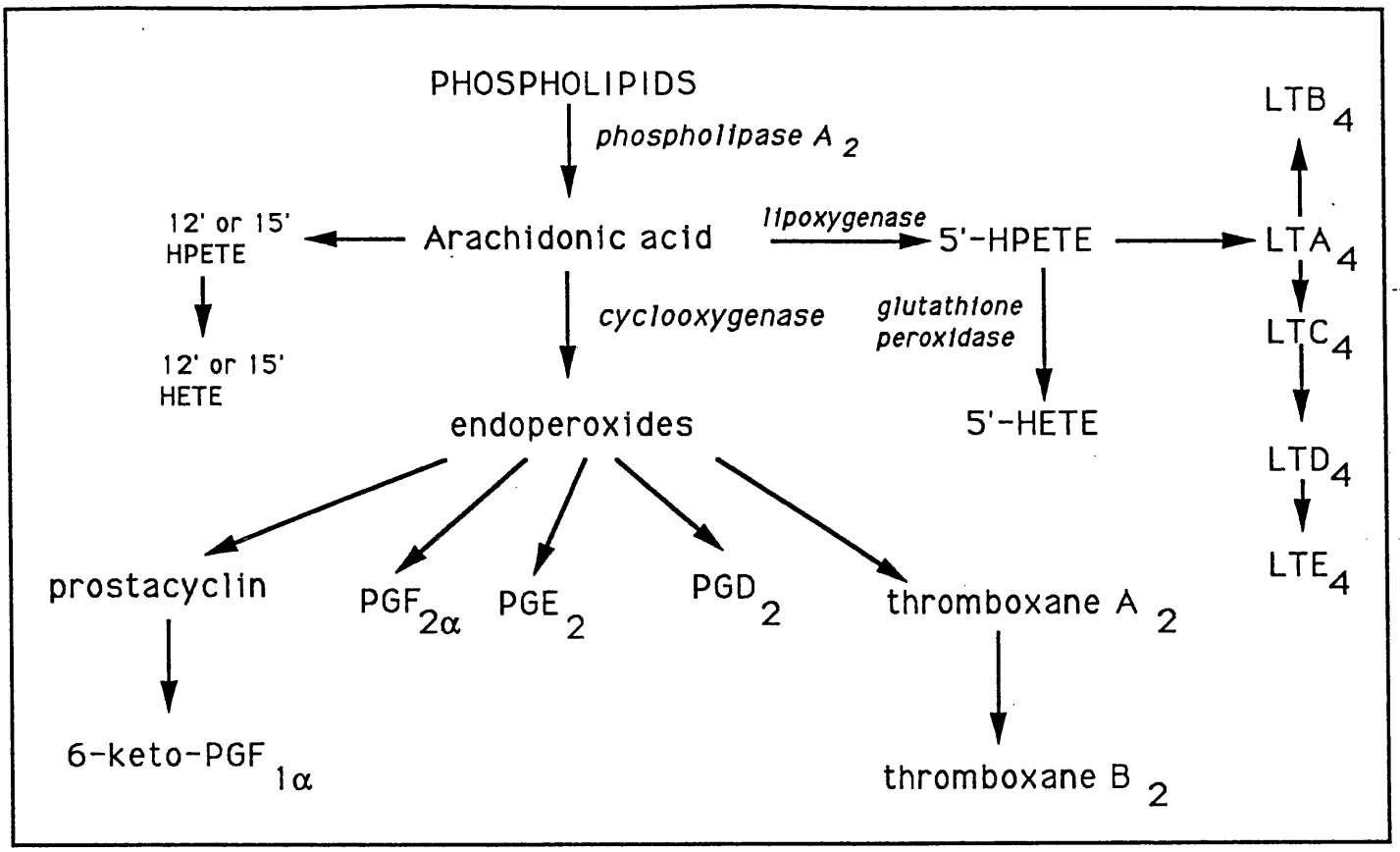

Figure 54. Schematic representation of myocardial arachidonic acid metabolism.

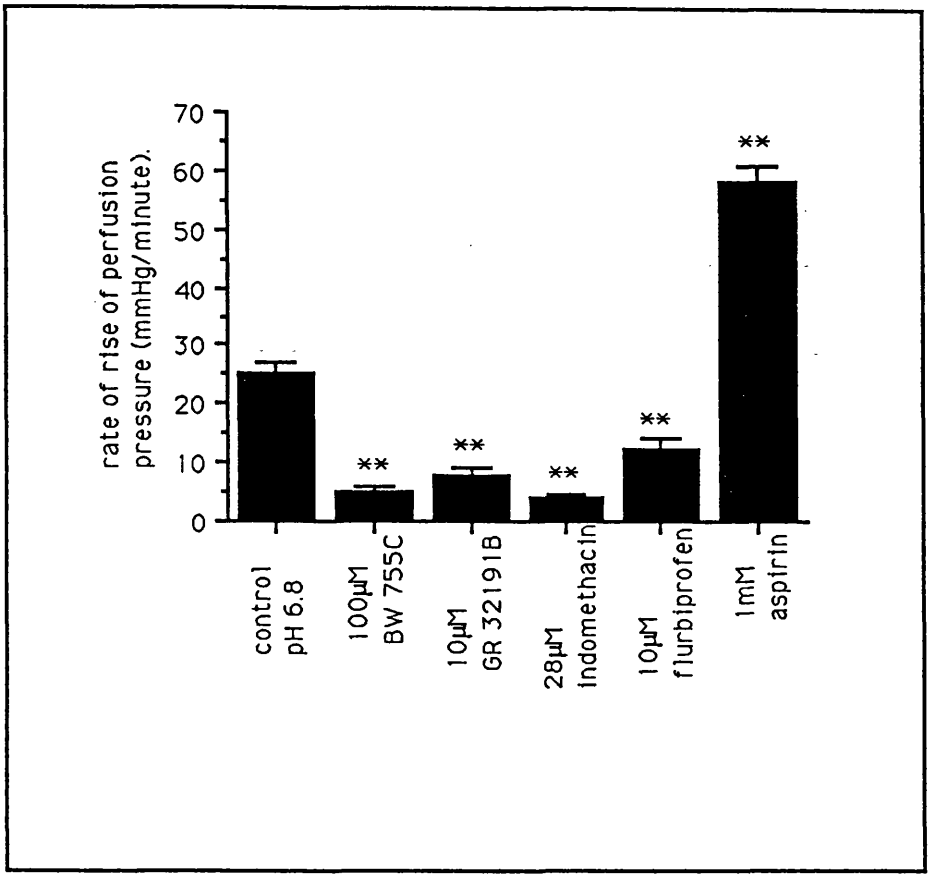

Figure 55. Effect of BW 755C (100µM), GR 32191B (10µM), indomethacin (28µM), flurbiprofen (10µM) and aspirin (1mM) on the rate of rise of perfusion pressure at pH 6.8 in the isolated perfused rat heart. Vertical bars represent ±s.e.mean. (n=4, **p<0.01 cw controls).

at pH 7.4 resulted in a transient and significant fall in coronary perfusion pressure of 22.5±4.6 mmHg that was not significantly different to the effect of indomethacin on resting pressure at pH 7.4. The subsequent perfusion of 10μg/ml indomethacin at pH 6.8 resulted in a rise in perfusion pressure at a rate of 4.3±1.6 mmHg/minute, which represented a significant reduction in the rate of coronary constriction compared to control responses in the absence of indomethacin (figure 55).

In contrast to flurbiprofen and indomethacin, acetylsalicylic acid (aspirin) is a non-reversible cyclooxygenase inhibitor. The effect of aspirin on the pH-dependent vasoconstriction was studied using the same protocol as for the studies with flurbiprofen and indomethacin. Unlike the two reversible cyclooxygenase inhibitors studied, the perfusion of 1mM aspirin at pH 7.4 resulted in a sustained rise in perfusion pressure of 11.7±1.1 mmHg. The perfusion of 1mM aspirin at pH 6.8 resulted in a potentiation of the vasoconstrictor response such that in response to acidosis the perfusion pressure rose at 57.5±3.2 mmHg/minute (figure 55).

9.1.1.6 Effects of dual cyclooxygenase/lipoxygenase inhibition.

The effect of the dual cyclooxygenase/lipoxygenase inhibitor BW 755C on the physiological response of the coronary vasculature to a reduction in pH was also investigated. Perfusion of 100μM BW 755C at pH 7.4 resulted in a significant transient vasodilation of 21.3±2.4 mmHg from a basal resting pressure of 49.8±1.9 mmHg which was not significantly different to the vasodilation at pH 7.4 reported above with 10μM flurbiprofen and 28μM indomethacin. The subsequent perfusion of 100μM BW 755C at pH 6.8 resulted in a significant attenuation of the pH-dependent vasoconstriction. Compared to a rise in perfusion pressure in response to acidosis of 25.75±2.2 mmHg/minute in control hearts, 100μM BW 755C significantly reduced this effect such that prefusion pressure rose at 5.25±1.1 mmHg/minute (figure 55).

9.1.1.7 Effects of thromboxane A_2 receptor antagonism.

In view of the observation that flurbiprofen and indomethacin both significantly

attenuated the rise in coronary perfusion pressure in response to acidosis, the potential involvement of thromboxane A_2 in this response was investigated. This was approached by the use of GR 32191B, a potent and selective thromboxane A_2 receptor antagonist (*Ritter et al., 1990*). Perfusion of 10μM GR 32191B at pH 7.4 resulted in a transient but significant fall in coronary perfusion pressure of 13.5±5.1 mmHg from a resting pressure of 48.5±5.6 mmHg. The subsequent perfusion of 10μM GR 32191B at pH 6.8 resulted in a significant attenuation of the pH dependent vasoconstriction such that in response to acidosis the perfusion pressure rose at 8.25±1.18 mmHg/minute (figure 55).

9.1.1.8 Effects of reduced glutathione and related compounds.

The physiological response of the heart to acidosis in the presence of reduced glutathione and related compounds was investigated and compared to the responses observed in control hearts to see if GSH might affect Na/H exchange which is involved in the physiological response of the heart to acidosis.

9.1.1.9 Reduced glutathione.

As previously described, the perfusion of 1mM reduced glutathione (GSH) at pH 7.4 resulted in a sustained and significant fall in perfusion pressure of 32.8±3.4 mmHg from a resting pressure of 55.5±2.9 mmHg. The subsequent perfusion of 1mM GSH at pH 6.8 resulted in a significant potentiation of the constrictor response to acidosis (figure 56). In the presence of 1mM GSH at pH 6.8, the coronary perfusion pressure rose at 90.0±7.07 mmHg/minute. Figure 57 shows that the efficacy of reduced glutathione to potentiate the pH-dependent vasoconstriction was concentration-dependent within the range 0.25-1.0 mM.

9.1.1.10 Glycine, cysteine and glutamate.

Reduced glutathione is a tripeptide consisting of the amino acid residues glycine, cysteine and glutamate (figure 1). For this reason, the effect of these individual constituent amino acids on the hearts physiological response to acidosis were investigated.

