Myocardial lipolysis in the ischaemically-perfused rat heart.

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Award date:
1975

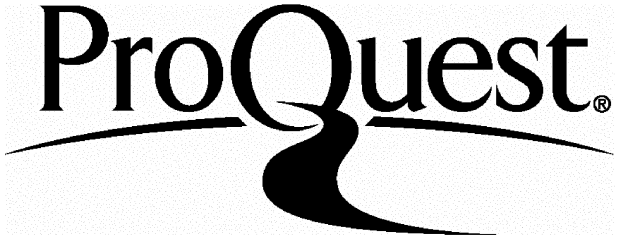
Awarding institution:
University of Bath

MYOCARDIAL LIPOLYSIS IN THE ISCHAEMICALLY - PERFUSED RAT HEART

Submitted by Roger William Brownsey for the degree of Ph.D. of the University of Bath.

1975

R. Brownsey



To Mum and Dad

Between the conception

And the creation

Between the emotion

And the response

Falls the shadow.

T.S.Eliot

Acknowledgments

I am greatly indebted to Dr.R.V.Brunt for advice, encouragement and criticism throughout the project. I would also like to thank Professor L.Broadbent and Professor A.H.Rose for the provision of facilities in the school of biological sciences and in the department of biochemistry. For financial support, I thank the Science Research Council.

The CASE award involved valuable collaboration with Pfizer Limited, which included two six-week periods of work in the laboratories at Sandwich. During these and other visits, I always received willing support and advice and I am particularly indebted to Dr. K.Blackburn, Mr. R.Burges, Dr. M.Morville, Mr. A.Higgins and Mr. S.Tickner.

In addition I would like to thank Dennis for prompting a valuable interest in G.L.C., Dr. B.Woodward for help with the electrocardiograms, my sister for undertaking the onerous duty of typing the manuscript and my wife for support in those dark moments.

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SUMMARY

The investigation was undertaken to study the effect of free fatty acids (FFA) on the performance of the ischaemic myocardium and the role of myocardial lipolysis in contributing to tissue FFA concentration.

An initial investigation using heart muscle extracts demonstrated cyclic-AMP stimulation of lipase hydrolysis of triglycerides but not monoglyceride. Subsequently, an investigation of the role of myocardial lipolysis in the ischaemically-perfused rat heart was begun.

The results demonstrated the deleterious effects on heart performance of FFA generated from myocardial triglyceride. Exogenous FFA had much less effect, arguing for a more potent effect or greater availability of endogenously-generated FFA. With hearts from normally-fed rats, adrenaline induced inotropic and chronotropic (first phase) responses. These were followed by marked (second phase) deteriorations in performance - decline in beatrate and developed tension, increases in resting tension and incidence of arrhythmias - which were proportional to the rates of lactate and FFA release. The deteriorations were greatly reduced by the abolition of the lipolytic component of the adrenaline challenge with nicotinic acid, at concentrations which did not inhibit the first phase responses. Reduction of FFA release also encouraged glucose oxidation.

With hearts from fat-fed rats (depending exclusively upon endogenous lipid as an energy source) performance was depressed whilst lipolysis was stimulated relative to the normally-fed series. In addition, the performance of these hearts declined more markedly after adrenaline - the high incidence of ventricular fibrillation (V.F.) being particularly marked. Nicotinic acid again offered protection - in particular, abolishing V.F.

In both series, adrenaline challenge increased the tissue FFA concentration and this was offset with nicotinic acid. In addition, adrenaline greatly stimulated the accumulation of FFA within the myocardium. The deteriorative effects on performance could not be ascribed solely to FFA since lactate was usually increased. However, several observations suggest that FFA are of primary significance and these factors are discussed.

INTRODUCTION

SECTION A The Mammalian Heart

1. The organ and its Function

(a) Double Circulation The heart is a constantly-acting muscular pump which drives blood to the lungs and body in the pulmonary and systemic circulations respectively. The pump is divided into two halves to provide this double circulation. The left side receives oxygenated blood from the lungs, delivering this to the systemic circulation; the right side receives deoxygenated blood from the body to deliver to the lungs (fig 1a). Each side of the heart is equipped with two chambers, the atria which receive blood are delicate compared to the muscular ventricles which drive blood out into the circulations.

The two sides of the heart must pump at an exactly-matched rate in order that blood should not accumulate in one circulation at the expense of the other. This balance is achieved despite a great difference in volume capacity and pressure develpment in the two circulations. The left ventricle is much more powerful (with thicker walls) than the right ventricle to enable it to develop the high pressure necessary to drive blood into the aorta. Left ventricular pressure rises to a peak of about 100mm mercury during contraction (systole) compared to about 25 mm mercury in the right ventricle. Of the total heart muscle, about two-thirds is represented by left ventricular muscle.

The pumping action of the heart is achieved not by contractions in a single phase as with most skeletal muscle but instead, a convoluted fibre arrangement is displayed (figure 1b). A wave of contraction spreads upwards from the apex of the ventricles forcing blood upwards and out through the arterial valves. Similar valves between auricles and ventricles oppose reverse flow of blood into the auricles. The sequence of contraction (systole) and relaxation (diastole) is described as the cardiac cycle, shown in figure 2.

(b) Coronary - Circulation The heart tissue, as any other highly active tissue, requires an extensive supply of blood to deliver substrates and remove products. The chamber blood contributes relatively little to the needs of the heart which are met, instead, by a discrete coronary circulation. The circulation begins with coronary arteries which branch from the aorta very near the aortic valve, thus delivering highly oxygenated blood to the myocardium. The circulation begins at the outer surface or subepicardial region from which the vessels branch into the mid- and subendocardial regions. An important aspect of the coronary circulation is the relationship between coronary flow and the phase of the cardiac cycle. The flow is restricted mainly to the diastolic period since during systole the increase in myocardial wall tension causes extensive occlusion of the coronary vessels. The occlusion is most extensive and of longest duration in the subendocardial region, figure 3. It can be seen that the supply of blood to the subendocardium is subject to two major limitations.

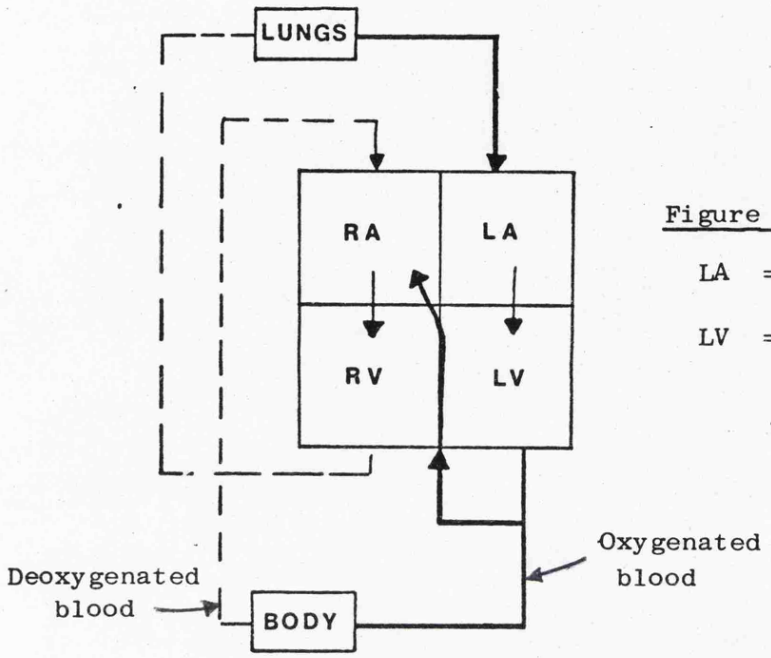


Figure 1a

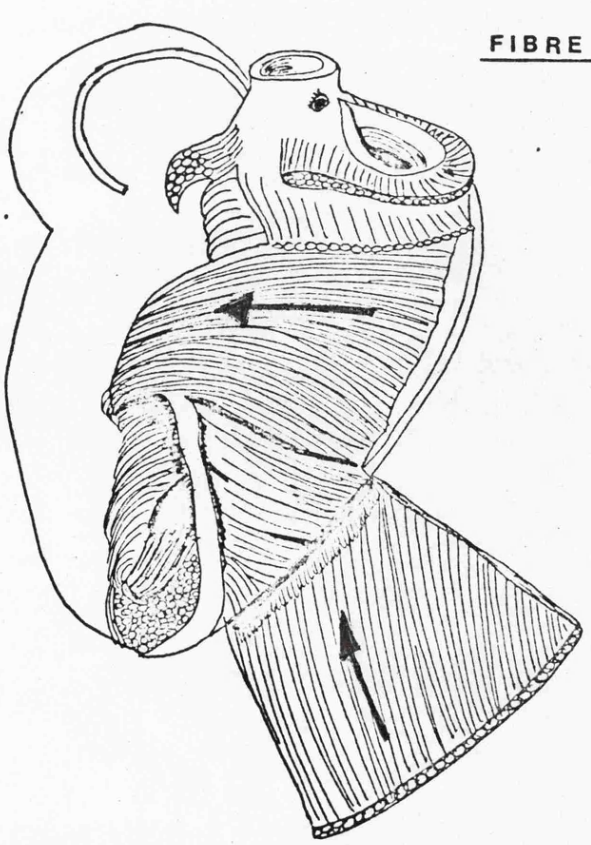
LA = left atrium

LV = left ventricle

Double circulation

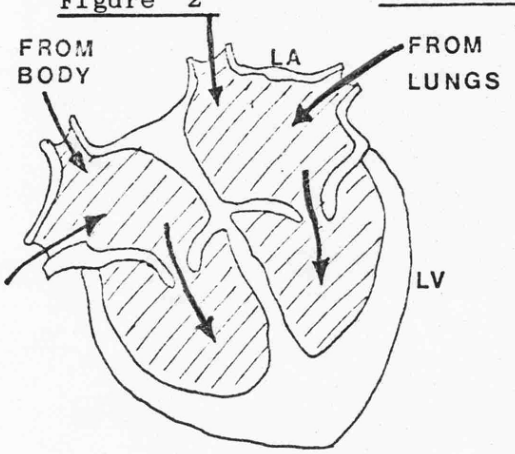
Figure 1b

FIBRE ARRANGEMENT

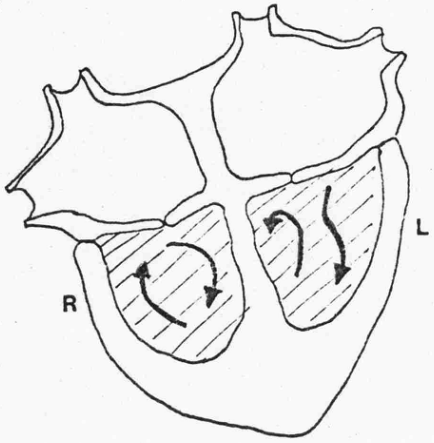


Adapted from Muir, 1971.

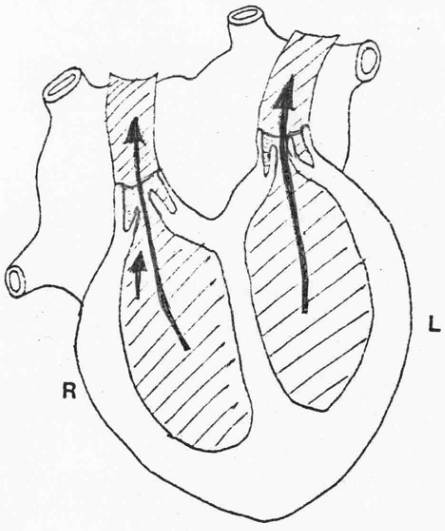
Figure 2 CARDIAC CYCLE



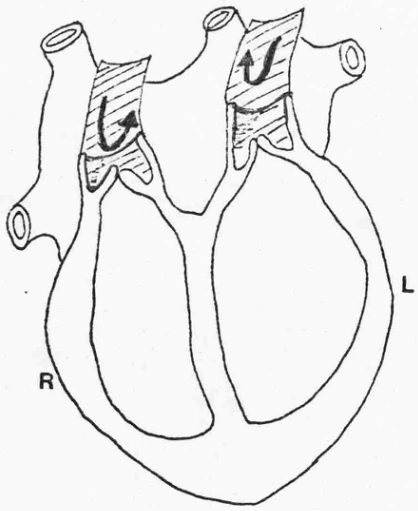
1. Atrial, ventricular filling



2. Vent. contraction begins



3. Vent. ejection



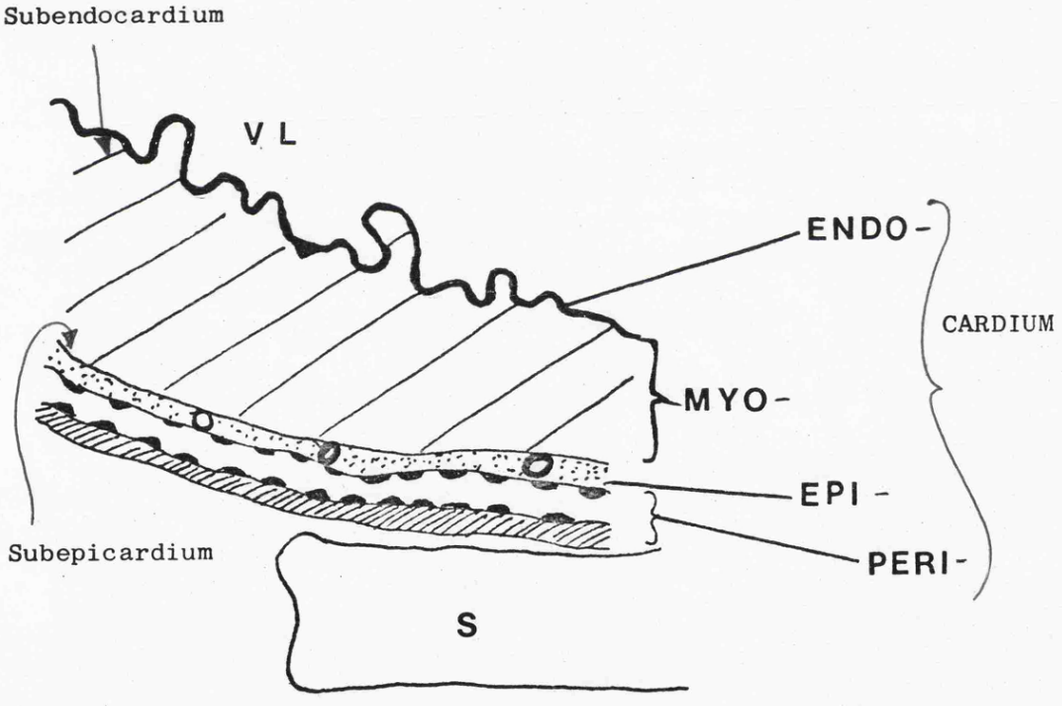
4. Vent. relaxation

Adapted from Selkurt, 1971.

Figure 3

CROSS-SECTION OF HEART WALL

VL = ventricular lumen
S = Sternum



Adapted from Muir, 1971.

These are (i) that the region is subject to the greatest degree of occlusion and (ii) that it is most distal from the point of initial (aortic) supply. The consequences of this situation will be discussed later.

The venous system of the myocardium conveys most of the deoxygenated blood to the right atrium via the coronary sinus although a small but significant drainage of blood occurs directly into the chambers through the Thebesian and other small veins.

Another feature of the coronary circulation is the relatively limited extent of cross-perfusion afforded by anastomoses. The effect is that in the event of occlusion of a major vessel, there is only a limited capacity for the deprived tissue to draw upon supply from other vessels. Improvement of cross-perfusion can occur with the development of collateral vessels but a particular stimulus is required to increase vascularisation. It can occur in response to coronary artery ligation (Charlier, 1972) and to physical training (Penpargkul and Scheuer, 1970).

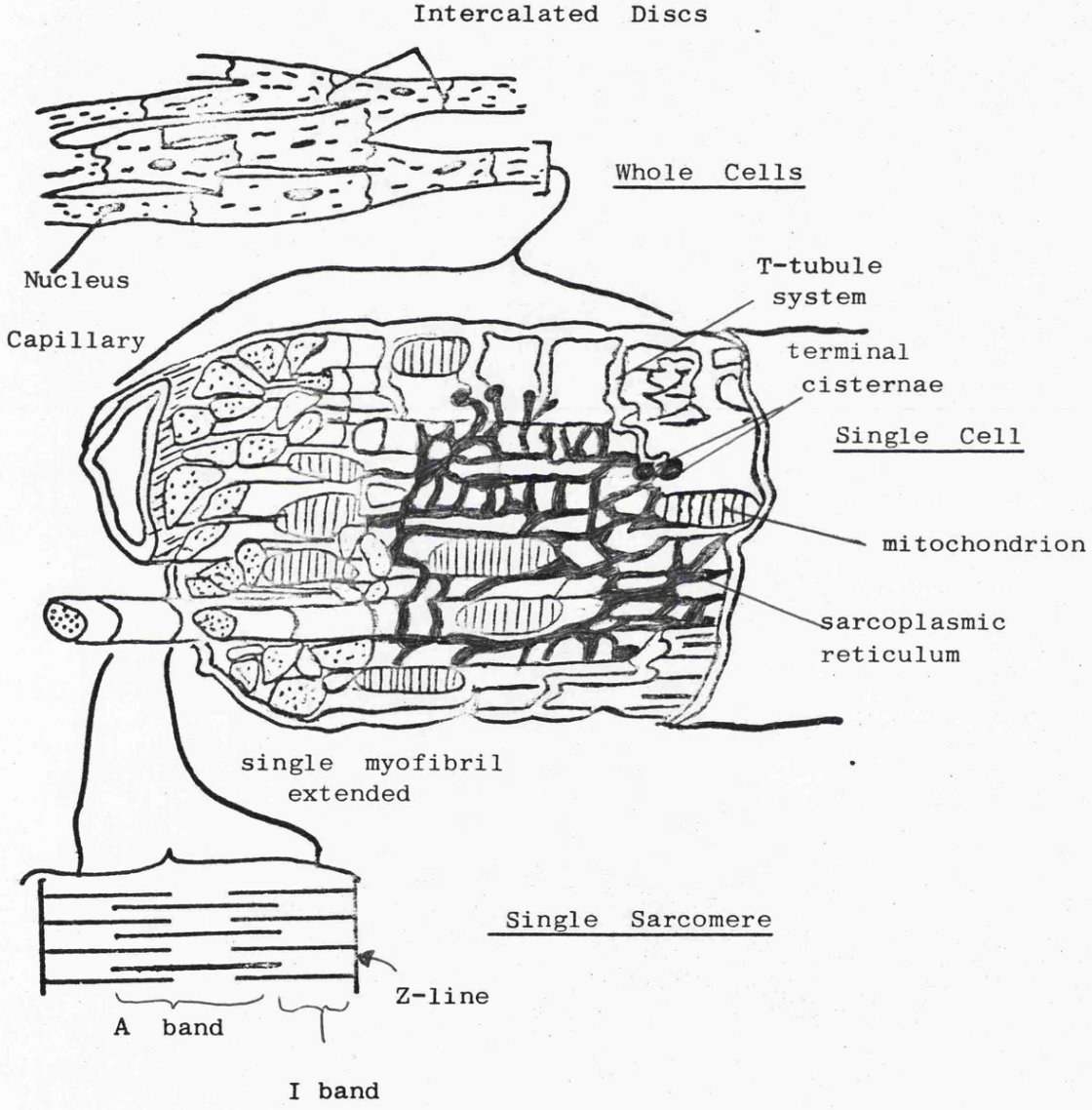
2. The heart as a muscle

(a) Ultrastructure The essential features of the ultrastructure of the myocardium are shown in figure 4. The myofibrils show the striated nature (as skeletal muscle) and the mechanism of contraction in the two types of muscle is essentially the same (Sonnenblick 1962, Huxley 1975). The myofibrils are in intimate contact with sarcoplasmic reticulum - consistent with the involvement of the latter in the release -sequestration of calcium ions required for contraction. (Naylor, 1967; Rasmussen and Tenenhouse, 1968; Katz and Repke, 1973). The large number of mitochondria and their highly cristate structure illustrates the highly oxidative nature of myocardial metabolism - in marked contrast to the glycolytically-oriented fast skeletal muscle. Glycogen granules and lipid droplets represent internal stores of energy substrates (Stein and Stein, 1963).

(b) Performance of the muscle The classical work of Frank (1895) and Starling (1918) using the heart-lung preparation, first described the characteristics of the working heart which provided such subtle adaptability to the changing needs of the body. The essential nature of the contractility which they described was the ability to increase cardiac output in response to a rise in venous (i.e. filling) pressure. Increased ventricular filling caused an increase of diastolic fibre length which in turn induced increase in strength of contraction. (Up to a point beyond which a further increase in diastolic length

Figure 4

Ultrastructure of the myocardial cell



Adapted from Kelman, 1971.

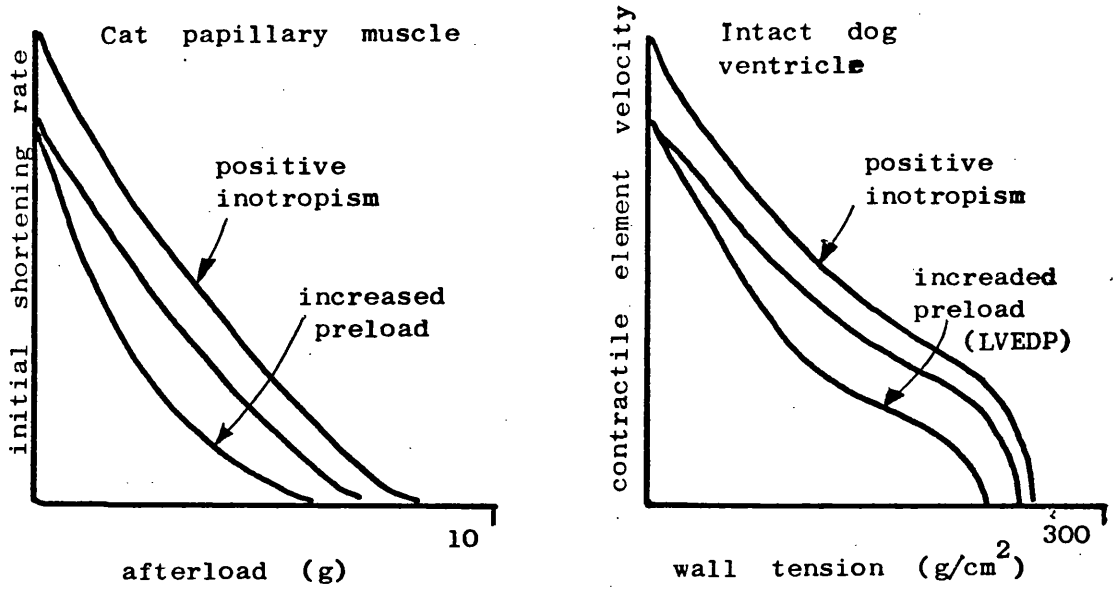
induces no further increase in output but instead induces failure). This is described as the length-tension relationship.

In addition to the effect of atrial filling pressure (preload) upon performance, subsequent studies have shown the importance of two other major factors - aortic pressure (afterload) and contractile state of the muscle. The studies have mainly employed the isolated papillary muscles of rats and cats. Papillary muscles are the cone -shaped muscles which project from the inner, endocardial surface of the ventricles and anchor the valves via their extensions - the cordae tendonae. The work has led to an elegant description of myocardial contractility and the factors which influence it. These are described in the force - velocity relationship of Sonnenblick (1962, 1974), Parmley and Sonnenblick (1969). This relationship is derived by the study of the velocity of shortening of muscle over a range of imposed afterloads. Contractility is further described in the three - dimensional force - velocity - length relationship of Henderson and Brutsaert (1973) - figure 5.

These relationships are difficult to apply directly to the intact heart since they have been established using isolated muscle preparations. Attempts have therefore been made to test the validity of these relationships in a more intact preparation. Sonnenblick, Parmley, Urschel and Brutsaert (1970) have been able to show the adherence to

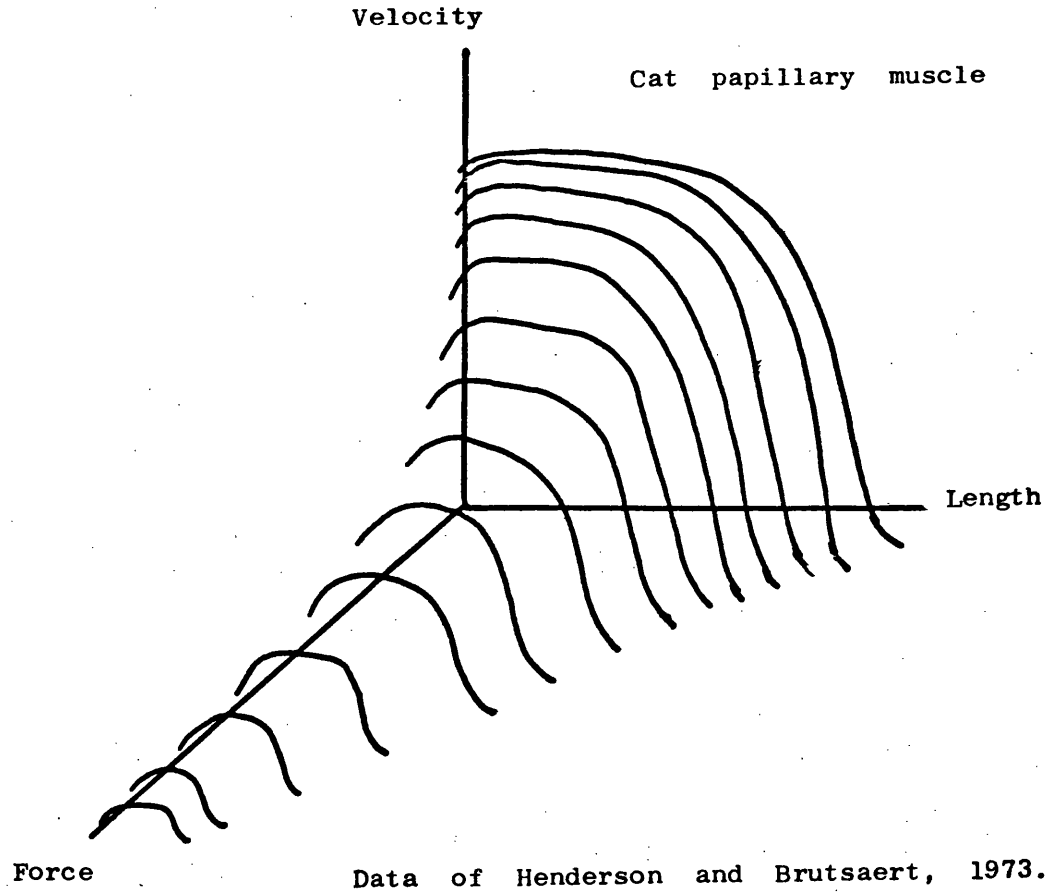
Figure 5

Myocardial Mechanics - force-velocity



Data of Sonnenblick et al (1970).

- force-velocity-length



Data of Henderson and Brutsaert, 1973.

these relationships of the intact, non-working, dog ventricle. Further extensions of these indices of function to the working heart in situ are greatly complicated by the external factors which affect performance. Because of these difficulties a number of parameters have been studied to evaluate their possible use as indices of performance. Thus shortening velocity (Tomoda and Sasamoto, 1973) atrial-ventricular systolic time intervals (Scully, Bello, Beierholm, Fredericksen, Weisfeldt and Doggett, 1973), peak left ventricular ejection velocity (Sonnenblick and Skelton 1971), maximal rate of rise of ventricular pressure (Mjøs, 1971) have all been used to indicate contractility. It is important to determine when investigations really do attempt to elucidate contractility since the rather less definitive term "function" often appears. This is usually a more general term than contractility and indicates the overall competence of the muscle.

An important aspect of the investigations into cardiac performance has been the indication of the major factors contributing to MVO_2. These factors (Sonnenblick and Skelton, 1971; Burns and Covell 1972) are as follows:

(i) Wall tension (dependent upon ventricular pressure, intraventricular volume and myocardial mass).

(ii) Contactility (or contractile state) - see force-velocity relationship.

(iii) Heart rate.

(iv) Metabolic rate.

There are two minor contributors to MVO_2; firstly, energy required for external work or active ejection (about 5 - 10% of MVO_2) and secondly, energy required for activation of contraction and relaxation (about 1% of MVO_2).

Another problem which has been solved by these investigations concerns the mechanism of contraction of cardiac muscle. On the basis of lower maximum force development per unit cross-sectional area (compared to skeletal muscle) and other factors such as apparent immunity to tetanisation, a discrete mechanism of action peculiar to cardiac muscle had been a possibility. The differences were explained, instead, by different control of contraction in cardiac muscle. Thus the lower force developed was explained by the operation of a smaller proportion of the contractile elements at any given time. The time of active contraction (twitch time) in cardiac muscle was limited by an extended refractory period, thus reducing the possibility of tetanisation. (Sonnenblick, Parmley, Buccino and Spann, 1968).

3. Non-contractile properties

In addition to the classical muscular function of contraction, the heart exhibits other properties which can be described as automatacity (the ability to contract independently of external stimulation), conductivity (electrical impluses passed from one muscle cell to another without the necessary intervention of nervous tissue) and sensitivity to modulation of performance at the muscle cell site as well as sensitivity to nervious control.

(a) Electrical Properties Unlike skeletal muscle, the myocardium acts as a syncytium allowing co-ordination of contractility vital to the pumping action. Electrical impulses originate in a group of specialised cells called the sinus node, located on the surface of the right atrium near its junction with the superior vena cava. The node depolarises and repolarises cyclicly at a rate which (usually) determines the overall heart rate. The sinus node provides the usual dominant heart rhythmn but automaticity is also exhibited by ventricular muscle itself in the absence of sinus rhythmn. Indeed single-layers of myocardial cells in tissue culture exhibit synchronous beating - Glick, Burns and Reddy, (1974).

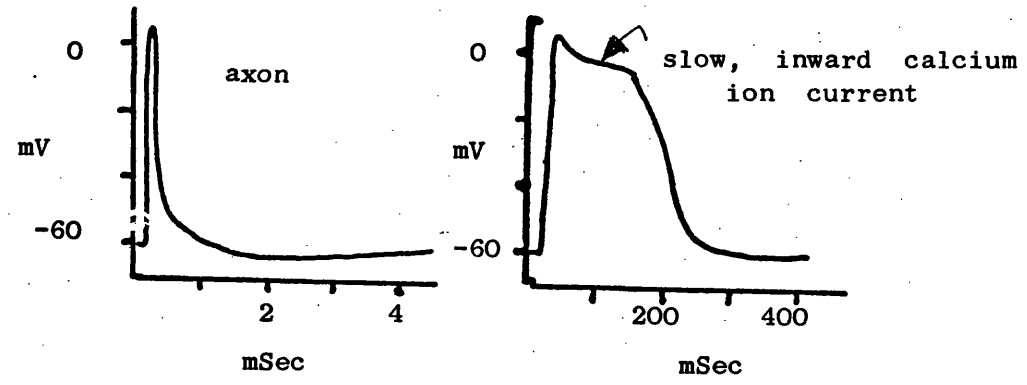
The impulse from the sinus node spreads across the surface of the atria, causing them to contract and then passes rapidly to the apex of the ventricles via the atrioventricular node and the Purkinje fibres. These are

muscle cells which, through specialisation, have lost the ability to contract but instead conduct electrical impulses more rapidly than normal myocardial cells. From the apex the impulse passes back up through the bulk of the muscle mass, initiating the rising wave of ventricular contraction. The electrical wave passes from one muscle cell to the next in a way analagous to nervous transmission along axons. The cells communicate at sites called inter-calated discs. The nature of the discs and thus the extent of communication is not clear.. The differences between nervous conductivity and myocardial conductivity are demonstrated by differences in the action potentials (figure 6a).

(b) The Electrocardiogram (ECG) The co-ordinated electrical activity of the muscle produces a strong and rapidly changing electrical potential which can be detected at the body surface, by the application of electrodes at suitable points. It should be stressed that the ECG is the resultant of the summation of the action potentials of all the individual myocardial cells. A typical complete wave (figure 6b) contains components associated with depolarisation and repolarisation of both atria (P wave) and ventricles. Ventricular depolarisation gives rise to the QRS complex of the electrocardiogram. The plateau of the action potential, during which the muscle is depolarised, produces the ST segment. The T wave is due to ventricular repolarisation. Paradoxically, the deflection of both depolarisation and repolarisation waves

Figure 6(a)

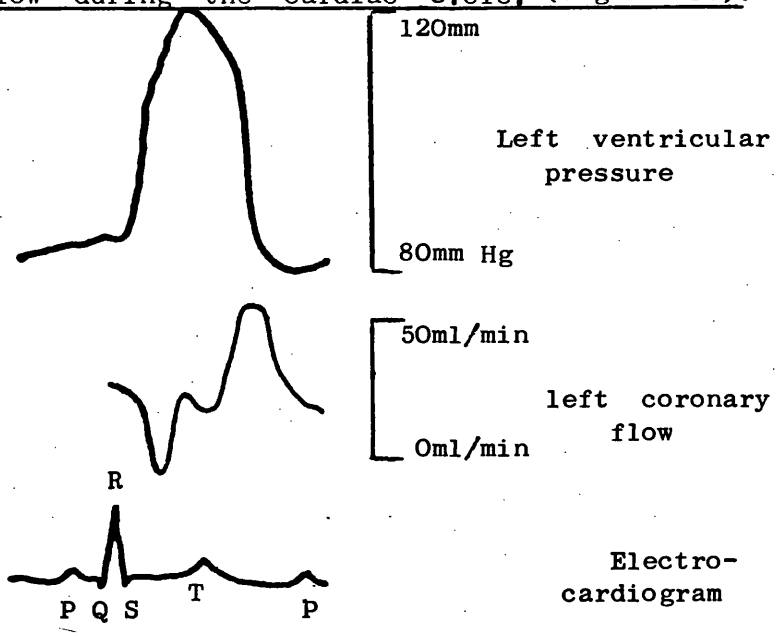
Conduction spikes of nerve axon and myocardial cell contrasted.



Adapted from Kelman, 1971.

Figure 6(b)

Time-course of ECG, ventricular pressure development and coronary flow during the cardiac cycle, (dog heart).



is in the same direction. This may be explained by the dependence of the pathway of depolarisation upon factors other than just cell - to - cell conduction (e.g. wall tension, Kelman, 1971).

Electrocardiography has proved extremely useful in both clinical and experimental situations since it gives valuable information concerning the performance of the heart by non-invasive means (Julian, 1973; Schamroth, 1973). Characteristic changes are found with the electrocardiogram coincident with various disturbances in performance and the procedure thus has valuable diagnostic uses. The particular uses of importance here are the characterisation of disturbances in atrial and ventricular rhythm and also the indication of myocardial ischaemia which is manifested as changes in ST segment and T wave (Brooks, 1974; Angell, Lakatta, Weisfeldt and Shoch, 1975). The physio-chemical basis of the electrocardiogram is described in detail by Schaefer and Haas (1962).

(c) Sensitivity to humoral influences Heart performance is influenced not only by nervous control (internal and external) but also by direct action of particular agents on the muscle cells. Thus a number of hormones (including catecholamines, ACTH, TSH and glucagon) influence contractility and rate of beating. At least two major modes of action have been proposed to describe the actions of these and other agents.

The first major theory (Sutherland, Robinson and Butcher, 1968) considers that the hormones act at the plasma membrane and/or intracellular membranes, modifying the activity of adenylate cyclase and hence the intracellular concentration of adenosine - 3' - 5' - cyclic monophosphate (cyclic AMP). The level of cyclic AMP is thought to influence contactility by as yet undefined mechanism(s). One possibility is the modulation of intracellular calcium ion av ailability through the action of cyclic AMP on the sarcoplasmic reticulum (Epstein, Levey and Skelton, 1971).

The second major theory considers the effect of hormones and other agents such as cardiac glycosides on the membrane systems involved in the release and sequestration of calcium ions and other ions involved in the excitation and contraction processes. (Katz and Repke, 1973; Nayler, 1967). The actions may involve no rise in cellular cyclic AMP concentration (Venter, Ross and Kaplan, 1975).

The basic information quoted in this section has been drawn largely from the texts of Kelman (1971) and Selkurt (1971).

SECTION B The Metabolism of the Heart

1. Substrates utilised

The heart is a constantly active muscular tissue and is therefore a centre of intense metabolic activity. The substrates required for combustion to provide the energy needed for mechanical and electrical activity are supplied largely by the blood. The heart thus normally depends largely upon exogenous substrates. Under normal conditions the glycogen stored within the myocardium (endogenous substrate) is not extensively utilised. Under certain conditions the balance between utilisation of exogenous and endogenous fuels can shift markedly. In particular, the utilisation of endogenous lipid may become significant.

Of the exogenous substrates, glucose was the first shown to be utilised by the heart (Locke and Rosenheim, 1907) using the isolated perfused rat heart preparation of Langendorff (1895). Since that time the importance of lipid as the other major fuel has been described. Lipid is supplied as free fatty acids (FFA) in the form of a complex with serum albumin (Solomon, 1968; Spector, John and Fletcher, 1969) and also as esterified fatty acids i.e. triglyceride - fatty acids (TGFA). TGFA are present in the blood in the form of chylomicra and very low density liprporteins (VLDL). Both these forms are complexes containing triglyceride (60%), phospholipid (15%), cholesterol (10%), cholesterol esters (5%) and protein (10%) - data of Fredrickson (1974). The chylomicra or primary particles

derive their lipid directly from dietary lipid in the intestinal mucosa and are discharged mainly into the lymph. VLDL or secondary particles, by contrast, are synthesised in the liver and are discharged into the blood. The depletion of lipid from these particles by extrahepatic tissue leads to the formation of low density lipoproteins (LDL) containing only about 10% triglyceride (Fredrickson, 1974). The potential importance of TGFA as a fuel is described by Delcher, Fried and Shipp (1965) who pointed out that, in those mammals studied, over 90% of the fatty acid content of the blood is esterified.

The utilisation of FFA by the heart has been shown by Shipp, Opie and Challoner (1961) and Scheuer and Olson (1967) with the isolated rat heart and by Most, Brachfeld, Gorlin and Wahren (1969) and Gousious, Felts and Havel (1963) who studied the utilisation of radioactive FFA in man. Similar studies have been performed with the perfused monkey heart (Crass, Tullis, McCaskill and Shipp, 1970) and with the heart of the conscious dog (Cowley, Scott and Spitzer, 1969). The uptake and oxidation of TGFA has also been shown by Gousious et al (1963); Enser et al (1967); Delcher et al (1965); Scheuer et al (1967) and Most et al (1969). The utilisation of FFA has been further investigated to elucidate any possible selectivity with regard to chain length or degree of unsaturation. Willebrands (1964) and Evans (1963) showed selective uptake in the order : 18:1 < 18:2 < 16:0 < 18:0 in the isolated rat heart.

Similar evidence has been provided for the human heart by Rothlin and Bing (1961) and Harris, Chlouverakis, Gloster and Jones (1962). These latter studies based their findings on difference in FFA composition of the blood measured at the coronary sinus compared to that at the aorta. Such use of arterio-venous (a - v) differences has been criticised by Stein and Stein (1963) since the compositions may be altered by exchange reactions catalysed by lipoprotein lipase (LPL - see below) with no necessity for nett FFA uptake. This criticism appears valid for in vivo investigations but seems less appropriate for the studies using the isolated perfused hearts which received no esterfied lipid. This factor could, however, explain the contradiction of the above results by Stein and Stein (1963) and Most et al (1969) who found no selectivity with regard to uptake in the group 16 : 0, 18 : 0, 18 : 1. There was selectivity of utilisation, however, with palmitate entering triglyceride more readily than linoleate - conversely, linoleic acid entered lecithin more readily than did palmitic acid.

TGFA utilisation proceeds not by direct lipid uptake (Crass et al 1966) but by hydrolysis at the cell surface to release FFA which then enter the cell. This hydrolysis is catalysed by LPL (as described later). Crass et al (1966) showed that the oxidation of TGFA by the isolated rat heart was accompanied by and indeed lagged behind a transient rise in FFA concentration of the perfusate.

Both these effects were stimulated by heparin - a known activator of LPL. Further, the oxidation of TGFA could be depressed by preperfusion with heparin in order to elute LPL from the tissue as shown by Enser et al (1967).

