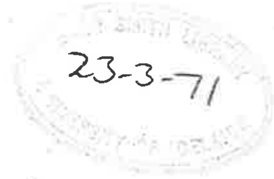
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LIPID METABOLISM BY MACROPHAGES AND BY ISOLATED FOAM CELLS

A thesis submitted for the degree

of

Doctor of Philosophy

by

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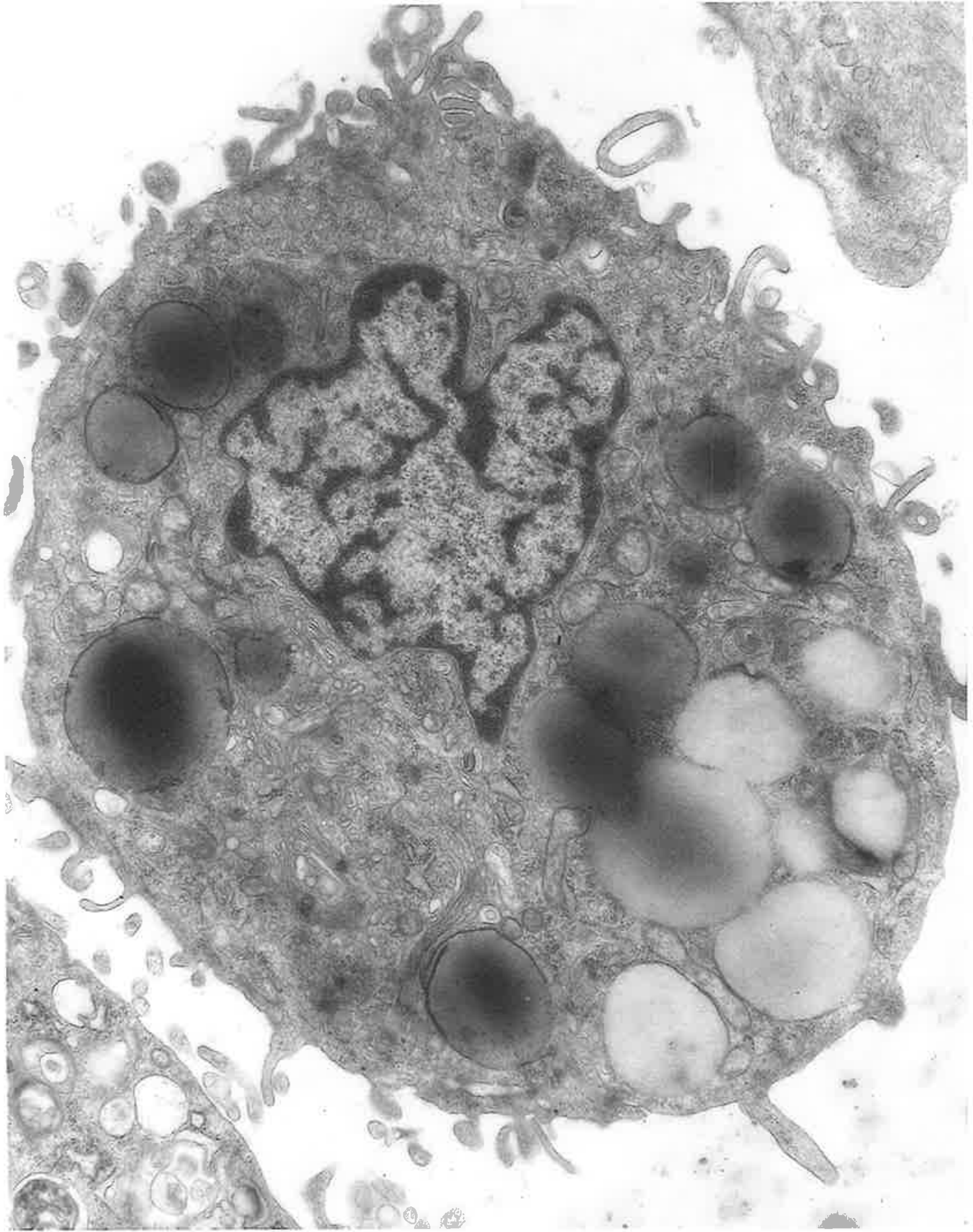
PREFACE

Atherosclerosis is associated with the deposition of lipid in the arterial intima. Since much of the lipid in early human lesions and in experimental lesions is present in foam cells, it has been suggested that these cells play an important role in the deposition of lipid in the intima. The object of the work reported in this thesis was to investigate the possible role of the foam cells in bringing about lipid deposition in the arterial wall. Initial work was carried out using reticuloendothelial cells obtained either from the peritoneal cavity or lungs of normal rabbits, and certain aspects of the metabolism of lipid by these cells investigated. This work was then extended to investigate directly the metabolism of lipid by foam cells isolated from atherosclerotic lesions.



Electron micrograph of an isolated rabbit foam cell (x33,000)

SUMMARY

The ability of macrophages and foam cells to metabolize lipid *in vitro* was investigated. Macrophages were obtained from the peritoneal cavities of rabbits injected with paraffin and from the lungs of rabbits injected with B.C.G. vaccine. Foam cells were isolated by enzymic disruption of atherosclerotic rabbit aortas. Studies were carried out on both cell-free preparations of cells, and on intact cells.

The results of the experiments described were as follows:-

1. The esterification of ^{14}C-labelled cholesterol by cell-free preparations of peritoneal macrophages was investigated. The cofactors ATP and CoASH were not required for the esterification of cholesterol, and the cholesterol-esterifying enzyme displayed a pH optimum of 6.3. The mechanism of the reaction was investigated, and it was concluded that the reaction does not proceed via a lecithin: cholesterol acyltransferase reaction, but by the direct acylation of cholesterol with free fatty acid.

2. The esterification of ^{14}C-labelled cholesterol by both intact alveolar macrophages and cell-free preparations of these cells was investigated. Apart from displaying a pH optimum of pH 4.5, the properties of the cholesterol-esterifying enzymes were found to be similar to those described for peritoneal macrophage homogenates.

3. The formation of glycerolipids by cell-free preparations and particulate fractions of peritoneal macrophages was investigated. The cofactors ATP, CoASH and Mg^{++} ions were required for the acylation of ^{14}C-labelled sodium palmitate, α-glycerophosphate serving as a fatty acid acceptor for both neutral lipid and phospholipid formation. Another pathway which involved the direct esterification of palmitic acid with lysolecithin in the presence of the cofactors was also found.

4. An investigation of the fatty acid patterns of the major lipids of foam cells was carried out. Gas-chromatographic analysis revealed

that the fatty acid patterns of cholesterol esters, phospholipids and triglycerides differed markedly. Cholesterol oleate accounted for 60% of the total cholesterol esters, while phospholipid and triglyceride-fatty acids were predominantly palmitic, stearic and oleic.

5. The potential for growth of isolated foam cells was studied using cell culture techniques. Attempts to grow the cells in media with various serum supplements, in conditioned media, and on fibroblastic feeder layers, were unsuccessful. It was concluded that mature foam cells did not divide under the conditions tested, and the significance of these observations has been discussed with respect to the origin of the foam cell.

6. Foam cells incubated with ^{14}C-labelled sodium oleate took up fatty acid and subsequently incorporated it into other lipid fractions. Most of the fatty acid was incorporated into phospholipid and cholesterol ester, with lesser amounts into triglyceride. Specific activity data suggested that

the phosphlipid had the greatest fractional turnover.

7. Cell-free preparations of foam cells incorporated ^{14}C-labelled sodium oleate in the presence of cofactors into phospholipids and cholesterol esters, with lesser amounts into the glyceride fractions. A consideration of the recovery of radioactivity suggests that up to 50% of the added label was lost as $^{14}CO_2$.

8. The uptake of various preparations of ^{14}C-labelled cholesterol by foam cells and its subsequent esterification was investigated. Whereas all preparations were avidly taken up, no esterification could be detected. Evidence that the cells were actually taking up the particulate cholesterol was demonstrated by experiments showing that the cells were phagocytic.

The results were discussed in relation to the events occurring within the arterial wall. Because of the unique patterns of lipid metabolism displayed by foam cells, it is suggested that these cells are responsible for the deposition and metabolism of lipid within the wall.

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DECLARATION

I declare that the material presented in this thesis is original work and has not been accepted for the award of any other degree or diploma in any University, and that, to the best of my knowledge and belief, contains no material previously published or written by another person, except when due reference is made in the text.

Signed

R.K. Tume

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INTRODUCTION

A. Atherosclerosis and Lipid Deposition

Atherosclerosis is characterized by the accumulation of lipid in the arterial wall, though it is not known whether the lipid deposition causes the early changes associated with the development of the lesion, or results from these changes. Several theories have been suggested to account for the lipid deposition.

1. Lipid Filtration

Since the greater part of the arterial wall is without a vascular supply of its own, its survival depends upon both the infiltration of nutritive materials from the blood stream, and the removal of waste materials through the lymphatics. It is known that serum lipoproteins can pass across the endothelial wall (Buck, 1958; Courtice & Garlick, 1962), and it is believed that some of these lipoproteins are retained in the intima, thus producing an accumulation of lipids (Page, 1954).

Such a theory is supported by a number of investigations. Weinhouse and Hirsch (1940) found a

remarkable similarity between the lipids of plasma and atherosclerotic plaques, and more recently, labelled cholesterol injected intravenously into patients was found in various tissues including aortic walls (Field, Swell, Schools & Treadwell, 1960). Finally, β-lipoprotein has been isolated in greater quantity from human aortic plaques than from either normal aorta, pulmonary artery, or vena cava (Tracy, Merchant and Kao, 1961; Gëro, Gergely, Jakab, Székely and Virág, 1961).

That lipid filtration is responsible for the presence of lipid in the arterial wall is further suggested by the correlation between abnormally high blood lipid levels and atherosclerosis (Keys, 1961), and by the production of experimental atherosclerosis in animals as a result of cholesterol feeding (Anitschkow, 1913).

As previously stated, it was believed that the lipids of the plasma and atherosclerotic plaque were identical (Weinhouse & Hirsch, 1940), but as more refined methods for lipid analysis became available, it became apparent that the composition of

the lipids in the atheromatous lesions differed from those present in the plasma. Although the major lipid groups were similar in both areas, analysis revealed that the fatty acids making up these groups differed depending upon the location (Swell, Law, Field & Treadwell, 1960; Böttcher, Woodford, Ter Haar Romeny-Wachter, Boelsma-Van Houte & Van Gent, 1960; Zilversmit, Sweeley & Newman, 1961). Therefore either the infiltrating lipoprotein is metabolized during the process of deposition, or the arterial wall is contributing to the lipid deposits by net synthesis.

2. Lipophage Migration

It has been postulated that plaques arise from lipid-filled circulating macrophages which have become detached from the reticuloendothelial (RE) system. The cells become attached to the arterial endothelium, and either become overgrown with endothelium or penetrate through it into the subendothelial space. Leary (1941) proposed, on the basis of work with cholesterol-fed rabbits, that Kupffer cells become detached from the liver and are

transported via the blood to the arterial intima. Lipophages (lipid-containing macrophages) have been found in the blood of lipaemic rabbits and rats (Rannie, 1956; Simon, Still & O'Neal, 1961), and some workers have demonstrated the passage of macrophages across the aortic endothelium in cholesterol-fed rabbits (Poole & Florey, 1958; Still & O'Neal, 1961). However, from such studies it is not possible to say in which direction the cells are moving. Other workers have labelled cells of the RE system of cholesterol-fed rabbits, but have been unable to find labelled foam cells either in plaques or in the blood (Simonton & Gofman, 1951; Duff, McMillan & Lautsch, 1954).

Although lipophages circulate in the blood of lipaemic animals, and have been seen to pass through the aortic endothelium, there is no conclusive evidence that these circulatory cells contribute significantly to the lipid build-up in the atherosclerotic plaque.

3. Lipid Synthesis

Some of the lipid that accumulates in atherosclerotic plaques may arise by net synthesis in

intimal cells which have undergone a metabolic derangement. Constantinides (1965) has pointed out that this situation is analogous to the events occurring in hepatic tissue in "glycogen storage disease", where abnormally large amounts of glycogen are synthesized.

During the past 10 years a great volume of information has been collected regarding the ability of the arterial wall to synthesize lipids both *in vivo* and *in vitro* (Werthessen, Milch, Redmond, Smith & Smith, 1954; Stein & Stein, 1962; Day & Wilkinson, 1967; Wahlqvist, Day & Tume, 1969). It has been shown that most of the phospholipid which accumulates in the lesion does so as a result of synthesis rather than by infiltration from the blood (Zilversmit, McCandless, Jordon, Henley & Ackerman, 1961). About half of the cholesterol ester-fatty acid in the experimental rabbit lesion is synthesized within the lesion (Dayton & Hashimoto, 1968).

Morphological observations of early human lesions by Geer, McGill and Strong (1961) suggest that lipid is synthesized within foam cells in the

intima. They observed that lipid sometimes appeared inside the cytoplasm of these cells before it was found extracellularly. In experimental atherosclerosis, and in the early fatty streak lesion of man, most of the lipid is present within foam cells. The presence of lipid within these cells suggests that they are playing an active role in the turnover of lipid within the arterial wall.

B. Foam Cells and Lipid Metabolism in the Arterial Wall

Foam cells are defined as "cells with a peculiar vacuolated appearance due to the presence of complex lipoids" (Dorland, 1965). Such a definition includes a number of different cells without regard to cell origin. Even in the atherosclerotic plaque at least two morphologically different types of foam cells have been described. Both types are grossly engorged with lipid vacuoles but one type has myofibrils in its cytoplasm, a basement membrane and numerous pinocytotic vesicles along the plasma membrane (Parker, 1960; Geer, McGill & Strong, 1961; Parker & Odland, 1966). These are commonly termed smooth muscle foam cells. The second

type has no myofibrils, no basement membrane, and a paucity of pinocytotic vesicles and membrane-bound intracellular lipid droplets.

1. Origin of Foam Cells

Whilst the basic morphology of foam cells gives some clue to their origin, this still remains unknown despite numerous studies. Leary (1941) proposed that when the RE system became filled with lipid after feeding a cholesterol diet, some of the lipid-engorged Kupffer cells were freed into the blood stream, and eventually penetrated the endothelium of blood vessels forming foam cell lesions. Rannie and Duguid (1953) suggested that foam cells originated as proposed by Leary, but that they were deposited in aggregates on the surface of the vessel, and were finally overgrown by endothelium. Endothelial cells were also suggested as precursors of foam cells (Altschul, 1950), and it has been shown that they can acquire phagocytic ability (Buck, 1958a; Cotran, 1965; Still & Prosser, 1964), particularly when overlying foam cell areas. Recently, additional morphological evidence has been proposed (Friedman, Byers & St.George, 1966; Balis, Chan & Conen, 1968)

to suggest that foam cells are of endothelial origin.

Duff, McMillan & Ritchie (1957) found no evidence to support these theories, and proposed that a mononuclear cell entered the intima from the blood. This theory has been supported by other workers (Gonzales & Furman, 1965; Robertson, 1965), and evidence has been presented that blood monocytes and lymphocytes are capable of transforming into macrophages (Ebert & Florey, 1939; Robbins, 1964). It has also been shown that macrophages frequently breach the endothelium (Poole & Florey, 1958; Still, 1964), and that lipophages are demonstrable in the blood of animals fed a high-fat diet (Suzuki & O'Neal, 1964; Marshall & O'Neal, 1966). Whilst it has been claimed that lipophages are morphologically similar to foam cells (Marshall & O'Neall, 1966), more recent work has demonstrated that they do not contain membrane-bound lipid vacuoles as do macrophage-foam cells (Balis, Chan & Conen, 1968). It is most likely that at least some of the foam cells develop from the blood monocytes which penetrate the intima of a vessel. However, in a study to link blood cholesterol levels with numbers of circulating leukocytes, Spraragen (1968)

found no correlation. A correlation would have suggested that more monocytes were available to the artery, but a lack of correlation in a study which does not take into account dynamic factors, by no means excludes the possible role of monocytes in the development of the lesion.

The most recently proposed theory, as a result of electron microscopic studies of various lesions, is that foam cells originate from smooth muscle cells (Parker, 1960; Geer, McGill & Strong, 1961; Balis, Haust & More, 1964; Luginbuhl, Jones & Detweiler, 1965). There is no question that some foam cells resemble smooth muscle cells, and in human lesions it is believed that about half of the foam cells are of smooth muscle origin (Geer, 1965).

2. Mitosis in Foam Cells

Irrespective of the origin of foam cells, it is of interest to know whether they can divide within the lesion, as local division would significantly alter the size of the plaque. Mitotic figures have been observed in developed foam cells in experimental lesions *in situ* (McMillan & Duff,

1948), and further, ^{3}H-labelled thymidine is incorporated into nuclei of foam cells in developed plaques, suggesting DNA synthesis (Spraragen, Bond & Dahl, 1962; McMillan & Stary, 1968). However, both isolated foam cells (Tume, Bradley & Day, 1969) and foam cell explants of atherosclerotic lesions (Kokubu & Pollak, 1961) showed no proliferative activity in tissue culture.

3. Studies with Isolated Foam Cells

Foam cells have been isolated from rabbit atherosclerotic lesions (Day, Newman & Zilversmit, 1966), and some initial metabolic studies have been carried out with these cells. It has been shown that foam cells are capable of synthesizing phospholipids when supplied with ^{32}P-labelled ortho-phosphate (Day, Newman & Zilversmit, 1966), the major phospholipids to be synthesized being lecithin and Phosphatidylinositol. ^{14}C-labelled-acetate was also taken up by these cells and incorporated predominantly into the phospholipid and cholesterol ester-fatty acid fractions (Day & Wilkinson, 1967). Thus these initial studies

demonstrate that foam cells have the necessary metabolic machinery for the synthesis of fatty acids from acetate, and for the incorporation of the synthesized fatty acid into phospholipids and cholesterol ester.

C. The Reticuloendothelial System and Lipid Metabolism

Whilst there are a number of possible precursor cells that form foam cells, it seems likely that some at least are of macrophage origin (Geer, 1965). In view of this relationship, much of the initial work on lipid metabolism by RE cells from other situations is of possible relevance to the arterial lesion, and in fact much of this work has been carried out with this relationship clearly in mind. Since a considerable body of work has accumulated relating to the metabolism of lipid by these RE cells, this aspect will now be reviewed in some detail as a more specific background for some of the work reported in this thesis.

Metchnikoff (1905) established that vertebrates possess a system of specialized cells which are capable of recognizing and ingesting foreign materials. These cells constitute the

fixed and free mononuclear phagocytes of the RE system. The fixed macrophages are generally referred to as tissue macrophages and are found throughout the body, predominantly in the spleen, lymph nodes, lung, liver, bone marrow and skin. Fixed macrophages are mainly responsible for the removal and destruction of bacteria, damaged cells and foreign materials from the blood and lymph. The free macrophages are mononuclear phagocytes or monocytes found in the peripheral blood, but the function and fate of these cells is not clearly understood. It is known that monocytes are capable of transforming into cells with characteristics of tissue macrophages both in vivo (Ebert & Florey, 1939) and in vitro (Bennet & Cohn, 1966). Recently it has been postulated (Van Furth & Cohn, 1968) that tissue macrophages in the mouse arise originally from promonocytes in the bone marrow which become monocytes in the peripheral blood, and in turn become tissue macrophages.

All macrophages, regardless of origin, have in common by definition the ability to ingest foreign material by a process known as phagocytosis. The foreign particle is engulfed by the phagocytic cell

and eventually is completely surrounded by a cytoplasmic membrane. Smaller particles are ingested by a process known as pinocytosis (cell drinking), and larger particles by phagocytosis. It is thought that both processes may be identical, although phagocytosis is generally referred to as the ingestion of particles or particulate aggregates which are visible with the light microscope.

The basic cellular mechanism of phagocytosis is unknown. However, ingestion of a particle represents a form of active transport, since mechanical work is performed by the phagocytic cell and energy is expended. It is known that macrophages depend on the supply of metabolic energy. Baldridge and Gerard (1933) demonstrated that respiration of a suspension of leucocytes increased immediately bacteria were added, and this has been confirmed by Karnovsky (1962). Recent studies have indicated that phagocytosis by peritoneal macrophages and polymorphonuclear leucocytes depends on energy derived from anaerobic glycolysis, whereas phagocytosis by alveolar macrophages is dependent upon energy derived from oxidative phosphorylation (Oren, Farnham, Saito, Milofsky & Karnovsky, 1963). It has

been suggested that the mechanisms involved in phagocytosis are located near the periphery of the cell, as it is here where the mechanical movements of phagocytosis are performed, and it is possible that the expenditure of energy in the form of ATP is by a similar process to that in the contraction and relaxation of muscle (North, 1968). North further suggested that since evidence has been produced that plasma membranes of phagocytic cells possess calcium- and magnesium-dependent ATPase activity (Woodin & Wieneke, 1966; North, 1966), and in view of the association of ATPases in cellular mechanical activities, it is likely that the plasma membrane plays a central mechanical role in phagocytosis.

Some of the metabolic functions attributed to the RE system are briefly mentioned. The phagocytic cells are responsible for the ingestion and destruction of invading micro-organisms and as such are the body's first line of defence against infection. Besides playing an important role in the immune response (Marshall, 1956), macrophages

enter damaged tissue by slow amoeboid movement, and ingest and digest cellular debris including fragments and fibrin and damaged blood cells (Florey, 1962). RE cells are responsible for the removal of damaged erythrocytes from the circulating blood. Although it was thought that the splenic RE cells were responsible for the phagocytosis of whole erythrocytes which had reached the end of their life span, it is now believed that the old cells haemolyze in the blood stream and the debris is removed by phagocytic cells throughout the body (Knisely, 1936). Since Ribbert (1904) demonstrated that macrophages have the ability to take up and store a number of foreign materials including lipid, iron and micro-organisms, interest has been aroused regarding their uptake and storage capacity.

1. The Uptake of Lipid by Reticuloendothelial Cells

In recent years many investigators have been concerned with the processes by which lipids are taken up by various cells, and it has become apparent that this occurs by pinocytosis or phagocytosis (Palay

and Revel, 1964). However, the involvement of lysosomal enzymes in the degradation of the ingested lipid is not known with any certainty. It is thought that the only lipolytic enzymes present are the slowly acting esterases (Straus, 1967). Nilsson (1962) and Fuxe and Nilsson (1963), in investigations on the role of lysosomes in lipid decomposition in mouse uterine epithelium, suggested that acid phosphatase might participate in the degradation of phospholipids, and esterase in the degradation of glycerides.

Investigations of the uptake of lipids by cells are difficult because of the rapid metabolic changes which the ingested lipid might undergo. Also the physical state of the lipid being investigated is important. For example, cholesterol uptake may be investigated by adding cholesterol in any one of the following forms; as a particulate suspension, as a non-aqueous solution, as a micellar solution or as a lipoprotein preparation, and each could be taken up differently. Thus comparisons of results from different workers should be closely examined.

Ribbert (1904) was first to demonstrate the uptake of fat by RE cells. Other workers found fat

droplets in RE cells of liver and spleen (Fischer, 1912; Landau & McNee, 1914; Kusunoki, 1914), and it was suggested that such cells had an abnormal lipid metabolism. However, Arndt-Marburg (1925) demonstrated the presence of fat in Kupffer cells of normal animals and Slotwinsky (1929) demonstrated fat in the lymph nodes of normal dogs. Intravenous injections of artificial triglyceride emulsions resulted in the deposition of fat in all parts of the RE system (Saxl & Donath, 1925), and this was confirmed by Jaffe and Berman (1928), who demonstrated its uptake by Kupffer cells in the liver of dogs and rabbits. The uptake of emulsified lipid preparations including cedar-wood oil and olive oil by phagocytic cells of connective tissue was described by Kedrowski (1933). Whereas colloidal carbon, silver or trypan blue often appeared in the same granules of macrophages and fibroblasts after simultaneous injection, the ingested fat was localized in separate granules, suggesting that fat was taken up differently from the other foreign particles. Murray & Freeman (1951) demonstrated that whereas artificial triglyceride emulsions were taken up by Kupffer cells, homologous

preparations of chylomicra were taken up, not by RE cells, but directly by the liver parenchymal cells. More recently it has been shown (French & Morris, 1960) that intraperitoneal injections of homologous chylomicra were relatively resistant to uptake by RE cells. However, chylomicra preparations which had been modified by the addition of Triton WR-1339 or heterologous serum were taken up more readily. Using ^{14}C-labelled triglyceride emulsions and ^{14}C-labelled chylomicron fat, Day (1960a, 1961) demonstrated that isolated peritoneal macrophages incubated _in vitro_ will ingest triglyceride emulsions more readily than chylomicron fat. Waddell, Geyer, Clarke and Stare (1953) observed the clearance of artificially prepared triglyceride emulsions from the blood stream of eviscerated animals, and suggested that the RE system might be of importance in the removal of blood fat particles, treating them essentially as foreign particles. However, in a follow up study (Waddell, Geyer, Clarke & Stare, 1954), it was shown that the RE system plays only a minimal role in the removal of fat particles from the blood stream, since "blocking" of the RE system with lithium carmine, trypan blue or

carbon, had little effect on the clearance rate compared with that of "unblocked" animals.

Weinhouse & Hirsch (1940) demonstrated that fine suspensions of cholesterol or cholesterol oleate introduced into the blood stream were taken up very rapidly by cells of the RE system. Subcutaneous injections of cholesterol were also taken up by macrophages (Tompkins, 1946), and it was shown that the fine cholesterol suspensions were deposited as discrete acicular crystals which were attracted to the surface of macrophages. It was postulated that the cholesterol crystals were converted to cholesterol esters on the surface of the macrophages, which then entered the cell. The esters were modified within the peripheral part of the cytoplasm, and it was suggested that these cells combined cholesterol ester with protein to form a diffusable complex which was then returned to the body fluids.

Intraperitoneal injections of cholesterol and cholesterol oleate particles were taken up by the RE cells of the sternal lymph nodes (French & Morris, 1960;

Day & French, 1961), following the same route as that described for non-toxic foreign particles such as carbon, colloidal iron and trypan blue (Kettler, 1936). Initially the lipid was present mainly in the free macrophages and RE cells of the marginal and cortical part of the intermediary sinuses. The bulk of the lipid-containing cells was found to migrate to the medullary lymph cords, where these cells aggregated to form compact masses of material stored at these sites. It appears that cholesterol ester, once taken up by these cells, is removed with difficulty (Day & French, 1961), in contrast to triglyceride emulsions which are removed quite rapidly (French & Morris, 1960). In vitro studies with peritoneal macrophages have also indicated that cholesterol suspensions are taken up by these cells (Day, 1961; Day & Gould-Hurst, 1961). More recently (Casley-Smith & Day, 1966) it has been shown, using both radiochemical and electron microscopic techniques, that suspensions of cholesterol and triglyceride are taken up by peritoneal macrophages in vitro by a process which appears to be energy dependent, since only a fraction of the normal uptake is found at low temperatures.

It has also been demonstrated that cholesterol and artificial triglyceride preparations are present in large membrane-bound vesicles within the cells. Lipoprotein preparations were found in small vesicles and their uptake was not energy dependent.

Following the feeding of cholesterol to animals, it was possible to demonstrate, using RE blocking agents, that the Kupffer cells are responsible for the removal of cholesterol from the circulation (Friedman, Byers & Rosenman, 1954; Rice, Schotz, Powell and Alfin-Slater, 1954). However, Di Luzio and Riggi (1962) failed to show increased uptake of injected cholesterol in chylomicra following the use of RE stimulating agents. They suggested that the Kupffer cells might play some role in cholesterol metabolism but not in its uptake from the blood.

The uptake of phospholipids by RE cells was demonstrated by Magat (1928) following intra-peritoneal injection into guinea-pigs. Sphingomyelin, injected intravenously into monkeys or rabbits, produced foam cell infiltration of the liver, spleen,

kidney and bone marrow (Ferraro & Jervis, 1940). Blocking the activity of the RE system with Congo red prevented the formation of foam cells, and it was therefore concluded that the foam cells had arisen from the RE cells. Injections of lecithin into animals produced only a temporary infiltration of foam cells (Sjövall, 1935), while injection of cephalin failed to produce any reaction at all (Ferraro & Jervis, 1940).

The ability of the RE cells to take up homologous lipoprotein is of some relevance in relation to the events occurring in the atheromatous arterial wall. Whilst it has been shown that β-lipoprotein can infiltrate the arterial intima (Page, 1954), it has been suggested, following electron microscopic studies (Buck, 1958), that lipoprotein enters the endothelial cells from the lumen as small vesicles. The lipoprotein is taken into the large cisternas of the endoplasmic reticulum where the protein fraction remains, but the lipid passes through the outer cytoplasmic membrane into the extracellular spaces, where it is phagocytozed by foam cells. Thus it is suggested

that the protein is split off prior to ingestion of the lipid by the foam cells.

As mentioned earlier, Casley-Smith & Day (1966) demonstrated the uptake of lipoprotein *in vitro* by peritoneal macrophages. It was observed that lipoprotein was present within the cells in small vesicles (about 50mμ diameter). Sometimes large vesicles of lipoprotein aggregates were observed, and it is believed that these may have formed by the direct ingestion of aggregates. Courtice & Schmidt-Diedrichs (1962) have shown that lipoprotein-lipid is demonstrable within intimal macrophages following infiltration from the blood stream in areas of injured arterial wall. Where the lipoprotein was modified by the injection of Triton WR-1339, lipoprotein filtered into the intima but was not taken up, suggesting that the properties of the lipoprotein influence its uptake by macrophages.

2. Lipid Synthesis by Reticuloendothelial Cells

Peritoneal macrophages have been shown to synthesize lipid from non-lipid precursors. Day and Fidge (1964) incubated macrophages *in vitro* with ^{14}C-labelled acetate and demonstrated that about one third of the acetate taken up was incorporated into

cellular lipid. Of this, about 80% was incorporated into the non-saponifiable fraction, 20% into fatty acids, and 1% into lipid glycerol. Similarly, where macrophages were incubated _in vitro_ with ^{14}C-labelled glucose, about one third of that taken up was converted into cellular lipid. Label was found predominantly in the triglyceride and phospholipid fractions, in accordance with the observation that greater than 95% of the label is present in the lipid glycerol fraction (Day & Fidge, 1965).

Peritoneal macrophages are capable of incorporating ^{32}P-labelled orthophosphate into lecithin and sphingomyelin (Day, Fidge & Wilkinson, 1966). Stimulation of the synthesis of phospholipid was observed when the cells were presented with cholesterol particles. Since there was little change in the respiration rate of the cells on addition of the particles, and since addition of carbon particles caused no stimulation of phospholipid synthesis, it was concluded that the increased synthesis was not due to increased phagocytosis. The authors suggest that cholesterol might initiate increased phospholipid synthesis as a specific response for the synthesis of lipoprotein. In a similar way it is believed that

cholesterol may be removed from the lipid-laden macrophages within the arterial wall.

The ability of phagocytic cells to synthesize lipid has been considered from the aspect of cellular membrane turnover as a result of phagocytosis. In experiments with granulocytes, it has been observed that the incorporation of ^{14}C-labelled acetate (Elsbach, 1959; Sbarra & Karnovsky, 1960), ^{14}C-labelled glucose (Sbarra & Karnovsky, 1959), ^{32}P-labelled ortho-phosphate (Karnovsky & Wallach, 1961) and ^{3}H-labelled inositol (Sastry & Hokin, 1966) was stimulated during phagocytosis, whereas the incorporation into lipid of both ^{14}C-labelled fatty acid (Elsbach, 1963) and ^{32}P-labelled α-glycerophosphate (Sastry & Hokin, 1966) was not stimulated during phagocytosis. However, Oren, Farnham, Saito, Milofsky and Karnovsky (1963) failed to demonstrate a greater incorporation of ^{14}C-labelled acetate or ^{32}P-labelled ortho-phosphate into lipids during phagocytosis by macrophages. Recently, Elsbach (1968) has demonstrated increased incorporation of both ^{32}P-labelled lysolecithin and ^{32}P-labelled lysophosphatidylethanolamine into lecithin and phosphatidylethanolamine, respectively, during

phagocytosis by rabbit granulocytes and alveolar macrophages. It is suggested that lysolecithin, a normal constituent of plasma, can act as a substrate in lecithin synthesis by phagocytic cells.

3. Lipid Metabolism by Reticuloendothelial Cells

Evidence that macrophages can metabolize ingested lipid was obtained by Schonheimer and Yuasa (1929), who demonstrated by histological and chemical techniques, the partial conversion of ingested cholesterol to cholesterol ester. More recent work has established the presence in RE cells of enzymes capable of mediating both the synthesis and hydrolysis of cholesterol ester. Intraperitoneal injection of aqueous suspensions of free cholesterol or cholesterol esters into rats resulted in uptake of free cholesterol by the RE cells of the sternyl lymph nodes, and was followed by an increase in the local concentration of cholesterol ester. Similarly, when cholesterol ester was taken up, there was a local increase in free cholesterol (Day & French, 1959). It was further shown that ^{14}C-labelled cholesterol was taken up and esterified by rat lymph nodes and by peritoneal macrophages *in vitro* (Day & Gould-Hurst, 1961).

French & Morris (1960) showed that suspensions of cholesterol and cholesterol oleate were only slowly removed after ingestion by RE cells of rat lymph nodes, and small quantities of cholesterol ester could be detected 18 months after ingestion. Also Day, Gould-Hurst, Steinborner and Wahlqvist (1965), demonstrated that cholesterol was removed by these cells much more slowly than triglyceride or phospholipid emulsions. Interestingly, it was observed using doubly-labelled lipoprotein preparations, that the phospholipid portion was removed more rapidly than the cholesterol portion.

The ability of macrophages to take up serum lipoprotein was described earlier (Day, Gould-Hurst & Wahlqvist, 1964), where it was observed that appreciable hydrolysis of lipoprotein-cholesterol ester occurred. Where synthetic lecithin or purified animal lecithin was added to homogenates of macrophages, there was an accelerated hydrolysis of cholesterol ester. However, these preparations had an inhibitory effect on the esterification of cholesterol (Day & Gould-Hurst, 1963). The fatty acid pattern of the cholesterol esters formed by RE

cells of rat lymph nodes was determined by gas-liquid chromatography (Day, Fidge, Gould-Hurst and Risely, 1963), and cholesterol was found to be esterified predominantly with saturated fatty acids. Where either corn oil or coconut oil was administered together with cholesterol, the pattern of the synthesized cholesterol esters became more unsaturated or more saturated respectively. The effect of corn oil or coconut oil on cholesterol esterification and removal from RE cells was investigated by Day (1960b), and it was found that the uptake and esterification was significantly greater when oil was added, but that there was no significant difference in the rates of removal of cholesterol from the cells. However, it has been shown that cholesterol is removed from lung macrophages of essential fatty acid deficient animals when the animals are given linoleate (Bernick & Alfin-Slater, 1963).

Rabbit peritoneal macrophage homogenates have been shown to possess both lipase and esterase activity (Day & Harris, 1960). Chylomicron triglyceride, tributyrin and β-napthyl esters were hydrolyzed. These findings for peritoneal macrophages were confirmed by Cohn & Weiner (1963), who also demonstrated the same activities in alveolar macrophages, showing the activity

to be located in the lysosomal fraction.

