

PROTECTION OF THE ISCHAEMIC HEART

by

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ABSTRACT

The primary purpose of the present study was to investigate the effects of ischaemia and reperfusion on developed and resting tension, tissue content of ATP and CP and mitochondrial respiratory function of isolated and perfused rabbit hearts. Three degrees of ischaemia lasting for 30, 60 and 90 minutes followed by reperfusion have been investigated. Ischaemia caused a decline of developed tension and a rise in resting tension. ATP and CP also declined during ischaemia, whilst mitochondrial function was reduced only after a prolonged period of severe ischaemia. Reperfusion resulted in a recovery of mechanical function or in a further worsening of mechanical and mitochondrial function, depending upon the degree and duration of flow reduction.

To establish if it is possible to reduce the damage induced by severe ischaemia and reperfusion, rabbits were treated with verapamil, nifedipine or propranolol before the isolation of their hearts. In these experiments measurements of mitochondrial ATP production and mitochondrial and tissue calcium were also made. Ninety minutes of severe ischaemia induced an alteration of intracellular calcium homeostasis. Reperfusion at the end of this period resulted in an increase of tissue and mitochondrial calcium. Treatment of the rabbit with verapamil, nifedipine or propranolol partially reduced ischaemic and reperfusion damage. Their cardioprotective effects appear to involve a common mechanism: an ability to slow the rate of depletion of ATP reserves during ischaemia.

Finally, the relation between mitochondrial calcium transport and oxidative phosphorylation has been examined. These experiments have been carried out using mitochondria from freshly-excised hearts, measuring rates of Ca^{2+} accumulation and ATP production and making simultaneous and continuous measurements of mitochondrial respiration and Ca^{2+} and H^{+} movements. It was concluded that mitochondrial Ca^{2+} transport and ATP synthesis compete for respiratory energy. These results are of physiological importance during post-ischaemic reperfusion, when the mitochondria in an attempt to buffer the continuously rising cytosolic calcium are using respiratory energy for calcium transport rather than for ATP production.

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LIST OF ABBREVIATIONS

$P\ddot{O}_2$	oxygen tension
Kg	kilogram
mm Hg	millimetre of mercury
ml	millilitre
μ	micro metre
ms	milliseconds
v	volt
g	gram
mM	millimolar
EDTA	ethylenediaminetetracetic acid
BSA	bovine serum albumin
tris	tris(hydroxymethyl)amino methane
μ l	microlitre
nm	nano metres
QO_2	oxygen quotient (mitochondrial oxygen consumption in the presence of ADP and phosphate)
RCR	respiratory control ratio
n atoms	nano atoms
ADP	adenosine-5'-diphosphate
mm	millimetre

LIST OF ABBREVIATIONS (Cont'd)

ATP	adenosine-5'-triphosphate
CP	creatine phosphate
G-6-P	glucose-6-phosphate
HK	hexokinase
G6P-DH	glucose-6-phosphate dehydrogenase
NADP	nicotinamide adeninedinucleotide phosphate
NADPH	reduced form of nicotinamide adeninedinucleotide phosphate
mU	milli units
CK	creatine kinase
prot.	protein
AMP	adenosine-5-monophosphate
wt	weight
A	anstrong
S.E.	standard error
b	beats
KEA	mitochondrial extraction medium, containing: potassium chloride, ethylenediaminetetracetic acid and bovine serum albumin
n moles	nano moles
μ M	micro molar

LIST OF ABBREVIATIONS (Cont'd)

NTA	nitrotriactic acid
vol.	volume
CPK	creatinphosphokinase
K _m	Michaelis constant

C H A P T E R 1

GENERAL INTRODUCTION

A. HISTORICAL INTRODUCTION

B. DEFINITION AND CLINICAL RELEVANCE OF MYOCARDIAL ISCHAEMIA

C. AIM OF THE THESIS

A. HISTORICAL INTRODUCTION

Atherosclerosis of the coronary arteries has been recognised as one of the pathological bases of coronary flow reduction leading to myocardial ischaemia. Such pathological findings have been known in man for over three thousand years (Long, 1931).

Czermak (1852) was the first author who demonstrated, histologically, sclerosis of the aorta in Egyptian mummies (an elderly woman and a boy 15 years old). Then, in 1909, Shattock in a more detailed paper, described the aorta of King Merneptah, a famous Pharaoh, "as affected by severe atheromatous disease, large calcified patches being distinctly visible".

However, it was in 1931 when Long, examining the heart of a 50 years old female mummy of the twenty-first dynasty (about 1000 B.C.), described for the first time marked fibrous thickening (chiefly of the intima) and calcification of the coronary artery.

All these findings establish the antiquity of degenerative vascular disease, but they do not prove that the existence of ischaemic heart disease was known at that time. This evidence must come from literary sources. This, of course, is complicated by the difficulties in deciphering the Egyptian hieroglyphs and, also, by the fact that the word "heart" in Egyptian, Greek, Hebrew and Arabic not only means the organ responsible for the circulation of the blood, but also means the stomach. Thus the Greek "Kardialgia" in the sources may be precordial pain as much as a gastric condition.

Nevertheless, the famous Ebers' Papyrus (1875) contains a description of what has been interpreted to be angina pectoris by Ebbell (1937) and Grapow (1935). This is the translated text:

"When you examine a man for illness in his cardia he has pain in his arm, in his breast, on the side of his cardia, it is said thereof: this is the w³d-illness. Then you shall say thereof: it is something which entered his mouth; it is death which approaches him".

It seems not unreasonable to find in this text features and elements of Herberden's angina. Pain in the arm, sensation in the left side of the breast and death threatening the patient are clearly outlined.

During the period of ancient Greece and Rome, Hippocrates, in his writings, is probably the first who described clinical allusions to coronary artery disease connected with biological ideas concerning impediment in the blood flow. The following passage is a translation from Hippocrates' "Regime in Acute Disease" Appendix, Chapter 5 (Littre II, 407; Adams I, 136; translated by Jones, 1923):

"With this alteration in the blood, air (Spiritus Vitalis, Pneuma) cannot pervade the natural passages. This stasis produces a cooling down, obscuring of the sight, loss of speech, heaviness of the head and convulsions as soon as the stasis reaches the heart".

"Alteration in the blood" apparently means a kind of deterioration of its normal state which leads to an obstruction of the blood vessel. The notion of air pervading the natural passages conforms with the

so-called "pneuma" which, according to the ancient biological theories, provides the organs with the necessary substance for survival. This is not very far from the modern concept of oxygenation.

The heart was already recognised as a vital organ, its dysfunction producing symptoms such as aphasia, and presumably, brain disorder. But what is extraordinary about this passage of Hippocrates' is that it is possible to recognise a description of what was later known as thrombosis. According to the classical definition by Virchow (1910) for thrombo-embolic processes, the factors involved are:

(1) hypercoagulability of the blood, (2) retardation of the blood flow and (3) lesion located in the coat of the vessel. Of these three factors, the first two are referred to in the above passage.

Knowledge about coronary disease increased with the numerous works of Galen, a Greek physician who lived in the great Roman metropolis. He provided anatomical descriptions and physiological significance of the coronary vessels. He described in "Anatomical Procedures", Book VII, Chapter 10 (translated by Singer and Rabin, 1946) very precisely the function of blood supplying the heart muscle through the coronary arteries and differentiated between arterial and venous function. Galen speculated about the quantity of the blood supplied to the heart and its ultimate distribution. He also gave an insight into the role of oxygenation with the following phrase "when the heart is deprived of respiration it dies instantaneously" (Kühn, V, 229; Daremberg, II, 626).

A much later author, Caelius Aurelianus, who lived in the Fifth Century wrote a long chapter in Latin, translated by Siegel (1961) where

there are a few allusions to coronary artery disease with a description of a morbid state (now termed as "shock") which may accompany a coronary event, but which can also be provoked by other etiological factors.

To the genius of Leonardo da Vinci (1452-1519) we owe the pictorial representation of the coronary arteries. For the first time he depicted the origins of the coronary vessels from the coronary sinus. He was also interested in the changes produced by the aging process. His drawings from "Anatomy of the Old Man" are, in fact, the first to give a representation of sclerotic and tortuous coronary vessels and he stated that the "vessels in the elderly, through the thickening of their tunnels, restrict the transit of the blood".

The next steps were made in the Sixteenth Century. Antonio Benivieni from Florence (1507) described extremely well a clinical case of pain in the heart, while the pathological findings are not significant. Also the description by Amatus Lusitanus (1560) of sudden death due to an obstruction in the heart lacks anatomical verification. Another Renaissance physician, Realduus Columbus (from Padua) (1559) provided a more precise understanding of coronary physiology, coming closer to the modern concept of oxygenation. In his book "De re Anatomica" he said that "the coronary arteries vivify the heart substance by the vital heat thereby conveyed to it". However the "vital spirits" are not engendered in the heart (as Galeno thought) but in the lungs. He also argued that if the heart vivified the blood, then there would be no need for the coronary artery to supply vital spirit to the heart substance.

The Seventeenth Century is characterised by the description of William Harvey (1649) of a "third and extremely short circulation" through the coronary arteries and veins. Bellini in 1683 described calcification of the coronary vessels with occlusion and appropriate clinical signs; and Thebesius (1708) not only referred to calcifications of the coronaries in his book, but also provided one of the earliest pathological illustrations of the condition.

With the advent of the Eighteenth Century a considerable body of facts and concepts have been elaborated. This was mainly because of the technique of history-taking and recording reaching high perfection and also because of the dissection of human bodies, especially in Italy becoming more common, often supported by the Church. Thus, Lancisi (1707), a papal physician in Rome, presented two books, one on sudden death (*De Subitaneis Mortibus*) and the other on aneurysm (*De Aneurysmatibus*, 1728) with clinical data and detailed autopsy reports. Great progress in correlating clinical and pathological data was made by Morgagni (1761). He studied, from the clinical-pathological point of view, almost 700 cases and speculated that the replacement of the myocardium by fibrous tissue may weaken the force generated by the heart. However, it was not until 1772 that the first organised and lucid clinical description of angina pectoris was presented by William Heberden (1772) in a paper entitled "Some Account of a Disorder of the Breast" and published four years later. Later clinicians were unable to add much to Heberden's description of the clinical features of angina pectoris. He recognised that exertional angina pectoris was

more readily brought on after a meal or walking up a hill. He realised the predominance of angina in males and appreciated that it may be terminated by sudden death. His case material also included a description of what, nowadays, we would qualify as unstable angina. However, he did not attempt to speculate on the physiological or anatomical basis of angina pectoris but, indeed, having established precordial pain as a working diagnosis stimulated several researches on ischaemic heart disease, particularly in England. During the early part of the Nineteenth Century the coronary theory of the disease, so vigorously expanded by the British school, gained international acknowledgement. An early criticism, however, was already expressed by Warren (1812) who felt that the ossification theory is "apt to simplify too much".

The last part of the Nineteenth Century is characterised by major contributions from English, French, German and Italian pathologists who put together the entire pathological picture of coronary artery disease. In 1895, Langendorff described a technique to perfuse the isolated heart and since that time the first experiments on glucose metabolism started (Locke and Rosenheim, 1907; Knowlton and Starling, 1912; Evans, 1914). In 1939, Evans reviewed the metabolism of the isolated heart.

Herrick, in 1912, published the first ECG tracing from a patient with myocardial infarction. The major interest in the second half of the Twentieth Century included epidemiological studies (Kannel et al., 1962), identification of risk factors (Rinzler, 1968) as well as the concept of coronary care unit (Day, 1962).

One of the most significant advances in the understanding of the natural history of myocardial ischaemia has been the introduction of selective angiography. Forssman in 1929 introduced a rubber catheter from the left cephalic vein into the right atrium of his heart. Only four years later Rousthoi utilised Forssman's technique to opacify the coronary artery tree.

But it was only in 1959 that Sones described a direct, straightforward approach for opacification of the coronary arteries and since then coronary arteriography made possible the development of a new surgical approach to ischaemic heart disease. Furthermore, the technique of cardiac catheterisation made possible the first studies on the heart metabolism in patients by means of the catheterisation of coronary sinus (Bing, 1954; 1965).

B. DEFINITION AND CLINICAL RELEVANCE OF MYOCARDIAL ISCHAEMIA

Within the body, tissues are constantly supplied with oxygen and nutrients necessary to maintain cell life and function through the blood vasculature. Tissue activities are not constant and as they increase, blood flow also increases. The ability to adapt blood supply to cell activity is particularly marked in muscle tissue where great extremes in oxygen uptake exist, from the resting to the fully activated state. The heart is primarily an aerobic organ and, as its oxygen extraction at rest is almost maximal, an increase of oxygen uptake is met by an increase of coronary flow more than by enhancing extraction. The predominance of aerobic metabolism in the heart is reflected, structurally, by the abundant mitochondria which occupy over 35% of the myocardial volume.

Fractional uptake of oxygen in the heart, therefore, is very high (77.9%), while fractional uptake of the different substrates is low (18.2% for FFA, 2.6% for glucose, 15.7% for lactate) (Bergman et al., 1980).

In addition within the myocardium there are relatively great stores of carbohydrates and lipid, while there is enough oxygen in the muscle to support only 5 active beats. Therefore a decrease in flow becomes rate-limiting for oxygen supply long before it does for glucose or FFA.

The term myocardial ischaemia describes a condition due to a reduction in coronary blood flow severe enough to have metabolic consequence such as depletion of ATP and anaerobic metabolism (Jennings, 1970). It is difficult to quantify the degree of coronary flow reduction

required to induce ischaemia, as it is relative to the uptake of oxygen. In men, myocardial ischaemia usually is secondary to coronary arterial disease and, if it is severe and prolonged enough to produce irreversible damage and development of tissue necrosis, leads to a myocardial infarction.

Ischaemic heart disease is the most common serious health problem of contemporary society. Braunwald (1980) reported that in the U.S., alone, almost one million patients die each year from ischaemic heart disease and its complications; approximately 1,300,000 patients have myocardial infarction and countless more suffer from congestive heart failure secondary to ischaemic myocardial damage.

It is therefore not surprising that the understanding of biochemical disturbances produced by myocardial ischaemia has become one of the most important topics in cardiological research.

C. AIM OF THE THESIS

The primary aim of the thesis has been to study the effect of gradual reduction in coronary flow and subsequent reperfusion on developed tension, resting tension, tissue concentrations of ATP and CP and mitochondrial respiratory function.

These experiments have been carried out on the isolated, perfused rabbit heart.

A number of interventions have been used to modify the effects of ischaemia and reperfusion. These comprise: the use of the "calcium antagonists" verapamil and nifedipine and of the β -adrenergic receptor antagonist, propranolol.

Many of the harmful effects of ischaemia and reperfusion appear to be related to abnormalities of calcium homeostasis and, in these experiments, measurements of tissue and mitochondrial calcium were also made.

The final section of the thesis examines the relation between mitochondrial calcium transport and oxidative phosphorylation.

C H A P T E R 2

THE EFFECTS OF GRADED REDUCTION OF CORONARY FLOW AND SUBSEQUENT
REPERFUSION IN THE ISOLATED LANGENDORFF PERFUSED RABBIT'S HEART

A. INTRODUCTION

B. EXPERIMENTAL

- (a) Perfusion of the hearts
- (b) Resting and developed force measurements
- (c) Mitochondrial enriched fraction preparation
- (d) Electron microscopy
- (e) Protein determination
- (f) Polarographic determination of mitochondrial respiration
- (g) ATP and CP determination
- (h) Statistical analysis
- (i) Animals and materials
- (j) Protocol

C. RESULTS

- (a) Effect of different degrees of ischaemia and subsequent reperfusion on developed tension
- (b) Effect of different degrees of ischaemia and subsequent reperfusion on resting tension
- (c) Effect of oxygen and glucose content of the perfusate on developed and resting tension during low flow ischaemia and subsequent reperfusion
- (d) Effect of different degrees of ischaemia and subsequent reperfusion on ATP and CP tissue concentration

C. RESULTS (cont'd)

- (e) Effect of oxygen and glucose content of the perfusate on tissue ATP and CP after 30 and 60 minutes of low flow ischaemia and subsequent reperfusion
- (f) Effect of different degrees of ischaemia and subsequent reperfusion on mitochondrial function
- (g) Effect of oxygen and glucose content of the perfusate on mitochondrial function after 30 and 60 minutes of low flow ischaemia and subsequent reperfusion

D. DISCUSSION

- (a) Effect of ischaemia on developed tension
- (b) Effect of ischaemia on resting tension
- (c) Effect of post-ischaemic reperfusion on developed and resting tension
- (d) Effect of ischaemia and reperfusion on tissue levels of ATP and CP
- (e) Effect of ischaemia and reperfusion on mitochondrial function

A. INTRODUCTION

Many authors have pointed out that there are wide differences in flow across an infarcting area of myocardium (Hood, 1971; Vokonas et al., 1974; Waters et al., 1977; Opie, 1980). The ischaemic damage, also, has been shown to be not homogeneous (Jennings et al., 1960; Hearse et al., 1977). It has been suggested that surrounding the severely damaged area (where coronary flow can be reduced to zero) may exist a region of moderate damage (where coronary flow is moderately reduced) separating it from the normally perfused non-ischaemic tissue. Histological analyses have been shown that adjacent individual heart cells may be differently affected. A severely damaged cell may lie next to one which still appears to be normal. An ischaemic zone, therefore, is a mixture of dead, dying and normal cells, subjected to a wide range of reduction in blood flow. The degree of coronary flow reduction during ischaemia may influence the degree of ischaemic damage, as it can provide some oxygen supply to the ischaemic cell and remove metabolic products from the interstitial space.

In this Chapter the effects of different degrees and duration of coronary flow reduction on force development, tissue ATP and CP content and isolated mitochondrial function have been studied.

The experiments have been carried out using isolated Langendorff perfused rabbits' hearts in which the rate of coronary flow can be precisely regulated and held constant. Three different degrees of ischaemia lasting for 30, 60 and 90 minutes have been

investigated, after which the effects of post-ischaemic reperfusion have been followed.

Finally, in a reduced number of experiments, PO_2 and glucose content of the perfusate was changed in order to investigate the relevance of aerobic or anaerobic metabolism during ischaemia.

B. EXPERIMENTAL

Adult, New Zealand white rabbits (2.5 to 3.0 Kg) were used for this study.

(a) Perfusion of the hearts

Rabbits were killed by a blow on the neck and the hearts rapidly removed and immersed in ice-cold Krebs-Henseleit solution (Krebs and Henseleit, 1932) containing (in millimolar): sodium chloride, 115.0; sodium bicarbonate, 25.0; potassium chloride, 4.0; potassium dihydrogen phosphate, 0.9; magnesium sulfate, 1.1; calcium chloride, 1.5 and glucose, 11.0, with oxygen equilibrated to room air. After the extraneous fat and connective tissue were removed, the aorta was exposed and suspended on a metal cannula. The tip of the cannula was positioned above the coronary ostia and the aortic valve. The hearts were perfused by the non-recirculating Langendorff technique (Langendorff, 1895) and the perfusion was started at constant flow, regulated by a Watson Marlow (MHRE MK3, Marlow Buckinghamshire, U.K.) rotary pump which was interposed between the reservoir and the aortic root. The initial perfusion pressure in the system was adjusted to approximately 30 mmHg and, after the first minutes of perfusion, it was increased to reach 60 to 70 mmHg.

This provided a mean coronary flow of 25 ± 1.72 ml/min. The perfusion pressure was measured at the head of the aortic inflow cannula either with a Statham P23 DB transducer or an aneroid manometer and the coronary flow by timed collection of the coronary

effluent into glass vials.

The perfusate was Krebs-Henseleit buffer solution equilibrated at atmospheric pressure with a gas mixture containing 95% O_2 - 5% CO_2 ; pH 7.4. It was delivered to the heart from a jacketed reservoir which was continuously warmed by water circulating from a bath adjusted to 40°C. This provided a constant wall temperature of the heart of 37°C as measured by means of a micro-thermistor inserted in the wall of the left ventricle. Before being delivered to the aortic cannula the perfusion fluid passed through cellulose-acetate filters, pore size 5.0 μ (Millipore Ltd., Bedford, Massachusetts, U.S.A.). A jacketed bubble-trap was also included in the perfusion line. The hearts were contained in a glass-jacket connected with a circulating water bath (Fig. 2.1).

A stab wound was made in the apex of the left ventricle to drain excess volume arising from the Thebesian circulation.

The hearts were paced, using suprathreshold rectangular pulses of 0.1 ms duration delivered from a Tektronix square wave generator assembly at a frequency of 180 per minute. The stimuli were delivered via two platinum electrodes, one attached to the metal inflow cannula and the other to the ventricular apex. Threshold was determined by slowly increasing the stimulator voltage until capture occurred. The stimulator voltage was then adjusted to 0.5 V above threshold. Under aerobic conditions capture was achieved with less than a 3 V impulse.

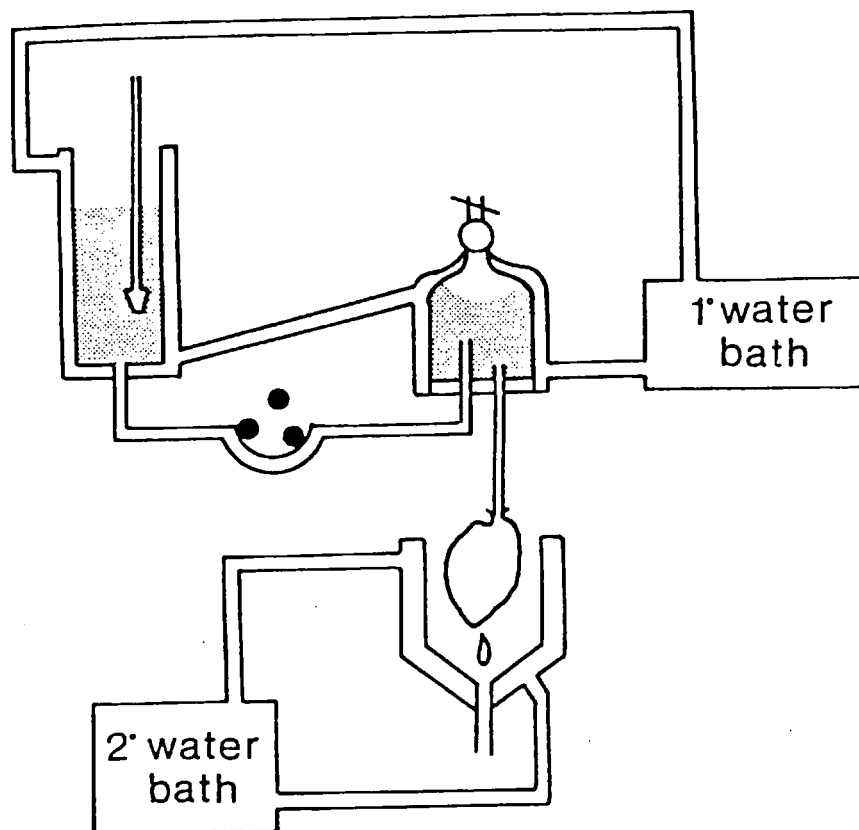


FIGURE 2.1 SCHEMATIC REPRESENTATION OF THE PERFUSION APPARATUS

(b) Resting and developed force measurements

Force development was monitored with a Grass FT03 force displacement transducer (Grass Inst., Mass., U.S.A.) attached via a nylon ligature to the ventricular apex. The output from the transducer was displayed on a Devices direct writing recorder (MX 12).

At the start of each experiment resting tension was adjusted until the developed tension reached its peak. This involved applying a resting load between 2 and 5 g weight.

The increase in resting tension was calculated as the difference between the resting tension recorded at a given time and the pre-set tension.

(c) Mitochondrial enriched fraction preparation

The isolation procedure was taken from that described by Williams and Barrie (1978).

After the rabbits were stunned by a blow on the neck, or at the end of perfusion, the hearts were removed from the chest, or from the Langendorff apparatus and quickly placed in an ice-cold medium containing 180 mM KCl, 10 mM ethylenediaminetetracetic acid (EDTA) and 0.5% bovine serum albumin (BSA), adjusted to pH 7.2 with TRIS-[hydroxymethyl]amino methane (TRIS) (Medium 1). The vessel trunk, the auricles and the ring of fat around the ventricles were removed. The ventricles were washed in medium 1 to remove excess perfusate, finely chopped with a razor blade into small pieces and weighed.

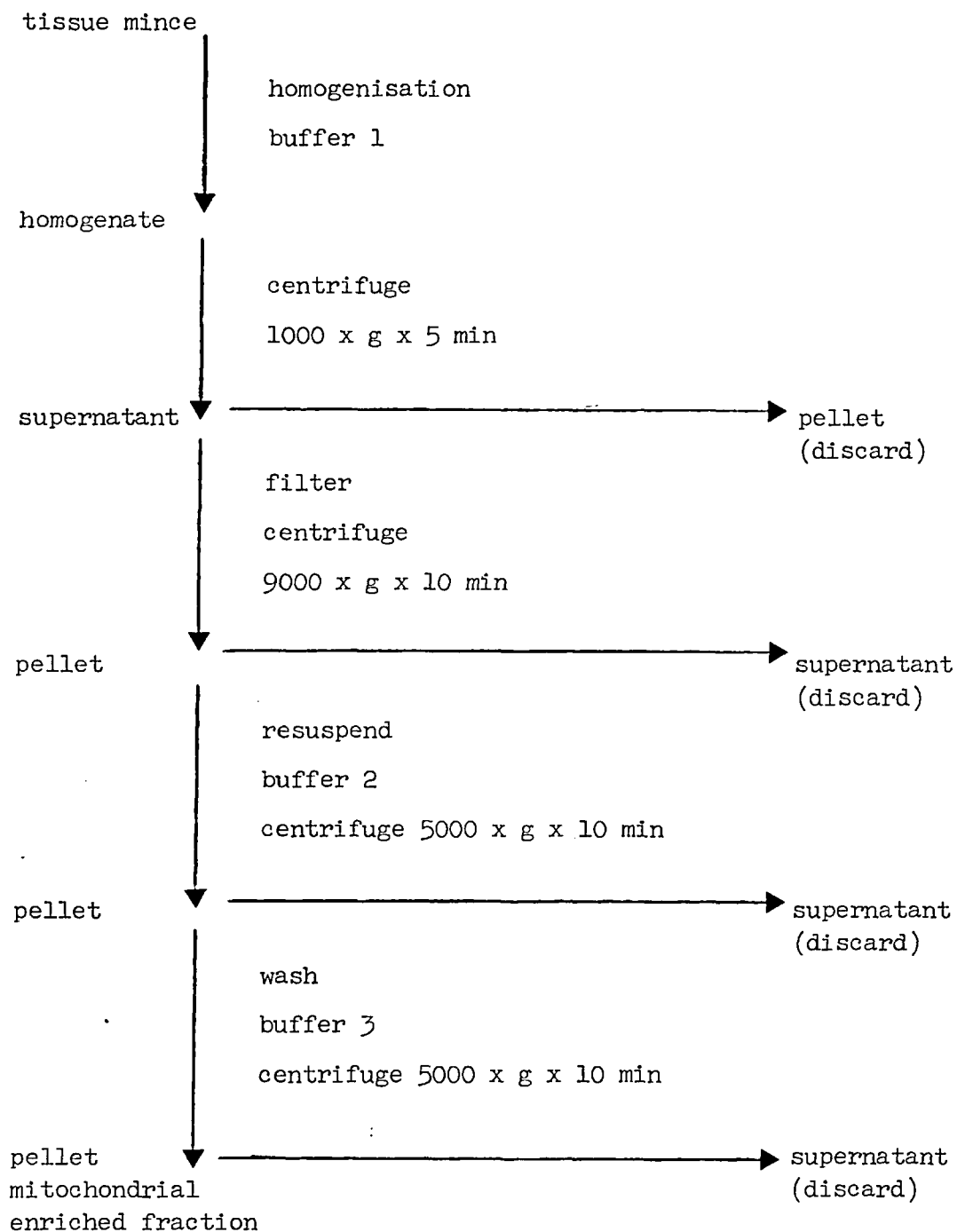


FIGURE 2.2 SCHEME FOR ISOLATION OF A MITOCHONDRIAL ENRICHED FRACTION FROM RABBIT VENTRICULAR TISSUE.

Details are under experimental procedures.

The tissue, after resuspension in the same buffer, was then homogenised using an ultra turrax homogeniser at speed setting 4.5 for 10 seconds. The homogenate was centrifuged at 1000 g at 4°C in an M.S.E. high speed 18 centrifuge for 5 minutes. The supernatant was filtered and recentrifuged for 10 minutes at 9000 g at 4°C. The resulting crude mitochondrial pellet was resuspended in 2 ml of a medium containing 180 mM KCl, 1 mM EDTA, 0.5% BSA, adjusted to pH 7.2 (medium 2) and centrifuged for 10 minutes at 5000 g at 4°C. The mitochondrial pellet obtained from this centrifugation was washed again in 2 ml of a medium containing 180 mM KCl and 0.5% BSA (medium 3) and centrifuged for 10 minutes at 5000 g at 4°C. The final pellet was collected and resuspended in 0.5 ml of medium 3 (Fig. 2.2).

The composition of the mitochondrial pellets was checked by electron microscopy.

(d) Electron microscopy

Negatively stained samples were prepared by either of the following procedures:

50 µl of freshly made 1% phosphotungstic acid, pH 7.0 were mixed with 20 µl of membrane preparation (20 - 30 mg protein/ml) on a glass slide. After mixing with a pasteur pipette, celloidin-coated copper grids were floated on the surface, removed after a few seconds and allowed to dry.

Alternatively, 20 µl of membrane preparation were dropped on to a glass slide and a celloidin-coated grid was applied to the drop.

Then the grid was inverted and dried on the edge of a filter paper. The grid was then placed on a drop of 1% phosphotungstic acid for 3 to 5 seconds and allowed to dry before examination.

Samples were examined with a Philips 301 electron microscope. A representative example is given in Fig. 2.3.

(e) Protein determination

Mitochondrial protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

3 ml of a solution containing 100 mg cromassie brilliant blue G, 50 ml 95% ethanol and 100 ml of orthophosphoric acid in a volume of 250 ml of distilled water were added to the samples, shaken and allowed to stabilize for 10 minutes. Absorbance was measured at 595 nm. Blanks and bovine serum albumin standards were assayed at the same time.

(f) Polarographic determination of mitochondrial respiration

Mitochondrial respiration was measured polarographically at 25°C using a Gilson oxygraph and a Clark type oxygen electrode (Clark et al., 1953).

This type of oxygen electrode consists of a platinum cathode and silver anode in saturated potassium chloride solution. When a potential (between 0.5 and 0.8 volt) is applied across the two electrodes, immersed in an oxygen-containing solution, oxygen is electrolytically reduced and the current generated is directly proportional to the oxygen concentration of the solution.

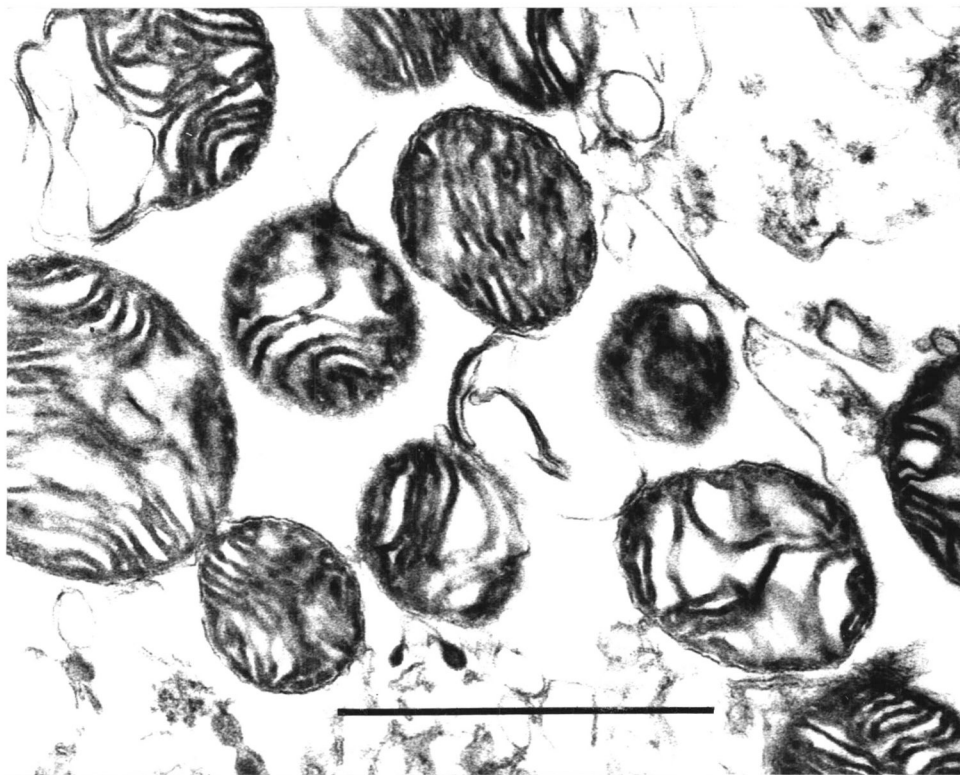


FIGURE 2.3 MICROGRAPH OF ISOLATED MITOCHONDRIAL FRACTIONS.

High power magnification: 50,000.

In the Gilson oxygraph, the electrode assembly is enclosed in a glass vessel of 2 ml capacity and surrounded by a water jacket which was continuously warmed by circulating water from a bath adjusted to 25°C.

The electrode is separated from the bulk of the solution by a polyethylene membrane to prevent deposition of materials on the electrode surfaces and a new membrane was used every day. The reaction medium was continuously mixed using a magnetic stirrer and the reagents were added through a small hole on the top of the glass vessel.

The reaction medium consisted of 250 mM sucrose, 3 mM KH_2PO_4 , 0.5 mM EDTA and 3 mM K glutamate, pH 7.4 adjusted with tris buffer. Mitochondria (to provide a final concentration of 1.25 mg mitochondrial protein/ml reaction medium) were added to the reaction chamber and allowed to equilibrate and respire. State 3 respiration was initiated by adding 0.25 mM ADP, pH 7.4.

Mitochondrial function was assessed in terms of QO_2 , RCR and ADP/O ratio, where QO_2 is n atoms of oxygen used/mg of mitochondrial protein/min in response to the addition of ADP. RCR (respiratory control ratio) is the ratio of oxygen used in the presence of ADP to that used in the absence of ADP. The ADP/O ratio is the nanomoles ADP used/n atoms of oxygen consumed.

Figure 2.4 shows an example of how these indices can be directly calculated from a typical oxygen electrode tracing. A and C are the rates of the respiration in the steady state and B the rate of

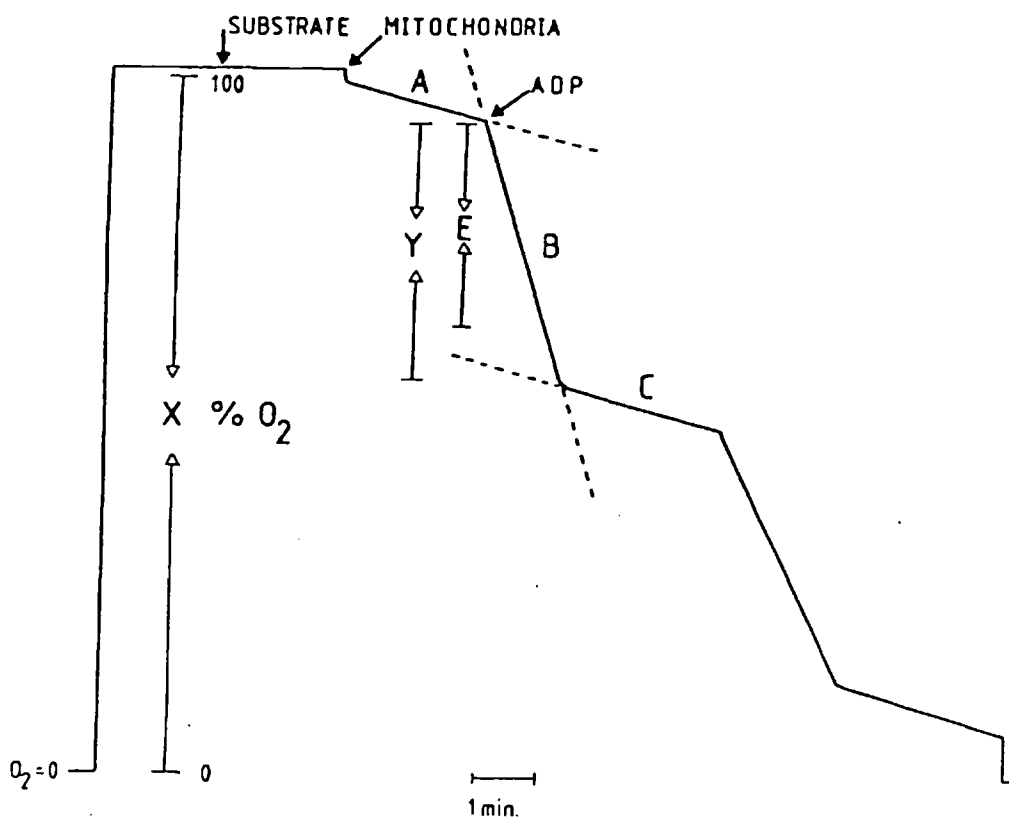


FIGURE 2.4 TYPICAL CURVE OF OXYGEN UPTAKE BY MITOCHONDRIA

Calculations:

$$\text{Respiratory control ratio (RCR)} = \frac{\text{respiration rate in presence of ADP}}{\text{respiration rate in absence of ADP}} = \frac{\text{slope B}}{\text{slope C}}$$

QO_2

$$\text{n atoms of oxygen/min/mg protein in the presence of ADP} = \frac{480 \cdot \frac{E}{X} \cdot \text{ml medium}}{\text{mg protein}}$$

$$\text{ADP/O} = \frac{\text{n moles ADP added}}{\text{n moles oxygen utilised}} = \frac{\text{n moles ADP added}}{480 \cdot \frac{Y}{X} \cdot \text{ml medium}}$$

phosphorylating respiration caused by the addition of ADP.

The distance Y is measured by extending the slopes of lines A, B and C and measuring the number of recorder divisions (or mm) from the intersect of the curves A - B and B - C. This is a measure of the extent of ADP - stimulated respiration (state 3). The deflection X expresses the total oxygen content of the reaction medium and in our experiments the solubility of oxygen in the air saturated, temperature-equilibrated medium was taken to be 480 n atoms ml⁻¹ at 25°C and 760 mmHg

The deflection E expresses the extent of ADP-stimulated respiration in 1 minute.

After each mitochondrial extraction, duplicate mitochondrial respiration measurements were established, so that each estimation could be made in duplicate and the results averaged.

(g) ATP and CP determination

The method was taken from that described by Lamprecht and Trautschold (1974).

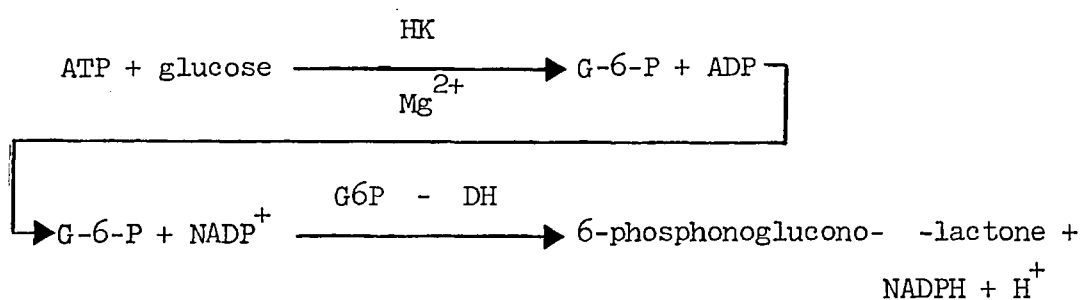
The perfusion of hearts in which adenosine-5'-triphosphate (ATP) and creatinine-phosphate (CP) concentrations were to be measured was terminated by freeze-clamping between large aluminium tongs precooled in liquid nitrogen as described by Wollenberg et al. (1960).

Only the tissue samples which were quickly pressed between the jaws of the quick-freeze tongs were used for the assay and the portion of the sample projecting over the edges of the blocks were

used to determine the wet to dry weight ratio.

The sample for determination, (approximately 200 mg) was quickly weighed and powdered in a steel percussion mortar with repeated addition of liquid nitrogen. Then the tissue powder was transferred into an homogeniser tube maintained at the temperature of liquid nitrogen, and was deproteinised by adding 6% perchloric acid in a ratio of 4:1. The mixture was then allowed to become warm and, just as it turned into fluid, was homogenised for 30 seconds while being kept cool in ice. The homogenisation was carried out using an ultra-turrax homogeniser at setting 4.5. The resultant suspension was centrifuged at 2000 g for 10 min at 0°C and the supernatant adjusted to pH 7.4, by adding 30% KOH saturated with KCl. The solution was then centrifuged at 2000 g for 10 minutes at 0°C and the supernatant used for the determination of ATP and CP.

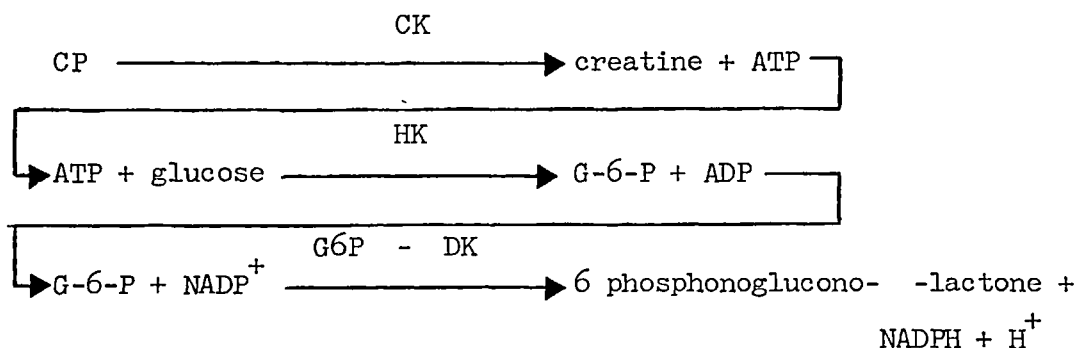
The ATP assay was carried out enzymatically by a spectrophotometric method. The reaction is based on the principle that ATP phosphorylates glucose to glucose-6-phosphate (G-6-P) in the presence of hexokinase (HK) and Mg^{2+} . Glucose-6-phosphate dehydrogenase (G6P-DH) catalyses the dehydrogenation of G-6-P with NADP to give 6-phosphonoglucono- γ -lactone and $NADPH + H^+$:



Therefore, for each mole of ATP, 1 mole of NADPH is formed and this product was determined by measurements of its extinction at 365 nm.

The reaction was carried out in cuvettes at 25°C by adding 0.1 ml of heart extract (or standard or blank) to 2.9 ml of assay medium containing 38 mM TRIS, 0.33 mM NADP, 6.66 mM MgCl_2 , 50 mM glucose, 462 mU/ml of G6P-DH suspension and finally, 1.8 U/ml of hexokinase suspension, pH 7.4. The small changes in extinction that occurred on adding the enzyme were determined once for each batch of enzyme and the values were corrected by this amount. The change in extinction was completed 15 minutes after the addition of hexokinase suspension and standards and blanks were determined with every assay.

When the change in extinction was established, 0.02 ml of 22 mM ADP (final conc. 0.15 mM) was added to provide excess ADP for the subsequent assay of creatine-phosphate and any further change in extinction was noted. The CP assay was based on the principle that the enzyme creatine kinase (CK) catalyses the transfer of phosphate from creatine phosphate to adenosine diphosphate to give creatine and adenosine triphosphate as:



One mole of NADPH therefore, is formed per mole of creatinine phosphate.

The reaction was initiated by adding 306 mU/ml of creatine kinase solution and reading the changes of extinction which followed. The change of extinction was completed in 20 minutes and standards and blanks were determined with every assay. Creatine kinase was made fresh every day. The dilution, due to the pretreatment of the sample during the extracting procedure was taken into account in the calculations. The wet to dry weight ratio was calculated by drying at 100°C to constant weight, the weighed tissue samples projecting over the edges of the quick-freeze tongs.

(h) Statistical analysis

Results are expressed as mean $\pm$ S.E. of n experiments. Tests of significance were made using Student's "t" test, taking $P = 0.05$ as the limit of significance.

(i) Animals and materials

New Zealand white rabbits were obtained from Hylyne, Cheshire, U.K. Standard laboratory chemicals of analar grade were purchased from BDH Ltd., Poole, Dorset, U.K. All enzymes were obtained from either Sigma Chemical Co. (London), Poole, Dorset, U.K. or Boehringer Corporation (London).

(j) Protocol

A period of 30 minutes after cannulation was allowed for

equilibration before any experimental intervention. During this time, hearts were beating spontaneously and were perfused with aerobic Krebs-Henseleit buffer. Hearts were then electrically paced to beat at 180 beats/min, as described.

Hearts were then divided into two major groups. In one group the hearts were perfused aerobically at a coronary flow of 25 ± 1.72 ml/min as described above for 30, 60, 90, 120 minutes. The hearts of the second group were subjected to three different degrees of global ischaemia. In one sub-group moderate ischaemia was induced by reducing the coronary flow rate to 3 ml/min (12% of control value). In a second sub-group severe ischaemia was induced by reducing the coronary flow to 1 ml/min (4% of control value). In a third sub-group the coronary flow was completely abolished to provide no flow ischaemia (or total ischaemia). When low flow or severe low flow ischaemia was induced, the perfusate consisted of Krebs-Henseleit buffer, equilibrated with 95% O_2 - 5% CO_2 and with glucose as substrate (11 mM).

In order to prevent cooling when the flow was discontinued or reduced, the temperature of the water jacket surrounding the heart was increased. To achieve that, the water jacket was connected via a two-way stop cock with a second bath where water was maintained at a temperature between $65^{\circ}C$ and $78^{\circ}C$ according to the degree of ischaemia (Fig. 2.1). Previous experiments showed that the temperature required in the surrounding water jacket to prevent cooling of the hearts was $65^{\circ}C$ for low flow ischaemia, $73^{\circ}C$ for severe low flow

ischaemia and 78°C for total ischaemia. During unrestricted perfusion and during reperfusion, the surrounding water jacket was connected with the circulating water bath of the reservoir (adjusted to 40°C).

At the onset of ischaemia the water jacket was abruptly connected with the second water bath by means of the two way stop cock. In this way, the wall temperature of hearts could be maintained at $36 \pm 0.5^{\circ}\text{C}$ irrespective of coronary flow.

After 30, 60, 90, 120 minutes of unrestricted or ischaemic perfusions the hearts were used either for ATP and CP determination or for the isolation of their mitochondria. In separate groups of experiments the hearts were reperfused after 30, 60, 90 minutes of ischaemia. Reperfusion was for 30 minutes at a coronary flow of 25 ml/min with Krebs-Henseleit solution before the assay of ATP and CP or the isolation mitochondria. Developed and resting tension were monitored in all the experiments.

A separate series of experiments was also undertaken in order to study the relevance of aerobic and anaerobic metabolism during ischaemia.

For this purpose hearts were perfused at low flow (3 ml/min) using the Krebs-Henseleit buffer equilibrated with 95% N_2 and 5% CO_2 to provide $\text{PO}_2 < 6$ mmHg with or without glucose.

Substrate-free perfusate was prepared by replacing the glucose in the Krebs-Henseleit solution with an equimolar (11 mM) concentration of mannitol. The data on mechanical function, ATP and CP concentrations and mitochondrial function obtained after 30 and 60 minutes of low

perfusion using these buffers were compared with those obtained when ischaemia was induced simply by reducing the coronary flow rate without changing the PO_2 or glucose content of the perfusate.

In a separate group of experiments, hearts were perfused for 30 and 60 minutes low flow conditions, either with aerobic perfusate or hypoxic perfusate with or without glucose after which they were reperfused for 30 minutes with aerobic perfusate containing glucose at a coronary flow rate of 25 ml/min before the assay of ATP and CP or the isolation of mitochondria.

C. RESULTS

(a) Effect of different degrees of ischaemia and subsequent reperfusion on developed tension

Figure 2.5 (A) shows that, in the isolated rabbit hearts perfused under aerobic conditions for 120 minutes at coronary flow of 25 ± 1.72 ml/min with oxygenated Krebs-Henseleit buffer, the developed tension remained constant for the first 60 minutes of perfusion and was only slightly decreased after 120 minutes.

Figure 2.5 (A) also shows that, when coronary flow was reduced from the unrestricted rate of 25 ml/min to 3 ml/min, developed tension rapidly fell to close to zero. A slightly faster fall ($P < 0.05$) in developed tension occurred when the coronary flow was reduced to 1 ml/min (Fig. 2.5, B) and 0 ml/min (Fig. 2.5, C).

Contractile activity of the hearts had completely ceased ten minutes after reducing the coronary flow to 1 ml/min or 0 ml/min despite an increase in the pacemaker stimulus to 5 V. Forty minutes after the reduction of coronary flow to 3 ml/min the tension developed was still 6% of control values and ventricular activity ceased completely only 60 minutes after the onset of low flow perfusion (Fig. 2.5, A).

Reperfusion of ischaemic hearts resulted in some restoration of their capacity to generate active tension (Fig. 2.5, A, B and C), the percentage of recovery being inversely proportional to the duration of the ischaemic period in all three groups (Table 2.1).

Table 2.1 and Figure 2.5 (A), (B) and (C) show that the degree

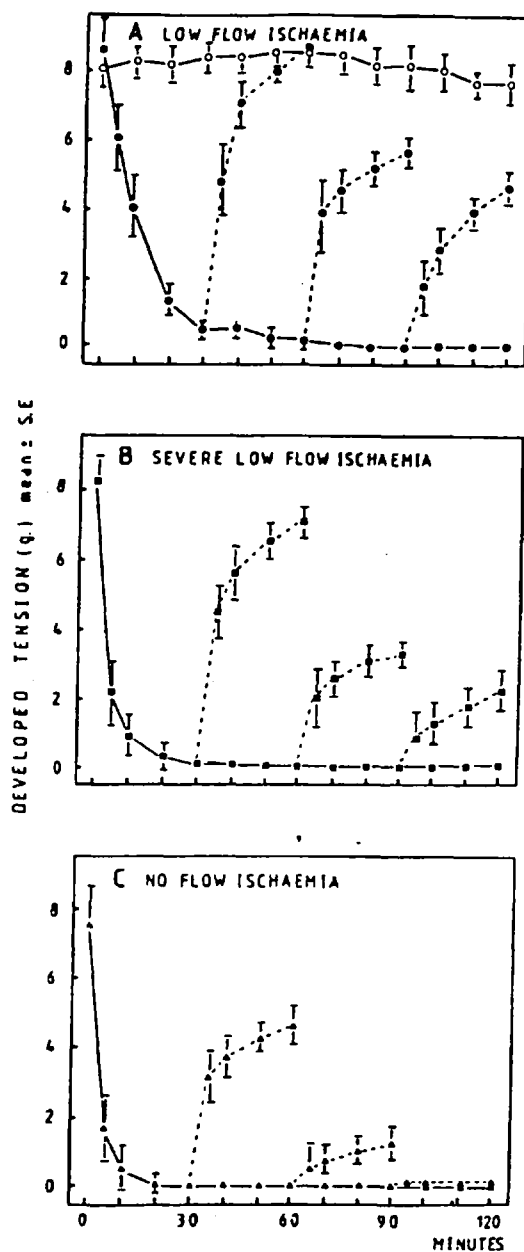


FIGURE 2.5 EFFECT OF DIFFERENT DEGREES OF ISCHAEMIA AND SUBSEQUENT REPERFUSION ON DEVELOPED TENSION

Solid lines represent developed tension during ischaemia and broken lines developed tension during reperfusion.

