

THE PASSAGE OF FATTY ACID
IN THE MYOCARDIAL CELL

by

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ABSTRACT

Two parallel and complementary studies have been carried out to examine the short-term biochemical and micro-anatomical pathways of radioactive oleic acid and erucic acid after pulse perfusion in the isolated, perfused guinea pig heart. Perfusions were carried out at 37°C and, with oleic acid only, also at 5°C and after 30 minutes hypoxia at 37°C.

Firstly, the incorporation of the radioactive fatty acid into tissue lipids was studied using freeze-clamping followed by lipid analysis. $^{14}\text{CO}_2$ production was studied as a measure of fatty acid oxidation. Secondly, the subcellular distribution of the fatty acid was determined by EM autoradiography. In preliminary experiments several methods of processing tissue for electron microscopy were assessed to maximise lipid retention within the tissue. Water-soluble Durcupan was found to be the most suitable resin. The autoradiographs were analysed using a modified "hypothetical grain" method.

Using oleic acid, triacylglycerol and phospholipid radioactivity increased with time at 37°C but not at 5°C when esterification was inhibited. Autoradiography showed that oleic acid moves rapidly to the mitochondria but incorporation into lipid droplets occurs more gradually and persistently. After 30 minutes hypoxia incorporation of radioactive oleic acid into acylglycerol was earlier and more intense. The well-oxygenated hearts produced approximately eleven times more $^{14}\text{CO}_2$ than the hypoxic hearts. Autoradiography showed a decreased incorporation of radioactivity into the mitochondria and earlier and more intense incorporation into the lipid droplets. These experiments showed the rapid switch from oxidation to esterification when the cells become hypoxic.

Erucic acid, a cardiotoxic fatty acid found in rapeseed oil, was metabolised much slower than oleic acid. Less oxidation occurred although erucic acid was transported to the mitochondria. The rate-limiting step was thought to be the activation of erucic acid to erucyl-CoA. A novel mechanism of expulsion of lipid droplets from the cells of hearts perfused with erucic acid was seen and assessed quantitatively.

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Radioactive oleic acid from the Radiochemical Centre (now Amersham International, Amersham, Bucks). Radioactive erucic acid from C.E.A. (France).

Bovine serum albumin, erucic acid, all lipid standards for thin-layer chromatography and para-phenylenediamine from Sigma (London) Chemical Co. Ltd. (Poole, Dorset).

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Objectives

- (1) To trace the biochemical and microanatomical pathways of oleic acid in the isolated, perfused guinea pig myocardium in the first two minutes after entry into the cells in well-oxygenated conditions at 37°C.
- (2) To compare these pathways with those occurring in hypothermic conditions (5°C).
- (3) To compare the pathways in (1) with those in hearts subjected to 30 minutes of hypoxic perfusion.
- (4) To study the biochemical and microanatomical distribution of the cardiotoxic fatty acid erucic acid, up to 30 minutes after entry into the cells and to compare these pathways with those of oleic acid.

The experiments described in this thesis were designed to answer the question of what happens to fatty acids taken up by the cells of the isolated, perfused guinea pig heart in the first minutes after uptake - where do they go to and in what form? As fatty acids are the main fuel for oxidation in the myocardium, their movements are of considerable importance and interest and the ability of the myocardial cell to direct fatty acids to the site of oxidation is crucial to its normal functioning. The study arose from some previous work in our laboratory in which the incorporation of radioactive palmitic acid and oleic acid into tissue lipids was examined in the isolated, perfused rat heart (Gloster et al., 1978). The method used was homogenisation of the hearts and separation of the subcellular fractions by differential centrifugation. The radioactivity in different lipid classes was then determined in each fraction by thin layer chromatography. These authors recognised, and stated clearly in their publications the limitations in their methods (Fields et al., 1975, Gloster et al., 1978). Considerable esterification and transfer between fractions occurred during the procedure. Although, using this method, results can still be compared in different conditions if the assumption is made that the degree of esterification and transfer between fractions remains constant, an absolute measure of the incorporation of the radioactive fatty acid into tissue lipids or subcellular fractions cannot be obtained unless any further metabolism, after the requisite time interval, is arrested. This was achieved in the present study by the introduction of the freeze-clamping method whereby the tissue was frozen almost instantaneously at the end of the perfusion and the lipids were extracted directly from the frozen tissue. The problem of the subcellular location of the radioactive fatty acid was approached in a different way. Instead of using cell fractions, the technique of electron

microscope autoradiography was applied which has the advantage of allowing the experimenter to look at intact tissue rather than disrupted tissue. A study of this sort was undertaken in 1968 by Stein and Stein who used subcellular fractions as well as EM autoradiography to determine the fate of ^3H oleic acid in the isolated, perfused rat heart. However the methods used for this autoradiographic study are also subject to criticism. Firstly, the method of analysis of autoradiographs did not take into account the effect of 'cross-fire' from one organelle to another, and the ascribing of any grain overlapping the mitochondria by one third to the sarcoplasmic reticulum whether or not elements of sarcoplasmic reticulum could be seen, was considered to be suspect.

Three parallel and complementary studies were therefore carried out in order to extend these experiments using more reliable techniques. Firstly the incorporation of ^3H oleic acid into tissue lipids was studied up to two minutes after a 15 second pulse with the radioactive fatty acid. This was achieved using freeze-clamping followed by lipid extraction and thin layer chromatography to separate the main lipid classes. Secondly, the degree of oxidation of the radioactive fatty acid was assessed by perfusing with ^{14}C - labelled oleic acid and measuring the $^{14}\text{CO}_2$ produced. Thirdly, using the same perfusion times as in the biochemical studies, EM autoradiography was undertaken to locate the subcellular sites of the radioactive fatty acid. The resulting autoradiographs were analysed using the hypothetical grain method of Blackett and Parry (1973). Before embarking on this third part of the study, considerable preliminary work had to be performed in order to find a method of processing cardiac muscle for electron microscopy which gave adequate lipid retention to make the autoradiography feasible.

Using these techniques a comprehensive picture emerged of the biochemical and anatomical fate of the radioactive fatty acid up to ten minutes after entering the cells. The degree to which the results were dependent on temperature was examined by repeating the series at 5°C. Having achieved this objective the next logical step seemed to be to see how this short-term metabolism differed when the cells were made hypoxic as this is one condition which is known to drastically alter the oxidative metabolism of the cell, in particular fatty acid oxidation which is highly dependent on a constant supply of oxygen. The length of hypoxia (30 minutes) was determined as one in which marked biochemical changes occurred but few ultrastructural changes so that the analysis of autoradiographs would be carried out on tissue which was morphologically similar to the well-oxygenated series in order to highlight any changes in the distribution of the radioactive fatty acid.

Finally, it was decided to study a cardiotoxic fatty acid, erucic acid, using the same methods but prolonging the maximum perfusion to 30 minutes when it emerged that erucic acid is metabolised very slowly compared with oleic acid. Erucic acid can be found in margarines made from rapeseed oil although in recent years an attempt has been made to keep the erucic acid content of rapeseed oil used for margarine to a minimum. There has been considerable interest in erucic acid recently and a fuller understanding of its metabolism is needed to explain its cardiotoxic effect.

PART ONE

THE INCORPORATION OF ^3H OLEIC ACID INTO TISSUE LIPIDS

CHAPTER 1 - INTRODUCTION

Fatty acid metabolism in the heart - a brief biochemical outline

Cardiac muscle can adapt to the use of a variety of different substrates as sources of energy. Intermittent feeding and changes in nutritional composition have brought about this versatility. Fatty acids, glucose, lactate, pyruvate, ketone bodies and, to a lesser extent, amino acids may all be used for energy production and, in a well-oxygenated heart, all of these substrates may be completely oxidised.

It was first recognised in 1954 by Bing and his associates that the human heart prefers fatty acids to any other substrate in the post-absorptive state. Under most conditions fatty acids are oxidised in preference to carbohydrates (Shipp et al., 1961, Bowman, 1962, Newsholme et al., 1962, Neely et al., 1969) and their oxidation normally accounts for approximately 60 - 70% of oxidative phosphorylation in the heart (Evans, 1964, Bing, 1965, Opie, 1968).

This introduction describes the major pathways of fatty acid metabolism in the heart and the factors which regulate or alter this metabolism. The pathways are summarised in Figure 1.

Fatty acids in the blood.

As lipids are virtually insoluble in aqueous media their transport in the blood is accomplished by binding to a hydrophilic protein. Fatty acids are carried in a fatty acid-albumin complex and to a much lesser extent by lipoproteins, which are also the transport vehicles for triacylglycerol, phospholipid and cholesterol.

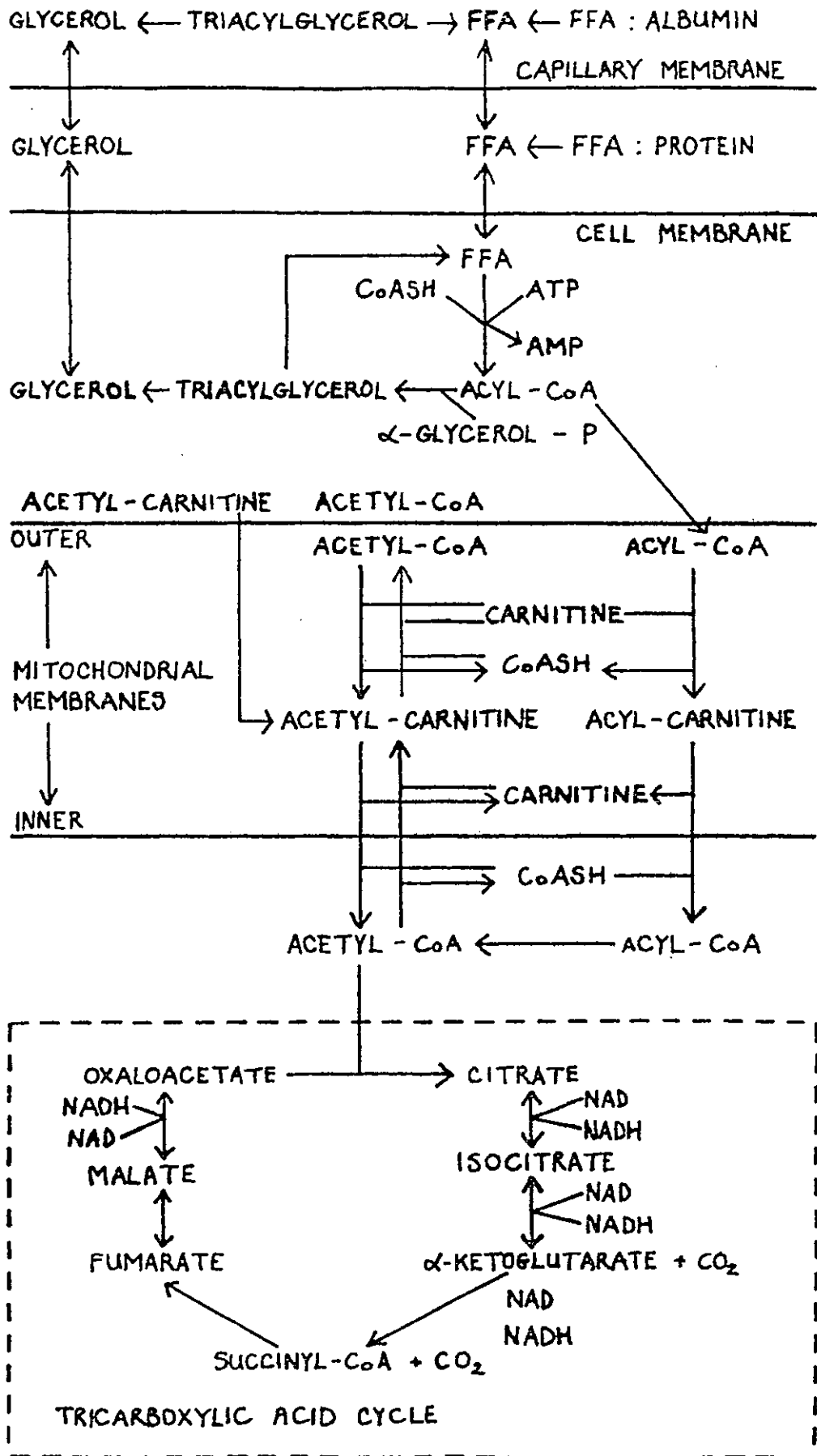


Figure 1. Fatty acid pathways in the heart. (Neely and Morgan, 1974)

Almost all the free fatty acid released into the blood from adipose tissue is bound to albumin. Albumin can increase the solubility of free fatty acid, in a solution with a salt concentration similar to that of plasma, by as much as 500 times. The interaction between long chain fatty acids and human serum albumin has been described by Goodman (1958) and Arvidsson (1965). The binding properties of long chain fatty acids to bovine serum albumin, the carrier protein most often used in experimental work, have been described by Spector et al. (1969). Their data showed that each molecule of bovine serum albumin contains six high affinity binding sites for long chain free fatty acids and a large number of weaker binding sites. Under normal, physiological conditions however, the molar ratio of free fatty acid to albumin rarely exceeds 3.

Although it is tightly bound to albumin, the free fatty acid can be taken up rapidly by mammalian cells. The half life of circulating free fatty acids is very short, ranging from 1 to 3.9 minutes (Fredrikson and Gordon, 1958, Eaton et al., 1969).

Esterified fatty acids are carried in the blood as lipoprotein complexes of which there are four main classes:

1. Chylomicrons, which are the vehicles for transporting dietary fat from the intestine, mainly to the liver and adipose tissue. They consist of large amounts of triacylglycerol enclosed in a protein and phospholipid coat.
2. High density lipoproteins which carry phospholipids and cholesterol.
3. Low density lipoproteins which are the main cholesterol carriers of the blood.
4. Very low density lipoproteins which transport triacylglycerols which have been synthesised by the liver.

Fatty acid uptake

Dissociation of the fatty acid - albumin complex and binding of the fatty acid to a site on the cell membrane is extremely rapid, in the order of milliseconds (Spector et al., 1965, Donabedian and Karman, 1967) and has been shown to be a reversible process (Donabedian and Karman, 1967). It is thought that the unbound fatty acid is in equilibrium with the fatty acid bound to albumin. As unbound molecules are taken up by the cells the unbound pool is replenished by the dissociation of albumin - free fatty acid complexes to maintain equilibrium (Spector, 1968).

Uptake of fatty acids by the cells is thought to be mainly an energy-independent process (Stein and Stein, 1963, Evans, 1964, Vaughan et al., 1964, Strauss, 1966) although an enzyme mediated, energy-dependent process was suggested by Shohet et al. (1968). Munday et al. (1969) observed that both energy-independent and energy-dependent transport processes occur with the latter dependent on the presence of calcium.

Phospholipids may be taken up intact (Stein and Stein, 1967, Marcus and Cass, 1969, Peterson and Rubin, 1969, 1970). Triacylglycerols are hydrolysed either in the serum (Howard and Kritchevsky, 1969) or on the cell surface or capillary wall (Higgins, 1967, Kuhl and Spector, 1970) and it is the fatty acids released by the hydrolysis which are taken up by the cells.

There are several factors which regulate the uptake of fatty acids. These are:

1. The total concentration of circulating fatty acids. This has been found to be true in both 'in vivo' studies (Ballard et al., 1960, Carlsten et al., 1961, Scott et al., 1962) and in isolated, perfused heart preparations (Evans et al., 1963, Willebrands, 1964). When the

concentration of free fatty acid in the perfusate is low, free fatty acid is released from the heart (Willebrands, 1964) which suggests that an equilibrium exists between free fatty acids inside and outside the cells.

2. The molar ratio of free fatty acid to albumin. As this ratio increases more free fatty acid is taken up by the cells (Evans et al., 1963, Shtacher and Shafrir, 1963, Spector, 1968, Spector et al., 1970).

3. The molecular structure of the fatty acids. Stein and Stein (1963) found that there was no difference in the extraction of palmitate, stearate, oleate or linoleate from the medium by isolated, perfused rat hearts. However this has not been confirmed by other workers and it is generally recognised that the uptake of fatty acids increases as chain length increases. There have been several conflicting reports on the effect of the degree of saturation of the fatty acid on its uptake by the cells. Various isolated cells in culture take up saturated fatty acids in preference to unsaturated ones (Spector and Steinberg, 1967, Donabedian and Karman, 1967, Spector et al., 1970). Studies on isolated organs, including the heart, have shown a preference for the uptake of unsaturated fatty acids, although there is some dispute as to whether monounsaturated fatty acids are taken up in preference to either saturated or polyunsaturated fatty acids (Rothlin and Bing, 1961, Evans, 1964, Willebrands, 1964) or whether polyunsaturated fatty acids are taken up to a greater extent than saturated or monounsaturated (Vasdev and Kako, 1977).

4. The rates of oxidation and esterification of fatty acids. These may affect uptake by changing the rate of clearance of fatty acid from intracellular binding sites (Evans, 1964). Oram et al. (1973) found that, in isolated, perfused rat hearts, the rate of palmitate uptake at high levels of exogenous palmitate was limited by the rate of acetyl-CoA oxidation through the tricarboxylic acid cycle.

5. The pH of the perfusing medium. A decrease in pH from 7.4 to 6.5 decreased uptake and this is thought to be a result of a pH - induced weakening of the fatty acid binding to albumin (Spector et al., 1969, Spector, 1969).
6. The presence of calcium ions. This may increase fatty acid uptake (Munday et al., 1969).

Fatty acid binding proteins

Proteins are thought to be involved in the transport of fatty acids not only in the plasma, but also within the cells. A protein with a high affinity for binding fatty acids has been identified in several tissues including myocardium and skeletal muscle (Ockner et al., 1972, Mishkin et al., 1972). It is thought that there may be more than one protein involved, all having a molecular weight of approximately 12,000. These are generally referred to collectively as Z protein.

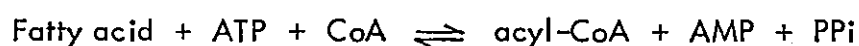
Although the binding of fatty acids to intracellular protein is not an obligatory step in their uptake (Mishkin et al., 1975) the presence of the binding protein has been found to increase the uptake of fatty acids by isolated hepatocytes (Wu-Rideout et al., 1976). In the liver, Z protein has been found to play a part in the regulation of fatty acid synthesis (Lunzer et al., 1976) and it stimulates the esterification of fatty acids to triacylglycerol in the liver and intestinal mucosa (Mishkin and Turcotte, 1974, O'Doherty and Kuksis, 1975). Fournier et al. (1978) showed that, in a solution containing fatty acids bound to rat heart Z protein and isolated rat heart mitochondria, fatty acids were transferred to the mitochondria. It was therefore suggested that, 'in vivo', fatty acids may be transferred from the cytoplasm to the mitochondrial β -oxidative system by the Z protein.

Fatty acid utilisation

Those fatty acids taken up by the cells which are not oxidised immediately are incorporated into tissue lipids, which include acyl-glycerols, phospholipids and cholesterol esters. Whichever pathway is entered the fatty acid must first be 'activated' to a molecule of higher energy.

Fatty acid activation

The first stage in both the β -oxidation pathway and the esterification pathway is the activation of the fatty acid to form the higher energy acyl-CoA derivative. The mechanism of this activation has been demonstrated by Berg (1956), Boyer et al. (1959) and Webster (1963) and can be represented by the following equation:-



The reaction is catalysed by an acyl-CoA synthetase and requires Mg^{++} . It is stimulated by other cations, especially K^+ . The reaction is reversible and involves the formation of an enzyme-bound acyl adenylate intermediate (Bar-Tana et al., 1972). At least six acyl-CoA synthetases, both soluble and membrane bound, have been described. These are distinguished from one another on the basis of the chain length of the fatty acid to be activated although these enzymes seem to have a broad range of specificity with respect to the fatty acid substrate. For example, palmityl-CoA synthetase activates fatty acid of chain length from C_{12} to C_{22} , both saturated and unsaturated. It is so named because its activity is greatest with palmitate as substrate. Palmityl-CoA synthetase is situated mainly on the outer surface of the outer mitochondrial membrane in the heart and to a lesser extent on the sarcoplasmic reticulum (De Jong and Hülsmann, 1970, Pande and Blanchaer, 1970)

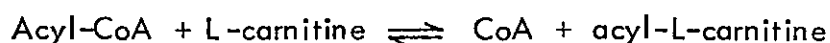
The role of carnitine

Acyl-CoA, formed by the activation of fatty acids, is the substrate for β -oxidation, which occurs in the mitochondrial matrix. As acyl-CoA cannot cross the inner mitochondrial membrane the acyl group is transferred to carnitine (β -hydroxy- γ -trimethylamino-butyric acid) which acts as a carrier across the inner membrane.

In heart muscle, which uses fatty acids as the major fuel for oxidation, carnitine is present in very high concentrations when compared with other tissues (Broekhuysen et al., 1965, Pearson and Tubbs, 1967). Fritz (1955) discovered that carnitine stimulates the oxidation of long chain fatty acids in rat liver slices and homogenates. This was also shown to be true in heart muscle (Fritz et al., 1962). Short and medium chain fatty acids can cross the inner mitochondrial membrane and are activated within the inner mitochondrial matrix. Their subsequent oxidation is carnitine independent (Fritz, 1968, Bressler, 1970). However, oxidation of long chain fatty acids by heart muscle is completely dependent on the presence of carnitine (Fritz et al., 1962, Bode and Klingenberg, 1964). It has been shown (Bressler and Wittels, 1965) that carnitine depletion leads to triacylglycerol accumulation, as available free fatty acid cannot be used for β -oxidation and is consequently esterified.

The exact mechanism by which carnitine transports acyl groups across the inner mitochondrial membrane has been the subject of a great deal of research and controversy in recent years. A few points have been generally accepted.

The transfer of the acyl group from CoA to carnitine can be summarised as follows:-



The reaction is catalysed by a carnitine acyltransferase. Two carnitine

acyl-transferases have been purified and characterised. These are carnitine acetyl-transferase (Chase et al., 1965) and carnitine palmityl-transferase (Kopec and Fritz, 1973). There is evidence for the existence of a third carnitine acyl-transferase with an optimal substrate chain length of C₇₋₈ (Solberg, 1970).

Yates and Garland (1966) postulated that if acyl-carnitines carry acyl units across the inner mitochondrial membrane, there must be an 'inner' and an 'outer' location for the carnitine acyl-transferases. This was verified by Yates and Garland (1970). They found that the activity of the enzyme with acyl-CoA as substrate was greatly increased by the sonication of rat liver and beef heart mitochondria indicating that the bulk, but not all, of the activity was present in a compartment not normally accessible to acyl-CoA. It is not at present known whether the 'inner' and 'outer' carnitine palmityl-transferases are two different enzymes or one enzyme in two locations.

A second transport system for fatty acids into the mitochondria has been put forward in recent years. It is based on the observation that ox heart mitochondria contain carnitine within the matrix (Ramsay and Tubbs, 1974) and a carnitine-acyl carnitine exchange carrier - 'carnitine translocase' (Ramsay and Tubbs, 1975, Pande, 1975). The translocase acts by exchange diffusion and exports carnitine to the outer carnitine acyl-transferase while transporting acyl-carnitine to the inner carnitine acyl-transferase. A rapid exchange of cytosolic and mitochondrial matrix carnitine takes place in a 1:1 ratio (Pande and Parvin, 1976).

The transport mechanisms are summarised in Figure 2.

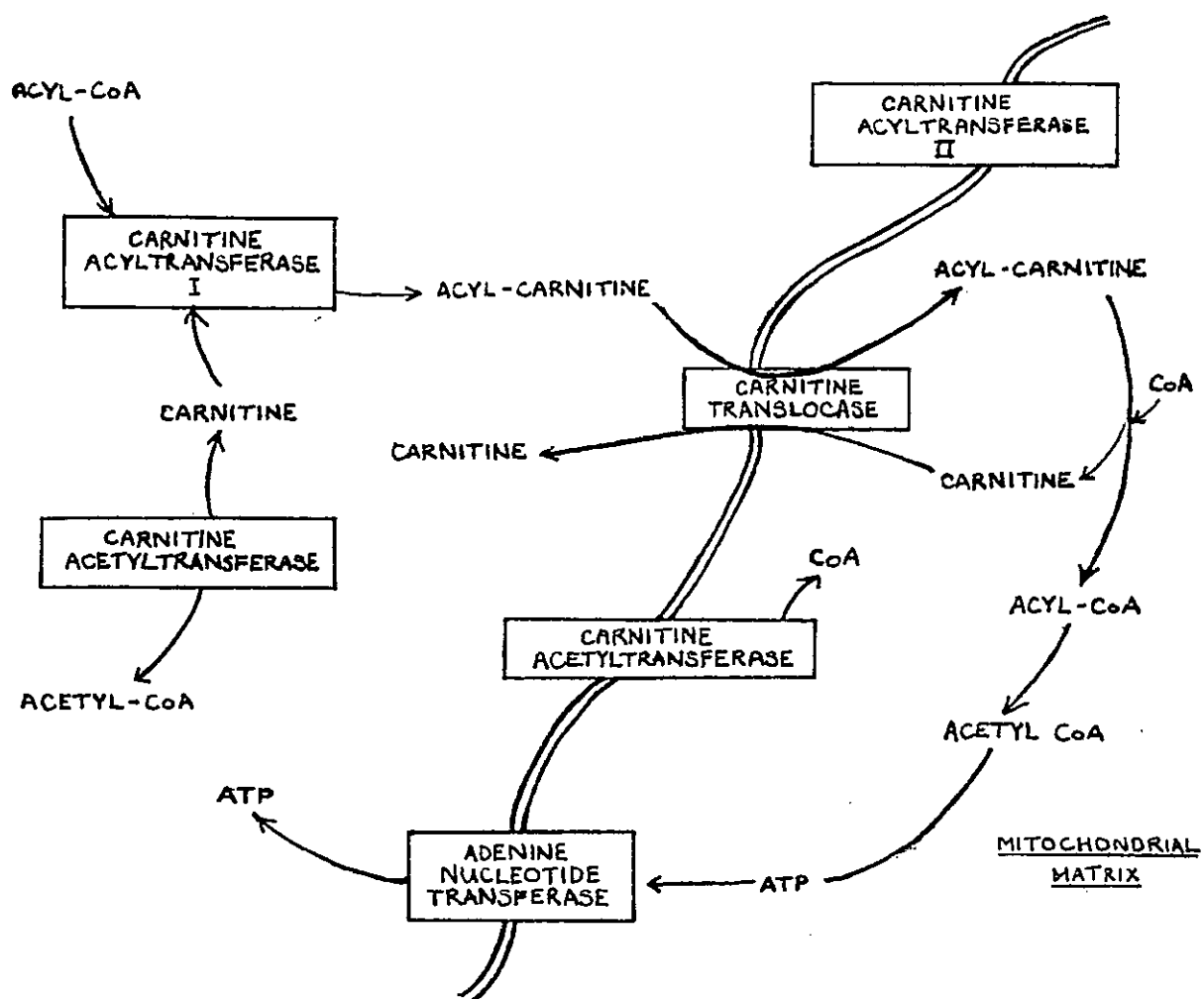


Figure 2

The transfer of fatty acid into the mitochondria (from Opie, 1979)

The double line represents the inner mitochondrial membrane.

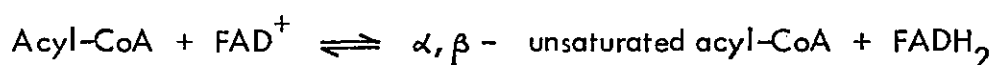
β -oxidation

In 1904, Knoop, a German biochemist, observed that when rabbits were fed an even carbon fatty acid which was labelled with a phenyl group at the methyl terminal, phenylacetic acid, $((C_6H_{12}) - CH_2 - COOH)$ was excreted in the urine. This occurred regardless of the chain length of the fatty acid. On the other hand, when he fed the rabbits odd chain fatty acids, again with a phenyl group at the methyl end of the molecule, benzoic acid $((C_6H_{12}) - COOH)$ was excreted. This suggested that phenyl-labelled fatty acids are degraded by oxidative removal of successive 2-carbon fragments starting from the carboxyl end. From these experiments Knoop suggested that fatty acids are oxidised by β -oxidation, which is so named because cleavage of the molecule occurs at the β -carbon.

β -oxidation was first demonstrated in 1943 by Munoz and Leloir. Shortly afterwards Lehninger (1945) showed that ATP is required for the oxidation of fatty acids. He also showed (Lehninger, 1946) that fatty acids are oxidised to yield 2-carbon units that can enter the tri-carboxylic acid cycle. In 1948 Kennedy and Lehninger showed that oxidation of fatty acids occurs exclusively in the mitochondria. Lynen and Ochoa (1953) found that after activation of the fatty acid to form acyl-CoA, all subsequent oxidative steps take place with the fatty acid in the form of its CoA thioester.

The pathway of β -oxidation consists of four steps:-

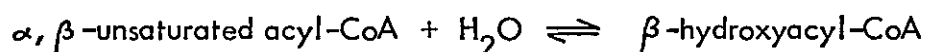
1. First dehydrogenation



The fatty acyl-CoA is dehydrogenated by the removal of two hydrogen atoms from the α and β carbon atoms to yield α, β -unsaturated acyl-CoA.

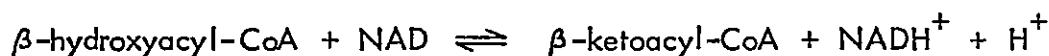
This reaction is catalysed by an acyl-CoA dehydrogenase, of which four have been described, each specific for a given range of fatty acid chain lengths. All contain the coenzyme FAD tightly bound. The FAD becomes reduced at the expense of the acyl-CoA substrate and is re-oxidised by the respiratory chain.

2. Hydration



The double bond is hydrated to form β -hydroxyacyl-CoA. This is catalysed by the enzyme enoyl-CoA hydratase.

3. Second dehydrogenation



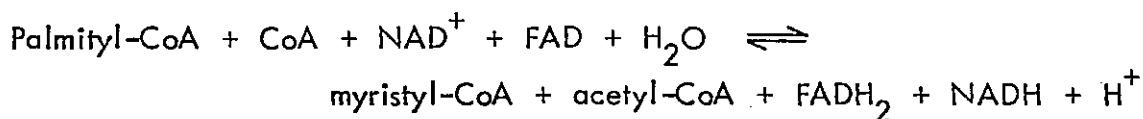
The β -hydroxyacyl-CoA undergoes further dehydrogenation on the β -carbon. NAD^+ is the electron acceptor. The enzyme involved is β -hydroxyacyl-CoA dehydrogenase, which is relatively non-specific with respect to fatty acid chain length.

4. Cleavage



The β -ketoacyl-CoA undergoes cleavage by interaction with a molecule of free CoA to yield a 2 carbon fragment from the carboxyl end as acetyl-CoA which enters the tricarboxylic acid cycle. The cleavage reaction is catalysed by acetyl-CoA acetyltransferase which is commonly known as thiolase. The remaining fatty acid is now shorter by 2 carbon atoms and reenters the β -oxidation pathway.

The overall equation for the pathway with palmityl CoA as substrate is as follows:-



The 16-carbon palmitic acid undergoes a total of 7 such cycles, to yield 8 molecules of acetyl-CoA. The palmitate is activated once only, since at the end of each cycle the shortened fatty acid appears as its CoA thioester.

Regulation of β -oxidation

The overall rate of β -oxidation in the heart is determined by the supply of exogenous fatty acid and by the energy demands of the tissue. When the rate of energy utilisation is constant, an increased supply of fatty acids results in a limited increase in uptake. The upper limit is reached when the supply of fatty acids exceeds the capacity of the cells to oxidise the fatty acids or to convert them to complex lipids (Neely et al., 1972). Conversely, at a constant level of exogenous fatty acid, increased cardiac work results in (a) a faster rate of oxidative phosphorylation indicated by increased oxygen consumption, (b) an acceleration of the tricarboxylic acid cycle, indicated by increased carbon dioxide production. Both of these factors result in (c) an increase in fatty acid uptake and β -oxidation. (Oram et al., 1973).

The concentrations of both carnitine and CoA affect the rate of β -oxidation. Fritz et al., (1962) showed that the oxidation of palmitate by whole heart homogenates was stimulated 3-fold by an increase in CoA concentration from 0.01 to 0.1 mM at a constant carnitine level. Pande, (1971) demonstrated that 1.5 mM carnitine produced the maximum rate of acyl-CoA oxidation by isolated rat heart mitochondria. Oram et al., (1973) suggested that the capacity to translocate acyl units across the inner mitochondrial membrane limits the oxidation of long chain

acyl-carnitine. This was based on the observation that an increase in oxygen consumption and in β -oxidation, caused by an increase in cardiac work, resulted in an accumulation of long chain acyl-carnitine in isolated, perfused rat hearts.

The rate of β -oxidation is closely linked to the rate of the tricarboxylic acid cycle. Oram et al., (1973) proposed that the carnitine acetyl-transferase system plays an important role in coupling the rate of fatty acid uptake to the tricarboxylic acid cycle. By transferring acetyl units formed in the mitochondrial matrix from mitochondrial to cytosolic CoA, the carnitine acetyl-transferases may limit the rate of oxidation of acetyl units through the tricarboxylic acid cycle and the rate of fatty acid activation in the cytosol.

Although β -oxidation and the tricarboxylic acid cycle are closely linked, there is competition between the two pathways for CoA, NAD^+ and FAD^+ (Hochachka et al., 1977). The rate of β -oxidation may exceed the rate of the tricarboxylic acid cycle, for example in the liver, where this leads to the production of ketone bodies. On the other hand, the tricarboxylic acid cycle activity may exceed that of β -oxidation in many tissues, including cardiac muscle, in situations where the supply of glucose is high and levels of circulating fatty acids are low.

When the oxygen supply to the heart is low, NADH and FADH_2 accumulate (Boxer and Devlin, 1961). This causes a reduction of β -oxidation at the FAD^+ - and NAD^+ dependent dehydrogenase reactions and long chain acyl-CoA, β -hydroxypalmitate and short chain hydroxy fatty acids accumulate (Shug et al., 1975, Hull et al., 1975, Neely et al., 1976).

Fatty acid esterification

Triacylglycerols and phospholipids

The pathways of acylglycerol and phospholipid synthesis are initially the same and can be summarised as follows:-

1. Glycerol-3-phosphate + acyl-CoA $\longrightarrow$ monoacylglycerol-3-phosphate
2. Monoacylglycerol-3-phosphate + acyl-CoA $\longrightarrow$
1,2 diacylglycerol phosphate (phosphatidic acid)
3. 1,2 diacylglycerol phosphate + H_2O $\longrightarrow$ 1,2 diacylglycerol + P_i

Two molecules of acyl-CoA combine with one of glycerol-3-phosphate to form 1,2 diacylglycerol phosphate (phosphatidic acid). In many tissues such as liver, kidney and intestinal mucosa the glycerol-3-phosphate for this reaction is supplied by the phosphorylation of glycerol which is catalysed by the enzyme glycerokinase. However, the activity of this enzyme is very low in heart muscle (Newsholme and Taylor, 1969) and the glycerol-3-phosphate is derived from dihydroxyacetone phosphate, an intermediate of the glycolytic pathway. This reaction is catalysed by glycerol-3-phosphate dehydrogenase.

The acylation of glycerol-3-phosphate occurs in a stepwise manner with the addition of one acyl group at a time from long chain acyl-CoA. Addition of each group is catalysed by a separate enzyme (Monroy et al., 1973). The first acylation is catalysed by glycerol-3-phosphate acyltransferase, which is specific for position one on glycerol-3-phosphate. Monoacylglycerol-3-phosphate acyltransferase catalyses acylation of position two to form 1,2 diacylglycerol phosphate (phosphatidic acid). This is converted by a diacylglycerol phosphate phosphatase to a 1,2 diacylglycerol.

Triacylglycerol synthesis

An acyl group is transferred from acyl-CoA to position three of the 1,2 diacylglycerol. This is catalysed by a diacylglycerol acyltransferase.

Triacylglycerols form the major energy stores in mammals. Glucose ingested in excess of immediate energy needs, and the limited storage capacity in the form of glycogen, is converted by glycolysis into pyruvate and then acetyl-CoA, from which fatty acids are synthesised. These in turn are converted to triacylglycerols, which have a much higher energy content than polysaccharides and may be stored in very large quantities in adipose tissue.

Phospholipid synthesis

The most commonly occurring phospholipids in the heart are phosphatidylcholine (lecithin) and phosphatidylethanolamine (cephalin). These are formed by the reaction of cytidine diphosphate choline or cytidine diphosphate ethanolamine with 1,2 diacylglycerol so that a phosphorylated base, either choline phosphate or ethanolamine phosphate, is transferred to the diacylglycerol. Phosphatidylserine is synthesised from phosphatidylethanolamine by direct reaction with serine, thereby releasing ethanolamine. Phosphatidylinositol is formed by the reaction of phosphatidic acid with cytidine triphosphate to form a cytidine diphosphate diacylglycerol which then reacts with inositol. Cardiolipin is a phospholipid which occurs in mitochondrial membranes and it is synthesised by the reaction of phosphatidyl glycerol with cytidine diphosphate diacylglycerol. The remaining phospholipid which is of importance in the heart is sphingomyelin. It is synthesised by the reaction of sphingosine, a complex amino alcohol, with cytidine diphosphate choline.

The cytidine phosphorylcholine so formed is acylated at the amino group by long-chain acyl-CoA to form sphingomyelin.

The relative amounts of free fatty acids incorporated into phospholipids as compared with neutral lipids vary from tissue to tissue. The ratio (phospholipid/neutral lipid) was found to be about 0.2 in rat heart slices (Fritz 1964). Differences in relative incorporation also occur among the individual fatty acids. Vasdev and Kako (1977) studied the incorporation of different ^{14}C labelled fatty acids. Labelling of triacylglycerol was highest with oleic acid as precursor, but phospholipid labelling was highest with linoleic or linolenic acid.

Synthesis of cholesterol esters

Fatty acids may form esters with cholesterol. The activated fatty acids react with the 3-hydroxyl group of cholesterol. This is catalysed by acyl-CoA cholesterol-acyl transferase.

Regulation of esterification

The rates of both uptake and esterification of free fatty acids increase as the free fatty acid concentration of the medium increases (Eaton and Steinberg, 1961), and in particular depend on the molar ratio of free fatty acid to albumin in the perfusing solution (Shtacher and Shafrir, 1963). As the molar ratio of free fatty acid to albumin increases, relatively more free fatty acid is incorporated into triacylglycerol than into phospholipid. This has been demonstrated in several tissues including heart muscle (Shtacher and Shafrir, 1963). This is probably because triacylglycerols form a reservoir for the storage of fatty acids which are incorporated into triacylglycerol when the supply of free fatty acid exceeds the immediate metabolic requirement for oxidation.

The rate of esterification is inversely related to the rate of β -oxidation. When β -oxidation is reduced, for example in ischaemia or hypoxia, triacylglycerol synthesis is increased. This will be discussed in more detail later (see page 35).

Another factor regulating esterification is the concentration of available glucose. An increase in glucose concentration stimulates the incorporation of fatty acids into phospholipid, and to a lesser extent into triacylglycerol (Spector and Steinberg, 1966). Glucose does not affect the steady state uptake of free fatty acid but does stimulate esterification of free fatty acid in the myocardium even though free fatty acid oxidation is not decreased (Shipp, 1964). This may be due to the glycolytic production of glycerol-3-phosphate which stimulates triacylglycerol formation (Wood et al. 1972). It has recently been suggested (Opie, 1979) that glycerol-3-phosphate can act as a trap for acyl-CoA because glycerophosphate acyl transferase has a lower K_m for acyl-CoA than has carnitine acyltransferase (Bremer, 1976).

The rate of incorporation of labelled free fatty acid increases as the extracellular pH is lowered from 7.4 to 6.7 (Spector, 1969). Fasting increases free fatty acid oxidation but not esterification in rat diaphragm (Bodel et al. 1962). Free fatty acid esterification 'in vitro' is not altered by the addition of adrenaline. The administration of adrenaline or noradrenaline causes the deposition of triacylglycerol in muscle and liver 'in vivo'. This probably results from free fatty acid mobilisation from adipose tissue, causing an increase in plasma free fatty acid levels rather than a direct effect of these hormones on the esterification process (Eaton and Steinberg, 1961, Feigelson et al. 1961). Insulin added 'in vitro' increases palmitate incorporation into acylglycerols in diaphragm, and this is enhanced further when glucose is added (Bodel et al., 1962). Growth hormone does

not affect esterification (Bodel et al., 1962). Low incubation temperatures decrease palmitate incorporation into acylglycerols to a greater extent than into phospholipids in the perfused rat heart (Stein and Stein, 1963).

Lipolysis

Triacylglycerols are hydrolysed to fatty acids and glycerol. Removal of the first fatty acid by triacylglycerol lipase is rate-limiting (Denton and Randle, 1967). For this reason most of the neutral lipid present in heart muscle is triacylglycerol, the ratio being 13:0.1:0.6 for tri-, di- and monoacylglycerols respectively.

Compared with the liver, heart muscle has relatively little glycerokinase activity (Robinson and Newsholme, 1967). It is therefore assumed that very little of the glycerol produced by lipase action is reutilised and that release of glycerol from the tissue can be used as an index of lipolysis.

With increased cardiac work, accelerated acylglycerol utilisation occurs in isolated hearts perfused without exogenous fatty acids. The mechanism of this acceleration may involve decreased tissue levels of fatty acids and fatty acyl-CoA (Oram et al., 1973). Since fatty acids inhibit lipase activity (Crass, 1972), increased lipolysis may result from a faster removal of the product.

Lipolysis is stimulated by catecholamines in association with elevated levels of cyclic AMP (Christian et al., 1969). In heart muscle a cyclic 3'5' - AMP - sensitive lipase is present (Christian et al., 1969). This enzyme is similar to the hormone-sensitive lipase in adipose tissue in that both are activated by catecholamines and glucagon and the

activation of both systems may be inhibited by adrenergic blockade and nicotinic acid. The perfused heart system differs from adipose tissue preparations in that insulin has been found to be without anti-lipolytic activity in the heart although it inhibits activation of lipolysis in adipose tissue (Havel and Carlson, 1963, Havel, 1965).

Biosynthesis of fatty acids

Most mammalian cells can synthesise fatty acids from non-lipid precursors or by the elongation of existing fatty acids by the addition of 2-carbon atom units. The synthesis is particularly active in liver, adipose tissue and mammary gland, but is of very little importance in heart muscle. In heart muscle the system is much more effective for the elongation of existing fatty acids than for 'de novo' synthesis (Christ, 1968).

The source of all the carbon atoms of fatty acids is acetyl-CoA, formed in the mitochondria by the oxidative decarboxylation of pyruvate, by the degradation of amino acids or by the β -oxidation of long chain fatty acids.

Fatty acid synthesis in the heart takes place in the mitochondria (Hulsman, 1960). Whereat et al. (1969) separated outer and inner membranes of rabbit heart mitochondria and found fatty acid synthesising activity in both membranes. A 'de novo' system responsible for the complete synthesis of C_{14} to C_{18} fatty acids was contained in the inner membrane. The outer membrane possessed a chain elongation system which added 2-carbon acetate units to form C_{14} to C_{24} fatty acids.

Fatty acid metabolism in hypoxia

The normal myocardium is highly dependent on the continuous delivery of oxygen to the cells to maintain contraction. In a mild hypoxia, energy levels are maintained, firstly by the presence of myoglobin, which can take up oxygen from haemoglobin when oxygen is freely available and releases it only when the oxygen tension in the cell becomes low. Secondly, the heart has stores of glycogen and creatine phosphate both of which are used for ATP production during an oxygen shortage.

In a severe hypoxia, the electron transport chain is impeded and the formation of ATP by oxidative phosphorylation is blocked (Jöbsis, 1964). This causes an increase in the $\text{NADH} : \text{NAD}^+$ ratio (Lai and Scheuer, 1975, Sylvia et al., 1975) and an accumulation of NADH and FADH_2 in the mitochondria (Jöbsis, 1964).

The rate of β -oxidation is linearly related to the rate of oxidative phosphorylation (Oram et al., 1973) and it is inhibited by NADH and FADH_2 . The capacity for fatty acid oxidation is impaired due to a decrease in the activity of the mitochondrial respiratory chain and not to a decrease in the activity of the fatty acid oxidising enzymes (Kinnula and Hassinen, 1978). β -oxidation is also limited prior to the tricarboxylic acid cycle during hypoxia. The evidence for this is the build-up of long chain acyl-CoA and long chain acyl-carnitine (Neely and Morgan, 1974, Shug et al., 1975, Whitmer, 1978). Hypoxic hearts have decreased levels of acetyl-CoA, the end product of β -oxidation, and of acetyl-carnitine (Bowman, 1966). Carnitine acyl-transferase activity, and hence transport of fatty acids into the mitochondria are reduced (Wood et al., 1973).

The changes in tricarboxylic acid cycle intermediates in hypoxia are not fully understood although it is known that acetyl-CoA, citrate and α -ketoglutarate decrease and malate increases (Bowman, 1966). There is also a large amount of succinate formed (Cascarano, 1974) and the presence of succinate inhibits the overall rate of β -oxidation of fatty acids in the mitochondria, apparently by competing for FAD^+ .

The anaerobic pathway of energy production by glycolysis is increased during hypoxia. Increased glucose transport into the myocardial cells and increased glycogen breakdown make more carbohydrate available for energy utilisation (Morgan et al., 1961, Klarwein et al., 1961). The increase in glycolysis causes an increase in pyruvate production while the inhibition of the tricarboxylic acid cycle causes a decrease in pyruvate utilisation. The accumulation of pyruvate causes an increase in the conversion of pyruvate to lactate (Krasnow et al., 1962).

The rate of fatty acid synthesis in the heart, which is normally slow when compared with other tissues, increases under hypoxic conditions (Whereat et al., 1969) and is directly proportional to lactate production (Harris and Gloster, 1971/1972). There is also an increased incorporation of fatty acids into tissue lipids (Evans, 1964, Scheuer and Brachfeld, 1966, Gloster and Harris, 1972). The increase in glycolysis causes an increase in glycerol-3-phosphate which, as well as being a by-product of glycolysis, is a precursor of the glycerolipids and stimulates the esterification of fatty acids (Eaton and Steinberg, 1961).

CHAPTER 2 - METHODS

The Langendorff perfusion

The original, and most widely used, method of perfusing the isolated heart is that of Langendorff (1895) and, with minor modifications introduced by Morgan et al. (1961) is the method used in the present study.

The pressure of the perfusion medium entering the heart in Langendorff's method was exerted by running water from a tap into a reservoir which was connected to the outflow of a Marriot flask containing the perfusate. Morgan et al. (1961) introduced the use of a peristaltic pump to give better control of flow and pressure. A bubble trap is included in the system to absorb wave forms caused by the pump as well as preventing air bubbles from entering the heart.

The Langendorff method is also known as the 'retrograde' perfusion technique as the hearts are cannulated through the aorta and the direction of flow of the perfusate in the aorta is therefore opposite to that of the normal blood flow. The method relies on the efficiency of the aortic valve. The coronary arteries which supply the myocardium arise just above the aortic valve and provide the only possible outflow when a pressure is exerted on the valve from above.

The coronary veins drain into the coronary sinus which opens into the right atrium. After perfusing the heart, the solution drips from the right atrial openings, the superior and inferior vena cavae. The muscle of both ventricles is perfused but only the left ventricle produces any tension by contracting against the closed aortic valve.