The ability of the RE cells to take up triglycerides has been described. The ingested triglyceride does not remain unaltered within the cell but is rapidly metabolized to other lipids, and in this way is removed from the cell (French & Morris, 1960).

It has been shown (Fidge, 1964) that peritoneal macrophages will partly incorporate triglyceride-fatty acid into phospholipids, and RE cells of lymph nodes will hydrolyze the triglyceride and incorporate the released fatty acids into phospholipids and cholesterol esters (Day, Fidge, Gould-Hurst, Wahlqvist & Wilkinson, 1966).

The incorporation of fatty acids into cell lipids has been investigated by a number of workers. Day & Fidge (1962) reported that ^{14}C-labelled fatty acids were readily taken up by peritoneal macrophages _in vitro_, and were subsequently incorporated into phospholipids and triglycerides. Similar findings were found by Elsbach (1965) for alveolar macrophages. More

recently Elsbach (1966) has compared the incorporation of fatty acids into phospholipids by homogenates of either rabbit polymorphonuclear leukocytes or of alveolar macrophages. It is apparent from this study that the incorporation of fatty acids into phospholipid takes place by different pathways depending upon the type of phagocytic cell investigated.

In this thesis the ability of macrophages from various locations to synthesize and metabolize lipids, with particular emphasis on the synthesis and metabolism of cholesterol ester, has been studied.

Firstly, the properties of cholesterol-esterifying enzymes of peritoneal macrophage homogenates are reported.

These studies are extended in the second section to the investigation of cholesterol-esterifying activity of alveolar macrophages.

Investigations on fatty acid incorporation into glycerolipids by peritoneal macrophage homogenates and by subcellular fractions of these cells, are reported in the third section.

In all of the succeeding sections, foam cells isolated from atherosclerotic lesions have been studied. Whilst the main emphasis was on the metabolism of these isolated foam cells, preliminary studies were carried out in order to provide analytical information about the cells, and in an attempt to grow the foam cells by tissue culture techniques.

In the final sections, the metabolism of

^{14}C-labelled oleic acid and ^{14}C-labelled cholesterol by intact foam cells and by foam cell homogenates was investigated.

MATERIALS AND METHODS

A. Materials

1. Radioactive Substrates

(a) ^{14}C-labelled Sodium Palmitate

[1-^{14}C] Palmitic acid (specific activity 143 or 214μCi/mg, Radiochemical Centre, Amersham, England), was dissolved in 0.05N sodium hydroxide solution to give a stock solution of sodium palmitate containing 20μCi/ml.

(b) ^{14}C-labelled Sodium Oleate

[1-^{14}C] Oleic acid (specific activity 202μCi/mg, Radiochemical Centre, Amersham, England), was dissolved in 0.05N sodium hydroxide solution to give a stock solution of sodium oleate containing 20μCi/ml.

(c) ^{14}C-labelled Cholesterol

[4-^{14}C] Cholesterol (specific activity 144μCi/mg) was obtained from the Radiochemical Centre, Amersham, England.

(i) Particulate Cholesterol Suspension

20μCi of [4-^{14}C] cholesterol (140μg) was dissolved in about 0.2ml warm acetone, and was transferred quantitatively to 5ml of water in a 10ml stoppered test tube. Acetone was removed by boiling,

and any cholesterol that separated from the solution was removed by filtration through glass wool. The filtrate was concentrated by further boiling to give a suspension containing about 10µCi cholesterol/ml.

(ii) Micellar Cholesterol Solution

20µCi [4-^{14}C] cholesterol (140µg) was dissolved in 2ml ethanol containing 20mg Tween 20 (Chemical Materials Ltd., Bayswater, Vic. , Aust.). The ethanol was removed by evaporation, and the residue was taken up in 2ml of water with vigorous shaking.

(iii) Non-aqueous Cholesterol Preparations

Non-aqueous preparations were obtained by dissolving [4-^{14}C] cholesterol in acetone.

2. Thin-Layer Chromatographic Standards

(a) Neutral Lipids

(i) Monopalmitin, Hormel Institute, Austin, Minn. U.S.A.

(ii) α and β-Dipalmitin, Hormel Institute, Austin, Minn., U.S.A.

(iii) Triolein (L.R.), British Drug Houses Ltd., Poole, England.

(iv) Palmitic Acid, British Drug Houses Ltd., Poole, England.

(v) Cholesterol Oleate, Applied Sci. Labs., State College, Penna., U.S.A.

(vi) Cholesterol, Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.

(b) Phospholipids

(i) Lecithin (Phosphatidylcholine), Applied Sci. Labs., State College, Penna., U.S.A.

(ii) Lysolecithin, Applied Sci. Labs., State College, Penna., U.S.A.

(iii) Phosphatidylethanolamine, Applied Sci. Labs., State College, Penna., U.S.A.

(iv) Phosphatidylserine, Applied Sci., Labs., State College, Penna., U.S.A.

(v) Sphingomyelin, Applied Sci. Labs., State College, Penna., U.S.A.

(vi) Phosphatidylinositol, Dr. M. Fauré, Paris.

(c) Cholesterol Esters

(i) Cholesterol Stearate, Applied Sci. Labs., State College, Penna, U.S.A.

(ii) Cholesterol Oleate, Applied Sci. Labs., State College, Penna., U.S.A.

(iii) Cholesterol Linoleate, Applied Sci., Labs., State College, Penna., U.S.A.

3. Bovine Serum Albumin Free of Long-Chain Fatty Acids

Bovine serum albumin, Cohn fraction V (Armour Pharmaceutical Co., Chicago, Ill., U.S.A.) was purified by the method of Goodman (1957). Lyophilized albumin (50g) was covered with 300ml of a mixture of glacial acetic acid:iso-octane (5:95, v:v). This mixture had previously been dried with anhydrous sodium sulphate

to remove traces of water. The extraction was carried out at 2°C without agitation for 18h. After this time the extraction mixture was decanted and discarded, and the albumin washed twice with 50ml of iso-octane. The extraction was repeated but using 300ml of the extraction mixture. Following this extraction the albumin was washed twice with 50ml of iso-octane, and dried in a vacuum oven at 20°C for 8h to remove the remaining iso-octane and acetic acid.

The resulting albumin preparation was dissolved in 250ml of distilled water. The remaining traces of acetic acid and iso-octane were removed by dialysis against 20 litres of distilled water per day for 4 days at 2°C. The albumin solution was then lyophilized and stored at -15°C.

The amount of total fatty acid, both unesterified and esterified, present in the albumin before and after extraction was determined. 80mg of each batch of albumin was extracted with 10ml chloroform:methanol (2:1, v:v), by the method of Folch, Lees and Sloane-Stanley (1957). Standard heptadecanoic acid was added to each extract. The resultant lipid extracts were taken to dryness and the fatty acids methyl-esterified as described later. Aliquots were then taken for analysis of methyl-fatty acids by gas-liquid chromatography.

Analysis showed that the original albumin contained 0.33 moles total fatty acid per mole of albumin, compared with 0.013 moles total fatty acid per mole albumin after extraction. Since Cohn fraction V often contains small amounts phospholipids, unesterified fatty acid of the albumin may be even lower than that measured, but this in no way affects the results of those experiments in which this preparation was used.

It has been shown (Goodman, 1957) that during the extraction procedure there is no change in the electrophoretic mobility, optical rotation, sedimentation rate or immunological characteristics.

4. ^{14}C-labelled Fatty Acid-Lecithin

Lecithin labelled in the β-position with ^{14}C-labelled fatty acid was required as substrate for the investigation of the presence of lecithin:cholesterol acyltransferase activity in homogenates of peritoneal and alveolar macrophages. This was prepared biosynthetically using macrophage homogenates.

Homogenates of peritoneal macrophages (see later for preparation) in 0.05M Tris-HCl buffer at pH 7.4, were incubated with ^{14}C-labelled fatty acid at $37^{o}C$ for 90min in 12ml centrifuge tubes with shaking. Each tube contained in a total volume of 3ml, 600μmoles Tris-HCl buffer at pH 7.4, 1ml homogenate (8-12mg protein), 30μmoles ATP, 3μmoles CoASH, 15μmoles $MgCl_2$, 15μmoles

α-glycerophosphate and 50μl of a known amount of [1-^{14}C] palmitic or [1-^{14}C] oleic acid in 0.05N NaOH. The reaction was stopped and the lipid extracted with chloroform: methanol (2:1, v:v) as described by Folch, Lees and Sloane-Stanley (1957). The whole extract was chromatographed by thin-layer chromatography (T.L.C.) to separate the phospholipid from neutral lipid as described later. The origin containing the mixed phospholipids was then eluted from the silicic acid with chloroform:methanol:water (75:25:2, v:v:v), and the eluted phospholipids separated into the individual phospholipids by T.L.C. by the method of Skipski, Peterson and Barclay (1964). The area corresponding to lecithin as determined by comparing the R_f values of the radioactive spots located by autoradio-chromatography with standard plant lecithin, was scraped from the plate and eluted as above.

The position of the labelled fatty acid was determined by splitting off the β-fatty acid of the lecithin with phospholipase A as described by Long and Penny (1957).

An aliquot of the biosynthetically prepared lecithin (1.5-2.0μmoles) in chloroform:methanol (2:1, v:v) was added to a 10ml stoppered test tube and taken to dryness. 1ml of diethylether together with 10μl of 0.005M calcium chloride and 10μl of phospholipase A (0.5mg/ml) (Commonwealth Serum Laboratories, Parkville, Vic., Aust.) was added to the residue and left to stand at room temperature (about 20°C) for 8h. Control incubations containing 10μl of water instead of phospholipase A were incubated under identical conditions. At the end of the incubation, the contents of each tube were taken to dryness and the residue taken up in 4ml chloroform:methanol (2:1, v:v). A suitable aliquot of each extract was taken for the separation of phospholipids by T.L.C. as described by Skipski, Peterson and Barclay (1964).

Areas of silica corresponding to lysolecithin, lecithin and neutral lipids (solvent front) were scraped into counting vials and counted in Snyders scintillator (Snyder, 1964).

Counting revealed that 80% of the lecithin added was broken down to lysolecithin and free fatty acid during the course of the incubation, whereas in the

control incubations less than 1% was broken down. Of that lecithin broken down, approximately 60-65% of the label was present in the free fatty acid area, demonstrating that 60-65% of the lecithin was labelled in the β-position with ^{14}C-labelled fatty acid.

B. Cell Isolation Techniques

1. Rabbit Peritoneal Macrophages

(a) Collection of Cells

Rabbit peritoneal macrophages were obtained by the method of Lucke, Strumia, Mudd, McCutcheon and Mudd (1933), as modified by Mackaness (1952). Adult rabbits were injected intra-peritoneally with 40ml of sterile liquid paraffin (Nujol, Plough Pty. Ltd., Richmond, Vic. Aust.). After 5 days the rabbits were killed by exsanguination under ether anaesthesia. Three 50ml washes of sterile 0.9% sodium chloride solution (containing 0.14% disodium ethylenediamine-tetraacetic acid to prevent macrophage aggregation), were introduced into the peritoneal cavity through a small midline incision. The peritoneal washings were removed into a separating funnel through a sheathed cannula by means of a vacuum pump. The paraffin was allowed to separate as an upper phase, and the lower

aqueous phase containing the suspended cells was then taken off into 50ml centrifuge tubes through a gauze filter to remove giant cells, cellular aggregates and debris. The cells were deposited by centrifuging at 220xg for 5min. The deposited cells were resuspended in Hanks solution (Hanks, 1948), and the number of cells counted in a haemocytometer counting chamber.

The cell population obtained by this method was found (Fidge, 1964) to consist of 85-90% large mononuclear cells identical with tissue macrophages, together with 10-15% of small mononuclear cells. Very few polymorphonuclear leukocytes were present.

(b) Preparation of Homogenate

Cells were suspended in 4 volumes of 0.05M Tris-HCl buffer pH 7.4 and homogenized for 1-2min in a Potter-Elvehjem type glass homogenizing tube with a teflon plunger. Disruption was checked microscopically and generally less than 10% of the cells remained intact after homogenization. The whole homogenate was centrifuged at 200xg for 5min to remove whole cells, nuclei and cellular debris. The supernatant, termed the "homogenate" fraction, was removed and dialyzed against 5l. of 0.9% NaCl solution at $4^{o}C$ for 6h. In those experiments in Section I where the esterification of both ^{14}C-labelled oleic acid and ^{14}C-labelled cholesterol was investigated, the homogenate was dialyzed against 4 x 1l.

changes of 0.9% NaCl solution at 4°C for a total period of 4h.

In some cases, the non-dialyzed homogenate was further fractionated as described below.

(c) <u>Subcellular Fractionation</u>

The homogenate was centrifuged at 5,000xg for 20min in a Sorvall RC2 Centrifuge. The supernatant resulting from this spin was centrifuged at 104,000xg for 45min in a Spinco (model L) preparative ultracentrifuge, using the swing-out head (SW-39). The thin skin of "fat" which formed on the top surface of the supernatant was carefully removed and discarded. The supernatant resulting from this high speed spin was removed and stored at 2°C until required. The 5,000xg and 104,000xg pellets were each taken up in 5ml of 0.44M sucrose containing 0.05M Tris-HCl buffer at pH 7.4, and centrifuged again at the appropriate speeds. This washing procedure was repeated a further two times, the supernatants being discarded. The final washed 5,000xg and 104,000xg pellets were reconstituted to their original volumes in 0.44M sucrose containing 0.05M Tris-HCl buffer at pH 7.4. At all times, fractions were maintained at 2°C.

Electron microscopy of the washed pellets revealed that the 5,000xg pellet consisted predominantly of mitochondria (70%), with a small amount of mitochondrial fragments and unidentifiable organelles. The 104,000xg pellet appeared as a typical microsomal preparation.

(d) Assessment of Purity of Isolated Subcellular Fractions

(i) Localization of Cytochrome *c* Oxidase Activity

Cytochrome *c* obtained from the Sigma Chemical Co., St. Louis, Mo., U.S.A., was predominantly in the oxidised form. Before use, it was necessary to reduce the cytochrome *c* by a chemical procedure. 5mg cytochrome *c* were dissolved in 2.5ml of 0.01M phosphate buffer pH 7.0, and placed in an ice bath at $0^{o}C$. Approximately 5mg of palladium on carbon (100mg) was added to the solution and nitrogen gas was bubbled through for 5min. Hydrogen gas was then passed through the solution for 2h and finally nitrogen for 5min. The reduced cytochrome *c* solution was kept at $2^{o}C$ under nitrogen until required for use. The extent of the reduction was determined by adding 250mμmoles of cytochrome *c* before and after the reduction step to 3ml of 0.05M phosphate buffer in $1cm^2$ silica cuvettes, and the change in optical density was recorded against changing wavelength over the range 250mμ to 600mμ using a Unicam SP700 recording spectrophotometer. Fig. 1 shows the absorption peak at 550mμ, typical of reduced cytochrome *c*.

The rate of change in optical density at 550mμ was investigated for each of the subcellular fractions

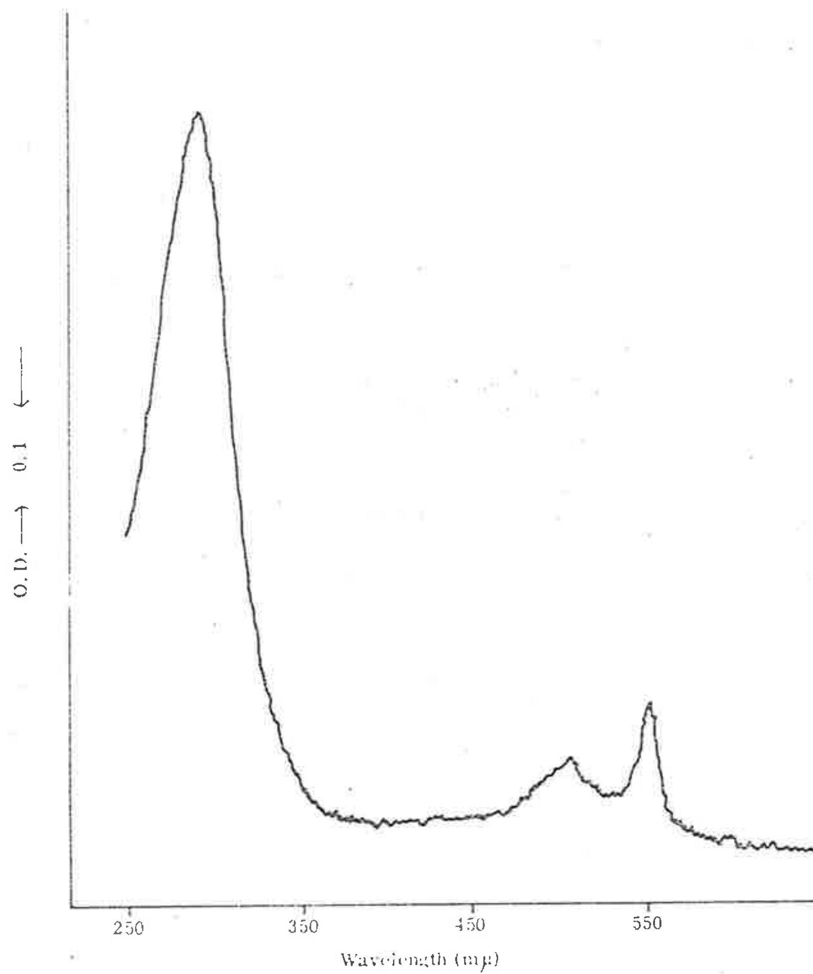


Fig.1 Absorption spectrum of reduced cytochrome c.

and for the original homogenate. The reactions were carried out at 25°C in silica cuvettes containing 3ml of 0.05M phosphate buffer at pH 7.4, containing 250mμ-moles reduced cytochrome c. The non-enzymic oxidation of the substrate was followed for several minutes prior to the addition of 20μl of the subcellular preparation. The change in optical density was followed for 10min, but all calculations were based on the initial rate for the first 2min (Fig.2). The change in optical density due to non-enzymic reactions was subtracted from the rate values obtained for each reaction.

Table 1 shows the rate of oxidation of cytochrome c with each subcellular fraction for 3 experiments. The rate is expressed as the change in optical density at 550mμ per min per ml of subcellular fraction. All fractions had been reconstituted to their initial concentrations prior to the incubation. It can be seen that the majority of the activity is in the 5,000xg pellet, with negligible amounts in the 104,000xg pellet and supernatant. The greater activity of the 5000xg pellet than the homogenate may be a result of removing an inhibitor during the purification step.

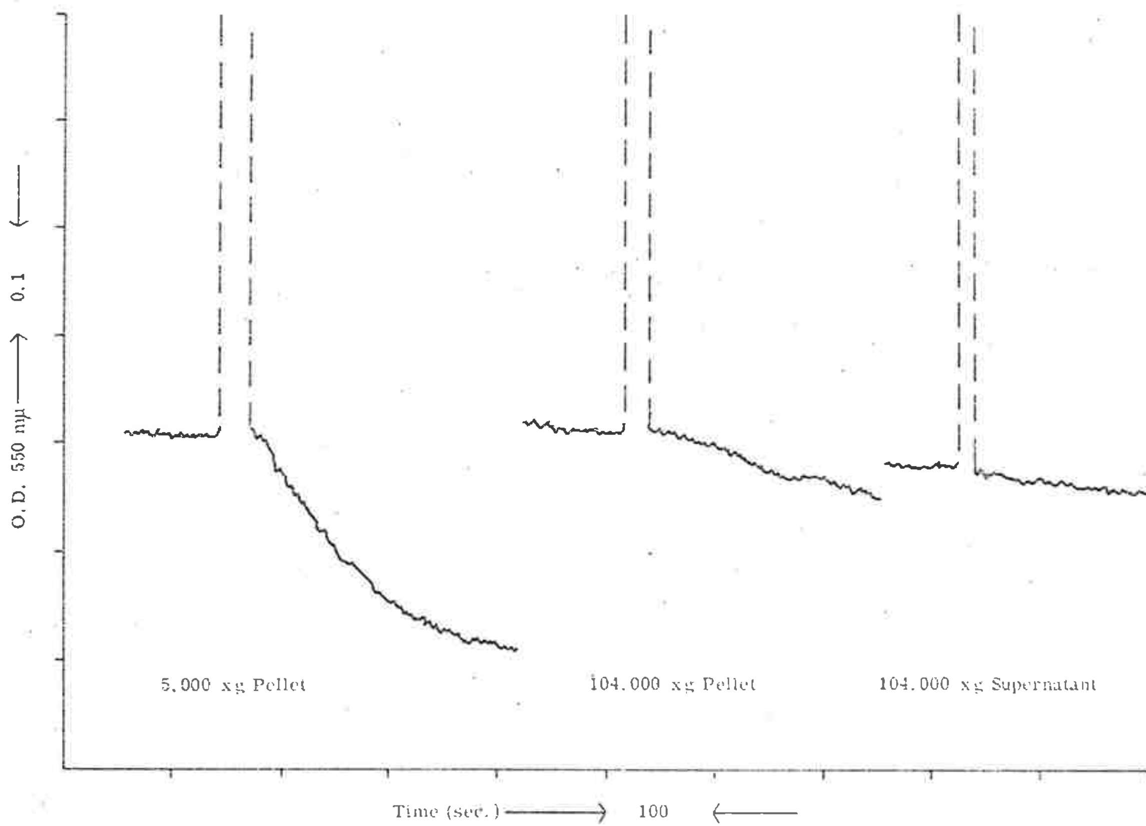


Fig.2 Localization of cytochrome c oxidase activity.

TABLE 1

OXIDATION OF CYTOCHROME c

Change in OD550 mμ (units/ml fraction)

Subcellular Fraction	Expt 1	Expt 2	Expt 3
Homogenate	3.20	1.63	0.95
5,000xg pellet	4.80	3.90	1.94
104,000xg pellet	0.60	0.50	0.14
Supernatant	0.20	0.075	0.040

It was not possible to add a larger volume of each fraction investigated, except that of the supernatant, because flocculation of the protein occurred in the cuvette. However, when 20µl or less of each fraction was added, the suspension remained homogenous throughout the period investigated.

It is assumed that the enzymes are located in one intracellular site, and that populations of subcellular fractions are enzymically homogenous.

(ii) Localization of RNA - Extraction and Estimation of RNA

The purity of the subcellular fractions was further checked by determining the RNA content of each fraction.

RNA was extracted from the subcellular fractions by the method of Woodin and Wieneke (1966). Generally, 0.5ml of a 25% tissue homogenate or 1.0ml of a reconstituted subcellular fraction was heated at 90°C for 15min with perchloric acid (0.45N final concentration), in a total volume of 2.5ml of water. The precipitate was centrifuged at 200xg for 5min and 1ml of the supernatant removed for RNA estimation by the orcinol colourimetric method (Dische, 1955).

1.0ml of RNA extract was mixed with 6ml of orcinol

reagent and heated in a boiling water bath for 25min. Orcinol reagent was prepared just prior to use by mixing 10ml of 1% orcinol with 40ml concentrated HCl and 1ml of 10% $FeCl_3.6H_2O$. The tubes were cooled and the optical density was measured at 660mμ in a Unicam SP600 spectrophotometer against a reagent blank. The standard curve is shown in Fig.3.

Whilst DNA will also give the orcinol reaction, it is likely that it will have little or no effect on the estimation of RNA, since the greatest fraction of DNA is removed with the cell nuclei. Proteins and other cell constituents also interfere, and it was therefore necessary to purify the RNA before pentose estimations.

It was necessary to dialyze those subcellular preparations that were made up in 0.44M sucrose containing 0.05M Tris-HCl buffer pH 7.4, since sucrose also interfered with the orcinol reactions.

Table 2 shows the RNA content of the whole homogenate, and the individual subcellular fractions expressed as μg RNA in 1ml of original 25% tissue homogenate. Both homogenates had about the same total amount of RNA present, although the distribution between the subcellular fractions varied. In Expt.1 most of the

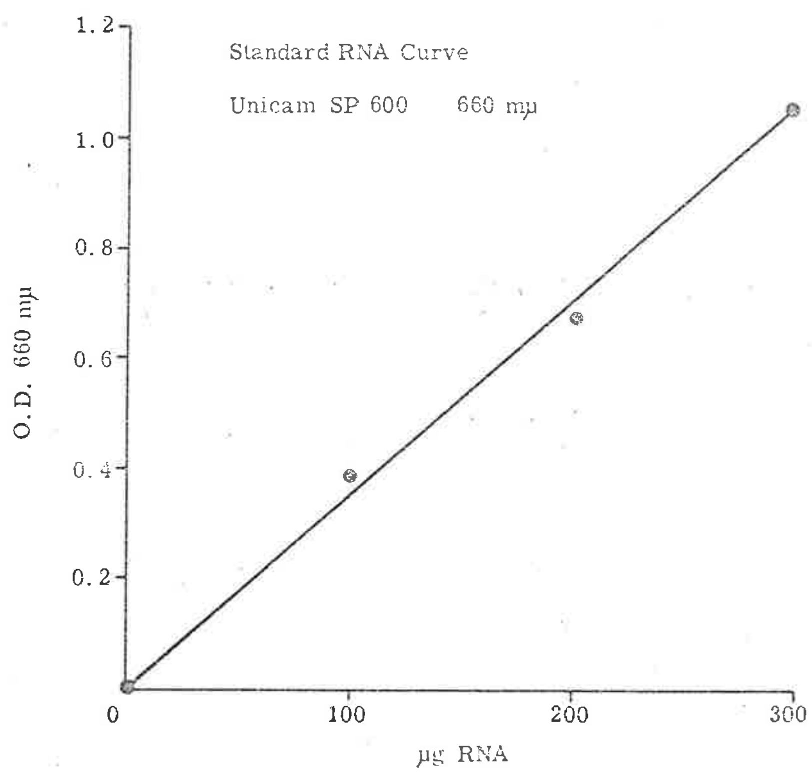


Fig.3

TABLE 2

RNA CONTENT OF SUBCELLULAR FRACTIONS

μg RNA in 1.0 ml of original 25% tissue homogenate

Subcellular Fraction	Expt 1	Expt 2
Homogenate	457	500
5,000xg pellet	105	112
104,000xg pellet	105	320
Supernatant	220	225
% Recovery	94%	130%

activity was present in the supernatant fraction, and not in the 104,000xg pellet as expected, and as found in Expt. 2.

One possible explanation for the apparent high RNA content of the supernatant fraction is that the sucrose had not been completely removed. Since both pellets can finally be resuspended in any desired buffer, it is possible to choose a medium which will not affect the orcinol reaction.

The results presented here do not confirm the purity of the subcellular fractions, possibly because of the limitations of the method as discussed above. Also, only 70% of the total RNA of liver is present in the microsomal fraction, the remainder being present in the supernatant fraction (Grant 1963).

2. Rabbit Alveolar Macrophages

Rabbit alveolar macrophages were stimulated by injecting adult rabbits with BCG (Bacillus Calmette-Guérin) vaccine by the method of Myrvik, Leake & Oshima (1962), as modified by Cohn and Wiener (1963). BCG vaccine was obtained as a lyophilized water-washed powder.

In order that it could be suspended readily it was ground with a pestle and mortar and passed through a fine wire sieve. The fine BCG powder was suspended in sterile 0.9% sodium chloride solution (20mg/ml) containing 0.01% Tween 80. 1ml of this preparation was injected into the marginal ear vein, followed by a second injection on the following day. After 4-5 weeks the rabbits were killed either by air embolism or by administering Nembutal (Abbott Laboratories, Sydney, Aust.) (60mg/kg body weight). The lungs were removed with the trachea intact. The macrophages were washed from the alveoli with four 50ml washes of 0.9% sodium chloride solution containing 0.14% disodium ethylenediaminetetraacetic acid, by syringe through the trachea.

The washings were collected in 50ml centrifuge tubes and the cells deposited by centrifuging at 220xg for 5min. The deposited cells were resuspended in Hanks solution, and the number of cells determined by counting using a haemocytometer chamber. 2-4gm wet weight of cells were obtained from each rabbit.

Whole homogenates of these cells were prepared as described above for peritoneal macrophages and were dialyzed against 4 x 1l. changes of 0.9% NaCl solution at $4^{o}C$ for a total period of 4 h.

3. Rabbit Foam Cells from Atherosclerotic Aortic Intimae

(a) Whole Cells

Foam cells were obtained by the method of Day, Newman and Zilversmit (1966). Male New Zealand albino rabbits were made atherosclerotic by feeding daily 100g rabbit chow containing 1gm of cholesterol and 3ml of peanut oil. Cholesterol was dissolved in peanut oil and ether, and the solution poured over the rabbit pellets (McMillan, Klatzo & Duff, 1954). The mixture was mixed thoroughly and air-dried to remove all traces of ether. The fatty acid composition of the peanut oil as determined by gas-liquid chromatography is shown in Table 3. The predominant fatty acid was oleic acid (56%), with lesser amounts of linoleic acid (27%) and palmitic acid (10%). After periods of 3-5 months rabbits were killed by administering Nembutal (60mg/kg body weight), and the thoracic aorta, including the arch, removed. The aorta was washed in 0.9% sodium chloride solution, the superficial fat removed and the aorta bathed in normal rabbit serum. The aorta was opened longitudinally and the intima stripped from the media and adventitia. The intima (approximately 1gm wet weight) was cut into fine pieces

TABLE 3

FATTY ACID COMPOSITION OF PEANUT OIL AS DETERMINED BY GAS-LIQUID CHROMATOGRAPHY

Fatty Acid	% Distribution
16:0	10.2
18:0	3.2
18:1	55.9
18:2	27.3
18:3	2.1
20:0	1.3

and incubated in 5ml Krebs-Ringer phosphate solution (pH 7.4), containing 4% bovine serum albumin (Commonwealth Serum Laboratories, Parkville, Vic., Aust.) (Cohn fraction $\bar{\underline{v}}$),15mg collagenase (Worthington Biochemical Corp., Freehold, New Jersey, U.S.A.), 4mg elastase (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) and 15μmoles glucose.

Incubation was carried out in 20ml plastic vials at 37°C for 2.5h, with gentle shaking. After 1h the intimal pieces were teased apart with forceps to speed up the disruption process. At the end of the incubation period, the digest was filtered through a fine wire sieve and then through a cotton gauze pad. The residue remaining on the sieve and gauze pad was washed with 1-2ml of 4% bovine serum albumin in Krebs-Ringer phosphate, and the washings combined with the original filtrate. The filtrate was centrifuged at 220xg for 5min, and the deposited cells washed twice with 0.9% sodium chloride solution and centrifuged. The washed cells were suspended in Hanks solution and an aliquot taken for cell counting in a haemocytometer chamber.

For metabolic studies, the cells were suspended

in Hanks solution containing 0.5% bovine serum albumin, dispensed into glass Leighton tubes (approximately $0.5x10^6$ cells in each), and incubated at 37°C for 60 min. During this period most of the foam cells adhered firmly to the glass, while other cells and particles remained in suspension or in loose contact with the glass. The medium was removed and the adhering foam cells were washed with warm 0.9% sodium chloride solution. Foam cells which did not adhere or were washed off were counted in order to determine the number of cells remaining.

All operations except for the final stage, were carried out in plastic containers.

The yield from such a preparation generally ranged from $2\text{-}4x10^6$ cells per rabbit.

For tissue culture studies the foam cells were isolated as described above. The operation and all subsequent manipulations, however, were carried out under sterile conditions. During removal of the aorta from the rabbit, care was taken to ensure that the oesophagous was not cut. The enzyme solution was prepared in sterile Krebs-Ringer phosphate containing 4% albumin, and then passed through a Millipore filter (pore size 0.45μ).

Incubation was carried out under the conditions described above, except that the intimal pieces were incubated in sterile plastic capped tubes. At the end of the incubation the residue was filtered through a sterile wire sieve and gauze pad, and the cells isolated by centrifugation. The cells were then suspended and washed twice in balanced salt solution containing 20 units penicillin and 0.1mg streptomycin sulphate per ml. The balanced salt solution was composed of sodium chloride (8g), potassium chloride (0.4g), glucose (1g) and 2ml of 1% aqueous phenol red solution per litre of water.

(b) Preparation of Foam Cell Homogenates

Foam cells were isolated from thoracic aortae of cholesterol-fed rabbits as described above. Generally the rabbits used had been on the cholesterol diet for 5-6 months, a greater number of cells being obtained from the more advanced lesions. The cells were homogenized in 6 volumes of 0.1M Tris-HCl buffer pH 7.4, using either a Potter Elvehjem-type homogenizer or an ultrasonicator. Complete disruption of the cells was obtained within 45sec by the mechanical method, or 10sec by ultrasonication. All procedures were carried

out at 2-4°C. The homogenate was centrifuged at 220xg for 5min and the pellet, consisting of nuclei and debris, was discarded. The supernatant was dialyzed against 3 changes of 1 litre 0.1M Tris-HCl buffer pH 7.4 for a total time of 3h, and finally against 1 litre 0.01M Tris-HCl buffer pH 7.4 for 1h.

C. Chromatographic Methods

1. Neutral-Lipid Thin-Layer Chromatography (T.L.C.)

Neutral lipids were separated on a 0.2mm thickness of Silica Gel G (Merck AG, Darmstadt, Germany) containing a fluorescent indicator Ultraphor (Badische-Anilin am Rhine, Cologne, West Germany) (5mg Ultraphor per 30g Silica Gel G). Plates were generally of the size 20x20cm, although plates 20x4.8cm were used when radioactive strip scanning was required. All plates were activated at 110°C for 60min prior to use. Lipid samples to be separated (maximum total lipid 1.5mg) were applied to the plate under a stream of nitrogen. Separation of the major classes of lipids (mono-, di- and triglycerides, phospholipids, cholesterol, fatty acids and cholesterol esters), was performed in sealed glass tanks using the developing solvent n-hexane:

diethylether:glacial acetic acid (100:38:3, v:v:v). The plates were developed by ascending chromatography for 20-25min, dried in air at room temperature, and the individual lipid spots shown up either under ultraviolet light or in iodine vapour.

2. Phospholipid Separations

(a) One-Dimensional T.L.C.

Lipid extracts were first run on a neutral lipid plate to separate neutral lipids from phospholipids, The phospholipids (origin) were scraped from the plate and eluted from the silica by the method of Arvidson (1967). The dried samples were then ready for phospholipid T.L.C.

Phospholipids were separated by T.L.C. by the method of Skipski, Peterson and Barclay (1964), using basic Silica Gel G (Camag, without $CaSO_4$ binder). The silica was made basic by preparing the slurry with 0.001M sodium carbonate solution and plates 20x20cm were coated with the slurry to a thickness of 0.5mm. The plates were activated at 110°C for 1h prior to use. The solvent system, chloroform:methanol:glacial acetic acid: water (25:15:4:1.9, v:v), allowed the complete separation of lysolecithin, sphingomyelin, lecithin, phosphatidylin-

ositol, phosphatidylserine and phosphatidylethanolamine. The plates were developed by ascending chromatography for 2h, dried in air at room temperature, and the individual lipid spots shown up with iodine vapour or with phosphate spray (Dittmer & Lester, 1964).

Adequate separations were obtained with up to 100µg of each individual phospholipid.

(b) Two-Dimensional T.L.C.

