LIBRARY OF THE UNIVERSITY OF ADELAIDE 8.2.71

ARTERIAL WALL METABOLISM

AND

ATHEROGENESIS

A thesis submitted for the degree
of
Doctor of Medicine
by

Mark Lawrence Wahlqvist
B.Med.Sc. (Hons.), M.B.,B.S.

at the
University of Adelaide

Department of Physiology
University of Melbourne

February, 1970

TO SOO AND INGMAR

PREFACE

Atherosclerosis and its complications account for a vast amount of human morbidity and mortality. It is a disease characterised by the accumulation of lipid, chiefly cholesterol ester and phospholipid, in the intima and inner media of the aorta and distributing arteries. Present information indicates that the lipid of the atherosclerotic lesion cannot arise unmodified from the blood. Further, it has become apparent that the arterial wall has a metabolic capacity of its own.

In order to clarify the likely role of metabolic processes in early atherogenesis, findings relating to the lipid chemistry of the arterial wall in childhood are reported in this thesis. The difference in handling of fatty acid by normal and atherosclerotic intima is examined. Sites of fatty acid and of choline incorporation into lipid in the atherosclerotic lesion are assessed by autoradiography. The differences in arterial handling of fatty acids according to their degree of unsaturation are considered. Also presented is a study to determine whether there is differential uptake by the atherosclerotic lesion of different forms of cholesterol. Finally, some of the work is elaborated using acetate as substrate for arterial lipids.

Evidence is adduced that phospholipid and cholesterol ester metabolism in the arterial wall are important pathogenetic, but not necessarily aetiological, considerations in the evolution of the atherosclerotic plaque and that most of this metabolism takes place in intimal foam cells.

CONTENTS

PREFACE i
CONTENTS ii
DECLARATION ix
ACKNOWLEDGEMENTS x
GENERAL INTRODUCTION
A. General Remarks 1
B. Lipids and Atherosclerosis in Early Life 16
C. Lipid Composition of the Arterial Wall 21
D. Lipid Entry into the Arterial Wall 28
E. Arterial Wall Metabolism 32
1. Lipid Metabolism 34
(1) Fatty Acid 34
(2) Cholesterol 37
(3) Cholesterol Ester 39
(4) Phospholipid 42
(5) Triglyceride 47
2. Carbohydrate Metabolism 48
3. Protein Metabolism 49
4. Metabolism of other Substances 50
5. Factors Affecting Arterial Wall Metabolism 51
(1) Diabetes and Insulin 51
(2) Hypertension 55
(3) Therapeutic Agents 56
F. Intimal Cells 58
G. Removal of Lipid from the Arterial Wall 61
MATERIALS AND METHODS
A. Materials 65
1. Radioactive substrates 65
(a) ^{14}C-labelled Sodium Palmitate 65

(b) ^{14}C-labelled Sodium Oleate 65
(c) ^{14}C-labelled Sodium Linoleate 66
(d) ^{3}H-labelled Sodium Oleate 66
(e) ^{14}C-labelled Cholesterol 66
(f) ^{3}H-labelled Cholesterol 67
(g) ^{14}C-labelled Choline 67
(h) ^{14}C-labelled Sodium Acetate 67
(i) ^{32}P-labelled Ortho-Phosphate 67
2. Thin-layer chromatographic standards 67
(a) neutral lipids 67
(b) phospholipids 68
(c) cholesterol esters 68
(d) methyl esters 68
3. Internal Standards 69
(a) Heptadecanoic Acid 69
(b) Cholesterol heptadecanoate 69
(c) ^{14}C-labelled cholesterol 70
4. Lipoprotein ^{3}H/^{14}C-labelled Cholesterol 71
5. Insulin and Anti-Insulin 72
6. Chlorophenoxyisobutyric Acid 76
B. Arterial Tissues 76
1. Rabbit arteries 76
2. Human arteries 79
(a) Metabolic Studies 79
(b) Chemical Studies 79
C. Lipid Extraction 82
D. Chromatographic Methods 82
1. Thin-layer chromatography 82
(a) neutral lipid separation 83
(b) separation of methyl esters from neutral lipids 84
(c) phospholipid separation 84
(d) methyl ester separation 86
(e) cholesterol ester separation 86

2. Alumina Column Chromatography 87

3. Paper Chromatography of Choline 88

4. Gas-Liquid Chromatography 88

(a) preparation of methyl esters for gas liquid chromatography 88

(b) separation of methyl esters 90

(c) quantitation of lipids 91

(d) gas-liquid radiochromatography 91

E. Radio-Assay 94

F. Chemical Assay 96

1. determination of phospholipid 96

2. determination of cholesterol 98

3. determination of free fatty acid 100

G. Autoradiography 101

1. ^{14}C-labelled oleic acid 101

2. ^{3}H-labelled oleic acid 103

3. ^{14}C-labelled choline 103

H. Ultracentrifugation 104

I. Electrophoresis 105

J. Isolation of Human Intimal Cells 105

Section 1.

ARTERIAL LIPID COMPOSITION AND ITS RELATIONSHIP TO SERUM LIPIDS

A. Introduction 111

B. Human Artery in Early Life 113

1. Morphological Considerations 113

2. Free and Ester Cholesterol Content 113

3. Cholesterol Ester Fatty Acid Composition 120

4. Phospholipid Content 126

5. Phospholipid Fatty Acid Composition 129

C. Normal and Cholesterol-Fed Rabbit 133

D. Discussion 137

Section 2.

INCORPORATION IN VITRO OF ^{14}C-LABELLED OLEIC ACID INTO COMBINED LIPID BY THE RABBIT ARTERIAL WALL.

A. Introduction 144

B. Uptake of ^{14}C-labelled Oleic Acid by Normal and Atherosclerotic Rabbit Aortic Intima 145

C. Incorporation of ^{14}C-labelled Oleic Acid into Combined Lipid by Normal and Atherosclerotic Rabbit Aortic Intima 146

D. Specific Activity of ^{14}C-labelled Oleic Acid Incorporated into Combined Lipid 152

E. Autoradiographic Localisation of ^{14}C-labelled Oleic Acid in Rabbit Atherosclerotic Lesions 154

F. Discussion 157

Section 3.

INCORPORATION IN VITRO OF ^{14}C-LABELLED OLEIC ACID INTO COMBINED LIPID BY THE HUMAN ARTERIAL WALL.

A. Introduction 165

B. Uptake and incorporation of ^{14}C-labelled Oleic Acid into Combined Lipid by Human Arterial Intima 165

C. Specific Activity of ^{14}C-labelled Oleic Acid Incorporated into Combined Lipid 169

D. Autoradiographic Localisation of ^{14}C-labelled Oleic Acid in Human Atherosclerotic Lesions 172

E. Discussion 180

Section 4.

PHOSPHOLIPID SYNTHESIS BY RABBIT ATHEROSCLEROTIC AORTA IN VITRO

A.	Introduction	184
B.	Uptake and Incorporation of ^{14}C-labelled Choline into Phospholipid	184
C.	Autoradiographic Localisation of Phospholipid Formation from ^{14}C-labelled Choline in Rabbit Atherosclerotic Lesions	186
D.	Uptake and Incorporation of ^{3}H-labelled Oleic Acid into Combined Lipid and Extraction of Lipid other than Phospholipid	189
E.	Autoradiographic Localisation of Phospholipid Formation from ^{3}H-labelled Oleic Acid in Rabbit Atherosclerotic Lesions	194
F.	Discussion	198

Section 5.

PHOSPHOLIPID SYNTHESIS BY THE HUMAN ARTERIAL WALL IN VITRO

A.	Introduction	203
B.	Uptake and Incorporation of ^{14}C-labelled Choline into Phospholipid	203
C.	Autoradiographic Localisation of Phospholipid Formation from ^{14}C-labelled Choline in Human Atherosclerotic Lesions	204
D.	Uptake and Incorporation of ^{3}H-labelled Oleic Acid into Combined Lipid and Extraction of Lipid other than Phospholipid	211
E.	Autoradiographic Localisation of Phospholipid Formation from ^{3}H-labelled Oleic Acid in Human Atherosclerotic Lesions	213
F.	Discussion	213

Section 6.

INCORPORATION OF DIFFERENT FATTY ACIDS INTO COMBINED LIPID BY RABBIT ATHEROSCLEROTIC AORTA IN VITRO

A. Introduction 220

B. Incubations with ^{14}C-labelled Palmitic Acid and ^{14}C-labelled Linoleic Acid Separately 222

C. Incubations with ^{14}C-labelled Palmitic, ^{14}C-labelled Oleic and ^{14}C-labelled Linoleic Acids Together 227

D. Re-incubation of Aortae Labelled with ^{14}C-labelled Palmitic, ^{14}C-labelled Oleic and ^{14}C-labelled Linoleic Acids in non-labelled Incubation Media 232

E. Discussion 235

Section 7.

DIFFERENTIAL UPTAKE OF CHOLESTEROL AND OF DIFFERENT CHOLESTEROL ESTERS BY RABBIT ATHEROSCLEROTIC AORTA IN VIVO AND IN VITRO.

A. Introduction 244

B. Entry of ^{3}H-labelled Cholesterol into Atherosclerotic Aorta In Vivo 245

C. Removal In Vitro of ^{3}H-labelled Cholesterol from the Atherosclerotic Aorta, Labelled In Vivo 253

D. Entry of ^{3}H/^{14}C-labelled Cholesterol into the Atherosclerotic Aorta In Vitro 257

E. Discussion 263

Section 8.

LIPID SYNTHESIS FROM ^{14}C-LABELLED ACETATE BY THE ARTERIAL WALL IN VITRO AND FACTORS AFFECTING IT

A. Introduction 271

B. Lipid Synthesis from ^{14}C-labelled Acetate by the Human Arterial Wall, in Isolated Intimal Cells, and the Effect of Chlorophenoxyisobutyric Acid (CPIB) 273

C. Lipid Synthesis from ^{14}C-labelled Acetate by the Rabbit Atherosclerotic Aorta and the Effect of Chlorophenoxyisobutyric Acid (CPIB) 281

D. Lipid Synthesis from ^{14}C-labelled Acetate by the Rabbit Atherosclerotic Aorta and the Effects of Insulin and of Anti-Insulin 283

E. Lipid Synthesis from ^{14}C-labelled Acetate by Human Aortic Segments Proximal and Distal to Coarctation 286

F. Discussion 294

GENERAL CONCLUSIONS, PROBLEMS, AND FUTURE INVESTIGATIONS 297

BIBLIOGRAPHY 303

DECLARATION

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

Signed

Mark L. Wahlqvist

ACKNOWLEDGEMENTS

I am indebted to Professor R.D. Wright in whose department it was possible to carry out these investigations.

Professor Allan J. Day supervised this work throughout and to him I am especially grateful.

I also wish to thank Mr R.K. Tume for assistance with sections 3 and 6 of this thesis and Mr D.J. Campbell for assistance with section 7.

The technical assistance at various times of Misses J. Anderson, J. Coldwell and J. Dare, Mrs G.M. Neill, Miss H.A. Lawrence and Mr. D. Vickery is also gratefully acknowledged.

The use of human material was made possible through the cooperation of surgeons at the Royal Melbourne and Royal Children's Hospitals and the Pathology and Biochemistry departments at the Royal Children's and Royal Women's Hospitals.

The work was supported by grants from the National Heart Foundation of Australia, the Australian Research Grants Committee and by U.S. Public Health Service Grant R05-TW 00318.

GENERAL INTRODUCTION

LIBRARY OF THE UNIV. OF ADELAIDE

A. General Remarks

Of the several changes evident in "atherosclerosis", lipid accumulation in the large and distributing arteries is the sine qua non. Not so for "the arterioscleroses" or arterial hardenings in general, of which atherosclerosis is one. The World Health Organisation (1958) has given the definition "Atherosclerosis is a variable combination of changes of the intima of arteries (as distinct from arterioles) consisting of the focal accumulation of lipids, complex carbohydrates, blood and blood products, fibrous tissue and calcium deposits, and associated with medial changes". The organisation recognises, however, several forms in which lipid may be present

"(a) The term 'fatty streak or spot' is applied to superficial yellow or yellowish-grey intimal lesions which are stained selectively by fat stains. It is not synonymous with 'atheroma'

(b) The term 'fibrous plaque' is applied to a circumscribed, elevated intimal thickening which is firm, and grey or pearly white

(c) The term 'atheroma' is applied to an atherosclerotic plaque in which fatty softening is predominant

(d) Complicated lesions are defined as lesions with additional changes or alterations such as haemorrhage, thrombosis, ulceration, and calcareous deposits."

It is these morphological descriptions which are used in this thesis.

Two systems of grading atherosclerotic lesions are now in current use. That which arose from WHO proposals (Uemura, Sternby, Vanecek, Vihert and Kagan, 1964) implies a sequence of events which is not altogether proven, but is the more helpful in considering individual lesions:

Stage 0	No lesions discernible at a magnification of 10
Stage I	Fatty streaks and/or spots present
Stage II	Discrete or confluent plaques and/or atheroma, but no evidence of ulceration or other complications
Stage III	Complicated lesions present e.g. haemorrhage, thrombosis, ulceration.

The system proposed by the American Heart Association (McGill, Brown, Gore, McMillan, Paterson, Pollak, Roberts and Wissler, 1968), using a panel of photographs, takes both lesion type and extent of vessel involvement into account.

At all times, the clinical sequelae of atherosclerosis must be distinguished from the disease itself. The increased mortality rates and incidence of morbidity from cardiovascular disease, especially coronary heart disease, in the Western world (Reader and Wynn, 1966: Biörck, 1968: Hundley, 1968) do not necessarily mean that there is a greater incidence of atherosclerosis. In other words, arterial occlusion could be on the increase rather than mural disease (Morris and Crawford, 1961). The rationale for an endeavour to reduce the incidence of atherosclerosis per se is that vessels free of the disease are less likely to undergo occlusion.

Another distinction also needs to be drawn and that is between the ageing process and atherosclerosis. There is a general intimal thickening with age (Dock, 1946; Movat, More and Haust, 1958) which occurs irrespective of focal lipid deposits. Confusion arises because atherosclerosis is age-related; the longer we live the more atherosclerosis we have (McGill, Geer and Strong, 1963). Further, the lipid content of the macroscopically normal intima increases with age, but the type of lipid is different from that in the atherosclerotic lesion (Buck and Rossiter, 1951; Smith, 1968). The subject of intimal lipids in early life is investigated in this thesis. A factor operating on atherosclerosis may operate in the same or opposite direction or not at all on senescence.

Unlike many other diseases, there is in the 20th century probably not a single human being who escapes atherosclerosis; it could now be regarded as "normal" (Holman, McGill, Strong and Geer, 1958). The disease did occur among the ancients who appear to have recognised some of its consequences (Sandison, 1967). Studies of Egyptian mummies (Ruffer, 1911; Smith 1912) have shown that atherosclerosis occurred during the period 1580 B.C. to 525 A.D. Atherosclerosis was probably first recognised by sixteenth and seventeenth century anatomists (Long, 1967). "Atheroma" was coined from the Greek by Haller in 1768, "arteriosclerosis" by Lobstein in 1833 and "atherosclerosis" by Marchand in 1904.

In recent years a number of reviews relating to the arterial wall and the pathogenesis of atherosclerosis has appeared (Lansing, 1959; Branwood, 1963; Jones, 1963; Moses 1963; Sandler and Bourne, 1963; Adams, 1964; Kummerow, 1965;

Constantinides, 1965; Mitchell and Schwartz, 1965; Roberts and Straus, 1965; French, 1966; Blumenthal, 1967; Day, 1967; Solomon, 1967; Haimovici, 1968; Miras, Howard and Paoletti, 1968; Lenègre, Scebat and Renais, 1968; Somlyo and Somlyo, 1968; Wissler and Vesselinovitch, 1968; Schettler and Boyd, 1969). Therefore, in this thesis, atherogenesis (the pathogenesis of atherosclerosis) is reviewed so as to bring the question of arterial wall metabolism, in particular lipid metabolism, and how it relates to the disease, into focus.

There are several ways in which lipid could accumulate in the arterial intima and inner media :

1. By Encrustation or Thrombosis

Formed elements, platelets, red cells or white cells and plasma protein could be deposited at the intimal surface. This was the encrustation view propounded by Von Rokitansky in 1846 and 1852. It was revived as the thrombogenic theory of the pathogenesis of atherosclerosis by Duguid in 1946 and 1948. The proposition was that arterial thrombi became overgrown with endothelium, organized with fibrous tissue and that a certain amount of fatty degeneration took place. The theory has been controversial in so far as it required the initial event in atherogenesis to be the formation of a thrombus. More recently, Mustard, Rowsell, Murphy and Downie (1963) have considered the particular role of the platelet as a source of intimal lipids. Chandler and Hand (1961) have shown that monocytes can become transformed into foam cells after the phagocytosis of platelets.

2. By Increased Endothelial Permeability

Endothelial permeability could be increased so allowing an increased migration of blood cells or serum lipoproteins or both into the intima. The permeability of the inner arterial wall to Evans blue labelled albumin increases with the development of atherosclerotic lesions (Howard, Gresham, Bowyer and Lindgren, 1967). Since very little is known about the lymphatic drainage of arteries (French, 1966), it is not known what plasma protein actually crosses the endothelium and passes through the arterial wall. Kellner (1954) did, however demonstrate the passage of lipoprotein across capillary endothelium by using the technique of lymphatic cannulation. Certainly, the passage of protein of low-density lipoprotein (1.019 - 1.063) labelled with radioiodine, through the normal canine aorta from endothelium to adventitia has been shown (Duncan, Buck and Lynch, 1963) as has the passage of labelled cholesterol (Adams, Bayliss, Davison and Ibrahim, 1964). Although, an investigation of ^{3}H-labelled cholesterol, ^{125}I-labelled albumin, ^{125}I-labelled globulin and ^{32}P-labelled lipid concentration gradients, led Adams, Virag, Morgan and Orton (1968) to suggest that plasma lipoproteins may not be the vehicles for transport of cholesterol across normal and slightly atherosclerotic arteries. The same workers adduced that plasma protein leaks into lesions once they have reached a certain critical size. Increased permeability could occur for mechnical reasons, for example, elevated blood pressure, bending of the vessel, vessel bifurcation or for metabolic or other reasons. The same factors could, of course, affect vessel properties other than permeability.

Where Evans blue labelled albumin penetrates the endothelium, there are platelet aggregates (Packham, Rowsell, Jørgensen and Mustard, 1967), but it is not clear whether increased permeability or platelet aggregation comes first or whether both happen for some common reason. Duncan (1963) has reported that hypertension increases the entry of albumin by stretching the aortic wall, rather than by an increased head of pressure.

The point is that if for any reason the permeability to lipid, in one form or another, increases so as to allow more lipid to enter the artery than can be removed, lipid will accumulate.

3. By Restriction of Lipid Movement

Changes in the intima or inner media could prevent the normal movement of lipoprotein through the arterial wall. This is one of the aspects of the filtration concept of atherosclerosis crystallized by Page in 1954. The origins of this theory, however, were with Virchow in 1856 who considered that there was a chronic inflammatory reaction to the presence of imbibed lipid. Aschoff (1924) belonged to the Virchow school, arguing that there was a mechanical basis of overstretching of elastic tissue and loosening of ground substance for the initial retention of lipid by the intima. The theory was helped by the chemical analyses of Weinhouse and Hirsch in 1940 when they showed that, in plaque lipid, the major lipid classes were present in about the same proportions as in plasma. The studies of Page (1941) also bore this out. But that the hold-up of lipid was in itself responsible for lipid accumulation was

questioned when Buck and Rossiter (1952) showed plaque lipid to have relatively more free cholesterol and sphingomyelin than plasma and when several groups of workers (see below) found differences in fatty acid composition between the atherosclerotic lesion and plasma. Nevertheless, the filtration concept was rationalized by the findings of Gofman, Lindgren, Elliott, Mantz, Hewitt, Strisower, Herring and Lyon in 1950 regarding the atherogenicity of different lipoprotein classes in the cholesterol - fed rabbit, later extended to man (Gofman and Young, 1963). Further, Courtice and Garlick (1962) found that different lipoprotein fractions cross capillaries according to size. Courtice and Schmidt-Dietrichs (1962) showed in the injured rabbit carotid artery that different lipoproteins were deposited to different extents. The implications of the investigations around different lipoprotein fractions are that the intima or intimo-medial junction may alter in a way which affects one lipoprotein fraction and not another or that the characteristics of the lipoprotein may change so that it is no longer possible for it to negotiate the artery in the ordinary way. Regarding the former implication, Gofman and Young (1963) have argued for a relatively impenetrable internal elastic membrane in the early stages of atherogenesis. Also, thickening of the intima for any reason would lessen the chances of a successful transit of lipoprotein through the arterial wall (Page, 1954). Much attention has been directed to possible changes in ground substance which might result in interaction between it and lipid or lipoprotein. In 1953, Taylor and in 1956, Noble, Boucek, Kao and Partin described changes in arterial mucopolysaccharides in association with atherosclerosis. It has been claimed

(Moon and Rinehart, 1952; Moon, 1957, Noble, Boucek and Kao, 1957) that connective tissue and ground substance changes precede lipid deposition. On the hand, Zugibe (1963) in a histochemical study of coronary artery from foetal life to seventy years of age, found no consistent relationship between lipid and acid mucopolysaccharide content. A vacuolar oedema has been found in the intimae of infants (Hieronymi, 1956). Provided this is not artefactual, the presence of an excess of tissue fluid raises the possibility that plasma proteins, and therefore lipoproteins, are present in increased amounts. Amenta and Waters (1960) showed that the density 1.006 - 1.019 and, to a lesser extent, 1.019 - 1.063 fractions of human plasma protein formed precipitates *in vitro* with sulfated mucopolysaccharides extracted from the intima and media of normal human aorta. Gerö, Gergely, Devenyi, Jakab, Szekely and Virag (1961) showed a similar formation of complexes *in vitro* between atherosclerotic human intimal mucopolysaccharide and plasma β-lipoprotein and also fibrinogen.

An interesting observation which is made by vascular surgeons is that they can identify the site of a bothersome atherosclerotic plaque by the presence of adventitial adhesions. While these may be secondary to the atherosclerotic process, it is interesting to speculate whether an adventitial change might restrict the outward flow of lipoprotein through the vessel wall.

4. Because of Increased Blood Lipid

Lipid could also accumulate in a normal intima because of

increased circulating levels of lipid and resultant overload of the normal mechanisms for lipid removal. This possibility is taken into consideration in the filtration concept of Page (1954). Impetus was given to the idea when Anitschow and Chalatow (1913) fed rabbits cholesterol dissolved in vegetable oil and produced hyperlipaemia and atherosclerosis. Actually, Ignatowski had succeeded in producing experimental atherosclerosis in rabbits in 1908 when feeding protein-rich foodstuffs, but he did not isolate the factor or factors in the diet responsible. There is no doubt that a lot of important information about the atherosclerotic process has been obtained from studies of the cholesterol-fed rabbit. But there has to be caution in extrapolating to the disease in man, since the rabbit only rarely develops the disease spontaneously. The lesions resulting from cholesterol-feeding alone do not assume the cellular complexity of the fibro-fatty lesion in man, and the lesions are seldom complicated by thrombosis, haemorrhage, ulceration or calcification. Modifications of the dietary regimen, however, can lead to advanced lesions in the rabbit (Malmros and Wigand 1959; Gresham and Howard, 1962; Constantinides, 1965). Even though the atherosclerotic lesion in the cholesterol-fed rabbit may not have the same multi-factorial basis as that in man, it has been used in this thesis to develop ideas of atherogenesis and, where possible, these have been followed up with experiments on human material.

In man, hyperlipidaemias can be associated with an increased tendency to premature clinical manifestations of atherosclerosis (Albrink and Man, 1959; Schettler, Kahlke and Schlierf, 1967). The suggestion is that, in

these conditions, the intima is overloaded with lipid, although other mechanisms could be operative.

Lipid may be present in increased amount in blood monocytes (Suzuki and O'Neal, 1964; Marshall and O'Neal, 1966); and Leary (1941) proposed that Kupffer cells, laden with cholesterol esters, migrated in the circulation from the liver to the arterial wall. Simonton and Gofman (1951) and Harris (1952) have had difficulty in demonstrating the migration of labelled macrophages into atherosclerotic lesions. Further, McMillan and Stary (1968) have demonstrated, by thymidine - ^{3}H autoradiography, DNA synthesis in endothelial, foam and medial smooth muscle cells of the rabbit atherosclerotic lesion. The general consensus of opinion is that circulating lipid-laden cells contribute little lipid to the lesion.

5. <u>Because of Unusual Blood Lipid</u>

Another way in which intimal lipid accumulation could come about is for the artery to be presented with unusual lipids, which it is not equipped to handle. It is generally felt that the important potential advantage of diets with a high ratio of polyunsaturated to saturated fatty acids is their hypocholesterolaemic effect, probably consequential on increased cholesterol excretion (Keys, 1967). But it could well be that the transport of lipids by the artery differs according to the degree of unsaturation of the fatty acids with which they are esterified. This question is taken up in this thesis.

6. By Intimal Vascularisation and Intramural Haemorrhage

Intimal vascularisation and intramural haemorrhage have been advanced as, among other things, ways of contributing lipid to the intima (Winternitz, Thomas and LeCompte 1938; Horn and Finkelstein, 1940). Haemosiderin deposition in early atherosclerotic lesions was shown by Paterson, Moffatt and Mills (1956), using the Prussian blue reaction, but Schwartz, Ardlie, Carter and Paterson (1967) could not find any evidence of haemosiderin deposition in normal aortic intima.

7. Because of Arterial Wall Metabolism

For two decades now, there has been increasing interest in the role that arterial wall metabolism might play in atherogenesis, whether by new formation of lipid in the artery or by alteration of lipids derived the serum. Constantinides (1965) has reasoned that a primary metabolic disorder in the arterial wall is unlikely, since genetic defects of this kind usually lead to early death and are therefore very rare, yet atherosclerosis is a common disease and most of its effects are seen late in life. What he does not take into account is that considerable environmental change, in the short space of time since the industrial revolution, may well have outstripped the evolutionary potential of the human organism. So that a fundamental metabolic problem in atherogenesis is still conceivable. It may well be, though, that changes in lipid metabolism have occurred because lipid has accumulated for some other reason. In other words, the dilemma of resolving cause and effect arises. For this reason, the distinction is made between aetiology and pathogenesis.

There are several reasons for the current interest in arterial wall metabolism and its relationship to atherogenesis;

(1) Diabetes was recognised as a metabolic disease and known to be associated with an increased tendency to atherosclerosis. (Stout and Vallance - Owen 1969)

(2) A series of papers by Chernick, Chaikoff and co-workers (Briggs, Chernick and Chaikoff, 1949; Chernick, Srere and Chaikoff, 1949; Siperstein, Chaikoff and Chernick, 1951) drew attention to the fact that arterial tissue had a metabolism of its own.

(3) The delicate state of oxygenation of the inner arterial wall was appreciated (Whereat, 1967). In normal arteries the vasa vasorum or capillaries do not extend towards the lumen beyond the inner third of the media (Woerner, 1951). Thus the intima and inner media must be supplied with oxygen from the lumen. In human aorta, a thickness of only 0.91 - 1.00 mm, of the same order as the avascular layer, can be supplied with oxygen (Kirk and Laursen, 1955) so that even small changes in respiratory rate, diffusion coefficient of oxygen, oxygen partial pressure or avascular layer thickness might not be tolerated.

(4) The lipid composition of atherosclerotic intima was found to be different from that in the serum (Buck and Rossiter, 1951), especially with respect to its fatty acid composition, both in the rabbit (Zilversmit, Sweeley and Newman, 1961; Evrard, Van Den Bosch, De Somer and Joossens, 1962; Moore, 1967;

Mizuguchi, 1968) and in man (Luddy, Barford and Riemenschneider, 1958; Swell, Field, Schools and Treadwell, 1960; Smith, 1962 and 1965).
Also fatty acid composition of normal intima, rabbit or human as the case may be, was found to be different from that in the rabbit atherosclerotic lesion (Zilversmit, Sweeley, and Newman, 1961) and from that in the human fatty streak (Nelson, Werthessen, Holman, Hadaway and James, 1961; Geer and Guidry, 1964; Smith, 1965; Smith, Evans and Downham, 1967). Further, the cholesterol ester fatty acid composition of human fatty streaks was found to differ from that in fibrous lesions (Smith, Slater and Chu, 1968). These differences have been thought good reason to look for peculiarities in intimal metabolism.

(5) Knowledge of the fine structure of arterial lipid accumulation has also raised the possibility that metabolic factors might be important. The first ultrastructural appearance of lipid seems to be intracellular, in cells with smooth muscle characteristics (McGill and Geer, 1963; Balis, Haust and More, 1964) rather than in macrophage - like cells or extracellularly. Also, the lipid which first appears is not membrane - bound so that it is unlikely to have been phagocytosed. Hence the suggestion that some metabolic anomaly of the smooth muscle - like cells has resulted in detectable lipid in their cytoplasm. It still could be, nevertheless, that the first lipid excess is extracellular and not detectable with the electron-microscope and that this lipid is taken up, other than by phagocytosis, by the

cells. It may or may not then be metabolised.

(6) Some of the lipid-containing or foam cells of the atherosclerotic lesion have macrophage-like characteristics, both in the rabbit (Imai, Lee, Pastori, Panlilio, Florentin and Thomas, 1966) and in man (Ghidoni and O'Neal, 1967). Day and co-workers have shown that macrophages have considerable lipid metabolic ability (Day, 1967). They can esterify cholesterol and the cholesterol esters so formed are mainly saturated and mono-unsaturated, although the relative proportions of these two classes of cholesterol ester depend on the type of fatty acid available (Day, Fidge, Gould-Hurst and Risely 1963). Lecithin inhibits esterification of free cholesterol by macrophages (Day and Gould-Hurst, 1963). At the same time, cholesterol stimulates lecithin synthesis in macrophages (Day, Fidge and Wilkinson, 1966). Macrophages can hydrolyse lipoprotein cholesterol ester and this is promoted by lecithin (Day, Gould-Hurst and Wahlqvist, 1964). The turnover of phospholipid in macrophages appears to be greater than that of triglyceride or cholesterol, (Day, Gould-Hurst, Steinborner and Wahlqvist, 1964; Day, Fidge, Gould-Hurst, Wahlqvist and Wilkinson, 1966). Palmitic and oleic acids are the major fatty acids labelled when macrophages are incubated with ^{14}C-labelled acetate (Day, Fidge and Wilkinson, 1964). These various findings have stimulated interest in the possible metabolic role of macrophage-like cells in atherosclerotic lesions.

The preferred view of atherogenesis would now be that none of the above explanations for lipid accumulation is the sole explanation, but rather that several processes, local and systemic, may operate together to produce the atherosclerotic lesion. Further, factors reckoned to affect the incidence of atherosclerosis, such as hypertension, sexual differences, endocrine disorders, diet, exercise, stress, family history and obesity, could be expected to act finally through one or more of the basic mechanisms postulated.

Page (1968) in "Atherosclerosis. A Personal Overview" makes a plea for a revision of policy on atherosclerosis research. Among the points he makes are these

> "Emphasise the basic mechanism................."
> "Define the systems for the storage, transport and degradation of lipids by intimal cells................."
> "Delineate the relationship of polyunsaturated fatty acids, hypertension, salt and insulin to atherogenesis"

This thesis seeks to endorse these principles.

B. Lipids and Atherosclerosis in Early Life

Investigations with young arterial tissue should help provide an answer to the question of the origin of early arterial lipid deposits. As already discussed, the question is, in fact, does the first lipid deposit have its origin in plasma lipoproteins or formed elements or in local synthesis in the arterial wall or in the local metabolic alteration of blood lipid? In order to answer such a question it is necessary to know, firstly, the state of lipid metabolism in early life; secondly, the lipid composition of the plasma in early life; thirdly, the changes that take place in the lipid composition of the normal vessel from an early age; and fourthly, the point at which the first atherosclerotic lesion arises, its morphology and its lipid composition. If it were possible to obtain young arterial tissue suitable for metabolic studies this would also be particularly useful.

Placental lipid metabolism and transport have recently been reviewed by Robertson and Sprecher (1968).

Fatty acid can be synthesized in placenta (Hosoya, Hagerman and Villee, 1960). It seems unlikely that the placenta or foetal tissues synthesize essential fatty acids, but they may convert linoleic to arachidonic acid (Robertson, Sprecher and Wilcox, 1968). Presumably, foetal linoleic acid is derived from the mother. Transport of essential and non-essential fatty acids across the placenta has been documented (Portman, Behrman and Soltys, 1969; Szabo, Grimaldi, and Jung, 1969), seemingly as free fatty acid, but also possibly via esterification and hydrolysis. Triglyceride probably does not pass intact across the placenta, but can be synthesized and hydrolysed by it

(Robertson and Sprecher, 1968). The Lands' pathway (Lands, 1960) in which lysolecithin is esterified with fatty acid has been shown to exist in placental tissue (Robertson and Sprecher, 1966). Maternal lysolecithin can be precursor for placental lecithin (Eisenberg, Stein and Stein, 1967a). But there is no good evidence that placental lecithin or lysolecithin are contributed to the foetal circulation. Cholesterol can be synthesized from acetate or mevalonate by placental tissue (Zelewski and Villee, 1966). Cholesterol is also transported across the placenta (Goldwater and Stetten, 1947). Cholesterol can be esterfied with fatty acid in the placenta, but cholesterol esters do not appear to be hydrolysed (Pritchard, Armstrong and Wilt, 1968). It is not known whether neonatal serum cholesterol esters have part of their origin in the placenta, but the lecithin: cholesterol acyltransferase enzyme is present in neonatal blood.

Little is known about foetal lipid metabolism, but the suggestion is that there is a dependency on maternal lipids in early pregnancy (Robertson and Sprecher, 1968). The acceleration of lipogenesis during the last term of foetal life and its relationship to caloric balance in the neonate has been reviewed by Shafrir (1968).

With the exception of lysolecithin (Vikrot, 1964; Zee, 1967) umbilical cord blood lipid levels are lower than maternal levels (Zee, 1968). There is no fall in neonatal serum lipid levels after delivery. In fact, there is a rise in free fatty acid and triglyceride levels by twelve hours. This suggests that lipolytic and triglyceride synthetic mechanisms are active in the neonate.

There are interesting differences between neonatal and adult or maternal fatty acid patterns. Umbilical cord serum cholesterol ester fatty acids have a lower linoleic acid, but a higher palmitic and arachidonic acid composition than does maternal serum cholesterol ester (Renkonen, 1966; Zee, 1968). The major cholesterol ester fatty acid in maternal or adult serum is linoleic acid, whereas in umbilical cord serum it is oleic acid (Muldrey, Hamilton, Wells, Swartwout and Miller, 1961, Zöllner, Wolfram, Londong and Kirsch, 1966). Morever, the foetal cholesterol ester fatty acid pattern is maintained in the face of moderate, but not severe changes (feeding single fatty acids) in prenatal diet, suggesting the existence of homeostatic mechanisms to maintain the pattern (Lopez-S, Miller and Muldrey, 1965). Also, umbilical cord phospholipid fatty acids have a lower linoleic acid and a higher arachidonic acid composition than those of maternal phospholipid (Renkonen, 1966; Zee, 1967).

The cholesterol ester fatty acid pattern has not changed at 12 hours after birth (Zee, 1968), but the serum free fatty acid pattern has become more like that of neonatal adipose tissues at 2 to 4 hours (Robertson and Sprecher, 1968). Cholesterol arachidonate levels decrease over the first few weeks of life, but cholesterol linoleate levels increase for about 2 years although this can be accelerated by a diet rich in linoleic acid (Zöllner, Wolfram, Londong and Kirsch, 1966). Total serum saturated and monounsaturated fatty acid levels fall from infancy to ten years of age, whereas total linoleic and arachidonic acid levels increase over the same period (Wiese, Bennett, Braun, Yamanaka and Coon, 1966). To prevent the emergence of an essential fatty acid deficiency pattern in the serum, infants require about 1.4% of their caloric intake as

essential fatty acids (Holman, Caster and Wiese, 1964). After childhood, it appears that serum cholesterol oleate rises again and cholesterol linoleate falls with advancing years (Swell, Field and Treadwell, 1960); Lopez-S, Krehl and Hodges (1967), however, report a continuing increase in the proportion of cholesterol linoleate with age.

Adipose tissue fatty acid composition also changes with age (Insull and Bartsch, 1967; Baker, 1969). The proportions of palmitic and palmitoleic acid fall and the proportion of oleic acid rises with age.

The earliest that lipid accumulation has been recognised in the human arterial wall is in the foetal aorta (Zugibe and Brown, 1960). In a study of aortae of individuals 1 to 40 years of age reported by Holman, McGill, Strong and Geer (1958), every individual aged 3 years or more had at least minimal sudanophilic intimal deposits and these appeared first in the region of the aortic ring. Schwartz, Ardlie, Carter and Paterson (1967) found definite fatty streaking in aortae of subjects 1 - 12 months of age; over the age of 1 year, definite fatty streaking was universal. Zeek (1930), reviewing juvenile atherosclerosis, raised the question of a pubertal peak in the incidence of atherosclerosis and McGill, Strong and Geer (1958) demonstrated that, at puberty, fatty streaks advance more rapidly than at any other period under 40 years of age. Fatty streaks are rare in coronary arteries before the age of 10 years, but most persons have them in their coronary arteries by the age of 20 (Strong and McGill, 1962 and 1969). Fibrous plaques first appear in the aorta in the second decade of life and, after 30 years of age, 90% of aortae have them (Holman, McGill, Strong and Geer, 1958). The extent to which fibrous plaques develop, however, appears to depend on ethnic and geographical factors (Holman, 1961).

Holman deduces from the universality of fatty streaking and the lack of correlation with nutritional state, that aortic fatty streaks occur independent of diet. On the other hand, Osborn (1968) in a retrospective study, found more coronary artery disease in individuals who were artificial-fed than in those who were breast-fed.

The incidence of atherosclerosis in childhood is increased in progeria, xanthomatosis, essential hyperlipaemia, myxoedema and juvenile diabetes (Keith, Rowe, and Vlad, 1967) and, decreased in cystic fibrosis of the pancreas (Holman, Blanc and Andersen, 1959). Yet it is noteworthy that, in cystic fibrosis of the pancreas, serum levels of linoleic acid are lower than in controls and there appears to be less efficient conversion of linoleic to arachidonic acid (Wiese, Bennet, Braun, Yamanaka and Coon, 1966).

Evidence has been produced by Friedman and Byers (1962) that young vascular endothelium has a permeability which is greater than that of older endothelium. Milch (1965) reports that endothelium usually does not have a basement membrane in young infants and that it appears and thickens with age. In themselves, these findings do not account for early focal deposits of lipid in the arterial wall.

The lipid composition of children's coronary arteries has been investigated by Scott, Florentin, Daoud, Morrison, Jones and Hutt (1966) and that of children's aortae by Wiese, Coon, Yamanaka, Barber and Johnson (1967). Both studies used arteries from which adventitia had been removed. No distinction was made between normal and atherosclerotic tissue. In both the coronary arteries and the aortae, there is possibly a fall in total

cholesterol concentration over the first years of life, but from the age of 5 years cholesterol values tend to rise in Americans, but not, for the coronary arteries, in East Africans. Aortic total fatty acid values also decrease until 5 years and then increase. Because of the small amounts of cholesterol, it was not possible to demonstrate the presence of cholesterol ester in coronary arteries of children under 10 years of age, nor to detail cholesterol ester fatty acid patterns. For the aortae, Wiese et al reported no fatty acid patterns for cholesterol ester below the age of one year; the findings relating to cholesterol ester content (determined by a weighing procedure) for the same aortae should therefore be viewed with a certain scepticism. But thereafter, for the whole aortic wall, the percentage saturated cholesterol ester decreased with age to 20 years, monounsaturated did not change and cholesterol linoleate rose. For children's aortae and coronary arteries, phospholipid concentration showed no convincing trend with age. In the case of aortic phospholipid, the proportion of fatty acid which was monounsaturated decreased slightly after 10 years of age and the arachidonic acid decreased slightly from 1 to 22 years of age. Sphingomyelin has been found in children's aortic tissues (Böttcher and Van Gent, 1961; Böttcher and Woodford, 1962).

C. Lipid Composition Of The Arterial Wall

Cholesterol has long been recognised as an important component of the atherosclerotic lesion (Vogel, 1847; Aschoff, 1906; Windaus, 1910). Phospholipid was also found to be a constituent of the lesion by Schoenheimer (1926, 1928).

With each advance in the techniques of lipid analysis has come a reappraisal of arterial lipids. The way in which the similarity or dissimilarity of intimal and serum lipids has led to divergent concepts of atherogenesis has been referred to earlier in this thesis.

Kritchevsky (1967) has averaged the findings of Weinhouse and Hirsch (1940), Page (1941) and Mead and Gouze (1961) for major lipid class composition of human atherosclerotic lesions as follows: free cholesterol 19.2%, cholesterol ester 37.6%, phospholipid 18.5% and neutral fat 23.9%. Cholesterol ester is generally regarded as the major lipid of the atherosclerotic lesion. Buck and Rossiter (1951), however, found similar proportions of free and ester cholesterol in advanced atherosclerotic lesions.

The cholesterol ester content of normal intima increases at a greater rate than does that of free cholesterol, phospholipid or triglyceride (Smith, 1965). There is, nevertheless, very little cholesterol ester in normal intima (Smith 1965; Kritchevsky, 1967). Cholesterol ester is, in fact not normally found in concentrations greater than free cholesterol, except in the adrenal gland and in the serum (Cook, 1958; Gould and Cook, 1958). Insull and Bartsch (1966) have shown that, for the human fatty streak, the increase in cholesterol ester lacks correlation with any other lipid class, although the other lipid classes also increase. Thus, the mechanism of cholesterol ester accumulation is one of the most important problems to solve in relation to atherogenesis.

There are species differences in the extent to which individual phospholipids are represented in the lesion. In

man, sphingomyelin is the major phospholipid at all stages of the disease (Weinhouse and Hirsch, 1940; Böttcher, 1963; Smith, 1965). Sphingolipids other than sphingomyelin, namely galactoceramides and sulfatides are also important plaque constituents, especially in stage II and stage III lesions (Böttcher, 1963). Sphingolipids from human aortic atherosclerotic lesions have been shown to be quite heterogeneous with respect to their long-chain bases (Panganamala, Geer and Cornwell, 1969). Lecithin is the phospholipid present in second largest quantities in human fatty streaks (Smith, 1960; Böttcher, 1963). Lysolecithin, phosphatidyl serines, phosphatidyl ethanolamines, lysocephalins and plasmalogens are other phospholipids found in normal and atherosclerotic human intima. In the rabbit aorta, sphingomyelin concentration increases more than lecithin on cholesterol feeding, but in both normal and cholesterol-fed animals, the aortic concentration of lecithin exceeds that of sphingomyelin (McCandless and Zilversmit, 1956).

Arterial free fatty acids are present in only small amounts (Böttcher, Woodford, Ter Haar Romeny, Boelsma and Van Gent, 1960; Böttcher, Woodford, Ter Haar Romeny-Wachter, Boelsma-Van Houte and Van Gent, 1960). Nevertheless, fatty acid esterified with cholesterol and as phospholipid and triglyceride is an important consideration in arterial wall chemistry, since its chain length and degree of unsaturation may affect the behaviour of a given combined lipid.

The studies of Böttcher and co-workers (Böttcher, Woodford, Ter Haar Romeny-Wachter, Boelsma-Van Houte, and Van Gent, 1960; Böttcher, Boelsma-Van Houte, Ter Haar Romeny-Wachter, Woodford and

Van Gent, 1960) on whole artery led to the view that in aorta, coronary and cerebral arteries, cholesterol ester fatty acids became more polyunsaturated as the severity of the atherosclerotic lesion increased and this was taken as evidence that the cholesterol ester was derived from the plasma. Luddy, Barford and Riemenschneider (1958) on the other hand, had shown that atherosclerotic human aorta had a high cholesterol oleate and a relatively low cholesterol linoleate content. However, when the human fatty streak was separated from underlying media it was found to have a cholesterol oleate composition greater and a cholesterol linoleate composition lower than that in the plasma (Smith, 1962 and 1965; Swell, Field, Schools and Treadwell, 1960b, and Swell, Schools and Treadwell, 1962). The same was found true of cholesterol - fed rabbit lesions when their cholesterol ester fatty acid composition was compared with the plasma (Zilversmit, Sweeley and Newman, 1961; Evrard, Van den Bosch, De Somer and Joossens, 1962). Also, the human lesion had a higher cholesterol oleate and lower cholesterol linoleate composition than underlying media (Swell, Field, Schools and Treadwell, 1960a and 1960b; Nelson, Werthessen, Holman, Hadaway and James, 1961) and surrounding normal intima (Geer and Guidry, 1964; Geer and Malcolm, 1965; Smith, 1965). It is also now clear that it is the fatty streak which is particularly different in its cholesterol ester fatty acid composition, and not the fibrous lesion which has a cholesterol ester fatty acid pattern resembling that in the serum (Smith, 1965; Smith, Slater and Chu, 1968).

According to Swell, Field, Schools and Treadwell, (1960b) the human atherosclerotic lesion has relatively more long chain phospholipid fatty acid than the serum. The long chain fatty

acid is probably present as sphingomyelin (Böttcher and Van Gent, 1961). A comparison of the human lesion phospholipid fatty acid patterns reported by Böttcher and Van Gent (1961) with the serum patterns reported by Schrade, Biegler and Bohle (1961) suggests there may be less polyunsaturated fatty acid in lesion phospholipid. The phospholipid of rabbit atherosclerotic lesions is less polyunsaturated than that in the serum (Swell, Law, Schools and Treadwell, 1961; Zilversmit, Sweeley and Newman, 1961).

Triglyceride fatty acid composition does not seem to be modified by the atherosclerotic process either in man (Swell, Field, Schools, Treadwell, 1960; Nelson, Werthessen, Holman, Hadaway and James, 1961) or in the rabbit (Zilversmit, Sweeley and Newman, 1961).

The question arises as to whether the atherosclerotic artery is deficient in essential fatty acids (Alfin-Slater and Aftergood, 1968). While increased triene: tetraene ratios have been observed in the lesion, the triene isomer concerned appears to be 20 : 3 ω 6 rather than 20 : 3 ω 9 (Geer and Guidry, 1964; Smith, 1965). It is the latter isomer which is characteristic of essential fatty acid deficiency. Nevertheless, mitochondrial change is thought by some to be the most sensitive indication of essential fatty acid depletion (Wilson and Le Duc, 1963; Leduc and Wilson, 1964) and mitochondrial change has been described in human and experimental atherosclerosis (Murray, Schrodt and Berg, 1968).

Lipoperoxides or ceroids have been demonstrated in atherosclerotic lesions, but there is controvery as to the amounts present (Glavind, Hartmann, Clemmensen, Jessen and Dam, 1952; Woodford, Böttcher, Oette and Ahrens, 1965;

Hartroft and Porta, 1965). These substances are polyunsaturated fatty acid derivatives and are therefore particularly interesting (Barber and Bernheim, 1967). There is evidence that life span is decreased by both increased amounts of unsaturated dietary fat and it is suggested that free radicals, formed during the transformation of unsaturated fatty acids to lipid peroxides, may play a role in reducing life span (Harman, 1969). It may be important to take into account the effects of polyunsaturated fatty acids on both the ageing process and on atherosclerosis.

The lipid compositon of the atherosclerotic lesion may bear an important relation to the extent to which fibrous change occurs. Free cholesterol and monounsaturated cholesterol esters are more sclerogenic than polyunsaturated cholesterol esters (Abdulla, Adams and Morgan, 1967).

Lipoproteins in the arterial wall could be plasma lipoproteins, cellular lipoproteins or locally-formed transport lipoproteins. One of the difficulties in interpreting arterial lipoproteins chemically is that, even if in every way they resembled plasma lipoproteins, this would not mean that they had definitely come from the plasma. The presence in human atherosclerotic lesions of lipoprotein, resembling serum β-lipoprotein electrophoretically and/or immunoelectrophoretically, has been shown by Kayden and Steele (1956), by Ott, Lohss and Gergely (1958), by Gerö, Gergely, Jakab, Székely and Virág (1961) and by Hollander (1967). Ott et al. (1958) also found α_2 (or pre-β) lipoprotein in human atherosclerotic lesions, but neither β nor α_2 - lipoprotein in normal aorta. β-lipoprotein was found in normal human aortic intima, and in relatively greater amounts in atherosclerotic lesions by Tracy, Merchant and Kao (1961). Gerö et al

(1961) found β-lipoprotein in every human atherosclerotic aortic intima, but not in pulmonary arteries or inferior vena cavae; they also could find no evidence of α-lipoprotein in any of the vessels studied. In ultracentrifugal studies by Hanig, Shainoff and Lowy (1956) lipoproteins of the Sf 12 - 100 (very low density lipoprotein or VLDL) class have been found in atherosclerotic lesions. Hollander (1967) has compared the ultracentifugal, electrophoretic, and immunological characteristics as well as the lipid compositions of the different ultracentrifugal fractions of lipoproteins from human atherosclerotic lesions with those in the serum (reported by other workers). He found that β-lipoprotein or 1.006 - 1.063 or low density lipoprotein (LDL) was the major lipoprotein of the lesion and also that the < 1.019 (VLDL) had a higher percentage of cholesterol and a lower percentage of triglyceride than the corresponding serum fraction. Another approach to the study of arterial lipoprotein chemistry has been that of immunofluorescent immunochemical localization. Watts (1963) has demonstrated that fluorescent anti-β lipoprotein, and Kao and Wissler (1965) that anti-low density lipoprotein, associates with lipid staining areas of the atherosclerotic lesion and, in particular, with intimal cells. Also, ferritin-labelled anti-β-lipoprotein associates with ground substance, cell membranes, intracellular lipid droplets and even mitochondria in ultrastructural studies (Watts, 1963).

Of interest, too, is the finding of Mathur, Sharma, Kashyap and Sapru (1964) of a lipid-binding capacity by globulins from human atherosclerotic intima.

That much of the phospholipid in rabbit and human atherosclerotic lesions is located in the foam cells has been shown by Day (1962) and Dunnigan (1964), respectively. Portman,

Alexander and Maruffo, (1967) and Portman, Alexander and Osuga (1969) have investigated the subcellular distribution of aortic phospholipid in monkeys. Sphingomyelin is poorly represented in the cell sap, but well-represented in the plasma membrane and it is suggested that changing ratios of subcellular fractions to one another may be responsible for the increased representation of sphingomyelin in atherosclerotic lesions.

Weller, Clark and Oswald (1968) have proposed from ultrastructural, polaroid light, histochemical and ultracentrifugal studies that intracellular lamellated droplets containing phospholipid and cholesterol are transformed into amorphous globules of cholesterol ester, but much of the evidence is circumstantial.

D. Lipid Entry Into The Arterial Wall

Several facets of the entry of lipid into the arterial wall have already been referred to under "General Remarks". Phospholipid appears to accumulate in atherosclerotic lesions because of synthesis in situ rather than because of increased entry from the plasma (Zilversmit, 1959). By corn oil feeding in man (Farquhar, Hirsch, and Ahrens, 1960) and elaidic acid feeding in cholesterol-fed rabbits (Dayton and Hashimoto, 1968) and subsequent fatty acid pattern analyses of the atherosclerotic lesion, it has been demonstrated that at least part of the lesion fatty acid (triglyceride fatty acid, in particular, in man; cholesterol ester and lecithin fatty acid and free fatty acid in the rabbit) arises from the serum. On the other hand, even though cholesterol synthesis can occur in the arterial wall

(see below), the observations of Newman, McCandless and Zilversmit (1961) on the formation of cholesterol from ^{14}C-labelled acetate, and those of Newman and Zilversmit (1962) relating to cholesterol flux, indicate that most of the sterol (in both the free and ester form) in the rabbit atherosclerotic lesion must arise from the plasma rather than from synthesis in the wall. The findings of Dayton (1959) on turnover of cholesterol in normal chicken arterial wall support this view.

The mechanism of entry of cholesterol into the arterial wall is not clear, however. There is controversy as to whether it may be active or passive, or both. The work of Jensen (1967 and 1969) on normal rabbit aorta suggests that there are two processes for cholesterol uptake, one temperature dependent and the other not, and that there is an active process at the endothelial surface. Also, Werthessen (1959) has related aortic glucose consumption to the increase in aortic cholesterol content. It is impossible to say, however, whether this is related to transport of cholesterol or some other metabolic process involved in cholesterol accumulation; or whether, indeed, it is not secondary to cholesterol accumulation for some non-metabolic reason. Newman and Zilversmit (1966) found that boiling the aorta did not stop cholesterol influx although esterified cholesterol influx was reduced to a greater extent than free cholesterol influx. Hashimoto and Dayton (1966) found that boiling did not affect the uptake of free cholesterol by normal rat aorta *in vitro*. Also, Newman and Zilversmit (1966) found little effect by the metabolic inhibitor cyanide on cholesterol uptake by rabbit atherosclerotic aortae *in vitro*.

For rabbit atherosclerotic lesions (Newman and Zilversmit, 1962 and 1966) and for the inner aortic wall of normal rats

(Hashimoto and Dayton, 1966; Dayton and Hashimoto 1966) it has been demonstrated that the influx of radioactively labelled free cholesterol from the plasma is greater relative to cholesterol ester than would be expected on the basis of their relative concentrations in the plasma. There are several possible explanations for this finding:

(1) That cholesterol does not enter the inner arterial wall as plasma lipoprotein, but in some other way which involves a different free:ester cholesterol ratio. The fact that there is more cholesterol ester than free cholesterol in the lesion might then be accounted for by differences in removal.

(2) That labelled free cholesterol in the plasma exchanges more readily with intimal free cholesterol than does plasma cholesterol ester for intimal cholesterol ester. Radioactively labelled free cholesterol exchanges readily with plasma lipoprotein, whereas cholesterol ester does not (Roheim, Haft, Gidez, White and Eder, 1962). The exchange of free cholesterol between the plasma and intima may therefore account for the entry of the radioactive free cholesterol into the intima, but exchange of cholesterol ester is unlikely to account for the entry of radioactive cholesterol ester. Cholesterol ester may therefore enter as lipoprotein.

(3) That free cholesterol, and not cholesterol ester, enters the inner artery and that it is converted, in part, to cholesterol ester. The entry of cholesterol ester in this case is more apparent than real. Most workers have had difficulty in achieving esterification of labelled free

cholesterol by the atherosclerotic lesion (Day and Gould-Hurst, 1966; Newman and Zilversmit, 1966; Newman, Gray and Zilversmit, 1968). At first, then, it would seem that this is an unlikely explanation. However, labelled free fatty acids are readily incorporated into lesion cholesterol ester (see below). The discrepancy may be accounted for either by the inaccessibility of sites of esterification to plasma free cholesterol or by a greater dilution of labelled cholesterol than of free fatty acid in the atherosclerotic lesion. Thus, the possibility of esterification of labelled free cholesterol as a basis for influx differences remains open.

(4) That cholesterol ester and not free cholesterol enters the inner artery and is hydrolysed to free cholesterol. Hydrolysis of cholesterol ester is known to occur in the atherosclerotic lesion (Day and Gouldhurst, 1966; Patelski, Bowyer, Howard and Gresham, 1968), but there is no information as to whether it accounts for the apparently greater influx of labelled cholesterol over cholesterol ester. This possibility is explored in this thesis.

The concept of a barrier to cholesterol influx which breaks down as the atherosclerotic lesion progresses has been put by Zilversmit (1968). A point in favour of the proposition is that free and ester cholesterol in early lesions fail to equilibrate with serum cholesterol whereas they do in later lesions (Newman and Zilversmit, 1962).

Attention has already been drawn to the fact that cholesterol oleate is the major cholesterol ester in rabbit atherosclerotic and human fatty streak lesions. This could come about in one of several ways and these are depicted in Fig. 1 by the heavy lines. Relevant to the present discussion is the possibility that the influx of monounsaturated cholesterol ester into the atherosclerotic lesion could be greater than that of saturated or polyunsaturated cholesterol ester. Swell, Law and Treadwell, (1963) reported that this, indeed, was the case in the cholesterol-fed rabbit. A similar study is reported in this thesis and an attempt made to determine what relationship this bears to serum concentrations of individual cholesterol esters. The same difficulties apply, however, in interpreting findings on the entry of individual cholesterol esters as in interpreting the differences in entry of free and ester cholesterol. Is the basis for "selective entry" of monounsaturated cholesterol ester really selective esterification of free cholesterol or perhaps interconversion of the various cholesterol esters, rather than selective transport per se?

E. Arterial Wall Metabolism

There have been several approaches to the study of arterial wall metabolism. Inferences have been made from arterial wall chemistry; the fate of various radioisotopically labelled substances has been determined; and enzymological studies have been undertaken, either histoenzymologically or on homogenates of the arterial wall or parts thereof. The enzyme biochemistry of the arterial wall has recently been reviewed by Zemplenyi (1968).

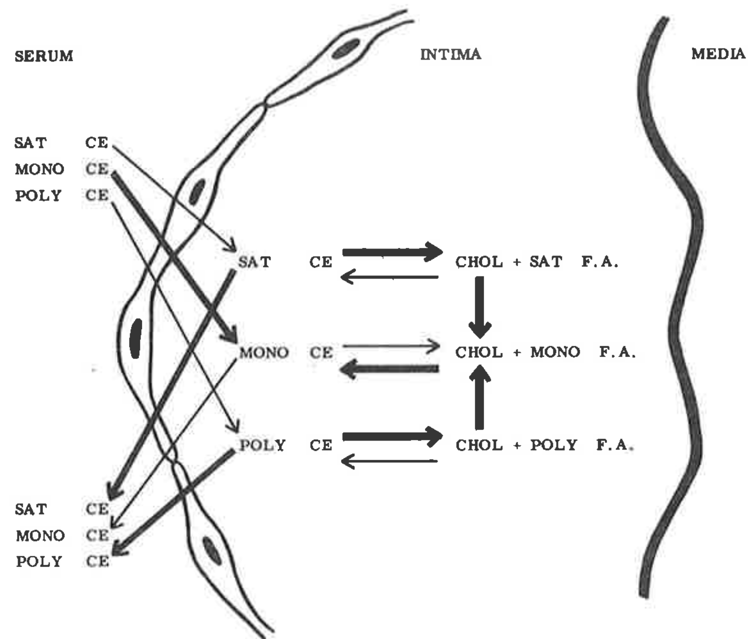

Figure 1

Arrow thickness, broad or narrow, indicates whether an event is more or less likely to account for the accumulation of monounsaturated cholesterol ester in atherosclerotic lesions. "CHOL" is free cholesterol "F.A." is free fatty acid and "CE" is cholesterol ester. The degree of unsaturation is indicated by "SAT", saturated, "MONO", monounsaturated and "POLY", polyunsaturated.

Various possible metabolic pathways in the arterial wall, some better documented than others, some more active than others and some changing their degree of representation with atherogenesis, are depicted in Fig. 2.

1. Lipid Metabolism

(1) Fatty Acid

That about half of the fatty acid in rabbit atherosclerotic lesions arises by local synthesis has been concluded from the elaidic acid feeding experiments of Dayton and Hashimoto (1967).

