MACROPHAGES

AND

LIPID SYNTHESIS

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Doctor of Philosophy

by

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PREFACE

The association of atheroma with lipid deposition has been observed by workers for more than half a century and the intracellular presence of much of this lipid within macrophages in the lesion has suggested that these cells may play a part in the deposition or removal of such lipid. The work reported in this thesis is concerned with one aspect of this possibility, namely the synthesis of lipids by macrophages and deals with the incorporation of various radioactive metabolites into the lipids of rabbit macrophages. These experiments were carried out using intact cells incubated *in vitro* and represent the first stage of an investigation into the synthesis of lipid by macrophages. Evidence will be presented that these cells are able to synthesise lipid and information concerning the isolation, identification and quantitation of the lipids synthesised will be described.

CONTENTS

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SUMMARY

Macrophages obtained from the peritoneal cavities of rabbits were incubated *in vitro* with various substrates as listed below in order to investigate the synthesis of lipid by these cells.

a. Sodium palmitate-1-^{14}C

^{14}C-labelled sodium palmitate-albumin complex was taken up by macrophages and incorporated into the lipid fraction of the cells. Up to 21% of the ^{14}C-labelled sodium palmitate added to the incubation medium was taken up by the macrophages. Most of this was converted to triglyceride and phospholipid, but a small amount was converted to cholesterol ester and to mono- and diglyceride.

b. Glyceryl tri(palmitate-1-^{14}C)

It was shown that the uptake of tripalmitin emulsion by macrophages was associated with little incorporation of the labelled fatty acid portion into other fatty acid esters, although in some preparations the phospholipid fraction did become labelled.

c. Acetate-1-^{14}C

Incorporation of sodium acetate-1-^{14}C into macrophage lipid was observed, the specific activity of the cholesterol labelled being ten to fifteen times that of the fatty acid. Fractionation of the labelled lipid demonstrated the incorporation of ^{14}C-labelled acetate into cholesterol, cholesterol ester, triglyceride, mono- and diglyceride and phospholipid. The importance of lipid synthesis by macrophages was indicated by the conversion to lipid of 27% of the acetate taken up by the macrophages. Of this, 70-80% was incorporated into

the nonsaponifiable fraction, 20-30% into fatty acid and approximately 1% into lipid glycerol.

The fatty acids labelled from acetate-1-^{14}C by macrophages were investigated by gas phase chromatography. 37.3% of the ^{14}C-labelled acetate was incorporated into palmitic acid, 18.4% into oleic acid and smaller amounts into myristic, stearic and linoleic acid. The specific activity of myristic acid was high relative to palmitic acid, but that of stearic acid and oleic acid similar to that of palmitic. Linoleic acid was only labelled at low specific activity.

d. ^{32}P-orthophosphate

Significant incorporation of ^{32}P into the phospholipid of macrophages occurred. Fractionation of the labelled phospholipids demonstrated that lecithin and sphingomyelin were most heavily labelled, but appreciable incorporation into phosphatidyl ethanolamine and inositol phosphatide also occurred. The concurrent uptake of cholesterol by the macrophages stimulated the incorporation of ^{32}P into total phospholipid. This increased incorporation was shared by all the phospholipids labelled and was not due to specific increase in any particular fraction.

e. Glucose-1-^{14}C

Appreciable conversion of glucose-1-^{14}C into macrophage lipid was shown to occur. Fractionation of the labelled lipid demonstrated incorporation of ^{14}C mainly into triglyceride and phospholipid with

only small amounts of cholesterol, mono- and diglyceride being labelled. Of the glucose-1-^{14}C converted to lipid, 94-96% was present in the lipid glycerol moiety, 4-6% being incorporated into cholesterol and fatty acid. The incorporation of glucose-1-^{14}C to lipid varied from 17.4 to 33.0% of that incorporated into all cell components; in addition more than half the glucose was oxidised to $^{14}CO_2$ by macrophages.

The results were discussed in relation to the role of macrophages in the synthesis of lipid in the arterial wall. It was concluded that macrophages might influence the deposition and metabolism of lipid in this situation.

DECLARATION

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

Signed

N.H. Fidge.

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INTRODUCTION

There are several possibilities which have been suggested to account for the presence of lipid in the arterial wall in atherosclerosis. Early investigations (Page 1954; Weinhouse and Hirsch 1940), have shown a similar composition of serum lipid and plaque lipid suggesting that lipid in the plaque arises by infiltration from the blood. But when more refined methods for lipid analysis became available it was apparent that the composition of lipids in atheromatous lesions differed from blood lipid, particularly regarding the fatty acid composition (Swell, Field, Schools and Treadwell, 1960; Bottcher, Woodford, Ter Haar Romeny-Wachter, Boelsma-Van Houte and Van Gent, 1960; Zilversmit, Sweeley and Newman, 1961). Therefore the infiltrating serum lipid must have been metabolised in some way during the process of deposition. Alternatively the lipid may have arisen by synthesis in the arterial wall itself.

There is evidence that lipid is synthesised in the arterial wall in vitro (Loomeijer and Van Der Veen 1962; Lazzarini-Robertson 1962; Stern and Stein 1962; Zsoldos and Heinemann 1963; Hollander 1963) and also that lipid can be synthesised in vivo in the arterial wall (Chernick, Srere and Chaikoff 1949; Azarnoff 1958). In addition it has been shown that phospholipid accumulates in experimental and human atherosclerosis as a result of synthesis in the lesion rather than infiltration from the blood, (Shore, Zilversmit and Ackerman, 1955; Zilversmit and McCandless, 1959; Zilversmit, McCandless, Jordan, Henley and Ackerman, 1961).

In human atheroma macrophages containing lipid material are a constant feature of the lesion, although they may not be present in the earliest lesion. They are also prominent in experimental atherosclerosis and it can be shown in this condition that they contain much of the lipid present in the affected arterial wall (Anitschkow 1913 and Duff 1935).

The presence of lipid within macrophages in the atheromatous lesion together with evidence that lipid is metabolised by these cells (discussed later) suggests that macrophages may play some role in the deposition or removal of lipid in atherosclerosis. There are three possible ways by which macrophages may influence such deposition or removal of lipid in the arterial wall.

1. Uptake of lipid or lipoprotein by macrophages.
2. Metabolic changes of this ingested lipid by macrophages.
3. Synthesis of lipid by macrophages.

Another alternative is that macrophages may ingest lipid at some site distant from the artery and then carry it into the arterial wall. Leary (1949), and Gordon (1947) have suggested that the macrophages in the lesion arise as Kupffer cells (hepatic reticulo-endothelial cells) in the liver and transport lipid from this site to the arterial wall.

The deposition of lipid filled blood macrophages on the arterial wall in cholesterol fed rabbits has been confirmed by Mannie and Dugaid (1953). They suggested that as the lesion developed the macrophages were covered with endothelium and the lipid became part of

the atheromatous plaque. Poole and Florey (1958) demonstrated the passage of macrophages across the aortic endothelium in cholesterol fed rabbits but it was not possible to show in which direction the cells were moving. Simon, Still and O'Neal (1961) and Still and O'Neal (1962) showed by electron microscopy the accumulation of lipid filled macrophages in the sub-endothelial space and penetrating the endothelium in rats. There is however much evidence (McMillan and Duff 1948; Kuntz and Sulkin 1948; Simonton and Gofman 1951), that "foam cells" i.e. macrophages arise *in situ*, rather than by migration from the blood.

The further possibility that the lipid present in macrophages in atherosclerotic lesions might arise by synthesis of such lipids by the macrophage itself forms the subject of this thesis. The experiments reported later although concerned with the physiology and biochemistry of macrophages *in vitro* carry the implication that such changes may occur *in vivo* in the arterial wall and so may influence the lipid picture of the arterial wall in atheroma.

On this background, I will turn now to consider the macrophage system in its physiological and biochemical aspects, and in particular those aspects which relate to lipid metabolism. These considerations will form an immediate background to the experiments described subsequently in connection with the synthesis of lipid by these cells.

The "Macrophage" System.

One of the primary physiological roles of macrophages is the phagocytosis of particulate material and substances foreign to the body. The phenomenon of phagocytosis which is characteristic of amoeboid feeding, was recognized in certain cell types of a variety of animals by Metchnikoff (1905). He suggested that digestion and phagocytosis of particulate matter had evolved from a feeding mechanism of unicellular organisms to become an important part of the defence mechanism of the more complex animal body. Later, Aschoff and Landau in 1913 suggested that the widely distributed mesenchymal cells present in the body and which are capable of taking up and concentrating vital dyes be grouped together and defined as the "Reticulo-Endothelial System" (see Aschoff 1924). A detailed description of the reticulo-endothelial system will not be given here, but brief descriptions are considered in text books of pathology (Payling Wright 1958, Florey 1958).

Physiology of the Reticulo-Endothelial System.

The reticulo-endothelial system is concerned with the phagocytosis and destruction of invading micro-organisms and also plays an important part in the production of immunity responses (Payling Wright 1958; Marshall 1956). These aspects are irrelevant to this report and will not be discussed.

Among the many metabolic functions attributed to the reticulo-endothelial system is the removal of damaged red blood cells from the circulating blood. It was once thought that reticulo-endothelial cells

phagocytose erythrocytes at the end of their life span but it now seems as though the majority of erythrocytes haemolyse in the circulation and only the debris of these cells are removed by the reticulo-endothelial system. In 1904 Ribbert showed that reticulo-endothelial cells could take up lipid and iron as well as micro-organisms and this shed new light on their storage capacity. In certain hyperlipaemic conditions the macrophages increase greatly in size, become filled with lipid droplets and acquire a foamy appearance (Thannhauser 1958).

When inert material is taken up by cells of the reticulo-endothelial system the material (such as carbon) remains unchanged intracellularly for some time. This is noticeable with Indian Ink particles deposited in tattoos. It is possible however that such particles may be moved from one site to another by migration of macrophages which may leave the body altogether. It has been demonstrated for example that macrophages laden with carbon particles are eliminated via the sputum (Quensel 1932).

The Uptake of Lipid by the Reticulo-Endothelial System.

After Ribbert (1904) demonstrated the ingestion of fat by reticulo-endothelial cells, other authors reported the occurrence of fat droplets in reticulo-endothelial cells of human liver and spleen (Fischer 1912; Landau and McNee 1914 and Kusunocki 1914). These findings were thought to be evidence of abnormal lipid metabolism, but fat has been found deposited in the Kupffer cells of normal animals

(Gilbert and Jomier 1908; Arndt 1925) and in the reticulo-endothelial cells of lymph nodes in dogs (Sletvinski 1929). Sherrington (1925) demonstrated the uptake of olive oil droplets by alveolar phagocytic cells while Saxl and Donath (1925) observed the uptake of injected triglyceride emulsions in all parts of the reticulo-endothelial system. Jaffe and Berman (1928) also describe the uptake of triglyceride by Kupffer cells in the liver of dogs and rabbits. Chylomicron fat, however, was not taken up by the Kupffer cells whereas artificial emulsions were (Murray and Freeman 1951). Triglyceride emulsions, when injected intraperitoneally, are taken up by the reticulo-endothelial cells of the regional lymph nodes in the thorax (French and Morris 1960). Day (1960 a, 1961) has demonstrated that macrophages incubated in vitro will ingest triglyceride emulsions more readily than chylomicron fat.

It is known that fine suspensions of cholesterol or cholesterol ester are readily taken up by cells of the reticulo-endothelial system, (Hirsch and Weinhouse 1949). Tompkins (1946) showed that subcutaneous injections of cholesterol resulted in the deposition of cholesterol as acicular crystals which are attracted to the surfaces of the macrophages. She postulated that the acicular crystals are converted to cholesterol esters which then entered the cell and that this esterification occurred at the cell surface and not in the interspaces. Tompkins concluded that the cells of the reticulo-endothelial system react to cholesterol in one way when it is presented in solid form and in another when it is given as a colloidal suspension. It

is in this latter form that cholesterol is removed from the t
modified by the reticulo-endothelial cells and then returned to t
body fluids.

French and Morris (1960) demonstrated that the uptake and storage of cholesterol and cholesteryl oleate by the reticulo-endothelial cells in lymph glands in the rat follow the same pattern described for non-toxic foreign particles such as carbon, colloidal iron and trypan blue. They observed that the initial uptake occurs predominately in the free macrophages and reticular cells of the marginal and cortical part of the intermediary sinuses. Following up these observations Day (1960b) observed the uptake and subsequent removal from sternal lymph nodes of suspensions of cholesterol and various cholesteryl esters injected intraperitoneally into rats. Using more refined chemical analyses he showed that cholesterol was removed more rapidly from the nodes than had been indicated earlier by histological observations. Moreover, the uptake of cholesterol and cholesteryl oleate also resulted in accumulation of phospholipids and total esterified fatty acids in the nodes (Day 1960b).

Kupffer cells of the rat were found to contain cholesterol within six hours after the oral administration of a cholesterol suspension (Friedman, Byers and Rosenmann 1954). This amount increased with time and by 24 hours, more cholesterol was found in the Kupffer cells and by this time had appeared in parenchymal cells. Peripheral or portal intravenous injections of various colloidal suspensions

which blocked the reticulo-endothelial system inhibited the deposition of cholesterol in both reticulo-endothelial and parenchymal cells and resulted in hypercholesteraemia (Feigenbaum, Byers and Friedman (1954).

When ^{14}C-labelled cholesterol was fed it was possible to demonstrate the presence of the labelled material in Kupffer cells isolated from the liver (Byers, St. George and Friedman 1957) and it was concluded that dietary cholesterol which is present in the blood as chylomicra, is taken up by the Kupffer cells and passed onto the liver cells for metabolism. Di Luzio (1959) isolated Kupffer cells from the liver of normal dogs and rats by magnetic means (St. George 1954). He was able to show a much higher concentration of both cholesterol and cholesterol ester in the Kupffer cells than in the parenchymal cells.

It has been shown that administration of zymosan, which stimulates the reticulo-endothelial system, causes decreased plasma and liver accumulation of cholesterol ester (Di Luzio 1960a; Di Luzio, Houston and Elko 1961). Further studies by Riggi and Di Luzio (1962) reconfirmed their previous observations and in addition they showed that zymosan administered intravenously produced stimulation of the reticulo-endothelial system and a reduction of plasma and liver cholesterol ester, whereas zymosan given orally was ineffective. They suggested that this reduction of plasma and liver cholesterol was due to an acceleration in the breakdown of cholesterol to bile

acids, brought about by the reticulo-endothelial cells (Di Luzio 1960b). In support of this concept the above authors site evidence relating to a hyperactive reticulo-endothelial system and the low serum cholesterol levels in many leukemic patients (Bases, Krakoff and Ellison 1961). More recently Ashworth, Di Luzio and Riggi (1963) have observed the effects of glucan, another reticulo-endothelial stimulating agent, on Kupffer cells of rats. When glucan was given intravenously, carbon particles and lipid emulsions were removed more rapidly and effectively in the glucan treated animals and most of this removal was due to actual phagocytic uptake by the Kupffer cells.

There are various reports in the older literature which suggest that lung macrophages ingest lipid. Seemann (1930) administered cholesterol into peripheral arteries and the portal vein and found that the primary site of cholesterol deposition was the lung, even if it had to pass through the liver first. Cioni (1932) suggested that the lung macrophages were important in removal of cholesterol from the body. More recently, this hypothesis was confirmed by Dougherty and Berliner (1959) who demonstrated by means of radioautographs that lung macrophages remove ^{14}C-cholesterol from the blood. Bernick and Patek (1961) fed rats on cholesterol diets for five months and showed that pulmonary macrophages ingested lipid droplets three days after commencement of the cholesterol feeding and that the uptake increased with time. More recently Bernick and

Alfin-Slater (1963) suggested that lung macrophages were active in maintaining the lipid and cholesterol levels of plasma. They showed that in the early stages of essential fatty acid deficiency the pulmonary macrophages phagocytosed the lipid droplets. The uptake of lipids and cholesterol could be reversed by the addition of the essential fatty acid, linoleic acid, to the otherwise fat-free diet.

Lipid Metabolism by Reticulo-Endothelial Cells.

The lipid which is phagocytosed by reticulo-endothelial cells does not remain unchanged for there is an increasing amount of evidence that reticulo-endothelial cells are able to metabolise the lipid they ingest. Schonheimer and Yuasa (1929) observed an increased affinity for Sudan IV in the phagocytic cells following implantation of cholesterol crystals in the skin or peritoneal cavity. Because pure cholesterol does not stain with Sudan, it follows that there is some conversion of cholesterol to stainable esters or else there is a simultaneous accumulation of other stainable lipids in the cell. It was later confirmed (Schonheimer and Yuasa 1929) by chemical means that partial esterification of cholesterol implanted in the skin occurred concurrently with its ingestion by macrophages. Day and French (1959) working with lymph nodes, showed that the uptake of cholesterol by reticulo-endothelial cells in the sternal lymph nodes in rats was followed by a rise in cholesterol ester which increased with time. It was suggested that esterification facilitates the elimination of cholesterol by the cells. French and Morris

(1960) showed that both cholesterol and cholesteryl oleate, after ingestion by cells of the lymph glands, were removed only slowly and in both cases were still detectable after three to four months (see also Day and French 1961).

Day and Gould-Hurst (1961) traced the esterification of cholesterol by rat lymph nodes using cholesterol-4-^{14}C. They found a high specific activity of the ester in the nodes this ruling out the possibility that esterification occurred at some other site prior to its uptake by cells of the lymph nodes. Rabbit macrophages were also incubated in vitro and shown to take up ^{14}C-labelled cholesterol and partially convert it to ^{14}C-labelled cholesterol ester. Previously cholesterol esterase activity had been demonstrated in macrophage homogenates (Day 1960c).

Day, Gould-Hurst and Wahlqvist (1964) using serum lipoprotein labelled with cholesterol-^{3}H demonstrated the partial uptake of lipoprotein by macrophages. This uptake was followed by appreciable hydrolysis of the ^{3}H-labelled lipoprotein cholesterol ester. Macrophage homogenates were also shown to hydrolyse lipoprotein cholesterol ester. The addition of synthetic lecithin or purified animal lecithin to this cell free system accelerated the hydrolysis. On the other hand Day and Gould-Hurst (1963) showed that in the presence of either synthetic lecithin or animal lecithin the esterification of the ^{14}C-labelled cholesterol brought about by macrophage homogenates was appreciably reduced.

The fatty acid pattern of the cholesterol ester synthesised by reticulo-endothelial cells has been investigated by gas liquid chromatography (Day, Fidge, Gould-Hurst and Risely 1963). Esterification normally occurs with saturated fatty acids when cholesterol alone is ingested by reticulo-endothelial cells, but if corn oil or coconut oil are administered with the cholesterol suspension, the fatty acids of the cholesterol esters can be influenced towards the polyunsaturated or saturated respectively. The effect of corn oil and coconut oil on the esterification and subsequent removal of cholesterol by reticulo-endothelial cells has been studied by Day (1960d). Both oils increased the amount of cholesterol taken up and both influenced the rate of esterification, but the rate of removal of cholesterol from the nodes was not significantly affected by the presence of either coconut or corn oil.

Tompkins (1946) suggested that cholesterol taken up by macrophages is modified in some way, possibly by combination with protein and then returned to the body fluids. This hypothesis was further investigated by Day, Gould-Hurst and Wilkinson (1964). They found that after phagocytosis of cholesterol suspensions by rabbit macrophages incubated in vitro, the cholesterol is incorporated into the serum lipoprotein at a more rapid rate than when cholesterol suspension is incubated with serum alone. They suggest that macrophages bring about removal of cholesterol from a site by converting it to the lipoprotein form.

Macrophages obtained from the peritoneal cavity of rabbits have been shown to possess both esterolytic and lipolytic activity (Day and Harris 1960). Using cell free systems, the esterase activity was demonstrated by the hydrolysis of both tributyrin and -naphthyl

acetate, and the presence of lipase demonstrated by the hydrolysis of chyle triglycerides and by the increased hydrolysis of β-naphthyl laurate in the presence of sodium taurocholate.

The oxidation of cholesterol-26-^{14}C, palmitate-1-^{14}C and tripalmitin-1-^{14}C by macrophages *in vitro* has recently been investigated (Day 1960a, 1961). Appreciable oxidation of ^{14}C-labelled fatty acids and triglycerides could be brought about by these cells but under similar circumstances, no oxidation of the cholesterol side-chain could be demonstrated.

Recently the question of lipid synthesis by phagocytic cells, notably polymorpho-nuclear leucocytes has been investigated. It has been found by electron microscopy studies that during the process of phagocytosis, the lipid rich cellular membrane is internalised (Goodman and Moore 1956). During this process there is considerable breakdown of cellular membrane and a build up of new lipid rich membrane surrounding ingested particles. An increased incorporation of acetate-1-^{14}C, glucose-1-^{14}C and ^{32}P-labelled phosphate into lipids during phagocytosis by polymorpho-nuclear leucocytes was observed by Sbarra and Karnovsky (1960). Elsbach (1959) also showed labelling of the lipids of rabbit polymorpho-nuclear leucocytes using acetate-1-^{14}C following uptake of heat killed streptococci. Sbarra and Karnovsky (1960) suggested that the cause of the increased synthesis of lipid during phagocytosis is an increased penetration of labelled precursors into the cells during the process of particle ingestion. Lipid synthesis by polymorpho-

nuclear leucocytes has been observed by Buchanan (1960), Marks, Gellhorn and Kidson (1960) and Rowe, Allison and Lovelock (1960) and it appears that the leucocytes are responsible for most of the lipid synthesis brought about by blood cells.

Recently synthesis of lipid by leucocytes has been confirmed by Malamos, Miras, Levis and Mantzos (1962) who demonstrated incorporation of acetate-1-^{14}C into six lipid fractions by leucocytes in vitro.

Macrophages in the Arterial Wall.

The possible role of arterial macrophages in the deposition, removal and metabolism of lipid has been discussed. It was also pointed out that macrophages may effect lipid deposition by transporting it through the endothelial wall after taking it up at some site. Much of the information reported above is concerned with the uptake and metabolism of reticulo-endothelial cells in other situations than in the arterial wall; however some information is available which suggests that macrophages present in the arterial wall are biochemically active.

Some interesting observations on the enzymes of macrophages in the aorta of normal and cholesterol fed rabbits were made by Adams, Bayliss and Ibrahim (1963). Using staining techniques they demonstrated lactic dehydrogenase (L.D.), NADH-tetrazolium reductase (NADH-TR) and adenosine triphosphatase (ATPase) activity in smooth-muscle fibres between elastic lamellae in aortae of normal fed rabbits. After four

weeks on a cholesterol fed diet, macrophages proliferated in the thickened intima of the aorta and showed strong L.D., NADH-TR and ATPase activity. After ten to twelve weeks on the diet the macrophages in the subendothelial zone continued to show some enzyme activity although large spaces devoid of enzyme activity were seen in the intima. Also noticed was a focal increase of enzyme activity in the intimal macrophages of the fatty streak lesion in the aorta of the human adolescent but the media retained its normal enzyme activity. Variable numbers of macrophages were seen on the inner aspect of the plaques in older subjects and these were usually enzymically active even when the adjacent media was devoid of enzyme. The authors also cite evidence provided by Lodja and Pelt (1960) and Lodja (1962) who reported a similar increase in enzyme activity apparently confined to lipophages and fibrocytes in the intima of atheromatous rabbit aorta.

No information is available regarding the synthesis of lipids by macrophages. In the experiments described in this thesis, this aspect of lipid metabolism by macrophages has therefore been investigated.

MATERIALS AND METHODS.

A. Radioactive Substrates

1. ^{14}C-labelled sodium palmitate-albumin complex.

Palmitic acid-1-^{14}C (specific activity, 7.8 μc/mg, Radiochemical Centre, Amersham) was dissolved in 0.05 N sodium hydroxide to give a stock solution of sodium palmitate containing 20 μc (2.5mg) /ml. Prior to use, this was diluted one in ten with 5% bovine albumin solution.

2. Glyceryl tri-(palmitate-1-^{14}C) emulsion.

Glyceryl tri-(palmitate-1-^{14}C) (specific activity 16.2 μc/mg) was obtained from the Radiochemical Centre. This preparation was found to contain appreciable amounts of di- and mono-glyceride and was therefore purified on silicic acid columns (described later). Radioactive emulsions were prepared in small volumes by dissolving tripalmitin-1-^{14}C (about 20 μc) in two ml ethanol and adding three drops of Tween 20. The ethanol was removed by evaporation and the residue was taken up in five ml of water with vigorous shaking.

3. Sodium acetate-1-^{14}C.

Also obtained from the Radiochemical Centre, Amersham (specific activity 278 μc/mg). This was dissolved in water to give a stock solution containing 100 μc/ml. Periodic purity tests were made by paper chromatography as follows. 10 μl were spotted onto Whatman No.1 filter paper and chromatograms were developed by descending

chromatography using ethanol, water, ammonia (95:1:5 v/v/v) as the developing solvent. After about twelve hours, the paper was dried and a radioautograph prepared by exposing the chromatogram to X-ray film overnight. The heavy label enabled easy detection of contaminating material. If impurities were present, the acetate-1-^{14}C was concentrated by lyophilization and purified by paper chromatography on Whatman 3MM paper using the solvent system described above. After development, the acetate zone was cut out and eluted, concentrated and distilled into dilute sodium hydroxide. The distillation procedure is one based on Bartley's (1953) method for the collection of acetic acid and utilises principles introduced by Conway and Downey (1950) and Kirk (1950), viz., that the addition of anhydrous sodium sulphate increases the tension of acetic acid in solution and therefore facilitates its distillation in distillation-diffusion units. Fig.1 illustrates the apparatus used for the collection of the ^{14}C-labelled acetic acid. Details are as follows. The sodium acetate-1-^{14}C was reconstituted in 0.2 to 0.5 ml of water and loaded on the surface of the anhydrous sodium sulphate in the distillation bulb. Dilute sodium hydroxide was placed in the collection tube (in excess of the amount required) together with a drop of indicator and both the tube and side arm were frozen by immersion in ethanol containing dry ice. The side arm stopper was then removed, and 2N sulphuric acid was added (the amount of acid depending on the amount of sodium acetate present) and the tube evacuated rapidly for two minutes. After

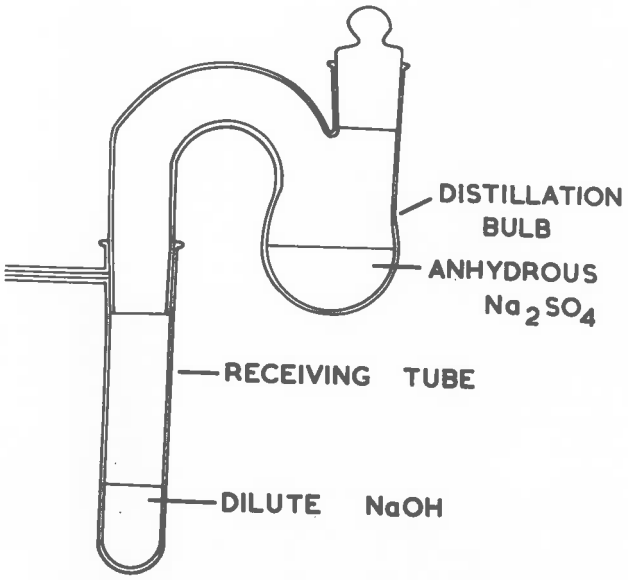


Fig.1 Apparatus used for distillation and collection of ^{14}C-labelled sodium acetate.

sealing the tube, the tube contents were allowed to thaw out. The side arm was immersed in a water bath and the tube immersed in an ice bath. The acetic acid was distilled over by raising the temperature of the water bath to about 40° and leaving it for 30-40 minutes. The recovery of sodium acetate-1-^{14}C was then checked by counting a known amount of the dilute sodium hydroxide. If much less than the expected radioactivity was recovered, more 2N sulphuric acid was added to the sidearm and the process repeated. The contents of the tube should remain alkaline throughout the distillation (thus the presence of indicator) to guard against loss of acetic acid on opening up the tube.

4. ^{32}P-labelled orthophosphate.

Obtained from the Radiochemical Centre Amersham (specific activity varied with batch and time, due to decay).

5. Glucose-1-^{14}C.

Glucose-1-^{14}C of specific activity 143 μc/mg was obtained from the Radiochemical Centre, Amersham. The preparation was dissolved in distilled water, stored deep frozen and dispensed with aseptic precautions.