In addition to the two major substrates, the heart can also utilise lactate, pyrurate, ketones and amino acids but under most conditions their availability is not great (Olson, 1962).

2. Quantitative significance of Substrates

(a) Exogenous substrates Since the heart is able to consume a number of different substrates, the relative importance of these will depend upon availability (plasma concentration) and preference. To determine substrate preference of the heart, a number of competition studies have been performed, in which the uptake and/or oxidation of a given substrate is studied in the presence and absence of potentially competitive substrates. Extensive reviews have been published by Opie (1968) and by Neely and Morgan (1974). The general conclusion from the investigations reviewed is that in sufficiently high concentration any of the major substrates (glucose, FFA, lactate, ketones or pyruvate) can dominate the oxidative pathways of the heart. At physiological concentration, however, the balance lies between glucose and FFA. In general, FFA are able to pre-empt glucose oxidation rather than the reverse. Thus Shipp et al (1961) showed extensive inhibition of oxidation of glucose (5 - 10mM) by palmitate (0.3mM) in the isolated perfused rat heart. Conversely, high glucose concentrations gave only a limited inhibition of FFA oxidation. Crass et al (1970) showed similar inhibition of glucose oxidation by pamitate in the perfused monkey heart; the inhibition probably being effected at several points in the pathway of glucose metabolism. One can regard the circulating FFA concentration as a vital control element, particularly since glucose uptake is insulin-sensitive (Morgan, Henderson, Regan,

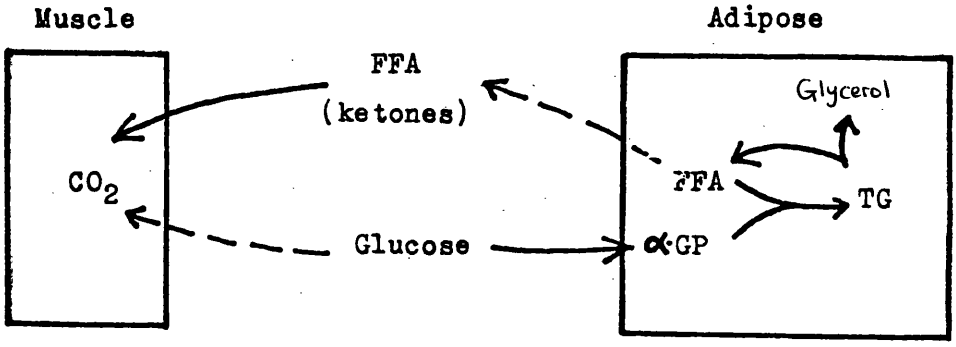
and Park 1961; Morgan, Neely, Wood, Liebecq, Liebermeister, Park 1965), whilst FFA uptake seems to be dependent only upon arterial concentration (Most et al 1969). The relationship between glucose and FFA utilisation is described by the glucose - FFA cycle (Randle, Garland, Hales and Newsholme, 1963). Thus during starvation and diabetes the circulating FFA concentration is elevated and the uptake and oxidation of glucose is depressed. The roles of the two substrates can be reversed by perfusion of hearts from starved or alloxan-diabetic rats with medium containing glucose and insulin. Similar evidence was provided by Kreisberg. (1966).

In man during periods of fasting, lipid appears to be the major oxidiseable substrate (Most et al 1969), accounting for 70% of the myocardial oxygen consumption (MVO_2) whilst glucose accounted for the remaining 30%. Similarly, in healthy fasting men, Lassers, Wahlqvist, Kaijser and Carlson, (1971); Lassers, Kaijser and Carlson, (1972) have shown that a significant negative correlation exists between FFA concentration and myocardial extraction of glucose, lactate and pyruvate. The depression of FFA concentration with the antilipolytic agent nicotinic acid stimulated myocardial extraction of the carbohydrates, (Lassers et al, 1972).

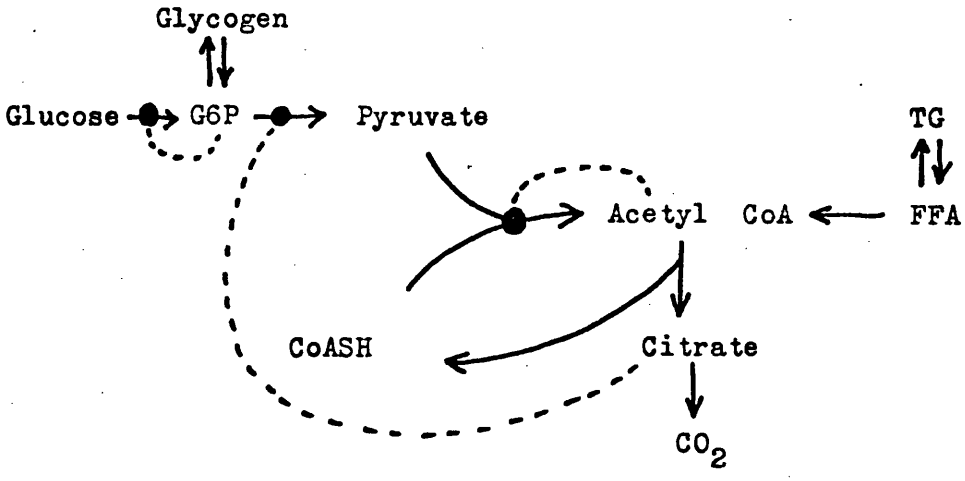
The studies above have indicated situations in which FFA are the preferred substrate. Hasselblat (1970) described the converse situation of extensive glucose utilisation during the post-prandial period. A high glucose load

Figure 7

Glucose-FFA Cycle (Randle et al, 1963).



Detail of glucose-FFA cycle in muscle - principal control points.



= inhibition

GP = sn-glycerol-1-phosphate

mimics the effect of antilipolytic agents in depressing nett lipolysis in adipose tissue, thus reducing the circulating FFA concentration. In addition, pancreatic insulin secretion is stimulated and glucose utilisation by peripheral tissues is stimulated.

Similar though less direct evidence for the balance between glucose and FFA utilisation is provided by the respiratory quotient (RQ) which tends towards unity during carbohydrate oxidation but falls during lipid oxidation. During the post-prandial period RQ = 1.0 (Goodale and Hackel, 1953), after an overnight fast it falls to 0.8 and during diabetes and starvation to 0.7 (Goodale, Olson and Hackel, 1959).

Gold, Scott and Spitzer (1967) showed that the elevation or depression of plasma FFA concentration in hyper - or hypothyroid dogs respectively, was accompanied by changes in myocardial FFA oxidation, RQ and MVO_2. During hyperthyroidism, FFA oxidation and MVO_2 were enhanced but RQ was depressed.

Olson (1962 [b]) showed that high concentrations of pynvate or acetoacetate (both 10mM) were required to influence the utilisation of 0.3mM palmitate. Uptake of palmitate was inhibited 50% but oxidation was inhibited by 75% thus a four-fold increase of cellular FFA concentration occurred despite a compensatory rise of FFA esterification into triglyceride. Little, Goto and Spitzer (1970) showed

that β-hydroxybutyrate and acetoacetate (10mM) inhibited the oxidation of radioactive palmitate in the dog heart in situ. The ketones accounted for about 80% of the myocardial CO_2 production.

Miller, Keuk and Durham (1971) studied metabolism in conscious dogs. Myocardial extraction of FFA was directly proportional to the arterial concentration and oxidation accounted for all the FFA uptake. FFA contributed 35% of the CO_2 production during rest, 75% during exercise or infusion of noradrenaline but only 25% during infusion of glucose. Lactate oxidation became significant during exercise.

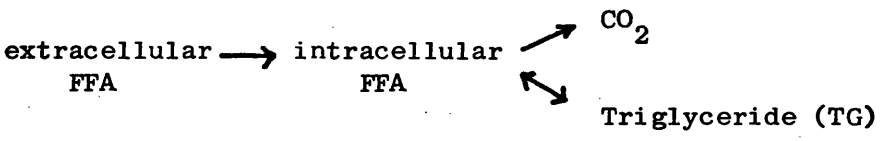
(b) Endogenous Substrates As can be seen from the preceeding evidence, exogenous substrates can usually account for most of the oxidation carried out in myocardium. Despite these findings, the utilisation of the endogenous reserves has also been demonstrated. Mayer, Namm and Hickenbottom (1969) showed that the activation of glycogen phosphorylase occurred in heart muscle as had previously been described for liver and skeletal muscle (for review see Fischer, Pocher and Saari 1970). Glycogen has also been suggested to be directly involved in fuelling contractility on the basis of the presence of glycogen granules near the contractile elements (Saidrasulov, 1963). The significance of this is uncertain in view of the inhibition of glycogen utilisation found by Ribeilima, Wendt, Ramos, Gudbjarnason, Bruce and Bing (1964) and by

Neely, Whitfield and Morgan (1970). The latter group in particular showed that glycogenolysis in the isolated rat heart became significant only when FFA were omitted from the perfusate. It could be stimulated in the working rat heart by elevating pressure development but during fasting, with elevated plasma FFA concentration, cardiac glycogen content actually rose (in contrast to liver glycogen).

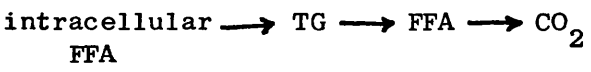
The utilisation of endogenous fuel was demonstrated by substrate-free perfusion of the isolated rat heart. (Shipp et al, 1964; Olson and Hoeschen, 1967). Glycogen was depleted within 5min but further contraction was maintained by endogenous lipid for up to 45min. This was confirmed by the production of $14\text{-}CO_2$ by perfused hearts in which the lipids had been pre-labelled with 1 - C - 14 - palmitic acid (Shipp, Thomas and Crevasse, 1964). Phospholipid fatty acid oxidation was observed in addition to triglyceride fatty acid oxidation, but this was not confirmed by Crass, McCaskill and Shipp (1969) and Crass, McCaskill, Shipp and Murthy (1971). In the latter studies TGFA oxidation was stimulated by increasing pressure development but the total phospholipid content of the muscle remained constant throughout the perfusions (Some evidence for transesterification between phospholipid and triglyceride was presented). Endogenous lipolysis was also found to be stimulated by adrenaline in the potassium - arrested rat heart (Challoner and Steinberg, 1966 [b]); and during diabetes and starvation (Kreisberg, 1966) with

consequent depression of utilisation of exogenous TGFA by isolated rat hearts. Endogenous lipolysis was found to contribute significantly to energy metabolism in the pig heart (Karpiak, 1968).

The involvement of endogenous lipid turnover in the utilisation of exogenous FFA is not clear. One would anticipate a pathway of the form:



This is supported by the sparing effect of exogenous FFA on endogenous TGFA utilisation (Crass et al, 1972). An alternative has been suggested by Shipp et al, (1964) whereby



This would require the compartmentation of the two intracellular pools. Such a system would offer control of FFA utilisation at the level of lipolysis and the evidence of Masters and Glainano (1969 and 1972) is consistent with this. Their work shows that FFA uptake is related to the rate of lipolysis (being stimulated by catecholamines and inhibited by β-blockade). This evidence, however, is open to the alternative explanation that the agents act simply by increasing and decreasing the demand for both substrates in parallel. To overcome this criticism, the work load

in the presence of β-blockade was increased by atrial pacing and the uptake of FFA but not of glucose remained inhibited. Despite these conflicting views, it is clear that the endogenous TG pool is active and contributes significantly to the FFA level of the cell. Thus Kong and Friedberg (1971) showed biphasic kinetics for the oxidation of labelled palmitic acid which had been infused into the coronary artery of the dog. A rapid phase of 14 - CO_2 release was consistent with direct oxidation of FFA whilst a slow phase of 14 - CO_2 release was consistent with oxidation preceeded by an intermediate esterification step. The speed of entry of exogenous labelled FFA into TG droplets was as rapid as the entry into mitochondria and 14 - CO_2 (Stein and Stein 1968). The location of lipid droplets near the oxidation sites (mitochondria) - Stein and Stein, 1968 - may support the pathway for FFA oxidation proposed by Shipp et al (1964) by offering a spatial explanation. The entry of FFA in this study was followed autoradiographically. The extent of involvement of myocardial TG reserves in the utilisation of FFA is further demonstrated by Masters and Glaviano (1972) using open-chest dogs, who showed that 30% of palmitate uptake entered TG. In addition, the <u>total</u> TG content of the tissue remained constant, thus indicating the turnover of the TG reserve.

Table 1 summarises the results of the above investigations. The common interventions and the consequent contributions to overall MVO_2 of the major substrates are described.

TABLE 1

Post-prandial	glucose	90-100%	FFA	0-10%
Overnight fast	"	10-20%	FFA	80-90%
Starvation	"	0 - 5%	FFA +endog TG	90-100%
Diabetes	"	0 - 5%	"	90-100%
Catecholamine stress	"	10-30%	"	70-90%
Hyperthyroidism	"	10-30%	"	70-90%
Exercise	glucose + lactate	30%	"	70%

"FFA" represents the sum of the contributions of plasma FFA, TGFA and ketones. The concentration of ketones in the blood is related to that of FFA since they are released from the liver from FFA precursors.

3. The effect of work on substrate utilisation

The work load of the heart dictates the level of substrate utilisation. Thus stimulation of contractility of the isolated rat heart with Ouabain (Kreisberg and Williamson, 1964; Gousious, Felts and Havel, 1967) stimulates the uptake and oxidation of glucose. Stimulation of the isolated rat heart with adrenaline (Kreisberg, 1966[a]) and of the dog heart with noradrenaline (Cowley et al, 1969) enhances the oxidation of both glucose and FFA. Neely, Liebomeister, Battersby and Morgan (1967); Neely, Bowman and Morgan (1969) and Neely et al (1970) showed that increased pressure development of the isolated, perfused working rat heart stimulated MVO_2, glucose uptake, FFA uptake and glycogenolysis. Crass et al (1969, 1970) compared the substrate utilisation of the working and non-working (Langendorff) isolated rat hearts. Glucose uptake was stimulated by imposition of work but FFA uptake was not: instead, the oxidation of FFA was stimulated at the expense of esterification to TG.

4. Metabolic Pathways

(a) Major Routes. The highly aerobic nature of myocardial metabolism exhibited under all but extreme conditions, is demonstrated by the large number of mitochondria, which make up about 30% of the cell volume (Sobel, 1974). Other indices of the aerobic metabolism are the capacity of the heart to utilise lactic acid (Opie, 1968) and the high concentration of oxidative enzymes, particularly cytochromes (Penpargkul and Scheuer, 1970). Although nett uptake of lactate is normal, there is still release of lactate across the heart as assessed by the reduction in the specific activity of lactate infused into the dog heart (Leunissen and Piatnek-Leunissen, 1973).

The pathways for generation of energy in the myocardium are those classically illustrated in standard texts (Mahler and Cordes, 1969) and will not be described in detail here (figure 8). Glucose is utilised by the Embden-Meyerhof-Parnas pathway, producing pyruvate which enters the mitochondrion. Pyruvate dehydrogenase catalyses the activation of pyruvate to acetyl CoA (figure 9) which is oxidised by the tricarboxylic acid cycle (TCA), producing reducing equivalents to be oxidised by the terminal oxidation chain. Cytosolic reducing equivalents enter the mitochondrion mainly by the malate-aspartate shuttle. FFA are first activated to the CoA esters and then enter the mitochondrion for oxidation by the beta-oxidation spiral which produces acetyl CoA units.

Legend for Figure 8

1. Glucose kinase, hexokinase.
2. Phosphoglucose isomerase.
3. Phosphoglucomutase.
4. Phosphofructokinase.
5. Aldolase.
6. Triosephosphate isomerase.
7. Glycerolphosphate dehydrogenase.
8. Glycerol kinase.
9. Lipase(s).
10. Glyceride synthesis.
11. Fatty acid thiokinases.
12. Glyceraldehyde-3-phosphate dehydrogenase (phosphorylating)
13. Phosphoglycerate kinase.
14. Phosphoglycerate mutase.
15. Enolase.
16. Pyruvate kinase.
17. Lactic dehydrogenase.
18. Carnitine acyl transferase (2 forms).
19. Pyruvate dehydrogenase.
20. Beta keto acyl CoA thiolase.
21. 3-keto acid-CoA transferase.
22. Betahydroxybutyrate dehydrogenase.
23. Beta oxidation spiral.

Figure 8. Major pathways for AcCoA production in the mammalian heart.

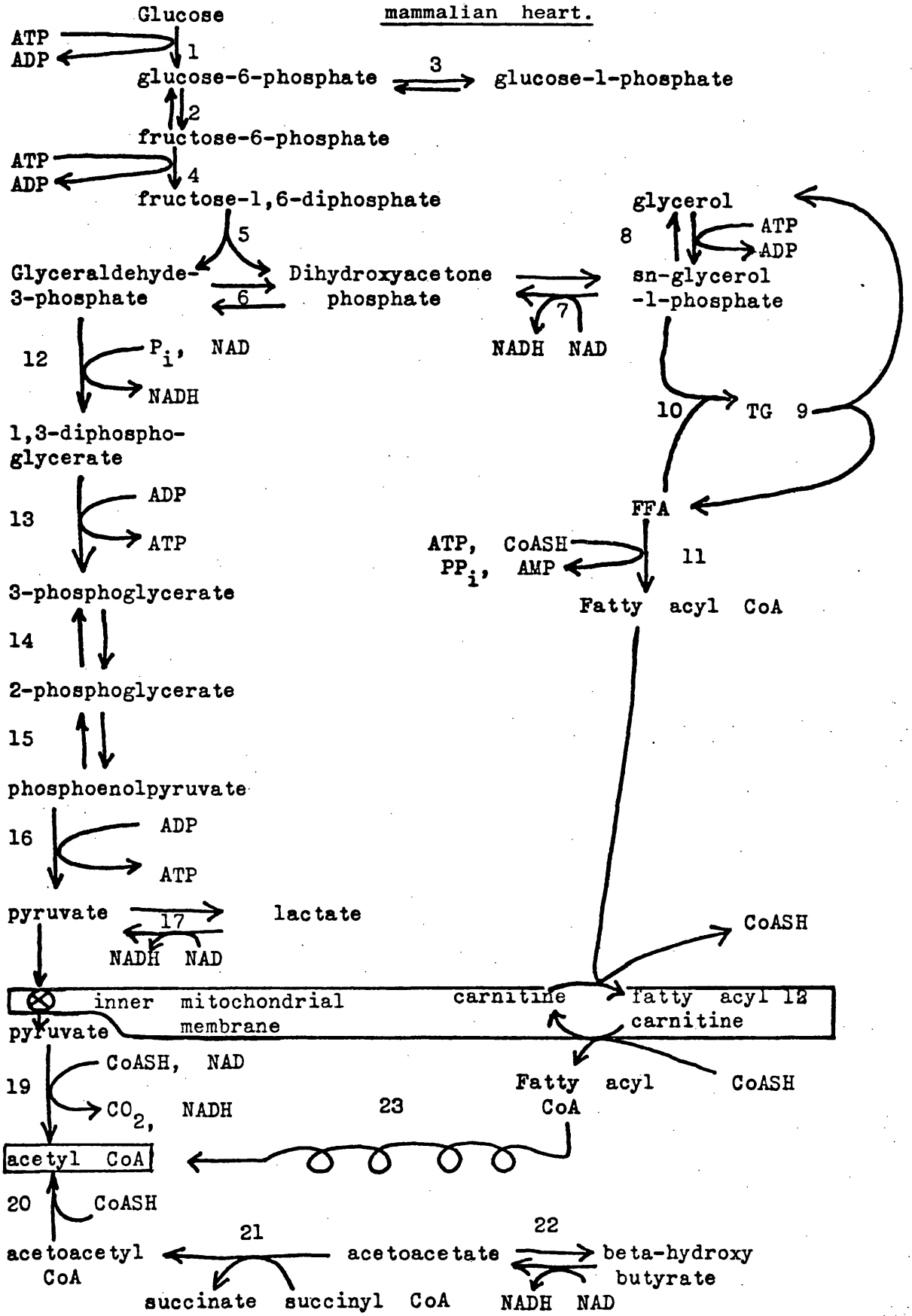
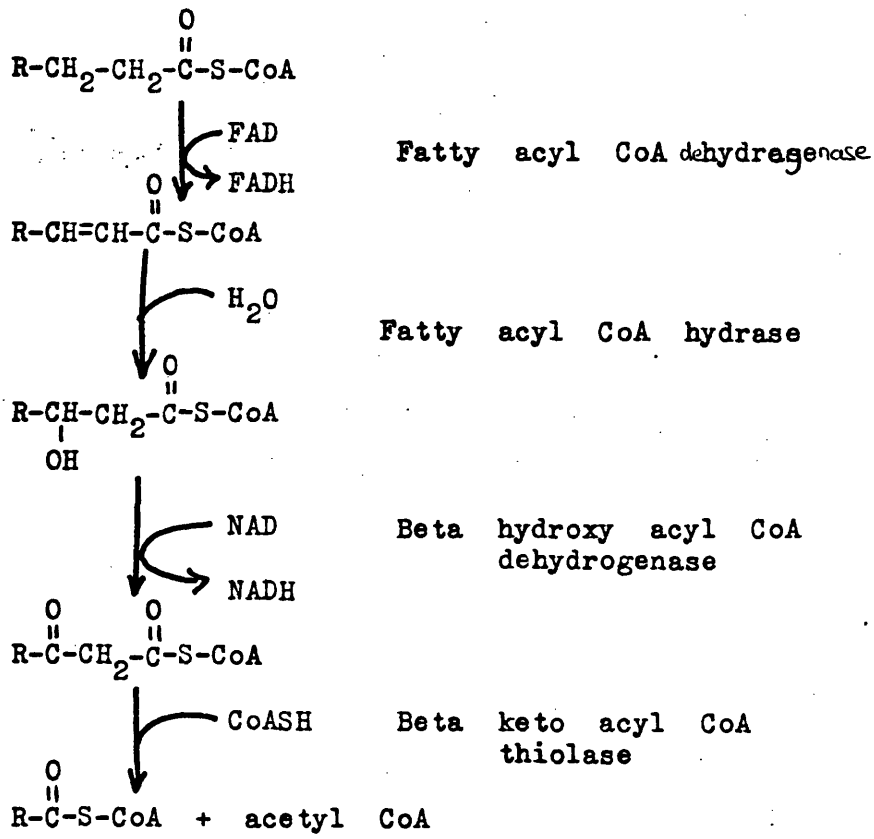
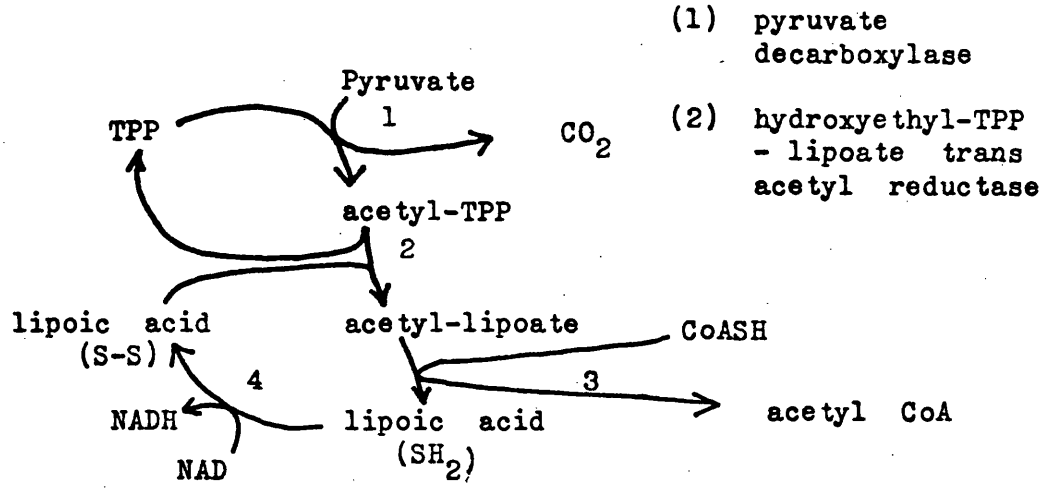


Figure 9

(a) Reactions of the Fatty Acid (beta) Oxidation Spiral



(b) Reactions of pyruvate dehydrogenase complex



(1) pyruvate decarboxylase

(2) hydroxyethyl-TPP - lipoate trans acetyl reductase

(3) dihydroacetyllipoate CoA transacetylase

(4) dihydrolipoate dehydrogenase

Activated fatty acid units enter the mitochondria by the carnitine-cycle, under the influence of carnitine-acyltransferase which is located at the inner mitochondrial membrane. Lactic acid is utilised by conversion to pyruvate with lactic dehydrogenase. A specific heart muscle isoenzyme of lactic dehydrogenase exists which, unlike the skeletal muscle isoenzyme, is normally sensitive to inhibition by high pyruvate concentrations, (Everse and Kaplan, 1973). This sensitivity must be lost during hypoxia or ischaemia to enable extensive lactate release by the myocardium, (unless a significant amount of the skeletal muscle isoenzyme is also present).

The final aspect of energy metabolism concerns energy transport. The three major aspects of this process are - (i) oxidative phosphorylation (Racker, 1970) and ATP transport through the inner mitochondrial membrane (Klingenberg, 1970); (ii) delivery of energy equivalent to the active sites of actinomyosin - largely as creatine phosphate, which obtains phosphate from ATP under the influence of creatine phosphokinase - (Saks, Chernousova, Voronkov, Smirnov and Chazov, 1974); (iii) consumption of energy in the processes of contraction, ion transport etc.

(b) Minor Routes

Glucose synthesis from lactate and pyruvate can be observed in heart muscle (Stadie, Haugaard and Perlmutter, 1947) but the key enzymes of the Utter-Ochoa pathway

(malic enzyme, pyruvate decarboxylase, PEP carboxykinase) are of relatively low activity and the pathway is not of great quantitative significance (Racker, 1954). The same is true of the key enzymes of the hexose monophosphate shunt (glucose - 6 - phosphate dehydrogenase and 6 - phosphogluconate dehydrogenase) - as shown by Glock and McLean (1954).

Lipid synthesis is active in the myocardium as described earlier. Fatty acyl CoA esters react with sn - glycerol - 1 - phosphate to produce mono, - di - and, principally, triglycerides (Denton and Randle, 1965). Phospholipid metabolism is believed to occur by classical routes (for review, see Dawson, 1966). The origin of sn - glycerol - 1 - phosphate from glycerol or glucose is debated since the quantitative significance of glycerol kinase in the heart is disputed. Scheuer and Olson (1967) could find no glycerol uptake by the isolated rat heart but Robinson and Newsholme (1967) found significant activity of glycerokinase in rat heart extracts.

Protein synthesis and degradation are obviously of great significance because of the constant need for structural, contractile and enzymic protein (particularly exaggerated with hypertrophy) but will not be described here.

5. Metabolic Control

It is possible to distinguish between control factors which operate essentially outside the tissue and those which operate within the tissue. The former group include factors which modify the circulating concentrations of essential substrates, such as the dietary status of the animal and the presence of lipolytic or antilipolytic hormones. This group will not be discussed further here.

(a) Respiratory Control The major element of metabolic control in the heart as in other tissue is the well-documented respiratory control exerted through the maintenance of a particular energy status in the cell (usually expressed as the extent of phosphorylation of the ATP - ADP - AMP system - Chance and Williams, 1956; Klingenberg, 1970). As the work of the heart increases from a particular steady state, ATP and creatine phosphate (CP) are consumed and the energy status shifts to the right in the above equation. The operation of adenine nucleotide translocase (Klingenberg, 1970) ensures that the energy status of the cytoplasm is reflected by that of the mitochondrion. The decline of ATP relative to ADP and AMP in the mitochondria stimulates oxidative phosphorylation. The consequent consumption of reducing equivalents and energy substrates provides for greater production of these units via activity of the TCA cycle, β-oxidation and glycolysis. This has been demonstrated in the rat heart by Neely, Denton, Randle and England (1972).

It is through this mechanism that agents such as catecholamines, cardiac glycosides, ACTH, β-blockers can affect metabolism. By stimulating contractility they secondarily stimulate the metabolic routes to accommodate the extra work load. Vital evidence concerning the order of action of catecholamines on contractility and metabolic responses was provided by Øye (1965) and Cheung and Williamson (1965) who showed a significant time-interval between the onset of intropism and the activation of glycogen phosphorylase. In addition, the direct action of catecholamines upon phosphorylase appears to be ruled out by the finding that glycogenolysis is stimulated by metabolite stimulation and depression of phosphorylase b rather than b → a conversion (Neely et al, 1967; Dhalla and McLain, 1967).

(b) Key enzymic Control Points Given the basis of respiratory control, there are a range of subtle cellular controls which dictate the relative contributions of different substrates under different conditions.

(i) Uptake of Substrates Glucose uptake occurs by a specific carrier mechanism as judged by kinetic characteristics (Morgan et al, 1961, 1965). Uptake is normally limited at this site but can be stimulated by insulin and hypoxia (Morgan et al, 1961; Morgan, Randle and Regen, 1959) so that the subsequent phosphorylation step becomes rate-limiting for glucose uptake.

FFA uptake is proportional to plasma concentration (Evans, 1964). In addition the albumin : FFA ratio is a determinant of uptake (Goodman, 1958; Evans, Opie and Shipp, 1963), but only differs from concentrations if albumin is varied from physiological concentrations of 2 - 4g%. One molecule of albumin binds two molecules of FFA with high affinity and up to 20 molecules with lower affinity (Solomon, 1968).

The uptake of lactate, pyruvate and ketones also appears to be limited by plasma concentrations (see Section B.2.). The uptake of TGFA is additionally dependent upon the activity of lipoprotein lipase.

(ii) Pyruvate Dehydrogenase The work of Randle, England and Denton (1970), Denton, Randle and Martin (1972), Martin, Denton, Pask and Randle (1972), Shaw and Boder (1972), Severson, Denton, Pask and Randle (1974) and Whitehouse, Cooper and Randle (1974) has shown the central role of pyruvate dehydrogenase (PDH) in the regulation of energy metabolism. The enzyme exists in dephosphorylated (active) and phosphorylated (inactive) forms, the interconversion of the two forms is catalysed by kinase and phosphatase which are part of the PDH complex. PDH activity is stimulated by insulin, calcium ions, magnesium ions and oral hypoglycaemics such as dichloroacetic acid. The activity is inhibited by catecholamines, acetyl CoA (Garland and Randle, 1963) and NADH (Garland, 1964).

This control offers an explanation for the facility with which FFA can pre-empt the oxidation of glucose since the generation of acetyl CoA units from FFA would shut down PDH activity. By contrast, few points exist for glucose units to interfere with the oxidation of FFA. One possibility is a mass action effect of reducing CoASH availability and depressing the access of fatty acylcarnitine to CoASH at the inner mitochondrial membrane. (Vahouny, Katzen and Entenman, 1967; Olson, 1962; Evans, Opie and Shipp, 1963).

(iii) Phosphofructokinase (PFK). PFK represents a most important point of control of glycolysis in heart. It is subject to inhibition by citrate - thus restricting the glycolytic rate when sufficient substrate is already available for TCA activity (this represents another likely contribution to the depression of glucose utilisation by FFA). PFK is also inhibited by ATP - this inhibition being lifted by inorganic phosphate (P_i), AMP, cAMP, ADP and fructose diphosphate. The enzyme is thus sensitive to the energy status of the cell (Mansow, 1963). The effect of inhibition of PFK is the accumulation of fructose - 6 - phosphate and hence glucose - 6 - phosphate (since phosphoglucose isomerase is balanced near to equilibrium). Accumulation of glucose - 6 - phosphate inhibits glucose phosphorylation and glucose uptake (England and Randle, 1967) and in addition causes inhibition of glycogenolysis by its inhibitory effect upon glycogen phosphorylase b (Øye, 1967).

High concentrations of glucose - 6 - phosphate cause glucose - 1 - phosphate accumulation by mass action effect on phosphoglucomutose. Accumulation of glucose - 1 - phosphate promotes glycogen synthesis.

(iv) Other Control Points The stimulation of PFK to a sufficient degree passes control of the rate of glycolysis to glyceraldehyde - 3 - phosphate dehydrogenase or pyruvate kinase - the other major control points of glycolysis in the heart (Williamson, 1965).

The combination of increased flux through glycolysis and sufficient cytosolic reducing equivalents, results in an increase in sn - glycerol - 1 - phosphate concentration. The increased capacity for esterification of FFA may be reflected in an increase in cellular lipid concentration. During hypoxic perfusion, therefore, associated with rapid glycolytic flux and a high level of reducing equivalents in the cell, triglyceride accumulation occurs rapidly (Scheuer and Brachfeld, 1966; Evans, 1964). The contribution of glycerol kinase to sn - glycerol - 1 - phosphate may also be significant (Robinson and Newsholme, 1967). Under normoxic conditions, increased glycolytic flux and capacity for esterification may provide a mechanism for removal of FFA and stimulation of glucose utilisation.

Section C The Heart in Ischaemia

1. General Ischaemia is the reduction of the flow through tissue of blood (or perfusate in the experimental situation). It is distinguished from hypoxia (or the extreme, anoxia) which describes a reduction of the oxygen content of blood or perfusate. Both terms describe a situation of depressed oxygen delivery but ischaemia is additionally complicated by depression of the delivery of other substrates and, more crucially as will be seen, depression of the removal from the tissue of metabolic products.

The onset of myocardial ischaemia in the clinical sense usually represents the result of a progressive chronic reduction of blood flow caused by occlusion of one or more of the major coronary arteries. Ischaemia may also occur as a transient episode caused by temporary oxygen deficit due to excessive cardiac activity. Chronic occlusion is the result of the degeneration of the intimal lining of the major vessels described as atherosclerosis (Schettler and Boyd, 1969). The degeneration is provoked by undefined primary factor(s) and leads to the accumulation of lipid and fibrous material at the intimal lining (for reviews see Shimamoto, 1972; Ross and Glomset, 1973). The accumulations have a two-fold effect; firstly to depress flow per se by the narrowing of arteries and secondly, to reduce flow by depressing the

elasticity of the vessels. Competent vessels expand during the peak of ventricular ejection (absorbing some of the cardiac output) and then contract again during diastole to sustain diastolic flow. The significance of the loss of this elastic capacity in coronary vessels is very great since the heart tissue is critically dependent upon diastolic flow.

The heart can respond to chronic ischaemia by revascularisation and the development of anast omoses (Charlier, 1971). No such response is possible to acute ischaemia caused by events such as coronary thrombosis - the blockage of a vessel by a fragment of solid material, usually sloughed off from atheromatous accumulation. The tissue affected by acute restriction of flow is rapidly depressed mechanically (within minutes) and suffers irreversible damage if flow is not restored within 30 - 60 minutes (Oliver, 1972). This is the process of acute myocardial infarction. Evidence has been presented which shows that myocardial infarction may also occur in the absence of thrombosis (Erhart, Lundman and Mellsteat,1973).

The effects of chronic ischaemia have received little experimental attention because of the time required and uncertainty concerning the nature of the causative factors. Attempts have been made, however, to mimic the clinical situation (Aubert, Ferrand, Lacaze, Pepin, Panak and Podesta, 1974). The major tools for the experimental

investigation of myocardial ischaemia include acute occlusion of vessels - usually in the dog heart in situ (Braunwald, Maroke and Libby 1974) but also, more recently, using rat hearts (Kannengeiser, Lubbe and Opie, 1975). Another technique is the imposition of whole heart ischaemia by reduction of gross coronary flow. In addition, studies of hypoxia have provided a considerable amount of information although the interpretation of this in relation to ischaemia is not straightforward, as will be seen below.

2. Mechanical responses of the myocardium to ischaemia

The onset of ischaemia is accompanied by a marked depression of the performance of the affected tissue. This has been observed in human anginal patients (Cohen, Elliot, Rolet and Gorlin, 1965) as assessed by ventricular pressure development and cardiac output. In the perfused rat heart subjected to ischaemia, systolic ventricular pressure (and tension) and cardiac output decline, and end diastolic pressure rises, (Opie, 1965; Neely, Rovetto, Whitner and Morgan, 1973; Kannengiesser, et al, 1975; Fisher, Martino, Harris and Kavaler, 1969). Similar responses to hypoxia and anoxia have been described by Fisher and Williamson (1961); Sonnenblick (1973); Henderson and Brutsaert (1973); Scheuer (1972); Su and Friedman (1973); Maher, Goodman, Bowers, Hartley and Angelakos (1972).

In the dog heart Theroux, Franklin, Ross and Kemper (1974) and Pirzada, Hood, Messer and Bing (1975) have shown depression of ventricular wall tension development using sutured strain guages. Bishop, Kaspar, Barnes and Kardon (1974) showed depression of systolic pressure development and cardiac output and a rise in wall stiffness and diastolic pressure, in the heart of the conscious dog. Similar studies have been reported by Redwood, Smith and Epstein (1972) and Regan, Markov, Burke and Oldewurtel (1970).

Despite the depressed mechanical performance of ischaemic tissue, the overall performance of the heart as assessed by cardiac output may be maintained by compensatory mechanisms. This has been shown in human patients (Hamosh and Cohn, 1971) and in experiemntal studies (Feola, Haiderer and Kennedy, 1971). This compensation was studied in the heart of the anaesthetised dog by Lekven, Mjøs and Kjekshus (1973). Total coronary flow could be reduced by 20% before cardiac output declined. The compensation involved reduction of contractility (measured by the rate of rise of ventricular pressure - dP/dt) but also ventricular dilation which provided increased ventricular diastolic filling and hence maintenance of cardiac output. Following these primary responses, extensive and persistant ischaemia leads to cardiac failure.

3. Metabolic Responses to Myocardial Ischaemia

(a) Carbohydrate Utilisation In clinical studies, a shift of myocardial carbohydrate metabolism to the glycolytic mode has been described as one of the major responses to ischaemia (Himwich, Goldford and Nahum, 1934; Dennis and Moore, 1938). The shift is described as a rise in the lactate/pyruvate ratio of coronary venous blood, as the appearance of nett lactate release or as a change to a less positive arterio-venous difference of lactate (Krasnow, Neill and Messer, 1962; Bowansa, Arbogast, Goulet, Campeau and David, 1973 and Neill, Kremkau, Oxendine and Phelps, 1974). These methods have been criticised by Kubler (1974) since the concentrations of metabolites in the blood may be a poor reflection of those in the tissue.

The responses of the heart to ischaemia and hypoxia differ markedly with respect to glycolysis, as described in experiemntal studies. With hypoxia and anoxia, glucose uptake (Scheuer, 1967, 1972) and lactate release (Morgan et al, 1961) rise sharply. The importance of glycolysis in the preservation of function and the ability to recover from hypoxic interventions has been described by Weissler, Altschuld, Gibb, pollack and Kruger (1973); Weissler, Kruger, Baba, Scorpelli, Leighton and Gallimore, (1968) in the rat heart and by Muller Ruchholtz, (1973) and Henry, Sobel and Braunwald, (1974) in the guinea pig heart.

The importance of glycogen has also been implicated by the ability of the turtle heart to withstand hypoxic perfusion more effectively than the rat heart due to more extensive glycogenolysis (Brachfeld, Ohtaka, Klein and Kawade, 1972; Bing, Brooks, Inamadar and Messer, 1972).

The effects of ischaemia upon substrate utilisation have been contrasted with the effects of hypoxia by Rovetto et al (1973). Although a transient rise in glucose uptake occurred, it was maintained for only a short period. Extensive lactate accumulation occurs within the tissue since little capacity for release into perfusate is provided (unlike hypoxic challenge) and this could account for the inhibition of glucose utilisation at two metabolic sites. The decline in cellular pH would tend to inhibit PFK and the incapacity to reoxidise NADH by the lactic dehydrogenase reaction would cause inhibition of glyceraldehyde-3-phosphate dehydrogenase by depressing the availability of NAD. Such inhibition depresses the utilisation of both glucose and glycogen. This evidence seems to be contradicted by the evidence of Opie, Owen and Riemersma (1973) which shows a rise of glucose uptake in the ischaemic segment of the dog heart. In this study, however, glucose uptake was expressed as μmole/ml blood, with no measurement of coronary flow. It is not possible, therefore, to calculate the true rate of glucose uptake in μmole/g/min.