Fatty acid can, indeed, be formed from ^{14}C-labelled acetate in the arterial wall of the rat (Chernick, Srere and Chaikoff, 1949; Feller and Huff, 1955; Loomeijer and Van der Veen, 1962) of the human foetus (Paoletti, Paoletti and Savi, 1958) and of adult humans (Chobanian and Hollander, 1963). Succinate has been shown to provide protons, during the synthesis of long-chain fatty acids by aortic mitochondria, in studies using succinate-2-3-^{3}H (Whereat, 1965) and the spectrum of fatty acids labelled is similar to that labelled when ^{14}C-labelled acetate is used as substrate, thus indicating a way in which Krebs cycle intermediates might influence fatty acid synthesis. Also, glucose-U-^{14}C has been shown to serve as substrate for fatty acid synthesis in the intima and media of normal rabbit aorta, although most of the label was found in non-fatty acid lipid moieties (Vost, 1969). Lofland and Clarkson (1965) found no conversion of glucose-1-^{14}C to fatty acid in the atherosclerotic pigeon aorta, however. The difference in the two studies might be accounted for by loss of

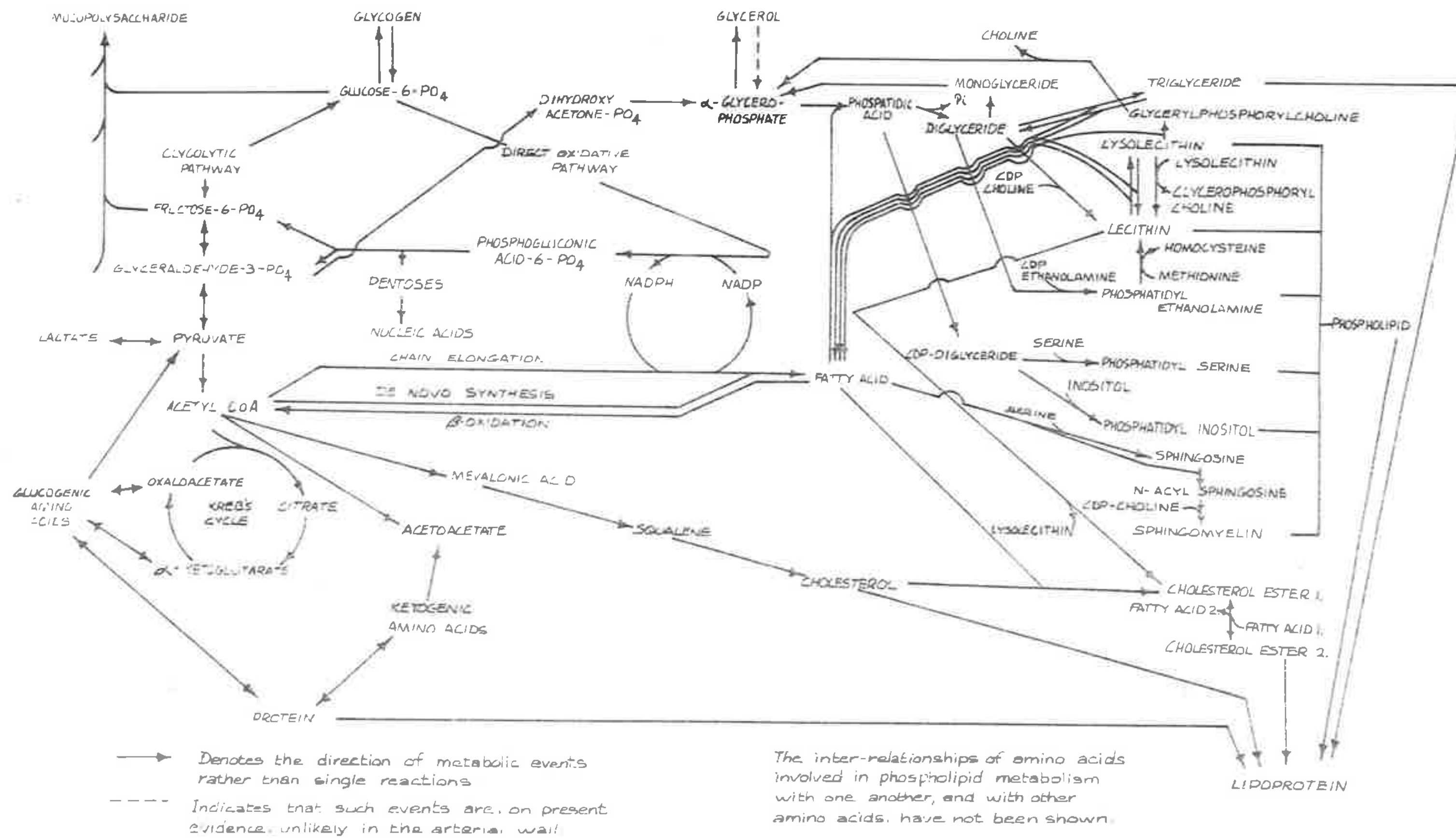

Figure 2

labelled CO_2 from glucose-1-^{14}C in the direct oxidative pathway.

There is disagreement as to whether the mitochondrial chain elongation pathway or the non-mitochondrial de novo synthetic pathway, or both are operative in fatty acid synthesis in the arterial wall. Whereat (1966) found that in the normal and atherosclerotic rabbit arterial wall, mitochondria were the major site of fatty acid synthesis from ^{14}C-labelled acetate and collateral studies demonstrated that malonyl-CoA was not an intermediate (as it is for the cytoplasmic de novo synthetic system elsewhere). For monkey aorta, Howard (1968) demonstrated both a microsomal de novo synthetic pathway and a mitochondrial chain elongation pathway. Gas liquid radiochromatography revealed that, for both systems, saturated and monounsaturated fatty acids were the main fatty acids formed. The chain length of fatty acids formed in the microsomes indicated that chain elongation could also take place in that site. The study of Vost (1969) of ^{14}C-labelled acetate metabolism in the perfused normal rabbit aorta suggested that there was little chain elongation in the media (intimal results were not reported), but that there was considerable de novo synthesis.

There is little information regarding the catabolism of fatty acids in the arterial wall. Chobanian and Hollander (1963) have reported that,

of ^{14}C-labelled palmitic, stearic, oleic and linoleic acids, only the saturated acids, palmitic and stearic, were degraded to smaller fatty acid intermediates. But findings reported at the same time, relating to the synthesis of fatty acids of the saturated variety only and to a greater fatty acid synthesis by the normal than by the atherosclerotic arterial wall, are in complete contrast to those of other workers for the experimental animal.

An increase in arterial wall fatty acid synthesis *in vitro* with the development of atherosclerosis has been described by Lofland, Moury, Hoffman and Clarkson, (1965), by Whereat (1966) and by Whereat and Orishimo (1968). Whether the increase in fatty acid synthesis is a primary metabolic derangement or a secondary effect is a matter for speculation. However, Whereat (1967) has pointed out that, if the pentose pathway (direct oxidative pathway) accounts for a significant proportion of glucose oxidation and if anaerobic conditions prevail, then the NADPH formed may well be reoxidized by fatty acid synthesis.

(2) Cholesterol

It has already been indicated that most of the cholesterol in the atherosclerotic lesion has its origin in the blood. But even if there were a slow rate of cholesterol synthesis *in situ*, it

would, over a long period of time, become quite important. The entry of cholesterol from the serum could be in exchange for what was originally intimal cholesterol. The synthesis of cholesterol from ^{14}C-labelled acetate in the arterial wall has been demonstrated by Siperstein, Chaikoff and Chernick (1951), Schwenk and Werthessen (1952), Werthessen, Milch, Redmond, Smith and Smith (1954), Eisley and Pritham (1955), Azarnoff (1958), Foster and Siperstein (1960) and Daly, Deming, Raeff and Brun (1963). Nevertheless, as has been pointed out by St. Clair, Lofland, Prichard and Clarkson (1968), digitonin precipitable material is not necessarily cholesterol. These workers have, in fact, established that squalene is part of the digitonin precipitable fraction formed from mevalonic-2-^{14}C acid by human and pigeon arteries and that even the labelled alumina column fraction corresponding to cholesterol (squalene being separated by this procedure) is only about 1% cholesterol on further purification and chromatography. Neither was labelled cholestanol found on gas liquid radiochromatography in contrast to the reports of Chobanian (1968a) that this is the major sterol synthesized from acetate and mevalonate in the human arterial intima. It is also of interest that squalene synthesis from mevalonate was positively correlated with the severity of pigeon atherosclerosis. The suggestion is made that squalene might behave as an irritant to the intima as might other sterol-like fractions formed.

(3) Cholesterol Ester

The incorporation of fatty acid, formed in the atherosclerotic arterial wall from ^{14}C-labelled acetate, into cholesterol ester is now documented for the rabbit (Day and Wilkinson, 1967; Newman, Gray and Zilversmit, 1968) and the pigeon (Lofland, Moury, Hoffman and Clarkson, 1965; St. Clair, Lofland, and Clarkson, 1968). Its incorporation into a combined neutral fat and cholesterol ester fraction of the human arterial wall has been shown by Maggi (1964). Also, the incorporation of labelled fatty acids into cholesterol ester of the rabbit atherosclerotic lesion has been reported (Bowyer, Howard and Gresham, 1967; Newman, Gray and Zilversmit, 1968).

That, *in vivo*, locally-formed fatty acid is likely to be an important source of cholesterol ester fatty acid is indicated by experiments with eviscerated cholesterol-fed rabbits into which ^{14}C-labelled acetate was injected intravenously. The specific activity of intimal cholesterol ester fatty acid was found to be many times greater than that in the serum (Newman, Gray and Zilversmit, 1968). These findings are supported by the work of Werthessen (1963) in the normal baboon.

Studies of fatty acid incorporation into the combined lipid of normal arteries have revealed that the major lipids labelled are phospholipid and triglyceride (see below) rather than cholesterol ester. Further, it has been shown that as the pigeon

aorta becomes more atherosclerotic, more newly synthesized fatty acid becomes esterified to cholesterol (Lofland, Moury, Hoffman and Clarkson, 1965).

In the rabbit atherosclerotic lesion, the newly synthesized fatty acid esterified to cholesterol is equally distributed among saturated, monounsaturated and polyunsaturated varieties according to Day and Wilkinson (1967), but mainly of the saturated and monounsaturated variety according to Newman, Gray and Zilversmit (1968) and, in the pigeon, mainly of the monounsaturated variety (St. Clair, Lofland and Clarkson, 1968). Bowyer, Howard and Gresham (1967) have reported, in an abstract, that the labelling of cholesterol ester with oleic acid-1-^{14}C is greater than with palmitic, stearic or linoleic acids-1-^{14}C when atherosclerotic aortae are perfused with these fatty acids separately. There are, therefore, metabolic reasons why cholesterol oleate should accumulate in atherosclerotic lesions, as shown in Fig. 1.

It has been of concern to many workers that while labelled fatty acid is readily incorporated into cholesterol ester in the atherosclerotic lesion, labelled free cholesterol is not (Day and Gould-Hurst, 1966; Newman, Zilversmit, 1966; Newman, Gray and Zilversmit, 1968). However, Felt and Beneš (1969) have now reported that in normal rat aorta [4-^{14}C] cholesterol is esterified _in vitro_ and that mainly saturated and monounsaturated cholesterol

esters are formed. Free cholesterol must also be esterified in the lecithin : cholesterol acyltransferase reaction described for rabbit and human atherosclerotic lesions (Abdulla, Orton and Adams, 1968). Unpublished observations of Rossiter, Tume and Day indicate that there is an enzyme which will esterify free cholesterol with free fatty acid in rabbit intimal foam cell homogenates.

Three mechanisms for cholesterol esterification in the arterial wall are proposed and these are shown in Fig. 2.

(a) The esterification of free fatty acid with free cholesterol

(b) The replacement of one cholesterol ester fatty acid with another fatty acid. In this case, net synthesis of cholesterol ester could not take place, but the cholesterol ester fatty acid pattern could change.

(c) The lecithin : cholesterol acyltransferase reaction as reviewed by Glomset (1968) for plasma.

The location of cholesterol esterification in the atherosclerotic lesion is one of the particular problems investigated in this thesis. Foam cells (lipid-containing mononuclear cells) isolated from rabbit atherosclerotic lesions have been shown to incorporate fatty acid synthesized from ^{14}C-labelled acetate (Day and Wilkinson, 1967) and ^{14}C-labelled oleic acid (Day and Tume, 1968) into cholesterol ester. More recently, foam cells isolated from rabbit atherosclerotic aortae, after prior incubation

with ^{14}C-labelled oleic acid, have been shown to have a higher cholesterol ester specific activity than any other portion of the arterial wall, suggesting that the foam cells are the major sites of cholesterol esterification (Day and Tume, 1970). Of the fatty acids synthesized from ^{14}C-labelled acetate by isolated rabbit foam cells, relatively more polyunsaturated seemed to be incorporated into cholesterol ester than was the case with the whole atherosclerotic intima (Day and Wilkinson, 1967). However, no information is available concerning the pattern of labelled cholesterol ester fatty acids in foam cells isolated after prior incubation of the arterial wall with ^{14}C-labelled acetate, so that it can not be asserted that their metabolism is necessarily different *in situ*.

The hydrolysis of cholesterol ester by normal and atherosclerotic aortae has been reported (Day and Gould Hurst, 1966; Howard and Portman, 1966; Shyamaza, Nichols and Chaikoff, 1966; Patelski, Bowyer, Howard and Gresham 1968). Bowyer, Howard, Gresham, Bates and Palmer (1968) claim the normal rabbit aorta hydrolyses cholesterol-1-^{14}C oleate less readily than cholesterol-1-^{14}C palmitate, stearate or linoleate and that the atherosclerotic rat aorta has less cholesterol oleate hydrolytic ability than the normal.

(4) Phospholipid

The earliest account of phospholipid formation in the arterial wall is that of Chernick, Srere and Chaikoff

(1949) when ^{32}P-labelled phosphate was the precursor.

A series of reports by Zilversmit and co-workers has established that the phospholipid of the atherosclerotic lesion in the cholesterol-fed rabbit (Zilversmit, Shore and Ackerman, 1954; Shore, Zilversmit and Ackerman, 1955; McCandless and Zilversmit, 1956; Zilversmit and McCandless, 1959; Zilversmit, 1959) and in man (Zilversmit, McCandless, Jordan, Henly and Ackerman, 1961), arises principally through synthesis *in situ*. The studies involved comparisons of specific activities of phospholipids, especially the choline-containing lysolecithin, lecithin and sphingomyelin, in plasma and arterial wall following the injection of radioactive phosphate or labelled phospholipids intravenously. That phospholipid fatty acid is also derived mainly by synthesis in the atherosclerotic lesion has been shown in experiments in rabbits *in vivo* using ^{14}C-labelled acetate (Newman, McCandless and Zilversmit, 1961).

Phospholipid formed from radioactive phosphate by rabbit (Newman, Day and Zilversmit, 1966) and human (Chobanian and Hollander, 1966) arterial wall *in vitro* resembles that for the rabbit *in vivo*. There is a relatively greater formation from radioactive phosphate of cephalin or of phosphatidyl inositol by normal than there is by atherosclerotic aorta (McCandless and Zilversmit, 1956; Newman et al, 1966). In atherosclerotic aorta, more lecithin is labelled with radioactive phosphate than any other phospholipid. Differences in radioactive phosphate incorporation

between aorta and coronary artery and between aortae of different species have also been shown (Nakatani, Sasaki, Miyasaki, Nakamura, 1967a).

Labelled fatty acid has also been incorporated into phospholipid by the normal arterial wall in the experimental animal (Stein and Stein, 1962; Stein, Stein and Shapiro, 1963; Parker, Schimmelbusch and Williams, 1964; Parker, Ormsby, Peterson, Odland and Williams, 1966) and by the human arterial wall (Parker et al, 1964; Chobanian and Hollander, 1966). Lecithin is the major phospholipid labelled. The same is also true of phospholipid formed from ^{14}C-labelled acetate in the rabbit (Day and Wilkinson, 1967) and in the human (Chobanian and Hollander, 1966) arterial wall. ^{14}C-labelled choline has been incorporated into phospholipid in normal and atherosclerotic rabbit lesions (Newman et al, 1966), but individual phospholipids were not assessed. Morin (1968), however, found choline-1-2-^{14}C to be incorporated principally into lecithin in normal and cholesterol-fed rabbits, although ^{14}C-labelled choline uptakes were much lower than those reported by Newman et al (1966). The finding by Morin that the non-choline containing phospholipids, phosphatidyl serine and phosphatidyl ethanolamine, are also labelled following incubations of rabbit aortae with choline-1-2-^{14}C suggests that the arterial wall is capable of catabolising choline to serine and ethanolamine. This could not have occurred had the choline been labelled in the methyl groups since these are lost in the course of choline catabolism. Morin also

demonstrated that ethanolamine-1-2-^{14}C was incorporated into phosphatidyl ethanolamine, lecithin, lysolecithin and phosphatidyl serine. It is not possible to say from these findings whether ethanolamine is converted to choline or whether phosphatidyl ethanolamine is converted to lecithin (phosphatidyl choline). The attempt by Morin to compare the incorporation of different precursors into phospholipid on the basis of specific activities in the incubation medium is invalid since, in that way, no account is taken of intermediate pools.

There are several possible pathways for lecithin synthesis in the arterial wall and these are depicted in Fig. 2.

(a) Phosphorylated choline (CDP choline, cytidine diphosphate choline) could be transferred to α, β diglyceride (Kennedy and Weiss, 1956)

(b) Phosphatidyl ethanolamine could be methylated to form phosphatidyl choline. (Bremer and Greenberg, 1959 and 1961)

(c) Two lysolecithin molecules could condense to yield lecithin and glycerophosphoryl choline (Erbland and Marinetti, 1962)

(d) The acylation of lysolecithin could take place (Lands, 1960).

Phosphatidyl ethanolamine could be formed from CDP-ethanolamine and diglyceride (Kennedy and Weiss, 1956). Phosphatidyl serine and phosphatidyl inositol could be formed from CDP diglyceride and serine or inositol (Paulus and Kennedy, 1960). The depiction in Fig. 2 of sphingomyelin formation from

phosphorylated choline and N-acylsphingosine (ceramide) is not meant to imply that this is the only pathway available to the arterial wall for sphingomyelin synthesis, as the acylation of sphingosyl phosphorylcholine is possible (Rossiter, 1968). It is noteworthy that a peptide bond, rather than an ester bond, links fatty acid to sphingol in sphingomyelin.

Stein, Stein and Shapiro (1963) demonstrated in rabbit and dog aortic homogenates that both lysolecithin and α-glycerophosphate stimulate ^{14}C-labelled linoleic acid incorporation into phospholipid. In the case of lysolecithin, labelled fatty acid was incorporated into the β-position of lecithin and in the case of α-glycerophosphate, into both the α and β positions. Portman (1967) also demonstrated the stimulating effect of lysolecithin on ^{14}C-labelled linoleic acid incorporation into lecithin in aortic homogenates. Also the difference in lecithin formation between normal and atherosclerotic vessels seemed related to endogenous levels of lysolecithin (Portman, 1967). The incorporation of labelled lysolecithin into arterial lecithin has also been shown in human umbilical artery, dog carotid artery (Eisenberg, Stein and Stein, 1967b) and atherosclerotic monkey aorta (Portman and Alexander, 1969).

On the question of location of phospholipid synthesis in the atherosclerotic lesion, ^{32}P-labelled phosphate ^{14}C-labelled acetate and ^{14}C-labelled oleic acid have been shown to be incorporated into phospholipid by foam cells isolated from rabbit atherosclerotic

lesions (Day, Newman and Zilversmit, 1966; Day and Wilkinson, 1967; Day and Tume, 1968). Also, after prior incubation of rabbit atherosclerotic aorta with ^{14}C-labelled oleic acid, foam cell phospholipid has a higher specific activity than phospholipid elsewhere in the artery (Day and Tume, 1970).

The breakdown of phospholipid may also be important in relation to lipid accumulation in the arterial wall. Phospholipase A, which converts lecithin to lysolecithin, has been demonstrated in pig (Patelski, Waligora and Szulc, 1967), in rabbit and rat (Patelski, Bowyer Howard and Gresham, 1968) and in rat and human aortae (Eisenberg, Stein and Stein, 1968). Lysolecithinase activity has also been demonstrated (Eisenberg et al. 1967b; Eisenberg, Stein and Stein, 1969). The presence of sphingomyelinase (sphingomyelin cholinephosphohydrolase), whose products are ceramide and phosphorylcholine (see Fig. 2), has also been shown in aortic homogenates and the suggestion made that failure of the atherosclerotic artery to increase its sphingomyelinase activity may account for the relatively greater concentration of sphingomyelin in the lesion (Rachmilewitz, Eisenberg, Stein and Stein, 1967; Eisenberg et al. 1969).

(5) Triglyceride

The studies of arterial wall metabolism using labelled acetate and fatty acid as precursors in which cholesterol ester and phospholipid formation have been demonstrated (see above), have also generally shown triglyceride formation. Zilversmit, Newman

and McCandless (1961) have presented a case for triglyceride fatty acid being synthesized in situ, although, as has been pointed out previously, triglyceride fatty acids are not very different from those in the serum.

Triglyceride synthesis in the arterial wall is stimulated by α-glycerophosphate and when it is omitted from homogenate systems little synthesis of neutral lipid occurs (Stein, Stein and Shapiro, 1963). Thus, it is unlikely that the conversion of glycerol to glycerophosphate by glycerokinase takes place in the arterial wall.

Lipolytic activity has been found in the arterial wall by some workers (Drury, 1961; Zsoldos and Heinemann, 1964; Leites and Fedoseev, 1965; Zemplenyi, 1968), but not by others (Maier and Haimovici, 1965).

2. Carbohydrate Metabolism

Early studies of arterial respiration were carried out by Lazovskaya (1943) and Briggs, Chernick and Chaikoff (1949). The respiration and carbohydrate metabolism of arterial tissue was extensively reviewed by Kirk (1963), largely on the basis of his and his co-workers findings. He found that normal aorta had a respiratory quotient of 0.91 in accord with the view that arterial wall metabolism is mainly carbohydrate in type.

Glycolysis occurs, but is relatively independent of oxygen tension. The weak Pasteur effect may lead to lactic

acid accumulation and a low arterial pH. While the pentose pathway is operative in the arterial wall (Kirk, 1963; Zemplenyi, 1968) there is disagreement as to how much glucose it metabolises relative to the glycolytic pathway (Whereat, 1967).

There is evidence that glycogenesis and glycogenolysis can take place in the arterial wall (Kirk, 1963).

Not all of the enzymes of the Krebs cycle and tricarboxylic acid cycle have been demonstrated in arterial tissue (Kirk, 1963; Maier, 1968; Zemplenyi, 1968) and its role in arterial wall metabolism must await further investigation.

Mucopolysaccharide synthesis from ^{35}S-labelled sulphate, ^{14}C-labelled acetate and ^{14}C-labelled glucose has been demonstrated in arterial tissue (Dyrbe, 1959; Hauss, Junge-Hülsing and Holländer 1962; Höllander, Yagi and Kramsch, 1964; Khokhar and Hilker, 1967; Hastings, Booth and Hopwood, 1968). Further, ^{35}S-labelled sulphate has been localized by autoradiography to intimal and medial cells (Bleyl and Wegener, 1969) or to foam cell and fibroblast areas of atherosclerotic lesions (Buck, 1955). As far as mucopolysaccharide catabolism is concerned, only very low levels of β-glucuronidase have been found in human arteries (Kirk, 1963).

3. Protein Metabolism

The incorporation of ^{14}C-labelled glycine into arterial proteins *in vivo* has been investigated in monkeys fed atherogenic diets (Lee, Jones, Kim, Florentin, Coulston and Thomas, 1966). A comparison of overall

protein synthesis and synthesis per unit DNA suggested different cells were synthesizing protein at different rates. ^{14}C-labelled glycine and ^{14}C-labelled leucine have been incorporated into protein of the inner human, canine and rat arterial wall (Chobanian, 1968b). ^{14}C-labelled lysine has been incorporated into elastin of the growing chick aorta (Sandberg and Cleary, 1968). Grigorian, Lysenko and Sokolova (1969) have shown alterations in electrophoretic patterns of human aortic proteins with the development of atherosclerosis and correlate them with RNA and DNA changes as a way of assessing changes in protein metabolism.

Alterations of protein metabolism could affect arterial lipids in a variety of ways, but the ability of the wall to form transport lipoproteins may be particularly important. Unfortunately, as has been mentioned previously, it is not simple to distinguish between cellular and transport lipoproteins. Hollander (1967) has reported that ^{14}C-labelled acetate is incorporated into both lipid and protein components of arterial lipoproteins and that the 1.019 - 1.063 density fraction contains most of the label, whether lipid or protein. Also, ^{14}C-labelled leucine is incorporated mainly into the 1.019 - 1.063 density fraction. Lipid in the form of soluble lipoprotein is more likely to be removed from the arterial wall and Hollander (1967) has shown that this is so for cholesterol using an intramural injection technique.

4. Metabolism of Other Substances

The metabolism of the arterial wall is not limited to lipid, carbohydrate and protein and this is reflected in the extensive documentation of arterial enzymes by

Zemplenyi (1968).

Not surprisingly, ^{14}C-labelled glycine has been shown to be incorporated in to RNA (Chobanian, 1968b) and studies of cellular division demonstrated the utilization of ^{3}H-labelled thymidine in DNA synthesis (McMillan and Stary 1968). Ribonuclease has been isolated from bovine aorta (Gamble, Hoelscher Nayar, and Kiersky, 1967).

In view of the effects of sex hormones on arterial wall metabolism (Malinow, 1960; Malinow and Moguilevsky, 1961; Chobanian, 1968), it is interesting that these substances are themselves metabolised by the arterial wall (Chobanian, Brecher, Lille and Wotiz, 1968). Whether fibroblast-like cells in the arterial wall metabolise corticosteroids in the same way as connective tissue fibroblasts (Dougherty and Berliner, 1959) is not known.

5. Factors Affecting Arterial Wall Metabolism

(1) Diabetes and Insulin

The increased incidence of atherosclerosis in diabetic patients has been referred to already. In the light of this it was somewhat surprising to find that alloxan diabetic rabbits developed less atherosclerosis when fed a cholesterol diet than did non-diabetic rabbits fed the same diet (Duff and McMillan, 1949; McGill and Holman, 1949). Insulin ameliorated the diabetic state and increased the severity of atherosclerosis, but possibly not significantly more than in the non-diabetic cholesterol-fed controls (Duff, Brechin, and Finkelstein, 1954). On the other hand, alloxan diabetic rats on an atherogenic diet

develop as much atherosclerosis as their non-diabetic controls (Still, Martin and Gregor, 1964). The finding of high levels of circulating insulin in persons with abnormal glucose-tolerance tests (Yalow and Berson, 1960; Hales, Walker, Garland and Randle, 1965); the knowledge that juvenile diabetics are treated with insulin (and that, in order to achieve a given portal concentration, using exogenous insulin, a much higher systemic concentration is necessary than is the case with endogenous insulin); and the described effect of insulin in the alloxan diabetic rabbit, raise the question of the effect of insulin itself on the metabolism of the arterial wall, as opposed to the effect of the diabetic state.

Aortae from alloxan diabetic rabbits do not appear to exhibit a reduction in glucose entry into cells, nor is the entry affect by insulin *in vitro* (Yalcin and Winegrad, 1963). The findings of Mulcahy and Winegrad (1962) in a previous study, of impaired glucose utilization in the aorta of the alloxan diabetic rabbit (reflected in reduced production of lactate, of CO_2, of glycogen and of total lipid), were probably based on reduced glucose phosphorylation or hexokinase activity. But though insulin is thought to increase the activity of glucokinase elsewhere, it exhibited no such effect on rabbit aorta *in vitro*. Nevertheless, there was a slight increase in glycogenesis in normal and diabetic rabbit aortae *in vitro* but this effect could be due to insulin acting on an enzyme specifically concerned with glycogenesis. However, after a prolonged period, insulin *in vivo* produced

a marginal effect on glucose utilization by the aorta *in vitro*. A reduction in lipogenesis from ^{14}C-labelled acetate by the alloxan diabetic rat aorta *in vitro* was found by Foster and Siperstein (1960). Again, no effect of insulin *in vitro* on glucose utilization by normal or diabetic rat aorta could be demonstrated by Urrutia, Beavan and Cahill (1962). In contrast to these findings, Wertheimer and BenTor (1962) have shown that insulin *in vivo* and *in vitro* restores glucose and galactose uptake by alloxan diabetic rat aortae *in vitro* to normal.

The observed decrease in glucose utilization and the reports of decreased lipogenesis in the aortae of diabetic animals *in vitro* are consistent with the known effects of insulin-lack in other tissues, but the failure of insulin *in vitro* to rectify these effects is difficult to understand unless it acts very slowly on arterial enzyme systems or unless insulin-lack in the animals concerned produces irreversible changes.

The inhibition of tissue lipase in arterial tissue by insulin (Mahler, 1966) is consistent with the known lipogenetic actions of insulin elsewhere. But Chmelař and Chmelařova (1968) found that, in the the normal rat, guinea-pig, rabbit and pig aorta, insulin stimulated lipase activity and also enhanced the lipolytic effect of ACTH and of norepinephrine.

Chattopadhyay and Martin (1969) have been unable to find an effect of insulin *in vitro* on ^{14}C-

labelled acetate incorporation into fatty acid and non-saponifiable lipid of the normal rat aorta. Glucose *in vitro*, however, stimulated synthesis of fatty acid and non-saponifiable lipid from ^{14}C-labelled acetate and its effect was not modified by insulin. Thus, the finding of Stout (1968) that insulin *in vivo* increases lipogenesis in the normal rat aorta from acetate-1-^{14}C and glucose-U-^{14}C *in vivo* is unlikely to represent a direct action of insulin on the arterial wall. The report by Stout (1969) that insulin *in vivo* increases cholesterogenes is in normal rat aorta from acetate-1-^{14}C *in vivo* is also unlikely to be related to a direct action of insulin. Furthermore, the conclusion by Stout that cholesterol is synthesized is based on insufficient evidence as no account is taken of high-counting companions. Neither, for that matter, is it clear whether the findings relate to total cholesterol, free cholesterol or cholesterol ester, so that fatty acid rather than sterol synthesis could have been under investigation.

In alloxan diabetic rats, the synthesis of monounsaturated fatty acids from acetate-1-^{14}C and stearic acid-1-^{14}C by epididymal fat pads *in vitro* is markedly depressed. Insulin causes an increase in saturated fatty acid synthesis alone in the diabetic fat pads with no associated increase in monounsaturated fatty acid synthesis (Benjamin and Gellhorn, 1963). The possibility that the pattern of fatty acid synthesis in the arterial wall might be altered by insulin has not been explored.

(2) Hypertension

The prospective Framingham study (Kannel, Dawber, Kagan, Revotskie and Stokes, 1961) has demonstrated that there is a direct correlation between hypertension and the incidence of coronary heart disease. In 1940, Davis and Klainer had shown a correlation between essential hypertension and coronary atherosclerosis, but argued that hypertension per se had not been an aetiological factor. Bruger and Chassin (1941) produced evidence that the cholesterol content of the thoracic aorta and renal arteries was greater in hypertensive than in normotensive patients. Also, Bronte-Stewart and Heptinstall (1954) found that in the cholesterol-fed rabbit the severity of the atherosclerotic lesions increased with hypertension. Moreover, the aortic cholesterol concentration of hypertensive rats is greater than that of normotensive controls (Daly, Deming, Raeff and Brun, 1963).

There are basically three ways in which hypertension might result in increased lipid deposition in the arterial wall (a) increased lipid filtration with overload of removal mechanisms (b) mechanical damage to the vessel with resulting trapping of lipid (c) concomitant effects of increased levels of vasoactive substances such as the catecholamines. Metabolic change in the arterial wall could follow any of these processes.

High levels of catecholamines result in the mobilization of free fatty acids from adipose tissue and increased arterial blood concentrations of free

fatty acids (Carlson, 1968). It is possible that, under such circumstances, intimal free fatty acid concentrations also rise and lead to changes in local lipid metabolism. Phospholipid synthesis from radioactive phosphate has been enhanced in normal rabbit aorta (Drury, 1955) and in normal rat aorta (Nakatani, Sasaki, Miyazaki and Nakamura, 1967) following epinephrine administration. Catecholamines inhibit the incorporation of ^{14}C-labelled acetate, ^{14}C-labelled mevalonate and ^{14}C-labelled glucose into arterial fatty acids and sterols (Hollander, 1963a, and 1963b) and of ^{35}S-labelled sulphate, ^{14}C-labelled acetate and ^{14}C-labelled glucose into arterial acid mucopolysaccharides (Hollander, Yagi, and Kramsch, 1964). No effect could be demonstrated by Zsoldos and Heinemann (1964) of epinephrine on the lipolytic activity of rabbit aorta.

In the hypertensive segment above a surgical narrowing of the dog aorta, mucopolysaccharide formation from ^{35}S-labelled sulfate increases, but there is no change in the influx of ^{14}C-labelled cholesterol (Hollander, Madoff, Kramsch and Yagi, 1964).

(3) Therapeutic Agents

Since the arterial wall clearly has a metabolism of its own, it becomes important in the evaluation of any drug with a hypolipidaemic action or potentially beneficial effect in the management of atherosclerosis, to consider its effects on that metabolism.

The ethyl ester of chlorophenoxyisobutyric

acid (CPIB, Clofibrate or Atromid-S) was developed by Thorp and Waring in 1962 and is known to be hypolipidaemic (Oliver, 1967; Sachs, 1968).

Ethyl p-chlorophenoxyisobutyrate and p-chlorophenoxyisobutyric acid have been found to inhibit the incorporation of mevalonic acid-2-^{14}C into cholesterol by cell-free extracts of bovine aorta (Teal and Gamble, 1965). Subsequent investigations in the same bovine aortic system (Walsh, Teal and Gamble, 1969) have shown that chlorophenoxyisobutyric acid inhibits mevalonate kinase, the biosynthesis of fatty acids and the conversion of acetate to carbon dioxide.

There is no information at the moment to indicate that the atherosclerotic process in man is reduced or reversed by CPIB. For cholesterol-fed rabbits, CPIB reduces the severity of lesions in the arch of the aorta, but not in the thoracic aorta (Kritchevsky, Sallata and Tepper, 1968), but for rabbits with arterial lesions induced by a semi-synthetic diet, CPIB has no effect on the course of the disease (Jones, Gresham, and Howard, 1963).

CPIB reduces the serum cholesterol linoleate and increases the serum cholesterol oleate and palmitate levels in patients with coronary artery disease (Hagopian and Robinson, 1968). This could reflect a lowering of total body cholesterol linoleate and an increase in total body cholesterol oleate and cholesterol palmitate; or it could mean that cholesterol

linoleate has moved into the tissues and that cholesterol oleate and cholesterol palmitate have moved from the tissues into the serum. Yet another explanation might be that the activity of the serum lecithin: cholesterol acyltransferase enzyme, known to have a preference for polyunsaturated fatty acid transfer (Portman and Sugano, 1964), has been depressed. It is important to know, firstly, the changes that take place in arterial cholesterol ester fatty acid composition following CPIB treatment and, secondly, the sequelae of those changes. If CPIB alters the composition of fatty acids synthesized in the arterial wall and esterified to cholesterol, then it is important to know this as well.

F. Intimal Cells

Cytologists have been learning that it is often misleading to classify cells too rigidly on morphological grounds and thence to extrapolate to origin, function and potential. This is probably being learned about intimal cells and atherogenesis.

The normal intima consists of little more than a layer of endothelium with occasional cells in the subendothelial zone, superficial to the internal elastic lamina. Depending on the species, age and whether the cells are assessed by light or electron-microscopy, the subendothelial cells have been found to consist of, variously, small round cells, macrophages, fibrocytes, stellate cells of Langhans, and smooth muscle cells (French, 1966).

The population of cells in the atherosclerotic lesion has for a long time been divided into cells containing an excess of lipid (foam cells) and those with a normal amount of lipid.

Foam cells differ from adipose cells in having more than one cytoplasmic drop of lipid. They are not confined to the atherosclerotic lesion, but are seen in xanthomata, the bone marrow, spleen, lungs, intestine, liver, lymph nodes, cerebrospinal fluid, thymus and glomeruli in the lipidoses (Schettler, 1968). Of particular interest to the investigator of atherosclerosis are those which contain relatively large amounts of cholesterol ester. Some xanthomata in familial hypercholesterolaemia (low density lipoproteinaemia; Type II hyperlipidaemia of Fredrickson et al) consist mainly of cholesterol ester (Glomset and Williams, 1968). Foam cells in Tangier disease (familial high density lipoprotein deficiency; hypo-α lipoproteinaemia), especially evident in the tonsils, also have a high cholesterol ester content (Schettler, 1968). Foam cells form in the Wallerian degeneration of leprosy (Khanolkar, 1960) and it is noteworthy that cholesterol ester formation takes place during Wallerian degeneration (Johnson, McNabb and Rossiter, 1950).

As has been discussed earlier, the first ultrastructural appearance of lipid in the inner arterial wall is in cells with myofilaments and a basement membrane (McGill and Geer, 1963; Balis, Haust and More, 1964), but macrophage -like foam cells also appear (Ghidoni and O'Neal, 1967). It is possible that these two foam cell types only represent the ends of a spectrum of cells and not two distinct classes, since when reviewing the available electronmicrographs, it is sometimes difficult to place cells into one or other category.

The presence of myofilaments in foam cells should not be taken as proof that the cells originate from smooth muscle cells. They may, as Wissler (1967 and 1968) suggests, originate from a multifunctional medial mesenchyme cell, or for that matter a

multifunctional subendothelial or even endothelial cell. The same could be said of the macrophage-like foam cell, that it need not arise from a blood monocyte or a histocyte. Leary (1941) considered that intimal foam cells were derived as such from the blood and ultimately from the liver. The macrophages that Poole and Florey (1958) observed between endothelial cells could have been moving into or out of the intima.

That intimal cells, whether foam cells, stellate cells or endothelial cells, proliferate has been demonstrated by inspection for mitotic figures (McMillan and Duff, 1948; Duff, McMillan and Lautsch, 1954) and by use of ^{3}H-labelled thymidine (McMillan and Stary, 1968).

The isolation of rabbit intimal foam cells has been described (Day, Newman and Zilversmit, 1966). The high proportion of foam cells isolated is probably more a reflection of their strong representation in the intimal cell population than of any cell-selection by the procedure. Endothelial cells and mesenchymal cells are known to stick to glass as do the foam cells. In fact, Day, Bradley and Tume (1969) observed considerable proliferation of fibroblast-like cells when "rabbit intimal foam cells" were followed in tissue culture. The foam cells themselves did not appear to proliferate. The advantage of the isolation procedure for the rabbit foam cells seems to be that a viable cell population is obtained from among intimal debris and dying cells. The isolation of particular cell types from human atherosclerotic lesions, where foam cells are often not as strongly represented as in the rabbit, in sufficient numbers for metabolic experiments, would be of considerable value.

The relevance to atherogenesis of metabolic information

obtained from intimal cells in tissue culture is open to question since characteristics may change; the organ culture approach is perhaps an advance on the tissue culture methods (Robertson, 1965).

G. Removal of Lipid from the Arterial Wall

The case for reversibility of the atherosclerotic lesion has been put by Katz and Pick (1963) and is based on the fate of the fatty streak of early childhood, the altered incidence of atherosclerosis *in man* with altered nutritional state, and the regression of atherosclerotic lesions in experimental animals. They stress that it is the lipid accumulation which is essentially reversible and this accords with the view of Holman, McGill, Strong and Geer (1958) and of Constantinides (1965) that it is the fatty streak rather than the fibro-fatty lesion which is reversible.

Schwenk and Stevens (1960) found that rabbit atherosclerotic lesions, labelled with cholesterol-^{14}C, lost their label when the rabbits were fed non-labelled cholesterol, whereas Connor, Armstrong, Jackson and Ali (1966) found that cholesterol-4-^{14}C persisted in rabbit atherosclerotic aortae. Zilversmit (1968) suggests that one explanation for the discrepancy might be that fibrosis had occurred in the experiments of Connor et al.

Both free and ester ^{14}C-labelled cholesterol effluxed *in vitro* from rabbit atherosclerotic lesions into hypercholesterolaemic and normal serum in the studies of Newman and Zilversmit (1966).

Hollander (1967) found that intramural implants of ^{14}C-labelled cholesterol as lipoprotein into the femoral artery of the dog were removed more rapidly than ^{14}C-labelled cholesterol suspensions in saline. It is possible thqt the increased formation

of phospholipid with atherogenesis (Zilversmit, 1959) represents an attempt by the arterial wall to solubilize cholesterol for removal. However, sphingomyelin accumulation may be a reflection of a relative lack of sphingomyelinase activity (Rachmilewitz, Eisenberg, Stein and Stein 1967).

Processes of lipid removal, or lack of them, may be responsible for the selective accumulation of cholesterol oleate in rabbit atherosclerotic and human fatty streak lesions (Fig. 1). It is known that the catabolism and biliary excretion of polyunsaturated cholesterol esters by the liver is greater than that of saturated cholesterol ester (Lewis, Pilkington and Hodd, 1961). Also, when more polyunsaturated fatty acid is available, the severity of atherosclerosis is diminished (Kritchevsky, Moyer, Tesar, McCandless, Logan, Brown and Englert, 1956; Bortz, Larsen and Civin, 1958; McMillan, Wegensberg and Ritchie, 1960). Polyunsaturated cholesterolesters are removed more readily from granulomatous lesions than other cholesterol esters (Abdulla, Adams, and Morgan, 1967). From studies of the net accumulation and influx of individual cholesterol esters into rabbit atherosclerotic lesions, Swell, Law and Treadwell (1963) have calculated that saturated cholesterol esters are retained more than the mono-unsaturated and these, in turn, more than the polyunsaturated. Reference has already been made to the report by Bowyer, Howard, Gresham, Bates and Palmer (1968) of low cholesterol oleate hydrolytic activity in the arterial wall.

It is interesting to speculate from the finding of Abdulla, Adams and Morgan (1967) that cholesterol and cholesterol oleate are more sclerogenic than other forms of cholesterol, that the necrosis of foam cells may result in the conversion of the fatty streak into the fibro-fatty lesion. Why, then, should the fibrofatty lesion have

a more polyunsaturated cholesterol ester fatty acid pattern (Smith, Slater and Chu, 1968)? It was suggested by Smith et al (1968) that the origin of cholesterol ester in the fibrous lesion was in the serum, but could it be a reflection of one cellular metabolic activity imposing itself on another?

Knowledge of the mechanisms for lipid removal from the arterial wall will aid not only an understanding of lipid accumulation, but also of ways in which the lesion might be reversed.

MATERIALS AND METHODS

A. Materials

1. Radioactive Substrates

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

Palmitic acid-1-^{14}C (specific activity 55.2 mCi/mM, Radiochemical Centre, Amersham, U.K.) was dissolved in 0.05N sodium hydroxide to give a stock solution of sodium palmitate containing 20μCi/ml. Radiochemical purity was checked by neutral lipid thin-layer chromatography, as described below, when 98% ran with an Rf corresponding to free fatty acid; and by gas liquid radiochromatography, as described below, when ^{14}C was found as a single peak with a retention time corresponding to 16:0.

In order to complex this and other labelled fatty acids to serum albumin it was heated in a boiling water-bath and then quickly added to the serum of the incubation medium in preparation.

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

Oleic acid-1-^{14}C (specific activity 32.5, 43.2, 57.2, or 57.8 mCi/mM, Radiochemical Centre, Amersham, U.K.) was dissolved in 0.05N sodium hydroxide to give stock solutions of sodium oleate containing approximately 20 μCi/ml. Radiochemical purity was checked by neutral lipid thin-layer chromatography when 98% ran with an Rf corresponding to free fatty acid; and by gas liquid radiochromatography when ^{14}C was found as a single peak corresponding to 18:1.

(c) <u>^{14}C-labelled Sodium Linoleate</u>

Linoleic acid-1-^{14}C (specific activity 52.9 mci/mM, Radiochemical Centre, Amersham, U.K.) was dissolved in 0.05N sodium hydroxide to give a stock solution of sodium linoleate containing 20 μCi/ml. Radiochemical purity was checked by neutral lipid thin-layer chromatography when 98% ran with an Rf corresponding to free fatty acid; and by gas liquid radiochromatography when ^{14}C was found as a single peak corresponding to 18:2.

(d) <u>^{3}H-labelled Sodium Oleate</u>

Oleic acid-9-10-^{3}H (specific activity 3020 mCi/mM, Radiochemical Centre, Amersham, U.K.) was dissolved in 0.1N sodium hydroxide to give a stock solution of sodium oleate containing 250 μCi/ml. Radiochemical purity was checked by neutral lipid thin-layer chromatography when 99% ran with an Rf corresponding to free fatty acid; and, as the methyl ester, by silver nitrate-impregnated silicic acid thin-layer chromatography, as described below, when 99% of the ^{14}C was found with an Rf corresponding to that of monounsaturated fatty acid.

(e) <u>^{14}C-labelled Cholesterol</u>

Cholesterol-4-^{14}C (specific activity 55.8 mCi/mM) was obtained from the Radiochemical Centre, Amersham, U.K.

A micellar cholesterol solution was prepared by dissolving 10 μCi in 1 ml ethanol containing 30 mg of Tween 20 (Chemical Materials Ltd., Bayswater, Vic.,

Aust.) The ethanol was removed by evaporation under a stream of nitrogen. The residue was taken up in 1 ml of water with vigorous shaking (Siperstein and Chaikoff, 1952). Serum lipoprotein was labelled with this Tween 20 preparation according to the method of Whereat and Staple (1960).

(f) ^{3}H-labelled Cholesterol

Cholesterol-T (G) (specific activity 2370 mCi/mM) was obtained from the Radiochemical Centre, Amersham, U.K.

(g) ^{14}C-labelled Choline

Choline Chloride (methyl-^{14}C) (specific activity 32 mCi/mM) was obtained from the Radiochemical Centre, Amersham, U.K. Purity was checked by paper chromatography as described below.

(h) ^{14}C-labelled Sodium Acetate

Sodium acetate-1-^{14}C (specific activity 52.9 mCi/mM) was obtained from the Radiochemical Centre, Amersham, U.K. This was dissolved in water to give stock solutions of 50 or 100 μCi/ml.

(i) ^{32}P-labelled Orthophosphate

^{32}P-labelled orthophosphate was obtained from the Australian Atomic Energy Commission, Lucas Heights, Sydney. Specific activity varied with batch and time, due to decay.

2. Thin-Layer Chromatographic Standards

(a) Neutral Lipids

(i) α and β-Dipalmitin, Hormel Institute, Austin Minnesota, U.S.A.

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

(iii) Palmitic acid, British Drug Houses Ltd., Poole, England.

(iv) Cholesterol Oleate, Applied Science Labs., State College, Pennsylvania.

(v) Cholesterol U.S.P., Nutritional Biochemical Corporation, Cleveland, Ohio, U.S.A.

(b) <u>Phospholipids</u>

(i) Lecithin (Phosphatidylcholine), Applied Science Labs, State College, Pennsylvania, U.S.A.

(ii) Lysolecithin, Applied Science Labs, State College, Pennsylvania, U.S.A.

(iii) Sphingomyelin, Applied Science Labs, State College, Pennsylvania, U.S.A.

(iv) Phosphatidylethanolamine, Applied Science Labs, State College, Pennsylvania, U.S.A.

(v) Phosphatidylserine, Applied Science Labs, State College, Pennsylvania, U.S.A.

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

(c) <u>Cholesterol Esters</u>

(i) Cholesterol Stearate, Applied Science Labs, State College, Pennsylvania, U.S.A.

(ii) Cholesterol Oleate, Applied Science Labs, State College, Pennsylvania, U.S.A.

(iii) Cholesterol Linoleate, Applied Science Labs, State College, Pennsylvania, U.S.A.

(d) <u>Methyl Esters</u>

(i) Methyl Palmitate, Applied Science Labs, State College, Pennsylvania, U.S.A.

(ii) Methyl Oleate, Applied Science Labs, State College, Pennsylvania, U.S.A.

(iii) Methyl Linoleate, Applied Science Labs, State College, Pennsylvania, U.S.A.

(iv) Methyl Linolenate, Applied Science Labs, State College, Pennsylvania, U.S.A.

3. Internal Standards

(a) Heptadecanoic Acid

Heptadecanoic acid was obtained from the Hormel Institute, Austin Minnesota, U.S.A. It is found in only trace amounts in mammalian tissues and was therefore used as an internal standard for free fatty acid methyl esterification followed by gas-liquid chromatography.

(b) Cholesterol Heptadecanoate

Cholesterol heptadecanoate is also found in only trace amounts in mammalian tissues. It could thus serve as an internal standard for the cholesterol ester of lipid extracts.

Cholesterol heptadecanoate was synthesized chemically by the acyl chloride method of Pinter, Hamilton and Muldrey (1964). Heptadecanoic acid was obtained from the Hormel Institute, Austin, Minnesota and cholesterol U.S.P. from Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A. The fatty acid was placed in the centre well of a Warburg flask and oxalyl chloride added in a molar ratio of about 3:1. The flask was closed immediately

with a ground glass stopper and the air evacuated by a water aspirator connected to the side arm. The flask was then placed in a water bath at 65°C and the mixture allowed to boil for 5 mins to drive off the excess oxalyl chloride. Oxalyl chloride was added a further two times and the procedure repeated. Then free cholesterol, in a molar ratio of 2:1 with respect to the fatty acid, was dissolved in 5 ml of dry diisopropyl either and added to the acyl chloride in the flask. With a vacuum applied, the mixture was allowed to boil at 60°C until no solvent was visible. The crude cholesterol ester was taken up in hexane and purified by neutral lipid and silver nitrate thin-layer chromatography as described below. Fatty acid purity was checked by gas liquid chromatography. The final product was assayed for cholesterol by the method of Zlatkis, Zak and Boyle (1953) following saponification by the method of Abell, Levy, Brodie and Kendall, (1952). It was evaluated as an internal standard by assaying against a known standard of cholesterol stearate (Applied Science Labs, State College, Pennsylvania, U.S.A.). The amount of cholesterol heptadecanoate was determined by comparison of 17:0 and 18:0 gas liquid chromatogram peaks. The coefficient of variation (n=4) was 1.1%. The colorimetric and internal standard methods agreed to within 7%.

(c) <u>^{14}C-labelled Cholesterol</u>

A ^{14}C-labelled cholesterol standard was incorporated into total lipid extracts to determine free cholesterol recovery. Free cholesterol, separated by thin layer chromatography, was eluted with chloroform : methanol, 2 :1. Eluants were made up to

a known volume, aliquots then being taken for counting and for cholesterol determination. As indicated by the ^{14}C-labelled cholesterol internal standard, recoveries were between 80 and 90%, but in any case cholesterol values were adjusted according to recovery.

4. Lipoprotein ^{3}H/^{14}C-labelled Cholesterol

To assess the role of cholesterol ester hydrolysis in the apparent uptake of free cholesterol by the atherosclerotic aorta, ^{3}H/^{14}C-labelled lipoprotein was prepared. Under light ether anaesthesia, an infant feeding tube was passed into the stomach of a male New Zealand white rabbit, fed cholesterol for 4 months. 3.5 mCi of ^{3}H-labelled cholesterol in 2 ml of corn oil were given, followed by 100 mg sodium taurocholate in 0.9% sodium chloride solution. 42 hours after intubation, the animal was exsanguinated by cardiac puncture. To the serum, ethylenediaminetetracetic acid (1 mg/ml) and penicillin (about 0.1 mg/ml) and streptomycin (about 0.1 mg/ml) were added. The ^{3}H-labelled cholesterol was present in the serum in the ester (82.1%) and free form. In order to exchange the ^{3}H-labelled free cholesterol with non-labelled free cholesterol and thereby increase the percentage of ^{3}H-labelled cholesterol ester, the serum was incubated with normal rabbit erythrocytes according to the method of Hashimoto and Dayton (1966). Following this procedure, 90.9% of the ^{3}H-labelled cholesterol was found to be in the ester form. This serum (17 ml) was then incubated *in vitro* for 3 hours with 0.5 ml of micellar ^{14}C-labelled cholesterol prepared as described above. In this way, a preparation of hypercholesterolaemic rabbit serum was obtained in which the lipoprotein cholesterol ester was labelled almost exclusively with ^{3}H and the lipoprotein free cholesterol labelled with both ^{14}C and ^{3}H. Its free and

ester cholesterol content and their respective specific activities (^{3}H and ^{14}C) were determined, as described in Section 7, and this information is presented in Table 1. The $^{3}H/^{14}C$ ratio of the free cholesterol was 1.26 and that of the cholesterol ester 804. The $^{3}H/^{14}C$ ratio of the free cholesterol was obtained by replicate determinations with standard deviations of 0.03 (n=8). In addition, the distribution among lipoprotein fractions of the ^{3}H and ^{14}C in the free cholesterol was determined after ultracentrifugation using densities of 1.006, 1.019, 1.063, 1.21, as described below. This information is shown in Table 2. The percentage of ^{3}H-labelled cholesterol as ester was similar in each of the lipoprotein fractions (85-88%). The percentage of ^{14}C-labelled cholesterol as ester ranged from 1% for the <1.006 fraction to 4% for the 1.063-1.21 fraction. Electrophoresis was performed on cellulose acetate as described below. It was found that both the ^{3}H and ^{14}C were present as single peaks with migrations corresponding to the pre β - lipoprotein region as demonstrated by Oil Red O Staining.

5. Insulin and anti-insulin

(a) Insulin

Insulin BP "Wellcome" (40 units/ml) was used for incubations *in vitro*.

(b) Anti-insulin

Anti-insulin was prepared according to the method of Armin, Grant and Wright (1960). Crystallised bovine insulin (strength 24.2 units/mg) was obtained from Commonwealth Serum Laboratories, Parkville, Victoria. 10 mg were dissolved in 10 ml aqueous phenol, 0.3%

TABLE 1

In Vivo Cholesterol - T (G) / In Vitro Cholesterol - 4 - ^{14}C labelled Lipoprotein from Cholesterol-fed Rabbits

	Cholesterol mg/ml	Specific Activity dpm ^{3}H/mg	Specific Activity dpm ^{14}C/mg	$^{3}H/^{14}C$ Ratio
Free cholesterol	1.9	255,300	199,000	1.26
Cholesterol ester	8.4	56,800	717	804
Cholesterol ester				
- Saturated	1.8	716,200	-	-
- Monounsaturated	4.4	413,500	-	-
- Polyunsaturated	2.1	744,000	-	-

"dpm" means disintegrations/minute

TABLE 2

Lipoprotein Analysis of $^{3}H/^{14}C$ Cholesterol-Labelled Hypercholesterolaemic Serum by Ultracentrifugation

	FREE CHOLESTEROL		
	% ^{3}H	% ^{14}C	$^{3}H/^{14}C$ Ratio
< 1.006	59.1	49.6	2.29
1.006 - 1.019	25.3	29.5	1.47
1.019 - 1.063	13.2	17.3	1.46
1.063 - 1.21	2.5	3.6	1.32

(w/v), acidified with hydrochloric acid to a pH of 2.6. This in turn was mixed with 7 ml liquid paraffin BP and 3 ml melted anhydrous lanoline (Adeps lanae BP, May Baker) and the mixture emulsified with the aid of a Waring blender. Male guinea-pigs were injected with a total of 2 ml of the preparation, 1 ml between the shoulders and 0.5 ml into the inner aspect of each thigh. A control group of guinea-pigs was set up at the same time. Injections were repeated at 3 to 4 weekly intervals and 2 weeks after the third or later injections about 10 ml blood was obtained by cardiac puncture from each animal. It was usually possible to collect blood two or three times from any one animal. Blood was centrifuged in an M.S.E. Mistral 2L centrifuge at 3000 rpm for 15 minutes at 0°C, and the pooled serum stored with penicillin (about 0.1 mg/ml) and streptomycin (about 0.1 mg/ml) at -20°C. Crude biological assay of the preparation was achieved by injecting 2.5 ml into a fasted rabbit via a marginal ear vein. Dextrostix monitoring demonstrated that the blood sugar level rose from the 65 to 90 mg % range before injection to a maximum of 150 to 200 mg% at 35 mins after injection where it remained for 75 mins. It did not return to the pre-injection levels until 8 hours after injection.

The anti-insulin activity of the prepared serum was determined by radioimmunoassay, by courtesy of Miss Margaret Pearson of the Department of Biochemistry, Royal Melbourne Hospital. The method was based on the insulin assay of Herbert, Lau, Gottlieb and Bleicher

(1965) using ^{125}I-labelled insulin, instead of anti-insulin. The preparation was found to have anti-insulin activity equivalent to 50 - 100 micro units of pork insulin per ml, while the control had no detectable activity.

6. Chlorophenoxyisobutyric Acid (CPIB)

The sodium salt of chlorophenoxyisobutyric acid was obtained from ICI, Pharmaceutical division, Macclesfield, Cheshire, U.K. This was used in concentrations of 500 μg per ml in incubations *in vitro*.

B. Arterial Tissues

1. Rabbit Arteries

Thoracic aortae were obtained from male New Zealand white rabbits fed a diet of poultry growers' pellets, (Barastoc, Kensington, Victoria) with or without cholesterol. Pellet ingredients were bran, pollard, wheat, sorghum, rye, oats, barley, meat meal, lucerne meal, soyabean oil meal, rice pollard, sodium chloride, rock phosphate, calcium carbonate, stabilised vitamin A, stabilised vitamin D_3, riboflavin and calcium pantothenate. Stated pellet analysis was:

Minimum Crude Protein...............15.0%
Minimum Crude Fat.....................3.5%
Maximum Crude Fat.....................5.5%
Maximum Crude Fibre..................8.0%
Maximum Salt.........................0.75%
Maximum Fluorine.....................0.9975%
Minimum Vitamin A....................7.5 I.U./g
Minimum Vitamin D_3...................1.5 I.U./g
Minimum Riboflavin....................5.0 μg/g
Minimum Pantothenic Acid............15 μg/g

Cholesterol-feeding was based on the method of McMillan, Klatzo and Duff (1954) where the pellet diet contained 1% (w/w) cholesterol and 3% (v/w) peanut oil. Batches of 10 Kg of feed were made up every few days. 100 g cholesterol were dissolved in 300 ml peanut oil and 2 litres of commercial ether added. 10 kg of rabbit pellets were evenly impregnated with the solution and the ether allowed to evaporate. Each rabbit received approximately 100 g of feed per day, from the age of 6 weeks for up to 5 months (except for one case reported in Section 8).

The fatty acid composition of the pellets and of the peanut oil, as determined by gas liquid chromatography is shown in Table 3.

Rabbits were killed by ether or Nembutal (Abbott Laboratories, Sydney, Australia) anaesthesia prior to removal of their thoracic aortae. Adherent fat was removed from the adventitial side of the aorta, the vessel opened longitudinally and washed in physiological saline.

The severity of the atherosclerosis was assessed macroscopically and ascribed a grade from 0-5, according to the method of Day and Wilkinson (1956). Grade 0 was a normal aorta and grade 5 gross involvement of the aorta in its whole extent with much heaping up of the lesions and a surface area involvement of over 60% of the total. Animals fed cholesterol 3-5 months had grades 3-5 atherosclerosis.

Where duplicate or triplicate specimens were required from one animal, the aorta was divided longitudinally. Unless otherwise indicated, incubations were carried out at pH 7.4 in equal parts of Hanks' solution (Hanks, 1948) and rabbit serum

TABLE 3

Fatty Acid Composition of Rabbit Diet

% Distribution

Fatty Acid	Pellets			Peanut Oil (Triglyceride)
	Phospholipid	Triglyceride	Cholesterol Ester	
14:0	Trace	Trace	1.9	0
16:0	20.4	17.7	28.6	10.2
16:1	1.4	1.4	4.9	0
18:0	2.6	4.3	5.7	3.3
18:1	18.6	28.7	18.4	55.9
18:2	52.7	44.8	31.8	27.3
20:0	4.4	3.2	5.3	2.1
20:4	Trace	0	3.6	1.3

"Trace" signifies < 1%

with added penicillin (about 0.1 mg/ml) and streptomycin (about 0.1 mg/ml) in an atmosphere of air.

Tissue for histology was fixed in 1% calcium chloride and 4% formaldehyde in physiological saline.

Intima was stripped from media with fine dissecting instruments for lipid analysis. The plane of separation was generally along the internal elastic lamina, but "intima" did include a small portion of the inner media.

2. Human Arteries

(a) Metabolic Studies

Fresh human arteries were obtained from renal transplant donors, at the time of vascular surgery, or when aortic coarctations were resected. This enabled arteries to be set up for incubation *in vitro* with various substrates within 1 or 2 hours of removal from the patient.

Conditions of incubation were similar to those for rabbit arteries, with normal human serum replacing rabbit serum. Histology was also done in the same way. Atherosclerotic lesions were dissected from normal artery before intima was separated from media for lipid analysis.

(b) Chemical Studies

Ascending aorta or aortic arch was obtained from children 8 - 36 hours post mortem for analysis of lipid composition. The common causes of death were congenital malformations, infectious diseases, neoplasia and trauma; arteries from children with

known metabolic disorders were excluded from the series. The youngest patients were a few hours old. Upon removal, the specimen was deep frozen. After thawing, adherent fat was removed from the adventitial side of the vessel. To remove, as far as possible, traces of serum, the specimen was washed several times in 0.9% saline over a period of about 30 minutes. Where atherosclerotic lesions were macroscopically visible, these were dissected from the normal surrounding tissue with the aid of a dissecting microscope (X 10.5 magnification). Intima and as little media as possible were stripped from underlying media and adventitia with the aid of fine dissecting instruments. Stripping technique was checked histologically and found to provide, for normal intima, an intima/media preparation of about one tenth the overall wall thickness (Fig. 3). In the age groups under one year,it was found necessary to pool aortic intima or media, or, where present, atherosclerotic intima, or media underlying involved intima, from 5 children. These were collected into chloroform : methanol 2 :1 for final processing as a group. Tissues from older children were processed individually. Lipid extracts were prepared by the method of Folch, Lees and Sloane Stanely (1957).