B. Preparation of Non-Radioactive Suspensions

1. Cholesterol Suspension.

500mg of cholesterol was dissolved in 30ml of warm acetone; this solution was added dropwise to 100 to 200ml of boiling water which was stirred constantly. The acetone was removed by boiling and porcelain chips were added to prevent bumping. In order to obtain a suspension containing about 10mg/ml the volume was reduced to 40 to 50 ml by boiling, this final suspension then being filtered through glass wool to remove any precipitated cholesterol. The cholesterol content of the suspension was determined; a concentration between nine to ten mg/ml was satisfactory for the experiments concerning the uptake of palmitate-1-^{14}C and ^{32}P-labelled phosphate.

2. Carbon Suspension.

Carbon C11/1431a (Gunther Wagner, Hanover, Germany) in 2% gelatin was prepared in suspension at a concentration of 32 mg/ml. This was diluted with water as required. The particle size was less than 500 Angstrom units.

C. Paper Chromatographic Standards

1. Non-labelled Neutral Lipids.

a. Cholesterol — Obtained from the Nutritional Biochemicals Corporation.

b. Cholesterol Oleate — B.D.H. (L.R.)

c. Palmitic Acid — B.D.H. (L.R.)

d. Tripalmitin — B.D.H. (L.R.)

e. Dipalmitin — Obtained as a hydrolytic by-product from the B.D.H. tripalmitin.

2. ^{14}C-labelled Neutral Lipids.

All of the labelled compounds listed below were obtained from the Radiochemical Centre, Amersham and details of their specific activities (except cholesterol-4-^{14}C) have been given above. They were dissolved in benzene, each standard containing 0.4 μc/ml.

a. Glyceryl tri-(palmitate-1-^{14}C)

b. Palmitic acid-1-^{14}C

c. Glyceryl di-and mono-(palmitate-1-^{14}C) were obtained by column purification of the glyceryl tri-(palmitate-1-^{14}C).

d. Cholesterol-4-^{14}C (specific activity 63.4 μc/mg).

3. Phospholipid Standards (Non-labelled).

a. Phosphatidyl choline — Synthetic dipalmitoyl lecithin obtained from Nutritional Biochemicals Corporation.

b. Phosphatidyl ethanolamine — Obtained from the Nutritional Biochemicals Corporation.

c. Phosphatidyl serine — Obtained from the Nutritional Biochemicals Corporation.

d. Sphingomyelin — Sphingomyelin was prepared from beef lung (Thannhauser, Benotti and Bonsodde 1946) as follows. Fifty pounds of fresh beef lung were obtained from the local Abattoirs, minced and washed twice with three gallons of acetone, filtered and dried at 60°. The tissue was ground to a powder and divided into three equal portions which were extracted with ether for three days. After standing in the refrigerator overnight, the extracts were filtered and the precipitate, in several lots, re-extracted in a Soxhlet apparatus for twenty hours. The residue was re-extracted with four litres of light petroleum: methanol (9:1 v/v) and filtered and the filtrate concentrated to a thin syrupy liquid and precipitated with 1500ml of acetone. This was allowed to stand in the refrigerator overnight and the precipitate was collected next morning by filtration. The residue weighed about 30g and was taken up in 300ml of glacial acetic acid (after slightly warming). This extract was kept at room temperature overnight and filtered the next day. A small amount of insoluble residue was re-extracted with ten

volumes of acetic acid, filtered and the filtrates combined. This acetic acid extract was concentrated to a small volume and the dissolved material precipitated with one litre of acetone. The suspension was filtered after standing in the refrigerator overnight and the precipitate dried at 37°. 10g of this material were ground to a paste with a small volume of water. Then 200ml of 0.25 N sodium hydroxide were added and the suspension shaken at 37° for three days. It was acidified with glacial acetic acid and filtered, washed with acetone and ether and the precipitate extracted in a Soxhlet apparatus for two days with ether to remove fatty acids. The contents of the thimble were dialysed against running tap water for 24 hours to remove inorganic material and the dialysed suspension was filtered and washed with acetone. The precipitate left after standing in the refrigerator overnight was taken up in warm ethyl acetate and stored at 4° until used. It gave a positive test for sphingosine (Saito 1950) and choline (described later) and had the same Rf value as sphingomyelin supplied by the Commonwealth Serum Laboratories, Melbourne.

e. Inositol Phosphatide — Synthetic inositol phosphatide was obtained from the Nutritional Biochemicals Corporation.

f. Lysolecithin — Lysolecithin was prepared by the hydrolysis of dipalmitoyl lecithin on an aluminium oxide column.

(Renkonen 1962). 48μmoles of lecithin were loaded on a four g aluminium oxide column, washed on with 1oml of chloroform and left in contact with the adsorbent for 48 hours at room temperature. It was then eluted successively with 20ml chloroform, 10ml of chloroform: methanol 9:1, then 8:2 (10ml), 7:3 (10ml), 6:4 (10ml), 5:5 (10ml), 2:8 (10ml) and each fraction was examined by paper chromatography. The last three fractions yielded lysolecithin. The Rf value was compared with that of an authentic sample of lysolecithin obtained from the Commonwealth Serum Laboratories, Melbourne and showed the presence of choline by a specific spot test (described later).

D. Collection of Free Macrophages from Rabbits

Fig. 2 shows the apparatus used for collecting macrophages from the peritoneal cavity of adult rabbits. In this method (Lucke, Strumia, Madd, McCutcheon and Madd (1933) as modified by Mackaness (1952)) forty ml of sterile liquid paraffin were injected intraperitoneally into adult rabbits. After five days, sterile 0.9% sodium chloride solution containing .004M E.D.T.A. (ethylene-diaminetetraacetic acid, disodium salt) was introduced into the peritoneal cavity through a small midline incision and the exudate transferred through a sheathed cannula into a separating funnel by means of a filter pump. These saline washings were allowed to stand for five minutes to separate the cells from the paraffin. The bottom phase was passed through a gauze filter to remove giant cells and debris and collected in fifty ml centrifuge tubes. They were spun at 220g (approximately 800 rev./min.) for three minutes, the supernatant poured off, and the macrophage deposit resuspended in Hanks' solution pH 7.4 (Hanks 1948). Smears were prepared and stained with Leishman's stain for differential counting and the number of cells estimated, after suitable dilution, using a haemocytometer counting chamber. The exudates contained 85-90% of large mononuclear cells which were identical with tissue macrophages. Some of the cells contained droplets of paraffin. In addition, the exudates contained approximately 10% small mono-nuclear cells, but rarely any polymorphs.

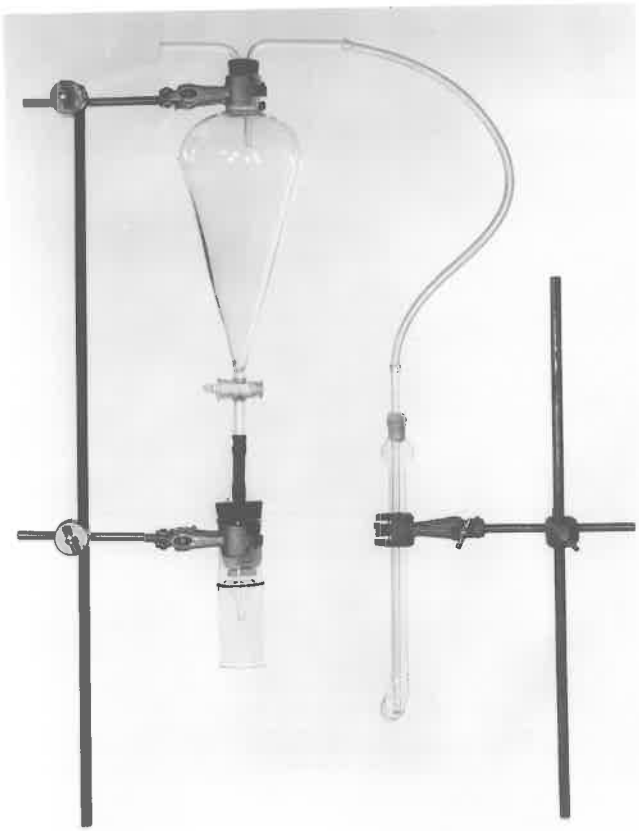


Fig.2 Apparatus used for the collection of free macrophages from the peritoneal cavity of rabbits.

E. Incubation of Macrophages and Extraction of Lipid

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

1. Incubation in McCartney bottles.

a. Sodium palmitate-1-^{14}C and Glyceryl tri-(palmitate-1-^{14}C) as substrates.

A known number of macrophages (approximately 100 x 10^6) were suspended in 45ml of Hanks' solution pH 7.4 containing 0.5% bovine albumin and were dispensed into 20 oz. McCartney bottles (Fig.3). These were incubated on their sides at 37° for 60 minutes. After this period, the macrophages had firmly adhered to the glass while any contaminating cells remained free in the fluid media. The Hanks' solution was removed and the adherent film of macrophages washed with 0.9% warm sodium chloride solution to remove any contaminating cells. Thirty ml of medium containing Hanks' solution - old rabbit serum - new rabbit serum (4:1: v/v/v) was then added to the cells together with a known amount (approximately 2 μc) of ^{14}C-labelled sodium palmitate-albumin solution. Streptomycin and penicillin were added to the medium to prevent bacterial growth. Controls were set up containg medium and ^{14}C-labelled palmitate but no macrophages. In all experiments, paired incubations were set up and the duplicates incubated for either four or twenty hours. The medium was transferred to a fifty ml centrifuge tube. The cells still adherent to the glass were washed twice with

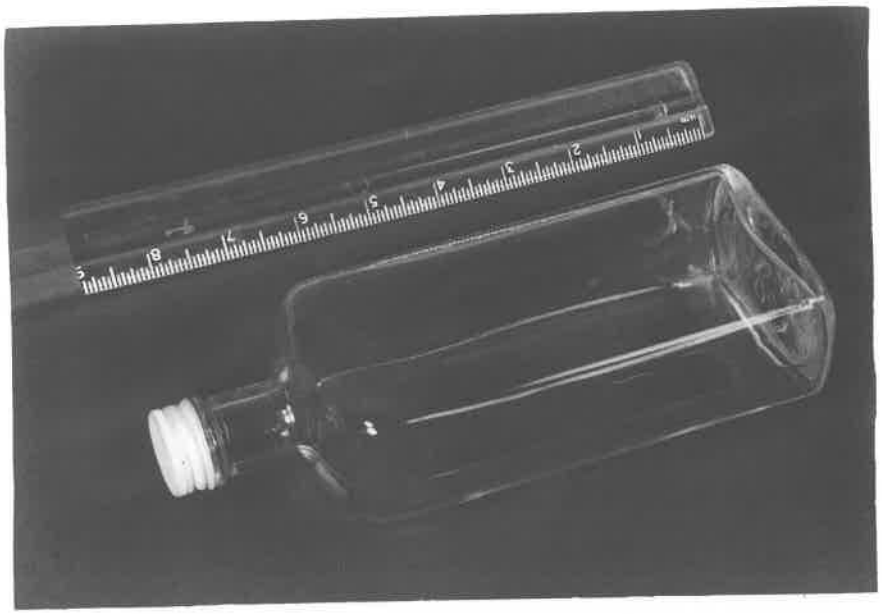


Fig.3 20oz. McCartney bottle used for incubating macro hages.

ten ml. of warm 0.9% sodium chloride solution; these washings were pooled, added to the medium and spun at 2,000 r.p.m. for ten minutes. This deposited any cells, or cell debris that had become detached from the glass during the incubation. The residue was washed again with 0.9% sodium chloride solution. The cells still adherent to the glass were extracted with ten ml of alcohol:ether (3:1 v/v) by standing the McCartney bottle in a water bath at 70°. Glass beads were added to the bottle and after the solvent had boiled, the bottles were laid down on their side with the cells adjacent to the bench. The solvent continued to boil for a few minutes. Then the extract was combined with the cell residue obtained from the medium and reheated at 70° to complete the extraction. After cooling, the extract was filtered through Whatman No. 1 filter paper and the filtrate made up to ten ml.

Five ml of the supernatant solution obtained after centrifuging the medium was extracted with 100 ml of alcohol:ether (3:1 v/v) at 70° and filtered. A known amount of this extract (80-90ml) was evaporated to near dryness in a stoppered test tube, two ml of water was added and the lipid extracted by a technique known as "phasing out". This was necessary because the medium contained considerable inorganic material, some of which dissolved in the alcohol:ether extract. Ten ml of 15% alcohol in light petroleum (b.p.60-80°) were added to the stoppered test tube which was shaken vigorously. The upper phase (light petroleum fraction) was removed and the bottom phase washed twice with ten ml of 15% ethanol in light petroleum. The washings were pooled and made up to a known volume.

b. Sodium acetate-1-^{14}C as substrate.

A known amount (approximately 1uc) of sodium acetate-1-^{14}C was added to the medium and incubation was carried out in pairs for four hours at 37°. After incubation the medium was removed from the McCartney bottles and centrifuged to deposit any detached cells. These macrophages were washed with 0.9% sodium chloride solution and respun. The macrophages still adherent to the glass were detached using three ml of 1% detergent solution (Teepol, Shell Oil Co. Ltd.) and combined with those that had been deposited after centrifuging the medium. The cell preparations were then lyophilized. One preparation of an incubation pair was reconstituted with five ml of water, a portion of which was counted by liquid scintillation counting using Dioxan Scintillator. This determined the total uptake of ^{14}C. The other member of the pair was extracted with 20ml of alcohol-ether (3:1 v/v) at 70° and the extract filtered. This filtrate was transferred to a stoppered test-tube and evaporated to near dryness. One ml of 1M unlabelled sodium acetate was added, and the lipid "phased out" with 15% ethanol in light petroleum as set out above. These light petroleum washings were combined and a portion counted using the Dioxan Scintillator.

F. Incubations using the Manometric Technique

Warburg cups were calibrated and siliconised. To each cup was added a known number of macrophages suspended in Hanks' solution ($30-50 \times 10^6$), one ml of rabbit serum and a known amount of the substrate. 0.2 ml of 4N NaOH was added to the centre well together with a small piece of fluted filter paper. The final volume was adjusted to five ml with Hanks' solution and the cups incubated at 37°. They were shaken at an optimum speed (previously determined) for oxygen exchange and duplicate flasks were removed after the appropriate incubation time. Manometric readings were taken every twenty minutes. Penicillin and streptomycin were added to prevent bacterial growth and controls were included which contained medium and substrate, but no cells. When necessary, some incubations were carried out in siliconised conical flasks, but at least two Warburgs were included in each of these runs for measuring the oxygen uptake. After incubation the cells and medium were transferred to centrifuge tubes and spun at 1500 rpm for five minutes. The cells were washed twice with five ml of 0.9% sodium chloride solution the cells being resuspended between each washing by gently shaking. The cells were then extracted by homogenising with chloroform: methanol (2:1 v/v) and the lipid extract was washed according to the method of Folch, Lees and Sloane-Stanley (1957). The efficiency of the washing procedure in removing acetate-1-^{14}C and inorganic ^{32}P from the lipid extract was checked by extracting macrophages with a small amount of chloroform:methanol (2:1 v/v) and

then adding 1 μc of acetate-1-^{14}C or 10 μc ^{32}P-labelled orthophosphate to the extract. After washing with 0.73% sodium chloride solution and pure solvents upper phase, less than 0.01% of the added counts were found in the lipid extract.

G. Analytical Procedures

1. Collection and Assay of $^{14}CO_2$.

Incubations were carried out in 30 ml siliconised conical flasks sealed with a rubber serum stopper in which was mounted a stainless steel coil supporting a collection vial as shown in Fig. 4 (Cuppy and Crevasse 1963). After incubations 0.3ml of 1M Hyamine hydroxide (a high molecular weight quaternary amine compound for CO_2 trapping) was injected into the vial with a tuberculin syringe. This was followed by the injection of 0.3ml of 6N sulphuric acid into the medium to terminate the reaction and evolve the $^{14}CO_2$ dissolved in the medium. After two hours equilibration the vial was removed, the contents diluted with toluene and a portion counted by liquid scintillation counting.

2. Hydrolysis of Lipid Extracts.

a. Basic Hydrolysis (Saponification). Entenman (1957).

Lipid extracts were evaporated to dryness under nitrogen and saponified by refluxing with 0.5ml of 30% potassium hydroxide and 0.5ml of ethanol, for two hours at 80-100°. After cooling to room temperature, the non-saponifiable material was removed by extracting three times with five ml of light petroleum (bp. 60-80°). The combined

Fig.4 Apparatus for collecting $^{14}CO_2$ evolved during incubation of macrophages with glucose-1-^{14}C.

light petroleum extracts were washed twice with small portions of 2N potassium hydroxide followed by a further four washings with water. The potassium hydroxide washings and the first two water washings were returned to the aqueous phase. The latter was acidified with 6N hydrochloric acid (using phenolphthalein as indicator) and the fatty acids thus liberated from their sodium salts were extracted with three washings of light petroleum.

The efficiency of the saponification procedure was checked using known amounts of either glyceryl tri-(palmitate-1-^{14}C) or cholesterol-4-^{14}C.

b. Acid Hydrolysis.

For the purpose of separating glycerol and glycerophosphate by paper chromatography, it was undesirable to have large quantities of salt left in the residue which was to be spotted onto papers. Therefore HCl methanol was used as a hydrolytic agent. The lipid to be hydrolysed was transferred into small glass vials, the solvent removed under nitrogen and one ml of 1.7 N HCl in methanol was added. The vials were sealed and placed in an oven at 110° for five hours. After cooling to room temperature the contents were transferred to small stoppered tubes and the lipid extracted.

3. Determination of Cholesterol Specific Activity.

The non-saponifiable fraction obtained as described above was dried over sodium sulphate:sodium bi-carbonate (4:1 w/w), filtered and evaporated to dryness. The residue was taken up in fifty µls of light petroleum and the cholesterol was separated from any impurities by chromatography on silicic acid impregnated paper (described later). The cholesterol zone located by radio-autography was cut out and eluted with alcohol-ether (3:1 v/v) at 70°. This purified cholesterol extract was filtered through Whatman No. 40 filter paper to remove silicic acid powder. A portion of the filtrate was counted by scintillation counting and the cholesterol in the remainder determined chemically by the method of Zlatkis, Zak and Boyle (1953). Blanks were included in each assay, in which a piece of silicic acid impregnated paper corresponding in size to the zones eluted was also extracted with alcohol:ether and filtered as above. The specific activity was expressed as cpm/mg of cholesterol.

The digitonin precipitation method was found to be unsuitable for the purification of cholesterol in these experiments, in view of the small amounts of cholesterol available from the macrophage lipid extract.

4. Determination of Fatty Acid Specific Activity.

The fatty acid fraction obtained after saponification was evaporated to dryness under nitrogen, dissolved in 0.5ml of benzene and the methyl esters were prepared as described later. The specific

activity was then determined by gas liquid chromatography using a Pye Argon Chromatograph with polyethyleneglycol adipate (10% on Embacel, 100-120 mesh) at 180° as stationary phase. The fatty acid mass was then determined by measurement of the total peak area by planimetry while the labelled fatty acids were collected for scintillation counting at the column outlet in calcium chloride tubes containing cotton wool soaked in light petroleum. These tubes were connected by means of silicone rubber joints to an heated outlet tube fitted to the detector. Fig.3 shows the apparatus used. Columns were calibrated using ^{14}C-labelled methyl palmitate of known specific activity. The specific activity was expressed as cps/mg fatty acid.

In some experiments the specific activity of the individual fatty acids was obtained. This was done by collecting individual peaks in the calcium chloride tubes containing light petroleum. The area of each peak was determined by triangulation and the specific activity expressed as cps/mg fatty acid obtained.

5. Glycerol Purification.

The ^{14}C-labelled glycerol in the lipid extracts was determined by purification and counting as follows. After removal of the fatty acid, 3umoles of unlabelled glycerol were added as carrier and the solution made alkaline with a few drops of ammonium hydroxide. This solution was deionised by passage firstly, through a column containing six g of Dowex 1 Cl. After carefully washing the Dowex with glass distilled water, about 40 ml of eluant were collected and

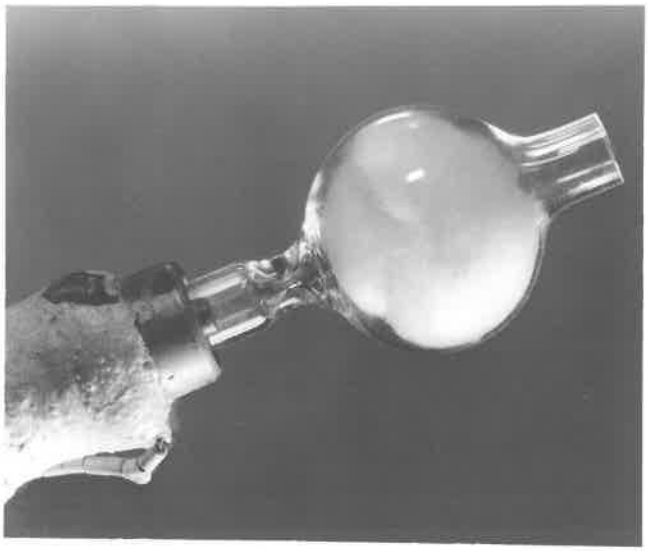


Fig.5 Apparatus used for collecting the eluant vapour containing ^{14}C labelled fatty acids after gas liquid chromatography. The calcium chloride tube is fitted into the outlet tube by means of a silicone rubber joint.

reduced to a smaller volume (approximately five ml) using a rotary evaporator. This solution was passed through a column of ten g of AG 50 W (H^+) (200-400 mesh, Bio-Rad, Laboratories). After washing with distilled water, approximately 80 ml of eluent was collected and evaporated to dryness in a rotary evaporator. The glycerol remaining was dissolved in 3.5 ml of pyridine and purified as the glycerol tribenzoate by the method of Rose, Kellermeyer, Stjernholm and Wood (1962), the amounts being scaled down for this preparation. Details are as follows. 2.4 ml of benzoyl chloride was added dropwise to the pyridine solution (in a 50 ml centrifuge tube) which was kept at 0° in an ice bath. The mixture was left in the ice bath for 20 minutes and after this time 5.5 ml of ice cold water was added to the mixture, which precipitated the glycerol tribenzoate as a yellow oil. The mixture was centrifuged, the aqueous solution removed and the oil washed twice with 4.4 ml of water, then once with 5.5 ml of 0.2 N hydrochloric acid. A stirring rod was used each time to disperse the oil. The oil was then dissolved in a minimal amount of hot ethanol (usually only a few ml) and then allowed to cool. Crystallization occurred after leaving in the refrigerator at 2° for 24 hours. However when crystals failed to appear, the sides of the centrifuge tube were scratched vigorously which always induced crystal formation.

The crystals were then washed with 1.5 ml of 80% methanol (in the cold in order to prevent the crystals melting). The glycerol tribenzoate was hydrolysed by adding 13.6 ml of 1N ethanolic potassium hydroxide and heating at 60° for 80 minutes in a water bath.

Ten ml of water were added and the ethanol carefully removed by evaporation. The aqueous solution remaining was acidified and the benzoic acid removed by extracting five times with five ml of diethyl-ether. After neutralization with alkali, the solution was deionised by passing through Dowex 1 Cl^- and then through AG 50 W (H^+) as described previously. The solution was evaporated to dryness using a rotary evaporator and finally dried over P_2O_5 in a vacuum dessicator.

The glycerol was taken up in five ml of redistilled ethanol and 0.5 ml was counted by scintillation counting. To check recovery the glycerol was determined quantitatively before and after purification. Generally, about 70% was recovered and correction was made for the loss in determination of the ^{14}C incorporated into the glycerol.

6. Quantitative Separation of Glycerol and Glycerophosphate.

The isolation of glycerol as the tribenzoate derivative is only satisfactory for a quantitative recovery of neutral glyceride glycerol. Phospholipids yield mainly glycerophosphate even after harsh hydrolysis and this is effectively removed during the initial attempt at purification of the glycerol by ion exchange chromatography. Another disadvantage is the long time required for the isolation. In order to quantitatively separate glycerol and glycerophosphate for radio assay the following method was used.

The macrophage lipid extract was hydrolysed with 1.7 N HCL in methanol as described above. After removal of the lipid the aqueous phase was concentrated to a small volume (approximately 50 µls) in vacuo. This was hastened by placing the tubes in warm water. The residue was spotted onto Whatmann No.1 filter paper and separated by descending chromatography using the solvent system n-propanol: ethyl acetate:water (7:1:2 V/V/V) (Polonovski, Payaant, Wald 1962). Using this system it was possible to separate glycerol from glycerophosphate and then to detect the glycerol with ammoniacal silver nitrate (Partridge 1948) and the glycerophosphate with the molybdic acid spray for phosphate esters (Hanes and Isherwood 1949) as modified by Bandurski and Axelrod (1951). The papers were cut into 1½" strips and scanned using a Nuclear Chicago 4 II Actigraph Paper Scanner. Only two peaks, identified as glycerol and glycerophosphate were labelled. The areas of each were determined by triangulation, and combined. The recovery in these two peaks varied between 80-90% of the total counts in the aqueous phase. This small loss was presumably due to the extreme volatility of glycerol. A scan is shown in Fig.6 which is representative of scans obtained in each experiment.

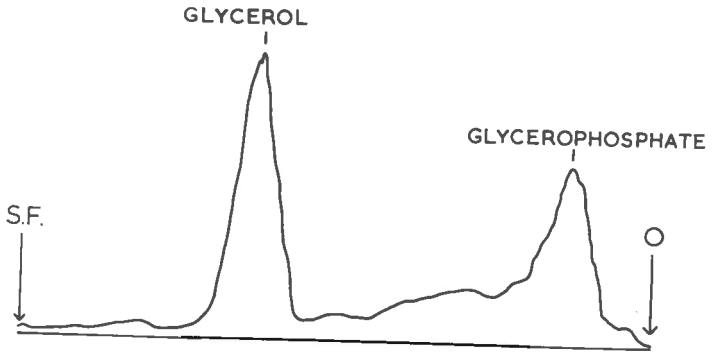


Fig.4 A scan of radioactivity obtained after separation of ^{14}C-labelled glycerol and glycerophosphate by paper chromatography.
Solvent system: n-propanol:ethyl acetate: water (7:1:2; v/v/v).
Scan Details: 45"/hr : $\frac{1}{2}$" slit.
Sensitivity: 0-100
Mean probable error 5%.

H. Chromatographic Methods

1. Paper Chromatography.

Lipids were separated on silicic acid impregnated paper using the solvent systems described by Marinetti, Erbland and Kochen (1957), Marinetti and Stotz (1960) and Marinetti (1961). Whatman No.1 filter paper was cut into sheets of two sizes, viz., 19 x 22 cm and 54 x 19 cm and dipped into a solution of sodium silicate (104g of silicic acid dissolved in 330ml of 7.2 N Sodium Hydroxide). The papers (impregnated individually in batches of fifteen) were hung for five minutes to remove excess sodium silicate and then immersed in 6N hydrochloric acid for 30 minutes. They were then washed in running tap water for two hours and finally in distilled water for one hour and allowed to dry at room temperature.

Separation of the five major classes of lipids (cholesterol, cholesterol ester, di, and tri-glycerides, phospholipid and fatty acid), was best performed in two quart wide mouthed Kilner (preserving) jars. The developing solvent used was light petroleum (b.p. 60-80°): diisobutylketone (96:6 V/V) and the papers were developed by ascending chromatography for 1½-1¾ hours. After this time, the papers were dried at room temperature and either exposed to X-ray film for the preparation of radioautographs, or dipped in 0.001% Rhodamine 6G and viewed under ultra-voilet light, where the lipid was detected as yellowish fluorescent spots.