(b) Energy Status During hypoxia the creatine phosphate content of guinea pig heart (Feinstein, 1962) and rat heart (Scheuer, 1972; Neeley et al, 1973) declines rapidly but the content of ATP is maintained at a high level. Despite the high ATP content, contractility is still depressed. Two possible explanations for this observation have been suggested. Firstly, ATP may be depleted near the site at which it is required for muscular contraction (Gercken and Schlette, 1968; Gudbjamason, Mathes and Ravens, 1970) or, secondly, some restriction is exerted at the level of energy utilisation rather than energy production (Katz and Hecht, 1969). In ischaemically-perfused isolated rat hearts (Rovetto et al, 1973) the ATP content of myocardial cells declined more rapidly and more extensively than during hypoxic perfusion. This is likely to be a consequence of inhibition of glycolysis during ischaemia which plays a significant role in maintenance of ATP content of hypoxic tissue. This observation offers an explanation of the ability of perfused rat heart papillary muscles and the intact dog heart to withstand periods of hypoxia more effectively than periods of ischaemia (Pirzada et al, 1975).

(c) Enzyme Release. The release of enzymes from the myocardium has been used as an index of ischaemic damage. (Whitby, 1968). The enzymes studied include lactic dehydrogenase, creatine phosphokinase, glutamate - oxaloacetate

transaminase, malate dehydrogenase, aldolase and others (Logan and Murdock, 1966). The use of enzyme release in this way has been criticised by Kubler (1974) on the basis of evidence which shows rapid release of enzymes (w ithin 2 minutes of the onset of anoxia) from the dog heart, presumably before irreversible damage could have occurred. Despite this, the release of enzymes is of considerable diagnostic value and has also recently been studied in acute experimental ischaemia using the isolated rat heart (Leiris, Opie and Lubbe, 1975).

(d) Ionic Disturbances The loss of certain ions (particularly potassium and inorganic phosphate) has been observed from ischaemic tissue (Regan, Markov, Oldewurld and Burke, 1970; Opie , Thomas, Owen and Shulman, 1972). These ionic disturbancess are probably a reflection of the disturbed energy balance of the affected tissue. The elevation of external potassium may be an important factor contributing to the high incidence of arrhythmias in severely ischaemic tissue, particularly after acute myocardial infarction (Brachfeld, 1973).

Disturbances to the ionic balance of ischaemic tissue are reflected in the gross electrical activity of the heart as shown by the ECG. Characteristic changes in ECG (principally of the S-T segment and T wave) are of considerable diagnostic value clinically (Schaefer and Haas, 1962; Julian, 1973; Schamroth, 1973) and of particular use experimentally in the estimation of the extent of ischaemic

injury (Braunwald, et al, 1974; Lekven and Semb, 1974; Mjøs, Kjekshus and Lekven, 1974). The use of ECG in this way is further validated by the finding that the more rigorous estimate of ischaemia of tissue oxygen tension relates closely to observed changes in S-T segment (Angell, et al, 1975).

(e) Lipid Accumulation . The onset of ischaemia with the accompanying increase in the concentration of reducing equivalents within the cell and also decrease in availability of CoASH units provides stimulation of fatty acid esterification. Fatty acids accumulate because of depressed oxidation and depressed capacity for release from the tissue. The reducing equivalents tend to force the production of sn - glycerol - 1 - phosphate by the reaction of glycerol - 1 - phosphate dehydrogenase. Both substrates for glyceride synthesis are thus made available and triglyceride soon accumulates, explaining the appearance of lipid droplets in ischaemic and hypoxic tissue (Bryant, Thomas and O'Neal, 1958; Evans, 1964; Scheuer and Brachfeld, 1966).

(f) Release of endogenous catecholamines Catecholamines stored in preterminal sympathetic fibres of the myocardium are released during ischaemia (Brown and Malliana, 1971; Wollenberger, Krause and Shabab, 1967). The catecholamine stores are depleted as soon as one hour after coronary artery ligation in the dog heart, reaching 75% depletion after 24 hrs. (Russel, Crafoord and Harris, 1961).

The release of endogenous catecholamines (together with the generally high systemic sympathetic activity) following acute myocardial infarction may be implicated in the high mortality associated with the acute episode (Julian and Oliver, 1968). Further evidence is provided by the increase in the extent of ischaemic injury caused by catecholamines during acute coronary artery ligation in the dog heart. (Mjøs et al, 1974; Braunwald et al, 1974). In these studies, beta-blockade offset the effects of catecholamines. The tendency of ischaemic tissue to develop arrhythmias is related to the presence of catecholamines (Mailing and Moran, 1957) and is depressed by the removal of endogenous catecholamine release by denervation (Schaal, Wallace and Sealy, 1969). In other studies of acute ischaemia in dog hearts, the release of succinic and lactic dehydrogenases was abolished in the absence of catecholamines (Herbacznska - Cedro, 1970).

The mechanism by which catecholamines induce such effects is not known although there are several possible contributing factors. Thus catecholamines could act by enhancing MVO_2 and thereby exaggerating the already severe oxygen deprivation of the ischaemic tissue. MVO_2 can be increased by the stimulation of contractility, rate and tension development (Coleman, Sonnenblick and Braunwald, 1971). MVO_2 can also be elevated in the absence of mechanical effects and this can be explained by the increased delivery of FFA due to stimulation of cardiac lipolysis (Challoner and Steinberg, 1965). The significance of FFA in the ischaemic heart will be discussed below.

4. Ultrastructural changes in the ischaemic myocardium

Within minutes of the onset of acute ischaemia, glycogen granules disappear and myofibrils become relaxed when visualised by electron microscopy (Jennings, Baum and Herdson, 1965). Within one-two hours, cells and mitocondria were found to have swollen in the ischaemic rat heart (Bryant et al, 1958). Rather more rapid swelling of mitochondria was observed by Jennings and Ganote (1974) within 30-40 minutes of the onset of ischaemia in the dog heart. The swelling was accompanied by the accumulation of amorphous densities within the mitochondria. Within a similar time interval of 1-4 hours lysosmal disruption was observed (Ricciutti, 1972) and this was one of the earliest structural changes following the onset of ischaemia in the dog heart.

The functional capacity of sarcoplasmic reticulum, assessed by its capacity to take up calcium, was also depressed within 1-2 hours of the onset of ischaemia (Lee, Ladinsky and Stuckey, 1967).

SECTION D Effects of FFA in the Heart

Ischaemia presents conditions which encourage FFA accumulation within the myocardium. Both adipose (Vaughan and Steinberg, 1963) and cardiac lipolysis (Kruger and Leighty, 1967) are stimulated by the presence of catecholamines, thus delivering both exogenous and endogenous FFA to the myocardium. In addition, the capacity for removal of FFA by oxidation is depressed by oxygen limitation (Opie et al, 1973) The extensive lipid accumulation described earlier also indicates the presence of high cellular FFA levels during ischaemia.

1. Effects observed in vitro. With the above factors contributing to an accumulation of FFA within ischaemic tissue, the actions of FFA to be described below could contribute significantly to the deleterious effects of ischaemia.

It should be mentioned that although I have talked of FFA and will describe the effects of FFA as investigated experimentally, other species could contribute to the observed effects. In particular, the contribution of fatty acyl CoA is a possibility in view of the capacity of the cell to activate FFA in the presence of ATP which may not be completely depleted during ischaemia (Neely et al, 1973).

FFA have been shown to depress contractility in the hypoxic perfused rat heart and hypoxic papillary muscles but not in well-oxygenated preparations. (Henderson, Most and Sonnenblick, 1969; Henderson, Most, Parmley, Gorlin, Sonnenblick, 1970; Henderson, Craig, Gorlin and Sonnenblick, 1970; Gmeiner, Apstein and Brachfeld, 1975). The effects were attributed to physio-chemical action of the free acids rather than some oxidation product since the non-oxidiseable pent - 4 - enoic acid had similar actions to palmitic and linoleic acids. Most, Szydlik and Sorem (1972) similarly observed depression of developed tension and elevation of resting tension in isolated rat papillary muscles subjected to periods of hypoxia. The action of FFA was abolished by the presence of glucose in the perfusate. The effects of FFA on contractility and rhythmn of the isolated rat heart were found to be due to molar excess of FFA over albumin in the perfusate, rather than simply to the concentration of FFA alone (Willebrands, Ter, Welle and Tasseron, 1973).

FFA require greater oxygen consumption for each unit of metabolic energy produced - expressed by the P/O ratio - than do carbohydrates. The P/O ratio describes the number of high-energy phosphate bonds produced per unit of oxygen consumed (Mahler and Cordes, 1969). The ratios are 2.8 for FFA but 3.0 for glucose. In addition, FFA oxidation may be still less efficient due to the uncoupling of oxidative phosphorylation (as described by Borst, Christ, Slater and Loos, 1962).

FFA have been found to have a fairly non-specific "detergent" effect which causes enzyme inhibition (Pande and Mead, 1968; Wills, 1961). In more specific investigations hexokinase (Borreback and Spydevold, 1970) and glyceraldehyde - 3 - phosphate dehydrogenase (Nagradova, Psasolova and Kriukova, 1972) were inhibited by FFA.

FFA at low absolute concentrations (μM) but high FFA : albumin ratio (6 : 1) were found to labilise lysosomes and mitochondria obtained from cultured myocardial cells (Acosta and Wenzel, 1974). Labilisation was assessed by the release of marker enzymes. These effects could be explained by interference with membrane integrity - this is consistent with the hydrophobic characteristics of FFA and offers a possible general explanation for the range of apparent toxic effects. The labilisation of lysosomes could be a vital factor since this could eventually induce cell lysis.

FFA and fatty acyl CoA esters were found to inhibit mitochondrial ademine nucleotide translocase at concentrations below those required to induce uncoupling of oxidative phosphorylation (Shug and Shrago, 1973). The significance of this effect during ischaemia is uncertain since FFA seem to exert an oxygen-wasting effect (see below) which is inconsistent with a simple inhibition of energy transport. With effective coupling of oxidation and phosphorylation, this inhibition would be expected to depress oxygen consumption.

2. Effects in intact tissue

The actions of FFA which have been described in the above experimental investigations offer a possible explanation of the gross actions of FFA indicated by the clinical and whole organ studies described below.

FFA have been argued to contribute to the incidence of ventricular arrhythmias and death after acute myocardial infarction (Oliver, Kurien and Greenwood, 1968; Julian and Oliver, 1968; Gupta, Young, Jewitt, Hartog and Opie, 1969; Oliver, 1974) and to ventricular arrhythmias in the dog heart after acute coronary artery occlusion (Kurier, Yates and Oliver, 1971). The protective effect of an antilipolytic agent (5 - fluoro - 3 - hydroxymethyl - pyridine) against ventricular arrhythmias after myocardial infarction was significant provided the control of plasma FFA was good (Rowe, Neilson and Oliver, 1975). FFA have also been shown to reduce the electrical threshold required to provoke ventricular fibrillation in normal dogs and those suffering from experimental myocardial infarction (Kostis, 1973).

FFA have been found to enhance MVO_2 in the heart of the rat (Challoner and Steinberg, 1966[a]) and in the dog heart in situ, where lipolytic agents enhanced MVO_2 (Mjøs, 1971, 1973; Ilebekk and Mjøs, 1973; Ilebekk and Lekven, 1974). The lipolytic agents used - isoproterenol and nicotine - also directly affect contractility but this contribution to MVO_2 was accounted for by the use of an

antilipolytic agent, β-pyridylcarbinol. The abolition of the lipolytic component of the nicotine and isoproterenol challenges significantly reduced the rise in MVO_2. An extension of these studies has shown that lipolytic intervention extends the area of ischaemic injury during acute coronary artery ligation in dogs. The converse saving effect of antilipolytic treatment is also reported (Kjekslus and Mjøs, 1973; Kjekshus, 1974; Lekven, Kjekshus and Mkøs, 1973, 1974). A recent report has shown that the presence of FFA accentuates the release of lactic dehydrogenase from the isolated perfused rat hearts during coronary artery ligation (Leiris et al, 1975).

A number of reports have appeared which contradict the findings reported above. Rutenberg, Pamintuan and Soloff , (1969) and Bucher, (1973) concluded from studies on human patients that FFA levels during the first 24 hours after infarction were not related to arrhythmias, late death or cardiogenic shock. Russo, Friesinger, Margolis and Ross (1970) reported that the elevation of plasma FFA concentration by heparin injection (in 20 patients after acute myocardial infarction) failed to increase the incidence of ventricular arrhythmias. Similarly, FFA were argued not to be arrhythmogenic in dog hearts after coronary artery occlusion (despite the occurrance of ectopic activity in 4 dogs, ventricular tachycardia in 2 and ventricular fibrillation in 2 - 8 of a total of 30). FFA were elevated by the infusion of adrenaline (Opie, Norris, Thomass, Holland, Owen and Van Noorden, 1971).

In other studies, FFA were found not to influence the degree of ischaemic injury produced by coronary artery occlusion in pigs (Most, Capone, Seydlik, Bruno and De Vona, 1974) or to influence MVO_2 in the intact anaethetised dog (most, Lipsky, Szydlik and Bruno, 1973).

The discrepencies between the effects of FFA observed by different workers may be resolved in several cases by the presence or absence of catecholamines together with FFA. The summation of the effects of both catecholamines and exogenous FFA may account for the different experimental results. A possible mechanism for the catecholamine effect is the stimulation of myocardial lipolysis. The possibility of contribution of both externally-applied and internally-generated FFA to the deleterious consequences of ischaemia has been indicated (Mjøs et al, 1973; Oliver, 1973) but no direct evidence for the involvement of myocardial lipolysis is available. The catecholamine effect could also be explained in terms of some other metabolic or ionic effects or by more direct action on the contractile processes.

SECTION E. Lipases

1. General Several discrete lipase activities have been characterised, in work mainly studying the activities of tissues of the rat, particularly adipose tissue. The main activities include lipoprotein lipase (LPL), hormone-sensitive lipase (HSL), monoglyceride lipase and acid lipase. (Pancreatic lipase and intestinal lipase will not be described here). In addition to these discrete activities other activities have been described which may or may not be unique. Thus tributyrinase may be a non-specific esterase (Biale, Gorin and Shafrir, 1968) and Tween hydrolase may be associated with monoglyceridase (Wallach, 1962). The position of diglyceridase activity is also not established (Buchet and Lauwerys, 1971) although recent evidence (Buchet, Roels and Lauwerys, 1974) indicates that in rat heart and adipose tissue monoglyceridase and diglyceridase activities are discrete.

The degree of fatty acid specificity of the lipases has not been investigated extensively. In the studies of Buchet et al (1974) both mono- and diglyceridases hydrolysed a range of substrates equally well, whereas Katocs, Gnewuch, Lech and Calvert (1972) report specific monoolein and monolaurin lipases in rat adipose tissue.

2. Discrete Lipases

(a) Lipoprotein Lipase Sometimes synonomously linked with clearing factor lipase, LPL is only a component (albeit the major component) of the total clearing factor activity. Clearing factor is found in the blood after heparin administration and is able to 'clear' lipaemic serum. Clearing factor contains at least one activity - (monoglyceridase) - which can be distinguished from LPL (Greten, Fredrickson and Leny, 1969).

LPL was first described by Hahn (1943) and was extracted from bovine heart by Overbeek and Van der Vies (1955) and Korn (1955 [a,b]) and from adipose by Korn and Quigley (1957). The enzyme is stimulated by heparin, inhibited by 0.5M sodium chloride and protamine sulphate and exhibits a pH optimum of 8.5. The activity is critically dependent upon the presence of serum protein factors - particularly $alpha_1$ - lipoprotein - pure glycerides being hydrolysed very slowly. The activator has been further characterised by LaRosa, Levy, Herbert, Lux and Fredrickson (1970). Borgstrom and Carlson (1957) found that LPL hydrolysed di- and triglycerides but not monoglycerides and also, using radioactively - labelled substrates, found that reversible exchange of fatty acyl residues was catalysed by LPL. The enzyme could be readily eluted from heart and adipose by medium containing heparin - suggesting a superficial cellular location, possibly in the capillary lining or extracellular space (Cherkes and Gordan, 1959; Feldman, 1960). Despite this, only 50% of

the total tissue activity of LPL was eluted by heparin (Mallov and Alousi, 1964) the rest remaining at intracellular sites. Further, Payza, Eiber and Walters (1967) showed that intracellular LPL was the precursor of the eluted fraction.

LPL is considered to be responsible for the hydrolysis of plasma lipid in order that TGFA may be utilised by peripheral tissues (Robinson and French, 1960). The sensitivity of LPL to the dietary status of the animal has been reported by Robinson and French (1960); Robinson and Jennings (1965) and Borensztajn, Otway and Robinson (1970). During fasting, adipose tissue LPL declines whilst that in the heart increases - consistent with nett lipolysis from adipose tissue but the consumption of TGFA by peripheral tissues. The oxidation of TGFA by isolated perfused hearts from fasted rats is greater than that of hearts from fed rats and further, is inhibited by pre-perfusion with medium which contains heparin (Borensztajn and Robinson, 1970). Further differences between cardiac LPL and adipose LPL with regard to control by glucagon (Borensztajn, Keig and Rubenstein, 1973) and insulin (Borensztajn, Samols and Rubenstein, 1972) have been described.

(b) Hormone-Sensitive Lipase (HSL) HSL differs from LPL in a number of properties. It is a tissue lipase and is not eluted by heparin. It is not stimulated by

heparin and has no requirement for serum factors. HSL exhibits a pH optimum of 6.8 and is inhibited by sodium fluoride but not sodium chloride or protamine sulphate (Rizack, 1961; Ho, Ho and Meng, 1967; Bjorntorp and Furman, 1962).

HSL as rapidly stimulated in the presence of cAMP and ATP or calcium and ATP (Rizack, 1964). It is also activated by incubation of fat pads with medium which contains adrenaline, noradrenaline, glucagon, TSH, ACTH (Vaughan and Steinberg, 1963; Vaughan, Berger and Steinberg, 1964).

The similarity of cardiac hormone-sensitive lipolysis to that of adipose tissue has been demonstrated by studies of heart homogenates and acetone powders (Leighty, 1967; Kniger, Leighty, Schreibman and Weisler, 1968; Mallov and Alousi, 1969) and of the perfused rat heart by Leighty (1967) and Christian, Kilsheimer, Pettett, Paradise and Ashmore, (1968). The inhibition characteristics of the adipose and heart forms of HSL have been shown to be similar (Shimoda, 1968).

The purification of rat adipose tissue HSL has enabled further characterisation. The enzyme appears to be lipid-rich (Huttunen, Ellingboe, Pittman and Steinberg, 1970,a,b) and may well exist in more than one molecular form (Pittman, Golanly and Steinberg, 1972) although the human

adipose enzyme was not thus resolved by affinity chromatography (Verine, Giudicelli and Boyer, 1974).

Studies on the purified lipase of rat adipose have shown (by analogy to glycogen phosphorylase) that the lipase is activated by a cyclic AMP-stimulated protein kinase (Corbin, Reimann, Walsh and Krebs, 1970; Huttunen, Steinberg and Mayer, 1970). Similar results were obtained with lipase from human adipose tissue (Khoo, Fong and Steinberg, 1972). Further, the activation has been shown to be acompanied by phosphorylation of the purified rat adipose enzyme (Huttunen and Steinberg, 1971).

In contrast to the above studies, a novel system of activation has been suggested by Okuda, Yanagi, Sele and Fujii (1970) and Saito, Matsuoka, Okuda and Fujii, (1975) whereby activation is achieved by facilitation of the substrate-enzyme interaction rather than by activation of the enzyme per se. In addition, Okuda and Fujii (1973) present evidence which suggests that the protein content of HSL and LPL are immunologically indistinguishable and that the two activities differ instead in lipid content.

(c) Monoglyceride Lipase Adipose tissue monoglyceridase has been distinguished from LPL by inhibitor and stimulator studies and from HSL on the basis of non-activatability by hormones or cAMP (Vaughan et al, 1964; Charbonnier, Arnaud and Boyer, 1973; Kupieki, 1966; Tsai and Vaughan, (1970). The adipose tissue monoglyceridase was

further differentiated from HSL on the basis of different pH optima and heat-sensitivity (Heller and Steinberg, 1972). The heart monoglyceridase has been studied by Okamoto, Glaviano and Pindoh, (1971) and Yamamoto and Drummond (1967).

(d) Acid Lipase(s) Lipase activity with pH optimum in the region 4.0 - 4.5 have been studied in adipose (Schatz, 1965), liver and kidney (Mahadevan and Tappell, 1961; Guder, Weiss and Wieland, 1969; Teng and Kaplan, 1974) and heart (Weglicki, Owens, Rugh and Sonnenblick, 1974). The enzymes are closely associated with lysosomes and have been termed "cellular clearing factor".

METHODS

SECTION A MYOCARDIAL LIPASES

1. Preparation of Lipases

(a) Pig heart lipases The method is based on that of Reed (1975). Hearts were placed on ice within 15 min of the killing of the animals at the slaughterhouse; a further 45 min was required for transport to the laboratory. Operations were subsequently carried out at 4°. Only the central region of ventricular myocardium was used. The atria and vessels were discarded together with the epicardial and endocardial margins of ventricular muscle which were trimmed away. The remaining bulk of ventricular muscle was cut into small cubes with scissors, rinsed with chilled buffer - potassium dihydrogen orthophosphate (0.15M); disodium hydrogen orthophosphate (0.15), pH 6.8; containing sucrose (o.25M) - and hereafter called Sorensen-sucrose - lightly blotted on tissue and weighed. Approximately 100g of fresh tissue was obtained from each heart. The fresh tissue was mixed with Sorensen-sucrose (100g to a final volume of 500ml) and homogenised in a Braun blendor for a total of 2 min in 15 sec bursts.

The crude homogenate was strained through a single layer of muslin and then centrifuged for 30 min at 1000g and at 4°. The pellet of cell debris and nuclei was discarded after washing with a further 100ml Sorensen-sucrose and the supernatant freed of suspended material by straining through two layers of muslin. The pooled 1000g supernatants

were recentrifuged at 4° for 30min at 105,000g. The supernatant when used, was stored at 4°, the pellet was washed with Sorensen-sucrose and finally Sorensen-sucrose containing 4mg/ml low-fat bovine serum albumin to remove FFA. The pellet was finally suspended in 10ml Sorensen-sucrose at a protein concentration of 40-60mg/ml and stored at 4°. The lipase activity was stable for about one week.

(b) Rat Heart Lipases Male Wistar rats weighing 180-250g were fed ad libitum on laboratory chow (Oxo diet 41B - modified) and killed by decapitation between 9 a.m. and 11 a.m. The heart was quickly removed, rinsed with cold Sorensen-sucrose and the atria and vessels removed. The ventricular muscle was cut finely with scissors and homogenised by 10 passes of a motor-driven teflon pestle (Potter-Elvejelm at 2,000rpm). The clearance between pestle and mortar was 0.0075". The mortar was supported in an ice bath. The subsequent procedure was the same as that described for the pig heart, up to the 100,000g pellet which was used as the enzyme preparation. Two or three rat hearts were required to give sufficient lipase activity.

2. Preparation of Lipid Substrates

(a) 'Ediol' A commercial coconut oil emulsion, 'Ediol' was diluted either 1 volume in 10 or 1 volume in 20 with Sorensen-sucrose and gently homogenised with a motor-driven pestle and mortar (500rpm, clearance of 0.0075").

(b) Pure glyceride substrates The solid (or liquid) substrate was added to Sorensen-sucrose, heated until molten (about 60°) and homogenised by 10 passes of a motor-driven pestle and mortar at 3000rpm with a clearance of 0.0075". The pestle was then driven at about 500rpm until the emulsion had cooled to about 30°. Such emulsions were stable for only 2-3hr, so the possibility of further stabilising them was investigated. The final procedure adopted involved the addition of Tween-60 (0.5-1.0mg/ml) to the substrate mixture prior to homogenisation. (Gum acacia at 2.5g% did not stabilise the emulsions). After homogenisation the emulsions were further treated by ultrasonication.

The emulsion was sonicated at 17kcs for a total of 45 sec in 15 sec bursts. The cooling of the emulsion during sonication and treatment for more than 90sec were both found to adversely affect the stability of the emulsions.

Monoglyceride substrates were usually prepared without the necessity for Tween or sonication since they are themselves good emulsifying agents, with the necessary balance of both hydrophilic and lipophilic moieties.

The presence of Tween required an additional control experiment since it was itself hydrolysed by the lipase preparation, releasing FFA. (The Tweens are a series of polyhydric alcohols esterified with long-chain fatty acyl residues).

3. Determination of Lipase Activity

(a) Conditions of Incubation Four component solutions, each made up in Sorensen-sucrose, were mixed in equal volumes (1ml) to give the final incubation mixture. Figures in parentheses indicate the final concentration in the incubation mixture as opposed to the concentration in that particular component solution.

(i) low-fat bovine serum albumin (5mg/ml), ATP (0.5mM), theophylline (4mM), $MgCl_2$ (1mM).

(ii) Substrate (10-12mg/ml).

(iii) Lipase preparation (100,000g pellet diluted either 1 volume in 5 or 1 volume in 10 with Sorensen-sucrose).

(iv) Sorensen-sucrose containing any additional factors e.g. cAMP (50μM)

The reaction was started by the addition of lipase. Reagents were pre-equilibrated at 37° and the incubations were carried out aerobically at 37° with shaking in stoppered flasks. The final volume was usually 5ml and 0.5-1.0ml aliquots were withdrawn at various times for analysis of glycerol release or, more commonly, FFA release.

(b) Glycerol Determination The method is based on that of Turner (1973), glycerol being oxidised directly by glycerol dehydrogenase (from Aerobacter aerogenes) in the presence of NAD^+.

$$\text{Glycerol} + NAD^+ \xrightarrow[\text{GDH}]{} \text{dihydroxyacetone} + NADH + H^+$$

The reaction was followed by the rise in absorbance at 340_{nm} due to the production of NADH. The rate of production of NADH was measured over the first 3-5min of reaction at room temperature with an automatic recording spectrophotometer, which gave a full-scale chart deflection with 0.100 A_{340nm}. The curvette contained:-

100μl samle (or 0.1 - 1.0mM stock glycerol)

100μl NAD (18mM)

1.8ml buffer (0.05M glycine pH 9.5)

The reaction was started by the addition of 25μl of GDH (4mg/ml freeze-dried powder). The enzyme is absolutely

dependent upon the presence of K^+ and thus potassium hydroxide and not sodium hydroxide must be used to adjust the pH of the glycine buffer. The GDH was a gift from R.S. Eisenthal of this department. The method, whilst less convenient for large numbers of assays than that of Wieland (1962) is convenient for small numbers and is very much less expensive. The method is sensitive to glycerol concentrations of 0.1 - 1.0μmoles/ml. The variability between duplicates is ± 6%.

(c) Determination of FFA Compared to many standard enzymic assays (e.g. those for glucose and lactate) the methods available for the estimation of FFA are rather less reproducible or considerably less convenient to perform. Beacuse of this, several methods of assay have been tested in the course of the work in order to achieve the most convenient and the most reproducible.

(i) The first assay system used was the Mosinger (1965) colourimetric modification of the titrimetric procedure of Dole and Meinertz (1960). The FFA was extracted as in the Dole method but then was reacted with sodium barbitone to produce diethylbarbituric acid which converted the indicator phenol red into its yellow acid form. A fall in absorbance measured at 560nm indicated the presence of FFA. The great disadvantage of this method was interference from atmospheric carbon dioxide which also converted the indicator to its acid form. This necessitated the purging of the final coloured solution with nitrogen - ideally in the

cu vette. This was a tedious procedure and also introduced the errors from vaporisation of the final mixture. The zero - FFA value (in this case the highest observed absorbance) was difficult to determine, being rather variable (variability between duplicates was commonly $\pm$ 15%). The assay procedure was as follows:- 1ml sample of lipase incubation (or standard 0.05 - 0.5 mM palmitic acid in heptane) was mixed with 5ml of extraction medium and shaken for 2 min. The extraction medium was propan - 2 - ol : n-heptane : sulphuric acid (1N) in the volume ratio 40 : 10 : 1. A further 3ml n-heptane and 2ml water were then added to the mixture and the whole shaken for 2 min. The mixture was allowed to settle and 2 x 1ml aliquots of the upper (heptane) layer were mixed with 1.5ml of the colour reagent. After 10min the mixture was purged with N_2 (scrubbed in sodium hydroxide (1N) and then heptane) and the absorbance measured at 560nm against n - heptane: ethanol (2:1, V:V). The colour reagent was made up as a stock solution from which the working solution was diluted just before use. The stock solution was prepared by mixing 100mg phenol red (suspended in about 1ml ethanol) with 9ml water, containing 0.25g sodium barbitone. The working solution was prepared by mixing 1ml stock colour reagent with 99ml ethanol and 200ml n-heptane.

(ii) The second method used was that of Duncombe (1963) modified by the phase-inversion solvent of Lauwerys (1969). The estimation is based on the formation of copper soaps of the FFA which are extracted into chloroform and thus

draw copper into this organic phase. The copper content of the chloroform is an estimate of FFA and is detected with a highly-coloured copper-sensitive reagent. The copper reagent employed was diethyldithiocarbamate.

(iii) A modification of the above procedure using the more sensitive diphenylcarbazide as copper reagent was described by Mikac-Devic, Stankovic and Boskovic (1973). This method is the one used most extensively and it is described as follows: to 0.2ml of sample (or standard 0.05-0.5mm palmatic acid in n-heptane) is added 1ml of sodium chloride solution (5g/100ml water) and 1ml copper reagent, which is freshly prepared by mixing equal volumes of two stock solutions - aqueous triethanolamine (M) and cupric nitrate trihydrate (0.22M). The mixture is shaken with an automatic tube-shaker for 2min in stoppered glass tubes, 4ml of chloroform added and the mixture shaken again for 5min. The mixture is centrifuged at 1000g for 5min to effect a clear separation of the chloroform and aqueous phases (breaking any emulsion which may have formed during the shaking) and compacting the intermediate layer of precipitated protein. The copper layer floats on the chloroform in this method (unlike the Lauwerys phase-inversion, so-called because the aqueous phase contains a very high salt concentration to make it more dense than chloroform) and is thus removed by suction. 2ml of the chloroform is carefully pipetted into a tube containing 0.2ml colour reagent (100mg depheylcarbazide in 10ml acetone) and after 10min the absorption at 550nm is determined. The assay is sensitive to FFA at a

concentration of 20 - 500nmole/ml. The variability between duplicates is $\pm$ 5%.

It is most important to acid-wash the glassware to remove conta minating lipid and FFA (Chromic acid was used routinely). Certain commercial detergents are difficult to remove without extensive rinsing and thus contribute to a high blank. In addition, great care must be taken to avoid contamination of the chloroform by 'wetting' with aqueous copper phase; both centrifugation and careful pipetting - to avoid touching the sides of the tubes - are important in this respect. Tests of recovery using 1 - C - 14 - palmitic acid, gave values close to 100%.

(iv) For the continuation of the lipase investigations a continuous assay would be a great advantage. Thus both control and stimulated (or inhibited) rates could be followed in one reaction without the necessity for multiple sampling. The possible use of a pH stat assay was investigated since the lipolytic reaction involves the production of a proton (or more correctly a hydronium ion H_3O^+). The proton would be available to titration by added alkali to give a measure of the extent of reaction. For the investigation, the model reaction of pancreatin with 'ediol' was used. The reaction indeed proceeded measureably but when aliquots were assayed for FFA the pH stat was found to underestimate the activity. It was thought that the FFA may not have been made available for titration but instead were being held in the lipid micelles. This was confirmed by the addition of agents

such as calcium and serum albumin which were able to expose added FFA for titration. (FFA added in the absence of any such solubilising agent produced virtually no release of titrant). The full theoretical titration of FFA was achieved with 4mM calcium chloride. Magnesium chloride up to 20mM was not fully effective, neither was serum albumin at 10mg/ml. Other emulsifying agents (e.g. deoxycholates) should be tested since it is desirable to eliminate calcium in view of its potential stimulation of hormone-sensitive lipase. The sensitivity of the assay can be made adequate by the use of the appropriate strength of titrant and size of titrant syringe.

(d) Preparation of FFA - serum albumin complex The solid fatty acid is dissolved by heating in one equivalent of sodium hydroxide solution. The hot solution of the sodium salt of the fatty acid is then added dropwise to a stirred solution of defatted serum albumin. A molar ratio of FFA to albumin of two to one should be regarded as maximal, since each albumin molecule has two high-affinity sites for long-chain fatty acids (Solomon, 1968). Solutions are stable at 4^{o} for several weeks. If the advised maximal molar ratio is exceeded, precipitation of the free acid becomes noticeable.

(e) Protein Determination The protein content of lipase preparations was determined by the modified biuret method of Haurowitz (1963), using bovine serum albumin as standard. A linear calibration over the range 0.2 - 2.0mg protein was obtained and the variability between duplicates

was $\pm$ 2%. 1.5ml sample is incubated with 1.5ml reagent for 10min at room tempterature and the absorance at 540nm determined. The reagent is prepared by dissolving 0.75g cupric sulphate pentahydrate and 3.0g sodium potassium tartrate tetrahydrate in 250ml water. To this is added 150ml of 10% ($^{w}/v$) potassium hydroxide and the final volume is adjusted to 500ml with water.

4. Preparation of Protein Kinase from rat heart

The first two steps in the method of Corbin, Reimann Walsh and Krebs (1970) were used. Rat hearts were obtained as for the rat heart lipase preparation and homogenised in buffer at 2^{o} with 10 passes of a motor-driven pestle and mortar. (3,000rpm, clearance of 0.0075".) The buffer used was potassium orthophosphate (10mM pH 6.5), containing EDTA (10mM) sodium chloride (0.5M) and theophylline (1mM). The homogenate was centrifuged at 2^{o} for 30min at 27,000g and the supernutant used as the protein kinase preparation.

SECTION B PERFUSION OF ISOLATED RAT HEARTS

1. Perfusate

(a) Inorganic Salts The following salts were added in the order reported to double-distilled water, each salt being fully dissolved before the addition of the next. The final component, calcium chloride dihydrate, was dissolved in water before adding to the final mixture; thus preventing the mixing of calcium ions and phosphate ions at high concentration. The components were: sodium chloride (118.4mM), sodium hydrogen carbonate (24.9mM), glucose (11.1mM), potassium chloride (4.6mM), magnesium sulphate heptahydrate (1.1mM), sodium dihydrogen orthophosphate dihydrate (1.0mM) and calcium chloride dihydrate (1.9mM). The final ionic concentrations are similar to those reported for rat serum (Krebs, 1950) except calcium (which will be discussed later) and orthophosphate which, if increased, caused precipitation of calcium phosphate.

(b) Serum albumin The perfusate also contained low-fat serum albumin at 2g/100ml. The albumin was dissolved in perfusate and dialysed against a large volume of the same for at least 60hr at 4^{o}. 1g albumin was dialysed against 1 litre perfusate. This extensive dialysis was required to allow complete equilibration of calcium with binding sites on the albumin. Insufficient dialysis gave a calcium-depleted perfusate to which a calcium chloride supplement had to be added in order to maximise the contractility of the perfused

heart. A marked time-dependence was observed; thus after 24hr dialysis a 2mM supplement of calcium chloride was required and after 72hr no supplement was required.(After 48hr the supplement required was 1mM).

(c) Reduction of Lipid content of serum albumin Albumin was washed by the charcoal method of Chen (1967). A solution of 50g albumin in 500ml water was cooled to 2^{o}, 25g of activated charcoal added and the pH adjusted to 3.0 with hydrochloric acid (1N). The mixture was stirred magnetically at 2^{o} for 1hr and the charcoal then removed by centrifugation at 2^{o} for 20min at 20,000g. The charcoal pellet was washed with 100ml acidified water (pH 3.0, 2^{o}). The pooled supernatant fractions were filtered by membrane filtration (pore size 0.45μ) to remove the last traces of charcoal and the pH adjusted to 7.0 with 2N sodium hydroxide. The solution was freeze-dried for storage. The washing procedure reduced the FFA concentration of albumin solution (2g%) from about 30nmole/ml to less than 5nmole/ml - not detectable by the chemical method but by G.L.C.

After dialysis against perfusate, the albumin was filtered by membrane filtration, as above, just before use.

2. Perfusion and analysis of perfusate

(a) Method of Perfusion and design of apparatus The perfusion apparatus is described in figures 10 and 11. It is composed of standard water-jacketed glassware connected by translucent vinyl tubing (i.d. 0.63mm o.d. 1.4mm) and maintained at 37° by a thermostat recirculating pump.

Male albino rats of Wistar strain, weighing 180 - 250g and fed ad libitum (for diet see later) were killed by decapitation. The heart was quickly removed into albumin-free perfusate (ph 7.4, 10°) to arrest heartbeat, rinsed free of blood and the aorta attached to a vinyl cannula with a bulldog clip. The cannula was grade 80 vinyl tubing (i.d. 0.63mm, o.d. 1.4mm) approximately 10mm long, cut obliquely at the end inserted into the aorta (for ease of insertion) and notched 3-5mm from the same end. The heart was then immediately mounted into the perfusion circuit to begin washout perfusion with albumin-containing perfusate, maintained at 37° and gassed with 95% oxygen : 5% carbon dioxide. The time taken betweeen decapitation and the start of washout perfusion was kept as short as possible and was usually about 90sec. During the 10min washout perfusion, the aorta was secured to the cannula with thread (tied just above the notch to prevent it slipping) and the bulldog clip removed. Thread was also tied through the apex of the ventricular muscle and in addition, extraneous tissue carefully dissected away from the atria and aorta. Recirculating perfusion was started by inverting

Recirculating perfusion apparatus for the perfusion of isolated rat hearts by a modified Langendorff method. A = aerator, G = gas flow (95% O_2 + 5% CO_2), P = peristaltic pump, B = bubble trap, M = mercury manometer, C = warming coil, O_2 = oxygen electrodes, E = ECG electrodes, H = heart chamber, T = tension transducer, ST = sampling tap, F = glass fibre filter.

Arrows indicate the direction of flow of perfusate. The perfusate was physiological bicarbonate buffer, containing 11mM glucose and 2g% low-fat serum albumin, pH 7.4, 37^{o}. The total recirculating volume was 30-35ml.