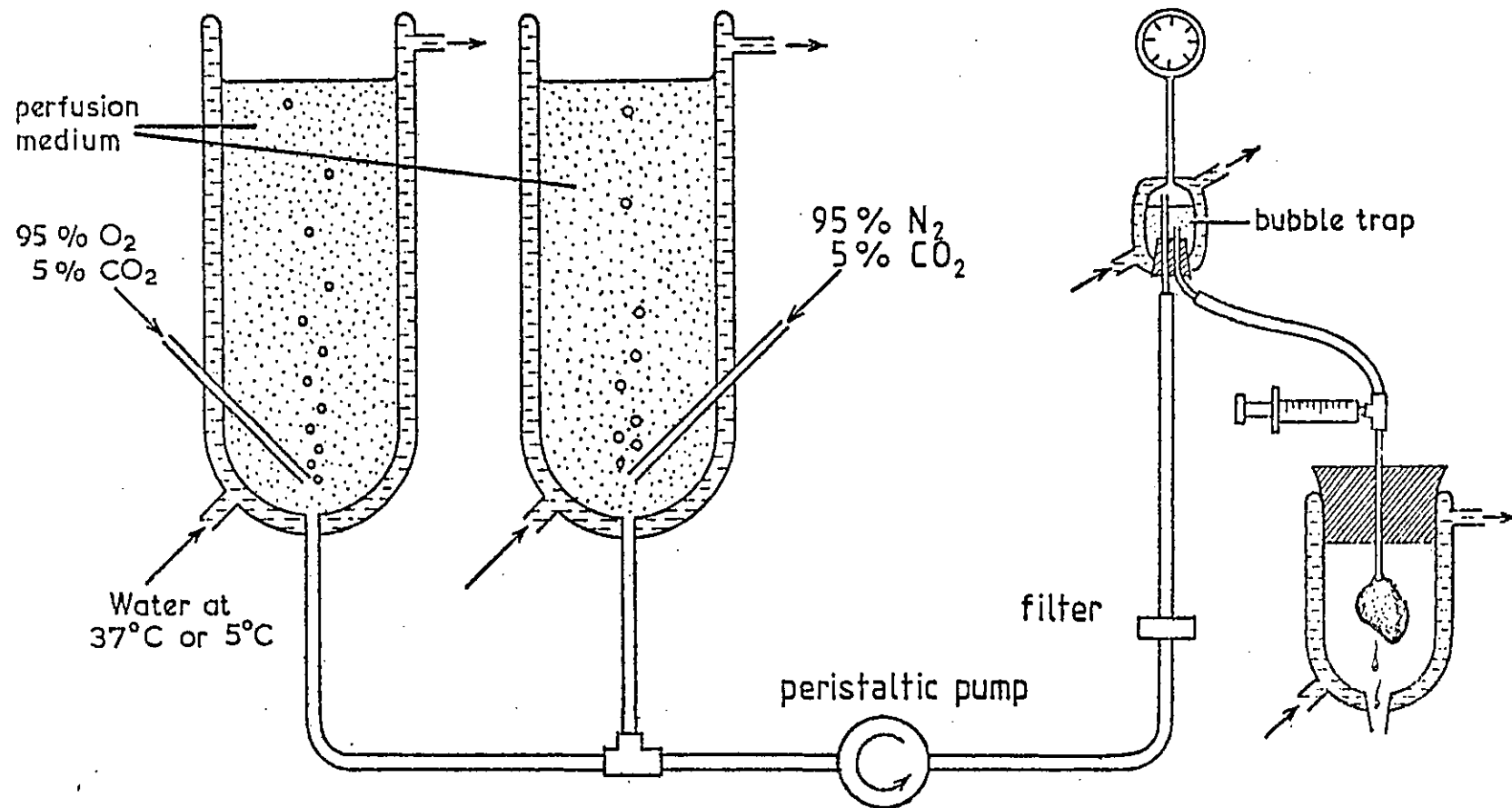


Figure 3. Perfusion apparatus

Perfusion apparatus

A simplified version of the perfusion apparatus is shown in Figure 3. The apparatus consisted of a reservoir for the perfusate which was pumped by a peristaltic pump to the heart via the bubble trap. Each piece of glassware was enclosed in a water jacket through which water was circulated from a thermostatically controlled water bath so that the temperature of the perfusate could be kept constant at 37°C or at 5°C according to the experiment. The perfusion pressure was monitored by a pressure gauge and a Millipore filter was included in the system to remove particulate matter. The perfusate reservoir contained anti-foam fibres to prevent frothing, which occurs when a solution containing albumin is bubbled with a gas.

In the well-oxygenated experiments, only one perfusate reservoir was used in which the solution was equilibrated with 95 % O₂: 5 % CO₂ for at least 20 minutes prior to use (to give $> 1000 \text{ nmoles} \cdot \text{O}_2 \text{ per ml}$; $p\text{O}_2 > 600 \text{ mm Hg}$). In the hypoxia experiments a second reservoir was used. In this reservoir the perfusate, which was of identical chemical composition to that in the first (see page 39), was bubbled with 95% N₂: 5% CO₂ (to give $< 200 \text{ nmoles O}_2 \text{ per ml}$; $p\text{O}_2 < 6 \text{ mm Hg}$). Both reservoirs were connected to the tubing passing through the peristaltic pump by a 3-way tap. By this means a rapid change over from one reservoir to the other could be implemented.

Perfusion solutions

The perfusate throughout these experiments was Krebs Henseleit bicarbonate buffer (pH 7.4) (Krebs and Henseleit 1932) which had the following composition:

	Concentration (mM)	
	Stock solution	Working solution
Sodium chloride	2369	118
Sodium bicarbonate	500	25
Potassium chloride	475	4.75
Potassium dihydrogen phosphate	215	2.15
Magnesium sulphate	65	6.5
Calcium chloride	180	1.8

The stock solutions were stored at 4°C and the working solution was prepared as required.

0.9g of glucose was added to each litre to make a final glucose concentration of 5mM.

Non - radioactive perfusate

0.2 mM oleic acid bound to 0.2mM dialysed bovine serum albumin by the method described below was added to the Krebs Henseleit buffer.

Radioactive perfusate

This was prepared in the same way as the non-radioactive perfusate with the addition of [9,10 -³H] oleic acid with a specific activity of 2.4 Ci per mmol. This was used at a final activity of either 50 or 100 μ Ci per ml of perfusate. The radiochemical purity of the ³H oleic acid was checked by thin layer chromatography and 96% of the counts were associated with the free fatty acid band on the thin layer plates.

Binding of oleic acid to albumin

The oleic acid was complexed to bovine serum albumin by the method of Fredrikson and Gordon(1958). 0.2mM oleic acid (final concentration) was dissolved in a small volume of aqueous ethanol containing a molar equivalent of K₂CO₃ and warmed to approximately 45°C in order to evaporate the ethanol. A current of air was applied to assist the evaporation. The solution was left at 37°C overnight

before it was added drop by drop, with shaking, to a 0.2mM solution of albumin in Krebs Henseleit buffer containing 5mM glucose.

Animals

Male Dunkin Hartley guinea pigs of approximately 300 – 350g body weight were used throughout these studies. The animals were allowed free access to both food and water prior to the experiments.

Perfusion procedure

The guinea pigs were killed by cervical dislocation and the hearts were rapidly removed. Each was washed briefly in a beaker of ice cold Krebs Henseleit buffer and transferred to a pre – weighed beaker of the buffer which had been equilibrated with 95% O₂ : 5% CO₂. The beaker was reweighed in order to obtain the heart weight. A cannula, filled with Krebs Henseleit buffer, was inserted into the aorta and tied in place with a ligature. The hearts were then transferred to the specimen chamber of the perfusion apparatus and perfused using a non – recirculating or 'once – through' system maintained at a pressure of 60 mm of mercury. The coronary flow rate was approximately 10 ml per minute.

Well – oxygenated perfusions

An initial period of five minutes perfusion with the unlabelled medium was followed by a 15 second pulse perfusion with the radioactive perfusate. Both solutions had been equilibrated with 95% O₂ : 5% CO₂ for at least 20 minutes. The radioactive solution was contained in a syringe and attached to the aortic cannula by a 3-way tap. The pulse perfusion was followed by an immediate return to the unlabelled perfusate for a period of 15, 30, 60 or 120 seconds. This will be referred to subsequently as the 'chaser' perfusion. Four guinea pig hearts were used for each of the perfusion times at 37°C and these were repeated

at 5°C, at which temperature oxidation and esterification of fatty acids are inhibited.

Hypoxic perfusions (see * page 48)

The initial period of five minutes perfusion with the oxygenated solution was followed by 30 minutes perfusion with the solution from the second reservoir which had been bubbled with 95% N₂ : 5% CO₂. The radioactive perfusate which had also been equilibrated with 95% N₂ : 5% CO₂ was given at the end of the 30 minutes hypoxic perfusion. The 15 second radioactive pulse was followed by an immediate return to the hypoxic, unlabelled perfusate for a period of 15, 30, 60 or 120 seconds. Four guinea pig hearts were used for each of the perfusion times. The perfusions were carried out at 37°C. The details of these experiments are summarised in the following table.

Table 1 A summary of the experimental design

	Temperature	Length of chaser (s)
Well - oxygenated	37°C	15
		30
		60
		120
	5°C	15
		30
		60
		120
Hypoxic	37°C	15
		30
		60
		120

For each of the conditions four animals were used.

Freeze - clamping

At the end of the chaser perfusion the hearts were frozen by clamping them between two aluminium blocks which had been pre - cooled in liquid nitrogen (Wollenberger et al., 1960). The frozen hearts were wrapped in aluminium foil and rapidly shattered with a hammer.

Lipid extraction

The lipids were extracted from the frozen heart tissue by the method of Folch et al. (1957). Each powdered, frozen heart was transferred to 90 ml of a chloroform : methanol mixture (2 : 1 by volume). The tissue was left in this mixture overnight at room temperature. After filtering the extract, 20 ml of 0.1M NaCl was added to each filtrate and the mixture was left overnight at 4°C to allow the phases to separate. The lipid-containing chloroform phase was taken to dryness using a rotary evaporator and redissolved in 2 ml of chloroform.

Thin layer chromatography of the lipid extracts

Lipids extracted from whole tissue by the above method are mixtures of individual lipid classes and several methods of separating these classes by chromatography have been described. Often more than one method of chromatography must be used in order to achieve total separation.

In these experiments a simple method of separating the lipids by thin layer chromatography was used, resulting in the separation of the lipid mixture into cholesterol esters, free fatty acids, mono- and di-acylglycerols (together), triacylglycerols and phospholipids.

* The length of hypoxic perfusion was chosen as one in which there were marked biochemical changes with little or no change in ultrastructure.

Preparation of the plates

Glass plates, 20 cm square, were washed and dried. The adsorbent used was a fine grade silicic acid (Merck silica gel H) which is a partially hydrated silicon dioxide. It was applied to the plates as a slurry formed by mixing 40g of silica gel with 95 ml of distilled water. The plates were coated using a spreader manufactured for the purpose, so that a uniform layer of 0.25mm thickness was obtained. The plates were dried at room temperature and activated by heating in an oven at 110°C for one hour before use. Prepared plates, if not for immediate use, were stored in an air-tight box with desiccant and were activated immediately before they were used.

Separation of the lipids

100 µl duplicates of each lipid extract were applied to the plates as a narrow streak about 2 cm from the bottom. Known standards of the main lipid classes were applied separately on each plate in order to aid identification. Each plate was placed in a glass tank containing the eluting solvent which consisted of light petroleum ether (boiling range 40°C - 60°C) : diethyl ether : glacial acetic acid (85 : 15 : 2 by volume). When the solvent neared the top of the plate, the plate was removed from the tank and allowed to dry. The lipid bands were stained by placing the plate in a glass tank containing a few crystals of iodine so that the lipids were stained by the iodine vapour. The lipid bands were marked and the iodine was then allowed to sublime completely before scraping each band into a scintillation vial. This is because the brown colouring caused by the iodine may cause quenching during scintillation counting. Elution of the lipids from the adsorbent is not necessary for scintillation counting as the adsorbent does not cause quenching.

Scintillation counting

To each sample, 10 ml of Bray's scintillant (Bray, 1960) were added. This was composed of the following:

Naphthalene	60g
PPO (2,5-diphenyloxazole)	4g
POPOP (1,4-di-2-(5-phenyloxazdyl)-benzene)	0.2g
Methanol	100 ml
Ethylene glycol	20 ml

To these was added 1,4 dioxane to give a final volume of 1 litre.

The samples were counted in a Packard Tricarb 3380 liquid scintillation spectrometer. Two blanks were prepared with each batch to monitor background radiation. These contained scintillant with adsorbent from the edge of the plates which had been in the eluting solvent but had not been in contact with the lipid mixture.

Quenching

This term is applied to any factor which reduces the light (photon) output of the scintillant and can occur in several ways (Kobayashi and Maudsley, 1974) :-

1. The sample itself may absorb light given off by the scintillator or may absorb some of its own radiation.
2. The solvent may not transfer the energy of the β -particles efficiently to the scintillator.
3. The scintillator may absorb some of its own fluorescence.
4. Chemical interaction of some of the components in the solution to be counted may result in reduced photon output.

Quench correction

Quenching was corrected by estimating the counting efficiency of each sample. Counts per minute (cpm) were converted to disintegrations per minute (dpm) by the following calculation:-

$$\text{dpm} = \frac{\text{observed mean cpm}}{\text{counting efficiency}}$$

Counting efficiency was estimated using the automatic external standard (A.E.S.) method. This involves the use of an external gamma radiation source which is built into the scintillation counter. Irradiation of the sample by the gamma source produces Compton electrons which behave in a similar manner to the β -particles emitted by the radioactive sample in that they produce photons in the scintillant. Any quenching in the sample results in a proportionate quenching in the Compton electrons and this is recorded as the A.E.S. count.

To determine the counting efficiency of each sample a 'quench curve' was constructed (see Figure 4). This was prepared from a set of 'quenched standards' which consisted of a calibrated series of scintillation vials containing a known number of cpm (100,000) with a different amount of a 'quenching substance' added to each. The efficiency of each sample is calculated by dividing the cpm obtained (minus background counts) by 100,000. The efficiency is plotted against the A.E.S. reading for each sample.

Thin layer chromatography of acyl-CoA and acyl-carnitine

In order to assess the contribution of radioactive acyl-CoA and acyl-carnitine to the lipid separation, [1 - ^{14}C] palmityl-CoA and [1 - ^{14}C] palmityl-carnitine were each added to a chloroform : methanol mixture (2 : 1 by volume). After separation of the phases as described previously, aliquots of both aqueous and chloroform phases were taken for scintillation counting. To prevent quenching the chloroform samples were taken to dryness in a current of air before the addition of the scintillant. It was found that only 33% of the palmityl-CoA was recovered in the lipid-containing chloroform phase, while 97% of the palmityl-carnitine partitioned into the chloroform phase.

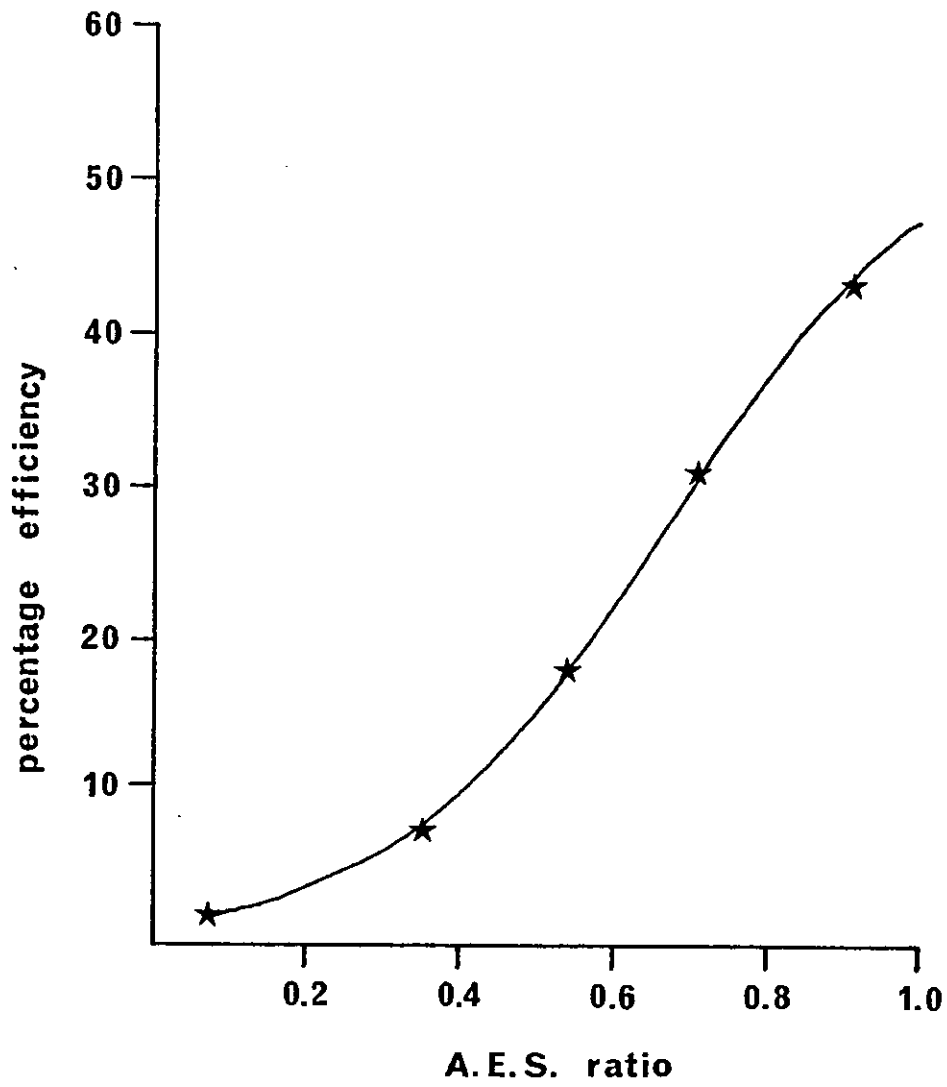


Figure 4

Tritium quench curve

On thin layer plates, using the solvent system described previously, $[1 - ^{14}\text{C}]$ palmityl-CoA migrated with the mono- and diacylglycerol fraction while the $[1 - ^{14}\text{C}]$ palmityl-carnitine remained at the origin with the phospholipids.

In order to determine the degree to which acyl-carnitine might contribute towards the radioactivity found in the phospholipid band, the individual phospholipid classes were separated by thin layer chromatography using the solvent system chloroform : methanol : glacial acetic acid : water (65 : 45 : 1 : 4 by volume). This separation was carried out using the lipid extracts from the well-oxygenated perfusions at 37°C with 120 seconds chaser perfusion. These extracts were chosen as this experimental condition was the one in which radioactivity in phospholipid was found to be maximal. There was not a clear separation of phosphatidylcholine from sphingomyelin, which is the region to which acyl-carnitine migrates in this system. However the radioactivity recovered from the phosphatidylcholine and sphingomyelin bands together amounted to only 5.9% of the total phospholipid radioactivity and only 1.8% of the total lipid radioactivity. The contribution of acyl-carnitine to the total phospholipid radioactivity was therefore assumed to be relatively small. The data for phospholipids are therefore included in the results section.

An attempt to assess the contribution of acyl-CoA to the radioactivity of mono- and diacylglycerol was not successful and these data are therefore not included.

Measurement of $^{14}\text{CO}_2$ production

In order to measure the carbon dioxide produced by the oxidation of the free fatty acid, perfusions were carried out using $[10, ^{14}\text{C}]$ oleic acid.

Apart from the change of isotope, the radioactive perfusate was prepared as before and duplicate aliquots were taken for scintillation counting before each experiment.

The hearts were perfused under liquid paraffin to prevent the diffusion of $^{14}\text{CO}_2$ into the surrounding atmosphere. Four guinea pig hearts were used for well-oxygenated perfusion and four for hypoxic perfusion. The well-oxygenated hearts were perfused for five minutes with the unlabelled solution which had been bubbled with 95% O_2 : 5% CO_2 . This was followed by a 15 second pulse with the perfusate containing the ^{14}C oleic acid. A 120 second chaser period with unlabelled perfusate followed. This was the only chaser period used to measure $^{14}\text{CO}_2$ production and was chosen as the one most likely to show a difference between the $^{14}\text{CO}_2$ produced by the well-oxygenated and hypoxic hearts.

After the initial five minutes perfusion with oxygenated perfusate, four hearts were made hypoxic by 30 minutes perfusion with the unlabelled solution which had been bubbled with 95% N_2 : 5% CO_2 . This was followed by the radioactive pulse and a chaser period of 120 seconds with the unlabelled, hypoxic solution.

The effluent from the pulse and chaser from each heart was collected under liquid paraffin in a flask containing 10 ml of 0.1M KOH ($\text{pH} > 13$). The alkaline medium ensures that all the free $^{14}\text{CO}_2$ produced is converted to bicarbonate and therefore remains in solution.

At the end of the 120 second chaser, the hearts were freeze-clamped. The frozen tissue was wrapped in aluminium foil and rapidly shattered with a hammer. It was immediately transferred to a stoppered separating funnel containing 100 ml of chloroform : methanol (2 : 1 by volume) and left overnight at room temperature. The aqueous phase and the

lipid-containing chloroform phase were separated by the introduction of 20 ml of an alkaline 0.1 M NaCl solution ($\text{pH} > 13$) through a 3-way tap at the top of the funnel. To facilitate this addition, the tap at the bottom of the funnel was opened and a similar volume of the chloroform : methanol mixture was removed. The chloroform : methanol which was removed from the funnel was discarded after the exact volume had been measured. The extract was then shaken and the phases allowed to separate overnight at 4°C .

The method of determining the amount of $^{14}\text{CO}_2$ present in the various solutions is that of Vasdev and Kako (1976). Aliquots of the following were taken for scintillation counting:-

1. The lipid-containing chloroform phase. The chloroform was evaporated off in a current of air to prevent quenching before the addition of the scintillant.
2. The alkaline aqueous phase.
3. The aqueous phase after acidification with 1N HCl to release $^{14}\text{CO}_2$. The HCl was added dropwise until a pH of less than 3 was reached.
4. The alkaline effluent.
5. The effluent after acidification to release $^{14}\text{CO}_2$.

The counts were corrected for quenching. The amount of $^{14}\text{CO}_2$ in the aqueous phase and in the effluent was calculated by subtracting the counts after acidification from the counts before acidification.

CHAPTER 3 - RESULTS

Well-oxygenated hearts

The radioactivity present in total lipid, free fatty acid, triacylglycerol and phospholipid after pulse perfusion with ^3H oleic acid is shown in Tables 2 and 3. The results are expressed in disintegrations per minute per mg wet weight. Table 2 gives the results for a 15 second pulse perfusion followed by a 15, 30, 60, or 120 second chaser perfusion at 37°C . Table 3 shows the results when these perfusions were repeated under hypothermic conditions at 5°C .

At 37°C the total lipid radioactivity and the free fatty acid radioactivity decreased rapidly after the 15 second chaser. These values continued to decrease throughout the total two minute washout period. At 5°C the radioactivity in total lipid and free fatty acid decreased by 45% between 15 and 30 seconds chaser but thereafter remained constant. There was little incorporation of radioactivity into triacylglycerol or phospholipid in the cold. At 37°C however, the radioactivity in triacylglycerol increased nearly three times and phospholipid nearly two times.

Hypoxic hearts

These results are given in Table 4. Radioactivity recovered in total lipid after the shorter perfusion times was three times higher in the hypoxic hearts than in the well-oxygenated hearts and more than four times higher after the longer times. Most of this increase was caused by a rise in the free fatty acid and triacylglycerol radioactivity. After 120 seconds the increase in triacylglycerol radioactivity was particularly great, being more than six times higher in the hypoxic hearts than in the well-oxygenated hearts. The increase in phospholipid radioactivity was small throughout the time course studied.

Table 2

The distribution of radioactivity in myocardial total lipid, free fatty acid, triacylglycerol and phospholipid after a 15 second perfusion with ^3H oleate at 37°C . *

Time of unlabelled chaser (seconds)	Radioactivity (dpm per mg wet weight)			
	Total lipid	Free fatty acid	Triacylglycerol	Phospholipid
15	3078 (± 1314)	2590 (± 924)	114 (± 81)	163 (± 159)
30	1999 (± 725)	1309 (± 576)	213 (± 31)	186 (± 168)
60	1591 (± 346)	676 (± 113)	339 (± 86)	230 (± 86)
120	1113 (± 398)	289 (± 102)	343 (± 69)	276 (± 121)

*Followed by periods of chaser perfusion with unlabelled oleate.

Each figure is the mean $\pm$ standard deviation of four hearts.

Table 3

The distribution of radioactivity in myocardial total lipid, free fatty acid, triacylglycerol and phospholipid after a 15 second perfusion with ^3H oleate at 5°C . *

Radioactivity (dpm per mg wet weight)				
Time of unlabelled chaser (seconds)	Total lipid	Free fatty acid	Triacylglycerol	Phospholipid
15	2750 (± 299)	2394 (± 127)	122 (± 39)	92 (± 55)
30	1513 (± 505)	1348 (± 437)	27 (± 11)	38 (± 16)
60	1722 (± 399)	1439 (± 315)	55 (± 7)	111 (± 39)
120	1820 (± 406)	1529 (± 362)	85 (± 21)	136 (± 83)

*Followed by periods of chaser perfusion with unlabelled oleate.

Each figure is the mean $\pm$ standard deviation of four hearts.

Table 4

The distribution of radioactivity in myocardial total lipid, free fatty acid, triacylglycerol and phospholipid after 30 minutes hypoxia followed by a 15 second perfusion with ^3H oleate at 37°C .*

Time of unlabelled chaser (seconds)	Radioactivity (dpm per mg wet weight)			
	Total lipid	Free fatty acid	Triacylglycerol	Phospholipid
15	9990 (± 1116)	8431 (± 842)	390 (± 161)	210 (± 11)
30	6269 (± 1184)	4392 (± 844)	715 (± 52)	216 (± 58)
60	7518 (± 1133)	3753 (± 621)	2079 (± 262)	352 (± 64)
120	4911 (± 2154)	1298 (± 558)	2244 (± 902)	390 (± 187)

*Followed by periods of chaser perfusion with unlabelled oleate.

Each figure is the mean $\pm$ standard deviation of four hearts.

Table 5

Percentage distribution of radioactivity in
free fatty acid, triacylglycerol and phospholipid.

Time of unlabelled chaser (seconds)		Free fatty acid	Triacylglycerol	Phospholipid
37°C	15	85.4 ($\pm$ 5.4)	3.5 ($\pm$ 1.0)	4.5 ($\pm$ 2.8)
	30	64.2 ($\pm$ 6.3)	11.2 ($\pm$ 2.5)	9.9 ($\pm$ 2.3)
	60	42.7 ($\pm$ 6.6)	21.2 ($\pm$ 3.3)	14.2 ($\pm$ 3.4)
	120	25.8 ($\pm$ 4.3)	31.8 ($\pm$ 4.8)	24.4 ($\pm$ 4.1)
5°C	15	87.4 ($\pm$ 2.7)	4.3 ($\pm$ 1.5)	3.3 ($\pm$ 1.4)
	30	88.9 ($\pm$ 1.7)	1.7 ($\pm$ 0.3)	2.5 ($\pm$ 0.3)
	60	83.9 ($\pm$ 4.1)	3.1 ($\pm$ 0.5)	6.3 ($\pm$ 2.6)
	120	85.5 ($\pm$ 2.9)	5.3 ($\pm$ 1.5)	4.7 ($\pm$ 0.7)
Hypoxia	15	84.0 ($\pm$ 1.6)	4.4 ($\pm$ 1.6)	2.1 ($\pm$ 0.2)
	30	61.2 ($\pm$ 1.6)	10.1 ($\pm$ 1.1)	3.0 ($\pm$ 0.4)
	60	46.0 ($\pm$ 2.2)	25.6 ($\pm$ 0.6)	4.6 ($\pm$ 0.8)
	120	25.0 ($\pm$ 10.8)	41.9 ($\pm$ 6.4)	7.1 ($\pm$ 1.2)

Percentage incorporation

Table 5 shows each fraction expressed as a percentage of the total lipid radioactivity. At 37°C the percentage of the radioactivity present in the free fatty acid fraction decreased with the length of the chaser. As free fatty acid radioactivity decreased, so the proportion of radioactivity in triacylglycerol and phospholipid increased. At 5°C, esterification was inhibited and most of the radioactivity was found in the free fatty acid fraction regardless of the length of the chaser perfusion.

Although the radioactivity from all the fractions from the hypoxic hearts had increased, there was a marked change in the proportion of radioactive fatty acid incorporated into each fraction. The percentage remaining in free fatty acid was the same in the hypoxic hearts as in the well-oxygenated hearts. There was however a shift towards triacylglycerol production with 41.9% of the total radioactivity present as triacylglycerol after 120 seconds compared with 31.8% in the well-oxygenated hearts. This shift seems to be at the expense of phospholipid production as after 120 seconds chaser perfusion only 7.1% of the radioactivity was found in the phospholipid fraction compared with 24.4% in the well-oxygenated hearts.

Two fractions are not included in Table 5 but account for the remaining percentages. Firstly, cholesterol esters, which contributed less than 5% to the total lipid radioactivity and did not appear to change after hypoxic perfusion. Secondly the percentage of mono- and diacylglycerol was not included because, as described previously (page 53) the contribution of acyl-CoA to this fraction could not be assessed.

$^{14}\text{CO}_2$ production

The counts obtained (expressed as disintegrations per minute per mg wet weight) and the calculation of radioactivity present as $^{14}\text{CO}_2$ are given in Table 6. The counts have been corrected for the volumes from which the aliquots were taken.

The $^{14}\text{CO}_2$ occurred mainly in the effluent. In total, the $^{14}\text{CO}_2$ produced by the well-oxygenated hearts after a 120 second chaser was approximately eleven times more than that produced by the hypoxic hearts after the same length of perfusion.

Table 6

$^{14}\text{CO}_2$ production 120 seconds after pulse perfusion with ^{14}C oleic acid

	Radioactivity (dpm per mg wet weight)	
	Well - oxygenated	Hypoxic
Lipid containing chloroform phase	78.2 (± 6.0)	235.1 (± 110.2)
Aqueous phase	4.8 (± 3.5)	2.4 (± 1.7)
Aqueous phase after acidification	3.0 (± 2.7)	2.1 (± 1.6)
Calculated $^{14}\text{CO}_2$ in aqueous phase	1.8 (± 1.3)	0.3 (± 0.1)
Effluent	2042.8 (± 479.3)	1783.6 (± 606.7)
Effluent after acidification	1931.7 (± 461.2)	1773.7 (± 610.4)
Calculated $^{14}\text{CO}_2$ in effluent	111.1 (± 18.9)	9.9 (± 2.0)
Total $^{14}\text{CO}_2$	112.9 (± 20.1)	10.2 (± 2.1)
Recovery of counts	87.4% ($\pm 9.4\%$)	83.3% ($\pm 11.9\%$)

Each figure is the mean $\pm$ standard deviation of four hearts.

PART TWO

LIPID LOSSES DURING THE PROCESSING OF
CARDIAC MUSCLE FOR ELECTRON MICROSCOPY

CHAPTER 4 - INTRODUCTION

The following part of this thesis describes the incorporation of radioactive oleic acid into various parts of the myocardial cell using the technique of EM autoradiography. The processing of biological material for electron microscopy generally involves dehydrating the fixed tissue with organic solvents before infiltrating with the embedding medium. Solutions containing increasing concentrations of ethanol followed by acetone or propylene oxide are frequently used for this purpose. These substances are all excellent lipid solvents, so some lipids are almost certain to be removed from the tissue at this stage (Ashworth et al., 1966, Carlier et al., 1968, Cope and Williams, 1968). Although this may not always matter to the morphologist, it can present problems when the intracellular localisation of lipids is to be studied by EM autoradiography.

Aldehydes, such as glutaraldehyde, are efficient cross-linking agents for proteins but seem to have little effect on most lipids (Carlier et al., 1968, Idelman, 1965). As much as 95% of total lipid can be lost during ethanolic dehydration following fixation with glutaraldehyde alone (Korn and Weisman, 1966). The use of a double fixation procedure in which glutaraldehyde is followed by osmium tetroxide markedly reduces the amount of lipid lost from the tissue. Osmium tetroxide reacts with the double bonds in the lipids (Bahr, 1955) resulting in the formation of a glycol. Almost complete fixation of phospholipids can be achieved using osmium tetroxide which reduces the subsequent solubility in ethanol and epoxy resin (Korn and Weisman, 1966, Cope and Williams, 1969,a,b). The retention of phospholipid is not improved further by preceding the osmium treatment by aldehyde fixation.

Osmium tetroxide may also reduce the subsequent solubility of neutral lipids although these present a greater problem of preservation. The formation of glycols alters the properties of the neutral lipids so that they appear more polar on silicic acid chromatography, less soluble in ethanol and more soluble in methacrylate and Araldite (Cope and Williams, 1968).

Neutral lipid droplets however, are not completely penetrated even by prolonged treatment with osmium tetroxide (Bahr, 1955) so that unfixed lipid at the centre of the droplet may still be removed by dehydration in ethanol.

Several authors have assessed the 'lipid-retaining properties' of some of the more widely used embedding media. As with fixation procedures the main problem has been with the preservation of neutral lipids. Ashworth et al. (1966) reported a 92.8% retention of neutral lipid in rat liver using Epon embedding, but these results were not supported by the observations of Korn and Weisman (1966) or Cope and Williams (1968) and to date this is the only reported study in which such a high preservation of neutral lipid has been found. None of the embedding procedures described has resulted in the total quantitative preservation of neutral lipid and special processing schedules have been suggested to overcome this problem. Procedures using ethanolic dehydration seem generally best avoided if lipids are to be examined although Idelman (1965) suggested the use of Epon embedding omitting both the 100% ethanol and propylene oxide stages. Stein and Stein (1968) reported a 78% retention of total lipid using this method.

Polymers of glutaraldehyde and urea mixtures were described by Pease and Peterson (1972) and Peterson and Pease (1972) in the context of lipid retention and this method was developed further by Heckman

and Barnett (1973) who described a 92% retention of [1 - ^{14}C]palmitic acid in rat liver using a solution of carbohydrazide in glutaraldehyde (GACH) for both dehydration and embedding.

The water-soluble methacrylates have been found to be of little use in neutral lipid retention even at -20°C (Cope and Williams, 1968, 1969,a,b). Gibbons (1959) described the separation of Aquon, the water-soluble phase of Epon resin. Dilutions of this can be used for dehydration and the undiluted product for embedding. The separation of Aquon from Epon is a complicated and lengthy procedure but a similar product, described by Stäubli (1960), later became available commercially. This is the water-soluble Durcupan. Casley-Smith's (1962) observations on the electron microscopic appearance of chylomicrons and lipoproteins suggested that water-soluble Durcupan might be of value for the retention of neutral lipid in tissues.

The purpose of the experiments described in the following chapters was to determine which of the methods previously described could be applied to cardiac muscle. A comparison was made of some of the above methods using the isolated guinea pig heart which was pulse perfused with [9, 10 - ^3H] oleic acid. The lipid retaining properties of para-phenylenediamine were also investigated. This is a stain which is used to enhance the reduction of osmium tetroxide (Ledingham and Simpson, 1972).

It has been suggested that frozen sections would have been the answer to the retention problem in these experiments. Indeed in many studies this would have been so but the resolution obtained with frozen sections, at the present time, would not have been adequate to identify some of the smaller structures within the cell, such as the sarcoplasmic reticulum.

CHAPTER 5 - METHODS

Perfusions

The perfusates, both non-radioactive and radioactive, were prepared as described on page 45, and were maintained at 37°C.

The hearts were cannulated and perfused by the method described before (page 46) using a non- recirculating system maintained at a perfusion pressure of 60mm of mercury. All the experiments commenced with five minutes perfusion with the non-radioactive solution which was followed by a 15 second pulse perfusion with the radioactive solution. This was contained in a syringe and attached to the aortic cannula by a 3-way tap. At the end of the pulse, ice cold Krebs Henseleit buffer from a second syringe in the 3-way tap was passed through the heart to remove radioactive material from the vascular space.

Fixation

The hearts were transferred immediately to a second, adjacent perfusion apparatus where they were perfused for 10 minutes with 4% glutaraldehyde in 0.1M sodium cacodylate (pH 7.3) at room temperature.

Tissue samples

After fixation, the hearts were removed from the cannula and pieces of tissue each of approximately 20 mg were removed, blotted dry and weighed. Two samples were taken from the left ventricle wall and two from the interventricular septum in each heart. One piece of muscle from each site was transferred immediately to chloroform : methanol (2 : 1 by volume) for lipid extraction (see page 48). The second piece from each site was cut into approximately 1mm cubes with a razor blade and these pieces were processed for electron microscopy using one of the experimental procedures as follows:-

Tissue processing

The initial stages of processing were identical for all the samples and are given in the following table.

Table 7

The initial stages of processing for electron microscopy

Following perfusion fixation:-

1. A further fixation by immersion in 4% buffered glutaraldehyde	1 - 2 hours
2. Buffer wash	10 minutes
3. Buffered 1% osmium tetroxide	2 - 3 hours
4. Buffer wash	10 minutes

The buffer used throughout was 0.1M sodium cacodylate (pH 7.3).

The embedding procedures investigated were as follows:-

1. Epon. The rapid ethanolic dehydration suggested by Idelman (1965) was combined with Epon embedding. 10 tissue samples from 5 hearts were used.
2. Para-phenylenediamine. 8 samples from 4 hearts were processed using Epon embedding but with the inclusion of para-phenylenediamine in the schedule. This substance enhances the reduction of osmium tetroxide (Ledingham and Simpson, 1972)

3. GACH. 20 tissue samples from 10 hearts were processed in a glutaraldehyde - carbohydrazide polymer (GACH) described by Heckman and Barnett (1973).

4. Water-soluble Durcupan. 16 tissue samples from 8 hearts were dehydrated and embedded in water-soluble Durcupan (Stäubli, 1960).

Details of these embedding procedures are given in Tables 8 - 11.

Table 8

Schedule for Epon processing

1. 70% ethanol 1	5 minutes
2. 70% ethanol 2	5 minutes
3. 95% ethanol 1	5 minutes
4. 95% ethanol 2	5 minutes
5. Epon 812, resin only	1 hour
6. Epon full mix (without accelerator)	Overnight

Epon full mix consisted of 2.25 ml Epon 812 resin, 1.0 ml DDSA (dodecenylsuccinic anhydride) and 1.75 ml MNA (methyl nadic anhydride).

Table 9Schedule for para-phenylenediamine with Epon processing

1. 1% para-phenylene diamine in 70% ethanol	30 minutes
2. 70% ethanol 1	5 minutes
3. 70% ethanol 2	5 minutes
4. 95% ethanol 1	5 minutes
5. 95% ethanol 2	5 minutes
6. Epon 812, resin only	1 hour
7. Epon full mix (without accelerator)	Overnight

Epon full mix was as in Table 8.

Table 10Schedule for GACH processing

1. 20% GACH in distilled water	2 - 4 hours
2. 50% GACH	2 - 4 hours
3. 80% GACH	Overnight
4. 100% GACH	4 - 6 hours

100% GACH consisted of 150mg carbonylhydrazide per ml of 50% glutaraldehyde

Table 11

Schedule for water-soluble Durcupan processing

Durcupan is supplied as four separate components, Durcupan A, B, C and D. Durcupan A is the basic resin, B is a hardener, C an accelerator and D is a plasticizer.

1. 70% Durcupan A	30 minutes
2. 90% Durcupan A	30 minutes
3. 100% Durcupan A 1	45 minutes
4. 100% Durcupan A 2	45 minutes
5. Durcupan full mix	Overnight

Durcupan full mix consisted of 5 ml Durcupan A, 13 ml Durcupan B, 1 ml Durcupan C and 0.2 ml Durcupan D.

The fluids used at each stage of processing were monitored for loss of radioactive lipid from the tissue by scintillation counting. The Pasteur pipettes used to transfer the processing fluids were rinsed with scintillant before discarding to ensure that a minimum of the radioactive material was left behind. Lipids remaining in the tissue after processing were extracted in chloroform : methanol (2 : 1 by volume) as described on page 43.

Calculation of percentage lipid loss

Radioactivity (in counts per minute) lost from the tissue at each stage of processing, was converted to disintegrations per minute by correcting for quenching by the method described on page 50.

Radioactivity per unit weight of tissue in the processing fluids and that extracted from the processed tissue was expressed as a percentage of the total initial radioactivity per unit weight in the tissue. This was determined from those pieces of tissue which were extracted directly after perfusion fixation.

Alkaline hydrolysis of lipids from para-phenylenediamine treated tissue

Electron micrographs of cardiac muscle 'stained' with osmium tetroxide followed by para-phenylenediamine show densely stained lipid droplets (see Figure 25, page 147) unlike the lipid droplets seen with Epon processing after osmium tetroxide alone which appear pale or empty.

An experiment was carried out to investigate the possibility that lipids in tissue treated with osmium tetroxide and para-phenylenediamine were so well fixed that they were not removed by chloroform : methanol extraction. Alkaline hydrolysis of the lipids in such tissue was carried out in an attempt to extract any remaining fatty acids from the tissue.

Four guinea pig hearts were used. Each was rapidly removed from the animal and three pieces of left ventricle were excised and weighed. All three were cut into approximately 1mm cubes before being treated as follows:-

1. Sample 1 was transferred unfixed to 20 ml of chloroform : methanol (2 : 1 by volume).
2. The second sample was fixed for two hours in 4% glutaraldehyde in 0.1M sodium cacodylate and then transferred to 20 ml of chloroform : methanol (2 : 1 by volume).
3. Sample 3 was processed in Epon through to Epon full mix and including 30 minutes in 1% para-phenylenediamine (as in Table 9). The pieces of tissue were then transferred to chloroform : methanol (2 : 1 by volume).

After overnight extraction the tissue fragments were removed by filtration and retained. The phases were separated by the addition of 4 ml of 0.1M NaCl to the chloroform : methanol mixture. The lipid-containing chloroform phase was taken to dryness using a rotary evaporator and 10 ml of 5M KOH were added to each, as well as to each of the samples of tissue pieces.

Isolation of fatty acids

(a) Alkaline saponification

When lipids are hydrolysed by heating them under reflux with dilute, aqueous, ethanolic alkali, fatty acids are obtained in the form of soaps. The reaction conditions are controlled so that complete saponification occurs without altering the structure of any of the fatty acids concerned.

An air condenser was placed in each of the six tubes and these were left to stand in water at 80°C for five hours.

(b) Petroleum ether extraction

5 ml of distilled water and 5 ml of petroleum ether (boiling range 40°C - 60°C) were added to each sample to separate the soaps from the unsaponifiable material. The tubes were shaken and centrifuged at 2,000 rpm for approximately 5 minutes. The top layer, which consisted of petroleum ether and unsaponifiable material was discarded. This extraction was then repeated without the addition of the distilled water.

(c) Reconversion of soaps to free fatty acids

The samples were acidified by the addition of 5N HCl which liberates the free fatty acids. The petroleum ether extraction was repeated and the free fatty acids partitioned into the petroleum ether layer.

Colorimetric method for free fatty acid determination (Duncombe, 1964)

The petroleum ether in the saponified samples was evaporated off using a rotary evaporator and 5 ml of chloroform was added to each tube. A series of palmitic acid standards was prepared containing 0.025, 0.050, 0.075 and 0.1 mg of palmitic acid, each in 5 ml of chloroform. Two blanks containing chloroform only were also prepared. 2.5 ml of a copper reagent were added to each of the samples, standards and blanks and mixed well. The copper reagent consisted of 45 ml aqueous 1M triethanolamine, 5 ml 1N acetic acid and 50 ml 6.45% copper nitrite. The tubes were centrifuged for 10 minutes at 2,000 rpm at 25°C after which the upper phase was removed by suction and discarded. 3 ml of the lower phase was pipetted into a clean tube and 0.5 ml of a colour reagent (0.1% diethyldithiocarmate in n-butanol) was added to each. The optical density of each sample was determined at a wavelength of 440 nm using a Unicam SP 600 spectrophotometer.

A linear result was obtained for the palmitic acid standards when optical density readings were plotted against concentration (Figure 5).

As Beer's Law was obeyed the concentration of free fatty acid in each sample could be calculated as follows:-

$$\text{Concentration of sample} = \frac{\text{optical density of sample} \times \text{concentration of sample}}{\text{optical density of standard}}$$

Estimation of the washout of radioactive lipid during perfusion fixation.

The glutaraldehyde solution used to perfuse four of the hearts was collected over one minute intervals after dripping from the heart. Aliquots of the fixative were taken for scintillation counting to determine whether there was any loss of radioactivity from the tissue during the

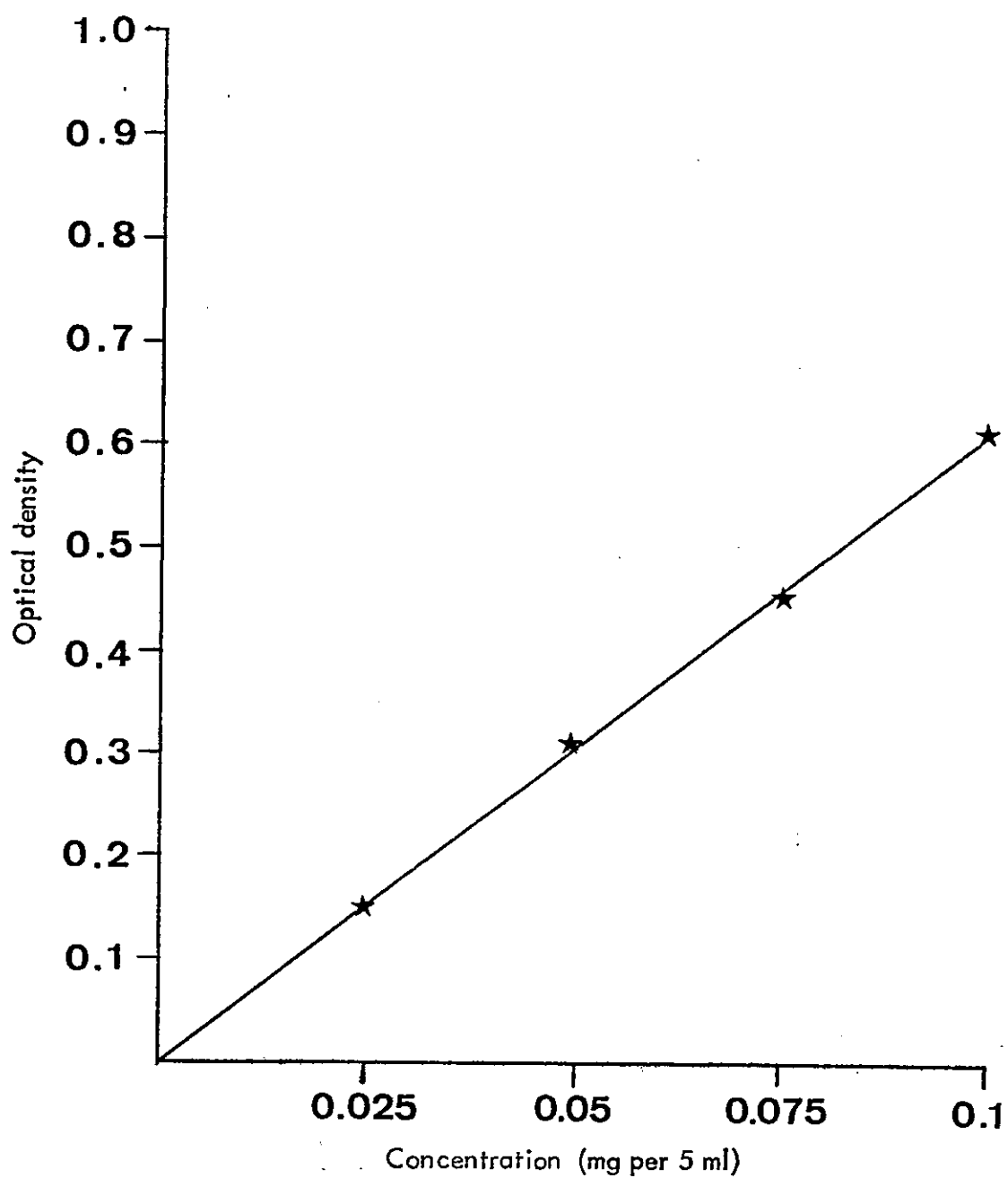


Figure 5

Optical density of palmitic acid standards

fixation period. This could be due either to the loss of intracellular radioactive lipid or to any radioactive oleic acid remaining in the vascular space after the 15 seconds perfusion with cold Krebs Henseleit buffer.