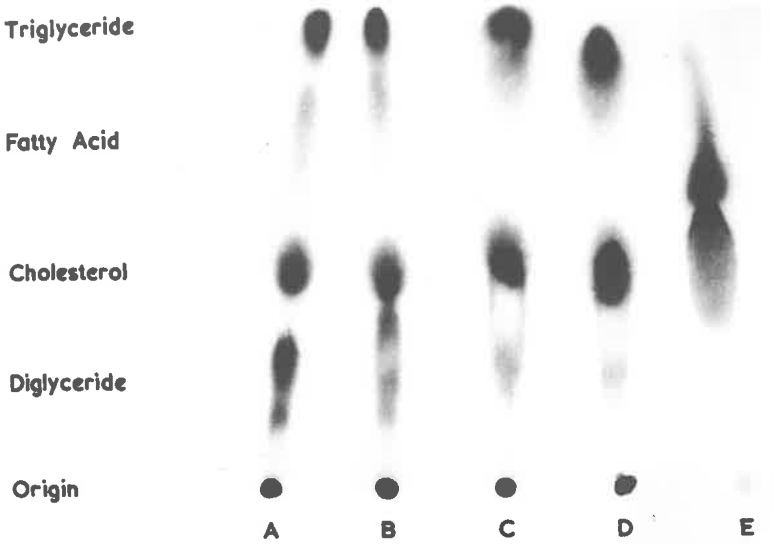
The loading capacity of the paper when separating neutral lipids was investigated by the use of radioautography and the

results of such an investigation are shown in Fig.7. It can be seen that when the loading capacity exceeds 30 µg per spot, the separation becomes unsatisfactory and the spots overlap. Therefore the amount of lipid per spot was always kept below 30 µg.

Separation of the phospholipids was also possible using silicic acid impregnated paper. The solvent system consisted of diisobutylketone, acetic acid and water (40:25:5 V/V/V). In some cases papers were developed with this solvent system in the proportion 40:20:3 or 40:30:7. Papers were supported by glass rods and developed for 16-20 hours by ascending chromatography in large chromatographic tanks.

2. Radio-Chromatography of ^{32}P-labelled Phospholipids.

In experiments in which ^{32}P-labelled phosphate was incorporated into lipid it was necessary to identify the phospholipids and to measure the amount of ^{32}P incorporated into each phospholipid. This was done as follows. The lipid extracts, after small portions had been removed for determination of phospholipid phosphorus and for radioassay of ^{32}P, were divided into two portions, evaporated to dryness and chromatographed on silicic acid impregnated paper to separate phospholipids. After development for sixteen to eighteen hours the papers were cut into strips (1½" wide) and scanned on a Nuclear Chicago 4 π Actigraph Paper Chromatograph Scanner. The areas of the peaks obtained were determined by triangulation which gave a quantitative assessment of the distribution of counts.



Radioautograph demonstrating the effect of Increasing the amount of Lipid on the Separation of Lipid Components by Silicic Acid Paper Chromatography.

Solvent System — Petroleum Spirit (60°-80°C) [96]
Di-isobutyl Ketone [6]

A. 3c.p.s./ 15µg./spot.
B. 3c.p.s./30µg./spot.
C. 3c.p.s./65µg./spot.
D. 3c.p.s./115µg./spot.
E. 3c.p.s./515µg./spot.

Fig.7 Separation of increasing amounts of neutral lipids by paper chromatography on silicic acid impregnated paper.

Radioautographs were prepared from each strip, indicating the position of each compound. In some experiments these zones were cut out, duplicates combined and extracted with four ml of 1 N hydrochloric acid in methanol for thirty minutes at 60°. However it was found that some of the labelled spots did not provide enough phosphorus for accurate estimations and specific activity determination by this method was not used. Specific activity of individual phospholipids was therefore expressed as cps of individual phospholipid per µg of total lipid phosphorus in the extract.

3. Identification of Labelled Phosphatides.

In order to identify the ^{32}P-labelled phospholipids a mixture of standard phospholipids was made. This contained equal amounts of phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine, inositol phosphatide, sphingomyelin and lysolecithin, and the position of each component separated by the solvent system (diisobutyl ketone:acetic acid:water; 40:25:5 V/V/V) was carefully marked. A comparison of Rf values of the radioactive spots with those of the standard mixture could then be made. The identification of the various compounds in the standard mixture was made by comparing their mobilities with those of the individual reference compounds run singly (Fig.8).

In addition the radioactive spots were identified by specific spot tests (described later). The identification of the labelled compounds was also confirmed by cochromatography (Hokin and Hokin

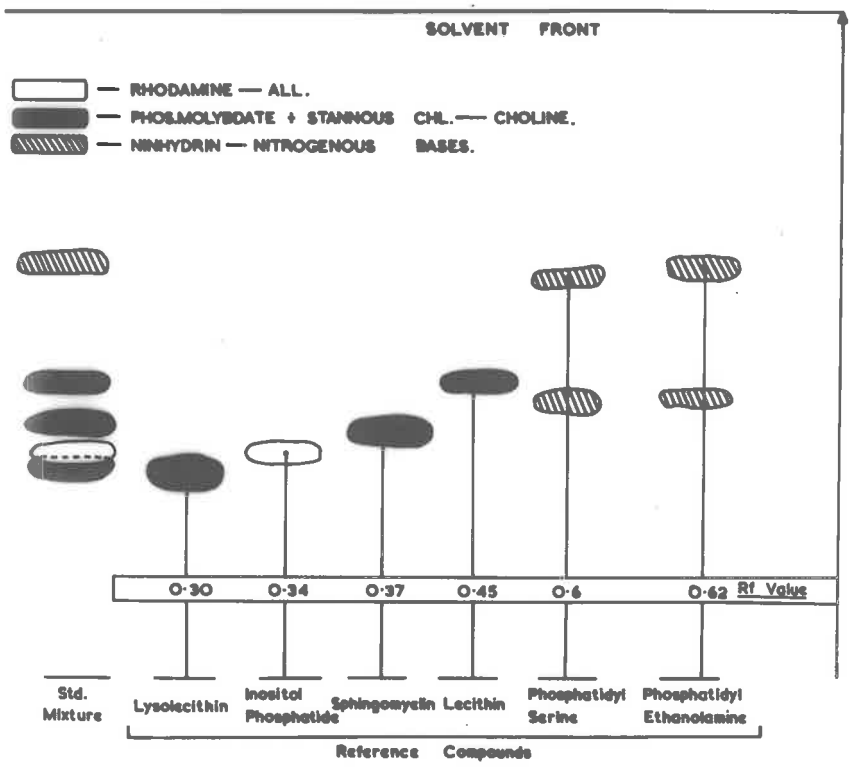


Fig.8 Separation and identification of phospholipids on silicic acid impregnated paper.
Solvent: diisobutyl ketone : acetic acid : water - 40:25:5.

1958). In this procedure the radioactive band was cut out and eluted at 37° with three five ml portions of chloroform:methanol:water (75:25:2 V/V/V) for three successive twenty four hour periods. Corresponding bands from several papers were combined. The eluates were pooled and centrifuged to remove particles derived from the paper. The solvent was transferred to clean tubes and removed under a stream of nitrogen and the residue was cochromatographed with an authentic sample of a reference phospholipid. The radioactive compounds were visualised by autoradiography and the reference compounds by specific spot tests as described below. If the two zones coincided accurately, the radioactive material was considered to be identical with the reference compound.

4. Detection of Compounds and Specific Staining Reactions.

All lipids could be detected by staining with Rhodamine 6G and the chromatograms were immersed in a 0.001% solution of Rhodamine 6G for two minutes and then observed while still wet under ultra-violet light and the spots outlined in pencil. Most lipid spots gave a yellowish fluorescence, but inositol phosphatide fluoresced with a distinct bluish color. For the detection of the amino phosphatides, 0.25% ninhydrin in acetone:lutidine (10:1 v/v) was sprayed onto the papers which were allowed to dry at room temperature. The amine phosphatides were detected by the appearance of a blue color. Choline containing phosphatides were demonstrated using the phosphomolybdate-stannous chloride reagent. The chromatograms were first washed with

distilled water for ten minutes and then immersed in a 1% aqueous solution of phosphomolybdic acid for ten minutes. The papers were then washed three times for ten minutes each in distilled water and then immersed in a 1% solution of stannous chloride in 3 N hydrochloric acid. Spots which contained choline appeared blue (Marinetti 1962).

5. Column Chromatography.

The separation of lipids on silicic acid columns was carried out using the method of Hirsch and Ahrens (1958). In order to reproduce the stepwise elution pattern described as Scheme A of the above authors, it was necessary to carry out the following procedures. 18g of silicic acid (obtained from the Bio-Rad Laboratories Richmond, California) was taken from a batch of absorbent which had been heated at 115° overnight and dusted into the jacketed columns. The constant temperature circulation was then started immediately and the silicic acid activated further by dehydrating washes with ten ml of diethyl ether (distilled over sodium); 30 ml of acetone-diethyl ether (1:1 v/v) and 20 ml of diethyl ether. The dehydrating solvents were removed by slowly washing the column with light petroleum (b.p. 60-80°) overnight and on the following day, the column was loaded with the sample in approximately ten ml of light petroleum. When the charge had passed into the column, the polarity of the solvent was increased by abrupt stepwise changes, the order of which are shown in Table 1. Initially the order of elution of lipids was established

TABLE 1

The Stepwise elution Scheme A of Hirsch and Ahrens which was used to fractionate lipids by silicic acid column chromatography.

Fraction	Solvent	
I	1% diethyl ether in light petroleum (b.p. 60-80°)	50 ml
II		75 ml
III a		225 ml
b	4% diethyl ether in light petroleum (b.p. 60-80°)	60 ml
IV a		240 ml
b	8% diethyl ether in light petroleum (b.p. 60-80°)	200 ml
V a		450 ml
b	25% diethyl ether in light petroleum (b.p. 60-80°)	50 ml
VI		200 ml
VII	diethyl ether	300 ml
VIII	absolute methanol	400 ml

.....

by running five separate columns, each loaded with a known amount of lipid. The eluant was collected in fractions and each fraction was evaporated to dryness and weighed. In addition portions were taken for determination of triglyceride, free fatty acid, cholesterol and phospholipid phosphorus. Another column was loaded with a mixture of 25 mg each of the lipids mentioned above, and approximately 20 ml fractions were collected. These were evaporated to dryness and the contents weighed.

Separations based on this gravimetric analysis are shown in Figs. 9a and b. Fig. 9a illustrates a separation on silicic acid which was not heated overnight at 115° and it can be seen that the lipids are eluted from the column before the start of each fraction change. Fig. 9b illustrates how this was corrected by activating the silicic acid overnight at 115° and the elution pattern is essentially the same as that reported by Hirsch and Ahrens.

Since it was necessary to separate fatty acid from triglyceride in some experiments, one column was loaded with approximately equal amounts of non-labelled tripalmitin and palmitic acid and also palmitic acid-1-^{14}C. Each tube collected after separation was concentrated to dryness, transferred to planchets and counted by G.M. counting. After weighing, the contents of each planchet were assayed for esterified fatty acid. The results of these assays are shown in Fig. 9c. The triglyceride and fatty acid peaks were extremely close however and for the sake of reproducibility, another column was loaded with a mixture of glyceryl tri-(palmitate-1-^{14}C) and palmitic acid-

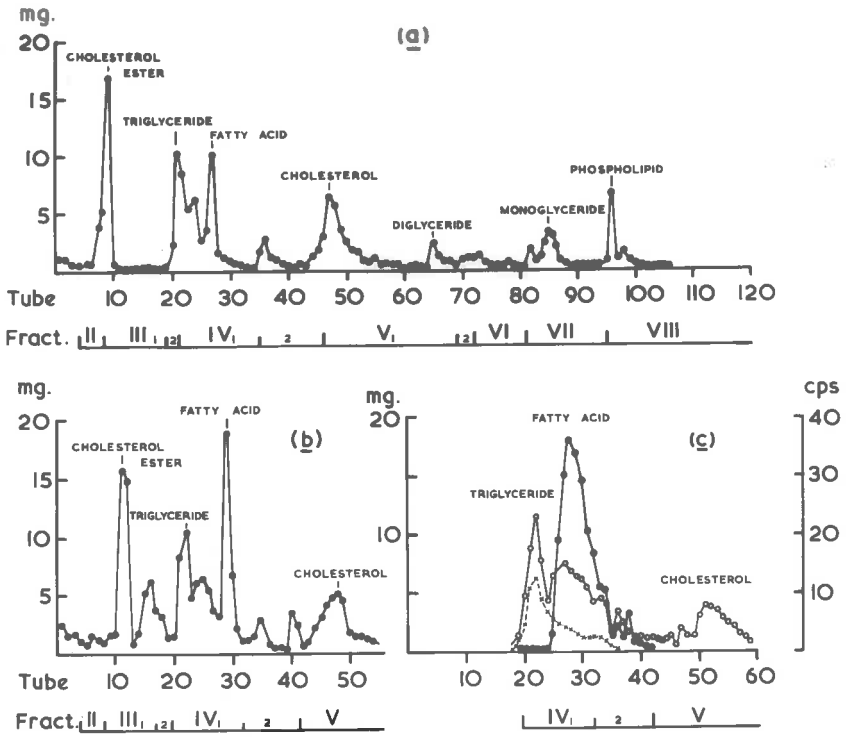


Fig.9a Gravimetric analysis of tubes following fractionation of known lipid mixture on non activated silicic acid columns.

b As in a, except that silicic acid was activated at 115° overnight.

c Separation of mixture of non labelled tripalmitin, palmitic acid and ^{14}C palmitic acid. Each tube assayed:

o____o gravimetrically

●____● G.M. counting

x----x esterified fatty acid

1-^{14}C, plus ten mg of each (non-radioactive) as carrier. The separation is shown in Fig. 10. In experimental runs because of the small quantities of lipid extracted from the macrophages, it was necessary to add ten mg each of cholesterol oleate, glyceryl tripalmitate, palmitic acid, cholesterol and lecithin as carrier.

The tubes obtained after fractionation were evaporated to dryness and the contents quantitatively transferred to weighed planchets by three 0.4ml washings with light petroleum, the solvent being evaporated between each transfer by means of a heat lamp. After G.M. counting, the planchets were weighed for self absorption correction.

In later experiments the fractions were radioassayed by liquid scintillation counting. In this procedure, the tube contents after evaporation to dryness were transferred by three 0.5ml light petroleum washings to counting pots containing the liquid scintillator.

6. Separation of Fatty Acids by Gas Liquid Chromatography.

a. Super Dry Methanol.

This was prepared by refluxing 50-75ml of methanol with five g of clean dry magnesium turnings and 0.5g of resublimed iodine in a two litre round bottomed flask. The mixture was warmed on a water bath until the iodine disappeared. 900ml of methanol was then added and the mixture refluxed for thirty minutes. The product was then distilled with the exclusion of moisture (calcium chloride tube) the first 20-30ml of distillate being discarded.

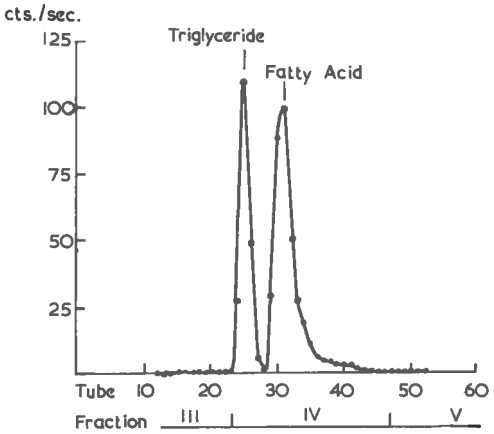


Fig.10 Separation on an 18g silicic acid column of a standard mixture of tripalmitin-1-^{14}C and palmitic acid-1-^{14}C. Ten mg each on non-labelled tripalmitin and palmitic acid added as carrier.

b. Dry HCl/Methanol.

Hydrogen chloride gas was evolved from ammonium chloride by concentrated sulphuric acid in a Kipps' apparatus. It was dried by passing through concentrated sulphuric acid and then collected in a known volume of weighed dry methanol. 5% HCl in methanol was obtained by calculating the weight gain.

c. Preparation of Methyl Esters of Fatty Acids.

(Stoffel, Chu and Ahrens 1959). The fatty acids of esters to be methylated (1-10mg) were dissolved in 0.5ml of dry benzene in a 15 ml micro-sublimation tube and four ml of 5% HCl in methanol was added. The mixture was refluxed at 80-100° for two hours with frequent shaking at the start to dissolve the lipid mixture. A calcium chloride moisture trap was used to prevent water from entering the mixture. After this procedure the mixture was cooled to room temperature, two volumes of water were added and the methyl esters were extracted three times with three ml of light petroleum (b.p. 60-80°). The pooled extracts were dried over a mixture of sodium sulphate:sodium bicarbonate (4:1 w/w) for one hour and then the esters were quantitatively transferred with light petroleum (b.p. 40-60°) to a second microsublimation tube. The contents of this tube were evaporated to dryness by a stream of nitrogen in a water bath. The microsublimation tube was then fitted to a cold finger and a vacuum of 0.2 $\pm$ 0.15 mm Hg was produced. Iced water at approximately 5° was pumped through the cold finger and the tube

was lowered into a water bath at 60 ± 2° for one hour. After this time the tube was allowed to cool, the assembly was disconnected, and the sublimed methyl esters, now collected on the cold finger, were carefully washed off into a glass stoppered tube with light petroleum (b.p. 40-60°). By removing the solvent the preparations were ready for the gas liquid column but they could be stored in light petroleum (b.p. 40-60°) in the refrigerator until required.

d. Preparation of Gas Liquid Chromatography Columns.

i. Apiezon M. One g of Apiezon was dissolved in 30-40ml of diethyl ether. This was transferred to a round bottomed flask and nine g of Embacel (100-120 mesh) was poured into the flask with gentle shaking. The solvent was then removed in a warm water bath and finally dried off in an oven at 100°.

ii. Ethylene Glycol Adipate Polyester. One g of E.G.A. was dissolved in 40 ml of methylene chloride to which was added nine g of Embacel (100-120 mesh) and the same procedure as described for the Apiezon preparation was carried out.

iii. Packing of Columns. The column, made of Pyrex was a 4ft. x 4mm bore column, with a sintered glass disc fitted into the bottom. After a piece of glass yarn had been pushed to the bottom the column was carefully packed with the prepared compound using a vibrator, or by gently tapping the column with a ruler. Only a small amount was added each time, with packing after each addition until the packing was about five cm from the top. A glass yarn plug was placed

on the top of the packing. The columns were then preheated by allowing the temperature of the heating jacket to remain above the maximum specified for the operational temperature, with a continual flow of Argon passing through them. This preheating was carried out in a specially provided side tube within the heating jacket so that any "bleeding" of the columns brought about by the preheating procedure would not contaminate the ionization detector. The Apiezon columns were preheated at 200° for 24 hours and the P.E.G.A. columns at 225° for 48 hours before use. The instrument used in this laboratory was a Pye Argon Chromatogram 12001 which incorporates an ionization detector based on the principle described by Lovelock (1958). The procedure for calibration of the columns has been described.

I. Radioactive Assay

1. G.M. Counting.

In earlier experiments with ^{14}C, G.M. counting was adopted using a thin mica end window G.M. tube for measuring the activity of samples labelled with ^{14}C. Most of the samples counted by this means were lipid and were therefore dissolved in lipid solvents. A known amount of the samples was plated onto aluminium planchets, (duplicates were taken whenever possible) and the solvents were evaporated using a heat lamp. For counting aqueous suspensions

of lipids a measured amount was pipetted onto the planchet to which 0.1ml of 0.1% Teepol had been added to facilitate spreading of the sample. The sample was then mixed with the Teepol and spread over the well of the planchet, a lens tissue of the same diameter as the well being carefully placed on the top (Entenman, Lerner, Chaikoff and Dauben 1949). The samples were counted at an efficiency of approximately 3%, sufficient activity being counted to give an error of less than $\pm$ 3% in all but the very low activity samples. When necessary the planchets were weighed before and after addition of the samples to allow a correction for self absorption. Self absorption curves were prepared for correcting samples containing cholesterol, triglyceride and fatty acid (Fig.11).

2. Scintillation Counting.

In later experiments all counting was done by liquid scintillation counting using an Ecko N662 Liquid Scintillation Counter. Two liquid scintillators were used.

a. 2:5 diphenyloxazole (0.3% w/v) in toluene was used for lipid extracts and column eluates.
Counting efficiency - 75%.

b. 2:5 diphenyloxazole (0.4% w/v) in dioxane as described by Bray (1960) was used for aqueous preparations at a counting efficiency of 67%.

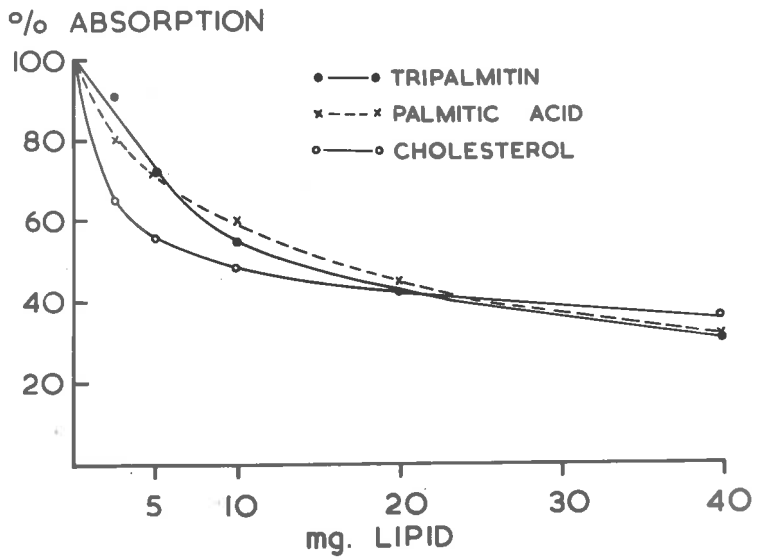


Fig.11 Self Absorption curve for correction in radioassay of ^{14}C lipids by G.M. counting.

J. Assay Procedures

1. Determination of Cholesterol. (Zlatkis, Zak and Boyle 1953)

In some determinations the same procedure as described by the above authors were used. In others however, (specific activity measurement) the method was slightly modified to accommodate smaller samples. For the ordinary method, the cholesterol extracts (between 50 and 100µg) were evaporated to dryness and dissolved in three ml of glacial acetic acid in 1" x 6" test tubes. The color reagent was prepared as follows. 0.5ml of 10% ferric chloride ($FeCl_3 6H_2O$) in glacial acetic acid was added to a 250ml conical flask, mixed with a few ml of concentrated sulphuric acid. The reagent was at first slightly yellowish, but turned colorless later. Two ml of color reagent were added carefully down the side of the tubes containing the samples so that two layers were formed. These were mixed thoroughly to obtain even heat distribution and allowed to cool. Standards (which contained 50, 100 and 150µg) were included: a blank with three ml of acetic acid alone was also prepared. The red color which developed was read against the blank at 550mµ in a Hilger Bio Chem. H 810 Absorptiometer. The samples were poured carefully into the cuvettes to avoid bubbling and stream formation. In the more sensitive method, the same procedure was carried out, except that half quantities of all the reagents were used. Standards containing 25µg, 50µg and 75µg of cholesterol were used and this method

was sensitive enough to detect cholesterol in the order of 10-20 µg per sample. Standard curves are shown in Fig.12a and b.

2. Determination of Total (Esterified) Fatty Acid.

(Stern and Shapiro 1953)

Extracts of macrophages were made in ethanol:ether (3:1 v/v) both of these reagents being redistilled before use. Three ml of the extract was pipetted into $\frac{3}{4}$" test tubes for the reaction; a blank containing three ml of ethanol:ether (redistilled) was also included. 0.5ml of hydroxylamine hydrochloride (14% in water) and 0.5ml of 3.5 N sodium hydroxide solution was added the tubes mixed, stoppered and allowed to stand for twenty minutes. After this time 0.6ml of 3.5 N hydrochloric acid was added, mixed and followed by 0.5ml of 0.37 M ferric chloride in 0.1 N hydrochloric acid. The color developed was read at 520mµ in a Hilger Biochem. Absorptiometer and standards were set up which contained 1, 2 and 3µmoles of fatty acid (see Fig.13).

This method measures both triglyceride and phospholipid esterified fatty acid but not cholesterol ester fatty acid. Large quantities of cholesterol ester interferred with the reaction causing a turbid final solution. Where this occurred it was cleared by adding up to 1.5ml of diethyl ether after the reaction had been completed. The sample was then corrected for dilution.

3. Determination of Free Fatty Acid. (Grossman 1955)

The lipid extracts were evaporated to dryness and two ml of 95% ethanol were added. The tubes were heated in a water bath at

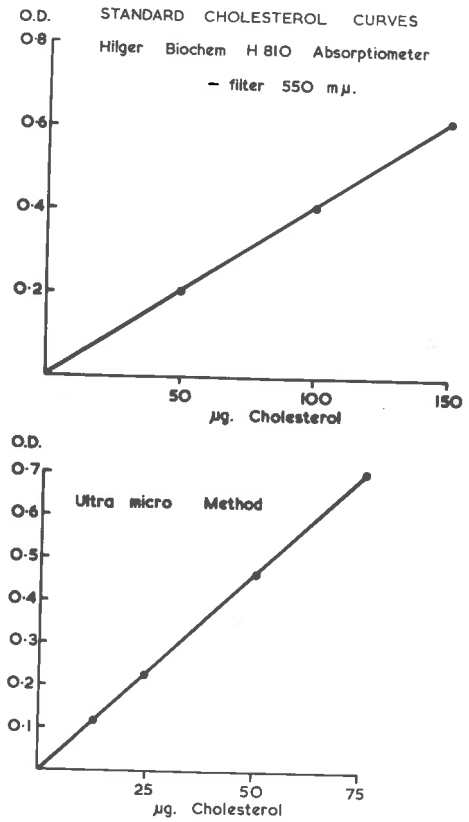

O.D.
STANDARD CHOLESTEROL CURVES
Hilger Biochem H 810 Absorptiometer
– filter 550 mμ.
0·8
0·6
0·4
0·2
50
100
150
μg. Cholesterol
O.D.
0·7
0·6
0·5
0·4
0·3
0·2
0·1
Ultra micro Method
25
50
75
μg. Cholesterol

Fig.12

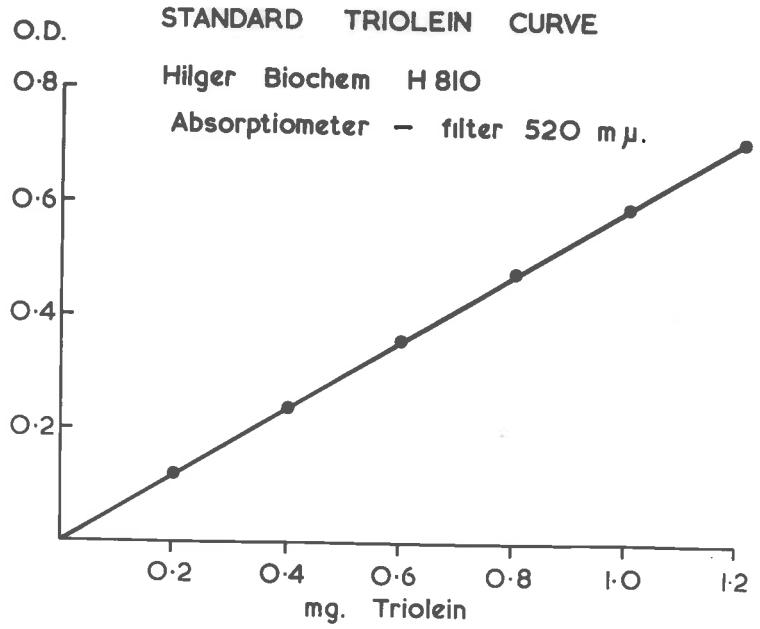

O.D.
STANDARD TRIOLEIN CURVE
Hilger Biochem H 810
Absorptiometer – filter 520 mμ.
0·8
0·6
0·4
0·2
0·2
0·4
0·6
0·8
1·0
1·2
mg. Triolein

Fig.13

90° for one minute and titrated while still hot with 0.02 N aqueous sodium hydroxide using thymol blue as indicator. A micro-buret was used for the titration and CO_2 free air was bubbled through the samples during titration. A water blank was included with each determination.