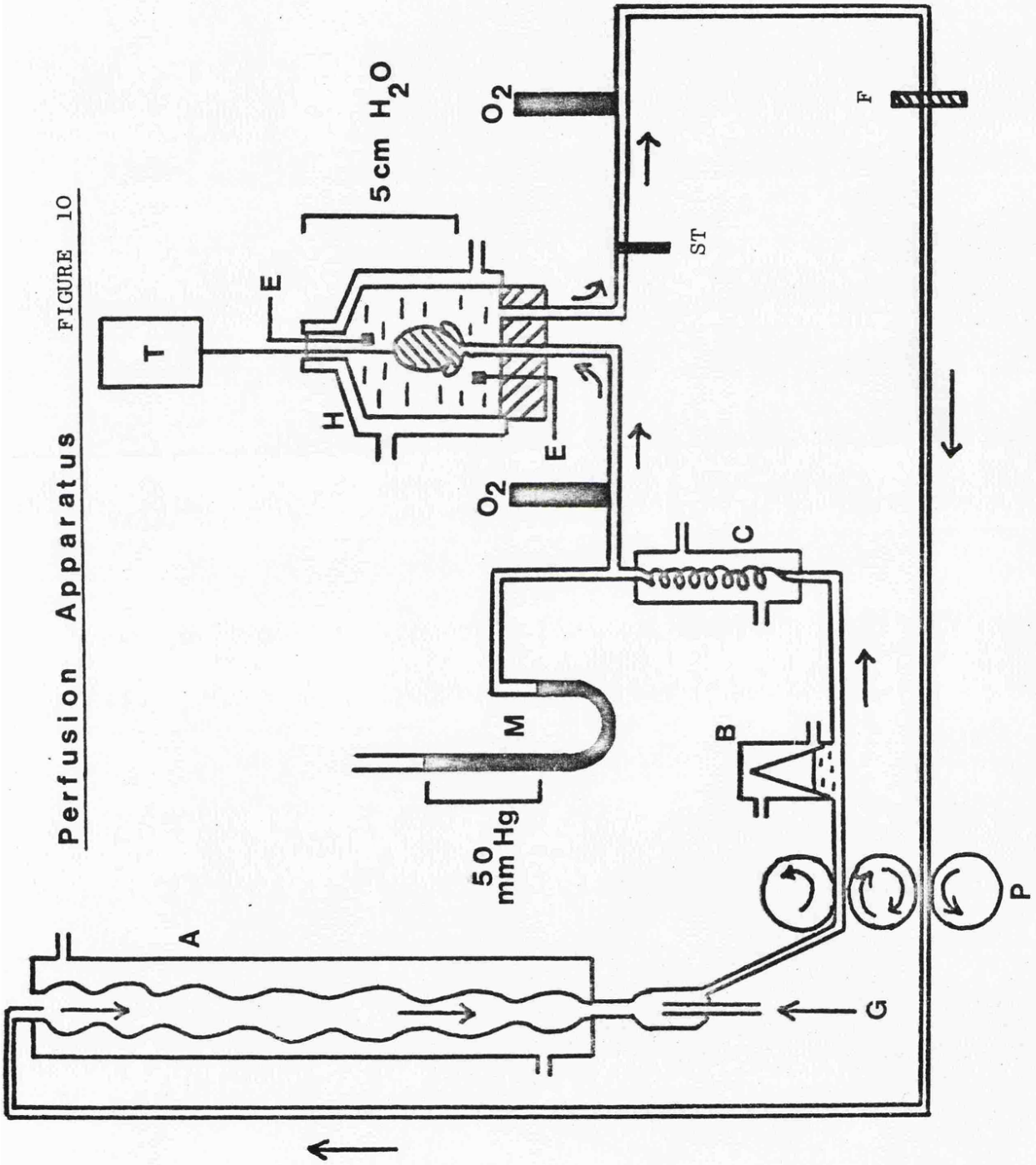
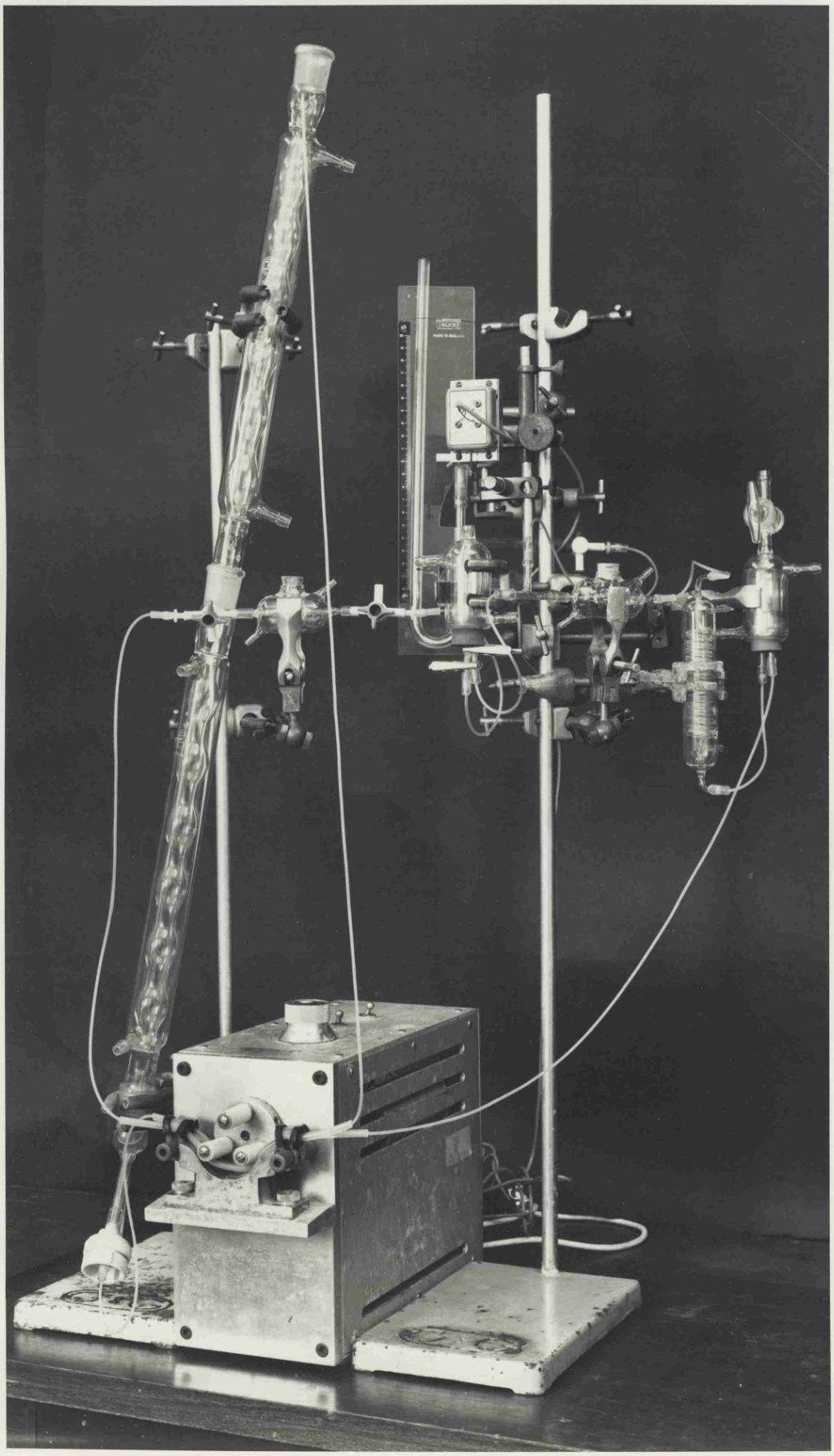


FIGURE 10

Figure 11



the heart into the perfusion chamber and securing the thread from the ventric le to a Dynamometer UF2 strain guage situated above the perfusion chamber. The resting tension of the muscle was adjusted to about 2g when the perfusion chamber had filled with perfusate. Above 2g resting tension there is little further rise in developed tension per unit rise in resting tension. The heart was thus supported both by the fluid in the perfusion chamber and by the strain guage thread. The recirculating volume of perfusate was about 30ml. Agents to be investigated were infused at the point in the circuit most distal from the heart (the aerator column) in order to allow dilution to a known concentration throughout the perfusate. Samples of perfusate were withdrawn from a 3-way tap situated on the 'venous' side of the heart - fresh perfusate was added back to maintain the recirculating volume. For the investigation of tissue metabolites, hearts were removed, blotted on tissue and quickly dropped into liquid nitrogen. The hearts were stored in screw-topped vials at -20^{o}. Extractions and estimations were usually performed within two weeks so that extensive metabolite changes should not have occurred over this period (Mayer, Stull and Wastila, 1974).

(b) Measurement of Mechanical Performance Tension development and ECG were recorded continuously with a two-pen chart recorder. (The tension recorded is essentially isometric or rather isovolumic and the resting tension is taken to be an index of the end-diastolic pressure function).

From the pen deflections heart rate was calculated manually using a stopwatch. An instantaneous ratemeter was used when available. The two methods gave values which agreed within $\pm$ 5 beats/min, up to about 300 beats/min above which rate the manual method was unreliable. A bi-polar ECG was recorded by means of silver electrodes attached to epoxy - resin- coated stainless steel thread. One electrode was situated below the right atrium and the other above the ventricles. The oxygen tension of the perfusate was monitored immediately before and immediately after the heart chamber with modified Clarke oxygen electrodes (Chappell, 1961) and recorded continuously. Electrodes were calibrated before and after perfusions with zero oxygen solution (Radiometer "sulphite" solution) and water gassed with 95% oxygen : 5% carbon dioxide.

Coronary flow was measured by rapidly removing a 1ml sample of perfusate from the sampling tap immediately after the perfusion chamber (thus reducing the level of perfusate in the chamber) and noting the time taken for the level in the chamber to return to its original position. The method was validated by comparison with the values obtained with an electromagnetic flowmeter (used throughout the series in which hearts from fat-fed rats were perfused) - values agreed within $\pm$ 0.2ml/min. Coronary flow measured in this way and perfusion pressure registered on the mercury manometer were noted frequently.

Intraventricular pressure was determined in several experiments by inserting a needle (guage 23G) through the ventricular wall, which sealed around it. The needle was connected by fluid-filled tubing to a pressure transducer and intraventricular pressure recorded continuously.

Arrhythmias (that is, ventricular arrhythmias) became apparent from the tension and ECG traces. The definition of arrhythmias for these purposes was any period during which the time interval between contractions (or ECG spikes) was irregular. Such periods were also manifested by fluctuation in peak developed tension so that an irregular tension trace was obtained. Atrial fibrillation with 3 to 1 or 4 to 1 atrio-ventricular block appeared to be quite common by inspection of the hearts during the perfusion but was difficult to record except with the ECG. Ventricular fibrillation was quite apparent both from chaotic ECG traces and also very poor tension development as well as quite overt rippling of the epicardial surface of the ventricles.

(c) Bacterial Contamination The contamination of the apparatus with bacteria was found to contribute significantly to glucose uptake (Burges and Blackburn, personal communication). To eliminate this interference the apparatus was filled overnight with an antibiotic mixture containing penicillin and streptomycin (each 300U/ml). In addition, the apparatus was washed with chromic acid after 6-8 perfusions and the vinyl tubling replaced.

(d) Determination of the uptake of glucose and the release of FFA, glycerol and lactate Perfusate samples were stored at -20° until analysis (usually within one week). Determinations were performed in duplicate.

(i) Glucose was determined by the glucose oxidase method of Huggett and Dixon (1957), using the Roche test kit. A 20µl sample of perfusate was added to 2ml reagent, incubated for 45min at room temperature and the absorption at 450nm determined. The reagent was 0.12M phosphate pH 7.0, containing O-dianisidine (66mg/ml), glucose oxidase (0.25 mg/ml) and peroxidase (0.05mg/ml)

(ii) FFA) determined as described under
(iii) Glycerol) Section A - 3b and 3c.

(One advantage of glycerol determination by this method is that pretreatment with perchloric acid is unnecessary). Some oxidation of glucose is found in the assay but this is constant despite variation in glucose concentration. It seems, fortunately, that even the lowest glucose concentrations were above the Km of glycerol dehydrogenase for glucose.

(iv) Lactate was determined by the method of Hohorst (1962) modified for reaction rate analysis. The rise in absorbance at 340nm due to NADH production was monitored over the first 3-5 min of reaction at room temperature. The full scale of deflection of the automatic recording spectrophotometer was achieved at 0.1 A340nm.

The cu vette contained:

100µl perfusate (or 5mM lactate standard)

100µl NAD (12mg/ml, 18mM)

1.8ml buffer (0.1M glycine, pH 10)

The reaction was started by the addition of 10µl lactic dehydrogenase (0.1 mg/ml). The assay was sensitive to lactate at 50/500nmole/sample (0.5/5mM) and the variability between deplicates was $\pm$ 5%.

Alternative assays were utilised in the Pfizer laboratories so these are briefly described below.-

(i) The Boehringer test kit for glucose differed in the colour reagent used (being ABTS at 1mg/ml - as described by Werner et al 1970) and the absorbance was thus determined at 436nm.

(ii) Glycerol was determined by reaction rate analysis using the method of Eggstein (1966) with the reaction sequence:

$$\text{glycerol} + \text{ATP} \xrightarrow[\text{kinase}]{\text{glycerol}} \text{sn - glycerol - 1 - P} + \text{ADP}$$

$$\text{ADP} + \text{PEP} \xrightarrow[\text{kinase}]{\text{pyruvate}} \text{Pyruvate} + \text{ATP}$$

$$\text{Pyruvate} + \text{NADH}_2 \xrightarrow{\text{LDH}} \text{Lactate} + \text{NAD}^+$$

The decline in absorbance at 340nm was follwed for 4min at 30°. The full scale deflection of the automatic sampling and recording spectrophotometer was 0.05 A340nm. The cu vette

contained:

50µl perfusate (or 0.1mM standard)

1ml buffer

50µl glycerokinase injected automatically to start the reaction (60µg/ml)

The buffer was a mixture of the following:

20ml 0.1M triethanolamine, 4mM magnesium sulphate pH 7.6

250µl NADH (6mM), ATP (33mM) and PEP (11mM)

100µl pyruvate kinase (1mg/ml) and lactic dehydrogenase (2mg/ml)

Variability between duplicates was better than ± 5%.

(iif) Pyruvate was determined by the method of Bücher (1962). The decline in absorption at 340nm due to the oxidation of NADH in the presence of lactic dehydrogenase was followed for 4min at 30° with an automatic sampling and recording spectrophotometer. (Full scale deflection 0.05 A340nm). The cu vette contained :

100 - 200µl sample (or 50µM standard)

1.0ml buffer (0.4M triethanolamine pH 7.6, 40mM disodium EDTA, NADH (70µM)

50µl LDH (20µg/ml) injected automatically to start the reaction.

Variability between duplicates was ± 5%.

(e) Diet of animals

(i) Normally-fed In most cases animals were fed ad libitum on Oxo diet 41B (modified).

(ii) Fat-fed Rats of Wistar strain (as for the normally-fed group) were fed ad libitum on a diet containing a high proportion of the total calories as saturated fat. The diet was maintained for three weeks prior to use. The diet had no obvious gross effects upon the behaviour of the rats although the weights varied much more within any one group. Special care was therefore required to select rats in the weight range 180 - 250g normally used. The composition of the diet was :

Casein 540g/2kg total

Potato starch 60g

Saturated fat (dripping) 500g

Vitamin mixture 10g

Salt mixture 58g

Avicel (cellulose) bulk mixture 832g

The powdered components (for source of components see Appendix) were blended with molten fat (preheated to 60^{o}) and mixed thoroughly.

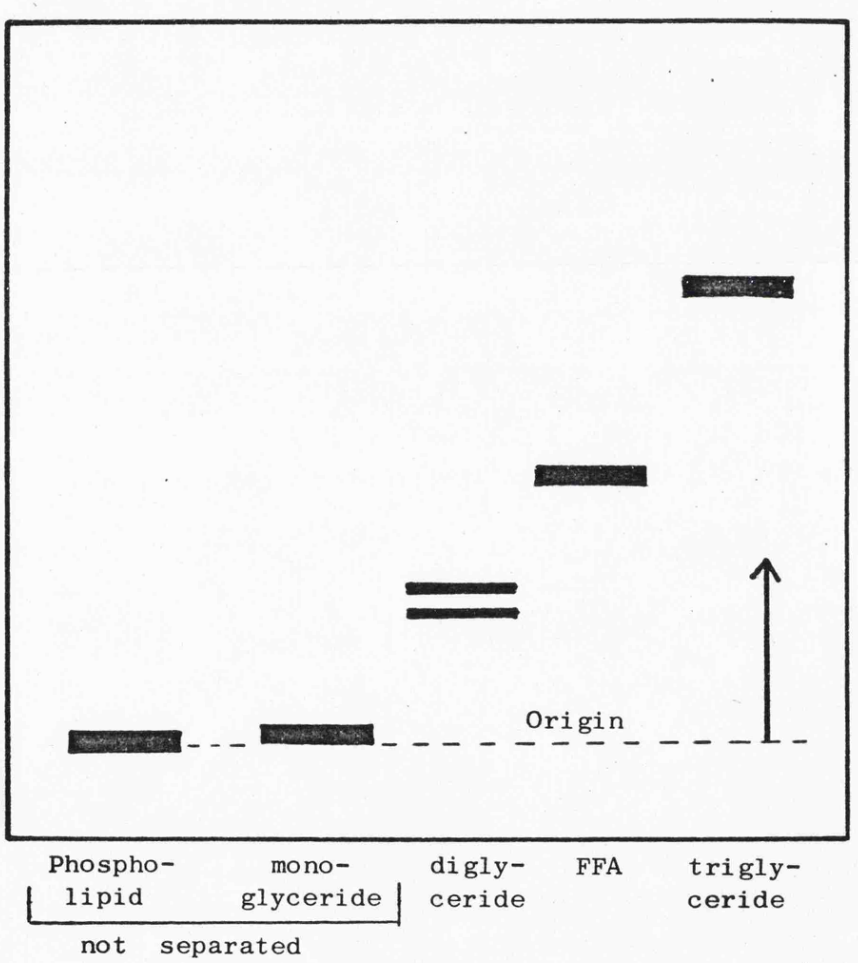
3. Analysis of the content of lipid and lactate of tissue

(a) Tissue Lipid Hearts were frozen immediately after perfusion as described earlier. Lipids were extracted and quantified by the method of Denton and Randle (1965). A weighed quantity of frozen tissue was extracted with 5ml of propan-2-ol : n-heptane : 1N sulphuric acid (in the volume ratio 40:10:1) by grinding in a pestle and mortar on an ice bath. A further 3ml n-heptane and water (0.8 ml/g tissue) were added and the mixture ground again. The total extract was centrifuged for 5min at 3,000g and the upper (heptane) layer was removed and reduced to dryness under a stream of nitrogen. The dried extract was taken up in about 100µl n-heptane and applied to a thin layer plate (20cm x 20cm) coated with a 0.5mm layer of silica gel G. The chromatogram was developed with n-hexane: diethyl ether:methanol:glacial acetic acid (90:20:2:3, v:v:v:v). The plates dried quickly in air at room temperature and were sprayed with dichlorofluorescein (200mg/100ml ethanol). Yellow spots on a purple background were visible under U.V. light (254nm). A separation is shown in figure 12. The spots were scraped from the plate and the lipids eluted from the gel by shaking with 4ml chloroform in stoppered tubes, for 5min. FFA were assayed directly in the chloroform extract as described previously. Glyceride extracts were reduced to dryness under a stream of nitrogen and hydrolysed prior to FFA determination. The extracts were hydrolysed for 4hr at 60°, in the presence of 5ml ethanol and 1ml potassium hydroxide (40% w/v).

Figure 12

Thin layer chromatogram of total lipid extract of rat heart ventricular muscle.

Lipids were extracted by the method of Denton and Randle (1965). Stationary phase was 0.5mm layer of silica



gel G. The chromatogram was developed with n-hexane: methanol:diethyl ether:glacial acetic acid (90:2:20:3 v/v).

The recoveries of known amounts of C^{14}- labelled FFA and triglyceride (as 1 - C - 14 - acid and 1 - C - 14 tripalmitin) added to the tissue prior to extraction were determined. The recovery of FFA was 60 $\pm$ 4% and that for triglyceride 78 $\pm$ 3% (four experiments in each case).

(b) Tissue Lactate Lactate was extracted by the method of Hohorst (1962) and assayed as described earlier. A weighed amount of frozen tissue was dropped into ice cold perchloric acid (6%, $^{w}/v$) and agitated with a glass rod until thoroughly dispersed. Precipitated protein was removed by centrifugation for 5min at 3,000g and washed with ice cold perchloric acid (6% $^{w}/v$). The pooled supernatants were made up to 8ml/g frozen tissue with the same acid. The pH of the supernatant was adjusted to 7-8 with potassium hydroxide (10 N), the solution allowed to stand on ice for 10min and then centrifuged for 10min at 3,000g. The clear supernatant was assayed for lactate. The recovery of lactate is complete (Hohorst, 1962).

(c) Tissue water Total tissue water was determined by comparing the weight of fresh blotted tissue to that of tissue which had been cut finely with scissors and taken to constant weight at 80°.

The total tissue water is distributed between the intra- and extracellular spaces and these two fractions were also estimated. Hearts were perfused with saline which contained 1 - C - 14 - Sorbitol (this is assumed to penetrate

only the extracellular space of the tissue and to equilibrate with the perfusate as a whole). Measurement of radioactivity in the perfusate and in the total tissue thus allows the calculation of the extracellular volume as follows :

$$\text{extracellular volume (ml/g wet weight of tissue)} = \frac{\text{cpm/g wet weight}}{\text{cpm/ml perfusate}}$$

Intracellular volume is then calculated by the difference between total water and extracellular water.

4. Characterisation of FFA and Glyceride - FA by Gas Liquid Chromatography

(a) Preparation of Samples Fatty acids were chromatographed as the methylesters, prepared by reacting and etherial solution of the acids with diazomethane. Perfusate samples (0.2ml) were extracted by shaking for 5min with 4ml chloroform, the organic phase taken to dryness under nitrogen and the dried residue dissolved in diethyl ether.

Tissue FFA and glycerides were extracted, chromatographed and eluted as described earlier. FFA were eluted with diethyl ether: glycerides were eluted, hydrolysed (as described earlier) extracted with chloroform, taken to dryness under N_2 and finally dissolved in diethyl ether.

(b) Preparation of diazomethane Considerable caution must be exercised when diazomethane is used because of its explosive nature. It is rendered harmless with acetic acid (forming methyl acetate) and so glassware is carefully rinsed with acetic acid after use.

Potassium hydroxide (0.4g in 9ml of 95% ethanol) is added to 2.4g N - meth - N - nitrosotoluene - 4 - sulphonamide (in 30ml diethyl ether). The reaction vessel is stood on ice during the addition of reagents but transferred to water (80^{o}) for the distillation of diazomethane which is collected in an ice-cool flask. The distillation is

allowed to proceed until the yellow colouration has disappeared from the reagent mixture. The diazomethane product is redistilled at 50° and collected in an ice-cool flask.

The fresh diazomethane is added dropwise to the etherial solution of FFA until yellow colour persists. The mixture is incubated at room temperature for 20min and then heated over steam to remove excess diazomethane.

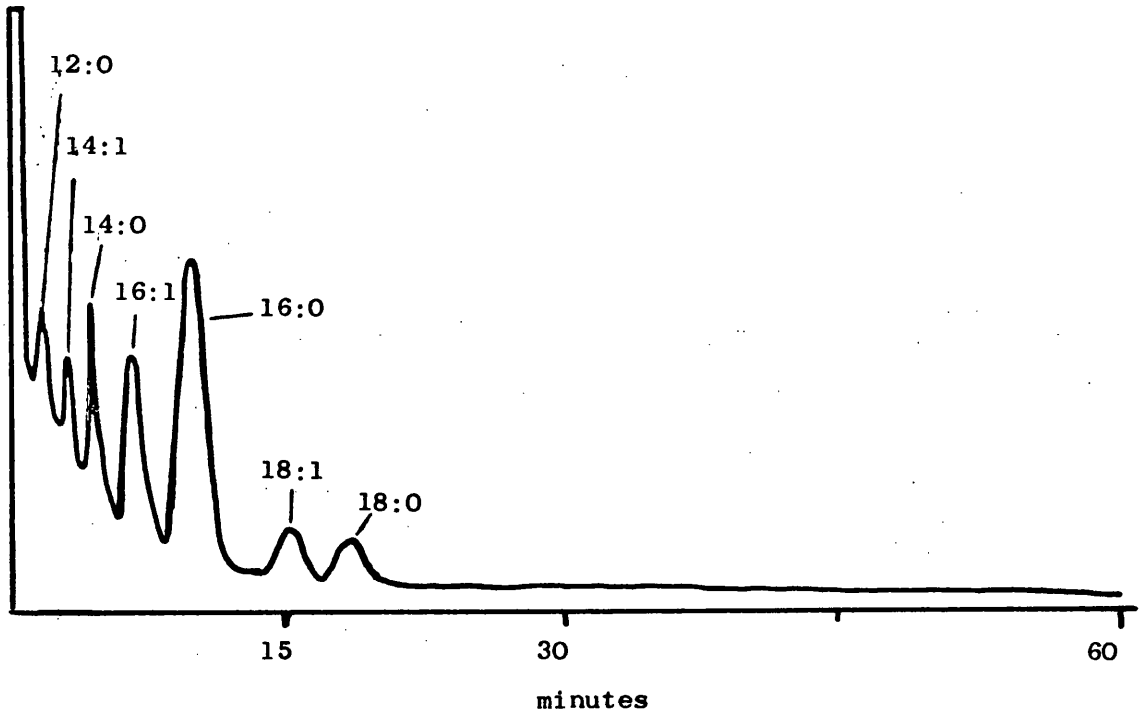
(c) Chromatography The glass column, 3m in length with an internal diameter of 4mm, was packed with stationary phase of Apiezon grease L at 10% on chromosorb 'W' support. The carrier gas was nitrogen. Detection was by flame ionisation. Hydrogen and oxygen were used at 30psi vs 15psi for ignition (temperature 210 - 220°) and at 24psi vs 24psi for operation (at 210°). The total gas flow was 60ml/min.

The samples were injected as 1µl aliquots in n-hexane and compared to pure methyl ester standards. Individual fatty acids were quantified by peak area - assessed as the product of peak height and peak width at half height. This calculation gave a linear calibration for standards over the range 0.1 - 2.5µg fatty acid.

Figure 13

Gas-liquid chromatogram of fatty acid methyl esters.

The esters (approximately 1µg FFA) were applied in 1µl n-hexane. Stationary phase was Apiezon grease L (10%) on Chromosorb W. Carrier gas was nitrogen, total gas flow was 60ml/min. Detection was by flame ionisation.



5. Preparation of Cell-free Haemoglobin solution

The method of Rabiner, Helbert, Lopas and Friedman (1968) was used. Out-dated bank blood was obtained from the local transfusion unit and kept on ice during transport. The whole blood was centrifuged at 4^{o} for 15min at 6,000g. The packed cells were washed three times with sodium chloride solution (1.6% $^{w}/_{v}$) and then lysed by mixing one volume of packed cells with 4 volumes of hypotonic sodium phosphate pH 7.6 (5 milliosmolar or 0.2mM). The mixture was allowed to stand on ice for 1hr with occasional gentle mixing and the cell debris then removed by centrifugation (4^{o} for 30min at 40,000g). The crude haemoglobin solution was dialysed against perfusate for 24hr at 4^{o} and finally filtered by membrane filtration (pore size 0.45μ) before mixing with albumin - containing perfusate to give a final haemoglobin concentration of 5g/100ml (albumin at 2g/100ml as usual).

6. Analysis of Results

(a) Statistical analyses were performed according to the usual formulae for Students t-test and regression analysis as described by Bailey (1968).

(b) Individual calculations

(i) When calculating the uptake of glucose and the release of products (all being reduced to unit wet weight of tissue) it was necessary to account for the nett addition of glucose to the recirculating perfusate involved in the sampling technique (i.e. fresh perfusate is added back to maintain the recirculating volume). Similarly a nett removal of FFA, glycerol and lactate occurs. Thus, for example, glucose uptake (μmole) =

$$(gi - gt+3) . V + (gi - gt+2) + (gi - gt+1)$$

gi = initial glucose concentration (μmole/ml) - first sample of perfusate.

gt+3 (gt+2, gt+1) = glucose concentration of fourth (third, second) perfusate sample.

(ii) Myocardial oxygen consumption, MVO_2, (μl oxygen/min/g wet weight of tissue) is calculated from the equation.

$$MVO_2 = \left[\begin{array}{c} \text{"arterial" oxygen} \\ (\mu l/ml) \end{array} - \begin{array}{c} \text{"venous" oxygen} \\ (\mu l/ml) \end{array} \right] \times \begin{array}{l} \text{coronary flow (ml/} \\ \text{min/g wet} \\ \text{weight)} \end{array}$$

The oxygen concentration in the perfusate is calculated from the Bunsen coefficient, which describes the solubility of oxygen in water at S.T.P. as 0.023ml oxygen/ml water, when water is exposed to pure oxygen. This value is corrected for temperature and solutes in the perfusate and finally the

partial pressure of oxygen which were determined with oxygen electrodes. The "arterial" concentration of oxygen usually 16.2µl/ml perfusate with these corrections made.

An additional point concerning the calibration of the oxygen electrodes is that the electrodes are not exposed to 95% oxygen : 5% carbon dioxide as the highest standard but this gas mixture saturated with water vapour. This reduces the oxygen tension from a theoretical 725mm mercury (95% of 760mm mercury) to 685mm mercury according to Umbreit, Burris and Stauffer (1964).

RESULTS

SECTION A. Myocardial Lipases

1. Summary

The aim of this study was to try to confirm the presence in myocardium, of an activatable lipase system, as previously described by Leighty et al (1968) for a rat heart preparation.

The present investigation confirmed the existence of a lipase system in pig heart homogenate which could be stimulated by cyclic AMP (50μM). The stimulation was apparent with triglyceride but not with monoglyceride as substrate. With rat heart preparations no activation of lipolysis with cyclic AMP could be found unless an addition of protein kinase was made.

2. Pig Heart Lipases

(a) Location of Lipase activity A crude fractionation of the homogenate was performed, giving 600g and 100,000g fractions. The activity of the various fractions towards 'Ediol' is shown in Table 2.

Activity was present in all the fractions tested but only the 100,000g pellet showed an increased rate of lipolysis in the presence of cyclic AMP (50μM). This fraction also gave the most conveniently concentrated form

TABLE 2

Location of cyclic AMP-sensitive lipase of pig heart homogenate. Partial cellular fractionation.

Fraction	Volume (ml)	protein (mg/ml)	specific activity (μmole FFA/hr/mg protein	total activity (μmole FFA/hr)	concentration of activity (μmole FFA/hr/ml)
Homogenate	500	not useful preparations because of high FFA blank values and viscosity			
600g pellet	350				
600g supernat.	150	18	0.062	167.0	1.1
100,000g pellet	10	40	0.058 (0.088)*	23.2 (35.2)*	2.3 (3.5)*
100,000g supernat.	150	8	0.016	30.0	1.3

* plus 50μM cyclic AMP

of activity as judged by activity/ml extract. This fraction was used as the lipase preparation in subsequent studies.

(b) Nature of Activation The typical reaction progress shown in figure 14 reveals the transient nature of activation found with cyclic AMP. The activation was marked for the first 15 min of incubation but fell away - often to rates slower than in the non-cAMP control. This is precisely the effect observed by Leighty (1968) and evidence in that case indicated that a concurrent stimulation of glycogenolysis was providing units of glycerol - 1 - phosphate sufficient to allow re-esterification of some of the released FFA.

To begin to characterise the activatable activity, it was considered that the first step should be to determine the class of substrate(s) hydrolysed. Activity towards 'ediol' was activated but this crude substrate contains three main components - triglyceride, monoglyceride and Tween. Each of these substrates were tested in the pure form in the presence and absence of cyclic AMP (50μM). Glycerides and ediol were presented at 10mg/ml and Tween - 60 at 1mg/ml. It can be seen (Table 3) that activation by cyclic AMP was only detected towards tripalmitin.

Figure 14

Stimulation of lipolysis by cyclic AMP.

Hydrolysis of ediol was by pig heart 100,000g pellet at 37°. cyclic AMP was used at 50μM.

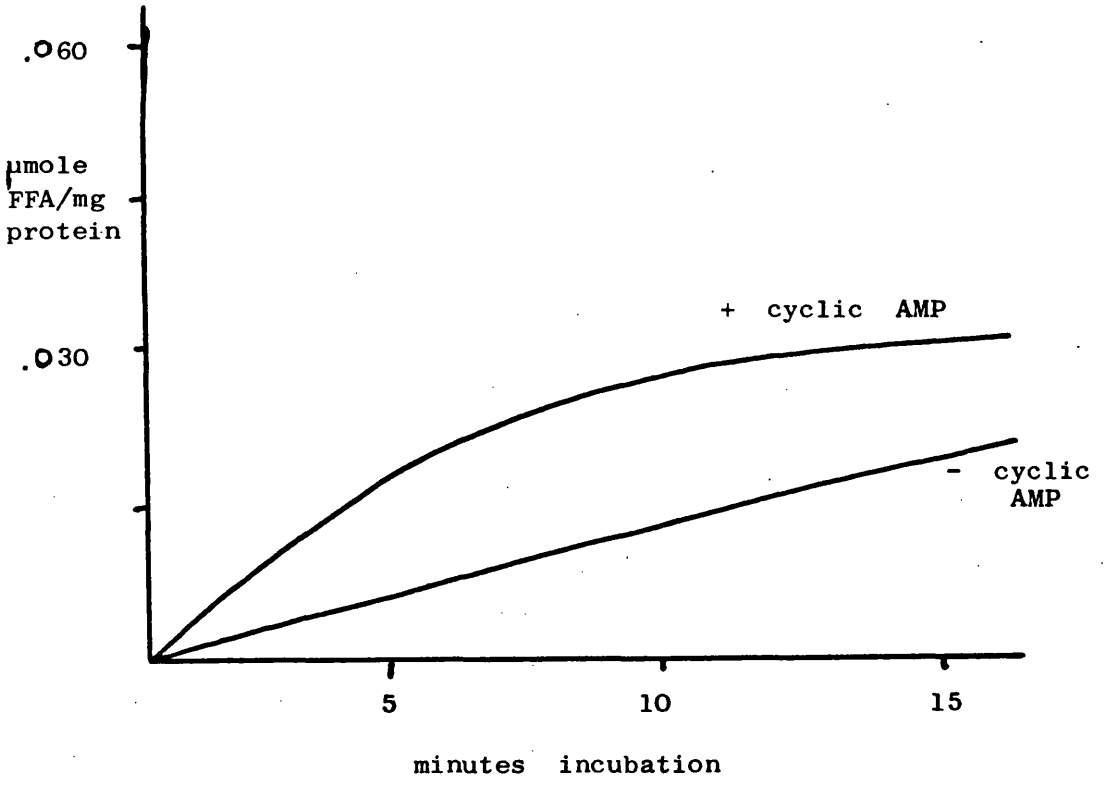


TABLE 3

Substrate specificity of cyclic AMP - stimulated lipase of pig heart homogenate (100,000g pellet)

Substrate	Activity (μmole FFA/hr/mg protein - mean ± S.E.	
	Control activity	+ cyclic AMP (50μM)
Tween - 60	0.032 ± 0.006	0.032 ± 0.006
Ediol	0.058 ± 0.006	0.088 ± 0.006
Monostearim	0.160 ± 0.008	0.160 ± 0.010
Tripalmitin	0.064 ± 0.020	0.168 ± 0.018

n = 5

Tripalmitin was emulsified with Tween - 60 (1mg/ml)

The Tween constribution to the overalll rate has been deducted.

2. Rat Heart Lipases

When pig hearts became unavailble due to the closure of the abbatoir, rat hearts were used instead. On the basis of the above results, the 100,000g pellet fraction was used. The range of activities was found to be similar to that in the pig heart preparation (Table 4) using substrates at the same concentrations as before.

Unlike the pig heart preparation, that of the rat heart was not sensitive to cyclic AMP, even up to 100μM. Since the rate of ediol hydrolysis was rather more rapid with the rat heart preparation it was thought that the lipase system may have been somewhat activated prior to incubation. This might be expected because the decapitation procedure would cause extensive catecholamine release due to sympathetic stimulation. By analogy to the method of Huttunen and Steinberg (1968) who preincubated adipose tissue prior to homogenisation in order to deactivate lipolysis, the heart tissue was incubated in perfusate at 37° for up to 60min before homogenisation. This preinc ubation - did not, however, reduce the lipolytic activity of the heart extract prepared subsequently. Conversely, prior activation of the lipase system by preincubation of intact tissue with (-) adrenaline and (-) noradrenaline (1μM) was similarly ineffective.

TABLE 4

Substrates hydrolysed by lipase preparation of rat heart homogenate

Substrate	Activity (μmole/hr/mg protein) - mean ± S.E.
Tween - 60	0.012 ± 0.002
Ediol	0.164 ± 0.008
Monostearin	0.218 ± 0.052
Tripalmitin	0.042 ± 0.006
Triolein	0.038 ± 0.006

n = 5

Tripalmitin (but not triolein) was emulsified with Tween (1mg/ml). The Tween contribution to the overall rate of lipolysis has been deducted.

To further test for the possibility of activation of the tissue extract, a protein kinase preparation was added in addition to cyclic AMP. The hydrolysis of 'ediol' was indeed stimulated. The rate with lipase and kinase incubated together was 0.196 μmole FFA/hr/mg protein, whilst the sum of the rates of the separately - incubated enzymes was 0.154 μmole FFA/hr/mg protein. The reaction progress was similar to that for the pig heart extract, showing only a short-lived activation at 37^{o}.

This work has since been confirmed in these laboratories (Handley, 1975).

SECTION B The Isolated Perfused Rat Heart as a Model of Ischaemia

1. Summary

The possible deleterious effects of FFA in the ischaemic heart were described in the introduction. The possible contribution of endogenous lipolysis to the FFA pool of the myocardium had been indicated. The presence of a hormone-sensitive lipase system in the heart had been reported previously and was confirmed in this study (Section A). It was considered that the study of the intact tissue would give insight into the involvement of myocardial lipolysis in the metabolism and performance of the heart. The investigation of myocardial lipolysis was particularly directed to a study of its importance in ischaemic tissue. For this reason it was necessary to develop a model system in which lipolysis could be studied and upon which an ischaemic challenge could be imposed. The model which was developed is described in this section. An essential feature of the system is the imposition of low coronary flow as opposed to the use of hypoxic perfusate. The differences between these challenges have been described in the introduction and will be pointed out where appropriate in the discussion.

The results in this section indicate that the model responds in the way expected of ischaemic tissue - as described for the clinical and experimental situations (see introduction). Thus heart rate and tension development are

depressed; metabolism shifts to a more glycolytic mode (and in addition absolute glucose uptake is depressed); characteristic changes in the electrocardiogram develop, particularly changes in T wave and ST segment.

Previous experimental work has investigated ischaemia in an essentially discontinuous manner, but in this model ischaemia of graded intensity is imposed and the corresponding graded responses observed.

2. General

The effects of ischaemia, imposed by reducing coronary flow, were studied in the isolated perfused rat heart. The method of Langendorff (1898) was modified (as described in 'Methods') to give a closed recirculating system in which the heart was immersed in perfusate. The oxygen tension of the perfusate was as high as the system would allow. The perfusate emerging from the bottom of the aerator contained oxygen at a partial pressure of 650 $\pm$ 30 mm mercury; at the point of entry of the perfusate to the heart, this had declined to 560 $\pm$ 30mm mercury.

The effects of coronary flow (expressed throughout as ml perfusate/min/g wet weight) on developed tension, heart rate, oxygen extraction, glucose uptake and lactate release are described. These indices of mechanical performance and metabolism were studied during control recirculating perfusion once the hearts had stabilised following the washout perfusion. Each heart was perfused at one flow rate only; thus each point of the graphs represents one heart. Hearts were perfused at coronary flow rates in the range 2 - 8 ml/min/g. This is a convenient range since below about 2ml/min/g contractility fails entirely, whilst rates above 10ml/min/g are commonly considered to give competent (non-ischaemic) preparations. (Fisher and Williamson, 1962; Shipp et al 1964).

3. Mechanical Reponses to Ischaemia

(a) Heart Rate and Developed Tension Both heart rate and developed tension were directly proportional to coronary flow over the range studied (see figures 15 and 16). The correlation coefficients and probability (P) values were +0.89 ($P < 0.001$) and +0.71 ($P < 0.001$) respectively. The equations which describe the relationships ($y = mx+c$) are as follows:

heart rate (beats/min) = (40.8 x coronary flow (ml/min/g)) + 10.0

developed tension (g) = (9.67 x coronary flow) + (-0.70)

(b) Resting Tension and Arrhythmias Resting tension was stable during control perfusion over the coronary flow range studied. Arrhythmias were infrequent during control perfusion and their occurrence did not show any relationship to coronary flow.

Figure 15

Effect of coronary flow on heart rate in the isolated perfused heart from normally-fed rats.