A. Open circles represent aerobic group (coronary flow 25 ± 1.72 ml/min)

Closed circles represent moderate low flow ischaemia (coronary flow 3 ml/min)

B. Severe low flow ischaemia (coronary flow 1 ml/min)

C. No flow ischaemia (coronary flow stopped)

Ischaemia was started at time 0

TABLE 2.1 PERCENTAGE RECOVERY OF DEVELOPED TENSION DURING REPERFUSION

Duration of Ischaemia (Min) before Reperfusion	30	60	90
Moderate low flow ischaemia (3 ml/min)	100	65	53
Severe low flow ischaemia (1 ml/min)	83	39	21
No flow ischaemia	62	20	0

The percentage of developed tension recovered after 30 minutes of reperfusion is calculated with respect to the tension developed before the onset of ischaemia.

of tension recovered during reperfusion was also inversely proportional to the degree of reduction of coronary flow, the highest percentage of tension recovery occurring in the group with coronary flow rate maintained at 3 ml/min and the lowest in the group with no coronary flow.

Reperfusion after 90 minutes of ischaemia resulted in some recovery of developed tension only in the 3 ml/min and 1 ml/min ischaemic groups (Fig. 2.5, A, B).

(b) Effect of different degrees of ischaemia and subsequent reperfusion on resting tension

Figure 2.6 (A) shows that the resting tension of hearts perfused with oxygenated Krebs-Henseleit buffer at a coronary flow of 25 ± 1.72 ml/min remained constant for 120 minutes. Figure 2.6 (A), (B) and (C) also shows that, during 120 minutes of ischaemic perfusion, all groups developed a significant increase in resting tension. When the coronary flow was reduced to 3 ml/min (Fig. 2.6, A), there was a transient initial reduction in the resting tension which was immediately followed by a progressive increase. By the end of 80 minutes the resting tension had risen to 6.3 ± 1.2 g, and by the end of 120 minutes of perfusion, it had increased to 7.2 ± 1.3 g. This pattern is in contrast to the pattern in 1 ml/min ischaemia (Fig. 2.6, B), or zero flow ischaemia (Fig. 2.6, C), in which resting tension began to rise only 20 minutes after the onset of ischaemia and its increase during the first 80 minutes of perfusion was less in extent. Resting tension values of the 1 ml/min and total ischaemia groups were, within the

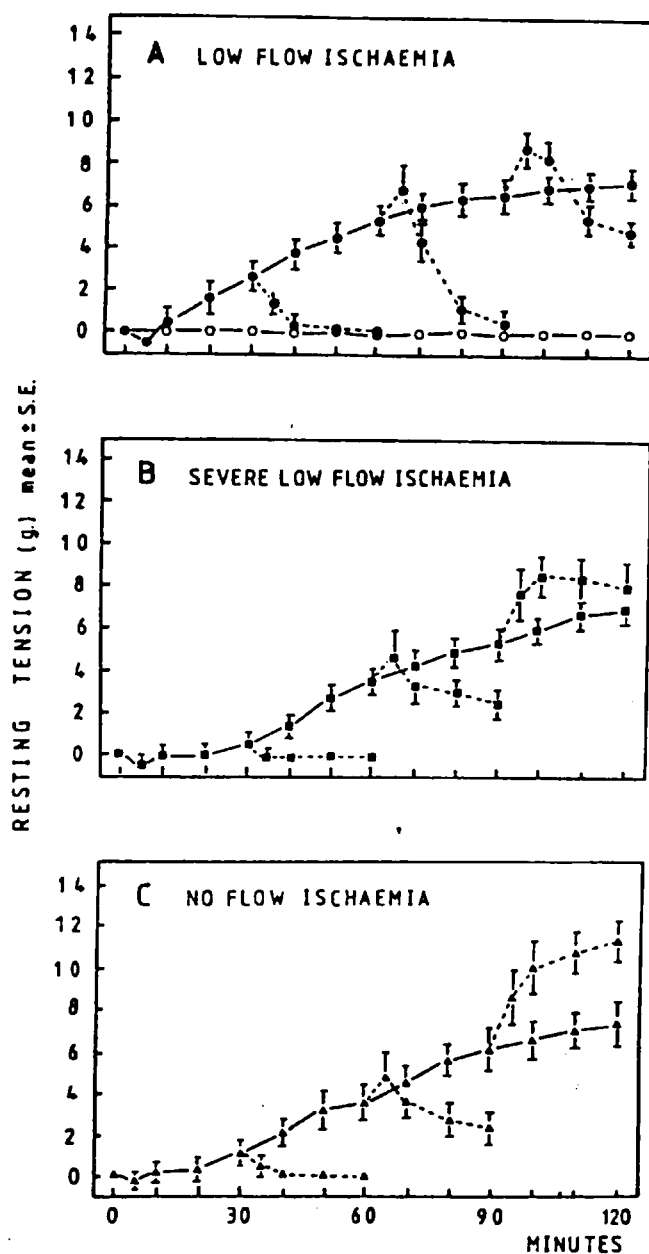


FIGURE 2.6 EFFECT OF DIFFERENT DEGREES OF ISCHAEMIA AND SUBSEQUENT REPERFUSION ON RESTING TENSION

Solid lines represent resting tension during ischaemia and broken lines resting tension during reperfusion.

A. Open circles represent aerobic group (coronary flow 25 ± 1.72 ml/min)

Closed circles represent moderate low flow ischaemia (coronary flow 3 ml/min)

B. Severe low flow ischaemia (coronary flow 1 ml/min)

C. No flow ischaemia (coronary flow stopped)

Ischaemia was started at time 0

first 60 minutes of perfusion, lower than those obtained when coronary flow was reduced to 3 ml/min. After 120 minutes, however, the severity of ischaemic-induced increase in resting tension was comparable in all groups.

Reperfusion after 30 minutes of ischaemia resulted in a rapid decline of resting tension to control values in all groups. When the ischaemic period was prolonged to 60 minutes, reperfusion resulted in a rapid further increase in resting tension, reaching a peak in about 5 minutes in all groups, followed by a decline towards control. However, after 30 minutes of reperfusion, following 60 minutes of ischaemia in the 1 ml/min or 0 ml/min groups, resting tension was still increased, while in the 3 ml/min group it was almost at the control values (Fig. 2.6, A, B and C).

Reperfusion after 90 minutes of ischaemia resulted in a marked increase in resting tension in the 1 ml/min and total ischaemia groups (Fig. 2.6, B and C). In the 3 ml/min group, resting tension, after an initial increase, started to decline and at the end of 30 minutes of reperfusion was 5 ± 0.9 g (Fig. 2.6, A).

(c) Effect of oxygen and glucose content of the perfusate on developed and resting tension during low flow ischaemia and subsequent reperfusion

The experiments illustrated in Figures 2.7 and 2.8 were performed to investigate the effect of oxygen and glucose content of the perfusate on mechanical function of the isolated rabbit's heart maintained at a low coronary flow rate (3 ml/min) for 90 minutes. For this purpose, hearts were perfused with Krebs-Henseleit buffer made hypoxic by

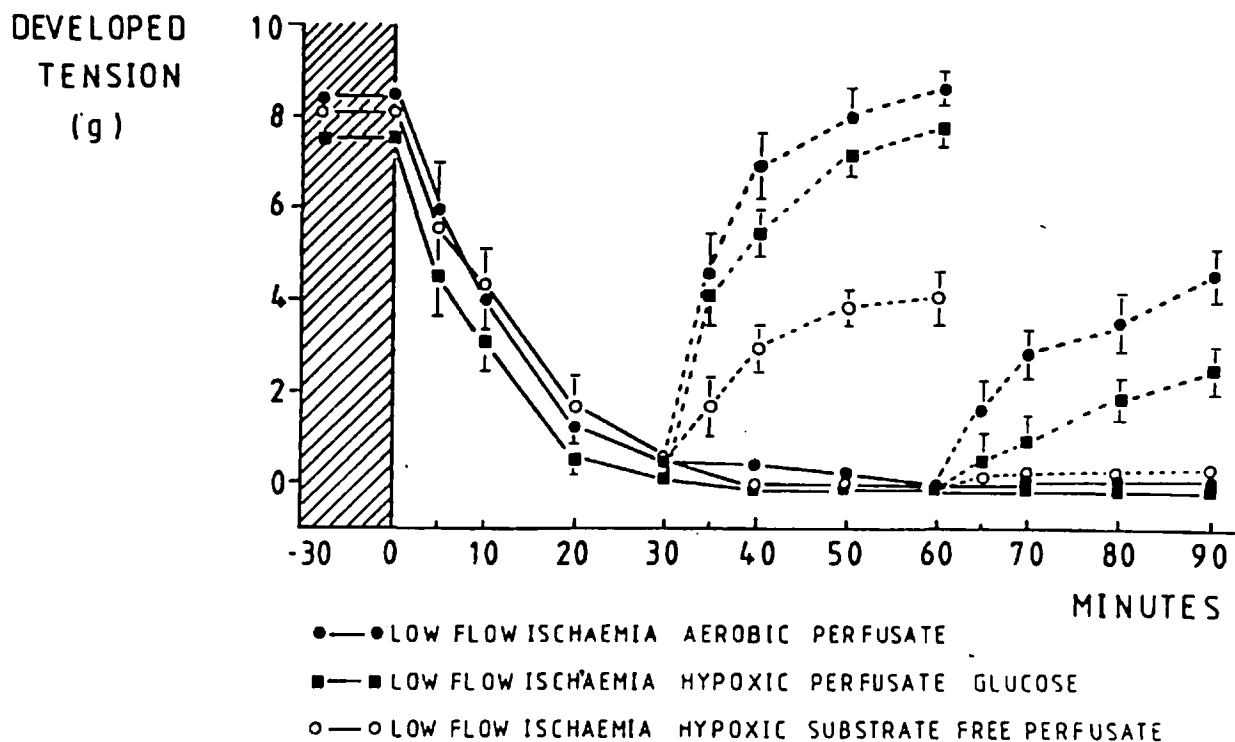


FIGURE 2.7 EFFECT OF OXYGEN AND GLUCOSE CONTENT OF THE PERFUSATE ON DEVELOPED TENSION AFTER 30 AND 60 MINUTES OF MODERATE LOW FLOW ISCHAEMIA AND SUBSEQUENT REPERFUSION

Solid lines represent developed tension during ischaemia and broken lines developed tension during reperfusion.

Ischaemia was started at time 0.

The results are expressed as mean $\pm$ S.E.

substituting N_2 for O_2 in the gas mixture with or without glucose as substrate.

The effects of reperfusion with oxygenated Krebs-Henseleit solution after 30 and 60 minutes of ischaemia have also been investigated.

Figure 2.7 shows that the presence of oxygen and glucose in the perfusate did not make any significant difference in the decline of developed tension during 3 ml/min ischaemia.

Figure 2.8, however, shows that presence of oxygen and glucose in the perfusate significantly modified the extent of rise in resting tension. When an hypoxic, substrate-free medium was used, resting tension increased rapidly during the first 60 minutes to 13 ± 1.3 g and then remained almost constant. At the end of 90 minutes it had increased to 13.5 ± 0.9 g. The presence of glucose in the hypoxic perfusate was able to delay the onset and to reduce the extent of the rise in resting tension. However, when glucose was present in the hypoxic medium the values of resting tension after 60 minutes of 3 ml/min perfusion were still higher than those obtained with the aerobic perfusate. There was a significant difference between the values obtained with hypoxic-substrate-free perfusate and those obtained with aerobic medium ($P < 0.01$) or with hypoxic medium containing glucose as substrate ($P < 0.05$). During reperfusion (with aerobic buffer at 25 ml/min) after 30 minutes of ischaemia, the hearts perfused with hypoxic, substrate-free medium recovered only 57% of developed tension (Fig. 2.7). In this group the resting tension

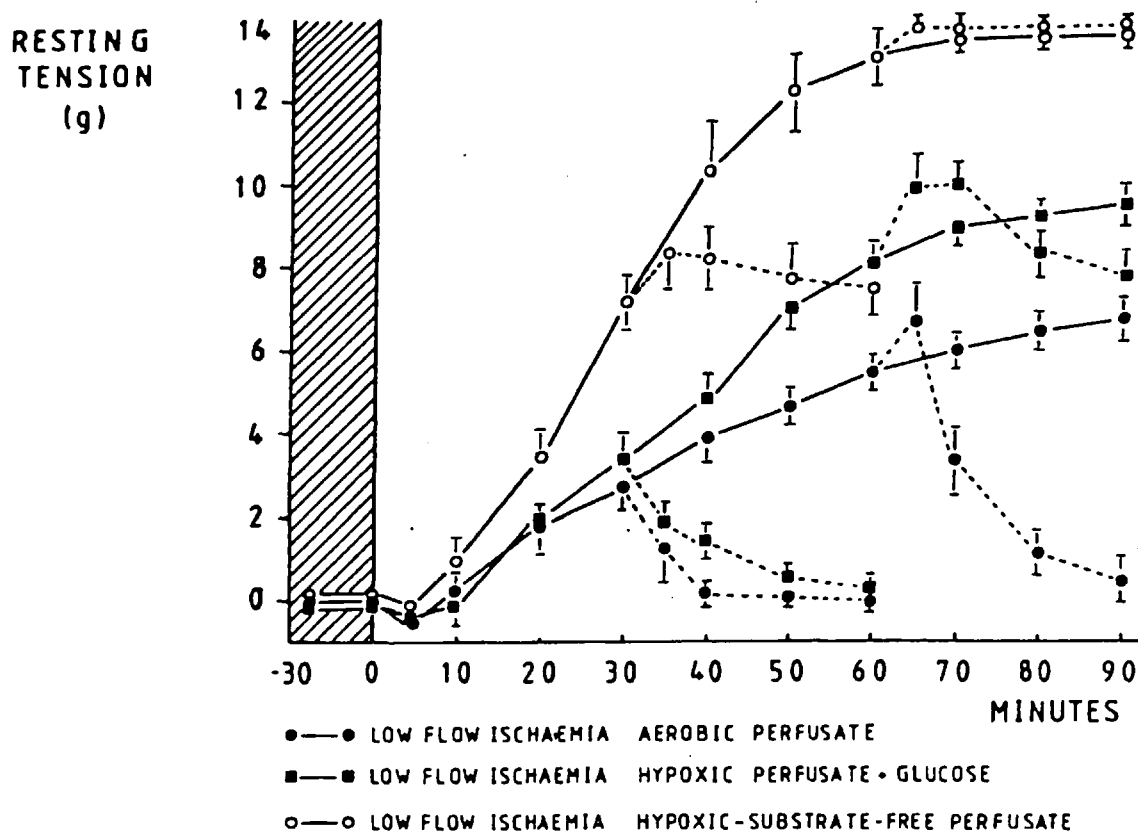


FIGURE 2.8 EFFECT OF OXYGEN AND GLUCOSE CONTENT OF THE PERFUSATE ON RESTING TENSION AFTER 30 AND 60 MINUTES OF MODERATE LOW FLOW ISCHAEMIA AND SUBSEQUENT REPERFUSION

Solid lines represent resting tension during ischaemia and broken lines resting tension during reperfusion.

Ischaemia was started at time 0.

The results are expressed as mean $\pm$ S.E.

increased slightly during the first 5 min of reperfusion and then, slowly, started to decline. At the end of 30 min of reperfusion, it had returned to the previous ischaemic value (Fig. 2.8).

If glucose was present in the hypoxic perfusate, recovery of developed tension on reperfusion after 30 minutes of low flow ischaemia was 100% (Fig. 2.7) and resting tension declined to control values (Fig. 2.8).

After 60 minutes of 3 ml/min ischaemia, with hypoxic-substrate-free medium, reperfusion failed to give rise to any recovery of developed tension (Fig. 2.7). The increased resting tension was also unchanged after 30 minutes of reperfusion (Fig. 2.8).

If glucose was present in the hypoxic medium, reperfusion with aerobic buffer after 60 min of ischaemia resulted in some recovery of developed tension (32% of the control values) (Fig. 2.7). In this group, resting tension increased during the first 10 minutes of reperfusion. Thereafter it slowly declined but, at the end of the reperfusion period, it was unchanged with respect to the previous ischaemic values.

(d) Effect of different degrees of ischaemia and subsequent reperfusion on ATP and CP tissue concentration

Tables 2.2 and 2.3 (Series A) show that tissue ATP and CP levels were virtually constant during 120 minutes of aerobic perfusion.

As expected, 30 minutes of ischaemia reduced ATP and CP levels significantly. The fall in ATP concentration after 30 min of ischaemia was proportional to the severity of the ischaemic condition. The ATP

TABLE 2.2 EFFECT OF DIFFERENT DEGREES OF ISCHAEMIA AND SUBSEQUENT REPERFUSION ON TISSUE ATP CONTENT

Duration of Ischaemia (Min)	30	60	90	120
<u>SERIES A: AFTER ISCHAEMIC PERFUSION</u>				
	ATP (μ moles/g dry wg)			
Aerobic	19.5 $\pm$ 1.8 (4)	16.7 $\pm$ 2.0 (3)	18.0 $\pm$ 1.9 (3)	18.4 $\pm$ 1.2 (3)
Moderate low flow ischaemia (3 ml/min)	12.8 $\pm$ 1.4 (6)	8.2 $\pm$ 0.6 (5)	8.6 $\pm$ 0.7 (6)	9.0 $\pm$ 1.2 (3)
Severe low flow ischaemia (1 ml/min)	7.2 $\pm$ 0.6 (5)	5.6 $\pm$ 0.6 (4)	4.8 $\pm$ 0.6 (6)	4.0 $\pm$ 1.2 (3)
No flow ischaemia	5.8 $\pm$ 0.5 (5)	4.1 $\pm$ 0.4 (5)	4.6 $\pm$ 0.7 (6)	4.1 $\pm$ 1.8 (3)
<u>SERIES B: 30 MIN AFTER REPERFUSION</u>				
Moderate low flow ischaemia (3 ml/min)	16.3 $\pm$ 1.0 (4)	14.4 $\pm$ 0.5 (4)	11.7 $\pm$ 0.9 (6)	
Severe low flow ischaemia (1 ml/min)	13.6 $\pm$ 1.6 (4)	6.9 $\pm$ 0.6 (4)	5.7 $\pm$ 0.7 (4)	
No flow ischaemia	10.7 $\pm$ 1.0 (5)	5.3 $\pm$ 0.8 (4)	4.2 $\pm$ 0.6 (6)	

The data are expressed as mean $\pm$ S.E. The number of experiments is reported in brackets.

content, in fact, was 83%, 69% and 55% of control aerobic values respectively for 3 ml/min, 1 ml/min and no flow ischaemia (Table 2.2, Series A). The fall in CP concentration during the first 30 minutes of ischaemia was greater than that for ATP in all groups, the CP content for each of the three groups being 34%, 18% and 19% of control values respectively (Table 2.3, Series A). After 30 minutes of ischaemia, there was no difference between the CP concentrations of the 1 ml/min ischaemic and totally ischaemic hearts (Table 2.3, Series A). There was, however, a significant difference ($P < 0.05$) when CP levels of these two groups were compared with those of the 3 ml/min group. Tables 2.2 and 2.3 (Series A) show that increasing the duration of the ischaemic episode to 60, 90 and 120 minutes resulted in a further reduction of ATP and CP content in all groups. The decline in ATP and CP concentrations, however, was not proportional to the duration of ischaemia as there was not a significant difference between the ATP and CP values after 60, 90 and 120 minutes of ischaemia for all groups.

The effects of reperfusion on the ATP and CP content of the hearts are shown in Tables 2.2 and 2.3 as Series B. The restoration of coronary flow, after 30 minutes of ischaemia, resulted in a significant ($P < 0.05$) increase of ATP and CP content in all groups when compared with the ischaemic values obtained before reperfusion. In none of the hearts, however, were the ATP and CP concentration restored completely to the aerobic control values. The ATP content of reperfused hearts after 30, 60 and 90 minutes of 3 ml/min

TABLE 2.3 EFFECT OF DIFFERENT DEGREES OF ISCHAEMIA AND SUBSEQUENT REPERFUSION ON TISSUE CP CONTENT

Duration Perfusion (Min)	30	60	90	120
<u>SERIES A: AFTER ISCHAEMIC PERFUSION</u>				
	CP (u moles/g dry wg)			
Aerobic	29.7 $\pm$ 2.7 (4)	28.1 $\pm$ 2.9 (3)	31.2 $\pm$ 2.0 (3)	32.8 $\pm$ 1.6 (3)
Moderate low flow ischaemia (3 ml/min)	10.0 $\pm$ 1.2 (6)	6.4 $\pm$ 1.6 (5)	6.0 $\pm$ 0.7 (6)	6.2 $\pm$ 0.9 (4)
Severe low flow ischaemia (1 ml/min)	5.4 $\pm$ 0.6 (6)	5.4 $\pm$ 1.2 (4)	5.5 $\pm$ 0.6 (6)	4.0 $\pm$ 1.2 (3)
No flow ischaemia	5.7 $\pm$ 0.4 (5)	5.1 $\pm$ 0.7 (4)	5.2 $\pm$ 0.6 (6)	4.6 $\pm$ 1.1 (3)
<u>SERIES B: 30 MIN AFTER REPERFUSION</u>				
Moderate low flow ischaemia (3 ml/min)	21.0 $\pm$ 1.4 (5)	17.0 $\pm$ 2.1 (4)	10.7 $\pm$ 1.25 (6)	
Severe low flow ischaemia (1 ml/min)	10.7 $\pm$ 1.4 (4)	5.5 $\pm$ 0.6 (4)	4.8 $\pm$ 0.6 (4)	
No flow ischaemia	10.8 $\pm$ 0.5 (6)	6.32 $\pm$ 0.7 (4)	6.6 $\pm$ 0.8 (6)	

The data are expressed as mean $\pm$ S.E. The number of experiments is reported in brackets.

ischaemia was 84%, 74% and 60% of the aerobic values, and the CP content was 72%, 58% and 34% of the aerobic values, respectively. When the recovery of ATP and CP on reperfusion was calculated as a percentage of the ischaemic value obtained immediately before reperfusion, however, there was no correlation with the length of ischaemic period.

From Tables 2.2 and 2.3 (Series B) it also appears that reperfusion of the hearts made ischaemic by means of severe reduction of coronary flow (1 ml/min) for 60 and 90 minutes resulted only in a small recovery of ATP, without any recovery of CP.

Reperfusion of hearts made totally ischaemic for 60 and 90 minutes failed to restore the ATP and CP content (Tables 2.2 and 2.3, Series B).

(e) Effect of oxygen and glucose content of the perfusate on tissue ATP and CP after 30 and 60 minutes of low flow ischaemia and subsequent reperfusion

Table 2.4 refers to experiments in which ischaemia was induced by reducing the coronary flow to 3 ml/min for 30 and 60 minutes. In order to evaluate the effect of oxygen and glucose content of the perfusate on tissue ATP and CP, the hearts were perfused at 3 ml/min with Krebs-Henseleit buffer made hypoxic by substituting N_2 for O_2 in the gas mixture with or without glucose as substrate. In Table 2.4. are also reported the data obtained using the aerobic perfusate to make the comparison between the results obtained with the different buffers easier.

TABLE 2.4 EFFECT OF OXYGEN AND GLUCOSE CONTENT OF THE PERFUSATE ON TISSUE ATP AND CP AFTER 30 AND 60 MINUTES OF MODERATE LOW FLOW ISCHAEMIA AND SUBSEQUENT REPERFUSION

Duration of Ischaemia (Min)	30		60	
	ATP	CP	ATP	CP
<u>SERIES A: AFTER ISCHAEMIC PERFUSION</u>				
Moderate low flow ischaemia (3 ml/min) aerobic perfusate	12.83 $\pm$ 1.4 (6)	10.06 $\pm$ 1.2 (6)	8.2 $\pm$ 0.6 (5)	6.4 $\pm$ 1.6 (5)
Moderate low flow ischaemia (3 ml/min) hypoxic perfusate + glucose (11 mM)	9.3 $\pm$ 1.1 (4)	9.8 $\pm$ 0.9 (4)	5.7 $\pm$ 2.1 (3)	5.8 $\pm$ 1.1 (3)
Moderate low flow ischaemia (3 ml/min) hypoxic perfusate - substrate free	5.4 $\pm$ 1.4 (4)	5.0 $\pm$ 2.1 (4)	3.1 $\pm$ 0.9 (3)	4.8 $\pm$ 1.2 (3)
<u>SERIES B: 30 MIN AFTER REPERFUSION</u>				
Moderate low flow ischaemia (3 ml/min) aerobic perfusate	16.3 $\pm$ 1.0 (4)	21.0 $\pm$ 1.4 (4)	13.6 $\pm$ 1.6 (4)	17.0 $\pm$ 2.1
Moderate low flow ischaemia (3 ml/min) hypoxic perfusate + glucose (11 mM)	16.2 $\pm$ 1.4 (4)	17.2 $\pm$ 2.1 (4)	7.5 $\pm$ 1.6 (3)	9.8 $\pm$ 2.1 (3)
Moderate low flow ischaemia (3 ml/min) hypoxic perfusate - substrate free	10.5 $\pm$ 1.6 (5)	5.4 $\pm$ 1.9 (5)	4.4 $\pm$ 2.1 (3)	4.2 $\pm$ 1.7 (3)

The ATP and CP data are expressed as μ moles/g dry weight. Values represent mean $\pm$ S.E. The number of experiments is reported in brackets.

Table 2.4, Series A, shows that when the ischaemic hearts were perfused using hypoxic substrate-free perfusate, their tissue values of ATP and CP were extremely reduced either after 30 and 60 minutes perfusion. Table 2.4, Series A, also shows that the presence of glucose in the hypoxic perfusate reduced the decline in ATP and CP content during 30 and 60 minutes of ischaemia. The values for ATP and CP obtained in the presence of glucose in the hypoxic buffer, however, were always lower than those obtained when low flow perfusion was achieved with the aerobic perfusate (Table 2.4).

Thirty minutes of reperfusion with aerobic buffer after 30 minutes of 3 ml/min ischaemia with hypoxic substrate-free perfusate, resulted in significantly ($P < 0.05$) higher ATP concentration than was found in hearts which were not reperfused. In these hearts, however, the CP levels after reperfusion were unchanged when compared with the ischaemic values (Table 2.4, Series B). If glucose was present in the hypoxic buffer, reperfusion after 30 minutes of 3 ml/min ischaemia, resulted in a significant recovery ($P < 0.05$) of both ATP and CP (Table 2.4, Series B).

When 3 ml/min ischaemia with hypoxic-substrate-free medium was extended to 60 minutes, the restoration of aerobic coronary flow failed to improve the ATP and CP content of the hearts. When glucose was present in the hypoxic buffer, reperfusion after 60 minutes of ischaemia resulted in higher ATP and CP concentrations than were found in hearts which were not reperfused, although the values were not statistically different. The amount of ATP and CP recovered on

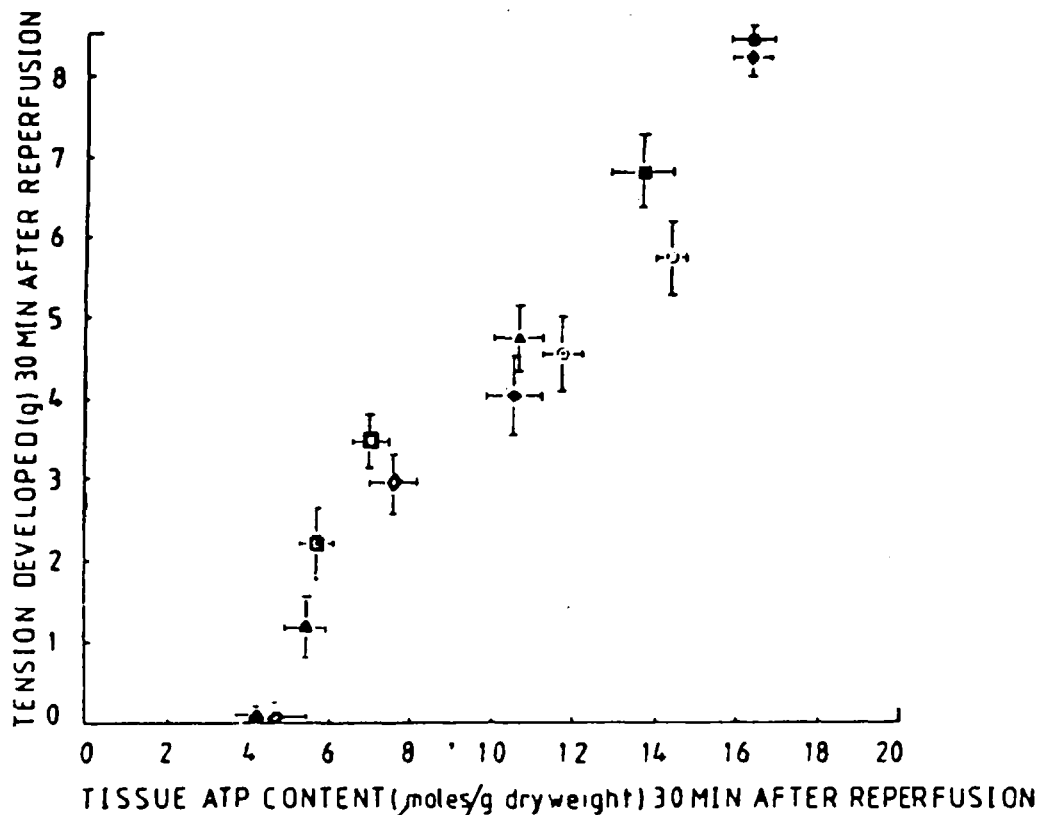


FIGURE 2.9 RELATION BETWEEN TISSUE ATP CONTENT AND RECOVERY OF DEVELOPED TENSION AFTER REPERFUSION

Reperfusion (30 min) followed:

● 30 min moderate low flow ischaemia (3 ml/min)

○ 60 " " " " " " " "

⊙ 90 " " " " " " " "

■ 30 min severe low flow ischaemia (1 ml/min)

□ 60 " " " " " " " "

⊠ 90 " " " " " " " "

▲ 30 min no flow ischaemia

△ 60 " " " "

▲ 90 " " " "

◆ 30 min moderate low flow ischaemia hypoxic perfusate + glucose

◇ 60 " " " " " " " "

◆ 30 min moderate low flow ischaemia hypoxic perfusate-substrate free

◇ 60 " " " " " " " "

Results represent mean $\pm$ S.E.

reperfusion after 30 and 60 minutes of 3 ml/min ischaemia with hypoxic buffer containing glucose as substrate was, however, always significantly lower ($P < 0.05$) than that recovered by the hearts perfused at 3 ml/min with aerobic buffer (Table 2.4, Series B).

Figure 2.9 shows that there was an association between the tissue ATP content measured at the end of 30 minutes of reperfusion and the maximum recovery of tension developed after 30 minutes of reperfusion. All the different conditions considered in this study have been included.

(f) Effect of different degrees of ischaemia and subsequent reperfusion on mitochondrial function

After the different experimental conditions, mitochondria were isolated and their function was measured by means of their capacity to utilise oxygen and was expressed in terms of RCR, QO_2 and ADP/O ratio calculated as previously described (Fig. 2.4). RCR values are reported in Table 2.5, QO_2 in Table 2.6 and ADP/O values in Table 2.7. The yield of mitochondria is reported in Table 2.8.

Tables 2.5, 2.6, 2.7 (Series A) show that RCR, QO_2 and ADP/O values were constant during 120 minutes of aerobic perfusion. Table 2.8, Series A, also shows that there was no difference in the mitochondrial yield during this time. After 30 minutes of ischaemia there was a small reduction of RCR (Table 2.5, Series A) and QO_2 values (Table 2.6, Series A) in all groups. The ADP/O values (Table 2.7, Series A) 30 minutes after the onset of ischaemia were unchanged with respect to the control pre-ischaemic values. The mitochondrial

TABLE 2.5 EFFECT OF DIFFERENT DEGREES OF ISCHAEMIA AND SUBSEQUENT REPERFUSION ON THE RESPIRATORY CONTROL RATIO (RCR) OF ISOLATED MITOCHONDRIA

Duration of Ischaemia (Min) before Reperfusion	30	60	90	120
	RCR			
<u>SERIES A: AFTER ISCHAEMIC PERFUSION</u>				
Aerobic	18.6 $\pm$ 1.9 (4)	16.6 $\pm$ 2.3 (4)	14.1 $\pm$ 2.6 (5)	16.0 $\pm$ 1.7 (5)
Moderate low flow ischaemia (3 ml/min)	14.9 $\pm$ 2.4 (6)	11.0 $\pm$ 1.0 (5)	12.2 $\pm$ 2.4 (6)	10.2 $\pm$ 1.8 (3)
Severe low flow ischaemia (1 ml/min)	13.2 $\pm$ 1.9 (5)	9.4 $\pm$ 1.3 (3)	9.8 $\pm$ 1.2 (4)	7.3 $\pm$ 1.7 (3)
No flow ischaemia	13.0 $\pm$ 0.7 (4)	9.9 $\pm$ 1.2 (5)	10.4 $\pm$ 0.9 (6)	8.4 $\pm$ 1.9 (3)
<u>SERIES B: 30 MIN AFTER REPERFUSION</u>				
Moderate low flow ischaemia (3 ml/min)	13.8 $\pm$ 1.8 (4)	12.6 $\pm$ 1.3 (4)	13.0 $\pm$ 2.1 (5)	
Severe low flow ischaemia (1 ml/min)	15.0 $\pm$ 2.1 (4)	10.9 $\pm$ 2.3 (4)	8.6 $\pm$ 1.2 (4)	
No flow ischaemia	15.6 $\pm$ 2.3 (3)	10.8 $\pm$ 1.2 (4)	5.8 $\pm$ 0.3 (6)	

The data are expressed as mean $\pm$ S.E. The number of experiments is reported in brackets.

TABLE 2.6 EFFECT OF DIFFERENT DEGREES OF ISCHAEMIA AND SUBSEQUENT REPERFUSION ON RATE OF OXYGEN CONSUMPTION OF ISOLATED MITOCHONDRIA

Duration of Ischaemia (Min) before Reperfusion	30	60	90	120
	QO ₂ (n atoms O ₂ /mg prot./min)			
<u>SERIES A: AFTER ISCHAEMIC PERFUSION</u>				
Aerobic	264 ± 21.7 (4)	275 ± 31.0 (4)	242 ± 22.0 (5)	251 ± 19.5 (5)
Moderate low flow ischaemia (3 ml/min)	232 ± 9.9 (6)	212 ± 6.9 (5)	203 ± 23.0 (6)	210 ± 31.2 (3)
Severe low flow ischaemia (1 ml/min)	227 ± 8.7 (5)	189 ± 21.1 (3)	197 ± 19.0 (4)	171 ± 21.0 (3)
No flow ischaemia	231 ± 13.1 (4)	202 ± 19.7 (5)	192 ± 20.1 (6)	159 ± 19.0 (3)
<u>SERIES B: 30 MIN AFTER REPERFUSION</u>				
Moderate low flow ischaemia (3 ml/min)	240 ± 11.0 (4)	227 ± 8.1 (4)	200 ± 15.2 (5)	
Severe low flow ischaemia (1 ml/min)	236 ± 12.1 (4)	176 ± 23.0 (4)	187 ± 17.3 (4)	
No flow ischaemia	249 ± 31.0 (3)	209 ± 13.3 (4)	121 ± 12.0 (6)	

The data are expressed as mean $\pm$ S.E. The number of experiments is reported in brackets.

TABLE 2.7 EFFECT OF DIFFERENT DEGREES OF ISCHAEMIA AND SUBSEQUENT REPERFUSION ON ADP/O RATIO OF ISOLATED MITOCHONDRIA

Duration of Perfusion (Min) before Reperfusion	30	60	90	120
	ADP/O			
<u>SERIES A: AFTER ISCHAEMIC PERFUSION</u>				
Aerobic	2.74 $\pm$ 0.07 (4)	2.75 $\pm$ 0.25 (4)	2.80 $\pm$ 0.22 (5)	2.78 $\pm$ 0.20 (5)
Moderate low flow ischaemia (3 ml/min)	2.85 $\pm$ 0.30 (6)	2.71 $\pm$ 0.05 (5)	2.65 $\pm$ 0.07 (6)	2.68 $\pm$ 0.04 (3)
Severe low flow ischaemia (1 ml/min)	2.73 $\pm$ 0.06 (5)	2.68 $\pm$ 0.12 (3)	2.59 $\pm$ 0.03 (9)	2.51 $\pm$ 0.12 (3)
No flow ischaemia	2.74 $\pm$ 0.12 (4)	2.69 $\pm$ 0.20 (5)	2.54 $\pm$ 0.17 (6)	2.55 $\pm$ 0.07 (3)
<u>SERIES B: 30 MIN AFTER REPERFUSION</u>				
Moderate low flow ischaemia (3 ml/min)	2.74 $\pm$ 0.09 (4)	2.79 $\pm$ 0.13 (4)	2.69 $\pm$ 0.09 (5)	
Severe low flow ischaemia (1 ml/min)	2.69 $\pm$ 0.07 (4)	2.68 $\pm$ 0.17 (4)	2.56 $\pm$ 0.09 (4)	
No flow ischaemia	2.72 $\pm$ 0.10 (3)	2.39 $\pm$ 0.03 (4)	2.25 $\pm$ 0.30 (6)	

The data are expressed as mean $\pm$ S.E. The number of experiments is reported in brackets.

TABLE 2.8 EFFECT OF DIFFERENT DEGREES OF ISCHAEMIA AND SUBSEQUENT REPERFUSION ON MITOCHONDRIAL YIELD

Duration of Perfusion (Min) before Reperfusion	30	60	90	120
MITOCHONDRIAL YIELD (mg prot./g wet wg)				
<u>SERIES A: AFTER ISCHAEMIC PERFUSION</u>				
Aerobic	10.9 $\pm$ 1.9 (4)	12.6 $\pm$ 1.2 (4)	10.9 $\pm$ 1.6 (4)	12.5 $\pm$ 1.2 (5)
Moderate low flow ischaemia (3 ml/min)	11.9 $\pm$ 1.2 (6)	12.1 $\pm$ 1.7 (5)	13.1 $\pm$ 1.1 (6)	10.9 $\pm$ 2.0 (3)
Severe low flow ischaemia (1 ml/min)	10.7 $\pm$ 1.3 (5)	12.4 $\pm$ 0.6 (3)	10.9 $\pm$ 1.3 (4)	11.7 $\pm$ 1.9 (3)
No flow ischaemia	11.9 $\pm$ 1.2 (4)	10.1 $\pm$ 1.4 (5)	8.1 $\pm$ 2.6 (6)	7.2 $\pm$ 2.3 (3)
<u>SERIES B: 30 MIN AFTER REPERFUSION</u>				
Moderate low flow ischaemia (3 ml/min)	12.1 $\pm$ 0.6 (4)	11.9 $\pm$ 2.1 (4)	13.9 $\pm$ 3.1 (5)	
Severe low flow ischaemia (1 ml/min)	11.7 $\pm$ 1.3 (4)	9.3 $\pm$ 2.1 (4)	9.0 $\pm$ 1.2 (4)	
No flow ischaemia	8.7 $\pm$ 1.4 (3)	5.4 $\pm$ 2.1 (4)	6.3 $\pm$ 1.9 (6)	

The data are expressed as mean $\pm$ S.E. The number of experiments is reported in brackets.

yield was also unchanged (Table 2.8, Series A). Increasing the duration of 3 ml/min ischaemia to 60, 90 and 120 minutes did not significantly modify the RCR, QO_2 and ADP/O values. The extension of 1 ml/min ischaemia and total ischaemia to 60, 90 and 120 minutes, however, resulted in a further slight reduction of RCR and QO_2 values (Tables 2.5, 2.6, Series A). The reduction in mitochondrial function for these groups, was not correlated with the duration of the ischaemic period, although the lowest values for RCR, QO_2 and ADP/O ratio were found after 120 minutes of ischaemia.

Table 2.8, Series A also shows that after 120 minutes of total ischaemia the mitochondrial yield was significantly decreased ($P < 0.05$). There were no significant changes for mitochondrial yield when measured after 30, 60, 90 and 120 minutes of 3 ml/min ischaemia or of 1 ml/min ischaemia (Table 2.8, Series A). Restoration of coronary flow after 30 or 60 minutes of ischaemia did not result in an amelioration of mitochondrial function in any group. The values for RCR, QO_2 and ADP/O ratio were unchanged when compared with those obtained for mitochondria from ischaemic non-reperfused hearts (Tables 2.5, 2.6, 2.7, Series B). Reperfusion after 90 minutes of ischaemia at 3 ml/min or 1 ml/min also resulted in no changes in RCR, QO_2 and ADP/O (Tables 2.5, 2.6, 2.7, Series B) and mitochondrial yield values (Table 2.8, Series B). In the total ischaemic group, however, reperfusion after 90 minutes of ischaemia resulted in a further reduction of mitochondrial function. The RCR, QO_2 and ADP/O values, in fact, were significantly lower ($P < 0.05$) when compared with those obtained

from mitochondria isolated from hearts after 90 and 120 minutes of total ischaemia. The yield of these mitochondria was also reduced (Table 2.8, Series B).

(g) Effect of oxygen and glucose content of the perfusate on mitochondrial function after 30 and 60 minutes of low flow ischaemia and subsequent reperfusion

In Tables 2.9 and 2.10, Series A, are reported the results of mitochondrial function obtained after 30 and 60 minutes of 3 ml/min ischaemia using aerobic perfusate with glucose, hypoxic perfusate with glucose (11 mM) or the hypoxic, substrate-free (11 mM mannitol) buffer.

When hypoxic, substrate-free medium was used as perfusate during 30 and 60 minutes of ischaemia, the RCR and QO_2 values were significantly lower than those obtained when the aerobic medium was used. However, the ADP/O ratio (Tables 2.9 and 2.10) was unchanged. Mitochondrial yield was also unchanged, being 10.1 ± 1.3 and 11.0 ± 1.2 mg protein/g wet weight after 30 and 60 minutes of perfusion, respectively.

The presence of glucose in the hypoxic perfusate did not significantly affect mitochondrial function after 30 minutes of ischaemia, but significantly increased ($P < 0.05$) the function of mitochondria isolated after 60 minutes of ischaemia (Table 2.10, Series A). The presence of glucose in the hypoxic perfusate also did not affect the mitochondrial yield, which was 11.3 ± 1.2 and 10.7 ± 0.9 mg protein/g wet weight after 30 and 60 minutes of low flow ischaemia.

TABLE 2.9 EFFECT OF OXYGEN AND GLUCOSE CONTENT OF THE PERFUSATE ON MITOCHONDRIAL FUNCTION (RCR, $\dot{Q}O_2$, ADP/O) AFTER 30 MINUTES OF MODERATE LOW FLOW ISCHAEMIA AND SUBSEQUENT REPERFUSION

Duration of Ischaemia (Min) before Reperfusion	30		
	RCR	$\dot{Q}O_2$	ADP/O
<u>SERIES A: AFTER ISCHAEMIC PERFUSION</u>			
Moderate low flow ischaemia (3 ml/min) aerobic perfusate	14.9 $\pm$ 2.4 (6)	232 $\pm$ 9.9 (6)	2.74 $\pm$ 0.07 (6)
Moderate low flow ischaemia (3 ml/min) hypoxic perfusate + glucose	10.9 $\pm$ 2.3 (5)	192 $\pm$ 12 (5)	2.78 $\pm$ 0.08 (5)
Moderate low flow ischaemia (3 ml/min) hypoxic - substrate free perfusate	8.9 $\pm$ 2.3 (6)	180 $\pm$ 15 (6)	2.80 $\pm$ 0.10 (6)
<u>SERIES B: 30 MIN AFTER REPERFUSION</u>			
Moderate low flow ischaemia (3 ml/min) aerobic perfusate	13.8 $\pm$ 1.8 (4)	240 $\pm$ 11.0 (4)	2.74 $\pm$ 0.09 (4)
Moderate low flow ischaemia (3 ml/min) hypoxic perfusate + glucose	9.9 $\pm$ 2.1 (4)	203 $\pm$ 21 (4)	2.75 $\pm$ 0.09 (4)
Moderate low flow ischaemia (3 ml/min) hypoxic - substrate free perfusate	7.7 $\pm$ 3.2 (3)	200 $\pm$ 27 (3)	2.76 $\pm$ 0.07 (3)

$\dot{Q}O_2$ is expressed as n atoms O_2 /mg prot./min. The data are expressed as mean $\pm$ S.E. The number of experiments is reported in brackets.

If glucose was present in the hypoxic perfusate, reperfusion with aerobic buffer after 30 and 60 minutes of low flow ischaemia did not make any difference in mitochondrial function when compared with the values obtained for mitochondria harvested from ischaemic non-reperfused hearts. Reperfusion of hearts made ischaemic with hypoxic substrate-free buffer for 30 minutes resulted in no change of mitochondrial function. When ischaemia was prolonged to 60 minutes, however, reperfusion resulted in a worsening of mitochondrial function (Table 2.10, Series B). The yield of these mitochondria was also very low: 4.2 ± 2.1 mg protein/g wet weight.

TABLE 2.10 EFFECT OF OXYGEN AND GLUCOSE CONTENT OF THE PERFUSATE ON MITOCHONDRIAL FUNCTION (RCR, QO_2 , ADP/O)
AFTER 60 MINUTES OF MODERATE LOW FLOW ISCHAEMIA AND SUBSEQUENT REPERFUSION

Duration of Ischaemia (Min) before Reperfusion	60		
	RCR	QO_2	ADP/O
<u>SERIES A: AFTER ISCHAEMIC PERFUSION</u>			
Moderate low flow ischaemia (3 ml/min) aerobic perfusate	11.01 ± 1.0 (4)	212 ± 6.9 (4)	2.75 ± 0.25 (4)
Moderate low flow ischaemia (3 ml/min) hypoxic perfusate + glucose	8.9 ± 2.1 (5)	180 ± 21 (5)	2.70 ± 0.12 (5)
Moderate low flow ischaemia (3 ml/min) hypoxic - substrate free perfusate	5.7 ± 2.2 (6)	120 ± 19 (6)	2.78 ± 0.09 (6)
<u>SERIES B: 60 MIN AFTER REPERFUSION</u>			
Moderate low flow ischaemia (3 ml/min) aerobic perfusate	12.6 ± 1.3 (4)	227 ± 8.1 (4)	2.79 ± 0.13 (4)
Moderate low flow ischaemia (3 ml/min) hypoxic perfusate + glucose	7.8 ± 2.3 (3)	191 ± 31 (3)	2.69 ± 0.25 (3)
Moderate low flow ischaemia (3 ml/min) hypoxic - substrate free perfusate	3.2 ± 0.9 (4)	106 ± 23 (4)	2.1 ± 0.13 (4)

QO_2 is expressed as n atoms O_2 /mg prot./min. The data are expressed as mean $\pm$ S.E. The number of experiments is reported in brackets.

D. DISCUSSION

(a) Effect of ischaemia on developed tension

The mechanical records obtained in this study during different degrees of ischaemia are similar to those obtained by Neely et al. (1975) and by Apstein et al. (1977) using an isolated and perfused rat heart preparation and to those of Shine et al. (1977) and Cobbe and Poole-Wilson (1979) using the isolated arterially perfused inter-ventricular septum of the rabbit.

Immediately after coronary flow was withdrawn or restricted, a precipitous decline in peak developed tension occurred (Fig. 2.5). The cause of the early, rapid decline in contractile function of the myocardium during ischaemia is controversial. The more likely causes include: a disturbance of the events involved in excitation-contraction coupling, changes in myofibrillar ATPase activity, depletion of high energy reserves, retention of products of anaerobic metabolism and acidosis.

Disturbance of events involved in excitation-contraction coupling

It is possible that an ischaemic-induced decrease in the duration of action potential could reflect an increased outward current (carried by K) and a decreased inward current (carried predominantly by Ca^{2+}) which, in turn, could account for the ischaemic-induced decline in tension. There is, however, evidence that excitability remains normal in the first minutes after induction of ischaemia (Kardesch et al., 1958; McDonald and McLeod, 1971), at a time when contractility is

already severely depressed.

It is possible that ischaemia reduces the availability of calcium bound to the mucopolysaccharide on the outer surface of the cell membrane (Nayler and Dunnett, 1974; Frank et al., 1977). It is suggested that it is a displacement of Ca^{2+} from these superficially-located stores which initiates the series of events resulting in contraction of normal myocardium. A reduction in the capacity of this site to store calcium would therefore result in a decline of tension. Experiments in which the trivalent earth ion lanthanum has been used to displace calcium from the basement coat of hypoxic heart muscle (Nayler and Merrilees, 1971) have shown what appears to be an hypoxic-induced reduction in the amount of lanthanum-displaceable calcium. These experiments, however, are difficult to interpret, since lanthanum has been found to increase the extracellular space (Wathen and Weston, 1978) and to penetrate into hypoxic muscle cells (Borton et al., 1976). Electron microscopic studies (Hatt and Moravel, 1971) failed to reveal any change in the organisation or localisation of the sarcolemma surface coat during the early phase of hypoxia.

Calcium movements across the sarcolemma can be reduced by ischaemia. It has been suggested that calcium movements are controlled by a cyclic 3,5 AMP-dependent phosphorylation of a protein constituent of the slow channels (Sperelakis and Schneider, 1976). This mechanism implies the maintenance of a localised supply of ATP or CP close to cell membrane. If this pool of ATP and CP is depleted early during ischaemia, then the calcium accumulating and transporting properties of the cell

membrane might be depressed. In addition the slow Ca^{2+} transport channels are inactivated by acidosis which always follows an ischaemic episode.

Finally, excitation-contraction coupling can also be impaired during ischaemia because of an alteration of the capacity of sarcoplasmic reticulum to release calcium. Whether this process is altered during ischaemia is still unknown. Nevertheless, as it is pH sensitive (Steenbergen et al., 1977; Nakamura and Schwartz, 1970) it could be depressed in vivo by acidosis during ischaemia.

Changes of myofibrillar ATPase activity

A change of myosin ATPase activity has been reported as one of the possible causes of the ischaemic-induced fall in tension development (Hillis and Branwald, 1977). However, anoxia of the heart has been shown to depress myosin ATPase activity (Luchi and Kritcher, 1967) or to have no influence on its activity (Dettli and Bing, 1956). Nevertheless, it is possible that myosin ATPase activity is depressed by lowered intracellular pH (Schädler, 1967; Cingolani et al., 1970; Williamson et al., 1976; Kentish and Nayler, 1976), a situation which arises during ischaemia.