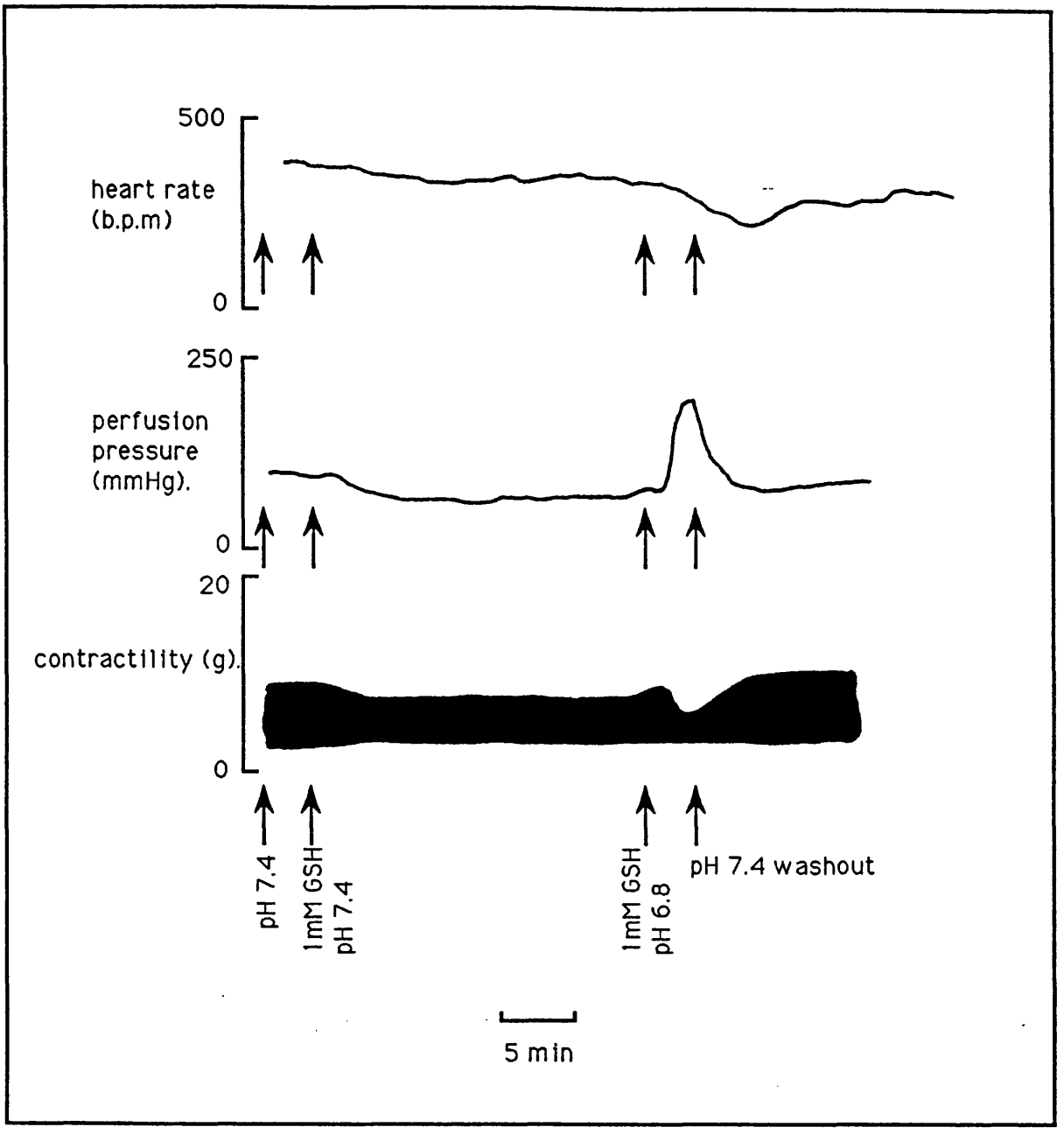

Figure 56. Typical trace of the effect of the perfusion of reduced glutathione (1mM) at pH 7.4 and pH 6.8 on coronary perfusion pressure, cardiac contractility and heart rate in the isolated perfused rat heart.

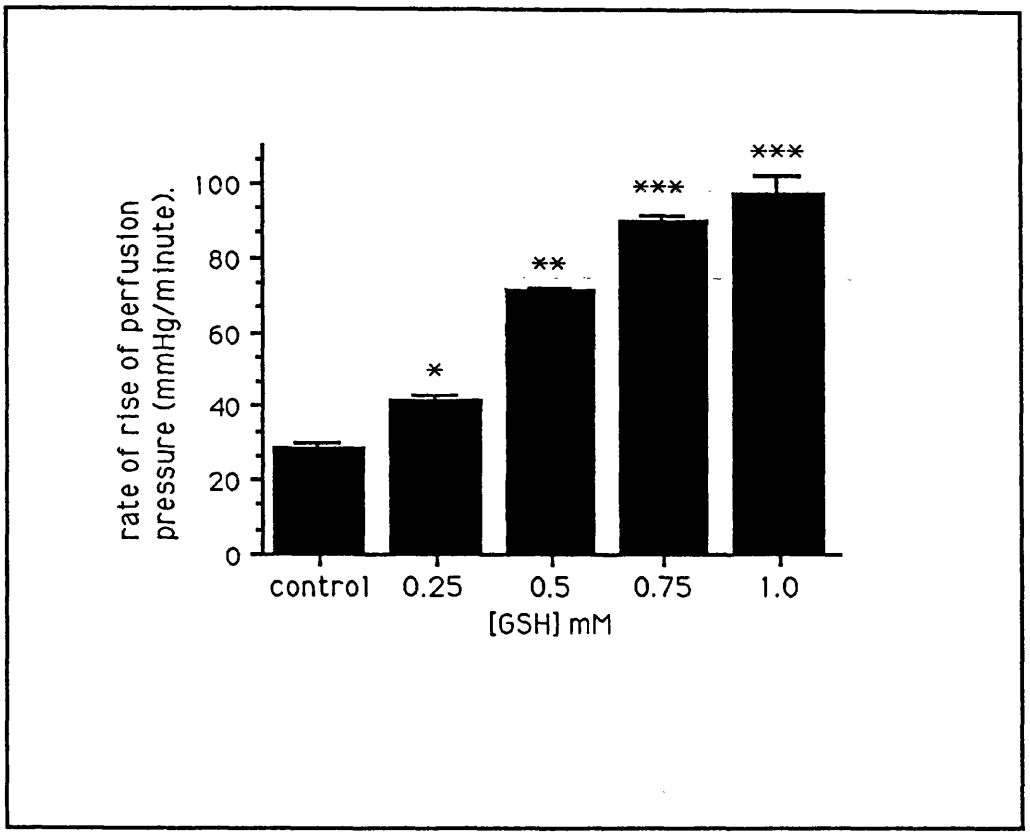

Figure 57. Effect of reduced glutathione (GSH) (0.25-1.0 mM), at pH 6.8, on the rate of rise of perfusion pressure in the isolated perfused rat heart. Vertical bars represent ±s.e.mean. (n=6, *p<0.05, **p<0.01, ***p<0.001 cw controls).

The perfusion of 1mM glycine at pH 7.4 resulted in no detectable change in basal coronary perfusion pressure. The subsequent perfusion of 1 mM glycine at pH 6.8 resulted in a constrictor response during which the perfusion pressure rose at 21.8±0.5 mmHg/minute. This was not significantly different to control responses to acidosis in the absence of glycine. In common with glycine, the perfusion of 1mM glutamate at pH 7.4 had no detectable effect on basal coronary perfusion pressure. The perfusion of glutamate under acidotic conditions resulted in a rise in perfusion pressure of 32.0±2.0 mmHg/minute representing a significant potentiation of the constrictor effect in response to acidosis (figure 58).

The pre-perfusion of 1mM cysteine at pH 7.4 resulted in a significant fall in perfusion pressure of 34.2±3.5 mmHg, from a resting pressure of 53.2±4.2 mmHg, that was not significantly different to the effect of the pre-perfusion of 1mM reduced glutathione at pH 7.4. The subsequent perfusion of 1mM cysteine at pH 6.8 resulted in no recordable change in coronary perfusion pressure in contrast to the pressor effect seen in response to acidosis in the absence of cysteine (figure 58).

In view of the observation that 1mM cysteine completely abolished the pH-dependent coronary vasoconstriction, concentration-effect studies were carried out within the range 0.25-1.0 mM. Figure 59 shows the results from these experiments. At a concentration of 0.25mM, cysteine still significantly attenuated the pH-dependent vasoconstriction. The minimum concentration of cysteine that could significantly attenuate the constrictor response to acidosis was approximately 0.07mM.

9.2 THROMBOXANE B_2 RELEASE IN RESPONSE TO MYOCARDIAL ACIDOSIS.

9.2.1 Control responses.

Figure 60 shows the release of thromboxane B_2, the relatively stable metabolite of thromboxane A_2, in response to two acidotic insults of 5 minutes duration at pH 6.8, separated by a 10 minute washout period at pH 7.4. A significant increase in

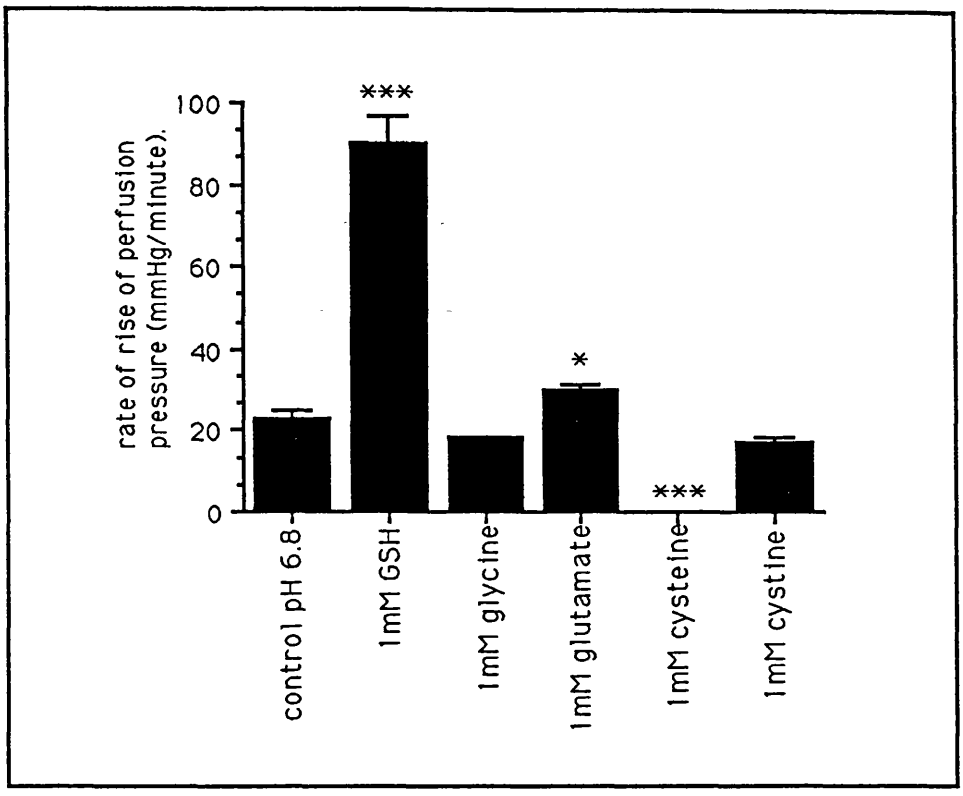

Figure 58. Effect of reduced glutathione (GSH) (1mM), glycine (gly) (1mM), glutamate (Glu) (1mM), cysteine (1mM), and cystine (1mM) on the rate of rise of perfusion pressure at pH 6.8 in the isolated perfused rat heart. Vertical bars represent ±s.e.mean. (n=4-6, *p<0.05, ***p<0.001 cw controls).

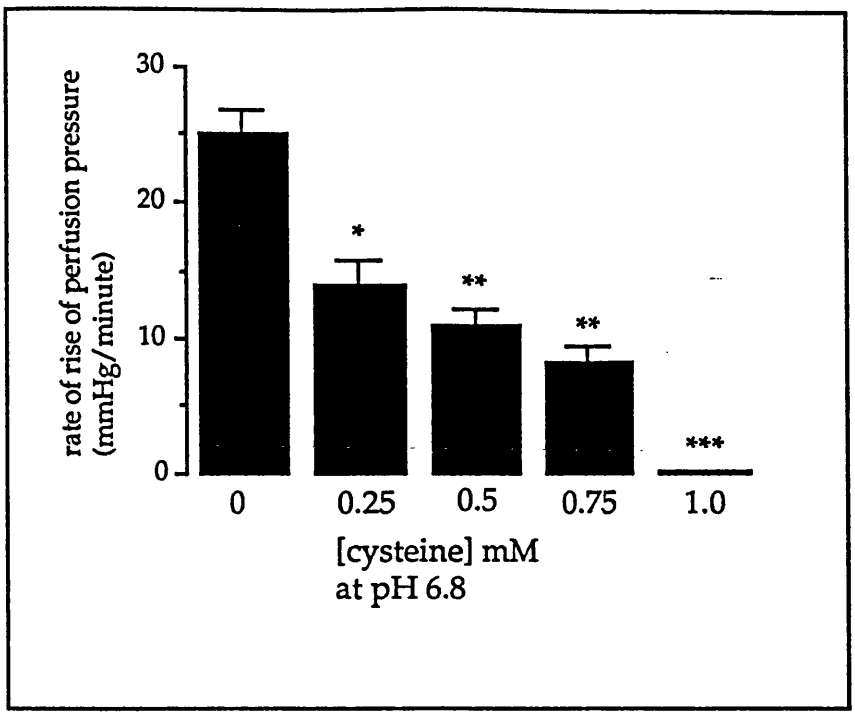

Figure 59. Effect of cysteine (0.25-1.0mM) on the rate of rise of perfusion pressure at pH 6.8 in the isolated perfused rat heart. Vertical bars represent ±s.e.mean. (n=4 hearts/group, *p<0.05, **p<0.01, ***p<0.001 cw 0mM cysteine controls).

