SOME ASPECTS OF LIPID METABOLISM BY CELLS OF THE RETICULO-ENDOTHELIAL SYSTEM.

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LIPID METABOLISM OF

THE RETICULO-ENDOTHELIAL SYSTEM.

PREFACE.

The accumulation of lipid in the wall of the atheromatous artery is associated with a degenerative reaction and the accumulation of macrophages. The part that these cells play in causing or preventing the disease process is not known but their presence in the lesion of this lipid-disorder of unknown aetiology makes knowledge of lipid metabolism by reticulo-endothelial cells of some importance.

In this thesis, after a discussion of the nature of the reticulo-endothelial system and the physiological properties of its cells, some discoveries of certain aspects of the lipid metabolism of these cells are described. The way in which these metabolic changes may influence the removal of lipid and in particular cholesterol, from the tissues is discussed in the light of the findings presented.

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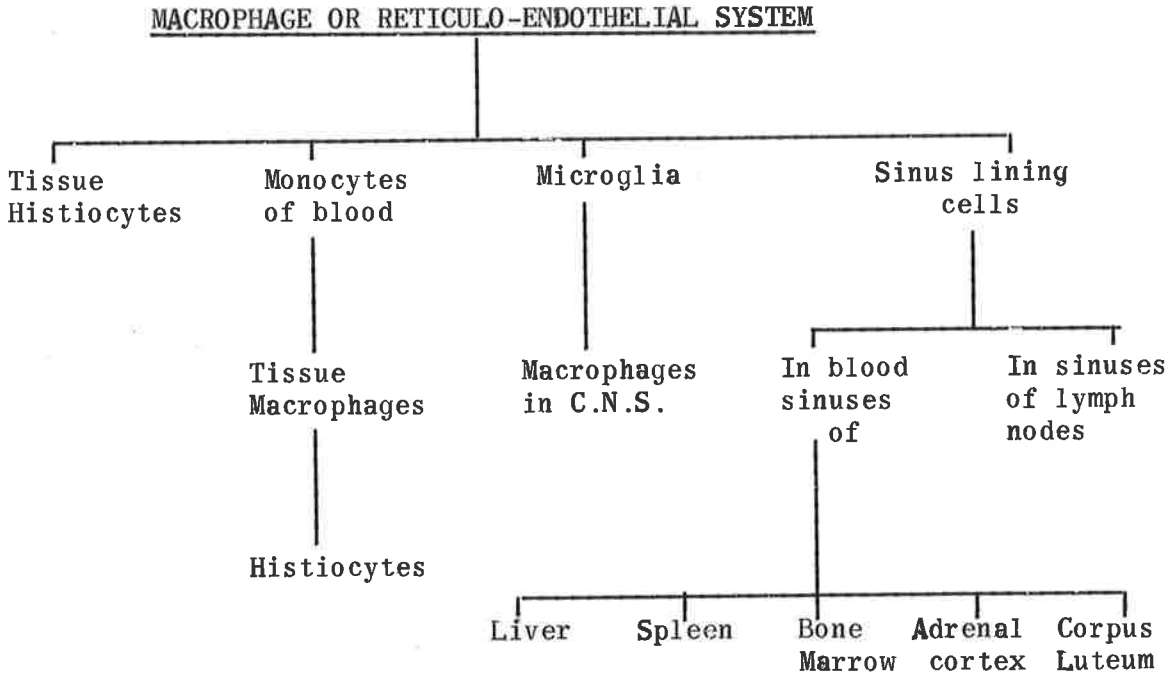
INTRODUCTION.

THE CONCEPT OF THE RETICULO-ENDOTHELIAL SYSTEM AND ITS HISTORICAL DEVELOPMENT.

The Reticulo-Endothelial System.

The reticulo-endothelial system is a group of cells widely distributed throughout the body having in common that their most notable function is the phagocytosis of particulate material.



(After Florey)
1962

The Concept of the Reticulo-Endothelial System. Historical Development.

Phagocytosis. The presence of cells in inflammatory exudates capable of the phagocytosis of invading micro-organisms was first described by Klebs and by Haeyem in 1870. Rosser in 1881 suggested that phagocytosis was not a means by which the bacteria disseminated themselves through the body as was thought at that time, but was rather a defence mechanism by which micro-organisms were destroyed. Metchnikoff in 1884 independently came to this same conclusion and propounded his "Theory of Phagocytosis" as the mechanism by which natural immunity operates. In a series of brilliant experiments on a variety of animals he investigated the response of the organism to irritation and, following ten years of violent controversy with the protagonists of the "humoral theory" of natural immunity, succeeded in gaining acceptance of his views. He showed that phagocytes of the blood, motivated by a positive chemotaxis (Leber, 1888; Massart and Bordet, 1890) pass through the vascular wall by diapedesis (Cohnheim, 1882) seize and engulf micro-organisms after the manner of the amoeba and digest them within vacuoles in their cytoplasm. As a zoologist working in the field of comparative pathology, Metchnikoff suggested that the phagocytosis and digestion of particulate matter which serves the nutritional needs of free living single-celled animals such as the amoeba, has become by evolutionary change, a means by which micro-organisms are combated.

Ehrlich in 1898 using aniline stains was able to arrange the phagocytic leucocytes of blood into two different groups which Metchnikoff later (1905) termed the microphages and macrophages. To the latter group of amoeboid cells he added the fixed amoeboid cells also of mesoblastic origin found in other tissues of the body, also the large cells of the splenic pulp, the Kupffer cells of the liver, certain connective tissue cells and the neuroglia of the central nervous system and named them "The Macrophage System". The microphages of the blood (which he named "polymorpho-nuclear" leucocytes) differ from the macrophages in being found only in vertebrates, in being short-lived and unable to reproduce, in appearing much more rapidly on the scene of invasion and playing their part especially in acute infection, engulfing the more virulent micro-organisms. The more slowly moving macrophages, he stated, appear much later on the scene and have a marked predeliction for animal-cells both introduced and endogenous, and for micro-organisms that set up chronic disease and for protozoan parasites.

Metchnikoff's theory of a division in function between the microphages and the macrophage-system of cells described above has not been substantiated by more recent work (Lucke et al 1933). It does seem to be agreed however that the reticulo-endothelial cells are the predominant defence in generalized infections whereas the polymorpho-nuclear leucocytes act primarily in localized foci of inflammation (Miles, 1957).

The concept of the macrophage system was greatly strengthened by Ribbert's discovery in 1904 of the vital dyes lithium and indigo carmine and the demonstration by Goldman working under Ehrlich's guidance, in 1909, using mainly Pyrrhol blue, of the distribution and limits of the cells of the "system". Goldman showed that the capacity to concentrate these dyes from dilute solution in the plasma into strikingly coloured granules within the cytoplasm was a property shared by all these diverse phagocytic cells.

Aschoff (1924) criticized Metchnikoff's concept of the macrophage system on the grounds that under certain circumstances the cells of almost every tissue of the body will take up vital dyes. He stated that the "frequency and intensity" of phagocytosis is that which enables certain body cells to be classified into a single system whose function is primarily phagocytic. He included in his own classification (A) the reticulum cells of the splenic pulp, cortical nodules and pulp cords of lymph nodes and (B) the blood sinuses of the spleen, bone marrow, adrenal cortex and hypophysis and the Kupffer cells of the liver. These two groups he named the reticulo-endothelial system and included loosely with them two other groups of even more intensely phagocytic cells - the histiocytes of connective tissue and the inflammatory macrophage. (See Table 1 page 12).

Aschoff's contribution was that he united the theories of Metchnikoff with the findings of histologists of the early twentieth century.

Many of the cell types making up the reticulo-endothelial system are related to each other morphologically and developmentally. This is supported by considerable evidence as will be described. Cells belonging to the reticulo-endothelial system whose appearances differ have been shown in some instances to be in fact similar cells, only differing in appearance because of their functional states or anatomical situations in the body. March and others more than thirty years ago showed that histiocytes of the loose connective tissue which are derived from connective tissue elements, in the presence of inflammation are awakened from their dormant state and become the more avidly phagocytic macrophages. Ebert and Florey in 1939 showed that blood monocytes may change into macrophages while Nicol and Bilbey in 1958 showed that macrophages may be shed from the tissues into the general circulation and can be traced to the lungs, lying free in the alveoli where they are indistinguishable from the phagocytic dust-cells. Aschoff (1924) suggested that the few macrophages seen in the systemic arteries are also derived from these tissue macrophages swept into the venous blood stream but managing to escape through the filter of the lung capillaries to the left side of the heart. Metchnikoff (1905) believed that the Kupffer cells of the liver which are not present in the newborn, are carried to the liver from the tissues by the portal veins and wedge in the hepatic sinusoids. This may not be true since electron microsopy reveals that the Kupffer cells do not sit on the endothelial cells lining the hepatic sinusoids but lie between them,

a fact suggesting that they may be derived from these endothelial cells (Casley-Smith, 1963).

These observations just described have served to strengthen Metchnikoff's and Aschoff's concept of a morphologically united system. On the other hand, some recent work studying reticulo-endothelial cells isolated from different organs and tissues and cultured in vitro has shown differences in function which suggest that the unity of the reticulo-endothelial system may be a useful rather than a correct concept. Rat alveolar macrophages have been shown to possess functional differences to rat peritoneal macrophages in their ability to kill certain bacteria and also in the enzymes they contain (Pavillard, 1963). Guinea pig peritoneal macrophages have a low resting oxygen uptake that increases greatly during phagocytosis and during glucose oxidation while in contrast, guinea pig alveolar macrophages have a very high resting oxygen uptake which does not increase significantly during the above functions. The former cell type depends on glycolysis alone for energy production while the latter cell meets its energy requirements mainly through anaerobic pathways (Karnovsky and Wallach, 1963). Finally, isolated reticulo-endothelial cells from the adrenal cortex and from the liver have been found to differ in their ability to carry out biosynthesis and biotransformation of steroids (Berliner, Nabors and Dougherty, 1964).

The concept of the reticulo-endothelial system is at present undergoing a revolutionary change due to discoveries to be described in the following sections of this introduction, discoveries which disclose an increasing importance in the part played by the system in the immune reaction. Since the system is defined in terms of its function, other cells which share the same functions must be considered candidates for inclusion in the system. Plasma cells and lymphocytes have been shown to be intimately involved with macrophages in the production of antibodies. Further, evidence will be described which suggests that macrophages as well as lymphocytes may give rise to plasma cells and that lymphocytes and plasma cells like reticulo-endothelial cells are considered to be derived from the primitive, phagocytic reticular-cells of the bone marrow, lymph nodes and spleen. Because of these facts, recent workers in the field of immunity have defined their concept of the reticulo-endothelial system to include plasma cells, lymphocytes and their precursors.

Humoral Immunity. The importance of the reticulo-endothelial system in resisting infection by micro-organisms has been known since the work of Metchnikoff. Metchnikoff's theories and discoveries were so extensive that subsequent work has largely been an elaboration and confirmation of his original findings.

The "humoral theory" of immunity obscured for many years the importance of the part played by cells of the body in combating

invasion. The humoral theory was founded on the discovery by Jenner (1796) and Pasteur (1880) that an acquired immunity could be generated in the body by innoculation with killed or attenuated micro-organisms. The beliefs of this school were strengthened by the demonstration of antitoxin by Von Behring and Kitasato (1890), of specific antibodies by Pfeiffer (1894) and of complement and the part it plays in the antigen-antibody reaction by Buchner (1893).

The cellular and humoral theories of immunity were eventually reconciled by the demonstration of the importance in immunity of both the cellular and the humoral factors. Wright and Douglas (1903) discovered that humoral factors, opsonins, are necessary even in what appears to be the purely cellular phenomenon of phagocytosis. Neufeld and Rimpau (1904) showed that the amount of opsonin increases on immunization and that while this substance fails to cause the death of bacteria it rapidly and specifically brings about their phagocytosis and destruction when leucocytes are present.

Deutsch in 1899 working under Metchnikoff showed that substances conferring an acquired immunity against infective organisms are produced by the bone marrow, lymphatic system and spleen. Metchnikoff suggested that since the microphages and macrophages emanated from these organs it is these cells that produced the "agglutinative substances and antitoxins". This assumption that the cells which take up particulate antigens are the source of antibody was logical but required independent experimental investigation and this was not

carried out until recent times. McMaster and Kruse in 1951 showed that soluble antigen (blue, azo-dye labelled, gamma-globulin) injected into mice, is taken up and localized in reticulo-endothelial cells. Other workers using radio-active labelled antigen (Libby and Madison, 1947), and fluorescent labelling (Coons, 1952) have produced similar results and shown that the antigens persist within the cells for considerable periods of time.

Jaffe in 1931 showed that antibody production is depressed after administration of substances which "blockade" the function of reticulo-endothelial cells. Since circulating antibodies are almost exclusively contained in the gamma-globulin fraction of the plasma, enquiry was made into which cells of the body synthesized this protein fraction. Miller and Bale (1954) showed that while most plasma proteins are synthesized by the liver, gamma-globulin is not. Coons, Leduc and Connolly (1955) and Ortega and Mellors in 1957 showed by fluorescent antibody technique the presence of intra-cellular gamma-globulin to be only in plasma cells and within certain cells of the germinal centres of lymphoid follicles and the red pulp of the spleen. The orthodox view on the origin of plasma cells is that they arise from primitive reticulum cells but Neil and Dixon (1959) have produced impressive evidence that they arise from the transformation of small lymphocytes.

The macrophage has recently been reinstated to its position of importance in the production of antibody by Fishman (1961) and

Fishman and Adler (1963) who showed that antibody formation is initiated through the sequential action of two cell types. The essential first step is the phagocytosis of the antigen by the macrophage. This gives rise within the macrophage to a "transformed" ribonucleic acid (probably containing antigenic fragments complexed with ribonucleic acid) and this substance then becomes incorporated into "lymph-node cells"and the latter give rise to specific antibody. Stimulation of the reticulo-endothelial system has been shown to result in a raised serum gamma-globulin and antibody level and to increase the protection of experimental animals against virulent infections (Nicol et al, 1963).

Knowledge regarding the origin and morphological-interrelationship of the cells involved in immune reactions is at present in a state of flux. The cells involved appear to possess marked mesenchymal potency and morphological polypotentiality. Holub and Riha (1960) concluded from experiments involving the transfer of lymphoid cells into X-irradiated or newborn recipients, that lymphocytes and macrophages become transformed, via a pyronin staining cell with an eccentric nucleus, into plasma cells. Roberts and co-workers (1957) showed that peritoneal exudate cells from immunized donor rabbits, of which 70 per cent were macrophages, gave rise to similar pyroninophilic cells. It is suggested too, (Wissler et al, 1957) that plasma cells after giving rise to antibody, may shed their cytoplasm and become lymphocytes. Further, several careful workers

have followed and described the development of cells usually classified as lymphocytes into monocytes and finally into macrophages (Conway, 1938; Taliaferro, 1949; Harper, 1942; De Bruyn, 1945; Rebuck, 1949; Rebuck and Crowley, 1955; Mackaness, 1963) but others who have also attempted to do so have failed to observe such changes (Ebert, Sanders and Florey, 1940).

The findings described above suggest that lymphocytes, plasma cells and macrophages have certain potentials in common and may be closely related ontogenetically. Because of this the definition of the reticulo-endothelial system may have to be changed or the concept abandoned. Taliaferro and Mulligan (1937) and other workers in fact now prefer and use the term "Lymphoid-Macrophage System".

TABLE I. MESENCHYMAL CELLS INVOLVED IN DEFENCE AND IMMUNE REACTIONS

After Taliaferro 1949.

1. Predominantly fixed cells
   - A. Fibroblasts and endothelial cells
   - B. Macrophages
     1. Reticular cells of reticular organs
     2. Littoral cells of sinuses of reticular organs and of sinusoids of liver, adrenal and hypophysis.
     3. Adventitial cells (Maximow's undifferentiated pericytes).
     4. Histiocytes of ordinary connective tissue e.g. macrophages of skin, stroma cells of intestine, septal cells of lung, and glial phagocytes of brain.

2. Free cells
   - A. Inflammatory macrophages
   - B. Intermediate polyblasts (cells transitional between nongranular leucocytes and inflammatory macrophages)
   - C. Nongranular leucocytes
     1. Monocytes
     2. Lymphocytes (including hemcytoblasts of myeloid tissue)
     3. Plasma cells
   - D. Granular leucocytes
     1. Heterophils
     2. Eosinophils
     3. Basophils

R.E.S. restricted sense (Aschoff)

R.E.S. broad sense (Aschoff)

Macrophage system (Metchnikoff)

Lymphoid-macrophage system (Taliaferro and Mulligan)

Microphages (Metchnikoff)

The Uptake and Removal of Ingested Material by Reticulo-Endothelial Cells.

Micro-organisms. Virchow, a hundred years ago (1860), saw cinnabar in the axillary lymph nodes of a heavily tatooed soldier and deduced that particulate matter is carried passively and deposited in the cells of the regional lymph nodes. Shortly after this observation, Wyssokowitch (1886) made his pioneering studies showing that micro-organisms carried in the circulation are removed by the liver and spleen. Histological studies made by Wright in 1927 and Sherman 1929 on animals and man at varying stages of this clearing process showed that the removal of circulating micro-organisms is brought about chiefly by cells of the reticulo-endothelial system lining sinuses and blood vessels of the liver, spleen, bone marrow and also the lungs.

The great bulk of phagocytic cells have been shown to be divided between three organs, liver, the bone marrow and the lungs, while a much smaller portion reside in the spleen (Drinker and Shaw, 1921; Haurowitz and Breine, 1932; Gaunt and Wright, 1940). A third of all the cells of the liver are littoral cells (Daoust, 1958).

When phagocytes ingest organisms which they cannot digest they may become vehicles for their dissemination (Goodpasture and Anderson, 1937). Occasionally highly virulent organisms may multiply within and destroy the cells which have ingested them to be released into the blood stream in vastly increased numbers.

The removal of invading micro-organisms from the blood by reticulo-endothelial cells forms the body's first line of defence against infection.

In the process of tissue healing recent direct observations (Florey, 1962) show that macrophages enter the wound from the periphery by slow amoeboid movement with cytoplasmic finger-like extensions in constant motion and ingest and digest red cells, fragments of fibrin and cellular debris - ingested material which no doubt is food for the macrophage. The cells increase in size and may reach a diameter of 30 microns or more (the phagocytic activity being directly correlated with the size of the cells, Taliaferro and Cannon, 1936) and may form multi-nucleated giant cells.

In later stages of the inflammation and in tissue culture phagocytic activity declines and the macrophages become transformed into fixed tissue histiocytes (Florey, 1962) or fibroblasts (Taliaferro, 1949).

Red blood cells. Erythrocytes live in the circulation for 90 to 120 days (Mollison, 1947). Serious doubt has been thrown on the old theory that reticulo-endothelial cells in the spleen phagocytose erythrocytes which have reached the end of their life span. It now appears likely (Knisley, 1936) that the majority of normal erythrocytes haemolyse in the circulation and that phagocytic cells throughout the body remove the debris.

LIPIDS.

Ribbert in 1902 showed that macrophages ingested other substances besides vital dyes and micro-organisms. He discovered that these cells take up iron and will also ingest and often are seen to contain lipids.

A great deal is known concerning the removal of triglyceride, cholesterol and phospholipid from the blood but it is difficult to interrelate the many isolated experimental findings some of which are contradictory.

The findings are as follows:-

(1) Triglyceride. Emulsions of triglyceride have been shown experimentally to be taken up and removed from the blood by reticulo-endothelial cells throughout the body (Saxl and Donath, 1925) but in particular in the liver where the lipid is found concentrated in both the parenchymal and Kupffer cells (Murray and Freeman, 1951). Waddell and co-workers (1954) confirmed these findings but found that blockade of the reticulo-endothelial system prevented the appearance of triglyceride in the Kupffer cells while not affecting its uptake by the parenchymal cells. This suggested that the transfer of triglyceride emulsions from the circulation to the liver cells does not involve Kupffer cells as was first suggested by Jaffe and Berman, (1928). Recent and comprehensive studies have shown that the reticulo-endothelial cells are in fact involved.

When the reticulo-endothelial system in live rats is stimulated by

glucan, both triglyceride emulsions and carbon particles are more rapidly removed from the circulation and are more completely localized in the liver (Di Luzio and Riggi, 1964 a). Histochemical and electron microscope studies have shown that the triglyceride injected as an emulsion is ingested by the hepatic reticulo-endothelial cells and increasingly so after glucan stimulation (Ashworth, Di Luzio and Riggi, 1963).

(2) Chylomicra. In histological studies by Murray and Freeman (1951), chylomicra were shown to be taken up directly by the hepatic parenchymal cells without appearing in the Kupffer cells. This finding is supported by Di Luzio and Riggi (1964 b) who showed that although hyperplasia of the reticulo-endothelial system brought about by glucan results in carbon particles being cleared from the blood ten times as rapidly as in normal rats, it does not effect the clearance rate of chylomicra labelled with cholesterol-4-^{14}C or 1-^{14}C tripalmitin. The carbon particles do not compete with or slow the removal rate of the labelled chyle. These findings have been confirmed by histo-chemistry and by the electron microscope (Ashworth, Di Luzio and Riggi, 1963) which show that chylomicra given by intravenous injection are removed primarily by hepatic parenchymal cells and are not detectable in the reticulo-endothelial cells even after glucan stimulation. Chyle is not readily taken up by reticulo-endothelial cells in rat lymph nodes (French and Morris, 1960) or by macrophages in vitro (Day, Gould-Hurst and Wahlqvist, 1964). This is in contrast to artificial fat emulsions and cholesterol

suspensions. Artificial fat emulsions are evidently not treated in the same way as chyle.

(3) Cholesterol. Friedman, Byers and Roseman (1954) showed that dietary cholesterol is removed by Kupffer cells, its appearance in both the parenchymal and Kupffer cells being depressed by reticulo-endothelial cell blocking agents. Di Luzio and Riggi (1962) however failed to show any increase in the uptake of injected cholesterol in chyle following the action of reticulo-endothelial system stimulating agents. Stimulation on the other hand does reduce chronic dietary hypercholesterolaemia.

Injected into the peritoneal cavity, cholesterol and its esters, like triglyceride and phospholipid, are absorbed through the diaphragmatic lymphatics and taken up by reticulo-endothelial cells in the thoracic lymph nodes (French and Morris, 1960). Cholesterol ester once taken up by these cells appears to be removed with difficulty, remaining in the cells for at least 18 months. Free cholesterol disappears much more rapidly than the ester form (Day and French, 1961) and triglyceride, as shown by histochemical means, remains more briefly still (French and Morris, 1960). Cholesterol suspensions are also readily phagocytosed by macrophages in vitro (Neveu et al, 1956). In lymph nodes the ingestion of cholesterol by reticulo-endothelial cells is followed by metabolic changes and the accumulation of cholesterol ester (Day and French, 1959).

