METABOLIC ASPECTS OF ACUTE MYOCARDIAL ISCHAEMIA

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I declare that this thesis has been composed by myself. I further declare that, as a member of a research group, I have made a substantial contribution to the work described in this thesis. I was the principal contributor for the following sections:-

1.1; 1.2; 2.1-5; 3.2; 3.3; 4.1; 5.1-4 and 6.

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Abbreviations

acetyl-CoA	coenzyme A ester of acetic acid
acyl-CoA	coenzyme A ester of long-chain fatty acids
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
CP	creatine phosphate
CPIB	p-chlorophenoxyisobutyrate
CK	creatine phosphokinase
CK MB	MB isoenzyme of CK
CK MM	MM isoenzyme of CK
cyclic-AMP	3'-5'-cyclic-adenosine monophosphate
DCA	dichloroacetate
FFA	free fatty acids
FFA/alb	free fatty acids to albumin molar ratio
GTP	guaninetriphosphate
LPLA	(lipoprotein) lipolytic activity
MBF	regional myocardial blood flow
NAD^+	oxidised nicotinamide adeninedinucleotide
NADH	reduced nicotinamide adeninedinucleotide
PDH	pyruvate dehydrogenase
PGE_1	prostaglandin-E_1
P_i	inorganic phosphate
$\overline{ST}$	mean ST-segment elevation
ΣST	sum of ST-segment elevation
TG	triglycerides
UTP	uridine triphosphate

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Abstract

Continuing metabolism is crucial for the function and cellular integrity of the ischaemic myocardium, and may be critically dependent on the substrate used. It can be manipulated by changing arterial substrate concentrations and/or by altering the metabolic regulation of the ischaemic myocardium directly, leading to a reduction in the severity of the ischaemic injury.

One approach to alter metabolism is the use of anti-lipolytic drugs, which reduced in a series of independent studies ischaemia induced ST-segment elevation.

On the other hand glucose utilisation may be enhanced, by a drug, dichloroacetate, shown to be effective in the normal myocardium. Enhanced glucose utilisation by the ischaemic zone after administration of dichloroacetate was associated with reduced ST-segment elevation, and a reduction in lactate release.

Intralipid/heparin as a model to raise plasma free fatty acids was re-examined with improved methodology avoiding in vitro lipolysis. Arterial plasma free fatty acid concentrations were in the physiological range and related to fatty acid turnover.

Plasma free fatty acid concentrations were raised without severe haemodynamic and arrhythmic side-effects by direct infusion of sodium oleate in normal dogs using a continuous blood cell separator. Sodium oleate was also infused during acute myocardial ischaemia, and did not precipitate serious ventricular arrhythmias. Ischaemic myocardial extraction of free fatty acids was raised, and did not influence that of glucose.

The reduction in ST-segment elevation and free fatty acid extraction by the ischaemic myocardium after the administration of anti-lipolytic drugs support and extend other evidence that they reduce the severity of myocardial ischaemic injury. The most likely mechanism is inhibition of ischaemia-induced myocardial lipolysis, in view of the lack of a detrimental effect of sodium oleate infusion. However, a synergistic effect of reduced plasma free fatty acids can not be excluded.

INTRODUCTION

There is probably no other disease to date, which challenges man in our western society more than coronary heart disease. No wonder many physicians and epidemiologists have been and are engaged in studies aimed to prevent this killer-disease. It is now accepted that one is not dealing with one particular form of illness, but more likely with a spectrum of different aetiologies: atherosclerosis, sudden death, thrombosis and possibly even coronary arterial spasm. Epidemiological studies have underlined the importance of a multitude of genetic, metabolic, dietary and environmental factors. It is believed that some of these factors may contribute to the narrowing of the coronary arteries by formation of atherosclerotic plaques or thrombi, and thereby ultimately impeding blood supply to the myocardium. During most of the time these underlying processes may go unnoticed to the person at risk and as a result it may be difficult to obtain his full cooperation in reducing his risk. Also the apparently constant changing environmental influences introduced by all kinds of changes (legislation, economic conditions, availability of raw products, etc)

makes it impracticable to examine the effect of one particular dietary or environmental change on the incidence of coronary heart disease. The speed of the development of new scientific methods and concepts in relation to the length of the primary prevention studies often make the best possible design and measurements at the start out-of-date or insufficient in some essential details on completion of those studies. And even if the incidence of ischaemic heart disease could be reduced, can long term treatment of an apparently healthy population or even those at higher risk, of whom some would never have suffered a heart attack, be justified by increasing the risk of inducing other diseases at the same time? These considerations raise doubt on the impact, which studies of primary prevention of ischaemic heart disease will have in the immediate future. Most likely therefore, patients will present themselves for treatment of acute myocardial infarction. The main challenge remains the early containment of the ischaemic changes, which so often lead to lethal ventricular arrhythmias.

In this thesis the role which myocardial metabolism of glucose and fatty acids plays in the early phase of acute myocardial ischaemia is examined using an experimental model in the dog.

AIM OF THE THESIS

Aim of the thesis

Acute myocardial infarction in man is accompanied by an increase in the plasma concentration of free fatty acids (Kurien and Oliver, 1966; Gupta et al, 1969; Rutenberg et al, 1969; Ravens and Jipp, 1972; Vetter et al, 1974; Christensen and Videbaeck, 1974). The rise, which may be observed as early as 1 hour after onset of symptoms (Vetter et al, 1974), is probably due to stimulated adipose tissue lipolysis as a result of increased activity of the sympathetic nervous system (Vetter et al, 1974; Christensen and Videbaeck, 1974). In support of this view is the observation that plasma glycerol concentrations were also raised after acute myocardial infarction (Carlström and Christensson, 1971) and raised plasma glycerol concentration often considered as a better index of adipose tissue lipolysis (Scow and Chernick, 1970). Those patients with the highest concentration of plasma free fatty acids have been reported to be at greater risk of developing serious ventricular arrhythmias and death (Oliver et al, 1968; Gupta et al, 1969), although not all investigations have confirmed this association (Rutenberg et al, 1969). In another study individual

free fatty acids were quantitated using gaschromatography and the concentration of plasma free linoleic acid, rather than the total free fatty acids concentration was associated with a bad prognosis (Ravens and Jipp, 1972).

A hypothesis was advanced that acute lipid mobilisation from adipose tissue may lead to the accumulation of 'unbound' free fatty acids within the ischaemic myocardium, where they exert direct detergent effects on the cell membrane, with cation loss and resultant development of ectopic pacemaker activity (Kurien and Oliver, 1970). This hypothesis has been supported by the demonstration that the incidence of ventricular arrhythmias during the early phase of acute myocardial infarction was reduced by effective antilipolytic therapy in the absence of changes in plasma catecholamine concentrations (Rowe et al, 1975) and may lead to decreased ischaemic injury (Kjekshus, 1978).

A toxic effect of FFA on the heart could be mediated through an alteration of membrane potentials (Wasilewska-Dziubinska et al, 1975) or an increase in the severity of the ischaemic injury to the myocardium (Mjøs, 1973).

Marked elevation of the plasma concentration of free fatty acids was associated with the development of arrhythmias during experimental coronary occlusion

in dogs (Kurien et al, 1971). In other experiments the severity of myocardial ischaemic injury was reduced by the administration of an antilipolytic drug - β-pyridyl-carbinol (Kjekshus and Mjøs, 1973), attributed to decreased oxygen requirements of the ischaemic tissue, in analogy with the reduced myocardial oxygen consumption observed in healthy dogs (Mjøs, 1971).

These results support the view that raised plasma concentrations of free fatty acids may have deleterious effects on the ischaemic myocardium, and are complemented by the beneficial effects claimed for the other major substrate: glucose.

It was suggested that 'raised blood glucose concentrations might benefit the ischaemic myocardium by increasing the rate of anaerobic glycolysis, by reversing ion losses, by a direct membrane effect, by altering the extracellular volume, and by decreasing the circulating free fatty acid concentrations' (Opie, 1970).

However it should be pointed out that the formulation of this hypothesis was largely based on

firstly - the extrapolation of observations made in anoxic, well perfused preparations.

secondly - on the extrapolation of reciprocal behaviour of fatty acid and glucose metabolism in well oxygenated heart muscle preparations (Randle, 1963) to the ischaemic myocardium.

Due to the complex changes occurring during acute ischaemia these extrapolations may not be justified. Therefore detailed studies of the interrelation between glucose and free fatty acid metabolism in the ischaemic myocardium are indicated.

THE WORKING HYPOTHESIS

Enhancement of glucose availability, or decrease in fatty acid utilisation might provide the ischaemic myocardium with greater cellular integrity, and therefore preserve the viability of potentially necrotic tissue.

Part 1

A SHORT REVIEW OF NORMAL AND ISCHAEMIC MYOCARDIAL METABOLISM

INTRODUCTION

It is not the intention to review all aspects of the metabolism of the normal and ischaemic myocardium. The reader is referred to several review articles and a monograph (Randle, 1963; Opie, 1968; Opie, 1969[a],[b]; Neely et al, 1972). However for sake of clarity a summary of the metabolism of normal and ischaemic myocardium is given.

SECTION 1.1:

METABOLISM OF THE NORMAL HEART

Metabolism of the normal heart

In order to satisfy the demand for energy required for the maintenance of contractile and cellular processes, the heart must generate ATP. In the heart most of the ATP is obtained from mitochondrial oxidative phosphorylation. Oxygen and substrates are extracted from the arterial circulation. Although the heart can use virtually any substrate, under normal physiological conditions two substrates contribute most to myocardial oxidative metabolism: plasma free fatty acids and glucose (85-90% of energy requirements), while others: lactate, triglycerides and ketonebodies play a definite but quantitatively less important role.

Fatty acid metabolism

Plasma free fatty acids utilised by the heart originate from adipose tissue triglycerides. During lipolysis fatty acids diffuse into the circulation where they are bound to albumin, which functions as the carrier for these water insoluble acids. Plasma free fatty acids are extracted by the myocardium in a process which does not require energy (Evans, 1964). The extraction is related to the arterial concentration, the nature of the fatty acids and the degree of binding to albumin, determined by the free fatty acid to albumin molar ratio. Within the cell the fatty acids are thought to be bound by a fatty acid binding protein (Ockner et al, 1972). Fatty acids can not be utilised within the cell directly, but must be 'activated' in the form of their coenzyme A ester (acyl-CoA). This process requires a phosphate transfer from ATP. Only then can they enter the various pathways: oxidation, incorporation into triglycerides or into phospholipids, synthesis of complex lipids.

Oxidation of acyl-CoA

Long chain acyl-CoA cannot penetrate directly into the mitochondrial matrix, where the integrated enzyme system for fatty acid oxidation is located adjacent to and functions in a closely-linked fashion with the respiratory chain. The transport of acyl-CoA into the mitochondria is achieved by a shuttle mechanism in which acyl-carnitine, which (in contrast to acyl-CoA) can cross the mitochondrial membrane, is the intermediate. Once inside the mitochondrion acyl carnitine is converted back into acyl-CoA. The stepwise degradation of acyl-CoA to acetyl-CoA yields reduced flavoprotein and NADH. Acetyl-CoA is further degraded by the enzymes of the Krebs cycle, also located in the mitochondrial matrix, leading to further formation of reduced coenzymes and 1 molecule of GTP. The reduced coenzymes are regenerated in the presence of oxygen in the respiratory chain under formation of ATP.

Synthesis of triglycerides

Experiments with radioactive fatty acids have demonstrated the synthesis of triglycerides. Activated fatty acids are esterified with α-glycerophosphate, derived from the glycolytic pathway. In the normal myocardium net synthesis of triglycerides is not observed and there must be a dynamic equilibrium between triglyceride synthesis

and hydrolysis of triglycerides by hormone-sensitive lipase and/or intracellular lipoprotein lipase, enzymes which are under hormonal control, and with activities which vary with the nutritional and hormonal status of the subject.

Control of plasma free fatty acid metabolism by the heart

The main control over the metabolism of plasma free fatty acids by the heart is exerted on the mobilisation of fatty acids from adipose tissue, which is the net result of lipolysis and re-esterification of hydrolysed fatty acids into adipose tissue triglycerides. Adipose tissue triglyceride lipase is thought to exist in enzymically interconvertible forms. The inactive form is phosphorylated by a cyclic-AMP sensitive protein-kinase to a catalytically active form. Catecholamines, growth hormone, glucagon all stimulate lipolysis, while insulin has a direct antilipolytic effect.

The rate of re-esterification is affected by the availability of glucose, providing α-glycerophosphate. Addition of fatty acids to isolated adipocyte stimulates esterification, but α-glycerophosphate levels in adipose tissue were unchanged. Thus there may be still unidentified factors which can regulate re-esterification and hence fatty acid mobilisation.

The cellular control of fatty acid metabolism in the

heart has not been widely investigated. The fate of fatty acid is determined by its availability, and that of other substrates, such as glucose. The rate of triglyceride formation is increased under conditions where fatty acid supply exceeds the rate of fatty acid oxidation. However much more work is needed to establish how control over this and other pathways is exerted. The reader is referred to a review article by Neely and Morgan (1974).

Myocardial Glucose metabolism

The other major substrate for the myocardium is glucose. It can provide the heart with 30-60% of its energy requirements. Transport of glucose across the cell membrane involves a specific carrier mechanism. No energy is required for this. Glucose is then, after phosphorylation to glucose-6-phosphate by ATP in a reaction catalysed by hexokinase, degraded to pyruvate in a series of enzymic conversions, which is known as the glycolytic pathway. It is important to point out that in addition to the formation of reduced NADH, in this pathway a net synthesis of 2 molecules of ATP per molecule of glucose is made. Another important feature is that a constant supply of NAD^+ is required in order to maintain the flux of glucose through the pathway. However glycolytic flux is not dependent on oxidative

regeneration of NADH in the mitochondrion, as is necessary to maintain fatty acid oxidation, since NAD^+ required for the dehydrogenation of glyceraldehyde-3-phosphate can be provided by conversion of pyruvate (+ NADH) to lactate ($+NAD^+$).

The fate of pyruvate

Normally pyruvate is further degraded to acetyl-CoA and CO_2 with the formation of NADH by mitochondrial pyruvate dehydrogenase. The fate of the formed acetyl-CoA is the same as that described for acetyl-CoA orginating from oxidation of fatty acids. The dehydrogenation of pyruvate and the metabolism of acetyl-CoA, which both are dependent on oxygen availability, constitute the energetically most important part of the pathway and lead to 95% of the ATP production.

Control of glucose metabolism

Glucose extraction by the heart is related to arterial glucose concentrations, the availability of insulin, pH and arterial concentrations of plasma free fatty acids. (Other factors such as workload are not considered here, and the effect of hypoxia will be discussed later).

Investigations of the intermediate metabolites and of the activities of the key enzymes of the glycolytic pathway suggest that phosphofructokinase is under most conditions a regulatory enzyme, controlling the overall

flux through the pathway (Newsholme and Start, 1973; Neely and Morgan, 1974).

Insulin stimulates glucose uptake by the heart, by direct enhancement of glucose transport, and intracellular free glucose accumulates. This is taken as evidence that under these conditions phosphorylation of glucose determines essentially the glycolytic flux. Insulin also stimulates glycogen synthetase, the regulatory enzyme for glycogen synthesis.

Catecholamines, by stimulating the formation of cyclic-AMP by adenylcyclase, affect the conversion of phosphorylase-b (inactive) to phosphorylase-a (active) and thereby increase glycogenolysis.

A low pH inhibits and a high pH increases the activity of phosphofructokinase and thereby influences glycolytic flux (Mansour, 1972).

The interrelation between fatty acid and glucose metabolism in the heart has been studied by Randle and colleagues (Randle, 1963). The glycogen sparing activity of fatty acids and inhibition of glycolysis was explained by increased citrate levels, inhibiting phosphofructokinase. As a result more of the glucose entering the cell is diverted to glycogen. The reduced glucose uptake seen under these conditions was related to increased levels of glucose-6-phosphate, which are inhibitory to hexokinase.

Glycogen metabolism

The heart stores some of the extracted glucose in the form of glycogen, serving as a fuel reserve. Three enzymic steps are involved in the conversion of glucose-6-phosphate into glycogen. In the first glucose-6-phosphate is converted into glucose-1-phosphate, a reaction catalysed by phosphoglucomutase. In the next step glucose is transferred to part of the nucleotide uridine-tri-phosphate (UTP). Only glucose moieties in this activated form (uridine-di-phosphate-glucose) can be transferred to glycogen by glycogen synthetase. The incorporation of 1 molecule glucose-6-phosphate into glycogen requires the hydrolysis of one high energy phosphate bond for the formation of uridine-di-phosphate-glucose.

Glycogenolysis is not simply the reversal of glycogen synthesis. The enzyme phosphorylase catalyses the cleavage of the glucose moiety from glycogen with the formation of glucose-1-phosphate, which is converted into glucose-6-phosphate, prior to entering the glycolytic pathway.

Control of glycogen metabolism

Glycogen synthetase controls the overall flux in the synthesis of glycogen. It occurs in two forms: I and D. The activity of the D form is dependent (D) on the glucose-6-phosphate concentration. This form is strongly

inhibited by ATP in such a way that under most physiological conditions it is considered to be inactive. The maximal activity of the I form is independent (I) of the level of glucose-6-phosphate, and this form is relatively insensitive to inhibition by ATP. Control of glycogen-synthesis depends on a phosphorylation-dephosphorylation mechanism. The D-form is phosphorylated and can be dephosphorylated by glycogen synthetase phosphatase, and as a result glycogen synthetase activity will increase. However glycogen inhibits the phosphatase, and as more glycogen accumulates a progressively lower percentage of glycogen synthase will be in the active I form. The conversion of the active (I) into 'inactive' (D) form is catalysed by a cyclic AMP dependent protein kinase.

Glycogenolysis is controlled by the activity of phosphorylase. This enzyme occurs in two forms, phosphorylase a and b. These two forms differ in their sensitivity to the inhibitors ATP and glucose-6-phosphate. Phosphorylase-b is only active when AMP and Pi levels are high enough to overcome the inhibitory effects of ATP and glucose-6-phosphate, while phosphorylase-a is also active at high levels of these inhibitors. The conversion to phosphorylase-a is regulated by phosphorylase-b kinase, which in its turn exists in an active and inactive form. A cyclic-AMP dependent protein kinase is

responsible for the formation of the active form. Thus stimulation of this protein kinase by cyclic-AMP will result in stimulation of phosphorylase-b-kinase, which will in its turn stimulate the formation of the active form of phosphorylase, leading to stimulation of glycogenolysis.

Thus myocardial glycogen synthesis and glycogenolysis are controlled in a complex but integrated manner, in which cellular levels of ATP, glucose-6-phosphate, glycogen, AMP, Pi and cyclic AMP all play a regulatory role.

Interrelation of glucose and fatty acid metabolism

Randle and collegues have extrapolated the findings from their extensive series of in vitro studies of heart, muscle and adipose tissue to the intact animal and man This concept is known as the glucose-fatty acid cycle (Randle, 1963), and gives an explanation for the well-known inverse relation between glucose and fatty acid metabolism. When concentrations of plasma free fatty acids are high, fatty acid utilisation is increased and glucose uptake by the heart (and muscle) is depressed. Consequently plasma glucose levels will increase, resulting in increased glucose uptake and glycolysis by adipose tissue. The provision of more α-glycerophosphate due to enhanced glycolysis will stimulate fatty acid reesterification

and as a result free fatty acid release will be diminished. Now glucose uptake by the heart (and muscle) is no longer inhibited and increased glucose uptake will result in lowered plasma glucose concentration. As a result increased mobilisation of fatty acids due to low glucose uptake by adipose tissue will cause plasma concentrations of free fatty acids to rise. Since this concept was proposed, evidence has been presented that high plasma concentrations of free fatty acids stimulate insulin release (Crespin et al, 1972), explaining the negative feedback of plasma free fatty acids on adipose tissue lipolysis. Similarly it is possible that fatty acids and ketone bodies exert an independent antilipolytic effect on adipose tissue. This additional information does not invalidate the original scheme, but extends the concept of the glucose-fatty acid cycle.

SECTION 1.2:

METABOLIC EVENTS DURING ACUTE MYOCARDIAL ISCHAEMIA

Metabolic events during acute myocardial ischaemia

The heart requires a constant supply of oxygen to maintain its functional activity. When its activity is increased the augmented oxygen demand is chiefly met by increased coronary flow, while the extraction of oxygen is almost unchanged. In patients with ischaemic heart disease with severe stenosis or occlusion of one or more coronary arteries, the heart is no longer capable of meeting the increased oxygen demand and acute ischaemia, which may or may not lead to infarction, ensues. It is apparent that different degrees of residual regional myocardial blood flow may occur. Reduction in flow will not necessarily correlate with the severity of ischaemia, and has to be considered against the background of oxygen demand of the heart.

In this section the metabolic consequence of acute ischaemia, but not those of anoxia will be reviewed. This distinction, which may seem rather arbitrary is made to avoid confusion. During anoxia, the heart compensates for the lack of oxygen supply by maximal vasodilation. Therefore this hypoxic condition is characterised by increased coronary flow rates, rather than a decrease, which is so characteristic for ischaemia. Marked differences between anoxia and ischaemia have emerged: the accumulation of 'waste' products (lactate, H^+ etc) occur in ischaemic, but not

in anoxic myocardium, which in turn appear to affect other processes within the cell. On the other hand the anoxic cell may be loaded with substrates (fatty acids) it can not utilise.

These considerations have to be taken into account when interpreting results obtained from different models of (experimental) ischaemia. It is conceivable that the reduction in coronary flow required to induce a given deficit in oxygen supply may be widely different. This may lead to differences in intracellular pH and/or accumulation of metabolites between models, which in turn may affect enzymic processes, etc. It is extremely difficult, if not impossible, to predict the ultimate outcome.

Animal experiments have contributed to most of our present knowledge of the effects of acute ischaemia. In such experiments localized ischaemia was induced by coronary ligation or global ischaemia was induced by a reduction in total coronary perfusion. While in the first model gradients of oxygen deficit and cellular impairment occur with or without functional impairment of the heart as a whole, the latter is characterised by a more homogeneously affected tissue, with impairment of left ventricular function. There is an increased tendency to develop differences in arrhythmias of the former model, which is presumably a reflection of the difference in metabolism. However since the effects of ischaemia on myocardial metabolism in these two preparations have not been studied systematically, they will

be discussed together.

The onset of myocardial ischaemia is characterised by a precipitous drop in myocardial oxygen tension in the area within the distribution of the ligated artery (Sayen et al, 1958; Gudbjarnason et al, 1970), despite increased oxygen extraction across this area (Opie et al, 1973; Opie et al, 1973[b]; Steenbergen et al, 1977). The lack of oxygen is reflected in an increase in NADH/NAD ratio. Contractility in the ischaemic area is depressed within seconds after occlusion (Puri, 1976; Smith, 1977) and the myocardium virtually ceases to contract at a time when the levels of ATP are not, but CP levels are decreased (Puri, 1976). It remains to be seen if the persistence of depressed contractile behaviour in the ischaemic area (Richardson et al, 1960) is solely responsible for the marked decline in ATP and CP immediately afterwards (Wollenberger and Krause, 1968; Kübler and Spieckermann, 1970; Gudbjarnason et al, 1970; Jones et al, 1976). AMP, ADP and inorganic phosphate levels rise, as well as those of the degradation products of adenine nucleotides, hypoxanthine and inosine (De Jong et al, 1977; Jones et al, 1976; Stam and De Jong, 1977). Cyclic AMP was also raised soon after induction of ischaemia (Wollenberger et al, 1972; Podzuweit et al, 1975; Podzuweit et al, 1978) possibly as a result of adenylcyclase stimulation by locally released noradrenaline (Shahab et al, 1969), due to enhanced activity of the myocardial sympathetic nervous system (Malliani et al, 1969).

Electrolyte gradients are no longer held and the ischaemic cell loses K^+ ions, while it gains Na^+ and Ca^{++} (Nayler et al, 1971; Flear et al, 1976), although this was not confirmed in another study (Whalen et al, 1974). These electrolyte changes might be a direct result of lack of ATP (Trump, 1976), since Na^+, K^+-ATP-ase might not be inhibited early on (Schwartz et al, 1973). The intracellular acidosis (Opie et al, 1975; Williamson et al, 1977) is probably responsible for the rise in pCO_2 (Khuri et al, 1975; O'Riordan et al, 1977), since CO_2 production is decreased during acute ischaemia (Opie et al, 1973[b]). All these changes are observed during the reversible phase of acute ischaemia with little or no histo-chemically demonstrable changes (Whalen et al, 1974; Jennings and Ganote, 1972).

However if the occlusion was maintained for longer periods (40-60 min) irreversible damage became apparent in a large proportion of ischaemic cells: swelling, disrupted mitochondria, with irregular dense amorphous matrix densities (Kloner et al, 1974; Jennings and Ganote, 1972). It is not clear whether the transition from reversible to irreversible damage is induced by an increase in permeability of the lysosomal membranes due to the lowered intracellular pH. However studies appear to exclude lysosomal involvement during the reversible

phase of ischaemia.

It should be emphasized that these histochemical observations of Jennings and colleagues were made on severely ischaemic tissue, (posterior papillary muscle) and that the transition of reversible to irreversible damage may be delayed in less severely ischaemic myocardium.

Mitochondrial function during ischaemia

Several groups have investigated the function of mitochondria, isolated from ischaemic myocardium (Jennings et al, 1969; Schwartz et al, 1973; Lochner et al, 1975). Mitochondria were almost in all cases isolated from irreversibly damaged tissue. They were fragile and yields were only half of those obtained for the isolation of mitochondria from the non-ischaemic myocardium (Jennings et al, 1969). When studied in vitro using manometric or polarographic techniques high oxygen tensions are used. Under these conditions the mitochondria exhibited a disturbed function: low phosphorylation/oxidation (P/O) ratios, low respiratory control by the addition of ADP and low oxygen consumption (Jennings et al, 1969; Schwartz et al, 1973; Lochner et al, 1975).

However there are two reasons, why these results should not be extrapolated to in vivo ischaemic myocardial mitochondrion:

1 - The homogenisation medium used during the isolation

procedure influenced markedly the results. Particularly the use of fat free albumin and EDTA could nullify the effect of ischaemia on P/C ratios and oxygen consumption (Lochner et al, 1975).

2 - With the present techniques (manometric and polarographic) high oxygen tensions are used. Reperfusion of ischaemic myocardium 40 min after coronary occlusion causes explosive cell damage: disrupted mitochondria intramitochondrial Ca deposits, etc (Kloner et al, 1974). This exacerbation of ischaemic myocardial damage during reoxygenation was confirmed with a different end point (enzyme release) in hypoxic perfused rat hearts (Hearse et al,1976). Thus it is conceivable that the exposure of isolated mitochondria from ischaemic myocardium to high oxygen tensions, necessary for the in vitro studies, contributes to the mitochondrial damage.

The effect of albumin and EDTA may tell us more of the likely causes of the mitochondrial disturbance in ischaemic tissue. The effect of albumin could be exerted by binding of fatty acids, formed from mitochondrial acyl-CoA (Idell-Wenger and Neely, 1977; Lochner et al, 1978). EDTA may counteract the accumulation of Ca, which is a known depressant of mitochondrial function. Although this suggests that fatty acids and Ca might be involved

in the disruption of mitochondrial function, it is almost impossible in these studies to be sure that the observations are not artifacts. A promising approach is the development of techniques designed to assess mitochondrial (and cytoplasmic) redox couples, using reflectance fluorescence NADH (Steenbergen et al, 1977). A first, exciting finding is that the ischaemic area of isolated rat heart was heterogeneous, with highly localised anoxic zones. These anoxic islands developed while the effluent oxygen tension was significantly greater than zero. Whether these results can be confirmed in blood perfused hearts remains to be seen.

However hopefully this technique will continue to contribute to the desperately needed information of mitochondrial function in ischaemic myocardium.

Substrate metabolism during acute ischaemia

The effect of acute ischaemia on the extraction and metabolism of fatty acids, glucose, lactate, pyruvate and ketone bodies has been investigated. The increase of glucose extraction across the ischaemic area is well established (Opie et al, 1973; Van der Vusse and Reneman, 1977; De Jong et al, 1977), and it appears that glucose uptake by the ischaemic myocardium is decreased despite the compensatory increase in extraction (Opie et al, 1973; Neely et al, 1973). Glycogenolysis leads to a rapid

decline of the glycogen stores (Braasch et al, 1968; Opie et al, 1973; Neely et al, 1973; Crass et al, 1976; Opie et al, 1975), although they do not appear to vanish completely (Kloner et al, 1974, Opie et al, 1973). The stimulation of glycogenolysis is thought to be mediated by raised intracellular levels of cyclic AMP (Wollenberger et al, 1967), possibly in combination with the increased levels of AMP, inorganic phosphate (Neely et al, 1973). Contrary to the widely held view the major fate of glucose is oxidation and not lactate formation (Opie et al, 1973; Neely et al, 1975). This is probably best illustrated in the recalculated data of Neely et al (1975), presented in Table 1.01. Even under the most severely ischaemic conditions (residual blood flow 4% of control values) most of the contribution of glucose to ATP formation is derived from its aerobic metabolism.

The effect of acute ischaemia on fatty acid metabolism has also been examined. Extraction of radioactive fatty acids may (Scheuer and Brachfeld, 1966) or may not be decreased (Riemersma et al, 1972). In an extension of the latter study Opie, Owen and Riemersma (1973) determined also the extraction of plasma free fatty acids by chemical methods. The observed decrease in net extraction was explained - in view of the unchanged extraction

Table 1.01

The contribution of aerobic and anaerobic glucose metabolism to ATP production in the ischaemically perfused rat heart

	Coronary flow*	ATP production*	% contribution to ATP production from glucose metabolism	
			Anaerobic	Aerobic
Control	100%	100%	5%	95%
ischaemic	34%	73%	13%	87%
	8%	37%	19%	81%
	4%	16%	19%	81%

*Results expressed as % from control. Data from Neely et al, 1975.

of radioactive fatty acids - by increased intramyocardial lipolysis, releasing free fatty acids into the ischaemic effluent. Oxidation of fatty acids is inhibited (Scheuer and Brachfeld, 1966; Riemersma et al, 1972; Idell-Wenger and Neely, 1977), and as a result intermediates of this pathway : acyl-CoA, acyl-carnitine, hydroxy-acyl-CoA all accumulate (Idell-Wenger and Neely, 1977; Bittar et al, 1976; Shug et al, 1978), but it remains to be seen whether acyl-CoA inhibits adenosine nucleotide translocase in ischaemic myocardium, which catalyzes the transport of ADP in and ATP out of the mitochondria (Shrago and Sul, 1977; Shug et al, 1978). The same reservations apply as those listed in the paragraph 'Mitochondrial function during ischaemia' (pp 47).

The synthesis of triglycerides is enhanced (Scheuer and Brachfeld, 1966; Crass et al, 1976; Riemersma, 1979), possibly due to increased levels of acyl-CoA and α-glycerophosphate, and probably exceeds the rate of lipolysis, in view of the well documented infiltration of neutral lipids in ischaemic myocardium (Bryant et al, 1958). Enhanced triglyceride synthesis in the presence of an increased rate of lipolysis indicates the operation of an energy wasting cycle.

Control of metabolism during acute myocardial ischaemia

It seems odd to consider control of biochemical pathways during acute ischaemia in view of the extensive derangement of the cell. However the reversibly damaged cell still generates and utilises energy in an attempt to maintain its integrity. Some enzymes will control the flux of substrates through the various pathways.

Several studies have recently been made, specifically designed to define which enzymes limit the utilisation of glucose and glycolysis in the ischaemic myocardium, others have looked at competition of substrates. Factors, which control the glucose utilisation in ischaemic myocardium are undoubtedly best, but by no means completely, understood. The early increase in glycolytic flux in the ischaemic myocardium is mainly due to enhanced glycogenolysis, due to stimulation of phosphorylase by increased levels of cyclic AMP (Wollenberger et al, 1967), in analogy with the effect of cyclic AMP in well oxygenated myocardium during catecholamine stimulation. In addition the increased levels of AMP and inorganic phosphate will enhance the activity of phosphorylase. Within minutes the rate of glycogenolysis slows down although it is actually never inhibited, as can be calculated from the data of Rovetto and Neely (1977). It is not clear,

which is the rate-limiting step of glycolysis during ischaemia. Rovetto et al (1973) suggested that either phosphofructokinase or glyceraldehyde-phosphate dehydrogenase limited the flux of glucose through glycolysis. Later Rovetto and Neely (1977) suggested that glyceraldehyde-3-phosphate dehydrogenase was most likely the enzyme which controlled glycolysis. On the other hand Opie (1975) suggested that glycolysis was restricted at the level of phosphofructokinase, like under aerobic conditions (Newsholme and Start, 1973; Neely and Morgan, 1974). It is acknowledged that studies using the cross-over theorem may provide suggestive evidence of exerted control, due to the fact that an apparent restriction could be due to an apparent build-up of difficult to measure product of the reaction.

The effect of pH has also been examined. Incorporation of a buffer in the perfusate of the ischaemic heart could overcome the inhibition of glycolysis (at the level of glyceraldehyde-3-phosphate dehydrogenase) and the rate limiting step appeared to be either pyruvate kinase or pyruvate-dehydrogenase (Rovetto et al, 1975). However more work is indicated, since modest elevation of H^+ concentration only caused inhibition when accompanied by oxygen deficiency (Rovetto and Neely, 1977), suggesting that other factors associated with ischaemia such as

high NADH levels, reduced phosphate potentials, etc. might inhibit glycolysis.

Control of fatty acid metabolism in ischaemic myocardium

Control of fatty acid metabolism during ischaemia has hardly been investigated. Apart from the effect of ischaemia on the passive process of extraction (see above) little attention has been given to its control of β-oxidation and triglyceride formation in ischaemic myocardium. Intracellular concentrations of free fatty acids might be raised (Lochner et al, 1978), however in view of the major technical difficulties of such measurements, they can only be considered with some reservations. Acyl-CoA accumulates, suggesting that activation of fatty acids might not be limiting for β-oxidation. In analogy with the requirements of the glycolytic pathway, the β-oxidation also requires constant supply of oxidised coenzymes NAD, flavoprotein. Thus the increased redox-state in the ischaemic mitochondrion (Steenbergen et al, 1977) might be responsible for the reduced oxidation of fatty acids (Idell-Wenger and Neely, 1977). As a result acyl-CoA levels rise, and acetyl-CoA levels fall (Idell-Wenger and Neely, 1977; Shug et al, 1978). Acyl-CoA accumulates intramitochondrially, and to a lesser extent in the cytoplasmic compartment (Idell-Wenger and Neely, 1977). It

is conceivable that raised acyl-CoA together with increased levels of α-glycerophosphate stimulate the formation of triglycerides, by a mass-action effect. Although the reasons are unclear, it has become evident that glucose competes more effectively with fatty acids for the residual oxygen (Opie et al, 1973); and that fatty acids may no longer be able to inhibit glucose utilisation (aerobic and anaerobic) in the ischaemic myocardium (Opie et al, 1973; Neely et al, 1975), however such observations need urgent confirmation. If true, they will markedly effect the thinking of the approach of biochemical intervention aimed to increase glucose and/or decrease fatty acid utilisation by the ischaemic heart.

PART 2

METHODS

SECTION 2.1:

EXPERIMENTAL MODEL

2.1.1. Considerations leading to the experimental design

Studies of myocardial metabolism have often been made using the isolated perfused 'non-working' heart preparation of Langendorff (1895). Its simplicity and the control of supply of substrates and hormones has particularly attracted many biochemists. However some rather striking differences emerge when this preparation is compared with in situ heart preparations:

- low work performance and hence low oxygen consumption.
- perfusates contain little or no protein (albumin).
- perfusates are usually free of red blood cells - hence low oxygen carrying capacity.
- extremely high coronary flow rates (retrograde) under conditions of optimal oxygenation.

The introduction of the working heart preparation by cannulation and perfusion of the left atrium by Neely et al (1967) was a noticeable improvement, but objections to its validity can still be raised on the basis of the unphysiologically high coronary flow rate. One interpretation of the observed coronary flow is that this preparation is hypoxic, despite maximal oxygenation of the perfusate. This view is supported by lactate and α-glycerophosphate release (O'Brien, 1969). But more convincing to the haemodynamically orientated investigators might be the observation that when red

blood cells were added to the perfusate of the isolated working rat heart, coronary blood flow decreased, while cardiac output was augmented (Duvelleroy et al, 1976). Such modifications have increased the sophistication of the isolated heart preparation, but at the same time it has reduced its major advantage: simplicity. In view of this and the limited experience with perfused heart preparations in our laboratory it was decided to use an in vivo dog heart preparation, with localised ischaemia. A similar preparation was extensively used by Opie et al and others for the study of myocardial metabolism during the first 2 hours of acute ischaemia (Owen et al, 1970; Opie et al, 1973; Marshall et al, 1974; Smith, 1977).

The metabolic response of the ischaemic myocardium was assessed by analysis of the various substrates in the effluent draining the ischaemic area, and was compared with the substrate extraction before coronary artery occlusion using the same cannula, as well as with the extraction across the whole myocardium in samples collected simultaneously from a coronary sinus catheter (draining predominantly the non-ischaemic myocardium).

This model is characterised by a relatively high variability of ischaemic injury (ST segment elevation, lactate release), despite careful standardisation of

the location of coronary occlusion, depth of anaesthesia, etc. Kjekshus and Mjøs (1973) observed that the average ST-segment elevation in the epicardial ECG of 10-15 sites, measured 15 min after coronary occlusion ranged from 1.5 to 9 mV, mean $\pm$ SD: 4.6 $\pm$ 2.8 (n = 12), showing a coefficient of variation of 60%.

Using their data (mean and SD) the number of experiments required to demonstrate a statistically significant reduction of an arbitrarily selected 30% was calculated (Statistical methods, Documenta Geigy). For a confidence limit of 1%, 20 experiments are required in the control and in the treated group. However only 14 experiments are needed if a model, which allows the animals to serve as their own control is used (paired Students t-test may be applied). The model of intermittent myocardial ischaemia, induced by occlusion of a branch of the left anterior descending coronary artery using a metal releasable clip as described by Kjekshus and Mjøs (1973) has this advantage, and the effect of treatment can be assessed during a repeat occlusion, made 30 min after release of the control occlusion and recovery. By combining this model and that of Opie and colleagues (1970; 1973) described above, using a small cannula inserted into the local vein draining the ischaemic area, substrate extraction across the ischaemic area should be possible.

This model should lead to reproducible effects during the successive occlusions. Irreversible myocardial injury does not occur within the first 20 min of myocardial ischaemia and cell death is not observed if ischaemia is reversed by restoring arterial flow (Jennings et al, 1969: Kloner et al, 1974). ST-segment elevation has been shown to be reproducible following reocclusion of the coronary artery provided a recovery period of 30 min was allowed (Maroko et al, 1971; Kjekshus and Mjøs, 1973; Berdeaux et al, 1976), but despite this was restudied to check whether the metabolic changes in the ischaemic myocardium were also reproducible. A depressant effect of induction of anaesthesia with pentobarbitone on the function and metabolism of plasma free fatty acids and glucose by the myocardium of the dog has been reported (Durham et al, 1970). No preferential effect on either fatty acid or glucose metabolism was observed. During anaesthesia additional injections of pentobarbitone had similar effects (Armstrong et al, 1961; Opie et al, 1973). The effect of other anaesthetics on particularly free fatty acid metabolism has apparently not been investigated. Pentobarbitone was adhered to, since the use of alternative anaesthetic agents would have required

a formal study of their effects on myocardial metabolism. However the acute, depressant activity of pentobarbitone by bolus injections was eliminated using constant infusions, inducing therefore a presumably constant anaesthesia without fluctuations in arterial concentrations of glucose and free fatty acids.

It is not claimed that this model represents a true description of the human myocardium during acute myocardial infarction. Apart from the effect of anaesthesia, mentioned above, other important differences are apparent:

- Acute myocardial ischaemia in man is set against the background of a gradual narrowing of the lumen of coronary arteries, with previous phases of ischaemia, glycogen depletion and lipid infiltration of the heart.
- Due to the surgical trauma heart rate and arterial blood pressure are high (Oliver, 1972).
- During the dissection of the coronary artery the nervous supply to the ischaemic myocardium might have been interrupted, leading to loss of noradrenaline stores (Oliver, 1972).
- Collateral circulation in the healthy myocardium is more developed in the dog than in man (Schaper, 1971).

However gradual obstruction of coronary flow during atherosclerosis may in itself lead to collateral

development, as was observed in dogs (Schaper, 1971).

Two factors largely determined the choice of the model:

1. the necessity to sample ischaemic effluent, excluding closed-chest preparations
2. the desire to use each animal as its own control.

Permanent occlusion by ameroid constrictors, thrombus formation, plug embolism can not be used. Balloon catheters and pump controlled restricted coronary flow to produce intermittent ischaemia were considered technically too difficult. Anaesthesia and local venous sampling is difficult in pig models, and dogs were preferred. This model should allow the study of effectiveness of treatment, designed to inhibit fatty acid utilisation or to enhance glucose metabolism by the ischaemic myocardium.

2.1.2. Modification of fatty acid utilisation

Myocardial utilisation of plasma free fatty acids is determined by its arterial concentration and the free fatty acid to albumin molar ratio (Ballard et al, 1960; Scott et al, 1962; Mjøs, 1971; Lassers et al, 1972; Miller et al, 1976). Several methods have been suggested to raise plasma concentrations of free fatty acids:

- direct infusion of fatty acids, unbound or partially bound to albumin (Hoak et al, 1964; Bezman-Tarcher, 1969; Greenough et al, 1969; Riemersma et al, 1974)
- induction of intravascular lipolysis by heparin injection during alimentary lipaemia (Schalch and Kipnis, 1965; Balasse and Ooms, 1968) or lipaemia caused by intravenous injection of triglycerides (Intralipid) (Kurien et al, 1971; Mjøs, 1971[b], Opie et al, 1972)
- stimulation of the adrenergic nerves, innervating adipose tissue (Rosell, 1966)

With the exception of the complicated model of Greenough et al (1969), direct infusion of fatty acids or their anions led to profound haemodynamic and arrhythmic disturbances in normal dogs (Hoak et al, 1972; Riemersma et al, 1974).

These findings are in sharp contrast with those obtained from experiments in which Intralipid and heparin was used as the model, when no adverse (haemodynamic) side effects were found. However this model was criticised rightly for the unphysiologically high concentration of plasma free fatty acids, ranging from 3 000 to 6 000 μEq/l (Mjös, 1971[b]; Kurien et al, 1971, Opie et al, 1972). However the actual concentration of plasma free fatty acids in these studies were almost certainly overestimated (Riemersma et al, 1977).

Electrical stimulation in vivo of the sympathetic nerve, innervating dog subcutaneous fat is too variable possibly due to the fact that release of free fatty acids from adipose tissue is counteracted by the vasoconstriction, induced by the concomittant release of noradrenaline to be of practical use.

The apparent discrepancy between toxic effects of free fatty acids, observed during direct infusions with the well documented lack of adverse side effects when Intralipid and heparin was used, is most likely due to circulating unbound plasma free fatty acids in the former model, which may not occur with Intralipid heparin since free fatty acids may remain within the triglyceride particle, once the high affinity sites of

albumin have been occupied (Goodman and Shafrir, 1959). In balance, therefore, the toxic effect of fatty acids during direct infusions are due to unbound and micellular anions, known detergents, and which are responsible for massive thrombosis, severe hypotension, arrhythmias and myocardial ST segment depression in normoxic animals (Hoak et al, 1972; Riemersma et al, 1974). Such method can't be used with confidence in normal dogs, but certainly not during acute myocardial ischaemia/hypoxia, where raised concentrations of free fatty acids bound to albumin exerted a depressive effect on myocardial contractility (Henderson et al, 1970; Kjekshus and Mjøs, 1972).

Of course concentrations of plasma free fatty acids are influenced by hormones and drugs (Galli et al, 1969). Kjekshus and Mjøs (1973) used a combination of catecholamines (isoprenaline, noradrenaline), to raise and an antilipolytic drug (β-pyridylcarbinol, Roche) to reduce the concentration of plasma free fatty acids. They showed that pretreatment with β-pyridylcarbinol did not affect the haemodynamic response of a 15-20 min infusion of the catecholamine, but effectively blocked the catecholamine-induced adipose tissue lipolysis.

This simple model was used to study the role of fatty acid and glucose metabolism of the ischaemic myocardium in the determination of the severity of acute myocardial ischaemia. The effect of the following

structurally unrelated antilipolytic drugs, alone or in combination with isoprenaline infusions, was examined:

- the active metabolite of clofibrate (p-chlorophenoxyisobutyrate, CPIB)
- a naturally occurring antilipolytic substance prostaglandin E_1 (PGE_1)
- a 'cardiospecific' β-blocker acebutolol
- nicotinic acid
- sodium salicylate.

It was appreciated that despite the wide range of structurally unrelated drugs, the drugs could all theoretically possess an unknown property affecting ischaemic metabolism or severity of myocardial ischaemia, in addition to their common antilipolytic activity. Therefore the effect of raised plasma free fatty acids concentration on ischaemic myocardial metabolism and severity of myocardial ischaemic injury using the complicated, but side-effect free system of Greenough et al (1969) was considered. This became more of practical proposition when it was learned that this system could be built for us by Dr N.McLeod, Department of Chemical Engineering, University of Edinburgh. In the original publication of Greenough et al (1969) haemodynamic parameters were not measured, and therefore the use of the blood cell separator required evaluation of haemodynamic effects in relation

to fatty acid (sodium oleate) infusions in control experiments. Both these experiments and those in which sodium oleate was infused during experimental coronary occlusion and its effect on ischaemic myocardium are described in detail in Part 5.

Originally it was not intended to use the model of Intralipid and heparin to raise plasma concentrations of free fatty acids. However when it was discovered, that plasma free fatty acids concentrations were seriously overestimated in lipaemic samples of heparinised dogs or men (Riemersma et al, 1977), a formal study of Intralipid-heparin as a model to raise plasma free fatty acid in anaesthetized dogs was made. The results of this study are reported in Part 5.

2.1.3. Stimulation of glucose utilisation

During experimental acute myocardial ischaemia the oxidation of plasma free fatty acids by the ischaemic myocardium is impaired to a greater extent than that of glucose, and the heart becomes more dependent upon both aerobic and anaerobic glucose metabolism for its energy requirements (Opie et al, 1973).
Early experiments, which showed that the perfused heart became totally reliant on anaerobic glycolysis and the rate of utilisation of glucose or glycogen could determine the rate of survival of the anoxic heart (Cascarano et al, 1968; Weissler et al, 1968; Scheuer and Stezoski, 1969), led to the now widely-held belief that measures promoting glucose utilisation by the ischaemic myocardium should decrease the extent of ischaemic injury (Opie, 1970). Although the early observations are undoubtedly valid and have been extended, serious doubt is raised to the validity of the extrapolation of the metabolic behaviour of the anoxic, well-perfused myocardium to situations of ischaemia (lack of bloodflow). The difference between these two preparations, with on the one hand in the anoxic model where coronary flow is increased with increased glucose supply and increased removal of lactate and H^+ and on the other hand the ischaemic

model with decreased glucose supply and accumulation of metabolites (lactate and H^+) falls outside the scope of this thesis. But it suffices that these reservations have been confirmed recently in comparative studies (Rovetto and Neely, 1977). Similarly it is not clear whether measures, which are known to enhance glucose utilisation of the normoxic heart such as increased glucose concentrations (Morgan et al, 1961; Opie et al, 1962; O'Brien, 1969), the addition of insulin (Morgan et al, 1959; Newsholme and Randle, 1964) will increase glucose utilisation by the ischaemic myocardium. The lack of effect of raised concentrations of plasma free fatty acids on glucose extraction by the ischaemic myocardium (Opie et al, 1973) in contrast to its effect on normoxic myocardium (Shipp et al, 1961; Newsholme and Randle, 1964) does support the view that control of ischaemic myocardial glucose metabolism has been modified. On the other hand high lactate concentrations (40 mmole/l) inhibit glucose utilisation of the ischaemically perfused rat heart (Rovetto et al, 1975). Clearly more work is indicated.

However, despite these theoretical objections animal studies have supported the hypothesis that raised glucose concentrations during intravenous infusion of glucose and insulin reduced myocardial ischaemic

injury following coronary occlusion (Maroko et al, 1972; Sybers et al, 1973; Haneda et al, 1974; Opie et al, 1975; Opie and Owen, 1976).

We tested the effect of enhanced glucose utilisation with another model, which had not previously been tested in dogs with acute myocardial ischaemia. This approach stemmed from the observation that di-isopropyl-ammonium dichloroacetate raised the respiratory quotient in alloxan-diabetic rats, suggesting an increased utilisation of glucose relative to that of (free) fatty acids (Lorini and Ciman, 1962). McAllister et al, (1973) showed that the active component dichloroacetate stimulated glucose uptake and oxidation, and inhibited fatty acid oxidation in the isolated perfused rat heart and in intact dog heart. It was suggested that dichloroacetate stimulated both glycolysis and glucose oxidation by increasing the activities of phospho-fructokinase and pyruvate dehydrogenase. The activation of pyruvate dehydrogenase in rat myocardium by dichloroacetate was confirmed by Whitehouse and Randle (1973).

Therefore experiments were conducted of the metabolic and haemodynamic effects of dichloroacetate during experimental acute myocardial ischaemia in dogs. The possibility that the stimulation of myocardial glucose

and inhibition of fatty acid utilisation might limit the severity of acute myocardial ischaemia was examined.

The results are presented in Part 4.

2.1.4. Surgical procedures and animal preparation

Mongrel dogs of either sex, weighing 12-25 kg were obtained from the Animal Breeding Centre, University of Edinburgh, The Bush. After overnight fast anaesthesia was induced by intravenous injection of sodium pentobarbitone (Sagatal, May and Baker, Dagenham) approximately 25-30 mg/kg. The depth of anaesthesia was judged by the presence of corneal and paw reflexes. The animal was intubated, using a cuffed endotracheal tube (size 7.5 - 9) and the cuff inflated using 12 - 14 ml air. Respiration using air (15 cycles/min) was maintained by a positive pressure respirator (Harvard Apparatus Co., Mass., USA). Stroke volume was adjusted according to nomogram (made by Kleinman and Radford for Harvard Apparatus Co., Mass., USA). Ventilation was monitored using arterial blood gas determinations kindly analyzed for us by the Blood Gas Laboratory, Department of Medicine, The Royal Infirmary, Edinburgh.

The left femoral vein was dissected and cannulated using a sterile polyethylene cannula (6 FG, 30 cm; Portex Ltd, Hyphe, Kent) for the continuous infusion of maintenance sodium pentobarbitone anaesthetic (dose 4 mg/kg/hr at a rate of 10 ml/hr) using a Harvard

Infusion pump.

The left and right femoral arteries were cannulated using polyethylene catheters (8 FG, 30 cm, Portex) for the measurement of arterial blood pressure and for the collection of arterial blood samples.

The right femoral vein was cannulated using a sterile polyethylene catheter (6 FG, 30 cm; Portex) for the administration of drugs.

The cannulae were filled with saline (0.9%), except in heparinised dogs where heparinised saline 5 U/ml was used.

If neccessary, the left jugular vein was dissected and left with a loose ligature around the vessel until required for the introduction of the coronary sinus catheter after opening of the chest (see later).

The chest was opened through the 5th intercostal space for the studies in which myocardial ischaemia was induced or sham occlusions were made. Pectoral muscles were tied using 2/0 silk and then transsected. In studies on heparinised dogs diathermy was adopted, to avoid bleeding.

A small opening was carefully made in the pleural space using a finger (no sharp instruments to avoid damage to the lung). The thorax was then further opened and the ribs were spread. After thoracotomy ventilation was increased by 20 ml/stroke to avoid

underventilation (empirical adjustment found in early studies with Dr R.G.Talbot).

The base of the heart was exposed. The pericardium was opened near the root of the aorta and a transsection was made parallel to the phrenic nerve, followed by a diagonal transsection to the apex. Fatty tissue overlying the pericardium was dissected, tied and cut.

A pericardial cradle was made using four 2/0 silk stitches tied to the ribspreader. Before the two ties near the base of the heart were made a small swab, soaked in saline was inserted between the pericardium and the lungs to avoid the threads cutting into the lungs. Care was taken not to obstruct the caval veins or to lift the cradle too high resulting in reduced arterial blood pressure.

A cannula (3 FG, 30 cm; Portex) was tied in the left atrium for injections of radioactive microspheres. The distal part or a branch of the left anterior descending coronary artery was dissected free over a distance of 0.5 - 1.0 cm, using two pairs of curved forceps (10 cm). A loose ligature was then placed around the vessel to facilitate later retrieval for occlusion.

A local vein draining the myocardium perfused by the dissected coronary artery was cannulated according

to a method suggested by Dr R.Norris, Auckland, New Zealand. A white needle (19 gauge, Yale microlance, was bent through a right angle 10 mm from the tip.

This needle was inserted into the (local) vein, preferably at a point at least 1-1½ cm away from any confluences, in order to avoid problems in advancing the cannula past these points. It is important to make a clean, full puncture with the needle. This is difficult since the view is partially obscured by a sudden squirt of blood before a complete puncture has been made. However the needle will check the bleeding once inserted, allowing inspection of the puncture. The needle was then pulled out and the matching cannula (3 FG, 30 cm; OD 1.02 mm; Portex) filled with 1 U/ml heparin inserted guided by the origin of the bleeding. Generally this caused no difficulty, except when a hole could only be made through a fine layer of fatty or connective tissue. In this case it was helpful to insert first a smaller cannula (3 FG, OD 0.75 mm; Portex) which was then replaced by the matching cannula (see above). The distal part of cannula is positioned away from curved sections or branches of the vein. A test collection of blood is made (under normal myocardial perfusion), and if found acceptable (more than 3-4 ml/min) the

cannula secured using the anchoring stitch, made whenever possible on the right ventricle.

Normally the cannula will stop the bleeding, but sometimes Sterispon (Allen and Hanburys Ltd, London) was applied to the punctured vessel to check the bleeding.

Occasionally the local anatomy was unsuitable for cannulation of a local vein, in this case cannulation was attempted in the great cardiac vein near the origin of the left anterior descending coronary artery. The cannula was then pushed in retrogradely to the desired site, using gentle finger pressure to guide it.

In 2 of 29 experiments it was impossible to obtain a suitable local venous cannulation.

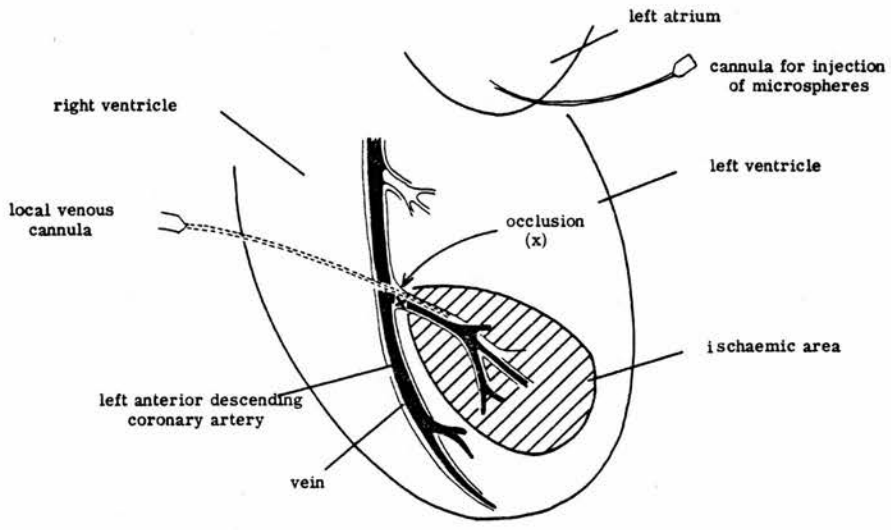
The coronary sinus polyethylene catheter (modified from a Bourassa-left coronary catheter (7 F; ESCI, USA)) was inserted using the exposed jugular vein and positioned under fluoroscopic guidance. When fluoroscopy was not available, the catheter was placed into the coronary sinus guided by the tip of a finger positioned at the orifice.

A schematic representation of the model is given in Fig 2.01.

The animals were allowed to recover from the surgical procedures for at least $\frac{1}{2}$ hour.

Figure 2.01

Schematic model of the experimental model



Not indicated are the coronary sinus catheter and the right atrial pacemaker electrodes.

2.1.5. Induction of acute myocardial ischaemia

The basic model for the induction and assessment of severity of myocardial ischaemia was taken from the work of Maroko et al (1971) and Kjekshus and Mjøs (1973).

Ischaemia was induced by occlusion of the distal part or a branch of the left anterior descending coronary artery using a Mayfield intracranial clip. Myocardial ischaemia was characterised by cyanosis, paradoxical contraction and ST segment changes in the epicardial electrocardiogram of the area supplied by the occluded coronary artery. The site of occlusion was selected to yield a relatively stable ischaemic preparation free of major haemodynamic disturbances and arrhythmias and would lead to an infarct, comprising 10-15% of the free wall of left ventricular myocardium (Owen et al, 1970). Despite the fact that in this model the site of occlusion is standardised the severity of myocardial ischaemia (ST-segment changes, lactate release) varies considerably from preparation to preparation. But if after release of occlusion a recovery period of 30 min is allowed a second occlusion leads to reproducible changes, provided the occlusion did not exceed 20 min, allowing each animal to serve as its own control. However, the exact time of onset of irreversible ischaemic changes is uncertain and

may depend on what criteria are used (morphological, biochemical or functional changes). ST-segment elevation is virtually constant between 10-20 min. We preferred to use occlusions of 10-15 min duration and thereby increase the margin of safety. The model we have adopted consists of 3 to 4 periods of myocardial ischaemia of 10-15 min duration, each separated by a recovery phase of at least 30 min. Usually studies were made both with and without isoprenaline stimulation.

The reproducibility of our model was demonstrated in a joint study with the University of Tromsø (Dr. H. Vik-Mo and Prof. O.D. Mjøs). The results are tabulated in Table 2.01. Due to the slow return of plasma glycerol concentrations to baseline values after cessation of an isoprenaline infusion, the interpretation of glycerol data, obtained during two repeated occlusions (basal conditions) is complicated, as glycerol extraction may depend on its arterial concentration. Therefore the model in this form is not suitable to study myocardial glycerol metabolism under basal conditions.

This model is versatile and allows randomisation of myocardial ischaemia with or without pre-treatment, provided that the half life of the drugs used is short.

Table 2.01

Experimental acute myocardial ischaemia: The reproducibility of the model

	Occlusion I (A)	Occlusion II (C)	P*	Isoprenaline + occlusion I (B)	Isoprenaline + occlusion II (D)	P
ΣST (mV)	25.3 ± 6.9	27.2 ± 7.3	N.S.	57.3 ± 10.6	57.6 ± 11.6	N.S.
ST> 2 mV	5.5 ± 0.8	6.2 ± 0.8	N.S.	9.0 ± 0.5	8.7 ± 0.4	N.S.
Heart rate (beats/min)	151 ± 9	150 ± 13	N.S.	189 ± 11	184 ± 12	N.S.
LVSP (kPa)	16.4 ± 1.1	15.8 ± 1.1	N.S.	15.8 ± 0.7	15.8 ± 0.9	N.S.
LV dP/dt max. (kPa/s)	208 ± 21	204 ± 18	N.S.	418 ± 37	395 ± 34	N.S.
$\overline{AP}$ (kPa)	15.1 ± 0.9	14.4 ± 0.8	N.S.	13.3 ± 0.5	13.3 ± 0.5	N.S.
Oxygen saturation (%)	98.9 ± 0.9	100 ± 0.9	N.S.	93.5 ± 2.5	95.8 ± 2.9	N.S.
Oxygen saturation$_{a-cs}$	59.2 ± 2.3	61.0 ± 3.1	N.S.	58.0 ± 2.5	56.5 ± 1.2	N.S.
Oxygen saturation$_{a-lv}$	71.8 ± 2.4	70.8 ± 3.3	N.S.	69.5 ± 2.4	68.5 ± 3.3	N.S.
Glucose$_a$ (mmole/l)	8.16 ± 0.42	7.55 ± 0.79	N.S.	12.71 ± 0.98	12.22 ± 1.11	N.S.
Glucose$_{a-cs}$	1.15 ± 0.67	0.77 ± 0.29	N.S.	0.36 ± 0.20	0.61 ± 0.46	N.S.
Glucose$_{a-lv}$	2.32 ± 0.27	1.75 ± 0.35	N.S.	2.65 ± 0.22	2.18 ± 0.32	N.S.
FFA$_a$ (μmole/l)	205 ± 29	194 ± 26	N.S.	1542 ± 258	1640 ± 274	N.S.
FFA$_{a-cs}$	59 ± 20	46 ± 7	N.S.	349 ± 69	351 ± 70	N.S.
FFA$_{a-lv}$	55 ± 19	57 ± 21	N.S.	525 ± 117	465 ± 71	N.S.
Lactate$_a$ (mmole/l)	1.87 ± 0.23	1.93 ± 0.29	N.S.	2.62 ± 0.38	2.31 ± 0.48	N.S.
Lactate$_{a-cs}$	0.64 ± 0.05	0.68 ± 0.09	N.S.	0.65 ± 0.14	0.24 ± 0.09	N.S.
Lactate$_{a-lv}$	-2.41 ± 0.69	-1.01 ± 0.62	N.S.	-2.86 ± 0.54	-2.39 ± 0.68	N.S.
Glycerol$_a$ (μmole/l)	33.5 ± 3.9	73.3 ± 13.6	‡	276.6 ± 32.8	398.5 ± 52.7	N.S.
Glycerol$_{a-cs}$	insufficient data		‡	23.6 ± 8.4	48.7 ± 15.5	‡
Glycerol$_{a-lv}$	-43.1 ± 16.4	-11.3 ± 5.9	‡	6.6 ± 9.9	3.9 ± 14.9	‡

The reproducibility of the effects of two intermittent coronary artery occlusions of 12 min duration was studied under basal conditions (A) and during isoprenaline infusion in 6 open-chest, anaesthetized dogs. The sequence of occlusions was: Occlusion I (A) Isoprenaline+occlusion I (B); Occlusion II (C) and Isoprenaline+occlusion II (D), each separated by a recovery period of 45 min after reflow. Myocardial ischaemic injury and haemodynamic parameters were recorded at 12 min of occlusion. Blood samples were obtained simultaneously from femoral arterial, coronary sinus and local venous cannulae between 6 and 12 min after occlusion.

Abbreviations: ΣST=sum of ischaemia-induced ST-segment elevation, ST>2 number of sites with ST-segment elevation > 2 mV, LVSP= syst left ventricular pressure, LV dP/dt max.= maximum of first derivation of left ventricular pressure, $\overline{AP}$=mean aortic blood pressure. Note: Arterial glycerol concentrations during Occlusion II(C) have not returned to basal values 45 min after cessation of the first isoprenaline infusion (Isoprenaline + occlusion I (B).

* Statistical analysis performed using the Wilcoxon's test for paired data.

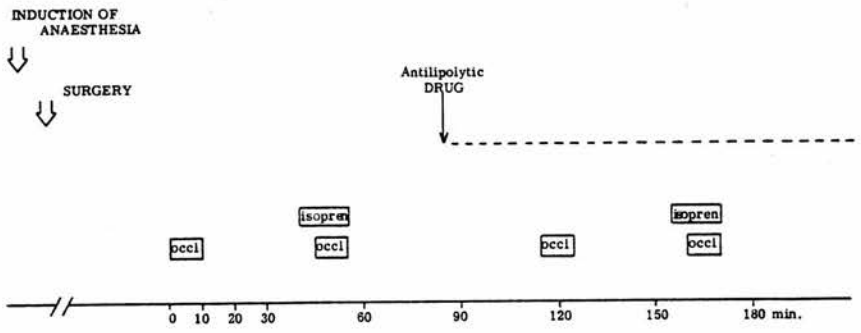
‡ Statistical analysis not made : number of experiments ≤ 5.

However, when the drug metabolism is slow (such as for CPIB, acebutolol, nicotinic acid, DCA) a fixed protocol of: control occlusion, control occlusion during isoprenaline infusion, repeated in the same sequence after the administration of the drug, was followed (see Fig 2.02).
In this way systematic factors (time, sequence) may operate. A check was made in a few studies on this by reversing the order, the two control occlusions were made after a delay sufficiently long to metabolise the drug.

In recent studies continuous monitoring of the epicardial ECG using a multiplex system (see Section 2.3) was possible. In some of these studies collection of a blood sample after 5 min of occlusion from the local vein reduced transiently ST-segment elevation. (The mechanism of this was not investigated but it seems likely that the negative venous pressure during sampling may have affected blood flow in the ischaemic area). In the same studies ST segment elevation was almost maximal at 5 min and were therefore used as the endpoint rather than ST-segment elevation at 10 or 15 min of occlusion.

In some studies drugs were administered during an established coronary occlusion, a more real-life situation. Under these circumstances drug induced

Figure 2.02. Experimental model : The basic procedure



Occl = occlusion; Isopren = isoprenaline infusion 0.1-0.2 µg/kg/min. In some experiments the administration of antilipolytic drugs preceded the first and the second occlusion (during isoprenaline infusion), and the 2 control occlusions were then made after a period sufficiently long to metabolise the drug.

alterations of myocardial metabolism before coronary occlusion, such as increased glycogen stores, which might influence the development of myocardial ischaemic injury are excluded.

Section 2.2:

BIOCHEMICAL TECHNIQUES

2.2.1. Biochemical techniques

Introduction

Measurements of differences in the concentrations of metabolites between arterial and coronary sinus blood have proved to be important in the study of myocardial metabolism *in vivo*. But alterations in the metabolism of small ischaemic areas, such as alterations in glucose extraction or lactate production, are too small effectively to modify the arterio coronary sinus difference of these metabolites, but are reflected in the arterio-venous difference of metabolites determined using blood, obtained from a cannulated local vein, draining that area.

An immediate implication of this observation is that preferably only micro(bio)chemical techniques should be used, because during ischaemia only small blood samples can be obtained from the local vein with difficulty and sometimes not at all.

Another condition which must be fulfilled by the various analytical techniques stems from the fact that it may not be sufficient to quantify the arterio-venous difference of a metabolite. It should well be that *the change* in arterio-venous difference may be more closely related to differences in myocardial ischaemic injury under varying conditions. Therefore methods with high precision, but not necessarily with a high accuracy are required

and this is particulary so for measurements of plasma glucose, where arterio-venous differences are small. It is obvious that simultaneous, preferably automatic analysis of all samples of a study will contribute to achievement of high precision. Therefore the basic condition in all methods described was the simultaneous analysis of samples from one experiment. This section is concerned with methodological aspects of the biochemical techniques. After a short summary of the principle of each method a description of the exact technique will be given, including details of the precision on individual estimates, the collection and storage of blood samples, and possible interference of other metabolites and drugs.

2.2.2. Estimation of plasma free fatty acid concentration

Introduction

Two methods for the measurement of plasma free fatty acid concentrations were used. Normally plasma free fatty acid concentrations were determined by the titrimetric method of Dole and Meinertz (1960), according to the modification of Trout et al (1960). Fatty acids were extracted from plasma aliquots and from standards (palmitic acid) into heptane and determined titrimetrically using a Metrohm micro-buret.

However in studies (described in section 5) when plasma free fatty acid concentrations were raised *in vivo* by heparin-induced intravascular lipolysis and by the administration of Intralipid* as the substrate the method of Dole is unsuitable, due to the continuation of lipolysis *in vitro*. Therefore another method was developed for those studies in which both Intralipid and heparin were administered. *In vitro* lipolysis was prevented by immediate denaturation of proteins in blood samples, after which fatty acids were extracted. Fatty acid concentrations were then determined using the method of Ho (1970). Its principle is the formation

* Intralipid is fractionated soya bean oil (Kabi Vitrum, Sweden).

of radioactive Ni-fatty acid salts, which, unlike free Ni^{2+}, are soluble in chloroform-heptane, and can be estimated by liquid scintillation counting.

The titrimetric method of Dole

The basic procedure of collection of blood samples was as follows:

Arterial and coronary sinus catheters and local venous cannula were cleared by withdrawal of 4-6 ml and 1 ml blood respectively. Blood samples were then collected into heparinized disposable plastic syringes (Becton, Dickinson Co Ltd., Ireland), transferred to heparinized tubes (Searle, Morpeth, Northumberland), and immediately cooled on ice. Blood cells and plasma were separated by centrifugation (10 min at 1 500 g) in a cooled (4°C) MSE 4L centrifuge within 15-30 min. Plasma (1 ml) was then transferred in duplicate to a stoppered thick walled glass tube (capacity 14 ml), containing 5 ml Dole's extraction mixture: isopropanol: n-heptane: H_2SO_4 (1 mole/l) 40:10:1 by vol., shaken vigorously and stored at -20°C until analysed.

Extraction

Free fatty acids were extracted using the method of Trout et al (1960), which includes a washing procedure to remove interference of lactic acid and short chain fatty acids.

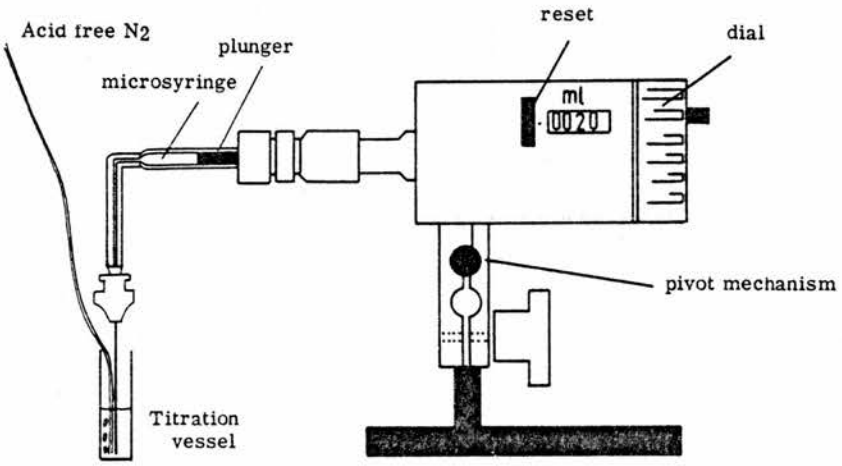
Titrimetric procedure

A Metrohm microtitrator (Metrohm, Switzerland) was used for the titrimetric step. The titrator, shown in Fig. 2.03 consists of a micro-syringe (1 ml capacity) which was filled with NaOH (approximate concentration 0.025 mole/l).

The location of the titrator is important as it is difficult to judge the end-point accurately under conditions of variable light intensity. Direct light into the laboratory was therefore excluded and the equipment was positioned far away from the windows.

Each extract was titrated in duplicate. Aliquots (1 ml) were transferred to a glass cuvette (3 ml) using a bulb pipette (Emil, Gold Line). Freshly redistilled ethanol (1 ml, J. Burrough, Analar) and 6 drops of phenol red indicator (0.79 mg/ml ethanol) were added. The end-point was considered to be reached when the yellow colour changed to pink.

Figure 2.03. The titrimetric analysis of plasma FFA: The microtitrator



The equipment used in the microtitration:
The microsyringe, which serves as the titrant reservoir, can be removed to be filled using a nut. Fine control of the titration is ensured by the use of a large dial moving the plunger attached to a micrometer. Acid free N_2 is used to expel CO_2 from the titration vessel and facilitates proper mixing. The pivot mechanism facilitates the insertion/removal of the hypodermic needle in and from the titration vessel (cuvette).

Interference of lactate, phospholipids and other compounds

Theoretical considerations

The specificity of the method is determined by two factors: the specificity of the extraction and the specificity of the titration.

The partition coefficients for lactate, phospholipids have been determined by Dole and are given in Table 2.02. The partition coefficients (r) were used to calculate the % extraction of the various fatty acids, lactate and lecithin.

The equations used for these calculations are derived as follows: if the fraction of extracted (fatty) acids is x, then (1-x) will remain in the water-phase. By definition r is the ratio between the acid concentration in the upper $[\,]_u$ and the lower phase $[\,]_l$:

$$r = \frac{[\,]_u}{[\,]_l} \qquad \text{Equation 1.}$$

Taking the volume of the upper phase (4 ml) and of the lower phase (7 ml) of the basic Dole's method into account, equation 1 can be rewritten as:

$$r = \frac{x \,/\, 4}{(1-x) \,/\, 7} \qquad \text{or} \qquad \frac{7\,x}{4\,(1-x)}$$

Table 2.02

Factors influencing the specificity of the titrimetric method. The effect of the Trout wash on lactate interference.

	partition coefficient)* r	Basic Dole's method		Dole's method + Trout wash
		% in extract	% in water phase	% in extract
FATTY ACIDS:				
C12:0	5.90	77	23	66
C14:0	8.50	83	17	74
C16:0	11.00	86	14	78
C18:0	13.60	89	11	83
C18:1	10.75	86	14	79
C18:2	10.30	86	14	78
C20:4	9.30	84	16	76
OTHERS:				
lactate	0.021	1.2	98.8	0.025
lecithin	0.07	3.8	96.2	0.25

* partition coefficient r, taken from Dole and Meinertz (1960).

Expressing the fraction extracted (x) as a function of r:

$$\text{Fraction extracted } (x) = \frac{4\,r}{4\,r + 7} \qquad \text{Equation 2.}$$

The volume of the Trout wash is 4 ml instead of 7 ml and as a result it can be shown that the fraction extracted during the Trout wash will be

$$x' = \frac{r}{1 + r} \qquad \text{Equation 3.}$$

The overall extraction including the Trout wash is x.x'. The results of the calculated percentages of fatty acids lactate and lecithin extracted, using the Dole method with or without the Trout wash are presented in Table 2.02.

A few points emerge:

a. The physiologically-occurring fatty acids (C16:0 - C18:2) are all extracted to the same degree, about 86% (justifying the use of palmitic acid as a standard)

b. Lactate and lecithin interference is small and is virtually eliminated using the Trout wash.

The specificity is also determined by the exact end-point used during the titration. The pKa values of the fatty acids, lactic acid, phospholipids etc in heptane-ethanol are likely to be different. Thus at a specific end point different degrees of neutralisation will have been achieved: Acids stronger than fatty acids will be fully dissociated and will therefore interfere more severely than weaker acids. However it is important to realise that the partition coefficients in Table 2.02 were almost certainly based on titrimetric estimations of these compounds using thymol blue as the indicator. Thus differences in interference caused by differences in pKa have already been taken into account. Nevertheless the registration of the titration curve (moles of NaOH vs pH) could reduce interference by weaker acids (but not by stronger ones). However recording pH accurately in heptane-ethanol mixtures is exceedingly difficult. An attempt was made, but unsatisfactory results (lack of stability of pH reading) were obtained. This was attributed to interference of static electricity (no proper earthing could be made) and unsuitable pH electrodes: electrolytes of the salt bridge were not soluble in heptane-ethanol, causing a poor continuity between the sample and pH electrode. Therefore this approach was abandoned.

Interference of lactate and phospholipids

The interference of lactate was measured by the extraction and titration of a lactate standard (100 mmole/l). The estimated lactic acid concentration was 0.051 ± 0.020 mmole/l, thus 99.95% of lactic acid present is eliminated.

The interference of phospholipids was not measured, due to the lack of phospholipid standards which would be fatty acid free.

Stability of stored samples

The stability of plasma samples or Dole extracts stored at -20°C has been investigated by several independent groups and plasma free fatty acid concentrations remain unchanged under these conditions for at least 8 months (Braun, 1971; Broechoven and Parijs, 1968). This aspect was not further explored.

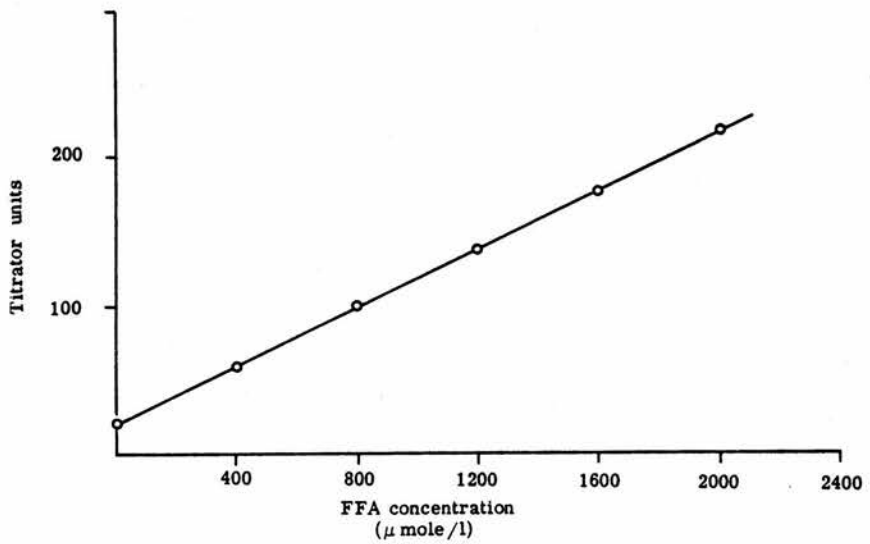
Calibration and precision of the method

A stock standard of palmitic acid (10 mmole/l, Analar 99%, British Drug Houses, Poole, Dorset) was made in redistilled n-heptane and stored at 4°C. Working standards (400-2400 μmole/l) were prepared every 10 days. Standards and blank (redistilled n-heptane) were simultaneously extracted with the samples in each run. A typical calibration graph is shown in Fig. 2.04. The blank reading is 20 titration units, equivalent to approximately 0.05 μmole/l of acid. The calibration graph was drawn by hand.

Samples which exceeded the range of the standards were diluted 1:1 with redistilled n-heptane and re-estimated.

The precision of the method, determined using 10 individual extracts of a canine pool was (mean ± SD): 475 ± 16.5 μmole/l (CV = 3.5%).

Figure 2.04. The titrimetric plasma FFA method: Calibration graph



Palmitic acid standards in heptane were 'extracted' and titrated with NaOH (about 0.025 moles/l) as described in the text.
Ten titrator units = 1 μl titrant.

2.2.2[b]. The measurement of free fatty acid concentration in heparinised animals

Preamble:

Originally the use of Intralipid and heparin to modify plasma free fatty acid concentrations in dogs was not planned as a part of the work concerning the thesis. However we were interested to re-examine the effect of heparin in patients admitted to the coronary care unit (CCU) with acute myocardial infarction. The reason for this was that if the original hypothesis was true: i.e. that raised plasma free fatty acid concentrations lead to serious ventricular arrhythmias, then the routine use of heparin in the CCU should be avoided in the prophylaxis of deep vein thrombosis. Indeed this was and is the official policy in the CCU in the Royal Infirmary for that reason. However heparin is routinely used in other countries (Holland, Germany and USA), and it seemed strange thet even incidental occurrence of serious ventricular arrhythmias after the administration of heparin would go unnoticed. Obviously this is not a simple problem, since the time of administration after the onset of symptoms might be crucial.

Unfortunately no double-blind trials of the effect of

heparin on the incidence of serious ventricular arrhythmias have been made, and therefore the intention was to re-examine this problem.

There was concern about the methodology used for the determination of plasma free fatty acid concentration in heparinised patients, particularly when it was likely that at least some samples would be taken during the night. The release of lipolytic enzymes (lipoprotein lipase, hepatic lipase and phospholipase) could artificially raise plasma free fatty acid concentrations, depending on the length of delay in deproteinising the samples. Therefore a pilot study was made in a closed-chest anaesthetised dog to examine the effect of time delay in deproteinisation of plasma samples on plasma free fatty acid concentration. The conditions were chosen to increase in vitro lipolysis, if any. Thus plasma triglycerides were raised by injection of Intralipid 20% (w/v) (dose 1 ml/kg body weight) and the collection of one blood sample (160 ml) was made 10 min after the injection of heparin (2 000 U), at a time of maximal lipolytic activity (see also Part 5, section 1). The blood was divided into two sets of heparinised tubes; one set was kept in ice and the other kept at room temperature. Plasma was obtained by a short (2 min) centrifugation at 1 500 g in a cooled centrifuge, and was immediately deproteinised

using Dole's extraction mixture. Plasma free fatty acid concentrations were then determined using the titrimetric procedure, described above.

The results are shown in Fig 2.05. Plasma free fatty acid concentrations in samples kept at room temperature rose quickly when the extraction/deproteinisation was delayed. The approximate rate calculated from the data is 4%/min of the original plasma free fatty acid concentrations. This process could be inhibited by storing the blood samples on ice, but an increase representing about 1%/min could still be demonstrated.

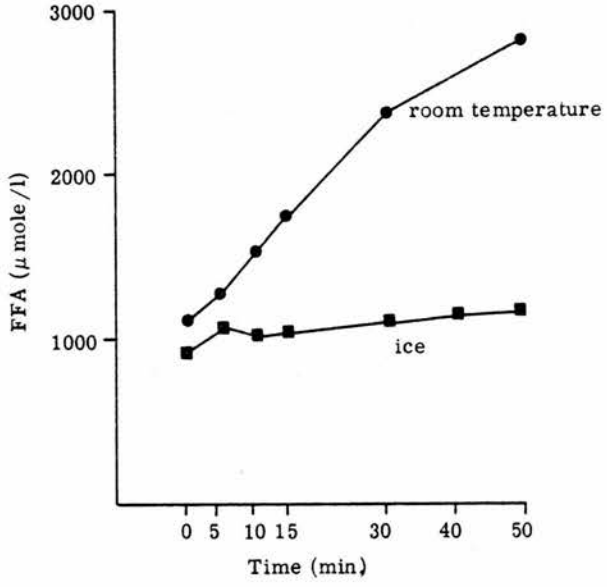
Similar results were found for ambulant ward patients given 12 500 U heparin 2 hours after a meal (Riemersma et al, 1977)

Previously the method of Intralipid/heparin to raise plasma free fatty acid concentrations in dogs was criticised amongst other thingsfor the unphysiologically high concentrations found (up to 6 000 µmole/l).

However the most important observation of the study of ambulant patients mentioned above, was that plasma free fatty acid concentrations were still in the physiological range, even after $\frac{1}{2}$ hour delay.

We therefore decided to reinvestigate the problems of the Intralipid-heparin model in the dog. We were particularly interested to measure plasma free fatty acid

Figure 2.05. The estimation of plasma free fatty acids in lipaemic samples from heparinised dogs: In vitro lipolysis in samples kept at room temperature and on ice



A blood sample (160 ml) was obtained 10 min after the intravenous injection of Intralipid/heparin, and divided in 14 aliquots, half stored at room temperature and the other half kept on ice. Plasma was obtained immediately and at 5-50 min after collection by a short centrifugation and plasma free fatty acids were extracted without delay.
For details see text.

concentrations in vivo and relate these to their turnover. Before we could do this we had to develop a practicable method for measuring plasma free fatty acid concentrations in heparinised subjects (dogs and patients with acute myocardial infarction).

Considerations leading to the FFA method, suitable for samples of heparinised dogs

As was demonstrated the in vitro lipolytic activity in lipaemic samples of heparinised dogs could not be blocked by cooling the samples on ice, the routine procedure in our laboratory. Addition of known inhibitors of heparin lipolytic activity (mainly lipoprotein lipase) to blood were tried in experiments identical to those described in the previous section, where storage at $0^{o}C$ and at room temperature were compared. EDTA, Triton X 100, alizuran blue and protamine sulphate were all ineffective in blocking in vitro lipolysis in samples stored on ice or at room temperature, and in the case of protamine sulphate (in presence of EDTA) clotting became a problem.

A more drastic approach was taken. It is well known that free fatty acids in blood are restricted to the plasma compartment, where they are bound to albumin. Only under situations of high FFA/albumin molar ratios exceeding, approx. 5 free fatty acids can be shown to bind with red cell membranes (Spector, 1969), but in quantitative

terms this is unimportant (less than 1% of the total).

Thus immediate extraction of blood, with the appropriate correction for blood cell volume, should be valid and overcome the artificially high free fatty acid concentrations caused by lipolysis in vitro.

Fatty acid extraction from blood samples

The extraction of free fatty acids, from different quantities of blood was checked in a pilot study, using albumin-bound H^3-palmitate (Radiochemical Centre, Amersham). The results showed that the volume of blood, ranging from 0.2 ml to 1.0 ml did not affect the amount of H^3-palmitate extracted by 5 ml Dole's extract (see Reagents, titrimetric FFA method). However when more than 0.75 ml blood was extracted the dead space of the cells aggregated by the procedure was large and it became difficult to work with the extracts. Therefore 0.75 ml blood was chosen as the maximum volume of blood to be extracted.

The extraction of H^3-palmitate from 0.75 ml human blood was checked. Using 8 different blood samples on average 100.0 $\pm$ 1.7 (SEM)% was recovered.

Interference by phospholipids

Normally 55% of blood phospholipids are located within the erythrocytes (Scientific Tables, Diem and Leutner (Eds), Ciba - Geigy, 7th Ed. pp 600) and the remainder

essentially in the plasma compartments. The implication is that if blood extracts are used for the determination of free fatty acids, phospholipid interference could be a much more serious problem than when plasma FFA are estimated by Trout's modification of the titrimetric method of Dole (see Table 2.02). Thus the required method should be free of interference by phospholipids.

Required sensitivity of the method

The method must also be more sensitive, since the extraction of 0.75 ml blood represents 0.3-0.35 ml of plasma instead of the usual 1 ml plasma used for the titrimetric procedure. On this basis the titrimetric procedure was ruled out, because one of its drawbacks is lack of sensitivity.

A wealth of methods for the determination of plasma free fatty acids have been described (Müller and Ulrich quoted 40 methods in 1966), most of them modifications of two basic procedures:

- (1) the titrimetric method of Dole
- (2) the colorimetric method of Duncombe (1963);

  or a combination of the two.

The colorimetric method of Duncombe (1963) which in itself was a modification of the method of Ayers (1956) was introduced as a more sensitive alternative to the titrimetric procedure. It is based on the principle that

metal ions (Cu^{2+}, Co^{2+}) form readily water-insoluble soaps when mixed with long chain fatty acids (14 C atoms or more). These metal soaps, unlike the metal ions, are soluble in chloroform and chloroform-heptane mixtures and are conveniently quantified as highly-coloured complexes with diethyldithiocarbamate (Duncombe, 1963); 1-nitrose-2-naphthol (Novak, 1965); diphenylcarbazide (Laurell and Tilbling, 1967); or tetra-ethylthiuram disulphide (Manasterski et al, 1972).