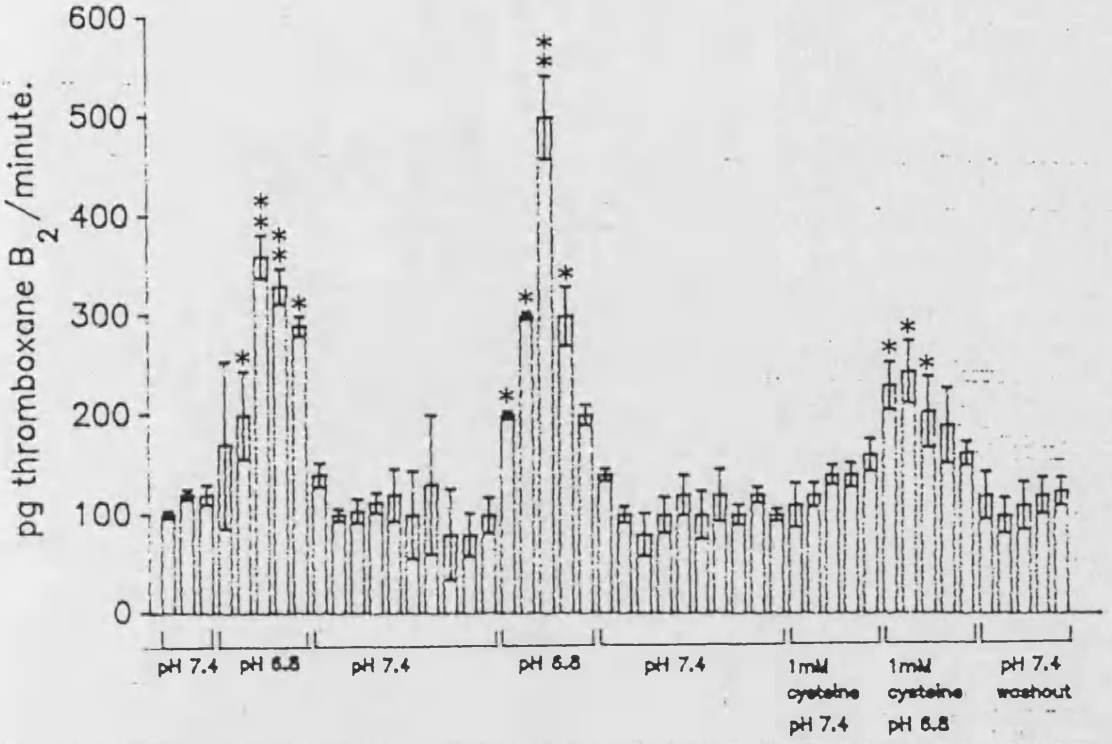

Figure 60. Effect of acidosis (pH 6.8), in the presence and absence of 1mM cysteine (cys), on thromboxane B_2 efflux from the isolated perfused rat heart. Vertical bars represent ±s.e.mean. (n=3, *p<0.05, **p<0.01 cw controls).

thromboxane B_2 was detected in coronary perfusate samples in response to acidosis, induced by perfusion of a crystalloid buffer at pH 6.8, compared to samples collected during control periods of perfusion at pH 7.4. Thromboxane B_2 release and, by inference, thromboxane A_2 release peaked 3 minutes after the onset of the acidotic insult and was not maintained; during the remainder of the acidotic insult, thromboxane A_2 synthesis decayed and returned to control levels upon switching to a perfusate at pH 7.4. There was no significant difference between the total thromboxane B_2 detected during the first acidotic insult and the second subsequent insult (figure 60).

9.2.2 Effect of cysteine on thromboxane B_2 perfusate levels in response to acidosis.

Figure 60 shows control levels of thromboxane B_2 detected in coronary perfusate samples during the perfusion of normal and acidotic buffer. The perfusion of 1mM cysteine at pH 7.4 had no significant effect on the peak level of thromboxane B_2 detected. However, at pH 6.8 the perfusion of 1mM cysteine significantly attenuated the peak level of thromboxane B_2 detected, as well as the total thromboxane B_2 released during the 5 minute acidotic insult. The peak level of thromboxane B_2 detected during acidosis in the presence of 1mM cysteine was still significantly greater than under conditions of normal pH.

9.1.1.11 INVESTIGATIONS INTO THE POSSIBLE MODES OF ACTION OF REDUCED GLUTATHIONE AND CYSTEINE UNDER ACIDOTIC CONDITIONS IN THE RAT CORONARY VASCULATURE.

9.1.1.11.1 Reduced glutathione.

In view of the observations that the cyclooxygenase inhibitors flurbiprofen and indomethacin both attenuated a pressor effect in response to acidosis in the coronary vasculature, their effect on the potentiation of the constriction by reduced glutathione was investigated. The rationale behind these experiments was that reduced gluathione may attenuate basal prostacyclin synthesis and release, or preferentially favour the synthesis of the vasoconstrictor compound, thromboxane A_2 (figure 54).

The perfusion of 10μM flurbiprofen at pH 7.4 resulted in a significant fall in perfusion pressure of 21.0±6.5 mmHg, as previously described. The addition of 1mM reduced glutathione resulted in a further dilation of 7.5±3.2 mmHg from a resting pressure of 40.2±5.2 mmHg. The subsequent and simultaneous perfusion of 10μM flurbiprofen and 1mM GSH at pH 6.8 resulted in a rise of perfusion pressure of 15.0±2.4 mmHg/minute (figure 61). This represents a significant reduction in the rate of constriction compared to the response to acidosis in the presence of 1mM reduced glutathione alone.

The perfusion of 10μg/ml indomethacin at pH 7.4 resulted in a significant fall in perfusion pressure of 25.2±3.4 mmHg. The addition of 1mM reduced glutathione resulted in no further detectable change in perfusion pressure. The subsequent perfusion of 28μM indomethacin and 1mM GSH at pH 6.8 resulted in a rise in perfusion pressure at 17.5±4.2 mmHg/minute (figure 61). This represents a significant reduction in the rate of constriction compared to the response to acidosis in the presence of 1mM reduced glutathione alone.

The perfusion of the dual cyclooxygenase/lipoxygenase inhibitor BW 755C (100 μM) at pH 7.4 resulted in a significant fall in perfusion pressure of 23.5±3.5 mmHg from a resting pressure of 55.2±6.2 mmHg. The subsequent addition of 1mM reduced glutathione resulted in a further dilation of 5.5±2.2 mmHg. The subsequent and simultaneous perfusion of 100μM BW 755C and 1 mM GSH at pH 6.8 resulted in a rise in perfusion pressure at 19.3±2.2 mmHg/minute (figure 61). This represents a significant reduction in the rate of coronary constriction compared to the response to acidosis in the presence of 1mM GSH alone.

The effect of a selective thromboxane A_2 antagonist, GR 32191B, on the potentiation of the pH-dependent vasoconstriction was also investigated. The perfusion of 10μM GR 32191B at pH 7.4 resulted in a significant fall in perfusion pressure of 10.6±3.5 mmHg from a resting pressure of 50.1±5.2 mmHg. The addition of 1mM reduced glutathione resulted in no further recordable change in perfusion pressure. The subsequent, simultaneous perfusion 10μM GR 32191B and 1mM GSH at pH 6.8 resulted in a rise in perfusion pressure of 16.2±2.6 mmHg/minute (figure 61). This represents a significant attenuation of the rate of constriction compared to the response to acidosis in the presence of 1mM GSH

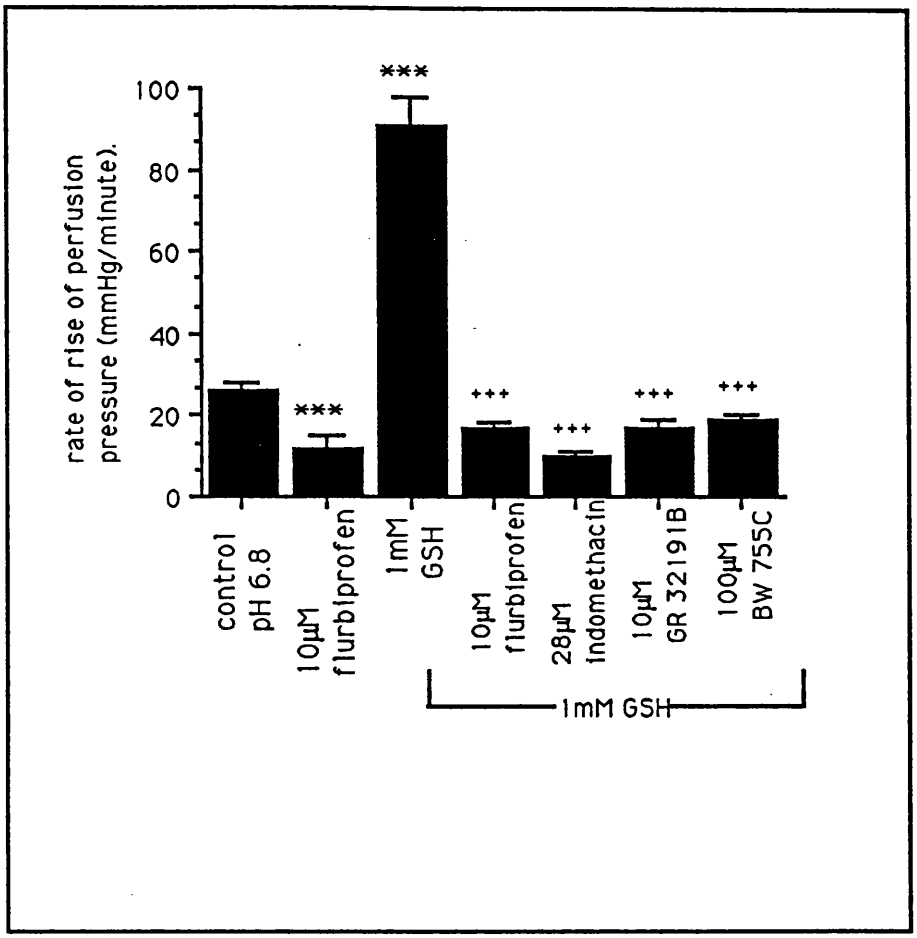

Figure 61. Effect of flurbiprofen (10mM), indomethacin (28mM), GR 32191B (10mM) and BW 755C (100mM) on the potentiation of the rate of rise of perfusion pressure by reduced glutathione (GSH) (1mM) at pH 6.8 in the isolated perfused rat heart. Vertical bars represent mean±s.e.mean. (n=4 hearts/group, ***$p<0.001$ cw control pH 6.8 perfusions, +++$p<0.001$ cw perfusions at pH6.8 in the presence of 1mM GSH).

alone.

There was no significant difference between the ability of flurbiprofen, indomethacin, BW 755C and GR 32191B, at the concentrations used, to attenuate the potentiation of the pH dependent vasoconstriction by 1mM GSH.

In view of the abiltity of the alpha-adrenoceptor antagaonist, phentolamine, to attenuate the physiological response of the coronary vasculature to an acidotic insult, its effect on the potentiation of this response by 1mM reduced glutathione was investigated. The perfusion of 1μM phentolamine at pH 7.4 resulted in a significant fall in perfusion pressure of 15.9±3.1 mmHg. Following the addition of 1mM reduced glutathione there was no further change in basal perfusion pressure. Under acidotic conditions the simultaneous perfusion of 1μM phentolamine and 1mM reduced glutathione resulted in a rise in perfusion pressure of 75.2±5.3 mmHg/minute that represents a significant attenuation of the rate of constriction compared to the response to acidosis in the presence of 1mM GSH alone (figure 62).

9.1.1.11.2 Cysteine.

As previously described, cysteine significantly attenuated the coronary vasoconstriction in response to acidosis in a concentration-dependent manner (0.25-1.0mM). Experiments were undertaken to investigate mechanisms by which cysteine may exert this effect. The possible involvement of endothelium-derived relaxant factor (EDRF) in the ability of cysteine to attenuate the pH dependent vasoconstriction was investigated by the use of L-N^G-nitro-arginine, a selective and potent inhibitor of EDRF synthesis (*Moore et al., 1990*). In this set of experiments an intermediate concentration of cysteine (0.1mM) was used so that any effect of L-N^G-nitro-arginine on the ability of cysteine to attenuate the pressor effect in response to acidosis could be observed.