All solvents used were redistilled and pre-checked by methyl esterification followed by gas liquid chromatography as described below. Instruments used were cleaned immediately before use with lipid-free solvents. Blanks were run throughout all steps and with each batch of samples. Internal standards were used for blanks in the same way as for

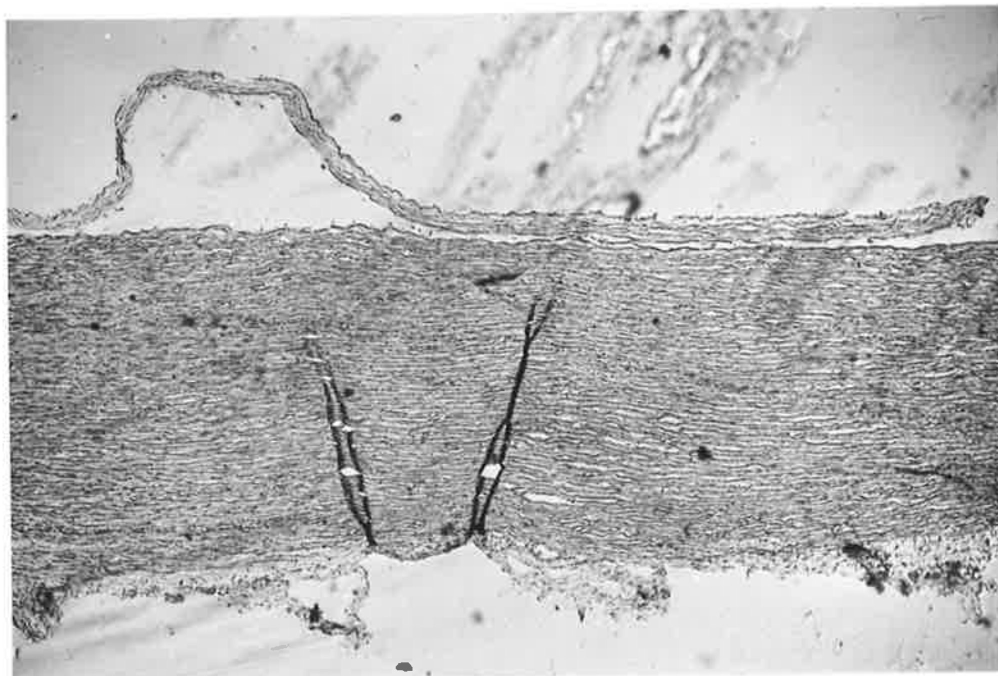

Figure 3

Aorta from a child aged 7 years 4 months showing an inner layer, consisting of intima with some media, stripped from the underlying media. Haematoxylin and Sudan IV, X 40.

samples so that cholesterol ester blanks were found to be 5 - 15% of sample values. Phospholipid fatty acid blanks were estimated to be less than 2%.

Defatted tissues were dried to constant weight to allow the expression of lipid values in terms of dried defatted weight. These weights ranged between 15 and 180 mg for the intima (although in one case the lesion intima was only 5.5 mg) and between 50 and 2000 mg for the media.

C. Lipid Extraction

Lipids were extracted from tissues and from serum by the method of Folch, Lees and Sloane Stanley (1957). Tissues were ground, using mortar and pestle, with chloroform : methanol 2 : 1 and left stand with 20 volumes of the solvent (assuming tissue specific gravity to be that of water) for at least 16 hours. Extracts were then filtered through sintered funnels or the tissue residue removed by centrifugation. The residue was washed 3 times with a small volume of chloroform : methanol 2 : 1. Then to the lipid extract, one fifth of its volume of 0.73% NaCl was added, the tube or container shaken and two phases allowed to develop. The upper phase was pipetted off and the lower phase washed three times with a few ml of "pure solvents upper phase containing salt" (2.8g NaCl, 30 ml chloroform, 480 ml methanol and 470 ml of water). The remaining upper phase was dissolved in the lower phase by the addition of a little methanol. The lower phase contained the lipid extract and was made up to a known volume in a volumetric flask and stored in the cold.

D. Chromatographic Methods

1. Thin Layer Chromatography (T.L.C.)

(a) Neutral Lipid Separation

Glass plates of 20 X 20 cm were spread with a thin layer of silica gel G (Merck AG, Darmstadt, Germany) containing a fluorescent indicator, Ultraphor (Badische - Anilin am Rhine, Cologne, West Germany) (5 mg Ultraphor / 30g Silica Gel G). Silica thickness was made 0.25 mm or 0.5 mm with the aid of a Quickfit Spreader. Plates were activated at 110°C for 60 min prior to use. Up to 1.5 mg of total lipid was applied to the plates under a stream of nitrogen in a spot of about 0.5 cm diameter or in multiples thereof, in a streak up to 4 cm. Plates were developed by ascending chromatography in sealed glass tanks using the solvent system diethyl ether : glacial acetic acid : n-hexane 38 : 3 : 100 (v/v/v) or, in the case of the children's arteries, 38 : 3 : 125 (v/v/v). Over 20 - 25 min good separation was achieved between phospholipid which remained at the origin, cholesterol (diglyceride ran with the same Rf), free fatty acid, triglyceride, and cholesterol ester, in that order from origin to solvent front. The presence of Ultraphor allowed visualisation of the individual lipid spots under ultraviolet light. On some occasions, where no further chromatography of the individual lipids was envisaged, spots were developed in iodine vapour.

Procedures for elution of lipids from neutral lipid T.L.C. plates are described later in this thesis.

^{14}C-labelled acetate was found to have an Rf corresponding to that of cholesterol.

(b) Separation of Methyl Esters from Neutral Lipids

T.L.C. plates were prepared in the same way as those for neutral lipid separation. Aliquots of lipid extracts whose free fatty acids had been methyl esterified by the diazomethane procedure (described below) were applied to the plates under a stream of nitrogen. The solvent system diethyl ether : acetic acid : n-hexane, 30 : 2 : 180 (v/v/v) was used and methyl esters were found to run with an Rf between those of triglyceride cholesterol ester. Methyl esters were eluted from the silica with 3 lots of 6-10 ml petroleum spirit (40°C - 60°C) and set aside for gas liquid chromatography.

(c) Phospholipid Separation

In all but the ^{14}C-labelled choline experiments, phospholipid was first separated by neutral lipid T.L.C. The method of Arvidson (1967) was used to recover the phospholipids from the neutral lipid plates. The phospholipid spots were scraped into sintered funnels and eluted with 3 washes of chloroform : methanol : acetic acid : water, 50 : 39 : 1 : 10, (v/v/v/v). To the eluate, 4N ammonium hydroxide was added in a ratio of 4 : 10 (v/v), the mixture shaken and allowed to develop 2 phases. The upper phase was removed and the interface washed with a few ml of an equilibrated mixture of 4 volumes 4N ammonium hydroxide : 10 volumes eluting solvent. The upper phase was again removed and the lower phase made up to a known volume with methanol. Recovery of phospholipid by this procedure was greater than 90%. Part or whole of the phospholipid so obtained was then evaporated to dryness under nitrogen and applied to the phospholipid

plate in a small volume of chloroform : methanol 2 : 1.

In the case of ^{14}C-labelled choline experiments, lipid extracts were applied directly to phospholipid T.L.C. plates.

The phospholipid T.L.C. method of Skipski, Peterson and Barclay (1964) was used. A basic Silica Gel G slurry was made by mixing 40g Camag (without $CaSO_4$ binder) with 6 mg Ultraphor and 90 ml of 0.001M sodium bicarbonate solution. This was applied to 20 x 20 cm plates at a thickness of 0.5 mm. Plates were activated at 110°C for 1 hour prior to spotting. The plates were developed in sealed tanks by ascending chromatography for 2 hours with the solvent system chloroform : methanol : glacial acetic acid : water, 25 : 15 : 4 : 1.9 (v/v/v/v). They were then allowed to dry in air at room temperature. The individual lipid spots were visualised under ultraviolet light or shown up in a tank containing iodine vapour. From origin to solvent front, spots were lysolecithin sphingomyelin, lecithin, phosphatidyl inositol, phosphatidyl serine and phosphatidyl ethanolamine. Origin and solvent front were taken off as additional spots and, on occasions, phosphatidylinositol and phosphatidylserine were combined together. Spots of about 0.5 cm diameter accommodated up to 100 μg of each phospholipid class.

^{14}C-labelled choline was found to run with an Rf corresponding to lysolecithin and ^{32}P-labelled phosphate to smear over the whole phospholipid lane,

although most ran with an Rf corresponding to lysolecithin.

(d) Methyl Ester Separation

Methyl esters were separated into polyunsaturated, monounsaturated and saturated classes, in that order from origin to solvent front, by thin layer chromatography according to the method of Morris (1964). Methyl linolenate was usually poorly separated from the origin. Plates were prepared with Silica Gel G in the way described for neutral lipid T.L.C. Then 3% silver nitrate in 83% methanol in water was allowed to ascend the plates for about 2 - 3 hours. The silver nitrate impregnated plates were dried and activated at 110°C for 20 min. Up to 1 mg total methyl ester could be applied in a spot of about 0.5 cm diameter. Application was made in a stream of nitrogen. Plates were finally developed in sealed tanks in a solvent system of diethylether : hexane, 10 : 90 (v/v) for 20 min. Spots were visualised by spraying the plates with 0.2% dichlorofluorescein in methanol and placing under ultraviolet light.

^{14}C-labelled acetate, methyl esterified as described below, was found to remain at the origin with this T.L.C. system.

(e) Cholesterol Ester Separation

Cholesterol esters were separated, according to their degree of unsaturation, by silver nitrate T.L.C. according to the method of Morris (1964). Plates were prepared, run and visualised in the way described for methyl ester separation. Poly-

unsaturated, mono-unsaturated and saturated cholesterol esters were separated in that order from origin to solvent front with up to 1 mg total cholesterol ester per cm plate. The presence of phospholipid, triglyceride, and free cholesterol did not affect the separation of the cholesterol ester classes from one another, but did obscure the separation between the origin and polyunsaturated cholesterol esters. This is of some importance since cholesterol ester from alumina columns was applied to silver nitrate T.L.C. plates. Distribution among the cholesterol ester classes determined by T.L.C. agreed with that found by gas liquid chromatography.

2. Alumina Column Chromatography

Free and ester cholesterol were separated on alumina columns by the method described by Deykin and Goodman (1962). Plugs of glass wool were placed in the bases of modified glass burettes. Petroleum ether was then added to the columns, followed by 1 g aluminium oxide (Savoury and Moore Ltd., London, U.K. "for chromatographic analysis standardised according to Brockmann"). Lipid for separation was applied to the column in a small volume of petroleum ether. Cholesterol ester was eluted from the column with 14 ml petroleum ether : benzene, 1 : 1 (v/v) and then free cholesterol with 10 ml acetone : ether, 1 : 1 (v/v). Adequate separations were possible with up to 10 mg total cholesterol per 1 g aluminium hydroxide, even when the cholesterol was associated with other lipids as in an extract of hypercholesterolemic serum. Separation between free and ester cholesterol was checked by adding tracer amounts of ^{14}C-labelled cholesterol and ^{14}C-labelled cholesterol palmitate to extracts of hypercholesterolemic

serum; 1.1% free cholesterol was found in the cholesterol ester fraction and 2.5% cholesterol ester in the free cholesterol fraction. Cleanness of separation was also checked by running a standard hypercholesterolaemic serum extract with each batch of samples and collecting the cholesterol ester in 4 fractions and free cholesterol in 3 fractions. Column fractions were made up to known volumes and samples taken for counting, for cholesterol determination and, in the case of cholesterol ester, for silver nitrate T.L.C.

3. Paper Chromatography of Choline

The purity of the ^{14}C-labelled choline was checked periodically by paper chromatography on Whatman No. 1 paper, using the solvent system n - butanol : water : acetic acid, 120 : 50 : 30 (v/v/v). The paper was developed by ascending chromatography for 6 inches, dried in air at room temperature, cut into half inch strips and counted in Gordon's scintillator, as described below. ^{14}C-labelled choline chromatographed as a single spot with an Rf of 0.4.

4. Gas-Liquid Chromatography (G.L.C.)

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

(i) Aliquots of lipid extracts were dried down in, or T.L.C. scrapes were transferred to, 5ml ampoules for preparation of methyl esters. 1 ml of 5% sulphuric acid in methanol was added to up to 10 mg lipid and the ampoules sealed. They were then kept at 60°C for 16 hours, cooled and opened. To each ampoule, 2 ml of petroleum ether (30°C - 40°C) and 1 ml water were added and the contents mixed. The upper phase of petroleum ether was

transferred to a small stoppered tube. The addition, mixing and removal of the petroleum ether was repeated 3 times and the combined petroleum ether washings stored at 2 - 4°C. Prior to each analysis on the gas liquid chromatograph, the sample was transferred to a special tapered tube and adjusted to a suitable volume. Duplicate aliquots of 1 - 5 μL were either injected directly into the injection port, or evaporated to dryness on stainless steel pellets for solid loading. All equipment was cleaned in lipid-free solvents immediately prior to use.

(ii) Methyl Esters of Non-Esterified Fatty-Acids

Non - esterified fatty acids, together with a heptadecanoic acid internal standard, were converted to their methyl esters with diazomethane prepared as described by De Boer and Backer (1954).

The apparatus for diazomethane preparation was small - sized Quickfit glassware. A dropping funnel with stop-cock was connected by a T-piece to both a long-necked distillation flask and a condenser. The condenser ended in a tapered delivery tube beneath the surface of ether in a collecting vessel. 0.49 ml carbitol (ethyldigol or diethyleneglycol monoethylether), 0.14 ml ether, 0.083g potassium hydroxide and 0.14 ml water were warmed together in the distillation flask at 70 - 75°C with the aid of a beaker of

hot water. Once the ether started to distil, 0.3g diazald (p-tolyl sulfphonyl methyl nitrosamide) in 1.95 ml ether was released over about 15 minutes from the dropping funnel. About 1 mM diazomethane is collected in ether.

Diazomethane in ether was added to dried lipid extracts in a molar ratio of about 5:1 for methyl esterification of free fatty acid. The ether was then blown off in a stream of nitrogen and the sample taken up in petroleum ether for storage. Methyl esters were separated from other lipids by neutral lipid thin layer chromatography as described above. The selectivity of the procedure for free fatty acid was checked by processing heptadecanoic acid with various combined lipids. Only 17 : 0 peaks appeared on gas liquid chromatograms.

(b) Separation of Methyl Esters

An F & M (Hewlett Packard) 5750 Gas Chromatograph was used for separation of fatty acid methyl esters. Diethylene glycol succinate on Gas Chrom P (Applied Science Lab, State College, Pennsylvania, U.S.A.) as the stationary phase was packed into 1/8" columns. Argon was the carrier gas. Column, injection port and detector temperatures were maintained at 185°C, 290°C and 250°C respectively.

Identification of peaks was on the basis of retention times relative to methyl palmitate. For 20 : 3ω6 and 20 : 3ω9, retention times were 0.9 and

0.8 as determined from the characteristic fatty acid pattern of phospholipid from essential fatty acid deficient rats (supplied by Dr F.D. Collins, Department of Biochemistry, Melbourne University). For each methyl ester, the product of peak height and retention time was calculated and this information was used to derive the chemical percentage distribution. Quantitative results with fatty acid standards KA, KB, KC and KD from Applied Science Laboratories, State College, Pennsylvania, agreed with the stated comparison data with a relative error less than 5% for major components (> 10% of total mixture) and less than 10% for minor components (< 10% of total mixture). Maximum column loading was found to be 100 μg methyl ester fatty acid.

(c) Quantitation of Lipids

The cholesterol ester of children's arteries was assayed by G.L.C., using cholesterol heptadecanoate as an internal standard (as described in "Materials and Methods", A3 and B2). A typical gas liquid chromatogram of the cholesterol ester fatty acids in the intima under one month of age, together with the heptadecanoate internal standard is shown in Fig. 4.

(d) Gas-Liquid Radiochromatography (G.L.R.C.)

The specific activity of ^{14}C-labelled methyl ester fatty acids was determined by G.L.R.C. using an F & M (Hewlett Packard) model 5750 Gas Chromatograph modified for solid loading. Argon was the carrier gas. The gas stream was split so that one fifth went through the detector for chemical analysis

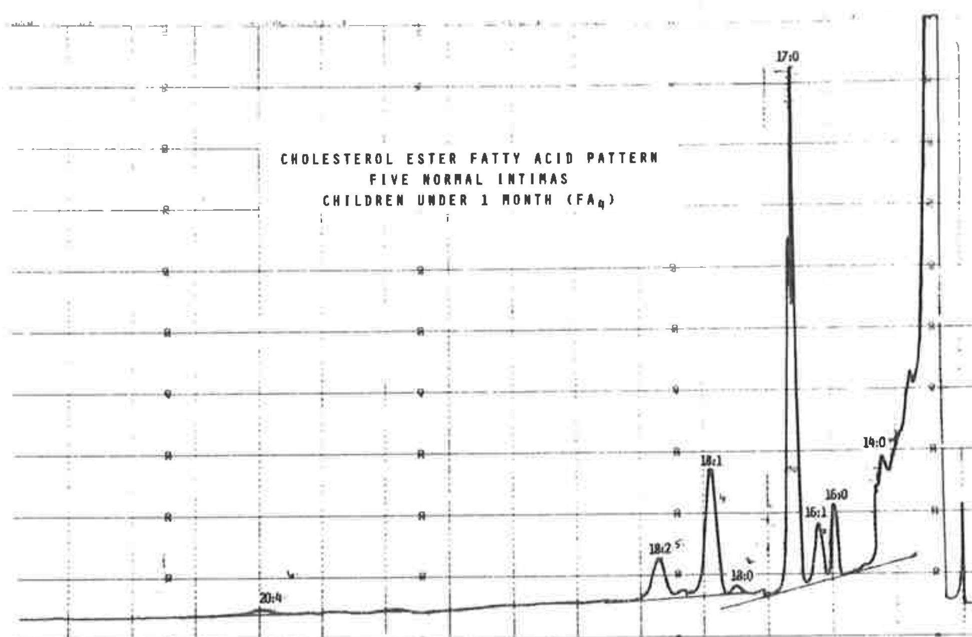

Figure 4

Gas-liquid chromatogram. Cholesterol heptadecanoate has been included as an internal standard in order to quantitate the cholesterol ester.

while the remainder was combusted to $^{14}CO_2$, and its ^{14}C content monitored with a Pye Radiochromatography Unit. In the series of experiments examining ^{14}C-labelled oleic acid metabolism in the human arterial wall some of the samples were processed on a Pye Argon Chromatograph coupled to the Radiochromatography Unit, as described by James and Piper (1961).

Fatty acid mass was measured as the product of peak height and retention time, while ^{14}C counts were recorded linearly against time by having the rate-meter in the integrating mode. Background was of the order of 20 integrated counts evolved in 1 minute. In most cases samples evolved more than 200 and up to 1000 integrated counts in 1 minute, and in no case less than 80, at flow rates of 12 - 15 ml /min for the F & M and 30 ml / min for the Pye Argon chromatograph. Duplicate determinations were routine. In the case of the human ^{14}C-labelled oleic acid experiments three samples did have counts less than twice background, but this is indicated in the relevant table. Further, two cholesterol oleate samples in the same series with low radioactivity, had their specific activities determined not by G.L.R.C., but by a combination of liquid scintillation counting of the methyl esters and G.L.C. quantitation using the internal standard cholesterol heptadecanoate. Again, this is indicated in the relevant table. Specific activities determined by the two methods agreed.

Specific activities were expressed as c.p.m./ mμmole fatty acid using methyl palmitate-1-^{14}C (35.2

c.p.m./mμmole or 0.0811 μCi/mg) as a standard. This standard yielded 700-1000 integrated counts in 1 minute. For the different series of experiments, the coefficients of variation (n=9) for methyl palmitate-1-^{14}C specific activity were 6.3, 7.0 and 7.2%.

E. Radio-Assay

All counting, apart from G.L.R.C., was by liquid scintillation in a Packard Tricarb Spectrometer Model 3375. Counts/min (c.p.m.) recorded were more than twice background. Counting was performed so that most samples were counted for a minimum of 2000 counts and in any case not less than 500 or 1000, depending on the background. Duplicate samples and duplicate counts of the one sample were routine. ^{3}H-labelled hexadecane or ^{14}C-labelled hexadecane standards were counted in the appropriate scintillator with every batch samples. Scintillators used were as follows:

(a) Snyder's Scintillator (Snyder, 1964)

^{14}C-labelled lipids, non-aqueous and solvent free, or silica scrapes from T.L.C. were counted directly. The scintillator was prepared by dissolving 10.5 g 2,5-diphenyloxazole (PPO), 0.45 g 1,4-bis-2-(4-methyl-5-phenoxazolyl)-benzene (dimethylPOPOP) and 150.0 g naphthalene in 1500 ml redistilled 1,4-dioxane. Water was added in the proportion 3 : 15 (i.e. 300 ml) to give a fully quenched solution. Up to 1 g silica /10 ml scintillator was used without quenching. Counting efficiency was 68 - 73%.

(b) Gordon's Scintillator (Gordon and Wolfe, 1960)

^{3}H and ^{14}C-labelled aqueous samples (up to 1 ml), tissue residues and paper chromatography strips were counted in

20 ml Gordon's scintillator.

40 g Cabosil (thixotropic gel powder, Packard Instrument Company, Illinois, U.S.A.) were dissolved in 385 ml 1,4 - dioxane, and this mixed with 385 ml xylene, 231 ml ethanol, 80 g naphthalene, 5 g PPO and 50 mg POPOP.

Counting efficiency was 65 - 70% for ^{14}C and 12 - 15% for ^{3}H.

(c) Toluene Scintillator

^{3}H alone, ^{3}H in association with ^{14}C, and ^{14}C in association with ^{32}P were counted in 10 ml toluene scintillator.

Labelled lipids were eluted from T.L.C. scrapes for counting. Phospholipids were eluted by the Arvidson method (1967); lipid on silver nitrate impregnated plates was eluted with chloroform : petroleum spirit 9 : 1; and other lipids were eluted with chloroform : methanol 2 : 1.

The scintillator was prepared by dissolving 4 g PPO and 100 mg dimethyl POPOP in 1 litre toluene.

^{3}H was counted with an efficiency of 33 - 35%. ^{3}H in the presence of ^{14}C was counted with an efficiency of 27% (^{14}C counted with an efficiency of 11% on the same channel) and ^{14}C, in the presence of ^{3}H, was counted with an efficiency of 50% (no ^{3}H counts were recorded). ^{14}C in the presence of ^{32}P was counted with an efficiency of 67% (13% of the ^{32}P counts were recorded in the same channel) and no ^{14}C was recorded in the ^{32}P channel.

(d) Triton : Toluene Scintillator (Patterson and Greene 1965)
As an alternative to Gordon's scintillator (see above) a 2 : 1 (v/v) toluene : Triton X-100 (Packard Instrument Company, Illinois, U.S.A.) mixture containing 4 g PPO was sometimes used. Up to 2 ml aqueous preparations could be accommodated in 15 ml without quenching. Counting efficiency for ^{3}H was 23 - 24% and for ^{14}C, 78%.

F. Chemical Assay

1. Determination of Phospholipid

Lipid phosphorus determination was based on the methods of Bartlett (1959) and Morrison (1964). Aliquots of lipid extracts were taken so that 1 - 4 µg lipid phosphorus would be assayed. Duplicate or triplicate standards were of 0, 1, 2, 3 and 4 µg inorganic phosphorus (disodium hydrogen phosphate, Analar, British Drug Houses, Poole, U.K.). To each tube 0.2 ml concentrated sulphuric acid was added and the tubes placed in a heating block at 200°C for 1 hour. Hydrogen peroxide (phosphorus-free) was then added, one drop/min up to 3 drops, until no colour remained after which the tubes were heated for a further 40 min at 200°C to decompose the hydrogen peroxide. Once cool, 4-6 ml of 0.22% ammonium molybdate was added and the tubes immediately shaken. This was followed by 0.2 ml Fiske-Sabbarow solution. The tubes, loosely stoppered, were placed in a boiling water bath for 15 min to develop the blue colour. The cooled samples were read at 820 mµ (red cell) in an Unicam SP 600 spectrophotometer. A standard curve is shown in Fig. 5.

The Fiske-Subbarow reagent was prepared by mixing (a) 0.25 g 1-amino-2-naphthol-4-sulphonic acid (Hopkin and Williams Ltd., Essex, U.K) with (b) 100 ml freshly prepared

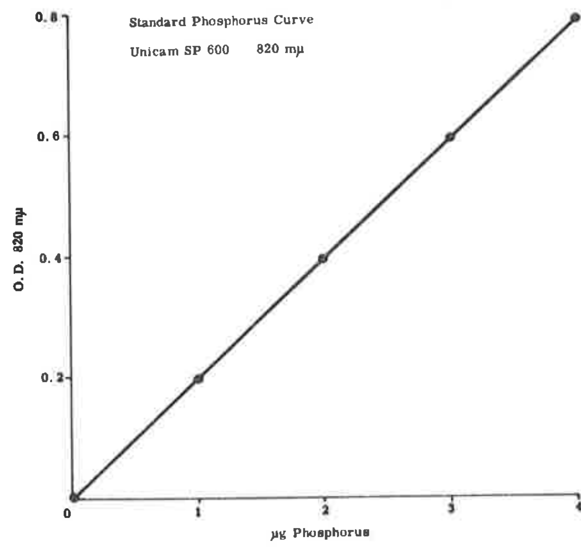

Figure 5

15% anhydrous sodium bisulfite solution and finally (c) 0.5 g anhydrous sodium sulfite. The reagent was allowed to stand for 1 hour, filtered and stored in a dark bottle at 4 - 6°C for not more than one week.

2. Determination of Cholesterol

Evaporated aliquots of lipid extracts, standards and blanks were first saponified by the method of Abell, Levy, Brodie and Kendall (1952). 5 ml of 2% potassium hydroxide in 95% ethanol was added and the stoppered test tubes heated in a water bath at 60°C for 60 mins. In this way, up to 5 mg cholesterol could be saponified. 10 ml petroleum ether (60 - 80°C) and 5 ml water were then added, with shaking. The upper phase which developed contained the cholesterol and of this a known aliquot, containing 10 - 150 μg cholesterol, was taken.

The amount of cholesterol present was determined by the method of Zlatkis, Zak and Boyle (1953). Aliquots were evaporated to dryness and taken up in 3 ml glacial acetic acid. Then, 2 ml ferric chloride colour reagent were slowly added. The colour reagent was prepared by adding 50 ml concentrated H_2SO_4 to 0.5 ml 10% $FeCl_3 \cdot 6H_2O$ in glacial acetic acid. After the reaction mixture had cooled, it was read at 550 mμ in an Unicam SP 600 spectrophotometer. The standard curve is shown in Fig. 6.

Because of the small amounts of cholesterol ester present in children's arteries and in order to obtain cholesterol ester fatty acid patterns as well, children's artery cholesterol ester was assayed by gas liquid chromatography, as described above. However, there was sufficient free cholesterol in any total tissue extract

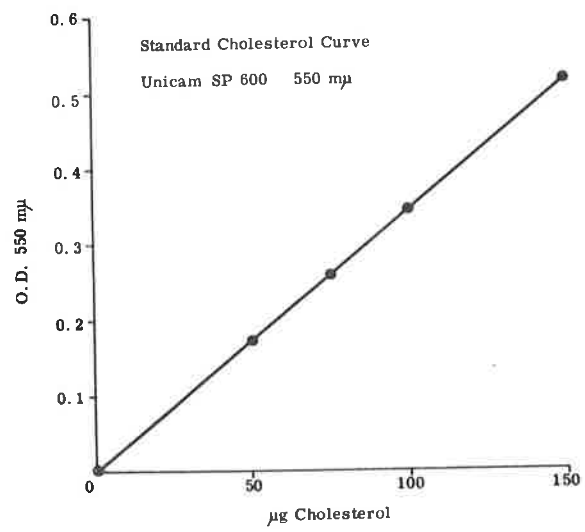

Figure 6

to assay by the method of Zlatkis, Zak and Boyle, provided it could be recovered. Thus, free cholesterol was separated from other lipids by neutral lipid thin layer chromatography and recovery monitored with a ^{14}C-labelled cholesterol internal standard, as described above.

3. Determination of Free Fatty Acid

The method of Dole (1956) was used for the determination of free fatty acid. Palmitic acid for use as a standard was obtained from Applied Science Labs, State College, Pennsylvania, U.S.A. and a 10 μEqu/ml stock solution in heptane prepared. This was diluted with heptane to a concentration of 1 μEqu/ml prior to use. Standards of 0.5, 1.0, and 2.0 μEqu were taken in triplicate and evaporated to dryness. To duplicate 1 ml samples of serum for assay, to blanks and to standards in stoppered test tubes were added 5 ml of a free fatty acid extraction mixture (40 parts isopropyl alcohol, 10 parts n-heptane, 1 part 1 N H_2SO_4). The tubes were shaken vigorously for at least 2 minutes and the mixture allowed to settle for at least 10 minutes. Then 3 ml n-heptane and 2 ml CO_2 free distilled water were added. Of the upper phases, 3 ml were transferred to other tubes.

A stock "titration mixture" of 0.1% thymol blue in water was prepared which was in turn diluted with 9 parts of redistilled ethanol to give a 0.01% thymol blue and 90% ethanol in water preparation. To each tube, 1 ml of the titration mixture was added. Nitrogen was delivered to the bottom of the tube to expel CO_2 and keep the two phases mixed. Alkali (about 0.018 N sodium hydroxide in CO_2 free distilled water) was titrated from a micro-burette at the surface of the liquid in the tube. As the end point was

LIBRARY OF THE UNIVERSITY OF ADELAIDE

approached, the gas stream was interrupted to enable examination of the colour in the lower alcoholic phase. A green-yellow end-point was observed. A typical standard curve is shown in Fig. 7.

G. Autoradiography

1. ^{14}C-labelled Oleic Acid

Arterial specimens, labelled with oleic acid-1-^{14}C, were fixed in 1% calcium chloride and 4% formaldehyde in physiological saline for at least 4 days. Sections (6μ) were cut, without prior embedding, using an International Cryostat Model C.T.I. and mounted on glass slides. Kodak AR 10 stripping film was supplied on glass plates as an emulsion layer 5 μ thick on a gelatine layer 10 μ thick. In a dark room, equipped with a 'Wratten' Series 1 (red) Safelight Filter and a 25 - watt bulb, the film was cut on the glass plates into convenient sized sections. It was then peeled off and the gelatine surface (which was in contact with the glass) placed uppermost as the film was floated in distilled water. Slides were then placed in the water and lifted up under the film which wrapped around them with the emulsion side next to the radio-active specimen. A specially made light-tight box fitted with an electric fan and air filters was used to dry the slides. Enough slides were set up in light-tight boxes so that batches could be taken off at regular intervals. The exposed slides were processed in Kodak D - 19b developer for 7 minutes, then rinsed in tap water, placed in 'Amfix' for 4 minutes, again rinsed in water and finally left to dry. Sections were stained through the film with haemalum for 5 minutes and Sudan IV for 2 hours. At all times the histological section remained in perfect register with the film.

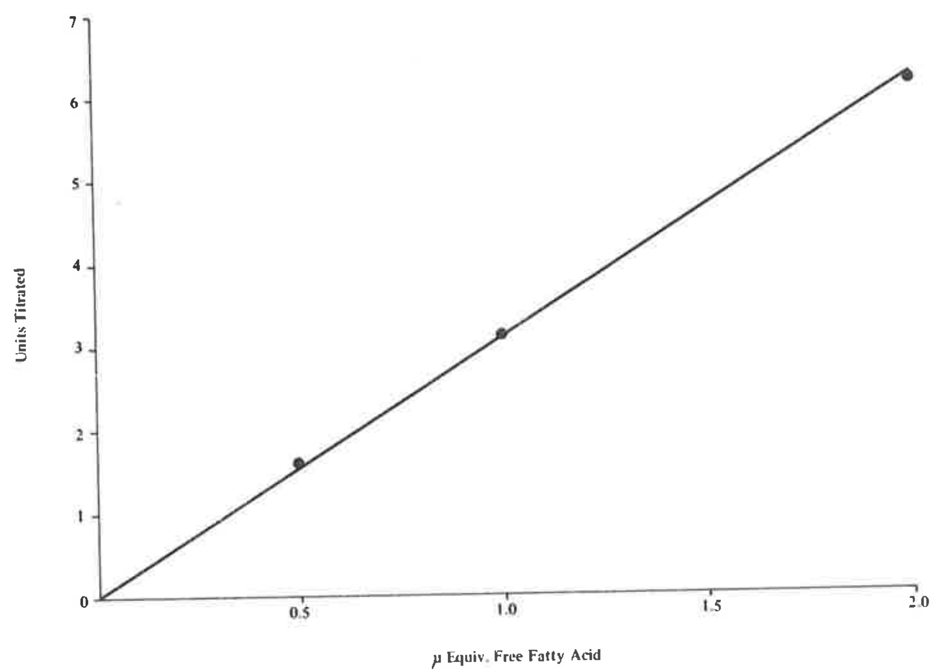

Figure 7

Exposure times varied from 1 to 6 weeks depending mainly on the radioactivity of the specimen. Grain counts were carried out at optimal exposures with the aid of a calibrated slide and eyepiece. At least $6000\mu^2$ were assessed for each feature and findings expressed as grains/$100\mu^2$.

2. <u>^3H-labelled Oleic Acid</u>

Oleic acid-9-10-^3H labelled tissues were processed in a way similar to that described for oleic acid-1-^{14}C. Arterial tissues were labelled so as to provide optimal exposures similar to those for ^{14}C. Silver grains however tended to be finer with ^3H than with ^{14}C. Sections (6μ) were mounted at both ends of slides. Those at one end were dipped into acetone at 4°C in order to remove lipids other than phospholipid. Enough slides were set up to determine, by means of thin-layer chromatography, the effect of acetone extraction on the distribution of ^3H-oleic acid among the arterial lipids. In the case of rabbit atherosclerotic aorta, the optimal time in acetone was found to be 25 min, 88.9% of the ^3H then being present as phospholipid.

3. <u>^{14}C-labelled Choline</u>

Arterial tissues labelled with choline chloride (methyl-^{14}C) were fixed in 1% calcium chloride in 4% formol saline for 4 days and then washed in running water for 24 hours to reduce the amount of non-lipid ^{14}C-labelled choline to a minimum. Tissues for radiochemical analysis were processed in the same way so that the amount of non-lipid ^{14}C-labelled choline remaining could be determined. Autoradiographs of 6μ sections were prepared and assessed in the way described for ^{14}C-labelled oleic acid. Optimal exposure times varied between 1 and 3 weeks.

H. Ultracentrifugation

Lipoproteins were separated in a Spinco Model L Preparative Ultracentrifuge with a type 40 fixed angle rotor according to the method of Havel, Eder and Bragdon (1955). Salt solution of various densities were prepared as follows:

Density	Composition
1.006	0.15M NaCl (0.9% saline)
1.350	354 g KBr and 153 g NaCl in one litre distilled water
1.210	4 parts 1.006 with 6 parts 1.350
1.063	5 parts 1.006 with 1 part 1.350
1.085	10 parts 1.006 with 3 parts 1.350
1.019	5 parts 1.006 with 1 part 1.085

Densities were checked with a pyknometer. Adjustment of non-protein solvent densities in the course of separation of serum lipoprotein fractions was made in the following way:

> 1 part of the 1.085 salt solution to 5 parts of the 1.006 infranatant gave the density 1.019, then 1 part of the salt solution 1.210 to 3 parts of the 1.019 infranatant gave the density 1.063; and finally 1.117 g KBr and 0.15 ml water to 4.5 ml of the 1.063 infranatant gave the density 1.210. Spins of serum at 40,000 r.p.m. for 16 hours at 10°C using densities of 1.006, 1.019, 1.063 and 1.21 provided the V.L.D.L. (very low density lipoprotein) fractions " < 1.006" and "1.006 - 1.019", the L.D.L. (low density lipoprotein) fraction "1.019 - 1.063" and the H.D.L. (high density lipoprotein) fraction "1.063 - 1.21".

The cellulose nitrate tubes used for centrifugation were sliced

horizontally at the end of a spin to recover the respective lipoprotein fraction.

I. Electrophoresis

Cellulose acetate strips 1" wide, with origins marked, were soaked in 0.1M barbitone acetate (Oxoid) at pH 8.6, the same buffer used in the electrophoretic chamber. They were then placed in the Shandon electrophoretic chamber. When strips were to be used for counting or stained for lipid, 5 µl hypercholesterolaemic rabbit serum were applied with the aid of a microsyringe. When strips were to be stained for protein, 2 µl were applied. Strips for both purposes were assembled side by side in the chamber. On each side of the origin, bromphenol blue was applied as a marker; it travelled 1 cm ahead of albumin. About 3 m amp/strip at a voltage of 180 - 200 volts was applied from a Gelman power supply for 2 hours, during which time albumin migrated 45 - 50 mm. Protein bands were identified with Amido Schwartz and lipid with Oil Red O. Strips were also cut into 2mm sections, the lipid extracted and counted in toluene.

J. Isolation of Human Intimal Cells

Human intimal cells were isolated by a method of enzymic disruption based on that described for the isolation of foam cells from rabbit atherosclerotic aorta by Day, Newman and Zilversmit (1966). Aortae were obtained fresh from renal transplant donors and intima stripped from underlying media. Intima so obtained was incubated with 5 ml Krebs Ringer phosphate solution containing 4% bovine albumin (Cohn fraction V, Commonwealth Serum Laboratories, Parkville, Victoria, Australia), 10 mg collagenase (Worthington Biochemical Corp., Freehold, New Jersey, U.S.A.), 5 mg elastase (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) and 15 µmoles glucose. Incubation was carried out in 20 ml plastic vials at 37°C for 2 hours

with agitation. After 1 hour the tissue was teased with fine forceps to facilitate disruption. At the end of the incubation, the digest was filtered through a gauze pad in the barrel of a plastic syringe, the residue washed with 1 - 3 ml of 4% bovine serum in Krebs Ringer phosphate and washings expressed from the pad from the syringe plunger. The washings were combined with the original filtrate. The filtrate was centrifuged at 220 g for 5 min, and the supernatant set aside. The deposited cells were washed with 0.9% saline and recentrifuged twice. The cells were then taken up in Hanks' solution containing 0.5% albumin and counted in a haemocytometer chamber. Between 0.6 and 0.8 X 10^6 cells were obtained from 1 g wet weight fibro-fatty intima.

Some of the cell suspension was dispensed into small beakers with glass cover slips at their bases and incubated at 37°C for 1 hour. Cover slips were removed, washed with saline and placed in an atmosphere of formaldehyde vapour. They were then stained with haematoxylin and Sudan IV and mounted on glass slides. This allowed an assessment of the proportion of cells which were sudanophilic and observation of their general morphology.

Aliquots of the supernatant, set aside at the time intimal cells were spun down, were either extracted by the method of Folch or centrifuged at 18,000 r.p.m. for 30 mins in a Spinco Model L Preparative Ultracentrifuge. The centrifuged deposit so obtained was referred to as "particles". Thus, in all, 4 intimal fractions came under consideration: (a) cells, (b) residue (c) supernatant and (d) the "particles" contained in the supernatant.

Figures 8 and 9 show cells isolated from human aortic intima affected with fibro-fatty atherosclerosis. The cells are all mononuclear, but vary in size and sudanophilia. The cells also tend to be more adherent to one another than cells isolated from the cholesterol-fed rabbit atherosclerotic aorta (fig. 10).

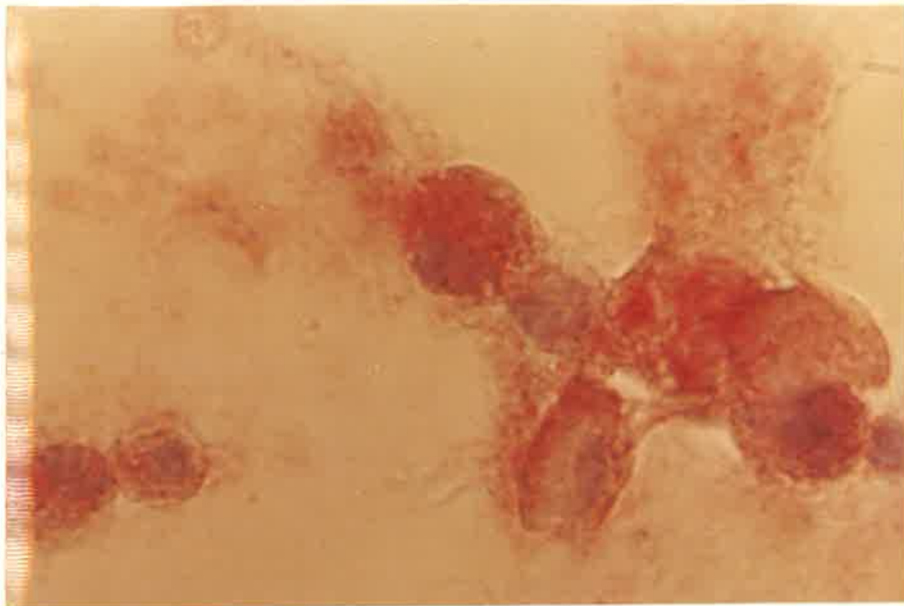

Figure 8

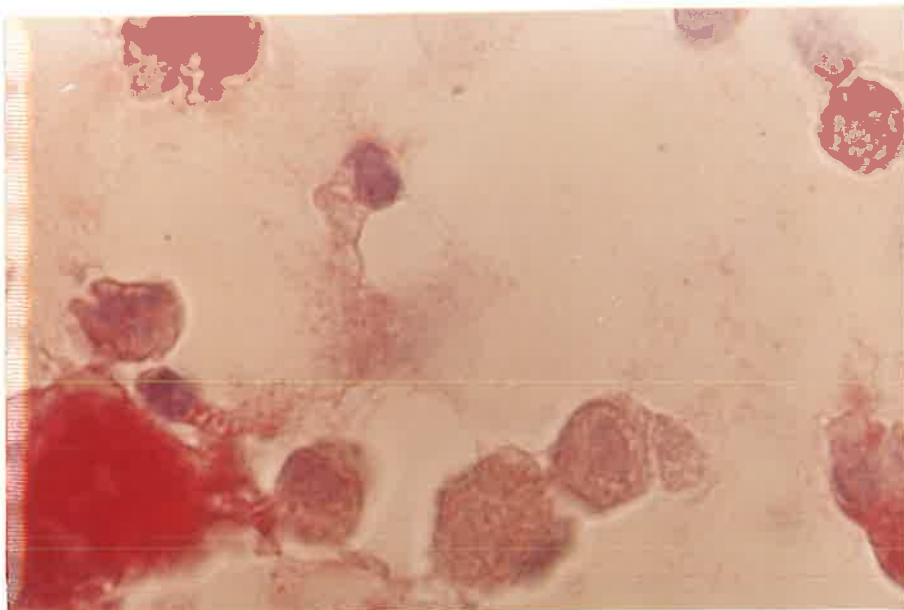

Figure 9

Figures 8 and 9

Cells isolated from human aortic intima affected with fibro-fatty atherosclerosis. Haematoxylin and Sudan IV, X 1000.

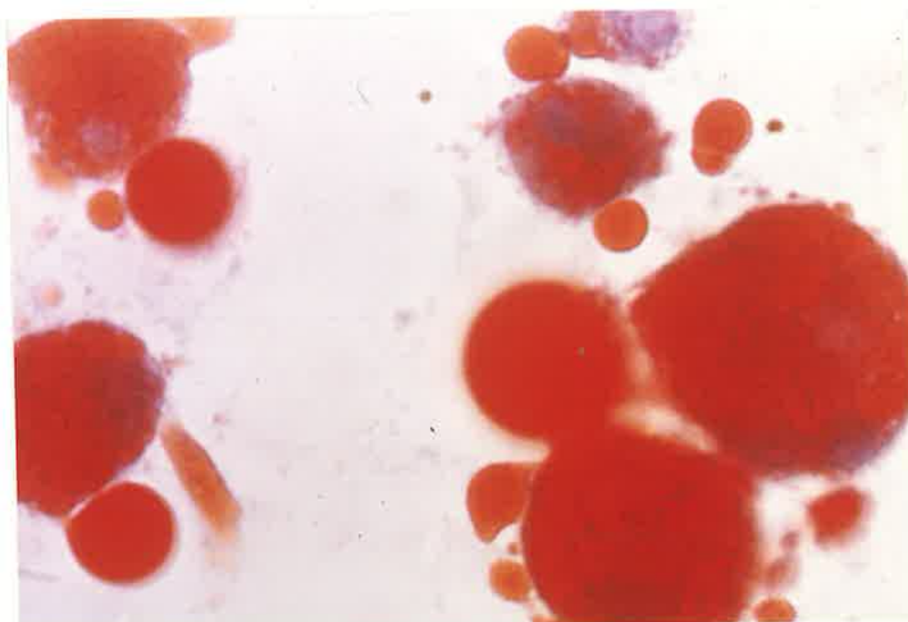

Figure 10

Cells isolated from cholesterol-fed rabbit atherosclerotic aorta. Haematoxylin and Sudan IV, X 1000.

An attempt to reduce the variability of cell type by prior removal of endothelium was not successful. The method for removal of endothelium is the one used by Dr. I. McKenzie, Department of Medicine, Royal Melbourne Hospital. Thoracic aorta was removed with the lumen intact, intercostals tied and the end clamped. The lumen was filled with 0.25% trypsin (Commonwealth Serum Laboratories, Parkville, Victoria, Australia) and incubated at 37°C for 30 mins. The luminal contents were then washed in and out with a syringe and a turbid fluid containing endothelial cells obtained. The vessel was then washed several times with 0.9% saline and processed as before.

The use of 7.5 mg / 5 ml of hyaluronidase (Sigma Chemical Co., St. Louis, Missouri, U.S.A.) during incubation of the intima increased cell yields and decreased cellular aggregation.

A metabolic study of interest using the isolation method in its original form is reported in this thesis, but until techniques are available to isolate particular cell types the method has limited application.

SECTION 1

ARTERIAL LIPID COMPOSITION AND ITS RELATIONSHIP TO SERUM LIPIDS

A. Introduction

In spite of the now considerable literature on arterial lipid composition, there is a relative paucity of information for children's arteries. Tuna and Mangold (1963) found, by viewing thin-layer chromatogram separations of major lipid classes, that there was relatively little cholesterol ester in the aorta of a six-hour-old baby. Using colorimetric methods, Scott, Florentin, Daoud, Morrison, Jones and Hutt (1966) were unable to demonstrate the presence of cholesterol ester in coronary arteries under the age of 10 years and, using a weighing procedure, Wiese, Coon, Yamanaka, Barber and Johnson (1967) were unable to quantitate it reliably in aortae under the age of 1 year. Furthermore, Wiese et al (1967) could not demonstrate the increase in aortic cholesterol ester content which they found from 10 years of age onwards, below that age. In contrast, Meyer, Meyer, Pepler and Theron (1966) have reported values for cholesterol ester, determined colorimetrically, for whole children's arteries, approaching those for free cholesterol. Hence, not only the cholesterol ester content, but also the cholesterol ester fatty acid composition, of arteries from children below the age of 10 years needed clarification.

While Scott et al (1966), Meyer et al (1966) and Wiese et al (1967) removed the adventitia from their arterial specimens, they did not attempt the separation of intima from media. This is understandable, since the intima in childhood is such a thin layer. Nevertheless, a satisfactory "inner arterial wall" preparation, consisting of intima and some inner media, can be obtained from children's arteries as described under "Materials and Methods" in this thesis, and, in the results to be reported, this is referred to as "intima"; "media", then, consists of the remaining media and adventitia with adherent fat removed.

With evidence that fatty streaks from the teens onwards have cholesterol ester fatty acid compositions different from normal intima and from the serum, evidence that fatty streaks occur in early childhood and evidence that serum and intimal fatty acid patterns change with advancing years (see "General Introduction"), it seemed important to analyse the fatty acid composition of childhood fatty streaks. Using a dissecting microscope, it has been possible to dissect macroscopic fatty streaks from the normal tissue of children's arteries. This is not to say that macroscopically normal tissue is free of excess lipid. It was hoped that even this problem could be overcome by Sudan staining of the intact artery with subsequent dissection of the sudanophilic from non-sudanophilic intima, but the staining materials seriously interfered with subsequent lipid analysis.

Aortae were collected randomly at autopsy from children without congential anomalies of their great vessels and no attempt was made to select for sex or race. As it transpired, all but one child was European and he was a Nauruan aged 9 years and 7 months.

Whatever the lipid chemistry of the arterial wall, the findings are best interpreted in the light of serum lipid chemistry. For children, serum lipid chemistry has been documented (Wiese, Bennett, Braun, Yamanaka and Coon, 1966; Zöllner, Wolfrom, Londong and Kirsch, 1966; Zee, 1967 and 1968). Nevertheless, in order to have information about the particular population whose arteries were being examined, and because of findings which emerged during the analyses of arterial tissues, serum fatty acid patterns of children in the state of Victoria, Australia, were determined.

In that the cholesterol-fed rabbit was to be used as an experimental animal its serum and intimal fatty acid patterns were

also assessed, in order to verify the findings of Swell, Law, Schools and Treadwell (1961), Zilversmit, Sweeley and Newman (1961) and Evrard, Van den Bosch, De Somer and Joossens (1962). The cholesterol ester fatty acid composition of normal rabbit intima, not reported by the above workers, has also been investigated.

B. Human Artery in Early Life

1. Morphological Considerations

Histological examination of macroscopically normal children's aorta revealed that, mostly, it was non-sudanophilic. However, occasionally, apparently extra-cellular lipid droplets were seen in the inner arterial wall (Fig. 11), as was lipid associated with cells and sudanophilic smudging along the endothelial surface (Fig. 12). Interestingly, two faintly sudanophilic round mononuclear cells can be seen on the luminal side of the endothelium in Fig. 11.

Of the 56 proximal aortic specimens processed, 11 had visible fatty streaking usually just above the aortic valves or adjacent to the origin of vessels. Where possible, a portion of the small amount of abnormal aorta was processed for histology. Figs 13 and 14 show extracellular and intracellular lipid in a fatty streak immediately above the aortic cusps in a nine-month-old infant.

2. Free and Ester Cholesterol Content

The cholesterol ester content of human intima and media from the age of a few hours to 10 years assayed against the internal standard cholesterol heptadecanoate by gas liquid chromatography, and expressed as μg cholesterol / 100 mg dry defatted tissue is shown in Table 4. For convenience, means

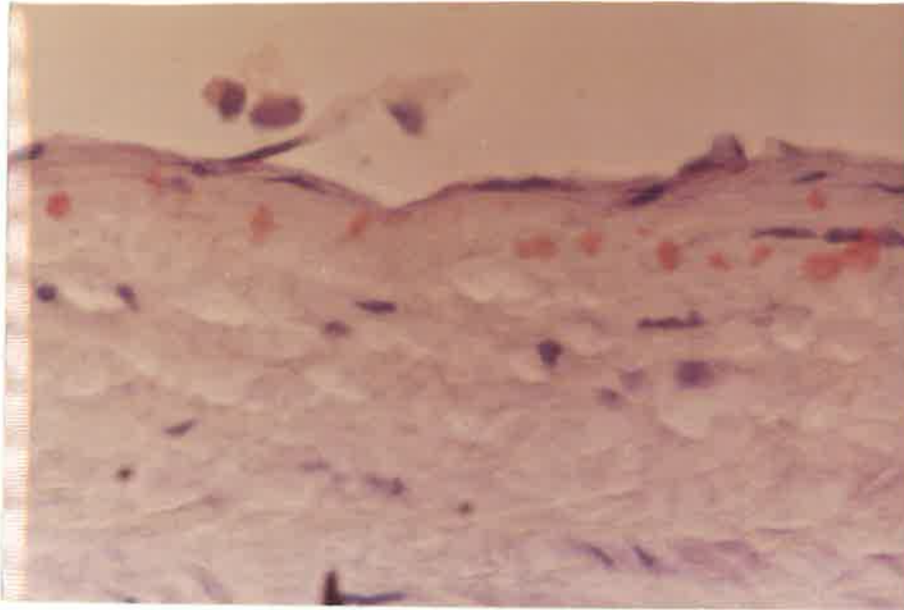

Figure 11

Proximal aorta with no visible fatty streaking, from a child aged 9 years 7 months. The inner arterial wall is sudanophilic and two luminal mononuclear cells exhibit faint sudanophilia. Haematoxylin and Sudan IV, X 600.

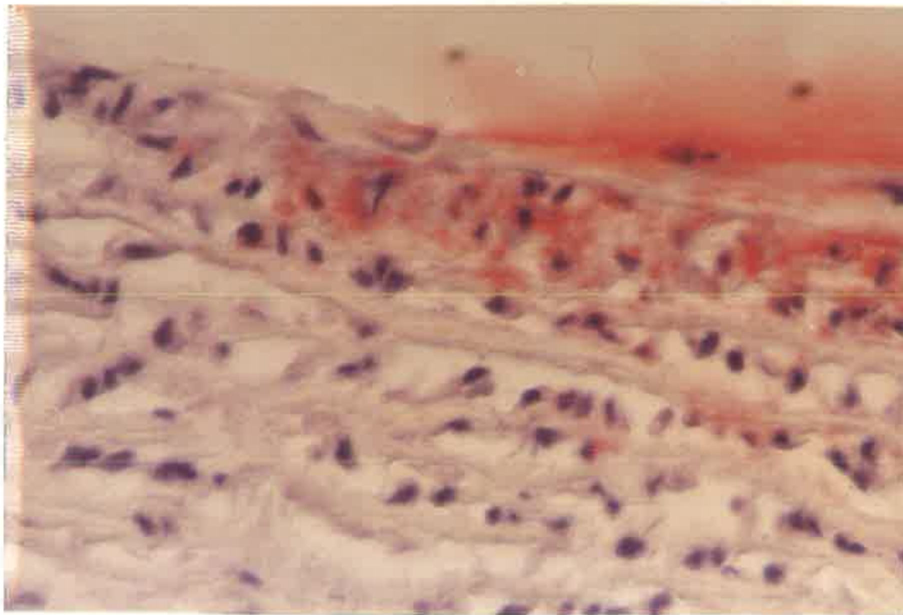

Figure 12

Proximal aorta with no visible fatty streaking, from a child aged 4½ months. There is a sudanophilic smudge along the endothelium and the inner arterial wall is sudanophilic. Haematoxylin and Sudan IV, X 600.

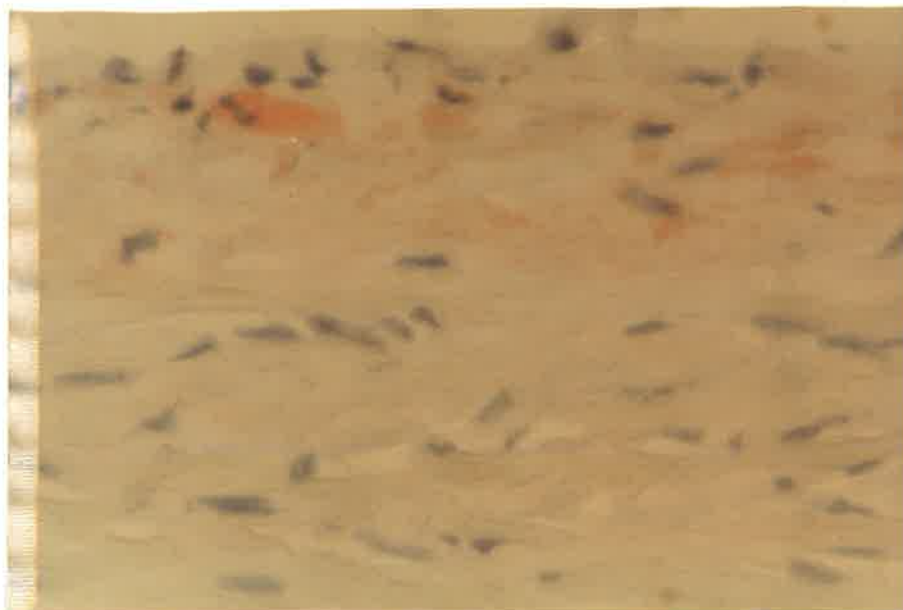

Figure 13

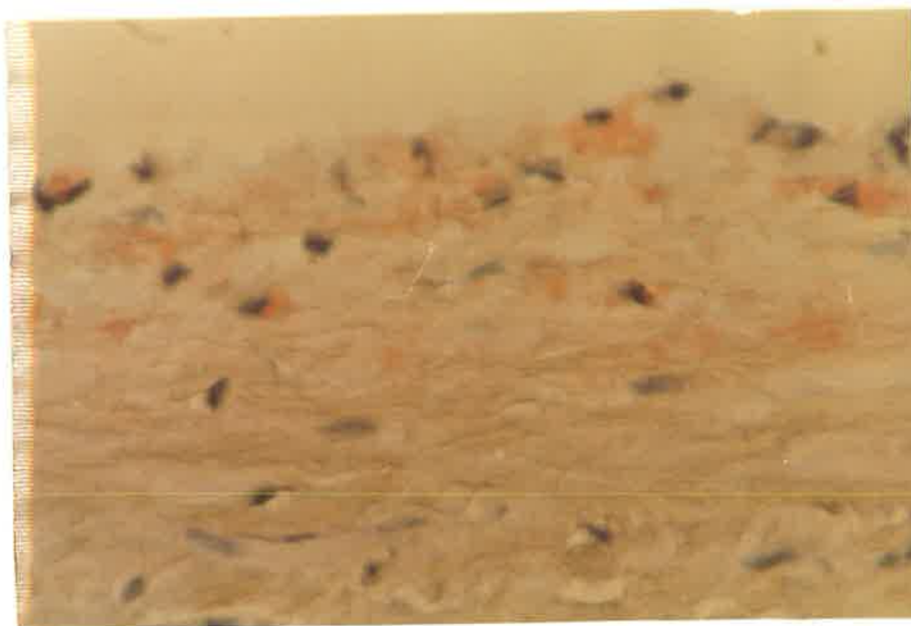

Figure 14

Figures 13 and 14

Photomicrographs of aortic fatty streaking just above the aortic cusps of a nine-month old child. Haematoxylin and Sudan IV, X 600.

TABLE 4

[a] CHOLESTEROL AND CHOLESTEROL ESTER CONTENT OF CHILDREN'S ARTERIES
(µg/100 mg dry defatted tissue)

	INTIMA								MEDIA							
	NORMAL				LESION				NORMAL				LESION			
	Free Cholesterol	Ester Cholesterol	% Total Cholesterol as Ester		Free Cholesterol	Ester Cholesterol	% Total Cholesterol as Ester		Free Cholesterol	Ester Cholesterol	% Total Cholesterol as Ester		Free Cholesterol	Ester Cholesterol	% Total Cholesterol as Ester
[b]0 - 1 month (3)	350.9 ±57.1	16.7 ±0.3	4.5 ±0.3					(3)	282.0 ±84.5	16.4 ±5.7	5.7 ±1.8				
[b]1 mth - 1 yr (3)	279.2 ±45.7	39.9 ±8.9	12.3 ±0.6	(3)	332.9 ±88.9	114.1 ±40.3	24.5 ±2.1	(3)	248.0 ±62.7	19.6 ±7.3	7.0 ±1.5	(3)	204.8 ±88.1	35.8 ±20.7	14.0 ±3.0
1 - 5 years (4)	497.8± 229.9	47.0± 20.9	10.3 ±4.3	(1)	1140.0	286.5	20.1	(3)	563.6± 341.2	116.6± 103.6	10.6 ±5.0	(1)	1246.0	323.8	20.6
5 - 10 years (5)	1159.5 ±415.7	127.0 ±27.5	15.0 ±4.5	(2)	641.9± 224.1	400.5± 231.5	35.5 ±6.7	(5)	275.5 ±53.3	43.4± 30.4	10.8 ±5.3	(3)	351.6 ±60.5	45.3 ±11.6	11.1 ±1.2
[c]Correlation Coefficient (15)	0.5966	0.7480	0.3606	(6)	0.4898	0.7445	0.6976	(14)	0.1570	0.4957	0.5983	(7)	0.1578	0.6741	-0.3182
[d]P	<0.05	<0.001	>0.1 (NS)		>0.1 (NS)	<0.1 (NS)	>0.1 (NS)		>0.1 (NS)	<0.1 (NS)	<0.05		>0.1 (NS)	<0.1 (NS)	>0.1 (NS)

Numbers of samples are shown in parenthesis.

a Ester cholesterol is expressed as cholesterol, conversion from G.L.C. data being made as though all cholesterol ester was cholesterol heptadeconoate.

b Each sample represents five pooled specimens.

c Correlation coefficients are calculated from the values of individual samples.

d NS means that age and cholesterol values are not significantly correlated.

and standard errors are given for the age groups "0 - 1 month", "1 month - 1 year", "1 - 5 years" and "5 - 10 years" and the number of samples in each group shown in parenthesis. It should be noted, however, that in the first two age groups each sample represents five pooled specimens from different children. The correlation coefficients calculated from the values of individual samples are also shown in Table 4. The possibility that any correlation between age and ester cholesterol value is due to chance is shown by "P". When P is < 0.05 a event is regarded as statistically significant. The rise in normal intimal ester cholesterol with age, shown graphically in Fig. 15, is highly significant. While there is apparently an even sharper rise in ester cholesterol in the fatty streaks with age (Table 3 and Figure 15), it is not statistically significant, perhaps because insufficient lesions were available for analysis. The media underlying normal intima or fatty streak exhibits no rise in ester cholesterol content in the first 10 years of life (Table 4 and Figure 16).

In the samples just considered free cholesterol was assayed colorimetrically using the radiotracer ^{14}C-labelled cholesterol as internal standard. There is a statistically significant rise in free cholesterol with age only in the normal intima (Table 4). The calculation of percentage of cholesterol as ester for the various tissues, also shown in Table 4, reveals that only the normal media exhibits a statistically significant rise, so that, presumably, the rises in free and ester cholesterol in normal intima with age are, in some way, related.