4. Determination of Lipid Phosphorus. (Brown 1954)

Three ml of the macrophage lipid extracts were evaporated to dryness in calibrated ten ml test tubes. 0.53ml of 11.7 N perchloric acid was added to the residue and allowed to digest on a sand bath at 250°, for 30 minutes or until the contents had become colorless. Then four ml of the color reagent was added and the volume made up to ten ml and shaken. This was placed in a boiling water bath for five minutes and read at 820mμ in a Unicam Spectrophotometer. The color reagent was prepared by dissolving (a) 60mg hydrazine sulphate in 1 N perchloric acid (b) 1.24g of sodium molybdate in 1 N perchloric acid. These solutions were dissolved individually by warming and then pooled and made up to 200ml with 1 N perchloric acid. Standards containing 2.5, 5, 7.5 and 10μg of phosphorus (from stock disodium hydrogen phosphate 100μg/ml) and blank tubes were prepared with 0.53ml perchloric acid (11.7 N) and these were also placed in the boiling water bath for five minutes. The Standard Curve is shown in Fig.14.

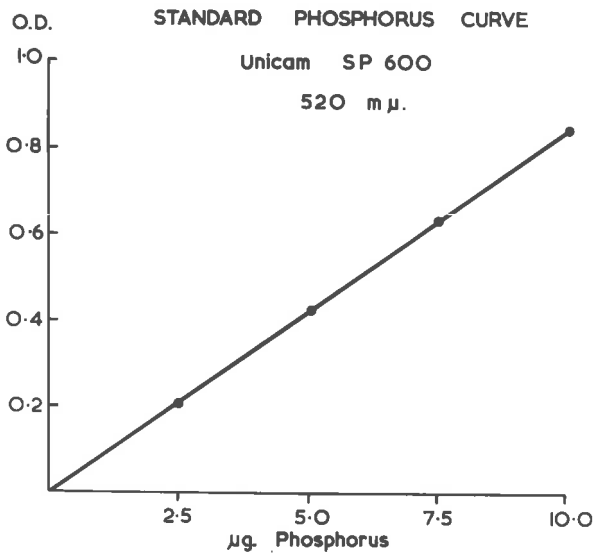

STANDARD PHOSPHORUS CURVE
Unicam SP 600
520 mμ.
O.D.
1·0
0·8
0·6
0·4
0·2
2·5
5·0
7·5
10·0
μg. Phosphorus

Fig.14

5. Determination of Glycerol. (Van Handel and Zilversmit 1957)

The procedure was based on a modification of the Lambert and Neish method (1950) which is sensitive enough to measure .005 µmole of glycerol. Approximately 0.1ml aliquots (or samples containing 5-10µg of glycerol) were pipetted into 0.1ml of 0.05 N sulphuric acid in ten ml marked test tubes ($\frac{3}{4}$"). 0.1ml of 0.05 N sodium periodate was added, left for five minutes, followed by 0.1ml of 0.5 N sodium arsenite to reduce the excess periodate. After an additional ten minutes, nine ml of chromotropic acid reagent was added. This reagent was prepared by dissolving one g of 1:8 dihydroxynaphthalene-3, 6-disulphonic acid, in 100ml of distilled water. A solution of sulphuric acid, prepared by the addition of 300ml of concentrated sulphuric acid to 150ml of water followed by cooling was added to the sulphonic acid solution to make 500ml. This reagent was stored in a brown glass stoppered bottle and prepared fresh every two or three weeks.

Following the addition of color reagent the tubes were stoppered and placed in a boiling water bath for 30 minutes away from the direct light. The cooled tubes were adjusted to a volume of ten ml with water and the absorbancy read at 570mµ in a Unicam Spectrophotometer. A Standard Curve (Fig.15) was prepared by measuring the optical density of standards containing 2.5, 5 and 7.5µg of glycerol.

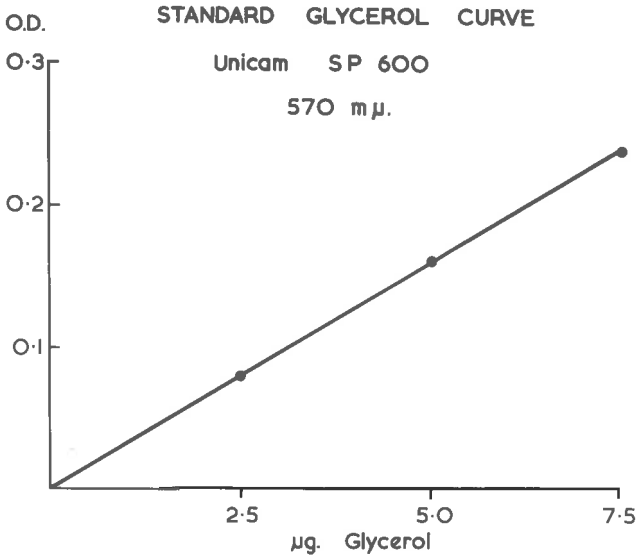

STANDARD GLYCEROL CURVE
Unicam SP 600
570 mμ.
O.D.
0·3
0·2
0·1
2·5
5·0
7·5
μg. Glycerol

Fig.15

6. Glucose Estimation.

The determination of glucose was carried out using the Somogyi-Nelson Method, (1944:1952). To measure the glucose concentration of Hanks':serum 2:1, one ml of the medium was pipetted into a $\frac{3}{4}$" test tube to which was added two ml of zinc sulphate (5%) and two ml of 0.3 N barium hydroxide. The tube contents were mixed and filtered through Whatman No.1 filter paper to remove the precipitated protein. Two ml of the filtrate or standard glucose solution was mixed with two ml of the Somogyi reagent (see reference), covered and boiled for ten minutes. After cooling to room temperature two ml of Nelson reagent was added, followed by shaking. The absorbancy was read at 530mu in a Unicam Spectrophotometer. The color was calibrated by reference to a standard curve (Fig.16) prepared on dilutions of the standard glucose solution (0, 20, 40, 60,120ug glucose).

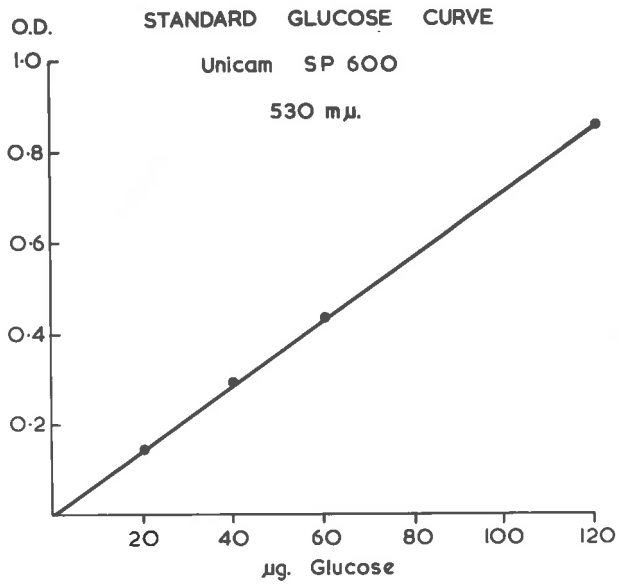

STANDARD GLUCOSE CURVE
Unicam SP 600
530 mμ.
O.D.
1·0
0·8
0·6
0·4
0·2
20
40
60
80
100
120
μg. Glucose

Fig.16

THE UPTAKE AND INCORPORATION OF SODIUM PALMITATE-1-^{14}C BY MACROPHAGES *in vitro*

A. Introduction

Evidence has been described which indicates that macrophages can participate actively in lipid metabolism and in particular that rabbit macrophages incubated *in vitro* can esterify cholesterol (Day 1960c; Day and Gould-Hurst 1961) and oxidise fatty acids and triglycerides to carbon dioxide (Day 1960a, 1961). There has been no evidence, however, of specific esterifying systems other than cholesterol esterase reported in macrophages and in order to obtain some information about the lipid synthesising systems in these cells, the fate of fatty acid following its uptake by macrophages was first investigated.

B. Uptake of Sodium palmitate-1-^{14}C.

Approximately 100×10^6 macrophages and 30 ml of medium (Hanks' serum, 2:1 v/v) were incubated at 37° with approximately 2μc of ^{14}C-labelled sodium palmitate-albumin solution in 20 oz. McCartney bottles. In order to investigate the influence of cholesterol on fatty acid incorporation into macrophages, 8mg of non-labelled cholesterol suspension were added to the medium in some experiments. After four or twenty hours incubation the cells and medium were separated and the lipid of both extracted with alcohol:ether (3:1 v/v). To determine the uptake of ^{14}C-labelled palmitate portions of both the cell and medium extracts were taken for G.M. counting. A portion of the lipid extracted from a control medium which was incubated with the substrate (palmitate-1-^{14}C) but no cells, was also counted.

Table 2 shows the results of these experiments. The recoveries of ^{14}C in the medium and cells is shown in the last two columns while the other columns summarize the experimental data. The percentage recovery of ^{14}C in the cells and medium is illustrated in Table 3. It can be seen that after a four hour incubation, the macrophages had taken up and incorporated intracellularly between 5 and 7% of the ^{14}C labelled sodium palm-

TABLE 2 The Uptake of Sodium Palmitate-1-^{14}C by Macrophages in vitro

Expt.	No. of Cells	Substrate	^{14}C Added cps	Incubation Time(hr)	Recovery of ^{14}C Cells cps	Recovery of ^{14}C Medium cps
G2	90×10^6	Palmitate-1-^{14}C	3220	4	214	2263
	90×10^6	Palmitate-1-^{14}C	3220	20	589	1905
	No cells	Palmitate-1-^{14}C	3220	20	-	2510
G4	37×10^6	Palmitate-1-^{14}C	2800	20	132	1877
	No cells	Palmitate-1-^{14}C	2800	20	-	2410
G5 A	63×10^6	Palmitate-1-^{14}C	3260	4	164	2455
	63×10^6	Palmitate-1-^{14}C	3260	20	535	2075
	No cells	Palmitate-1-^{14}C	3260	20	-	2680
B	63×10^6	Palmitate-1-^{14}C with 8mg cholesterol suspension added	3260	20	408	2200
	No cells	Palmitate-1-^{14}C with 8mg cholesterol suspension added	3260	20	-	2750
G6 A	90×10^6	Palmitate-1-^{14}C	3260	4	166	2090
	90×10^6	Palmitate-1-^{14}C	3260	20	693	1850
	No cells	Palmitate-1-^{14}C	3260	20	-	2420
	90×10^6	Palmitate-1-^{14}C with 8mg cholesterol suspension added	3260	20	389	2160
	No cells	Palmitate-1-^{14}C with 8mg cholesterol suspension added	3260	20	-	2660
G8	100×10^6	Palmitate-1-^{14}C	3525	4	254	+
	100×10^6	Palmitate-1-^{14}C	3525	20	506	-

+ Extraction of medium not carried out in this experiment.

54a

TABLE 3 Percentage Recovery of ^{14}C in the Cells and Medium after Incubation of Macrophages with Sodium Palmitate-1-^{14}C

Expt.	Incubation Time (hr.)	% recovery in cells	% recovery in medium	Total Recovery (%)
G2	4	6.7	70.5	77.2
	20	18.3	59.1	77.4
	20	+	78.0	78.0
G4	20	4.7	67.0	71.7
	20	+	86.1	86.1
G5	4	5.0	75.4	80.4
	20	16.4	63.7	80.1
	20	+	82.2	82.2
	20 chol.	12.5	67.5	80.0
	20 added	+	84.5	84.5
G6	4	5.1	64.1	69.2
	20	21.3	56.8	78.1
	20	+	74.3	74.3
	20 chol.	11.9	66.3	78.2
	20 added	+	81.6	81.6
G8	4	7.2	++	-
	20	14.3	++	-

\+ Controls - no cells present

++ Extraction of medium not carried out

itate added to the medium; after twenty hours incubation up to 21% of the sodium palmitate in the medium had been taken up. In the presence of cholesterol the total uptake was less but not significantly so. However it was noticed that more cells became detached from the glass in the presence of cholesterol so the difference is probably only a spurious one.

The total recovery of ^{14}C varied between 69.2-89.4% of the palmitate-1-^{14}C added to the medium at the start of the incubation, while the total recovery of ^{14}C in the control medium (no cells) varied between 78 and 86.1%.

C. Fractionation of Lipid Extracts

Of more interest was the incorporation of palmitate-1-^{14}C into the various lipids of macrophages and for this purpose, portions of the lipid extracts of both cells and medium were examined by paper chromatography on silicic acid impregnated paper. Two solvent systems were used, one of which separated the neutral lipids, the other separating the phospholipids. Radioautographs were prepared by exposing Ilfex X-ray film to the developed chromatograms in X-ray cassettes. The exposure varied from five to ten days. A mixture of radioactive marker standards was used to identify the neutral lipids and in the case of the phospholipid separation, non radioactive synthetic DL-dipalmitoyl lecithin was

run as a standard and its position determined by staining the paper chromatogram with Rhodamine 6G and marking the radioautographs. Figs.17 and 18 below represent radioautographs prepared from paper chromatograms of lipid extracts from experiments G2 and G6 respectively. In both, labelled triglyceride shows up strongly after four hours and in one radioautograph (Fig.17) the presence of labelled diglyceride is also shown after 20 hours. In G2 (20 hours) appreciable ^{14}C-labelled fatty acid can be seen in the cell extract in addition to the di- and triglyceride but in all other experiments, there was little remaining ^{14}C-labelled fatty acid in the cells. Fig.18 is a more representative radioautograph and little detectable ^{14}C unesterified fatty acid was present after either four or twenty hours incubation. The medium extracts in all cases showed up only ^{14}C-labelled free fatty acid and so also did the control medium (Fig.17).

Incorporation into phospholipid after four and twenty hour incubations is shown in Fig.19. In each case the fatty acid has been taken up by two phospholipid fractions (as separated by this method). The faster moving spot corresponds to lecithin (as compared to the mobility of the unlabelled marker) and the other is probably sphingomyelin, but definite identification was not attempted in these experiments. There was no labelled phospholipid present in any of the medium extracts.

More quantitative information about the incorporation of ^{14}C-labelled palmitate into the various types of lipid classes was achieved by chromatography on silicic acid columns. The separations of macro-

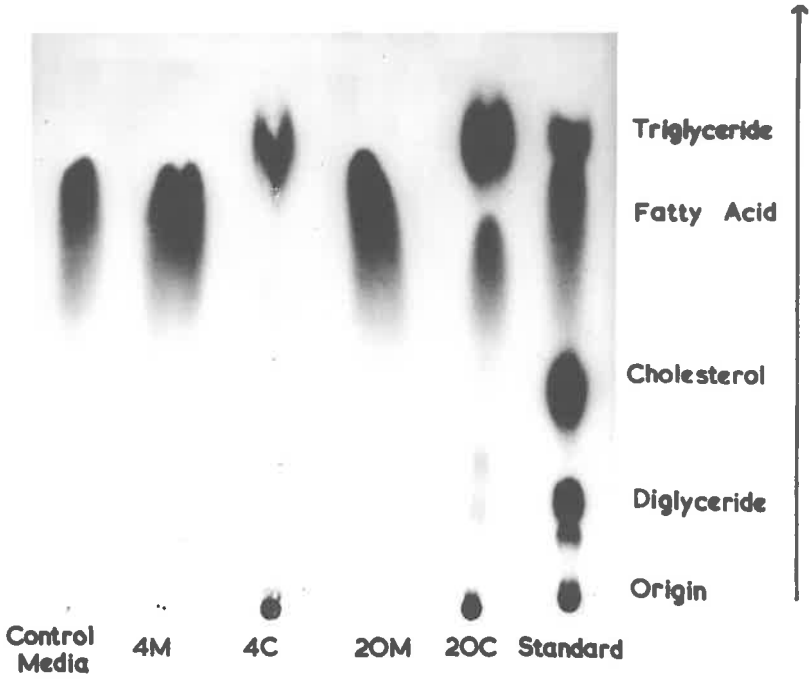


Fig.17 Fractionation of lipids from macrophages and medium extracts on silicic acid-impregnated paper. Radioautogram prepared from the chromatogram in Exp.62. Mobile phase: light petroleum -diisobutly ketone 96:6. Control media: incubation of sodium palmitate-1-^{14}C in the absence of cells. 4M and 4C: corresponding medium and cell extracts after 4-hr incubation with sodium palmitate-1-^{14}C, 20M and 20C: corresponding medium and cell extracts after 20-hr incubation with sodium palmitate-1-^{14}C. The presence of triglyceride and diglyceride in addition to unesterified fatty acid in the cell extracts is demonstrated.

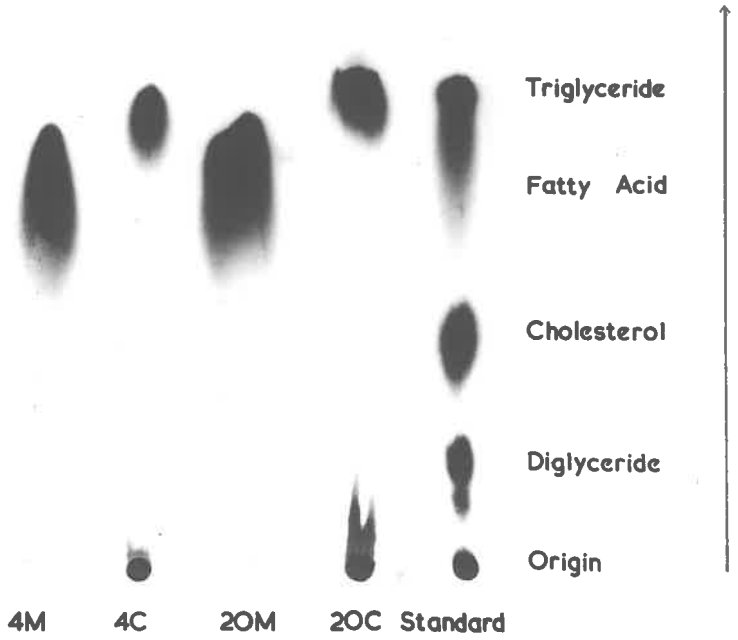


Fig.18 Fractionation of lipids from macrophage and medium extracts on silicic acid impregnated paper. Radioautogram prepared from the chromatogram in Exp.G6. Mobile phase: light petroleum-diisobutyl ketone 96:6. 4M and 4C: corresponding medium and cell extracts after 4-hr incubation with sodium palmitate-1-^{14}C. 20M and 20C: corresponding medium and cell extracts after 20-hr incubation with sodium palmitate-1-^{14}C. Mainly triglyceride is demonstrated in the cell extracts.

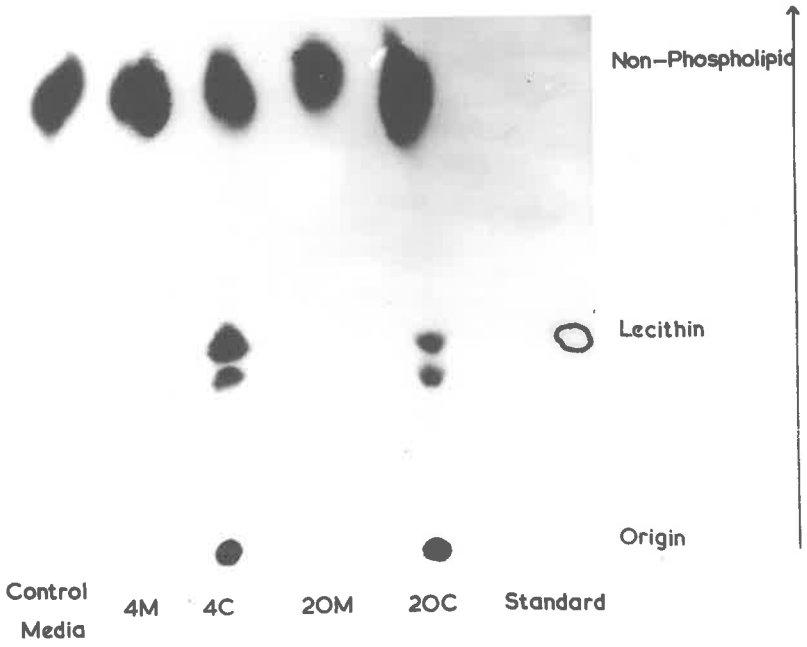


Fig.19 Separation of phospholipids from macrophage and medium extracts on silicic acid impregnated paper. Radioautogram prepared from the chromatogram on Exp.G2. Mobile phase: diisobutyl ketone-acetic acid-water 40:20:3. Control media: incubation of sodium palmitiate-1-^{14}C in the absence of cells. 4M and 4C: corresponding medium and cell extracts after 4-hr incubation with sodium palmitate-1-^{14}C. 20M and 20C: corresponding medium and cell extracts after 20-hr incubation with sodium palmitate-1-^{14}C. The position of the non-radioactive DL-dipalmitoyl lecithin as determined by staining with Rhodamine 6G is marked on the radioautogram. The presence of lecithin and one other undentified phospholipid in the cell extracts is demonstrated.

phage and medium lipid extracts on such columns is shown for one experiment (G6) in Fig.20. In the cell extracts there is a relatively large incorporation of ^{14}C-labelled palmitate into triglyceride and phospholipid with smaller amounts incorporated into the cholesterol ester, mono and diglyceride. Very little of the ^{14}C-labelled fatty acid remained after its uptake by the macrophages. The medium extract contained traces of mono- and diglyceride and of phospholipid but 99% of ^{14}C was still present as unesterified fatty acid. In these columns the elution of the palmitic acid peak occurred earlier in fraction IV than that shown for mixed non-esterified fatty acids by Hirsch and Ahrens (1958). However the reproducibility of this separation was exhaustively confirmed and the elution of labelled tripalmitin and labelled palmitic acid has already been recorded (Fig.10).

Fig.21A and B show the separation on silicic acid columns of lipids from macrophage and medium extracts in an experiment in which 8mg of non-labelled cholesterol (in suspension) were added to the incubation medium in addition to the labelled palmitate. The experiment was carried out at the same time and with the same batch of macrophages as that for the chromatogram above (Expt. G6 Table 2). The relative conversion to triglyceride and phospholipid was essentially the same and some incorporation of labelled fatty acid into cholesterol ester and into mono- and diglyceride also occurred.

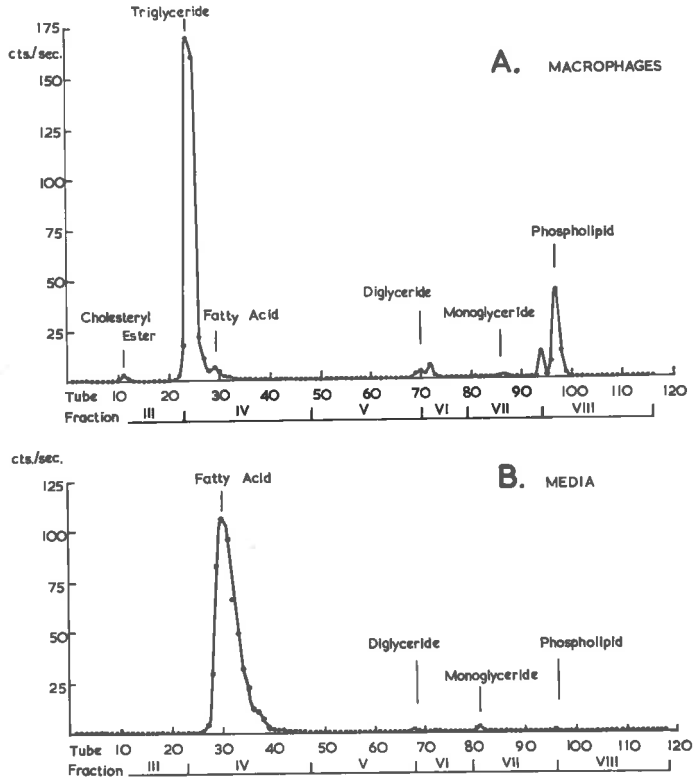


Fig.20 Separation on silicic acid columns of the lipid components of macrophages and medium after 20-hr incubation with ^{14}C-labelled sodium palmitate.
A. Macrophage extract. Column load: 630cps ^{14}C with 10mg cholesterol ester, 10mg tripalmitin, 10mg palmitic acid, 10mg cholesterol and 15mg lecithin added as carrier.
B. Medium extract. Column load: 500cps ^{14}C and carrier lipids as in A.

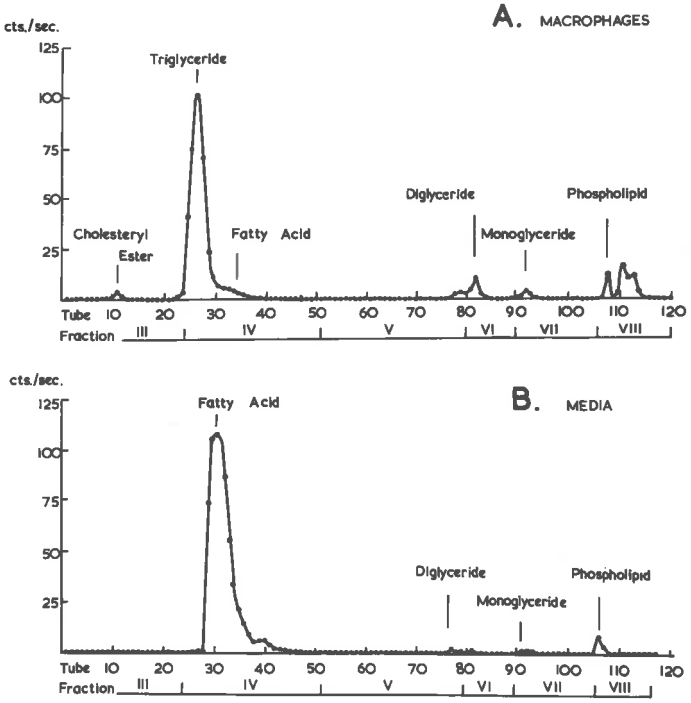


Fig.21 Separation on silicic acid columns of the lipid components of macrophages and medium after 20-hr incubation with ^{14}C-labelled sodium palmitate and non-labelled cholesterol suspension.

A. Macrophage extract. Column load: 570cps ^{14}C, with 11mg cholesterol ester, 18mg tripalmitin, 11mg palmitic acid, 11mg cholesterol and 18mg lecithin added as carrier.

B. Medium extract. Column load: 480cps ^{14}C and carrier lipids as in A.

D. Discussion

The recovery of ^{14}C from the control medium in all experiments is not quantitative, but it is reasonable to expect some loss during the many steps involved in the extraction procedure. However when compared with the total recoveries of cells and medium in corresponding experiments it becomes apparent that more activity is lost when cells are present with the palmitate-1-^{14}C during the incubation. This loss could probably be accounted for as $^{14}CO_2$. However, no determinations of $^{14}CO_2$ were made in these experiments because Day (1960a) has previously shown that macrophages incubated in vitro with sodium palmitate-1-^{14}C can oxidise the fatty acid with the production of $^{14}CO_2$.

The uptake of fatty acid and its conversion to triglyceride has been shown to occur in many other tissues than macrophages. Adipose tissue (Shapiro, Chowers and Rose 1957) liver slices (Masoro and Felts 1957) and ascites tumor cells (Fillerup, Knauf and Mead 1960) have all been shown to take up ^{14}C-labelled fatty acid from fatty acid albumin complexes. In the present experiments up to 21% of the fatty acid added to the medium has been taken up by the macrophages. The enzymic conversion of the fatty acid to triglyceride and phospholipid which has been shown to occur in the cells, would probably facilitate this uptake by transferring fatty acid to the water-insoluble phase. In fact the situation is

similar to that described by Shapiro, et.al. (1957) for adipose tissue incubated in vitro, where ready uptake of fatty acid and its conversion to triglyceride has been shown to occur, although there is no evidence of conversion of fatty acid to phospholipid in adipose tissue.