The method of Duncombe works well when standards are made up in heptane or if plasma extracts are analysed. However, the water-phase containing the excess copper ions forms above the organic phase (extract), and this gives rise to occasional spurious results. An important improvement was made by reversing the water and chloroform phases, by adding salts to the water and heptane to the chloroform phase (Novak, 1965), because this allowed direct analysis of the metal ions in the upper phase. Interference of phospholipids was eliminated by preferential adsorbtion of extracted phospholipids to silicic acid (Laurell and Tibling, 1967).

Finally the use of radioactive tracers (Co^{60}, Cu^{60} and Ni^{63}) led to a further increase in sensitivety (Ho and Meng, 1969;Ho, 1970). Ho incorporated the modification of Novak (1965) and of Laurell and Tibling (1967) and his

method is practical and highly specific (no phosphopipid interference). It seemed therefore that this method was the best available. Ni^{63}, rather than Co^{57}, Co^{60} and Cu^{60} was selected because it was noticed that the Co reagent spontaneously oxidized. Cu^{60} was not considered due to its hard γ-emitting properties.

The radiochemical analysis of fatty acids in heptane extracts

The method of Ho

In the method of Ho for estimating plasma free fatty acids, free fatty acids are extracted using the extraction procedure of Dole (1955), scaled down to allow extraction of 5 - 10 µl plasma samples. Ni^{63} soaps are then formed by shaking the extracts (final mixture chloroform: heptane 8:7) with alkaline Ni-reagent, triethanolamine is added to form a Ni-complex and thereby prevents the precipitation of $Ni(OH)_2$ under alkaline conditions used. Phospholipids are adsorbed to silicic acid, as was suggested by Laurell and Tibling (1967).

The development of the Ni^{63}-method

We increased the scale of the method in order to handle 1 ml of heptane extract, rather than 80 µl as was originally used by Ho. The reason for this was that it became apparent that handling small quantities of these highly volatile solvents was extremely difficult

and was not really necessary as it would be impossible to extract quickly and accurately correspondingly small volumes of blood (5-10 µl).

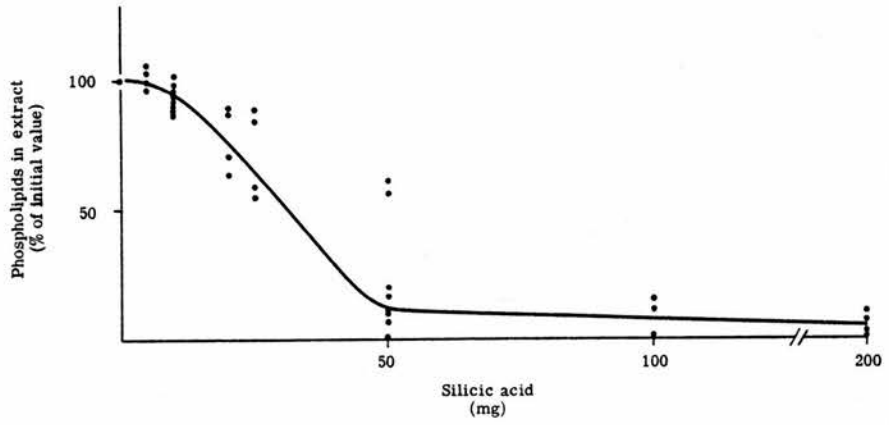
The adsorption of phospholipids

The conditions for the preferential adsorption of phospholipids was also investigated. A solution of lecithin in heptane in a concentration about equal to that of the total phospholipids in blood (2.75 mg/ml) was extracted using 5 ml of Dole's extract. The extracts which were diluted with an equal volume of redistilled chloroform were shaken for 5 min in the presence of varying quantities (0-500 mg) of activated silicic acid (heated overnight at 110°C). The supernatant (3 ml) was evaporated using a stream of N_2(60°C) and the residue was redissolved in 1.5 ml of heptane.

The heptane-soluble acids (phospholipids and free fatty acids) were then determined by the previously described titrimetric procedure.

The results (mean of 4 experiments; Fig 2.06) show that between 50 and 100 mg activated silicic acid are necessary for the complete adsorption of phospholipids. (The low amount of titratable acids not removed were assumed to be free fatty acids contaminating the phospholipids, about 6% of the titre if allowance is made for the difference in extraction of phospholipids.

Figure 2.06. The estimation of plasma free fatty acids: Adsorption of phospholipids (lecithin) to silicic acid



Phospholipids (lecithin) were adsorbed to varying amounts of silicic acid. The phospholipids in the silicic acid treated extract were determined by a titrimetric procedure and expressed as a % of the value found for the silicic acid free extract. Results of 4 experiments. For further details see text.

Alternatively it could denote incomplete adsorption of phospholipids, however this may be less likely in view of the monophasic curve.)

However palmitic acid was also adsorbed (up to 50% with 300 mg silicic acid) but, in agreement with Laurell and Tibling (1967), this could be prevented by adding 2% (v/v) methanol. The procedure using 300 mg silicic acid was therefore adopted.

Interference by lactic acid

In the titrimetric procedure lactic acid interferes and is removed using a washing procedure (Trout wash). We wanted to establish whether this step was still necessary since the soaps of short chain fatty acids are not soluble in organic solvents. For this a solution of lactic acid (0.8 mmole/l) dissolved in chloroform-heptane (8:7 v/v, plus 2% methanol) and blanks were mixed with the Ni^{63} reagent. Lactate failed to solubilise any Ni^{63} into the chloroform-heptane mixture. Therefore the washing procedure of Trout was omitted.

Preliminary method

The method which was tried for routine use was as follows:

Blood samples (0.75 ml) were extracted using standard Dole's extraction procedure: 5 ml Dole's extract (see

above) and the addition of 3 ml heptane and 2 ml double-distilled water to separate the two phases. The heptane extract (1.5 ml) was mixed with 3 ml chloroform-heptane-methanol (26:6:1 by vol.) and 300 mg activated silicic acid was added using a scoop. The samples were shaken for 5 min, and silicic acid precipitated by short centrifugation (5 min, 1 000 g). The supernatant (3 ml) was then shaken with the Ni^{63} reagent (as described by Ho, see Reagents) for the formation and extraction of Ni-fatty acid salts (5 min). After another short centrifugation the radioactive salts in 1 ml of the organic upper phase was determined by liquid scintillation spectrometry. For this 9 ml liquid scintillation solution (NE 260, New England Nuclear, Winchester, Hants.) was used.
Samples were counted for 10 min or until 10 000 counts had been accumulated.

Initial experience with the preliminary method

The method was tested (precision, standards) and most of this work went without problems, except that suddenly spontaneous inversion of the two phases during the formation of Ni^{63} salts was noted in about 60% of all samples. This was frequently associated with the presence of white crystals (presumably Na_2SO_4 from the Ni-reagent) in the test tubes. Reproducible results could not be obtained.

At first it was thought that a decrease in the temperature caused the crystallisation of the salts in the Ni^{63} reagent and that this process at times was initiated by the presence of grains of silicic acid. However it turned out to be a complicated problem: and although the temperature did play a role it was obviously not the sole factor.

The most important factor was the methanol which had been added to the chloroform-heptane mixture to avoid the adsorption of palmitic acid to the silicic acid. This final concentration of 2% methanol may seem low, but it constitutes a volume of 60 µl, which diluted the 200 µl saturated salt solution of the Ni^{63} reagent to such a degree, that its final spec. density was lower than that of the chloroform-heptane mixture. Indeed, inversion could be induced by addition of 60 µl methanol to tubes not containing methanol. This problem was avoided by taking the methanol/chloroform/heptane mixtures to dryness (the residue was redissolved in 3 ml chloroform:heptane 8:7 (v/v)); and the crystallisation problem was overcome by replacing the saturated Na_2SO_4 solution by an unsaturated solution of KBr (450 g/l).

It was also noticed that a number of factors

affected the blank values:

- the amount of radioactive Ni used per test
- the addition of silicic acid
- the batch of silicic acid
- the evaporation of solvents
- the glassware

The most important contributor to the blank was silicic acid. Blank values of 10000 cpm were observed with a particular batch. Washing the silicic acid with chloroform or methanol reduced the blank by some 20%.

Separate experiments demonstrated the effect of evaporating and then redissolving the FFA extracts and of washing the glassware used with chloroform or KOH. We were unable to reduce the blank to those observed when redistilled chloroform-heptane (8:7 v/v) was directly shaken with Ni^{63} reagent (200-400 cpm) by a combination of chloroform rinsed glassware; choice of suitable silicic acid batch and washing silicic acid with chloroform; distillation of solvents. The blank was usually 1900-2000 cpm. The top-standard (600 µmole/l) gave some 60000 cpm. Although we realised that further reduction of the blank value might be possible by avoiding the evaporation step. During the evaporation step

fatty acids contaminating the redistilled solvents are concentrated and lead to higher blank values. Thus avoiding this step, used to eliminate the methanol should lead to lower blank values. However we were concerned that spontaneous reversal of the phases would occur in the presence of methanol (see above). Since the actual blanks are not excessive and it was exceedingly difficult to make small improvements, ways of reducing blanks were not further explored.

The reproducibility of individual estimates was steadily improved by selecting different pipettes (Pettor; Alpha Laboratories, London) which were more suitable for the pipetting of organic solvents than the previously used Oxford pipettors.

The procedure finally adopted

Blood (0.75 ml) was extracted using 5 ml Dole's extraction mixture as described for the titrimetric method in Section 2.2.2, except that the Trout wash was omitted (see above). Phospholipids were removed in a 2 ml aliquot of the heptane extract by preferential adsorption to silicic acid after adding 2.5 ml chloroform-heptane methanol (231:9:9 by vol). Silicic acid (200 mg) was added using a scoop and the sample shaken for 5 min. After a short centrifugation (5 min at 1000 g), 3 ml of the supernatant was removed using a Pettor and was evaporated under a stream of nitrogen in a heating block (WTW, Weilheim Obb., Germany) set at 60°C. The fatty acids were redissolved in 3 ml chloroform-heptane 8:7 (v/v) and the tubes were stoppered. The tubes were left for 30 min, which is claimed to improve the reproducibility of the results (Elving and Carlson, personal communication).

The Ni^{63} salts were prepared by addition of 200 µl Ni^{63} reagent and the samples were mixed for 15-20 s using a Vortex. After a short centrifugation (5 min, 1000 g) the radioactive Ni-salts in the chloroform-heptane phase were analysed in 1 ml sample added to 9 ml liquid scintillation solution (NE 260, New England, Nuclear) using a liquid scintillation counter.

Calibration of the method

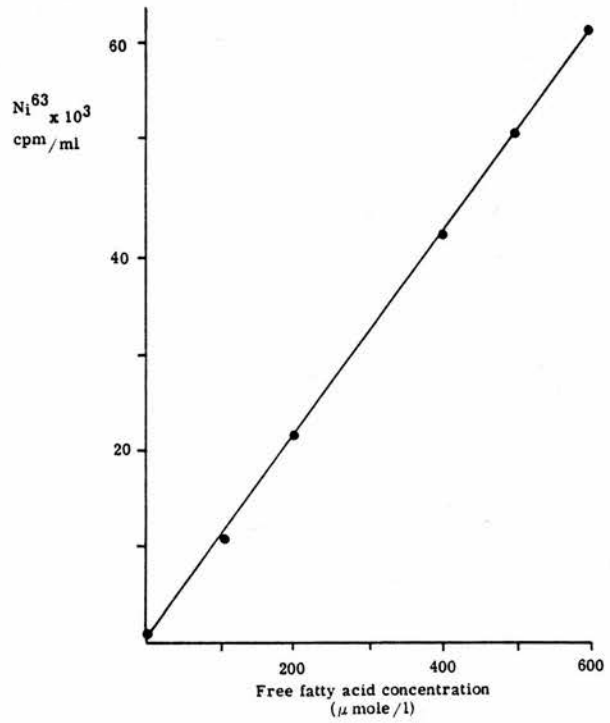
The method was calibrated using palmitic acid standards dissolved in redistilled heptane, of which 3 ml were taken through the extraction procedure. The concentrations were 0-600 μmole/l.

A representative calibration graph is shown in Fig 2.07.

Calculations

The fatty acid concentrations of the unknown samples were read from the hand-drawn calibration line. Allowance was made for the fact that only 0.75 ml blood was extracted, and usually the results were converted to plasma free fatty acid concentration by multiplying by a factor correcting for the packed cell volume (PCV) (100/(100-PCV)).

Figure 2.07. The estimation of free fatty acids in blood extracts: Calibration graph



Palmitic acid standards in heptane were 'extracted' and analysed by the formation of the Ni^{63}-salt. On the ordinate : Ni^{63} concentration in the chloroform-heptane phase.

Reagents

All organic solvents (isopropanol, heptane, chloroform, methanol) were redistilled. The composition of the Ni^{63} reagent was modified from the method of Ho (1970) to increase its specific gravity by replacing the saturated Na_2SO_4 solution with accurate solution of KBr (This had no effect on either the blank or the standards).

The following stock solutions were prepared:

Solution a. Nickel nitrate: 2 mmoles of $Ni(NO_3)_2$ and 0.8 ml of glacial acetic acid were made up to 100 ml with double-distilled water.

Solution b. KBr solution: 45 g/100 ml in double-distilled water.

Solution c. $Ni(NO_3)_2$/KBr solution:
2.5 ml solution a and 7.5 ml triethanolamine were added to 90 ml solution b.

Solution d. Working Ni^{63} reagent:
To 10 ml of solution c 0.1 ml of $Ni^{63}Cl_2$ in 0.1 mole/l HCl were added (20 μCi Ni^{63}, NBS 1, The Radiochemical Centre, Amersham). This solution is stable for at least 3 months. Two hundred μl of this working Ni^{63} reagent mixed with 9 ml of scintillant solution NE 260

gave 350 000-400 000 cpm.

The original Ni^{63} reagent (Method of Ho, 1970) was made by addition of the following stock reagents:

10 ml $Ni(NO_3)_2$ in a saturated aqueous solution of sodium sulphate

8.5 ml saturated aqueous solution of potassium sulphate

1.5 ml triethanolamine.

This solution contained 5 μCi Ni^{63}/ml.

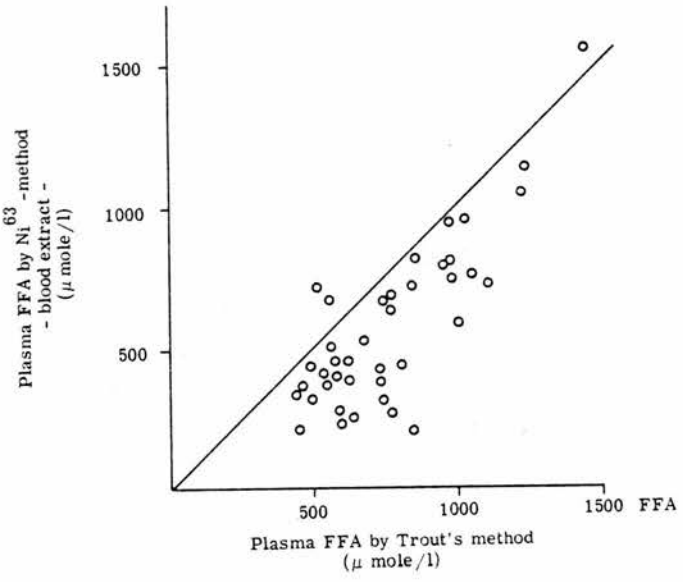
Stability of the extracts

Stability of free fatty acids in plasma or plasma extracts has been well documented by other groups, (Braun, 1971; Broechoven and Parijs, 1968) and this aspect of the method was not investigated.

Comparison with the titrimetric method

The method was compared with the standard titrimetric method of Trout. Blood samples were obtained from blood donors at the Blood Transfusion Service, Edinburgh. No heparin was administered to the donors during this procedure. The samples were collected into heparinised tubes and stored on ice. Fatty acids in each whole blood sample were extracted in duplicate and the remainder was used for the preparation of plasma. Plasma concentrations of free fatty acids were analysed in duplicate. A separate sample was collected in a small EDTA tube (2.5 ml, Stayne Laboratories, London) for the determination of packed cell volume in quadruplicate, using a Hawksley micro-haematocrit centrifuge. The concentrations of fatty acids in blood extracts were expressed in terms of plasma concentrations to allow direct comparison of the two methods. The results of the comparison are presented in Fig. 2.08 , and show that the titrimetric method overestimated plasma free fatty acid

Figure 2.08. The estimation of free fatty acids in blood extracts: Comparison with the titrimetric method of Trout



Plasma concentrations of free fatty acids were estimated in blood samples from healthy volunteers (40) using the radio-chemical method and the titrimetric method of Trout. For the radiochemical method 0.75 ml blood was extracted and the results were expressed as plasma free fatty acid concentration by multiplying by a factor correcting for the packed cell volume. Note the overestimation of plasma free fatty acid concentration by the method of Trout. For details see text.

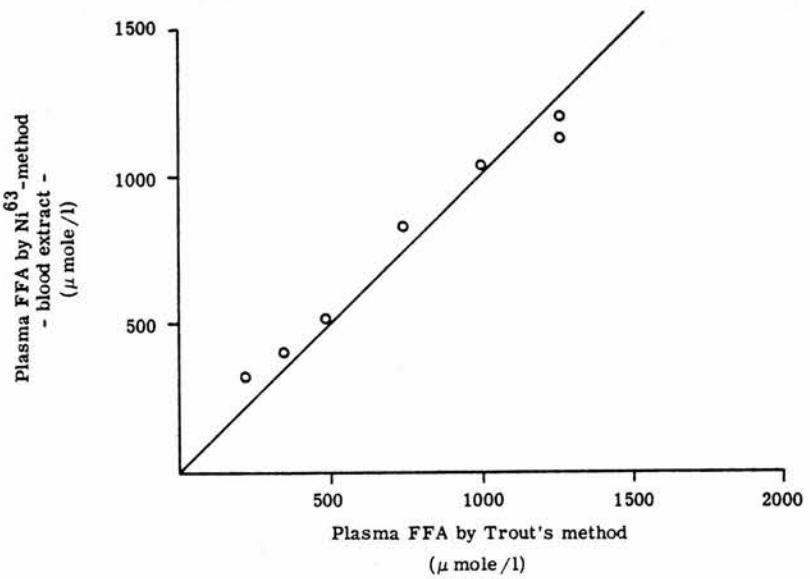
concentrations by about 300 μmole/l. This almost certainly was due to the interference of plasma phospholipids in that method, whereas in the radiochemical method phospholipids were removed by silicic acid. In line with this is the fact that the slope of the regression is not significantly different from 1.00 (line of identity).

This was further examined by treating plasma extracts of 7 volunteers with silicic acid in an identical manner to that described for the whole blood extracts, to remove interfering phospholipids. The purified extracts were evaporated under a stream of nitrogen, redissolved in heptane, and analysed titrimetrically. Both methods give similar, but not quite identical results (see Fig. 2.09), the reason for this is not clear, but could be explained by a more efficient adsorption of phospholipids from the plasma extracts due to a lower phospholipid content. Novak found that the colorimetric analysis of plasma FFA concentrations (which did not use silicic acid to adsorb phospholipids) gave similar results to the titrimetric method of Trout et al (1960).

The precision of the individual estimate

The precision of the method was assessed by repeatedly analysing a palmitic acid standard (400 μmole/l) 10 times on two occasions. The CV was 6% and 8.5% respectively.

Figure 2.09. The estimation of free fatty acids in blood extracts: Comparison with the titrimetric method of Trout using silicic acid treated extracts



Blood samples were obtained from healthy volunteers (7). Phospholipids interfering in the method of Trout were removed by adsorption with silicic acid (300 mg). Note similarity in results of the two methods. For details see text.

2.2.3. The estimation of plasma glucose

At times insufficient thought is given to the special requirements of plasma glucose methods, suitable for the determination of glucose extraction by the heart in vivo. The reason for this may be that glucose methods are considered to be well characterized. Therefore they may be accepted without considering what effect methodological errors have on the determination of arterio-venous differences of glucose across the heart. Indeed it is surprising that this problem appears to have been ignored, or at least not been published formally.

The effect of methodological errors on the determination of arterio-venous differences of plasma glucose will be considered in some detail, before describing the actual methods which were adopted with this particular purpose in mind.

Theoretical consideration on the measurements of arterio-coronary sinus differences of glucose

The effect of the precision of the estimation of glucose on that of the determination of its arterio-venous difference was studied by computer simulation using a Hewlett Packard computer Model 9821A and by theoretical consideration.

At a physiologically normal arterial glucose

concentration of 5 mmole/l the arterio-coronary sinus difference of glucose is about 0.3 mmole/l. This was used as the basis for generation of 20 random arterial (5.0 mmole/l) and coronary sinus (4.70 mmole/l) concentrations. The computer programme used (Pseudorandom numbers, Hewlett Packard Statistics Package 9820A/9821A, Programme no.0516, written by J.Gabriel) generates pseudo-numbers with a mean and a standard deviation which can be declared. Two coefficients of variation were tested: 3 and 1% (the former is frequently published for manual methods, and the latter can be achieved using Autoanalyzers). From the generated "arterial" and "coronary sinus" glucose concentrations arterio-coronary sinus differences were computed and were sequentially put into 4 groups, each comprising 5 replicate determinations and the mean was determined for each group. The results (mean $\pm$ SD) are presented in Table 2.03.

The arterio-venous difference of the 4 groups ranged from 0.15 - 0.43 mmole/l (CV = 3%) or from 0.28 - 0.32 mmole/l (CV = 1%). The means of these 4 average arterio-venous differences, were 0.31 $\pm$ 0.25 and 0.31 $\pm$ 0.07 mmole/l. The means are not different from one another, or from the the theoretical value of 0.3, but the standard deviations are. If we accept the mean of the 4 calculated standard deviations (which is

Table 2.03

Effect of precision in glucose measurement on the determination of an arterio-venous difference of glucose concentration of 0.3 mmole/l

Arterial and venous glucose concentrations were simulated using a computer programme generating pseudo-random numbers. Arterio-venous differences were computed, and results expressed as the mean ± SD of 5 calculations. The arterio-coronary sinus difference was also determined using normally-distributed random numbers and the theoretically-expected standard deviations.

Arterio-coronary sinus difference

Calculation	Data by computer simulation		Data from random numbers	
	CV = 3%	CV = 1%	CV = 3%	CV = 1%
1 (n=5)	0.38 ± 0.26 (127%)*	0.28 ± 0.09 (92%)	0.25 ± 0.13 (83%)	0.28 ± 0.04 (93%)
2 (n=5)	0.26 ± 0.18 (88%)	0.32 ± 0.09 (105%)	0.28 ± 0.17 (93%)	0.29 ± 0.06 (97%)
3 (n=5)	0.15 ± 0.24 (50%)	0.31 ± 0.06 (102%)	0.50 ± 0.28 (167%)	0.37 ± 0.09 (123%)
4 (n=5)	0.43 ± 0.32 (144%)	0.31 ± 0.03 (103%)	0.28 ± 0.14 (93%)	0.29 ± 0.05 (97%)
Mean‡	0.31 ± 0.25 (102%)	0.31 ± 0.07 (101%)	0.33 ± 0.18 (109%)	0.31 ± 0.06 (103%)

( )* Mean computed arterio-venous difference, expressed as a percentage of the theoretical arterio-venous difference of 0.3 mmole/l.

‡ Expressed as the mean of the calculated arterio-venous differences ± the average SD.

doubtful on small numbers), one can predict that the coefficient of variation of estimating arterio-coronary sinus differences using a glucose method with a CV = 3% will be about 80% ($\frac{0.25}{0.30}$ x 100%).

A much more acceptable figure for this will be obtained with a more precise method (CV = 1%): but it will still be about 20% ($\frac{0.07}{0.30}$ x 100%).

Similar results were obtained by calculation of the standard deviation of the arterio-venous differences using the equation $\delta a\text{-}cs = \sqrt{(\delta a)^2 + (\delta cs)^2}$ where δa, δcs, $\delta a\text{-}cs$ is the standard deviation of arterial, coronary sinus and of arterio-coronary sinus difference respectively (Scientific Tables, Documenta Geigy, 7th Ed.).

The standard deviation of the arterio-coronary sinus difference estimated using a method with a CV of 3% and 1% is 0.2058 ($= \sqrt{(0.15)^2 + (0.141)^2}$) and 0.0686 ($= \sqrt{(0.05)^2 + (0.047)^2}$) respectively. The CV of the arterio-coronary sinus difference is thus about 70 or 20% depending on the coefficient of variation of the glucose estimations.

Using these calculated standard deviations random arterio-coronary sinus differences can be derived from tables of random normal deviates (Nixon and Massey, 1969). These tables provide random, normally distributed data

with a known mean and standard deviation. The random numbers were transformed to data with the required mean arterio-coronary sinus difference of 0.3 and a standard deviation of 0.2058 or 0.0686.

The average arterio-coronary sinus difference of 5 consecutive estimations was then calculated (Table 2.03). Like in the simulation experiment the data shows that although the mean of the calculated arterio-coronary sinus differences, based on data with a CV = 3%, is not different, the variability is higher.

It is clear therefore that a method with high precision is required, since the alternative of analysing many replicates is not possible due to the limited amount of blood available.

In establishing the finally adopted method improvements in CV % of the method have played an important, at times dominating role.

At the beginning of this project an automated method of using glucose oxidase had been established for studies on glucose turnover in man and glucose utilisation in the perfused rat heart in collaboration with Drs. Nimmo and O'Brien, Department of Biochemistry, University of Edinburgh. It was therefore only natural to adopt their method as the basic method. The stability of the reagents, optimal pH and reaction time etc were not only well known, but had been thoroughly tested over a period of years. We will quote here the relevant information at the time available to us. For full details the reader is referred to the thesis of O'Brien (1969).

The method of O'Brien and its modifications

O'Brien (1969) developed his method for glucose utilisation studies of the perfused rat heart. The principle of the method is the enzymically catalysed oxidation of β-glucose using glucose oxidase (EC 1.1.3.4.):

$$\beta\text{ - D (+) glucose} + O_2 \longrightarrow \text{D - glucono-5-lactone} + H_2O_2$$

Hydrogen peroxide is quantified by the indicator reaction catalysed by peroxidase (EC 1.11.1.7), in which a compound from gum guaiacum is oxidized to a product absorbing at 625 nm:

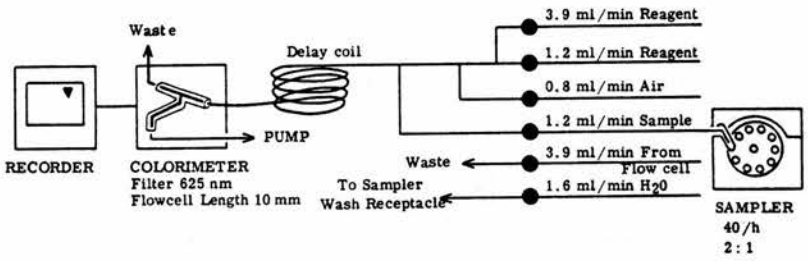
$$\text{gum guaiacum} + H_2O_2 \longrightarrow \text{oxidized gum guaiacum} + H_2O$$

The reaction was carried out using an Autoanalyzer. The flow diagram of the system is shown in Fig 2.10. Standards and unknown samples were analysed in palindromic sequence, in order to nullify the influence of any regular change in the baseline (O'Brien, 1969).

Calibration of the method

Glucose standards (concentration 0 - 25 μg/ml) were prepared according to O'Brien (1969), except that an aqueous solution saturated with benzoic acid was used. The justification for the inclusion of the bacteriostat was shown in an experiment summarised in Table 2.04.

Figure 2.10. AAI method for glucose/glucose oxidase: Manifold



The composition of the reagent is presented in the text.

Table 2.04

The effect of benzoic acid on the estimation of glucose

Standards were prepared in distilled water according to O'Brien (1969) or in a solution saturated with benzoic acid and analysed using an Autoanalyzer. The enzyme reagent contained 10 ml Fermcozyme and 10 mg peroxidase per litre.

The value of the standards was also calculated back as unknowns using the aqueous standards for calibration.

Standards		Aqueous standards		Benzoic acid standards	
		O.D.	Concentration	O.D.	Concentration
mg/100 ml	mmole/l		mmole/l		mmole/l
50	2.78	.162	2.80	.162	2.80
		.160	2.78	.163	2.80
100	5.55	.292	5.55	.288	5.50
		.296	5.63	.292	5.55
150	8.32	.410	8.33	.415	8.44
		.413	8.38	.411	8.33
200	11.10	.503	11.05	.502	11.05
		.504	11.10	.500	10.99

Reagent

Stock gum guaiacum in sodium acetate buffer was prepared according to O'Brien (1969).

The properties of this stock dilution vary with different batches of the resin. O'Brien found that the relation of glucose concentration with extinction was only linear after the stock solution had been left for 1 to 10 days at room temperature. Therefore the stock solution was always left for 14 days before use.

The enzyme reagent (1 litre), containing 5 ml Fermcozyme (Hughes and Hughes Ltd., Brentwood, U.K.) and 5 mg horse radish peroxidase (Hughes and Hughes Ltd., Brentwood, U.K.) was made the day before use and left overnight in a fridge. The enzyme concentrations were modified from the original method in order to obtain a linear relation between extinction and glucose concentration up to 11.10 mmole/l (200 mg/100 ml), equivalent to 25 ug/ml in Autoanalyzer cup.

Preparation of plasma samples

Blood samples were collected as described in Section 2.2.2. and immediately transferred to plastic fluoride-oxalate tubes (1 ml), cooled on ice. Blood cells and plasma were separated by centrifugation (10 min at 1500 g) in a cooled (4^{o}C) MSE 4L centrifuge

within 15-30 min. Plasma was then transferred to a clean plastic tube and stored at -20°C until analysed. Under these conditions plasma samples are stable for at least 128 days (Nimmo, personal communication).

Originally plasma samples (25 µl) were deproteinised with perchloric acid and neutralised with K_2CO_3 according to the method of Nimmo (1970). It was noted that this ultra-micro method consistently overestimated plasma glucose concentrations by about 10%. The coefficient of variation was also considered slightly too large (1.2%). Therefore this approach was abandoned. Plasma samples (50 µl) were diluted with 4 ml distilled water, saturated with benzoic acid, acting as a bacteriostat.

Special attention was paid to factors causing variability of the results. Oxford pipettors can be used in two ways: dispensing with the 'forward' or with the 'reverse' technique. In the forward technique, which was originally recommended by the manufacturer, the exact quantity of a sample is sucked into the pipette tip, and then expelled using an excess of air to displace it. Thus in this manner the volume of the sample sucked into the pipette is equal to the volume of displaced air prior to sampling.

On the other hand one can suck as much as possible into the pipette tip by maximal displacement of air. The required volume can then be dispensed by displacement of the exact volume of air, leaving some sample in the tip

behind. Since in this fashion the order of moving the plunger is reversed to that as originally recommended, it has been called the 'reverse' technique.

It was found that the method of pipetting protein free samples was unimportant, both in regard to mean quantity dispensed and precision of dispensing. However this did not hold for plasma samples. Pipetting of small quantities (50 µl) was checked using a plasma pool to which potassium chromate was added. Plasma samples were then dispensed using the forward or reverse technique into plain plastic tubes, containing 4.0 ml double-distilled water. The extinction (320 nm, light path 10 mm) was measured using a Pye Unicam SP 1800 Ultraviolet spectrophotometer and the results were recorded on a Pye Unicam AR 25 linear recorder. Using the forward technique more plasma was dispensed as indicated by the higher extinction (see Table 2.05). The precision of the forward technique was 1.1% and of the reverse technique 0.7% (n= 10) and could be further improved when a fresh tip was used with the reverse technique CV= 0.5% (n= 10).

Pipetting was also checked by weighing and the variability was smallest when the 'reverse' technique with a fresh tip each time was used.
The results are tabulated in Table 2.05.

Therefore in the finally adopted method the reverse technique with a fresh tip for each pipetting was used.

Table 2.05

The effect of pipetting on the CV of glucose estimation

Canine plasma pool was repeatedly pipetted using the 'forward', 'reverse' and the 'reverse' technique with a fresh tip each time and the amount dispensed estimated by extinction measurement (plasma + chromate) or weighing. For further details see text (n = 10).

pipetting technique	Extinction	Weight mg
'Forward'	1.016 ± 0.011 CV= 1.1%	49.6 ± 0.4 CV= 0.9%
'Reverse'	0.951 ± 0.007 CV= 0.7%	48.6 ± 0.3 CV= 0.6%
'Reverse + fresh tip'	0.946 ± 0.005 CV= 0.5%	49.0 ± 0.1 CV= 0.2%

Calculation of plasma glucose concentration

The percentage transmissions of standards and unknown samples were read from the recorder charts, and were converted to optical densities. A line y= a + bx was fitted to the data of the standards, using a least square line fit computer programme, and the optical densities of unknown samples were then converted to plasma glucose concentrations using the coefficients of the calculated line. All calculations were made using a computer programme, written by Dr J.O'Brien, Department of Biochemistry, University of Edinburgh, for a desk top computer (Advanced Programmable Calculator 700 B, Wang Laboratories Inc., Mass., USA).

Precision and accuracy of the glucose oxidase method

A canine plasma pool was repeatedly analysed (mean ± SD): 6.98 ± 0.03 mmole/l (CV = 0.4%, n= 20). Recovery of added glucose 2.78 mmole/l was 96.5 ± 1.5% (n= 4).

Quality control samples (Wellcome Laboratories Ltd., Beckenham, Kent), assigned value 5.93 mmole/l were analysed during each run. In 11 consecutive runs plasma glucose was (mean ± SD): 5.65 ± 0.11 mmole/l.
The mean glucose concentration is 5% lower than the specified value for automated glucose oxidase methods and is most likely caused by unidentified reducing agents in the quality control sample or by an error in the assigned value.

The measurement of plasma glucose in haemolysed samples

In FFA infusion experiments using the blood cell separator (Part 5) haemolysis, caused by the blood cell separator was observed. Using the glucose oxidase method lowered plasma glucose concentrations were found. The reason for this is most likely a reduction in H_2O_2 (formed by glucose oxidation) by glutathione, released from haemolysed red blood cells. Interference of glutathione can be eliminated by preincubation of samples with 0.25 mole/l sodium carbonate (Technicon, Clinical method 02, 1972). However, despite this precaution, artificially low results were still observed, and may be due to the oxidation of haemoglobin to methaemoglobin by H_2O_2 (Makarem, 1974). Thus incorporation of a dialysis step in the manifold for plasma glucose seemed inevitable for haemolysed samples despite the fact that the incorporation of a dialyser would certainly lead to more practical problems and decreased precision. Two alternatives were considered : purchase of a dialyser unit for the existing method (glucose oxidase) or purchase of the newly developed cartridge for the measurement of glucose using hexokinase and glucose-6-phosphate dehydrogenase, yielding NADPH as the end point. The advantage of the latter method would be the increased specificity due to hexokinase/glucose-6-phosphate

dehydrogenase. The disadvantage was obviously capital expenditure and higher running cost. The factor which pushed the balance towards purchase of the new system was the improved performance of the new chemical cartridges, including miniature mixing coils, heating bath and dialyser unit. This improved performance expresses itself in diminished sample-to-sample interaction, despite higher sampling rates. NADPH formed on oxidation of glucose-6-phosphate is quantified using a double-beam colorimeter at 340 nm. The output potential is proportional to optical density.

Other automated hexokinase methods (Bernt and Lachenicht, 1970) have used fluorimetry for the determination of NADPH (CV $\pm$ 2%), but despite the lower sensitivity of the colorimeter in the present method colorimetry was prefered for its stability. Manual methods using hexokinase were really never considered due to their lack of precision.

Glucose by hexokinase/glucose-6-phosphate dehydrogenase

The principle of the method is as follows: Glucose is phosphorylated using ATP, a reaction catalysed by hexokinase (EC 2.7.1.1.):

glucose + ATP $\longrightarrow$ glucose-6-phosphate + ADP

and glucose-6-phosphate is oxidized in the presence

of $NADP^+$ by glucose-6-phosphate dehydrogenase (EC 1.1.1.49) to 6-phosphogluconate and NADPH:

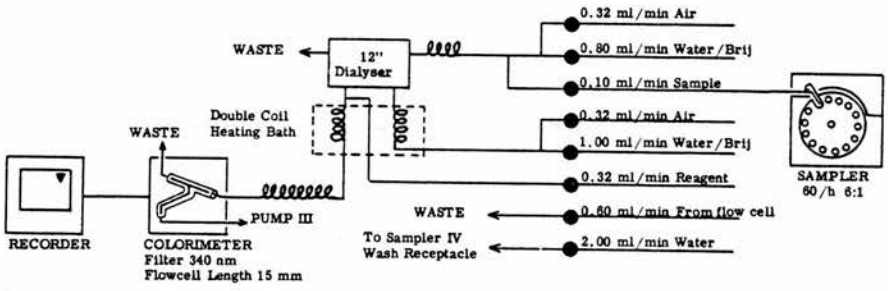
$$\text{glucose-6-phosphate} + NADP^+ \longrightarrow \text{6-phosphogluconate} + NADPH+H^+$$

The pH is optimal at 7.5 - 7.6. The equilibrium of the indicator reaction is moved far to the right, and this reaction drives the phosphorylation of glucose by continuous removal of glucose-6-phosphate.

Although hexokinase catalyzes also the phosphorylation of fructose and mannitol, the specificity of the method is ensured by the specificity of glucose-6-phosphate dehydrogenase for glucose-6-phosphate (Bergmeyer et al, 1970). This sequence of reactions is achieved using a manifold as depicted in Fig 2.11.

The sample is added to an air-segemented stream of distilled water. After mixing it passes through a 12 inch dialyser where some of the glucose is dialysed into an air-segmented, concurrent recipient stream of distilled water. The enzyme reagent, containing hexokinase, glucose-6-phosphate dehydrogenase, ATP and $NADP^+$ is then added to the recipient stream and incubated at about 37^oC. The absorbance at 340 nm is measured using a double beam colorimeter.

Figure 2.11. AAII method for glucose/hexokinase: Modified manifold



The official Technicon cartridge (No. 933-0453-01) was modified to increase the sensitivity of the method by replacement of the 3 inch dialyser by a 12 inch dialyser. The composition of the reagents is presented in the text.

Establishment of an automated glucose method using hexokinase

The Autoanalyzer system was tried as recommended by the manufacturer (AA II method for glucose/hexokinase, Technicon International Div., 1976). Under these conditions a linear response for glucose concentrations up to 27.75 mmole/l (500 mg/100 ml) is claimed. We wanted to check this for our working range (4-8 mmole/l) with standards ranging from 0 to 10 mmole/l . At this time the Technicon enzyme reagent was not available. Although the concentration of hexokinase, glucose-6-phosphate dehydrogenase, NADP and ATP were known, the nature and the strength of the buffer and the $MgSO_4$ concentration were not (classified information). We therefore prepared our own enzyme reagent on the basis of the available Technicon information, but the buffer system (0.3 mole/l triethanolamine with 4 mmole/l $MgSO_4$, pH 7.6) was taken from another automated method (Bernt and Lachenicht, 1970). The change in optical density was very small: equivalent to 0.090 O.D. units for the 10 mmole/l standard. Increasing the concentration of ATP, NADP and the enzymes did not increase the peak height, excluding the nature of the enzyme mixture as the cause for small peaks. In the mean time we had learned from the manufacturer that our response was similar to that

usually found by others.

Peak height could be electronically enlarged, but it was felt that this would be compensating for the bad chemistry and would not lead to high precision. Only a small amount of glucose is dialysed (± 3%) and substitution of the 3 inch dialyser by a 12 inch dialyser should lead to greater sensitivity, possibly with more sample to sample interaction.

Replacement of the dialyser resulted in a 3 fold increase in peak height: ΔO.D. of 0.270 for 10 mmole/l standard.

The precision of the method was assessed on two occasions by sequentially analyzing 20 samples of a canine plasma pool of 5.8 mmole/l glucose. In this way sample to sample interaction as a source of variability is excluded, especially since the first peak was ignored. The coefficient of variation was rather high 1.4% and 1.3% (n= 19).

A continuous aspiration of a glucose standard did not result in a steady reading, but in a reproducible oscillating trace with a cycle of about 4 min. The flow rate through the system was constant, and therefore variations in flow (surging) could not explain this phenomenon.

It is known that the dialysis is variable if the donor and the acceptor fluid segments are travelling

through the dialyser at slightly different rates. Only maximal and constant dialysis can be obtained, when the fluid segments are exactly opposite each other and remain in phase. The flow in the donor and receptor stream was matched by calibration and selection of pump tubes. The phasing of the fluid segments was achieved by shortening the transmission tube, connecting the mixing coil with the dialyser. Despite this the oscillating trace remained, leaving variability of the temperature as its only possible cause.

The temperature in the heating bath was checked using a digital thermometer (DU-3, Ellab A/S, Copenhagen, Denmark). A variation between 37.0 and 37.6°C was found with a periodicity of ± 4 min. The stability of a replacement bath was checked and found to be virtually constant temperature variations between 35.5 and 35.6°C (only one decimal point could be recorded). A steady trace was found during constant aspiration of a sample. The reproducibility also improved: coefficient of variation of 0.4% (n= 19). A noticeable drift up was observed in the peak heights over a period as little as 20 min (0.3%/hour). Sample-to-sample interaction was examined using standards. Standards were analysed in palindromic sequence (0, 2, 4, 6, 8, 10, 10, 8, 6, 4, 2, 0 mmole/l). Peak heights obtained during the descending

part of the palindrome were higher than those measured in ascending order; normally indicative for carry-over. Carry-over is less with lower sampling-rates. Therefore standards were analysed in palindromic sequence with different sampling rates and sample-to-wash ratios: 60 samples/hour; sampling time 6 parts wash 1 part (60 6:1), 50 6:1 and 50 8:1. Carry-over expressed as a percentage of the difference in concentration between two adjacent peaks was less with 50 6:1 or 50 8:1 than 60 6:1 : 0.7-0.8 vs 0.9% respectively. However during these experiments we observed that in addition to the earlier baseline drift the sensitivity of the method also increased with time. Therefore the observed 'carry-over' could partly be explained by the increase in sensitivity with time. It became apparent that for highest precision corrections were necessary for baseline and sensitivity drift, as well as for sample-to-sample interaction (see Calculations of results, pp 150-154). We therefore decided to study whether different sampling rates were associated with different degrees of precision, in a fashion which eliminated effects of baseline and sensitivity drift (appropriate corrections were made). The results are summarized in Table 2. 06 . No major differences were found. Therefore in the finally adopted method the higher sampling rate was preferred for economic reasons.

Table 2.06

Plasma glucose by hexokinase

The effect of sampling rate and sample-to-wash ratio on the precision (CV %) of the method. Canine plasma samples were repeatedly (n= 20) analysed using different cams used for the timing of the sampler. Two observations were made using each cam. Results were corrected for baseline and sensitivity drift.

CAM*	Precision %
50 5:1	0.14 , 0.26
50 6:1	0.13 , 0.32
60 6:1	0.23 , 0.24

* Sampling rate per hour; sample-to-wash ratio.

Reagent mixture

The hexokinase reagent mixture was purchased from Technicon (Catalogue number T 11-1049-56) and stored in a fridge until used. The reagent is provided in a lypophilized form. Immediately before use it was reconstituted by addition of 80 ml double-distilled water. The mixture was stored in an ice bath, in which it is kept during the analysis, during the working day (Temperature 0 - 0.5°C).

The concentration of the active ingredients were:

NADP	0.67	mmole/l
ATP	0.40	mmole/l
Hexokinase	1 000	U/l
G-6-P DH	1 000	U/l

The nature of the buffer (pH 7.5) is unknown. For some experiments (interference of glucose-6-phosphate) the enzyme reagent was made up with or without hexokinase. The concentrations of the active ingredients were:

NADP	0.67	mmole/l
ATP	0.40	mmole/l
Hexokinase	1 000	U/l (if required)
G-6-P DH	1 000	U/l

The buffer was triethanolamine 0.3 mole/l, 4 mmole/l $MgSO_4$, pH 7.6. The reagent was immediately used and kept on ice. A 0.03% (w/v) Brij-35 solution (Technicon Chemicals, Belgium) was used as the diluent.

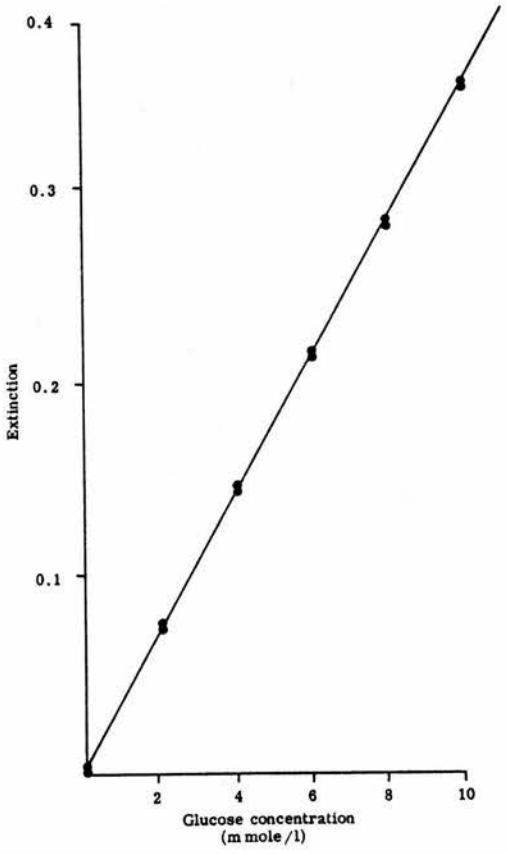
Standards

Standards (0-10 mmole/l) were prepared in a bovine albumin (fraction V) solution, (55.55 g/l). The concentration of albumin was selected on the basis of the average canine plasma albumin concentration and the albumin/total protein ratio. The reason for inclusion of albumin is the fact that rate of dialysis is claimed to be protein dependent (AA II method for glucose/ hexokinase, Technicon International Div., 1976). Batches of albumin were checked for glucose contamination prior to use. No glucose was found. Sodium azide (0.1 g/l) which did not affect peak heights was included as a bacteriostat. Aliquots were frozen at -20°C.
A typical calibration graph is shown in Fig. 2.12.

Calculation of results

Baseline drift during the run was recorded and assumed to be constant. Sensitivity drift was assessed as the difference in slopes of calibration graphs analysed in the beginning and at the end of the run. The change in sensitivity was assumed to increase linearly with time. Carry-over was determined as follows: a drift standard

Figure 2.12. AAII method for glucose/hexokinase: Calibration graph



Peak heights were converted into their corresponding optical density readings using a Technicon calibration chart. The plotted optical density measurements were corrected for drift and carry-over. Standards made in bovine albumin (55.55 g/l).

was analysed three times in a row. It was assumed that the sample-to-sample interaction is constant after the second peak. The height of the 3rd peak (R3; see Fig 2.13) is the sum of the true peak height h plus the contribution (c) due to carry-over. The value of c is also equal to the difference between the observed baseline and the baseline which would have been observed without carry-over (see Fig 2. 13). The factor which characterizes carry-over is expressed as:

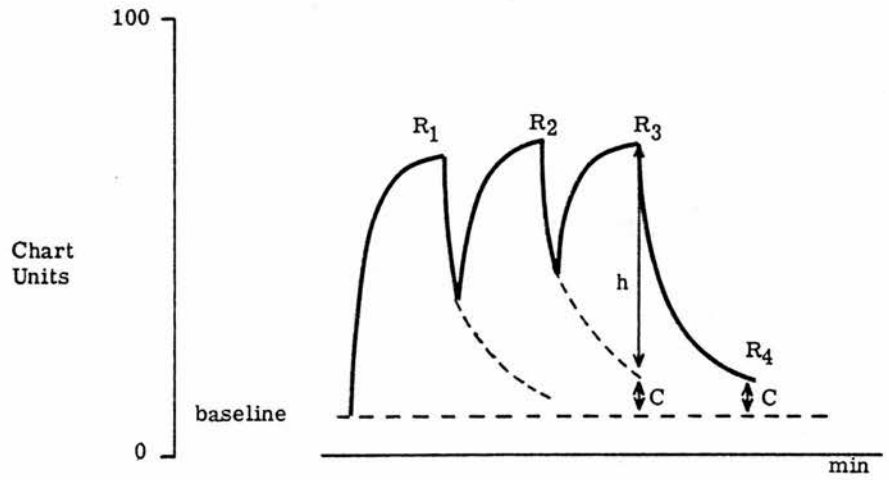
$$= \frac{R_4 \text{ - baseline}}{(R_3\text{-baseline})-(R_4\text{-baseline})} \quad \text{or}$$

$$= \frac{R_4 \text{ - baseline}}{R_3 - R_4}$$

The magnitude of this factor is considered to be a constant both with respect to time and to the difference in peak height between samples.

A computer programme was made for the Hewlett Packard Computer Model 9821A in which the above 3 corrections were applied. The effect of carry-over corrections was assessed by analysis of two drift standards 4 and 6 mmole/l respectively inserted after every 10 samples in a normal run with unknown samples. The coefficient of variation, without correction was 1.8 and 2.0% respectively. Much

Figure 2.13. AAII method for glucose/hexokinase: Carry-over corrections



This is a hypothetical trace to illustrate the method used for the calculation of the sample-to-sample interaction (carry-over). True peak height = h; contribution in peak height due to carry-over = c, R_{1-4} are the uncorrected chart readings. During repeated aspirations of the same sample c is assumed to be constant after the first two peaks and is equal to R_4—baseline. The mathematical equation expressing the carry-over factor is presented in the text.

more acceptable results were obtained after correction: 0.35 and 0.50%.

Interference in haemolysed samples

Haemolysis can theoretically cause interference in the glucose by hexokinase method by releasing

- compounds which yield NADPH, directly or after conversion of an endogenous substrate
- compounds which oxidize the formed NADPH
- compounds which absorb light at 340 nm.

The most obvious compound in the first category is glucose-6-phosphate. However it was doubtful whether glucose-6-phosphate could be dialysed, due to the net charge of the molecule. Nevertheless interference of glucose-6-phosphate was checked. A solution of glucose-6-phosphate (5 mmole/l) was analysed using an enzyme reagent with glucose-6-phosphate dehydrogenase, but without hexokinase. Under these conditions glucose standards can not be used, since in the absence of hexokinase glucose is not converted to glucose-6-P and no peaks are observed. Therefore the response was related to glucose standards analysed after the addition of hexokinase to the reagent. On this basis only 0.46 mmole/l glucose-6-phosphate was found (9.2% of theoretical value of 5 mmole/l). This taken together with published glucose-6-phosphate concentrations of (human) whole

blood (Scientific Tables, Documenta Geigy, 1970) it is impossible for glucose-6-phosphate to interfere with glucose measurements.

It is conceivable that there are substrates which would be able to dialyse. However most substrate concentrations in red blood cells would be too low to cause serious interference, even if the enzyme reagent is contaminated with the relevant NADP utilising enzyme.

One of compounds which might utilize the formed NADPH is oxidized glutathione (Bergmeyer, 1970). However the enzyme reagent did not contain glutathione reductase, and therefore even if oxidized glutathione could dialyse no interference can be expected. Haemolysed blood showed no significant absorption at 340 nm.

2.2.4. Plasma glycerol estimation

Principle

The concentration of plasma glycerol was estimated using a modification of the fluorimetric enzymic method of Chernick (1969). The principle of the method is the phosphorylation and then dehydrogenation of glycerol. The first reaction is catalysed by glycerokinase (EC 2.7.1.30):

$$\text{glycerol} + \text{ATP} \longrightarrow \underset{(\alpha\text{-glycerophosphate})}{\text{L-glycerol-3-phosphate}} + \text{ADP}$$

The indicator reaction is catalysed by glycerophosphate dehydrogenase (EC 1.1.1.8):

$$\text{L-glycerol-3-phosphate} + \text{NAD} \rightleftharpoons \text{dihydroxyacetone-phosphate} + \text{NADH} + \text{H}^+$$

The equilibrium of the indicator reaction lies to the left, but by removal of dihydroxyacetone-phosphate and H^+ by hydrazine and alkaline conditions (pH 9.25) respectively, the reaction is shifted far to the right. NADH is quantified by fluorimetry.

This method was introduced by the late Dr B.W.Lassers in our laboratory for the measurement of glycerol in perfusates of isolated rat hearts and in plasma ultrafiltrates. The basic validation of it will not be presented, except

where modifications were introduced.

Sample preparation

Blood samples were collected and processed as described in Section 2.2.2.

Deproteinisation of plasma

In the original method glycerol was estimated in samples deproteinised by ultrafiltration. Ultrafiltrates were prepared by centrifugation of 2 ml plasma in Amicon filter cones (Amicon Ltd., High Wycombe) for 40 min at 1 400 g in a refrigerated centrifuge. The ultrafiltrates were stored at 4°C and analysed the next day.

As this method does not lend itself to the analysis of glycerol in small blood samples alternative techniques were examined. Acid deproteinisation (perchloric acid, trichloroacetic acid) was unsatisfactory, due to occasional inactivation of the assay enzymes, which could not be prevented by the inclusion of 0.02% (w/v) bovine albumin in the assay buffer.

Heat denaturation of plasma proteins proved to be satisfactory, provided plasma was first diluted and subsequently frozen. Both the effect of time and temperature on this process were examined.
In the method that was finally adopted canine plasma (250 µl) was diluted with 350 µl double-distilled water and heated for 15 min at 138°C in a stoppered tube

(nitric acid washed, bacteriological tube 50 x 6 mm, McKay and Lynn, Edinburgh). The tubes were stored overnight at - 20°C. The denatured proteins were precipitated by centrifugation for 30 min at 1 300 g.
The clear supernatant (100 µl) was used for the analysis that day.

It was possible to use smaller plasma samples (50 µl). However the procedure was tedious and only with difficulty could one single analysis be made. Therefore the methods described above using 250 µl plasma was preferred, despite its requiring more plasma.

I am grateful for the cooperation of Mr J.Simpson M.Phil., who developed the deproteinisation procedures.

Reagents

Glycerokinase, from Candida mycoderma, specific activity 85 U/mg and glycerol-3-phosphate dehydrogenase from rabbit muscle, specific activity 170 U/mg were obtained from Boehringer Corporation London Ltd.

The following stock solutions in double-distilled water were prepared using nitric acid washed glassware:

-glycine 0.2 mole/l

-$MgSO_4$ 0.3 mole/l

-hydrazine hydrate 1 mole/l

-EDTA 0.1 mole/l

Bulk buffer was prepared weekly by mixing 100, 6.06, 100 and 3.03 ml of the above stock solutions respectively. The pH was adjusted to 9.25 with NaOH (1 mole/l prepared fresh fortnightly). The bulk buffer was stored at 4°C. Working buffer was made daily by addition of NAD^+ (Grade I, Boehringer Corporation London Ltd.) and glycerol-3-phosphate dehydrogenase to bulk buffer. Final concentrations in the cuvette were:

- glycine 70 mmole/l
- NAD^+ 0.4 mmole/l
- glycerol-3-phosphate dehydrogenase 1 000 U/l
- hydrazine hydrate 340 mmole/l
- $MgSO_4$ 6 mmole/l
- EDTA 1 mmole/l

The glycerokinase/ATP reagent was prepared daily using working buffer.

Final concentrations in the cuvettes were:

- glycerokinase 50 U/l
- ATP 0.6 mmole/l

Enzymic conversion

The enzymic conversion of glycerol was directly carried out in the fluorimetry cuvettes. To each cuvette, containing 100 µl standard or deproteinised sample, was added 900 µl glycine buffer (containing NAD^+ and glycerol-3-phosphate dehydrogenase, see Reagents). Cuvettes, sealed

with Parafilm, were incubated for 25 min at 36 - 38°C in a bacteriological incubator (Townson and Mercer, Edinburgh). The fluorescence due to oxidation of endogenous glycerol-3-phosphate was determined. Phosphorylation and oxidation of glycerol was then initiated by addition of 25 µl glycerokinase/ATP (see Reagents). After a second incubation for 25 min the final fluorescence was determined.

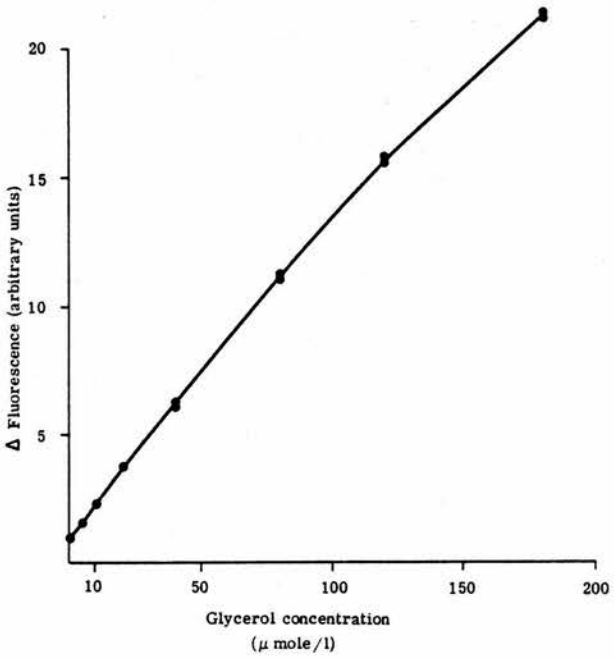
Calibration of the method

An aqueous stock standard (10 mmole/l) was prepared from glycerol (Analar, British Drug Houses, Poole). The purity of glycerol was determined by measuring its specific gravity at 20°C (Handbook of Chemistry and Physics, pp D-181, 51st Edition).

Working standards (7 concentrations, ranging from 5-180 µmole/l) were prepared from this stock standard, divided into quantities sufficient for one analytical run and stored at -20°C. Under these conditions glycerol solutions are stable for at least 1 year. On the day of the analysis the working standards were thawed out, 100 µl was analysed directly in the cuvette and the remainder discarded. Standards and blanks were analysed in duplicate.

A representative calibration graph (increase in fluorescence after the addition of glycerokinase vs glycerol concentration) is shown in Fig 2.14.

Figure 2.14. Plasma glycerol method: Calibration graph



The line ($y = a + bx + cx^2$) was calculated using a polynomial computer program.
Ordinate: Increase in fluorescence (Δ) at 415 nm after the addition of glycerokinase.

Calculation of plasma glycerol concentration

A line ($y = a+b\,x+ c\,x^2$) was fitted to the data of the calibration graph using a least squares line fit-computer programme, written by Dr J O'Brien, Department of Biochemistry, University of Edinburgh. A desk top computer (Advanced Programmable Calculator 700B, Wang Laboratories, Inc., Mass., USA) was used.

The increase in fluorescence after the addition of glycerokinase to plasma samples was not directly converted into corresponding glycerol concentration. The reason for this is that initial fluorescence readings for de-proteinised plasma samples were invariably higher than those for standards. Therefore for a given glycerol concentration the increment in fluorescence would be less for samples than for standards, due to the tailing off of the calibration graph at higher glycerol concentrations and direct conversion would lead to underestimation of plasma glycerol concentration. This is avoided if fluorescence readings (initial and final) are both expressed as 'glycerol' using the calibration graph. Plasma glycerol concentration is then taken as the difference between the two 'glycerol' concentrations.

Validation of the method

A main obstacle in the validation of the modified plasma glycerol method was the lack of a suitable plasma quality control sample, containing in addition to free

glycerol physiological levels of esterified glycerol. Plasma lipids are not stable and may hydrolyse slowly to free fatty acids and glycerol (Lindlar, 1969). Therefore our main concern was that this process might be heat-catalysed during the deproteinisation procedure. On the other hand albumin, which acts as the binding protein for the water-insoluble part of the lipids (the fatty acids), will be denatured and it is conceivable that it would therefore lose its binding capacity and hence its property to facilitate the lipolytic formation of glycerol.

The validity of the method was checked by two different and independent series of experiments. First of all the results obtained using heat-denatured, deproteinised plasma samples were compared with those obtained from plasma ultrafiltrates prepared at 4°C (Reference method). The results were similar, provided corrections were made for the loss of glycerol in the preparation of the ultrafiltrates (see Table 2.07). These data, however, should be interpreted with caution, because the recovery of glycerol using the reference method could vary considerably.

A more direct approach checking the occurrence of the hydrolysis of esterified glycerol (α glycerophosphate, β-glycerophosphate, lecithin and triolein, all from Sigma Ltd., London) was made. Both α glycerophosphate, β-glycero-

Table 2.07

Comparison of 2 plasma glycerol methods

	Glycerol (µmole/l)*	
Pool	Ultrafiltration	Heatdenaturation
1	100.8 (77)	91.3 (95)
2	75.6 (99)	87.2 (103)
3	93.4 (92)	91.4 (105)
Average:	89.9	90.0

* The pools were analysed in triplicate, using ultrafiltrates (Reference method) and heat-denatured deproteinised samples. Recovery of glycerol, presented in brackets, was determined on separate aliquots. The glycerol results were corrected for recovery: observed value x (100/recovery).

phosphate, lecithin and triglycerides were stable during the heat-denaturation procedure.

The recovery of 50 nmoles glycerol added to 1 ml canine plasma was 104% (mean ± SD: 52.0 ± 1.0 nmoles; n=7). The recovery was slightly, but significantly higher than 100% ($p < 0.01$, Student's t-test).

On the basis of these findings on accuracy, lack of hydrolysis of esterified glycerol and glycerol recovery, the heat-denaturation deproteinisation procedure was adopted for the analysis of plasma glycerol concentrations.

The recovery was a marked improvement upon the original method (ultrafiltration). In that technique varying amounts of distilled water adhering to the Amicon cones caused dilution of the ultrafiltrates ('loss' of glycerol). Even with much care the mean recovery was 89.5 ± 12.3%, giving a CV of 14%.

Quality control of the plasma glycerol methods

Both in the original method, using ultrafiltrates, and in the modified method (heat-denatured samples) the performance of the enzymic conversion was always checked. For this glycerol quality control samples at a concentration of 10 and 100 µmoles/l, made in double-distilled water and stored in aliquots at -20°C, were analysed during each run. The increase in fluorescence after the addition of glycerokinase was remarkably constant over long

periods. The results obtained from one set of quality control samples analysed over a 9 month period are summarised in Table 2.08. The mean glycerol concentration (± SD) of the low quality control sample was not statistically significantly different from the theoretical values, but the high quality control sample gave results which were slightly, but significantly too low ($p < 0.01$, Student's t-test).

For the monitoring of glycerol-3-phosphate dehydrogenase a quality control sample was prepared using L-glycerol-3-phosphate in double-distilled water and stored at -20°C.

The results are presented in Table 2.08.

Recovery of plasma glycerol

The recovery of glycerol added to plasma samples prior to deproteinisation was determined on some randomly selected samples during each run. The mean ± SD recovery recorded over a 9 month period (same as above) was

98.7 ± 3.9% (n=31), CV= 4.0% (see Table 2.08)

The precision of the individual estimate

The precision of the individual estimate was analysed by repeated analysis of a canine pool,
mean ± SD : 92.1 ± 1.3 (n=7) µmole/l, CV= 1.4%.

Table 2.08

Quality control of plasma glycerol method

Sample	Value		
	Theory	Observed (mean ± SD)	CV
glycerol(μmole/l)	10	10.1 ± 0.4 (n=40)	4.0%
	100	97.9 ± 2.5 (n=32)	2.6%
Recovery*(%)	100	98.7 ± 3.9 (n=31)	4.0%
L-glycerol-3-phosphate (μmole/l)	-	126.9 ± 5.1 (n=23)	3.0%

* Samples were deproteinised by heat denaturation.

2.2.5. The estimation of plasma lactate concentration

Principle:

Plasma lactate concentrations were determined using an enzymic fluorimetric method (Passonneau, 1970). Lactate dehydrogenase (L-lactate: NAD oxidoreductase, EC 1.1.1.27) is used to catalyze the reaction:

$$\text{L-lactate} + NAD^+ \rightleftharpoons \text{pyruvate} + NADH + H^+.$$

The equilibrium lies to the left; however, the reaction can proceed quantitatively to the right provided the end products of the reaction are removed: pyruvate as pyruvate hydrazone, and H^+ by selection of a pH of 10 of the reaction mixture. The NADH formed is measured fluorimetrically.

Sample preparation

Initially, blood was collected into a heparinised tuberculin syringe (1 ml) and immediately transferred into 4 micro haematocrit capillary tubes. The tubes were quickly sealed and centrifuged for 1 min in a micro-haematocrit centrifuge (Hawksley, London) at room temperature. The plasma freed from red blood cells was recovered by breaking the tube, and transferred to a small plastic conical tube (Beckman, Glenrothes, Fife), frozen and stored at -20°C until analyzed (within one

week of collection). More recently, blood was centrifuged in the small conical tubes described above in a Jobling micro centrifuge Model 320 (Townson and Mercer, Edinburgh) until full speed (10 000 rpm, 9 000 g) was reached (10 s). Plasma was then immediately transferred to another tube, frozen and stored at -20°C (as above). The procedure (i.e. sampling until plasma was separated) normally took $\frac{3}{4}$ min and during this time a small change in lactate concentration was found. This was also checked by delaying the processing of blood samples (Table 2.09).

On the day of the analysis, plasma samples were allowed to thaw on the bench. Plasma samples (50 µl) were diluted with 2.45 ml fresh double distilled water using Oxford pipettes, mixed and 100 µl of the diluted sample was used for the analysis. A higher dilution was used (50 µl plasma plus 4.95 ml double distilled water) when plasma samples with high lactate concentrations were processed.

The enzymic conversion of lactate

The enzymic conversion of lactate to pyruvate and NADH was carried out directly in the fluorimetric cuvettes. To 1 ml incubation buffer 100 µl of diluted samples (unknown and standards) were added, and the initial fluorescence was determined. The fluorimetric equipment and procedures were identical to those described in

Table 2.09

Plasma lactate estimation: The effect of time delay in processing ofblood samples on the plasma lactate concentration

A canine blood sample (10 ml) was collected and plasma was obtained by short centrifugation with a delay as indicated.

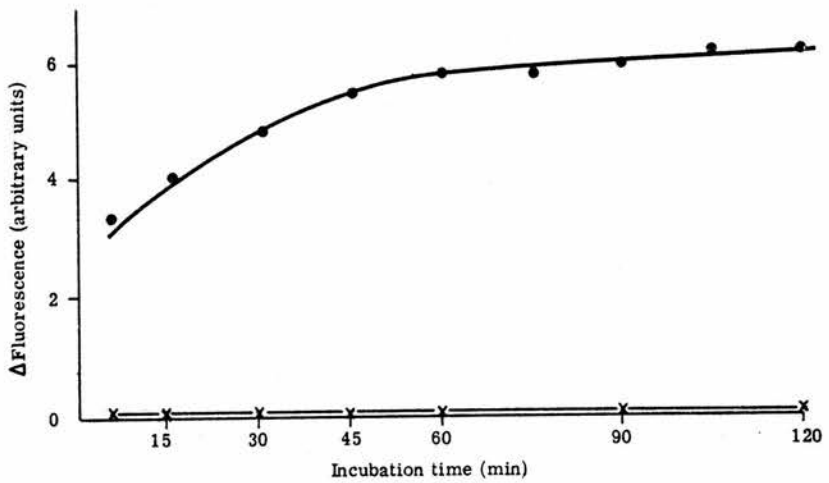
Delay (min)	Plasma Lactate (mmole/l)	% of 'initial'value
0.45	1.90	100.0
0.95	1.89	99.5
1.17	1.95	102.6
1.75	2.07	108.9
2.42	2.11	111.1
3.08	2.03	106.8
3.75	2.12	111.6
4.62	2.24	117.9
5.28	2.25	118.4
6.00	2.29	120.5
6.67	2.28	120.0

Section 2.2.4. (fluorimetric analysis of glycerol). The enzymic conversion was then initiated by the addition of 20 U lactate dehydrogenase. The cuvettes were sealed with Parafilm and incubated for 60 min in a bacteriological incubator set at 37°C.

In the original method (Passonneau, 1970) the necessary incubation time for complete conversion of lactate to pyruvate was given as 20-30 min (temperature unspecified). We were unable to reproduce this. At least 90 to 105 min was necessary to reach steady fluorescence reading when the samples were incubated at room temperature (Fig 2.15). Increasing the incubation temperature to 37°C and/or doubling the amount of enzyme did not change the time course of the reaction. Passonneau had suggested that the enzyme is not very stable at high pH, and theoretically this could cause the decline in reaction rate with time as more and more enzyme would be inactivated. However even if this possibility were operational, it could not have been the major factor, since the addition of lactate to lactate dehydrogenase (incubated in buffer of pH 10 for 60 min) resulted in a marked increase in fluorescence. Increasing the hydrazine concentration resulted in increased conversion of lactate during a 45 min incubation period (37°C)(Fig 2.16).

As a result, the concentration of hydrazine in the

Figure 2.15. Plasma lactate method: Timecourse of the enzymic conversion of lactate (Original Passonneau method)

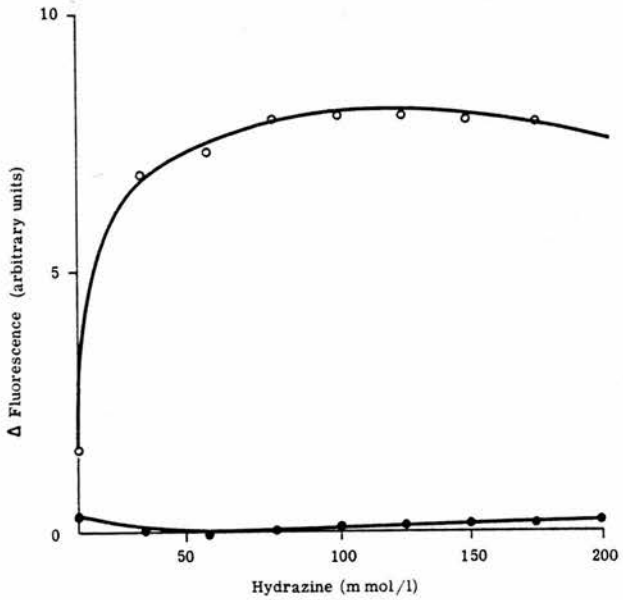


The enzymic conversion of 10 nmoles lactate was followed fluorimetrically. Cuvettes were incubated for times varying from 5-120 min at 25°C. Appropriate blanks were also carried through. Each point represents the mean of 4 observations.
Ordinate : increase in fluorescence (Δ) at 415 nm after the addition of LDH.

× ——— × blank

● ——— ● lactate standard (10 nmoles/cuvette).

Figure 2.16. Plasma lactate method: The effect of hydrazine on lactate conversion



The effect of hydrazine (final concentration 0-200 m moles/l) on the enzymic conversion of 10 nmoles lactate was analysed. The increase in fluorescence (△) after the addition of LDH and incubation of the cuvettes for 45 min at 37°C was determined (Ordinate). Each point is the average of 4 cuvettes. The appropriate blanks were also carried through.

● ——— ● blank

o ——— o lactate standard (10 nmoles/cuvette).

Note: Optimal hydrazine concentration of 125 mmol/l.

incubation mixture was increased from 50 to 125 mmole/l. Increasing the concentration of NAD was considered but in view of its price not explored.

Calibration of the method

Standards were prepared freshly from a commercial stock solution (1.00 mmole/l), Boehringer Corporation, London). A working stock solution of 1 mmole/l was prepared by diluting 100 µl of the stock solution with 100 ml double distilled water. The secondary standard was then further diluted to obtain standards with 2, 4, 6, 8, and 10 nmoles per 100 µl respectively.

A representative calibration graph is shown in Fig 2.17. The curve was calculated using the same programme as described in detail in Section 2.2.4.

The precision of the individual estimate

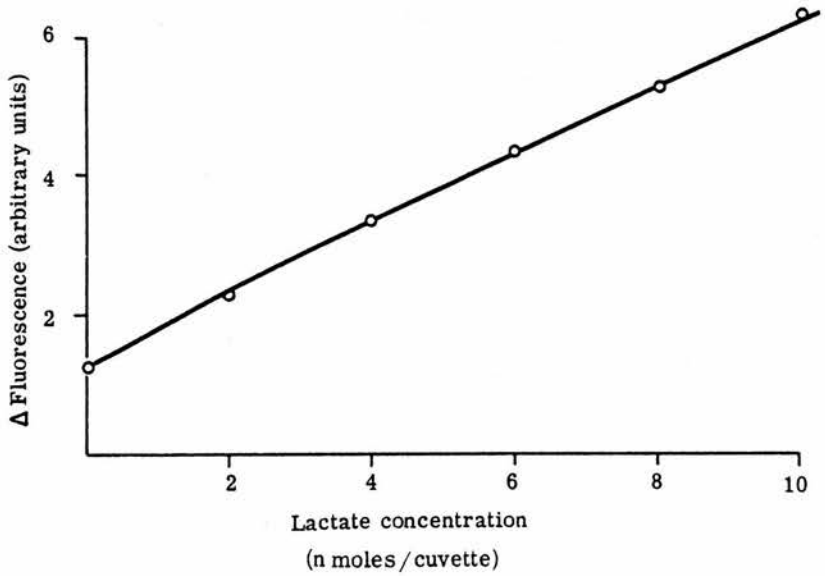
Aliquots of a frozen canine plasma pool were repeatedly analysed. The mean ± SD concentration of plasma lactate was 4.58 ± 0.11 mmole/l; the coefficient of variation ranged from 1-3.1%, mean 2.5% (see Table 2.10).

The accuracy of the individual estimate

The specificity of lactate dehydrogense ensures that only lactate is converted (Bergmeyer, 1970). The enzyme used was of the highest purity available.

A source of inaccurate results which is more difficult to assess is related to the method of collection of blood

Figure 2.17. Plasma lactate method: Calibration graph



The line ($y = a + bx + cx^2$) was calculated using a polynomial computer programme.
Ordinate : increase in fluorescence (Δ) at 415 nm after addition of lactate dehydrogenase.
Blank set at 1.0.

samples and the preparation of plasma. The increase in lactate concentrations in vitro is due to glycolysis, which takes place in the cells. Quick separation of cells from plasma is essential. Using the finally adopted method we found a small significant increase in plasma lactate concentrations of about 3%/min, since delaying the processing up to 7 min did increase lactate concentrations (see Table 2.09). However separate blood samples were collected over a short period, usually about 30 s, simultaneously from the arterial, coronary sinus and local venous catheters. Therefore the estimation of arterio-venous differences of lactate concentration should be correct.

Stability of lactate in stored canine plasma (at -20°C)

A canine plasma pool was analysed on the day of collection and the remainder was divided into 7 aliquots and stored at -20°C. There was no deterioration within 10 days (Table 2.10).

Reagents

Buffer: 2-amino-2 methyl-1-propanol (1 mole/l, 9.5 ml/100 ml double-distilled water; Sigma, London), pH adjusted to 10 with 4.2 ml HCl concentration.

Hydrazine hydrate (2 mole/l) was prepared by 1:10 dilution of 20 mole/l stock (B.D.H., Poole).

Table 2.10

Plasma lactate method: Stability of plasma lactate samples

A canine plasma pool was collected and analysed on day 0. The remainder was divided into aliquots and stored at -20°C for later analysis.

On each day samples were analysed in 5 fold. The results ± SD are presented (n= number of replicates).

Day of analysis	Plasma lactate (mmole/l)	CV %
0	4.54 ± 0.14	3.1
1	4.45 ± 0.06	1.2
2	4.66 ± 0.08	1.8
3	4.66 ± 0.06	1.2
4	4.52 ± 0.05	1.1
8	4.57 ± 0.04	1.0
10	4.66 ± 0.10	2.1
Grand mean (n= 35)	4.58 ± 0.11	2.5

Nicotinamide adenine dinucleotide (NAD) 0.1 mole/l, 70 mg/ml double-distilled water (Boehringer Corporation, London) stored in 100 µl aliquots at -20°C.

The reaction mixture finally adopted:

10 ml buffer plus 6.25 ml hydrazine hydrate and 50 µl NAD in 100 ml double-distilled water, prepared immediately before use.

Lactate dehydrogenase (Boehringer Corporation, London, pig heart, cat. no 107 042) suspension in ammonium sulphate solution, 5 mg protein/ml.

2.2.6. The estimation of plasma albumin concentration

Principle:

Plasma albumin concentrations were determined using a manual colorimetric method, based on the method of Northam and Widdowson (1967). When a solution containing albumin is added to a buffered solution of bromocresol green (3, 3', 5, 5',-tetrabromo-m-cresolsulfonphthalein, Fig 2.18) an increase in optical density at 637 nm is observed, which is proportional to the concentration of albumin.

Sample preparation

Heparinised plasma samples were prepared as described for the determination of plasma free fatty acid concentrations (Section 2.2.2.) and stored at -20°C until analysed.

Reagent

Working bromocresol green solution was prepared by addition of:

8.0 ml stock bromocresol green solution (50 mmole/l, BDH, clinical reagent)

5.08 g sodium citrate dihydrate (BDH, Analar)

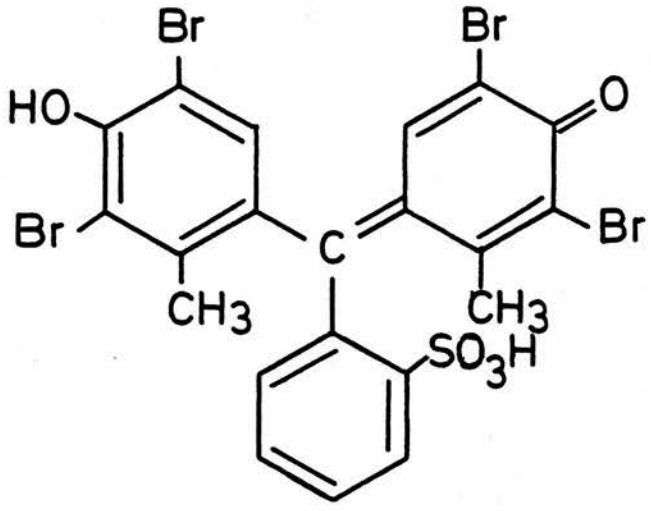
6.85 g citric acid (BDH,Analar)

0.1 g sodium azide (BDH, Analar)

1 ml 30% Brij-35 (Technicon Chemicals, Belgium).

The pH was adjusted to 3.80 using sodium citrate or citric acid (but not NaOH or HCl), and the volume made up to 1 litre using double distilled water. Final concentration

Figure 2.18. The chemical structure of bromocresol green



of bromocresol green is 0.40 mmole/l, the strength of the citric acid buffer about 50 mmole/l.

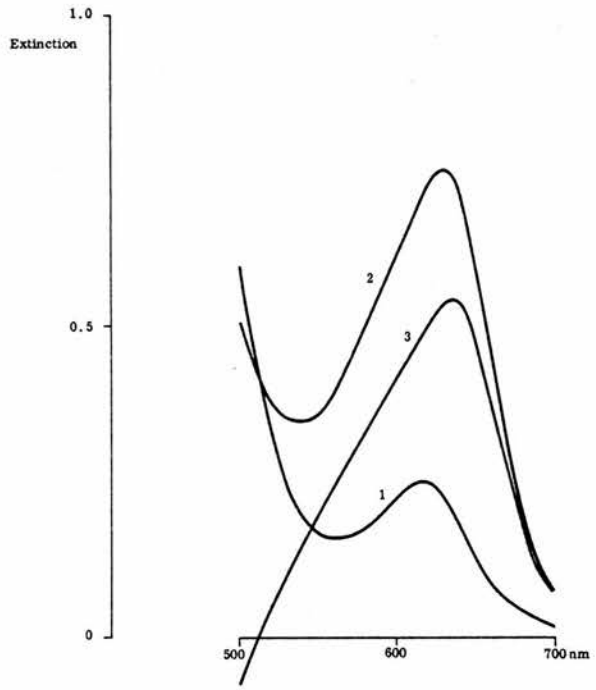
Procedure

Bromocresol green reagent (5.0 ml), dispensed using an Oxford Pipettor (Boehringer Corporation, London) was mixed with 50 µl of double distilled water (blank), albumin standard (Bovine albumin Cohn's fraction V, Sigma, London) or samples. Optical density measurements are made using an Unicam SP 1800 ultraviolet spectrophotometer (Pye Unicam, Cambridge) and recorded on an Unicam AR 25 linear recorder (Pye Unicam, Cambridge).

Spectra obtained from a blank and from a canine plasma sample are shown in Fig 2.19. It was found that albumin not only increased the optical density in the region of 580 to 680 nm, but also shifted the wave length of the absorption maximum from 620 to 630 nm. The maximal difference in optical density between samples and blanks was at 637 nm. This was the wavelength used in the finally adopted method.

The stability of the developed colour was examined and found to be stable after 5 min. Therefore optical densities were measured 5 min after albumin samples were mixed with bromocresol green reagent. Optical densities were measured using disposable plastic cuvettes (optical path 10 mm). The slitwidth was 0.2 nm.

Figure 2.19. Plasma albumin method: The spectrum of bromocresol green - albumin complex



Blank and plasma were mixed with bromocresol green reagent (for details see text) and their spectrum determined 5 min later (scanning speed 1 nm/s, path length 10 mm, reference double distilled water).
Spectrum 1: blank; 2: canine plasma (albumin concentration, 33.8 g/l). The difference between plasma and blank (in reference position) is presented as spectrum 3, note maximal difference is observed at 637-638 nm.

Calibration of the method

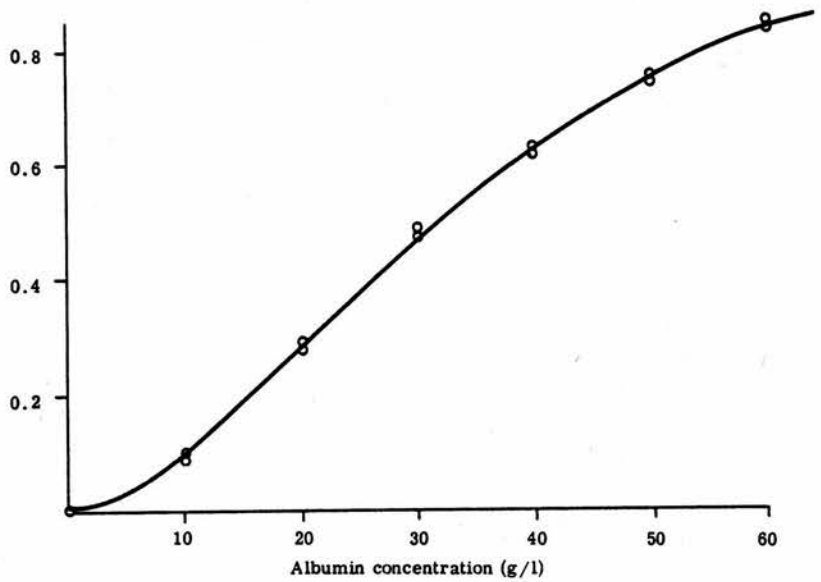
There are considerable difficulties in obtaining absolute albumin standards due to varying water content and electrophoretic homogeneity (Doumas et al, 1971). Standards could only be made with great difficulty of canine albumin (Cohn's fraction V, Sigma, London), quite unlike our experience with bovine albumin (Cohn's fraction V, Sigma, London). The resultant solution was more viscous and had a gelatinous character. No stable optical density readings could be obtained due to floating flocculent material in the cuvettes. We never encountered similar experiences with bovine albumin.

The physiological role and chemical structure of albumin in different species is similar. E.g. we are not aware of difference in binding affinity of bromocresol green to albumin of different species. Therefore despite the theoretical advantage of standardizing against canine albumin, plasma albumin measurements were standardized against bovine albumin in view of the mentioned problems. A calibration graph is shown in Fig 2.20.

The precision of the individual estimate

The precision of the method was determined using a canine plasma pool: mean $\pm$ SD 32.0 $\pm$ 0.8 g/l (n=10) (CV = 2.5%).

Figure 2.20. Plasma albumin method: Calibration graph



Standards were made of crystallised bovine albumin.
The graph was drawn by hand.
For details of the method see text.

Interference by free fatty acids

Uptake of plasma free fatty acids (FFA) by the heart is dependent on the FFA/albumin molar ratio rather than on the FFA concentration (Spector et al, 1965; Evans et al, 1963). Meaningful FFA/albumin molar ratios can only be obtained, if plasma FFA don't interfere with the bromocresol green albumin method. Generally speaking dye binding albumin methods are subject to interference by compounds competing for albumin binding sites (Watson, 1969). E.g. it has been suggested that the low albumin values (using eosin as the dye) were caused by a FFA induced reduction in eosin binding to plasma albumin.

Therefore the effect of raised plasma FFA concentrations on the estimation of albumin using bromocresol green was examined. Appr. 4% (w/v) albumin solution (Bovine albumin, Cohn's fraction V, essentially fatty acid free, Sigma, London) in 0.9% w/v saline was prepared. The FFA concentration was 275 μmole/l. To one part potassium linoleate (99% pure, Sigma, London) was added, final concentration 1815 μmole/l. Albumin concentrations were 6% lower: 48.3 ± 0.1 and 44.8 ± 0.1 g/l respectively.

The effect of haemolysis on albumin determination

In experiments using the blood cell separator haemolysis was at times observed (see Part 5). Therefore the effect of haemolysis on albumin estimations was studied.

Dog red blood cells (one volume) were washed 7 times with one volume of 0.9% w/v sodium chloride, after which the cells were haemolysed by addition of one volume double distilled water. The haemoglobin concentration of the haemolysate was kindly measured by Mr. P.Newman, Department of Haematology, The Royal Infirmary, Edinburgh.