Depletion of high energy reserves

A reduction of ATP and CP stores could be responsible for the ischaemic-induced decrease in tension, particularly with respect to the energy required to modulate the interaction between actin and myosin (Mommaerts, 1969). Although tissue content of ATP and CP is reduced during ischaemia, it appears that the decline in total tissue

ATP develops relatively slowly (Reibel and Rovetto, 1978) and the size of the fall is insufficient to depress contractile function until several minutes of ischaemia have elapsed (Wollenberger and Krause, 1968; Gudbjarnason et al., 1970). CP declines more rapidly than ATP during ischaemia. Because CP is localised in the cytosol, its concentration, rather than total tissue ATP, may provide a more reliable index of energy available for muscle contraction. Nevertheless, the size and rate of CP decline are also unlikely to account for the rapid contractile failure (Covell et al., 1967).

Retention of products of anaerobic metabolism and acidosis

It has been suggested that a retention and redistribution of the products of anaerobic metabolism as phosphate ions (Kübler and Katz, 1977), lactate ions (Tennant, 1935), hydrogen ions or CO_2^- (Katz and Hecht, 1969) can cause the ischaemic-induced tension decline. An increased concentration of phosphate might diminish the intracellular free calcium ion activity (Kenyon and Gibbons, 1977; Kübler and Katz, 1977) and, therefore, the amount of free calcium available for interaction with contractile protein. Lactate may also reduce the concentrations of free calcium in the cytosol. Accumulation of lactate also reduces aerobic metabolism by decreasing mitochondrial oxidative phosphorylation (Mukherjee et al., 1979) and anaerobic metabolism by inhibiting glyceraldehyde-3-P dehydrogenase activity (the rate-limiting enzyme for glycolysis during ischaemia). Finally, a fall in intracellular pH due to retention of hydrogen ions is known to block the slow calcium current (Chesnais et al., 1976; Sperelakis and Schneider,

1976) to reduce calcium influx and efflux (Poole-Wilson and Langer, 1975), to decrease the sensitivity of the cardiac myofibrillar ATPase to Ca^{2+} (Williamson et al., 1976; Kentish and Nayler, 1976) and to affect Ca^{2+} transporting capacity of the SR (Huxtable and Bressier, 1974; Sorenson and De Meis, 1977). Measurement of intracellular pH during ischaemia presents methodological difficulties (Poole-Wilson, 1976, 1978), and in particular, few techniques have sufficient time resolution to follow events in the first minute. Recently Cobbe and Poole-Wilson (1979) using rapidly responding interstitial pH electrodes suggested that the fall of pH during ischaemia is sufficiently large and rapid in onset to account for most of the early decline in tension. However, the existence of other concurrent mechanisms has not been excluded.

Evidence from our studies

From the present study it seems that the perfusate composition did not affect the decline in tension development induced by ischaemia (Fig. 2.6). This suggests that the fall in developed tension was not related to availability of oxygen and glucose to maintain some aerobic or anaerobic ATP production.

However, the fall in developed tension was related to the severity of coronary flow restriction, being more rapid during total and 1 ml/min ischaemia, than during 3 ml/min ischaemia (Fig. 2.5). This suggests a dependence on the rate of coronary perfusion and washout. Accumulation of metabolic products such as lactate, H^+ and CO_2 in the total and 1 ml/min ischaemia groups is expected to be more pronounced than in the

3 ml/min ischaemia group. This should result in a greater fall in intracellular pH, which could account for the difference in the fall of developed tension we found.

(b) Effect of ischaemia on resting tension

The experiments reported in this study (Fig. 2.6) show that ischaemia induced, in all groups, an increase in resting tension. The cause of ischaemic-induced contracture of the myocardium is still controversial. The most likely explanation for this phenomenon is a reduction of intracellular availability of energy with a concomitant intracellular redistribution of calcium ions.

It is generally accepted that ATP not only supplies energy for contraction, but also dissociates the myosin-actin complex (Weber and Murfay, 1973). Under normal aerobic conditions, with adequate supply of ATP, the events of muscle contraction involve the combination of a myosin head with a molecule of ATP. The myosin-ATP intermediate binds to an actin molecule of a thin filament. Hydrolysis of ATP then occurs producing adenosine diphosphate (ADP) and inorganic phosphate liberating energy for the process of muscle contraction. The myosin-actin complex remains tightly bound as a cross-bridge until a new ATP molecule binds to the myosin.

Under conditions of ischaemia, the progressive reduction in availability of ATP in the presence of an excess of calcium in the vicinity of the myofilaments could result in the formation of increasing numbers of cross-bridges and contracture.

Evidence for an increased cytosolic concentration of Ca^{2+} is indirect. As will be discussed in detail in Chapter 3, total tissue calcium was unchanged during total ischaemia, while mitochondrial calcium was increased. Because the external mitochondrial calcium concentration is the major factor determining mitochondrial calcium uptake (Carafoli and Lehninger, 1971; Scarpa and Graziotti, 1973), an increase in mitochondrial calcium implies an increased concentration of cytosolic calcium.

Our experiments show that, during total or 1 ml/min ischaemia, the onset and the rate of increase in resting tension was delayed and reduced when compared with that of 3 ml/min ischaemia (Fig. 2.5). There are two possible explanations for this phenomenon. The more severe intracellular acidosis expected to occur during total and 1 ml/min ischaemia, compared with 3 ml/min ischaemia could account for the delay in the rise in resting tension. Alternatively, the more rapid decline in developed tension of the hearts made totally ischaemic could account for a reduced rate of ATP hydrolysis during the early stage of ischaemia assuring therefore more ATP available which, in turn, reduces the onset of contracture. However, this can account only for the early stage of ischaemia because after 30 minutes the ATP and CP levels of the 1 ml/min and total ischaemic groups were more reduced (Tables 2.2 and 2.3) than those of the 3 ml/min ischaemic group.

From our experiments (Figs. 2.7 and 2.8), also, it appears that the oxygen and glucose content of the perfusate were important in

reducing ischaemic contracture. When ischaemia was induced by reducing coronary flow to 3 ml/min using hypoxic substrate-free perfusate, resting tension increased rapidly to high values (Fig. 2.8). The presence of glucose in the hypoxic medium to support anaerobic ATP production (Table 2.4) reduced the extent of the increase in resting tension. When oxygen was also present in the perfusate the increase in tension was further reduced and ATP and CP stores better maintained. This is in favour of the hypothesis that ischaemic contracture is dependent on the availability of energy.

(c) Effect of post-ischaemic reperfusion on developed and resting tension

From the present results it appears that the capacity of the cell to develop active tension and to relax during reperfusion depends upon the degree of reduction of coronary flow and on the duration of the ischaemic episode.

Development of contracture on reperfusion or re-oxygenation has been already described in the isolated heart preparation (Neely et al., 1975; Apstein et al., 1977; Cobbe and Poole-Wilson, 1979; Lewis et al., 1980) and the results presented here are, basically, in agreement with the previous findings, considering the existing species differences in development of hypoxic or ischaemic contracture (Lewis et al., 1980) between rats and rabbits.

Reperfusion to 25 ml/min resulted in a considerable increase in the perfusion pressure, particularly after a prolonged period of ischaemia, when ischaemic contracture had developed (Fig. 2.6). This

is presumably due to an increased coronary vascular resistance associated with contracture. Perfusion may have small mechanical effects on the resting tension due to erectile forces in the coronary circulation. These forces may be the explanation of the small transient decrease in resting tension observed when coronary flow was abruptly reduced at the beginning of the ischaemic period (Fig. 2.6, A). Such small effects are, however, unlikely to play a part in the more substantial changes in resting tension which developed on reperfusion.

From our results it appears that reperfusion after 60 minutes of ischaemia resulted in a temporary increase in resting tension followed by a decrease, while, after 90 minutes of ischaemia, reperfusion resulted in a further increase of the ischaemic contracture (Fig. 2.6, B and C). The most likely explanation for this phenomenon is that, with reperfusion after a prolonged period of ischaemia, cytosolic Ca^{2+} concentration increases as a result of restored availability of exogenous Ca^{2+} with the coronary flow. If, at the same time, the reoxygenation allows the regeneration of sufficient ATP, the cells' homeostatic mechanisms may be able to handle the excess calcium and reduce the cytosolic concentration of Ca^{2+} , thus leading to a subsequent decrease in resting tension. If reoxygenation fails to restore ATP content (as in the case of the 1 ml/min and total ischaemia groups) then the high calcium content and low ATP level in the cytosol may cause a progressive contracture. The accumulation of calcium during reperfusion will be discussed in the next chapter.

The capacity of the myocardial cell to develop tension has been found by others to be correlated with the ATP content of the cell (Gudbjarson et al., 1970; Reibel and Rovetto, 1978). We found an association between developed tension and tissue ATP content after 30 minutes of reperfusion (Fig. 2.9). In all groups, the highest values for both ATP and developed tension occurred during reperfusion after 30 minutes of ischaemia. In some groups of experiments developed tension was reduced to zero when ATP was still present and in other groups the same amount of ATP was associated with a small recovery of developed tension. This finding provides additional support for the hypothesis that the maintenance of mechanical function in cardiac muscle is dependent on a portion rather than the total muscle ATP content. Alternatively, it is possible that destruction of myofilaments rendered them unable to contract during the ischaemic period.

(d) Effect of ischaemia and reperfusion on tissue levels of ATP and CP

The results reported in this study on changes in ATP and CP concentrations after different degrees of ischaemia are, basically, in agreement with those of other studies (Rovetto et al., 1973; Neely et al., 1975; Hearse et al., 1976; Bricknell and Opie, 1978), although isolated rabbit hearts instead of rat hearts have been used. The depletion of ATP and CP during ischaemia reflects the balance between their utilisation and production. ATP utilisation during the early phase of ischaemia, results from the reduced but continued contractile activity. ATP is also used for cellular anabolic and catabolic

processes and for maintenance of various transmembrane ionic gradients (particularly those for sodium, potassium and calcium). ATP supply, during total ischaemia, theoretically, should be derived by the anaerobic glycolysis of endogenous glycogen. During low flow ischaemia, however, anaerobic glycolysis is also supported by exogenous glucose and ATP can derive from aerobic metabolism, depending on the degree of reduction of coronary flow and, therefore, supply of oxygen.

The rate of anaerobic glycolysis during ischaemia is regulated by the activity of glyceraldehyde-3-P-dehydrogenase (Rovetto et al., 1975). This enzyme is inhibited by increased tissue levels of lactate, H^+ , ATP, ADP and AMP. Increased tissue ADP and AMP levels would function to activate phosphofructokinase (the key enzyme of glycolysis under aerobic conditions), whereas these compounds inhibit glyceraldehyde-3-P-dehydrogenase just as effectively as does ATP. In addition, glyceraldehyde-3-P-dehydrogenase is much more sensitive to inhibition by adenine nucleotides at pH 6.8 than it is at pH 7.4.

It has been argued, therefore, that during ischaemia anaerobic glycolysis should be inhibited, particularly, by the development of intracellular acidosis. From the studies of Rovetto et al. (1973) and Neely et al. (1975), designed to measure the rate of glycolysis during ischaemia, it appears that severe reduction of coronary flow rates (to 7% of the control values) resulted in a marked acidosis (pH 6.7) with an inhibition of the glycolytic pathway, while reduction of coronary flow rate above 7% of control values, accelerated the rate of anaerobic glycolysis. From our results it appears that there was

no difference between the ATP and CP content of the 1 ml/min and total ischaemia groups (Tables 2.2 and 2.3). This indicates that the supply of glucose and oxygen delivered at the rate of 1 ml/min was unable to support aerobic or anaerobic metabolism to any significant degree. As the flow was reduced to 4% of control values in this group, we would expect an inhibition of the glycolytic pathway.

In the group of 3 ml/min ischaemia with a coronary flow rate of 12% of control values, however, ATP and CP values were always higher than in the other groups. From our results, also, it appears that during 3 ml/min ischaemia some of the ATP maintained derives from oxidative metabolism. The substitution of N_2 for O_2 in the perfusate resulted in a reduction of the ATP and CP levels (Table 2.4, A). Substituting mannitol for glucose in the hypoxic perfusate resulted in further reduction of ATP and CP (Table 2.4, A) demonstrating the role of anaerobic glucose utilisation under these circumstances. In addition, in the 3 ml/min ischaemic group, ATP utilisation was higher than in the other groups as developed tension decreased more slowly during the first minutes of ischaemia (Fig. 2.5). Thirty minutes after the reduction of coronary flow these hearts were still producing some active tension and they stopped contracting only after 60 minutes.

On reperfusion, if the ischaemic period was not prolonged over 30 minutes, there was a recovery of ATP and CP in all groups, indicating that the cellular energy-producing pathways were still functional. Our data is in agreement with the results of Reibel et al. (1978), Kübler and Spieckerman (1970) and Benson et al. (1961) in the sense that,

after 30 minutes of ischaemic perfusion, ATP concentrations were not fully restored by subsequent reperfusion (Table 2.2).

As suggested by Reibel et al. (1978), incomplete restoration of ATP concentration on reperfusion after a short period of ischaemia (50 minutes) can be due to insufficient purine base for phosphorylation more than to an impaired capacity of the cell for the phosphorylation processes. Loss of purine base may be due either to a release from the ischaemic cells or to an increased hydrolysis of ADP and AMP. Loss of total purine from oxygen deficient tissue has been well documented (Goldthwait, 1957; Benson et al., 1961) and the rate of de novo synthesis of adenine nucleotides, even though increased by oxygen deficiency, is so slow that only about 0.04% of the total tissue ATP pool can be synthesised in one hour (Zimmer et al., 1973). From our results on mitochondrial function it appears that the phosphorylating capacity of mitochondria isolated from ischaemic and reperfused hearts was unchanged from control values (Table 2.7, A).

In our study also, CP values were not restored to the control value during reperfusion. This is in agreement with Kübler and Spieckerman (1971) who reported incomplete synthesis of CP in reperfused hearts and suggested that this could be due either to a loss of creatine and creatine-kinase from the ischaemic cell or to a reduction of creatine-kinase activity. The failure in ATP and CP recovery after prolonged periods of total or 1 ml/min ischaemia may also represent an alteration of mitochondrial function, as will be discussed later.

(e) Effect of ischaemia and reperfusion on mitochondrial function

In this chapter, mitochondrial function was determined as an index of ischaemic damage. No attempt was made to investigate the cause of ischaemic-induced damage to mitochondria or if the function of isolated mitochondria was representative of their function in the cell.

Mitochondria isolated from control-aerobic perfused hearts (Tables 2.5, 2.6, 2.7) showed a respiratory control ratio greater than 10 and an ADP/O ratio close to the theoretical values of 3 in all instances. These results are not different from those obtained for mitochondria from freshly-excised non-perfused rabbit hearts and are in agreement with those of other laboratories (Lindenmayer et al., 1968; Jennings et al., 1969; Schwartz et al., 1973; Sordal et al., 1973; Lochner et al., 1976).

When calculated from the yield, the total mitochondrial content of control ventricular tissue was 32.7 mg mitochondrial protein per g wet weight. This value is similar to that reported by Kleitke et al. (1963) for left ventricular tissue and by Murfit et al. (1978) for the anterior papillary muscle of the dog. Although we have not carried out a quantitative study, the electron micrographs of mitochondrial suspensions from isolated perfused hearts (Fig. 2.2) show that in our preparation the majority of mitochondrial profiles were reasonably intact and of similar appearance. Most mitochondria appeared rounded in cross-section; almost 50% of them showed margination of cristae with clear central areas, whereas the remainder showed more closely packed

cristae. These findings are in agreement with those of Jennings et al. (1969).

This indicates that the conventional technique we used, based on homogenisation and differential centrifugation in the absence of proteolytic enzymes, produces mitochondria qualitatively and quantitatively similar to those obtained by others using different techniques (Safer and Schwartz, 1967; Vercesi et al., 1978; Mela and Seitz, 1979). In addition, our mitochondria oxidised NADH in the presence of ADP at low rates (QO_2 values 24% of that obtained after addition of glutamate). Such low rates are indicative of structural integrity of the isolated organelles.

Studies of the biochemical and structural integrity of mitochondria isolated from ischaemic myocardium have indicated considerable disagreement. Some workers (Jennings et al., 1969; Lindenmayer et al., 1968; Schwartz et al., 1973; Peng et al., 1977) have reported depressed State 3 respiration using succinate, glutamate or pyruvate as substrate in mitochondria from ischaemic tissue, while others (Calva et al., 1976) have reported no changes. Respiratory control ratios of mitochondria isolated from ischaemic tissue have been reported to be depressed by most workers using various substrates, although one report indicated no significant change (Jennings et al., 1969). The reported decreases in RCR after periods of ischaemia have resulted from a decrease in State 3 respiration and not from an increased State 4 respiration rate as is traditionally encountered in uncoupled mitochondria. Peng et al. (1976), however, found a

marked increase in State 4 respiration of ischaemic mitochondria.

Most previous investigators have found the ADP/O ratio to be unchanged, even after a prolonged period of ischaemia (Lochner et al., 1976; Weismaar et al., 1979). In one study the ratio was severely depressed (Schwartz et al., 1973). Mitochondrial yield after 60 minutes of ischaemia, using a very similar model of coronary flow reduction in dogs, has been found unchanged by Murfit et al. (1978) but significantly reduced by Jennings et al. (1969). This is probably because of the different experimental conditions, animal species and procedure for the mitochondrial extraction.

Our results indicate that the degree of coronary flow reduction affects mitochondrial function, as the QO_2 and RCR values were significantly reduced after 90 and 120 minutes of total and 1 ml/min ischaemia, while there was only a slight reduction (not significant) after the same periods of 3 ml/min ischaemia. The ADP/O ratio, however, was unchanged in all groups even after prolonged periods of ischaemia. This indicates that the ischaemic mitochondria maintained the capacity to phosphorylate ADP even if at a reduced rate (as suggested by the reduced RCR and QO_2).

Because of the greater fragility of ischaemic mitochondria, it is possible that changes in their function represent excessive fragmentation during the isolation procedure. It is also possible that a fraction of intact mitochondria, not representative of the entire ischaemic population, is selected during the extraction procedure. The composition of the extraction medium may also influence

the respiratory function of isolated mitochondria (as will be discussed in Chapter 3).

There are some reasons for believing that the changes we found are not the results of excessive fragmentation during the extraction procedure. Electromicrographs of the pellets consistently showed that intact mitochondria were obtained from aerobic or ischaemic mitochondria. When performed (in 3 experiments after 60 minutes of 3 ml/min ischaemia and in 3 after 60 minutes of no flow ischaemia) the NADH test produced results similar to those obtained for aerobic mitochondria (25% and 28% reduction of QO_2 , respectively). The yield of these mitochondria was unchanged. If the mitochondria were fragmented we should expect larger % reduction of QO_2 induced by addition of NADH and a decrease of the yield. This was the case of mitochondria extracted after 120 minutes of total ischaemia. The mitochondrial yield was significantly reduced and NADH stimulation reduced the QO_2 of these mitochondria by 45% (means of 3 experiments), indicating that not only few mitochondria can be harvested, but also, few of them are intact.

Restoration of coronary flow did not modify mitochondrial function when measured in vitro. However, reperfusion of the 3 ml/min group resulted in some recovery of tissue ATP indicating that, in vivo, the readmission of oxygen stimulated the function of the still viable mitochondria. Reperfusion of the total or 1 ml/min ischaemia groups did not improve the function of isolated mitochondria, which remained reduced as it was after the ischaemic period. This is not surprising.

The most likely causes of a reduced function of isolated ischaemic mitochondria are reported to be loss of Krebs cycle intermediates, nicotinamide, adenine nucleotides and electron transport chain component (Calva et al., 1966; Jennings et al., 1969; Schwartz et al., 1973) and a disorganisation of normal structure.

Thirty minutes of readmission of coronary flow is not expected to restore the ultrastructure of the mitochondria as the half-life of mitochondria, in the cell, is 6 to 10 days (Fletcher and Sanadi, 1961; Swick et al., 1968; Meinzeis and Gold, 1971). The poor recovery of tissue ATP during reperfusion of these groups also suggests some damage of mitochondria. Reperfusion of the hearts made totally ischaemic for 90 minutes resulted in a further deterioration of mitochondrial function. There is not a simple explanation for this phenomenon. An increased accumulation of calcium and granular dense bodies of calcium phosphate have been found in mitochondria from myocardium reperfused after prolonged periods of ischaemia (Jennings and Ganote, 1976). This suggests that calcium can be involved in this phenomenon. This possibility will be discussed in detail in the next chapter.

C H A P T E R 3

THE EFFECTS OF VERAPAMIL AND NIFEDIPINE ON
THE ISCHAEMIC AND REPERFUSED MYOCARDIUM

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A. INTRODUCTION

A regulated supply of calcium is required for aerobic heart muscle for a variety of purposes. They include energy production and utilisation (Carafoli and Crompton, 1976), tissue growth and repair (Nayler et al., 1980), maintenance of membrane selective permeability (Zimmerman and Hulsman, 1960; Frank et al., 1977), regulation of glycolytic flux (Mayer, 1974) and of oxidative phosphorylation in mitochondria (Lehninger; 1974, Carafoli, 1975) as well as the activation and termination of the mechanical response (Weber and Herz, 1963; Ebashi and Endo, 1968; Langer, 1980).

In ischaemic heart muscle, however, factors modulating energy metabolism and consumption appear to play an important role in determining cell death. Ca^{2+} is of particular importance since the intracellular concentration of free calcium is believed to control major steps of synthesis and hydrolysis of ATP (Maroko et al., 1971; Carafoli and Crompton, 1976) as well as myocardial contractility (Gergely, 1976).

Cellular damage due to myocardial ischaemia followed by reflow has been found to be associated with a 10 to 20 fold increase in intracellular calcium concentration (Shen and Jennings, 1972). An increase of intracellular calcium is thought to potentiate ischaemic cell injury by increasing energy utilisation and decreasing tissue ATP. Increased intracellular free Ca^{2+} could also lead to cell death by damaging mitochondria (Dhalla et al., 1978) and impairing their function, since the respiration of isolated mitochondria is

inhibited by high levels of calcium (Carafoli et al., 1976; Ferrari et al., 1979; Williams et al., 1980).

These considerations have stimulated, during the last few years, a growing interest in treatments or interventions designed to reduce the influx of calcium during and after ischaemic injury. Therefore, it is not surprising that a new group of agents, commonly referred to as "calcium-antagonists" (Fleckenstein et al., 1969), have been considered for the pharmacological management of angina pectoris, ischaemic heart disease, cardiac arrhythmias and, more recently, for the treatment of essential hypertension (Krikler, 1974; Sing et al., 1978; Parodi et al., 1979; Rowland et al., 1979; Nayler, 1980; Opie, 1980).

Verapamil (Fig. 3.1), a drug which had been developed in the early 1960s as a coronary vasodilator, is the prototype of these substances (Fleckenstein, 1970). Other drugs included in this group are nifedipine (Fig. 3.1), fendiline, D600, a methoxy derivative of verapamil, and diltiazem (Fleckenstein, 1977; Nayler, 1980). The term "calcium antagonist" for these substances was introduced on the basis of the following four separate observations (Fleckenstein, 1969; 1970; 1971; Beeler and Reuter, 1970; Kohlhardt et al., 1973) - that (a) ionised calcium reversed the cardiodepressant effect of verapamil and D600; (b) that verapamil and nifedipine depressed the calcium-induced developed tension in potassium depolarised muscle (c) that these drugs despite the absence of beta-adrenoreceptor blocking activity prevented the myocardium from becoming overloaded

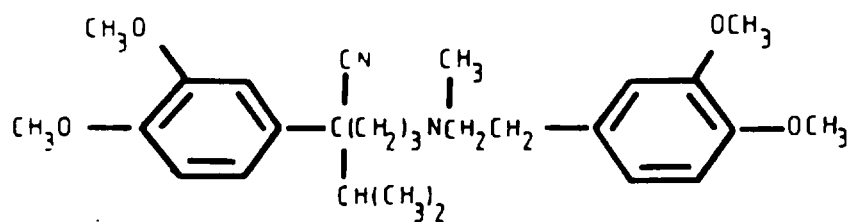
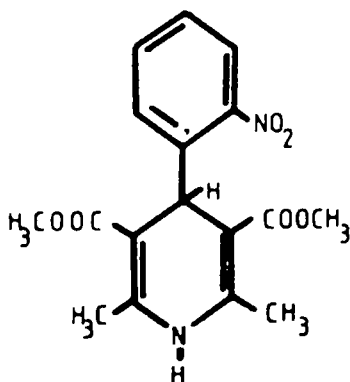
VERAPAMILNIFEDIPINE

FIGURE 3.1 FORMULA OF VERAPAMIL AND NIFEDIPINE

with calcium in response to the in vivo administration of large amounts of isoproterenol and (d) that they selectively inhibited the Ca^{2+} -dependent component of the cardiac action potential.

However, the term "calcium antagonist" to describe these drugs lacks specificity since there are at least three ways in which calcium can enter the myocardium. This can be (a) by passive diffusion down its electrochemical gradient; (b) in exchange for internal sodium and for K^+ (c) through voltage-activated ion-selective channels that are activated in response to membrane depolarisation and which carry the slow inward Ca^{2+} current (Beeler and Reuter, 1970; Kohlhardt et al., 1973). Of these three pathways only that which involves the inward displacement of calcium through the voltage-dependent channels has been shown to be sensitive to verapamil and to nifedipine (Kohlhardt et al., 1972; Fleckenstein, 1977). It is generally accepted that it is the excitation-induced inward displacement of calcium through the voltage-activated channels that mediates the controlled transition from the resting to the active state in cardiac and smooth muscle cells (Reuter, 1979). It follows that this can explain the negative inotropic effect of this group of drugs and their ability to reduce vascular tone (Fleckenstein, 1977).

In skeletal muscle, on the contrary, the activation of contraction is dependent on the ability of the depolarising current to initiate a release of "activator calcium" from the internal stores, without any inward displacement of calcium from the extra- to the intracellular phase. This explains why "calcium antagonists" do not affect the

functioning of skeletal muscle. To account for the clinical usefulness of these drugs in the treatment of ischaemic heart diseases we have to consider that they may affect the functioning of cardiac and smooth muscle cell and the cells of pacemaker, nodal and conducting tissue. It is uncertain if the ability of this group of substances to reduce the severity of ischaemic-induced damage results directly from their ability to impede slow channel transport in the myocardium or if other mechanisms are involved.

From the positive results obtained using intact animals (Smith et al., 1975; Henry et al., 1978; Christlieb et al., 1978; Bier et al., 1978; Selwin et al., 1979), it is impossible to determine whether the protection described is due to a direct effect on myocardium. It may be due to a reduced afterload and/or to a reduced heart rate, resulting in a decrease in the oxygen requirements of the hearts. It may also be due to a coronary vasodilatation improving oxygen delivery to the heart.

In addition, if their protective effect is a direct action on the myocardium, the mechanism is still unknown, as the increase of intracellular calcium which may occur during and after a period of ischaemia is not necessarily dependent upon the entry of calcium through the slow channel.

The aim of the present study was to investigate if pre-treatment with either verapamil or nifedipine is able to reduce some of the deleterious effects caused by ischaemia and reperfusion in the isolated Langendorff perfused rabbit's heart. The use of an isolated heart preparation is particularly valuable for the evaluation of the direct effects of agents or interventions on the myocardium, since peripheral

and hormonal influences are eliminated. The use of a model of no-flow ischaemia, as has been used in this study, offers also the advantage of eliminating the effects of agents on coronary flow as well as on the collateral circulation.

Because recovery of function is associated with high levels of cellular ATP (Chapter 2) and the respiration of isolated mitochondria from severely ischaemic hearts is depressed, disruption of mitochondrial function has been suggested as a major cause of irreversible ischaemic damage (Dhalla et al., 1978). Consequently, the maintenance of mitochondrial structure and function may be important for the preservation of the ischaemic myocardium (Trump et al., 1976). The present study was designed to determine whether pre-treatment with either verapamil or nifedipine was able to reduce the deterioration in mitochondrial function caused by ischaemia and reperfusion. For this purpose mitochondria were isolated and their function assessed by measuring their calcium content, oxygen consumption and the capacity to phosphorylate adenosine diphosphate (ADP). As a corollary to these studies, the tension developed by the isolated hearts was monitored and the stores of adenosine triphosphate (ATP) and creatine phosphate (CP) assayed.

B. EXPERIMENTAL

(a) Treatment of the animal

Adult male New Zealand white rabbits (2.5 to 3.0 Kg) maintained on a standard diet were used for this study. The control rabbits received 2 mg/Kg of 0.9% sodium chloride solution subcutaneously twice daily for 4-5 days. The other rabbits (treated groups) received either 2 mg/Kg of racemate verapamil or nifedipine subcutaneously, twice daily, for the same period of time, and dissolved in the same volume of sodium chloride solution.

Nifedipine is difficult to dissolve and it is light-sensitive (Vatery et al., 1972). To avoid this problem nifedipine was dissolved by warming the saline solution to 30° while stirring for 45 minutes in a dark-room. The syringe used for the injection was also covered with opaque material.

All animals received the last injection two hours before the experiment. The doses of verapamil and nifedipine selected for this study were employed after an initial period of testing several different doses. Doses of verapamil and nifedipine of 6.0 and 8.0 mg/Kg twice daily killed the animals. Doses of either drug below 1 mg/Kg did not exert any noticeable effect on the tension development or heart rate of the isolated rabbit hearts. The dose regime selected for this study, is the same as that used in other studies (Nayler et al., 1978) where it provided plasma levels which are comparable with those used clinically.

(b) Protocol

Each rabbit was stunned by a blow on the head. The heart was rapidly excised and perfused as has been described in detail in Chapter 2.

A period of at least 30 minutes after cannulation was allowed for equilibration of all hearts before any experimental intervention. After the spontaneous heart rate was determined, they were electrically stimulated at a constant rate of 180 beats/min as described in detail in Chapter 2. The hearts, both from treated or untreated rabbits, were then divided into three groups (Fig. 3.2). The hearts of the first group were perfused under aerobic conditions with oxygenated Krebs-Henseleit buffer solution at a mean coronary flow of 25 ± 1.2 ml/min for another 30 or 120 minutes. The hearts of the second group were made ischaemic by stopping the pump for 90 minutes. The third group of hearts were made ischaemic for 90 minutes and then reperfused with aerobic Krebs-Henseleit buffer solution for another 30 minutes. During reperfusion the coronary flow was readjusted to the 25 ml/min control level. Left ventricular wall temperature was maintained at between 36 and 37°C, irrespective of coronary flow as described in Chapter 2.

(c) Force measurements

The procedure for developed and resting tension measurements has been described in detail in Chapter 2.

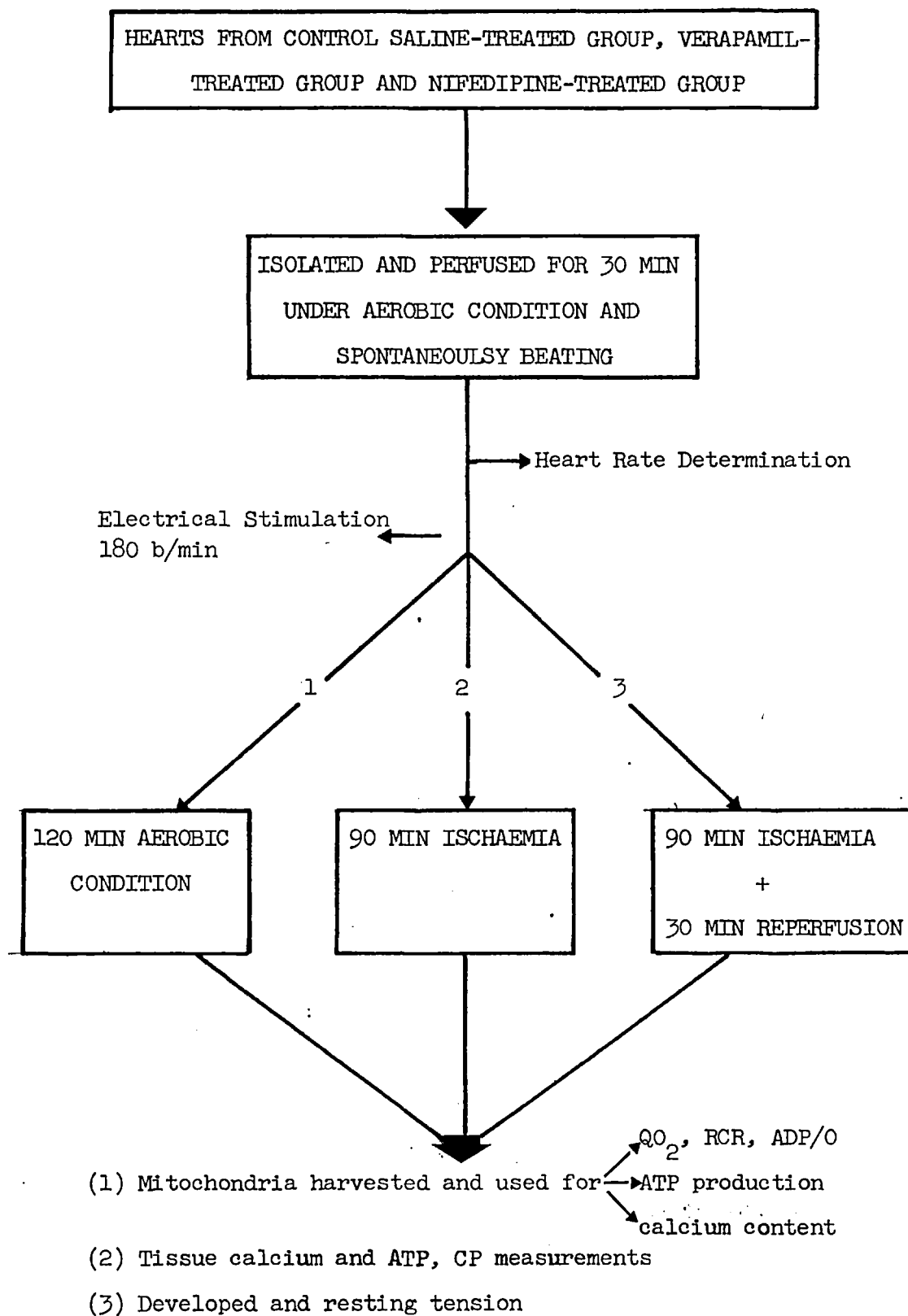


FIGURE 3.2 SCHEMATIC REPRESENTATION OF EXPERIMENTAL SCHEDULE

(d) ATP and creatine phosphate determination

The procedure for ATP and creatine phosphate determination has been described in detail in Chapter 2.

(e) Tissue calcium measurements

At the end of the perfusion time, duplicate samples of left ventricular muscle (excluding septum and apex) were taken. They were dried on the edge of a filter paper, weighed and digested in 1 ml/g wet wt of nitric acid (HNO_3) for at least 12 hours.

The extracts were diluted in 25 ml of de-ionised water and the calcium content was assayed spectrophotometrically using a Perkin Elmer absorption spectrometer (Model 306, Perkin Elmer) at a wavelength of 4227 Å, using calcium carbonate as the standard. LaCl_3 was also added to provide a final concentration of 0.1% in order to inhibit chemical interference. Total tissue water was obtained by drying to constant weight at 95°C.

(f) Mitochondrial preparation

The isolation procedure was taken from Williams and Barrie (1978) and has been described in detail in Chapter 2. According to the purpose for which the mitochondria were being isolated, two different isolation media were used. Those required for oxygen consumption studies were isolated in the medium described by Sordahl et al. (1973) which contains, in addition to potassium chloride and bovine serum albumin, ethylenediamine tetraacetic acid. This medium is described in detail in

Chapter 2 (medium I) (KEA). The mitochondria isolated for the determination of their endogenous calcium and their ATP-producing capacity were extracted in a medium based on that described by Peng et al. (1976) which contained 250 millimolar sucrose, 50 millimolar tris-buffer, pH 7.2. This medium was modified by us by the addition of 5 micromolar ruthenium red (medium II). Ruthenium red, which is an inhibitor of mitochondrial calcium uptake (Drahota et al., 1965; Rossi et al., 1973; Thakar et al., 1973; Henry et al., 1977), was included to prevent the mitochondria from accumulating Ca^{2+} during the isolation procedure.

Unless a separate series of experiments was carried out, the hearts were disconnected from the Langendorff-apparatus and quickly placed in cold medium (0°C) containing sucrose, tris buffer and ruthenium red. The ventricles were removed, washed and finely chopped. Then the tissue mince was divided into two portions, each one was weighed and resuspended using either the extraction medium I or II. The homogenisation and the differential centrifugation extraction were carried out as previously described (Chapter 2). The final crude mitochondria pellet was resuspended in 0.5 ml of medium I or II.

(g) Protein determination

Mitochondrial protein concentration was determined by the method of Bradford (1976) as described in detail in Chapter 2.

(h) Mitochondrial respiration

Mitochondrial respiration was determined polarographically as described in detail in Chapter 2.

(i) Mitochondrial ATP production

The amount of ATP produced from isolated mitochondria after the addition of ADP was measured using the enzymatic method of Lamprecht and Trautschold (1974) described in Chapter 2. Mitochondria, for this purpose, were extracted at the end of each perfusion in the EDTA-free medium in the presence of 5 μ M of ruthenium red (medium II). 1.25 mg/ml of mitochondrial protein was suspended in 2 ml of 250 mM sucrose, 3 mM KH_2PO_4 , 3 mM K.glutamate solution adjusted to pH 7.2 with tris-buffer and maintained at 25°C. They were allowed to equilibrate for one minute. Following this period, a first sample was taken before adding a final concentration of .5 mM ADP to stimulate phosphorylating respiration. Samples were then taken after 6, 15, 30, 45, 60, 120 and 180 seconds and vigorously mixed with 50 μ l of 10% perchloric acid on ice using a vortex mixer. Precipitated protein was separated by centrifugation and the ATP content of the supernatant (200 μ l) determined enzymatically as described in Chapter 2.

A standard ATP calibration curve was obtained for each assay by adding different known concentrations of ATP to the 2 ml of incubation medium. These standard samples were processed as described for the test samples. The mitochondrial ATP content before the addition of ADP was taken into account during the calculation.

(j) Mitochondrial calcium measurements

Mitochondria for this purpose were extracted in the EDTA-free medium in the presence of 5 μ M of ruthenium red (medium II). 250 μ l of the final crude mitochondrial pellet resuspended in .5 ml of extraction medium were digested overnight in 500 μ l of HNO_3 and their calcium content was determined by atomic absorption spectrometry as previously described.

(k) Statistical analysis

Results are expressed as mean $\pm$ S.E. of n experiments. Tests of significance were made using Student's "t" test, taking $P = 0.05$ as the limit of significance.

(l) Animals and materials

New Zealand white rabbits were obtained from Hylyne, Cheshire, U.K. Standard laboratory chemicals of analar grade were purchased from BDH HD, Poole, Dorset, U.K. Ruthenium red and all enzymes were obtained from either Sigma Chemical Co. (London), Poole, Dorset, U.K. or Boehringer Corporation (London). Verapamil was obtained as the hydrochloric salt from Knoll AG, Ludwigshafen, Germany and nifedipine (AS BAY 1040) from Bayer AG, Germany.

C. RESULTS

(1) FORCE DEVELOPMENT

(a) Effects of prolonged perfusion and treatment with verapamil or nifedipine on heart rate and tension

Control studies, using spontaneously beating and electrically paced (180 b/min) rabbit hearts, showed that developed tension, resting tension, heart rate and coronary flow remained constant during 120 minutes of aerobic perfusion at pH 7.4, T 37⁰ (Table 3.1, Fig. 3.3, tracing A).

Table 3.1 also shows that the treatment of rabbits with verapamil or nifedipine induced a reduction of the spontaneous rate at which hearts were beating during the first 30 minutes of perfusion under aerobic conditions (Fig. 3.1). The decrease in heart rate, however, was statistically significant only for the verapamil treated group.

After 30 minutes of aerobic perfusion, hearts in the verapamil and nifedipine groups developed significantly less tension than those from the control group (Table 3.1). For the verapamil group, the reduction in tension development was still present after 120 minutes of perfusion under aerobic conditions. In contrast, the hearts from the nifedipine-treated group increased the tension developed after 80 minutes of aerobic perfusion and, by the end of 120 minutes, the values were not significantly different from those of the control group (Table 3.1).

Treatment with verapamil or nifedipine did not alter the resting tension values.

TABLE 3.1 EFFECT OF TREATMENT WITH VERAPAMIL OR NIFEDIPINE ON HEART RATE AND DEVELOPED TENSION OF THE ISOLATED LANGENDORFF-PERFUSED RABBIT HEARTS

Groups	Heart Rate Beats/Min	Developed Tension g 30 Min after Aerobic Perfusion	Developed Tension g 120 Min after Aerobic Perfusion
Control	142 $\pm$ 13 (n = 37)	7.5 $\pm$ 1.9 (n = 19)	7.1 $\pm$ 1.1 (n = 8)
Verapamil	99 $\pm$ 19 * (n = 16)	4.9 $\pm$ 0.9 * (n = 6)	4.3 $\pm$ 0.9 * (n = 6)
Nifedipine	122 $\pm$ 20 (n = 14)	4.0 $\pm$ 1.0 * (n = 5)	6.8 $\pm$ 1.3 (n = 5)

* P < 0.05

Results are expressed as mean $\pm$ S.E. The number of experiments is reported in brackets.

Tests of significance calculated with respect to the difference from the control group.

(b) Effect of ischaemia and reperfusion in control hearts

Figure 3.3, tracing B, shows the mechanical records of a typical experiment during ischaemia and reperfusion from control rabbits. The mean results of at least 6 experiments are illustrated in Fig. 3.4 and Fig. 3.5.

When coronary flow was reduced to zero, a rapid decrease in developed tension occurred (Fig. 3.3 and Fig. 3.4). The continuous monitoring of tension development in the control group showed a slowing of heart rate after the first minutes of ischaemia (5.6 min), despite an increase of pacemaker stimulus to 5 V. Ten minutes after the onset of ischaemia it was impossible to record any ventricular activity. Resting tension began to rise 20 minutes after the onset of ischaemia and by the end of 90 minutes it was increased to 6 ± 0.9 g (Fig. 3.5). During the first 5 minutes of reperfusion, resting tension rapidly rose, from 6 ± 0.7 g to 8.7 ± 1.12 g and at the end of 30 minutes reperfusion had increased to 11 ± 1.1 g. The recovery of developed tension during reperfusion was almost negligible in 5 experiments and only in 2 of the 7 control experiments was there as much as a 10% recovery in tension developed.

(c) Effects of ischaemia and reperfusion in verapamil and nifedipine treated groups

Figure 3.3 also shows the typical mechanical records of two hearts subjected to exactly the same protocol, except that they were isolated from rabbits treated either with verapamil (tracing C) or

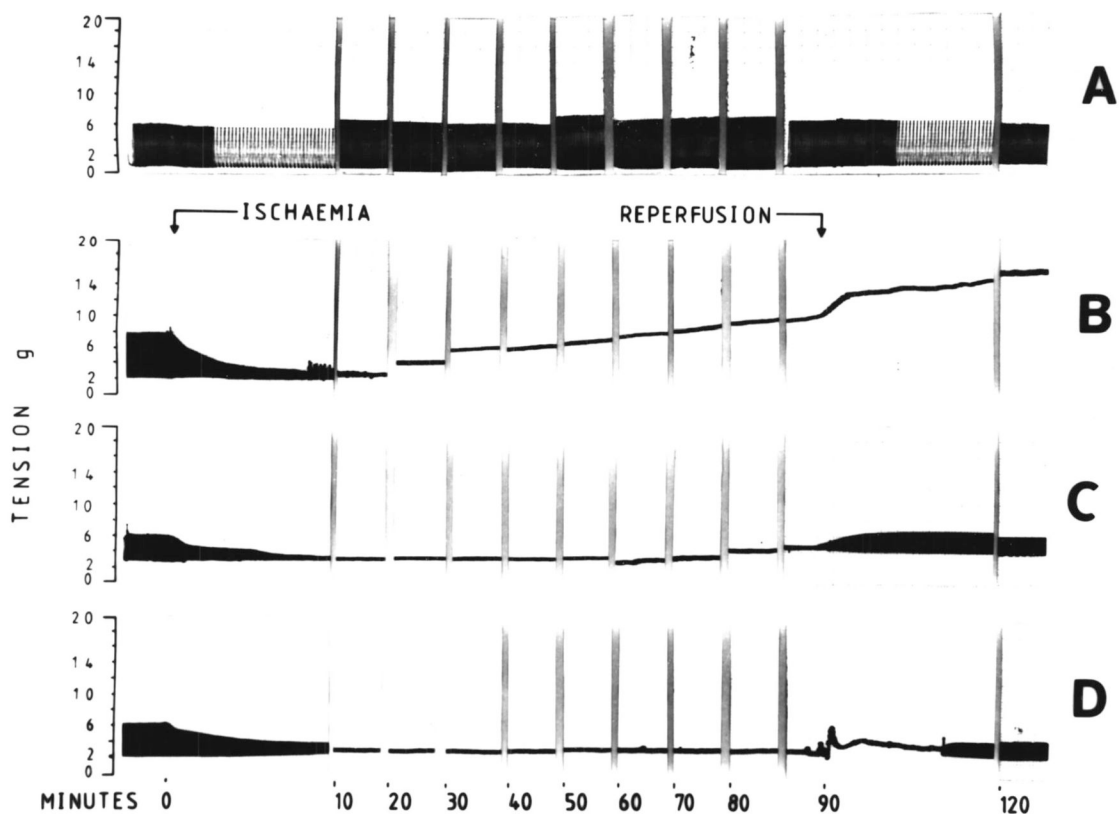


FIGURE 3.3 MECHANICAL RECORDS FROM FOUR ELECTRICALLY PACED (180 b/min) LANGENDORFF-PERFUSED RABBIT HEARTS

- A. Control hearts perfused for 120 min under aerobic conditions
- B. Control hearts made totally ischaemic for 90 min and reperfusion for another 30 min
- C. Verapamil-treated hearts made totally ischaemic for 90 min and reperfusion for another 30 min
- D. Nifedipine-treated hearts made totally ischaemic for 90 min and reperfusion for another 30 min

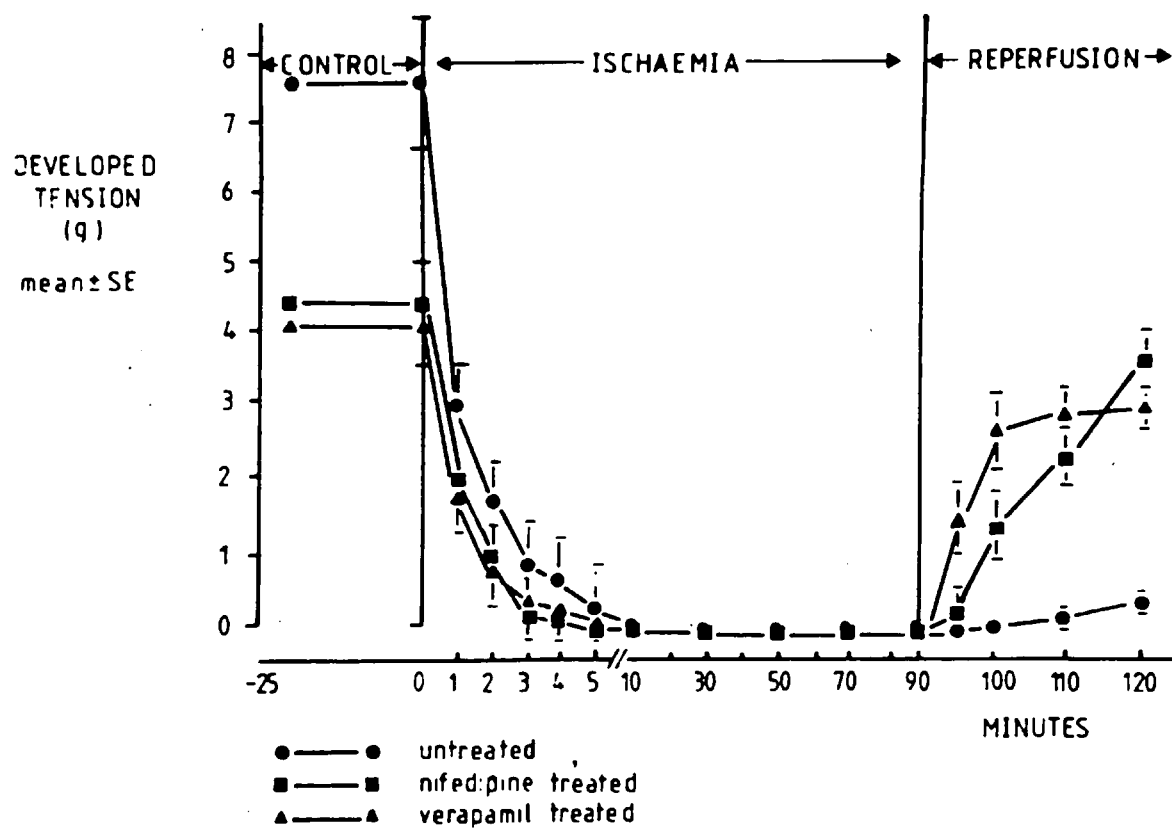


FIGURE 3.4 EFFECT OF TREATMENT WITH VERAPAMIL OR NIFEDIPINE ON DEVELOPED TENSION DURING ISCHAEMIA AND REPERFUSION

with nifedipine (tracing D). The difference in tension developed just before the onset of ischaemia is due to the effect of treatment. The early decline in tension, cardiac slowing and absence of ventricular activity 10 minutes after abolition of coronary flow, were similar to those observed in the control group (Fig. 3.3 and 3.4). However, in the hearts from treated rabbits (either with verapamil or nifedipine) the onset and extent of ischaemic-induced increase in resting tension were significantly delayed and reduced (Fig. 3.5). On reperfusion, the verapamil-treated group showed an increase in resting tension from 1.7 ± 0.5 to 2.8 ± 1 g and a progressive recovery in developed tension with a regular, paced rhythm (Fig. 3.3, tracing C). In this group the percentage of developed tension recovered was 72% of the tension developed by the verapamil-treated hearts immediately before the onset of ischaemia and 37.3% of the tension developed by hearts from the control group just before the onset of ischaemia. Because the reduction in tension development induced by verapamil treatment was still present after 120 minutes of aerobic perfusion (Table 3.1), we should not expect that these ischaemic hearts would recover as much developed tension as is shown by control hearts.