But that any such relationship is incomplete is demonstrated by an analysis of the significance of the

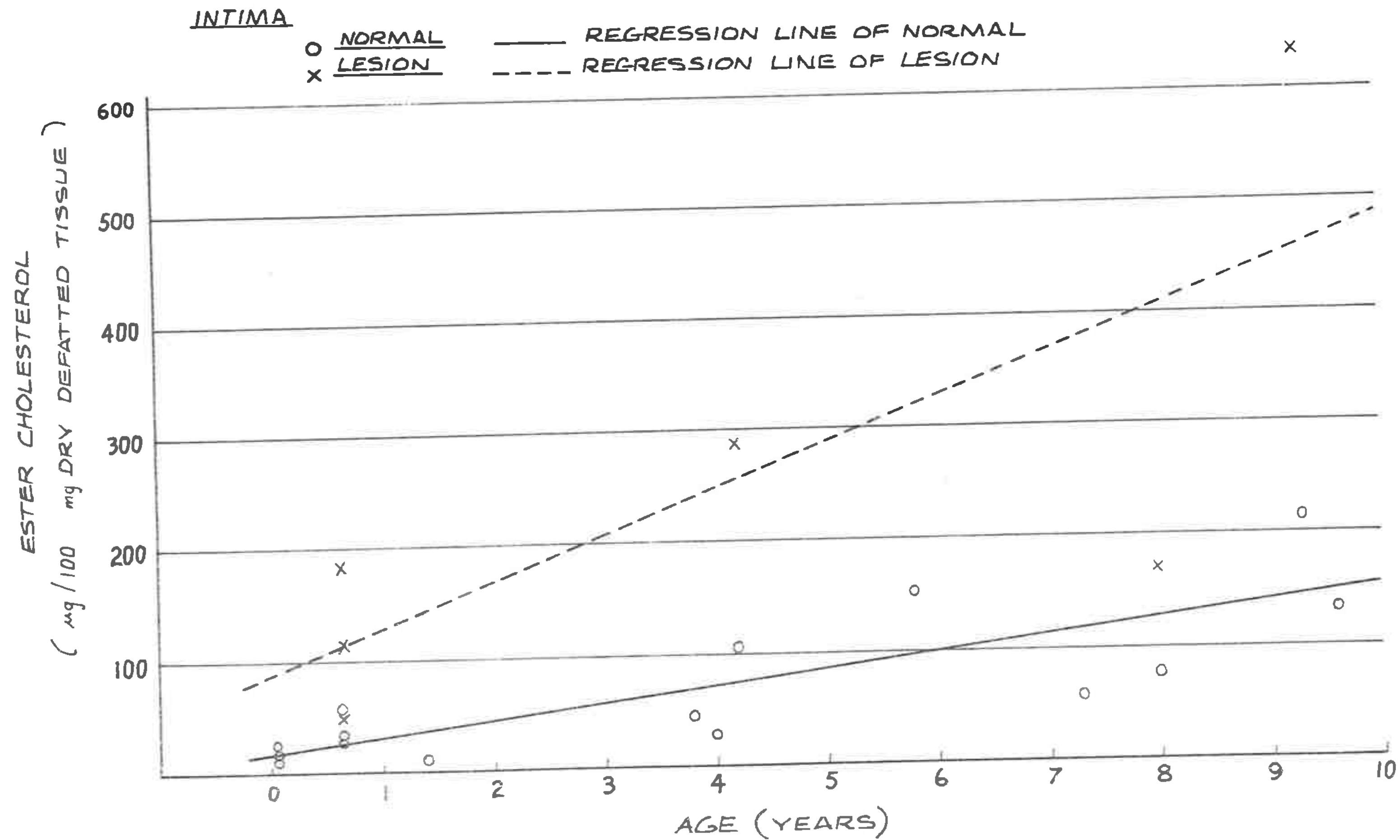

Figure 15

Ester cholesterol content of normal and atherosclerotic human aortic intima, plotted as individual samples according to age. Linear regression lines are also shown (normal, $P<0.001$; lesion, $P<0.1$)

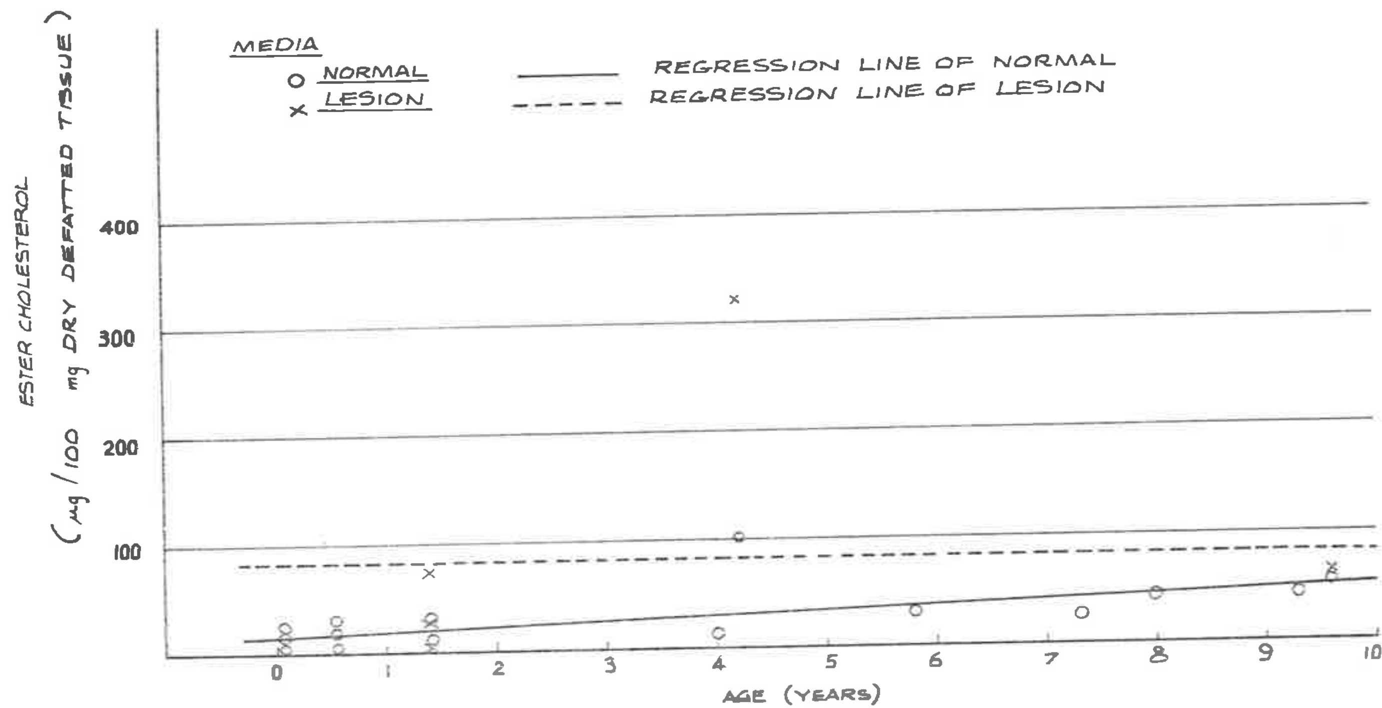

Figure 16

Ester cholesterol content of human aortic media, underlying normal and atherosclerotic intima, plotted as individual samples according to age. Linear regression lines are also shown (normal, P<0.1; lesion, P<0.1)

difference of their respective regression coefficients. Students' t is 2.3570 with 26 degrees of freedom and P is < 0.05. It is not valid to make the same comparison between normal media free and ester cholesterol since neither have correlation coefficients which are significant.

While free and ester cholesterol in the normal intima have significant correlation coefficients and in the abnormal intima they do not, it is interesting from the point of view of future investigation, to perform an analysis here. There is no significant difference between age-related changes for free cholesterol in normal and abnormal intima, but for ester cholesterol the difference between normal and abnormal intima has a significance of $P < 0.05$.

3. Cholesterol Ester Fatty Acid Composition

Aortic cholesterol ester fatty acid patterns, determined at the time of quantitation of ester cholesterol, by gas liquid chromatography are shown in Table 5 for the intima and in Table 6 for the media. The normal intima shows a significant negative correlation for cholesterol palmitate with age and significant positive correlations for cholesterol linoleate and cholesterol arachidonate with age. That the ratio of cholesterol oleate to cholesterol linoleate (18 : 1 / 18 : 2) falls significantly with age must be essentially a reflection of events for cholesterol linoleate, since cholesterol oleate shows no trend with age. Although, the absolute amount of cholesterol oleate would be rising, since the total ester cholesterol content increases with age. For the abnormal intima, trends similar to the normal intima are evident for cholesterol linoleate and the 18 : 1 / 18 : 2 ratio. Events in the normal media correspond with those in the normal intima except that the 18 : 1 / 18 : 2 ratio does not change

TABLE 5

INTIMAL CHOLESTEROL ESTER FATTY ACID PATTERNS
(% Distributions)

	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:3ω6	20:4	18:1/18:2
NORMAL										
[a] 0 - 1 month (5)	1.2 ± 1.1	29.4 ± 3.1	11.0 ± 2.2	7.3 ± 1.7	36.6 ± 2.9	9.5 ± 2.3	5.2 ± 2.2	0	0	4.90 ± 1.23
[a] 1 month - 1 year (4)	1.1 ± 1.1	20.3 ± 4.7	10.6 ± 0.9	4.2 ± 1.2	42.0 ± 3.7	12.9 ± 2.8	2.1 ± 1.2	0	1.1 ± 1.1	4.23 ± 0.90
1 - 5 years (5)	0.3 ± 0.3	19.5 ± 1.5	5.6 ± 0.5	7.5 ± 2.0	40.2 ± 2.6	20.9 ± 3.8	0	0	6.2 ± 1.5	2.39 ± 0.07
5 - 10 years (6)	0.6 ± 0.6	17.4 ± 2.0	7.5 ± 1.2	5.1 ± 0.9	37.1 ± 2.2	26.5 + 4.0	0.2 ± 0.2	0	5.5 ± 1.1	1.59 ± 0.28
[b] Correlation coefficient (20)		-0.5031	-0.3592		-0.2317	0.6939			0.6345	-0.5902
P		<0.05	>0.01 N.S.		>0.1 N.S.	<0.001			<0.01	<0.01
LESION										
1 month - 1 year (3)	0.8 ± 0.8	18.5 ± 1.6	6.9 ± 1.0	5.8 ± 1.4	50.9 ± 2.3	11.4 ± 1.3	4.1 ± 2.1	0	1.7 ± 1.7	4.62 ± 0.67
1 - 5 years (1)	0	20.2	6.3	7.1	32.4	24.5	0	0	9.8	1.32
5 - 10 years (4)	2.0 ± 2.0	18.9 ± 5.7	6.8 ± 1.8	4.5 ± 2.4	35.4 ± 4.7	25.8 ± 6.6	0.5 ± 0.5	0.7 ± 0.7	5.3 ± 1.3	1.52 ± 0.28
[b] Correlation coefficient (8)		-0.1880	-0.2590		-0.6371	0.8485			0.5885	-0.8565
[c] P		>0.1 N.S.	>0.1 N.S.		>0.1 N.S.	<0.5			>0.1 N.S.	<0.05

Numbers of samples are shown in parenthesis.

a Each sample represents five pooled specimens

b Correlation coefficients are calculated from the values of individual samples

c N.S. means age and cholesterol ester fatty acid are not significantly correlated.

TABLE 6

MEDIAL CHOLESTEROL ESTER FATTY ACID PATTERNS
(% Distributions)

	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:3ω6	20:4	18:1/18:2
NORMAL										
[a] 0 - 1 month (5)	0.8 ± 0.5	23.4 ± 1.7	11.4 ± 1.3	3.5 ± 0.2	39.9 ± 1.0	11.8 ± 2.2	0 ± 0	0 ± 0	9.3 ± 1.6	3.92 ± 0.75
[a] 1 month - 1 year (3)	0.7 ± 0.7	19.0 ± 1.3	9.8 ± 1.0	3.6 ± 0.7	48.8 ± 4.0	14.1 ± 2.3	0	0	4.1 ± 0.6	3.76 ± 0.93
1 - 5 years (5)	0.5 ± 0.5	24.3 ± 1.7	6.8 ± 1.8	9.4 ± 1.0	41.0 ± 3.8	13.9 ± 4.0	0	0	4.4 ± 1.0	5.44 ± 2.73
5 - 10 years (6)	0.3 ± 0.3	17.2 ± 1.0	6.3 ± 0.3	5.4 ± 1.7	37.4 ± 1.3	27.5 ± 3.0	0.1 ± 0.1	0	6.2 ± 0.9	1.47 ± 0.21
[b] Correlation Coefficient (19)		-0.4915	-0.5044		0.3824	0.6692			-0.1757	-0.3346
P		<0.05	<0.05		>0.1 N.S.	<0.1			>0.1 N.S.	>0.1 N.S.
LESION										
1 month - 1 year (3)	0	22.9 ± 9.4	6.5 ± 0.5	5.5 ± 1.3	49.2 ± 2.1	11.9 ± 2.0	0	0	4.3 ± 0.5	4.60 ± 1.03
1 - 5 years (1)	0	15.3	5.4	3.6	34.1	32.7	0	0	9.3	1.04
5 - 10 years (4)	0.8 ± 0.7	17.4 ± 1.1	6.0 ± 0.5	4.5 ± 0.6	38.7 ± 2.6	28.3 ± 2.9	0.1 ± 0.1	0.4 ± 0.4	4.3 ± 1.0	1.45 ± 0.26
[b] Correlation Coefficient (8)		-0.4623	-0.2882		-0.5618	0.6943			-0.0590	-0.7127
[c] P		>0.1 N.S.	>0.1 N.S.		>0.1 N.S.	<0.1 N.S.			>0.1 N.S.	<0.1 N.S.

Numbers of samples are shown in parenthesis.

a Each sample represents five pooled specimens

b Correlation coefficients are calculated from the values of individual samples

c N.S. means age and cholesterol ester fatty acid are not significantly correlated

significantly. There is no significant change with age in cholesterol ester fatty acid patterns in the media underlying fatty streaks.

What is of particular interest is that, for those cholesterol ester fatty acid changes which are significantly correlated with age (cholesterol linoleate and 18 : 1 / 18 : 2), there are no significant differences between the two corresponding regression coefficients for normal and abnormal intima, as determined by Student's t test.

This point is borne out by Fig. 17 in which each normal intimal sample with a lesion to correspond has its cholesterol palmitate (16:0), oleate (18:1) and linoleate (18:2) plotted together. There is close correspondence between the normal and abnormal values.

No cholesterol ester of the 20:3ω9 type was found in any of the arterial tissues.

In order to understand better the cholesterol ester fatty acids changes, these were also assessed in the serum of 55 children and young adults drawn from the same population as the arteries. Again, for convenience, these data have been arranged as means with standard errors of means for the ages "0" (umbilical cord blood), "1 day - 1 year", "1 - 5 years", "5 - 15 years" and "15 - 30 years" and correlation coefficients computed for individual fatty acids. This information is given in Table 7. With age, there are significant falls in the percentage of cholesterol ester as palmitate, palmitoleate, oleate and arachidonate and a significant rise in cholesterol linoleate. The 18 : 1 / 18 : 2 ratio falls significantly. So that, with the

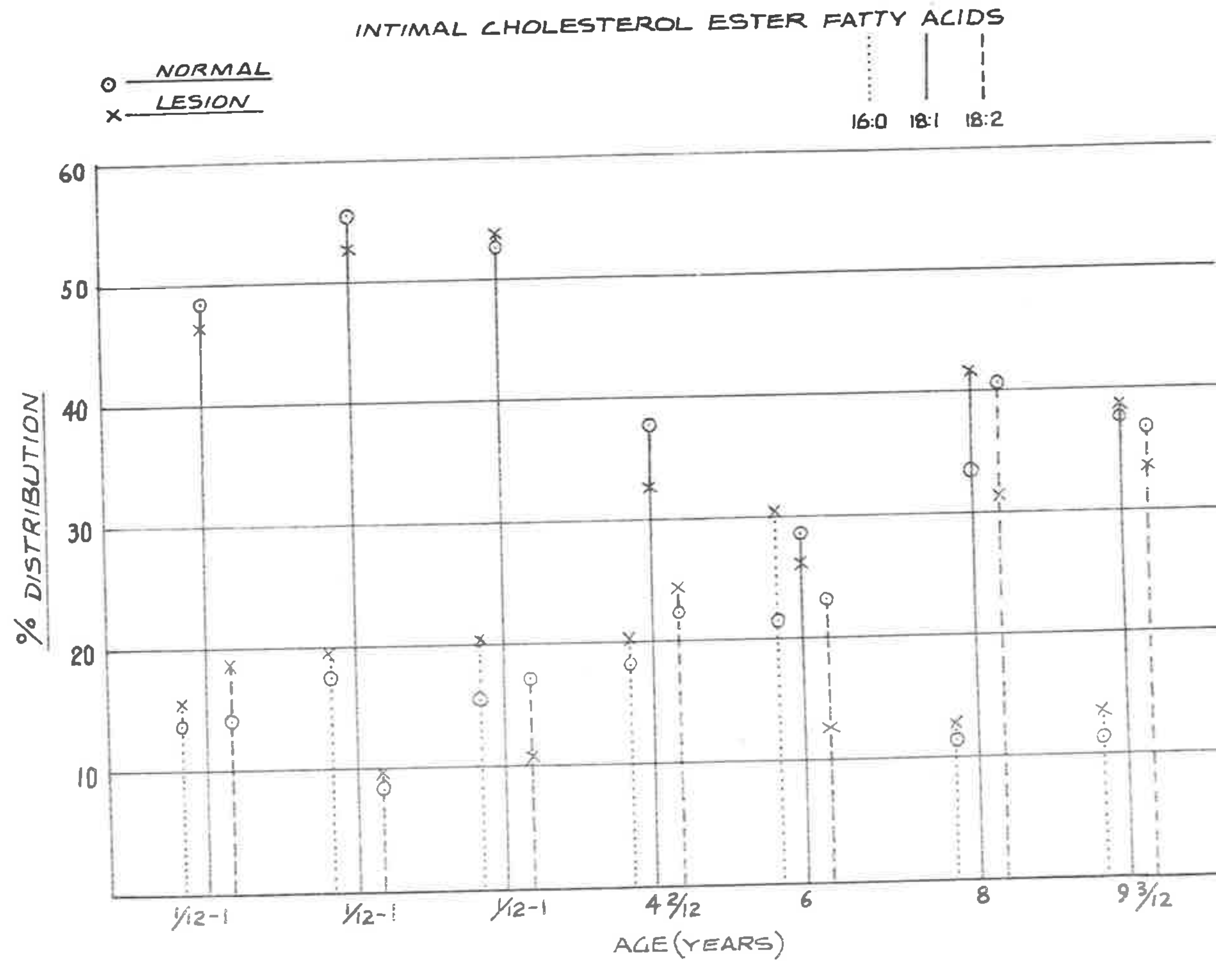

Figure 17

A comparison of the cholesterol palmitate, oleate and linoleate compositions of normal and atherosclerotic human aortic intimae at various ages.

TABLE 7

HUMAN SERUM CHOLESTEROL ESTER FATTY ACID PATTERNS
(% Distributions)

	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:3ω9	20:3ω6	20:4	18:1/18:2
Umbilical (10)	0 ±0	20.0±0.5	11.3±0.6	3.6±0.3	40.3±1.9	13.2±1.0	0.2±0.1	0 ±0	0 ±0	0 ±0	11.7±1.5	3.27±0.41
1 Day - 1 Year (10)	1.1±0.2	18.0±1.4	12.2±1.0	2.0±0.4	42.5±2.3	17.9±3.1	0.8±0.2	0.7±0.2	0 ±0	0 ±0	5.7±0.9	3.24±0.60
1 - 5 Years (17)	0.8±0.1	14.2±0.7	6.0±0.4	2.0±0.2	35.2±1.4	35.1±2.4	1.1±0.2	0.7±0.1	0 ±0	0.1±0.1	6.4±0.5	1.12±0.13
5 - 15 Years (9)	0.9±0.1	12.7±0.7	4.8±0.3	1.3±0.2	31.1±1.3	42.7±2.0	1.3±0.2	0.7±0.2	0 ±0	0.2±0.1	5.4±0.6	0.75±0.07
15 - 10 Years (9)	0.5±0.0	12.0±0.3	4.7±0.6	1.3±0.1	31.9±1.5	43.8±1.9	1.0±0.3	0.7±0.1	0 ±0	0.1±0.1	5.5±0.4	0.75±0.08
[a] Correlation Coefficient (55)		-0.5161	-0.5606		-0.4292	0.6125					-0.2678	-0.4815
P		<0.001	<0.001		<0.01	<0.001					<0.05	<0.001

Figures in parenthesis are numbers of samples in each group.

a Correlation coefficients are calculated from the values of individual samples.

exception of cholesterol arachidonate which moves in opposite directions in normal intima and serum, the direction of significant cholesterol ester fatty acid changes in the aorta mirror those in the serum. For the percentage cholesterol oleate, there is no significant age-related change in the aorta, but there is in the serum.

The trends for the serum and normal intimal cholesterol ester fatty acids, palmitic, oleic, linoleic and arachidonic are evident from Fig. 18 where both individual sample values and linear regression lines have been plotted. In actual fact, peak changes in serum cholesterol ester fatty acids seem to occur at about 2 years of age. Those regression lines which show a significant correlation between a parameter of interest and age can be determined by referring to Tables 5 and 7. For both serum and normal intima, cholesterol palmitate and cholesterol linoleate are significantly correlated with age and the differences between their serum and intimal regression coefficients have P values of < 0.01 and < 0.05 respectively. The 18 : 1 / 18 : 2 ratios of normal intima and serum also have regression coefficients significantly different ($P < 0.05$).

4. Phospholipid Content

Lipid phosphorus was determined colorimetrically after quantitative recovery from neutral - lipid T.L.C. plates. Results are expressed, however, as µg phospholipid / 100mg dry defatted tissue since the correction factor, 25, was applied to lipid phosphorus values. In Table 8, the children's arteries have been grouped for phospholipid content in the same way as for free and ester cholesterol content and the correlation coefficients and P values are also shown. There is generally considerable variation within groups and the

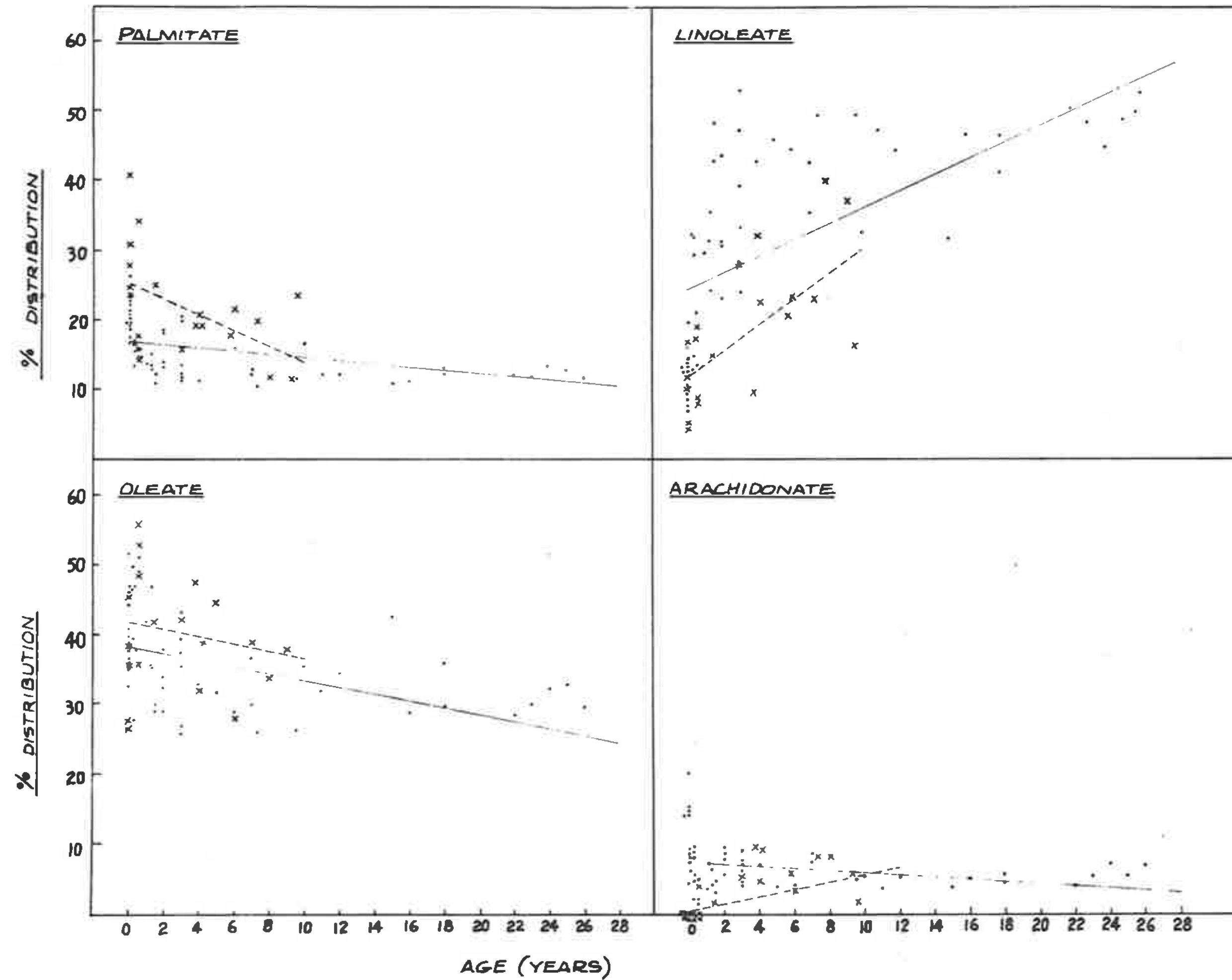

Figure 18

Cholesterol ester fatty acid compositions of serum and normal intima, plotted as individual samples according to age. Linear regression lines are also shown (P values are shown in tables 5 and 7)

TABLE 8

PHOSPHOLIPID CONTENT OF CHILDREN'S ARTERIES
(μg/100 mg dry defatted weight)

		INTIMA				MEDIA		
		NORMAL		LESION		NORMAL		LESION
[a] 0 - 1 month	(3)	840 ± 252			(3)	1100 ± 318		
[a] 1 month - 1 year	(3)	1201 ± 1037	(3)	1765 ± 632	(3)	953 ± 360	(3)	1339 ± 844
1 - 5 years	(4)	1129 ± 211	(1)	909	(4)	718 ± 368	(1)	280
5 - 10 years	(5)	1310 ± 505	(3)	1148 ± 244	(5)	386 ± 113	(3)	784 ± 86
[b] Correlation Coefficient	(15)	0.2282	(7)	-0.3593	(15)	-0.5309	(7)	-0.2749
[c] P		>0.1 N.S.		>0.1 N.S.		<0.05		>0.1 N.S.

Numbers of samples are shown in parenthesis.
Values shown are as phospholipid and not as lipid phosphorus

a Each sample represents five pooled specimens

b Correlation coefficients are calculated from the values of individual samples

c N.S. means that age and phospholipid values are not significantly correlated

only significant correlation with age is a negative one for the phospholipid of the normal media.

5. Phospholipid Fatty Acid Composition

The phospholipid fatty acid patterns, with correlation coefficients and P values where relevant, are shown for the intima in Table 9 and for the media in Table 10.

Values for both the 20:3ω9 and 20:3ω6 isomers of eicosatrienoic acid are shown because of the possibility of essential fatty acid deficiency in the arterial wall. However, no more than an occasional trace of 20:3ω9 was found and this is reflected in the 20:3ω9 / 20:4 ratios. There is certainly no relationship evident between this ratio and age, neither is there any difference between the ratio in normal and abnormal tissue.

No significant correlation between age and phospholipid fatty acids of normal intima was revealed. For the abnormal intima, however, linoleic acid increased with age and the 18 : 1 / 18 : 2 ratio fell. In the normal media, palmitic acid fell with age.

Differences in the behaviour of the phospholipid fatty acids, palmitic (16:0), oleic (18:1), linoleic (18:2) and arachidonic (20:4) between normal and abnormal intima, plotted for individual samples in Fig. 19, are scarcely evident, however. An analysis of regression coefficients, although invalid because of correlation coefficients not being statistically significant, also revealed no difference between the behaviour of phospholipid fatty acids in normal and abnormal intima.

TABLE 9

NTIMAL PHOSPHOLIPID FATTY ACID PATTERNS
% Distributions)

	16:0	16:1	18:0	18:1	18:2	20:3ω9	20:3ω6	20:4	18:1/18:2	20:3ω9/20:4
					NORMAL					
0 - 1 month (3)	26.0 ± 1.5	3.1 ± 0.6	20.8 ± 1.0	20.6 ± 0.3	2.2 ± 0.7	0	4.5 ± 0.6	21.7 ± 0.9	12.2 ± 4.4	0
1 mth - 1 yr (3)	23.2 ± 0.6	2.0 ± 0.2	22.3 ± 1.1	20.8 ± 0.6	3.7 ± 1.5	0.5 ± 0.5	5.0 ± 0.8	22.6 ± 1.9	7.2 ± 2.1	0.020 ± 0.020
1 - 5 years (4)	25.1 ± 0.4	2.1 ± 0.1	22.5 ± 0.7	22.2 ± 1.6	3.7 ± 0.3	0	3.1 ± 0.5	21.6 ± 2.0	6.1 ± 0.7	0
5 - 10 years (6)	26.7 ± 1.9	2.6 ± 0.7	21.3 ± 0.7	21.6 ± 1.6	4.3 ± 0.2	0	2.4 ± 0.6	21.4 ± 3.0	5.2 ± 0.5	0
Correlation Coefficient (16)	0.3858			0.3812	0.4219			-0.2949	0.4230	
P	>0.1 N.S.			>0.1 N.S.	>0.1 N.S.			>0.1 N.S.	>0.1 N.S.	
					LESION					
1 mth - 1 yr (3)	25.8 ± 3.4	1.8 ± 0.2	24.5 ± 1.6	22.7 ± 1.4	2.4 ± 0.2	0	2.3 ± 0.6	20.7 ± 4.5	9.5 ± 0.7	
1 - 5 years (1)	25.3	3.9	19.8	20.6	4.6	0	1.2	24.7	4.5	
5 - 10 years (4)	27.8 ± 2.2	1.9 ± 0.2	21.6 ± 0.6	21.0 ± 1.3	5.4 ± 0.4	0	3.1 ± 0.9	19.3 ± 2.3	3.9 ± 0.1	
Correlation Coefficient (8)	0.2398			-0.2745	0.9491			-0.1637	-0.9109	
P	>0.1 N.S.			>0.1 N.S.	<0.001			>0.1 N.S.	>0.01	

Numbers of samples are shown in parenthesis

a Each sample represents five pooled specimens

b Correlation coefficients are calculated from the values of individual samples

c N.S. means age and phospholipid fatty acid are not significantly correlated

TABLE 10

:DIAL PHOSPHOLIPID FATTY ACID PATTERNS
; Distributions)

	16:0	16:1	18:0	18:1	18:2	20:3ω9	20:3ω6	20:4	18:1/18:2	20:3ω9/20:4
					NORMAL					
0 - 1 month (3)	26.6 ± 2.1	2.3 ± 0.2	22.0 ± 1.2	22.0 ± 0.6	2.1 ± 0.2	0	2.8 ± 0.3	22.4 ± 3.2	10.7 ± 1.3	0
1 mth - 1 yr (3)	25.3 ± 0.8	2.3 ± 0.3	20.8 ± 0.7	24.6 ± 0.9	3.7 ± 1.4	0.4 ± 0.4	2.6 ± 0.2	20.4 ± 1.4	8.4 ± 2.5	0.019 ± 0.019
1 - 5 years (4)	23.9 ± 0.9	1.7 ± 0.2	23.7 ± 1.9	23.9 ± 1.3	4.1 ± 0.4	0.3 ± 0.3	2.6 ± 0.6	20.8 ± 2.4	6.0 ± 1.8	0.012 ± 0.012
5 - 10 years (6)	22.7 ± 1.8	1.9 ± 0.4	22.7 ± 1.2	21.8 ± 1.4	4.1 ± 0.6	0	2.7 ± 0.6	22.0 ± 3.6	6.0 ± 1.0	0
Correlation Coefficient (16)	-0.5080			-0.4277	0.3491			-0.0610	-0.4631	
P	<0.05			<0.1 N.S.	>0.1 N.S.			>0.1 N.S.	<0.1 N.S.	
					LESION					
1 mth - 1 yr (3)	21.7 ± 0.5	1.6 ± 0.3	22.6 ± 1.2	25.7 ± 1.7	3.3 ± 0.4	0.5 ± 0.5	2.7 ± 0.1	22.1 ± 0.8	8.3 ± 0.6	0.020 ± 0.020
1 - 5 years (1)	31.3	8.1	18.2	21.8	5.3	0	1.5	13.9	4.1	0
5 - 10 years (4)	25.5 ± 2.1	2.4 ± 0.8	21.7 ± 0.9	24.8 ± 3.9	3.7 ± 0.4	0	2.9 ± 0.9	18.8 ± 4.9	7.2 ± 1.8	0
Correlation Coefficient (8)	0.4259			0.1120	0.0973			-0.2879	0.1338	
P	>0.1 N.S.			>0.1 N.S.	>0.1 N.S.			>0.1 N.S.	>0.1 N.S.	

Numbers of samples are shown in parenthesis.

a Each sample represents five pooled specimens

b Correlation coefficients are calculated from the values of individual samples

c N.S. means age and phospholipid fatty acid are not significantly correlated

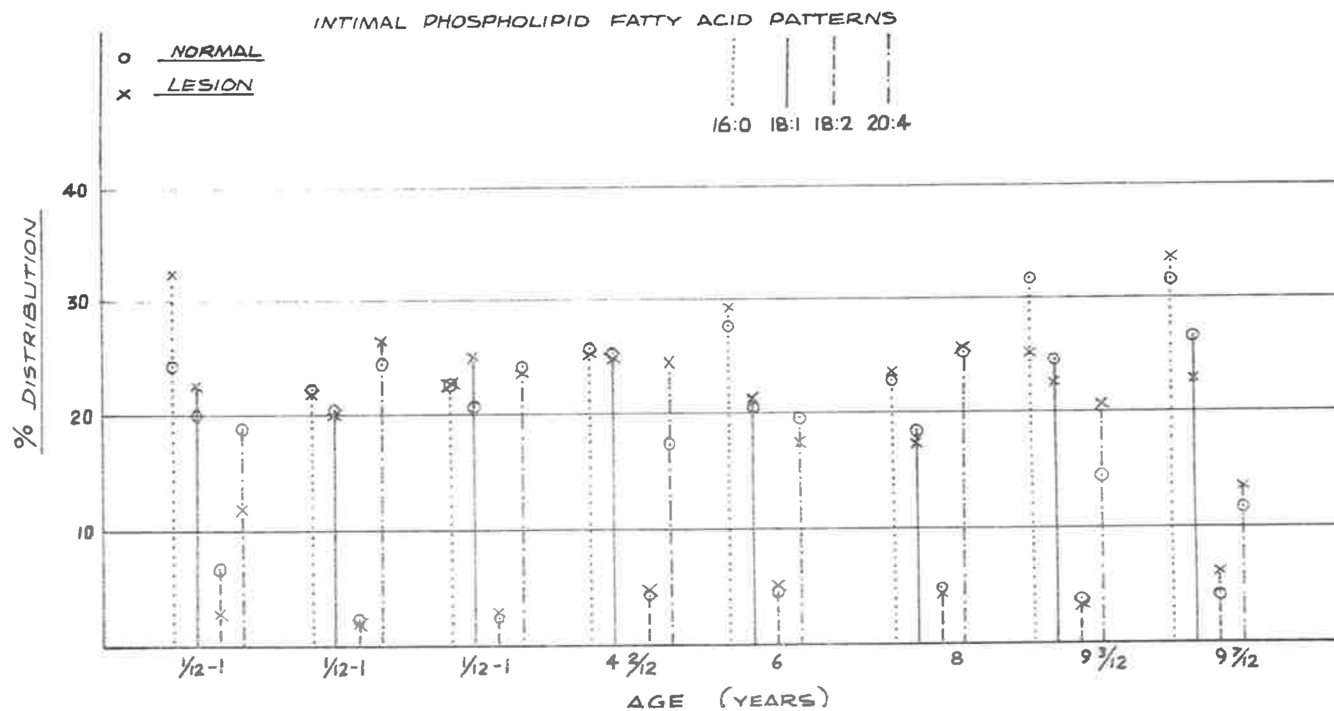

Figure 19

A comparison of the phospholipid fatty acid compositions of normal and atherosclerotic human aortic intimae at various ages.

Serum phospholipid fatty acids (Table 11) show a negative correlation with age in the case of arachidonic acid and a positive correlation in the case of linoleic acid. The 18 : 1 / 18 : 2 ratio falls significantly with age.

With few significant age-related changes in phospholipid fatty acids of normal intima and of serum, there is little room for a valid comparison of events. Even so, normal intimal and serum phospholipid palmitic, oleic, linoleic and arachidonic acids percentage distribution have been plotted as individual samples, and as linear regression lines (Fig. 20). There is, in fact, no significant difference in the behaviour of the normal intimal and serum phospholipid fatty acids depicted in Fig. 20. The 18 : 1 / 18 : 2 ratios, nonetheless, do differ significantly ($P < 0.01$).

C. Normal and Cholesterol - Fed Rabbit

The serum cholesterol ester fatty acid patterns of five rabbits fed cholesterol for 3 months and of their controls are shown in Table 13. There is relatively more cholesterol oleate and less cholesterol linoleate in the serum from the cholesterol-fed animals. In the same table, the cholesterol ester fatty acid patterns of intimae from three rabbits, fed cholesterol for the same period as rabbits for serum analysis, and their normal controls are shown. There is relatively more cholesterol oleate in the intimae from the cholesterol-fed animals than in the intimae from the normal animals, but no significant difference in the representation of cholesterol linoleate. A comparison of serum and intimal cholesterol ester fatty acids from the same cholesterol-fed rabbits is reported in Section 7 of this thesis (Table 44). The finding of a higher proportion of cholesterol oleate in the atherosclerotic intima than in the hypercholesterolaemic serum is

TABLE 11

HUMAN SERUM PHOSPHOLIPID FATTY ACID PATTERNS
(% Distributions)

	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:3ω9	20:3ω6	20:4	20:4	18:1/ 18:2	20:3ω9/ 20:4
Umbilical (10)	0.3±0.1	29.0±0.8	2.0±0.2	16.7±0.6	14.3±0.9	6.6±0.5	0.3±0.1	0 ±0	1.5±0.3	5.1±0.3	20.1±1.1	4.8±0.7	2.30±0.23	0.083± 0.018
1 Day - 1 Year (10)	0.1±0.1	33.9±2.0	3.1±0.2	15.9±0.6	23.3±1.8	8.2±1.1	0.8±0.1	0.1±0.1	1.1±0.4	3.1±0.3	8.7±1.4	1.7±0.4	3.32±0.44	0.166± 0.079
1 - 5 Years (17)	0.2±0.1	32.3±0.7	2.5±0.2	15.8±0.5	19.5±0.8	13.6±0.9	0.7±0.1	0.2±0.1	0.4±0.1	3.6±0.2	10.5±0.8	1.6±0.5	1.62±0.19	0.042± 0.012
5 - 15 Years (9)	0.3±0.1	30.8±0.9	2.0±0.3	16.5±0.7	18.6±1.8	18.6±1.2	0.5±0.0	0.3±0.1	0.3±0.1	4.0±0.6	9.0±0.8	0.6±0.3	0.90±0.11	0.029± 0.010
15 - 30 Years (9)	0.4±0.1	30.7±0.7	1.7±0.2	16.5±0.7	17.8±1.0	19.8±0.9	0.6±0.1	0.5±0.1	0.2±0.1	3.7±0.3	9.1±0.6	0.5±0.3	0.89±0.10	0.023± 0.018
[a] Correlation Coefficient (55)		-0.0796			-0.0977	0.6817					-0.2993		-0.2541	-0.5038
[b] P		>0.1 N.S.			>0.1 N.S.	<0.001					<0.05		<0.001	<0.1 N.S.

Figures in parenthesis are numbers of samples in each group.

a Correlation coefficients are calculated from the values of individual samples

b N.S. means that age and particular phospholipid fatty acid are not significantly correlated

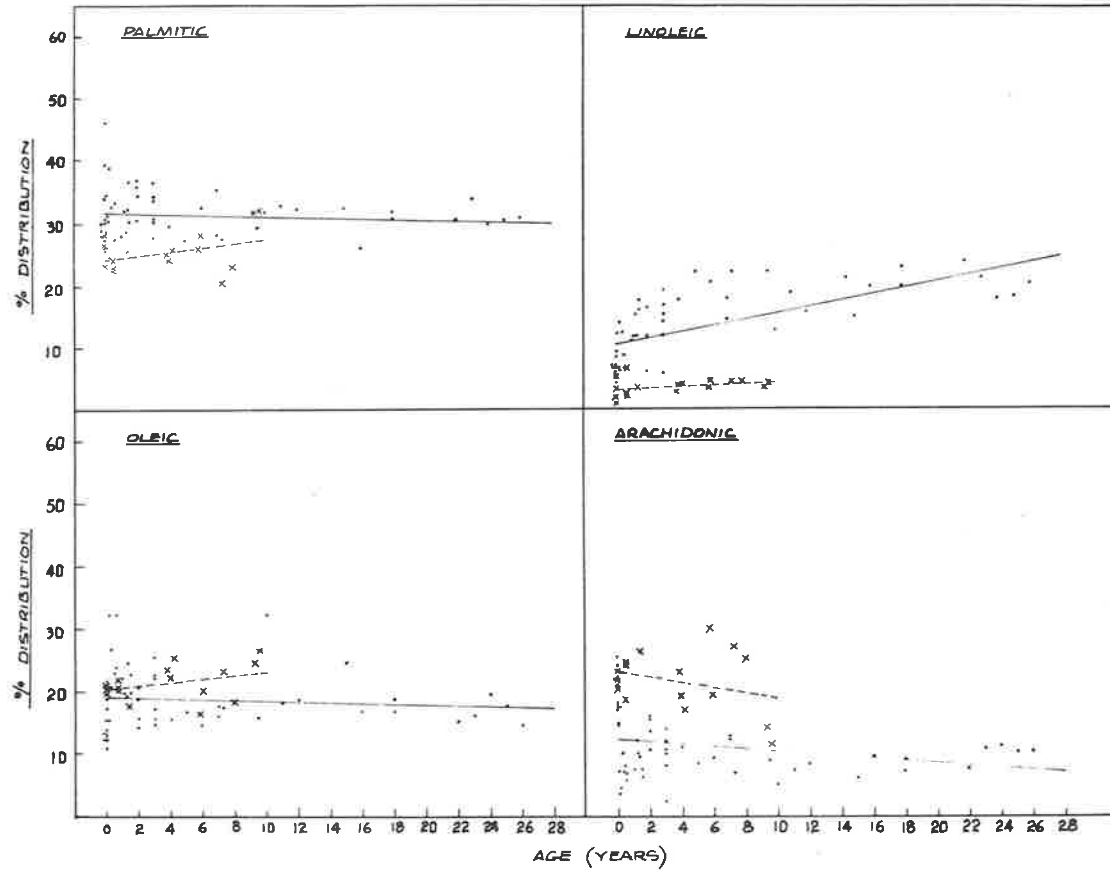

Figure 20

Phospholipid fatty acid compositions of serum and normal intima, plotted as individual samples according to age. Linear regression lines are also shown (P values are shown in tables 9 and 11).

TABLE 12

[a] RABBIT CHOLESTEROL ESTER FATTY ACID PATTERNS

	NORMAL		CHOLESTEROL-FED	
	[b] SERUM	[c] AORTIC INTIMA	[b] SERUM	[c] AORTIC INTIMA
14:0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0
16:0	19.4 ± 0.5	21.6 ± 3.0	19.2 ± 1.5	12.0 ± 0.5
16:1	5.4 ± 0.4	5.6 ± 0.6	8.0 ± 0.5	3.9 ± 0.4
18:0	4.1 ± 0.3	8.6 ± 0.9	3.9 ± 0.4	3.7 ± 0.5
18:1	32.9 ± 1.1	40.1 ± 1.4	48.3 ± 2.3	58.1 ± 2.1
18:2	38.4 ± 1.2	20.3 ± 3.6	20.6 ± 1.0	18.1 ± 1.6
18:3	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0
20:0	0.5 ± 0.0	1.1 ± 0.1	0.5 ± 0.0	2.6 ± 0.4
20:3	-	0.5 ± 0.0	-	0.5 ± 0.0
20:4	-	2.9 ± 0.5	-	0.7 ± 0.0

[a] Serum and intimal samples are not from the same rabbit

[b] Means and standard errors of means of serum samples from five cholesterol-fed rabbits and their normal controls

[c] Means and standard errors of means of aortic intimae from three cholesterol-fed rabbits and their normal controls.

common to both this study and the one of Section 7, but in this study, the finding of a lower intimal cholesterol linoleate is not evident. For the normal animals there is also a greater percentage of cholesterol oleate and a lesser percentage of cholesterol linoleate in the intima than in the serum. So that for the rabbit it is possible that intimal properties apart from those related to atherosclerosis induced by cholesterol-feeding are important in determining intimal cholesterol fatty acid composition.

The phospholipid fatty acid compositions of normal and cholesterol-fed rabbit serum are substantially the same (Table 13). The monounsaturated fatty acids have a similar representation in normal and atherosclerotic rabbit (Table 13) and so, apparently, do the total polyunsaturated fatty acids, but there is much less phospholipid arachidonic acid in the atherosclerotic intima and more linoleic. There is about the same order of polyunsaturated phospholipid fatty acid in the normal intima as in the normal serum, but there is less polyunsaturated fatty acid in the atherosclerotic intimal phospholipid than there is in the serum. Also, the way in which the polyunsaturated fatty acids are represented, whether mainly as linoleic or arachidonic acid, differ markedly between serum and intima, irrespective of cholesterol - feeding.

D. Discussion

The intimal cholesterol ester fatty acid pattern of the cholesterol-fed rabbit has been found to differ from the serum pattern in essentially the way reported by Zilversmit et al (1961), Swell et al (1961) and Evrard et al (1962). The possibility has been raised in the present study, however, that normal rabbit intima, like atherosclerotic intima, has the capacity to determine its own cholesterol ester fatty acid composition. Proper assessment must await studies of serum and intima from the same normal animals.

TABLE 13

RABBIT PHOSPHOLIPID FATTY ACID PATTERNS[a]

	NORMAL		CHOLESTEROL-FED	
	SERUM[b]	AORTIC INTIMA[c]	SERUM[b]	AORTIC INTIMA[c]
14:0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0
16:0	24.8 ± 0.4	19.8 ± 1.4	27.3 ± 1.2	27.6 ± 3.9
16:1	1.5 ± 0.1	1.9 ± 1.4	2.0 ± 0.2	1.8 ± 0.1
18:0	19.0 ± 0.3	21.2 ± 0.3	17.3 ± 0.4	16.6 ± 0.9
18:1	17.5 ± 0.5	14.4 ± 0.9	15.4 ± 0.3	16.2 ± 1.3
18:2	33.1 ± 0.3	5.6 ± 1.1	32.4 ± 1.5	16.2 ± 2.4
18:3	0.5 ± 0.0	2.8 ± 1.6	0.5 ± 0.0	1.6 ± 0.1
20:0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0
20:3	0.5 ± 0.0	3.7 ± 0.5	0.5 ± 0.0	2.5 ± 0.2
20:4	4.3 ± 0.2	21.8 ± 1.1	5.8 ± 0.5	10.2 ± 1.7
20:4	-	-	-	6.5 ± 0.7

[a] Serum and intimal samples are not from the same rabbit.

[b] Means and standard errors of means of serum samples from five cholesterol-fed rabbits and their normal controls.

[c] Means and standard errors of means of aortic intimae from three cholesterol-fed rabbits and their normal controls.

The differences in phospholipid fatty acid composition between rabbit serum and intima and between normal and atherosclerotic rabbit intima also confirm, in general terms, the work of Zilversmit et al (1961), which was an analysis of individual phospholipid classes, and of Swell et al (1961). The latter workers did not, however, find any differences between the phospholipid fatty acid patterns of normal and atherosclerotic rabbit aorta, while in the present study, relatively more linoleic acid has been found in atherosclerotic intimal phospholipid. There is good reason, then, to consider the role of arterial lipid metabolism and of the possible selective entry of various lipids into the arterial wall in the pathogenesis of experimental atherosclerosis in the rabbit. Together with the provision of a background for interpreting results of metabolic and flux experiments, this was the rationale for these chemical analyses in the rabbit.

The studies with children's arteries fill some important gaps in the literature of human arterial lipid composition, which is reviewed in the general introduction to this thesis and the introduction to this section.

There is a small amount of cholesterol ester, about 15 - 40 µg/ 100 mg dry tissue, in the inner wall of children's aortae. Smith (1965) found that, from the age of 10 years, the normal intima accumulates increasing amounts of free and ester cholesterol, with ester increasing at a greater rate than free cholesterol. The same can now be said to be the case in the first 10 years of life. The amount of cholesterol ester at age 10 years in the present study is similar to that found by Smith (1965) for that age. It seems likely that, in the first years of life, fatty streak cholesterol ester accumulates at an even greater rate than that in normal intima, but this has not been validated statistically. For adult arteries, this is known to be so (Insull and Bartsch, 1966; Smith, Evans and

Downham, 1967). The differences in cholesterol and cholesterol ester accumulation in normal intima and media are also of interest. There is little change in medial content of either moiety with age in contrast to the increases of both in the intima. This also confirms the histological view that different tissues have been analysed as "intima" and "media".

Unlike cholesterol, phospholipid content did not change significantly with age in the normal intima. Yet, Smith (1965) has shown an increase from the age of 10 years onwards. The phospholipid content of the normal media does decrease from 0 - 10 years and it is interesting to speculate what relationship this might have to later intimal phospholipid changes or to parallel increases in intimal cholesterol ester - especially since some workers consider that medial cells become involved in early atherosclerotic lesions (Wissler, 1967 and 1978).

Knowing that human fatty streaks differ in their cholesterol ester fatty acid composition from normal intima and from serum in having a relatively high oleate and low linoleate content (Smith, 1962 and 1965; Swell et al 1960 and Swell et al 1962) the findings in children's arteries were somewhat surprising. The juvenile fatty streak does have a low cholesterol linoleate content, relative to cholesterol oleate, but so does the normal intima and the serum. Also, the same trends, up to the age of 10 years, in serum cholesterol linoleate composition and in the serum 18:1 / 18:2 ratio are reflected in the normal intima and in the juvenile fatty streak. In fact, the real difference between children's and adults' intimal cholesterol ester fatty acids almost appears to be in normal rather than fatty streak tissue. So that, apparently, no selective intimal transport or metabolic process needs to be invoked to account for the early lesion. When the extent of trends in serum, normal intima and lesion intima are compared there is, indeed, no

significant difference in the behaviour of linoleate. Yet, there is a significant difference between the regression coefficient of the normal intimal 18:1 / 18:2 ratio and that in the serum and between the lesion intimal 18:1 / 18:2 ratio regression coefficient and that in the serum, but not between normal intimal 18:1 / 18:2 and lesion intimal 18:1 / 18:2. Another difference between intima and serum is that while the proportion of cholesterol ester as arachidonate increases with age in the intima, it decreases with age in the serum. The findings, therefore, do not provide evidence that cholesterol ester is deposited in the juvenile intima only from the serum. If intimal cholesterol ester is derived from the serum, then presumably some alteration takes place both in normal and in abnormal intima.

While it would be difficult to be sure that the procedure for washing the aortae in this study removed all traces of blood serum, those differences observed between intima and serum lipids lend support to the view that the procedure was adequate.

The normal intimal phospholipid fatty acid patterns are similar to those reported by Böttcher, Woodford, Ter Haar Romeny-Wachter, Boelsma-Van Houte and Van Gent (1960) and Böttcher and Van Gent (1961) for whole adult aorta, except for arachidonic acid which is relatively higher in the children's intimae. The proportion of phospholipid fatty acid as arachidonic acid which Wiese et al (1967) found in whole children's arteries is, indeed, similar to that in the children's intimae of the present study.

There is a lack of correlation between normal intimal phospholipid fatty acid patterns and age in the present study. Nevertheless, in Fig. 20, normal intimal linoleic acid composition is consistently lower than is its composition in the serum and the regression coefficients of 18:1 / 18:2 ratios of intima (not

age-correlated) and serum (age-correlated) are significantly different. Phospholipid linoleic acid in the lesion intima and in the serum are age-correlated, and, as in the normal intima, the percentage lesion phospholipid linoleic acid is consistently less than that in the serum. This is consistent with the view that phospholipid is formed locally (Zilversmit, 1959), but does not prove it. Unlike the adult lesion (Böttcher and Van Gent, 1961), the juvenile fatty streak does not appear to have a greater percentage saturated and a lower percentage polyunsaturated phospholipid than the normal intima (Fig. 19).

In conclusion, children's aortae do contain small quantities of cholesterol ester and the amount of cholesterol ester increases with age. The fatty acid studies lead to the view that juvenile serum and aortic lipids are different from those in the adult, and that the reasons for lipid accumulation in arteries may be different in childhood and adulthood.

SECTION 2

INCORPORATION IN VITRO OF ^{14}C-LABELLED OLEIC ACID INTO COMBINED LIPID BY THE RABBIT ARTERIAL WALL

A. Introduction

The incorporation of radioisotopically - labelled fatty acid into triglyceride and phospholipid in the normal arterial wall of experimental animals has been documented (Stein and Stein, 1962; Stein, Stein and Shapiro, 1963; Parker, Schimmelbusch and Williams, 1964; Parker, Ormsby, Peterson, Odland and Williams, 1966). For the atherosclerotic arterial wall, fatty acid is also incorporated into cholesterol ester (Bowyer, Howard and Gresham, 1967; Newman, Gray and Zilversmit, 1968). Moreover, fatty acid, newly synthesized from ^{14}C-labelled acetate in the rabbit atherosclerotic lesion, is incorporated into combined lipids (Day and Wilkinson, 1967; Newman et al, 1968). As the atherosclerotic process becomes more severe, so the amount of newly-formed fatty acid incorporated into cholesterol ester increases (Lofland, Moury, Hoffman and Clarkson, 1965; Whereat, 1966). From the latter studies it is not possible to decide, however, whether fatty acid synthesis or the process of esterification is responsible for the increased incorporation. In this section, a comparison of the uptake and incorporation of ^{14}C-labelled fatty acid into the combined lipid of normal and atherosclerotic rabbit intima is reported.

If there are differences in the way fatty acid is metabolised by normal and by atherosclerotic intima, then it is of particular interest to find the components of the atherosclerotic lesion responsible for such differences. Geer and Guidry (1964) and Smith (1965) have demonstrated that the human atherosclerotic lesions which contain large numbers of foam cells have a cholesterol ester fatty acid pattern which differs markedly from that of the serum, being high in oleic acid relative to linoleic acid. Parker et al (1966), in a combined biochemical and morphological study, have shown that rabbit atherosclerotic lesions take up and incorporate linoleic acid into phospholipid and that the magnitude of such

incorporation is related to the increasing number of myointimal cell membranous organelles in the developing lesion. Also, foam cells isolated from rabbit atherosclerotic lesions incorporate fatty acid into combined lipid (Day and Wilkinson, 1967; Day and Tume, 1968). These various studies indicate that foam cells may be at least partly responsible for the handling of fatty acid by the atherosclerotic lesion. In the present study, the sites of uptake and incorporation of ^{14}C-labelled oleic acid into combined lipid have been investigated directly by a combined biochemical and autoradiographic approach.

B. Uptake of ^{14}C-labelled Oleic Acid by Normal and Atherosclerotic Rabbit Aortic Intima

Normal and atherosclerotic rabbit thoracic aortae were obtained as described in the "Materials and Methods" section of this thesis. The cholesterol-fed animals received their diet for 4 months while the normal group had an ordinary diet.

Two series of experiments were carried out. In the first, aortic halves were incubated separately in 5 ml of medium (50 : 50 Hanks' solution : normal rabbit serum) containing a known amount of sodium 1-^{14}C oleate: albumin for 4 hours. Also, atherosclerotic aortic thirds were incubated for 1, 2 or 4 hours. The uptakes of oleic acid from the incubation medium are shown in Table 14. For the normal intima, a mean of 0.830% of the oleic acid was taken up and most of this (0.702%) as incorporated into combined lipid. There appeared to be a greater uptake of oleic acid by the atherosclerotic intima (4.48%) but in the absence of information about oleic acid specific activities in the different incubation media, this is not proven. Again, however, most of the oleic acid taken up (3.51%) was incorporated into combined lipid. Both the uptake from a common incubation medium of ^{14}C-labelled oleic acid and its incorporation

into lipid were approximately linear over the 4-hour period (Table 14).

In the second series of experiments, normal and atherosclerotic aortae were incubated together in 10 ml of medium (50 : 50 Hanks' solution : normal rabbit serum) to which had been added a known amount (8.2 μCi) of ^{14}C-labelled sodium oleate. The excess serum albumin was used to bind the tracer amount of ^{14}C-labelled sodium oleate, so that no additional albumin was required. The fatty acid content of the serum used was determined by the method of Dole (1956) and the proportion of free fatty acid as oleic acid was assessed by gas liquid chromatography following separation by neutral lipid T.L.C. Thus, the specific activity of ^{14}C-labelled oleic acid in the incubation medium was derived. The specific activity was, of course, common to both normal and atherosclerotic aorta, since they were paired, so that the percentage uptakes shown in Table 17 can be compared. There is about five times as much ^{14}C-labelled oleic acid taken up by the atherosclerotic intima as by the normal intima.

C. Incorporation of ^{14}C-labelled Oleic Acid into Combined Lipid by Normal and Atherosclerotic Rabbit Aortic Intima

The percentage distribution of ^{14}C-labelled oleic acid among intimal phospholipid, diglyceride, free fatty acid, triglyceride and cholesterol ester, separated by neutral lipid T.L.C., is shown for the first series in Table 15. In the normal artery very little cholesterol ester (2.4%) was labelled; most of the oleic acid taken up was incorporated into phospholipid, and to a lesser extent into triglyceride. In the atherosclerotic artery, however, a mean of 29.4% of the ^{14}C-labelled fatty acid was incorporated into the cholesterol ester fraction. The pattern of incorporation of oleic acid into cholesterol ester and phospholipid at 4 hours is similar

TABLE 14

Uptake of ^{14}C-labelled Oleic Acid and Its Incorporation into Combined Lipid by Normal and Atherosclerotic Rabbit Intimae

	Incubation time	^{14}C Present initially in incubation medium (cpm x 10^{-6})	% Uptake	% ^{14}C Initially present incorporated into lipid
Normal *	4 hr	6.5	0.830 ± 0.126	0.702 ± 0.115
Atherosclerotic +	4 hr	5.83 ± 1.00	4.48 ± 0.80	3.51 ± 0.60
Atherosclerotic ‡	1 hr	1.19	0.77	0.46
		6.1	1.2	0.71
	2 hr	1.19	1.39	1.09
		6.1	2.3	1.67
	4 hr	1.19	2.05	1.78
		6.1	4.2	3.42

* Mean of four experiments (duplicate aortic halves) with SE of mean; + mean of six experiments (five with duplicate aortic halves) with SE of mean; ‡ two experiments as shown.

TABLE 15

Percentage Distribution of ^{14}C-labelled Oleic Acid Among Lipid Fractions After Incubation with Normal or Atherosclerotic Rabbit Intimae

	Incubation time	Phospholipid	Diglyceride	Fatty Acid	Triglyceride	Cholesterol ester
Normal *	4 hr	48.1 ± 5.0	8.3 ± 1.7	15.8 ± 1.4	25.5 ± 3.4	2.4 ± 0.6
Atherosclerotic +	4 hr	32.2 ± 1.2	2.8 ± 0.3	21.4 ± 2.4	14.2 ± 2.3	29.4 ± 3.3
Atherosclerotic ‡	1 hr	28.2	3.7	40.8	5.9	21.5
	2 hr	37.8	3.1	24.6	5.7	29.1
	4 hr	35.3	2.3	16.1	11.9	34.7

* Mean of four experiments (duplicate aortic halves) with SE of mean; + mean of six experiments (five with duplicate aortic halves) with SE of mean; ‡ mean of two experiments (both with single aortic thirds).

to that at earlier intervals. There is, however, relatively more unesterified oleic acid present at the earlier time intervals.

Table 16 shows the distribution of the label between individual phospholipids for both the normal and atherosclerotic intimae. In both groups the major phospholipid labelled is lecithin. No significant differences between normal and atherosclerotic intima are evident in the labelling of the various phospholipid fractions. Distribution of label was similar over the 4-hour period studied.

At the end of a control or actual incubation more than 97% of the label in the medium was still present as free fatty acid, so that unless the intima exhibits a highly selective uptake mechanism for combined lipid, free fatty acid must have been incorporated into combined lipid in the intima itself and not in the incubation medium.

In the second series of experiments, as in the first, the atherosclerotic intima incorporated a higher proportion of the label into cholesterol ester and a lower proportion into phospholipid and triglyceride than did the normal (Table 17).