The two-dimensional separation of phospholipids was carried out using the method of Rouser, Kritchevsky, Gelli and Heller (1965). Glass plates, 20x20cm, were spread with Silica Gel G (Merck) to a thickness of 0.2mm and activated at 110°C for 60min. The sample to be chromatographed was applied to the lower right hand corner, and the plate developed in chloroform:methanol: water (65:35:5, v:v:v) for about 1h. The plate was removed from the tank, air dried quickly and developed at right angles to the original development in chloroform:methanol:acetone:glacial acetic acid (5:1:1:0:5, v:v) for about 2h. The plate was dried and set up for autoradiography using Agfa-Gevaert Osray film. The film was exposed overnight (approx. 15,000 d.p.m. total radioactivity was present) and the plate stained for phosphorus (Dittmer and Lester, 1964).

(c) Paper Chromatography

In early experiments (Section 3) phospholipids were separated on silicic acid-impregnated paper as described by Marinetti, Erbland and Kochen (1957), Marinetti and Stotz (1960) and Marinetti (1961). Whatman No.1 filter paper was cut into sheets 19x54cm and dipped into a solution of sodium silicate (104g of silicic acid - Mallinckrodt Chemical Works, N.Y., U.S.A., dissolved in 330ml of 7.2N sodium hydroxide). The papers were dipped individually and drained for 5min to remove excess sodium silicate and then immersed in 6N hydrochloric acid for 30min. Finally the papers were washed in running tap water for 2h, rinsed in distilled water for 1h, and allowed to dry at room temperature.

Separation of phospholipids was carried out in large chromatographic tanks by ascending chromatography for 16-20h. The solvent system consisted of di-isobutylketone:glacial acetic acid: water (40:25:5, v:v:v). Loading of the paper was kept below 30ug total phospholipid per spot (Fidge, 1964).

(d) Silicic Acid Column Chromatography

Where larger amounts of phospholipids were fractionated, silicic acid column chromatography was used, as described by Newman, Ching-Tong Liu and Zilversmit (1961). Silicic acid (Bio-Rad Laboratories, Richmond, Calif., U.S.A.) was activated at 120-130°C for 48h prior to use. The silicic acid was cooled, slurried with chloroform and transferred to a column of 1cm diameter. The flow rate was adjusted to 0.5ml/min. Loads of up to 1.4mg phospholipid-phosphorus per 4g silicic acid were added in chloroform and allowed to enter the column. Neutral lipids were eluted with 30ml chloroform. Non-choline containing phospholipids were eluted with 50ml chloroform:methanol (4:1, v:v), and lecithin with 130ml chloroform:methanol (3:2, v:v). 70ml of chloroform:methanol (2:3, v:v) were required to elute sphingomyelin and finally, lysolecithin was eluted with 50ml methanol.

All fractions were checked for purity by thin-layer chromatography (Skipski, Peterson & Barclay, 1964).

3. Separation of Free and Esterified Cholesterol

(a) T.L.C. on Microscope Slides

Excellent separations of free and esterified cholesterol could be obtained rapidly by T.L.C. on microscope slides (25x75mm) by modification of the technique described by Vahouny, Borja and Weersing (1963) and Kelly and Newman (1964). Two slides were placed "back to back" and dipped into a homogeneous suspension of Silica Gel G in water, (25g/100ml water) containing a fluorescent indicator, Ultraphor. The slides were separated and activated at 110°C for 60min. The sample to be chromatographed (maximum 200ug lipid) was spotted 1cm from the bottom of the plate and developed for 10min in Coplin jars containing 3ml xylene. The spots were visualized under ultraviolet light. Cholesterol ester ran with the solvent front while free cholesterol ran with an R_f of 0.2. More reproducible separations were obtained by preparing the silica slurry with water instead of using organic solvents as described by the above authors.

(b) Alumina Column Chromatography

The separation of free and esterified cholesterol on alumina columns was carried out as described by Kerr and Bauld (1953). Columns 3/16" in diameter and 4" long with a 12ml reservoir at the upper end were packed to a height of 2.5cm with a suspension of washed aluminium oxide (Savory and Moore Ltd., London, England, "for chromatographic

analysis standardized according to Brockmann") in petroleum spirit (B.P. 60-80°C). The sample containing free and esterified cholesterol was applied to the column in 0.5ml petroleum spirit and petroleum spirit was added to the reservoir and 18ml of column effluent collected. This fraction contained esterified cholesterol. The excess petroleum spirit in the reservoir was replaced with 6ml benzene which eluted the free cholesterol. The column fractions were taken to dryness and saponified (Abell, Levy and Brodie, 1952), and cholesterol determined by the method of Zlatkis, Zak and Boyle (1953) as described below.

4. Separation of Cholesterol Esters by T.L.C. on Silver Nitrate-Impregnated Silicic Acid

Isolated cholesterol esters (obtained by neutral lipid T.L.C.) were separated into groups depending on the degree of unsaturation of the fatty acid. Plates were coated with Silica Gel G as previously described, and when dry were placed in a tank containing 3% silver nitrate in methanol, and the solution was allowed to run up the plate. Plates were developed in the silver nitrate solution for

approximately 2-3h. The impregnated plates were dried and activated at 110°C for 20min. Samples (containing up to 0.5mg total esters per spot) were applied to the plate under a stream of nitrogen, and the plate developed in n-hexane:diethylether (90:10, v:v) for 20min.

Visualization of the individual cholesterol esters was obtained by spraying the plate with 0.2% dichlorofluorescein in ethanol and viewing under ultraviolet light.

5. Gas-Liquid Chromatography of Methyl-Fatty Acids

(a) Preparation of Methyl Esters for G.L.C.

Methyl esters of fatty acids in lipid fractions were prepared for G.L.C. analysis by heating lipid samples (up to 10mg) in 1ml of 5% H_2SO_4 in methanol for 16h at 65°C, in sealed 5ml ampoules. Then 2ml of petroleum spirit (30-40°C) and 1ml water were added and mixed. The petroleum spirit was removed and transferred to a small stoppered tube. This extraction procedure was repeated three times and the petroleum spirit washings combined and stored at 2-4°C. Prior to each analysis, samples were blown down with nitrogen to a suitable volume, and injection volumes

ranging from 1-5μl were either injected directly into the injection port, or evaporated to dryness on a stainless-steel pellet for solid loading.

(b) Separation of Methyl Esters

The separation of fatty acid-methyl esters was carried out on 1/8" columns using an F & M (Hewlett Packard) 5750 Gas Chromatograph with argon as carrier gas, and diethylene glycol succinate on Gas Chrom P (Applied Science Laboratories, State College, Pennsylvania, U.S.A.) at 180°C, as the stationary phase. The injection port and the detector temperatures were maintained at 295°C and 250°C respectively.

(c) Gas-Liquid Radiochromatography

Lipid fractions for radiochromatography were methyl-esterified as described above, and gas-liquid radiochromatography of the ^{14}C-labelled methyl oleate from each fraction performed using an F & M (Hewlett Packard) 5750 Gas Chromatograph modified for solid loading, and using argon as carrier gas. Conditions were as set out above. The ^{14}C-labelled methyl-fatty acid in the column effluent was combusted and measured

as $^{14}CO_2$ using a Pye Radio-Chromatograph. A splitter was located in the column ahead of the detector, allowing one fifth of the column effluent for chemical analysis, and the remainder for ^{14}C-analysis. Fatty acid mass was measured as peak height by retention time, while the amount of ^{14}C-label present was recorded linearly using the integrated range of the rate meter. Unless otherwise indicated, total counts ranging from 50-440 were recorded and duplicate runs were routine. Calibration with methyl-[1-^{14}C]-labelled palmitate (specific activity 0.0811μCi/mg), enabled measurements of specific activity of the ^{14}C-labelled oleate from cholesterol ester, phospholipid and triglyceride of the foam cell extracts to be carried out.

D. Radioactive Assay

All counting was performed on ^{14}C-labelled isotopes.

1. Radioactive Scanning

Early experiments were analyzed using a Nuclear Chicago 4π Actigraph II Chromatogram Scanner, Model 1030, modified to take both paper chromatographic strips and thin-layer chromatographic plates. Where

thin-layer chromatographic plates were scanned, of course only one side of the plate was counted. The areas of the peaks obtained were determined by triangulation, and by comparison with a known standard, a quantitative assessment of the distribution of radioactivity could be obtained.

Certain limitations are applicable to the use of this method for counting T.L.C. plates, since any variation in the thickness of the silica produced a marked effect on the number of counts recorded. However, this technique was used for those experiments described in Section 3. Repeat experiments using a more reliable method of counting (liquid scintillation counting of silica scrapes) revealed the same overall pattern, although individual lipids differed by about 10% from the original experiments.

2. Liquid Scintillation Counting

(a) Early scintillation counting was performed on an Ecko N662 Liquid Scintillation Counter. Scintillator used for lipid extracts was 5ml of 2,5-diphenyloxazole (PPO)(0.3% w/v) in toluene. Counting efficiency was 75%.

(b) In later experiments all liquid scintillation counting was performed on a Packard Tri-Carb Liquid Scintillation Spectrometer, Model 3375.

Scintillators used were:

(i) Toluene scintillator

PPO (0.4% w/v) and 1,4-bis-2-(4-methyl-5-phenoxazolyl)-benzene (dimethyl-POPOP) 0.01% w/v POPOP in toluene were used for non-aqueous preparations.

(ii) Gordons scintillator (Gordon & Wolfe, 1960).

Gordons scintillator was used for counting up to 1ml of aqueous solution per 20ml scintillator with an efficiency of 65%. The scintillator was prepared by mixing 80g napthalene, 5g PPO, 50mg POPOP with 385ml xylene, 385ml 1,4-dioxane and 231ml absolute ethanol.

(iii) Snyders scintillator (Snyder, 1964).

Snyders scintillator was used for counting T.L.C. scrapes directly without eluting the labelled lipids from the silica. The scintillator was prepared by dissolving 10.5g PPO, 0.45g dimethyl-POPOP and 150g napthalene in 1,4-dioxane, and made up

to 1,500ml with 1,4-dioxane. 300ml of water was added to give a fully quenched solution. Up to 1g of silica has been used per 10ml of scintillator without further quenching, and an efficiency of 70% was routinely obtained.

E. Chemical Assay

1. Phosphorus Determination

Phosphorus was determined by the methods of Bartlett (1959) and Morrison (1964). Aliquots of lipid extracts for phosphorus determination (1-4μg phosphorus) were evaporated to dryness. A series of standard phosphorus solutions were prepared from disodium hydrogen phosphate (Analar, British Drug Houses, Poole, England) containing 0, 1, 2, 3 and 4μg phosphate-phosphorus. 0.2ml concentrated sulphuric acid was added to all tubes, followed by heating at 200°C for 60min. 1 drop of 30% hydrogen peroxide (phosphorus-free) was added and the tubes heated for a further 40min at 200°C to decompose the hydrogen peroxide. After cooling, 4.6ml of 0.22% ammonium molybdate solution was added to all tubes and immediately shaken, followed by 0.2ml of Fiske-Subbarow solution.

Tubes were covered and placed in boiling water for 15min, cooled and read at 820mμ on a Unicam SP600 spectrophotometer. The standard curve is shown in Fig.4.

Fiske-Subbarow solution was prepared by mixing the following reagents in the order of (a) 0.25g 1-amino-2-napthol-4-sulphonic acid (Hopkin & Williams Ltd., Essex, England, (b) 100ml of 15% anhydrous sodium bisulphite solution, and (c) 0.5g anhydrous sodium sulphite solution. The mixure was shaken, filtered and stored in the refrigerator.

2. Cholesterol Determination

For determination of total cholesterol, samples were first saponified by the method of Abell, Levy and Brodie (1952). 1.0ml of an evaporated lipid extract (up to 5mg cholesterol) was heated at 60°C for 60min with 5ml of 2% KOH in 95% ethanol in a stoppered test tube. Standards and blanks were included. The saponified cholesterol was extracted with 10ml petroleum spirit (BP 60-80°C). An aliquot of the extract was taken for cholesterol determination by the method of Zlatkis, Zak and Boyle (1953).

Aliquots (containing 10-150μg cholesterol) were evaporated to dryness and taken up in 3ml glacial

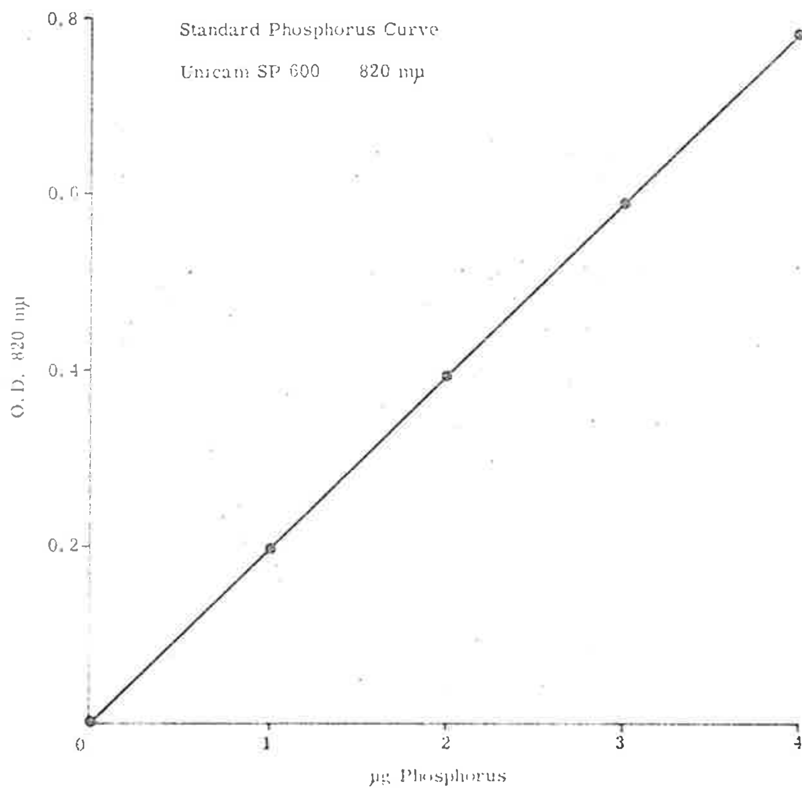


Fig.4

acetic acid. 2ml of ferric chloride reagent was added and mixed thoroughly. The ferric chloride reagent was prepared by slowly adding 99ml of concentrated sulphuric acid to 1ml of 10% $FeCl_3.6H_2O$ in glacial acetic acid. The optical density was measured against the saponified "blank" at 550mμ in a Unicam SP600 spectrophotometer. The standard curve is shown in Fig.5.

3. Protein Determination

Protein was determined using the Folin-Ciocalteu reagent (Folin & Ciocalteu, 1927) as described by Lowry, Rosebrough, Farr and Randall (1951).

Folin-Ciocalteu phenol reagent (May & Baker, Dagenham, England) was diluted with an equal volume of distilled water prior to use. A second reagent was prepared freshly by mixing 100ml of 2% sodium carbonate in 0.1N sodium hydroxide with 1ml of 2% sodium-potassium tartrate in water, and 1ml of 1% hydrated copper sulphate in water. 6.5ml of this reagent mixture was added to 1.0ml protein solution containing up to 300μg protein, mixed and allowed to stand for 10min. Folin-Ciocalteu reagent

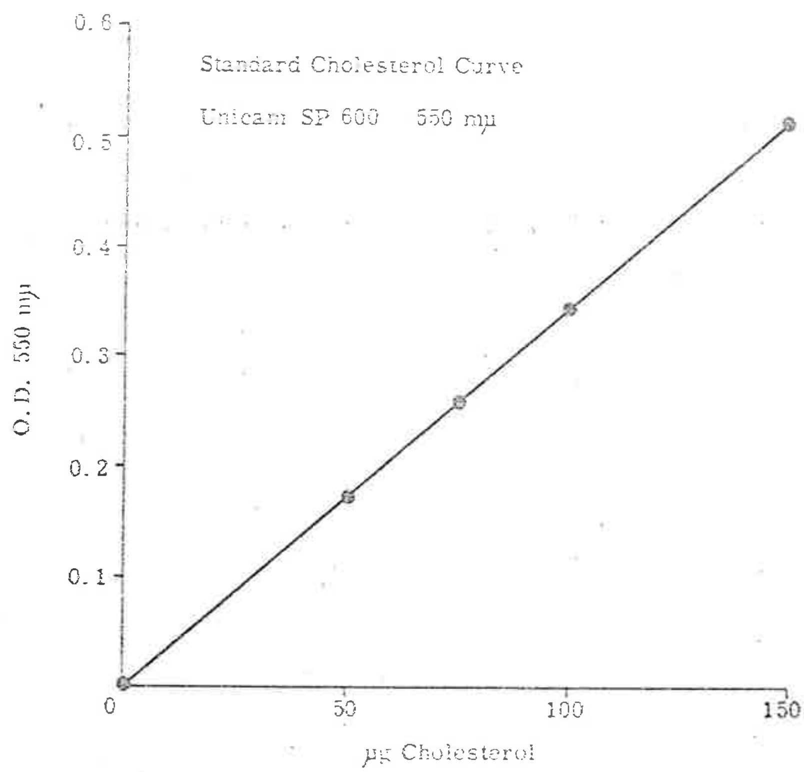


Fig.5

(0.6ml) was then added and mixed immediately. The optical density at 750mμ, measured against a reagent blank, was determined after 1h and compared with a set of standard protein solutions. Human serum albumin (Commonwealth Serum Laboratories, Parkville, Vic., Aust.) was used as a standard. The standard curve is shown in Fig.6.

4. Histochemical Determination of Acid Phosphatase Activity

Acid phosphatase activity was determined by the simultaneous coupling azo-dye method using a substituted naphthol as described by Burstone (1958), and modified by Barka and Anderson (1963). Approximately 2×10^5 isolated foam cells were incubated for 1h at 37°C on glass coverslips in perspex slide chambers, with 0.3ml of substrate solution at pH 5.0.

The substrate solution was prepared from four stock solutions.

(1) 4% para-rosanalin-HCl (British Drug Houses, Poole, England) in 2N HCl. A solution was obtained by heating gently, cooling and filtering.

(2) 4% sodium nitrite solution

(3) 0.14M Michaelis-veronal-acetate buffer pH 5.0.

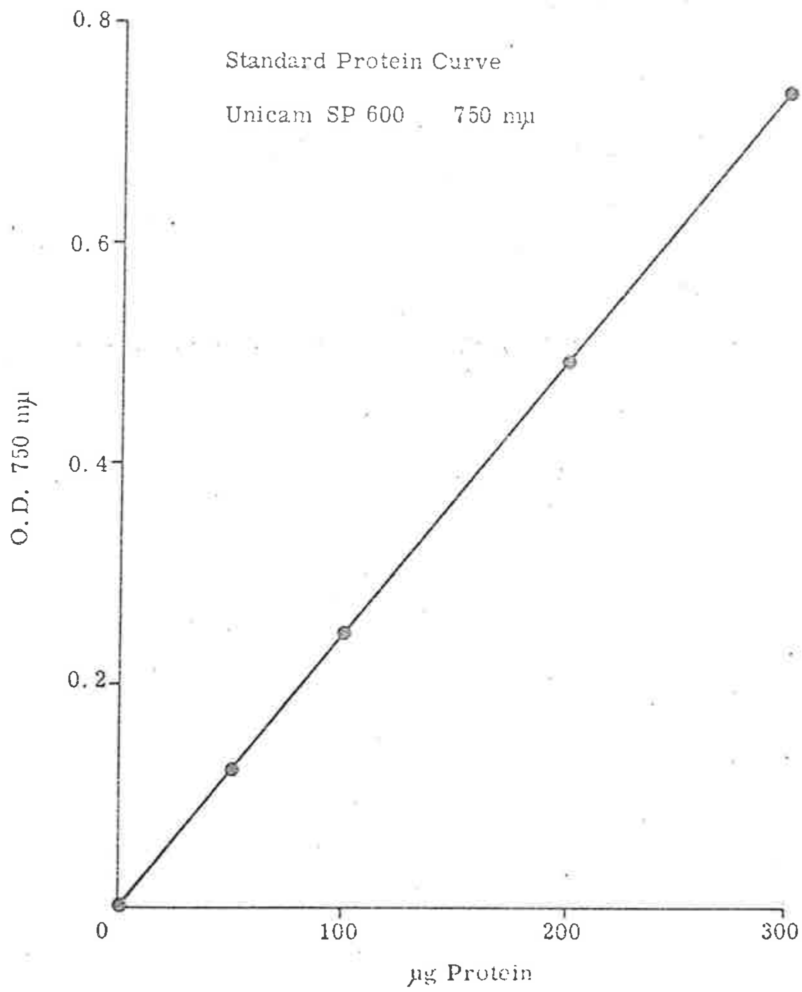


Fig. 6

(4) 1% naphthol AS-TR phosphate (Sigma Chemical Co., St.Louis, Mo., U.S.A.), in N,N-dimethylformamide. 5ml of buffer solution (3) was diluted to 17ml with distilled water, and was mixed with 1ml of stock solution (4). 0.8ml of stock solutions (1) and (2) were mixed together and then poured into the buffered substrate solution.

The pH of the final substrate solution was adjusted to pH 5.0 with 1N NaOH.

At the end of the incubation, the cells adhering to the coverslips were rinsed in 0.9% sodium chloride and viewed microscopically as an aqueous preparation. Control incubations of cells were set up in which either the substrate, naphthol AS-TR phosphate, had been omitted, or the cells had been maintained at 60°C for 10min prior to incubation.

SECTION 1.

CHOLESTEROL ESTERIFYING ACTIVITY OF CELL-FREE PREPARATIONS OF RABBIT PERITONEAL MACROPHAGES

A. Introduction

The presence in various types of RE cells of enzymes capable of synthesizing and hydrolyzing cholesterol ester has now been well established. Intraperitoneal injection of suspensions of either free cholesterol or cholesterol ester into rats is followed by the uptake of such suspensions by the RE cells of the sternyl lymph nodes, (Day & French 1959; Day & Gould-Hurst, 1961). Where cholesterol uptake was studied, an increase in the cholesterol ester content of the cells occurred; where cholesterol ester uptake was investigated, there was an increase in the free cholesterol content, indicating that these RE cells had the capacity to both hydrolyze and to synthesize cholesterol ester.

Rabbit peritoneal macrophages incubated _in vitro_ have also been shown to take up and esterify ^{14}C-labelled cholesterol preparations (Day & Gould-Hurst, 1961), and preliminary experiments carried out with cell-free preparations of these cells have confirmed the presence of an enzyme or enzymes capable of esterifying free cholesterol, and of hydrolyzing cholesterol esters (Day, 1960; Day & Gould-Hurst, 1963;

Day, Gould-Hurst & Wahlqvist, 1964). In this section the cholesterol esterifying enzyme(s) of peritoneal macrophages has been further studied and its characteristics with respect to pH optimum, cofactor requirements and possible mechanism of action in homogenates and its subcellular localization are described.

B. pH Optimum for Cholesterol Esterification

The effect of pH on the esterification of cholesterol was investigated by incubating ^{14}C-labelled cholesterol with the macrophage homogenate. The system contained 0.7ml of 0.2M citrate-phosphate buffer over the range pH 5.5-7.2, 0.2ml dialyzed homogenate (containing a known amount of protein, 1-2mg), and 50µl of acetone containing 1mg oleic acid, 250µg cholesterol together with a known amount of ^{14}C-labelled cholesterol. The system was made up to a total volume of 1.1ml with 0.2M citrate-phosphate buffer, and duplicate incubations were carried out in 12ml centrifuge tubes at 37°C for 15h with continuous shaking. This procedure was used in the remainder of the experiments described in this section except where otherwise indicated. The pH of the incubation mixture was measured before and after incubation, and the pH of the medium was found to remain

constant throughout the incubation. In each experiment controls with no homogenate were set up, and in some experiments controls containing homogenate which had been heated at 100°C for 10min were tested.

The incubations were stopped by the addition of chloroform:methanol (2:1, v:v), and the lipid extracted according to the method of Folch, Lees and Sloane-Stanley (1957). Duplicate aliquots of the lipid extracts were taken for separation of cholesterol and cholesterol ester by thin-layer chromatography, as described under Methods. Areas corresponding to free and ester cholesterol were scraped from the thin-layer plate and counted for radioactivity.

The cholesterol esterifying enzyme activity of peritoneal macrophage homogenates is expressed as mμmoles cholesterol esterified per mg protein per h. Thus for each experiment, knowing the percentage esterification of cholesterol and the amount of cholesterol present, it is possible to determine the amount of cholesterol esterified in mμmoles. In each experiment the total cholesterol contributed by the homogenate was determined, and was found to be approximately 50μg (85% free cholesterol). This figure

was considered in the total cholesterol present when calculating activity, although it is probable that most of the endogenous cholesterol is firmly bound within subcellular membranes. However, the 250μg of exogenous cholesterol added is greatly in excess of any free endogenous cholesterol present, so that any difference in calculating with the endogenous cholesterol omitted would be minimal.

No significant esterification of cholesterol occurred in controls incubated with ^{14}C-labelled cholesterol in the absence of homogenates, or in controls where the homogenate was boiled prior to incubation. The small control values obtained however were subtracted from the percentage esterification values of the test samples.

Investigation of the esterification of cholesterol at various pH values over the range pH 5.5-7.2 is given for one representative experiment in Fig.7. Maximal esterification occurred at pH 6.3 and fell off sharply on either side. There was negligible esterification above pH 7.0. It will be noted that the esterifying activity is directly proportional to the pH of the medium over the range pH 5.4-6.2.

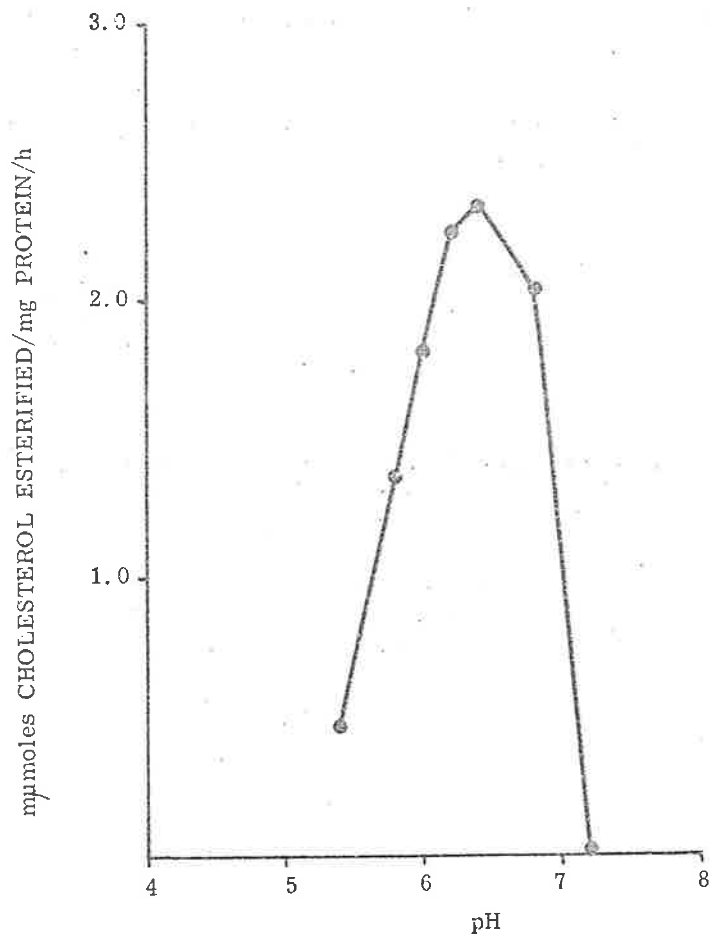


Fig.7 Effect of pH upon the rate of cholesterol esterification by peritoneal macrophage homogenates.

C. Effect of Cholesterol Concentration on the Rate of Esterification

The amount of cholesterol esterified after 0, 5, 10, 15 and 20h incubations with various amounts of carrier cholesterol added is given in Fig.8. These experiments were carried out at pH 6.3 as described above. Esterification was essentially linear over the first 15h in each case, except where no carrier cholesterol was added. In this case the rate of esterification fell off after the initial 5h. Where 1,000μg cholesterol was added the reaction was linear over the whole 20h period. In these and all other experiments the amounts of substrates and cofactors stated refer to those present in the incubation.

D. Effect of Oleic Acid Concentration on Rate of Esterification

Fig.9 shows the effect of increasing the amount of oleic acid added on the rate of esterification of cholesterol. In this single experiment, incubation was carried out for 15h under the conditions described previously, with 250μg of cholesterol and various amounts of oleic acid added. The rate of esterification of cholesterol was calculated as mumoles esterified per mg protein per h, assuming linearity over this period. The endogenous free fatty acid was determined in similar homogenate preparations by the method of Dole (1956),

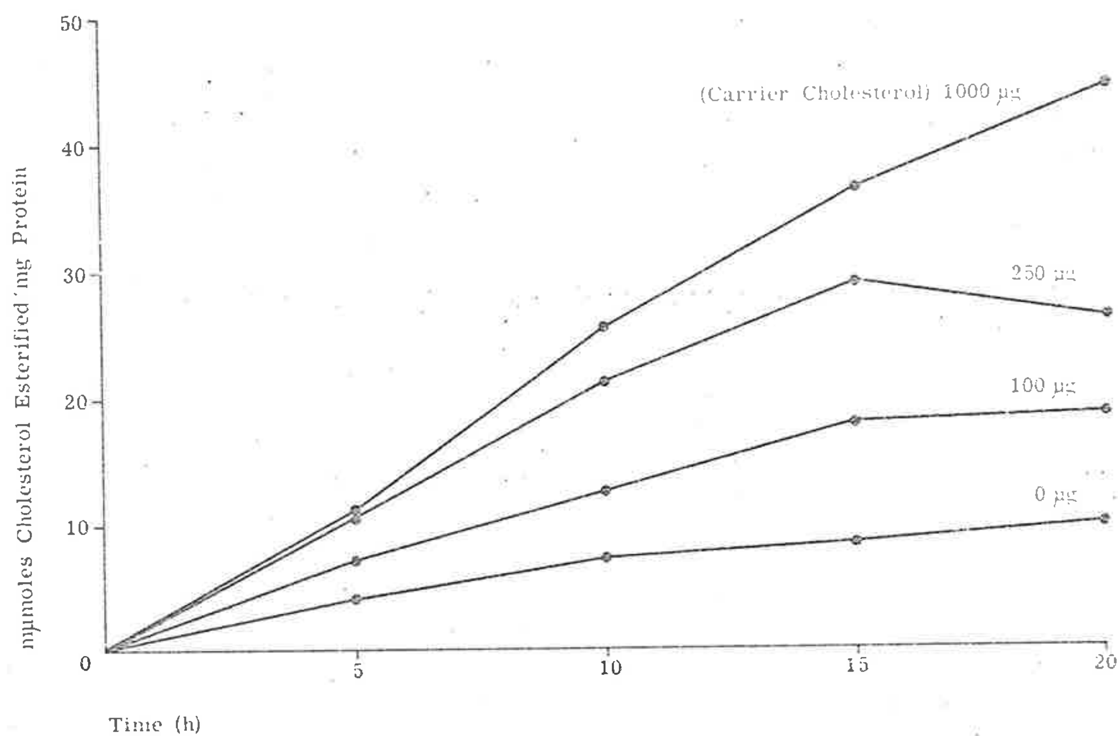


Fig.8 Effect of time upon the esterification of cholesterol at various cholesterol concentrations by peritoneal macrophage homogenates.

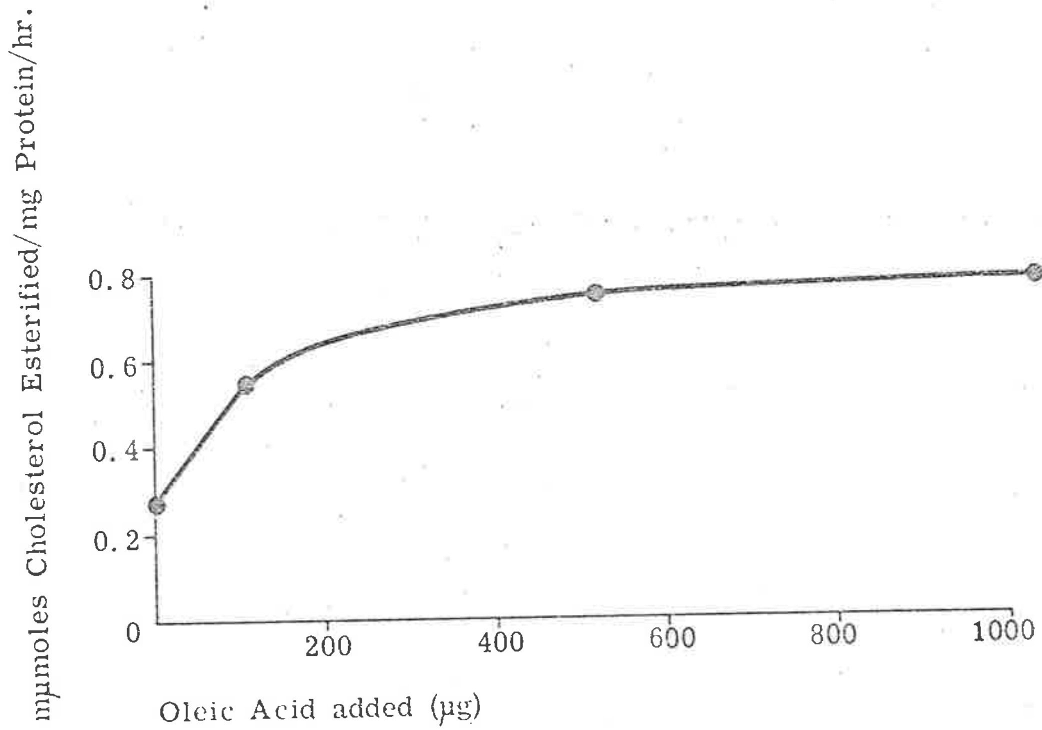


Fig. **9** Effect of addition of oleic acid upon the rate of cholesterol esterification by peritoneal macrophage homogenates.

and was found to be approximately 0.7μeq free fatty acid (equivalent to 200μg long-chain free fatty acid) per incubation.

Considerable esterification occurred without the addition of exogenous free fatty acid, and the rate of esterification increased up to a maximum with 500μg of added oleic acid. Amounts of up to 1,000μg did not inhibit the rate of esterification.

E. Effect of Cofactors ATP and CoASH on the Rate of Cholesterol Esterification

In the experiments described above, cholesterol esterification proceeded in the absence of added cofactors. That ATP and CoASH had been removed from the homogenates during dialysis was checked by investigating the ability of such homogenates to incorporate ^{14}C-labelled fatty acid into glycerolipids in the presence and absence of added cofactors. The ability of peritoneal macrophages to incorporate ^{14}C-labelled palmitic acid into combined lipids in the presence of ATP and CoASH is described in a later section of this thesis. Where both or either cofactors were omitted from the incubation system, the homogenate failed to incorporate fatty acid into combined lipids.

Use was therefore made of this property to check that the cofactors had been completely removed from the dialyzed homogenates in the cholesterol

esterification experiments. A fraction of each dialyzed homogenate was taken for (1) fatty acid esterification and (2) cholesterol esterification in the presence and absence of cofactors ATP and CoASH. In this way, it was possible to have a direct control; where no fatty acid esterification occurred without cofactors added, it could be assumed that endogenous cofactors had been completely removed during the dialysis period.