The nifedipine-treated group also showed a recovery of tension development on reperfusion, but only after 5 minutes of irregular, unpaced rhythm (Fig. 3.3, tracing D). During the first 5 minutes of reflow, resting tension increased and then remained constant. At the end of reperfusion, the tension recovered was 83% of that developed by nifedipine-treated hearts before the onset of ischaemia and 46% of the

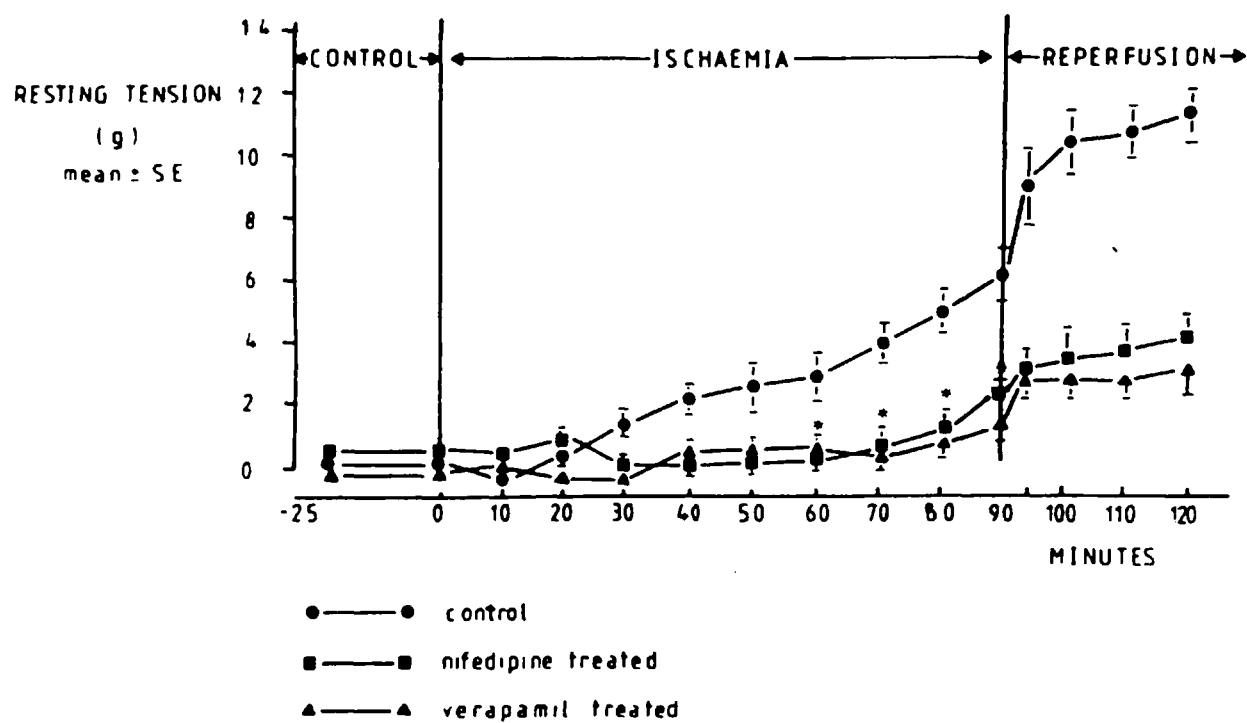


FIGURE 3.5 EFFECT OF TREATMENT WITH VERAPAMIL OR NIFEDIPINE ON RESTING TENSION DURING ISCHAEMIA AND REPERFUSION

tension developed by control-saline-treated hearts. The effect of nifedipine treatment on developed tension, however, was no longer present after 120 minutes of aerobic perfusion (Table 3.1), so that these hearts should be able to recover the same degree of tension as that achieved by control hearts.

(2) ISOLATED MITOCHONDRIAL FUNCTION

(a) Effects of different extraction media on mitochondrial function from aerobically perfused control hearts

Tables 3.2 and 3.3 summarise the data obtained from mitochondria isolated by using two different extraction media as described in the Methods section. A typical oxygen electrode tracing of mitochondrial respiration is also shown in Fig. 3.6 (A).

The data reported in Tables 3.2 and 3.3 as Series I refer to mitochondria isolated in a medium containing KCl, EDTA and albumin (medium I) (KEA) which has been proposed (Lindenmayer et al., 1968) to be ideal for isolation of tightly coupled heart mitochondria. The data reported in Tables 3.2 and 3.3 as Series II refer to those mitochondria isolated in an EDTA-free medium (medium II) containing sucrose and ruthenium red, included to prevent the mitochondria from accumulating Ca^{2+} during the isolation procedure. Mitochondrial respiration was measured using glutamate as substrate and the reaction mixture is described in the Methods. The same reaction mixture was used irrespective of which medium was used for the extraction. The results show that there is no significant difference between RCR,

TABLE 3.2 EFFECT OF DIFFERENT EXTRACTION MEDIA ON MITOCHONDRIAL RCR, QO_2 , ADP/O

Experiments	Series	RCR	QO_2 n moles O_2 /mg/prot.	ADP/O
Freshly Excised	I	19.2 ± 1.7 (4) $P_1 < 0.001$	291 ± 8.3 (4) $P_1 < 0.05$	2.9 ± 0.1 (4) $P_1 < 0.05$
	II	7.6 ± 1.7 (4)	238 ± 11.5 (4)	2.3 ± 0.1 (4)
Aerobic Perfusion 30 Min	I	18.6 ± 1.9 (4) P_2 NS. $P_1 < 0.001$	264 ± 21.7 (4) P_2 NS. $P_1 < 0.05$	2.7 ± 0.1 (4) P_2 NS. $P_1 < 0.05$
	II	6.2 ± 0.3 (4) P_2 NS.	196 ± 11.0 (4) P_2 NS.	2.3 ± 0.2 (4) P_2 NS.
Aerobic Perfusion 120 Min	I	16.0 ± 1.7 (5) P_2 NS. $P_1 < 0.001$	251 ± 19.5 (5) P_2 NS. $P_1 < 0.05$	2.8 ± 0.2 (5) P_2 NS. $P_1 < 0.05$
	II	5.6 ± 1.2 (8) P_2 NS.	194 ± 13.1 (8) P_2 NS.	2.3 ± 0.4 (8) P_2 NS.
Ischaemia 90 Min	I	10.7 ± 0.4 (7) $P_2 < 0.05$ $P_1 < 0.001$	187 ± 8.7 (7) P_2 NS. P_1 NS	2.5 ± 0.5 (7) P_2 NS. $P_1 < 0.05$
	II	3.4 ± 2.0 (7) $P_2 < 0.05$	124 ± 3.0 (7) $P_2 < 0.05$	1.9 ± 0.1 (7) $P_2 < 0.05$
Ischaemia 90 Min Reperfusion 30 Min	I	5.8 ± 0.3 (6) $P_2 < 0.01$ $P_1 < 0.001$	121 ± 12.0 (6) $P_2 < 0.01$ $P_1 < 0.01$	2.3 ± 0.3 (6) $P_2 < 0.05$ $P_1 < 0.01$
	II	1.1 ± 0.1 (6) $P_2 < 0.01$	62.5 ± 4.0 (6) $P_2 < 0.01$	1.5 ± 0.6 (6) $P_2 < 0.01$

The data are expressed as mean $\pm$ S.E. Series I refers to results obtained for KEA medium, Series II to those obtained using sucrose-ruthenium red medium. P_1 relates to the significance of the difference between the values obtained using medium I and II in each experimental condition. P_2 relates to the significance of the difference between the values obtained under the experimental conditions and those obtained after 120 minutes of aerobic perfusion for medium I and for medium II.

TABLE 3.3 EFFECT OF DIFFERENT EXTRACTION MEDIA ON MITOCHONDRIAL CALCIUM AND YIELD

Experiments	Series	Mitochondrial Calcium n moles/mg/prot.	Yield mg/prot./g wt weight
Freshly Excised	I	6.3 $\pm$ 2.0 (4)	12.4 $\pm$ 2.3 (4)
	II	$P_1 < 0.001$ 15.1 $\pm$ 2.1 (4)	$P_1 < 0.05$ 7.02 $\pm$ 0.9 (4)
Aerobic Perfusion 30 Min	I	5.7 $\pm$ 1.8 (3) P_2 N.S.	10.9 $\pm$ 1.9 (4) P_2 N.S.
	II	$P_1 < 0.001$ 16.6 $\pm$ 3.9 (4) P_2 N.S.	$P_1 < 0.05$ 6.12 $\pm$ 0.5 (4) P_2 N.S.
Aerobic Perfusion 120 Min	I	5.0 $\pm$ 1.0 (3) P_2 N.S.	10.6 $\pm$ 1.2 (3) P_2 N.S.
	II	$P_1 < 0.001$ 16.5 $\pm$ 2.3 (5) P_2 N.S.	$P_1 < 0.05$ 6.94 $\pm$ 1.1 (8) P_2 N.S.
Ischaemia 90 Min	I	5.9 $\pm$ 2.0 (4) P_2 N.S.	8.1 $\pm$ 2.6 (7) P_2 N.S.
	II	$P_1 < 0.001$ 23.2 $\pm$ 2.2 (7) $P_2 < 0.05$	$P_1 < 0.05$ 5.06 $\pm$ 0.6 (7) P_2 N.S.
Ischaemia 90 Min Reperfusion 30 Min	I	7.2 $\pm$ 3.1 (4) P_2 N.S.	6.3 $\pm$ 1.9 (6) $P_2 < 0.05$
	II	$P_1 < 0.001$ 42.0 $\pm$ 1.9 (6) $P_2 < 0.001$	$P_1 < 0.05$ 3.77 $\pm$ 1.0 (6) $P_2 < 0.05$

The data are expressed as mean $\pm$ S.E. Series I refers to results obtained for KEA medium, Series II to those obtained using sucrose-ruthenium red medium. P_1 relates to the significance of the difference between the values obtained using medium I and II in each experimental condition. P_2 relates to the significance of the difference between the values obtained under the experimental conditions and those obtained after 120 minutes of aerobic perfusion for medium I and for medium II.

QO_2 , ADP/O yield and calcium content values of mitochondria isolated from hearts after 30 or 120 minutes of aerobic perfusion and those obtained for mitochondria from freshly-excised non-perfused hearts (Tables 3.2 and 3.3, Series I and II).

The RCR, QO_2 and ADP/O values obtained for mitochondria isolated in medium I (Tables 3.2, Series I), however, were always significantly higher than those obtained from mitochondria extracted after the same experimental conditions using medium II (Table 3.2, Series II). The difference for the RCR values, between Series I and II, was always higher than that for the QO_2 values. This suggests that the difference between the mitochondria isolated in mediums I and II was higher for State 4 respiration than for State 3, as also appears from the tracing illustrated in Fig. 3.6 (A). State 3 respiration represents the rate of oxygen consumption in the presence of ADP, while State 4 respiration is the rate of oxygen consumption in the absence of ADP and represents the energy consumed to maintain ion gradients in the face of ion leakage across the membrane. In addition, the yield of mitochondria extracted in medium I was higher than that obtained for those extracted in medium II, indicating that fewer mitochondria can be isolated using this medium (Table 3.3). As expected, the calcium content of mitochondria from Series I (Table 3.3) was always significantly lower than that of mitochondria isolated in the presence of ruthenium red and absence of EDTA (medium II, Table 3.3).

The values of mitochondrial calcium content obtained in the present studies, using medium II, are in agreement with those found by Carafoli and Lehninger (1971) and by Peng et al. (1977).

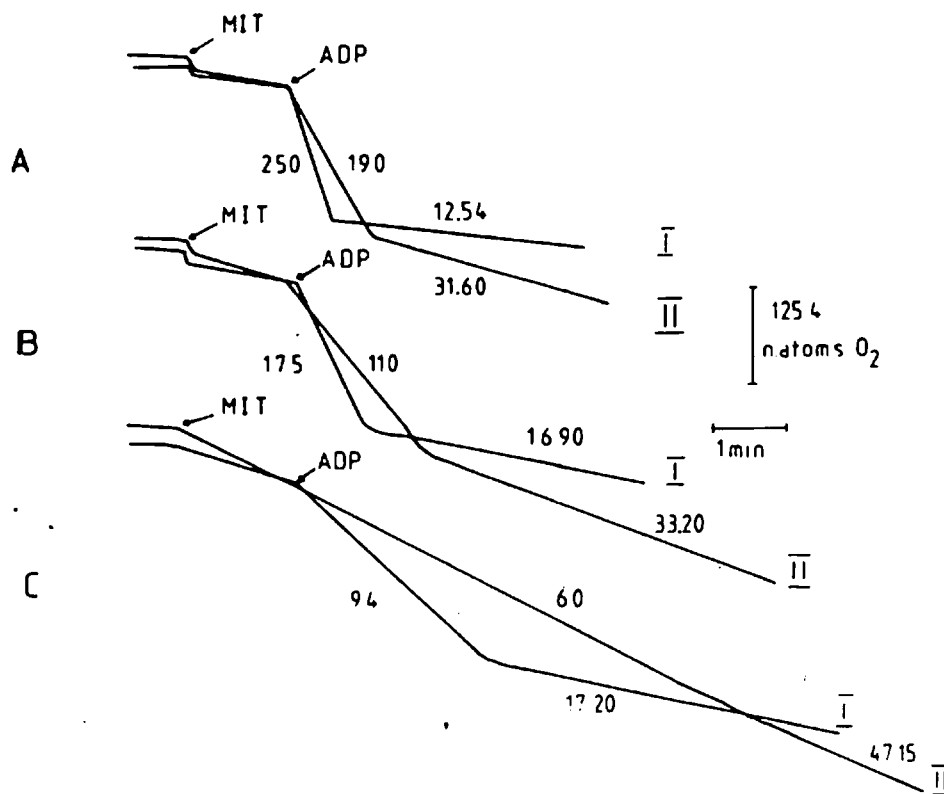


FIGURE 3.6 OXYGEN ELECTRODE TRACING OF TYPICAL EXPERIMENTS

Oxygen electrode tracings showing the effect of the two isolation media (I and II) on respiration of mitochondria isolated from hearts perfused under aerobic condition for 120 minutes (A), or after 90 minutes of ischaemia (B) or after 90 minutes ischaemia - followed by 30 minutes of reperfusion (C). Experimental conditions are described in the methods section.

Series I refers to mitochondria isolated with medium I (KEA) and Series II to mitochondria isolated using medium II (sucrose-ruthenium red).

(b) Effects of different extraction media on mitochondrial function from ischaemic or reperfused control hearts

The mitochondria isolated after 90 minutes of ischaemia, using either medium I or II, had significantly reduced values of RCR, and QO_2 (Table 3.2, Fig. 3.6). The ADP/O ratio was reduced only for ischaemic mitochondria isolated in medium II (Table 3.2). This suggests that the composition of medium I was able to maintain the phosphorylating capacity (ADP/O) of the ischaemic mitochondria despite the reduction in the respiration rate (QO_2 - RCR). The RCR, QO_2 and ADP/O of mitochondria isolated after 30 minutes of post-ischaemic reperfusion were also significantly reduced, irrespective of which medium was used for the extraction. In addition, in 2 out of 6 experiments, the mitochondria isolated with medium II were completely uncoupled. When the RCR, QO_2 and ADP/O values were expressed as % reduction of the values of mitochondria from aerobic-perfused hearts (Table 3.4) it appears that the percentage mitochondrial function reduction, either after ischaemia or after reperfusion, was always higher for those mitochondria harvested using medium II. This indicates that the isolation medium had an important effect on the degree of depression of mitochondrial respiration and phosphorylation, induced by ischaemia and reperfusion.

Table 3.3 also shows that the calcium content of mitochondria isolated in medium II (Series II) from ischaemic myocardium was increased.

A highly significant increase of calcium content was also found

TABLE 3.4 PERCENTAGE REDUCTION IN MITOCHONDRIAL FUNCTION INDUCED BY ISCHAEMIA AND REPERFUSION.

Experiments	RCR		QO ₂		ADP/O	
Series	I	II	I	II	I	II
90 Min Ischaemia.	3.3	39.3	25.4	36.0	10.0	18.4
90 Min Ischaemia + 30 Min Reperfusion	63.7	80.3	51.7	67.7	19.0	35.6

The percentage reduction in RCR, QO₂ and ADP/O is calculated with respect to the value obtained from mitochondria isolated after 120 minutes of aerobic perfusion. Series I refers to mitochondria isolated in KEA medium and Series II to those isolated in sucrose-ruthenium red medium.

TABLE 3.5 EFFECT OF THE ADDITION OF 5 μ M RUTHENIUM RED TO THE ISOLATION MEDIUM ON THE RCR, QO_2 , ADP/O VALUES OF MITOCHONDRIA ISOLATED FROM FRESHLY EXCISED NON-PERFUSED HEARTS

	ISOLATION MEDIUM	
	250 mM Sucrose 50 mM Tris 5 μ M Ruthenium Red	250 mM Sucrose 50 mM Tris
RCR	7.6 $\pm$ 1.7	7.3 $\pm$ 2.0
QO_2	238 $\pm$ 11.5	221 $\pm$ 21.4
ADP/O	2.3 $\pm$ 0.1	2.3 $\pm$ 0.1

The data represent the mean $\pm$ S.E. of 4 experiments with ruthenium red present in the isolation medium and of 3 without ruthenium red in the isolation medium.

The procedure for the isolation of mitochondria was identical for the two groups. Mitochondrial respiration was measured using glutamate as substrate and the reaction mixture described in the methods section.

There is no difference between the two groups.

in the mitochondria isolated in medium II after reperfusion. In contrast, the calcium content of mitochondria from the same ischaemic or reperfused hearts, but isolated in medium I, was the same as it was in aerobically perfused hearts. This finding indicates that the EDTA content of the medium I was able to buffer all the Ca^{++} during the extraction procedure. Finally, Table 3.3 shows that the yield of mitochondria isolated from reperfused myocardium was significantly lower than that of mitochondria from either ischaemic or aerobic myocardium. However, the yield of mitochondria harvested in medium II was always less than that of mitochondria isolated in medium I (Table 3.3).

Table 3.5 shows that the presence of $5\text{ }\mu\text{M}$ ruthenium red during the isolation procedure did not significantly affect the respiration rates, respiratory control ratios and ADP/O ratios of mitochondria from fresh non-perfused rabbit hearts.

(c) Effect of treatment with verapamil or nifedipine on mitochondrial function

Table 3.6 shows that treating the rabbits with either verapamil or nifedipine made no difference to the oxidative phosphorylating activity of mitochondria harvested from either freshly excised or aerobically-perfused hearts irrespective of which extraction medium was used. The yield of mitochondria was also unchanged. When verapamil and nifedipine were added directly to the isolated mitochondria in the reaction medium to provide a final concentration of $5\text{ }\mu\text{M}$ there was no significant change in the oxidative phosphorylating

TABLE 3.6 EFFECT OF PRETREATMENT WITH EITHER VERAPAMIL OR NIFEDIPINE ON THE RCR, QO_2 AND ADP/O VALUES OBTAINED FOR MITOCHONDRIA HARVESTED FROM FRESHLY EXCISED AND AEROBICALLY PERFUSED HEARTS (30 AND 120 MIN) USING KEA EXTRACTION MEDIUM (SERIES I) OR SUCROSE-RUTHENIUM RED MEDIUM (SERIES II)

Experiments	RCR		QO_2		ADP/O	
Series	I	II	I	II	I	II
<u>Freshly Excised, Non-perfused Rabbit Hearts</u>						
Control	19.2 $\pm$ 1.7	7.6 $\pm$ 1.7	291 $\pm$ 8	238 $\pm$ 11	2.9 $\pm$ 0.1	2.3 $\pm$ 0.1
Verapamil	19.9 $\pm$ 1.3	7.3 $\pm$ 1.4	298 $\pm$ 25	231 $\pm$ 18	2.9 $\pm$ 0.1	2.5 $\pm$ 0.1
Nifedipine	18.6 $\pm$ 1.2	7.4 $\pm$ 0.9	288 $\pm$ 16	234 $\pm$ 14	2.8 $\pm$ 0.2	2.5 $\pm$ 0.1
<u>After 30 Min Aerobic Perfusion</u>						
Control	18.6 $\pm$ 1.9	6.2 $\pm$ 0.3	264 $\pm$ 21	196 $\pm$ 11	2.74 $\pm$ 0.1	2.35 $\pm$ 0.1
Verapamil	18.9 $\pm$ 1.3	6.0 $\pm$ 1.1	265 $\pm$ 12	204 $\pm$ 13	2.7 $\pm$ 0.1	2.3 $\pm$ 0.3
Nifedipine	17.6 $\pm$ 0.9	6.6 $\pm$ 1.3	248 $\pm$ 14	214 $\pm$ 9	2.4 $\pm$ 0.3	2.3 $\pm$ 0.4
<u>After 120 Min Aerobic Perfusion</u>						
Control	6.0 $\pm$ 1.7	5.6 $\pm$ 1.2	251 $\pm$ 19	194 $\pm$ 13	2.78 $\pm$ 0.2	2.33 $\pm$ 0.3
Verapamil	15.0 $\pm$ 1.1	4.8 $\pm$ 1.1	255 $\pm$ 21	190 $\pm$ 39	2.75 $\pm$ 0.4	2.39 $\pm$ 0.5
Nifedipine	16.9 $\pm$ 1.3	5.1 $\pm$ 0.9	258 $\pm$ 23	199 $\pm$ 22	2.82 $\pm$ 0.1	2.37 $\pm$ 0.3

Results are presented as mean $\pm$ standard error of at least 3 separate experiments.

TABLE 3.7 EFFECT OF DIRECT ADMINISTRATION OF VERAPAMIL AND NIFEDIPINE TO THE ISOLATED MITOCHONDRIA AFTER 120 MINUTES AEROBIC PERFUSION, 90 MINUTES ISCHAEMIA AND 90 MINUTES ISCHAEMIA + 30 MINUTES REPERFUSION

Experiments	RCR		QO ₂		ADP/O	
Series	I	II	I	II	I	II
Control 120 Min Aerobic Perfusion	16.0	5.6	251	194	2.78	2.33
+ Verapamil	15.9	5.2	248	190	2.77	2.31
+ Nifedipine	15.9	5.2	250	192	2.77	2.33
Control 90 Min Ischaemia	10.7	3.4	187	124	2.52	1.91
+ Verapamil	10.4	3.2	187	120	2.53	1.78
+ Nifedipine	10.1	3.1	188	121	2.45	1.90
Control 90 Min Ischaemia + 30 Min Reperfusion	5.8	1.1	121	62	2.25	1.55
+ Verapamil	5.6	1.0	122	62	2.24	1.53
+ Nifedipine	5.5	1.2	120	62	2.22	1.45

The data represent the mean of at least 3 experiments. Series I refers to results obtained with KEA medium, Series II to mitochondria extracted using sucrose-ruthenium red medium. Verapamil and nifedipine were added to the incubation medium to provide a final conc. of 5 uM. Because there was no difference after the addition of verapamil or nifedipine, standard errors and statistical analysis are not reported.

activity of the mitochondria measured in terms of RCR, QO_2 and ADP/O (Table 3.7).

In Figures 3.7, 3.8 and 3.9 are shown the RCR, QO_2 , ADP/O data of mitochondria from control animals (A) or animals treated with verapamil (B) or nifedipine (C). The mitochondria have been extracted after 120 minutes of aerobic perfusion (1) or after 90 minutes of ischaemia (2) or after 90 minutes of ischaemia followed by 30 minutes reperfusion (3) using either extraction medium I (in the presence of EDTA) or medium II (EDTA-free).

As previously described, when hearts from control rabbits were made totally ischaemic for 90 minutes the yield of mitochondria was unchanged (Table 3.2), but RCR and QO_2 were significantly reduced. Reperfusion after 90 minutes of ischaemia resulted in a significant decline of RCR, QO_2 and ADP/O irrespective of which extraction medium was used (Figs. 3.7, 3.8 and 3.9, Series A).

Pretreatment with verapamil or nifedipine reduced significantly the deterioration in mitochondrial respiration and phosphorylating activity caused by 90 minutes of ischaemia and by reperfusion. This effect was evident for RCR (Fig. 3.7, Series B and C), QO_2 (Fig. 3.8, Series B and C) and ADP/O ratio (Fig. 3.9, Series B and C), irrespective of which extraction medium was used. Nevertheless the differences in RCR, QO_2 and ADP/O values between mitochondria from rabbits treated with verapamil or nifedipine and those from the control rabbits after reperfusion, was always significantly higher when the extraction was carried out with medium II ($P < 0.01$).

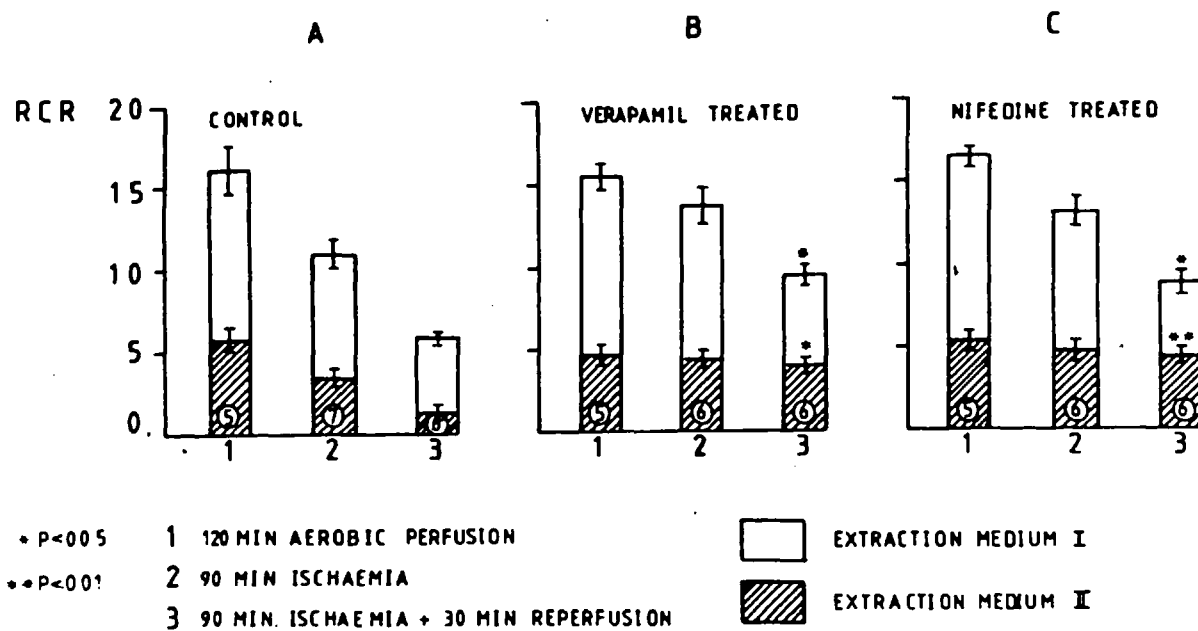


FIGURE 3.7 EFFECT OF TREATMENT WITH VERAPAMIL AND NIFEDIPINE ON MITOCHONDRIAL RCR

The results are expressed as mean $\pm$ S.E. of the mean.

The encircled number of each column represents the number of experiments.

P relates to the significance of the difference between the control and the relative verapamil or nifedipine-treated groups.

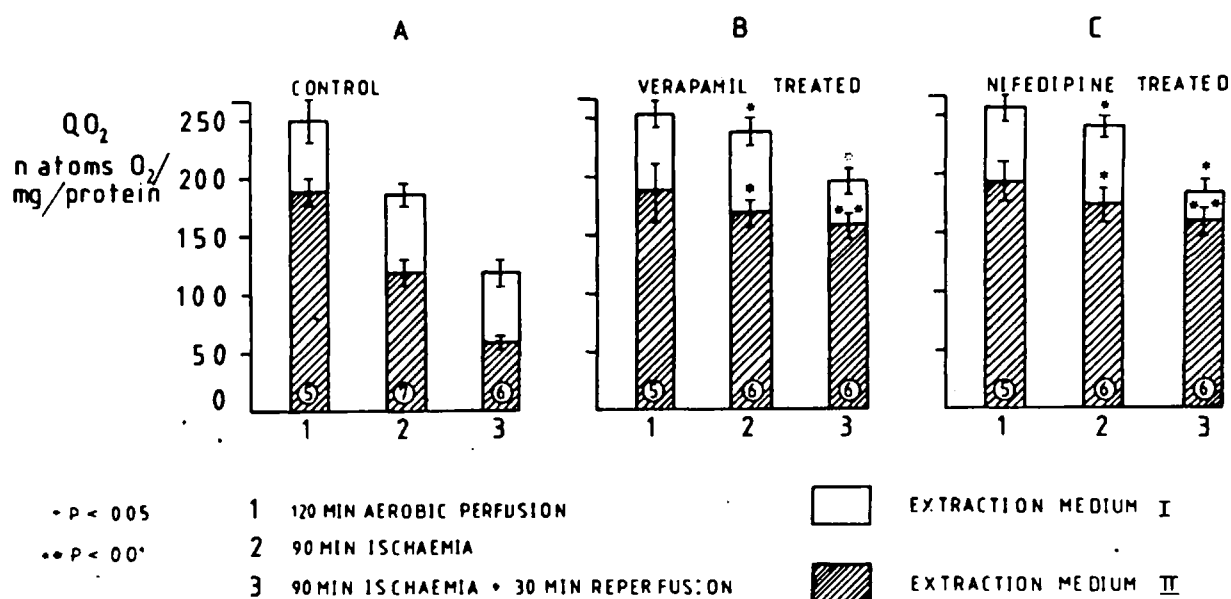


FIGURE 3.8 EFFECT OF TREATMENT WITH VERAPAMIL OR NIFEDIPINE ON
MITOCHONDRIAL QO₂

The results are expressed as mean $\pm$ S.E. of the mean.

The encircled number of each column represents the number of experiments.

P relates to the significance of the difference between the control and the relative verapamil or nifedipine-treated groups.

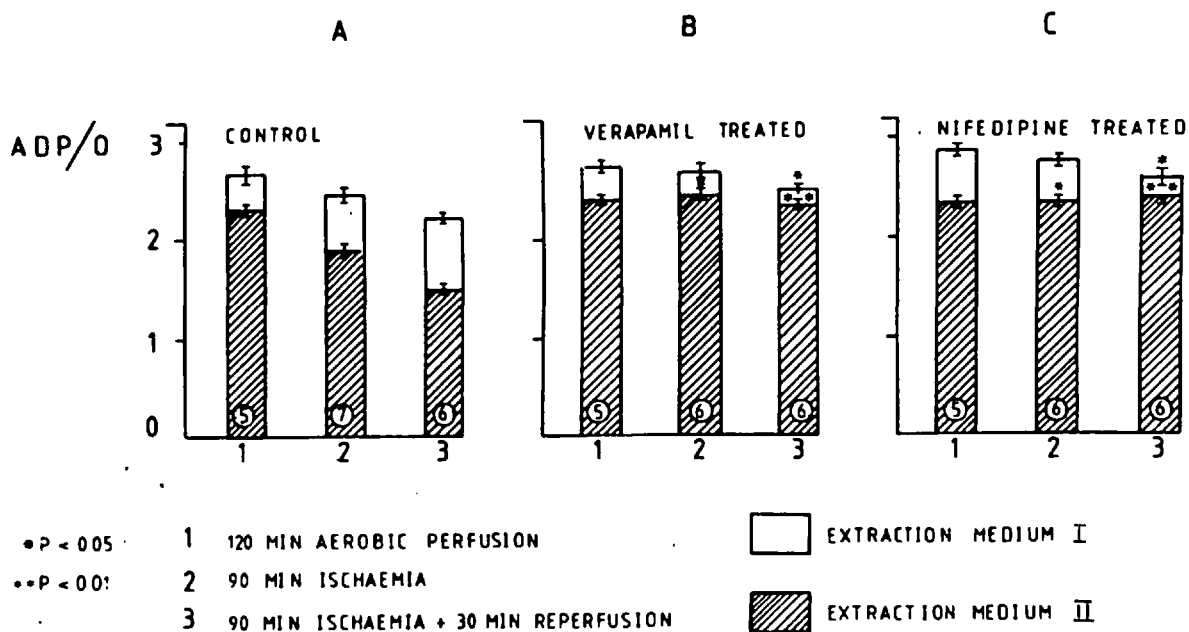


FIGURE 3.9 EFFECT OF TREATMENT WITH VERAPAMIL AND NIFEDIPINE ON MITOCHONDRIAL ADP/O

The results are expressed as mean $\pm$ S.E. of the mean.

The encircled number of each column represents the number of experiments.

P relates to the significance of the difference between the control and the relative verapamil or nifedipine-treated groups.

instead of with medium I ($P < 0.05$). The yield of mitochondria from verapamil or nifedipine-treated rabbits was unchanged after ischaemia or reperfusion.

(d) Effect of ischaemia and reperfusion on mitochondrial ATP production

Figure 3.10 shows a typical example of the time course of ATP production and O_2 utilisation by cardiac mitochondria from a freshly excised, non-perfused rabbit heart.

The relationship of ATP production and O_2 consumption was investigated using the medium described in the experimental section and glutamate (3 mM) as respiratory substrate. Oxygen consumption was initiated by adding 400 n moles ADP/mg protein. The addition of ADP induced an immediate production of ATP and an associated consumption of oxygen.

About 1 minute after the addition of ADP, 390 n moles of ATP/mg of protein were produced and 144 n atoms of oxygen/mg of protein were consumed, yielding an ATP/O ratio of 2.70. The ATP produced by the mitochondria was not significantly changed when measured 2 or 4 minutes after the addition of ADP.

The rate of ATP production was constant over the first 30 seconds of reaction in all the experiments and was taken as the initial rate of ATP synthesis.

Figures 3.11 and 3.12 (A) show the total amount and the initial rate of ATP production of mitochondria isolated from control saline-treated rabbits after 120 minutes aerobic perfusion or 90 minutes of

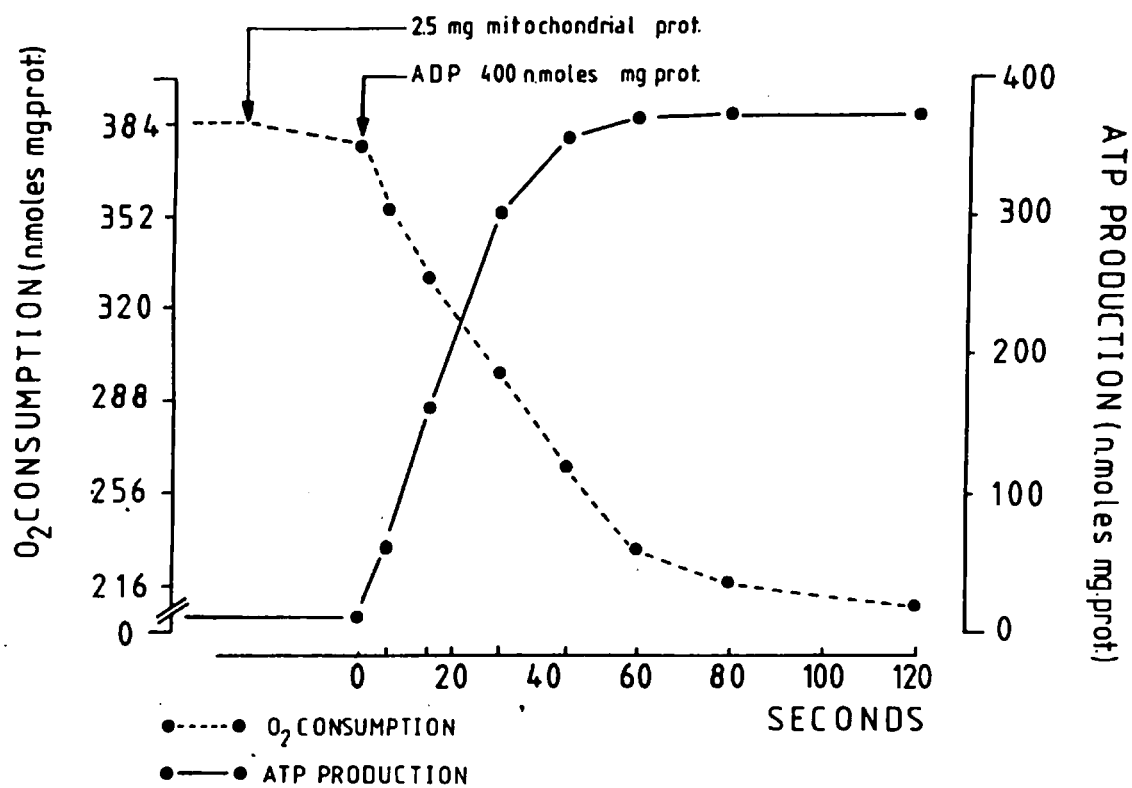


FIGURE 3.10 TYPICAL EXAMPLE OF TIME COURSE OF ATP PRODUCTION AND O₂ CONSUMPTION BY CARDIAC MITOCHONDRIA FROM A FRESHLY EXCISED, NON-PERFUSED RABBIT HEART

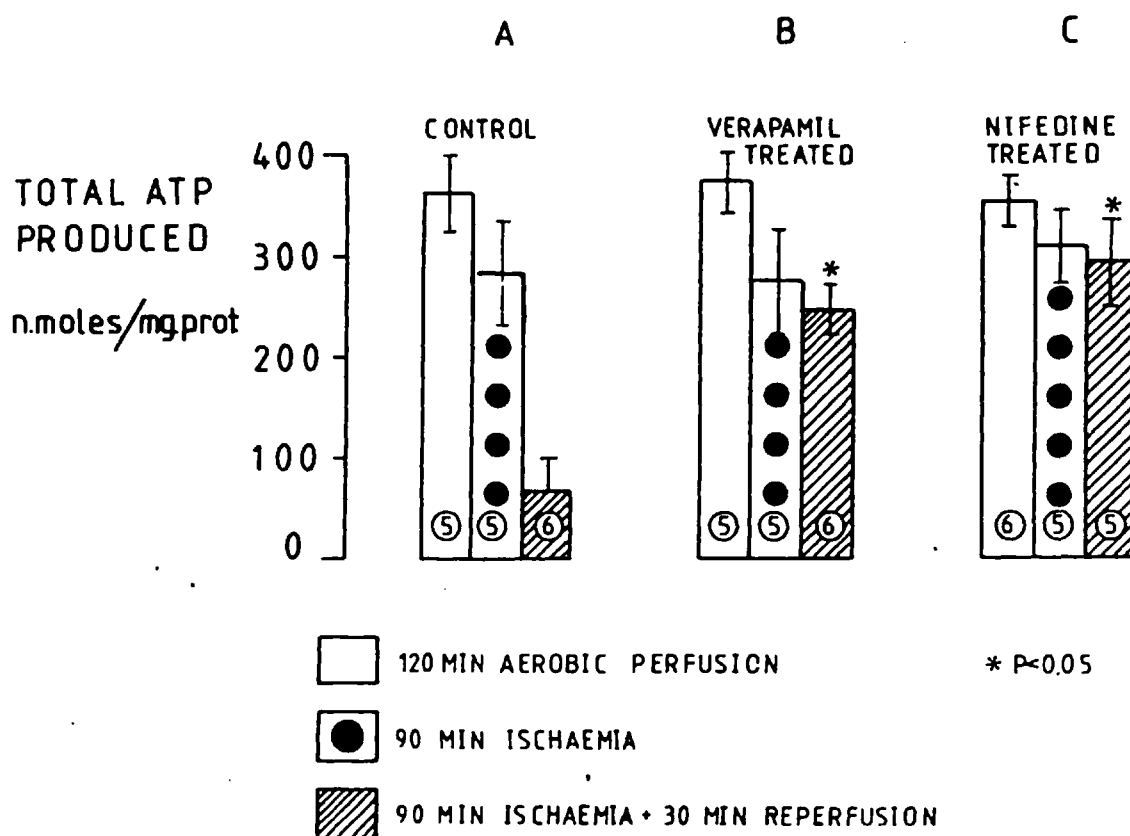


FIGURE 3.11 EFFECT OF TREATMENT WITH VERAPAMIL AND NIFEDIPINE ON TOTAL MITOCHONDRIAL ATP PRODUCTION

Results are expressed as mean $\pm$ S.E. of the mean.

The encircled number of each column represents the number of experiments.

P relates to the significance of the difference between the control and the relative treated groups.

ischaemia or after reperfusion. For this purpose, the mitochondria were isolated using medium II (EDTA-free) so that they would retain calcium.

The total amount of ATP produced by the mitochondria was calculated as the ATP present in the reaction chamber 15 seconds after the transition from State 3 to State 4 respiration. The rate of respiration was monitored by measuring the oxygen consumption.

Figure 3.11 shows that 90 minutes of ischaemia induced a decline of mitochondrial ATP production, although the reduction was not statistically significant.

The mean ATP/O ratio was 2.5 ± 0.3 in mitochondria from aerobic perfused hearts and only 1.5 ± 0.2 in mitochondria from ischaemic hearts, indicating that the mitochondria from ischaemic hearts used oxygen to phosphorylate ADP less efficiently. Figure 3.12 shows that the initial rate of ATP production of these mitochondria was also reduced.

Mitochondria from ischaemic-reperfused hearts had a considerably reduced rate of ATP production and also a significant reduction in the total amount of ATP produced during State 3 respiration (Figs. 3.11 and 3.12). The ATP/O value of these mitochondria was 0.33 ± 0.15 .

(e) Effect of treatment with verapamil or nifedipine on mitochondrial ATP production

Treatment of the rabbits, either with verapamil (Figs. 3.11 B and 3.12 B) or with nifedipine (C), had no effect on the total amount

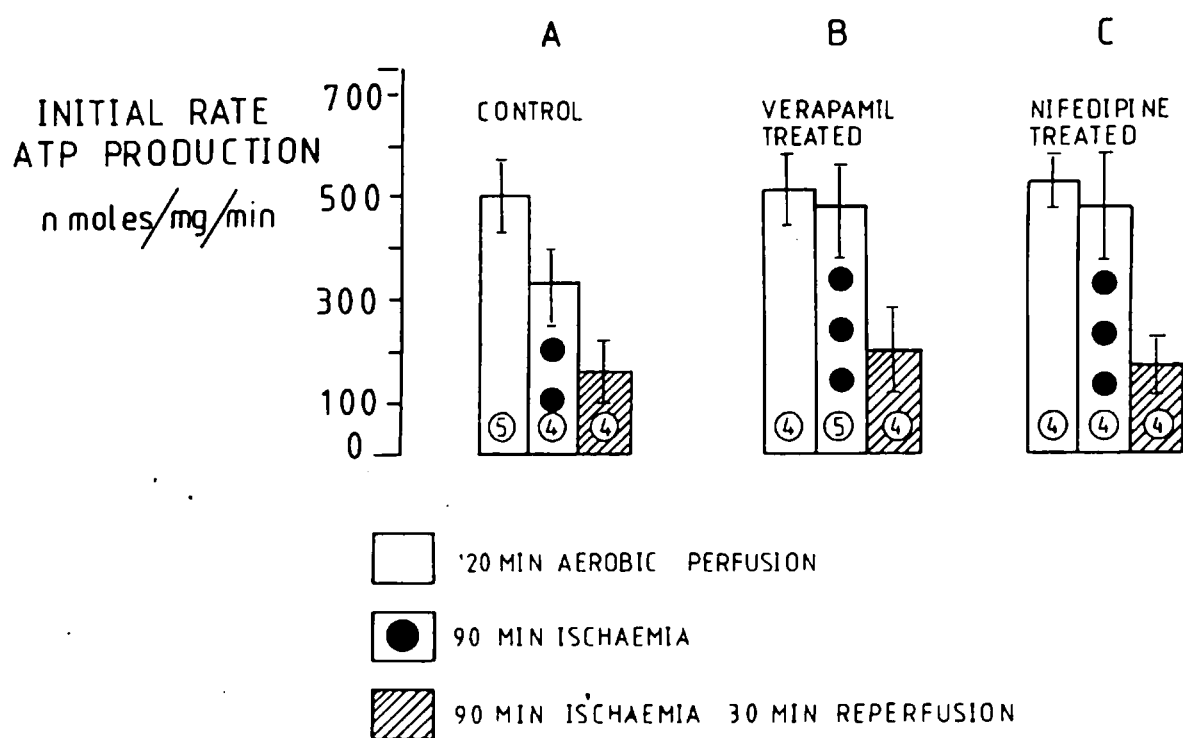


FIGURE 3.12 EFFECT OF TREATMENT WITH VERAPAMIL AND NIFEDIPINE ON MITOCHONDRIAL INITIAL RATE OF ATP PRODUCTION

Results are expressed as mean $\pm$ S.E. of the mean.

The encircled number of each column represents the number of experiments.

or the initial rate of ATP production from mitochondria isolated from aerobic-perfused hearts. Treatment also had no significant effect on the total ATP produced by the mitochondria isolated after the ischaemic period, but the initial rate of ATP production was increased (Fig. 3.12).

Treatment with verapamil or nifedipine significantly increased the amount of ATP produced by mitochondria isolated after 30 minutes of post-ischaemic reperfusion (Fig. 3.11) but failed to increase the initial rate at which ADP was phosphorylated to ATP (Fig. 3.12). The ATP/O ratio of mitochondria from the verapamil-treated group was 2.7 ± 0.3 after aerobic perfusion, unchanged after ischaemia and 1.78 ± 0.1 after reperfusion. For the nifedipine-treated group, the ATP/O ratio was 2.5 ± 0.3 after aerobic perfusion, 2.2 ± 0.2 after ischaemia and 2.2 ± 0.17 after reperfusion.

(f) Effect of treatment with verapamil or nifedipine on mitochondrial calcium

Mitochondria used for the determination of their calcium content were extracted in medium II, which contained ruthenium red and lacked EDTA, so they would be prevented from accumulating calcium during the extraction procedure. Preliminary control studies showed that the mitochondrial calcium concentration remained relatively constant during 120 minutes of aerobic perfusion (Table 3.2).

Figure 3.13, Series A, shows, however, that the calcium content of mitochondria from control saline-treated rabbits was increased after 90 minutes of ischaemia and that a large accumulation of calcium

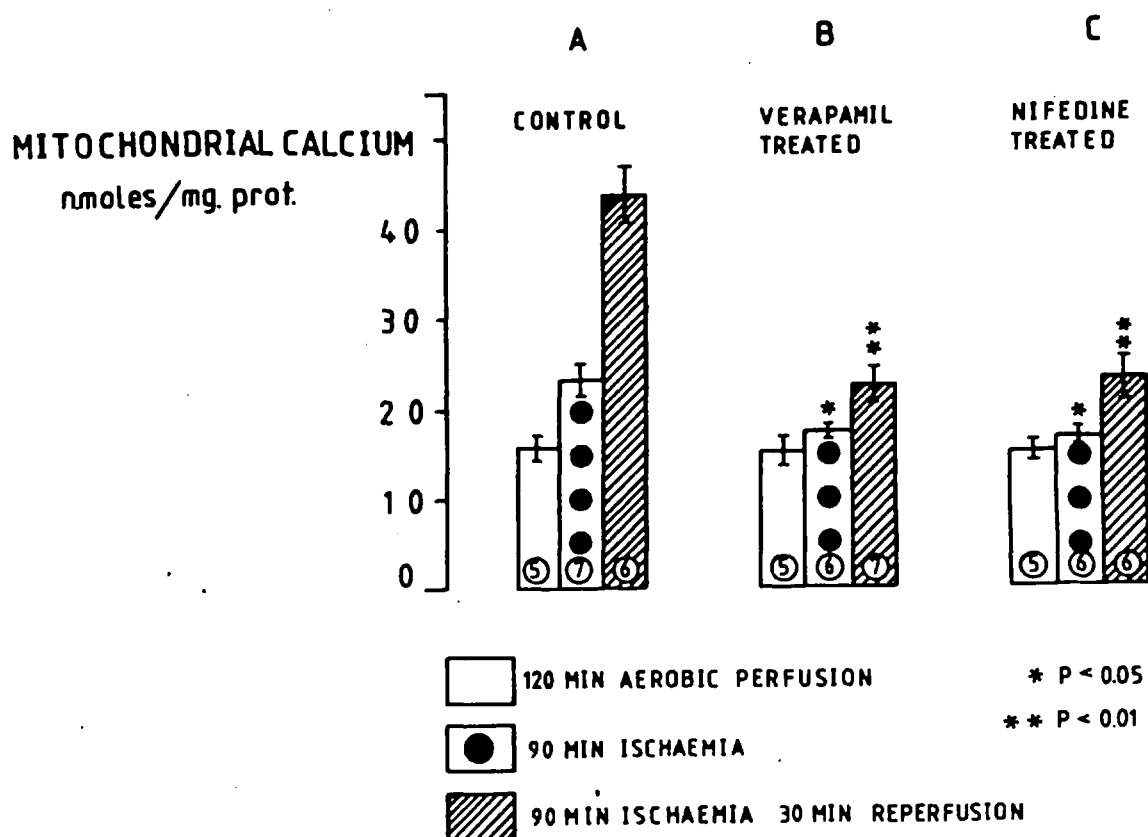


FIGURE 3.13 EFFECT OF TREATMENT WITH VERAPAMIL AND NIFEDIPINE ON MITOCHONDRIAL CALCIUM CONTENT

Results are expressed as mean $\pm$ S.E. of the mean.

The encircled number of each column represent the number of experiments.

P relates to the significance of the difference between the control and the relative treated groups.

occurred in the mitochondria from the reperfused myocardium.

Figure 3.13 also shows that treatment of the rabbits with verapamil (B) or nifedipine (C) had no effect on the calcium content of mitochondria from aerobically perfused hearts, but significantly reduced the increase in mitochondrial calcium content caused by the episodes of ischaemia and reperfusion.

(3) TISSUE CALCIUM

(a) Effect of treatment with verapamil or nifedipine on total tissue calcium

The values of total tissue calcium concentration have been expressed in nmol.Kg^{-1} dry tissue, as the dry weight is not influenced by different degrees of oedema and the variable blotting of the specimen before weighing.

The results are shown in Fig. 3.14. The calcium concentration in the homogenate of control saline-treated rabbits (A) was 25.1 ± 3.9 . This value is in agreement with previously reported values (Harding, 1980). Although ischaemia induced only a small, non-significative gain in tissue calcium, reperfusion resulted in a large and significant ($P < 0.01$) increase of tissue calcium content.

Pretreating the rabbits with verapamil (Series B) or nifedipine (Series C), had no effect on the calcium content of the aerobically perfused and ischaemic hearts. However, it significantly decreased the large gain induced by reperfusion.

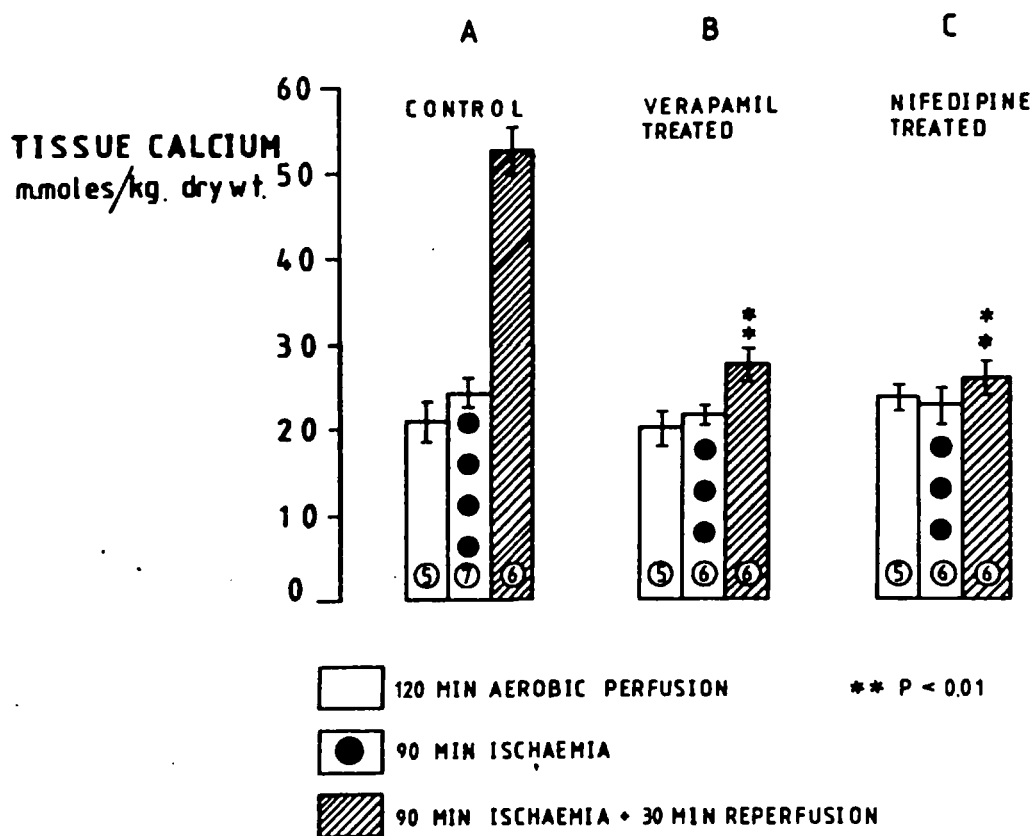


FIGURE 3.14 EFFECT OF TREATMENT WITH VERAPAMIL AND NIFEDIPINE ON
TISSUE CALCIUM

Results are expressed as mean $\pm$ S.E. of the mean.