$P < 0.001$

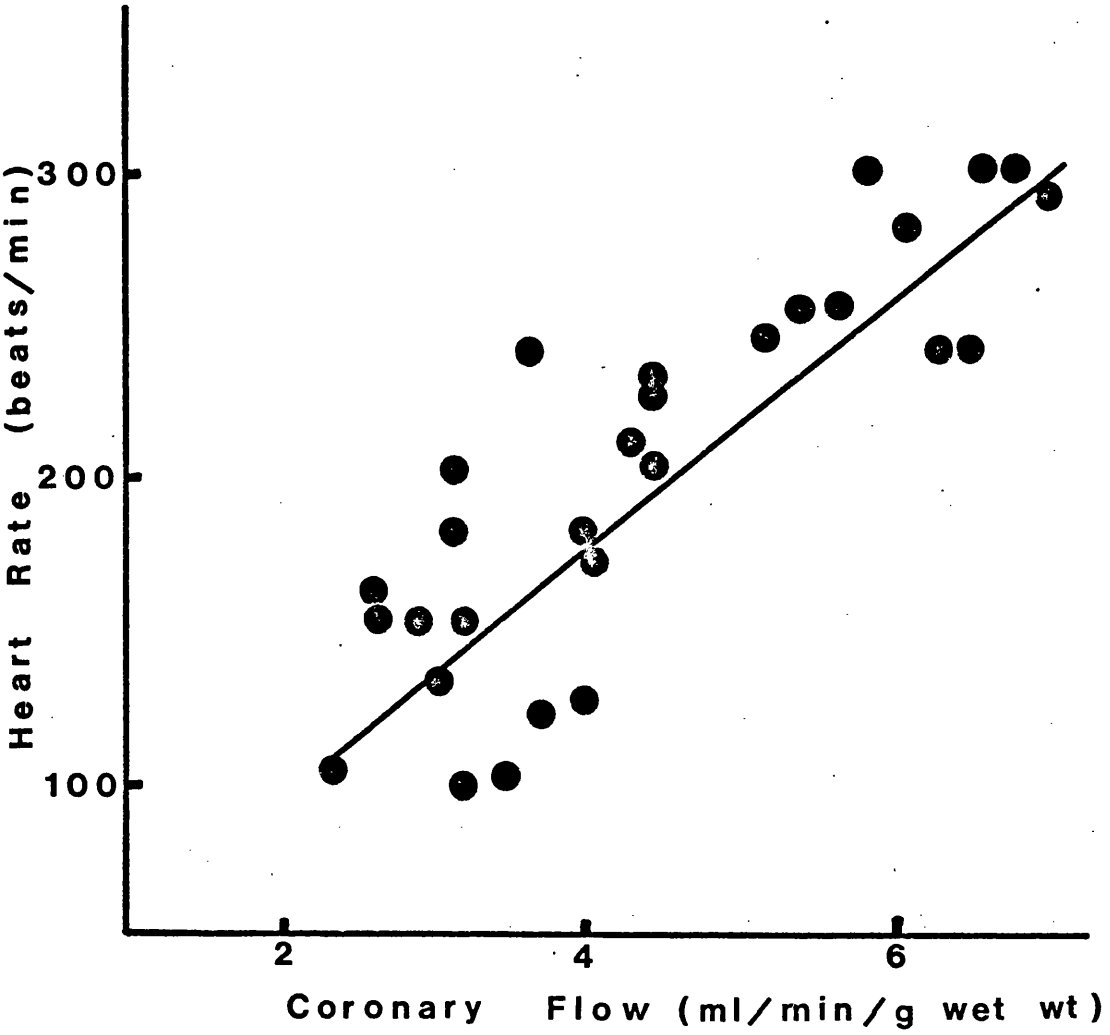
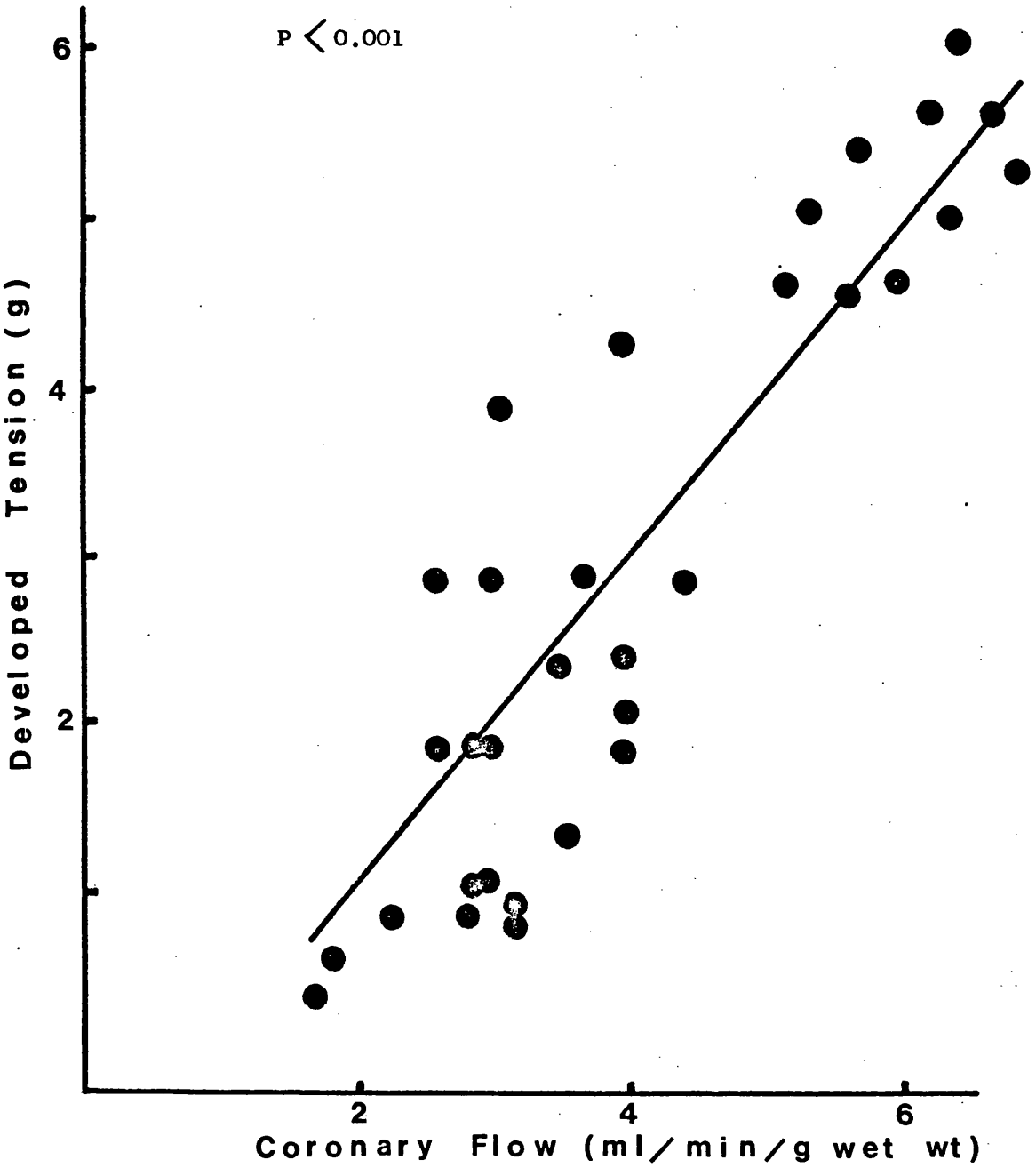


Figure 16

Effect of coronary flow on the tension development of isolated perfused hearts from normally-fed rats.



4. Metabolic Responses to Ischaemia

(a) Oxygen Consumption Hearts responded to the decline in coronary flow by extracting a greater percentage of the oxygen being delivered.

$$\text{Percent oxygen extraction} = \frac{\text{"arterial" } pO_2 - \text{"venous" } pO_2}{\text{"arterial" } pO_2} \times 100$$

The relationship between percent oxygen extraction and coronary flow is described by the equation:

$$\text{Percent oxygen extraction} = (-6.72 \times \text{coronary flow (ml/mn/g)}) + 106.3$$

(n = 24, correlation coefficient = -0.66, $P < 0.02$). Thus oxygen extraction reached 100% when the coronary flow had been reduced to about 2ml/min/g. See figure 17.

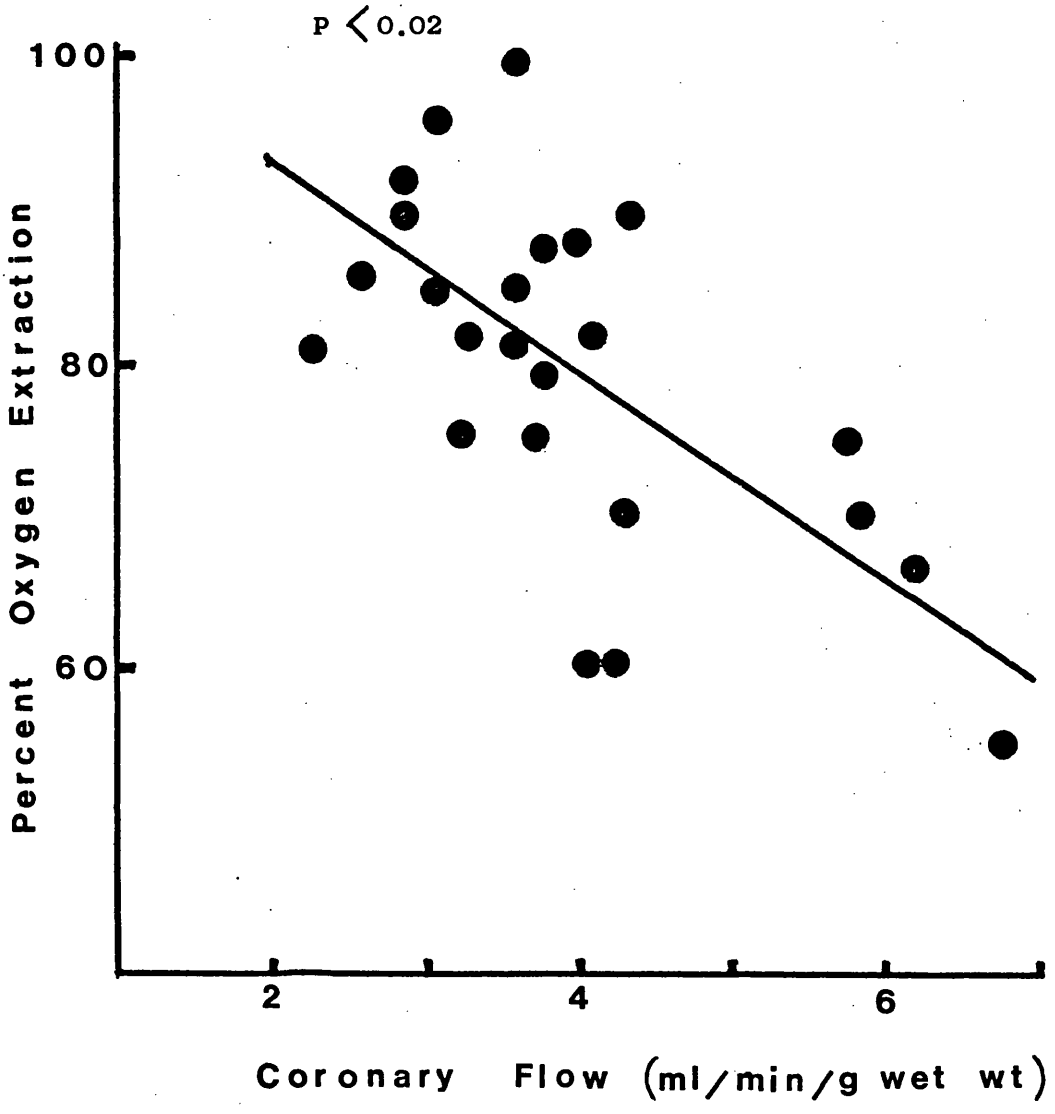
The rise in the percent oxygen extraction was not sufficient to compensate for the decline in delivery of oxygen as the coronary flow was reduced. Thus the total oxygen consumption of the hearts - MVO_2 (µl/min/g) - declined in proportion to the coronary flow (figure 18). The regression equation is :

$$MVO_2 = (8.33 \times \text{coronary flow}) + 4.5$$

(n = 30, correlation coefficient = 0.87, $P < 0.001$). The predominant role of coronary flow over percent oxygen extraction is predictable since the extraction can only rise from 60% to 100% whilst coronary flow is reduced

Figure 17

Effect of coronary flow on the percent oxygen extraction of isolated perfused hearts from normally-fed rats.

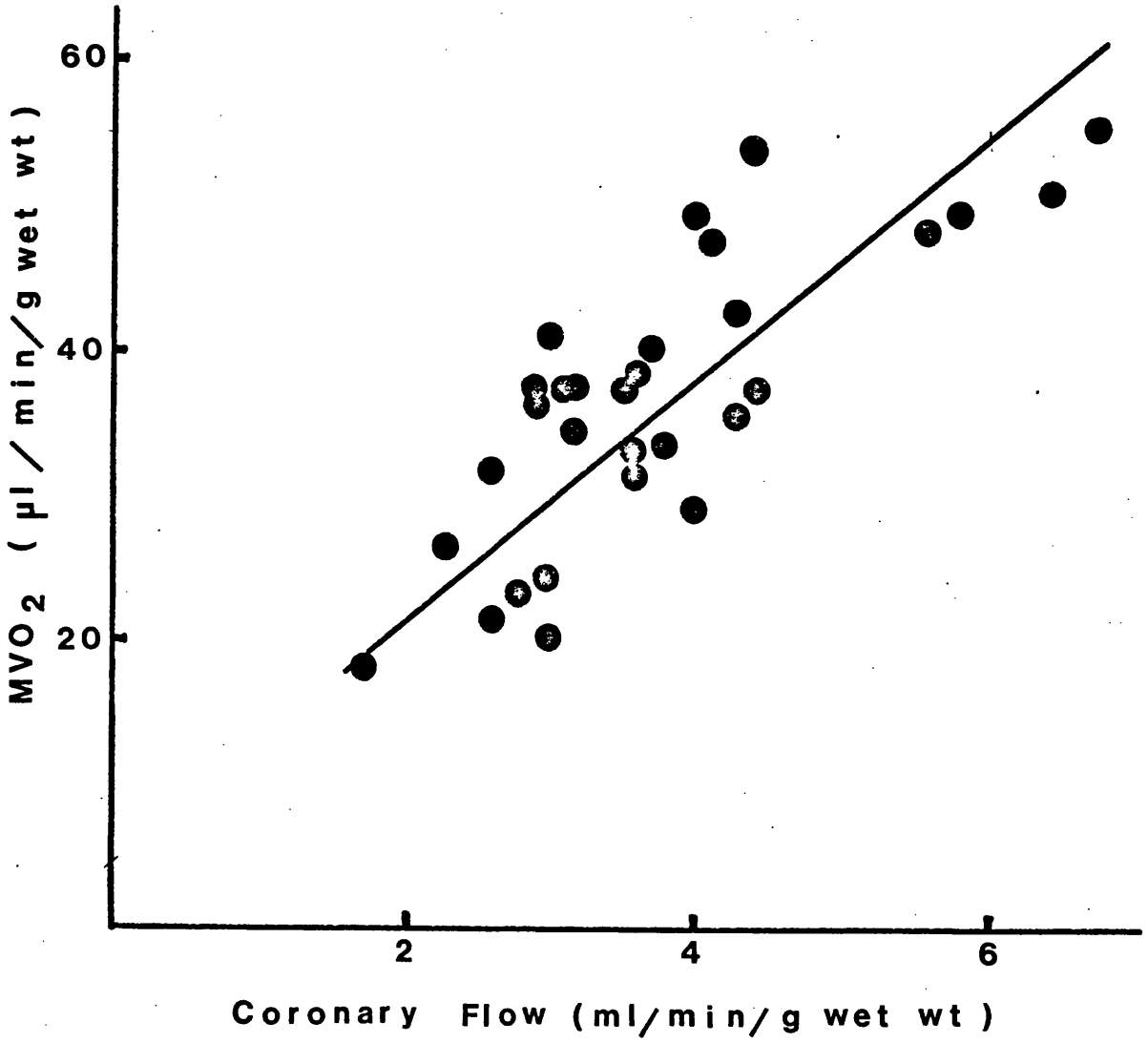


$$\text{Percent extraction} = \frac{\text{"arterial" } pO_2 - \text{"venous" } pO_2}{\text{"arterial" } pO_2} \times 100$$

Figure 18

Effect of coronary flow on MVO_2 in the isolated perfused hearts from normally-fed rats.

$p < 0.001$



more extensively - in this case to about 20% of the peak value.

(b) Lactate Release. Ischaemic tissue is considerably dependent upon glycolysis for energy production (Opie et al, I973). Under our perfusion conditions, lactate release into the perfusate was extensive (mean ± S.E. = 44.1 ± 2.1 μmole/hr/g wet weight). The release of lactate was not statistically related to coronary flow over the range studied. The release at a coronary flow of 2.0 - 3.5 ml/min/g (44.8 ± 7.0 μmole/g/hr) was not significantly different to that observed at a coronary flow of 5.5 - 7.0 ml/min/g (40.0 ± 5.0 μmole/g/hr).

(c) Glucose Utilisation. The effect of coronary flow rate on glucose uptake is shown in figure I9. As coronary flow is reduced, glucose uptake declines according to the equation ;

glucose uptake (μmole/hr/g) = (9.7 x coronary flow (ml/min/g)) + 13.6

(correlation coefficient = 0.57, $P < 0.05$, n = 26).

The decline of glucose uptake over a range of coronary flow which exhibits a more constant lactate release indicates a rise in the fraction of the glucose uptake which can be accounted for by lactate release. The relationship is shown in figure 20, where :

percent conversion of glucose to lactate = (-7.2 x coronary flow) + 64.2

This parameter expresses the fraction of the energy production from glucose which is derived from glycolysis (and

by difference the fraction from glucose oxidation) and is calculated from the equation :

$$\text{percent conversion of glucose to lactate} = \frac{\text{lactate release } (\mu\text{mole/hr/g}) \times 100}{\text{glucose uptake } (\mu\text{mole/hr/g}) \times 2}$$

The calculation does not allow for the contribution of endogenous glycogen, which is expected to be small in the absence of catecholamine stimulation (Gartner and Vahouny, 1969; Shipp et al 1970). The contribution of glycogen may be more significant following adrenaline infusion. By discounting the contribution of glycogen to lactate release, the fractional conversion of glucose to lactate may be overestimated.

(d) Lipolysis Neither the release of FFA nor that of glycerol showed a significant linear relationship to coronary flow over the range studied.

Figure 19

Effect of coronary flow on the glucose uptake of isolated perfused hearts from normally-fed rats.

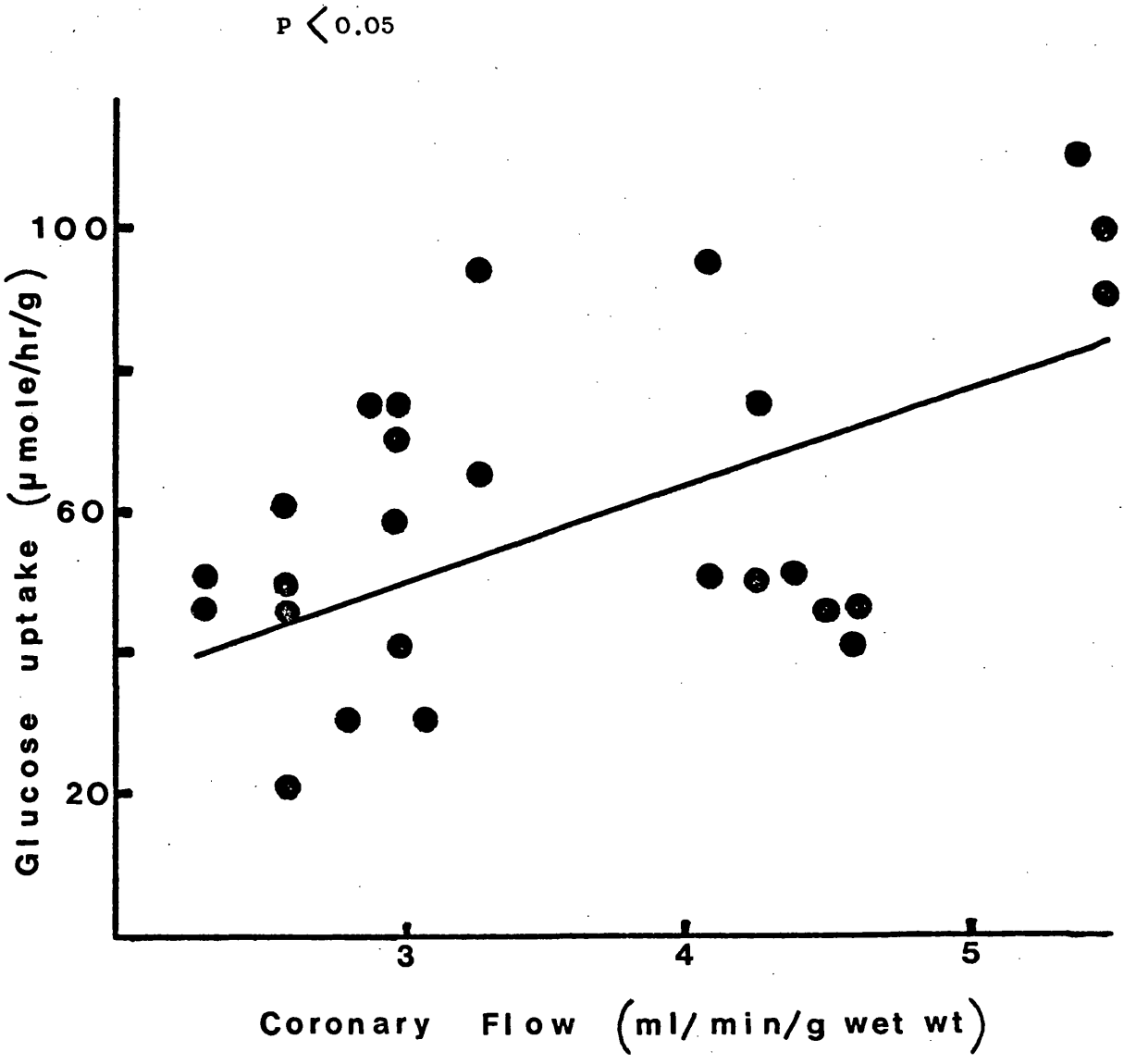
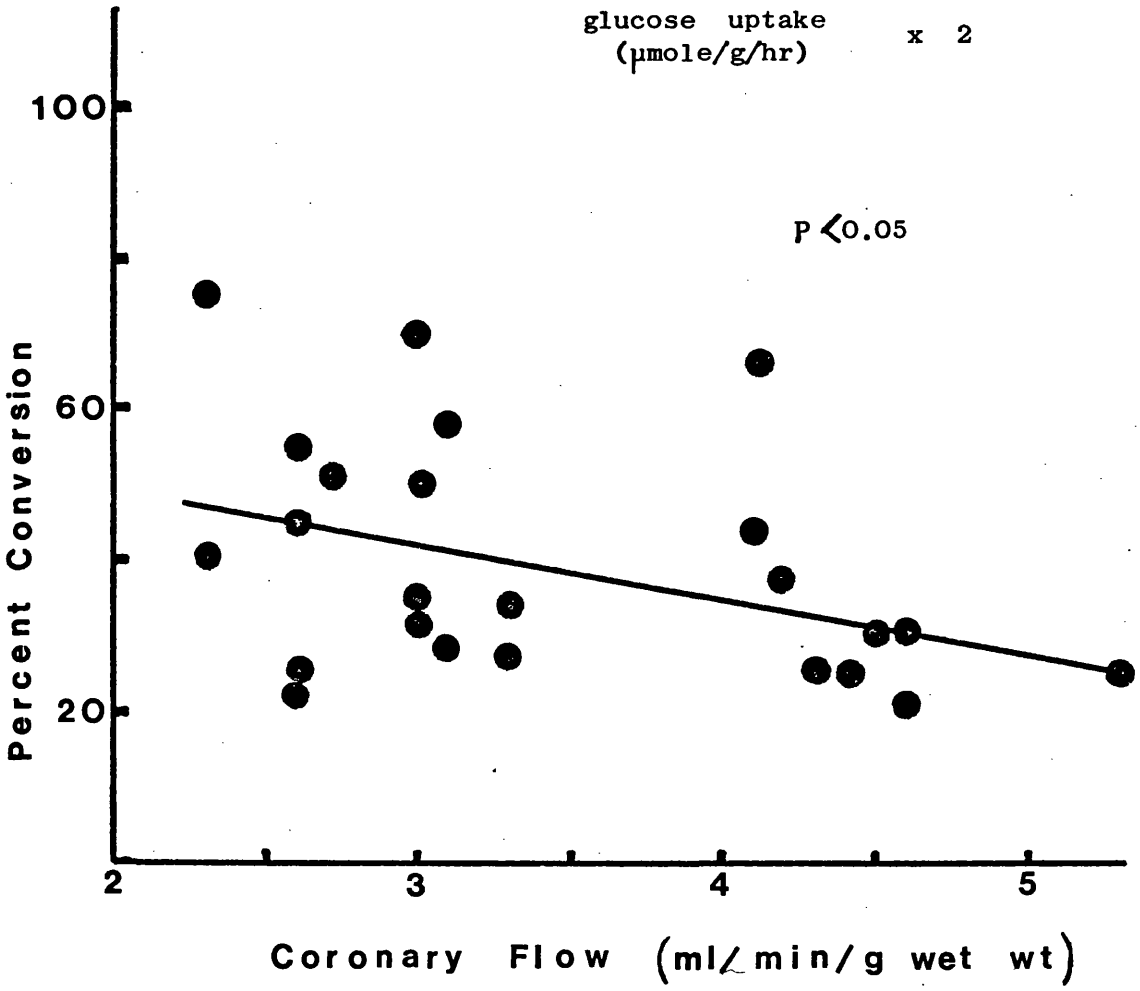


Figure 20

Effect of coronary flow on the fractional conversion of glucose to lactate by isolated perfused hearts from normally-fed rats.

$$\% \text{ conversion of glucose to lactate} = \frac{\text{Lactate release } (\mu\text{mole/g/hr})}{\text{glucose uptake } (\mu\text{mole/g/hr})} \times 100 \times 2$$



5. Electrocardiographic Responses to Ischaemia

Preliminary data are available concerning the effects of coronary flow on the electrocardiogram (ECG). The effects of coronary flow on T wave and S-T segment are shown in Table 5. It can be seen that both factors become more negative as the coronary flow is reduced.

Typical traces are shown in figure 21. These illustrate a reservation concerning the significance of these results - that of signal size, which is rather small using the electrode arrangement described. This could possibly be improved by the use of an epicardial probe electrode to avoid the resistance imposed by the perfusate.

The changes in the T wave were easily measured but those in the S-T segments were more ambiguous considering the small changes involved. The procedure of Schaefer and Haas (1962) was employed to determine the height of the S-T segment. It was assessed by the difference in height between the QR upstroke and the RS downstroke. The zero line was taken as the TQ interval since P and Q waves were not usually observed.

Despite the reservation concerning signal size, the results indicate a marked effect of coronary flow upon the ECG pattern.

TABLE 5

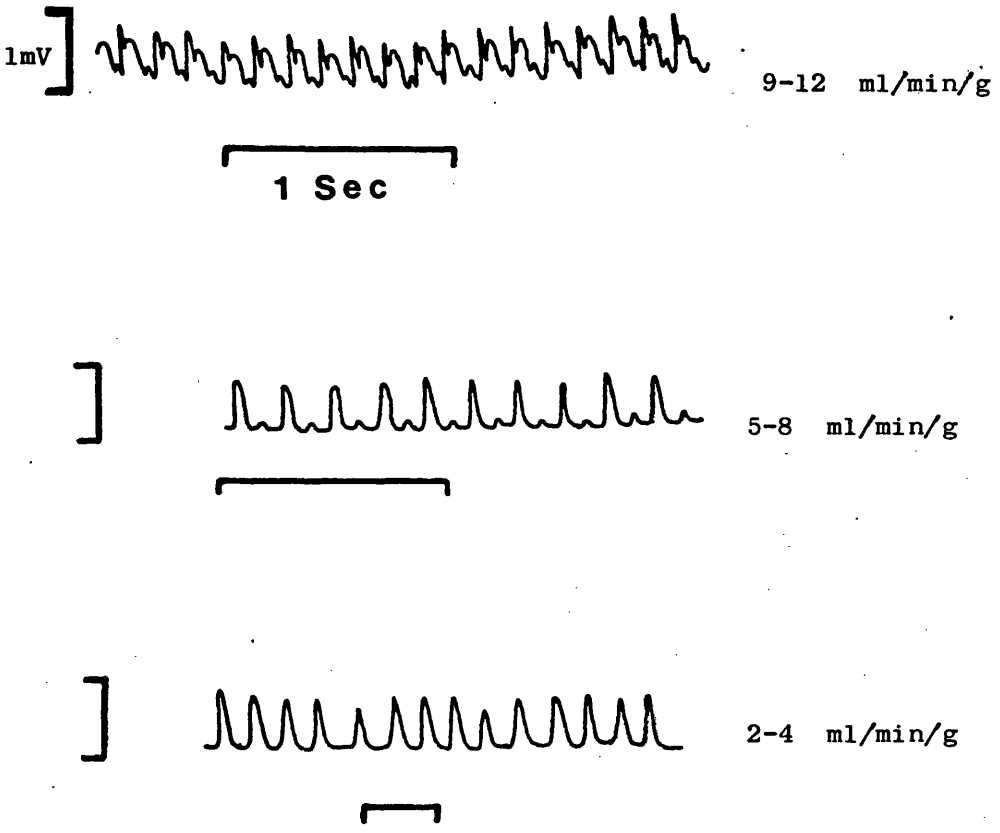
Effects of coronary flow on ST segment and T wave of the ECG of isolated perfused rat hearts.

Coronary flow (ml/min/g)	T wave (mV)	ST segment (mV)
8 - 11 (n = 3)	+ 0.14 $\pm$ 0.06	+ 0.10 $\pm$ 0.01
5 - 8 (n = 9)	+ 0.08 $\pm$ 0.03	- 0.01 $\pm$ 0.01
2 - 4 (n = 9)	- 0.02 $\pm$ 0.03	- 0.03 $\pm$ 0.01
P for difference 2 - 4 vs 5 - 8	$<$ 0.001	$<$ 0.002
2 - 4 vs 10-14	$<$ 0.001	$<$ 0.001

Figure 21

Effect of coronary flow on the bi-polar ECG recorded from the isolated perfused hearts from normally-fed rats.

The traces were recorded in three broad coronary flow ranges : 2-4, 5-8 and 9-12 ml/min/g wet weight.



SECTION C The Role of Myocardial Lipolysis in the ischaemically-perfused rat heart

1. Summary

Having established that the isolated perfused rat heart preparation could be used as a suitable model of ischaemia, the effects on lipolysis in this model were investigated. The results have been divided into four sub-sections. In the first sub-section the effects of adrenaline stimulation are described. The hearts exhibited a biphasic response : in the first phase (2 - 8 min) positive inotropic and chronotropic effects were observed; in the second phase (8 - 30 min) the performance of the hearts deteriorated, thus heart rate and developed tension returned to or declined below the pre-adrenaline control, resting tension rose and the incidence of arrhythmias increased. The rise in resting tension was linearly correlated to the release of FFA and lactate. The decline in developed tension was linearly correlated to the release of FFA.

In the second sub-section the contribution of the recirculating perfusate to the deleterious (second phase) consequences of the adrenaline challenge were investigated. The recirculating perfusate was shown to contribute significantly to the second-phase responses of the hearts to adrenaline, since these deteriorations in performance were greatly reduced by the imposition of non-recirculating

perfusion. The imposition of non-recirculating perfusion was also accompanied by an increased release of FFA and a lower fractional conversion of glucose to lactate.

In the third sub-section the effects of exogenous FFA and lactate are reported. The increase in FFA and lactate concentrations represented the major metabolic changes observed in the recirculating perfusion following adrenaline challenge but neither exogenous FFA nor exogenous lactate induced any marked effects upon mechanical performance of the hearts.

In the final sub-section the effects of antilipolytic treatment (with nicotinic acid) upon the responses of the heart to adrenaline are described. The first-phase responses to adrenaline were unaffected by nicotinic acid but the second-phase deteriorations were reduced. Thus the rises in resting tension, the decline in developed tension and the incidence of arrhythmias were reduced. Glucose oxidation was encouraged by nicotinic acid.

The results indicate that both FFA and lactate contribute to the decline in mechanical performance of the perfused hearts after adrenaline challenge. The possible primary role of FFA is discussed.

2. Effects of adrenaline in the recirculating perfusion

Hearts were perfused at 3-4 ml/min/g to impose a marked degree of ischaemia as indicated in Section B. In preliminary experiments the chronotropic and inotropic responses of the heart were found to be maximal at

10^{-6} M (-) adrenaline. The hearts were thus perfused for 30 min control period and then adrenaline was infused to a final concentration of 10^{-6} M. The effects were observed during the following 30 min.

(a) Major Mechanical and Metabolic Responses to Adrenaline

A biphasic response to adrenaline was observed. The first phase lasted 2-8 min during which time heart rate and developed tension were elevated above the pre-adrenaline control values. During the second phase from 8-30 min after adrenaline infusion, the resting tension increased, arrhythmias developed and heart rate and developed tension returned to or declined below the pre-adrenaline control. Typical responses to adrenaline are shown in figure 22.

During the second phase of the adrenaline response, changes in the metabolic as well as the functional characteristics of the heart became apparent. The glucose uptake, lactate release, fractional conversion of glucose to lactate and lipolysis (as assessed by FFA and glycerol release) were all stimulated.

The effects of adrenaline are shown in table 6. The major statistically significant changes detected during the second phase response to adrenaline were: the rise in resting tension ($109.7 \pm 20.0\%$), the rise in the incidence of arrhythmias ($48.7 \pm 8.3\%$), the stimulation of the release of lactate ($150 \pm 42\%$) and FFA ($146 \pm 53\%$), and the rise in the fractional conversion of glucose to lactate ($30.1 \pm 3.7\%$).

Figure 22

Effects of adrenaline (10^{-6}M) on the performance of isolated perfused hearts from normally-fed rats. Hearts were perfused at the ischaemic flow of 3-4ml/min/g.

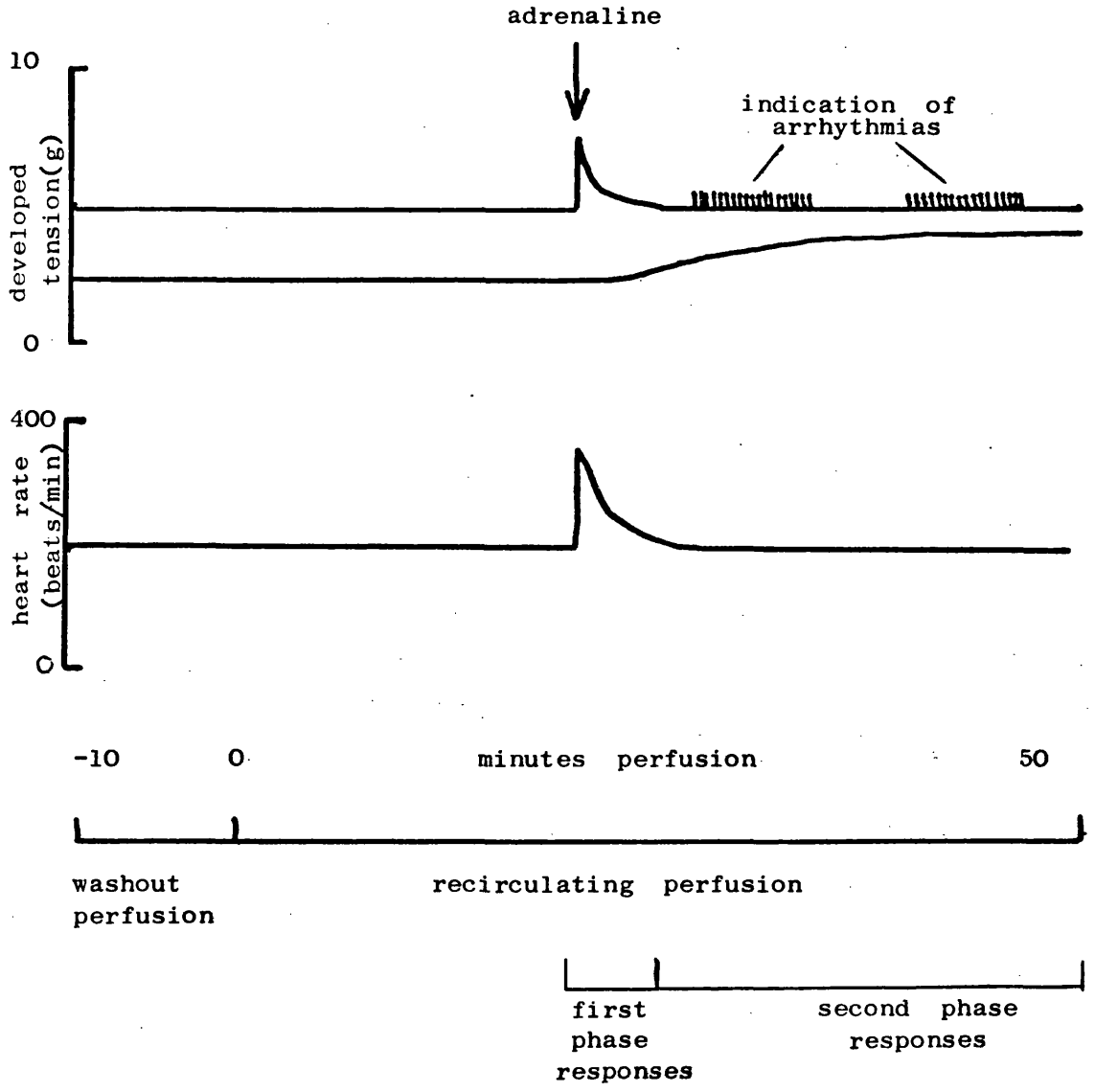


TABLE 6

Biphasic response to adrenaline

First Phase

Function	Control	Peak response	% change
Heart rate (b/min)	194 ±16	273 ±16	48 ± 12 *
Developed Tension(g)	3.4 ±0.5	5.6 ±0.6	52 ± 13 *

Second Phase

Function	Control	30 min post-adrenaline	% change
Heart rate (b/min)	194 ±16	188 ±20	11.2 ±9.5
Developed tension(g)	3.4 ±0.5	2.6 ±0.3	-15.0 ±20.3
Resting tension(g)	1.9 ±0.3	3.7 ±0.4	109.7 ±20.0 *
arrhythmias	2.3 ±0.4 min (7.8 ±1.5% of time)	16.3 ±3.5 min (56.5 ±9.4% of time)	- 48.7 ±8.3% *
glucose uptake (µmole/g/hr) wet weight	62.2 ±6.4	74.9 ±10.4	26.8 ±20.9
lactate release (µmole/g wet weight/hr)	65.0 ±12.5	129.3 ±18.3	150.5 ±42.4 *
% glucose to lactate	49.4 ±7.8%	79.5 ± 7.6%	30.1 ± 3.7 *
FFA release (µmole/g wet wt/ hr)	1.9 ±0.4	3.6 ± 0.75	146.0 ±53.4 *
glycerol release (µmole/g wet wt/ hr)	1.6 ±0.6	3.5 ± 1.4	104.2 ±32.3

(n = 11, * = significantly different to control)

(b) Relationship between Mechanical and Metabolic Responses to Adrenaline

It was considered that the metabolic and mechanical responses to adrenaline may be related. An analysis was therefore performed of the possible relationships between the major metabolic changes (stimulation of the release of FFA and lactate) and the deterioration in performance over the second phase of the adrenaline challenge. It was considered that a direct toxic effect of adrenaline or one (or more) of its breakdown products was unlikely with the low concentration used. The rates of release of FFA and of lactate correlated linearly to the rise in resting tension. The equations which describe the relationships are:

$$\text{rise in resting tension (g)} = (0.24 \times \text{FFA release } (\mu\text{mole/g/hr})) + 0.96$$

(correlation coefficient = 0.66, $P < 0.05$ $n = 9$).

$$\text{rise in resting tension (g)} = (0.01 \times \text{lactate release } (\mu\text{mole/g/hr})) + (-0.12)$$

(correlation coefficient = 0.64, $P < 0.05$, $n = 11$). See figure 23. In addition, the rate of release of FFA (but not that of lactate) was significantly related to the decline in developed tension after adrenaline. The relationship is described by the equation:

$$\text{developed tension (g)} = (-0.17 \times \text{FFA release}) + 2.25$$

(correlation coefficient = - 0.78, $P < 0.02$, $n = 9$). See figure 24.