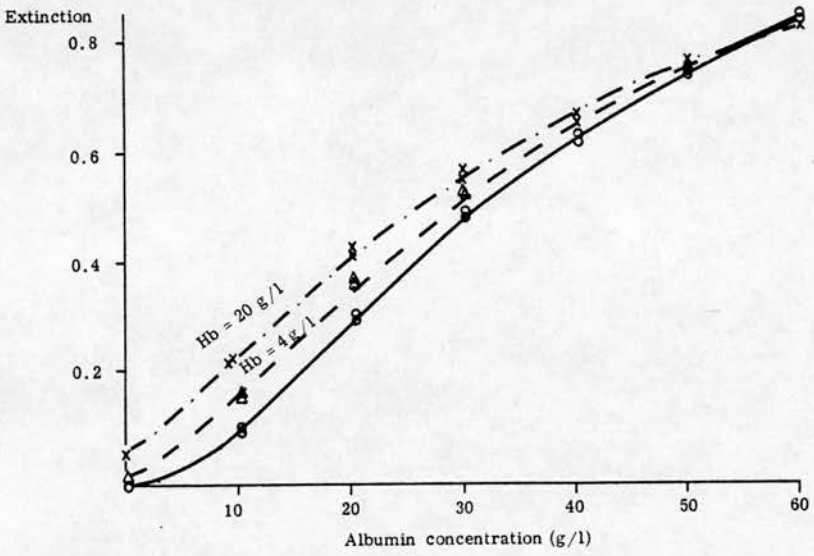
The effect of different degrees of haemolysis expressed as haemoglobin g/l, on the bovine albumin calibration curve is shown in Fig 2.21. (Haemolysis of 1% of circulating red blood cells would result in free haemoglobin concentration of about 2 g/l)

The interference of haemoglobin is related to the haemoglobin/albumin ratio, rather than the absolute amount of haemoglobin. A hyperbolic relation between the error in albumin measurement and haemoglobin/albumin ratio (w/v) was found (not shown): for a given haemoglobin concentration a marked interference at low and no interference at high albumin concentrations. The nature of the interference is therefore almost certainly binding of bromocresol green by haemoglobin.

Haemoglobin did not absorb light at 637 nm (not shown).

Our experimental animals usually have albumin concentrations ranging from 25 - 35 g/l, and as a result the problem is more serious than it would have been for human samples range 50 - 65 g/l. However the high free

Figure 2.21. Plasma albumin method: The effect of haemolysis



The effect of haemolysis (haemoglobin) on the determination of plasma albumin concentration was studied by addition of albumin free haemolysed red blood cells. The amount of haemoglobin added was determined separately.

O——O albumin standards (control)
Δ— — —Δ albumin standards plus 4 g/l haemoglobin
X——•——X albumin standards plus 20 g/l haemoglobin

High haemoglobin concentrations were selected to demonstrate its effect. For further details and implications see text.

haemoglobin concentrations used were chosen to demonstrate an effect, and they are much higher than observed in our experiments: 1.0 - 3.0g/l. At this level of haemolysis errors in plasma albumin concentrations were estimated in the order of 1.5- 2.0g/l, which could represent an error of 4-8% depending on the actual albumin concentration.

2.2.7. The estimation of lipolytic activity in canine plasma

Normal blood does not contain appreciable lipolytic activity, but after the injection of heparin the activity is markedly increased, as evidenced by the clearing of any lipaemia. It is now clear that a number of lipolytic enzymes are released by heparin from various organs: including lipoprotein lipase and other triglyceride- and phospholipases (Jansen and Hülsmann, 1974; Vogel and Bierman, 1965).

This post-heparin lipolytic activity in the presence of lipaemia (Intralipid infusion) was used to raise the concentration of plasma FFA in vivo. The lipolytic activity in plasma samples was estimated to relate this on a semi-quantitative basis to changing plasma FFA concentrations after heparin administration. The conditions employed to assay the lipolytic activity were optimal (but not specific) for the expression of lipoprotein lipase (EC 3.1.1.3) activity (Riley and Robinson, 1974).

Principle:

Plasma lipolytic activity was estimated using a triglyceride emulsion, Intralipid, preincubated with canine serum apolipoprotein lipase activators, as the substrate. This is in contrast to the conditions in vivo,

when Intralipid does not require pre-activation. This is due to the presence of circulating apolipoprotein-lipase activators (La Rosa, 1970).

The activity was expressed as free fatty acids released during incubation for 60 min at 37°C, in Tris buffer of pH 8.31 (1 Unit= umole/ml/60 min). The enzymic reaction was initiated by adding the plasma sample to the reaction mixture and terminated by deproteinisation/extraction of an aliquot into Dole's extraction mixture. Free fatty acid concentrations were analysed as described in Section 2.2.2. using the titrimetric method of Dole. This lipoprotein lipase method, established in our laboratory by Mr James Simpson, M.Phil., is essentially the same as described by Riley and Robinson (1974).

Preparation of plasma samples

Blood samples were collected in EDTA tubes (Stayne Laboratories, London), and stored on ice. Plasma and red blood cells were separated by centrifugation at 4°C (10 min, 150 g) and the plasma was transferred to another tube kept on ice. The assay was started immediately after the end of the experiment (less than 2 hours after the first samples had been collected.

The incubation

The lipolytic activity was assayed in Tris buffer, pH 8.31 with 5.7% (w/v) bovine albumin (Cohn's fraction

V, Sigma, London). The substrate Intralipid (20% (w/v), Kabi-Vitrum, Ealing, UK) was preincubated with canine serum. For this fresh blood (25 ml) was collected from the non-heparinised dog early during the experiment and mixed with 0.380% (w/v) trisodium citrate. Citrate plasma was obtained by centrifugation (15 min, 1 500 g) in a cooled MSE 4L centrifuge at 4°C. The plasma (1 ml) was recalcified by adding 25 µl of 1 mole/l $CaCl_2$ and left to clot for 30 min at 37°C. The clear re-calcified serum, containing apolipoprotein lipase-activators (La Rosa et al, 1970), was stored at 4°C until used later that day for the activation of Intralipid. For this Intralipid, double-distilled water and the recalcified serum (1:3:4 by vol) were mixed and incubated at 37°C for 30 min.

Immediately before lipolytic activity was estimated 1 ml activated substrate was added to the buffer (5.8 ml) dispensed in incubating flasks (glass liquid scintillation vials). The flasks were then transferred to a shaking waterbath set at 37°C (Grants Instruments Ltd., Cambridge), and allowed to equilibrate for 5 min.

The enzymic reaction was initiated by addition of 0.2 ml plasma, mixed and immediately three 1 ml aliquots were taken and transferred to tubes each containing 5 ml Dole's extraction mixture for the estimation of free

fatty acid concentration at time zero.

The incubation was terminated by transferring another three 1 ml aliquots of the reaction mixture after exactly 60 min to tubes containing Dole's extraction mixture. The extracts were then stored at -20°C until analysed.

Results are expressed as FFA released (μmole $ml^{-1}h^{-1}$).

The amount of free fatty acids released is said to be linear with time (Robinson, 1963) and no extensive tests were made to confirm this. However, during each run one post-heparin sample was also incubated for 30 min. The results were similar in 4 experiments (mean ± SEM): 28 ± 5 and 26 ± 2 U for 30 min and 60 min incubations respectively.

The effect of heparin

Samples were analysed from dogs before and after the intravenous injection of 2 000U heparin. Therefore the effect of additional heparin (10 U/7 ml reaction mixture) was investigated. On average there was a small, but insignificant increase (mean ± S.E.M.): 11 ± 14%(n=4) in post-heparin lipolytic activity.

The effect of serum activation

The addition of extra serum activator (+ 75%) increased in3 out of 4 experiments the post-heparin lipolytic activity:8 ± 5% (mean ± S.E.M.; n=4).

Discussion

The lipolytic activity is strongly influenced by the substrate used in the assay (Riley and Robinson, 1974). We have not used the physiological substrates chylomicrons or VLDL triglycerides, but a stabilised triglyceride emulsion (Intralipid). On the other hand it was exactly the same Intralipid and not chylomicrons or VLDL triglycerides which was infused to serve as the substrate of heparin-induced lipolytic activity.

Heparin also releases phospholipase activity (Vogel and Bierman, 1965) and as Intralipid contains 1.2% (w/v) lecithin, it is not clear to what extent lecithin and plasma phospholipids were hydrolysed in vitro.

It is of course quite impossible to extrapolate from the in vitro activity to the intact animal:

1. The activity was estimated at pH 8.31.
2. Incubations in vitro were carried out with excess albumin to bind fatty acids released.

Dog plasma albumin concentrations are much lower than observed in man, ranging from 25-35 g/l (2.5-3.5%). Thus in vivo, the fate of fatty acids originating from hydrolysed triglycerides might be expected to play (indirectly) a role in determining the overall enzyme activity too. However the in vitro activity will indicate the relative activities in vivo in a qualitative manner.

2.2.7. The estimation of plasma triglyceride concentrations

Principle:

Plasma triglyceride concentrations were determined on plasma extracts in isopropanol using an automated fluorimetric technique (Kessler and Lederer, 1966). All chemical reactions were carried out using an Autoanalyzer system (depicted in Fig 2.22), the hydrolysis to free glycerol:

$$\text{Triglycerides} + KOH \longrightarrow \text{free glycerol} + K^{+} \text{ soaps}$$

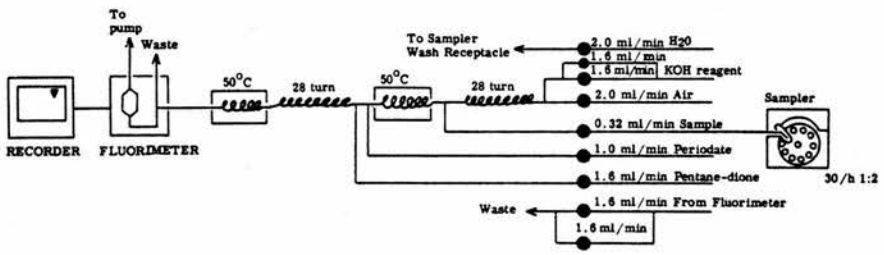
the oxidation of free glycerol:

$$\text{glycerol} + 2\ NaIO_4 \longrightarrow 2\ \text{formaldehyde} + \text{formic acid} + 2\ NaIO_3$$

the condensation of formaldehyde to 3,5-diacetyl-1, 4-dihydrolutidine (see Fig 2.23) using pentane-2, 4-dione 3, 5-diacetyl-1, 4-dihydrolutidine is quantified fluorimetrically.

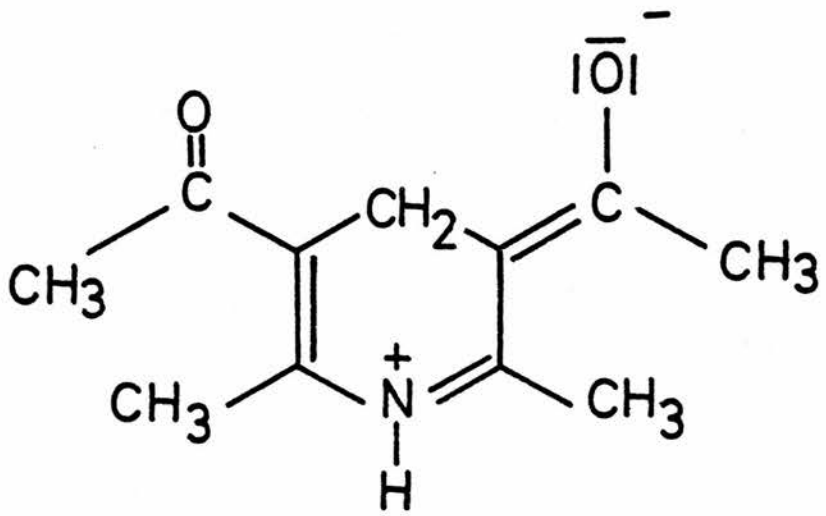
The oxidation is specific for 2 cis hydroxyl groups, as a result most carbohydrates will yield formaldehyde. Therefore the extract must be purified prior to analysis: carbohydrates (free glycerol,

Figure 2.22. AAI Method for plasma triglycerides: Manifold



The sample line was changed each day.

Figure 2.23. The chemical structure of 3,5-diacetyl-1,4-dihydrolutidine



The CH_2-group in the ring, opposite the $\equiv N^+_H$ group originates from formaldehyde.

glucose etc) are removed by adsorption to $CuSO_4$ and phospholipids are adsorbed by zeolite (sodium aluminium silicate).

Preparation of lipaemic plasma samples

Samples were collected in heparinised syringes as described in Section 2.2.2. Plasma was obtained by centrifugation in a cooled centrifuge for 10 min at 150 g. In hyperlipaemic samples part of the triglycerides were floating on the surface and were then resuspended, using a glass rod and plasma was then transferred, immediately extracted (see below).

Extraction and treatment with zeolite

A plasma sample (0.5 ml) was extracted into 9.5 ml redistilled isopropanol using glass stoppered Quick fit tubes. Zeolite (about 2 g, Harleco, Didcot, Berks.) was added using a scoop, the tubes thoroughly mixed on a Vortex and left overnight at 4°C. Zeolite was then packed into a pellet by centrifugation (10 min at 1 000 g) and the clear supernatant transferred to another stoppered tube. Extracts were allowed to reach room temperature before analysis using the Autoanalyzer.

Reagents

Isopropanol (Analar, British Drug Houses, Poole) was redistilled prior to use. Pentane-2,4-dione obtained from the same manufacturer was redistilled and the

clear distillate was stored in a brown bottle at 4°C. Even under these conditions it would slowly turn yellow and then high baseline fluorescence was observed. Therefore it was kept for about 1 month, after which it was discarded.

The isopropanol/KOH mixture was prepared fresh:
5 g KOH in aqueous isopropanol, 75% (v/v).
For the analysis of blanks the KOH was omitted from the KOH/isopropanol reagent. This did not influence the final pH of the reaction mixture (flowcell effluent).
The sodium periodate reagent:
5.4 g sodium periodate,
115 ml glacial acetic acid (Analar, B.D.H.), made up to 1 litre with double-distilled water.

Ammonium acetate 154 g/l adjusted with approx. 200 ml 2 moles/l HCl to pH 6.0 (stable for at least 1 month).

The pentanedione in ammonium acetate reagent was prepared fresh daily: 65 ml of a mixture of pentane-2,4-dione/isopropanol 15:50 (v/v) was added to 1 litre ammonium acetate reagent.

Analysis of extracts using the Autoanalyzer

Under laboratory conditions a noticeable evaporation of isopropanol was observed. Therefore samples were loaded on sampling plates in small batches of 5 extracts

(in duplicate), separated by drift standards. Standards were analysed after every 40 extracts. They were loaded in palindromic sequence to check on carry-over between samples. Usually no carry-over was observed, but if present the manifold was checked for bad connections and/or bubble pattern,and changed. Provided the precautions mentioned above were adhered to, no corrections for drift were necessary.

Samples which exceeded the range of the standards were diluted 1:1 with 95% isopropanol and reanalysed.

Analysis of blanks

For the analysis of blank values of unknown samples the same extracts used for triglyceride analysis, were reanalysed in an identical manner, except that the KOH necessary for the hydrolysis step was omitted.

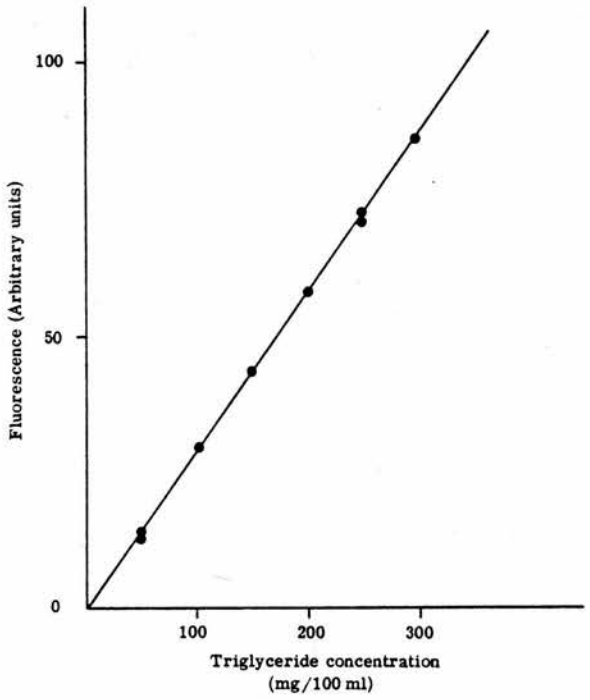
Calibration of the method

Standards made of triolein(99%+, Sigma, London) in redistilled isopropanol were made and stored at $4^{o}C$.

The standards were 'extracted' and treated with Zeolite after the addition of 0.5 ml double-distilled water (in stead of plasma) in an identical manner as was used for unknowns.

A representative graph is shown in Fig 2.24. The graph was drawn on a transparent plastic comparator

Figure 2.24. Plasma triglyceride method: Calibration graph



(Technicon, Basingstoke), which was then used to convert peak heights of unknowns directly into triglyceride concentrations.

The precision of the individual estimate

Precision of the method was determined using 7 individual extracts of a human quality control sample (Technicon): mean ± SD was 2.40 ± 0.20 mmole/l (CV=8.2%). These pools have a considerable amount of free glycerol. Corrections were made for this, by omitting the plasma triglyceride hydrolysis step. The mean (± SD) plasma triglyceride concentration was then: 1.82 ± 0.03 mmole/l (CV=1.7%).

Specificity of the method

Despite the use of zeolite blank readings were observed for certain quality control samples (with unphysiologically high free glycerol concentrations) and some dog samples, obtained after the injection of Intralipid (stabilised with glycerol). However, if results were corrected for blank readings, comparison with an automated enzymic method(SMAC, Department of Clinical Chemistry, The Royal Infirmary, Edinburgh) were good, and the precision was also better. The results suggest that in situations with high free glycerol or glucose concentrations (Intralipid-heparin studies, injection of glucose etc) zeolite adsorbs in an irreproducible manner interfering carbohydrates and individual corrections have to be made.

SECTION 2.3:

ELECTROCARDIOGRAPHIC AND HAEMODYNAMIC MEASUREMENTS

2.3.1. The recording of epicardial electrocardiograms (ECG)

Since the publication of Maroko et al (1971) ST-segment elevation in epicardial ECG's has been widely used as an index of ischaemic injury. The methods used vary, mainly in the use of different exploring electrodes: a looped metal wire (Opie et al, 1975); a steel ball electrode (Smith, 1977) or cotton-wick electrodes (Maroko et al, 1971; Kjekshus and Mjøs, 1973). Moreover the different techniques appear not to have been compared systematically. The same pragmatic approach as other workers before us was adopted. The acceptance criteria were that ECG's should be reproducible and the technique did not irritate the myocardium: e.g. induced ST-segment elevation in normal myocardium or caused arrhythmias.

The cotton-wick fulfilled these criteria. Reproducible tracings were obtained if the electrode was loosely held and allowed to move with the moist surface of the heart. However ECG complexes were always inspected immediately and recordings were repeated if necessary.

The actual technique

The epicardial ECG was recorded using a cotton-wick

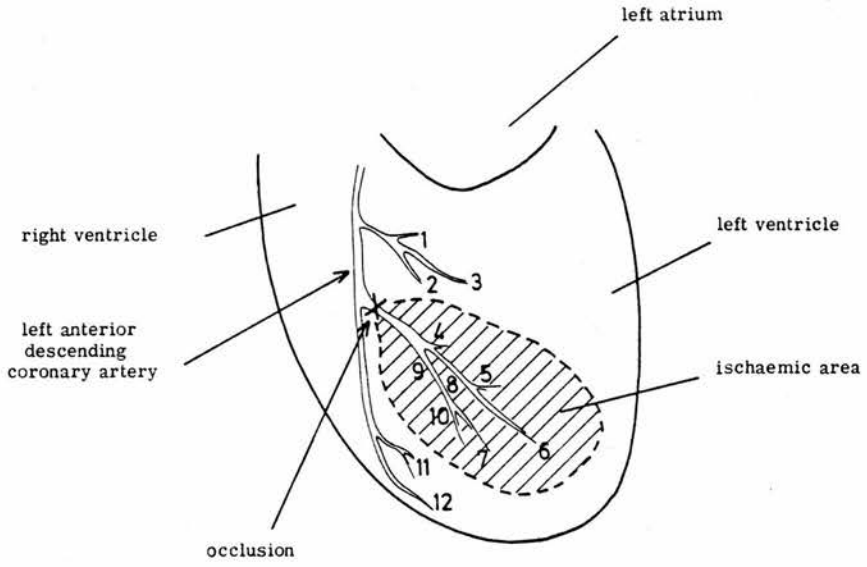
electrode, according to the method of Maroko et al. (1971). The cotton-wick electrode was constructed using a piece of Autoanalyzer tube (length 5-7 cm, diameter 2 mm). A U shaped thread of cotton was twisted around the exposed metal of an insulated wire and pulled through the tube. Only the cotton loop (wick) was allowed to emerge. Good contact between the exposed wick and the wire was ensured by storing the electrode immersed in saline (0.9% w/v) until used.

The combined limb leads served as the reference electrode (see Appendix A, Fig 1).

Location of epicardial sites: Ten-15 anatomically well defined sites were selected within the area supplied by the coronary artery to be occluded as well as in areas remote from the occlusion (see Fig 2.25). Sites were at least 1 cm away from the anterior descending coronary artery where ECG changes due to conduction delay are common. For each experiment a map was drawn showing the selected sites in relation to the site of occlusion and anatomical features.

The epicardial ECG's were recorded on a Devices M8 recorder with a sensitivity of 10 mV/cm and a paper speed of 25 mm/s. Recording of all 10-15 sites took about 1 min. Unless otherwise specified, recordings were made immediately before the start of infusion of

Figure 2.25. Monitoring of epicardial electrocardiogram: Selected sites in relation to local anatomy



A schematic representation of the selected sites used for the recording of epicardial electrocardiograms. The ischaemic myocardium is indicated by the hatched area. The numbers relate to the sites in the sequence which was used during the epicardial ECG mapping.

drugs, and prior to, and at 5, 10 and 15 min of coronary occlusion.

ST-segment elevation was determined at mid ST-segment and the exact point was marked on each complex using a divider. The average of 4-6 ECG complexes recorded from each site was calculated. The results were expressed as ischaemia induced ST-segment elevation and the values for all sites were summed (ΣST). ΣST was used as an estimate of the severity of myocardial ischaemia (Maroko et al, 1971).

The evaluation of the results was time consuming, and because of its nature observer bias could possibly affect the results. Added to this was the relatively long time required to make the recordings (1 min) during a period of ischaemia of not more than 15 min. Alternative methods devoid of these problems were considered. The solution was found in a 10-16 lead epicardial grid and an electronic averaging device (see Appendix A, Figure 2), which allowed continuous monitoring of mean ST-segment elevation.

The epicardial grid was made of thin latex rubber with a suture ring made of gauze. Silver electrodes (diameter 4 mm) soldered to 36 Swg insulated copper wire, were glued to the grid using latex rubber. The grid

is positioned loosely over the area within the distribution of the coronary artery to be occluded. It is secured with 3-4 stitches (4-O silk) well outside the area of interest.

The leads are connected to a 16 lead selection box. The right leg electrode served as the earth lead and the combined right and left front leg (RA, LA) and the left hind leg (LL) were used as the reference electrode (see Appendix A, Figure 2). During the experiment only those leads, showing ST segment elevation during the 1st coronary occlusion, were selected for the computation of mean ST segment elevation. During the rest of the experiment the validity of this assumption was regularly checked for all selected leads. Correction was made for ST-segment depression, if necessary.

This system proved adequate and superior to the above described manual technique since it offered continuous information, less time required for evaluation of results, and elimination of observer bias. However, the basic information of a particular individual lead was lost. Transient changes in some of the leads might have gone undiscovered. Only continuous sampling of all leads would overcome this problem and was thought to lead to the insight required to decide whether recording of averaged ECG would always give the right answer.

This led to the development of a multiplexer system.

In the multiplexer each of the fifteen epicardial signals is amplified and then they are arranged into three groups of five, i.e. channels 1-5, 6-10, 11-15.

The five channels in each group are then sequentially sampled at a rate of 300 samples/second/channel. The three time division multiplexed data channels along with the synchronizing pulse channel are recorded on a four channel F.M. tape recorder running at 15"/second, the bandwidth and track configuration of this recorder dictating to a large extent the design of the complete system. The magnetic tape was replayed and decoded. ST-segment elevation was computed using a purpose built ST-segment computer (Neilson et al, 1968) and recorded on a Devices M4 recorder with a sensitivity of 1 or 2 mV/cm. ST-segment elevation was calculated at 70 ms after the onset of the QRS complex (Appendix A, Fig 3).

The early method of recording epicardial ECG's (Phase 1) should be considered essentially relative to one another within a particular experiment. The reason for this is the lack of input resistors for the limb leads. (Appendix A, Fig 1A). Thus the exact signal obtained would be dependent on the resistance of the individual limb leads, which might have varied from experiment to experiment, depending on the actual contacts

made. However as the limb electrodes were intramuscular, variations in input resistance of the limb leads within an experiment seem unlikely.

This problem was rectified by a modification to the attenuator box (Phase 2).

Epicardial ST-segment elevation reflects the severity of experimentally induced myocardial ischaemia (Wegria et al, 1949; Kjekshus et al, 1972; Smith, 1977) and correlates with the local reduction in myocardial oxygen tension produced by coronary artery occlusion (Angell et al, 1975).

However ST-segment elevation is an index of myocardial ischaemic injury, and its limitations, particularly when precordial mapping in man is used have been reviewed (Ross, 1976). The technique cannot be used when marked conduction delay is present (Muller et al, 1975), as occurred in our studies with proximal occlusions (Section 5.4).

2.3.2. Haemodynamic measurements

Arterial blood pressure was measured from a catheter introduced into a femoral artery. A Statham pressure transducer (P23Db) calibrated using a mercury manometer was used. Airbubbles were carefully excluded. The cannula was kept patent by intermittent flushing with saline. Heparin was not used, except in heparinised animals when the cannula was filled with heparinised saline 5 U/ml.

The signal from the transducer was amplified using a Devices direct-coupled DC2 amplifier and recorded on a Devices M 8 recorder, with a sensitivity of 5 mm Hg/mm.

The overall frequency response of the system was flat within 5% at 40 Hz and is mainly determined by the response of the heated stylus, which was determined using a sinusoidal wave form of fixed amplitude, generated by a wave generator (Wavetek, Model 112).

In the colaborative studies with Dr. H. Vik-Mo and Prof. O.D. Mjøs, University of Tromsø, as well as in those with Dr. A. Ungar, Department of Pharmacology, University of Edinburgh, it was also possible to measure left ventricular pressure and its first derivative (dP/dt). A short cannula was inserted into the left ventricle through the dimple of the apex and directly

connected to the pressure transducer (a Statham P23Gb in Tromsø; a L223 (Consolidated Electro dynamics, England) in Edinburgh). The left ventricular pressure and its first derivative were recorded in Tromsø on a Dynograph recorder and Edinburgh on a UV-recorder (Honeywell Ltd, Holytown). In Edinburgh the frequency response of the overall system was also flat within 5% at 40 Hz.

SECTION 2.4:

THE MEASUREMENT OF REGIONAL MYOCARDIAL BLOOD FLOW

2.4. The measurement of regional myocardial blood flow

Introduction

Accurate knowledge of regional myocardial blood flow in the non-ischaemic and ischaemic area is a keystone in the interpretation of the effect which enhanced glucose availability, or decreased fatty acid utilisation has on the severity of myocardial ischaemia.

Essentially two methods are most frequently used for the determination of regional (myocardial) blood flow. In the first, the initial uptake of a diffusible, flow-limited tracer is followed after a bolus injection, and measured before the onset of the wash-out phase. Without going into details of the problems of this method, it can be said that this method has a limited range of applications, due to the fact that the heart has to be excised shortly after the injection during the wash-in phase. This restricts the method to one observation per experiment.

The principle of the microsphere method is not all that different: the distribution of a non-diffusible tracer (microsphere) in a region or an organ after an arterial injection is determined by flow. However there is one essential difference and that is that unlike the diffusible tracer the microsphere with a diameter of 15 μ becomes lodged during its first transit in the

precapillary bed. It is therefore not necessary to excise the heart immediately and provided different labels for microspheres are used for successive injections several determinations can be made.

For this reason the microsphere technique was selected.

2.4.1. The Method

The adopted procedure is based on the method of Utley et al (1974).

The characteristics of the microspheres used are summarised in Table 2.11.

Prior to injection 1.5 - 3.0 x 10^6 microspheres were subjected to ultrasound in 10% dextran for 5-10 min to dispel aggregates (Dawe Sonicleaner, type 6441A), and drawn into a syringe containing 0.1 mg 5% (v/v) Tween-80 in saline (9 g/l) to prevent reaggregation. The suspension was diluted with 10% dextran to give a final concentration of Tween-80 of less than 0.5% (v/v). A small air bubble was taken into the syringe, allowing continuous agitation until injected.

Ten seconds prior to the injection of the microspheres withdrawal of femoral arterial blood for the estimation of reference blood flow (RBF) was commenced. Reference blood was collected into a weighed heparinized syringe at a constant rate (approximate 9 ml/min) for a period of 2 min using a Harvard infusion/withdrawal pump.

Table 2.11

Characteristics of the radioactive microspheres

Manufacturers: 3M; NEN

General properties Carbonized microspheres;

Nominal diameter 15 ± 3 μ(Mean ± SD)

Nominal specific activity: 10 mCi/g

Density : 1.3 - 1.6 g/ml

Tendency to aggregate

Plastic: polystyrene (3M)

styrene-divinyl copolymer (NEN)

Radioisotope labels used:

	Isotope	Half-life (days)	Principal energy (keV)	Manufacturer
Group 1:	Gd^{153}	242	97 - 103	NEN
	Co^{57*}	271	122 - 136	NEN
	Ce^{141}	32.5	145	3M, NEN
	Yb^{169}	31	110 - 177 - 198	3M
Group 2:	Sn^{113}	115	393	NEN
Group 3:	Ru^{103}	40	497	NEN
	Sr^{85}	64.5	514	3M
Group 4:	Nb^{95}	35	765	3M
	Sc^{46}	84	889 - 1120	3M, NEN

* contained free Co^{++}, washed before use.

The microspheres were injected through a left atrial cannula over a period of about 10 sec, and flushed with 5 ml 10% Dextran.

It was observed that despite the Dextran flush, some microspheres were left behind in the tap and/or the Luer-lock connection of the cannula. Therefore the tap was replaced after microspheres had been removed from the connector. Not more than 4 injections were made per experiment.

Between 5 and 30 min after the last injection the dog was sacrificed with sodium pentobarbitone. The heart was excised, the free wall of the left ventricle dissected out, and visual fat and large vessels removed.
Full thickness biopsies (10-20) were made and divided into epicardial and endocardial layers by mid-line transsection. (This was easier if the heart was allowed to go into rigor mortis overnight and this was adopted generally. The heart was stored in a closed, moist chamber at $4^{o}C$ to prevent dehydration.)

The epicardial and endocardial layers were transferred to a plastic tube (18 x 63 mm, Ravens, Haverhill, Suffolk, when the Gamma Guard; and 15 x 950 mm when the Wallac gamma counter was used). The layers were divided into 10-20 blocks to produce relatively constant geometrical factors for the measurement of radioactivity.

2.4.2. The measurement of radioactivity

The radioactivity was originally measured using a three channel gamma counter (Gamma Guard, Tracerlab Inc., USA), equipped with a three inch NaI crystal. The counting times ranged from 100 - 600 sec and were approximated to yield 10 000 counts in samples with low counting rates. The windows were selected so that no isotope contributed in a significant degree (less than 0.1%) to an energy window, selected for an isotope with a higher energy (Table 2.12). This was an essential condition to allow the calculation of the results (see Calculations).

The contribution of the isotopes was determined at the start of each counting procedure using the reference blood samples as standards in a similar geometrical setting. The underlying assumption of this is that only the one isotope, injected during the collection of the reference blood sample is present, and that no microspheres from the previous injection recirculate.

This was checked on a few occasions by placing a shielded counter over the withdrawal line before and during a 5 min collection of arterial blood in an otherwise identical manner as for the collection of reference blood. The results showed that most microspheres had been removed from the arterial blood $1\frac{1}{2}$ min after injection, and possibly very few were circulating for

Table 2.12

Selected energy windows (Gamma Guard)

Isotope	Window (keV)	Relative contribution to channel			
		I^{125}	Ce^{141}	Yb^{169}	Sr^{85}
I^{125}	25 - 47.5	1.00	0.001	0.001	0.001
Ce^{141}	122.5 - 152.5	0.03	1.00	0.001	0.001
Yb^{169}	180 - 310	N.D.	0.20	1.00	0.001
Sr^{85}	480 - 620	0.09	0.27	0.35	1.00

N.D. Not determined, combination not used.

another $\frac{1}{2}$-$\frac{3}{4}$ min.

It has also been checked by bleeding and collection of blood into a donor bag at least 30 min after the last injection of microspheres. Using the whole body counter (Department of Medical Physics, The Royal Infirmary, Edinburgh), only background counts were obtained.

Later the Gamma Guard could no longer be used due to mechanical fault in the sample changer. Radioactivity was then analysed using a two channel gamma counter (Wallac, 80 000 Gamma sample counter, LKB-Wallac, Finland), equipped with a two inch NaI crystal. Samples were counted in 2 cycles if necessary. The results were printed on a paper tape. For selected windows see Table 2.13.

Electronic stability of the gamma counters

The gamma counters were checked for stability over periods similar to those used for the counting of samples. For this a mixed source containing 3 isotopes: Sn^{113}, Sr^{85} and Nb^{95} was repeatedly (46 times) counted. The variability of the count rates was almost identical to the theoretically predicted values. Thus the stability of the counters has a small effect on the accuracy of the count rates, if anything.

Table 2.13

Selected energy windows (Wallac)

Isotope	Window(keV)	Relative contribution to channel*			
		Group I	Group II	Group III	Group IV
GroupI:					
Gd^{153}	78-108				
Co^{57}	110-140	1.00	0.00	0.00	0.00
Ce^{141}	95-135				
GroupII:					
Sn^{113}	365-465	0.16	1.00	0.04	0.00
GroupIII:					
Sr^{85}	460-680				
Ru^{103}	470-560	0.21	0.10	1.00	0.00
GroupIV:					
Nb^{95}	670-850	0.13	0.23	0.22	1.00
Sc^{46}	650-1200				

*Approximate values for groups I, III and IV.

2.4.3. Calculation of regional myocardial blood flow

In the early experiments, using the Gamma Guard, windows were selected to avoid contributions of an isotope with a low energy to a channel selected for an isotope with a higher energy. Thus the channel of the isotope with the highest energy was free from interference by other isotopes. The net count rate of the lower energy channels was obtained after stepwise correction for the contributing counts, originating from isotopes with a higher energy. When the Wallac was used the contribution of isotopes to a higher channel was not zero and the stepwise correction could not be applied. The net count rates of the 4 channels were computed by solving 4 simultaneous equations, each describing the respective contributions of the 4 isotopes to a particular channel.

These equations were solved using

a computer programme (Gelg, I.B.M.-mathematical package). Regional myocardial blood flow (MBF) was calculated for each biopsy using the formula:

$MBF = \frac{CM}{CR} \times RBF$, where MBF is expressed in ml blood g^{-1}. min^{-1}. CM is the biopsy radioactivity in cpm/g wet weight, CR is the total cpm in the reference blood sample. Reference blood flow (RBF) was calculated from the weight and timing of the reference blood collection, using an assumed blood specific gravity of 1.05 g/ml.

Corrections

Corrections for decay during counting procedures were not made. It can be theoretically shown that even for Ce^{141} and Nb^{95} (with the shortest half lives of 32 and 35 days) inaccuracies in counting rate due to decay do not exceed 0.7%; for other isotopes Sr^{85}, Sc^{46}, this is $<$0.4% (total counting time 10 hours).

The errors in the calculation of contributing factors of one isotope to other channels due to decay are of the same order of magnitude.

The influence of geometrical factors on the estimation of radioactivity was explored by Dr P.Tothill, Department of Medical Physics, The Royal Infirmary, Edinburgh. Using the same counter (Wallac) and sample tube, he studied the height corrections for the counting of two isotopes (in the extremes of the energy range we were using

Co^{57} and Sc^{46}).

- It was found that the counting efficiency was height dependent.
- The relation between height and efficiency was dependent on the selection of the enery windows. E.g. Sc^{46} counting was more sensitive to variations in height in its own window than in the window selected for Co^{57} (see Appendix B-1). In view of the complexity of this problem it is not possible to use simple height correction factors. Besides it was found that the corrections were small in our working range 1-3 cm and therefore corrections were not made.

The calculations described above were made using a computer programme written by Dr J.O'Brien for a desk-top computer (Advanced programmable calculator 700B, Wang Laboratories Inc., Mass.) or a Fortran programme (see Appendix B-2) written by Dr K.Boardman for the PDP-12. The former was used for the data obtained using the Gamma Guard and the latter using the data on a paper tape from the Wallac.

2.4.4. The reproducibility of the method

The reproducibility of the method was checked in 4 experiments. Physiological variation in coronary blood flow as a cause of variability in regional myocardial blood flow was excluded by a simultaneous injection of 1.5×10^6 microspheres labelled with 3 different isotopes

(Co^{57}, Ru^{103}, Sc^{46}). In one experiment a 4th isotope (Sn^{113}) could be injected as well. The left atrial injection was made 5 min after coronary artery occlusion, made to reproduce the conditions under which microspheres are normally injected. In addition this would ensure that the reproducibility of the method could be checked over a range of regional myocardial blood flows. The influence of the reference blood flow determination was studied by two simultaneous collections of reference blood from the left and the right femoral artery using the same Harvard withdrawal pump (9 ml/min).

The myocardial biopsies (endocardial and epicardial) were taken as described above. Three sets of (4) artificial standards containing one isotope only were made and the breakthrough of the isotopes into other energy windows determined. The net radioactivity (per isotope) of the real reference blood samples collected during the experiment was then calculated using the Fortran programme (described above), and they were used to calculate the regional myocardial blood flow.

The mean results ($\pm$ SEM) obtained from epicardial and endocardial layers of the normal border and ischaemic area as determined by Co^{57} (Sn^{113} 1 experiment), Ru^{103} and Sc^{46} labelled microspheres are tabulated in Appendix B-3. The reference sample which was used for the calculation

is referred to as A and B.

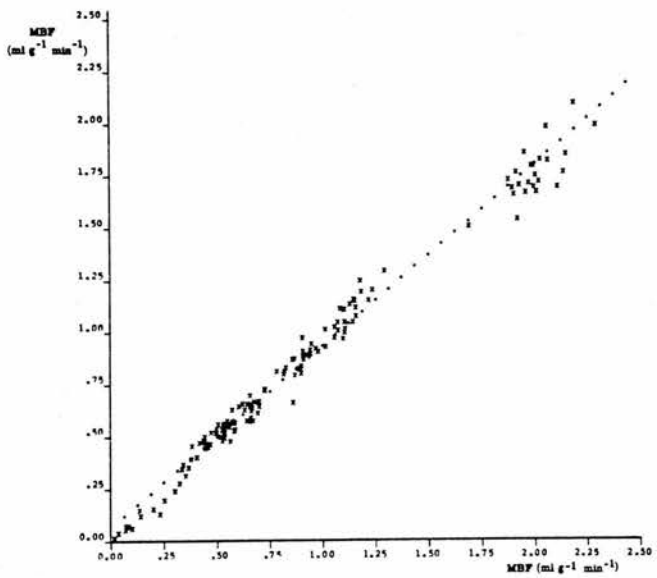
The data of one particular experiment (1A, 1B, 2A, etc) was analysed using paired analysis. Highly significant differences between the determinations with two isotopes were found in most cases (19 out of 24 possible combinations). Only in 1 experiment (4B) was there no significant difference between the measurements obtained with the three isotopes. However there was no systematic difference between blood flow determination made with the different isotopes.

Thus as an example in experiment 1A the highest flow was determined with Sc^{46} microspheres and using the same tissue but a different reference blood sample (B) the highest flow was estimated with microspheres labelled with Ru^{103}.

The average of the mean data of the 4 experiments as determined by Co^{57}, Ru^{103} and Sc^{46} microspheres are the same: 0.918; 0.890 and 0.888 ml g^{-1} min^{-1}. ($p > 0.05$, paired t - test). The individual results obtained with the various microspheres were inspected graphically and a representative example is given in Fig 2.26 A/B, Co^{57} vs Ru^{103} microspheres.

These graphs underline the importance of the reference blood sample in the determination of regional myocardial blood flow. E.g. when the results were calculated on the

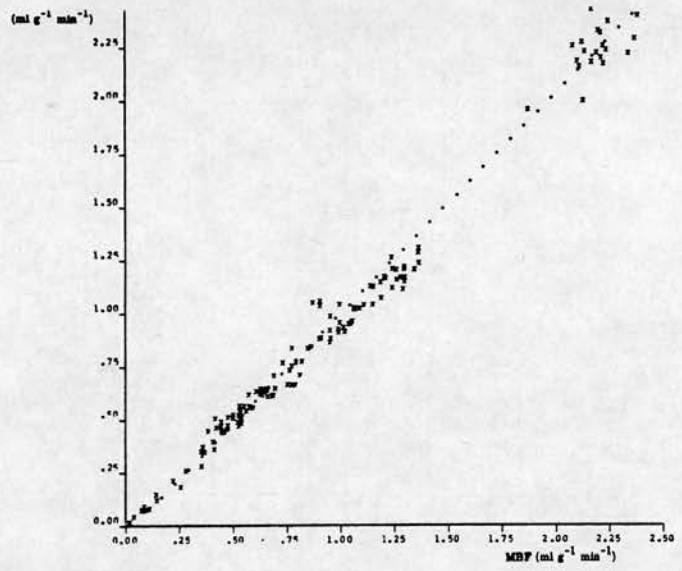
Figure 2.26 A. Determination of myocardial blood flow (MBF): The reproducibility of the method



The reproducibility of the determination of regional myocardial blood flow in epi- and endocardial layers of the ischaemic and non-ischaemic area of the left ventricle was determined 5 min after coronary occlusion using a simultaneous injection of different microspheres (Co^{57}, Ru^{103} and Sc^{46}) in 4 experiments.

Results based on reference blood sample A.
On the abscissa: MBF determined with Co^{57} microspheres.
On the ordinate: MBF determined with Ru^{103} microspheres.
The calculated, dotted line is:
$y = 0.065 + 0.872\ x$ ($r = 0.992$, $n = 144$, $P < 0.001$)

Figure 2.26 B. Determination of myocardial blood flow (MBF): The reproducibility of the method



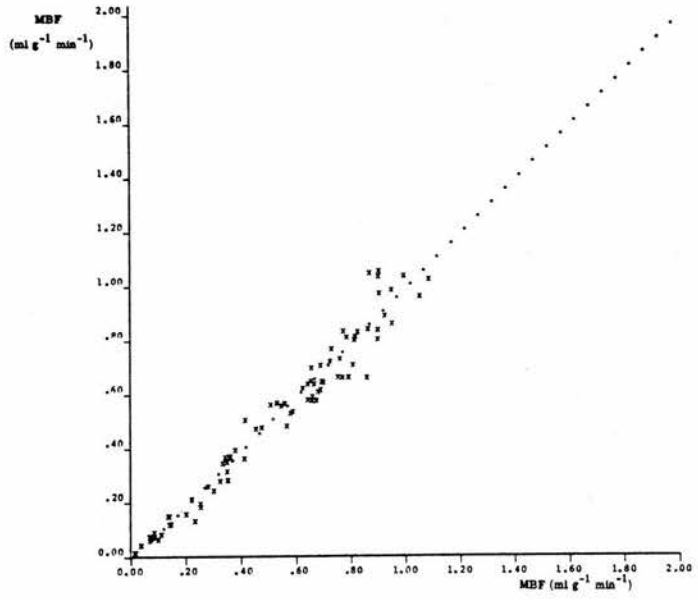
Details as in Figure 2.26 A.
Results based on reference blood sample B.
The calculated, dotted line is:
$y = -0.042 + 1.038\ x$ ($r = 0.993$, $n = 144$, $P < 0.001$)

basis of reference blood sample A regional myocardial blood was underestimated with Co^{57} microspheres (Fig 2.26 A), but not if reference blood sample B was used (Fig 2.26 B). On close inspection of the data it was observed that this error was mainly due to the results of experiment 1, in which myocardial blood flow in the non-ischaemic myocardium was high. Nevertheless it is worrying that the discrepancy between the relative amount of different microspheres in simultaneously withdrawn reference blood samples can (occasionally) be as large as was observed.

A separate statistical analysis was made of the blood flow obtained from epi-and endocardial samples taken from the border and ischaemic zone. (In experiment 4 myocardial blood flow was low and the effect of coronary artery occlusion was hardly detectable. For this reason it was excluded for this analysis.) The mean results $\pm$ SEM are tabulated in Appendix B-4 and show in agreement with the overall data that no systematic effects were operative. The results were also graphically examined and an example (Co^{57} vs Ru^{103} microspheres) is presented in Fig 2.27.

Finally the endocardial/epicardial ratios of regional myocardial blood flow were also analysed using paired t-tests. (Only one estimate per experiment is obtained, since in this ratio the radioactivity and the weight of

Figure 2.27.Determination of myocardial blood flow (MBF): The reproducibility of the method, applied to ischaemic and border zone



The reproducibility of the determination of regional myocardial blood flow in epi- and endocardial layers of the ischaemic and border zone was determined 5 min after coronary occlusion using a simultaneous injection of different microspheres (Co^{57}, Ru^{103} and Sc^{46}) in 4 experiments.

On the abscissa : MBF determined with Co^{57} microspheres.
On the ordinate : MBF determined with Ru^{103} microspheres.
The calculated, dotted line is :
$y = -0.020 + 1.009\ x$ ($r = 0.983$, $n = 88$, $P < 0.001$).

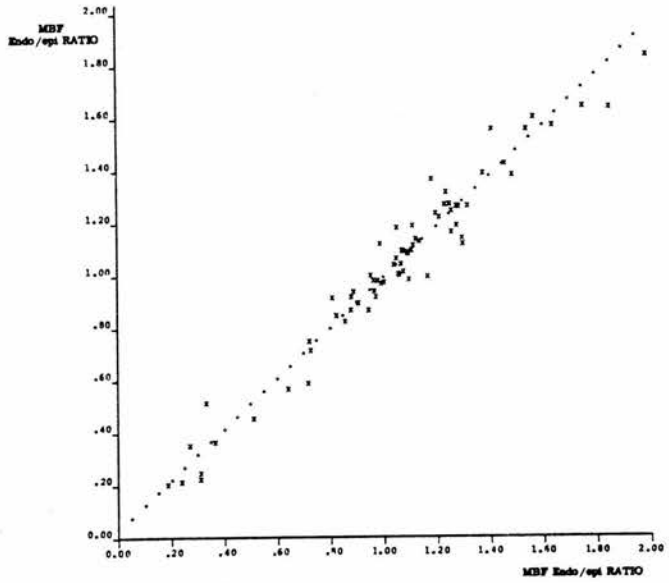
the reference blood sample drop out as can be seen in the MFB equation on page 223).

The results of the overall data (normal plus ischaemic tissues) are tabulated in Appendix B-5and an example of a typical correlation is presented in Fig 2.28. The results of the ischaemic and border zone biopsies are similar (Appendix B-6 and Fig 2.29).

In one study (experiment 3) the difference in duplicate estimation of regional myocardial blood flow (expressed as a percentage of the mean result) was analysed and related to the weight of the biopsy. Comparing the data from all tissues (ischaemic plus non-ischaemic) as well as those of non-ischaemic biopsies only no significant correlation was found (see Fig 2.30).

This and the above data show that there is no <u>systematic</u> error in the method of blood flow determination with differently labelled microspheres (Co^{57}, Ru^{103},Sc^{46}) or their endo-epicardial distribution; as could be operative due to differences in geometrical factors and/or decay during counting. There was a wide range of isotope $\frac{1}{2}$ lives and energies of the gamma rays, suggesting that within these boundaries with similar isotopes these results may be considered as universal. The biggest source of variability is the composition of the reference blood sample. We used left atrial

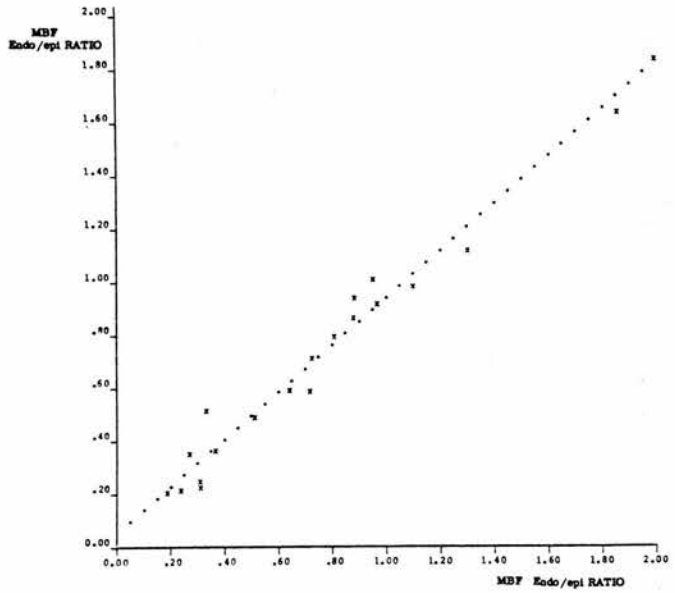
Figure 2.28. Determination of endo-/epicardial ratio of regional myocardial blood flow (MBF): Reproducibility of the method



The reproducibility of the method was checked in 4 experiments. The ratio of regional myocardial blood flow in epi- and endocardial biopsies of the ischaemic and non-ischaemic areas of the left ventricle was determined 5 min after coronary occlusion with a simultaneous injection of different microspheres (Co^{57}, Ru^{103}, Sc^{46}).

On the abscissa: Endo-/epi ratio of MBF measured with Co^{57} microspheres; on the ordinate determined with Ru^{103} labelled spheres.
The calculated, dotted line is : $y = 0.0264 + 0.962\ x$
($r = 0.976$, $n = 71$, $P < 0.001$)

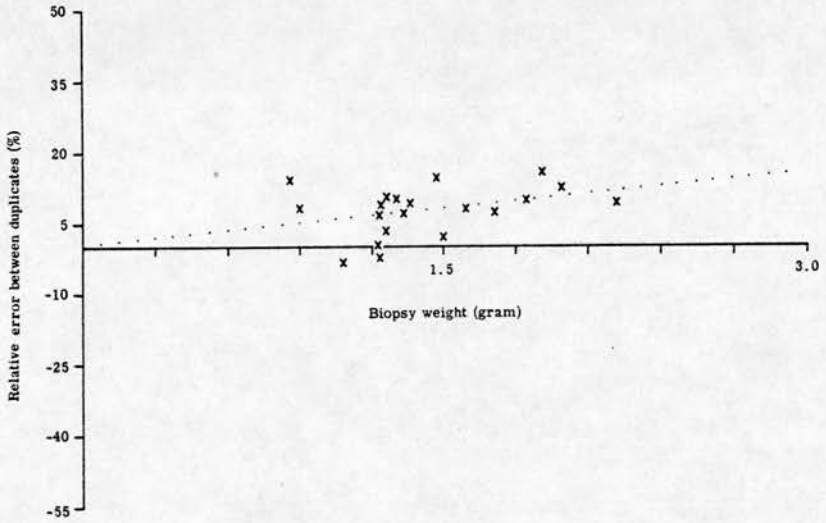
Figure 2.29. Determination of endo-/epicardial ratio of regional myocardial blood flow (MBF): The reproducibility of the method, applied to the ischaemic and border zone



Ischaemic and border zone biopsies only.
Details as in Figure 2.28.
The calculated dotted line is:
$y = 0.049 + 0.890\ x$ ($r = 0.987$, $n = 20$, $P < 0.001$)

Note that some endo-/epicardial ratios of MBF $>$ 1 due to a reduction in epicardial, but not endocardial MBF (border zone).

Figure 2.30. Determination of myocardial blood flow: Relative error between duplicate determination with different microspheres (Co^{57}, Sc^{46}) in relation to sample weight



Regional myocardial blood flow was estimated using a simultaneous injection of different microspheres (Co^{57}, Ru^{103}, Sc^{46}).

The error between duplicate determination of regional blood flow in non-ischaemic myocardium was calculated from the Co^{57} and Sc^{46} microsphere data, selected to obtain the maximal difference in gamma energy used in our experiments. No significant correlation was found between the relative error between duplicate estimation, expressed relative to the mean and the weight of the biopsy. ($r = 0.345$, dotted line, $n = 20$, NS).

injections as suggested by Kaihara et al (1968) to ensure adequate mixing, but despite this streaming of microspheres in the aorta must have occurred (Phibbs et al, 1967).

SECTION 2.5:

THE USE OF ENZYME RELEASE IN THE ASSESSMENT OF SEVERITY OF MYOCARDIAL ISCHAEMIA

2.5.1. Biochemical assessment of severity of myocardial ischaemia

It is well established that myocardial proteins and enzymes (myoglobin, creatine kinase, lactate dehydrogenase etc) are released from the infarcting myocardium into the circulation during acute myocardial infarction. The plasma activity ofcreatine kinase (CK) and its MB isoenzyme (CK-MB), which is more cardiospecific, are the first to increase significantly at about 4-6 hours after the onset of symptoms. These enzyme tests are therefore commonly used for the confirmation of a clinical diagnosis of myocardial infarction. Plasma CK-MB activity seems to be a particularly sensitive and specific index of myocardial infarction (Varat et al, 1975; Galen et al, 1975; Smith et al, 1976).

Therefore the use of CK release, in addition to electrocardiographic evidence, as an index of severity of myocardial ischaemia was considered. In a model of intermittent coronary artery occlusion this could be done by measurement of myocardial CK activity in successive biopsies or alternatively by estimation of the CK release into the venous effluent of the ischaemic myocardium (by measurement of the arterio-venous difference of plasma CK).

There were likely to be difficulties with the first procedures since it is conceivable that repeated biopsies might have deleterious effects on the model. It would also be difficult to quantify CK loss against the high background of myocardial CK activity. On the other hand, local venous catheters draining the ischaemic area were used and therefore estimation of CK activity in the effluent from the ischaemic area seemed to be the better approach. However plasma CK activity might become raised in the open-chest model due to release from skeletal muscle cut during the operation, rather than from the myocardium. We had no data on this, although it is recognised that surgical operations are associated with a rise in plasma CK activity (Schmidt and Schmidt, 1976). Since skeletal muscle contains predominantly CK-MM, with little or no CK-MB, it was hoped to overcome this problem by the use of isoenzyme studies.

While setting up the electrophoretic method for the separation and quantification of CK isoenzymes, it was reported that dog myocardium had little or no CK-MB (Yasmineh et al, 1975; Ahmed et al, 1976). The following experiments were performed in order to verify these findings.

Samples of myocardium were obtained from 5 dogs. A 10% (w/v)myocardial homogenate in 10% (w/v) sucrose

plus 2 mmole/l glutathione was prepared using a Douce-Potter homogeniser (Unicorn, Hemel-Hempstead). The heart and homogenates were kept on ice throughout.

The CK isoenzymes were quantitated by electrophoresis on agarose films (Corning Eel, Halstead, Essex) followed by visualisation of the separated isoenzymes (Wong and Smith, 1975). A fresh 1:50 dilution of the homogenates was made, and 1 µl applied to the film. After electrophoresis, the CK isoenzymes were visualised by the following reaction sequence:

$$\text{ADP + creatine phosphate} \rightleftharpoons \text{ATP + creatine}$$

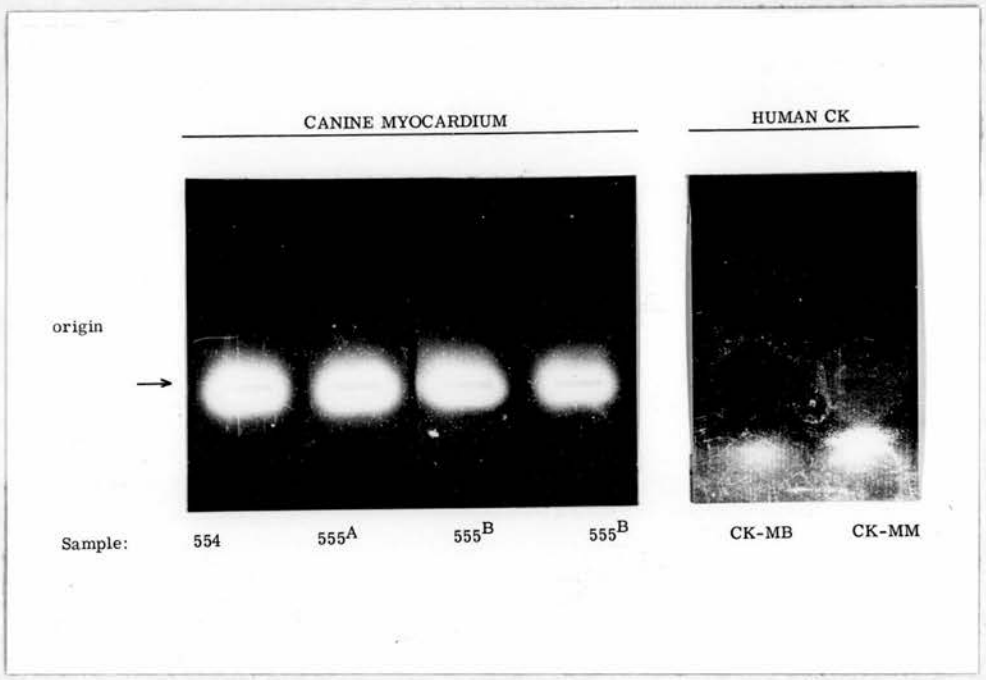
$$\text{ATP + glucose} \longrightarrow \text{ADP + glucose-6-phosphate}$$

$$\text{glucose-6-phosphate + } NADP^{+} \longrightarrow \text{NADPH + } H^{+} \text{+ 6-phosphogluconate}$$

NADPH fluorescence was used for the visualisation and quantification of the isoenzyme bands with a Vitatron TLD 100 densitometer (Fisons Scientific Apparatus, Loughborough).

In agreement with the reports mentioned above, a small percentage ranging from 0-1.2% of CK-MB was found. A representative photograph taken of the fluorescent bands is shown in Fig 2.31. The photograph was deliberately over-exposed to show up the faint MB isoenzyme bands present. A similar photograph

Figure 2.31. The occurrence of creatine kinase isoenzymes in canine myocardium



Creatine kinase isoenzymes from dog myocardium were separated electrophoretically using agarose sheets. A homogenate sample, equivalent to 10 µg tissue was applied. The isoenzyme bands were visualised using an enzymatic staining procedure yielding NADPH.
Top: Anode Bottom: Cathode
The isoenzyme bands of three homogenates, prepared from 2 hearts 554 and 555 (biopsy A and B) are shown.

For comparison a similar photograph of purified human CK-MB and CK-MM (at the origin) after electrophoresis is mounted to the right. Note a barely detectable CK-MB band in the canine samples (photo deliberately overexposed).

(kindly made available by Dr A.F.Smith, Department of Clinical Chemistry, The Royal Infirmary, Edinburgh) mounted to the right shows the fluorescent bands of purified human CK-MM (on the origin) and CK-MB.

In view of small amounts of CK-MB in dog myocardium it was decided to measure total plasma CK activity. Creatine kinase activity was determined using the method of Rosalki (1967). Measurements were made in the Department of Clinical Chemistry, The Royal Infirmary, by arrangement with Dr A.F.Smith. The coefficient of variation of this method is 6-7% for samples with plasma CK activity in the range 100-1 000 IU/l.

The effect of surgery on femoral arterial plasma CK activity was studied in 8 anaesthetized dogs. Samples were collected at frequent intervals (at least $\frac{1}{2}$ hourly) throughout the experiment. Arterial plasma CK activity rose continuously from 311 $\pm$ 91 I.U./l at about 30 min after completion of the surgical procedures to 600— 1 500 I.U./l $3\frac{1}{2}$ to $4\frac{1}{2}$ hours later.

In the same experiments the effect of the first coronary artery occlusion on myocardial CK release was studied. Arterial, coronary sinus and local venous blood samples were taken simultaneously immediately before and at 6-10 min after occlusion. No sham occlusion was made. The results are expressed as the difference

between arterial and coronary sinus (draining predominantly non-ischaemic myocardium) or arterial and local venous (draining ischaemic myocardium) plasma CK activities.

Prior to coronary artery occlusion no CK release could be demonstrated: both the arterio-coronary sinus and the arterio-local venous difference of plasma CK activity were positive and undistinguishable from each other using a Wilcoxon paired ranktest Table 2.14, ($P > 0.05$). However during the occlusion the plasma CK activity in the effluent from ischaemic myocardium did exceed the arterial CK activity: resulting in a negative arterio-venous difference of -57 ± 16 I.U./l. This gradient across the ischaemic area was significantly different from the one observed before the occlusion ($p < 0.01$) (Wilcoxon paired-rank test). Although the arterio-coronary sinus difference of plasma CK activity also became negative (-28 ± 13 I.U./l), it was not significantly different from the arterio-coronary sinus difference measured prior to the occlusion (control).

These results suggest that CK may be released from ischaemic myocardial cells, before irreversible damage can be demonstrated histochemically - usually after 20-30 min of occlusion (Jennings et al, 1969).

Table 2.14

The effect of experimental coronary occlusion (6-10 min) on arterio-venous differences of creatine kinase activity (Mean ± S.E.M.)

Difference in plasma creatine kinase activity (I.U./l)=	Control	Occlusion	P value from Control**
arterio-local venous	10 ± 10	-57 ± 16*	<0.01
arterio-coronary sinus	31 ± 24	-28 ± 13	N.S.

** A paired Wilcoxon rank test was used (one tail value).

* p = 0.025 vs arterio-coronary sinus difference (Occlusion).

Alternatively it is possible to explain the negative arterio-venous difference of CK activity in terms of transfer of plasma water into damaged cells.

Whatever the explanation may be, it was evident that a precise estimation of CK release could not be obtained in this model, and this approach was abandoned.

Part 3

THE EFFECTS OF REDUCTION OF PLASMA CONCENTRATIONS OF FREE FATTY ACIDS ON MYOCARDIAL METABOLISM AND ST-SEGMENT ELEVATION DURING EXPERIMENTAL MYOCARDIAL ISCHAEMIA IN DOGS

INTRODUCTION

In order to test the hypothesis, which is the basis of this thesis, that fatty acid and glucose metabolism affect the severity of myocardial ischaemic injury, the effects of altering myocardial metabolism are examined. This part of the thesis is confined to the effect of reducing plasma free fatty acid concentrations on ischaemic myocardial metabolism, and will be related to changes in ischaemia-induced ST-segment elevation in epicardial electrocardiograms. As a tool to lower plasma concentrations of free fatty acids, a series of drugs were used. Their effect was studied under basal conditions and during stimulation of adipose tissue lipolysis using isoprenaline infusion. The effect of the following drugs, which were all structurally unrelated, were tested:

- p-chlorophenoxyisobutyrate (sodium salt)
- prostaglandin-E_1
- acebutolol
- nicotinic acid
- sodium salicylate

The selection of each drug was not solely based on their ability to lower plasma concentrations of free

fatty acids, and the reasons will be given in each of the sections, dealing with a particular drug.

SECTION 3.1:

EFFECTS OF P-CHLOROPHENOXYISOBUTYRATE

Effects of p-chlorophenoxyisobutyrate

Introduction

Substantial reductions in plasma concentrations of free fatty acids have been reported in animals treated with the active metabolite of clofibrate: p-chlorophenoxyisobutyrate (Barrett and Thorp, 1968; Cenedella et al, 1968), although it is not known whether this is accompanied by a reduction in the utilisation of free fatty acids by the myocardium (Thorp, 1973).

The effect of p-chlorophenoxyisobutyrate on the extraction of plasma free fatty acids by the heart and magnitude of epicardial ST-segment elevation during experimental coronary artery occlusion in dogs was examined. The effect of this antilipolytic drug on regional myocardial blood flow during coronary artery occlusion was also investigated, with the aid of microspheres.

We were interested in the effect of this active metabolite of clofibrate, since it might be relevant to the acute phase in patients with acute myocardial infarction, pretreated with clofibrate.

3.1.1. Methods

Animal preparation

Experiments were carried out in 23 mongrel dogs of both sexes (15-20 kg body weight), fasted for 12-15 hours, as described in Part 2 (Section 2.1) of this thesis.

Regional Myocardial Blood Flow

Regional myocardial blood flow was measured by means of radioactive microspheres as described in Section 2.4.

Biochemical analyses

Blood samples were collected 15 min after each occlusion and processed as described in Section 2.2.2.

Plasma concentrations of p-chlorophenoxyisobutyrate were kindly analysed by Dr. Thorp, ICI Pharmaceuticals Div., Alderley Edge, Manchester, using a spectrophotometric method (Barrett and Thorp, 1968). Plasma concentrations of free fatty acids were assayed in duplicate by the titrimetric method of Trout et al (1960), as described in Section 2.2.2. Corrections for the presence of p-chlorophenoxyisobutyrate,

which interferes in the titrimetric method, were made according to Barrett and Thorp (1968). Plasma free glycerol were determined in duplicate on deproteinised samples, prepared by ultrafiltration,using the fluorimetric enzymic method described in Section 2.2.4.

3.1.2. Experimental design

Effects of pretreatment with p-chlorophenoxyisobutyrate on subsequent coronary artery occlusion

The effect of pretreatment with p-chlorophenoxyisobutyrate on plasma free fatty acid concentrations and on the ST-segment elevation during subsequent coronary artery occlusion was studied in 10 dogs. In 7 animals the basic procedure of 4 intermittent periods of ischaemia (each of 15 min duration), separated by a recovery period of at least 30 min was used (see Fig. 2.02). After the recovery period of the second control occlusion (in the presence of isoprenaline (0.2-0.3 μg/kg/min)) p-chlorophenoxyisobutyrate was administered as the sodium salt by slow intravenous injection in 0.9% (w/v) saline at the dose of 20 mg/kg body weight. Thirty minutes later the two test occlusions were made at first in the absence and then in the presence of isoprenaline.

In the remaining 3 dogs (307, 309 and 313) studies were carried out either in the absence or presence of isoprenaline only.

In five additional dogs the effect of p-chlorophenoxyisobutyrate on regional myocardial blood flow during coronary artery occlusion was assessed. Measurements were made during an initial control occlusion (in 3 dogs only), during a second occlusion in the presence of isoprenaline and during a third occlusion performed in the presence of isoprenaline 30 min after the intravenous injection of p-chlorophenoxyisobutyrate. The effect of p-chlorophenoxyisobutyrate on myocardial blood flow was assessed in the presence of isoprenaline, rather than under basal conditions, since it had previously been found that the reduction in epicardial ST-segment elevation by p-chlorophenoxyisobutyrate was more marked in the former situation.

Effects of p-chlorophenoxyisobutyrate administration during established coronary artery occlusion

In six dogs an i.v. infusion of isoprenaline (0.2-0.3 μg/kg/min) was maintained for the duration of the study. Five minutes after its commencement a branch of the left anterior descending coronary artery was permanently occluded. Five and ten minutes later recordings were made of epicardial electrocardiograms, aortic blood pressure and heart

rate. p-Chlorophenoxyisobutyrate was then administered as previously described, and the recordings repeated after further intervals of 5 and 10 minutes. Simultaneously with each recording, arterial blood was sampled for the measurement of plasma free fatty acids, drug levels and free glycerol concentrations. In five of these dogs, and in an additional two animals, coronary sinus blood was also sampled immediately before and ten minutes after p-chlorophenoxyisobutyrate administration for the determination of the arterial-coronary sinus concentration differences of plasma free fatty acids.

Statistics

Each dog served as its own control. Student's t-test for paired data was used to calculate probability values. $P > 0.05$ was regarded as not statistically significant.

3.1.3. Results

Effects of pretreatment with p-chlorophenoxyisobutyrate

The effects of p-chlorophenoxyisobutyrate on the response to subsequent coronary occlusion are summarised in Tables 3.01 and 3.02. Occlusion of a branch of the left anterior descending coronary artery was followed by marked changes in epicardial ST-segments. After 15 minutes of occlusion ΣST averaged 26 $\pm$ 6 mV (mean $\pm$ SEM). After p-chlorophenoxyisobutyrate administration re-occlusion of the artery resulted in a much smaller ΣST, averaging 14 $\pm$ 3 mV ($P < 0.03$). p-Chlorophenoxyisobutyrate also reduced the number of sites with evidence of ischaemic injury (ST > 2 mV) from an average of 4.9 $\pm$ 1.1 to 2.9 $\pm$ 1.0 ($P < 0.05$). These effects of p-chlorophenoxyisobutyrate occurred in the absence of any changes in mean aortic blood pressure and heart rate, or blood glucose concentration (Table 3.01). However, arterial concentrations of plasma free fatty acids were reduced from 466 $\pm$ 41 to 221 $\pm$ 44 μmol/l ($P < 0.001$; Table 3.01).

Prior to p-chlorophenoxyisobutyrate the infusion of isoprenaline increased occlusion-induced ΣST from 26 $\pm$ 6 to 74 $\pm$ 11 mV ($P < 0.001$), and the number of sites with ischaemic injury from 4.9 $\pm$ 1.1 to 9.0 $\pm$ 0.9 ($P = 0.02$; Tables 3.01 and 3.02). After p-chlorophenoxyisobutyrate

Table 3.01

Effect of pretreatment with p-chlorophenoxyisobutyrate (CPIB) on epicardial ST-segment elevation, arterial concentration of plasma free fatty acids (FFA), mean aortic blood pressure ($\overline{AP}$), heart rate (HR) and blood glucose concentration (Bd gluc) at 15 min after coronary artery occlusion.

	ΣST* (mV)		ST > 2mV** (number of sites)		FFA (μmole/l)		$\overline{AP}$ (mm Hg)		HR (beats/min)		Bd gluc (mg/100 ml)		Plasma CPIB concentration (μg/ml)
Dog	Occ	Occ CPIB	Occ	Occ CPIB	Occ	Occ CPIB	Occ	Occ CPIB	Occ	Occ CPIB	Occ	Occ CPIB	Occ CPIB
303	8	5	1	1	340	105	98	90	143	138	—	—	139
304	33	32	7	7	440	130	82	92	133	139	61	47	129
305	17	10	4	1	510	105	100	107	145	147	52	60	148
306	5	3	0	0	320	150	102	104	143	142	66	65	125
307	20	17	3	2	460	245	103	97	156	142	66	53	159
308	26	10	5	0	420	340	112	105	162	164	65	64	194
309	31	18	7	4	390	150	75	75	111	109	58	58	166
312	62	19	8	8	630	265	86	92	111	104	75	71	208
315	34	12	9	3	680	500	177	116	100	95	34	46	166
Mean	26	14	4.9	2.9	466	221	97	98	134	131	60	58	159
SEM	6	3	1.1	1.0	41	44	5	4	7	8	4	3	9
P	< 0.03		< 0.05		< 0.001		NS		NS		NS		—

* Sum of ST-segment elevations at 10-15 sites

** Number of sites at which ST-segment elevation exceeded 2 mV.

NS = $P > 0.05$

Abbreviations: Occ = occlusion; IP = isoprenaline

Table 3.02

Effects of pretreatment with p-chlorophenoxyisobutyrate on epicardial ST-segment elevation, arterial concentration of plasma free fatty acids, mean aortic blood pressure, heart rate and blood glucose concentration at 15 min after coronary artery occlusion performed during a continuous intravenous infusion of isoprenaline.

	ΣST* (mV)		ST > 2mV** (number of sites)		FFA (μmole/l)		$\overline{AP}$ (mm Hg)		HR (beats/min)		Bd gluc (mg/100ml)		Plasma CPIB concentration (μg/ml)
Dog	Occ IP	Occ CPIB	Occ IP	Occ CPIB	Occ IP	Occ CPIB	Occ IP	Occ CPIB	Occ IP	Occ CPIB	Occ IP	Occ CPIB	Occ CPIB
303	19	5	4	1	1300	975	82	83	185	180	___	___	116
304	71	43	11	11	1930	1275	90	92	185	185	81	60	111
305	92	65	9	6	1560	945	93	97	132	185	80	70	122
306	58	40	10	9	2820	2315	82	82	175	176	95	82	127
308	64	17	7	4	1880	1160	98	90	175	170	77	75	166
312	124	54	9	9	1880	1360	83	80	154	149	136	141	185
313	79	34	12	11	2640	2400	102	105	200	198	52	60	139
315	87	58	10	10	1720	1000	95	90	155	148	42	54	166
Mean	74	40	9.0	7.6	1966	1429	91	90	176	174	80	77	142
SEM	11	7	0.9	1.3	183	209	3	3	6	6	12	11	10
P	<0.005		<0.05		<0.001		NS		NS		NS		___

* Sum of ST-segment elevation at 10-15 sites.

** Number of sites at which ST-segment elevation exceeded 2 mV.

NS = $P > 0.05$

For abbreviations see Table 3.01

ΣST associated with subsequent coronary occlusion and isoprenaline infusion was markedly reduced to 40 ± 7 mV ($P < 0.005$), while the number of sites with ischaemic injury was reduced to 7.6 ± 1.3 ($P < 0.05$). Intravenous infusion of isoprenaline before p-chlorophenocyisobutyrate reduced mean aortic blood pressure from 97 ± 5 to 91 ± 3 mm Hg ($P < 0.05$), and increased heart rate from 134 ± 7 to 176 ± 6 beats/min ($P < 0.001$), blood glucose concentration from 60 ± 4 to 80 ± 12 mg/100 ml ($P < 0.02$) and arterial concentrations of plasma free fatty acids from 466 ± 41 to 1966 ± 183 μmole/l ($P < 0.001$). These effects of isoprenaline (reduction in mean aortic blood pressure, increase in heart rate and increase in blood glucose concentration) were not altered by the administration of p-chlorophenoxyisobutyrate, except that isoprenaline's lipolytic effect was reduced, evidenced by reduced arterial plasma free fatty acid concentrations (now 1429 ± 209 μmole/l, $P < 0.001$).

Myocardial blood flow during coronary artery occlusion

When the tissue of the free wall of the left ventricle distant from the occluded artery was taken to represent normal myocardium, blood flow to the ischaemic zone was reduced by about 40% at 10-13 min after occlusion. The reduction in blood flow was greater in the endocardial than

in the epicardial layers, resulting in a significant increase in the epicardial/endocardial blood flow ratio ($P < 0.01$; Table 3.03).

The effect of isoprenaline on myocardial blood flow

Isoprenaline significantly increased the blood flow to the non-ischaemic left ventricle, but had no effect on that to the ischaemic areas (Table 3.03).

The effect of p-chlorophenoxyisobutyrate on myocardial blood flow during isoprenaline infusion

It was of interest to note that pretreatment with p-chlorophenoxyisobutyrate did not alter blood flow to the ischaemic and non-ischaemic layers of the free wall of the left ventricle.

Effects of p-chlorophenoxyisobutyrate administration during established coronary artery occlusion

The acute effects of p-chlorophenoxyisobutyrate when given during an established coronary artery occlusion and isoprenaline infusion are summarised in Table 3.04. Five and ten minutes after giving (i.e. 15 and 20 minutes following coronary artery occlusion) values for ΣST and arterial concentration of plasma free fatty acids were both significantly reduced relative to those recorded immediately before treatment, while mean aortic blood pressure and heart rate remained unchanged. ΣST has previously been

Table 3.03

Effect of coronary artery occlusion, isoprenaline and p-chlorophenoxyisobutyrate on regional myocardial blood flow in non-ischaemic and ischaemic layers of the free wall of the left ventricle 10-13 min after coronary artery occlusion in 5 dogs

		MBF ($ml.g^{-1}.min^{-1}$)				
		Occ †	P	Isoprenaline+ Occ	P	Isoprenaline+ CPIB + Occ
Non-ischaemic myocardium*	Epi	1.30 ± 0.09 (n = 21)	< 0.001	1.88 ± 0.09 (n = 34)	NS	1.85 ± 0.07 (n = 34)
	Endo	1.36 ± 0.10 (n = 21)	< 0.001	1.62 ± 0.07 (n = 34)	NS	1.61 ± 0.05 (n = 34)
	Epi/Endo	0.97 ± 0.04 (n = 21)	< 0.001	1.17 ± 0.04 (n = 34)	NS	1.16 ± 0.03 (n = 34)
Ischaemic myocardium**	Epi	0.86 ± 0.16 (n = 6)	NS	0.89 ± 0.08 (n = 11)	NS	0.94 ± 0.10 (n = 11)
	Endo	0.71 ± 0.14 (n = 6)	NS	0.71 ± 0.11 (n = 11)	NS	0.76 ± 0.12 (n = 11)
	Epi/Endo	1.32 ± 0.12 (n = 6)	NS	1.45 ± 0.16 (n = 11)	NS	1.50 ± 0.12 (n = 11)

Results are expressed as mean ± SEM (n = number of biopsies)

NS = $P > 0.05$

*Myocardium distant from the occluded artery.

** Myocardium within the area of distribution of the occluded artery.

† Three experiments only

Abbreviations: MBF = myocardial blood flow; epi = epicardial; endo = endocardial.

Table 3.04

Acute effects of p-chlorophenoxyisobutyrate (CPIB) on ST-segment elevation, mean aortic blood pressure, heart rate and arterial concentrations of plasma free fatty acids and free glycerol when administered during an established coronary artery occlusion and isoprenaline infusion

Time (min)	0	5	10	15	20	25
	Isoprenaline infusion (0.2 - 0.3 µg/kg/min)					
			Coronary artery occlusion			
					CPIB (20 mg/kg)	
Σ ST (mV)		11 ± 4 (6)*	74 ± 12 (6)	81 ± 13 (6)	57 ± 8 (6)*	53 ± 6 (6)*
ST > 2mV (number of sites)		1.4 ± 0.7 (6)*	9.6 ± 0.9 (6)	9.2 ± 0.9 (6)	8.4 ± 0.7 (6)	8.4 ± 0.8 (6)*
$\overline{AP}$ (mm Hg)		82 ± 4 (6)	84 ± 5 (6)	84 ± 5 (6)	86 ± 5 (6)	85 ± 5 (6)
HR (beats/min)		154 ± 13 (6)	152 ± 13 (6)	150 ± 13 (6)	148 ± 12 (6)	146 ± 12 (6)
FFA (µmole/l)		—	1540 ± 20 (2)	1993 ± 259 (6)	1218 ± 155 (6)**	1108 ± 156 (6)*
Glycerol (µmole/l)		—	—	254 ± 31 (6)	—	185 ± 21 (6)**
Arterial CPIB (µg/ml)		0	0	0	251 ± 15 (6)	223 ± 12 (6)

Results are expressed as mean ± SEM (number of dogs).
Statistical comparisons were performed by paired t-test analysis against those results (3rd column) obtained immediately before the administration of CPIB.
*P < 0.025
**P < 0.01
Other differences were not statistically significant.
Abbreviations see Table 3.01.

found to increase or remain unchanged between 10 and 20 min following coronary occlusion during isoprenaline infusion in the absence of treatment (Mjøs, unpublished results). The reduction in arterial concentration of plasma free fatty acids was associated with a proportionate decrease in the arterial-coronary sinus difference (Table 3.05). No significant uptake of p-chlorophenoxyisobutyrate by the myocardium could be detected: mean arterial and coronary sinus p-chlorophenoxyisobutyrate concentrations were 225 ± 18 and 218 ± 21 μg/ml respectively: p-chlorophenoxyisobutyrate reduced the arterial free glycerol concentration from 254 ± 31 to 185 ± 21 μmole/l ($P < 0.01$).

Table 3.05

Effects of p-chlorophenoxyisobutyrate (CPIB) on arterial concentration of plasma free fatty acids (FFA) and myocardial free fatty acid extraction during established coronary artery occlusion (Occ)

	Arterial FFA concentration (μmole/l)		Arterial-coronary sinus FFA concentration difference (μmole/l)		Extraction ratio * (%)	
Dog	Occ	Occ** CPIB	Occ	Occ CPIB	Occ	Occ CPIB
without isoprenaline						
323	760	365	360	50	47	14
324	460	370	60	30	13	8
during isoprenaline 0.2-0.3 μg/kg/min						
321	3020	1125	750	155	25	14
323	2800	2155	450	400	16	19
324	1210	920	360	300	30	33
325	1070	730	120	105	11	14
326	2250	1780	300	180	13	10
327	1850	950	420	375	23	41
328	1890	850	420	315	22	37
Mean	1701	1022	260	212	22	21
SEM	297	199	67	46	3.8	4.2
P	<0.01		<0.01		NS	

*Extraction ratio= $\left(\frac{\text{arterial concentration-coronary sinus concentration}}{\text{arterial concentration}}\right) \times 100\%$

**Values obtained 10 minutes after intravenous CPIB (20 mg/kg).

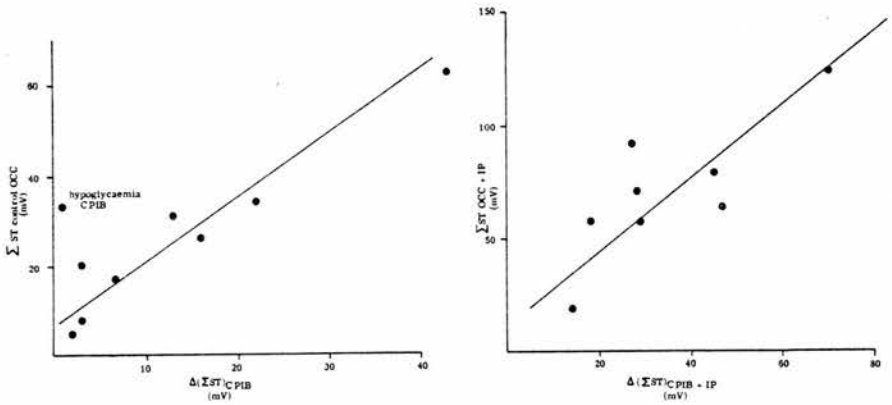
3.1.4. Discussion

The present observations demonstrate that pretreatment with p-chlorophenoxyisobutyrate substantially reduced the extent and magnitude of epicardial ST-segment elevation after experimental coronary artery occlusion in dogs, and reduces the increase in ischaemia-induced ST-segment elevation by isoprenaline infusion. The reduction in ST-segment elevation, achieved by pretreatment with p-chlorophenoxyisobutyrate was related to the initial magnitude of ST-segments during the control occlusions. This was observed both in the absence and in the presence of isoprenaline (see Fig. 3.01). The absolute reduction in ST-segment elevation during isoprenaline infusion was thus greater than that recorded during occlusion in its absence. This difference was also observed when p-chlorophenoxyisobutyrate was given during an occlusion established 10 min earlier.

These findings probably indicate a limitation of the acute myocardial ischaemic injury by p-chlorophenoxyisobutyrate.

The severity of acute myocardial ischaemic injury has been shown to be influenced by factors, which alter the oxygen requirements of the heart, relative to oxygen supply (Maroko et al, 1971). The major determinants of

Figure 3.01. The relation between initial ST-segment elevation and the effect of p-chlorophenoxyisobutyrate (CPIB) on ST-segment elevation during experimental myocardial ischaemia (basal and isoprenaline stimulated state).



Panel A: Basal state.
The effect of p-chlorophenoxyisobutyrate on ST-segment elevation expressed as the difference between $\Sigma ST(\text{control occlusion}) - \Sigma ST_{(CPIB + \text{occlusion})} = \Delta(\Sigma ST)_{CPIB}$, was plotted against ST determined during the control occlusion. One experiment did not adhere to the general trend. Data from Table 3.01.
Panel B: Isoprenaline stimulated state.
The effect of p-chlorophenoxyisobutyrate on ST-segment elevation $\Delta(\Sigma ST)_{CPIB + IP}$ was plotted against ΣST determined during the occlusion, preceded by a continuous isoprenaline (IP) infusion. Data from Table 3.02.
Occ = occlusion.

myocardial oxygen requirements include the mechanical factors of contractility, heart rate and wall tension (Sonnenblick et al, 1968). However, the lack of any effect of p-chlorophenoxyisobutyrate on mean aortic blood pressure or heart rate in this investigation renders it unlikely that there was a reduction in ischaemic injury due to altered mechanical activity, but the fact is acknowledged that necessary sophisticated haemodynamic measurements to prove this could not be made. An effect of p-chlorophenoxyisobutyrate on coronary collateral circulation can be excluded from the results of the microsphere studies, which demonstrated that blood flow to both the ischaemic and non-ischaemic areas of the left ventricular free wall during isoprenaline infusion was unaltered by treatment. Furthermore the failure of p-chlorophenoxyisobutyrate to alter regional ischaemic myocardial blood flow might suggest that the reduction in ST-segment elevation was also unrelated to its known effects on platelet aggregation, blood clotting mechanisms and blood viscosity. This is supported by the observation that platelet aggregation in the ischaemic myocardium after coronary artery occlusion does not impair coronary circulation (Vik-Mo, 1978).

p-Chlorophenoxy(iso)butyrate reduced the arterial concentration of plasma free fatty acids by some 40%, and this was

associated with a proportionate decrease in the arterial-coronary sinus difference of plasma free fatty acid concentration. Myocardial uptake of radiolabelled palmitate was also reduced by p-chlorophenoxyisobutyrate (Miller and Mjós, unpublished observations). It could not have been predicted that fatty acid extraction would still relate to arterial fatty acid concentrations after the administration of p-chlorophenoxyisobutyrate, in view of reports that it may displace fatty acids to weaker binding sites on the albumin molecule (Thorp, 1973). The fact that plasma free glycerol concentrations were already reduced after 10 min post p-chlorophenoxyisobutyrate suggests that the effect of the drug stemmed at least in part from an inhibition of lipolysis, in accordance with in vitro studies of adipose tissue metabolism (Speake, 1967; Curtis-Prior, 1974).

It is also possible that ischaemia induced myocardial lipolysis may have been inhibited by p-chlorophenoxyisobutyrate. However in absence of arterio-venous difference of glycerol concentrations across the ischaemic myocardium this study cannot confirm or deny this possibility. Nor can it elucidate whether the effect of p-chlorophenoxyisobutyrate on ST-segment elevation was associated with increased glucose utilisation in the ischaemic area. In this context

it is of interest that in one study where the effect of p-chlorophenoxyisobutyrate on ST-segment elevation was negligible (Dog 304), this was associated with a hypoglycaemic response.