Thin layer chromatography of glutaraldehyde fixed tissue

Four guinea pig hearts were perfused for 15 seconds with 2.5 ml of 0.2mM [9, 10 - ^3H]oleic acid (50 μCi per ml), followed by 15 seconds with ice cold Krebs Henseleit buffer. They were fixed by perfusion with 4% glutaraldehyde for ten minutes as before. The heart muscle was then chopped finely with a razor blade and the lipids extracted with chloroform :methanol (2 : 1 by volume). After separation of the phases aliquots of the upper aqueous phase were taken for scintillation counting.

The lipid-containing chloroform phase was taken to dryness using a rotary evaporator and the residue was redissolved in 2 ml of chloroform. Duplicate 30 μl aliquots of each sample were applied to thin layer plates to determine the degree of esterification of the ^3H oleate. A mixture of known lipid standards containing a cholesterol ester, di- and triacylglycerol, free fatty acid and a phospholipid was run on each plate to aid identification. The solvent system used was the same as that described previously (page 49). The bands were stained with iodine and scraped into scintillation vials for counting.

Thin layer chromatography of the processing fluids from the Durcupan schedule

To determine which classes of lipid were lost from the tissue at each stage of processing, four guinea pig hearts were perfused with 2.5 ml of 0.2 mM [9, 10 - ^3H]oleic acid (100 μCi per ml) for 15 seconds and fixed by a 10 minute period of perfusion with 4% glutaraldehyde. A sample from the left ventricle of each heart was cut into 1mm cubes and processed according to the water-soluble Durcupan schedule (Table 11).

2 ml of processing fluid were used each time. After each stage the processing fluid was transferred to 20 ml of chloroform : methanol (2 : 1 by volume) to extract any lipids removed from the tissue by the procedure. The processed tissue itself was also extracted in chloroform : methanol. To facilitate the analysis, the glutaraldehyde and subsequent buffer wash were combined and analysed together as were the osmium tetroxide and buffer wash. After separation of the phases, the lipid-containing chloroform phase was taken to dryness using a rotary evaporator. In the case of Durcupan A and the full mix samples, a viscous residue remained after the evaporation of the chloroform. All the samples were redissolved in 2 ml of chloroform and aliquots were taken for thin layer chromatography as before. As several extra bands appeared, the plates were scraped according to the positions of the lipid standards.

Thin layer chromatography of Durcupan processing fluids with radioactive lipid standards

To assess whether the processing fluids which were extracted into the chloroform phase along with the lipids were affecting the polarity of the lipids and their migration on thin layer plates, the following was carried out.

1 ml each of glutaraldehyde, osmium tetroxide, 100% Durcupan A and Durcupan full mix were added to 10 mg oleic acid containing trace amounts of $[1 - ^{14}\text{C}]$ oleic acid and 10 mg of trioleylglycerol containing trace amounts of $[1 - ^{14}\text{C}]$ trioleylglycerol. These were left for 1 hour at room temperature. The radiochemical purity of the labelled standards was assessed by thin layer chromatography and was found, in both cases, to be better than 96%.

The samples were extracted with chloroform : methanol and the phases separated as previously. The chloroform phases were taken to dryness using a rotary evaporator and the residues were dissolved in 2 ml of chloroform. 50 μ l aliquots were applied to thin layer plates with triacylglycerol and oleic acid standards. As distinct bands were not visible after treatment with any of the processing fluids, the plates were divided into three for scraping according to the untreated standards. The three regions scraped were the lowest third, including the origin, which was called the 'origin' zone, the central zone which included the position of the free fatty acid standard and was therefore called the 'free fatty acid' zone and the upper third including the position of the triacylglycerol standard, referred to as the 'triacylglycerol' zone.

CHAPTER 6 - RESULTS

The retention of lipids during EM processing

The loss of radioactive material from the tissue was monitored by measuring the radioactivity present in the processing fluids at each stage in the different techniques studied. Measurements were also made of the radioactivity remaining in the tissue after all the processes had been completed. The results in each case are expressed as the percentage ($\pm$ standard deviation) of the total initial radioactivity. This latter was determined from those pieces of tissue which were extracted directly after perfusion fixation.

The recovery of counts was calculated by comparing the counts from processing fluids and processed tissue together, with counts from tissue extracted after perfusion fixation. If these were equal, recovery was 100%.

Each result is the mean $\pm$ standard deviation of all the samples used for that particular method.

Epon

The results from the Epon processing are given in Table 12. Although the dehydration process was considerably shortened from that generally used with Epon, 33.7% of the initial radioactivity was extracted from the tissue in 70% and 95% ethanol. When the dehydration was reduced further to only one change each of 70% and 95% ethanol, ultrastructural preservation of the tissue was impaired. 9.8% of the radioactivity was lost in the Epon resin and full mix stages together and only 20.6% of the initial radioactivity was extracted from the tissue after processing. The overall recovery of counts using the chloroform : methanol extraction was 73.9%.

Table 12Lipid losses with Epon processing

Stage of processing	% of initial radioactivity ($\pm$ SD)
Glutaraldehyde	3.4 ($\pm$ 1.3)
Buffer 1	2.6 ($\pm$ 0.7)
Osmium tetroxide	0.7 ($\pm$ 0.6)
Buffer 2	1.8 ($\pm$ 1.6)
70% ethanol 1	7.5 ($\pm$ 3.9)
70% ethanol 2	7.5 ($\pm$ 3.8)
95% ethanol 1	9.2 ($\pm$ 4.6)
95% ethanol 2	9.5 ($\pm$ 5.3)
Epon resin	5.4 ($\pm$ 2.4)
Epon full mix	4.4 ($\pm$ 2.3)
Total processing fluid	53.3 ($\pm$ 11.9)
Tissue	20.6 ($\pm$ 8.1)
Total recovery	73.9 ($\pm$ 19.6)

Table 13Lipid losses with GACH processing

Stage of processing	% of initial radioactivity ($\pm$ SD)
Glutaraldehyde	2.6 ($\pm$ 1.8)
Buffer 1	1.3 ($\pm$ 0.6)
Osmium tetroxide	0.2 ($\pm$ 0.1)
Buffer 2	0.3 ($\pm$ 0.2)
20% GACH	1.3 ($\pm$ 0.7)
50% GACH	2.8 ($\pm$ 1.9)
80% GACH	2.3 ($\pm$ 1.3)
100% GACH	2.3 ($\pm$ 0.5)
Total processing fluids	12.1 ($\pm$ 2.8)
Tissue	56.5 ($\pm$ 13.6)
Total recovery	68.6 ($\pm$ 12.7)

Table 14Lipid losses with Durcupan processing

Stage of processing	% of initial radioactivity ($\pm$ SD)
Glutaraldehyde	4.2 ($\pm$ 1.5)
Buffer 1	1.5 ($\pm$ 0.3)
Osmium tetroxide	0.5 ($\pm$ 0.4)
Buffer 2	0.6 ($\pm$ 0.5)
70% Durcupan A	12.6 ($\pm$ 6.7)
90% Durcupan A	8.2 ($\pm$ 2.4)
100% Durcupan A 1	4.3 ($\pm$ 3.0)
100% Durcupan A 2	1.8 ($\pm$ 0.5)
Durcupan full mix	0.4 ($\pm$ 0.1)
Total processing fluid	34.1 ($\pm$ 8.6)
Tissue	65.9 ($\pm$ 14.3)
Total recovery	100.0 ($\pm$ 5.4)

Para-phenylenediamine

It was not possible to monitor losses of radioactivity using this method as para-phenylenediamine produces a dark orange colour in solution which was not affected by treatment with hydrogen peroxide. The degree of quenching was so great that quench correction became inaccurate. However, it was still possible to compare counts extracted from the tissue after processing with those extracted after fixation only. Only 17.3% of the radioactivity present in the tissue could be extracted from it after processing.

GACH (glutaraldehyde - carbohydrazide)

The results from the GACH processing are given in Table 13. Only 12.1% of the lipid radioactivity originally present in the tissue appeared in the processing fluids. However the radioactivity extracted from the muscle after processing amounted to only 56.5% of the initial lipid radioactivity. The total recovery of counts was therefore only 68.6%.

Water-soluble Durcupan

The results from the Durcupan processing are given in Table 14. With this method, although there was a 34.1% loss of radioactivity during processing, 65.9% remained in the tissue. Total recovery of counts was 100%.

Alkaline hydrolysis of para-phenylenediamine treated tissue

The total fatty acid content of the chloroform extract and of the residual tissue after alkaline hydrolysis are shown in Table 15. There was little difference between the total fatty acids recovered from unfixed tissue, glutaraldehyde fixed tissue and para-phenylenediamine with Epon processed tissue. Most of the fatty acids present were found in the chloroform : methanol extract and very little remained in the tissue after it had been treated with the chloroform : methanol in all

three cases. Thus the poor recovery of counts from the tissue after treatment with para-phenylenediamine could not be related to poor extraction of lipid by the chloroform : methanol method.

Table 15

Alkaline hydrolysis of para-phenylenediamine treated tissue

Sample		Total fatty acid (mg)
Unfixed tissue	chloroform extract	0.121
	tissue	0.015
Glutaraldehyde fixed tissue	chloroform extract	0.124
	tissue	0.004
Tissue processed with para-phenylenediamine and Epon	chloroform extract	0.123
	tissue	0.012

Washout of radioactive lipid during perfusion fixation

The disintegrations per minute in 10 samples of glutaraldehyde each collected over one minute of a 10 minute fixation perfusion are shown in Table 16. 83% of the counts were recovered during the first 3 minutes of the 10 minute fixation period and 94% in the first 5 minutes.

Thin layer chromatography of glutaraldehyde fixed tissue

When the lipid extracts from the glutaraldehyde fixed hearts were separated on thin layer plates, several additional bands appeared which did not correspond to any of the lipid standards. A sample of glutaraldehyde which had been extracted and chromatographed under the same conditions showed similar bands. It appears that the glutaraldehyde

TABLE 16

Washout of radioactive lipid during perfusion fixation

Time (mins)	dpm	%
0 - 1	116838	43.9
1 - 2	80537	30.2
2 - 3	24430	9.2
3 - 4	13476	5.1
4 - 5	13983	5.2
5 - 6	6471	2.4
6 - 7	3581	1.3
7 - 8	2491	0.9
8 - 9	1383	0.5
9 - 10	2990	1.1

used for fixation is extracted into the chloroform phase of a chloroform : methanol extraction. These additional bands obscured the positions of the lipid bands and the plates were therefore scraped according to the positions occupied by the lipid standards without reference to the extra bands.

After a 15 second pulse perfusion with [9, 10 - ^3H] oleic acid followed by a 15 second chaser with ice cold Krebs Henseleit buffer and 10 minutes perfusion with 4% glutaraldehyde, an average of 50.7% of the lipid radioactivity was present as free fatty acid, 16.7% as triacylglycerol, 21.5% as mono- and diacylglycerol and 7.3% as phospholipid. However, this may not represent the true distribution of the radioactivity in the fixed tissue as glutaraldehyde was found to affect the migration of lipids on thin layer plates as demonstrated below.

Thin layer chromatography of processing fluids from the Durcupan schedule

The processing fluids and processed tissue after the Durcupan technique were extracted with chloroform : methanol and the material partitioning into the chloroform phase was separated by thin layer chromatography. In this way it was hoped to identify the classes of lipid lost from the fixed tissue at each stage. On thin layer chromatography however, the position of the neutral lipid zones was obscured by wide bands visible after exposure to iodine vapour. Because of the difficulties in identifying these bands, the material on the plates was again scraped according to the positions of known standards run simultaneously.

In all the samples, except those from the glutaraldehyde and Durcupan full mix, more than 75% of the radioactivity occurred at the bottom of the plate, at or just above the origin. The results are shown in detail in Table 17.

TABLE 17

Thin layer chromatography of processing fluids from the Durcupan schedule

disintegrations per minute (+ %)

	cholesterol esters	triacylglycerol	free fatty acid	mono- and diacylglycerol	phospholipid
Glutaraldehyde	179 (2.8)	201 (3.1)	2421 (37.8)	2520 (39.4)	1074 (16.8)
Osmium tetroxide	170 (27.5)	90 (14.6)	61 (9.9)	87 (14.1)	208 (33.8)
70% Durcupan A	83 (5.0)	66 (4.0)	86 (5.2)	601 (36.1)	831 (49.8)
90% Durcupan A	70 (2.6)	208 (7.6)	105 (3.8)	1147 (42.0)	1198 (43.9)
100% Durcupan A	54 (3.5)	41 (2.7)	218 (14.2)	531 (34.5)	694 (45.1)
Durcupan full mix	40 (2.3)	62 (3.6)	647 (37.9)	804 (47.1)	154 (9.0)
Tissue	151 (1.2)	458 (3.5)	1990 (15.2)	7124 (54.5)	3372 (25.8)

The most likely explanation for the radioactivity remaining at the bottom of the plate was that the polarity of the lipids and consequently their rates of migration had been modified during processing. This view was confirmed by the results of the following experiment.

Thin layer chromatography of Durcupan processing fluids with radioactive lipid standards

Glutaraldehyde, osmium tetroxide, Durcupan A and Durcupan full mix were chromatographed with $[1 - ^{14}\text{C}]$ oleic acid or $[1 - ^{14}\text{C}]$ trioleylglycerol. The results are given in Table 18.

After glutaraldehyde treatment, seven distinct bands of material were seen on the thin layer plates after they had been exposed to iodine vapour. With osmium tetroxide and both Durcupan A and full mix, wide, densely staining bands were seen just above the origin. As no clear lipid bands could be determined the plates were divided into three portions, the 'triacylglycerol', 'free fatty acid' and 'origin' zones as described in the previous chapter.

With glutaraldehyde, only 78.6% and 86.0% of the counts were associated with the free fatty acid or triacylglycerol bands respectively. After treatment with osmium tetroxide the free fatty acid and triacylglycerol radioactivities were found just above the origin and not in the 'free fatty acid' and 'triacylglycerol' zones respectively. With Durcupan A, 86.2% of the radioactive triacylglycerol was found in the 'triacylglycerol' zone but only 55.3% of the radioactive free fatty acid occurred in the 'free fatty acid' zone. Similarly, with the Durcupan full mix only 48.2% and 13.8% were found in the appropriate zones while most of the rest of the radioactivity was found in the 'origin' zone.

Table 18

Thin layer chromatography of Durcupan processing fluids
with radioactive lipid standards

Sample	Zone	% of counts with free fatty acid	% of counts with triacylglycerol
Lipid standard alone	'triacylglycerol'	1.3	97.1
	'free fatty acid'	96.8	1.9
	'origin'	1.9	1.0
Glutaraldehyde	'triacylglycerol'	7.1	86.0
	'free fatty acid'	78.6	7.7
	'origin'	14.3	6.3
Osmium tetroxide	'triacylglycerol'	3.1	4.0
	'free fatty acid'	9.2	23.2
	'origin'	87.7	72.8
Durcupan A	'triacylglycerol'	6.7	86.2
	'free fatty acid'	55.3	9.5
	'origin'	38.0	4.3
Durcupan full mix	'triacylglycerol'	9.0	13.8
	'free fatty acid'	48.2	51.0
	'origin'	42.8	35.2

PART THREE

THE ULTRASTRUCTURAL LOCATION, BY EM
AUTORADIOGRAPHY, OF ^3H OLEIC ACID

CHAPTER 7 - INTRODUCTION

The ultrastructure of cardiac muscle with relation to its function

The micrographs on the following pages are all of guinea pig left ventricle wall embedded in water-soluble Durcupan.

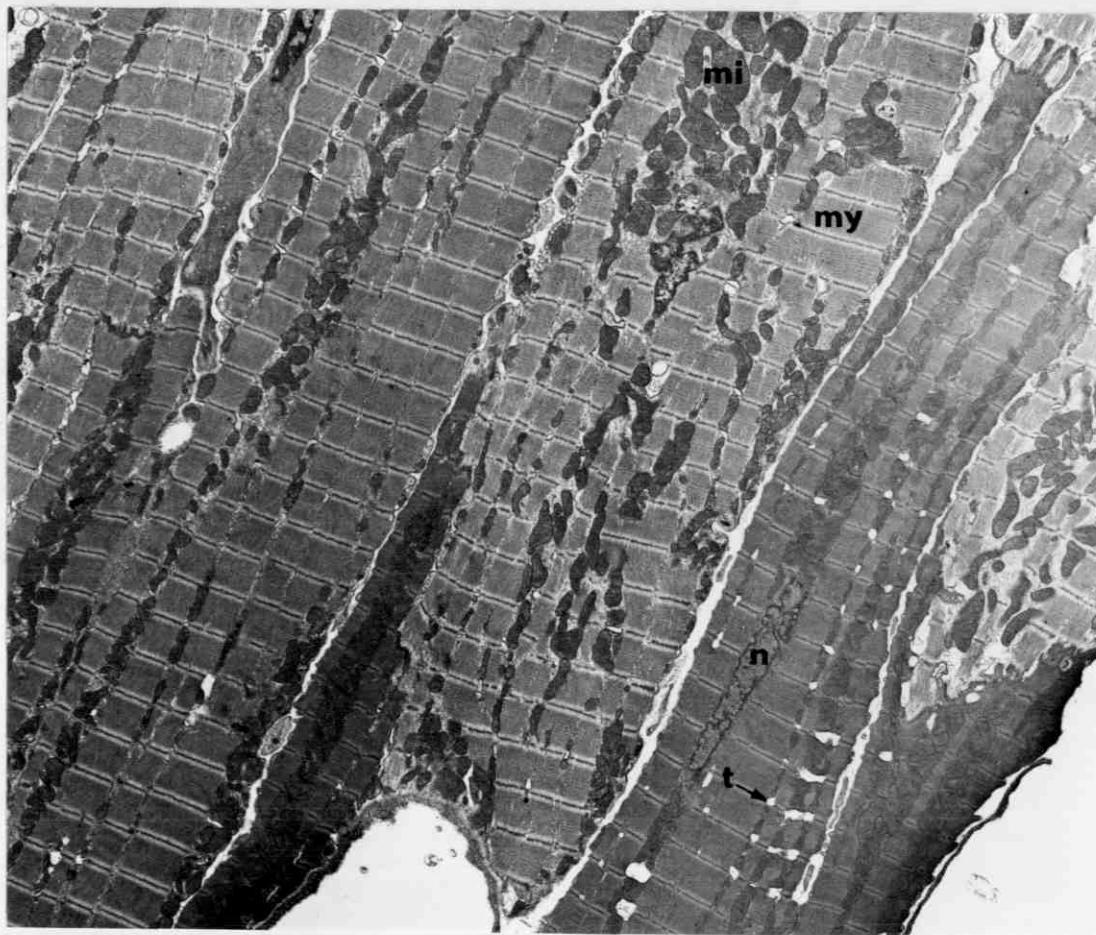


Figure 6

Longitudinal section. Magnification 7,000. my = myofibrils,
mi = mitochondria, n = nucleus, t = T-tubule.



Figure 7

Longitudinal section. Magnification 30,000. my = myofibrils, mi = mitochondrion, n = nucleus, t = T-tubule, sr = sarcoplasmic reticulum, es = extracellular space.

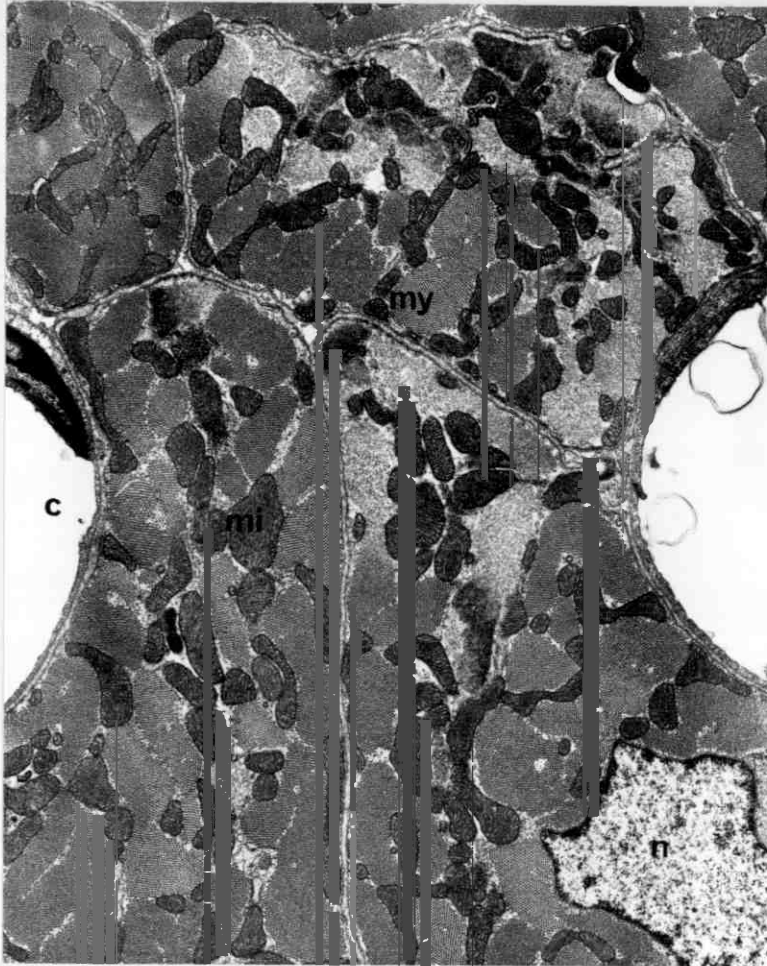


Figure 8

Transverse section. Magnification 15,000. my = myofibrils,
mi = mitochondria, n = nucleus, c = capillary.

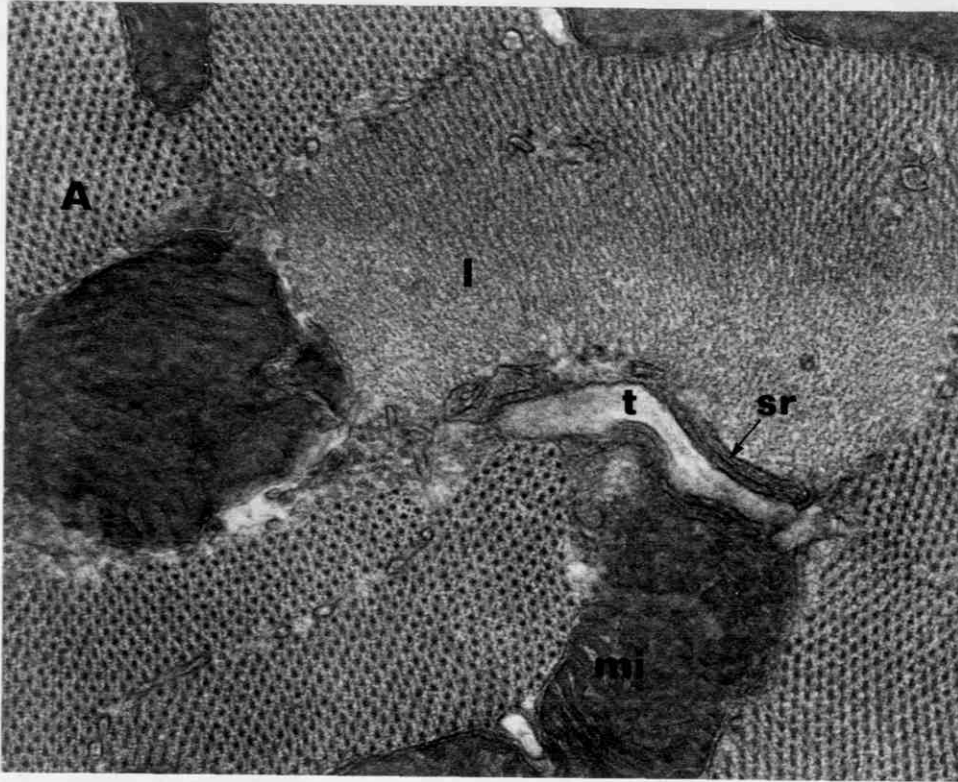


Figure 9

Transverse section. Magnification 48,000. I = I band, A = A band, mi = mitochondrion, t = T-tubule, sr = sarcoplasmic reticulum.

The mammalian myocardium is a functional network of small cells, approximately 10 to 15 microns in diameter (compared with about 100 microns in skeletal muscle). Every myocardial cell is capable of a rhythmic contraction and relaxation by converting chemical energy to mechanical work and also of the conduction of electrical impulses from cell to cell to coordinate the mechanical activity throughout the heart. This is facilitated by the gap junctions which are part of the specialised regions of attachment between cells, known as the intercalated discs, a specific feature of cardiac muscle. The main function of most of the cells is contraction, while a small number, in the nodes and Purkinje system, are specialised for the initiation and rapid conduction of electrical impulses throughout the heart.

The contractile apparatus

The contraction of the muscle cells is brought about by a system of sliding filaments (Figure 10). Each muscle cell contains a large number of longitudinally arranged rod-like myofibrils which run the length of each muscle cell. Each myofibril consists of a long succession of partially overlapping, interdigitating arrays of thick and thin protein filaments, each thick filament being surrounded by six thin filaments in a hexagonal pattern. The most prominent bands of the muscle are the A-band (A = anisotropic), which stains densely with heavy metals, and the I-band (I = isotropic). In the I-band only thin filaments are present while the A-band consists of the thick filaments and the overlapping portions of the thin filaments. Muscle shortening results from the relative sliding of the two sets of filaments (Huxley H. and Hanson, 1954, Huxley A.F. and Niedegerke, 1954).

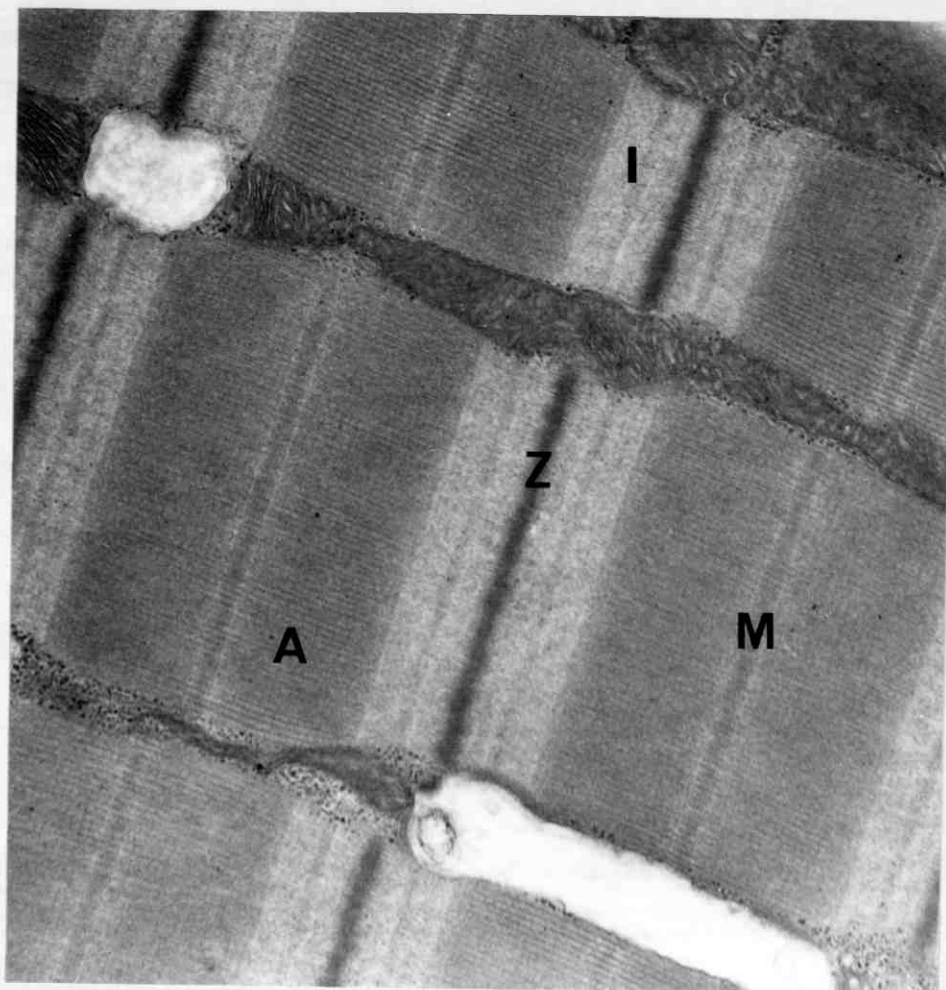


Figure 10

The contractile apparatus. Magnification 48,000.

I = I band, A = A band, Z = Z band, M = M band.

In the centre of each I-band is an electron-dense, cross-linking structure called the Z-band which links one set of thin filaments to another. The repeating unit between one Z-band and the next is known as the sarcomere.

The thick filaments are 15 nm in diameter and about 1.5 μ m in length. More than 95% of the thick filament consists of the protein myosin (Huxley, 1963). Each myosin molecule consists of a rod-shaped region about 150 nm by 2 nm and a "head" region consisting of two globular units. The rod-like "tails" form the relatively inactive, rigid backbone of the thick filament. Negative staining of the thick filament has highlighted numerous projections located on either side of a central bare zone (Huxley, 1963). These are the myosin "heads" which form the "cross-bridges" which are seen in micrographs spanning most of the 1.3 μ m gap from the thick to the thin filaments in muscle in rigor. This model of the thick filament in which only the head of the myosin molecule is biologically active is consistent with results from biochemical experiments in which selective enzymatic digestion of myosin molecules was used to delineate the properties of each part of the molecule (Szent-Györgyi, 1953, Mueller and Perry, 1962). These studies showed that the fragment which forms most of the myosin tail consists of an α -helical rod, light meromyosin (LMM) which is insoluble at low ionic strengths (< 200 mM). The insolubility of LMM causes myosin molecules to aggregate spontaneously if the ionic strength is low enough. The fragment forming the part of the tail close to the globular heads is composed of heavy meromyosin subfragment 2 (HMM S2) whilst the heads are composed of heavy meromyosin subfragment 1 (HMM S1). The two biochemical activities of myosin are ATPase activity (Engelhardt and Ljubimova, 1939) and the ability to combine with actin (Straub, 1942).

Other proteins occur in the thick filaments. In particular, C-protein which binds strongly to LMM and so is probably associated with the thick filament backbone (Offer, 1972). Electron micrographs of muscle treated with antibody to C-protein have shown the C-protein molecules to be arranged in seven stripes about 43 nm apart in either half of the A-band (Offer, 1972).

The central, bare region of the thick filament where no heads are present is approximately 160 nm long. In the centre of the bare zone the thick filaments are cross-linked to each other in a structure called the M-band. Probably at least two proteins, apart from myosin, comprise the M-band since the band appears to consist of (1) "M-bridges" and (2) "M-filaments" lying parallel to the thick filaments (Knappeis and Carlson, 1968). The M-bridges link any thick filament to its six neighbours in the hexagonal filament array. There is a maximum of five M-bridges each separated by about 22 nm thus giving the M-band a maximum width of about 88 nm. The M-bridges and filaments are composed of the so-called M-proteins, one of which has been identified in skeletal muscle as creatine kinase (Turner et al., 1973). This creatine kinase, in combination with cytosolic creatine phosphate, forms a powerful ATP regenerating system located only a short distance ($<0.8 \mu\text{m}$) from the site of ATP hydrolysis by actomyosin.

The thin filament has a backbone consisting of globular actin monomers (G-actin) polymerised together to form filaments (F-actin which are coiled into a double helix of pitch $2 \times 37 \text{ nm}$ (Hanson and Lowy, 1963, Huxley, 1963). Each thin filament is about $1.0 \mu\text{m}$ long and terminates at the Z-band where it is joined to four other thin filaments by inter-connecting Z-filaments in a tetragonal lattice or "basket-weave" structure (Knappeis and Carlson, 1962). Between the two actin chains of the thin filament are two grooves, in each of which is localised a tropomyosin filament.

Thus both actin and tropomyosin extend along the length of the thin filament. The troponin complex, which consists of one molecule each of troponin C, troponin I and troponin T, is bound to tropomyosin at intervals of approximately 38 nm, i.e. one for every seven actin monomers (Head and Perry, 1974) and about 13 nm from the end of each tropomyosin molecule (Ohtsuki et al., 1969). This has been demonstrated in electron microscope studies using ferritin-labelled antibody to the troponin complex (Ohtsuki, 1974) and confirmed by X-ray diffraction studies (Rome et al., 1973). Troponin and tropomyosin are together known as the "regulatory proteins" and confer calcium sensitivity on the interaction between actin and myosin.

Mitochondria

Figure 11

(Magnification 40,000)



Each mitochondrion is composed of two separable membrane systems, the limiting or outer membrane and the inner membrane which encloses an inner space known as the matrix. The inner membrane is thrown into numerous folds known as cristae. These are sheet-like structures which appear as tubes in cross-section, extending into the matrix which are closed on their interior end and open on the end which fits into the inner membrane. The space within the crista is therefore continuous with the space between the two boundary membranes.

The inner and outer membranes may be separated by chemical and physical methods and this has made it possible to pinpoint in which membranes the mitochondrial functions are localised. The outer membrane is more penetrable by small molecules than is the inner membrane. It is also the site of some of the mitochondrial enzymes including monoamine oxidase, fatty acyl-CoA synthetase, hexokinase 2 and phospholipase A2. The inner membrane has characteristic repeating units, connected by a cylindrical stalk to a basepiece. These structures can be seen in negatively stained preparations. The mechanism of oxidative phosphorylation is totally dependent on the presence of these repeating units. The four complexes of the electron transfer chain have been identified in size, shape and properties (e.g. the 'de novo' membrane formation) with the basepieces of these repeating structures. The headpieces and stalks play no part in the electron transfer process which still occurs if the headpieces and stalks are stripped off. However they are essential for the synthesis of ATP. Isolated headpieces have an ATPase activity and when isolated this ATPase has the same size and shape as the headpieces. The stalk consists of a single protein of about 25,000 molecular weight. Korman et al (1970) described an "energy cycle" in mitochondria which consists of a series of events which occurs

when mitochondria in the "non-energized" state (a state in which they are not capable of performing work) are energized, either by oxidation of oxidisable substrates or by the hydrolysis of ATP, to an energized state (a state in which they are capable of doing work) and then discharged back to the non-energized state by the performance of work (e.g. ion translocation or synthesis of ATP) or by uncouplers. During the energy cycle the stalk changes from an extended (energized) to a relaxed (non-energized) state. As well as the respiratory chain and ATP synthesising enzymes, the inner membrane contains a fatty acyl-transferase for the transfer of fatty acids into the matrix. This process is described in more detail on page 27.

The matrix contains small granules, 30 to 40 nm in diameter which are thought to be sites of calcium storage. Smaller, less conspicuous granules are also present in the matrix and may be the nucleoprotein which is known to be present. The matrix is the site of both the tricarboxylic acid cycle and of fatty acid oxidation and therefore contains the enzymes involved in these processes.

The mitochondria are closely applied to the myofibrils. This minimises the distance that energy rich compounds must be transported, from the mitochondria where they are synthesised to the myofibrils where they are used. Although ATP is synthesised in the mitochondrial matrix, its high energy phosphate is transferred to creatine to form creatine phosphate by the action of a creatine kinase. Another creatine kinase is located within the myofibrils, in the M-band, and this catalyses the reformation of ATP.

Nucleus

There is usually a single elongated nucleus in each cell, situated near the centre and oriented along the cell's axis. Two nuclei per cell have however been reported in the myocardium of the dog (Muir, 1965), mouse (Kogan et al., 1976), rat (Katzburg et al., 1977), pig (Pfitzer, 1971) and human (Schneider and Pfitzer, 1973). The myocardial nuclei differ from those of other tissues in that during the embryonic and foetal period in most animal species, they undergo mitosis, and cell proliferation occurs, but soon after birth the cells cease to divide and the growth of the heart takes place by the increase in size of the existing cells.

Although mammalian myocardial cells are usually thought to be diploid, polyploid cells have been found in the hypertrophied human heart (Sandritter and Scmazzone, 1964) and more abundantly in the myocardium of the monkey and pig (Pfitzer, 1971, Pfitzer et al, 1977), the mouse (Peterson and Baserga, 1965, Arefyeva, 1978) and in the rat (Kuhn et al., 1974). Ya Brodsky et al. (1980) suggested that the binuclear cells are formed by an acytotic mitosis in the neonatal mouse heart while the polyploid condition is due to other types of incomplete mitosis.

The nucleus is enclosed in a membranous nuclear envelope which appears as two membranes separated by a narrow space, the perinuclear cisterna. Nuclear pores occur at intervals in the envelope. These are round or octagonal structures approximately 80 nm in diameter. At these points the outer and inner nuclear membranes join to form a non-membranous annular structure consisting of three rings, each of which consists of 8 electron dense granules with one central granule. The rings are joined by electron-dense fibrillar material. It is thought that these pores are the sites of the exchange of substances between the cytoplasm and the nucleoplasm. Close to the nuclear envelope on the inside of the nucleus is a thin layer of condensed chromatin (hetero-chromatin) which is inactive.

Sarcolemma

The bounding membrane of cardiac muscle cells, the sarcolemma, consists of (1) a plasma membrane which is 8 - 9 nm thick and (2) an extracellular glycoprotein layer - the basement coat, which is approximately 50 nm thick.

The plasma membrane is enzymatically active and it acts as a selective barrier to the free flow of low molecular weight substances and ions such as sodium, potassium and calcium. It contains a Na^+ - K^+ - activated ATPase (Dietze and Hepp, 1971) enzyme. The plasma membrane displays an active pinocytotic activity and subsarcolemmal vesicles are often seen.

The glycoprotein coat is thought to play a part in influencing the local environment at the plasma membrane because of its fixed negative charges. Using the electron-dense ion lanthanum to displace calcium from the basement coat, Langer and Frank (1972) showed that this layer forms a Ca^{2+} reservoir.

Intercalated discs

Cardiac muscle cells are attached to one another by a specialised region known as the intercalated disc where the two cell membranes are closely apposed. Over much of the intercalated disc the two membranes lie parallel and are separated by a 20-30 nm gap containing proteinaceous material which binds the membranes together. This region is known as the fascia adherentes. The cytoplasm close to these membranes often contains a dense network of fine filaments. The thin filaments of the I-band enter this network and so attach the myofibrils to the plasma membrane. Specialised areas known as desmosomes (or spot desmosomes or macula adherens) occur where again the plasma membranes are parallel and separated by a 25-30 nm gap filled with proteinaceous

material, but in the desmosomes, this proteinaceous material often exhibits an electron-dense central layer. The cytoplasmic side of each membrane has an electron-dense thickening. The desmosomes are round areas approximately 200 - 300 nm in diameter.

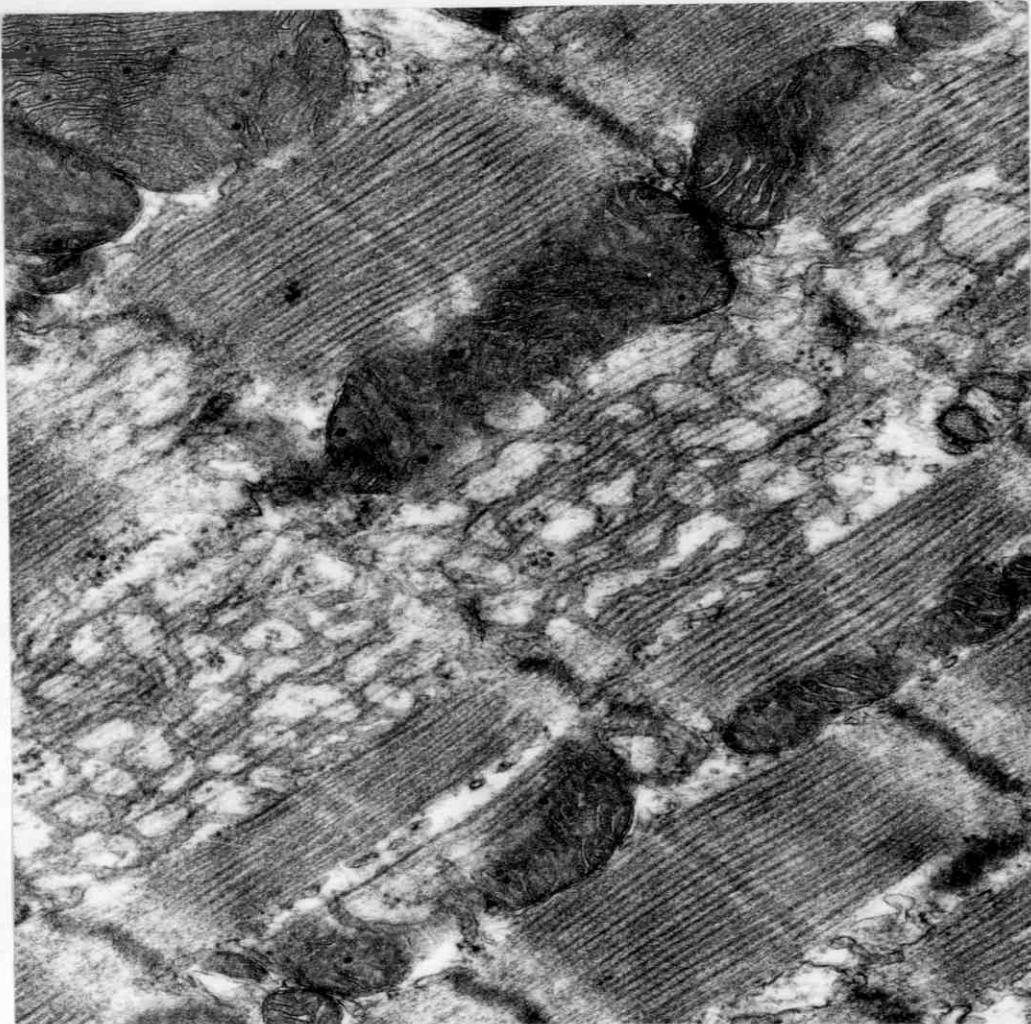
Another specialised region of the intercalated disc is the gap junction or nexus, first described by Barr et al. (1965). This is probably the site at which waves of membrane depolarization pass from one cell to the next as the membranes are in close apposition with a gap between them of only 2 nm. The gap junction membranes are composed of globular subunits arranged in a hexagonal array with 9 to 10 nm spacing (Revel and Karnovsky, 1967, McNutt and Weinstein, 1970).

Transverse tubules

Tubular invaginations of the plasma membrane extend into the interior of the cell. These invaginations form long, slender tubules which are individually called transverse or T - tubules, and collectively, the T - system. The lumen of the T - tubule is continuous with the extracellular space and the tubules extend deep into the cells running mainly transversely but also longitudinally. This has been shown with the use of electron-dense, extracellular markers such as colloidal lanthanum, horseradish peroxidase and ferritin (Huxley, 1965, in skeletal muscle, Forssman and Giradier, 1970, Nayler and Merrillees, 1971). The longitudinal elements have been called 'labyrinths' and may span the length of a sarcomere or more (Forbes and Sperelakis, 1977).

The T - tubules usually occur at the level of the Z-band in cardiac muscle. They increase the surface area to volume ratio and therefore facilitate the rapid distribution of the excitatory stimulus throughout the cell (Bastian and Nakajima, 1974) as well as providing a large area for the exchange of metabolic substrates and ions. The T - tubules are lined with the glycoprotein basement coat.

Sarcoplasmic reticulum (Figure 12)



The endoplasmic reticulum of muscle cells is generally lacking in ribosomes and is called the sarcoplasmic reticulum. It consists of a three-dimensional network of branching and anastomosing tubules approximately 20 - 40 nm in diameter, closely applied to the myofibrils. It covers many sarcomeres without interruption and approaches closely to the plasma membrane. Isolated fragments of sarcoplasmic reticulum are able to accumulate calcium against a concentration gradient (Martonosi, 1972). The rate of calcium accumulation is dependent

on the rate of hydrolysis of ATP (Makinose and Hasselbach, 1965). There is no continuity between the lumen of the sarcoplasmic reticulum and the extracellular space (Forssman and Girardier, 1970). The tubules of the sarcoplasmic reticulum generally appear unspecialised but where these tubules approach the plasma membrane at the cell periphery they form flattened cisternae which are in close proximity to the plasma membrane. These cisternae presumably facilitate the exchange of material between the lumen of the sarcoplasmic reticulum and the cell surface. The cisternae may be involved in the coupling of the action potential to released calcium from the sarcoplasmic reticulum. Sarcoplasmic reticulum cisternae are also seen close to the T - tubules. In thin sections of skeletal muscle, cisternae can often be seen on both sides of a T - tubule forming a "triad" but in cardiac muscle these cisternae occur less frequently and a "dyad" is more often seen where a single cisterna is applied to the T - tubule. However in some mammals such as the mouse or ferret where the heart beats rapidly, many cisternae may be seen apposed to the T - system (Simpson and Rayns, 1968). The gap between the cisternal membrane and the T - tubule membrane is approximately 20 nm. Regularly arranged electron-dense 'feet' are sometimes seen (Figure 13) projecting from the facing membranes of the sarcoplasmic reticulum. These feet are approximately 15 nm long. They do not therefore span the entire gap between the membranes (Franzini-Armstrong, 1973, Nayler, 1975). The flattened lumen of the subsarcolemmal cisternae contains a granular dense material (Sommer and Johnson, 1970). The main function of the sarcoplasmic reticulum is the uptake of calcium ions from the myofibrils and the release of calcium to initiate contraction.

A number of vesicles, which are an integral part of the sarcoplasmic reticulum, occur at the level of the Z-line. These were first described



Figure 13

Transverse tubule with elements of sarcoplasmic reticulum

t = T-tubule, sr = sarcoplasmic reticulum, f = feet.

Magnification 110,000.

by Fawcett and McNutt (1969, McNutt and Fawcett, 1969) and more recently by Sommer and Waugh (1976) and Sommer and Johnson (1979). These have been called "coated dense vesicles", "corbular sarcoplasmic reticulum" or "coated reticulum". The lumen of these vesicles is electron opaque and the coating layer is made up of membranous elements similar to those seen in the junctional regions of the sarcoplasmic reticulum. These specialised regions of the sarcoplasmic reticulum are thought to be instrumental in the mobilization and utilization of calcium ions within the cell either by releasing calcium into the cytoplasm to initiate contraction or by pumping calcium in from the cytoplasm to cause relaxation or possibly by both processes (Langer, 1964, Reuter, 1974, Fabiato and Fabiato, 1977).

In some species a specialised tubule of the sarcoplasmic reticulum has been described (Simpson, 1965, Simpson and Rayns, 1968) which surrounds the myofibrils following the course of the Z-band and it has therefore been called the Z-tubule. These Z-tubules often appear to be connected to the myofibrillar surface at the Z-line by strands of opaque material. It is thought that the Z-tubules follow the Z-line because they are held together by this material (Forbes and Sperelakis, 1980). Z-tubules frequently lead into the coated dense vesicles and junctional sarcoplasmic reticulum close to the T-tubules.