The perfusion of 0.1mM cysteine at pH 7.4 resulted in a significant fall in perfusion pressure of 21.8±1.2 mmHg. The subsequent perfusion of the same concentration of cysteine at pH 6.8 resulted in a rise in perfusion pressure at

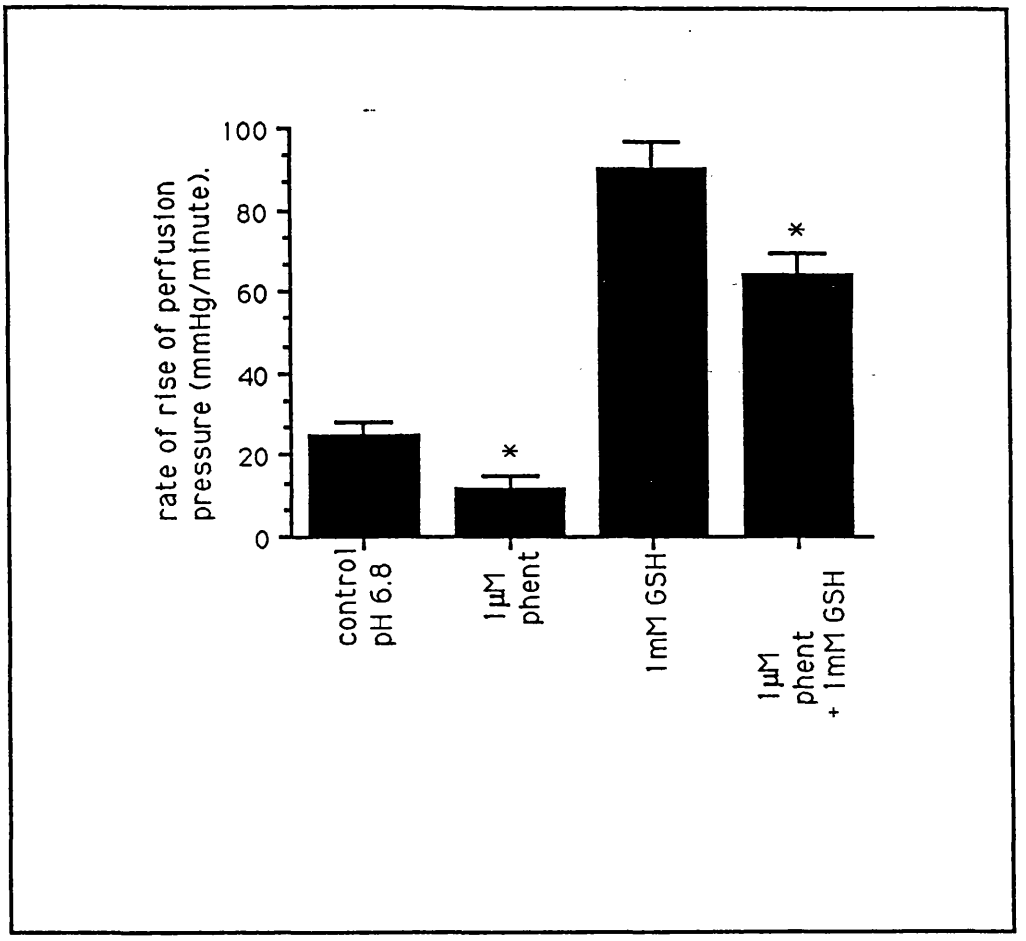

Figure 62. Effect of phentolamine (1μM) on the potentiation of an acidosis-induced coronary constriction by reduced glutathione (GSH) (1mM), in the isolated perfused rat heart. Vertical bars represent ±s.e.mean. (n=4, *p<0.05 cw controls).

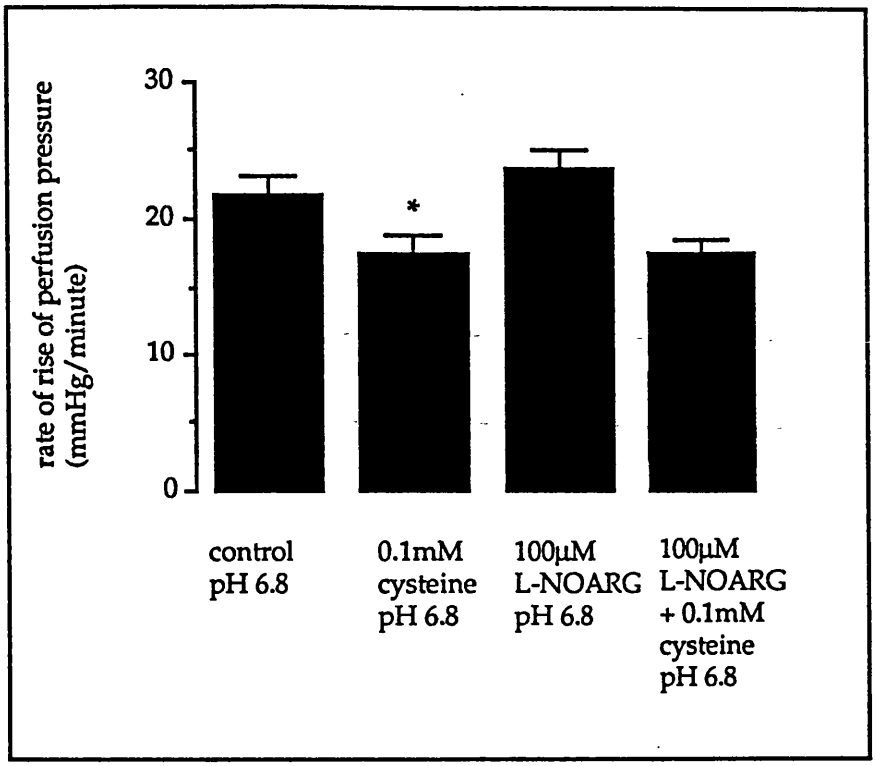

Figure 63. Effect of L-N^G-nitro-arginine (L-NOARG) (100mM), in the presence and absence of cysteine (0.1mM), on the rate of rise of perfusion pressure at pH 6.8 in the isolated perfused rat heart. Vertical bars represent ±s.e.mean. (n=4 hearts/group, *$p<0.05$ cw control pH 6.8 perfusion group).

17.5±1.4 mmHg/minute, which was significantly less than in the absence of 0.1mM cysteine (figure 63). The perfusion of 100μM L-N^G-nitro-arginine at pH 7.4 resulted in a significant rise in perfusion pressure of 23.8±1.3 mmHg. Perfusion of the same concentration of L-N^G-nitro-arginine at pH 6.8 resulted in a rise in perfusion pressure at 23.3±1.3 mmHg/minute. This was not significantly different to the response of coronary perfusion pressure to acidosis in the absence of L-N^G-nitro-arginine. In hearts in which the coronary tone had been artifically raised by 23.8±1.3 mmHg by the perfusion of 100μM L-N^G-nitro-arginine at pH 7.4, the perfusion of 0.1mM cysteine resulted in a significant fall in perfusion pressure of 22.5±1.4 mmHg. The subsequent and simultaneous perfusion of 100μM L-N^G-nitro-arginine and 0.1mM cysteine at pH 6.8 resulted in a rise in perfusion pressure of 17.4±1.1 mmHg/minute. This was not significantly different from the effect of 0.1mM cysteine alone at pH 6.8 (figure 63). Therefore it appears unlikely that EDRF is involved in the ability of cysteine to significantly attenuate a pH-dependent vasoconstriction in the isolated coronary vascular bed of the rat.

Experiments were also undertaken in order to assess whether cysteine exerts its ability to attenuate a pH-dependent coronary constriction by acting as a calcium channel antagonist. The isolated guinea-pig taenia-coli preparation was used for these studies described below.

9.1.1.11.2.1 Effects of cysteine on calcium-induced contraction.

The isolated guinea-pig taenia-coli was used to assess whether cysteine exerts an antagonistic effect on contractile responses of this preparation to calcium. Cumulative concentration-response curves were constructed to calcium (0.1-2.7mM) in potassium-depolarised (110mM K^+) preparations. Following the washout of the calcium, the effect of cysteine on calcium-induced contraction was investigated by suspending the tissue in 1mM cysteine and repeating the cumulative addition of calcium in the presence of cysteine. 1mM cysteine had no significant effect on the contractions induced by calcium (27-2700 μM) (figure 64).

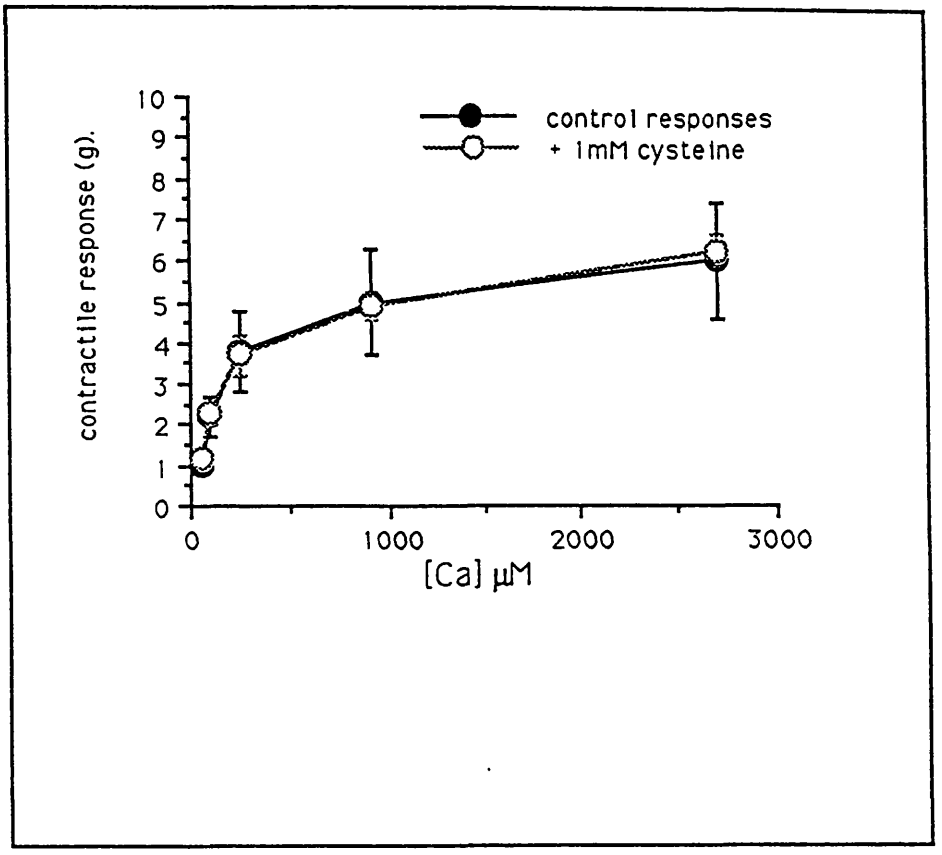

Figure 64. Comparison of the effects of cysteine (1mM) on the contractile response of isolated guinea-pig taenia coli with that of time-matched controls. Vertical bars represent ±s.e.mean. (n=4, *p<0.05 cw controls).

9.1.1.11.3 EFFECT OF CYSTEINE ON THE POTENTIATION OF A pH-DEPENDENT VASOCONSTRICTION BY REDUCED GLUTATHIONE IN THE CORONARY VASCULATURE.

In view of the observation that reduced glutathione potentiated while cysteine attenuated the vasoconstriction of the coronary vasculature in response to acidosis, the effect of cysteine on the potentiation of the pressor response to acidosis by reduced glutathione was investigated. The rat coronary vasculature was perfused at pH 7.4 with varying concentrations of cysteine (0.25-1.0mM) and any changes in perfusion pressure recorded. Further perfusion pressure changes following the addition of 1mM reduced glutathione were also recorded. Subsequently, varying concentrations of cysteine (0.25-1.0mM) and 1mM reduced glutathione were perfused simultaneously at pH 6.8 and the rate of rise of perfusion pressure determined.