3.1.5. Summary

Pretreatment with p-chlorophenoxyisobutyrate reduced ischaemia-induced ST segment elevation both in the basal state and during infusion of isoprenaline. p-Chlorophenoxyisobutyrate was also effective, when administered during an established coronary artery occlusion.

This beneficial effect of p-chlorophenoxyisobutyrate was not related to changes in heart rate, blood pressure or regional myocardial blood flow, but was associated with a reduction in arterial concentrations and myocardial extraction of plasma free fatty acids. These observations support the view that the severity of myocardial ischaemic injury can be reduced by effective antilipolytic treatment.

SECTION 3.2:

EFFECTS OF PROSTAGLANDIN-E1

Effects of prostaglandin-E1

Introduction

Prostaglandins are naturally occurring substances with wide-ranging haemodynamic (Horton and Ungar, 1975) and metabolic effects (Bergström et al, 1968). Prostaglandins E and A increase coronary blood flow in normal dogs (Horton and Ungar, 1975) and it has been suggested that they might be used clinically to increase blood flow to the ischaemic myocardium (Bloor et al, 1973).

In addition to increasing coronary blood flow prostaglandin E_1 also reduces adipose tissue mobilisation of free fatty acids. As was discussed in Part 1 of this thesis, this might be desirable. Prostaglandin E_1 has also been claimed to be anti-arrhythmogenic (Vergroesen et al, 1972), presumably by controlling the equilibrium between sympathetic and parasympathetic nervous system. It is also possible that prostaglandin-E_1 could inhibit platelet aggregation during acute myocardial ischaemia.

These beneficial effects of prostaglandin-E_1 stimulated us to study its role on plasma free fatty acid concentration, myocardial blood flow and ST-segment elevation in dogs with experimental myocardial infarction, since it was realised that all possible beneficial effects could

not be investigated. Prostaglandin-E_1 in low concentrations however actually may increase plasma concentrations of free fatty acids, and therefore a dose which has been shown to be consistently antilipolytic was selected (Galli et al, 1969).

3.2.1. Methods

Animal preparations

Experiments were carried out in 21 mongrel dogs of both sexes (12-25 kg body weight), using in 17 experiments the procedures described in Part 2 (Section 2.1) of this thesis.

A different preparation was used in four dogs whereby heart rate, left ventricular end diastolic pressure and mean aortic pressure could be kept constant (Kane and Ungar, 1976). The heart was exposed by a mid-sternal split. The left atrium was cannulated and connected to a reservoir after heparinisation of the dogs (2000 U). Left ventricular end diastolic pressure was computed on-line using an analogue computer and maintained constant by varying the height of the fluid column in the left atrial reservoir. Mean aortic blood pressure was kept constant using a pressurised flask connected to both femoral arteries.

If changes in peripheral resistance occurred, equilibrium between two reservoirs was maintained by pumping blood from one to the other with a Watson-Marlow MHRE

roller pump. A water jacket and a heated, siliconised glass coil in the reservoir were used to maintain the fluid in the system near 37°C. The system was primed with a solution of 2.5% (w/v) dextran, which was allowed to mix with the blood in the dog in order to minimise later changes in haematocrit during the observation periods. Right atrial pacing was used to keep the heart rate constant. Arterial pressure was measured through a carotid arterial cannula. Left ventricular pressure was measured using a metal cannula inserted through the dimple of the apex and directly connected to the pressure transducer (L223, Consolidated Electrodynamics, England). The frequency response of the overall system was flat within 5% at 40 Hz. dP/dt max. was derived by the analogue computer. The initial values of heart rate, mean aortic blood pressure and left ventricular end diastolic pressure were chosen close to those observed prior to the first infusion and then maintained constant during the observation periods. After release of the occlusion heart rate, left ventricular end diastolic pressure and mean aortic blood pressure were no longer controlled.

Measurements

Electrocardiographic recordings were performed with a mobile cotton wick electrode at 10-15 anatomically well defined sites on the epicardial surface as described in Section 2.3.1.

Regional myocardial blood flow was measured using radioactive microspheres, as described in Section 2.4.

Biochemical analyses

Plasma concentrations of free fatty acids (Section 2.2.2), glucose (Section 2.2.3) and free glycerol (Section 2.2.4) were determined according to the standard procedures.

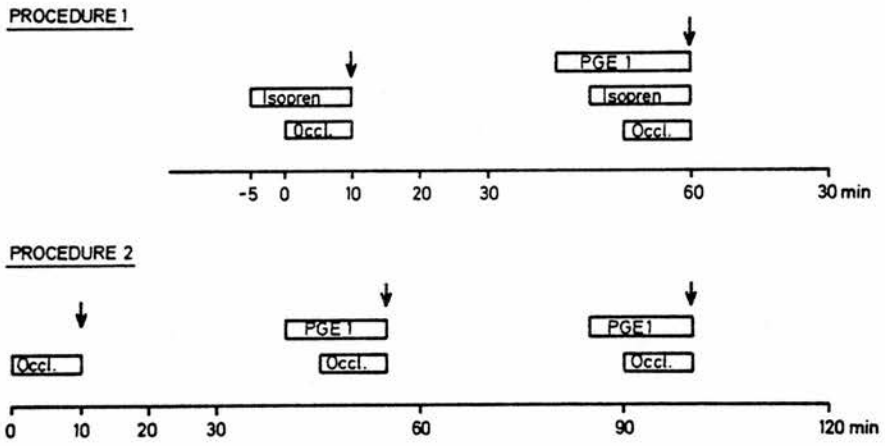
In the 4 heparinised dog studies the blood samples were centrifuged immediately after collection and fatty acids were extracted without delay, to prevent in vitro lipolysis. This precaution was taken despite the fact, that plasma triglyceride levels are too low to cause significant - in vitro - increase in free fatty acid concentration.

3.2.2. Experimental procedures

The basic design was to induce two or three discrete periods of myocardial ischaemia each of 10-14 min duration, separated by a recovery period of 30 min, as described in Section 2.1.3. Three series of experiments were performed.

In the first series (Procedure 1, Fig 3.02) the effect of prostaglandin-E_1 was studied on ST segment elevation during lipolysis stimulated by a continuous infusion of isoprenaline in nine dogs (dogs 1-9). Intravenous isoprenaline 0.1-0.2 $\mu g\ kg^{-1}min^{-1}$ was commenced 5 min prior to the control occlusion and maintained throughout. At 10 min of ischaemia the epicardial electrocardiogram, heart rate and mean aortic blood pressure were recorded and microspheres injected into the left atrium for measurement of regional myocardial blood flow. An arterial blood sample was taken for the determination of plasma concentration of free fatty acids. The occlusion was released and the isoprenaline infusion stopped. After a 30 min recovery period an intravenous infusion of prostaglandin-E_1 0.6 $\mu g\ kg^{-1}min^{-1}$ was given, followed 5 min later by an isoprenaline infusion. Both infusions were maintained during this occlusion. All measurements: epicardial ECG (ΣST), heart rate, mean aortic blood pressure and regional myocardial blood flow were

Figure 3.02 Protocol for the study of the effect of prostaglandin E_1 (PGE_1) on ST-segment elevation during isoprenaline-stimulated and basal lipolysis



The basic experimental design consisted of two or three intermittent occlusions (Occl). Prior to release, recordings of epicardial electrocardiograms were made, and an arterial blood sample was obtained for biochemical parameters (arrows). Regional myocardial blood flow was determined by injection of 1.5-3.0 x 10^6 microspheres into the left atrium immediately before the release of the occlusion.
Further details see text.

repeated (Fig 3.02).

In two experiments (dogs 7 and 9) the sequence was reversed, isoprenaline and prostaglandin-E_1 being infused during the first occlusion period. After release and recovery, isoprenaline alone was infused for the second occlusion period. This was done in order to check whether the effects of prostaglandin-E_1 were related to the time of administration.

In the second series (Procedure 2, Fig 3.02) the effect of prostaglandin-E_1 administered intravenously (0.6 μg $kg^{-1}min^{-1}$) or intra-atrially (0.03 μg $kg^{-1}min^{-1}$) was studied in eight dogs (dogs 10-17). Three occlusions of approximately 10 min duration were made as described above. The first occlusion served as a control. The other occlusions were made 5 min after the start of either a continuous prostaglandin-E_1 infusion into the femoral vein or into the left atrium. The infusion of prostaglandin-E_1 into the left atrium was made in order to check whether prostaglandin-E_1 or its metabolites were the active components. Normally up to 90% of prostaglandin-E_1 is removed on a single passage through the lungs. The order in which the prostaglandin-E_1 infusions were administered was randomised. ΣST segment elevation, heart rate and mean aortic blood pressure were measured immediately prior to the release of the occlusion. In

five dogs, microspheres were injected for measurement of regional myocardial blood flow after 10 min of occlusion during each of the three experimental occlusions described above. Isoprenaline was not given to dogs in the second series.

In the third series the effect of intravenous prostaglandin-E_1 (0.6 $\mu g\ kg^{-1}min^{-1}$) was studied in four dogs (dogs 18-21), whilst heart rate, mean aortic blood pressure and left ventricular-end diastolic pressure were maintained constant.

Two occlusions of approximately 10 min duration were made as described above (Procedure 2, Fig 3.02), each 5 min after the start of a continuous infusion of either prostaglandin-E_1 or saline. The order of these infusions was randomised. Epicardial ECG (ΣST-segment elevation) heart rate, mean aortic blood pressure, left ventricular end diastolic pressure and left ventricular dP/dt max were measured immediately before and at 5 min intervals during the occlusion. Arterial (carotid artery) blood samples were obtained for the estimation of plasma free fatty acids.

Statistics

Each dog served as its own control. Student's t-test for paired data was used to calculate probability values.

3.2.3. Results

The effects of prostaglandin-E_1 during isoprenaline infusions

After the occlusion of a distal branch of the left anterior descending coronary artery, the onset of acute myocardial ischaemia was characterised by localised cyanosis, contractile asynergy and ST-segment elevation in the distribution of the ligated artery.

When the induction of myocardial ischaemia was made in the presence of isoprenaline ΣST segment elevation averaged 46 $\pm$ 6 mV (mean $\pm$ SEM) after 10 min of occlusion (Table 3.06). When the isoprenaline infusion was preceded by a continuous infusion of prostaglandin-E_1 ΣST averaged 34 $\pm$ 6 mV ($P < 0.001$). This effect of prostaglandin-E_1 was independent of the sequence of administration. The data from all dogs were pooled.

The lipolytic activity of isoprenaline was apparent from the high plasma free fatty acids concentrations (1925 $\pm$ 150 μmole/l). This was partly inhibited when prostaglandin-E_1 was simultaneously administered (1320 $\pm$ 220 μmole/l: $P < 0.005$) (Table 3.06).

The intravenous administration of prostaglandin-E_1 did not significantly alter mean aortic blood pressure

Table 3.06

The effect of intravenous prostaglandin-E_1 (PGE_1) on ischaemia and isoprenalin-induced ST-segment elevation (ΣST), mean aortic blood pressure ($\overline{AP}$) heart rate (HR) and arterial plasma concentrations of free fatty acids (FFA) during experimental myocardial ischaemia of 10 min duration

	ΣST (mV)		FFA (μmole/l)		$\overline{AP}$ (mmHg)		HR (beats/min)	
Dog no.	Occl. + Isopr.	Occl. + Isopr. + PGE_1	Occl. + Isopr.	Occl. + Isopr. + PGE_1	Occl. + Isopr.	Occl. + Isopr. + PGE_1	Occl. + Isopr.	Occl. + Isopr. + PGE_1
1	38	27	2100	1190	103	90	175	172
2	36	25	1290	520	69	55	172	151
3	42	35	1860	1550	103	109	195	189
4**	21	14	2650	2550	73	81	150	150
5	36	24	2520	2040	85	72	164	146
6[M]	78	62	2065	790	110	96	169	164
7[M]	74	62	1650	1070	130	130	173	175
8[M]	53	36	1600	750	105	88	142	141
9[M]	39	25	1580	1400	106	106	147	140
Mean	46	34	1925	1320	98	92	165	159
± SEM	6	6	150	220	6	7	6	6
P*	<0.001		<0.005		NS		<0.05	

Isoprenaline was administered intravenously at a dose of 0.1-0.2 μg $kg^{-1}min^{-1}$. PGE_1 at 0.6 μg $kg^{-1}min^{-1}$.

In dogs 1 -6 and 8 the first occlusion was made during isoprenaline infusion, followed by an occlusion made during an isoprenaline plus PGE_1 infusion, whereas in dog 7 and 9 the first occlusion was made during an isoprenaline plus PGE_1 infusion, followed by an occlusion during an infusion of isoprenaline alone.

*Probability values for comparison of paired data of occlusion + isoprenaline vs. occlusion + isoprenaline + PGE_1. NS = not significant ($P > 0.05$).

**Dose of PGE_1 = 0.3 μg $kg^{-1}min^{-1}$.

[M]Regional myocardial blood flow estimation (see Table 3.07).

during isoprenaline infusions, but a small and significant decrease in heart rate was observed (Table 3.06). Prostaglandin-E_1 administration had no effect on regional myocardial blood flow in the endocardial and epicardial layers of the ischaemic and non-ischaemic part of the left ventricle during the isoprenaline infusions (Table 3.07).

Effects of prostaglandin-E_1 infusions alone

In the absence of isoprenaline, prostaglandin-E_1 did not reduce plasma free fatty acid concentrations (Table 3.08), or alter plasma free glycerol and glucose concentrations.

The haemodynamic effects of prostaglandin-E_1 alone, were also different from those observed during isoprenaline infusions. Prostaglandin-E_1 alone did not change heart rate. Prostaglandin-E_1 reduced mean aortic blood pressure significantly, irrespective of which route was used (Table 3.08). This drop in mean aortic blood pressure was associated with a marked reduction in regional myocardial blood flow in epicardial and endocardial layers of the non-ischaemic myocardium (Table 3.09. The reduction was slightly more marked in the endocardial layers of the non-ischaemic myocardium, giving rise to a significant decrease in the endocardial-

Table 3.07

The effect of intravenous prostaglandin-E_1 (PGE_1) on regional myocardial blood flow in non-ischaemic and central plus peripheral ischaemic left ventricle during continuous isoprenaline infusion in four dogs with experimental acute myocardial ischaemia of 10 min duration

	Non-ischaemic			Ischaemic		
	Endocardium ($ml\ g^{-1}min^{-1}$)	Epicardium ($ml\ g^{-1}min^{-1}$)	Endocardial/epicardial ratio	Endocardium ($ml\ g^{-1}min^{-1}$)	Epicardium ($ml\ g^{-1}min^{-1}$)	Endocardial/epicardial ratio
Occlusion + isoprenaline	1.29 ± 0.06 (24)	1.45 ± 0.05 (24)	0.89 ± 0.03 (24)	0.82 ± 0.08 (20)	1.08 ± 0.09 (20)	0.76 ± 0.04 (20)
Occlusion + isoprenaline + PGE_1 i.v.	1.32 ± 0.06 (24)	1.47 ± 0.05 (24)	0.90 ± 0.03 (24)	0.84 ± 0.09 (20)	1.08 ± 0.10 (20)	0.78 ± 0.04 (20)
P*	NS	NS	NS	NS	NS	NS

*Probability values for comparison of paired data of occlusion + isoprenaline vs occlusion + isoprenaline + PGE_1 i.v.
NS = not significant ($P > 0.05$).

Table 3.08

The effect of prostaglandin-E_1 (PGE_1), administered intravenously (i.v.) or intra-atrially (i.a.) on ischaemia-induced ST-segment elevation (ΣST), mean aortic blood pressure ($\overline{AP}$), heart rate (HR) and arterial concentrations of plasma free fatty acids (FFA) during experimental myocardial ischaemia of 10 min duration

	ΣST (mV)			FFA (μmole/l)			$\overline{AP}$ (mmHg)			HR (beats/min)		
Dog no.	Occl.	Occl. + PGE_1 i.v.	Occl. + PGE_1 i.a.	Occl.	Occl. + PGE_1 i.v.	Occl. + PGE_1 i.a.	Occl.	Occl. + PGE_1 i.v.	Occl. + PGE_1 i.a.	Occl.	Occl. + PGE_1 i.v.	Occl. + PGE_1 i.a.
10	15	11	-	780	700	-	145	125	-	168	175	-
11	27	24	21	640	600	660	100	75	80	132	125	128
12	23	29	31	500	520	400	147	133	133	159	164	160
13**	8	-	8	400	-	460	135	-	124	-	-	-
14**	3	8	6	350	370	270	155	123	125	-	-	-
15**	27	9	19	370	440	565	115	88	86	190	182	184
16**	13	6	8	1050	-	880	112	97	100	218	220	224
17**	24	-	16	410	-	440	141	-	118	186	-	180
Mean	17	14	15	560	525	525	131	108	109	176	173	175
± SEM	3	4	3	85	60	75	7	10	8	12	15	16
P*		NS	NS		NS	NS		<0.001	<0.001		NS	NS

The first occlusion was a control occlusion. In dogs 11, 12 and 15 the second occlusion was made during an intravenous infusion of PGE_1, followed by a third occlusion during an intra-atrial infusion of PGE_1. In dogs 13, 14, 16 and 17 the second occlusion was made during an intra-atrial infusion of PGE_1, followed by a third occlusion during intravenous infusion of PGE_1 (dogs 14 and 16).

*Probability value for comparison of paired data vs. occlusion. NS = not significant ($P>0.05$).

**Regional myocardial blood flow estimation (see Table 3.09).

Table 3.09

The effect of prostaglandin-E_1, administered intravenously (i.v.) or intra-atrially (i.a.) on regional myocardial blood flow in non-ischaemic and central plus peripheral ischaemic left ventricle in five dogs with experimental acute myocardial ischaemia of 10 min duration

	Non-ischaemic			Ischaemic		
	Endocardium (ml $g^{-1}min^{-1}$)	Epicardium (ml $g^{-1}min^{-1}$)	Endocardial/epicardial ratio	Endocardium (ml $g^{-1}min^{-1}$)	Epicardium (ml $g^{-1}min^{-1}$)	Endocardial/epicardial ratio
Control occlusion	1.76 ± 0.08 (42)	1.62 ± 0.07 (42)	1.09 ± 0.02 (42)	0.95 ± 0.13 (20)	1.07 ± 0.11 (19)	0.85 ± 0.08 (20)
Occlusion + PGE_1 i.v.	1.12 ± 0.10 (17)	1.15 ± 0.09 (17)	0.96 ± 0.02 (17)	0.56 ± 0.13 (8)	0.74 ± 0.16 (8)	0.78 ± 0.10 (8)
P*	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.05
Occlusion + PGE_1 i.a.	1.18 ± 0.04 (33)	0.99 ± 0.03 (33)	1.02 ± 0.02 (33)	0.54 ± 0.08 (16)	0.71 ± 0.08 (16)	0.73 ± 0.07 (16)
P*	< 0.001	< 0.001	< 0.001	< 0.005	< 0.001	NS

Results are expressed as mean ± SEM (number of observations).

*Probability value of comparison of paired data vs control. NS = not significant ($P > 0.05$).

epicardial blood flow ratio ($P < 0.005$). Prostaglandin-E_1 (both administered intravenously or intra-atrially) also reduced myocardial blood flow in the ischaemic epi- and endocardial layers ($P < 0.005$), and the reduction was possibly more pronounced in the endocardial layers. However, no change was observed in ST-segment elevation after 10 min of occlusion (Table 3.08). Therefore the studies were extended to another series in which the blood pressure as well as heart rate and left ventricular end diastolic pressure were controlled. Under these conditions intravenous prostaglandin-E_1 reduced ΣST in three out of four experiments. This effect was not related to plasma free fatty acid concentrations, which tended to increase (Table 3.10).

Table 3.10

The electrocardiographic, haemodynamic and metabolic effects of intravenous prostaglandin-E_1 during coronary artery occlusion under conditions of controlled preload, afterload and heart rate

	ΣST (mV)		dP/dt max. (mmHg s^{-1})		$\overline{AP}$ (mmHg)		LVEDP (mmHg)		FFA (μmole/l)	
Dog no.	Occl.	Occl. + PGE_1	Occl.	Occl. + PGE_1	Occl.	Occl. + PGE_1	Occl.	Occl. + PGE_1	Occl.	Occl. + PGE_1
18	148	136	3520	3720	96	84	3.9	3.0	360	260
19*	202	155	1250	3300	67	68	2.0	2.5	360	460
20*	151	122	3060	2790	80	83	9.0	8.9	410	600
21*	59	74	1750	1740	77	73	4.3	5.5	455	520

*In dog 18 an occlusion of 10 min duration was made during an infusion of saline followed by a second occlusion of 10 min during an infusion of PGE_1, whereas in dog 19, 20 and 21 the first occlusion was made during infusion of PGE_1 and the second occlusion during the infusion of saline.

dP/dt max. = peak of the first derivative of left ventricular pressure.

LVEDP = left ventricular end diastolic pressure.

Other abbreviations as in Table 3.06.

3.2.4. Discussion

The effect of prostaglandin-E_1 during isoprenaline infusions

During experimental myocardial ischaemia under conditions of high adrenergic drive (isoprenaline infusions), prostaglandin-E_1 reduced ST-segment elevation, which is thought to reflect the severity of acute myocardial ischaemia (Wegria et al, 1949).

Arterial concentrations of plasma free fatty acids were lowered by prostaglandin-E_1 due presumably to its inhibitory effect on the lipolytic activity of isoprenaline. This is likely to have led to reduced myocardial extraction of free fatty acids. Arterial-coronary sinus differences were not measured but proportionality between arterial concentration and myocardial extraction has been found (see Part 1). This applies also to the extraction of free fatty acids by the ischaemic myocardium (Riemersma et al, 1972). In the presence of acute myocardial ischaemia, such reduction in plasma concentrations of free fatty acids after the administration of antilipolytic drugs was also associated with reduced signs of ischaemia, such as ΣST (Kjekshus and Mjøs, 1973; Lekven et al, 1973; Mjøs et al, 1974; Ilebekk and Mjøs, 1974).

Factors changing myocardial oxygen requirements relative to oxygen supply, such as heart rate and blood pressure, also influence the extent of ST-segment elevation (Maroko et al, 1971; Redwood et al, 1972). In this study during isoprenaline infusions, prostaglandin-E_1 did not change mean aortic blood pressure, and the small reduction in heart rate which was observed seems too small to account for the reduction in ST-segment elevation.

Direct effects of prostaglandin-E_1 have also to be considered. ST-segment elevation can occur when resting membrane potential is reduced (Prinzmetal et al, 1961). Prostaglandin-E_1 had no effect on resting membrane potential in mammalian, well oxygenated myocardial cells and, when used in very high concentrations, caused a decrease (Kecsmeti et al, 1974). This would tend to increase ST-segment elevation. Since prostaglandin-E_1 reduced ΣST, it seems unlikely that an effect on the resting membrane potential could explain our findings. Prostaglandin-E_1 could have exerted its effect by inhibiting platelet aggregation in the ischaemic myocardium. Although platelet aggregation at 15 min of occlusion in this model was demonstrated (Vik-Mo, 1978), it is of insufficient magnitude to cause severe impair-

ment of blood flow to the ischaemic myocardium (Vik-Mo, 1978). The fact that in our experiments during isoprenaline infusion prostaglandin-E_1 did not change myocardial blood flow, supports the view that inhibition of platelet aggregation was not its mode of action.

The effects of prostaglandin-E_1 in the absence of isoprenaline

During experimental myocardial ischaemia, but in the absence of isoprenaline, the effect of prostaglandin-E_1 on occlusion-induced ST-segment elevation was dependent on the experimental conditions. In the main series of experiments during basal lipolysis, prostaglandin-E_1 effected no change in occlusion-induced ST-segment elevation. But a marked reduction was observed in mean aortic blood pressure and regional myocardial blood flow both in the ischaemic and non-ischaemic areas of the myocardium. Similar haemodynamic effects have been reported, when prostaglandin-E_1 was given to cats with acute myocardial ischaemia (Hutton et al, 1973), but ST-segment measurements were not made. Since hypotension has been shown to increase occlusion-induced ST-segment elevation (Maroko et al, 1971; Redwood et al, 1972), the observation of unaltered ST-segments by prostaglandin-E_1 needs comment. It cannot be explained by increased myocardial

uptake of free fatty acids, since plasma concentrations of fatty acids were unaltered by prostaglandin-E_1. A possible explanation was that the expected increase in ΣST due to hypotension was counteracted by some opposing influence. To test this hypothesis further studies were made. In these studies, conducted with controlled mean aortic blood pressure and left ventricular end diastolic pressure, prostaglandin-E_1 reduced ST-segment elevation. While these studies do not explain the nature of this opposite action, they appear to exclude mechanisms based on changes in metabolism of plasma free fatty acids, but ischaemia-induced myocardial lipolysis, thought to be mediated by noradrenaline release, may have been inhibited.

Patients with acute myocardial infarction have increased adrenergic drive. During nervous stimulation (Wennmalm and Stjärne, 1971) and hypoxia (Wennmalm et al, 1974) endogenous prostaglandins are released from the sympathetically innervated heart and prevent excessive stimulation of the myocardium by noradrenaline. Indomethacin, which inhibits prostaglandin synthesis, increased infarct size after experimental coronary artery occlusion in conscious dogs (Jugdutt et al, 1978). Their and our findings suggest that prostaglandins play a role in de-

termining the viability of the ischaemic myocardium.

It needs to be stressed that the effect of intravenous prostaglandin infusions may be quite different from that of locally released prostaglandin from the ischaemic myocardium. In the latter situation this may be devoid of an effect on adipose tissue lipolysis.

In the present study pharmacological doses of prostaglandin-E_1 were used and due to its side effects (Bergström et al, 1959) may not be useful to reduce myocardial infarct size in man.

3.2.5. Summary

Prostaglandin-E_1 reduced isoprenaline-stimulated myocardial ischaemia, through its antilipolytic action, without affecting regional myocardial blood flow. However in the absence of catecholamine stimulation the main effect is to reduce blood pressure, resulting in reduced regional myocardial blood flow, and thereby counterbalancing any potential beneficial metabolic effects on the ischaemic myocardium.

SECTION 3.3:

EFFECTS OF ACEBUTOLOL

Effects of a 'cardioselective' β-blocker: acebutolol

Introduction

Patients with acute myocardial infarction have increased activity of the sympathetic nervous system, as evidenced by increased plasma concentrations of catecholamines (Gazes et al, 1959; Valori et al, 1967; McDonald et al, 1969; Christensen and Videbaeck, 1974; Vetter et al, 1974) or by increased urinary catechol excretion (Jewitt et al, 1969; Prakash et al, 1972). The origin of raised catecholamines is complex, but appears to stem from the adrenals (Ceremużyński et al, 1969) and from the ischaemic myocardium (Shahab et al, 1969). Raised catecholamines are commonly considered to be detrimental to the acutely ischaemic heart and thus prevention of their (haemodynamic and electrophysiological) effects should be beneficial. This has stimulated the search for drugs which by their haemodynamic and vascular action would specifically reduce the consumption of oxygen by the ischaemic heart; 'cardioselective' β-blocker (e.g. practolol, acebutolol).

It was of interest whether the beneficial effect of β-blockade was due to their heart rate lowering property, or whether non-'cardioselective' effects on

fat and glucose metabolism were also contributory. Therefore the effects of acebutolol on the severity of myocardial ischaemia, arterial concentrations of free fatty acids and glucose and on myocardial metabolism were investigated during experimental myocardial ischaemia in dogs.

3.3.1. Methods

Animal preparation

Experiments were carried out in 11 mongrel dogs of both sexes (11-21 kg body weight), fasted for 12-15 hours, as described in Part 2 (Section 2.1) of this thesis.

Electrocardiographic monitoring

In this study electrocardiograms were recorded using an epicardial grid with 10-15 silver electrodes. Initially the mathematical mean electrocardiogram was computed by an electronic averaging device, and more recently (experiments without pacing) using a multiplexer system, as described in Section 2.3.1.

Acebutolol hydrochloride (May and Baker) was a gift from Dr. Khambatta, was administered in slow intravenous injection over 5 min (dose 0.3 mg/kg).

Biochemical analyses

Standard procedures were used for the processing of blood samples and analyses of plasma free fatty acids (Section 2.2.2), plasma glucose (Section 2.2.3) and plasma glycerol (Section 2.2.4). Insulin was measured by a radio-immunoassay in the laboratory of Dr. E. Cameron, Regional Hormone Laboratory, Department of Clinical Chemistry, The Royal Infirmary, Edinburgh. Dog insulin (Novo, Denmark) was used as the standard.

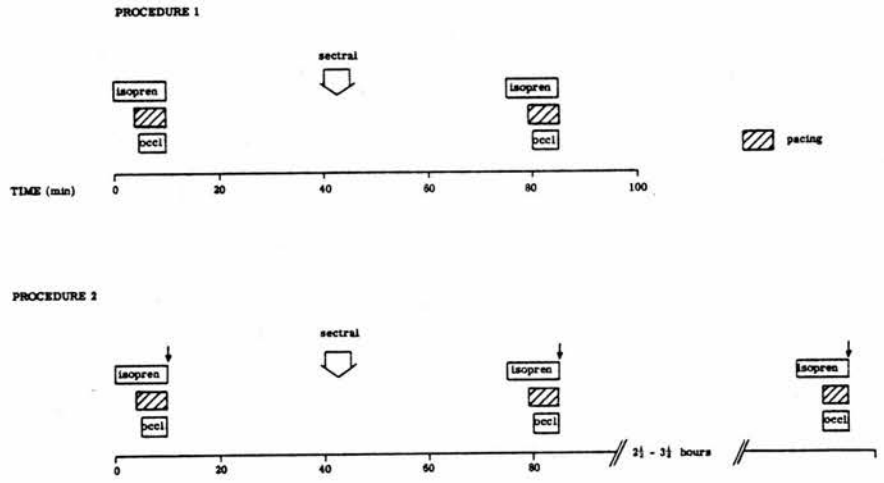
3.3.2. Experimental procedures

The basic experimental design was two to four discrete periods of ischaemia of 5-7 min duration, separated by a recovery period of 30 min, as described in Section 2.1.3. The protocol used is presented in Fig 3.03.

Two series of experiments were performed. In the first the effects of acebutolol was examined in dogs, whilst heart rate was maintained constant by right atrial pacing. Pacing frequencies were 10 above basal heart rate for both the control occlusion and the occlusion after acebutolol. For the occlusions during isoprenaline infusions (control and acebutolol) the heart rate was kept at 10 above the heart rate after 5 min isoprenaline infusion in the absence of acebutolol. $\overline{ST}$ segment elevation was recorded before and at 5 min of coronary artery occlusion. Blood samples for the biochemical analyses was obtained between 5-7 min of occlusion, at the same relative time during control and sectral occlusions.

In three dogs, two in the presence of and one in the absence of isoprenaline, the experimental design was modified to check on the effect of the sequence of administration. In these experiments a first occlusion (control) and a second occlusion (acebutolol) were made,

Figure 3.03. Experimental procedures for assessing the effect of acebutolol on the response to subsequent coronary artery occlusion



Four identical occlusions of 5-7 min duration were performed in each animal, two in the absence of acebutolol (0.3 mg/kg) and two in its presence. One pair of occlusions was performed during a continuous infusion of isoprenaline (0.1 μg/kg/min), started 5 min before the occlusion. Heart rate was maintained constant by right atrial pacing. Recovery periods of 30 min were allowed between successive occlusions. Immediately before the release of each occlusion (indicated by vertical arrows) haemodynamic measurements were recorded and blood samples were collected. Sectral = acebutolol.

followed by a period sufficiently long for the effects of acebutolol on heart rate to wear off ($2\frac{1}{2}$ to $3\frac{1}{2}$ hours). At this time a second control occlusion was made. The results of the first and second control occlusion were the same and the effects of acebutolol in these experiments were similar to those performed according to the basic procedure. Therefore the effects of acebutolol were assessed by paired analysis of all data from the 9 experiments.

In the second series the effects of acebutolol were examined in 5 dogs, without pacing. Two occlusions (5 dogs) were made 5 min after commencement of an intravenous infusion of isoprenaline (0.1 μg/kg/min), the first was the control and the second was made 30 min after acebutolol. In one of these another control occlusion was made (during isoprenaline) after a period sufficiently long for the effect of acebutolol on heart rate to wear off. The results of this study were similar and all results were pooled.

Statistics

Each dog served as its own control. Student's t-test for paired data was used to calculate probabilities. $P > 0.05$ was regarded as not statistically significant.

3.3.3 Results

The effects of acebutolol in dogs with constant heart rate

When myocardial ischaemia was induced in dogs in which heart rate was maintained at a constant level by right atrial pacing, acebutolol did not affect $\overline{ST}$-segment elevation in the absence or in the presence of isoprenaline infusion (Table 3.11). Mean aortic blood pressure was also not significantly different.

The effects of acebutolol on isoprenaline induced tachycardia and ST-segment elevation in unpaced dogs

Acebutolol inhibited the increase in heart rate in all but one experiment, on average by some 45 $\pm$ 8%. In the 4 experiments, in which heart rate was not maintained at a constant level, a marked reduction in $\overline{ST}$-segment elevation was observed : isoprenaline + occlusion : 8.9 $\pm$ 2.0 and acebutolol + isoprenaline + occlusion 5.3 $\pm$ 1.8 mV($P < 0.05$). Mean aortic blood pressure was not significantly changed but heart rate was (Table 3.12).

The metabolic effects of acebutolol (Table 3.13)

The effects of acebutolol in paced and unpaced experiments were similar and the data was pooled. Acebutolol reduced arterial concentrations of plasma free fatty acids, but not glycerol during basal and isoprenaline stimulated conditions. Arterial glucose concentrations were also reduced. These reductions were reflected in similar reductions

Table 3.11

The effect of acebutolol on ischaemia-induced mean ST-segment elevation ($\overline{ST}$) and mean aortic blood pressure ($\overline{AP}$) at constant heart rate* and during basal and isoprenaline-stimulated lipolysis

	Basal state					Isoprenaline infusion			
	$\overline{ST}$(mV)		$\overline{AP}$(mmHg)			$\overline{ST}$(mV)		$\overline{AP}$(mmHg)	
DOG	control	acebutolol	control	acebutolol	DOG	control	acebutolol	control	acebutolol
617	4.1	6.0	145	133	616	11.1	20.8	118	97
653	7.1	3.5	112	120	617	6.7	7.5	133	132
654	5.3	3.4	122	115	620	4.6	1.7	115	97
661	2.6	2.1	105	100	624	7.0	6.5	133	120
	3.6	2.1^{R}	97	100		5.2	6.5^{R}	118	120
Mean ± SEM	4.5 ± 0.8	3.4 ± 0.7	116 ± 8	114 ± 6		6.9 ± 1.1	8.6 ± 3.2	123 ± 4	113 ± 7
P**	NS		NS			NS		NS	

* Mean (±SEM) heart rate: basal state .175 ± 5; during isoprenaline infusion: 178 ± 5 beats/min.

** Probability value for comparison of paired data; acebutolol vs control. NS = not significant ($P > 0.05$).

R = reversed procedure.

Table 3.12

The effect of acebutolol on ischaemia-induced ST-segment elevation, mean aortic blood pressure ($\overline{AP}$) and heart rate (HR) during isoprenaline infusion (UNPACED experiments)

	$\overline{ST}$(mV)		$\overline{AP}$ (mmHg)		HR (beats/min)	
Dog	control	acebutolol	control	acebutolol	control	acebutolol
699	6.5	3.5	94	94	154	105
702	14.5	10.7	121	103	183	151
705	9.2	3.7	122	120	176	119
706	5.4	3.3	111	122	168	136

$\overline{ST}$ = average ST-segment elevation.

Table 3.13

The effect of acebutolol on art. concentrations and arterio-venous differences of plasma free fatty acids (FFA), glucose and glycerol during experimental coronary occlusion and isoprenaline infusion

	Occlusion			Occlusion + isoprenaline		
	n	control	acebutolol	n	control	acebutolol
FFA_a (μmole/l)	n = 8	510 ± 40	440 ± 25*	n = 12	990 ± 65	590 ± 45***
FFA_{a-cs}	n = 5	170 ± 20	60 ± 25***	n = 12	275 ± 20	160 ± 20***
FFA_{a-lv}	n = 5	180 ± 25	90 ± 40*		365	165
$Glucose_a$ (mmole/l)	n = 8	5.40 ± 0.17	4.96 ± 0.16***	n = 12	7.36 ± 0.25	6.59 ± 0.19**
$Glucose_{a-cs}$	n = 4"	0.51 ± 0.07	0.20 ± 0.08	n = 12	0.81 ± 0.22	0.26 ± 0.09*
$Glucose_{a-lv}$	n = 5	0.91 ± 0.10	0.55 ± 0.03**	n = 3"	1.66 ± 0.09	1.48 ± 0.11
$Glycerol_a$ (μmole/l)	n = 7	59 ± 16	49 ± 12	n = 10	138 ± 17	122 ± 18
$Glycerol_{a-cs}$	n = 3"	-2 ± 6	-10 ± 12	n = 9	17 ± 8	8 ± 4
$Glycerol_{a-lv}$	n = 4"	-2 ± 6	- 3 ± 4		-	-
$Insulin_a$ (ng/ml)	n = 3"	0.9 ± 0.3	0.4 ± 0.1	n = 8	2.8 ± 0.5	2.1 ± 0.3

"Probability values were calculated for comparison of paired data appropriate control only if 5 pairs or more were available

* $P<0.05$; ** $P<0.01$ and *** $P<0.001$.

Abbreviations: arterial concentrations, arterio-coronary sinus differences (across the predominantly non-ischaemic myocardium) and arterio-local venous differences (across the ischaemic zone) are indicated by the subscript a, a-cs and a-lv respectively.

in extraction of plasma free fatty acids and glucose across the non-ischaemic myocardium (arterio-coronary sinus difference). Arterial insulin levels tended to decrease, however this effect did not reach a level of statistical significance.

The effects of acebutolol on substrate extraction by the ischaemic myocardium

The extraction of metabolites across the ischaemic myocardium was studied by analysis of the effluent from the area supplied by the occluded artery. In this study however, difficulties were experienced in obtaining a complete set of blood samples (control and after acebutolol), particularly when isoprenaline infusions were used. As a result the interpretation of the data is restricted to the observations made in the basal state. The reduction in arterial plasma free fatty acid concentration by acebutolol was associated with a decrease in fatty acid extraction by the ischaemic myocardium. The ischaemia-induced increase in glucose extraction (compare control occlusion:glucose a-cs 0.51 ± 0.07 vs. glucose a-lv: 0.91 ± 0.10 mmole/l) was reduced by the administration of acebutolol. However during isoprenaline this effect may not be observed (no statistical test performed).

3.3.4. Discussion

These studies show that acebutolol reduced evidence of myocardial ischaemic injury in dogs, which were not electrically paced. This observation is in agreement with those of others using other β-blockers such as propranolol (Maroko et al, 1971; Serrano et al, 1971; Becker et al, 1975) and using the 'cardioselective' drug practolol (Libby et al, 1973[b]). The mechanism of this beneficial effect appears to be complex. A reduction in heart rate limits the extent of myocardial ischaemia per se (Maroko et al, 1971). However propranolol can not be equated with the 'cardioselective' β-blockers. In an elegant study Marshall and Parratt (1976) showed that, although propranolol and practolol both depressed myocardial function and oxygen demand, practolol unlike propranolol, did not reduce myocardial blood flow to the ischaemic area. This was explained by an increase in left ventricular end diastolic pressure after propranolol, which was much less after practolol. The reduction in coronary perfusion pressure after practolol was balanced by an increase in the period, during which the coronary perfusion pressure exceeds the trans-ventricular pressure leading to myocardial perfusion (effective perfusion period). As a result oxygen supply

to the ischaemic myocardium was not depressed and was apparently sufficient to meet the oxygen demand in view of the ceased lactate production.

On the other hand, they showed that practolol caused a pronounced deterioration of cardiac function in 2 experiments in which heart rates were maintained constant by electrical pacing. In our studies acebutolol did not effect a beneficial(nor an overt detrimental) effect, despite the fact that the reduced extraction of plasma free fatty acids was not modified by pacing.

Therefore the contribution of reduced fatty acid metabolism to the development of ischaemic injury may superficially appear insignificant. However acebutolol also reduced myocardial extraction (Δ arterio-local vein) of glucose by the ischaemic myocardium (in the absence of isoprenaline)and therefore the relative contributions of each substrate to total myocardial metabolism was unaltered. The effect of acebutolol on glucose extraction by the ischaemic myocardium was not apparent during isoprenaline infusion. However, due to technical difficulties insufficient observations were obtained.

The reduced glucose extraction is possibly due to the reduction of arterial glucose and insulin concentrations. Similarly the decreased arterio-venous difference of free fatty acids across the ischaemic myocardium

is likely to be caused by reduced plasma concentrations of free fatty acids due to inhibition of catecholamine-induced lipolysis.

The effects are similar to those of propranolol (Opie and Thomas, 1976). They showed reduced arterial concentrations of glucose and free fatty acids. However despite the reduction in glucose concentration, propranolol increased the extraction of glucose by the ischaemic myocardium. The discrepancy between their and our results can not be explained. Neither they nor we measured regional myocardial blood flow in the ischaemic area and it may well be the residual blood flow which determines glucose uptake by the ischaemic myocardium, as demonstrated using an ischaemically perfused rat heart preparation (Neely et al, 1975) could have been an important difference.

If the results of our experiments of experimental myocardial ischaemia in anaesthetised dogs can be extrapolated to patients with acute myocardial infarction at all, then it appears that the predominant effect of acebutolol is reduction of heart rate. Although metabolic effects can not be excluded, their contribution to the severity of ischaemic injury may be of lesser importance.

3.3.5. Summary

In experiments with controlled heart rate, acebutolol did not reduce the severity of acute myocardial ischaemic injury, despite the fact that the extraction of plasma free fatty acids by the ischaemic myocardium was reduced. However glucose extraction was also diminished, and the relative contribution of glucose and free fatty acids to myocardial metabolism was unaltered. However when heart rate was allowed to decline, a marked reduction in ST-segment elevation and a similar reduction in plasma free fatty acid concentrations was observed. Thus the beneficial effect of acebutolol appears to be due predominantly to the reduction in heart rate.

SECTION 3.4:

EFFECTS OF SODIUM SALICYLATE

INTRODUCTION

Sodium salicylate has been shown to reduce the extent of myocardial ischaemic injury in experimental studies (Vik-Mo, 1977; Vik-Mo and Mjøs, 1977). This was related to its plasma free fatty acids lowering effect. However extraction of free fatty acids and of glucose by the ischaemic myocardium could not be determined in these studies. Therefore the effect of sodium salicylate on ischaemic myocardial metabolism was examined.

3.4.1. Methods

Animal preparation

Studies were performed in 7 overnight-fasted mongrel dogs of either sex, with an average weight of 22 kg, as described in Part 2 (Section 2.1) of this thesis. The coronary sinus was catheterised without fluoroscopic control as described in Section 2.1.4.

Haemodynamic measurements

Mean aortic blood pressure ($\overline{AP}$) and left ventricular pressure were recorded on A Dynograph recorder (Type 411, Beckman Instruments Inc., Schiller Park, Ill., USA), as described in Appendix E.

Metabolic measurements

Plasma concentrations of free fatty acids were analysed using the radiochemical method of Ho (1970) and plasma glucose was estimated by a manual glucose oxidase method using a commercial kit (Boehringer Mannheim Gmbh, Mannheim, Germany) in Tromsø. Frozen plasma samples for the analyses of lactate were shipped by air in dry-cold (-56°C) to Edinburgh, where they were estimated using the enzymic fluorimetric method described in Section 2.2.5.

Packed cell volumes were determined by a micro-haematocrit centrifuge, and oxygen saturations were measured spectrophotometrically (The Oxygen Saturation Meter, type OSM 1, Radiometer, Copenhagen, Denmark).

In Tromsø all analyses were performed in triplicate. The coefficients of variations for the plasma free fatty acid method and glucose method were 3% and 2% respectively.

3.4.2. Experimental procedure

Experiments were carried out both under basal conditions and during continuous intravenous infusion of isoprenaline at a rate of 0.10 - 0.20 µg/min/kg. Generally four intermittent occlusions were performed in each animal (see Section 2.1.5). This allowed each animal to serve as its own control for the assessment of the effect of treatment during basal and isoprenaline stimulated conditions. Haemodynamic measurements were performed immediately before and 12 min following the occlusion of the coronary artery. Blood samples were obtained from 6 to 12 min after induction of myocardial ischaemia. The coronary artery occlusion was released after 12 min and a recovery period of 45 min was allowed.

A second occlusion was performed after 10 min of isoprenaline infusion when the haemodynamic variables had stabilised. All measurements were repeated. After release and recovery, sodium salicylate (60 mg/kg as a slow injection followed by a continuous infusion at a dose of 0.15 mg/min/kg dissolved in 0.9% (w/v) saline, pH 7.4) was started. After 30 min a third occlusion was made, followed after release and recovery by a fourth occlusion during isoprenaline. Care was taken that the haemodynamic and electrocardiographic measurements were always done and blood samples were always obtained in the

same order, and at the same time after occlusion.

In two of the seven dogs given sodium salicylate only experiments without infusion of isoprenaline were conducted.

Statistics

Each dog served as its own control. Wilcoxon's non-parametric test (two-tailed) for paired data was used to calculate probabilities. Statistical comparisons are not performed between samples of 5 or less. $P > 0.05$ was regarded as not statistically significant. Standard error of the methods was calculated from triplicate analyses of 4 randomly selected experiments, and is expressed relative to the mean as coefficient of variation.

3.4.3. Results

Metabolic and haemodynamic effects of sodium salicylate

In the basal state sodium salicylate administration reduced arterial concentrations of plasma free fatty acids ($P < 0.05$, $n = 7$), whereas no changes in arterial oxygen saturation or plasma concentrations of glucose and lactate were observed. The reduced arterial concentration of plasma free fatty acids was associated with a reduction of fatty acids across the ischaemic and non-ischaemic myocardium ($P < 0.05$, $n = 7$, Table 3.14).

Sodium salicylate did not modify the haemodynamic state: no significant differences were observed in heart rate, left ventricular systolic pressure or the maximum of its first derivative.

During isoprenaline infusion sodium salicylate reduced arterial concentrations of plasma free fatty acids in all 5 experiments, and the free fatty acid extraction (a-lv) was consistently reduced. Arterial plasma lactate concentrations were not affected by sodium salicylate (5 experiments), and although an average lactate release by the ischaemic myocardium increased after sodium salicylate it actually was reduced in 2 out of 3 experiments. However these observations were very limited due to difficulties in obtaining complete sets of blood

Table 3.14

The effects of sodium salicylate on haemodynamic and biochemical measurements during coronary artery occlusion under control conditions (n=7) and during isoprenaline infusion (n=5)

	Occlusion	Salicylate + occlusion	Isoprenaline + occlusion	Salicylate + Isoprenaline + occlusion
HR (beat/min)	151 ± 8	157 ± 6	183 ± 4	183 ± 6
LVSP (kPa)	17.4 ± 0.7	16.9 ± 1.1	16.9 ± 0.7	18.1 ± 0.5
LV dP/dt max. (kPa/s)	293 ± 30	302 ± 32	411 ± 14	387 ± 18
$\overline{AP}$ (kPa)	15.5 ± 0.7	15.5 ± 1.1	13.3 ± 0.3	13.7 ± 0.8
$Oxygen_a$(%)	97.8 ± 1.5	94.8 ± 3.0	98.3 ± 1.8	96.0 ± 3.1
$Oxygen_{a-cs}$	60.5 ± 3.6	55.8 ± 4.5	59.2 ± 2.5	50.5 ± 5.8
$Oxygen_{a-lv}$(%)	63.9 ± 0.9	56.1 ± 6.4	60.6 ± 3.6	56.9 ± 5.1
$Glucose_a$(mmole/l)	7.34 ± 0.52	7.99 ± 0.82	8.35 ± 1.02	8.76 ± 0.09
$Glucose_{a-cs}$	0.63 ± 0.25	0.99 ± 0.34	0.63 ± 0.37	0.94 ± 0.65
$Glucose_{a-lv}$	1.12 ± 0.20	1.35 ± 0.31	1.11 ± 0.55	1.28 ± 0.66
$Lactate_a$**(nmole/l)	1.89 ± 0.35	1.39 ± 0.29	1.81 ± 0.57	1.81 ± 0.34
$Lactate_{a-cs}$	0.64 ± 0.09	0.27 ± 0.19	–	–
$Lactate_{a-lv}$**	-1.75 ± 0.54	-1.73 ± 0.60	-0.90 ± 0.47	-1.47 ± 0.87
FFA_a(µmole/l)	340 ± 41	233 ± 39*	1764 ± 357	801 ± 312
FFA_{a-cs}	108 ± 21	66 ± 13*	206 ± 100	111 ± 28
FFA_{a-lv}	96 ± 16	40 ± 8*	321 ± 84	121 ± 36

Abbreviations: HR= heart rate, LVSP= left ventricular systolic pressure, LV dP/dt max.= the maximum of the first derivative of the left ventricular pressure, FFA= plasma free fatty acids.

The subscripts a, a-cs and a-lv refer to the arterial concentration, arterio-coronary sinus difference and arterio-local venous difference in concentration respectively.

Probability values were calculated using the Wilcoxon's non-parametric test for paired data. $P > 0.05$ was regarded as not significant.

*P values for comparison vs control occlusion < 0.05.

The lactate data and the observations made during isoprenaline were not examined by statistical analysis ($n \leq 5$, see text).

**In three experiments with isoprenaline.

samples in the experiments. The utilisation of oxygen and glucose did not appear to be modified by sodium salicylate, and the mechanical activity of the heart was mainly unaltered.

3.4.4. Discussion

The administration of sodium salicylate before the induction of experimental acute myocardial ischaemia reduced the magnitude and the extent of myocardial ischaemic injury in dogs (Vik-Mo, 1977; Vik-Mo and Mjøs, 1977). The effect of sodium salicylate was assumed to be due to its antilipolytic property, since blood flow to the ischaemic myocardium was not affected (Vik-Mo, 1977). In the present study we have demonstrated that the extraction of plasma free fatty acids by the ischaemic myocardium is indeed substantially reduced by sodium salicylate. This was not associated with reduced lactate release. It should be noted that these findings occurred without significant change in glucose extraction by the ischaemic heart, although glucose extraction tended to increase. Glucose extraction by the non-ischaemic myocardium followed a similar pattern. This raises the possibility that the manual glucose method might not have been precise enough to pick-up a small increase in glucose extraction by the ischaemic heart after sodium salicylate, although the results did demonstrate the well-known increase in glucose extraction across the ischaemic heart (a-lv) in comparison to that by the non-ischaemic myocardium (a-cs).

This most probably means that during myocardial ischaemia salicylate lowers fatty acid supply to the ischaemic cell, and thereby reduces the α-glycerophosphate (and ATP) requirements for triglyceride synthesis and more glucose may be available for immediate energy purposes.

However a direct inhibition of ischaemia-induced myocardial lipolysis (Opie et al, 1973; Hough and Gevers, 1975) by sodium salicylate cannot be excluded.

It could be argued that sodium salicylate could have effected the observed reduction in severity of myocardial ischaemic injury by inhibiting prostaglandin synthesis (Vane, 1971). This is unlikely, however, since prostaglandins prevent excessive stimulation of adrenergic receptors by noradrenaline release (Wennmalm, 1976), and therefore inhibition of prostaglandin synthesis by sodium salicylate would lead to unopposed stimulation of the myocardium by noradrenaline, released from the ischaemic myocardium (Shahab et al, 1969). Indomethacin, which also inhibits prostaglandin synthesis, has been shown to increase myocardial ischaemic injury (Jugdutt et al, 1978).

In high doses salicylates increase total body oxygen consumption in dogs and man (Tenney and Miller, 1955) and may uncouple oxidative metabolism in vitro (Brody, 1956).

However in this study and in a recent study using a similar dose, myocardial oxygen consumption was not raised (Vik-Mo and Mjøs, 1976).

The results of experimental models should only be extrapolated to the clinical situation in patients with acute myocardial infarction with great caution. The biochemical effects of this study and the evidence of reduced myocardial ischaemic injury in a previous study (Vik-Mo and Mjøs, 1977), support the view that ischaemic myocardial metabolism may influence the viability of the heart.

3.4.4. Summary

Sodium salicylate reduced arterial concentrations of plasma free fatty acids and their extraction by the ischaemic and non-ischaemic myocardium, during basal and isoprenaline stimulated lipolysis. The extraction of glucose and oxygen and the production of lactate were not affected. Nor was the haemodynamic state altered.

These results and the evidence of reduced myocardial ischaemic injury in a previous study, support the view that ischaemic myocardial metabolism may influence the viability of the heart.

SECTION 3.5:

EFFECTS OF NICOTINIC ACID

INTRODUCTION

Nicotinic acid is known to have antilipolytic activity. Catecholamine-induced lipolysis can completely be abolished (Kjekshus and Mjøs, 1973), which was not observed with the other drugs used to lower plasma concentrations of free fatty acids. Thus the impression is that nicotinic acid is most effective, although admittedly, a formal comparative study has not been made.

Nicotinic acid and its derivative β-pyridylcarbinol reduced myocardial ischaemic injury during experimental coronary occlusion in dogs (Vik-Mo, 1977; Kjekshus and Mjøs, 1973) and in patients with acute myocardial infarction (Russell and Oliver, 1978; Kjekshus, 1978). Reduction in myocardial oxygen requirements, due to reduced plasma concentrations and uptake of free fatty acids, was assumed to be the mode of action. However, the metabolism of the ischaemic myocardium could not be assessed. Therefore the effect of nicotinic acid on the metabolism of plasma free fatty acids, glucose and lactate by the ischaemic myocardium was examined. This was accomplished by measurement of arterio-venous differences of plasma concentrations of free fatty acids, glucose and

lactate across the ischaemic and non-ischaemic myocardium during basal and isoprenaline-stimulated lipolysis, preceding and after administration of nicotinic acid.

Furthermore, since glycerol is not utilised in the myocardium to any significant amount (Robinson and Newsholme, 1967), the effect of nicotinic acid on myocardial lipolysis was studied by measurement of glycerol release.

3.5.1. Methods

Animal preparation

Studies were performed on 7 overnight-fasted mongrel dogs of either sex, weighing on average 22 kg as described in Part 2 (Section 2.1) of this thesis.

Haemodynamic measurements

Mean aortic blood pressure ($\overline{AP}$), left ventricular pressure, dP/dt were recorded on a Dynograph recorder (Type 411, Beckman Instruments Inc., Schiller Park, Ill., USA) as described in Appendix E.

Metabolic measurements

The biochemical procedures were identical to those described in Section 3.4.1., except that plasma glycerol was also analysed in Edinburgh, using the fluorimetric enzymic method presented in Section 2.2.4.

3.5.2. Experimental procedure

Experiments were carried out both under basal conditions and during continuous intravenous infusion of isoprenaline at a rate of 0.10 - 0.20 μg/min/kg. Generally four intermittent occlusions were performed in each animal (see Section 2.1.3). This allowed each animal to serve as its own control for the assessment of the effect of treatment during basal and isoprenaline stimulated conditions. Haemodynamic measurements were performed immediately before and 12 min following the occlusion of the coronary artery. Blood samples were obtained from 6 to 12 min after induction of myocardial ischaemia. The coronary artery occlusion was released after 12 min and a recovery period of 45 min was allowed.

A second occlusion was performed after 10 min of isoprenaline infusion when the haemodynamic variables had stabilised. All measurements were repeated. After release and recovery, the infusion of nicotinic acid dissolved in 0.9% (w/v) saline at a dose of 0.3 mg/kg/min was started. After 30 min a third occlusion was made, followed after release and recovery by a fourth occlusion during isoprenaline. Care was taken that the haemodynamic and electrocardiographic measurements were always done and blood samples were always obtained in the same order, and

at the same time after occlusion.

Saline (0.9%, w/v), at a rate of 10 ml/kg/hr was given throughout the experimental procedure.

Glycerol measurements were made in 5 experiments without and in 6 experiments with isoprenaline infusion.

Statistics

Each dog served as its own control. Wilcoxon's non-parametric test (two-tailed) for paired data was used to calculate probabilities. Statistical comparisons are not performed between samples of 5 or less. $P > 0.05$ was regarded as not statistically significant.

3.5.3. Results

Effects of nicotinic acid during basal lipolysis

Nicotinic acid significantly reduced the arterial concentrations of plasma free fatty acids during basal lipolysis (i.e. in the absence of isoprenaline); $P < 0.05$, $n = 7$, whereas no other significant changes in arterial levels of oxygen, glucose or lactate were observed. The extraction of free fatty acids by the ischaemic and non-ischaemic myocardium were both reduced by nicotinic acid in all experiments, but no change in arterio-local venous (across the ischaemic myocardium) or arterio-coronary sinus difference (draining mainly non-ischaemic myocardium) for oxygen, glucose and lactate was observed. Arterial plasma glycerol concentrations were reduced after administration of nicotinic acid in only 2 out of the 5 experiments, where glycerol was measured. In 4 out of 5 experiments coronary occlusion induced glycerol release into the local venous effluent. After administration of nicotinic acid this was observed in 3 experiments. Due to the preceding isoprenaline infusion (see Fig 2.02) arterial glycerol levels were still falling. Therefore, in order to cancel the effect of the increased arterial glycerol concentrations, the exchange of glycerol was also calculated from arterio-local venous differences

Table 3.15

The effects of nicotinic acid on haemodynamic and metabolic measurements during coronary artery occlusion under control conditions and during isoprenaline infusion in 7 open-chest, anaesthetized dogs

	Control		Isoprenaline infusion	
	Occlusion	Nicotinic acid + Occlusion	Occlusion	Nicotinic acid + Occlusion
Heart rate (beats/min)	150 ± 11	150 ± 13	188 ± 10	184 ± 9
LVSP (kPa)	17.4 ± 1.2	17.1 ± 1.2	17.6 ± 1.6	16.7 ± 1.4
LV dP/dt max.(kPa/s)	252 ± 23	261 ± 27	419 ± 30	409 ± 28
$\overline{AP}$ (kPa)	16.5 ± 1.3	15.3 ± 1.3	14.9 ± 1.1	14.9 ± 1.2
$Oxygen\ saturation_a$(%)	97.1 ± 1.1	97.6 ± 1.3	95.1 ± 2.5	97.4 ± 1.5
$Oxygen\ saturation_{a-cs}$	54.3 ± 2.8	60.3 ± 2.6	53.6 ± 3.8	50.1 ± 3.5
$Oxygen\ saturation_{a-lv}$	60.6 ± 2.8	60.9 ± 3.4	58.8 ± 3.4	53.4 ± 2.7*
$Glucose_a$(mmole/l)	6.96 ± 0.27	6.64 ± 0.42	9.44 ± 0.79	9.13 ± 0.75
$Glucose_{a-cs}$	0.27 ± 0.07	0.19 ± 0.08	0.29 ± 0.15	0.65 ± 0.28
$Glucose_{a-lv}$	1.40 ± 0.35	0.97 ± 0.27	1.44 ± 0.43	1.40 ± 0.19
$Lactate_a$(mmole/l)	1.30 ± 0.21	1.98 ± 0.28	1.49 ± 0.24	1.83 ± 0.34
$Lactate_{a-cs}$	0.68 ± 0.21	0.52 ± 0.48	0.03 ± 0.12	-0.11 ± 0.46
$Lactate_{a-lv}$	-1.22 ± 0.91	-0.59 ± 0.82	-2.46 ± 0.79	-1.56 ± 0.60
FFA_a(μmole/l)	331 ± 43	212 ± 13*	1580 ± 236	296 ± 23*
FFA_{a-cs}	91 ± 41	30 ± 12*	261 ± 65	45 ± 17*
FFA_{a-lv}	100 ± 19	33 ± 11*	342 ± 43	57 ± 18*
$Glycerol_a$(μmole/l)	56.5 ± 12.9	73.6 ± 25.5	271.0 ± 37.3	56.0 ± 15.1*†
$Glycerol_{a-cs}$	1.3 ± 3.8	-7.2 ± 8.6	6.1 ± 8.4	-7.2 ± 8.6†
$Glycerol_{a-lv}$	-20.9 ± 8.0	13.7 ± 13.4	22.0 ± 13.7	-2.8 ± 11.0†

Abbreviations: LVSP= left ventricular systolic pressure, LV dP/dt max.= the first maximal derivation of the left ventricular pressure, $\overline{AP}$= mean aortic blood pressure, FFA= plasma free fatty acids.
The subscripts a, a-cs and a-lv refer to the arterial concentration, arterio-coronary sinus difference and arterio-local venous difference in concentration respectively.
Probability values were calculated using the Wilcoxon's non-parametric test for paired data.
$P > 0.05$ was regarded as not significant.
*P values for comparison vs appropriate occlusion < 0.05.
The glycerol data (in the absence of isoprenaline) was not examined by statistical analyses (n=5, see text).
† 6 experiments.

measured immediately before (not shown in Table 3.15) and during coronary artery occlusion. The change in arterio-local venous differences in glycerol concentrations induced by myocardial ischaemia were then -30.9 ± 12.9 and -6.5 ± 10.2 μmole/l, measured before and after the administration of nicotinic acid respectively.

Nicotinic acid did not alter the haemodynamic parameters measured in this study.

The effects of nicotinic acid during isoprenaline infusion

During isoprenaline infusion the arterial concentrations of plasma free fatty acids ($P < 0.05$, $n = 7$) and glycerol ($P < 0.05$, $n = 7$), measured at 6-12 min of coronary artery occlusion, were substantially reduced by nicotinic acid, whereas those of the other substrates and oxygen were unaltered. Extraction of plasma free fatty acids and oxygen by the ischaemic myocardium were markedly reduced (both $P < 0.05$, $n = 7$). The arterio-coronary sinus difference of plasma free fatty acids was also significantly reduced, but this change in free fatty acid metabolism by the predominantly normal myocardium was not expressed in a reduction in oxygen extraction. Nicotinic acid appeared to unmask glycerol release by the ischaemic myocardium, however this change in arterio-local venous difference of glycerol was not significant,

nor could it be distinguished from zero or from pre-occlusion values, which were 4.7 $\pm$ 4.6 µmole/l (n = 6). Nicotinic acid did not modify the heart rate, left ventricular pressure and left ventricular dP/dt max during isoprenaline infusion in these dogs with coronary artery occlusion.

3.5.4. Discussion

Nicotinic acid substantially reduced the plasma concentrations and extractions of free fatty acids by the ischaemic myocardium, and thus almost certainly net uptake of free fatty acids, since regional myocardial blood flow in the ischaemic area is not effected by the drug (Vik-Mo, 1977). It should be noted that these changes in fatty acid metabolism occurred without significant changes in arterio-local venous difference of glucose and lactate, although a tendency of reduced lactate release was noticed.

Therefore the well established beneficial effect of treatment with nicotinic acid or its analogues in experimental acute myocardial ischaemia (Kjekshus and Mjøs, 1973; Vik-Mo, 1977); in patients with acute myocardial infarction (Rowe et al, 1975; Russell and Oliver, 1978; Kjekshus, 1978) or with angina pectoris (Luxton et al, 1976) may indeed have a metabolic basis. It needs to be stressed that the actual demonstration of reduced ischaemic myocardial metabolism of fatty acids is crucial, since one cannot reasonably extrapolate from the normal to the ischaemic tissue.

The possibility that inhibition of ischaemia-induced myocardial lipolysis by nicotinic acid partly or entirely accounts for its beneficial effect should be considered. In

the absence of isoprenaline, occlusion of the left anterior descending coronary artery induced glycerol efflux from the ischaemic myocardium in all experiments (5), where glycerol was measured and was observed after nicotinic acid in 4 out of 5 experiments. Mean arterio-local venous difference of glycerol increased from -20.9 ± 8.0 to -13.7 ± 13.4 μmole/l (n = 5). It is therefore tempting to interpret this as an inhibition of ischaemia-induced myocardial lipolysis by nicotinic acid. However such interpretation is hampered by the fact that

- a trend of reduced glycerol release was observed during repeated (control) occlusions in our model (results reported in Table 2.01).
- arterial concentrations of glycerol were higher during nicotinic acid infusion*, and may be associated with increased extraction of glycerol, in analogy with similar, well-documented relations between arterial concentrations and arterio-venous differences observed for other substrates.

*The reason for the apparent failure of nicotinic acid to reduce arterial glycerol concentrations, while plasma free fatty acids were lowered, is most likely an effect of the preceding isoprenaline infusion (see Section 2.1.5).

Therefore in view of these problems our results can not substantiate whether nicotinic acid inhibited ischaemia-induced lipolysis or not*, but it has been shown to inhibit glycerol release from the ischaemically perfused rat heart (Brownsey and Brundt, 1977). However it may be that this study cannot be compared directly, due to the absence of free fatty acids in the perfusate of the isolated rat heart study, since fatty acids themselves affect lipolysis. Further studies are needed to establish firmly whether nicotinic acid, apart from its effect on fatty acid extraction, also inhibits ischaemia-induced lipolysis, and thereby prevent the energy-wasting recycling of myocardial triglycerides on account of these two effects. The reduced oxygen requirements by the ischaemic, but not by the non-ischaemic myocardium, could be explained by such a mechanism (Observations during isoprenaline infusion). It is of interest to note that this was not observed with sodium salicylate, which had less marked anti-lipolytic effects (Section 3.4).

These results support the view that ischaemic myocardial metabolism may influence the viability of the compromised heart, but a distinction between the effects of inhibition of ischaemia-induced myocardial lipolysis and adipose tissue lipolysis can not as yet be made.

*The extrapolation of the antilipolytic effect of nicotinic acid on catecholamine induced lipolysis in normoxic tissue to the ischaemic myocardium is deliberately not made.

3.5.5. Summary

The mechanism of nicotinic acid's beneficial effect during acute myocardial ischaemia was examined. A marked reduction in fatty acid extraction by the ischaemic myocardium was found. It is not clear whether the reduction in severity of acute myocardial ischaemic injury is also due to an inhibition of ischaemia-induced myocardial lipolysis. Nicotinic acid did not affect glucose extraction by the ischaemic heart, but reduced that of oxygen, suggesting that ischaemic myocardial metabolism became more efficient.

Part 4

THE EFFECTS OF ENHANCED GLUCOSE UTILISATION ON MYOCARDIAL METABOLISM AND ST-SEGMENT ELEVATION DURING EXPERIMENTAL MYOCARDIAL ISCHAEMIA IN DOGS

SECTION 4.1:

DICHLOROACETATE, MYOCARDIAL METABOLISM AND ISCHAEMIA

Introduction

The hypothesis that fatty acid and glucose metabolism of the ischaemic myocardium influences the severity of acute myocardial ischaemic injury was also tested by enhancing glucose utilisation.

The effect of glucose, often in combination with insulin and potassium, on the severity of myocardial ischaemic injury has been demonstrated by others (Maroko et al, 1972; Sybers et al, 1973; Haneda et al,1974;Opie and Owen, 1976). Dichloroacetate which increases the consumption of glucose by the normal heart, by stimulating phosphofructokinase and pyruvate-dehydrogenase was used (McAllister et al, 1973). Its use during acute ischaemia might have an advantage over glucose infusions, in that it could theoretically prevent at the same time the intracellular acidosis (lactate accumulation), although this is not certain.

4.1.1. Methods

Animal preparation

Experiments were performed on 29 healthy mongrel dogs of both sexes (12-19 kg body weight) as described in Part 2 (Section 2.1) of this thesis. Coronary sinus blood was sampled through a catheter inserted under fluoroscopic guidance. In 10 experiments the local vein draining the ischaemic area was cannulated for the assessment of metabolic changes in the ischaemic myocardium.

Measurements

Epicardial ST-segment mapping

The severity of acute myocardial ischaemic injury was assessed by epicardial ST-segment mapping using a mobile cotton wick electrode, as described in Section 2.3.1 of this thesis.

Regional myocardial blood flow

Regional myocardial blood flow was measured with Ce^{141}- and Sr^{85}-labelled microspheres (nominal diameter 15 μ), as described in Section 2.4.

Biochemical analyses

Standard procedures for the collection and processing of blood samples and for the estimation of plasma free fatty acids (Section 2.2.2) and plasma glucose (Section 2.2.3) were used. Blood lactate was measured in duplicate according to

the method of Hohorst (1970), or when local venous plasma lactate concentrations were estimated as described in Section 2.2.5. Plasma lipids were extracted in duplicate and radioactive free fatty acids isolated by thin layer chromatography as described by Boberg (1966). Radioactivity was measured using a Packard Liquid Scintillation spectrometer Model 3390. Internal standards were used to correct for quenching.

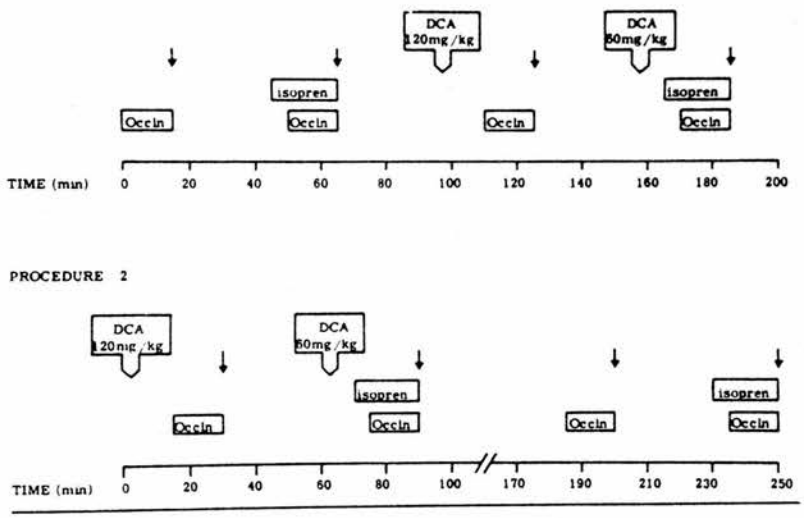
4.1.2. Experimental design

Effects of dichloroacetate (DCA) on the response to subsequent coronary occlusion

Experiments were carried out both under basal conditions and during a continuous intravenous infusion of isoprenaline. In six animals the experimental design conformed to procedure 1 of Fig 4.01. In one animal a similar sequence of occlusions was performed without the use of isoprenaline in another animal isoprenaline was given during all four occlusions. In three animals the experimental design was reversed in accordance with procedure 2 of Fig 4.01. In these experiments sodium dichloroacetate was given before the first and second occlusions, and a period of 95 min was then allowed for the metabolic effects of dichloroacetate to diminish before repeating the occlusion. The effects of dichloroacetate were assessed by paired analysis of the data for all 11 experiments.

In five separate animals the effect of dichloroacetate (120 mg/kg) on the metabolic pattern across the ischaemic zone was studied during basal lipolysis. A constant infusion of

Figure 4.01 Experimental procedure for assessing the effect of dichloroacetate on the response to subsequent coronary artery occlusion



Four identical occlusions of 15 min duration were performed in each animal, two in the absence of dichloroacetate (DCA) and two in its presence. Immediately before the release of each occlusion (indicated by vertical arrows) epicardial electrocardiograms, mean aortic pressure and heart rate were recorded, and blood was sampled from a femoral artery and the coronary sinus.
Note in procedure 2 the sequence of two control and two test (+ dichloroacetate) occlusions was reversed.
Occln = coronary artery occlusion.
Isopren = isoprenaline infusion.

albumin-bound H^3-oleate (Radiochemical Centre, Amersham), prepared as described by Opie et al (1973) was commenced at least 90 min before blood samples were collected and maintained throughout the experiment. Between 5 and 15 min after the occlusion of the branch of the LAD coronary artery, arterial, local venous, and coronary sinus blood samples were taken for measurements of FFA, H^3-FFA, glucose and lactate. After release and recovery DCA was administered and the occlusion repeated 10 min later. Care was taken that blood samples were obtained at the same relative times during the two occlusions. In another five animals the identical procedure was repeated except that now the occlusions were preceded by a continuous isoprenaline infusion (0.1-0.15 $\mu g\ kg^{-1}\ min^{-1}$).

In four additional animals the effects of DCA on regional myocardial blood flow 15 min after coronary occlusion were assessed by means of radioactive microspheres. Measurements were made during an initial control occlusion, and then during a second occlusion performed 10 min after administration of sodium dichloroacetate (120 mg/kg). The order of injection of the differently labelled microspheres was varied to avoid systematic errors due to possible differences in their behaviour.

Effects of dichloroacetate administration during established coronary occlusion

In another four animals iv infusion of isoprenaline (0.2-0.3 $\mu g.kg^{-1}min^{-1}$) was maintained for the duration of the study. Five minutes after its commencement control recordings of the epicardial electrocardiogram, arterial blood pressure and heart rate were made before a branch of the left anterior descending coronary artery was permanently occluded. Further electrocardiographic and haemodynamic measurements were made 5, 10, and 15 min following coronary occlusion. Sodium dichloroacetate (120 mg/kg iv) was then infused over 2 min and the electrocardiographic and haemodynamic recordings repeated 5, 10, 15, and 20 min later. Arterial and coronary sinus blood was sampled immediately before and 20 min after the injection of dichloroacetate for measurement of plasma free fatty acids and glucose concentrations.

In the same animals the effect of dichloroacetate on the total (a-cs) myocardial extraction of radiolabelled palmitate was also examined. For this purpose a continuous intravenous infusion of albumin-bound (9,10(n)-^{3}H) palmitate (Radiochemical Centre, Amersham) was commenced at least 90 min before blood samples were collected. Arterial and coronary sinus blood for measurement of plasma

free fatty acid radioactivity was sampled immediately before and 20 min after dichloroacetate injection.

Statistics

Each dog served as its own control. Student's t-test for paired data was used to calculate probabilities. $P > 0.05$ was regarded as not statistically significant.

4.1.3. Results

Effects of dichloroacetate on the response to subsequent coronary occlusion

The effects of dichloroacetate on the response to subsequent coronary occlusion under basal conditions are summarised in Table 4.01. Coronary occlusion raised epicardial ST segments in all experiments. In the absence of dichloroacetate, ΣST averaged 18 $\pm$ 3 mV (mean $\pm$ SEM) 15 min after coronary occlusion. Values for ΣST were significantly less when coronary occlusion was performed after the administration of dichloroacetate (ΣST 6 $\pm$ 2 mV; $P < 0.005$). Heart rate was decreased from 136 $\pm$ 8 to 127 $\pm$ 8 beats/min ($P < 0.05$), while $\overline{AP}$ was unchanged.

Arterial blood lactate concentration was reduced by dichloroacetate from 1.10 $\pm$ 0.24 to 0.28 $\pm$ 0.03 mmole/l ($P < 0.01$). The arterial-coronary sinus difference ($Lactate_{a-cs}$) in lactate concentration was reduced from 0.32 $\pm$ 0.07 to 0.08 $\pm$ 0.02 mmole/l ($P < 0.02$), while that

Table 4.01

Effects of pretreatment with sodium dichloroacetate (120 mg/kg) on epicardial ST-segment elevation and haemodynamic and biochemical measurements 15 min after coronary occlusion under basal conditions

Measurements	n*	Mean ± SEM		P**
		Control	Dichloroacetate	
ΣST(mV)	10	18±3	6±2	<0.005
HR(beats/min)	10	136±8	127±8	<0.05
$\overline{AP}$(kPa)	10	15.3±0.8	15.3±0.8	NS
FFA_a	10	0.45±0.05	0.46±0.06	NS
FFA_{a-cs}	10	0.09±0.03	0.06±0.02	NS
$Glucose_a$	9	5.22±0.22	5.22±0.28	NS
$Glucose_{a-cs}$	9	0.25±0.08	0.55±0.06	<0.005
$Lactate_a$****	7	1.10±0.24	0.28±0.03	<0.01
$Lactate_{a-cs}$****	7	0.32±0.07	0.08±0.02	<0.02
Series II***				
FFA_a(umole/litre)	5	0.54±0.04	0.54±0.06	NS
H^3-$FFA_{(a-lv)/a(\%)}$	3	38±6	28±12	NS
$Glucose_a$	5	6.11±0.72	5.56±0.67	<0.05
$Glucose_{a-lv}$	4	0.99±0.46	1.12±0.50	NS
$Lactate_a$	4	0.78±0.13	0.50±0.11	NS
$Lactate_{a-lv}$	4	-2.38±0.71	-1.72±0.44	NS

ΣST=sum of ST-segment elevation at 10-15 epicardial sites; HR=heart rate; $\overline{AP}$=mean aortic pressure; H^3-FFA=radioactive FFA.The subscripts indicate:a=arterial concentration; a-cs=arteriocoronary sinus concentration difference; a-lv=arterio-local venous concentration difference (local vein, draining the ischaemic area);(a-lv)/a=extraction in the ischaemic area. Concentrations and arterio-venous differences in mmole/l.
*Number of dog studies.
**P values were obtained by the paired t test; NS=not statistically significant($P > 0.05$).
***Separate study, observations were made between 5-15 min after coronary occlusion.
****Blood lactate concentrations.
Conversion: SI to traditional units: 1 kPa ≃ 7.5 mmHg.

in glucose concentration ($Glucose_{a-cs}$) was increased from 0.25 ± 0.08 to 0.55 ± 0.06 mmole/l ($P < 0.005$). Other metabolic measurements were unchanged.

The effect of dichloroacetate on the a-v differences in concentration of metabolites across the ischaemic zone is tabulated in Table 4.01 (series II). There was a tendency for arterial-local venous difference in plasma glucose concentration ($Glucose_{a-lv}$) to increase (three out of four experiments), although arterial glucose concentration tended to decrease and the decrease in glucose a-lv actually occurred when arterial glucose concentration decreased as much as 18%. In three out of four experiments lactate release was diminished. No marked effect of dichloroacetate on the extraction of radioactive free fatty acids in the ischaemic area was found.

In the absence of dichloroacetate, isoprenaline infusion increased ΣST from 18 ± 3 to 62 ± 8 mV (Table 4.01 and 4.02; control occlusions; $P < 0.001$). This was associated with a reduction in $\overline{AP}$ ($P < 0.005$), and increase in heart rate ($P < 0.001$), and increases in the arterial concentrations of free fatty acids ($P < 0.001$), glucose ($P < 0.01$) and lactate ($P < 0.025$).

The effects of dichloroacetate on the response to subsequent coronary occlusion during isoprenaline infusion appear in Table 4.02.

Table 4.02

Effect of pretreatment with sodium dichloroacetate (60 mg/kg) on epicardial ST-segment elevation and haemodynamic and biochemical measurements 15 min after coronary occlusion during a continuous intravenous infusion of isoprenaline (0.2-0.3 μg/kg/min)

Measurements	n	Isoprenaline (mean±SEM)		P
		Control	Dichloroacetate	
ΣST(mV)	10	62±8	45±7	<0.001
HR(beats/min	10	162±8	155±6	<0.05
$\overline{AP}$(kPa)	10	11.7±0.93	12.2±0.80	<0.02
FFA_a	9	1.72±0.18	1.72±0.17	NS
FFA_{a-cs}	9	0.31±0.40	0.19±0.02	<0.005
$Glucose_a$	9	6.78±0.61	6.67±0.56	NS
$Glucose_{a-cs}$	9	0.39±0.11	0.68±0.14	0.02
$Lactate_a$**	7	1.67±0.35	0.69±0.10	0.02
$Lactate_{a-cs}$	6	0.42±0.16	0.12±0.03	NS
Series II*				
FFA_a	4	1.36±0.12	1.37±0.19	NS
H^3-$FFA_{(a-1v)/a(\%)}$	4	26.4±15.0	28.5±16.7	NS
$Glucose_a$	5	6.39±0.48	5.83±0.59	NS
$Glucose_{a-1v}$	5	1.28±0.21	1.66±0.39	NS
$Lactate_a$	4	1.32±0.15	0.80±0.19	<0.01
$Lactate_{a-1v}$	4	-2.67±0.63	-1.88±0.51	<0.05

All abbreviations as in Table 4.01. Concentrations and arteriovenous differences in mmole/l.

*Separate study: dose of sodium dichloroacetate 120 mg/kg; dose of isoprenaline 0.1-0.15 $\mu g.kg^{-1}.min^{-1}$; observations were made between 5-15 min after coronary occlusion.

**Blood lactate concentrations.

Pretreatment with dichloroacetate reduced isoprenaline-induced increase in ΣST from 62 ± 8 to 45 ± 7 mV ($P < 0.001$). Heart rate was reduced from 162 ± 6 to 155 ± 6 beats/min ($P < 0.05$), while $\overline{AP}$ was increased from 11.7 ± 0.93 to 12.3 ± 0.80 kPa (88 ± 7 to 92 ± 6 mmHg) ($P < 0.02$). The effects of dichloroacetate on lactate and glucose metabolism during isoprenaline resembled those observed under basal conditions: i.e. decreases in arterial lactate concentration ($P < 0.02$) and a decrease in the arterio-venous difference of lactate concentration ($P < 0.10$), and an increase in the $glucose_{a-cs}$ of 0.29 ± 0.09 mmole/l ($P < 0.02$). In addition, the arterio-venous difference in free fatty acid concentration was decreased from 0.31 ± 0.04 to 0.19 ± 0.02 mmole/l ($P < 0.005$) in the absence of change in arterial free fatty acid concentration.

The effects of dichloroacetate on the a-v differences of substrates across the ischaemic area during isoprenaline infusion are summarised in Table 4.02 (series II). Dichloroacetate had a consistent and significant lowering effect on lactate release from the ischaemic left ventricle. The arterio-venous difference of glucose increased in four out of five experiments, and arterial plasma glucose concentrations tended to decrease; when arterial glucose concentrations decreased by as much as

25%, glucose extraction by the ischaemic area actually decreased after dichloroacetate (one experiment). The extraction of (^{3}H) free fatty acid appeared to be unchanged by dichloroacetate.

The results of the radioactive microsphere studies are summarised in Table 4.03. Non-ischaemic tissue of the free wall of the left ventricle distant from the occluded artery was taken to represent normal myocardium. On this basis coronary occlusion produced an average decrease of approximately 75% in blood flow to the ischaemic zone, the reduction in flow being greater in the endocardial (mean reduction, 0.95 $ml.g^{-1}.min^{-1}$) than in the epicardial (0.87 $ml.g^{-1}.min^{-1}$) layers. Dichloroacetete increased blood flow to both the epicardial (+0.19 $ml.g^{-1}.min^{-1}$ or 16%; $P < 0.005$) and endocardial (+0.16 $ml.g^{-1}.min^{-1}$ or 13%; $P < 0.025$) layers of the non-ischaemic myocardium, but had no effect on that to the ischaemic zone.

Effects of dichloroacetate administration during established coronary occlusion

The effects of dichloroacetate administration during established coronary occlusion and isoprenaline infusion are presented in Table 4.04 and the changes in epicardial ST segments in one experiment are illustrated in Fig 4.02. Ten, 15, and 20 min after dichloroacetate values for ST

Table 4.03

Effects of sodium dichloroacetate, 120 mg/kg, on regional myocardial blood flow in ischaemic and non-ischaemic free left ventricular wall 15 min after coronary occlusion in four dogs

	Myocardial blood flow ($ml.g^{-1}.min^{-1}$)		
	Occlusion	Occlusion plus dichloroacetate	P*
Non-ischaemic myocardium**			
Epicardial	1.16±0.05 (25)	1.35±0.06 (25)	<0.005
Endocardial	1.25±0.05 (25)	1.41±0.06 (25)	<0.025
Ischaemic myocardium***			
Epicardial	0.29±0.07 (11)	0.34±0.07 (11)	NS
Endocardial	0.30±0.08 (11)	0.35±0.10 (11)	NS

Results are expressed as means ± SEM. The figures in parentheses indicate the number of biopsies.

*Paired t test. NS: P>0.05.

**Myocardium distant from the occluded artery.

***Myocardium within the area of distribution of the occluded artery.

Table 4.04

Acute effects of sodium dichloroacetate (DCA), 120 mg/kg, on epicardial ST-segment elevation, haemodynamic measurements, and myocardial substrate utilisation in four dogs when administered during an established coronary artery occlusion and isoprenaline infusion

	0	5	10	15	20	25	30	35	40
	Isoprenaline infusion (0.2 - 0.3 $\mu g.kg^{-1}$/min)								
		Coronary artery occlusion							
					Dichloroacetate (120 $mg.kg^{-1}$ iv)				
ΣST (mV)		2 ± 1	48 ± 8	65 ± 9	70 ± 10	56 ± 15	49 ± 11"	48 ± 9**	44 ± 6*
$\overline{AP}$ (kPa)		13.8±1.5	13.7±1.5	14.0±1.5	13.7±1.5	14.2±1.3	14.2±1.2	14.0±1.2	14.0±1.2
HR (beat/min)		188± 8	190± 9	188± 11	187± 14	184± 12	184± 13	184± 13	179± 13""
FFA_a					1.26±0.26				1.13±0.36
FFA_{a-cs}					0.27± 0.08				0.20±0.07
H^3-FFA_a					2483± 1240				2426± 1340
H^3-FFA_{a-cs}					629± 366				444± 391*
$Glucose_a$ ♣♣				9.56	8.28	8.44	8.28		8.56
$Glucose_{a-cs}$ ♣♣				0.17	0.11	0.78	0.50		0.61

Results are expressed as mean ± SEM.

All abbreviations are as in Table 4.01. Concentrations and arterio-venous differences in mmole/l, except for H^3-FFA (a and a-cs) which are expressed in dpm/ml.