The distribution of the label between the various moieties together with the specific activity of oleic acid in the incubation medium has been used to calculate the amount of oleic acid incorporated into phospholipid, triglyceride and cholesterol ester by the normal and atherosclerotic intima (Table 18). This calculation makes the assumption that there is no intervening intimal free fatty acid pool in which the label might be diluted. The dry defatted weight of the normal intima was similar to that of the atherosclerotic intima, but the amount of oleic acid incorporated

TABLE 16

Percentage Distribution of ^{14}C-labelled Fatty Acid Among Individual Phospholipids After Incubation with Normal or Atherosclerotic Rabbit Intimae

	Incubation time	Origin	Lyso-lecithin	Sphingo-myelin	Lecithin	Phosphatidyl inositol	Phosphatidyl ethanolamine	Solvent front
Normal *	4 hr	0.9 ± 0.1	0.7 ± 0.1	1.4 ± 0.1	66.1 ± 1.1	9.5 ± 2.7	15.7 ± 0.8	6.2 ± 1.1
Atherosclerotic +	4 hr	0.2 ± 0.0	0.4 ± 0.1	0.9 ± 0.1	65.5 ± 1.2	11.9 ± 1.0	14.5 ± 0.9	7.1 ± 1.2
Atherosclerotic ‡	1 hr	0.1	0.1	1.6	66.1	11.2	15.9	5.2
	2 hr	0.1	0.1	0.7	66.9	11.4	18.6	2.3
	4 hr	0.2	0.1	1.0	67.6	9.6	18.6	3.2

* Mean of four experiments (duplicate aortic halves) with SE of mean; + mean of five experiments (duplicate aortic halves) with SE of mean; ‡ mean of two experiments (single aortic thirds).

TABLE 17

Percentage Distribution of ^{14}C-labelled Oleic Acid Among Lipid Fractions After Incubation with Normal and Atherosclerotic Rabbit Intimae

	% Uptake of oleic acid	Phospholipid	Diglyceride	Fatty Acid	Triglyceride	Cholesterol ester
Normal	0.756	49.3	4.2	24.5	15.2	7.2
Atherosclerotic	4.076	30.1	1.9	21.1	9.6	37.5

Data are means of three paired experiments

into all of the lipid moieties was considerably greater in the atherosclerotic intima than it was in the normal. The amount of oleic acid incorporated into phospholipid and triglyceride was an average of about twice that in the normal. The amount of oleic acid incorporated into cholesterol ester was increased an average of some twenty times. Most of the increased incorporation of oleic acid into lipid in the atherosclerotic intima is therefore accounted for by its increased incorporation into cholesterol ester.

D. Specific Activity of ^{14}C-labelled Oleic Acid Incorporated into Combined Lipid

To determine the specific activity of oleic acid incorporated into intimal cholesterol ester and phospholipid these lipid fractions were first separated by neutral lipid T.L.C. and then methyl esterified for gas-liquid radiochromatography (G.L.R.C.) as described under "Materials and Methods". No radioactive peak other than that of oleic acid was found on G.L.R.C. The specific activities of oleic acid in the phospholipid and cholesterol ester of the normal and atherosclerotic intimae are shown in Table 18. These are expressed as cpm/mμmole oleic acid present. Biologically occurring phospholipids usually have different fatty acids in the α and β positions so that it can be assumed that only one fatty acid position of the lecithin (the major phospholipid labelled) would be occupied by oleic acid. The specific activities of the cholesterol ester and phospholipid, as expressed in Table 18, are therefore directly comparable on a molar basis. For phospholipid, the specific activity in the atherosclerotic intima is of the same order as, although less than, it is in the normal. The specific activity of the cholesterol ester fatty acid in the atherosclerotic intima is about one fifth that in the normal intima.

TABLE 18

Amount of Oleic Acid Incorporated into and Specific Activities of Oleic Acid as Combined Lipids for Normal and Atherosclerotic Rabbit Intimae Incubated in Vitro * +

Exp. no.		Dry defatted weight (mg)	mμmoles oleic acid incorporated/g dry defatted intima			Specific activity (cpm/mμuole oleic acid)	
			Phospholipid	Triglyceride	Cholesterol ester	Phospholipid	Cholesterol ester
1	Normal	79.6	58.6	12.6	12.0	68.9	47.2
	Atherosclerotic	94.5	335.2	102.1	424.5	57.9	11.7
2	Normal	72.2	116.8	44.2	3.0	92.6	64.6
	Atherosclerotic	102.2	281.4	118.1	339.1	75.2	11.9
3	Normal	54.8	156.8	52.0	30.8	153.0	87.3
	Atherosclerotic	80.8	152.1	34.8	192.5	42.3	11.5

* Four-hour incubations carried out in pairs; normal and atherosclerotic intimas incubated in same medium.
\+ All incubations had 12.7 X 10^6 cpm oleic acid added. There was a total of 1.94 μEq. oleic acid present in 10 ml of medium.

E. Autoradiographic Localisation of ^{14}C-labelled Oleic Acid in Rabbit Atherosclerotic Lesions

In these studies the atherosclerotic aortic halves were incubated for 3 hours in radioactive medium and then for a further hour in non-radioactive medium. The artery was washed in saline and finally in a solution of unlabelled sodium oleate in 15% albumin (5 mg oleate / ml 15 % albumin solution) for 1 hour at 4°C. Both halves were fixed in 1% calcium chloride and 4% formaldehyde in physiological saline. A lipid extract of the intima of one aortic half was prepared for radiochemical analyses and the other aortic half used to prepare autoradiographs as described under "Materials and Methods".

Most of the ^{14}C-labelled oleic acid was incorporated into phospholipid and cholesterol ester, as in the experiments already described, and only 12.2% of the label was free fatty acid.

The localisation of ^{14}C-labelled lipid by the atherosclerotic rabbit artery is shown in Figures 21 to 25. No significant film background granulation was present in any of the autoradiographs. Early sudanophilic cellular lesions with associated fragmentation of the internal elastic lamina are shown in Figure 21. Granulation, representing essentially ^{14}C-labelled oleic acid and the cholesterol ester and phospholipid formed from it, overlies the foam cell areas. There is little label in intervening relatively normal intima, and little in underlying media. The higher magnification of a similar lesion in Figure 22 confirms localisation of ^{14}C to intimal foam cells.

The more established lesion shown in the coloured autoradiograph of Figure 23 shows a concentration of silver grains in the region of sudanophilic cells and little label in non-cellular areas of the intima or in the region of the intimo-medial junction.

Figure 21

Autoradiograph of an early atherosclerotic lesion consisting of several closely related foam cells. Granulation representing ^{14}C-labelled oleic acid and its metabolic derivatives is confined to foam cell areas. Aorta from rabbit aged 21 weeks and cholesterol-fed for 15 weeks. Haematoxylin and Sudan IV. Exposure time 7 days.

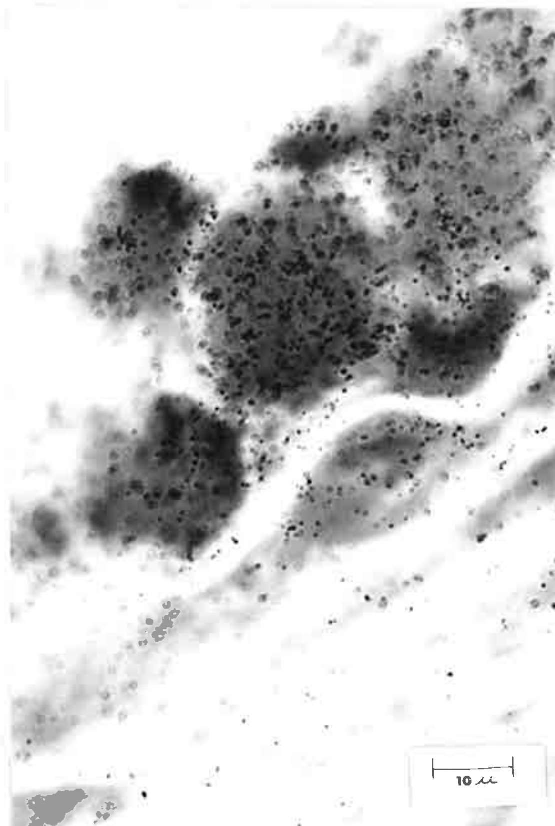

Figure 22

Early atherosclerotic lesion from the same rabbit as in Figure 21 viewed under oil immersion confirming the relationship of autoradiograph granules to foam cells. Haematoxylin and Sudan IV. Exposure time 8 days.

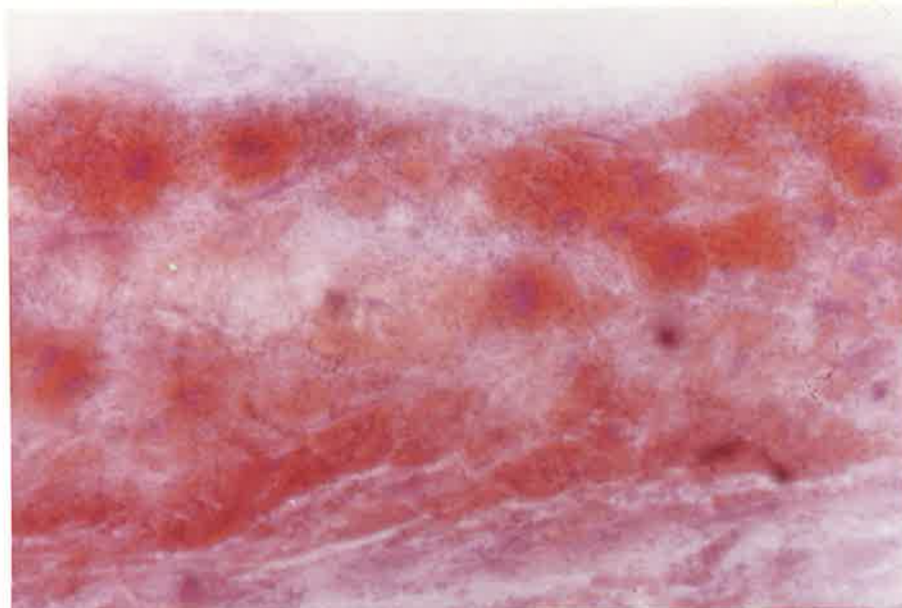

Figure 23

Established atherosclerotic lesion from the same rabbit as in figure 21, showing stippling or silver grains in the region of sudanophilic cells. The endothelial border is uppermost and the intimo-medial junction towards the lower border of the photomicrograph. Haematoxylin and Sudan IV, X 600. Exposure time 12 days.

An advanced lesion with grossly thickened intima is shown in Figure 24. Localisation of ^{14}C to the foam cells scattered throughout the intima is apparent and there is little evidence of label in other areas of the thickened intima. Figure 25 is a high-power coloured autoradiograph of foam cells in an advanced lesion which again confirms that silver grains are concentrated in the region of these cells.

To obtain a more quantitative assessment of localisation of ^{14}C-labelled oleic acid, grain counts were performed and these are shown in Table 19 as grains / 100 μ^2. The intimal foam cells of early and advanced lesions clearly localise more label than do extra-cellular areas of the intima and underlying arterial media.

F. Discussion

It has been shown that oleic acid is taken up by both normal and atherosclerotic rabbit intimae and incorporated into various lipids. In the normal intima, fatty acid was incorporated mainly into phospholipid and triglyceride, as reported by others. The diversion of oleic acid to cholesterol ester in the atherosclerotic intima, however, was in marked contrast to events in the normal.

The incorporation of ^{14}C-labelled oleic acid into cholesterol ester in the atherosclerotic intima was approximately linear over the 4-hour incubation period studied. If the normal intima behaved in the same way with respect to time, the relative fractional turnover of the phospholipid and cholesterol ester fatty acid pools in the intima can be estimated. The specific activity of the phospholipid fatty acid after incubation for 4 hours was roughly of the same order in the atherosclerotic as that in the normal. The small cholesterol ester fatty acid pool in the normal

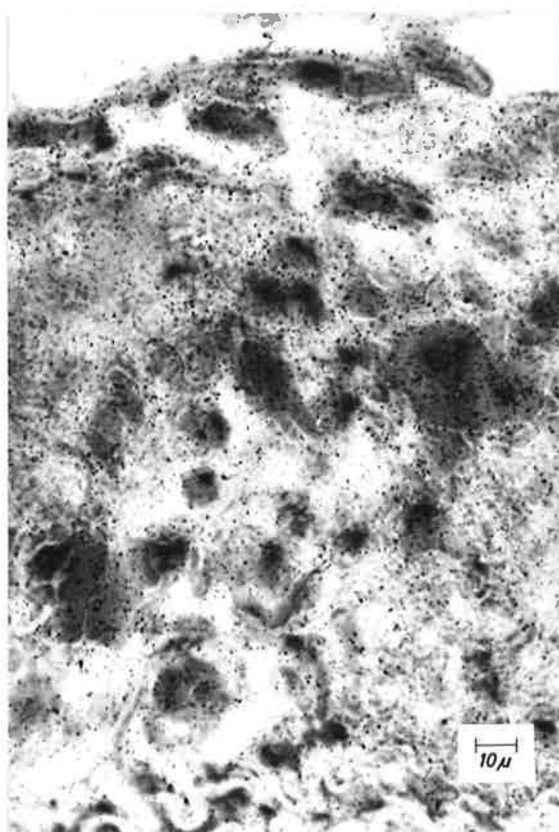

Figure 24

Autoradiograph of an advanced atherosclerotic lesion with much extracellular lipid. ^{14}C-labelled oleic acid has been taken up and metabolised by the foam cells. The luminal aspect is uppermost and the intimo-medial junction is visible at the lower border of the photomicrograph. Aorta is from a rabbit aged 21 weeks and cholesterol-fed for 15 weeks. Haematoxylin and Sudan IV. Exposure time 38 days.

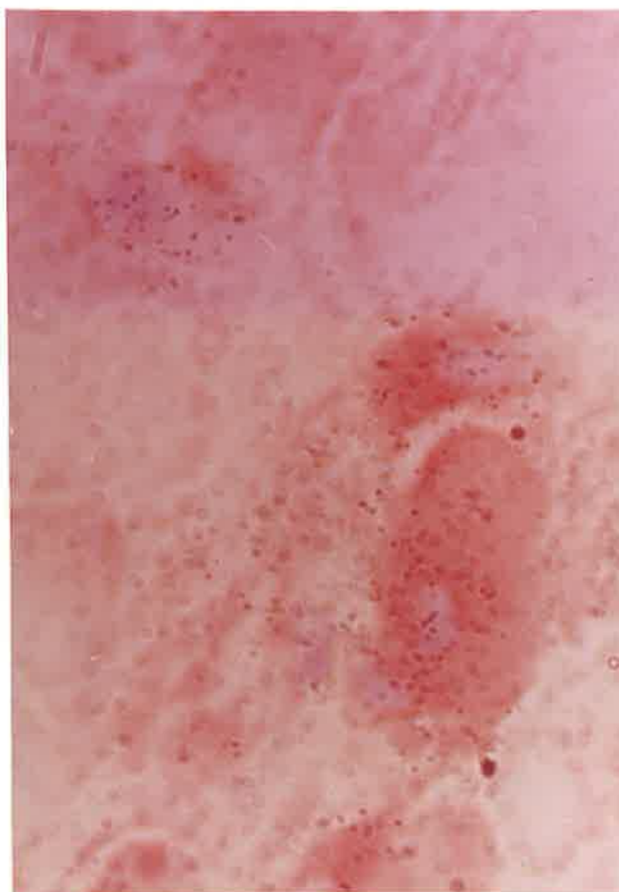

Figure 25

Advanced atherosclerotic lesion from the same rabbit as in Figure 24, viewed under oil immersion, confirming the relationship of autoradiograph granules to foam cells. Haematoxylin and Sudan IV, X 1000. Exposure time 38 days.

TABLE 19

RABBIT ATHEROSCLEROTIC LESIONS

^{14}C-OLEIC ACID AUTORADIOGRAPHS

* GRAIN COUNTS (No./100 μ^2)

LESION	INTIMA		MEDIA
	FOAM CELLS	EXTRA-CELLULAR	
ADVANCED	16.8	2.8	0.8
EARLY	21.0	3.2	2.2

* At least 6000 μ^2 assessed for each feature

intima had a specific activity considerably higher than that of the atherosclerotic intima, so that although the total amount of fatty acid incorporated per unit time into cholesterol ester in the atherosclerotic intima was many times that in the normal, the fractional turnover rate was much less.

The pathway by which fatty acid is incorporated into cholesterol ester in the atherosclerotic intima in the present experiments is not known. As discussed in the "General Introduction", either direct esterification of cholesterol with fatty acid, enzymic exchange with rearrangement of the large cholesterol ester fatty acid pool in the intima, or the lecithin : cholesterol acyl transferase reaction might occur. The findings that most oleic acid is incorporated into lecithin and that phospholipid oleic acid has a higher specific activity than cholesterol ester oleic acid means that the transferase mechanism is a possibility in the normal and the atherosclerotic intima. The specific activity of phospholipid relative to cholesterol ester is considerably higher in the atherosclerotic intima than in the normal. However, this might not represent a difference in transferase enzyme activity, but rather be a reflection of an inert cholesterol ester pool. Nevertheless, Abdulla, Orton and Adams (1968) have demonstrated the presence of lecithin : cholesterol acyl transferase in rabbit atherosclerotic lesions.

The incorporation of ^{14}C-labelled oleic acid into individual phospholipids is similar in the normal and atherosclerotic artery. This is in contrast to previous studies in vitro (Newman, Day and Zilversmit, 1966) in which it has been shown that, after 4 hours of incubation, ^{32}P-labelled phosphate is incorporated mainly into phosphatidyl inositol in the normal artery and into lecithin in the atherosclerotic artery. In the present experiments, oleic acid is incorporated mainly into lecithin by both normal and atherosclerotic

intimae. Of the several pathways by which fatty acid could be incorporated into lecithin (see "General Introduction") in the normal artery, one is that it could be incorporated by the Lands pathway, lysolecithin serving as a fatty acid acceptor (Stein, Stein and Shapiro, 1963; Eisenberg, Stein and Stein, 1967b). It is therefore possible that, for the normal intimae of the present experiments, the fatty acid was taken up and incorporated directly into phospholipid via the lysolecithin pathway. For the atherosclerotic arteries, however, more information is required before the importance of this pathway can be determined.

In the arteries examined by autoradiography, the possibility that some of the radioisotope demonstrable was not lipid was excluded by determining, in some of the experiments, the ^{14}C present in both the upper phase of the Folch wash and the tissue residue of the ^{14}C-labelled intima following lipid extraction. More than 97% of the ^{14}C present in the intima was recovered in the lipid extract. Phospholipid and cholesterol ester, present in approximately equal amounts, together accounted for over 70% of this lipid ^{14}C. Nevertheless, the possibility remained that the ^{14}C localised over foam cells might have represented essentially the uptake of unesterified oleic acid and not the localisation of phospholipid and cholesterol ester formation. That this is very unlikely is indicated by the studies of Day and Tume (1969) in which isolated foam cells were found to incorporate most of the ^{14}C-labelled oleic acid taken up into combined lipid. Further, it has now been shown that foam cells isolated from rabbit atherosclerotic intimae incubated *in vitro* with ^{14}C-labelled oleic acid have only about 4% of the label as free fatty acid (Day and Tume 1970). Yet again, the specific activity of oleic acid in both cholesterol ester and phospholipid in the isolated foam cells is five to six times that in other intimal components, so that foam cell ^{14}C-labelled cholesterol ester and phospholipid are unlikely to have been formed elsewhere.

Oleic acid was chosen as a precursor to study cholesterol ester and phospholipid formation by autoradiography so that there would be no nonlipid ^{14}C and little ^{14}C lipid precursor present. Even so, the findings are presumably applicable to fatty acid formed in the atherosclerotic lesion from acetate.

The lack of localisation of the ^{14}C in the medial cells is striking. Since aortae were incubated suspended freely in the incubation medium and some areas of media were covered only by a thin layer of normal intima, this finding is presumed not to be accounted for by reduced access of labelled oleic acid to the medial cells. Clearly uptake and synthesis of lipid is a particular property of the intimal foam cells so that, if these arise from medial cells (Wissler, 1967), their metabolism must change. At the light microscope level it is not possible to say whether the foam cells which take up the label in the more superficial portions of the intima represent smooth muscle-like or macrophage-like foam cells, or both.

Although localisation of ^{14}C-labelled oleic acid is related to its uptake and conversion to, principally, phospholipid and cholesterol ester by intimal foam cells, there may be other factors which encourage its retention within the cells. The low rate of hydrolysis of cholesterol oleate relative to other cholesterol esters shown by atherosclerotic intima (Howard, Bowyer and Gresham, 1967) and the slow catabolism of unsaturated fatty acids by this tissue (Chobanian and Hollander, 1963) could both be foam cell phenomena and partly explain the localisation of label.

Thus there is now direct evidence that the foam cells are the principal sites in rabbit atherosclerotic lesions for the incorporation of fatty acid into combined lipid.

SECTION 3

INCORPORATION IN VITRO OF ^{14}C-LABELLED OLEIC ACID INTO COMBINED LIPID BY THE HUMAN ARTERIAL WALL

A. Introduction

Just as labelled fatty acid has been shown to be incorporated into combined lipid by arteries from experimental animals, so also has it been shown to be incorporated into the phospholipid of human umbilical arteries (Stein, Selinger and Stein, 1963), of normal adult human arteries (Parker, Schimmelbusch and Williams, 1964) and of atherosclerotic human artery (Chobanian and Hollander, 1966). Stein, Selinger and Stein (1963) have also demonstrated the incorporation of labelled fatty acid into glyceride. The pathways described by Kennedy and Weiss (1956) and by Lands (1960) for lecithin formation may both be operative in the human arterial wall (Parker et al, 1964; Stein, Selinger and Stein, 1963). Since, as shown in Section 2 of this thesis, fatty acid is incorporated into cholesterol ester to a greater extent in rabbit atherosclerotic lesions than in normal intima, it seemed important to examine the human lesion for cholesterol ester formation.

In this section, then, the uptake and metabolism of ^{14}C-labelled oleic acid by human atherosclerotic lesions and adjacent normal intima have been investigated in vessels obtained from cadaveric renal transplant donors. The localisation of uptake and of metabolic events in the human atherosclerotic lesion has been studied by autoradiography in a way similar to that described for the rabbit.

B. Uptake and Incorporation of ^{14}C-labelled Oleic Acid into Combined Lipid by Human Arterial Intima

Portions of thoracic, iliac or renal arteries with uncomplicated atherosclerotic lesions were set up in an incubation medium containing equal volumes of Hanks' solution and normal human serum, together with a known amount (about 0.5 to 2.0 µCi/ml

incubation medium) of sodium oleate-1-^{14}C : albumin. After incubation for 4 hours a segment of the vessel was taken for autoradiography. The remainder was dissected into macroscopically normal and atherosclerotic artery and intimal extracts prepared.

The clinical data for the four cases from which arteries were obtained for metabolic studies _in vitro_ are given in Table 20. The causes of death were not primary metabolic disorders, and the lesions were either fatty-streak (W.H.O. Grade I) or fibro-fatty lesions (W.H.O. Grade II). The relative amounts of normal and atherosclerotic artery varied considerably in different experiments and, in experiment 2, as most of the artery used was atherosclerotic, no separation into normal and atherosclerotic was attempted. From case 4, renal and iliac arteries were obtained in addition to aorta.

The percentage distribution of ^{14}C-labelled oleic acid among phospholipid, diglyceride, fatty acid, triglyceride, and cholesterol ester is shown in Table 21. Since the relative amount of normal and atherosclerotic tissue varied considerably in the different experiments, no comparison of the amount of ^{14}C-labelled oleic acid taken up and incorporated into combined lipid by normal and atherosclerotic portions of the intimae can be made. In both the normal intima and atherosclerotic lesion, most of the ^{14}C-labelled oleic acid was incorporated into phospholipid. There is, however, a higher proportion of ^{14}C-labelled oleic acid incorporated into cholesterol ester in all of the atherosclerotic intimal lesions than for the corresponding normal intima. This is particularly evident for the fatty-streak lesion where the normal intima incorporated 1.9% of the oleic acid into cholesterol ester compared with the adjacent atherosclerotic intima in which 15.6% of the oleic acid was diverted to cholesterol ester.

TABLE 20

Clinical Data Related to Arteries Obtained for In-Vitro Studies

Case No.	Cause of death	Sex	Age	Artery obtained	Lesion type
1	Head injury	Male	18	Thoracic aorta	Fatty Streak
2	Cerebral tumor	Male	48	Thoracic aorta	Extensive confluent fibrofatty
3	Subarachnoid hemorrhage	Male	42	Thoracic aorta	Fibrofatty plaques
4	Head and chest injuries	Male	32	Thoracic aorta Renal artery Iliac artery	Fibrofatty plaques

TABLE 21

Percentage Distribution of ^{14}C-Labelled Oleic Acid Among Intimal Lipid Fractions after Incubation in Vitro

Case no.	Lesion type	Incubation medium (cpmx10^{-6}/ml)	% Uptake	Phospholipid	Diglyceride	Fatty acid	Triglyceride	Cholesterol ester
				Aorta				
1	Normal	3.7	0.260	48.7	5.8	32.1	11.1	1.9
	Fatty streak	3.7	0.080	37.5	3.6	30.3	12.7	15.6
2	Fibrofatty	0.77	0.540	72.1	4.2	6.1	11.0	6.2
3	Normal	2.61	0.580	40.4	4.6	38.8	11.8	4.4
	Fibrofatty	2.61	0.071	26.0	4.1	50.7	10.1	9.1
4	Normal	1.45	0.171	34.4	6.3	33.4	18.0	8.1
	Fibrofatty	1.45	0.359	29.7	7.1	26.4	22.1	14.8
				Renal Artery				
4	Normal	1.45	0.163	40.6	6.4	32.2	15.3	5.6
	Fibrofatty	1.45	0.138	39.2	7.0	19.0	21.7	13.2
				Iliac Artery				
4	Normal	1.45	0.260	35.5	6.4	35.4	18.3	4.8
	Fibrofatty	1.45	0.320	33.0	5.3	16.4	26.3	19.1

The incorporation of ^{14}C-labelled oleic acid into individual phospholipids is shown in Table 22. Most of the oleic acid has been incorporated into lecithin in both the normal and atherosclerotic portions in all of the cases studied. Further, the overall pattern of incorporation into individual phospholipids is similar for the two portions.

C. Specific Activity of ^{14}C-labelled Oleic Acid Incorporated into Combined Lipid

The specific activity of ^{14}C-labelled oleic acid incorporated into intimal phospholipid, triglyceride and cholesterol ester was determined by gas liquid radiochromatography, as described under "Materials and Methods", following separation of the lipid fractions by neutral lipid T.L.C. As also pointed out in "Materials and Methods", two cholesterol oleate samples with low radioactivity had their specific activities determined by a combination of liquid scintillation counting and G.L.C. quantitation. Specific activities are shown in Table 23. In most of the experiments the specific activity for the phospholipid, triglyceride, and cholesterol ester of the atherosclerotic lesion is of the same order as that for the corresponding normal intima. For the fatty-streak lesion (case I), the specific activities of both triglyceride and cholesterol ester are lower for the lesion than for the intima. It can be seen also from Table 23 that the specific activity of the phospholipid oleic acid is several times higher than that for the cholesterol ester oleic acid in the equivalent portion of the artery whether normal or atherosclerotic.

TABLE 22

Percentage Distribution of ^{14}C-Labelled Oleic Acid Among Individual Intimal Phospholipids

Case no.	Lesion type	Origin	Lysolecithin	Sphingomyelin	Lecithin	Phosphatidyl inositol	Phosphatidyl ethanolamine	Solvent front
				Aorta				
1	Normal	1.0	0.6	2.0	60.9	15.5	6.1	14.0
	Fatty streak	3.9	1.0	2.6	55.1	11.2	7.2	18.9
2	Fibrofatty	1.5	1.3	1.7	68.3	9.3	11.3	6.8
3	Normal	0.2	0.3	0.5	69.0	16.9	8.8	4.4
	Fibrofatty	0.3	0.4	0.9	69.3	11.1	12.2	5.8
4	Normal	0.5	0.8	0.4	72.7	9.7	8.4	7.5
	Fibrofatty	0.2	0.4	0.9	72.5	9.7	9.5	7.0
				Renal Artery				
4	Normal	0.0	0.0	0.0	76.7	13.0	5.7	4.5
	Fibrofatty	0.0	0.0	0.0	78.1	7.0	8.3	6.5
				Iliac Artery				
4	Normal	0.5	0.9	0.5	72.4	11.2	8.3	5.0
	Fibrofatty	0.3	0.4	0.2	74.2	8.8	9.1	7.0

TABLE 23

Specific Activities of Phospholipid, Triglyceride, and Cholesterol Ester (cpm/mμmole oleic acid)

Case no.	Lesion type	Phospholipid	Triglyceride	Cholesterol ester
		Aorta		
1	Normal	154	54	93*
	Fatty streak	169	34	18
3	Normal	213	194	30
	Fibrofatty	338	116	42*
4	Normal	232	225	<36+
	Fibrofatty	163	103	32
		Renal Artery		
4	Normal	141	47	<35+
	Fibrofatty	204	50	20
		Iliac Artery		
4	Normal	110	18	<75+
	Fibrofatty	299	51	27

* Determined by direct counting and separate assay by gas liquid chromatography. + Counts less than twice background, precluding accurate determination.

D. Autoradiographic Localisation of ^{14}C-labelled Oleic Acid in Human Atherosclerotic Lesions

Localisation of ^{14}C-labelled lipid to sudanophilic mononuclear cells was evident in all lesions studied. This is illustrated in Figures 26 to 32. Figure 26 is an autoradiograph of a lesion from case 1, where there was visible fatty streaking in the thoracic aorta. This lesion was essentially cellular though less so at the intimomedial junction. Most of the ^{14}C present is localised in the sudanophilic mononuclear cells. A cluster of nonsudanophilic or faintly sudanophilic mononuclear cells in the upper right-hand corner of the photomicrograph shows little localisation. The coloured autoradiograph of Figure 27 is a high-power view of the same fatty streak lesion, and is further evidence of the close relationship of ^{14}C-labelled oleic with foam cells.

In Figure 28 a more fibrous lesion from case 4 is shown. Spindle-shaped cells can be seen which have few related silver grains, in contrast to the sudanophilic mononuclear cells with which more label is associated. On the luminal side of the lesion shown in Figure 29, large foam cells have localised ^{14}C-labelled oleic acid and its metabolic derivatives; there is some localisation by cells with pyknotic nuclei and fragmented cytoplasm deep in the lesion. Figure 30 makes the comparison, at greater magnification and in colour, of the localisation of label by foam cells and the failure to localise by spindle-shaped cells. Again, in Figures 31 and 32 coloured autoradiographs of lesions from case 4, viewed under oil immersion, demonstrate the concentration of ^{14}C-labelled oleic in the region of intimal foam cells.

Grain counts carried out for representative sections from all the cases studied are shown in Table 24. This data confirms quantitatively the findings illustrated in Figures 26 to 32.

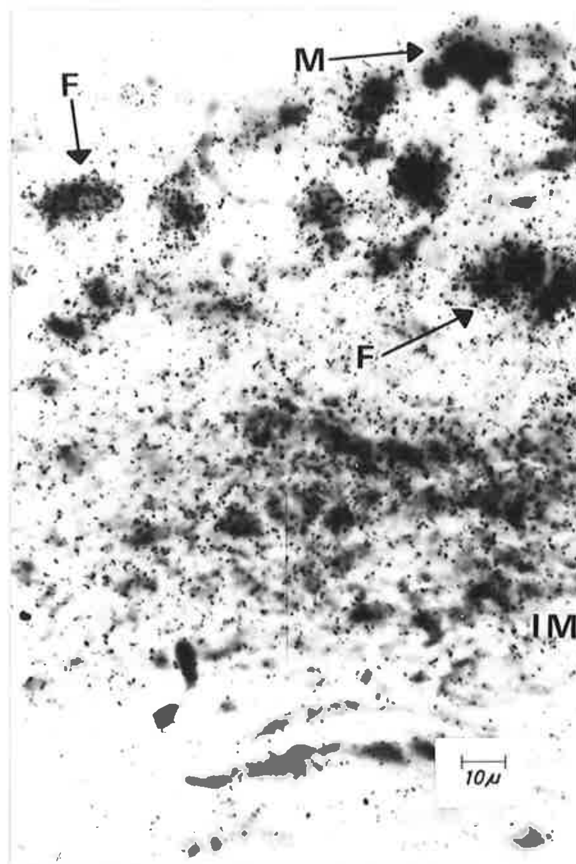

Figure 26

Autoradiograph of an aortic fatty streak, incubated with ^{14}C-labelled oleic acid, from an 18-year-old male (case 1). The luminal side of the vessel is uppermost. Localisation of the ^{14}C-labelled fatty acid by intimal foam cells (F) is apparent. There is little label associated with a cluster of non-sudanophilic or faintly sudanophilic mononuclear cells (M). The region of the intimo-medial junction is denoted IM. Haematoxylin and Sudan IV. Exposure time, 10 days.

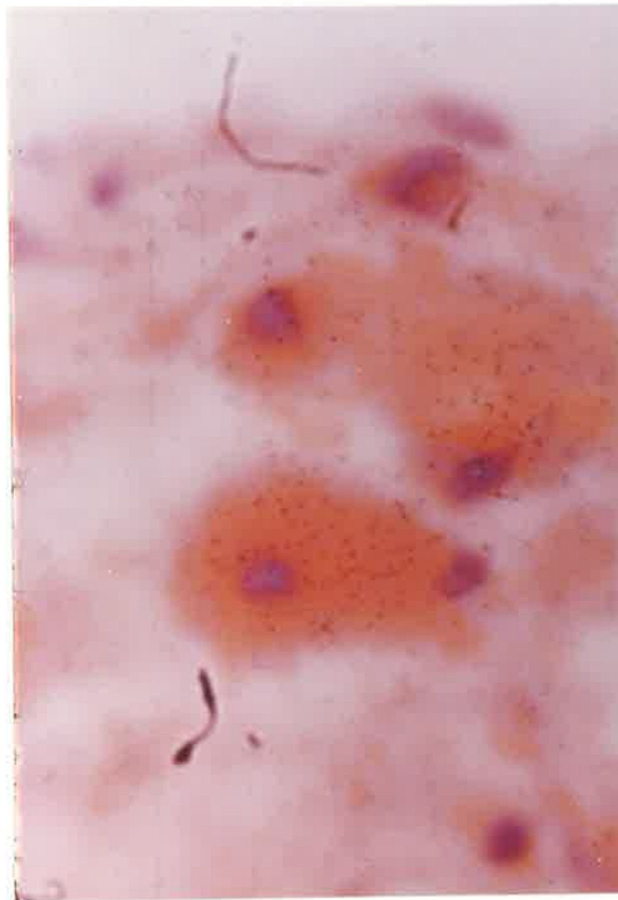

Figure 27

The same fatty streak lesion as in figure 26, viewed under oil immersion, confirming the localisation of silver grains to foam cells. Haematoxylin and Sudan IV, X 1000. Exposure time 8 days.

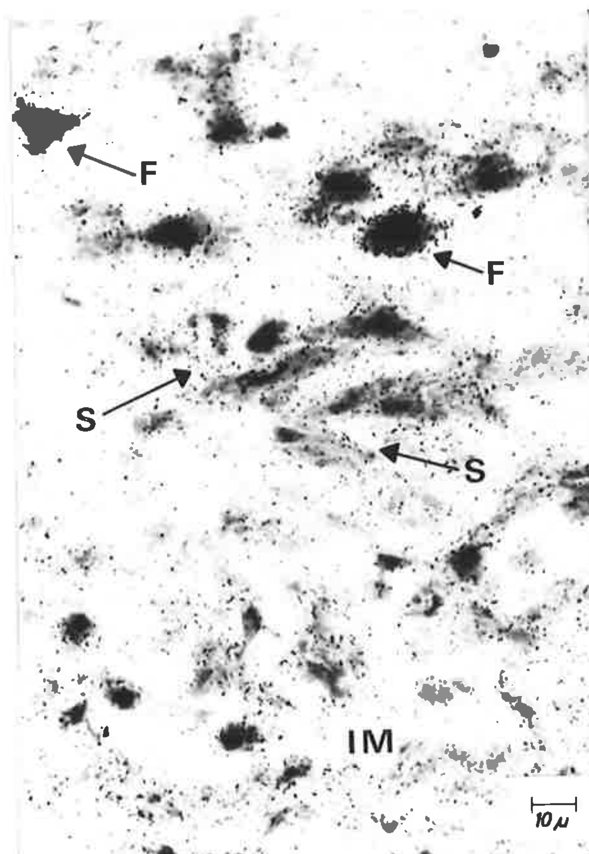

Figure 28

Autoradiograph of an aortic fibro-fatty lesion from a 32-year-old male (case 4). The luminal side is uppermost. There is less localisation by spindle-shaped cells (S) than by sudanophilic monoculears (F). The intimomedial junction is denoted IM. Haematoxylin and Sudan IV. Exposure time, 8 days.

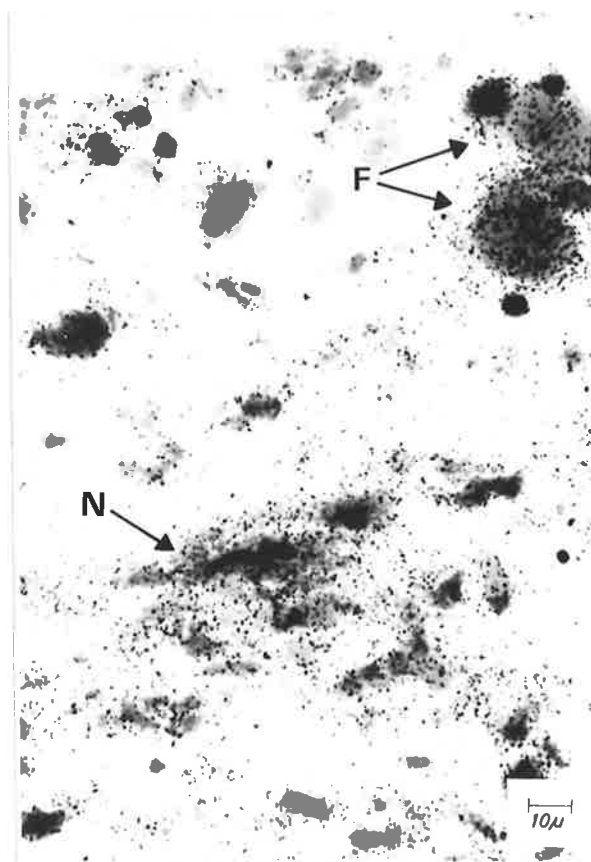

Figure 29

Autoradiograph of a lesion from the same vessel as in Figure 28. Localisation of ^{14}C to large foam cells (F) on the luminal side of the lesion (upper aspect of figure) is marked. Somewhat necrotic cells (N) are seen deeper in the lesion. Haematoxylin and Sudan IV. Exposure time, 8 days.

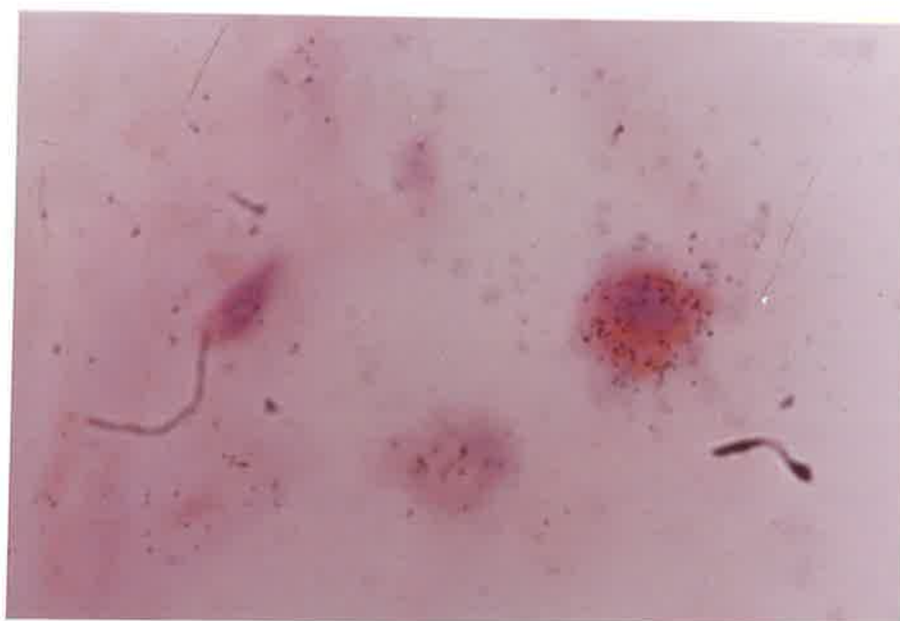

Figure 30

Autoradiograph of an aortic fibro-fatty lesion, incubated with ^{14}C-labelled oleic acid, from a 48-year-old male (case 2), viewed under oil immersion. Localisation of silver grains to a foam cell is apparent, but there is little lable in the region of a spindle - shaped cell. Haematoxylin and Sudan IV, X 1000. Exposure time, 36 days.

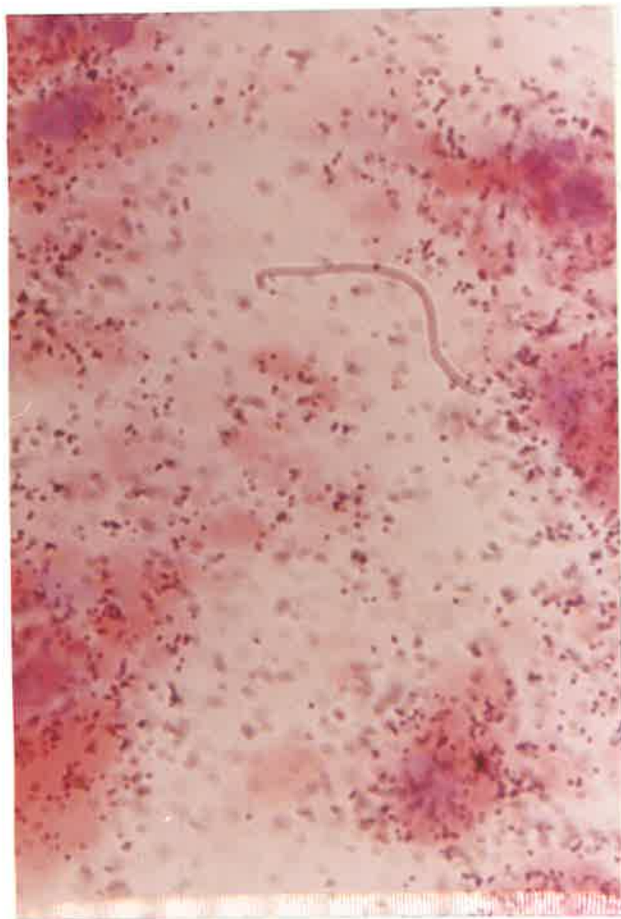

Figure 31

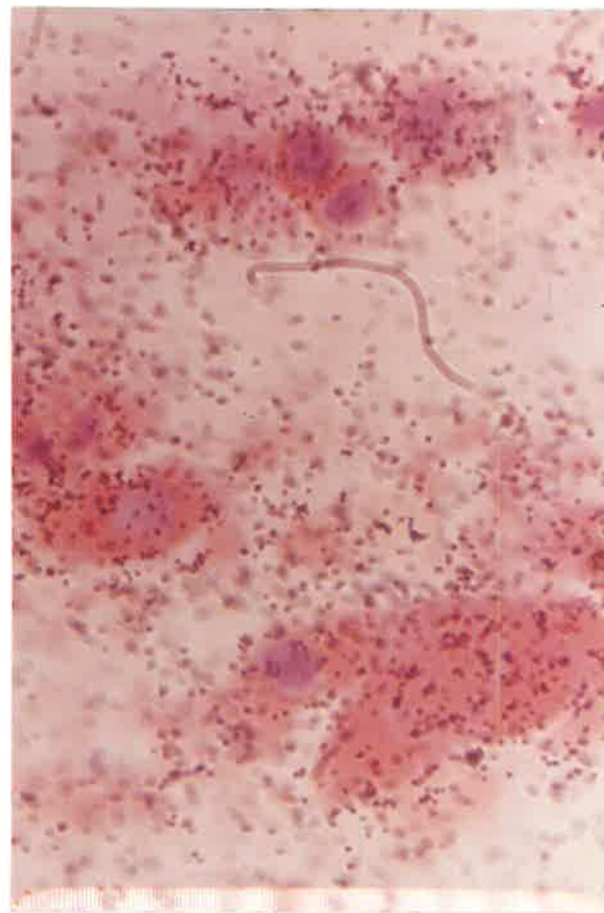

Figure 32

Figures 31 and 32

Autoradiographs of aortic lesions from case 4, viewed under oil immersion. Localisation of ^{14}C-labelled oleic acid by intimal foam cells is evident. It is possible that it is a giant cell rather than a cell cluster which is seen in the upper right-hand corner of Figure 31. Haematoxylin and Sudan IV, X 1000. Exposure time, 8 days.

TABLE 24

Autoradiograph Grain Counts (no./100 μ^2)[a]

Case no.	Lesion type	Intima: Foam cells	Intima: Nonsudanophilic round mononuclear cells	Intima: Spindle-shaped cells	Intima: Extracellular	Media
			Aorta			
1	Fatty streak	15.6	5.0		2.9	1.1
2	Fibrofatty	15.3	4.9	4.1	2.5	1.1
3	Fibrofatty	17.7	4.0	2 .6	2.2	1.2
4	Fibrofatty	18.7	5.8	4.7	4.1	4.4
			Renal Artery			
4	Fibrofatty	15.2	3.6	4.5	2.4	3.6
			Iliac Artery			
4	Fibrofatty	12.6	2.7	1.8	1.1	1.1

a At least 6000μ^2 assessed for each feature

I. Discussion

Most of the investigations in which human arterial wall has been incubated *in vitro* have used arteries obtained either post mortem or by surgery. Post-mortem material is usually not available immediately after death and post-mortem changes make interpretation of metabolic data uncertain. Surgically removed arteries commonly contain advanced and complicated lesions, so that use of this material to study metabolic changes in the early atherosclerotic lesion in man is limited. The availability of vessels obtained from renal transplant donors provided an opportunity to study the lipid metabolism of early atherosclerotic lesions *in vitro* within 1 to 2 hours of clinical death. Thus most of the advantages in studying experimental lesions were retained while the ultimately important human lesion was studied.

The data indicate that in the early human atherosclerotic lesion, fatty acid is actively taken up and incorporated into phospholipid, triglyceride, and cholesterol ester. The percentage of fatty acid diverted to cholesterol ester was higher in the atherosclerotic lesion than in the adjacent normal intima, and this was more marked in the fatty-streak lesion than in the fibrofatty lesion. The specific activity of the oleic acid in the cholesterol ester was not greater in the lesion than in the normal intima. Such data are available for only one time interval in each experiment, however, so that an adequate comparison of the fractional turnover of cholesterol ester in the lesion and normal intima cannot be made. However, as far as they go, the specific activity data provide no indication of increased fractional turnover time of cholesterol ester in the lesion as compared with the normal. Presumably, the amount of ^{14}C-labelled oleic acid incorporated into cholesterol ester *in vitro* per unit weight of intima was increased in the lesion.

The data presented is inadequate to assess the route of incorporation of fatty acid into either phospholipid or cholesterol ester. The specific activity of the phospholipid was much higher than that of the cholesterol ester at all time intervals, so that lecithin : cholesterol acyl transferase activity cannot be excluded as a source of cholesterol ester fatty acid.

The findings reported in this section relating to the incorporation of oleic acid into various combined lipids are essentially similar to those reported in the previous section for cholesterol-fed rabbits. The type of phospholipid into which the oleic acid was incorporated in the present study also closely parallels that demonstrated for the rabbit intima, whether normal or atherosclerotic.

The study in vitro of atherosclerotic aortae from cholesterol-fed rabbits indicated that most of the uptake and incorporation into lipid occurs in intimal foam cells. This observation has been confirmed for both the human fatty-streak and fibro-fatty lesion. Most of the ^{14}C present in the lesion after incubation was present as phospholipid, but varying amounts of labelled triglyceride and cholesterol ester were also present, together with a significant amount of unesterified ^{14}C-labelled oleic acid. Localisation by autoradiography, therefore, cannot provide information regarding incorporation into a single lipid moiety, but only the total process of fatty acid uptake and its incorporation into phospholipid, triglyceride, and cholesterol ester. It must be further assumed that, if the oleic acid is present mainly as combined lipid in the foam cells, the combined lipids were not formed elsewhere and transferred to the foam cells. In the previous section of this thesis, these possibilities have been considered and excluded as far as the rabbit atherosclerotic lesion is concerned.

It is likely, then, that the incorporation of fatty acid into phospholipid, triglyceride, and cholesterol ester by the lesion is a foam cell phenomenon. Whether fatty acid synthesized in the human atherosclerotic lesion is subsequently incorporated into combined lipid by foam cells is a question left unanswered.

SECTION 4

PHOSPHOLIPID SYNTHESIS BY RABBIT ATHEROSCLEROTIC AORTA IN VITRO

A. Introduction

The phospholipid content of the rabbit atherosclerotic intima increases as the lesion progresses (McCandless and Zilversmit, 1956) and much of this phospholipid is located in foam cells (Day, 1962). Further, the phospholipid of the rabbit atherosclerotic lesion arises principally through synthesis *in situ* (Zilversmit, 1959). Thus, the question arises as to whether the foam cells are responsible for phospholipid synthesis in the atherosclerotic lesion. Macrophages incorporate ^{32}P-labelled phosphate into phospholipid and this incorporation is stimulated by the uptake of cholesterol (Day, Fidge and Wilkinson, 1966). Foam cells isolated from rabbit atherosclerotic aortae taken up various lipid precursors and incorporate these precursors into phospholipid and other lipid fractions (Day, Newman and Zilversmit, 1966; Day and Wilkinson, 1967; Day and Tume, 1969). The autoradiographic studies already reported in this thesis also indicate that foam cells may be responsible for fatty acid incorporation into phospholipid in the atherosclerotic lesion.

In the investigations reported in this section ^{14}C-labelled choline was used as a precursor for phospholipid synthesis and for related autoradiographic studies. In addition, by using ^{3}H-labelled oleic acid as a precursor, and subjecting the tissues to acetone extraction, all the lipid moieties, with the exception of phospholipid, could be removed, allowing assessment of sites of formation of phospholipid itself.

B. Uptake and Incorporation of ^{14}C-labelled Choline Into Phospholipid

Three experiments were carried out using choline chloride (methyl-^{14}C) as precursor.

In the first experiment, thoracic aortae were obtained from three rabbits fed cholesterol for one month, two months and three months respectively. The aortae were divided longitudinally into halves and each half incubated in 5 ml of medium (50 : 50 Hanks' solution : normal rabbit serum) to which was added a tracer dose of ^{14}C-labelled choline (3.04×10^{6} cpm). Incubation was carried out for four hours. After this period, the aortic halves were washed thoroughly in 0.9% sodium chloride solution and both halves from each of the three rabbits were fixed in 1% calcium chloride in 4% formol saline for four days and finally washed in running water for a further period of twenty-four hours. One aortic half from each animal was reserved for autoradiography (see below), the other half being reserved for radioassay. In the latter half, the intima was stripped from the media and adventitia and lipid extracts of the two portions prepared separately. In order to determine the amount of ^{14}C-labelled choline present in non-lipid components, the upper phase of the Folch wash and the subsequent washings, together with the tissue residue were collected and counted separately. The lipid extract was counted and the distribution of label among individual phospholipids, separated by thin layer chromatography, determined.

In the second experiment, the thoracic aorta obtained from a rabbit fed cholesterol for three months was divided longitudinally into thirds, each third being incubated in a medium containing 50 : 50 Hanks' solution ; normal rabbit serum, to which had been added 7×10^{6} cpm of ^{14}C-labelled choline. Incubation was carried out for 1, 3 or 4 hours respectively for each of the thirds. A segment of each third was reserved for autoradiography (see below) and the remainder used to prepare lipid extracts of intima and media for counting of ^{14}C, phospholipid T.L.C. and lipid phosphorus determination. The specific activity of the total phospholipid at the three time intervals for both media and intima was calculated.

In the third experiment, duplicate halves of a thoracic aorta were obtained from a rabbit fed cholesterol for 4 months and incubated for the short periods of 15 and 30 minutes respectively. Incubation was carried out in 5 ml of Hanks' solution : normal rabbit serum containing 25 x 10^6 cpm of ^{14}C-labelled choline. Most of the artery was used for radioassay, but portions were taken for autoradiography (see below).

The uptake of ^{14}C-labelled choline and its incorporation into phospholipid in atherosclerotic intima and underlying media is shown in Table 25. ^{14}C-labelled choline was taken up by both intima and media and incorporated into phospholipid in each of the three experiments. In the second series of experiments, the specific activity of the phospholipid labelled was determined in relation to time. In the intima, phospholipid specific activity increases with time. Some levelling-off of specific activity in the media occurred after 3 hours.

The distribution of ^{14}C-labelled choline between individual phospholipids in the intima and media for the three experiments is shown in Table 26. Practically all of the ^{14}C-labelled choline was incorporated into choline containing phospholipids, mainly into lecithin, but partly into sphingomyelin and lysolecithin. The pattern of incorporation is similar in the intima and the media, and in all of the arteries studied.

C. Autoradiographic Localisation of Phospholipid Formation from ^{14}C-labelled Choline in Rabbit Atherosclerotic Lesions

The arterial specimens set aside for autoradiography in the three experiments described above were processed in the way described under "Materials and Methods".

TABLE 25

Uptake and Conversion of ^{14}C-labelled Choline to Phospholipid by Rabbit Atherosclerotic Aortae

Period rabbit cholesterol fed (months)	Incubation time (hours)	Intima: % of Incubation medium ^{14}C-choline present as: Lipid	Intima: Nonlipid	Intima: Phospholipid specific radioactivity (cpm /μg lipid P)	Media: % of Incubation medium ^{14}C-choline present as: Lipid	Media: Nonlipid	Media: Phospholipid specific radioacticity (cpm/μg lipid P)
1	4	10.8	0.19	-	-	-	-
2	4	11.3	0.31	-	-	-	-
3	4	10.8	0.16	-	-	-	-
3	1	1.21	-	2830	0.81	0.064	4560
3	3	1.31	0.083	6130	2.61	0.131	12200
3	4	2.69	0.089	8360	1.79	0.115	12500
4	0.25	0.13	0.006	-	0.022	0.002	-
4	0.5	0.39	0.011	-	0.088	0.004	-

TABLE 26

Percentage Distribution of Lipid ^{14}C-Labelled Choline in Rabbit Atherosclerotic Aortae

Period rabbit cholesterol fed (months)	Incub-ation time (hours)	Intima				Media			
		Phospholipid			Other lipids	Phospholipid			Other lipids
		Lyso-lecithin	Sphingo-myelin	Leci-thin		Lyso-lecithin	Sphingo-myelin	Leci-thin	
1	4	6.5	5.0	87.8	0.7	-	-	-	-
2	4	6.4	3.8	89.2	0.7	-	-	-	-
3	4	6.8	4.2	88.3	0.8	-	-	-	-
3	1	13.4	2.6	81.9	2.3	9.0	4.1	83.7	3.2
3	3	12.2	1.2	84.7	2.0	7.2	3.0	87.2	2.7
3	4	7.5	3.2	86.6	2.8	11.7	2.4	83.9	2.1
4	0.25	7.2	2.5	87.5	2.6	6.0	4.2	81.9	7.7
4	0.5	6.5	2.0	89.1	2.4	5.9	2.8	88.9	2.4

Determination of the amount of non-lipid ^{14}C-labelled choline in portions of aortae subjected to the same washing procedures as those investigated autoradiographically was necessary if a valid assessment of sites of phospholipid synthesis was to be made. Choline is itself non-lipid, has non-lipid derivatives such as acetylcholine and, labelled in the methyl position, is a potential source of labelled labile methyl groups for other non-lipids. In fact, very little non-lipid choline remained in the vessels after the washing procedure and this data is given in Table 25. Therefore, autoradiographic localisation of ^{14}C-labelled choline in the arteries can be taken to indicate localisation of synthesized phospholipid.

From the chromatographic analyses, it is apparent that the chief phospholipid whose synthetic site was examined autoradiographically was lecithin.

Localisation of the ^{14}C-labelled phospholipid synthesized in the aorta is illustrated in Figures 33 to 36. Much of the ^{14}C is concentrated over the foam cell areas. There is some radioactivity, however, in areas between the cells, which are at times closely applied to one another, and also in the media. This is shown quantitatively by grain counts in Table 27. In all cases, the concentration of grains over foam cells exceeds that over other areas of the intima and, in all but one case, exceeds that over the media.

D. Uptake and Incorporation of ^{3}H-labelled Oleic Acid into Combined Lipid and Extraction of Lipid other than Phospholipid

The thoracic aorta obtained from a rabbit fed cholesterol for three months was divided longitudinally into halves and both

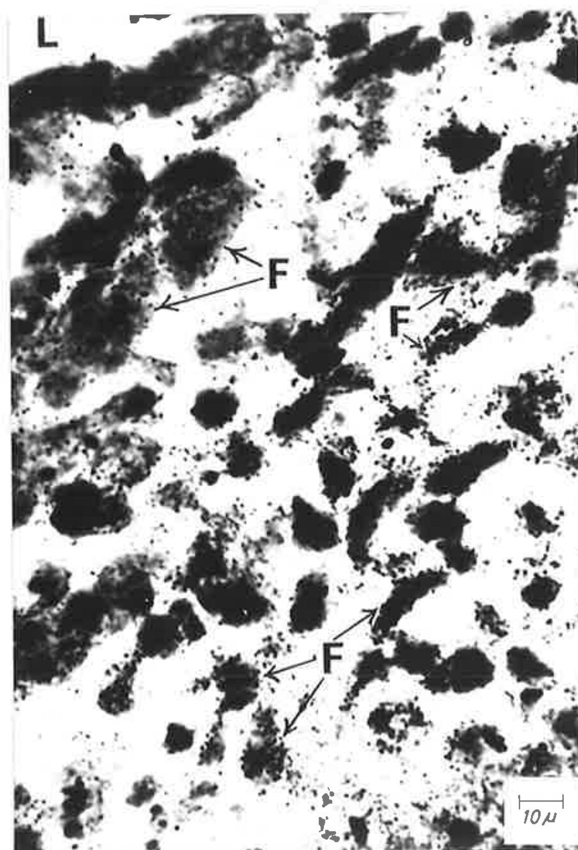

Figure 33

Autoradiograph of an atherosclerotic lesion incubated for 30 mins with ^{14}C-labelled choline. The aorta was obtained from a 23-week-old rabbit fed cholesterol for 17 weeks. The luminal aspect (L) is uppermost in the figure. Localisation of silver grains representing essentially choline-containing phospholipids, to variously shaped foam cells (F) is shown. Haematoxylin and Sudan IV. Exposure time 8 days.

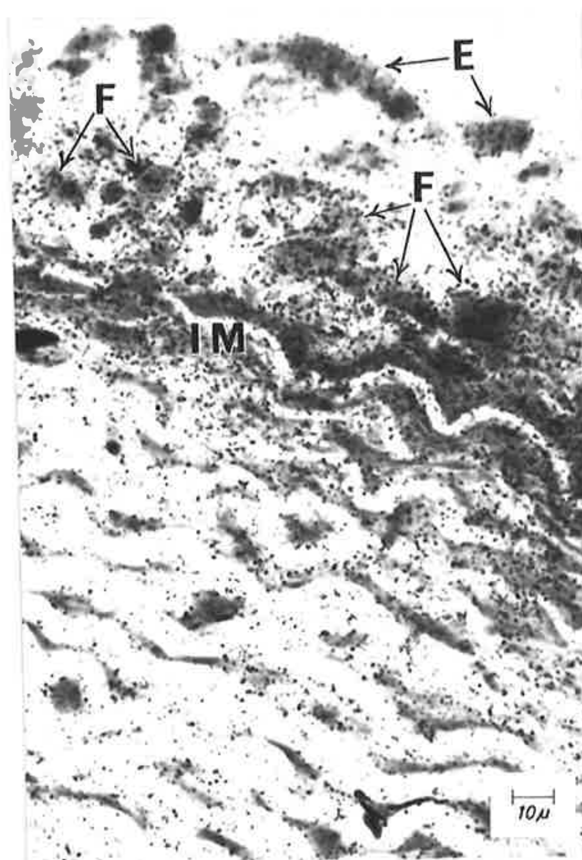

Figure 34

Autoradiograph of an atherosclerotic lesion incubated for 4 hours with ^{14}C-labelled choline. The aorta was obtained from a 10-week-old rabbit fed cholesterol for 4 weeks. Cells at the luminal border, possibly endothelial (E), exhibiting some sudanophilia, have localised ^{14}C-labelled choline. Foam cells (F) in the intima have also localised label. The intimo-medial junction is denoted IM. Some granulation is also evident in the media. Haematoxylin and Sudan IV. Exposure time 12 days.