Figure 65 shows that cysteine attenuated, in a concentration-dependent manner (0.25-1.0mM), the potentiation of the constrictor response to acidosis by 1mM reduced glutathione; 1mM cysteine completely abolished the pressor effect of 1mM GSH.

9.1.1.12 EFFECT OF REDUCED DITHIOTHREITOL, DL-PENICILLAMINE, CAPTOPRIL, OXIDISED GLUTATHIONE, CYSTINE AND OXIDISED DITHIOTHREITOL ON THE PHYSIOLOGICAL REPONSE OF THE CORONARY VASCULATURE TO ACIDOSIS.

In common with reduced glutathione, DL-penicillamine, reduced dithiothreitol and captopril all contain a free sulphydryl group. Oxidised glutathione (*N,N'*-[dithiobis[1-[(carboxymethyl)carbamoyl]ethylene]]diglutamine), oxidised dithiothreitol (trans-4,5-dihydroxy-1,2-dithiane) and cystine are the oxidised, disulphide forms of reduced glutathione, reduced dithiothreitol and cysteine, respectively. With the above in mind, the effects of these compounds on the physiological response of the coronary vasculature to acidosis was investigated.

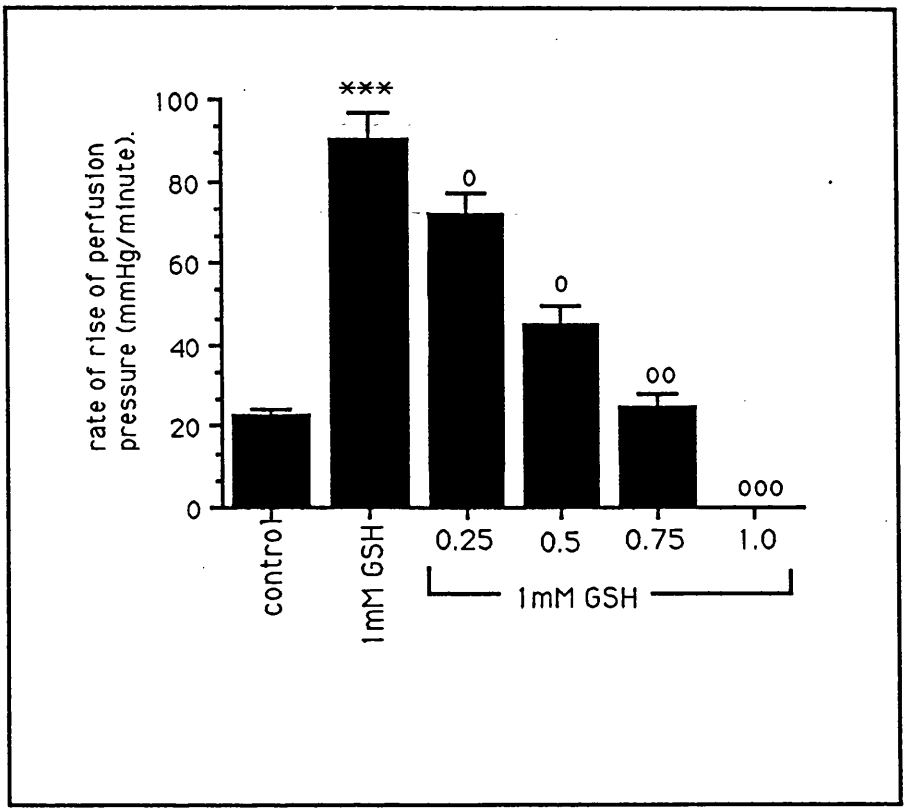

Figure 65. Effect of cysteine (0.25-1.0mM) on the potentiation of an acidosis-induced coronary constriction by reduced glutathione (1mM) (GSH). Vertical bars represent ±s.e.mean. (n=4, $^{o}p<0.05$, $^{oo}p<0.01$, $^{ooo}p<0.001$, ***$p<0.001$ cw controls).

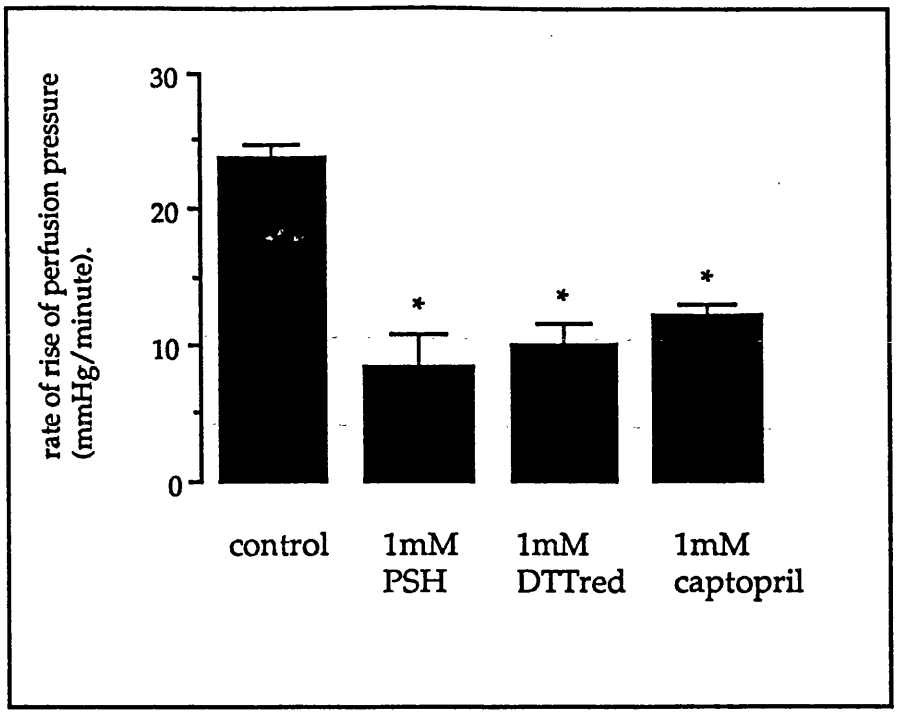

Figure 66. Effect of DL-penicillamine (PSH) (1mM), reduced dithiothreitol (DTTred) (1mM) and captopril (1mM) on the rate of rise of perfusion pressure in the isolated perfused rat heart at pH 6.8. Vertical bars represent $\pm$s.e.mean. (n=6 hearts/group, *$p<0.05$ cw control group).

9.1.1.12.1 DL-penicillamine, reduced dithiothreitol and captopril.

The perfusion of 1mM DL-penicillamine (DL-PSH) at pH 7.4 resulted in a significant fall in perfusion pressure of 7.5±2.5 mmHg, indicative of coronary vasodilation. In this set of experiments the basal perfusion pressure was 45.2±5.2 mmHg. Perfusion pressure rose at 8.5±2.3 mmHg/minute following the subsequent perfusion of 1mM DL-PSH at pH 6.8 (figure 66). In a separate group of hearts, a fall in perfusion pressure of 21.5±2.2 mmHg followed the perfusion of 1mM reduced dithiothreitol (DTT_{red}) at pH 7.4. The perfusion of the same concentration of DTT_{red}, at pH 6.8, resulted in a rise in perfusion pressure of 10.0±1.5 mmHg/minute (figure 66). In another group of hearts, the perfusion of 1mM captopril, at pH 7.4, caused a significant fall in perfusion pressure of 15.2±4.2 mmHg. The subsequent perfusion of 1mM captopril at pH 6.8 resulted in a rise of perfusion pressure of 12.2±0.8 mmHg/minute that was significantly less than the response to acidosis in control hearts in the absence of captopril (figure 66).

This data shows that in contrast to the effects of the free sulphydryl containing compound reduced glutathione, DL-penicillamine, reduced dithiothreitol and captopril all significantly attenuated the rise in coronary perfusion pressure in response to acidosis.

9.1.12.2 Oxidised dithiothreitol, cystine and oxidised glutathione.

The perfusion of 1mM oxidised dithiothreitol (DTT_{oxid}) at pH 7.4 had no significant effect on basal perfusion pressure. Perfusion of the same concentration of DTT_{oxid} at pH 6.8 resulted in a rise in perfusion pressure of 28.0±1.7 mmHg/minute (figure 67). At pH 7.4, the perfusion of 1mM cystine resulted in no significant change in perfusion pressure. The perfusion pressure rose at a rate of 22.6±4.3 mmHg/minute following the subsequent perfusion of the same concentration of cystine at pH 6.8. (figure 67). The perfusion of 1mM oxidised glutathione (GSSG) at pH 7.4 had no significant effect on perfusion pressure. At pH 6.8, the perfusion of 1mM GSSG resulted in a rise in perfusion pressure of 25.6±5.1 mmHg/minute (figure 67). The effects of DTT_{oxid}, cystine and GSSG at pH 6.8 were not significantly different to the changes in coronary perfusion

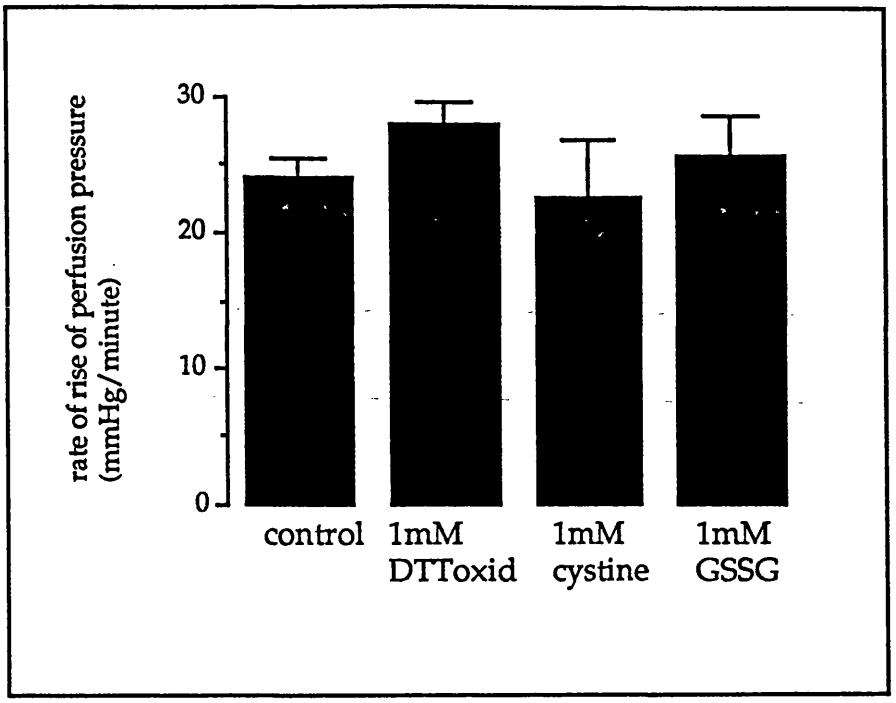

Figure 67. Effect of oxidised dithiothreitol (DTToxid) (1mM), cystine (1mM) and oxidised glutathione (GSSG) (1mM) on the rate of rise of perfusion pressure at pH 6.8 in the isolated perfused rat heart. VErtical bars represent $\pm$s.e.mean. (n=6 hearts/group).

pressure during acidosis in the absence of these compounds.

9.1.2 ISOLATED RAT MESENTERIC VASCULAR BED.

9.1.2.1 Control responses to acidosis.

In addition to the physiological response of the rat coronary vasculature to acidosis, the response of the isolated rat mesenteric bed to an acidotic insult, in the presence and absence of reduced glutathione was investigated.

From figure 68 it can be seen that upon changing the perfusate from pH 7.4 to 6.8 there was no significant change in perfusion pressure. The viability of each preparation was confirmed in control and experimental tissues both prior to and after the acidotic insult by the perfusion of 10μM phenylephrine which resulted in submaximal vasoconstriction. In addition, acidosis had no effect upon the perfusion pressure of tissues pre-contracted with 10μM phenylephrine (figure 68).

9.1.2.2 Effect of reduced glutathione.

The perfusion of 1mM reduced glutathione at pH 7.4 had no significant effect on basal perfusion pressure in both control tissues and tissues pre-contracted with 10μM phenylephrine. The perfusion of 1mM reduced glutathione at pH 6.8 resulted in no detectable change in perfusion pressure in control tissues. In preparations pre-contracted with phenylephrine (10μM), the addition of 1mM reduced glutathione under acidotic conditions resulted in a further significant constriction, indicated by a rise in perfusion pressure of 18.2±3.2 mmHg (figure 68).