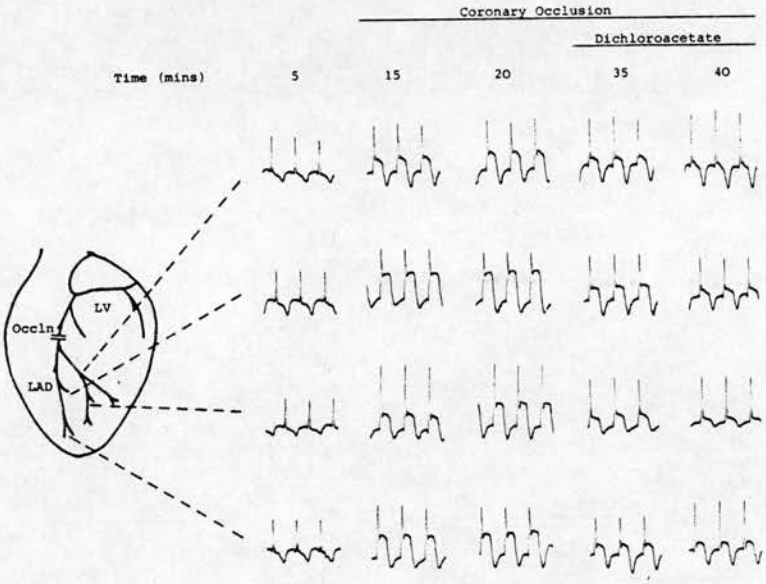
H^3-FFA_a = arterial FFA radioactivity as (9, 10(n)-H^3)palmitate; H^3-FFA_{a-cs} = arterial-coronary sinus concentration difference in FFA radioactivity.

Statistical comparisons were performed by paired t test against those results obtained immediately before DCA (time 20 min).

*$p < 0.05$; ♣$P < 0.01$; ""$P < 0.005$; **$P < 0.001$.

♣♣ One dog only.

Figure 4.02. The effect of dichloroacetate during an established coronary artery occlusion on ST-segments in epicardial electrocardiograms



Epicardial electrocardiographic recordings from four of 11 sites in one experiment. Five minutes after the commencement of an intravenous infusion of isoprenaline (0.2-0.3 $\mu g.kg^{-1}/min$) a branch of the left anterior descending coronary artery was permanently occluded. Fifteen min later sodium dichloroacetate was given by intravenous injection at the dose of 120 mg/kg. Epicardial electrocardiograms were recorded immediately before coronary occlusion (time, 5 min), and subsequently at 5 min intervals until 20 min after the administration of dichloroacetate. LV = left ventricle; LAD = left anterior descending coronary artery; Occln = coronary occlusion.

were significantly lower than those observed immediately before treatment ($P < 0.05$). Mean aortic pressure and heart rate at these times were similar to those recorded before treatment, with the exception that heart rate had decreased by 8 $\pm$ 2 beats/min ($P < 0.01$) after 20 min. The arterio-venous difference$_{(a-cs)}$ in glucose concentration was increased from 0.11 to 0.61 mmole/l (one experiment), while that in free fatty acid concentration was decreased ($P < 0.05$) in the presence of unchanged arterial concentrations. Dichloroacetate also reduced the arterio-venous difference in free fatty acid radioactivity in these experiments ($P < 0.05$).

4.1.4. Discussion

The most notable finding of the present study was that dichloroacetate reduced the degree of epicardial ST-segment elevation following acute coronary occlusion in dogs, and reduced also the augmentation of ST-segment elevation induced by isoprenaline. This latter effect was observed both when dichloroacetate preceded subsequent reocclusion of the artery and when given during an occlusion established 15 min earlier.

Epicardial ST-segment elevation has been shown to reflect the severity of myocardial ischaemia (Wegria et al, 1949), and to correlate with the local changes in tissue

oxygen tension (Sayen et al, 1958) and cellular membrane potential(Prinzmetal et al, 1961) during coronary occlusion. The degree of ST-segment elevation 15 min after coronary occlusion has been correlated with the subsequent depletion of myocardial creatine kinase activity (Maroko et al, 1971; 1972; Kjekshus and Mjøs, 1973) and the development of histological, histochemical, and ultrastructural evidence of cellular necrosis (Maroko et al, 1972; Libby et al, 1973) after 24 h of sustained occlusion. It is likely, therefore, that the reduction in ST-segment elevation produced by dichloroacetate in the present study reflected a limitation of the acute myocardial ischaemic injury.

The severity of myocardial ischaemic injury is influenced by factors which alter myocardial oxygen requirement relative to oxygen supply. The major determinants of myocardial oxygen requirement are heart rate, wall tension, and contractility (Sonnenblick et al, 1968), and the possibility must be considered that the reduction of ST-segment elevation by dichloroacetate may have been secondary to a change in one of these parameters. Although dichloroacetate reduced mean heart rate in all experiments, this effect averaged only 5%, and was probably insufficient to account for the mean reduction

in ST-segment elevation of 44% (Maroko et al, 1971; Wendt et al, 1974). Furthermore, in six instances dichloroacetate administration was associated with an unchanged or increased heart rate, but nevertheless ΣST was significantly reduced. Changes in $\overline{AP}$, when present, were also insufficient to explain the reduction in ST segment elevation. An effect of dichloroacetate on myocardial oxygen supply is unlikely as the results of the microsphere studies showed that blood flow to the ischaemic zone was unchanged by dichloroacetate.

The effect of dichloroacetate on non-ischaemic myocardial metabolism

The use of arterial-coronary sinus differences has been shown to be justified for the study of non-ischaemic myocardial metabolism when small ischaemic lesions are produced (Owen et al, 1970; Opie et al, 1973). Changes in arterial-coronary sinus concentration differences have indicated that dichloroacetate had marked effects on the pattern of substrate utilisation by the non-ischaemic left ventricle. In all experiments dichloroacetate raised the total myocardial extraction of glucose relative to that of free fatty acid. In most instances this reflected both an increase in the arterio-venous difference in

glucose concentration and a decrease in that of free fatty acid, although the latter reached statistical significance only in the presence of isoprenaline. In all but one of seven animals dichloroacetate also reduced the arterial lactate concentrations and the arterio-venous difference in lactate concentrations. The decrease in arterio-venous difference in free fatty acid (^{3}H-palmitate experiments and free fatty acid during isoprenaline) can be interpreted either as a decrease in the extraction of free fatty acid or an increase in intramyocardial lipolysis. The latter does not seem likely, since dichloroacetate exerted an antilipolytic effect in rats (Blackshear et al, 1974). Another possibility could be that dichloroacetate behaves in a similar fashion as a non-readily oxidisable acid, such as hypoglycin or 4-pentenoic acid (Bressler, 1970). These acids lower tissue levels of free coenzyme A, and thereby leading to a situation in which extracted fatty acids cannot be readily activated and may diffuse back into the plasma free fatty acid pool.

The stimulation of glucose metabolism by dichloroacetate is in agreement with the observations of McAllister et al. (1973) who studied alloxan-diabetic dogs and healthy dogs in which free fatty acid concentrations had

been raised by infusions of a triglyceride emulsion and heparin. The increase in myocardial glucose utilisation was attributed by these workers to a stimulation of phosphofructokinase and to the activation of pyruvate dehydrogenase. The reduction in arterial lactate concentrations is also in agreement with earlier studies in dogs and rats (McAllister et al, 1973; Blackshear et al, 1974). In functionally hepatectomised rats it was shown that the decrease in arterial lactate concentrations is due to a reduced release of lactate from extrahepatic tissues (Blackshear et al, 1974). This and the observation that dichloroacetate increases pyruvate dehydrogenase activity in muscle (Whitehouse and Randle, 1973) may explain the decrease in arterial lactate concentration. The decrease in total lactate extraction by the heart accords with its known arterial concentration-extraction relationship (Kaijser et al, 1972).

The effect of dichloroacetate on ischaemic myocardial metabolism

These metabolic findings cannot be extrapolated to the metabolism in the ischaemic area and therefore should not be directly related to the electrocardiographic changes recorded over that area. It is necessary therefore to

make comparable measurements using local vein sampling techniques, thereby deriving arterial-local vein concentration differences which mostly reflect metabolic changes in the ischaemic tissue (Owen et al, 1970; Opie et al 1973). Dichloroacetate increased a-lv concentrations of glucose, even when arterial glucose concentrations fell. Lactate release was decreased in the isoprenaline stimulated situation.

It is possible that apparent changes in local venous concentrations of metabolites are entirely due to a change in the relative proportions of blood originating from non-ischaemic and ischaemic myocardium. However, having acknowledged the existence of this problem, there are no methods to our knowledge which would define the particular admixture of blood sampled from the local vein.

With this reservation in mind, it appears that the local metabolic effects; increased arterio-venous difference in glucose concentration and reduced lactate release could be related to the reduction in ΣST in the ischaemic area.

This interpretation is supported by the observation that dichloroacetate reduced ischaemia induced enzyme release from the isolated perfused rat heart (Burges , personal communication). However the effect of dichloroacetate on myocardial metabolism could not be assessed in

those studies. On the other hand our findings have not been confirmed by another study of coronary artery occlusion induced ischaemia in an anaesthetized dog preparation (Burges , personal communication). He used however 1/10 of the dose, which was used in this study and did not measure the effect on myocardial metabolism.

Mechanism of dichloroacetate

The metabolism of the acutely ischaemic myocardium has been reviewed (Opie, 1970; Oliver, 1972; Opie, 1972; Opie, 1975; Rovetto and Nealy, 1977; this thesis). The oxidation of glucose, free fatty acids and lactate is immediately impaired. However, for unexplained reasons glucose competes better with fatty acids for the residual oxygen than it normally does. There is evidence that catecholamines are locally released, and this may explain the early rise in cyclic AMP levels in the ischaemic myocardium, which are thought to stimulate glycogenolysis. Other factors which may play a role are the enhanced breakdown of ATP and the accumulation of AMP and inorganic phosphate under these conditions. However within minutes glycolysis is inhibited, presumably due to inhibition of glyceraldehyde-3-phosphate dehydrogenase or of phosphofructokinase, which are both sensitive to the intracellular acidosis caused by lactate accumulation.

It is now clear that a considerable degree of glucose oxidation persists in the ischaemic myocardium (Opie et al, 1973; Neely et al, 1975). It is possible that dichloroacetate in our experiments might have prevented the intracellular acidosis, by enhancing the activity of pyruvate dehydrogenase. This could possibly then have prevented the inhibition of phosphofructokinase and/or glyceraldehyde-3-phosphate dehydrogenase. A similar mechanism appeared to operate in ischaemic perfused rat hearts, in which acidosis was counteracted by the use of buffers. Control of glycolysis in ischaemic myocardium under these conditions was shifted to pyruvate kinase or pyruvate dehydrogenase (Rovetto et al, 1975). Thus dichloroacetate might have reduced ischaemic injury by increasing the capacity of the ischaemic cells to metabolise glucose aerobically, and its effectiveness may be dependent on the presence of residual oxygen supply.

Other mechanisms whereby dichloroacetate may have reduced ischaemic injury include the inhibition of free fatty acid metabolism and an enhancement of lactate removal from the ischaemic cell secondary to the reduction in arterial lactate concentration.

However, this latter possibility seems remote in view of the high gradient of lactate across the ischaemic membrane (Rovetto and Neely, 1977). Therefore,

no certain conclusions concerning the precise mechanisms whereby dichloroacetate reduces ST-segment elevation can be drawn at this stage.

Application of dichloroacetate in the clinical setting of acute myocardial infarction

Dichloroacetate has not been used in the early treatment of acute myocardial infarction. Although it has been used alone or in combination with buformin in maturity-onset of diabetics (Stacpoole et al, 1978; Standl et al, 1977). Other workers have suggested that dichloroacetate could lead to oxalate (derived from dichloroacetate) nephrotoxicity and that 2-chloropropionate, which also activates pyruvate dehydrogenase may be devoid of this severe side effect (Crabb and Harris, 1978)*. It is often said that increased myocardial glycogen stores enhance the chances of survival during ischaemia. If this is true, then depletion of glycogen stores after the administration (Hülsmann, personal communication) could have serious repercussion for patients with acute myocardial infarction, treated with these compounds which have an extension of their infarct. Therefore, alternative combinations such as PDH activators plus pyruvate or glucose could offer potential advantages. Clearly more work with non-toxic drugs is indicated.

*Note in print: Dichloroacetate has been withdrawn after neurological disorders were observed in patients during chronic treatment (Stacpoole, personal communication to Prof. K.G.M.M. Alberti).

4.1.5. Summary

Acute administration of or pretreatment with dichloroacetate reduced ischaemia induced ST-segment elevation. This effect could not be explained by changes in mean aortic blood pressure, heart rate or regional myocardial blood flow. Increased glucose and decreased free fatty acid extraction by the non-ischaemic myocardium was observed. Lactate release by the ischaemic zone was reduced and glucose extraction tended to increase. These results support that ischaemic myocardial metabolism influences the cellular viability.

However the use of pyruvate dehydrogenase activators can not yet be recommended for the treatment of patients with acute myocardial infarction, since they may deplete myocardial glycogen stores and dichloroacetate can certainly not be used due to its recently discovered toxicity.

Part 5

THE EFFECTS OF RAISED PLASMA CONCENTRATIONS OF FREE FATTY ACIDS ON NORMAL AND ISCHAEMIC MYOCARDIAL METABOLISM

INTRODUCTION

In this section we will examine the effects of raised plasma concentrations of free fatty acids on metabolism of the normal and ischaemic myocardium. The considerations leading to the choice and development of the two models used is given in Section 2.1. In the first model, for sake of convenience referred to as Intralipid/heparin, plasma free fatty acids are raised by intravascular lipolysis of triglycerides (Intralipid), induced by heparin. In the second model plasma concentrations of free fatty acids were raised using a blood cell separator. This centrifuge separates blood into a stream of blood cells and cell free plasma. Sodium salts of fatty acids can then be infused and bound directly to the plasma albumin of the dog, which is then returned to the animal reconstituted with the blood cells.

The validity of the former model was queried due to its very high arterial plasma concentrations of free fatty acids, reported by our group and others, using Intralipid/heparin as the model (Mjøs, 1971[b]; Kurien et al, 1971; Opie et al, 1972). Therefore a formal study of the effect of Intralipid/heparin as a model was undertaken in closed-chest anaesthetised dogs (Section 5.1).

The effects of raised plasma free fatty acids, using the blood cell separator, was also examined in normal dogs (control studies) and in dogs with coronary artery occlusion. The results are reported in Section 5.3. and 5.4. respectively, after the development of the blood cell separator is first described in Section 5.2.

SECTION 5.1:

THE EFFECTS OF RAISED PLASMA CONCENTRATION OF FREE FATTY ACIDS ON MYOCARDIAL METABOLISM AND FUNCTION, USING INTRALIPID/HEPARIN AS A MODEL

The effects of Intralipid/heparin

Introduction

Heparin has been widely used in studies of the effects of raised plasma concentrations of free fatty acids on various aspects of general metabolism in man or in animals. In some studies heparin was used to induce intravascular lipolysis during alimentary lipaemia (Schalch and Kipnis, 1965; Greenough et al, 1967; Balasse and Ooms, 1968; Crespin et al, 1969). In other studies the complicating effects associated with alimentation were avoided by injection of Intralipid (Meng and Edgren, 1963; Kurien and Oliver, 1970; Mjøs, 1971[b]; Opie et al, 1972).

There can be no doubt that this is the most convenient system, available to those who aim to raise plasma concentrations of free fatty acids and who can not or do not want to use lipolytic drugs, with their cardiovascular and haemodynamic effects. Despite this we did not intend to use Intralipid/heparin as a model, mainly due to our concern over the unphysiologically high plasma free fatty acid concentrations.

Nor were we unduly concerned about the use of heparin, since this drug could be administered as a continuous infusion and thereby be a presumably constant factor common to the control and observation periods. However, it is acknowledged that heparin may have effects which change the background conditions, under which the experiments were made.

When it was learned that in vitro lipolysis in lipaemic samples of heparinised dogs or patients could be as high as 1-4%/min, a formal study of Intralipid/heparin as a model was planned, in order to reappraise its biochemical effects.

5.1.1. Methods

Animal preparation

Seven mongrel dogs of either sex, weighing between 13 and 18 kg, were anaesthetised after overnight fasting by an intravenous injection of sodium pentobarbitone (about 25 mg/kg). Anaesthesia was maintained using a constant infusion of pentobarbitone at a dose of 3-4 mg/kg/hr. Ventilation was maintained through a cuffed endotracheal tube with a positive pressure respirator (Harvard Apparatus Co, Mass., USA). Both femoral veins were cannulated as a route of infusion, and the left femoral artery was cannulated for the monitoring of aortic blood pressure with a Statham P23Db transducer.

A catheter was introduced via the left jugular vein into the coronary sinus under fluoroscopic guidance as described in Section 2.1.4, and kept patent using a slow drip of 0.9% (w/v) saline.

Biochemical analyses

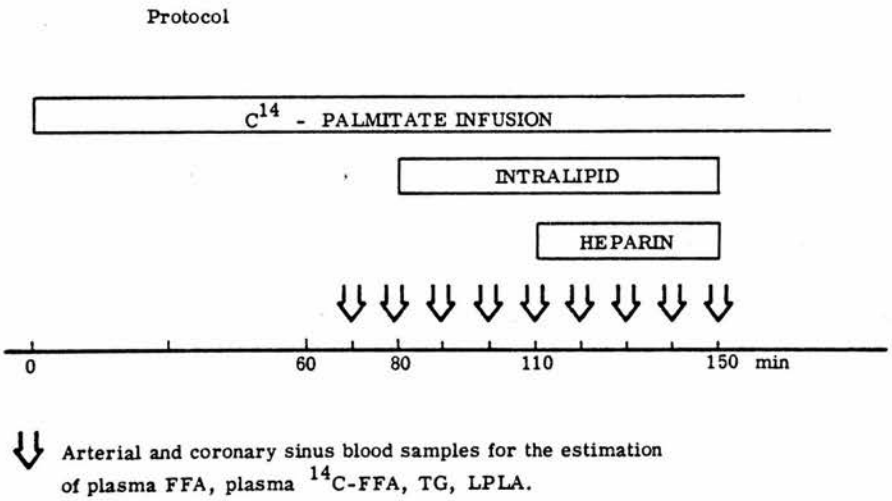
Blood samples were collected simultaneously from the arterial and coronary sinus catheter. In addition to our standard procedure of keeping samples on ice, special care was taken with the processing in this study to avoid flotation of triglycerides during centrifugation and to avoid artifacts due to in vitro lipolysis.

Free fatty acid concentrations were estimated on blood samples, immediately extracted, as described in Section 2.2.2[b]. The results were expressed in terms of plasma concentrations by correcting for the dead space occupied by the packed cells, which were measured in quadruplicate using a Hawksley microhaematocrit centrifuge. Plasma triglycerides were immediately extracted after centrifugation (usually within 15 min) and their concentration was determined using the automated fluorimetric method with blank corrections, as described in Section 2.2.7. Radioactive free fatty acid concentrations were determined in duplicate by liquid scintillation counting after immediate extraction and later purification by thin layer chromatography according to the method of Boberg (1966). Free fatty acid turnover was calculated from the arterial plasma specific activity and the infusion rate of ^{14}C-palmitate,(bound to bovine albumin – Fatty acid poor Cohn's fraction V - prepared according to Opie et al (1973). Final FFA/albumin molar ratio 0.20). Plasma albumin was measured according to the method, described in Section 2.2.6.

5.1.2. Experimental procedure (Fig 5.01).

Two series of experiments were performed. In the first group comprising 4 dogs, a bolus injection of

Figure 5.01. Experimental procedure for the study of myocardial metabolism and whole body turnover of free fatty acids using Intralipid/heparin as the model



Abbreviations: FFA = plasma free fatty acid concentration; ^{14}C-FFA = ^{14}C-radioactivity in free fatty acids fraction; TG = triglycerides; LPLA = plasma (lipoprotein) lipase activity.

1-^{14}C-palmitate, sufficient to raise arterial plasma ^{14}C- fatty acids at the required level of 2000 dpm/ml was given (about 0.3 μCi/kg), followed by a continuous infusion of ^{14}C-palmitate, calculated to maintain this level of radioactive fatty acids (about 1.2 μCi/kg/hr). The bolus injection was given to ensure that a steady state was quickly reached (Dr. G.L. Atkins, personal communication). The infusion was maintained throughout the experiment. The first blood sample for the analysis of arterial and coronary sinus concentration of plasma free fatty acids, triglycerides, ^{14}C-free fatty acids and lipolytic activity was taken at 70 min of infusion and at 80 min of infusion (control observations). An intravenous bolus injection of Intralipid 20% (w/v), (Kabi-Vitrum, Ealing) at a dose of 1.0 ml/kg, followed by a continuous infusion of 30 ml/hr was given throughout the rest of the experiment. Blood samples were taken 10, 20 and 30 min later (Intralipid observations). After the collection of the last samples heparin was administered as an intravenous bolus injection (100 U/kg), followed by a continuous infusion at a dose of 50 U/kg/hr, and another set of blood samples were taken at 10 min intervals.

The second series (3 dogs), was identical to the first except that plasma albumin levels were raised (from an

average 31.5 to 42.7 g/l) 70 min after the start of the ^{14}C-palmitate infusion. Blood samples were collected 10, 20 and 30 min later (control samples), after which the Intralipid bolus injection and infusion were commenced. From this moment the procedure was identical to that of the first series.

5.1.3. Results

In series I, initially plasma free fatty acid and triglyceride concentrations were low, averaging 0.35 ± 0.05 and 0.85 ± 0.07 mmole/l respectively (Table 5.01, Fig 5.02). A steady state of arterial ^{14}C-free fatty acid concentration had been reached (not shown) and the turnover of plasma free fatty acids was on average 138 ± 10 µmole/min. No lipolytic activity could be detected in plasma samples.

The infusion of Intralipid increased arterial triglyceride levels in all experiments (mean ± SEM) 2.52 ± 0.27 mmole/l. No systematic effect of Intralipid alone on arterial plasma concentrations of free fatty acids and their turnover was observed, but both tended to increase slightly. Lipolytic activity in plasma remained virtually undetected. Ten min after the administration of heparin plasma concentration of free fatty acids were increased and remained elevated during the rest of the 40 min observation period. The concentrations of ^{14}C-free

Table 5.01

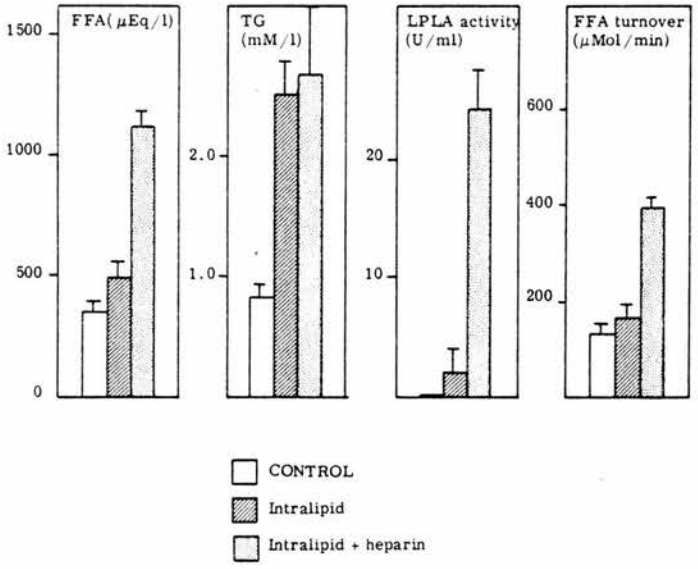
The effect of Intralipid and heparin on plasma concentrations of free fatty acids and triglycerides, lipolytic activity and whole-body free fatty acid (FFA) turnover in anaesthetized dogs

	Plasma free fatty acids (µmole/l)				FFA-turnover (µmole/l)				Plasma triglycerides (mmole/l)				Lipolytic activity (U/ml)			
Dog series I:	pre-albunin	control	Intra-lipid	Intra-lipid + Heparin	pre-albumin	control	Intra-lipid	Intra-lipid + Heparin	pre-albumin	control	Intra-lipid	Intra-lipid + Heparin	pre-albumin	control	Intra-lipid	Intra-lipid + Heparin
605	—	345	560	1230	—	112	174	398	—	0.76	2.03	1.80	—	—	0.2	27.2
606	—	270	250	975	—	147	117	396	—	0.97	2.52	1.65	—	—	0.9	26.3
609	—	485	575	1030	—	137	176	329	—	0.95	3.29	4.44	—	—	0.0	21.0
612	—	340	560	1250	—	137	205	446	—	0.71	2.26	2.94	—	0.8	6.6	21.9
Mean±SEM	—	350±50	490±80	1120±70	—	138±10	168±18	395±22	—	0.85±0.07	2.52±0.27	2.70±0.65	—	-1.3	2±2	24±3
													—	-0.2		
Dog Series II:																
644	605	880	1180	1855	218	277	342	483	1.19	1.20	3.63	4.00	—	—	0.2	12.5
647	1545	1845	1925	2370	426	561	534	575	1.67	1.55	3.26	4.35	—	-2.1	1.1	19.0
649*	1010	1245	1255	1450	386	408	432	437	1.36	1.20	3.44	5.17	—	4.4	0.4	3.3
Mean±SEM	1055±270	1325±280	1455±235	1890±265	343±64	415±82	436±55	419±41	1.41±0.14	1.32±0.12	3.44±0.11	4.51±0.35	—	1.2	0.6±0.3	11.6±4.5

For conditions see text. *Note in dog 649, heparin did not release lipolytic activity.

Concentrations were determined in arterial plasma. Average results of observation periods(3-4 measurements/period).

Figure 5.02. The effect of Intralipid and Intralipid/heparin on plasma free fatty acid concentration, triglycerides, (lipoprotein) lipase activity and free fatty acid turnover in 4 anaesthetised dogs



Effects of Intralipid and Intralipid/heparin on arterial plasma free fatty acids (panel 1); plasma triglycerides (panel 2) on plasma lipolytic activity (panel 3) and on free fatty acid turnover (panel 4).
Abbreviations as in Figure 5.01.

fatty acids remained constant throughout the heparin-period. Thus within 10 min a new steady state was obtained, and the increase in free fatty acid turnover is valid. The lipolytic activity of plasma was also increased after heparin and remained constant during the rest of the observation period. The results of all observations during heparin were pooled. No systematic effect of triglycerides was observed: in experiments 609/612 levels were rising, whilst in the other 2 concentrations fell, but not below the initial values.

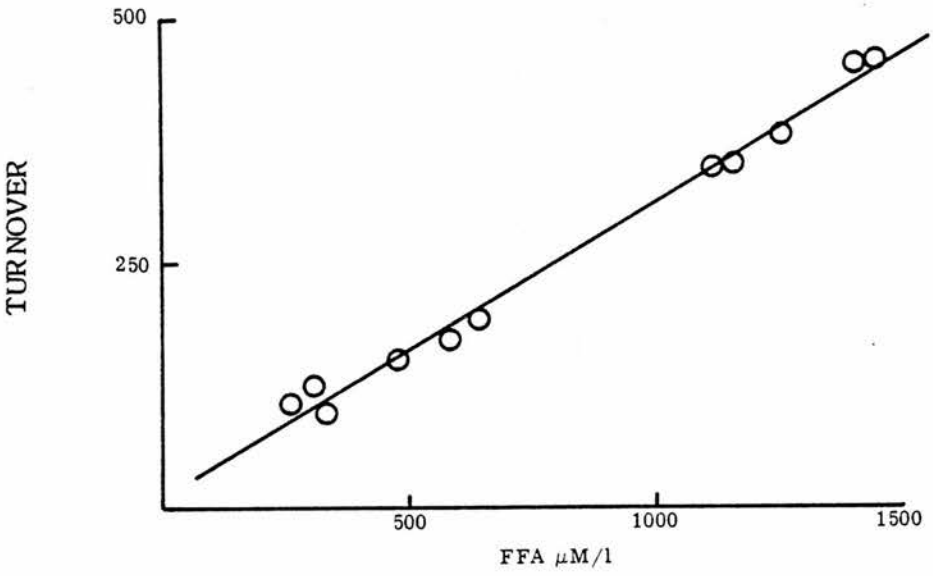
The increase in plasma fatty acid turnover was always related to its arterial concentration and the results of a representative study is shown in Fig 5.03.

The increase in arterial plasma free fatty acid concentration was associated with an increased arterial-coronary sinus difference of free fatty acids, and the results of all dogs are shown in Fig 5.04.

In series II the effects of Intralipid/heparin was examined in 3 anaesthetised dogs, after their plasma albumin concentrations were increased from a mean of 31.5 to 42.7 g/l. The results are summarised in Table 5.01.

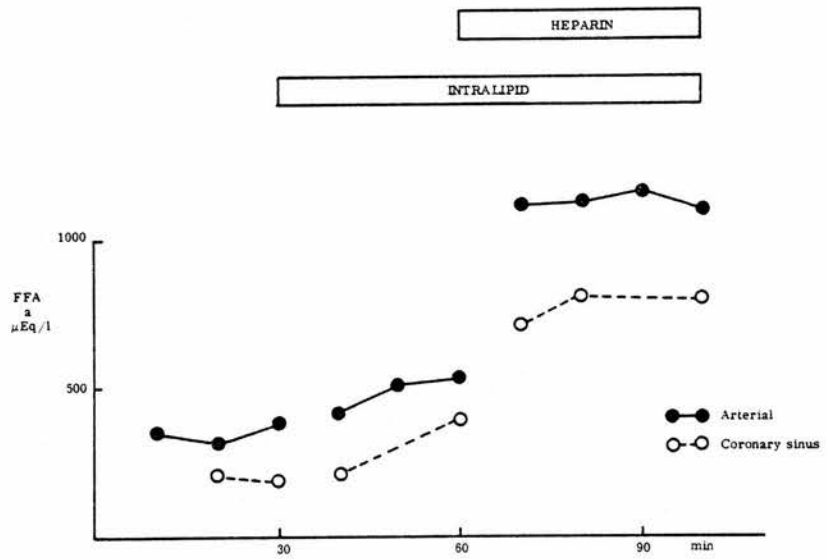
Plasma free fatty acid concentrations in this sub-group were higher than those of series I. As a result

Figure 5.03. The relation between arterial concentrations of plasma free fatty acids and their turnover. Intralipid/heparin study (Dog 605)



Fatty acid turnover was measured before, during Intralipid and during Intralipid/heparin (Combined results). A similar relation was found between arterial free fatty acid concentration and turnover in all dogs.
Abbreviations: Turnover = free fatty acid turnover in μmole/min; FFA = arterial concentration of plasma free fatty acids in μmole/l.

Figure 5.04. The effects of Intralipid and Intralipid/heparin on arterial and coronary sinus concentrations of plasma free fatty acids in 4 anaesthetised dogs



Abbreviations as in Fig 5.01.
Note: increase in difference between arterial and coronary sinus concentration of free fatty acids during Intralipid/heparin.

whole-body turnover rates of free fatty acids were also higher. Albumin increased plasma concentrations of free fatty acids in all three dogs, and despite the increase in albumin concentration free fatty acid/albumin molar ratio also increased (but less marked than the concentration of fatty acids) from an average of 2.36 to 2.65.

The effects of Intralipid and heparin were similar but less marked in 2 out of the 3 experiments as in the experiments of Series I, except that in dog 649 heparin did not release lipolytic enzymes and did not change the free fatty acid turnover. The reasons for this abnormal behaviour could not be revealed.

Intralipid/heparin did not affect heart rate and mean aortic blood pressure.

5.1.4. Discussion

This study was undertaken to check the validity of the Intralipid/heparin model, to raise plasma free fatty acid concentrations. If in vitro lipolysis was prevented by immediate extraction of whole blood samples, plasma concentrations of free fatty acids increased after the administration of Intralipid and heparin, but remained well within the physiological range. Those results are in sharp contrast with those of others, who reported

plasma free fatty acid concentrations ranging from 3000-6000 μmole/l (Mjøs, 1971[b]; Kurien et al, 1971; Opie et al, 1972). This difference is almost certainly due to the in vitro lipolysis in blood samples taken from lipaemic, heparinised dogs. Of course it is difficult to reconstruct later the exact conditions used in the processing of laboratory samples. However, Mjøs (1971[b]) appeared to take more precautions and extracted fatty acids quicker than Kurien et al (1970); and it is therefore of interest that he found much lower values than Kurien in Edinburgh.

It is not clear whether the raised free fatty acids were solely bound to albumin or whether a substantial amount was still associated with the triglyceride particles. However even if they were they appear to be utilised in the usual way. This conclusion is based on the fact that no discontinuity in the relation between arterial free fatty acid concentration and the turnover (Fig 5.03) was observed. (In this context it is important to note that the measurements for this graph were made with wide ranging triglyceride and free fatty acid concentrations). Secondly the extraction of plasma free fatty acids by the heart increased, with increasing arterial concentrations (Fig 5.04).

Therefore the model of Intralipid/heparin is sound from the point of view of its effect on plasma free fatty acids and their turnover. These results eliminate a severe criticism against the work of Mjøs (1971[b]), Kurien et al (1971) and Opie et al (1972) which was the apparent unphysiologically high plasma free fatty acid concentration. Thus the increase in serious ventricular arrhythmias in dogs with experimental coronary occlusion, occurred most likely during plasma free fatty acid concentrations in the high physiological range ($\sim$1200 µmole/l). It is of interest that these workers suggested from clinical experience that raised plasma concentrations of this order or higher were detrimental. It also means, that the depressant effect of Intralipid/heparin on the ischaemic dog heart was probably due to a physiological increase in fatty acid metabolism (Kjekshus and Mjøs, 1972). Our observations add to the strength of their argument, that raised plasma concentrations of free fatty acids may be detrimental to the electrical stability and funtion of the ischaemic heart.

However another point, concerning Intralipid/heparin as a model to study the effects of raised concentrations of plasma free fatty acids, has emerged. It has been reported that lysolecithin levels in ischaemic myocardium are raised (Sobel et al, 1978), and that perfusion of

Purkinje fibers with lysolecithin predisposed them to arrhythmias. During Intralipid/heparin plasma lysolecithin is raised, due to the effect of heparin-released phospholipase activity (Vogel and Bierman, 1965; Jansen and Hülsmann, 1974) on plasma phospholipids or lecithin, originating from Intralipid*. However the concentrations of lysolecithin, observed in effluents of anoxic hearts were much lower (2 μmole/l) than the high concentrations used in the perfusion studies (1-3 mmole/l). Thus, although there is ground for some doubt as to whether raised plasma free fatty acids or lysolecithin may be detrimental to the ischaemic heart, either alone or in combination with one another, at present evidence seems to point at free fatty acids rather than lysolecithin. Until this question is satisfactorily solved, no definite conclusions of the mechanism involved can be drawn from results obtained in studies of experimental myocardial ischaemia, employing Intralipid/heparin as a model.

*Intralipid contains egg lecithin (1.2 g/100 ml).

5.1.5. Summary

Heparin releases lipolytic enzymes in vivo and these enzymes continue to degrade triglycerides in vitro, leading to serious overestimation of plasma concentrations of free fatty acids. Immediate deproteinisation of blood overcomes this problem, and a new method for the measurement of free fatty acids on whole blood extracts was developed (Section 2.2.2^{b}). Using the improved methodology, the use of Intralipid/heparin as a model to raise plasma free fatty acids was reinvestigated in 7 anaesthetised dogs. Arterial plasma free fatty acid concentrations were raised during Intralipid/heparin well within the physiological range : 1120 $\pm$ 70 μmole/l (n = 4). The increase in arterial plasma free fatty acids was associated with increased extraction by the heart and whole-body FFA-turnover. The results show that Intralipid/heparin may be a convenient model to raise plasma free fatty acids, but previously insufficient attention has been paid to the technical problems of measuring plasma free fatty acids in studies, using Intralipid/heparin as a model.

SECTION 5.2:

THE DEVELOPMENT OF THE BLOOD CELL SEPARATOR FOR DIRECT INFUSION OF SODIUM OLEATE IN DOGS

The blood cell separator

Introduction

The direct infusion of free fatty acids to raise plasma concentrations, has an advantage over the use of drugs, and this has been discussed in Section 2.1.2. However desirable this approach may be it has been severely hampered by the fact that it can only successfully be done with the complicated system of the blood cell separator (Greenough et al, 1969). Briefly, the principle is as follows: Blood is continuously separated by a centrifuge into a stream of cell free plasma and another, containing all blood cells (packed cell volume of about 70%). Fatty acids, dissolved as their sodium salt in distilled water (pH $\simeq$10) are infused directly into the plasma line, where they bind instantaneously to the dogs own albumin. The fatty acid enriched plasma is then returned to the animal together with the blood cells. Although this system was published as early as 1969, we have been hesitant to use it for a long time, due to its complexity. However, it became increasingly apparent that direct infusions of fatty acid anions, even when 'stabilised' with albumin* were toxic to normal dogs (Hoak et al, 1972; Riemersma

*Final fatty acid/albumin molar ratio was 55.

et al, 1974). On the other hand these problems were not encountered when fatty acid anions were infused using the blood cell separator. Fortunately a simplified version of the blood cell separator could be built for us by Dr. N. McLeod, Department of Chemical Engineering, Kings Buildings, University of Edinburgh, at little expense.

In this section of the thesis some of the problems encountered in the introduction of this technique of raising plasma concentrations of free fatty acids are presented. But, first a detailed description of the design and the functioning of the blood cell separator is presented.

5.2.1. The blood cell separator

The blood cell separator consists of 4 essential components:

- the centrifuge bowl
- the face seal
- the pumps and tubing connections
- the centrifuge

The centrifuge bowl

The bowl consists of 3 separate parts, all made of perspex (Fig. 5.05-5.06):

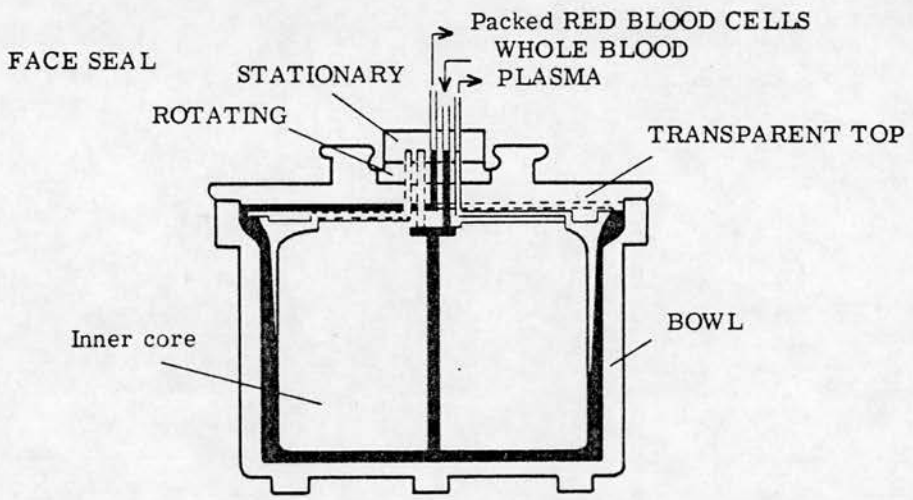
- a transparent top containing the entrance port for whole blood, and the exit ports for the separated components
- a solid inner core with the entrance channel for whole blood through its centre
- the bowl

The effective radius is 2.5 inches and the depth of the bowl is 4 inches. The inner core is screwed to the top and the distance between it and the sides and the bottom of the bowl is 0.04 inches.

The face seal

The most important part of the blood cell separator is the face seal (see Fig 5.05) through which whole blood and the separated components must pass. It is

Figure 5.05. The blood cell separator: Schematic representation of the centrifuge bowl



A diagonal view through the centrifuge bowl. The perspex bowl consists of 5 parts: the bowl; the inner core, filling the dead space; the transparent top and the rotating and stationary part of the face seal. Blood enters through the face seal and is separated into its components while travelling up through the channel between the inner core and the outer shell of the bowl.
Effective radius 2½ inch; 160 g at 1500 rpm.
(Simplified diagram).

Figure 5.06. The blood cell separator: The centrifuge bowl

The face seal, which rests on the concentric silastic rubber rings, has been removed to show the concentric collection channels.

composed of 2 matching parts:

- the upper part is a stainless steel adaptor (stationary)
- the lower part made of ceramic, rotates with the centrifuge bowl

The upper part contains the connections for the input and output ports: whole blood input, plasma and red blood cell output; and 3 ancillary ports one to lubricate the seal and two connecting to the irrigation channel (see later). (On the actual face seal there is another port for the collection of the buffy coat, but as it is never used in our experiments, it is not shown for the sake of simplicity in Fig 5.05 nor is it referred to in the text). The seal is designed to keep the components separated as they travel through it. This is achieved by concentric islands of pressurised saline, between all channels. The outer channel is irrigated with saline to cool the seal. It also prevents air entering it, as there is a negative pressure in the bowl due to the action of the plasma and red blood cell pumps.

Pumps and tubing

The separated components (plasma and (red) blood cells) are pumped from the centrifuge bowl using two identical roller-type blood pumps (5M 1154, Travenol Laboratories, Inc., Deerfield, Ill., USA). Their action generates a

small negative pressure within the centrifuge bowl, which causes the whole blood to enter. Silicone tubing (Ø 2.0 mm) is used for the pumps, whilst all other tubing is pyrogen-free polyethylene tubing. The tubing manifold is sterilised by ethylene oxide.

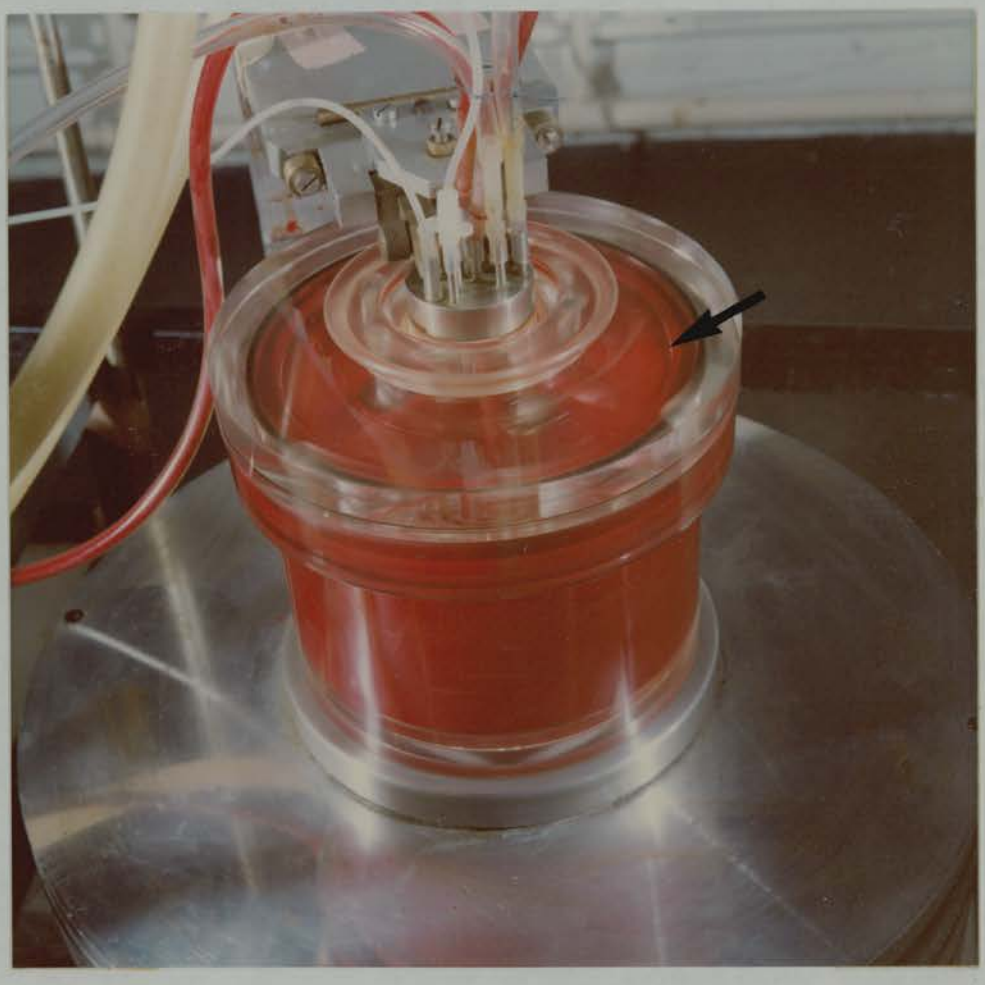
The centrifuge

The centrifuge of the blood cell separator was made from a simple laboratory centrifuge (MSE Minor, MSE, London). The original bowl of the centrifuge was covered with a basement plate (see Fig 5.07) with a central opening for the spindle. A flywheel was mounted on the emerging spindle. Lateral movement greater than 0.001 inch was prevented by stabilising the shaft using a ball bearing around it.

5.2.2. Operation and functioning of the blood cell separator

The centrifuge bowl is stored in a sterilising solution (Cidex). On the day of the experiment the cidex is removed, and the bowl flushed 6-9 times with sterile saline. The bowl is then primed with heparinised saline (5 U/ml) and mounted on the centrifuge flywheel. The venous withdrawal line is primed with heparinised donor blood (1 U/ml) and connected to the face seal port. The centrifuge bowl is primed with donor blood from a blood donor set, using the blood cell pump at a rate of 5-10 ml/min. All air is removed. The venous withdrawal

Figure 5.07. The blood cell separator: close up photograph



The centrifuge was covered with a basement plate. The upper face seal is held in position by a brass pivot mechanism, which allows the seal to find the position of least friction. The interface between cell free plasma and packed cells is indicated by the arrow (Simulated run).

cannula is inserted into the left femoral vein and the primed return cannula is inserted into the left jugular vein. The dog is heparinised by an intravenous injection of 2000 U heparin, followed by a slow drip of heparinised saline (80 U/ml at a rate of about 1 ml/min) into the withdrawal line is started. The centrifuge is switched on and slowly taken to the desired speed (1500 rpm, 160 g). Blood cell pump is started and whole blood enters the machine via the upper stainless steel face seal, and passes through the seal and downward through the center of the solid inner core. Centrifugal force is exerted on the blood as it travels across the bottom and up the sides of the bowl. The red blood cells are collected at the top from 4 ports near the wall of the bowl. When the separation is achieved the plasma pump is started and plasma is pumped away from 4 centrally located ports. The separated components then travel through the rotating and stationary parts of the face seal.

After plasma has gone through the pump it passes through a mixing chamber, positioned immediately behind an infusion port. It is then recombined with the red blood cells in an air bubble trap, before it is returned to the dog. The total flow rate was about 90-100 ml/min: plasma flow 30-40 ml/min and packed red cells

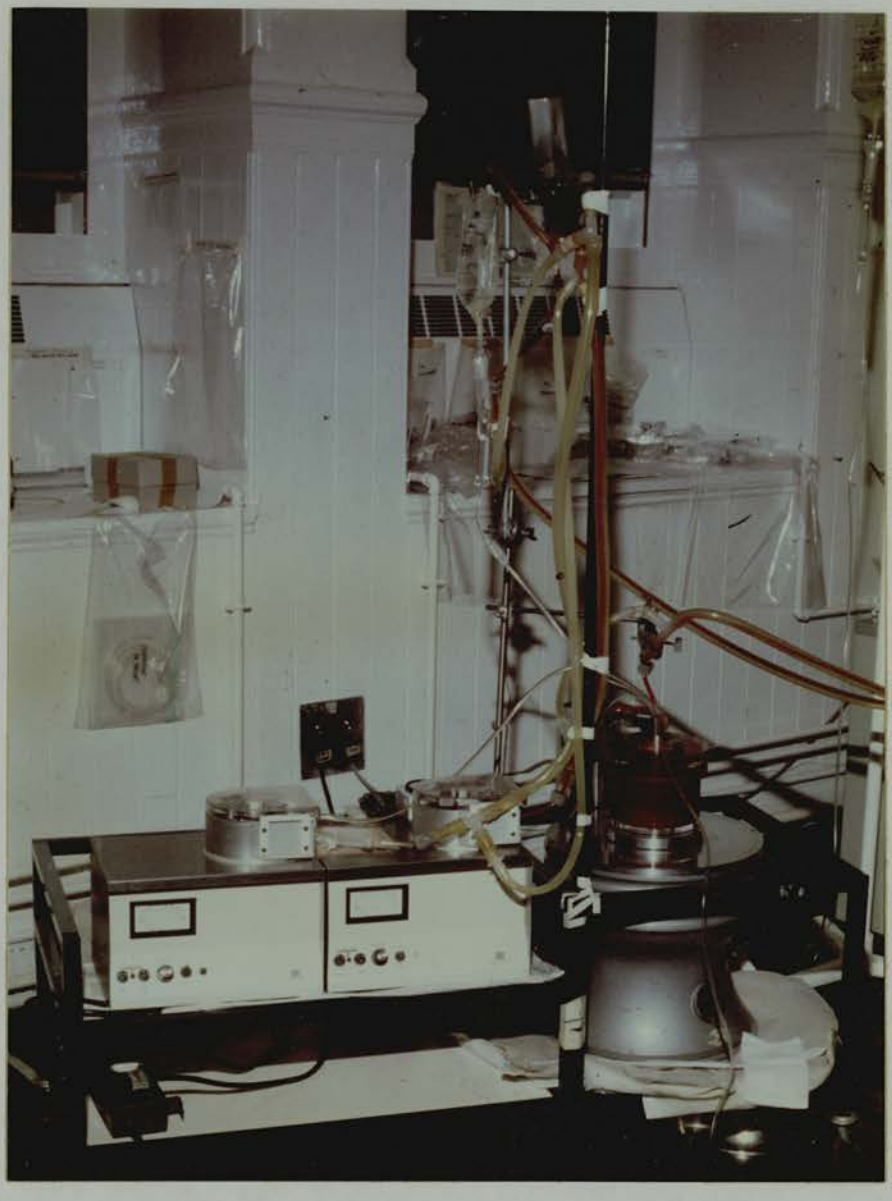
60-70 ml/min.

All tube lines, except the silastic tubes in the pumps, are water-jacketed (temperature 39°C) to prevent heat loss. The total volume in the bowl and tubing is about 220 ml. A photograph of the complete system is presented in Fig 5.08.

5.2.3. Early developmental experiments

Many technical problems were encountered during the early blood cell separator runs, performed in 23 anaesthetised dogs (surgical procedures as described in Section 2.1.4). Blood withdrawal from the arterial side, as advocated by Greenough et al (1969), resulted in wild blood pressure oscillations. Therefore blood was withdrawn from a venous cannula, and arterial blood pressure was stable, provided the level of blood in the bubble trap remained constant. The problems met in the pilot studies were all technical by nature and were solved in due time. The main problem was air entering the system, causing a high initial failure rate of about 50% in the first dozen pilot studies. This problem was traced to the fine adjustment of the face-seal and of the fitting of the centrifuge bowl on the turntable of the centrifuge, necessary to ensure that the upper and lower part of the face-seal were dead opposite each other, while spinning. The tolerance

Figure 5.08. The blood cell separator: overview



Note: the thermostated polyethylene tubes around the withdrawal and return lines. The bubble trap is barely visible at the top. The polyethylene tube in front of the centrifuge drains the irrigation, coolant-saline to waste.

allowed is extremely small.

Heat loss

Another problem related to heat loss, which was initially overcome by using pediatric extracorporeal heat exchangers. But these proved to be cumbersome and difficult to clean. The tubes were therefore thermostated using water-jackets made of heavy polyethylene tubes. This had the additional advantage of reducing the extracorporeal volume, which was determined to a large extent by the heat exchangers.

Inadequate blood cell separation and pumps

Adjustment to the degree of separation of blood cells and plasma proved to be difficult, due to interaction of several factors. Intuitively centrifugal force is increased to obtain better separation. However paradoxically the effectiveness of separation became worse under these conditions, due to the fact that the increased spinning speed of the centrifuge increased the flow rate of the system and hence the transit time of blood in the bowl was reduced. Therefore the speed of the centrifuge was always kept constant (1450-1550 rpm), and the degree of separation was adjusted in the first instance by the relative flow rates of plasma and packed red cells; and failing this by reducing total flow rate (increase in transit time). This was very much an empirical affair

and was extremely difficult when initially two different peristaltic pumps (with different calibrations) were used. However, with the two roller-type blood pumps this problem is less, but improvements could be made by electrically linking the two pumps. The advantage of 2 linked pumps is that although the relative flow rates of the pumps can be varied at will, total flow remains constant.

Haemolysis during centrifugation

Haemolysis during the centrifugational procedure was observed, particularly when the centrifuge speeds exceeded 1700 rpm. However, when the speed was well controlled below 1550 rpm, only a slight degree of haemolysis was noticeable. Donor blood was occasionally haemolysed.

Mixing of sodium oleate with plasma

Problems were also encountered during the infusion of sodium oleate. The specific gravity of the infusate was greater than plasma, and as a result the infusate flowed through the tube without proper mixing. Centrifugal pumps are ideal for instantaneous mixing of liquids, since the fluid is forced at high speed from the center of the pump to its perimeter. However a pilot study showed that plasma proteins underwent denaturation. A much better and simple solution was found. The plasma and the infusate were forced to flow through the fine network of a plastic filter holder (Millipore-Filter unit Ø 1 cm) resulting in adequate mixing.

Acute septicaemia

During the design of the centrifuge bowl a choice of plastics had to be made. The commercial bowl was made of polycarbonate, which can be heat sterilised. However, a block of polycarbonate of high quality to make the bowl, was not available. More experience with machining of perspex, which was necessary to fabricate the bowl, had been obtained in the workshop of the Department of Chemical Engineering. Therefore the fact that perspex can not be heat sterilised seemed irrelevant, since if necessary, gas sterilisation could be used. But this was not considered seriously, due to the long, necessary delay of 7 days, which this process requires. However when it became apparent that acute septicaemia did - unpredictably, but sporadically occur (and was confirmed in one case by bacterial growth examination kindly performed for us by Dr. R. Tonkin, Department of Bacteriology, The Royal Infirmary). The following preventative measures were taken:

- the complete plastic tube manifold was gas sterilised
- the centrifuge bowl and face seals were sterilised using Cidex
- an antibiotic (Keflin, 1 g) was administered intravenously before the procedure

- all plastic tubes were disposed of at the end of each experiment

Since these steps were taken no adverse side effects, due to the actual blood cell separating procedure have been observed.

5.2.4. Discussion

Many technical problems were encountered during the introduction of the blood cell separator technique. In 13 out of 23 pilot experiments complete data could not be obtained, often due to combinations of technical problems. Similar difficulties were also experienced by other workers, while developing the blood cell separating technique in dogs (Buckner et al, 1968). Their initial failure rate in a series of 140 experiments was 40%. After that, they still experienced technical problems in 3% of the final studies. Five dogs died during their experiments: one each of infection, haemorrhage, carotid artery thrombosis, pulmonary oedema and unknown cause.

In our pilot studies 4 dogs died due to embolism. Blood cultures could not routinely be made, but early signs of septicaemia were encountered. These findings illustrate the difficulty of the technique, but in the recent - definite experiments such problems were not

encountered, due to experience in the operation of the machine and also due to improvements in equipment. Despite this, further improvements could be made: (1) introduction of electronic control of blood return to eliminate the risk of air embolism (2) a modification of the speed control of the centrifuge, which will make it independent of the flow rate through the system (3) electric linking of the two peristaltic pumps which will allow fine regulation of the blood cell separation without affecting the total flow rate through the system.

5.2.5. Summary

A procedure has been developed, which allows infusion and binding of fatty acid anions to plasma albumin. Many technical problems were encountered and incomplete data was obtained in many studies. Four animals died and others may have suffered from mild to severe septicaemic shock. However all these problems could be solved and the technique in its present form should allow the infusion of fatty acid anions into dogs.

SECTION 5.3:

DIRECT INFUSION OF SODIUM OLEATE AND ITS EFFECTS ON ARTERIAL PLASMA CONCENTRATIONS OF FATTY ACIDS, MYOCARDIAL METABOLISM AND FUNCTION IN NORMAL DOGS

The infusion of sodium oleate in normal dogs

Introduction

The effects of elevated plasma concentrations, of free fatty acids, raised by direct infusion of sodium oleate were studied in normal dogs. These studies were designed as a control series for those, in which the effects of raised plasma free fatty acids were examined during experimental myocardial ischaemia.

5.3.1. Methods

Animal preparation

Mongrel dogs of both sexes, were anaesthetised after overnight fast by an intravenous injection of sodium pentobarbitone (about 25 mg/kg), followed by a continuous infusion of pentobarbitone at a dose of 4 mg/kg/hr. Ventilation was maintained through a cuffed endotracheal tube with a positive pressure respirator (Harvard Apparatus Co, Mass., USA). Thoracotomy was performed through an incision in the left fifth intercostal space using diathermy, and the heart was suspended in a pericardial cradle. The left anterior descending coronary was dissected free for a distance of 0.5-1.0 cm. distally to the first diagonal branch and a ligature placed loosely around it. However in this series of experiments, which served as controls for those in which the effects of sodium oleate were examined during coronary occlusion (Section 5.4.), the coronary artery was not occluded.

The right femoral vein was cannulated as a route for infusion. The right femoral artery was cannulated for measurement of mean aortic blood pressure ($\overline{AP}$), monitored with a Statham P23 Db transducer. The left femoral artery was cannulated for collection of arterial blood

samples. The jugular vein was dissected high in the left neck immediately below the mandible, at which point there is a bifurcation. The superior branch was used for the insertion and placement of the coronary sinus catheter as described in Section 2.1.4. (without fluoroscopic guidance). The connection of the dog to the blood cell separator was initiated after the intravenous administration of heparin (2000 U) and Keflin (1 g), as described in Section 5.2.2.

Biochemical procedures

Arterial and coronary sinus blood samples were obtained simultaneously, for the measurement of plasma concentrations of free fatty acids, glycerol, blood gases albumin and haemoglobin. Plasma free fatty acids were analysed by the titrimetric method, described in Section 2.2.2. Glycerol was determined using an enzymic fluorimetric method (Section 2.2.4.) and plasma albumin was measured with a manual colorimetric technique (Section 2.2.6.). Blood gas analyses were kindly performed for us by the Blood Gas Laboratory, Department of Medicine, The Royal Infirmary, using an IL313 blood gas analyser (Instrumentation Laboratory) and oxygen contents were determined using a LexO_2con (Lexington Instr. Corp., Waltham, Mass., USA). Plasma haemoglobin was measured spectrophotometrically at a wavelength of 540 nm, using a Unicam SP1800 ultraviolet

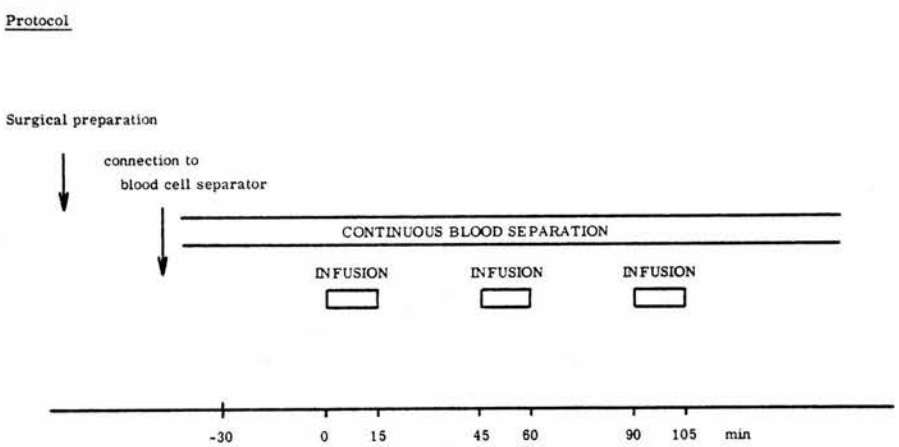
spectrophotometer (Pye Unicam, Cambridge). Haemoglobin concentrations were calculated from the observed extinctions using the molecular extinction coefficient for haemoglobin of 11000 Mole/cm^2.

5.3.2. Experimental procedure (Fig 5.09)

After the surgical procedures were completed the dog was connected to the blood cell separator. A surface electrocardiogram (lead III) and arterial blood pressure were continuously recorded on electromagnetic tape, for later arrhythmia analysis. Thirty min after steady blood cell separation was achieved, arterial and coronary sinus blood samples were collected; and blood pressure and heart rate were recorded. Sodium hydroxide (0.111 mole/l) was then infused for 15 min at a rate of 4.5 ml/min. Arterial blood samples were collected at 5 and 10 min of infusion, and arterial and coronary sinus blood at 15 min. Arterial plasma free fatty acid concentrations were also measured 4 and 8 min post-infusion.

Thirty min after the end of this infusion, this procedure of blood sampling, recording of heart rate and blood pressure was repeated in an identical manner this time during a 15 min infusion of sodium oleate (0.106 mole/l in 0.111 mole/l NaOH) at the same rate. Another infusion of sodium hydroxide (second control infusion) was made again in an identical manner 30 min after cessation of the 2nd infusion in 5 dogs. In one dog the sequence of infusion of NaOH and sodium oleate was reversed: 1st infusion sodium oleate, 2nd infusion NaOH. The results were similar and the data of the experiments were pooled.

Figure 5.09. Experimental procedure for assessing the effect of sodium oleate in openchest, anaesthetised dogs (control experiments)



Three infusions were made; NaOH (0.111 mole/l) was infused during the first and the third period, and sodium oleate (0.106 mole/l in 0.111 mole/l NaOH) during the second. Blood samples were collected immediately before, and at 5, 10 and 15 min during the infusion. Arterial plasma free fatty acids were measured also 4 and 8 min post infusion.

In a subgroup of 3 experiments the effect of sodium oleate on myocardial blood flow was examined. Radioactive microspheres were injected at 15 min of the first (control) and second (sodium oleate) infusion.

The effect of blood sampling on total blood volume was annulled by transfusing an identical volume of heparinised blood (1 U/ml), each time after collection.

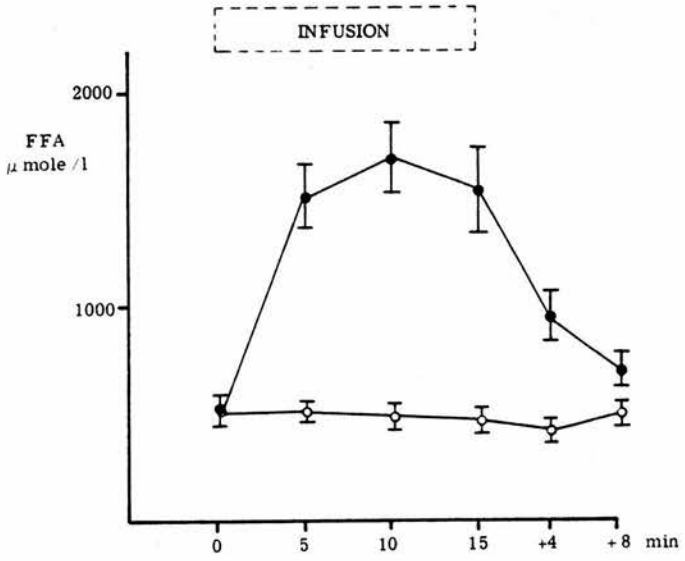
Statistical analysis

Each dog served as its own control. Probability values were calculated using a Student's t-test for paired data. The observations during the two control infusions were similar and the data was pooled. $P > 0.05$ = not significant. Statistical calculations were not performed for 5 or less pairs.

5.3.3. Results

During the infusion of sodium oleate, arterial concentrations of plasma free fatty acids rose quickly from a baseline value of 544 ± 46 to 1530 ± 141 μmole/l (mean ± SEM, n = 8) at 5 min of infusion. In all but one experiment arterial concentrations of plasma free fatty acids peaked at 10 or 15 min of infusion: with values of 1710 ± 154 and 1561 ± 190 μmole/l, respectively, and returned quickly to pre-infusion levels (Fig 5.10; Table 5.02). In contrast, during NaOH infusion (control) plasma concentrations of free fatty acids remained steady. The increase in arterial concentration of plasma free fatty acids was reflected in an increased myocardial extraction. Also, since arterial plasma albumin levels did not change during the control and oleate infusions, the increase in arterio-venous difference of plasma free fatty acids across the myocardium was related to an increased ratio in the molar concentrations of free fatty acids and albumin (Fig 5.11). Arterial glycerol levels were measured in 7 experiments, no effect of NaOH or of sodium oleate was found (Table 5.02). Complete data of myocardial glycerol extraction was obtained in 4 experiments and was reduced in 3 (Data not shown). The infusion of sodium oleate did not effect arterial blood gases when

Figure 5.10. The effect of sodium oleate on arterial plasma free fatty acid concentrations in 8 dogs (control experiments)



● ——————— ● sodium oleate infusion

o ——————— o combined NaOH infusions

Table 5.02

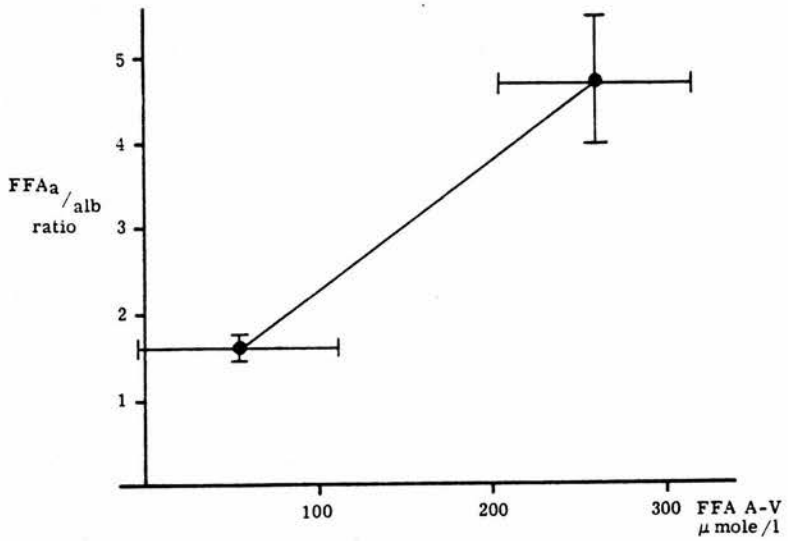
The haemodynamic and biochemical effects of sodium oleate infusion in 10 dogs

	control	control infusion	control sodium oleate	
Heart rate (beats/min)	152±6	153 ± 6	157±8	140 ± 7**
Mean aortic blood pressure (mmHg)	130±6	116 ± 6	122±7	103 ± 7
Free fatty acids$_a$ (μmole/l)	511±23	483 ± 40	544±46	1561 ± 190**
Free fatty acids$_{a-cs}$	42±21	-28 ± 51	54±58	260 ± 56**
Free fatty acid/alb.ratio	1.38±0.09	1.43 ± 0.12	1.62±0.14	4.72 ± 0.75*
Glycerol$_a$ (μmole/l)	67±7	71 ± 7	66±9	68 ± 8
Glycerol$_{a-cs}$	–	0 ± 6	–	15 ± 8
Albumin$_a$ (g/l)	26.5±0.7	25.6 ± 0.6	24.5 ± 0.8	24.4 ± 1.0

Probability values were calculated using a Student's t-test for paired data,

* $P < 0.05$; ** $P < 0.01$

Figure 5.11. The effect of sodium oleate on arterial plasma free fatty acid to albumin molar ratio (FFA_a/alb) and arterio-venous difference of free fatty acids (FFA A-V) across the normal myocardium



The standard errors of the mean are indicated by the bars.
Lower point: during NaOH infusion.

measured at 15 min of infusion (Table 5.03). Complete data for oxygen extraction (arterio-coronary sinus difference) was obtained in 3 experiments (yielding 6 pairs) and no difference between control and infusion of sodium oleate was observed: control 11.2 ± 0.6 and sodium oleate 11.4 ± 0.6 ml O_2/100 ml ($P > 0.05$).

The effect of sodium oleate on arterial blood pressure, heart rate and blood flow

During the blood cell separation procedure heart rate and mean aortic blood pressure remained constant (not shown). The infusion of sodium hydroxide (control) did not affect mean aortic blood pressure (Fig 5.12) nor heart rate (Fig 5.13). The infusion of sodium oleate reduced heart rate at 15 min, but not at 10 min of infusion in 4 dogs, and mean heart rate* of all dogs was reduced ($P < 0.01$; Fig 5.13). This phenomenon was unrelated to the molar ratio of fatty acids to albumin (not shown). Mean arterial blood pressure fell during sodium oleate in 3 experiments, but the average mean arterial blood pressure of all experiments was unchanged. The reductions in heart rate and blood pressure (when observed) were transient and returned to preinfusion levels on termination of the infusion. Sodium oleate increased myocardial blood flow, particularly to the epicardial layers (Table 5.04).

*at 15 min of infusion

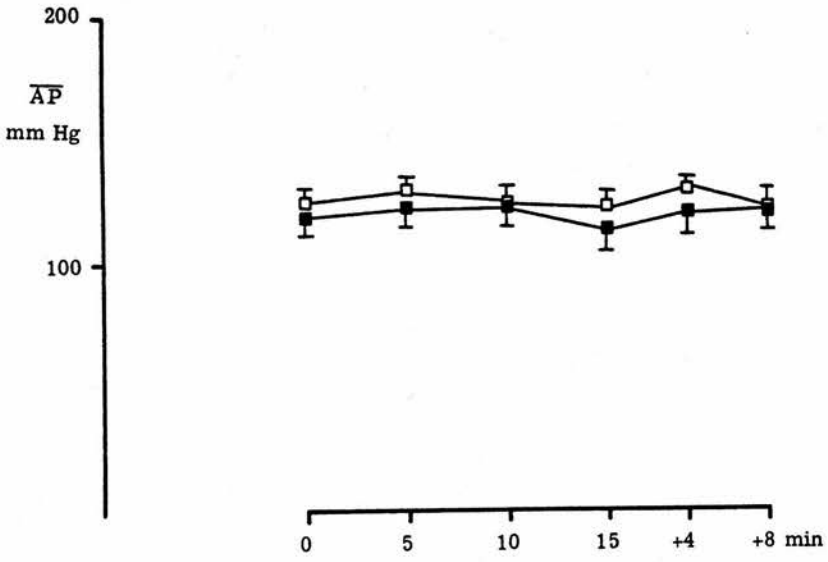
Table 5.03

The effect of sodium oleate infusion on arterial pH, pCO_2 and O_2 content in 4 anaesthetized, open-chest dogs (mean ± SEM)

	Infusion	
	control	sodium oleate
pH	7.40 ± 0.02	7.39 ± 0.01
pCO_2 (mmHg)	35 ± 2	34 ± 4
O_2 (ml/100 ml)	14.6 ± 1.2	14.7 ± 0.3

Measurements at 15 min of control or sodium oleate infusion. For details of composition of the infusates see text.

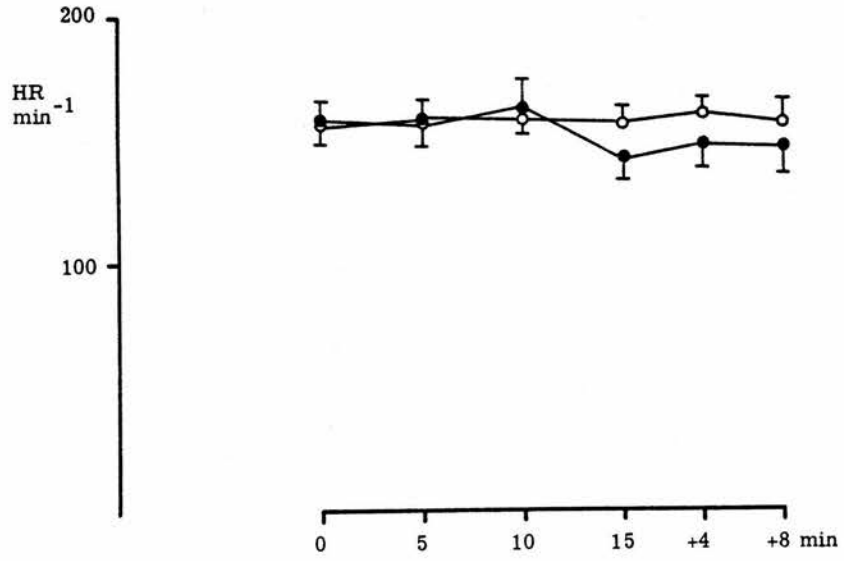
Figure 5.12. The effect of sodium oleate on mean aortic blood pressure ($\overline{AP}$) in 8 open chest, anaesthetised dogs (control experiments)



■ ——— ■ sodium oleate infusion

□ ——— □ combined NaOH infusions

Figure 5.13. The effect of sodium oleate on heart rate in 8 open chest, anaesthetised dogs (control experiments)



●——● sodium oleate infusion.

○——○ combined NaOH infusions.

Note significant reduction in heart rate after sodium oleate.

Table 5.04

The effect of sodium oleate infusion on regional myocardial blood flow in 3 dogs

	control	sodium oleate
Endocardium (N=52)	0.68 ± 0.01	0.79 ± 0.02*
Epicardium (N=52)	0.56 ± 0.01	0.74 ± 0.03*
Endo/epicardium ratio (N=52)	1.22 ± 0.03	1.08 ± 0.02*

N= number of biopsies.

Probability values were calculated using a Student's t-test for paired data.

* $P < 0.001$.

The effect of sodium oleate on serious ventricular arrhythmias

In all (10) experiments the electrocardiogram was recorded on magnetic tape for later analysis of arrhythmias. In one case the recording had failed and the record was of poor quality in another. Of the remaining 8 experiments no arrhythmias were found in 5 and occasional ectopics in 3. Ectopic beats were not associated with the infusion of sodium oleate, and their rate never exceeded 2 min^{-1}.

5.3.3. Discussion

Direct infusion of sodium oleate, with the aid of the blood cell separator, resulted in raised arterial concentrations of plasma free fatty acids. The increments attained were in the same order of magnitude as those in the study of Greenough et al (1969). In the present study we have extended their observations and showed that - as expected - the increased arterial concentrations were reflected in increased arterio-venous difference of fatty acid concentration across the myocardium. Their observations of unchanged arterial blood gas results have been confirmed. However, it may be that the infusion of sodium oleate also affected heart rate and blood pressure in their studies. The reason for this effect of sodium oleate could be due to the high free fatty acid to albumin molar ratio attained both in their and in the

present study. The most likely explanation for the high ratios is the low albumin concentration, ranging from 20-30 g/l in these dogs, much lower than in well-fed greyhounds (Opie et al, 1973). But this can not be the complete explanation since the reduction in heart rate in our studies was not related to the attained free fatty acid to albumin molar ratio. Nor could it be explained by other factors: pH, haemolysis etc. The response to infusions of fatty acid emulsions was also variable and unpredictable in normal dogs (Riemersma et al, 1974). It is of interest that heparin increased the sensitivity of rabbits to direct injection of arachidonic acid (Debye et al, 1978), although it is not clear whether this effect of heparin is specific for the response to this particular fatty acid. More work is indicated and at present it is difficult to say with confidence, whether mild haemodynamic effects observed are not artifacts.

The high concentrations of plasma free fatty acids were not associated with the development of serious ventricular arrhythmias. These findings do not agree with those of Hoak et al (1972), who claimed arrhythmias*. However, it should be pointed out that the occurrence of arrhythmias in normal dogs after infusions of free fatty acids is not well documented. The effects of infusion

*after infusion of 'unbound' fatty acids

of fatty acid anions on arterial blood pressure for instance, are not presented. This lack of information is serious, since the crucial distinction between a primary onset of arrhythmias and a hypotension, followed by arrhythmias can not be made. In a previous study arrhythmias were in most instances seen after hypotension (Riemersma et al, 1974). And it may well be that the same applies for the studies of Hoak et al (1972).

The limitation in raising arterial levels of plasma free fatty acids was mainly determined by the plasma line flow rate, which in turn is dictated by the haematocrit. Obviously with higher flow rates more fatty acids can be infused, while they remain properly bound. It has been suggested that the volume load might be another limiting factor in reaching higher arterial concentrations. However the sodium oleate infusate prepared by our method was much more stable, since saline was replaced by distilled water, and the infusate could have been more concentrated. We have also attempted direct injection of oleic acid through a fine hypodermic needle directly into the plasma line (30 times as concentrated as the present infusate). However the quality of our pumps was insufficient to control the infusion rate under the high pressures required for this. This approach would have

had the additional advantage that it can be used for the infusion of polyunsaturated fatty acids, which are prone to isomerisation and autooxidation under alkaline conditions. Our vein to vein procedure might be expected to be less efficient than an artery to vein procedure (Greenough et al, 1969). Some of the fatty acid enriched blood might have been recirculating in the blood cell separator. Although we cannot exclude this in our experiments, the withdrawal cannula is positioned far from the cannula used for the return of fatty acid enriched blood, and recirculation seems unlikely.

The blood cell separator requires full anticoagulation of the blood to prevent blockage of the narrow channels in the face seal. Heparin was used, despite its well-known effects on lipid-metabolism (releasing lipoprotein lipase, phospholipase and hepatic lipase (Vogel and Bierman, 1965; Jansen and Hülsmann, 1974). However fasting triglyceride concentrations in dogs are low, and as a result the effect of heparin on plasma free fatty acids might be expected to be transient. Initial concentrations of free fatty acids measured 30 min after heparinisation could not be distinguished from those of our previous non-heparinised studies. The effect of heparin on myocardial triglyceride utilisation may not be

transient due to the presumed heparin-induced depletion of myocardial endothelial lipoprotein lipase. However it is difficult, if not impossible, to predict the overall effect of heparin on myocardial fat metabolism (free fatty acids and triglycerides), since the presumed reduction in triglyceride metabolism may be balanced by increased utilisation of free fatty acids. Furthermore alternative drugs such as coumarin, with its uncoupling activity on oxidative phosphorylation, provide other problems, particularly when used during ischaemia. However the fact is and should be acknowledged that the use of heparin (as any other anticoagulant for that matter) is not ideal. Particularly, since it made the prevention of bleeding from the chest wounds extremely difficult, and in some experiments we had to take refuge to use of blood donation (up to 100 ml) to maintain constant arterial pressure. Possibly, the use of chemically-bonded heparin in the extracorporeal system may provide the best alternative.

Plasma concentrations of glycerol are a good index of adipose tissue lipolysis (Scow and Chernick, 1970). High concentrations of free fatty acids inhibited glycerol release from adipose tissue (Scow and Chernick, 1970). There is also some evidence that free fatty acids may

inhibit adipose tissue lipolysis in vivo (Bezman-Tarcher, 1969). Also the release of insulin by raised plasma free fatty acids (Greenough et al, 1969) would be expected to inhibit adipose tissue lipolysis mainly by the effect of insulin on reesterification (Scow and Chernick, 1970). In our experiments, which were not designed especially to study adipose tissue lipolysis, the infusion of sodium oleate did not reduce arterial glycerol concentrations. Thus it appears that adipose tissue lipolysis was not inhibited. However it should be pointed out that glycerol was measured 15 min after the infusion of oleate, and in view of the slow turnover of glycerol, this might have been too early to notice any effect. Another explanation may be that adipose tissue lipolysis was not stimulated in the present experiments by catecholamines, and that raised free fatty acid concentrations inhibit only stimulated adipose tissue lipolysis. The data on myocardial glycerol release was incomplete, a rather unfortunate fact, particularly since more knowledge of the effects of free fatty acids on myocardial lipolysis is urgently required. Clearly more studies designed to examine the possible modulating effect of free fatty acids on myocardial and/or adipose tissue lipolysis are indicated. The blood cell separator may provide us with a useful tool for these studies.

5.3.4. Summary

Plasma free fatty acids were raised in anaesthetised dogs using a blood cell separator. This machine allowed the binding of free fatty acids to the dog's own albumin prior to recombination and infusion into the dog. Plasma levels rose from 544 ± 46 µmole/l to 1530 ± 141 µmole/l within 5 min of infusion. Increased arterial free fatty acid concentrations, resulted in increased extraction by the heart. Arterial blood gas values and plasma glycerol concentrations remained unchanged. Haemodynamic effects were observed in some dogs, but were not related to arterial free fatty acid to albumin molar ratios. No arrhythmias were observed. These results show that the blood cell separator may be a useful, although technically complicated tool in metabolic studies in dogs.

SECTION 5.4:

THE EFFECTS OF SODIUM OLEATE ON MYOCARDIAL METABOLISM AND FUNCTION DURING EXPERIMENTAL CORONARY ARTERY OCCLUSION IN DOGS

5.4.1. Methods

Animal preparation

Mongrel dogs of both sexes, weighing 11.0-19.5 kg were anaesthetised after overnight fast with sodium pentobarbitone (about 25 mg/kg). Anaesthesia was maintained by continuous infusion of pentobarbitone, at the dose of 4 mg/kg/hr. Ventilation was maintained through a cuffed endotracheal tube with a positive pressure respirator (Harvard Apparatus Co, Mass). All surgical procedures: dissection and cannulation of veins and arteries, thoracotomy, suspension of the heart and dissection of the left anterior descending coronary artery at a point immediately behind the first diagonal branch, were identical to those of the control series (normal dogs; see Section 5.3). In addition to this, a small cannula was inserted into the local vein, draining the myocardium perfused by the dissected coronary artery. A cannula was inserted into the left atrium for the injection of microspheres to estimate regional myocardial blood flow. A 15 electrode epicardial grid was positioned over the area supplied by the dissected coronary artery, and secured by 3-4 small stitches (4/O silk) outside the area of interest.

Induction of myocardial ischaemia

Intermittent myocardial ischaemia of 10-15 min duration was induced by occlusion of the dissected coronary artery using a metal, releasable clip, as described in Section 2.1.5. The first was a sham occlusion of 10 min duration made at the end of the surgical procedures. A recovery period of 30 min was allowed between successive coronary artery occlusions.

Electrocardiographic and haemodynamic monitoring

Three of the 15 epicardial electrodes were selected for continuous monitoring. The signals were pre-amplified* (see Section 2.3.1.), and recorded on a 3 channel magnetic tape recorder (speed: $\frac{1}{4}$ in/s), for later analysis of ST-segment elevation and arrhythmias. The proximal occlusion used in these studies caused marked conduction delay, and measurements of ST-segment elevation could not be made with confidence. The use of the epicardial rather than surface leads (for the measurement of ST-segment elevation) made the analysis of arrhythmias

*The multiplexer was used as a three channel ECG amplifier, by inhibiting electronically its multiplexing function.

with the arrhythmia computer impracticable, due to constant changing configuration of the electrocardiogram. Therefore the tapes were frequently replayed to scan visually whether arrhythmias occurred. Arrhythmias were inspected by replaying the concerned section of the magnetic tape on a chart recorder. Mean aortic blood pressure was monitored from the right femoral arterial cannula with a Statham P23 Db pressure transducer and recorded on a Devices M8 recorder.

Regional myocardial blood flow

Regional myocardial blood flow was measured by means of radioactive microspheres (nominal diameter 15 μ), as described in detail in Section 2.4. Regional myocardial blood flow was calculated for each biopsy by relating its radioactivity to that of a reference arterial blood sample, withdrawn at a known fixed rate (reference flow). The calculations were made using a computer programme written by Dr. Boardman for a PDP-12 computer (Appendix B).

Biochemical analyses

Arterial and local venous blood samples were collected simultaneously immediately before, at 5 min and at 12-15 min of the successive infusions (= 7-10 min of occlusion). Plasma free fatty acids were estimated using a titrimetric

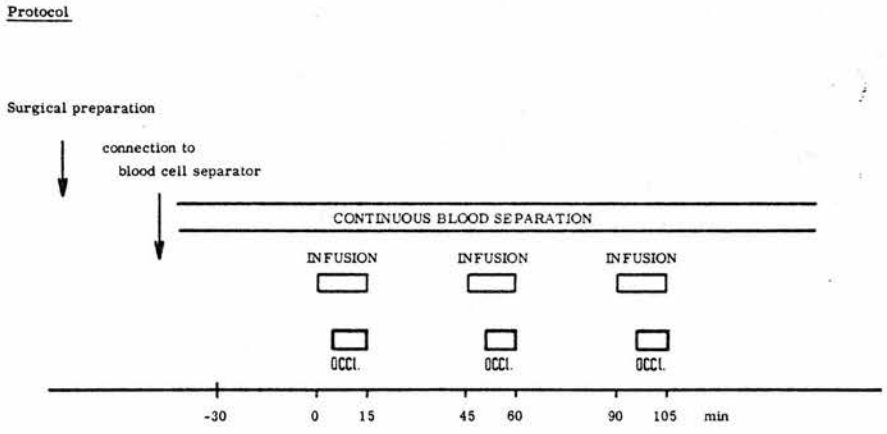
method, described in Section 2.2.2. Plasma glucose was determined using the automated hexokinase method (Section 2.2.3). Plasma glycerol was analysed by the manual enzymic fluorimetric method of Passonneau (Section 2.2.4). Plasma albumin was determined by a dye-binding technique (Section 2.2.6). K^+ was estimated by flamephotometry in the Medical Renal Unit, The Royal Infirmary, Edinburgh. Haemoglobin was estimated spectrophotometrically as described in Section 5.3.1.

5.4.2. Experimental design

The design was the same as that used for the normal (control) dogs, described in Section 5.3 , except that after an initial sham occlusion 3 intermittent occlusions of 10 min duration, each separated by a recovery period of 30 min, were superimposed on the basic protocol (see Fig 5.14. Note that the sham occlusion is not indicated). Haemodynamic measurements were recorded pre-infusion, 5 min post-infusion and at 5 min of occlusion. Regional myocardial blood flow was determined 5 min after coronary artery occlusion. Heart rate was controlled during infusions.

The frequency of the blood sampling procedure was reduced, in view of the expected difficulties in collecting local venous samples during ischaemia. Thus arterio venous differences in concentrations of fatty acids,

Figure 5.14. Experimental procedure for assessing the effect of sodium oleate on the response to subsequent coronary artery occlusion



A sham occlusion (10 min) was made at the end of the surgical procedures. Thirty min after attaining steady blood cell separation and collection of blood samples, an infusion of NaOH (0.111 mole/l) was commenced (Control I). Myocardial ischaemia was induced 5 min later after collection of pre-occlusion blood samples. Regional myocardial blood flow (5 min of occlusion) and arterio-local venous differences (7-10 min of occlusion) were then assessed. After release and recovery (30 min) the procedure was repeated except that sodium oleate 0.106 mole/l in 0.111 mole/l NaOH was infused. A second control infusion followed by occlusion was made 30 min post sodium oleate. Occl. = occlusion.
Note: during all infusion periods the heart rate was maintained constant by right atrial pacing.

glycerol and glucose were estimated: (1) immediately before infusion; (2) 5 min later, just before occlusion and (3) at 7-10 min of occlusion during the sustained infusion. Total blood volume taken over the course of the experiment was 240 ml. The influence of this was annulled by transfusing an identical volume of heparinised donor blood, each time after collection.