To investigate the fatty acid esterification, the system used was essentially that described in Section 3. The complete system contained in a total volume of 1.2ml, 200μmoles Tris-HCl buffer pH 7.4, 0.3ml dialyzed homogenate (2.4mg protein), 10μmoles ATP, 1μmole CoASH, 5μmoles $MgCl_2$, 5μmoles DL-α-glycerophosphate, and 25μl of a known amount of [1-^{14}C]-labelled oleic acid in 0.05N NaOH. Incubations were performed at 37°C for 90min. The reactions were then stopped and the lipid extracted and separated as described under Methods.

Table 4 shows that such homogenates incorporate ^{14}C-labelled oleic acid into both phospholipid and

TABLE 4

(a) INCORPORATION OF [1-^{14}C] OLEIC ACID INTO COMBINED LIPID FRACTIONS

% distribution of lipid

	Expt 1*					Expt 2*				
System	PL	MG & DG	FA	TG	CE	PL	MG & DG	FA	TG	CE
Complete†	51	6.7	13	24	5.2	49	9.9	12	28	2.6
- ATP	2.3	2.2	95	0.4	0.5	2.2	2.5	94	0.5	1.0
- CoASH	11	1.8	79	7.6	1.0	5.5	3.1	89	1.3	1.2
- ATP & CoASH	1.9	2.0	96	0.3	0.5	1.8	2.8	93	0.9	1.2
Control	1.7	2.5	96	0.2	0.2	1.1	2.4	95	1.1	0.6

(b) ESTERIFICATION OF [4-^{14}C] CHOLESTEROL

mμmoles cholesterol esterified/mg protein/h

No additions ϕ	1.76	1.45
+ ATP (10 μmoles), + CoASH (1μmole), + Mg^{++} (5 μmoles)	1.92	1.55
+ ATP (10 μmoles), + Mg^{++} (5 μmoles)	1.42	1.17
+ CoASH (1 μmole)	1.69	1.70

* Phospholipid (PL), monoglyceride (MG), diglyceride (DG), fatty acid (FA), triglyceride (TG), and cholesterol ester (CE).

† System as given in text, page 76 Incubations performed at pH7.4

ϕ System as given in text, page 70 Incubations performed at pH6.3

triglyceride in the presence of ATP and CoASH. Where both or either of these cofactors were absent there was no significant incorporation of ^{14}C-labelled oleic acid into glycerolipids. It was therefore concluded that no significant amounts of ATP or CoASH remained in the homogenate after dialysis, or were synthesized during the incubation.

The experiments described above using ^{14}C-labelled oleic acid were carried out to demonstrate that the cofactors ATP and CoASH had been completely removed from the homogenate. This same homogenate which was proved to be free from cofactors and cofactor synthesizing systems was then used in the second part of the experiment to demonstrate that cholesterol esterification does not require these cofactors.

Table 4 also shows the effect of cofactors on the esterification of ^{14}C-labelled cholesterol by the same homogenates used to test the esterification of oleic acid. The addition of the cofactors, either alone or together, produced an insignificant change in the rate of esterification.

Increasing the concentration of either ATP or CoASH at constant levels of the other, failed to increase the amount of cholesterol esterified (Fig.10).

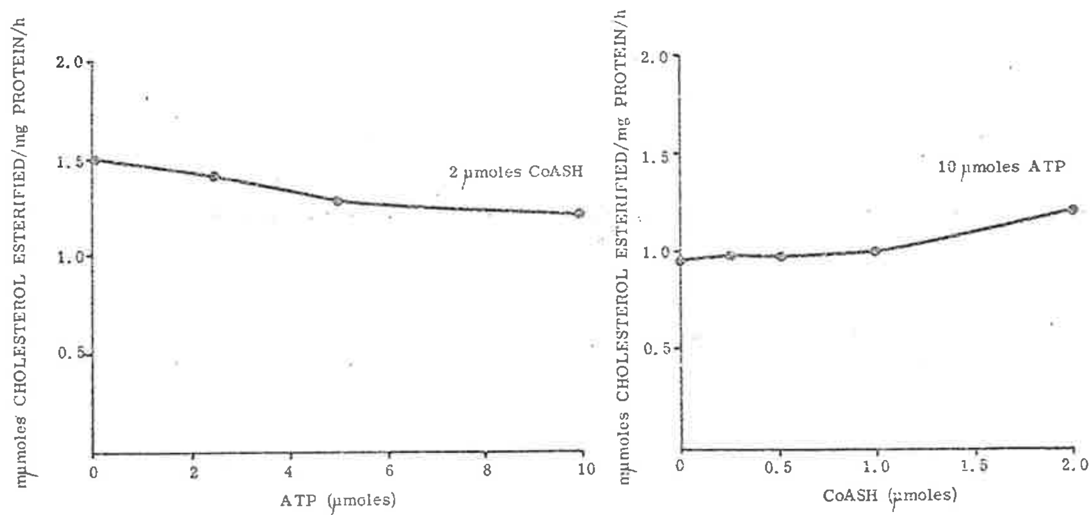


Fig. 10 Effect of various cofactor concentrations upon the esterification of cholesterol by peritoneal macrophage homogenates.

(a) Shows the esterification at constant CoASH concentration and varying ATP amounts.

(b) Shows the esterification at constant ATP concentration and varying CoASH amounts.

F. Investigation of Lecithin:Cholesterol Acyltransferase Type Reaction

The preceding experiments have established that cholesterol esterification can proceed in the absence of ATP and CoASH, and it is assumed that the free fatty acid present provides the source of fatty acid for such esterification. However, it is possible that the reaction is of the transesterification type described by Glomset (1962) for serum cholesterol esterifying activity, lecithin providing the source of fatty acid. To test this possibility, lecithin labelled predominantly in the β-position with ^{14}C-labelled fatty acid was prepared biosynthetically using peritoneal macrophage homogenates as described under Methods.

For these experiments, triplicate incubations were set up containing 0.2ml homogenate (1-2mg protein) in a final volume of 1.1ml of 0.2M citrate phosphate buffer pH 6.3. In these experiments, lecithin (300μg) was added to each tube in petroleum spirit and the solvent was removed by evaporation. 0.8ml of citrate-phosphate buffer pH 6.3 was then added and each tube was sonicated for 1 min to form a suspension of lecithin in buffer. Cholesterol (250μg) and oleic acid

(100µg) were added in 50µl acetone. The first tube contained in addition a tracer dose of ^{14}C-labelled cholesterol, the second a tracer dose of ^{14}C-labelled oleic acid and the third, a tracer dose of ^{14}C-labelled fatty acid labelled lecithin. In each case where required, the labelled substrate was added with its unlabelled substrate. After incubation for 15h at 37°C, the reaction was stopped and the lipid extracted as described under Methods. The lipid extracts were separated into cholesterol, fatty acid, phospholipid and cholesterol ester by thin-layer chromatography, and counted for radioactivity. Table 5 gives the results for two such experiments. In the first experiment, lecithin was labelled in the β-position with (1-^{14}C)-labelled palmitic acid, and in the second experiment with (1-^{14}C)-labelled oleic acid. The (1-^{14}C)-labelled oleic acid and the (1-^{14}C)-labelled fatty acid-lecithin were added to the respective incubation medium in approximately equimolar amounts. In each experiment both ^{14}C-labelled cholesterol and ^{14}C-labelled oleic acid were converted to cholesterol ester. There was almost a mole for mole conversion of cholesterol and oleic acid to cholesterol ester. However, there was no appreciable incorporation of the ^{14}C-labelled fatty acid from the lecithin into

TABLE 5

INCORPORATION OF ^{14}C-LABELLED SUBSTRATES INTO CHOLESTEROL ESTER BY PERITONEAL MACROPHAGE HOMOGENATES

(mμmoles of cholesterol, oleic acid or fatty acid from lecithin converted to cholesterol ester/mg protein/h)

Labelled Substrate	Expt 1	Expt 2
(1) [4-^{14}C] cholesterol	0.32	0.55
(2) [1-^{14}C] oleic acid[1]	0.23	0.46
(3) Lecithin[1,2]	0.0097	0.030

1. No allowance has been made in the calculation of the amounts of oleic acid or lecithin fatty acid converted to cholesterol ester for the endogenous fatty acid or endogenous lecithin present in the homogenate itself. See Discussion.

2. Lecithin labelled with [1-^{14}C] palmitic acid in Experiment 1 and [1-^{14}C] oleic acid in Experiment 2. Approximately 60% of the label is present in the β-position in both cases.

cholesterol ester in either experiment. It is possible that in these experiments, the failure to detect transfer of fatty acid from lecithin into cholesterol ester might be due to the physical form in which lecithin was added. Therefore these negative results need to be interpreted with caution.

G. Subcellular Localization of Cholesterol Esterifying Activity

Subcellular fractions of peritoneal macrophages were prepared as described under Methods. Each fraction was reconstituted to its original concentration, and dialyzed against 5 litres of 0.9% sodium chloride solution for 6h to remove sucrose.

An aliquot of each fraction was set up for investigation of cholesterol esterifying activity as described in part B of this section. The reaction was carried out at pH 6.3 and 37°C, and was stopped at 15h by the addition of chloroform:methanol (2:1; v:v), and the lipid extracted as described.

Because the concentrations of endogenous cholesterol differed in each subcellular fraction (Table 6), the extent of dilution of ^{14}C-labelled cholesterol by the endogenous cholesterol was therefore different for each fraction. Assuming complete mixing

TABLE 6

Cholesterol content of subcellular fractions of peritoneal macrophages expressed in terms of protein present in the original homogenate. In Experiment 1 there was 5.6 mg protein/ml homogenate and in Experiment 2 8.7mg protein/ml homogenate.

Subcellular Fraction	μg total cholesterol/1 mg protein Expt 1	Expt 2
Homogenate	26	26
5,000xg pellet	5.9	5.4
104,000xg pellet	13	11
104,000xg supernatant	2.2	2.1

of the added free cholesterol with all of the endogenous free cholesterol, it was possible to calculate the amount of free cholesterol esterified by 1ml of each subcellular fraction. The calculated values obtained for two experiments are shown in Table 7, from which it is apparent that the supernatant fraction had the highest cholesterol esterifying activity.

H. Discussion

Cholesterol esterifying enzyme activity has been found in a number of tissues, including pancreas (Swell & Treadwell, 1950: Hernandez & Chaikoff, 1957), intestine (Nieft & Deuel, 1949: Swell, Byron & Treadwell, 1950), serum (Sperry, 1935; Glomset, 1962), liver (Mukherjee, Kunitake & Alfin-Slater, 1958; Goodman, Deykin & Shiratori, 1964), and adrenals (Longcope & Williams, 1963; Shyamala, Lossow & Chaikoff, 1965). The mechanism of action of the enzymes responsbile for this activity differs in different tissues. Enzymes found in pancreatic and intestinal tissue bring about the esterification of free cholesterol with fatty acid without any requirement for ATP or CoASH (Murthy & Ganguly, 1962; Lossow, Migliorini, Brot & Chaikoff, 1964), but there is an

TABLE 7

CHOLESTEROL ESTERIFICATION BY SUBCELLULAR FRACTIONS

(mμmoles cholesterol esterified/ml of fraction)

Subcellular Fraction	Expt 1	Expt 2
Homogenate	10.5	19.6
5,000xg pellet	1.35	1.91
104,000xg pellet	1.06	1.83
104,000xg supernatant	4.13	6.46

absolute requirement for bile salt. It has previously been reported that bile salt is not an absolute requirement, as very small amounts of synthesis have been reported in the absence of added bile salts (Hernandez & Chaikoff, 1957). It is possible that trace amounts of bile salt might be associated with the enzyme, thus enabling a low level of esterification to occur. In the liver, the cholesterol esterifying enzyme activity is dependent upon the presence of ATP and CoASH, and therefore prior formation of the acyl-CoA derivative is a necessary preliminary to esterification (Mukherjee, Kunitake & Alfin-Slater, 1958). The enzyme found in serum transfers the β-fatty acid of lecithin to the 3β-ol position of the cholesterol, synthesizing cholesterol ester and lysolecithin (Glomset, 1962).

The peritoneal macrophage homogenates esterify cholesterol by direct combination of oleic acid with cholesterol in the absence of the cofactors ATP and CoASH. The pH optimum of 6.3 for such homogenates is another property which resembles fairly closely that reported for pancreatic cholesterol esterifying enzyme by Hernandez and Chaikoff (1957).

Shyamala, Lossow and Chaikoff (1965) have demonstrated the presence of an esterifying enzyme

in homogenates of bovine adrenal glands and their subcellular components. They found that esterification had an optimum pH of 5.0, cofactors ATP and CoASH were not required and that their addition did not stimulate the esterification, and finally that the subcellular fraction with the greatest activity was the 104,000xg supernatant. In each of these respects the peritoneal macrophage homogenates and subcellular fractions are similar. However, canine adrenal homogenates and cell fractions differ markedly (Swell, Dailey & Treadwell, 1965). In this tissue, the major proportion of the activity was found to be associated with the mitochondria, and was dependent upon ATP and CoASH for activity.

Unlike liver and adrenal gland homogenates, macrophage homogenates were not inhibited by high levels of oleic acid (up to 1mg/ml of incubation medium). Goodman, Deykin and Shiratori (1964), found cholesterol esterification by liver mitochondria to be inhibited by addition of potassium oleate, to a final concentration of 10^{-5}M or greater. The inhibition could be reversed by adding albumin to the medium. When the mole ratio of fatty acid anion to serum albumin exceeded 7, marked inhibition occurred (Goodman, 1958).

Similarly, Longcope and Williams (1963) showed that esterification was partly inhibited by the addition of 0.2umole palmitic acid to a 3ml incubation. In the present studies it is possible that sufficient protein was present in the homogenate to bind the fatty acid.

In view of the cofactor independence of these macrophage homogenates with respect to esterifying activity, it is difficult to explain the source of energy for the formation of the ester bond. As pointed out by Goodman (1965), more detailed information is required about the role of the physical state of the substrate, and of the substrate-enzyme complex during cholesterol esterification, in order that the mechanism can be determined. The possibility that the esterifying activity in the macrophage homogenates was due to a lecithin:cholesterol acyltransferase type reaction, as found in serum (Glomset, 1962), was not substantiated in these studies. These negative findings need cautious interpretation as any of a number of factors might be responsible for the inability to detect activity. The physical form in which the lecithin was added may not have been suitable for the enzyme. The lecithin substrate used by Glomset (1962) where activity was detected was a lipoprotein preparation. In that work

reported here and work by Shyamala, Lossow and Chaikoff (1966) on rat adrenal homogenates, where "non-lipoprotein" preparations are used, no activity could be detected. However, activity was detected in homogenates of human and rabbit atherosclerotic lesions using a two phase system of di-isopropylether and water (Abdulla, Orton and Adams, 1968).

Alternatively, the low recorded activity of lecithin:cholesterol acyltransferase may have been due to dilution of the labelled lecithin with that present in the homogenate. Although the exact amount of endogenous lecithin present was not measured in the experiments reported, it is possible to derive an approximate value. Analyses of peritoneal cells have shown there to be 1µg of total cholesterol and 0.7µg of lipid phosphorus per 10^6 cells (Day & Fidge, 1966). In the present studies the cholesterol content of the homogenate added to each incubation was measured, and found to be approximately 50µg of total cholesterol, and thus by deduction there would be approximately 35µg of lipid phosphorus. Hence, there is about 700µg of total phospholipid present in each incubation, of which 500-600µg would be lecithin. Since 300µg of lecithin was added to each incubation, the maximum dilution of

added label would be only three times. Thus, even assuming the greatest conversion of fatty acid from lecithin to cholesterol ester per mg protein per h, there would be only 0.029 and 0.090 conversion for experiments 1 and 2 of Table 5 respectively, which is considerably less than the incorporation of oleic acid into cholesterol ester. The negative results, in view of the limitations of the experimental design, are therefore invalid and only a positive finding would be meaningful. Therefore it can be concluded that though these results do not rule out the presence of this enzyme, they make its presence unlikely.

In those experiments where ^{14}C-labelled fatty acid was incubated with macrophage homogenates, in the presence or absence of ATP and CoASH, in order to check the removal of these cofactors from the dialyzed homogenate, it was noticed that the incorporation of fatty acid into cholesterol ester was absolutely dependent upon the presence of ATP and CoASH at pH 7.4. It is likely then that a second cholesterol esterifying enzyme which is

present is active in a higher pH range to that described, and dependent on the prior formation of an acyl-CoA derivative of fatty acid. Further work is necessary, however, to confirm this possibility.

Esterifying activity was chiefly associated with the nonparticulate fraction, although the particulate fractions did show a small amount of activity. That the subcellular fractions had been adequately separated from each other was checked by electron microscopy, cytochrome c oxidase activity, and RNA determination of each fraction. Whilst the data obtained from the RNA determinations was not clear cut, the other investigations revealed that a satisfactory separation is obtainable by the methods described, and thus although possible, it is unlikely that those "soluble" enzymes found in the supernatant originated from a particulate fraction or membrane-bound fraction of the cells.

SECTION 2.

CHOLESTEROL ESTERIFYING ACTIVITY OF RABBIT ALVEOLAR MACROPHAGES

A. Introduction

In the preceding section, the presence and some of the properties of the cholesterol esterifying enzyme(s) present in peritoneal macrophages has been described. In this section, the presence of a cholesterol esterifying enzyme in macrophages obtained from another source (viz. the lungs) was investigated, and its properties compared with those found for peritoneal macrophages.

B. Uptake and Esterification of [4-^{14}C]-labelled Cholesterol Suspension by Whole Cells

The uptake and subsequent esterification of cholesterol by alveolar macrophages was investigated at various times of incubation. Isolated alveolar macrophages ($27x10^6$ cells per incubation) were incubated in duplicate in 20ml plastic vials in 2ml medium containing Hanks: heat-inactivated normal rabbit serum (1:1, v:v) (serum maintained at 55°C for 10min), together with a tracer dose of [4-^{14}C]-labelled cholesterol (980,000cpm) added as an aqueous suspension. The vials were gassed with 95% oxygen and 5% carbon

dioxide, sealed, and incubated at 37°C for either 0, 5, 10 or 20h with gentle shaking. At the completion of the incubation period, the cells were separated from the medium by centrifugation (220xg for 5min), and washed three times with 5ml 0.9% sodium chloride solution. The cells and the media were extracted with chloroform:methanol (2:1, v:v) as described by Folch, Lees and Sloane-Stanley (1957).

The percentage of ^{14}C-labelled cholesterol suspension at various time intervals is shown in Fig.11. Approximately 3% of the ^{14}C-labelled cholesterol suspension added was associated with the cells at zero time. This may represent uptake occurring during the cell centrifugation and washing period, even though this was carried out at 2°C, or it may represent label which was not removed by the washing procedure. Following this inital adsorption process, the uptake of cholesterol proceeded rapidly for 10h and then the rate fell off. Approximately 16% of the cholesterol present in the medium had been taken up at this time, and no further uptake occurred after 10h incubation.

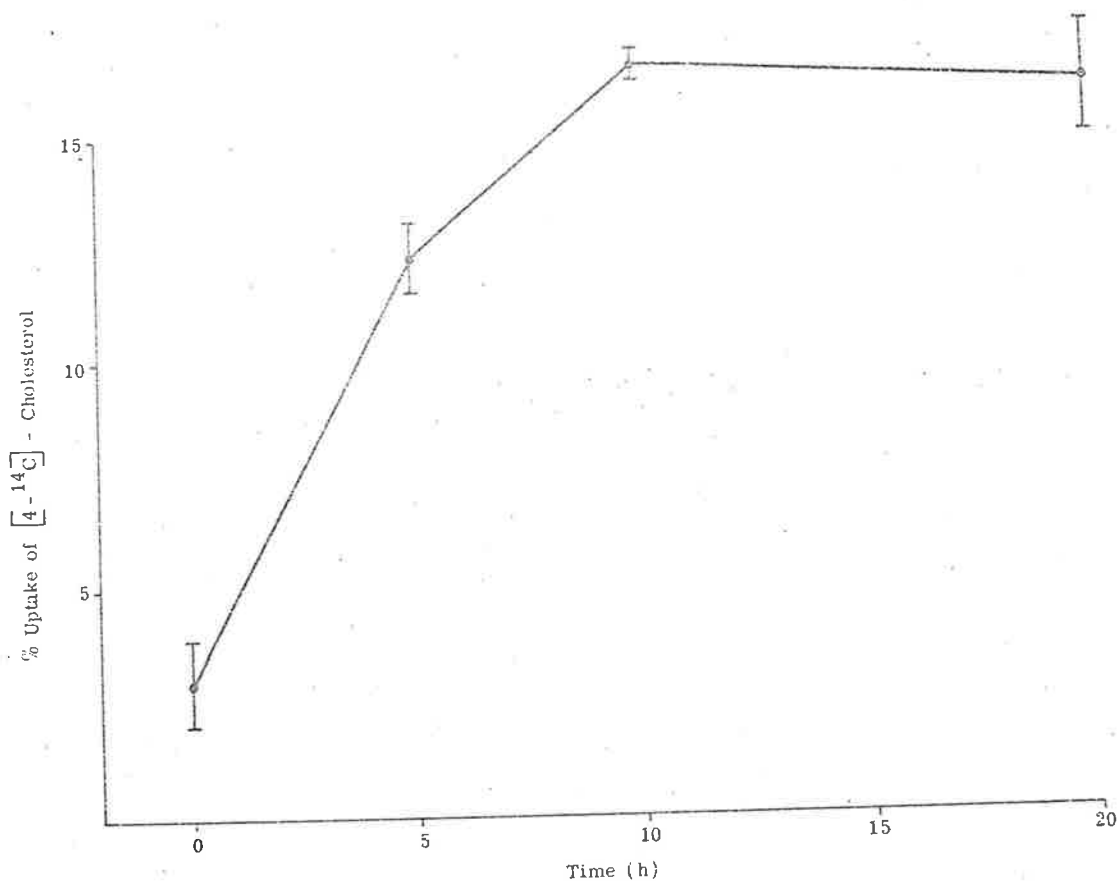


Fig. 11 Percentage uptake of $[4-^{14}C]$ cholesterol suspension by intact alveolar macrophages. Each point represents the mean of three experiments, each with duplicate incubations, showing the standard deviations of the values obtained.

Investigation of the esterification of the cholesterol taken up by the macrophages revealed that a small amount was esterified. Fig.**12** shows the esterification of cholesterol after 0, 5, 10 and 20h incubation. Even after 20h, less than 2% of that taken up was esterified; however, the reaction was approximately linear over the period studied and had not reached its maximum. Less than 0.1% of the cholesterol present in the medium at 20h was present as cholesterol ester.

C. pH Optimum for Cholesterol Esterification by Cell-Free Preparations

The effect of pH on the esterification of cholesterol by cell-free preparations of alveolar macrophages was investigated. For these studies, homogenates were prepared in a similar way to that described under Methods for peritoneal macrophages. Duplicate incubations were carried out in 12ml centrifuge tubes with continuous shaking at 37°C for 15h. The incubation system contained 0.7ml of 0.2M citrate-phosphate buffer (pH 3.0-7.2), 0.2ml dialyzed homogenate (containing a known amount of

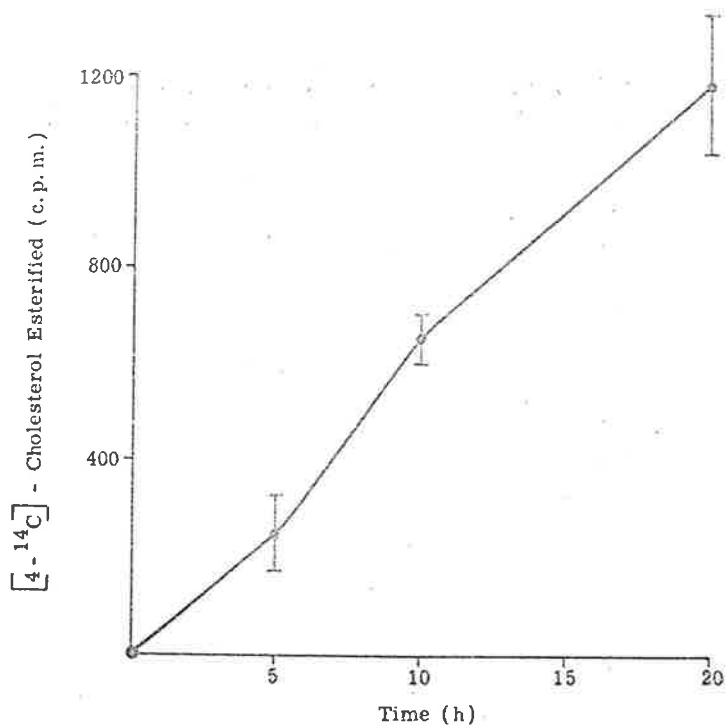


Fig. 12 Effect of time on the esterification of [4-^{14}C]cholesterol by intact alveolar macrophages. Each point represents the mean of three experiments, each with duplicate incubations, showing the standard deviations of the values obtained.

protein, 1-2mg), and 50μl of acetone containing 1mg oleic acid together with a tracer amount of [4-^{14}C]-labelled cholesterol. The reaction mixture was made up to a total volume of 1.1ml with water. The pH of some reaction mixtures was measured before and after incubation and was found to remain at the original value. In each experiment controls with no homogenate added were set up, and in some experiments controls containing homogenate which had been heated at 100°C for 10 min were tested. At the end of the incubation period the lipid was extracted, cholesterol and cholesterol ester separated, and then counted for radioactivity as described under Methods.

No significant esterification occurred in controls in which no homogenate was added, or in which the homogenate had been boiled prior to incubation. Fig. **13** shows the effect of pH on the esterification of cholesterol over the range pH 3.0-7.2 for one representative experiment. Appreciable esterification was obtained over the range pH 4.0-6.0 with an optimum at pH 4.5. All subsequent experiments were therefore carried out at pH 4.5.

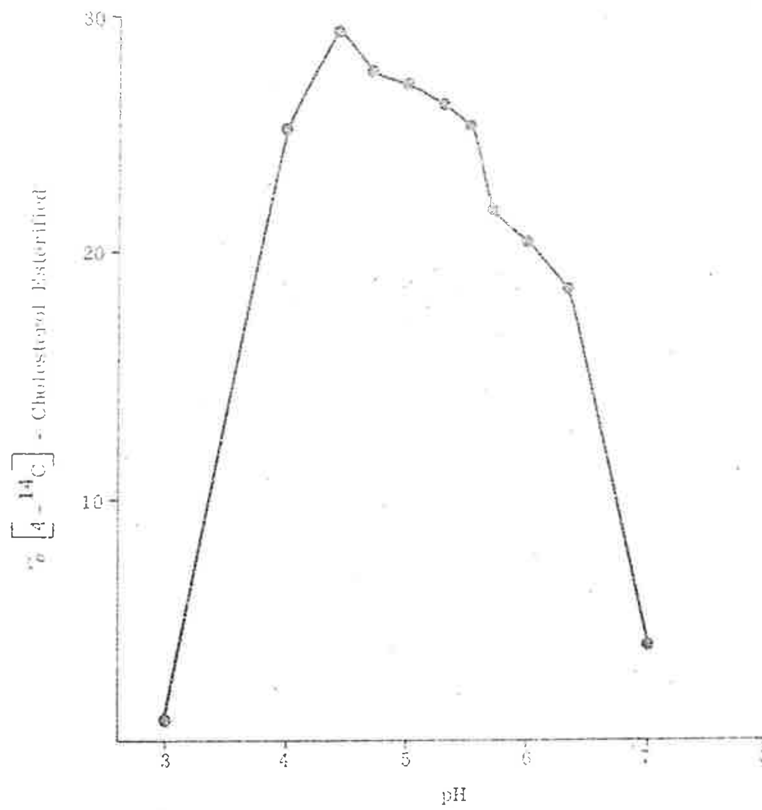


Fig.**13** Effect of pH upon the esterification of [4-^{14}C] cholesterol by alveolar macrophage homogenates.

The basic incubation system described above was used in the remainder of the experiments performed in this section unless otherwise indicated. Each incubation contained approximately 200μg total cholesterol (90% free cholesterol) supplied by the homogenate. Whilst it is likely that the majority of the endogenous cholesterol is firmly bound within subcellular membranes, being part of the structural integrity of the membranes and thus unavailable for esterification, this cannot be known with any certainty. In order to ignore the endogenous contribution of cholesterol, it would be necessary to add at least 1,000μg cholesterol, but with such a low specific activity of exogenous cholesterol, it would be difficult to detect any significant esterification. Because of this, it was decided not to add exogenous carrier cholesterol, but high specific activity ^{14}C-labelled cholesterol; thus the results can only be expressed as percentage of added cholesterol esterified. In some cases, however, sufficient exogenous cholesterol was added to be able to ignore the contribution from the homogenate, and so obtain a more quantitative estimate (mμmoles/mg protein/h) of the amount of esterification brought about.

D. Time Curve for Cholesterol Esterification

Fig.14 shows the effect of time of incubation on the esterification of cholesterol over a 20h period with various amounts of carrier cholesterol added. With either 0, 250, 620 or 1,000μg carrier cholesterol added the reaction was linear over the period investigated.

E. Effect of Oleic Acid Concentration on Rate of Esterification

Fig.15 shows the effect of increasing the amount of oleic acid added on the rate of cholesterol esterification. Each point represents the average results of two separate experiments, each with duplicate incubations, and the range of values obtained is shown. The endogenous free fatty acid present was determined in similar homogenate preparations by the method of Dole (1956), and was found to be approximately 3μeq free fatty acid (equivalent to 800μg long-chain fatty acid) per incubation.

Considerable esterification occurred without addition of exogenous free fatty acid, as was found with peritoneal macrophage homogenates. Similarly, the percentage esterification increased up to a maximum with 500μg of oleic acid added, and amounts of up to 1,000μg did not inhibit the esterification. In some experiments the distribution

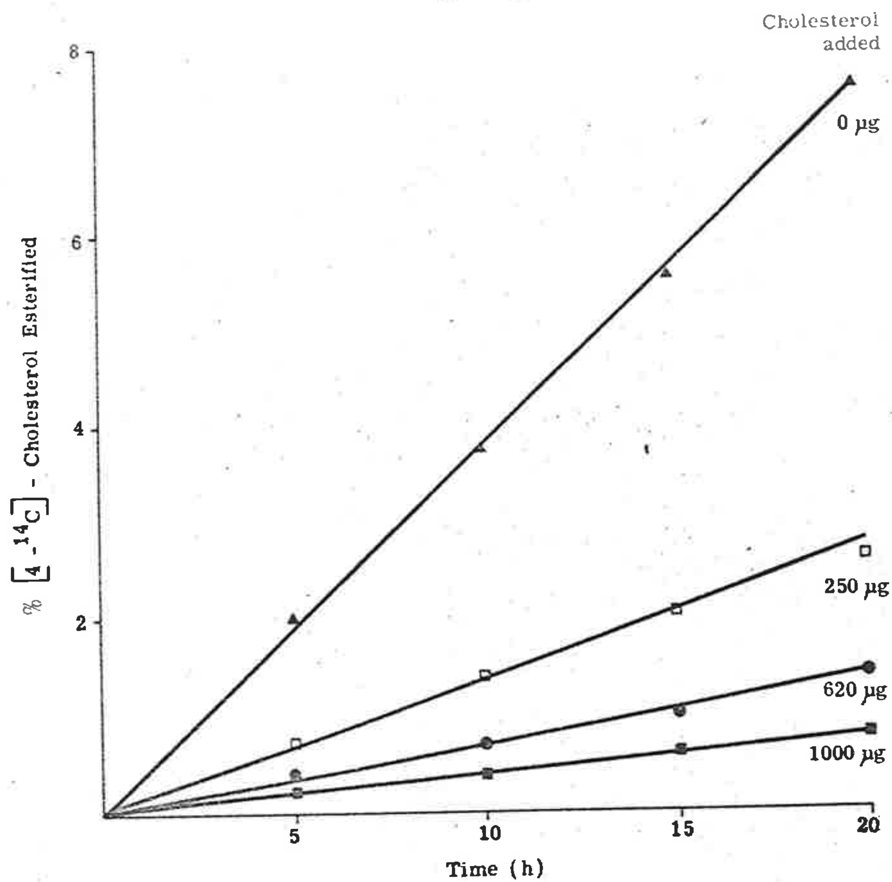


Fig. 14 Effect of time upon the esterification of $[4-^{14}C]$ cholesterol at various cholesterol concentration, by alveolar macrophage homogenates.

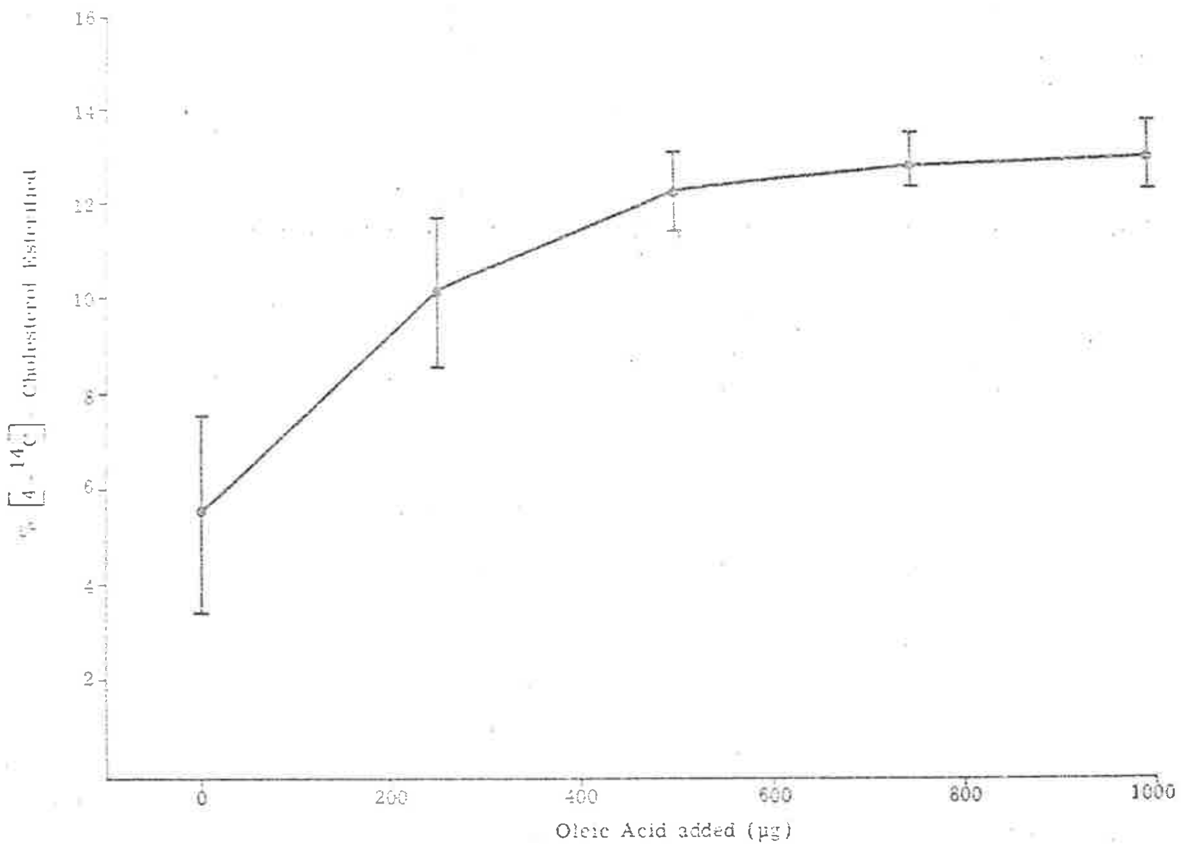


Fig. 15 Effect of addition of oleic acid upon the esterification of [4-^{14}C] cholesterol by alveolar macrophage homogenates. Each point represents the mean of two experiments each with duplicate incubations, showing the range of values obtained.

of the ^{14}C-labelled cholesterol in individual cholesterol esters was determined by argentation thin-layer chromatography as described under Methods. Cholesterol ester-fatty acid analysis revealed that 26% was saturated, 53% monounsaturated and 21% polyunsaturated, suggesting that the endogenous fatty acids play an active part in the esterification of cholesterol.