The enzymic conversion of fatty acid to triglyceride and phospholipid by macrophages requires more detailed study with intact cells as well as homogenates and sub-cellular components. For instance the data presented in these experiments does not indicate the sequential pathway of incorporation of labelled palmitate as it enters the macrophage; but as mentioned previously this work was carried out mainly to establish the presence of esterifying or lipid synthesising mechanisms in the macrophage. There have been several possibilities suggested for fatty acid incorporation into triglyceride and phospholipid. Many workers (Borgstrom, Borgstrom and Rottenburg, 1952; Stein and Shapiro, 1959) postulated that in liver, free fatty acid is first incorporated into triglyceride and then transferred to phospholipid. Although no specific mechanisms were proven, they suggested that trans-esterification might be involved. Neptune, Sudduth, Foreman and Pash (1960) showed that labelled palmitate was more rapidly incorporated into neutral lipid than into phospholipid of rat diaphragm and from these findings they postulated that triglyceride was a precursor of phospholipid. Later however they reported that they had incorrectly designated their neutral lipid fraction as

"triglyceride" and so their hypothesis proposed from earlier data was not tenable. In subsequent experiments with rat diaphragm Neptune, Sudduth, Colodzin and Reish (1962) convincingly demonstrated that it was in fact the diglyceride fraction which was rapidly labelled at first and with increasing incubation time, the specific activity of triglyceride gradually approached that of diglyceride. They also showed that the fatty acids of diglyceride were turned over at a much higher rate than those of the triglyceride. Finally they demonstrated that the specific activity of diglyceride always exceeded that of the glycerol phosphatides and that the specific activity of the phosphatides approached that of the diglyceride with increasing time of incubation. They concluded that their data was in agreement with Kennedy's proposition (Kennedy 1957) that diglyceride served as a precursor for either triglyceride or the glycerol phosphatides. The synthesis of both triglyceride and phospholipid by macrophages, together with the appearance of appreciable quantities of diglyceride also suggests a pattern of synthesis similar to that shown by Kennedy for rat liver.

The small amount of monoglyceride found in macrophages is possibly due to the enzymic breakdown of some of the triglyceride with the formation of mono- and diglyceride in addition to unesterified fatty acid. That lipase is present in macrophage homogenates has already been shown (Day and Harris 1960). However the presence of these smaller units cannot be enlarged upon. Within the cells there is surely an extensive turnover of glyceride components with

the fatty acid units being shuffled about in response to the cell requirements. The labelled fatty acid molecule either becomes esterified as a prerequisite for entry as it passes into the cell, or is phagocytosed by the macrophage and becomes part of the fatty acid pool either before or after esterification. Therefore the presence of labelled free fatty acid in some of the cell lipid extracts could be accounted for as part of the free fatty acid pool, the specific activity of which has increased after twenty hours.

It is significant that a small amount of the fatty acid taken up by the macrophages was incorporated into cholesterol ester. It is known that macrophages can bring about esterification of cholesterol and the present finding that exogenous fatty acids can be incorporated into the cholesterol ester renders it likely that the fatty acid composition of the cholesterol ester, produced by macrophages, can be influenced by varying the fatty acid available to the cell. This work has been further investigated by Day et.al. (1963) with rat lymph nodes. They showed that the cholesterol esters synthesised by reticulo-endothelial cells in rat lymph nodes were influenced by the type of fatty acid available. Where corn oil or coconut oil was administered together with cholesterol, the fatty acid pattern of the cholesterol esters synthesised became more unsaturated or more saturated respectively.

The uptake of a cholesterol suspension by the macrophages failed to influence appreciably the uptake of fatty acid and its incorpor-

etion into triglyceride or phospholipid. In other systems however, investigators, have shown that cholesterol influences lipid metabolism. Day (1960b) has shown that the uptake of cholesterol suspensions by reticulo-endothelial cells in rat lymph nodes was followed by triglyceride and phospholipid accumulation, presumably the result of synthesis by the cells. The phospholipid synthesis that occurs in the arterial wall (Zilversmit, Shore and Ackerman 1954) is also accelerated in cholesterol-fed rabbits as compared with normally fed animals.

The physiological role of macrophages regarding the uptake and metabolism of fatty acids is not clear. It has been suggested (Jaffe and Berman 1928; Friedman, Byers and Rosenman 1954) that reticulo-endothelial cells in the liver are involved in the uptake of lipid from the blood and its subsequent metabolism. Perhaps macrophages play a similar role in the tissues, e.g. the arterial wall, at least as far as removing fatty acids from the blood and storing them as cholesterol ester, glycerides and phospholipids.

THE UPTAKE AND INCORPORATION OF GLYCERYL TRI-(PALMITATE-1-^{14}C) BY MACROPHAGES *in vitro*

A. Introduction

Having shown that non-esterified fatty acid can be taken up and incorporated into triglyceride, phospholipid, cholesterol ester, mono- and diglycerides by macrophages it was considered worthwhile to investigate the fate of triglyceride following its ingestion by these cells. Many investigators have reported the uptake of triglyceride emulsions by the reticulo-endothelial system (see p.6 in INTRODUCTION) and some work has already been done on the oxidation of triglyceride by macrophages (Day 1960a).

The experiments to be described in this section are concerned with the intracellular incorporation of glyceryl tri-(palmitate-1-^{14}C), following its uptake by macrophages incubated *in vitro*. Other facets of triglyceride metabolism by macrophages have not been investigated and only a few experiments were performed in order to obtain additional information about the incorporation of esterified fatty acid as compared with unesterified fatty acid.

B. Uptake of Glyceryl tri-(palmitate-1-^{14}C)

Essentially the same techniques were employed as when palmitate-1-^{14}C was the substrate. Macrophages (approximately 90 x 10^6) were incubated with lpc tripalmitin-1-^{14}C emulsion (preparation described on p.16) in McCartney bottles at 37° for twenty hours. After the appropriate incubation time, the cells and medium were extracted with

alcohol:ether (3:1 v/v) and portions of these lipid extracts were counted by G.M. counting to determine the uptake of the tripalmitin-1-^{14}C. The results are shown in Table 4. It can be seen that there is much variation in uptake of tripalmitin-1-^{14}C, between the different cell batches which were all incubated for twenty hours. Previous determinations of the uptake of tripalmitin-1-^{14}C at four hours showed negligible activity in the cell lipid extracts. These four hour incubations were not pursued further due to the amount of tripalmitin-1-^{14}C required to increase the activity of the four hour samples and the radioassay procedure (G.M. counting) available at that time.

In Table 5 recovaries of ^{14}C in cells and medium extracts are expressed as percentages of the total tripalmitin-1-^{14}C added to the medium at the start of the incubation. The recovery in the cells varies between 1.2 and 10.5% and the medium extracts, when cells were present, accounts for approximately 51-76% of the label. The recovery when no cells were present is 98%.

C. Fractionation of Lipid Extracts

Some lipid extracts were fractionated by column chromatography on silicic acid in order to examine the incorporation of tripalmitin-1-^{14}C into other lipid classes. Figs.22 and 23 show the radioactive peaks obtained in such separations. In the cell extracts most of the label has remained in the triglyceride fraction with some activity in the phospholipid and diglyceride fractions. In the medium extract

TABLE 4 Uptake of Glyceryl tri-(palmitate-1-^{14}C) by Macrophages in vitro

Expt.	No. of Cells	^{14}C Added cps	Incubation Time (hr.)	Recovery of ^{14}C	
				Cells cps	Medium cps
G33	90×10^6	1600	20	54	820
	90×10^6	1600	20	54	820
	90×10^6	1600	20	54	830
	90×10^6	1600	20	72	860
G34A	90×10^6	1250	20	35	795
	90×10^6	1250	20	21	765
	90×10^6	1250	20	23	820
	90×10^6	1350	20	19	915
	90×10^6	1390	20	52	825
G34B	90×10^6	1250	20	82	815
	90×10^6	1250	20	132	945
	90×10^6	1250	20	96	835
	No cells	1250	20	-	1230

TABLE 5 Percentage recovery of ^{14}C in the Cells and Medium after Incubation of Macrophages with Glycerol tri-(palmitate-1-^{14}C).

Expt.	Incubation Time (hr.)	% recovery in cells	% recovery in medium	Total recovery (%)
G33	20	3.4	51.3	54.7
	20	3.4	51.3	54.7
	20	3.4	51.9	55.3
	20	4.5	53.8	58.3
G34a	20	2.8	63.6	66.4
	20	1.7	61.2	62.9
	20	1.8	65.6	67.4
	20	1.2	59.0	60.2
	20	3.7	59.4	63.1
G34B	20	6.6	65.2	71.8
	20	10.5	75.5	86.0
	20	7.7	66.8	74.5
	20	+	98.5	98.5

+ Control - No cells.

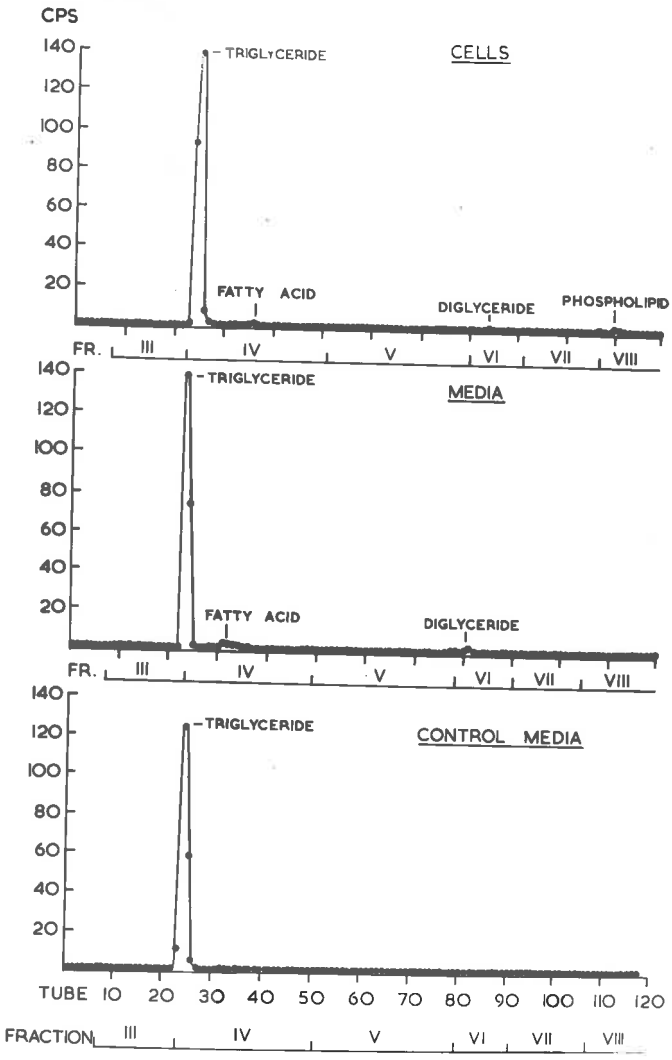


Fig.22 Separation on silicic acid columns of lipids of macrophages, medium and control medium after 20-hr incubation with ^{14}C-labelled tripalmitin. 10mg cholesteryl ester, 10mg tripalmitin, 10mg palmitic acid, 10mg cholesterol and 10mg lecithin added as carrier.

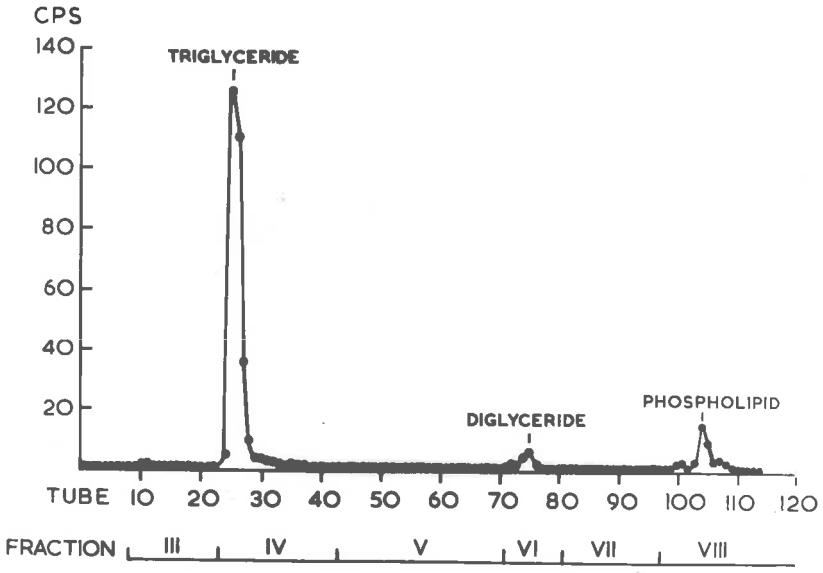


Fig.23 Separation on silicic acid column of lipid components of macrophages after 20-hr incubation with ^{14}C-labelled tripalmitin. 10mg cholesteryl ester, 10mg tripalmitin 10mg palmitic acid, 10mg cholesterol and 10mg lecithin added as carrier.

(Fig.22) there is some labelling of the diglyceride fractions, while most of the radioactivity is present in the triglyceride peak. In the control medium the only label present is in the triglyceride peak.

D. Discussion

Most of the label in the macrophage lipid extract is present in the triglyceride fraction with only small amounts of ^{14}C present in the diglyceride and phospholipid peaks. This suggests that very little of the esterified palmitate-1-^{14}C was available for esterification, at least into phospholipid or cholesterol ester. However Day and Harris (1960) have previously demonstrated that lipase is present in macrophages so it would be expected that some of the tripalmitin-1-^{14}C entering the cell would be hydrolysed, with the release of free fatty acid. The small amount of ^{14}C-labelled cholesterol ester found in the medium could be due to release of free fatty acid from the cell surface followed by esterification with cholesterol in the medium.

It is evident from Table 4 that a quantitative recovery of radioactive material in the control medium (no cells) was obtained. However, when cells are incubated with this medium the recovery of label is significantly decreased, yet only a small amount is present in the cells. This loss in radioactivity cannot all be accounted for as $^{14}CO_2$ produced by oxidation of the labelled triglyceride,

because Day (1960a) has provided evidence that only a small amount of tripalmitin-1-^{14}C glyceride is oxidised to $^{14}CO_2$ by macrophages. It is just as unlikely that such a considerable amount of labelled material could be lost so consistently during the extraction procedure, even though there were many steps involved. Besides the extraction procedure has been proved quantitative with preliminary experiments, and is borne out by quantitative recovery of the label in the control medium. The only other possible chance of loss was the removal of tripalmitin-1-^{14}C emulsion (loosely attached to the macrophage surface) during the washing procedure preceeding the extraction of lipid from the cells.

With this in mind it is interesting to discover that Elsbach (1962) working with polymorphonuclear leucocytes from rabbit peritoneal exudates found a similar situation with ^{14}C-labelled triglycerides. No release of $^{14}CO_2$ was found and the label did not appear in other lipid fractions, suggesting that triglycerides did not enter the cells, but adhered to surface sites. He concluded that lipid uptake by leucocytes is apparently selective, and not dependant upon pinocytosis or phagocytosis.

The possibility of a similar situation then arises with macrophages. However these conclusions are based only on negative evidence and it is clear that there are many aspects of the metabolism of triglyceride by macrophages in vitro which require more detailed study. However the pursuance of these studies would not fall within the scope visualised for presentation of this thesis and it is hoped that more investigations on the lines discussed above will be carried out in the future.

THE INCORPORATION OF SODIUM ACETATE-1-^{14}C

INTO LIPID BY MACROPHAGES *in vitro*

A. Introduction.

It has long been known that fat may be formed after the administration of carbohydrate or of protein to an animal. In the metabolic conversion of the carbon atoms of glucose to fatty acids, acetyl CoA is known to be an essential intermediate and the early work of Stadtman and Barker (1951) showed that two C_2 units condense to form a C_4 compound, which in turn condenses with another C_2 unit to form the C_6 acid. Evidence that the higher fatty acids of animal fat could be biosynthesised from C_2 units, was provided by Rittenberg and Bloch (1948), who showed that both carbon atoms of doubly labelled acetic acid were incorporated into the fatty acids. The formation of long chain fatty acids from acetyl CoA, no matter what the source, is termed lipogenesis and represents a de novo synthesis. Apparently a multi-enzyme system is involved in elongation of the fatty acid chain. However, it is also known that some of the very long chain fatty acids can be built up by the addition of a C_2 unit to a preformed fatty acid of shorter chain length.

More recently the existence of two systems involved in the biosynthesis of fatty acids has become apparent. The malonyl CoA incorporating system was earlier investigated by Wakil (1958), Wakil and Ganguly (1959) and the enzyme system was found to be biotin dependant. Essentially the same results were obtained

with rat liver (Brady 1958) and yeast systems (Lynen 1959). This system appears to be located in the soluble ("supernatent") portion of the cytoplasm and is the system responsible for net palmitate synthesis, whereas the other system is present in the mitochondria which is the site of most of the fatty acid oxidation enzymes. (Seubert, Freull and Lynen 1957). This mitochondrial system could convert β-keto, β-hydroxy and α-β-unsaturated acyl derivatives into their corresponding saturated counterparts and it has also been suggested that the mitochondrial system is responsible for lengthening the carbon chain of palmitate or of the polyunsaturated fatty acid series (Green and Wakil 1960; Wakil 1961).

The biosynthesis of cholesterol also involved the condensation of C_2 units. Bloch and Rittenberg (1944) and Bloch (1954) showed that both carbon atoms of doubly labelled acetic acid ($^{13}CH_3{}^{14}COOH$ and $^{14}CH_3{}^{13}COOH$) were used for cholesterol biosynthesis in rat liver. Since then extensive investigations by many workers have contributed to the unravelling of the pathway leading to the formation of cholesterol.

In order to investigate further the lipid synthesising mechanisms of macrophages, experiments were carried out using ^{14}C-labelled acetate as substrate.

B. Total Intracellular Incorporation of Sodium Acetate-1-^{14}C and Proportion Converted to Lipid

In these experiments, macrophages were incubated at 37° in 20 oz. McCartney bottles with 1μc of sodium acetate-1-^{14}C for four hours. The technique used was similar to that described in the previous sections. In order to compare the total incorporation of ^{14}C with the extent of labelling of the lipid, the cells from paired incubations were lyophilized, one of each pair being reconstituted with water and counted, while the lipid was extracted from the other. Details of the procedures involved are described on p.27.

The total ^{14}C present in macrophages in all forms after four hour incubation with sodium acetate-1-^{14}C is compared with the amount present as lipid in Fig.24. This represents the data from five paired experiments. The total ^{14}C present varied from 265cps to 880cps. In four experiments, 27-31% is present in the lipid fraction; in one experiment 52% of the total ^{14}C is present as lipid ^{14}C indicating that a considerable proportion of the acetate taken up by the macrophages has been diverted to lipid metabolism.

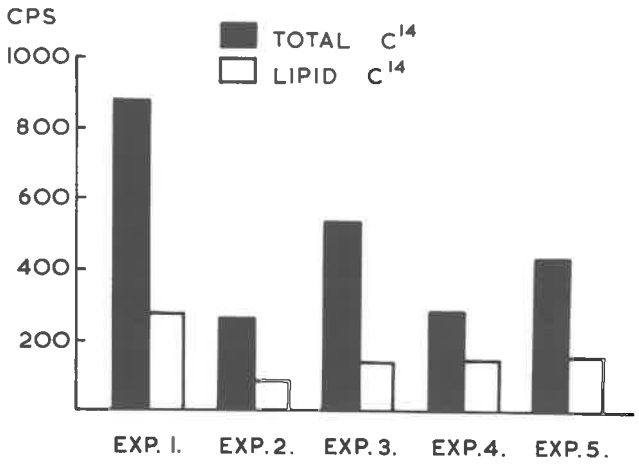


Fig. 24 The total ^{14}C present in all forms in macrophages after 4-hr incubation with sodium acetate-1-^{14}C compared with the amount present as lipid. Data from five paired experiments.

C. Rate of Incorporation

To obtain information about the rate of incorporation of acetate-1-^{14}C into macrophage lipid, incubations were carried out at varying time intervals as described on p.28. 30-50 x 10^6 macrophages were incubated in siliconised Warburg cups with 10µc of sodium acetate-1-^{14}C for ½, 1, 2, 4 and 6 hours, during which the oxygen uptake was measured manometrically. After incubation the cells were separated from the medium, washed, and the lipid extracted with chloroform:methanol (2:1 v/v) and counted.

D. Total Lipid Specific Activity Determinations

The total counts in the lipid extracted from macrophages incubated with acetate-1-^{14}C were expressed for the various time intervals as cpe/mg of total esterified fatty acid. For this purpose about 100 x 10^6 macrophages from each experimental batch were reserved for determination of total esterified fatty acid by the method of Stern and Shapiro (1953). Incorporation of acetate-1-^{14}C into total lipid is shown in Fig.23. It can be seen that the specific activity increased progressively with time to a mean of 2600cpe/mg after six hours. This represents about 0.4% of the acetate-1-^{14}C added to the medium which had been incorporated into macrophage lipid. The pattern of incorporation of acetate in relation to time was similar in all experiments although the amount

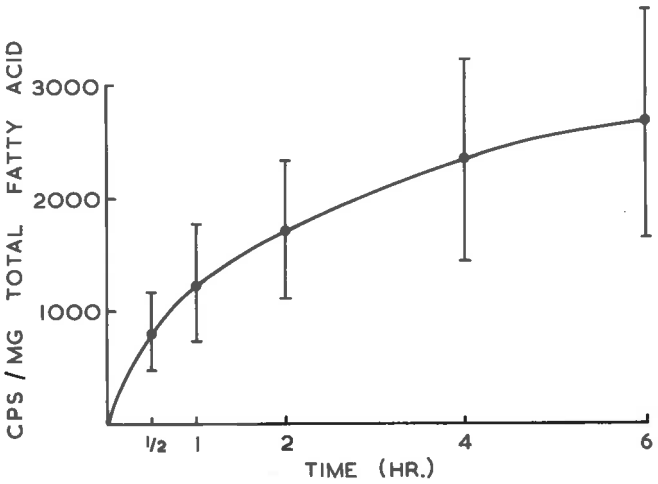


Fig.25 Specific activity of total lipid (expressed as counts/second/mg of total esterified fatty acid) following incubation of macrophages with sodium acetate-1-^{14}C plotted against time. The mean of six experiments together with the standard deviation of the mean is shown.

incorporated varied with different batches of cells. Fig.26 shows the oxygen uptake plotted against time for the experiments in which data related to lipid synthesis from acetate is reported. The cells took up oxygen steadily at a rate of 5.0μmole/10^8 cells/hour, indicating that the cells remained alive during the experiment. Therefore, the flattening of the curve in Fig.25 is not a decreased rate of incorporation of acetate-1-^{14}C into lipid due to cell death.

E. Distribution of Radioactivity in Saponifiable and Non-Saponifiable Fractions of Total Lipid Extract

The total lipid extract remaining after a small portion had been taken for counting purposes was saponified as described on p.29. The percentage distribution of the ^{14}C between the non-saponifiable and saponifiable fractions is shown in Fig.27 for the five time intervals investigated. Approximately 80% was present in the non-saponifiable fraction and only 20% was present in the fatty acid fraction. This distribution of ^{14}C did not vary significantly at the different time intervals studied.

F. Fatty Acid and Cholesterol Specific Activity

The information presented above which deals with the proportion of radioactivity present in the non-saponifiable and saponifiable fractions gives only a rough idea of the distribution of ^{14}C. More

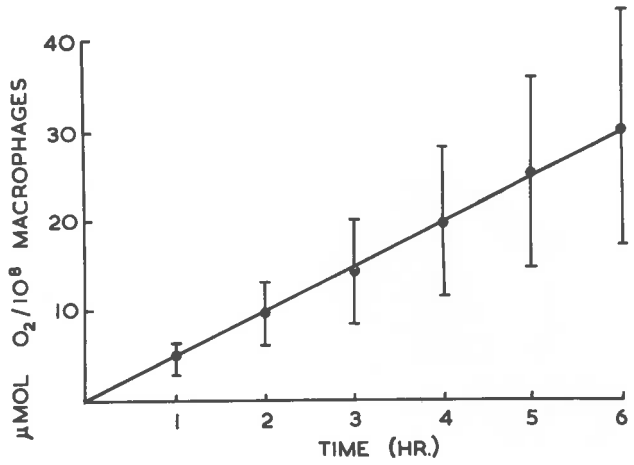


Fig.26 Oxygen uptake of macrophages incubated *in vitro*. The mean O_2 uptake together with the standard deviation of the mean is plotted against time for the six experiments for which data on lipid synthesis are presented.

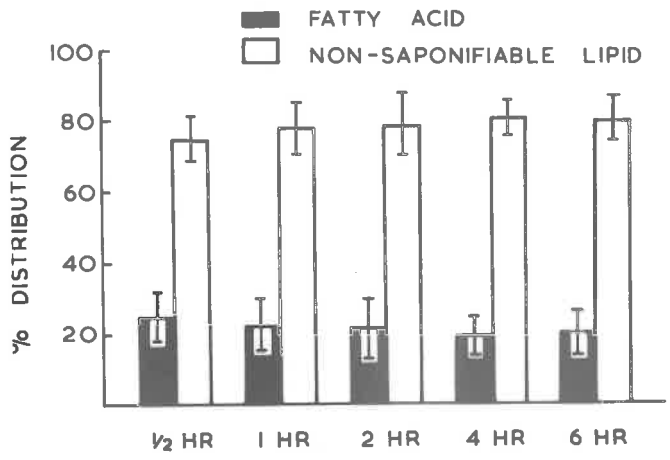


Fig.27 Percentage distribution of the total ^{14}C-labelled lipid between the nonsaponifiable and fatty acid fractions at various time intervals following incubation of macrophages with sodium acetate-1-^{14}C. The mean of six experiments together with the standard deviation of the mean is shown at each time interval.

detailed information was provided by further purifying these non-saponifiable and saponifiable extracts.

The fatty acids present in the saponifiable extract were converted to their corresponding methyl-esters and fractionated by gas liquid chromatography as described on p.42. The specific activity of the cholesterol in the non-saponifiable extract was obtained by isolating the cholesterol on silicic acid impregnated paper. The cholesterol zone was eluted, a portion of this extract counted and the cholesterol content was determined on the remainder (see p.47).

The rate of incorporation into cholesterol and fatty acid is shown in Fig.28. The specific activity rises to a mean of 8890cps/mg at six hours for cholesterol and of 680cps/mg at six hours for fatty acid. It can been seen that there is a wide variation in the extent of labelling between individual experiments which is evident from the standard deviations plotted at each time interval. It has been pointed out that mainly cholesterol was labelled with a smaller amount of fatty acid labelling and for this reason it was not possible to determine the specific activity of the fatty acid in all six experiments as was the case with cholesterol. The graph in Fig.28 given for the mean specific activity of fatty acid in relation to time, represents the mean data from only three experiments, in which the total lipid synthesis was maximal. For this reason the contrast in specific activity between cholesterol and fatty acid should be even more marked than is indicated by Fig.28.

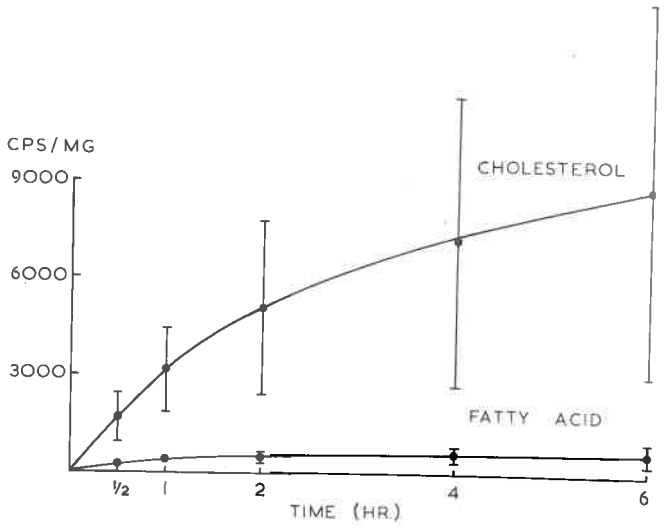


Fig.28 Specific activity of cholesterol and fatty acid (expressed as counts/sec/mg of cholesterol and fatty acid respectively) following incubation of macrophages with sodium acetate-1-^{14}C plotted against time and to the same scale. The mean of six experiments is given for the cholesterol data, and of three for the fatty acid data. The standard deviation in each case is shown.