An interesting isolated observation is that cholesterol oleate suspension injected intravenously into rats depresses the phagocytic

activity of the reticulo-endothelial system. Small doses stimulate activity but larger doses depress phagocytosis and clearing of particles from the blood for long periods of time (Stuart and Davidson, 1963). This finding may explain why cholesterol oleate is removed so slowly from the reticulo-endothelial cells in lymph nodes. This fact also agrees with the theory of Sinclair (1956) that the esterification of cholesterol particularly with saturated fatty acids, decreases its ease of transport and predisposes to its deposition in the tissues.

(4) Phospholipids. Given by mouth or parenterally, phospholipids are partly taken up and removed from the body fluids by reticulo-endothelial cells and if certain of the phospholipids are given in large amounts the reticulo-endothelial cells increase in number and size forming foam-cell lesions. Sphingomyelin injected intravenously into monkeys or rabbits causes foam-cell infiltration in the liver, spleen, kidney, marrow and adrenals but not the brain (Ferraro and Jervis, 1940). Since these workers found that blocking the activity of the reticulo-endothelial system by injecting congo red prevented the formation of foam-cells it was concluded that the foam-cells had arisen from reticulo-endothelial cells. Kimmelstiel and Lass (1934) produced foam-cell lesions by injecting cerebrosides into the peritoneal cavity and Christianson (1941) produced similar lesions by injecting into the peritoneal cavity and by feeding cerebrosides to dogs. Kimmelstiel and Lass (1934) found that when lecithin was given in the same way a lesser reaction consisting of a few histiocytes and epithelial cells

resulted but no foam-cell lesions were produced. When Sjövall (1935) injected lecithin by the intravenous route or into the peritoneal cavity foam-cell lesions in the lungs, liver and spleen were produced but these were not as striking or long-lasting as when sphingomyelin was given. Cephalin injected intravenously by Ferraro and Jervis (1940) failed to produce foam-cell lesions.

Uptake of lipid in Lipoidoses. In those lipoidoses in which serum lipid levels are elevated, it is likely that the lipid found within the reticulo-endothelial cells (which is a characteristic feature of the disease) results from its uptake by ingestion from the serum.

Uptake and transport of lipid by macrophages in Atherosclerosis. It has been postulated that macrophages take up and transport lipid from the liver and deposit it in the arterial wall, thereby being involved in the pathogenesis of atheroma (Gordon, 1947; Leary, 1949; Simon, Still and O'Neal, 1961).

Histological studies have shown that following intimal injury in cholesterol fed rabbits there is infiltration of the intima with lipoprotein lipid, most of which is seen to be ingested by macrophages (Courtice and Schmidt-Diedrichs, 1962). In naturally occurring atheroma, macrophages are also seen with their cytoplasm engorged with lipid. It is generally accepted that much of this lipid taken up by the macrophages also originates from the plasma lipoprotein (Buck, 1958; Botcher et al, 1960; Gero et al, 1960 and 1961). The protein

component is thought to be split off prior to the ingestion of the lipid (Buck, 1958). Lipoprotein can be ingested by macrophages in vitro but does not appear to be concentrated in their cytoplasm as are artificial lipid emulsions and suspensions, (Day, Gould-Hurst and Wahlqvist, 1964).

In addition to the role played by reticulo-endothelial cells in the uptake of lipid by the liver and arterial wall, other roles have been attributed to macrophages. Alveolar macrophages have been shown (Dougherty and Berliner, 1959) to remove cholesterol from the circulation and it has been suggested (Quensel, 1932) that lipid filled phagocytes eliminated in the sputum represent one pathway of lipid excretion.

The influence of polyunsaturated fatty acids on the uptake and removal of lipid. Experimental findings suggest that polyunsaturated fats can influence the uptake by and storage of lipids by macrophages. Alveolar macrophages of rats on a diet deficient in essential fatty acids show an accumulation of lipids including cholesterol. This lipid accumulation can be reversed when the deficient polyunsaturated fatty acids are replaced in the diet (Bernick and Alfin-Slater, 1963). The removal rate of cholesterol from reticulo-endothelial cells in rat lymph nodes on the other hand is not influenced by the administration of either saturated or unsaturated triglycerides, (Day, 1960 a).

Circulating lipophages in cholesterol fed rats vary in number with the serum cholesterol level (Simon, Still and O'Neal, 1961).

When oil containing polyunsaturated fatty-acids is substituted for butter in the diet the number of lipophages in the blood falls by half (Hartcroft and O'Neal, 1962). Oil containing polyunsaturated fatty-acids in the diet also results in a fall in the level of cholesterol and the percentage of esterified cholesterol in the serum while in the liver the total cholesterol and per cent of esterified cholesterol increase (Bloomfield, 1964). The significance of this association between the dietary fats, the number of circulating lipophages and the serum cholesterol is not known.

In summary, reticulo-endothelial cells in many tissues of the body have been shown at times to ingest, store, remove and sometimes transport lipid. The metabolism of the ingested lipid by these cells will be described in the next section.

Metabolic Functions of Reticulo-Endothelial Cells.

Reticulo-endothelial cells have been shown to possess many important metabolic functions some of which will now be described.

Haemoglobin. Most red blood cells lyse in the circulation and reticulo-endothelial cells then remove the debris (Knisley, 1936). Haemoglobin from the cell fragments and free haemoglobin in the plasma is broken down by reticulo-endothelial cells to biliverdin and bilirubin. Haemosiderin is also formed in the cells (Rich, 1924; Lemberg, 1937).

Prothrombin. This protein and also proconvertin (Factor VII) are thought to be synthesized by reticulo-endothelial cells, primarily those in the liver but also in other parts of the body as well (Slatis, 1958; Adlercreutz and Petterson, 1963).

LIPID METABOLISM.

Although it has been known for many years that reticulo-endothelial cells can take up lipids (as described in the last section) little was known about the lipid metabolism of these cells and only recently have significant investigations been carried out in this field.

Triglyceride. Hass in 1938 injected triglycerides and fatty acids into the skin of various animals and observed a local reaction in which granulocytes, macrophages and giant cells converged and ingested the lipid. Unsaturated fats caused a more marked reaction than saturated fats. Hydrolysis of the esters occurred, identified by changes in the physical properties of the fat. Hydrolysis was greater when

methyl esters of fatty acids were injected than when glycerol esters were given. Methyl esters also caused the greatest accumulation of macrophages, their cytoplasm foamy in appearance due to the large amount of fat they had ingested. Hydrolysis of lipid infiltrating the atheromatous arterial wall may also occur since Gomori (1946) has shown by histochemical means the presence of non-specific lipase in fat-laden macrophages and other cells in the lesion.

Macrophages from the peritoneal cavity of rabbits cultivated in vitro have been shown by Day (1961) using ^{14}C-labelled preparations, to be capable of oxidizing sodium palmitate and glycerol tripalmitate with the production of $^{14}CO_2$. Labelled tripalmitate in chyle presented to macrophages was similarly oxidized.

Macrophages have recently been shown to be capable of incorporating various substrates into lipids. Day and Fidge (1962) showed that macrophages from the peritoneal cavity of rabbits ingested ^{14}C-labelled sodium palmitate and incorporated it into triglyceride and phospholipid and a small amount into mono and diglyceride. The same authors have shown (Day and Fidge, 1964) that ^{14}C-labelled acetate ingested by these cells is incorporated into the fatty acids of mono, di, and triglycerides. ^{14}C-labelled glucose is incorporated by these cells into the glycerol fraction of glycerides (Day and Fidge, 1965 a).

Cholesterol. Suspensions of cholesterol injected beneath the skin cause macrophages to accumulate (Tompkins, 1946). These cells have an affinity for cholesterol whose needle shaped crystals are seen

to congregate on the cells' surface where they exhibit an increasing sudanophilia suggesting that esterification of the cholesterol is taking place, probably due to surface acting enzymes. The cholesterol is later seen within the cells from where it gradually disappears and as it does so it increasingly stains with neutral red. Tompkins interpreted this latter staining reaction as indicating that the esterified cholesterol had combined with protein in the cells and suggested that this may have aided its suspension and dispersion.

Reticulo-endothelial cells in rat lymph nodes as well as macrophages in the skin appear to esterify ingested cholesterol since French and Morris (1960) showed an increasing staining of these cells with Sudan IV after the uptake of cholesterol. It was found (Day and French, 1961; Day, 1960 b) that esterified fatty acids and phospholipid accumulate in reticulo-endothelial cells after the uptake of cholesterol or cholesterol oleate but the Sudanophilia developing after free cholesterol uptake was shown to be due, not merely to this accumulation of other lipids but to the synthesis of cholesterol ester. This esterification was demonstrated by Day and French (1959) to be occurring using chromatographic separation and chemical estimation. They also showed that hydrolysis of cholesterol oleate occurred in these cells with the production of free cholesterol. (Day and French, 1959). Macrophages from the peritoneal cavity of rabbits possess the necessary enzymes to esterify and hydrolyze cholesterol and its ester; they have been shown to contain cholesterol-esterase both synthetic and hydrolytic (Day, 1960 c).

There is some evidence that cholesterol is esterified more rapidly by reticulo-endothelial cells when esterified with unsaturated rather than saturated fatty acids (Day, 1960 a; Day, Fidge, Gould-Hurst and Risely, 1963).

Cholesterol is excreted from the body unchanged or as bile salts, the cyclic structure of the molecule remaining unbroken. The side chain is oxidized and removed in the formation of bile salts but apparently cannot be oxidized by macrophages (Day, 1961). This was shown when cholesterol preparations labelled in the side chain were presented to and ingested by rabbit peritoneal macrophages in vitro. No $^{14}CO_2$ was evolved. Under similar circumstances triglyceride and fatty acid ingested were rapidly oxidized.

Rabbit peritoneal macrophages are able to synthesize cholesterol. When ^{14}C-labelled acetate was taken up by these cells 27 per cent of the acetate was incorporated into lipid, emphasizing the significance of these cells in lipid synthesis. The labelled acetate was incorporated into cholesterol and cholesterol ester as well as other lipids (Day and Fidge, 1964). Free fatty acid presented to these cells is partly incorporated into cholesterol ester (Day and Fidge, 1962).

The influence of cholesterol on the synthesis of phospholipid by macrophages will be described in the next section.

Phospholipid. The demonstration that phospholipid accumulates in reticulo-endothelial cells which have ingested cholesterol (Day, 1960 b; Day and French, 1961) suggested that these cells may be able to

synthesize phospholipid. Day and Fidge showed in 1962 that macrophages in vitro after ingesting ^{14}C-labelled fatty acid contain lecithin and sphingomyelin labelled with the ^{14}C. The same authors in 1964 showed that ^{14}C-labelled acetate is partly incorporated into phospholipid by these cells and ^{14}C-labelled glucose is partly incorporated into the glycerol fraction of phospholipid (Day and Fidge, 1965 a). ^{32}P-labelled phosphate taken up by the macrophages is incorporated (Day and Fidge, 1965 b) into phospholipids, mainly lecithin and to a lesser extent sphingomyelin but also phosphatidyl ethanolamine and inositol phosphatide. The uptake of cholesterol (but not carbon) was shown to increase the incorporation of the ^{32}P into phospholipid.

Sphingomyelin and lecithin increase in amount in the arterial wall as atheroma develops (Kayden, Seegal and Hsu, 1959; Smith, 1959) and are in fact synthesized in the arterial wall (Chernicks, Srere and Chaikoff, 1949; McCandless and Zilversmit, 1956; Zilversmit, McCandless, Jordan et al, 1961). Since it has been shown, as described above, that macrophages synthesize phospholipid from a number of precursors and since macrophages are a prominent part of the atheromatous lesion, Day (1962) examined the aorta of cholesterol-fed rabbits by histochemical means in an attempt to detect the presence of phospholipid in macrophages in atheroma. As described earlier, cholesterol stimulates the synthesis of phospholipid by macrophages and the macrophages in atheroma are filled with cholesterol. The aortic macrophages were found to contain phospholipid and indeed to be the main elements in the

lesion to have accumulated it. This was evidence that the foam-cells in atheroma synthesize the phospholipid which increases in the wall in this condition. More recently Dunnigan (1964) and Day and Gould-Hurst (1964, unpublished observations) have similarly demonstrated by histochemistry that phospholipid is present in macrophages in human atheroma (see fig 11 and 12 after page 90).

Reticulo-endothelial cells have been shown as described in this section to be active and important cells in many aspects of the metabolism of lipid.

Reticulo-Endothelial Cells and Atheroma.

Adams (1963) has defined atherosclerosis as a multifocal degenerative and proliferative condition affecting the intima and inner media of certain large and medium sized muscular arteries. The degenerative changes are characterized by lipid accumulation, hyalinization and fragmentation of connective and elastic tissues while the proliferative element of the disease is a sclerotic reaction of intimal connective tissue.

The majority of human atherosclerotic plaques contain lipid deposits in their base at the outer intima but a purely fibrous plaque is not uncommonly encountered. In cholesterol-fed rabbits cellular proliferation over the aortic intima forming a pallisade of cells occurs in the earliest lesions and usually contain droplets of lipid; however such proliferation may also be seen in the absence of the lipid (Friedman, 1963). In both species degenerative changes of the internal

elastic lamina accompany the earliest lesions (Moon and Rinehart, 1952) and changes in and increase of the intimal mucoid ground substance occur and both may precede deposition of lipid (Gero, Gergley and Farkas, 1962). Degeneration may occur in adjacent muscle fibres sometimes independently of any intimal alteration. These facts suggest that there may be early lesions which precede and cause the lipid accumulation.

Experimental rabbit atheroma is characterized by accumulation beneath the endothelium of large numbers of macrophages containing lipid filled vacuoles (fig 1). As the lesion progresses these foam-cells may enlarge and undergo necrosis releasing their lipid content (McMillan and Duff, 1951). In early human lesions on the other hand the lipid accumulation is first seen partly within subintimal cells thought to be partly derived from smooth muscle-cells (Geer, McGill and Strong, 1961) and partly extracellularly. Fewer lipid filled macrophages are present than in the cholesterol-fed rabbit (Thomas, Jones and Scott et al, 1963). A pooling of lipid, mainly phospholipid, also occurs around elastic fibres of the intima and inner part of the media and this latter may be independent of trans-intimal lipid infiltration, not always present in early plaques (Adams and Tuquan, 1961). These differences between the early human and rabbit lesion may be due to differences in the anatomy of the human and rabbit aorta or to the enormous levels of serum lipids and the

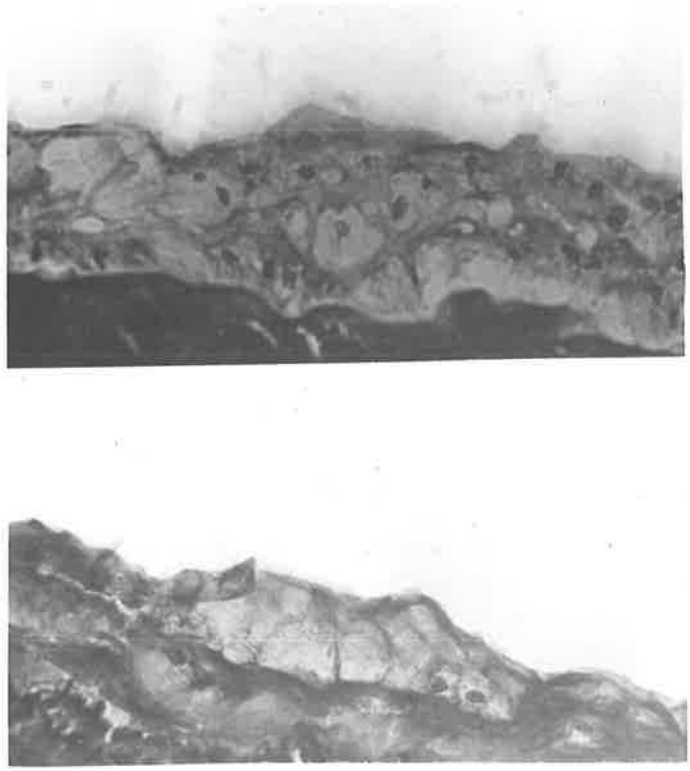


Fig 1. "Foam-cell lesions" in the aortic-intima of a rabbit fed cholesterol for 6 weeks.

Neutral red stain 400 x mag.

Frozen section.

rapid development of rabbit lesions. Rabbit atheroma can be made to resemble human lesions by intermittent cholesterol feeding carried on for several years (Constantinides, 1961).

In the cholesterol-fed rabbit the elastic lamina of early plaques invariably exhibits staining changes, fraying and fragmentation while sites of foam-cell multiplication invariably overlie such elastic changes. As the elastica degenerates further, smooth muscle-cells from the media erupt through it and become vacuolated (Friedman, 1963).

Summarizing the above description; degeneration of the elastic laminae and adjacent smooth muscle-cells and changes in the mucoid ground substance appear early in human and rabbit atherosclerosis. These changes may precede intimal changes and lipid deposition and so possibly constitute the initial lesions. The earliest lipid deposits in the human are seen in the sub-intima within cells and as a pool adjacent to the media. In the cholesterol-fed rabbit the earliest lipid appears within the intimal cells and in the pallisade of cells often seen above them and is followed by the appearance of lipid-filled sub-endothelial macrophages. Subsequent to these changes fibroblastic proliferation, calcification and ulceration may occur but these secondary changes are not relevant to the present work and will not be further described.

Stimulation of the reticulo-endothelial system in rabbits by

injecting sulphated polymannuronides during cholesterol feeding has been shown by Constantinides and Saunders (1958) to stimulate the phagocytosis of lipid by macrophages and to arrest the progress of aortic atherosclerosis by the reduction of serum lipid levels.

In contrast to the above Patek and Bernick (1959 and 1961) produced aortic lesions in animals by giving reticulo-endothelial cell blocking agents. The cells which accumulated in the aortic lesions ingested cholesterol to become foam-cells when cholesterol was added to the diet. The fact that stimulating the reticulo-endothelial cells lowers the serum lipid level and arrests the progress of atheroma while blocking the cells causes an atheromatous lesion to appear, suggests that in atheroma these cells play a protective role concerned with the removal of accumulating lipid.

As has just been described, high doses of the mucopolysaccharide, polymannuronide, a heparin-like substance, decreases the accumulation of cholesterol in the arteries of hypercholesterolaemic rabbits. This may not only be due to reticulo-endothelial cell hypertrophy and hyperplasia but also to the following: Gero et al (1960) have shown that mucopolysaccharides which accumulate in early atheroma (Taylor, 1953) react with lipoprotein to form a single compound. Day (1964) suggested that this compound may be more readily phagocytosed by reticulo-endothelial cells than lipoprotein alone. If this is so then the mucopolysaccharides which accumulate in

atheroma may stimulate and increase the uptake of infiltrating lipoprotein by macrophages in the lesion.

Courtice and Schmidt-Diedrichs (1962) have shown that following intimal injury in cholesterol-fed rabbits there is infiltration of the intima with lipoprotein lipid most of which is found in macrophages. In rabbits with hypercholesterolaemia due to the injection of triton (WR-1339), infiltration of the intima by lipoprotein is not followed by uptake of the lipoprotein by the macrophages suggesting that the properties of the lipoprotein may influence its uptake by the macrophages. In further experiments the same workers (Schmidt-Diedrichs and Courtice, 1963) introduced into injured carotid arteries in live rabbits various lipoprotein fractions separated by the ultracentrifuge and also introduced hypercholesterolaemic serum. They described a subsequent accumulation of macrophages which ingested the lipoprotein and disappeared. There was no difference in uptake or removal between any of the lipoprotein fractions.

Lipoprotein injected directly into the arterial wall rapidly disappears (Hollander, Kaplan and Sherwin 1962) but when a crystalline suspension of cholesterol is injected it remains in the wall and is only very slowly removed. This however is probably related to the solubility of the preparation rather than to its ingestion by macrophages.

There is some evidence (Courtice and Schmidt-Diedrichs, 1963; Schmidt-Diedrichs and Courtice, 1963) that lipid laden macrophages in the atheromatous arterial wall find themselves trapped in the intima between the hydrostatic pressure of the circulation and the elastic laminae of the arterial wall. In animal experiments, returning a raised serum cholesterol level to normal only resulted in the very slow disappearance of foam-cells while on the other hand, lowering the luminal blood pressure resulted in their rapid disappearance as also occurred if the elastic laminae were fragmented or capillaries were made to grow into the wall from the media and adventitia providing apparently an escape route for the cells.

The nature and origin of foam-cells in atheroma. Until recently it was universally believed that the foam cells in the wall of the atheromatous arteries are cells of the reticulo-endothelial system. As a result of studies with the electron microscope it is now thought that some of these cells at least may be derived from other cellular elements. This is one of many facets of the disease of which the exact morphology is not known. The foam-cells are believed by some workers to originate from cells of the blood and by others to originate from mitosis of cells of the arterial wall. The theory that foam-cells originate from the blood is supported by observations of lipophages seen adhering to the luminal surface of the endothelium, seen immediately beneath the endothelium and caught in the process of penetrating the endothelium (Poole and Florey, 1958; Rannie and Duguid, 1958; Still

and O'Neil, 1962; Hartcroft and O'Neal, 1962; Still, 1963). It was suggested by Gordon (1947) and Leary (1949) that these lipophages arise from reticulo-endothelial cells in the liver where they ingest lipid, and in particular cholesterol esterified by the liver and deposit it after being carried by the circulation to the intima of an artery into which they penetrate.

There is, however, no evidence to prove that the lipophages seen passing through the endothelium are in fact penetrating into, rather than escaping from, the arterial wall. Workers who have labelled reticulo-endothelial cells to try to determine the direction in which they pass through the endothelium have not seen these labelled cells appear in the intima (Simonton and Gofman, 1951; Day and Gould-Hurst, unpublished work 1961).

Other workers who also believe that foam-cells originate from the circulation have produced evidence that lipid-laden macrophages fall out of the arterial stream of blood, adhere to the endothelium and are covered over by proliferating endothelial cells. Numerous coats of cells applied as just described become the atheromatous plaque (Rannie and Duguid, 1953; Still and O'Neal, 1962).

A further group believe that foam-cells arise by division of cells of the arterial wall. Mitosis has been observed in the walls of affected arteries occurring in "foam-cells" (McMillan and Duff, 1948) and in "macrophages" (Poole and Florey, 1958). Other workers

believe that foam-cells arise from the proliferation of endothelial cells (Hueper 1941; Altschul 1944; Kuntz and Sulkin, 1948; Duff and McMillan, 1951; Friedman, 1963) a theory denied by Poole and Florey (1958).

Electron microscope studies have disclosed morphological evidence that smooth muscle-cells accumulate lipid to become foam-cells (Altschul, 1950; Geer, McGill and Strong, 1961). This discovery is supported by evidence from workers who however see two distinct types of foam-cell in atheroma, one derived from smooth muscle and the other, in the more superficial layers of the lesion, from lipid filled macrophages of the reticulo-endothelial system (Still, 1963). Other workers show that smooth muscle-cells proliferate but deny that they are the origin of the foam-cells (Thomas, Jones, Scott and Morrison, 1963).