F. Effect of Cofactors ATP and CoASH on the Esterification of Cholesterol

In the experiments described above, cholesterol esterification proceeded in the absence of added cofactors, and it was therefore necessary to check that the dialyzed homogenate preparations were free of such cofactors. The method used for checking the alveolar macrophage homogenates was essentially the same as that described for peritoneal homogenates in the previous section. A fraction of each dialyzed homogenate was taken for (1) fatty acid esterification, and (2) cholesterol esterification, in the presence and absence of the cofactors ATP and CoASH. In this way it was possible to have a direct control. Where no fatty acid

esterification occurred without cofactors added, it could be assumed that endogenous cofactors had been completely removed during the dialysis period.

Tables 8 and 9 show the results of three experiments. It can be seen that such homogenates incorporate ^{14}C-labelled oleic acid into phospholipids in the presence of ATP and CoASH, but where both or either of these cofactors are absent, there is no significant incorporation of label into phospholipids. Although the total esterification of oleic acid was low in these experiments compared with peritoneal macrophage homogenates, it could be concluded that no significant amounts of ATP or CoASH remained in the homogenate after dialysis, or were synthesized during the incubation.

Table 9 shows the effect of the addition of cofactors on the esterification of ^{14}C-labelled cholesterol by the same homogenates used to test the esterification of oleic acid in Table 8 . These experiments were carried out at pH 4.5 using the basic incubation system described above. The addition of the cofactors, either alone or together, caused no significant change in the amount of esterification of

TABLE 8

INCORPORATION OF [1-^{14}C] OLEIC ACID INTO COMBINED LIPID FRACTIONS

The complete system contained in a total volume of 1.2 ml, 200μmoles Tris-HCl buffer pH 7.4, 0.3 ml dialyzed homogenate (2-3mg protein), 10μmoles ATP, 1μmole CoASH, 5μmoles $MgCl_2$, 5μmoles ∝-glycerophosphate and 25μl of a known amount of [1-^{14}C]oleic acid in 0.05 N NaOH. Incubations were performed at 37°C for 90 min.

% Distribution of Lipid ^{14}C

	Expt 1*					Expt 2*					Expt 3*				
System	PL	MG DG	FA	TG	CE	PL	MG DG	FA	TG	CE	PL	MG DG	FA	TG	CE
Complete	8.5	3.2	87	0.5	0.5	23	5.4	71	0.8	0.8	13	4.3	82	0.7	0.6
- ATP -Mg^{++}	2.4	3.2	94	0.2	0.2	1.9	3.0	95	0.2	0.2	1.9	3.1	95	0.2	0.1
- CoASH	1.2	3.0	95	0.4	0.6	3.0	3.0	93	0.3	0.3	3.0	2.9	94	0.3	0.3
- ATP) - CoASH) - Mg^{++})	2.8	2.6	94	0.6	0.4	3.7	3.0	93	0.7	0.3	2.6	2.7	94	0.6	0.2
Control	1.8	3.2	95	0.4	0.1	1.8	3.2	95	0.4	0.1	1.8	3.2	95	0.4	0.1

* Phospholipid (PL), monoglyceride (MG), diglyceride (DG), fatty acid (FA), triglyceride (TG), and cholesterol ester (CE).

TABLE 9

ESTERIFICATION OF [4-^{14}C] CHOLESTEROL

The basic system contained in a total volume of 1.1 ml, 0.7 ml of 0.2 M citrate-phosphate buffer pH 4.5, 0.2 ml dialyzed homogenate (1-2 mg protein) and 50 µl of acetone containing 1 mg oleic acid together with a tracer amount of[4-^{14}C]cholesterol. Incubations were performed at 37°C for 15 h.

% [4-^{14}C] Cholesterol Esterified

	EXPERIMENT 1	EXPERIMENT 2	EXPERIMENT 3
No additions	25	15	15
(+ ATP (10 µmoles) (+ CoASH (1 µmole) (+ Mg^{++} (5 µmoles)	24	14	15
(+ ATP (10 µmoles) (+ Mg^{++} (5 µmoles)	20	15	15
+ CoASH (1 µmole)	24	15	15
Control (no homogenate)	0.6	0.6	0.6

cholesterol, so that in these homogenates with both CoASH and ATP removed, cholesterol esterification proceeded normally and was not accelerated by returning ATP and CoASH to the incubation system.

The effect of the addition of cofactors over a range of pH values was investigated to see if there were other cholesterol esterifying enzymes present which were dependent upon the presence of ATP and CoASH. In these experiments it was necessary to alter the reaction mixture, because high levels of free fatty acids inhibit cholesterol esterifying activity of those enzymes that are ATP and CoASH dependent (Goodman, Deykin & Shiratori, 1964). Hence, instead of adding the ^{14}C-labelled cholesterol and oleic acid as an acetone solution, a tracer dose of ^{14}C-labelled cholesterol was added as an aqueous suspension, and 100μg of oleic acid was added as the sodium salt, bound to 3.5mg albumin. The remainder of the reaction mixture was as described previously, and when added, the amounts of cofactors were 10μmoles ATP, 1μmole CoASH and 5μmoles $MgCl_2$.

There was no observable difference between the two series of experiments (Fig.16) over the range pH 3.0-7.8, suggesting that only one type of enzyme is present. However, the possibility exists that the particulate cholesterol must first be converted to a "molecular" form which is suitable for esterification and thus may be the rate limiting step. In which case, the addition of cofactors would be ineffective. Therefore, it is not possible to exclude the presence of a second enzyme. That (4-^{14}C)-labelled cholesterol was present as an aqueous suspension did not alter the pH optimum for esterification.

G. Investigation of a Lecithin:Cholesterol Acyltransferase Type Reaction

The preceding experiments have established that cholesterol is esterified with fatty acid in the absence of ATP and CoASH, and it is assumed that the free fatty acid present provided the source of fatty acid for such esterification, as was the case for peritoneal macrophage homogenates. Again it was necessary to investigate the possibility that the reaction was of the transesterific-ation type described by Glomset (1962) for serum cholesterol esterifying activity.

Alveolar macrophage homogenates were tested by the method described for peritoneal macrophage homogenates

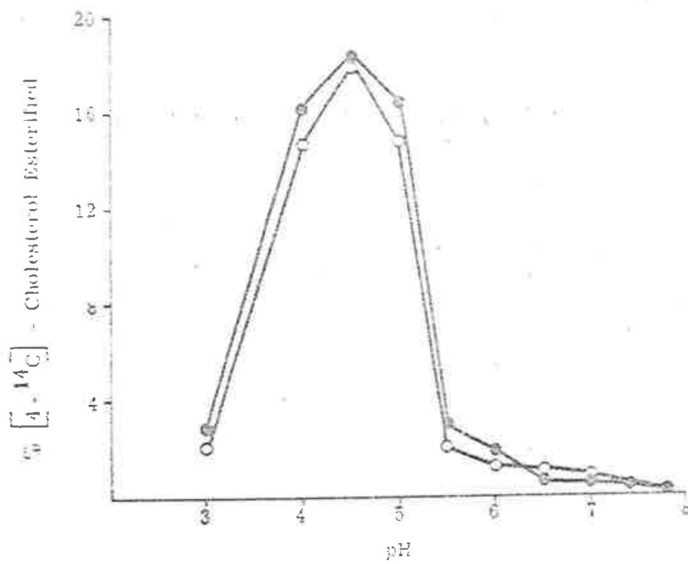


Fig. **16** Effect of pH on the esterification of [4-^{14}C] cholesterol in the presence and absence of cofactors ATP, CoASH and $MgCl_2$.

O = Cofactors added. ● = No cofactors added.

(previous section), using biosynthetically prepared lecithin labelled in the β-position with a ^{14}C-labelled fatty acid. The reaction mixture differed only in that carrier cholesterol was not added and the reaction was carried out at pH 4.5. Table 10 gives the results for two such experiments. In the first experiment lecithin was labelled in the β-position with (4-^{14}C)-labelled palmitic acid, and in the second with (4-^{14}C)-labelled oleic acid. The (4-^{14}C)-labelled oleic acid and the (4-^{14}C)-labelled fatty acid-lecithin were added to the respective incubation media in approximately equimolar amounts. In each experiment both ^{14}C-labelled cholesterol and ^{14}C-labelled oleic acid were incorporated into cholesterol ester. However, unlike peritoneal macrophage homogenates, between 2-3% of the labelled fatty acid of the lecithin was converted to cholesterol ester. The amount of lecithin already present in the homogenate as distinct from that added was not measured, however, and it is possible that the fatty acid from the lecithin contributed rather more to the cholesterol ester than indicated by the data of Table 10. ^{14}C-label analysis of the neutral lipids present after the 15h incubation revealed

TABLE 10

INCORPORATION OF ^{14}C-LABELLED SUBSTRATE INTO CHOLESTEROL ESTER BY ALVEOLAR MACROPHAGE HOMOGENATES

% of cholesterol, oleic acid or fatty acid from lecithin converted to cholesterol ester.

LABELLED SUBSTRATE	EXPERIMENT 1	EXPERIMENT 2
[4-^{14}C] cholesterol	14	2.3
[1-^{14}C] oleic acid	8.8	3.4
^{14}C lecithin*	2.8	1.4

* Labelled in the β-position with [1-^{14}C] palmitic acid in Expt 1, and [1-^{14}C] oleic acid in Expt 2.

that 60% of the lecithin had been hydrolyzed, releasing free fatty acid which would then be available for esterification by the direct pathway, so that evidence of lecithin:cholesterol acyltransferase activity could not be obtained for this reason.

H. Discussion

It has previously been shown that whole cell preparations of peritoneal macrophages take up and esterify particulate preparations of labelled cholesterol (Day & Gould-Hurst, 1961), and the work presented here demonstrates that alveolar macrophages have the same ability. During the 20h incubation period, less than 2% of that cholesterol taken up by the cells was esterified. However, this was significantly greater than in the control incubations and in the media after the cells had been removed. Heat-inactivated serum was used in these experiments so that the serum transesterifying enzyme (Glomset, 1962) would not be operative, and control incubations indicated that less than 0.1% of the cholesterol in the medium was converted to ester. The cholesterol esterifying activity is therefore associated with the cells. Since no labelled cholesterol ester was found

in the media, it can be assumed that no ester was lost from the cells. In view of the relatively large amount of ^{14}C-labelled cholesterol remaining in the medium at the end of the 20h incubation, it would be difficult to detect re-entry if it did occur. The labelled cholesterol ester found in the media of the experiments reported by Day and Gould-Hurst (1961) may have resulted from active transesterifying enzymes present in fresh serum, and not from leakage from the cells as suggested.

The work presented in this section demonstrates that homogenates also have the ability to esterify cholesterol. These homogenates esterify cholesterol by a direct combination with fatty acid in the absence of the cofactors ATP and CoASH, and in this respect are similar to peritoneal macrophage homogenates. However, whereas the pH optimum was 6.3 for peritoneal macrophage homogenates, it was 4.5 for alveolar macrophage homogenates.

The experiments in which the incorporation of ^{14}C-labelled oleic acid into phospholipid was investigated, were undertaken primarily to verify that CoASH and ATP had been removed from the homogenate during dialysis. The absence of significant incorporation of ^{14}C-labelled oleic acid into

phospholipid, unless CoASH or ATP was replaced indicated that the cofactors had been removed. The uptake of fatty acids by alveolar macrophages has been studied by Elsbach (1965 & 1966), and he has adduced evidence that lecithin is formed in these cells by acylation of lysolecithin, and that such acylation is dependent on ATP and CoASH. The incorporation of fatty acid into phospholipid by alveolar macrophages, and its dependence on CoASH and ATP, has been confirmed in the present study. It is presumed that such incorporation occurs by the Lands pathway (Lands, 1960), as described for these cells by Elsbach.

Homogenates of alveolar macrophages displayed an independence for cofactors with regard to cholesterol esterification. Where cofactors were added over a range of pH values, only one peak was obtained at pH 4.5, suggesting that macrophages do not have an enzyme which depends on the presence of ATP and CoASH for activity, as found in liver (Goodman, Deykin & Shiratori, 1964). The failure of excess fatty acid to inhibit esterification supports this conclusion.

Most of the cholesterol esterifying activity can be accounted for by direct esterification of free fatty acid with cholesterol. In the experiments reported, ^{14}C-labelled oleic acid was shown to be converted directly to cholesterol ester. Since these experiments were carried out at pH 4.5 and in the absence of CoASH and ATP, the prior conversion of oleic acid to lecithin, with subsequent esterification of cholesterol by lecithin:cholesterol acyltransferase, can be excluded.

The exclusion of significant lecithin: cholesterol acyltransferase activity is not quite so straightforward. Phospholipase A activity has been demonstrated by Elsbach (1965) in alveolar macrophage homogenates with maximal activity at acid pH. Hydrolysis of the acyl-^{14}C-labelled lecithin in the experiments described in the present work occurred presumably due to the activity of this enzyme. The incubations were, of course, carried out at pH 4.5. It was impossible therefore to avoid releasing ^{14}C-labelled fatty acid from the lecithin for esterification with cholesterol by the direct pathway. 60% of the ^{14}C-labelled fatty acid

originally present combined with lecithin was present as free fatty acid at the end of the incubation. It appears unlikely therefore that significant conversion of the ^{14}C-labelled lecithin to cholesterol ester by lecithin:cholesterol acyltransferase occurs. The possibility that some lecithin:cholesterol acyltransferase activity is present in the homogenates, however, cannot be excluded.

SECTION 3.

ESTERIFICATION OF ^{14}C-LABELLED PALMITIC ACID BY HOMOGENATES AND SUBCELLULAR FRACTIONS OF PERITONEAL MACROPHAGES

A. Introduction

It has been shown (Day & Fidge, 1962) that isolated cell preparations of peritoneal macrophages take up ^{14}C-labelled palmitic acid and incorporate it predominantly into triglyceride and phospholipid, with relatively little incorporation into cholesterol ester. In the present section, the esterification of ^{14}C-labelled fatty acid by peritoneal macrophage homogenates has been investigated, both as an extension of these initial studies, and as a corollary of the work described in Section 1 on cholesterol esterifying enzymes in peritoneal macrophage homogenates. In order to examine more closely the formation of the glycerolipids by peritoneal macrophages, and to obtain some information regarding the pathways involved in their synthesis, the following investigations were carried out using homogenates and subcellular fractions of these cells.

B. Effect of Time of the Incubation on the Incorporation of Fatty Acid into Glycerolipids

The effect of time of incubation on the incorporation of ^{14}C-labelled palmitic acid into glycerolipid by macrophage homogenates was investigated over a 4h period. For these studies

homogenates were prepared by ultrasonication using a Mullard MSE Magnetostrictive Ultrasonicator. Ultrasonication was carried out for 20sec in an ice bath, the preparation being checked microscopically every 5sec for the percentage disruption. The cell debris and nuclei were removed by centrifugation (220xg for 5min) and the supernatant removed and dialyzed for 15h against 5 litres 0.05M Tris-HCl buffer pH 7.4 containing 5×10^{-4}M cysteine.

Incubations were performed at 37°C for 90min in duplicate continuously agitated 12ml centrifuge tubes. Each tube contained a total volume of 1.0ml, 200μmoles Tris-HCl buffer pH 7.4, 10μmoles ATP, 2μmoles CoASH, 5μmoles α-glycerophosphate, 5μmoles $MgCl_2$, 0.3ml dialyzed homogenate and a tracer amount of ^{14}C-labelled sodium palmitate (330,000cpm - 143μCi/mg). At times 0, 5, 10, 30, 60, 120 and 240min, duplicate tubes were removed and the lipid extracted with chloroform:methanol (2:1, v:v). Thin-layer chromatography on neutral lipid plates was carried out as described under Methods, except that each sample was spotted on to a thin-layer plate of dimensions 20x4.8cm so that

the plates could be scanned for radioactivity using a Nuclear Chicago 4π Actigraph Paper Chromatograph Scanner, modified for use with thin-layer plates. The percentage distribution of the ^{14}C-labelled fatty acid in the various lipid fractions after incubation was calculated by measuring the areas of the radioactive peaks obtained after scanning. The total recovery of counts in all fractions was found to account for the ^{14}C-labelled palmitate added, suggesting that none was oxidized to $^{14}CO_2$ under these conditions.

The total fatty acid incorporated into lipid and that incorporated specifically into phospholipids and triglycerides at each time interval is given in Fig.17. The incorporation is expressed as mumoles fatty acid incorporated into each individual lipid fraction, assuming the ^{14}C-labelled fatty acid not to be significantly diluted by endogenous fatty acids. These figures therefore represent minimal incorporation.
Both phospholipid and triglyceride were rapidly labelled, but with longer incubation times there was a decrease in the labelling. This might either be due to a decrease in the specific activity of the

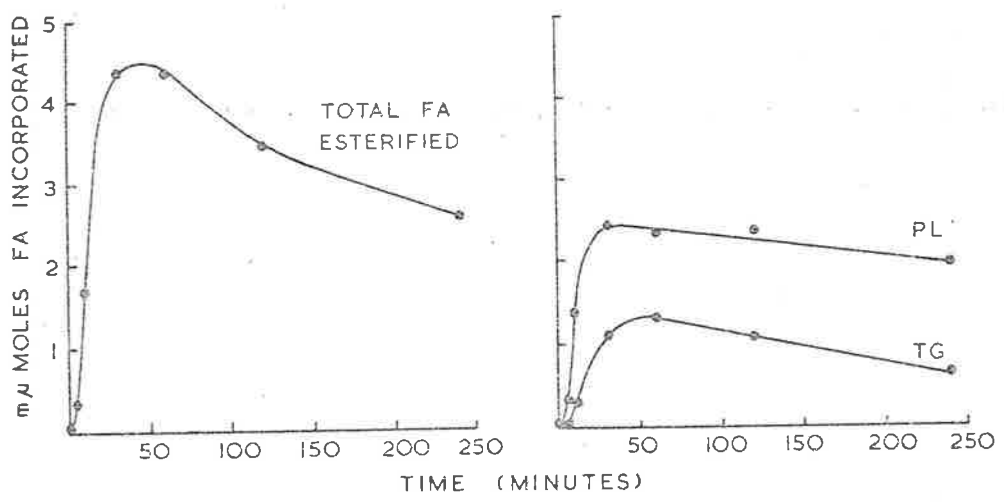


Fig. 17 Effect of time on the total fatty acid incorporation and on the individual incorporation into phospholipid and triglyceride by peritoneal macrophage homogenates.

precursor free fatty acid pool as a result of lipid hydrolysis or due to hydrolysis of the labelled phospholipid or triglyceride or both. All future incubations were of 90min duration.

C. Effect of Cofactors on the Incorporation of Fatty Acid into Glycerolipids

In the experiments described above, cofactors were added to the incubation mixture. The effect of removal of these from the incubation system was therefore investigated in order to ascertain the basic cofactor requirements for the incorporation of fatty acid into glycerolipids by the macrophages. Incubations were set up as described above with omissions as shown in Table 11. The percentage distribution of ^{14}C-labelled lipid after incubation of either fresh homogenate, dialyzed homogenate, or homogenate which had been stored during the dialysis period is also shown in Table 11. Storage of the homogenate for 15h produced little change in its ability to incorporate fatty acid into glycerolipid as compared with that of homogenate investigated immediately. Dialysis

TABLE 11

% DISTRIBUTION OF LIPID ^{14}C AFTER INCUBATION OF HOMOGENATE WITH ^{14}C-LABELLED PALMITATE

Complete system contained in a total volume of 1.0 ml, 200 μmoles Tris-HCl buffer pH 7.4, 10 μmoles ATP, 2 μmoles CoASH, 5 μmoles α-glycerophosphate, 5 μmoles $MgCl_2$, 0.3 ml dialyzed homogenate (containing a known amount of protein, 1.4-2.5 mg protein) and a tracer amount of ^{14}C-labelled sodium palmitate. Incubations were performed in duplicate at 37°C for 90 min.

		Expt 1	Expt 2	Expt 3
Complete system with freshly prepared homogenate	FA	18.0	6.4	2.0
	PL	44.3	49.0	51.3
	Glyc.	37.7	44.6	46.7
Complete system with dialyzed homogenate	FA	29.2	11.5	6.5
	PL	70.8	42.7	42.3
	Glyc.	0	45.8	51.2
Complete system with homogenate stored during dialysis period at 2°C	FA	Not done	5.0	7.4
	PL		47.0	30.2
	Glyc.		48.0	62.4
Complete system with dialyzed homogenate - ATP	FA	100	Not done	100
	PL	0		0
	Glyc.	0		0
Complete system with dialyzed homogenate -CoASH	FA	100	96.5	100
	PL	0	2.4	0
	Glyc.	0	1.1	0
Complete system with dialyzed homogenate - α-glycerophosphate	FA	45.9	60.5	72.1
	PL	54.1	28.8	18.4
	Glyc.	0	10.7	9.5
Complete system with dialyzed homogenate - $MgCl_2$	FA	64.1	58.6	27.3
	PL	35.9	36.7	56.2
	Glyc.	0	4.7	16.5
Homogenate protein added (mg)		1.4	2.5	2.4

also produced no change except in Experiment 1 where apparently no glycerides were formed. Where either ATP or CoASH were omitted, there was virtually no esterification of fatty acid, demonstrating an absolute requirement for these cofactors. Omission of α-glycerophosphate resulted in a marked decrease in incorporation into both total glycerides and phospholipids. However, phospholipids appeared to be less affected than the glycerides. Similarly, omission of $MgCl_2$ had a more marked effect on the glyceride.

The effect of increasing concentrations of ATP on total fatty acid incorporation and on individual incorporation into phospholipids and triglycerides are shown in Fig.18. All other cofactors were present in the reaction mixture at the concentrations given above. The maximum amount of total fatty acid esterified occurred with 10µmoles of ATP present, although this was not much greater than with 5µmoles of ATP.

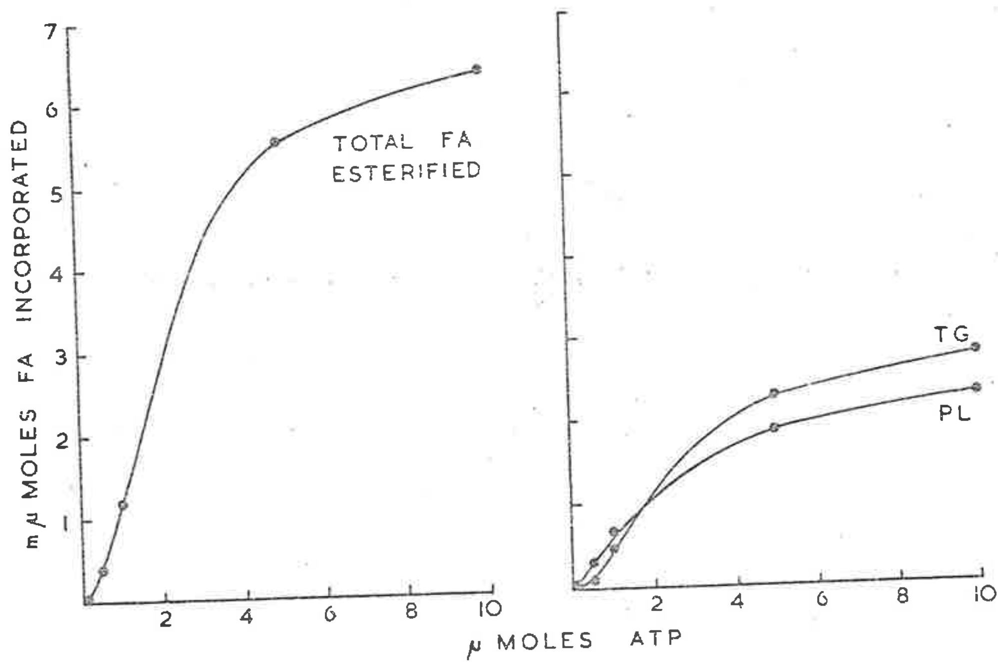


Fig. 18 Effect of increasing concentrations of ATP on total fatty acid incorporation and on individual incorporation into phospholipid and triglyceride by peritoneal macrophage homogenates.

Fig.19 shows the effect of increasing CoASH concentration on the incorporation of fatty acid. The concentrations of the other cofactors were as described above. At these low concentrations of CoASH there was greater incorporation of fatty acid into phospholipid than into triglyceride. Where the effect of CoASH over the concentration range of 0-2μmoles was investigated (Fig.20), it was found that there was a crossover point at about 0.2μmoles CoASH: below this level the incorporation of fatty acid into phospholipids exceeded the incorporation into triglycerides, but above it the pattern was reversed.

A similar situation occurred when α-glycerophosphate was added in increasing amounts to the incubation system (Fig.21). The incorporation of fatty acid into triglycerides exceeded the incorporation into phospholipids when α-glycerophosphate was present in amounts greater than 2μmoles. Maximal esterification of fatty acid was observed with 1μmole of α-glycerophosphate present. If both

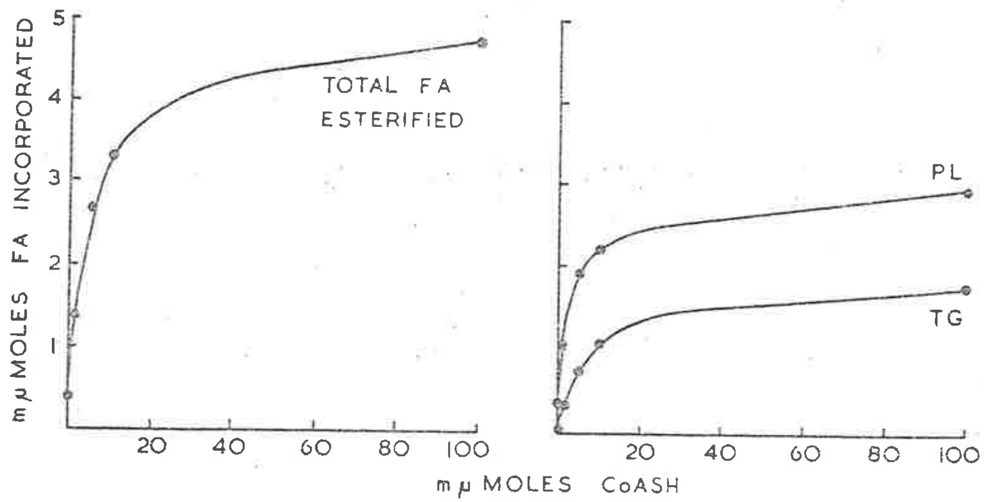


Fig. 19 Effect of increasing concentrations of CoASH on the total fatty acid incorporation and on the individual incorporation into phospholipid and triglyceride by peritoneal macrophage homogenates.

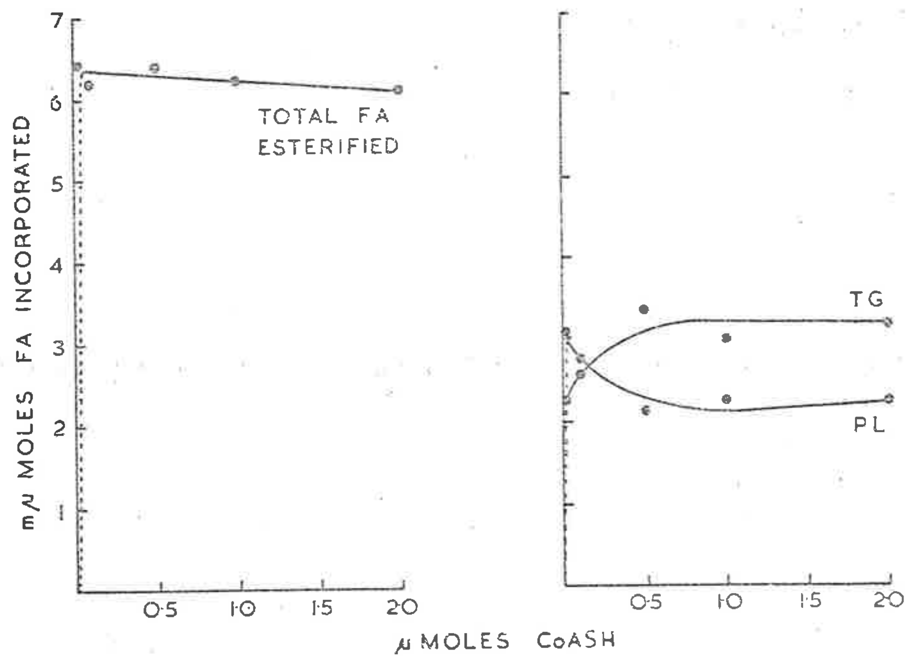


Fig. 20 Effect of increasing concentrations of CoASH on the total fatty acid incorporation and on the individual incorporation into phospholipid and triglyceride by peritoneal macrophage homogenates. A higher range of CoASH concentrations was used here than in Fig. 19.

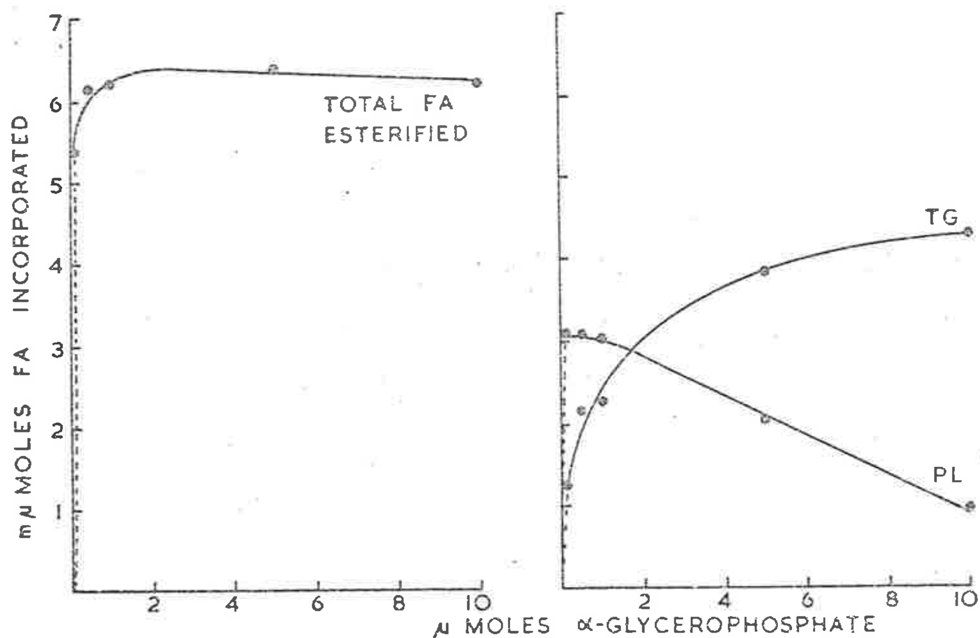


Fig. 2·1 Effect of increasing concentrations of α-glycerophosphate on the total fatty acid incorporation and on the individual incorporation into phospholipid and triglyceride by peritoneal macrophage homogenates.

triglyceride and phospholipid are formed via α-glycerophosphate according to the Kennedy and Weiss pathway (Kennedy & Weiss, 1956), it would be expected that both products would increase together when α-glycerophosphate is added, and not one increase at the expense of the other. It seems probable therefore that an alternative pathway may be involved in the incorporation of fatty acids into phospholipid by the macrophages. Such a pathway has been described in liver by Lands (1960), in which fatty acid is esterified with lysolecithin to form lecithin. This reaction described initially by Lands (1958) in lung and in liver (Lands, 1960) has since been described in aortic homogenates (Stein, Stein & Shapiro, 1963), brain homogenates (Webster, 1965) and in liver and yeast preparations (Van den Bosch, Bonte & Van Deenan, 1965). In view of the above findings it was of interest to see whether the Lands pathway was demonstrable in macrophage homogenates.

D. Investigation of the Presence of the Lands Pathway in Macrophage Homogenates

In order to determine the presence of the

Lands pathway, the effect of adding α-glycerophosphate or lysolecithin on the formation of phospholipid and triglyceride by the macrophage homogenate was determined. While the addition of α-glycerophosphate gives no direct insight into the presence or absence of the Lands pathway it was included in these experiments so that a comparison of the incorporation of fatty acid into lecithin could be made with either acceptor, i.e. α-glycerophosphate or lysolecithin. Both α-glycerophosphate and lysolecithin were added in some experiments to see if the incorporation was additive. Dialyzed homogenate preparations were incubated as described in part B of this section, except that α-glycerophosphate was omitted from the basic system. Varying amounts of α-glycerophosphate and lysolecithin were added to some incubations as indicated in Table 12. The lipid classes were separated by thin-layer chromatography and the phospholipids further fractionated on silicic acid impregnated paper to separate the lecithin.

Table 12 shows the amount of fatty acid incorporated into lecithin and triglyceride for each of 5 experiments. In 4 of the 5 experiments, addition of lysolecithin alone caused an increased incorporation of fatty acid into lecithin. The incorporation of fatty acid into triglyceride, however, was inhibited by the presence of lysolecithin. Similarly this inhibition was apparent

TABLE 12

EFFECT OF VARIOUS ACCEPTORS ON THE INCORPORATION OF ^{14}C-LABELLED PALMITATE

The basic system contained in a total volume of 1.0 ml, 200 μmoles Tris-HCl buffer pH 7.4, 10 μmoles ATP, 2 μmoles CoASH, 5 μmoles $MgCl_2$, 0.3 ml dialyzed homogenate and a tracer amount of ^{14}C-labelled sodium palmitate. Incubations were performed in duplicate at 37°C for 90 min.