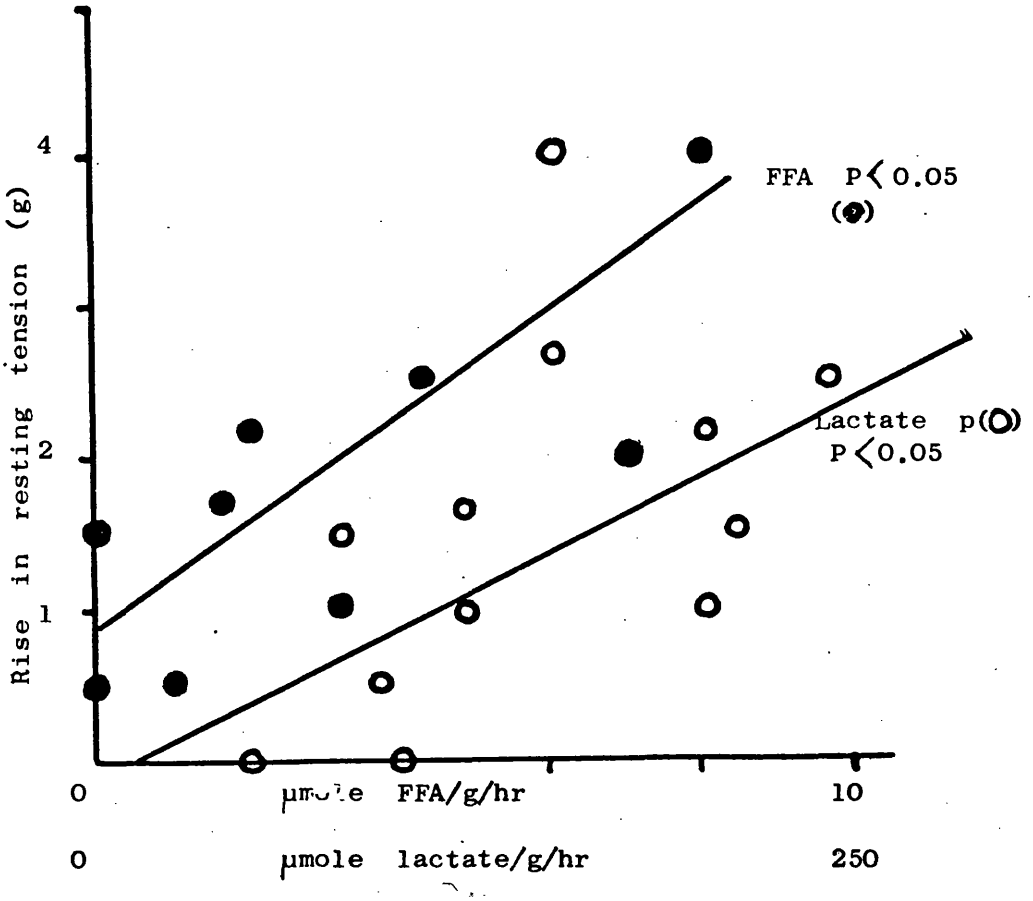
Neither the rate of release of FFA nor that of lactate was significantly related to either heart rate or the incidence of arrhythmias after adrenaline.

Since rates of release of FFA and lactate were usually linear both before and after adrenaline infusion, the rates of release of the metabolic products is also an index of their concentration in the perfusate.

Figure 23

Relationship between the release of lactate and FFA and the rise in resting tension in isolated hearts from normally-fed rats.

Hearts were perfused with adrenaline (10^{-6}M) during ischaemic perfusion (3-4ml/min/g).

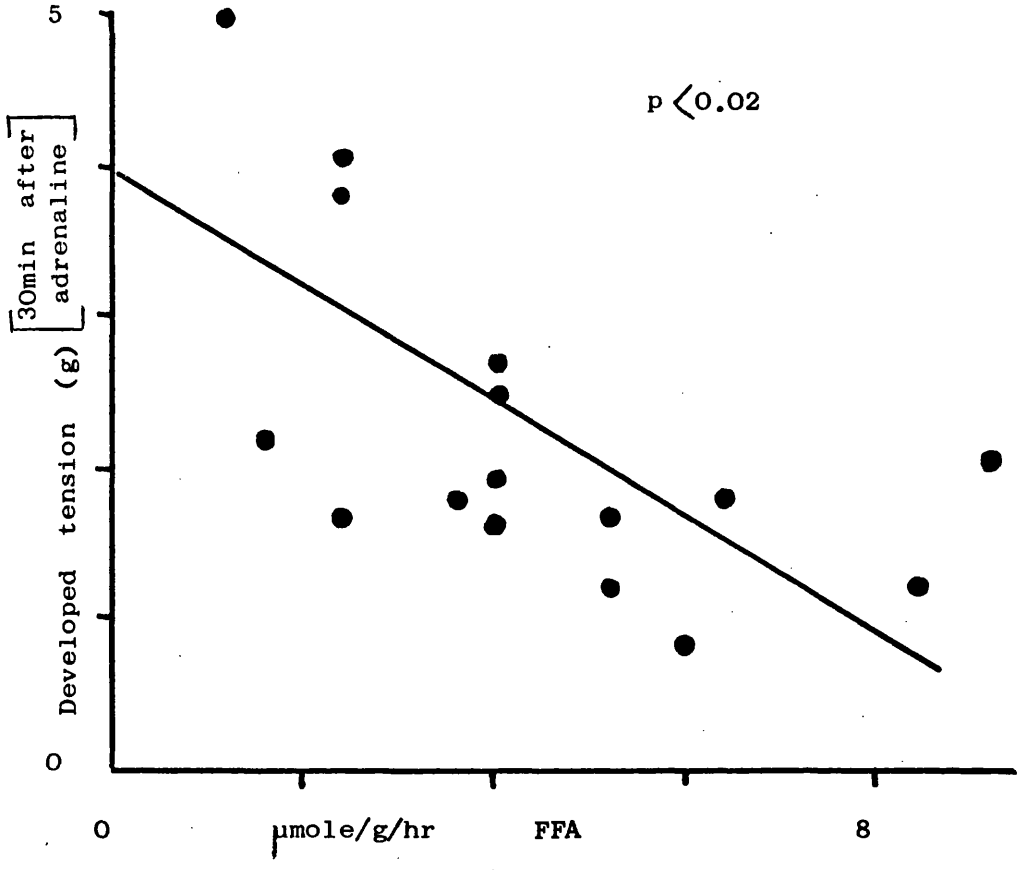


This represents a second phase response to adrenaline, resting tension being determined 30min after adrenaline infusion.

Figure 24.

Relationship between release of FFA and the decline in developed tension in isolated hearts from normally-fed rats.

Hearts were perfused with adrenaline (10^{-6}M) during ischaemic perfusion (3-4ml/min/g). This represents a second phase response to adrenaline, during the 30min immediately after infusion.



3. Effects of Adrenaline - Comparison between recirculating and non-recirculating perfusion

Experiments were performed in order to investigate the possible role of the recirculating metabolic products in the adrenaline-induced deterioration in mechanical performance observed in sub-section A. The conditions of perfusion were identical in the two series except that for non-recirculation perfusions a large reservoir of perfusate was incorporated prior to the aerator column. In addition, perfusate was collected after a single pass through the heart. (This allowed direct measurement of coronary flow in this series). (-) Adrenaline (10^{-6}M) was infused for periods of about 10 min and the results are compared to the measurements of adrenaline effect 10 min after infusion in the recirculating system.

The phase I responses of the hearts were not significantly different in the two modes of perfusion but marked differences in the second phase response were apparent as shown in Table 7.

Significant differences in metabolism are also apparent when a comparison is made between recirculating and non-recirculating perfusion systems. The rates of release of FFA and glycerol are greater in the non-recirculating perfusion; in addition, glucose uptake is greater and lactate release is smaller. Thus the fractional conversion of glucose to lactate is smaller in the non-recirculating perfusion, indicating that the metabolism is more extensively

TABLE 7

Second phase response of hearts to (-) adrenaline (10^{-6}M) infusion in non-recirculating (NR) and recirculating (R) perfusion.

Function	NR		R		
	Control	10min post-adrenaline	Control	10 min post-adrenaline	
Resting tension(g)	1.1 ±0.2	1.1±0.2	1.9 ±0.3	3.0 ±0.3	*
Developed tension(g)	2.5 ±0.4	2.3±0.4	3.4 ±0.5	3.6 ±0.4	
Arrhythmias(min)	0	0	2.3 ±0.4min (7.8 ±1.5% of time)	16.3 ±3.5min (56.5 ±9.4% of time)	*
FFA release (μmole/g/min)	0.44±0.10	1.42±0.59	0.03 ±0.01	0.06 ±0.01	ø
Glycerol release (μmole/g/min)	0.15±0.06	0.16±0.10	0.03 ±0.01	0.06 ±0.02	ø
glucose uptake (μmole/g/min)	3.74±0.85	7.92±2.01	1.04 ±0.11	1.25 ±0.17	ø
lactate release (μmole/g/min)	2.02±0.40	3.50±0.72	1.08 ±0.21	2.16 ±0.31	
% glucose conversion to lactate	27.6±5.0	32.8±13.5	49.4 ±7.8%	79.5 ±7.6%	* ø

(n = 6, * = adrenaline induced a significantly greater change in R than NR.

ø = pre-adrenaline control is significantly greater in NR than in R.

aerobic. Adrenaline induced a similar rise in the rate of release of FFA and glycerol in the two systems but the effect on glucose and lactate was such that the fractional conversion of glucose to lactate rose more markedly in the recirculating perfusion.

The results indicate that the recirculating perfusate contributes significantly to the adrenaline - induced deterioration. The major metabolic changes to be detected were a greater release of FFA and glycerol into the non-recirculating perfusate and a greater contribution from glucose oxidation to the overall energy production. One could postulate that these two observations are related, thus the greater clearance of FFA from the tissue (possibly explained by the supply of fresh albumin containing no bound FFA) could explain the greater oxidation of glucose in the non-recirculating perfusion. The elimination or reduction of FFA fragments pre-empting the terminal oxidation sequence beyond the level of pyruvate dehydrogenase would allow the more extensive oxidation of glucose carbon (Randle et al, 1963). Such a hypothesis would thus attempt to explain the improved mechanical response to adrenaline in the non-recirculating perfusion in terms of improved clearance of metabolites from the tissue.

In order to further test the involvement of the recirculating perfusate, the factors presumed to be of significance (lactate and FFA) were applied directly as described in the following section.

4. Effects of exogenous FFA and Lactate in the Ischaemically-perfused Rat Heart.

Palmitic acid (bound to low-fat serum albumin) and lactic acid were infused into hearts for 10 min following a 30 min control perfusion. All hearts were perfused by the non-recirculating method at a coronary flow of 3-4 ml/min/g. The concentrations of lactic acid and palmitic acid were more than double those previously measured in the recirculating system. Thus the maximum FFA concentration previously measured was 0.2 mM whilst palmitic acid was presented at 0.5 mM and 1.0 mM in these experiments. The maximum lactic acid concentration previously measured was 5mM so the concentrations used in these experiments were 5mM and 10mM.

Lactic acid at 5mM had no observable effects at all and even the effects seen with 10 mM lactic acid were not statistically significant. Lactic acid (10mM) had no effect on heart rate, resting tension, developed tension, incidence of arrhythmias, glucose uptake or FFA release. Similarly, little evidence for a direct action of applied palmitic acid could be seen. At 0.5mM no significant changes were observed, at 1.0mM the heart rate was significantly depressed (from 170 ± 16 to 106 ± 12 beats/min, $P < 0.002$, $n = 6$) but the depression of developed tension was not significant (from 2.6 ± 0.4 to 1.3 ± 0.5g $n = 6$ $P = NS$). No changes were observed in resting tension,

incidence of arrhythmias or in any of the metabolic parameters studied (glucose uptake, lactate release, fractional conversion of glucose to lactate).

The results show that the effects of lactic acid and palmitic acid as applied externally are not sufficient to explain the damaging effects of adrenaline found in the recirculating perfusion. To complete this investigation one would need to apply both FFA and lactic acid together in order to exclude the possibility of a synergistic action, although the possible mode of action of such synergism is difficult to predict.

The only indication of direct action is that of 1.0mM palmitic acid on heart rate (and possibly developed tension) but the concentration required is far above that normally present in the recirculating perfusate. The important effects of the recirculating perfusate shown in sub-section 3 cannot, therefore, be explained simply by the concentration of lactate and FFA presented. Assuming that other components in the perfusate do not contribute, an alternative explanation such as that suggested earlier concerning the effect of the recirculating perfusate upon metabolite clearance from the heart tissue becomes more likely. It is possible that a relatively small concentration of FFA or lactate in the perfusate may be sufficient to significantly disturb the outflow of metabolic products whilst exerting no direct effect.

To further investigate the role of endogenously-generated FFA and lactic acid, recirculating perfusions were

performed in which the adrenaline challenge was modified by the presence of an antilipolytic agent. Nicotinic acid was the main antilipolytic agent used although insulin was also applied in two experiments.

5. Effects of antilipolytic treatment on the responses of the isolated perfused rat heart to adrenaline

(a) General Hearts were perfused for a 20 - 30 min control period before the infusion of nicotinic acid (5×10^{-6}M). After a further 20 min, adrenaline (10^{-6}M) was infused. All hearts were perfused at the ischaemic coronary flow rate of 3 - 4 ml/min/g.

Preliminary experiments had shown that 5×10^{-6}M - 10^{-5}M nicotinic acid was a convenient antilipolytic concentration. At concentrations below 10^{-6}M, nicotinic acid had no antilipolytic effect. At 10^{-4}M nicotinic acid was apparently toxic, causing cardiac arrest - this was a time-dependent effect, occurring after about 20 min perfusion (and interstingly only in the recirculating perfusion, not in the non-recirculating system). Insulin was used at 100 μU/ml which represents the upper range of serum concentration in vivo (Selkurt, 1971). Nicotinic acid alone did not affect any of the metabolic or mechanical parameters which were measured during control perfusion.

(b) Effects of nicotinic acid on the first phase responses to adrenaline. Comparison of these values with those reported for the effects of adrenaline alone

(Tables 6,8) shows that nicotinic acid did not significantly affect the mechanical response of the hearts to adrenaline. In fact the percent rise of developed tension following adrenaline with nicotinic acid (145 ± 46%) is greater than that seen with adrenaline alone (52 ± 13%). This, however, is rather a reflection of the difference in control developed tension which coincidentally was higher in the group treated with adrenaline alone. The results demonstrate that nicotinic acid has not acted as a negatively inotropic or chronotropic agent.

(c) Effects of nicotinic acid on the second phase responses to adrenaline The effects of nicotinic acid on the second phase response of hearts to adrenaline are shown in table 9 and figure 25. In this phase, nicotinic acid exerted a marked saving effect upon the subsequent deterioration in performance following adrenaline. Thus developed tension was maintained better (177 ± 17% of control) in the presence of nicotinic acid than with adrenaline alone (85 ± 20% of control). Resting tension was stable in the presence of nicotinic acid but rose 109 ± 20% with adrenaline alone. The incidence of arrhythmias was much reduced in the presence of nicotinic acid (19.2 ± 6.3% of time) compared to adrenaline alone (56.5 ± 9.4% of time). Heart rate returned to pre-adrenaline control similarly with both treatments.

The metabolic responses of the heart to adrenaline were also modified by the presence of nicotinic acid. Thus FFA release rose only 5.8 ± 3.4% with nicotinic acid but

TABLE 8

Effect of nicotinic acid (5.10^{-6}M) on the first phase responses of hearts to adrenaline.

Function	Control (pre-adren, + nicotinic acid)	+nicotinic acid + adrenaline	% change
Heart rate (b/min)	171 ± 18	210 ± 16	+42.1 ± 17.5
Developed tension(g)	1.8 ± 0.4	3.5 ± 0.7	+ 145 ± 46

Data are for hearts from normally-fed rats perfused for 20min prior to nicotinic acid infusion and then a further 20min prior to adrenaline (10^{-6}M) infusion.

TABLE 9

Second phase responses to adrenaline + nicotinic acid

Function	Control (Pre-adren + nicotinic acid)	30 min post - adrenaline + nicotinic acid	% change after adrenaline
Heart rate (beats/min)	171 ±18	150 ±18	-11.7 ±15
Developed Tension(g)	1.8 ±0.4	3.2 ±0.3	177 ±17 *
glucose uptake (μmole/g/hr)	47.6 ±3.5	57.3 ±9.3	45.7 ±31.4
lactate release (μmole/g/hr)	40.6 ±3.8	68.4 ±8.9	79.0 ±27.6
% conversion of glucose to lactate	42.1 ±5.4	52.9 ±10.4	12.0 ± 6.5 *
FFA release (μmole/g/hr)	2.7 ±0.3	2.8 ±0.3	5.8 ± 3.4 *
Glycerol release (μmole/g/hr)	1.8 ±0.4	1.0 ±0.4	-0.6 ± 37.5*

(n = 8, * = change is significantly different to that obtained with adrenaline alone)

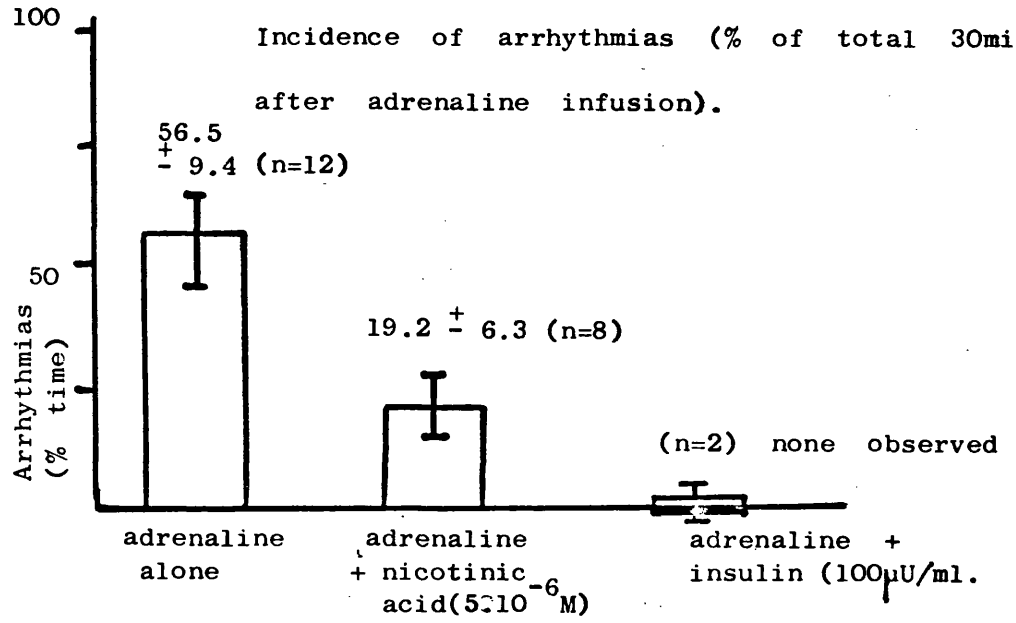
Measures of performance were taken 30 min after adrenaline (in the presence of nicotinic acid). Metabolic parameters represent the linear rates of uptake or release over the 30 min period after adrenaline.

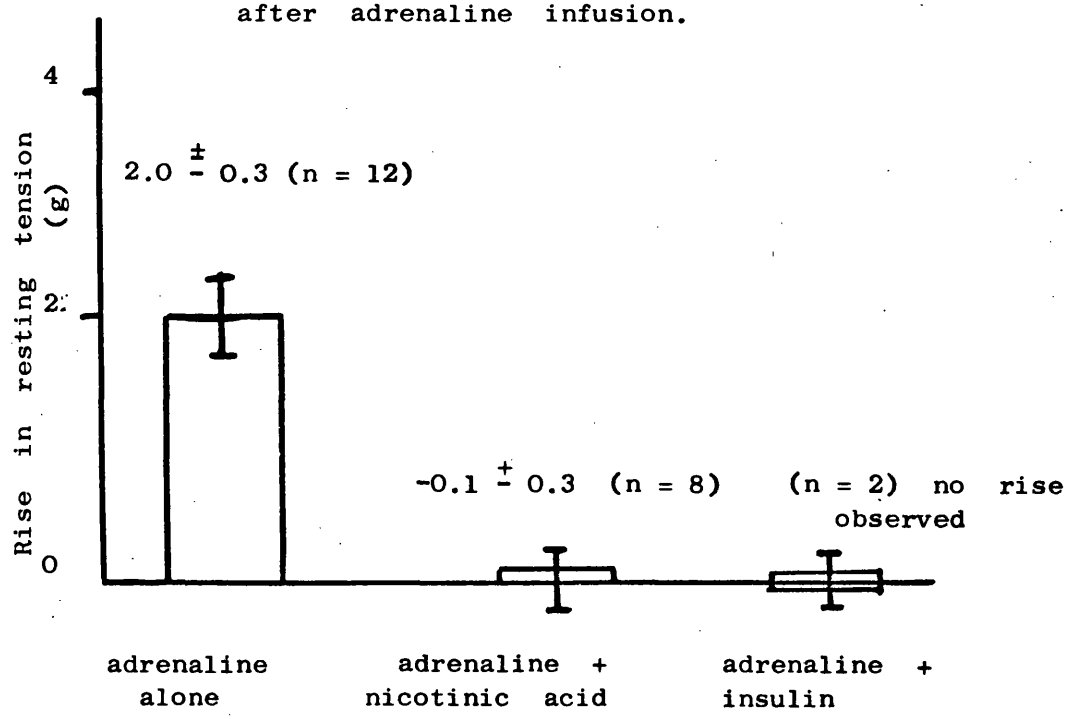
Adrenaline was used at 10^{-6}M after a prior 40min perfusion, the latter 20min in the presence of 5.10^{-6}M nicotinic acid.

Figure 25

Effects of antilipolytics on the second phase responses of isolated perfused hearts from normally-fed rats.

Hearts were perfused at the ischaemic flow 3-4ml/min/g. Adrenaline was used at 10^{-6}M. Values are mean ± S.E.





rose 146 ± 53% with adrenaline alone. In addition the stimulation of lactate release induced by adrenaline alone (150 ± 43%) was markedly reduced by the presence of nicotinic acid (to 79 ± 28%). The response of glucose uptake in the two groups was similar, showing no significant increase with adrenaline, and so the fractional conversion of glucose to lactate rose less in the presence of nicotinic acid (12.0 ± 6.5%) than with adrenaline alone (30.1 ± 3.7%).

(d) Effects of Insulin on the responses of the heart to adrenaline. In two experiments insulin (100μU/ml) was used as an alternative antilipolytic agent. It had no effect on any of the mechanical or metabolic parameters measured during the pre-adrenaline control perfusion, including glucose uptake. The second phase responses to adrenaline (Table 10, figure 25) in the presence of insulin were similar to those observed in the presence of nicotinic acid. Thus no arrhythmias were observed and no rise in resting tension occurred after adrenaline in the presence of insulin. Developed tension was maintained above the pre-adrenaline control level (425 ± 78%) but heart rate declined below the control (-47.5 ± 6.6%) - in contrast to the values obtained with adrenaline alone (-15 ± 20% and 11 ± 9%).

Insulin also modified the metabolic responses of the heart to adrenaline. The release of FFA and glycerol were not stimulated at all and the uptake of glucose was similarly unaffected. Lactate release rose less in

the presence of insulin (23 ± 23%) than with adrenaline alone (150 ± 42%). The fractional conversion of glucose to lactate also rose less in the presence of insulin (13 ± 13%) compared to 30 ± 4% with adrenaline alone.

The overall effect of nicotinic acid (and insulin) was to abolish the lipolytic component of the adrenaline challenge and at the same time to greatly offset the deteriorations in performance associated with the second phase adrenaline responses. In particular, the rise in resting tension and in the incidence of arrhythmias associated with adrenaline challenge were significantly reduced. In addition, the developed tension was maintained above the pre-adrenaline control level for longer in the presence of the antilipolytics (although with insulin there was a converse, more marked, decline in heart rate than with adrenaline alone). Antilipolytic treatment also offset the rise in the fractional conversion of glucose to lactate previously observed with adrenaline alone. This indicates a greater degree of oxidation of glucose after adrenaline in the presence of antilipolytics.

TABLE 10

Effects of insulin (100μU/ml) on the response of ischaemically-perfused hearts to adrenaline (10^{-6}M)

Function	Contol (pre-adren, + insulin)	30 min post - adrenaline + insulin	% change
First Phase response			
Heart rate (beats/min)	230 ±10	325 ±25	41.5 ±4.5
Developed tension(g)	1.0 ±0.0	3.5 ±0.5	250 ±50 *
Second Phase response			
Heart rate (b/min)	230 ±10	120 ±20	-47.5 ±6.6 *
Developed tension(g)	1.0 ±0	5.3 ±0.8	425 ± 78 *
glucose uptake (μmole/g/hr)	100.0 ±10	100.0 ±10	0 ± 0
lactate release (μmole/g/hr)	130.0 ±30	175.0 ±75	23.0 ± 23 *
% conversion of glucose to lactate	64.0 ±9	78.0 ±22	13.0 ± 13 *
FFA release (μmole/g/hr)	5.3 ±0.9	4.0 ±0.6	-25.0 ± 14 *
Glycerol release (μmole/g/hr)	5.3 ±1.1	5.3 ±1.1	0 ± 0 *

(n = 2, * = difference is significantly different to that observed with adrenaline alone),

SECTION D. Perfusion of Hearts from Fat-Fed Rats

In order to obtain further information concerning the role of myocardial lipolysis during ischaemic perfusion, hearts from rats fed on a diet containing a high proportion of saturated fats were studied. It was anticipated that the hearts from fat-fed rats would exhibit higher total lipid stores and/or a higher lipolytic capacity than hearts from normally-fed rats (Agostini and Angelotti, 1973). It would therefore be anticipated that more exaggerated responses to the mobilisation of endogenous lipids would be found in such hearts.

Hearts from fat-fed rats were perfused at coronary flow rates over the range 2-14ml/min/g. After a control perfusion of 30min, adrenaline (10^{-6}M) was infused and the perfusion continued for a further 30min. In a separate group of hearts (all perfused at 5-7ml/min/g) nicotinic acid (5×10^{-6}M) was infused after 20min control perfusion. After 20min in the presence of nicotinic acid, adrenaline (10^{-6}M) was infused and the perfusion continued for a further 20min. Comparisons have thus been drawn between the hearts from fat-fed and normally-fed rats with respect to : (1) control perfusion - metabolism and performance

(2) responses to adrenaline

For simplicity, the two groups are referred to as "fat-fed hearts" and "normally-fed hearts" in the text.

1. Control Perfusion (Fat-fed vs Normally-fed)

(a) Mechanical properties The hearts from both groups were perfused by the recirculating method. The fat-fed hearts were perfused over a wider range of coronary flow in an attempt to maximise performance. Even up to 14ml/min/g the improvement in performance with the rise in coronary flow was not as marked as had been observed with the normally-fed hearts. For most comparisons, the highest flow rates of the fat-fed series have been eliminated so that the mean coronary flow rates of the two groups are more similar (normally-fed = 4.9 $\pm$ 0.8 ml/min/g, fat-fed = 6.9 $\pm$ 0.7 ml/min/g). Even so the direction of error is to permit more adequate performance of the fat-fed hearts. Significant differences between the fat-fed and normally-fed hearts were observed with regard to both performance (Table 11, figures 26-28) and metabolism (Table 12).

The factors used to describe mechanical performance are those used in the normally-fed series with two additions. Firstly, dT/dt (the rate of rise of developed tension) was determined with the use of an electronic differentiator in the fat-fed series but by direct measurement from the tension trace in the normally-fed series. The second additional factor is the oxygen-cost of "work" as described below.

The results show that heart rate (148 $\pm$ 9 beats/min) and dT/dt (58 $\pm$ 13 g/sec) are both markedly depressed in the fat-fed hearts compared to the normally-fed hearts

Table 11

Performance of isolated perfused rat hearts from fat-fed and normally-fed rats.

Function	Fat-fed (n = 20)	Normally-fed (n = 24)
Heart rate (beats/min)	148 ± 9	194 ± 16 **
Developed tension (g)	2.4 ± 0.6	2.9 ± 0.3
Rise in resting tension (g)	1.70 ± 0.4	0.1 ± 0.4 **
MVO_2 (µl/min/g)	54.0 ± 6.4	36.2 ± 1.8 **
% oxygen extraction	62.4 ± 8.0	57.2 ± 7.4
dT/dt (g/sec)	58.3 ± 12.9	173.6 ± 17.4 **
MVO_2/beat	0.34 ± 0.03	0.21 ± 0.02 **

Values are mean ± S.E.

** indicates that difference between the means is significant.

Data are for the stable 30min control perfusion.

(194 ± 16 beats/min and 173 ± 17 g/sec). Developed tension was not significantly different in the two groups. The rise in resting was very marked in the fat-fed hearts (1.7 ± 0.4g) but was constant during control perfusion in the normally-fed hearts. Although the percent extraction of oxygen was not significantly different in the two groups, the total MVO_2 was higher in the fat-fed hearts (54 ± 6 µl/g/min) than in the normally-fed hearts (36 ± 2 µl/g/min). From a consideration of heart rate and MVO_2 it can be seen that the oxygen-cost of "work" is higher in the fat-fed hearts (0.34 ± 0.03 µl/g/beat) than in the normally-fed hearts (0.21 ± 0.02 µl/g/beat). MVO_2/beat is derived from the equation;

$$MVO_2/\text{beat} = \frac{\mu l \text{ oxygen/g/min}}{\text{beats/min}}$$

The use of this term is justified by the relationship of heart rate with MVO_2. The relationship is described by the regression equations:

MVO_2 (µl/g/min) = (0.84 x beats/min) + (-76.3)

(correlation coefficient 0.62, $P < 0.01$, $n = 20$), in fat-fed hearts.

MVO_2 (µl/g/min) = (0.09 x beats/min) + 21.4

(correlation coefficient = 0.50, $P < 0.02$, $n = 20$), in normally-fed hearts.

This evidence is in agreement with the argument of Sonnenblick and Skelton (1971) that heart rate contributes significantly to MVO_2.

Of the parameters described in table 11, only percent oxygen extraction and resting tension showed a dependence

Figure 26

Effect of coronary flow on the percent oxygen extraction of isolated perfused hearts from normally-fed and fat-fed rats.

See figure 17 for derivation of % oxygen extraction. Data are for the stable 30min control perfusion

Points represent "fat-fed hearts" only.

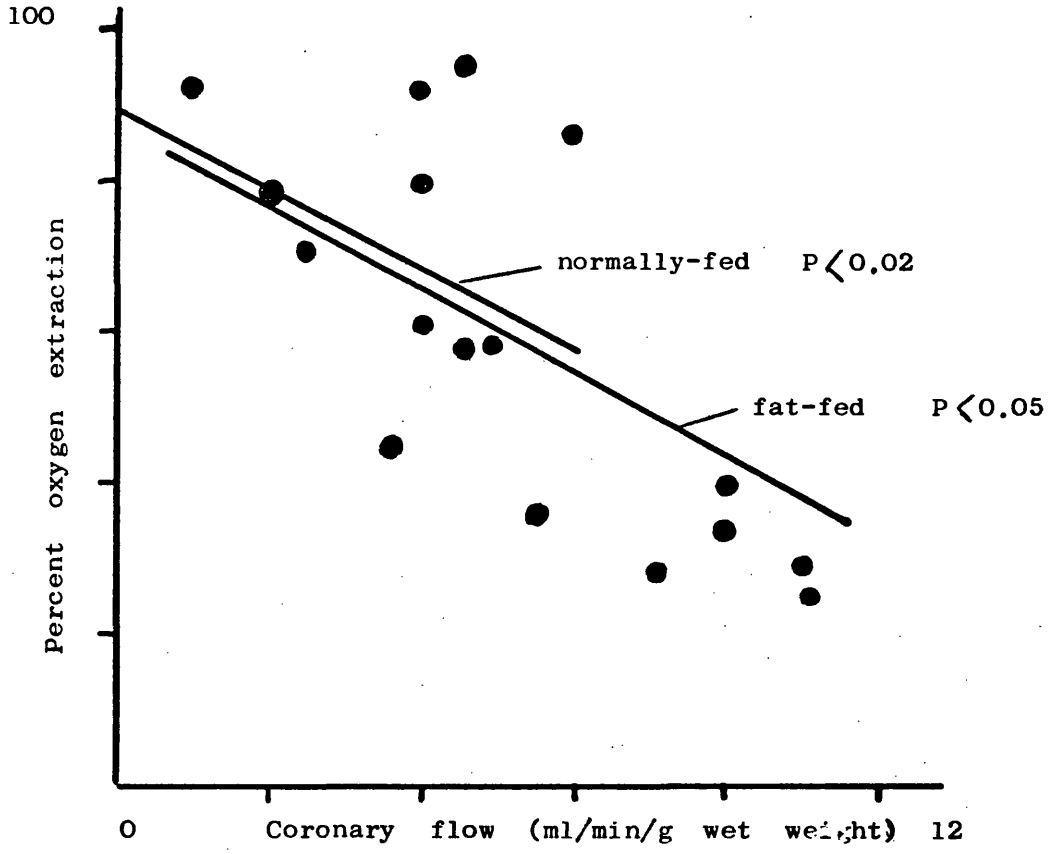


Figure 27

Effect of coronary flow on resting tension in isolated perfused hearts from normally-fed and fat-fed rats.

The points represent hearts from fat-fed rats only. Data are for the stable 30min control perfusion.

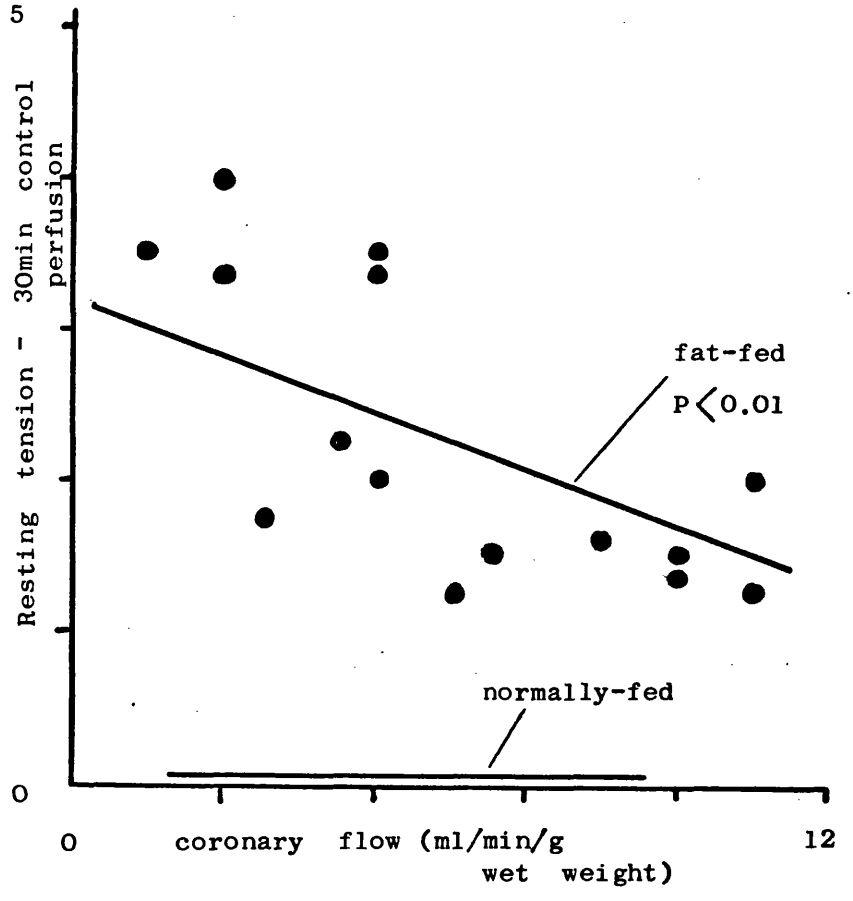
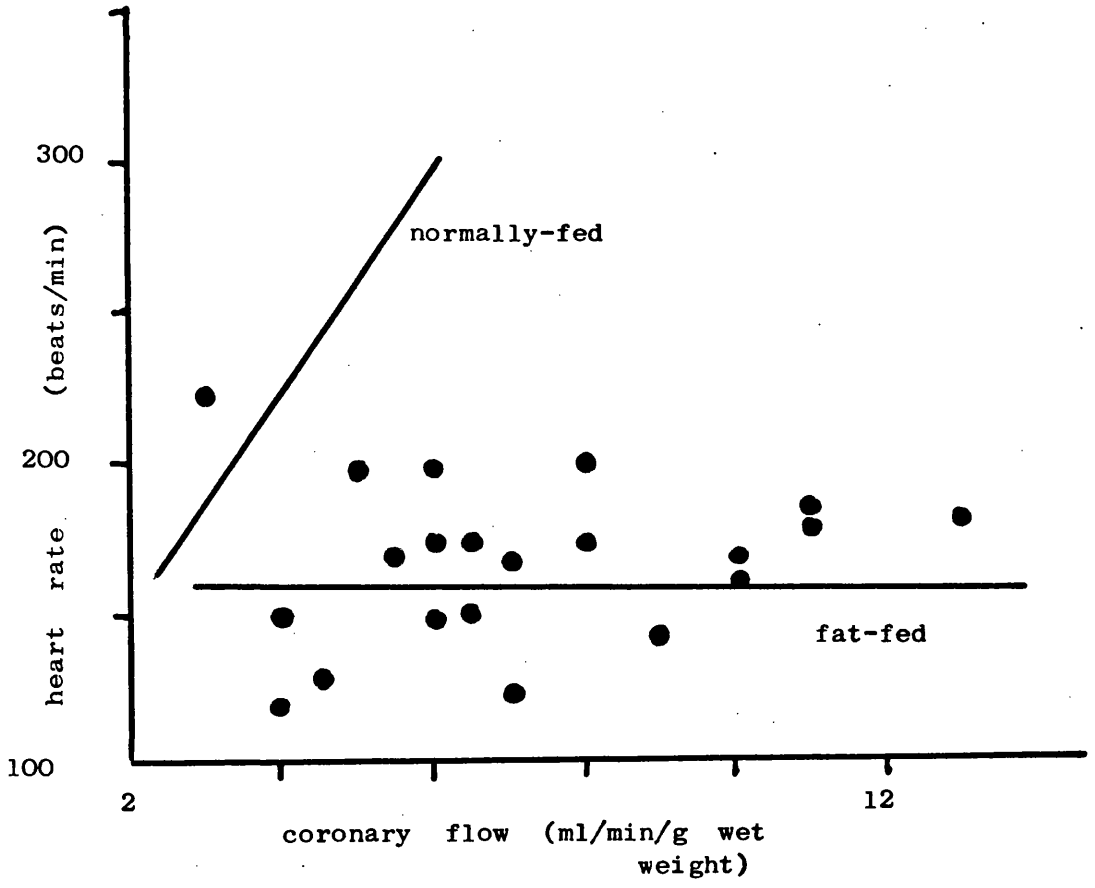


Figure 28

Effect of coronary flow on heart rate in isolated perfused hearts from normally-fed and fat-fed rats. Data are for the first stable 30min control perfusion. Points represent hearts from fat-fed rats only.



upon coronary flow (figures 26, 27). The lack of dependence of heart rate upon coronary flow (figure 28) is a sharp contrast to the relationship observed in the normally-fed hearts.

(b) Metabolic properties. The differences between the fat-fed and normally-fed hearts is principally expressed as an almost complete dependence upon endogenous lipid for energy production (table 12) in the former group.

The results show a significantly increased rate of FFA release from fat-fed hearts (4.2 ± 0.5 μmole/g/hr) compared to normally-fed hearts (2.3 ± 0.3 μmole/g/hr) and a shift in the extent of FFA release relative to glycerol release in the fat-fed hearts (FFA/glycerol = 2.8 ± 0.4 compared to 1.3 ± 0.5 in the normally-fed hearts). Glucose uptake was very low in the fat-fed hearts (2.9 ± 0.4 μmole/g/hr compared with a value of 55.7 ± 10.0 μmole/g/hr in normally-fed hearts) but a significant lactate release was still observed in the fat-fed hearts (20.7 ± 3.5 μmole/g/hr). This lactate may arise from glucose uptake since the error of measurement of the latter is large enough to account for the lactate. Alternatively, the degradation of glycogen could contribute to the release of lactate.

Table 12

Metabolism of isolated perfused rat hearts from fat-fed and normally-fed rats.

Function	Fat-fed (n = 20)	Normally-fed (n = 24)
Glucose uptake	2.9 ± 0.2	55.7 ± 10.0 **
Lactate release	20.7 ± 3.5	54.2 ± 5.8 **
FFA release	4.2 ± 0.5	2.3 ± 0.3 **
glycerol release	1.5 ± 0.2	1.8 ± 0.7
FFA/glycerol	2.8 ± 0.4	1.3 ± 0.5 **

Values are mean ± S.E. expressed as μmole/hr/g wet weight.
** indicates that the difference between the means is significant.

Data are for the stable 30min control perfusion.

2. Responses to Adrenaline (Fat-Fed vs Normally-Fed)

(a) First phase responses. The results are presented in table 13. In both absolute and percent terms, the chronotropic and inotropic responses of the two groups were not significantly different.