At the present time it is most widely believed that foam-cells are derived both from smooth muscle-cells and from macrophages. If macrophages penetrate into the arterial wall from the blood it seems unlikely that they carry with them more than a small part of the lipid deposited in the lesion. It seems more likely that the part they play in the pathogenesis of atheroma is related to the metabolism of the lipid which they ingest or to the synthesis of lipid within the arterial wall (Day, 1964).

The manner in which reticulo-endothelial cells handle ingested

lipid both from the aspect of chemical metabolism and that of physical transport can be seen from what has been said so far to be of great interest and importance. The difficulty of studying these cells in the living tissues is great and particularly so in the arterial wall, However, since they can be harvested and studied in vitro and also are accessible in lymph nodes which are easily removed it has been possible to carry out experiments with these living cells which it is hoped may add something to the growing body of knowledge regarding the general functions of the cells and their relation to lipids and to atheroma. These experiments will now be described.

MATERIALS AND METHODS.

MATERIALS AND METHODS.

Lipid Preparations for Intraperitoneal Injection into Rats.

(1) Suspensions of Cholesterol.

Aqueous suspensions of cholesterol were used for intraperitoneal injection. One gm of cholesterol was dissolved in 100 ml of warm acetone and this solution was added slowly with constant stirring to 400 ml of warm water. The acetone was driven off by heating, porcelain chips being added to prevent bumping. The resulting suspension was concentrated to about 100 ml by boiling and then filtered through glass wool to remove any precipitate. The cholesterol concentration of the suspension was estimated and its composition analysed by chromatography which confirmed that no cholesterol ester was present. Prior to injection the suspension was diluted with water to give the required concentration and bovine albumin (Commonwealth Serum Laboratories) in water was added to give a final concentration of 2.5 per cent albumin in the final mixture.

Albumin was added because it has been shown to increase the uptake of cholesterol suspension by reticulo-endothelial cells in lymph nodes (Day and French, 1959).

Cholesterol-4-^{14}C Suspension. Cholesterol-4-^{14}C with a specific

activity of 63.4 micro curie per mg was obtained from the Radiochemical Centre, Amersham, England. Aqueous suspensions were prepared by adding to a small volume of water an acetone solution of the cholesterol-4-^{14}C and driving off the acetone by heating. The resulting stock suspension was estimated by radio-assay and was used either unchanged or diluted with a more concentrated non radio-active cholesterol suspension. In certain experiments a homogenous suspension was made by dissolving the radio-active and the non-radio-active cholesterol together in acetone before preparing the suspension.

(2) Double-labelled Lipoprotein.

Two preparations were used in which the serum lipoprotein from cholesterol-fed rabbits was labelled in the phospholipid with ^{32}P and in the cholesterol with either ^{14}C-labelled or ^{3}H-labelled cholesterol.

The procedure for labelling of lipoprotein was essentially that described by Shore, Zilversmit and Ackerman (1955) and was performed as follows. A rabbit fed a cholesterol-rich diet was given intravenously 4 to 9 milli curie of ^{32}P-labelled orthophosphate. Forty-eight hours later the rabbit was bled by cardiac puncture and the serum collected and dialysed against saline to remove any remaining labelled orthophosphate.

In the first preparation the cholesterol in the serum lipoprotein was then labelled with ^{14}C-labelled cholesterol by incubating the serum in vitro with an aqueous preparation of ^{14}C-labelled cholesterol as described by Whereat and Staple (1960). Under these circumstances most

of the labelled phospholipid and cholesterol were present in the beta-lipoprotein fraction of the serum, this being the major lipoprotein fraction present in the cholesterol-fed rabbit serum. The incorporation of label was checked by paper electrophoresis of the serum (for details of method see below) and scanning of the resultant paper electrophoretogram using a Nuclear-Chicago 4Pi Radiochromatography Scanner. After incubating the serum with ^{14}C-labelled cholesterol, electrophoresis showed this, as well as the ^{32}P to be incorporated mainly into the beta-lipoprotein fraction (see fig 2).

A second preparation of double-labelled beta-lipoprotein was also used in these experiments. Cholesterol-fed rabbits were given 2.5 milli curie of ^{3}H-labelled cholesterol by gastric intubation. Two days later the incorporation of the ^{3}H-labelled cholesterol into beta-lipoprotein was confirmed by electrophoresis and scanning, and then ^{32}P-labelled orthophosphate was injected intravenously to label the phospholipid in the lipoprotein. The serum, now double-labelled, was collected on the fourth day. Confirmation of the labelling of the beta-lipoprotein by ^{32}P was now carried out by electrophoresis and scanning. The data for the two preparations used in the experiments with double-labelled lipoprotein are given in Table II.

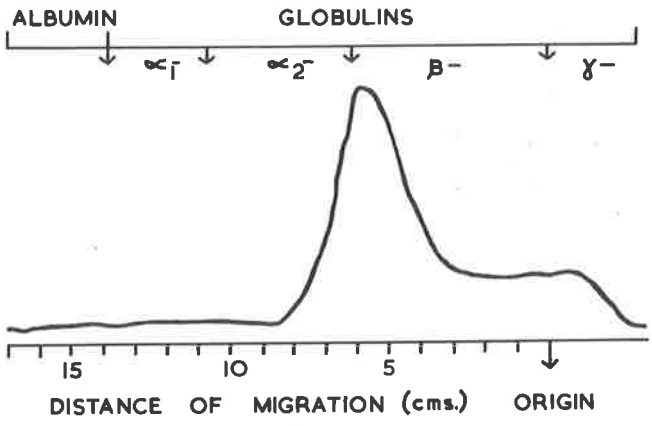


Fig 2. The distribution of ^{32}P-labelled phospholipid in the lipoprotein of rabbit serum after intravenous injection of ^{32}P-labelled orthophosphate.

Paper electrophoresis.

TABLE II.

COMPOSITION OF THE TWO PREPARATIONS OF DOUBLE-LABELLED LIPOPROTEIN FOR INTRAPERITONEAL INJECTION INTO RATS.

		Concentration (mg/ml serum)	Radio-activity (dps/ml serum)	Sp.Activity (dps/mg)
1.	^{14}C-labelled cholesterol	12.6	18000	1430
	^{32}P-labelled phopholipid	4.7	6800	1440
2.	^{3}H-labelled cholesterol	6.6	49250	7450
	^{32}P-labelled Phospholipid	3.1	10750	3470

Paper electrophoresis

Separation of radio-active labelled lipoprotein fractions was carried out by Mrs. G. K. Wilkinson using the apparatus of Flynn and De Mayo with "Veronal" buffer (pH 8.5) as the conducting agent. The serum was applied to Whatman 3 mm paper strips measuring 38 by 3 centimetres. Runs of approximately 20 hours at room temperature at 180 volts produce a 12 to 13 centimetre displacement of albumin toward the anode. Control papers were stained with bromphenol blue and sudan black to locate the position of the protein and lipoprotein bands

respectively. After drying, the experimental papers were either scanned as just described or in later experiments were cut transversely, either into 1 cm strips over the whole area of migration or into sections corresponding to the origin and to areas occupied by the alpha-1 and alpha-2 globulin and the beta-globulin fraction. The area covered by albumin was included in the alpha-1 fraction. The papers were then extracted either by boiling with alcohol-ether (3:1) or in cold chloroform-menthanol(2:1) and the labelled cholesterol present in the extracts counted by scintillation counting. In some instances the lipid extracts were separated into free and ester cholesterol before counting. Recovery from the paper was of the order of 80 per cent.

(3) Triglyceride emulsions

Corn Oil emulsion was made with the following composition: Tween 60, 0.5gm; glucose 5gm; corn oil 10 ml; water 100 ml. The Tween 60 was dissolved by warming, mixed with a few ml of hot water and diluted to 20 to 30 ml before adding the oil and emulsifying in a Waring Blender. The glucose, dissolved in water was added and the volume made up to 100 ml. The emulsion was finally homogenized for 5 minutes in the Waring Blender.

Coconut oil emulsion was prepared in the same way as were corn oil emulsions. Because of the high melting point of the coconut oil, hot water was used while preparing the suspension.

(4) Phospholipid emulsions

Lecithin emulsion. Animal lecithin ("90 per cent pure") and pure

synthetic DL alpha-lecithin were obtained from the Nutritional Biochemicals Incorporation. Suspensions were prepared by two methods:

Method A The lecithin was dissolved in a small volume of warm ethanol, diluted with water and the ethanol removed by heating under a stream of air.

Method B 100 mg lecithin together with 10 drops of Tween 20 were dissolved in ethanol. The ethanol was removed by heating and the solution of lecithin in Tween 20 which remained was diluted with water. This method was based on that described for cholesterol by Meier, Siperstein and Chaikoff (1952).

Lecithin suspensions prepared by either method were diluted to contain 10 mg per ml.

The fatty acid composition of both lecithin preparations was determined by Dr. A. J. Day using gas phase chromatography and shown to be as follows. The animal lecithin used contained 53.6 per cent oleic and 31.5 per cent palmitic acids with small amounts of stearic, palmitoleic and myristic acids. The synthetic lecithin contained 98.5 per cent palmitic acid.

^{32}P-labelled phospholipid emulsion. This was prepared from rabbit liver following the intravenous injection into the rabbit of ^{32}P-labelled orthophosphate. The rabbit, which had received a diet containing 0.75 gm of cholesterol and 4.5 gm peanut oil daily for 17 days was given, intravenously, 9 milli curie of ^{32}P-labelled orthophosphate. Forty-eight hours later the animal was killed, the

liver removed and the liver lipid extracted with chloroform-methanol (2:1). The phospholipid was separated from the neutral lipid by chromatography on silicic acid columns and the combined phospholipid was then emulsified using Tween 20 as emulsifying agent as described above. The distribution of the ^{32}P between the different phospholipid fractions was determined by paper chromatography on silicic acid impregnated paper, and found to be 68.3 per cent lecithin, 5.6 per cent sphingomyelin and 2.5 per cent "cephalin". The silicic acid column and paper chromatography were carried out by Mr. N. H. Fidge.

The Experimental Procedure for Intraperitoneal Injection into Rats.

Male Wistar-strain of Norwegian hooded tree rats, weighing between 180 and 220 gm, were injected with 2 ml of an aqueous suspension of cholesterol either alone or together with animal lecithin prepared as described above. Under ether anaesthesia the muscle of the abdominal wall was exposed through a small skin incision and the injection made through the muscle into the peritoneal cavity using a blunt serum needle. The skin incision was then closed by a suture. This technique avoided damage to the bowel and loss of injected material into the skin and subcutaneous tissue.

The Extraction of Lipid from Rat Lymph Nodes.

Rats were killed with ether. The sternum and the sternal end of the ribs were cut out and discarded and the remainder of the thoracic

cage and mediastinum were removed from the body and pinned to a dissecting board. The thoracic lymph nodes were carefully dissected from the adjacent mediastinal tissue, removed, cleaned of adherent fat and fibrous tissue and homogenized in a lipid solvent. In early experiments the nodes were homogenized by grinding in a pestle and mortar with washed sand. In later experiments a high speed micro-blender or an "all-glass rotary tissue grinder" (Kontes Glass Co., Vineland, N.J.) was used. One of the three following methods for the extraction of lipid was used in different experiments:

(1) Acetone-Ethanol extraction (Schoenheimer and Sperry, 1934)

The homogenate was boiled in a minimum of 50 times its volume of a mixture containing equal parts of acetone and ethanol (v/v). On cooling, the extract was made up to a known volume, filtered and a volume of the filtrate taken for estimation or analysis. This method has been shown (Jesting and Bang, 1963) to extract a larger proportion of non-lipid and to extract a smaller proportion of the lipid present than the methods used here in later experiments. The lipid extract produced by this method was in some experiments further purified by phase separation; the extract was evaporated to near dryness, 2 ml of water was added and the lipid re-extracted 3 times with 10 ml washings of 15 per cent ethanol in petroleum spirit (B.P. 60-80^{o}).

(2) Ethanol-Ether extraction (Bloor, 1928). This method is similar to that described above except that the solvent mixture used contains ethanol and diethyl-ether in the proportions 3 to 1 (v/v). This solvent mixture is miscible with the toluene used in the liquid scintillator used for radio-isotope counting. This permitted direct counting of lipid extracts.

(3) Cold Chloroform-Methanol extraction (Folch, Lees and Sloane-Stanley, 1957). The tissue was homogenized in 20 times its volume of chloroform and methanol mixed in the ratio of 2 to 1 (v/v). The extract was filtered and a known volume of the filtrate purified by phase separation. This was achieved by the addition of 0.73 per cent aqueous sodium-chloride solution, the volume of saline added being one-fifth the volume of the filtrate. Two phases formed, the lower containing the lipid. The upper phase was discarded and the interface washed three times with "pure solvent upper phase" composed of chloroform, methanol and 0.58 per cent aqueous sodium chloride solution in the proportions 3:48:47 (v/v/v).

The Separation of Free and Ester Cholesterol by Column Chromatography.

Aluminium oxide was used as adsorbent as described by Kerr and Bauld (1953). The columns consisted of 3/16ths" glass tubing expanded at the upper end to form a reservoir and tapering below to retain a small cotton wool plug. The columns were filled to a height of 2.5 cm with aluminium oxide washed in petroleum spirit (B.P. 60-80^{0}). The

samples containing cholesterol, free and ester, were dissolved and applied to the columns in 0.5 ml of petroleum spirit followed by two 0.5 ml washings of petroleum spirit. The cholesterol ester was eluted with 18 ml of petroleum spirit after which the free cholesterol was eluted from the alumina with 18 ml of benzene.

Chemical Assay of Cholesterol.

The method of Zlatkis, Zak and Boyl (1953) was used. The sample of free or ester cholesterol was dissolved in 3 ml of acetic acid. To this was added 2 ml of colour reagent (1 ml of 10 per cent ferric chloride in acetic acid and 100 ml of concentrated sulphuric acid). The port wine colour which developed was read using a Hilger Biochem H 810 Absorptiometer at a wave length of 550 microns against a blank of glacial acetic acid and colour reagent. The colour was found to be stable for 24 hours and the method was sufficiently sensitive to detect amounts of cholesterol as small as 10 to 20 micrograms.

Radio Activity Assay.

(1) G.M. tube counting. Counting was performed at infinite thinness using a thin mica end-window G.M. tube, sufficient counts being made to give an error of less than $\pm$ 5 per cent. Stock Cholesterol-4-^{14}C suspensions and suspensions for injection were counted by direct plating into aluminium planchets as described by Entenman, Lerner,

Chaikoff and Dauben (1949). Other samples were evaporated to dryness and the deposit taken up in 0.5 ml of petroleum ether. After application to the planchet the petroleum ether was evaporated under a heat lamp.

(2) Liquid scintillation counting. This was carried out using an Ecko N 662 Liquid-Scintillation counter. The liquid scintillator used was 0.3 per cent 2,5-diphenyloxazole in toluene. ^{14}C samples were counted at ambient temperature at a counting efficiency of 75 per cent; tritium was counted at 5^{o} at a counting efficiency of 33 per cent.

(3) Procedure for Counting Double-labelled Compounds. Radio assay was carried out using an Ecko N 664 A scintillation counter together with a pulse height analyser. Each sample was counted under two conditions in order to favour in turn one of the isotopes by eliminating partially or completely the counts contributed by the other. By this means it was possible to determine the separate levels of activity of each of the isotopes in the sample. Correcting for internal quenching was made for each isotope. The activity was expressed for the purpose of comparison as disintergrations per second.

The Collection of Free Macrophages from Rabbits.

Macrophages were obtained from the peritoneal cavity of rabbits using the method of Lucke, Strumia, Mudd, McCutcheon and Mudd (1933),

as modified by Mackaness (1952). Forty ml of medicinal paraffin (Harrington or Nujol), sterilized by heating to 120°, was injected into the peritoneal cavity of rabbits anaesthetized with ether. After 5 days the rabbits were killed by cardiac puncture and exsanguination under anaesthesia. The abdomen was opened and the peritoneum washed three times with chilled 0.9 per cent sodium chloride solution containing 0.004M E.D.T.A. (ethylene-diamine-tetra-acetic acid, disodium salt). The saline washings were removed by suction through a sheathed cannula into a separating funnel (see fig 3). In the separating funnel the macrophages were allowed to settle from the paraffin into the saline below and the latter was then run off through a gauze filter which removed giant cells, cellular aggregates and debris. The macrophages were deposited by centrifuging for 3 minutes at 800 revolutions per minute (approximately 220 g) and resuspended in Hanks solution (Hanks, 1948) or homogenized in a phosphate buffer solution of pH6.

The number of macrophages harvested was calculated in a haemocytometer counting chamber using standard white-count fluid or the volume of packed cells was measured using a conical, graduated centrifuge tube. Smears prepared for differential counting were dried in air and stained with Leishmann's Stain. The exudate contained 85 to 90 per cent of cells morphologically similar to tissue macrophages. Some of the cells contained droplets of

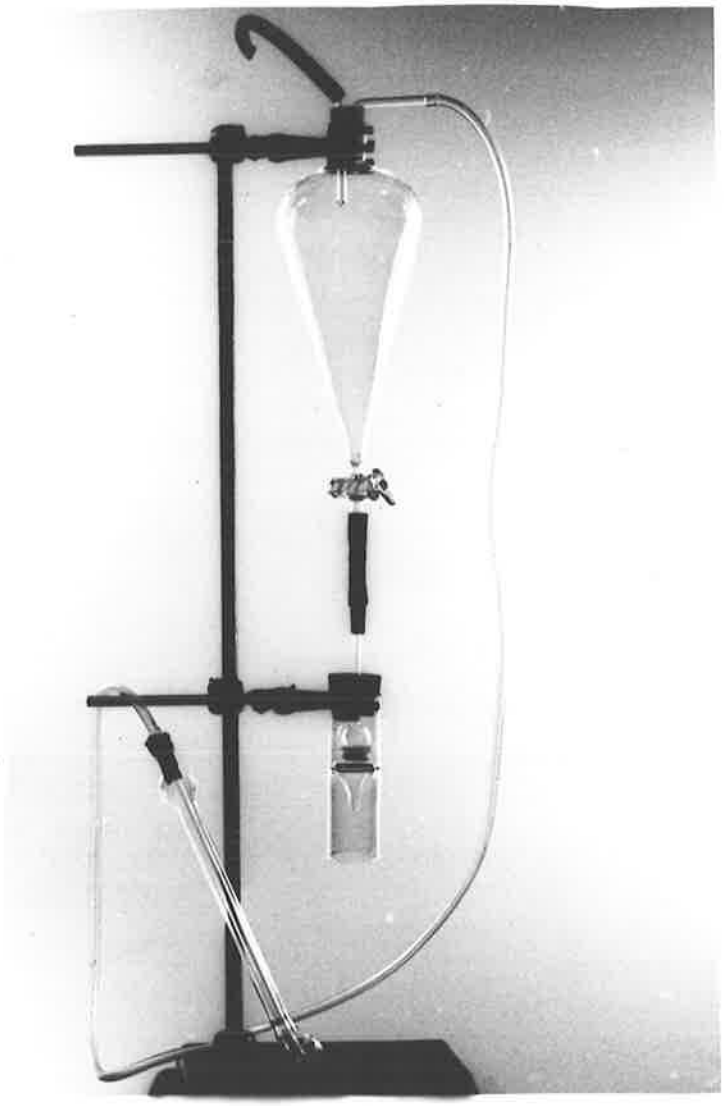


Fig 3. Apparatus for collecting rabbit macrophages. The sheathed cannula was used to suck saline washings of effusion of macrophages from the peritoneal cavity into the flask. After the macrophages had settled from the paraffin layer they were run off through the gauze filter into centrifuge tubes.

paraffin. In addition the exudate contained approximately 10 per cent of small mono-nuclear cells but rarely any polymorphs.

The Incubation of Rabbit Macrophages.

The incubation and extraction procedures varied with different experiments as follows.

(1) Incubation in McCartney bottles. The following procedure was used in the experiments designed to investigate the esterification of cholesterol by rabbit peritoneal macrophages. The macrophages were counted and approximately 60 million cells (suspended in 45 ml of Hanks solution containing 0.5 per cent bovine albumin) were dispensed in 20 ounce McCartney bottles (see fig 4). These were incubated at 37^{o} for 60 minutes to allow the macrophages to adhere to the glass. Contaminating dead and foreign cells were then removed by washing once with 0.9 per cent sodium chloride solution. Thirty ml of media containing Hanks solution, fresh serum and old serum (4:1:1 v/v/v) was then added to the cells together with a known amount (1500 to 2000 counts per second) of cholesterol-4-^{14}C stock suspension. The presence of fresh serum in the media was designed to provide opsonins and facilitate phagocytosis of the particulate suspension. The cells were incubated for a further 60 minutes and the radioactive media was then removed, the cells washed once with dilute non radioactive

Fig 4. A 20 ounce screw-topped (McCartney) bottle in which free macrophages were incubated.

cholesterol suspension and once with warm saline. One bottle was set aside for immediate extraction of the washed cells with 10 ml of alcohol-acetone (1:1) in order to determine the uptake of ^{14}C-labelled cholesterol by the cells during the 60 minute initial incubation. To the remaining bottles was added 30 ml of non radioactive media (Hanks solution, fresh serum and old serum, 4:1:1). These bottles were then incubated for a further 20 hours at 37^{o}. At the end of this period the media were removed and the contained dead cells and cell debris deposited by centrifuging at 2000 revolutions per minute for 10 minutes. The cells which were still attached to the glass were washed once with warm 0.9 per cent sodium chloride solution and the further cell debris removed from the saline washings by centrifuging. The cell debris from the media and washings were combined and extracted with 10 ml of alcohol-acetone. The cells still attached to the glass after washing were similarly extracted. Five ml of the supernatent solution obtained after centrifuging the media was extracted with 100 ml of alcohol-acetone. Ninety ml of this extract was evaporated to near dryness, 2 ml of water added and the lipid re-extracted 3 times with 10 ml of 15 per cent ethanol in petroleum ether (B.P. $60-80^{o}$).

Portions of the respective extracts from the cells, the cell debris and the media were evaporated to dryness and the free and ester cholesterol separated on alumina columns for radio assay by G.M. counting as described above. Carrier, free and ester

cholesterol (200 micro gm of each) were added to the cell and cell debris extracts prior to chromatography.

A control McCartney bottle was also set up with each incubation run. This contained no macrophages but to 30 ml of media as above was added 20 to 30 counts per second of the cholesterol-4-^{14}C stock suspension, i.e. an amount similar to that taken up by the macrophages. The control was incubated for 20 hours and the media extracted and assayed as described above.