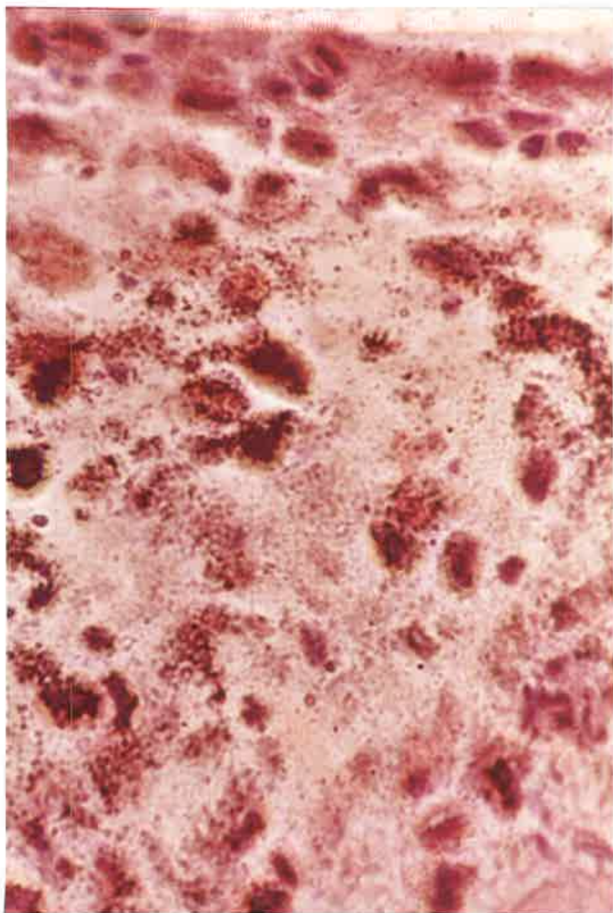

Figure 35

Colour autoradiograph of a lesion from the same rabbit as in figure 33, incubated with ^{14}C-labelled choline for the same time. Localisation of silver grains to foam cells is again apparent. Haematoxylin and Sudan IV, X 600. Exposure time, 8 days.

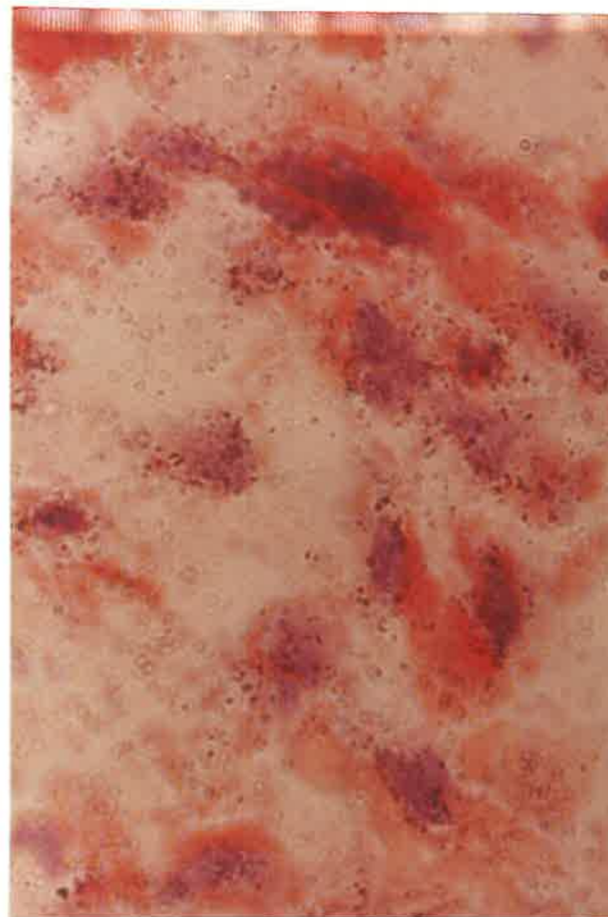

Figure 36

A high-power view of ^{14}C-labelled choline localisation by foam cells. Details as for Figure 33. Haematoxylin and Sudan IV, X 1000. Exposure time, 8 days.

TABLE 27

Grain Counts (no. grains/100 μ^2)[a] in Autoradiographs Prepared from Rabbit Aortae Incubated in Vitro with ^{14}C-labelled Choline

Period rabbit cholesterol fed (months)	Incubation time (hours)	Intima		Media
		Foam cells	Extracellular	
1	4	8.3	5.4	6.2
2	4	8.3	4.5	3.2
3	4	10.7	6.7	9.9
3	1	6.5	1.1	2.0
3	3	11.3	4.6	5.9
3	4	9.6	3.2	5.4
4	0.25	7.3	2.2	14.5
4	0.5	5.9	1.9	7.3

a At least 6000 μ^2 assessed for each feature.

halves incubated in 5.5 ml of medium containing 50 : 50 Hanks' solution : normal rabbit serum, together with a known amount (34.4 x 10^6 cpm) of ^{3}H-labelled oleic acid. After incubation for three hours, the aortic halves were washed thoroughly in 0.9% sodium chloride solution and reincubated in non-labelled 50 : 50 Hanks' solution : normal rabbit serum for a further period of 1 hour. One half was reserved for autoradiography. In the other half the intima was stripped from the media/adventitia and lipid extracts of the two portions prepared.

The uptake of ^{3}H-labelled oleic acid and its incorporation into different lipid fractions is shown in Table 28. In the intima, most of the oleic acid has been taken up and incorporated into cholesterol ester and phospholipid with a lesser amount incorporated into triglyceride. In the media, most of the oleic acid has been taken up and incorporated into triglyceride with smaller amounts incorporated into phospholipid and cholesterol ester.

E. Autoradiographic Localisation of Phospholipid Formation from ^{3}H-labelled Oleic Acid in Rabbit Atherosclerotic Lesions

The aortic half labelled with ^{3}H-labelled oleic acid and reserved for autoradiography as described above was processed in the way detailed under "Materials and Methods". Thus, on the same glass slide were mounted control sections containing various labelled lipids and sections extracted with cold acetone containing, almost exclusively, labelled phospholipid.

The localisation of ^{3}H-labelled oleic acid in the lesion is shown in the coloured autoradiograph of Figure 37 and the black and white autoradiograph of Figure 38. Most of the radioactivity is concentrated over foam cells especially towards

TABLE 28

Uptake and Incorporation of ^{3}H-labelled Oleic Acid into Lipids of Atherosclerotic Rabbit Aorta Incubated in Vitro

	Percentage uptake from incubation medium	Percentage distribution				
		Phospholipid	Diglyceride	Fatty Acid	Triglyceride	Cholesterol ester
Intima	2.69	28.3	3.2	10.7	5.7	52.2
Media	2.15	14.5	15.6	7.4	54.3	8.1

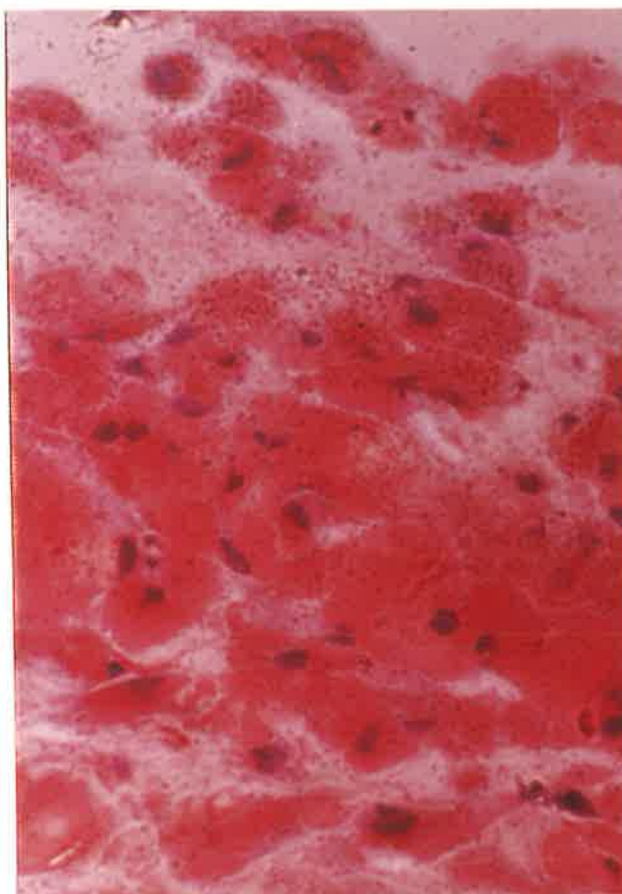

Figure 37

Autoradiograph of an atherosclerotic lesion incubated with ^{3}H-labelled oleic acid. The aorta was obtained from a rabbit aged 18 weeks and fed cholesterol for 12 weeks. The luminal side is uppermost. The more superficially-situated foam cells localise ^{3}H. Haematoxylin and Sudan IV, X 600. Exposure time, 9 days.

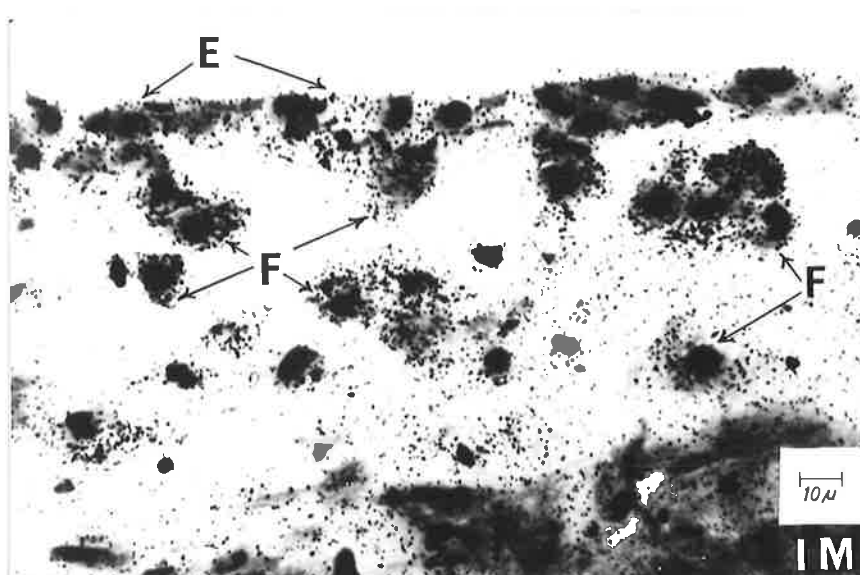

Figure 38

Autoradiograph of an atherosclerotic lesion incubated with ^{3}H-labelled oleic acid. The aorta was obtained from a rabbit aged 18 weeks and fed cholesterol for 12 weeks. Some endothelial cells (E) localise the label representing chiefly free fatty acid, phospholipid, and cholesterol ester. So also do superficial intimal foam cells (F). There is less label in the region of cells deeper in the intima. Intimo-medial junction is denoted IM. Haematoxylin and Sudan IV. Exposure time 9 days.

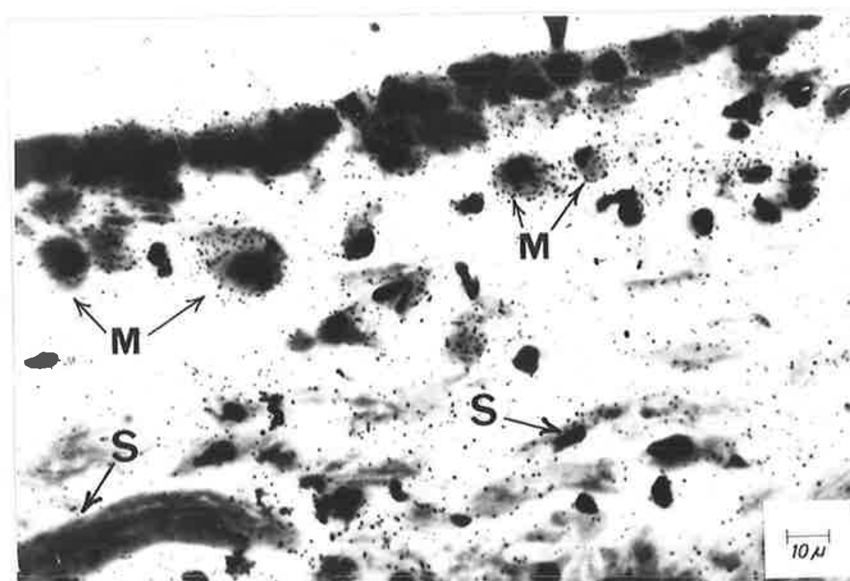

Figure 39

An adjacent section of the same lesion as in Fig. 38, but extracted with acetone in the cold. It is apparent that phospholipid formed from oleic acid is localised to superficial intimal round mononuclears (M) with little label in the region of spindle-shaped cells (S). Haematoxylin and Sudan IV. Exposure time 9 days.

the luminal aspect. However, since silver grains represent unesterified ^{3}H-labelled oleic acid as well as that incorporated into cholesterol ester and phospholipid, specific information about phospholipid synthesis is not provided. This is the reason sections obtained from the same aorta were subjected to acetone extraction. Under these circumstances, most of the free fatty acid, triglyceride and cholesterol ester was removed and, in the sections studied, 88.9% of the ^{3}H-labelled oleic acid was present in phospholipid. Thus, the autoradiographs prepared from these sections, relate to the localisation of ^{3}H-labelled oleic acid uptake and its specific incorporation into phospholipid. Figure 39 is an autoradiograph of a section so treated. The localisation to round mononuclear cells, presumed to be foam cells, is still apparent, although, of course, the total amount of radioactivity present is not as great as in the section shown in Figure 38. Grain counts, given in Table 29, provide quantitative information about the autoradiographs from both the control and acetone-extracted sections. Considerable concentration or label occurs over intimal foam cells mainly nearer the luminal aspect. In the acetone-extracted sections, the grain counts over the foam cells are approximately half those in the controls, but still considerably above those in the other cells present in the intima and above those in the media.

F. Discussion

The uptake of ^{14}C-labelled choline by rabbit atherosclerotic aortae and its incorporation into phospholipid confirms previously reported work (Newman, Day and Zilversmit, 1966). The incorporation of choline into the choline-containing phospholipid, lecithin, with lesser amounts into sphingomyelin and lysolecithin, is the expected product of such incorporation. It will be noted, as has been observed for ^{32}P-labelled phosphate (Newman, Day and Zilversmit,

TABLE 29

Grain Counts (No. Grains/100 μ^2)[a] in Autoradiographs Prepared from Rabbit Aortae Incubated with ^{3}H-labelled Oleic Acid

	Intima						Media
	Foam cells			Non-sudan-ophilic mononu-clears	Spindle-shaped cells	Extra cellular	
	Luminal	Inter-mediate	Deep/inti-momedial				
Control [b]	23.3	4.2	1.0	0.9	-	2.4	0.6
Acetone ex-tracted [c]	11.2	0.7	0.6	-	0.7	0.6	0.7

a At least 6000 μ^2 counted for each feature.

b "Foam cells" include lipid-containing cells of all kinds, including those of a more spindle shape. Endothelial cells are at times somewhat sudanophilic and localize label.

c In the case of the acetone-extracted section there is scarcely any sudanophilia and therefore "foam cells" are not recognizable by this feature. Round mononuclears are assessed instead and those spindle-shaped cells which were sudanophilic are now grouped under "spindle-shaped cells". Endothelial cells again apparently localise label in the acetone-extracted section.

1966) and for ^{14}C-labelled fatty acid (this thesis) that the formation of sphingomyelin is considerably less than that of lecithin, assuming both are formed from a common precursor pool. In the atherosclerotic intima, the amount of sphingomyelin increases considerably and in the rabbit lesion comprises approximately one third of the phospholipid present in the intima (McCandless and Zilversmit, 1956). It seems possible that the accumulation of sphingomyelin is not associated with its increased synthesis in the wall, but rather with reduced removal by hydrolysis, and there is some experimental evidence to support this view (Rachmilewitz, et al, 1967). In a recent paper by Morin (1968) it has also been shown that ^{14}C-labelled choline is taken up by normal and atherosclerotic rabbit aortae and incorporated into choline-containing phospholipids. The amount of choline taken up in these experiments, however, is very low and a significant proportion of the choline appears in the non-choline containing phospholipids, phosphatidyl serine and phosphotidyl ethanolamine. The difference between the present study and that of Morin, as far as the type of phospholipids formed is concerned, might be accounted for by the fact that Morin used choline-1-2-^{14}C rather than choline chloride (methyl-^{14}C).

The main objective of the present work was to find whether phospholipid synthesis was localised in intimal foam cells. Although isolated foam cells are able to incorporate ^{32}P-labelled phosphate, ^{14}C-labelled acetate and ^{14}C-labelled oleic acid into phospholipid, such synthesis may not represent more than a small proportion of the phospholipid synthesis which takes place in the atherosclerotic arterial wall. The present study does, however, provide definitive evidence for the view that foam cells are responsible for much of the phospholipid synthesis which occurs in the atherosclerotic intima.

The localisation to foam cells of phospholipid synthesized from choline was not as clear-cut as that when oleic acid was used as precursor. It is apparent from Table 27 that there is some label present in areas of the intima other than the foam cells and also in the media. Parker _et al_. (1966) have shown that the incorporation of fatty acid into phospholipid by rabbit atherosclerotic aortae correlates closely with the formation of membranous organelles. It is likely that some choline is incorporated into phospholipid in membranes of cells other than foam cells both in the intima and in the media and that some spread of label occurs for this reason. Again, the more decisive localisation of phospholipid synthesis in the intima when oleic acid was used as precursor may reflect the different pathways taken by the two precursors in their incorporation into phospholipid. These pathways are detailed in the "General Introduction".

SECTION 5

PHOSPHOLIPID SYNTHESIS BY THE HUMAN ARTERIAL WALL IN VITRO

A. Introduction

In the preceding section it has been shown by autoradiography that the phospholipid synthesis which occurs in rabbit atherosclerotic intima incubated *in vitro* takes place essentially in foam cells. In the work reported in the present section, the precursors ^{14}C-labelled choline and ^{3}H-labelled oleic acid have again been used to investigate, by the combined biochemical and autoradiographic approach, sites of phospholipid formation in human atherosclerotic lesions.

B. Uptake and Incorporation of ^{14}C-labelled Choline into Phospholipid

Abdominal aorta used was obtained from a male renal transplant donor, aged 20 years, who died of head injuries. Fatty streak lesions (W.H.O., Grade I; American Heart Association Grading Committee, Grade 2) were present. Femoral artery was obtained at the time of mid-thigh amputation from a 74 year old female with peripheral vascular disease. The atherosclerotic lesion was complicated by an organized thrombus with a further super-added foam cell lesion (W.H.O., Grade III; American Heart Association Grading Committee, Grade 5 - 6).

Portion of the abdominal aorta was incubated in 5 ml 50 : 50 Hanks' solution : normal human serum containing 1.62 x 10^{6} cpm of ^{14}C-labelled choline, the femoral artery in 10 ml of the same medium containing 3.20 x 10^{6} cpm ^{14}C-labelled choline. Following incubation for 2 hours, the arterial specimen was washed thoroughly in saline and then fixed in 1% calcium chloride in 4% formol saline for 4 days. This was followed by a further wash in running water for 24 hours. A representative piece of tissue was then reserved for autoradiography (see below). Lipid extracts were prepared from the remaining tissue, lesion being dissected

from normal and intima stripped from media. The amount of non-lipid ^{14}C-labelled choline was assessed by counting the upper phase of the Folch wash, together with the residue as described in Section 4.

^{14}C-labelled choline was taken up and incorporated into phospholipid by both intima and media and by normal and atherosclerotic portions of the two arteries studied (Table 30). Up to 13% of the choline present in the incubation medium was taken up and incorporated into phospholipid by the various portions of the artery. Since the area of artery involved varied considerably no significance can be attached to the relative uptake and incorporation in the lesion compared with the normal artery; the difference indicated in Table 30 reflected by and large and difference in relative size of tissue or lesion. The individual phospholipids labelled in the various portions of the two arteries studied are also shown in Table 30. Most of the choline was incorporated into lecithin with smaller amounts of label incorporated into the other choline containing phospholipids, sphingomyelin and lysolecithin. Very little label appeared in the non-choline containing phospholipids. There were no obvious differences in the pattern of labelling of individual phospholipids between intima and media or between the two types of lesion studied, although there is no sufficient data to assess less than gross differences.

C. <u>Autoradiographic Localisation of Phospholipid Formation from ^{14}C-labelled Choline in Human Atherosclerotic Lesions</u>

Autoradiographs were prepared as described under "Materials and Methods".

The localisation of ^{14}C-labelled phospholipid formation in

TABLE 30

Percentage Distribution of Lipid ^{14}C-Choline in Human Arteries

Vessel	Lesion type	Intima				
		% Conversion of medium ^{14}C-choline to phospholipid	Lysolecithin	Sphingomyelin	Lecithin	Other lipids
Abdominal aorta	Normal	2.30	4.0	8.4	82.1	6.0
	Fatty streak	0.10	8.2	10.1	75.1	6.8
Femoral	Normal	0.17	3.0	2.3	92.4	2.5
	Complicated (organized thrombus)	1.69	3.0	4.6	91.4	1.1
		Media				
Abdominal aorta		13.1	8.9	4.7	82.1	4.5
Femoral	Normal	0.16	2.7	1.8	94.3	1.2
	Complicated (organized thrombus)	1.17	4.1	4.0	91.5	0.5

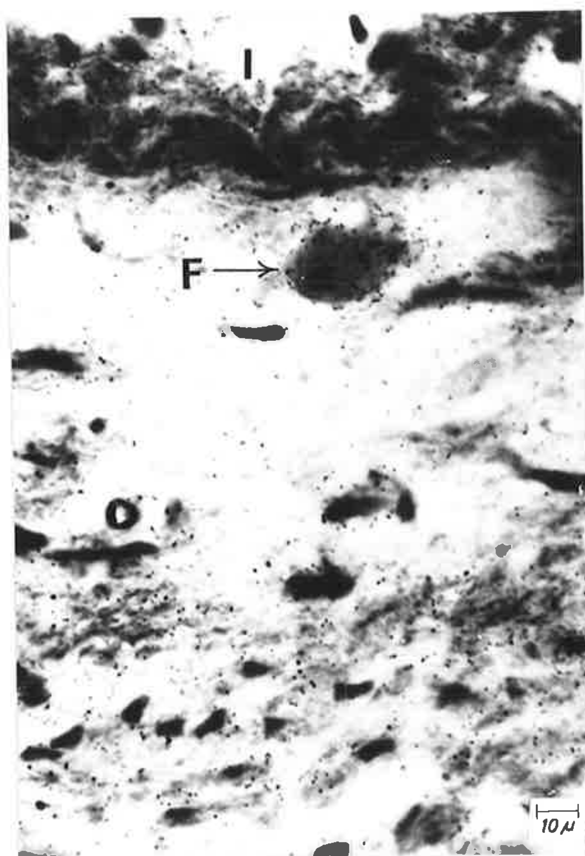

Figure 40

Autoradiograph of an early fatty streak in the abdominal aorta of a 20-year-old man. Incubated with ^{14}C-labelled choline *in vitro*. The intima (I) is uppermost. In the region of the intimo-medial junction, a sudanophilic round mononuclear (F) has localised phospholipids containing ^{14}C-labelled choline. Haematoxylin and Sudan IV. Exposure time 7 days.

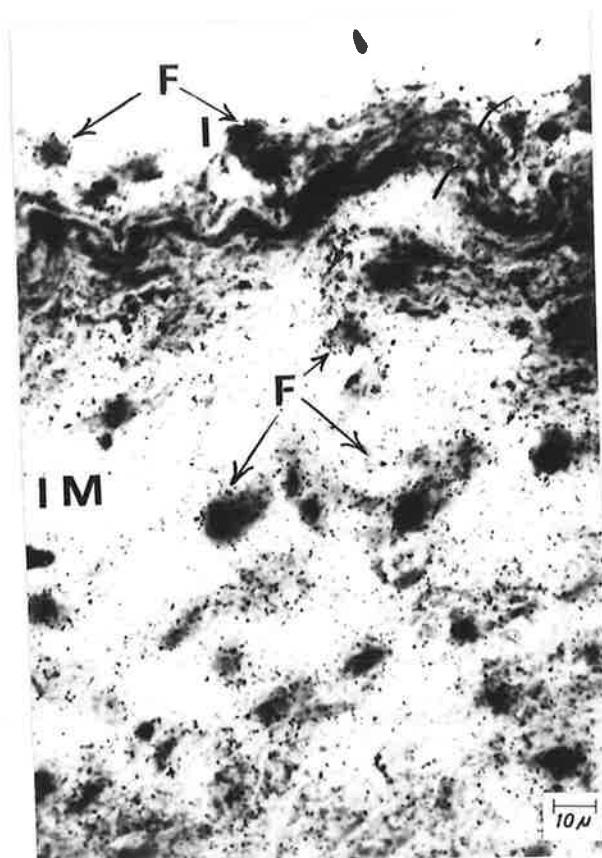

Figure 41

Autoradiograph of a lesion from the same vessel as Fig. 40. Sudanophilic cells (F) in the intima (I) and disorganised intimo-medial junction region (IM) have in their vicinity a concentration of silver grains representing ^{14}C-choline-containing phospholipids. Haematoxylin and Sudan IV. Exposure time 19 days.

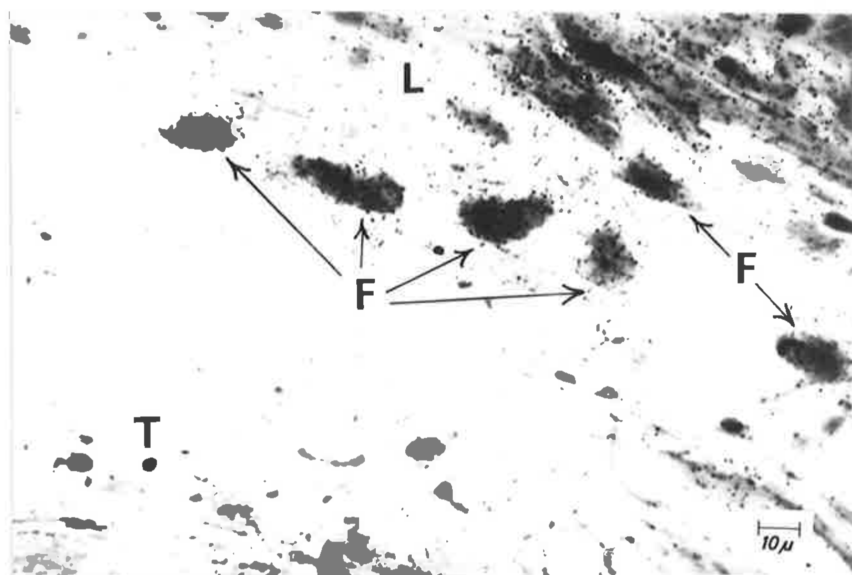

Figure 42

Autoradiograph of an atherosclerotic lesion complicated by an organised thrombus (T) and a super-added foam cell lesion (L) in the femoral artery from a 74-year-old female. Foam cells (F) have localised phospholipids formed from the ^{14}C-labelled choline with which the artery was incubated. Haematoxylin and Sudan IV. Exposure time 19 days.

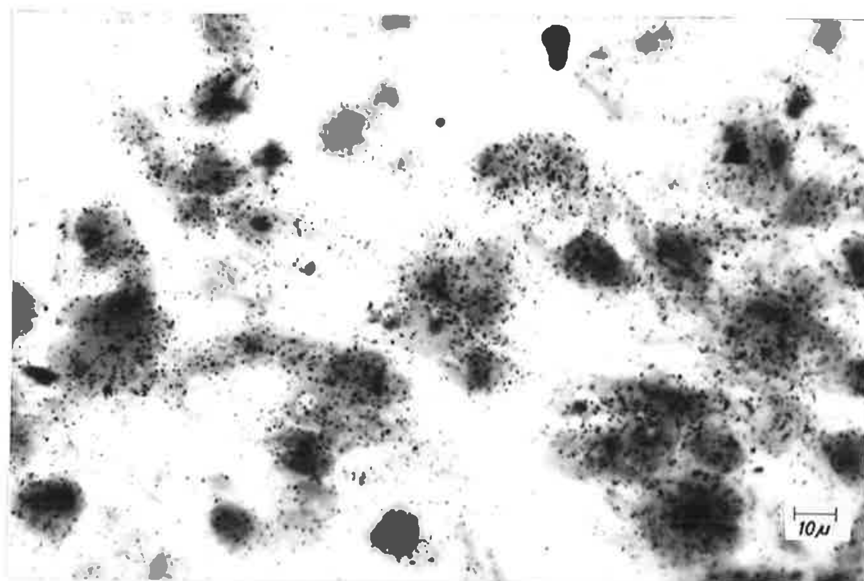

Figure 43

Autoradiograph of another part of the lesion shown in Fig. 42, showing the localisation of ^{14}C-labelled lipid in foam cells. Haematoxylin and Sudan IV. Exposure time 20 days.

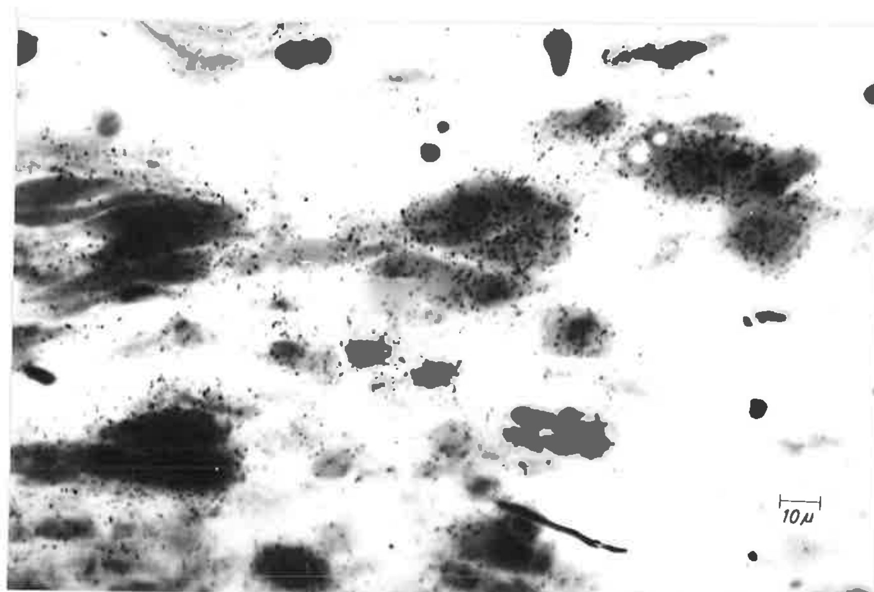

Figure 44

Autoradiograph of the atherosclerotic intima of the femoral artery showing concentration of silver grains in the region of variously-shaped foam cells. The vacuoles with no overlying silver grains in the cell in the upper right hand corner are presumably artifacts. Haematoxylin and Sudan IV. Exposure time 20 days.

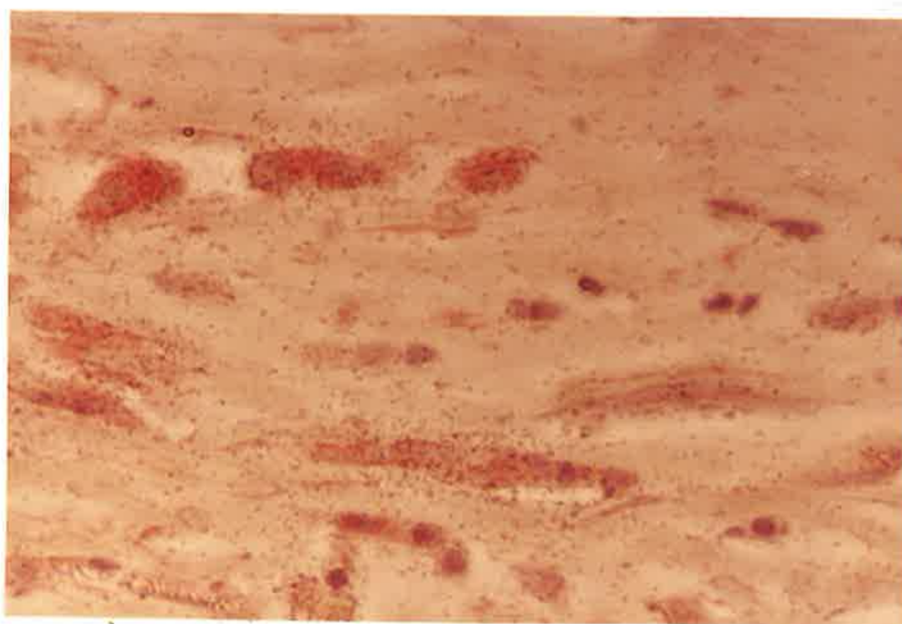

Figure 45

Colour autoradiograph of another part of the lesion shown in Figure 42. Silver grains representing ^{14}C-labelled choline are localised in the region of variously-shaped foam cells. Non-sudanophilic or faintly sudanophilic spindle-shaped and small round cells have little label associated with them. Haematoxylin and Sudan IV, X 600. Exposure time, 20 days.

the artery to foam cells is shown in the autoradiograph of Figures 40 - 45. More than 90% of the ^{14}C-labelled choline in the arteries used for autoradiography was present as phospholipid so that localisation of phospholipid formation in the artery can be determined from silver grain localisation of the radioactivity present.

Localisation to foam cells in both the aortic fatty streak and in the complicated lesion in the femoral artery is confirmed by the more quantitative information given in Table 31. Grain counts in both cases studied show clear localisation of ^{14}C to the foam cells present. The other cell types present in the intima and the media do not exhibit the same localisation.

D. Uptake and Incorporation of ^{3}H-labelled Oleic Acid into Combined Lipid and Extraction of Lipid Other than Phospholipid

In the case of the abdominal aorta a segment was also incubated with 34.4 x 10^{6} cpm of oleic acid-9-10-^{3}H complexed to the human serum albumin of the incubation medium. The medium consisted of 0.5 ml sodium oleate-9-10-^{3}H, and 5 ml of 50 : 50 Hanks' solution : normal human serum. After incubation for 3 hours the artery was thoroughly washed in 0.9% sodium chloride solution and reincubated in non-labelled medium for 1 hour to reduce the amount of free ^{3}H-labelled oleic acid.

Significant uptake of oleic acid, followed by incorporation into phospholipid and cholesterol ester, by both lesion and normal intima occurred as reported for ^{14}C-labelled oleic acid in Section 3. Some of the sections cut, were exposed to acetone extraction in the way that rabbit sections were (Section 4) in order to remove lipids other than phospholipid and so study specifically the localisation of phospholipid formation in the artery. The

TABLE 31

Grain Counts (no. grains/100 μ^2) on Autoradiographs Prepared from Human Atherosclerotic Lesions Incubated with ^{14}C-labelled choline [a]

Lesion	Intima				Media
	Foam cells	Nonsudanophilic round mononuclears	Spindle-shaped cells	Extra-cellular	
Complicated	14.8	1.5	0.3	0.2	0.1
Fatty streak	14.1	3.0	1.6	2.0	1.6

a At least 6000 μ^2 counted for each feature

distribution of label in control and in acetone-treated sections is shown in Table 32. The acetone treatment left most of the radioactivity as phospholipid. It was not possible, however, to remove all of the other lipid.

E. Autoradiographic Localisation of Phospholipid Formation from ^{3}H-labelled Oleic Acid in Human Atherosclerotic Lesions

Autoradiographs were prepared as described under "Materials and Methods".

Figures 46 and 47 show autoradiographs from control and acetone extracted sections of the atherosclerotic artery incubated with ^{3}H-labelled oleic acid. In both types of sections, the ^{3}H-labelled lipid is present mainly over the foam cells and this is confirmed by the grain counts shown in Table 33. There is little activity in other cells or in the normal intima or in the media.

F. Discussion

Atherosclerotic human arteries investigated *in vivo* (Zilversmit, McCandless, Jordan, Henly and Ackerman, 1961) or *in vitro* (Chobanian and Hollander, 1966) have been shown to incorporate ^{32}P-labelled phosphate into phospholipid. ^{14}C-labelled acetate and fatty acid have also been incorporated *in vitro* into phospholipid in atherosclerotic human arteries (Maggi, 1964; Parker *et al*. 1964: Chobananian and Hollander, 1966; this thesis section 3). Also, human umbilical artery has been found to take up and incorporate ^{14}C-labelled linoleic acid (Stein, Selinger and Stein, 1963) and labelled lysolecithin (Eisenberg, Stein and Stein, 1967) into phospholipid.

TABLE 32

Percentage Distribution of ^{3}H-labelled Oleic Acid Among Human Arterial Lipids of Autoradiograph Sections

	Phospho-lipid	Diglyceride	Fatty acid	Triglyc-eride	Choles-terol ester
Control	18.6	9.6	59.9	5.7	5.9
Acetone-extracted	50.7	20.3	25.0	3.9	0.0

TABLE 33

Grain Counts (no. grains/100 μ^2) on Autoradiographs Prepared from Human Fatty Streak Lesion Incubated with ^{3}H-labelled Oleic Acid

	Intima				Media
	Foam cells	Nonsudanophilic mononuclears	Spindle-shaped cells	Extracellular	
Control	13.2	2.7	2.9	2.7	0.6
Acetone-extracted	7.6[a]		2.2	1.6	0.6

a Where the section has been acetone extracted, it is not possible to distinguish sudanophilic from nonsudanophilic cells.

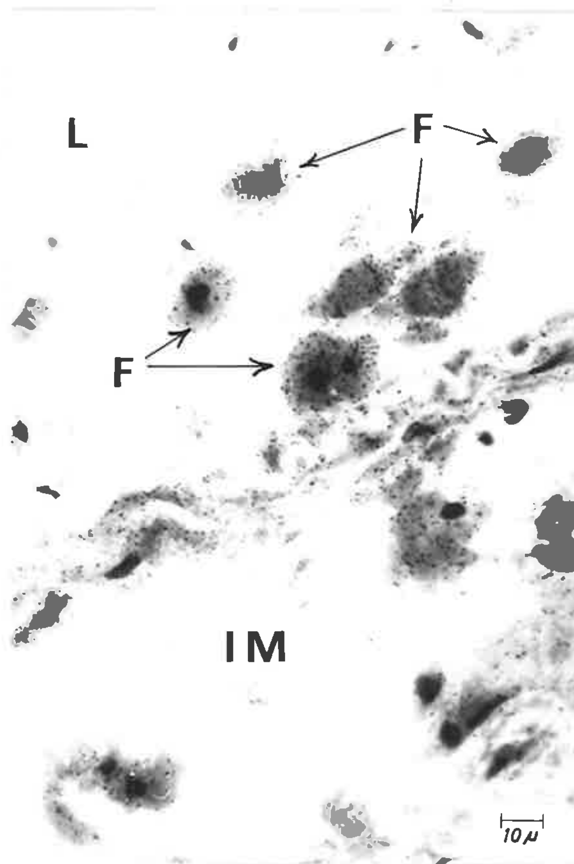

Figure 46

Autoradiograph of the same lesion as in Fig. 40, this time incubated with ^{3}H-labelled oleic acid. Localisation of ^{3}H-labelled oleic acid and its metabolic derivatives, phospholipid, triglyceride, and cholesterol ester to intimal foam cells (F) is apparent. The luminal border (L) is uppermost and the region of the intimo-medial junction is denoted IM. Haematoxylin and Sudan IV. Exposure time 30 days.

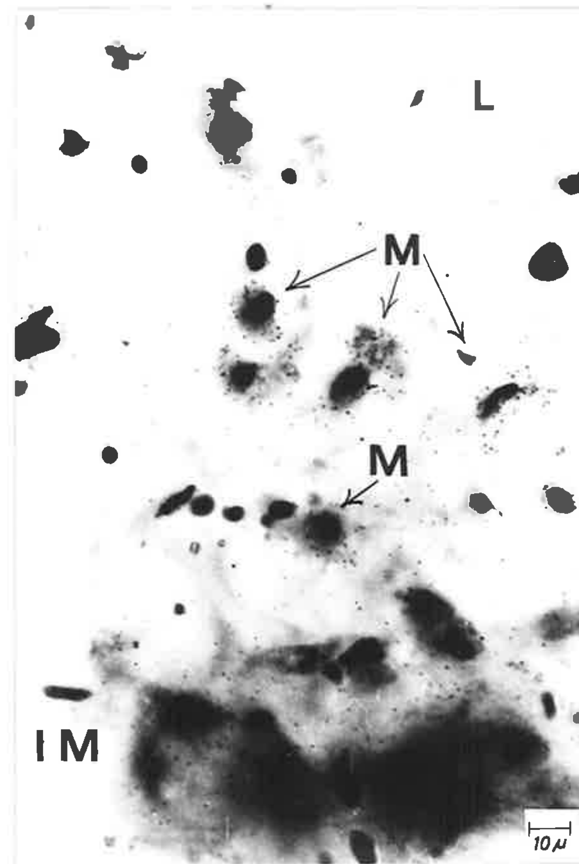

Figure 47

An adjacent lesion to that shown in Fig. 46, but extracted with cold acetone so that most of the ^{3}H-labelled lipid is phospholipid. Localisation of silver grains to intimal mononuclear cells (M) is evident. The luminal border (L) is uppermost and the region of the intimo-medial junction is denoted IM. Haematoxylin and Sudan IV. Exposure time 30 days.

The study reported here indicates that choline chloride (methyl-^{14}C) is incorporated into the phospholipid lecithin, in particular, in normal and atherosclerotic human intima and media. Very recently, and after the completion of this study, Morin (1969) has reported the incorporation of choline-1-2-^{14}C into the choline containing phospholipids, lecithin, lysolecithin and sphingomyelin and also into phosphatidyl serine and phosphatidyl ethanolamine of human arteries with advanced atherosclerotic lesions. As explained earlier in this thesis, the failure of labelled choline to label lipids other than choline-containing phospholipids in the present work presumably reflects the way in which the choline was labelled.

In the short term incubations of the present experiments steady-state conditions are presumed to be present. The net synthesis of phospholipid has not been examined. Exchange of non-labelled for labelled choline without phospholipid synthesis, as discussed for oleic acid incorporation into cholesterol ester in rabbit atherosclerotic lesions, is therefore a possibility. However, Zilversmit (1961) has provided evidence that the phospholipid of the human atherosclerotic lesion arises <u>in situ</u>. It is likely, therefore, that the incorporation of ^{14}C-labelled choline into phospholipid in this study is relevant to phospholipid accumulation in the human artery.

Dunnigan (1964) has shown histochemically that most of the phospholipid of the human atherosclerotic lesion is located in foam cells and Boelsma-Van Houte and Böttcher (1966) have shown, also histochemically, that phospholipids containing choline are present in normal intimal and medial cells, in intimal foam cells and in the region of elastic lamellae. Despite the apparent wide distribution of choline-containing phospholipids in the human artery, ^{14}C-labelled choline was localised to intimal foam cells in the

present study.

The localisation of the uptake and incorporation of ^{14}C-labelled oleic acid into phospholipid, triglyceide and cholesterol ester by foam cells in human atherosclerotic lesions was reported in Section 3. ^{14}C-labelled choline, however, is a specific phospholipid precursor and, in the present autoradiographic study, the formation of choline-containing phospholipids has been localised to foam cells. The acetone-extraction studies using ^{3}H-labelled oleic acid have confirmed this observation. When the relative amount of phospholipid is increased in adjacent sections by the removal of free fatty acid and cholesterol ester, the localisation to intimal foam cells as demonstrated by autoradiography is still evident.

The general conclusion appears to be, therefore, that the human atherosclerotic artery is capable of forming phospholipid and that this formation takes place essentially in foam cells.

SECTION 6

INCORPORATION OF DIFFERENT FATTY ACIDS INTO COMBINED LIPID BY RABBIT ATHEROSCLEROTIC AORTA IN VITRO

A. Introduction

Studies of the fatty acid composition of lipids in the atherosclerotic lesion, in normal intima and in the serum have led to the view that lesion phospholipid and cholesterol ester are not derived in simple fashion from the blood, but that triglyceride could be (see "General Introduction"). While differences in transport of lipids into and out of the atherosclerotic lesion might explain the phospholipid and cholesterol ester fatty acid patterns, there is now ample evidence that phospholipid, triglyceride and cholesterol ester metabolism take place in the arterial wall. The question therefore arises as to whether one fatty acid is incorporated into or removed from a particular combined lipid in the lesion in the same way as another. If not, this might explain the divergence of serum and lesion phospholipid and cholesterol ester fatty acid patterns.

Various workers have examined the incorporation of the saturated fatty acid palmitic acid (Chobanian and Hollander 1966; Newman, Gray and Zilversmit, 1968), the monounsaturated fatty acid oleic acid (this thesis, Sections 2 - 5) and the polyunsaturated fatty acid linoleic acid (Stein, Selinger and Stein, 1963; Parker, Ormsby, Peterson, Odland and Williams, 1966) into arterial lipids. Stein and Stein (1962) have made a comparison of the separate incorporations of palmitic acid-1-^{14}C and linoleic acid-1-^{14}C into the combined lipids and individual phospholipids of rabbit, dog, rat and baboon aortic slices and find no differences. Parker, Schimmelbusch and Williams, (1964) have also compared the separate incorporation of palmitic acid-1-^{14}C and linoleic acid-1-^{14}C into the phospholipid of the normal rabbit and human aorta and again find no difference. Normal rabbit aortae have been perfused with ^{14}C-labelled palmitic, stearic, oleic and linoleic acids

separately by Bowyer, Howard, Gresham, Bates and Palmer (1968). Because of the minimal incorporation into cholesterol ester, they were unable to demonstrate any preferential acylation, but did report differences in incorporation of the different fatty acids into lecithin, triglyceride and diglyceride. Bowyer, Howard and Gresham (1967) have also reported in an abstract that the labelling of cholesterol ester with oleic acid-1-^{14}C is greater than with palmitic, stearic or linoleic acids-1-^{14}C when atherosclerotic rabbit aortae are perfused with these fatty acids separately and that the rate of labelling is correlated with the cholesterol content of the tissue. However, there is no report in the literature of a comparison of the incorporation of palmitic, oleic and linoleic acids together into the same atherosclerotic intima. In the studies using ^{14}C-labelled acetate as a lipid precursor (Lofland, Moury, Hoffman and Clarkson, 1965; Day and Wilkinson, 1967; Newman, Gray and Zilversmit, 1968) it was not possible to distinguish between differences in fatty acid synthesis and differences in incorporation.

Selective breakdown of a given combined lipid, according to its fatty acid composition, could also account for the peculiar fatty acid patterns of the atherosclerotic lesion. Normal rabbit aorta is claimed to hydrolyse cholesterol-1-^{14}C oleate less readily than cholesterol-1-^{14}C palmitate, stearate or linoleate (Bowyer, Howard, Gresham, Bates and Palmer, 1968).

The findings reported in this paper relate firstly to the separate metabolism of ^{14}C-labelled palmitic and ^{14}C-labelled linoleic acid by rabbit atherosclerotic aorta and chiefly to the question of whether conversion of one labelled fatty acid to another takes place. It has been shown for the same preparation (Section 2) that oleic acid-1-^{14}C is not converted to any other

labelled fatty acid. Secondly, the incubation of ^{14}C-labelled palmitic, ^{14}C-labelled oleic and ^{14}C-labelled linoleic acids together with the same rabbit atherosclerotic aorta and the determination of specific activities of intimal free fatty acid, phospholipid, triglyceride and cholesterol ester by gas liquid radiochromatography is reported. The incorporation of fatty acid into combined lipid is calculated from a knowledge of intimal free fatty acid specific activity and the radioactivity of each of the combined lipids. In a third series of experiments, aortic thirds from cholesterol-fed rabbits were labelled with palmitic acid-1-^{14}C, oleic acid-1-^{14}C and linoleic acid-1-^{14}C. Then two of the thirds were re-incubated in non-labelled medium for 2 and 4 hours respectively. A comparison of the specific activities at 2 and 4 hours with those at change-over from labelled to non-labelled medium was made in an effort to assess the relative removals of combined lipids themselves and of combined lipids according to their degree of fatty acid unsaturation.

B. Incubations with ^{14}C-labelled Palmitic Acid and ^{14}C-labelled Linoleic Acid Separately

Atherosclerotic aortae were obtained from rabbits fed cholesterol for 5 months. The aortae were divided longitudinally into halves. One half was incubated with ^{14}C-palmitic acid-1-^{14}C (12.2 µCi) and the other with linoleic acid-1-^{14}C (17.4 µCi) complexed to the serum albumin of the incubation medium. In each case, the incubation medium was 10 ml of 50 : 50 Hanks' solution : normal rabbit serum and the time of incubation 4 hours. At the conclusion of the experiment, the tissues were washed in 0.9% saline and lipid extracts of the intima prepared.

The intimal uptakes of ^{14}C-labelled palmitic acid and of

TABLE 34

Uptake and Incorporation into Intimal Combined Lipid of Palmitic Acid-1-^{14}C and Linoleic Acid-1-^{14}C Incubated Separately with Rabbit Atherosclerotic Aortae

Experiment	Fatty acid	% Uptake from incubation medium	% Incubation medium ^{14}C incorporated into combined lipid
1	16:0	3.9	2.5
	18:2	3.3	2.9
2	16:0	2.1	1.3
	18:2	2.0	1.8

^{14}C-labelled linoleic acid are shown for two experiments in Table 34. Uptakes for the two fatty acids were of the same order, although it is not strictly valid to make such a comparison since the specific activities in the incubation medium differed. More than 85% of the ^{14}C-labelled linoleic acid and more than 60% of the ^{14}C-labelled palmitic acid taken up from the incubation medium has been incorporated into combined lipid. This can also be seen in Table 35 which shows the percentage distribution of label among the intimal lipids.

Phospholipid, diglyceride, unesterified fatty acid, triglyceride and cholesterol ester were separated from one another by neutral lipid T.L.C. Each fatty acid was incorporated mainly into phospholipid, with lesser amounts incorporated into cholesterol ester and triglyceride (Table 35).

Table 36 shows the percentage distribution of ^{14}C-labelled palmitic or ^{14}C-labelled linoleic acid among individual phospholipids separated by T.L.C. Lecithin was the major phospholipid formed. It is noteworthy that there appeared to be more incorporation of palmitic acid than of linoleic acid into sphingomyelin, although the "spingomyelin" spot was not characterized further.

In this same series of experiments, phospholipid, triglyceride, and cholesterol ester fatty acids were methyl esterified and assessed by gas liquid radiochromatography. In the palmitic acid experiments, radioactivity was found only in the position of 16:0 and, in the linoleic acid experiments, only in the position of 18:2, so that conversion to other labelled fatty acids had not occurred.

TABLE 35

Percentage Distribution of either Palmitic Acid-1-^{14}C or Linoleic Acid-1-^{14}C among Intimal Lipids of Different Rabbit Atherosclerotic Aortic Halves

Experiment	Fatty acid	Phospholipid	Diglyceride	Free fatty acid	Triglyceride	Cholesterol ester
1	16:0	41.8	3.8	32.1	11.2	11.2
	18:2	49.2	3.8	13.7	13.7	19.7
2	16:0	41.6	3.6	38.4	8.7	7.7
	18:2	49.0	4.3	14.4	10.8	21.5

TABLE 36

PERCENTAGE DISTRIBUTION OF EITHER PALMITIC ACID-1-^{14}C OR LINOLEIC ACID-1-^{14}C AMONG INDIVIDUAL INTIMAL PHOSPHOLIPIDS OF DIFFERENT RABBIT ATHEROSCLEROTIC AORTIC HALVES

Experiment	Fatty acid	Origin	Lyso-lecithin	Sphingo-myelin	Lecithin	Phosphatidyl inositol and phosphatidyl serine	Phosphatidyl Ethanolamine	Solvent front
1	16:0	0.1	0.5	8.0	72.7	6.0	5.7	7.0
	18:2	0.1	0.3	1.5	74.2	7.7	6.1	10.2
2	16:0	0.1	0.6	6.9	75.7	5.5	4.8	6.4
	18:2	0.1	0.3	0.5	72.3	10.2	6.3	10.4

C. Incubations with ^{14}C-labelled Palmitic, ^{14}C-labelled Oleic and ^{14}C-labelled Linoleic Acids together

Because interconversion of palmitic acid-1-^{14}C, oleic acid-1-^{14}C and linoleic acid-1-^{14}C was not evident, it was possible to incubate them together in one experiment.

Rabbits were fed cholesterol for 4 months. Atherosclerotic aortae were divided longitudinally into halves, only one half being used in this series of experiments. Incubation was in a 10 ml medium of 50 : 50 Hanks' solution : normal rabbit serum together with sodium palmitate-1-^{14}C (13.3 μCi), sodium oleate-1-^{14}C (4.3 μCi) and sodium linoleate-1-^{14}C (5.1 μCi) for a period of 4 hours. As in the separate incubations, intima was stripped from media and lipid extracts prepared. Dry defatted tissues were weighed.

Free fatty acid was methyl esterified by the diazomethane procedure and then isolate from other lipids by T.L.C. prior to gas liquid radiochromatography. Total lipid methyl esters were prepared in the usual way with 5% sulphuric acid in methanol, as were those of phospholipid, triglyceride and cholesterol ester after separation of these lipid classes by neutral lipid T.L.C. Gas liquid radiochromatography allowed assessment of (a) the percentage distribution of label among the three fatty acids as either total lipid, free fatty acid, phospholipid, triglyceride or cholesterol ester and (b) the specific activities of the different fatty acids as either free fatty acid, phospholipid, triglyceride or cholesterol ester.

For intimal total lipid and free fatty acid, their radioactivities together with the distribution of label between palmitic, oleic and linoleic acid, allowed the calculation of uptake and incorporation into combined lipid for each of the fatty

TABLE 37

Uptake and Incorporation of ^{14}C-labelled Fatty Acids by the same Atherosclerotic Rabbit Intima *

Fatty acid	% Uptake	% Incorporation into Lipid
16:0	2.03 ± 0.44	1.71 ± 0.32
18:1	3.37 ± 0.90	2.70 ± 0.75
18:2	4.63 ± 1.15	3.86 ± 1.10

* Means and standard errors of means of three experiments

acids as shown in Table 37. Figures are the means and standard errors of the means of three experiments (three different animals).

Biologically occurring phospholipids and triglycerides usually have different fatty acids in the α and β positions so that it can be assumed that the molar ratio of a given fatty acid to the lipid with which it is combined is 1 : 1. By the same token, one triglyceride molecule, for example, could be labelled with two different fatty acids. However, for a given fatty acid it is possible to compare specific activities on a molar basis (Table 38). For palmitic, oleic and linoleic acids, the free fatty acid specific activity was greater than that of phospholipid, and its specific activity in turn, is greater than that of cholesterol ester. The specific activity of triglyceride did not appear significantly different from that of phospholipid or cholesterol ester. Without making allowance for the different specific activities of the intimal free fatty acids, it is not valid to make comparisons within a given lipid class i.e. phospholipid, triglyceride or cholesterol ester.

Incorporation of different intimal free fatty acids into combined lipids in the same vessel was expressed as mμmoles/g dry defatted tissue. The calculation of incorporation required knowledge of intimal free fatty acid specific activities and intimal phospholipid, triglyceride or cholesterol ester radioactivities. The derived data are shown in Table 39. For palmitic and linoleic acids more was incorporated into phospholipid than into triglyceride or cholesterol ester. For oleic acid, however, more was incorporated into cholesterol ester than into phospholipid or triglyceride. It is valid here to make comparisons within a lipid class. The incorporation of the three fatty acids into phospholipid and triglyceride was similar, but the incorporation of oleic acid into cholesterol ester was greater than that of palmitic or linoleic acid.

TABLE 38

Specific Activities of Lipids in the same Atherosclerotic Rabbit Intima (cpm/mμmole fatty acid) *

	Free fatty acid	Phospholipid	Triglyceride	Cholesterol ester
16:0	1024 ± 157	67 ± 18	73 ± 33	14 ± 1
18:1	366 ± 62	43 ± 11	29 ± 12	5 ± 1
18:2	1182 ± 313	107 ± 13	67 ± 33	19 ± 3

* Means and standard errors of means of three experiments

TABLE 39

Incorporation of ^{14}C-labelled Intimal Free Fatty Acids into Combined Lipids of the same Atherosclerotic Rabbit Intima and Pool Sizes of Combined Lipids

	Incorporation (mμmoles/g dry defatted weight)			Pool size (mμmoles fatty acid)		
	Phospholipid	Triglyceride	Cholesterol ester	Phospholipid	Triglyceride	Cholesterol ester
16:0	4843 ± 1171	1277 ± 341	1423 ± 387	3.49 ± 1.39	0.85 ± 0.20	4.34 ± 1.56
18:1	5243 ± 1196	1645 ± 399	7370 ± 1854	2.10 ± 0.85	0.96 ± 0.18	21.77 ± 7.96
18:2	4530 ± 2194	790 ± 360	3237 ± 1984	1.90 ± 0.85	0.55 ± 0.08	4.76 ± 1.93

Means and standard errors of means of three experiments

Also in Table 39, the μmoles of each fatty acid in phospholipid, triglyceride and cholesterol ester are shown. These were calculated from the specific activity and radioactivity of each moiety. Cholesterol oleate had a greater pool size than any other lipid moiety examined.

Incorporation into a given lipid has been expressed in terms of the amount of that lipid in Table 40. It is evident that, expressed this way, the incorporation of each fatty acid into phospholipid is greater than into cholesterol ester, and that there is no difference in incorporation of different fatty acids into their corresponding cholesterol esters.

D. Re-incubation of Aortae Labelled with ^{14}C-labelled Palmitic, ^{14}C-labelled Oleic and ^{14}C-labelled Linoleic Acids in Non-labelled Incubation Media

In this series, rabbits were fed cholesterol for 5 months. Atherosclerotic aortae were incubated with ^{14}C-labelled palmitic (23.9 μCi), Oleic (7.8 μCi) and linoleic (9.2 μCi) acids together as described above. However, each vessel was divided longitudinally into thirds and all tissues removed from the labelled incubation medium at 2 hours. They were then thoroughly washed in 0.9% saline. One third was taken as the "0 hours" specimen and its intimal lipids extracted. The other two thirds were re-incubated in 5 ml non-labelled 50 : 50 Hanks' solution : normal rabbit serum for 2 and 4 hours respectively. Intima was again stripped from media and lipid extracts prepared. Phospholipid, triglyceride and cholesterol ester were separated by neutral lipid T.L.C. and methyl esterified for gas-liquid radiochromatographic determination of specific activities.

TABLE 40

* mμmoles Fatty Acid Incorporated/μmole Combined Lipid

	Phospholipid	Triglyceride	Cholesterol ester
16:0	66 ± 15	86 ± 52	14 ± 2
18:1	118 ± 19	99 ± 60	15 ± 2
18:2	101 ± 22	90 ± 64	18 ± 2

* Means and standard errors of means of three experiments

TABLE 41

Specific Activities of Different Fatty Acids in Combined Lipids of Rabbit Atherosclerotic Aortic Thirds. "0 Hours" Represents the time of Transfer from Labelled to Non-Labelled Incubation Medium. (cpm/mμmole fatty acid)

	EXPT 1			EXPT 2			EXPT 3		
	0 Hr	2 Hr	4 Hr	0 Hr	2 Hr	4 Hr	0 Hr	2 Hr	4 Hr
Phospholipid									
16:0	20.9	31.5	19.0	85	70	80	39.5	34.0	32.2
18:1	14.2	17.4	9.9	49	37	36	13.9	16.8	14.0
18:2	43.2	44.6	24.4	96	78	77	44.6	31.2	21.3
Cholesterol Ester									
16:0	6.5	8.4	7.7	26	23	32	10.4	10.4	16.8
18:1	1.8	1.5	2.2	8.2	5.4	7.2	2.1	2.5	3.5
18:2	7.1	16	8.8	16	14.6	17	11.8	14.2	18.8
Triglyceride									
16:0	58	42	49	49	46	12	40.9	39.1	23.8
18:1	21	18	17	9.8	11	3.4	11.5	13.7	7.7
18:2	118	62	71	19	17	4.4	32.1	24.9	13.4

The specific activities of the three fatty acids in the combined lipids phospholipid, triglyceride and cholesterol ester at 0, 2, and 4 hours are shown in Table 41 for each of three experiments. The changes in specific activity with time are better appreciated by plotting them relative to specific activities at 0 hours. This has been done in Figure 48. In all three experiments, the relative specific activity of cholesterol palmitate, cholesterol oleate and cholesterol linoleate at 4 hours was greater than that of the corresponding phospholipid or triglyceride. This applies even in the case of the cholesterol oleate whose relative specific activity at 4 hours was less than 1. Also, apart from this one particular cholesterol ester, the specific activity of cholesterol ester, irrespective of the fatty acid incorporated, was greater at 4 hours than it was at 0 hours. Phospholipid specific activity at 4 hours was either the same or less than at 0 hours. Triglyceride specific activity at 4 hours was consistently less than that at 0 hours.

E. Discussion

A comparison of the incorporation of more than two different fatty acids into intimal combined lipid of the same tissue simultaneously has been made possible by gas-liquid radiochromatography. Also, in previous comparisons of the incorporation of different fatty acids into arterial lipids (see introduction), the intimal free fatty acids were not isolated. These may be immediate precursors of combined lipid and their specific activities are used to calculate fatty acid incorporation in the work reported here.