(b) Second phase responses - mechanical. One would anticipate a worsening of mechanical performance after adrenaline in the hearts from fat-fed rats compared to those from normally-fed rats. The results are not entirely consistent but significant factors support the original contention that fat-feeding may predispose hearts to poor performance following adrenaline challenge during ischaemic perfusion.

Heart rate declined more rapidly (returning to the pre-adrenaline rate in 8.9 $\pm$ 2.4 min compared to 14.1 $\pm$ 4.0 min with normally-fed hearts) and more extensively (to 112 $\pm$ 11 beats/min or 75 $\pm$ 6% of control by 30 min after adrenaline, in contrast to the values for the normally-fed hearts - 188 $\pm$ 20 beats/min or 111 $\pm$ 10% of control) in the fat-fed hearts. Although there was no significant difference between the two groups with regard to the incidence of arrhythmias, there were significantly more ventricular fibrillations in the fat-fed hearts (23.3 $\pm$ 8.3% of the time after adrenaline) than in the normally-fed hearts (5.9 $\pm$ 5.9% of time).

The two groups did not differ significantly in their responses to adrenaline with regard to either developed tension or resting tension.

Table 13

Responses of isolated perfused rat hearts to adrenaline (10^{-6}M).

The responses are the initial (first phase) inotropic and chronotropic responses of hearts from fat-fed and normally-fed rats.

	Fat-fed		Normally-fed	
Function	Control	Adrenaline	Control	Adrenaline
Heart rate (beats/min)	148 ± 9	229 ± 22	194 ± 16	273 ± 16
% change heart rate	53 ± 12		48 ± 12	
Developed tension (g)	2.2 ± 0.7	3.6 ± 0.9 **	3.4 ± 0.5	5.6 ± 0.6
% change developed tension	95 ± 33		52 ± 13	

Values are means ± S.E.

** indicates that the peak adrenaline-induced tensions are significantly different.

The interpretation of the rise in resting tension in the fat-fed hearts is complicated by the high levels obtained during the control perfusion. There may exist a finite limit to which the final approach is non-linear. This would increase the significance of the relatively smaller changes found with the fat-fed hearts.

Considering the mixed evidence it is necessary to decide which parameters are most significant in order to clearly differentiate between the responses to adrenaline of the two groups of hearts. In this respect, the incidence of ventricular fibrillation should be firmly emphasised since this is the most critical single event in the determination of the survival of the heart. Thus catecholamine challenge during ischaemic perfusion may depress performance to a certain degree, beyond which the maintainance of contractility is jeapardised. The additional stress of an increased rate of endogenous lipolysis may be sufficient to enforce such irreversible responses. See table 14.

(c) Second phase - metabolic. The small glucose uptake observed during the control perfusion of the fat-fed hearts was not stimulated by adrenaline. The percent stimulation of lactate release by adrenaline was greater in the fat-fed hearts ($365 \pm 85\%$ compared to $150 \pm 42\%$ with the normally-fed hearts) but the absolute lactate release was much reduced (47.3 ± 4.4 μmole/g/hr compared to 129.3 ± 18.3 μmole/g/hr in the normally-fed hearts). The fractional

Table 14

Second phase mechanical responses to adrenaline (10^{-6}M) of isolated perfused rat hearts, from fat-fed and normally-fed rats.

Function	Fat-fed (n = 16)	Normally-fed (n = 11)
Heart rate (beats/min)	112 ± 11	188 ± 20 **
Heart rate (% control)	75 ± 6	111 ± 10 **
Heart rate (min to return to control)	8.9 ± 2.4	14.1 ± 4.0
Resting Tension (g)	4.5 ± 0.4	3.7 ± 0.4
Resting tension (% control)	127 ± 8	210 ± 20 **
Ventricular fibrillation (% total time)	23.3 ± 8.2	5.9 ± 5.9 **
Arrhythmias (% total time)	43.5 ± 9.4	56.5 ± 9.4
MVO_2 (µl/g/min)	68.6 ± 7.8	33.0 ± 5.2 **
MVO_2 (% control)	132.3 ± 10.2	124.8 ± 7.3
MVO_2/beat	0.56 ± 0.07	0.12 ± 0.01 **
MVO_2/beat (% control)	177 ± 20	143 ± 10 **

Values are mean ± S.E.

Parameters were measured 30min after adrenaline infusion. Arrhythmias and fibrillations are expressed as % of the total 30min. ** indicates that the difference between the means is significant.

conversion of glucose to lactate is not meaningful in the fat-fed hearts because of the low glucose uptake. The rate of release of FFA after adrenaline was more rapid from fat-fed hearts (6.5 ± 1.3 μmole/g/hr compared to 3.0 ± 0.7 μmole/g/hr in the normally-fed hearts) but the percent stimulations were not significantly different. Neither the rates of release of glycerol nor the stimulation of the rates of glycerol release by adrenaline were significantly different in the two groups. See table 15.

Table 15

Second phase metabolic responses to adrenaline (10^{-6}M) of isolated perfused hearts from fat-fed and normally-fed rats.

Function	Fat-fed (n = 16)	Normally-fed (n = 11)
Glucose uptake (μmole/g/hr)	2.9 ± 0.2 *	74.9 ± 10.4 **
Glucose uptake (% control)	100 *	126.8 ± 20.9
Lactate release (μmole/g/hr)	47.3 ± 4.4	129.3 ± 18.3 **
Lactate release (% control)	465 ± 85	250 ± 42 **
% conversion of glucose to lact.	100 *	79.5 ± 7.6
FFA release (μmole/g/hr)	6.5 ± 1.3	3.0 ± 0.7 **
Glycerol release (μmole/g/hr)	1.9 ± 0.3	3.5 ± 1.4

Values are mean ± S.E. representing the linear rates of release or uptake during the 30min after adrenaline infusion. ** indicates that the difference between the means is significant. * indicates approximate values due error in determination of small glucose changes.

3. Effects of Nicotinic acid

(a) During control perfusion. The effects of antilipolytic treatment on the responses to adrenaline of hearts from fat-fed rats were investigated.

As in the normally-fed hearts, nicotinic acid(5.10^{-6}M) had no effects on the indices of mechanical performance during the control perfusion. Unlike the normally-fed hearts, however, there were changes in metabolism with nicotinic acid (table 16).

The pre-adrenaline rate of lipolysis was depressed by nicotinic acid. Thus FFA release was reduced from 4.3 ± 1.2 μmole/g/hr to 1.9 ± 1.2 μmole/g/hr, glycerol release was reduced from 1.8 ± 0.3 μmole/g/hr to 0.6 ± 0.3 μmole/g/hr. Glucose uptake rose in the presence of nicotinic acid from 6.0 ± 4.0 to 45.1 ± 23.1 μmole/g/hr but the large standard errors mean that the rise is not significant ($P = 0.1$). The lactate release was not affected by the presence of nicotinic acid so the fractional conversion of glucose to lactate was reduced from about 100% to 53 ± 19%.

(b) Effects on the first phase responses to adrenaline. The first phase responses of the hearts from fat-fed rats to adrenaline were not restricted by the presence of nicotinic acid. Heart rate was not affected at all and in fact the developed tension (7.8 ± 1.7g) and dT/dt (185 ± 40g/sec) were significantly higher after adrenaline in the presence of nicotinic acid than with adrenaline alone (3.6 ± 0.9g and 86 ± 18g/sec). The percent stimulations

Table 16

Effects of nicotinic acid (5.10^{-6}M) on the metabolism of the isolated ischaemically-perfused hearts from fat-fed rats.

Function	Control	+ Nicotinic acid
Glucose uptake (μmole/g/hr)	6.0 ± 4.0	45.1 ± 23.1
Lactate release (μmole/g/hr)	31.5 ± 5.9	32.8 ± 13.2
% conversion of glucose to lact.	100 *	53.0 ± 18.8 **
FFA release (μmole/g/hr)	4.3 ± 1.2	1.9 ± 1.0 **
Glycerol release (μmole/g/hr)	1.8 ± 0.3	0.6 ± 0.3 **

Values are mean ± S.E. (n = 6).

** indicates that the difference between the means is significant. * approximate value due to error of determination of small glucose changes.

of these functions by adrenaline was not affected by the presence of nicotinic acid.

(c) Effects of nicotinic acid on the responses to adrenaline - second phase, mechanical.

Hearts from fat-fed rats treated with either adrenaline alone or with adrenaline in the presence of nicotinic acid did not differ significantly in their second phase responses to adrenaline with regard to developed tension. Resting tension rose more in the presence of nicotinic acid (136 $\pm$ 61%) than with adrenaline alone (27 $\pm$ 8%). Both these results contradict the expected saving effect of nicotinic acid. The other data do suggest beneficial effects of the antilipolytic agent. Thus heart rate declined more slowly (22.2 $\pm$ 5.3 min to return to pre-adrenaline control rate, compared with 8.9 $\pm$ 2.4 min to return to control with adrenaline alone) and less extensively (214 $\pm$ 8 b/min or 104 $\pm$ 10% of control compared with 112 $\pm$ 11 b/min or 75 $\pm$ 6% of control by 30min after adrenaline)than the group treated with adrenaline alone. In addition, the group of hearts which received nicotinic acid developed significantly fewer arrhythmias (19 $\pm$ 9% of time compared with 43 $\pm$ 9% of time for adrenaline alone) and more importantly developed no ventricular fibrillations at all. By contrast, the group of hearts which received adrenaline alone developed ventricular fibrillation frequently (23 $\pm$ 9% of the 30 min period after adrenaline.

The data concerning MVO_2 and MVO_2/beat should be assessed with caution since both control levels were significantly higher in the nicotinic acid - treated group. There was no obvious explanation for this difference. The difference in control values may explain the significantly smaller rise in MVO_2/beat observed with the nicotinic acid - treated group.

(d) Effects of nicotinic acid on the responses to adrenaline - second phase, metabolic.

Of the metabolic parameters studied, only FFA release after adrenaline was significantly affected by the presence of nicotinic acid. FFA release rate rose from 3.9 ± 0.6 μmole/g/hr to 6.5 ± 1.3 μmole/g/hr with adrenaline alone but did not change significantly from the control in the presence of nicotinic acid (2.4 ± 1.8 μmole/g/hr).

Table 17

Effects of nicotinic acid (5.10^{-6}M) on the first phase responses to adrenaline (10^{-6}M) of isolated perfused hearts from fat-fed rats.

Function	Adrenaline	Adrenaline + nicotinic acid
Heart rate (beats/min)	229 ± 22	236 ± 13
Heart rate (% control)	153 ± 12	128 ± 10
Developed tension (g)	3.6 ± 0.9	7.8 ± 1.7 **
Developed tension (% control)	195 ± 33	195 ± 23

Values are mean ± S.E. representing peak responses to adrenaline during the first 2-8 min after challenge.

** indicates significant difference between the means.

Table 18

Effects of nicotinic acid (5.10^{-6}M) on the second phase mechanical responses to adrenaline (10^{-6}M) of isolated perfused hearts from fat-fed rats.

Function	Adrenaline (n = 16)	Adrenaline + nicotinic acid (n = 6)
Heart rate (beats/min)	112 ± 11	214 ± 8 **
Heart rate (% control)	175 ± 6	215 ± 10 **
Heart rate (time to return to control)	8.9 ± 2.4	22.2 ± 5.3 **
Resting Tension (g)	4.5 ± 0.4	6.3 ± 1.5
Resting tension (% control)	127 ± 8	239 ± 61 **
Arrhythmias (% total time)	43.5 ± 9.3	18.7 ± 8.5 **
Ventricular fibrillations (% total time)	23.0 ± 8.5	none observed **
MVO_2 (µl/g/min)	68.6 ± 7.8	106.3 ± 12.3 **
MVO_2 (% control)	132 ± 10	123 ± 7
MVO_2/beat	0.56 ± 0.07	0.48 ± 0.04
MVO_2/beat (% control)	177 ± 20	101 ± 6 **

Values are mean ± S.E. representing parameters determined 30min after adrenaline infusion (arrhythmias and fibrillations as % of this 30min). ** indicates significant difference between the means.

Table 19

Effects of nicotinic acid (5.10^{-6}M) on the second phase metabolic responses to adrenaline (10^{-6}M) of isolated perfused hearts from fat-fed rats.

Function	Adrenaline (n = 16)	Adrenaline + nicotinic acid (n = 6)
Glucose uptake (μmole/g/hr)	2.9 ± 0.2 *	45.0 ± 22.7
Glucose uptake (% control)	100 *	100 ± 0
Lactate release (μmole/g/hr)	47.3 ± 4.4	66.3 ± 11.5
Lactate release (% control)	466 ± 85	241 ± 48 **
FFA release (μmole/g/hr)	6.5 ± 1.3	2.0 ± 1.2 **
FFA release (% control)	280 ± 81	-17 ± 19 **
Glycerol release (μmole/g/hr)	1.9 ± 0.3	1.6 ± 0.3
Glycerol release (% control)	197 ± 37	230 ± 48

Values are mean ± S.E. representing rates of uptake or release during the 30 min period after adrenaline infusion.

** indicates a significant difference between the means.

* indicates approximate value due to error in the determination of small glucose changes.

Section E. Tissue Analysis

(I) General The investigation of the metabolism of the perfused hearts has depended on the measurement of changes in the perfusate. The procedure gives an indication of tissue substrate utilisation and product release, but the validity of the procedure as a means of determining the tissue concentration of metabolic products is less certain. For example, accumulation of FFA during recirculating perfusion is suggested by the relatively small FFA release compared to that observed during non-recirculating perfusion. Glycerol release was not similarly increased in the non-recirculating perfusion, showing that the extra FFA release was not due simply to a greater degree of lipolysis. In addition, a large accumulation of lactate in ischaemic tissue was reported by Rovetto et al (1973). Thus although the perfusate may reflect in proportional terms the status of the tissue, the absolute relationship between tissue and perfusate concentrations is likely to be less predictable. For this reason, the concentration of FFA in the tissue has been measured directly.

It was anticipated that several questions could be answered :

(i) does fat-feeding result in a higher level of stored lipid in the tissue?

(ii) is the concentration of FFA within the tissue similar to that in the perfusate ?

(iii) do the various conditions of challenge alter tissue FFA concentrations in a way consistent with the hypothesis that the adrenaline-induced effects could

be explained, at least in part, by the accumulation of endogenously-generated FFA.

(2) Effect of fat-feeding on ventricular muscle lipids

Hearts were removed immediately after decapitation, rinsed with perfusate and frozen in liquid nitrogen. The results (table 20) show that hearts from fat-fed rats contained significantly more TGFA (4.14 ± 0.50 μmole/g wet ventricle) than did hearts from normally-fed rats (2.36 ± 0.40 μmole/g wet ventricle). The two groups of hearts did not differ significantly with regard to DGFA or FFA.

(3) Relationship between tissue and perfusate concentrations of FFA. The relationships for the hearts from both fat-fed and normally-fed rats are shown in figure 29. Both sets of data contain results from both adrenaline-treated and adrenaline + nicotinic acid-treated hearts. The sets of data appear to belong to single families, thus the two challenges do not appear to impose different relationships although they do impose different absolute concentrations.

The relationships are described by the equations :

$$\underset{\text{(nmole/ml)}}{\text{perfusate FFA}} = (0.95 \times \underset{\text{(nmole/g)}}{\text{tissue FFA}}) + (-0.5)$$

for the normally-fed group (correlation coefficient = 0.85, $n = 15$, $P < 0.001$).

$$\underset{\text{(nmole/ml)}}{\text{perfusate FFA}} = (0.1 \times \text{tissue FFA}) + 48.8$$

for the fat-fed group (correlation coefficient = 0.66, $n = 16$, $P < 0.01$).

Table 20

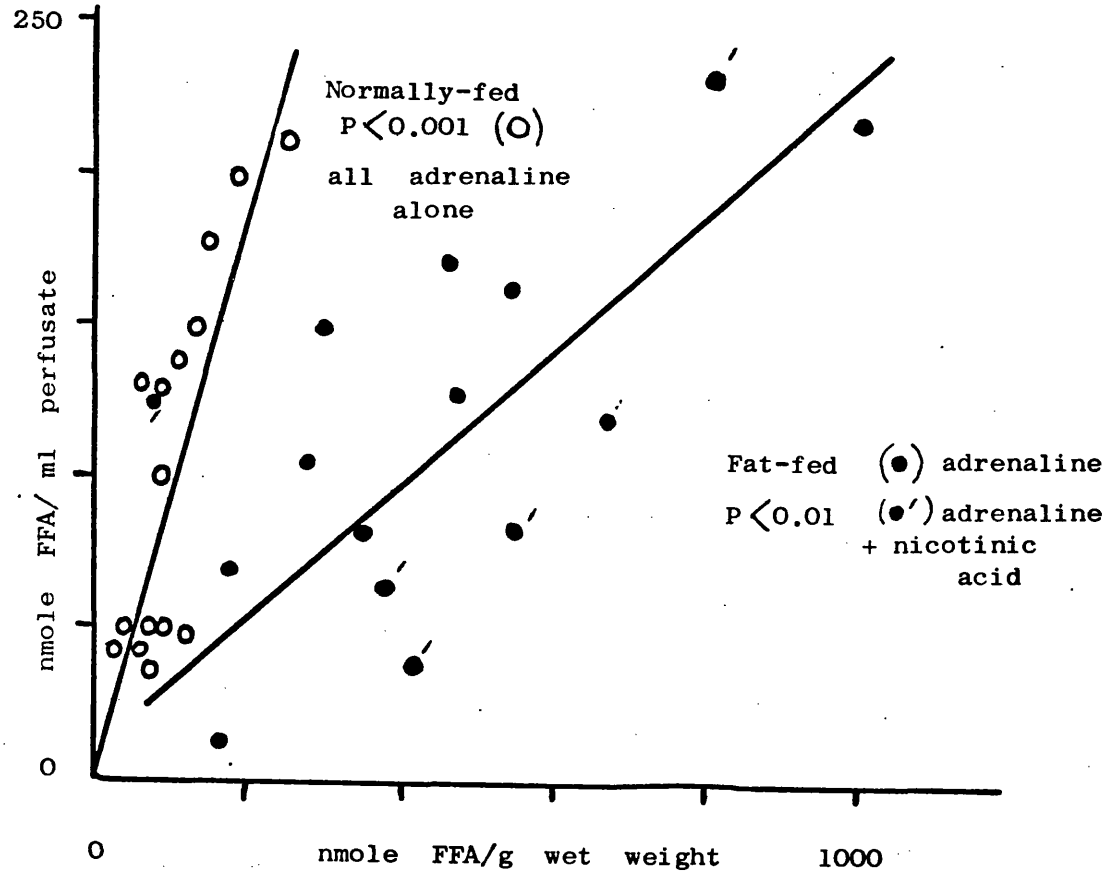
Effects of fat-feeding on the lipid content of rat hearts

Lipid	Fat-fed	Normally-fed
Triglyceride (μmole TGFA/ g wet weight)	4.16 ± 0.45 (n = 4)	2.36 ± 0.48 ** (n = 6)
Diglyceride (μmole DGFA/ g wet weight)	0.20 ± 0.05 (n = 6)	0.22 ± 0.03 (n = 6)
FFA (μmole/ g wet weight)	0.43 ± 0.11 (n = 4)	0.28 ± 0.08 (n = 2)

Values are mean ± S.E. Hearts were removed immediately after decapitation, rinsed in saline and dropped into liquid nitrogen. Results are for ventricular muscle only. ** indicates that the difference between the means is significant.

Figure 29

Relationship between tissue (ventricular muscle) and perfusate FFA concentrations with isolated perfused hearts from normally-fed and fat-fed rats.



Both lines seemed to deviate from linearity at the highest FFA concentrations but this was not a significant trend (the correlation coefficients for logarithmic plots were smaller than those obtained for the direct linear analysis). The relationships should not be greatly influenced by lack of binding capacity for FFA in the perfusate over the range of concentrations studied. The highest perfusate concentrations of FFA studied were about 300nmole/ml (0.3mM) but the albumin concentration of 2g% (0.3mM) should provide a binding capacity for FFA of 600nmole/ml (0.6mM).

(4) Effect of challenge on tissue FFA concentration.

The elevation of tissue FFA concentration by adrenaline was highly significant in hearts from fat-fed rats ($0.83 \pm 0.13 \longrightarrow 1.33 \pm 0.23$ µmole/g wet ventricle) but was not significant in hearts from normally-fed rats ($0.15 \pm 0.02 \longrightarrow 0.24 \pm 0.05$ µmole/g wet ventricle) - P = 0.1-0.05. Similarly, nicotinic acid significantly reduced the effect of adrenaline in the fat-fed group ($1.33 \pm 0.23 \longrightarrow 0.59 \pm 0.12$ µmole/g) but the change in the normally-fed group was not significant ($0.24 \pm 0.05 \longrightarrow 0.15 \pm 0.02$ µmole/g, P = 0.1 -0.05). See table 21.

(5) Effect of coronary flow on tissue FFA concentration.

As for section (c), data were drawn from hearts perfused both with adrenaline alone and with adrenaline + nicotinic acid. The different treatments do not appear to affect the relationship studied. With hearts from fat-fed rats there was no significant relationship between coronary flow

Table 21

Effects of adrenaline (10^{-6}M) and adrenaline + nicotinic acid (5.10^{-6}M) on the FFA concentration in the isolated perfused hearts from fat-fed and normally-fed rats.

Challenge	Fat-fed	Normally-fed
Adrenaline	1.33 ± 0.23 (n = 10) ++,*,ø	0.24 ± 0.05 (n = 6) øø
Adrenaline + nicotinic acid	0.59 ± 0.12 (n = 6)	0.15 ± 0.02 (n = 7) øø
No addition	0.83 ± 0.13 (n = 22)	0.15 ± 0.02 (n = 8) ++,øø

Values are mean $\pm$ S.E. Hearts were perfused for 30min control period followed by 30min in the presence of adrenaline. Nicotinic acid, when used, was added 20min prior to adrenaline. * indicates significant difference between the two challenges. ø indicates significant difference between challenge and control. All values were obtained by direct analysis of the (ventricular) muscle except ++ which were calculated from perfusate concentration and the known relationship between tissue and perfusate concentrations. These latter values should therefore be regarded as tentative. All values are μmole/g wet ventricle. øø indicates significant difference between fat- and normally-fed.

and tissue FFA concentration (correlation coefficient = 0.35, P = NS). With hearts from normally-fed rats, tissue FFA was significantly related to coronary flow :

Tissue FFA (nmole/g) = (-87.0 x coronary flow (ml/min/g)) + 374.6

(correlation coefficient = -0.70, n = 15, $P < 0.01$). This is consistent with an increased oxidation of the available FFA brought about by the increase in oxygen delivery to the tissue. See figure 30.

(6) Lactate concentration of ischaemically-perfused hearts. As yet, data are only available from studies of the hearts of fat-fed rats. Several observations have been made :

(i) no statistically significant relationship was observed between the tissue and perfusate concentrations of lactate. Unlike the situation with FFA, therefore, it is not legitimate to attempt to predict the tissue concentration of lactate from the knowledge of perfusate concentration.

(ii) the concentration of lactate in the tissue was found to decline progressively as coronary flow was increased (figure 31). The regression equation describing the relationship is as follows :

Tissue lactate (μmole/g wet ventricle) = (-0.71 x coronary flow (ml/min/g)) + 11.53

(correlation coefficient = -0.71, n = 13, $P < 0.01$, all hearts were treated with adrenaline alone). This is consistent with a shift to a more oxidative mode of metabolism with the increase in oxygen delivery to the

Figure 30

Effect of coronary flow on the FFA concentration of ventricular muscle of isolated perfused hearts from normally-fed rats.

Hearts were perfused for 30min control and then 30min with adrenaline (●) or adrenaline (10^{-6}M) + nicotinic acid (5.10^{-6}M) (●′).

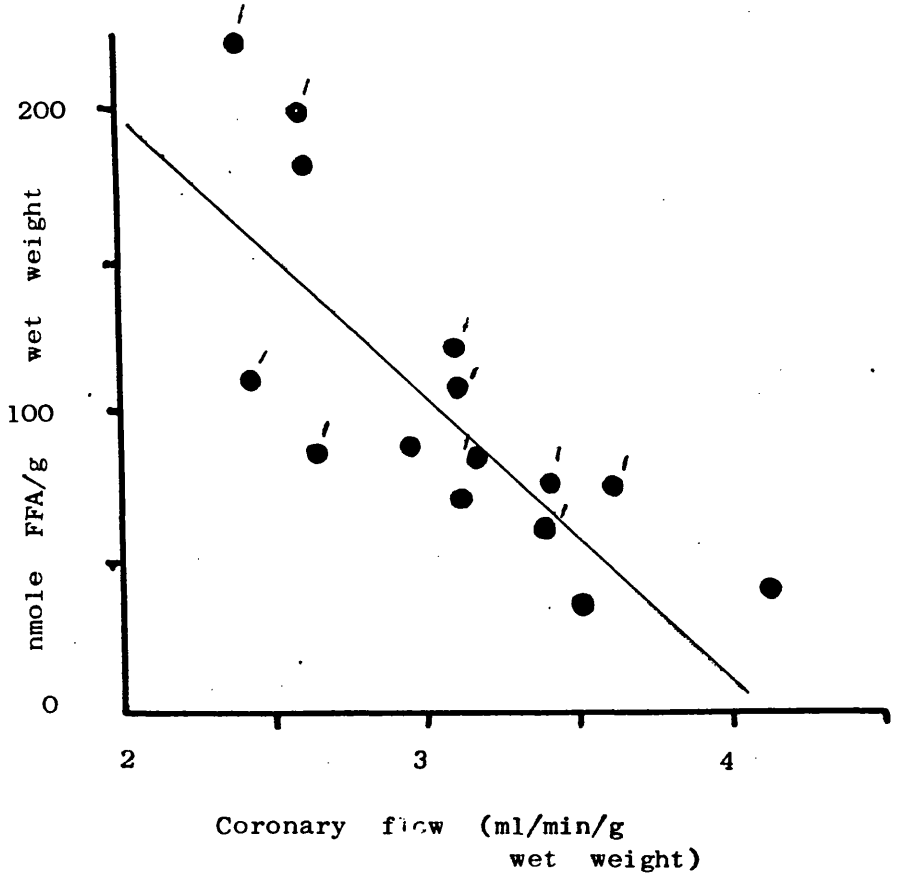
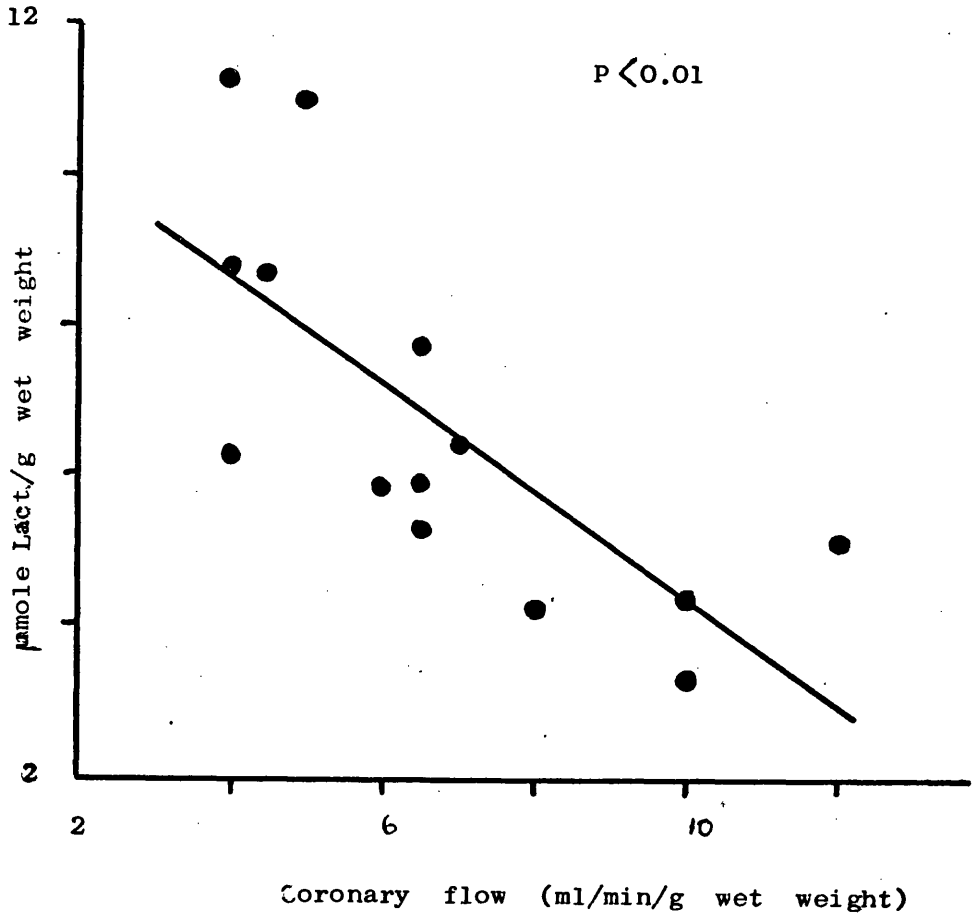


Figure 31

Effect of coronary flow on the lactate concentration of ventricular muscle of isolated perfused hearts from fat-fed rats.



Hearts were from fat-fed rats only, being perfused for 30 min control and then 30min in the presence of adrenaline (10^{-6}M).

tissue. It should be noted that the absolute concentration of lactate in the tissue (even with the very low glucose uptake in the fat-fed series) is of the order 3-10 μmole/g wet ventricle, which is considerably higher than that of FFA (0-0.3 μmole/g wet ventricle in the fat-fed series).

(iv) Lactate concentration after adrenaline was not significantly affected by the presence of nicotinic acid. Thus with adrenaline alone the concentration of lactate was 7.2 ± 0.6 μmole/g ($n = 13$) but was 9.3 ± 1.1 μmole/g ($n = 6$) in the presence of nicotinic acid. Thus the observed saving effects of nicotinic acid were accompanied by a reduction of FFA concentration in the tissue but not by a reduction in the level of lactate. This offers further evidence for the dominant role played by FFA in the adrenaline-induced decline in performance in the ischaemically-perfused hearts used in the study. It should be stressed that the fat-fed series differs from the normally-fed series in that glucose uptake and lactate release are slower. Thus the relative contribution of lactate to adrenaline-induced deterioration may be considerably greater in the hearts from normally-fed rats. This observation suggests that it will be necessary to measure tissue lactate concentration in hearts from normally-fed rats.

(7) Intracellular concentration of lactate and FFA

The values previously reported as μmole/g wet weight have been corrected for the intracellular volume.

Total tissue water = 852 ± 10 μl/g wet weight (n = 5)
Of the total water, 621 ± 32 μl/g is extracellular and thus 231 μl/g is intracellular.

In hearts from fat-fed rats treated with adrenaline (table 22) a very large excess of both lactate (50x) and FFA (46x) developed, compared to the perfusate concentration. The molar concentrations in the intracellular water were 31.00 ± 2.45 mM (lactate) and 5.70 ± 1.02 mM FFA. This represents values of the extreme of fat-fed rats with hearts stimulated with adrenaline. The other extreme of normally-fed rats with hearts in control perfusion and adrenaline + nicotinic acid (FFA values only) is described in table 23. Here the FFA concentration is much lower, being 0.29 ± 0.07 mM (control) and 0.24 ± 0.06 mM (adrenaline + nicotinic acid). The excess of tissue concentration over perfusate is 4:1 and 2:1 respectively.

Table 22

Intracellular concentration of FFA and Lactate in the ventricular muscle of isolated perfused hearts of fat-fed rats.

Product	Lactic acid (n = 16)	FFA (n = 10)
Perfusate concentration (μmole/ml)	0.63 ± 0.05	0.13 ± 0.01
Tissue concentration (μmole/g wet weight)	7.21 ± 0.60	1.33 ± 0.24
Intracellular concentration (μmole/ml)	31.00 ± 2.45	5.70 ± 1.02
Perfusate/intracellular concentrations (both μmole/ml)	1/49.9	1/46.3

Values are mean ± S.E. Intracellular concentration was calculated on the basis of measurements which showed that intracellular volume was 221μl of a total 852μl tissue water/g wet weight. Hearts were perfused for 30min control period, followed by 30min in the presence of adrenaline (10^{-6}M) and the frozen in liquid nitrogen.

Table 23

Intracellular FFA concentration of the ventricular muscle of isolated perfused hearts from normally-fed rats.

Challenge	Control (n = 8)	Adrenaline + nicotinic acid (n = 7)
Perfusate concentration (μmole/ml)	0.08 ± 0.01	0.14 ± 0.03
Tissue concentration (μmole/g wet weight)	0.15 ± 0.02	0.15 ± 0.02
Intracellular concentration (μmole/ml)	0.29 ± 0.07	0.24 ± 0.06
Perfusate/intracellular concentrations (both μmole/ml)	1/3.5	1/1.7

Values are mean ± S.E. Hearts were perfused for 60min, or for 30min followed by 30min in the presence of adrenaline (10^{-6}M) and nicotinic acid (5.10^{-6}M). Nicotinic acid was added 20min before adrenaline. At the end of perfusion, hearts were frozen in liquid nitrogen prior to FFA analysis.

Section F. Nature and Location of Cardiac Lipid.

(1)Location of Lipid. It had been assumed that the major lipid reserve of the heart was located in the ventricular muscle, and that this reserve was mobilised during the perfusion. This assumption has been validated.

The total heart tissue was divided by gross inspection, into 5 discrete types of tissue. The wet weights of these different types are shown in table 24. The ventricular muscle represented about 80% of the total heart weight.

Several freshly excised hearts were perfused in the usual way, but with medium containing dye (bromocresol green). The dye rapidly stained the entire ventricular muscle (within 1-2sec). The atria and aorta also became stained but the extraneous tissue did not, suggesting that the latter was unperfused. The lack of perfusion restricts the contribution of this tissue to the overall observed metabolism during perfusion. The only contribution will be by diffusion across the surface into the perfusate which bathes the heart.

The lipid content of the ventricular and "atrial" (fractions 2-5 pooled to provide sufficient material for analysis) fractions of the heart were measured before and after perfusion. The results in table 25 confirm that the triglyceride reserve of the ventricular muscle is significantly depleted ($2.36 \pm 0.48 \longrightarrow 1.45 \pm 0.28$ μmole/g, $n = 11$, $P < 0.05$) whilst that of the atrial fraction is not ($3.20 \pm 0.80 \longrightarrow 4.80 \pm 1.70$ μmole/g) during perfusion lasting 1hr with adrenaline present for

the final 30min. All hearts were from normally-fed rats. The results thus confirm the major contribution made by the ventricular muscle reserve. The decline in the triglyceride is insufficient, however, to account for the observed FFA release. The control rate of lipolysis is 1.9 μmole/g/hr, stimulated to 3.6 μmole/g/hr by adrenaline. The total FFA release in 1hr with 30min adrenaline is thus expected to be 2.75 μmole. This casts doubt on the lipid extraction procedure and the values reported here are indeed smaller than those of Crass et al (1969). Taking these latter values, which are about 100% greater than the ones reported, the decline in ventricular TG would be about 4.7 → 1.9 μmole/g or 2.6 μmole. This is close to the observed FFA release.

(2) Nature of lipid. The fatty acid compositions of perfusate and tissue FFA pools have been studied by gas-liquid chromatography of the methyl esters of FFA. Quantitation of FFA was obtained by calculating the area under the chromatogram trace, having deducted the control (albumin solution, for the perfusate samples). Individual FFA were characterised by comparison of retention times to those of known fatty acid methyl esters.

FFA compositions of the tissue and perfusate have been expressed as percent composition of the total content. The tissue contained significantly more of FFA of chain length above C-18 but significantly less of C-16:1. The other detected FFA were similar in both pools. i.e. 12:0, 14:0, 14:1, 16:0 and 18:0,:1,:2.

Table 24

Relative weights of different component tissues of the rat heart.

Tissue type	mg/g wet weight total heart.
1. Ventricular muscle	778 ± 20
2. Atrial muscle	61 ± 5
3. Aorta	26 ± 4
4. Connective tissue *	84 ± 10
5. Extraneous tissue ø	50 ± 12

Values are mean ± S.E. (n = 6). Freshly excised hearts from normally-fed rats were rinsed with saline and divided by dissection into anatomically discrete tissues. * was essentially the atrio-ventricular septum and valves. ø was largely adipose tissue situated near the atria and aorta.

Table 25

Fatty acid compositions of the FFA pools of perfusate and ventricular muscle of isolated perfused hearts from normally-fed rats.

Individual FFA (retention time - min)	Tissue FFA (n = 4)	Perfusate FFA (n = 8)
12:0 (1.5)	1.8 ± 0.9	4.3 ± 1.7
14:1 (2.5)	0.0 ± 0.0	1.6 ± 0.6
14:0 (3.4)	10.3 ± 5.0	11.8 ± 6.0
16:1 (5.5)	0.8 ± 0.8	17.5 ± 4.3 **
16:0 (8.6)	27.0 ± 13.8	34.5 ± 10.0
18:0,:1,:2 (12-19)	13.3 ± 4.2	29.0 ± 13.4
C-19 and above (30 -)	45.3 ± 18.0	0.5 ± 0.5 **

Values are mean ± S.E. representing the percent contribution of the individual FFA to the total composition. ** shows significant difference between tissue and perfusate. Hearts were perfused for 30min control period followed by 30min in the presence of adrenaline (10^{-6}M) and then frozen in liquid nitrogen prior to lipid extraction, purification and gas-liquid chromatography.

DISCUSSION

SECTION A Myocardial Lipases

1. Activation

The main aim of the investigation was achieved in that an activatable lipase system (sensitive to cyclic AMP) was demonstrated in both the pig heart and rat heart extracts. It should be emphasised that this tissue lipase was assayed under conditions which virtually exclude interference from lipoprotein lipase. In particular, the low pH and absence of serum activating factor would prevent lipoprotein lipase activity.

2. Location

No positive conclusion can be drawn concerning the intracellular location of the lipases in general but it does appear that the 100,000g pellet is the only fraction studied which contains the complete system for activation. By analogy with adipose tissue this implies that both hormone-sensitive lipase and cAMP-dependent protein kinase are present in the 100,000g pellet but are not both present in such useful concentration in the other fractions studied.

3. Complexity of the "lipase"

The results indicate that there are at least two discrete lipases present in the 100,000g pellet. One

activity hydrolyses tripalmitin (and the TG component of ediol) and can be stimulated by cyclic AMP. Another activity must be capable of monoglyceride (and possibly Tween) hydrolysis but is insensitive to cyclic AMP. It is not possible to say to what extent the monoglyceride lipase might contribute to the non-cyclic AMP hydrolysis of triglyceride. It is safe to assume, however, that the triglyceride lipase is unable to hydrolyse monoglyceride, unless one imagines some change in substrate specificity coincident with cyclic AMP - induced activation. The latter possibility may be more feasible in the light of evidence from Okuda and Fujii (1975). Studying the adrenaline-stimulation of lipolysis in adipose tissue lipid micelles, they have presented evidence that the activation involves stimulation of the interaction between substrate and enzyme rather than stimulation of the enzyme per se. This may offer another explanation for the evidence of Huttenen and Steinberg (1968) and others who have shown activation to be coincident with phosphorylation of the lipase itself. Previously, this had been assumed to stimulate lipase activity in a way analagous to the activation of glycogen phosphorylase.

4. Further Work

(a) Purification. Further progress would seem to require some degree of purification of the lipase preparation. This should lead to information concerning the nature of activation and its possible similarity to

that of adipose tissue lipase. The requirement for protein kinase as suggested by the experiments with rat heart lipases - and the phosphorylation of the lipase may be confirmed. Purification should also open the way to investigation of the multiplicity of otherwise of the "lipase system". In particular the number of discrete lipases and their specificity with respect to both lipid class (mono-di-triglycerides) and the nature of the fatty acid residues may be elucidated.

(b) Endogenous Substrates The presentation of substrates has been and remains a problem in the investigation of lipases. Even with apparently good activity, it is not possible to determine if the substrate presentation is entirely satisfactory. An interesting approach has been used by Okuda and Fujii (1973) using endogenous substrate in the form of lipid micelles prepared from adipose tissue. The micelles respond both lipolytically and lipogenically to hormones. Endogenous lipolysis has also been studied in rat heart extracts by Sonnenblick et al (1974) so it seems likely that there is sufficient substrate to make this measureable. The adequate supply of substrate is confirmed by the results to be shown later in this thesis. The endogenous substrate is largely excluded in the present method of lipase preparation by the removal of any floating material. The specificity of activity would be more difficult to test using endogenous substrate, requiring quantitation of triglyceride, diglyceride and monoglyceride

and also characterisation of FFA released.