Other intracellular inclusions

In close proximity to the mitochondria are situated the reserves of fuel for oxidation, namely lipid droplets and glycogen granules. The lipid droplets are spherical bodies, approximately $0.1\ \mu\text{m}$ in diameter and are closely apposed to the mitochondria. They contain large amounts of triacylglycerol of which there is a dynamic turnover as triacylglycerol is constantly hydrolysed to provide free fatty acid especially when substrates in the blood are depleted. The triacylglycerol is increased when free fatty acid levels in the blood are high.

Glycogen granules are abundant in normal myocardial cells with a plentiful supply of oxygen and are seen more in cardiac muscle than in skeletal muscle. The electron-dense granules are approximately 15 - 30 nm in diameter and are distributed singly in the cytoplasm in the region of the mitochondria although occasionally aggregates can be seen. These two large stores of energy-rich substances, the lipid droplets and the glycogen granules, ensure against substrate deprivation.

Other cytoplasmic inclusions occur, especially in the regions of cytoplasm at either end of the nucleus. These include the Golgi apparatus which consists of flattened membranous sacs which occur close to the nuclear envelope. The sacs are slightly dilated at each end and numerous vesicles occur close to these ends. Some of these are smooth vesicles limited by a single unit membrane and may sometimes be seen fusing with the nuclear envelope and sometimes with the Golgi apparatus (McNutt and Fawcett, 1974) implying a transport function. Other vesicles in this region are the "coated" vesicles which are better named "bristle-coated" vesicles to avoid confusion with the coated dense vesicles of the sarcoplasmic reticulum. The bristle-coated vesicles have a 20 nm layer of electron-dense material which thickens the limiting unit membrane and has a bristly appearance. The function of these vesicles is unclear although they may play a part in transporting proteins to lysosomes which also occur in the perinuclear region. Also in this part of the cell lipofuchsin bodies are sometimes seen consisting of heterogeneous electron-dense material enclosed by a membrane. They consist of an insoluble lipoprotein pigment which may be left after the lysosomal enzymes have completed their digestion in autophagic vacuoles. The pigment increases in amount with the age of the animals.

Occasionally, a collection of fibrillar material, the "leptofibrils" is seen, particularly in transverse sections. They display a distinct banding pattern with a periodicity of about 180 nm which has led to the suggestion that they may represent abortive attempts at sarcomere formation (Walker et al., 1975). The striations often appear to merge with the Z-line material indicating a possible origin from it. The function of the leptofibrils, if any, is unknown.

"Intermediate filaments", having a diameter intermediate between actin and myosin were first described by Hueson-Stiennon in 1965 and have recently been studied by Forbes and Sprerelakis (1980). These are filaments collected into discrete bundles, running along the Z-lines at right angles to the long axes of the myofibrils. The average diameter of these filaments is 10 nm and they have a relatively translucent centre. It is thought that these filaments contribute towards the formation of the cell's internal supporting framework or cytoskeleton in various muscle cell types (Cooke, 1976, Eriksson and Thornell, 1979). In adult myocardial cells these filaments are nearly always oriented along the transverse plane of the cell, passing across the sarcomeres at the Z-line. This is in contrast to myocardial microtubules, the circular profiles of which may be seen in transverse sections (Ferrans and Roberts, 1973). It is thought that the intermediate filaments may form the bulk of the cytoskeleton in some species (e.g. man: Ferrans and Roberts, 1973, rat: Behrendt, 1977, mouse: Forbes and Sperelakis, 1980) while the microtubules predominate in other species (e.g. dog: Goldstein and Entman, 1979).

Ultrastructure in hypoxia

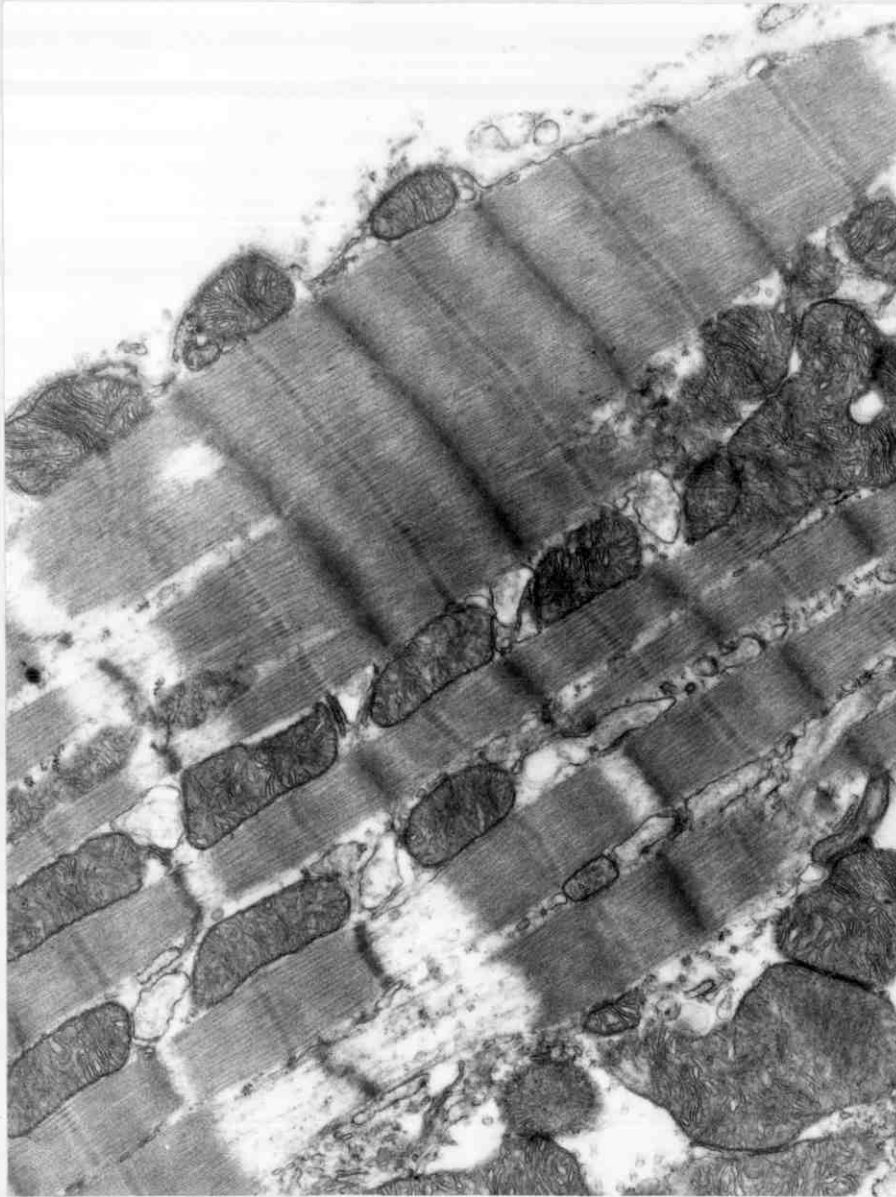


Figure 14

Guinea pig left ventricle after 30 minutes hypoxic perfusion showing loss of glycogen and intramitochondrial particles, contraction of the myofibrils and disruption of the sarcolemma. Many cells appear unaffected after this length of perfusion. Magnification 30,000.

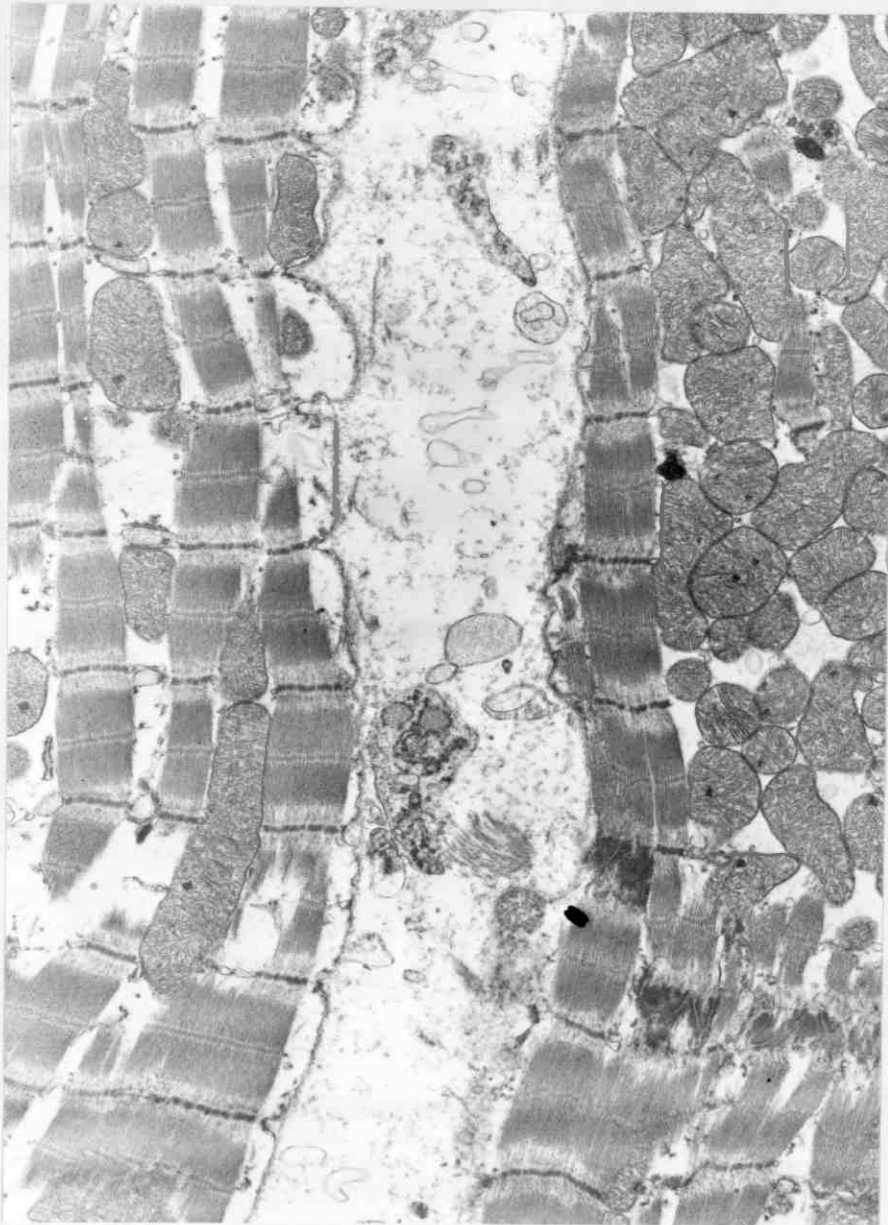


Figure 15

Guinea pig left ventricle after 90 minutes of hypoxic perfusion (without substrate for oxidation) showing severe intracellular oedema and disruption. Magnification 18,000. Micrograph courtesy of A. Slade. Araldite embedding.

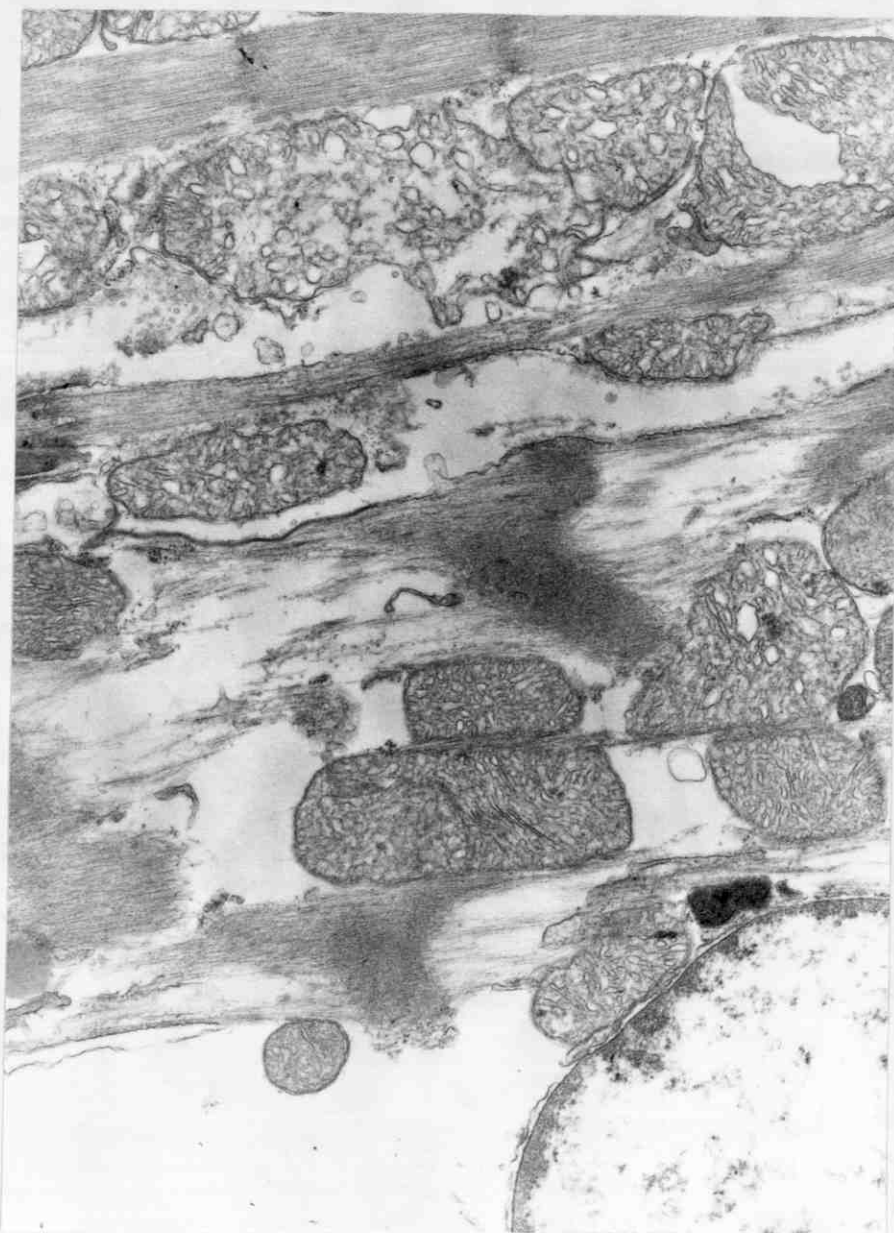


Figure 16

Guinea pig left ventricle after 120 minutes of hypoxic perfusion (without substrate for oxidation) showing irreversible damage (see page 116). Magnification 43,000. Micrograph courtesy of A. Slade. Araldite embedding.

Various methods of producing acute myocardial hypoxia have been studied in animal models. These include inhalation of nitrogen, constriction of the trachea or perfusion of isolated hearts with solutions equilibrated with a nitrogen and carbon dioxide mixture. All these methods result in similar effects on the ultrastructure of the myocardium.

Firstly, there are changes in mitochondrial structure which occur after a few minutes of severe hypoxia. These include the disappearance of the intramitochondrial granules (Hausamen and Poche, 1965, Poche et al., 1969). In isolated rat papillary muscles bathed in oxygenated Tyrode's solution, cross-sectional profiles of the entire muscle showed a gradient of intramitochondrial granules from the surface to the centre with the mitochondria in the centre having fewer granules than those near the periphery (Goldstein et al., 1977). In chronic hypoxia the mitochondria may appear swollen (Sulkin and Sulkin, 1965, Bischoff et al., 1969) and "giant" mitochondria, extending over several sarcomeres, are seen and appear to originate from the fusion of several mitochondria. Intermembranous inclusions may be seen in the mitochondria. These may be unilaminar or bilaminar plate-like structures or of paracrystalline nature or they may be amorphous. These inclusions are thought to be of protein nature as shown by enzyme digestion of inclusions with similar appearance in ischaemic heart muscle (Hanzlikova and Schiaffino, 1977, Schiaffino et al., 1979) and are thought to be due to the aggregation of mitochondrial enzymes such as creatine kinase which is abundant in mitochondria and known to aggregate readily. The cristae of the mitochondria appear reduced in number and may appear irregular or fragmented.

Other ultrastructural changes due to acute hypoxia include the depletion of the glycogen stores, first described by Caulfield and Klionski (1959). The T-tubules and sarcoplasmic reticulum are enlarged and spaces appear between the myofibrils which are attributed to intracellular oedema

(Hasper, 1964, Swynghedauw et al., 1967). The myofibrils may be disrupted (Bahr and Jennings, 1961, Hasper, 1964, Pentilla and Liesto, 1980) and contraction bands may be seen (Schmalbruch, 1970). The intercalated discs may separate (Buchner and Onishi, 1967, Poche et al., 1969). Lipid droplet accumulation occurs in chronic hypoxia but there is no evidence of this in acute hypoxia. In the nucleus, clumping and margination of the nuclear chromatin may occur (Swynghedauw et al., 1967, Pentilla and Liesto (1980) assessed the time course of ultrastructural myocardial changes in the Langendorff perfused rat heart when perfused with Krebs solution equilibrated with 95% N₂: 5% CO₂. As criteria of irreversible damage they took : swelling and stretching of fibres associated with loss of staining intensity and loss of register of band areas, granular sarcoplasm, breaks in the sarcolemma and extracellular detachment of sarcoplasmic organelles, swelling and disorganisation of the mitochondrial membrane system with the presence of amorphous intramitochondrial densities. Using these criteria they found that after 30 minutes of hypoxic perfusion, approximately one tenth of the myocytes showed irreversible changes; after 60 minutes approximately one third to one half and after 120 minutes practically all the cells were irreversibly damaged.

The autoradiographic technique

Historical background

The discovery of the autoradiographic process occurred many years before the discovery of radioactivity itself. In 1867 Niepce de St. Victor published his findings that uranium nitrate and tartrate were capable of producing a blackening effect on emulsions of silver chloride or iodide. He found that this blackening occurred even when the uranium salts and the silver halide emulsion were separated by sheets of glass of different colours. He explained these results in terms of luminescence.

It was not until twenty years after Niepce de St. Victor's publication that the work of Henri Becquerel, and later of the Curies, led to the discovery of radioactivity. Becquerel (1896) repeated Niepce de St. Victor's work and found that, after exposure to sunlight, crystals of uranyl sulphate could blacken a photographic plate even when there were several layers of black paper between the two. During his experiments several days elapsed when the weather remained cloudy and the uranyl sulphate crystals and photographic plate were left together in a closed drawer in readiness for the next sunny day. This photographic plate too was found to have blackened in spite of the fact that the crystals had not previously been exposed to sunlight. Two years later, in 1898, Marie Curie demonstrated the existence of radioactivity.

In 1904, London exposed a frog, which was contained in a hermetically sealed bottle, to a solution of radium. He placed the animal, after death, on a photographic plate and, after suitable exposure and photographic development, obtained an image of the whole frog in silvery grains.

The first real use of the autoradiographic process to study the distribution of radioactive substances was by Lacassagne and Lattes (1924). They assessed the distribution of polonium in various tissues embedded in paraffin wax by its reaction with a photographic emulsion.

From this time, until the early 1950's, very little use was made of the autoradiographic technique in the field of biology, although the study of radioactivity by physicists progressed greatly during the 1930's and 1940's. The main reason for this was that the few naturally occurring radioactive substances, namely thorium, radium and uranium, were of little interest or importance to biologists. However a few studies were carried out on the distribution of the salts of these elements in a few animals and plants. One such study which is worthy of note is that of Hamilton et al. (1940) who showed that the thyroid gland would take up radioactive iodine. This led Leblond (1943) to prepare and publish autoradiographs showing the distribution of radioactive iodine within the gland.

With the invention of the atomic bomb the need to assess the distribution of fission products in radioactive fall-out in plant and animal tissues arose. At this time many new radioactive isotopes became available for biological research and the process of autoradiography was rapidly expanded and refined. In 1949, Belanger and Leblond published a technique for autoradiography which gave better resolution than that used previously. They removed the molten emulsion from a lantern plate and brushed it on to the specimen using a fine paint brush. The stripping film method which is still sometimes used today involved stripping the emulsion from the plate as a solid and applying it directly to the specimen. This method was described by McDonald et al. (1948) and by Boyd and Williams (1948). The widely used technique of dipping slides into molten emulsion was first described by Joffes and Warren in 1955.

In 1957, Taylor et al. introduced tritium as a biological marker and were able to produce higher resolution autoradiographs. The β -particles emitted from tritiated compounds are of much lower energy than those of other isotopes and for this reason do not travel very far from their source.

Autoradiography was first coupled with electron microscopy by Liquier-Milward in 1956 but the resolution of the micrographs he published was little better than that already achieved by light microscopy. He used a preparation of isolated liver cell nuclei labelled with cobalt-60, a high energy β -particle emitter. The samples were placed on a glass slide and coated with Ilford G-5 emulsion. Exposure was carried out in a coal mine at a depth of 600 metres to avoid 'background effects'.

O'Brien and George (1959) were the first to demonstrate the real potential of autoradiography in electron microscopy in a study of yeast cells using the α emitter polonium-210. The emulsion was applied directly on to the grids and ultra-thin sections were used. The marker was found on the surface of the cells showing that yeasts are impermeable to polonium.

The first EM autoradiographs using tritium were published by van Tubergen in 1961. He labelled bacteria of the Escherichia coli strain with tritiated thymidine and used Kodak V-1055 emulsion which contained smaller silver halide crystals than those used previously. The combination of the lower energy isotope with the smaller crystals gave considerably better resolution. Van Tubergen applied the emulsion with a loop. He dipped the loop into the emulsion and allowed it to gel in the loop before applying it to the specimen.

A resolution of 0.3 microns was achieved by Caro (1961) using 60 nm sections of guinea pig pancreas labelled with tritiated leucine and emulsified with Kodak K-5 emulsion. In the same year a new emulsion, Ilford L-4 was introduced with a crystal diameter of about 0.12 microns. This is the emulsion which is generally used today for EM autoradiography.

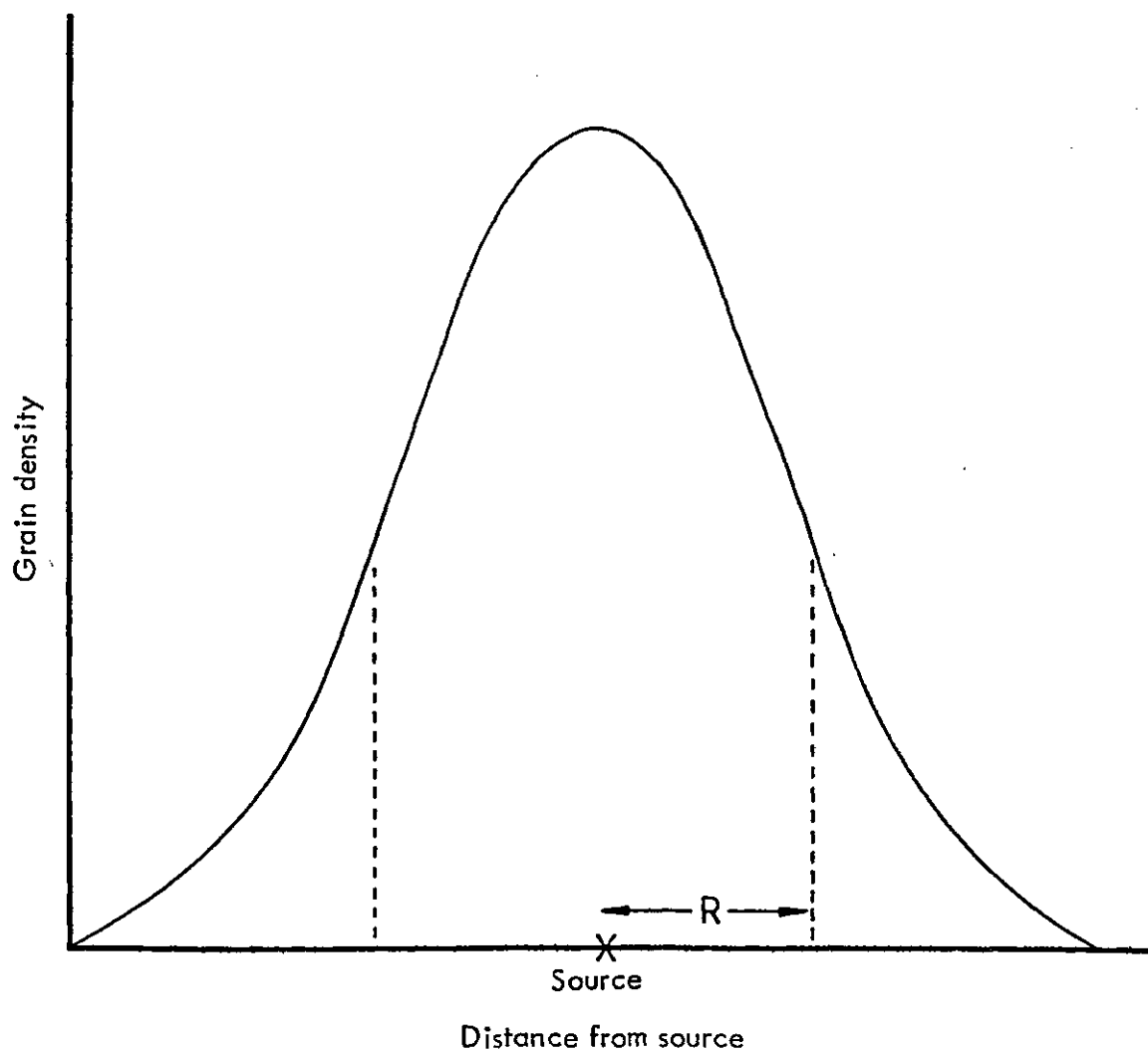
The technique has changed very little since the 1960s. Most of the recent developments have been in the field of statistical analyses of the autoradiographs.

The photographic process

The photographic emulsion, used in plates and films as well as that used for autoradiography, consists of a suspension of silver bromide crystals in gelatin. When light falls on such an emulsion a change is produced in the silver bromide crystals. The light, or radioactive particle, increases the energy of the outermost electron of a bromide ion so that it leaves its orbit. This electron travels through the crystal from one ion to the next until it becomes trapped at one of the defects in the lattice. These defects are known as 'sensitivity specks' and usually consist of silver sulphide. At this site an ion of silver becomes converted to an atom of silver by accepting the electron. A latent image is formed when enough atoms of silver are present at a sensitivity speck to bring about conversion of the entire crystal to metallic silver on development. The developer is a reducing agent and results in silver deposition catalysed by the silver present at the sensitivity speck. An elongated ribbon is formed by the addition of silver to the latent image spot. Fixation dissolves away the crystals which have not been exposed and leaves the gelatin clear, interrupted only by the silver grains.

Resolution

A point source of β -particles emits particles in all directions from the source. The density of silver grains decreases with increasing distance from the source. Figure 8 represents the distribution of silver grains around a point source. The grain density in the emulsion is highest immediately over the source and falls off symmetrically as the distance away from it increases.

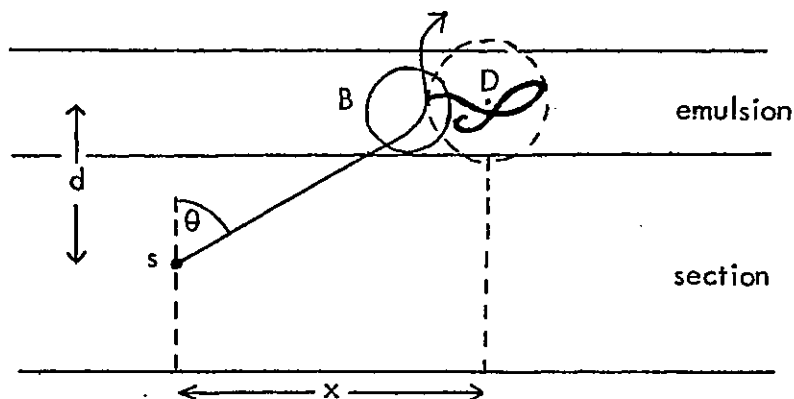


The distribution of grains around a point source

In the figure on the preceding page, R indicates the resolution which may be defined as the distance from the source at which the grain density falls to half that directly over the source. This is difficult to measure as the distribution of grains rarely forms a distinct circular area when the autoradiograph is viewed from above. The grains further away from the source merge into background levels rather than falling away completely as in the diagram on the preceding page.

There have been several other definitions of resolution but the most useful in the analysis of autoradiographs has been that of Bachmann and Salpeter (1965). They defined resolution as the radius of a circle around a point source which contains half of the silver grains produced by that source. This is known as the half-radius (HR).

Salpeter et al. (1969) described the following theoretical autoradiograph of a point source emitting particles upwards into an emulsion within a cone of half angle θ . Thus if viewed from above, a circular area of grains would be seen with the source in the centre.



From Salpeter
et. al. (1969)

x is the horizontal distance from the source (s) to a developed grain whose position is defined as the centre (D) of the smallest circumscribed circle. d is the distance along a vertical line from the source to the horizontal mid-plane of the emulsion. B is a silver bromide crystal.

From this model Salpeter et al. (1969) derived an equation to describe the grain density ' f_p ' at a horizontal distance ' x ' from a point source.

$$f_p = \frac{1}{\left[1 + (x/d)^2\right]^{3/2}} \quad \text{Equation 1}$$

In practice a point source cannot be produced in the laboratory. Bachmann and Salpeter (1965) therefore introduced the concept of the half-distance (HD), the distance from a line source which encloses half of the grains formed from it, to measure the resolution of an autoradiographic system.

Salpeter et al. (1969) evaluated the relationship between HR and HD. They found that a circle of 1.7 HD has a 50% probability of enclosing a point source if the circle is drawn around a grain so that the centre of the circle coincides with the mid-point of the longest axis of the grain. The circle is known as a 50% probability circle, i.e. if drawn around a grain it has a 50% probability of enclosing the source or conversely, if drawn around a point source it will enclose 50% of the grains formed by that source.

Salpeter et al. (1969) obtained the theoretical density distribution ' f_l ' around a line source by integration of Equation 1 above (and normalisation to give $f_l = 1$ at $x = 0$).

$$f_l = \frac{1}{1 + (x/d)^2} \quad \text{Equation 2}$$

However, Salpeter et al. (1969) found that this grain density function did not accurately fit the experimentally observed distribution of grains about a radioactive line source prepared with tritium. They therefore added an experimental correction for tritium which was derived by trial and error until a good fit with the experimental data was obtained. This gave:

$$f_1 = \frac{0.982}{1 + (x/d)^2} + \frac{0.45}{25 + (x/d)^2} \quad \text{Equation 3}$$

The analysis of autoradiographs

It has already been mentioned that β -particles from a radioactive source are emitted in all directions. A particle originating from a source in one structure may cause the formation of a silver grain overlying another structure, a phenomenon known as 'cross-fire'. Although it is not possible to determine the source of any one grain, with a sufficiently large number of grains it is possible to analyse the overall distribution of radioactive sources if the distribution of grains around a point source is known.

The current methods of analysis are based on the work of Bachmann and Salpeter (1965) who defined resolution in terms of the radius of a circle around a point source. This became known as the 50% probability circle. Salpeter et al. (1969) derived grain distribution curves for radioactive sources of various simple geometric shapes. These 'universal curves' can be used to compare grain distributions around certain structures in autoradiographs providing these structures conform to one of these simple shapes.

The 50% probability circle was used by Williams (1969) to describe a method of analysing autoradiographs. In this method a screen of 50% probability circles is applied to each of a large number of micrographs. The structures falling within the circles are noted and this gives a measure of the relative area of each structure or an estimate of where the radioactivity would occur if the labelling were uniform throughout the tissue. A circle is then centred on each silver grain and the structures within the circles are noted. The two distributions can then be compared using the Chi-squared test to determine if the radioactivity is distributed uniformly or non-uniformly.

An advance in methods of analysis of autoradiographs was the development of the 'hypothetical grain' method of Blackett and Parry (1973, Parry and Blackett, 1973). The method uses the experimentally observed distribution of grains about a thin line source and the calculated distribution of grains about a point source (Salpeter et al., 1969). This distribution is used to give the cross-fire which can be expected from each structure.

A transparent overlay screen of 50% probability circles, arranged regularly, is prepared. Each circle represents the site of a hypothetical grain when applied to a micrograph. The position of a hypothetical source of each grain is determined by making use of a list of 'distances' and 'directions' generated by computer. The distances are "obtained on a random basis such that the frequency with which any distance occurs in the list is equal to the probability of a disintegration from a point source producing a grain at this distance" (Blackett and Parry, 1973). The directions are obtained

from a randomised list of 16 compass points. Each hypothetical source is represented by a dot on the screen. By applying the screen to a micrograph a cross-fire table can be constructed in which each row represents the position of the source of the hypothetical grain. For example, a circle (hypothetical site) falling on a mitochondrion with the associated point falling on the cytoplasm would be recorded in the square marked below.

	mitoch.	nucleus	cyto.
mitochondria			
nucleus			
cytoplasm	X		

The sites of the real silver grains are then recorded by placing a 50% probability circle over each grain so that the centre of the circle coincides with the mid-point of the longest axis of the grain. Structures enclosed within the circle are noted. The distribution of the real grains is then compared with that of the hypothetical grains using the Chi-squared test to determine whether the actual distribution of grains is different from that which would be expected if the radioactivity were distributed uniformly throughout the tissue. If a bad fit, i.e. a high value of Chi-squared, is obtained, a computer program which includes a minimising routine is used. This manipulates the hypothetical grain distribution by multiplying each row by an appropriate number.

The new values in each column are then added up and compared with the real grains using the Chi-squared test. A set of multipliers which gives a low Chi-squared value represents a distribution of radioactivity which can account for the distribution of grains within the autoradiographs. These multipliers are termed 'grains per grid point' by Parry and Blackett (1975).

A simplified version of the hypothetical grain analysis has been developed in this laboratory by Peter Harris and simultaneously by Downs and Williams (1978, Williams and Downs, 1978). In this method the hypothetical grain table is derived in the same way as that of Blackett and Parry (1973) and is used to calculate the relative intensity of sources (i.e. intensity of sources per unit area) in each of a number of organelles by solving a set of x simultaneous equations where x is the number of possible radioactive sources. The equations are as follows:-

$$R_a = (H_{a,a}/S_a)A + (H_{a,b}/S_b)B + (H_{a,c}/S_c)C \text{ etc.}$$

$$R_b = (H_{b,a}/S_a)A + (H_{b,b}/S_b)B + (H_{b,c}/S_c)C \text{ etc.}$$

R_a is the total number of real grains with their centres on organelle a .
 $H_{b,a}$ is the number of hypothetical grains occurring in organelle b which had their origin from sources in organelle a . This figure is obtained from the cross-fire table. S_a is the total number of hypothetical sources in organelle a . A is the number of real sources in organelle a . By solving the simultaneous equations A/S_a , B/S_b etc. are obtained. This gives the relative intensity of sources in each organelle.

CHAPTER 8 - METHODS

Perfusion procedure

Well-oxygenated hearts

The animals, perfusion media, both radioactive and non-radioactive and the perfusion procedure were as described previously (pages 44-46). Perfusions were carried out at 37°C or at 5°C. An initial period of five minutes perfusion with non-radioactive medium was followed by a 15 second pulse perfusion with 2.5 ml of the radioactive solution which was contained in a syringe and attached to the aortic cannula by a 3-way tap. The pulse perfusion was followed by an immediate return to perfusion with the unlabelled solution for a period of 15, 30, 60 or 120 seconds. Four guinea pig hearts were used for radioactive perfusion plus one in which the radioactive pulse was omitted, for each of the perfusion times at each of the perfusion temperatures.

Hypoxic hearts

A second reservoir of non-radioactive perfusate was used in these experiments in which the perfusate was equilibrated with 95% N₂ : 5% CO₂ for at least 20 minutes prior to use. The initial period of five minutes perfusion with the oxygenated perfusate was followed by 30 minutes of perfusion with the solution which had been bubbled with the nitrogen mixture. The radioactive solution was also bubbled with 95% N₂ : 5% CO₂ and was given at the end of the 30 minutes hypoxic perfusion. The radioactive pulse (2.5 ml over 15 seconds) was followed by an immediate return to the hypoxic, non-radioactive perfusate for a period of 15, 30, 60 or 120 seconds. The experimental design was the same as that summarised in Table 1 (page 47), except that four experimental animals were used plus one control animal in each group. The control was perfused for 30 minutes with the hypoxic medium followed by the appropriate chaser period. The radioactive pulse was omitted. This heart was used to ensure that any tissue changes observed

were due to hypoxia and not to poor fixation. The omission of the radioactive pulse enabled the same heart to be used as the control for positive chemography which will be described later.

Processing for electron microscopy

All the hearts were fixed immediately after the chaser perfusion by perfusion with 4% glutaraldehyde in 0.1M sodium cacodylate (pH 7.3), for 10 minutes. The perfusion fixation was carried out at 5°C in order to inhibit any further esterification of the radioactive fatty acid during fixation.

After fixation, pieces of tissue were excised from the free wall of the left ventricle and from the interventricular septum and cut into pieces approximately 2mm by 1mm, oriented with the aid of a dissecting microscope with the fibres running longitudinally. The tissue was processed for electron microscopy using the Durcupan schedule (Table 11, page 72). Staining was carried out 'en bloc' with 1% para-phenylenediamine in 0.1 M sodium cacodylate (pH 7.3) immediately prior to dehydration. Although it had not proved possible to assess the effect of para-phenylenediamine on lipid retention during processing, it was hoped that by dissolving the stain in buffer instead of in ethanol additional lipid losses would be kept to a minimum.

Ultrathin sections

Sections of pale gold interference colour were cut from the polymerised blocks on an LKB ultratome III ultramicrotome using glass or diamond knives. The sections were transferred to 300 mesh gold or nickel grids which had previously been coated with a support film prepared from a 1% solution of celloidin in amyl acetate. One grid from each block was examined in the electron microscope to ensure that the tissue was structurally well preserved. This grid was discarded. Enough sections to cover at least ten grids were taken from each block and blocks from all four hearts were sectioned in each group. A thin layer of carbon

(just visible on filter paper) was evaporated on to the sections, using an NGN coating unit.

One micron sections

Sections approximately one micron thick were cut from the same blocks as were used for thin sectioning. The sections were picked up with a nickel loop and transferred to a 3 x 1 inch glass microscope slide and allowed to dry on to the slide. The sections were stained with a warmed 1% aqueous solution of toluidine blue diluted 1 + 20 with 2.5% sodium bicarbonate. It has been reported (Rogers, 1973) that toluidine blue causes positive chemography but the controls carried out for this phenomenon (page 133) did not show any such reaction. The staining was for approximately one minute and the sections were washed well in distilled water and air-dried. One micron sections were used to determine the length of exposure required for the ultrathin sections. The length of exposure required for one micron sections emulsioned with Ilford K-2 emulsion is approximately one tenth of that of ultrathin sections emulsioned with Ilford L-4.

Application of the emulsion

Thin sections Ultrathin sections were emulsioned for autoradiography by a modified version of the loop technique (Caro and van Tubergen, 1962). The grids were mounted on glass microscope microscope slides and held in place by strips of double sided tape so that only the edges of the grids were in contact with the tape.

In darkroom conditions, using only dark red safelight safe-light filters, 10g of Ilford L-4 nuclear emulsion, diluted 1+2 with distilled water, were allowed to melt at 45°C with gentle stirring. After 15 minutes the liquid emulsion was transferred to ice for four minutes then allowed to stand at room temperature to gel. To test whether the emulsion had gelled, a 3 cm. nichrome loop was dipped into the emulsion

and withdrawn slowly. When the gel state had been reached no movement could be detected when a loop of emulsion was held up to the safelight and rotated. A loop of gelled emulsion was applied to each slide so that the emulsion dropped on to the sections. The slides were stored at 4°C with silica gel present in each box as a desiccant.

One micron sections

One micron sections were coated with emulsion using the dipping technique. In darkroom conditions, 15 ml of molten Ilford K-2 nuclear emulsion were diluted 1 + 1 with distilled water with the addition of 0.3 ml of glycerol. The emulsion was transferred to a water bath at 43°C and left for 10 minutes with occasional gentle stirring to mix the emulsion and water. The diluted emulsion was left to stand for a further 2 minutes at room temperature to allow any bubbles to disperse. To check that the emulsion was ready for use a clean slide was dipped in to it and held up to the safelight. If the slide was evenly coated and free of bubbles the emulsion was considered to be ready for use.

The slides were dipped using a dipping apparatus (modified from Kopriwa, 1966). The apparatus facilitated the removal of the slide from the emulsion at an even speed by attaching it to a lead weight falling through a cylinder of glycerol. The slides were left to dry in a vertical position before storing in light tight boxes at 4°C. Silica gel was again used as a desiccant in each of the boxes.

Exposure and development

The one micron sections, used to determine the length of exposure required for the ultrathin sections, were developed after 8, 10 or 12 days exposure. The exposed slides were developed for 3 minutes in undiluted Kodak D-19 developer. The slides were washed briefly in distilled water and fixed in 25% sodium thiosulphate for 8 minutes.

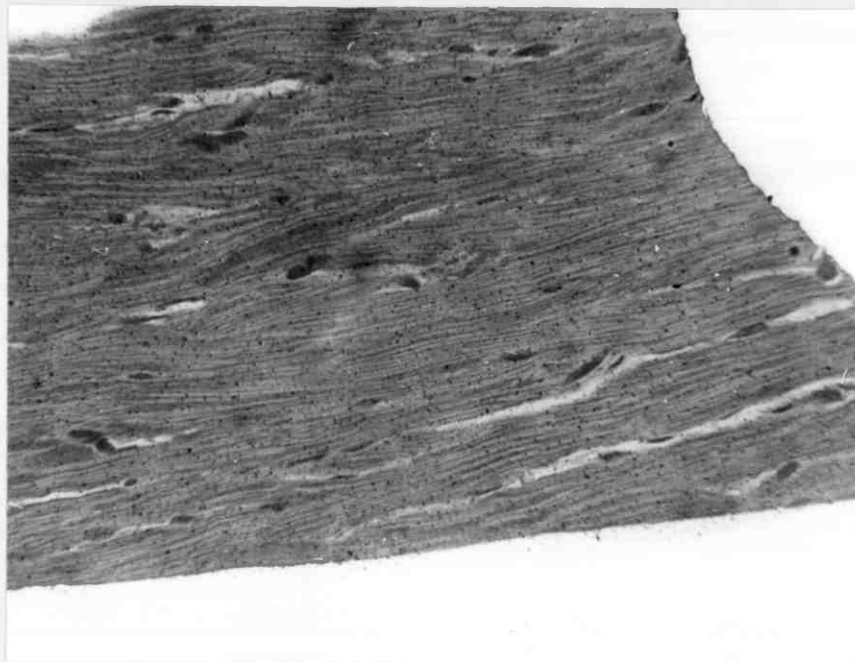


Figure 17

A light microscope autoradiograph of a one micron thick section of a heart perfused for 120 seconds after pulse perfusion with ^3H oleic acid at 37°C in well oxygenated conditions.

Magnification 1,000.

Both development and fixation were carried out at room temperature. The sections were washed in running tap water for 10 - 15 minutes, rinsed in distilled water and allowed to dry. A good cover of silver grains over the tissue but not over the surrounding resin was found after 10 or 12 days (Figure 7). The exposure time for the thin sections was therefore chosen in the region of 100 - 120 days.

Development of the ultrathin section autoradiographs was for 3 minutes in undiluted Kodak D-19 developer at room temperature. The sections were washed in distilled water and fixed for 3 minutes in 25% sodium thiosulphate. They were then washed in several changes of distilled water and allowed to dry.

Controls (Numbers 2 and 3 below apply to both light and EM autoradiographs, number 1 to EM only).

1. Monolayer

With each batch of emulsion two or three grids bearing a support film but no sections were emulsioned. These grids were transferred without development to the electron microscope to ensure that the crystals were applied to the grids as a closely packed monolayer. The appearance of such a monolayer is shown in Figure 18.

2. Positive chemography

This control was carried out in order to show that all the silver grains present were produced by radioactivity alone and not by any chemical reaction between the tissue or the resin and the emulsion. Sections from the non-radioactively labelled hearts, which had been processed in an identical way to the radioactive hearts, were emulsioned with each batch. These were exposed for exactly the same length of time as the radioactive sections and were developed at the same time. The number of background grains was found to be less than one per 30 microns square in all the experiments.

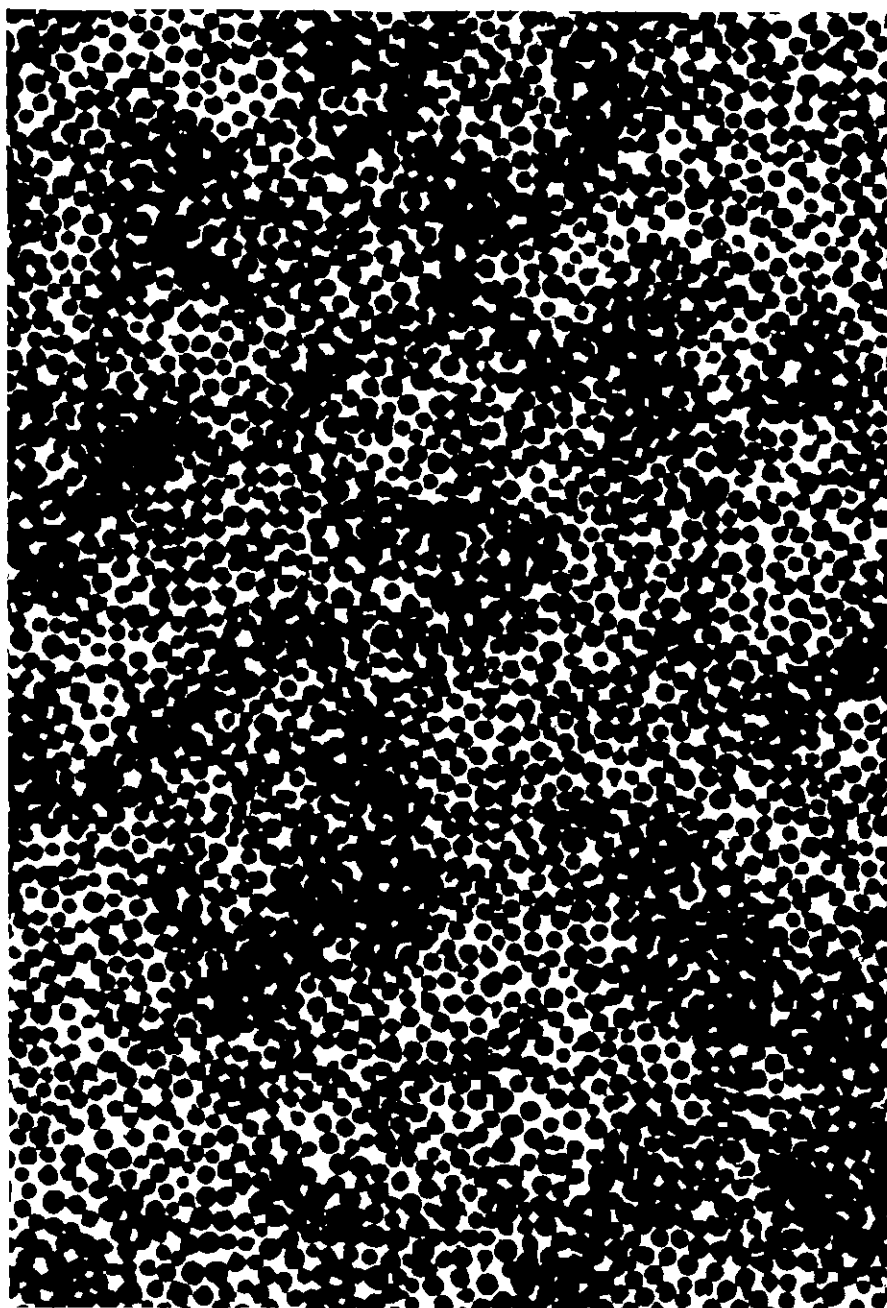


Figure 18

'Monolayer' of Ilford L-4 nuclear emulsion

x 15,000

3. Negative chemography and latent image fading

This was to determine whether there was any chemical effect of the tissue on the emulsion which prevented a latent image from being formed or whether a latent image faded before development. A number of radioactive sections were emulsioned and then exposed to light. They were stored with the experimental sections at 4°C for the exposure period. After development a dense even layer of grains was seen indicating that there had been no negative chemography or latent image fading.