(2) Incubation of rabbit peritoneal macrophages in Leighton Tubes. In the experiments designed to investigate the incorporation of cholesterol into lipoprotein by macrophages, the following procedure was used. Macrophages (10 to 20 million) suspended in Hanks solution containing 0.5 ml of fresh rabbit serum in a final volume of 2.2 ml were incubated for 30 minutes at 37^{0} in an agitating water bath with a known amount of cholesterol-4-^{14}C suspension. The macrophages now having ingested ^{14}C-labelled cholesterol were deposited by centrifugation (1000 revolutions per minute for 3 minutes), washed twice with saline and resuspended in Hanks solution containing 0.5 per cent bovine albumin. One and a half ml of this cell suspension was dispensed into several Leighton tubes so that each tube contained an equal number of cells (varying from 1 to 2 million in different experiments). The Leighton tubes were made from test tubes in which an area approximately 12 mm by 35 mm had been flattened to facilitate microscopic observations of the living cells (see fig 5). The tubes

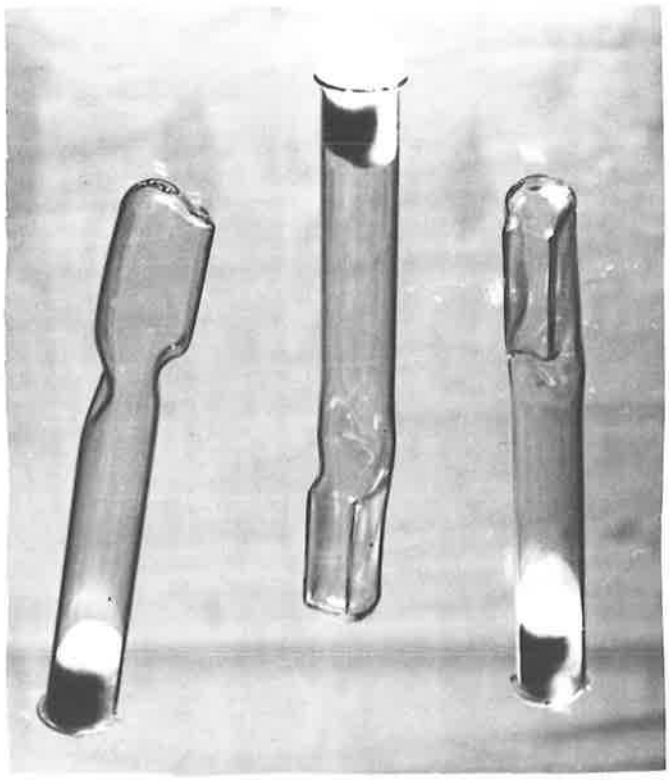


Fig 5. Leighton tubes in which free macrophages were incubated.

were incubated at 37° to allow the macrophages to adhere to the flattened glass surface. After 60 minutes incubation the medium from each of the tubes was removed and discarded and the cells, which now were adhering to the glass, were washed with warm saline. One ml of fresh medium (Hanks solution and fresh rabbit serum in equal parts) was added to the washed cells in the Leighton tubes and these were now incubated at 37° for a further period. Two tubes with adherent cells were reserved and extracted immediately with alcohol-ether (3:1) to determine the amount of ^{14}C-labelled cholesterol ingested by the cells. An amount of ^{14}C-labelled cholesterol equal to half that taken up by the cells i.e. the amount it was estimated would be released by the cells into the medium in 20 hours, was added directly to two tubes containing control media. To the first, ^{14}C-labelled cholesterol suspension was added while to the second was added ^{14}C-labelled cholesterol dispersed with Tween 20. The two controls together with the tubes containing macrophages and medium were incubated at 37°. After various time intervals (up to 72 hours) the media were removed from the tubes containing cells, centrifuged to remove debris and studied by comparing them with the control media in the following three ways:

(1) The protein fractions in the media were separated and examined by paper electrophoresis.

(2) An aliquot of each medium was extracted directly with

alcohol-ether (3:1) and the ^{14}C-labelled free and ester cholesterol content of the lipid extract determined after chromatography by scintillation counting. The macrophages adherent to the glass incubation-tubes were washed with saline and extracted with alcohol-ether (3:1) for determination of their ^{14}C-labelled free and ester cholesterol content as for the medium.

(3) A portion of each test and control medium was taken, such that each contained an equal number of counts per second. To each sample, half a ml of fresh serum was added and Hanks solution to a final volume of 2.2 ml. An equal number of fresh rabbit macrophages were added to each sample and the cells and media were then incubated with agitation at 37^{o} for 30 minutes. Following incubation the cells were deposited and extracted as described above and the amount of ^{14}C-labelled cholesterol taken up by the cells from each test and control medium was measured by direct scintillation counting.

The Preparation of Homogenate of Rabbit Macrophage.

Macrophages from the peritoneal cavity of rabbits collected by a procedure already described, were packed in a graduated conical centrifuge tube by centrifuging at 220 g for 3 minutes and resuspended in ten times the packed cell volume of 0.2M phosphate buffer at pH6.

The pH was checked using a model C. Jones pH Electrometer. The cells were homogenized by one of the following three methods:- In the first, the suspended cells were agitated in the vibrating flask of a Mickel's homogenizer containing glass sand previously wetted with buffer solution; in the second method, a high speed, bladed rotary micro-blender was used; in the third method, a rotating, conical, frosted all-glass homogenizer (Kontes Glass Co., Vineland, N.J.) was used. Homogenization was carried out at 5^{o} until complete disruption of the cells had occurred as judged by microscopic examination.

Substrates for Incubation with Rabbit Macrophage Homogenate.

(1) Cholesterol-4-^{14}C Aqueous Suspension. This was prepared as already described. Two preparations were used; in the first, non-labelled cholesterol was added to the cholesterol-4-^{14}C prior to suspension while in the second, cholesterol-4-^{14}C was suspended without addition of non-labelled cholesterol. The specific activities of the two preparations were 10 and 50.1 micro curie per mg of cholesterol respectively.

(2) Lipoprotein labelled with cholesterol-^{3}H. A rabbit, fed a diet containing 0.75 gm of cholesterol and 4.5 ml of peanut oil daily for 6 weeks was given a single dose of 2.5 milli curie of ^{3}H-labelled cholesterol by intragastric intubation. Three days later the rabbit was exanguinated by cardiac puncture under ether

anaesthesia and the labelled lipaemic serum used as a source of lipoprotein. The radio-chemical composition of the lipid in the lipoprotein was verified by silicic acid chromatography (Hirsch and Ahrens, 1958) performed by Mr. N. H.Fidge. This showed that approximately 80 per cent of the ^{3}H-label was present in the cholesterol ester fraction, 18 per cent in free cholesterol and only 2 per cent in all other lipid fractions. The distribution of the cholesterol-^{3}H in the serum lipoprotein was determined by paper electrophoresis (see fig 6) and almost all of the ^{3}H-labelled cholesterol was found to have been incorporated into the beta-lipoprotein.

(3) Lecithin emulsions. Emulsions of animal lecithin and synthetic lecithin in aqueous Tween 20 solution were prepared and the fatty acid composition defined as already described. In control incubations to which lecithin was not added aqueous Tween 20 solution alone was added in its place.

(4) Sodium oleate. This was dissolved in 2.5 per cent bovine albumin solution to give a final concentration of 10 mg per ml of sodium oleate.

The Incubation of Macrophage Homogenates.

In each experiment three incubations were set up, each in stoppered tubes in duplicate. All contained 0.3 ml of a 1 in 10 macrophage homogenate in 0.2 M phosphate buffer of pH6, 0.1 ml of

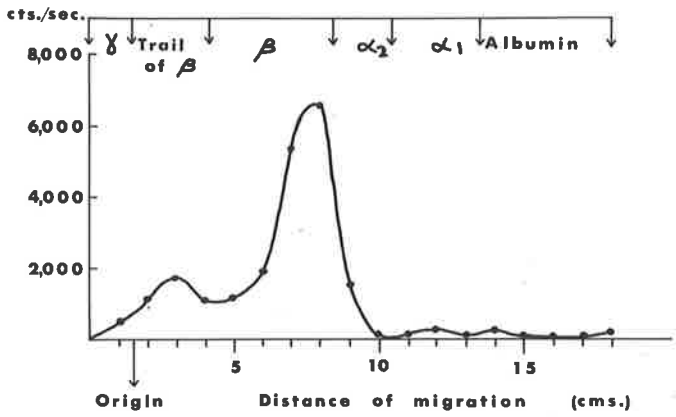


Fig 6. The distribution of ^{3}H-labelled cholesterol in serum lipoprotein as shown by paper electrophoresis. The serum is from a rabbit given cholesterol-^{3}H by gastric intubation.

sodium oleate solution (10 mg per ml) together with a known amount of cholesterol-4-^{14}C suspension or ^{3}H-labelled lipoprotein. In addition, 0.1 ml of animal lecithin suspension, or of synthetic lecithin suspension, was added to two of the incubations in each experiment, the third serving as control. Penicillin and streptomycin were added. Two series of experiments were carried out. In the first, a known amount, approximately 8×10^{-2} micro curie (109 to 170 counts per second by G.M. counting) of cholesterol suspension of specific activity 10 micro curie per mg was used and the lecithin prepared by Method A described earlier(page 41). Incubation was carried out at 37^{o} for 20 hours after which the incubation was stopped and the labelled free and ester cholesterol extracted with acetone-ethanol followed by petroleum spirit phase separation (page 43). The labelled free and ester cholesterol in the extracts were separated on alumina columns after the addition of carrier cholesterol and the labelled free and ester cholesterol in the eluates counted by plating on aluminium planchettes and counting at infinite thinness using a thin mica end-window G.M. tube.

In the second series of experiments a known amount approximately 8×10^{-2} micro curie (2200 to 2500 counts per second by scintillation counting) of cholesterol suspension (specific activity 50.1 micro curie per mg) was added and the

lecithin suspensions prepared by Method B was used. After incubation the lipid was extracted using 2:1 chloroform-methanol. Following separation of the extracts on alumina columns the free and ester cholesterol were counted directly using an Ecko N664A scintillation counter.

In experiments in which the hydrolytic activity of macrophage homogenate was studied, tritiated-cholesterol labelled lipoprotein was used as a source of cholesterol ester. Incubations were set up in duplicate containing 0.3 ml of macrophage homogenate, 0.1 ml of oleate-albumin solution, 0.02 ml of 1 in 10 diluted labelled lipoprotein, together with 0.1 ml of animal or synthetic-lecithin solution prepared by Method B above. Controls were set up containing no homogenate, consisting of phosphate buffer solution, sodium oleate and dilute Tween 20 equivalent to that present in the lecithin preparations. After 20 hours incubation at 37^{o}, chloroform-methanol extracts were separated into free and ester cholesterol and estimated by scintillation counting as described above.

EXPERIMENTAL RESULTS.

THE ESTERIFICATION OF CHOLESTEROL BY RETICULO-ENDOTHELIAL CELLS

During the last thirty years there has been an increasing amount of evidence that lipid ingested by reticulo-endothelial cells is metabolized within their cytoplasm. In the preceding pages it has been related how macrophages in the peritoneal cavity, skin and lymph nodes become increasingly sudanophilic after ingesting cholesterol, suggesting that they may be accumulating cholesterol ester. This change was shown to be in fact due to the appearance of cholesterol ester by Day and French (1959) who carried out chromatographic separation and chemical analysis of the lipid from lymph nodes after the introduction of free cholesterol. In support of this evidence that cholesterol is esterified by reticulo-endothelial cells, cholesterol esterase activity has been shown to be present in homogenate of peritoneal macrophage (Day, 1960 c).

In the experiments now to be described, radio-isotope-labelled cholesterol has been used to confirm the above findings and to seek further related information.

The Esterification of Cholesterol-4-^{14}C by Reticulo-Endothelial Cells of Rat Lymph Nodes.

When finally divided particulate suspensions or emulsions are injected into the peritoneal cavity of the rat, they are absorbed through the lymph channels of the disphragm and are ingested by reticulo-endothelial cells in the thoracic lymph nodes as was demonstrated by histochemical means by French and Morris (1960).

Subsequent change in the ingested substance reflects the metabolic activity of these cells.

The results from four experiments in which cholesterol-4-^{14}C in aqueous suspension was injected into the peritoneal cavity of rats (see page 42) are given in Table III. The ^{14}C-labelled ester and free cholesterol and the specific activity of these two fractions in the thoracic lymph nodes at intervals after the injection of the ^{14}C-labelled cholesterol suspensions is shown. Four hours after injection, appreciable amounts of ^{14}C-labelled cholesterol were present in the nodes but only a trace of ^{14}C-labelled cholesterol ester. Four days after injection, the ^{14}C-labelled cholesterol ester content had increased so that the amount of labelled ester present was approximately the same as the amount of labelled free cholesterol.

The rising percentage of the total cholesterol present as ester is shown for one experiment (Exp 2 of Table III) in fig 7. The percentage ester increased progressively so that 4 days after injection 24.0 per cent of the total cholesterol in the nodes as determined by chemical assay and 46.7 per cent of the ^{14}C-labelled cholesterol in the nodes as determined by radio assay was present as esterified cholesterol. The difference in percentage ester as determined by the two methods is partly due to the free cholesterol present in the uninjected nodes and partly due to the rapid fall in the specific

TABLE III ESTERIFICATION OF CHOLESTEROL-4-^{14}C FOLLOWING ITS UPTAKE BY RETICULO-ENDOTHELIAL CELLS IN RAT LYMPH NODES

Four Experiments. Mean of eight observations, together with the estimated standard error.

Exp N°	Cholesterol-4-^{14}C suspension injected			Radioassay (cts/sec in sternal nodes)		Specific activity (cts/sec/mg cholesterol)	
	Amount (cts/sec per rat)	Specific activity (cts/sec/mg)	Time after injection	Ester cholesterol	Free cholesterol	Ester cholesterol	Free cholesterol
1	500	60	4 hrs	... ...	10.5 ± 2.32	... ...	21.0 ± 0.72
			4 days	3.3 ± 0.67	3.7 ± 0.50	21.6 ± 2.12	9.2 ± 1.20
2	500	50	4 hrs	0.07 ± 0.01	8.71 ± 1.52	... ± ...	20.3 ± 3.03
			24 hrs☆	2.86 ± 0.35	13.97 ± 1.15	17.4 ± 0.91	15.9 ± 0.66
			4 days	4.95 ± 1.04	5.31 ± 0.69	32.1 ± 3.22	11.3 ± 1.36
3	750★	80	4 hrs	0.33 ± 0.03	12.03 ± 0.80	... ...	26.4 ± 0.87
			24 hrs	2.76 ± 0.39	5.98 ± 0.81	25.4 ± 1.92	17.5 ± 1.66
			3 days	1.42 ± 0.17	1.94 ± 0.17	20.9 ± 1.83	6.0 ± 0.40
4	500	10000	4 hrs	0.17 ± 0.02	8.30 ± 1.06	... ...	... ...
			24 hrs	0.88 ± 0.09	5.24 ± 0.60	... ...	... ...
			4 days	0.66 ± 0.17	1.52 ± 0.26	... ...	... ...

★ Homogeneous suspension used.

☆ Seven rats only.

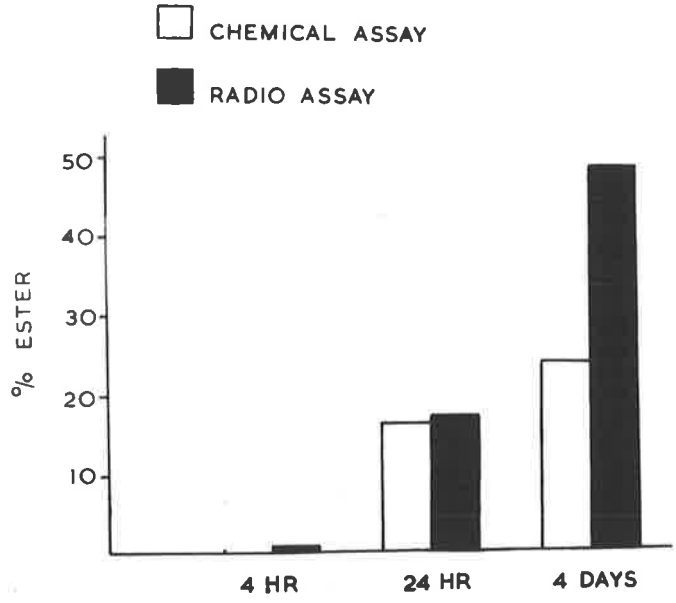


Fig 7. The percentage of the total cholesterol present as ester in rat lymph nodes at intervals after the uptake of free cholesterol-4-^{14}C.

activity of free cholesterol compared to that of the cholesterol ester.

The specific activity of the cholesterol suspensions injected in the first two experiments was 60 and 50 cts per sec per mg respectively. The specific activity of the ^{14}C-labelled cholesterol present in the nodes 4 hours after injection, was 21.0 and 20.3 cts per sec per mg. This level fell progressively so that 4 days after injection the specific activity in the two experiments was 9.2 and 11.3 cts per sec per mg respectively. In contrast to this the specific activity of the labelled ester formed remained relatively high so that at the end of 4 days the specific activity of the ester present was 2 to 3 times that of the ^{14}C-labelled cholesterol suggesting that the cholesterol is made less exchangeable by its esterification.

It has been shown that relatively large amounts of ^{14}C-labelled cholesterol ester appear in rat lymph nodes following the uptake of cholesterol-4-^{14}C. The high specific activity of the ester present in the nodes excludes the possibility that the esterification occurred in some other site with subsequent deposition of the labelled ester in the nodes. Since the cholesterol suspensions are taken up and stored within the reticulo-endothelial cells in the nodes it can be concluded that esterification of cholesterol can be brought about within these cells.

The fall in specific activity of the free cholesterol in the

nodes was not accompanied by a similar fall in specific activity of the cholesterol ester. In all experiments the specific activity of the cholesterol ester 3 to 4 days after injection of cholesterol-4-^{14}C was 2 to 3 times that of the free cholesterol. The fall in specific activity of the free cholesterol in the nodes was presumably partly due to exchange with non-labelled cholesterol and partly due to net removal of labelled cholesterol or its conversion to cholesterol ester, all of which would decrease the proportion of injected cholesterol to endogenous cholesterol. Since there is no ester present in the normal nodes there would not be a corresponding proportional alteration in the specific activity of the ester formed. However, it is doubtful whether the endogenous tissue cholesterol in the nodes (about 0.2 mg per rat) as it became an increasing percentage of the total cholesterol during the slow removal of the injected cholesterol would account for more than a small part of the difference in fall in specific activity of cholesterol and cholesterol ester. If this is so, it is reasonable to suggest the alternative that when cholesterol is esterified it becomes fixed and non-exchangeable in the cells so that its specific activity remains high, while that of cholesterol falls progressively.

The increase in specific activity of the cholesterol ester from 24 hours to 4 days in Experiment 2 Table III needs some comment;

One possible explanation is that the ^{14}C-labelled suspension and the non-labelled suspension, which were mixed prior to injection, did not behave homogenously. If the non-labelled suspension were taken up faster than the labelled suspension (due to differences in physical characteristics) the specific activity of the cholesterol and consequently of the ester formed would rise during the later absorption of the labelled preparation. The high specific activity would be maintained by the ester if it is non-exchangeable, whereas that of the cholesterol would be reduced by exchange with non-labelled cholesterol in the blood and lymph. However where a homogenous suspension was used in which the radio-tracer and carrier cholesterol were mixed prior to suspension (Experiment 3) there was no rise in ester specific activity but the other findings were similar to the previous experiments and consistent with the suggestion that cholesterol ester becomes fixed in the reticulo-endothelial cell.

The Esterification of Cholesterol-4-^{14}C by Macrophages from the Peritoneal Cavity of Rabbits.

Having confirmed that reticulo-endothelial cells in lymph nodes esterify ingested cholesterol, experiments were performed to see whether reticulo-endothelial cells from another source also possess this capacity.

Injection of liquid paraffin into the peritoneal cavity of

the rabbit causes a sterile irritation and an outpouring of inflammatory cells most of which are free macrophages. These cells can be conveniently collected as described on page 46.

A known number of rabbit peritoneal macrophages were incubated in 20 ounce McCartney bottles with media containing a known amount of cholesterol-4-^{14}C in aqueous suspension. After incubation for an hour the medium was replaced by fresh medium containing no cholesterol. At this stage one flask was set aside to provide a control both of the amount of cholesterol taken up by the cells and the percentages ester initially in the cells. The remaining flasks were incubated for 20 hours after which time the lipid was extracted from the cells and from the media and assayed after separation into free and ester cholesterol. Details of the procedure are given on page 48.

The results of one such experiment is reported in full in Table IV. In this experiment 1500 counts per second of ^{14}C-labelled cholesterol suspension was added to the media in the three McCartney bottles.

TABLE IV. COUNTS PER SECOND OF CHOLESTEROL-4-^{14}C AS ESTER OR FREE CHOLESTEROL IN THE CELLS AND IN THE MEDIUM AFTER INCUBATION OF RABBIT MACROPHAGES CONTAINING CHOLESTEROL-4-^{14}C.

Detailed data from one experiment

Experimental details	Incubation Flask No.	Incubation Time (hr)	Cells (cts per sec)		Medium (cts per sec)	
			Ester Chole	Free Chole	Ester Chole	Free Chole
$54x10^6$ cells	1	1	0.07	39.2	...	...
	2	20	1.57	20.54	0.34	9.30
	3	20	1.31	18.24	0.34	9.95
Control (no cells)	4	20	...	...	0.27	21.5

After incubation for 1 hour the medium was removed from the first flask and the uptake of the ^{14}C-labelled cholesterol by the cells was determined. 39.2 counts per second of cholesterol-4-^{14}C was found to have been taken up by the cells but no ^{14}C-labelled ester was detected. The incubation of the duplicate second and third flasks, in which the cells now contained about 40 counts per second of ingested 4-^{14}C-cholesterol, was continued for 20 hours with fresh, non-labelled medium. After this time the ^{14}C-labelled cholesterol ester in the cells was found to have increased so that 1.57 and 1.31 counts per second were present as ester. Approximately 10 counts per second of the ^{14}C-labelled cholesterol also appeared in the medium after 20 hours incubation presumably by breakdown of dead cells or by exchange with the non-labelled serum cholesterol in the medium. This accounted for about 30 per cent of the original ^{14}C-labelled cholesterol taken up by

the cells but in the media only 0.34 counts per second was present as ester. In the control, which contained no cells, 21.8 counts per second of cholesterol-4-^{14}C was present but only 0.27 counts per second was converted to cholesterol ester after 20 hours incubation.

Table V gives the percentage of the cholesterol-4-^{14}C present in the ester form in three experiments.

TABLE V. PERCENTAGE ESTERIFICATION OF CHOLESTEROL-4-^{14}C BY MACROPHAGES IN VITRO

Experiment	Number of cells	Uptake of cholesterol-4-^{14}C (Cts/sec)	Incubation time (hours)	Percentage cholesterol-4-^{14}C present as ester in the cells and media	
				Cells	Medium
1	63 x 10^6	36.1	1	0.3	...
			20	7.7	3.4
2	54 x 10^6	39.3	1	0.2	...
			20	6.9	3.4
	0 (control)	...	20	...	1.2
3	60 x 10^6	55.9	1	0.1	...
			20	5.6	3.2
	0 (control)	...	20	...	1.1

In the cells the percentage of the cholesterol present as ester rose from a mean of 0.2 per cent one hour after incubation to a mean of 6.7 per cent 20 hours after incubation. In the media a mean of only 3.3 per cent esterified ^{14}C-labelled cholesterol was detected. Controls in which no cells were present, but an

approximately equivalent amount of ^{14}C-labelled cholesterol was added, showed only 1.1 and 1.2 per cent esterification in the 20 hours incubation period. This suggests that most of the esterification in the media occurred within the macrophages.