The encircled number of each column represent the number of experiments.

P relates to the significance of the difference between the control and the relative treated groups.

(4) ATP AND CP STORES.

(a) Effect of treatment with verapamil or nifedipine on tissue ATP and CP

As for the total tissue Ca^{2+} concentration, the adenosine triphosphate (ATP) and creatine phosphate (CP) values have been expressed in $\mu\text{moles/g/dry weight}$. Results are shown in Table 3. There was only a small, non significant, loss of ATP and CP during 120 minutes of aerobic perfusion. As expected, hearts from the control group after 90 minutes of ischaemia were depleted of endogenous stores of ATP and CP. Readmission of coronary flow failed to restore the ATP and CP values, which were unchanged after 30 minutes of post-ischaemic reperfusion.

Treatment with either verapamil or nifedipine had no significant effect on the tissue levels of ATP and CP when determined in fresh non-perfused hearts or after perfusion under aerobic conditions. Nevertheless, in the treated hearts the content of ATP and CP was significantly maintained after the period of ischaemia. Reperfusion of the ischaemic hearts treated with verapamil or nifedipine resulted in a partial recovery of the CP level, although the ATP level was not significantly increased over that observed in ischaemic hearts.

TABLE 3.8 EFFECT OF VERAPAMIL OR NIFEDIPINE TREATMENT ON TISSUE ATP AND CP CONTENT

Perfusion	120 min aerobic	90 min ischaemia	90 min ischaemia + reperfusion
<u>ATP micromoles/g dry weight</u>			
<u>Treatment</u>			
Control	18.4 $\pm$ 1.2	4.6 $\pm$ 0.8	4.2 $\pm$ 0.3
Verapamil	19.1 $\pm$ 1.8	14.8 $\pm$ 0.9*	15.6 $\pm$ 1.2*
Nifedipine	19.3 $\pm$ 1.1	14.3 $\pm$ 1.0*	15.9 $\pm$ 0.8*
<u>CP micromoles/g dry weight</u>			
Control	32.8 $\pm$ 1.6	5.2 $\pm$ 0.6	6.6 $\pm$ 0.8
Verapamil	31.6 $\pm$ 2.7	14.6 $\pm$ 1.6*	19.6 $\pm$ 0.4*
Nifedipine	32.1 $\pm$ 1.8	14.2 $\pm$ 2.4*	19.1 $\pm$ 0.8*

Results are expressed as mean $\pm$ S.E. of the means of 6 individual experiments. * P < 0.001

Tests of significance calculated with respect to the difference between the control and the relative treated groups.

D. DISCUSSION

(a) General conclusions on the effects of verapamil and nifedipine

These results indicate that treatment of the rabbits with verapamil or nifedipine was able to reduce some of the damage caused by ischaemia and subsequent reperfusion in the isolated, Langendorff perfused rabbit heart.

There are three confirmatory groups of evidence in support of this general conclusion:- (1) The rise in resting tension during ischaemia was diminished with a greater percentage recovery of developed tension during reperfusion (Figs. 3.4 and 3.5). (2) The oxygen consumption and ATP generating capacity of mitochondria (Figs. 3.7, 3.8, 3.9 and 3.11) were well maintained despite the prolonged period of ischaemia and subsequent reperfusion. (3) Associated with this preservation of mitochondrial function was the maintenance of near normal levels of mitochondrial and tissue calcium and the maintenance of endogenous ATP and CP stores (Figs. 3.13, 3.14 and Table 3.8).

(b) Effect of extraction media on mitochondria

The data from this study (Tables 3.2 and 3.3) indicate that the mitochondrial isolation medium is of importance in the interpretation of the results:- (1) The calcium content of mitochondria isolated from freshly excised or aerobically perfused hearts with sucrose-ruthenium red was twice that found when potassium-EDTA-albumin medium was used. (2) A five-fold increase

in calcium content was found in mitochondria harvested with sucrose-ruthenium red after reperfusion (Table 3.3, Series II). No significant change in calcium content was present when mitochondria were isolated in KEA medium after reperfusion. (3) The yield, RCR, QO_2 and ADP/O values were always significantly less when sucrose-ruthenium red was used as extraction medium. (4) The phosphorylating capacity of ischaemic mitochondria (measured by means of ADP/O ratio) was maintained when isolated in KEA medium, while it was reduced when sucrose-ruthenium red was used as isolation medium (Table 3.2). (5) The percentage of mitochondrial function reduction after ischaemia and reperfusion was higher when medium II was used (Table 3.4).

It is known that KEA is, together with the Nagarse technique (Tyler and Gonze, 1967), an ideal medium for isolation of tightly coupled heart mitochondria (Lindemayer et al., 1968; Lochner et al., 1975). Bovine serum albumin has an important role in this medium, maintaining the phosphorylating activity of the isolated mitochondria (Lindenmayer et al., 1968). This is mainly because albumin can avidly bind free fatty acids (Pullman and Racker, 1956) which might be responsible for a defect in phosphorylation. Albumin also lessens the uncoupling action of lysosomes on mitochondria (Mellors et al., 1967).

The presence of EDTA is important for the removal of Ca^{++} as well as several other divalent cations during the isolation procedure. Peng et al. (1977) has suggested that endogenous Ca^{2+} plays an important role in the reduction of ATP production and efficiency

of oxidative phosphorylation of heart mitochondria. In his studies, the removal of ionic Ca^{2+} by EDTA in the medium was able to improve the inhibition of oxidative phosphorylation in ischaemic and ischaemic-reperfused mitochondria. The presence of KCl in the medium could also be important because mitochondria isolated in 0.18 M KCl were better preserved than those in sucrose (Jennings et al., 1966).

Because of these differences in mitochondrial behaviour, it is always difficult and almost impossible to be sure of what actually is the "in vivo" mitochondrial state in the myocardial cell, particularly after a period of ischaemia or reperfusion.

Extraction media like KEA which are designed to protect the mitochondria during the extraction period can be useful for studying the behaviour of mitochondria "in vitro" or for comparing the behaviour of mitochondria isolated from different "in vivo" conditions as we did in these experiments. It is very unlikely, however, that the measurements of mitochondrial function obtained using this isolation medium simulate the state of mitochondria in the cell.

In this study, an attempt has been made to preserve mitochondrial calcium content in order to know how much calcium was accumulated by the mitochondria after ischaemia and reperfusion. For this reason we used a medium containing just sucrose and ruthenium red, a polysaccharide dye which inhibits Ca^{2+} transport and binding by liver and heart mitochondria without interfering

with energy conservation which is necessary for retention of Ca^{2+} by mitochondria (Drahota et al., 1961; Rossi et al., 1973; Henry et al., 1977).

The dose of ruthenium red we used is double that used by Thakar et al. (1973) to inhibit Ca^{2+} accumulation of guinea-pig mitochondria and 100 times less than that used by Henry et al. (1977). This is because it has been shown by Vasington et al. (1972) that at high concentration (710 mMoles/mg mitochondrial protein) ruthenium red inhibited State 4 respiration and decreased State 3. The results of Table 3.4 indicate that, at the concentration we used, ruthenium red did not interfere with State 3 or 4 respiration.

The results of Henry et al. (1977) also indicate that at high concentration ruthenium red failed to reduce Ca^{2+} release of isolated mitochondria.

Our calcium values for mitochondria from fresh myocardium are in agreement with those obtained by other investigators (Carafoli and Lehninger, 1971; Peng et al., 1977) and higher than those obtained by Henry et al. (1977). Although the possibility that mitochondria lost some calcium during their preparation cannot be ruled out, the data described in Table 3.3 and Fig. 3.13 show that, during ischaemia and reperfusion, an increase of mitochondrial calcium took place and this data should reflect the situation in the tissue at the end of the perfusion period.

(b) Effect of ischaemia and reperfusion in the control saline-treated group

The results of this study indicate that tissue ATP and CP levels were significantly reduced after 90 minutes of ischaemia (Table 3.8). At this time tissue calcium concentration was increased by 9% (Fig. 3.14) and mitochondrial calcium content by 40% (Table 3.2). Isolated mitochondria had a reduced rate of respiration and a reduced rate of ATP production (Figs. 3.7, 3.8, 3.9 and 3.12), but the total amount of ATP produced during State 3 respiration was unchanged (Fig. 3.11). Ischaemia also induced a rapid decline of developed tension and, at the end of 90 minutes, resting tension was increased (Figs. 3.4 and 3.5). After 30 minutes of reperfusion, tissue and mitochondrial calcium was greatly increased (147% and 168% respectively) (Figs. 3.13 and 3.14). Isolated mitochondria were unable to use oxygen for phosphorylation (Fig. 3.16) and their ATP generating capacity was reduced. The ATP and CP tissue reserves were not restored (Table 3.7) and there was no recovery of active tension development (Fig. 3.4).

In these experiments coronary flow during ischaemia was reduced to zero. The calcium available to enter the myocardium, therefore, was limited to that of the extracellular space. This may explain why the net increase in tissue calcium was only 9%. In contrast, the mitochondrial concentration of calcium increased as much as 40%. Thus, the ischaemic period induced a redistribution of intracellular calcium rather than an accumulation.

A rise in mitochondrial calcium concentration implies an increased content of the cytosolic calcium because the major factor determining Ca^{2+} uptake by the mitochondria is the extra-mitochondrial Ca^{2+} concentration (Carafoli and Lehninger, 1971; Scarpa and Graziotti, 1973).

Because of the existence of Ca^{2+} gradients across the cell and subcellular membranes, maintenance of the normal Ca^{2+} homeostasis implies movements of Ca^{2+} against these gradients and therefore it is an energy-dependent process (Nayler et al., 1981). Since the severe impairment of oxygen delivery which occurs during ischaemia results in a rapid depletion of high energy phosphate reserves, it is quite likely that during an ischaemic episode the tissue level of ATP would fall beyond the level required for the maintenance of cytosolic Ca^{2+} .

It has been reported (Harris, 1975) that the Mikaelis constants of different animals for the various fractions which influence calcium movement in the myocardium lie in the range of 5 - 25 μM and the highest is that of Ca^{2+} -activate-ATPase of the sarcolemma. This function is, therefore, likely to be the most sensitive to a fall in the concentration of ATP.

During ischaemia, a redistribution of intracellular ATP can also take place (Gudbjarnason et al., 1970) with an increased ATP concentration within the mitochondria and a relative decrease of the cytosolic pool. The reason for this probably is that adenine nucleotide translocase, the enzyme which regulates the movement of

ATP from the mitochondria to the cytoplasm is inhibited by ischaemia (Shug et al., 1975; Shrago et al., 1976) and by an increased mitochondrial Ca^{2+} concentration. Total tissue ATP measurements, as were carried out in the present study, therefore, provide no direct information as to which ATP-dependent process will fail first, or whether processes which fail do so because of an inadequate supply of ATP. Nevertheless, an indirect evidence of relationship between ATP and CP content and Ca^{2+} homeostasis is furnished by our data with treated animals where the ATP and CP content were maintained and mitochondrial calcium overload did not occur (Fig. 3.13). An increase of cytosolic calcium during ischaemia can also be the result of an increased intracellular Na^{+} concentration (Nayler et al., 1979) which will displace Ca^{2+} ions from mitochondria (Reuter, 1974; Carafoli et al., 1976). In addition, increased ADP and phosphate concentrations will favour the escape of calcium ions from the sarcoplasmic reticulum (Hasselbach and Suko, 1974).

An increase of cytosolic calcium, together with a decrease of ATP can explain the increase of resting tension after 90 minutes of ischaemia (Nayler et al., 1979).

An increased Ca^{2+} content is thought to damage the mitochondria (Parr et al., 1975; Hearse, 1977; Henry et al., 1977). This possibility will be discussed in more detail in Chapter 5. Indirect evidence of the deleterious effect of calcium on the oxidative phosphorylating capacity of mitochondria is provided by the data of Table 3.2. These data show that, in mitochondria extracted from

hearts subjected to various conditions, the RCR, QO_2 and ADP/O values were always lower in the presence of the chelating agent EDTA.

Similar results were obtained by Peng et al. (1977).

During reperfusion with the readmission of coronary flow there was clearly an abundant supply of external Ca^{2+} and, after 30 minutes, tissue and mitochondrial calcium was two to three fold increased.

From experiments where radioactive Ca^{++} influx was monitored during reoxygenation and reperfusion (Shen and Jennings, 1972; Harding and Poole-Wilson, 1980; Bourdillon and Poole-Wilson, 1981) it appears that calcium influx increased as soon as flow and oxygen was reintroduced. Several hypotheses have been put forward to account for the increased Ca^{2+} influx during reperfusion, including a mechanical effect (Ganote and Kaltenbach, 1979), changes in sarcolemma due to small changes of Ca^{2+} homeostasis during ischaemia (Hearse, 1977; 1978; Watts et al., 1980) or sarcolemmal damage due to a direct effect of oxygen or radicals formed from oxygen (Chance et al., 1979; Harding and Poole-Wilson, 1980; Guarnieri et al., 1980). From our results it is not possible to speculate on these early events of reperfusion. On the other hand, once a net gain of calcium has occurred, it seems likely that the mitochondria become overloaded with Ca^{2+} . This would impair their respiratory function and capacity to produce ATP so that a vicious circle is probably established preventing restoration of the already depleted energy reserves.

(c) Effect of treatment with verapamil or nifedipine on ischaemia and reperfusion

These results show that pre-treatment with verapamil or nifedipine was able to reduce the deterioration of mitochondrial function (measured in terms of oxygen consumption, Ca^{2+} content and capacity to phosphorylate ADP) caused by ischaemia and reperfusion (Figs. 3.7, 3.8, 3.9, 3.11 and 3.13).

Verapamil and nifedipine, when added directly to mitochondria which had been extracted from aerobically perfused, or ischaemic or reperfused hearts, failed to alter the oxidative phosphorylating activity of the mitochondria (Table 3.6). This indicates that the effect resulted from their *in vivo* administration.

Neither is it possible to explain these results in terms of a peripheral dilatation or a reduction of heart rate or an increase of coronary flow because an isolated heart preparation was used in which temperature, cardiac frequency, coronary flow and composition of perfusate were controlled. Furthermore, during ischaemia, coronary flow was reduced to zero.

Since the animals received their last injection of verapamil or nifedipine 2 hours before they were killed and there was no further addition of these substances to the isolated hearts, we must assume either that their effect results from their ability to bind to cardiac tissue or that the prior administration had an effect which persisted through 90 minutes of ischaemia and reperfusion.

The rate of mitochondrial respiration and ATP and CP content

of the myocardium were unchanged by pre-treatment with verapamil or nifedipine in the fresh non-perfused hearts or after 30 and 120 minutes of aerobic perfusion. It is, therefore, unlikely that the effect of treatment with these substances could be explained by an altered availability of energy (ATP and CP) before the onset of the ischaemic period. The possibility of a redistribution of ATP within the cell, however, cannot be ruled out.

From our results, also, it appears that the reduction in tension development induced by the treatment with verapamil was present after 120 minutes of aerobic perfusion (Table 3.1). It has been shown that verapamil binds tightly to carbohydrate-containing residues of the sarcolemma (Mas-Oliva et al., 1980). It is possible, therefore, that, despite the high rate of coronary flow, there is still some verapamil (or a derivative) bound to the sarcolemma after 120 minutes of aerobic perfusion. Similar information is not available for nifedipine and in our experiments the effect of nifedipine on tension development lasted for only 60 minutes of aerobic perfusion.

We think that an explanation for these results lies in the fact that the rate of fall in CP and ATP during ischaemia is dependent on the physical activity and the rate of ATP hydrolysis existing before ischaemia (Kubler and Spierckerman, 1970; Reibel and Rovetto, 1978).

From Table 3.2 it appears that ATP and CP stores after 90 minutes of ischaemia were higher in the groups treated with verapamil or nifedipine. It is unlikely that the maintenance of ATP that we found is because these substances increased the rate of anaerobic

metabolism. The availability of glucose was, in fact, severely reduced by the abolition of coronary flow. In addition, during severe ischaemia, because of the fall of intracellular pH (Neely et al., 1975) the enzymes regulating the anaerobic glycolysis are inhibited (Rovetto et al., 1975).

The most likely explanation of the beneficial effect of verapamil and nifedipine seems, therefore to be that, by reducing tension development before the onset of ischaemia, they decrease the rate of consumption of ATP. Thus sufficient ATP remains available during the early stages of ischaemia to maintain membrane ultrastructure and intracellular Ca^{2+} homeostasis (Reibel and Rovetto, 1978; Nayler, 1981). Under these circumstances, cytosolic calcium would not increase and mitochondria would not become overloaded with Ca^{2+} (Fig. 3.13) while resting tension would not rise (Nayler et al., 1979) (Fig. 3.5).

A rise in the cytosolic calcium concentration will induce a further wasting of the tissue stores of ATP, through activation of the various Ca^{2+} -sensitive ATPases and a loss of membrane ultrastructure through the activation of the Ca^{2+} -sensitive phospholipases (Parr et al., 1975; Nayler, 1981).

As will be discussed in detail in Chapter 5, Ca^{2+} ions play an important role in the regulation of mitochondrial metabolism (Parr et al., 1975; McCormack and Denton, 1980). The capacity to oxidise oxoglutarate and other substrates diminishes when they are overloaded with calcium (Denton et al., 1980). An increased mitochondrial

calcium concentration has been shown to inhibit the activity of adenine-nucleotide translocase which results in a reduced capacity for ATP transport from the mitochondria to the cytosol with a further decrease in the cytosolic ATP pool.

From our experiments also it appears that the decline in mitochondrial function (obtained using medium II) was always associated with an increased mitochondrial calcium content. In addition, maintenance of mitochondrial function in the treated group (which was significantly higher when medium II was used) was always associated with a significant reduction in mitochondrial calcium content induced by the treatment.

If Ca^{2+} homeostasis has been maintained through the ischaemic period, the readmission of oxygen during reperfusion should result in a rapid re-energisation of the electron transport pathway, restoration of oxidative phosphorylation and ATP production and translocation into the cytosol with a resumption of contractile activity. On the other hand, when energy reserves are depleted and mitochondria already overloaded with Ca^{++} , reperfusion results in a massive respiration-linked uptake of Ca^{2+} and its irreversible sequestration into mitochondria as Ca^{2+} phosphate (Jennings and Ganote, 1976; Hearse, 1977). The respiration-linked accumulation of Ca^{2+} in heart mitochondria occurs as an alternative to oxidative phosphorylation (see Chapter 5) and, therefore, would lead to a further inhibition of ATP production and prevent the re-establishment of the cytosolic ATP level and recovery of mechanical function.

An alternative explanation, suggested by Henry et al. (1977), is that the excessive mitochondrial calcium content which occurs during ischaemia and reperfusion involves the entry of Ca^{2+} through the slow channels of the sarcolemma, in which case verapamil and nifedipine might act directly to slow this excessive slow channel Ca^{2+} transport.

There are reasons for rejecting the idea that the alteration in Ca^{2+} homeostasis induced by ischaemia and reperfusion involves Ca^{2+} transport through slow channel. There is evidence (Sperelakis and Schneider, 1976) that ischaemia by itself and the acidosis subsequent to ischaemia cause a rapid deactivation of Ca^{2+} channels. From our experiments it appears that after ischaemia there was a redistribution of intracellular calcium rather than an accumulation. In addition, during reperfusion, the massive entry of Ca^{2+} is too large to be accounted for by the slow channel current unless there is an accompanying prolongation of the action potential, which does not happen (Sperelakis and Schneider, 1976; Bourdillon and Poole-Wilson, 1981).

Indirect evidence that the effect of these substances during ischaemia is not dependent on their capacity to reduce the slow channels Ca^{2+} transport is that, when verapamil is given during reperfusion, it does not have the beneficial effects which occur when it is given to the animal before death (Bourdillon and Poole-Wilson, 1980; Watts et al., 1980). This also supports the view that the action of verapamil requires its presence before ischaemia is

induced. At this time it is able to influence the slow calcium channels. As soon as ischaemia is induced the slow channels are deactivated and unable to respond to the drug.

Nifedipine, on the other hand, has been shown to be beneficial when added at the time of reperfusion (Henry et al., 1978). These experiments, however, were performed in intact dogs. It is impossible to conclude whether the results obtained were a consequence of a direct effect of the drug on cardiac cells or whether they were indirectly due to its effect on smooth muscle cells causing peripheral and coronary vasodilatation.

C H A P T E R 4

THE EFFECT OF PROPRANOLOL ON THE ISCHAEMIC AND REPERFUSED MYOCARDIUM

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A. INTRODUCTION

ADRENERGIC BLOCKING SUBSTANCES

Beta ~~adrenergic~~ ~~receptor~~ antagonists have received major attention in the last decade because of their utility in the management of cardiovascular disorders, including hypertension, angina pectoris, cardiac arrhythmias and, possibly, myocardial infarction. Recent reviews have been presented by Shand (1975), Prichard (1978) and Opie (1980).

The first substance shown to produce a selective blockade of β adrenergic receptor of the heart was dichloroisoproterenol (Powell and Slater, 1958). Studies with this substance made a considerable contribution to the understanding of effects mediated by β receptors, but it was not used clinically, largely because it has a prominent β -receptor stimulant action (intrinsic sympathomimetic action). Propranolol was the first beta adrenergic receptor antagonist to come into wide clinical use and probably it remains the most important of these compounds. It is a highly potent, nonselective β adrenergic receptor antagonist agent with no intrinsic sympathomimetic activity (Weiner, 1980). The chemical formula of propranolol is shown in Fig. 4.1.

The basic observation that the β effects of catecholamines could precipitate features of ischaemia in experimental preparations (Rona, 1959; Raab, 1962; Opie, 1980) and that the rate of catecholamine secretion is increased in patients with acute myocardial infarction (Vetter, 1974) or in animals after coronary artery ligation (Herbaczynska-Cedro, 1970) made it logical to search for the effects

PROPRANOLOL

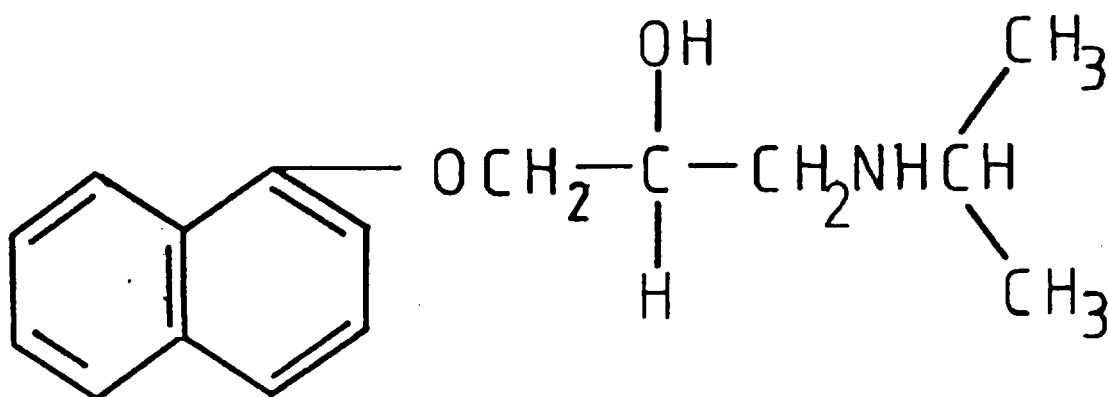


FIGURE 4.1 FORMULA OF PROPRANOLOL

of propranolol or other β -blocking agents on ischaemic myocardial damage.

Thus several authors have shown that propranolol reduced the damage induced by experimental ischaemia (Parrat, 1969; Reimer et al., 1973; Opie and Thomas, 1976; Rasmussen et al., 1977; Nayler et al., 1980; Kissin and Cavander, 1981; Manning et al., 1981). There are two discordant studies in which propranolol even in high doses had no effect (Peter, 1978; Edoute, 1981). Several hypotheses have been proposed, but the exact mechanism whereby propranolol exerts its effect on the ischaemic myocardium is still unknown. Theoretically, it could be secondary to (1) a reduction of heart rate and force of contraction (and, therefore, to a reduction of the cellular oxygen need) (2) a favourable redistribution of blood supply to the ischaemic area (3) a metabolic action, resulting in reduced free fatty acid uptake with an increased uptake of glucose by the ischaemic zone and (4) a direct stabilising effect of the heart membrane (Opie, 1980).

The aim of the experiments described in this chapter was to investigate if propranolol is able to modify some of the deleterious effects of ischaemia and reperfusion in isolated rabbit hearts. Some rabbits were treated with propranolol before the isolation of the heart. In other experiments, propranolol was delivered directly to the isolated hearts at the onset of ischaemia or during reperfusion.

Ischaemia was induced by reducing coronary flow from 25 to 1 ml/min and ischaemic and reperfusion damage was determined using the same parameters described in Chapter 3.

B. EXPERIMENTAL

Adult, New Zealand white rabbits (2.5 to 3.0 Kg) maintained on a standard diet were used for this study.

(a) Protocol

The rabbits were divided into four groups (Fig. 4.2). The control rabbits (A) either received 2 mg/kg of 0.9% sodium chloride solution injected subcutaneously twice daily for 5 - 6 days or were used without any previous treatment. The rabbits in the second group (B) were injected subcutaneously twice daily for the same period of time with 2 mg/kg of racemate propranolol dissolved in the same volume of sodium chloride solution. Treated rabbits were always used 2 hours after the last injection. Rabbits in the third and fourth groups were used without any treatment. Those in the third group (C) received racemate propranolol (1 mg/l) delivered to the isolated heart in the perfusate at the start of low flow perfusion for the whole ischaemic period and during reperfusion. Those in the fourth group (D) received the same dosage of racemate propranolol delivered in the perfusate only during post-ischaemic reperfusion (Fig. 4.2). Each rabbit was killed by a blow on the neck and the heart was rapidly excised and perfused as has been described in detail in Chapter 2. A period of 30 minutes after cannulation was allowed for equilibration before any experimental intervention. Hearts were then electrically paced to beat at 180 beats/min as described in Chapter 2. The hearts of each group (A,B,C,D) were perfused under (a) aerobic conditions for 120 minutes; (b) ischaemic

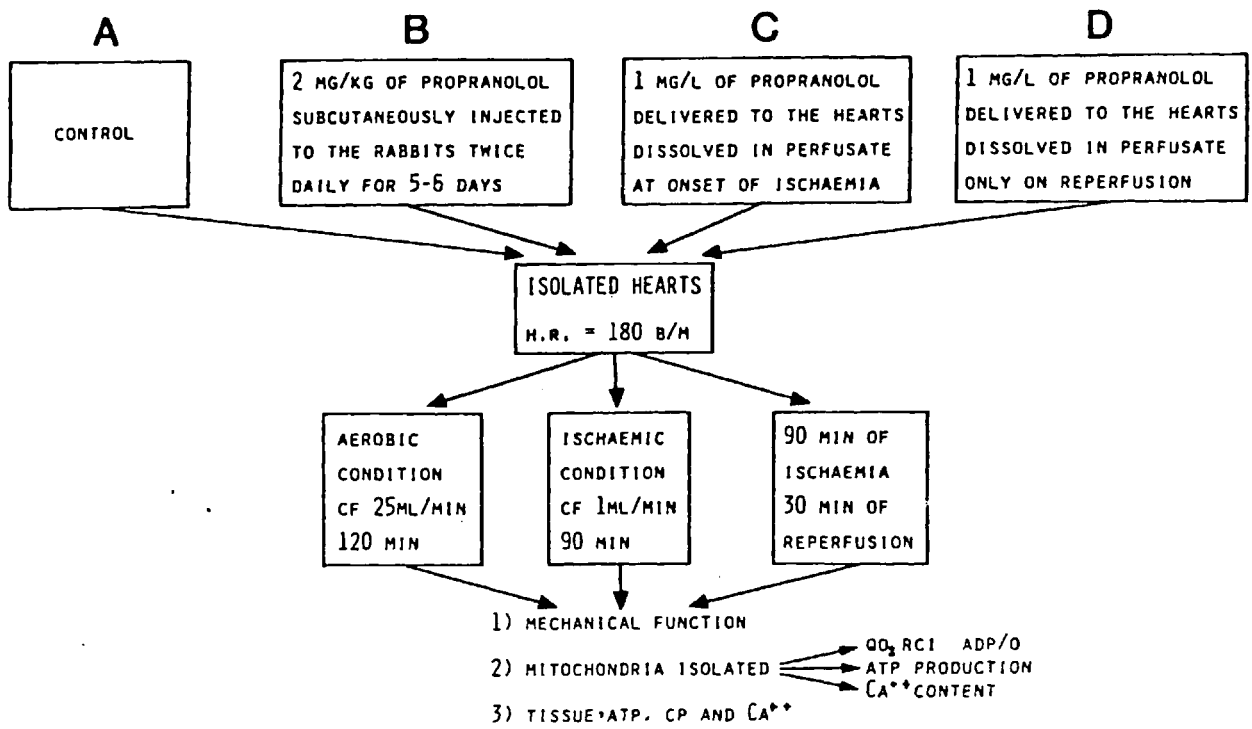


FIGURE 4.2 SCHEMATIC REPRESENTATION OF EXPERIMENTAL SCHEDULE

conditions for 90 minutes and (c) ischaemic conditions for 90 minutes + 30 minutes of reperfusion. Aerobic perfusion was at a mean coronary flow of 25 ± 1.2 ml/min with oxygenated Krebs Henseleit buffer solution. Ischaemia was induced by reducing coronary flow to 1 ml/min (4% of control value). During reperfusion, coronary flow was readjusted to 25 ml/min control level. Left ventricular wall temperature was maintained at between 36° and 37°C , irrespective of coronary flow as described in Chapter 2.

(b) Methods

The methods used for developed and resting tension measurements, ATP and CP determination and mitochondrial preparation have been described in detail in Chapter 2, while those for tissue and mitochondrial calcium determination, and for mitochondrial ATP production are described in Chapter 3.

(c) Statistical analysis

Results are expressed as mean $\pm$ S.E. of n experiments. Tests of significance were made using Student's "t" test, taking $P = 0.05$ as the limit of significance.

(d) Material

Rabbits, standard laboratory chemicals and enzymes were purchased as previously described in Chapters 2 and 3. Racemate propranolol was obtained from Imperial Chemical Industries, England.

C. RESULTS

(a) Effects of treatment with propranolol on heart rate and tension under well perfused conditions

The dose of propranolol selected for the animal treatment has been shown by Vaughan Williams (1975) to provide plasma levels comparable with those observed clinically.

Table 4.1 shows that treatment of rabbits with propranolol induced a non-significant reduction of the heart rate (recorded in the first minutes of perfusion under aerobic conditions when the hearts were still beating spontaneously) and a reduction of developed tension. The tension developed by the hearts of propranolol-treated rabbits increased during the 120 minutes of aerobic perfusion. After 60 or 120 minutes the values of developed tension of the control and propranolol-treated groups were almost identical (Table 4.1). Treatment with propranolol did not alter the resting tension values.

(b) Effects of propranolol on developed and resting tension during ischaemia and reperfusion

Figures 4.3 and 4.4 show that, when coronary flow was reduced to 1 ml/min, a rapid decrease in developed tension occurred in all groups. During the first four minutes of ischaemia, the decline in developed tension was more rapid for the hearts of propranolol-treated animals (Group B) or for the hearts receiving propranolol at the onset of ischaemia (Group C) (Fig. 4.4). Figures 4.3 and 4.5 show that in the hearts of Groups B and C the extent of ischaemic-induced increase in

TABLE 4.1 EFFECT OF IN VIVO TREATMENT WITH PROPRANOLOL ON HEART RATE AND DEVELOPED TENSION OF ISOLATED LANGENDORFF PERFUSED RABBIT HEARTS

Groups	Heart Rate beats/min	Developed Tension (g)	Developed Tension (g)	Developed Tension (g)
		30 min Aerobic Perfusion	60 min Aerobic Perfusion	120 min Aerobic Perfusion
A. Control	142 $\pm$ 13 (n = 37)	8.0 $\pm$ 1.6 (n = 7)	8.2 $\pm$ 1.3 (n = 7)	7.4 $\pm$ 0.9 (n = 6)
B. Propranolol Treated	132 $\pm$ 19 (n = 12)	6.2 $\pm$ 1.0* (n = 6)	7.9 $\pm$ 1.1 (n = 6)	7.2 $\pm$ 0.8 (n = 6)

* P < 0.05

Results are expressed as mean $\pm$ S.E. of the mean. The number of experiments is reported in brackets. P relates to the significance of the difference between the control and the relative treated series of experiments.

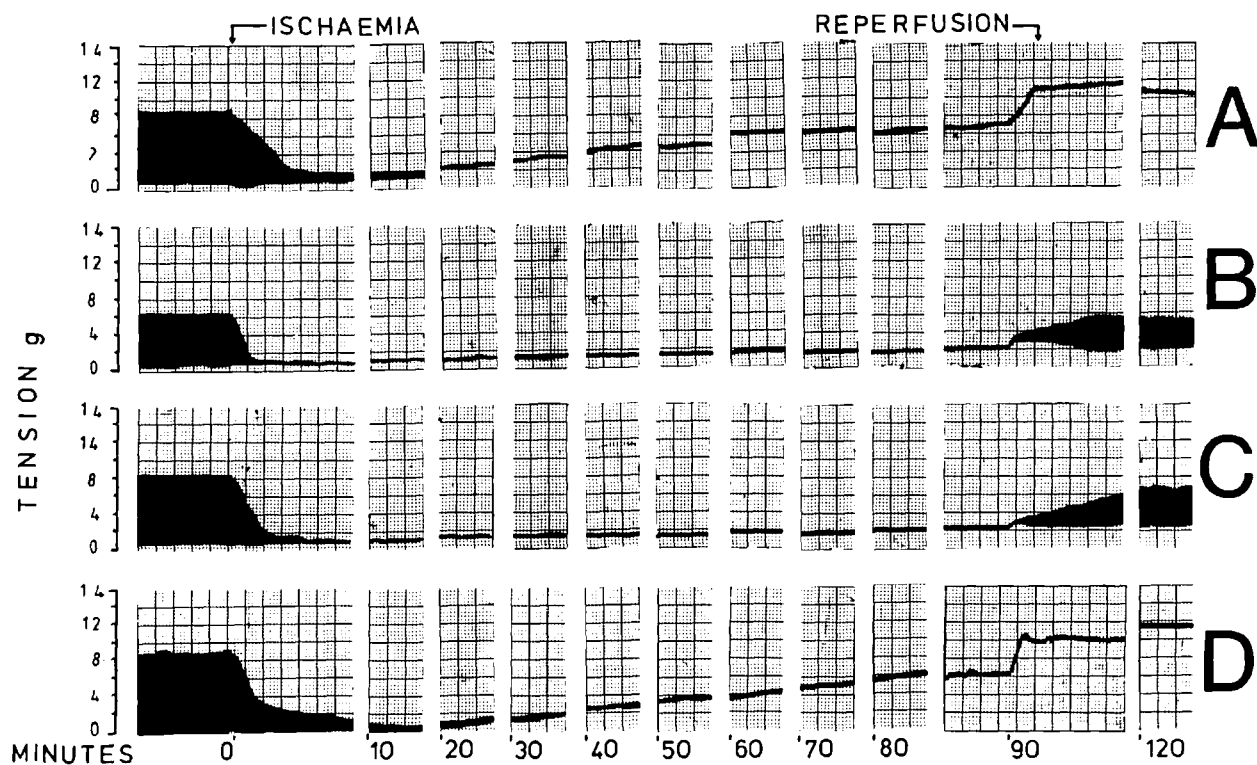


FIGURE 4.3 MECHANICAL RECORDS FROM FOUR ELECTRICALLY PACED (180 b/min) LANGENDORFF-PERFUSED RABBIT HEARTS

- A Control.
- B Propranolol treated.
- C Propranolol during ischaemia and reperfusion.
- D Propranolol during reperfusion.

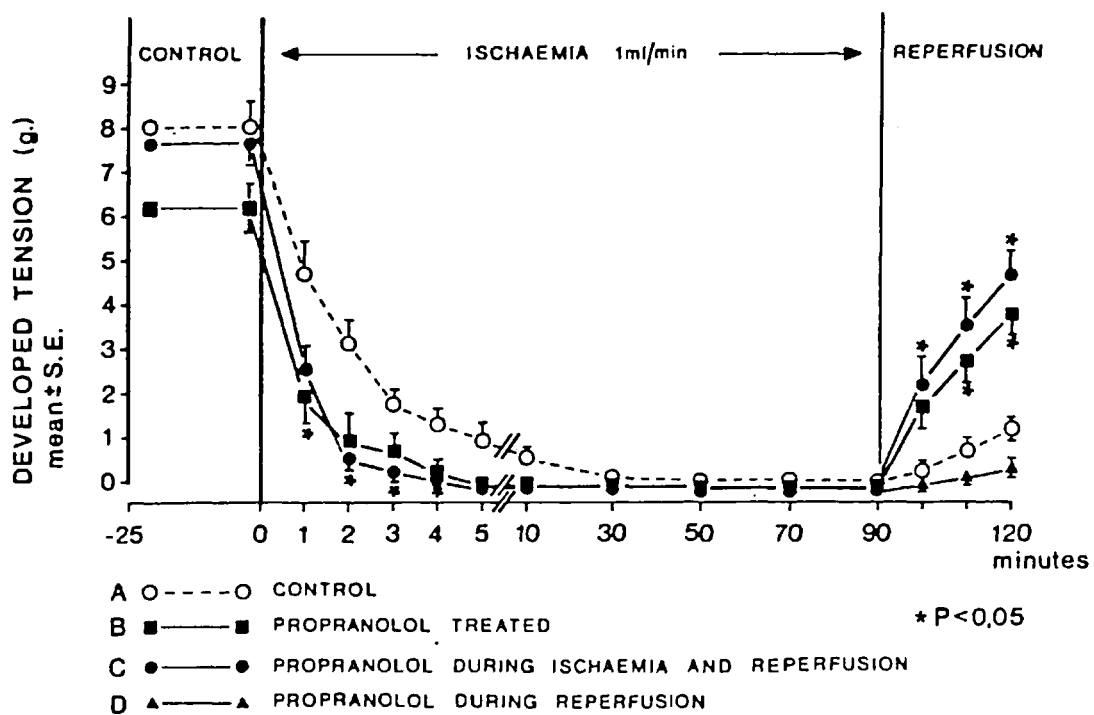


FIGURE 4.4 EFFECT OF PROPRANOLOL ON DEVELOPED TENSION DURING ISCHAEMIA AND REPERFUSION

Each point is the mean of at least five separate experiments.

P relates to the significance of the difference for the control and the relative treated series of experiments.

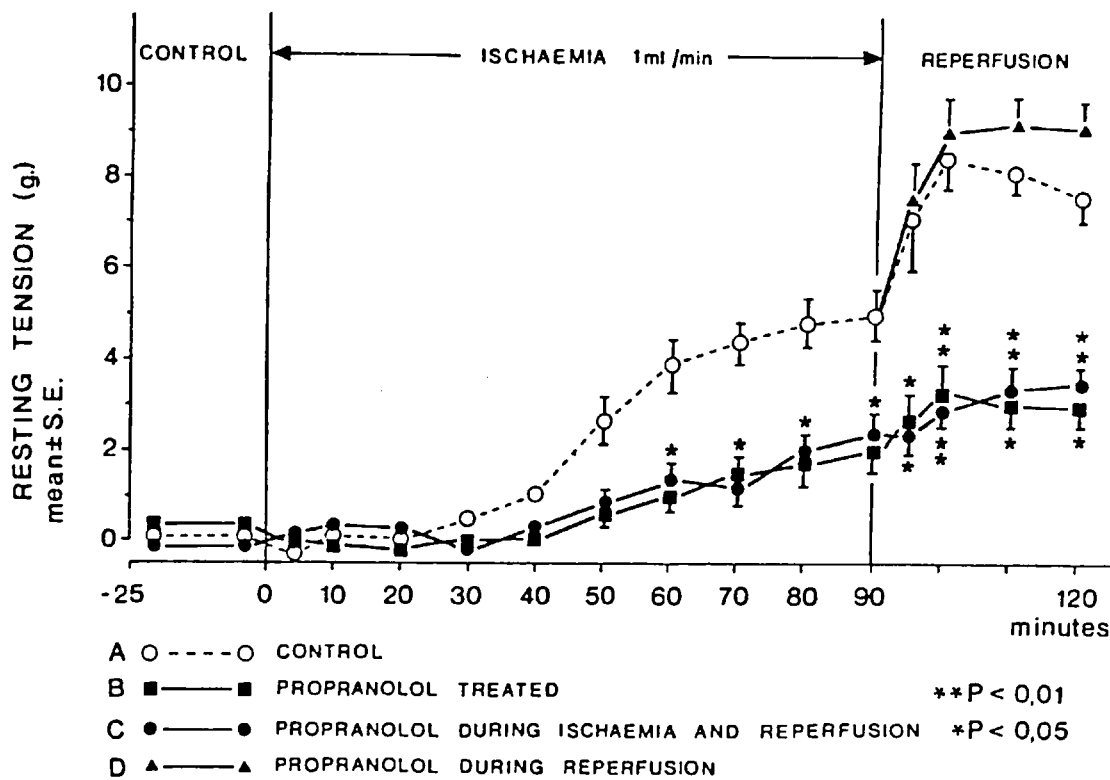


FIGURE 4.5 EFFECT OF PROPRANOLOL ON RESTING TENSION DURING ISCHAEMIA AND REPERFUSION

Each point is the mean of at least five separate experiments.

P relates to the significance of the difference for the control and the relative treated series of experiments.

resting tension was reduced when compared with that of control hearts (Group A). During the first 10 minutes of reperfusion, in control rabbits (Group A) resting tension rapidly rose from 5 ± 1.3 g to 8.5 ± 2.0 g and then started to decline. In the two groups receiving propranolol before or during ischaemia (Groups B and C), resting tension for the first 10 minutes of reperfusion increased to 3.2 ± 1.2 g and 3.0 ± 1.5 g, respectively and then remained constant (Fig. 4.5). At the end of reperfusion, recovery of developed tension for control hearts was 18% of the tension developed before coronary flow reduction, while for hearts from rabbits treated with propranolol (Group B) or hearts receiving propranolol during ischaemia and reperfusion (Group C) recovery of developed tension was 61% and 57% respectively. However, when the recovery of tension for Group B was calculated as a percentage of the tension developed by hearts of Group A just before the onset of ischaemia, it was as much as 47%. Figures 4.3 and 4.5 also show that, when propranolol was delivered in the perfusate only during post-ischaemic reperfusion (Group D), it failed to reduce the reperfusion-induced increase in resting tension. In addition, in four of these experiments there was no recovery of developed tension during reperfusion and in three there was only 10% recovery of developed tension (Fig. 4.4).

(c) Effects of propranolol on mitochondrial function

Table 4.2 summarises the data obtained from mitochondria isolated with medium I (containing KCl, EDTA and BSA, as described in Chapter 3),

TABLE 4.2 EFFECT OF PROPRANOLOL ON MITOCHONDRIAL FUNCTION DURING ISCHAEMIA AND REPERFUSION

Groups	PERFUSION		
	Aerobic (120 min)	Ischaemic (90 min)	Ischaemic (90 min)+ Reperfusion (30 min)
RCR			
A	16.8 $\pm$ 1.6 (5)	9.9 $\pm$ 1.3 (7)	6.8 $\pm$ 1.7 (7)
B	17.1 $\pm$ 2.3 (4)	14.3 $\pm$ 1.0 (6)*	12.9 $\pm$ 1.3 (5)**
C	16.7 $\pm$ 1.9 (4)	12.4 $\pm$ 1.2 (5)	10.01 $\pm$ 1.2 (5)**
D	16.8 $\pm$ 1.6 (5)	9.9 $\pm$ 1.3 (7)	5.9 $\pm$ 0.9 (7)
QO ₂ (n atoms O ₂ /mg prot./min)			
A	259 $\pm$ 16.6 (5)	186 $\pm$ 7.9 (7)	161 $\pm$ 9.9 (7)
B	246 $\pm$ 17.1 (4)	201 $\pm$ 13.7 (6)	205 $\pm$ 7.6 (5)*
C	239 $\pm$ 19.9 (4)	197 $\pm$ 12.3 (5)	199 $\pm$ 6.7 (5)*
D	259 $\pm$ 16.6 (5)	186 $\pm$ 7.9 (7)	149 $\pm$ 14.9 (7)
ADP/O			
A	2.81 $\pm$ 0.3 (5)	2.69 $\pm$ 0.2 (7)	2.37 $\pm$ 0.61 (7)
B	2.70 $\pm$ 0.2 (4)	2.70 $\pm$ 0.3 (6)	2.70 $\pm$ 0.9 (5)*
C	2.70 $\pm$ 0.2 (4)	2.72 $\pm$ 0.1 (5)	2.68 $\pm$ 0.3 (5)*
D	2.81 $\pm$ 0.3 (5)	2.69 $\pm$ 0.2 (7)	2.25 $\pm$ 0.29 (7)
YIELD (mg/prot./g wt weight)			
A	10.9 $\pm$ 1.2 (5)	8.6 $\pm$ 2.6 (7)	6.0 $\pm$ 1.9 (7)
B	11.1 $\pm$ 1.9 (4)	10.7 $\pm$ 1.8 (5)	10.3 $\pm$ 1.4 (5)*
C	11.9 $\pm$ 1.8 (4)	10.8 $\pm$ 2.1 (5)	10.0 $\pm$ 1.7 (5)*
D	10.9 $\pm$ 1.2 (5)	8.6 $\pm$ 2.6 (7)	5.8 $\pm$ 2.9 (7)

A:Control; B:2 mg/kg of propranolol subcutaneously injected into the rabbits twice daily for 5-6 days; C:1 mg/l of propranolol delivered to the hearts dissolved in the perfusate from the onset of ischaemia; D:1 mg/l of propranolol delivered to the hearts dissolved in the perfusate only during reperfusion. Data are expressed as mean $\pm$ S.E. The number of experiments is reported in brackets. Mitochondria were isolated using medium I. *P<0.05; **P<0.01. P relates to the significance of the difference between the control and the relative treated series of experiments.

TABLE 4.3 EFFECT OF PROPRANOLOL ON MITOCHONDRIAL FUNCTION DURING ISCHAEMIA AND REPERFUSION

Groups	PERFUSION		
	Aerobic (120 min)	Ischaemic (90 min)	Ischaemic (90 min)+ Reperfusion (30 min)
RCR			
A	5.6 $\pm$ 1.2 (8)	3.7 $\pm$ 1.9 (3)	1.7 $\pm$ 0.4 (3)
B	5.9 $\pm$ 0.6 (4)	5.6 $\pm$ 0.2 (6)*	5.2 $\pm$ 0.1 (5)**
C	5.3 $\pm$ 0.9 (4)	4.9 $\pm$ 0.3 (5)	5.4 $\pm$ 0.3 (5)**
D	5.6 $\pm$ 1.2 (8)	3.7 $\pm$ 1.9 (3)	2.0 $\pm$ 0.5 (7)
QO ₂ (n atoms O ₂ /mg prot./min)			
A	194 $\pm$ 13 (8)	136 $\pm$ 18 (3)	59.1 $\pm$ 7 (3)
B	180 $\pm$ 13 (4)	172 $\pm$ 15 (6)	158 $\pm$ 14 (5)**
C	176 $\pm$ 17 (4)	159 $\pm$ 13 (5)	146 $\pm$ 16 (5)**
D	194 $\pm$ 13 (8)	136 $\pm$ 28 (3)	61.2 $\pm$ 4 (7)
ADP/O			
A	2.31 $\pm$ 0.3 (8)	1.9 $\pm$ 0.1 (3)	1.50 $\pm$ 0.6 (3)
B	2.46 $\pm$ 0.2 (4)	2.3 $\pm$ 0.3 (6)	2.47 $\pm$ 0.3 (5)**
C	2.43 $\pm$ 0.6 (4)	2.3 $\pm$ 0.7 (5)	2.39 $\pm$ 0.3 (5)**
D	2.31 $\pm$ 0.3 (8)	1.9 $\pm$ 0.1 (3)	1.40 $\pm$ 0.2 (7)
YIELD (mg/prot./g wt weight)			
A	6.9 $\pm$ 1.1 (8)	5.0 $\pm$ 0.6 (3)	3.8 $\pm$ 1.0 (3)
B	7.3 $\pm$ 0.7 (4)	7.0 $\pm$ 1.0 (6)	6.5 $\pm$ 1.1 (5)*
C	6.7 $\pm$ 0.4 (4)	6.9 $\pm$ 1.1 (5)	6.4 $\pm$ 1.7 (5)*
D	6.9 $\pm$ 1.1 (8)	5.0 $\pm$ 0.6 (3)	3.1 $\pm$ 1.3 (7)

A:Control; B:2 mg/kg of propranolol subcutaneously injected into the rabbits twice daily for 5-6 days; C:1 mg/l of propranolol delivered to the hearts dissolved in the perfusate from the onset of ischaemia; D:1 mg/l of propranolol delivered to the hearts dissolved in the perfusate only during reperfusion. Data are expressed as mean $\pm$ S.E. The number of experiments is reported in brackets. Mitochondria were isolated using medium II. *P < 0.05; **P < 0.01. P relates to the significance of the difference between the control and the relative treated series of experiments.

while Table 4.3 presents the data obtained from mitochondria extracted from the same hearts, but using medium II (containing sucrose and ruthenium red), as described in Chapter 3.

Treatment of the rabbits with propranolol (Group B) or addition of propranolol to the perfusate (Group C) made no difference to the oxidative phosphorylating capacity of mitochondria isolated after 120 minutes of aerobic perfusion using either medium I or II. The mitochondrial yield was also unchanged (Tables 4.2 and 4.3).

Tables 4.2 and 4.3 also show that propranolol, either injected into the rabbits (Group B) or delivered in the perfusate (Group C) reduced the deterioration in mitochondrial function caused by 90 minutes of severe coronary flow reduction and by subsequent reperfusion. This effect was evident irrespective of which extraction medium was used. The difference in RCR, QO_2 and ADP/O values between mitochondria from control hearts (Group A) and those receiving propranolol (Groups B and C) was higher when the extraction was carried out with medium II instead of medium I.