Microscopy

Micrographs for analysis were taken on an AEI 801 electron microscope (well-oxygenated series) or on a Philips 301 (hypoxia series). An accelerating voltage of 60 kV was used and a plate magnification of 10,000 or 9,800. A diffraction grating was used with each cassette to check the magnification and any alteration was corrected in the final printing. The plates were enlarged on printing to a final magnification of 30,000. The micrographs were taken in a non-selective manner around the edges of the grid squares, to eliminate bias. A minimum of 500 silver grains was photographed for each set of perfused hearts.

Determination of resolution

Resolution is usually defined as the radius of a circle around a point source which encloses one half of the silver grains formed by that source (Bachmann and Salpeter, 1965). As it is not possible to prepare a point source in the laboratory, resolution is measured in terms of the scatter of grains on either side of a line source (Salpeter et al., 1969).

A line source was prepared by pipetting one drop of [9, 10 - ^3H] oleic acid in benzene (1 mCi per ml) on to half a block of polymerised Durcupan. The benzene was allowed to evaporate and a second layer of Durcupan full mix was added. The blocks were polymerised at 60°C.

The blocks were sectioned at right angles to the line and emulsioned as previously. Approximately 1500 grains were photographed at a final print magnification of 30,000 (Figure 19). The perpendicular distance of each grain from the line was measured. Distances were measured in millimetres (mm) on the prints and divided by 30,000 to obtain actual measurements which were expressed in nanometers (nm). Grains at a distance of more than 2000 nm (or 6mm on the print) were not included in the count. At this distance grain densities approach background levels. Figure 20 shows the histogram obtained from these measurements. From the integrated curve (Figure 21), the half-distance (HD), the distance from the line within which half the grains lie, was derived. This was found to be 120 nm.

Hypothetical grain analysis

Using Salpeter et al's equation for the distribution of grains around a line source, with the correction for tritium (see Equation 3, page 124) a computer was programmed to supply a number of points to give a theoretical grain density curve. This was compared with the curve from the experimental data from the radioactive line source. The distance from the source to the mid-point of the emulsion (represented by d in the equation on page 123) varies according to the distance from the source to the emulsion, i.e. its location in the section, and to variations in section thickness, crystal size or carbon layer thickness. As most of these variables were not known, a mean value of d could not be obtained. For this reason a number of theoretical curves were obtained with different values of d ranging from 110 nm to 150 nm. The best fit with the experimental data was obtained when d was equal to 125 nm (Figure 22)

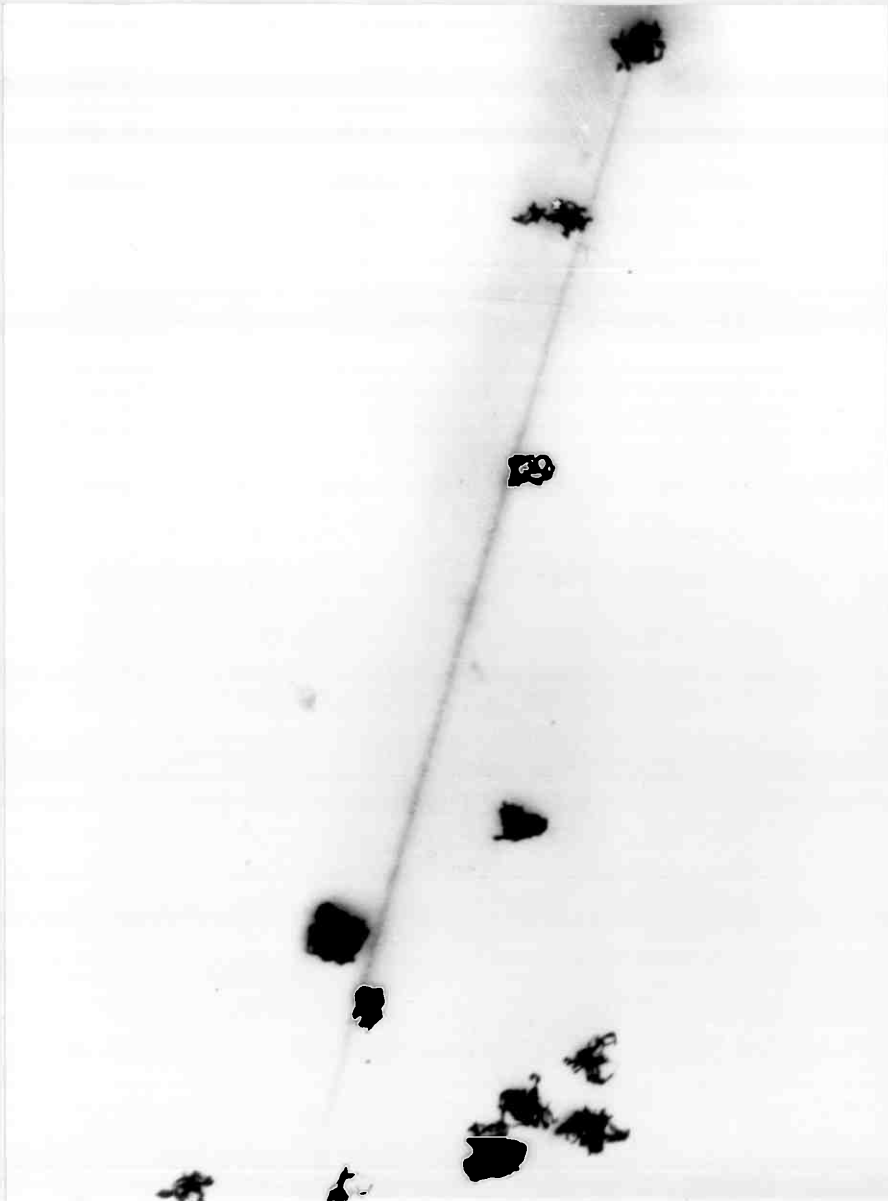


Figure 19

An autoradiograph of a tritium line source

x 30,000

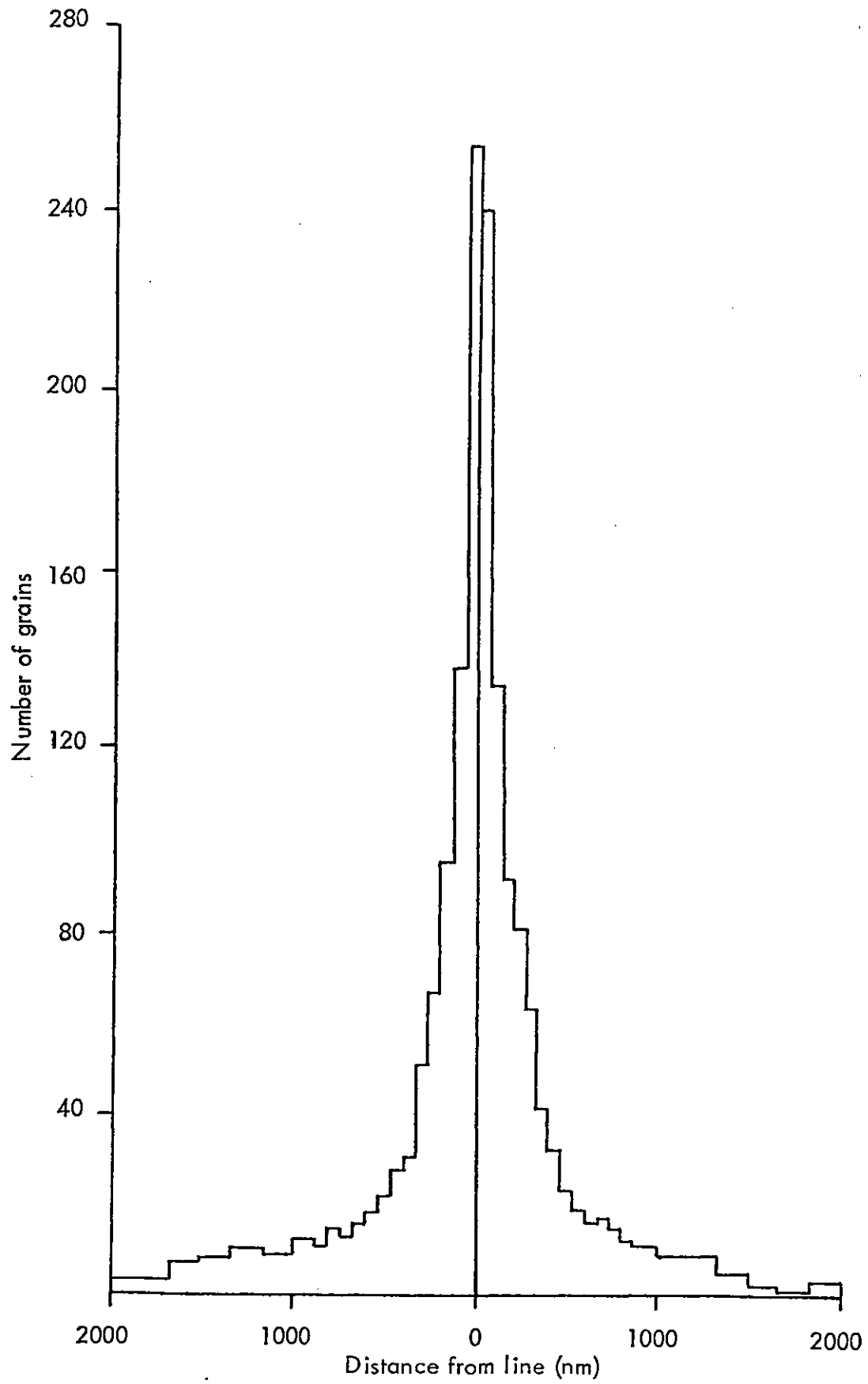


Figure 20

Histogram of grain density on either side of a line source

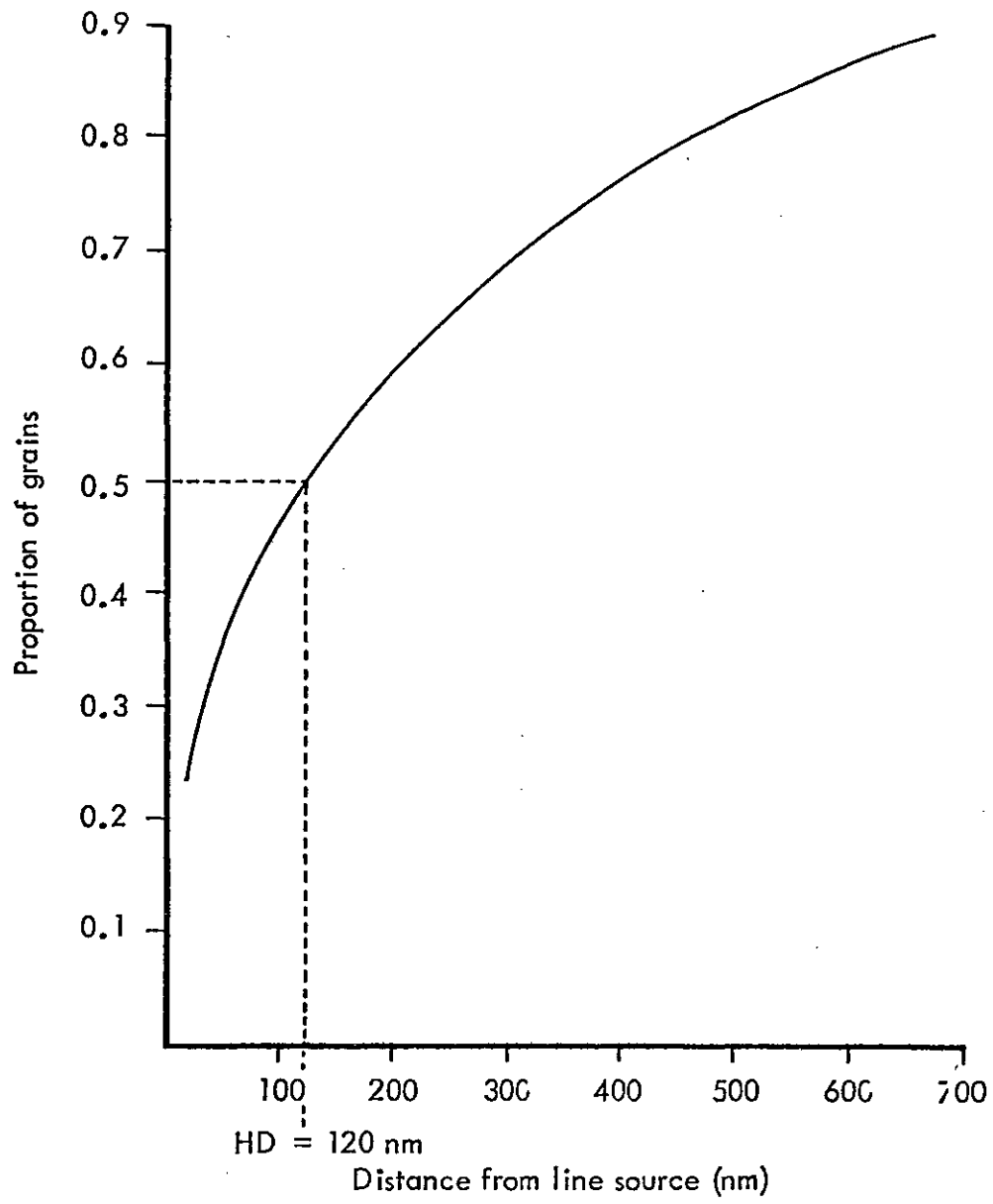


Figure 21

Proportion of grains within different distances from a line source

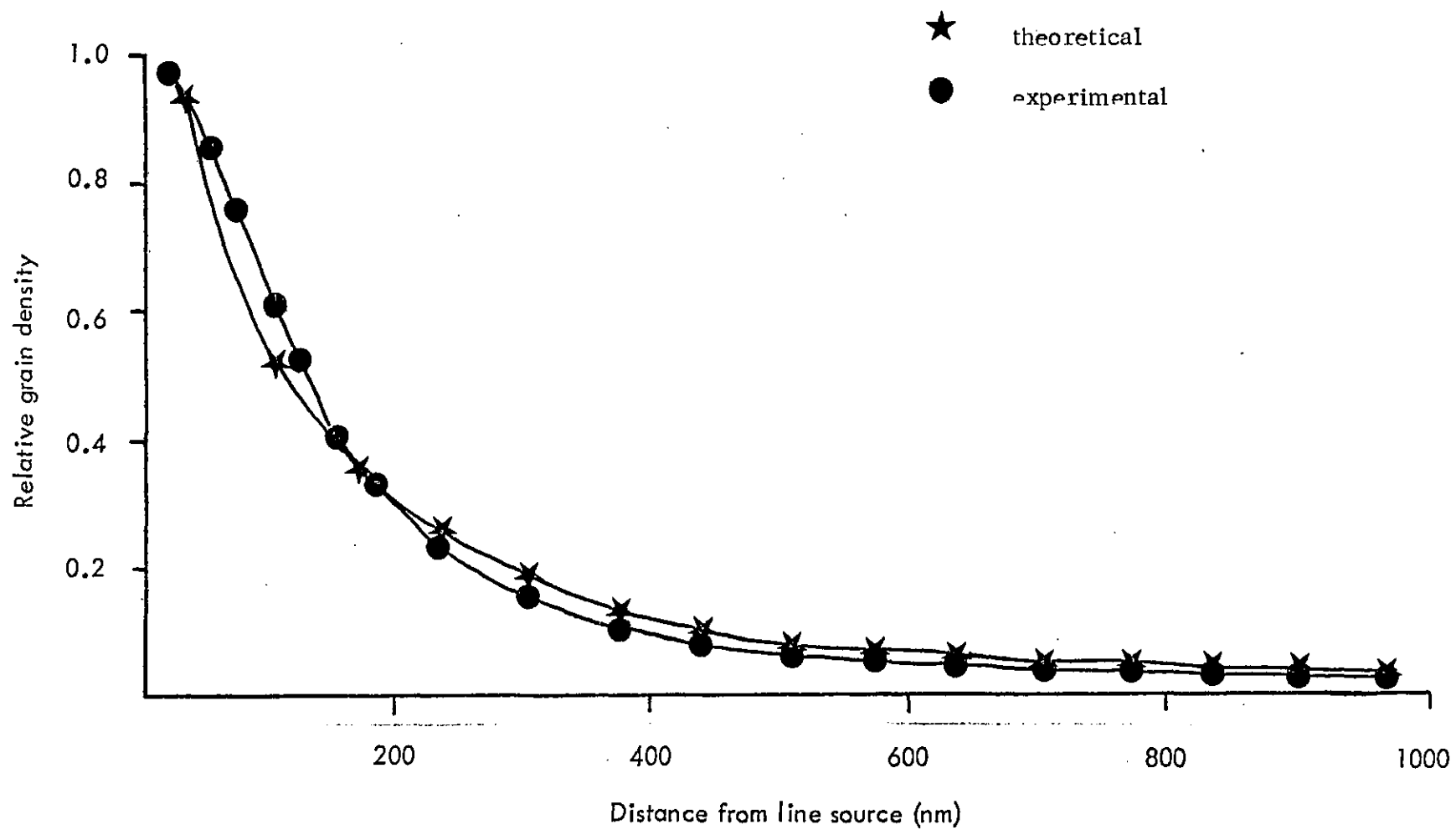


Figure 22. Experimental and theoretical grain density functions for a line source

The overlay screen for the hypothetical grain analysis was prepared from the same random circles used for the circle analysis. Each circle represented the site of a hypothetical grain. A point was placed at a particular distance and direction from each circle. The distances and directions were derived as follows. Differentiation of the density function for a line source with the correction for tritium (Equation 3, page 124) gives the corrected function for a point source (Salpeter et al., 1969)

$$f_p = \frac{0.9964}{\left[1 + (x/d)^2\right]^{3/2}} + \frac{0.45}{\left[25 + (x/d)^2\right]^{3/2}}$$

Using this function with $d = 125$ nm, a computer was programmed to generate a list of distances representing the distribution of grains around a point source, the frequency of occurrence of each distance in the list being proportional to the probability of a grain being formed at that distance.

A randomised list of 16 compass points was also obtained by computer. Using these two lists the screen was completed by marking a point at a distance and direction from the centre of each circle from the two lists. Each dot represented the position of a hypothetical radioactive source of the hypothetical grain.

A preliminary analysis using this screen gave a list of over 40 possible combinations of single and junctional items. These often had 3 or 4 structures falling within the circle so that it was impossible to associate the radioactivity with a particular structure. The 50% probability circle has been adopted by most workers as a convenient size for the analysis but a smaller circle size can be used.

In this case it was decided to omit the circles completely and carry out a 'point to point' analysis. This meant that the analysis was limited to single items only and there were no junctional items.

A new screen was constructed placing a dot in the centre of each circle in the previous screen. This represented the site of the centre of each hypothetical grain. The position of the hypothetical sources was added as described above but each was depicted by a tiny circle the central enclosed area of which was no bigger than the dot. The centre of the circle represented the hypothetical source. A reduced copy of the screen is shown in Figure 23.

For each set of micrographs a cross-fire table was constructed using this screen. For the real grains, only the structure underlying the mid-point of the longest axis of the grain was noted. A Chi-squared test was first carried out to determine whether or not the distribution of real grains was random by adding up each column of hypothetical grains and comparing these totals with the real grains. In all cases a non-random distribution of silver grains was found ($p < 0.005$).

An estimate of the relative intensity of sources (or grains per grid point as it is termed by Blackett and Parry, 1975) in each subcellular structure was obtained. Both the Blackett and Parry (1975) method and the simultaneous equations method were used and these gave very similar results.

The subcellular regions chosen for counting were myofibrils, 'cytoplasm' (i.e. any areas of cytoplasm not occupied by myofibrils, which included areas of glycogen granules, coated vesicles and any other cytoplasmic structure not otherwise classified), mitochondria, T-tubules, sarcoplasmic reticulum, lipid droplets, nucleus and

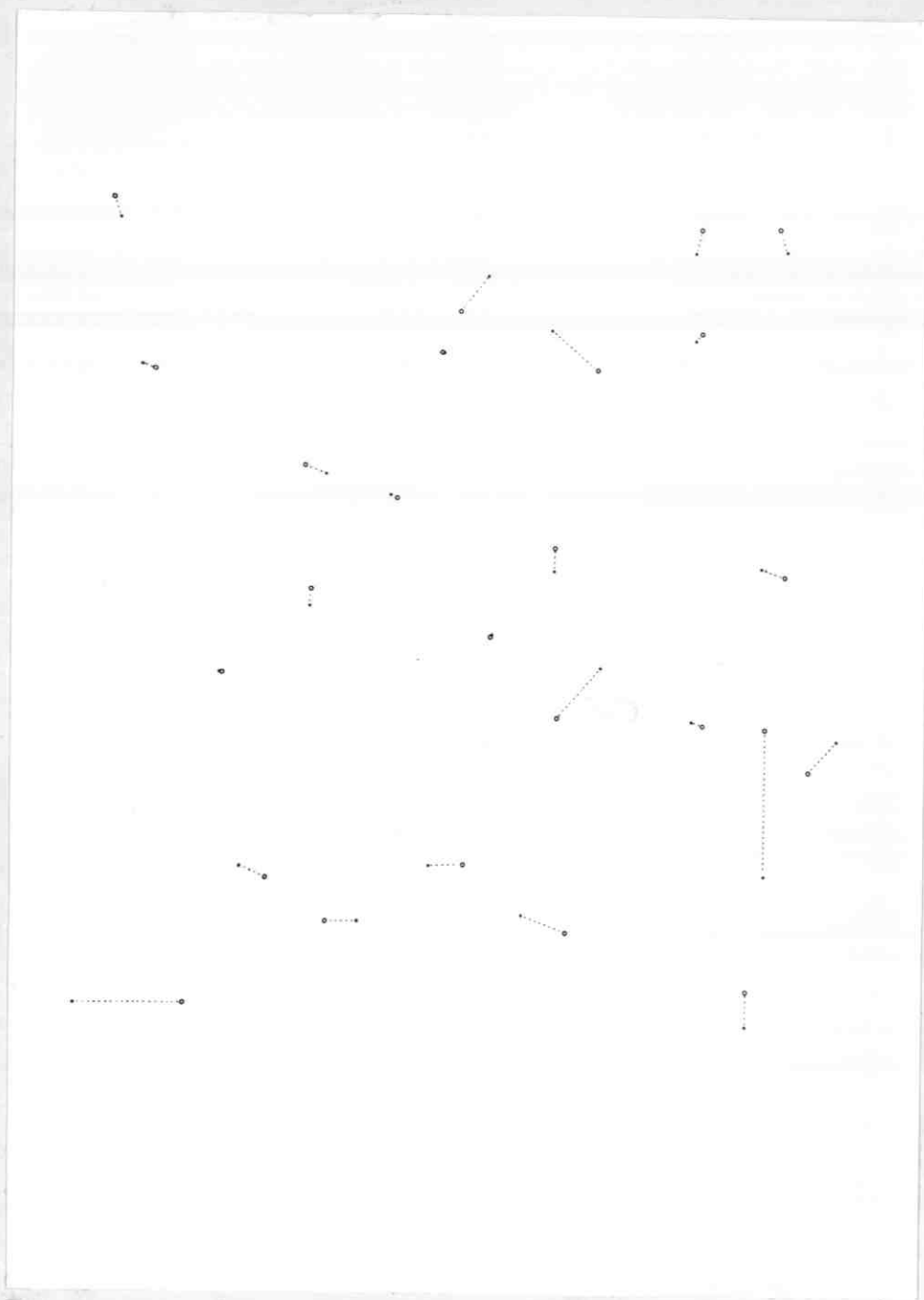


Figure 23

Overlay screen used for the hypothetical grain analysis

(reduced by approximately half)

'extracellular space'. 'Extracellular space' consisted of any true extracellular space and capillaries as well as any areas of micrograph occupied by blank areas of resin. Originally for the 37°C and the 5°C well-oxygenated series, 'cytoplasm' and myofibrils were counted together under the heading cytoplasm. It was later decided that these areas may be of interest separately and the 37°C micrographs were reanalysed with 'cytoplasm' and myofibrils counted as separate items. As no difference was found between the activities of these two areas the 5°C series was not reanalysed and so they appear under the heading of cytoplasm in the results section.

Hypothetical grain analysis was carried out on micrographs of both left ventricle and interventricular septum after a 120 second chaser at 37°C (well-oxygenated). This perfusion time was chosen for the comparison as it is the longest and is therefore the one in which the greatest difference between the two regions of the heart would be expected. As there was no significant difference (see Results, page 152) between the two sets of micrographs it was decided to photograph and analyse autoradiographs from the left ventricle only, in the subsequent experiments, to reduce the enormous number of micrographs involved.

Estimation of relative areas

The construction of the hypothetical grain data tables enabled an estimate of the relative areas of each structure within the cell to be obtained. This was done by totalling each column, with the exception of the 'extracellular space' and expressing each as a percentage of the total.

CHAPTER 9 - RESULTS

Ultrastructure

The ultrastructure of the myocardium was well preserved throughout the perfusion procedures. Figures 24 to 27 show representative micrographs from the well-oxygenated and hypoxic series. The good preservation of the control hearts in the hypoxia series indicated that any ultrastructural changes or oedema were caused by the hypoxia and not by poor fixation.

Hypothetical grain analysis

Left ventricle and interventricular septum

Tables 19 and 20 are examples of the constructed hypothetical cross-fire tables and are from the analysis of autoradiographs of the left ventricle and interventricular septum, respectively, after 120 seconds chaser perfusion at 37°C. All of these perfusions were carried out under well-oxygenated conditions. Each column was totalled to give the hypothetical grains for each subcellular region. These were normalised so that the total number of hypothetical grains equalled the number of real grains. The two grain distributions, real and hypothetical, could then be compared using the Chi-squared test.

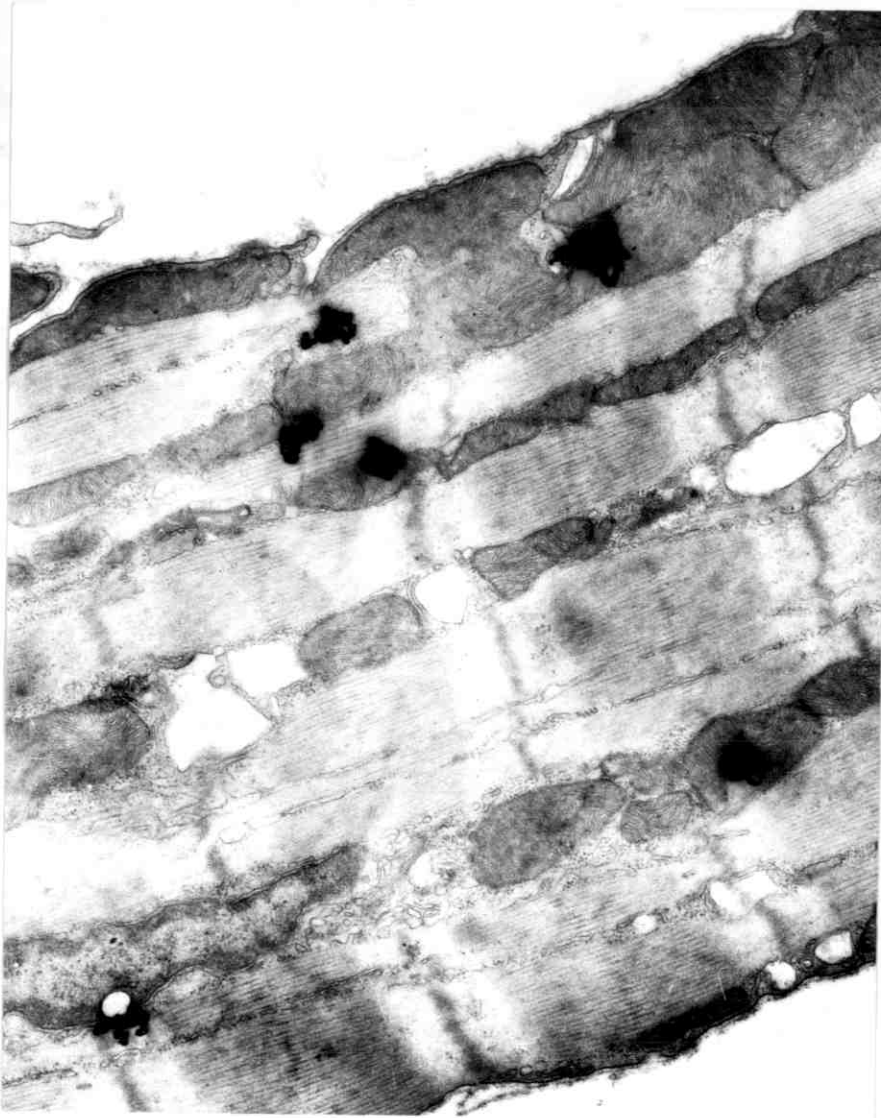


Figure 24

An autoradiograph of a heart perfused at 37°C in well-oxygenated conditions with a 30 second chaser perfusion after the radioactive pulse
Magnification 17,500.

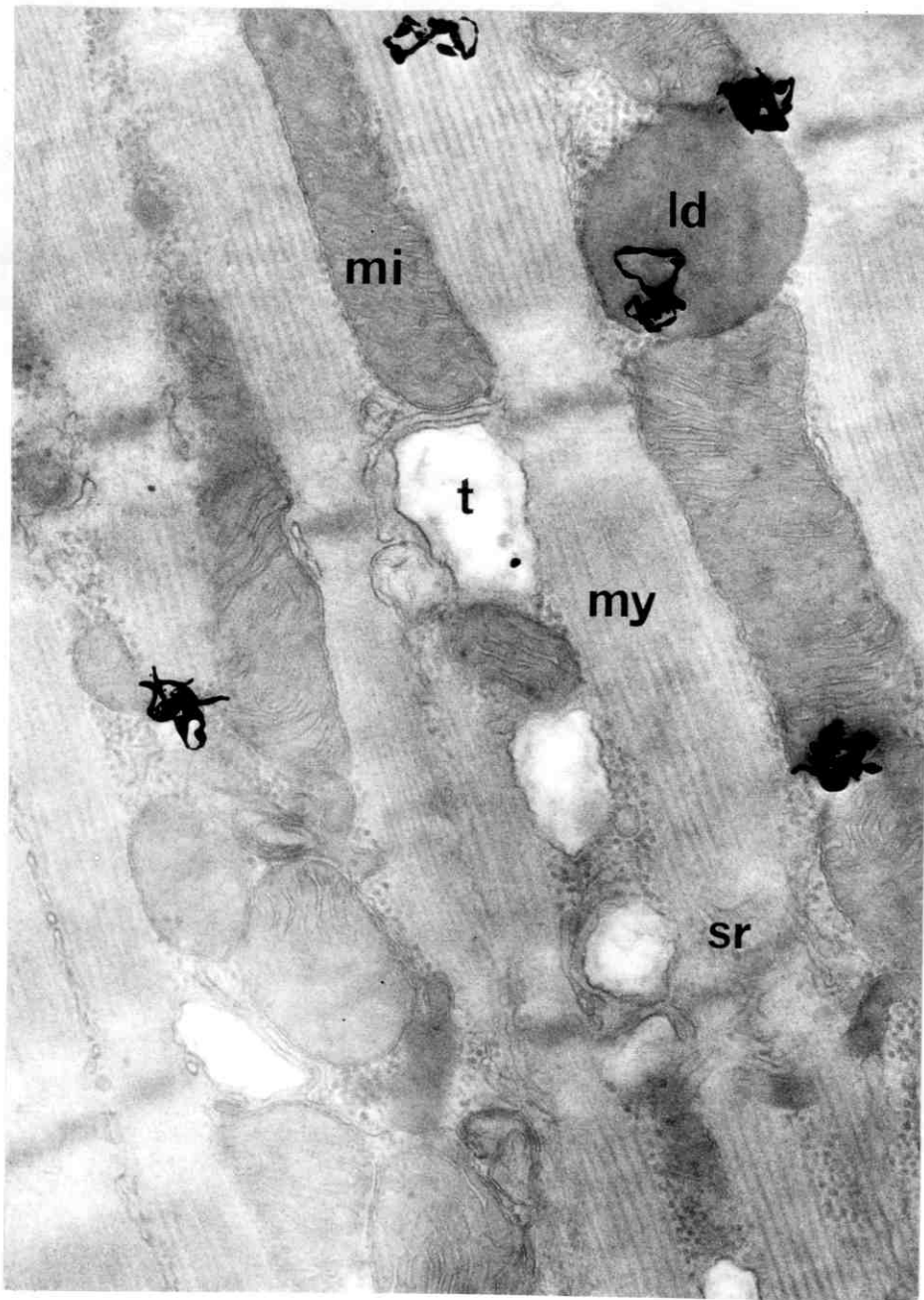


Figure 18

An autoradiograph of a heart perfused at 37°C in well-oxygenated conditions with a 60 second chaser perfusion after the radioactive pulse

ld = lipid droplet, my = myofibrils, sr = sarcoplasmic reticulum,
mi = mitochondrion, t = transverse tubule. Magnification x38,000

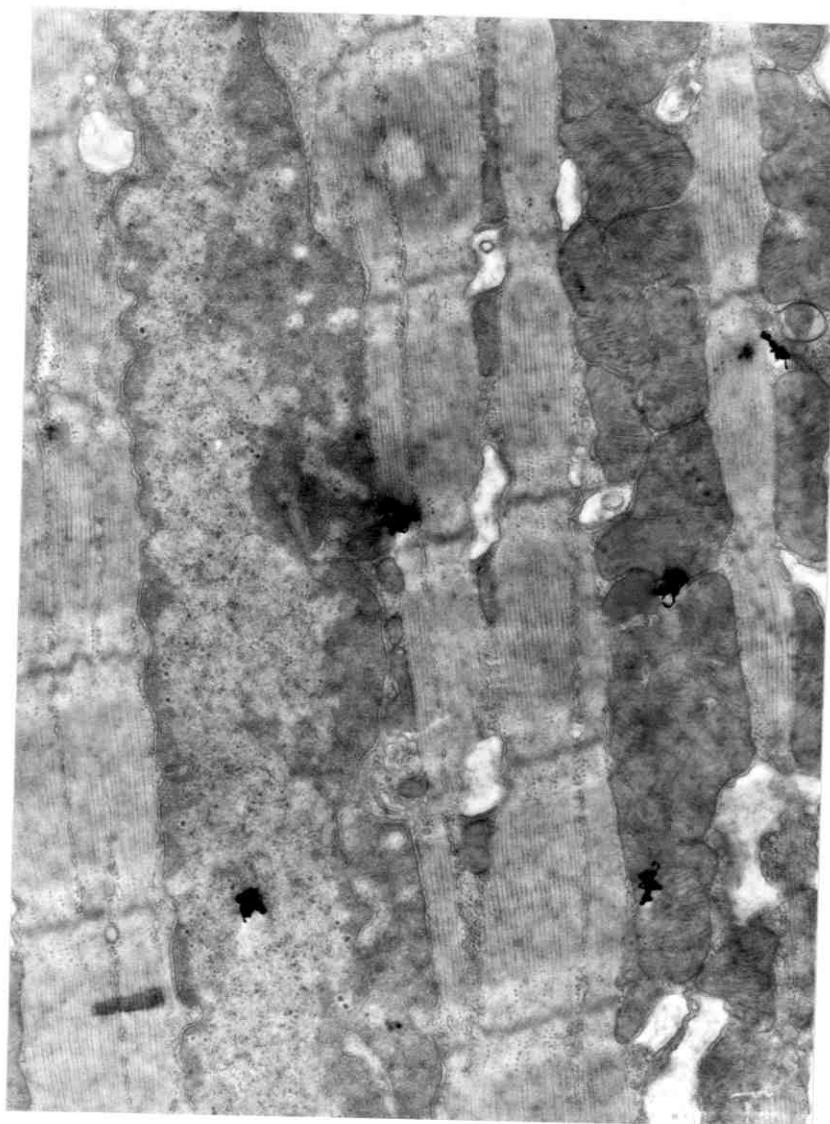


Figure 26

An autoradiograph of a heart perfused for 30 minutes with the hypoxic solution followed by the radioactive pulse and a 30 second chaser perfusion
Magnification 15,000.

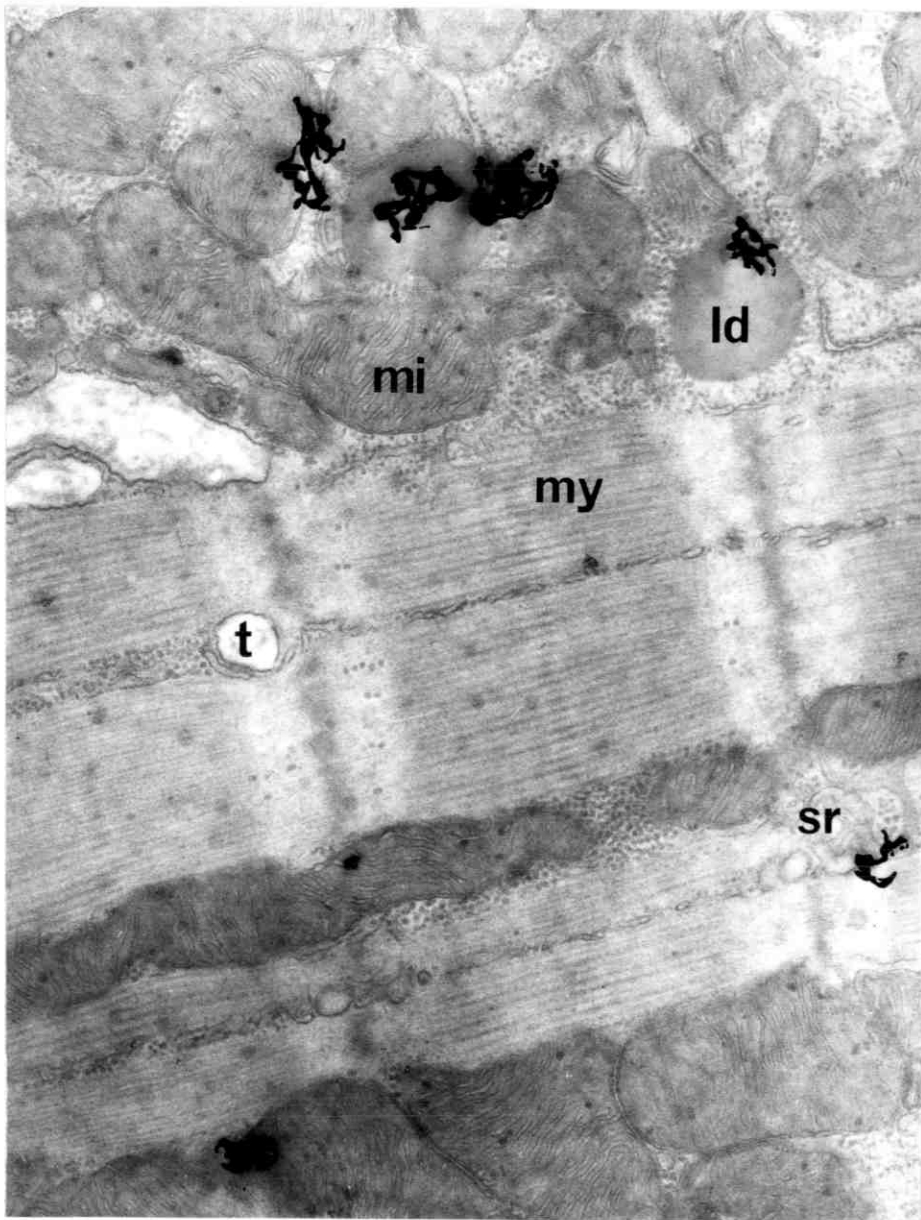


Figure 19

An autoradiograph of a heart perfused for 30 minutes with the hypoxic solution followed by the radioactive pulse and a 15 second chaser perfusion

ld = lipid droplet, my = myofibrils, sr = sarcoplasmic reticulum,
mi = mitochondrion, t = transverse tubule. Magnification $\times 38,000$.

TABLE 19

Cross-fire table from the analysis of autoradiographs from the left ventricle of hearts perfused for 120 seconds after the radioactive pulse, at 37°C.

Hypothetical grain site								
		Cytoplasm	Mitochondria	T-tubules	Sarcoplasmic reticulum	Lipid droplets	Nucleus	'Extracellular space'
Hypothetical source	Cytoplasm	883	121	34	26	8	7	0
	Mitochondria	140	401	10	8	2	1	0
	T-tubules	40	15	18	4	1	0	0
	Sarcoplasmic reticulum	9	6	1	12	0	0	1
	Lipid droplets	3	8	3	0	14	0	2
	Nucleus	12	6	0	1	0	87	0
	'Extracellular space'	6	5	0	1	0	0	110
Total hypothetical grains		1093	562	66	52	25	95	113
Hypothetical grains (normalised)		228	117	14	11	5	20	24
Real grains		169	148	11	6	69	5	11

TABLE 20

Cross-fire table from the analysis of autoradiographs from the interventricular septum of hearts perfused for 120 seconds after the radioactive pulse, at 37°C.

Hypothetical grain site								
		Cytoplasm	Mitochondria	T-tubules	Sarcoplasmic reticulum	Lipid droplets	Nucleus	'Extracellular space'
Hypothetical source	Cytoplasm	1027	143	42	25	10	8	0
	Mitochondria	156	442	10	11	2	1	0
	T-tubules	47	17	21	4	1	0	0
	Sarcoplasmic reticulum	10	6	3	15	0	0	2
	Lipid droplets	5	14	4	2	20	0	2
	Nucleus	12	7	0	1	0	98	0
	'Extracellular space'	5	6	0	0	0	0	95
Total hypothetical grains		1262	635	80	58	33	107	99
Hypothetical grains (normalised)		244	123	15	11	6	21	19
Real grains		177	161	14	8	66	4	10

The distribution of radioactivity in both left ventricle and interventricular septum after 120 seconds chaser perfusion at 37°C was non random ($\chi^2 = 151.9$ and 170.4 respectively, $p < 0.005$ in both cases). The Chi-squared test was also used to compare the total hypothetical grains (i.e. relative area) for each organelle from the left ventricle sample with the hypothetical grains from the septum sample. The real grains from the left ventricle were then compared with the real grains from the septum. There was no significant difference between the hypothetical grains (relative area) in the two sites or between the two distributions of real grains.

Table 21 gives the relative intensity of sources as obtained from the simultaneous equations computer program and the 'grains per grid point' from the Blackett and Parry (1975) program for both left ventricle and interventricular septum. The two programs gave almost identical results. As there was no significant difference between the results for the two regions of the heart, only micrographs from the left ventricle were analysed subsequently. In the same manner, as the two computer programs gave such similar results, for the sake of simplicity only the results from the simultaneous equations program are given in the following sections.

In the analysis of all the autoradiographs the cross-fire tables were constructed as in Tables 19 and 20 and a Chi-squared test carried out as described to determine whether or not the distribution of radioactivity was random. In all cases the values of Chi-squared were high enough to show that the distribution of observed grains was not random, with probability values less than 0.005.

Table 21

Relative intensity of sources in left ventricle and interventricular septum after 120 seconds chaser perfusion. A comparison of the simultaneous equations and 'grains per grid point' computer programs.

<u>Left ventricle</u>	Relative intensity of sources	Grains per grid point
Cytoplasm	.174	.144
Mitochondria	.241	.233
T-tubules	-.668	.001
Sarcoplasmic reticulum	.177	.016
Lipid droplets	4.842	4.486
Nucleus	.041	.019
'Extracellular space'	.047	.055
<u>Interventricular septum</u>		
Cytoplasm	.091	.121
Mitochondria	.310	.227
T-tubules	.003	.000
Sarcoplasmic reticulum	.037	.000
Lipid droplets	4.018	4.024
Nucleus	.004	.006
'Extracellular space'	.081	.042

Well-oxygenated perfusions at 37°C

Table 22 and Figure 28 show the changes with perfusion time of the calculated relative intensity of sources in the mitochondria, T-tubules, lipid droplets and cytoplasm (including myofibrils) at 37°C. No detectable activity was associated with the nucleus, sarcoplasmic reticulum or 'extracellular space'.

There was a rapid incorporation of tritium into the mitochondria, which was apparent after only 15 seconds chaser. This incorporation increased during 60 seconds of chaser perfusion but dropped dramatically between 60 and 120 seconds. Activity was detected in the T-tubules during the shorter perfusion times but not after 60 or 120 seconds chaser. There was some activity detected in the cytoplasm at all the perfusion times but this remained relatively low throughout. Incorporation of radioactivity into the lipid droplets was first seen after 60 seconds of chaser perfusion and was still increasing rapidly after 120 seconds.

Well-oxygenated perfusions at 5°C

At 5°C (Table 22 and Figure 29) radioactivity in the mitochondria was considerably lower than at 37°C and was still rising after 120 seconds chaser. Radioactivity in the cytoplasm was low although, after the shorter perfusion times at 5°C, it was higher than at 37°C. Similarly, the radioactivity in the T-tubules was higher than at 37°C after the 15 seconds chaser and was still detectable after 60 seconds but not after 120 seconds. There was no radioactivity associated with the lipid droplets after any of the perfusion times at 5°C.

As in the 37°C perfusions there was no radioactivity detected in the sarcoplasmic reticulum, nucleus or 'extracellular space' at any of the perfusion times.