C. Labelling of Glycerol

Preliminary experiments had shown that glycerol was labelled to a very small extent as compared with the non-saponifiable and fatty acid fractions when acetate-1-^{14}C was the substrate. It was necessary therefore to use lipid extracts with higher activities for glycerol studies. Thus the extent of glycerol labelling from acetate-1-^{14}C was investigated in experiments in which the specific activity of individual fatty acids was determined. These experiments are dealt with in the next section and it is sufficient to say at this point that 20μc of acetate-1-^{14}C was used in each Flask instead of 10μc. After six hours incubation, the cells from five or six incubation flasks were combined in order to obtain a total lipid extract with a relatively high radioactivity. The glycerol was isolated as the tribenzoate (see p.32), dissolved in alcohol and counted by liquid scintillation counting. Glycerol determinations were carried out before and after purification to check for any losses incurred during the lengthy procedures involved and correction was made for this loss. The incorporation of acetate-1-^{14}C into lipid glycerol by macrophages is shown in Table 6. Some labelling of glycerol occurred but the amount only accounts for 1-1.3% of the acetate-1-^{14}C incorporated into the total lipid.

TABLE 6

Incorporation of Acetate-1-^{14}C into Lipid Glycerol by Macrophages

Total Lipid cps	Glycerol cps	% of Counts incorporated into Glycerol
1,050	12.6	1.2
11,000	127	1.2
8,800	89	1.0
15,860	175	1.1

H. Fractionation of Lipid Extracts

Having shown that fatty acid and cholesterol were labelled from acetate it was next decided to investigate the type of major lipid classes into which the label had been incorporated. For this purpose some lipid extracts from various experiments involving macrophages and ^{14}C-acetate were fractionated by paper chromatography on silicic acid impregnated paper and also by column chromatography on silicic acid. Both of these methods have been discussed in previous sections. Fig.29 represents a radioautograph prepared from a paper chromatogram on which a macrophage lipid extract (labelled from acetate-1-^{14}C) and a mixture of ^{14}C labelled standards have been separated. Most of the ^{14}C is present in cholesterol and triglyceride which are clearly shown as two heavily labelled spots. Some labelling of cholesterol ester and of fatty acid were also shown on the radioautograph but are not visible in this photograph.

These findings were confirmed by column separation of the macrophage lipid extracts (Figs.30, 31, 32). This method gives more quantitative information regarding the distribution of ^{14}C between the various lipid fractions. Again the bulk of the ^{14}C-labelled lipid is present in the triglyceride and cholesterol fractions with smaller amounts in the cholesterol ester, free fatty acid and di and monoglyceride fractions. Appreciable labelling of phospholipid was also demonstrated. However by calculating the number of counts in the cholesterol peaks and also in the esterified fatty acid peaks it

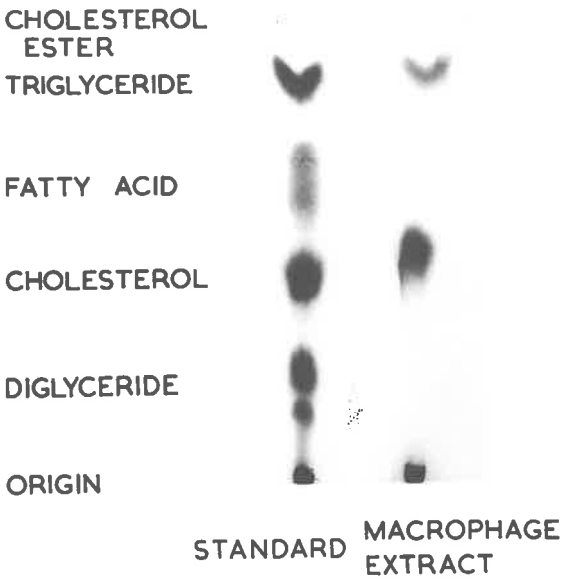


Fig.29 Radioautograph showing fractionation of lipids from macrophages after 4-hr incubation with sodium acetate-1-^{14}C, on silicic acid impregnated paper. Mobile phase: light petroleum:diisobutyl ketone, 96:6.

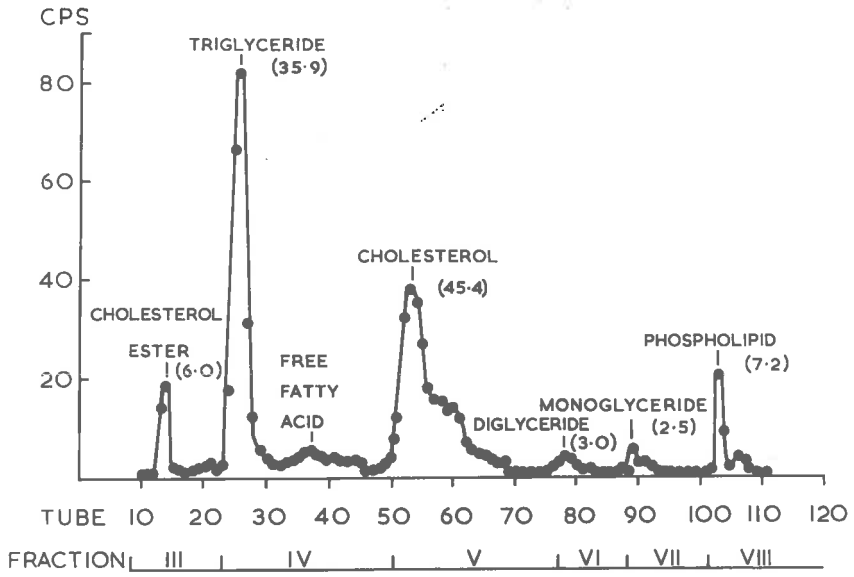


Fig.30 Fractionation on a silicic acid column of the ^{14}C-labelled lipid components of macrophages after 4-hr incubation with sodium acetate-1-^{14}C. Approximately 10mg each of unlabelled cholesterol ester, tripalmitin, palmitic acid, cholesterol and lecithin added as carrier. The percentage recovery in each peak is shown in parenthesis.

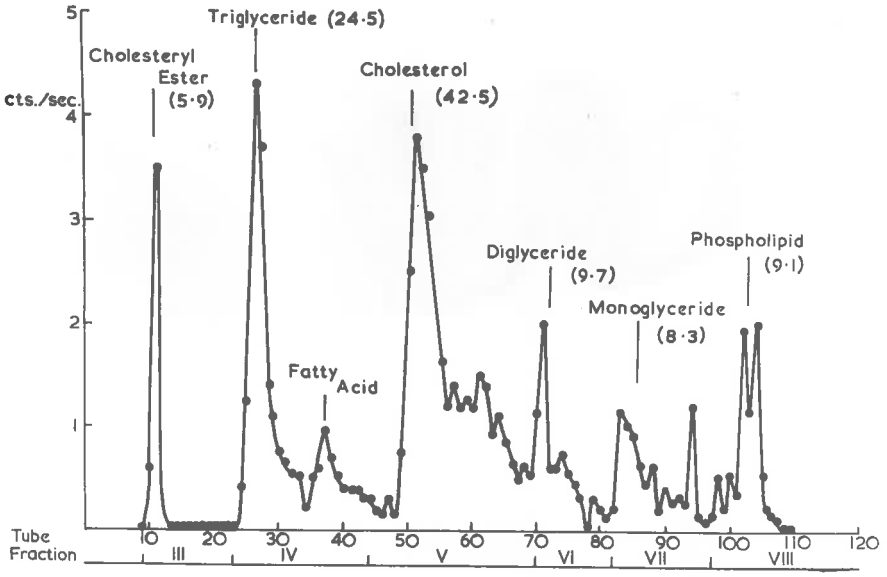


Fig.31 Fractionation on a silicic acid column of the ^{14}C-labelled lipid components of macrophages after 4-hr incubation with sodium acetate-1-^{14}C. Approximately 10mg each of unlabelled cholesterol ester, tripalmitin, palmitic acid, cholesterol and lecithin added as carrier. The percentage recovery in each peak is shown in parenthesis. Radioassayed by G.M. counting.

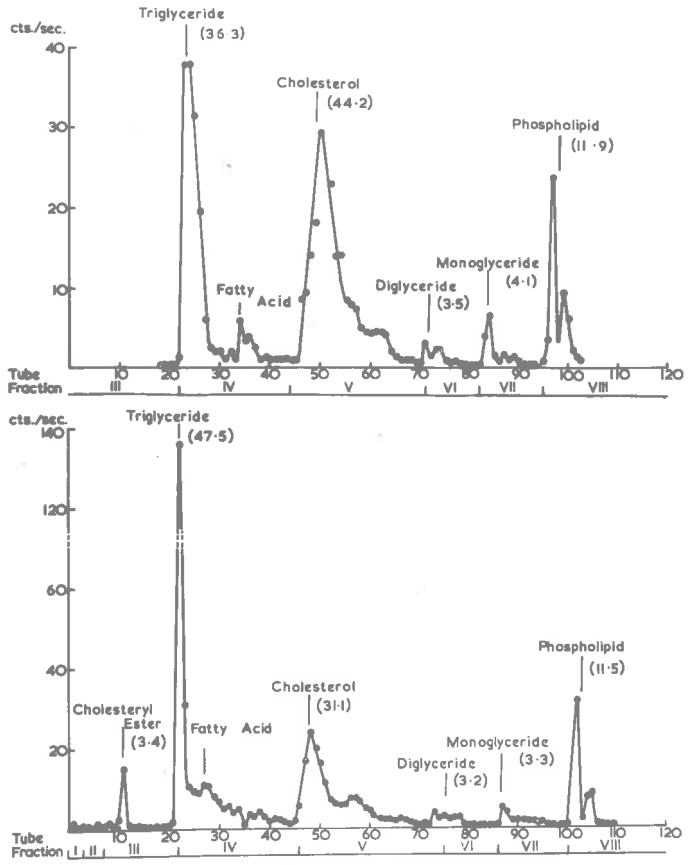


Fig.32 Fractionation on two silicic acid columns of the ^{14}C-labelled lipid components of macrophages after 4-hr incubation with sodium acetate-1-^{14}C in two experiments. Approximately 10mg each of unlabelled cholesterol ester, tripalmitin, palmitic acid, cholesterol and lecithin added as carrier. The percentage recovery in each peak is shown in parenthesis.

became obvious that the distributions found by column fractionation are variable and differ from the percentage distribution of counts found in the non-saponifiable and saponifiable fractions after saponification of the total lipid extract. In some column separations (e.g. Fig.30) almost half the radioactivity is present as esterified fatty acid and the other half is present in the cholesterol fraction. However there is appreciable variation in the different column separations as to the percentage distribution of counts as shown in Figs.31 and 32. The reason for these anomolous findings is not apparent. Samples of both alcohol:ether and chloroform:methanol extracts were used both for fractionation on columns as well as for saponification, so that the differences were not due to different extraction procedures. The reproducibility of the saponification procedure has been well established as described in the Methods section (p.29) and good recoveries after fractionation of lipid samples by column chromatography have been achieved. However no quantitative interpretations have been based on either the column separations or the saponification data. The conclusions (discussed later) concerning the relative labelling of cholesterol and fatty acid from acetate-1-^{14}C were made from measurements in specific activities which were obtained by purification of the cholesterol and fatty acid in the non-saponifiable and saponifiable fractions respectively.

I. Discussion

The oxygen uptake, which was constant for the six hour period of incubation indicated that the cells were in a steady metabolic state during this time. Other workers have reported oxygen uptakes which compare closely with the rate found in these experiments, (Harris and Barclay, 1955; Stobelin, Karnovsky and Suter, 1956; Pavillard and Rowley, 1962). The uptake and incorporation of the two carbon unit, sodium acetate, into lipid by macrophages *in vitro* indicates that these cells have the mechanisms necessary for them to synthesise lipid from smaller units. These findings could merely indicate general lipid metabolism of the cells, or else the pattern of incorporation may represent a special function of the macrophages. Under the experimental conditions reported, about 0.1% of the acetate-1-^{14}C in the medium was converted to lipid by the cells after thirty minutes and 0.4% after six hours incubation. More valid however is the proportion taken up and channeled into lipid synthesis. The results show that up to a third of the acetate-1-^{14}C taken up was converted into lipid. This conversion to lipid seems to be higher than that reported for other tissues. Holdsworth and Neville (1963) for example using sheep heart preparations incubated with ^{14}C-labelled acetate *in vitro* have shown that less than 1% of the acetate that is incorporated into the preparation is present as long chain fatty acids, the bulk of the acetate being incorporated into non lipid products.

Possibly then, the higher conversion of acetate to lipid found in macrophages represents a more specific function of these cells which is of great interest since they appear to be involved in the lipid metabolism in atheroma.

The distribution of label between the cholesterol and fatty acid fractions is also of interest. Only 20% of the total lipid labelled is present in the free fatty acid fraction. In contrast polymorpho-nuclear leucocytes which have also been shown to synthesis lipid from acetate, (Pastore and Lionetti, 1959; Elsbach 1959; Sbarra and Karnovsky, 1960) channel most of the acetate to fatty acid, (Pastore and Lionetti, 1959). A more important consideration is the comparison between the specific activities of cholesterol and fatty acid. O'Donnell, Ottolenghi, Malkin, Dendstedt and Heard (1958) demonstrated the biosynthesis of fatty acids and cholesterol in monocytes obtained from the pleural cavity of rabbits. Although they demonstrated a greater specific activity of labelled fatty acid than of cholesterol it is not possible to calculate the proportion of ^{14}C-labelled acetate directed into the monocyte lipids from their results. The relatively higher activity in the cholesterol fraction found in macrophages indicates that the smaller cholesterol pool is turning over much more rapidly than is the fatty acid pool. Some labelling of lipid glycerol has occurred, but does not represent a significant amount.

The chromatographic investigations indicate that most of the labelled lipid is present in the triglyceride and cholesterol fractions but that some labelling of cholesterol ester and phospholipid occurs. These findings are consistent with the qualitative information found in the studies using ^{14}C-labelled palmitic acid. Again, most of the fatty acid which has been labelled has been directed into triglyceride molecules and to a fair degree into the phospholipid fraction.

THE INCORPORATION OF SODIUM ACETATE-1-^{14}C INTO LONG CHAIN FATTY ACIDS BY MACROPHAGES *in vitro*

A. Introduction

The investigations with acetate-1-^{14}C and macrophages described in the preceeding section were extended in order to obtain information about the types of fatty acids present in macrophages as well as the labelling of individual fatty acids. These experiments were carried out with 20μc of ^{14}C-labelled acetate using the same technique as described in the last section. After incubation, the cells from six incubation vessels (siliconised conical flasks) were combined and the lipid extracted. The total lipid counts were obtained and expressed as cps/mg triglyceride fatty acid and after saponification of the lipid extracts the methyl esters of the fatty acids were prepared (see p.42). Individual peaks were collected in the calcium chloride tubes containing cotton wool soaked in light petroleum and the area of each peak was determined by triangulation and the specific activity expressed as cps/mg of fatty acid.

B. Fatty Acid Present in Macrophages.

A typical fatty acid pattern of the macrophage extract is shown in Fig.33. It consists mainly of palmitic acid with considerable amounts of linoleic, oleic and stearic acids present. Smaller amounts of lauric, myristic, myristoleic and palmitoleic acids were also found. Two unidentified peaks with retention times of 0.70 and 0.88 relative to palmitic acid were also demonstrated in all the extracts chromatographed.

C. Labelling of Macrophage Fatty Acids

Fig.34 shows the mean percentage distribution of fatty acids together with the mean percentage distribution of counts, following incubation with acetate-1-^{14}C. Most of the acetate has been incorporated into palmitic and oleic acid after six hours incubation but appreciable labelling of stearic and myristic acids has also occurred, with less incorporation into linoleic and palmitoleic. Very little of the acetate has been incorporated into lauric acid. A mean of 11.3% of the acetate-1-^{14}C has been incorporated into myristic acid even though it constitutes only 1.4% of the fatty acids present in the extract.

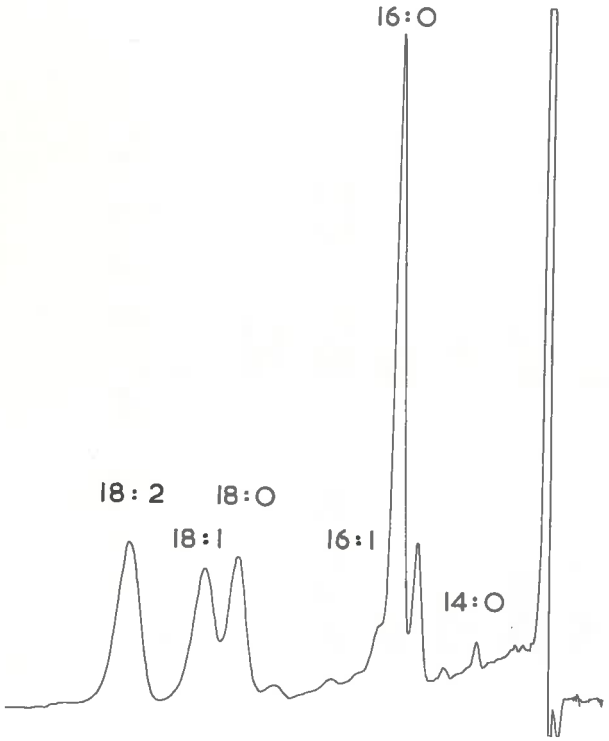


Fig.33 Typical fatty acid pattern of macrophage lipid extracts. 10% polyethyleneglycol adipate on Embacel 100-120 mesh at 180°. Argon flow 90ml/min. Methyl esters applied to the column in 0.1 µl of chlorobenzene.

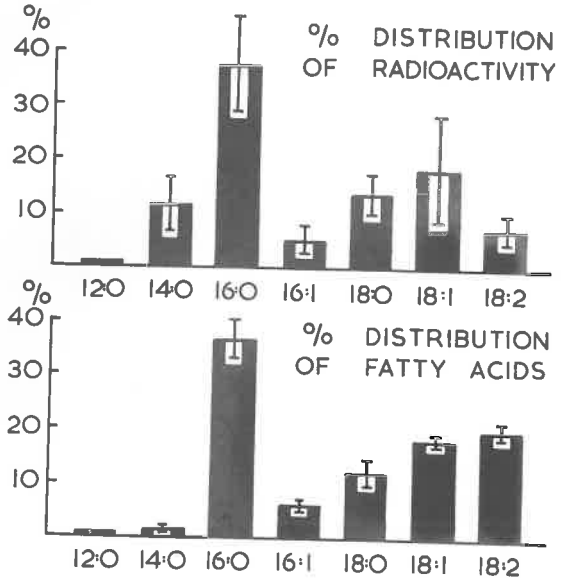


Fig. 34 Percentage of distribution of ^{14}C and of fatty acids in macrophage lipid extracts after 6-hr incubation with ^{14}C-labelled sodium acetate. The mean of 6 experiments together with the standard deviation of the mean is shown.

Information about the specific activities of the individual fatty acids obtained after incubating macrophages with ^{14}C-labelled-acetate for six hours is shown in Table 7. As a unit of measurement, the specific activity of palmitic acid at six hours is taken as 1 and specific activities of the other fatty acids expressed in relation to this figure. Mean figures calculated on this basis are given for the six experiments performed. Stearic, oleic and palmitic acid have similar specific radioactivities after six hours incubation with ^{14}C-labelled-acetate, whereas linoleic acid is lower than palmitic and myristic acid considerably higher than palmitic acid.

D. Time Study of the Incorporation of Acetate-1-^{14}C into Individual Fatty Acids of Macrophages

The specific activity of each individual fatty acid was also obtained at various time intervals but in order to obtain enough activity for specific activity determinations, macrophages (50×10^6) were incubated in siliconised conical flasks with 50mc of acetate-1-^{14}C for ½, 1, 3 and 6 hours. Duplicate flasks were prepared and after incubation for the appropriate time, the cells were combined and the lipid extracted with chloroform:methanol

TABLE 7 Specific Activity of ^{14}C-labelled Fatty Acids Relative to Palmitic Acid. Mean of six experiments with the standard deviation of the mean is given.

Fatty Acid	Relative Specific Activity
Myristic	7.5 (2.2)
Palmitic	1.0
Palmitoleic	0.7 (0.3)
Stearic	1.1 (0.3)
Oleic	1.1 (0.7)
Linoleic	0.4 (0.2)

(2:1 v/v). Gas liquid chromatography was carried out, the peaks measured and collected as described previously.

The rise in specific activity of the individual fatty acids in relation to time is shown in Fig.35. Myristic acid is rapidly labelled and is always above that of the other fatty acids. Palmitic acid rises next while stearic acid rises more slowly but subsequently rises above palmitic acid. The specific activity of stearic acid is above that of oleic acid over the whole time range studied; linoleic acid is not markedly labelled when compared with the others and stays low for six hours.

B. Discussion

The fatty acid pattern of macrophages is similar to the type and proportion of fatty acids present in rabbit leucocytes, (Elsbach 1959) and does not seem to present any unusual features. The macrophage extracts do contain more palmitic acid and less linoleic acid than was the case with the leucocyte extracts however.

The incorporation of acetate-1-^{14}C into palmitic, oleic, stearic and myristic acids indicates a relatively rapid turnover of these fatty acids and this is particularly true for myristic. Bloch, Baronowsky, Goldfine, Lennarz, Light, Norris and Scheuerbrandt (1961) have postulated as one pathway of biosynthesis of the

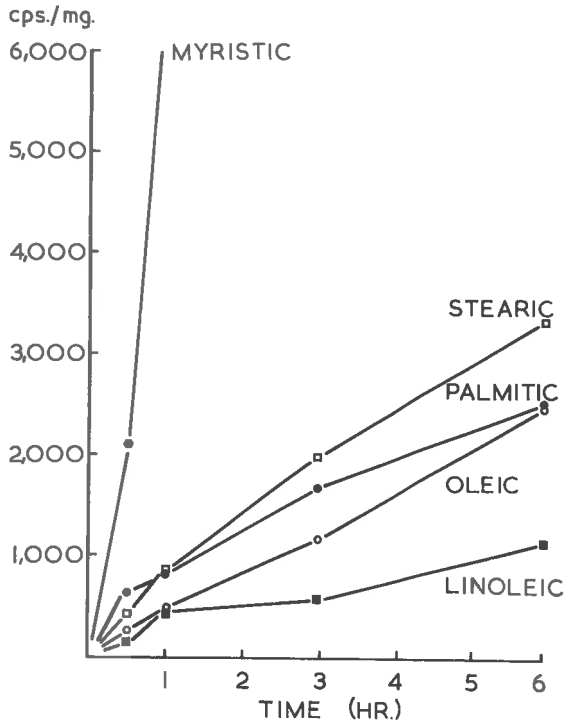


Fig. 35 Specific activity of different fatty acids (counts/second/mg fatty acid) plotted against time following incubation of macrophages with ^{14}C-labelled sodium acetate.

mono-unsaturated fatty acids, a scheme involving desaturation of the corresponding saturated fatty acid. The observation made from the labelling pattern in these experiments is consistent with the sequence of labelling.

palmitic ⟶ stearic ⟶ oleic acid

which is in agreement with Bloch's postulate. Other workers have reported different pathways for the synthesis of mono-unsaturated fatty acids in certain other tissues which takes place independently of the corresponding saturated fatty acid (James 1962; Getz 1962).

Mead and Howton (1960) and Mead (1960) also suggested that palmityl CoA gives rise to the longer chain saturated fatty acids and the mono-unsaturated fatty acid series but it is possible that there are two separate enzyme pathways involved; one for palmitate synthesis <u>de novo</u> from acetyl CoA and another for the addition of acetyl units to palmitate and other long chain fatty acids. According to Mead (see references above) the more saturated fatty acids can give rise to a less saturated series but the reverse process does not occur. More detailed study however is required before it is possible to speculate on the involvement of various pathways in macrophages.

THE INCORPORATION OF ^{32}P-LABELLED ORTHOPHOSPHATE INTO LIPID BY MACROPHAGES *in vitro*

A. Introduction

The possibility that macrophages are concerned with phospholipid accummulation in the arterial wall in atheroma is of special interest in these current investigations.

Shore, Zilversmit and Ackerman, (1955) and Zilversmit and McCandless (1959) demonstrated that phospholipid is actively synthesised in the arterial wall in both experimental and human atheroma. Day (1962) then showed that in the arterial wall of rabbits with equivalent lesions to those produced by the above workers, all of the phospholipid was present intracellularly in the intimal macrophages. Similar observations were made by Dunnigan (1964) who found that phospholipid, when present in human atherosclerotic plaques, was localised within macrophages. An interesting feature of the experiments concerning the uptake of sodium palmitate-1-^{14}C and incorporation of acetate-1-^{14}C into macrophage lipid was the consistent labelling of the phospholipid fraction. This information together with additional evidence that macrophages incorporated glucose-1-^{14}C into phospholipid (described later) indicates that macrophages have the ability to synthesise phospholipids. More detailed information concerning the rates of synthesis and types of phospholipids present in macrophages was obtained by studies in which macrophages were incubated with ^{32}P-labelled orthophosphate.

B. Total Phospholipid Specific Activity Measurements

Approximately 50 x 10^6 cells were incubated at 37° with 60-80uc of ^{32}P-labelled orthophosphate in siliconised Warburg cups for 1, 2, 4, 6 and 12 hours, during which time the oxygen uptake was recorded. After incubation the cell lipid was extracted with chloroform:methanol (2:1 v/v) and the extracts made up to ten ml. Half ml portions were taken for scintillation counting and one ml portions for determination of the phospholipid phosphorus. In this way the rise in specific activity of the total phospholipid, expressed in cps/µg lipid P_4 was obtained over a time period and is demonstrated by the curve in Fig.36. This curve shows the mean specific activity of seven experiments together with standard deviations. From 12.9 cps/µg lipid P_4 at 1 hour, the specific activity has risen to 110 cps/µg at six hours but starts to fall off over the next six hours. It is interesting to note that the oxygen uptake which was recorded also starts to fall off after six hours and this data is given in Fig.37 in which the mean oxygen uptake is shown with standard deviations expressed as µmole O_2 per 10^8 cells at various time intervals. It is apparent that the respiration rate recorded in these experiments is similar to that already demonstrated previously (Fig.26).

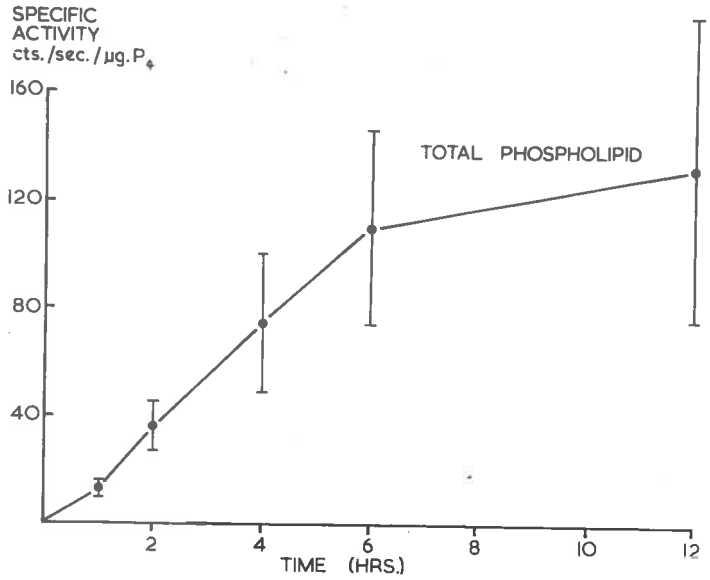


Fig. 36 Specific activity of total phospholipids (expressed as counts/second/µg of lipid phosphorus) following incubation of macrophages with ^{32}P-labelled phosphate plotted against time in hours. The graph represents a mean plot with standard deviations of the mean included.

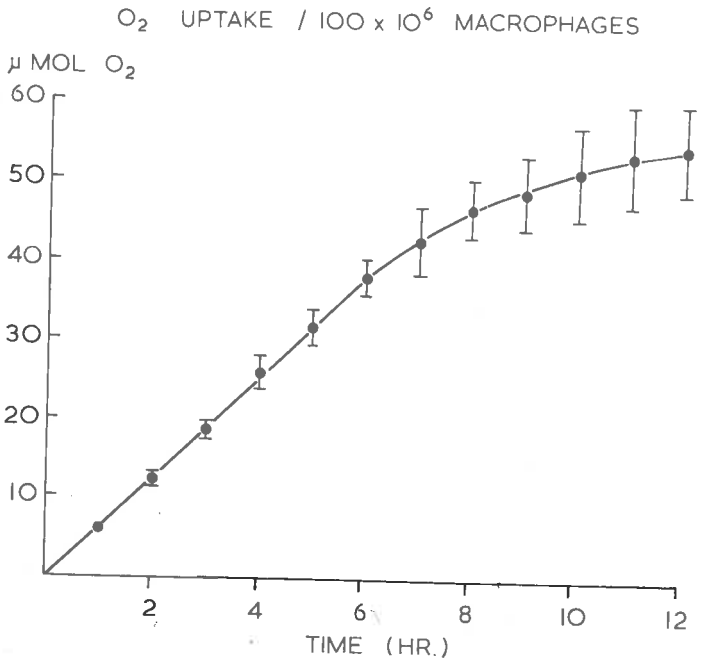


Fig.37 Oxygen uptake of macrophages incubated with ^{32}P-labelled phosphate *in vitro*. The mean O_2 uptake together with the standard deviation of the mean is plotted against time.