A time run was carried out in which rabbit peritoneal-macrophages were incubated with medium containing serum. This was performed in Leighton tubes into which the cells were dispensed after having taken up cholesterol-4-^{14}C suspension. At time intervals during the incubation period pairs of the tubes were taken down and the lipid in the cells and media extracted, separated into free and ester cholesterol by chromatography and the radio-activity of each sample assayed.

The results are given in Table VI. The results of two similar experiments are presented in which 1 and 1.5 million rabbit peritoneal-macrophages in each tube had ingested and contained initially 200 to 400 and 1000 counts per second of cholesterol-4-^{14}C respectively.

TABLE VI. THE ESTERIFICATION OF INGESTED CHOLESTEROL-4-^{14}C BY RABBIT MACROPHAGES INCUBATED IN VITRO.

First Experiment

Incubation Time (hrs)	Cholesterol in cells: Total (cts/sec)	Cholesterol in cells: Percentage ester	Cholesterol in media: Total (cts/sec)	Cholesterol in media: Percentage ester
1	238	4.6	...	...
	216	4.2	...	...
4	378	6.6	17	12.1
	299	7.4	13	19.4
10	225	8.4	11	31.6
	239	8.8	18	31.3
24	162	9.0	28	40.0
	132	8.3	24	40.4
48	217	15.0	195	75.0
	137	15.6	...	83.0

Second Experiment

Incubation Time (hrs)	Cholesterol in cells: Total (cts/sec)	Cholesterol in cells: Percentage ester	Cholesterol in media: Total (cts/sec)	Cholesterol in media: Percentage ester
1	1106	3.1	24	...
	1121	2.8	25	...
4	932	8.0	66	27.3
	937	5.0	59	27.2
8	676	10.3	196	30.4
	781	9.9	106	14.7
24	351	10.8	578	58.6
	481	11.5	534	63.8
48	313	9.3	559	76.4
	258	10.6	580	75.0

As can be seen the percentage esterification in the cells continued to increase rapidly for 8 to 10 hours after which it increased much more slowly. The medium shows a more steady rise in the percentage ester present concurrent with the release of cholesterol from the cells. Part of the esterification of the cholesterol in the medium was undoubtedly brought about by the esterase in the serum in the medium - the serum having been added to obtain maximal viability of the cells during their prolonged cultivation.

It was shown in the last experiment that the rising percentage of ester in the cells cultured in this way is not due to the uptake of ester from the medium; in fact, cholesterol escaping from these cells is not readily taken up a second time by the cells, as will be shown by experiments to be described later.

The Esterification of Cholesterol-4-^{14}C by Homogenate of Rabbit Peritoneal Macrophages

Homogenates of macrophages in phosphate buffer at pH 6 were incubated together with cholesterol-4-^{14}C and sodium oleate at 37^{o} for 20 hours. At the end of this time the free and ester cholesterol was extracted from the mixture, separated by chromatography on alumina columns and assayed for radioactivity in each fraction.

After incubating the ^{14}C-labelled cholesterol suspensions with homogenate of rabbit macrophage, 12 to 30 per cent ester was present

in five similar experiments. Table VII shows the results of five further experiments in which high specific activity cholesterol suspensions were used. The cholesterol suspension contained no ^{14}C-labelled cholesterol-ester and its incubation alone for 20 hours at 37^{o} did not result in the appearance of any ester. Following incubation with the macrophage homogenate 24 to 44 per cent of the cholesterol was present in the form of ester.

The Fatty Acid Pattern of Cholesterol Ester Synthesized by Reticulo-Endothelial Cells

Having shown that reticulo-endothelial cells from two different sites esterify ingested cholesterol, the following experiments were carried out to explore the nature of the fatty acids of the cholesterol ester formed. Cholesterol in aqueous suspension was injected into the peritoneal cavity of rats and after 24 hours recovered from their thoracic lymph nodes. The lipid extract was separated into its lipid components on silicic acid columns and the fatty acids of the cholesterol ester fraction were identified by gas phase chromatography. In addition to injecting a cholesterol suspension other groups of rats had injected, together with the cholesterol, emulsions of oils containing either saturated or poly unsaturated fatty acids.

The results presented in Table VIII give the amount of ester synthesized by the nodes in all the experiments. No ester

TABLE VII. THE ESTERIFICATION OF CHOLESTEROL-4-^{14}C BY HOMOGENATE OF RABBIT MACROPHAGES

Second Series of Experiments

To each tube, 2230 counts per second of cholesterol-4-^{14}C suspension was added

Exp No	Experimental details	Incubation time	Ester cts per sec	Total cts recovered	Per cent ester
1	Control. (suspension alone)	0 hrs	5	2200	...
			1	2100	...
	"	20 hrs	1	2200	...
			2	2240	...
	Test. (suspension + homogenate)	"	1000	2270	44.0
			885	2075	42.7
2	"	"	719	2171	33.1
			680	2184	31.1
3	"	"	680	2220	30.3
			757	2188	34.8
4	"	"	776	2089	37.2
			730	2099	34.5
5	"	"	497	2166	23.0
			515	2176	23.7

TABLE VIII. THE AMOUNT OF CHOLESTEROL ESTER PRESENT IN RAT LYMPH NODES 24 HOURS AFTER THE INJECTION OF FREE CHOLESTEROL EITHER ALONE OR TOGETHER WITH CORN OR COCONUT OIL

Experiment number	Number of Rats	Amount of ester (mg)	Percentage ester
		CHOLESTEROL ALONE	
B6	28	1.04	12
B7	29	2.28	18
B17	24	2.77	16
B21	21	2.04	16
B22	19	1.84	20
		CHOLESTEROL WITH CORN OIL	
B9	25	2.01	24
B10	25	1.90	18
B11	20	1.01	25
B14	18	1.43	46
		CHOLESTEROL WITH COCONUT OIL	
B13	22	1.88	25
B15	25	1.89	25
B16	24	2.25	29
B19	24	2.65	13

cholesterol was present in the nodes prior to their uptake of free cholesterol but the percentage ester present rose following cholesterol uptake to a mean of 16 per cent in the group receiving cholesterol alone. When either corn oil or coconut oil was given with the cholesterol the percentage esterification was higher than when cholesterol was given alone. The fatty acid composition of the corn and coconut oil used is shown in Table IX.

Table X gives the percentage distribution of fatty acids from cholesterol esters synthesized by the nodes under the three experimental conditions. In the group receiving cholesterol alone the principal fatty acids esterified with the cholesterol were palmitic, oleic, and linoleic, but predominantly palmitic. Where corn oil was given with the cholesterol, the same three fatty acids predominate but considerably more oleic acid and linoleic acid were present. Although linoleic acid was the main fatty acid in the corn oil given, in the cholesterol ester formed, oleic acid was predominant.

Where coconut oil (containing a high proportion of lauric acid) was given, the fatty acids of the cholesterol esters became still more saturated and of shorter chain length. Less linoleic acid was present than in the other two groups and a considerable amount of myristic acid appeared in the cholesterol ester fatty acid. Little increase in the lauric acid was apparent. Figure 8 shows the proportion of saturated, monounsaturated and polyunsaturated fatty

TABLE IX. PERCENTAGE DISTRIBUTION OF FATTY ACIDS IN CORN OIL AND COCONUT OIL USED FOR EXPERIMENTS

	Corn Oil	Coconut Oil
12 : 0 (Lauric)	0.6	47.0
14 : 0 (Myristic)	0.6	26.7
16 : 0 (Palmitic)	20.6	12.4
16 : 1 (Palmitoleic)	...	...
18 : 0 (Stearic)	1.3	3.4
18 : 1 (Oleic)	30.1	8.4
18 : 2 (Linoleic)	46.7	1.9
18 : 3 (Linolenic)	trace	...
20 : 4 (Arachidonic)	...	...

TABLE X. PERCENTAGE DISTRIBUTION OF FATTY ACIDS IN CHOLESTEROL ESTERS SYNTHESIZED BY RETICULO-ENDOTHELIAL CELLS

(mean of four or five experiments together with the estimated standard error is shown)

Fatty acid	cholesterol alone (mean of five experiments)	cholesterol + corn oil (mean of four experiments)	cholesterol + coconut oil (mean of four experiments)
12 : 0 (Lauric)	0.7 ± 0.2	0.7 ± 0.1	1.6 ± 0.3
14 : 0 (Myristic)	4.2 ± 1.0	1.1 ± 0.2	14.4 ± 3.3
16 : 0 (Palmitic)	39.1 ± 4.4	21.0 ± 0.9	28.9 ± 1.3
16 : 1 (Palmitoleic)	8.1 ± 1.7	5.6 ± 0.6	5.6 ± 0.4
18 : 0 (Stearic)	5.2 ± 0.5	4.6 ± 1.2	6.6 ± 0.7
18 : 1 (Oleic)	28.7 ± 5.2	42.6 ± 1.3	31.3 ± 1.5
18 : 2 (Linoleic)	10.7 ± 1.5	20.4 ± 0.7	7.3 ± 1.1
18 : 3 (Linolenic)	... ...	..trace..	... ...
20 : 4 (Arachidonic)	3.1 ± 0.8	3.9 ± 1.4	4.4 ± 1.3

% DISTRIBUTION OF MONO, DI, AND POLY UNSATURATED FATTY ACID IN CHOLESTEROL ESTER AFTER THE UPTAKE OF CHOLESTEROL BY R.E. CELLS

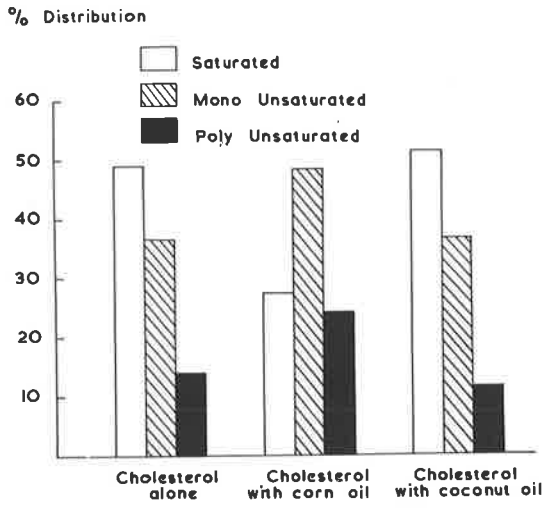


Fig 8.

acids ester under the three conditions. In the nodes in which cholesterol alone was taken up 49.3 per cent of the cholesterol-ester fatty-acids was saturated and only 13.9 per cent poly-unsaturated. There was little difference in this pattern when coconut oil was administered, although a higher proportion of the saturated fatty acids were shorter chain acids. Where corn oil was injected only 27.4 per cent was saturated and 24.3 per cent polyunsaturated and even in this case the pattern contained less polyunsaturated fatty acids than one would expect.

The Exchange Rate of Free Cholesterol Relative to that of Ester Cholesterol in the Aorta of the Living Rabbit

In experiments presented it has been shown that when reticulo-endothelial cells in rat lymph nodes esterify ingested cholesterol it becomes less freely exchangeable. The present experiments were performed to study the atheromatous arterial wall where there is a continual exchange of cholesterol with the plasma (Newman and Zilversmit, 1959: Schwenk and Stevens, 1960) and where reticulo-endothelial cells are abundant. The purpose was to discover whether in atheromatous aorta, cholesterol ester is less exchangeable than free cholesterol. This was done by measuring the specific activity of labelled-cholesterol, free and ester, in aorta and serum at intervals after introducing labelled cholesterol. Live, cholesterol-fed

rabbits were used and were given 500 micro curies of tritiated cholesterol in olive oil with sodium taurocholate, administered by gastric intubation.

At various intervals after the intubation, blood was collected from the marginal ear vein and the changing specific activity in the serum estimated. The animal was finally killed and the terminal specific activity of the aorta free and ester cholesterol was calculated by homogenizing and extracting the aorta in a Kontes all-glass homogenizer with chloroform-methanol, followed by separation of free and ester cholesterol and radio and chemical assay of the fractions.

In all the experiments performed the specific activity of the plasma cholesterol ester increased more rapidly, rose higher and fell more rapidly than that of the free cholesterol. In one experiment the fall of the specific activity of the ester cholesterol overtook that of the free and in the remaining experiments the curves suggest that a similar event would have occurred if the experiments had been extended in duration. Figure 9 shows in graph form the results from four representative experiments. Contrasted with the similarities in pattern is the striking difference between rabbits in velocity of change. The rate and amount of the intubated cholesterol absorbed from the gut varied greatly from one animal to another and so did the

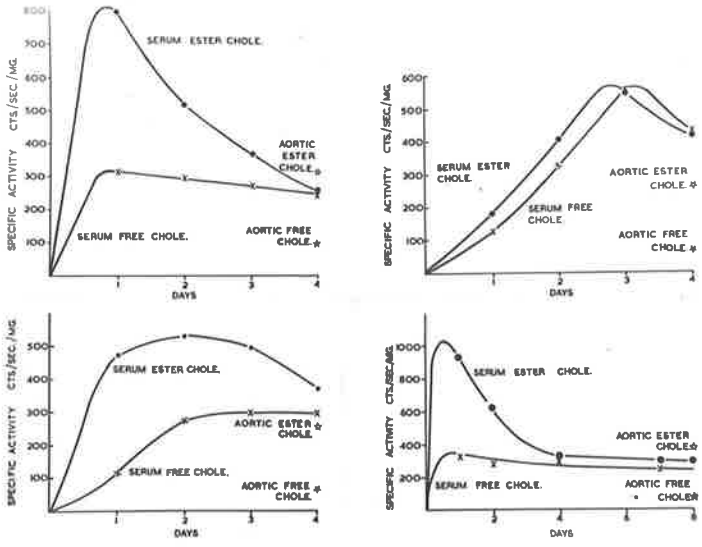


Fig 9. The specific activity of free and ester cholesterol in the serum and aorta of cholesterol-fed rabbits after the gastric intubation of tritiated cholesterol.

percentage of the cholesterol subsequently esterified and also the rate at which both the free and the ester cholesterol were excreted.

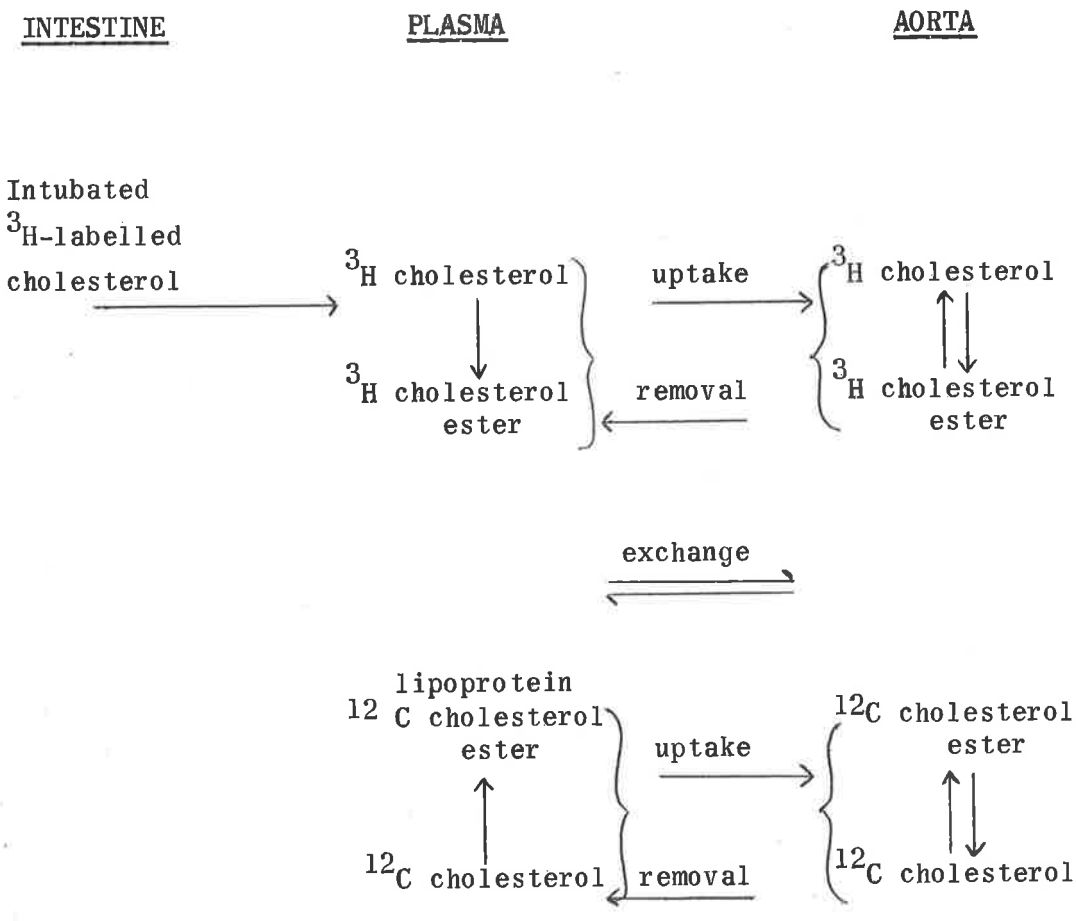
Also shown in fig 9 is the specific activity of the free and the ester cholesterol in the aorta on the day on which the animals were killed. In all experiments the specific activity of the ester cholesterol was higher than that of the free. There was however no constant ratio of ester to free specific activity in the aorta of different animals and there was no constant relationship between the specific activities in the aorta and in the serum.

The dissimilarities between each animal in the velocity and amplitude of changes in the serum and the lack of obvious or constant relationship between aortic and serum patterns, ruled out any comparison between different animals and the drawing of a composite graph in which the rate of change of specific activities of free and ester cholesterol in the aorta could be followed, superimposed on a background picture of changing serum specific activities.

In all experiments the specific activity of the aorta ester cholesterol was higher than the free at the time of death but there is no indication whether the isolated values determined were situated on a rising or falling curve of changing specific activity. Further, since the free and ester cholesterol were taken up from the serum

during the experiment at continually changing specific activity, another unknown variable was introduced. Lastly, once the serum free and ester cholesterol had infiltrated the aortic wall in these experiments not only "exchange" occurred but also net removal back into the circulation and possibly hydrolysis or further esterification of the cholesterol was occurring in the wall (fig 10). Because of this large number of variables and the lack of a single fixed value on which to found a formula, no conclusions on the relative exchange rate of free and ester cholesterol in the aorta could be derived from this experiment.

Fig 10. SCHEME SHOWING FACTORS RESULTING IN VARIABLE LEVELS OF RADIOACTIVE LABELLED AND UNLABELLED CHOLESTEROL, FREE AND ESTER, IN THE PLASMA AND AORTA AFTER GASTRIC INTUBATION OF ^{3}H CHOLESTEROL.



Discussion

The experiments described so far have shown the following facts. Reticulo-endothelial cells in rat lymph nodes and macrophages from the rabbit peritoneum partially esterify ingested cholesterol. Macrophage homogenate was also shown to bring about esterification of cholesterol and a time run showed that this esterification under the experimental conditions used is a relatively slow process, preliminary steps before examining the enzyme system involved in this process.

Results of experiments by other workers support the findings described here that the esterification of cholesterol produces a decrease in its exchange rate between tissues and plasma and include the following:

(1) After prolonged tracer-cholesterol feeding of an animal with xanthoma, the ratio of the specific activities in the xanthoma to that in the serum is greater for free cholesterol than for esterified cholesterol (Wilson, 1963). The cholesterol in the xanthoma is mainly within reticulo-endothelial cells.

(2) The esterified cholesterol in plasma lipoprotein is not easily exchangeable as is the free cholesterol in the lipoprotein. Radio-active labelled cholesterol ester incubated with plasma lipoprotein in vitro does not exchange with the lipoprotein ester cholesterol (Roheim, Haft, Gidez, White and Eder, 1963) while

radio-active free cholesterol similarly incubated is rapidly exchanged with and labels the lipoprotein cholesterol; this is followed by its esterification (Whereat and Staple, 1960).
(3) After the intravenous injection of ^{14}C-labelled cholesterol or mevalonate, an early precursor in the synthesis of cholesterol, the specific activity of free cholesterol in the liver equals that of the plasma in one hour, while the specific activity of ester cholesterol in the liver does not rise to that of the plasma for 12 hours (Sugano and Portman, 1964).

The fact that esterification of cholesterol decreases the isotopic exchange-rate of the cholesterol between compartments does not necessarily mean that the removal rate is also decreased. However, if the esterification of cholesterol does decrease its removal rate, then this would result in and explain a decreased exchange rate.

It is not easy to demonstrate whether reticulo-endothelial cells in the atheromatous artery esterify cholesterol. No specific stain is known for histochemical demonstration of this fact. An attempt has been made in this laboratory to examine the cholesterol esterase activity of the whole arterial wall but has not been described fully because the experimental work is not yet completed. Briefly, the experiments

consisted of incubating pieces of aorta from cholesterol-fed rabbits in a physiological medium with cholesterol-4-^{14}C. No esterification of the cholesterol was observed under the conditions used. On the other hand, when 4-^{14}C-labelled cholesterol oleate was taken up by the aortic pieces considerable hydrolysis occurred as compared with controls. The possible significance of this will be discussed in a later section.

If esterification or hydrolysis of cholesterol and its esters occurs in the atheromatous aorta, whether or not the foam cells are involved in the process, a change in exchange rate with the plasma cholesterol and possibly a change in ease of removal from the tissue may result. An attempt has been made and described to discover whether this is in fact so. The specific activity of free and ester cholesterol in the aorta was measured after feeding labelled cholesterol. However it was not found possible to assess the relative exchange rate of free and of ester cholesterol in the aorta as was intended. A background of changing serum specific activities and many other variables were involved and obscured the significance of the experimental findings.

The fatty acid pattern of cholesterol ester synthesized by reticulo-endothelial cells in rat lymph nodes was shown, in experiments just described, to be predominantly saturated.

The fatty acid patterns of cholesterol ester in the tissues tend to be more saturated than that of the serum. Rat liver cholesterol ester has been shown to have a pattern similar to that shown here in reticulo-endothelial cells (Swell, Law, Field and Treadwell, 1960) while up to 70 per cent of the cholesterol ester fatty acids of serum of normal rats is polyunsaturated. This latter figure is in marked contrast to the 13.9 per cent polyunsaturated fatty acid in the cholesterol ester synthesized by reticulo-endothelial cells. This is not merely a reflection of the type of fatty acid available for synthesis because even where, as here, large amounts of polyunsaturated fatty acid were present, the amount of polyunsaturated fatty acid in the cholesterol ester was only elevated to 24 per cent of the total. This implies that cholesterol may be selectively esterified with "more-saturated" fatty acids by reticulo-endothelial cells.