9.1.3 ISOLATED RAT THORACIC AORTA PREPARATION.

Acidosis has been recently reported in the literature to cause vasodilation of the isolated rat aorta using a HEPES-buffered physiological salt solution (PSS), the compostion of which is described in the methods (section 2.8) (*Loutzenhiser et al., 1990*). Experiments were undertaken to investigate the effect of acidosis, in the presence and absence of reduced glutathione, on aortic rings using the

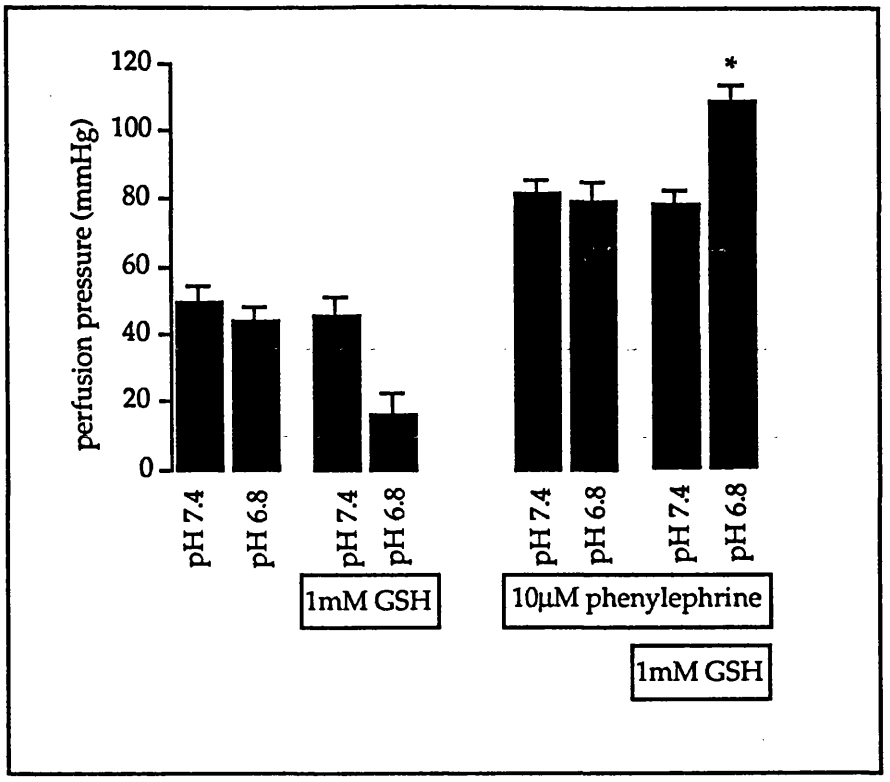

Figure 68. Effect of acidosis, in the presence and absence of reduced glutathione (GSH) (1mM), on perfusion pressure in untreated and pre-contracted (phenylephrine, 10μM) isolated rat mesenteric beds. Vertical bars represent ±s.e.mean. (n=4, *$p<0.05$ cw respective controls).

Krebs-Henseleit buffer used in the acidosis experiments in the isolated rat heart and the alternative HEPES-buffered PSS described in the literature (*Loutzenhiser et al., 1990*). The aim was to determine if a feature of the buffer was responsible for the contrasting responses of acidosis in the coronary vasculature and larger vessels, such as the aorta.

Figure 69 shows the effects of lowering the pH from 7.4 to 6.8 on the tone of aortic rings pre-contracted with 10μM noradrenaline, using both perfusates described in the methods (section 2.1 & 2.8). In pre-contracted rings, acidification resulted in a transient reduction in tension of 28.0±2% (basal tension, +5.1±0.3g) using Krebs-Henseleit buffer and 26.0±2% using the HEPES-buffered PSS (basal tension, +5.2±0.3g). There was no significant difference between these two responses, which are in agreement with Loutzenhiser *et al.* (1990). At pH 7.4, 1mM reduced glutathione caused a significant increased relaxation of preparations pre-contracted with 10μM noradrenaline using both types of buffer. Using normal Krebs buffer this relaxation was 36.0±3% (100% = 5.1±0.2g) and with the HEPES-buffered PPS, 38±2% (100% = 5.6±0.3g). Acidosis, in the presence and absence of 1mM GSH using both types of buffer, had no effect on the tone of aortic rings that had not been previously pre-contracted with 10μM noradrenaline.

9.2 RADIOIMMUNOASSAY OF CORONARY PERFUSATE LEVELS OF CYCLIC GMP (cGMP) IN RESPONSE TO ACIDOSIS IN THE PRESENCE AND ABSENCE OF CYSTEINE.

Figure 70 shows that under resting conditions there was a relatively low detectable level of cGMP in coronary perfusate samples. The perfusion of 1mM cysteine, at pH 7.4, resulted in a significant rise in perfusate cGMP levels. During acidosis, in the presence of 1mM cysteine, the amount of cGMP detected in perfusate samples was significantly raised in comparison to levels during acidosis in the absence of cysteine. The subsequent washout with buffer at normal pH resulted in a return of perfusate cGMP levels to within control values.

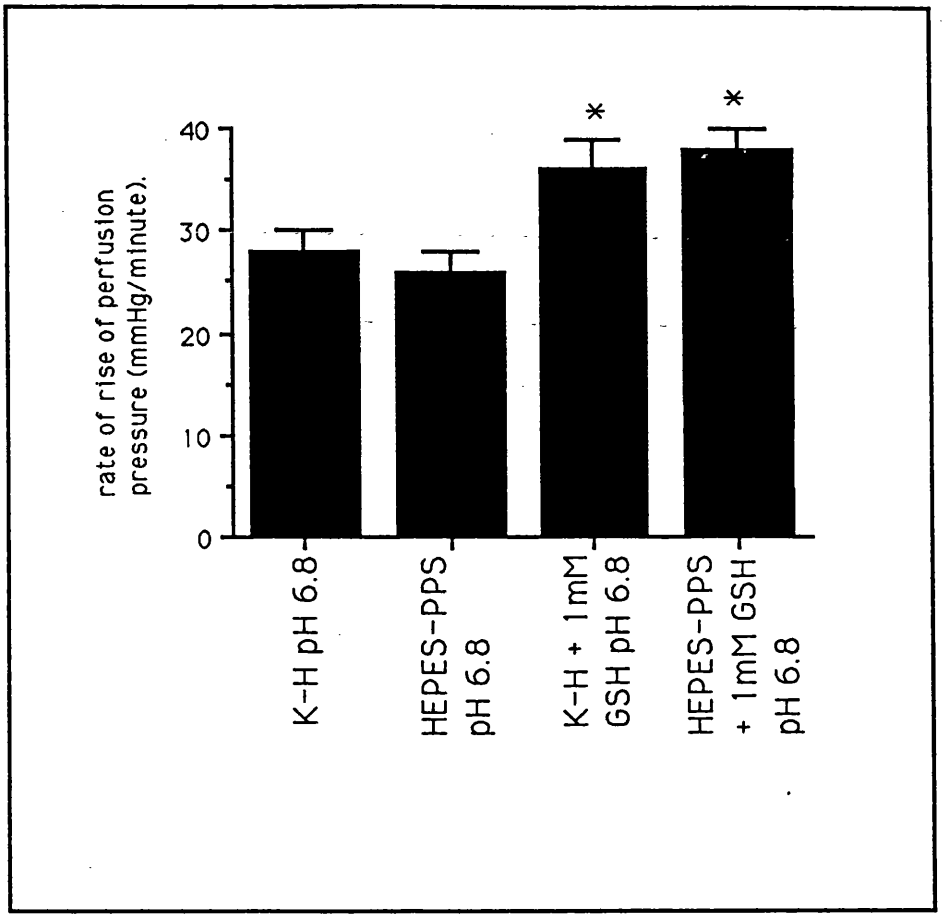

Figure 69. Effect of Krebs-Henseleit buffer (K-H) and HEPES-buffered physiological salt solution (HEPES-PPS), in the presence and absence of reduced glutathione (GSH) (1mM), at pH 6.8 on the tone of pre-contracted (10μM noradrenaline (NA)) isolated rat thoracic aortae. The composition of the buffers is described in the methods (sections 2.1 & 2.8). Vertical bars represent ±s.e.mean. (n=4, *$p<0.05$ cw controls).

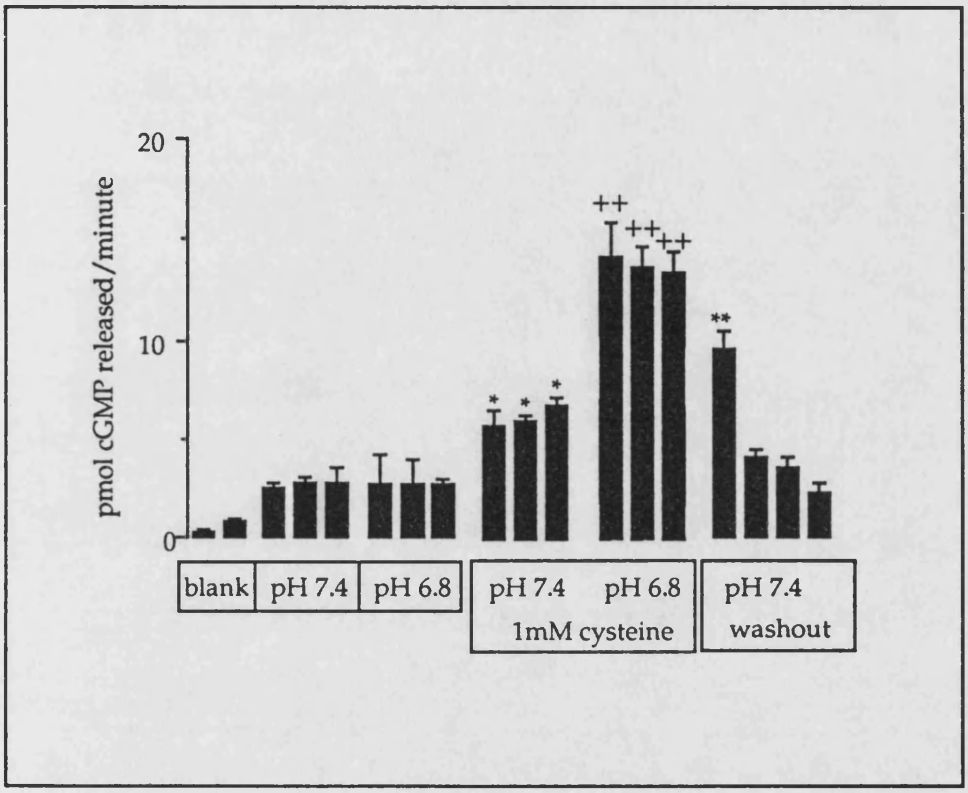

Figure 70. Effect of cysteine (1mM), under normal and acidotic conditions, on coronary perfusate cyclic GMP (cGMP) levels in the isolated perfused rat heart. Vertival bars represent ±s.e.mean. (n=3, *p<0.05, **p<0.01, ++p<0.01 cw respective controls).

CHAPTER 10.

DISCUSSION.

The ability of reduced glutathione and related compounds to modulate Na/K ATPase activity has been demonstrated. The physiological response of the coronary vasculature to acidosis, in the presence and absence of reduced glutathione and related compounds, was studied to investigate the effect of these compounds on the Na/H exchange mechanism as another example of an intrinsic, membrane-bound protein that is essential for the maintainance of cellular function and ionic homeostasis under normal and/or ischaemic conditions (*Lazdunski et al., 1985; Tani, 1990*). The present investigations have shown that acidosis causes a pH-dependent rise in perfusion pressure that is indicative of coronary vasoconstriction in the constant flow Langendorff rat heart model. This is in contrast to a vasodilator response to acidosis in the isolated, pre-contracted rat thoracic aorta and the absence of any change in tone in response to acidosis in the isolated rat mesenteric bed. Of all the compounds investigated containing a free sulphydryl group, only GSH potentiated, in a concentration-dependent manner, the acidosis-induced rise in perfusion pressure, whilst cysteine, reduced dithiothreitol, penicillamine and captopril all attenuated this response. The role of cyclooxygenase products and catecholamines in the response of the coronary vasculature to acidosis in the presence and absence of GSH was investigated. With respect to the effect of cysteine under acidotic conditions, the involvement of endothelium-dependent relaxant factor (EDRF) and the release of cGMP and thromboxane A_2 were studied. In addition, the guinea-pig taenia coli preparation was used to quantify any calcium-antagonist activity of cysteine.