C. Separation and Identification of Labelled Phospholipids

The remainder of the lipid extract after counting and phosphorus analysis was divided into two equal portions and chromatographed by paper chromatography (see p.37). With such investigations, a portion of the extract containing approximately 16-20µg of phospholipid phosphorus was applied in a line one inch wide at the origin, four samples being loaded onto each paper. After development, the papers were cut into strips 1½" wide and scanned on a Nuclear Chicago 4 π Actigraph Paper Chromatograph Scanner in order to determine the distribution of radioactivity in each spot separated.

The identification of the ^{32}P-labelled phospholipids separated by paper chromatography consisted of three stages all of which have been described on p.38. The results are discussed as follows. A comparison of the Rf values of the labelled phospholipids with the standards is shown in Fig.38 which indicates that spot 2 may be lysolecithin or inositol phosphatide; spot 3 runs with sphingomyelin; spot 4 with lecithin and spot 5 with the "cephalin" mixture. No standards were available with which to compare Rf values of the other labelled phospholipids.

The results of specific staining reactions are also shown in Fig.38. With such investigations a portion of the extract containing approximately 16-20µg of phospholipid phosphorus was applied in a line one inch wide at the origin adjacent to another one inch line

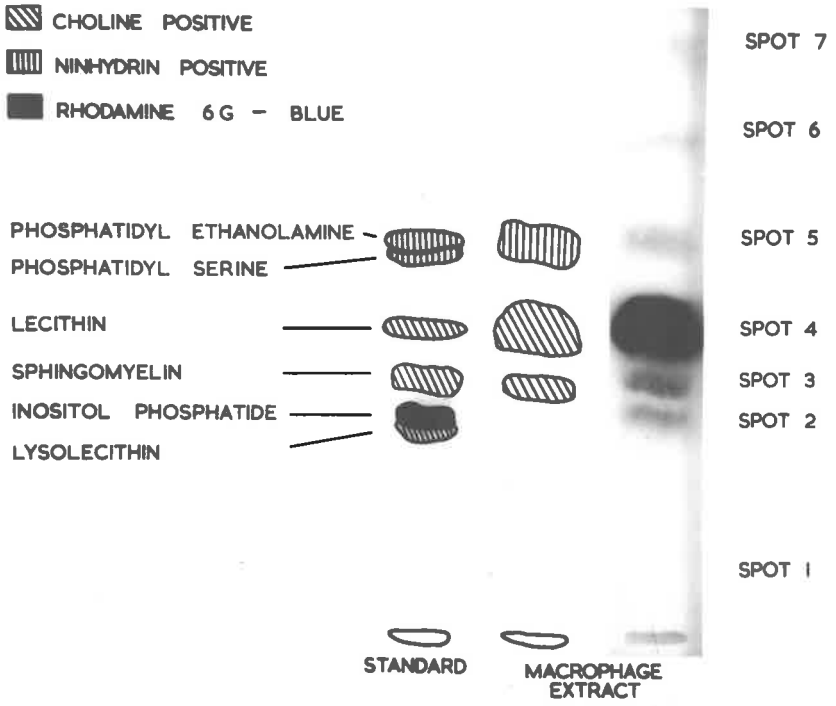


Fig. 8 Separation of phospholipids by paper chromatography. Separation of a macrophage lipid extract is shown on the right together with a radioautograph prepared from the paper chromatogram. The shaded areas represent staining characteristics which can be identified from the legend.
Solvent: diisobutyl ketone:acetic acid:water ; 40:25:5.

loaded with the appropriate marker standard. Three such chromatograms were developed with diisobutylketone:acetic acid:water (40:25:5) and then dried at room temperature. One paper was then stained with Rhodamine 6G (detects all phospholipids under ultra-violet light); another with 0.25% ninhydrin to detect amino containing phospholipids and the remainder was stained with the choline detecting agent (phosphomolybdate:stannous chloride). From Fig.38 it can been seen that spots 3 and 4 gave positive color tests for choline and spot 5 gave a positive reaction to ninhydrin. The technique of cochromatography (described on p.38) was next performed which confirmed the identifications.

The identification of radioactive bands 2 and 5 are worthwhile discussing here. Band 2 did not contain enough material to give a spot test upon staining with either three of the color reagents. By comparing its mobility with that of the standards, it could not be distinguished from inositol phosphatide or lysolecithin, which overlapped when developed with this solvent system (diisobutylketone: acetic acid:water (40:25:2 v/v/v). Cochromatography was carried out with both lysolecithin and inositol phosphatide using the solvent system described above but in the proportion 40:30:7 as well as 40:25:5. This system more adequately separates (under the conditions found here) inositol phosphatide from lysolecithin and also phosphatidyl serine from phosphatidyl ethanolamine. Similarly, spot 5 which could have been either phosphatidyl serine or phos-

phatidyl ethanolamine according to its Rf value (when using the solvent in the ratio 40:25:5) and staining reaction, was cochromatographed with phosphatidyl serine or phosphatidyl ethanolamine as the reference phospholipid using the solvent system in the ratio 40:30:7. On this basis it can be concluded that phosphatidyl ethanolamine was labelled but not phosphatidyl serine. (see Fig. 39B). However in some extracts, a second unidentified radioactive spot was present, which ran above the phosphatidyl ethanolamine, as shown in Fig.39A.

The results of cochromatography of spot 2 with the two reference compounds lysolecithin or inositol phosphatide is shown in Fig.40. Thus this labelled spot was identified as inositol phosphatide. Spot 1 and 6, which contained small and somewhat variable counts (and in some extracts not detectable) were not identified.

D. Distribution of ^{32}P within Phospholipids of Macrophages

The percentage distribution of counts between each component in relation to time was obtained after chromatography and scanning the paper stripe. The scan shown in Fig.41 is representative of the scans obtained from various experiments. After determining the peak areas the percentage distribution was calculated and is shown in Table 8. When each spot is considered individually for

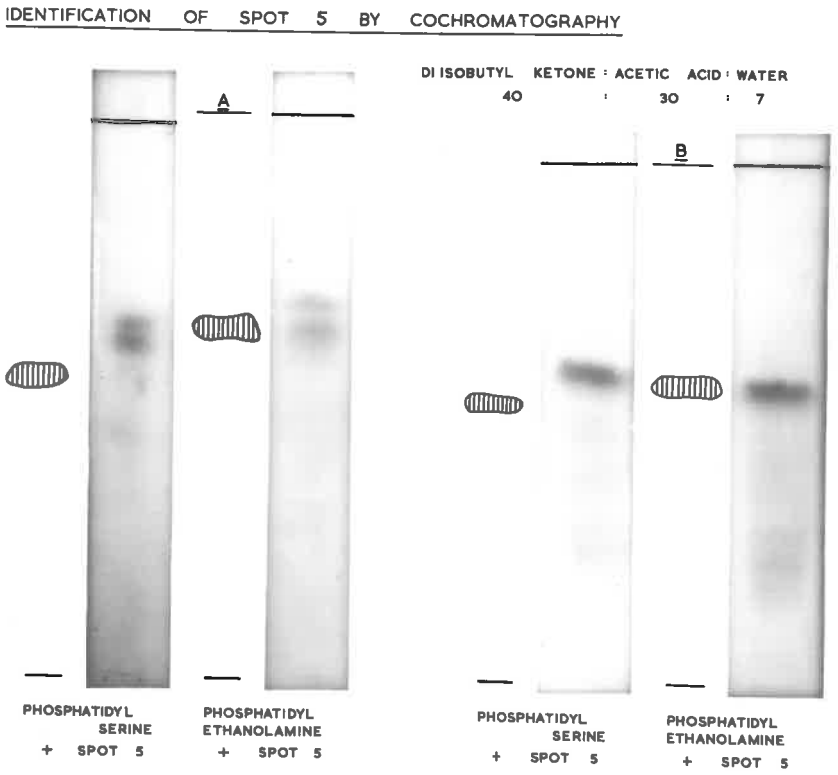


Fig. 39 Identification of spot 5 by cochromatography. The shaded areas represent the positive ninhydrin staining spots, on the chromatogram strips, and to the right of each is shown the radioautographs prepared by exposing each strip to X-ray film. A and B are representative of two cochromatography identifications. Both show the presence of labelled phosphatidyl ethanolamine, but in A two radioactive spots can be seen, the faster moving spot probably being a plasmalogen derivative.

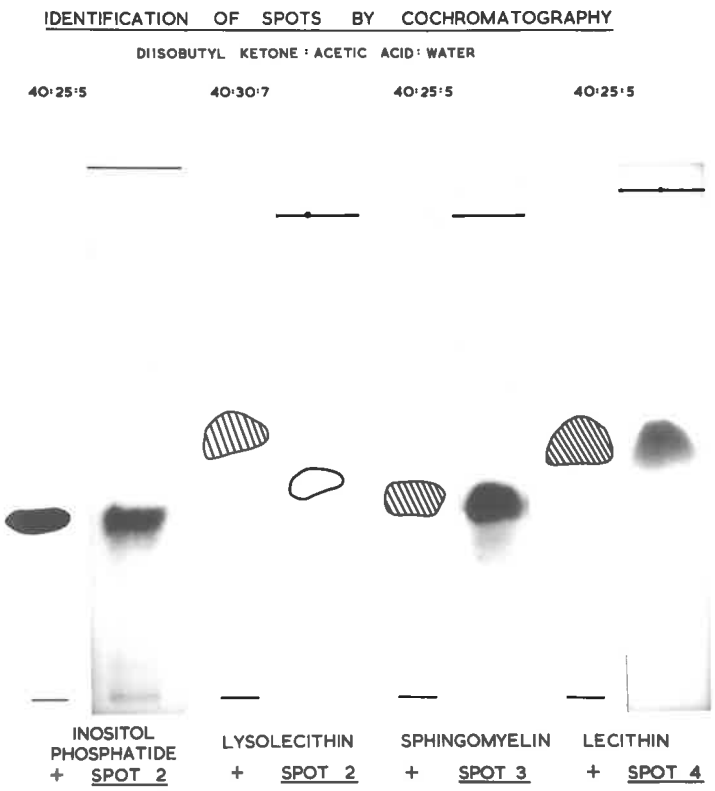


Fig.40 Identification of spots 2 and 3 by cochromatography. The solvent ratios are shown above. The shaded areas represent positive Rhodamine 6G staining for inositol phosphatide, and positive choline tests for lysolecithin, sphingomyelin and lecithin. Radioautographs prepared from each paper chromatogram are shown to the right of each corresponding stained strip. In the case of lysolecithin, a circle was drawn around the exposed area, which was not intense enough to show up in this picture. The identification of spot 4 as lecithin is shown on the right of the figure.

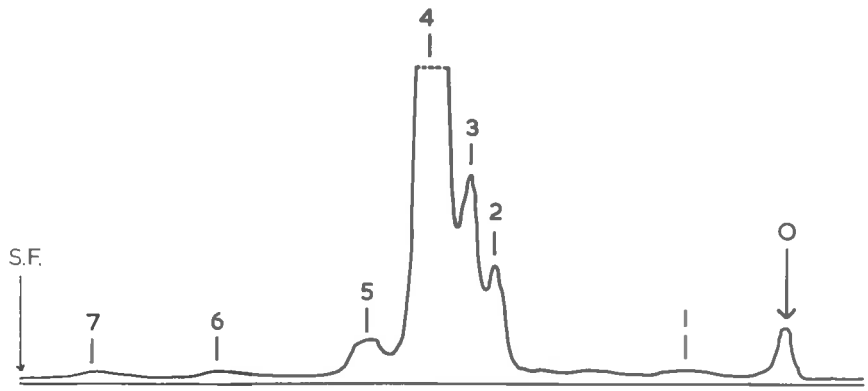


Fig.41 A scan of radioactivity obtained after separating ^{32}P-labelled phospholipids on silicic acid impregnated paper. The identification of the numbered spots has been described in the text.
Solvent System: diisobutyl ketone:acetic acid: water (40:25:5 ; v/v/v).
Scan Details: 6"/hr 1/8" slit.
Sensitivity: 0-100
Mean probable error 3%.

TABLE 8 Percentage Distribution of Counts between Individual Phospholipids following incubation of ^{32}P-labelled phosphate with Macrophages in vitro. Mean Distribution for Various Incubation Times in 5 Experiments together with the Standard error of the mean is shown.

Time	Spot 1 Unidentified	Spot 2 Inositol Phosphatide	Spot 3 Sphingomyelin	Spot 4 Lecithin	Spot 5 "Cephalin"	Spot 6 Unidentified	Spot 7 Phosphatidic Acid
1 hr.	2.4 ± 0.7	5.2 ± 0.9	10.0 ± 2.6	68.8 ± 6.2	4.0 ± 2.0	1.0 ± 0.2	8.6 ± 1.4
2 hr.	1.0 ± 0.2	5.6 ± 0.3	9.1 ± 2.9	76.9 ± 4.1	3.0 ± 0.8	0.7 ± 0.2	3.8 ± 0.7
4 hr.	-	5.8 ± 0.3	9.3 ± 2.5	79.4 ± 3.1	3.2 ± 0.7	0.5 ± 0.1	1.6 ± 0.3
6 hr.	-	5.5 ± 0.3	10.6 ± 2.5	77.9 ± 3.1	3.4 ± 0.7	0.6 ± 0.2	1.7 ± 0.3
12 hr.	-	7.4 ± 1.6	15.3 ± 2.5	71.9 ± 1.8	4.3 ± 1.0	0.5 ± 0.2	0.4 ± 0.1

the time period investigated it can be seen that the percentage distribution of counts of spots 1-6 did not vary significantly with time. Spot 7 however did fall off from 8.6% at one hour to 0.4% at six hours. Although not positively identified, it was the fastest moving spot and was probably phosphatidic acid. Spot 4 (lecithin) contained 69-79% of the label; sphingomyelin (spot 3) varied between 9 and 15%; phosphatidyl ethanolamine (spot 5) contained 3-4% of the label and between 5.2 and 7.4% was present in the inositol phosphatide spot.

E. Rate of ^{32}P Incorporation into Individual Phospholipids of Macrophages

The rise in specific activities (cps/µg total lipid P_4) of the individual phospholipids in relation to time are shown in Figs. 42 and 43. ^{32}P was increasingly incorporated into lecithin, sphingomyelin, phosphatidyl ethanolamine and inositol phosphatide over the time period studied. Lecithin was labelled more rapidly than the other phospholipids and at six hours had a specific activity of 97 cps/µg of total lipid P_4 compared with 12.5, 6.7 and 4.1 cps/µg lipid P_4 for sphingomyelin, inositol phosphatide and phosphatidyl ethanolamine respectively.

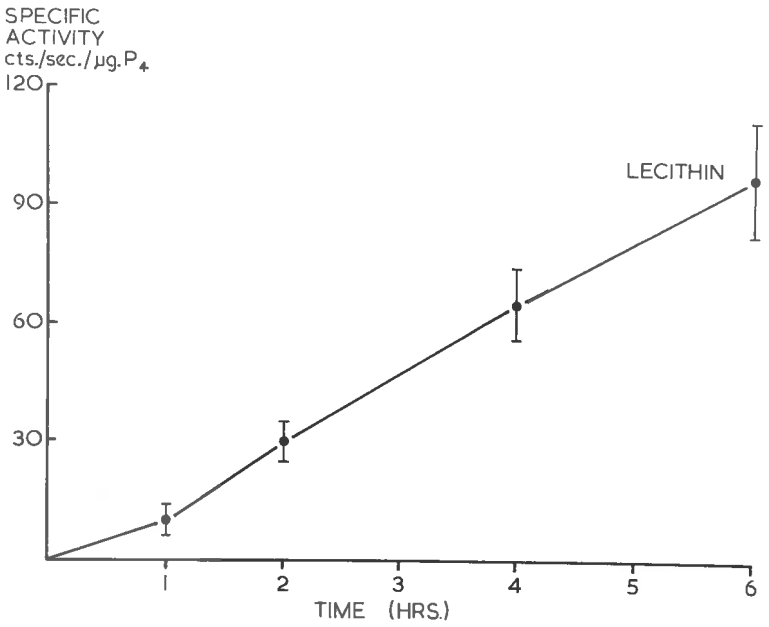


Fig.42 Specific activity of lecithin (expressed as counts/second/µg of total lipid phosphorus) following incubation of macrophages with ^{32}P-labelled phosphate plotted against time. The graph shows the mean plot together with standard deviations from the mean.

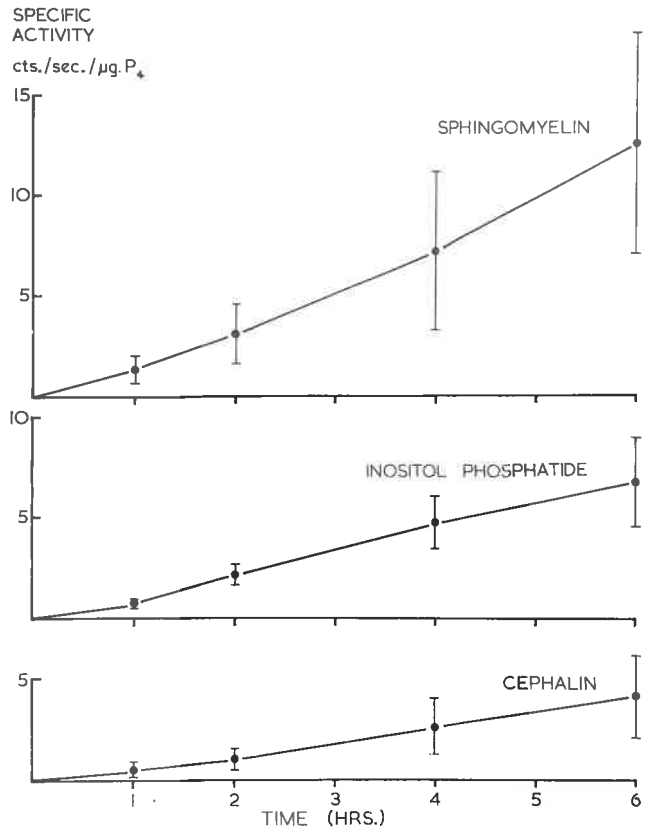


Fig.43 Specific activity of sphingomyelin, inositol phosphatide and "cephalin" (expressed as counts/second/µg of total lipid phosphorus). Each graph represents a mean plot and is shown together with the standard deviations of the mean.

F. Effect of Cholesterol and Carbon Suspensions on the Incorporation of ^{32}P into Phospholipids of Macrophages

It has been suggested (Zilversmit, Shore and Ackerman (1954) Zilversmit and McCandless 1959) that phospholipid is synthesised in the arterial wall in response to accumulation of cholesterol. This phenomenon has been explained as a mechanism for disposing of large quantities of unwanted cholesterol by cells. This is achieved by forming phospholipid/cholesterol complexes which are more water soluble than cholesterol alone and are therefore more readily dispersed in the various body fluids (Dixon, 1958; Ladd, Kellner and Correll, 1949). In experiments reported elsewhere (Day, Gould-Hurst and Wilkinson, 1964) it has been shown that cholesterol suspensions taken up by macrophages *in vitro* is incorporated into lipoprotein in the medium more readily than where such uptake does not occur. The synthesis of phospholipid in response to such uptake and its role in forming soluble lipoprotein complexes with the cholesterol would explain such a dispersion.

In order to obtain some information about the effect of cholesterol on phospholipid synthesis by macrophages, 2.5mg of cholesterol suspension was added to the medium in one set of a triplicate series containing 80uc of ^{32}P phosphate, 50 x 10^6 macrophages and 2.4ml Hanks' serum (2:1 v/v) and made up to a final volume of four ml. To observe whether the act of phagocytosis has some effect on the rate of incorporation of ^{32}P into phospholipid, 2.5mg of carbon

particles (C11/1431a: Gunther Wagner, Hanover, Germany, particle size less than 500 Å) was included in another series. The third (control) series contained neither cholesterol nor carbon suspension. Incubations were carried out at 37° in Warburg cups for 1, 2, 4 and 6 hours and the oxygen uptakes recorded. That carbon particles are phagocytosed by macrophages is well known and it has also been shown by Day (1961) that cholesterol in suspension is taken up by these cells under similar conditions to those reported in this work.

From Fig.44B which illustrates the oxygen uptake recorded in each of the three series, it can be seen that the presence of 2.5mg carbon significantly increased the oxygen uptake over that of the control series but however killed the cells after two hours. Cholesterol did not significantly alter the oxygen uptake, although some decrease in metabolic activity is evident after four hours. The effect of the presence of carbon and cholesterol on the incorporation of ^{32}P into phospholipid is shown in Fig.44A. Cholesterol, which did not significantly alter the oxygen uptake, has, however, stimulated the incorporation of ^{32}P into macrophage phospholipid. Of considerable interest is the fact that the presence of 2.5 mg carbon although it has a marked effect on the respiration rate, has not significantly altered the labelling of phospholipid up to the two hour period before arrest of respiration.

A similar series of experiments were next performed except that in the carbon treated series, 0.5mg of carbon suspension was used

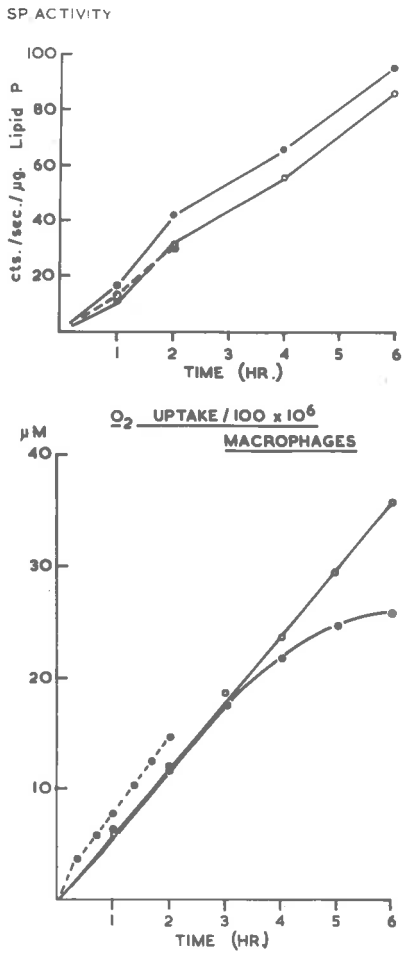


Fig.44 A Specific activity of total phospholipid (expressed as counts/second/µg of total lipid phosphorus) following incubation of macrophages with ^{32}P-labelled phosphate.
B Oxygen uptake of macrophages from experiments recorded in A.

o——o normal
●——● Carbon (2.5mg)
•——• Cholesterol (2.5mg)

instead of 2.5mg. This was considered to be a better control, since it did not kill the cells as did the presence of 2.5mg of carbon and the respiration response observed in the cells was no different to that observed when cholesterol (2.5mg) was present (see Fig.45B). Thus conditions were produced under which the incorporation of ^{32}P into phospholipid could be studied independantly of differences in the respiration rate.

In each experiment the specific activity of the control run at six hours was taken as 100% and the specific activities at the other time intervals and of the time interval in the runs containing carbon and cholesterol respectively were expressed in relation to this. The mean of three experiments is shown in Fig.45A. In these experiments there was some loss of metabolic activity after four hours in the incubations to which the cholesterol suspension had been added as evidenced by a reduction in oxygen uptake. However, during the total period of incubation, the rate of incorporation of ^{32}P into phospholipid was about thirty per cent. faster than in the control or in the preparation containing the carbon suspension. The average rates of increase in specific activity up to the various times of measurement were analysed statistically on a logarithimic scale. Analysis indicated that changes in the average rates of increase at different times were the same for all these treatments and that there were no significant differences between the two controls. The average rate of increase in specific activity was significantly higher however for the chol-

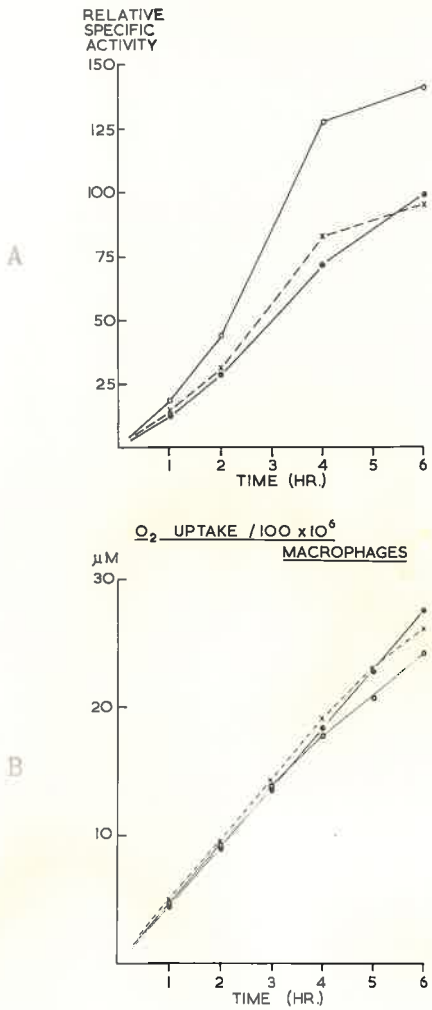


Fig.45 A The effect of cholesterol and of carbon suspension on the incorporation of ^{32}P-labelled phosphate into phospholipid by macrophages *in vitro*. Mean of three experiments in which the relative specific activity (percentage of that present in the content at 6-hr) is plotted against time.

B Oxygen uptake of macrophages from experiments recorded in A.

●——● normal
x-----x Carbon (0.5mg)
o——o Cholesterol (2.5mg)

esterol treatment than for either control ($p < 0.1\%$).

The effect of the uptake of cholesterol on the percentage distribution of radioactivity between individual phospholipids as determined following paper chromatography and scanning is shown for one experiment in Table 9. The percentage distribution of counts in the various phospholipids at the different time intervals is essentially the same, whether cholesterol has been taken up or not, so that although the total phospholipid synthesis is increased as a result of cholesterol uptake it appears that such increase is shared by all fractions.

G. Discussion

The phospholipids of macrophages which were labelled with ^{32}P in the experiments described above were lecithin, sphingomyelin, inositol phosphatide, phosphatidyl ethanolamine and probably phosphatidic acid. It is interesting to find that Karnovsky and Wallach (1961) have fractionated phospholipids synthesised by polymorphonuclear leucocytes and identified the components as phosphatidic acid, phosphatidyl ethanolamine, inositol phosphatide, phosphatidly serine, lecithin and sphingomyelin.

The metabolic response to the addition of particulate matter by macrophages is of particular interest in this work. Some comparative

TABLE 9 The Effect of Cholesterol Uptake on the Percentage Distribution of Counts Between Individual Phospholipids following Incubation of ^{32}P-labelled Phosphate with Macrophages.

Data from one Experiment

Time	Spot 1 Unidentified	Spot 2 Inositol Phosphatide	Spot 3 Sphingomyelin	Spot 4 Lecithin	Spot 5 "Cephalin"	Spot 6 Unidentified	Spot 7 Phosphatidic acid
1 hr. Control	2.1	8.9	9.3	66.8	2.2	1.6	9.3
Cholesterol	1.8	7.1	8.6	67.6	1.3	1.5	12.1
2 hr. Control	1.1	5.0	6.4	79.5	2.1	0.7	5.1
Cholesterol	1.8	6.3	8.1	76.2	1.4	0.8	5.4
4 hr. Control	-	4.9	8.0	81.6	2.3	0.6	2.5
Cholesterol	1.3	7.2	8.2	75.9	2.1	0.9	4.3
6 hr. Control	0.7	5.2	8.9	80.0	2.3	0.3	2.5
Cholesterol	1.0	7.6	7.3	79.5	1.6	0.5	2.3

observations on the respiratory rate of various phagocytes have been made by several workers and the results have been summarised by Karnovsky (1962). During phagocytosis, respiration increases about 2.5 fold in polymorpho-nuclear leucocytes, 3.5 fold for monocytes and only about twenty per cent. for alveolar macrophages. The expired $^{14}CO_2$ liberated from ^{14}C-labelled glucose was also measured at the resting level or during phagocytosis in each of the above mentioned phagocytes and it was found that in monocytes and alveolar macrophages no changes in liberation of $^{14}CO_2$ occurred during phagocytosis.