In atherosclerosis in both the human and the rabbit the plasma cholesterol ester fatty acids differ from those of the early plaque in that the latter contain more-saturated fatty acids (Zilversmit, Sweeley et al 1961; Tuna et al, 1958; Lewis, 1958; Bottcher et al, 1960; Swell, Field et al, 1960). There are two possible sources of this predominantly saturated cholesterol ester in the early atheromatous lesion. The first is from plasma lipoprotein-cholesterol infiltrating the arterial wall

and being esterified (or alternatively plasma ester-cholesterol infiltrating and being transesterified). The second possible source is from cholesterol synthesized de novo in the arterial wall. Field, Swell, Schools and Treadwell (1960) have shown cholesterol to be synthesized in the arterial wall and to account for 30 to 40 per cent of the cholesterol present in the lesion. Cholesterol and fatty acid can also be synthesized by reticulo-endothelial cells as discovered by Day and Fidge (1964) who showed that macrophages in vitro synthesized lipids from ^{14}C-labelled acetate, including cholesterol and fatty acid.

Whatever the source of the cholesterol in atheroma, the reticulo-endothelial cells in the lesion, which as has been stated earlier contain much of the cholesterol, may be involved in the esterification of the cholesterol. Reticulo-endothelial cells have been shown in these experiments to esterify cholesterol with predominantly saturated fatty acids just as the cholesterol ester of fatty acids in the atheromatous artery is also known to be predominantly saturated. It is tempting to postulate that this similarity is due to reticulo-endothelial cells being involved in the esterification in the arterial wall.

Deykin and Goodman (1962) and Pinter, Miller and Hamilton (1964) have shown that cholesterol esters are hydrolyzed less

rapidly by cholesterol esterase when they are esterified with saturated, as opposed to unsaturated, fatty acids. This may result in a rising percentage of esterified cholesterol in tissues and cells where cholesterol esterase is present and a tendency for cholesterol to accumulate if ester-cholesterol is less readily removed from the tissues than free cholesterol.

The consequence of increasing saturation of the cholesterol ester fatty acid in the arterial wall which it has been suggested here may be brought about by reticulo-endothelial cells, is possibly, as Sinclair (1956) has shown to be the case in the skin, an accumulation of the cholesterol in the artery, due to its decreased rate of catabolism and decreased rate of removal.

SUMMARY

Reticulo-endothelial cells from two sites were shown to esterify ingested cholesterol. The esterification of cholesterol was found to decrease its exchange rate and an attempt was described to discover whether esterification of cholesterol in the arterial wall also results in a decreased exchange rate.

The fatty acid pattern of cholesterol esterified by reticulo-endothelial cells in rat lymph nodes was found to contain predominantly saturated fatty acids. This was so even when polyunsaturated fatty acids were taken up with the cholesterol suggesting that reticulo-endothelial cells selectively esterify cholesterol with more saturated fatty acids.

THE EFFECT OF PHOSPHOLIPID ON CHOLESTEROL-ESTERASE ACTIVITY OF RETICULO-ENDOTHELIAL CELLS

INTRODUCTION

The association between phospholipid, cholesterol, macrophages and atheroma is described here in some detail because, as will become apparent, they are functionally related in many ways and the relationship is particularly relevant to this thesis.

Phospholipid and plasma cholesterol. Lipid, insoluble in aqueous medium, is maintained in suspension in the plasma by protein (Ludlum, Taft and Nugent, 1929) which may form a coat on the lipid particle or a core within it (Cook and Martin, 1962). Phospholipid is necessary to maintain this suspension (Ahrens and Kunkel, 1949).

Sera with a high total lipid content are optically clear if the phospholipid level is predominant over the neutral fat level but, if not, the serum shows a milky lipaemia (Ahrens and Kunkel, 1949). Lecithin, the major plasma phospholipid and the most active, is a surface-active agent which stabilizes oil in water emulsions (Seifriz, 1923). In the serum the lecithin probably binds with the protein and enmeshes cholesterol and triglyceride in the expanded hydrophobic fatty-acid portion of its molecule (Ahrens and Kunkel 1949).

Ahrens and Kunkel also suggested in 1949 that the ratio between plasma phospholipid and cholesterol (P/C ratio) may be

implicated in the pathogenesis of atheroma. In certain diseases characterized by hyperlipaemia and in which atheroma is predisposed a P/C ratio of less than one is found while in diseases associated with hypercholesterolaemia, but a P/C ratio of more than one, there is no such predisposition. Administration of oestrogens to men raises the P/C ratio. Oestrogen may therefore be responsible for the lower incidence of coronary disease in premenopausal women who have a high natural level of circulating oestrogen. Later studies (Jackson and Wilkinson, 1952) have tended to decry significance of the P/C ratio as an aetiological factor in atheroma and as a diagnostic index. There is nevertheless support for the theory that a relationship exists between the serum phospholipid level, stabilization of lipids in the serum and their fixation in the intima of the arterial wall, including the following findings:

(1) Intravenous administration of synthetic detergents - Tween 80 and Triton which increase the phospholipid cholesterol ratio reduce the incidence and severity of atheroma in cholesterol-fed rabbits (Kellner, Correll and Ladd, 1949). Further, intravenous injection of synthetic di-myristoyl lecithin (Maurukas and Thomas, 1960), brain, soya-bean or "90 per cent pure, animal-lecithin" (Byers and Friedman, 1960) but not inositol-phosphatide (Sacks,

Danielson and Leiter 1960) produce a rise in serum cholesterol (by mobilizing stores and deposits) and result in prevention or partial resolution of aortic atheroma. (2) Serum, in very small amounts, has been shown in vitro to stabilize and to slow the crystallization rate of supersaturated solutions of cholesterol in triglyceride. This effect is due to lecithin in the serum; cephalin and sphingomyelin have no such effect and lysolecithin actually increases the rate of crystallization. The slowing of crystallization is halved when the serum is taken from patients showing evidence of atherosclerotic disease (Wilkens and Krut, 1963). This finding may explain how infusion of lecithin into cholesterol-fed rabbits prevents the development of atheroma i.e. by stabilizing the high concentration of cholesterol in the plasma and preventing its precipitation and crystalline deposition in the arterial wall. Further (3) whether removal or deposition of lipid takes place in the wall may depend on whether the intracellular lipid is dispersed as invisible micellar fat or present as globular deposits. Phospholipid and protein maintain such micellar dispersion while cholesterol antagonizes their action (Dixon, 1961). Infusion of lecithin may therefore prevent crystallization of suspended cholesterol by assisting the dispersion of globular lipid deposits, thereby preventing the

deposition and assisting in the removal of cholesterol from the arterial wall.

The plasma phospholipid is found chiefly in the alpha-lipoprotein fraction. In patients with coronary disease the alpha-lipoprotein as a percentage of the total lipoprotein has been shown to be less than half that in normal controls (Allard and Choquette, 1962). This also suggests a relationship between plasma phospholipid, cholesterol and atheroma.

Phospholipid and the esterification of cholesterol. The cholesterol in plasma is mainly in the ester form. When serum is incubated in vitro the percentage of esterified cholesterol increases due to the action of esterase normally present in the serum (Wagner and Rogalski, 1952). The addition of cholesterol to the incubation does not alter this increase in ester but addition of lecithin increases both the speed and amount of such ester formed. This effect is only brought about by certain lecithins; plant phosphatide (Wagner and Rogalski, 1952) and synthetic di-myristoyl lecithin but not di-oleyl or di-stearoyl lecithin (Wagner, 1959). Glomset (1963) showed that when human serum is incubated there is a decrement in lecithin and an increment of lysolecithin in the high density lipoprotein while the greatest cholesterol esterification occurs in the low density lipoprotein. It appears from this that lipoprotein lecithin acts

as a fatty acid donor to lipoprotein cholesterol (Glomset, 1963; Rowen and Martin, 1963) and suggests why addition of an emulsion of cholesterol (Wagner and Rogalski, 1952) or free fatty acid (Glomset, 1963) to the incubation does not enhance the reaction i.e. because the added lipid is not lipoprotein lipid.

Phospholipid in the arterial wall. Phospholipid constitutes an important part of atheromatous-plaque lipids, the nature of the phospholipids changing as atheroma develops. Sphingomyelin constitutes the major part of the plaque phospholipid (Smith, 1959) in contrast to that in the plasma where lecithin predominates and possesses a different fatty acid pattern (Böttcher et al, 1960). It has been shown that in the cholesterol-fed rabbit the phospholipid in the aortic wall increases to 4 times that normally present, to become 14 per cent of the total plaque lipid.(Zilversmit, Shore and Akerman, 1954 and Smith, 1959). At the same time sphingomyelin increases from 40 to 50 per cent of the phospholipid present to over 80 per cent (Smith, 1959).

The aorta of normal rats (Chernicks, Srere and Chaikoff, 1949), cholesterol-fed rabbits (McCandless and Zilversmit, 1956) and man (Zilversmit, McCandless et al, 1961) have been shown actually to synthesize much of the phospholipid they contain, the accumulation of phospholipid in atheroma being due to this synthesis rather than infiltration from the plasma. There is a fivefold increase

in phospholipid synthesis in the aorta of cholesterol-fed rabbits compared with the normal rabbit aorta (Zilversmit, Morris, Shore and Ackerman, 1954). Macrophages, present in the early atheromatous lesions, have been shown in vitro to synthesize lecithin and sphingomyelin from fatty acids and from acetate (Day and Fidge, 1962 and 1964).

Recently the phospholipid in the rabbit aorta (Day, 1962) and the human aorta (Dunnigan, 1964; Day and Gould-Hurst, 1964, unpublished observations) in early atheroma has, as described earlier, been shown by histochemistry to be mainly intracellular within the macrophages. These cells have taken up the accumulating cholesterol to become foam cells (see fig 11 and 12) so that the accumulation of phospholipid in the macrophages may represent a response to their ingestion of cholesterol. Phospholipid synthesis by macrophages in vitro has in fact been shown to be stimulated by cholesterol ingestion (Day and Fidge, 1956 b) (see also fig 13).

Since phospholipid is necessary for the suspension of lipid in the plasma this suggests that the synthesis of phospholipid may take part in the resuspension of cholesterol deposits and help mobilize and remove cholesterol from the arterial lesion.

Fig 11. Macrophages in the endothelium of the aorta of a rabbit fed cholesterol for 4 weeks.

Positive stain for phospholipid.

Bakers acid-haematin 400 x mag.

Frozen section.

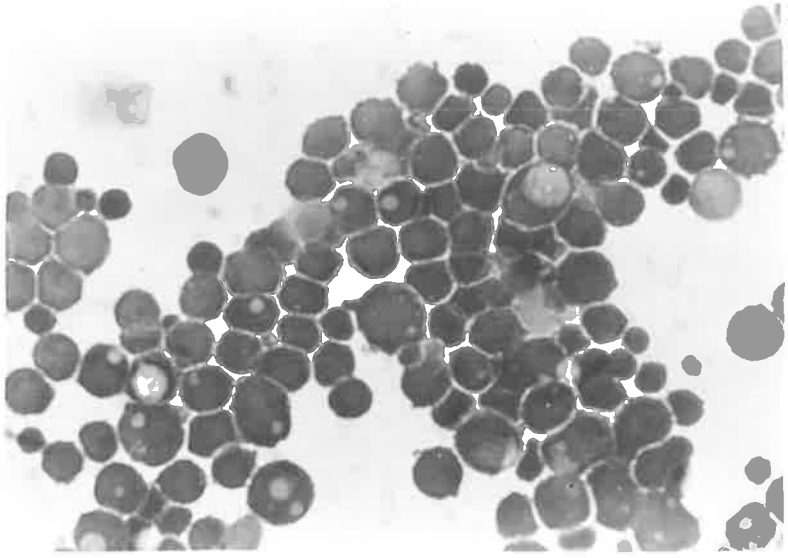


Fig 13. Macrophages from the peritoneal cavity of a rabbit 4 days after the injection of cholesterol into the peritoneal cavity. Positive reaction for phospholipid.

Bakers acid-haematin stain 400 x mag.

Smear fixed in formalin vapour.

Since lecithin stimulates the activity of cholesterol esterase in the serum the important possibility suggests itself that it may also have an influence on the cholesterol esterase of reticulo-endothelial cells. This has been investigated and the experiments and their results are described here. Reticulo-endothelial cells themselves synthesize phospholipid and increasingly so after they have ingested cholesterol. Discovering the effect of lecithin on the cholesterol esterase of these cells may therefore suggest what the relationship is between the phospholipid synthesized by macrophages and the cholesterol metabolism of the cells.

An effect of lecithin on cholesterol-esterase activity may result from either inhibition or stimulation of esterification or hydrolysis. If two separate enzymes carry out these functions in reticulo-endothelial cells then lecithin may inhibit one, stimulate the other or effect both. This was investigated using labelled lipoprotein-cholesterol which was nearly 80 per cent esterified and was prepared as described on page 53. This as a source of cholesterol ester has the advantage over artificial aqueous-suspensions of cholesterol ester of simulating the form in which cholesterol

is thought to be presented to reticulo-endothelial cells in the body.

Lecithin is known to be taken up by reticulo-endothelial cells in vivo (Sjövall, 1935) as are other phospholipids (Ferraro and Jervis, 1940; Christianson, 1941) an example of this is shown in figure 14. In the experiments to be described now, an investigation was made into the effect of lecithin on the cholesterol-esterase of reticulo-endothelial cells in rat lymph nodes and its effect on the cholesterol-esterase of rabbit macrophage homogenate, as well as on the cholesterol-esterase of rabbit serum.

The Effect of Lecithin on the Esterification of Cholesterol by Reticulo-Endothelial cells of Rat Lymph Nodes

Into the peritoneal cavity of each of a number of rats was injected a suspension of cholesterol-4-^{14}C together with either a solution of animal lecithin or, in the case of controls, an equal volume of sodium chloride solution. Lecithin or saline was injected initially with the cholesterol as just described and was injected each day subsequently until the animals were killed. Their thoracic lymph nodes were then removed, lipid was extracted from the nodes and the free and ester cholesterol

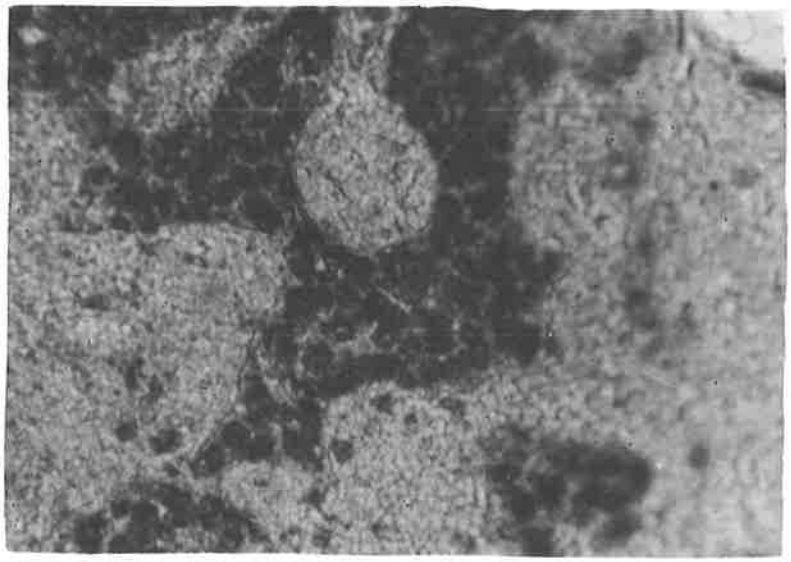


Fig 14. Reticulo-endothelial cells in a rat lymph node 8 hours after the intraperitoneal injection of animal lecithin.

Positive staining reaction for phospholipid.

Bakers acid-haematin stain 400 x mag.

Frozen section.

separated on alumina columns. These fractions were then assayed by scintillation counting and by chemical estimation.

The results of three experiments are shown in Table XI. The percentage of cholesterol-4-^{14}C in the nodes esterified at 24 hours and at 4 days after the intraperitoneal injection of labelled cholesterol are given. The table shows at both times the percentage ester present after cholesterol plus lecithin was given and that present when cholesterol was given alone.

TABLE XI

THE EFFECT OF LECITHIN ON THE ESTERIFICATION OF CHOLESTEROL-4-^{14}C BY RETICULO-ENDOTHELIAL CELLS OF RAT NODES

Experiment number	Time after injection	Percentage Ester* Cholesterol Alone	Cholesterol + Lecithin
38	24 hrs	25.9 ± 2.00	24.0 ± 1.56
	4 days	47.9 ± 1.23	32.4 ± 3.06
43	24 hrs	... ...	... ...
	4 days	43.6 ± 2.34	22.6 ± 2.74
46	24 hrs	31.0 ± 1.68	23.3 ± 2.47
	4 days	47.2 ± 2.74	39.4 ± 1.92

* Each figure represents the mean of 8 rats, together with standard error.

The lecithin is seen to have caused a decrease in the percentage esterification as compared with the controls. By four days this difference has become very marked.

The esterification of cholesterol by reticulo-endothelial cells

in lymph nodes was earlier shown to decrease its rate of exchange. Observations were now made to see whether lecithin altered this effect. Table XII shows the specific activity of free and of ester cholesterol in the nodes at intervals after the uptake of cholesterol-4-^{14}C. Where cholesterol was injected alone the specific activity of the ester cholesterol in the nodes did not decrease rapidly as did that of free cholesterol. Where lecithin was injected with the cholesterol the specific activity of the cholesterol ester decreased similarly to the free cholesterol. This suggests that lecithin increases the exchange rate of cholesterol ester.

Table XIII shows the rate of removal of free and of ester cholesterol from the nodes and the effect of lecithin on the removal rate when it was injected with the cholesterol. The removal rate of ester cholesterol but not free cholesterol from the nodes appears to have been increased by the addition of lecithin. This complements the finding that lecithin increases the exchange rate of ester cholesterol. However, lecithin has been shown earlier to decrease the esterification of cholesterol by reticulo-endothelial cells so that the apparent increase in removal rate of cholesterol ester shown here may in fact be due to a decrease in the rate of esterification of cholesterol.

No conclusion can therefore be drawn from this finding.

TABLE XII. THE SPECIFIC ACTIVITY OF FREE AND ESTERIFIED CHOLESTEROL IN RAT LYMPH NODES AFTER THE UPTAKE OF CHOLESTEROL-4-^{14}C AND THE EFFECT OF ANIMAL LECITHIN ON CHANGE IN THE SPECIFIC ACTIVITY

Exp N°	Group	Time	SPECIFIC ACTIVITY: Ester Cholesterol counts per second per mg	Ester Cholesterol percentage fall from 1st to 4th day	Free Cholesterol counts per second per mg	Free Cholesterol percentage fall from 1st to 4th day
38	cholesterol alone	24 hrs	28.8 ± 1.49	10.7	23.4 ± 0.72	72.2
		4 days	25.7 ± 4.1		6.5 ± 0.31	
	cholesterol + lecithin	24 hrs	28.7 ± 1.37	62.4	19.9 ± 1.68	72.4
		4 days	10.8 ± 1.90		4.50 ± 0.50	
46	cholesterol alone	24 hrs	26.5 ± 1.27	- 2.64	20.8 ± 1.16	51.7
		4 days	27.2 ± 1.33		8.99 ± 0.48	
	cholesterol + lecithin	24 hrs	22.9 ± 1.38	21.8	20.0 ± 0.89	75.0
		4 days	17.9 ± 1.16		5.4 ± 0.67	
67	cholesterol alone	24 hrs	33.0 ± 1.58	....	34.0 ± 0.98	44.4
		4 days	✱17.0 ± 0.81		8.90 ± 0.56	
	cholesterol + lecithin	24 hrs	33.0 ± 1.65	59.2	28.0 ± 1.05	41.3
		4 days	14.5 ± 1.37		6.40 ± 0.99	

✱This figure is low because of high chemical assay figure for two rats in the group. This has resulted in the large apparent percentage fall in specific activity between 24 hours and 4 days.

TABLE XIII. THE REMOVAL OF ^{14}C-LABELLED FREE AND ESTER CHOLESTEROL FROM RETICULO-ENDOTHELIAL CELLS OF RAT LYMPH NODES AFTER THE INGESTION OF CHOLESTEROL-4-^{14}C ALONE OR TOGETHER WITH ANIMAL LECITHIN

Exp N°	Group	Time	Ester Cholesterol		Free Cholesterol	
			counts per second	percentage removed between 1st & 4th day	counts per second	percentage removed between 1st & 4th day
38	cholesterol alone	24 hrs	3.51 ± 0.26	61.8	10.27 ± 0.79	86.2
		4 days	1.34 ± 0.21		1.41 ± 0.16	
	cholesterol + lecithin	24 hrs	2.35 ± 0.46	75.9	7.49 ± 1.30	85.5
		4 days	0.57 ± 0.12		1.08 ± 0.14	
46	cholesterol alone	24 hrs	3.80 ± 0.57	49.4	8.46 ± 1.08	77.3
		4 days	1.93 ± 0.27		1.92 ± 0.15	
	cholesterol + lecithin	24 hrs	2.53 ± 0.56	68.6	7.46 ± 1.79	85.8
		4 days	0.79 ± 0.13		1.20 ± 0.14	
67	cholesterol alone	24 hrs	3.96 ± 0.25	55.5	17.8 ± 1.56	84.8
		4 days	1.79 ± 0.32		2.71 ± 0.28	
	cholesterol + lecithin	24 hrs	4.43 ± 0.61	71.0	14.8 ± 1.80	87.8
		4 days	1.26 ± 0.15		1.85 ± 0.29	

Free and ester cholesterol figures are the average for a group of 8 rats and are given together with the standard errors for the group.

Having shown the effect of lecithin on reticulo-endothelial cells in lymph nodes, the effect of lecithin on cholesterol-esterase activity of macrophages from the rabbit peritoneal cavity was investigated.

The Effect of Lecithin on the Esterification of Cholesterol by Homogenate of Rabbit Macrophage and by the Cholesterol-Esterase of Rabbit Serum

Homogenate of macrophage suspended in phosphate buffer at pH_6 was incubated for 20 hours with cholesterol-4-^{14}C, sodium oleate and either animal lecithin, synthetic (di-palmitoyl) lecithin or the Tween 20 solution in which the lecithin was dissolved. A control was incubated which contained cholesterol-4-^{14}C and sodium oleate but no homogenate. After incubation the lipid was extracted, the free and ester cholesterol separated by alumina chromatography, assayed by scintillation counting and the percentage ester calculated.