Additions	(μmoles)	mμmoles fatty acid incorporated into Lecithin	Triglyceride	mμmoles FA added
Expt 1	No additions	3.9	2.5	19.9
	+ 0.5 LL	5.3	0.86	
	+ 2 LL	5.0	0.14	
	+ 5 GP	6.1	6.0	
	+ 5 GP + 1 LL	6.6	0.62	
Expt 2	No additions	2.7	1.6	19.9
	+ 0.5 LL	3.3	1.3	
	+ 2 LL	10.7	0.26	
	+ 5 GP	4.4	4.2	
	+ 5 GP + 1 LL	4.7	0.92	
Expt 3	No additions	4.7	3.7	20.0
	+ 1 LL	9.9	0	
	+ 4 LL	6.3	0	
	+ 5 GP	6.1	7.1	
	+ 5 GP + 4 LL	6.7	0	
Expt 4	No additions	3.7	2.3	20.0
	+ 1 LL	14.7	0.38	
	+ 4 LL	16.7	0.21	
	+ 5 GP	4.3	5.9	
	+ 5 GP + 4 LL	17.6	0.18	
Expt 5	No additions	2.4	0.86	14.8
	+ 0.05 LL	2.6	0.77	
	+ 0.1 LL	2.4	1.3	
	+ 0.5 LL	3.5	0.74	
	+ 1 LL	3.1	0.28	
	+ 2 LL	1.9	0.15	
	+ 5 GP	3.8	3.3	

FA = fatty acid LL = lysolecithin GP = α-glycerophosphate

where α-glycerophosphate was also present together with the lysolecithin, and in these incubations also, the lysolecithin depressed the formation of triglyceride to a considerably lower level than when α-glycerophosphate was added in the absence of lysolecithin. The addition of α-glycerophosphate alone produced an increase in the formation of both lipid fractions. No additive effect of the lysolecithin and α-glycerophosphate was apparent, however, on lecithin formation; i.e., while both α-glycerophosphate and lysolecithin increased the formation of lecithin by the macrophage homogenate, this increase did not exceed that produced by either acceptor when added separately.

E. Subcellular Localization of Glycerolipid Synthesizing Enzymes

The incorporation of ^{14}C-labelled palmitic acid into glycerolipids by the various subcellular fractions of peritoneal macrophages was investigated. As this work was carried out at an early stage, the purity of the subcellular fractions used was not established. The same procedure, however, was used for the preparation as that described under Methods, except that the cells were disrupted by ultrasonication.

The basic incubation system described above was used for these studies, the subcellular fractions

reconstituted to their original homogenate volumes being added. The incorporation of ^{14}C-labelled palmitic acid into glycerolipids by the various subcellular fractions is shown in Table 13. The particulate fractions were most active in esterifying palmitic acid to form both phospholipids and glycerides. The 104,000xg supernatant fraction, however, incorporated some label into phospholipid but not into glycerides. The rate of incorporation of palmitic acid by each subcellular fraction is given in Fig. 22, and in this experiment the incorporation into phospholipids by each fraction occurred rapidly and was almost maximal by 1h. The incorporation into glycerides was considerably less than for phospholipids, the maximum level being reached by 30min. Again, there was only very low incorporation of label by the supernatant into the glyceride fraction.

F. Discussion

Several pathways have now been described for the biosynthesis of triglycerides in mammalian tissues. Triglycerides may be synthesized from L-α-phosphatidic acids and D-α, β-diglycerides, as

TABLE 13

% DISTRIBUTION OF LIPID ^{14}C AFTER INCUBATION OF ^{14}C-LABELLED PALMITATE WITH SUBCELLULAR FRACTIONS

The system used in these experiments consisted of 200 μmoles Tris-HCl buffer pH 7.4, 10 μmoles ATP, 2 μmoles CoASH, 5 μmoles α-glycerophosphate, 5 μmoles $MgCl_2$, 0.3 ml dialyzed subcellular preparation and a tracer amount of ^{14}C-labelled sodium palmitate, in a total volume of 1.0 ml. Incubations carried out at 37°C for 90 min.

		Expt 1*	Expt 2	Expt 3	Expt 4
Homogenate	FA	7.1	5.5	18.7	12.1
	PL	41.0	52.5	40.0	36.3
	Glyc.	51.9	42.0	41.3	51.6
5000xg pellet	FA	14.6	21.9	13.1	7.0
	PL	55.1	52.3	62.2	44.8
	Glyc.	30.3	25.8	24.7	48.2
104,000g pellet	FA	11.8	13.4	10.2	5.9
	PL	62.9	73.9	57.8	49.4
	Glyc.	25.3	12.7	32.0	44.7
104,000xg supernatant	FA		69.6	76.5	23.0
	PL	Not done	30.4	23.5	74.9
	Glyc.		-	-	2.1

* Particulate fractions not washed.

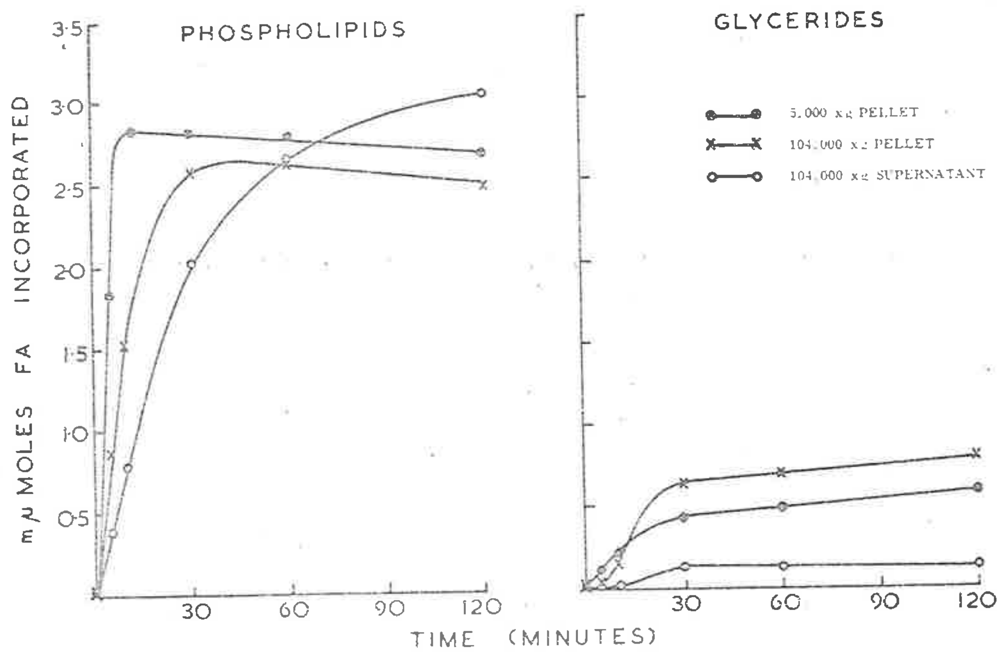


Fig. **22** Effect of time on the incorporation of fatty acid into phospholipid and glycerides by various subcellular fractions.

has been described in liver (Smith, Weiss & Kennedy, 1957; Stein & Shapiro, 1957), and in adipose tissue (Steinberg, Vaughan & Margolis, 1961). A second pathway for the synthesis of glycerides has been found in liver, kidney (Hubscher, 1961), and intestinal mucosa (Clark & Hubscher, 1961; Senior & Isselbacher, 1961; Johnston & Brown, 1962), and involves the direct acylation of monoglycerides.

The biosynthesis of phospholipids also takes place via the first pathway described above, using L-α-glycerophosphate as a precursor. The existence of other pathways by which fatty acid is incorporated into phospholipids has been described, but in such cases, there is no net synthesis of phospholipid; for example, the acylation of lysophospholipids (Lands, 1960), and the condensation of two lysolecithins to give lecithin and glycerophosphorylcholine (Erbland & Marinetti, 1962).

The data presented using fatty acid acceptors indicates that the biosynthesis of phospholipids and glycerides in macrophages homogenates can take place via the common pathway involving the acylation of α-glycerophosphate according to the Kennedy scheme. The fact that the

esterification of fatty acid was absolutely dependent upon the presence of the cofactors ATP, CoASH and Mg^{++} is consistent with the cofactor requirements for triglyceride and phospholipid synthesis found by other workers (Stein, Stein & Shapiro, 1963; McBride & Korn, 1964).

The existence of other pathways for phospholipid synthesis became apparent when it was found that omission of α-glycerophosphate from the incubation system decreased the formation of glycerides, but had a lesser effect on the incorporation of fatty acids into phospholipids. The possibility that direct esterification of fatty acid with lysolecithin to form lecithin occurred, was confirmed in the experiments where the system was manipulated by adding either lysolecithin or α-glycerophosphate. Addition of lysolecithin to the system results in almost exclusive formation of lecithin.

Elsbach (1966) has described the acylation of ^{32}P-labelled lysolecithin to form lecithin by homogenates of rabbit polymorphonuclear leukocytes and by alveolar macrophages. Such acylation of

lysolecithin by leukocytes did not depend upon the presence of ATP and CoASH, whereas macrophages had an absolute requirement for these cofactors. This lack of stimulation by ATP and CoASH of lecithin formation in leukocytes is explained by the pathway which involves the transfer of a fatty acid from one lysolecithin molecule to another (Erbland & Marinetti, 1962). It is apparent then that macrophages are unable to use this pathway. However, at high concentration of ^{14}C-labelled fatty acid lysolecithin, Elsbach (1966) found that macrophage homogenates would incorporate lysolecithin-fatty acid into lecithin. He suggested that the lysolecithin was being broken down, releasing fatty acid which was then available for the acylation of lysolecithin.

It is difficult to assess the optimum concentration of lysolecithin in the experiments reported here and in those described by other workers, since the same amounts were toxic in some experiments, yet stimulated lecithin formation in others. At high concentrations of lysolecithin an appreciable portion of the lysolecithin is broken down with the release

of free fatty acid (Elsbach, 1966). The inhibitory effect of lysolecithin on fatty acid esterification observed in some of the experiments described, may be due to the dilution of the ^{14}C-labelled fatty acid with the fatty acid released from the lysolecithin. Other workers have found that certain concentrations of lysolecithin inhibited fatty acid esterification by aortic homogenates (Stein, Stein & Shapiro, 1963), whereas the same concentrations stimulated lecithin formation in liver systems (Lands, 1960). In the experiments described here, the amount of protein present in each incubation ranged from 2-3mg, which is about one quarter of the amount present in the aortic and liver systems described above for the same amount of lysolecithin. It is known that albumin serves as a carrier for circulating lysolecithin (Switzer & Eder, 1965) and therefore the relative amounts of protein to lysolecithin are critical, and also that an excess of lysolecithin will possibly cause a marked inhibition of fatty acid esterification. The lysolecithin used in these experiments was isolated from a commercial preparation of lecithin, and not by

enzymically removing the β-fatty acid of lecithin with phospholipase A. It is not known if the fatty acid was present in the α- or β-position of this lysolecithin preparation. This may account for the differences mentioned in the above discussion.

In those experiments where the subcellular localization of the enzymes responsible for fatty acid esterification were investigated, it was found that all fractions were equally capable of synthesizing phospholipids, although the initial rate of esterification was much slower with the supernatant fraction than with the particulate fractions. On the other hand, the supernatant fraction was almost incapable of synthesizing glycerides, whereas both particulate fractions were quite active. This study is supported by the work of Stein and Shapiro (1958) using subcellular preparations of liver. They found that the mitochondrial and microsomal fractions were most active in esterifying fatty acid, and that there was an absolute requirement for ATP, CoASH and Mg^{++}.

That peritoneal macrophages can bring about the esterification of fatty acid to form

predominantly phospholipids and triglycerides is evident from these studies. Under the experimental conditions used there was negligible esterification of fatty acid to form cholesterol ester, the major lipid to accumulate in macrophages of atherosclerotic lesions.

SECTION 4.

THE ISOLATION AND COMPOSITION OF FOAM CELLS

A. Introduction

In the preceding sections of this thesis some of the metabolic properties of macrophages isolated from the peritoneal cavity or lungs of rabbits have been described.

This work was undertaken not so much as a primary study of the metabolism of RE cells, but as part of a wider study concerned with the role of RE cells in lipid deposition in atherosclerosis. It was originally considered that the lipid-containing foam cells present in atherosclerotic lesions were modified tissue macrophages, and their metabolism of lipid might therefore be intimately associated with the deposition of lipid in the arterial wall. Whether the lipid metabolism of normal macrophages resembles that of the aberrant foam cells cannot, however, be concluded, so that the extrapolation of data obtained from peritoneal and lung macrophages to foam cells in the arterial wall may not be justified. Further, it has recently been found that many of the foam cells in atherosclerotic lesions arise from smooth muscle cells (Parker, 1960; Geer, McGill & Strong, 1961;

Balis, Haust & Moore, 1964; Luginbuhl, Jones & Detweiler, 1965), and not from macrophages, so that an investigation of the metabolism of the foam cell becomes an essential to studying its role in lipid deposition in the atherosclerotic arterial wall.

Since it is now possible to isolate homogeneous preparations of foam cells from cholesterol-fed rabbits (Day, Newman and Zilversmit, 1966), the metabolic properties of these cells can be investigated directly. The main object of the work described in the following sections was to investigate lipid metabolism by foam cells. However, in this section and the one that follows, some of the basic properties of these cells and their behaviour in tissue culture have been described.

B. Isolation of Foam Cells

The method described by Day, Newman and Zilversmit (1966) was slightly modified in order to provide a greater number of cells than could otherwise be obtained. The method used, as described under Methods, differed from the original in that

15mg instead of 10mg collagenase per aortic intima was used. Teasing the intima at the time of stripping the intima from the media gave a greater amount of available substrate for the enzymes to act upon. This together with a slightly increased time of incubation resulted in an increase in the yield of cells.

The pH of the medium was checked at pH 7.4 at the start of the incubation. During the incubation the pH tended to decrease, and was readjusted with 0.2M Na_2HPO_4 solution. Collagenase and elastase have pH optimum ranges of 6.7-7.8 and 8.0-9.0 respectively (Gallop, Seifter & Meilman, 1957). The incubations were not carried out under optimal conditions for elastase activity, as it was considered more important to have optimal conditions for collagenase activity, since there is less elastin than collagen present in the intima. Also, collagenase preparations are highly specific for collagen whereas elastase demonstrates unspecific proteolytic activity.

C. Fatty Acid Composition of Lipid Fractions of Isolated Foam Cells

The fatty acid composition of the cholesterol ester, triglyceride and phospholipid fractions of the foam cells were determined by gas-liquid chromatography. Foam cells were isolated and chloroform:methanol (2:1, v:v) extracts prepared as described previously. The extracted lipids were separated by neutral lipid thin-layer chromatography into the major fractions - phospholipids, cholesterol esters and triglycerides. Each fraction was scraped from the plate and their fatty acid-methyl esters prepared as described under Methods. Suitable aliquots of the fatty acid-methyl esters were taken for separation by gas-liquid chromatography. The tracing obtained was quantitated by multiplying peak height by retention time for each fatty acid, and the percentage distribution of each fatty acid obtained.

The fatty acid composition of the three lipid fractions of isolated foam cells is shown in Table **14**. The cholesterol ester-fatty acids were

TABLE 14

FATTY ACID COMPOSITION OF VARIOUS LIPID FRACTIONS OF ISOLATED FOAM CELLS

% Distribution

Fatty Acid	Phospholipid*	Cholesterol Ester **	Triglyceride***
14:0	Trace	Trace	1.7 + 1.4
16:0	24.0 ± 5.5	14.1 ± 2.8	29.6 ± 5.8
16:1	1.6 ± 1.1	2.3 ± 1.5	5.5 ± 0.8
18:0	18.5 ± 2.8	5.3 ± 1.2	17.6 ± 5.8
18:1	18.2 ± 3.7	58.5 ± 3.0	29.0 ± 2.4
18:2	14.3 ± 3.3	14.2 ± 2.9	10.9 ± 2.2
18:3	Trace	Trace	1.5 ± 1.0
20:0	Trace	2.8 ± 1.7	1.9 ± 1.1
20:3	2.6 ± 1.9	0.99± 0.8	0
20:4	13.0 ± 4.6	1.8 ± 1.1	3.4 ± 4.7
?	3.6 ± 2.2	0	0

* Mean ± Standard Deviation - 18 samples

** Mean ± Standard Deviation - 15 samples

*** Mean ± Standard Deviation - 5 samples

mainly monounsaturated with significantly lower proportions of saturated and polyunsaturated fatty acids. In contrast, both the phospholipid and triglyceride contained higher amounts of saturated fatty acids, and in the case of phospholipid, appreciable amounts of arachidonic acid.

The fatty acid patterns of the lipid fractions resembles quite closely that of the whole intima (Table 15). Much of the lipid in the rabbit atherosclerotic lesion is present of course in foam cells, so that one might expect a close correlation. The cholesterol ester-fatty acid acid patterns of human foam cell lesions (Smith, Evans & Downham, 1967; Smith, Slater & Chu, 1968), are also similar to those found for the rabbit foam cells.

TABLE 15

FATTY ACID COMPOSITION OF VARIOUS LIPID FRACTIONS
OF AORTIC INTIMA FROM CHOLESTEROL-FED RABBITS

% Distribution

Fatty Acid	Phospholipid*	Cholesterol Ester*	Triglyceride**
14:0	Trace	Trace	Trace
16:0	27.6 ± 4.0	11.8 ± 0.8	25.9 ± 3.2
16:1	2.4 ± 0.6	4.2 ± 0.7	4.1 ± 0.6
18:0	15.9 ± 2.0	3.2 ± 0.7	11.0 ± 1.5
18:1	17.6 ± 1.7	60.8 ± 3.6	34.7 ± 1.3
18:2	16.2 ± 2.3	16.5 ± 2.5	21.7 ± 3.4
18:3	1.4 ± 0.3	Trace	1.2 ± 1.2
20:0	Trace	2.7 ± 0.5	0.7 ± 0.9
20:3	1.6 ± 1.1	Trace	0
20:4	10.0 ± 2.3	0.9 ± 0.8	0
?	6.6 ± 0.9	0	0

* Mean ± Standard Deviation - 8 samples

** Mean ± Standard Deviation - 5 samples

SECTION 5.

TISSUE CULTURE OF FOAM CELLS

A. Introduction

Although the major emphasis in the investigations with foam cells was placed on the biochemical properties of these cells, the opportunity arose to make an attempt at culturing them. This approach was of interest for two reasons; firstly, yields of foam cells were relatively low and therefore any method which increased their yield would be welcomed; secondly, there is conflicting evidence regarding the ability of foam cells to divide *in situ*. The availability of homogeneous preparations of mature foam cells provided an opportunity to determine whether multiplication of established foam cells took place.

In this section therefore work is described in which an attempt has been made to grow foam cells in tissue culture.

B. Liquid Culture Media

Foam cells were isolated from thoracic aortas of rabbits fed a cholesterol diet for 12-16 weeks as described under Methods. Approximately $250x10^3$ foam cells were dispensed into each of a

series of 30mm petri dishes, containing in a total volume of 2ml, Eagle's M.E.M. (Eagle, 1959), supplemented with pyruvate (1mM), serine (0.2mM), soy broth (Baltimore Biological Laboratories - 10%), and serum. Sera tested for possible growth-inducing activity were 10% foetal calf (FCS), 10% normal rabbit, 10% cholesterol-fed rabbit, 10% horse, 10% FCS plus 5% normal rabbit, 10% FCS plus 5% cholesterol-fed rabbit, and 10% FCS plus 5% horse. All cultures were incubated at 37°C in 5% carbon dioxide in air in a humidified incubator. Cultures were carried out in triplicate. The progress of the cultures was followed on an inverted microscope by observation of specifically marked areas of the dishes so that changes in the areas from day to day could be recorded.

In all dishes the foam cells rapidly stuck to the glass (Fig.23), and at 24h the cultures were predominantly populated by typical lipid-filled foam cells with a few spindle shaped fibroblasts (Fig.24). The "non-foam" cells at the time of plating represented about 5% of the total number of cells plated. At the eighth day after seeding, fibroblast proliferation was obvious, and foam cells were

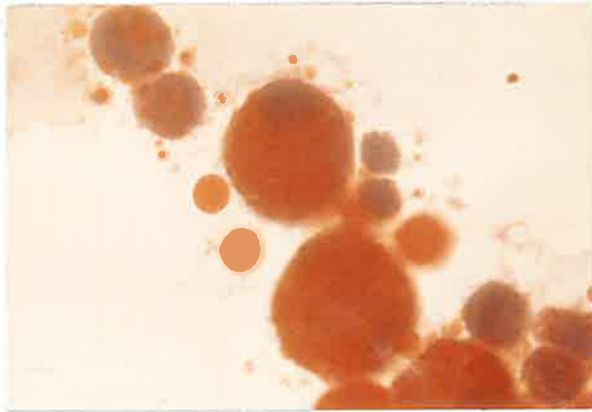


Fig. 23

Foam cells adhered to glass at time of plating. Stained with Haematoxylin and Sudan IV. (X1000).

Fig. 24

Portion of a rabbit foam cell culture, typical of that seen 24h after inoculation of cells into the dish. Unstained. (X400).

observed as small clusters, mostly on or near the fibroblastic cells (Fig.25 & 26).

The cultures were maintained for periods of up to 28 days and during this time there was no marked change, except that the fibroblasts continued to proliferate until almost a complete cover of the surface was obtained (Fig.27). Foam cells remained without any obvious change in numbers, and there was only a slight tendency for the cells to float off into the medium with increasing culture time. Whilst the majority of foam cells appeared intact near the end of the culture period, a number were obviously broken down and had formed a lipid mass which was completely surrounded by fibroblasts (Fig.28). Although clusters or aggregates of foam cells were seen throughout the culture period, no indication was found of increasing size of these aggregates.

No differences were observed between the serum supplements and in subsequent experiments 10% foetal calf serum was used routinely.

The effect of cell density at the time of seeding was investigated by plating cells at densities

Fig.25

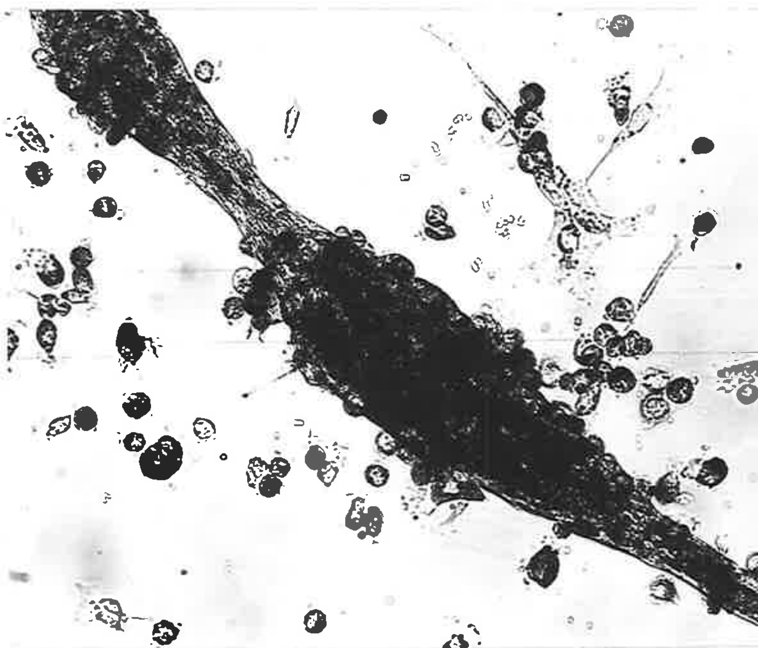
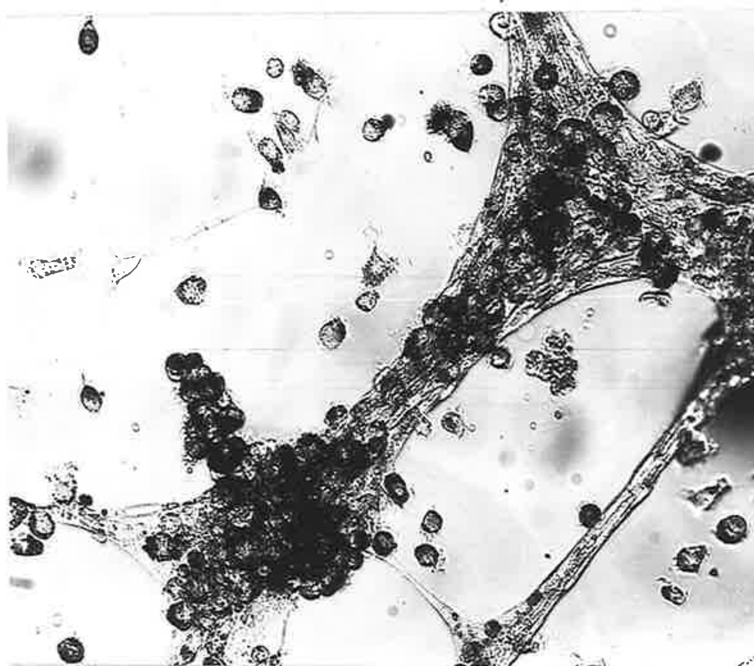


Fig.26



Figs. 25 & 26

Aggregate of foam cells adhering to fibroblasts as typically seen in an 8 day culture. Unstained. (X400).

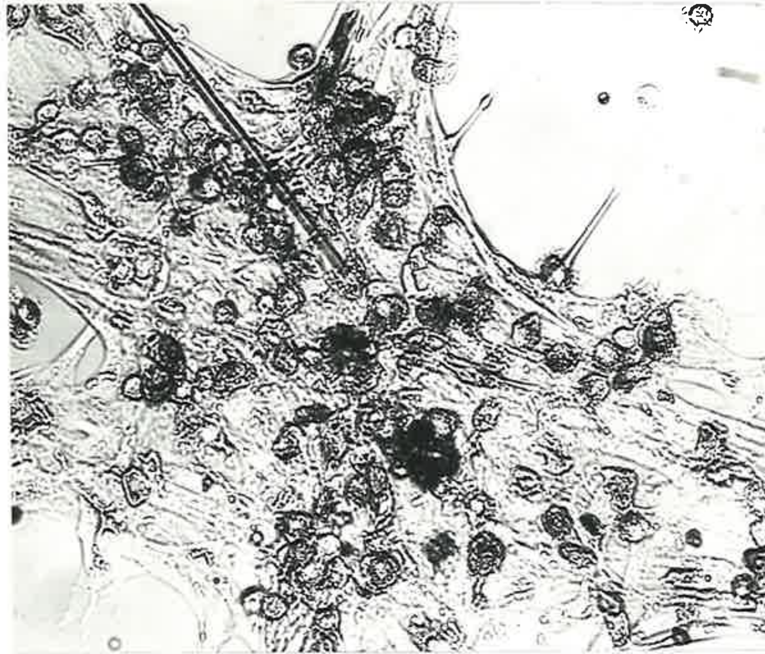


Fig. 27

Portion of a rabbit foam cell culture, typical of that seen after 28 days of culture. Unstained (X400).

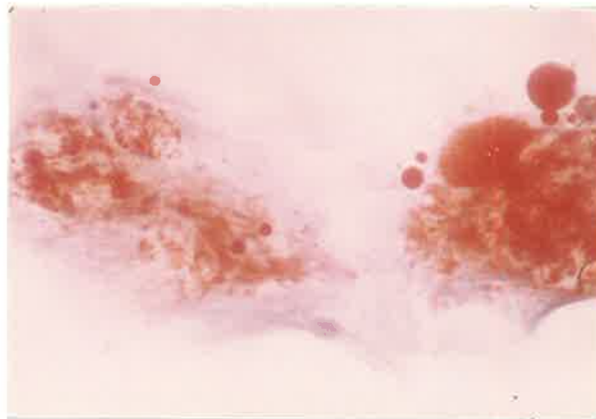


Fig. 28

Broken-down foam cells after 28 days of culture. Lipid masses are surrounded by aggregates of fibroblasts. Stained with Haematoxylin and Sudan IV. (X600).

ranging from $25x10^3$ to $500x10^3$ cells per 30mm glass petri dish. Within the limits used, the size of the inoculum did not change the qualitative behaviour of the culture. However, at higher densities, the fibroblasts rapidly developed to give a complete layer with a few foam cell aggregates adhering to the fibroblast layer.

C. Fibroblast "Feeder Layers"

As there was a tendency to find foam cell aggregates in areas where fibroblasts were proliferating, some cultures were set up using fibroblasts obtained from previous cultures as "feeder layers". In these experiments freshly isolated foam cells were inoculated on to the "feeder layers", either in a liquid medium or in a semi-solid 0.3% agar medium. The cultures were maintained for up to 10 days, and in the case of the liquid cultures the added foam cells adhered to the fibroblast layer specifically as single cells, and did not change over the course of the incubation period (Fig. 29). The cultures were not continued after 10 days, because at this time the fibroblast layer tended to roll up,

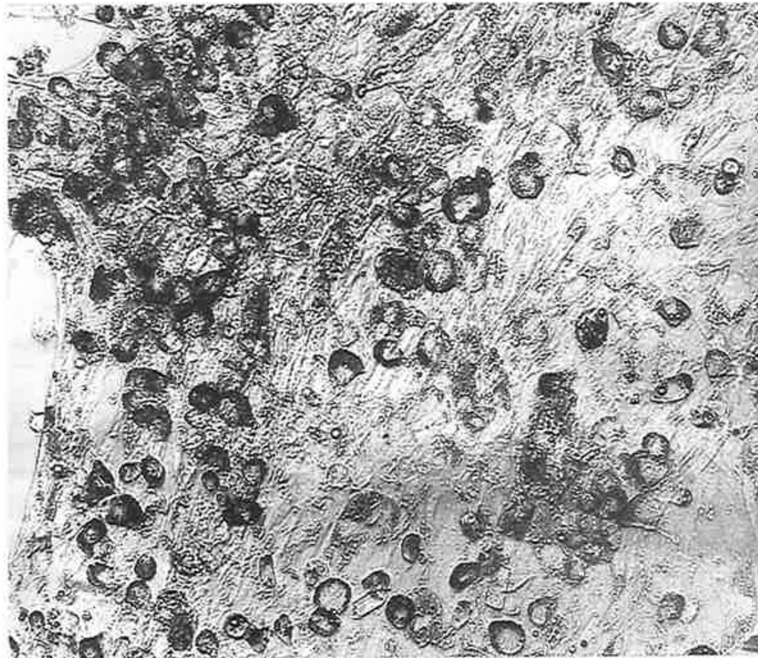


Fig. 29

Foam cells inoculated onto a feeder layer of rabbit fibroblasts. 10th day of incubation. Unstained. (X400).

suggesting that the cultures were terminal. Those cells added to the "feeder layer" in an agar medium also remained as single cells throughout the period investigated.

D. "Conditioned" Media

In view of recent results demonstrating the ability of "conditioned" media to stimulate clonal growth of various cell types *in vitro* (Konigsberg, 1961; Eagle, 1965; Pluznik & Sachs, 1966; Virolainen & Defendi, 1967; Bradley & Sumner, 1968), some cultures were set up with medium that had previously been incubated for 7 days with foam cell populations, or with medium conditioned by mouse embryo cells, which has been found suitable for colony induction of bone marrow cells (Bradley & Sumner, 1968). In these experiments 0.5ml of conditioned medium was added to each 30mm petri dish together with 1.5ml of freshly prepared liquid culture medium, or semi-solid agar containing 500×10^3 foam cells. The control incubations contained no conditioned media. Again, both liquid and agar cultures were investigated, and in each case there was no difference observed between

cultures with either type of conditioned media, and the control (non-conditioned medium) cultures.

E. Uptake of Carbon Particles

The phagocytic characteristics of cells after 10 days of incubation *in vitro* in liquid culture was tested by investigating their ability to take up fine carbon particles. 0.2ml carbon suspension (approximately 10mg) (C11-1431-a Guenther & Wagner, Pelikan Werke, Hanover, Germany), was added to each petri dish and incubated for a further 24h. The medium was removed and the plated cells washed thoroughly with 0.9% sodium chloride solution to remove any adhering carbon. Figs. 30, 31, 32 and 33 show that massive particle uptake occurred in the foam cells, but little or no uptake occurred in the fibroblasts, demonstrating that foam cells are still phagocytic after 10 days of incubation. Cultures stained at this time with Haematoxylin and Sudan IV showed typical foam cell staining, with no observable differences from that obtained when the cells were stained immediately after isolation from the lesions (Figs. 34 & 35).

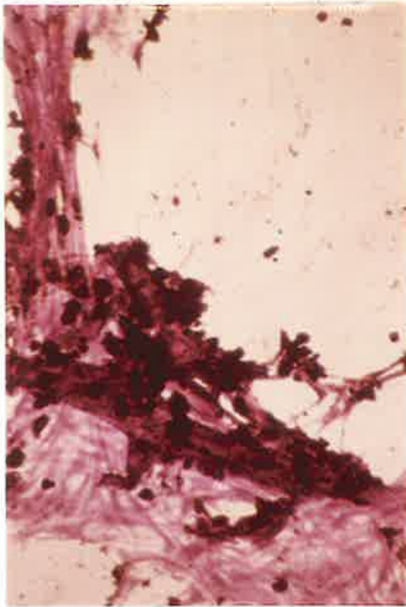


Fig. 30

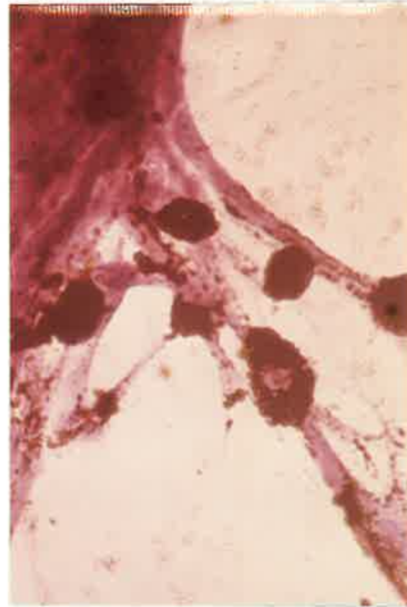


Fig. 31

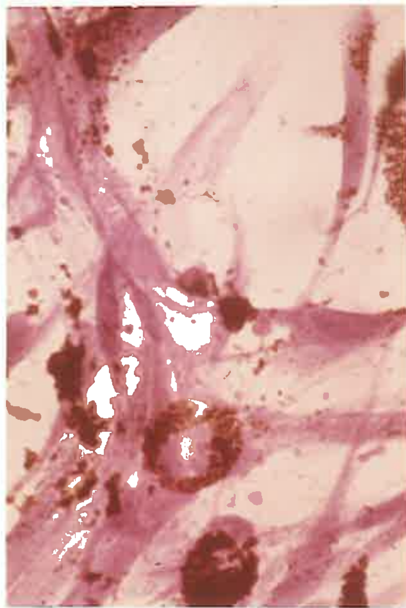


Fig. 32

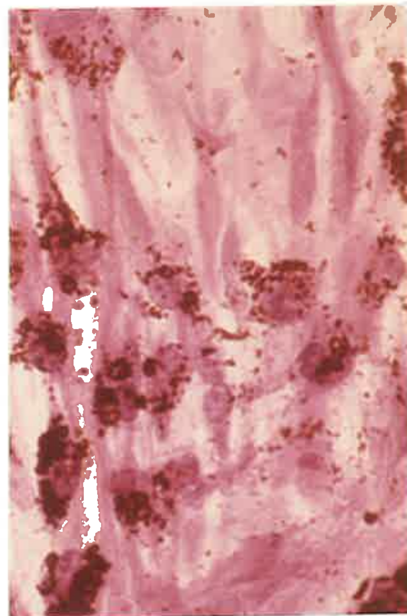


Fig. 33

Figs. 30, 31, 32 & 33

Carbon uptake by foam cells in a 10th day culture. Note lack of uptake by fibroblasts. Stained with Haematoxylin and Eosin. Fig. 30 (X87). Figs. 31, 32 & 33. (X600).