It is well documented that decreased oxygen delivery and the accumulation of metabolic end products are components of an ischaemic insult. However, the relative contribution of acidosis to ischaemically-induced myocardial damage is unclear. Acidosis has been reported to cause coronary vasodilation (*Jerusalem and Starling, 1910; Patterson, 1914; Gremels and Starling, 1926),* whereas Steenburgen *et al.* (1977) observed a decline in coronary flow following acidosis that is indicative of increased vascular resistance in the constant pressure Langendorff model. The data presented in the present investigations, using a constant flow model, is in agreement with this observation. There are, however, a diverse array of reports on the effects of acidosis on other isolated vessels and

tissues. Rinaldi *et al.* (1987) and Rooke and Sparks (1981) have used isolated dog coronary arteries to investigate the effects of both acidosis and alkalosis on contactile tension; acidosis relaxed depolarised tissues whereas alkalosis increased coronary tone. Therefore, in a whole organ, such as the heart that contains both relatively large and fine resistance vessels, there is a danger of extrapolating results obtained with isolated vessels to isolated perfused organs. This is because in the coronary vascular bed the reponse to selected conditions or agents is recorded as a net change in perfusion pressure a result of the net 'tone' of the vasculature as a whole. The tone of selected areas of coronary vasculature cannot be determined using this preparation.

In the experiments, described here using a constant flow Langendorff heart preparation, acidosis (over the range pH 7.2-6.8) resulted in a rise in perfusion pressure that is indicative of coronary vasoconstriction in this model. This is in contrast to the vasodilator effect of acidosis in other vessels, such as the rat aorta (*Loutzenhiser et al., 1990*) and the absence of any effect in the rat mesenteric bed observed in the present investigations. This difference in response is thought to be due to the tissue type and not to differences between experimental protocols and buffers described by other groups.

Kwan *et al.* (1990) recently reported the effects of simulated ischaemia on the tone of isolated sheep circumflex coronary artery rings. The conditions chosen to mimic ischaemia were hypoxia, a raised potassium and lactate concentration (10mM and 1mM respectively), a reduced glucose concentration (4mM) and a reduction of extracellular pH (pH 6.85±0.05). These conditions resulted in a transient relaxation of the preparations lasting 5 minutes followed by a sustained contraction. The authors concluded that the 'ischaemic contraction' was mediated by substances released from the endothelium, including a product of the lipoxygenase pathway (figure 54). Furthermore, they provided evidence that simulated ischaemia causes the release of noradrenaline and 5-hydroxytryptamine, both of which cause coronary constriction. These observations may be significant in view of the ability of selected cyclooxygenase and lipoxygenase inhibitors and a thromboxane A_2 receptor antagonist to attenute a pH-dependent coronary constriction in the present investigations. Kwan *et al.* (1990) did not investigate the role of catecholamines in

the two phases of the response to acidosis, whereas the efficacy of alpha-adrenoceptor antagonism in attenuating a pH-dependent constriction in the experiments described here suggests that catecholamine release may have a role in this response. The initial brief relaxation phase in response to simulated ischaemia described by Kwan *et al.* (1990) was not endothelium dependent; it was antagonised by a product of the phospholipase A_2 pathway and possibly caused by a vasodilator released immediately after the onset of simulated ischaemia. In the experiments reported here, acidosis caused a transient rise and fall in perfusion pressure followed by a sustained constriction (figure 51). This response and the effect of selected pharmacological antagonists on this constriction are in agreement with the findings of Kwan *et al.* (1990). In view of the difficulty in removing the endothelium of the coronary vasculature whilst maintaining the viability of the heart, no evidence for the endothelium-dependence of the coronary constriction in response to acidosis was obtained in this set of experiments.

There is little evidence in the literature for a vasoconstrictor response of the coronary vasculature in response to acidosis. The present investigations provide evidence for the involvement of cyclooxygenase products in the mediation of the response of the coronary vasculature to acidosis, although the exact mechanism is by not clear. It seems unlikely that the ability of reduced glutathione to potentiate the acidosis-induced coronary vasoconstriction is related to its free sulphydryl group in view of the opposing effect of cysteine. In contrast to GSH, 1mM cysteine completely abolished the pressor response to acidosis whilst both 1mM cysteine and GSH both caused a similar vasodilation when perfused at pH 7.4. Experiments using the sulphur containing compounds methionine and taurine are required to determine whether the presence of a free -SH group is essential for these effects or whether this is just incidental. This is also in view of the observation that both methionine and taurine attenuate stress-induced hypertension in rats (*Yamori et al., 1983*) and that taurine can protect against the calcium paradox (*Chapman and Rodrigo, 1990*). In addition to cysteine, the effect of the other component amino acids of glutathione on the physiological response of the heart to acidosis were investigated and it was shown that glutamate potentiated the acidosis-induced coronary vasoconstriction. It would, therefore, be of interest to further investigate

the effect of other excitatory and non-excitatory amino acids, such as aspartate and methionine, under similar conditions.

The data presented here shows that the acidosis-induced coronary constriction was significantly attenuated by a non-specific alpha-adrenoceptor antagonist, phentolamine, as was the reduced glutathione-induced potentiation of the constriction. These results suggest that acidosis may cause catecholamine release mediating coronary constriction and that GSH may exert its effect, at least in part, via increasing catecholamine release or by potentiating the effects of catecholamines. There is also the possibility that GSH affects the uptake mechanisms that remove catecholamines thereby potentiating their effect. From the preliminary experiments presented in this thesis, GSH was shown to have no effect on noradrenaline overflow during myocardial ischaemia and, therefore, it is unlikely that it has any effect on catecholamine uptake. A reduction in the activity of the uptake mechanisms would not be expected in the absence of an increase in noradrenaline overflow during acidosis. Acidosis, in the presence and absence of GSH, may increase receptor sensitivity resulting in an increased pressor response in the absence of an increase in noradrenaline release. Dart and Riemersma (1989) were unable to detect any direct effect of acidosis (pH 6.5) on noradrenaline release from the isolated perfused heart. Although they showed catecholamine release to be increased during acidosis this was found to be statisically insignificant. Therefore, local concentrations of catecholamines may be high enough to elicit a coronary vasoconstriction in the absence of significant net increase in noradrenaline overflow in response to acidosis. Alternatively, the actual bioassay system (i.e. coronary vasoconstriction) may be more sensitive than the physiochemical assay (HPLC of perfusate noradrenaline levels). Therefore, a relatively small and undetectable increase in local noradrenaline concentrations may elict coronary constriction. There is also the possibility that the effect of phentolamine may be independent of alpha-adrenoceptor blockade and due to other unknown non-specific effects. It would be of interest to investigate whether cysteine attenuates basal myocardial catecholamine release which may represent a method by which it attenuates the acidosis-induced coronary vasoconstriction. Catecholamine release from rat myocardium in response to acidosis was not

assessed in the present investigations which would help resolve the mechanism of this response. In addition, the effect of GSH and cysteine on the response of the coronary vasculature to bolus injections of noradrenaline should be investigated.

In vitro, neuropeptide Y (NPY) has a vasoconstrictor action in the coronary circulation and isolated blood vessels. It is a co-transmitter with noradrenaline and potentiates the action of other vasoactive substances, such as noradrenaline, phenylephrine, histamine and angiotensin II, at lower doses than are required for a direct response. Recently it has been shown that NPY is co-released with noradrenaline from the human heart during exercise and hypoxia and, therefore, may contribute to an acidosis-induced coronary constriction either directly or by potentiating the pressor effect of noradrenaline. (*Walker et al., 1991*). There is also the possibility that acidosis may evoke a general release of neurotransmitters. Acetylcholine, for example, causes both vasodilation and vasoconstriction in the coronary circulation (*Kalsner, 1989*) although the muscarinic constrictor action is predominant. Whether acetylcholine, neuropeptide Y or other potent coronary vasoconstrictors, such as endothelin, contribute significantly towards the acidosis-induced constriction remains to be seen.

Selected cyclooxygenase/lipoxygense inhibitors attenuated both the constrictor response to acidosis and the potentiation of this response by GSH. Therefore, the involvement of thromboxane A_2 in this response was investigated using a potent and selective thromboxane A_2 receptor antagonist, GR 32191B. The hypothesis was that acidosis may increase basal thromboxane A_2 synthesis and release which has a constrictor action on the coronary vasculature. This, linked with a possible reduction in prostacyclin release (which causes coronary vasodilation) may contribute to the coronary constriction in response to acidosis. 10μM GR 32191B attenuated the acidosis-induced coronary constriction, indicating that thromboxane A_2 is released under acidotic conditions and contributes to an increase in coronary tone. Acidosis may increase thromboxane A_2 synthesis or increase its half-life resulting in coronary constriction. In a similar set of experiments, cysteine was found to attenuate thromboxane A_2 release during myocardial acidosis. This may be due to cysteine reducing the activity of thromboxane synthase and/or increasing prostacyclin synthesis resulting in a net attenuation of the pH-dependent

vasoconstriction. Further experimental analysis of the effects of acidosis on thromboxane synthesis and perfusate-levels of the stable prostacyclin metabolite, 6-keto $PGF_{1\alpha}$, are required to clarify the role of these vasoactive substances in this response. A change in pH may affect the activity of a diverse array of intra- and extracellularly located enzymes which makes the situation complex. Under these conditions the cell may lose its ability to maintain ionic homeostasis due to a change in the activity of key enzyme systems involved in cell regulation. In a separate part of these investigations the acidosis-induced inhibition of Na/K ATPase was demonstrated. In the absence of any other effects of acidosis on other enzyme systems, a reduction in Na/K ATPase activity may lead to an increase in intracellular sodium that may exchange for calcium leading to a concomitant increase in coronary tone.

L-N^G-nitro-arginine is a novel, potent and reversible inhibitor of endothelium-dependent vasodilation *in vitro* (*Moore et al., 1990*) and it was for this reason that it was used to investigate the possible involvement of EDRF in the ability of cysteine to attenuate acidosis-induced coronary constriction. 100μM L-N^G-nitro-arginine had no effect on the coronary constriction alone or on the attenuation of the constriction by 0.1M cysteine. Therefore, the involvement of EDRF in the action of cysteine in this model is unlikely. In addition, the guinea-pig taenia coli preparation was used to investigate whether cysteine has any L-type calcium channel antagonist activity. These experiments show that cysteine (1.0mM) has no effect on the contractile response of this preparation to an increasing calcium concentration (27-2700 μM). The mode of action by which cysteine attenuates a pH-dependent vasoconstriction of the coronary vasculature remains unclear. At the present time there is no data available on the effect of cysteine on prostacyclin release during acidosis or on the synthesis and release of other endogenous vasoconstrictors or vasodilators. The data presented here shows that cysteine increases cGMP release during acidosis. Cysteine is involved in the regulation of guanyl cyclase (*Needleman et al., 1973*) and so its inhibitory effect on the acidosis-induced vasoconstriction could be a consequence of guanyl cyclase activation.

Acidosis, in the presence and absence of GSH, may cause coronary constriction in this model by the activation of L-type calcium channels in the vascular smooth

muscle or by alterations in the Na/Ca exchange process resulting in calcium influx. Vogel and Sperelakis (1977) have shown L-type calcium channels to be inhibited in the acidotic myocardium. Further experiments need to be performed to investigate the effect of verapamil and nifedipine on the physiological response of the heart to acidosis and whether these calcium channel antagonists can reverse an established acidosis-induced coronary constriction. GSH potentiates the pH dependent vasoconstriction and, therefore, it would also be of interest to investigate whether GSH potentiates the effect of the calcium channel activator BAY K8644 which, as a bolus injection, produces a concentration-dependent coronary vasoconstriction (Mestre et al., 1985).

If myocardial acidosis causes coronary constriction *in vivo* during ischaemia, the effect of GSH may be beneficial. Increased coronary constriction would result in a relatively slow restoration of oxygen and washout of metabolic substrates that has been shown to be protective against reperfusion-induced arrhythmias. These observations are of interest as they indicate that the amino acid composition of the plasma and the glutathione or thiol status of the heart could have a role in the modulation of cardiovascular responses under normal and ischaemic/acidotic conditions.

CHAPTER 11.

CONCLUSIONS.