Statistical analysis

Each dog served as its own control. Probability values were calculated using a Student's t-test for paired data. The observations made immediately before and during the first and the second infusions of NaOH (control periods) and coronary occlusion were not statistically significantly different and all control data were pooled. Probability values of > 0.05 were not regarded as statistically significant (N.S.).

Statistical calculations for 5 or less pairs were not performed.

5.4.2. Results

The effects of sodium oleate in the absence of ischaemia

During the control infusion of NaOH (0.111 mole/l) mean aortic blood pressure was stable. When plasma free fatty acids were raised by sodium oleate infusion, mean aortic blood pressure fell slightly, but significantly:

control: 106 ± 8 mmHg; sodium oleate: 100 ± 8 mmHg ($P < 0.01$, $n = 7$, Table 5.05). The increase in arterial concentrations of free fatty acids from 575 ± 43 to 1531 ± 111 μmole/l ($P < 0.01$, $n = 6$), was reflected in an increase in the molar ratio of free fatty acids to albumin. Myocardial extraction of free fatty acids was also increased. Arterial plasma glucose concentrations tended to fall during the experiment, but this effect did not reach a level of significance. During sodium oleate infusion glucose extraction (arterio-local venous difference) was 0.48 ± 0.09 mmole/l. This value was not significantly different from that obtained immediately before the sodium oleate infusion (0.55 ± 0.05 mmole/l). It could also not be distinguished from myocardial glucose extraction measured during the NaOH infusion (Comparison vs combined data of NaOH Infusion I + II).

Arterial glycerol concentrations tended to increase throughout the experiment, the pre-infusion observations were: 38 ± 9; 58 ±12 and 68 ± 14 μmole/l. (No statistical analysis was performed: only 5 observations during the second NaOH infusion). There was insufficient data for the statistical analysis of the effect of sodium oleate on arterial glycerol concentrations, but in the 4 experiments with complete data no effect was observed:

Table 5.05

The haemodynamic and biochemical effects of sodium oleate infusion in 7 dogs with experimental coronary artery occlusion

	Control infusion (I)			Sodium oleate			Control infusion (II)		
	control	pre.occl.	occlusion	control	pre.occl.	occlusion	control	pre.occl.	occlusion
Mean aortic blood pressure (mm Hg)	109±8	110±8	107±8	106±8	100±8‡	93±7*	111±9	111±10	103±12
Free fatty acids$_a$ (µmole/l)	448±33	469±36	523±93	575±48	1531±111‡	1786±157*	717±57	587±102	662±40
Free fatty acids$_{a-lv}$	65±15	21±49	92±30	158±29	558±29‡	603±69*	208±66	170±62	198
Free fatty acid/alb.ratio	1.92±0.39	1.53±0.12	1.76±0.27	2.12±0.21	5.26±0.39‡	6.57±0.60*	2.71±0.28	2.59±0.21	2.61±0.27
Albumin$_a$ (g/l)	26.9±0.8	—	25.4±0.6	24.3±0.8	—	24.3±1.0	22.8±0.8	—	22.6±1.4
Glucose$_a$ (mmole/l)	5.36±0.11	5.33±0.16	5.39±0.23	5.20±0.23	5.07±0.16	5.47±0.28	5.27±0.41	4.96±0.33	4.76±0.38
Glucose$_{a-lv}$	0.57±0.06	0.79±0.25	1.19±0.30‡	0.55±0.05	0.48±0.09	1.14±0.21‡	0.36±0.11	0.39±0.15	0.64
Glycerol$_a$ (µmole/l)	38±9	38±7	37±7	58±12	60±5	49±9	68±14	84±22	66±26
Glycerol$_{a-lv}$	-13	9±4	-13	15±7	22	-13	11	21	12
Potassium$_a$ (mmole/l)	4.0±0.2	3.9±0.1	3.8±0.1	3.9±0.1	3.8±0.1	3.8±0.1	3.9±0.2	3.9±0.1	3.9±0.2
Haemoglobin$_a$ (mg/100ml)	194±29	187±37	190±52	263±63	245±70	272±86	415±120	397±134	422±137

Heart rates were kept constant by right atrial pacing.

* $P < 0.01$ vs combined control occlusion.

‡ $P < 0.01$ vs control of the same infusion (preinfusion data).

control 58 ± 12 vs 60 ± 5 μmole/l.

Plasma K^+ concentrations remained constant throughout the procedure.

The effects of sodium oleate and coronary artery occlusion

During the infusion of NaOH (control) occlusion of the coronary artery did not reduce mean aortic blood pressure. However, when sodium oleate was infused the decline in mean aortic blood pressure before coronary occlusion was continued. Mean aortic blood pressure during sodium oleate pre- and during occlusion were 100±8 and 93±7 mmHg respectively ($P < 0.01$, $n = 12$).

During ischaemia arterial concentrations of plasma free fatty acids remained high (1786 ± 157 μmole/l), while those of glucose, glycerol and potassium were unaltered. The increased arterial fatty acid concentration resulted in an increased myocardial extraction.

Coronary occlusion (during NaOH infusion) increased extraction of glucose across the ischaemic myocardium. Arterio-local venous difference of glucose , pre-occlusion: 0.79 ± 0.25; during occlusion 1.19 ± 0.30 mmole/l. This effect of ischaemia was not changed by the high arterial plasma concentrations and ischaemic myocardial utilisation of free fatty acids: arterio-local venous difference of glucose pre-occlusion: 0.48 ± 0.09; during occlusion 1.14 ± 0.22 mmole/l.

The effect of sodium oleate on regional myocardial blood flow

When the myocardium distant from the ischaemic area was taken to represent normal myocardium, then sodium oleate tended to decrease myocardial blood flow in the endocardial and increase it in the epicardial layers (Both effects not statistically significant). However the distribution of blood flow was significantly changed: control endo/epi MBF ratio 1.17 $\pm$ 0.02 and sodium oleate 1.08 $\pm$ 0.02 ($P < 0.001$, $n = 77$). Sodium oleate did not alter ischaemic myocardial blood flow, nor did it change the blood flow distribution (Table 5.06).

The effect of sodium oleate on arrhythmias

During coronary artery occlusion ectopic beats, often coupled were seen in all but one experiment (Table 5.07/8). Spontaneous ventricular fibrillation was observed on 5 occasions, and all were during the NaOH infusion (control).

Table 5.06

The effect of sodium oleate infusion on regional myocardial blood flow in non-ischaemic and ischaemic free ventricular wall 5 min after coronary occlusion in 7 dogs

	control	sodium oleate
Non-ischaemic area (N=77):		
MBF endocardium	0.73 ± 0.03	0.69 ± 0.02
MBF epicardium	0.63 ± 0.03	0.67 ± 0.02
endo/epicardium ratio	1.17 ± 0.02	1.08 ± 0.02*
Ischaemic area (N=39)		
MBF endocardium	0.27 ± 0.03	0.24 ± 0.03
MBF epicardium	0.36 ± 0.04	0.34 ± 0.03
endo/epicardium ratio	0.80 ± 0.09	0.83 ± 0.12

N= number of biopsies. MBF = myocardial blood flow. Probability values were calculated using a student's t-test for paired data.

*$P < 0.001$; all other observations not significantly different from control.

Table 5.07

The effect of sodium oleate on the occurrence of arrhythmias during experimental coronary artery occlusion

	Coronary artery occlusion		
Exp.	Control infusion	Sodium oleate	Control infusion
1	Ectopics	none	Ectopics
2	Ectopics	none	none
3	Ectopics → VF(5 min)	Ectopics	Ectopics → VF(6 min)
4	Ectopics → VF(5 min)	Ectopics	Ectopics → VF(6 min)
5	none	none	—
6	Ectopics → VF($5\frac{1}{2}$ min)	Ectopics	Ectopics
7	Ectopics	none	Ectopics

For details see text.

Table 5.08

The effect of sodium oleate on the occurrence of ectopic beats and ventricular fibrillation (VF) during experimental coronary occlusion in 7 dogs

	Ectopic beats	VF
Control infusion (N=13)	11	5
Sodium oleate infusion (N=7)	4	0

N= number of observations.

Probability values calculated using Fisher's exact test were >0.05.

For details see text.

5.4.3. Discussion

Direct infusion of sodium oleate in order to raise plasma free fatty acid concentrations, was associated with increased extraction of plasma free fatty acids across the ischaemic myocardium. Since regional myocardial blood flow was not changed, nor its distribution in the ischaemic area, fatty acid uptake by the ischaemic myocardium was also increased. Another notable finding was that increased utilisation of free fatty acids did not reduce glucose extraction by the ischaemic myocardium, in agreement with the findings of Opie et al (1973). They measured glucose extraction by the ischaemic myocardium 20 min after injection of heparin to induce plasma lipolysis and Intralipid as the substrate. Although their plasma free fatty acid concentrations during Intralipid/heparin were overestimated, their glucose data should be valid. Results of anoxic perfused rat hearts can not readily be extrapolated to the ischaemically perfused - in vivo - dog heart. However it is certainly of interest that raised concentrations of palmitate did not inhibit glucose uptake by the anoxic perfused rat heart (Randle et al, 1964). It should be pointed out that raised plasma free fatty acids also did not inhibit glucose extraction before coronary artery occlusion, against what is usually accepted.

Indeed palmitate and oleate inhibit glucose utilisation by the well perfused rat heart (Randle et al, 1964); but the actual decrease is small: 34 and 27% respectively. We observed a non-significant reduction of about 13%. It may be that our failure to demonstrate any (significant) effect is purely methodological. Errors in quantifying arterio-venous differences are large (Section 2.2.3.), but with our method, with a coefficient of variation 0.2-0.5%, we should have observed a 30% reduction. A more likely explanation is therefore, that insulin release by sodium oleate (Crespin et al, 1969) counteracted partially its inhibition of glucose extraction.

Arrhythmia analysis was hampered by the fact that two modifications in our techniques were introduced. An epicardial rather than a surface lead was used, in order to obtain information both on ST-segment elevation and arrhythmias. Another difference was the more proximal site of occlusion, this to enhance the yield of arrhythmias. The epicardial lead was not compatible with the Neilson arrhythmia computer, due to rapid changes in the configuration of the epicardial electrocardiogram. Unfortunately a separate triggering channel was not used, and could have solved the problem. Therefore the tapes were frequently scanned and the occurrence of arrhythmias

was checked by write-out of the relevant sections of the recording.

The more proximal site of occlusion caused marked conduction delay and ST-segment mapping would have been meaningless. However, despite these problems, the results are of interest. On occasions ventricular fibrillation was observed and all were during the infusion of NaOH. Although this effect was not statistically significant, these findings clearly show that raised plasma free fatty acids per se are not arrhythmogenic.

These results contrast to earlier studies of our unit, employing other methods of raising plasma concentrations of free fatty acids (Oliver and Yates, 1972). In the present study experiments were designed to allow each dog to serve as its own control. Myocardial ischaemia is reversible up to 20 min and leads to reproducible ST-segment elevation (Maroko et al, 1971; Berdeaux et al, 1976; this thesis Section 2.1.5) and disturbance of ischaemic myocardial metabolism (Section 2.1.5), provided a recovery period of 30-45 min is allowed. The price to pay for superior control of the study had to be a reduction in the observation period (10 min of occlusion). However VF was frequently observed. Furthermore myocardial extraction was fully stimulated before coronary occlusion.

This would favour the observation of a detrimental effect of fatty acids. The difference between the present and the early fatty acid infusion studies (Oliver and Yates, 1972) may never be explained, but reduced tendency to develop arrhythmias of open-chest dog preparation (Oliver and Yates, 1972), does not appear to be the explanation, in view of the incidence of ventricular fibrillation and coupled ectopic beats in the present studies. Nor was the heart rate different. The same source of dogs was still used, and potassium and albumin levels were similar. Therefore the results of the present study raise doubt to the validity of the early observations, as has arisen before (Riemersma et al, 1974).

Other studies during experimental coronary occlusion have also provided evidence that raised plasma free fatty acids - per se - may not be arrhythmogenic (Opie et al, 1971, Opie and Lubbe 1974), or reduce the ventricular fibrillation threshold in dogs with acute myocardial ischaemia (Kostis et al, 1973). In contrast, antilipolytic drugs have been associated with reduced incidence of arrhythmias in dogs (Smith and Duce, 1974) and in patients with acute myocardial infarction (Rowe et al, 1975). These and our findings tend to favour the possibility that increased myocardial lipolysis might

enhance, and its inhibition might decrease, the incidence of ventricular arrhythmias. The mechanism of this hypthesized, deleterious effect of myocardial lipolysis could be due to the fact that it appears to control the overall rate of the triglyceride fatty acid energy wasting cycle (Fig 6.02). Increased myocardial fat mobilisation has been linked with increased contractility (Stam and Hülsmann, 1978). This effect of myocardial lipolysis was explained by the Ca^{++}-ionophoric properties of intracellular fatty acids. If the reverse is also true and can be applied to ischaemic tissue, then inhibition of lipolysis would lead to reduced contractile behaviour and thereby reducing the oxygen requirements of the ischaemic cell. It is clear that more attention should be paid to myocardial lipolysis.

One question springs to mind: 'would raised concentrations of plasma free fatty acids increase the incidence of arrhythmias when local ischaemic myocardial lipolysis is stimulated by sympathetic nerve stimulation?' Studies, along this line of investigation should provide an important area of future research.

5.4.4. Summary

Concentrations of plasma free fatty acids were raised in dogs with experimental acute myocardial ischaemia, by infusion of sodium oleate. Arterial free fatty acids levels rose from 575 ± 48 to 1786 ± 157 μmole/l. Extraction of plasma free fatty acid by the ischaemic area was also increased, but glucose extraction was unchanged. Sodium oleate did not change significantly regional myocardial blood flow in the non-ischaemic and ischaemic layers, but did increase the distribution of normal blood flow. Mean aortic blood pressure fell from 106 ± 8 to 93 ± 7 mm Hg. Ventricular arrhythmias tended to occur during control occlusions and infusions, and no ventricular fibrillation was observed during sodium oleate. These results and those of others support the view that myocardial lipolysis, rather than raised concentrations of plasma free fatty acids, may be detrimental to the ischaemic myocardium.

Part 6

GENERAL DISCUSSION

Introduction

The outcome of acute myocardial infarction is not predetermined from its onset, but can be influenced when the balance between oxygen supply and demand is modified*. Oxygen consumption is increased when fatty acid metabolism is stimulated (Mjós, 1973). Thus alteration of myocardial metabolism should be beneficial. Two metabolically orientated hypotheses have been proposed, one claims that raised plasma free fatty acids have a detrimental (Kurien and Oliver, 1970) and the other that glucose has a beneficial effect to the ischaemic heart (Opie, 1970). Both hypotheses are critically dependent on the assumption that ischaemic myocardial metabolism can be influenced by its environment. This is well established for the normoxic heart, but extrapolations to the ischaemic myocardium should not be made.

*For a recent review of haemodynamic interventions, see Hillis and Braunwald, 1977.

Extraction and utilisation of fatty acids by the ischaemic myocardium

Normally myocardial fatty acid metabolism is positively related to the arterial fatty acid concentrations or more correctly FFA/alb ratio (Ballard et al, 1960; Scott et al, 1962; Evans et al, 1963; Spector et al, 1968). This relation is also observed in all studies where arterio-venous differences across the ischaemic myocardium were measured and arterial fatty acid concentrations were altered using lipolytic and antilipolytic drugs (Sections 3.3, 3.4 and 3.5), infusion of glucose (Russell et al, 1979, Haneda et al, 1974) or when raised directly by sodium oleate infusion (Section 5.4).

Evidence is accumulating that ischaemia induces myocardial lipolysis (Opie et al, 1973; Brownsey and Brundt, 1977, Appendix E). The stimulus is presumably cyclic-AMP, formed by adenylcyclase, stimulated by locally released noradrenaline (Shahab et al, 1969). The normal regulatory mechanisms appear to apply: since ischaemia-induced lipolysis was inhibited by an antilipolytic drug (Brownsey and Brundt, 1977).

Glucose metabolism in the ischaemic myocardium

The increased glucose extraction by the ischaemic myocardium has been well established (Opie et al, 1973;

De Jong et al, 1977; Jageneau et al, 1975; Vik-Mo et al, Appendix E; Russell et al, 1979). This observation fits well with the observation that glucose transport is accelerated in the anoxic, well perfused heart (Morgan et al, 1961), but no explanation can be given for the finding of Rovetto et al, (1973) that glucose transport of the ischaemic perfused rat heart was not accelerated. Glucose and insulin can stimulate the glucose uptake by the ischaemic heart (Rovetto et al, 1973), and both could be responsible for increased glucose extraction, after glucose infusions in dogs (Russell et al, 1979). An important finding is that raised plasma free fatty acids do not inhibit glucose uptake during ischaemia (Neely et al, 1975; this thesis, Section 5.4). This is to the contrary of what was generally assumed from observations on normoxic myocardium (Kurien and Oliver, 1970; Opie, 1970), underlining that extrapolation from the normoxic myocardial metabolism to ischaemic tissue can not be made.

Increased glycolysis and glycogenolysis ceases early during ischaemia and the actual glycolytic flux becomes even inhibited, at the level of phosphofructokinase (Opie, 1975) or glyceraldehyde-phosphate dehydrogenase (Rovetto et al, 1975) due to a decrease in cellular pH

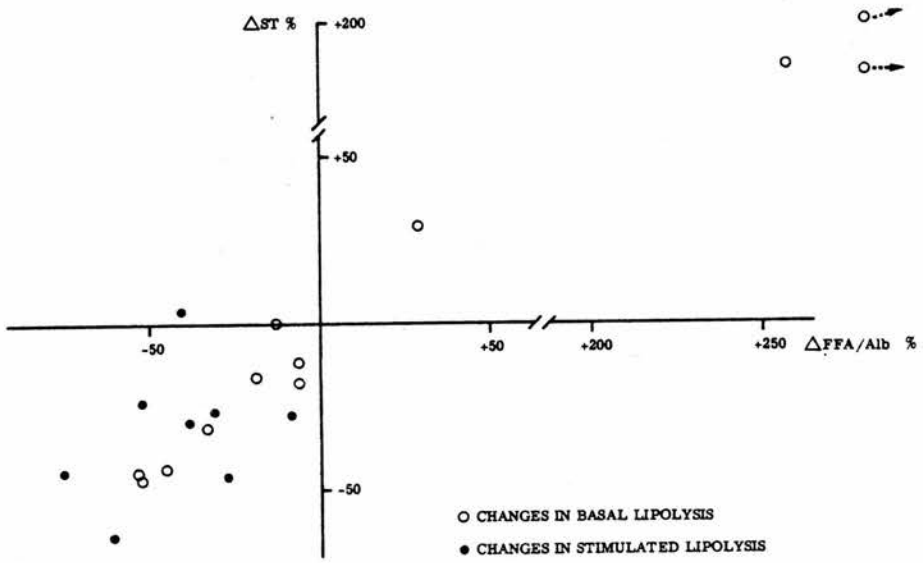
and phosphate potential (ATP/ADP) and/or an increase in $NADH/NAD^+$ ratio. And when the intracellular acidosis is prevented, pyruvate dehydrogenase may become rate-limiting (Rovetto et al, 1975). This together with the fact that the major fate of glucose in the ischaemic heart (Opie et al, 1973; Neely et al, 1975) is oxidation and not lactate formation underlines the importance of the residual oxidative phosphorylation for the survival of the ischaemic cell.

Metabolic intervention and severity of myocardial ischaemic injury

It is now well established that effective antilipolytic therapy reduces the extent and magnitude of ST-segment elevation after experimental coronary artery occlusion. Reduction in epicardial ST-segment elevation after anti-lipolytic treatment has been correlated with preservation of myocardial creatine kinase during a sustained occlusion. Alternatively stimulation of lipolysis with isoprenaline or nicotine increases the severity of myocardial ischaemia. The results of 20 independent experiments using a wide range of anti-lipolytic or lipolytic drugs are summarised in Fig 6.01*.

*For details: Appendix G.

Figure 6.01 Effect of changing free fatty acid to albumin molar ratio (FFA/alb) on alteration of ischaemia induced ST-segment elevation (ΔST) using lipolytic and anti-lipolytic drugs



The effect of lipolytic or anti-lipolytic treatment during basal and isoprenaline stimulated lipolysis on plasma free fatty acid to albumin molar ratio and on ST-segment elevation, were both expressed as a percentage of the initial value.
For further details, see text.

Decreased fatty acid extraction by the ischaemic myocardium after anti-lipolytic treatment was associated with a reduction in ST-segment elevation, but glucose extraction was not changed. The beneficial effect of anti-lipolytic treatment could not be explained by changes in haemodynamic state or regional myocardial blood flow.

Antilipolytic treatment also reduced ST-segment elevation in patients with acute myocardial infarction (Russell and Oliver, 1978; Kjekshus, 1978).

During experimental myocardial ischaemia ST-segment elevation was reduced after the administration of glucose (Maroko et al, 1972, Ueno et al, 1976); glucose-insulin-potassium (GIK) (Maroko et al, 1972; Sybers et al, 1973; Haneda et al, 1974, Opie and Owen, 1976: Ueno et al, 1976). It is of interest that this decrease in ST-segment elevation was associated with increased glucose and decreased free fatty acid utilisation across the ischaemic myocardium (Haneda et al, 1974). Similar effects have been observed in patients with coronary artery disease (Chiong et al, 1976) and may also be related to a change in metabolism (Stanley et al, 1975).

When glucose uptake by the ischaemic myocardium was stimulated by a variety of drugs: dichloroacetate (Section 4.1), a similar compound UK 25842 (Burges, 1977),

and propranolol (Opie and Thomas, 1976) or by infusion of pyruvate and a buffer (Liedtke et al, 1976[b]) ST-segment elevation was also reduced. The beneficial effect of enhanced glucose utilisation was reflected in reduced enzyme loss (Maroko et al, 1972; De Leiris and Opie, 1978) and preservation of mitochondrial function (Calva et al, 1965), reduction in histochemical evidence of necrosis (Sybers et al, 1973). During severe ischaemia glucose may be ineffective (Opie et al, 1975; Liedtke et al, 1976).

Metabolic intervention and arrhythmias

Intravascular lipolysis of triglyceride emulsion (Intralipid) induced by heparin increased the incidence of ventricular arrhythmias (Kurien et al, 1971), presumably due to a lowering of ventricular fibrillation threshold (Takano, 1976). Other workers were unable to reproduce these results (Opie et al, 1972; Kostis et al, 1973; Mjøs and Kjekshus, 1972; Opie and Lubbe, 1975; Mbuyama, 1976). The significance of raised plasma free fatty acids for the induction of arrhythmias after administration of heparin to patients with acute myocardial infarction is also doubtful (Nelson, 1970; Russo et al, 1970; Riemersma et al, 1977), although arrhythmias have been reported in another study (Reimann and Schwandt, 1971).

Raised plasma free fatty acid concentrations by direct infusion of fatty acids also did not precipitate arrhythmias during the early phase of ischaemia (Section 5.4). Earlier preliminary reports (Oliver and Yates, 1971) were probably due to toxic effects of such infusions/in normal dogs (Riemersma et al, 1974). Arrhythmias also seen can be reduced by anti-lipolytic treatment in patients with acute myocardial infarction (Rowe et al, 1975). The difference between the results of heparin or Intralipid/heparin and antilipolytic treatment could be explained by inhibition of myocardial lipolysis in the latter situation, either alone or in conjunction with lowering of plasma free fatty acids.

The effect of raised glucose concentrations on the incidence of arrhythmias

A reduction in arrhythmias after glucose infusion was only observed during the early period of ischaemia (Russell and Oliver, 1979), and a similar transient increase in glucose extraction by the ischaemic myocardium was noted. Ventricular fibrillation was reduced in dogs with coronary artery occlusion after infusion of glucose-insulin-potassium (Ueno et al, 1976), however in the baboon, which has more severe ischaemia after coronary occlusion no effect was observed (Opie et al, 1975). Thus the severity of myocardial ischaemia may be a critical factor

in determining the efficacy of glucose treatment. This view is supported by electrophysiological (Russell et al, 1979) and clinical studies (Rogers et al, 1976).

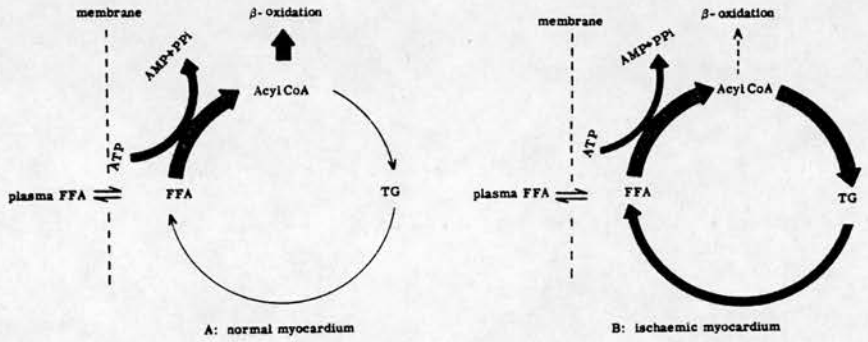
Mechanism of metabolic effects

Myocardial oxygen consumption is raised during enhanced fatty acid utilisation, and has been suggested as a mechanism for the detrimental effect of free fatty acids (Mjøs, 1973). Indeed ischaemic myocardial oxygen consumption was increased after the administration of Intralipid/heparin (Liedtke et al, 1978), and could be reduced by nicotinic acid (Section 3.5). Free fatty acids and acyl-CoA uncouple oxidative phosphorylation in vitro, but these studies may bear no relation to the in vivo situation, due to the absence of fatty acid binding proteins in vitro assays (Ockner et al, 1972).

Oxygen wasting could also be explained by the operation of an energy-wasting cycle (Schematically depicted in Fig 6.02). On entry into the cell free fatty acids are immediately esterified as their co-enzyme-A ester under hydrolysis of ATP, and since they cannot readily be oxidised during acute ischaemia (Scheuer and Brachfeld, 1966; Riemersma et al, 1972; Opie et al, 1973; Neely et al, 1975), they accumulate (Shug et al, 1975; Idell-Wenger and Neely, 1977; Liedtke et al, 1978). The formation of triglycerides is enhanced

Figure 6.02

Proposed FFA-TG energy wasting cycle



The metabolism of the normal and ischaemic myocardium is represented in a schematic form in panels A and B respectively. The flux through a pathway is indicated by the width of the arrows. The energy wasted in the provision of ∝-glycero-phosphate is not indicated. One full cycle would consume 4 molecules ATP (3 for the activation of FFA and 1 for glycerol).

possibly due to a mass-action effect and increased availability of α-glycero-phosphate, and a net accumulation of triglycerides (neutral fat) is observed (Bryant et al, 1958; Scheuer and Brachfeld, 1966), despite the fact that myocardial lipolysis is stimulated (Opie et al, 1973; Brownsey and Brundt, 1977; Appendix E). Thus lipolysis appears to be the rate limiting step in the cycle (since both acyl-CoA and triglycerides accumulate). The completion of one full turn of the cycle (the synthesis and degradation of 1 molecule of triglyceride) requires 4 molecules of ATP, and will be directly related to the lipolytic activity. Controlling the entry of fatty acids (by reducing plasma free fatty acid concentrations) may have little effect on the energy wasted, since the activity of the lipolysis is rate-limiting. Stimulation of myocardial lipolysis by enhanced activity of the myocardial sympathetic nervous system will initiate a further decline in ATP, and thus initiate a vicious circle. This hypothesized mechanism could also explain the observation that raised levels of cyclic AMP relate to the incidence of ventricular fibrillation (Podzuweit et al, 1978), since cyclic AMP, local noradrenaline release (Shahab et al, 1969) and ischaemia-induced lipolysis are most likely closely related.

When plasma free fatty acids are raised, the level of acyl-CoA in the ischaemic myocardium is further stimulated (Liedtke et al, 1978), and may inhibit adenine nucleotide translocase and thereby impeding the transport of ADP into and ATP from the ischaemic mitochondrion, leading to an immediate interruption of energy production (Bittar et al, 1976; Shrago and Sul, 1977). Acyl-carnitine does not inhibit adenine nucleotide translocase, and provision of L-carnitine could theoretically lower acyl-CoA levels. Intracoronary L-carnitine (hyperosmolar) has been reported to reduce the severity of ischaemic injury (Shrago and Sul, 1977). There is serious doubt about the interpretation of the results: hyperosmolar solutions can reduce infarct size by reducing cell swelling (Opie, 1975). No in vivo evidence of carnitine uptake was presented, and extraction of L-carnitine by the ischaemic myocardium could not be demonstrated (Riemersma, unpublished results). Thus it is not clear whether inhibition of adenine nucleotide translocase is involved in the detrimental effects of fatty acids.

Non-specific effects of fatty acids or their derivatives on (cytoplasmic) enzymes have been proposed (Kurien and Oliver, 1970). Fatty acids inhibit Na^+, K^+-ATP-ase in vitro (Lamers and Hülsmann, 1977), but it remains to be

seen whether these studies have a physiological meaning in view of the high free fatty acid to albumin molar ratios used. An interesting observation was made by Stam and Hülsmann (1978[b]) who showed that free fatty acids might lead to noradrenaline release due to their Ca^{++} ionophoric properties. If this were to hold during ischaemia, a self-perpetuating stimulation of noradrenaline could be initiated. That ionophoric properties of fatty acids do play a role follows from the observations that the accumulation of intra-mitochondrial calcium is associated with lipolytic material of unidentified nature (Jennings, personal communication).

Anaerobic glycolysis can not contribute significantly to the energy production of the ischaemic heart (Opie et al, 1973; Neely et al, 1975), thus it is more likely that the apparent beneficial effect of glucose is mediated or associated with a change in its relative contribution to oxidative metabolism, leading to more efficient oxygen utilisation possibly due to the higher P/O ratio of glucose in comparison to fatty acids. Glycolytic flux is inhibited by the early accumulation of H^{+} ions, and the residual ischaemic blood flow is an important regulator of glycolytic flux (Rovetto et al, 1975). Acidosis can reduce further impeded coronary flow, while oxygen is still available and thereby aggravate the compromised

situation further (Steenbergen et al, 1977). This may help to explain why the effects of glucose are dependent on the severity of myocardial ischaemia in experimental (Opie et al, 1976; Russell et al, 1979) and clinical settings (Rogers et al, 1976), since residual flow will determine both oxygen supply and removal of H^+ ions.

Direct beneficial effects of glucose, such as a reduction of potassium loss from the ischaemic myocardium have been suggested (Opie, 1970). However enhanced glucose utilisation by the ischaemic myocardium was not associated with reduced potassium loss, while spontaneous arrhythmias were less frequent (Russell and Oliver, 1979). On the other hand K^+ loss may not be related to subsequent development of arrhythmias (Thomas et al, 1970).

Glycolytically produced ATP might be essential for the maintenance of the transmembrane potential in anoxic preparations (see Opie, 1970), but data on ischaemic myocardium is lacking. It would be surprising, that during ischaemia the small amount of glycolytically produced ATP could not be furbished. Provision of glucose reduced plasma free fatty acids and hence indirectly ischaemic myocardial free fatty acid utilisation. Whether increased glycolytic flux within the ischaemic

myocardium reduces the accumulation of intracellular free fatty acids or their acyl-CoA esters by stimulating esterification has not been studied. However, even if it did the use of antilipolytic drugs may have the additional advantage of inhibiting ischaemia-induced lipolysis, and thereby preventing the initiation of an energy-wasting cycle depicted in Fig 6.02. A precise mechanism of the detrimental effects of enhanced myocardial lipolysis and raised plasma concentrations of free fatty acids, and of the beneficial properties of increased glucose utilisation by the ischaemic myocardium can not be given and more basic research in the controlling factors of ischaemia-induced lipolysis is indicated.

The acquired knowledge on control of ischaemic metabolism will then help to define new therapeutic approaches to the management of acute myocardial infarction.

The use of pyruvate and buffer infusion to reduce the severity of myocardial ischaemic injury is a direct result of the increased knowledge of ischaemic-myocardial metabolism. Pyruvate serves a dual purpose: to act as an intracellular H^+ scavenger and to provide the cell with an alternative substrate. Glycolysis and pyruvate oxidation were both stimulated and a reduction in the severity of ischaemic injury was observed (Liedtke et al, 1976: Liedtke and Nellis, 1978).

Main conclusions

- Effective antilipolytic treatment reduces the severity of myocardial ischaemic injury (Part 3).
- The reduction of evidence of ischaemic injury after antilipolytic treatment is associated with a reduction in free fatty acid extraction, but glucose and lactate exchange are not affected (Part 3).
- The reduction in ST-segment elevation is associated with a reduction in oxygen extraction across the ischaemic myocardium (Section 3.5).
- The extraction of plasma free fatty acids across the ischaemic myocardium is related to the arterial concentration (Section 5.4).
- Increased arterial concentrations of plasma free fatty acids (oleic acid) do not precipitate ventricular fibrillation (Section 5.4).
- Raised concentrations of plasma free fatty acids do not inhibit glucose utilisation by the ischaemic heart (Section 5.4).
- Enhanced glucose utilisation by the ischaemic myocardium limits the degree of ST-segment elevation. This effect may be dependent on residual blood flow (Section 4.1).

- During acute myocardial ischaemia the enhanced triglyceride formation and degradation, establishes an energy-wasting cycle. The rate limiting step is the lipolytic activity (Part 6).
- Local metabolic and hormonal changes in the ischaemic myocardium deserve more attention, since they may be of crucial importance to the development of myocardial ischaemic injury.

References

Ahmed, S.A., Williamson, J.R., Roberts, R., Clark, R.E. et al (1976) Circulation 54, 189

Angell, C.S., Lakatta, E.G., Weisfeldt, M.L. and Shock, N.W. (1976) Cardiovasc Research 9, 12

Armstrong, D.T., Steele, R., Altszuler, N., Dunn, A. et al (1961) Am J Physiol 201, 9

Ayers, C.W. (1956) Anal Chim Acta 15, 77

Balasse, E., and Ooms, H.A. (1968) Rev Fr Etud Clin Biol 13, 62

Ballard, F.B., Danforth, W.H., Naegle, S., and Bing, R.J. (1960) J Clin Invest 39, 717

Barrett, A.M., and Thorp, J.M. (1968) Br J Pharmacol 32, 381

Becker, L.C., Ferreira, R., and Thomas, M. (1975) Cardiovasc Research 9, 178

Berdeaux, A., Coutte, R., Giudicelli, J.-F., and Boissier, J.-R. (1976) Eur J Pharmacol 39, 287

Bergmeyer, H.U. (1970) Methoden der enzymatischen Analyse (2nd Ed) Verlag Chemie, Weinheim

Bergström, S., Carlson, L.A., and Weeks, J.R. (1968) Pharmacol Rev 20, 1

Bernt, E., and Lachenicht, R. (1970) in: Methoden der enzymatischen Analyse (2nd Ed) Verlag Chemie, Weinheim, p 1168

Bezman-Tarcher, A. (1969) J Lip Research 10, 197

Bittar, N., Shug, A.L., Koke, J.R. and Folts, J.D. (1976) Rec Adv Studies Card Struct Metab 7, 137

Blackshear, P.J., Holloway, P.A.H., and Alberti, K.G.M.M. (1974) Biochem J 142, 279

Bloor, C.M., Waite, F.C., and Sobel, B.E. (1973) Cardiovasc Research 7, 156

Boberg, J. (1966) Clin Chim Acta 14, 325

Braasch, W., Gudbjarnason, S., Puri, P.S. and Ravens, K.G. (1968) Circ Research 23, 429

Braun, J.S. (1971) Z klin Chem klin Biochem 9, 387

Bressler, R. (1970) in: Comprehensive biochemistry - 18 Elsevier, Amsterdam p 331

Brody, T.M. (1956) J Pharmacol Exp Ther 117, 39

Broechoven, Ch., and Parijs, J. (1968) Clin Chim Acta 20, 530

Brownsey, R.W., and Brundt, R.V. (1977) Clin Sci Molec Med 53, 513

Bryant, R.E., Thomas, W.A., and O'Neal, R.M. (1958) Circ Research 6, 699

Buckner, D., Eisel, R., and Perry, S. (1968) Blood 31, 653

Burges, R. (1977) Application for Clinical trial certificate, MLA 202, Pfizer Ltd, Kent.

Calva, E., Mujica, A., Bisteni, A., and Sodi-Pallares, D. (1965) Am J Physiol 209, 371

Carlström, S., and Christensson, B. (1971) Br Heart J 33, 884

Cascarano, J., Chick, W.L., and Seidman, I. (1968) Proc Soc Exp Biol Med 127, 25

Cenedella, R.J., Jarrell, J.J., and Saxe, L.H. (1968) J Atheroscler Research 8, 903

Ceremużyński, L., Staszewska-Barczak, J. and Herbacyczyńska-Cedro, K. (1969) Cardiovasc Research 3, 190

Chernick, S.S. (1969) in: Methods in enzymology - 14 Academic Press, New York, p 627

Chiong, M.A., West, R., and Parker, J.O. (1976) Circulation 54, 37

Christensen, N.J., and Videbaeck, J. (1974) J Clin Invest 54, 278

Crabb, D.W., and Harris, R.A. (1978) New Eng J Med 299, 255

Crass III, M.F., Holsinger Jr, J.W., Shipp, J.C., Eliot, R.S. et al.(1976) Rec Adv Studies Card Struct Metab 7, 225

Crespin, S.R., Greenough III, W.B., and Steinberg, D. (1969) J Clin Invest 48, 1934

Crespin, S.R., Greenough III, W.B., and Steinberg, D. (1972) Diabetes, 21, 1179

Curtis-Prior, P.B. (1974) Atherosclerosis 19, 463

Debye, C., Van Caneghem, P., and Bacq, Z.M. (1978) Biochem Pharmacol 27, 613

De Jong, J.W., Verdouw, P.D., and Remme, W.J. (1977) J Molec Cell Cardiol 9, 297

De Leiris, J., and Opie, L.H. (1978) Cardiovasc Research 12, 585

Dole, V.P., and Meinertz, H. (1960) J Biol Chem 235, 2595

Doumas, B.T., Watson, W.A., and Biggs, H.G. (1971) Clin Chim Acta 31, 87

Duncombe, W.G. (1963) Biochem J 88, 7

Durham, B.C., Prestileo, P.W., Mulford, A.M. and Miller, H.I. (1970) Physiologist 13, 185

Duvelleroy, M., Martin, J.L., Duruble, M. and Teisseire, B. (1976) Proc Myocardial Ischaemia-Enzyme Release-Exp and Clin Aspects, Orsay, p. 32

Evans, J.R. (1964) Circ Research 14-15 (Suppl), II-96

Evans, J.R., Opie, L.H., and Shipp, J.C. (1963) Am J Physiol 205, 766

Flear, C.T.G., Riemersma, R.A., Nandra, A., Nandra, G. et al (1976) Rec Adv Studies Card Struct Metab 7, 297

Galen, R.S., Reiffel, J.A., and Gambino, S.R. (1975) J Am Med Assoc 232, 145

Galli, C., Sirtori, C.R., and Paoletti, R. (1969) in: Structural and functional aspects of lipoproteins in living systems, Academic Press, London, p. 517

Gazes, P.C., Richardson, J.A., and Woods, E.F. (1959) Circulation 19, 657

Goodman, D.S., and Shafrir, E. (1959) Am J Chem Soc 81, 346

Greenough III, W.B., Crespin, S.R., and Steinberg, D. (1969) J Clin Invest 48, 1923

Gudbjarnason, S., Mathes, P., and Ravens, K.G. (1970) J Molec Cell Cardiol 1, 325

Gupta, D.K., Young, R., Jewitt, D.E., Hartog, M. et al (1969) Lancet ii, 1209

Hearse, D.J., Humphrey, S.M., and Garlick, P.B. (1976) J Molec Cell Cardiol 8, 329

Haneda, T., Ganz, W., Lee, T., and Katz, J. (1974) Circulation 49/50, Suppl. III, 102

Henderson, A.H., Most, A.S., Parmley, W.W., Gorlin, R. et al (1970) Circ Research 26, 439

Hillis, L.D., and Braunwald, E. (1977) New Eng J Med 296, 1093

Ho, R.J. (1970) Anal Biochem 36, 105

Hoak, J.C., Connor, W.E., Eckstein, J.W. and Warner, E.D. (1964) J Lab Clin Med 63, 791

Hoak, J.C., Warner, E.D., and Connor, W.E. (1972) Rec Adv Studies Card Struct Metab 1, 127

Hohorst, H.-J. (1970) in: Methoden der Enzymatischen Analyse (2nd Ed), Verlag Chemie, Weinheim pp 1425

Horton, E.W., and Ungar, A. (1975) in: Modern trends in cardiology-3, Butterworths, London p 67

Hough, F.S., and Gevers, W. (1975) S Afr Med J 49, 538

Hutton, I., Parratt, J.R., and Lawrie, T.D.V. (1973) Cardiovasc Research 7, 149

Idell-Wenger, J.A., and Neely, J.R. (1977) in: Patho-physiology and therapeutics of myocardial ischaemia , Spectrum Publications, Inc., p 227

Ilebekk, A., and Mjøs, O.D. (1974) Scand J Clin Lab Invest 33, 145

Jageneau, A.H.M., Van Gerven, W., Kruger, R., Van Belle, H. et al (1975) Rec Adv Studies Card Struct Metab 10, 331

Jansen, H., and Hülsmann, W.C. (1974) Biochim Biophys Acta 369, 387

Jennings, R.B., Herdson, P.B., and Sommers, H.M. (1969) J Lab Invest 20, 548

Jennings, R.B., and Ganote, C.E. (1972) in: Effect of acute ischaemia on myocardial function, Churchill Livingstone, Edinburgh, p 50

Jewitt, D.E., Mercer, C.J., Reid, D., Valori, C. et al (1969) Lancet, i, 635

Jones, C.E., Thomas, J.X., Parker, J.C. and Parker, R.E. (1976) Cardiov Res 10, 275

Jugdutt, B.I., Becker, L.C., Bulkley, B.H. and Hutchins, G.M. (1978) Am J Cardiol 41, 359

Kaijser, L., Lassers, B.W., Wahlqvist, M.L., and Carlson, L.A. (1972) J Appl Physiol 32, 847

Kane, K.A., and Ungar, A. (1976) Q J Exp Physiol 61, 35

Kecsmeti, V., Keleman, K., and Knoll, J. (1974) Pol Pharmacol Pharm 26, 171

Kessler, G., and Lederer, H. (1966) in: Automation in Analytical Chemistry, Mediad, New York, p 341

Khuri, S.F., Flaherty, T.J., and O'Riordan, J.B. (1975) Circ Res 37, 455

Kjekshus, J.K. (1978) Proc VIIIth World Conference Cardiology, Tokyo, p. 72

Kjekshus, J.K., and Mjøs, O.D. (1972) J Clin Invest 51, 1767

Kjekshus, J.K., and Mjøs, O.D. (1973) J Clin Invest 52, 1770

Kjekshus, J.K., Maroko, P.R., and Sobel, B.E. (1972) Cardiovasc Research 6, 490

Kloner, R.A., Ganote, C.E., Whalen, Jr., D.A., and Jennings, R.B. (1974) Am J Pathology 74, 399

Kostis, J.B., Mavrogeorgis, E.A., Horstmann, E., and Gotzoyannis, S. (1973) Cardiology 58, 89

Kübler, W., and Spieckermann, P.G. (1970) J Mol Cell Cardiol 1, 351

Kurien, V.A., and Oliver, M.F. (1966) Lancet ii, 122

Kurien, V.A., and Oliver, M.F. (1970) Lancet i, 813

Kurien, V.A., Yates, P.A., and Oliver, M.F. (1971) Eur J Clin Invest 1, 225

Lamers, J.M.J., and Hülsmann, W.C. (1977) J Molec Cell Cardiol 9, 343

Langendorff, O. (1895) Pflügers Arch Ges Physiol 61, 225

La Rosa, J.C., Levy, R.I., Herbert, P., Lux, S.E. et al (1970) Biochem Biophys Res Comm 41, 57

Lassers, B.W., Carlson, L.A., Kaijser, L., and Wahlquist M. (1972) in: Effect of acute ischaemia on myocardial function, Churchill Livingstone, Edinburgh, p. 200

Laurell, S., and Tibbling, G. (1967) Clin Chim Acta 16, 57

Lekven, J., Kjekshus, J.K., and Mjøs, O.D. (1973) Scand J Clin Lab Invest 32, 129

Libby, P., Maroko, P.R., Bloor, C.M., Sobel, B.E. et al (1973) J Clin Invest 52, 599

Libby, P., Maroko, P.R., Covell, J.W., Malloch, C.I. et al (1973[b]) Cardiovasc Research 7, 167

Liedtke, A.J. and Nellis, S.H. (1978) Circ Research 43, 189

Liedtke, A.J., Hughes, H.C. and Neely, J.R. (1976) Am J Cardiol 38, 17

Liedtke, A.J., Nellis, S.H., and Neely, J.R. (1978) Circ Research 43, 652

Liedtke, A.J., Nellis, S.H., Neely, J.R. and Hughes, H.C. (1976[b]) Circ Research 39, 378

Lindlar, F. (1969) Beitr Gerichtl Med 26, 71

Lochner, A., Kotzé, J.C., Benade, A.J., and Gevers, W. (1978) J Molec Cell Cardiol 10, 857

Lochner, A., Opie, L.H., Owen, P., Kotzé, J.C.N. et al (1975) J Molec Cell Cardiol 7, 203

Lorini, M., and Ciman, M. (1962) Bioch Pharmacol 11, 823

Luxton, M.R., Miller, N.E., and Oliver, M.F. (1976) Br Heart J 38, 1204

Makarem, A. (1974) in: Clinical chemistry-Principles and techniques (2nd Ed), Harper Row, Hagerstown p 1145

Malliani, A., Schwartz, P.J., and Zanchetti, A. (1969) Am J Physiol 217, 703

Manasterski, A., Watkins, R., and Zak, B. (1972) Microchem J 17, 682

Mansour, T.E. (1972) Curr Top Cell Regul 5, 1

Maroko, P.R., Kjekshus, J.K., Sobel, B.E., Watanabe, T. et al (1971) Circulation, 43, 67

Maroko, P.R., Libby, P., Sobel, B.E., Bloor, C.M. et al (1972) Circulation 45, 1160

Marshall, R.J., and Parratt, J.R. (1976) Br J Pharmacol 57, 295

Marshall, R.J., Parratt, J.R. and Ledingham, I.McA. (1974) Cardiovasc Research 8, 204

Mbuyamba, P.W. (1976) Arch Int Physiol Bioch 84, 305

McAllister, A., Allison, S.P., and Randle, P.J. (1973) Biochem J 134, 1067

McDonald, L., Baker, C., Bray, C., McDonald, A. et al (1969) Lancet ii, 1021

Miller, N.E., Mjøs, O.D. and Oliver, M.F. (1976) Clin Science Molec Med 51, 209

Mjøs, O.D. (1971) J Clin Invest 50, 1869

Mjøs, O.D. (1971[b]) J Clin Invest 50, 1386

Mjøs, O.D. (1973) Biomedicine 18, 9

Mjøs, O.D., and Kjekshus, J.K. (1972) in: Effect of acute ischaemia on myocardial function, Churchill Livingstone, Edinburgh, p. 197

Mjøs, O.D., Kjekshus, J.K. and Lekven, J. (1974) J Clin Invest 53, 1290

Morgan, H.E., Randle, P.J., and Regen, D.M. (1959) Biochem J 73, 573

Morgan, H.E., Henderson, M.J., Regen, D.M., and Park, C.R. (1961) J Biol Chem 236, 253

Müller, G., and Ulrich, E. (1966) Dtsch Zeitschr Verdauungs-Stoffw krankh 26, 271

Muller, J.E., Maroko, P.R., and Braunwald, E. (1975) Circulation 52, 16

Nayler, W.G., Stone, J., Carson, V., and Chipperfield, D. (1971) J Molec Cell Cardiol 2, 125

Neely, J.R. and Morgan, H.E. (1974) Ann Rev Physiol 36, 413

Neely, J.R., Rovetto, M.J., Oram, J.F. (1972) Prog Cardiovasc Dis 15, 289

Neely, J.R., Whitmer, J.T., and Rovetto, M.J. (1975) Circ Research, 37, 733

Neely, J.R., Liebermeister, H., Battersby, E.J., and Morgan, H.E. (1967) Am J Physiol 212, 804

Neilson, J.M.M., Davies, C.T.M. and Kitchin, A.H. (1968) Br Heart J 30, 872

Nelson, P.G. (1970) Br Med J 3, 735

Newsholme, E.A., and Randle, P.J. (1964) Biochem J 93, 641

Newsholme, E.A. and Start, C. (1973) Regulation in Metabolism, John Wiley and Sons, London

Nimmo, I.A. (1970) PhD thesis, University of Edinburgh

Nixon, W. and Massey, F.J. (1969) Introduction in statistical analysis (3rd Ed), McGraw-Hill

Northam, B.E., and Widdowson, G.M. (1967) Assoc Clin Biochem Technical Bull 11, 1

Novak, M. (1965) J Lipid Research 6, 431

O'Brien, J.A. (1969) Ph. D Thesis, University of Edinburgh

Ockner, R.K., Manning, J.A., Poppenhausen, R.B. and Ho, W.K.L. (1972) Science, 177, 56

Oliver, M.F. (1972) Circulation 45, 491

Oliver, M.F., and Yates, P.A. (1972) Cardiology 56, 359

Oliver, M.F., Kurien, V.A., and Greenwood, T.W. (1968) Lancet, i, 710

Opie, L.H. (1968) Am Heart J 76, 685

Opie, L.H. (1969[a]) Am Heart J 77, 100

Opie, L.H. (1969[b]) Am Heart J 77, 383

Opie, L.H. (1970) J Molec Cell Cardiol 1, 107

Opie, L.H. (1972) Circulation 45, 483

Opie, L.H. (1975) Am J Cardiol 36, 938

Opie, L.H. and Lubbe, W.F. (1975) J Molec Cell Cardiol 7, 155

Opie, L.H. and Owen, P. (1976) Am J Cardiol 38, 310

Opie, L.H., and Thomas, M. (1976) Postgr Med J 52 (Suppl. 4), 124

Opie, L.H., Bruyneel, K., and Owen, P. (1975) Circulation, 52, 49

Opie, L.H., Owen, P., and Riemersma, R.A. (1973) Eur J Clin Invest 3, 419

Opie, L.H., Owen, P., Thomas, M., and Samson, R. (1973[b]) Am J Cardiol 32, 295

Opie, L.H., Shipp, J.C., Evans, J.K., and Leboeuf, B. (1962) Am J Physiol 203, 839

Opie, L.H., Norris, R.M., Thomas, M., Holland, A.J. et al (1972) Lancet ii, 818

O'Riordan, J.B., Flaherty, J.T., Donahoo, J.S., and Gott, V.L. (1977) Am J Cardiol 39, 529

Owen, P., Thomas, M., Young, R., and Opie, L.H. (1970) Am J Cardiol 25, 562

Passonneau, J. (1970) in: Methoden der enzymatischen Analyse (2nd Ed), Verlag Chemie, Weinheim, p 1430

Phibbs, R.H., Wyler, R.H., and Neutze, J. (1968) Nature (London) 216, 1339

Podzuweit, T., Dalby, A.J., and Opie, L.H. (1975) S Afr Med J 21, 585

Podzuweit, T., Dalby, A.J., Cherry, G.W., and Opie, L.H. (1978) J Molec Cell Cardiol 10, 81

Prakash, R., Parmley, W.W., Horvat, M., and Swan, H.J.C. (1972) Circulation 45, 735

Prinzmetal, M., Toyoshima, E., Ekmekci, A., Mizano, Y. et al (1961) Am J Cardiol 8, 493

Puri, P.S. (1976) Rec Adv Studies Card Struct Metab 7, 161

Randle, P.J. (1963) Ann Rev Physiol 25, 291

Randle, P.J., Newsholme, E.A., and Garland, P.B. (1964) Biochem J 93, 652

Ravens, K.G., and Jipp, P. (1972) Arzneim Forsch 22, 1831

Redwood, D.R., Smith, E.R., and Epstein, S.E. (1972) Circulation 46, 323

Reimann, R., and Schwandt, P. (1971) Dtsch Med Wochenschr 96, 93

Riemersma, R.A. (1979) Unpublished observations

Riemersma, R.A., Logan, R.L., and Oliver, M.F. (1977) J Molec Cell Cardiol 9, Suppl p.45

Riemersma, R.A., Talbot, R.G., and Oliver, M.F. (1974) Eur J Clin Invest 4, 350

Riemersma, R.A., Holland, A., Owen, P., Lewis, B. et al (1972) Cardiology 56, 364

Riley, J. and Robinson, D.S. (1974) Biochim Biophys Acta 369, 371

Robinson, D.S. (1963) Adv Lip Research 1, 133

Robinson, J., and Newsholme, E.A. (1967) Biochem J 104, 2C

Rogers, W.J., Stanley, A.W., Breinig, J.B., Prather, J.W. et al (1976) Am Heart J 92, 441

Rosalki, S.B. (1967) J Lab Clin Med 69, 696

Rosell, S. (1966) Acta Physiol Scand 67, 343

Ross Jr., J. (1976) Circulation 53 Suppl I - 73

Rovetto, M.J., and Neely, J.R. (1977) in: Pathophysiology and therapeutics of myocardial ischaemia, Spectrum Publications, Inc., p. 169

Rovetto, M.J., Lamberton, W.F., and Neely, J.R. (1975) Circ Research, 37, 742

Rovetto, M.J., Whitmer, J.T., and Neely, J.R. (1973) Circ Research, 32, 699

Rowe, M.J., Neilson, J.M.M., and Oliver, M.F. (1975) Lancet i, 295

Russell, D.C., and Oliver, M.F. (1978) Br Heart J 40, 117

Russell, D.C., and Oliver, M.F. (1979) J Molec Cell Cardiol 11, 31

Russell, D.C., Smith, H.J., Riemersma, R.A., and Oliver, M.F. (1979) submitted for publication

Russo, J.V., Friesinger, G.C., Margolis, S., and Ross, R.S. (1970) Lancet ii, 1271

Rutenberg, H.L., Pamintuan, J.C., and Soloff, L.A. (1969) Lancet ii, 559

Sayen, J.J., Sheldon, W.F., Peirce, G., and Kuo, P.T. (1958) Circulation Research 6, 779

Schalch, D.S., and Kipnis, D.M. (1965) J Clin Invest 44, 2010

Schaper, W. (1971) The collateral circulation of the heart, North Holland Publishing Co., Amsterdam

Scheuer, J., and Brachfeld, N. (1966) Metabolism 15, 945

Scheuer, J., and Stezoski, S.W. (1969) Circulation 40, Suppl III, 179

Schmidt, E., and Schmidt, F.W. (1976) in: Brief guide to practical enzyme diagnosis (2nd Ed), Boehringer Mannheim GmbH, Mannheim

Schwartz, A., Wood, J.M., Allen, J.C., Bornet, E.P. et al (1973) Am J Cardiol 32, 46

Scott, J.C., Finkelstein, L.J., and Spitzer, J.J. (1962) Am J Physiol 203, 482

Scow, R.O., and Chernick, S.S. (1970) in: Comprehensive Biochemistry, Volume 18, Lipid Metabolism, Elsevier, Amsterdam, p. 19

Serrano, P.A., Chavez-Lara, B., Bisteni, A., and Sodi-Pallares, D. (1971) J Molec Cell Cardiol 2, 91

Shahab, L., Wollenberger, A., Haase, M., and Schiller, U. (1969) Acta Biol Med Germ 22, 135

Shipp, J.C., Opie, L.H., and Challoner, D. (1961) Nature 189, 1018

Shrago, E., and Sul, H.S. (1977) in: Pathophysiology and Therapeutics of myocardial ischemia, Spectrum Publications Inc., p. 239

Shug, A.L., Thomsen, J.H., Folts, J.D., Bittar, N. et al (1978) Arch Biochem Biophys 187, 25

Smith, A.F., Radford, D., Wong, C.P., and Oliver, M.F. (1976) Br Heart J 38, 225

Smith, E.R., and Duce, B.R. (1974) Cardiovasc Res 8, 550

Smith, H.J. (1977) M.D. Thesis, University of Otago, Dunedin, New Zealand

Sobel, B.E., Corr, P.B., Robinson, A.K., Goldstein, R.A. et al (1978) J Clin Invest 62, 546

Sonnenblick, E.H., Ross, J., and Braunwald, E. (1968) Am J Cardiol 22, 328

Speake, R.N. (1967) Progr Biochem Pharmacol 2, 372

Spector, A.A. (1969) J Lipid Research 10, 207

Spector, A.A., Steinberg, D., and Tanaka, A. (1965) J Biol Chem 240, 1032

Stacpoole, P.W., Moore, G.W., and Kornhauser, D.M. (1978) New Eng J Med 298, 526

Stam, H., and De Jong, J.W. (1977) J Mol Cell Cardiol 9, 633

Stam, H., and Hülsmann, W.C. (1978) Biochem Biophys Res Comm 82, 609

Stam, H., and Hülsmann, W.C. (1978b) Bas Res Cardiol 73, 208

Standl, E., Janka, H.-U. and Standl, A. (1977) Diabetologia 13, 433

Stanley, A.W., Moraski, R.E., Russell, R.O., Rogers, W.J. et al (1975) Am J Cardiol 36, 929

Steenbergen, C., Deleeuw, G., Barlow, C., Chance, B. et al (1977) Circ Res 41, 606

Sybers, H.D., Maroko, P.R., Ashraf, M., Libby, P. et al (1973) Am J Pathology 70, 401

Takano, S. (1976) Jap Circ J 40, 287

Tenney, S.M., and Miller, R.M. (1955) Am J Med 19, 498

Thomas, M., Shulman, G., and Opie, L.H. (1970) Cardiovasc Research 4, 327

Thorp, J.M. (1973) in: Lipid Metabolism and Atherosclerosis, Excerpta Medica, Amsterdam, p. 90

Trout, D.L., Estes Jr., E.H., and Friedberg, S.J. (1960) J Lipid Research 1, 199

Trump, B.F. (1976) Circulation 53, Suppl 1, I-52

Ueno, T., Tshiyama, T., Morita, Y., Hatanaka, Y. et al (1976) Proc VIIIth Int Meet Int Study Gr Res Card Metab p. 100

Utley, J., Carlson, E.L., Hoffman, J.I.E., Martinez, H.M. et al (1974) Circ Research 34, 391

Valori, C., Thomas, M., and Shillingford, J.P. (1967) Am J Cardiol 20, 605

Van der Vusse, G.J., and Reneman, R.S. (1977) J Molec Cell Cardiol 9, Suppl p.59

Vane, J.R. (1971) Nature 231, 232

Varat, M.A., and Mercer, D.M. (1975) Circulation 51, 855

Vergroesen, A.J., Zijlstra, W.G., Brunsting, J.R. and Ten Hoor, F. (1972) in: Effect of acute ischaemia on myocardial function, Churchill Livingstone, Edinburgh p 315

Vetter, N.J., Strange, R.C., Adams, W., and Oliver, M.F. (1974) Lancet i, 284

Vik-Mo, H. (1977) Scand J Clin Invest 37, 697

Vik-Mo, H. (1978) M D Thesis, University of Tromsø

Vik-Mo, H., and Mjøs, O.D. (1976) Scand J Clin Lab Invest 36, 763

Vik-Mo, H., and Mjøs, O.D. (1977) Scand J Clin Lab Invest 37, 287

Vogel, W.C., and Bierman, E.L. (1965) Fed Proc 24, 439

Wasilewska-Dziubinska, E., Czarnecka, M., Beresewicz, A., and Lewartowski, B. (1975) Acta Physiol Pol 26, 1

Watson, D. (1969) Adv Clin Chem 8, 272

Wegria, R., Segers, M., Keating, R.P., and Ward, H.P. (1949) Am Heart J 38, 90

Weissler, A.M., Kruger, F.A., Baba, N., Scarpelli, D.C. et al (1968) J Clin Invest 47, 403

Wendt, R.L., Canavan, R.C., and Michalak, R.J. (1974) Am Heart J 87, 468

Wennmalm, A. (1976) Proc 8th Int Meeting of Int Study Gr Res Card Metab, 279

Wennmalm, A., and Stjarne (1971) Life Sci 10, 471

Wennmalm, A., Huu-Chanh, P., and Junstad, M. (1974) Acta Physiol Scand 91, 133

Whalen Jr., D.A., Hamilton, D.G., Ganote, C.E. and Jennings, R.B. (1974) Am J Pathology 74, 381

Whitehouse, S., and Randle, P.J. (1973) Biochem J 134, 651

Williamson, J.R., Steenbergen, C., Rich, T., Deleeuw, G. et al (1977) in: Pathophysiology and therapeutics of myocardial ischemia, Spectrum Publications Inc. p. 193

Wollenberger, A., and Krause, E.-G. (1968) Am J Cardiol 22, 349

Wollenberger, A., Krause, E.-G., and Heier, G. (1972) Rec Adv Studies Card Struct Metab 1, 27

Wong, P.C.-P., and Smith, A.F. (1975) Clin Chim Acta 65, 99

Yasmineh, W.G., and Hanson, N.Q. (1975) Clin Chem 21, 381

Appendix A

THE RECORDING OF EPICARDIAL ELECTROCARDIOGRAMS

APPENDIX A. The recording of epicardial electrocardiograms. Electronic equipment and block diagrams

Manual recording of epicardial electrocardiograms:

Phase 1 — The original attenuator box/cotton wick electrode.

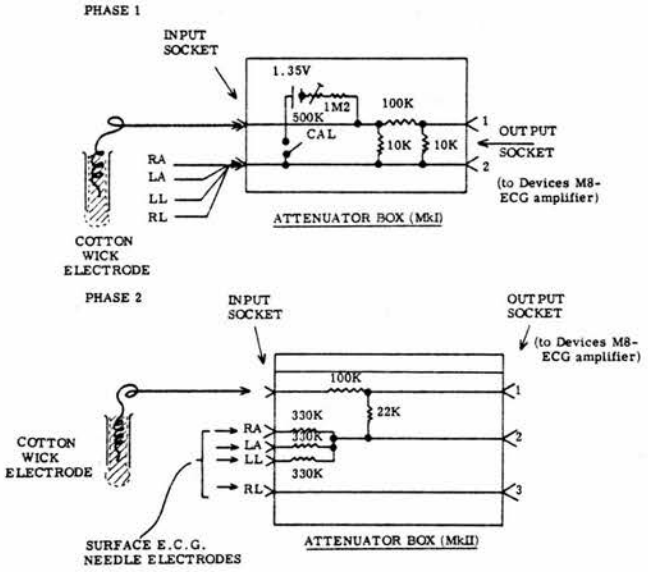
Phase 2 — Improved attenuator box.

Continuous monitoring:

Phase 3 — Recording of average epicardial electrocardiogram.

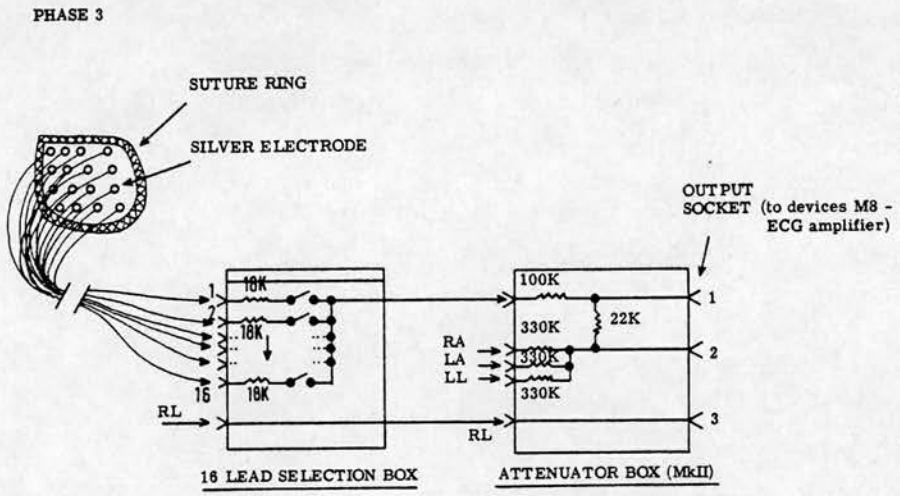
Phase 4 — Continuous monitoring of 15 electrocardiograms, using a multiplexer system.

Figure 1: Phase 1-2 Manual recording of epicardial electrocardiograms using a cotton-wick electrode



The epicardial electrocardiogram was recorded using a cotton-wick electrode. The combined limb leads served as the reference electrode. The epicardial signal was too large for the Devices AC amplifier and was first suitably attenuated using the attenuator box. The equipment was calibrated prior to each experiment. RA = right arm, LA = left arm; LL = left leg and RL = right leg. The attenuator box was improved (Phase 2) by the insertion of input resistors for the limb leads. Attenuation x 10.

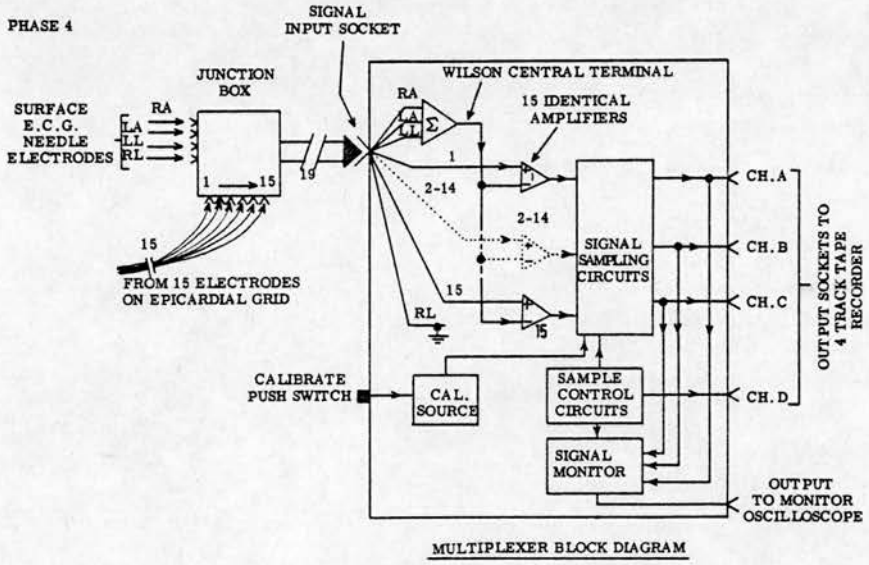
Figure 2 : Phase 3 Continuous monitoring of the epicardial electrocardiogram



The electrocardiogram of 16 epicardial electrodes, attached to a thin latex grid, were continuously monitored. Electrocardiograms could be monitored either individually or as the average of a number of selected sites using a 16 lead selection box. The resulting complex was suitably attenuated using the attenuator box (Mark II) and recorded on a Devices M8 recorder.

Figure 3 : Phase 4 Continuous monitoring of epicardial electrocardiograms using a multiplexer system

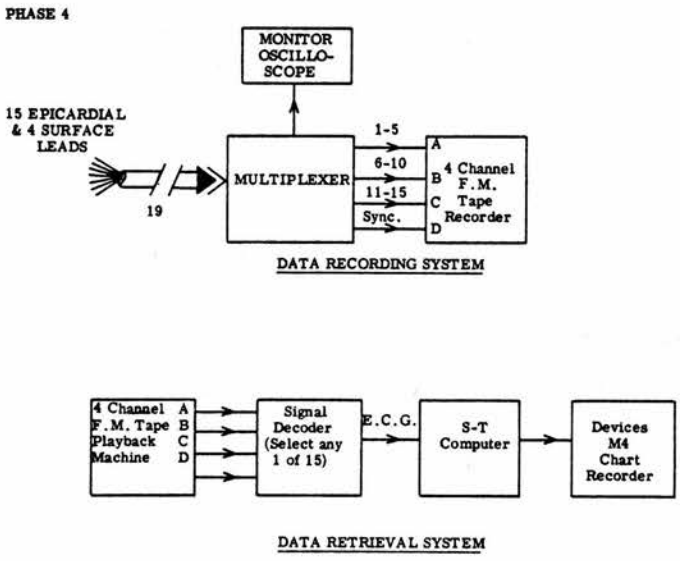
PANEL A:



Multiplexer block diagram (Panel A and B). Electrocardiograms, obtained from 15 electrodes on an epicardial grid were continuously monitored. After suitable amplification using 15 identical amplifiers, the multiplexer sampled the signals in three groups; group A: electrode 1-5, group B: 6-10 and group C: 11-15. The sampling rate for each complex was 300 samples/sec. The 3 resultant multiplexed signals (Groups A, B and C) were recorded on a tape recorder. The individual signals could be retrieved using a signal decoder (Panel C), and the selected epicardial electrocardiogram was analysed using a purpose built ST-segment computer (Neilson et al, 1968). For further details see text.

Figure 3 : Phase 4 Continuous monitoring of epicardial electrocardiograms using amultiplexer system

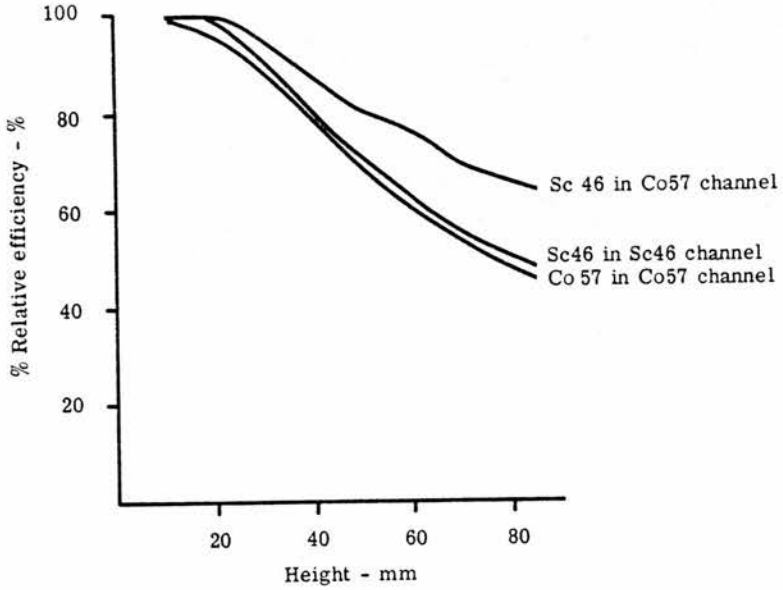
PANEL B:



Appendix B

THE MEASUREMENT OF REGIONAL MYOCARDIAL BLOOD FLOW

APPENDIX B 1 Geometrical aspects of gamma counting



The effects of geometrical factors on the counting efficiency of two isotopes Co^{57} and Sc^{46}, with the maximal difference in gamma energy used in our experiments was examined by Dr. P. Tothill, Department of Medical Physics, The Royal Infirmary, Edinburgh. Water soluble standards of Co^{57} and Sc^{46} were repeatedly counted in both channels, each time after the addition of water to increase the sample height, while the amount of radioactivity was kept constant. The efficiency of each isotope was always expressed relative to the maximal count rate in its own channel (height O).
The relative efficiency of Co^{57} and Sc^{46} decreases slightly in our working range (2-4 cm).
Note that the decrease in Sc^{46} efficiency in its own channel is not identical to that observed in the Co^{57} channel. No counts of Co^{57} were observed in the Sc^{46} channel.

APPENDIX B 2

```
FORTRAN IV  4BAAA   28-MAR-79                PAGE  ONE

C       RUDI.FT
C
C
C       CARDIAC BLOOD FLOW MEASUREMENTS USING MICROPSHERES.
C
C
C CALLS GELG
002     -       DIMENSION STAND(4,4,4),DOG(5,4,4,2),STANDX(4,4,4),BGD(4,2)
003             DIMENSION SETNO(4),FACT(4,4),GELGA4(4,4),GELGB(4),GELGA3(3,3)
004             DIMENSION REFWT(4),TIME(4),STANWT(4,4),SAMWT(5,4,2),FACTOR(4)
005             DIMENSION FILE1(3),FILE2(3),FILE3(3),GELGA2(2,2),RBF(5,4,4)
006             DIMENSION L(64),TEXT(12)
007             EQUIVALENCE (DOG(1,1,1,1),STANDX(1,1,1))
010             INTEGER ENERGY,SPEC,SET,ROW,COL,ENDEPI,CHANEL
011             DATA INDEV/4/,OUTDEV/3/
012             DATA YES/"Y"/,NO/"N"/,AB/"A"/,RE/"R"/,EPI/"EP"/,ENDO/"EN"/
013             DATA EPS/0.1E-5/
        C
014             DATA REV/"B"/
        C
015             CALL DATE(MTH,IDAY,IYR)
016             IYR=IYR-1900
017             WRITE(OUTDEV,2)REV,IDAY,MTH,IYR
020         2   FORMAT(1H0,"RUDI (REV ",A1,")  RUN ON ",I2,"-",I2,"-",I2)
021         1   WRITE(4,3)
022         3   FORMAT(1H0,"WALLAC FILE 1 ",$)
023             READ(INDEV,4) FILE1
024         4   FORMAT(3A6)
025             CALL USR(6,FILE1,2,IERR)
026             IF(IERR.NE.0) GO TO 999
027             WRITE(4,5)
030         5   FORMAT(1H ,"WALLAC FILE 2 ",$)
031             READ(INDEV,4) FILE2
032             CALL USR(7,FILE2,2,IERR)
033             IF(IERR.NE.0) GO TO 999
034             WRITE(4,6)
035         6   FORMAT(1H ,"WEIGHTS FILE  ",$)
036             READ(INDEV,4) FILE3
037             CALL USR(8,FILE3,2,IERR)
040             IF(IERR.NE.0) GO TO 999
041             WRITE(4,7)
042         7   FORMAT(1H ,"IDENTIFICATION ",$)
043             READ(INDEV,8) TEXT
044         8   FORMAT(12A6)
045             WRITE(OUTDEV,9) TEXT
046         9   FORMAT(1H0,"IDENTIFICATION: ",12A6//)
        C
        C       READ STANDARDS DATA
        C
047             DO 10 SET=1,4
050             DO 10 SPEC=1,4
051             READ(6,12) (STANDX(ENERGY,SPEC,SET),ENERGY=1,2)
052             READ(7,12) (STANDX(ENERGY,SPEC,SET),ENERGY=3,4)
053        10   CONTINUE
054        12   FORMAT(27X,I6,3X,I6)
```

```
      C
      C       READ IN BACKGROUND DATA
      C
0055          DO 15 SET=1,2
0056          READ(6,12) (BGD(ENERGY,SET),ENERGY=1,2)
0057     15   READ(7,12) (BGD(ENERGY,SET),ENERGY=3,4)
      C
      C       AVERAGE BACKGROUNDS FOR EACH ENERGY
      C
0060          DO 17 ENERGY=1,4
0061     17   BGD(ENERGY,1)=(BGD(ENERGY,1)+BGD(ENERGY,2))/2.
      C
      C       TEMPORARY DUMP OF BACKGROUND MATRIX
      C
      C       WRITE(OUTDEV,18)
      C    18 FORMAT(1H0,'AVERAGE BACKGROUNDS FOR EACH ENERGY'/)
      C       WRITE(OUTDEV,44) (BGD(ENERGY,1),ENERGY=1,4)
      C
      C       FIND IN WHICH CHANNEL THE MAXIMUM OCCURS FOR EACH SET OF DATA.
      C       FIRST DETERMINE HOW MANY ISOTOPES.
      C
0062          ISOTOP=0
0063          SPEC=1
0064          DO 22 SET=1,4
0065          TEMP=0.0
0066          DO 20 ENERGY=1,4
0067     20   TEMP=TEMP+STANDX(ENERGY,SPEC,SET)
0070          IF(TEMP.GT.2000) ISOTOP=ISOTOP+1
0071     22   CONTINUE
0072          DO 25 SET=1,ISOTOP
0073          TEMP=0.0
0074          DO 25 ENERGY=1,ISOTOP
0075          IF(STANDX(ENERGY,SPEC,SET).LT.TEMP) GO TO 25
0076          TEMP=STANDX(ENERGY,SPEC,SET)
0077          SETNO(SET)=ENERGY
0100     25   CONTINUE
      C
      C       THE ARRAY SETNO CONTAINS THE CHANNELS IN WHICH MAXIMUM COUNTS OCCUR.
      C       USING THIS INFORMATION RESHUFFLE THE DATA INTO STAND SO THAT:
      C       SET 1 HAS MAX ENERGY 1
      C       SET 2 HAS MAX ENERGY 2   ETC...
      C
0101          DO 30 SET=1,ISOTOP
0102          CHANEL=SETNO(SET)
0103          DO 30 SPEC=1,4
0104          DO 30 ENERGY=1,4
0105          STAND(ENERGY,SPEC,CHANEL)=STANDX(ENERGY,SPEC,SET) - BGD(ENERGY,1)
0106          IF(STAND(ENERGY,SPEC,CHANEL).LT.0.0) STAND(ENERGY,SPEC,CHANEL)=0.0
0107     30   CONTINUE
      C
      C       TEMP DUMP OF DATA.
      C
      C       DO 40 SET=1,ISOTOP
      C       WRITE(OUTDEV,42) SET
```

```
C    42  FORMAT(1H0,'SET ',I1,5X,'SPECIMENS 1 -> 4'/)
C        DO 40 SPEC=1,4
C    40  WRITE(OUTDEV,44) (STAND(ENERGY,SPEC,SET),ENERGY=1,4)
0110    44  FORMAT(1H ,4I10)
C
C        CONTRIBUTION FACTORS.
C
0111        DO 49 SET=1,4
0112        DO 49 ENERGY=1,4
0113    49  FACT(SET,ENERGY)=0.0
0114        DO 50 SET=1,ISOTOP
0115        DO 50 ENERGY=1,ISOTOP
0116        DO 50 SPEC=1,4
0117    50  FACT(SET,ENERGY)=FACT(SET,ENERGY)+STAND(ENERGY,SPEC,SET)
0120        DO 52 SET=1,ISOTOP
0121        TEMP=FACT(SET,SET)
0122        DO 52 ENERGY=1,ISOTOP
0123    52  FACT(SET,ENERGY)=FACT(SET,ENERGY)/TEMP
C
C        WRITE OUT FACTOR MATRIX
C
0124        DO 55 SET=1,4
0125        WRITE(OUTDEV,53)  SET
0126    53  FORMAT(1H0,'CONTRIBUTION FACTORS FOR STANDARD ',I1/)
0127    55  WRITE(OUTDEV,58) (FACT(SET,ENERGY),ENERGY=1,4)
0130    58  FORMAT(1H ,4F10.4)
0131        WRITE(OUTDEV,59) ISOTOP
0132    59  FORMAT(1H0,I1,' ISOTOPES IN THIS STUDY'/)
C
C        READ IN DATA MATRIX
C
0133        DO 60 ROW=1,5
0134        DO 60 COL=1,4
0135        DO 60 ENDEPI=1,2
0136        READ(6,12) (DOG(ROW,COL,ENERGY,ENDEPI),ENERGY=1,2)
0137    60  READ(7,12) (DOG(ROW,COL,ENERGY,ENDEPI),ENERGY=3,4)
C
C        SUBTRACT BACKGROUND FROM DATA
C
0140        DO 66 ENDEPI=1,2
0141        DO 66 ENERGY=1,ISOTOP
0142        DO 66 COL=1,4
0143        DO 66 ROW=1,5
0144        DOG(ROW,COL,ENERGY,ENDEPI)=DOG(ROW,COL,ENERGY,ENDEPI)
          +   - BGD(ENERGY,1)
0145        IF(DOG(ROW,COL,ENERGY,ENDEPI).LT.0.0) DOG(ROW,COL,ENERGY,ENDEPI)=0.0
0146    66  CONTINUE
C
C        TEMPORARY DUMP OF DATA
C
C        DO 70 ENDEPI=1,2
C        WRITE(OUTDEV,72) ENDEPI
C    72  FORMAT(1H0,'END/EPI ',I1/)
C        DO 70 ENERGY=1,ISOTOP
C        WRITE(OUTDEV,77) ENERGY
```

```
C    77  FORMAT(1H0,'ENERGY ',I1/)
C        DO 70 ROW=1,5
C    70  WRITE(OUTDEV,44) (DOG(ROW,COL,ENERGY,ENDEPI),COL=1,4)
C
C        FOR EACH TISSUE SAMPLE CORRECT THE COUNTS FOR CROSS CONTRIBUTIONS USING
C        THE FACT MATRIX DEVELOPED FROM THE STANDARDS.
C
0147         IF(ISOTOP.EQ.1) GO TO 82
0150         DO 86 ENDEPI=1,2
0151         DO 86 COL=1,4
0152         DO 86 ROW=1,5
0153         DO 83 SET=1,ISOTOP
0154         DO 83 ENERGY=1,ISOTOP
0155         IF(ISOTOP.EQ.2) GELGA2(ENERGY,SET)=FACT(SET,ENERGY)
0156         IF(ISOTOP.EQ.3) GELGA3(ENERGY,SET)=FACT(SET,ENERGY)
0157         IF(ISOTOP.EQ.4) GELGA4(ENERGY,SET)=FACT(SET,ENERGY)
0160    83   CONTINUE
0161         DO 84 ENERGY=1,ISOTOP
0162    84   GELGB(ENERGY)=DOG(ROW,COL,ENERGY,ENDEPI)
0163         IF(ISOTOP.EQ.2) CALL GELG(GELGB,GELGA2,2,1,EPS,IER)
0164         IF(ISOTOP.EQ.3) CALL GELG(GELGB,GELGA3,3,1,EPS,IER)
0165         IF(ISOTOP.EQ.4) CALL GELG(GELGB,GELGA4,4,1,EPS,IER)
0166         IF(IER.NE.0) WRITE(OUTDEV,87) IER,ROW,COL,ENDEPI
0167    87   FORMAT(1H0,'***WARNING ',I1,' - POSSIBLE LOSS OF SIGNIFICANCE AT
           $  ROW ',I1,' COLUMN ',I1,3X,'END/EPI OF ',I1)
0170         DO 86 ENERGY=1,ISOTOP
0171         IF(GELGB(ENERGY).LT.0.0) GELGB(ENERGY)=0.0
0172    86   DOG(ROW,COL,ENERGY,ENDEPI)=GELGB(ENERGY)
C
C        DUMP THE CORRECTED TISSUE DATA.
C
0173    82   WRITE(OUTDEV,88)
0174    88   FORMAT(1H0//T10,'ENDOCARDIUM CORRECTED COUNTS',
           $  T61,'EPICARDIUM CORRECTED COUNTS'/)
0175         DO 90 ENERGY=1,ISOTOP
0176         WRITE(OUTDEV,93) ENERGY,ENERGY
0177         DO 90 ROW=1,5
0200    90   WRITE(OUTDEV,92) ((DOG(ROW,COL,ENERGY,ENDEPI),COL=1,4),ENDEPI=1,2)
0201    92   FORMAT(1H ,4I10,10X,4I10)
0202    93   FORMAT(1H0,T21,'ENERGY ',I1,T71,'ENERGY ',I1/)
C
C        CALCULATE FACTOR ELEMENTS. ALLOCATE STREAM 8 TO WEIGHT FILE.
C
0203         DO 100 SET=1,4
0204         READ(8,102) REFWT(SET)
0205   100   READ(8,102) TIME(SET)
0206   102   FORMAT(F6.4)
C        READ STANDARD WEIGHTS (4 SAMPLES FOR EACH ISOTOPE)
0207         DO 105 ENERGY=1,4
0210         DO 105 SET=1,4
0211   105   READ(8,102) STANWT(SET,ENERGY)
C        READ SPECIMEN WEIGHTS
0212         DO 107 ROW=1,5
0213         DO 107 COL=1,4
0214         DO 107 ENDEPI=1,2
```

```
      C
0262            DO 145 ENERGY=1,ISOTOP
0263            DO 145 ROW=1,5
0264            DO 145 COL=1,4
0265            IF(DOG(ROW,COL,ENERGY,2).EQ.0.0) GO TO 144
0266            RBF(ROW,COL,ENERGY)=DOG(ROW,COL,ENERGY,1)/DOG(ROW,COL,ENERGY,2)
0267            GO TO 145
0270       144  RBF(ROW,COL,ENERGY)=0.0
0271       145  CONTINUE
0272            WRITE(OUTDEV,148)
0273       148  FORMAT(1H0//'   RATIOS OF ENDOCARDIUM/EPICARDIUM BLOOD FLOWS'/)
0274            DO 150 ENERGY=1,ISOTOP
0275            WRITE(OUTDEV,151) ENERGY
0276       151  FORMAT(1H0,T21,'ENERGY ',I1/)
0277            DO 150 ROW=1,5
0300       150  WRITE(OUTDEV,152) (RBF(ROW,COL,ENERGY),COL=1,4)
0301       152  FORMAT(1H ,4(6X,F6.3))
0302            WRITE(OUTDEV,153)
0303       153  FORMAT(1H1)
         C
         C      DISPLAY SCALING ETC....
         C
0304            TOP=DOG(1,1,1,1)
0305            BOT=DOG(1,1,1,1)
0306            DO 170 ENDEPI=1,2
0307            DO 170 ENERGY=1,ISOTOP
0310            DO 170 ROW=1,5
0311            DO 170 COL=1,4
0312            IF(DOG(ROW,COL,ENERGY,ENDEPI).GT.TOP)
              +  TOP=DOG(ROW,COL,ENERGY,ENDEPI)
0313            IF(DOG(ROW,COL,ENERGY,ENDEPI).LT.BOT)
              +  BOT=DOG(ROW,COL,ENERGY,ENDEPI)
0314       170  CONTINUE
0315            TVSC1=16./(TOP-BOT)
0316            TOPRBF=RBF(1,1,1)
0317            BOTRBF=RBF(1,1,1)
0320            DO 180 ENERGY=1,ISOTOP
0321            DO 180 ROW=1,5
0322            DO 180 COL=1,4
0323            IF(RBF(ROW,COL,ENERGY).GT.TOPRBF) TOPRBF=RBF(ROW,COL,ENERGY)
0324            IF(RBF(ROW,COL,ENERGY).LT.BOTRBF) BOTRBF=RBF(ROW,COL,ENERGY)
0325       180  CONTINUE
0326            TVSC2=16./(TOPRBF-BOTRBF)
         C
         C      DISPLAY REQUEST
         C
0327       185  WRITE(4,190)
0330       190  FORMAT(1H0,'TV DISPLAY (Y/N) ? ',$)
0331            READ(4,4) ANS
0332            IF(ANS.EQ.NO) STOP
0333            IF((ANS.NE.NO).AND.(ANS.NE.YES))  GO TO 185
0334            CALL TVCLR
0335            DO 191 COL=1,64
0336       191  L(COL)=1
0337            DO 192  COL=1,8
```

```
0340            L(1)=COL*2
0341            L(2)=L(1)
0342            DO 192 SET=1,8
0343            LINE=8*(COL-1)+SET
0344      192   CALL TV(LINE,L)
0345      194   WRITE(4,195)
0346      195   FORMAT(' ENERGY (1,2,3,4) ? ',$)
0347            READ(4,196), ENERGY
0350      196   FORMAT(I1)
0351            IF(ENERGY.LE.ISOTOP) GO TO 198
0352            WRITE(4,197) ISOTOP
0353      197   FORMAT(1H0,'**ONLY ',I1,' ISOTOPES IN THIS STUDY')
0354            GO TO 194
0355      198   WRITE(4,199)
0356      199   FORMAT(' ABSOLUTE OR RELATIVE BLOOD FLOW (A/R) ? ',$)
0357            READ(4,4) ANS
0360            IF(ANS.EQ.AB) GO TO 200
0361            IF(ANS.EQ.RE) GO TO 300
0362            GO TO 198
          C
          C     DISPLAY ABSOLUTE BLOOD FLOWS
          C
0363      200   WRITE(4,205)
0364      205   FORMAT(' ENDO OR EPI (EN/EP) ? ',$)
0365            READ(4,4) ANS
0366            IF(ANS.EQ.ENDO) ENDEPI=1
0367            IF(ANS.EQ.EPI) ENDEPI=2
0370            IF((ANS.NE.ENDO).AND.(ANS.NE.EPI)) GO TO 200
0371            DO 215 ROW=1,5
0372            DO 212 COL=1,4
0373            DO 212 SET=1,8
0374            L(16+8*(COL-1)+SET)=TVSC1*DOG(ROW,COL,ENERGY,ENDEPI)+1
0375            IF(L(16+8*(COL-1)+SET).GT.16) L(16+8*(COL-1)+SET)=16
0376      212   CONTINUE
0377            L(1)=16-2*ROW
0400            L(2)=L(1)
0401            DO 215 SET=1,8
0402            LINE=57-8*(ROW-1)-SET
0403      215   CALL TV(LINE,L)
0404            GO TO 400
          C
          C     RELATIVE BLOOD FLOW DISPLAY
          C
0405      300   DO 315  ROW=1,5
0406            DO 312 COL=1,4
0407            DO 312 SET=1,8
0410            L(16+8*(COL-1)+SET)=TVSC2*RBF(ROW,COL,ENERGY)+1
0411            IF(L(16+8*(COL-1)+SET).GT.16) L(16+8*(COL-1)+SET)=16
0412      312   CONTINUE
0413            L(1)=16-2*ROW
0414            L(2)=L(1)
0415            DO 315  SET=1,8
0416            LINE=57-8*(ROW-1)-SET
0417      315   CALL TV(LINE,L)
          C
```

```
0420      400   WRITE(4,401)
0421      401   FORMAT(1H0,'DISPLAY MORE (Y/N) ? ',$)
0422            READ(4,4) ANS
0423            IF(ANS.EQ.YES)  GO TO 194
0424            IF(ANS.EQ.NO) STOP
0425            GO TO 400
          C
0426            STOP
          C
0427      999   WRITE(OUTDEV,9999)
0430     9999   FORMAT(1H0,'**CHECK LAST FILE ENTRY '/)
0431            GO TO 1
0432            END
```

APPENDIX B 3/6 The measurement of regional myocardial blood flow: The reproducibility of the method (Tables)

Appendix B 3

Mean (± SEM) regional myocardial blood flow (ml $g^{-1}min^{-1}$) determined simultaneously with different microspheres

Regional myocardial blood flow was determined in epicardial and endocardial layers of ischaemic and non ischaemic myocardium 5 min after coronary occlusion using 3 differently labelled radioactive microspheres (Co^{57}, Ru^{103}, Sc^{46}). In experiment 2, 4 different microspheres were used. A simultaneous left atrial injection was made during the withdrawal of two reference blood samples from the left and right femoral artery.