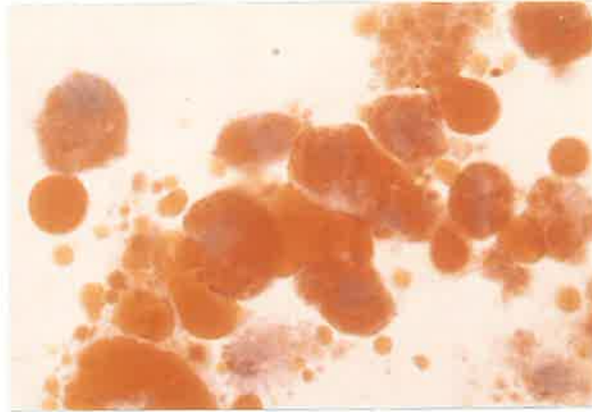


Fig.34

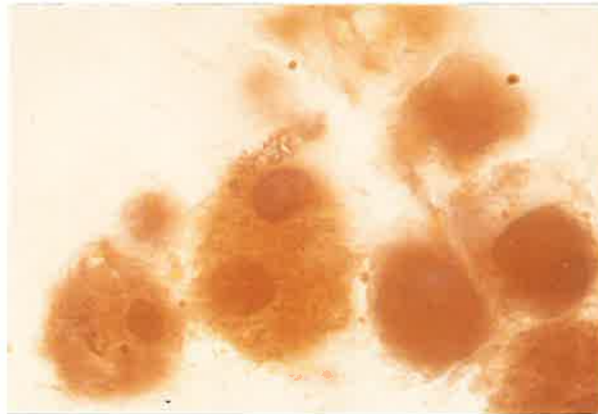


Fig.35

Figs.34 & 35

Foam cells after 10 days of culture show no observable differences from those stained immediately after isolation. (Fig.23). Stained with Haematoxylin and Sudan IV. Fig.34 (X600), Fig.35 (X1000).

F. Discussion

No other indication has been found in the present experiments that the developed foam cells, isolated from rabbit lesions 16 weeks after starting the cholesterol diet, will multiply in tissue culture under any of the conditions studied. The cells used were active metabolically as indicated by their capacity to take up and incorporate oleic acid into combined lipid (Section 6), and by their ability to phagocytoze carbon particles. It is possible that the foam cells were unable to divide due to the effects of collagenase and elastase used during their isolation. However, it is known that mammalian lung cells proliferate after isolation using the proteolytic enzymes trypsin and collagenase (Hinz & Syverton, 1959). Similarly, the contaminating fibroblasts in the foam cell preparations reported in the present paper divided rapidly. Thus a general effect of proteolytic enzymes on cell proliferation seems unlikely, but a specific effect on foam cells cannot be excluded. Other work indicating that foam cells are incapable of extensive multiplication has

been described by Kokubu and Pollak (1961), using explants of cholesterol-induced rabbit atherosclerotic lesions; the present observations are essentially in agreement with this.

Contrary to these observations, McMillan and Duff (1948) have observed the presence of mitotic figures in foam cells present in rabbit lesions after cholesterol feeding for 11-13 weeks. Also Spraragen, Bond and Dahl (1962) found that tritiated thymidine was incorporated into the nuclei of some foam cells, but not into smooth muscle cells and fibroblasts in experimental rabbit lesions observed 5-13 weeks after starting the cholesterol diet. In each of these studies the mitotic index was not computed by the authors. However, in both cases the number of mitotic figures or labelled cells is obviously low compared with the total cell population examined. It is therefore unquestionable that certain foam cells can divide at some time during the development of the lesions, but the question of the number of cells showing proliferative activity throughout the course of development of a lesion is one which is still to be

settled by a thoroughly quantitative study utilizing tritium-labelled thymidine uptake.

The present results from the in vitro culture study may not be incompatible with these observations, if it is recognized that (a) foam cells isolated from more developed lesions (as used in this section) no longer exhibit proliferative activity, and that (b) even when a mitotic figure is observed in a foam cell, it may be nearly the last generation of cells capable of showing mitosis, in which case in vitro cultures could be expected to show only small "clones", and would not be readily recognized or able to be quantitated in a mixed culture with a high density of cells.

In the present studies, although aggregates of foam cells are seen on some areas of the dishes, it is suggested that these aggregations result from the proliferation of the 5% contamination of fibroblasts in the foam cell preparation, giving rise to the typical "strands" of fibroblasts observed, with the foam cells aggregated or stuck to the surface of the fibroblasts as shown in Fig.**26**. This suggestion

is supported by other cultures where isolated foam cells were incubated on an already established complete feeder layer of fibroblasts. Here, no such aggregation occurred, the foam cells remaining as single cells only. Essentially the same behaviour was shown when the cells were plated in agar, a situation in which cell movement would not be possible, but in which clones should form if the cells were capable of extensive proliferation.

The atherophil or potential foam cell described by Robertson (1963) can actively multiply in culture with a generation time of 28-39h. Such cells have been shown to take up lipoprotein, and this uptake resulted in cell death with subsequent reutilization of the lipid present into other cells in culture. This effect of lipid uptake on cells in culture has been confirmed by Branwood (1964). Thus, it is possible that lipid-laden foam cells become terminal cells as the lesion progresses, although in the early lesion they may exhibit some mitotic activity.

The question of the origin of foam cells in the atherosclerotic lesion is still unanswered. Whether the foam cell lesions are generated by a single cell type or not, and whether the foam cells developed are derived from local pluripotential tissue cells or from cells circulating in the blood, has not been resolved at the present time.

SECTION 6.

IN VITRO INCORPORATION OF ^{14}C-LABELLED OLEIC ACID INTO COMBINED LIPID BY ISOLATED FOAM CELLS

A. Introduction

Aortic tissue, like virtually all other tissues, possesses the ability to carry out numerous metabolic reactions. Both the quantitative and qualitative aspects of the synthesis and metabolism of lipid by aortic tissue and by aortic preparations of cells is of great importance in view of the presence of large amounts of lipid in atherosclerotic lesions. A number of workers have investigated the incorporation of fatty acids into combined lipids by aortic homogenates (Stein & Stein, 1962; Stein, Stein & Shapiro, 1963; Parker, Schimmelbusch & Williams, 1964; Parker, Ormsby, Petersen, Odland & Williams, 1966; Portman, 1967). Most of this work has been concerned with normal tissue. However, Parker and coworkers (1966) compared the incorporation of ^{14}C-labelled linoleic acid into phospholipids by homogenates of aortas from normal rabbits and from those fed a cholesterol diet. They observed that the homogenates obtained from cholesterol-fed rabbits incorporated significantly more label into phospholipid than did homogenates of normal aortas.

Recently it has been shown using rabbit aortic lesions (Day & Wahlqvist, 1968), and human aortic lesions (Wahlqvist, Day & Tume, 1969) incubated *in vitro* with ^{14}C-labelled oleic acid that incorporation of label occurred primarily into cholesterol ester and phospholipid, and evidence was presented that foam cells were responsible for these reactions.

In this section, the ability of isolated foam cells to take up and incorporate oleic acid into combined lipids, is investigated.

B. Uptake of Oleic Acid

The uptake of oleic acid by foam cells was investigated over a 4h period. A known number of washed foam cells (approximately 0.5×10^6 cells) were dispensed into Leighton tubes and incubated for either 1, 2 or 4h at 37°C in 1ml Hanks solution:normal rabbit serum (1:1, v:v), containing approximately 2μCi ^{14}C-labelled sodium oleate, 1mg non-radioactive sodium oleate, and 35mg bovine serum albumin (fatty acid-poor). The pH was adjusted to pH 7.4 and all tubes were gassed with 5% carbon dioxide in oxygen and stoppered prior to incubation. Control media were incubated for 4h in the absence of cells. All incubations were

carried out in duplicate.

Following incubation the cells were separated from the medium (including both cells adherent to the glass and those free in the medium) by centrifugation (220xg for 5min), and washed twice with 1ml 0.9% sodium chloride solution. Both cells and medium were extracted with chloroform:methanol (2:1, v:v) according to the method of Folch, Lees and Sloane-Stanley (1957).

Table 16 shows the uptake of oleic acid by the isolated foam cells at various time intervals. The amount of oleic acid taken up (expressed as mμmoles per 10^6 cells) is calculated from the amount of ^{14}C label present in the cells at the respective time intervals, and the specific activity of the oleic acid originally present in the medium. Since 1mg of oleic acid was added to the medium, an amount well in excess of that contributed by either the serum or the free fatty acid in the albumin present, these latter contributions were ignored in calculation of uptake. During the first hour of the incubation uptake was rapid, but little further increase was observed during

TABLE 16

UPTAKE AND INCORPORATION OF ^{14}C-LABELLED OLEIC ACID INTO COMBINED LIPIDS BY ISOLATED FOAM CELLS

Expt	Amount of ^{14}C-labelled oleic acid added cts/min	No. of cells per incubation tube	Uptake (mμmoles oleic acid/10^6 cells)			*Incorporation into combined lipids (mμmoles oleic acid/10^6 cells)		
			1 h	2 h	4 h	1 h	2 h	4 h
1	3.73×10^6	0.5×10^6	50.7	71.0	57.4	13.1	21.6	34.8
2	4.05×10^6	0.4×10^6	19.5	26.6	34.8	7.82	13.2	23.6
3	3.73×10^6	0.5×10^6	25.2	29.4	38.0	9.04	12.7	23.0

* Incorporation into combined lipid equals that fraction of the total ^{14}C-labelled oleic acid taken up by the cells and incorporated into phospholipid, mono- and diglyceride, triglyceride and cholesterol ester, viz. total uptake equals free fatty acid plus combined lipid.

the next 3h even though 99% of the added oleic acid was still available in the medium.

C. Incorporation of Oleic Acid into Combined Lipid

Table 16 also shows the total incorporation of ^{14}C-labelled oleic acid into combined lipids of foam cells, expressed as mumoles oleic acid esterified per 10^6 cells. These values were obtained from the percentage distribution of ^{14}C-label between the various lipid fractions as determined by thin-layer chromatography. The Table shows that most of the oleic acid taken up after 4h was esterified.

Phospholipid, triglyceride and cholesterol ester were the major lipid fractions to become labelled, and Fig.36 shows the rate of oleic acid incorporated into each fraction. The incorporation was approximately linear over the 4h period studied. Phospholipid and cholesterol ester became labelled at approximately the same rate, whereas triglyceride became labelled at about half their rate. No information is available regarding the dilution of the ^{14}C-labelled oleic acid taken up from the medium by any free fatty acid within the cells. Thus, these calculated values of oleic

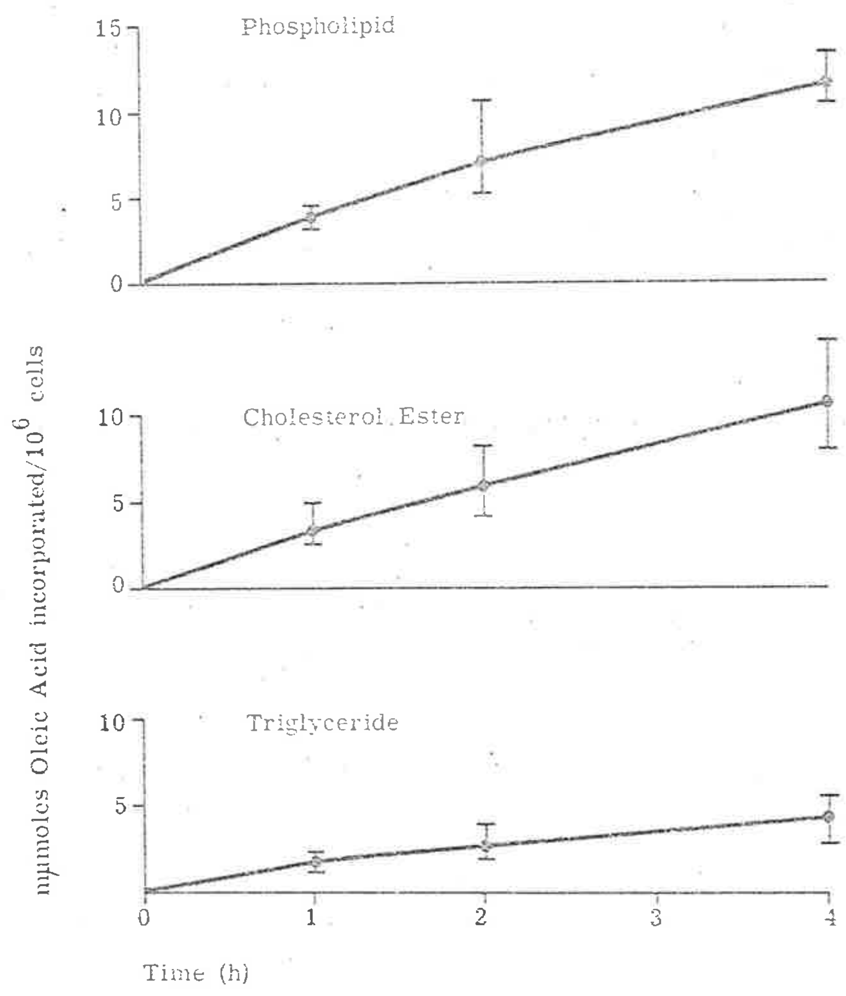


Fig. **36** The rate of incorporation of oleic acid into individual lipid fractions of foam cells. Each point represents the mean of three experiments each with duplicate incubations, showing the range of values obtained.

acid incorporation into the lipid fractions represent minimal figures. No incorporation of oleic acid into combined lipids occurred in either the control medium or in the medium in which the foam cells were incubated.

Fig. 37 shows the incorporation of ^{14}C-labelled oleic acid into the individual phospholipids. Most of the label was incorporated into lecithin (phosphatidylcholine) (50-60%), with lesser amounts into phosphatidylethanolamine, phosphatidylinositol and an unknown lipid or lipids running near the solvent front. The latter spot was not identified but possibly represented phosphatidic acid. There was little incorporation of the ^{14}C-labelled oleic acid into lysolecithin and sphingomyelin. The incorporation rate into lecithin was linear over the 4h period investigated. Phosphatydlethanolamine and phosphatidylinositol were linear for the inital 2h, but then levelled off.

D. Specific Activity of Oleic Acid Incorporation

The specific activity of the oleic acid in the phospholipid, cholesterol ester and trigylceride fractions was determined by gas-liquid

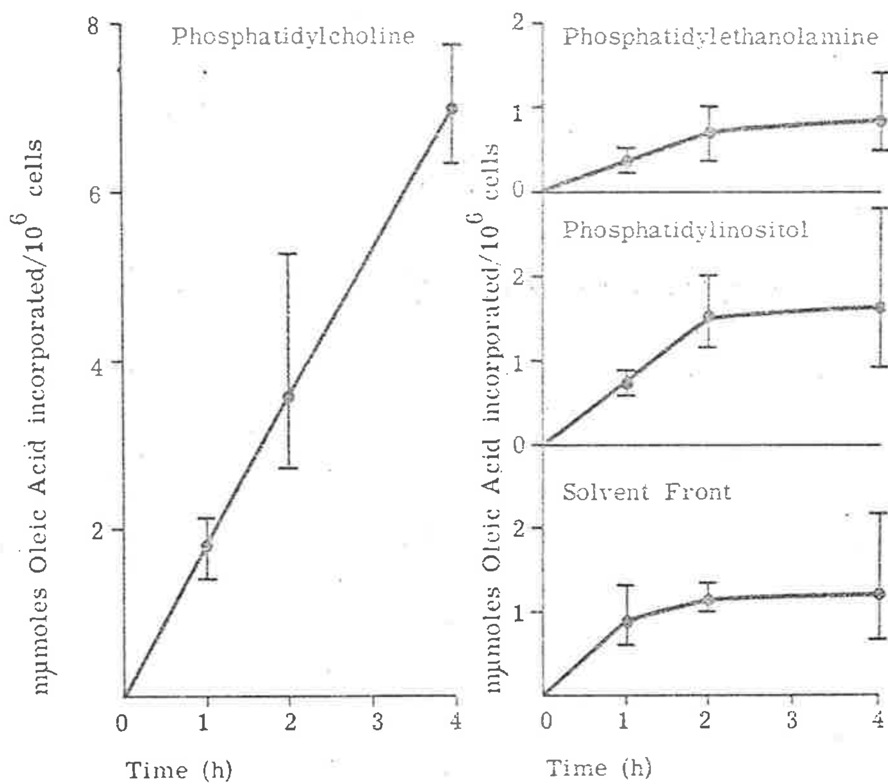


Fig. 37 The rate of incorporation of oleic acid into individual phospholipid classes of foam cells. Each point represents the mean of three experiments each with duplicate incubations, showing the range of values obtained.

radiochromatography as described under Methods. Table 17 shows the specific activity of the oleic acid in counts per minute per mumole oleic acid at time 1, 2 and 4h for the three experiments. In each experiment and for each lipid fraction there was an approximately linear increase in specific activity over the 4h period studied. However, the specific activity of the phospholipid was considerably greater than that of cholesterol ester at all time intervals in all experiments. In the third experiment the specific activity of the triglyceride was also measured, and was also found to be in excess of that for the cholesterol ester, but less than that recorded for the phospholipid in the same samples. There is no apparent reason for the decreased phospholipid specific activity at 4h in experiment 1. The chemical amount (mμmoles) present in this sample was similar to that found in the other phospholipid samples in this experiment, but the amount of label present at 4h was less than at 2h.

E. Discussion

The data presented shows that isolated foam cells incubated *in vitro* take up and incorporate ^{14}C-labelled oleic acid into cholesterol ester phospholipid and triglyceride. This study demonstrates only that such metabolic pathways are operative in foam cells, and does not necessarily

TABLE 17

SPECIFIC ACTIVITY OF ^{14}C-LABELLED
OLEIC ACID IN COMBINED LIPID FRACTIONS

(cts/min/mμmole oleic acid)

Experiment	Time (h)	LIPID FRACTION		
		Phospholipid	Cholesterol Ester	Triglyceride
1	1	130	46	
	2	415	62	-
	4	263	104	
2	1	125	17	
	2	186	34	-
	4	439	75	
3	1	75	8*	44*
	2	184	11*	78
	4	253	28	111*

* Total number of counts recorded was between 35-50 per run in the samples marked. In all other cases between 50 and 440 counts per run were recorded.

indicate that these cells are responsible for the accumulation of phospholipids and cholesterol esters in the atherosclerotic lesion. However, in view of the studies of Day and Wahlqvist (1968) and Wahlqvist, Day and Tume (1969), where autoradiographic localization of ^{14}C-labelled oleic acid and its metabolic products (cholesterol ester and phospholipid) was demonstrated over foam cell areas in the intima, it seems likely that foam cells do represent the major lipid synthesizing cells in the lesion.

The uptake of fatty acid and its incorporation into cell lipid has been observed for a number of cell types, including tumour cells in tissue culture (Spector & Steinberg, 1965), peritoneal macrophages (Day & Fidge, 1962), alveolar macrophages (Elsbach, 1965) and blood cells (Winterbourn & Batt, 1968). In all of these cases, phospholipid and triglyceride were the major lipid fractions into which the fatty acid was incorporated, and there was a minimal incorporation into cholesterol ester. In the present studies however, a considerable proportion of the fatty acid taken up was incorporated into cholesterol ester; in fact there was about equal incorporation of label into phospholipid and cholesterol ester. Because the amount of

cholesterol ester formed in these cells was about equal to the amount of phospholipid formed. Because the amount of cholesterol ester present in the cells was far in excess of any other lipid (Day, Newman & Zilversmit, 1966), specific activity data were required to provide some indication of the fractional turnover of cholesterol ester-fatty acid relative to phospholipid-fatty acid. Assuming that the fatty acid in the two lipid fractions arises from a common fatty acid precursor pool, then some indication of the relative fractional turnover can be assessed from a comparison of the relative specific activities at corresponding time intervals. In all cases the specific activity of phospholipid-oleic acid was considerably in excess of that of the cholesterol ester-oleic acid. The units for the specific activity data were given as counts per min of ^{14}C-label per mμmole oleic acid in the respective lipid fractions, and thus assuming that only one of the two positions of the phospholipid is occupied by oleic acid, direct comparison of specific activities of cholesterol ester and phospholipid is valid

It is of interest to speculate on the possible metabolic pathways available for the incorporation of fatty acid into phospholipid and cholesterol ester by foam cells. Labelled fatty acid may be incorporated into cholesterol ester by several pathways:

(1) by direct esterification of cholesterol with fatty acid. The work presented in earlier sections of this thesis suggests that peritoneal and alveolar macrophages can bring about a direct esterification of fatty acid with cholesterol, without any cofactor requirements. That foam cells incorporate fatty acid into cholesterol ester specifically by this pathway cannot, however, be concluded from the data presented;

(2) by an enzyme exchange reaction, whereby the fatty acid of endogenous cholesterol ester is exchanged with the labelled fatty acid. In such a case there would be no net synthesis. That foam cells carry out such an exchange reaction is supported by work presented later, where it was

not possible to demonstrate significant conversion of ^{14}C-labelled cholesterol to cholesterol ester when supplied to foam cells either as cholesterol particles or as micelles;

(3) by lecithin:cholesterol acyltransferase reaction. It is possible that the labelled fatty acid could be initially incorporated into lecithin, and then esterified with cholesterol by means of this reaction. There is no direct evidence of the presence of an acyltransferase reaction in this situation, but the specific activity of the phospholipid is considerably in excess of that of cholesterol ester and thus makes this a possible mechanism. This pathway was not found to be of significance in peritoneal macrophage homogenates; however, it has recently been claimed to be operative in both human and rabbit aortic homogenates (Abdulla, Orton & Adams, 1968).

Lecithin was the major phospholipid fraction to become labelled. Again, no direct conclusions regarding its formation can be made. In order to investigate the pathways involved in cholesterol ester and phospholipid synthesis, it was necessary

to turn to subcellular preparations of foam cells and so investigate directly the individual reactions. Preliminary studies using such subcellular preparations are described in the next section.

SECTION 7.

ESTERIFICATION OF ^{14}C-LABELLED OLEIC ACID

BY FOAM CELL HOMOGENATES

A. Introduction

The investigations with ^{14}C-labelled oleic acid and intact foam cells described in the preceding section were extended in the work described in the present section. Foam cell homogenates were prepared and these preparations used to investigate further the incorporation of oleic acid into combined lipids. These studies, although still preliminary, form a starting point for determining the pathways involved in the formation of cholesterol ester and phospholipid by the foam cells. The elucidation of these pathways makes their manipulation by chemical means a possible approach to modifying the deposition of lipid in the atherosclerotic wall.

B. Incorporation of Oleic Acid and its Possible Oxidation to CO_2 by Foam Cell Homogenates

Isolated foam cells were homogenized and dialyzed as described under Methods. All reactions were carried out in duplicate in 12ml centrifuge tubes with moderate shaking for 15h, (except for Experiment 2 which was incubated for 90min only), at 37°C in an atmosphere of air. Each tube contained

0.6ml 0.2M citrate-phosphate buffer pH 7.4, 0.3ml dialyzed foam cell homogenate containing a known amount of protein (1-2mg), and a tracer dose of [1-^{14}C]-labelled sodium oleate (containing a known number of counts), complexed with an excess amount of albumin. Where added, cofactors were present in the following amounts: ATP (10μmoles), CoASH (1μmole), Mg^{++} (5μmoles), and α-glycerophosphate (5μmoles), and the reaction mixture was made up to total volume of 1.2ml with buffer. Control incubations, containing either no homogenate or boiled homogenate, were included.

Following the incubation, lipid extracts of each reaction mixture were obtained, and aliquots taken for radioactive counting as described under Methods. Table 18 shows the recovery of label from each of four experiments. In each experiment there was greater than 95% recovery in the control incubations. Where cofactors were added, there was a considerable loss of label - up to 49% of that added in Experiments 1 and 3. Where no cofactors were added, in only Experiment 1 was there any appreciable loss of label. All other experiments

TABLE 18

LOSS OF ^{14}C-LABEL FROM INCUBATION MEDIUM

	cpm Added x1000	cpm Recovered x1000	% Added Label Lost
Expt 1			
Complete System †	1010	516	49
- Cofactors	1010	585	42
Control	1010	980	3.0
Expt 2*			
Complete System	560	540	3.6
- Cofactors	560	552	1.4
Control	560	558	0.4
Expt 3			
Complete System	298	152	49
- Cofactors	298	285	4.4
Control	298	285	4.4
Expt 4			
Complete System	518	445	14
- Cofactors	518	570	0
Control	518	496	4.2

* Incubated 90 min.

† Complete system contained in a total volume of 1.2ml, 0.6ml 0.2M citrate-phosphate buffer pH 7.4, 0.3ml dialyzed foam cell homogenate containing a known amount of protein (1-2mg), a tracer dose of ^{14}C-labelled sodium oleate and 10μmoles ATP, 1μmole CoASH, 5μmoles Mg^{++} and 5μmoles α-glycerophosphate. Incubated at 37°C for 15 h.

gave a greater than 95% recovery. It is thus apparent that the addition of cofactors to the dialyzed homogenate preparation brings about a series of reactions which result in the loss of ^{14}C-labelled lipid from the incubation system. Presumably the label is lost as $^{14}CO_2$ by oxidation of [1-^{14}C]-labelled oleic acid, a series of reactions which are dependent upon the presence of cofactors ATP and CoASH.

C. Incorporation of Oleic Acid into Combined Lipids

The incorporation of ^{14}C-labelled oleic acid into phospholipids, mono- and diglycerides, triglycerides and cholesterol esters in the presence and absence of cofactors is shown in Table 19. In each experiment where cofactors were added, there was appreciable incorporation of label into phospholipids and cholesterol esters with lesser amounts into the glyceride fractions, but where cofactors were omitted there was negligible esterification, except in Experiment 1 where the dialysis was apparently incomplete. There was no esterification in those tubes containing boiled homogenate, or where no homogenate was added.

TABLE 19

ESTERIFICATION OF [1-^{14}C] OLEIC ACID
(% Distribution of Lipid ^{14}C-Remaining)

	PL	MG & DG	FA	TG	CE
Expt 1					
Complete system†	52	1.1	20	3.9	20
- Cofactors	11	0.7	83	0.9	5.5
Control	2.1	1.4	96	0.2	0.2
Expt 2*					
Complete system	10	1.0	79	1.6	8.1
- Cofactors	1.7	0.8	97	0.3	0.2
Control	1.2	1.5	97	0.1	0.1
Expt 3					
Complete system	63	7.0	9.6	4.0	16
- Cofactors	0.6	2.5	96	0.2	0.2
Control	1.7	3.7	94	0.2	0.1
Expt 4					
Complete system	27	8.2	61	0.6	2.6
- Cofactors	1.4	1.3	96	1.2	0.1
Control (boiled homogenate)	0.8	1.3	97	1.0	0

† Complete system as given in Table 18

* Incubate 90 min.

Table 20 shows the incorporation of ^{14}C-labelled oleic acid into the individual phospholipids. Whereas with intact foam cells lecithin was the major phospholipid to become labelled, here, incorporation was predominantly into a spot having the same R_f as phosphatidylethanolamine. This labelled phospholipid was rechromatographed with phosphatidylethanolamine by two dimensional thin-layer chromatography (Rouser, Kritchevsky, Galli & Heller, 1965), as described under Methods. The radioactive areas were located by autoradiography, and those areas containing phosphorus localized by spraying the plate with acid molybdate solution (Dittmer & Lester, 1964). Fig. 38 shows a typical separation - the overlying dark areas indicate regions of radioactivity. The autoradiograph demonstrates clearly that most of the label is present as phosphatidylethanolamine. A small amount of radioactivity was present in each of three further spots corresponding to lecithin, phosphatidylserine and an unknown lipid. Although no phosphatidic acid was available as a standard for chromatography, it

TABLE 20

INCORPORATION OF [1-^{14}C] OLEIC ACID INTO PHOSPHOLIPIDS

% Distribution of Phospholipid ^{14}C

<table>
<tr><th>Expt.</th><th>Origin</th><th>Lyso-Lecithin</th><th>Sphingo-Myelin</th><th>Lecithin</th><th>Phosphatidyl-Inositol</th><th>Phosphatidyl-Serine</th><th>Phosphatidyl-Ethanolamine</th><th>Solvent Front</th></tr>
<tr><td>1</td><td>0.3</td><td>0.07</td><td>0.03</td><td>0.1</td><td>0.7</td><td>0.3</td><td>77</td><td>22</td></tr>
<tr><td>2</td><td>0.2</td><td>0.3</td><td>0.4</td><td>22</td><td colspan="2">26</td><td>17</td><td>35</td></tr>
<tr><td>3</td><td>0.08</td><td>0.2</td><td>0.2</td><td>1.1</td><td>0.7</td><td>15</td><td>63</td><td>20</td></tr>
<tr><td>4</td><td>0.3</td><td>0.3</td><td>0.6</td><td>14</td><td>2.0</td><td>8.5</td><td>66</td><td>8.7</td></tr>
</table>

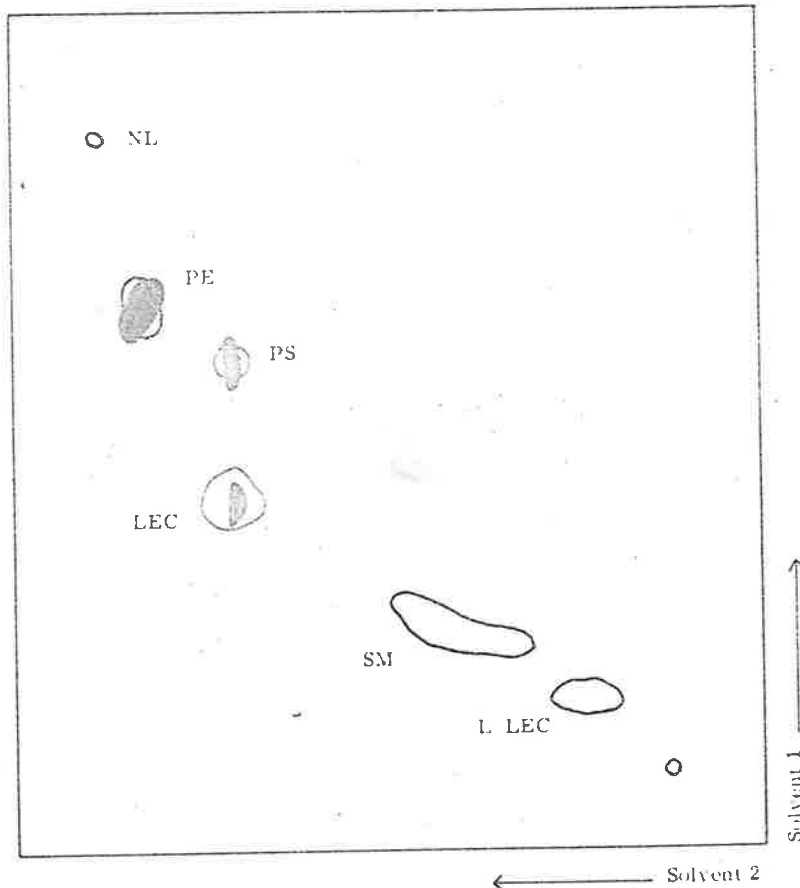


Fig. **38** Identification by two-dimensional thin-layer chromatography of ^{14}C-labelled phospholipids synthesized by foam cell homogenates. The diagram shows the radioactive areas (as determined by autoradiography) overlying the chemical pattern.

O = origin, L/Lec = lysolecithin, SM = sphingomyel

Lec = lecithin, PS = phosphatidylserine,

PE = phosphatidylethanolamine, NL = neutral lipi

Radioactive spot near centre is unknown.

can be assumed that in the experiments described here, negligible ^{14}C-labelled fatty acid was incorporated into phosphatidic acid as phosphatidic acid runs to the left of and below sphingomyelin, and there was no label in this area at all.

D. Discussion

The incorporation of fatty acid into combined lipids by cell-free preparations of aortas has been described by a number of workers (Stein, Stein & Shapiro, 1963; Parker, Schimmelbusch & Williams, 1964; Portman, 1967). In these studies, fatty acid was incorporated into phospholipids and glycerides, and the reaction was absolutely dependent upon the presence of ATP and CoASH for activity. Manipulation of the reaction by the addition of lysolecithin or α-glycerophosphate caused marked changes in the patterns of fatty acid incorporation. Addition of lysolecithin produced an increase in the incorporation of fatty acid into lecithin, whilst addition of α-glycerophosphate increased incorporation into glycerides (Stein, Stein & Shapiro, 1963).

The cell-free system of isolated foam cells used in the present study actively incorporated labelled fatty acid into phospholipids and cholesterol esters when supplemented with the cofactors ATP and CoASH. Unlike normal aortic homogenates, these homogenates incorporated negligible amounts of label into triglycerides, but considerable amounts into cholesterol esters.

It is apparent from the data presented (Table 18) that adequate recovery of label was only obtained in the control incubations and in those incubations which did not have cofactors added, suggesting that ^{14}C label was being lost from the lipid fractions through an ATP, CoASH dependent reaction. Presumably the ^{14}C-labelled fatty acids were oxidised, releasing the [1-^{14}C]-labelled carbon as $^{14}CO_2$. In future studies it will be necessary to collect and count the $^{14}CO_2$ liberated during the incubation, and in this way the loss could be measured directly.

Whereas the major phosphilipid to become labelled in the whole cell studies (previous section), and in homogenates of whole rabbit aorta (Stein, Stein

& Shapiro, 1963), was lecithin, it was phosphatidylethanolamine in the foam cell homogenates. Fatty acid may first be incorporated into phosphatidylethanolamine, either by de novo synthesis (Kennedy & Weiss, 1956), or by acylation of lysophosphatidylethanolamine (Merkl & Lands, 1963). Alternatively it is possible that fatty acid is first incorporated into phosphatidylserine, which is then decarboxylated to form phosphatidyl-ethanolamine (McMurray, 1964; Sherr & Law, 1965; Donisch & Rossiter, 1965).

The possibility exists that foam cells synthesize much of their lecithin by methylation of phosphatidylethanolamine with S-adenosylmethicnine, a pathway which was first described in vitro in liver by Bremer and Greenberg (1961). The dialyzed cell-free preparations of foam cells may be unable to bring about such methylation of the phosphatidyl-ethanolamine, resulting in accumulation of labelled phosphatidylethanolamine. This possibility could be investigated by adding S-adenosylmethionine to the system, and subsequently measuring the conversion of labelled phosphatidylethanolamine to lecithin.

Additional studies on foam cell homogenates are restricted by the small number of cells obtainable. Since only 1-2ml crude homogenate (4-6mg protein/ml) can be obtained from a batch of foam cells from 4 rabbits, progress is relatively slow, and careful experimental design is required. However, such studies provide the only method of investigating the detailed pathways of lipid metabolism within the foam cells, and hence this work must be extended if a clear picture is to be ultimately achieved.

SECTION 8.

UPTAKE AND ESTERIFICATION OF CHOLESTEROL BY FOAM CELLS

A. Introduction

It is apparent from the work described in the previous sections that oleic acid can be incorporated into cholesterol ester by both intact foam cells and by foam cell homogenates.

The role of foam cells in cholesterol ester formation in the atherosclerotic artery has been suggested by several workers. Geer and Guidry (1964) have demonstrated that plaques containing the highest ratio of oleic acid to linoleic acid are also those which show the highest proportion of foam cells. Smith, Evans and Downham (1967) have demonstrated an association between fat-filled cells and the low linoleic, high oleic acid pattern of cholesterol esters, and they suggest that the cholesterol esters which form the major lipid component of the fat-filled cells are wholly or partly synthesized *in situ*. On this basis, then, it is postulated that at least part of the cholesterol esterification in the atherosclerotic arterial wall occurs within foam cells.