The work described here has shown that reduced glutathione (GSH) and other related compounds may be involved in the modulation of several cardiovascular regulatory systems under normal and ischaemic conditions. Although at the present time the mode(s) of action are unclear, the intracellular ratio of reduced glutathione : oxidised glutathione (GSH/GSSG) may be a critical factor, especially under ischaemic/reperfusion conditions when oxidising/reducing species may also cause deleterious conformational changes in ion translocating systems.

The current research provides the first evidence that GSH attenuates ischaemic contracture, possibily via indirect interaction with the Na/K pump. In whole hearts, GSH attenuated reperfusion-induced ^{86}Rb efflux indicating a potassium sparing action which may be accounted for by a stimulatory action on the Na/K pump. However, interactions with other myocardial potassium channels, such as ATP-dependent potassium channels (*Noma et al., 1990*), cannot be ruled out and should be a focus of future investigations.

Although the literature contains information on the structure of the Na/K pump and locality of free sulphydryls and disulphides within this intrinsic protein, it is difficult to determine where GSH and GSSG are acting from the preliminary data described here. A potential experimental approach to elucidate these sites is the isoelectricfocusing of Na/K pump proteins, previously exposed to varying concentrations of GSH/GSSG, and the determination of whether Na/K pump is S-thiolated under normal and/or ischaemic conditions. This is a prime objective for future work. One complication is the determination of any sidedness of interactions of GSH and GSSG with the Na/K pump. Basal intracellular levels of GSH are within the 0.5-10mM range and, therefore, the removal of intracellular GSH by dialysis and replacement with known GSH and/or GSSG concentrations is a method of investigating how changes in cellular redox state affect the structure and activity of membrane bound proteins. Using compounds such as glutathione, diamide, *p*HMPSA and diethylmaleate as pharmacological tools may help elucidate how this ion pump is regulated *in vivo* under normal and ischaemic conditions. It is unlikely that the Na/K pump is the only ion translocating mechanism whose activity can be modulated by cellular redox or glutathione status. It seems reasonable to suggest that the Na/Ca and Na/H excange mechanisms, as well as

other selected receptors and ion channels, may also be affected by similar conditions. Therefore, a profile of the effect of GSH, and related compounds, on a range of ion translocating mechanisms, channels and receptors under normal and ischaemic conditions is a priority.

In preliminary studies to investigate whether GSH affects the Na/H mechanism, the physiological response of the heart to acidosis, in the presence and absence of GSH, was investigated. In contrast to its vasodilator action at pH 7.4, 1mM GSH potentiated an acidosis-induced coronary vasoconstriction at pH 6.8 and evidence has been provided for the potential involvement of cyclooxygenase metabolites in this response. Acidosis has contrasting effects in several isolated vascular beds and tissues. Therefore, preparations such as the aorta, mesentery and isolated heart may be useful in the elucidation of the mechanism(s) underlying the hearts physiological response to acidosis. The mechanism by which cysteine attenuates an acidosis-induced coronary constriction and the potentiation of this constriction by GSH remains unclear. There is some evidence for a reduction of acidosis-induced thromboxane A_2 release in the presence of cysteine, although this cyclooxygenase metabolite is not entirely responsible for the pressor response. In addition, cysteine increased cGMP release under basal and acidotic conditions and, therefore, its inhibitory effect on the acidosis induced vasoconstriction could be a consequence of guanyl cyclase activation. Clearly, *in vivo*, the situation is more complex due to the presence of circulating platelets that also release several vasoactive compounds which may influence vessel tone under control and acidotic conditions. Future work should include studies on the effect of extracellular acidosis, in the presence and absence of GSH and related compounds, on cyclooxygenase metabolism and aggregation in isolated cell types, such a platelets. These observations are of considerable interest as they indicate that the amino acid composition of the plasma, and the glutathione or thiol status of the heart could play an important role in modulating cardiovascular responses under normal and ischaemic/acidotic conditions.

Questions that need to be resolved in subsequent investigations are, firstly, how does the glutathione status of the heart affect the ability of membrane-bound proteins involved in ionic homeostasis to function normally? It is possible to

manipulate the glutathione content of whole hearts, atria, myocytes, aorta and the mesenteric vasculature by the use of selective pharmacological agents. *N*-ethylmalemide, diethylmaleate, *t*-butyl hydroperoxide, diamide and buthionine sulphoximine all deplete GSH, while intracellular GSH can be increased by the use of *N*-acetylcysteine, L-2-oxothiazolidine-4-carboxylate or glutathione isopropyl ester. By modulating the GSH/GSSG ratio using a combination of these agents, the effect of cellular thiol status on physiological responses to acidosis can be investigated in a variety of tissue and cell types.

Secondly, how do GSH, thiols and amino acids modulate the response of the heart and other vascular preparations to acidosis? In the isolated heart it is possible that other amino acids, as well as cysteine, could affect vascular responses to acidosis which may be due to the release of one or more vasoactive substances. Any future investigations into the effect of amino acids on coronary tone during acidosis should include radioimmuno- and bioassays of thromboxane A_2, prostacyclin and catecholamine release as a starting point.

Finally, myocytes, vascular smooth muscle cells, platelets and neutrophils, for example, are exposed to acidotic conditions and an altered thiol status during ischaemia *in vivo* and it is, therefore, important to determine how GSH/GSSG, thiol-containing compounds and amino acids affect ion movements in these individual isolated cells. The preliminary, fluorimetric data obtained has shown that GSH reduces while GSSG increases intracellular free calcium, in a concentration-dependent manner. These studies had one major experimental draw back in that it was not posssible to alter the composition of the suspension medium during the course of an experiment. One approach to overcome this problem in the future will be to use adherent myocytes in a superfusion set-up linked to a microscope attachment on the fluorimeter, allowing the composition of the superfusate to be changed. With this apparatus it would be possible to alter the GSH status of cells and isolated atria and simultaneosly monitor intracellular calcium, pH and sodium under acidotic and simulated-ischaemic conditions. These investigations would yield data on the actions of GSH and amino acids on cardiac contractility and ion movements under acidotic/ischaemic conditions, independent of vascular changes.

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APPENDIX 1.

INDIVIDUAL RUBIDIUM EFFLUX PROGRAMME.

This computer programme was used tc calculate ^{86}Rb efflux rate coefficients (erc's) with data obtained from liquid scintillation counting of perfusate and tissue samples. The programme was modified by Dr C. Blackwell from that originally devised by Dr M. Coldwell (SmithKline Beechams Pharmaceuticals, Harlow, Essex) and was written for use on the BBC Master Series microcomputer.

```
10
20  REM   INDIVIDUAL RUBIDIUM EFFLUX PROGRAMME
30  REM
40  @%=10
50  CLEAR
60  CLS
70  VDU2
80  PRINT TAB(12)"RUBIDIUM EFFLUX"
90  PRINT TAB(12)STRING$(15,"=")
100 VDU3
110 INPUT"DATE (DD-MM-YY) "DATE$
120 VDU2
130 PRINT"DATE  "DATE$
140 VDU3
150 REM         DATA ENTRY
160 REM      AND CALCULATIONS
170
180 INPUT "CONDITIONS " CONDITIONS$
190 VDU2
200 PRINT"CONDITIONS " CONDITIONS$
210 VDU3
220 INPUT "NUMBER OF EFFLUX TUBES" E
230 DIM A(E),B(E),D(E),L(E),M(E),P(E),R(E)
240 INPUT"START TIME(MINS)" T
250 *FX229,1
260 PRINT"ENTER EFFLUX COUNTS"
265 PRINT"AND COLLECTION PERIOD....."
270 FOR N=1 TO E
280   PRINT N;
290   INPUT A(N);L(N)
300   R(N)=R(N-1)+L(N)
310 NEXT
320 REM   INCORRECT VALUE TRAP
330
340 CLS
350 INPUT "ARE THE VALUES CORRECT?(Y/N) " YES$
360 IF YES$= "Y" THEN 490
370 CLS
380 INPUT "WHICH SAMPLE NUMBER IS WRONG? "WRONGSAMP
390 INPUT "WHAT IS THE CORRECT VALUE?  " RIGHTVAL
400 A(WRONGSAMP)=RIGHTVAL
410 CLS
420 FOR N=1 TO E
430   PRINT N,A(N)
440   IF N=20 REPEAT:PRINT"PRESS SPACE BAR TO CONTINUE":UNTIL GET=32:CLS
450 NEXT N
460 INPUT "NOW ARE THE VALUES CORRECT? (Y/N) "YES2$
```

```
470  IF YES2$="Y" THEN 490
480  GOTO 370
490  CLS
500  FOR N=1 TO E
510    B(N)=B(N-1)+(A(N)*L(N))
520  NEXT
530  PRINT'
540  INPUT "TISSUE COUNTS " C
550  PRINT
560  INPUT "TISSUE WEIGHT (mg)    " W
570  PRINT
580
590  *FX229,0
600  @%=&20309
610  FOR N=1 TO E
620    D(N)=(B(E)+C)-B(N-1)
630    P(N)=A(N)/D(N)
640  NEXT
650
660  REM         PRINTING
670  REM        OF RESULTS
680
690  VDU2
700  PRINT
710  PRINT"      EFFLUX ";"      CUMUL. ";"      TOTAL   ";"   erc  "
720  PRINT"      COUNTS ";"      TOTALS ";"      COUNTS   ";" (/min)  "
730 PRINT"     (cpm)  ";"     (cpm) ";"     (cpm)    "
740  FOR N=1 TO E
750    PRINT TAB(5);A(N);TAB(17);B(N);TAB(28);D(N);TAB(40);P(N)
760  NEXT
770  PRINT
780  PRINT
790  PRINT"TISSUE COUNTS  ";C;" cpm";"     TISSUE WEIGHT  ";W;" mg"
800  PRINT
810  VDU3
820  @%=10
830  PRINT
840  PRINT:PRINT:PRINT:PRINT
850  INPUT"DO YOU WANT THE EFFLUX CURVE PLOTTED (ON THE SCREEN)?(Y/N)"YES3$
860  IF YES3$="Y" THEN 940
870  PRINT:@%=10:VDU3
880  PRINT"DO YOU HAVE FURTHER DATA?(Y/N)"
890  INPUT A$
900  IF A$="Y" THEN 10
910  PRINT"THE END"
920  END
930
940  REM     CURVE PLOTTING
```

```
 950  MODE4
 960  VDU24,0;0;1279;1023;
 965  VDU28,11,1,39,0
 970  X=20
 980  XLENGTH=1100+100
 990  MOVE 100,100:DRAW XLENGTH,100
1000  MOVE 100,100:DRAW 100,940
1010  FOR Y=1 TO 4
1020    MOVE 100,Y*210+100:DRAW 116,Y*210+100
1030  NEXT Y
1040  FOR N=1 TO E
1050    MOVE 100+(T*X)+(R(N)*X),100
1060    DRAW 100+(T*X)+(R(N)*X),80
1070    MOVE (T+R(1))*X+100,(P(1)*7000)+100
1075  NEXT N
1080  FOR N=2 TO E
1090    DRAW (T+R(N))*X+100,(P(N)*7000)+100
1100  NEXT N
1110  VDU5
1120  PLOT 4,0,1000:PRINT"erc(/min)"
1130  PLOT 4,0,952:PRINT".12"
1140  PLOT 4,0,742:PRINT".09"
1150  PLOT 4,0,532:PRINT".06"
1160  PLOT 4,0,322:PRINT".03"
1170  PLOT 4,0,112:PRINT".00"
1180  Z=R(E) DIV 10
1190  FOR N=1 TO Z
1200    MOVE 100+(N*X*10),100
1210    DRAW 100+(N*X*10),70
1215    PRINT;
1220    PRINT;N*10
1230  NEXT N
1240  PLOT 4,425,32:PRINT"TIME(min)"
1250  VDU4
1260  PRINT"PRINT GRAPH ";:INPUT GRAPH$
1270  PRINT STRING$(14," ")
1280  PRINT"erc   :  PER TIME PERIOD"
1290
1300  IF GRAPH$="Y" OR GRAPH$="y" PROCSDUMP ELSE GOTO 1330
1310  PRINT"PRESS SPACE BAR TO CONTINUE"
1320  REPEAT UNTIL GET =32
1330  PRINT
1340  @%=10
1350  VDU3
1360  MODE7:GOTO 880
1370  DEF PROCSDUMP
1380 *SDUMP
1390  ENDPROC
```