TABLE 22

Relative intensity of sources in subcellular regions after well-oxygenated perfusion at 37°C and 5°C
and after 30 minutes hypoxia

	Seconds of chaser											
	Well-oxygenated 37°C				Well-oxygenated 5°C				Hypoxic			
	<u>15</u>	<u>30</u>	<u>60</u>	<u>120</u>	<u>15</u>	<u>30</u>	<u>60</u>	<u>120</u>	<u>15</u>	<u>30</u>	<u>60</u>	<u>120</u>
Myofibrils	.033	.111	.124	.073	.335	.243	.226	.198	.166	.073	.154	.125
'Cytoplasm'	.033	.058	.099	.100					.265	.233	.364	.269
Mitochondria	.697	.861	1.100	.202	.398	.461	.591	.589	.379	.317	.643	.486
T-tubules	.235	.225	-.112	.075	.584	.277	.301	-.028	-.348	.123	.018	-.552
Sarcoplasmic reticulum	.003	.206	.233	.250	-.602	-.342	-.656	-.141	-.830	.205	-.120	.232
Lipid droplets	.058	.085	.786	4.160	.023	-.132	.100	-.079	4.678	6.968	7.354	8.276
Nucleus	.058	.009	-.017	.024	.027	.056	.042	.013	-.651	.119	.216	.170
'Extracellular space'	.054	.085	.080	.105	-.005	.024	.013	-.003	.140	.127	.114	.048

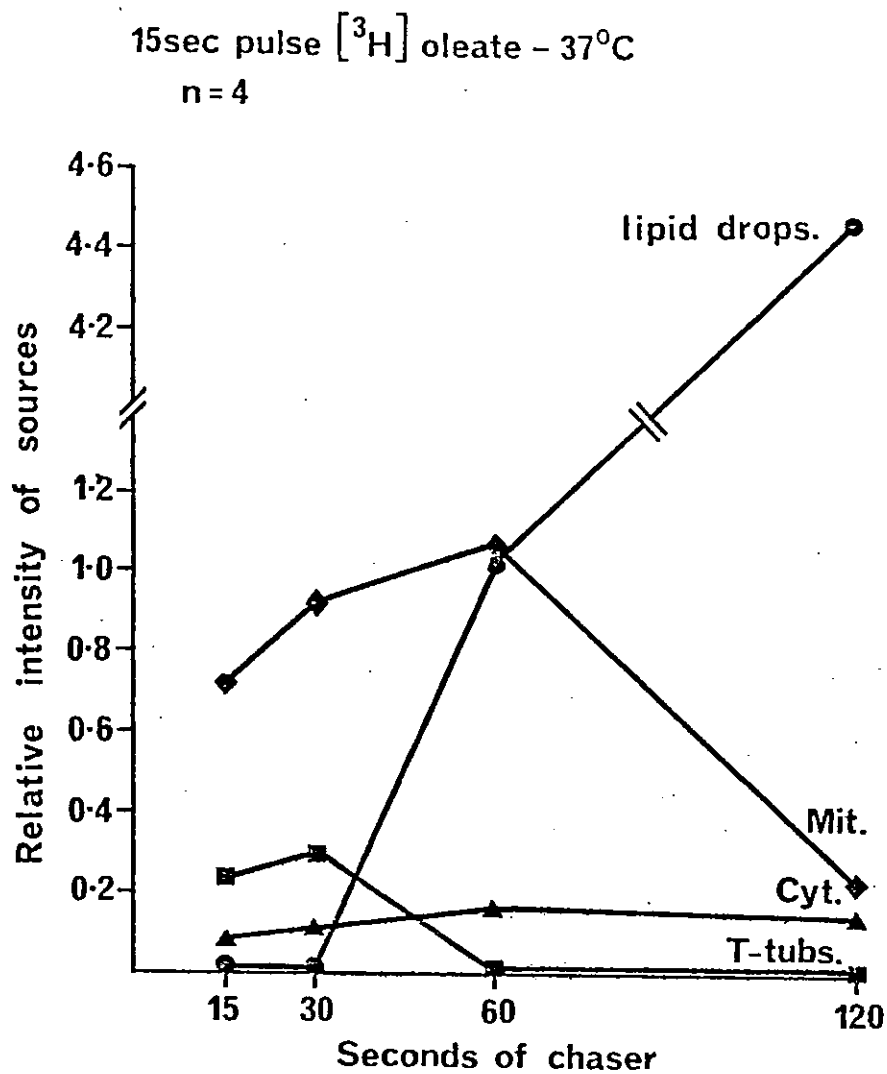


Figure 28

Relative intensity of sources in mitochondria, cytoplasm, T-tubules
and lipid droplets after well-oxygenated perfusion at 37°C

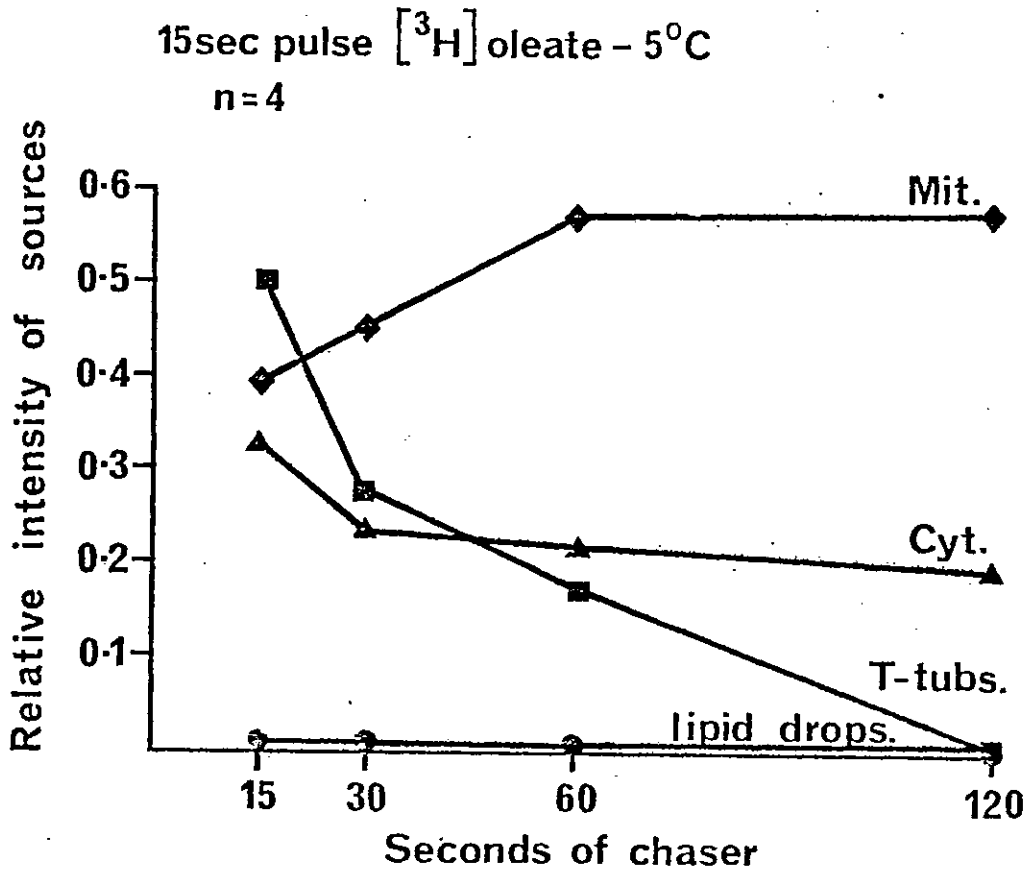


Figure 29

Relative intensity of sources in mitochondria, cytoplasm, T-tubules
and lipid droplets after well-oxygenated perfusion at 5°C

Hypoxic perfusions at 37°C

The relative intensity of sources in the various subcellular regions studied are given in Table 24 and some of these in Figure 30. Figures 31 - 34 show a comparison between the hypoxic hearts and the well-oxygenated hearts at 37°C.

In the well-oxygenated perfusions there was no difference between the radioactivity in the myofibrillar area and that in the 'cytoplasm'. After 30 minutes hypoxia however, radioactivity in the 'cytoplasm' was 2 - 3 times higher than in the myofibril region after all four chaser times. Incorporation of tritium into the mitochondria during hypoxic perfusion was considerably lower than in the well-oxygenated series but in a similar way it increased up to 60 seconds and decreased between 60 and 120 seconds, although this decrease was not as marked as in the well-oxygenated hearts. Activity in the lipid droplets was higher after only 15 seconds of hypoxic chaser perfusion and it increased to very high levels after 120 seconds. Little or no radioactivity was associated with the transverse tubules, sarcoplasmic reticulum or nucleus at any of the perfusion times.

Estimation of relative areas

Table 23 shows the relative area and hence volume, of each region for each chaser time in both well-oxygenated and hypoxic conditions. Although there appeared to be a small increase in the mitochondrial volume and a decrease in the volume of the lipid droplets in the hypoxic hearts, analysis of variance showed that there was no overall difference between the well-oxygenated and hypoxic hearts in terms of the relative areas of the subcellular components ($p > 0.05$).

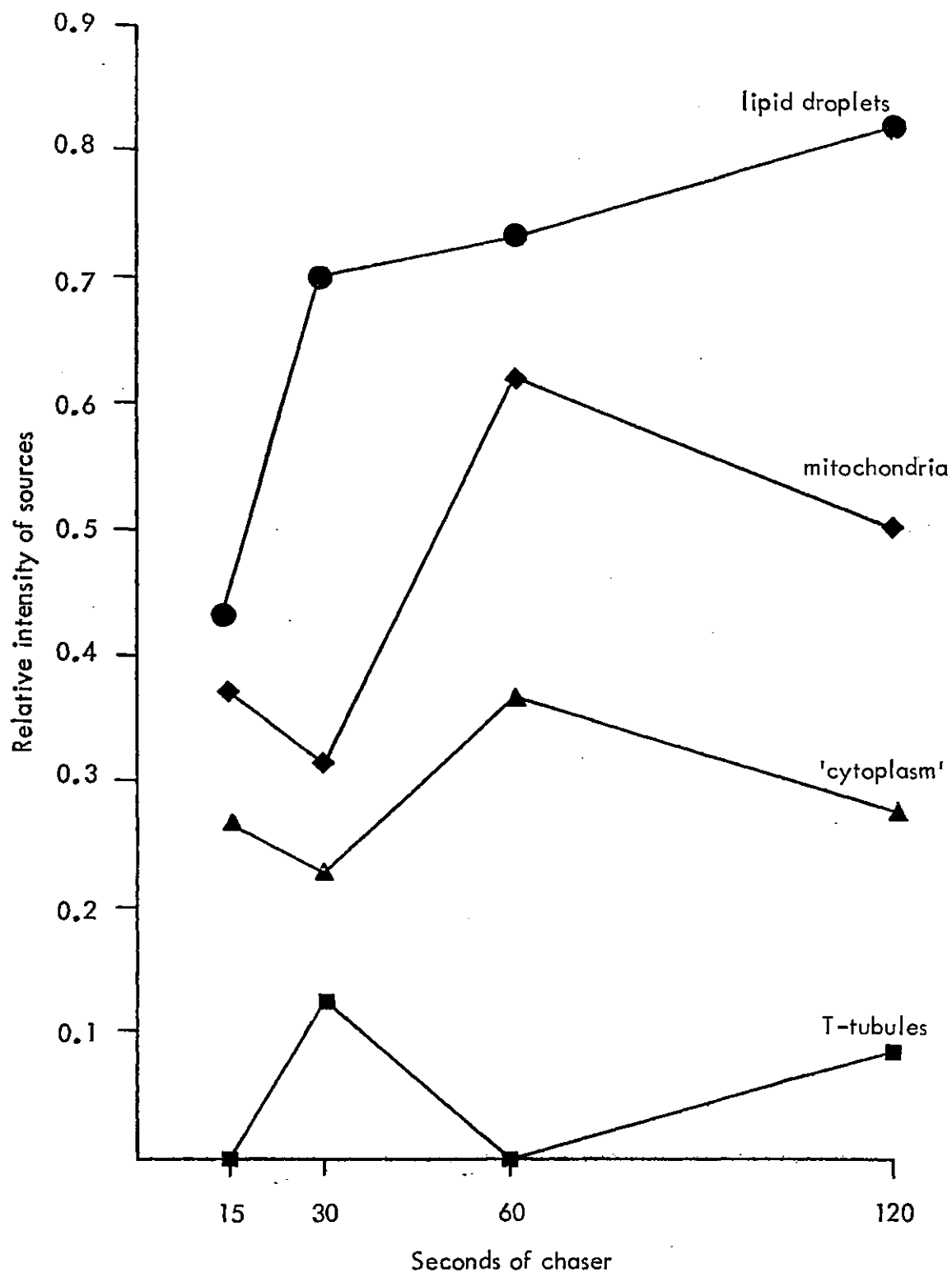


Figure 30

Relative intensity of sources in lipid droplets, 'cytoplasm',
mitochondria and T-tubules after 30 minutes hypoxia

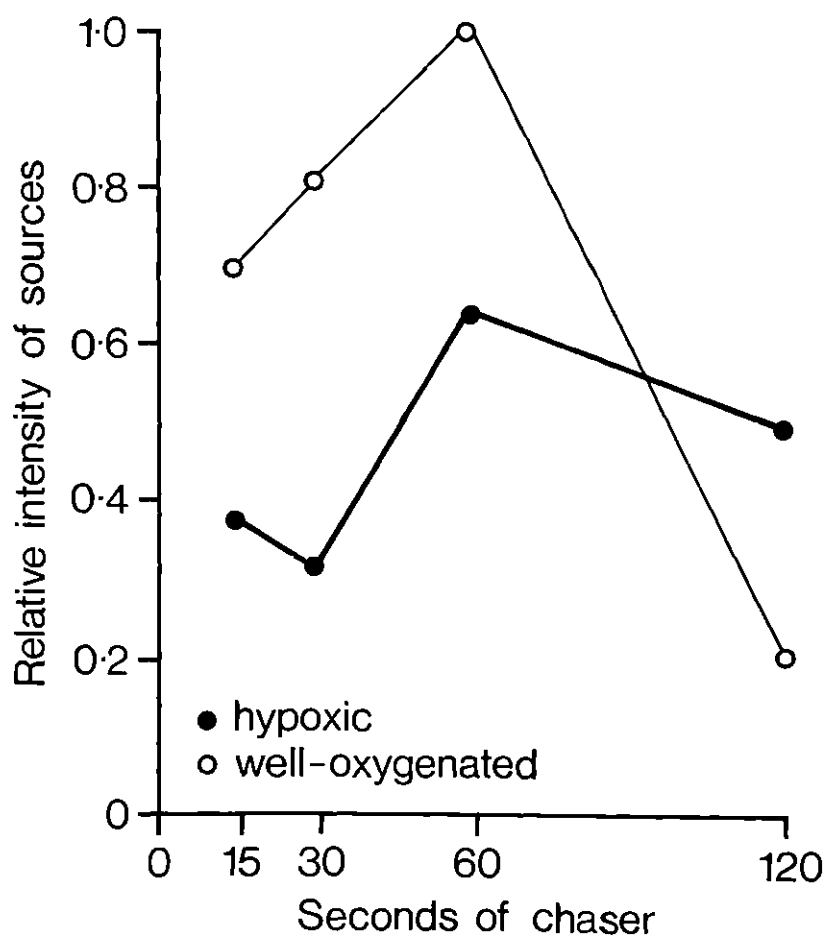


Figure 31.

Relative intensity of sources in the mitochondria

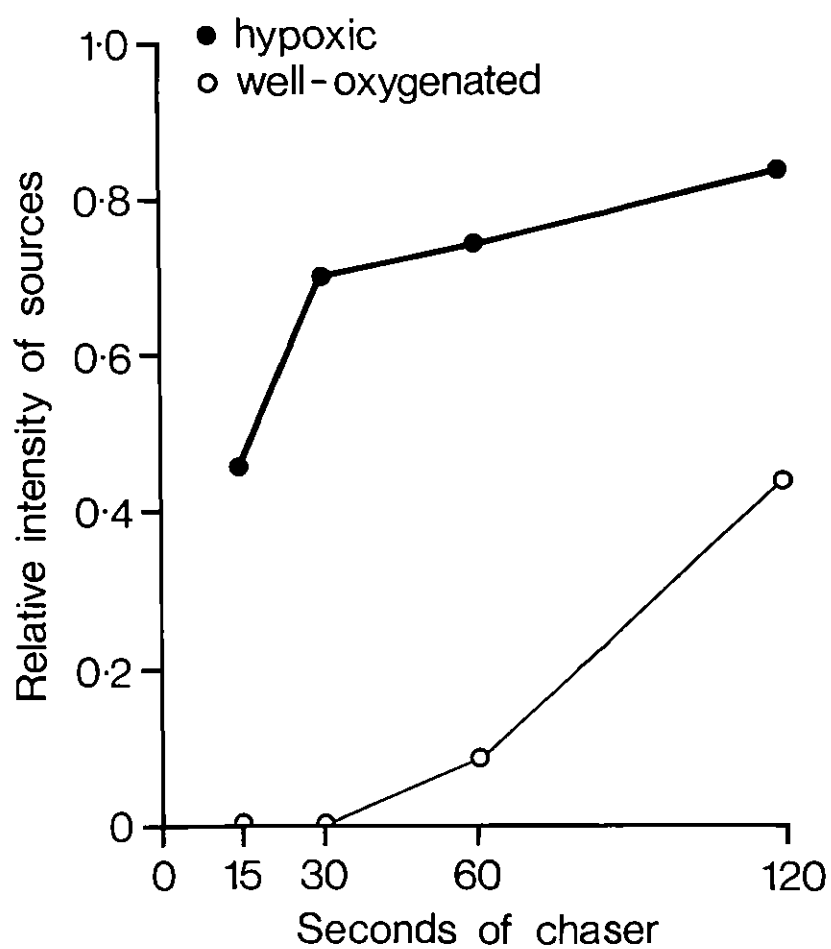


Figure 32

Relative intensity of sources in the lipid droplets

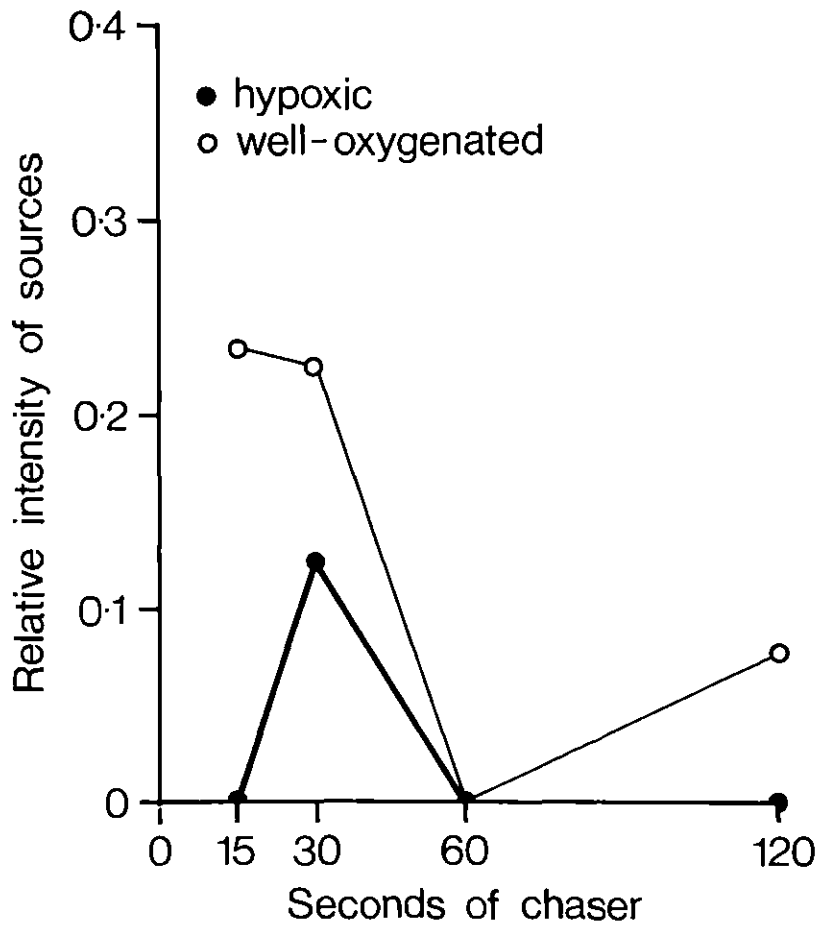


Figure 33

Relative intensity of sources in the T-tubules

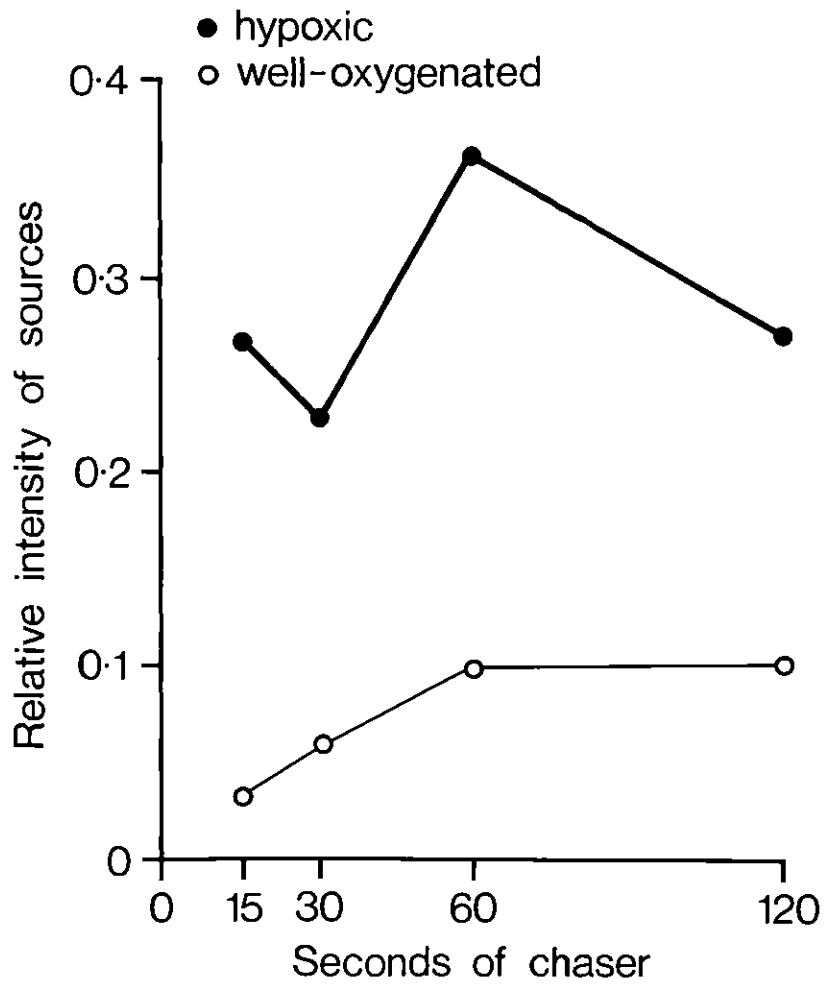


Figure 34

Relative intensity of sources in the 'cytoplasm'

Table 23

Relative area of subcellular organelles (expressed as percentage of whole cell).

Organelle	Well-oxygenated				Hypoxic			
	seconds of chaser							
	15	30	60	120	15	30	60	120
Myofibrils	47.2	47.1	47.9	41.4	49.6	48.8	44.9	46.6
'Cytoplasm'	15.5	13.3	13.9	19.1	12.1	11.4	16.0	15.0
Mitochondria	23.9	25.1	26.0	28.0	28.2	28.9	29.1	29.1
Transverse tubules	5.3	6.3	4.8	3.7	5.0	4.7	4.6	5.6
Sarcoplasmic reticulum	3.5	3.8	1.9	1.9	2.9	3.1	1.9	1.0
Lipid droplets	1.0	1.3	1.4	1.7	0.4	0.3	0.4	0.5
Nucleus	3.6	3.1	4.1	4.2	1.7	2.4	3.0	2.2

PART FOUR

THE INCORPORATION OF ^3H ERUCIC ACID INTO TISSUE LIPIDS
AND ITS ULTRASTRUCTURAL LOCATION BY EM AUTORADIOGRAPHY

CHAPTER 10 - INTRODUCTION

Erucic acid is the main constituent fatty acid of rapeseed oil, an oil which has been extensively used in animal feed products and, after the mid 1950's, was used regularly in large quantities in the margarine industry where it accounted for approximately 30% of the fats in margarine. No action was taken on human consumption until 1970, although growth retardation and changes in testes, adrenals and liver were reported since 1940 in animals fed rapeseed oil. In 1970 the Swedish National Board of Health and Welfare, at the request of the Swedish margarine industry, appointed a committee to examine the evidence for a toxic effect of rapeseed oil in man. This committee reported that there was no direct evidence for a toxic effect in man. However, since then, both in Sweden and elsewhere, research has shown that there is a toxic effect due to the erucic acid content of rapeseed oil. Although this oil is still extensively used, the Brassicas from which it is extracted have been selectively bred to produce a rapeseed oil which is low in erucic acid.

Erucic acid is not usually found in mammalian body fat but when the diet contains a high erucic acid rapeseed oil, erucic acid is found in depot fat, organ fat and milk fat. The quantities of erucic acid in these tissues however, is not as high as would be expected from the amounts in the diet and quantities of oleic acid are found to be higher than in controls. The metabolic conversion of long chain fatty acids from dietary rapeseed oil to oleic acid has been demonstrated experimentally (Carreau et al., 1968, a, b, Lapou et al., 1970).

The pathological effects of erucic acid can be summarised as follows (Borg, 1975). Firstly it has a growth retarding effect in animals. Secondly, rats fed rapeseed oil have larger adrenal glands with more fat and the synthesis of prostaglandins is reduced.

Cholesteryl erucate cannot be used efficiently for the synthesis of steroid hormones, especially at low temperatures (4°C), and plasma corticosterone levels in rats fed erucic acid are low. The dietary intake of erucic acid is associated with low survival rates in the cold.

In reproduction, the mortality of offspring is high because of deficient mammary development and lactation in the mother. Rats fed rapeseed oil high in erucic acid produce offspring which are fewer in number and smaller in size. Erucic acid causes fatty infiltration and degeneration of the liver and in the spleen it causes lipidosis and atrophy of the red pulp in ducks and guinea pigs but has no effect in the rat. In rats the renal concentrating capacity is nephrosis occurs characterised by vacuolation of the tubular epithelium, tubular dilation and connective tissue proliferation.

In skeletal muscle, a reversible fatty infiltration occurs in rats fed rapeseed oil (Abdellatif and Vles, 1970, a, b, 1971). The muscles appear pale after 2 weeks, normal colour after 8 weeks and after 16 weeks the morphology is normal indicating an increased tolerance to erucic acid with increased time of feeding. This has also been seen in cardiac muscle where, again in rats, intracellular lipidosis occurs after a few hours feeding, reaches a maximum after 3-6 days, then decreases rapidly (Abdellatif and Vles, 1970 b, Houtsmuller et al., 1970, Beare-Rogers et al., 1971). Engfeldt and Brunius (1975) found that 2% erucic acid in the diet causes myocardial lipidosis but 1% does not. In the heart, unlike in skeletal muscle, the lipidosis does not disappear completely.

The effects of dietary erucic acid on the rat heart can thus be seen in two stages. Firstly, acute lipid accumulation occurs in the first three days of feeding (Abdellatif and Vles, 1970, a, b, Beare-Rogers et al., 1971). This is ascribed to increased delivery of erucic acid

to the heart in serum VLDL (very low density lipoprotein) triacylglycerol (Thomassen et al., 1979) and myocardial lipoprotein lipase is enhanced (Jansen et al., 1975, Hülsmann et al., 1979). Also, the increased erucic acid is oxidised at a lower rate (Swarttouw, 1974, Cheng and Pande, 1975) and erucic acid inhibits the β -oxidation of other fatty acids (Heijensköld and Ernster, 1975, Christophersen and Bremer, 1972, Vasdev and Kako, 1976, Christiansen et al., 1977, Osmundsen and Bremer, 1978). This leads to increased esterification mainly to triacylglycerol.

The second stage occurs after prolonged feeding. Triacylglycerol levels fall almost to normal values (Abdellatif, 1972). Focal myocardial lesions occur after 40 days (in rats, Engfeldt and Brunus, 1975). These consist of histiocytic infiltration, myolysis, proliferation of fibroblasts and scarring. Lipid-filled, membrane bound bodies have been seen (Stam et al., 1980) which are thought to represent secondary lysosomes or autophagosomes.

In the isolated perfused rat heart, erucic acid was taken up at the same rate as palmitic acid but incorporation into tissue lipids was higher and CO_2 production lower than with palmitic acid (Vasdev and Kako 1976) These authors used a continuous perfusion with the radioactive fatty acid.

The experiments described in the following chapters were undertaken to compare the subcellular myocardial distribution of erucic acid with that of oleic acid after pulse perfusion with the radioactive fatty acid. The complementary techniques of freeze-clamping followed by thin layer chromatographical separation of the lipids and EM autoradiography were used as before, but the maximum perfusion time was extended from 2 minutes to 30 minutes as it was found that erucic acid was metabolised extremely slowly when compared with oleic acid.

CHAPTER 11 - METHODS

The animals used and the perfusion procedure were as described on page 46. The perfusion media consisted of Krebs Henseleit bicarbonate buffer (pH 7.4) containing 5 mM glucose and 0.2 mM erucic acid or oleic acid complexed to 0.2 mM bovine serum albumin by the method of Fredrikson and Gordon (1958). Radioactive perfusion media contained 100 $\mu\text{M}/\text{ml}$ $[9, 10 - ^3\text{H}]$ oleic acid or $[11, 12 - ^3\text{H}]$ erucic acid. All the perfusates were equilibrated with 95% O_2 : 5% CO_2 for at least 20 minutes prior to use.

Freeze-clamping

After an initial 5 minutes perfusion with the non-radioactive solution, the hearts were given a 15 second pulse perfusion with one of the radioactive solutions which was contained in a syringe and attached to the aortic cannula by a 3-way tap. The pulse perfusion was followed by an immediate return to perfusion with the relevant non-radioactive solution (i.e. perfusate containing erucic acid after the ^3H erucic acid or oleic acid after the ^3H oleic acid). This chaser perfusion was maintained for 2 minutes in the first experiments but, as it was found that very little of the erucic acid had been metabolised after this time, this was extended to 15 minutes and for the same reason it was again extended to 30 minutes in subsequent experiments. Unfortunately, due to the limited availability of the tritiated erucic acid, carbon-14 labelled erucic acid was used in the 15 and 30 minute experiments. The results cannot therefore be compared with those of the oleic acid hearts for these two perfusion times but the experiments were carried out in order to assess when a fairly high proportion of radioactive erucic acid had been incorporated into tissue lipids. This length of perfusion (30 minutes) was then used for the autoradiographic studies. Four guinea pig hearts were used for each fatty acid after each period of chaser.

At the end of the chaser period the hearts were frozen between two aluminium blocks, the lipids extracted and the main lipid classes were separated by thin layer chromatography. These methods are described in more detail on page 48.

$^{14}\text{CO}_2$ production

Four hearts were used for each perfusion time (2 minutes or 30 minutes chaser) with oleic acid and with erucic acid to measure the carbon dioxide produced by the oxidation of the radioactive fatty acid. The radioactive perfusate was prepared as before, using $[10\text{-}^{14}\text{C}]$ oleic acid or $[14\text{-}^{14}\text{C}]$ erucic acid. Duplicate aliquots of the radioactive perfusate were taken for scintillation counting before each experiment.

The method used was described in detail on page 53. In brief, the hearts were perfused under liquid paraffin to prevent the diffusion of $^{14}\text{CO}_2$ into the surrounding atmosphere. The effluent was collected in an alkaline medium. The hearts were freeze-clamped and the lipids were extracted as before (page 48) at the end of the appropriate chaser. The radioactivity in the effluent and extract was measured before and after acidification to release $^{14}\text{CO}_2$ (Vasdev and Kako, 1976).

EM autoradiography

In a further series of perfusions both fatty acids, oleic and erucic, were used with 2 minutes and 30 minutes chaser after the 15 second radioactive pulse. The hearts were fixed at the end of the chaser by perfusion with 4% glutaraldehyde in 0.1M cacodylate buffer for 10 minutes. Again four hearts were used for each fatty acid at each perfusion time. Pieces of left ventricle free wall were excised and processed for electron microscopy using water-soluble Durcupan (Stäubli, 1960, see page 72).

The method of autoradiography, including the analysis of autoradiographs by the hypothetical grain method was as described previously (pages 128-144). Using this method the "relative intensity of sources" was calculated in the myofibrils, mitochondria, T-tubules, sarcoplasmic reticulum, lipid droplets, nucleus and "cytoplasm" i.e. areas of cytoplasm not occupied by myofibrils. The calculation of the relative areas occupied by different regions of the cell was made from the data collected.

CHAPTER 12 - RESULTS

The results of the oleic acid perfusions with 2 minutes chaser have been given in a previous section (page 57) but have been included here for comparison with the erucic acid results.

Freeze-clamping

Table 24 shows the radioactivity (expressed as disintegrations per minute per milligram wet weight) in total lipid, free fatty acid, triacylglycerol and phospholipid respectively after 2 minutes chaser. Radioactivity in total lipid was slightly lower after two minutes chaser with erucic acid than with oleic acid. However the radioactivity in the free fatty acid fraction was almost three times as high with erucic acid ($p < 0.05$). At the same time the incorporation of radioactivity into triacylglycerol and phospholipid was much less with erucic acid than with oleic acid.

Table 25 gives the percentage incorporation of oleic acid and erucic acid into tissue lipids after 2, 15 and 30 minutes chaser. Almost 75% of the oleic acid present in tissue lipids was present in esterified lipid and only 25% was left as free fatty acid. This contrasts with the erucic acid where 85% was present as free fatty acid and this figure was still fairly high, at 46 %, even after 30 minutes. The two fractions not included in Table 25 are firstly cholesterol esters, which contributed less than 5% in all the experiments and secondly mono- and diacylglycerols because, as described previously (page 53), of the unknown quantity of acyl-CoA in this fraction.

$^{14}\text{CO}_2$ production

Table 26 shows the radioactivity (expressed as disintegrations per minute per milligram wet tissue) present as $^{14}\text{CO}_2$. The counts have been corrected for the volumes from which the aliquots were taken.

After 2 minutes chaser, the average total radioactivity recovered in CO_2 was 113 disintegrations per minute per milligram wet weight for oleic acid and 12 for erucic acid. After 30 minutes the radioactivity in CO_2 could not be measured as the effluent after acidification gave more counts than before acidification.

Ultrastructure and autoradiography

The analysis of autoradiographs, as well as giving an estimate of the 'relative intensity of sources' allows a measurement of the relative areas (and hence volumes) of the subcellular organelles to be made. These are given in Table 27. Analysis of variance revealed no significant difference of these areas between oleic acid and erucic acid or between 2 minutes and 30 minutes chaser.

On examination of several hundred autoradiographs, an interesting and unexpected phenomenon was noted. In the cells of the hearts perfused for 30 minutes with erucic acid, lipid droplets were seen apparently in various stages of emerging from the cells. Figure 35 show electron micrographs of this process. There was no evidence that this was due to exocytosis; the lipid droplets were not enclosed by a membrane but apparently released uncovered into the extracellular space. There also appeared to be more lipid droplets close to, or immediately beneath, the sarcolemma in these micrographs. In order to quantify this observation, the perpendicular distance of the centre point of each lipid droplet to the nearest sarcolemma was measured in all the autoradiographs from the 30 minute chaser with both erucic acid and oleic acid. The mean lipid droplet to sarcolemma distance of 570 lipid droplets (disregarding those outside the cell) was 1200 nanometers (± 726 nanometers standard deviation) in the erucic acid perfused hearts and 2600 (± 726) in those perfused with oleic acid (524 lipid droplets).

The relative intensity of the subcellular organelles is shown in Table 28 and a representative autoradiograph in Figure 36.

Chi-squared tests showed a significantly non-random distribution of radioactivity in each case. Both after 2 minutes and 30 minutes the radioactive erucic acid had penetrated to the myofibrillar area and was found here to a greater extent than in the areas of cytoplasm which do not contain myofibrils. The oleic acid radioactivity was greater in the 'cytoplasm' than in the myofibrillar region after both chaser times.

Mitochondrial radioactivity was considerably higher in the erucic acid perfused hearts than in the oleic acid hearts after both chaser times. Lipid droplet activity was high in the oleic acid hearts especially after 2 minutes, but was virtually absent in the erucic acid hearts.

TABLE 24

Distribution of radioactivity in myocardial total lipid, free fatty acid, triacylglycerol and phospholipid 2 minutes after a 15 second perfusion with ^3H oleate or erucate at 37°C

	Radioactivity (dpm per mg wet weight)			
	Total lipid	Free fatty acid	Triacylglycerol	Phospholipid
Oleate	950 ($\pm$ 188)	247 ($\pm$ 36)	293 ($\pm$ 73)	235 ($\pm$ 47)
Erucate	801 ($\pm$ 141)	682 ($\pm$ 112)	48 ($\pm$ 7)	7 ($\pm$ 1)

Each figure is the mean $\pm$ standard deviation of four hearts.

Table 25

Percentage distribution of radioactivity in
free fatty acid, triacylglycerol and phospholipid

Time of unlabelled chaser (seconds)		Free fatty acid	Triacylglycerol	
Oleate	2	25.8 ($\pm$ 4.3)	31.8 ($\pm$ 4.8)	24.4 ($\pm$ 4.1)
	15	16.3 ($\pm$ 1.9)	33.2 ($\pm$ 6.1)	23.6 ($\pm$ 3.8)
	30	15.5 ($\pm$ 4.1)	24.5 ($\pm$ 8.1)	17.6 ($\pm$ 2.0)
Erucate	2	85.5 ($\pm$ 3.4)	6.1 ($\pm$ 3.9)	0.9 ($\pm$ 0.3)
	15	52.0 ($\pm$ 2.8)	16.2 ($\pm$ 5.6)	8.6 ($\pm$ 1.7)
	30	46.0 ($\pm$ 7.9)	12.1 ($\pm$ 3.1)	16.1 ($\pm$ 3.1)

Table 26

$^{14}\text{CO}_2$ production 2 minutes after pulse perfusion with ^{14}C oleic acid
or ^{14}C erucic acid

Lipid containing chloroform phase	78.2 ($\pm$ 6.0)	65.8 ($\pm$ 25.2)
Aqueous phase	4.8 ($\pm$ 3.5)	8.8 ($\pm$ 2.9)
Aqueous phase after acidification	3.0 ($\pm$ 2.7)	6.6 ($\pm$ 2.2)
Calculated $^{14}\text{CO}_2$ in aqueous phase	1.8 ($\pm$ 1.3)	2.3 ($\pm$ 1.3)
Effluent	2042. ($\pm$ 479.3)	2118.1 ($\pm$ 321.6)
Effluent after acidification	1931.7 ($\pm$ 461.2)	2108.4 ($\pm$ 328.1)
Calculated $^{14}\text{CO}_2$ in effluent	111.1 ($\pm$ 18.9)	9.7 ($\pm$ 2.6)
Total $^{14}\text{CO}_2$	112.9 ($\pm$ 20.1)	11.9 ($\pm$ 3.9)
Recovery of counts	87.4% ($\pm$ 9.4%)	92.3% ($\pm$ 8.4%)

Each figure is the mean $\pm$ standard deviation of four hearts.

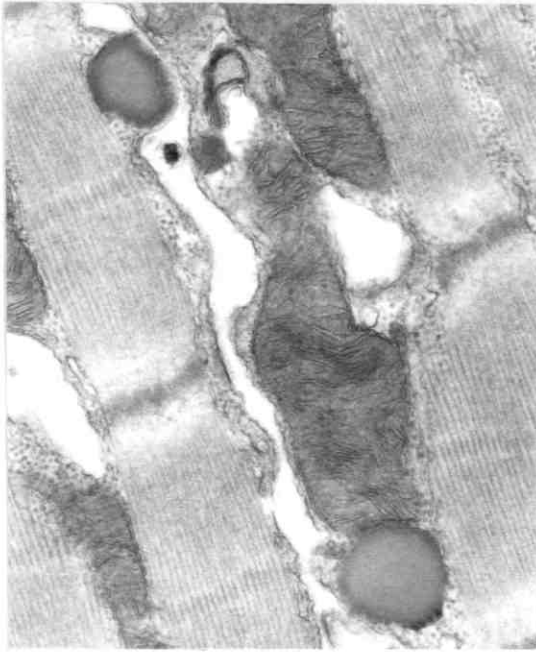
Table 27Relative areas of subcellular organelles (expressed as percentage of whole cell)

	Oleic acid		Erucic acid	
	2 mins	30 mins	2 mins	30 mins
Myofibrils	41.4	41.1	45.9	40.9
'Cytoplasm'	19.1	18.2	17.6	19.2
Mitochondria	28.0	28.9	26.2	25.7
T- tubules	3.7	4.9	5.8	5.6
Sarcoplasmic reticulum	1.9	1.6	1.7	1.4
Lipid droplets	1.7	1.5	0.3	1.9
Nucleus	4.2	3.8	2.5	5.3

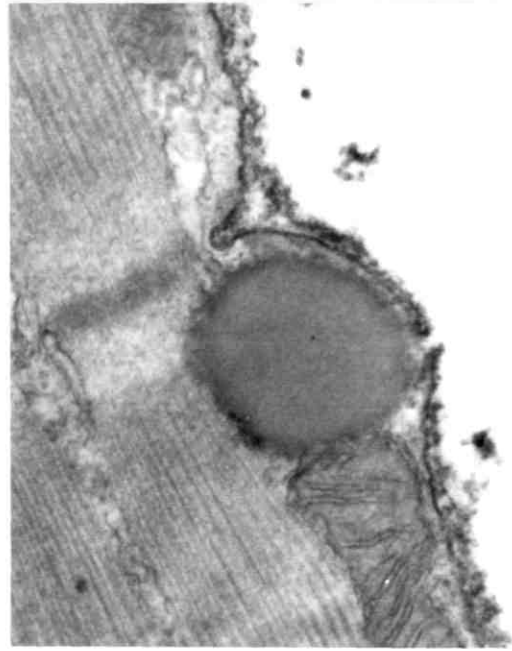
Table 28

Relative intensity of sources in subcellular organelles after
pulse perfusion with ^3H oleic acid or with ^3H erucic acid

	Oleic acid		Erucic acid	
	2 mins	30 mins	2 mins	30 mins
Myofibrils	.073	-.329	.251	.227
'Cytoplasm'	.100	.417	.095	-.114
Mitochondria	.202	.218	.488	.402
T-tubules	.075	.127	.127	.129
Sarcoplasmic reticulum	.250	-.064	-.485	-.106
Lipid droplets	4.160	.477	-.796	.185
Nucleus	.024	.076	.132	-.002



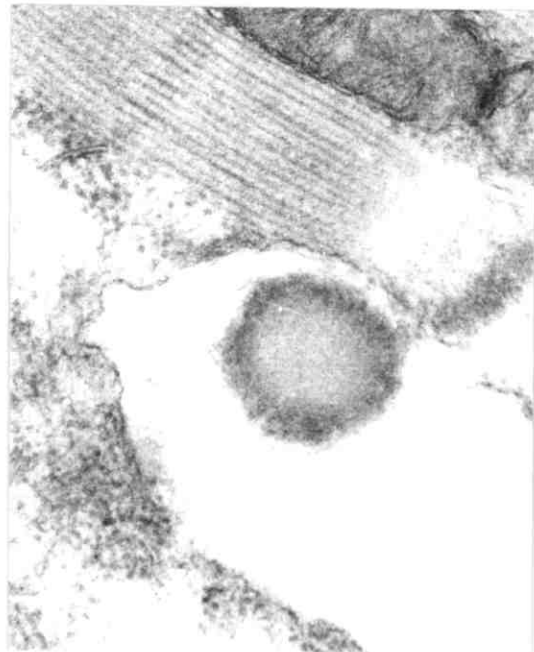
x 30,000



x60,000



x 50,000



x 65,000

Figure 35

The expulsion of lipid droplets from myocardial cells

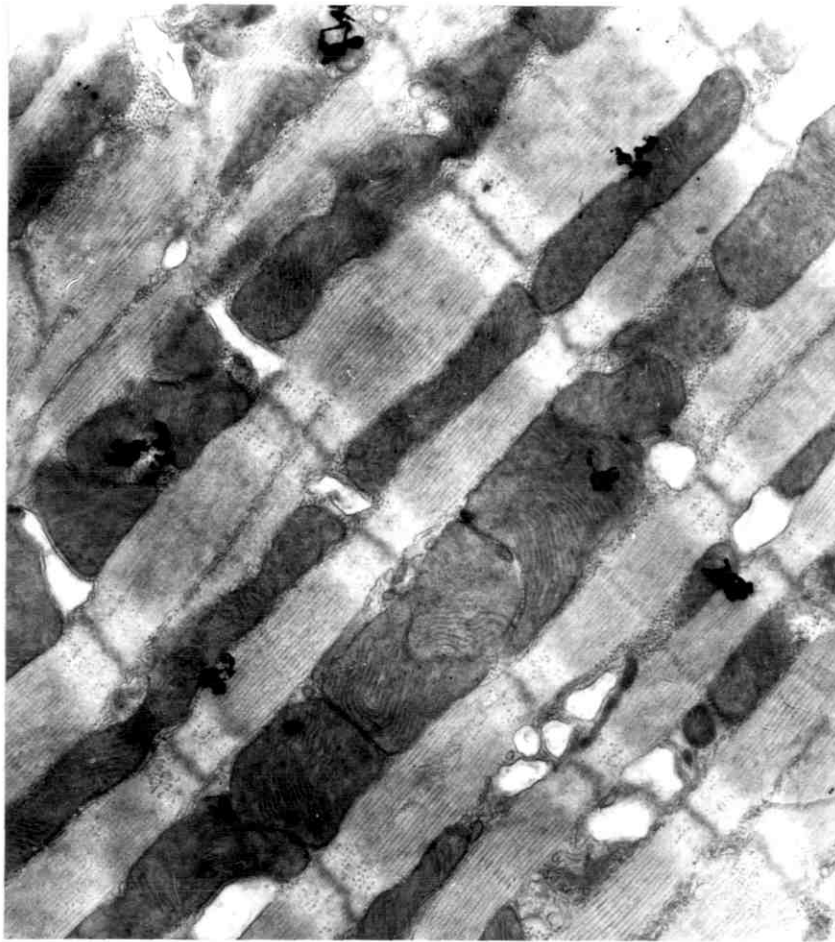


Figure 36

A representative autoradiograph of a heart perfused with ^3H erucic acid followed by 30 minutes chaser with non-radioactive erucic acid

x 20,000

PART FIVE

DISCUSSION

General

This thesis has described the incorporation of tritiated oleic acid into the cells of the isolated, perfused guinea pig myocardium, up to 120 seconds after the introduction of the radioactive fatty acid. By using the techniques of lipid analysis and electron microscope autoradiography, two separate pictures have emerged. Firstly of the biochemical fate of the fatty acid after each time interval studied, and secondly of its spatial distribution within the cell. Although it is not possible to attribute the formation of any one silver grain in an autoradiograph to a particular lipid class, the overall distributions, both biochemical and anatomical, can be interpreted, each in relation to the other. Having done this, the changes occurring in these distributions after the hearts had been subjected to 30 minutes of hypoxic perfusion will be discussed. The measurement of radioactivity present in $^{14}\text{CO}_2$ allows a comparison of the oxidation of the radioactive fatty acid in well-oxygenated and in hypoxic conditions to be made.

An unsaturated fatty acid was used in this study rather than a saturated fatty acid such as palmitic acid, because unsaturated fatty acids are more readily fixed, particularly by osmium tetroxide.

Methodology

(i) A comparison of freeze-clamping and fixation

At the end of the prescribed chaser period, metabolic activity in the hearts was arrested, either by freeze-clamping or by glutaraldehyde fixation. As there was, in some cases, as little as 15 seconds difference between the lengths of the chaser perfusions it was important to arrest all the metabolic activity at a precise time at the end of the chaser.

The rapid freezing method (Wollenberger et al., 1960) which involves clamping the hearts between two aluminium blocks precooled in liquid nitrogen does precisely this. The procedure adds at the most one second to the chaser period. Stein and Stein (1968) described the incorporation of tritiated oleic acid into the cells of the isolated, perfused rat heart. They used various lengths of perfusion with the radioactive fatty acid, from 15 seconds to 20 minutes and a post-perfusion period of from 0 seconds to 20 minutes. The results of these experiments will be discussed later in comparison with my own, but it is worth noting at this stage that, instead of freeze-clamping, Stein and Stein (1968) preceded their lipid analyses by homogenisation of the whole heart in a 0.25 M sucrose solution at room temperature. Some of the homogenates were separated into cell fractions by differential centrifugation. It has subsequently been shown (Fields et al., 1975) that considerable exchange of radioactivity between subcellular fractions and from free fatty acid into acylglycerols and phospholipids can occur during the process of cell fractionation even at 4°C.