Treatment with propranolol in Groups B and C also had an effect on the yield of mitochondria. In the control group (A) the yield became reduced during ischaemia and more so during reperfusion. On the other hand the yield of mitochondria from hearts of Groups B and C after ischaemia or reperfusion was unchanged. Although there was not a significant difference between the two groups, the RCR and QO_2 values after ischaemia and reperfusion of Group B were higher than those of Group C.

TABLE 4.4 EFFECT OF DIRECT ADMINISTRATION OF PROPRANOLOL TO MITOCHONDRIA ISOLATED AFTER 120 MIN AEROBIC PERFUSION, 90 MIN ISCHAEMIA AND 90 MIN ISCHAEMIA + 30 MIN REPERFUSION

Experiments	RCR		QO ₂ n atoms O ₂ /mg/min		ADP/O	
	I	II	I	II	I	II
Control - 120 min aerobic perfusion	16.8	5.6	257	194	2.8	2.30
+ propranolol	16.9	5.6	256	192	2.8	2.31
Control - 90 min ischaemic (1 ml/min) perfusion	11.0	3.7	189	136	2.6	2.0
+ propranolol	11.3	3.9	179	137	2.6	2.0
Control - 90 min ischaemic (1 ml/min) perfusion + 30 min reperfusion	6.8	1.7	141	5.1	2.3	1.5
+ propranolol	6.7	1.7	140	5.1	2.3	1.4

The data represent the mean of at least 4 experiments. Series I refers to results obtained with medium I (KEA), Series II to mitochondria extracted using medium II (sucrose-ruthenium red). Propranolol was added to the incubation medium to provide a final concentration of 5 μ M.

When propranolol was delivered to the isolated hearts after ischaemia, during the reperfusion period (Group D), it failed to improve mitochondrial function since RCR, QO_2 , ADP/O and yield values obtained after 30 minutes of reperfusion were not significantly different from those obtained for the control group (A) (Tables 4.2 and 4.3).

Finally, Table 4.4 shows that when propranolol was added directly (final concentration of $5\text{ }\mu\text{mol}$) to mitochondria isolated from either aerobic, ischaemic and reperfused myocardium there was no change in their oxidative phosphorylating activity.

(d) Effects of propranolol on mitochondrial calcium content and ATP production

The data on mitochondrial calcium content and ATP production are shown in Fig. 4.6. These mitochondria were isolated in medium II (Chapter 3) and ATP production was calculated as the ATP present in the reaction chamber 15 seconds after the transition from state 3 to state 4 respiration (as described in detail in Chapter 3).

Figure 4.6 shows that, in control hearts (Group A), 90 minutes of ischaemia at a coronary flow rate of 1 ml/min induced a significant increase of mitochondrial calcium content and a small, non-significant decrease of mitochondrial ATP-producing capacity. Reperfusion of the ischaemic hearts resulted in a large accumulation of calcium in the mitochondria associated with a severe reduction in the total amount of ATP produced during state 3 respiration. Propranolol either injected into the rabbit before the isolation of the heart (Group B) or delivered directly to the isolated heart (Group C) had no effect on the calcium

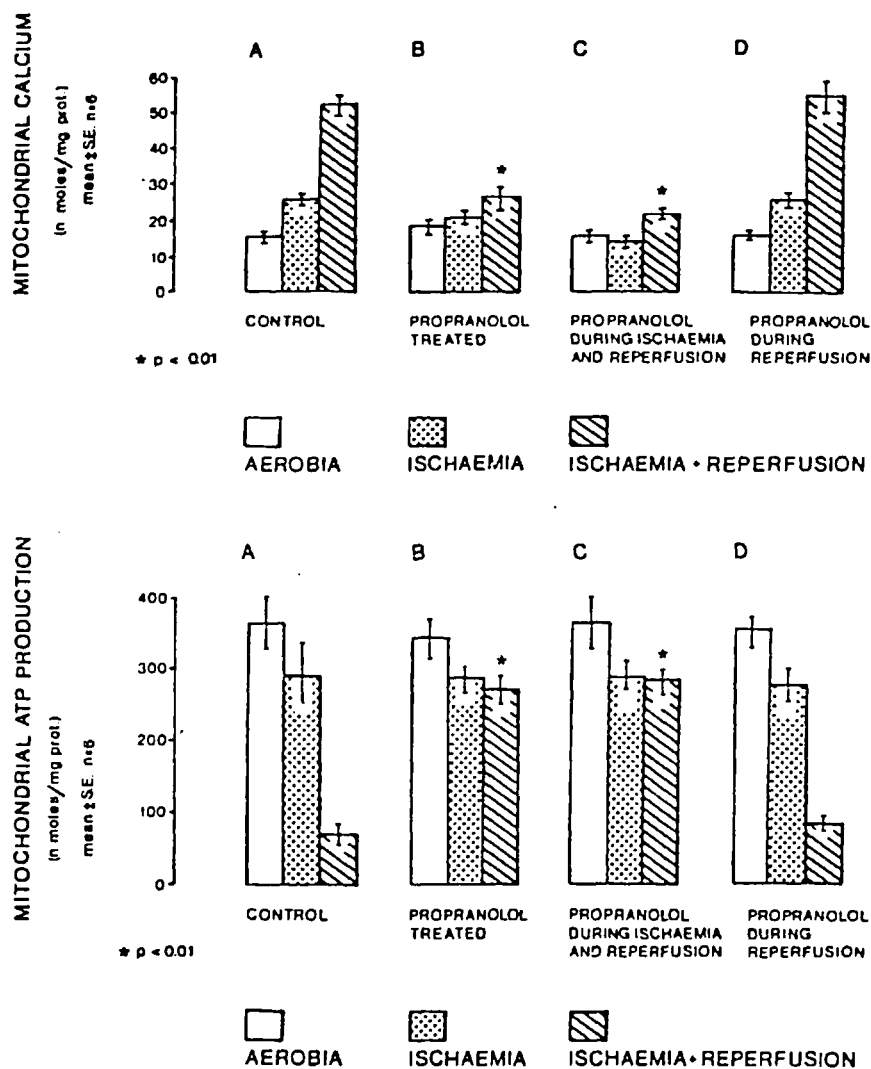


FIGURE 4.6 EFFECT OF PROPRANOLOL ON MITOCHONDRIAL CALCIUM CONTENT AND ON MITOCHONDRIAL ATP PRODUCTION

P relates to the significance of the difference for the control and the relative treated series of experiments.

content or ATP production of mitochondria isolated from aerobic-perfused hearts. However, it reduced the increase in mitochondrial calcium content caused by ischaemia and prevented the large mitochondrial calcium accumulation occurring on reperfusion. After reperfusion, in Groups B and C, mitochondrial ATP production was also significantly improved when compared with the values of control hearts.

When delivered to isolated hearts only during reperfusion (Group C), propranolol failed to reduce the increase in mitochondrial calcium content and to improve the capacity of the mitochondria to produce ATP (Fig. 4.6).

(e) Effects of propranolol on total tissue calcium

The results are shown in Fig. 4.7. In control hearts (Group A), 90 minutes of ischaemia induced a small, not significant gain in tissue calcium, while reperfusion resulted in a large and significant increase of myocardial calcium content. Propranolol (Groups B and C), had no effect on tissue calcium content of the hearts perfused under aerobic or ischaemic conditions but it reduced the large gain induced by 30 minutes of reperfusion. When delivered after ischaemia, during reperfusion (Group C), propranolol was unable to reduce the increase in myocardial calcium content.

(f) Effects of propranolol on tissue ATP and CP content

The results are shown in Table 4.5. In control hearts (Group A), as expected, 90 minutes of ischaemia induced a severe depletion of endogenous stores of ATP and CP. Readmission of coronary flow failed

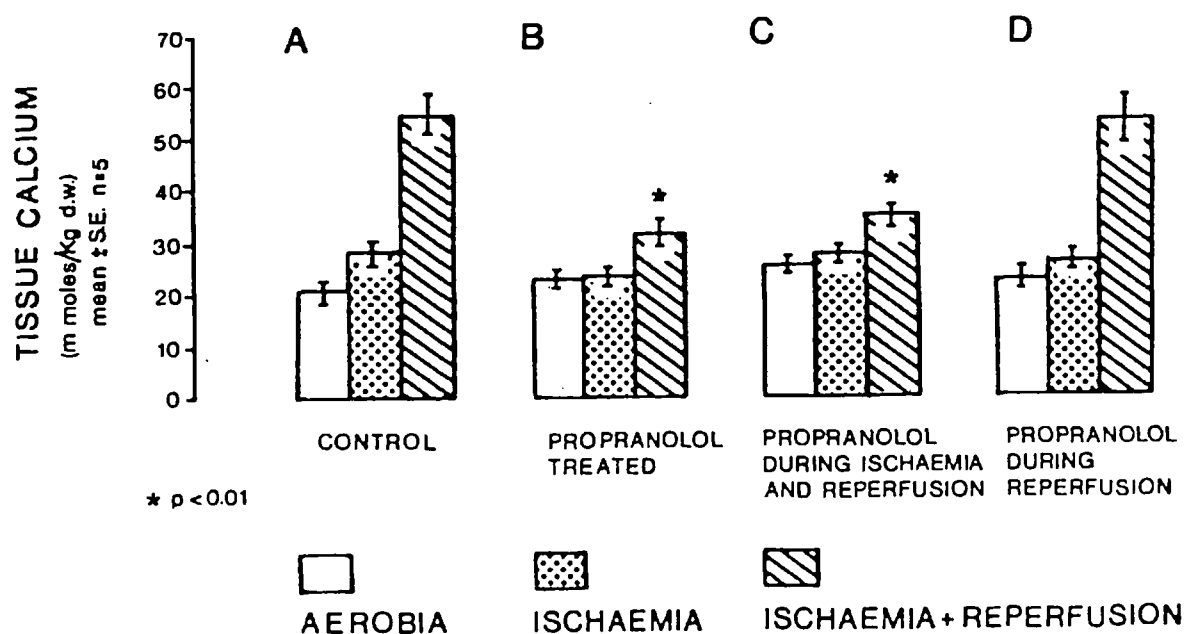


FIGURE 4.7 EFFECT OF PROPRANOLOL ON TISSUE CALCIUM CONTENT

P relates to the significance of the difference between the control and the relative treated series of experiments.

to restore the ATP and CP values, which were unchanged after 30 minutes of post-ischaemic reperfusion.

Propranolol (Groups B and C) had no effect on the tissue levels of ATP and CP after 120 minutes of aerobic perfusion. In the hearts receiving propranolol either before their isolation (Group B) or during the entire ischaemic period (Group C) ATP and CP contents were significantly maintained after the ischaemic episode. Reperfusion of these hearts (Groups B and C) resulted in partial recovery of ATP and CP content. ATP and CP levels obtained after reperfusion, however, were not significantly increased over those observed after ischaemia, except for the CP level of Group B.

When added during reperfusion, propranolol had no significant effect on the energy stores.

TABLE 4.5 EFFECT OF PROPRANOLOL ON MYOCARDIAL ATP AND CP CONTENT DURING ISCHAEMIA AND REPERFUSION

Groups	PERFUSION		
	Aerobic (120 min)	Ischaemic (90 min)	Ischaemic (90 min)+ Reperfusion (30 min)
ATP (μ moles/g dry weight)			
A	18.4 $\pm$ 1.2 (4)	4.2 $\pm$ 0.8 (5)	5.2 $\pm$ 1.4 (6)
B	18.8 $\pm$ 2.0 (4)	13.6 $\pm$ 1.2 (6)**	15.2 $\pm$ 1.3 (5)**
C	18.4 $\pm$ 2.6 (4)	10.9 $\pm$ 2.1 (5)**	12.1 $\pm$ 2.3 (5)**
D	18.4 $\pm$ 1.2 (4)	4.2 $\pm$ 0.8 (5)	3.9 $\pm$ 0.7 (5)
CP (μ moles/g dry weight)			
A	34.9 $\pm$ 2.1 (3)	5.9 $\pm$ 0.9 (5)	6.6 $\pm$ 2.9 (6)
B	30.2 $\pm$ 2.1 (4)	13.3 $\pm$ 1.6 (6)*	18.6 $\pm$ 2.2 (5)**
C	32.8 $\pm$ 1.6 (4)	12.9 $\pm$ 2.1 (5)*	14.7 $\pm$ 3.2 (5)*
D	34.9 $\pm$ 2.1 (4)	5.9 $\pm$ 0.9 (6)	7.3 $\pm$ 0.9 (3)

A:Control;

B:2 mg/kg of propranolol subcutaneously injected into the rabbits twice daily for 5-6 days;

C:1 mg/l of propranolol delivered to the hearts dissolved in the perfusate from the onset of ischaemia;

D:1 mg/l of propranolol delivered to the hearts dissolved in the perfusate only during reperfusion.

Data are expressed as mean $\pm$ S.E. The number of experiments is reported in brackets.

*P<0.05; **P<0.01.

P relates to the significance of the difference between the control and the relative treated series of experiments.

D. DISCUSSION

(a) General conclusions on the effects of propranolol

These results indicate that treatment of the rabbits with DL propranolol or its administration to the isolated hearts at the onset of the ischaemic period was able to reduce some of the damage caused by 90 minutes of restricted coronary flow and subsequent reperfusion. The rise in resting tension during ischaemia and reperfusion was diminished (Fig. 4.5) and there was a greater percentage recovery of developed tension after 30 minutes of reperfusion (Fig. 4.4). Mitochondrial respiration and ATP generating capacity were better maintained (Tables 4.2, 4.3, Fig. 4.6), overloading with calcium was reduced (Figs. 4.6, 4.7) and the endogenous stores of ATP and CP were preserved (Table 4.5). These two different modalities of propranolol administration, in vivo to the animals (Group B) or in vitro to the isolated hearts (Group C), did not produce substantially different results, although isolated mitochondrial function and endogenous stores of ATP and CP either after ischaemia or reperfusion were better maintained when propranolol was given to the rabbits for several days before excising the hearts.

When administered to the isolated hearts after the ischaemic period, during reperfusion, propranolol failed to modify the exacerbation of ischaemic damage caused by the readmission of coronary flow.

(b) Effect of pretreatment of rabbits with propranolol

Five or six days of treating the rabbits with propranolol failed to alter the rate of mitochondrial respiration or the ATP and CP content of the hearts perfused under aerobic conditions. Although an intracellular redistribution of high energy stores as a result of propranolol treatment cannot be excluded, this suggests that the effects resulting from in vivo administration of propranolol cannot be accounted for by a direct action on mitochondrial metabolism or by an improved supply of high energy phosphate groups.

In addition, when added directly to the mitochondria isolated from either aerobic, ischaemic or reperfused myocardium (Table 4.4), propranolol did not modify their oxidative phosphorylating capacity. These results conflict with those reported by Sakurada et al. (1972), who found that propranolol reduced oxidative phosphorylation of isolated rat heart mitochondria. The dosage of propranolol they used, however, was very high (1.44×10^{-3} M) and the authors themselves concluded that their experimental data were of no relevance to the in vivo state because of the high dosage. There is no evidence that propranolol is able to cross the myocardial cell membrane and act at the mitochondrial level. In addition, Sobel et al. (1966), using a similar dosage to that used by Sakurada found that propranolol does not affect isolated mitochondrial oxidative capacity.

In our experiments, propranolol treatment led to a reduction of the tension developed by the isolated hearts during the first minutes of aerobic perfusion, together with a small reduction of heart rate

(Table 4.1). These data are in agreement with those reported by Nayler et al. (1980) using the same experimental preparation, but not with those reported by Manning et al. (1981).^A The latter authors showed that the isolated and working hearts from rats receiving oxprenolol or propranolol administered orally for 3 weeks ($4 \text{ mg} \cdot \text{kg}^{-1} \text{ body wt. day}^{-1}$) developed a significantly higher peak systolic pressure and aortic output compared with untreated hearts. In their study no chronotropic alterations were observed. They concluded that the increase in contractility represents an adaptation of the myocardium to the direct in vivo effects of persistent beta-blockade such as lowered blood pressure. If it is so, then the different period and way of administration of propranolol (orally for three weeks in the Manning study and intraperitoneally for 5 days in ours) may account for these discrepancies.

There is no simple explanation for the decline in developed tension which we found after propranolol treatment of the rabbits.

It seems most likely that the explanation lies in its property of beta-adrenoceptor blockade. The rabbits were always killed 1 - 2 hours after the administration of the last dose of propranolol when our previous experiments (Nayler, 1978) had shown that the inotropic response to isoprenaline was blocked. Although in the isolated heart preparation adrenergic stimulation is extremely limited, it is possible that some endogenous catecholamines are released from the myocardium during the necessary manipulation for the isolation of the heart and during the early period of perfusion. During the first minutes of perfusion the hearts often present irregularities of rhythm such as extra beats, tachycardia

and ventricular fibrillation. In addition, our hearts were electrically paced (Fig. 4.2). It is known that the artificial stimulation of the hearts leads to a release of endogenous catecholamines (Arnim and Welman, 1981; Manning et al., 1981).

Another possible explanation for these results is that propranolol treatment caused a reduced availability of norepinephrine for release. Recently, Raine and Chubb (1977) reported reduced tyrosine hydroxylase and dopamine-beta-hydroxylase activities in cervical ganglia of rabbits treated for 6 days with a dose regime of propranolol closely resembling that used in our study. Because tyrosine hydroxylase is the rate-limiting enzyme in the biosynthesis of norepinephrine, it is possible that less endogenously stored norepinephrine was available in propranolol-treated than in control rabbit hearts.

It is unlikely that the decline in developed tension after propranolol treatment of the rabbits is due to a membrane stabilising action of the drug. As will be discussed later in this chapter, the dosage of propranolol used is not accompanied by a significant membrane stabilising action.

As already discussed in Chapter 3, the reduced physical activity of the hearts from propranolol-treated rabbits before the onset of ischaemia, may explain the better preservation of ATP and CP content after ischaemia (Table 4.5). A reduced rate of ATP hydrolysis (consequent to the reduced contraction) before ischaemia, however, cannot be the only explanation for the beneficial effects of propranolol in our experiments. Similar effects were also found in Group C,

when propranolol was delivered to the isolated hearts at the beginning of coronary flow reduction. In these experiments the tension developed by the hearts before ischaemia was almost identical to that developed by control hearts (Fig. 4.4).

(c) Effect of propranolol delivered during ischaemia and reperfusion

In this group of experiments (C), propranolol was delivered to the isolated hearts only during ischaemia and reperfusion. Its beneficial effect, therefore, cannot be related to changes occurring before the ischaemic period, but has to be related to changes occurring during ischaemia and/or reperfusion. The presence of propranolol in the ischaemic perfusate resulted in a more rapid fall of developed tension during ischaemia (Fig. 4.4). This effect was similar to that observed in the hearts of rabbits pre-treated with propranolol (Group B) (Fig. 4.4). The hearts of Groups B and C completely ceased their contractile activity 5 minutes after coronary flow reduction, while control hearts continued beating for 10 minutes after coronary flow reduction.

This effect can be explained by the β -receptor antagonist properties of propranolol. It is known that endogenous catecholamines are released in response to myocardial oxygen deprivation (Wollenberger and Shahab, 1965; Shonig et al., 1981). Wollenberger and Shahab (1965) using an isolated rabbit heart preparation similar to the one which we used showed that during the first four minutes of ischaemia there is a mean noradrenaline release of $0.46 \mu\text{g/g}$. This amount represents nearly one

fourth of the noradrenaline normally present in the rabbit's heart.

There is evidence that the sarcolemmal calcium channels are activated when the membrane channel protein is phosphorylated by a cyclic-AMP dependent protein kinase (Reuter, 1979). This explains why a rise of cyclic AMP concentration increases the slow inward calcium current and Ca^{2+} influx in a beating cardiac preparation (Reuter, 1979). The inward movement of calcium leads ultimately to the positive inotropic effect of sympathomimetic amines (Reuter, 1979).

The release of noradrenaline induced by the ischaemic episode leads to an increase in the intracellular level of cyclic AMP. This results in an activation of the slow calcium current. During ischaemia, however, the slow calcium current may, at the same time, be inhibited by the fall in intracellular pH.

The net inward movement of calcium during ischaemia is therefore influenced by two opposing mechanisms.

The presence of propranolol in the early stage of ischaemia (Groups B and C) removes the cyclic AMP dependent activation of slow calcium channels by blocking the effects of noradrenaline. The net inward movement of calcium is therefore reduced by protons. This may explain why the ischaemic-induced fall in developed tension was faster in Groups B and C than in Group A.

The more rapid decline in developed tension of the hearts receiving propranolol (either before their isolation, or at the time of ischaemia, Groups B and C) may account for the preservation of ATP and CP found in these hearts after 90 minutes of ischaemia. About 75% of the total

ATP production is normally used by the myocardium to support active contraction. Under the severe coronary flow reduction used in this study, ATP synthesis is supported only by the small rate of oxygen uptake, the rate of anaerobic metabolism being severely reduced as a consequence of intracellular acidosis (see Discussion in Chapter 2). Thus the rate of synthesis of ATP is greatly diminished and a reduction in mechanical activity spares ATP for the maintenance of membrane structure and intracellular ionic homeostasis. The finding that in Groups B and C mitochondrial oxidative phosphorylation was maintained and calcium accumulation did not occur during ischaemia indicates that subcellular membrane integrity and Ca^{2+} homeostasis was maintained in these hearts.

An alternative explanation of these results might be that they are due to the membrane stabilising action of propranolol.

Recently Arnim and Welman (1981) reported that in the isolated "working" rat heart preparation propranolol protects against the deleterious effects of ischaemia by a mechanism unrelated to its beta-adrenergic blocking action. The membrane stabilising activity or "quinidine like" effect of propranolol was suggested to be the major active property by which it exerted its beneficial effect. Against this hypothesis is the finding that when D-propranolol (the isomer that has a membrane stabilising effect but little B adrenergic blocking activity) was given to isolated rabbit hearts, or to dogs during ischaemia it was almost ineffective (Nayler et al., 1975; Kloner et al., 1978). In addition, Manning et al. (1981) repeated the study of Armin and Welman (1981) in the presence of exogenous catecholamine drive. These authors

concluded that only the L-isomer of propranolol is beneficial during ischaemia, suggesting that the beta-adrenergic blocking action of propranolol is responsible for these effects.

Finally, in our experiments, propranolol was used at a concentration of 1 mg/l at which the B-adrenergic receptor antagonising effect of the drug is not accompanied by a significant membrane stabilising action. It has been shown that the "quinidine-like effect" of propranolol on cardiac transmembrane potential is present only at concentrations of 3 to 10 mg/l (Fitzgerald et al., 1974; Davis and Temte, 1968; Kissin and Cavender, 1981).

From our results it appears that pretreatment of the rabbits before the onset of ischaemia (Group B) is not essential for propranolol to have its effect. Similar results were obtained when propranolol was delivered to the isolated hearts at the onset of ischaemia (Group C). However, the ATP and CP content and the mitochondrial oxidative-phosphorylating capacities after 90 minutes of ischaemia of hearts of Group B were better preserved than those of Group C. This effect may be secondary to the in vivo treatment of the animals. The mechanism involved may be the reduction of myocardial contraction and therefore of ATP hydrolysis before the onset of the ischaemic episode.

(d) Effect of propranolol delivered only during reperfusion

When delivered only during post-ischaemic reperfusion, propranolol had no effect on the damage induced by reperfusion. This suggests that the improvements in developed tension, mitochondrial function and

ATP and CP contents found in Groups B and C after reperfusion, are related to the effects of propranolol during the ischaemic episode and not to a specific effect of propranolol during reperfusion.

These results are in agreement with those of Reimer et al. (1973) who reported that propranolol, when given to anaesthetised dogs at the time of reperfusion, failed to alter significantly myocardial necrosis. However, Reynolds et al. (1981) reported recently that propranolol administered after initiation of reperfusion, was able to limit myocardial infarct size measured in dogs by the nitroblue tetrazolium staining method. These authors suggested that the protective effect of propranolol may involve a reduction in the intracellular calcium overload occurring on reperfusion.. Myocardial calcium concentrations, however, were not measured in their study. From the results of our study, it appears that propranolol, when added during reperfusion, did not reduce tissue and mitochondrial calcium overload.

As already discussed in Chapter 3, the molecular mechanism of the net gain in tissue calcium occurring on reperfusion after a period of ischaemia is still unknown, although several authors (Watts et al., 1980; Bourdillon and Poole-Wilson, 1981) have suggested that readmission of flow may induce a specific abnormality of the cell membrane related to calcium transport. This suggestion and the finding that the massive entry of calcium during reperfusion does not necessarily happen through the slow calcium channel, may provide an explanation why propranolol in our study, failed to reduce tissue and mitochondrial calcium accumulation during reperfusion. Unless high dosages with membrane

stabilising effects are used, the B-blocking effects of propranolol will reduce only calcium movement through the slow channel and not the bulk movement of calcium which occurs on reperfusion.

In Groups B and C, propranolol was able to reduce the rate of depletion of the energy-rich phosphate reserves during ischaemia. In this way, sufficient ATP may have remained to ensure the integrity of the cell membrane. This may explain why propranolol in these two groups restricted the entry of calcium during reperfusion. Under these circumstances, as already discussed in Chapter 3, mitochondria would not become overloaded with calcium, their ATP producing capacity would be maintained (Fig. 4.6), and resting tension would not rise (Fig. 4.3).

Thus, although verapamil, nifedipine (Chapter 3) and propranolol have different pharmacological properties, their ability to reduce reperfusion damage may involve a common mechanism: an ability to slow the rate of depletion of ATP reserves during ischaemia.

C H A P T E R 5

THE RELATIONSHIP BETWEEN CARDIAC MITOCHONDRIAL
Ca²⁺ TRANSPORT AND OXIDATIVE PHOSPHORYLATION

A. INTRODUCTION

B. EXPERIMENTAL

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- (b) Isotopic calcium transport
- (c) Mitochondrial ATP production
- (d) Simultaneous measurements of mitochondrial respiration and ion movements
- (e) Animals and materials

C. RESULTS

- (a) Respiration-dependent Ca²⁺ transport
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D. DISCUSSION

- (a) Properties and preparation of the mitochondria
- (b) Ca²⁺ efflux from respiring mitochondria
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- (d) Effects of ATP production and Ca²⁺ uptake on proton movements

A. INTRODUCTION

That mitochondria can accumulate certain ions such as K^+ or Mg^{++} from the suspending medium as long as respiration is occurring, was first observed in the early 1950s by Spector (1953), MacFarlane and Spencer (1953) and Bartley and Davies (1954). Later Werkheiser and Bartley (1957) suggested that mitochondria may contain two internal water compartments, thus anticipating the now widely accepted concept that it is the inner mitochondrial membrane across which energy-dependent ion translocation occurs. Little progress, however, was made in analysing ion transport processes quantitatively until Vasington and Murphy (1961) and, independently, DeLuca and Engstrom (1961), discovered that isolated mitochondria can accumulate large net amounts of Ca^{2+} from the suspending medium. It was found that no oxidative phosphorylation of ADP occurs when Ca^{2+} is accumulated.

It was shown later that the experimental conditions used were somewhat unfavourable for studying this process in detail because of mitochondrial damage incurred by exposure to a concentration of Ca^{2+} in the test medium which was as much as 200 times greater than the endogenous Ca^{2+} .

Later it was demonstrated by Rossi and Lehninger (1964) and confirmed by Brierley et al. (1964) that Ca^{2+} and phosphate accumulation can occur in the absence of electron transport, supported by hydrolysis of ATP.

It has also been reported that mitochondria are able to bind Ca^{2+} in the absence of an energy source, but the relation between binding

and the energy-linked massive accumulation is not clear, nor is it clear whether the binding is on the surface of the membrane or inside the mitochondria (Reynafarje and Lehninger, 1969).

During recent years there has been a growing interest in research of the respiration-linked transport of ions by isolated mitochondria and, particularly, of Ca^{2+} (Chance, 1965; Lehninger, 1970; Carafoli, 1975; Harris, 1977; Nicholls and Crompton, 1980). It is not surprising that different results have been produced because there are so many related effects of the rapid accumulation of Ca^{2+} by mitochondria, such as: stimulated respiration, shift of internal and external pH, shift of the redox state (Chance, 1965; Lehninger, 1970; Harris, 1977), delocalisation of respiratory components (Vinogradov et al., 1972; Azzi et al., 1975) and uptake of other ions (Harris and Berent, 1969; Brand et al., 1976). Some of these aspects will be discussed in this chapter.

Almost all of the work on mitochondrial Ca^{2+} transport has been focused on the molecular mechanism of energy coupling during electron transport, especially on the dilemma posed by the chemical-coupling and chemiosmotic hypothesis for oxidative phosphorylation (Mitchell, 1966). But, there is also another side to the importance of mitochondrial Ca^{2+} transport, namely its physiological role in the cell. It is well known that the cytosolic calcium concentration is the factor which regulates the state of the contractile apparatus of the myocardial cell via its interaction with the regulatory protein molecule troponin. The intracellular calcium concentration varies between approximately 10^{-7} molar and 10^{-5} molar during relaxation and contraction respectively.

There is little doubt that within this range, the beat to beat fluctuation of cytosolic calcium is controlled by the sarcoplasmic reticulum. However, there is now a considerable body of evidence to support the theory that the mitochondria of the myocardial cell may have an important long term buffering effect on the intracellular calcium concentration. It has also been shown that mitochondrial Ca^{2+} concentration controls a number of enzymes of the tricarboxylic acid cycle (Denton et al., 1980; McCormack and Denton, 1980).

It is now apparent that the energy derived from mitochondrial respiration may be employed to drive a number of energy-requiring mitochondrial reactions. The two principle reactions are the synthesis of ATP and the transport of ions across the inner mitochondrial membrane (Slater, 1977). Probably, the most physiologically significant ion movement is the transport of Ca^{2+} into the mitochondrial matrix against a concentration gradient. The mechanisms by which electron transfer along the respiratory chain is coupled to these energy requiring processes has been, and still is, the subject of considerable discussion. The most widely accepted explanation is that ATP synthesis and cation transport are driven by electron transfer via an electrochemical proton gradient, or proton motive force, as suggested in Mitchell's chemiosmotic hypothesis (Mitchell, 1966). ATP synthesis is then, theoretically, driven by the back flow of protons through a specific proton channel associated with the mitochondrial ATPase F_1 . Calcium ions appear also to be transported into the mitochondrial matrix in response to the membrane potential component or the respiration-generated proton motive force (Rottenberg, 1979; Nicholls

and Crompton, 1980). The apparently closely related driving force of both ATP synthesis and Ca^{2+} transport makes the relation between these two processes of interest both in terms of basic mitochondrial function and also physiologically. The physiological relevance of this relation is particularly important in certain pathological conditions such as cardiac hypoxia or ischaemia.

As described in the previous chapters cellular ATP and CP fall during ischaemia. As a result of this, a redistribution of intracellular Ca^{2+} occurs with an increase of the cytosolic and mitochondrial Ca^{2+} concentration, probably due either to an influx of calcium from the extracellular space or to a release of calcium from the intracellular stores such as sarcoplasmic reticulum.

It has been shown that, if the period of ischaemia or hypoxia has been prolonged enough, reperfusion and/or reoxygenation results in a further increase of the ischaemic or hypoxic-induced tissue damage (Chapters 3 and 4; Hearse, 1977).

From the previously described experiments, reperfusion after 90 minutes of total (Chapter 3) or 1 ml/min ischaemia (Chapter 4) resulted in a massive accumulation of Ca^{2+} into the mitochondria together with a loss of their capacity to use oxygen for producing ATP.

It has been proposed that the reintroduction of oxygen to the ischaemic or hypoxic myocardium provokes respiration-supported accumulation of Ca^{2+} into the mitochondrial matrix in preference to the synthesis of ATP. Such a sequence would result in mitochondrial Ca^{2+} overload which would, in turn, cause a further inhibition of ATP production and, therefore, prevent the re-establishment of intracellular

ATP levels and normal muscle function (Hearse, 1977).

The basic premise of this series of events, that mitochondrial Ca^{2+} transport takes precedence over ATP synthesis, has been the aim of the present investigation.

A number of conflicting reports have appeared in the literature (Rossi and Lehninger, 1963; Jacobus et al., 1975; Vercesi et al., 1978), some suggesting priority of calcium transport over ATP synthesis while others providing evidence of competition between the processes. In view of the physiological significance of the relation between mitochondrial Ca^{2+} transport and ATP synthesis, we have carried out a systematic analysis of the situation.

This has been done by using mitochondria from freshly excised, non-perfused, rabbit hearts. The effects of ATP synthesis on mitochondrial Ca^{2+} transport was determined over a range of free Ca^{2+} concentrations. The values of ATP production in the presence of the same range of free Ca^{2+} concentrations was also determined. Additionally, simultaneous and continuous measurements of mitochondrial respiration and H^+ and Ca^{2+} movements during ATP synthesis and Ca^{2+} transport were made. Finally, we have examined the effects of factors such as oligomycine and ruthenium red which may influence either Ca^{2+} transport or ATP production.

B. EXPERIMENTAL

(a) Mitochondrial extraction

Adult male New Zealand rabbits (2.5 - 3 kg) were used for this study. The rabbits were killed by a blow to the head, the hearts excised and mitochondria were isolated by differential centrifugation as described in Chapter 2. The second and the third washing was in a medium containing in mM 105 KCl, 10 Tris and 10 Hepes instead of in Medium II and III as reported in Chapter 2. The final pellet was suspended in 0.5 ml of the same medium. The protein concentration of the suspensions was determined as described in Chapter 2.

(b) Isotopic calcium transport

Respiration-supported Ca^{2+} transport was determined using an adaptation of the ruthenium red, EGTA quench technique of Reed and Bygrave (1975).

1.25 mg of mitochondrial protein were suspended in 2 ml of 250 mM sucrose, 3 mM K_2PO_4 , 40 mM Tris, 10 mM nitrotriactic acid adjusted to pH 7.2 at 25°C with HCl.

The medium was continually stirred by a magnetic stirrer and was surrounded by a jacket of circulating water maintained at 25°C. Following one minute's incubation, potassium glutamate was added to a final concentration of 3 mM to stimulate respiration. Ca^{2+} transport was then initiated by adding a known quantity of CaCl_2 containing 0.2 m Curies $^{45}\text{Ca}^{2+}$ /ml. The rate of calcium transport was monitored by withdrawing 200 μl samples of the mixture after 6, 15, 20, 30, 45,

60 and 90 seconds. Each sample was immediately mixed with 500 μ l of a quench solution containing 40 mM EGTA and 3 μ M ruthenium red, pH 7.4 at 4°C in order to stop mitochondrial Ca^{2+} transport. The samples were then immediately filtered through 0.45 μ m pore size Millipore filters and washed with a total of 10 ml of 250 mM sucrose, 10 mM Tris, pH 7.4 at 4°C. The filters were dried, placed in scintillation vials, mixed with a scintillant and counted in a Packard 3380 liquid scintillation counter.

The rate of Ca^{2+} transport over the first 15 seconds of the reaction was taken to represent the initial rate. Experiments were carried out at free Ca^{2+} concentrations ranging from 6 to 150 μ M. Ca^{2+} concentrations of 50 μ M and below were controlled by the inclusion of the calcium buffering system, nitrotri-acetic acid (NTA, 10 mM), in the reaction medium. A computer program, designed to calculate ion-complexes in solution was then employed to determine the free Ca^{2+} concentration of the reaction medium using the available stability constants (Sillen and Martell, 1971).

(c) Mitochondrial ATP production

The reaction media and experimental conditions were identical to those previously described for the measurements of rates of Ca^{2+} transport. Reactions were initiated either by adding ADP or ADP plus Ca^{2+} . Samples were then withdrawn at 6, 15, 30, 60 and 90 seconds and mixed with 50 μ l of 10% perchloric acid on ice. Precipitated protein was separated by centrifugation and the ATP content of the supernatant determined enzymatically using hexokinase and glucose-6-phosphate dehydrogenase

as described in Chapter 2.

(d) Simultaneous measurements of mitochondrial respiration and ion movements

Experiments were carried out in a water jacketed perspex chamber (vol. 2.5 mls) maintained at 25°C containing a Clark type oxygen electrode (Rank Bros., Bottisham, Cambridge). A glass pH electrode (401 M5 Ingold Gallenkamp) and a Ca^{2+} electrode (Band et al., 1978) were inserted through the air-tight fittings. A common reference was used for the Ca^{2+} and pH electrodes and their outputs connected to the inputs of high input impedance operational amplifiers. The input of the amplifiers together with that of O_2 electrode were recorded on a Watanabe pen recorder (Multicorder MC 641). Additions to the reaction medium were made through a small hole in the top of the cuvette. The Ca^{2+} and pH electrodes were calibrated by adding known amounts of CaCl_2 and HCl.

Mitochondria (approximately 2 mg protein) were added to the reaction chamber and allowed to equilibrate. The reaction medium consisted of 120 mM KCl, 3 mM KH_2PO_4 , 2 mM Hepes, pH 7.2. Respiration was initiated by adding 3 mM K glutamate. Respiration and ion movements were then monitored following the addition of ADP or Ca^{2+} or a combination of the two.

(e) Animals and materials

New Zealand white rabbits were obtained from Hylyne, Cheshire, U.K. Standard laboratory chemicals of analar grade were purchased from BDH,

Poole, Dorset, U.K. Rotenone, nitrotri-acetic acid, oligomycin and all enzymes were obtained from either Sigma Chemical Co. (London) Ltd., or Boehringer Corporation (London) Ltd. All reaction media were made up in analar water supplied by BDH, Poole, Dorset, U.K. This ensured a reproducible low contaminant Ca^{2+} level in unbuffered solutions, usually in the range 10 - 20 μM as determined with a Ca^{2+} -sensitive electrode. The oxygen electrode and the chamber were supplied by Rank Bros., Buttisham, Cambridge, U.K.

C. RESULTS

(a) Respiration-dependent Ca^{2+} transport

In Figure 5.1 is shown a typical experiment on the effect of increasing the external free Ca^{2+} concentration on mitochondrial Ca^{2+} accumulation. The mitochondrial Ca^{2+} accumulation has been determined for a period of 90 seconds in the absence (Fig. 5.1 A) or presence (Fig. 5.1 B) of 475 mmol ADP in the reaction medium. Glutamate was used as respiratory substrate.

In the absence of ADP, mitochondria accumulated calcium progressively over a period of 90 seconds when exposed to a concentration of $6.03\ \mu\text{M}$ Ca^{2+} . When external Ca^{2+} concentrations were increased to $12.8\ \mu\text{M}$ or $28.6\ \mu\text{M}$, an initial period of rapid accumulation of about 30 seconds was succeeded by a release of the previously transported calcium from the mitochondria back to the medium. Further increase of external free Ca^{2+} concentration to 47.9; 74.0; $158.0\ \mu\text{M}$ resulted in a proportionally increased accumulation of Ca^{2+} for the first 20 seconds which was followed by a rapid release of the transported Ca^{2+} into the medium. 90 seconds after the addition of Ca^{2+} , there was little difference in the amount of Ca^{2+} retained by the mitochondria, irrespective of the external Ca^{2+} concentrations (Fig. 5.1 A).

When ADP (475 mmol) was added simultaneously with each different Ca^{2+} concentration to initiate the reaction (Fig. 5.1 B), the amount of Ca^{2+} accumulated during the first 20 seconds of the reaction was less than that which occurred in the absence of ADP (Fig. 5.1 A). However, Ca^{2+} accumulation increased with increasing the external Ca^{2+}

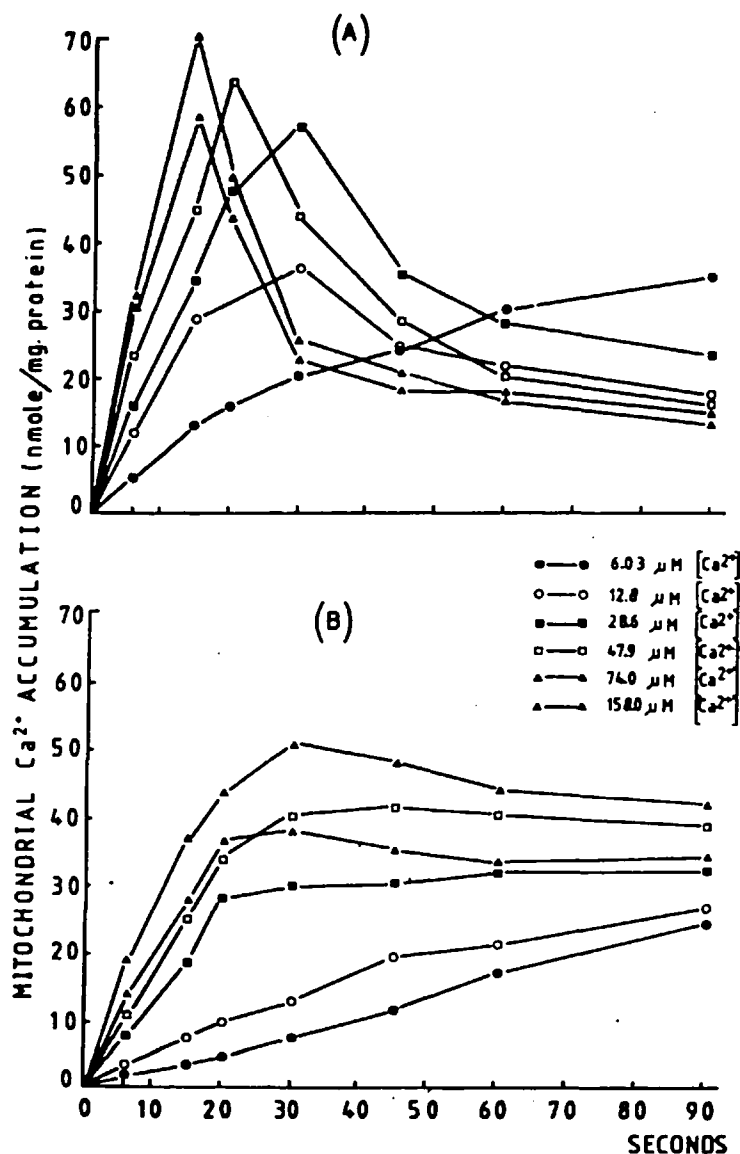


FIGURE 5.1 RESPIRATION DEPENDENT MITOCHONDRIAL Ca^{2+} TRANSPORT IN PRESENCE OR ABSENCE OF ADP

Accumulation of $^{45}\text{Ca}^{2+}$ into mitochondria respiring with glutamate as substrate was determined by means of the Quench technique as described in the experimental section. Free Ca^{2+} concentrations of 50 μM and below were controlled by adding 10 mM of nitroacetic acid.

(A) Ca^{2+} transport was initiated by adding $^{45}\text{Ca}^{2+}$ at each different concentration.

(B) Ca^{2+} transport was initiated by adding $^{45}\text{Ca}^{2+}$ at each different concentration plus ADP (475 nmol).

concentration. When the isolated mitochondria were exposed to the two lower external Ca^{2+} concentrations (6.03 and 12.8 μM), they accumulated Ca^{2+} constantly for 90 seconds. When exposed to higher free Ca^{2+} concentrations, mitochondria accumulated Ca^{2+} mainly during the first 30 seconds of the reaction after which their Ca^{2+} content remained constant.

Figures 5.1 A and B also show that calcium accumulation during the first 20 seconds was relatively linear at all the external Ca^{2+} concentrations examined, whether ADP was present or not.

(b) Effect of ADP and oligomycin on the initial rate of Ca^{2+} accumulation

The relation between the initial rate of Ca^{2+} transport and external free Ca^{2+} concentration is shown in Fig. 5.2.

As expected, increasing the external Ca^{2+} concentration leads to an increased initial rate of Ca^{2+} transport, the system tending to saturate at high external Ca^{2+} concentrations (Nicholls and Crompton, 1980). This was true for all the conditions we examined (in the presence or absence of ADP, with or without oligomycin).

Initial rates of Ca^{2+} transport, however, were markedly decreased if ADP (475 mmol) was added to respiring mitochondria simultaneously with Ca^{2+} (Fig. 5.2). Inhibition occurred over the whole range of Ca^{2+} concentrations examined. If these data are expressed by a Lineweaver-Burk plot (Fig. 5.2, upper panel), it appears that ADP produced a mixed type inhibition of mitochondrial Ca^{2+} accumulation.

When oligomycin was added to the system in a dose sufficient to inhibit oxidative phosphorylation (8 $\mu\text{g}/\text{mg}$ protein), the inhibitory

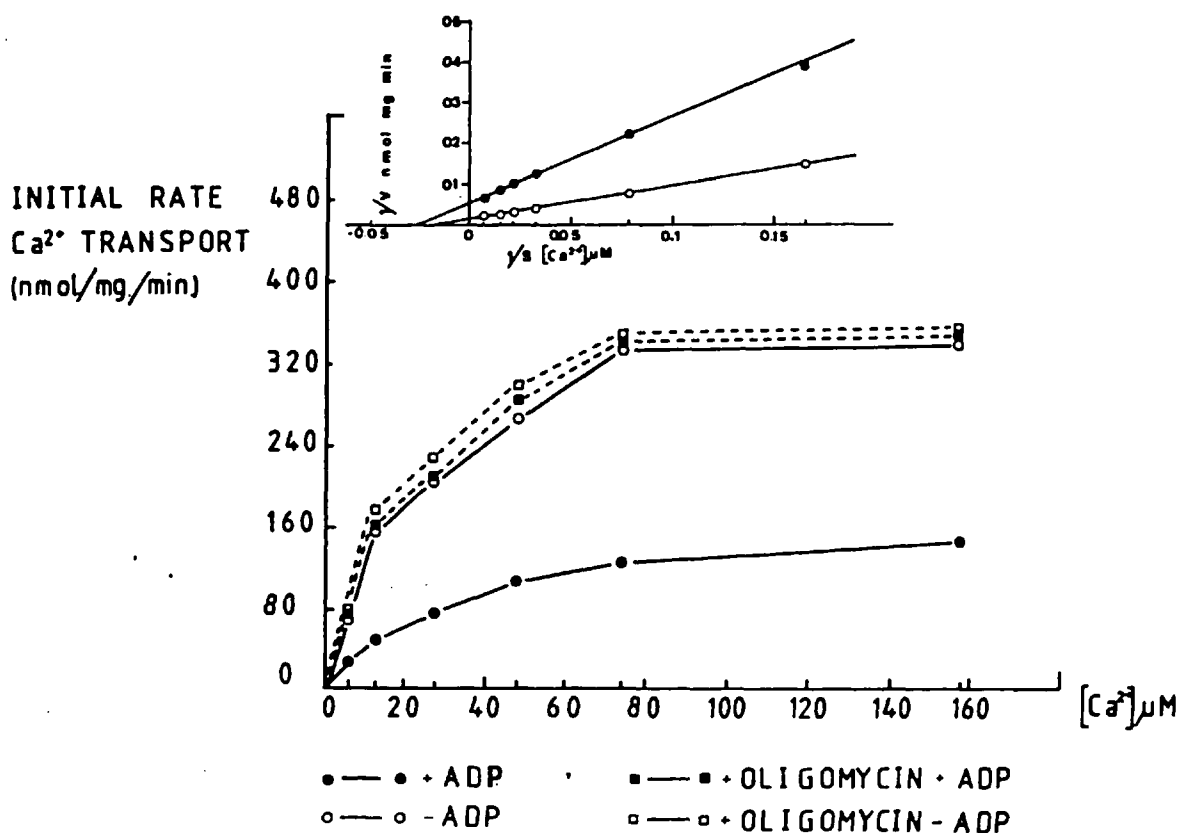


FIGURE 5.2 INHIBITION OF MITOCHONDRIAL Ca²⁺ TRANSPORT BY SIMULTANEOUS ADDITION OF ADP

Initial rate of Ca²⁺ transport was determined as described in the experimental section. Transport was initiated by adding either ⁴⁵Ca²⁺ or ⁴⁵Ca²⁺ plus ADP (475 nmol) to mitochondria respiring with glutamate as substrate.

Oligomycine was added at the dose of 8 μg/mg mit.prot. prior to addition of mitochondria.

effect of ADP on the initial rate of calcium transport disappeared. Figure 5.2 also shows that, in the absence of added ADP, oligomycin did not affect the rate of respiration-supported Ca^{2+} transport.

The presence of oligomycin in the system, either in the presence or absence of ADP, did not modify the kinetic of the calcium transport reaction (data not shown).

(c) Effect of Ca^{2+} accumulation on ATP synthesis

The effect of Ca^{2+} accumulation on ATP synthesis has been determined by adding simultaneously various concentrations of Ca^{2+} together with ADP (final concentration 0.34 mM) to initiate the reaction. Figure 5.3 (A) shows the effect of different Ca^{2+} concentrations on the total amount of ATP produced by the isolated mitochondria. The total amount of ATP produced was calculated as the ATP present in the reaction chamber 15 seconds after the transition from State 3 to State 4 respiration. Figure 5.3 (B) shows the effect of the same Ca^{2+} concentrations on the initial rate of ATP synthesis.

In the absence of added Ca^{2+} , that is with a buffered free Ca^{2+} concentration of approximately 10^{-9} M, almost all the added 272 nmoles/mg protein ADP were converted to ATP (260 nmoles/mg protein) and the initial rate was 580 nmoles/mg protein/min (Fig. 5.3 A and B). As Ca^{2+} ($6.03 \mu\text{M}$) was added together with ADP to start the reaction, the net amount of ATP produced declined (Fig. 5.3) as well as the initial rate of ATP synthesis. Increasing the quantity of Ca^{2+} added together with ADP induced a further decrease of the initial rate of ATP synthesis and of the net amount of ATP produced.