In these investigations the peritoneal macrophages of rabbits demonstrated an increase in respiration when 2.5mg of carbon was present, but not when 2.5mg of cholesterol or 0.5mg of carbon suspension was present. It is to be remembered that these cells were harvested in response to liquid paraffin. Stahelin, Suter and Karnovsky (1956) found that differences in QO_2 of leucocyte cells were dependant on the irritant material used to elicit the exudate and also on the time which had elapsed between injection of the irritant and collection of the cells. The ingestion of paraffin by these cells has possibly stimulated their metabolic activity to a maximum and although small amounts of cholesterol and carbon (when present at the lowest concentration) are taken up, they do not affect the respiration rate further.

It is not clear why the presence of higher concentrations of carbon caused increased respiration and then arrest in respiration.

Perhaps clumping occurred which increased the particle size and it is known that large doses of carbon suspensions will block phagocytosis.

There are two other alternatives which must briefly be considered. It is possible that cholesterol does not illicit a respiration response because it is not phagocytosed by the macrophage but is merely absorbed onto the cell surface and in this situation still provokes the stimulation of phospholipid synthesis. Although Day (1961) has demonstrated the esterification of cholesterol by macrophages (intact cells) it is possible that such ester formation was the result of cell surface acting enzymes. A more entertaining hypothesis concerning particle size can be considered in an attempt to explain the negative respiration response induced by carbon. The particle size employed here was less than 500 Å and at this size it might not stimulate metabolic activity, although it is phagocytosed readily. There is some evidence to support this suggestion. Strauss, in a personal communication to Karnovsky (1962) reported that she was able to demonstrate a respiration response in leucocytes which was proportional to the particle size of the polystyrene spherules when used within the range of 0.25μ to 1.2μ in diameter. With particles less than 0.2μ in diameter no respiration response occurred at all. Perhaps a similar situation applies to macrophages since the carbon particles were less than 500 Å (less than .05μ) in diameter.

Although there is no other information concerning the types of phospholipid present in macrophages or concerning the synthesis of phospholipids by these cells, there have been many reports in the literature about lipid synthesis in polymorphonuclear leucocytes. Elsbach (1959) demonstrated the synthesis of lipid from ^{14}C-labelled acetate by these cells and was also able to show that phagocytosis of bacilli stimulated this synthesis. Similarly, Sbarra and Karnovsky (1960) demonstrated an increased incorporation of acetate-1-^{14}C into leucocyte lipid when using inert polystyrene latex spherules. More relevant is the increased incorporation of ^{32}P into phospholipids of polymorpho-nuclear leucocytes as shown by the above authors and in more recent work by Karnovsky and Wallach (1961). They associate this stimulation of phospholipid synthesis with the manufacture of new membrane required to enfold the ingested particle.

These aspects of phagocytosis although relevant to the present discussion do not form the subject of this investigation. Under the conditions reported above, it was shown that phospholipid synthesis is stimulated by cholesterol but not by carbon. It is possible then that phospholipid is synthesised in response to cholesterol accumulation, which has already been suggested earlier as being one way for dispersing large amounts of unwanted cholesterol.

The separation of individual phospholipids has demonstrated that the label is distributed mainly to lecithin and to a lesser extent to sphingomyelin. It has been shown that sodium palmitate-

1-^{14}C is incorporated into these two phospholipids by macrophages and it is of interest to point out the relationship of these observations to phospholipid accumulation in the arterial wall in atheroma. McCandless and Zilversmit (1956) have shown that lecithin and sphingomyelin are turned over by the atheromatous arterial wall at a more rapid rate than is "cephalin" while other workers (Smith 1960) have demonstrated that the increase in phospholipid content of the atheromatous artery is mainly due to an increase in the sphingomyelin level. Adams and Bayliss (1963) and Adams, Bayliss and Ibrahim (1963) have also shown by histochemical means that in experimental and human atheroma sphingomyelin is present as the major phospholipid but they consider this to arise from elastic tissue breakdown rather than to macrophage activity.

THE CONVERSION OF GLUCOSE-1-^{14}C TO LIPID BY MACROPHAGES *in vitro*

A. Introduction

Animals which are fed on a fat free diet deposit fat in the tissues and therefore must synthesise it from carbohydrate as the major carbon source. In an earlier section in this thesis it was reported that macrophages incubated *in vitro* could incorporate the C_2 unit acetate-1-^{14}C into lipid and it was shown that fatty acids and cholesterol were prominently labelled during such experiments.

When considering carbohydrate as a source of carbon for lipid manufacture in any tissue it must be recognised that there are two possibilities of contribution. Either carbohydrate can be converted to fatty acids and sterols or else it can be converted to glyceride-glycerol. The extent of contribution in either direction will obviously be dependant on regulatory processes imposed as part of the internal or external environment. Some of these aspects will now briefly be discussed.

The coupling of carbohydrate oxidation with fatty acid synthesis has been recognised for some time but Stetten and Boxer (1944) were the first to call attention to the quantitative significance of this conversion in the normal animal. They showed that in the rat fed on a high carbohydrate diet approximately ten times as much dietary glucose was used to synthesise fatty acids as was used for the synthesis of glycogen.

A study of the synthesis of lipid under abnormal conditions, such as in the diabetic, depancreatised or alloxan treated animals has

helped in understanding the inter-relationships of carbohydrate metabolism and lipid synthesis in normal animals. The importance of lipogenesis is illustrated by the fact that liver preparations from diabetic animals are unable to synthesise fatty acids although they are capable of oxidising fatty acids to ketone bodies. It is known that in the diabetic animal the conversion of glucose to glucose-6-phosphate is markedly depressed. However, when carbohydrates that can enter the glycolytic pathway and be oxidised (e.g. fructose and glucose-6-phosphate) are administered the diabetic animal regains its ability to synthesis fatty acids. The effect of fasting or restricting the caloric intake stops the deposition of fat in animal tissues (Boxer and Stetten 1944). Livers taken from rats after a 24 hour fast show a diminished ability to synthesise fatty acids in vitro from both ^{14}C-labelled glucose and acetate (Masoro, Chaikoff, Chernick and Felts, 1950; Bloch 1948).

Although there is much information available regarding the incorporation of glycerol into triglycerides and phospholipids, there seems to have been little investigation into the origin of the glyceride-glycerol. The earliest studies of the conversion of glucose to lipid glycerol using animal tissue were made by Popjak, Glascock and Folley (1952). These will be discussed later in this section.

The following investigations were carried out using glucose-1-^{14}C as substrate in order to obtain information concerning the conversion of carbohydrate to lipid by macrophages.

B. Determination of Total Intracellular ^{14}C, ^{14}C-labelled Lipid and $^{14}CO_2$ Production

Paired incubations were performed in groups of three in siliconised thirty ml conical flasks. Each flask contained a known number of rabbit macrophages ($30-90 \times 10^6$), 0.9ml dialysed rabbit serum, approximately 2μc of glucose-1-^{14}C and were made up to a final volume of four ml with glucose free Hanks' solution.

After two hours incubation at 37° the cells from 2 groups of flasks were separated from the medium, washed twice with 0.9% sodium chloride and then lyophilised. The lipid was then extracted with chloroform:methanol (2:1 v/v) from one pair and the two members of the other pair were reconstituted with water. Portions of both were counted by scintillation counting using the Dioxan scintillator.

The remaining pair of the group were used to measure $^{14}CO_2$ produced from glucose-1-^{14}C by macrophages. The results of these three investigations are combined in Table 10. The proportion of glucose-1-^{14}C incorporated into the lipid fraction of the macrophages varies between 17 and 33% of the total ^{14}C present in the cells. About 50% of the glucose added to the medium has been converted to $^{14}CO_2$ by the cells in each experiment. Controls were included in which medium was incubated in the presence of glucose-1-^{14}C but no cells. There was no $^{14}CO_2$ produced or ^{14}C-labelled lipid in the control medium after two hours.

TABLE 10 The Conversion of Glucose-1-^{14}C to Total Cell ^{14}C, ^{14}C-labelled Lipid and $^{14}CO_2$ following Incubation with Macrophages in vitro.

Expt.	No. of cells	Amount of glucose-1-^{14}C added	Total Cell ^{14}C cps	Lipid ^{14}C cps.	Lipid ^{14}C % of total ^{14}C	$^{14}CO_2$ cps
1	90×10^6	2.0μc	4275	1235	29	* -
2	55×10^6	1.6μc	1550	510	33	24,000
3	30×10^6	2.0μc	1880	440	23.4	32,300
4	40×10^6	2.0μc	670	176	26.3	33,000
5	30×10^6	2.0μc	2740	469	17.4	21,000

* not determined

C. Total Lipid Specific Activity Determination

Incubations were carried out as described above for 5, 10, 30, 60 and 120 minutes. After this time the flask contents were quickly transferred to chilled centrifuge tubes in an ice bath. The cells were separated by centrifuging (1500rpm for five minutes) at 2° and washed twice with cold 0.9% sodium chloride solution; the lipids were extracted with chloroform:methanol (2:1 v/v) and a portion was counted by scintillation counting.

The specific activities were expressed as cps/mg of total esterified fatty acid, the latter being determined in each experiment on a batch of cells reserved for this purpose.

The conversion of glucose-1-^{14}C into total lipid is shown in Fig.46. The graph represents the mean plot of four experiments and is shown with standard deviations from the mean. There is a rapid rise in specific activity of the total lipid for the first five minutes which increased progressively with time to a mean of 905cps/mg at thirty minutes. In each experiment the pattern of labelling was the same as can be seen from the standard deviations.

The amount of glucose-1-^{14}C converted to lipid, however, varied with different cell batches.

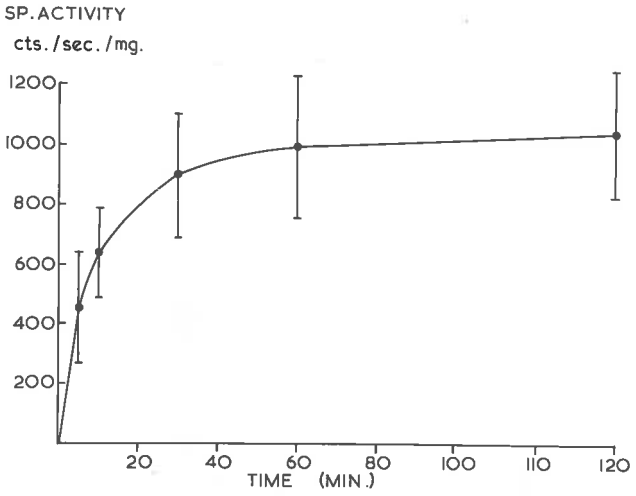


Fig.46 Specific activity of total lipid (counts/second/mg of total esterified fatty acid) following incubation of macrophages with glucose-1-^{14}C plotted against time. The mean of four experiments together with the standard deviation of the mean is shown.

D. Distribution of Radioactivity in Macrophage Lipid After Incubation with glucose-1-^{14}C

The lipid extracts from cells which had been incubated with glucose-1-^{14}C for two hours were used for hydrolysis in each experiment. The lipids were hydrolysed with 1.7 N HCl in methanol in sealed tubes at 110° for five hours as described on p.30. The lipid was extracted with light petroleum (b.p. 60-80°) and counted and a method was developed for the quantitative analysis of glycerol and glycerophosphate remaining in the aqueous extracts (see p.34). Thus glycerol and glycerophosphate were separated by paper chromatography using n-propanol:ethyl acetate:water (7:1:2) as the developing solvent and the papers were scanned. The distribution of radioactivity was determined by measuring the area of the peaks. These were added and the total counts are presented in Table 11 for four such experiments. 94-96% of the activity is present as glycerol and glycerophosphate while the fatty acid and cholesterol only accounts for 4-6% of the total.

TABLE 11 Incorporation of Glucose-1-^{14}C into Lipid Glycerol and Cholesterol/Fatty Acids after two hour Incubation with Macrophages.

Exp.	Glycerol/ Glycerophosphate		Cholesterol/ fatty acid	
	cps	%	cps	%
1	694	94	30	4.0
2	449	95	24	5.0
3	378	96	15	4.0
4	406	94	27	6.0

E. Fractionation of Lipid

The fractionation on silicic acid columns of lipid extracts of macrophages which had been incubated with glucose-1-^{14}C is shown in Fig.47 which shows the seperation of lipid from two experiments. Most of the activity is present in the triglyceride and phospholipid fractions and some labelled diglyceride is present. Only a few counts were present in the cholesterol fraction.

F. Discussion

The rapid rise in specific activity of the total lipid fraction of macrophages after incubation with glucose-1-^{14}C provides evidence for the enzymic conversion of carbohydrate to fat in these cells. Of the total ^{14}C present intracellularly after two hours about twenty-five percent is present as lipid in macrophages. This is similar to the amount of acetate-1-^{14}C converted to lipid under similar conditions by these cells. Most of the label was present in the triglyceride and phospholipid fractions i.e. in the glyceride fractions, with only a few counts in the cholesterol fraction. Further hydrolysis of the lipids provided evidence that 95% of the labelling occurred in the glyceride-glycerol moiety. There is considerable evidence (Chernick, Masoro and Chaikoff 1950; Baker, Chaikoff and

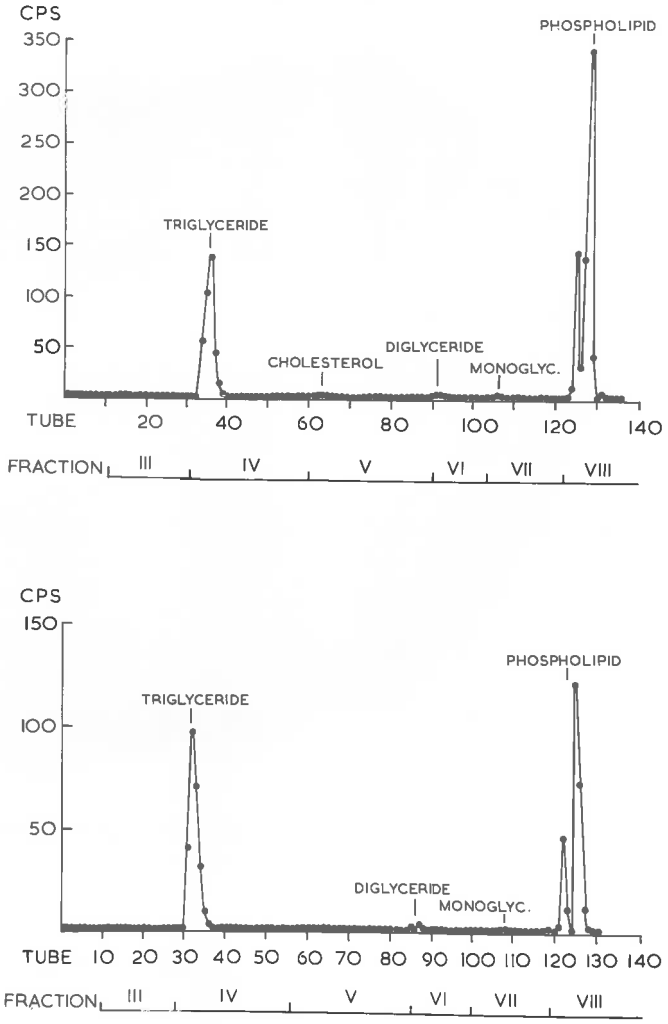


Fig.47 Fractionation on silicic acid columns of the ^{14}C-labelled lipid components of macrophages after 2-hr incubation with glucose-1-^{14}C in two different experiments. Each column loaded with 10mg each of unlabelled cholesterol ester, tripalmitin, palmitic acid, cholesterol and lecithin

Schusdek 1952) for the conversion of carbohydrate, e.g. glucose and fructose to fatty acid and cholesterol all of which presumably occur via the anaerobic breakdown of carbohydrate and then through acetyl CoA to fatty acids and cholesterol. The present experiments show that the contribution of glucose to "fat" in macrophages is more extensive through its conversion to the glycerol part of the glyceride molecule than to the fatty acid fraction.

The labelling of the glycerol portion of lipids from glucose-1-^{14}C was first demonstrated in animal tissue by Popjak, Glascock and Folley (1952) who investigated the conversion of carbohydrate to lipid in milk fat. Polonovski, Payeant and Wald (1962) demonstrated that almost the total radioactivity introduced into spleen lipids after incubation with glucose-1-^{14}C for two hours was found in the glycerol of triglycerides and phospholipids.

Terner and Korah (1962) demonstrated that glycerol and glucose were of equal potential as glyceride glycerol sources in bull spermatozoa and also that acetate was incorporated into fatty acids. However, the incorporation of fatty acids (labelled from acetate-1-^{14}C) into glycerides proceeded at a considerably slower rate than might be expected from the rate of labelling of the glycerol moiety. Similar observations were also made by Cahill, Lebeouf and Renold (1959) who found that the rate of glyceride-glycerol synthesis greatly exceeded that of the fatty acids. The present study of glucose to glycerol conversion by macrophages similarly indicates a more rapid turnover of the glycerol moiety than of the fatty acids.

The conversion of glucose-1-^{14}C almost exclusively to glycerol by macrophages supports conclusions reached by other authors that glycerol as such is not the immediate precursor of glyceride glycerol. Buell and Reiser (1959) have shown that the conversion of ^{14}C-labelled fructose 1:6 diphosphate into fat glyceride by pig intestinal mucosa was not altered by the presence of glycerol; however in the presence of dihydroxyacetone phosphate and glycerophosphate the incorporation was reduced suggesting that these latter two were precursors of glyceride glycerol. They suggested that glycerophosphate was probably the immediate precursor.

There are several pathways known in animal tissue by which glycerol can be formed from glucose. The enzyme aldolase catalyses the formation of dihydroxyacetone-phosphate and glyceraldehyde phosphate from fructose1:6 diphosphate. Although the equilibrium constant of the isomerase enzyme (which catalyses the interconversion of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate) favours the formation of dihydroxyacetone phosphate, the rapid removal of glyceraldehyde-3-phosphate from the reaction would tend to convert nearly all the dihydroxyacetone phosphate to glyceraldehyde phosphate. Thus only a small amount of dihydroxyacetone phosphate would be available for glycerol formation. The former is converted to glycerol phosphate and according to Kennedy (1957) this then forms phosphatidic acid which is dephosphorylated to form 1,2-diglyceride. This may then react with cytidine diphosphate-choline, ethanolamine etc. to form phospholipids or with long chain fatty esters of CoA to form

triglycerides. These reactions which are carried out at the expense of ATP involve the expenditure of energy. In the experiments with macrophages it has been found that more than half the glucose added to the medium has been oxidised to carbon dioxide which indicates that pathways of glucose oxidation are available for energy production. It is known that glycolysis proceeds under aerobic or anaerobic conditions in these cells together with the production of lactic acid in the medium (Harris and Barclay 1955).

There are other pathways by which glycerophosphate can be formed. The pentose phosphate cycle is one such alternative. It is also known that recycling of glycolytic pathway intermediates such as glyceraldehyde-3-P, dihydroxyacetone phosphate and fructose 1:6 diphosphate can occur in the pentose pathway. Therefore, it is not possible to speculate on the extent of glucose available for glycerol formation in macrophages without obtaining more information about existing pathways in these cells. The observation made that only a small amount (0.3-2.0%) of the glucose-1-^{14}C added to the medium is converted to lipid glycerol is in accord with the fact that only a small amount of dihydroxyacetone phosphate is produced during oxidation of glucose. Similar results have been found by Stjernholm and Noble (1961) who incubated polymorpho-nuclear leucocytes with ^{14}C-labelled glucose. Only 0.05 to 0.07% of the initial radioactivity of the labelled glucose entered the lipids of leucocytes and half of this was in the glycerol molecule. This is considerably less than the amount of glucose converted to lipid glycerol by macrophages.

However they did not take into account the labelled glycerol, if any, present as phosphatide glycerol. After degradation of the labelled glycerol and determination of the distribution of ^{14}C, Stjernholm and Noble found a randomization of ^{14}C in the glycerol which indicated a moderate recycling in the pentose pathway. In these experiments glucose-1-^{14}C was the only substrate available and therefore no attempts were made to investigate the various pathways of glucose oxidation in these cells.

GENERAL DISCUSSION AND CONCLUSIONS

The information accumulated as a result of the investigations described in this thesis are of interest from two points of view, one concerning the lipid biochemistry of macrophages and the second being that these investigations might also provide information in relation to the deposition of lipid in the arterial wall in atheroma. Both points are considered seperately below.

Lipid Biochemistry of Macrophages

It is assumed that the palmitate-albumin complex and the triglyceride emulsion ingested by rabbit peritoneal macrophages were taken up as a result of phagocytosis. The mechanisms involved in the transport of the smaller molecules viz. acetate, orthophosphate and glucose into macrophages, however, are not known. The subsequent incorporation of all these radioactively labelled compounds into the lipid fraction of macrophages formed the major part of these studies which were carried out with intact cells. Although this approach using intact cells was necessary initially, such a system obviously has distinct disadvantages when attempting to interpret results pertaining to synthesis of lipid. The fact that no net synthesis of lipid was demonstrated in any of these experiments does not rule out the possibility that the reactions reflect merely isotope exchange rather than true synthesis. In many of the investigations performed however, labelled compounds were isolated from macrophages

which could be identified as intermediates in pathways of lipid synthesis already explored and confirmed in other animal tissues by other investigators. Therefore, from the results of these investigations it is possible to speculate that macrophages have the enzymic ability to manufacture such lipid components.

A point of favour in the use of intact cells and an important aspect to be considered when studying synthesis of any kind is the realisation that reactions such as esterification of fatty acids, synthesis of fatty acids and cholesterol and incorporation of phosphate into phospholipid do not take place unless energy is supplied via coupled reactions. This supply problem i.e. presence of cofactors, metals etc., is eliminated when using intact animals or cells.

In most cases it was apparent that no matter what the carbon source of the lipid, that triglycerides and phospholipids were predominantly labelled. This is not unexpected since it has been pointed out by Folley and French (1950) and Balmain and Folley (1951) that in fat synthesis by animal tissues, free fatty acids do not detectably accumulate, but are immediately transformed into glycerol esters. This appears to have been the situation in macrophages since palmitate-1-^{14}C was incorporated mostly into the triglyceride and phospholipid fractions and the fatty acids synthesised from acetate-1-^{14}C were also incorporated into these two fractions. A comparison of the incorporation of acetate-1-^{14}C into cholesterol and fatty acids with increasing time indicated that the cholesterol pool is turned over at a much greater rate than is the fatty acid pool. The significance of this difference is not clear. Both fatty acids and chol-

esterol are synthesised from C_2 units but it is apparent that there are regulatory conditions which affect in different ways the synthesis of cholesterol and fatty acids from acetate-1-^{14}C. One such instance is that the synthesis of cholesterol in rat liver, unlike that of fatty acids, is not dependent directly upon the glycolytic activity of the cells (Tomkins and Chaikoff 1952).

The labelling of phospholipid after incubation of macrophages with ^{32}P-labelled phosphate indicates that these cells can synthesise phospholipid. The probable presence of phosphatidic acid, a known phospholipid precursor, which was rapidly labelled at first and then decreased with time as the specific activity of the other phosphatides increased, adds weight to this suggestion. Of more interest was the fact that the presence of cholesterol stimulated the incorporation of ^{32}P phosphate into phospholipid. In order to synthesise phospholipid on demand, such as in this situation, the coupling of energy producing reactions and formation of new phospholipid components must be a function of these cells. The controlling rates in such reactions would be the carbohydrate oxidation rate as well as the formation of fatty acids and glycerol. That these latter two substances are synthesised by macrophages has been indicated by investigations with acetate-1-^{14}C and glucose-1-^{14}C as substrates. It can be concluded therefore that rabbit peritoneal macrophages possess the necessary enzyme and energy requirements for synthesis of cholesterol, fatty acids and glycerol.

Macrophages, Lipid Synthesis and Atheroma

If it can be inferred that the results of the *in vitro* investigations with macrophages will apply to the *in vivo* situation, then macrophages will be capable of synthesising lipid in the arterial wall as long as the necessary nutriments are provided.

It was shown that macrophages can take up palmitic acid and synthesise triglyceride and phospholipid from this fatty acid *in vitro*. In the arterial wall, such a situation could occur, since unesterified fatty acids are present in the plasma although only to a quantitatively small extent (2-5%). This would result in an accumulation of triglycerides and phospholipids, so long as the macrophages can provide the other lipid components necessary for esterification. This would appear to be so since compounds such as phosphatidic acid, glycerophosphate, di- and monoglyceride were isolated from macrophages and had become labelled, possibly as intermediates.

The nature of the fatty acids synthesised from acetate-1-^{14}C by macrophages *in vitro* resembles the composition of fatty acids in most animal tissues studied, including the arterial wall. However, it is unwise to speculate on the composition of fatty acids of macrophages and their role in lipid metabolism of the arterial wall because such a composition will be dependent on external conditions and the presence of other lipids. Some of the cholesterol which accumulates in the atheromatous lesion can be attributed to the accumulation of chol-

esterol in macrophages in the arterial wall. Since a high level of cholesterogenesis was observed in macrophages incubated *in vitro* with acetate-1-^{14}C the possibility that some of this accumulation has arisen as a result of synthesis of cholesterol by macrophages *in situ* cannot be overlooked.

That macrophages are partly responsible for the accumulation of phospholipid in the arterial wall has been considered in earlier discussions. A survey of each observation adds weight to the above hypothesis, since it has been shown that phospholipid has been labelled from palmitate-1-^{14}C, tripalmitin-1-^{14}C, acetate-1-^{14}C, ^{32}P-phosphate and glucose-1-^{14}C by macrophages *in vitro*. More interesting is the fact that in each case, lecithin and sphingomyelin were preminently labelled and it is known that lecithin and sphingomyelin both increase in the arterial wall in atherosclerosis.

Before arriving at any conclusions concerning the role of macrophages in the synthesis of lipid in the arterial wall, it is necessary to consider the many inter-relationships of lipid metabolism already studied. The inter-relationships which are closely connected with this problem are summarised as follows. It has been found that the ingestion of cholesterol or cholesterol ester by reticulo-endothelial cells is associated with accumulation of various other lipids such as triglyceride and phospholipid (Day 1960b). The fatty acid pattern of cholesterol esters are influenced by the type of triglyceride ingested (Day et.al. 1963) which also influences the rate of esterification of cholesterol (Day 1960d). Bernick and Alfin-Slater (1963) observed

that cholesterol accumulates in lung macrophages when animals are kept on an essential fatty acid deficient diet and observed that mobilisation of cholesterol occurred when linoleate was fed. It has also been shown that phospholipid promotes the hydrolysis of lipoprotein cholesterol ester and inhibits esterification of free cholesterol by macrophages (Day and Gouldhurst 1963). The stimulation of phospholipid synthesis by macrophages in the presence of cholesterol has been discussed. After cholesterol ester (labelled in the fatty acid molecule) was ingested by reticulo-endothelial cells of rat lymph nodes, an appreciable amount of the label was found in the phospholipid fraction (including lecithin and sphingomyelin). However, little of the fatty acid was incorporated into the triglyceride fraction as was expected (current investigations). In many tissues, lipogenesis and cholesterogenesis are differently affected by certain conditions such as carbohydrate and fat feeding or the administration of insulin (Hill, Webster, Linazero, Chaikoff, 1960; Williams, Hill and Chaikoff, 1960). Thus the synthesis of lipids such as fatty acids and cholesterol by macrophages in the arterial wall will also be controlled by the presence of extracellular fat deposits, the supply of oxygen, energy requirements and the carbon source.

These considerations emphasise the complexities involved in the situation involving macrophages, lipid synthesis and the arterial wall and make it clear that there is much work to be done before there is a complete understanding of the problem.

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