The percentage 4-^{14}C-labelled cholesterol present as ester following incubation of the cholesterol suspension with macrophage homogenate, both alone and together with animal lecithin or synthetic lecithin, is shown in fig 15 and 16. Figure 15 shows in graphic form the results of the experiments in the first series in which cholesterol suspensions of lower specific activity were

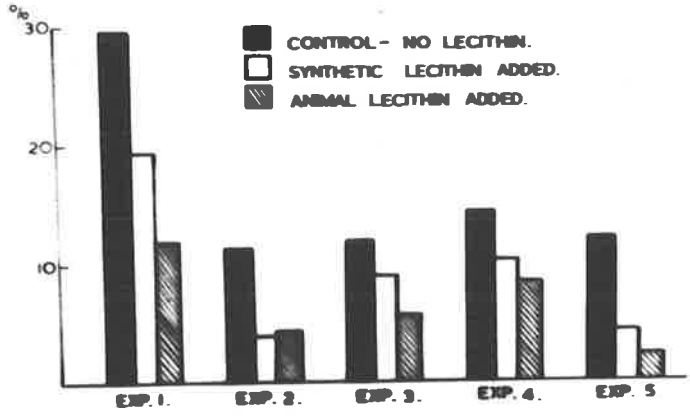


Fig 15. The percentage of cholesterol-4-^{14}C esterified after incubation with macrophage homogenate alone or with added lecithin.

First series of experiments.

used and fig 16 shows the results of the experiments in the second series using higher specific activity suspension.

The results show that in the presence of macrophage homogenate esterification of cholesterol-4-^{14}C occurred. In both series the presence of either animal lecithin or of synthetic lecithin partially inhibited this esterification.

The total esterification was somewhat less in the first series than the second, presumably because of the lower specific activity of the cholesterol suspension used but no difference between the two series with respect to the effect of phospholipid on such esterification was apparent. The inhibitory effect of synthetic lecithin on cholesterol esterase activity was less than that of the animal lecithin in most of the experiments. However, as some difficulty was experienced in keeping the former preparation in suspension no significance can be attached to this difference.

Fresh rabbit serum was substituted for the macrophage homogenate in an additional series of experiments and fig 17 shows the result of incubation of the serum with ^{14}C-labelled cholesterol suspension either alone or in the presence of added animal or synthetic lecithin. As was the case when macrophage homogenates were used, the lecithin can be seen to have partially inhibited the cholesterol-esterase activity demonstrated in the serum.

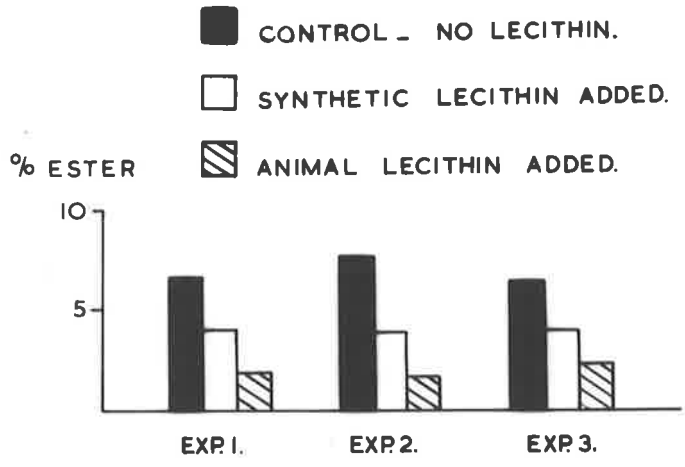


Fig. 17. The percentage of cholesterol-4-^{14}C esterified after incubation with rabbit serum alone or with added lecithin.

The effect of lecithin of decreasing the percentage esterification of cholesterol could be due either to the lecithin inhibiting esterification or to the lecithin enhancing the hydrolysis of cholesterol ester formed. The latter possibility is examined in the next experiment.

The Hydrolysis of Cholesterol Ester by the Rabbit Peritoneal Macrophage and the Effect of Lecithin

Cholesterol-H^3 labelled plasma-lipoprotein was made by gastric intubation of a cholesterol-fed rabbit with tritiated cholesterol. The distribution of the cholesterol-^3H amongst the lipoprotein fractions and the distribution of the ^3H-label amongst the lipid components of the lipoprotein are described on page 53.

Incubations were set up containing macrophage homogenate and ^3H-labelled lipoprotein as a source of cholesterol ester. After incubation with suitable controls (see page 56) the free and ester cholesterol in lipid extracts were separated and estimated.

Detailed data from one experiment showing the effect of macrophage homogenate on cholesterol ester are given in Table XIV. The effect of added lecithin is shown.

TABLE XIV

HYDROLYSIS OF LIPOPROTEIN H^3-CHOLESTEROL ESTER BY MACROPHAGE HOMOGENATE AND THE EFFECT OF ADDED LECITHIN

Details from one experiment

	Ester Chole (cts/sec)	Free Chole (cts/sec)	Ester (per cent)
Control: no homogenate (0 hours)	35.0	11.5	75.4
Control: no homogenate (20 hours)	37.3	12.6	74.8
Homogenate alone (20 hours)	29.0	15.7	65.0
Homogenate plus animal lecithin (20 hours)	24.5	27.0	47.7
Homogenate plus synthetic lecithin (20 hours)	29.2	22.2	56.9

The mean change in percentage ester together with the standard error for 6 similar experiments performed is shown in fig 18 and includes the control group containing no homogenate and the groups containing homogenate alone or homogenate with synthetic or animal lecithin added. In the control where lipoprotein was incubated alone no significant change in percentage ester occurred. Where macrophage homogenates were present a fall in the cholesterol ester content of the lipoprotein from a mean percentage of 74.6 ± 1.6 at 0 hours to 65.7 ± 1.5 at 20 hours occurred. When either animal lecithin or synthetic lecithin was added, the hydrolysis was significantly increased so that the percentage of ester fell to 56.7 ± 3.0 and 57.6 ± 2.2 respectively.

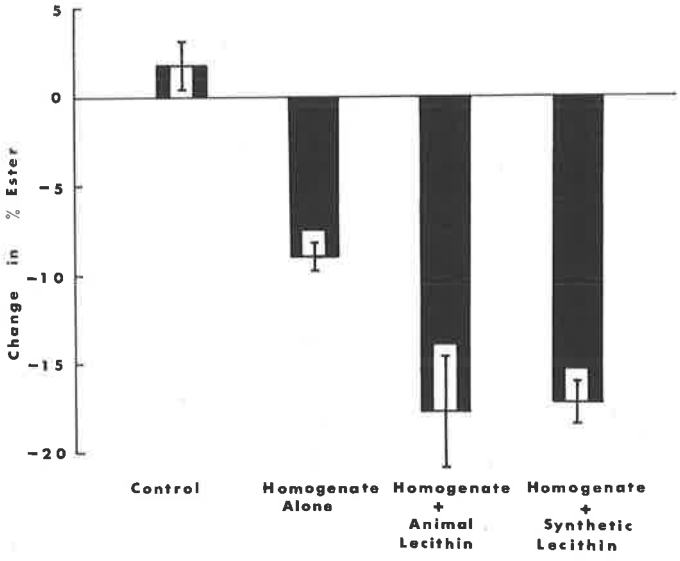


Fig 18. The change in the percentage of cholesterol esterified in ^{3}H-cholesterol-labelled lipoprotein after incubation with homogenate of macrophage, alone or with added lecithin.

Discussion

Hydrolytic as well as synthetic cholesterol-esterase activity has now been demonstrated in rabbit macrophages and lecithin has been shown not only partially to inhibit the esterification but also to enhance the hydrolysis of cholesterol-ester brought about by these cells.

In view of the varying effect of different synthetic lecithins on cholesterol-esterase activity (Wagner, 1959) it appears that their fatty-acid composition influence the "lecithin effect". In the present work the fatty-acid composition of the lecithin used was established by gas phase chromatography. As a result it is possible to say that synthetic di-palmitoyl lecithin and also animal lecithin with a 2:1 ratio of oleic and palmitic acid both inhibit cholesterol-esterase synthetic activity and enhance its hydrolytic activity.

The findings of Glomset (1963) and Rowen and Martin (1963) suggest that in the serum, lecithin acts as a fatty-acid donor for the esterification of cholesterol. The fatty-acid composition of the phospholipid therefore becomes still more important and it may be concerned in trans-esterification as well as esterification of free cholesterol.

In experiments not reported in this work, uptake of

^{3}H-labelled lipoprotein by intact macrophages in vitro was demonstrated (Day, Gould-Hurst and Wahlqvist, 1964). The uptake was so small as to suggest diffusion rather than active phagocytosis. The hydrolysis of the cholesterol ester however was greater than occurred here using a homogenate of these cells; the intact cells causing a fall from 80 per cent esterification to 25 per cent. It is interesting to compare this degree of hydrolysis of cholesterol ester with the degree of esterification of free cholesterol by macrophages in vitro. In the "time run" reported earlier in this work (see page 67) the average percentage ester rose to only 12.6. However the cholesterol preparation used in the two experiments was not the same and the equilibrium point for the esterase system cannot in any case be easily discovered since the optimum pH of the esterifying and hydrolytic activity may differ and the pH they encounter in vivo is not known. It is tempting to suggest on the grounds of function that the system may be basically hydrolytic in action. Studies of the system are at present in progress in this laboratory to determine whether more than one enzyme is involved and in which direction the reaction would proceed under physiological conditions.

Other experiments at present being carried out but not completed at the time of writing this thesis were designed to investigate the existence and to study the action of cholesterol-

esterase in the atheromatous aorta of the cholesterol-fed rabbit. The results so far available have shown that when radio-tracer cholesterol is added to medium in which pieces of atheromatous aorta are cultured, 50 to 90 per cent of the cholesterol is taken up by the aorta. This large uptake was thought to be due mainly to isotopic exchange. No evidence of esterification of the cholesterol was observed following incubation but when labelled cholesterol-oleate was added to the medium and similar uptake occurred, 20 to 30 per cent of the cholesterol ester taken up was hydrolyzed. No hydrolysis took place in controls. It is planned to investigate the effect of lecithin and other substances on this hydrolysis which may in part be brought about by foam cells in the lesion. Radio-autographs of the aortic strips following incubation may show in what cells of the wall the hydrolysis is brought about and it is intended to compare atheromatous artery with normal.

The fatty-acid pattern of the cholesterol ester synthesized by reticulo-endothelial cells has been shown to be predominantly and preferentially saturated. It is probable therefore that, following the hydrolysis of cholesterol ester, equilibrium between synthesis and hydrolysis will be set up with a changing fatty-acid pattern. Such changes brought about by macrophages which take up infiltrated lipoprotein cholesterol ester in

arterial lesions would satisfactorily explain the increased free to ester cholesterol ratio and the more saturated fatty-acid pattern of the cholesterol esters present compared with that in the plasma (Weinhouse and Hirsch, 1940; Zilversmit, Sweeley et al, 1961).

Many inter-relationships between phospholipid and cholesterol in the body exist and these include their involvement in the pathogenesis of atheroma. Certain facets of the role played by phospholipid may be clarified by the findings of these experiments. Reticulo-endothelial cells have been shown to esterify cholesterol and in rat lymph nodes this esterification has been shown to result in a decreased exchange rate and a probable decrease in mobility. If the phospholipid synthesized in the arterial wall inhibits the esterification of cholesterol and increases the exchange rate of the cholesterol ester which is formed it may enhance the mobility of the cholesterol in the vessel wall. The phospholipid synthesized by macrophages in atheroma in response to cholesterol accumulation may therefore be a defence reaction on the part of the reticulo-endothelial system stimulated by cholesterol infiltration. This is in keeping with the role of reticulo-endothelial cells in other situations.

The effect of phospholipid on the cholesterol-esterase activity of serum as reported by Wagner and Rogalski (1952)

and Wagner (1959) differs from the effect of lecithin on the cholesterol-esterase activity of macrophages just described. They claimed that serum cholesterol-esterase does not esterify added extrinsic cholesterol but only intrinsic, plasma-lipoprotein cholesterol and that the esterification of the latter is accelerated by lecithin. In view of the findings presented above using macrophage-homogenate as a source of esterase, their experiments were repeated but using labelled cholesterol. The results given in fig 17 show that when ^{14}C-labelled cholesterol suspension was incubated with the serum some esterification of the extrinsic cholesterol was observed, certainly not as much as with macrophage homogenate (see fig 16) but providing definite evidence that esterification of added cholesterol by serum does occur. Further, inhibition of this serum cholesterol-esterase activity by phospholipid to a similar degree to the inhibition of the cholesterol-esterase activity of macrophage homogenate, was discovered to occur. The radio-active cholesterol which here was added to the serum was no doubt incorporated into the plasma lipoprotein by isotopic exchange. Only a very small fraction of the relatively large amount of non-labelled cholesterol which Wagner and Rogalski added to the serum could have been incorporated into the lipoprotein. This is possibly why their findings differ from ours.

If phospholipid has the same effect on serum cholesterol-esterase in vivo as in vitro and decreases the amount of plasma

cholesterol esterified, this may also result in an increase in its ease of exchange and mobility. This may explain why an increase in ratio of serum phospholipid to cholesterol in disorders, associated with hypercholesterolaemia tends to protect against atheroma and why intravenous infusions of phospholipid results in the regression of, or prevents the development of, atheroma in cholesterol-fed rabbits (Byers and Friedman, 1960; Maurukas and Thomas, 1960). Such infusions initially cause a rise in serum cholesterol suggesting that the phospholipid is causing a mobilization of cholesterol deposits from the tissues including those from the atheromatous arteries.

Summary

Hydrolytic as well as synthetic cholesterol-esterase activity has been demonstrated in reticulo-endothelial cells. Lecithin was shown partially to inhibit the esterification and to enhance the hydrolysis brought about by the cells.

Esterification of cholesterol was earlier shown to decrease the isotopic exchange rate of cholesterol. Lecithin has been shown here to restore the exchange rate of esterified cholesterol to that of the free form.

The possible significance of these findings was discussed particularly in relation to the increased transport of cholesterol and the mobilization of cholesterol from the tissues.

THE REMOVAL OF CHOLESTEROL FROM RETICULO-ENDOTHELIAL CELLS

The Removal of Cholesterol from Reticulo-Endothelial Cells

If reticulo-endothelial cells as part of the defence mechanism of the body influence the removal of lipid deposits, including cholesterol, from the tissues, they may do this either by effecting the catabolism of the lipid or by facilitating its physical removal. The part played by these cells in removing lipid from the tissues may vary depending on the nature of the lipid concerned. It has been shown by histochemical and chemical means that cholesterol and its esters unlike triglyceride and phospholipid remain in reticulo-endothelial cells of lymph nodes for relatively long periods of time (French and Morris, 1960).

In the present experiments the rate of removal of individual lipids was measured after the uptake of lipid-mixtures by reticulo-endothelial cells. This was done so that the relative removal rates of the different lipids could be studied. A mixture of radio-tracer labelled cholesterol and labelled triglyceride or labelled cholesterol and labelled phospholipid was injected and taken up by reticulo-endothelial cells in rat thoracic lymph nodes. The subsequent removal rates were followed. The radio-tracer labelled lipids were injected either in the form of mixtures of lipid emulsions or as lipoprotein in which two of the lipids were labelled (see pages 37 to 46 for details of the procedure used).

The results are shown in fig 19 of a representative experiment in which the rate of removal of ^{3}H-labelled cholesterol and ^{14}C-labelled triglyceride from the cells were compared. The lipid was injected in aqueous suspension. The amount of each lipid remaining in the nodes at intervals after injection is shown. It will be seen that both the triglyceride and cholesterol were removed from the nodes but the triglyceride disappeared more rapidly than the cholesterol. At the end of 7 days about 4 times as much cholesterol remained in the nodes as triglyceride. It may be noted that the number of counts of radioactivity of the cholesterol and triglyceride present in the nodes at 4 hours was not the same. The curves of the graph have nevertheless been drawn diverging from the same point because the amount of the two lipids in mg present in the nodes at this time was the same; it was the specific activity of the two injected lipids which differed.

Fig 20 shows the results of the same experiment but presented in a more informative way by showing the relative removal rates of cholesterol and triglyceride from the nodes, the graph line representing a changing ratio. The initial portion of the graph line represents a period during which lipid was still being taken up by lymph nodes and by the

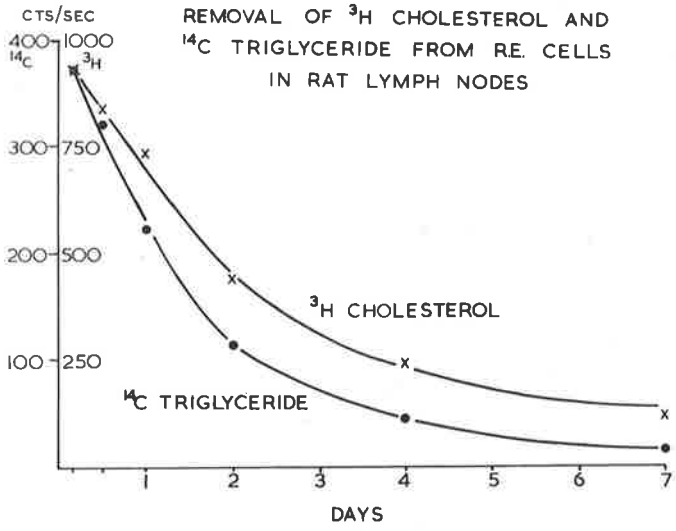


Fig 19. The cholesterol-^{3}H and triglyceride-1-^{14}C remaining in rat lymph nodes at intervals after their intraperitoneal injection.

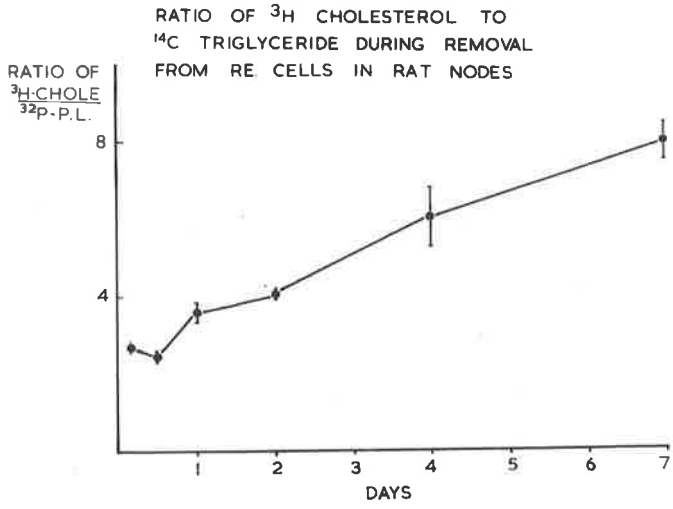


Fig 20. The ratio of cholesterol-^{3}H to triglyceride-1-^{14}C remaining in the nodes at intervals after injection.

Each point represents the mean value for 8 rats with standard error shown.

reticulo-endothelial cells in the nodes. Subsequent to this, while the lipid was leaving the nodes, it can be seen that the proportion of cholesterol to triglyceride remaining did not stay in the same ratio as when initially injected and taken up (which would have resulted in a horizontal graph line) but steadily increased.

In a further series of experiments labelled cholesterol was again ingested by reticulo-endothelial cells in rat lymph nodes but this time together with phospholipid. Subsequently, a change in the ratio of cholesterol to phospholipid remaining in the nodes occurred, both where the lipids were in the form of aqueous emulsion (Table XV) and where they were injected as double-labelled lipoprotein (Table XVI). In both cases there was an increase in the cholesterol:phospholipid ratio indicating that the phospholipid was removed from the nodes more rapidly than the cholesterol.

TABLE XV REMOVAL OF CHOLESTEROL-4-^{14}C AND PHOSPHOLIPID-^{32}P FROM RETICULO-ENDOTHELIAL CELLS IN RAT LYMPH NODES AFTER INJECTION OF A DOUBLE-LABELLED MIXTURE

Mean of 8 rats together with the standard error of the mean is shown

Time (hrs) after injection	Cholesterol-4-^{14}C (d.p.s.)	Phospholipid-^{32}P (d.p.s.)	^{14}C : ^{32}P ratio
4	156 ± 25	108 ± 19	1.49 ± 0.06
12	244 ± 28	76 ± 11	3.41 ± 0.32
24	171 ± 28	52 ± 8	3.27 ± 0.10
48	71 ± 10	15 ± 2	4.86 ± 0.26
96	32 ± 4.1	4.9 ± 0.4	6.51 ± 0.53

Suspension injected. Cholesterol-4-^{14}C, 15920 d.p.s. and 5.7 mg plus phospholipid-^{32}P, 9080 d.p.s. and 7.5 mg per rat

TABLE XVI REMOVAL OF LIPOPROTEIN LABELLED WITH CHOLESTEROL-4-^{14}C (OR ^{3}H) AND PHOSPHOLIPID-^{32}P FROM RETICULO-ENDOTHELIAL CELLS IN RAT LYMPH NODES

Mean of 8 rats together with the standard error of the mean is shown

Exp N°	Time (hrs) after injection	Cholesterol-4-^{14}C or ^{3}H (d.p.s.)	Phospholipid-^{32}P (d.p.s.)	^{14}C : ^{32}P ratio or ^{3}H : ^{32}P ratio
1	4	115 ± 24	21 ± 4.3	5.6 ± 0.5
	12	165 ± 30	32 ± 6	5.7 ± 0.5
	24	117 ± 15	19 ± 2	6.2 ± 0.2
	48	84 ± 6	10 ± 0.8	8.7 ± 0.3
	96	27 ± 6	0.9	... ...
	7 days	11 ± 2	0.5	... ...
	Lipoprotein injected.	Cholesterol-4-^{14}C, 5750 d.p.s. and 4.03 mg plus phospholipid-^{32}P, 2180 d.p.s. and 1.52 mg per rat		
2	4	620 ± 70	52 ± 5	11.9 ± 0.4
	12	890 ± 94	66 ± 7	13.6 ± 0.4
	24	474 ± 64	29 ± 3	16.3 ± 0.6
	48	247 ± 32	14 ± 2	19.1 ± 1.3
	96	66 ± 10	2.2 ± 0.2	30.3 ± 3.1
	Lipoprotein injected.	Cholesterol-^{3}H, 29000 d.p.s. and 3.9 mg plus phospholipid-^{32}P, 6350 d.p.s. and 1.81 mg per rat		

Discussion

In these experiments, triglyceride and phospholipid have been shown to be more rapidly removed from reticulo-endothelial cells in rat lymph nodes than cholesterol. Triglyceride is readily oxidized by these cells unlike cholesterol (Day, 1961) and this may partly account for its more rapid removal. Also, triglyceride taken up by macrophages is partly incorporated into phospholipid (Day, Fidge, Gould-Hurst, Wahlqvist and Wilkinson, 1965) and this may have two results: firstly, the rise in ratio of cholesterol to triglyceride remaining in the cells in these experiments may be greater than is apparent since some of the radioactivity attributed to triglyceride may no longer be present in triglyceride but be present in phospholipid. Secondly, this transformation may represent another mechanism not available to cholesterol by which triglyceride is removed from reticulo-endothelial cells.

The fact that phospholipid is more rapidly removed than cholesterol is not surprising when the ease of diffusion of phospholipid, which is water soluble, is contrasted with that of hydrophobic cholesterol.