In the first series of experiments, in which palmitic acid-1-^{14}C and linoleic acid-1-^{14}C were incubated separately, and, in the oleic acid-1-^{14}C study reported in Section 2, it was shown that each fatty acid retained its radioisotopic identity in rabbit atherosclerotic

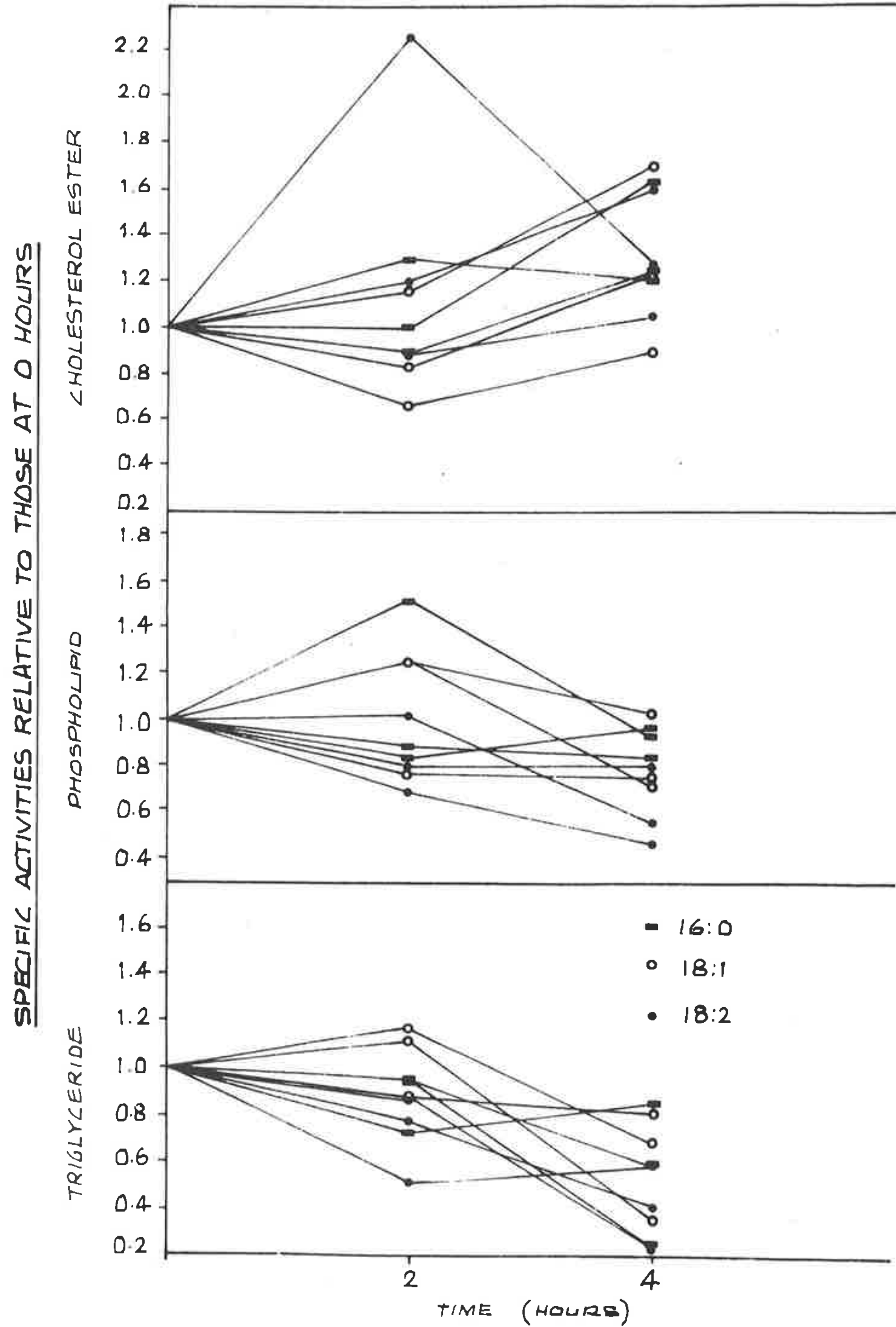

Figure 48

Changing specific activities of phospholipid, triglyceride and cholesterol ester in rabbit atherosclerotic lesions, labelled with palmitic acid-1-^{14}C, oleic acid-1-^{14}C and linoleic acid-1-^{14}C together. "0 hours" is the point at which aortae were transferred from labelled to non-labelled incubation media. Three experiments are shown in which intimal thirds from the one animal are compared at 0, 2 and 4 hours.

aortic intima. Were this not so, incubations of the three fatty acids together would not have provided a valid means of comparing their separate behaviours. At the same time, the fact that chain elongation was not observed, needs to be reconciled with studies of fatty acid formation from acetate-1-^{14}C in the arterial wall (Lofland, Moury, Hoffman and Clarkson, 1965; Day and Wilkinson, 1967; Newman, Gray and Zilversmit, 1968). Whereat (1966) has shown that fatty acid synthesis in the whole rabbit aorta is chiefly by the mitochondrial chain elongation pathway and not by *de novo* synthesis in the cytoplasmic system. Howard (1968) has found both *de novo* synthesis and chain elongation of fatty acids by subcellular fractions of monkey aorta. However, Vost (1969) reports that in normal rabbit media, *de novo* synthesis is the major pathway for incorporation of ^{14}C-labelled acetate into fatty acid. If β-oxidation of labelled fatty acid took place, it might be expected that labelled two-carbon fragments would be available for use in either *de novo* synthesis or chain elongation. Further work is needed to clarify these anomalies. At all events, the present study is not complicated by recognisable fatty acid synthesis and the specific problem of fatty acid incorporation is examined.

The percentage distribution of palmitic acid-1-^{14}C and linoleic acid-1-^{14}C among major lipid classes in the rabbit atherosclerotic aorta (Table 35) is similar to that reported for oleic acid-1-^{14}C in Section 2, but detailed comparisons cannot be made for these incubations of fatty acids separately. The greater percentage of labelled sphingomyelin in the palmitic acid-1-^{14}C study than in the linoleic acid-1-^{14}C study (Table 36) is consistent with the fact that palmitic acid is the major sphingomyelin fatty acid in the rabbit atherosclerotic aorta (Zilversmit, Sweeley and Newman, 1961). As in the oleic acid study (Section 2), lecithin was the major phospholipid labelled when either palmitic or linoleic acid was the precursor.

In the second series of experiments where palmitic, oleic and linoleic acids were incubated together, the fact that the specific activities of the intimal free fatty acids were greater than those of the combined lipids is consistent with the view that these fatty acids are the precursors of the combined lipids. However, the higher specific activities of the phospholipids than of the cholesterol esters favours the possibility that phospholipid may be a donor of cholesterol ester fatty acid. Abdulla, Orton and Adams (1968) have produced evidence that a lecithin : cholesterol acyl transferase enzyme is present in rabbit atherosclerotic lesions. The serum enzyme of the same kind, however, has a preference for polyunsaturated fatty acid transfer (Portman and Sugano, 1964). Calculation of incorporation of fatty acid into cholesterol ester from phospholipid as precursor (22,200 $\pm$ 6100, 63,500 $\pm$ 15,700, 17,900 $\pm$ 2,500 mμmoles/g dry defatted tissue for 16:0, 18:1 and 18:2 respectively) does not support the view that all cholesterol ester fatty acid in the atherosclerotic lesion is derived by transacylation, if the arterial enzyme is the same as that in the serum.

Newman, Gray and Zilversmit (1968) have shown that ^{14}C-labelled acetate is incorporated mainly into the saturated and monounsaturated fatty acids of cholesterol ester by the rabbit atherosclerotic aorta _in vivo_. From their studies, however, it is not possible to say whether the preference for saturated and monounsaturated cholesterol esters is a reflection of fatty acid synthesis or of cholesterol esterification. The same is true of the investigation of ^{14}C-labelled acetate metabolism by atherosclerotic rabbit intima _in vitro_ by Day and Wilkinson (1967) when ^{14}C-labelled acetate was distributed equally among saturated, monounsaturated and polyunsaturated cholesterol esters, but was found mainly in saturated fatty acids for phospholipid. St. Clair,

Lofland and Clarkson (1968) have examined ^{14}C-labelled acetate metabolism in the pigeon atherosclerotic aorta and found the major newly synthesized fatty acids to be stearic in phospholipid, stearic, palmitic and oleic in glyceride, and oleic in cholesterol ester.

As mentioned in the introduction, Bowyer et al (1968) perfused normal rabbit aortae with albumin-bound ^{14}C-labelled palmitic, stearic, oleic and linoleic acids separately. They found little incorporation into cholesterol esters and therefore were unable to demonstrate any preferential acylation. They report that lecithin synthesis was greatest from palmitic acid and that triglyceride and diglyceride synthesis were least from stearic acid. However, they give no information about the intermediate aortic free fatty acid pools and, therefore, their findings must be viewed cautiously.

The only study reported of successful esterification of labelled free cholesterol with fatty acid in arterial tissue is that of Felt and Beneš (1969). They found, for normal rat aorta, that the rate of esterification for saturated and monounsaturated cholesterol esters was greater than that for polyunsaturated cholesterol esters.

In the present study, ^{14}C-labelled oleic acid was incorporated into cholesterol ester to a greater extent than was either ^{14}C-labelled palmitic or ^{14}C-labelled linoleic acid in the same vessel. However, this was directly related to the amount of the different cholesterol esters present. There are two ways of viewing this finding. One is that the increased incorporation into cholesterol ester of oleic acid relative to palmitic and linoleic acid is due to the larger amount of cholesterol oleate present. The other is

that there is more cholesterol oleate because of the greater incorporation of oleic acid. St Clair, Lofland and Clarkson (1968) found that the pattern of fatty acid synthesis from acetate resembled the actual fatty acid composition of the atherosclerotic pigeon aorta. Another possibility related to the second view is, therefore, that local fatty acid synthesis determines the fatty acids with which combined lipids are esterified, differences in esterification being secondary phenomena and the accumulation of cholesterol oleate being a tertiary phenomenon.

When incorporation is expressed in terms of the amount of lipid present, it is apparent that, for palmitic, oleic and linoleic acids, incorporation into phospholipid exceeds that into cholesterol ester. However, it is known that the rate of accumulation of cholesterol ester in atherosclerotic lesions is greater than that of phospholipid (Insull and Bartsch, 1966) and it is likely, therefore, that the turnover of phospholipid is much greater than that of cholesterol ester. For oleic acid (Section 2) it has been shown that its incorporation into phospholipid and cholesterol ester in the rabbit atherosclerotic lesion is linear over 4 hours and, if it be assumed that the same is true of palmitic and linoleic acids, the comparisons of incorporation into phospholipid and cholesterol ester just made are valid.

The fatty acid composition of the atherosclerotic lesion could be influenced by selective transport into the arterial wall and evidence is presented in Section 7 that the influx of monounsaturated cholesterol ester into the rabbit atherosclerotic aorta is greater than that of polyunsaturated cholesterol ester. Again, this may not be a primary change in transport, but a consequence of the increased amount of cholesterol oleate already present and this in turn may reflect increased cholesterol oleate formation from free cholesterol or from other cholesterol esters.

Yet another reason for differences in fatty acid composition between atherosclerotic lesion and serum is that there may be differences in rates of removal. Such differences could depend on actual transport mechanisms or on hydrolysis or on fatty acid catabolism. Bowyer et al (1968) have reported that the normal rabbit aorta hydrolyses cholesterol-1-^{14}C oleate less readily than cholesterol-1-^{14}C palmitate, stearate or linoleate and that the atherosclerotic rat aorta has less cholesterol oleate hydrolytic ability than the normal. However, in their studies no account was taken of the specific activities of the various cholesterol esters after they had been taken up by the arteries, hydrolysis being expressed as a percentage of incorporated radioactivity found in the free fatty acids. In the present study, changes in specific activity of phospholipid, triglyceride and cholesterol ester labelled with palmitic acid-1-^{14}C, oleic acid-1-^{14}C and linoleic acid-1-^{14}C after reincubation in non-labelled medium have been assessed. There is no evidence of a difference in behaviour between the various fatty acids in any of phospholipid, triglyceride, or cholesterol ester. But a comparison of the specific activities at 4 hours with these at 0 hours reveals that, while those of triglyceride fall and those of phospholipid are similar or fall, those of cholesterol ester tend to rise. It is recognised that incorporation of labelled fatty acid may still be proceeding as it is removed, but the trends suggest that cholesterol ester is not removed from the atherosclerotic lesion as readily as phospholipid or triglyceride, whatever the mechanism of such removal. This is further evidence that the turnover of phospholipid is greater than that of cholesterol ester in the lesion.

In conclusion, the greater incorporation into cholesterol ester of the monounsaturated fatty acid oleic acid, over that of the saturated fatty acid palmitic acid, and that of the poly-unsaturated fatty acid, linoleate acid, seems to be related to

the chemical amounts of individual cholesterol esters in the atherosclerotic lesion. It has not been possible to demonstrate the preferential removal of one or other fatty acid. The turnover of phospholipid in the rabbit atherosclerotic lesion, however, appears to be greater than that of cholesterol ester.

SECTION 7

DIFFERENTIAL UPTAKE OF CHOLESTEROL AND OF DIFFERENT CHOLESTEROL ESTERS BY RABBIT ATHEROSCLEROTIC AORTA IN VIVO AND IN VITRO

A. Introduction

Various aspects of lipid entry into the arterial wall have been discussed in the "General Introduction". The cholesterol of the atherosclerotic lesion, in the free or ester form, appears to arise essentially from the plasma (Dayton, 1959; Newman, McCandless and Zilversmit, 1961; Newman and Zilversmit, 1962) rather than by local synthesis although this can take place (Siperstein, Chaikoff and Chernick, 1951; Azarnoff, 1958). Of the several possible explanations for the apparently greater influx of free cholesterol than of cholesterol ester into the intima than would be expected from their relative plasma concentrations (Newman and Zilversmit, 1962 and 1966; Hashimoto and Dayton, 1966; Dayton and Hashimoto, 1966), one that has not been excluded is that free and ester cholesterol enter according to their plasma concentrations, but that ester cholesterol is hydrolysed in the intima. This possibility has been investigated in the present work.

Another question which arises is whether or not there is more monounsaturated cholesterol ester than other cholesterol ester in the lesion because of a greater entry of monounsaturated cholesterol ester. Swell, Law and Treadwell (1963) have reported that the influx of monounsaturated cholesterol ester, labelled with cholesterol-4-^{14}C, into the whole aorta of the cholesterol-fed rabbit *in vivo* is greater than that of saturated cholesterol ester or of cholesterol linoleate. An attempt had been made in a similar study, reported in this section, to find what relationship this bears to serum concentrations of individual cholesterol esters.

The efflux of cholesterol from the atherosclerotic aorta has been investigated in order to confirm the findings of Newman and Zilversmit (1966) and Dayton and Hashimoto (1966) and to clarify the question of different intimal cholesterol pools and the mode of exit of cholesterol.

B. Entry of ^{3}H-labelled Cholesterol into Atherosclerotic Aorta in Vivo

Rabbits fed a cholesterol diet for 3 to 4 months were used. They were incubated with an infant feeding tube under light ether anaesthesia and given 500 μCi of cholesterol-T (G) (2370 mCi/mM, Radiochemical Centre, Amersham) in 2 ml corn oil, followed by 100 mg sodium taurocholate in 0.9% sodium chloride solution. Blood samples, taken at 12, 24, 48, 72 and 96 hours, were centrifuged at 3000 rpm for 15 min and lipid extracts of the serum prepared. The animals were killed by ether anaesthesia at 96 hours and their thoracic aortae removed. The severity of atherosclerosis was assessed macroscopically and ascribed a grade from 0 - 5, according to the method of Day and Wilkinson (1956). Aortae used had grades from 3 to 5. The adventitia was freed of superficial fat and the aorta thoroughly washed in 0.9% sodium chloride solution. The vessel was then divided longitudinally into halves, one half being reserved for efflux studies. The other half was separated into intima and media/adventitia and lipid extracts prepared.

Lipid extracts of serum and aortic tissues were separated into cholesterol and cholesterol ester and then the cholesterol ester was separated into individual cholesterol esters for determination of the specific activity of these fractions as set out below. In order to calculate the influx of each component into the aortic intima or media an expression indicating mean exposure of the aorta to ^{3}H-labelled cholesterol (or cholesterol ester) over the 96 hour period was required. To this end, the median specific activity of each serum cholesterol component was determined using Simpson's rule where the curve of changing specific activities is broken up into polygons.

$$\text{Median Specific Activity} = \frac{\tfrac{1}{2}t_1\, x + \tfrac{1}{2}(t_1 + t_2)\, y + \tfrac{1}{2}t_2\, z}{\Sigma\, t}$$

Where x = the specific activity at time 0 hours

y = the specific activity after time t_i

z = the specific activity after a further interval of time t_2, etc.

A Digital Equipment Corporation Data Processor PDP-8 was programmed to derive this information.

Free and ester cholesterol were separated on alumina columns by the method described by Deykin and Goodman (1962). Column fractions were made up to known volumes, samples taken for counting and for cholesterol determination, and specific activities calculated. The cholesterol ester was further fractionated by thin layer chromatography on silver nitrate impregnated silicic acid. Polyunsaturated, monounsaturated and saturated cholesterol esters were eluted from the plates and aliquots taken for radioassay. Cholesterol ester was separated from other lipid fractions prior to gas liquid chromatography, by neutral lipid thin layer chromatography. G.L.C. allowed determination of fatty acid composition and in turn the amount of monounsaturated and saturated cholesterol esters present in the total cholesterol ester. Specific activities were then derived from silver nitrate T.L.C. and G.L.C. data.

The ingestion of 500 μc of ^{3}H-labelled free cholesterol was followed by labelling of both free and ester cholesterol in the serum. The change in specific activity of these fractions is shown in Figure 49 for the four day period studied. There is appreciable labelled cholesterol and cholesterol ester present in the serum at 12 hours. The specific activity of both fractions rises together reaching a maximum 48 hours after ingestion. Over the whole period of the experiment the specific activity of the cholesterol ester exceeds that of the free cholesterol.

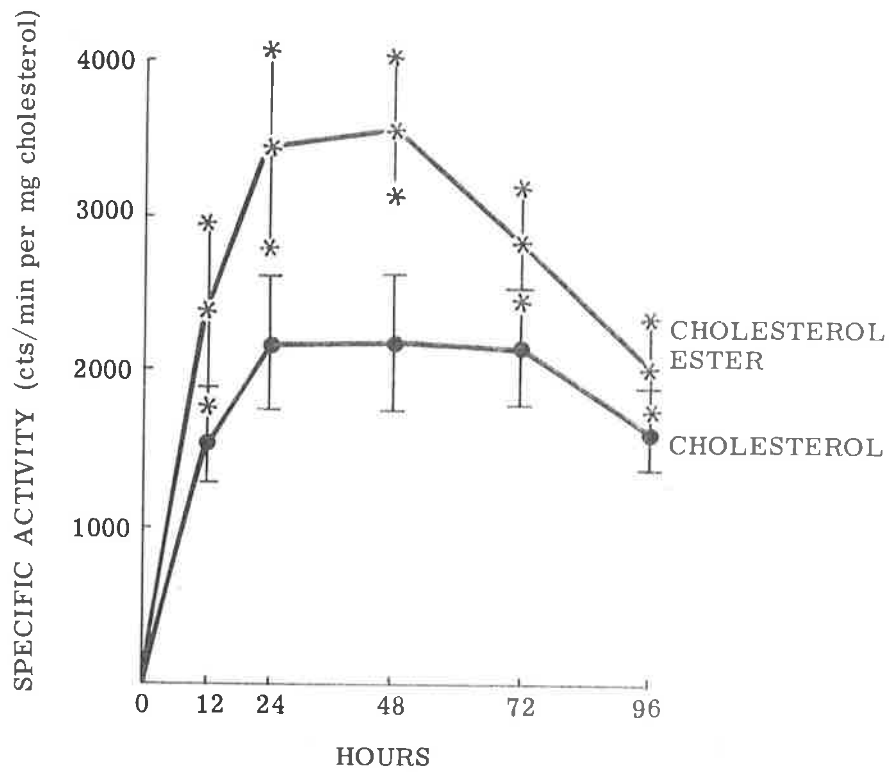

Figure 49

Specific activities of serum free and ester cholesterol following ingestion of ^{3}H-labelled cholesterol. Means of six experiments and standard errors of means are plotted.

The changes in specific activity of the individual groups of cholesterol esters over the four day period are shown in Figure 50. The pattern of labelling, with respect to time, of the saturated, monounsaturated, and polyunsaturated cholesterol ester is similar. It also resembles that of the total cholesterol ester shown in Figure 49. The specific activities of poly-unsaturated and of saturated cholesterol ester exceed those of the monounsaturated cholesterol ester. The amount of monounsaturated cholesterol ester present in the serum, however, is more than twice that of the other two fractions (see Table 42).

The median specific activities of the serum cholesterol and cholesterol ester and of the individual cholesterol esters were derived from the specific activities of these fractions at the different time intervals, as described above. This median specific activity was then used to calculate the influx of the various fractions into the arterial wall as follows. It was assumed that, over the four day period studied, the fractions were passing into the intima from common plasma pools at their respective median specific activities. By dividing the cpm of a given cholesterol fraction which accumulated in the intima or media over the four day period by the median specific activity in the serum, influx was derived. Influx was expressed as μg/day.

The influx of free and ester cholesterol into the intima, together with the influx of the individual cholesterol esters, saturated, monounsaturated and polyunsaturated, is given in Table 42. This table also shows the mean content of free cholesterol and of cholesterol esters in the serum and the intima, together with the median specific activities of the various fractions in the serum and, in the intima, the specific activities 4 days after the ingestion of labelled cholesterol. The influx of free cholesterol

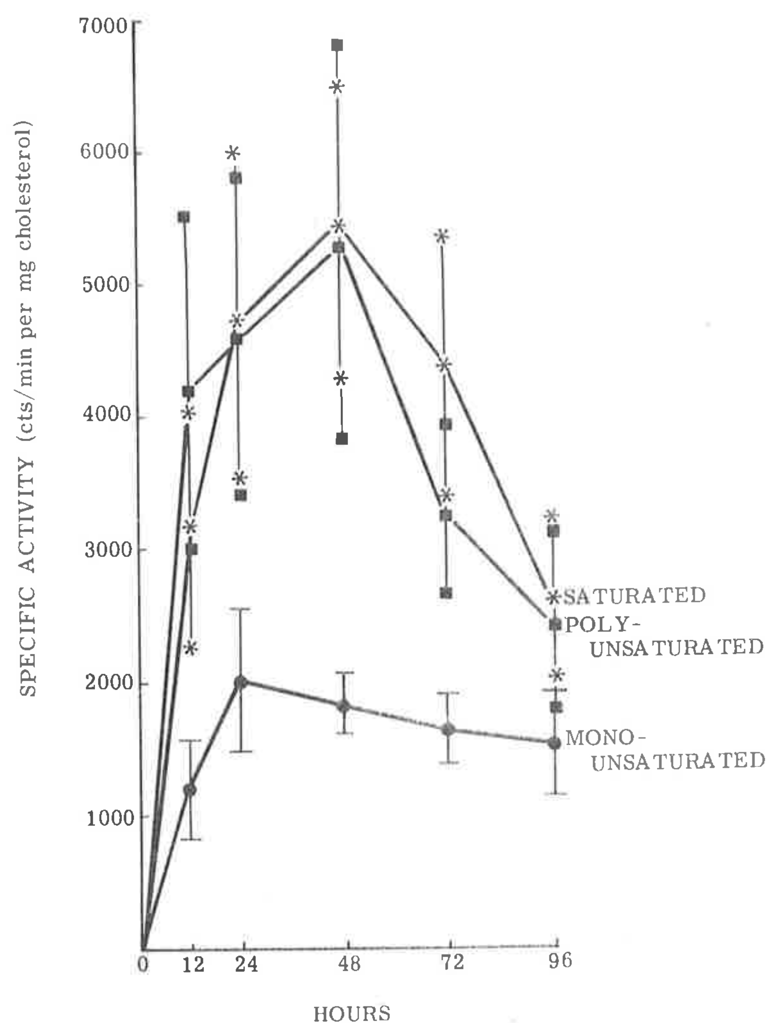

Figure 50

Specific activities of saturated, monounsaturated, and polyunsaturated cholesterol esters in the serum following ingestion of ^{3}H-labelled cholesterol. Means of four experiments and standard errors of the means are plotted.

TABLE 42

Influx of Lipoprotein ^{3}H-labelled Cholesterol and Cholesterol Esters into Cholesterol-Fed Rabbit Intimal Halves *In Vivo*

	Cholesterol content		Specific activity (cpm/mg)		Influx		
	Serum (mg/ml)	Intima (mg in toto)	Serum	Intima	μg/day	μg/day per mg/ml	[c]Relative influx
[a] Free cholesterol	5.10 $\pm$ 0.41	5.35 $\pm$ 0.95	1881 $\pm$ 322	269 $\pm$ 74	179.1 $\pm$ 46.3	34.1 $\pm$ 6.8	1
[a] Cholesterol ester	11.61 $\pm$ 1.30	10.36 $\pm$ 1.62	2797 $\pm$ 397	256 $\pm$ 80	224.8 $\pm$ 76.2	18.5 $\pm$ 4.2	[d]0.54 $\pm$ 0.02
[b] Cholesterol ester							
- saturated	2.23 $\pm$ 0.23	-	4091 $\pm$ 938	-	33.0 $\pm$ 5.1	15.4 $\pm$ 3.1	[e]0.78 $\pm$ 0.11
- monounsaturated	5.82 $\pm$ 0.78	-	1631 $\pm$ 294	-	108.0 $\pm$ 4.4	20.1 $\pm$ 3.8	1
- polyunsaturated	2.28 $\pm$ 0.22	-	3880 $\pm$ 908	-	30.6 $\pm$ 5.1	14.0 $\pm$ 3.5	[f]0.68 $\pm$ 0.06

[a] Means and standard errors of means of six experiments.

[b] Means and standard errors of means of four experiments.

[c] Relative influx is the ratio of the influx per mg/ml of serum cholesterol and cholesterol ester with respect to free cholesterol; or of the individual cholesterol esters with respect to monounsaturated cholesterol ester.

[d] Significant at $< 0.1\%$ level.

[e] No significant difference

[f] Significant at $< 2\%$ level

and of cholesterol ester into the intima from the serum is approximately the same. However, about twice as much cholesterol ester as free cholesterol is present in the serum and a more comparable figure can be derived by comparing the influx of the different fractions entering the intima in relation to their concentration in the serum. The influx per mg/ml of the corresponding fraction in the serum is given in Table 42. From this expression for the individual experiments, the mean relative influx has been calculated. It can be seen that the entry into the intima of free cholesterol relative to cholesterol ester is about twice as much as might be expected from their respective concentrations in the serum.

The influx of the individual cholesterol esters, saturated, monounsaturated and polyunsaturated, into the intima is also given in Table 42. Most of the cholesterol ester entering the artery is monounsaturated. However, the concentration of monounsaturated cholesterol ester in the serum is much greater than that of the other two groups. But even when this is taken into account the relative influx of monounsaturated cholesterol ester is greater than that of saturated or polyunsaturated cholesterol ester. The difference between monounsaturated and saturated, however, is not statistically significant.

In four of the six experiments, the influx of free and ester cholesterol into the aortic media was also determined, and this data is provided in Table 43, together with the mean data for the chemical content of the serum and its median specific activity over the time period studied. The influx of cholesterol and cholesterol ester into the media was calculated assuming that it came directly from the serum rather than from the intima. The influx of both free cholesterol and cholesterol ester is considerably less than that into the intima. The relative influx, however, indicates that the entry of free cholesterol exceeds that of cholesterol ester by

TABLE 43

Influx of Lipoprotein ^{3}H-labelled Cholesterol and Cholesterol Esters into Cholesterol-fed Rabbit Aortic Medias *in Vivo* [a]

	Cholesterol content	Specific activity cpm/mg	Influx		
	Serum mg/ml	Serum	µg/day	µg/day per mg/ml	Relative influx
Free cholesterol	4.95 ± 0.40	1590 ± 359	40.8 ± 7.5	8.6 ± 2.0	1
Cholesterol ester	10.40 ± 1.13	2529 ± 553	26.5 ± 8.6	2.5 ± 0.7	[b] 0.29 ± 0.02

[a] Means and standard errors of means of four experiments

[b] Significant at < 0.1% level

an even greater margin than that for the intima. In view of the very small influx of cholesterol ester into the media in these experiments *in vivo*, it was not possible to assess the influx of the individual cholesterol esters into the media. The chemical content of the individual cholesterol esters in the media is low and was not determined in these experiments.

Since the relative influx of ^{3}H-labelled monounsaturated cholesterol ester into the arterial intima over the four day period exceeded that of the polyunsaturated ester, an attempt was made to confirm by gas-liquid chromatography whether there were differences in the cholesterol ester fatty acid composition of the aortic intima relative to the serum. This data is shown for two rabbits in Table 44. In both rabbits, the percentage of cholesterol oleate in the intima exceeds that in the serum, whereas that of cholesterol linoleate in the intima is less than that in the corresponding serum.

C. Removal In Vitro of ^{3}H-labelled Cholesterol from the Atherosclerotic Aorta, Labelled In Vivo

The half of the thoracic aorta removed from the cholesterol-fed rabbits incubated with 500 µCi ^{3}H-labelled cholesterol and reserved for efflux studies as described above, was incubated with shaking for 4 hours in 5 ml of medium containing 50 : 50 Hanks' solution : hypercholesterolaemic rabbit serum. The serum used was between 2 and 8 weeks old having been kept at 4°C with penicillin and streptomycin (about 0.1 mg/ml of each). After 4 hours, the aorta was washed in 0.9% sodium chloride solution and treated in the same manner as for the influx experiments.

The efflux of cholesterol and cholesterol ester from the intima into the incubation medium over a four hour period is presented

TABLE 44

Percentage Distribution of Serum and Intimal Cholesterol Ester Fatty Acid Patterns of Individual Rabbits used for Studies _In Vivo_

	16:0	16:1	18:0	18:1	18:2	20:0
Serum	19.1	7.2	3.9	51.9	18.0	-
Intima	18.9	5.2	5.2	58.1	9.6	2.3
Serum	19.7	5.7	5.4	44.2	27.5	-
Intima	15.0	3.2	4.8	56.7	18.6	1.8

in Table 45. Efflux from the artery into the incubation medium, may have occurred from both intimal and medial aspects of the vessel, but since a mean of 81.6% of the ^{3}H-labelled total cholesterol in these aortic halves was present in the intima, the effluxes shown in Table 45 can be considered to have come essentially from the intima. The assumption is made that the free and ester cholesterol were removed from a single pool at the specific activity of the intima. The efflux was calculated from the cpm of free and ester cholesterol in the incubation medium divided by the specific activity in the intima. It has been expressed as µg/day. Efflux, calculated in this way, is considerably in excess of that for influx in the equivalent artery. It appears likely, therefore, that there may be more than one intimal pool. However, if one can assume that the relative specific activities of the free and ester cholesterol in the more active pool are similar to those for the whole intima then the comparison of efflux data is valid. A greater relative efflux of cholesterol than of cholesterol ester is apparent from Table 45.

In one experiment, a cholesterol-fed rabbit was intubated with 3.5 mCi cholesterol - T (G), in order to obtain ^{3}H-labelled serum for the in-vitro experiments set out below. The thoracic aorta of this rabbit was removed 2 days after ingestion of cholesterol, divided longitudinally into halves and the efflux of cholesterol over a 4 hour period determined. However, one half was incubated in Hanks' solution alone and the other half in 50 : 50 Hanks' solution : hypercholesterolaemic serum. The medium was changed at 10 - 30 min intervals in order to determine efflux in relation to time. The lipids of intima and media and the series of incubation media were extracted and radioassayed.

TABLE 45

Efflux into Hanks' Solution : Hypercholesterolaemic Serum of ^{3}H-labelled Cholesterol from Cholesterol-fed Rabbit Aortic Halves Labelled *In Vivo* [a]

	Cholesterol content	Specific activity cpm/mg	Efflux [c]		
	Intima (mg in toto)	Intima	μg/day	μg/day per mg in toto	Relative efflux
Free cholesterol	4.47 ± 0.69	256 ± 70	7401 ± 1448	1925 ± 466	1
Cholesterol ester	10.01 ± 1.63	209 ± 46	9873 ± 3453	915 ± 216	[b]0.54 ± 0.11

[a] Means and standard errors of means of six experiments

[b] Significant at 1% level

[c] Values on caluculated assuming efflux from a single pool. This assumption is probably incorrect as discussed in the text.

The efflux of ^{3}H-labelled cholesterol with time is shown in Figure 51. The ^{3}H-labelled cholesterol was assumed to be coming solely from the intima since more than 90% of the total ^{3}H in the artery was in the intima. In Figure 51A the total cpm remaining in the intima of each half of the aorta has been plotted on a logarithmic scale against time. The curves represent the disappearance of ^{3}H-labelled cholesterol from the intima and can be resolved into two separate components, suggesting two separate pools in the intima. After 120 min, both curves are essentially straight and represent the exit of ^{3}H-labelled cholesterol from the less active pool. From the gradient of the straight section, the pool half life can be calculated. It is 25 hours in the case of the Hanks' : serum incubation medium and 50 hours in the case of the Hanks' only incubation medium. Extrapolation of this line back to the ordinate and subtraction from the disappearance curve, resolves the second component (Figure 51B). That this component can be represented by a straight line suggests that it is a single more active pool, the half life of which is 20 min for the Hanks' : serum and 19 min for the Hanks' only incubation medium. 19.1% of the ^{3}H-labelled cholesterol present initially in the artery was removed over 4 hours into the medium containing Hanks' solution and hypercholesterolaemic serum, and 17.9% of the ^{3}H-labelled cholesterol present in the artery into the medium containing Hanks' solution only, so that in both cases over 80% of the label was still present in the artery at this time.

D. Entry of $^{3}H/^{14}C$-labelled Cholesterol into the Atherosclerotic Aorta in Vitro

Hypercholesterolaemic rabbit serum containing lipoprotein doubly-labelled with ^{14}C and ^{3}H-labelled cholesterol and cholesterol ester was prepared as described under "Materials and Methods".

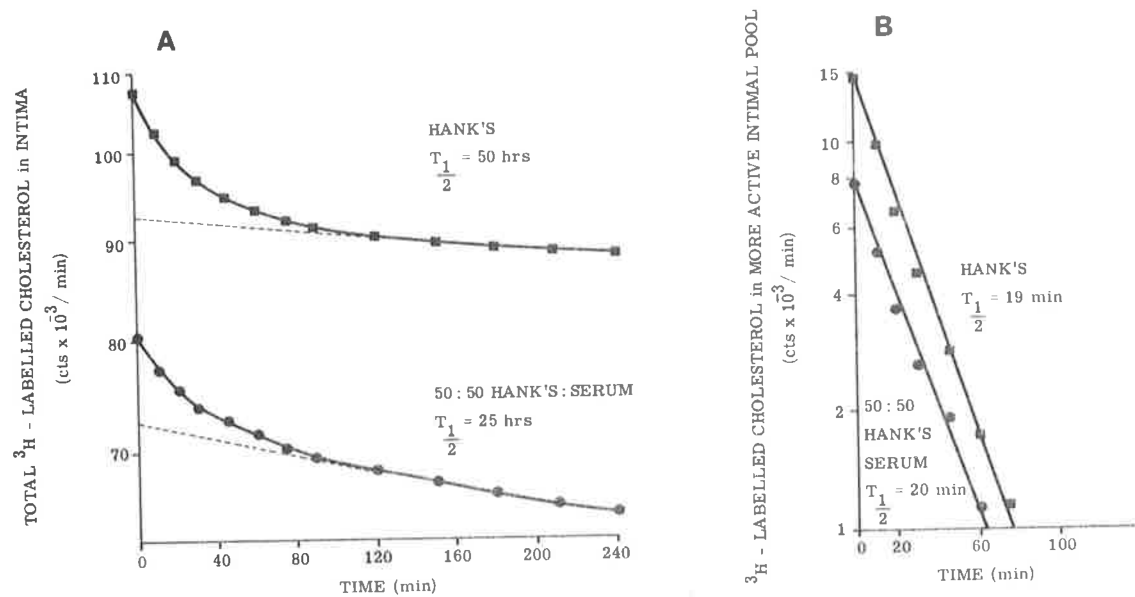

Figure 51

Efflux of ^{3}H-labelled cholesterol from atherosclerotic intimal halves into either an incubation medium of Hanks'solution or 50 : 50 Hanks' : hypercholesterolaemic serum. Counts/min remaining in the intima are plotted logarithmically in both A and B. Curves in A have been resolved into two components. The more slowly removed component (dotted line) has been subtracted from the composite curves to yield a plot of the more rapidly removed component in B.

A preparation of hypercholesterolaemic serum was obtained in which the lipoprotein cholesterol ester was labelled almost exclusively with ^{3}H and in which lipoprotein free cholesterol was labelled with both ^{14}C and ^{3}H. The cholesterol content, specific activities (^{3}H and ^{14}C), and $^{3}H/^{14}C$ ratios of this preparation are shown in Table 1. The free cholesterol distribution of ^{3}H and ^{14}C and $^{3}H/^{14}C$ ratios among the lipoprotein fractions, separated by ultracentrifugation, are shown in Table 2.

The influx *in vitro* of cholesterol and cholesterol esters was determined using whole thoracic aortae obtained from rabbits fed cholesterol for 4 months. The aorta was removed following killing by ether anaesthesia, cleaned and then incubated for 4 hours in 4.5 ml of the $^{3}H/^{14}C$ double - labelled serum. Serum was incubated alone, also, to observe possible esterification and hydrolysis in the medium. After incubation, the lipids of the intima and the media/adventitia and of the incubation medium were extracted. Free and ester cholesterol were then separated as for the *in vivo* experiments. Specific activities of individual cholesterol esters were, however, determined directly on the eluates from silver nitrate T.L.C., by radioassay and the colorimetric assay of cholesterol following saponification.

The influx of free and ester cholesterol and of the various cholesterol esters into the intima in the *in-vitro* experiments is shown in Table 46. The influx was calculated on the same basis as that for the *in-vivo* experiments, except that in this case the specific activity of the serum bathing the artery was constant throughout the experiment. In addition, since the free cholesterol was labelled with both ^{3}H and ^{14}C, it was possible to calculate the influx of free cholesterol from the ^{3}H-labelled and the ^{14}C-labelled cholesterol present. The amount of free cholesterol and of

TABLE 46

Influx of Lipoprotein $^{3}H/^{14}C$-labelled Cholesterol and Cholesterol Esters into Cholesterol-fed Rabbit Aortic Intimae In Vitro [a,b,c]

		^{3}H-DATA				^{14}C-DATA		
	Cholesterol content (mg)	Specific activity (dpm/mg)	Influx µg/day	Influx µg/day per mg/ml	Relative influx	Specific activity (dpm/mg)	Influx µg/day	Influx µg/day per mg/ml
Free cholesterol	6.96 $\pm$ 4.02	1258 $\pm$ 436	144.4 $\pm$ 21.2	67.1 $\pm$ 9.9	1	1316 $\pm$ 430	181.8 $\pm$ 34.3	84.5 $\pm$ 16.0
Cholesterol ester	14.71 $\pm$ 6.80	2559 $\pm$ 605	315.4 $\pm$ 82.1	36.5 $\pm$ 9.5	[d]0.53 $\pm$ 0.09	-	-	-
Cholesterol ester								
- saturated	4.32 $\pm$ 1.86	2198 $\pm$ 532	68.6 $\pm$ 11.1	34.8 $\pm$ 5.6	0.82 $\pm$ 0.12	-	-	-
- nonunsaturated	6.19 $\pm$ 3.09	2691 $\pm$ 524	204.0 $\pm$ 58.4	45.8 $\pm$ 13.1	1	-	-	-
- polyunsaturated	4.21 $\pm$ 1.85	3050 $\pm$ 463	96.2 $\pm$ 26.4	44.0 $\pm$ 11.8	0.97 $\pm$ 0.03	-	-	-

[a] Means and standard errors of means of three experiments

[b] Data for serum used given in Table 5

[c] Whole aortae (not halves) were used in these experiments

[d] Significant at < 5% level

cholesterol ester entering the intima (μg/day) in these experiments was similar to the figures obtained for the in-vivo experiments, both with respect to the free cholesterol and cholesterol ester and with respect to the individual groups of cholesterol esters. It needs to be borne in mind, however, that in these experiments influx into whole aortae and not into aortic halves is being calculated. The relative influx of free cholesterol was greater than that of cholesterol ester as in the experiments in vivo. There was no significant difference, however, between the relative entry of the individual groups of cholesterol esters. It will be observed from Table 46 that the influx of free cholesterol as calculated from the ^{14}C data was about 20% higher than that calculated from the ^{3}H data. This difference suggested that the lipoprotein labelling produced by the ^{14}C differed somewhat from that produced by the ^{3}H, one being labelled in vivo and the other in vitro. In order to determine whether ^{3}H and ^{14}C-labelled cholesterol were distributed in the same proportion in the various lipoprotein fractions of the serum, the serum lipoproteins were separated by ultracentrifugation, extracted, and the ^{3}H and ^{14}C present in the free cholesterol of the various fractions determined. This data is shown in Table 2. It can be seen that while the bulk of both the ^{3}H-labelled and the ^{14}C-labelled cholesterol is present in the fraction with a density less than 1.006, there is some difference in relative distribution of the label; slightly more ^{14}C and less ^{3}H-labelled free cholesterol is present in the higher density lipoproteins. This skewing of distribution of ^{14}C compared with ^{3}H may explain the slight difference in the influx data calculated from the two labels, presumably by increased entry of the smaller lipoprotein particles labelled with ^{14}C.

Influx of cholesterol, cholesterol ester and the individual groups of cholesterol ester into the media in the experiments in vitro is shown in Table 47. It will be noted that the influx

TABLE 47

Influx of Lipoprotein $^{3}H/^{14}C$-labelled Cholesterol and Cholesterol Esters into Cholesterol-fed Rabbit Aortic Media <u>In Vitro</u> [a,b,c]

		^{3}H-DATA				^{14}C-DATA			
	Cholesterol content (mg)	Specific Activity (dpm/mg)	Influx µg/day	Influx µg/day per mg/ml	Relative influx	Specific Activity (dpm/mg)	Influx µg/day	Influx µg/day per mg/ml	Relative influx
Free cholesterol	2.14 ± 0.40	10152 ± 1472	558 ± 81	259 ± 38	1	10872±2222	752 ± 127	350 ± 59	-
Cholesterol ester	5.75 ± 1.30	20832 ± 4761	1198 ± 212	139 ± 24	0.53 ± 0.04	-	-	-	-
Cholesterol ester									
- saturated	1.49 ± 0.50	29210 ± 7120	279 ± 51	142 ± 26	0.87 ± 0.03				
- monounsaturated	2.81 ± 0.47	22070 ± 6050	732 ± 153	164 ± 34	1				
- polyunsaturated	1.45 ± 0.34	35350 ± 10030	334 ± 71	150 ± 32	0.91 ± 0.04				

[a] Mean and standard error of mean of three experiments

[b] Data for serum used given in Table 5

[c] Whole aortas not halves were used in these experiments

into the media in these experiments was considerably higher than that into the media in the experiments *in vivo*, and exceeded that into the intima in the corresponding experiments *in vitro*. The influx of free cholesterol relative to ester cholesterol, however, was similar to that in the intima. That is, more free cholesterol entered the media than would have been expected on the basis of its concentration in the serum of the incubation medium (Table 1). The entry of individual groups of cholesterol esters into the media is also shown in Table 47. The relative influx of monounsaturated exceeded that of both saturated and polyunsaturated but differences are not significant. Thus, although influx into the media in the experiments *in vitro* was greatly in excess of that in the experiments *in vivo*, the relative influxes of free and ester cholesterol were essentially those shown for the experiments *in vivo*.

The contribution which hydrolysis of cholesterol ester in the artery might make to the apparent influx of free cholesterol is shown in the data given in Table 48. Hydrolysis of the predominantly ^{3}H-labelled cholesterol ester in the intima would have led to an elevation of the $^{3}H/^{14}C$ ratio of free cholesterol and so a sensitive index of cholesterol ester hydrolysis in the artery has been provided. No hydrolysis occurred in the incubation medium over 4 hours, the $^{3}H/^{14}C$ ratio altering from 1.26 to 1.21 (Table 48). For the three experiments the $^{3}H/^{14}C$ ratio of free cholesterol fell slightly in both intima and media. Under the circumstances of the present experiments, then, no evidence of hydrolysis of lipoprotein cholesterol ester by the arterial wall was obtained.

E. Discussion

The entry of cholesterol and cholesterol ester into the normal and

TABLE 48

Esterification and Hydrolysis of Lipoprotein $^{3}H/^{14}C$-labelled Cholesterol by Atherosclerotic Rabbit Aortae *In Vitro*

	Time (hours)	Experiment	% Cholesterol ester		$^{3}H/^{14}C$ free cholesterol
			^{3}H	^{14}C	
Serum	0		90.9	1.6	1.26
	4		[a] 91.1 ± 0.2	[a] 1.4 ± 0.3	[a] 1.21 ± 0.03
Intima	4	1	82.8	2.1	0.97
	4	2	87.6	15.3	0.86
	4	3	83.1	1.5	0.97
Media	4	1	85.8	1.4	0.86
	4	2	82.9	1.4	1.00
	4	3	83.3	1.1	1.00

[a] Mean of eight determinations with standard deviations

atherosclerotic arterial intima has been the subject of a number of studies (Newman and Zilversmit, 1962 and 1966; Hashimoto and Dayton, 1966; Dayton and Hashimoto, 1966; Jensen, 1969). The results reported in the present paper confirm those of Newman and Zilversmit for the atherosclerotic intima in that the entry, both *in vivo* and *in vitro*, of radioactively labelled free cholesterol in relation to cholesterol ester is twice that expected on the basis of their respective plasma concentrations. The reason for the apparently greater relative influx of free cholesterol, however, is open to question. Hydrolysis of cholesterol ester has been reported for both the normal and atherosclerotic arterial wall (Day and Gouldhurst 1966; Patelski, Bowyer, Howard and Gresham, 1968) and it seemed possible that this mechanism might affect the calculation of cholesterol influx. In the present work, this possibility has been examined by using double-labelled lipoprotein cholesterol and cholesterol ester. It can be seen from a consideration of the properties of the double-labelled lipoprotein used in these experiments that it is possible to detect very small amounts of hydrolysis of the cholesterol ester entering the arterial intima. 5% hydrolysis of the ^{3}H-labelled cholesterol ester would produce an increase in the $^{3}H/^{14}C$ ratio of the free cholesterol of about 50%. No such shift occurred, in fact a slight fall in the ratio was observed. This should not be taken to mean that cholesterol ester hydrolysis cannot occur in the arterial wall, for, indeed, it has been shown to occur. The point being made is that, for all intents and purposes, the calculated influx of free cholesterol cannot be accounted for by hydrolysis of labelled cholesterol ester which has entered the wall. It should be noted, that the $^{3}H/^{14}C$ ratios of the individual lipoprotein fractions are consistently above the overall serum ratio and it is possible, therefore that not all of the ^{14}C-labelled cholesterol was complexed to lipoprotein. Nevertheless, it is

the overall ratios which are compared and these provide no evidence of hydrolysis. There is a case, however, for extending the present approach to individual lipoprotein fractions, especially as Okishio (1961) has reported that the entries of low and high density lipoproteins labelled with ^{131}I into the rabbit atherosclerotic lesion are different.

Even though in the experments *in vitro* (Table 48) and in those of other workers (Day and Gouldhurst, 1966; Newman, Gray and Zilversmit, 1968) esterification of radioactive cholesterol has been found difficult to demonstrate in the intima, this is still a possible explanation for the origin of cholesterol ester as discussed in the "General Introduction." Entry of cholesterol ester directly as plasma lipoprotein is also a possibility since, unlike free cholesterol, cholesterol ester does not readily exchange off lipoprotein (Roheim, Haft, Gidez, White and Eder, 1962).

The finding, from the *in-vivo* studies, that most of the aortic labelled cholesterol, both free and ester, was in the intima, is in accord with the autoradiographic findings of Adams, Bayliss, Davison and Ibrahim (1964) although, in a later paper, using a multiple-layering technique, Adams, Virag, Morgan and Orton (1968) report differences in distribution of labelled cholesterol across the aortic wall according to the degree of atherosclerosis. It should be noted that, in the present work, calculation of influx of cholesterol and cholesterol ester into the arterial media *in vivo* is based on the assumption that the cholesterol entering the media has the same specific activity as plasma cholesterol. If radioactive cholesterol in the arterial media was derived from the vasavasorum, the calculation is valid. If the radioactive cholesterol was derived from the intima, however, the calculated influx could be an under-estimate because of the

lower intimal specific activities. The influx of radioactive cholesterol into the intima, is similar *in vivo* and *in vitro*. However, the influx of cholesterol into the media was much greater *in vitro* than *in vivo*, probably due to the more direct exposure of the arterial media/adventitia to radioactive cholesterol from the incubation medium.

The efflux of labelled cholesterol from the aorta, in μg/day, exceeded influx by some fifty times. Since more than 80% of the radioactivity in the aorta was present in the intima, it is reasonable to assume that efflux of labelled cholesterol *in vitro* was mainly from the intima. The high apparent efflux may be explained if a cholesterol pool of high specific activity were present in the intima, as suggested by Newman and Zilversmit (1966). To investigate this possibility the efflux of ^{3}H-labelled cholesterol from the artery was studied in relation to time. Data from only one experiment is available, but with this limitation in mind it is possible, using a semilogarithmic plot, to resolve the efflux into two components. In this experiment, efflux of the more rapidly removed component of labelled cholesterol did not appear to be dependent on the availability of lipoprotein in the incubation medium. The observation that efflux of labelled cholesterol occurred into incubation medium containing Hanks' solution alone, is in contrast to the observation of Dayton and Hashimoto (1966) for normal rat aorta, in which relatively little efflux into buffer solution, but considerable efflux into a solution containing serum lipoprotein took place. The divergent findings may be related to a difference between normal and atherosclerotic intima. For example, greater amounts of plasma lipoprotein may be present in the atherosclerotic intima because of surface disorganisation or increased permeability. This lipoprotein would then have been removed into an aqueous medium over a period of incubation *in vitro*. Another possible source of

labelled cholesterol in the aqueous medium might have been the sloughing-off of cells and cell breakdown during incubation. Efflux of the more slowly removed component was appreciably greater, as indicated by the $T_{1/2}$ when Hanks' solution : serum was used rather than Hanks' solution alone. This difference is likely to represent exchange of radioactive intimal cholesterol for that in the serum lipoprotein of the Hanks' : serum medium.

In the timed efflux experiment, as in all the efflux experiments, more than 80% of the labelled cholesterol was present in the intima at the end of the four hour incubation *in vitro* and this amount contained both free and ester labelled cholesterol suggesting that efflux *in vivo* might not have affected significantly, calculated influx. However, since the $T_{1/2}$ for the efflux of ^{3}H-labelled cholesterol from the larger less active pool into the Hanks' : serum incubation medium was 25 hours, there is the possibility of considerable recycling of ^{3}H-labelled cholesterol during the 96 hours study of influx *in vivo*. The influx figures as calculated from the cpm accumulated in the intima over the 96 hour period may therefore be rather low. However, the validity of the comparisons, on a relative basis, of cholesterol and cholesterol ester is not necessarily influenced by such considerations. The relative efflux of free cholesterol was twice that of cholesterol ester as was the case for the relative influx. The cholesterol of the intima, therefore, seems to be turning over more rapidly than the cholesterol ester. This may simply reflect a process of physico-chemical exchange, however, and need not necessarily reflect the mechanism of the overall accumulation of cholesterol ester in the atherosclerotic lesion.

The other aspect of the present work was to obtain some information regarding the relative entry of different groups of

cholesterol esters into the atherosclerotic arterial intima. There is now considerable evidence that the atherosclerotic intima differs from the serum in its fatty acid composition, both in man and experimental animals, in that it contains a higher proportion of cholesterol oleate and a lower proportion of cholesterol linoleate (see "General Introduction"). In the present work, this has been confirmed in that the aortic intima contained more cholesterol oleate and less cholesterol linoleate than did the terminal plasma. The experiments reported *in vivo* are acute in the sense that the entry of ^{3}H-labelled cholesterol esters into an already atherosclerotic intima was observed. It was shown, nonetheless, that the influx in μg/day of monounsaturated cholesterol ester exceeded that of polyunsaturated and saturated cholesterol ester. This agreed with the findings of Swell, Law and Treadwell (1963). Further, when serum concentrations of individual cholesterol esters were taken into account, the relative influx of ^{3}H-labelled monounsaturated cholesterol esters was still significantly in excess of that of the polyunsaturated cholesterol esters. This finding may, however, only be a reflection of cholesterol oleate accumulation, rather than the reason for it. That is, the increased entry of monounsaturated cholesterol ester could have a metabolic basis. It could even be that, as discussed for the comparison of free and ester cholesterol entry, the increased entry of monounsaturated cholesterol ester is more apparent than real and depends on intimal interconversion of cholesterol esters or on esterification of free cholesterol.

SECTION 8

LIPID SYNTHESIS FROM ^{14}C-LABELLED ACETATE

THE ARTERIAL WALL IN VITRO AND

FACTORS AFFECTING IT

A. Introduction

In the arterial wall of the experimental animal, ^{14}C-labelled acetate has been shown to be incorporated into cholesterol (Siperstein, Chaikoff and Chernick, 1951; Azarnoff, 1958) and into fatty acid (Chernick, Srere and Chaikoff, 1949; Feller and Huff, 1955; Loomeijer and Van der Veen, 1962). There is evidence for both the de novo and the chain elongation pathways of fatty acid synthesis (Whereat, 1966; Howard, 1968; Vost, 1969), as detailed in the "General Introduction". Fatty acid synthesized from acetate in the rabbit atherosclerotic lesion can be incorporated into phospholipid, triglyceride and cholesterol ester (Day and Wilkinson, 1967). Saturated, monounsaturated and polyunsaturated fatty acids are synthesized from acetate in monkey and rabbit arterial wall (Howard, 1968; Whereat and Orishimo, 1968). Yet, for the human arterial wall, there is only a limited amount of information. Paoletti, Paoletti and Savi (1958) have shown that fatty acid can be formed from ^{14}C-labelled acetate in the arterial wall of the human foetus; Chobanian and Hollander (1963) have reported in an abstract that palmitic and stearic acids, but not unsaturated fatty acids are synthesized in normal and atherosclerotic human vessels; Maggi (1964) has shown that ^{14}C-labelled acetate is incorporated into phospholipid and into a combined neutral fat and cholesterol ester fraction of atherosclerotic human arteries; and Chobanian and Hollander (1966) have produced evidence that the phospholipids cephalin, lecithin and sphingomyelin are synthesized from ^{14}C-labelled acetate in normal and atherosclerotic human intima.

It seemed worthwhile to re-investigate lipid metabolism by the human intima using ^{14}C-labelled acetate as precursor especially as synthesis of cholesterol ester itself had not been documented and as the fatty acids found synthesized in human

artery by Chobanian and Hollander were very different to those which have been found to be synthesized by the artery of the experimental animal.

In the present study, too, an effort has been made to see whether intimal cells behave differently to non-cellular intimal components with respect to lipid formation from ^{14}C-labelled acetate. Robertson and Insull (1967) and Robertson and Page (1967) have isolated several different human intimal cells with a proteolytic enzyme technique, an advance on the enzyme disruption method employed in this thesis which does not appear to discriminate between different cells. But, as yet, no metabolic studies have been reported with the different cell types.

^{14}C-labelled acetate has been used as a precursor to assess the effect of a given factor on arterial lipid metabolism. The effects of chlorophenoxyisobutyric acid (CPIB) and of aortic coarctation, with proximal hypertension and distal hypotension or normotension, (see "General Introduction") on lipid metabolism by the human arterial wall are reported. At the same time, the control studies provide an extension to the basic studies of human arterial acetate metabolism.

The opportunity has also been taken to examine the effects of CPIB and of insulin and anti-insulin (see "General Introduction") on lipid metabolism by the rabbit atherosclerotic aorta.

In the case of the human coarctation experiments, and in the case of the rabbit CPIB experiments, the vessels were incubated with ^{32}P-labelled phosphate as well as ^{14}C-labelled acetate to determine if these factors had effects on phospholipid metabolism from one precursor and not another.

B. Lipid Synthesis from ^{14}C-labelled Acetate by the Human Arterial Wall, in Isolated Intimal Cells, and the Effect of Chlorophenoxyisobutyric Acid (CPIB)

Human vessels were obtained from five patients whose clinical details are shown in Tables 49 and 52. In all but the first case of Table 49, where thoracic aorta was obtained from a renal transplant donor, specimens were obtained at the time of vascular surgery. The object was to compare, *in vitro*, acetate-1-^{14}C metabolism in normal intima and in different lesion types from the one patient under the same incubation conditions. This was only possible for the first case of Table 52.

Incubations were carried out for 4 hours in a medium consisting of equal parts of Hanks' solution and normal human serum with a known amount of ^{14}C-labelled acetate (Tables 49 and 52). Normal artery and different lesion types were dissected from one another, intima stripped from media, and lipid extracts prepared. 1-2% acetate-1-^{14}C was found to remain in the lower lipid-containing phase of a Folch wash. In that the volume of incubation medium varied from experiment to experiment (according to the amount of tissue to be incubated), comparisons of percentage uptake and conversion to lipid of acetate-1-^{14}C per mg dry defatted intima are only valid within the one experment. The opportunity was taken in the second experiment shown in Table 49 to incubate saphenous vein as well as femoral artery with ^{14}C-labelled acetate. The uptake and conversion to lipid was less for the venous intima than for the arterial intima. While in experiment 1, Table 52, the uptake and conversion to lipid was greater in the normal intima than in the various lesions, no conclusion can be drawn from a single experiment.

Major lipid fractions were separated on neutral lipid T.L.C. Since acetate-1-^{14}C was found to run in the region of cholesterol

TABLE 49

CLINICAL DATA OF HUMAN VESSELS INCUBATED IN VITRO WITH ACETATE-1-^{14}C AND THE INTIMAL UPTAKE AND CONVERSION TO LIPID OF ACETATE-1-^{14}C

Experiment	Patient's Age	Clinical Details	Vessel	State of Vessel	Incubation Medium µCi/ml	% Uptake and Conversion to Lipid/mg dry Defatted Tissue
1	48	Cerebral Tumour; Renal Transplant Donor	Thoracic Aorta	Fibro-Fatty	30.0	-
2	48	Traumatic Thrombosis of Internal Iliac and Femoral Artery	Femoral Artery	Fibro-Fatty	8.8	0.0034
			Saphenous Vein	Normal	8.8	0.0008
3	42	Coronary Artery Disease; Peripheral Vascular Disease	Iliac Artery	Fibro-Fatty & Calcified	4.2	0.0007

and diglyceride, it is possible that some of the label in this spot was precursor rather than lipid. In all intimae, whether arterial or venous, whether aortic, femoral or iliac, whether normal or atherosclerotic, the fractions into which most label was incorporated, were phospholipid and triglyceride, less being incorporated into free fatty acid and cholesterol ester (Tables 50 and 53).

It is noteworthy that, in the aortic fibro-fatty lesion which was separated into the intimal fractions, cells, residue, supernatant and particles (see "Materials and Methods") after incubation with acetate-1-^{14}C, relatively more cholesterol ester was labelled in the cells than in any other intimal fraction (Table 50).

Intimal total fatty acid was methyl esterified and separated on silver nitrate T.L.C. as described in "Materials and Methods". "Origin" and "polyunsaturated" spots were combined. Because methyl acetate was found to remain at the origin, "polyunsaturated" fatty acid may include same precursor acetate. An index of the upper limits of the amount of ^{14}C-labelled methyl acetate in the "polyunsaturated" fatty acid methyl esters is given by the percentage of label as cholesterol/diglyceride in neutral lipid separations (refer Tables 50 and 53). The percentage distributions of label among human intimal fatty acids are given in Tables 51 and 54. Most of the label is present as polyunsaturated fatty acid and as saturated fatty acid with a lesser amount as monounsaturated fatty acid.

The effect <u>in vitro</u> of the sodium salt of chlorophenoxyisobutyric acid, in concentrations of 500 µg/ml, on acetate-1-^{14}C metabolism by the human arterial intima is shown in Tables 52 - 54. Human

TABLE 50

PERCENTAGE DISTRIBUTION OF LABEL AMONG INTIMAL LIPIDS FOLLOWING INCUBATION OF HUMAN VESSELS WITH ACETATE-1-^{14}C IN VITRO

Experiment	Vessel	State of Vessel	*Intimal Fraction	LIPID FRACTIONS				
				Phospholipid	Cholesterol/ Diglyceride	Fatty Acid	Triglyceride	Cholesterol Ester
1	Thoracic Aorta	Fibro-Fatty		61.8	7.8	4.8	20.9	4.4
			Cells	55.6	5.7	3.1	19.6	16.1
			Residue	61.6	5.8	1.9	22.4	8.3
			Supernatant	62.6	19.6	7.3	5.4	5.3
			Particles	62.5	19.6	7.3	5.4	5.3
2	Femoral Artery	Fibro-Fatty		49.4	7.0	7.0	27.9	8.8
	Vein	Normal		50.8	13.2	10.4	20.8	4.9
3	Iliac Artery	Fibro-Fatty and Calcified		46.0	17.5	9.9	20.8	5.9

* Eplanation under "Materials and Methods" and in text

TABLE 51

PERCENTAGE DISTRIBUTION OF LABEL AMONG INTIMAL TOTAL FATTY ACIDS ACCORDING TO THEIR DEGREE OF UNSATURATION, FOLLOWING INCUBATION OF HUMAN VESSELS WITH ACETATE-1-^{14}C IN VITRO

			FATTY ACIDS			
Experiment	Vessel	State of Vessel	*Polyunsaturated	Monounsaturated	Saturated	Solvent Front
1	Thoracic Aorta	Fibro-Fatty	31.6	9.0	59.2	0.3
2	Vein	Normal	57.2	11.1	29.0	2.9
3	Iliac Artery	Fibro-Fatty and Calcified	36.0	23.1	39.1	2.0

* The "origin" of the silver nitrate T.L.C. plate has been included with "polyunsaturated".