The ability of both normal and athero-sclerotic aortic tissue to bring about cholesterol esterification has been described by several workers. Stein and Stein (1962) demonstrated that normal aorta incorporated ^{14}C-labelled linoleic acid into cholesterol ester. Using ^{14}C-labelled acetate as precursor, Lofland, Moury, Hoffman and Clarkson (1965) demonstrated that atherosclerotic pigeon aorta converted more label into cholesterol ester than did the grossly normal aorta.

However, although the fatty acid has been shown to be readily incorporated into cholesterol ester by the arterial wall, attempts to incorporate ^{14}C-labelled free cholesterol into cholesterol ester have been negative (Day & Gould-Hurst, 1966; Newman, Gray & Zilversmit, 1968). This failure to esterify cholesterol might be due to several possible factors. Cholesterol may not be taken up readily by the arteries, or may fail to penetrate to esterification sites, including foam cells. The preparation of isolated foam cells enables a more direct investigation to be carried out of the ability of these cells to take up and esterify cholesterol preparations. In an artery, cholesterol must first penetrate through

the endothelium into the intima before it is in contact with the foam cells, whereas with isolated foam cells, these other penetration steps are eliminated.

In the present section the ability of foam cells to take up and esterify particulate and micellar preparations of labelled cholesterol is therefore investigated.

B. Uptake and Esterification of Particulate Cholesterol

The ability of foam cells to take up and esterify particulate cholesterol was investigated at either 1 or 20h incubation, and in the presence and absence of serum. Generally 0.5×10^6 foam cells were incubated in duplicate in Leighton tubes containing either 1ml Hanks:normal rabbit serum (1:1, v:v), or 1ml of Hanks solution alone. A tracer amount of (4-^{14}C)-labelled cholesterol suspension, prepared as described under Methods, was added to each tube and all tubes were then gassed with 95% oxygen and 5% carbon dioxide. In those tubes where serum was added, the reaction was either allowed to proceed

for 20h at 37°C, or the medium was removed from the cells at 1h, and the cells washed three times with 1ml warm 0.9% sodium chloride solution, and reincubated for a further 19h in 1ml Hanks solution: normal serum (1:1, v:v). In all cases, at the end of incubation the cells were separated from the media by centrifugation at 220xg for 5min, and the cells washed three times with 1ml 0.9% sodium chloride solution. In each experiment control incubations were performed which contained no cells. Lipid extracts of the cells and media were prepared as described by Folch, Lees and Sloane-Stanley (1957), and aliquots taken for radioactive counting and for separation of cholesterol from cholesterol esters by thin-layer chromatography.

Table **21** shows the results of a series of experiments which were incubated with labelled medium for 1h and then transferred to unlabelled media for a further 19h. In the 6 experiments performed, an average of 16% of the added ^{14}C-labelled cholesterol suspension had been taken up at 1h. However, values ranged from 8.6 to 27.5%. The amount of cholesterol

TABLE 21

UPTAKE OF PARTICULATE ^{14}C-LABELLED CHOLESTEROL

0.5×10^6 Cells incubated 1 h at 37°C with ^{14}C-labelled cholesterol suspension and then incubated in unlabelled Hanks:serum (1:1) for a further 19 h at 37°C.

Expt.	cpm added	cpm in cells 1 h	% uptake	cpm in ester 20 h in cells	% of added label as cholesterol ester in	
					cells	medium
1	38200	5070	13.3	126	0.33	-*
2	77500	21300	27.5	729	0.94	0.13
3	294000	53700	18.3	470	0.16	0.45
4	294000	25400	8.6	279	0.095	0.45
5	1.78×10^6	260000	14.6	516	0.029	0.07
6	1.78×10^6	221000	12.4	214	0.012	0.08

* Not measured

ester formed by these cells was low, representing less than 1% of the total label present in the cells. Expressing the results as the percentage of added label present as cholesterol ester in the cells and in the medium demonstrates that in most cases the amount of cholesterol ester formed by the cells is small compared with that synthesized by the medium.

In those experiments where the cells were incubated for 20h at 37°C with ^{14}C-labelled cholesterol suspension in Hanks:normal rabbit serum (1:1, v:v), approximately 60% of the added label was taken up by the cells (Table 22). However, again less than 1% of that cholesterol taken up by the cells was esterified. The percentage of the added label present as cholesterol ester in the medium was considerably higher (2.5%), suggesting that the serum was active in esterifying the cholesterol (Glomset, 1962).

Table 23 shows the uptake and esterification of cholesterol by foam cells incubated for 20h at 37°C in Hanks solution alone. 75% of the added label was taken up by the cells, but less

TABLE 22

UPTAKE OF PARTICULATE ^{14}C-LABELLED CHOLESTEROL

0.5×10^6 Cells incubated for 20 h at 37°C in Hanks:normal serum (1:1)

Expt.	cpm added $\times 10^3$	cpm in cells $\times 10^3$	% uptake	cpm in ester 20 h	% of added label as cholesterol ester in	
					cells	medium
1	115	62.2	54.1	483	0.42	2.44
2	115	77.6	67.4	564	0.49	2.65

TABLE 23

UPTAKE OF PARTICULATE ^{14}C-LABELLED CHOLESTEROL

0.5×10^6 Cells incubated for 20h at 37°C in Hanks solution (no serum)

Expt.	cpm added $\times 10^3$	cpm in cells 20 h $\times 10^3$	% uptake	cpm in ester 20 h	% of added label as cholesterol ester in	
					cells	medium
1	398	328	82.5	338	0.085	*
2	398	364	91.5	366	0.092	*
3	190	142	74.8	532	0.28	0.38
4	190	141	74.1	627	0.33	0.38
5	290	138	47.6	928	0.32	0.30
6	580	456	78.5	3074	0.53	0.30

* Counts not significantly greater than background.

than 1% of that label was present as cholesterol ester.

It is apparent from these studies that foam cells can readily "take up" particulate cholesterol from a medium containing Hanks solution alone or Hanks:normal rabbit serum (1:1, v:v). It cannot be said if this uptake of label represents actual ingestion or merely adsorption of labelled material to the cells. Because significant esterification of cholesterol could not be detected it is possible that labelled cholesterol is only adsorbed to the cells.

C. Uptake and Esterification of Micellar Cholesterol

Because of the inability of foam cells to esterify cholesterol when it was presented as a particulate suspension, it was decided to investigate the effect of adding cholesterol in a different physical form, namely, as a micellar solution stabilized with Tween 20. The micellar solution of (4-^{14}C)-labelled cholesterol was prepared as described under Methods. A known amount of ^{14}C-labelled cholesterol was added to 0.5×10^6 foam cells in 1ml Hanks solution containing 0.5% albumin (pH 7.4). The reactions were carried out in Leighton tubes under an atmosphere of 95%

oxygen and 5% carbon dioxide for 1h at 37°C. At this time the radioactive medium was removed, the cells washed three times with 1ml warm 0.9% sodium chloride solution, and reincubated for a further 19h in 1ml Hanks solution alone. The cells were separated from the medium, washed, extracted and analyzed as described above.

The uptake of the micellar solutions of labelled cholesterol is given in Table **24**. In these experiments the cells were reincubated in Hanks solution alone. The cpm taken up at 1h by the cells was determined by measuring the cpm in the cells and the medium at 20h, since those cpm in the medium at 20h represent label that has been lost from the cells during the reincubation period. In the 3 experiments performed, each with duplicate incubations, an average of about 9% of the added label was taken up by the cells during the first hour of incubation. During the reincubation period, about 1% of the added label was returned to the medium in Experiments 1 and 3, but about 8% in Experiment 2, in this case representing about 60%

TABLE 24

UPTAKE AND ESTERIFICATION OF MICELLAR ^{14}C-LABELLED CHOLESTEROL

0.5 x 10^6 cells incubated for 1 h at 37°C in 1 ml Hanks solution containing 0.5% albumin. At 1 h medium removed, cells washed and reincubated 20 h at 37°C in unlabelled Hanks solution

Expt.		cpm added to incubation	cpm taken up by cells at 1 h (cells 20 h + medium 20 h)	% uptake 1 h	% of label returned to medium during 20 h	% ester-ification in cells 20 h	% of added label as ester in cells	% ester-ification in medium 20 h	% of added label as ester in medium
1	a	180,000	11,950	6.7	0.97	2.3	0.13	0.23	0.022
	b	180,000	15,690	8.7	1.4	2.8	0.24	0.27	0.037
	Control	180,000	-	-	-	-		-	0.19
2	a	978,500	142,650	14.6	9.0	0.51	0.028	0.11	0.010
	b	978,500	124,350	12.7	7.4	0.40	0.021	0.09	0.007
	Control	978,500	-	-	-	-		-	0.11
3	a	973,600	40,650	4.2	1.0	0.33	0.011	0.75	0.074
	b	973,600	93,900	9.7	1.2	0.15	0.012	0.41	0.005
	Control	973,600	-	-	-	-		-	0.09

of the label taken up by the cells.

Table **24** also shows the amount of labelled cholesterol ester present in the cells and medium at 20h, expressed as both the percentage of added label, and as the percentage of total radioactivity in either the cells or media at 20h. In each experiment there was insignificant esterification of cholesterol by the cells, as compared with the control incubations which contained no cells.

It is apparent from the work described in the previous sections that cholesterol esters can be formed by foam cells or foam cell homogenates when supplied with oleic acid. However, in the present study it was not possible to esterify free cholesterol supplied either as a particulate suspension, or as a micellar solution, even though a large percentage appeared to be taken up by the cells.

There are several possibilities which would account for the inability of exogenous cholesterol to become esterified. The foam cells may not be actually taking up cholesterol, but adsorbing it to the cell surface. Alternatively, the cholesterol may be present

within the cell in a pool which is unavailable for esterification. That the foam cells were capable of taking up the particulate cholesterol by phagocytosis was therefore investigated as follows.

Firstly, the effect of a phagocytic inhibitor on the uptake of particulate cholesterol by foam cells was investigated. Secondly, the ability of foam cells to take up carbon particles was determined, and thirdly, the presence of acid phosphatase, an enzyme associated with phagocytosis, was determined.

D. Inhibition of Phagocytosis

Since phagocytosis is an energy dependent process and depends on energy released from glycolysis, inhibitors of glycolysis will inhibit phagocytosis. Evidence that foam cells are phagocytic was obtained by investigating the effect of a phagocytic inhibitor (sodium azide) on the rate of uptake of particulate cholesterol. Particulate cholesterol was used in these studies because the particles are of a size which would be taken up by phagocytosis, (approximately 1μ diameter). The uptake can most readily be studied using ^{14}C-labelled cholesterol.

Initial experiments were carried out in order to determine the time course of particulate cholesterol uptake by the foam cells. Isolated foam cells ($0.5x10^6$ cells) were incubated in 1.0ml of Hanks solution, pH 7.4, containing 0.5% albumin and a known amount of ^{14}C-labelled cholesterol suspension. Incubations were carried out in 12ml centrifuge tubes in an atmosphere of air, and incubated at $37^{\circ}C$ for various times of up to 2h. At each time period investigated, duplicate tubes were removed and placed in ice. The cells were separated from the medium by centrifugation at 220xg for 5min and washed three times with 5ml 0.9% sodium chloride solution, and the cells and media extracted with chloroform:methanol (2:1, v:v), as described under Methods. Aliquots were then taken for radioactive counting.

The results of one representative experiment are given in Fig.39. Cholesterol uptake occurred rapidly in the first 30min and then levelled off. There was little further uptake during the remainder of the period investigated. Future experiments were therefore carried out for 30min.

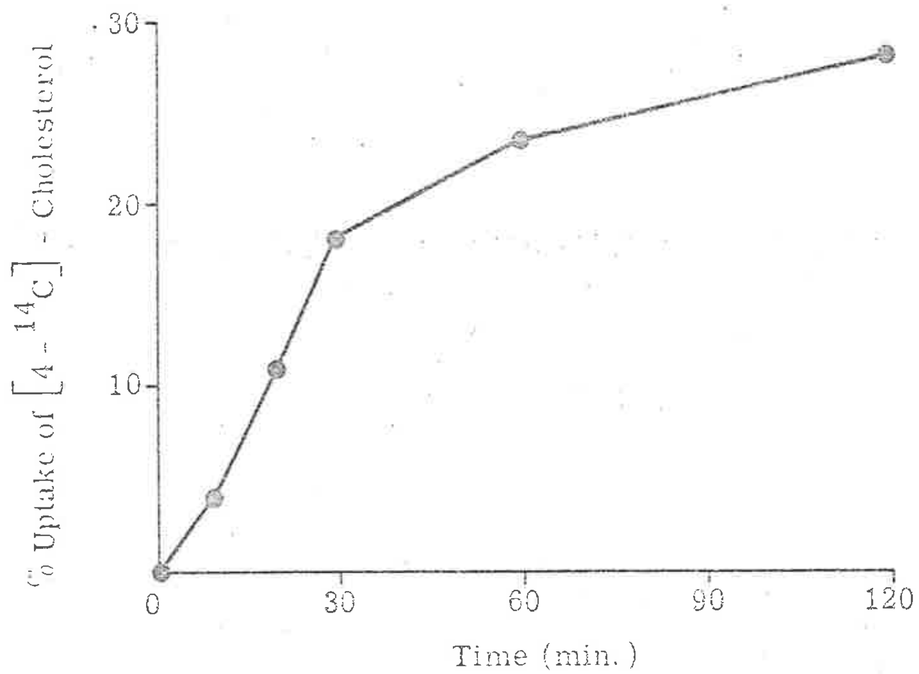


Fig.**39** Effect of time on the uptake of particulate cholesterol by foam cells.

The effect of sodium azide on the uptake of particulate cholesterol by the foam cells was investigated over a range of cholesterol concentrations. ^{14}C-labelled cholesterol suspensions of various specific activities were prepared from [4-^{14}C]-labelled cholesterol by diluting with non-labelled cholesterol as described under Methods.

Foam cells (0.5×10^6 cells) were incubated in 0.9ml Hanks solution pH 7.4, containing albumin and either 0.1ml of 10^{-3}M sodium azide or 0.1ml of water for 30min at 37°C, prior to the addition of ^{14}C-labelled cholesterol suspensions of various specific activities. ^{14}C-labelled cholesterol suspensions containing amounts of cholesterol ranging from 1 to 39 mμmoles cholesterol in Experiment 1, and 0.1 to 46mμmoles cholesterol in Experiment 2, were added to each series of tubes and incubated for a further 30 min at 37°C with gentle shaking (10 oscillations/min). At the completion of the incubation, the cells were separated from the media, washed and extracted as described above. Aliquots of each extract were taken for counting using toluene scintillator (see Methods).

Table **25** shows the effect of addition of 10^{-4}M sodium azide on the uptake of particulate cholesterol supplied at various concentrations to the cells. In each experiment, regardless of the presence or absence of azide, increasing the amount of cholesterol supplied to the cells produced an almost linear increase in the rate of uptake over the range investigated. However, preincubation of the cells with azide caused the cells to take up particulate cholesterol at less than half of the control rate, demonstrating that the uptake of particulate cholesterol occurs at least in part by an energy dependent process.

E. Uptake of Colloidal Carbon Particles

The ability of foam cells to phagocytoze carbon particles was further investigated by incubating approximately $2x10^5$ foam cells on cover slips, mounted in perspex slide chambers with 0.3ml of Hanks:rabbit serum (1:1, v:v), containing 25μl of colloidal carbon C11-1431-a (Guenther, Wagner, Pelikan-Werke, Hanover, Germany). The particle size was approximately 500Å. Incubation was carried out for 4h at 37°C, and during

TABLE 25

EFFECT OF SODIUM AZIDE ON THE UPTAKE OF PARTICULATE CHOLESTEROL BY ISOLATED FOAM CELLS

	Cholesterol Added (mμmoles)	Uptake of Cholesterol (mμmoles/30 min/0.5 x 10^6 Cells)	
		No Azide	+ 10^{-4}M Azide
Expt 1	1.01	0.14	0.07
	2.17	0.27	0.16
	4.10	0.53	0.13
	19.6	3.00	1.46
	38.7	10.8	4.45
Expt 2	0.077	0.02	0.01
	7.82	2.01	1.34
	15.6	4.91	2.19
	23.3	6.23	1.87
	31.0	7.28	3.26
	34.9	8.63	4.26
	38.7	10.9	4.80
	46.4	11.7	4.37

this time the foam cells had firmly adhered to the glass coverslip. The excess carbon particles were removed by rinsing the coverslips gently in 0.9% sodium chloride solution. Following staining of the lipid-containing cells with Sudan IV, the preparations were inverted on to a glass microscope slide and viewed microscopically as an aqueous preparation.

Fig. 40 shows two foam cells laden with carbon particles. Fig. 41 demonstrates that sudanophilic lipid-containing cells (foam cells), have taken up carbon, whereas another contaminant cell, containing no lipid, has no intracellular carbon particles.

F. Acid Phosphatase Activity of Foam Cells

Acid phosphatase activity was determined by the simultaneous coupling azo-dye method using a substituted naphthol as described under Methods. Approximately $2x10^5$ foam cells were incubated with the substrate solution at pH 5.0 for 1h at 37°C. During this time, foam cells displayed acid phosphatase activity. The cells were quite densely laden with red azo-dye throughout the whole of the cytoplasm (Fig. 42), suggesting that lysosomal bodies

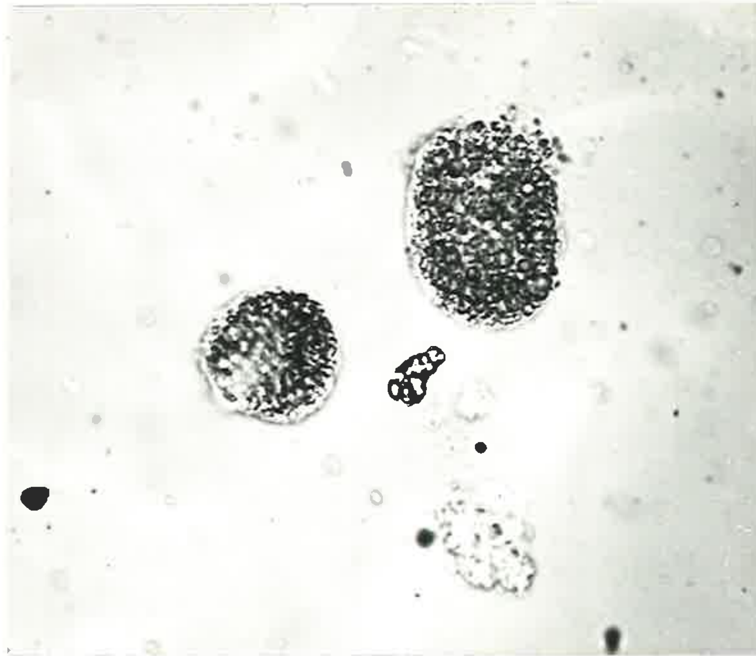


Fig. 40

Foam cells laden with carbon particles. Unstained. (X1000).

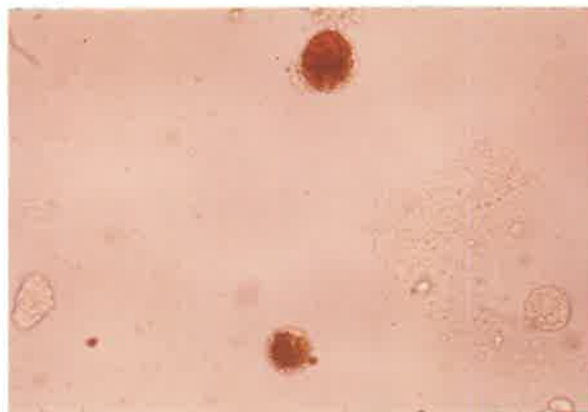


Fig. 41

Sudanophilic lipid-containing foam cells have taken up carbon, whereas contaminant cell (lower right corner) containing no lipid has no intracellular carbon particles. Stained with Sudan IV. (X400).

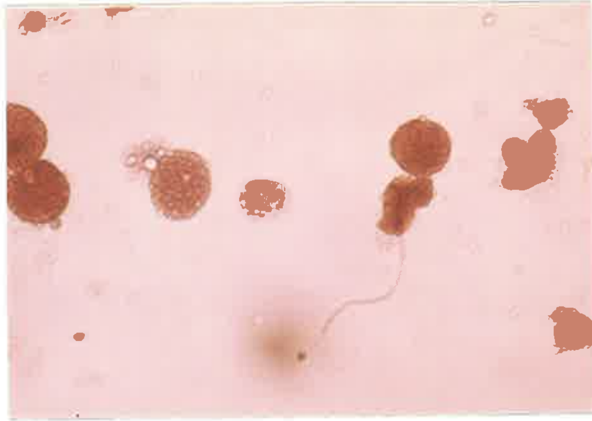


Fig. 42

Foam cells demonstrating acid Phosphatase activity. (X600).

Fig. 43

Control incubation for acid phosphatase. (X600).

are present in large numbers within the cells. Investigation of cells that had been heat-inactivated (Fig. 43), or incubated without naphthol phosphate ester, revealed no deposition of dye within the cells.

G. Discussion

It has been reported that plasma cholesterol, upon entering the aorta of cholesterol-fed rabbits, is not readily converted into cholesterol ester (Newman & Zilversmit, 1966; Newman, Gray & Zilversmit, 1968). *In vitro* studies with rabbit aortas have also shown that free cholesterol is not esterified (Day & Gould-Hurst, 1966). In the experimental work reported in this section, although considerable uptake of cholesterol by foam cells occurred, neither preparation was significantly esterified.

In view of the inability of these cells to esterify free cholesterol it was considered important to see whether the cholesterol was actually being taken up by the cells, or merely being adsorbed to the cell surface. Studies by other workers investigating the uptake of lipoprotein cholesterol from medium containing serum by lymphoblasts in tissue culture, suggests that the uptake occurred by physical

adsorption, since the uptake was both concentration and temperature dependent, and heat-inactivated cells were still able to take up cholesterol (Rothblat, Hartzell, Mialhe & Kritchevsky, 1967). Newman & Zilversmit (1966) showed that boiling or treatment of rabbit aorta with KCN or NaF had no effect on the uptake of lipoprotein cholesterol, and Hashimoto and Dayton (1966) demonstrated that metabolic activity was not required for the transport of cholesterol into rat aorta *in vitro*. Although the uptake of cholesterol from a medium containing serum is believed to occur by a non-enzymic process, there does exist a certain structural specificity in that the side chain of the steroid molecule is essential for uptake (Rothblat, Hartzell, Mialhe & Kritchevsky, 1967). It is thus apparent that lipoprotein preparations of free cholesterol are taken up by a physical process and therefore the cells would have no control over the amount taken up, unless an excretory process was present. Bailey (1965) demonstrated that such a process was operative in mouse fibroblasts and lymphoblasts, and that

an α-globulin was required as an acceptor in the medium.

The uptake of particulate cholesterol by foam cells during a 20h incubation remained approximately the same regardless of the presence or absence of serum. It is interesting to note that about 60% of the added particulate cholesterol was taken up by the cells in the presence of serum, even though a large amount of unlabelled cholesterol was present in the serum as lipoprotein cholesterol. Had the added particulate cholesterol been freely mixed with the serum cholesterol, such a great uptake of cholesterol could not have occurred, suggesting that the added cholesterol did not rapidly become associated with the lipoprotein in the medium.

That the cholesterol particles were actually being taken up by foam cells was demonstrated by inhibiting phagocytosis. A number of chemical substances have been described which inhibit phagocytosis, an example being sodium azide, and iodoacetate (Sbarra & Karnovsky, 1959). It is

believed that the initiation of phagocytosis is accompanied by an elevation of the respiration and lactate formation, whether glucose is present or not, so that apparently phagocytosis initiates an active type of metabolism (Becker, Munder & Fischer, 1958). Energy for phagocytosis is dependent upon active glycolysis (Sbarra & Karnovsky, 1959), and glycolysis is inhibited by iodoacetate. However, whereas polymorphonuclear leukocytes and peritoneal macrophages were dependent only on glycolysis for energy supply during phagocytosis, alveolar macrophages depended to a large extent on oxidative phosphorylation (Oren, Farnham, Saito, Milofsky & Karnovsky, 1963).

The evidence presented in the experiments described in this section demonstrates that the uptake of particulate cholesterol was dependent upon a supply of energy. Inhibition of the processes supplying the energy resulted in a marked decrease in the uptake of particulate cholesterol, suggesting that foam cells are phagocytic. Serum was not added to the incubation medium in these experiments because of the possibility of the labelled cholesterol

exchanging with the lipoprotein cholesterol, and in turn exchanging with the cell cholesterol. Such an exchange process would have resulted in cholesterol uptake by a method other than phagocytosis.

Gonzalez and Furman (1965) have demonstrated histochemically the presence of a number of hydrolytic lysosomal enzymes, including acid phosphomonoesterases, lipases, esterases and peroxidases, within lipid-laden foam cells of sections of human and rabbit atheromatous lesions. Other cells in the lesion, namely histiocytes, endothelium and smooth muscle cells, showed little or no hydrolytic activity. The presence of acid phosphatase within isolated foam cells provides additional evidence that these cells are phagocytic, since all phagocytic cells contain lysosomes and the complement of lysosomal enzymes. However, just because lysosomal enzymes are present, the cell need not be phagocytic, but if they are absent, then it is very likely that the cell is not actively phagocytic.

That foam cells can take up carbon particles, possess acid phosphatase activity, and that sodium azide decreases the uptake of particulate cholesterol, together reinforce the conclusion that the foam cells are phagocytic.

It is therefore apparent that the particulate cholesterol supplied to foam cells is actually being taken up by the cells, but is not esterified. The cholesterol may either be within the cell at a site unavailable for esterification, or the labelled cholesterol, upon entering a certain pool within the cell, is diluted to such an extent that esterification cannot be detected.

GENERAL DISCUSSION

General Discussion

It was the aim of the experiments described in this thesis to give some insight into the role of foam cells in the atherosclerotic lesion. In view of the large quantities of lipids which these cells contain, it was of interest to see if the cells were capable of synthesizing these lipids. As it was not possible to isolate foam cells when this work was initiated, and because it is considered that some foam cells originate as reticuloendothelial cells, such cells readily available from other locations were therefore used. When a method for isolation of arterial foam cells became available (Day, Newman & Zilversmit, 1966), it was possible to look directly at the metabolism within these cells and this was described in the latter Sections.

As the major lipid to accumulate within foam cells is cholesterol oleate, much of the work has related to the esterification of cholesterol to delineate the properties of the enzyme(s) responsible for its formation. The cholesterol esterifying enzyme(s) found in both peritoneal and alveolar macrophages have similar properties. They were optimally active in a medium of acid pH, and in fact were completely inactive

above pH 7.0. It was not possible to show any dependence for the cofactors ATP and CoASH, suggesting that the esterification involved the direct interaction of free fatty acid with the 3β-ol group of cholesterol. The majority of the activity in peritoneal macrophage homogenates resided in the soluble fraction, as was found for alveolar macrophage homogenates (Day, Tume & Kefford, 1969*). In each of these respects, the cholesterol esterifying enzyme(s) reported here is similar to that described for bovine adrenals (Shyamala, Lossow & Chaikoff, 1965).

It is unfortunate that a similar study could not be made of foam cell homogenates, but this of course was limited by the small numbers of cells that were obtainable. However, several limited experiments were performed on the esterification of cholesterol by foam cell homogenates (Day, Tume & Rossiter, 1969*), giving good indication that the enzyme behaved similarly to those found in macrophage homogenates. It had a pH optimum near pH 4.5, did not require ATP and CoASH for activity and it failed to demonstrate lecithin-cholesterol acyltransferase activity, using the incubation system of Abdulla, Orton and Adams (1968).

* Unpublished observations

As has been implied by the macrophage work described, and borne out by preliminary experiments with foam cells, it would seem that the foam cells comprising the atherosclerotic lesion are capable of esterifying cholesterol. The difficulty encountered in demonstrating the esterification of cholesterol by intact foam cells may have been attributable to the physical form in which the cholesterol was added, and thus its failure to reach the actual sites of esterification.

Even though the presence of lecithin-cholesterol acyltransferase activity has been demonstrated in homogenates of human and rabbit atherosclerotic lesions (Abdulla, Orton & Adams, 1968), it does not necessarily mean that this enzyme originated there. It is in fact possible, in view of a more recent report (Abdulla, Adams & Bayliss, 1969), that the lecithin-cholesterol acyltransferase activity results in the intima by influx from the serum. These workers find that the greatest activity is in lesions and is located in the outer layers of the lesion, diminishing in deeper layers. This is consistent with a lipoprotein infiltration process which is known to be more rapid in atherosclerotic vessels than in normal vessels (Tracy,

Merchant & Kao, 1961; Gero, Gergely, Jakab, Szekely & Virag, 1961).

The possible pathways by which lipid might accumulate in foam cells are summarized in Fig. 44. Peritoneal macrophages (Day & Gould-Hurst, 1961) and alveolar macrophages (Section 2) have been shown to take up and esterify particulate cholesterol preparations. Foam cells however failed to esterify such cholesterol preparations, even though large percentages were actually "taken up" by an energy dependent process. It is believed that the uptake of free cholesterol from serum (i.e. lipoprotein cholesterol) by either rabbit or rat aorta is not dependant upon metabolic activity, as boiling or treatment with KCN or NaF failed to reduce its uptake (Newman & Zilversmit, 1966; Hashimoto & Dayton, 1966). Also, work with lymphoblasts in tissue culture has indicated that the uptake of cholesterol from serum occurs by a physical adsorptive mechanism (Rothblat, Hartzell, Mailke & Kritchevsky, 1967). A certain structural specificity was found to exist, suggesting that the side chain of cholesterol was essential for its uptake. These workers have suggested that because the uptake occurs by a physical process, the cell

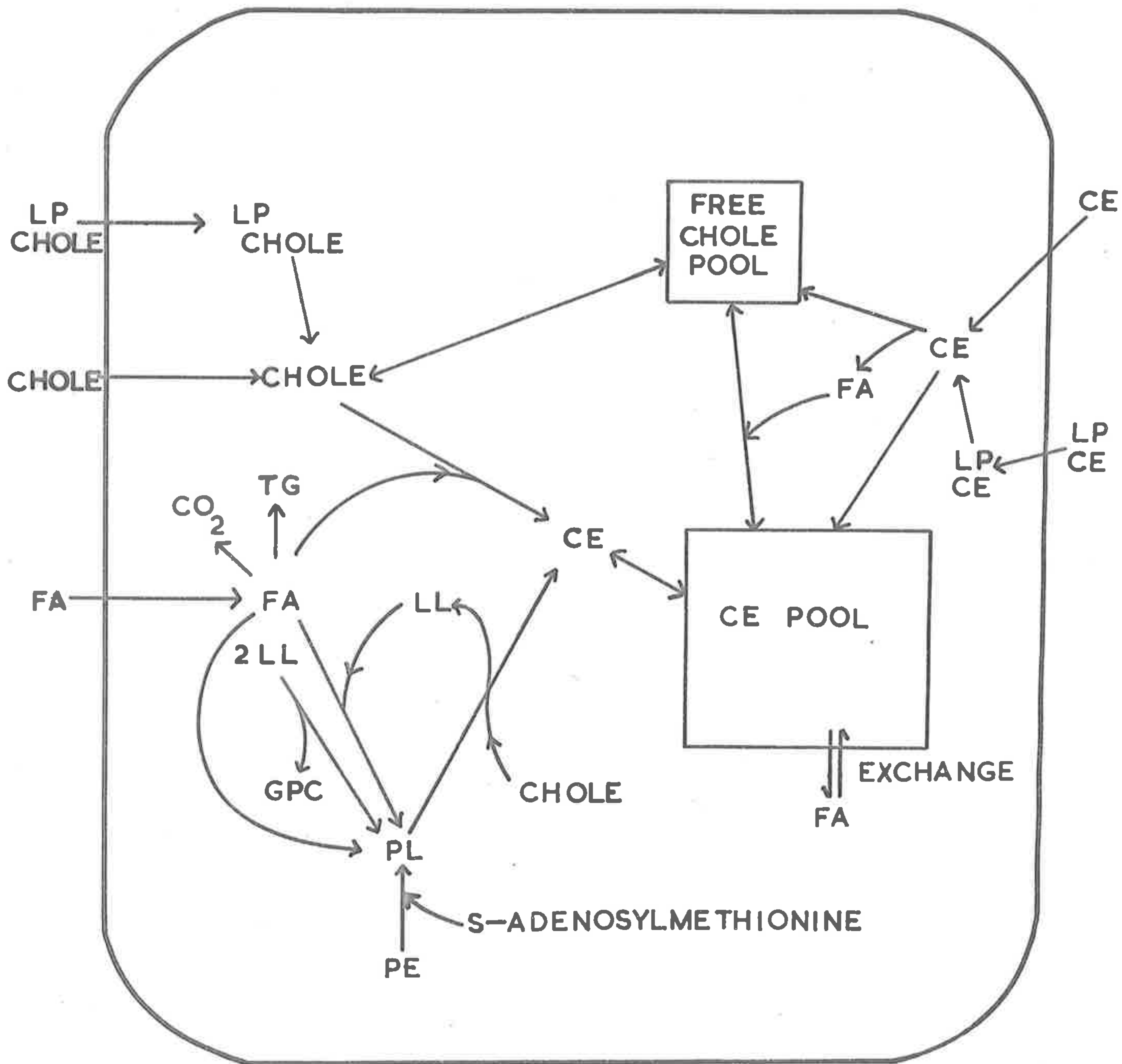


Fig. 44 Possible pathways for lipid accumulation in the foam cell.

would have little control over the amount of cholesterol adsorbed. Therefore, in a cell there must be control at some other metabolic level, e.g. active excretion of cholesterol. In the case of foam cells it is possible that, in view of the large amounts of cholesterol ester present, this excretion mechanism is inoperative or proceeding only at a very low rate.

The uptake of cholesterol ester from serum-containing media by rat aorta (Hashimoto & Dayton, 1966) was about equal to the rate of free cholesterol uptake, even though 60-80% of cholesterol was in the esterified form in serum. Thus free cholesterol appears to be taken up more readily. Since the cholesterol ester-fatty acid pattern of the foam cells is different from that of the serum, then assuming that the cholesterol fraction originates from the serum, it follows that the esterified cholesterol is hydrolyzed and then re-esterified. This more unsaturated fatty acid released by this "exchange" process might then be available for further membrane formation via its incorporation into phospholipid. In view of the rapid increase in bulk of atherosclerotic lesions, membrane synthesis must be of prime importance.

The uptake and esterification of fatty acid

was investigated and discussed in Sections 3, 6 and 7 of this thesis. Some of the pathways by which fatty acid might be interrelated with cholesterol ester and phospholipid are also shown in Fig. 44.

In conclusion, the work reported here along with other work from this laboratory, demonstrates that foam cells are indeed metabolically active, and that they have the necessary metabolic machinery to at least qualitatively account for the formation of lipid within these cells. The value of such an approach to the problem of atherosclerosis is evident when considered in relation to future quantitative assessment of lipid accumulation studies, to be carried out under a variety of experimental conditions.

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