In the hearts perfused for autoradiography, it is unlikely that metabolic activity is arrested at the exact moment that fixation with glutaraldehyde begins. To minimise these differences between the fixed and the freeze-clamped hearts, glutaraldehyde fixation was carried out at 5°C to inhibit or decrease the rate of further esterification or oxidation of the radioactive fatty acid, although the perfusions at 5°C showed that some uptake and transport of fatty acids do occur at this temperature.

A further problem arises when comparing freeze-clamped hearts with hearts which have been processed for electron microscopy. Hearts which have been freeze-clamped contain all the radioactive lipid present at the end of the chaser period and the lipid extracts from these hearts contain all the radioactive lipid except the water-soluble products.

In contrast, approximately 35% of the total lipid radioactivity present at the end of perfusion fixation is lost during tissue processing with water-soluble Durcupan and a considerable loss will have already occurred during the perfusion fixation itself. This loss is of lipid which would be extracted into the chloroform phase of a chloroform : methanol extraction and is quite separate from the loss of water-soluble products such as acyl-CoA and intermediates and by-products of the β -oxidation pathway which would be lost both from the freeze-clamped hearts during lipid extraction and from the fixed hearts during processing for electron microscopy. As it proved impossible to assess which classes of lipid were lost at each stage of processing, there was no way of telling whether there was a preferential loss of a particular lipid class or classes.

The freeze-clamped hearts, especially after the shorter chaser perfusions, will contain radioactive fatty acid in the perfusate which had remained in the vascular space at the time of freeze-clamping. In these hearts the proportion of free fatty acid to other lipid classes will be higher than in the fixed hearts, in which the ten minute fixation period will have removed the radioactive free fatty acid from the vascular space.

(ii) Measurement of radioactivity in $^{14}\text{CO}_2$ -

The method used to determine ^{14}C incorporation into carbon dioxide was as described by Vasdev and Kako (1976) with one or two modifications. The hearts were perfused under the surface of liquid paraffin to prevent diffusion of any $^{14}\text{CO}_2$ produced into the surrounding atmosphere. Both the effluent and the aqueous phase of the lipid extraction were collected in an alkaline medium ($\text{pH} > 13$) before contact with the air. In this way any free $^{14}\text{CO}_2$ was converted to bicarbonate and kept in solution. On acidifying these solutions the $^{14}\text{CO}_2$ was released and the quantity produced was assessed from the difference in radioactivity between the alkaline and acidified solutions.

This method does not give any measure of the overall rate of β -oxidation but allows a comparison of the well-oxygenated and the hypoxic hearts to be made in terms of the oxidation of the radioactive fatty acid.

(iii) EM autoradiography and hypothetical grain analysis using simultaneous equations

Most methods of studying the entry and fate of metabolites in cells involve destructive processes such as homogenisation and cell fractionation before biochemical analysis. Although chemical fixation and dehydration of the tissue are also destructive processes from the biochemical viewpoint, the structural integrity of the cell is maintained. Thus the autoradiographic technique enables the electron microscopist to follow the movement of metabolites in intact tissue. However, autoradiography does have its limitations. Because of the large size of the silver bromide crystals in the nuclear emulsion, the resolution of the autoradiographs is approximately 100 times poorer than the resolution of the electron microscope. Although the results of some of the larger subcellular structures can be regarded with some confidence, others, such as that of the sarcoplasmic reticulum, cannot be relied on as a good estimate of the relative intensity of sources in that structure but only as a rough guide. A grain overlying a small portion of sarcoplasmic reticulum may completely obscure the sarcoplasmic reticulum and may therefore be attributed to another structure.

The hypothetical grain analysis of Blackett and Parry (1973, Parry and Blackett, 1973, 1975) provides a method of analysing autoradiographs at the EM level taking into account the 'cross-fire' effect, i.e. the possibility that a source in one structure may give rise to a silver grain overlying another structure. The use of simultaneous equations has simplified the approach to the final processing of the collected data although in theory the two methods are much the same. The 'grains per grid point' in Blackett and Parry's analysis are obtained by a computer manipulation of the hypothetical grain distribution. Each row of the hypothetical grain table is multiplied by an appropriate number. The new values are added up and compared with the real grains using the Chi-squared test.

This is, in effect, the same as solving a set of simultaneous equations. The set of multipliers which gives the lowest Chi-squared value represents the distribution of radioactivity which can most closely account for the distribution of the real grains. The simultaneous equations program is, in practice, a simpler method of obtaining the same result, i.e. the relative intensity of radioactive sources in each subcellular structure.

The retention of lipids during EM processing

The lipid-retaining properties of several methods of processing tissue for electron microscopy were compared. These experiments were carried out as a preliminary to the autoradiography in order to find a reproducible method of processing which would minimise lipid losses from the tissue. To compare the different procedures a radioactive label was introduced into the lipids of the guinea pig heart by a brief period of perfusion with 9, 10 - ^3H oleic acid. A 15 second perfusion with ice cold Krebs Henseleit buffer followed which was intended to remove radioactive material from the vascular space. The hearts were then fixed by perfusion with 4% glutaraldehyde for ten minutes and processed using one of the experimental methods. The processing fluids were retained for scintillation counting.

The perfusion effluent from the glutaraldehyde fixation was collected at one minute intervals over the ten minute fixation period. The radioactivity present in these samples indicated that the 15 second washout with Krebs Henseleit buffer was not long enough to remove all the labelled material from the vascular tree. During the 10 minute fixation 83% of the radioactivity which was washed into the glutaraldehyde effluent, was lost from the hearts in the first three minutes and 94% in the first five minutes.

In the second half of the fixation period the loss of radioactivity from the hearts was minimal. It was concluded that the large loss of radioactivity in the first five minutes of perfusion fixation was mainly due to washout of radioactive fatty acid from the vascular space. After the full ten minute perfusion it can be assumed that virtually all the labelled lipid remaining will be intracellular although some albumin bound fatty acid may be fixed in situ in the T-tubules by the glutaraldehyde fixation of the albumin. The loss of counts during processing for EM would therefore be due mainly to the loss of intracellular lipid.

It was originally intended that not only the amount of radioactive lipid lost at each stage of processing would be established, but also which classes of lipid were lost to see whether there was any preferential extraction by the processing fluids. Unfortunately this did not prove possible as the processing fluids partitioned into the chloroform phase of a chloroform : methanol extraction together with the lipids. These processing fluids obscured the positions of the lipid bands on the thin layer plates. This meant that lipids extracted from processed tissue could not be successfully separated on thin layer chromatography plates. This difficulty was further demonstrated when radioactive oleic acid and trioleylglycerol of high radiochemical purity were treated with glutaraldehyde, osmium tetroxide and water-soluble Durcupan. In addition to a number of unidentified bands occurring on thin layer chromatographical separation after this treatment, the distribution of radioactivity was significantly altered when compared with the untreated lipid standards. This indicates the inherent error in the method used by Cope and Williams (1968), in a study of lipid-retaining processing methods, of dividing thin layer plates according to the position of lipid standards in cases where definite bands cannot be seen.

As a result of these findings the loss of radioactivity reported in the procedures examined was described as that which partitions into the chloroform phase during lipid extraction. This material is described as 'total lipid'. However, as the administration of the radioactive label was rapidly followed by a chaser of ice cold Krebs Henseleit buffer and then immediate fixation with glutaraldehyde it is unlikely that esterification of the free fatty acid would have proceeded to any significant degree. Most of the radioactivity would therefore be present in the free fatty acid fraction. No appreciable radioactivity could be detected originating from the water-soluble derivatives of fatty acid metabolism.

The lipid-retaining properties of each method of tissue processing were assessed by comparing the radioactivity present in the tissue at the end of processing with the radioactivity in the tissue immediately after perfusion fixation with glutaraldehyde. The processing fluids at each stage were monitored for radioactivity. In all the experiments a small loss of radioactivity was observed during immersion fixation in glutaraldehyde, amounting to an average of 3.4% of the total lipid radioactivity present. Losses in osmium tetroxide and its subsequent buffer wash were even smaller.

At the end of the Epon schedule, with the shortened ethanolic dehydration (Idelman, 1965), only 20.6% of the initial radioactivity was retained in the tissue and 15.0% and 18.7% were lost in 70% and 95% ethanol respectively. This differs markedly from the results of Stein and Stein (1968) who reported only 0.5% and 0.6% loss in 70% and 95% ethanol respectively and a retention of 78.2% in the tissue. The difference in the species used (Stein and Stein used the rat) is unlikely to have played a significant part in this enormous difference. As ethanol is

such an excellent lipid solvent it seems likely that lipid losses during these stages would be large, and ethanolic dehydration is probably best avoided in cytochemical studies of lipids by electron microscopy.

The addition of para-phenylenediamine to the Epon processing schedule did not appear to improve lipid retention. The retention of 17.3% obtained using para-phenylenediamine was slightly lower than the 20.6% retention obtained using the Epon schedule without para-phenylenediamine. This is probably due to the increased time in which the tissue was in 70% ethanol. The experiment in which the total fatty acids were extracted from the unfixed tissue, glutaraldehyde fixed tissue and tissue processed with Epon including para-phenylene diamine showed that para-phenylenediamine did not affect the extraction of lipids by chloroform : methanol. Para-phenylenediamine does, however, have advantages as a stain as it enhances the reduction of osmium tetroxide and eliminates the necessity of staining sections with a substance such as lead citrate in order to improve contrast. Uranyl acetate was not included as a stain in any of the processing schedules. This was because uranyl acetate is a powerful α -particle emitter and, although the energy of the particles is too high to have much effect on an emulsion, it may marginally increase background grains and was therefore considered to be best omitted.

Clearly, those methods of processing which offer most scope to electron microscopists interested in lipids, are those which use water-miscible components. The dehydration occurs during immersion of the tissue in increasing concentrations of the embedding medium so that the use of organic solvents for dehydration is avoided.

The highest lipid retention was found using the GACH (glutaraldehyde/carbohydrazide) polymer where 82% of the counts recovered remained in the tissue after processing. Recovery of counts, however, was low when the lipids were extracted in chloroform : methanol. In a similar experiment, when the tissue was completely solubilised in Soluene (Packard) after processing in GACH, the recovery of counts was nearly 100% and the lipid retention was nearly 90%. The low recovery of counts with chloroform : methanol is therefore caused by lipid bound in the tissue by the presence of the GACH prepolymer so that it is not extracted by the chloroform : methanol mixture.

Second to GACH in terms of lipid retention was water-soluble Durcupan with a retention of 69.9%. Recovery of counts was 100% using chloroform : methanol extraction. In spite of its poorer lipid retention, water-soluble Durcupan has several advantages over GACH when considering ease of handling. Preparation of GACH is a lengthy and critical procedure. A temperature of 0°C - 3°C must be maintained throughout the preparation to prevent premature polymerisation. At this temperature the carbohydrazide does not dissolve readily in the 50% glutaraldehyde. Durcupan is supplied as four liquid components which require only to be mixed in the correct proportions.

Another disadvantage of GACH is that the tissue must be embedded in tiny drops of resin so that polymerisation can occur. When hardened these droplets must then be cemented on to blank blocks before they can be sectioned. With water-soluble Durcupan the tissue can be embedded directly into capsules which are the correct size for sectioning.

The polymerised GACH resin is extremely brittle, making it difficult to section. The addition of plasticisers such as dibutylphthalate, renders

the resin more likely to extract lipid from the tissue (Heckman and Barnett, 1973). The sectioning problems are particularly relevant when working with muscle as this is a tissue which is very difficult to infiltrate. GACH may be of value to workers using cell suspensions, cell fractions or tissues which are more readily infiltrated.

For the above reasons water-soluble Durcupan was chosen for use in the autoradiographic studies. This was combined with para-phenylenediamine as a stain as it was found that sections of Durcupan embedded tissue did not stain very readily with lead citrate. A 1% solution of para-phenylenediamine in 0.1M sodium cacodylate was included in the Durcupan schedule for 30 minutes immediately after the second buffer wash. Although para-phenylenediamine was not found to improve lipid retention using ethanolic dehydration and Epon embedding, there was no reason to believe that it had any detrimental effect on lipid retention, especially as its action is to increase osmium tetroxide reduction (Ledingham and Simpson, 1972). By using buffer as the solvent for the para-phenylenediamine instead of ethanol it was hoped that lipid loss during this stage would be minimal.

The incorporation of ^3H oleic acid into tissue lipids in well-oxygenated conditions

The amount of radioactivity in total lipid after 15 seconds of unlabelled chaser was similar at 37°C and at 5°C . By 30 seconds it had in each case decreased to almost one half its value at 15 seconds. Thereafter the level of radioactivity in total lipid remained unchanged at 5°C but continued to decrease at 37°C . It seems likely that the initial sharp decrease in lipid radioactivity found both at 37°C and at 5°C

was due to the mechanical washout of free fatty acid located in the vascular space, and possibly, to some extent, in the space between the blood vessels and the myocardial cells.

These changes in myocardial total lipid radioactivity were mainly due to changes in free fatty acid radioactivity, which showed similar courses of events. The decline in free fatty acid radioactivity after more than 30 seconds chaser at 37°C was partly due to the oxidation of the free fatty acid. The water-soluble products and intermediates of β -oxidation partition into the aqueous phase of a chloroform: methanol extraction and do not therefore, contribute toward the total lipid radioactivity. The decrease in total lipid radioactivity at 37°C but not at 5°C was also due in part, to the esterification of the free fatty acid to acylglycerol or to phospholipid. Both oxidation and esterification would be expected to be inhibited at 5°C. Consistent with this explanation is the increase in radioactivity in triacylglycerol and phospholipid with increasing time at 37°C but not at 5°C.

A study of the subcellular distribution of ^{14}C palmitate and ^{14}C oleate was carried out in the isolated perfused rat heart by Gloster et al. (1978). In some of their experiments the same perfusion times were used as in the present study, namely a 15 second radioactive pulse followed by 15, 30, 60 or 120 seconds chaser. Although these authors looked at the total lipid, free fatty acid and triacylglycerol in mitochondrial, microsomal and cytoplasmic fractions separately, their results show a similar decrease in total lipid and free fatty acid with time and an increase in triacylglycerol with time.

At 5°C more than 80% of the total lipid radioactivity was still present in free fatty acid after 120 seconds. The high level of total

lipid radioactivity at all the chaser times at 5°C indicates that the uptake of free fatty acids into the cells continues even at this low temperature. This again agrees with Gloster et al. (1978).

The degree of incorporation of radioactivity into esterified lipids was considerably less than that reported by Stein and Stein (1968) in rat hearts. These authors found that as much as 70% of the total counts derived from radioactively - labelled fatty acid was present in triacylglycerol after 60 seconds chaser perfusion at 37°C. This contrasts with the results of my own experiments where only 21% was incorporated into triacylglycerol after the same length of time at 37°C. Part of this difference may be due to the different species used. Part, however, is probably methodological. Stein and Stein extracted lipids from tissue homogenised at room temperature, which might permit considerable esterification to occur during processing. Such esterification was prevented in the present study by freeze-clamping the hearts. Similar considerations apply to the findings of Stein and Stein (1968) that the majority of the radioactivity in isolated mitochondria and sarcoplasmic reticulum was found in acylglycerols. As mentioned previously, considerable exchange of radioactivity between organelles and from free fatty acid into acylglycerols and phospholipids can occur during the process of cell fractionation (Fields et al., 1975).

I was unable to separate either acyl-CoA or acyl-carnitine from the lipid extracts by any of the published methods. After 120 seconds chaser, only 1.8% of the total lipid radioactivity was found in the region of the thin layer plate containing the phosphatidylcholine and sphingomyelin which is the region to which acyl carnitine migrates. The contribution of acyl-carnitine to the total lipid radioactivity must

therefore be very small and unlikely to affect the overall results as given. The phospholipid results have therefore been included. The percentage of the total lipid radioactivity which was associated with the phospholipid was of a similar magnitude to that reported by Stein and Stein (1968) in the perfused rat heart. These workers too made no allowance for the possible presence of long chain acyl-carnitine in this fraction.

Acyl-CoA migrates to the same region of the thin layer plates as mono- and diacylglycerol. Although two thirds of the acyl-CoA partitions into the aqueous phase of a chloroform : methanol extract and would therefore not affect the lipid separation, the contribution of the remaining one third of the acyl-CoA to the radioactivity of the mono- and diacylglycerol band could not be assessed. The results for mono- and diacylglycerol have therefore not been given.

$^{14}\text{CO}_2$ production in hearts perfused with ^{14}C oleic acid in well-oxygenated conditions

These experiments were only carried out on hearts perfused for 120 seconds after the radioactive pulse as this was the longest perfusion time used and therefore the one in which most $^{14}\text{CO}_2$ production would be expected. The shorter chaser times were not studied as these $^{14}\text{CO}_2$ experiments were performed merely to compare the $^{14}\text{CO}_2$ produced by the hearts in well oxygenated and in hypoxic conditions. Most of the radioactivity present as $^{14}\text{CO}_2$ was found in the effluent, indicating that after 120 seconds some of the ^{14}C oleic acid had been completely oxidised to $^{14}\text{CO}_2$ and had passed out of the heart to be collected in the effluent. A much smaller amount of $^{14}\text{CO}_2$ was trapped in the heart at the time of freeze-clamping and

was therefore recovered in the aqueous phase of the lipid extraction.

It was not possible to assess the relative proportions of radio-active free fatty acid which was oxidised as opposed to that which was esterified, by a direct comparison of disintegrations per minute in $^{14}\text{CO}_2$ and in esterified lipid. This was because of the different specific activities of the isotopes used, ^{14}C oleic acid for the oxidation experiments and ^3H oleic acid for the measurement of esterification.

Ultrastructural location of ^3H oleic acid in the well-oxygenated hearts

It must first be emphasised that while freeze-clamping experiments reflect the distribution of the radioactivity in the lipids of the entire heart, the autoradiographic studies show the distribution of tritiated oleic acid in the left ventricle only. Although a comparison of the autoradiographs of left ventricle and interventricular septum after 15 seconds chaser showed no difference between these two sites it cannot be assumed that the distribution is the same throughout all the cells of the heart. With this reservation in mind, parallels may be drawn between the ultrastructural location of the radioactivity and the biochemical fate of the fatty acid.

(i) Nucleus and sarcoplasmic reticulum

No appreciable radioactivity was found to be associated with the nucleus or sarcoplasmic reticulum at any of the chaser times. It might be expected that there would be some incorporation of radioactive phospholipid into the membranes of these structures particularly after 120 seconds chaser as 24% of the tritiated oleic acid had been incorporated into phospholipid after this time. Furthermore, if, as has been suggested by Stein and Stein (1968), the sarcoplasmic reticulum is the site of esterification of fatty acids, some association of radioactivity with this structure would be expected. Stein and

Stein (1968) found a high degree of radioactivity in the sarcoplasmic reticulum of rat hearts. In the present study the intensity of sources in the sarcoplasmic reticulum remained low throughout. It seems likely that the difference is again methodological. Stein and Stein counted all grains which overlapped the mitochondrion by one third as belonging to the sarcoplasmic reticulum, whether or not they could see sarcoplasmic reticulum. In my analysis of the autoradiographs only separately identified sarcoplasmic reticulum was included in this category. My results therefore, do not bear out the suggestion of Stein and Stein (1968) that fatty acids travel along the longitudinal tubular system of the sarcoplasmic reticulum. The low intensity of sources in the sarcoplasmic reticulum would also appear to be inconsistent with the results of Gloster et al. (1978) who found a high level of radioactivity in the microsomal fraction after similar lengths of perfusion with radioactive oleic acid. However, this can be explained by the fact that electron microscopy revealed that their microsomal fraction, as well as membranes, contained a large number of lipid droplets (P. Harris, personal communication).

(ii) Myofibrils and 'cytoplasm'

Stein and Stein (1968) suggested that the relative lack of grains over myofibrils was also an indication that "fatty acid is channelled intracellularly through selective pathways and not by random diffusion". In the present study, in well-oxygenated conditions, there was no distinction between the intensity of sources in the myofibrils and in the 'cytoplasm', both of which were higher than the sarcoplasmic reticulum. This result is not consistent with the concept of an intracellular channel through the sarcoplasmic reticulum. This observation also suggests that the myofibrils do not present a physical

barrier to fatty acids whether or not they are bound to intracellular protein, even though the fatty acid binding proteins are relatively large molecules.

At 37°C there was some radioactivity in the 'cytoplasm' after all four chaser periods. At 15 and 30 seconds after the pulse perfusion the intensity of sources in the 'cytoplasm' was lower than in both the T-tubules and the mitochondria even though the 'cytoplasm' must be an intermediate site between the two in the transport of fatty acids. In interpreting the relative intensity of sources in the different regions, account must be taken of the fact that there may be some preferential loss of radioactivity during processing for electron microscopy. It is possible that some of the 'cytoplasm' radioactivity was present in acyl-CoA formed by the activation of the free fatty acid on either the sarcoplasmic reticulum or the outer mitochondrial membrane. Much of the acyl-CoA would have been removed during tissue processing. The membranes of the mitochondria may present a physical barrier so that more radioactivity is lost from the cytoplasm than from the mitochondria during processing, resulting in the lower intensity of sources in the 'cytoplasm'. The glutaraldehyde fixation of the albumin to which the fatty acids are bound in the perfusate, may prevent a similar washout of radioactive fatty acid from the T-tubules.

The differences between the relative intensity of sources in the T-tubules, 'cytoplasm' and mitochondria may also be explained in terms of bound and unbound fatty acid molecules. The transfer of fatty acid, for example into the mitochondria, will take place if the concentration of unbound fatty acid molecules in the mitochondria

is lower than that in the cytoplasm. This transport may take place even if the total fatty acid concentration (i.e. both bound and unbound) is high in the mitochondria (giving rise to a higher relative intensity of sources) and low in the cytoplasm.

(iii) Mitochondria

At 37°C there was an association of radioactivity with the mitochondria even after 15 seconds chaser. This indicates that oleic acid is taken up and transported rapidly to the site of its oxidation. Because of the invaginated nature of the inner membrane of the mitochondrion, it was not possible to determine whether the radioactive fatty acid associated with the mitochondria in the autoradiographs had actually crossed the inner membrane. However, the experiments in which $^{14}\text{CO}_2$ was measured after perfusion with ^{14}C oleic acid, showed that considerable oxidation of the fatty acid had occurred. It can therefore be concluded that at least some of the radioactivity found associated with the mitochondria was from free fatty acid molecules which had crossed the inner mitochondrial membrane to the mitochondrial matrix where oxidation takes place.

At 5°C it was assumed that β -oxidation was inhibited. However the transport of fatty acid to the mitochondria still occurred at this temperature, although the peak in the intensity of sources is lower and later than at 37°C. Free fatty acids have been found to bind to mitochondria even at 0°C although oxidation is greatly diminished (Reshef and Shapiro, 1965).

By 120 seconds at 37°C, the intensity of sources in the mitochondria had greatly decreased compared with after 60 seconds. Presumably this would be due to the formation within the mitochondria of water-soluble oxidation products, which would be lost during tissue processing for autoradiography. If this is so, this once again suggests that the radioactive fatty acid has crossed the inner mitochondrial membrane. At 5°C this decrease was not seen as both the movement of fatty acid to the mitochondria and β -oxidation were slowed down.

(iv) T-tubules

At 5°C radioactivity was detected in the T-tubules up to 60 seconds after the radioactive pulse, but at 37°C it was found only up to 30 seconds. It can be assumed that the uptake of free fatty acid is slower at 5°C and so remains in the T-tubules for longer. It might be expected that much of the free fatty acid remaining in the T-tubules would have been washed out by the ten minute fixation perfusion. However, glutaraldehyde fixation of the albumin to which the fatty acid was bound may render this less likely to occur.

(v) Lipid droplets

The appearance of a high intensity of sources in the lipid droplets after 60 and 120 seconds at 37°C reveals the rate of formation of acylglycerols. These findings run parallel with the findings made by the freeze-clamping experiments of an increase of triacylglycerol radioactivity with time. In well-oxygenated conditions the labelling of myocardial triacylglycerol is highest when labelled oleate is used as the precursor when compared with palmitate, linoleate and stearate (Vasdev and Kako, 1977).

At 5°C the freeze-clamping experiments showed that esterification was almost totally inhibited, and, at this temperature, the intensity of radioactive sources in the lipid droplets remained negligible. At 37°C the incorporation of radioactivity into the lipid droplets was slower than the incorporation into the mitochondria suggesting that it is only those fatty acid molecules which are not immediately required for β -oxidation which are incorporated into acylglycerols and stored for later use.

The incorporation of ^3H oleic acid into tissue lipids in hypoxic conditions

The purpose of this part of the study was to monitor the biochemical changes in fatty acid metabolism over the short chaser period studied, namely up to 120 seconds after the radioactive pulse. This was so, that any changes in the anatomical pathway of the fatty acid, as revealed by autoradiography, could be interpreted in terms of the biochemical changes caused by hypoxia.

Throughout this thesis I have compared the results of the hypoxic hearts with those from the well-oxygenated hearts. The perfusates (apart from the gas mixture used for equilibration) and periods of chaser perfusion were identical in the two series. However the experiments on well-oxygenated hearts were originally designed as a study in their own right and not as a control series for the hypoxia experiments. For this reason the total length of perfusion was some thirty minutes less in the well-oxygenated hearts than in the hypoxic hearts. Although this should not greatly affect the overall results, it should be borne in mind as there may be some loss of metabolites, e.g. ATP, due to the increased perfusion time.

The freeze-clamping experiments showed that after 30 minutes hypoxia there was a very large increase in total lipid radioactivity. It was more than three times higher than in the well oxygenated hearts at 37°C after the shorter chaser perfusion times and more than four times higher after the longer times. This is consistent with the findings of other workers (Evans, 1964, Scheuer and Brachfeld, 1969, Gloster and Harris, 1972). This observation reflects the decrease in α -oxidation of the fatty acid due to a reduced supply of oxygen. A similar increase in total lipid radioactivity might be expected in the well-oxygenated hearts perfused at 5°C compared with those perfused at 37°C, caused, not by a reduced oxygen supply, but by the low temperature giving rise to an inhibition of β -oxidation. This was not the case (the amount of total lipid radioactivity was similar at the two temperatures) which suggests either that the uptake of free fatty acid had been increased in hypoxia or greatly decreased at 5°C. The greater free fatty acid radioactivity in the hypoxic hearts, especially after the shorter chaser perfusions, indicated that the uptake of free fatty acid is decreased less by hypoxia (if it is decreased at all under the conditions used) than by the low temperature at 5°C. The decreased uptake at 5°C supports the view of Shohet et al. (1968) and Munday et al. (1969) that fatty acid uptake is an energy-dependent process.

The increase in total lipid radioactivity in hypoxia was also due to an increase in triacylglycerol radioactivity. The increased incorporation of fatty acids into tissue lipids is directly related to the increase in glycolysis which occurs in hypoxia. Glycerol-3-phosphate, which is a precursor of the acylglycerols, is a by-product of glycolysis and is thought to stimulate fatty acid esterification (Eaton and Steinberg, 1961).

Counts in phospholipid seemed to be little affected by the hypoxia. Free fatty acid in hypoxic hearts is more likely to be incorporated into triacylglycerol than into phospholipids because there is a lower requirement for high energy phosphates in the acylglycerol pathway. There may even be a decrease in phospholipid radioactivity in hypoxia if there was an increase in acyl-carnitine, as acyl-carnitine remained with the phospholipids on the thin layer plates, and unfortunately, could not be separated from them.

$^{14}\text{CO}_2$ production in hearts perfused with ^{14}C oleic acid in hypoxic conditions

Again, no conclusions can be drawn as to the proportion of radioactive fatty acid oxidised as opposed to esterified because of the different isotopes used. However the radioactivity in $^{14}\text{CO}_2$ in the hypoxic hearts, although ten times less than that produced by the well-oxygenated hearts, was significant. This shows that β -oxidation was not completely inhibited in the conditions used, but was reduced.

Ultrastructural location of ^3H oleic acid in hypoxic conditions

The relatively short time of perfusion with the hypoxic medium and the presence of both glucose and free fatty acid as substrates for oxidation were not sufficiently severe conditions to give rise to any ultrastructural changes, which was in fact the reason for choosing this length of hypoxic perfusion. The tissue appeared to be structurally well preserved and in most cases glycogen granules could still be seen (see figure , page).

(i) Mitochondria and 'cytoplasm'

The reduction in β -oxidation seen in the $^{14}\text{CO}_2$ experiments is consistent with the decrease in radioactivity associated with the mitochondria in the autoradiographs of the hypoxic hearts. The decline in mitochondrial radioactivity between 60 and 120 seconds is less marked in the hypoxic hearts than in the well-oxygenated hearts, in which β -oxidation will be occurring at a faster rate. The capacity for fatty acid oxidation by the mitochondria is impaired during hypoxia due to a decrease in the activity of the mitochondrial electron transport chain (Kinnula and Hassinen, 1978) and not, as was previously thought, to a decrease in the activity of the fatty acid oxidising enzymes. A proportion of the radioactivity associated with the mitochondria is probably in the form of acyl-CoA, most of which is found in the mitochondrial matrix (Idell - Wenger et al., 1978) although some of the acyl-CoA will have been removed during tissue processing. The tissue levels of both long chain acyl-CoA and long chain acyl-carnitine are known to increase in hypoxia (Whitmer, 1978). The rise in acyl-carnitine is probably the reason for the increase in the intensity of sources in the 'cytoplasm' area which was found to be twice as high in the hypoxic hearts as in the well-oxygenated hearts.

(ii) Lipid droplets

The analysis of autoradiographs of the hypoxic hearts showed a much higher level and a much earlier association of radioactivity with the lipid droplets than in the well-oxygenated hearts. This is consistent with the high level of triacylglycerol radioactivity in the freeze-clamped hearts. The relative area of lipid droplets in the

autoradiographs (and hence their volumes in the cells) would therefore be expected to increase, an occurrence often seen in hypoxic hearts. However, in the conditions used in these experiments, there appeared to be a decrease in the area of micrograph occupied by lipid droplets, although the analysis of variance showed that there was no overall difference between the relative areas in the well-oxygenated and in the hypoxic hearts. The apparent decrease in the lipid droplet area in the hypoxic heart autoradiographs, in spite of the greater incorporation of radioactivity into triacylglycerol, may indicate an increase in the hydrolysis of the endogenous triacylglycerol. It has been shown (Wollenberger and Shahab, 1965, Krause and Wollenberger 1965), that there is an increase in the release of catecholamines from storage sites soon after the onset of hypoxia which is responsible for the early increase in phosphorylase activity and glycolysis in the hypoxic myocardium (Wollenberger et al., 1964). A catecholamine-activated myocardial lipase has been identified (Kruger et al., 1967). It is possible that after longer periods of hypoxic perfusion, or in more severe conditions, such as hypoxia combined with substrate free perfusion, that an increase in lipid droplet volume would be seen. This would be caused by the inhibition of the lipase activity by further increased levels of free fatty acid (Crass, 1972).

The incorporation of ^3H erucic acid into tissue lipids in well-oxygenated conditions

There have been many studies of the effect of rapeseed oil, and, more specifically, of erucic acid on the mammalian myocardium. Most of these studies have involved either the administration to laboratory animals of a diet which is rich in erucic acid or experiments

on the effects of erucic acid on isolated, subcellular organelles. This study has used the isolated, perfused heart model and is a study of the short term incorporation of erucic acid into the cells. Vasdev and Kako (1976) studied the incorporation of erucic acid and palmitic acid in the isolated, perfused rat heart using a similar time sequence. Their experiments differed from mine in that they used a continuous perfusion with the radioactive fatty acid over the period studied. I, on the other hand, have used a 15 second radioactive pulse followed by a chaser period with non-radioactive fatty acid. I have therefore determined the fate of a number of radioactive erucic acid molecules 2 or 30 minutes after their entry into the cells as opposed to the cumulative fate of radioactive molecules entering the cells throughout the experimental period.

Vasdev and Kako (1976) reported a greater incorporation of erucic acid into tissue lipids than with palmitic acid. In my experiments, using freeze-clamping followed by lipid extraction, erucic acid was metabolised more slowly than oleic acid. At the end of two minutes after the radioactive pulse there was no difference in the incorporation of radioactivity into total tissue lipids. There was, however, a great increase in radioactivity in free fatty acid with erucic acid but incorporation into triacylglycerol and phospholipid was less than with oleic acid. The comparable amounts of total lipid radioactivity with the two fatty acids indicates that the uptake of erucic acid by the cells is comparable to the uptake of oleic acid. Gumpen and Norum (1973) suggested that there is an increase in erucic acid uptake in animals fed rapeseed oil and this is consistent with an increased delivery of erucic acid to the heart in serum very low density lipoprotein (Thomassen et al., 1979) and increased myocardial lipoprotein lipase activity (Jansen

et al., 1975, Hüllsmann et al., 1979). However, in these early stages of exposure to erucic acid there is no increase in uptake although clearly the cells do not recognize the cardiotoxicity of erucic acid at the site of uptake, the sarcolemma. Vasdev and Kako, (1976) found that erucic acid and palmitic acid were taken up by the cells at the same rate in the isolated, perfused rat heart.

Very little ^{14}C CO_2 was produced by the hearts perfused with ^{14}C erucic acid, either 2 minutes or 30 minutes after the pulse. This confirms the view that erucic acid is oxidised at a much slower rate than oleic acid.

Ultrastructural location of ^3H erucic acid in well-oxygenated hearts

The relative areas of organelles showed no significant effect of erucic acid. If anything, the volume occupied by lipid droplets was less after a pulse of erucic acid. Such a finding is not necessarily incompatible with the development of an excess of lipid droplets following longer term oral administration.

The incorporation of radioactivity into lipid droplets was clearly very much greater with oleic acid than with erucic acid. This is consistent with the difference which was observed in triacylglycerol radioactivity after two minutes. On the other hand, the intensity of sources of oleic acid in lipid droplets showed a rapid decrease by 30 minutes, by which time there had been a slight increase with erucic acid. This may suggest that the turnover was faster with oleic acid than with erucic acid.

There was clearly no hold-up in the transport of erucic acid to the mitochondria in spite of the low rate of oxidation of this fatty acid. It has been shown that erucic acid is preferentially incorporated into cardiolipin (Christophersen and Christiansen, 1975) and hence into the inner mitochondrial membrane. However, if this were so in these experiments, the radioactivity in the phospholipid fraction of the freeze-clamped hearts would be expected to be high and this was not the case.

The α -oxidation of erucic acid is thought to be limited by the decreased activity of acyl-CoA dehydrogenase in the mitochondria (Christophersen and Christiansen, 1975, Heijenskjöld and Ernster, 1975) and of carnitine acyl-transferase on the inner side of the inner mitochondrial membrane (Christophersen and Bremer, 1972, a,b). The decreased activity of these enzymes would bring about a build-up of either acyl-CoA or acyl-carnitine respectively. With the solvent system used in this study, acyl-CoA migrates with diacylglycerol on thin layer chromatography plates and acyl-carnitine with the phospholipids. However, the radioactivity in neither of these fractions was high enough to account for the high intensity of radioactive sources in the mitochondria.

The very high level of free fatty acid in the freeze-clamped hearts leads me to conclude that much of the radioactivity in the myocardial cell is present as non-esterified erucic acid and that the rate-limiting step in the oxidation pathway is probably the activation of the fatty acid to acyl-CoA. Swarttouw (1974) showed that long-chain acyl-CoA synthetase activates erucic acid slower than palmitic acid but concluded that, as triacylglycerol is known to accumulate in the cells, the activation process cannot be greatly

hampered. However, Lamarchal et al. (1974) postulated that the two acyl-CoA synthetases, one in the sarcoplasmic reticulum and the other in the outer mitochondrial membrane, do not have the same affinity for erucic acid, i.e. erucic acid is less suitable for the mitochondrial synthetase than for the sarcoplasmic reticulum synthetase. This may be so even though my results show more radioactivity associated with mitochondria compared with the sarcoplasmic reticulum after these relatively short perfusion times as this may indicate a bias in transport to the mitochondria rather than a difference in the activities of the two acyl-CoA synthetases. The development of increased numbers of lipid droplets in cardiac cells during feeding with erucic acid may be due to a decrease in lipolytic activity rather than an increase in esterification. This would lead to a slow turnover of erucic acid such as is suggested by the autoradiographic study.

Expulsion of lipid droplets

I do not believe that this phenomenon has been described in the heart before. The mechanism appears to be unusual. Most cells which secrete lipid do so by one of two processes. In sebaceous glands, for example, the cell becomes engorged with lipid droplets and the whole cell is released together with its contents. In the mammary gland, on the other hand, secretory cells package each lipid droplet separately in membrane by a mechanism involving the vesicles of the Golgi apparatus (Wooding, 1977). More recently Wooding (1980) suggested a third process in the Harderian gland which is found in the eye of rodents and lagomorphs in which lipid droplets are released by the "pinching-off" or local rupturing of the cell membrane. It is the last process which seems to be happening in these myocardial cells. The sarcolemma ruptures

and the lipid droplets are ejected without a surrounding membrane into the extracellular space.

My results give no indication of what stimulates the process. At the stage I have studied there is clearly no increase in the numbers or size of the droplets. They appear to migrate to the periphery of the cell before being expelled although it is possible that the droplets are being formed closer to the sarcolemma. The full expulsion is accompanied by a rupture of the cell membrane. Perhaps erucic acid or an erucyl phospholipid alter the physical properties of the plasma membrane or perhaps trierucylglycerol inserts itself more readily than other triacylglycerols into the lipid mosaic of the plasma membrane.

In conclusion

Fatty acids form the major fuel supply of the working myocardium. It is therefore of the utmost importance to understand the way in which fatty acids are taken up and used by the myocardial cells as any alteration in fatty acid metabolism will affect the working of the heart to a greater or lesser extent and, conversely, any other biochemical or physiological change may directly or indirectly affect fatty acid metabolism. I believe that this thesis has contributed a little towards this understanding.

Most methods of processing tissue for electron microscopy involve the use of organic solvents such as ethanol or acetone as dehydrating agents. As these are such good lipid solvents I first assessed some of the available processing methods which have been claimed to have good lipid retaining properties. This led me to choose water-soluble Durcupan for the subsequent studies and to recommend this method to others working in this field.

In the experiments reported here I have traced the pathways of oleic acid through the cells of the perfused heart, in the first two minutes after its uptake. I have established, by freeze-clamping the heart, to what extent the radioactive fatty acid remains unesterified and to what extent it is incorporated into acylglycerols and phospholipids. I have also determined the time-scale of this incorporation. These studies showed that, at 37°C, the majority of the radioactivity was in free fatty acid, 15 seconds after a radioactive pulse. By the end of two minutes,

radioactivity had declined in free fatty acid but had substantially increased in triacylglycerol and phospholipid. At 5°C very little radioactivity became incorporated into esterified lipid demonstrating that the enzymes in these pathways are temperature dependent.

I have also followed the anatomical distribution of radioactivity in the cell following pulse perfusion of tritiated oleic acid. This was achieved using EM autoradiography. Considerable importance was placed on using, and improving, the best available morphometric technique. Although one or two autoradiographs are included in this thesis as examples, the emphasis throughout has been on the numerical data gathered from many hundreds of micrographs.

These two sets of experiments together present a clear picture. The entry of free fatty acids into the myocardial cell is extremely rapid. They move rapidly across the cytoplasm predominately to the mitochondria where they are subsequently oxidised. Esterification into acylglycerols occurs more gradually and persistently.

The working myocardium is highly dependent on a continuous supply of oxygen. I have determined how the above pattern of incorporation of oleic acid can change when the oxygen supply is reduced by subjecting the hearts to a 30 minute hypoxic perfusion before studying the pathways of the radioactive oleic acid. There was a higher intensity of radioactivity in the tissue free fatty acid of hypoxic hearts. The incorporation of radioactivity into acylglycerol was earlier and considerably more intense in hypoxic hearts. Autoradiography showed a decreased incorporation of radioactivity into the mitochondria and a decreased washout of radioactivity from the mitochondria because of the reduction in

oxidation of the fatty acid. Radioactivity was found earlier and more intensely in lipid droplets. These experiments show how rapidly fatty acid metabolism can switch from oxidation to esterification when the cells become hypoxic.

Erucic acid is not one of the fatty acids which is regularly found in mammalian tissues but it still (alarmingly, as it is cardio-toxic) occurs in human foodstuffs particularly in margarines manufactured from rapeseed oil. It is therefore of importance to understand its metabolism so that, hopefully, any detrimental effects may ultimately be counteracted. I have demonstrated the short-term pathways of erucic acid in the perfused myocardium wherein erucic acid is metabolised very much slower than oleic acid. Less oxidation occurs although erucic acid is readily transported to the mitochondria. The rate-limiting step seems to be the activation of the fatty acid to acyl-CoA suggested by the high levels of free fatty acid in the erucic acid perfused hearts. Electron microscopy revealed a widespread process of expulsion of lipid droplets from the cells of the hearts perfused with erucic acid. This novel mechanism appears to be distinct from exocytosis. It was associated with a quantitatively demonstrable general movement or formation of lipid droplets towards the surface of the cells.

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Lipid losses during processing of cardiac muscle for electron microscopy

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SUMMARY

The lipid retaining properties of several methods of processing tissue for electron microscopy (EM) have been assessed quantitatively.

Guinea-pig hearts were perfused *in vitro* at 37°C with ^3H -oleic acid bound to albumin. The hearts were fixed by perfusion with 4% glutaraldehyde in 0.1 M cacodylate buffer. Pieces of left ventricle and interventricular septum were removed, weighed and processed for EM. The fluids used at each stage of processing were monitored for loss of radioactive lipid by scintillation counting. Lipids were extracted from the processed tissue immediately before the embedding stage using a mixture of chloroform:methanol (2:1 v/v). Counts from processed tissue were compared with counts from tissue extracted directly after perfusion fixation in order to monitor subsequent losses during processing.

A modified version of Epon processing, omitting 100% ethanol, acetone or propylene oxide, gave a lipid retention of only 20.6%. The addition of paraphenylenediamine to the procedure did not improve the retention although this has been shown to be a useful stain for intracellular lipid. Water soluble Durcupan which does not involve ethanol or acetone dehydration has an average retention of 63% with 100% recovery while the lesser known polymer GACH, a mixture of glutaraldehyde and carbohydrazide used both for dehydration and embedding showed a lipid retention of 82% of the counts recovered although recovery was only 69%.

An attempt was made to determine which classes of lipids were present in the tissue after perfusion fixation using thin layer chromatography. It was found that the presence of any of the processing fluids affected the polarity of the lipids and their rates of migration on thin layer plates.

INTRODUCTION

The processing of biological material for EM generally involves dehydrating the fixed tissue with organic solvents before infiltrating with embedding media. Solutions containing increasing concentrations of ethanol followed by acetone or propylene oxide are frequently used for this purpose. These substances are all excellent lipid solvents, so some lipids are almost certain to be removed from the tissue at this stage (Ashworth *et al.*, 1966; Carlier *et al.*, 1968; Cope & Williams, 1968). Although this may not always matter to the morphologist, it can present problems when the intracellular localization of lipids is to be studied by such techniques as EM autoradiography.

Aldehydes, such as glutaraldehyde, are efficient cross-linking agents for proteins but seem to have little effect on most lipids (Carlier *et al.*, 1968; Idelman, 1965). As much as 95% of total lipid can be lost during ethanolic dehydration following fixation with glutaraldehyde alone (Korn & Weisman, 1966). The use of a double fixation procedure in which glutaraldehyde is followed by osmium tetroxide markedly increases the amount of lipid retained by the tissue. Osmium tetroxide reacts with the double bonds in the lipid (Bahr, 1955) resulting in the formation of a glycol. Almost complete fixation of phospholipids can be achieved using osmium tetroxide which reduces the subsequent solubility in ethanol and in epoxy resin (Korn & Weisman, 1966; Cope & Williams, 1969a, b). The retention of phospholipid is not improved further by preceding the osmium treatment by aldehyde fixation.

Osmium tetroxide may also reduce the subsequent solubility of neutral lipids although these present a greater problem of preservation. The formation of glycols alters the properties of the neutral lipids so they appear more polar on silicic acid chromatography, less soluble in ethanol and more soluble in methacrylate and Araldite (Cope & Williams, 1968).

Neutral lipid droplets however, are not completely penetrated even by prolonged treatment with osmium tetroxide (Bahr, 1955) so that unfixed lipid at the centre of the droplet may still be removed by dehydration in ethanol.

Several authors have assessed the 'lipid-retaining' properties of some of the more widely used embedding media. As with fixation procedures the main problem has been with the preservation of neutral lipids. Ashworth *et al.* (1966) reported a 92.8% retention of neutral lipid in rat liver using Epon embedding, but these results were not supported by the observations of Korn & Weisman (1966) or Cope & Williams (1968) and to date this is the only reported study in which such a high preservation of neutral lipid has been found. None of the embedding procedures described has resulted in the total quantitative preservation of neutral lipid and special processing schedules have been suggested to overcome this problem. Procedures using ethanolic dehydration seem generally best avoided if lipids are to be examined although Idelman (1965) suggested the use of Epon embedding omitting both the 100% ethanol and propylene oxide stage. Stein & Stein (1968) reported a 78% retention of total lipid using this method.

Polymers of glutaraldehyde and urea mixtures were described by Pease & Peterson (1972) and Peterson & Pease (1972) in the context of lipid retention and this method was taken a step further by Heckman & Barnett (1973) who described a 92% retention of [1-¹⁴C] palmitic acid in rat liver using a solution of carbohydrazide in glutaraldehyde (GACH) for both dehydration and embedding.

The water soluble methacrylates have been found to be of little use in neutral lipid retention even at -20°C (Cope & Williams, 1968; 1969b).

Gibbons (1959) described the separation of 'Aquan', the water soluble phase of Epon resin. Dilutions of this can be used for dehydration and the undiluted product for embedding. The separation of 'Aquan' from Epon is a complicated and lengthy procedure but a similar product, described by Stäubli (1960), later became commercially available. This is the water soluble Durcupan. Casley-Smith's (1962) observations on the EM appearance of chylomicra and lipoproteins suggested that water soluble Durcupan might be of value for the retention of neutral lipid in tissues.

The purpose of the present investigation was to determine which of the methods described could be applied to cardiac muscle. A comparison was made of some of the above methods using the isolated guinea-pig heart which was pulse perfused with [9,10-³H]-oleic acid. The lipid retaining properties of para-phenylenediamine

were also investigated. This is a stain which is used to enhance osmium tetroxide reduction (Ledingham & Simpson, 1972).

MATERIALS AND METHODS

Animals

Male Dunkin-Hartley guinea-pigs were used in these studies. The animals were allowed free access to both food and water prior to the experiments.

Labelled substrate

[9,10-³H] oleic acid with a specific activity of 2.4 Ci per mmol was obtained from the Radiochemical Centre, Amersham, England. It was used at a final activity of 100 μ Ci per ml of perfusion fluid.

The radiochemical purity was checked by thin-layer chromatography when 96% of the counts were associated with the free fatty acid band on the thin-layer plate.

Substrates for perfusion

The oleic acid used was bound to bovine serum albumin by the method of Fredrickson & Gordon (1958). Both unlabelled and radioactive perfusion medium contained 0.2 mM oleic acid complexed to 0.2 mM albumin in Krebs bicarbonate buffer (pH 7.4) containing 5 mM glucose.

Perfusion

The guinea-pigs were killed by cervical dislocation. The hearts were rapidly removed and placed in ice-cold Krebs bicarbonate solution. A cannula was inserted into the aorta and the heart perfused by the Langendorff technique in a non-circulatory system maintained at a pressure of 60 mmHg. The fluids used for perfusion were equilibrated with 95% O₂:5% CO₂ and maintained at 37°C.