Epi- and endocardial biopsies were cut by mid-line transsection and counted using a gamma counter (Wallac). (for further details see Text)

Combined results of all biopsies (epi-and endocardial, ischaemic and non ischaemic) calculated on the basis of either reference sample A or B are presented. Number of biopsies (n =).

The mean blood flow is also expressed as a percentage of the average blood flow, measured with microspheres labelled with Co^{57} (values within brackets).

Experiment	RBS*		Co^{57} microspheres	Ru^{103} microspheres	Sc^{46} microspheres	Sn^{113} microspheres
1.	A	(n=20)	1.396 ± 0.124(100%)	1.236 ± 0.110(88.5%)	1.517 ± 0.134(108.7%)	—
	B	(n=20)	1.543 ± 0.137(100%)	1.605 ± 0.142(104.0%)	1.387 ± 0.122(89.9%)	—
2.	A	(n=16)	0.838 ± 0.043(100%)	0.788 ± 0.041(94.0%)	0.806 ± 0.042(96.2%)	0.810 ± 0.042(96.7%)
	B	(n=16)	0.984 ± 0.051(100%)	0.905 ± 0.047(92.0%)	0.931 ± 0.048(94.6%)	0.946 ± 0.049 (96.1%)
3.	A	(n=18)	0.753 ± 0.069(100%)	0.758 ± 0.069(100.7%)	0.688 ± 0.064(91.4%)	—
	B	(n=18)	0.789 ± 0.073(100%)	0.767 ± 0.070(97.2%)	0.705 ± 0.066(89.4%)	—
4.	A	(n=18)	0.518 ± 0.013(100%)	0.536 ± 0.012(103.5%)	0.542 ± 0.015(104.6%)	—
	B	(n=18)	0.521 ± 0.013(100%)	0.525 ± 0.012(100.8%)	0.528 ± 0.015(101.3%)	—
Mean (Exp 1-4)			0.918 ± 0.133(100%)	0.890 ± 0.129(97.6%) NS vs Co^{57}	0.888 ± 0.132(97.0%) NS vs Co^{57} NS vs Ru^{103}	

NS: $p > 0.05$, paired t-test.

RBS* Ref. Blood Sample

Appendix B 4

Regional myocardial blood flow (ml $g^{-1}min^{-1}$) in ischaemic and border zone, determined simultaneously with different microspheres

Regional myocardial blood flow was determined in the ischaemic and border zone 5 min after coronary occlusion. Combined results (mean ± SEM) of epi- and endocardial layers. Details and abbreviations as in Appendix B 3

Experiment	RBS*		Co^{57}microspheres	Ru^{103}microspheres	Sc^{46}microspheres	Sn^{113}microspheres
1.	A	(n=8)	0.484 ± 0.077(100%)	0.437 ± 0.075(90.3%)	0.538 ± 0.085(111.2%)	—
	B	(n=8)	0.535 ± 0.085(100%)	0.568 ± 0.097(106.2%)	0.492 ± 0.077(92.0%)	—
2.	A	(n=6)	0.618 ± 0.053(100%)	0.573 ± 0.052(92.7%)	0.586 ± 0.050(94.8%)	0.591 ± 0.049(95.6%)
	B	(n=6)	0.725 ± 0.062(100%)	0.657 ± 0.069(90.6%)	0.677 ± 0.058(93.4%)	0.690 ± 0.057(95.2%)
3.	A	(n=8)	0.370 ± 0.078(100%)	0.384 ± 0.081(103.8%)	0.322 ± 0.066(87.0%)	—
	B	(n=8)	0.388 ± 0.082(100%)	0.389 ± 0.082(100.3%)	0.330 ± 0.068(85.1%)	—
4.*						
Mean (Expl-4)			0.520 ± 0.056(100%)	0.501 ± 0.046(97.3%)	0.491 ± 0.058(93.9%)	
				NS vs Co^{57}	NS vs Co^{57} NS vs Ru^{103}	

NS: $p > 0.05$, paired t-test.

* The effect of coronary occlusion on ischaemic blood flow was hardly detectable. Results excluded.

RBS* Ref. Blood Sample

Appendix B 5

The determination of the endocardial/epicardial ratio of regional myocardial blood flow determined simultaneously with different microspheres

Regional myocardial blood flow was determined in epicardial and endocardial layers of ischaemic and non-ischaemic myocardium 5 min after coronary occlusion, using a simultaneous injection of different microspheres (Co^{57}, Ru^{103}, Sc^{46} and in experiment 2 Sn^{113}). The effect of the reference blood sample cancels out in the determination of the ratio (see Text). In view of the number of observations (4) a formal statistical analysis was not made.

Conditions and abbreviations as in Appendix B 3

Experiment	Co^{57} microspheres	Ru^{103} microspheres	Sc^{46} microspheres	Sn^{113} microspheres
1.	0.910 ± 0.084(100%)	0.883 ± 0.077(97.0%)	0.869 ± 0.083(95.5%)	—
3.	1.079 ± 0.133(100%)	1.075 ± 0.126(99.6%)	1.122 ± 0.107(104.0%)	—
2.	1.136 ± 0.088(100%)	1.116 ± 0.084(98.2%)	1.124 ± 0.083(98.9%)	1.131 ± 0.088(99.6%)
4.	1.185 ± 0.051(100%)	1.179 ± 0.055(99.5%)	1.241 ± 0.062(104.7%)	—
Mean (Exp 1-4)	1.078 ± 0.060(100%)	1.063 ± 0.064(98.6 %)	1.089 ± 0.078(100.8 %)	—

Appendix B 6

The determination of the endocardial/epicardial ratio of regional myocardial blood flow in the ischaemic and border zone, determined simultaneously with different microspheres

Regional myocardial blood flow was determined in epicardial and endocardial layers in the ischaemic and border zone 5 min after coronary occlusion, using a simultaneous injection of different microspheres. No effect of coronary occusion was observed in experiment 4 and the results were excluded.

Conditions and abbreviations as in Appendix B 3

In view of the number of observations a formal statistical test was not made.

Experiment		Co^{57} microspheres	Ru^{103} microspheres	Sc^{46} microspheres	Sn^{113} microspheres
1.	(n=8)	0.787 ± 0.205(100%)	0.712 ± 0.176(90.5%)	0.691 ± 0.191(87.8%)	—
2.	(n=6)	0.795 ± 0.075(100%)	0.800 ± 0.093(100.6%)	0.811 ± 0.084(102.0%)	0.794 ± 0.085 (99.9%)
3.	(n=8)	0.461 ± 0.143(100%)	0.473 ± 0.135(102.6%)	0.613 ± 0.119(133.0%)	—
4.		—	—	—	—
	Mean	0.681 (100%)	0.662 (97.9%)	0.705 (107.6%)	—

Appendix C

Effects of p-Chlorophenoxyisobutyrate on Myocardial Free Fatty Acid Extraction, Ventricular Blood Flow, and Epicardial ST-segment Elevation During Coronary Occlusion in Dogs

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Effects of p-Chlorophenoxyisobutyrate on Myocardial Free Fatty Acid Extraction, Ventricular Blood Flow, and Epicardial ST-segment Elevation During Coronary Occlusion in Dogs

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SUMMARY **The effect of p-chlorophenoxyisobutyrate (CPIB) on ST-segment elevation in epicardial electrocardiographic recordings was studied during coronary artery occlusion in dogs. Occlusion alone raised the sum of ST-segment elevations (ΣST) to 26 ± 6 mV (mean ±SEM). Intravenous (i.v.) administration of CPIB 30 min before re-occlusion reduced ΣST to 14 ± 3 mV ($P < 0.03$). A continuous i.v. infusion of isoproterenol increased ΣST to 74 ± 11 mV. Pretreatment with CPIB reduced ΣST during isoproterenol infusion to 40 ± 7 mV ($P < 0.005$). CPIB had no effect on mean aortic blood pressure, heart rate, or regional myocardial blood flow, as measured by radioactive microspheres. Arterial free fatty acid (FFA) concentrations were reduced by CPIB from 466 ± 41 to 221 ± 44 μEq/L ($P < 0.001$) in the basal state, and from 1966 ± 183 to 1429 ± 209 μEq/L ($P < 0.001$) during isoproterenol infusion. The reduction in arterial FFA concentration was associated with a proportionate decrease in the myocardial extraction of FFA. Similar changes were observed when CPIB was administered during an occlusion which had been established 10 min earlier. These observations support other evidence that the severity of acute myocardial ischemic injury in dogs is positively correlated with the myocardial extraction of FFA, and that the severity of the ischemic injury can be reduced by effective antilipolytic therapy.**

ACUTE MYOCARDIAL INFARCTION (AMI) in man is accompanied within one hour by an increase in the plasma concentration of free fatty acids (FFA), probably due to increased adipose tissue lipolysis as a result of enhanced sympatho-adrenal activity.[1-3] Those patients with the highest plasma FFA concentrations have been reported to be at the greatest risk of developing serious ventricular arrhythmias and death,[4, 5] although not all investigations have confirmed this association.[6] The hypothesis that this might reflect a toxic effect of FFA on the ischemic myocardium,[7] rather than a direct consequence of increased catecholamine activity, has been supported by the recent demonstration that the incidence of ventricular arrhythmias during the early phase of AMI in man can be reduced by effective antilipolytic therapy in the absence of changes in plasma catecholamine concentration.[8] A toxic effect of FFA on the heart could be mediated through an alteration of membrane potentials or an increase in the severity of the ischemic injury to the myocardium.

Increases in plasma FFA concentration similar to those observed in clinical studies have been reported to increase the frequency of ventricular arrhythmias[9] and the severity of myocardial ischemic injury[10] during experimental coronary occlusion in dogs. In healthy dogs, elevated plasma FFA enhance myocardial oxygen consumption (MVO_2) without improving the mechanical activity of the heart,[11, 12] suggesting that the deleterious effect of FFA during coronary occlusion may reflect an increase in the oxygen requirement of the ischemic tissue.

Such observations have raised the possibility that the survival of the ischemic cells following acute coronary occlusion may be enhanced by measures which decrease the delivery of FFA to the ischemic cells. Substantial reductions in plasma FFA concentrations have been reported in animals treated with p-chlorophenoxyisobutyrate (CPIB),[13, 14] although it is not known whether this is accompanied by a reduction in the utilization of FFA by the myocardium,[15] as has been demonstrated with β-pyridyl-carbinol and its active metabolite, nicotinic acid.[12, 16]

We have studied the effect of CPIB on the extraction of FFA by the heart and the extent and magnitude of epicardial ST-segment elevation during experimental coronary artery occlusion in dogs. The effect of antilipolytic therapy on regional myocardial blood flow during coronary occlusion was also examined, with the aid of radioactive microspheres.

Methods

Surgical Techniques

Experiments were carried out in 23 mongrel dogs of both sexes (15–20 kg body weight), fasted for 12–15 hours. The dogs were anesthetized with sodium pentobarbital, 25 mg/kg body weight, followed by maintenance doses of 30–40 mg. Ventilation was maintained through a cuffed endotracheal tube with a positive pressure respirator (Harvard Apparatus Co. Inc., Mass.). Thoracotomy was performed through an incision in the left fifth intercostal space, and the heart suspended in a pericardial cradle. A branch of the left anterior descending (LAD) coronary artery was dissected free for a distance of 0.5 cm and a ligature placed loosely around it. Subsequent occlusions of the artery were performed with a releasable metal clip. The left femoral artery was cannulated for measurement of mean aortic blood pressure ($\overline{AP}$), and the left femoral vein as a route of infusion. $\overline{AP}$ was monitored with a Statham P23Db transducer and recorded on a 4-channel recorder (Devices Ltd.,

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Herts, U.K.). Arterial blood was sampled through a catheter inserted into the right femoral artery. In seven experiments coronary sinus blood was withdrawn simultaneously through a catheter introduced via the left jugular vein under fluoroscopic guidance. Arterial oxygen saturation was monitored with a Kipp type M01 Hemoreflector, and pH and pCO_2 with a Type AME 1c Astrup Micro Equipment (Radiometer, Copenhagen).

Epicardial ST-segment Mapping

Epicardial electrocardiographic (ECG) measurements were performed with a mobile cotton-wick electrode, as described by Maroko et al.,[17] from 10–15 anatomically recognizable sites supplied by the dissected coronary artery and from the surrounding left ventricular tissue. Measurements were recorded, together with the standard limb leads, on a Devices 4-channel recorder at a paper speed of 25 mm/sec. The sensitivity of the epicardial recordings was set at 1mV/mm deflection. Those sites at which ST-segment elevation exceeded 2 mV were considered to be ischemic.[17] Results were expressed as the sum of ST-segment elevations recorded from all sites (ΣST), and as the number of sites at which ST elevation exceeded 2 mV[10, 17]

It should be noted that the values obtained with this technique are related not only to the severity of the ischemic injury, but also to the number and location of the recording sites, and cannot therefore be used to compare the results of different experiments. For such a purpose it would have been necessary to use a standardized grid system, which has disadvantages because of anatomical variations from dog to dog. In the present study, however, such a system was not required since each animal served as its own control, the electrode sites remaining constant throughout a given study and the effect of CPIB being assessed by paired analysis.

Regional Myocardial Blood Flow

Regional myocardial blood flow was measured by means of radioactive microspheres labelled with ^{125}I, ^{141}Ce, or ^{85}Sr (3M Riker Laboratories, Loughborough, U.K.: nominal diameter, 15μ).[18, 19] Prior to injection 2.0–2.5 × 10^6 microspheres were subjected to ultrasound in 10% dextrose for 10 or more min to dispel aggregates (Dawe Sonicleaner, type 6441A), and drawn into a syringe containing 0.1 ml 5% Tween 80 to prevent reaggregation. The suspension was diluted with 10% dextrose to give a final concentration of Tween 80 of less than 0.5% and then continuously agitated until injected. Ten to 13 min after each coronary occlusion blood for the estimation of reference blood flow (RBF) was withdrawn from a femoral artery into a weighed heparinized syringe at a constant rate (approx. 9 ml/min) for a period of 2 min using a Harvard infusion/withdrawal pump. Immediately after the commencement of blood withdrawal, the microspheres were injected through a left atrial cannula over a period of about 10 sec. Between 5 and 30 min after the last injection the dog was sacrificed with sodium pentobarbital. The heart was excised, the free wall of the left ventricle dissected out, and visual fat and large vessels removed. Between 10 and 14 full thickness biopsies were made and divided into epicardial and endocardial layers of wet weight 1.5–4.0 g. The method was designed to give a minimum of 1000 microspheres/g wet weight of ischemic myocardium. Each layer was then divided into 10–20 blocks to produce relatively constant geometrical factors for the measurement of radioactivity in a three channel gamma counter (Gamma-Guard, Tracerlab Ltd.). Approximately 27% and 9% of ^{85}Sr radioactivity was counted in the ^{141}Ce and ^{125}I channels respectively, while 3% of ^{141}Ce radioactivity was counted in the ^{125}I channel. These factors were determined during each counting procedure using the reference blood samples (containing only one isotope) as standards in a similar geometrical setting. Regional blood flow was estimated by weighing, using an assumed blood specific gravity of 1.05 g/ml. Regional myocardial blood flow (MBF) was calculated for each biopsy using the formula: $MBF = \frac{CM}{CR} \times RBF$, where MBF is expressed in ml g^{-1}, min^{-1}. CM is the biopsy radioactivity in cpm/g wet weight, CR is the total cpm in the reference blood sample, and RBF is expressed in ml/min.[18, 19]

Laboratory Procedures

Plasma CPIB concentrations were measured by a spectrophotometric method.[13] Plasma FFA concentrations were assayed in duplicate by the method of Dole[20] as modified by Trout et al.,[21] and were corrected for the presence of CPIB as described by Barrett and Thorp.[13] Plasma free glycerol was estimated in duplicate according to Chernick.[22] Blood glucose was measured in duplicate by an automated method.[23]

Design of Experiments

Effects of Pretreatment with CPIB on Subsequent Coronary Occlusion. The effects of pretreatment with CPIB on the response to subsequent coronary occlusion were studied in ten dogs. In seven animals the following experimental design was employed. Epicardial electrocardiograms were recorded before and 5, 10, and 15 min after an initial control occlusion. The clip was then removed and a recovery period of 30 min allowed. The arterial FFA concentration was then raised by a continuous intravenous (i.v.) infusion or isoproterenol (0.2–0.3 μg/kg/min). Coronary occlusion alone does not alter plasma FFA concentrations in anesthetized dogs.[9, 10] This was confirmed in five of the present experiments, in which arterial FFA concentrations 15 minutes after occlusion (414 ± 16 μEq/L, mean ±SEM) were not significantly different from those immediately before occlusion (406 ± 23 μEq/L). Five minutes after the start of the infusion (by which time a stable hemodynamic response had been attained) the artery was reoccluded. Epicardial ECG recordings were again made at 5 min intervals for 15 min, after which time the clip was released and the isoproterenol infusion discontinued. After another recovery period of 30 min CPIB was administered as the sodium salt by slow i.v. injection in 0.9% saline at the dose of 20 mg/kg body weight. Thirty minutes later the foregoing sequence was repeated, identical occlusions of the artery being induced at first in the absence and then in the presence of isoproterenol, and ST-segment elevation being mapped immediately before and 5, 10, and 15 min after each occlusion. In the remaining three dogs (307, 309, 313)

studies were carried out either in the absence or presence of isoproterenol only.

The reproducibility of the ST-segment mapping procedure was checked in the same experiments by performing repeated occlusions under identical conditions. In ten comparisons, values for ΣST measured 15 min after coronary occlusion agreed consistently within ± 7% (first occlusion: ΣST = 46 ± 12 mV, mean ± SEM: second occlusion: 47 ± 12 mV [paired *t*-test: $P > 0.30$]).

Measurements of $\overline{AP}$ and HR were made simultaneously with each ECG recording. Arterial blood was collected 15 min after each occlusion into a heparinized tube, and plasma for FFA and CPIB estimations separated immediately by centrifugation at 4°C. In nine dogs arterial blood was also collected for blood glucose determination.

In five additional dogs the effect of CPIB on regional myocardial blood flow during coronary occlusion was assessed by means of radioactive microspheres. Measurements were made during an initial control occlusion (three dogs), during second occlusion performed in the presence of isoproterenol (five dogs), and during a third occlusion performed in the presence of isoproterenol 30 min after i.v. CPIB (five dogs), the order of injection of the differently labeled microspheres being randomized in order to avoid systematic errors due to possible differences in their behavior. (The effect of CPIB on myocardial blood flow was assessed in the presence of isoproterenol, rather than under basal conditions, since it had previously been found that the reduction of epicardial ST-segment elevation by CPIB was more marked in the former situation.)

Effects of CPIB Administration during Established Coronary Occlusion. In six dogs an i.v. infusion of isoproterenol (0.2–0.3 μg/kg/min) was maintained for the duration of the study. Five minutes after its commencement a branch of the LAD coronary artery was permanently occluded. Five and ten minutes later recordings were made of epicardial ECG, $\overline{AP}$ and HR. CPIB was then administered as previously described, and the recordings repeated after further intervals of 5 and 10 minutes. Simultaneously with each recording, arterial blood was sampled for the measurement of plasma FFA, CPIB, and free glycerol concentrations. In five of these dogs, and in an additional two animals, coronary sinus blood was also sampled immediately before and ten minutes after CPIB administration for the determination of the arterial-coronary sinus concentration differences of FFA and CPIB.

Statistics

Each dog served as its own control. Student's *t*-test for paired data was used to calculate probability values. $P > 0.05$ was regarded as not statistically significant.

Results

Effects of Pretreatment with CPIB

The effects of CPIB on the response to subsequent coronary occlusion are summarized in tables 1 and 2. Occlusion of a branch of the LAD coronary artery was followed by marked changes in epicardial ST segments. After 15 minutes of occlusion ΣST averaged 26 ± 6 mV (mean ±SEM). After CPIB administration re-occlusion of the artery resulted in a much smaller ΣST, averaging 14 ± 3 mV ($P < 0.03$). CPIB also reduced the number of sites with evidence of ischemic injury (ST > 2mV) from an average of 4.9 ± 1.1 to 2.9 ± 1.0 ($P < 0.05$). These effects of CPIB occurred in the absence of any changes in $\overline{AP}$, HR, or blood glucose concentration (table 1). However, arterial FFA concentrations were reduced from 466 ± 41 μEq/L to 221 ± 44 μEq/L ($P < 0.001$) (table 1).

Prior to CPIB the infusion of isoproterenol increased occlusion-induced ΣST from 26 ± 6 mV to 74 ± 11 mV ($P < 0.001$), and the number of sites with ischemic injury from 4.9 ± 1.1 to 9.0 ± 0.9 ($P = 0.02$) (tables 1 and 2). After CPIB ΣST associated with subsequent coronary occlusion and isoproterenol infusion was markedly reduced to 40 ± 7 mV ($P < 0.005$), while the number of sites with ischemic injury was reduced to 7.6 ± 1.3 ($P < 0.05$). Intravenous infusion of isoproterenol before CPIB reduced $\overline{AP}$ from 97 ± 5 to 91 ± 3 mm Hg ($P < 0.05$), and increased HR from 134 ± 7 to 176 ± 6 beats/min ($P < 0.001$), blood

TABLE 1. *Effects of Pretreatment with CPIB on Epicardial ST-segment Elevation, Arterial FFA Concentration (FFA), $\overline{AP}$, HR, and Blood Glucose Concentration of 15 Minutes after Coronary Occlusion*

Dog	ΣST* (mV)		ST > 2 mV† (number of sites)		FFA (μEq/l)		$\overline{AP}$ (mm Hg)		HR (beats/min)		Bd gluc (mg/100 ml)		Plasma CPIB concentration (μg/ml)
	Occ	Occ CPIB	Occ	Occ CPIB	Occ	Occ CPIB	Occ	Occ CPIB	Occ	Occ CPIB	Occ	Occ CPIB	Occ CPIB
303	8	5	1	1	340	105	98	90	143	138	—	—	139
304	33	32	7	7	440	130	82	92	133	139	61	47	129
305	17	10	4	1	510	105	100	107	145	147	52	60	148
306	5	3	0	0	320	150	102	104	143	142	66	65	125
307	20	17	3	2	460	245	103	97	156	142	66	53	159
308	26	10	5	0	420	340	112	105	162	164	65	64	194
309	31	18	7	4	390	150	75	75	111	109	58	58	166
312	62	19	8	8	630	265	86	92	111	104	75	71	208
315	34	12	9	3	680	500	117	116	100	95	34	46	166
Mean	26	14	4.9	2.9	466	221	97	98	134	131	60	58	159
SEM	6	3	1.1	1.0	41	44	5	4	7	8	4	3	9
P	<0.03		<0.05		<0.001		NS		NS		NS		—

*Sum of ST-segment elevations at 10–15 sites.
†Number of sites at which ST-segment elevation exceeded 2 mV.
NS = $P > 0.05$.
Abbreviations: Occ = occlusion; IP = isoproterenol; FFA = free fatty acid; $\overline{AP}$ = mean arterial pressure; HR = heart rate; Bd gluc = blood glucose.

TABLE 2. *Effects of Pretreatment with CPIB on Epicardial ST-segment Elevation, Arterial FFA Concentration (FFA), $\overline{AP}$, HR and Blood Glucose Concentration 15 Minutes after Coronary Occlusion Performed during a Continuous i.v. Infusion of Isoproterenol*

Dog	ΣST* (mV)		ST > 2 mV† (number of sites)		FFA (μEq/l)		$\overline{AP}$ (mm Hg)		HR (beats/min)		Bd gluc (mg/100 ml)		Plasma CPIB concentration (μg/ml)
	Occ IP	Occ CPIB	Occ IP	Occ CPIB	Occ IP	Occ CPIB	Occ IP	Occ CPIB	Occ IP	Occ CPIB	Occ IP	Occ CPIB	Occ CPIB
303	19	5	4	1	1300	975	82	83	185	180	—	—	116
304	71	43	11	11	1930	1275	90	92	185	185	81	60	111
305	92	65	9	6	1560	945	93	97	132	185	80	70	122
306	58	40	10	9	2820	2315	82	82	175	176	95	82	127
308	64	17	7	4	1880	1160	98	90	175	170	77	75	166
312	124	54	9	9	1880	1360	83	80	154	149	136	141	185
313	79	34	12	11	2640	2400	102	105	200	198	52	60	139
315	87	58	10	10	1720	1000	95	90	155	148	42	54	166
Mean	74	40	9.0	7.6	1966	1429	91	90	176	174	80	77	142
SEM	11	7	0.9	1.3	183	209	3	3	6	6	12	11	10
P	<0.005		<0.05		<0.001		NS		NS		NS		—

*Sum of ST-segment elevations at 10–15 sites.
†Number of sites at which ST-segment elevation exceeded 2 mV.
NS = $P > 0.05$.
For abbreviations see table 1.

glucose concentration from 60 ± 4 to 80 ± 12 mg/100 ml ($P < 0.02$) and arterial FFA concentration from 466 ± 41 to 1966 ± 183 μEq/L ($P < 0.001$). None of these effects of isoproterenol was altered by CPIB other than the increase in arterial FFA concentration, which was reduced to 1429 ± 209 μEq/L ($P < 0.001$).

The results of the studies with radioactive microspheres are summarized in table 3. Nonischemic tissue of the free wall of the left ventricle distant from the occluded artery was taken to represent normal myocardium. On this basis coronary occlusion produced an average decrease of approximately 40% in blood flow to the ischemic zone. As reported by other investigators,[24] the reduction in flow was greater in the endocardial than in the epicardial layers, resulting in a significant increase in the epicardial/endocardial blood flow ratio ($P < 0.01$). Isoproterenol significantly increased the blood flow to the nonischemic left ventricle, as previously reported,[25] but had no effect on that to the ischemic zone. Values for regional myocardial blood flow in both the ischemic and nonischemic zones were unchanged by treatment with CPIB.

Effects of CPIB Administration During Established Coronary Occlusion

The acute effects of CPIB when given during an established coronary occlusion and isoproterenol infusion are summarized in table 4. Five and ten minutes after giving CPIB (i.e., 15 and 20 minutes following coronary occlusion) values for ΣST and arterial FFA concentration were both significantly reduced relative to those recorded immediately before treatment, while $\overline{AP}$ and HR remained unchanged. In contrast, ΣST has previously been found to increase or remain unchanged between 10 and 20 min following coronary occlusion during isoproterenol infusion in the absence of CPIB administration (10 min: 61 ± 8 mV: 15 min 67 ± 8 mV: N = 12, $P < 0.005$, 10 min: 94 ± 19 mV: 20 min: 98 ± 16 mV: N = 5, P = NS). The reduction in arterial FFA concentration was associated with a proportionate decrease in the arterial-coronary sinus FFA concentration difference (table 5). No significant uptake of CPIB by the myocardium could be detected: mean arterial and coronary sinus CPIB concentrations were 225 ± 18 and 218 ± 21

TABLE 3. *Effects of Isoproterenol and CPIB on Regional Myocardial Blood Flow in Nonischemic and Ischemic Free Ventricular Wall 10-13 Minutes after Coronary Occlusion*

		MBF (ml/g⁻¹ min⁻¹)				
		Occ (3)	*P*	IP occ (5)	*P*	IP occ CPIB (5)
Nonischemic myocardium*	Epi	1.30 ± 0.09 (N = 21)	<0.001	1.88 ± 0.09 (N = 34)	NS	1.85 ± 0.07 (N = 34)
	Endo	1.36 ± 0.10 (N = 21)	<0.001	1.62 ± 0.07 (N = 34)	NS	1.61 ± 0.05 (N = 34)
	Epi/Endo	0.97 ± 0.04 (N = 21)	<0.001	1.17 ± 0.04 (N = 34)	NS	1.16 ± 0.03 (N = 34)
Ischemic myocardium†	Epi	0.86 ± 0.16 (N = 6)	NS	0.89 ± 0.08 (N = 11)	NS	0.94 ± 0.10 (N = 11)
	Endo	0.71 ± 0.14 (N = 6)	NS	0.71 ± 0.11 (N = 11)	NS	0.76 ± 0.12 (N = 11)
	Epi/endo	1.32 ± 0.12 (N = 6)	NS	1.45 ± 0.16 (N = 11)	NS	1.50 ± 0.12 (N = 11)

Results are expressed as mean ± SEM (number of biopsies).
NS = $P > 0.05$.
*Myocardium distant from the occluded artery.
†Myocardium within the area of distribution of the occluded artery.
Abbreviations: MBF = myocardial blood flow; epi = epicardial; endo = endocardial.

TABLE 4. *Acute Effects of CPIB on ST, $\overline{AP}$, HR and the Arterial Concentrations of FFA and Free Glycerol when Administered during an Established Coronary Occlusion and Isoproterenol Infusion*

Time (min)	0	5	10	15	20	25
		Isoproterenol infusion (0.2 – 0.3 μg/kg/min)				
			Coronary artery occlusion			
						CPIB (20 mg/kg)
ΣST (mV)		11 ± 4 (6)†	74 ± 12 (6)	81 ± 13 (6)	57 ± 8 (6)*	53 ± 6 (6)*
ST > 2mV (number of sites)		1.4 ± 0.7 (6)†	9.6 ± 0.9 (6)	9.2 ± 0.9 (6)	8.4 ± 0.7 (6)	8.4 ± 0.8 (6)*
$\overline{AP}$ (mm Hg)		82 ± 4 (6)	84 ± 5 (6)	84 ± 5 (6)	86 ± 5 (6)	85 ± 5 (6)
HR (beats/min)		154 ± 13 (6)	152 ± 13 (6)	150 ± 13 (6)	148 ± 12 (6)	146 ± 12 (6)
FFA (μEq/L)		—	1540 ± 20 (2)	1993 ± 259 (6)	1218 ± 155 (6)†	1108 ± 156 (6)*
Glycerol (μmol/l)		—	—	254 ± 31 (6)	—	185 ± 21 (6)†
Arterial CPIB conc (μg/ml)		0	0	0	251 ± 15 (6)	223 ± 12 (6)

Results are expressed as mean ± SEM (number of dogs).
Statistical comparisons were performed by paired *t*-test analysis against those results (3rd column) obtained immediately before the administration of CPIB.
*$P < 0.025$.
†$P < 0.01$.
Other differences were not statistically significant.
Abbreviations: conc = concentration; for others see table 1.

μg/ml respectively: CPIB reduced the arterial free glycerol concentration from 254 ± 31 to 185 ± 21 μmol/L ($P < 0.01$).

Discussion

The present observations demonstrate that pretreatment with CPIB substantially reduced the extent and magnitude of epicardial ST-segment elevation after experimental coronary artery occlusion in dogs, and reduces the augmentation of ST-segment elevation induced by isoproterenol infusion. The absolute reduction in ST elevation during isoproterenol infusion was greater than that recorded during occlusion in the absence of an isoproterenol infusion. This difference was observed both when the administration of CPIB preceded a subsequent re-occlusion of the artery, and when the drug was given during an occlusion established 10 minutes earlier.

TABLE 5. *Effects of CPIB on Arterial FFA Concentration, Arterial-Coronary Sinus FFA Concentration Difference and FFA Extraction Ratio* during Established Coronary Artery Occlusion*

	Arterial FFA concentration (μEq/L)		Arterial-coronary sinus FFA concentration difference (μEq/L)		Extraction ratio (%)	
Dog	Occ	Occ† CPIB	Occ	Occ CPIB	Occ	Occ CPIB
Without isoproterenol						
323	760	365	360	50	47	14
324	460	370	60	30	13	8
During isoproterenol (0.2 – 0.3 μg/kg/min)						
321	3020	1125	750	155	25	14
323	2800	2155	450	400	16	19
324	1210	920	360	300	30	33
325	1070	730	120	105	11	14
326	2250	1780	300	180	13	10
327	1850	905	420	375	23	41
328	1890	850	420	315	22	37
Mean	1701	1022	260	212	22	21
SEM	297	199	67	46	3.8	4.2
P	<0.01		<0.01		NS	

*Extraction ratio = $\frac{\text{(arterial concentration} - \text{coronary sinus concentration)} \times 100\%}{\text{arterial concentration}}$
†Values obtained 10 minutes after i.v. CPIB (20 mg/kg).
Arterial FFA concentration versus arterial-coronary sinus FFA concentration difference: $r_s = +0.76$, $P < 0.01$.

Epicardial ST-segment elevation has been reported to reflect the severity of experimentally induced myocardial ischemia,[26] and to correlate with both the local reduction in myocardial oxygen tension produced by acute coronary occlusion[27] and the subsequent depletion of myocardial creatine kinase activity during sustained occlusion.[10, 17] The latter relationship has been shown to persist under a variety of conditions, including antilipolytic therapy.[10] Since irreversible myocardial injury does not occur during the first 20 minutes of ischemia,[28] and reproducible results for ΣST were obtained in the absence of intervention, the reduction in ST-segment elevation achieved with CPIB probably indicates limitation of the acute myocardial ischemic injury.

The severity of acute myocardial ischemic injury has been shown to be influenced by factors which alter the oxygen requirements of the heart relative to oxygen supply.[17] The major determinants of myocardial oxygen requirements include the mechanical factors of contractility, heart rate, and wall tension.[29] However, the lack of any effect of CPIB on $\overline{AP}$ or HR in the present investigation renders it unlikely that there was reduction in ischemic injury due to altered mechanical activity.

An effect of CPIB on the coronary collateral circulation can be excluded from the results of the microsphere studies, which demonstrated that blood flow to both the ischemic and non-ischemic zones of the left ventricular wall was unaltered by treatment. Furthermore, the failure of CPIB to alter regional myocardial blood flow suggests that the reduction in ST-segment elevation was also unrelated to its known effects on platelet aggregation, blood clotting mechanisms, and blood viscosity.[30-32]

CPIB reduced the arterial concentration of FFA by a mean of 40%, and this was associated with a proportionate decrease in the arterial-coronary sinus difference in FFA concentration. We have shown in other studies that CPIB also reduces the myocardial uptake of radioactive FFA from plasma during a continuous infusion of albumin-bound radiolabelled palmitate (Miller, N.E., Mjøs, O.D.: un-

published observations). The association of these changes with the failure of CPIB to alter myocardial blood flow indicates a reduction in the utilization of FFA by the heart as a consequence of the fall in arterial FFA concentration. Although proportionality between the arterial concentration and myocardial extraction of FFA has been well documented in other situations,[12, 16, 33, 34] it could not have been predicted that this would apply after CPIB administration in view of reports that CPIB may displace FFA to weaker binding sites on the albumin molecule.[15] The reduction in arterial glycerol concentration suggests that the FFA-lowering effect of CPIB derived, at least in part, from an inhibition of lipolysis, in accordance with *in vitro* studies of adipose tissue metabolism.[35, 36]

In 1965 Challoner and Steinberg[37] reported that high concentrations of FFA raised the oxygen requirements of the isolated perfused rat heart. This finding was extended by Mjøs[11] who reported that raised arterial FFA concentrations increased MVO_2 in healthy dogs without influencing the mechanical activity of the heart. Furthermore, by inhibiting catecholamine-induced lipolysis with β-pyridyl-carbinol, it was shown that as much as 30% of the rise in MVO_2 induced by catecholamines was attributable to FFA, and was independent of their inotropic and chronotropic actions.[12] β-pyridyl-carbinol was subsequently shown to reduce the severity of myocardial ischemic injury, as assessed by myocardial creatine kinase depletion, during experimental coronary occlusion in dogs,[10] and it was suggested that this reflected a decrease in myocardial oxygen demand as a result of the fall in arterial FFA. The present demonstration that the reduction of myocardial FFA extraction by CPIB is associated with a decrease in epicardial ST-segment elevation in the absence of changes in hemodynamics or regional myocardial blood flow is consistent with this proposal.

In addition to their calorigenic activity, FFA have other metabolic actions which might also be expected to increase the severity of acute myocardial ischemic injury. Thus, the inhibition of glycolysis by FFA in the normally perfused heart[38] raises the possibility that they may impair carbohydrate utilization during coronary occlusion. More recently it has been proposed that FFA may augment myocardial ischemic injury by increasing the intracellular concentration of long-chain acyl CoA esters, which have been shown *in vitro* to inhibit the translocation of adenine nucleotides across the mitochondrial membrane,[39] and which are known to accumulate within the heart during experimental coronary occlusion.[40]

Acute coronary occlusion has been shown to stimulate the release of catecholamine stores within the myocardium,[41] and this would be expected to enhance the hydrolysis of intramyocardial triglyceride with a local release of FFA.[42] Studies in this laboratory have indicated that CPIB inhibits catecholamine induced lipolysis in the isolated rat heart (de Deckere, E.A.M., Mjøs, O.D., Miller, N.E: unpublished observations). Thus, any limitation of myocardial ischemic injury achieved with CPIB could have been related not only to the fall in arterial FFA concentration but also to an inhibition of intramyocardial lipolysis within the ischemic zone.

Although it seems likely that the reduction in epicardial ST-segment elevation achieved with CPIB reflected a limitation of myocardial ischemic injury, there is also the possibility that it may have reflected, at least in part, a direct effect of FFA on cellular membrane potentials. An alteration in cellular action potentials by sodium palmitate has been noted in the isolated guinea pig heart,[43] an observation which may be relevant to the possible arrhythmogenic activity of FFA.[7-9] It was not established in these studies, however, whether the effect of FFA on action potentials was direct, i.e., at membrane level, or indirect and mediated through a disturbance of cellular metabolism.[43] In the present investigation it was not possible to examine the effect of CPIB on ventricular arrhythmias, since these were uncommon, probably due to the use of an open-chest preparation and to the production of relatively small areas of myocardial ischemia.

The antilipolytic activity of CPIB in most human subjects appears to be substantially less than that in animals,[13, 14, 44-46] and the results of the present study cannot be interpreted as indicating that CPIB might similarly reduce the consequences of acute myocardial ischemia in man. However, they strengthen the proposal[47] that effective antilipolytic therapy during the early phase of AMI in man, when FFA concentrations may be increased by three-fold or more,[1, 2, 4] might be of value in aiding the survival of the ischemic cells.

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References

1. Kurien VA, Oliver MF: Serum-free-fatty acids after acute myocardial infarction and cerebral vascular occlusion. Lancet **2:** 122, 1966
2. Vetter NJ, Strange RC, Adams W, Oliver MF: Initial metabolic and hormonal response to acute myocardial infarction. Lancet **1:** 284, 1974
3. Christensen NJ, Videbaeck J: Plasma catecholamines and carbohydrate metabolism in patients with acute myocardial infarction. J Clin Invest **54:** 278, 1974
4. Oliver MF, Kurien VA, Greenwood TW: Relation between serum-free-fatty-acids and arrhythmias and death after acute myocardial infarction. Lancet **1:** 710, 1968
5. Gupta DK, Young R, Jewitt DE, Hartog M, Opie LH: Increased plasma-free-fatty-acid concentrations and their significance in patients with acute myocardial infarction. Lancet **2:** 1207, 1969
6. Rutenberg HL, Pamintuan JC, Soloff LA: Serum-free-fatty-acids and their relation to complications after acute myocardial infarction. Lancet **2:** 559, 1969
7. Kurien VA, Oliver MF: A metabolic cause of arrhythmias during acute myocardial ischaemia. Lancet **1:** 813, 1970
8. Rowe MJ, Neilson JMM, Oliver MF: Control of ventricular arrhythmias during myocardial infarction by antilipolytic treatment using a nicotinic-acid analogue. Lancet **1:** 295, 1975
9. Kurien VA, Yates PA, Oliver MF: The role of free fatty acids in the production of ventricular arrhythmias after acute coronary artery occlusion. Europ J Clin Invest **1:** 225, 1971
10. Kjekshus JK, Mjøs OD: Effect of inhibition of lipolysis on infarct size following experimental coronary artery occlusion. J Clin Invest **52:** 1770, 1973
11. Mjøs OD: Effect of free fatty acids on myocardial function and oxygen consumption in intact dogs. J Clin Invest **50:** 1386, 1971
12. Mjøs OD: Effect of inhibition of lipolysis on myocardial oxygen consumption in the presence of isoproterenol. J Clin Invest **50:** 1869, 1971
13. Barrett AM, Thorp JM: Studies on the mode of action of clofibrate: Effects on hormone-induced changes in plasma free fatty acids,

cholesterol, phospholipids and total esterified fatty acids in rats and dogs. Br J Pharmacol **32:** 381, 1968
14. Cenedella RJ, Jarrell JJ, Saxe LH: Effects of ethyl p-chlorophenoxyisobutyrate, clofibrate, on the plasma and red blood cell free fatty acids of the rat. J Atheroscler Res **8:** 903, 1968
15. Thorp JM: Assessment of clinical trials results in relation to the mode of action of clofibrate. *In* Lipid Metabolism and Atherosclerosis. Amsterdam, Excerpta Medica, 1973, p 90
16. Lassers BW, Wahlqvist ML, Kaijser L, Carlson LA: Effect of nicotinic acid on myocardial metabolism in man at rest and during exercise. J Appl Physiol **33:** 72, 1972
17. Maroko PR, Kjekshus JK, Sobel BE, Watanabe T, Covell JW, Ross J, Braunwald E: Factors influencing infarct size following experimental coronary artery occlusions. Circulation **43:** 67, 1971
18. Domenech RJ, Hoffman JIE, Noble MIM, Saunders KB, Henson JR, Subijanto S: Total and regional coronary blood flow measured by radioactive microspheres in conscious and anesthetized dogs. Circ Res **25:** 581, 1969
19. Utley J, Carlson EL, Hoffman JIE, Martinez HM, Buckberg GD: Total and regional myocardial blood flow measurements with 25μ, 15μ, 9μ, and filtered 1-10μ diameter microspheres and antipyrine in dogs and sheep. Circ Res **34:** 391, 1974
20. Dole VP: A relation between nonesterified fatty acids in plasma and the metabolism of glucose. J Clin Invest **35:** 150, 1956
21. Trout DL, Estes EH, Friedberg SJ: Titration of free fatty acids of plasma: A study of current methods and a new modification. J Lipid Res **1:** 199, 1960
22. Chernick SS: Determination of glycerol in acyl glycerols. *In* Methods in Enzymology, edited by Lowenstein JM. New York, Academic Press, 1969, 14, p 627
23. Technicon Instruments Corporation, New York. Method N-9a
24. Becker LC, Ferreira R, Thomas M: Mapping of left ventricular blood flow with radioactive microspheres in experimental coronary artery occlusion. Cardiovasc Res **7:** 391, 1973
25. Hoffbrand BI, Forsyth RP, Melmon KL: Dose-related effects of isoprenaline on the distribution of cardiac output and myocardial blood flow in conscious monkeys. Cardiovasc Res **7:** 664, 1973
26. Wegria R, Segers M, Keating RP, Ward HP: Relationship between the reduction in coronary flow and the appearance of electrocardiographic changes. Am Heart J **38:** 90, 1949
27. Angell CS, Lakatta EG, Weisfeldt ML, Shock NW: Relationship of intramyocardial oxygen tension and epicardial ST segment changes following acute coronary artery ligation: Effects of coronary perfusion pressure. Cardiovasc Res **9:** 12, 1975
28. Jennings RB: Early phase of myocardial ischemic injury and infarction. Am J Cardiol **24:** 753, 1969
29. Sonnenblick EH, Ross J, Braunwald E: Oxygen consumption of the heart. Am J Cardiol **22:** 328,1968
30. Carson P, McDonald L, Pickard S, Pilkington T, Davies B, Love F: Effect of Atromid on platelet stickiness. J Atheroscler Res **3:** 619,1963
31. Ogston D, Bennett NB, Crawford GPM, Douglas AS: The effect of clofibrate on components of the fibrinolytic enzyme system. Atherosclerosis **16:** 273,1972
32. Dormandy JA, Gutteridge JMC, Hoare E, Dormandy TL: Effect of clofibrate on blood viscosity in intermittent claudication. Br Med J **4:** 259, 1974
33. Ballard FB, Danforth WH, Neagle S, Bing RJ: Myocardial metabolism of fatty acids. J Clin Invest **39:** 717,1960
34. Scott JC, Finkelstein LJ, Spitzer JJ: Myocardial removal of free fatty acids under normal and pathological conditions. Am J Physiol **203:** 482, 1962
35. Speake RN: The effects of 'Atromid-S' on adipose tissue metabolism. Progr Biochem Pharmacol **2:** 372, 1967
36. Curtis-Prior PB: Effect of the sodium salt and ethyl ester of α-p-chlorophenoxyisobutyric acid (clofibrate) on a fat-mobilizing factor in serum. Atherosclerosis **19:** 463, 1974
37. Challoner DR, Steinberg D: Effects of free fatty acids on the oxygen consumption of perfused rat heart. Am J Physiol **210:** 280, 1966
38. Randle PJ, Newsholme EA, Garland PB: Regulation of glucose uptake by muscle. 8. Effects of fatty acids, ketone bodies and pyruvate, and of alloxan-diabetes and starvation, on the uptake and metabolic fate of glucose in rat heart and diaphragm muscle. Biochem J **93:** 652, 1964
39. Shrago E, Shug AI, Elson C, Spenneta T, Crosby C: Regulation of metabolite transport in rat and guinea pig liver mitochondria by long chain fatty acyl coenzyme A esters. J Biol Chem **249:** 5269, 1974
40. Shug AL, Shrago E, Bittar N, Folts JD, Kokes JR: Long chain CoA inhibition of adenine nucleotide translocation in the ischemic myocardium. Am J Physiol **228:** 689, 1975
41. Shahab L, Wollenberger A, Krause E-G, Genz S: The effect of acute ischemia on catecholamines and cyclic AMP levels in normal and hypertrophied myocardium. *In* Effect of Acute Ischaemia on Myocardial Function, edited by Oliver MF, Julian DG, Donald KW. Edinburgh, Churchill Livingstone, 1972, p 97
42. Christian DR, Kilscheimer GS, Pettett G, Paradise R, Ashmore J: Regulation of lipolysis in cardiac muscle: a system similar to the hormone-sensitive lipase of adipose tissue. *In* Advances in Enzyme Regulation. New York, Pergamon, 1969, pp 7, 71
43. Wasilewska-Dziubinska E: Are free fatty acids arrhythmogenic? Effects of cellular cardiac action potentials. J Molec Cell Cardiol **7:** 153, 1975
44. MacMillan DC, Oliver MF, Simpson JD, Tothill P: Effect of ethyl chlorophenoxyisobutyrate on weight, plasma volume, total body-water, and free fatty acids. Lancet **2:** 924, 1965
45. Hunninghake DB, Azarnoff DL: Clofibrate effect on catecholamine-induced metabolic changes in humans. Metabolism **17:** 588, 1968
46. Wolfe BM, Kane JP, Havel RJ, Brewster HP: Mechanism of the hypolipemic effect of clofibrate in post-absorptive man. J Clin Invest **52:** 2146, 1973
47. Oliver MF: The vulnerable ischaemic myocardium and its metabolism. *In* Modern Trends in Cardiology, ed 3, edited by Oliver MF. London, Butterworths, 1975, p 280

Appendix D

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Effects of prostaglandin-E_1 on ST segment elevation and regional myocardial blood flow during experimental myocardial ischaemia in dogs

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Abstract. The effects of prostaglandin E_1 (PGE_1) on myocardial ischaemia (as measured by epicardial ST segment changes), myocardial flow and substrate exchange has been studied in dogs. Myocardial ischaemia was induced by intermittent external clipping of a branch of the left anterior descending coronary artery.

During occlusion with a continuous intravenous infusion of isoprenaline, elevated epicardial ST segments (ΣST), were raised to 46 ± 6 mV (mean ± SEM). Pretreatment with PGE_1 reduced ΣST to 34 ± 6 mV ($P < 0.001$), but had no effect on mean aortic blood pressure ($\overline{AP}$) or on regional myocardial blood flow. Isoprenaline infusion increased plasma free fatty acids (FFA) to 1925 ± 150 μmol/l and this was reduced to 1320 ± 220 μmol/l by PGE_1 ($P < 0.005$).

During occlusion without isoprenaline, PGE_1 did not effect ΣST or plasma FFA when infused intravenously or into the left atrium. Mean aortic blood pressure decreased from 131 ± 7 to 108 ± 10 (PGE_1 i.v.) or 109 ± 8 mmHg (PGE_1 i.a.) ($P < 0.001$). This was associated with a decrease in regional myocardial blood flow, both in the ischaemic and non-ischaemic myocardium. However, when blood pressure was maintained constant, intravenous PGE_1 tended to decrease occlusion-induced ST segment elevation.

These results suggest that PGE_1 can reduce isoprenaline-stimulated myocardial ischaemia through its antilipolytic action but in the absence of catecholamine stimulation the main effect is to reduce blood pressure thereby counterbalancing any potentially beneficial effects on the ischaemic myocardium.

Key words. ST segment elevation, free fatty acids, myocardial ischaemia, blood flow, prostaglandin E_1, dog.

Introduction

Prostaglandins are naturally occurring substances with wide-ranging haemodynamic [1] and metabolic effects [2]. Prostaglandins E and A increase coronary blood flow in normal dogs [1] and it has been suggested that they might be used clinically to increase blood flow to the ischaemic myocardium [3].

In addition to increasing coronary blood flow prostaglanding E_1 (PGE_1) also reduces tissue mobilization of free fatty acids (FFA) [4-6]. This might also be desirable since marked elevation of plasma FFA concentration in patients with acute myocardial infarction has been associated with a poor immediate prognosis, ventricular arrhythmias and death [7], and it is known that reduction of raised plasma FFA concentration by antilipolytic drugs during experimental myocardial ischaemia in dogs decreases electrocardiographic signs of myocardial ischaemia [8-12] and conserves the loss of myocardial creatinine kinase (EC 2.7.3.2) activity [8].

Other haemodynamic effects of PGE_1, such as decreased peripheral vascular resistance and increased cardiac work, may counteract or even outweigh the benefits to the ischaemic myocardium of its metabolic effects. Studies have therefore been made to assess the value of PGE_1 in experimental ischaemia by measuring its effects on metabolism, epicardial electrocardiographic changes and regional myocardial blood flow in dogs.

Methods

Animal preparations

Mongrel dogs weighing between 12 and 25 kg were anaesthetized with sodium pentobarbitone (25 mg/kg, i.v.) followed by a continuous infusion of sodium pentobarbitone (approx. 4 mg/h, i.v.) or occasionally by intravenous maintenance doses of 50 mg. Ventilation was maintained with a Harvard respirator and monitored using IL313 blood gas equipment. Arterial pO_2 was 89 ± 5 mmHg, pCO_2 33 ± 2 mmHg, pH 7.44 ± 0.01.

The left and right femoral veins were cannulated for the measurement of mean aortic blood pressure ($\overline{AP}$), which was monitored using a Statham Instruments pressure transducer (P23 Db) and recorded on a Devices Instruments M8 recorder.

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The heart was exposed through a left thoracotomy and suspended in a pericardial cradle. Care was taken not to obstruct the caval veins. A distal branch of the left anterior descending (LAD) coronary artery was dissected free over a distance of 0.5-1.0 cm. The left atrium was cannulated using a PE90 catheter for atrial infusions of PGE_1, since PGE_1 is up to 90% eliminated by passage through the pulmonary circulation [1].

Electrocardiographic recordings were performed with a mobile cotton wick electrode at ten to fifteen anatomically well-defined sites on the epicardial surface [13] and recorded with a sensitivity of 1 mV/mm deflection and a paper speed of 25 mm/s on a Devices Instruments M8 recorder. A 10 mV signal was used for calibration. The results were expressed as the sum of ST segment elevations of all sites (ΣST), and were corrected for ST segment elevation if any existed prior to the coronary artery occlusion.

Regional myocardial blood flow was measured according to the method of Utley *et al.* [14] as described previously [12]. Radioactive microspheres, 15 μm nominal mean diameter, labelled with ^{85}Sr, ^{141}Ce or ^{125}I (3M Company Ltd, Loughborough, U.K.) were used. In recent experiments, microspheres labelled with ^{169}Yb were used in preference to ^{125}I. Between 5 and 30 min after the last injection the dogs were killed with an intravenous overdose of sodium pentobarbitone. The heart was excised, the free wall of the left ventricle dissected out, visual fat and large vessels removed. Between ten and fourteen full thickness sections were made and separated into epicardial and endocardial layers of wet weight 1.5-4.0 g. Each section was then cut into ten to twenty-blocks to produce relatively constant geometric factors for the measurement of radioactivity. By comparison with the radioactivity collected in a reference arterial blood sample withdrawn at a known fixed rate, absolute flow was calculated. The sections were allocated to one of two groups: (1) normal myocardium; (2) peripheral plus central ischaemic myocardium. The results were expressed as the mean regional blood flow in the ischaemic area. Estimations of regional myocardial blood flow were made with differently labelled microspheres used at random in order to avoid systematic errors.

A different preparation was used in four dogs whereby heart rate, left ventricular end diastolic pressure (LVEDP) and mean aortic pressure could be kept constant [15]. The heart was exposed by a mid-sternal split. The left atrium was cannulated and connected to a reservoir. LVEDP was computed on-line using an analogue computer and maintained constant by varying the height of the fluid column in the left atrial reservoir. Mean aortic blood pressure was kept constant using a pressurized flask connected to both femoral arteries.

If changes in peripheral resistance occurred, equilibrium between two reservoirs was maintained by pumping blood from one to the other with a Watson-Marlow MHRE roller pump. A water jacket and a heated, siliconized glass coil in the reservoir were used to maintain the fluid in the system near 37°C. The system was primed with a solution of 2.5% w/v dextran, which was allowed to mix with the blood in the dog in order to minimize later changes in haematocrit during the observation periods. Right atrial pacing was used to keep the heart rate constant. Arterial pressure was measured through a carotid arterial cannula. Left ventricular pressure was measured using a metal cannula inserted through the dimple of the apex and directly connected to the pressure transducer (L223, Consolidated Electrodynamics, England). The frequency response of the overall system was flat within 5% at 40 Hz. dP/dt max. was derived by the analogue computer. The initial values of heart rate (HR), $\overline{AP}$ and LVEDP were chosen close to those observed prior to the first infusion and then maintained constant during the observation periods. After release of the occlusion HR, LVEDP and $\overline{AP}$ were no longer controlled.

PGE_1 dissolved in methanol was stored at −30°C. Prior to the administration the methanol was evaporated under a stream of nitrogen at room temperature and PGE_1 was immediately dissolved in 150 mmol/l sodium chloride solution.

Isoprenaline was freshly prepared in 150 mmol/l sodium chloride.

Plasma FFA concentrations were determined from femoral arterial plasma according to the titrimetric method of Trout *et al.* [16]. Arterial plasma glucose was determined using an automated technique using glucose oxidase [17], and arterial plasma free glycerol by an enzymatic method [18].

Experimental procedures

The basic design was to induce two or three discrete periods of myocardial ischaemia each of 10-14 min duration, separated by a recovery period of 30 min. Irreversible damage does not occur within the first 20 min of ischaemia [19]. The degree of ST segment elevation during three successive occlusions of the same artery has been shown to be reproducible, provided a recovery period of 30 min is allowed [8, 12, 13]. This design permits comparison of ischaemic electrocardiographic changes under different experimental conditions in the same animal. Three series of experiments were performed.

In the first series (Procedure 1, Fig. 1) the effect of PGE_1 was studied on ΣST segment elevation during lipolysis stimulated by a continuous infusion of isoprenaline in nine dogs (dogs 1-9). Isoprenaline 0.1-0.2 $\mu g\ kg^{-1}\ min^{-1}$ i.v. was commenced 5 min prior to the control occlusion and maintained throughout. At 10 min of ischaemia the epicardial electrocardiogram, HR and $\overline{AP}$ were recorded and microspheres injected into the left atrium for measurement of regional myocardial blood flow. An arterial blood sample was taken for the determination of plasma FFA concentration. The occlusion was released and the isoprenaline infusion stopped. After a 30 min recovery period an infusion of PGE_1 0.6 $\mu g\ kg^{-1}min^{-1}$ i.v. was given, followed 5 min later by an isoprenaline infusion. Both infusions were maintained

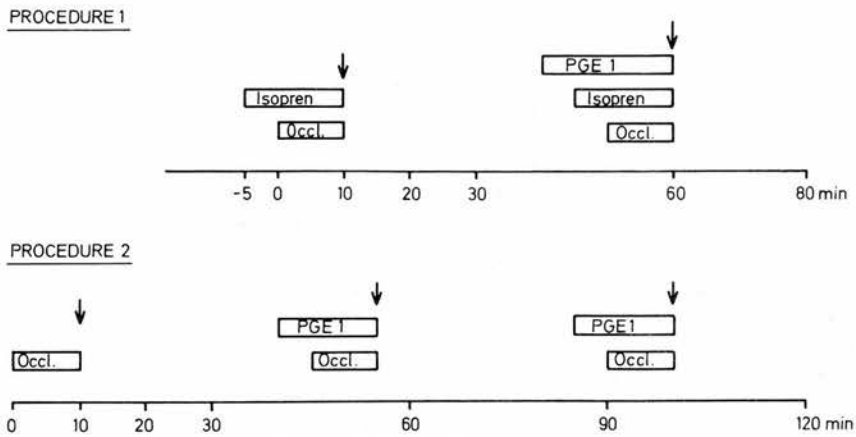

Figure 1. Protocol for the study of the effect of PGE_1 on ST segment elevation during isoprenaline-stimulated and basal lipolysis. The basic experimental design consisted of two or three intermittent occlusions (Occl.) of the terminal branch of the left anterior descending coronary artery of 10–14 min duration. Prior to release, recordings of epicardial electrocardiograms of ten to fifteen anatomically well defined sites were made, and an arterial blood sample was obtained for biochemical parameters (arrows). After release a 30 min recovery period was allowed for ST segments to revert to normal.

Procedure 1 was used to study the effect of PGE_1 (0.6 μg kg^{-1} min^{-1} i.v.) during isoprenaline (0.1–0.2 μg kg^{-1} min^{-1} i.v.) stimulated lipolysis. Regional myocardial blood flow was determined by injection of $1.5-3.0 \times 10^6$ microspheres into the left atrium immediately before the release of the occlusions.

Procedure 2 was used to study the effect of PGE_1, either infused into the left atrium (0.03 μg kg^{-1} min^{-1}) or intravenously (0.6 μg kg^{-1} min^{-1}) during basal lipolysis. Using three differently labelled radioactive microspheres, regional myocardial blood flow was estimated immediately prior to release of each occlusion.

during this occlusion. All measurements: epicardial ECG (ΣST), HR, AP and regional myocardial blood flow were repeated (Fig. 1).

In two experiments (dogs 7 and 9) the sequence was reversed, isoprenaline and PGE_1 being infused during the first occlusion period. After release and recovery, isoprenaline alone was infused for the second occlusion period. This was done in order to check whether the effects of PGE_1 were related to the time of administration.

In the second series (Procedure 2, Fig. 1) the effect of PGE_1, administered intravenously (0.6 μg kg^{-1} min^{-1}) or intra-atrially (0.03 μg kg^{-1} min^{-1}) was studied in eight dogs (dogs 10–17). Three occlusions of approximately 10 min duration were made as described above. The first occlusion served as a control. The other occlusions were made 5 min after the start of either a continuous PGE_1 infusion into the femoral vein or into the left atrium. The infusion of PGE_1 into the left atrium was made in order to check whether PGE_1 or its metabolites were the active components. Normally up to 90% of PGE_1 is removed on a single passage through the lungs. The order in which the PGE_1 infusions were administered was randomized. ΣST segment elevation, HR and $\overline{AP}$ were measured immediately prior to the release of the occlusion. In five dogs, microspheres were injected for measurement of regional myocardial blood flow after 10 min of occlusion during each of the three experimental occlusions described above. Isoprenaline was not given to dogs in the second series.

In the third series the effect of intravenous PGE_1 (0.6 μg kg^{-1} min^{-1}) was studied in four dogs (dogs 18–21), whilst HR, $\overline{AP}$ and LVEDP were maintained constant. Two occlusions of approximately 10 min duration were made as described above (Procedure 2, Fig. 1), each 5 min after the start of a continuous infusion of either PGE_1 or saline. The order of these infusions was randomized. Epicardial ECG (ΣST segment elevation) HR, $\overline{AP}$, LVEDP and LV dP/dt max were measured immediately before and at 5 min intervals during the occlusion. Arterial (carotid artery) blood samples were obtained for the estimation of plasma FFA and free glycerol.

Statistics

Each dog served as its own control. Student's *t*-test for paired data was used to calculate probability values.

Results

The effects of PGE_1 during isoprenaline infusions

After the occlusion of a distal branch of the left anterior descending coronary artery, the onset of acute myocardial ischaemia was characterized by localized cyanosis, contractile asynergy and ST segment elevation in the distribution of the ligated artery.

When the induction of myocardial ischaemia was made in the presence of isoprenaline ΣST segment elevation averaged 46 ± 6 mV (mean ± SEM) after 10 min of occlusion (Table 1). When the isoprenaline infusion was preceded by a continuous infusion of PGE_1 ΣST averaged 34 ± 6 mV ($P < 0.001$). This effect of PGE_1 was independent of the sequence of administration. The data from all dogs were pooled.

The lipolytic activity of isoprenaline was apparent

Table 1. The effect of PGE_1 i.v. on ischaemia and isoprenaline-induced ST segment elevation, mean aortic pressure, heart rate and arterial plasma FFA concentrations during experimental myocardial ischaemia of 10 min duration

	ΣST (mV)		FFA (μmol/l)		$\overline{AP}$ (mmHg)		HR (beats/min)	
Dog No.	Occl. + Isopr.	Occl. + Isopr. + PGE_1	Occl. + Isopr.	Occl. + Isopr. + PGE_1	Occl. + Isopr.	Occl. + Isopr. + PGE_1	Occl. + Isopr.	Occl. + Isopr. + PGE_1
1	38	27	2100	1190	103	90	175	172
2	36	25	1290	520	69	55	172	151
3	42	35	1860	1550	103	109	195	189
4†	21	14	2650	2550	73	81	150	150
5	36	24	2520	2040	85	72	164	146
6‡	78	62	2065	790	110	96	169	164
7‡	74	62	1650	1070	130	130	173	175
8‡	53	36	1600	750	105	88	142	141
9‡	39	25	1580	1400	106	106	147	140
Mean	46	34	1925	1320	98	92	165	159
± SEM	6	6	150	220	6	7	6	6
P*	< 0.001		< 0.005		NS		< 0.05	

Isoprenaline was administered intravenously at a dose of 0.1–0.2 μg kg^{-1} min^{-1}. PGE_1 at 0.6 μg kg^{-1} min^{-1}.

In dogs 1–6 and 8 the first occlusion was made during isoprenaline infusion, followed by an occlusion made during an isoprenaline plus PGE_1 infusion, whereas in dog 7 and 9 the first occlusion was made during an isoprenaline plus PGE_1 infusion, followed by an occlusion during an infusion of isoprenaline alone.

* Probability values for comparison of paired data of occlusion + isoprenaline vs. occlusion + isoprenaline + PGE_1. NS = not significant ($P > 0.05$).

† Dose of PGE_1 = 0.3 μg kg^{-1} min^{-1}.

‡ Regional myocardial blood flow estimation (see Table 2).

from the high plasma FFA concentrations (1925 ± 150 μmol/l). This was partly inhibited when PGE_1 was simultaneously administered (1320 ± 220 μmol/l: $P < 0.005$) (Table 1).

The intravenous administration of PGE_1 did not significantly alter $\overline{AP}$ during isoprenaline infusions, but a small and significant decrease in HR was observed (Table 1). PGE_1 administration had no effect on regional myocardial blood flow in the endocardial and epicardial layers of the ischaemic and non-ischaemic part of the left ventricle during the isoprenaline infusions (Table 2).

The reproducibility of the effects of isoprenaline was good in separate control experiments. In ten comparisons of two consecutive occlusions ΣST was 46 ± 12 mV (first occlusion) 47 ± 12 mV (second occlusion): $P > 0.30$.

Table 2. The effect of PGE_1 i.v. on regional myocardial blood flow in non-ischaemic and central plus peripheral ischaemic left ventricle during continuous isoprenaline infusion in four dogs with experimental acute myocardial ischaemia of 10 min duration

	Non-ischaemic			Ischaemic		
	Endocardium (ml g^{-1} min^{-1})	Epicardium (ml g^{-1} min^{-1})	Endocardial/epicardial ratio	Endocardium (ml g^{-1} min^{-1})	Epicardium (ml g^{-1} min^{-1})	Endocardial/epicardial ratio
Occlusion + isoprenaline	1.29 ± 0.06 (24)	1.45 ± 0.05 (24)	0.89 ± 0.03 (24)	0.82 ± 0.08 (20)	1.08 ± 0.09 (20)	0.76 ± 0.04 (20)
Occlusion + isoprenaline + PGE_1 i.v.	1.32 ± 0.06 (24)	1.47 ± 0.05 (24)	0.90 ± 0.03 (24)	0.84 ± 0.09 (20)	1.08 ± 0.10 (20)	0.78 ± 0.04 (20)
P*	NS	NS	NS	NS	NS	NS

* Probability values for comparison of paired data of occlusion + isoprenaline vs. occlusion + isoprenaline + PGE_1 i.v. NS = not significant ($P > 0.05$).

Table 3. The effect of PGE_1 i.v. or i.a. on ischaemia-induced ST segment elevation, mean aortic pressure, heart rate and arterial plasma FFA concentrations during experimental myocardial ischaemia of 10 min duration

	ΣST (mV)			FFA (μmol/l)			$\overline{AP}$ (mmHg)			HR (beats/min)		
Dog No.	Occl.	Occl. + PGE_1 i.v.	Occl. + PGE_1 i.a.	Occl.	Occl. + PGE_1 i.v.	Occl. + PGE_1 i.a.	Occl.	Occl. + PGE_1 i.v.	Occl. + PGE_1 i.a.	Occl.	Occl. + PGE_1 i.v.	Occl. + PGE_1 i.a.
10	15	11	–	780	700	–	145	125	–	168	175	–
11	27	24	21	640	600	660	100	75	80	132	125	128
12	23	29	31	500	520	400	147	133	133	159	164	160
13†	8	–	8	400	–	460	135	–	124	–	–	–
14†	3	8	6	350	370	270	155	123	125	–	–	–
15†	27	9	19	370	440	565	115	88	86	190	182	184
16†	13	6	8	1050	–	880	112	97	100	218	220	224
17†	24	–	16	410	–	440	141	–	118	186	–	180
Mean	17	14	15	560	525	525	131	108	109	176	173	175
± SEM	3	4	3	85	60	75	7	10	8	12	15	16
*P**		NS	NS		NS	NS		< 0.001	<0.001		NS	NS

The first occlusion was a control occlusion. In dogs 11, 12 and 15 the second occlusion was made during an intravenous infusion of PGE_1, followed by a third occlusion during an intra-atrial infusion of PGE_1. In dogs 13, 14, 16 and 17 the second occlusion was made during an intra-atrial infusion of PGE_1, followed by a third occlusion during intravenous infusion of PGE_1 (dogs 14 and 16).

* Probability value for comparison of paired data vs. occlusion. NS = not significant ($P > 0.05$).

† Regional myocardial blood flow estimation (see Table 4).

Effects of PGE_1 infusions alone.

In the absence of isoprenaline, PGE_1 did not reduce plasma FFA concentrations (Table 3), or alter plasma free glycerol and glucose concentrations.

The haemodynamic effects of PGE_1 alone, were also different from those observed during isoprenaline infusions. PGE_1 alone did not change HR. PGE_1 reduced $\overline{AP}$ significantly, irrespective of which route was used (Table 3). This drop in $\overline{AP}$ was associated with a marked reduction in regional myocardial blood flow in epicardial and endocardial layers of the non-ischaemic myocardium (Table 4). The reduction was slightly more marked in the endocardial layers of the non-ischaemic myocardium, giving rise to a significant decrease in the endocardial-epicardial blood flow ratio ($P < 0.005$). PGE_1 (both i.v. and i.a.) also reduced myocardial blood flow in the ischaemic epi- and endocardial layers ($P < 0.005$), and the reduction was possibly more pronounced in the endocardial layers. However, no change was observed in

Table 4. The effect of PGE_1 i.v. or i.a. on regional myocardial blood flow in non-ischaemic and central plus peripheral ischaemic left ventricle in five dogs with experimental acute myocardial ischaemia of 10 min duration

	Non-ischaemic			Ischaemic		
	Endocardium (ml g^{-1} min^{-1})	Epicardium (ml g^{-1} min^{-1})	Endocardial/epicardial ratio	Endocardium (ml g^{-1} min^{-1})	Epicardium (ml g^{-1} min^{-1})	Endocardial/epicardial ratio
Control occlusion	1.76 ± 0.08 (42)	1.62 ± 0.07 (42)	1.09 ± 0.02 (42)	0.95 ± 0.13 (20)	1.07 ± 0.11 (19)	0.85 ± 0.08 (20)
Occlusion + PGE_1 i.v.	1.12 ± 0.10 (17)	1.15 ± 0.09 (17)	0.96 ± 0.02 (17)	0.56 ± 0.13 (8)	0.74 ± 0.16 (8)	0.78 ± 0.10 (8)
*P**	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.05
Occlusion + PGE_1 i.a.	1.18 ± 0.04 (33)	0.99 ± 0.03 (33)	1.02 ± 0.02 (33)	0.54 ± 0.08 (16)	0.71 ± 0.08 (16)	0.73 ± 0.07 (16)
*P**	< 0.001	< 0.001	< 0.01	< 0.005	< 0.001	NS

Results are expressed as mean ± SEM (number of observations).

* Probability value of comparison of paired data vs. control. NS = not significant ($P > 0.05$).

Table 5. The electrocardiographic, haemodynamic and metabolic effects of PGE_1 i.v. during coronary artery occlusion under conditions of controlled preload, afterload and heart rate

	ΣST (mV)		dP/dt max. (mmHg s^{-1})		$\overline{AP}$ (mmHg)		LVEDP (mmHg)		FFA (μmol/l)	
Dog No.	Occl.	Occl. + PGE_1	Occl.	Occl. + PGE_1	Occl.	Occl. + PGE_1	Occl.	Occl. + PGE_1	Occl.	Occl. + PGE_1
18	148	136	3520	3720	96	84	3.9	3.0	360	260
19*	202	155	1250	3300	67	68	2.0	2.5	360	460
20*	151	122	3060	2790	80	83	9.0	8.9	410	600
21*	59	74	1750	1740	77	73	5.3	5.5	455	520

* In dog 18 an occlusion of 10 min duration was made during an infusion of saline followed by a second occlusion of 10 min during an infusion of PGE_1, whereas in dogs 19, 20 and 21 the first occlusion was made during infusion of PGE_1 and the second occlusion during the infusion of saline.

ST segment elevation after 10 min of occlusion (Table 3). Therefore the studies were extended to another series in which the blood pressure as well as HR and LVEDP were controlled. Under these conditions PGE_1 i.v. reduced ΣST in three out of four experiments. This effect was not related to plasma FFA concentrations, which tended to increase.

Discussion

The effect of PGE_1 during isoprenaline infusions

During experimental myocardial ischaemia under conditions of high adrenergic drive (isoprenaline infusions), PGE_1 reduced ΣST segment elevation. Epicardial ST segment elevation reflects the severity of acute myocardial ischaemia [20] and is inversely related to myocardial oxygen tension [21].

Arterial plasma FFA concentrations were lowered by PGE_1 due presumably to its inhibitory effect on the lipolytic activity of isoprenaline. This is likely to have led to reduced myocardial extraction of FFA. Arterial-coronary venous differences were not measured but proportionality between arterial concentration and myocardial extraction has previously been found [23-25]. Reduced plasma FFA and myocardial FFA uptake leads to reduced myocardial oxygen consumption (MVO_2) when the lipolytic action of isoprenaline is blocked by an antilipolytic agent [26]. In the presence of acute myocardial ischaemia, such reduction of MVO_2 would be expected to be associated with reduced signs of ischaemia, such as ΣST. Reduction of FFA uptake may also reduce their accumulation as long-chain acyl-CoA esters [27]. *In vitro* these inhibit adenine nucleotide translocase and impede transport of ADP across the inner mitochondrial membrane [28, 29]. A decrease in long-chain acyl CoA esters in the ischaemic myocardium might facilitate ADP transport and restore oxidative metabolism resulting in decrease ST segment elevation. Whatever the explanation, these effects of PGE_1 on induced myocardial ischaemia in the presence of isoprenaline are consistent with those previously reported for other antilipolytic drugs [8-12] and for lipid-free albumin [30].

Factors changing myocardial oxygen requirements relative to oxygen supply, such as heart rate and blood pressure, also influence the extent of ST segment elevation [13, 22]. In the present study $\overline{AP}$ was unchanged, and the small reduction in heart rate which was observed seems too small to account for the reduction in ST segment elevation.

Direct effects of PGE_1 have also to be considered. ST segment elevation can occur when resting membrane potential is reduced [31]. PGE_1 had no effect on resting membrane potential in mammalian, well oxygenated myocardial cells and, when used in very high concentrations, caused a decrease [32]. This would tend to increase ST segment elevation. Since PGE_1 reduced ΣST, it seems unlikely that an effect on the resting membrane potential could explain our findings.

The effect of PGE_1 in the absence of isoprenaline

During experimental myocardial ischaemia but in the absence of isoprenaline, the effect of PGE_1 on occlusion-induced ST segment elevation was dependent on the experimental conditions. In the main series of experiments during basal lipolysis, PGE_1 effected no change in occlusion-induced ST segment elevation. But a marked reduction was observed in $\overline{AP}$ and regional myocardial blood flow both in the ischaemic and non-ischaemic areas of the myocardium. Similar haemodynamic effects were reported while this study was in progress when PGE_1 was given to cats with acute myocardial ischaemia [33], but ST segment measurements were not made. Since hypotension has been shown to increase occlusion-induced ST segment elevation [13, 22] the observation of unaltered ST segments by PGE_1 needs comment. It cannot be explained by increased myocardial uptake of FFA since plasma concentrations of FFA were unaltered by PGE_1. A possible explanation was that the expected increase in ΣST due to hypotension was counteracted by some opposing influence. In studies conducted with con-

trolled $\overline{AP}$ and LVEDP, PGE_1 reduced ST segment elevation. While these studies do not explain the nature of this opposite action, they appear to exclude mechanisms based on changes in FFA metabolism.

Patients with acute myocardial infarction have increased adrenergic drive. During nervous stimulation and hypoxia endogenous prostaglandins are released and are thought to prevent excessive stimulation of the myocardium by noradrenaline.

In the present study pharmacological doses of PGE_1 were used and due to its side effects PGE_1 may not be useful to reduce myocardial infarct size in man.

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References

1 Horton E.W. & Ungar A. (1975) Prostaglandins and the cardiovascular system. *Modern Trends in Cardiology–3* (ed. by M. F. Oliver), pp. 67–68. Butterworths, London.
2 Bergstrøm S., Carlson L.A. & Weeks, J.R. (1968) The prostaglandins: a family of biologically active lipids. *Pharmacol Rev* **20**, 1–48.
3 Bloor C.M., Waite F.C. & Sobel B.E. (1973) Coronary and systemic haemodynamic effects of prostaglandins in the unanaesthetized dog. *Cardiovasc Res* **7**, 156–166.
4 Steinberg D., Vaughan M., Nestel P.J. & Bergstrøm S. (1963) Effects of prostaglandin E opposing those of catecholamines on blood pressure and on triglyceride breakdown in adipose tissue. *Biochem Pharmacol* **12**, 764–766.
5 Bergstrøm S., Carlson L.A. & Orø L. (1964) Effect of prostaglandins on catecholamine induced changes in the free fatty acids of plasma and in blood pressure in the dog. Prostaglandin and related factors. *Acta Physiol Scand* **60**, 170–180.
6 Steinberg D., Vaughan M., Nestel P.J., Strand O. & Bergstrøm S. (1964) Effects of the prostaglandins on hormone-induced mobilization of free fatty acids. *J Clin Invest* **43**, 1533–1540.
7 Oliver M.F. (1972) Metabolic response during impending myocardial infarction. II. Clinical implications. *Circulation* **45**, 491–500.
8 Kjekshus J.K. & Mjøs O.D. (1973) Effect of inhibition of lipolysis on infarct size after experimental coronary artery occlusion. *J Clin Invest* **52**, 1770–1778.
9 Lekven J., Kjekshus J.K. & Mjøs O.D. (1973) Effects of glucagon and isoproterenol on severity of acute myocardial ischemic injury. *Scand J Clin Lab Invest* **32**, 129–137.
10 Mjøs O.D., Kjekshus J.K. & Lekven J. (1974) Importance of free fatty acids as a determinant of myocardial oxygen consumption and myocardial ischemic injury during norepinephrine infusion in dogs. *J Clin Invest* **53**, 1290–1299.
11 Ilebekk A. & Mjøs O.D. (1974) Effect of nicotine on severity of acute myocardial ischemic injury in dogs. *Scand J Clin Lab Invest* **33**, 145–151.
12 Mjøs O.D., Miller N.E., Riemersma R.A. & Oliver M.F. (1976) Effects of p-chlorophenoxyisobutyrate on myocardial free fatty acid extraction, ventricular blood flow, and epicardial ST-segment elevation during coronary occlusion in dogs. *Circulation* **53**, 494–500.
13 Maroko P.R., Kjekshus J.K., Sobel B.E., Watanabe T., Covell J.W., Ross J. & Braunwald E. (1971) Factors influencing infarct size following experimental coronary artery occlusions. *Circulation* **43**, 67–82.
14 Utley J., Carlson E.L., Hoffman J.I.E., Martinez H.M. & Buckberg G.D. (1974) Total and regional myocardial blood flow measurements with 25μ, 15μ, 9μ, and filtered 1–10μ diameter microspheres and antipyrine in dogs and sheep. *Circ Res* **34**, 391–405.
15 Kane K.A. & Ungar A. (1976) Divergent changes in the rate of development of pressure in the left ventricle and in the performance of the heart as a pump. *Q J Exp Physiol* **61**, 35–44.
16 Trout D.L., Estes E.H. & Friedberg S.J. (1960) Titration of free fatty acids of plasma: a study of current methods and a new modification. *J Lipid Res* **1**, 199–202.
17 Nimmo I.A., Kirby B.J. & Lassers B.W. (1973) Rapid oscillations in blood glucose and plasma free fatty acid concentrations in man. *J Appl Physiol* **34**, 448–451.
18 Chernick S.S. (1969) Determination of glycerol in acyl glycerols. *Methods in Enzymology–14* (ed. by J. M. Lowenstein), pp. 627–630. Academic Press, New York.
19 Jennings R.B. (1969) Early phase of myocardial ischemic injury and infarction. *Am J Cardiol* **24**, 753–765.
20 Wegria R., Segers M., Keating R.P. & Ward H.P. (1949) Relationship between the reduction in coronary flow and the appearance of electrocardiographic changes. *Am Heart J* **38**, 90–96.
21 Angell C.S., Lakatta E.G., Weisfeldt M.L. & Shock N.W. (1975) Relationship of intramyocardial oxygen tension and epicardial ST segment changes following acute coronary artery ligation: Effects of coronary perfusion pressure. *Cardiovasc Res* **9**, 12–18.
22 Redwood D.R., Smith E.R. & Epstein S.E. (1972) Coronary artery occlusion in the conscious dog. Effects of alterations in heart rate and arterial pressure on the degree of myocardial ischaemia. *Circulation* **46**, 323–332.
23 Ballard F.B., Danforth W.H., Neagle S. & Bing R.J. (1960) Myocardial metabolism of fatty acids. *J Clin Invest* **39**, 717–723.
24 Carlsten A., Hallgren B., Jagenburg R., Svanborg A. & Werkø L. (1961) Myocardial metabolism of glucose, lactic acid, amino acids and fatty acids in healthy human individuals at rest and at different work loads. *Scand J Clin Lab Invest* **13**, 418–428.
25 Scott J.C., Finkelstein L.J. & Spitzer J.J. (1962) Myocardial removal of free fatty acids under normal and pathological conditions. *Am J Physiol* **203**, 482–486.
26 Mjøs O.D. (1971) Effect of inhibition of lipolysis on myocardial oxygen consumption in the presence of isoproternol. *J Clin Invest* **50**, 1869–1873.
27 Shug A.L., Shrago E., Bittar N., Folts J.D. & Koke J.R. (1975) Acyl-CoA inhibition of adenine nucleotide translocation in ischemic myocardium. *Am J Physiol* **228**, 689–692.
28 Pande S.V. & Blanchaer M.C. (1971) Reversible inhibition of mitochondrial adenosine diphosphate phosphorylation by long chain acyl CoA esters. *J Biol Chem* **246**, 402–411.
29 Shug A.L. & Shrago E. (1973) A proposed mechanism for fatty acid effects on energy metabolism of the heart. *J Lab Clin Med* **81**, 214–218.
30 Miller N.E., Mjøs O.D. & Oliver M.F. (1976) Reduction of myocardial free fatty acid extraction and epicardial ST segment elevation by albumin infusion during coronary occlusion in dogs. *Clin Sci Molec Med* **51**, 209–213.
31 Prinzmetal M., Toyoshima E., Ekmekci A., Mizano Y. & Nagaya T. (1961) Myocardial ischaemia. Nature of ischemic electrographic patterns in the mammalian ventricles as determined by intracellular electrographic and metabolic changes. *Am J Cardiol* **8**, 493–503.
32 Kecsmeti V., Keleman K. & Knoll, J. (1974) Microelectrophysiological analysis of the cardiac effects of prostaglandins E_2. *Pol Pharmacol Pharm* **26**, 171–176.
33 Hutton I., Parratt J.R. & Lawrie T.D.V. (1973) Cardiovascular effects of prostaglandin E_1 in experimental myocardial infarction. *Cardiovasc Res* **7**, 149–155.