Where cholesterol and phospholipid were presented to the cells in lipoprotein form, the resultant relative removal-rates probably represent more closely the picture of that which occurs

in tissues under physiological conditions. Although the lipid in the atheromatous arterial wall differs from that in the plasma both qualitatively and quantitatively, most of the cholesterol and triglyceride and some of the phospholipid in the atheromatous artery is believed to have infiltrated the wall as plasma lipoprotein (Buck, 1958; Bötcher et al, 1960; Gero et al, 1961). The subsequent differences between the lipid pattern in the plasma and that in the arterial wall is thought to be due to the metabolism of lipid in the arterial wall and to different removal rates of different lipids. If the lipoprotein-cholesterol is removed at a lesser rate than other lipids from the wall, then this would be one possible explanation of why it accumulates to a greater degree than other lipids. In the arterial wall as in the experiments described here, lipid is contained within macrophages so that in atheroma these cells, by the difference in their capacity to remove different lipids, may be partly responsible for the establishment of the final lipid pattern.

Summary

Double-labelled lipid mixtures and double-labelled lipoprotein were injected intra-peritoneally into rats. Following their uptake by reticulo-endothelial cells in the thoracic lymph nodes the removal of the different lipids were compared using counting

techniques designed for double-labelled compounds. Triglyceride and phospholipid were found to be more rapidly removed than cholesterol. The possible mechanisms and significance of these findings were discussed.

THE INCORPORATION OF CHOLESTEROL INTO LIPOPROTEIN BY MACROPHAGES

The Incorporation of Cholesterol into Lipoprotein by Macrophages.

Experiments have been described in which the incubation of ^{14}C-labelled cholesterol suspension with rabbit serum resulted in partial esterification of the cholesterol. The amount of cholesterol esterified did not exceed 5 to 10 per cent in any of the experiments (see page 98; fig 17). This amount of ester formed contrasts with the results of experiments in which labelled cholesterol was incubated with rabbit serum after having been taken up by macrophages and allowed to diffuse from these cells into the serum. In this case 70 to 80 per cent of the cholesterol in the medium was esterified. The percentage ester of the cholesterol remaining in the cells did not exceed 10 to 15 per cent (see page 67; fig 6).

A possible explanation for the increase in esterification brought about by the serum and cells together is that the cholesterol, ingested in the form of a particulate suspension by the macrophages, is altered in some way by these cells before its diffusion into the medium, to a form that is more readily acted on by the serum esterase. In blood plasma, cholesterol is held in solution and transported combined with water-soluble proteins. Macrophages may similarly disperse ingested particulate cholesterol by forming a combination of cholesterol with protein and this may result in it being more rapidly acted on by serum enzymes. Tomkins in 1946 showed by histochemical means that

when cholesterol suspensions are injected into the skin the cholesterol is dispersed, apparently by the formation of a soluble cholesterol-protein complex by macrophages.

The experiments now to be described were designed to investigate whether macrophages convert particulate cholesterol to a soluble lipoprotein form.

Rabbit macrophages which had ingested ^{14}C-labelled cholesterol suspension were incubated in vitro in fresh medium containing rabbit serum. The ^{14}C-labelled cholesterol which passed from the cells into the fresh medium was then compared with two controls with reference to the following properties: (1) its incorporation into the lipoprotein of the serum in the medium, investigated by electrophoresis, (2) the degree of its esterification in the medium brought about by the serum esterase, and (3) the readiness with which it was phagocytosed again by fresh macrophages. These three tests performed on the cholesterol taken up and then excreted by the cells into the medium were performed in order to assess whether any change in the form of the cholesterol had been brought about by the cells. The two control cholesterol preparations with which the properties of the excreted cholesterol were compared were incubated in media containing no cells and consisted of ^{14}C-labelled cholesterol in aqueous suspension and ^{14}C-labelled cholesterol in a dilute Tween 20 solution.

For a scheme of the experimental procedure, tabulated, see Table XVIII and for full details see page 50.

The initial uptake of ^{14}C-labelled cholesterol suspension by the macrophages is shown in Table XVII. An average of 21.6 per cent of the cholesterol in the medium was phagocytosed by the cells during this, their first incubation.

TABLE XVII

INITIAL UPTAKE OF ^{14}C-LABELLED CHOLESTEROL SUSPENSIONS BY MACROPHAGES IN VITRO

Experiment Number	Number of Cells (millions)	Cholesterol-4-^{14}C in medium (cts/sec)	Uptake (per cent)
K 1	10	44600	21.3
K 5	10	44600	15.9
K 7	12	89200	9.1
K 10	21	66900	20.2
K 11	12	60500	12.0
K 12	12	60500	45.7
K 13	20	60500	35.3
K 14	20	63800	13.0

TABLE XVIII

EXPERIMENTAL PROCEDURE USED TO INVESTIGATE THE INCORPORATION OF CHOLESTEROL INTO LIPOPROTEIN BY MACROPHAGES

	A. Test run	B. (Control)	C. (Control)
Stage I	Particulate-suspension of labelled cholesterol ingested by macrophages.	(No cells involved)	(No cells involved)
Stage II	The cells now containing particulate cholesterol were incubated in fresh medium containing serum. Cholesterol leaked out of cells into medium.	A particulate suspension of labelled cholesterol was incubated in medium containing serum.	Labelled cholesterol dissolved in a solution of Tween 20 in water was incubated in medium containing serum.
Stage III	Media analyzed and compared with reference to: (1) The amount of the labelled cholesterol now complexed with protein in the medium, estimated by electrophoresis. (2) The percentage esterification of the labelled cholesterol now in the medium. (3) The amount of labelled cholesterol from the medium taken up by fresh macrophages (i.e. ingested for a second time).		

Table XIX presents basic data for Stage 2 of the experiment; the incubation of the cholesterol-4-^{14}C suspension-containing cells in fresh medium and the return of the cholesterol from the cells to the medium. Also shown are the details of the controls in which cholesterol-4-^{14}C in aqueous suspension or in Tween 20 solution was added to medium followed by incubation. After 20 hours incubation, approximately half the label was found to have returned from the cells to the fresh medium. To correspond with this figure (which could only be surmised at the commencement of Stage 2) an amount equal to about half that taken up by the cells was added to the control media prior to incubation.

TABLE XIX ^{14}C-LABELLED CHOLESTEROL TAKEN UP INITIALLY BY MACROPHAGES AND RETURNED TO THE MEDIUM DURING 20 HOURS INCUBATION

Exp N°	Cholesterol-4-^{14}C initially present in cells (cts/sec)	Cholesterol-4-^{14}C returned to medium from cells during 20 hours incubation (cts/sec)	Cholesterol-4-^{14}C suspension added (cts/sec)	Cholesterol-4-^{14}C solution with Tween 20 added (cts/sec)
K 1 ✱	1430	600	1100	2160
K 5 ✱ ☆	1065	560	930	950
K 7 ☆	1210	570	620	630
K 10 ☆	2015	1260	1090	1100
K 11 ☆	1210	810	725	790
K 12 ✱ ☆	2770	1230	1165	1010
K 13	2390	1610	1220	1080
K 14 ✱ ☆	1250	970	600	545

✱ Free and ester cholesterol in lipoprotein fractions determined in these four experiments - see Table XX.

☆ Detailed electrophoretic patterns constructed for these six experiments - see fig 22.

The return of ^{14}C-labelled cholesterol into fresh medium during the incubation of the cells is shown in the relation to time for one experiment in figure 21. About three-quarters of this ^{14}C-labelled cholesterol has passed into the medium after 72 hours, while in most of the experiments this had occurred within 20 hours.

The incorporation of the ^{14}C-labelled cholesterol into the lipoprotein fractions of the serum in the media is shown in figure 22 the results of electrophoresis performed on the media. Little incorporation of ^{14}C-labelled cholesterol into lipoprotein has occurred where cholesterol as a particulate suspension was incubated with the medium, most of the label remained at the origin. However, where the cholesterol was first taken up by and then re-entered the medium from macrophages, a large proportion can now be seen to have travelled with the alpha- and beta-lipoprotein and no longer remained at the origin. In this it more closely resembled cholesterol dispersed as a solution with Tween 20.

The distribution of the free and ester cholesterol in the lipoprotein of the three media is presented in Table XX. The figures are the mean of four experiments in which incubation was carried out for 20 hours, the lipoprotein fractions then being separated and fractionated into free and ester cholesterol.

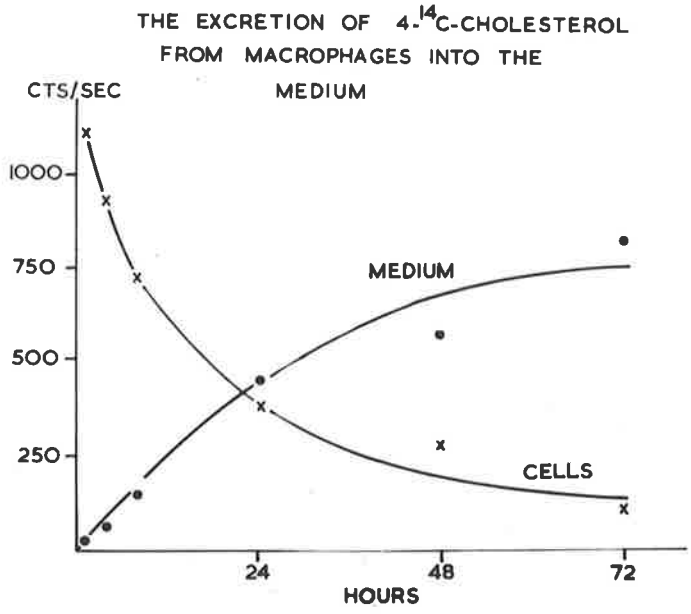


Fig 21. Cholesterol-4-^{14}C present in macrophages and medium at intervals during the incubation of the cholesterol-4-^{14}C laden macrophages.

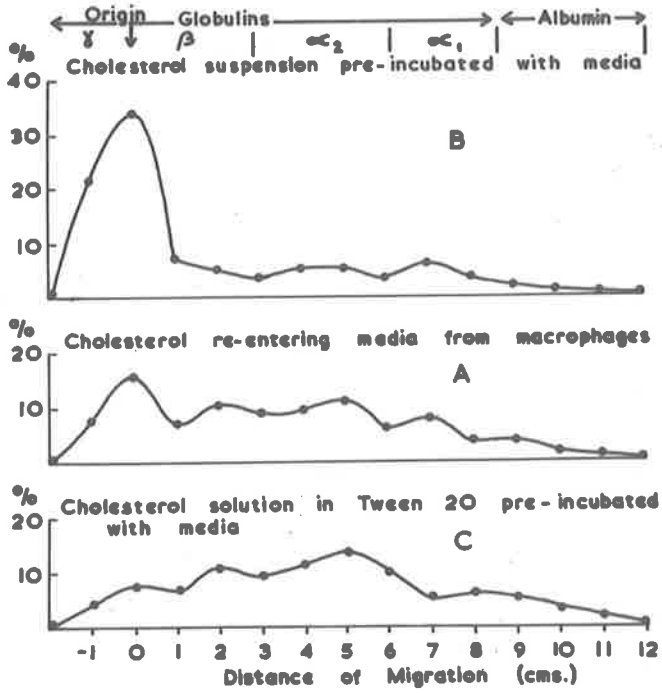


Fig 22. Percentage distribution of cholesterol-4-^{14}C after incorporation into the lipoprotein fractions of the media.
Paper electrophoretic separation.

TABLE XX

PERCENTAGE OF THE TOTAL ^{14}C-LABELLED CHOLESTEROL DISTRIBUTED IN THE MEDIA BETWEEN THE NON-LIPOPROTEIN FREE AND ESTER CHOLESTEROL AND THE LIPOPROTEIN FREE AND ESTER CHOLESTEROL

Incubation Period 20 hours

Mean of 4 experiments with standard errors

★ Electrophoretic fraction	^{14}C-labelled cholesterol suspension from macrophages (A)	^{14}C-labelled cholesterol suspension control (B)	^{14}C-labelled cholesterol dispersed with Tween 20 control (C)
Non-Lipoprotein			
Free cholesterol	20 ± 3	63 ± 3	8 ± 3
Ester cholesterol	6 ± 1	3 ± 1	10 ± 2
Lipoprotein			
Free cholesterol	38 ± 8	22 ± 7	32 ± 3
Ester cholesterol	36 ± 9	12 ± 4	50 ± 10

★ Non-lipoprotein cholesterol is the labelled cholesterol (free or ester) which remained at the origin during electrophoretic separation; lipoprotein cholesterol is that which travelled with the plasma proteins.

As can be seen from the above table, the medium incubated with cells releasing ^{14}C-labelled cholesterol contained significantly more of both free and ester cholesterol present in the lipoprotein fractions than in control (B) in which the medium was incubated with ^{14}C-labelled cholesterol suspension, which had been added directly to it. Esterification in both non-lipoprotein and

lipoprotein fractions is also shown to be greater in the medium incubated with cells and to approach the percentage esterification of the ^{14}C-labelled cholesterol which was added to the medium dispersed with Tween 20. In fig 23 the mean esterification figures for these experiments are presented and show that the esterification which has taken place in the medium, which has received the ^{14}C-labelled cholesterol from the macrophages, falls midway between that of the two controls.

An aliquot of each test and control medium was used to perform a "second-uptake test" as follows. A sample of each medium (A, B & C) was taken of such a volume that all contained the same number of counts of labelled cholesterol. An equal number of fresh macrophages was then added to each sample of medium and the final volume of the cells and medium made up to a constant volume. The samples were incubated, during which time the cells ingested varying amounts of the cholesterol depending on its physical form. The cells were then extracted and assayed quantitatively for radioactivity.

Table XXI shows the results of the "second-uptake" test.

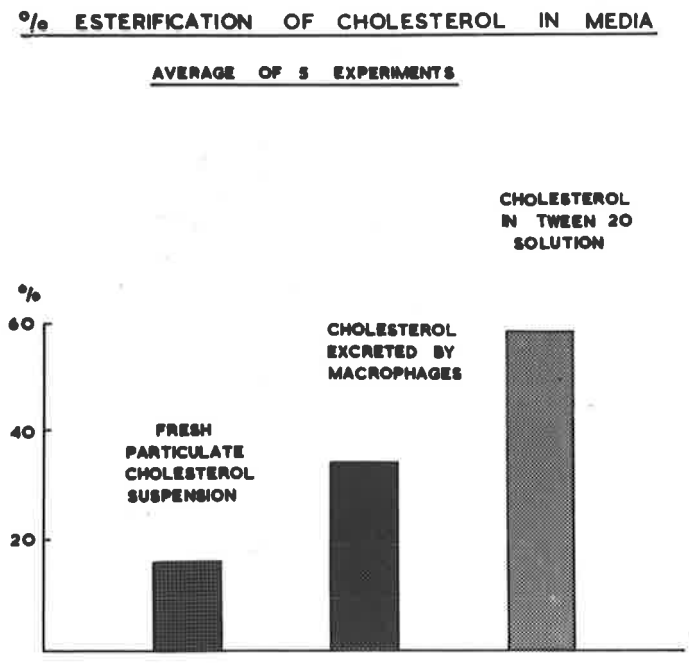


Fig 23. The percentage of the three preparations of cholesterol-4-^{14}C esterified by incubation with cholesterol esterase of serum.

TABLE XXI

SECOND UPTAKE EXPERIMENT

THE UPTAKE BY FRESH MACROPHAGES OF CHOLESTEROL-4-^{14}C FROM MEDIA

Exp No	★Medium	cts/sec present in the medium	cts/sec uptake by fresh macrophages	Percentage uptake
K12	A	340	23.2	6.8
	B	330	46.6	14.1
	C	305	11.2	3.7
K13	A	477	38.9	8.2
	B	468	97.9	21.0
	C	482	35.1	7.3
K14	A	388	56.0	14.4
	B	390	57.0	14.6
	C	382	11.0	2.9
K11	A	325	63.0	19.4
	B	325	26.1	8.0
	C	325	13.7	4.1

★ A = medium into which has passed ^{14}C-cholesterol from macrophages.

CONTROL MEDIA

B = medium incubated with an aqueous suspension of cholesterol-4-^{14}C.

C = medium incubated with a Tween 20 dispersion of cholesterol-4-^{14}C.

In three of the four experiments for which data is given, it is shown that the cholesterol suspension having been "passaged" through macrophages is less readily phagocytosed than a similar suspension presented directly to the fresh macrophages. Similar "less ready phagocytosis" i.e. decreased uptake, was found in seven out of ten similar experiments performed in this laboratory

by Dr. A. J. Day. A particulate suspension of cholesterol added to but not incubated with the medium was found to be even more readily phagocytosed, Labelled cholesterol, dispersed and made soluble with Tween 20 and incubated with the medium is, on the other hand, phagocytosed less readily than any other of the above preparations. In other words, passing a particulate suspension of cholesterol through macrophages tends to decrease its subsequent uptake by a fresh group of cells and appears to make it more closely resemble Tween 20 dispersed cholesterol than a particulate suspension.

The results of these experiments have shown that macrophages can alter particulate cholesterol which they have ingested so that subsequently the incorporation of the cholesterol into the lipoprotein fraction of serum is increased; it is more readily esterified by serum-esterase and it is less avidly phagocytosed by fresh macrophages.

All three of these findings indicate that the macrophages have altered the form of the particulate cholesterol suspension so that in the properties examined it behaves more nearly like a molecular dispersion of cholesterol than a particulate suspension. It might be argued that the increased incorporation of the cholesterol into lipoprotein is a result not of increased

dispersion brought about by the cells but as a result of the cells esterifying the cholesterol. However, evidence has been presented that not only labelled ester cholesterol is present in increased amounts in the lipoprotein but that the amount of labelled free cholesterol incorporated is also increased. A further reason why the esterification is not likely to be the predisposing cause of the increased incorporation of the cholesterol into the lipoprotein is the following fact confirmed in this laboratory: ester cholesterol in vitro does not exchange with and become incorporated into lipoprotein (Roheim, Haft, Gidez, White and Eder, 1963), unlike free cholesterol which does exchange with and is incorporated into lipoprotein in vitro followed by its being esterified (Whereat and Staple, 1960).

Discussion

The experimental evidence presented here suggests that dispersion of particulate cholesterol takes place within macrophages after they have ingested it. The way in which such dispersion occurs in other biological situations is by combination with protein and it seems likely that here partial conversion of the cholesterol suspension to lipoprotein in the cells has occurred.

What is the possible significance of these findings in relation to cholesterol and reticulo-endothelial cells in the atheromatous arterial wall? The experiments of Hollander, Kaplan and Sherwin (1962) showed the great mobility and rapidity of removal from the arterial wall of lipoprotein-cholesterol as contrasted with the immobility of non-protein-bound cholesterol. Hollander also showed (1963) that 94 per cent of all the lipid and 80 per cent of cholesterol synthesized by the atheromatous aorta is protein-bound and water-soluble. He suggests that the accumulation of lipid in atheroma is due to a failure in complexing all of the lipid with protein in the diseased artery. The function of reticulo-endothelial cells of defence and repair elsewhere in the body makes it not improbable that they play a similar role in the atheromatous lesion. If macrophages in the arterial wall also make cholesterol more soluble by converting it to lipoprotein, then they may play

an important part in facilitating the removal of particulate cholesterol from the atheromatous deposits and assisting in the resolution of the disease process.

Summary

Macrophages which had ingested an aqueous suspension of cholesterol-4-^{14}C were incubated in fresh medium containing serum. During incubation the cholesterol passed into the medium. The form of the cholesterol-4-^{14}C in the medium was then compared with controls and it was found that the passage of the cholesterol through the macrophages had resulted in certain changes in the properties of the cholesterol which were as follows: an increased amount of the cholesterol travelled with the lipoprotein on electrophoretic separation; the cholesterol was esterified more rapidly by the cholesterol-esterase of the medium; the cholesterol was less readily phagocytosed by fresh macrophages.

The findings were interpreted as showing that macrophages can alter the form of ingested particulate cholesterol and make it more finely dispersed, resulting in it becoming more soluble and being incorporated into lipoprotein.

GENERAL SUMMARY AND CONCLUSIONS

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Certain aspects of the lipid metabolism by reticulo-endothelial cells were studied. The cells used were the reticulo-endothelial cells of lymph nodes in live rats and macrophages from the peritoneal cavity of rabbits. The latter were studied in vitro both intact and as homogenate. Information was sought on the influence that the metabolism of lipid by these cells may have on the removal of cholesterol both from the reticulo-endothelial cells themselves and by these cells from other tissues.

The results of the experiments described were as follows:-

1. Reticulo-endothelial cells partly esterified ingested cholesterol-4-^{14}C and hydrolyzed cholesterol ester. Esterification was found to reduce the exchange rate of the cholesterol. An attempt was made to discover whether the esterification of cholesterol in the aorta of living rabbits also reduces the exchange rate of the cholesterol.
2. The fatty-acid pattern of the cholesterol esters synthesized by reticulo-endothelial cells was examined and it was discovered that the cells esterify cholesterol with predominantly saturated fatty-acids and do so selectively even when polyunsaturated fatty-acids are available.

3. A report is included of an experiment designed to investigate the cholesterol-esterase activity of rabbit aorta cultured in vitro of which preliminary results only are available.

4. Observations were made of the effect that lecithin has on the cholesterol-esterase activity of reticulo-endothelial cells in vivo, on the cholesterol-esterase of macrophages in vitro (intact and as homogenate) and on that of rabbit serum. In all cases it was found that lecithin partly inhibited the esterification by the esterase. The esterified cholesterol of serum lipoprotein was found to be hydrolyzed by the esterase of rabbit-macrophage homogenate and this hydrolysis was enhanced when lecithin was added. The exchange rate of ^{14}C-labelled cholesterol which was earlier shown to be decreased by esterification was found to be restored to that of free cholesterol by the addition of lecithin.

5. Using double-labelled lipid mixtures, cholesterol ingested by reticulo-endothelial cells was shown to be more slowly removed than either triglyceride or phospholipid ingested at the same time as the cholesterol.

6. The ingestion of cholesterol by macrophages was shown to facilitate the incorporation of the cholesterol into lipoprotein.

Further investigation suggested by the results of this

work is needed to establish the action of the cholesterol-esterase of reticulo-endothelial cells in the body and in particular in atheroma. The esterification of cholesterol by these cells with a resulting decrease in exchange rate may not occur in atheroma. Esterification was shown to be diminished by lecithin while hydrolysis of cholesterol ester, also demonstrated to be brought about by these cells, was increased. The fact that phospholipid is synthesized by macrophages in atheroma suggests that in this situation the action of the enzyme may be primarily hydrolytic. Cholesterol, thought to infiltrate the arterial wall from the plasma, is predominantly in the esterified form, so that since the enzyme apparently catalyzes a reversible reaction, hydrolysis of cholesterol is likely to be the primary process occurring. The results of preliminary experiments suggested that atheromatous aorta, studied in vitro, in fact hydrolyze cholesterol ester.

The slower removal of ingested cholesterol than other lipids from reticulo-endothelial cells may be more related to the different properties of the lipids than the function of the reticulo-endothelial cells. The incorporation of ingested cholesterol into lipoprotein which if it takes place in

atheromatous deposits may greatly influence the resuspension and transport of the cholesterol, accords well with the known and accepted role of reticulo-endothelial cells elsewhere in the body, namely of repair and defence.

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