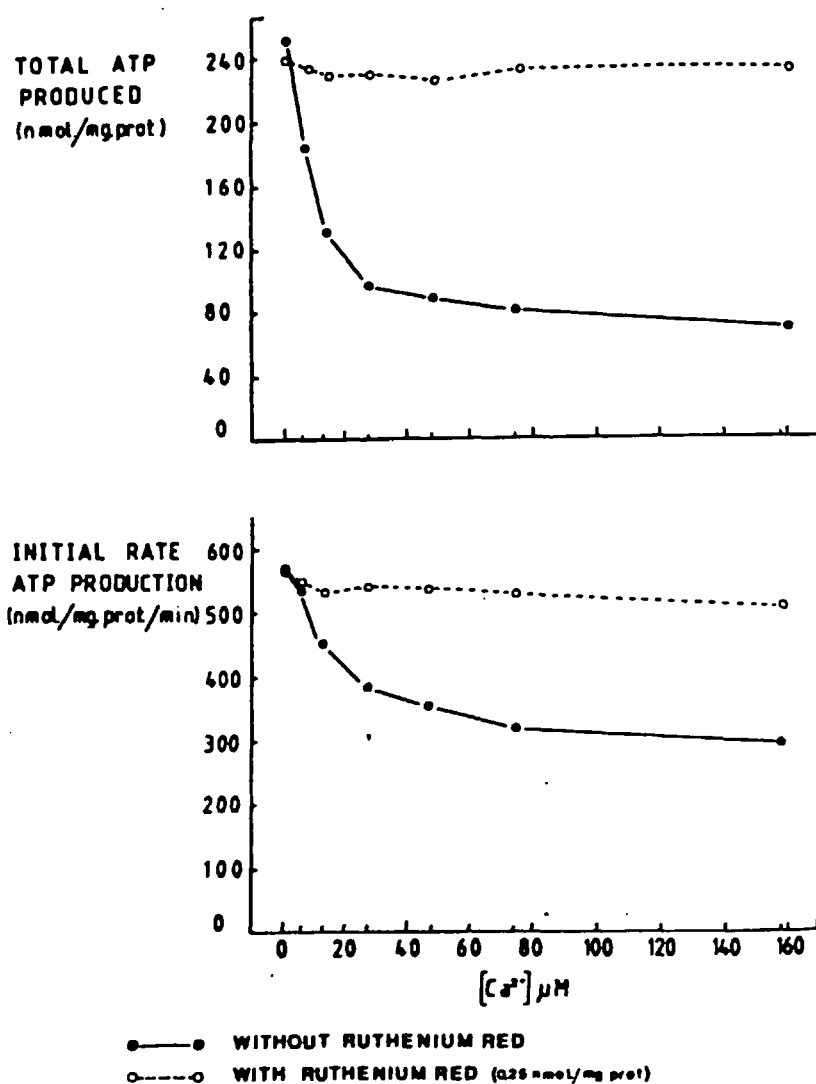


FIGURE 5.3 EFFECT OF MITOCHONDRIAL Ca^{2+} TRANSPORT ON ATP PRODUCTION

Total ATP produced (A) and initial rate of ATP production (B) were determined as described in the experimental section. ATP production was initiated by adding either ADP (0.34 ml final concentration) or ADP + Ca^{2+} to mitochondria respiring with glutamate as substrate. Free Ca^{2+} concentrations of 50 μM and below were controlled by including 10 mM of nitroacetic acid. The results concerning total amount of ATP produced are the mean of 7 experiments, while those on the initial rate of ATP production are the mean of 5 experiments.

The open circles and dashed lines in Figure 5.3 show the results obtained when ruthenium red (0.25 nmol/mg protein) was added to the medium 3 minutes before starting the reaction. In the presence of ruthenium red, increasing concentrations of Ca^{2+} failed to reduce the production of ATP.

(d) Simultaneous measurements of respiration and ion movements

The results shown in Figure 5.4 were obtained from a single, typical mitochondrial preparation. They show O_2 , H^+ and Ca^{2+} concentration tracings obtained simultaneously by means of the electrodes. A decrease of Ca^{2+} concentration in the medium means a transport of Ca^{2+} into the mitochondria.

The addition of ADP (475 nmol) to cardiac mitochondria respiring with glutamate produced the well-documented stimulation of oxygen consumption (Fig. 5.4 A) which was maintained until all the added ADP was phosphorylated, at which time the rate of oxygen consumption slowed to State 4 respiration, producing an ADP/O ratio of 3.0. Details of rates are given with the Figure 5.4.

During ATP synthesis there was a net uptake from the medium of 451 nmol H^+ and a small, but significant, decrease in the quantity of contaminant Ca^{2+} in the reaction medium. It is difficult to say if this represents a true mitochondrial Ca^{2+} accumulation, as it may be also due to a chelation of Ca^{2+} by the newly synthesised ATP.

The addition of Ca^{2+} (420 nMol) also produced an increased rate of respiration or respiratory jump (Fig. 5.4 B). However, oxygen consumption during Ca^{2+} accumulation was considerably slower than

during phosphorylation of ADP to ATP. A total of 69 n atoms of oxygen were consumed during the transport of 420 nMol of Ca^{2+} , while 158 n atoms of oxygen were consumed for phosphorylating 475 nMol of ADP (Fig. 5.4 A). The Ca/O activation ratio was 6.1 or 2.04 per site for a three site substrate such as glutamate.

These ratios are in good agreement with previously published data (Chance, 1965; Rossi and Lehninger, 1964; Vercesi et al., 1978). A net proton extrusion was observed during Ca^{2+} accumulation (Fig. 5.4 B) with a total of 476 nMol H^+ released into the medium during the transport of the added Ca^{2+} . In these experiments, it is not possible to determine the initial rate of Ca^{2+} accumulation, as this rate is masked by the rising phase of the Ca^{2+} electrode response following Ca^{2+} addition. However, it is possible to determine the total Ca^{2+} accumulation. As can be seen in Figure 5.4 (B), the Ca^{2+} content of the reaction medium returns to approximately the level seen before the Ca^{2+} addition. This indicates that virtually all the added 420 nMol of Ca^{2+} were accumulated with a H/Ca^{2+} ratio of 1.1.

In Figure 5.4 (C) are shown the effects of simultaneous addition of 475 nMol ADP and 420 nMol Ca^{2+} . As with the addition of either ADP or Ca^{2+} alone, this simultaneous addition produced an increased rate of respiration, the rate of oxygen consumption being intermediate between the rates achieved during ATP synthesis (Fig. 5.4 A) and Ca^{2+} transport (Fig. 5.4 B). Total oxygen consumption during this respiration jump was 229 n atoms. Following the addition of ADP and Ca^{2+} , there was an initial increase in the rate of proton extrusion (Fig. 5.4 C). This phase was then followed by a net proton absorption

and finally, a return to the base rate of proton extrusion seen prior to the addition of ADP plus Ca^{2+} . The majority of Ca^{2+} added to the system was accumulated during the proton release phase (Fig. 5.4 C) but Ca^{2+} transport was still occurring during the second net proton absorption phase. Total oxygen consumption was approximately equal to the sum of the oxygen consumption with ADP or Ca^{2+} given separately. Figure 5.4 (D) shows results obtained in an experiment in which only 210 nMol of Ca^{2+} were added with 475 nMol ADP. Oxygen consumption was increased to a rate greater than that produced by 475 nMol ADP plus 420 nMol Ca^{2+} , but slower than that produced by adding 475 nMol ADP alone. Total oxygen consumption during this respiration jump was 198 n atoms.

Addition of these proportions of ADP and Ca^{2+} produced no initial alteration in the rate of proton ejection. Eventually, a small net proton uptake occurred, followed, at the end of the respiratory jump, by a return to a basal low rate of proton extrusion. Ca^{2+} transport occurred through the duration of the respiratory jump, including the phase of net proton uptake.

In the presence of added ADP (Fig. 5.4 C and D) the mitochondria were able to lower the Ca^{2+} content of the reaction medium to a level below that possible in the absence of ADP (Fig. 5.4 B). As mentioned earlier, this may represent chelation of Ca^{2+} by synthesised ATP.

(e) Influence of ATP production on Ca^{2+} retention

It will be recalled that our previous experiments with isotopic calcium (Fig. 5.1) showed that, with higher concentrations of Ca^{2+} , uptake was followed by release. In the presence of ADP the uptake was reduced, but the subsequent release was abolished (Fig. 5.1).

The same phenomenons could be demonstrated by means of the Ca^{2+} electrode.

Figure 5.5 (A) shows that adding 600 nMol of Ca^{2+} to mitochondria respiring with glutamate as substrate produced an initial stimulation of respiration, a net ejection of H^+ and the accumulation of the majority of the added Ca^{2+} . This was followed by release of Ca^{2+} back into the medium. During this release phase the oxygen consumption declined as did the rate of H^+ ejection. The final stages of Ca^{2+} release were accompanied by a small net H^+ uptake. The simultaneous addition of ADP (475 nMol) with the same amount of Ca^{2+} prevented the release of this accumulated Ca^{2+} back into the medium.

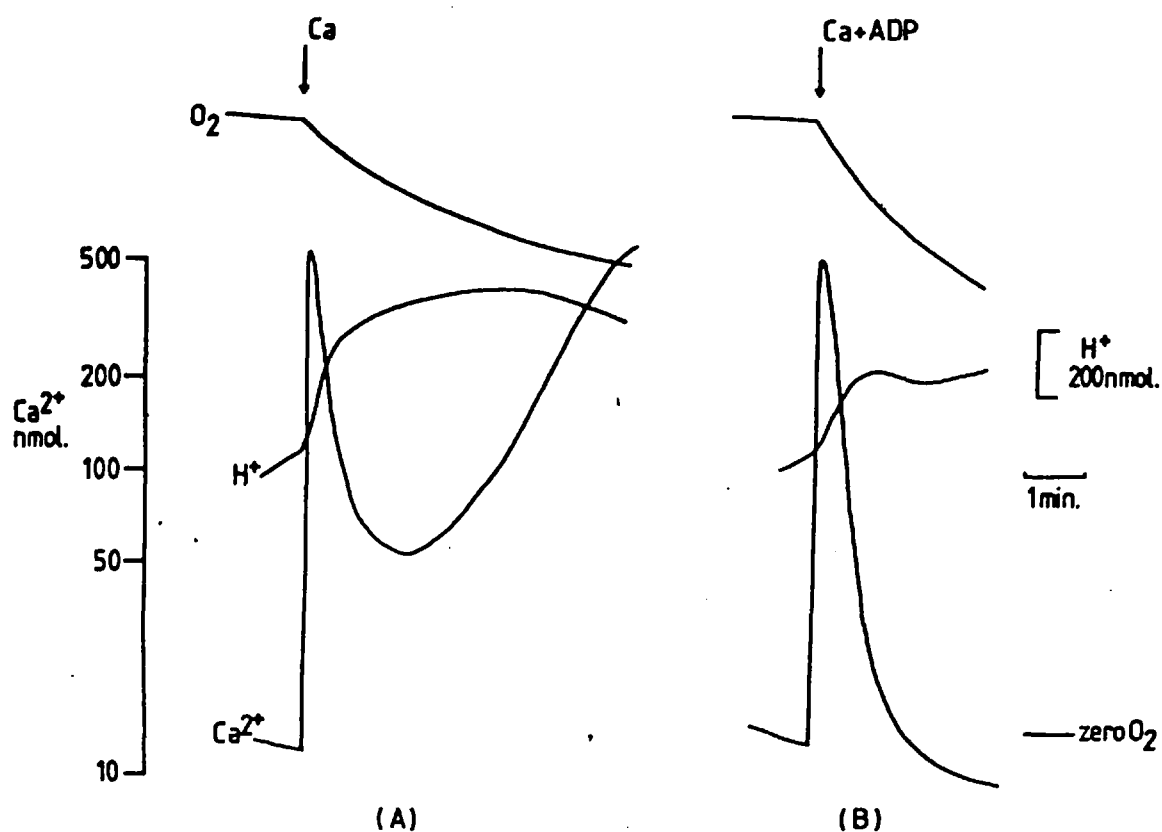


FIGURE 5.5 MITOCHONDRIAL Ca^{2+} MOVEMENTS IN PRESENCE OR ABSENCE OF ADP

Experiments were performed as described in the experimental section.

(A) 600 n mol Ca^{2+} added to mitochondria respiring with glutamate.

(B) 600 n mol Ca^{2+} plus 475 n mol ADP added simultaneously to mitochondria respiring with glutamate as substrate.

D. DISCUSSION

(a) Properties and preparation of the mitochondria

The original studies of the relation between mitochondrial Ca^{2+} transport and oxidative phosphorylation produced results which were interpreted as suggesting that Ca^{2+} transport took precedence over ATP production. Rossi and Lehninger (1964), using liver mitochondria exposed to a low Ca^{2+} concentration, concluded that the affinity of Ca^{2+} for the energy conserving sites of the respiratory chain is higher than that of ADP. When both Ca^{2+} and ADP were added simultaneously to the liver mitochondria, Ca^{2+} stimulated respiration occurred first and exclusively. After all the Ca^{2+} added had been accumulated, ADP activated respiration and was phosphorylated. The extrapolation of these conclusions from liver to heart mitochondria is of particular importance not only for the pathological implication described in the introduction, but also in view of the special role of both oxygen and calcium in the physiology of the heart cell.

A similar investigation of the relation between Ca^{2+} transport and oxidative phosphorylation in rat heart mitochondria, however, produced conflicting results (Jacobus et al., 1975). Using glutamate plus malate as the substrate, these authors measured rates of Ca^{2+} transport in the presence or absence of ADP, together with the associated H^+ movements. They found little or no respiratory stimulation by Ca^{2+} , low Ca^{2+} uptake rates and an inability of Ca^{2+} to compete effectively with ADP for respiratory energy. They concluded

that heart mitochondria differ considerably from liver mitochondria with respect to the transport of Ca^{2+} . More recently, Vercesi et al. (1978) has suggested that, as is the case with liver mitochondria, "well prepared" cardiac mitochondria will preferentially transport Ca^{2+} rather than phosphorylate ADP. Their conclusions were based on H^+ movements monitored following the initiation of the reaction with succinate in the presence of a high Ca^{2+} concentration (340 mg ions/mg protein).

Vercesi et al. (1978) suggested that the method of preparation of the mitochondria was important. In the procedure first reported by Chance and Hagihara (1961) and discussed in detail by Tyler and Gonze (1967) and Pande and Blanchaer (1971), the proteolytic enzyme nagarse was added to the homogenisation medium. Vercesi believed that this might be harmful to the mitochondria. In his preparation the minced heart was incubated with nagarse prior to homogenisation, and the nagarse was removed by washing before the tissue was disrupted and the mitochondria released. Jacobus et al. (1975) had isolated the mitochondria either without nagarse or using the method of Pande et al. (1971).

In view of the possible importance of the preparation procedure in the ability of the mitochondria to handle Ca^{2+} , we carried out these experiments on mitochondria isolated from rabbit hearts using nagarse following the method of Vercesi et al. (1978) and Mela and Seitz (1979). Mitochondria prepared in this way produced qualitatively and quantitatively similar results to those isolated without Nagarse.

All preparation showed Ca^{2+} induced respiratory jumps and Ca^{2+} transport rates comparable with those reported by Vercesi et al. (1978).

We concluded that Nagarse was not an important factor in the preparation of rabbit heart mitochondria and the experiments described in this thesis were carried out on mitochondria isolated in the absence of proteolytic enzymes.

(b) Ca^{2+} efflux from respiring mitochondria

It has been suggested that Ca^{2+} efflux from respiring mitochondria is energy-dependent and occurs by a route different from the pathway of influx (Lehninger et al., 1978; Nicholls, 1981).

Nicholls and Crompton (1980) suggested that, in addition to the calcium uniporter, the cardiac mitochondrial inner membrane also contains an independent calcium release mechanism which is stimulated by sodium. This system operates electroneutrally, two sodium ions entering the matrix in exchange for each calcium ion. Recently it has been demonstrated (Affulter, 1980) that during sodium-calcium exchange the mitochondrial membrane potential remains unchanged, again implying a two sodium-one calcium exchange. The carrier will also exchange matrix calcium for extramitochondrial calcium with a one-to-one stoichiometry. Potassium is not carried by the exchanger but recently it has been demonstrated that potassium may have some stimulatory effect on the exchanger (Crompton, 1980). A mechanism for the exchanger has been proposed (Nicholls and Crompton, 1980) in which the sodium-calcium antiporter is functionally linked to a sodium-proton antiporter

known to be present in the mitochondrial inner membrane. It has been suggested that two sodium ions enter the mitochondrial matrix in exchange for one calcium ion. Sodium ions are then recycled across the inner membrane in a one-to-one exchange with protons. In this way calcium efflux is driven initially by the sodium gradient across the membrane and the sodium gradient is maintained by the respiration generated proton gradient.

In addition, calcium can be released by the mitochondria through the uniport by collapsing the membrane potential. Generally this occurs along with structural damage, loss of NADH and adenine nucleotides and requires high levels of calcium. The existence in mitochondria of independent calcium influx and efflux systems convey on them the ability to act as calcium buffers.

It is, also, now well established that the mitochondrial Ca^{2+} storage capacity is influenced by respiratory substrate. Large quantities of Ca^{2+} transported by mitochondria with succinate as substrate are retained within the matrix (Williams et al., 1980). The accumulation of large quantities of Ca^{2+} with NADH linked substrates, such as glutamate, on the contrary, is followed by the release of this Ca^{2+} back into the reaction medium (Figs. 5.1 and 5.5) (Williams et al., 1980). It has been proposed by Lehninger et al. (1978) that net efflux of Ca^{2+} is induced by oxidation of the matrix piridine nucleotides. When mitochondrial NAD is in a reduced state (i.e. when the NADH/NAD^+ ratio is high, as would be expected with succinate as substrate in the presence of rotenone) the rate of mitochondrial Ca^{2+} efflux is minimal

and mitochondria tend to accumulate and retain Ca^{2+} . On the other hand when the NADH/NAD ratio is low (as would be expected with glutamate as respiratory substrate) the rate of Ca^{2+} efflux is maximal. This mechanism has been also proposed as a possible physiological method of regulating mitochondrial Ca^{2+} transport. In agreement with this hypothesis Sordahl and Stewart (1980) have reported that a rapid decrease in mitochondrial NADH accompanies the efflux of Ca^{2+} . Nicholls and Crompton (1980), however, have suggested that nicotinamide nucleotide oxidation makes the mitochondria more vulnerable to damage during Ca^{2+} uptake and that release of Ca^{2+} occurs as a result of the collapse of the membrane potential component of the proton motive force. They demonstrate that agents such as ATP and ADP which stabilise mitochondria against damage induced by high free Ca^{2+} concentrations, prevent membrane potential collapse and consequent Ca^{2+} release. The molecular basis of these effects is unknown.

The results of the present study support these findings. Ca^{2+} release occurred in the presence of glutamate which leads to an increased oxidative state of the nicotinamide nucleotide pool in the matrix. Simultaneous addition of ADP with Ca^{2+} prevented the subsequent release of Ca^{2+} (Fig. 5.1 B). Although the conditions used here are considerably different from those employed by Nicholls and Crompton (1980), it seems likely that Ca^{2+} retention reflects the maintenance of the membrane potential due to the presence of ADP (Fig. 5.1 B). From the results shown in Fig. 5.1 (A), also, it appears that Ca^{2+} release was dependent on the external free Ca^{2+} concentration, being higher and quicker when

mitochondria were exposed to the highest Ca^{2+} concentration. There was no Ca^{2+} release when mitochondria were exposed only to $6.03 \mu\text{M}$ of Ca^{2+} .

(c) Relation between mitochondrial Ca^{2+} transport and oxidative phosphorylation

The investigations of previous authors on the effect of mitochondrial Ca^{2+} transport on oxidative phosphorylation have been based on H^+ movements monitored following the initiation of respiration with a substrate in the presence of a high Ca^{2+} concentration and ADP (Verseci et al., 1978; Jacobus et al., 1975).

We have examined this situation more directly with measurements of Ca^{2+} transport (Figs. 5.1 and 5.2) and of ATP production (Fig. 5.3). Direct measurements of Ca^{2+} transport indicate that the initial rate of Ca^{2+} accumulation is reduced by the simultaneous addition of ADP. This is true not only when glutamate is used as respiratory substrate but also when succinate is used (Williams et al., 1980).

Inhibition is not produced simply by the presence of ADP, but requires the production of ATP. The inhibitory effect of ADP is completely overcome by the presence of sufficient oligomycin to completely inhibit phosphorylation of ADP (Brand et al., 1976) (Fig. 5.2).

Direct measurements of mitochondrial ATP production (Fig. 5.3 A) indicate that increasing levels of Ca^{2+} added to the reaction media simultaneously with ADP, decreased the total amount of ATP produced and the initial rate of ATP production. The experiments in which ruthenium red was included in the medium in a dose sufficient to

inhibit Ca^{2+} transport (Vasington et al., 1972; Takar et al., 1973; Rossi et al., 1973) show that the reduced mitochondrial capacity to phosphorylate was not caused by the presence of Ca^{2+} in the medium but by its transport into the mitochondria. Thus, on the one hand, oxidative phosphorylation decreases the rate of respiration-supported Ca^{2+} uptake, while, on the other hand, the inward movement of Ca^{2+} decreases the rate of production of ATP. These two observations lead to the conclusion that, with glutamate as substrate, ATP production and Ca^{2+} uptake compete for respiratory energy.

(d) Effects of ATP production and Ca^{2+} uptake on proton movements

According to Mitchell's chemiosmotic hypothesis (Mitchell, 1966), ATP synthesis is driven by the inward movement of protons through a specific proton channel located in the inner mitochondria membrane. It is now accepted that the carriers of the electron transport chain are positioned in the inner mitochondrial membrane such that the passage of electrons to oxygen is obligatorily linked with the transfer of protons from the mitochondrial matrix to the cytosolic side of the inner membrane. The electrogenic movements of protons across the inner membrane establishes a charge difference or membrane potential. The membrane potential and the accompanying chemical concentration gradient of protons result in the formation of the electrochemical gradient or proton motive force. One of the basic assumptions of this hypothesis is that the inner mitochondrial membrane is relatively impermeable to H^+ except at certain points where the protein $\text{F}_1\text{-ATPase}$ is located.

Here the H^+ expelled by the respiratory chain can re-enter the membrane when ADP and phosphate are present and in so doing the potential is dissipated and energy for ATP synthesis is provided. During ATP production, therefore, we would expect an absorption of H^+ from the medium into the mitochondria as shown in Fig. 5.4.

It is generally accepted that Ca^{2+} is transported into the mitochondrial matrix in response to the membrane potential component of the proton motive force (Rottenberg and Scarpa, 1974; Nicholls and Crompton, 1980). Because the calcium ions carry a positive charge, the membrane potential decreases as they enter the mitochondrion. This causes further stimulation of respiration and concomitant H^+ ejection. During Ca^{2+} transport in the absence of ATP production, therefore, we would expect an outward movement of H^+ from the mitochondria into the medium (Fig. 5.4 A).

Thus the addition of ADP to a mitochondrial suspension results in an absorption of protons by the mitochondria, while the addition of Ca^{2+} results in an extrusion of protons. When both ADP and Ca^{2+} are added together, the consequent movements of protons represent the net changes resulting from H^+ movements in opposite directions.

Evidence that H^+ movements observed in our experiments following the addition of Ca^{2+} and ADP are net movements, is given in Fig. 5.4 C and D. If the proportion of Ca^{2+} and ADP are altered, then the net H^+ movements are changed as expected. As decreasing amounts of Ca^{2+} are added, the initial amount of H^+ extrusion decreases and, under certain conditions (Fig. 5.4 D), Ca^{2+} accumulation is occurring during

a phase of net H^+ influx.

Therefore it is possible to conclude that the monitored H^+ movements accompanying these processes represent net changes.

These results confirm that Ca^{2+} transport and ATP synthesis reactions of isolated cardiac mitochondria compete for respiratory energy. The relation between cardiac mitochondrial Ca^{2+} transport and ATP production can be influenced by factors affecting either of these processes. For example, inhibition of ATP production by oligomycin obviously favours the Ca^{2+} transport reaction (Fig. 5.2) and inhibition of Ca^{2+} transport by ruthenium red favours the oxidative phosphorylating reaction (Fig. 5.3).

C H A P T E R 6

GENERAL DISCUSSION

A. EFFECTS OF ISCHAEMIA

- (a) General conclusions on the effects of ischaemia on the isolated and perfused rabbit heart
- (b) Events during the early stage of ischaemia
- (c) Events during prolonged periods of ischaemia
- (d) Mechanisms for maintenance of normal calcium homeostasis
- (e) Alteration of calcium homeostasis during ischaemia

B. EFFECTS OF REPERFUSION

- (a) General conclusions on the effects of reperfusion on the isolated and perfused rabbit heart
- (b) Events on reperfusion in relation to different period and degree of ischaemia
- (c) The role of mitochondria in reperfusion damage

C. PROTECTION OF THE ISCHAEMIC AND REPERFUSED MYOCARDIUM

- (a) General conclusions on the effects of verapamil, nifedipine and propranolol
- (b) Effects of verapamil and nifedipine
- (c) Effects of propranolol

D. CLINICAL RELEVANCE

A. EFFECTS OF ISCHAEMIA

(a) General conclusions on the effects of ischaemia on the isolated and perfused rabbit hearts

Under our experimental conditions, the reduction or abolition of coronary flow resulted in a rapid decline of developed tension, followed by a progressive rise in resting tension. Increasing the degree of coronary flow reduction induced a faster decline in developed tension and delayed the increasing of resting tension. The maintenance of some aerobic and/or anaerobic metabolism during restricted coronary perfusion (3 ml/min) was unable to modify the decline in developed tension, but it reduced the increase in resting tension.

During ischaemia, ATP and CP concentrations progressively fell, the fall in ATP and CP during the early stage of ischaemia being proportional to the severity of ischaemic condition. Under restricted coronary perfusion the presence of oxygen and glucose in the perfusate supported some aerobic and anaerobic ATP production.

When isolated from the ischaemic myocardium, the mitochondria were still able to utilise oxygen for oxidative phosphorylation, although the initial rate of ATP production was reduced after prolonged periods of severe ischaemia. The presence of oxygen and/or glucose in the perfusate during restricted coronary perfusion maintained the oxidative-phosphorylating capacity of the isolated mitochondria.

After 90 minutes of total ischaemia or severe coronary flow reduction (to 1 ml/min) the tissue calcium content of the myocardium was unchanged whilst mitochondrial calcium concentration was increased.

(b) Events during the early stage of ischaemia

During myocardial ischaemia, as a consequence of reduction or abolition of coronary flow, the rate of oxygen uptake in the cell decreases considerably. This causes an impairment in the oxidation of mitochondrial NADH or other reduced coenzymes, molecular oxygen being the terminal electron acceptor of the electron transport chain. There is, therefore, a reduction or, when taken to the extreme (as in the case of complete coronary flow abolition) an abolition of the passage of electrons to oxygen in the inner mitochondrial membrane. This leads to a reduction or abolition of the membrane potential, which, according to Mitchell's chemiosmotic hypothesis, is essential for ATP synthesis. The immediate result of this sequence of events is a reduced or abolished mitochondrial ATP synthesis, and an accumulation of metabolic products which cannot be oxidised and are not washed out of the tissue because of the coronary flow reduction. Their retention is accompanied by a fall of intracellular pH. Intracellular acidosis has two effects which very rapidly bring about an alteration of the normal contractile behaviour of the myocardial cell:- (1) protons decrease the affinity of the contractile apparatus for Ca^{2+} and (2) elevated intracellular H^+ inhibits the sarcolemmal channels responsible for the passage of Ca^{2+} ions into the cell during the action potential. Together these actions lead to a decline of active tension generation.

These changes are very rapid in the ischaemic myocardium, occurring with few minutes after coronary flow reduction (Jennings and Reimer,

1981). This, however, does not necessarily mean that tissue ATP content is reduced immediately after the onset of ischaemia. On the contrary, from our results (Chapter 2) it appears that after 30 minutes of ischaemia, the ATP content was as much as 83%, 69% and 55% of control values, depending on the degree of coronary flow reduction.

There are at least three reasons which can explain these findings:-

(1) The maintenance of contraction utilises approximately 75% of the total ATP production of the myocardial cell. Therefore the ischaemia-induced cessation of contraction removes an enormous energy drain on the cell, (2) a certain amount of ATP can be generated during the early phase of ischaemia by anaerobic glycolysis and by residual oxidative metabolism and (3) ATP can also be generated from CP. CP functions as a reservoir and as transmitter of energy. CP is formed by direct enzymatic transfer of a phosphate group from ATP to creatine when ATP is at a high concentration; for example, at the outer surface of the inner mitochondrial membrane under aerobic conditions. Whenever the ATP concentration falls, thus raising the ADP concentration, the phosphate groups of CP are transferred back to ADP, producing ATP. In addition, ATP is not free to diffuse into the cytosol. The ATP transported out of the mitochondria by the adenine nucleotide translocase is converted into CP by creatinphosphokinase (CPK). CP travels into the cytosol and is converted back to ATP at the site of ATP hydrolysis, for example, the sarcolemma or the contractile proteins (Moreadith and Jacobus, 1982). It can be argued, therefore, that myocardial CP levels, rather than ATP content, provide a more

reliable index of energy directly available for cell function during the early stage of ischaemia. In our experiments the decline of CP after 30 minutes of ischaemia was always greater than the decline of ATP (Chapter 2).

It should be emphasised here that, although these series of events are the consequence of a reduced or abolished rate of mitochondrial oxidation, this does not mean that the mechanisms of respiration or energy coupling in the mitochondrial respiratory chain are deficient. The cellular uptake of oxygen is abolished or severely reduced simply because of a deficient supply. In our experiments (Chapter 2) after 30 minutes of ischaemia, the mitochondrial yield was unchanged. Once isolated after 30 minutes of ischaemia, the mitochondria were still able to use oxygen for ADP phosphorylation. Reperfusion of the hearts made ischaemic for 30 minutes always resulted in some recovery of ATP and CP content suggesting that a significant proportion of the mitochondrial population, at least, have retained the capacity to oxidise substrate and to form ATP.

Similarly the non-contraction state of the ischaemic myocardial cell does not mean that the cell has lost the capacity of generating active force. It means only that the conditions to bring about an active contraction - a Ca^{2+} concentration of 10^{-5}M and sufficient ATP to support myosine-ATPase - are not met. Restoration of such conditions with reperfusion after 30 minutes of ischaemia resulted in a prompt recovery of developed tension.

(c) Events during prolonged periods of ischaemia

Extending the ischaemic period to 60, 90 and 120 minutes resulted in a progression of ischaemic damage, as resting tension further increased and ATP and CP levels further decreased (Chapter 2). The oxidative phosphorylation capacity of mitochondria isolated from hearts after prolonged periods of ischaemia was either unchanged or slightly reduced, depending on the degree of coronary flow reduction (Chapter 2).

The progression of ischaemic damage seems to be delayed by the presence of residual coronary flow. When coronary flow was reduced by 78% from control values for 120 minutes, ATP and CP levels together with the indices of isolated mitochondrial function were better maintained than when coronary flow was completely abolished for the same period of time.

It is possible that during low flow ischaemia (3 ml/min) the hearts reached a new state where the rate of cellular ATP hydrolysis is reduced by the depressed mechanical activity to match the lower ATP production. In this group, during the ischaemic period, there was some aerobic and anaerobic ATP production as shown from the data obtained by using hypoxic, substrate-free perfusate (Chapter 2). Under ischaemic conditions, however, several metabolic pathways continue to utilise the free energy provided by ATP. They include reactions involved in the maintenance of ion distribution and cell volume (Langer, 1974), enzyme reactions such as adenylcyclase (Dobson and Mayer, 1974), myokinase (Berne and Rubio, 1974), fatty acid

synthetase (Wood et al., 1972) and protein kinase (Larner, 1978).

In addition, ATP is essential for maintenance of normal cell structure, biosynthesis of proteins, nucleic acids, complex carbohydrates and lipids. It is also necessary indirectly for mitochondrial oxidative phosphorylation, as the entry of glucose into the glycolytic pathway and the activation of fatty acid require ATP.

In the 3 ml/min group, the residual ATP production may be just sufficient to maintain the structural integrity of the mitochondria which, when isolated after 90 or 120 minutes, had retained a normal capacity of oxidative phosphorylation. The same amount of ATP however may not be sufficient to prevent the increase in resting tension, an abnormality which depends on both ATP and cytosolic calcium (Chapter 2). Extending the period of severe ischaemia (1 ml/min or total ischaemia) from 30 to 60, 90 and 120 min, resulted in a further depletion of the high energy phosphate stores (Chapter 2). This suggests that, in these groups of experiments, ATP consumption during ischaemia exceeded ATP production. Under severe ischaemic conditions, the possibility of ATP production is almost non existent. Oxygen uptake is extremely reduced or abolished. Anaerobic glycolysis is severely inhibited by the intracellular accumulation of H^+ , NADH and lactate. Theoretically some ATP synthesis could be coupled with the reverse flux of ions through an ATP dependent ion pump such as, for example, the ATPase of the sarcoplasmic reticulum or the Na-K ATPase of the sarcolemma. It has been reported (Tada et al., 1977) that when the calcium

concentration is high inside the sarcoplasmic reticulum and ADP is present in the cytosol together with phosphate, the ATPase dependent calcium transport can be reversed so as to synthesise ATP. However, it is still uncertain if an ischaemic episode brings about the conditions necessary for reversing the ATPase-dependent calcium flux across the sarcoplasmic reticulum and, in any case, it is unlikely that the amount of ATP produced by this process is of great importance.

The RCR, QO_2 and initial rate of ATP production of mitochondria isolated from hearts made severely ischaemic for 90 minutes were slightly reduced, whilst the ATP/O ratio and total ATP producing capacities were unchanged. It has been reported that after 60 to 90 minutes of severe ischaemia, mitochondria may present ultrastructural alterations such as oedema of the matrix, breakage of the cristae and amorphous densities (Jennings and Ganote, 1976; Neely and Fewry, 1981). All these alterations, however, seem to be reversible.

It is interesting to note here that after 90 minutes of severe ischaemia (total ischaemia, Chapter 3 or 1 ml/min ischaemia, Chapter 4) mitochondrial calcium was slightly increased, when tissue calcium was unchanged. The finding that the ischaemic mitochondria had accumulated some calcium suggests that: (a) the ischaemic mitochondria are capable of maintaining a membrane potential, which is the driving force of mitochondrial calcium transport; (b) the cytosolic calcium concentration is somewhat elevated, the extra-mitochondrial calcium concentration being the major factor determining calcium accumulation. This also suggests that the ischaemic episode induces an alteration of the intracellular calcium homeostasis.

(d) Mechanisms for maintenance of normal calcium homeostasis

It is well known that in the myocardial cell the cytosolic calcium concentration is the factor which regulates the state of the contractile apparatus and of several enzymatic reactions (Langer, 1980). The regulation of cytosolic calcium concentration resides primarily with three organelles: the sarcoplasmic reticulum, the mitochondria and the sarcolemma. Sarcoplasmic reticulum and mitochondria are intracellular organelles capable of accumulation and release of calcium, thereby regulating the cytosolic levels of this ion. The sarcolemma has components which regulate the influx and efflux of calcium between the intracellular and extracellular space. The removal of cytosolic calcium by sarcoplasmic reticulum, mitochondria or, ultimately, calcium transport to the external medium through the sarcolemma is an energy requiring process since it implies movement of the ion against its electrochemical gradient.

In the heart the mechanisms involved in buffering cytosolic calcium are: (a) the ATP-dependent calcium pump of the sarcoplasmic reticulum (Tada et al., 1977), (b) the sarcolemmal sodium/calcium exchanger (Reuter, 1974), (c) the sarcolemmal ATP-dependent calcium pump (Caroni and Carafoli, 1981) and the respiration or energy dependent mitochondrial calcium transport (Chapter 5). A schematic representation of these mechanisms is reported in Fig. 6.1.

The sarcoplasmic reticulum calcium pump is a very complex protein system which transports two calcium ions into the SR for each ATP hydrolysed (Tada et al., 1977).

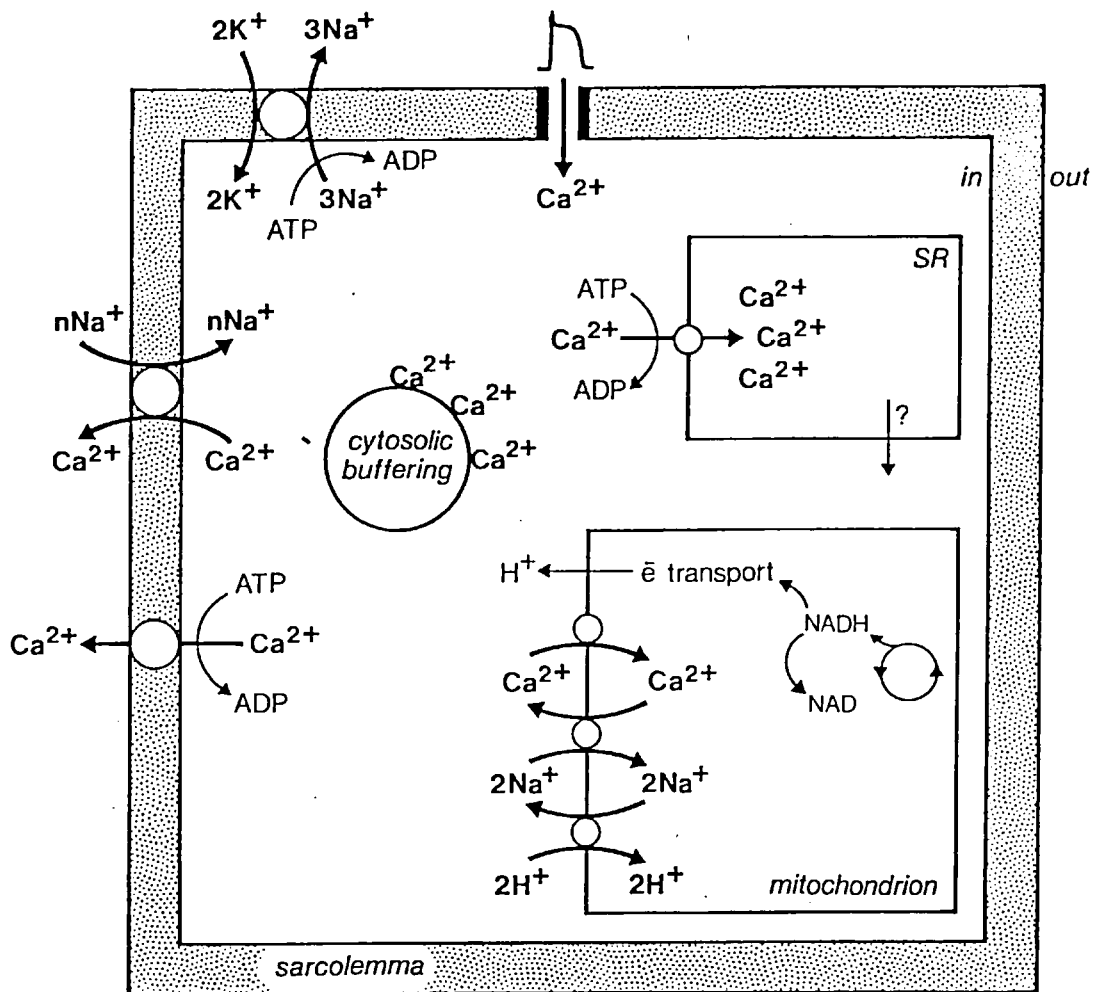


FIGURE 6.1 DIAGRAM OF THE PROPOSED MECHANISM OF CALCIUM
REGULATION IN THE CELL

The cell membrane has two quite different ATPases. A specific calcium ATPase which has very high affinity for calcium and a relatively low maximal pump rate. This ATPase presumably is responsible for the maintenance of calcium levels during diastole when high affinities, but not high pumping rate are required. The other mechanism is the sodium/calcium exchanger. A portion of the cytosolic calcium is pumped into the extracellular space by an exchange for sodium that is present at much higher concentration in the extracellular space. The energy used for calcium transport out of the myocardial cell by this mechanism is thus derived directly from the sodium gradient. The ultimate energy source for sodium/calcium exchange, however, is always ATP, which is hydrolysed by the sodium pump during the establishment and the maintenance of sodium gradient.

Finally the mitochondria which have independent calcium influx and efflux mechanisms (Chapter 5) provide an efficient and sensitive calcium buffering system in the cardiac muscle cell. Their capacity for calcium accumulation is at least several times that of sarcoplasmic reticulum (Lehninger, 1970; Carafoli, 1975). The rates of calcium movements carried out by mitochondria are relatively slow and consequently would probably not fluctuate from beat to beat. However the role played by mitochondria in buffering the calcium content of cells with elevated or continuously rising cytosolic calcium concentrations is likely to be extremely important as will be further discussed in this chapter. The energy source for mitochondrial calcium transport can be either the respiratory chain or the hydrolysis of ATP. Both the respiratory chain activity and ATP hydrolysis energise the

transport by generating a membrane potential across the inner membrane, which is the immediate pulling force for calcium accumulation (Rottemberg and Scarpa, 1974). Mitochondrial calcium transport does not require the simultaneous uptake of permeant anions, although the uptake of phosphate increases the total level of accumulated calcium (Carafoli et al., 1976).

(e) Alteration of calcium homeostasis during ischaemia

Whether or not these mechanisms for cytosolic calcium extrusion will be functioning under ischaemic conditions depends on the availability, in the ischaemic cells, of enough ATP to support them. The concentration of ATP (Michaelis constant, K_m) required for the half maximal activity of the sarcoplasmic reticulum ATPase is about 10^{-6} M, while that for the sarcolemmal ATPase is in the range of 10^{-3} M and that for the sarcolemmal sodium calcium exchanger is also 10^{-3} M (Harris, 1975). As discussed in Chapter 3, the sarcolemmal ATPase together with sodium/calcium exchanger, having the highest K_m for ATP are likely to be the most sensitive systems to the ischaemic-induced fall of ATP concentration. This should result in an increased cytosolic calcium and sodium concentration. The rise of intracellular sodium can induce a release of calcium from the mitochondria.

Mitochondria have two different systems for calcium release. Calcium can be released through the uniport resulting from a lowering of the respiratory generated membrane potential, or it can be released in an electroneutral exchange with sodium through the mitochondrial sodium-calcium antiporter (Nicholls, 1981).

The rise in cytosolic calcium resulting from a reduction of calcium extrusion across the sarcolemma during ischaemia, can be regulated only by the sarcoplasmic reticulum and by the mitochondria.

It may be possible that the residual ATP content of the ischaemic cell is sufficient and available to support the functioning of the sarcoplasmic reticulum ATPase. The sarcoplasmic reticulum, however, releases calcium with a mechanism still not completely known. There is evidence of a calcium induced release of calcium (Fabiato and Fabiato, 1977). An increased cytosolic concentration of ADP and phosphate also favour calcium release from the sarcoplasmic reticulum (Hasselbach and Suko, 1974). It is interesting that the concentration of ADP and phosphate found to stimulate calcium release from the sarcoplasmic reticulum are as much as might be expected to occur under ischaemic conditions. In addition the optimal pH for the ATP-dependent calcium pump of cardiac sarcoplasmic reticulum is above 7.5 (Shigekawa et al., 1976). The ischaemic episode, lowering the intracellular pH, should decrease the activity of this enzyme.

Therefore the alterations induced by an ischaemic episode, resulting in an increase in cytosolic calcium, ADP and phosphate concentrations, and in a decrease in intracellular pH and ATP content would be likely to favour calcium release from the sarcoplasmic reticulum into the cytosol and to reduce the net calcium uptake.

According to our data, this cytosolic calcium appears to be transported by the mitochondria as the mitochondrial calcium concentration increased after 90 minutes of severe ischaemia. As

previously suggested, this means that the ischaemic mitochondria were capable of maintaining a membrane potential. Under ischaemic conditions, however, the activity of the respiratory chain is reduced or abolished. During 1 ml/min ischaemia the reduced oxygen delivery may be sufficient to generate a membrane potential, which could support, although at reduced rate, some mitochondrial calcium accumulation. During total ischaemia, however, mitochondrial calcium accumulation has to be supported primarily by hydrolysis of residual ATP since there is no oxygen to maintain respiratory chain activity.

It is difficult to understand why the residual ATP should support calcium accumulation in the mitochondria and not, for example, in the sarcoplasmic reticulum. A possible explanation is related to the compartmentalisation of ATP during an ischaemic episode. As discussed in Chapter 3, there are several lines of evidence to suggest that the movement of ATP from the mitochondria to the cytosol is reduced during ischaemia as a consequence of an inhibition of the adenine nucleotide translocase. This will lead to a sequestration of the residual ATP in the mitochondrial matrix. In this case, some ATP could be available for the accumulation of calcium in the mitochondria and not in the sarcoplasmic reticulum.

B. EFFECTS OF REPERFUSION

(a) General conclusions on the effects of reperfusion on the isolated and perfused rabbit hearts

Reperfusion after 30 minutes of ischaemia resulted in a recovery of mechanical function and ATP and CP content. The recovery of developed tension and of high energy phosphate groups was inversely proportional to the severity of coronary flow reduction. ATP and CP were not completely restored to aerobic control levels (Chapter 2).

Reperfusion after 60 or 90 minutes of restricted coronary flow (3 ml/min) resulted in a restoration of active tension generation and of ATP and CP level, the recovery of developed tension being inversely correlated with the duration of ischaemic episode (Chapter 2). The presence of oxygen and/or glucose in the perfusate during 3 ml/min ischaemia was essential for the subsequent recovery on reperfusion. When the hearts were perfused at 3 ml/min with hypoxic-substrate free perfusate, for 30 and 60 minutes, reperfusion failed to restore mechanical activity or high energy phosphate groups. On the contrary, reperfusion of these hearts induced a further increase of resting tension and a further deterioration of isolated mitochondrial function.

Reperfusion of those hearts made ischaemic for 60 minutes by stopping or reducing coronary flow to 1 ml/min, resulted in only a small recovery of developed tension. Following a transient increase, resting tension remained elevated above the control levels. Mitochondria isolated after 30 minutes of post-ischaemic reperfusion showed only a slight reduction in RCR and QO_2 (Chapter 2).

Reperfusion after 90 minutes of total or 1 ml/min ischaemia, resulted in a massive tissue and mitochondrial calcium overload (Chapters 3 and 4). Mitochondria virtually lost their capacity to phosphorylate ADP. There was no recovery of ATP, CP or active tension generation. Resting tension, which rose rapidly during the early stages of reperfusion, remained elevated (Chapters 2, 3 and 4).

The degree of developed tension recovered during reperfusion was inversely proportional to the degree of reduction of coronary flow and to the duration of the ischaemic episode (Chapter 2). There was an association between the tissue ATP content measured at the end of reperfusion and the maximum recovery of developed tension after reperfusion (Chapter 2).

(b) Events on reperfusion in relation to different period and degree of ischaemia

From these results it would appear that whether or not reperfusion is beneficial or detrimental to the ischaemic myocardium depends upon the severity and duration of the ischaemic period. Increasing the severity and the duration of the ischaemic period reduces the recovery of normal myocardial function on reperfusion. A prompt recovery of mechanical and metabolic function on reperfusion always occurred after 30 minutes of severe coronary flow reduction (1 ml/min) or complete ischaemia. After this short period of ischaemia, there was some residual ATP content, ATP being considerably depleted only after 60 or 90 minutes of severe ischaemia. The presence of a residual coronary flow rate of 3 ml/min able to maintain some aerobic and anaerobic

metabolism enabled some recovery during reperfusion even after 60 - 90 minutes of ischaemia. In these groups, mitochondrial function was either unchanged or improved after reperfusion, but never worsened.

The most likely explanation for these results is that the residual ATP content of these ischaemic hearts was sufficient to support the basic cellular function such as control of cell volume, maintenance of ionic homeostasis and preservation of ultrastructure. The readmission of flow at this stage of the ischaemic damage, when cellular membranes are presumably still intact, some cellular supplies of ATP are available, and pathways of electron transport and oxidative phosphorylation are simply blocked and not damaged, should result in a rapid re-energisation of the electron transport pathway. This, in turn, leads to a restoration of oxidative phosphorylation and ATP production with a resumption of contractile activity.

The restoration of ATP content on reperfusion, however, was incomplete even after 30 minutes of ischaemia. This is probably due to a reduction of the adenine nucleotide pool of the ischaemic cell rather than to a defect of the mitochondrial ATP producing capacity. Jennings and Reimer (1981) reported that, during the early state of ischaemia, adenosine monophosphate (AMP) accumulates, while ATP decreases. AMP is then dephosphorylated to form adenosine which is lost from the cell and further degraded (Berne and Rubio, 1974). This results in a loss of much of the adenosine nucleotide pool of the ischaemic cell. Resynthesis of adenine nucleotides is limited under ischaemic condition since it is an ATP-requiring process.

Reperfusion of the ischaemic myocardium after 90 minutes of total or 1 ml/min ischaemia did not lead to a re-establishment of cell function. On the contrary, it produced massive tissue and mitochondrial calcium accumulation with a further decline in mitochondrial function. There was no recovery of active tension generation or of high energy phosphate groups (Chapters 2, 3 and 4).

A number of mechanisms have been suggested to explain these findings. In particular, it has been proposed that when given the choice of producing ATP via oxidative phosphorylation or accumulating calcium, cardiac mitochondria will preferentially perform the latter task, causing a further deterioration of ATP synthesis (Hearse, 1977). Thus it appears that mitochondrial function plays an important role in the molecular mechanism of reperfusion damage after prolonged period of ischaemia.

(c) The role of mitochondria in reperfusion damage

With the readmission of coronary flow there is clearly an abundant supply of external calcium to enter the myocardium. In our experiments the tissue calcium content was increased after 30 minutes reperfusion following prolonged ischaemia (total or 1 ml/min ischaemia) (Chapters 3 and 4). This indicates that there was an alteration of the cell membrane leading to a breakdown of the permeability barrier to ions such as calcium. The mechanism for this breakdown is unclear. It may be related to abnormalities occurring during the period of ischaemia which become manifest only during reperfusion. They include

disruption of sarcolemma as consequence of: (a) severe depletion in cytosolic ATP, (b) detergent effect of acyl CoA and free fatty acids accumulated during ischaemia and (c) activation of phospholipases (Chien et al., 1978; Vasdev et al., 1980). Alternatively, the increased permeability of the sarcolemma during reperfusion may result directly from the re-introduction of coronary flow. Ganote and Kaltenbach (1979) suggested that on reoxygenation contraction of the myocardial cell pulls apart the sarcolemmal membrane altering its permeability. The sudden readmission of oxygen to the cell after a period of ischaemia may generate free radicals leading to a peroxidation of membrane lipids.

Whichever mechanism is involved, the influx of calcium leads to an increased calcium concentration at the time when mitochondria are re-energised by readmission of O_2 . Cytosolic ADP concentration is also increased (Jennings et al., 1978). Under these circumstances the ischaemic mitochondria which are still able to function can use the restored membrane potential either to phosphorylate ADP or to accumulate calcium, thus buffering the cytosol. The amount of ATP produced or calcium accumulated will depend on the equilibrium between these two reactions.

From the results of Chapter 5, it appears that in cardiac mitochondria calcium accumulation does not necessarily take precedence over ATP synthesis, but, in fact, the two processes compete for respiratory energy. Furthermore, it has been shown by Williams et al. (1980) that the relationship of these two processes is influenced by a number of physiological factors including respiratory substrate and

intracellular effectors such as magnesium. In the presence of 1 mM free magnesium, which is a physiological concentration, the equilibrium between mitochondrial calcium transport and ATP synthesis seems to be shifted towards ATP synthesis. Magnesium acts as a physiological modulator of mitochondrial calcium transport. These results of Williams et al. (1980) may be interpreted as suggesting that, in the presence of magnesium, the rate of ATP synthesis would be maintained at free calcium concentrations far in excess of those found in the normal myocardial cell.

However, when considering the situation in vivo during post ischaemic reperfusion, we have to take into account that: (a) reperfusion induces a large release of magnesium with a consequent decrease of the cytosolic concentration of this ion (Shen and Jennings, 1972), (b) calcium enters the cell abnormally causing a very significant elevation of the cytosolic concentration and (c) the influx of calcium from the extracellular fluid is continuous. Under these circumstances the equilibrium between mitochondrial ATP production and calcium accumulation is very likely to be shifted towards calcium transport. Mitochondria would begin to accumulate calcium at maximal rate in an attempt to buffer the cytosolic calcium. With a continuous influx of calcium from the extracellular fluid, this would inevitably lead to a massive accumulation of calcium, causing mitochondrial damage.