Appendix E

EFFECT OF MYOCARDIAL ISCHAEMIA AND ANTILIPOLYTIC AGENTS ON LIPOLYSIS AND FATTY ACID METABOLISM IN THE IN SITU DOG HEART

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Key words: fatty acids, glycerol, isoprenaline, lipolysis, metabolism, myocardial ischaemia, nicotinic acid, sodium salicylate.

Running title: Myocardial ischaemia and antilipolytic agents.

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SUMMARY

Myocardial metabolism was studied in open-chest dogs before and during induction of myocardial ischaemia by coronary artery occlusion. Blood was sampled from a local coronary vein draining ischaemic tissue and from coronary sinus draining predominantly non-ischaemic tissue. In the basal state, induction of myocardial ischaemia stimulated myocardial lipolysis as shown by release of glycerol from the ischaemic zone. During isoprenaline infusion, free fatty acids (FFA) extraction across the ischaemic myocardium was substantially increased, but no glycerol release occurred.

Pretreatment with nicotinic acid or sodium salicylate markedly depressed FFA extraction across ischaemic myocardium both during basal and isoprenaline stimulated lipolysis and nicotinic acid most likely inhibited lipolysis in the ischaemic zone. Thus, reduced severity of acute ischaemic injury by antilipolytic treatment might be due to a combination of inhibited myocardial lipolysis and reduced FFA extraction.

INTRODUCTION

High supply of free fatty acids (FFA) to the ischaemic myocardium has been claimed to increase infarct size (25) and mortality (22) in patients with acute myocardial infarction. Antilipolytic treatment has recently been shown to reduce the ischaemic injury in these patients (11, 30) and to reduce the exercise-induced ST-segment depression in patients with angina pectoris (15). In experimental studies elevated plasma FFA concentrations enhance myocardial oxygen consumption (3, 17), suggesting that the deleterious effect of FFA during myocardial ischaemia may reflect an increased oxygen requirement in ischaemic tissue. Accordingly, during coronary artery occlusion the increased myocardial damage following catecholamine infusion is substantially limited when the rise in arterial FFA concentration is inhibited by β-pyridylacarbinol (12), p-chlorophenoxyisobutyrate (19), prostaglandin E_1 (29) or salicylates (39). In vitro experiments further suggest that infusion of catecholamines (6, 37, 42) or induction of myocardial ischaemia per se (9) might activate myocardial lipolytic activity with consequent hydrolysis of endogenous triglycerides. The beneficial effect of antilipolytic agents on myocardial ischaemia might therefore in part be due to inhibition of local release of FFA within the ischaemic tissue.

In the present study, the influence of arterial FFA availability on substrate exchange in ischaemic and non-ischaemic myocardium was investigated in open-chest, anaesthetized dogs following coronary artery occlusion. This was accomplished by measurement of arterio-venous differences of FFA, glucose and lactate across ischaemic and non-ischaemic myocardium both during basal and

isoprenaline-stimulated lipolysis, as well as after pretreatment with the antilipolytic agents nicotinic acid or sodium salicylate. Furthermore, since glycerol is not utilised in the myocardium to any significant amount (14, 20, 31), the possible role of endogenous lipolysis in the myocardium was studied by measurement of glycerol exchange.

METHODS

Animal Preparation:

Studies were performed on 20 overnight-fasted mongrel dogs of either sex on average 22 kg body weight (b.w.). They were anaesthetised with sodium pentobarbital, 25 mg/kg b.w., intravenously (i.v.), and followed by continuous infusion of 3.5 mg/h kg b.w. The dogs were ventilated with a volume controlled respirator (Servo Ventillator 900, Elema-Schönander AB, Solna, Sweden) through a cuffed endotracheal tube. A thoracomtomy was performed through the fifth left intercostal space. The pericardium was incised, and the heart suspended in a cradle. Care was taken not to obstruct central veins. A branch of the left anterior descending coronary artery was dissected for about 0.5 cm, and left in situ with a ligature placed loosely around it. Intermittent myocardial ischaemia was induced by coronary artery occlusion using a releasable metal clip.

A plastic catheter was inserted into a local vein draining the area supplied by the dissected coronary artery, and kept patent by flushing with a dilute heparinised saline solution (1 i.u./ml).

The coronary sinus was catheterised with a plastic catheter introduced through a jugular vein in 14 of the animals. At least 30 min recovery period was allowed from the end of surgical procedure and introduction of catheters to the first blood sampling.

Both femoral veins were cannulated for intravenous infusions. Saline (0.9%), at a rate of 10 ml/h kg b.w. i.v., was given throughout the experimental period.

Haemodynamic measurements:

Aortic blood pressure (AP) was recorded through a catheter introduced from a femoral artery with a Statham P23 Gb pressure transducer (Statham Instruments, Inc., Oxnard, Calif., USA). A short catheter through the ventricular apex permitted measurement of left ventricular pressure. The first derivative of left ventricular pressure with regard to time (LV dP/dt) was recorded from that pressure channel. LVSP, LVdP/dt, AP and heart rate (HR) were recorded on a Dynograph recorder (Type 411, Beckman Instruments, Inc., Schiller Park, Ill., USA).

Epicardial ST-segment mapping:

Epicardial electrocardiograms (ECG) were recorded using a cotton-wick electrode immediately before and 12 min after coronary artery occlusion from 10-15 anatomically well recognisable sites within and around the area supplied by the occluded artery, as described by Maroko et al. (16). The epicardial ECG was recorded with the limb leads serving as the ground electrode, and recorded with a sensitivity of 1 mV/mm and a paper speed of 25 mm/sec on a Dynograph recorder. The sum of all occlusion induced ST-segment elevations (ΣST, mV) and the number of sites at which ST-segment elevation exceeded 2 mV were used as an index of the severity of myocardial ischaemia in each dog (16, 32).

Metabolic measurements:

Myocardial metabolism was assessed by arterio-venous differences. Blood was drawn simultaneously from the aorta, coronary sinus and local coronary vein into heparinised syringes and immediately centrifuged at 4^{o}C. Plasma FFA concentrations were estimated

by a radio-chemical method (8), glucose concentrations by a glucose oxidase method using a commercial kit (Boehringer Mannheim GmbH, Mannheim, Germany), glycerol by an enzymatic method (4), and lactate concentrations according to the method of Passonneau (28). Packed cell volumes were determined by a micro-haematocrit centrifuge, and the oxygen saturations were measured spectrophotometrically (The Oxygen Saturation Meter, type OSM 1, Radiometer, Copenhagen, Denmark).

All analyses were performed in triplicate. The coefficients of variations for the assays are: FFA 3%, glucose 2%, glycerol 3% and lactate 6%.

Experimental procedure:

Experiments were carried out both under basal conditions and during continuous i.v. infusion of isoprenaline at a rate of 0.10 - 0.20 µg/min kg b.w. Generally four intermittent occlusions were performed in each animal. This allowed each animal to serve as its own control for the assessment of the effect of treatment during basal and isoprenaline stimulated conditions. Haemodynamic measurements were performed immediately before and 12 min following the occlusion of the coronary artery. Blood samples were obtained before and from 6 to 12 min after induction of myocardial ischaemia. The coronary artery occlusion was released after 12 min and a recovery period of 45 min was allowed.

A second occlusion was performed after 10 min of isoprenaline infusion when the haemodynamic variables had stabilised. All measurements were repeated. After release and recovery, nicotinic acid (0.3 mg/min kg b.w.; 7 dogs) or sodium salicylate (60 mg/kg b.w. as a slow injection followed by 0.15 mg/min kg b.w. dissolved

in 0.9% saline, pH 7.40; 7 dogs) or 0.9% saline (6 dogs) was started. After 30 min a third occlusion was made, followed after release and recovery by a fourth occlusion during isoprenaline. Care was taken that the haemodynamic and electrocardiographic measurements were always done and blood samples were always obtained in the same order, and at the same time after occlusion.

The three series of experiments were performed in sequence: firstly the sodium salicylate experiments, secondly the nicotinic acid experiments and thirdly the control experiments with saline. In three of the seven dogs given sodium salicylate only experiments without infusion of isoprenaline were conducted. Furthermore, in the salicylate experiments plasma glycerol concentrations were not measured. Epicardial ST-segment mapping was only performed in the control experiments.

Statistics:

Each dog served as its own control. Wilcoxon's non-parametric test (two-tailed) for paired data was used to calculate probabilities (36). Statistical comparisons are not performed between samples of 5 or less. $P>0.05$ was regarded as not statistically significant. Standard error of the methods was calculated from triplicate analyses of 4 randomly selected experiments, and is expressed relative to the mean as coefficient of variation.

RESULTS

Myocardial metabolism and performance before coronary artery occlusion. Effects of isoprenaline infusion (Table I)

Before coronary artery occlusion the arterio-coronary sinus (a-cs) and arterio-local venous (a-lv) differences of oxygen saturation, plasma glucose, lactate, FFA and glycerol concentrations were not significantly different either during basal or isoprenaline stimulated conditions. This indicates that before induction of myocardial ischaemia the a-lv differences were representative for myocardial metabolism in the whole left ventricle as represented by a-cs differences.

In the absence of isoprenaline the coronary artery occlusion did not change the arterial concentrations of any of the measured substrates - oxygen, glucose, lactate, FFA or glycerol. Induction of myocardial ischaemia produced release of glycerol from the ischaemic area, as reflected in negative a-lv differences of glycerol in all the experiments where plasma glycerol concentrations were measured. Compared with the a-lv differences before induction of myocardial ischaemia the glycerol release was significant ($p<0.02$, $n = 7$). This was also true when a-lv differences were compared with a-cs differences of glycerol measured simultaneously ($p<0.05$, $n = 7$). In contrast to the effect of coronary occlusion on glycerol release, no effect was observed on plasma FFA a-lv differences. As expected the extraction of oxygen and glucose increased across the ischaemic myocardium and lactate was produced. Coronary artery occlusion had no effect on a-cs differences of oxygen and substrates. HR, LVSP and LV dP/dt were not changed by the induction of myocardial ischaemia.

During isoprenaline infusion, a steady metabolic state had not been obtained after 10 min of infusion. Since the arterial concentrations of glucose, FFA and glycerol during myocardial ischaemia were constantly increasing compared with preocclusion values, only comparisons of a-lv and a-cs differences obtained simultaneously are valid. No glycerol release was observed from the ischaemic myocardium during isoprenaline infusion by comparison of a-lv and a-cs differences (n.s., n = 10), nor were the a-lv differences significantly different from zero (n.s., n = 10). The extraction of FFA across the ischaemic myocardium increased ($p<0.01$, n = 14). Comparison of a-lv and a-cs differences showed that after the induction of myocardial ischaemia glucose and oxygen extraction were increased and lactate was produced. Haemodynamic variables were unchanged by the induction of myocardial ischaemia during isoprenaline infusion.

Effects of two repeated coronary artery occlusions before and during isoprenaline infusion (Table II)

The reproducibility of our model was tested in 6 dogs. In absence of isoprenaline infusion the arterial oxygen saturation and concentrations of glucose, lactate and FFA during the repeated occlusion did not significantly differ from those during the first occlusion, and the a-lv and a-cs differences were not significantly altered. In all the 5 experiments where glycerol concentrations were measured induction of myocardial ischaemia produced release of glycerol from the ischaemic area, as reflected in negative a-lv differences of glycerol, while a-cs differences measured simultaneously remained positive. During the second of the two occlusions arterial concentrations were higher. Glycerol release was found in 4 of the 5 experiments during the repeated occlusion,

but no glycerol release was observed across the whole heart (a-cs).

The severity of the acute myocardial ischaemia expressed as ΣST was the same during the two repeated coronary artery occlusions (occlusion I: 25.3 $\pm$ 6.9, occlusion II: 27.2 $\pm$ 7.3 mV, n.s., n = 6) and the number of sites with ST-segments exceeding 2 mV was unchanged (occlusion I: 5.5 $\pm$ 0.8, occlusion II: 6.2 $\pm$ 0.8 sites, n.s., n = 6).

During infusion of isoprenaline arterial levels of oxygen, glucose, lactate, FFA and glycerol were not significantly different between the two repeated occlusions. The a-lv differences of glycerol, as well as of the other substrates and oxygen, were the same during the two occlusions. HR, LVSP and LV dP/dt were unaltered from first to second occlusion.

The electrocardiographic changes were reproduceable as shown by unchanged ΣST (isoprenaline + occlusion I: 57.3 $\pm$ 10.6, isoprenaline + occlusion II: 57.6 $\pm$ 11.6 mV, n.s., n = 6) and unchanged number of sites with ST-segments exceeding 2 mV (isoprenaline + occlusion I: 9.0 $\pm$ 0.5, isoprenaline + occlusion II: 8.7 $\pm$ 0.4, n.s., n = 6).

Metabolic and haemodynamic effects of nicotinic acid (Table III)

Nicotinic acid significantly reduced the arterial concentrations of FFA in absence of isoprenaline ($p = 0.03$, $n = 7$), whereas no other significant changes in arterial levels of oxygen, glucose of lactate were observed. The a-lv differences of FFA were significantly reduced by nicotinic acid ($p<0.02$, $n = 7$), but no change in a-lv differences for oxygen, glucose or lactate occurred. Arterial glycerol concentrations were reduced after administration of nicotinic acid in only 2 of the 5 experiments where glycerol concentrations were measured. In 4 out of 5 experiments coronary occlusion induced glycerol release into

the local vein. After administration of nicotinic acid this was observed in 3 experiments. In order to cancel the effect of the increased arterial glycerol concentrations, the exchange of glycerol was also calculated from a-lv differences before and during coronary artery occlusion. The change in a-lv differences induced by myocardial ischaemia were then -6.5 ± 10.2 µmol/l during nicotinic acid administration and -30.9 ± 12.9 µmol/l before drug administration. Nicotinic acid did not alter haemodynamic variables in the absence of isoprenaline.

During isoprenaline infusion and myocardial ischaemia the arterial concentrations of FFA ($p<0.02$, $n = 7$) and glycerol ($p<0.02$, $n = 7$) were substantially reduced by nicotinic acid, whereas the other substrate concentrations were unaltered. The a-lv differences of FFA ($p<0.02$, $n = 7$) and oxygen ($p<0.02$, $n = 7$) were markedly reduced. No significant change in the a-lv differences of glycerol occurred during administration of nicotinic acid and isoprenaline. The a-lv differences of glycerol during coronary artery occlusion were not different from preocclusion values (4.7 ± 4.6 µmol/l; n.s., $n = 6$), nor were they different from zero (n.s., $n = 6$). HR, LVSP and LV dP/dt were not changed by nicotinic acid during isoprenaline infusion.

Metabolic and haemodynamic effects of sodium salicylate (Table IV)

In the basal state sodium salicylate administration reduced arterial concentrations of FFA ($p<0.05$, $n = 7$), whereas no changes in arterial oxygen saturation or plasma concentrations of glucose and lactate were observed. Only extraction of plasma FFA across ischaemic myocardium was significantly reduced by sodium salicylate ($p = 0.03$, $n = 7$). The drug did not modify the haemodynamic state.

During isoprenaline infusion and sodium salicylate administration the arterial concentrations of FFA were reduced in all the 5 experiments, and the a-lv differences of FFA were also consistently reduced. During unaltered arterial lactate concentrations the average lactate release increased after sodium salicylate administration, but in 2 out of 3 experiments it was actually reduced. No other striking differences in arterial concentrations and a-lv differences were found, and the mechanical activity of the heart was mainly unaltered.

DISCUSSION

The method used in this study for induction of small ischaemic areas and local venous sampling from a vein draining the ischaemic area is a well-established procedure for obtaining blood for study of metabolic changes secondary to induction of ischaemia (23, 26, 27). Ischaemic areas involving less than 15% of the whole heart do not produce any significant changes in coronary sinus blood (24), as also demonstrated in this study. The experimental model therefore allows comparison of a-v differences across ischaemic tissue (a-lv differences) with non-ischaemic tissue (a-cs differences), as shown for oxygen, glucose, lactate, FFA and glycerol. The fact that during isoprenaline infusion a steady state for arterial concentrations of glucose, glycerol and FFA was not obtained, complicates the interpretations of the data. However, simultaneous use of a-lv and a-cs differences during myocardial ischaemia overcomes this possible criticism.

The severity of an acute myocardial ischaemia is reproduceable following several repetitive coronary artery occlusions (1, 16), as also demonstrated in the present study in the basal state as well as during isoprenaline infusion. The metabolic consequences of myocardial ischaemia were also found to be fully reproduceable for oxygen, glucose, lactate and FFA. In interpretation of the glycerol data, however, it should be noted that the arterial glycerol concentrations during the second occlusion in the basal state were higher than during the first occlusion, most likely related to an effect of the preceding isoprenaline infusion. But the arterial concentrations of glycerol were not altered by coronary artery occlusion in the basal state, thus allowing the

assessment of the effect of myocardial ischaemia also by comparing a-lv differences of glycerol before and after coronary artery occlusion. A comparison of the substrate exchanges across ischaemic myocardium following pharmacological interventions in this animal model should therefore be allowed. It should be pointed out, however, that the amount of uptake or release of substrates cannot be calculated from the a-v differences since blood flow is not known.

During coronary artery occlusion release of glycerol to the local vein was observed, whereas no significant a-v differences were found before induction of myocardial ischaemia. The glycerol released from the ischaemic area might come from endogenous myocardial triglycerides. The glycero-kinase activity is very low in the myocardium (31), and glycerol oxidation is presumed to be insignificant (14, 20). Consequently, the heart is unable to reutilise the glycerol in any significant amount, and mobilization of the myocardial triglyceride stores would release glycerol into the venous effluent. Alternatively, hydrolysis of plasma triglycerides by lipoprotein lipase in the endothelium of myocardial capillaries might be responsible for the release of glycerol into the local vein. It is, however, unlikely that contribution from this source is significant in these experiments since plasma triglycerides are low in overnight fasted dogs (13), and moreover, lipoprotein lipase activity is markedly depressed following myocardial ischaemia of short duration (10). Glycerol release in ischaemic myocardium might also be due to activation of lysosomal lipolytic activity. However, this possibility is also unlikely since lysosomal integrity is maintained up to 30 min after induction of anoxia (41). It is therefore reasonable to assume that the glycerol released

from the ischaemic myocardium is a measure of endogenous lipolysis within the tissue. Our finding of enhanced lipolysis in the myocardium during ischaemia, as evidenced by release of glycerol, is in accordance with Hough and Gevers (9), recently reporting increased activity of hormone-sensitive lipase in ischaemically perfused rat hearts. This increase was markedly reduced by reserpinisation, indicating that local release of noradrenaline within the ischaemic myocardium might be the stimulus for enhanced lipolysis (34).

Before induction of myocardial ischaemia we could not demonstrate any significant glycerol release from the myocardium either before or during isoprenaline infusion, although isoprenaline substantially increased the lipolysis in adipose tissue as evidenced by increased arterial FFA concentrations. Isoprenaline (37) or adrenaline (6, 42) stimulates myocardial lipolysis in rat hearts perfused without FFA. However, when fatty acids were included in the perfusate, Crass et al. (6) found that the stimulatory effect of adrenaline on mobilisation of triglyceride was abolished consistent with an inhibitory effect of exogenous FFA on myocardial lipolysis. At variance with the effect of myocardial ischaemia in the basal state, no release of glycerol into the local vein was observed during isporenaline infusion. In the present study, therefore, the high arterial FFA concentration during isoprenaline infusion might have effected an inhibition of myocardial lipolysis both in ischaemic and non-ischaemic myocardium. Alternatively, during isoprenaline infusion and high arterial glycerol concentration some glycerol might be taken up and metabolised in the myocardium, and thereby outbalancing the glycerol released from the myocardium during ischaemia.

During myocardial ischaemia the oxidation of fatty acids is

impaired (33), and the alternatives to FFA oxidation are accumulation of intracellular FFA either free or as long-chain acyl-CoA esters or esterification of FFA to triglycerides. Long-chain acyl-CoA esters are shown to accumulate in ischaemic myocardium (35), but although FFA are found in elevated concentrations in hypoxic perfused myocardium (7), as yet intracellular accumulation of FFA is not documented in ischaemic myocardium (23, 33). Esterification of FFA to triglycerides is stimulated in ischaemic myocardium (33). Therefore, increased esterification and the demonstration of enhanced lipolysis in this study might indicate a wasting of energy by recycling triglyceride. Thus, it has been shown that rat hearts with high triglyceride content exposed to ischaemic perfusion exhibit a more pronounced lipolytic activity and higher oxygen requirement than rats with normal triglyceride content (2).

The antilipolytic agents nicotinic acid or sodium salicylate have been shown to reduce the size of an acute myocardial ischaemic injury in experimental studies (12, 38, 39). In the present study we demonstrate that the extraction of FFA across ischaemic myocardium is substantially decreased by antilipolytic agents. Since blood flow in ischaemic area is not affected by any of the drugs (38), the reduced a-lv differences also reflect reduced uptake of FFA in ischaemic myocardium. It should be noted that these changes in FFA extraction by the antilipolytic agents occurred without any significant changes in glucose and lactate exchange. This most probably means that during myocardial ischaemia nicotinic acid lowers the α-glycerophosphate requirements for triglyceride synthesis and thereby making the glucose available for immediate energy purposes.

No consistent glycerol release across the ischaemic myocardium was found after administration of nicotinic acid either in the basal or isoprenaline stimulated state. The interpretation of this effect of nicotinic acid was hampered by the fact that a trend of reduced glycerol release was observed during two repeated occlusions. Furthermore, the arterial glycerol concentrations were higher during nicotinic acid in the basal state. The reason for the apparent failure of nicotinic acid to reduce arterial glycerol concentrations, while FFA concentrations were reduced, is most likely an effect of the preceding isoprenaline infusion. It has been shown that nicotinic acid inhibited glycerol release from ischaemically perfused rat heart (2, 5). These studies cannot be compared directly, however, since plasma FFA seems to have a modulating activity on myocardial lipolysis. If the only effect of nicotinic acid was to reduce plasma FFA concentrations, then an enhanced lipolysis in the myocardium might be expected. However, the finding of no glycerol release from ischaemic myocardium after administration of nicotinic acid, suggests that inhibition by nicotinic acid of lipolytic activity in myocardium overrules the effect of the concomitantly reduced exogenous FFA supply to the myocardium. Further studies, are, however, needed to investigate the regulatory role of plasma FFA in ischaemia-induced lipolysis.

This study was not designed to compare two antilipolytic agents. However, taken together with other investigations, nicotinic acid reduces myocardial uptake of FFA and oxygen more markedly than sodium salicylate (18, 40), probably as a consequence of a more potent lowering of arterial FFA concentrations (18, 40). In line with the possibility of a more effective lowering of

myocardial oxygen requirement by nicotinic acid than by sodium salicylate, Oliver (21) reported that the effect of different antilipolytic agents on myocardial ischaemia-induced epicardial ST-segment changes were closely associated with the change in fatty acid/albumin ratio obtained by the antilipolytic agent.

In conclusion, the results of this investigation show that myocardial ischaemia stimulates endogenous lipolysis. According to the present study, reduced severity of acute myocardial infarction by antilipolytic treatment shortly after onset of symptoms might be due to the combination of inhibited myocardial lipolysis and decreased FFA extraction from the circulation.

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Table I. The effects of coronary artery occlusion on myocardial metabolism in the control situation and during isoprenaline infusion in dogs. Means ± SEM of the observations are given.

		Control situation				Isoprenaline infusion		
		Before occlusion	P	Occlusion		Before occlusion	P	Occlusion
HR (beats/min)	n=14	151±5	n.s.	155±5	n=14	181±7	n.s.	188±5
LVSP (kPa)	n=14	16.7±0.7	n.s.	17.2±0.7	n=14	17.1±0.7	n.s.	17.2±0.9
LV dP/dt (kPa/sec)	n=14	251±18	n.s.	249±16	n=14	395±25	n.s.	405±20
Oxygen:	n=14				n=14			
Arterial saturation (%)		97.0±1.4	n.s.	97.0±0.9		96.0±0.9	n.s.	93.9±1.7
a-lv differences (%)		56.5±2.1	<0.01	65.8±2.2		57.5±2.3	<0.01	63.3±2.6
a-cs differences (%)		54.8±2.4	n.s.	57.8±1.7*		55.6±2.0	n.s.	56.6±1.7*
Glucose:	n=14				n=14			
Arterial plasma concentration (mmol/l)		7.12±0.32	n.s.	7.23±0.31		9.26±0.60	<0.01	10.11±0.81
a-lv differences (mmol/l)		0.38±0.07	<0.01	1.61±0.25		0.28±0.06	<0.01	1.81±0.31
a-cs differences (mmol/l)		0.24±0.08	n.s.	0.64±0.26*		0.49±0.23	n.s.	0.41±0.13*
Lactate:	n=13				n=13			
Arterial plasma concentration (mmol/l)		1.48±0.25	n.s.	1.68±0.22		1.99±0.31	n.s.	1.93±0.30
a-lv differences (mmol/l)		0.49±0.16	<0.01	-1.94±0.50		0.22±0.34	<0.01	-2.68±0.46
a-cs differences (mmol/l)		0.69±0.11	n.s.	0.69±0.08*		0.58±0.31	n.s.	0.34±0.11*
FFA:	n=14				n=14			
Arterial plasma concentration (µmol/l)		255±28	n.s.	287±33		1174±127	<0.01	1549±186
a-lv differences (µmol/l)		76±11	n.s.	78±14		246±28	=0.05	380±62
a-cs differences (µmol/l)		68±11	n.s.	85±14		257±28	n.s.	260±43*
Glycerol:	n=7				n=10			
Arterial plasma concentration (µmol/l)		37.4±3.8	n.s.	37.4±3.3		193.3±25.2	<0.01	245.2±22.5
a-lv differences (µmol/l)		3.5±4.9	<0.02	-25.3±7.9		25.2±12.6	n.s.	17.4±9.3
a-cs differences (µmol/l)		2.2±2.7	n.s.	- 0.1±2.9**		11.5±10.6	n.s.	12.7±5.9

*P<0.01 and **P<0.05 when comparisons are performed between a-lv and a-cs differences in the same experimental situation. HR = heart rate; LVSP = left ventricular systolic pressure; LV dP/dt = maximal rate of rise of left ventricular pressure; a-lv differences = arterio-local coronary vein differences; a-cs differences = arterio-coronary sinus differences. n.s. = not significant. n = number of experiments.

Table II. The effects of two repetitive coronary artery occlusions on haemodynamic and biochemical measurements under control conditions and during isoprenaline infusion in 6 open-chest dogs. Means ± SEM of observations are given.

	Occlusion I (A)	Isoprenaline+ occlusion I (B)	Occlusion II (C)	Isoprenaline+ occlusion II (D)	A - C	B - D
HR (beats/min)	151 ± 9	189 ± 11	150 ± 13	184 ± 12	n.s.	n.s.
LVSP (kPa)	16.4 ± 1.1	15.8 ± 0.7	15.8 ± 1.1	15.8 ± 0.9	n.s.	n.s.
LV dP/dt (kPa/sec)	208 ± 21	418 ± 37	204 ± 18	395 ± 34	n.s.	n.s.
$Oxygen_a$ (%)	98.9 ± 0.9	93.5 ± 2.5	100 ± 0.9	95.8 ± 2.9	n.s.	n.s.
$Oxygen_{a-lv}$ (%)	71.8 ± 2.4	69.5 ± 2.4	70.8 ± 3.3	68.5 ± 3.3	n.s.	n.s.
$Glucose_a$ (mmol/l)	8.16 ± 0.42	12.71 ± 0.98	7.55 ± 0.79	12.22 ± 1.11	n.s.	n.s.
$Glucose_{a-lv}$ (mmol/l)	2.32 ± 0.27	2.65 ± 0.22	1.75 ± 0.35	2.18 ± 0.32	n.s.	n.s.
$Lactate_a$ (mmol/l)	1.87 ± 0.23	2.62 ± 0.38	1.93 ± 0.29	2.31 ± 0.48	n.s.	n.s.
$Lactate_{a-lv}$ (mmol/l)	-2.41 ± 0.69	-2.86 ± 0.54	-1.01 ± 0.62	-2.39 ± 0.68	n.s.	n.s.
FFA_a (µmol/l)	205 ± 29	1542 ± 258	194 ± 26	1640 ± 274	n.s.	n.s.
FFA_{a-lv} (µmol/l)	55 ± 19	525 ± 117	57 ± 21	465 ± 71	n.s.	n.s.
$Glycerol_a$ (µmol/l)**	33.5 ± 3.9	276.6 ± 32.8	73.3 ± 13.6	398.5 ± 52.7	*	n.s.
$Glycerol_{a-lv}$ (µmol/l)**	-43.1 ± 16.4	6.6 ± 9.9	-11.3 ± 5.9	3.9 ± 14.9	*	n.s.

HR = heart rate; LVSP = left ventricular systolic pressure; LV dP/dt = maximal rate of rise of left ventricular pressure; a = arterial concentration; a-lv = arterio-local coronary vein difference. n.s. = not significant. n = number of experiments. *Statistical comparisons were not performed, $n \leq 5$. **Glycerol measurements were performed in 5 experiments in occlusion I and II.

Table III. The effects of nicotinic acid on haemodynamic and metabolic measurements during coronary artery occlusion under control conditions and during isoprenaline infusion in 7 open-chest dogs. Means ± SEM of the observations are given.

	Occlusion (A)	Isoprenaline+ occlusion (B)	Nicotinic acid+ occlusion (C)	Isoprenaline+ nicotinic acid+ occlusion (D)	A - C	B - D
HR (beats/min)	150 ± 11	188 ± 10	150 ± 13	184 ± 9	n.s.	n.s.
LVSP (kPa)	17.4 ± 1.2	17.6 ± 1.2	16.7 ± 1.2	16.7 ± 1.4	n.s.	n.s.
LV dP/dt (kPa/sec)	252 ± 23	419 ± 30	261 ± 27	409 ± 28	n.s.	n.s.
$Oxygen_a$ (%)	97.1 ± 1.1	95.1 ± 2.5	97.6 ± 1.3	97.4 ± 1.5	n.s.	n.s.
$Oxygen_{a-lv}$ (%)	60.6 ± 2.8	58.8 ± 3.4	60.9 ± 3.4	53.4 ± 2.7	n.s.	$p<0.02$
$Glucose_a$ (mmol/l	6.96 ± 0.27	9.44 ± 0.79	6.64 ± 0.42	9.13 ± 0.75	n.s.	n.s.
$Glucose_{a-lv}$(mmol/l)	1.40 ± 0.35	1.44 ± 0.43	0.97 ± 0.27	1.40 ± 0.19	n.s.	n.s.
$Lactate_a$ (mmol/l)	1.30 ± 0.21	1.49 ± 0.24	1.98 ± 0.28	1.83 ± 0.34	n.s.	n.s.
$Lactate_{a-lv}$ (mmol/l)	-1.22 ± 0.91	-2.46 ± 0.79	-0.59 ± 0.82	-1.56 ± 0.60	n.s.	n.s.
FFA_a (μmol/l)	331 ± 43	1580 ± 236	212 ± 13	296 ± 23	p=0.03	$p<0.02$
FFA_{a-lv} (μmol/l)	100 ± 19	342 ± 43	33 ± 11	57 ± 18	$p<0.02$	$p<0.02$
$Glycerol_a$** (μmol/l)	56.5 ± 12.9	271.0 ± 37.3	73.6 ± 25.5	56.0 ± 15.1	*	$p<0.02$
$Glycerol_{a-lv}$** (μmol/l)	-20.9 ± 8.0	22.0 ± 13.7	-13.7 ± 13.4	-2.8 ± 11.0	*	n.s.

Abbreviations as in Table II. *Statistical comparisons were not performed, $n \leq 5$. **Glycerol measurements were performed in 5 experiments without and 6 with isoprenaline.

Table IV. The effects of sodium salicylate on haemodynamic and biochemical measurements during coronary artery occlusion under control conditions (n = 7) and during isoprenaline infusion (n = 5) in open-chest dogs. Means ± SEM of the observations are given.

	Occlusion (A)	Isoprenaline+ occlusion (B)	Salicylate+ occlusion (C)	Isoprenaline+ salylate+ occlusion (D)	A - C	B - D
HR (beats/min)	151 ± 8	183 ± 4	157 ± 6	183 ± 6	n.s.	*
LVSP (kPa)	17.4 ± 0.7	16.9 ± 0.7	16.9 ± 1.1	18.1 ± 0.5	n.s.	*
LV dP/dt (kPa/sec)	293 ± 30	411 ± 14	302 ± 32	387 ± 18	n.s.	*
$Oxygen_a$ (%)	97.8 ± 1.5	98.3 ± 1.8	94.8 ± 3.0	96.0 ± 3.1	n.s.	*
$Oxygen_{a-lv}$ (%)	63.9 ± 0.9	60.6 ± 3.6	56.1 ± 6.4	56.9 ± 5.1	n.s.	*
$Glucose_a$ (mmol/l)	7.34 ± 0.52	8.35 ± 1.02	7.99 ± 0.82	8.76 ± 0.09	n.s.	*
$Glucose_{a-lv}$ (mmol/l)	1.12 ± 0.20	1.11 ± 0.55	1.35 ± 0.31	1.28 ± 0.66	n.s.	*
$Lactate_a$** (mmol/l)	1.89 ± 0.35	1.81 ± 0.57	1.39 ± 0.29	1.81 ± 0.34	n.s.	*
$Lactate_{a-lv}$** (mmol/l)	-1.75 ± 0.54	-0.90 ± 0.47	-1.73 ± 0.60	-1.47 ± 0.87	n.s.	*
FFA_a (µmol/l)	340 ± 41	1764 ± 357	233 ± 39	801 ± 312	$p<0.05$	*
FFA_{a-lv} (µmol/l)	96 ± 16	321 ± 84	40 ± 8	121 ± 36	p=0.03	*

Abbreviations as in Table II. n = number of experiments. *Statistical comparisons were not performed, $n \leq 5$. **Lactate measurements were performed in 3 experiments with isoprenaline.

REFERENCES

1. Berdeaux, A., Goutte, R., Giudicelli, J.-F. and Boissier, J.-R. Effects of verapamil on regional myocardial blood flow and ST segment. Role of the induced bradycardia. Europ. J. Pharmacol. 39, 287, 1976.

2. Brownsey, R.W. and Brundt, R.V. The effect of adrenaline-induced endogenous lipolysis upon the mechanical and metabolic performance of ischaemically perfused rat hearts. Clin. Sci. Molec. Med. 53, 513, 1977.

3. Challoner, D.R. and Steinberg, D. Effect of free fatty acid on the oxygen consumption of perfused rat heart. Amer. J. Physiol. 210, 280, 1966.

4. Chernick, S.S. Determination of glycerol in acyl glycerol. In Methods in Enzymology - 14. Edited by Lowenstein, M. Academic Press, New York, 1969.

5. Christian, D.R., Kilsheimer, G.S., Pettett, G., Paradise, R. and Ashmore, J. Regulation of lipolysis in cardiac muscle: A system similar to the hormone-sensitive lipase of adipose tissue. In Advances in Enzyme Regulation. Pergamon, New York, 1969.

6. Crass, M.F., III, Shipp, J.C. and Pieper, G.M. Effects of catecholamines on myocardial endogenous substrates and contractility. Amer. J. Physiol. 228, 618, 1975.

7. Evans, J.R. Importance of fatty acid in myocardial metabolism. Circulat. Res. 15, 96, 1964.

8. Ho, R.J. Radiochemical assay of long-chain fatty acids using ^{63}Ni as tracer. Analyt. Biochem. 36, 105, 1970.

9. Hough, F.S. and Gevers, W. Catecholamine release as mediator of intracellular enzyme activation in ischaemic perfused rat hearts. S. Afr. Med. J., 49, 538, 1975.

10. Kessler, J.I. and Senderoff, E. Effect of experimental infarction, manual massage, and electrical defibrillation on myocardial lipo-protein lipase activity of dogs. J. Clin. Invest. 41, 1531, 1962.

11. Kjekshus, J.K. and Grøttum, P. Modification of acute myocardial infarction by intravenous β-pyridylcarbinol. Acta med. Scand. In press.

12. Kjekshus, J.K. and Mjøs, O.D. Effect of inhibition of lipolysis on infarct size after experimental coronary artery occlusion. J. Clin. Invest. 52, 1770, 1973.

13. Kurien, V.A., Yates, P.A. and Oliver, M.F. The role of free fatty acids in the production of ventricular arrhythmias after acute coronary artery occlusion. Europ. J. Clin. Invest. 1, 225, 1971.

14. Lassers, B.W., Wahlquist, M.L., Kaijser, L. and Carlson, L.A. Effect of nicotinic acid on myocardial metabolism in man at rest and during exercise. J. Appl. Physiol. 33, 72, 1972.

15. Luxton, M.R., Miller, N.E. and Oliver, M.F. Antilipolytic therapy in angina pectoris. Reduction of exercise-induced ST segment depression. Brit. Heart J. 38, 1204, 1976.

16. Maroko, P.R., Kjekshus, J.K., Sobel, B.E., Watanabe, T., Covell, J.W., Ross, J. and Braunwald, E. Factors influencing infarct size following experimental coronary artery occlusion. Circulation 43, 67, 1971.

17. Mjøs, O.D. Effect of free fatty acids on myocardial function and oxygen consumption in intact dogs. J. Clin. Invest. 50, 1386, 1971.

18. Mjøs, O.D. Effect of inhibition of lipolysis on myocardial oxygen consumption in the presence of isoproterenol. J. Clin. Invest. 50, 1869, 1971.

19. Mjøs, O.D., Miller, N.E., Riemersma, R.A. and Oliver, M.F. Effects of p-chlorophenoxyisobutyrate on myocardial free fatty acid extraction, ventricular blood flow, and epicardial ST-segment elevation during coronary occlusion in dogs. Circulation 53, 494, 1976.

20. Most, A.S., Brachfeld, N., Gorlin, R. and Wahren, J. Free fatty acid metabolism of the human heart at rest. J. Clin. Invest. 48, 1177, 1969.

21. Oliver, M.F. The influence of myocardial metabolism on ischemic damage. Circulation 53, Suppl. I, 168, 1976.

22. Oliver, M.F., Kurien, V.A. and Greenwood, T.W. Relation between serum-free-fatty-acids and arrhythmias and death after acute myocardial infarction. Lancet I, 710, 1968.

23. Opie, L.H., Owen, P. and Riemersma, R. Relative rates of oxidation of glucose and free fatty acids by ischaemic and non-ischaemic myocardium after coronary artery ligation in the dog. Europ. J. Clin. Invest. 3, 419, 1973.

24. Opie, L.H., Owen, P., Thomas, M. and Samson, R. Coronary sinus lactate measurements in the assessment of myocardial ischaemia. Comparison with changes in the ratio lactate/pyruvate and beta-hydroxybutyrate/acetoacetate and with release of hydrogen, phosphate and potassium ions from the heart. Amer. J. Cardiol. 32, 295, 1973.

25. Opie, L.H., Tonsey, M. and Kennelly, B.M. Proposed metabolic vicious circle in patients with large myocardial infarcts and high plasma-free-fatty-acid concentrations. Lancet II, 890, 1977.

26. Owen, P., Thomas, M., Young, V. and Opie, L.H. Comparison between metabolic changes in local venous and coronary sinus blood after acute experimental coronary arterial occlusion. Amer. J. Cardiol. 25, 562, 1970.

27. Parratt, J.R., Ledingham, I.M. and McArdle, C.S. Effect of a coronary vasodilator drug (carbochromen) on blood flow and oxygen extraction in acute myocardial infarction. Cardiovasc. Res. 7, 401, 1973.

28. Passonneau, J.V. L-(+)-lactate. A fluorimetric method. In Methods of Enzymatic Analysis, 2nd ed. Edited by Bergmeyer, H.U. Academic Press, London, 1974.

29. Riemersma, R.A., Talbot, R.C., Ungar, A., Mjøs, O.D. and Oliver, M.F. Effects of prostaglandin -E_1 on ST segment elevation and regional myocardial blood flow during experimental myocardial ischaemia in dogs. Europ. J. Clin. Invest. 7, 515, 1977.

30. Russell, D.C. and Oliver, M.F. Effect of antilipolytic therapy on ST segment elevation during myocardial ischaemia in man. Brit. Heart J. 40, 117, 1978.

31. Robinson, J. and Newsholm, E.A. Glycerol kinase activities in rat heart and adipose tissue. Biochem. J. 104, 2c, 1967.

32. Sayen, J.J., Sheldon, W.F., Peirce, G. and Kuo, P.T. Polarographic oxygen, the epicardial electrocardiogram and muscle contraction and experimental acute regional ischemia of the left ventricle. Circulat. Res. 6, 779, 1958.

33. Scheuer, J. and Brachfeld, N. Myocardial uptake and fractional distribution of palmitate-1-C^{14} by the ischaemic dog heart. Metabolism 15, 945, 1966.

34. Shahab, L., Wollenberger, A., Krause, E.G. and Genz, S. The effect of acute ischemia on catecholamines and cyclic AMP levels in normal and hypertrophied myocardium. In Effect of Acute Ischaemia on Myocardial Function. Edited by Oliver, M.F., Julian, D.G. and Donald, K.W. Churchill Livingstone, Edinburgh and London, 1972.

35. Shug, A.L., Shrago, E., Bittar, N., Folts, J.D. and Koke, J.R. Acyl-CoA inhibition of adenine nucleotide translocation in ischemic myocardium. Amer. J. Physiol. 228, 689, 1975.

36. Snedecor, G.W. and Cochran, W.G. Statistical Methods, 6th ed. The Iowa State University Press, Ames. 1967.

37. Takenaka, F. and Takeo, S. Effects of isoproternol on myocardial lipid metabolism in rat hearts perfused with and without exogenous substrate. J. Molec. Cell. Cardiol. 8, 925, 1976.

38. Vik-Mo, H. Distribution of coronary blood flow during acute coronary occlusion in dogs. Effect of nicotinic acid and sodium salicylate. Scand. J. Clin. Lab. Invest. 37, 697, 1977.

39. Vik-Mo, H. and Mjøs, O.D. Effects of sodium salicylate and acetylsalicylic acid on epicardial ST-segment elevation during coronary artery occlusion in dogs. Scand. J. Clin. Lab. Invest. 37, 287, 1977.

40. Vik-Mo, H. and Mjøs, O.D. Myocardial metabolism and performance during sodium salicylate infusion in dogs. Scand. J. Clin. Lab. Invest. 36, 763, 1976.

41. Welman, E. Labilisation and stabilisation of lysosomes in anoxic guinea-pig heart. Brit. Heart J. 37, 781, 1975.

42. Williamson, J.R. Metabolic effects of epinephrine in the in the isolated, perfused rat heart. I. Dissociation of the glycogenolytic from the metabolic stimulatory effect. J. Biol. Chem. 239, 2721, 1964.

Appendix F

EFFECTS OF DICHLOROACETATE ON MYOCARDIAL SUBSTRATE EXTRACTION, EPICARDIAL ST-SEGMENT ELEVATION, AND VENTRICULAR BLOOD FLOW FOLLOWING CORONARY OCCLUSION IN DOGS

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Effects of dichloroacetate on myocardial substrate extraction, epicardial ST-segment elevation, and ventricular blood flow following coronary occlusion in dogs[1]

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AUTHORS' SYNOPSIS Glucose metabolism in the healthy heart is stimulated by dichloroacetate (DCA). The possibility has been examined in dogs that DCA, by increasing glucose utilization, might limit the severity of acute myocardial ischaemic injury.

Intravenous administration of DCA reduced the degree of epicardial ST-segment elevation induced by subsequent coronary occlusion, both under basal conditions and during isoprenaline infusion. A similar result was obtained when DCA was given during an established coronary occlusion. This effect could not be explained by changes in mean aortic blood pressure, heart rate, or regional myocardial blood flow as measured by radioactive microspheres. Measurements in arterial and coronary sinus blood demonstrated an increase in the extraction of glucose and a decrease in that of FFA by the heart. Glucose extraction also tended to be increased in the ischaemic zone, as shown by the differences in the concentrations of these substrates between arterial blood and blood obtained from the local vein draining that zone. Lactate release by the ischaemic zone was markedly reduced.

Under normal circumstances the energy requirements of the heart are derived principally from the oxidation of free fatty acids (FFA) (Lassers *et al*, 1972). During myocardial ischaemia the oxidation of FFA is impaired to a greater extent than that of glucose, and the heart becomes more dependent upon both aerobic and anaerobic glucose metabolism for its energy requirements (Opie *et al*, 1973). Under such circumstances an increased delivery of FFA to the heart has been associated experimentally with the development of ventricular arrhythmias (Kurien *et al*, 1971) and with further deterioration in cellular function (Kjekshus and Mjøs, 1972; de Leiris *et al*, 1974).

For these reasons it was proposed that the survival of the ischaemic myocardium might be improved by measures which promote the utilization of glucose and decrease that of FFA (Kurien and Oliver, 1970; Opie, 1970). Recent animal studies have supported this hypothesis, and intravenous (iv) infusions of glucose and insulin were reported by Maroko *et al* (1972) to

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reduce myocardial ischaemic injury following coronary occlusion in dogs; and similar results were obtained by Kjekshus and Mjøs (1973) when arterial FFA concentrations were lowered by β-pyridyl-carbinol and by Mjøs *et al* (1976) using clofibrate to reduce myocardial FFA extraction.

In 1962, Lorini and Ciman reported that di-isopropyl-ammonium dichloroacetate raised the respiratory quotient in alloxan-diabetic rats, suggesting an increased utilization of glucose relative to that of FFA. Stacpoole and Felts (1970; 1971) demonstrated that the active component was dichloroacetate (DCA), and that this stimulated glucose oxidation and inhibited FFA oxidation in rat diaphragm muscle. McAllister *et al* (1973) subsequently showed that DCA had similar effects on the metabolism of the isolated perfused rat heart and the intact dog heart. Associated changes in lactate and pyruvate metabolism and in intracellular intermediates suggested that DCA was stimulating both glycolysis and glucose oxidation by increasing the activities of phosphofructokinase and pyruvate dehydrogenase. An activation of pyruvate dehydrogenase in rat myocardium by DCA was observed by Whitehouse and Randle (1973).

In the present study we have investigated further the metabolic and haemodynamic effects of DCA in dogs, and have examined the possibility that the stimulation of myocardial glucose utilization by DCA might limit the severity of acute myocardial ischaemic injury during experimental coronary occlusion.

Methods

Animal preparation

Experiments were performed on 29 healthy mongrel dogs of both sexes (12–19 kg body weight). Each dog was fasted overnight and anaesthetized with sodium pentobarbitone (25 mg/kg, iv), followed by maintenance doses of 50 mg. Ventilation was maintained throughout a cuffed endotracheal tube with a positive pressure respirator (Harvard Apparatus Co Inc, Mass, USA). Thoracotomy was performed through the left fifth intercostal space and the heart suspended in a pericardial cradle. A branch of the left anterior descending (LAD) coronary artery was then dissected free for 0.5 cm. Subsequent occlusions of the artery were performed with a releasable metal clip. The left femoral vein was cannulated as a route for infusion, and the left femoral artery for measurement of mean aortic blood pressure ($\overline{AP}$). $\overline{AP}$ was monitored with a Statham P23Db transducer. A catheter for withdrawal of arterial blood was inserted into the right femoral artery. Coronary sinus blood was sampled through a catheter introduced via the left jugular vein under fluoroscopic guidance. In 10 experiments the local vein draining the ischaemic area was cannulated for the assessment of metabolic changes in the ischaemic myocardium.

Experimental designs

Effects of DCA on the response to subsequent coronary occlusion

Experiments were carried out both under basal conditions and during a continuous iv infusion of isoprenaline. In six animals the experimental design conformed to procedure 1 of Fig. 1. In one animal a similar sequence of occlusions was performed without the use of isoprenaline; in another animal isoprenaline was given during all four occlusions. In three animals the experimental design was reversed in accordance with procedure 2 of Fig. 1. In these experiments DCA was given before the first and second occlusions, and a period of 95 min was then allowed for the metabolic effects of DCA to diminish before repeating the occlusions. The effects of DCA were assessed by paired analysis of the data for all 11 experiments.

In five separate animals the effect of DCA (120 mg/kg) on the metabolic pattern across the ischaemic zone was studied during basal lipolysis. A constant infusion of albumin bound ^{3}H-oleate (Radiochemical Centre, Amersham), prepared as described by Opie *et al* (1973) was commenced at least 90 min before and maintained throughout the experiment. Between 5 and 15 min after the occlusion of the branch of the LAD coronary artery, arterial, local venous, and coronary sinus blood samples were taken for the measurement of FFA, ^{3}H-FFA, glucose, and lactate. After release and recovery DCA was administered and the occlusion repeated 10 min later. Care was taken that blood samples were obtained at the same relative times during the two occlusions. In another five animals the identical procedure was repeated except that now the occlusions were preceded by a continuous isoprenaline infusion (0.1–0.15 $\mu g \cdot kg^{-1}$/min).

In four additional animals the effects of DCA on regional myocardial blood flow 15 min after coronary occlusion were assessed by means of radioactive microspheres. Measurements were made during an

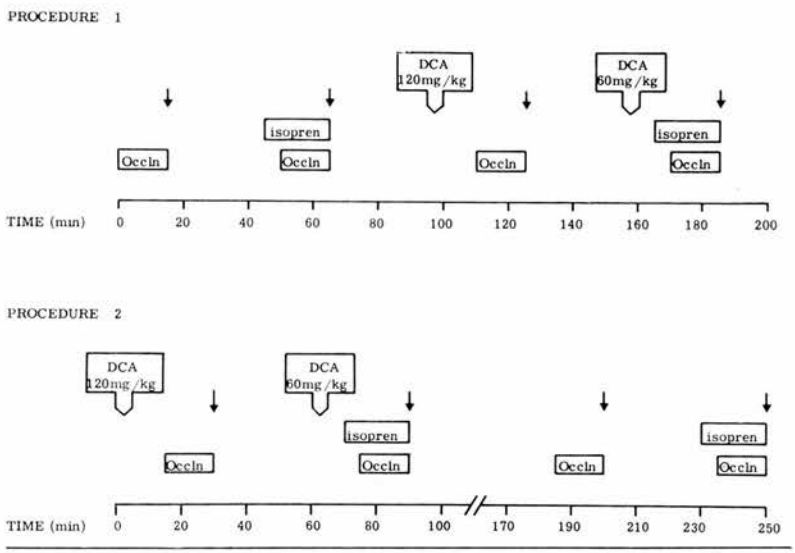

FIG. 1 *Experimental procedures for assessing the effect of dichloroacetate* (DCA) *on the response to subsequent coronary occlusion. Four identical occlusions of 15 min duration were performed in each animal, two in the absence of DCA and two in its presence. One of each pair of occlusions was performed during a continuous iv infusion of isoprenaline (0.2–0.3 μg·kg⁻¹/min), started 5 min before the occlusion. Recovery periods of 30 min were allowed between successive occlusions. Immediately before the release of each occlusion (indicated by vertical arrows) epicardial electrocardiograms from 10–15 sites in the ischaemic and non-ischaemic zones, mean aortic blood pressure and heart rate were recorded, and blood was sampled from a femoral artery and the coronary sinus. Sodium DCA was administered at the dose of 120 mg/kg body weight by iv injection over 5 min in 10 ml 0.9% saline.* Occln = *coronary occlusion.* Isopren = *isoprenaline infusion.*

initial control occlusion, and then during a second occlusion performed 10 min after iv administration of sodium dichloroacetate (120 mg/kg). The order of injection of the differently labelled microspheres was varied to avoid systematic errors due to possible differences in their behaviour.

Effects of DCA administration during established coronary occlusion

In another four animals iv infusion of isoprenaline (0.2–0.3 μg·kg⁻¹/min) was maintained for the duration of the study. Five minutes after its commencement control recordings of the epicardial electrocardiogram (ECG), $\overline{AP}$, and HR were made before a branch of the LAD coronary artery was permanently occluded. Further ECG and haemodynamic measurements were made 5, 10, and 15 min following coronary occlusion. Sodium dichloroacetate (120 mg/kg iv) was then infused over 2 min, and the ECG and haemodynamic recordings repeated 5, 10, 15, and 20 min later. Arterial and coronary sinus blood was sampled immediately before and 20 min after the injection of DCA for measurement of plasma FFA and glucose concentrations.

In the same animals the effect of DCA on the total myocardial uptake of radiolabelled palmitate was also examined. For this purpose a continuous iv infusion of albumin-bound (9,10 [n]-^{3}H) palmitate (Radiochemical Centre, Amersham). Arterial and coronary sinus blood for measurement of plasma FFA radioactivity was sampled immediately before and 20 min after DCA injection.

Measurements

Epicardial ST-segment mapping

The severity of the acute myocardial ischaemic injury was assessed by epicardial ST-segment mapping (Maroko *et al*, 1971). Epicardial electrocardiograms were recorded 15 min after coronary occlusion from 10–15 anatomically recognizable sites supplied by the occluded artery and within the adjacent area, using a mobile cotton-wick electrode (Devices M4 recorder; paper speed, 25 mm/s; sensitivity, 1 mV/mm deflection). The sum of the ST-segment elevations from all sites (ΣST) was used as an approximate index of the severity of ischaemic injury.

Regional myocardial blood flow

Regional myocardial blood flow was measured with ^{141}Ce- and ^{85}Sr-labelled radioactive microspheres (3M Riker Laboratories, Loughborough, UK; nominal diameter, 15 μ) (Utley *et al*, 1974). Before injection 2.0–2.5 × 10^{6} microspheres (sufficient to give at least 1000 microspheres/g of myocardium) were sonicated in 10% dextrose for 10 min to dispel aggregates (Dawe Sonicleaner, type 6441A), and drawn into a syringe containing 0.1 ml 5%

Tween 30. The suspension was diluted with 10% dextrose to a final concentration of Tween 80 of less than 0.5%, and then continuously agitated until injected. Fifteen min after coronary occlusion blood for estimation of reference blood flow (RBF) was withdrawn from a femoral artery into a weighed heparinised syringe at constant rate (approximately 9 ml/min) for 2 min. Immediately after the commencement of blood withdrawal, the microspheres were injected through a left atrial cannula over 10 s. After each experiment the heart was excised, the free wall of the left ventricle dissected out, and visual fat and large vessels removed. Between eight and 10 full thickness tissue samples were taken and divided into epicardial and endocardial layers (wet weight 1.5–4.0 g). Each layer was divided into 10–20 blocks to produce constant geometrical factors for measurements of radioactivity in a three channel gamma counter (GammaGuard, Tracerlab Ltd). Approximately 27% of ^{85}Sr radioactivity was counted in the ^{141}Ce channel. This factor was determined during each counting procedure using the reference blood samples as standards. RBF was estimated by weighing, using an assumed blood specific gravity of 1.05 g/ml. Regional myocardial blood flow (MBF) was calculated for each tissue sample using the formula:

$$\text{MBF} = \frac{\text{CM}}{\text{CR}} \times \text{RBF}$$

where MBF is expressed in $ml \cdot g^{-1}/min$, CM is the tissue sample radioactivity in cpm/g wet weight, CR is the total cpm in the reference blood sample, and RBF is expressed in ml/min (Utley *et al*, 1974).

Metabolic measurements

Plasma FFA concentrations were measured in duplicate by the method of Trout *et al* (1960). Plasma glucose was assayed in quadruplicate using a semi-automated glucose oxidase method (Nimmo *et al*, 1973). Blood lactate was measured in duplicate according to Hohorst (1963), or when local venous blood samples were obtained, plasma lactate concentrations were estimated in triplicate according to the method of Passonneau (1974). Plasma lipids were extracted in duplicate and radioactive FFA isolated by thin layer chromatography as described by Boberg (1966). Radioactivity was measured using a Packard Liquid Scintillation spectrometer Model 3390. Internal standards were used to correct for quenching. Arterial oxygen saturation was monitored with a Kipp type Mol Hemoreflector, and arterial pH and pCO_2 with a Type AME 1c Astrup Micro Equipment (Radiometer, Copenhagen).

Statistics

Each dog served as its own control. Student's t test for paired data was used to calculate probabilities. $P > 0.05$ was regarded as not statistically significant.

Results

Effects of DCA on the response to subsequent coronary occlusion

The effects of DCA on the response to subsequent coronary occlusion under basal conditions are summarized in Table 1. Coronary occlusion raised epicardial ST segments in all experiments. In the absence of DCA, ΣST averaged 18 ± 3 mV (mean ± SEM) 15 min after coronary occlusion. Values for ΣST were significantly less when coronary occlusion was performed after the administration of DCA (ΣST 6 ± 2 mV; $P < 0.005$). HR was decreased from 136 ± 8 to 127 ± 8 beats/min ($P < 0.05$), while $\overline{AP}$ was unchanged. Arterial blood lactate concentration was reduced by DCA from 1.10 ± 0.24 to 0.28 ± 0.03 mmol/litre ($P < 0.01$). The arterial-coronary sinus difference (Δa–cs) in lactate concentration was reduced from 0.32 ± 0.07 to 0.08 ± 0.02 mmol/litre ($P < 0.02$), while that in glucose concentration was increased from 4.5 ± 1.4 to 9.9 ± 1.0 mg/100 ml ($P < 0.005$). Other metabolic measurements were unchanged.

The effect of DCA on the a–v differences in concentration of metabolites across the ischaemic zone is tabulated in Table 1 (series II). There was a tendency for arterial-local venous difference (Δa–lv) in plasma glucose concentration to increase (three out of four experiments), although arterial glucose concentration tended to decrease and a decrease in Δa–lv of glucose actually occurred when arterial glucose concentration decreased as much as 18%. In three out of four experiments lactate release was diminished. No marked effect of DCA on the extraction of FFA in the ischaemic area was found.

The effects of DCA on the response to subsequent coronary occlusion during isoprenaline infusion appear in Table 2. In the absence of DCA, isoprenaline infusion increased ΣST from 18 ± 3 to 62 ± 8 mV ($P < 0.001$). This was associated with a reduction in $\overline{AP}$ ($P < 0.005$), and increase in HR ($P < 0.001$), and increases in the arterial concentrations of FFA ($P < 0.001$), glucose ($P < 0.01$) and lactate ($P < 0.025$). Pre-

TABLE 1

Effects of pretreatment with sodium dichloroacetate (120 mg/kg) on epicardial ST-segment elevation and haemodynamic and biochemical measurements 15 min after coronary occlusion under basal conditions

Measurement	*n**	*Mean ± SEM*		*P†*
		Control	*Dichloroacetate*	
ΣST (mV)	10	18 ±3	6 ±2	<0.005
HR (beats/min)	10	136 ±8	127 ±8	<0.05
$\overline{AP}$ (kPa)	10	15.3 ±0.80	15.3 ±0.80	NS
FFA_a (μmol/litre)	10	447 ±51	461 ±63	NS
FFAa-cs (μmol/litre)	10	85 ±29	63 ±15	NS
$Glucose_a$ (mg/100 ml)	9	94 ±4	94 ±5	NS
$Glucose_{a-cs}$ (mg/100 ml)	9	4.5 ±1.4	9.9 ±1.0	<0.005
$Lactate_a$ (mmol/litre)	7	1.10 ±0.24	0.28 ±0.03	<0.01
$Lactate_{a-cs}$ (mmol/litre)	7	0.32 ±0.07	0.08 ±0.02	<0.02
Series II‡				
FFA_a (μmol/litre)	5	537 ±43	543 ±61	NS
$^3H\text{-}FFA_{(a-lv)/a(\%)}$	3	38 ±6	28 ±12	NS
$Glucose_a$ (mg/100 ml)	5	110 ±13	100 ±12	<0.05
$Glucose_{a-lv}$ (mg/100 ml)	4	17.9 ±8.3	20.1 ±9.0	NS
$Lactate_a$ (mmol/litre)**	4	0.78 ±0.13	0.50 ±0.11	NS
$Lactate_{a-lv}$ (mmol/litre)**	4	−2.38 ±0.71	−1.72 ±0.44	NS

ΣST = sum of ST-segment elevations at 10–15 epicardial sites; HR = heart rate; $\overline{AP}$ = mean aortic pressure; ^3H-FFA = radioactive FFA. The subscripts indicate: a = arterial concentration; a–cs = arteriocoronary sinus concentration difference; a–lv = arterio-local venous concentration difference (local vein, draining the ischaemic area); (a–lv)/a = extraction in the ischaemic area.

*Number of dogs studies.

†P values were obtained by the paired t test; NS = not statistically significant (P > 0.05).

‡Separate study, observations were made between 5–15 min after coronary occlusion.

**Plasma lactate concentrations.

Conversion: SI to traditional units: 1 kPa ≃ 7.5 mmHg.

TABLE 2

Effects of pretreatment with sodium dichloroacetate (60 mg/kg) on epicardial ST-segment elevation and haemodynamic and biochemical measurements 15 min after coronary occlusion during a continuous intravenous infusion of isoprenaline (0.2–0.3 μg/kg/min)

Measurement	*n*	*Isoprenaline (mean ± SEM)*		*P*
		Control	*Dichloroacetate*	
ΣST (mV)	10	62 ±8	45 ±7	<0.001
HR (beats/min)	10	162 ±6	155 ±6	<0.05
$\overline{AP}$ (kPa)	10	11.7 ±0.93	12.2 ±0.80	<0.02
FFA_a (μmol/litre)	9	1716 ±177	1719 ±167	NS
FFA_{a-cs} (μmol/litre)	9	310 ±41	192 ±19	<0.005
$Glucose_a$ (mg/100 ml)	9	122 ±11	120 ±10	NS
$Glucose_{a-cs}$ (mg/100 ml)	9	7·1 ±1·9	12.3 ±2.5	<0.02
$Lactate_a$ (mmol/litre)	7	1.67 ±0.35	0.69 ±0.10	<0.02
$Lactate_{a-cs}$ (mmol/litre)	6	0.42 ±0.16	0.12 ±0.03	NS
*Series II**				
FFA_a (μmol/litre)	4	1363 ±118	1369 ±192	NS
$^3H\text{-}FFA_{(a-lv)/(\%)}$	4	26.4 ±15.0	28.5 ±16.7	NS
$Glucose_a$ (mg/100 ml)	5	115.0 ±8.7	105.0 ±10.6	NS
$Glucose_{a-lv}$ (mg/100 ml)	5	23.0 ±3.8	29.9 ±7.1	NS
$Lactate_a$ (mmol/litre)†	4	1.32 ±0.15	0.80 ±0.19	<0.01
$Lactate_{a-lv}$ (mmol/litre)†	4	−2.67 ±0.63	−1.88 ±0.51	<0.05

All abbreviations as in Table 1.

*Separate study: dose of sodium dichloroacetate 120 mg/kg; dose of isoprenaline 0.1–0.15 $\mu g \cdot kg^{-1}/min$; observations were made between 5–15 min after coronary occlusion.

†Plasma lactate concentrations.

treatment with DCA reduced isoprenaline-induced increase in ΣST from 62±8 to 45±7 mV ($P<0.001$). Heart rate was reduced from 162±6 to 155±6 beats/min ($P<0.05$), while $\overline{AP}$ was increased from 11.7±0.93 to 12.3±0.80 kPa (88±7 to 92±6 mmHg) ($P<0.02$). The effects of DCA on lactate and glucose metabolism during isoprenaline resembled those observed under basal conditions: ie, decreases in arterial lactate concentration ($P<0.02$) and in the Δa–cs in lactate concentration ($P<0.10$), and an increase in the Δa–cs in glucose concentration of 5.2±1.7 mg/100ml ($P<0.02$). In addition, the Δa-cs in FFA concentration was decreased from 310±41 to 192±19 μmol/litre ($P<0.005$) in the absence of change in arterial FFA concentration.

The effects of DCA on the a–v differences of substrates across the ischaemic area during isoprenaline infusion are summarized in Table 2 (series II). DCA had a consistent and significant lowering effect on lactate release from the ischaemic left ventricle. The Δa–lv of glucose increased in four out of five experiments, and arterial plasma glucose concentrations tended to decrease; when arterial glucose concentrations decreased by as much as 25%, glucose extraction by the ischaemic area actually decreased after DCA (one experiment). The extraction of (^{3}H) FFA appeared to be unchanged by DCA.

TABLE 3

Effects of sodium dichloroacetate, 120 mg/kg, on regional myocardial blood flow in ischaemic and non-ischaemic free left ventricular wall 15 min after coronary occlusion in four dogs

	Myocardial blood flow (ml·g^{-1}/min)		
	Occlusion	*Occlusion plus dichloroacetate*	*P**
Non-ischaemic myocardium†			
Epicardial	1.16±0.05 (25)	1.35±0.06 (25)	<0.005
Endocardial	1.25±0.05 (25)	1.41±0.06 (25)	<0.025
Ischaemic myocardium‡			
Epicardial	0.29±0.07 (11)	0.34±0.07 (11)	NS
Endocardial	0.30±0.08 (11)	0.35±0.10 (11)	NS

Results are expressed as means ± SEM. The figures in parentheses indicate the number of biopsies.
*Paired t test. NS: $P>0.05$.
†Myocardium distant from the occluded artery.
‡Myocardium within the area of distribution of the occluded artery.

The results of the radioactive microsphere studies are summarized in Table 3. Non-ischaemic tissue of the free wall of the left ventricle distant from the occluded artery was taken to represent normal myocardium. On this basis coronary occlusion produced an average decrease of approximately 75% in blood flow to the ischaemic zone, the reduction in flow being greater in the endocardial (mean reduction, 0.95 ml·g^{-1}/min) than in the epicardial (0.87 ml/g) layers. DCA increased blood flow to both the epicardial (+0.19 ml·g^{-1}/min or 16%; $P<0.005$) and endocardial (+0.16 ml·g^{-1}/min or 13%; $P<0.025$) layers of the non-ischaemic myocardium, but had no effect on that to the ischaemic zone.

Effects of DCA administration during established coronary occlusion

The effects of DCA administration during established coronary occlusion and isoprenaline infusion are presented in Table 4, and the changes in epicardial ST segments in one experiment are illustrated in Fig. 2. Ten, 15, and 20 min after DCA, values for ST were significantly lower than those observed immediately before treatment ($P<0.05$). Mean aortic pressure and HR at these times were similar to those recorded before treatment, with the exception that HR had decreased by 8±2 beats/min ($P<0.01$) after 20 min. The Δa–cs in glucose concentration was increased from 2 to 11 mg/100 ml (one experiment), while that in FFA concentration was decreased ($P<0.05$) in the presence of unchanged arterial concentrations. DCA also reduced the Δa–cs in FFA radioactivity in these experiments ($P<0.05$).

Discussion

The most notable finding of the present study was that DCA reduced the degree of epicardial ST-segment elevation following acute coronary occlusion in dogs, and prevented the augmentation of ST-segment elevation induced by isoprenaline. This latter effect was observed both when DCA preceded subsequent reocclusion of

TABLE 4

Acute effects of sodium dichloroacetate (DCA), 120 mg/kg, on epicardial ST-segment elevation, haemodynamic measurements, and myocardial substrate utilization in four dogs when administered during an established coronary artery occlusion and isoprenaline infusion

	Time (min)								
	0	*5*	*10*	*15*	*20*	*25*	*30*	*35*	*40*
	←—— *Isoprenaline infusion (0.2–0.3 μg·kg⁻¹/min)* ——→								
		←—— *Coronary artery occlusion* ——→							
						←— *Dichloroacetate (120 mg/kg, iv)* —→			
ΣST (mV)		2(1)	48(8)	65(9)	70(10)	56(15)	49(11)†	48(9)**	44(6)*
$\overline{AP}$ (kPa)		13.8(1.5)	13.7(1.5)	14.0(1.5)	13.7(1.5)	14.2(1.3)	14.2(1.2)	14.0(1.2)	14.0(1.2)
HR (beats/min)		188(8)	190(9)	188(11)	187(14)	184(12)	184(13)	184(13)	179(13)‡
FFA_a (μmol/litre)					1255(364)				1133(360)
FFA_{a-cs} (μmol/litre)					270(78)				198(68)*
$^3H\text{-}FFA_a$ (dpm/ml)					2483(1240)				2426(1340)
$^3H\text{-}FFA_{a-cs}$ (dpm/ml)					629(366)				444(391)*
$Glucose_a$ (mg/100 ml)††				172	149	152	149		154
$Glucose_{a-cs}$ (mg/100 ml)††				3	2	14	9		11

Results are expressed as means. The SEMs are given in parentheses.

All abbreviations are as in Table 1.

$^3H\text{-}FFA_a$ = arterial FFA radioactivity as (9, 10 (n)-^3H) palmitate; $^3H\text{-}FFA_{a-cs}$ = arterial-coronary sinus concentration difference in FFA radioactivity.

Statistical comparisons were performed by paired t test against those results obtained immediately before DCA (time 20 min).

*P < 0.05; †P < 0.01; ‡P < 0.005; **P < 0.001.

††One dog only.

the artery and when given during an occlusion established 15 min earlier.

Epicardial ST-segment elevation has been shown to reflect the severity of myocardial ischaemia (Wégria *et al,* 1949), and to correlate with the local changes in tissue oxygen tension (Sayen *et al,* 1958) and cellular membrane potential (Toyoshima *et al,* 1965) during coronary occlusion. The degree of ST-segment elevation 15 min after coronary occlusion has been correlated with the subsequent depletion of myocardial creatine kinase activity (Maroko *et al,* 1971; 1972; Kjekshus and Mjøs, 1973) and the development of histological, histochemical, and ultrastructural evidence of cellular necrosis (Maroko *et al,* 1972; Libby *et al,* 1973) after 24 h of sustained occlusion. It is likely, therefore, that the reduction in ST-segment elevation produced by DCA in the present study reflected a limitation of the acute myocardial ischaemic injury.

The severity of myocardial ischaemic injury is influenced by factors which alter myocardial oxygen requirement relative to oxygen supply. The major determinants of myocardial oxygen requirement are heart rate, wall tension, and contractility (Sonnenblick *et al,* 1968), and the possibility must be considered that the reduction of ST-segment elevation by DCA may have been secondary to a change in one of these parameters. Although DCA reduced mean HR in all experiments, this effect averaged only 5%, and was probably insufficient to account for the mean reduction in ST-segment elevation of 44% (Maroko *et al,* 1971; Wendt *et al,* 1974). Furthermore, in six instances DCA administration was associated with an unchanged or increased HR, but nevertheless ΣST was significantly reduced. Changes in $\overline{AP}$, when present, were also insufficient to explain the reduction in ST segment elevation. An effect of DCA on myocardial oxygen supply is unlikely as the results of the microsphere studies showed that blood flow to the ischaemic zone was unchanged by DCA.

The use of arterial-coronary sinus differences has been shown to be justified for the study of non-ischaemic myocardial metabolism when small ischaemic lesions are produced (Owen *et al,* 1970; Opie *et al,* 1973). Changes in arterial-coronary sinus concentration differences have

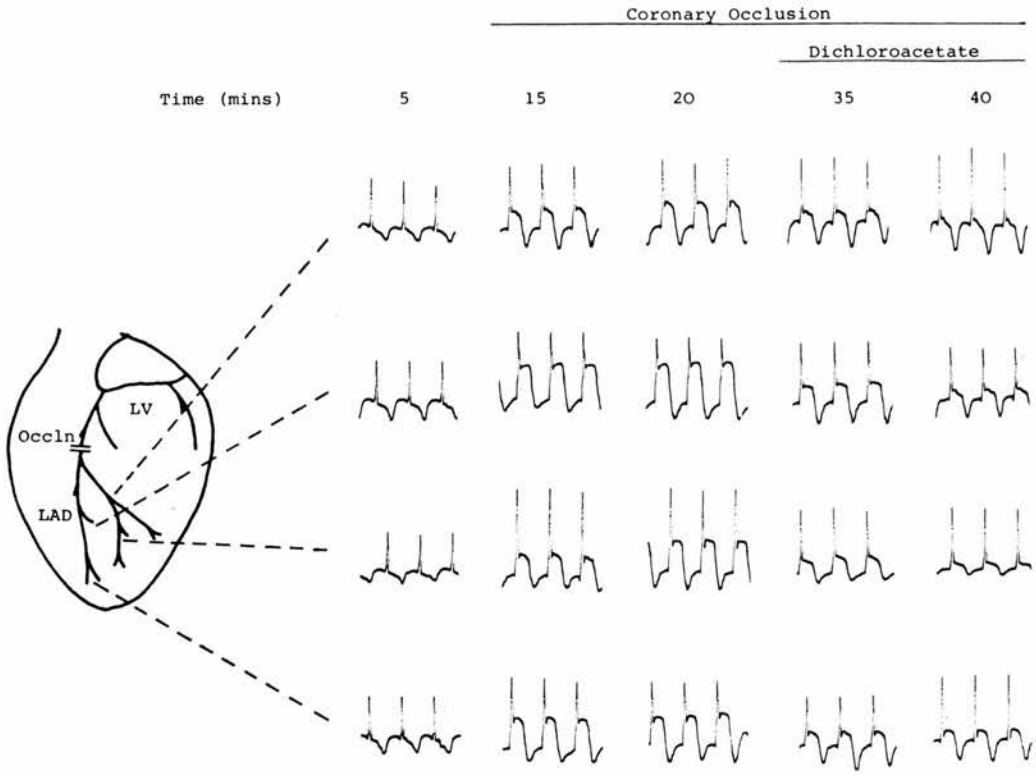

FIG. 2 *Epicardial electrocardiographic recordings from four of 11 sites in one experiment. Five minutes after the commencement of a continuous iv infusion of isoprenaline (0.2–0.3 $\mu g \cdot kg^{-1}/min$), a branch of the left anterior descending coronary artery was permanently occluded. Fifteen min later sodium dichloroacetate was given by iv injection at the dose of 120 mg/ kg. Epicardial electrocardiograms were recorded immediately before coronary occlusion (time, 5 min), and subsequently at 5 min intervals until 20 min after the administration of dichloroacetate.* LV = *left ventricle;* LAD = *left anterior descending coronary artery;* occln = *coronary occlusion.*

indicated that DCA had marked effects on the pattern of substrate utilization by the non-ischaemic left ventricle. In all experiments DCA raised the total myocardial extraction of glucose relative to that of FFA. In most instances this reflected both an increase in the Δa–cs in glucose concentration and a decrease in that of FFA, although the latter reached statistical significance only in the presence of isoprenaline. In all but one of seven animals DCA also reduced the arterial lactate concentrations and the Δa–cs in lactate concentrations. The decrease in Δa–cs in FFA (^{3}H-palmitate experiments and FFA during isoprenaline) can be interpreted either as a decrease in the extraction of FFA or an increase in intramyocardial lipolysis. The latter does not seem likely, since DCA exerted an antilipolytic effect in rats (Blackshear *et al,* 1974).

These findings cannot be extrapolated to the metabolism in the ischaemic area and therefore should not be directly related to the ECG changes recorded over that area. It is necessary therefore to make comparable measurements using local vein sampling techniques, thereby deriving arterial-local vein concentration differences which mostly reflect metabolic changes in the ischaemic tissue (Owen *et al,* 1970; Opie *et al,* 1973). DCA increased Δa–lv concentrations of glucose, even when arterial glucose concentrations fell. Lactate release was decreased. These findings permit us to postulate that the reduction of ΣST in the ischaemic area could be related to these local metabolic changes.

The stimulation of glucose metabolism by DCA is in agreement with the observations of McAllister *et al* (1973) who studied alloxan-diabetic dogs and healthy dogs in which FFA concentrations had been raised by infusions of a triglyceride emulsion and heparin. The increase in myocardial glucose utilization was attributed by these workers to a stimulation of phosphofructokinase and to the activation of pyruvate dehydrogenase. The reduction in arterial lactate concentrations is also in agreement with earlier studies in dogs and rats (McAllister *et al,* 1973; Blackshear *et al,* 1974). In functionally hepatectomized rats it was shown that the decrease in arterial lactate concentrations is due to a reduced release of lactate from extrahepatic tissues (Blackshear *et al,* 1974). This and the observation that DCA increases pyruvate dehydrogenase activity in muscle (Whitehouse and Randle, 1973) may explain the decrease in arterial lactate concentration. The decrease in total lactate extraction by the heart accords with its known arterial concentration-extraction relationship (Kaijser *et al,* 1972). The decrease in lactate release could also be explained by the increased activity of pyruvate dehydrogenase and/or decreased arterial lactate concentrations.

The metabolism of the acutely ischaemic or hypoxic myocardium has been reviewed (Opie, 1970; Kübler and Spieckermann, 1970; Oliver, 1972; Opie, 1972). In both situations the oxidation of glucose, FFA, and lactate by the heart is immediately impaired. The consequent decline in the intracellular concentrations of ATP and citrate activates phosphofructokinase, which in turn stimulates glycolysis, resulting in increased lactic acid and ATP production. During hypoxia but normal perfusion, when the excess lactic acid can be adequately cleared, the stimulation of anaerobic glycolysis in this way can supply up to 90% of myocardial energy requirements in the presence of extracellular glucose (Kübler and Spieckermann, 1970). Following coronary occlusion, however, lactic acid accumulates within the ischaemic tissue due to inadequate removal, and the consequent fall in intracellular pH produces a secondary inhibition of phosphofructokinase and a decline in ATP production. For this reason, glycolysis is normally unable to supply more than 70% of myocardial energy requirements during ischaemia (Kübler and Spieckermann, 1970).

A considerable degree of glucose oxidation persists in the ischaemic myocardium (Opie *et al,* 1973), and DCA could have increased the capacity of ischaemic cells to metabolize glucose aerobically. Other mechanisms whereby DCA may have reduced ischaemic injury include the inhibition of FFA metabolism and an enhancement of lactate removal from the ischaemic cell secondary to the reduction in arterial lactate concentration. Therefore, no certain conclusions concerning the precise mechanism whereby DCA reduces ST-segment elevation can be drawn at this stage.

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References

Blackshear, P. J., Holloway, P. A. H., and Alberti, K. G. M. M. (1974). The metabolic effects of sodium dichloroacetate in the starved rat. *Biochemical Journal,* **142,** 279–286.

Boberg, J. (1966). Separation of labeled plasma and tissue lipids by thin layer-chromatography. A quantitative methodological study. *Clinica Chimica Acta,* **14,** 325–334.

Hohorst, H. J. (1963). L-(+)-lactate determination with lactic acid dehydrogenase and DPN. In *Methods of Enzymatic Analysis,* ed. by H. U. Bergmeyer, pp. 266–270. Academic Press: London.

Kaijser, L., Lassers, B. W., Wahlquist, M. L., and Carlson, L.A. (1972). Myocardial lipid and carbohydrate metabolism in fasting men during prolonged exercise. *Journal of Applied Physiology,* **32,** 847–858.

Kjekshus, J. K., and Mjøs, O. D. (1972). Effect of free fatty acids on myocardial function and metabolism in the ischemic dog heart. *Journal of Clinical Investigation,* **51,** 1767–1776.

Kjekshus, J. K., and Mjøs, O. D. (1973). Effect of inhibition of lipolysis on infarct size after experimental coronary artery occlusion. *Journal of Clinical Investigation,* **52,** 1770–1778.

Kübler, W., and Spieckermann, P. G. (1970). Regulation of glycolysis in the ischemic and the anoxic myocardium. *Journal of Molecular and Cellular Cardiology,* **1,** 351–377.

Kurien, V. A., and Oliver, M. F. (1970). A metabolic cause for arrhythmias during acute myocardial hypoxia. *Lancet,* **1,** 813–815.

Kurien, V. A., Yates, P. A., and Oliver, M. F. (1971). The role of free fatty acids in the production of ventricular arrhythmias after acute coronary artery occlusion. *European Journal of Clinical Investigation,* **1,** 225–241.

Lassers, B. W., Carlson, L. A., Kaijser, L., and Wahlquist, M. (1972). The nature and control of myocardial substrate metabolism in healthy man. In *Effect of Acute Ischaemia on Myocardial Function,* ed. by M. F. Oliver, D. G. Julian, and K. W. Donald, pp. 200–212. Churchill Livingstone: Edinburgh.

Leiris, J. de, Bricknell, O., and Opie, L. H. (1974). Effect of substrate on enzyme release after coronary artery ligation in the isolated rat heart. *European Journal of Clinical Investigation,* **4,** 346.

Libby, P., Maroko, P. R., Bloor, C. M., Sobel, B. E., and Braunwald, E. (1973). Reduction of experimental myocardial infarct size by corticosteroid administration. *Journal of Clinical Investigation,* **52,** 599–607.

Lorini, M., and Ciman, M. (1962). Hypoglycaemic action of diisopropylammonium salts in experimental diabetes. *Biochemical Pharmacology,* **11,** 823–827.

McAllister, A., Allison, S. P., and Randle, P. J. (1973). Effects of dichloroacetate on the metabolism of glucose, pyruvate, acetate, 3-hydroxybutyrate and palmitate in rat diaphragm and heart muscle *in vitro* and on extraction of glucose, lactate, pyruvate and free fatty acids by dog heart *in vivo*. *Biochemical Journal,* **134,** 1067–1081.

Maroko, P. R., Kjekshus, J. K., Sobel, B. E., Watanabe, T., Covell, J. W., Ross, J., and Braunwald, E. (1971). Factors influencing infarct size following experimental coronary artery occlusions. *Circulation,* **43,** 67–82.

Maroko, P. R., Libby, P., Sobel, B. E., Bloor, C. M., Sybers, H. D., Shell, W. E., Covell, J. W., and Braunwald, E. (1972). Effect of glucose-insulin-potassium infusion on myocardial infarction following experimental coronary artery occlusion. *Circulation,* **45,** 1160–1175.

Mjøs, O. D., Miller, N. E., Riemersma, R. A., and Oliver, M. F. (1976). Effects of p-chlorophenoxyisobutyrate on myocardial free fatty acid extraction, blood flow and ischemic injury during experimental coronary occlusion in dogs. *Circulation.* (In press.)

Nimmo, I. A., Kirby, B. J., and Lassers, B. W. (1973). Rapid oscillations in blood glucose and plasma free fatty acid concentrations in man. *Journal of Applied Physiology,* **34,** 448–451.

Oliver, M. F. (1972). Metabolic response during impending myocardial infarction. II. Clinical implications. *Circulation,* **45,** 491–500.

Opie, L. H. (1970). The glucose hypothesis: relation to acute myocardial ischaemia. *Journal of Molecular and Cellular Cardiology,* **1,** 107–115.

Opie, L. H. (1972). Metabolic response during impending myocardial infarction. I. Relevance of studies of glucose and fatty acid metabolism in animals. *Circulation,* **45,** 483–490.

Opie, L. H., Owen, P., and Riemersma, R. A. (1973). Relative rates of oxidation of glucose and free fatty acids by ischaemic and non-ischaemic myocardium after coronary artery ligation in the dog. *European Journal of Clinical Investigation,* **3,** 419–435.

Owen, P., Thomas, M., Young, V., and Opie, L. (1970). Comparison between metabolic changes in local venous and coronary sinus blood after acute experimental coronary arterial occlusion. *American Journal of Cardiology,* **25,** 562–570.

Passonneau, J. V. (1974). L−(+)−Lactate. A fluorimetric method. In *Methods of Enzymatic Analysis,* 2nd edition, ed. by H. U. Bergmeyer, pp. 1468–1472. Academic Press: London.

Sayen, J. J., Sheldon, W. F., Peirce, G., and Kuo, P. T. (1958). Polarographic oxygen, the epicardial electrocardiogram, and muscle contraction in experimental acute regional ischemia of the left ventricle. *Circulation Research,* **6,** 779–798.

Sonnenblick, E. H., Ross, J., and Braunwald, E. (1968). Oxygen consumption of the heart: newer concepts of its multifactoral determination. *American Journal of Cardiology,* **22,** 328–336.

Stacpoole, P. W., amd Felts, J. M. (1970). Diisopropylammonium dichloroacetate (DIPA) and sodium dichloroacetate (DCA): effect on glucose and fat metabolism in normal and diabetic tissue. *Metabolism,* **19,** 71–78.

Stacpoole, P. W., and Felts, J. M. (1971). Diisopropylammonium dichloroacetate: regulation of metabolic intermediates in muscle of alloxan diabetic rats. *Metabolism,* **20,** 830–834.

Toyoshima, A., Prinzmetal, M., and Horiba, M., Koboyashi, T., Mizuno, Y., Nakayama, R., and Yamada, K. (1965). Nature of normal and abnormal electrocardiograms. VIII. Relation of ST segment and T-wave changes to intracellular potentials. *Archives of Internal Medicine,* **115,** 4–16.

Trout, D. L., Estes, E. H., and Friedberg, S. J. (1960). Titration of free fatty acids of plasma: a study of current methods and a new modification. *Journal of Lipid Research,* **1,** 199–202.

Utley, J., Carlson, E. L., Hoffman, J. I. E., Martinez, H. M., and Buckberg, G. D. (1974). Total and regional myocardial blood flow measurements with 25 μ, 15 μ, 9 μ and filtered 1–10 μ diameter microspheres and antipyrine in dogs and sheep. *Circulation Research,* **34,** 391–405.

Wégria, R., Segers, M., Keating, R. P., and Ward, H. P. (1949). Relationship between the reduction in coronary flow and the appearance of electrocardiographic changes. *American Heart Journal,* **38,** 90–96.

Wendt, R. L., Canavan, R. C., and Michalak, R. J. (1974). Effects of various agents on regional ischemic myocardial injury: electrocardiographic analysis. *American Heart Journal,* **87,** 468–482.

Whitehouse, S., and Randle, P. (1973). Activation of pyruvate dehydrogenase in perfused rat heart by dichloroacetate. *Biochemical Journal,* **134,** 651–653.

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Appendix G

Figure 6.01

Drugs used were:

β-pyridylcarbinol	Kjekshus and Mjøs, 1973
nicotine	Ilebekk and Mjøs, 1974
isoprenaline	Lekven et al, 1973
prostaglandin-E_1	Section 3.2
p-chlorophenoxyisobutyrate	Section 3.1
5-fluoronicotinyl-alcohol	Riemersma, unpublished results
noradrenaline	Mjøs et al, 1974
acebutolol	Section 3.3
nicotinic acid	Vik-Mo, 1977
sodium salicylate	Vik-Mo and Mjøs, 1977; Vik-Mo, 1977
fat free albumin	Miller et al, 1976

Antilipolytic agents were often tested in the presence and absence of isoprenaline.