SECTION B. The Isolated Perfused Rat Heart as a Model of Ischaemia

1. Ischaemia / Hypoxia

It is important to emphasise that normoxic perfusate has been used throughout and that the effects of ischaemia (induced by low coronary flow) rather than the effects of hypoxia have been studied. The terms "ischaemia" and "hypoxia" are used in a functional sense to describe the challenge imposed.

The extent to which even high coronary flow will still induce some degree of hypoxia in the isolated perfused rat heart has been discussed by Henderson et al (1969). The capacity of perfusate to carry oxygen is restricted to the solubility of oxygen whilst blood has a greatly increased capacity afforded by haemoglobin in addition to this simple solubility. Despite this an argument can be made to show the potential adequacy of oxygen supply in simple solution. Thus, on the basis of evidence which shows that the MVO_2 of the intact working heart in vivo (Selkurt, 1971) is about 100µl oxygen/g wet weight/min, the perfusate is theoretically able to deliver sufficient oxygen even at 10ml/min/g. When perfusate is exposed to a partial pressure of oxygen of about 550 mm mercury, the oxygen content is about 16 µl/ml. Thus at 10ml/min/g coronary flow the oxygen delivery is 160 µl/min which allows a considerable excess above the predicted requirement

of the tissue of 100 µl/min/g. However, the initial reservation must be considered when any attempt is made to extrapolate from results obtained in vitro to predict possible effects in vivo.

2. Graded Ischaemia

Previous reports have described the reduction in mechanical performance and changes in metabolism associated with the imposition of low coronary flow in the isolated perfused rat heart (Opie, 1965; Neely et al, 1973). Such changes have been confirmed in this investigation and in addition it has been possible to generate a system of graded ischaemia. The indices of ischaemia intensify progressively as coronary flow is reduced. Such a model system allows for comparative investigations without the necessity to consider the less secure definition of the absolute limits of ischaemic and non-ischaemic tissue.

3. Whole Heart Ischaemia

The use of whole-heart ischaemia as in this model, is restrictive if one is interested in investigating the effects of ischaemia on cardiac performance in the clinical sense. With clinically overt ischaemic disease there exists, within the myocardium, both tissue which has a severely restricted blood supply and tissue which is adequately perfused (i.e. "regional ischaemia"). With

imposed whole heart ischaemia, the gross effects upon rate and tension can be observed and in those studies in which cardiac output can be determined (Rovetto, et al, 1973) it is found that the primary measures of performance (rate, tension, dP/dt) reflect the nett effect of ischaemia upon cardiac output. With imposed regional ischaemia, however, the relationship between the primary indices of performance and cardiac output is not as clear. Severely ischaemic tissue exhibits depressed output as one would expect, but the remaining tissue can perform at a normal or supranormal level in order to compensate for the depressed region (Lekven et al, 1973). The overall effect of regional ischaemia upon cardiac output is thus very difficult to predict and will depend upon both the size and location of the area of depressed tissue.

Despite this disadvatage, the model which utilises whole-heart ischaemia offers a more straightforward interpretation of information about the metabolism of ischaemic tissue. Since the tissue is uniformly perfused, it can be expected to respond in a relatively uniform fashion. By contrast, with imposed regional ischaemia, the biochemical responses of ischaemic tissue are difficult to isolate from those of nearby non-ischaemic tissue. The reason is that it is difficult to distinguish between the blood (or perfusate) emerging from ischaemic tissue and that emerging from non-ischaemic tissue. Gross sampling at the coronary sinus is unlikely to be sufficiently sensitive since observations will be made upon perfusate

from ischaemic tissue which has been diluted by perfusate from normal tissue (unless the size of the ischaemic tissue is large compared to that of normal tissue and in the clinical setting this is extremely unlikely). Instead of sampling from the coronary sinus alone, the sinus site can be used as an index for normal tissue and is compared to perfusate drawn from a local vein emerging from the ischaemic tissue. Such local vein sampling requires a very delicate technique in a small experimental heart such as that of the rat. Alternatively, a more conveniently - sized preparation such as a dog heart is used. This means that the great advantage of the inexpensive rat heart preparation is lost.

In addition to the differences between the whole-heart and regional ischaemia models, results from both models should be assessed in the light of evidence which shows that differences in metabolic response may occur depending on the depth of tissue. Subepidardial muscle is likely to be more adequately perfused than subendocardial muscle (as described in the introduction). The differences in metabolism between the two levels of tissue are slight under normal conditions but become marked as the blood or perfusate supply is reduced. As total tissue coronary flow is reduced, the decline in flow to subendocardial tissue is greater than the decline experienced in the subepidardial tissue. The subendocardial tissue is thus relatively more ischaemic.

4. Oxygen extraction

The changes in oxygen utilisation with change in coronary flow suggest two interesting points. Firstly, although a straight line gives the best statistical fit of data to the line relating MVO_2 to coronary flow, there is a tendency for the MVO_2 to rise less per unit of coronary flow at the highest coronary flows studied. This indicated that approaching 7.8 ml/min/g the coronary flow may be almost sufficient to meet fully the oxygen requirements of the heart. This would agree with literature data concerning MVO_2 in vivo.

The second point arises from the relationship between coronary flow and the percent extraction of oxygen. At the lowest coronary flow used (2ml/min/g) the heart extracts almost all the oxygen delivered. It is difficult to be certain of extraction at this extreme since a finite error is involved in measurements due to the possibility of reoxygenation of the perfusate between the heart and the "venous" oxygen electrode (even with the closed system used). Despite the ability to eventually extract almost all the delivered oxygen at sufficiently low coronary flow, the extraction is still sub-maximal at flow rates where performance is depressed. Thus at 5 ml/min/g even though both heart rate and developed tension are depressed the oxygen extraction is not maximal. One might expect maximal oxygen extraction at such flow rates in order to maximalise performance but this does not appear to happen.

There are several possible explanations for this observation. One possibility is that as coronary flow is increased, the residence time of oxygen is sufficiently reduced to prevent maximal extraction. To test this possibility one would need to know the "mean residence time" of oxygen (i.e. perfusate) in the tissue (about 4 sec at 4 ml/min/g, 2 sec at 8 ml/min/g) and compare this to the time required for gaseous diffusion between perfusate and tissue—if the latter is of the order of seconds it could significantly affect extraction.

Another possible explanation for sub-maximal oxygen extraction is some restriction upon oxygen utilisation other than simply oxygen supply. For example if oxidative metabolism were inhibited then oxygen utilisation would be depressed, (see 5, substrate utilisation)

One final point suggested by the data concerning oxygen consumption illustrates a similarity of the model to the situation existing in vivo. In both cases, the major factor contributing to oxygen supply is the coronary flow and the maintenance of flow is crucial to the maintenance of function. There is some capacity to increase percent extraction of oxygen but this represents a comparatively small reserve.

5. Substrate Utilisation

The utilisation of glucose offers a sensitive index of the status of the tissue, particularly showing the difference

between the challenges of hypoxia and ischaemia. In hypoxic tissue one observes a faster response with a large increase in glucose uptake and lactate release. Thus the relatively inefficient glycolytic mode of energy production can proceed sufficiently rapidly to provide energy for contraction. In ischaemic tissue this response can occur for a short time (Rovetto et al, 1973) but soon declines. The coronary flow (unlike that in hypoxia) is insufficient to supply glucose and more particularly to remove lactate. Thus even the relatively inefficient glycolytic mode of energy production is inhibited in ischaemic tissue. This inhibition of glucose utilisation provides a possible explanation for the restriction of oxygen utilisation in the tissue.

The evidence of submaximal oxygen extraction suggests that there is still some unused capacity for oxygen utilisation in the perfused heart preparation. Interventions which are designed to release this unused oxygen capacity (thus encouraging greater substrate oxidation) might be expected to improve the performance of the heart during ischaemia (see Section C).

6. Electrocardiogram

The electrocardiographic evidence, though somewhat limited, indicates that the changes in T wave and ST segment commonly associated with ischaemia in the clinical sense can be observed in the isolated perfused rat heart. It is hoped that a direct epicardial electrode may provide a

larger and more consistent signal. A technique to achieve this has recently been suggested (Burges, 1975) whereby a light, coiled spring of stainless steel wire (offering very little mechanical resistance) would be attached to the apex of the ventricular musc le and coiled around the strain-guage thread.

The ECG is potentially a very useful immediate measure of the degree of ischaemia within the tissue, particularly when used in such an experimental situation which eliminates many of the anomolies observed with its clinical use (e.g. T wave changes are associated with conditions other than ischaemia - Armstrong, 1968). The application of the ECG in the perfused rat heart has suffered from a lack of knowledge as to the metabolic basis of the electrical changes. Recent evidence has begun to overcome this problem with the finding that the severity of the ST changes are quantitatively related to the depression of tissue pO_2 (Angell et al, 1975).

SECTION C The Role of Myocardial Lipolysis in the Ischaemically - Perfused Rat Heart

1. Adrenaline-induced Deterioration in mechanical performance

Adrenaline induced significant deteriorations in performance in the ischaemically-perfused hearts after initial (first phase) chronotropic and inotropic responses. The second phase deteriorations were principally: a rise in resting tension (taken to be an indication of a rise in end - diastolic pressure which is a sign of failure in the intact heart in vivo - Braunwald and Ross 1963), a decline in developed tension and a rise in the incidence of arrhythmias. Over the time-course of the changes in performance, marked changes in metabolism of the hearts were observed. In particular, endogenous lipolysis was stimulated as assessed by the release of FFA into the perfusate and the release of lactic acid was also increased. Moreover the lactic acid release occurred in the absence of a significant rise in glucose uptake, thus suggesting a decrease in the extent of glucose oxidation. This inference from the data may be questioned, however, since adrenaline challenge is likely to stimulate glycogolysis (by the stimulation of the conversion of phosphorylase 'b' into the more active 'a' form) and so the extra lactate output may be explained at least in part by glycogen degradation.

The adrenaline-induced rise in resting tension was significantly correlated to both the rate of release of endogenously-generated FFA and that of lactic acid. In addition, the decline in developed tension was significantly correlated to the release of FFA (though not to that of lactic acid). These relationships are consistent with the involvement of FFA (endogenous lipolysis) and lactate in the adrenaline-induced (second phase) deteriorations in performance.

2. Amelioration of adrenaline-induced deterioration with antilipolysis

To test the involvement of myocardial lipolysis in the adrenaline-induced deteriorations of the second phase reponse an antilipolytic agent (nicotinic acid) was applied during the adrenaline challenge. The results show that the lipolytic component (but not the mechanical component) of the adrenaline challenge was inhibited. At the same time the deteriorations in performance were greatly reduced. Thus the rise in resting tension was abolished and the incidence of arrhythmias was reduced. In addition, the decline in developed tension caused in the second phase of the adrenaline challenge was also significantly reduced. Two experiments using insulin as an alternative antilipolytic agent gave similar results. This evidence indicates that the stimulation of endogenous lipolysis by adrenaline contributes significantly to the deteriorations in performance observed.

A possible mode of action of the lipolytic factor (in addition to one of the several direct actions of FFA pointed out in the introduction) is suggested by the fact that the fractional conversion of glucose to lactate rises less with adrenaline in the presence of an antilipolytic agent. This would indicate either a smaller glycogenolytic response to adrenaline with nicotinic acid or a greater degree of glucose oxidation - made possible by the reduction in the availability of FFA for oxidation, which would be expected to release more oxidative capacity to glucose oxidation. This argument is based on the glucose - FFA cycle of Randle et al (1963).

3. Influence of Recirculating Perfusate

The imposition of a non-recirculating rather than the usual recirculating perfusion greatly reduces the deterioration in performance induced by adrenaline. This suggests that the recirculating perfusate contributes significantly to the adrenaline effects. It has been assumed that the likelihood of a direct action of adrenaline and/or its metabolites is not great considering the maximum concentration is 10^{-6}M. Ignoring this possible contribution, the major components of the recirculating perfusate likely to be disadvantageous are FFA and lactic acid. A contribution to deterioration by glycerol seems unlikely and the glucose concentration never fell sufficiently to limit the supply of that substrate.

4. Presentation of Exogenous Palmitate and Lactate

Experiments in which either lactic acid or palmitic acid (bound to defatted serum albumin) were presented to the heart showed that these acids had no apparent effect on performance or metabolism at concentrations well above those demonstrated in the recirculating perfusate.

The lack of effect of lactic acid and FFA as applied exogenously argues against a direct effect of the recirculating metabolic products upon heart performance but poses the problem concerning how the recirculating perfusate exerts its apparent effect. One possibility is that the rather low perfusate concentrations may reduce the clearance of metabolic products from the tissue rather than exerting a direct effect. This possibility is supported by the greater release of FFA into the non-recirculating perfusate than into the recirculating perfusate. This is understandable in that fresh albumin (unloaded with FFA) is continuously being presented in the non-recirculating perfusion whereas the binding capacity of the recirculating perfusate is progressively reduced and can be expected to exert some restriction upon FFA withdrawal from the tissue. In addition, the fractional conversion of glucose to lactate was significantly lower in the non-recirculating perfusion, indicating more extensive oxidation of glucose. This is consistent with a release of inhibition of glucose oxidation when FFA are more extensively removed from the oxidation sites (Randle et al, 1963).

5. Possible central role of FFA

The evidence taken in total indicates the involvement of both FFA and lactic acid in the deteriorations in performance induced by adrenaline in the ischaemically-perfused rat heart. The evidence available supports the contention that of these two factors the generation of FFA within the tissue may be the more primary factor. The rise in FFA production could exaggerate the tendency for lactic acid production by reducing the capacity of the tissue to oxidise glucose, according to the glucose - FFA cycle.

The effects have been developed in the absence of external FFA or lactate (except for relatively low recirculating levels which alone could not induce obvious deteriorations in performance) and were thus caused by endogenously generated metabolic products. This is not to deny an important role for recirculating products which may depress clearance of the endogenously-generated products as argued earlier. By comparison to externally-applied metabolites, endogenously-generated metabolites may be generated in a more damaging mode. For example, from the evidence of Stein and Stein (1968) FFA applied exogenously proceed across the cell wall and then through the sarcoplasmic reticulum to lipid droplets and mitochondria. This uptake was dependent upon plasma concentration. By contrast, endogenously-generated FFA released from lipid droplets (located very close to mitochondria) would be

immediately released into the vicinity of the mitochondria where localised effects on oxidative metabolism could become apparent. Such effects would be particularly exaggerated in ischaemic conditions where the capacity to remove FFA - by oxidation and by losses into perfusate - is reduced. By contrast, in hypoxia only the oxidative removal of FFA would be depressed.

The decline in performance following the adrenaline challenge during ischaemia could then be rationalised in two ways. Firstly, by the known metabolic and physio-chemical effects of FFA as described in the introduction; and secondly by generalised acidosis. Acidosis would be primarily a function of the accumulation of lactic acid rather than FFA. This is explained by the generation of a higher molar concentration of lactic acid than FFA (as will be seen later). In addition, lactic acid can be expected to be more available to the general aqueous phase than FFA (being more hydrophilic) and also as a stronger acid, would contribute more extensively to cellular hydrogen ion concentration.

SECTION D Perfusion of hearts from fat-fed rats

1. Control Perfusion

The results obtained are consistent with the hypothesis that myocardial lipolysis and the endogenously generated FFA can exert significant effects upon the performance of the ischaemically-perfused heart. Hearts from fat-fed rats showed significantly elevated rates of lipolysis compared to hearts from normally-fed animals. Evidence concerning tissue lipid and FFA concentrations also supports the evidence from analysis of the perfusate (see later). The glucose utilisation of the hearts from fat-fed rats was greatly depressed and lactate release was also reduced compared to hearts from normally-fed rats. The almost complete dependence upon endogenous lipid as an energy source was accompanied by a marked depression of performance and also an increase in the oxygen-cost of mechanical performance. The fat-fed series thus presents a situation in which the release of lactic acid is relatively low, yet performance is markedly depressed. This suggests that endogenously-generated FFA exert a more significant effect than lactic acid and may indicate that FFA also exert a more important role than lactic acid in the hearts from normally fed rats.

The elevation of oxygen-cost of performance was considerably more than would be expected if the only

reason were the relatively greater oxygen requirement for oxidation of lipid as opposed to carbohydrate. The expression of this function is the P : O ratio (see introduction) which is 3.0 for carbohydrate and 2.8 for lipid. The difference is about 6% only, much less than the difference between oxygen cost of performance in fat-fed and normally-fed series, which was about 50%. This suggests additional oxygen-wasting associated with lipid oxidation. The uncoupling of oxidative phosphorylation by FFA observed by Borst et al (1962) would offer an explanation for these results.

2. Effects of adrenaline

As in the normally-fed series, adrenaline induced marked deteriorations in performance during the second phase response (8 - 30 min after adrenaline infusion). One would predict a worsening of adrenaline response in view of the greater mobilisation of endogenous lipid and in some significant respects this was true. Thus the incidence of ventricular fibrillation and decline in heart rate were significantly increased in the fat-fed series compared to the normally-fed series. The adrenaline-induced incidence of arrhythmias, decline in developed tension and rise in resting tension were not further stimulated by the challenge of fat-feeding.

It should be remembered, particularly with respect to resting tension, that the fat-feeding induced a marked

response during control perfusion so that a further worsening with adrenaline may not have been possible.

Although the evidence is not entirely consistent it largely supports the hypothesis that the stimulation of endogenous lipolysis contributes significantly to the decline in performance observed in the second phase response to adrenaline. Of the individual parameters, the incidence of ventricular fibrillation may be the most significant single element considering the critical effect of such a response upon the potential survival of the heart.

3. Effects of Nicotinic acid

(a) Control Perfusion In the normally-fed series nicotinic acid had no effect upon the control rate of lipolysis, suggesting that a lipase was present which was insensitive to nicotinic acid (unlike the nicotinic acid-sensitive adrenaline-stimulated lipase). The effect of nicotinic acid in reducing control lipolysis in the fat-fed series indicates that at least part of control lipolysis is contributed by the adrenaline-stimulated lipase. The system of lipolysis may therefore be partly activated in the absence of exogenous catecholamines. This could be explained by a greater concentration and activity of endogenous catecholamines in the hearts from fat-fed rats (which could be tested by direct measurement) or perhaps by a more sustained modification of the enzymic machinery of the hearts accustomed to a more extensive metabolism of

lipid. Another consequence of the inhibition of control lipolysis might be the stimulation of the use of carbohydrate as an energy-yielding fuel. Evidence for this from this work is not convincing as yet but it is worth examining with further experiments. It would be particularly interesting to see if the performance of the hearts improved as the metabolism switched from lipid utilisation to carbohydrate utilisation. The lack of a significant effect in the experiments reported here may be due to the fact that insufficient perfusion time was used to allow full adaptation of the tissue to glucose utilisation.

(b) Adrenaline Challenge Nicotinic acid abolished the lipolytic response to adrenaline and at the same time exerted some saving effect upon the adrenaline-induced (second phase) deterioration in performance. Thus the incidence of both arrhythmias and ventricular fibrillations was significantly reduced. In addition, the decline in heart rate was slower and less extensive in the presence of nicotinic acid. The evidence is not entirely consistent however, since no improvement in developed tension and no reduction in the rise in resting tension were observed in the presence of nicotinic acid.

The oxygen cost of work (and to a lesser extent MVO_2 itself) do not rise with adrenaline in the presence of nicotinic acid, whilst a marked rise is found in the challenge with adrenaline alone. This is consistent with the depression

of FFA availability and a consequent increase in glucose oxidation in the nicotinic acid-treated group compared to the adrenaline group.

As with the perfusion of hearts from normally-fed rats, the results in this section largely indicate the important consequences of stimulation of endogenous lipolysis during ischaemia. The significance of the various parameters involved must be carefully assessed in order to draw conclusions as to the nett effect of the antilipolytic agent. In this respect the abolition of ventricular fibrillations in the presence of nicotinic acid may be crucial since it represents the most severe challenge to the survival of the tissue.

Section E. Tissue lipid and tissue lactate.

The metabolic studies had involved measurement of changes in the perfusate and these were assumed to reflect changes imposed by and within the tissue. The underlying assumption in particular, had been that the effects of endogenous lipolysis were mediated by the intracellular concentration of FFA and also that of lactic acid. It was necessary, therefore, to test this assumption. Evidence has been presented concerning the concentration of FFA within the tissue, but further work is required to complete the investigation of tissue lactate levels.

(1) Tissue TG elevated by fat-feeding. The finding of increased lipolytic rates in hearts of fat-fed rats compared to those of normally-fed rats was supported by the significant increase in the total triglyceride stores of the hearts from fat-fed rats. The levels of FFA and diglycerides were not significantly different in the two groups.

(2) Tissue FFA changes and perfusate changes. The evidence provided by changes in tissue FFA concentration with different challenges supports the hypothesis concerning the involvement of FFA in the effects of adrenaline. Thus adrenaline significantly increased the tissue concentration of FFA in hearts from fat-fed rats (the rise of FFA in the hearts of normally-fed rats after adrenaline was of marginal significance - $P = 0.1\text{-}0.05$). The presence of nicotinic acid depressed this rise of tissue FFA -

significantly in the hearts from fat-fed rats but not significantly in hearts from normally-fed rats (P = 0.1-0.05).

The relationship between perfusate and tissue concentrati on of FFA was linear and highly significant in the hearts from both fat-fed and normally-fed rats, over the range of concentrations studied. Changes in perfusate concentration of FFA thus accurately reflect the changes taking place in the tissue. Such a relationship did not exist between perfusate and tissue concentrations of lactate, indicating that lactate changes measured in the perfusate need not reflect change in the tissue.

(3) Intracellular accumulation of FFA and lactate. Although the perfusate concentration of FFA is an index of the tissue concentration, the two values differ in absolute terms. The difference is especially marked when the tissue content is converted into intracellular concentration. The evidence indicates, moreover that the pre-adrenaline control level (an excess in the tissue of 24x the perfusate concentration) is markedly shifted by adrenaline to a 46x excess. The challenge thus greatly accentuates the accumulation of FFA in the tissue. The shift in excess is abolished by nicotinic acid.

The concentration of FFA within the tissue changes as would be predicted if the effects of adrenaline (and the amelioration of those effects with antilipolysis) were to be explained, at least in part, by the tissue concentration of FFA. With the hearts from fat-fed rats, the

concentration of lactate does not change in the direction expected for its involvement in the adrenaline-induced effects. The lactate concentration after adrenaline is not reduced in the presence of nicotinic acid. This indicates a more significant contribution by FFA than lactate to the adrenaline-induced effects, in the hearts from fat-fed rats. It must be stressed that the glycolytic flux is lower in these hearts than those from normally-fed rats and therefore the relative importance of lactate as a contributing factor in the adrenaline effects may be greater in the latter group.

The intracellular concentration of lactate is very high in the ischaemically-perfused hearts of the fat-fed rats (up to 30mM). Such concentrations would be expected to significantly affect tissue pH and the consequences of such a change have been described by Rovetto et al (1973).

The poor relationship between the lactate concentration of the tissue and that of the perfusate indicates that the lactate concentration of perfusate cannot be legitimately used as an index of the tissue concentration during ischaemia. Further, the constant lactate release over a range of ischaemic flow rates indicates that the degree of lactate release is a poor index of the degree of ischaemia.

(4) Tissue binding capacity for FFA. The concentration of FFA and the accumulation of FFA in the intracellular space are both greater in the hearts from fat-fed rats

than those from normally-fed rats. It is not known why the accumulation in excess of the perfusate concentration should be so marked in such tissue. As was discussed earlier, the albumin content of the perfusate should be sufficient to allow a much greater FFA release than is actually observed. The evidence indicates, rather, that the binding capacity of the tissue for FFA is increased in the hearts from fat-fed rats. A cytoplasmic protein which binds FFA with high affinity has been described (Ochner, Manning, Poppenhausen and Ho, 1972). It is possible that this protein is present in large amounts in the tissue of fat-fed animals, in order to accommodate the greater dependence on lipid utilisation.

Another possibility for tissue binding of FFA is provided by the increase in the triglyceride content of the myocardium with fat-feeding. This possibility illustrates a reservation one must have when considering concentration terms for FFA. It is reasonable to assume a uniform dispersal of FFA in perfusate because of the binding capacity of the dispersed albumin. Adequate dispersal of FFA within the tissue is less certain, so the localised concentration of FFA is a possibility.

A further factor which indicates the presence of extensive tissue binding of FFA is the non-energetic nature of FFA uptake by the myocardium (Evans,1963). This requires equilibration of FFA concentration across the plasma membrane, which is far from the observed situation. Thus a large proportion of the total tissue FFA must be unavailable to this equilibrium pool.

Section 6.

(1) Location of lipid in heart. It was confirmed that the ventricular muscle provided the major part of the available cardiac TG reserve. The "atrial" fraction contained a significant amount of TG but this appeared to be inert during perfusion.

(2) Possible role for phospholipid. The depletion of the ventricular TG reserve was insufficient to account for the observed FFA release during perfusion. This observation casts doubt on the effectiveness of the lipid extraction procedure (tested by the recovery of isotopically-labelled FFA and TG). It is difficult to assess the adequacy of the control, since the labelled lipids (injected into the tissue just before extraction) are likely to be more available to the extraction medium than lipids buried within the depth of the tissue. The values reported above are about 50% of those reported by Crass et al (1969) and using these latter values, the amount of TG appears to be sufficient to account for the observed FFA release.

A factor not yet considered, is the contribution of FFA oxidation. The FFA which are oxidised should be added to the FFA released in order to assess the the total FFA liberation from the endogenous reserve. The FFA oxidation can be roughly estimated as below. With MVO_2 of 50µl/g/min (3µmoles oxygen/g/min) the heart could oxidise 30µmole glucose/g/hr. Glucose uptake is actually about 40µmole/g/hr of which about 50% appears

as lactate (assuming no glycogen degradation). Thus oxygen sufficient to oxidise 10 μmole glucose/g/hr may be available for FFA oxidation - sufficient to oxidise 3 μmole FFA/g/hr. This means that the total TGFA decline during 1hr (including 30min adrenaline) should be approximately 5-6 μmole/g in order that all FFA arise from TG.

The possible discrepency between FFA mobilisation and the TGFA reserve would suggest an alternative source for FFA. Since the contribution of DGFA and MGFA is small (Garland and Randle, 1963), this leaves phospholipid as the remaining large lipid reserve. This pool of lipid has previously been regarded as relatively inert, Opie (1968).

These points suggest further investigation to test
(i) the true extent of recovery of tissue lipid by the method used,
(ii) the possible role of phospholipid-fatty acid as a source of FFA within the tissue.

(3) Selectivity of FFA mobilisation. The investigation of the nature of the FFA released into the tissue and the perfusate has raised two main points. The first point arises from the observation that the composition of the perfusate and tissue FFA pools differs significantly with respect to 16:1 (higher in perfusate) and FFA of chain length above C-18 (higher in tissue). This suggests selectivity of transport from the tissue, assuming that all the FFA are equally available for transport. Such

selectivity has been suggested by Evans (1963) but contradicted by Stein and Stein (1963).

The second point concerns the difference in FFA composition of the FFA pools determined in this study compared to reported TGFA compositions (Gloster and Harris, 1973; Szuhaj and McCarl, 1973). This indicates the need to determine TGFA content of the hearts used in this study in order to account for any differences in the composition of the diets. Such an investigation of tissue FFA and TGFA compositions would also be revealing with regard to selectivity of FFA utilisation and mobilisation. Such selectivity of mobilisation is suggested by recent (unpublished) work in these laboratories, which have shown that the hormone-sensitive lipase of the rat heart exhibits a smaller degree of activatability (with cyclic AMP) towards triolein than to tripalmitin.

In experiments to test the effects of exogenous FFA, palmitic acid was used. These results suggest that the application of different exogenous FFA could be tested (in particular, 14:0 and 18:0,:1,:2, which are present in large amounts in the tissue). In addition, the delivery of different FFA from the endogenous reserve might be possible with the imposition of different lipid diets. The different composition of the TG reserve may modify the lipolytic response to adrenaline (on the basis that HSL attacks different TG to different degrees) and thus may modify the mechanical responses also.

Section G. Summary

The results presented demonstrate the damaging effects of FFA mobilised from endogenous reserves during ischaemic perfusion. The TG mobilised is located within the ventricular muscle itself. Exogenous FFA had much less effect, suggesting different availability and potency of endogenously-generated FFA.

With hearts from fat-fed rats and normally-fed rats adrenaline induced inotropic and chronotropic (first phase) responses which were followed (second phase) by marked deteriorations in performance. Thus heart rate and developed tension declined to/below the pre-adrenaline values. In addition resting tension and the incidence of arrhythmias both increased after adrenaline. In the normally-fed series, the rise in resting tension was directly proportional to the rates of release of lactate and FFA. The decline in developed tension was directly proportional only to the rate of FFA release. The second phase deteriorations were greatly reduced by the abolition of the lipolytic component of the adrenaline challenge with nicotinic acid. Preliminary results show the deteriorations were similarly reduced in the presence of an alternative antilipolytic - insulin. The concentrations of the antilipolytics was not sufficient to reduce the first phase responses of the hearts to adrenaline. The reduction of FFA release was accompanied by greater glucose oxidation, consistent with the glucose-FFA cycle

(Randle et al, 1963).

With hearts from fat-fed rats control lipolysis was significantly stimulated and control performance was depressed compared to the hearts from normally-fed rats. Thus heart rate and dT/dt were smaller, resting tension rose more and a greater incidence of arrhythmias was observed. These hearts were almost completely dependent upon endogenous lipid as an energy source. The performance of hearts from fat-fed rats also declined more markedly after adrenaline challenge (although the first phase responses were not significantly different) - in particular the development of ventricular fibrillation was a sharp contrast to the hearts from normally-fed rats. Nicotinic acid again offered protection from the second phase deteriorations, abolishing the ventricular fibrillations.

In both groups of hearts, adrenaline increased the tissue concentration of FFA and this was offset by the presence of nicotinic acid. The changes in tissue FFA were accompanied by much smaller changes in the perfusate. The excess tissue concentration of FFA over that in the perfusate thus increased markedly after adrenaline and the shift was abolished by nicotinic acid. The importance of the tissue FFA concentration is further, indirectly, indicated by the response to coronary flow. In the normally-fed series performance improves with a rise in coronary flow and the FFA concentration declines. In the fat-fed series, however performance does not improve greatly over the range of coronary flow studied and in

addition, the FFA concentration does not decline with increasing coronary flow. The validation of this argument is dependent on the confirmation that the control FFA concentration in the tissue responds to flow as does the concentration during adrenaline (and adrenaline + nicotinic acid) challenge.

The parallel involvement of lactate in the deteriorative effects of adrenaline is concluded from most of the evidence. The primary role of FFA in contributing to the accumulation and release of lactate (according to the glucose-FFA cycle) is suggested by the increase in glucose oxidation in the presence of antilipolytics. In addition, the relatively minor role of lactate in the fat-fed series is suggested by the fact that the saving effects of nicotinic acid are accompanied by a reduction of tissue FFA concentration but by no reduction of lactate concentration. This argument could, however, be used to opposite effect. Thus the maintainance of tissue lactate might explain the fact that not all the effects of adrenaline were abolished by nicotinic acid.

The effects of increasing the myocardial TG reserve, demonstrated by the differences between the hearts from fat-fed and normally-fed rats, raises the question of the significance of the lipid in those species in which it is present in greater amounts. Human myocardium contains significantly more lipid than rat myocardium (Gloster and Harris, 1973, 1974). In addition, the hearts of pigs and rabbits possess discrete fatty deposits on

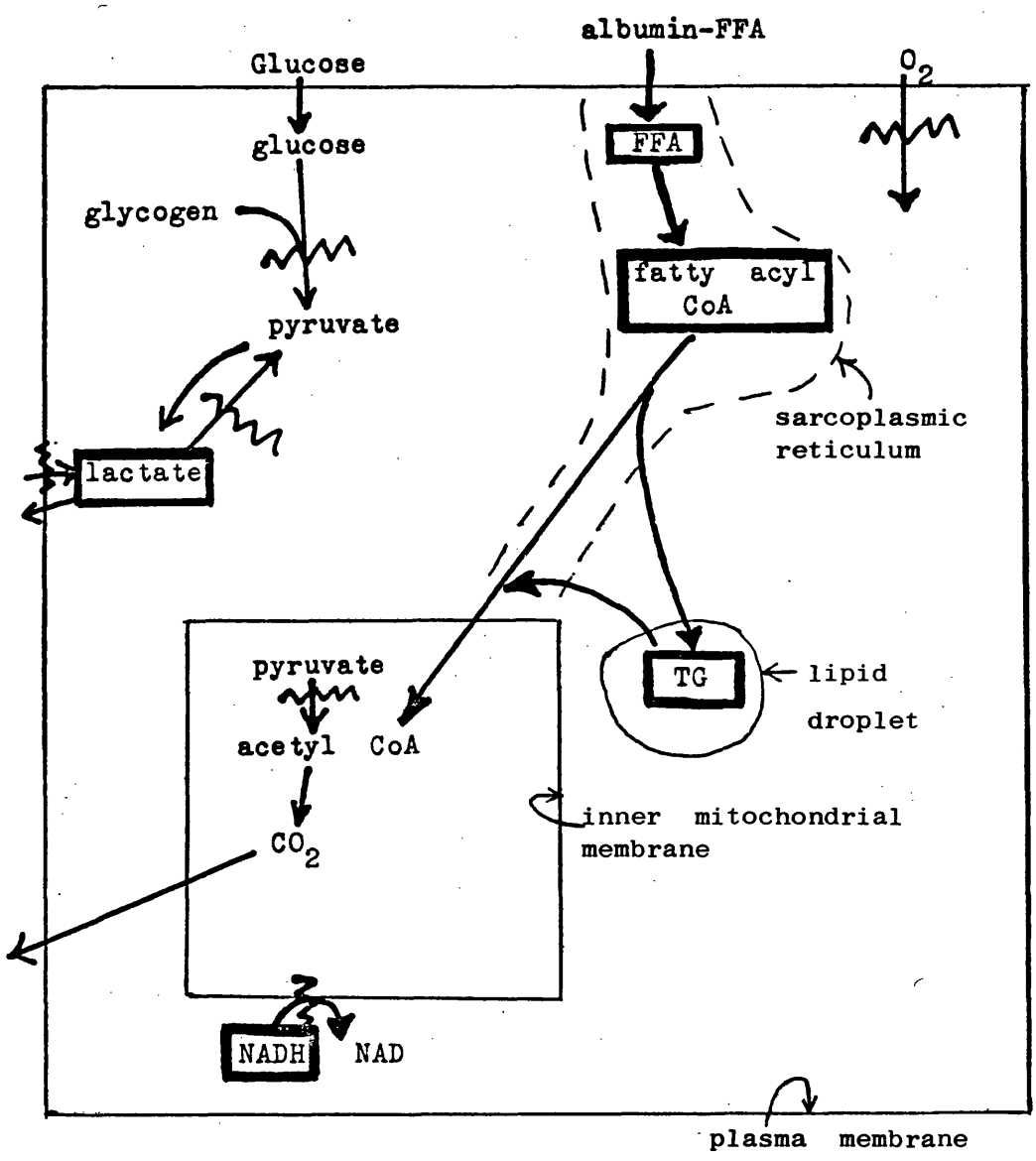
the epicardial surface, particularly in the region of the atrio-ventricular septum. It is reasonable to predict that in species with relatively more stored lipid, the stimulation of mobilisation is likely to be accompanied by more severe effects on performance during ischaemic perfusion.

The potential significance of endogenous lipid in vivo is questioned by the recent evidence of Crass et al (1975). In studies with perfused rat hearts, adrenaline stimulation of myocardial lipolysis was inhibited by about 75% by 0.6mM exogenous palmitic acid. The contribut ion of endogenous lipid to the intracellular pool of FFA was thus restricted by the presence of external FFA. It will be of great importance to investigate the extent of this effect during ischaemic perfusion. In addition and of crucial importance is to determine the overall effect upon tissue FFA concentration. Thus, does the presence of external FFA lead to an equal intracellular accumulation of FFA?

Another factor of importance in determining the importance of the myocardial lipid pool in vivo is the relative speed of delivery of exogenous and endogenous FFA to the tissue. The delivery of the endogenous FFA is likely to be considerably more rapid, particularly in ischaemic tissue - in which endogenous catecholamine activity is high and to which the delivery of external factors is, by definition, depressed.

Figure 32

Early Metabolic changes in ischaemic myocardium - a summary in schematic form .



= inhibition

= accumulation

Further work

The points raised in the individual discussion sections are summarised below.

Lipase (i) Further purification to investigate mode of activation and substrate specificity (lipid class and fatty acyl residue)

(ii) activity towards endogenous substrates

(iii) activity in hearts from fat-fed rats as compared to normally-fed rats - is this consistent with the higher pre-adrenaline lipolytic rates and susceptibility to nicotinic acid of the control lipolysis in the fat-fed series?

Perfusion (i)Investigation of the FFA composition of TGFA and FFA pools and the changes caused by perfusion with adrenaline $\pm$ antilipolytic treatment.

(ii) effects of imposition of different FFA composition in the endogenous lipid by modified diet - complimented by the application of different exogenous FFA.

(iii) investigation of the sensitivity of adrenaline-stimulated lipolysis to inhibition by external FFA and the overall effect of the modification of the lipolytic response on the tissue FFA concentration. Similarly, do external FFA generate high intracellular FFA concentrations as found with adrenaline?

(iv) investigation of the tissue lactate concentration of ischaemically-perfused tissue during pre-adrenaline and adrenaline phases in the hearts from normally-fed rats.

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APPENDIX

Appendix

1. Instruments Automatic recording spectrophotometers were from Unicam Instruments Ltd., England. (SP600, SP1800). Automatic sampling and recording spectrophotometer (8300) was from LKB instruments Ltd., Stockholm, Sweden.

Physiological recorders (MX2, M19) were from Devices Instruments Ltd., Welwyn Garden City, Herts., England.

Peristaltic pump and silicone tubing were from Watson Marlow Ltd., Falmouth, England.

Tri-carb liquid scintillation counter was from Packard Instruments Ltd., Caversham, Berks., England.

Potter-Elvejelm motor was from Electric Motors Ltd., Bournemouth, Hants., England.

Gas chromatograph was from Pye-Unicam Instruments Ltd., England.

Ultrasonicator was composed of a low heat-conducting probe (titanium-steel) powered by a 400watt amplifier and a model 512 oscillator (Waveforms Inc., New York).

2. Chemicals Laboratory chemicals were AnalAr grade from BDH Ltd., Poole, Dorset, England.

All biochemicals were from The Boehringer Corporation, Lewes, East Sussex, England, except those described below.

Sigma Chemical Co. (Kingston-upon-Thames, Surrey, England) supplied nicotinic acid, lactic acid, streptomycin sulphate, penicillin-G, lauric and myristic acids and their triglycerides.

Other pure glycerides and FFA were from Associated Scientific Ltd., Richmond, Surry, England.

Glucose oxidase test kit was from Roche products Ltd., Welwyn, Herts., England. Bovine serum albumin (fraction 5 powder) was from Armour pharmaceuticals Ltd., Eastbourne, Sussex, England. Ediol was from Calbiochem Ltd., Wyndham pl., London. Tween-60 was from Koch-Light Ltd., Colnbrook, Bucks., England. Radiochemicals were from the Radiochemical Centre, Amersham, Bucks., England.

3. Animals and Supplies Rats were obtained from Animal Supplies Ltd., Welwyn, Herts., England. Pig hearts were obtained from the factory of Spears and Son Ltd., Bristol, England. Laboratory chow (diet modified-41B) was from Oxo Ltd., London. Out-dated bank blood was obtained from the regional transfusion unit, Southmead Hospital, Bristol.

4. Miscellaneous Vinyl tubing was from Portex Ltd., Hythe, Kent, England. Specialised perfusion glassware was from Jencons Scientific Ltd., Hemel Hempstead, Herts., England. Vinyl sampling taps were from Travenol Labs. Ltd., Thetford, Norfolk, England. Medical supplies were from Gallenkamp, London.