All studies commenced with a period of perfusion with unlabelled 0.2 mM oleate of 5 min duration. This was followed by a 15 s pulse perfusion with 2.5 ml of a solution of 0.2 mM [9,10-³H]-oleate (100 μ Ci/ml) which was contained in a syringe attached via a three-way tap to the aortic cannula. At the end of the experiment ice-cold Krebs bicarbonate buffer from a second syringe was passed through the heart for 15 s to remove radioactive material from the vascular space.

Fixation

Immediately after the washout with Krebs bicarbonate buffer the heart was perfused with 4% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.3) (both EMScope Laboratories, London) for 10 min at room temperature.

In one experiment the glutaraldehyde solution perfusing through the heart was collected over 1 min intervals and aliquots were then taken for scintillation counting.

After fixation, pieces of tissue each of approximately 20 mg were removed and weighed. Two samples were taken from the left ventricular wall and two from the interventricular septum in each heart. One piece of muscle from each site was extracted directly with chloroform:methanol (2:1 v/v) for lipid analysis according to the method of Folch *et al.* (1957). The second sample from each site was cut into approximately 1 mm cubes with a razor blade and these segments were then processed for EM using one of the experimental procedures described below.

Embedding procedures

The fluids used at each stage of processing were monitored for loss of lipid from the tissue by scintillation counting.

The buffer used throughout was 0.1 M sodium cacodylate pH 7.3 and all processing was carried out at 4°C. In all cases the initial stages of processing were identical and consisted of a further fixation by immersion in 4% buffered glutaraldehyde (1–2 h), buffer wash (10 min), buffered 1% osmium tetroxide (EMscope Laboratories) (2–3 h) and a final buffer wash (10 min).

After this fixation stage various embedding procedures were carried out as given below.

Epon. Ten samples from five hearts were processed for embedding in Epon. Processing consisted of 70% ethanol 1 (5 min), 70% ethanol 2 (5 min), 95% ethanol 1 (5 min), 95% ethanol 2 (5 min), Epon resin alone (1 h) and Epon full mix without accelerator (overnight).

Epon full mix consisted of 2.25 ml Epon 812:1.0 ml DDSA (dodecenyl-succinic anhydride):1.75 ml MNA (methyl nadic anhydride) (all from EMScope Laboratories).

Para-phenylenediamine. Eight tissue samples from four hearts were used in this experiment. Processing was as for Epon with the addition of 30 min in a 1% solution of para-phenylenediamine (Sigma) in 70% ethanol preceding the 70% ethanol stages.

GACH (Glutaraldehyde/carbohydrazide). Twenty tissue samples from ten hearts were used for GACH processing. Processing consisted of 20% GACH (2–4 h), 50% GACH (2–4 h), 80% GACH (overnight) and 100% GACH (4–6 h). The 100% GACH contained 150 mg carbohydrazide (Eastman Kodak) per ml of 50% glutaraldehyde.

Water soluble Durcupan

Sixteen samples from eight hearts were processed with Durcupan. Durcupan is supplied as four separate components: A, B, C and D. Processing consisted of 70% Durcupan A (30 min), 90% Durcupan A (30 min), 100% Durcupan A1 (45 min), 100% Durcupan A2 (45 min) and Durcupan full mix (overnight). Durcupan full mix consisted of 5 ml Durcupan A:13 ml Durcupan B:1 ml Durcupan C:0.2 ml Durcupan D. All components were supplied by EMScope Laboratories.

Lipid extraction

Lipids were extracted from processed tissue and from tissue obtained directly after glutaraldehyde fixation. In both cases the tissue was chopped finely and extracted with twenty volumes of a mixture of chloroform:methanol (2:1 v/v) according to the method of Folch *et al.* (1957). The extraction mixture was allowed to stand at room temperature for 16 h with occasional shaking. After filtration, one-fifth of the volume of 0.1 M NaCl was added and the mixture kept overnight at 4°C to allow the phases to separate. Aliquots of the chloroform phase were taken to dryness directly in the scintillation vial. After the addition of 10 ml of a dioxane-based scintillant as described by Bray (1960) to each vial, radioactivity was determined in a Packard Tricarb liquid scintillation spectrometer. Quenching was corrected by the external standard method.

Thin layer chromatography

Glutaraldehyde fixed tissue. Four guinea-pig hearts were perfused for 15 s with 2.5 ml of 0.2 mM [9,10-³H]-oleate (50 µCi/ml) and fixed by perfusion with glutaraldehyde as previously described. The heart muscle was then chopped finely and the lipids extracted with chloroform:methanol (2:1 v/v) for 16 h at room temperature. The phases were separated by the addition of 20% by volume of 0.1 M NaCl. Aliquots of the upper aqueous methanolic phase were taken for scintillation count-

ing. The lower chloroform phase was taken to dryness in a rotary evaporator and the residue immediately redissolved in 2.0 ml chloroform. Duplicate 30 μ l aliquots of each sample were applied to a 0.25 mm thick layer of Merck silica gel H on a 200 $\times$ 200 mm glass plate, which had been activated for 1 h at 110°C before use. Known standard mixtures were also run on each plate to aid identification. The solvent mixture used for separation was light petroleum ether (40–60°C):diethyl ether:glacial acetic acid (85:15:2 by volume). In this system the neutral lipids separate into their main components while the phospholipids remain at the origin. The zones corresponding to the lipid classes were located by iodine vapour, and each band was scraped off into scintillation vials. Ten millilitres of Bray's solution was added to each vial and the activity determined.

Processing fluids from Durcupan embedding procedure. A further four guinea-pig hearts were perfused with 2.5 ml of 0.2 mM [9,10-³H]-oleate (100 μ Ci/ml) for 15 s and fixed by a 10 min period of perfusion with 4% glutaraldehyde. The left ventricle of each heart was then cut into 1 mm cubes and processed according to the Durcupan schedule. After each stage a 2 ml aliquot of the processing fluid was added to 20 ml chloroform:methanol (2:1 v/v) to extract any lipids removed from the tissue during the procedure. The processed tissue itself was also extracted with chloroform:methanol (2:1 v/v). To facilitate analysis the glutaraldehyde fixative and its buffer wash were combined and analysed together as were the osmium tetroxide and its buffer wash. After 2–3 h at room temperature one fifth of the volume of 0.1 M NaCl was added to the chloroform and methanol mixture and the phases were allowed to separate. The lower chloroform phase was taken to dryness using a rotary evaporator. In the case of Durcupan A and the full mix samples a viscous residue remained after evaporation of the chloroform. The samples were then redissolved in 2.0 ml chloroform and aliquots taken for thin-layer chromatographical separation using the solvent mixture light petroleum ether:diethyl ether:glacial acetic acid (85:15:2 by volume).

RESULTS

The loss of radioactive material from the tissue was monitored by measuring the radioactivity present in the processing fluids at each stage of preparation in the different techniques studied. Measurements were also made of the radioactivity present in the muscle tissue immediately after perfusion fixation and the radioactivity remaining in the tissue after all the processes had been completed. The results in each case are expressed as percentages of the total initial radioactivity in the tissue. This was determined from those pieces of tissue which were extracted directly after perfusion fixation.

Epon

The results from Epon processing are given in Table 1. Although the dehydration process was considerably shortened, 33.7% of the initial labelled lipid was extracted from the tissue in 70% and 95% ethanol. 9.8% was lost in the Epon resin and full mix stages together and only 20.6% of the initial activity was extracted from the tissue after processing. The overall recovery of counts from processed tissue was 73.9% using chloroform:methanol extraction.

Para-phenylenediamine

It was not possible to monitor losses of radioactivity using this method as para-phenylenediamine produces a dark orange solution which was not affected by treatment with hydrogen peroxide. The degree of quenching was so great that quench correction became inaccurate. However, it was still possible to compare

Table 1. Lipid losses with Epon processing

Stage of processing	Initial radioactivity (%)
Glutaraldehyde	3.4 ± 1.3
Buffer 1	2.6 ± 0.7
Osmium tetroxide	0.7 ± 0.6
Buffer 2	1.8 ± 1.6
70% ethanol 1	7.5 ± 3.9
70% ethanol 2	7.5 ± 3.8
95% ethanol 1	9.2 ± 4.6
95% ethanol 2	9.5 ± 5.3
Epon resin	5.4 ± 2.4
Epon full mix	4.4 ± 2.3
Total processing fluid	53.3 ± 11.9
Tissue	20.6 ± 8.1
Total recovery	73.9 ± 19.6

counts extracted from the tissue after processing with those extracted after fixation only. Only 17.3% of the radioactivity originally present in the tissue could be extracted from it after processing.

GACH

The results from GACH processing are given in Table 2. Only 12.1% of the lipid radioactivity originally present in the tissue appeared in the processing fluids. However, the radioactivity extracted from the muscle after processing amounts only to 56.5% of the initial lipid radioactivity. Thus the total recovery of counts was 68.6%.

Water soluble Durcupan

The results from Durcupan processing are given in Table 3. With this method, although there was a 34.1% loss of label during processing, 65.9% remained in the tissue. Total recovery of counts was 100%.

Wash-out of lipid radioactivity during perfusion fixation

Following a 15 s rinse perfusion with cold Krebs bicarbonate buffer, perfusion fixation with glutaraldehyde was carried out for 10 min. The perfusion effluent was collected each minute during this period and lipid radioactivity extracted with chloroform:methanol. The results are shown in Table 4. Eighty-three per cent of counts from the glutaraldehyde perfusion were recovered during the first 3 min of the 10 min fixation period, and 94% in the first 5 min.

Thin-layer chromatography

Glutaraldehyde fixed tissue. The degree of esterification of the [9,10-³H]-oleate used for perfusion of the guinea-pig hearts was determined by extracting the lipids from glutaraldehyde fixed muscle and separating the main classes by thin-layer

Table 2. Lipid losses with GACH processing

Stage of processing	Initial radioactivity (%)
Glutaraldehyde	2.6 ± 1.8
Buffer 1	1.3 ± 0.6
Osmium tetroxide	0.2 ± 0.1
Buffer 2	0.3 ± 0.2
20% GACH	1.3 ± 0.7
50% GACH	2.8 ± 1.9
80% GACH	2.3 ± 1.3
100% GACH	1.3 ± 0.5
Total processing fluid	12.1 ± 2.8
Tissue	56.5 ± 13.6
Total recovery	68.6 ± 12.7

Lipid losses during EM processing

Table 3. Lipid losses with Durcupan processing

Stage of processing	Initial radioactivity (%)
Glutaraldehyde	4.2 ± 1.5
Buffer 1	1.5 ± 0.3
Osmium tetroxide	0.5 ± 0.4
Buffer 2	0.6 ± 0.5
70% Durcupan A	12.6 ± 6.7
90% Durcupan A	8.2 ± 2.4
100% Durcupan A1	4.3 ± 3.0
100% Durcupan A2	1.8 ± 0.5
Durcupan full mix	0.4 ± 0.1
Total processing fluid	34.1 ± 8.6
Tissue	65.9 ± 14.3
Total recovery	100 ± 5.4

chromatography. In all cases there were several additional bands present which did not correspond to the lipid standards. A sample of glutaraldehyde which had been extracted and chromatographed under similar conditions showed similar bands. It appears that the glutaraldehyde used for fixing the tissue is extracted into the chloroform phase. The plates were scraped according to the position occupied by lipid standards without reference to the extra bands caused by the glutaraldehyde. After a 15 s pulse perfusion with [9,10- ^3H]-oleic acid followed by 15 s washout with cold Krebs and 10 min perfusion with 4% glutaraldehyde, an average of 50.7% of the lipid radioactivity was present as free fatty acid, 16.7% as triglyceride, 21.5% as diglyceride and 7.3% as phospholipid. However, this may not represent the true distribution of radioactivity in the fixed tissue as demonstrated later. Counts in the aqueous phase were not significantly higher than background.

Processing fluids from Durcupan embedding procedure. The processing fluids and processed tissue after the Durcupan technique were extracted with chloroform:methanol (2:1 v/v) and the material partitioning into the chloroform phase was separated by thin-layer chromatography. In this way it was hoped to identify the classes of lipids lost from the fixed tissue at each stage. On thin-layer chromatography however the position of the neutral lipid zones was obscured by wide bands visible after exposure to iodine vapour. Because of the difficulties in identifying these bands, the material on the plate was scraped according to the positions of known standards run simultaneously. In all the samples, except those obtained from glutaraldehyde more than 75% of the radioactivity occurred in the bottom part of the plate. The most likely explanation being that the polarity of the lipids and their rates of migration had been modified during processing. This view was confirmed by the results of an experiment in which 1 ml of glutaraldehyde, osmium tetroxide, 100% Durcupan A or Durcupan full mix were added to 10 mg oleic acid containing trace amounts of [1- ^{14}C]-oleic acid and 10 mg glycerol trioleate

Table 4. Washout of lipid radioactivity during perfusion fixation

Time (min)	dpm	(%)
0-1	116,838	43.9
1-2	80,537	30.2
2-3	24,430	9.2
3-4	13,476	5.1
4-5	13,983	5.2
5-6	6,471	2.4
6-7	3,581	1.3
7-8	2,491	0.9
8-9	1,383	0.5
9-10	2,990	1.1

Table 5. Thin layer chromatography of processing fluids with radioactive lipid standards

Sample	Zone	Counts with free fatty acid	Counts with triglyceride
		(%)	(%)
Lipid standard alone	'TG'	1.3	97.1
	'FFA'	96.8	1.9
	'Origin'	1.9	1.0
Glutaraldehyde	'TG'	7.1	86.0
	'FFA'	78.6	7.7
	'Origin'	14.3	6.3
Osmium tetroxide	'TG'	3.1	4.0
	'FFA'	9.2	23.2
	'Origin'	87.7	72.8
Durcupan A	'TG'	6.7	86.2
	'FFA'	55.3	9.5
	'Origin'	38.0	4.3
Durcupan full mix	'TG'	9.0	13.8
	'FFA'	48.2	51.0
	'Origin'	42.8	35.2

Note: In this experiment 'TG' includes all the material scraped from the top third of the thin-layer plate which includes the zone corresponding to the triglyceride standard. Similarly 'FFA' represents the central third of the plate including the free fatty acid band and 'origin' includes the material scraped from the lower third of the plate.

containing trace amounts of [^{14}C] glycerol trioleate. These samples were then extracted with chloroform:methanol (2:1 v/v) and the material partitioning into the chloroform phase after the addition of one-fifth of the volume of 0.1 M NaCl was separated on Silica gel H plates using the solvent mixture light petroleum ether (40–60°C):diethyl ether:glacial acetic acid (85:15:2 by volume). The radiochemical purity of the oleic acid and glycerol trioleate was shown to be better than 96% in both cases. However, after glutaraldehyde treatment seven distinct bands of material were seen on the thin layer plates after they had been exposed to iodine vapour. Only 78.6% and 86.0% of the counts were associated with the fatty acid or triglyceride bands respectively. After treatment with osmium tetroxide radioactivity from both oleic acid and triglyceride were found as broad zones just above the origin.

A similar effect was observed with 100% Durcupan A with free fatty acid and with Durcupan full mix where again most of the radioactivity occurred towards the bottom of the plate and not in the region corresponding to the oleic acid and glycerol trioleate standards. These results are shown in Table 5.

DISCUSSION

A radioactive label was introduced into the lipids of the guinea-pig heart by a brief period of perfusion with [$9,10\text{-}^3\text{H}$] -oleate. Measurements were made of the radioactivity removed during the washout with cold Krebs solution and during perfusion fixation with glutaraldehyde. The results indicated the 15 s washout with Krebs solution was not long enough to remove all the labelled material from the vascular tree and extracellular space and that some continues to be removed in the first 5 min of the glutaraldehyde perfusion. After the full 10 min perfusion it can be assumed that virtually all the labelled lipid present will be intracellular, although some exchange with the extracellular space will occur. The loss of counts occurring during processing for EM would therefore be due to loss of intracellular lipid.

The rate of esterification of free fatty acids, particularly of oleic acid, is very rapid (Fredrickson & Gordon, 1958; Baker & Schotz, 1967; Eaton *et al.*, 1969). To determine the distribution of label in the main lipid classes at the beginning of processing the counts associated with these classes were examined by thin layer

chromatography. After perfusion fixation with glutaraldehyde about half of the total radioactivity extracted from the tissue was associated with the free fatty acid band on the thin layer plate. It was found, however, that treatment with glutaraldehyde affected the migration of the lipids on silica gel.

Similar problems in identifying the lipid classes removed from the tissue by the various processing fluids were also encountered. This was particularly evident in all the stages following treatment with osmium tetroxide. These difficulties were well demonstrated when radioactive oleic acid and glycerol trioleate of high purity were treated with glutaraldehyde, osmium tetroxide and Durcupan. In addition to a number of unidentified bands occurring on thin layer chromatographical separation after treatment, the distribution of radioactivity was significantly altered. This indicates the inherent error in the method used by some authors of dividing thin layer plates according to standards where definite bands cannot be identified.

As a result of these findings the loss of activity reported in the procedures examined is taken as that which partitions into the chloroform phase during lipid extraction. This material is described as total lipid. No appreciable radioactivity could be detected originating from the water-soluble derivatives of fatty acid metabolism. In all our experiments we observed a small loss of radioactive lipid during fixation in glutaraldehyde amounting to an average loss of 3.4% of the total lipid present. Loss in osmium tetroxide and its subsequent buffer wash were even smaller.

During the shortened ethanolic dehydration used in the Epon schedule (Idelman, 1965) 33.7% of the total label recovered was extracted from the tissue in ethanol even though the absolute alcohol stage was omitted. This confirms the unsuitability of ethanolic dehydration in cytochemical studies of lipids by EM.

It is interesting to note the inability of para-phenylenediamine to fix lipids. This substance does, however, have advantages as a stain as it enhances osmium tetroxide reduction and eliminates the necessity of staining sections in order to improve contrast. The retention of 17.3% obtained using para-phenylenediamine was slightly lower than the 20.6% retention obtained using the Epon schedule. This is probably due to the increased time in which the tissue was in 70% ethanol.

Clearly, those methods of processing which offer the greatest scope to electron microscopists interested in lipids are those which use water-miscible components. The dehydration which occurs during immersion of the tissue in increasing concentrations of the embedding medium is generally sufficient so that the use of organic solvents for dehydration is avoided.

The highest retention was found using GACH when 82% of counts recovered remained in the tissue after processing. Recovery of counts, however, was low when the lipids were extracted in chloroform:methanol. In a similar experiment when the tissue was completely solubilized in Soluene (Packard) after processing the recovery of counts was considerably higher. The low recovery with chloroform:methanol is caused by lipid bound in the tissue by the presence of GACH prepolymer so that it is not extracted by the chloroform:methanol mixture. A similar result is found with Epon where again recovery of counts was greatly improved by dissolving the tissue in Soluene.

Second to GACH in terms of total lipid retention is water-soluble Durcupan with a retention of 65.9%. Recovery of counts was 100% using chloroform:methanol. In our laboratory we have favoured Durcupan processing as it has several advantages over GACH when considering ease of handling. The polymerized GACH resin is extremely brittle, making it difficult to section. The addition of plasticizers, such as dibutylphthalate renders the resin more likely

to extract lipid from the tissue (Heckman & Barnett, 1973). The sectioning problems are particularly relevant when working with muscle as this is a tissue which is very difficult to infiltrate. GACH may be of value to workers using cell suspensions, cell fractions or tissues which are more readily infiltrated. A possible disadvantage with GACH is that the tissue must be embedded in tiny drops of the resin so that polymerization can occur. When hardened these droplets must then be cemented on to blank blocks before they can be sectioned. Water soluble Durcupan is considerably easier to prepare than GACH and the tissue can be embedded directly into capsules which are the correct size for sectioning.

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THE INCORPORATION AND DISTRIBUTION OF ^3H OLEIC ACID IN THE ISOLATED PERFUSED GUINEA-PIG HEART: A BIOCHEMICAL AND EM AUTORADIOGRAPHIC STUDY

ABSTRACT. The incorporation of ^3H oleic acid into tissue lipids of guinea pig heart was studied after 15, 30, 60 or 120 sec perfusion using EM autoradiography with 'hypothetical grain' analysis and lipid analysis by thin-layer chromatography. Radioactivity in triacylglycerol and phospholipid increased and in free fatty acid decreased with time. This corresponded to an increase in radioactivity associated with lipid droplets in the autoradiographs. High levels of radioactivity were found associated with the mitochondria after only 15 sec. The movement of fatty acids is interpreted in terms of transport mechanisms, concentration gradients and bound and unbound molecules.

Summary

THE incorporation of ^3H oleic acid into tissue lipids of the isolated perfused guinea-pig heart was studied after 15, 30, 60 or 120 sec perfusion using freeze-clamping followed by lipid extraction and separation by thin-layer chromatography. A parallel study using EM autoradiography with the 'hypothetical grain' method of analysis showed the location within the cell of the radioactivity after the same lengths of perfusion. Perfusions were carried out at 37°C and repeated at 5°C when esterification and oxidation were inhibited.

The freeze-clamping experiments showed that at 37°C there was an increase of radioactivity in triacylglycerol and phospholipid with time and a decrease in free fatty acid with time. This corresponded to a time-related increase in radioactivity associated with the lipid droplets in the autoradiographs. High levels of radioactivity were found in association with the mitochondria even after

a 15 sec washout. The movement of radioactive fatty acid from a region of low radioactivity to a region of high radioactivity is interpreted in terms of specific transport mechanisms into the mitochondria, selective removal of water-soluble products during EM processing and concentrations of bound and unbound molecules within different cellular compartments. The results confirm the rapid entry of radioactive fatty acid into the mitochondria but give no evidence that there is a specific anatomical pathway through the sarcoplasmic reticulum.

At 5°C radioactivity in the mitochondria was considerably lower and none was found in association with the lipid droplets. This corresponded to a low incorporation of labelled fatty acid into triacylglycerol.

Introduction

Free fatty acid, taken up from the blood stream, follows a variety of biochemical and anatomical pathways within the myocardial cell. The present study has been designed to examine some of these pathways in the isolated heart perfused with tritiated oleic acid.

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The study is divided into two parts. In the first part the hearts have been freeze-clamped after varying periods of perfusion and the distribution of radioactivity within lipid classes determined. In the second part of the study, the spatial distribution of radioactivity within the myocardial cell has been examined by electron microscopy with autoradiography of the hearts after the same periods of perfusion as those used for freeze-clamping.

During the processing of tissue for electron microscopy the great majority of water-soluble products of the metabolism of free fatty acids are removed, but the method used (Stäubli, 1960) permits the retention of the majority of the water-insoluble lipid. Each of the two parts of the study is, therefore, concerned with the water-insoluble products of lipid metabolism: one part with the biochemical pathways, and the other part with the micro-anatomical pathways within the cell. In this way the results of the two parts of the study have been designed to complement each other.

In each part of the study, perfusions were carried out at 37°C and 5°C in order to distinguish between energy-dependent and energy-independent processes.

Materials and Methods

Perfusion procedure

Male Dunkin Hartley guinea-pigs of approximately 300 g body weight were used. The hearts were perfused using a non-recirculatory Langendorff method at a perfusion pressure of 60 mmHg.

The perfusion media, both unlabelled and radioactive, consisted of Krebs Henseleit bicarbonate buffer (pH 7.4) containing 5 mM glucose and 0.2 mM oleic acid complexed to 0.2 mM bovine serum albumin by the method of Fredrikson and Gordon (1958). The radioactive substrate contained 100 μ Ci/ml [9,10-³H] oleic acid (Radiochemical Centre, Amersham, England). The radiochemical purity was found to be 96% by thin-layer chromatography. The fluids for perfusion were equilibrated with 95% O₂:5% CO₂ for at least 20 min prior to use.

Perfusion was carried out at 37°C or at 5°C. The perfusion procedure consisted of an initial period of 5 min perfusion with unlabelled medium followed by a 15 sec pulse perfusion

with the radioactive solution which was contained in a syringe and attached to the aortic cannula via a three-way tap. The pulse perfusion was followed by an immediate return to perfusion with the unlabelled substrate for a period of 15, 30, 60 or 120 sec.

Freeze-clamping

The hearts were frozen between two aluminium blocks precooled in liquid nitrogen (Wollenberger *et al.*, 1960). Four guinea-pig hearts were used for each of the perfusion times at each of the perfusion temperatures. The lipids were extracted from the frozen heart tissue by adding 7 ml of methanol and macerating with a glass rod. Chloroform (14 ml) was then added to make a 2:1 chloroform:methanol mixture. The tissue was left overnight in chloroform:methanol at room temperature. After filtering the extract, 4 ml of 0.1 M NaCl was added to each sample and the mixture was left overnight at 4°C to allow the phases to separate (Folch, *et al.*, 1957). The lipid-containing chloroform phase was taken to dryness using a rotary evaporator and redissolved in 2.0 ml chloroform. Duplicate 0.1 ml aliquots were taken to dryness to determine the total lipid content by weight of each sample.

Thin-layer chromatography

Samples of each lipid extract (50 μ l) were applied to a 0.25 mm thick layer of Merck silica gel H. The solvent mixture was light petroleum ether (40–60°C):diethyl ether:glacial acetic acid in the ratio 85:15:2 by volume. Using this system the neutral lipids separate into their main classes while the phospholipids remain at the origin. The lipid zones were located with iodine vapour, which was allowed to sublime before the bands were scraped into scintillation vials. Dioxane-based scintillant (10 ml) (Bray, 1960) was added to each vial and radioactivity was determined using a Packard Tricarb liquid scintillation spectrometer. Quenching was corrected using the external standard method.

When [1-¹⁴C]palmitoyl-CoA and [1-¹⁴C]-palmitoylcarnitine were extracted with chloroform:methanol as above, only 33% of the palmitoyl-CoA was recovered in the lipid-containing chloroform phase, while 97% of the palmitoylcarnitine partitioned into the

Table 1. Distribution of radioactivity in myocardial total lipid, free fatty acid, triacylglycerol and phospholipid after a 15 sec perfusion with ^3H oleate at 37°C *

Time of unlabelled chaser (sec)	Radioactivity dpm/ μg total lipid			
	Total lipid	FFA	Triacylglycerol	Phospholipid
15	141.2 $\pm$ 56.5	122.7 $\pm$ 46.3	4.9 $\pm$ 2.0	6.4 $\pm$ 4.2
30	86.3 $\pm$ 19.9	56.1 $\pm$ 12.5	9.6 $\pm$ 2.1	8.4 $\pm$ 1.4
60	74.4 $\pm$ 30.9	31.3 $\pm$ 8.6	16.7 $\pm$ 9.1	11.4 $\pm$ 7.4
120	40.3 $\pm$ 12.9	10.9 $\pm$ 3.0	13.2 $\pm$ 2.2	10.4 $\pm$ 3.5

* Followed by periods of chaser perfusion with unlabelled oleate.

Each figure is the mean $\pm$ s.d. of four observations each representing a separate heart.

chloroform phase. When applied to the above thin-layer chromatographic system, acyl-CoA migrated with the monoacylglycerol and diacylglycerol fractions, while acylcarnitine stayed at the origin with phospholipid.

In order to determine the degree to which radioactivity from acylcarnitine might influence the radioactivity found in phospholipid, the individual phospholipids were separated by thin layer chromatography using chloroform : methanol : acetic acid : water (65:45:1:4 by volume) in the lipid extract from heart muscle after 120 sec chaser perfusion at 37°C . Only 1.8% of the total lipid radioactivity was found in the region of phosphatidylcholine and sphingomyelin which is the region to which acylcarnitine migrates in this system. Since, as will be seen from Tables 1 and 2, this experimental condition was the one in which phospholipid activity was maximal, it would seem that the contribution of acylcarnitine is relatively small and the data for phospholipid are, therefore, presented.

We have been unable to separate the contribution of acyl CoA to the radioactivity of mono- and diacylglycerol in a similar way and these data are, therefore, not presented.

Tissue processing for electron microscopy

The animals, substrates and perfusion procedure were as described in the freeze-clamping experiments. After the chaser perfusion of 15, 30, 60 or 120 sec, the hearts were fixed immediately by perfusion with 4% glutaraldehyde in 0.1 M sodium cacodylate pH 7.3 at 5°C for 10 min. Four guinea-pig hearts were used for each perfusion time at each temperature and one non-radioactive control heart was perfused with each group.

After fixation, pieces of tissue were taken from the free wall of the left ventricle of each heart. These were cut into pieces of approximately 1 mm³ and processed for electron microscopy using water soluble Durcupan (EMscope Laboratories) which has been found to possess good lipid-retaining properties (Ward and Gloster, 1976). The tissue was stained *en bloc* with 1% para-phenylene-

Table 2. Distribution of radioactivity in myocardial total lipid, free fatty acid, triacylglycerol and phospholipid after a 15 sec perfusion with ^3H oleate at 5°C *

Time of unlabelled chaser (sec)	Radioactivity dpm/ μg total lipid			
	Total lipid	FFA	Triacylglycerol	Phospholipid
15	162.9 $\pm$ 33.1	145.2 $\pm$ 25.2	7.0 $\pm$ 2.0	5.3 $\pm$ 1.9
30	84.6 $\pm$ 36.8	76.6 $\pm$ 15.9	1.5 $\pm$ 0.5	2.1 $\pm$ 0.7
60	73.3 $\pm$ 17.4	62.4 $\pm$ 14.0	2.4 $\pm$ 0.3	4.8 $\pm$ 1.6
120	74.3 $\pm$ 32.9	64.7 $\pm$ 28.4	3.8 $\pm$ 2.0	3.1 $\pm$ 1.1

* Followed by periods of chaser perfusion with unlabelled oleate.

Each figure is the mean $\pm$ s.d. of four observations each representing a separate heart.

diamine (Sigma) in 0.1 M sodium cacodylate pH 7.3 immediately prior to dehydration (Ledingham and Simpson, 1972).

Sections

Ultrathin sections of pale gold interference colour were cut and transferred to 300-mesh nickel or gold grids which had been previously coated with a celloidin support film. A thin layer of carbon (just visible on filter paper) was evaporated on to the sections to minimize positive chemography.

Emulsifying

Thin sections were emulsioned for autoradiography by a modified version of the loop technique (Caro *et al.*, 1962). The grids were mounted on glass microscope slides and held in place by strips of double-sided tape so that only the edges of the grids were in contact with the tape. Ilford L4 emulsion, diluted 1 + 2 with distilled water, was applied to the grids as a gel using a 3 cm diameter nichrome wire loop. The slides were stored in light-tight boxes at 4°C with silica gel present in each box as a desiccant.

Exposure and development

Thick sections (1 µm) emulsioned with Kodak K2 emulsion were used to determine the length of exposure required for the ultrathin sections. The 1 µm sections were developed after 8, 10 or 12 days in Kodak D-19 developer and fixed in 25% sodium thiosulphate. A good cover of silver grains over the tissue but not over the surrounding resin was found after 10 or 12 days. The exposure time for the thin sections was therefore chosen in the region of 100–120 days.

Controls were carried out to check that the emulsion was applied as a closely-packed monolayer. Several non-radioactive sections were emulsioned with each batch to test for positive chemography. The number of silver grains per unit area overlying the tissue was found to be no greater than at background levels. To ensure that there was no negative chemography or latent image fading a number of radioactive sections were emulsioned and then exposed to light. These were stored with the experimental sections at 4°C for the exposure period. After development an even distribution of silver grains was seen indicating that neither negative chemography nor latent image fading had occurred.

Microscopy

Micrographs for analysis were taken on an AEI 801 electron microscope and printed at a final magnification of 30,000. The micrographs were taken in a non-selective manner around the edges of each grid square to eliminate bias. There was no evidence from the monolayers, or from the grids which had been exposed to light before development, of any accumulation of silver grains close to the grid bars. At least 500 silver grains were photographed for each perfusion time.

Determination of resolution

The resolution of the autoradiographs, defined as the radius of a circle around a point source of radiation which encloses one half of the silver grains formed by that source, was determined. A radioactive line source was prepared using [9,10-³H]oleic acid and the distance ('half-distance') from the line within which 50% of the grains lay was measured. This distance was multiplied by 1.7 to obtain the resolution or 'half-radius' (Salpeter *et al.*, 1969) and was found to be 120 nm. Using Salpeter's density function for a line source with the correction for tritium (Salpeter *et al.*, 1969) a theoretical grain density curve for a line source was derived which was found to have a reasonably good fit with the experimental curve. Salpeter's equation for the distribution of grains around a point source was therefore used in the subsequent analysis described below.

Analysis of grains

The method used was a modification of the point-counting procedure described by Blackett and Parry (1973, 1977) and Parry and Blackett (1976). A preliminary analysis using 50% probability circles gave a list of over 40 possible combinations of single and junctional items. These often had three or four structures falling within the circle so that it was impossible to associate the radioactivity with a particular structure. The 50% probability circle has been adopted by most workers as a convenient size for the analysis but a smaller circle size can be used. In this case it was decided to omit the circles completely and carry out a 'point to point' analysis. This means that there were only single items in the analysis and no junctional items.

The overlay screen also differed from that of Blackett and Parry in that the hypothetical sources were arranged randomly by plotting coordinates from a list of random numbers. For the real grains only the structure underlying the mid point of the longest axis of the grain was noted.

A chi-squared test was first carried out to determine whether the distribution of real grains was random or not. The 'observed' in the chi-squared test was represented by the real grains and the 'expected' was calculated for each organelle from the formula $(Sa \times Rt)/St$ where Sa is the total hypothetical sources in organelle 'a' Rt is the total number of real grains and St the total number of hypothetical grains.

The 'relative intensity of sources' in each organelle was then calculated by solving a series of simultaneous equations, for example

$$Ra = (Ha, a/Sa)A + (Ha, b/Sb)B + (Ha, c/Sc)C, \text{ etc.}$$

where Ra is the number of real grains overlying organelle a , Ha, a is the number of hypothetical grains occurring in organelle a with their sources in a , Ha, b is the number of hypothetical grains in a with their sources in b , etc. Sa is the total hypothetical sources in a . Since the proportions $(Ha, a/Sa)$, etc., are known in each case, the equations can be solved for A, B, C , etc.

Finally the intensity of sources in different organelles is given by

Organelle a A/Sa

Organelle b B/Sb

Identical results were obtained using the computer program designed by Dr N. Blackett which was kindly given to us. The subcellular regions chosen for counting in the present study were: mitochondria, myofibrils, transverse tubules, sarcoplasmic reticulum, lipid droplets, nucleus and 'cytoplasm', i.e. areas of cytoplasm not occupied by myofibrils.

Results

Freeze-clamping

The radioactivity present in the total lipid, free fatty acid, triacylglycerol and phospholipid of the guinea-pig heart after pulse perfusion with ^3H oleic acid is shown in Tables 1 and 2. Table 1 gives the results for a

15 sec pulse perfusion followed by a 15, 30, 60 or 120 sec chaser at 37°C . Table 2 shows the results when the perfusions were repeated under hypothermic conditions. The total lipid radioactivity and free fatty acid activity decreased rapidly after the 15 sec chaser at 37°C . The values continued to decrease throughout the total 2 min washout period. At 5°C the radioactivity in total lipid and free fatty acid decreased by 45% during the first 15 sec of the washout, but thereafter the radioactivity remained constant. There was little evidence of triacylglycerol or phospholipid production in the cold (Table 2). At 37°C , however (Table 1), the radioactivity in triacylglycerol increased nearly three times and phospholipid nearly two times.

Autoradiography

The ultrastructure of the myocardium was well preserved throughout the perfusion procedures (Fig. 1). The values for chi-squared in individual samples of myocardium were all high enough to show that the distribution of observed grains was not random, with probability values less than 0.0005 in all cases.

No difference was found between the intensity of sources in 'cytoplasm' and in myofibrils. For the sake of simplicity of presentation, therefore, these two categories have been pooled under the title of cytoplasm. Fig. 2 shows the changes with perfusion time of the calculated intensity of real sources in the mitochondria, T-tubules, lipid droplets and cytoplasm at 37°C . No detectable activity was associated with the nucleus or sarcoplasmic reticulum at 37°C or at 5°C .

At 37°C there was a rapid incorporation of ^3H into the mitochondria. This incorporation increased during 60 sec of chaser perfusion but dropped dramatically between 60 and 120 sec. Activity was detected in the T-tubules during the shorter perfusion times but not after 60 or 120 sec chaser. There was some activity detected in the cytoplasm at all perfusion times, but this remained relatively low throughout. Incorporation of label into the lipid droplets was first seen after 60 sec of chaser perfusion and was still increasing rapidly after 120 sec.

At 5°C (Fig. 3) the intensity of sources in the mitochondria was considerably lower than at 37°C and was still rising after 120 sec chaser. Activity in the cytoplasm was low

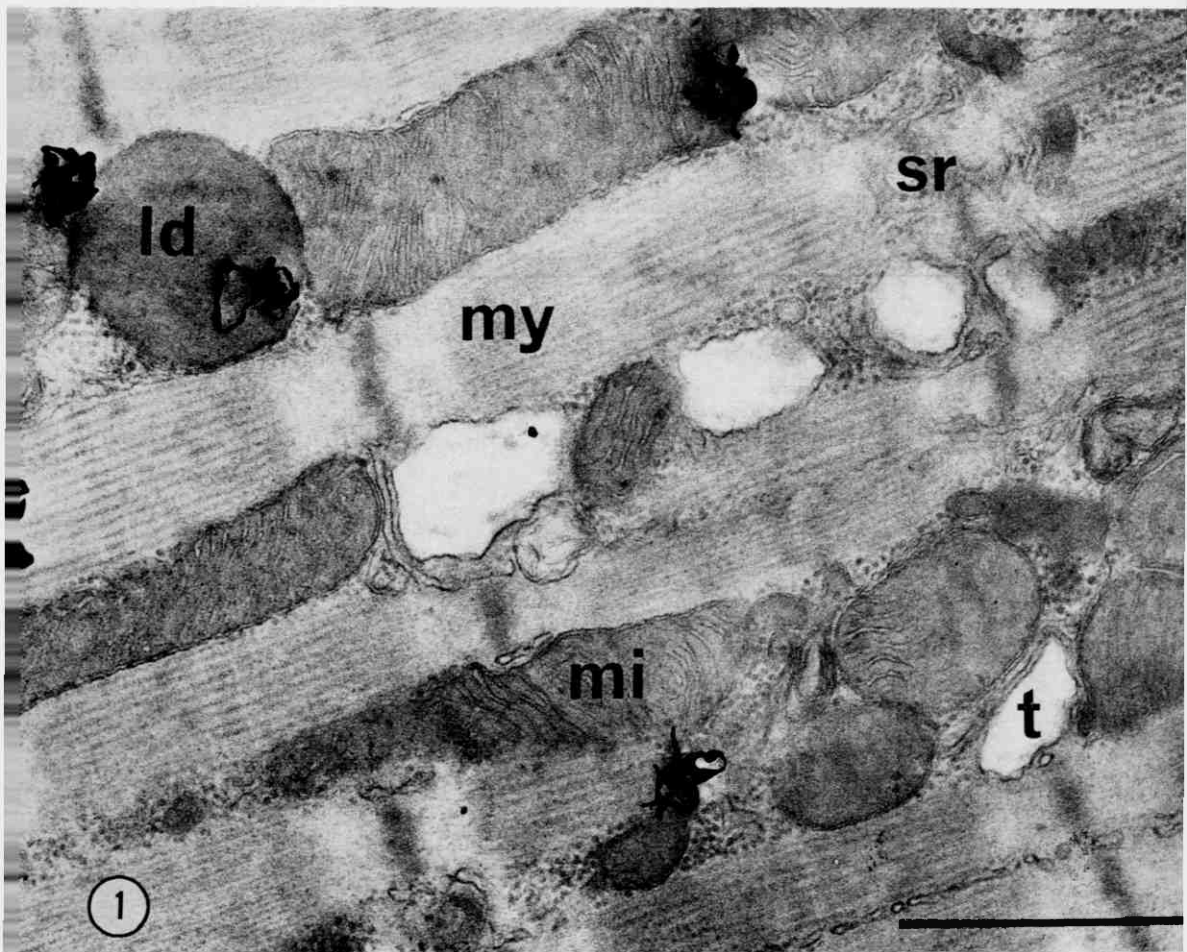


Fig. 1. Autoradiograph of a heart perfused for 60 sec after a 15 sec radioactive pulse with [^3H] oleic acid, showing myofibrils (my), mitochondria (mi), transverse tubules (t), sarcoplasmic reticulum (sr) and a lipid droplet (ld). Marker bar = $1\ \mu\text{m}$.

although, after shorter perfusion times at 5°C , it was higher than at 37°C . Similarly, the activity in the T-tubules was higher than at 37°C after the 15 sec chaser and was still detectable after 60 sec but not after 120 sec. There was no activity associated with the lipid droplets after any of the perfusion times at 5°C .

The use of point-counting made it possible to estimate simultaneously the relative areas of the different divisions of the myocardial cells. The cytoplasm and myofibrils occupied $59.7 (\pm 2.2 \text{ s.d.})\%$ of the cell area; mitochondria $27.2 (\pm 2.4)\%$; transverse tubules $4.6 (\pm 0.6)\%$; sarcoplasmic reticulum 2.1

$(\pm 0.3)\%$; and nuclei $4.9 (\pm 0.5)\%$. There was no difference in this respect between the hearts perfused at 37°C and those perfused at 5°C . The area occupied by cytoplasm (including myofibrils) was more than twice that occupied by mitochondria and this needs to be borne in mind when relating the data from autoradiography to the radioactivity of extracts from whole tissue.

Discussion

Freeze-clamping

The degree of radioactivity in total lipid after 15 sec of unlabelled chaser was similar

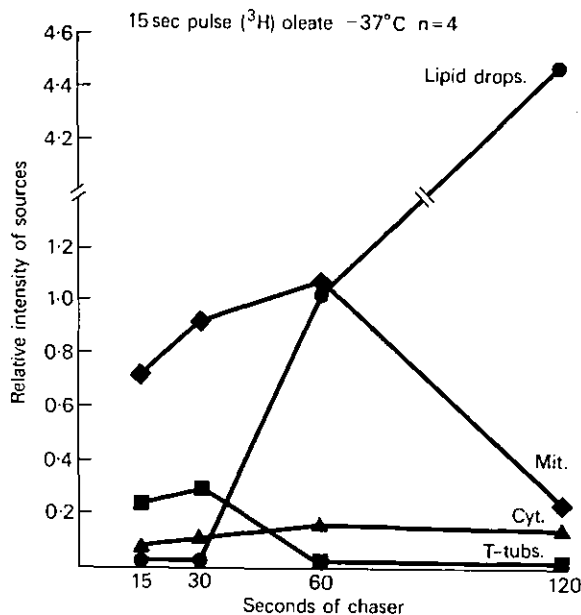


Fig. 2. Relative intensity of sources in lipid droplets, mitochondria, cytoplasm and T-tubules at 37°C .

at 37°C and at 5°C . By 30 sec it had in each case decreased to almost one half its previous value. Thereafter the level of radioactivity in total lipid remained unchanged at 5°C but continued to decrease at 37°C . These figures for total lipid were largely influenced by those for free fatty acid which showed similar courses of events. It seems likely that the

initial sharp decrease in lipid radioactivity found both at 37°C and 5°C was due to the mechanical washout of radioactive free fatty acid located in the vascular space and possibly to some extent, in the space between the blood vessels and the cells. The further decrease in radioactivity of free fatty acids found after 30 sec at 37°C was due either to

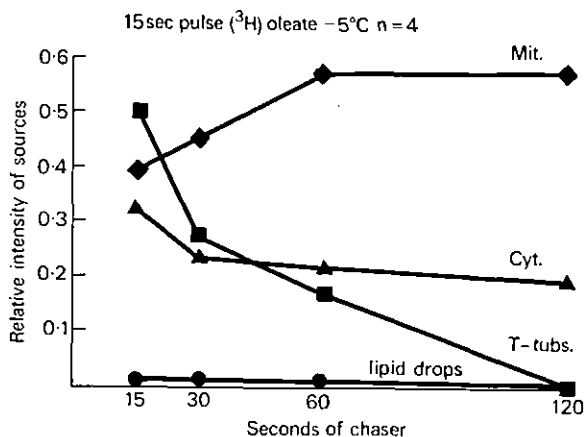


Fig. 3. Relative intensity of sources in lipid droplets, mitochondria, cytoplasm and T-tubules at 5°C .

the oxidation of free fatty acid or to esterification both of which processes would be expected to be inhibited at 5°C. Consistent with this explanation is the increase in radioactivity in triacylglycerol and phospholipid at 37°C but not at 5°C.

The degree of incorporation of radioactivity into esterified lipids was considerably less than that reported by Stein and Stein (1968) in rats. Part of this difference may be due to the different species. Part, however, is probably methodological. Those authors extracted lipids directly from the tissue with chloroform:methanol at room temperature, which might permit considerable esterification to occur during processing. Such esterification was prevented in the present study by the application of freeze-clamping. Similar considerations apply to the finding by Stein and Stein (1968) that the majority of radioactivity in isolated mitochondria and sarcoplasmic reticulum was present in acylglycerols. Previous work in this laboratory (Fields *et al.*, 1975) has shown that considerable exchange of radioactivity between organelles and from free fatty acid into acylglycerol and phospholipid can occur during the process of cell fractionation.

Autoradiography

The autoradiographic studies recorded a rapid entry of radioactivity into the mitochondria. At 15 and 30 sec after the pulse perfusion the intensity of sources in the mitochondria was, for instance, some three times that in the T-tubules. At this time, the intensity of sources in the cytoplasm was lower than either the mitochondria or the T-tubules.

At first sight it may seem surprising that labelled fatty acid is moving at that time from a region of low radioactivity to a region of high radioactivity. There are, however, three mechanisms which prevent the interpretation of such data simply in terms of concentration gradients of free fatty acids.

First, specific transport mechanisms occur across the mitochondrial inner membrane. Second, an unknown proportion of fatty acid in the cytoplasm and mitochondria will be present as fatty acyl CoA. Since these compounds are relatively soluble in water, they will be removed preferentially during tissue-processing. Third, movements along concentration gradients will be determined

by the concentration of unbound free fatty acids, while the total concentration of free fatty acids in a region is determined by the sum of the unbound and bound molecules. Where a region possesses a high concentration of high-affinity binding sites, the total concentration of free fatty acids may be high while the concentration of unbound free fatty acids is low.

The appearance of a high intensity of sources in the lipid droplets after 60 and 120 sec at 37°C reveals the rate of formation of acylglycerols. The findings run parallel with the observations made by freeze-clamping. By 120 sec at 37°C the intensity of sources in mitochondria had greatly decreased. Presumably this was due to the formation of water-soluble oxidation products which would be lost during tissue-processing. Under hypothermic conditions the intensity of sources in lipid droplets remained negligible while that in the mitochondria reached a lower and later peak than at 37°C.

In the experiments of Stein and Stein (1968) on rats a high degree of radioactivity was found in the sarcoplasmic reticulum. In the present experiments the intensity of sources in sarcoplasmic reticulum remained low. It seems likely that this difference is methodological. Stein and Stein counted all grains which overlapped the mitochondrion by less than one-third as belonging to sarcoplasmic reticulum. In our studies only separately identified sarcoplasmic reticulum was included in this category.

Our results, therefore, do not bear out the suggestion of Stein and Stein (1968) that fatty acids travel along the longitudinal tubular system. Stein and Stein suggested that the relative lack of grains over myofibrils was also an indication that 'fatty acid is channelled intracellularly through selective pathways and not by random diffusion'. In the present study there was no distinction between the intensity of sources in the myofibrils and that in the 'cytoplasm'.

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