Under these conditions ATP production would not be able to compete effectively for respiratory energy and the situation would be made worse by the fact that intracellular magnesium levels continuously decrease on

reperfusion, so favouring the accumulation of calcium. As a consequence of this series of events, little or no ATP will be produced during post-ischaemic reperfusion. Figure 6.2 shows an inverse correlation between mitochondrial calcium content and ATP production.

The excessive accumulation of calcium causes disruption of the normal mitochondrial structure with irreversible mitochondrial damage. Under these circumstances, reperfusion does not result in a recovery of myocardial function but, on the contrary, will eventually lead to cell death.

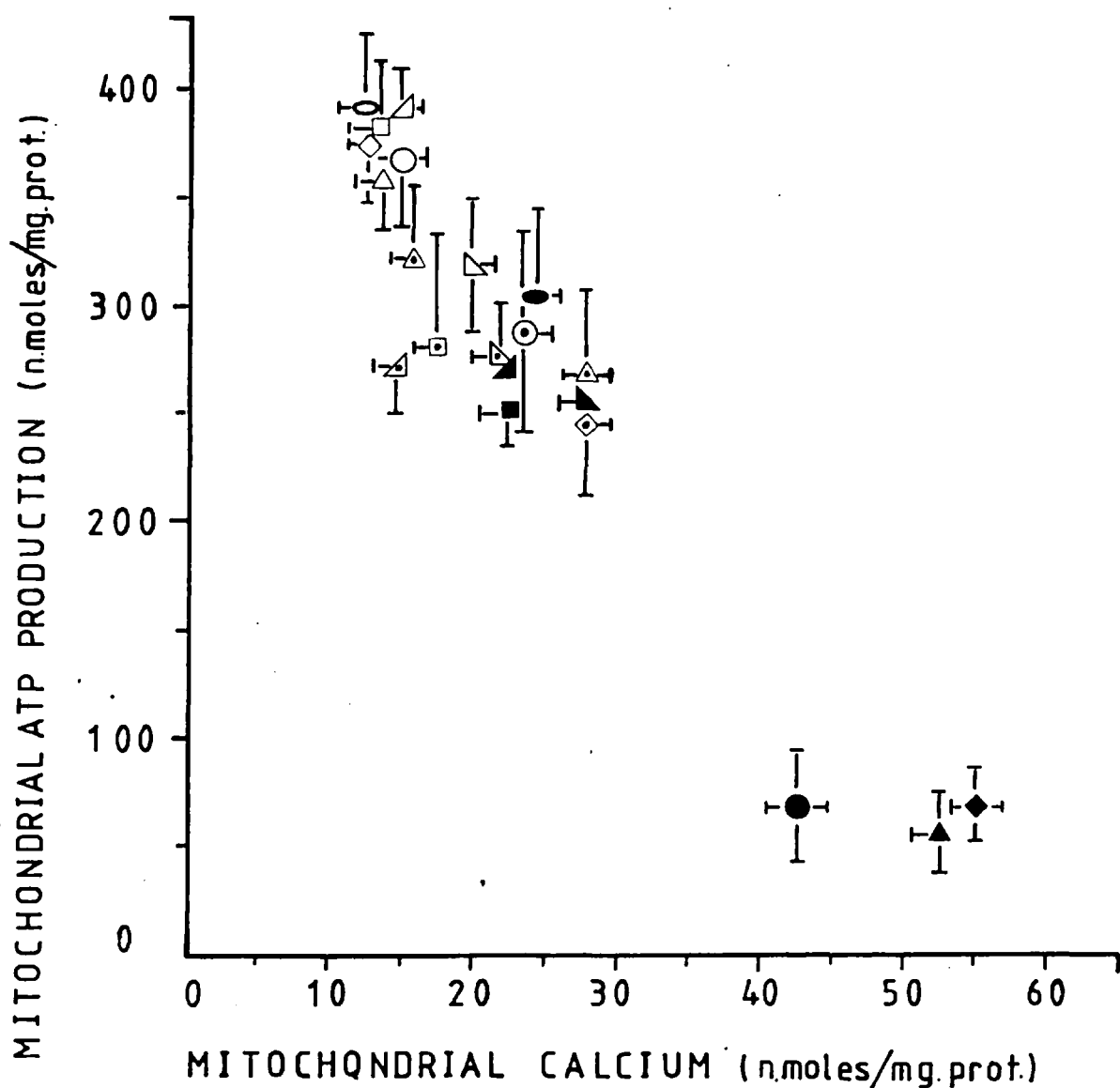


FIGURE 6.2 RELATIONSHIP BETWEEN MITOCHONDRIAL CALCIUM CONTENT AND MITOCHONDRIAL ATP PRODUCTION AFTER DIFFERENT CONDITIONS OF PERFUSION. Mitochondria were isolated in Medium II (Chapter 3). Results are expressed as the mean $\pm$ S.E.

○ 120' aerobic perfusion	Control (Chapter 3)	△ 120' aerobic perfusion	Group A (Chapter 4)
⊙ 90' total ischaemia		△ 90' 1 ml/min ischaemia	
● 90' total ischaemia + 30' reperfusion		▲ 90' 1 ml/min ischaemia + 30' reperfusion	
□ 120' aerobic perfusion	Verapamil Group (Chapter 3)	△ 120' aerobic perfusion	Group B (Chapter 4)
⊠ 90' total ischaemia		△ 90' 1 ml/min ischaemia	
■ 90' total ischaemia + 30' reperfusion		▲ 90' 1 ml/min ischaemia + 30' reperfusion	
○ 120' aerobic perfusion	Nifedipine Group (Chapter 3)	△ 120' aerobic perfusion	Group C (Chapter 4)
⊙ 90' total ischaemia		△ 90' 1 ml/min ischaemia	
● 90' total ischaemia + 30' reperfusion		▲ 90' 1 ml/min ischaemia + 30' reperfusion	

C. PROTECTION OF THE ISCHAEMIC AND REPERFUSED MYOCARDIUM

(a) General conclusions on the effects of verapamil, nifedipine and propranolol

The results of Chapters 3 and 4 showed that the pre-treatment of the rabbits with two distinctly different types of substances - Ca^{2+} antagonists (verapamil and nifedipine) and a beta adrenoceptor blocking drug (propranolol) - was able to reduce some of the damage caused by prolonged ischaemia and subsequent reperfusion in the isolated Langendorff perfused rabbit heart.

Evidence for this conclusion included improved recovery of mechanical function during reperfusion, maintenance of tissue stores of ATP and CP, maintenance of near-normal oxidative-phosphorylating and ATP-generating capacity of the mitochondria and absence of tissue and mitochondrial calcium overload during reperfusion.

Propranolol reduced ischaemic and reperfusion damage not only when given to the animals before death, but also when given to the isolated hearts at the onset of the ischaemic period. When administered during reperfusion, propranolol failed to modify the exacerbation of ischaemic damage caused by the readmission of coronary flow (Chapter 4).

(b) Effects of verapamil and nifedipine

Substances which inhibit the slow calcium channel may be useful in life to protect ischaemic or hypoxic myocardium by affecting the functioning of cardiac and smooth muscle cells and of the cells of the pacemaker, nodal and conducting tissue (Nayler and Poole-Wilson, 1981).

In the experiments described in Chapter 3, an isolated heart preparation, stimulated at constant rate, and a model of no flow ischaemia have been used. The effects of verapamil and nifedipine on coronary and systemic vasculature and on the cell of pacemaker and conducting tissue during the ischaemic episode, therefore, were completely eliminated. The protective effect which we found during ischaemia resulted from a direct action on the heart muscle.

It is well established that, when added to isolated or denervated heart muscle, verapamil and nifedipine are negatively inotropic and exert a cardioplegic effect on the myocardium (Magee et al., 1978; Nayler, 1980). The most likely explanation for the negative inotropic effect of these drugs on isolated heart muscle is that they reduce the inward calcium movements during the plateau phase of the action potential.

The negative inotropic effect of verapamil and nifedipine can explain the protection which they exerted in our experiments on the ischaemic and reperfused myocardium. A reduced or abolished contractile activity of the myocardial cell decreases the rate of ATP hydrolysis. Thus sufficient ATP may remain available during ischaemia to maintain membrane ultrastructure and intracellular Ca^{2+} homeostasis.

This mechanism requires the presence of the slow calcium channel inhibitors before or at the time of the onset of ischaemia. As soon as the cardiac cell becomes ischaemic the slow channels are deactivated by the accumulation of protons and developed tension starts to decline (Cobbe and Poole-Wilson, 1979). Evidence in favour of this hypothesis

has been obtained in experiments in which verapamil was introduced after developed tension had fallen during hypoxia (Nayler et al., 1978). These authors reported that the rise of resting tension was not prevented. This is in contrast to the delay in the rise of resting tension observed by the same authors when verapamil was introduced at the start of the hypoxic episode (Nayler et al., 1978).

In our experiments, verapamil and nifedipine have also been shown to prevent the massive overloading with calcium which occurred on reperfusion after 90 minutes of total ischaemia. Since calcium overload is complex and is not necessarily dependent on entry of calcium through the slow channels, verapamil and nifedipine may not directly prevent overload by impeding slow channel transport. Evidence in favour of this hypothesis has been obtained in experiments in which verapamil was given during post-ischaemic reperfusion (Bourdillon and Poole-Wilson, 1980; Watts et al., 1980). Under these circumstances it did not have the same beneficial effects which occurred when given before the onset of ischaemia.

We think that in our experiments the positive effects of verapamil and nifedipine during reperfusion were indirect, depending again on their ability to spare ATP during ischaemia.

If sufficient ATP remains available during ischaemia to maintain membrane ultrastructure and Ca^{2+} homeostasis, readmission of flow does not result in a massive influx of calcium into the cell. Figure 6.3 shows that an inverse relation existed between the ATP content at the end of the ischaemic period and tissue calcium accumulation after reperfusion. The greater the depletion in ATP content, the greater was

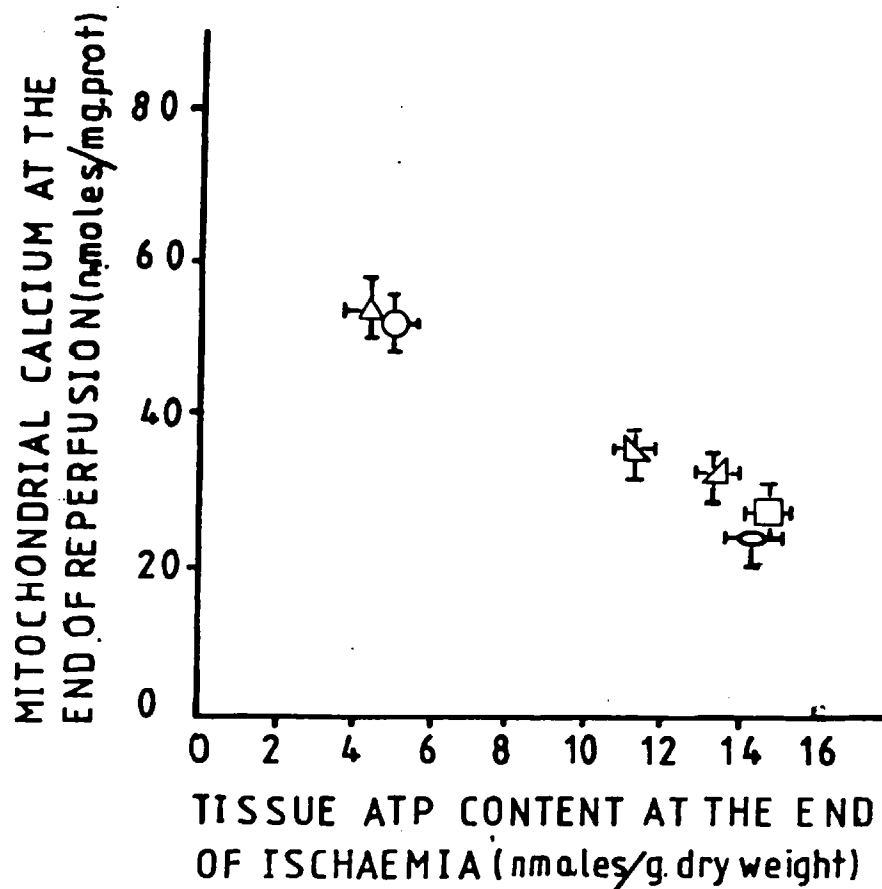


FIGURE 6.3 RELATIONSHIP BETWEEN TISSUE ATP CONTENT AT THE END OF THE ISCHAEMIC PERIOD AND MITOCHONDRIAL CALCIUM CONTENT AFTER 30 min OF POST-ISCHAEMIC REPERFUSION.

The results are expressed as mean $\pm$ S.E.

- No flow ischaemia: Control untreated group (Chapter 3).
- No flow ischaemia: Verapamil treated group (Chapter 3).
- ◐ No flow ischaemia: Nifedipine treated group (Chapter 3).
- △ 1 ml/min ischaemia: Control group (Chapter 4).
- ▵ 1 ml/min ischaemia: Propranolol treated group B (Chapter 4).
- ◁ 1 ml/min ischaemia: Group receiving propranolol in the perfusate during ischaemia and reperfusion. Group C (Chapter 4).

the reperfusion-induced tissue calcium accumulation. It is likely that ATP is essential for the maintenance of the permeability barrier of the sarcolemma. The molecular mechanism involved, however, is not known. In the presence of normal or slightly elevated cytosolic calcium concentration, mitochondria can use their restored membrane potential to phosphorylate ADP, thus producing ATP. Under these circumstances reperfusion results in a prompt recovery of mechanical function. In Chapter 2 a positive correlation between ATP content after reperfusion and recovery of developed tension has been shown.

It is difficult to establish the relevance of this mechanism of protection under in vivo conditions. The introduction of calcium antagonists into the circulation of intact animals has not always resulted in a decreased force of contraction, and may even result in an increased contractility. The reason for this is that these drugs dilate the peripheral vasculature. The fall in the intravascular pressure is sufficient to stimulate the atrial baroreceptors, leading to a stimulation of the sympathetic system and a release of catecholamines. As calcium antagonists lack β adrenoceptor blocking activity, catecholamines will stimulate the slow calcium current. Therefore the negative inotropic effect of calcium antagonists will be masked by the positive inotropic effect of catecholamines.

(c) Effect of propranolol

As suggested in Chapter 4, although propranolol has different pharmacological properties to verapamil and nifedipine, its ability

to reduce ischaemic and reperfusion damage may also involve a reduced rate of depletion of ATP reserves during ischaemia.

Administration to the animals before death is not essential for propranolol to have its effect. Propranolol reduced ischaemic and reperfusion damage also when delivered to the isolated hearts at the onset of ischaemia. The presence of propranolol during the ischaemic period, however, was essential for its protective action during reperfusion, since it had almost no effect when administered only during reperfusion.

From the results described in Chapter 4 it would appear that the mechanism involved in propranolol protection is related to its ability to block the effects of noradrenaline during the early phase of ischaemia. The release of endogenous noradrenaline induced by the ischaemic episode, leads to a maintenance of some contractile activity of the myocardium during the first minutes of coronary flow reduction. This, in turn, results in a rapid depletion of ATP and CP stores of the ischaemic cells.

In our experiments, propranolol, by blocking the effects of noradrenaline, induced a more rapid decline in developed tension during ischaemia, thus sparing ATP for the maintenance of membrane structure and intracellular ionic homeostasis. Under these circumstances reperfusion resulted in a recovery of mechanical and metabolic function.

D. CLINICAL RELEVANCE

Although caution must be taken in extrapolating experimental data to the clinical environment, the results reported in this thesis support the concept that reperfusion of the ischaemic myocardium may accelerate tissue damage, depending on the duration and severity of the ischaemic episode.

This finding may be of practical importance considering the growing interest for coronary artery recanalisation in patients with transmural myocardial infarction by using spasmolytic agents or streptokinase. The likelihood that the ischaemic myocardial cells will recover their metabolic and functional capacities during reperfusion will depend on the rapidity of the recanalisation.

Verapamil, nifedipine and propranolol may reduce the ischaemic and reperfusion damage by a direct action on the myocardium. The mechanism involved in their protective effect is an ability to slow the rate of depletion of ATP reserves during ischaemia consequent to a reduction of myocardial work. It is difficult, however, to establish the relevance of this mechanism of protection in clinical practice, particularly for drugs such as verapamil and nifedipine. It will be recalled that the use of calcium antagonists in intact animals or in man has little or no effect on myocardial contractility. This is because the intrinsic negative effect of these drugs is masked by the positive inotropic effect of catecholamines which are released in response to the dilatation of the peripheral vasculature.

Propranolol has a negative inotropic effect not only in isolated

preparations but also in intact animals and in man. It is likely therefore to reduce ischaemic and reperfusion damage through this mechanism also in vivo. If this is so, the timing of administration seems to be of critical importance for propranolol to have its effect.

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Protective Effect of Pretreatment With Verapamil, Nifedipine and Propranolol on Mitochondrial Function in The Ischemic and Reperfused Myocardium

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To establish whether the prophylactic use of verapamil, nifedipine or propranolol protects heart muscle against the deleterious effects of global ischemia and reperfusion, rabbits were injected subcutaneously twice daily with 2.0 mg/kg of one of these drugs for 4 to 5 days. The hearts were then isolated, paced and either perfused aerobically, made totally ischemic for 90 minutes or made ischemic for 90 minutes and then reperfused. At the end of this time some of the hearts were assayed for adenosine triphosphate (ATP), creatine phosphate (CP) and calcium (Ca^{++}). Other hearts were homogenized, the mitochondria harvested and monitored for oxidative phosphorylating and ATP-generating capacity and Ca^{++} content. The effect of Ca^{++} on the ATP-generating capacity of cardiac mitochondria was also determined.

Hearts that were made ischemic gained Ca^{++} . The endogenous stores of ATP and CP were depleted; the mitochondria accumulated Ca^{++} and the oxidative phosphorylating activity (respiratory control index and oxygen quotient) was impaired. During reperfusion, tissue and mitochondrial Ca^{++} was substantially increased, the capacity of the mitochondria to use O_2 for state III respiration was further impaired and the ATP-generating capacity reduced. Resting tension increased and there was only a small recovery of active tension generation.

Hearts from the rabbits treated with propranolol, verapamil or nifedipine were protected against ischemia, and ischemia with reperfusion-induced decline in the ATP-generating and O_2 -utilizing capacity of the mitochondria. There was also a less marked increase in tissue and mitochondrial Ca^{++} and the systolic tension-generating capacity of the hearts was better maintained.

Ischemia has two important consequences—a reduction in the supply of oxygen and substrate and a retention of the products of metabolism.¹ The resultant functional, metabolic and morphologic changes are complex, and if the supply of oxygen is not rapidly restored cell death and necrosis will result.²⁻⁵ Procedures that might reduce the severity of ischemia-induced damage include administration of agents that either facilitate better perfusion (hyaluronidase^{6,7}) and stabilize membrane ultrastructure (glucocorticosteroids⁸) or improve the balance between energy production and utilization (beta adrenergic receptor antagonists⁹ and inhibitors of slow channel calcium (Ca^{++})-transport such as verapamil^{10,11} and nifedipine.^{12,13}

The maintenance of mitochondrial function is of fundamental importance.¹⁴ Previously, we have shown that the prophylactic use of either verapamil¹⁵ or propranolol¹⁶ protects cardiac mitochondria against some of the damage caused by hypoxia (inadequate oxygenation in the presence of maintained coronary arterial flow). The purpose of this study was to determine whether verapamil, nifedipine or propranolol can be used prophylactically to protect cardiac mitochondria against deterior-

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ration in function caused by global ischemia and reperfusion.¹⁴

Methods

Animals: Adult male New Zealand white rabbits (2.5 to 3.0 kg body weight) were used. The control rabbits received 1 mg/kg of 0.9 percent sodium chloride solution subcutaneously twice daily, and the other rabbits 2.0 mg/kg of either racemate propranolol, verapamil or nifedipine twice daily for the same period of time. This regimen provided plasma levels comparable with those used clinically.^{15,16} Treatment was maintained for 4 to 5 days. The rabbits used for a particular experiment were chosen at random 2 hours after the last injection.

Perfusion of hearts: The rabbits received a standard diet of water and a Short Gamage diet. When required, each rabbit was stunned by a blow on the head. The heart was rapidly excised and perfused by the nonrecirculating Langendorff technique.¹⁵⁻¹⁷ Each heart was paced at 180 beats/min.^{15,16} Resting and developed systolic tension was monitored as previously described.¹⁷

After 20 to 30 minutes of aerobic perfusion (Krebs-Henseleit buffer solution gassed with 95 percent O₂ + 5 percent CO₂) the hearts were randomly separated into three groups (Fig. 1). In one group perfusion was continued as described for another 120 minutes. The second group (Fig. 1) were made totally ischemic for 90 minutes; the remainder were made totally ischemic for 90 minutes and then reperfused for another 30 minutes. Left ventricular wall temperature was maintained at 35 to 36°C, irrespective of coronary flow.

After the required period of perfusion, ischemia and reperfusion, the hearts were either assayed for adenosine triphosphate (ATP), creatine phosphate (CP) and Ca⁺⁺ or the mitochondria were harvested^{15,16} and assayed for O₂ consumption ATP generation and Ca⁺⁺ content (Fig. 1).

Mitochondrial studies: 1. *Oxidative phosphorylation:* The mitochondria were isolated by differential centrifugation^{15,16} using either the ethylenediaminetetraacetic acid (EDTA)-containing medium described by Sordahl et al.¹⁸ or the EDTA- and albumin-free medium described by Peng et al.¹⁹ The composition of the pellets was checked as previously described.^{16,16} Function was assessed in terms of *oxygen quotient* (n atoms O₂ used per milligram mitochondrial protein per minute in response to the addition of adenosine diphosphate [ADP]—that is, state III respiration); *RCI* (respiratory control index) (the ratio of O₂ used in the presence of ADP to that used after conversion of ADP to ATP), and the *ADP/O₂ ratio* (n moles ADP used/n atoms O₂ consumed).

O₂ consumption was monitored polarographically at 25°C using a Gilson oxygraph and a Clark O₂ electrode.¹⁶ The incubation medium contained 250 mM sucrose, 50 mM Tris, 3.0 mM Tris-glutamate (or succinate), 3.0 mM potassium dihydrogen phosphate and 0.25 mM ADP, pH 7.3 with or without 0.5 mM EDTA as indicated. Protein was added to provide a final concentration of 1.25 mg of mitochondrial protein per milliliter of reaction mixture, protein being determined by the method of Bradford,²⁰ standardized against crystalline bovine albumin.

2. *ATP generation and Ca⁺⁺ content:* The mitochondria for these studies had to be extracted under conditions that favored retaining of the endogenous Ca⁺⁺. The EDTA-free albumin-free extraction medium described by Peng et al.¹⁹ was therefore used, with the addition of 5 μM of ruthenium red, to prevent the mitochondria from exchanging Ca⁺⁺ with the extraction medium. ATP-generating capacity was monitored by adding freshly isolated mitochondria to a reaction medium containing 250.0 mM of sucrose, 12.5 mM of Tris-HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic

acid), pH 7.2; 3.0 mM of Tris-glutamate and 3.0 mM of potassium dihydrogen phosphate. Mitochondria were added to provide a final concentration of 2.5 mg protein/ml, and the reactions, which were performed at 25°C, started by adding ADP to provide a final concentration of 2.5 mM. Fifteen, 30, 45, 60 and 120 seconds later 50 μliter samples were removed from the reaction vessel, either added to 10 percent perchloric acid or boiled, and then assayed for ATP by measuring the change in absorbance at 340 nm caused by the reduction of nicotinamide-adenine dinucleotide phosphate (NADP) in a coupled system containing glucose, hexokinase and glucose-6-phosphate dehydrogenase.^{15,21}

Mitochondrial Ca⁺⁺ was assayed spectrophotometrically using digests of the pellets in an atomic absorption spectrometer (model 306, Perkin-Elmer, Buckinghamshire, United Kingdom) with limestone (CaCO₃) as the standard. Lanthanum trichloride (LaCl₃) was added to provide a final concentration of 0.1 percent.

Effect of Ca²⁺ on mitochondrial ATP production: To establish whether Ca⁺⁺ affected the ATP-generating capacity of cardiac mitochondria, mitochondria were harvested from freshly excised hearts as described by Sordahl et al.¹⁸ The mitochondria were added to a reaction medium containing 250 mmol sucrose, 10 mM of Tris buffer, pH 7.2, 3 mM of Tris glutamate, 3 mM of potassium dihydrogen phosphate and 10 mM of nitrilotriacetic acid (NTA) (added as a Ca⁺⁺-buffer). Calcium was added as calcium chloride (CaCl₂) to provide a free Ca⁺⁺ of between 0 and 150 μM as required; this amount of CaCl₂ was needed to provide the required free Ca⁺⁺ concentration being calculated using an ion-complexes in solution computer program and available stability constants.²² Mitochondrial protein was added to provide a final concentration

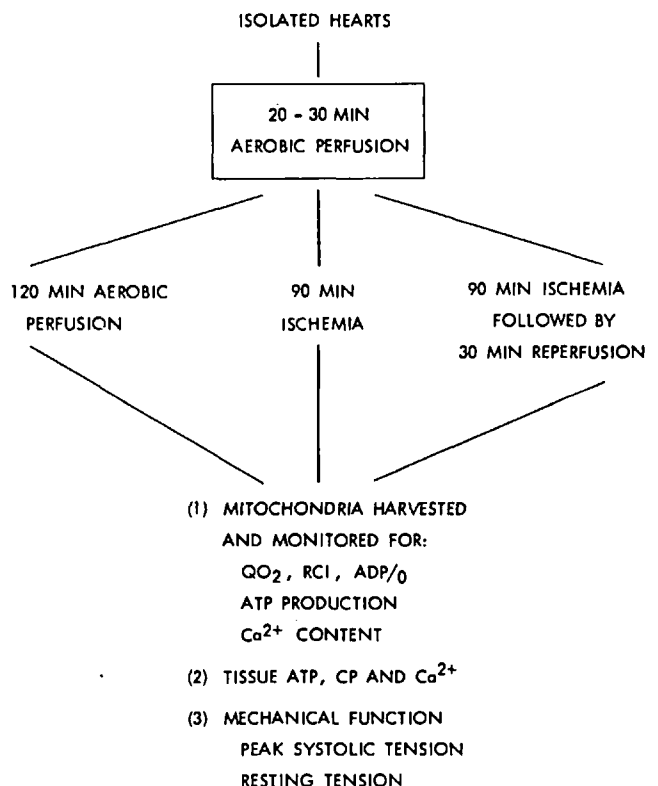


FIGURE 1. Schematic representation of experimental schedule. ADP/O = ratio of n moles adenosine diphosphate used to n atoms of oxygen consumed; ATP = adenosine triphosphate; CP = creatine phosphate; QO₂ = oxygen quotient; RCI = respiratory control index.

of 2.5 mg protein/ml. The reaction was performed at 25° C and was started by adding ADP to provide a final concentration of 2.5 μ M. Three minutes later (and hence at a time when state III respiration had stopped)²⁰ samples were taken, mixed with 10 percent perchloric acid or boiled, and assayed for ATP.²¹ In some experiments ruthenium red was added to the incubation medium to provide a final concentration of 5 μ M.

Tissue ATP, CP and Ca⁺⁺: The perfusion of hearts in which ATP and CP levels were to be measured was terminated by freeze clamping between large precooled stainless steel tongs.²³ Approximately 200 mg of the frozen muscle was pulverized and assayed for ATP and CP, as previously described. Tissue Ca⁺⁺ was measured by atomic absorption spectrometry as already described, using nitric acid (HNO₃) digests.

Statistical analysis: Results are expressed as mean $\pm$ standard error of *n* experiments. Tests of significance were made using either Student's *t* test or the paired *t* test as appropriate, taking *p* = 0.05 as the limit of significance.

Reagents: Analytic reagent grade chemicals were used. Propranolol, verapamil and nifedipine were used in the racemate form, and the nifedipine was not exposed to light. Propranolol (as the hydrochloride) was obtained from Imperial Chemical Industries, Macclesfield, England; verapamil (as the hydrochloride salt) from Knoll A.G., Ludwigshafen, Germany; and nifedipine (as Bay 1040) from Bayer, A.G., Germany.

Results

Oxidative phosphorylation studies: 1. Control aerobic perfusions (Table I): Irrespective of whether the mitochondria were harvested in the absence or presence (results not

shown) of EDTA, the respiratory control index (RCI), QO₂ and ADP/O₂ values obtained after 2 hours of aerobic perfusion were not significantly different from those exhibited by mitochondria that had been isolated after the initial 20 to 30 minutes of perfusion (Fig. 1). The RCI, QO₂ and ADP/O₂ values of mitochondria isolated in the absence of EDTA were always lower than those isolated in the presence of EDTA. However, irrespective of which extraction medium was used the RCI (*p* < 0.05) and QO₂ (*p* < 0.05) values obtained for mitochondria harvested from freshly excised nonperfused hearts were higher than those obtained for the mitochondria of the aerobically perfused hearts, although the yield was the same (12.6 $\pm$ 0.8 mg/g wet weight).

Pretreating the rabbits with verapamil, nifedipine or propranolol made no difference to the oxidative phosphorylating activity of the mitochondria irrespective of whether the hearts were perfused before the mitochondria were extracted and irrespective of which extraction medium was used. Verapamil, nifedipine and propranolol had no effect when added directly to the freshly isolated mitochondria.

2. Ischemia and reperfusion (Table I): When hearts from untreated rabbits were made totally ischemic for 90 minutes the yield of mitochondria was unchanged (*p* < 0.8) but QO₂, RCI and ADP/O₂ were reduced. Reperfusion after 90 minutes of ischemia resulted in a further decline in RCI and QO₂. These results relate to mitochondria extracted in the absence of EDTA and incubated in the presence of glutamate. Similar results were obtained if succinate was used as a substrate and if the extraction medium contained EDTA (results not shown).

Pretreatment with verapamil, nifedipine or propranolol protected the mitochondria against the deterioration in function caused by 90 minutes of ischemia and against the exacerbation of that damage during reperfusion. This protective effect was observed irrespective of the presence or absence of EDTA in the extraction medium and irrespective of whether glutamate or succinate was used as substrate.

TABLE I

Effects of Ischemia and Reperfusion on Oxidative Phosphorylation

Treatment	Perfusion		
	Aerobic (120 min)	Ischemic (90 min)	Ischemia (90 min) + Reperfusion (30 min)
RCI			
Saline control	5.7 $\pm$ 0.6	3.4 $\pm$ 0.2	1.8 $\pm$ 0.3
Verapamil	4.6 $\pm$ 0.5	4.2 $\pm$ 0.3 [†]	4.2 $\pm$ 0.5*
Nifedipine	5.2 $\pm$ 0.6	4.6 $\pm$ 0.6 [†]	4.4 $\pm$ 0.6*
Propranolol	5.9 $\pm$ 0.6	5.6 $\pm$ 0.2*	5.2 $\pm$ 0.5*
QO ₂			
Saline control	192 $\pm$ 16	148 $\pm$ 12	62.5 $\pm$ 0.3
Verapamil	190 $\pm$ 32	171 $\pm$ 25 [†]	162 $\pm$ 12*
Nifedipine	196 $\pm$ 23	181 $\pm$ 12*	160 $\pm$ 12*
Propranolol	180 $\pm$ 13	172 $\pm$ 15 [†]	158 $\pm$ 17*
ADP/O ₂			
Saline control	2.3 $\pm$ 0.2	1.9 $\pm$ 0.1	1.5 $\pm$ 0.3
Verapamil	2.4 $\pm$ 0.1	2.5 $\pm$ 0.1*	2.5 $\pm$ 0.3*
Nifedipine	2.4 $\pm$ 0.1	2.4 $\pm$ 0.3*	2.5 $\pm$ 0.2*
Propranolol	2.5 $\pm$ 0.1	2.6 $\pm$ 0.3*	2.4 $\pm$ 0.3*

* *p* < 0.001, relate to the significance of the difference between the results obtained with mitochondria from the hearts of the control rabbits treated with saline solution and those obtained for the drug-treated series. Results are presented as mean $\pm$ standard error of the mean of six separate experiments; [†] *p* < 0.01.

ADP/O₂ = adenosine diphosphate/oxygen; QO₂ = oxygen quotient; RCI = respiratory control index.

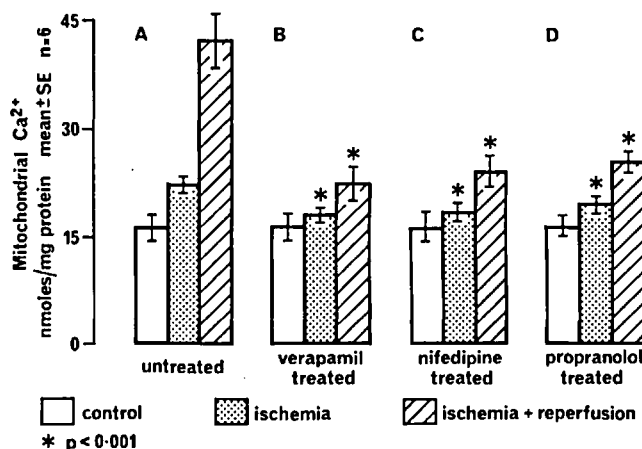


FIGURE 2. Effect of twice daily injections of 2.0 mg/kg verapamil, nifedipine or propranolol on ischemia and ischemia plus reperfusion-induced changes in mitochondrial Ca⁺⁺. The mitochondria were isolated in the absence of a chelating agent and in the presence of micromolar amounts of ruthenium red to ensure that they retained their endogenous Ca⁺⁺ during extraction. *n* = number of separate perfusions; *p* = probability that the difference between the results obtained for the untreated and the relative drug-treated series was significant; SE = standard error of the mean. Control refers to mitochondria harvested from hearts that had been perfused aerobically for 120 minutes. The hearts were all paced (see text).

Mitochondrial Ca^{++} (Fig. 2): Control studies showed that 2 hours of aerobic perfusion had no effect ($p < 0.8$) on mitochondrial calcium. Mitochondria gained some Ca^{++} ($p < 0.01$) during 90 minutes of ischemia and reperfusion caused a much larger increase ($p < 0.001$).

Although pretreatment with verapamil, nifedipine or propranolol did not effect the calcium content of mitochondria isolated from aerobically perfused hearts, such pretreatment reduced the gain in mitochondrial calcium caused by 90 minutes of ischemia and the exacerbation of that gain during reperfusion.

Mitochondrial ATP production (Fig. 3): ATP production was monitored using mitochondria obtained from the aerobic and ischemic reperfused hearts. The mitochondria were harvested in the medium that lacked EDTA and contained ruthenium red, so that they would retain their Ca^{++} . Pretreatment with verapamil, nifedipine or propranolol failed to alter the rate at which the mitochondria of the aerobically perfused hearts converted ADP to ATP. Also, mitochondria from the untreated ischemic and reperfused hearts converted ADP to ATP relatively slowly ($p < 0.001$).

Pretreating the rabbits with verapamil, nifedipine or propranolol resulted in the maintenance of a relatively fast rate of ATP production ($p < 0.001$) even after 90 minutes of ischemia and another 30 minutes of reperfusion. When these drugs were added directly to the incubation medium to provide a final concentration of $15 \mu\text{M}$ none of them altered the rate at which the mitochondria converted ADP to ATP (results not shown).

Tissue Ca^{++} , ATP and CP reserves (Fig. 4): Although 90 minutes of ischemia caused only a small gain in tissue Ca^{++} ($p < 0.05$), reperfusion resulted in a large gain ($p < 0.001$). Pretreating the rabbits with verapamil, nifedipine or propranolol had no effect on the Ca^{++} content of the aerobically perfused hearts but it reduced ($p < 0.001$) the gain in tissue Ca^{++} caused by the episodes of ischemia and reperfusion.

Further evidence of the protective effect of pretreatment with verapamil, nifedipine or propranolol was obtained in the experiments in which the perfused hearts were frozen and

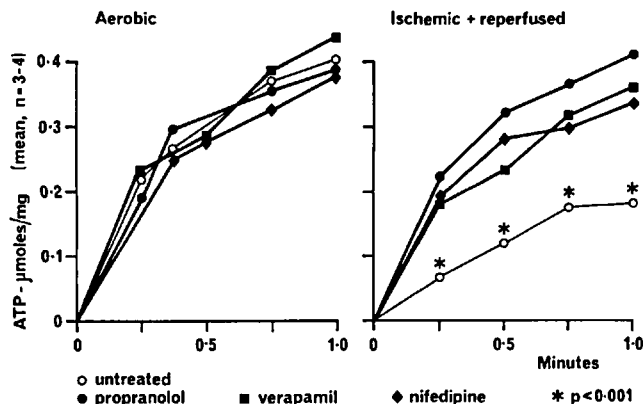


FIGURE 3. Effect of twice daily subcutaneous injections of 2.0 mg/kg verapamil, propranolol or nifedipine on the rate of ATP production by mitochondria that had been isolated after either 120 minutes of aerobic perfusion (aerobic) or 90 minutes of ischemia followed by 30 minutes of reperfusion. Each point is the mean of four separate experiments. The standard error bars have been omitted for purposes of clarity. p = probability that the results obtained for ischemic-reperfused drug-treated hearts differed significantly from those obtained for the untreated series. ATP production was monitored at the indicated points (see text for details of assay and reaction mixture). The mitochondria were isolated in the absence of a chelating agent so that they would retain their endogenous Ca^{++} .

TABLE II

Effect of Ischemia and Reperfusion on Myocardial Stores of Adenosine Triphosphate (ATP) and Creatine Phosphate (CP)

Treatment	Perfusion		
	Aerobic (120 min)	Ischemic (90 min)	Ischemia (90 min) + Reperfusion
ATP ($\mu\text{mol/g}$ dry weight)			
Control	18.4 ± 1.2	4.6 ± 0.8	4.2 ± 0.3
Verapamil	19.1 ± 1.8	$14.8 \pm 0.9^*$	$15.6 \pm 1.2^*$
Nifedipine	19.3 ± 1.1	$14.3 \pm 1.0^*$	$15.9 \pm 0.8^*$
Propranolol	18.8 ± 2.0	$13.6 \pm 1.2^*$	$15.2 \pm 1.3^*$
CP ($\mu\text{mol/g}$ dry weight)			
Control	32.8 ± 1.6	5.2 ± 0.6	6.6 ± 0.8
Verapamil	31.6 ± 2.7	$14.6 \pm 1.6^*$	$19.6 \pm 0.4^*$
Nifedipine	32.1 ± 1.8	$14.2 \pm 2.4^*$	$19.1 \pm 0.8^*$
Propranolol	30.2 ± 2.1	$13.3 \pm 1.6^*$	$18.6 \pm 2.2^*$

* $p < 0.001$.

Results are expressed as mean $\pm$ standard error of the means of six individual experiments. Tests of significance calculated with respect to the difference in test results from the control animals treated with saline solution (see text).

assayed for ATP and CP (Table II). As expected, ischemia and ischemia plus reperfusion depleted the endogenous stores of ATP and CP. Pretreating the rabbits with either verapamil, nifedipine or propranolol resulted in a significant ($p < 0.001$) preservation of the cardiac stores of ATP and CP in the ischemic hearts and those with ischemia plus reperfusion. Pretreatment with these drugs had no effect on the ATP and CP content of the aerobically perfused hearts.

Effect of Ca^{++} on mitochondrial ATP production (Fig. 5): The mitochondria used in these experiments were harvested in the EDTA-containing extraction medium (see Methods). The effect of Ca^{++} on ATP production was investigated over a free Ca^{++} range of 1 nM to $150 \mu\text{M}$, using NTA

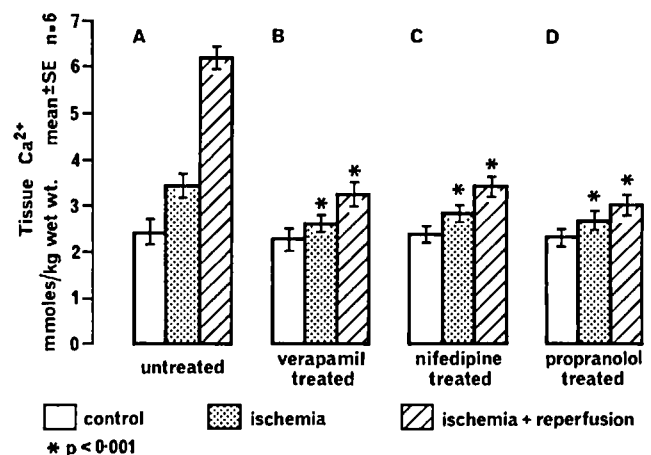


FIGURE 4. Effect of twice daily subcutaneous injections of 2.0 mg/kg of verapamil, nifedipine or propranolol on the Ca^{++} content of heart muscle after 120 minutes of aerobic perfusion (control), 90 minutes of ischemia or ischemia for 90 minutes followed by reperfusion. n = number of separate experiments made under the relevant conditions; p = probability that the Ca^{++} content of heart muscle from the untreated rabbits was significantly different from that of the relative treated rabbits; SE = standard error of the mean.

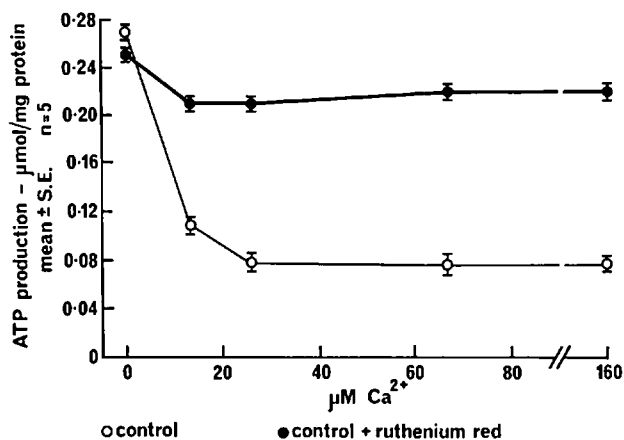
EFFECT OF Ca^{2+} ON MITOCHONDRIAL ATP PRODUCTION

FIGURE 5. Effect of Ca^{++} on mitochondrial ATP production. In some experiments ruthenium red was added to prevent the mitochondria from accumulating Ca^{++} from the isolation medium. The experiments were performed at 25° C using mitochondria obtained from untreated, non-perfused heart muscle. Nitrilotriacetic acid was used as a Ca^{++} buffer, to provide a concentration of free Ca^{++} ranging between 0 and 160 μmol . Abbreviations as in Figure 2.

as a Ca^{++} buffer. Increasing the free Ca^{++} slowed the rate of ATP production and this effect of Ca^{++} was blocked by ruthenium red.

Mechanical function studies (Tables III and IV): When paced, aerobically perfused hearts from the rabbits treated with propranolol had approximately the same peak systolic tension (in grams) (16.6 ± 5.0 , $n = 19$) as the control animals given saline solution (14.6 ± 3.4 , $n = 37$). By contrast the hearts of the rabbits treated with verapamil (9.4 ± 2.7 , $n = 16$) and nifedipine (10.6 ± 2.6 , $n = 14$) manifested less ($p < 0.01$) tension than the control animals treated with saline solution. Pretreatment with either propranolol, nifedipine or verapamil reduced the rise in resting tension that occurs during reperfusion and increased the percent recovery of peak systolic tension development.

Discussion

Myocardial Protective Effect

These results show that pretreatment with verapamil, nifedipine or propranolol protects the myocardium and

its mitochondria against some of the deterioration in function caused by prolonged ischemia and subsequent reperfusion. Mitochondrial respiration and ATP-generating capacity was better maintained, overloading with Ca^{++} was reduced, the endogenous stores of ATP and CP were better preserved and during reperfusion there was a greater percent recovery of systolic tension generation, with a smaller rise in resting tension. This evidence of protection agrees with that obtained in other studies^{9-13,24-28} in which the drugs have been given on a short-term basis. However, our method is distinctly different in that we gave the drugs for several days before excising the hearts and making them ischemic. There was no further addition of the drugs to the isolated hearts. Because we obtained clear evidence of protection under these conditions we can conclude that the protective effect of drugs like verapamil and nifedipine does not reside only in their ability to reduce "afterload."¹³ Possibly after 3 to 4 days of pretreatment the heart contains sufficient amounts of these drugs (or their metabolites) to allow it to survive 90 minutes of ischemia and reperfusion. Alternatively, but less likely, 3 to 4 days of treatment with these drugs may modify the myocardium in some way, making it more resistant to the effects of ischemia and reperfusion. However, if this is the case an improved endogenous supply of ATP and CP or an altered rate of mitochondrial respiration before the ischemia is apparently not involved.

Comparison of the various calcium antagonist drugs: In this study two distinctly different types of drugs were used—nifedipine and verapamil, which are " Ca^{++} antagonists"—and propranolol, a beta adrenergic receptor antagonist. The results obtained for verapamil and nifedipine closely resemble those described by Henry et al.¹² for nifedipine. These investigators added nifedipine directly to the coronary perfusion line of isolated rabbit hearts made partially ischemic under conditions of low flow; they observed a protective effect for nifedipine, including the absence of Ca^{++} overloading. This evidence of protection, and that described in this study, contrasts with the results recently described for diltiazem.²⁹ Diltiazem is usually classified as a " Ca^{++} antagonist,"³⁰ and Weishaar et al.²⁹ infused it into dogs 10 minutes after ligating several branches of the left anterior descending coronary artery.

TABLE III

Effect of Ischemia and Reperfusion on Resting Tension

Treatment	Perfusion		Ischemia (90 min) + Reperfusion
	Aerobic (120 min)	Ischemic (90 min)	
Control ($n = 17$)	0	1.7 ± 0.3	6.6 ± 0.7
Verapamil ($n = 10$)	0	$1.1 \pm 0.2^{\dagger}$	$3.5 \pm 0.5^*$
Nifedipine ($n = 10$)	0	$0.8 \pm 0.2^*$	$1.8 \pm 0.2^*$
Propranolol ($n = 14$)	0	$1.2 \pm 0.3^{\dagger}$	$1.3 \pm 0.1^*$

* $p < 0.001$; $^{\dagger} p < 0.01$.

Results are expressed as mean $\pm$ standard error of the mean of n experiments. Tests of significance calculated with respect to the difference in test results from the control animals treated with saline solution (see text).

TABLE IV

Percent Recovery of Developed Tension* During Reperfusion After 90 Minutes of Ischemia

Treatment	Hearts (n)	Recovery (%)
Control	11	24
Verapamil	7	72 [†]
Nifedipine	14	74 [†]
Propranolol	10	56 [†]

* Percent recovery calculated relative to the peak systolic tension that developed immediately before the start of the ischemic episode; $^{\dagger} p < 0.001$.

The hearts were reperfused with Krebs-Henseleit buffer solution at 37°C and gassed with 95 percent O_2 + 5 percent CO_2 as described in the text. Tests of significance calculated with respect to the difference in test results from the control animals treated with saline solution (see text).

Although these investigators found a preservation of the tissue stores of ATP, it was not accompanied by a preservation of mitochondrial function. There are several possible explanations for the difference between our results and those described by Weishaar et al.²⁹ First, different animal species were used. Second, damage caused by the global ischemia induced in our study may differ from that due to the regional ischemia induced by Weishaar et al.²⁹ Third, the Ca^{++} antagonist drugs are a heterogeneous group of compounds with other properties apart from their ability to impede the slow channel transport of Ca^{++} . The different drugs may therefore act differently, and different dose levels may be involved. Indeed it would be interesting to know the maximally effective dose for each of the compounds. Finally, and possibly of greatest importance, are the mode and time of administration of the drugs. In the study of Weishaar et al.²⁹ the infusion of diltiazem was not started until 10 minutes after occluding the branches of the left anterior descending coronary artery, whereas in our study pretreatment with nifedipine and verapamil had been started 4 to 5 days previously.

Mechanism of Protection

There is still no certain explanation as to why nifedipine, verapamil or propranolol should protect isolated heart muscle against the deleterious effects of ischemia and reperfusion.

Verapamil and nifedipine: In the case of these two agents we could envisage the sequence of events shown in Figure 6. These drugs inhibit slow channel transport³⁰ and therefore are negatively inotropic. Under these conditions the rate at which the ATP reserves are depleted during ischemia should be slowed, leaving ATP available for maintaining intracellular homeostasis, particularly with respect to Ca^{++} and Na^+ . Under these circumstances overloading of the mitochondria with Ca^{++} should be avoided, thereby possibly ensuring their functional survival.³¹⁻³³ Sufficient ATP might also remain available to ensure the continued functioning of the Na^+ , K^+ adenosine triphosphatase enzyme and an intracellular accumulation of Na^+ might therefore be avoided. If this happens then an enhanced rate of exchange between Na^+ and Ca^{++} might be avoided, particularly during reperfusion. This may explain why these drugs restrict the entry of Ca^{++} during reperfusion, for there is no evidence that the Ca^{++} entry that occurs during reperfusion is restricted to the slow channels.

Propranolol: Pretreatment with propranolol has no effect on the endogenous stores of ATP and CP or glycogen¹⁶; the protective effect described in our study

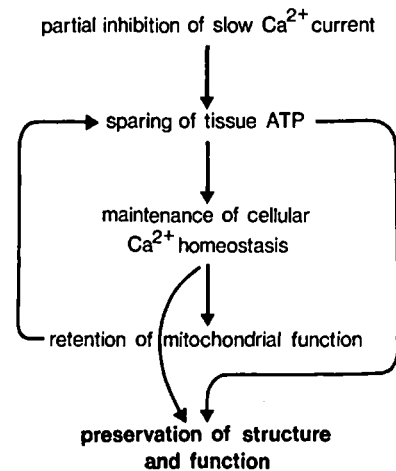


FIGURE 6. Schematic representation of the possible pathways involved in the protective effect of drugs inhibiting slow channel Ca^{++} transport (Ca^{++} antagonists).

cannot be explained therefore in terms of an improved endogenous supply of energy. Beta adrenergic blockade may be involved because under the dose regimen used in our study this persisted for at least 2 hours after excision of the heart.^{16,34} Endogenous catecholamines are released during the early stages of oxygen deprivation,³⁵ and because the catecholamines increase cardiac work they will accelerate the rate of depletion of the energy-rich phosphate reserves. Alternatively the "membrane-stabilizing" properties of the drug might be involved.

Implications: Our results show that *pretreatment* with propranolol, verapamil or nifedipine protects the myocardium against some of the mechanical and metabolic consequences of ischemia and reperfusion. Although these drugs differ in their pharmacologic properties (propranolol being a beta adrenergic receptor antagonist and nifedipine and verapamil inhibitors of slow channel Ca^{++} transport [Ca^{++} antagonists]), their ability to protect the ischemic and reperfused heart may involve a common mechanism—an ability to slow the rate of depletion of the ATP reserves. Under these conditions it is possible to envisage a sequence of events whereby intracellular homeostasis will be better maintained particularly with respect to Na^+ and Ca^{++} . Under these conditions we can anticipate that mitochondrial function might be preserved. The difference between the protection of mitochondrial function described in this study for certain " Ca^{++} antagonists" and the absence of a protective effect in other studies may indicate that the time of drug administration is of critical importance.

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