TABLE 52

CLINICAL DATA OF HUMAN ARTERIES INCUBATED IN VITRO WITH ACETATE-1-^{14}C AND THE EFFECT OF CPIB ON INTIMAL UPTAKE AND CONVERSION TO LIPID

Experiment	Patient's Age	Clinical Details	Vessel	State of Vessel	Incubation Medium μCi/ml	% Uptake and Conversion to Lipid/mg Dry Defatted Tissue	
						Control	CPIB
1	61	Ruptured Abdominal Aortic Aneurism	Abdominal Aorta	Normal	3.7	0.0094	0.0049
				Fatty Streak		0.0068	0.0122
				Fibro-Fatty		0.0028	0.0069
				Complicated		0.0039	0.0019
2	59	Mature-Onset Diabetic; Moderate Hyper-tension; Hypercholesterol-aemia (398 mg%)	Abdominal	Fibro-Fatty	3.6	0.0151	0.0162
			Femoral Artery	Fibro-Fatty		0.0096	0.0120

TABLE 53

PERCENTAGE DISTRIBUTION OF LABEL AMONG INTIMAL LIPIDS FOLLOWING INCUBATION OF HUMAN ARTERIES WITH ACETATE-1-^{14}C IN VITRO AND THE EFFECT OF CPIB

Experiment	Vessel	State of Vessel		LIPID FRACTIONS: Phospholipid	Cholesterol/ Diglyceride	Fatty Acid	Triglyceride	Cholesterol Ester
1	Abdominal Aorta	Normal	Control	47.8	8.7	3.2	34.0	6.4
			CPIB	48.9	7.1	2.2	38.5	3.4
		Fatty Streak	Control	50.3	6.9	4.0	31.7	7.2
			CPIB	53.3	4.8	3.4	34.3	4.2
		Fibro-Fatty	Control	44.4	5.8	7.4	31.6	10.8
			CPIB	51.0	5.2	3.4	35.3	5.2
		Complicated	Control	45.5	11.0	7.1	30.6	5.9
			CPIB	43.7	11.6	7.4	31.0	6.3
2	Abdominal Aorta	Fibro-Fatty	Control	31.6	30.9	10.1	22.9	4.7
			CPIB	37.0	21.6	14.6	19.8	7.1
	Femoral	Fibro-Fatty	Control	41.0	12.9	7.5	27.3	11.3
			CPIB	65.4	9.0	6.3	14.6	4.8

TABLE 54

PERCENTAGE DISTRIBUTION OF LABEL AMONG INTIMAL TOTAL FATTY ACIDS, ACCORDING TO THEIR DEGREE OF UNSATURATION, FOLLOWING INCUBATION OF HUMAN VESSELS WITH ACETATE-1-^{14}C IN VITRO AND THE EFFECT OF CPIB

Experiment	Vessel	State of Vessel		FATTY ACIDS			
				*Polyunsaturated	Monounsaturated	Saturated	Solvent Front
1	Abdominal Aorta	Normal	Control	33.6	29.4	35.6	1.5
			CPIB	39.4	12.0	56.9	1.5
		Fatty Streak	Control	38.1	17.0	44.5	0.6
			CPIB	40.0	12.0	47.4	0.7
		Fibro-Fatty	Control	46.0	14.6	36.8	2.8
			CPIB	36.8	18.5	43.1	1.7
		Complicated	Control	41.0	19.0	37.1	2.9
			CPIB	43.4	14.8	38.9	2.9
2	Abdominal Aorta	Fibro-Fatty	Control	45.2	14.1	33.1	7.7
			CPIB	36.7	23.2	32.0	8.4
	Femoral	Fibro-Fatty	Control	27.1	23.3	45.0	4.7
			CPIB	35.5	13.5	40.6	10.6

* The "origin" of the silver nitrate T.L.C. plate has been included with the "polyunsaturated".

arterial tissues were divided into nearly duplicate specimens so that incubations with a control medium (50 : 50 Hanks' solution : normal human serum) could be compared with those using the same medium containing CPIB. No consistent effect of CPIB on the intimal uptake and conversion of ^{14}C-labelled acetate to lipid (Table 52), the labelling of major lipid fractions (Table 53), or the labelling of fatty acid classes (Table 54) was observed.

C. Lipid Synthesis from ^{14}C-labelled Acetate by the Rabbit Atherosclerotic Aorta and the Effect of Chlorophenoxyisobutyric Acid (CPIB)

Atherosclerotic thoracic aortae were obtained from two rabbits fed a cholesterol diet for 4 and 1/2 months. The aortae were divided longitudinally into halves. One half was incubated in 5 ml of a control medium of equal parts of Hanks' solution and normal rabbit serum, containing 17.9 µCi acetate-1-^{14}C and about 20 µCi ^{32}P-labelled phosphate; the other half was incubated in 5 ml of the same medium containing 500 µg sodium chlorophenoxyisobutyrate/ml. After 4 hours incubation, the tissues were washed in 0.9% saline, intima stripped from media and lipid extracts prepared.

The uptake and conversion to lipid of ^{14}C-labelled acetate is shown for control and CPIB-treated aortic intimae in Table 55. There are no obvious differences.

Major lipid fractions were separated on neutral lipid T.L.C. and eluted for counting in toluene scintillator, since the two radioisotopes ^{14}C and ^{32}P were used (see "Materials and Methods"). The distribution of ^{14}C among intimal major lipid fractions is shown in Table 55. There are no obvious differences in labelling between the control and CPIB treated vessels.

TABLE 55

EFFECT OF CPIB ON THE UPTAKE AND CONVERSION TO LIPID OF ACETATE-1-^{14}C BY RABBIT ATHEROSCLEROTIC INTIMA IN VITRO AND ALSO ON THE PERCENTAGE DISTRIBUTION OF LABEL AMONG MAJOR LIPID FRACTIONS

Experiment		% Uptake and Conversion to Lipid/mg Dry Defatted Tissue	LIPID FRACTIONS				
			Phospholipid	Diglyceride/ Cholesterol	Fatty Acid	Triglyceride	Cholesterol Ester
1	Control	0.0039	59.0	8.5	3.3	20.1	9.2
	CPIB	0.0040	61.9	4.1	1.2	16.3	16.6
2	Control	0.0108	57.1	4.7	3.4	15.0	20.0
	CPIB	0.0101	55.4	4.8	2.8	15.4	21.7

Phospholipid was separated by neutral lipid T.L.C. and eluted by the method of Arvidson (1967) prior to separation of individual phospholipids, also by T.L.C. Control and CPIB samples were run on the same plates. Individual phospholipids were also eluted by the Arvidson method and counted in toluene scintillator. Essentially, the distribution of label among the individual phospholipids is the same in the control and CPIB-treated intimae, lecithin being the major labelled phospholipid, although there is, possibly, relatively more lecithin labelled in the CPIB-treated intima (Table 56). ^{32}P-labelled phosphate was used simultaneously with ^{14}C-labelled acetate as a precursor of phospholipid synthesis to assess whether CPIB affected one metabolic pathway and not another. The distributions of ^{32}P-labelled phosphate among individual phospholipids for the control and CPIB-treated intimae agreed essentially with those of ^{14}C-labelled acetate (Table 56).

In one of the two experiments, intimal total fatty acid was methyl esterified and these methyl esters separated from other lipids (in particular, those containing ^{32}P-labelled phosphate) by T.L.C. (see "Materials and Methods"). The total fatty acids were then separated by silver nitrate T.L.C. There was a similar order of labelling among the polyunsaturated, monounsaturated and saturated classes of fatty acid and no obvious difference between the control and the CPIB-treated intimae (Table 57).

D. Lipid Synthesis from ^{14}C-labelled Acetate by the Rabbit Atherosclerotic Aorta and the effects of Insulin and of Anti-Insulin

Atherosclerotic thoracic aortae were obtained from two rabbits fed cholesterol for 7 months. They were divided longitudinally into thirds.

TABLE 56

EFFECT OF CPIB ON THE PERCENTAGE DISTRIBUTION OF LABEL AMONG INDIVIDUAL PHOSPHOLIPIDS OF THE RABBIT ATHEROSCLEROTIC INTIMA INCUBATED IN VITRO WITH ACETATE-1-^{14}C AND ^{32}P-LABELLED PHOSPHATE

Experiment		Origin	Lysolecithin	Spingomyelin	Lecithin	Phosphatidyl Inositol	Phosphatidyl Ethanolamine	Solvent Front
				Acetate-1-^{14}C				
1	Control	1.8	3.7	7.8	58.9	8.2	6.9	12.8
	CPIB	1.3	2.7	4.8	73.3	7.2	1.8	10.1
2	Control	0.5	4.1	10.6	67.3	7.4	1.5	8.8
	CPIB	4.5	4.9	1.0	76.1	6.9	1.4	5.3
				^{32}P-Labelled Phosphate				
1	Control	3.8	4.6	6.2	68.5	6.9	5.8	4.1
	CPIB	2.9	3.7	4.2	75.1	6.3	4.5	3.4
2	Control	2.7	4.0	4.5	74.7	7.7	3.5	3.0
	CPIB	2.2	2.1	2.5	79.6	7.6	3.7	2.3

TABLE 57

EFFECT OF CPIB ON THE PERCENTAGE DISTRIBUTION OF LABEL AMONG TOTAL FATTY ACIDS OF RABBIT ATHEROSCLEROTIC INTIMA INCUBATED IN VITRO WITH ACETATE-1-^{14}C

Experiment		*Polyunsaturated	Monounsaturated	Saturated	Solvent Front
2	Control	33.5	21.5	44.3	0.9
	CPIB	36.8	23.8	38.9	0.6

* The "origin" of the silver nitrate T.L.C. plate has been included with "polyunsaturated".

Incubation media consisted of 2.5 ml Hanks' solution, 2.5 ml guinea-pig serum (control or anti-insulin), 0.5 ml saline, with or without 2.5 units of insulin, and 13 μCi acetate-1-^{14}C. Thus, for each vessel, three incubations were set up, control, insulin and anti-insulin, each with one aortic third. After 2 hours, the tissues were washed in physiological saline, intima stripped from media and lipid extracts prepared.

There was no effect of insulin or of anti-insulin on the uptake and conversion to lipid of ^{14}C-labelled acetate (Table 58).

The major lipid classes of control, insulin-treated and anti-insulin treated intimae were separated on the same neutral lipid T.L.C. plate (Table 58). Again, no differences were apparent.

Total fatty acid was separated into polyunsaturated, monounsaturated and saturated classes by silver nitrate T.L.C. Mainly polyunsaturated and saturated fatty acids were labelled in control, insulin-treated and anti-insulin-treated intimae (Table 59).

E. Lipid Synthesis from ^{14}C-labelled Acetate by Human Aortic Segments, Proximal and Distal to Coarctation

Clinical data relating to the four cases from which aortic coarctations were obtained are shown in Table 60. Material was obtained from two children aged 6 years and one aged 13 years so that same information relating to arterial wall lipid metabolism in children has also been obtained.

The resected specimen was incubated in a medium containing equal parts of Hanks' solution and normal human serum together with a known amount of ^{14}C-labelled acetate (7-11 μCi/ml) and, in the latter three experiments, of ^{32}P-labelled phosphate (about 3-30 μCi/

TABLE 58

EFFECT OF INSULIN AND ANTI-INSULIN ON THE UPTAKE AND CONVERSION TO LIPID OF ACETATE-1-^{14}C BY RABBIT ATHEROSCLEROTIC INTIMA IN VITRO AND ALSO ON THE PERCENTAGE DISTRIBUTION OF LABEL AMONG MAJOR LIPID FRACTIONS

Experiment	Factor	% Uptake and Conversion to Lipid/ Dry Defatted Tissue	LIPID FRACTIONS				
			Phospholipid	Diglyceride/ Cholesterol	Fatty Acid	Triglyceride	Cholesterol Ester
1	Control	0.0077	46.5	6.1	7.7	11.4	28.4
	Insulin	0.0073	51.7	3.9	5.9	11.3	27.4
	Anti-Insulin	0.0047	55.2	4.9	7.8	11.2	21.0
2	Control	0.0064	55.9	4.3	4.6	11.6	23.7
	Insulin	0.0063	57.0	3.8	3.7	12.1	23.5
	Anti-Insulin	0.0058	56.7	4.2	4.5	11.3	23.4

TABLE 59

EFFECT OF INSULIN AND OF ANTI-INSULIN ON THE PERCENTAGE DISTRIBUTION OF LABEL AMONG TOTAL FATTY ACIDS OF RABBIT ATHEROSCLEROTIC INTIMA INCUBATED IN VITRO WITH ACETATE-1-^{14}C

Experiment	Factor	*Polyunsaturated	Monounsaturated	Saturated	Solvent Front
1	Control	45.2	18.8	35.2	1.1
	Insulin	36.8	19.7	43.2	0.4
	Anti-Insulin	37.5	20.9	41.0	0.7
2	Control	38.0	17.4	44.3	0.5
	Insulin	35.6	15.1	49.0	0.4
	Anti-Insulin	37.0	24.2	38.0	0.9

* The "origin" of the silver nitrate T.L.C. plate has been included with "polyunsaturated"

ml). After 4 hours the specimen was removed, washed in saline, proximal and distal segments dissected, intima stripped from media and lipid extracts prepared.

The uptake and conversion to lipid of ^{14}C-labelled acetate and of ^{32}P-labelled phosphate in proximal and distal intima is shown in Table 60. In two experiments there was a greater uptake and conversion of acetate-1-^{14}C to lipid in the proximal segment than in the distal, but in the other two experiments the reverse was true. The findings were similar for ^{32}P-labelled phosphate.

Neutral lipid, phospholipid and silver nitrate T.L.C. was performed in the way described earlier in the section for ^{14}C/^{32}P double-labelled experiments in which the effect of CPIB on rabbit atherosclerotic intima was examined.

Of the major lipid fractions, those chiefly labelled were phospholipid and triglyceride. Diglyceride/cholesterol, fatty acid and cholesterol ester were labelled to a lesser extent except in the fourth experiment where diglyceride/cholesterol had much more label (Table 61). No differences between proximal and distal segments were apparent.

The major phospholipid labelled with ^{14}C-labelled acetate and with ^{32}P-labelled phosphate was lecithin (Table 62). Although, in the case of ^{14}C-labelled acetate, significant label appeared at the solvent front and it cannot be said what this represents. In the case of ^{32}P-labelled phosphate, significant label appeared in phosphatidyl inositol. There appeared to be no correlation, however, between the pattern of labelling of phospholipid and the source of the aortic segment.

In three of the four experiments, fatty acid classes were

TABLE 60

CLINICAL DATA OF RESECTED HUMAN AORTIC COARCTATIONS USED FOR METABOLIC STUDIES IN VITRO TOGETHER WITH THE INTIMAL UPTAKE AND CONVERSION TO LIPID OR ACETATE-1-^{14}C AND ^{32}P-LABELLED PHOSPHATE

Experiment	Patient's Age	Blood Pressures	Segment	% Uptake and Conversion to Lipid/mg Dry Defatted Tissue	
				^{14}C-Acetate	^{32}P-Phosphate
1	13	U.L. - 140/80	Proximal	0.0024	-
		A.P. - 175/105	Distal	0.0011	-
2	6	U.L. - 130/80	Proximal	0.0041	0.0010
			Distal	0.0068	0.0015
3	6	U.L. - 150/95	Proximal	0.0050	0.0018
			Distal	0.0002	0.0002
4	24	U.L. - 160/70	Proximal	0.0019	0.0041
		A.P. - 150/100	Distal	0.0067	0.0056

"U.L." upper limbs (sphygmomanometer reading); "A.P." aortic pressure (catheter reading)

TABLE 61

PERCENTAGE DISTRIBUTION OF LABEL AMONG INTIMAL LIPIDS FOLLOWING INCUBATION OF HUMAN AORTIC COARCTIONS WITH ACETATE-1-^{14}C IN VITRO

Experiment	Segment	Phospholipid	Diglyceride/Cholesterol	Fatty Acid	Triglyceride	Cholesterol Ester
1	Proximal	40.7	11.1	9.9	34.6	3.9
	Distal	38.0	12.5	10.7	32.2	6.7
2	Proximal	37.4	6.9	2.3	43.0	10.4
	Distal	48.0	13.1	4.3	23.9	10.8
3	Proximal	32.3	7.3	7.9	49.4	3.3
	Distal	44.0	11.4	7.7	29.1	7.9
4	Proximal	38.5	21.9	11.5	22.8	5.4
	Distal	29.7	45.3	7.3	16.1	1.8

TABLE 62

PERCENTAGE DISTRIBUTION OF LABEL AMONG INTIMAL PHOSPHOLIPIDS FOLLOWING INCUBATION OF HUMAN AORTIC COARCTIONS WITH ACETATE-1-^{14}C AND ^{32}P-LABELLED PHOSPHATE IN VITRO

Experiment	Segment	Origin	Lysolecithin	Sphingomyelin	Lecithin	Phosphatidyl Inositol	Phosphatidyl Ethanolamine	Solvent Front
				Acetate-1-^{14}C				
2	Proximal	2.7	2.6	2.1	55.2	17.6	17.8	2.1
	Distal	5.1	0.5	1.8	58.0	10.3	7.7	16.6
3	Proximal	0.3	2.1	3.0	48.7	8.6	9.9	27.5
	Distal	2.0	0.7	6.1	37.0	10.3	6.4	37.5
4	Proximal	3.6	7.7	9.5	34.3	10.0	7.8	27.3
	Distal	12.6	1.2	5.6	37.9	28.7	6.7	7.5
				^{32}P-Labelled Phosphate				
2	Proximal	3.2	2.1	1.8	72.1	8.4	0.6	11.9
	Distal	0.6	4.9	1.9	74.1	10.8	6.4	1.6
3	Proximal	0.2	1.0	1.3	68.4	22.9	4.1	2.2
	Distal	0.6	0.7	1.8	63.5	29.4	1.7	2.4
4	Proximal	0.4	2.5	19.2	67.4	4.7	2.8	3.2
	Distal	1.2	0.7	7.1	45.1	40.9	3.1	2.1

TABLE 63

PERCENTAGE DISTRIBUTION OF LABEL AMONG INTIMAL TOTAL FATTY ACIDS, ACCORDING TO THEIR DEGREE OF UNSATURATION, FOLLOWING INCUBATION OF HUMAN AORTIC COARCTATIONS WITH ACETATE-1-^{14}C IN VITRO

Experiment	Segment	*Polyunsaturated	Monounsaturated	Saturated	Solvent Front
2	Proximal	13.4	15.2	68.5	3.0
	Distal	38.7	6.2	49.6	5.7
3	Proximal	35.3	27.2	37.4	0.2
	Distal	49.1	18.6	25.1	7.3
4	Proximal	23.1	15.3	59.3	2.3
	Distal	32.5	18.1	49.0	0.5

* The "origin" of the silver nitrate T.L.C. plate has been included with "polyunsaturated".

separated by silver nitrate T.L.C. There is, in each experiment, less polyunsaturated and more saturated fatty acid labelled in the proximal segment than there is in the distal (Table 63). However, with significant label in the "diglyceride/cholesterol" zone, (Table 61) and, therefore, possibly of precursor acetate-1-^{14}C, there is a distinct possibility that "polyunsaturated" consists in part of labelled methyl acetate.

F. Discussion

In both rabbit and human atherosclerotic intima, ^{14}C-labelled acetate served as precursor for phospholipid, fatty acid, triglyceride and cholesterol ester synthesis, although the lipid most labelled was phospholipid. The major phospholipid formed was lecithin.

For the rabbit atherosclerotic intima, it has been found that polyunsaturated, monounsaturated and saturated fatty acids are synthesized from acetate-1-^{14}C and this is in accord with previous work (Day and Wilkinson, 1967; Newman, Gray and Zilversmit, 1968; Whereat and Orishimo, 1968). The finding that the human intima synthesizes polyunsaturated, monounsaturated and saturated fatty acids, however, is not in agreement with the brief report of Chobanian and Hollander (1963) in which only saturated fatty acid synthesis was found. It is not suggested that essential fatty acids are synthesized, since not all polyunsaturated fatty acids are essential; this is, therefore, not an objection to the present findings.

Despite the reports of the effect of CPIB on serum and tissue lipids (Oliver, 1967; Sachs, 1968), on serum cholesterol ester fatty acid patterns (Hagopian and Robinson, 1968) and on cholesterol synthesis in cell-free extracts of bovine aorta _in vitro_ (Teal and Gamble, 1965), it has not been possible in the present work to demonstrate an effect of CPIB _in vitro_ on arterial lipid metabolism

using ^{14}C-labelled acetate as precursor. Further work, using gas liquid radiochromatography to investigate the synthesis of particular fatty acids associated with a given combined lipid, especially cholesterol ester, is necessary. Also, arterial wall metabolism needs to be assessed after the administration of CPIB to experimental animals and to man.

The failure of insulin *in vitro* to alter the incorporation of ^{14}C-labelled acetate into lipid or to alter the pattern of lipids so-formed is consistent with the view that the reports of Stout (1968 and 1969) of an effect of insulin *in vivo* on acetate-1-^{14}C metabolism by the arterial wall *in vivo* are not related to a local effect of insulin on the arterial wall. The concentration of insulin used in the incubations *in vitro* was greater than that used by Mulcahy and Winegrad (1962) when slight increases of glucose carbon incorporation into glycogen and into total lipid of normal and diabetic rabbit aortae were observed. The insulin concentration was similar to that used by Wertheimer and BenTor (1962) when an effect of insulin on glucose uptake by diabetic rat aorta *in vitro* was demonstrated. Anti-insulin was used in case the aortic preparation was still affected by insulin associated with it on removal. However, with acetate-1-^{14}C as precursor, its use produced no change in the lipid metabolism of the rabbit atherosclerotic intima.

The only possible difference in acetate-1-^{14}C metabolism by human aortic segments proximal and distal to coarctation was in the synthesis of fatty acids. It would seem worth following up this observation in experimental coarctation. Of course, more extensive studies on normotensive and hypertensive individuals and experimental animals would be necessary to determine whether any differences were related to blood pressure differences or to some other factor. Nevertheless, human coarctation seemed a

convenient way of looking at the possible effects of blood pressure on arterial wall metabolism in the one individual.

GENERAL CONCLUSIONS, PROBLEMS and FUTURE INVESTIGATIONS

The lipid composition of the arterial wall has for a long time served as a background and stimulus for research into the aetiology and pathogenesis of atherosclerosis. In the first section of this thesis, knowledge of the lipid composition of children's arteries has been extended. Cholesterol ester has been found to be present in children's arteries and, in the normal intima, its concentration found to increase in the first 10 years of life. The increase for cholesterol ester in intima involved with fatty streaking was probably even greater. Early in life, both normal and atherosclerotic intimae were found to resemble serum in their cholesterol fatty acid composition, whereas it is known that later in life the fatty streak differs from the serum in this respect. It is not so much that the fatty streak cholesterol ester fatty acid pattern changes with age. It is as though the normal intima responds to the changing serum pattern while the intimal fatty streak does not. But even the normal intima in early life was not found to mirror exactly the serum changes in cholesterol ester fatty acid pattern.

Thus, transport or metabolic processes need to be considered if intimal cholesterol ester accumulation is to be explained satisfactorily, although the role of such processes may be different in childhood and adulthood.

Over two decades, there has been a growing interest in the metabolism of the arterial wall and the part it might play in atherogenesis. It would appear from Sections 2 and 3 of this thesis, that the rabbit and the human atherosclerotic lesion incorporate more fatty acid into cholesterol ester than do the corresponding normal intimae. This could, however, be related to the amount of cholesterol ester already present.

Nevertheless, it was thought of importance to know where such fatty acid incorporation took place in the atherosclerotic lesion and it has

been reported in Sections 2 - 5 that this takes place in intimal foam cells, as evidenced by autoradiography. Not that all foam cells localise fatty acid incorporation into cholesterol ester, and into phospholipid and triglyceride. Some faintly sudanophilic, some deep intimal, and some apparently degenerating foam cells do not localise labelled fatty acid very well or at all. It may be, therefore, that a particular type of cell, once it has a certain critical level of lipid, incorporates fatty acid into combined lipid until something results in its necrosis. The localisation to intimal foam cells of the formation, from radioisotopically-labelled choline and fatty acid, of phospholipid itself, and that mainly lecithin, has been dealt with in Sections 4 and 5. The significance of the foam cell in the metabolism of the atherosclerotic lesion is better appreciated in the human artery than in the rabbit, because of the greater diversity of cells in the human lesion. It should be possible to extend the localisation studies to the particular phospholipid, sphingomyelin, if the labelled precursors, sphingosine and very long chain fatty acids (e.g. lignoceric acid, 24:0) become available. Ultimately, electron-microscope autoradiography should allow localisation of metabolic events at an ultrastructural level.

The studies reported in Sections 6 and 7 relate to possible mechanisms for the accumulation of cholesterol oleate in rabbit atherosclerotic lesions. Both a greater incorporation into lesion cholesterol ester of monounsaturated fatty acid than of other fatty acid classes and a greater influx into the lesion of serum monounsaturated cholesterol ester than of serum polyunsaturated cholesterol ester were found. It has not been possible, however, to determine, firstly, whether the influx finding is more apparent than real and, secondly, whether metabolic differences precede transport differences, whether transport differences precede metabolic differences, or whether both metabolic and transport differences are consequent upon yet some other factor or factors.

That the apparently greater influx of serum free cholesterol than of serum ester cholesterol into the rabbit atherosclerotic lesion was not due to hydrolysis of cholesterol ester has also been shown in Section 7.

Phospholipid accumulation and metabolism in the atherosclerotic lesion may have a significance different from that for cholesterol ester accumulation and metabolism. The hallmark of the atherosclerotic lesion is cholesterol ester accumulation rather than phospholipid accumulation. Also, as pointed out in Section 6, the turnover of phospholipid in the atherosclerotic lesion appears to be greater than that of cholesterol ester. It is possible, therefore, that phospholipid metabolism and accumulation is a response of the arterial wall to cholesterol ester accumulation. Nevertheless, at the moment, there is insufficient evidence for such a view to be maintained.

The last section of this thesis, Section 8, has provided evidence that lipids other than phospholipid and fatty acid can be synthesized from acetate in human arterial intima and also that, besides saturated fatty acids, unsaturated fatty acids can be synthesized. Nothing positive emerged from an assessment _in vitro_ of the effects of chlorophenoxyisobutyric acid on rabbit and human intimal lipid metabolism. Neither was any significant effect found of insulin or of anti-insulin _in vitro_ on acetate metabolism by rabbit atherosclerotic lesions. With human aortic coarctation, the intima of the proximal hypertensive segment was found, possibly, to synthesize relatively less polyunsaturated fatty acid than the intima of the distal normotensive or hypotensive segment.

From the practical point of view, the researcher in the field of atherogenesis must repeatedly ask himself what possible prophylactic or therapeutic implications arise out of his work. Although the effects of new drugs on the atherosclerotic process must be understood, what

seems to be required is for new ways of rectifying the atherosclerotic process to be found. Since the intimal foam cell seems actively engaged in atherogenesis, the question arises as to whether it is a cell beneficial or detrimental to the inner arterial wall. A simple view is that, if by some means it could be eliminated in the experimental lesion, this question might be answered. If the cell were detrimental, then the way of eliminating the cell might be a useful tool in the treatment of atherosclerosis. Could an immunological approach, such as the use of anti-foam cell sera, provide a way of eliminating the foam cell? A more sophisticated immunological approach might be to define a detrimental enzyme system and reduce its activity with specific anti-enzymes (provided they could reach the relevant enzymes). Alternatively, specific metabolic inhibitors might be found. By the same token, if an enzyme system is insufficiently active, metabolic stimulators might be found. In any case, a more detailed knowledge of normal and atherosclerotic arterial wall metabolism would be required.

The role of fatty acid metabolism in atherogenesis is certainly worthy of closer attention. It has been pointed out in this thesis that fatty acid could determine cholesterol ester accumulation just as well as cholesterol. More should be known about the consequences of increased blood levels of free fatty acid on intimal fatty acid metabolism. Again, in the knowledge that prostaglandins are derivatives of essential fatty acids, that they affect lipid metabolism and that the degree of unsaturation of cholesterol ester fatty acid seems important in cholesterol ester accumulation, some information about prostaglandins in the arterial wall should probably be obtained.

While there seems to be room for optimism about the process of atherosclerosis being understood in the not-too-distant future, it is disturbing to think about what hope there is for successful control measures when such radical environmental changes have taken place in recent

times. It would not be surprising if the rate of change in such as the level of human physical activity, the nature of man's dietary habits and atmospheric pollution had already outstripped the evolutionary capacity of man's cardiovascular system and its protective mechanisms. Whether it will be possible to define the impact of environmental change on the cardiovascular system and reverse the relevant environmental trends before it becomes necessary to further complicate man's environment with prophylactic and therapeutic agents directed against atherosclerosis, remains to be seen.

BIBLIOGRAPHY

ABDULLA, Y.H., ADAMS, C.W.M. and MORGAN, R.S. : (1967) J. Path. Bact., 94 : 63

ABDULLA, Y.H., ORTON, C.C. and ADAMS, C.W.M. : (1968) J. Atheroscler. Res., 8 : 1967

ABELL, L.L., LEVY, B.B., BRODIE, B.B. and KENDALL, F.E. : (1952) J. Biol. Chem., 195 : 357

ADAMS, C.W.M. : (1964) "Arteriosclerosis in Man, Other Mammals and Birds", Biol. Rev. Cambridge Phil. Soc., 39 : 372

ADAMS, C.W.M., BAYLISS, O.B., DAVISON, A.N. and IBRAHIM, M.Z.M., : (1964) J. Path. Bact. 87 : 297

ADAMS, C.W.M., VIRÁG, S., MORGAN, R.S. and ORTON, C.C. : (1968) J. Atheroscler. Res. 8 : 679

ALBRINK, M.J. and MAN, E.B., : (1959) Arch. Intern. Med. 103 : 4

ALFIN-SLATER, R.B. and AFTERGOOD, L. : (1968) "Essential Fatty Acids Reinvestigated" in Physiological Reviews, 48 : 758

AMENTA, J.S. and WATERS, L.L. : (1960) Yale J. Biol. Med., 33 : 112

ANITSCHKOW, N.N. and CHALATOW, S. : (1913) Centralbl. f. allg. Path., u. pathol.Anat., 24 : 1

ARMIN, J., GRANT, R.T. and WRIGHT, P.H. : (1960) J. Physiol., 153 : 131

ARVIDSON, G.A.: (1967) J. Lipid Res. 8, 155

ASCHOFF, L. : (1906) Verhandl. d. deutsch. path. Gesellsch., 10 : 166

ASCHOFF, L. : (1924) Lectures on Pathology, Paul B. Hoeber Inc., New York

AZARNOFF, D.L. : (1958) Proc. Soc. Exptl Biol. Med., 98 : 680

BAKER, G.L. : (1969) Am. J. Clin. Nutrition., 22 : 829

BALIS, J.U., HAUST, M.D. and MORE, R.M. : (1964). Exptl Molec. Path., 3 : 511

BARBER, A.A. and BERNHEIM, F. : (1967) "Lipid Peroxidation: Its Measurement, Occurrence and Significance in Animal

Tissues" p. 355 In Advances in Gerontology, edited by B.L. Strehler, vol. 2, Academic Press, New York and London.

BARTLETT, G.R.: (1959) J. Biol. Chem., 234 : 466

BENJAMIN, W. and GELLHORN, A. : (1964) J. Biol. Chem., 239 : 64

BIÖRCK, G. : (1968) Circulation, 37 : 1071

BLEYL, U. and WEGENER, K. : (1969) Triangle 9 : 9

BLUMENTHAL, H.T. : (1967) Editor, "Cowdry's Arteriosclerosis, A Survey of the Problem", 2nd edition, Charles C. Thomas, Springfield, Illinois.

BOELSMA-VAN HOUTE, E. and BÖTTCHER, C.J.F. : (1967) J. Atheroscler. Res., 7 : 269

BORTZ, W.M., LARSEN, N.P. and CIVIN, W.H. : (1958) Arch. Path., 66 : 218

BÖTTCHER, C.J.F., WOODFORD, F.P., TER HAAR ROMENY, C. Ch., BOELSMA, E. and VAN GENT, C.M. : (1959) Nature 183 : 47

BÖTTCHER, C.J.F., BOELSMA-VAN HOUTE, E., TER HAAR ROMENY-WACHTER, C. Ch., WOODFORD, F.P. and VAN GENT, E.M., : (1960) Lancet, 2 : 1162

BÖTTCHER, C.J.F., WOODFORD, F.P., TER HAAR ROMENY-WACHTER, C. Ch., BOELSMA-VAN HOUTE, E. and VAN GENT, C.M., : (1960) Lancet 1 : 1378

BÖTTCHER, C.J. and VAN GENT, C.M., : (1961) J. Atheroscler. Res., 1 : 36

BÖTTCHER, C.J. and WOODFORD, F.P., : (1962) Federation Proc., 21 (Pt. II) : 15

BÖTTCHER, C.J.F., : (1963) "Phospholipids of Atherosclerotic Lesions in the Human Aorta", p. 109 in Evolution of the Atherosclerotic Plaque, edited by R.J. Jones, The University of Chicago Press, Chicago and London.

BOWYER, D.E., HOWARD, A.N. and GRESHAM, G.A. : (1967) Biochem. J., 103 : 54P

BOWYER, D.E., HOWARD, A.M., GRESHAM, G.A., BATES, D. and PALMER, B.V.: (1968) Progr. Biochem. Pharmacol., 4 : 235 (karger, Basel/ New York)

BRANWOOD, A.W.: (1963) "Modern Concepts of the Pathogenesis of Coronary Atherosclerosis", E. & S. Livingstone Ltd., Edinburgh and London

BREMER, J. and GREENBERG, D.M. (1959) Biochim. Biophys. Acta 35 : 287

BREMER, J. and GREENBERG, D.M. (1961) Biochim. Biophys. Acta 46 : 205

BRIGGS, F.N., CHERNICK, S. and CHAIKOFF, I.L.: (1949) J. Biol. Chem., 179 : 103

BRONTE - STEWART, B. and HEPTINSTALL, R.H.: (1954) J. Path. Bact., 68 : 407

BRUGER, M. and CHASSIN, M.R. (1941) Annals Int. Med., 14 : 1756

BUCK, R.C. and ROSSITER, R.J.: (1951) Arch. Path., 51 : 224

BUCK, R.C.: (1955) J. Histochem. Cytochem. 3 : 435

CARLSON, L.A. : (1968) p. 243 in Acute Myocardial Infarction, edited by D.G. Julian and M.F. Oliver, Edinburgh

CHANDLER, A.B. and HAND, R.A. : (1961) Science 134 : 946

CHATTOPADHYAY, D.P. and MARTIN, J.M. : (1969) J. Atheroscler. Res., 10 : 131

CHERNICK, S., SRERE, P.A. and CHAIKOFF, I.L. : (1949) J. Biol. Chem., 179 : 113

CHMELAŘ, M. and CHMELAŘOVÁ, M. : (1968) Experientia, 24 : 1118

CHOBANIAN, A.V. and HOLLANDER, W.: (1963) Clin. Res., 11 : 216

CHOBANIAN, A.V. and HOLLANDER, W. : (1966) J. Clin. Invest., 45 : 932

CHOBANIAN, A.V. : (1968a) J. Clin. Invest., 47 : 595

CHOBANIAN, A.V. : (1968b) J. Atheroscler. Res., 8 : 763

CHOBANIAN, A.V., BRECHER, P.I., LILLE, R.D. and WOTIZ, H.H. : (1968) J. Lipid Res., 9 : 701

CONNOR, W.E., ARMSTRONG, M.L., JACKSON, C.S. and ALI, M.A. : (1966) Circulation 33 and 34 Supplements : III - 5

CONSTANTINIDES, P. : (1965) "Experimental Atherosclerosis", Elsevier, Amsterdam

COOK, R.P. : (1958) p. 145 in "Cholesterol, Chemistry, Biochemistry

and Pathology", edited by R.P. Cook, Academic Press, New York

COURTICE, F.C. and GARLICK, D.G.: (1962) Quart. J. Exptl. Physiol., 47 : 221

COURTICE, F.C., and SCHMIDT-DIETRICHS, A. : (1962) Quart. J. Exptl. Physiol. 47 : 228

COURTICE, F.C., GOLDRICK, R.B., MADDOK, K., NESTEL, P.J., READER, R., WHYTE, H.M.: (1967) "Dietary Fat and Coronary Heart Disease: A Review", Med. J. Aust. I: 309

DALY, M.M., DEMING, Q.B., RAEFF, V.M. and BRUN, L.M.: (1963) J. Clin. Invest., 42 : 1606

DAVIS, D. and KLAINER, M.J.: (1940) Amer. Heart J. 19 : 185

DAVIS, D. and KLAINER, M.J. (1940) Amer. Heart J. 19 : 193

DAY, A.J. and WILKINSON, G.K.: (1956) Aust. J. Exptl. Biol. Med. Sci., 34 : 423

DAY, A.J.: (1962) J. Atheroscler. Res., 2 : 350

DAY, A.J., FIDGE, N.H., GOULD HURST, P.R.S. and RISELY, D.J.: (1963) Quart. J. Exptl. Physiol., 48 : 298

DAY, A.J. and GOULD HURST, P.R.S. (1963) Austral. J. Expt. Biol. Med. Sci., 41 : 323

DAY, A.J., FIDGE, N.H. and WILKINSON, G.K. (1964) Biochim. Biophys. Acta., 84 : 149

DAY, A.J., GOULD HURST, P.R.S. and WAHLQVIST, M.L.: (1964) J. Reticuloendothel. Soc., 1 : 40

DAY, A.J., GOULD HURST, P.R.S., STEINBORNER, R. and WAHLQVIST, M.L.: (1965) J. Atheroscler. Res., 5 : 466

DAY, A.J., FIDGE, N.H., GOULD HURST, P.R.S., WAHLQVIST, M.L. and WILKINSON, G.K.: (1966) Quart. J. Exptl. Physiol., 51 : 11

DAY, A.J., FIDGE, N.H. and WILKINSON, G.N. : (1966) J. Lipid Res., 7 : 132

DAY, A.J., and GOULD HURST, P.R.S.: (1966) Biochem. Biophys. Acta 116 : 169

DAY, A.J., NEWMAN, H.A.I., and ZILVERSMIT, D.B.: (1966) Circulation Res., 19 : 122

DAY, A.J.: (1967) "Lipid Metabolism by Macrophages and Its Relationship to Atherosclerosis" p. 185, in Advances in Lipid Research vol. 5 Academic Press, New York.

DAY, A.J. and WILKINSON, G.K.: (1967) Circulation Res., 21 : 593

DAY, A.J. and TUME, R.K.: (1969) J. Atheroscler. Res., 9 : 141

DAY, A.J. and TUME, R.K.: (1970) J. Atheroscler. Res., (In the press)

DAYTON, S.: (1959) Circulation Res., 7 : 468

DAYTON, S. and S. HASHIMOTO, : (1966) Circulation Res., 19 : 1041

DAYTON, S. and HASHIMOTO, S. : (1967) J. Atheroscler. Res., 8 : 555

DAYTON, S. and HASHIMOTO, S. : (1968) J. Atheroscler. Res., 8 : 555

DE BOER Th. J. and BACKER, H.J. : (1954) Receuil Trav. Chim., 73 : 229

DEYKIN, S. and GOODMAN, D.S. : (1962) J. Biol. Chem., 237 : 3649

DOCK, W. : (1946) J. Am. Med. Assoc., 131 : 875

DOLE, V.P. : (1956) J. Clin. Invest., 35 : 150

DOUGHERTY, T.F. and BERLINER, D.L. : (1959) p. 143 in Connective Tissue Thrombosis and Atherosclerosis, edited by I.H. Page Academic Press, New York.

DRURY, A. : (1955) Proc. Soc. Exptl. Biol. Med., 89 : 508

DRURY, A. : (1961) J. Gerontol., 16 : 114

DUFF, G.L. and McMILLAN, G.C. : (1949) J. Exptl. Med., 89 : 611

DUFF, G.L., BRECHIN, D.J.H. and FINKELSTEIN, W.E. : (1954) J. Exptl. Med., 100 : 371

DUFF, G.L. and McMILLAN, G.C. and LAUTSCH, E.V. : (1954) Amer. J. Path. 30 : 941

DUGUID, J.B. : (1946) J. Path. & Bact., 58 : 207

DUGUID, J.B. : (1948) J. Path. & Bact., 60 : 57

DUNCAN, L.E. Jr. : (1963) "Mechanical Factors in the Localization of Atheromata" in Evolution of the Atherosclerotic Plaque, edited by R.J. Jones, University of Chicago Press, Chicago, Illinois

DUNCAN, L.E., Jr. BUCK, K. and LYNCH, A.: (1963) Science, 142 : 972

DUNNIGAN, M.G. : (1964) J. Atheroscler. Res., 4 : 144

DYRBE, M.O.: (1959) J. Gerontol., 14 : 32

EISENBERG, S., STEIN, Y. and STEIN, O. : (1967a) Biochim. Biophys. Acta, 137 : 115

EISENBERG, S., STEIN, Y. and STEIN, O. : (1967b) Biochim. Biophys. Acta, 137 : 221

EISENBERG, S., STEIN, Y. and STEIN, O. : (1968) Biochim. Biophys. Acta, 164 : 205

EISENBERG, S., STEIN, Y. and STEIN, O. : (1969) Biochim. Biophys. Acta, 176 : 557

EISLEY, N.F. and PRITHAM, G.H. : (1955) Science, 122 : 121

ERBLAND, J.F. and MARINETTI, G.V. : (1962) Fed. Proc., 21 (No. 2) : 295

EVRARD, E., VAN DEN BOSCH, J., DE SOMER, P., JOOSSENS, J.V. : (1962) J. Nutrition, 76 : 219

FARQUHAR, J.W., HIRSCH, R.L. and AHRENS, E.H.: (1960) J. Clin. Invest. 39 : 984

FELLER, D.D. and HUFF, R.L. : (1955) Amer. J. Physiol. 182 : 237

FELT, V. and BENEŠ, P. : (1969) Biochim. Biophys. Acta, 176 : 435

FOLCH, J., LEES, M. and SLOANE STANLEY, G.H. : (1957) J. Biol. Chem. 226 : 497

FOSTER, D.W. and SIPERSTEIN, M.D. : (1960) Am. J. Physiol. 198 : 25

FRENCH, J.E. : (1966) "Atherosclerosis in Relation to the Structure and Function of the Arterial Intima, with Special Reference to the Endothelium" p. 253 in International Review of Experimental Pathology vol. 5, edited by G.W. Richter and M.A. Epstein, Academic Press, New York and London

FRIEDMAN, M. and BYERS, S.O. : (1962) Brit. J. Exptl. Path., 43 : 363

GAMBLE, W., HOELSCHER NAYAR, G. and KIERSKY, E.S. : (1967) Biochim. Biophys. Acta, 145 : 260

GEER, J.C. and GUIDRY, M.A. : (1964) Exptl. Mol. Path., 3 : 485

GEER, J.C. and MALCOLM, G.T.: (1965) Exptl. Med. Path. 4 : 500

GERŐ, S., GERGELY, J., DEVÉNYI, T., JAKAB, L., SZÉKELY, J. and VIRÁG, S. : (1961) J. Atheroscler. Res., 1 : 67

GERŐ, S., GERGELY, J., JAKAB, L., SZÉKELY, J. and VIRÁG, S. : (1961) J. Atheroscler. Res., 1 : 88

GHIDONI, J.J. and O'NEAL, R.M.: (1967) "Recent Advances in Molecular pathology. A Review. Ultrastructure of Human Atheroma". Exptl. Molec. Path., 7 : 378

GLAVIND, J., HARTMANN, S., CLEMMENSEN, J., JESSEN, K.E. and DAM, H. : (1952) Acta. Pathol. Microbiol. Scand., 30 : 1

GLOMSET, J.A. : (1968) J. Lipid Res., 9 : 155

GLOMSET, J.A. and WILLIAMS, R.H. : (1968) "Lipid Metabolism and Lipopathies", p. 1039 in Textbook of Endocrinology, edited by R.H. Williams, W.B. Saunders.

GOFMAN, J.W., LINDGREN, F., ELLIOTT, H., MANTZ, W., HEWITT, J., STRISOWER, B., HERRING, V. and LYON, T.P. : (1950) Science 111 : 166

GOFMAN, J.W. and YOUNG, W. : (1963) "The Filtration Concept of Atherosclerosis and Serum Lipids in the Diagnosis of Atherosclerosis" p. 197 in Atherosclerosis and its Origin, edited by Sandler, M. and Bourne, G.H. Academic Press, New York and London

GOLDWATER, W.H. and STETTEN, D., Jr., :(1947) J. Biol. Chem. 169 : 723

GOULD, R.G. and COOK, R.P.: (1958) p. 237 in "Cholesterol, Chemistry, Biochemistry and Pathology", edited by R.P. Cook, Academic Press, New York

GORDON, C.F. and WOLFE, A.L. : (1960) Analyt. Chem. 32 : 574

GRESHAM, G.A. and HOWARD, A.N. : (1962) Arch. Path. 74 : 1

GRIGORIAN, D.G., LYSENKO, V.B., SOKOLOVA, V.A. : (1969) Doklady Akademii Nauk SSSR 184 : 497

HAGOPIAN, M. and ROBINSON, R.W. (1968) J. Atheroscler. Res. 8 : 21

HAIMOVICI, H. : (1968) Editor, "Atherosclerosis: Recent Advances" Annals of the New York Academy of Sciences vol. 149 art. 2 pp. 585-1068

HALES, C.N., WALKER, J.B., GARLAND, P.B., and RANDLE, P.J. (1965) Lancet I : 65

V. HALLER, A. : (1768): Materia Ossium Morborosum, Observatio LIX, Opuscula Pathologica, Vol. 111 p. 361, Francisci Grasset.

HANIG, M., SHAINOFF, J.R., and LOWY, A.D. : (1956) Science, 124 : 176

HANKS, J.H. : (1948) J. Cell. Comp. Physiol., 31 : 235

HARMAN, D. : (1969) J. Am. Geriatrics Soc., 17 : 721

HARRIS, P.N. : (1952) Proc. Soc. Exp. Biol. Med. 79 : 455

HARTROFT, W.S. and PORTA, E.S. : (1965) Am. J. Med. Sci., 250 : 324

HASHIMOTO, S. and DAYTON, S. (1966) J. Atheroscler. Res., 6 : 580

HASTINGS, S.G., BOOTH, N.H. and HOPWOOD, M.L. : (1968) J. Atheroscler. Res. 8 : 29

HAVEL, R.J., EDER, H.A. and BRAGDON, J.H : (1955) J. Clin. Invest. 34 : 1345

HAUSS, W.H., JUNGE-HÜLSING and HOLLÄNDER, H.J. : (1962) J. Atheroscler. Res., 2 : 50

HERBERT, V., LAU, K.S., GOTTLIEB, C.W., and BLEICHER, S.J., : (1965) J. Clin. End., 25 : 1375

HIERONYMI, G. : (1956) "Uber den altersbedingten Formwandel elastischer und muskulärer Arterien" Springer, Heidelberg

HOLLANDER, W. : (1963a) Fed. Proc., 22 : 219

HOLLANDER, W. : (1963b) Circulation, 28 : 660

HOLLANDER, W., MADOFF, I.M., KRAMSCH, D.M. AND YAGI, S. : (1964) p. 191 _in_ Hypertension XIII, Proceedings of the Council for High Blood Pressure Research, American Heart Association, Cleveland

HOLLANDER, W., YAGI, S. and KRAMSCH, D.M. : (1964) Circulation 34 and 35, Suppl. II : 1

HOLLANDER, W. : (1967) "Recent Advances in Experimental and Molecular Pathology. Influx, Synthesis and Transport of Arterial Lipoproteins in Atherosclerosis" Exptl. Molec. Path., 7 : 248

HOLMAN, R.L., McGILL, H.C. STRONG, J.P., and GEER, J.C. : (1958) Am. J. Path., 34 : 209

HOLMAN, R.L., BLANC, W.A. and ANDERSEN, D. : (1959) Pediatrics, 24 : 34

HOLMAN, R.L. : (1961) Am. J. Clin. Nutrition, 9 : 565

HOLMAN, R.T., CASTER, W.O. and WIESE, H.F. : (1964) Am. J. Clin. Nutrition, 14 : 70

HORN, H. and FINKELSTEIN, L.E. : (1940) Am. Heart J. 19 : 655

HOSOYA, N., HAGERMAN, D. and VILLEE, C. : (1960) Biochem. J., 76 : 297

HOWARD, A.N., BOWYER, D.E. and GRESHAM, G.A. : (1967) Circulation, 36 (Suppl. II): II - 18

HOWARD, A.N., GRESHAM, G.A., BOWYER, D.E., and LINDGREN, F.N. : (1967) Progr. Biochem. Pharmacol., 4 : 438

HOWARD, C.F. Jr. and PORTMAN, O.W. : (1966) Biochim. Biophys. Acta, 125 : 623

HOWARD, C.F. Jr.: (1968) J. Lipid Res., 9 : 254

HUNDLEY, J.M. : (1968) J. Amer. Dietetic Assoc., 52 : 195

IGNATOWSKI, A. : (1909) Virchows Arch. f. path. Anat., 198 : 248

IMAI, H., LEE, K.T., PASTORI, S., PLANLILIO, E., FLORENTIN, R. and THOMAS, W.A. : (1966) Exptl. Molec. Path., 5 : 273

INSULL, W. Jr., and BARTSCH, G.E. : (1966) J. Clin. Invest., 45 : 513

INSULL, W. and BARTSCH, G.E. : (1967) Amer. J. Clin. Nutrition, 20 : 13

JAMES, A.T., and PIPER, E.A. : (1961) J. Chromatog., 5 : 265

JENSEN, J. : (1967) Biochim. Biophys. Acta, 135 : 544

JENSEN, J. : (1969) Biochim. Biophys. Acta, 173 : 71

JOHNSON, A.C., McNABB, A.R. and ROSSITER, R.J. : (1950) Arch. Neurology and Psychiatry, 64 : 105

JONES, D., GRESHAM, G.A. and HOWARD, A.N. : (1963) J. Atheroscler. Res., 3 : 716

JONES, R.J. :(1963) Editor, "Evolution of the Atherosclerotic Plaque", The University of Chicago Press, Chicago, Illinois

KANNEL, W.B., DAWBER, T.R., KAGAN, A., REVOTSKIE, N. and STOKES, J. : (1961) Ann. Int. Med., 55 : 33

KAO, V.C.K. and WISSLER, R.W. : (1965) Exptl. Molec. Path., 4 : 465

KATZ, L.N. and PICK, R. :(1963) "Reversibility of the Atherosclerotic Lesion" p. 251 in Evolution of the Atherosclerotic Plaque, edited by R.J. Jones, University of Chicago Press.

KAYDEN, H.J. and STEELE, J.M. : (1956) Circulation 14 : 482

KEITH, J.D., ROWE, R.D. and VLAD, P. : (1967) "Atherosclerosis in Childhood" chpt 47 in Heart Disease in Infancy and Childhood, 2nd edition, The MacMillan Company, New York.

KELLNER, A. : (1954) The Lipid and Protein Content of Tissue Fluid in Normal and Hyperlipemic Rabbits, p. 42 in Symposium on Atherosclerosis, National Academy of Sciences, National Research Council, Washington, D.C.

KENNEDY, E.P. and WEISS, S.B. : (1966) J. Biol. Chem. 222 : 193

KEYS, A. : (1967) "Dietary Factors in Arteriosclerosis" p. 576 in Cowdry's Arteriosclerosis. A Survey of the Problem, edited by H.T. Blumenthal, 2nd edition, Charles C. Thomas, Springfield, Illinois.

KHANOLKAR, V.R. : (1960) Triangle 4 : 251

KHOKHAR, S.A. and HILKER, D.M. : (1967) J. Atheroscler. Res., 7 : 767.

KIRK, J.E. and LAURSEN, T.J.S. : (1955) J. Gerontol., 10 : 288.

KIRK, J.E. : (1963) "Intermediary Metabolism of Human Arterial Tissue

and its changes with Age and Atherosclerosis" p. 67 in Atherosclerosis and its Origin, edited by M. Sandler and G.H. Bourne, Academic Press, New York and London.

KRITCHEVSKY, D., MOYER, A.W., TESAR, W.C., McCANDLESS, R.F.J., LOGAN, J.B., BROWN, R.A. and ENGLERT, M.E. : (1956) Am. J. Physiol. 185 : 279.

KRITCHEVSKY, D. :(1967) J. Dairy Science, 50 : 776.

KRITCHEVSKY, D., SALLATA, P., TEPPER, S.A. : (1968) J. Atheroscler. Res., 8 : 755.

KUMMEROW, F.A. : (1965) Editor, " Metabolism of Lipids As Related to Atherosclerosis: Charles C. Thomas, Springfield, Illinois.

LANDS, W.E.M. : (1960) J. Biol. Chem., 235 : 2233.

LANSING, A.I. : (1959) Editor, "The Arterial Wall, Aging, Structure and Chemistry", The William & Wilkins Co. Baltimore.

LAZOVSKAYA, L.N. : (1943) Biokhimya, 8 : 171

LEARY, T. : (1941) Arch. Path., 32 : 507.

LEDUC, E.H. and WILSON, J.W. : (1964) J. Natl. Cancer Inst. 33 : 721

LEE, K.T., JONES, R., KIM, D.N., FLORENTIN, R., COULSTON, F. and THOMAS, W.A. : (1966) Exptl. Molec. Path. Suppl. 3. Article IV

LEITES, F.L. and FEDOSEEV, A.N. : (1965) Fed. Proc., 24 : T829.

LENÈGRE, M.J., SCEBAT, L. and RENAIS, J. : (1967) "Le Rôle De La Paroi Artérielle Dans L'Athérogénèse" Vols. I & II Centre National De La Recherche Scientifique, Paris.

LEWIS, B., PILKINGTON, T.R.E., and HODD, K.A. : (1961) Clin. Science, 20 : 249.

LOBSTEIN, J.F.M. : (1829 - 1833) "Traité d'Anatomie Pathologique" 2 vols F.G. Lefrault, Paris.

LOFLAND, H.B., Jr. and CLARKSON, T.B. : (1965) Arch. Pathol., 80 : 291.

LOFLAND, H.B., Jr., MOURY, D.M., HOFFMAN, C.W., and CLARKSON, T.B. : (1965) J. Lipid Res., 6 : 112.

LONG, E.R. :(1967) "Development of Our Knowledge of Arteriosclerosis" chapter 1 in Cowdry's Arteriosclerosis. A Survey of the Problem, 2nd edition, Edited by H.T. Blumenthal, Charles C. Thomas, Springfield, Illinois.

LOOMEIJER, F.J. and VAN DER VEEN, K.J. : (1962) J. Atheroscler. Res., 2 : 478.

LOPEZ-S, A., MILLER, O.N. and MULDREY, J.E. : (1965) Proc. Soc. Exptl. Biol. Med., 118 : 829.

LOPEZ-S, S.A., KREHL, W.A. and HODGES, R.E. : (1967) Am. J. Clin. Nutr., 20 : 808.

LUDDY, F.E., BARFORD, R.A. RIEMENSCHNEIDER, R.W. and EVANS, J.D. : (1958) J. Biol. Chem., 232 : 843.

McCANDLESS, E.L. and ZILVERSMIT, D.B. : (1956) Arch. Bioch. Biophys., 62 : 402.

McGILL, H.C. and HOLMAN, R.L. : (1949) Proc. Soc. Exptl. Biol. Med. 72 : 72

McGILL, H.C. and GEER, J.C. : (1963) "The Human Lesion, Fine Structure" p. 65 in Evolution of the Atherosclerotic Plaque, edited by R.J. JONES, University of Chicago Press, Chicago and London.

McGILL, H.C. Jr., GEER, J.C. and STRONG, J.P. : (1963) "Natural History of Human Atherosclerotic Lesions" Chapter 2 in Atherosclerosis and Its Origin Edited by M. Sandler and G.H. Bourne, Academic Press, New York and London.

McGILL, H.C. JR., BROWN, B.W., GORE, I., McMILLAN, G.C., PATERSON, J.C., POLLAK, O.J., ROBERTS, J.C. and WISSLER, R.W. : (1968) Report of Committee on Grading Lesions, Council on Arteriosclerosis, American Heart Association, Circulation, 37 : 455

McMILLAN, G.C. and DUFF, G.L. : (1948) Arch. Path., 46 : 179.

McMILLAN, G.C., KLATZO, I. and DUFF, G.L.: (1954) Lab. Invest., 3 : 451.

McMILLAN, G.C., WEIGENSBERG, B.I., and RITCHIE, A.C. : (1960) Arch. Path., 70 : 220.

McMILLAN, G.C. and STARY, H.C. : (1968) "Preliminary experience with mitotic activity of cellular elements in the atherosclerotic plaques of cholesterol-fed rabbits studied by labeling with tritiated thymidine" p. 699 in Atherosclerosis, Recent Advances, edited by H. Haimovici, Annals of the New York Academy of Sciences, vol. 149 Art. 2.

MAGGI, V. : (1964) J. Atheroscler. Res., 4 : 469

MAHLER, R.F. : (1966) p. 41 in Diabetes Mellitus, edited by L.J.P. Duncan, Edinburgh.

MAIER, N. : (1968) "Metabolism of Arterial Tissue and its Relation to Atherosclerosis". p. 655 in Atherosclerosis, Recent Advances edited by H. Haimovici, Annals of the New York Academy of Sciences vol. 149, art. 2.

MALINOW, M.R. : (1960) Circulation Res., 8 : 506

MALINOW, M.R. and MOGUILEVSKY, J.A. : (1961) J. Atheroscler. Res., 1 : 417.

MALMROS, H. and WIGAND, G. : (1959) Lancet II, 749.

MARCHAND, F. : (1904) Über Arteriosklerose (Athero-Sklerose) p. 23 in Verhandl d. Kongr. f. Inn. Med., 21st Kongr.

MARSHALL, J.R. and O'NEAL, R.M. :(1966) Exptl. Molec. Path., 5 : 1.

MATHUR, K.S., SHARMA, R.D., KASHYAP, S.K. and SAPRU, R.P. : (1964) Circulation, 30 : 694.

MEAD, J.F. and GOUZE, M.L. : (1961) Proc. Soc. Exptl. Biol. Med., 106 : 4.

MEYER, B.J., MEYER, A.C., PEPLER, W.J., and THERON, J.J. : (1966) Am. Heart J., 71 : 68.

MILCH, R.A. : (1965) "Matrix properties of the Aging Arterial Wall": p. 261 in Monographs in the Surgical Sciences, Vol. 2, The Williams & Wilkins Co., U.S.A.

MIRAS, C.J., HOWARD, A.M., and PAOLETTI, R. :(1968) Editors "Recent Advances in Atherosclerosis", The Athens Symposium. 4th volume of Progr. Biochem. Pharmacol. S. Karger, Basel.

MITCHELL, J.R.A. and SCHWARTZ, C.J.: (1965) "Arterial Disease", Blackwell Scientific Publications, Oxford.

MIZUGUCHI, T. : (1968) Jap. Heart J., 9 : 34.

MOON, H.D. and RINEHART, J.F. : (1952) Circulation, 6 : 481.

MOON, H.D. : (1957) Circulation, 16 : 263.

MOORE, J.H. : (1967) Brit. J. Nutrition, 21 : 715.

MORIN, R.J. : (1968) Metabolism, 17 : 1051

MORIN, R.J. : (1969) Proc. Soc. Exp. Biol. Med., 131 : 880.

MORRIS, J.N. and CRAWFORD, M.D. : (1961) Lancet, I : 47.

MORRIS, L.J. : (1964) p. 295 in "New Biochemical Separations" edited by A.T. James and L.J. Morris, Van Nostrand, London.

MORRISON, W.R. : (1964) Anal. Biochem., 7 : 218.

MOSES, C. : (1963) "Atherosclerosis, Mechanisms as a Guide to Prevention", Lea and Febiger, Philadelphia.

MOVAT, H.Z., MORE, R.H. and HAUST, M.D. : (1958) Am. J. Path., 34 : 1023

MULCAHY, P.D. and WINEGRAD, A.I. : (1962) Am. J. Physiol. 203 : 1038.

MULDREY, J.E., HAMILTON, J.G., WELLS, J.A., SWARTWOUT, J.R. & MILLER, O.N. : (1961) Fed. Proc., 20 : 277

MURRAY, M.C., SCHRODT, G.R. and BERG, H.F. : (1968) Biochem. Med., 2 : 118.

MUSTARD, J.F., ROWSELL, H.C., MURPHY, E.A. and DOWNIE, H.G. (1963) "Intimal Thrombosis in Atherosclerosis" p. 183 in Evolution of the Atherosclerotic Plaque, Edited by R.J. Jones, The University of Chicago Press, Chicago, Illinois.

NAKATANI, M., SASAKI, T., MIYAZAKI, T. and KANAMURA, M. : (1967a) J. Atheroscler. Res., 7 : 747.

NAKATANI, M., SASAKI, T., MIYAZAKI, T., and NAKAMURA, M. : (1967b) J. Atheroscler. Res. 7 : 759.

NELSON, W.R., WERTHESSEN, N.T., HOLMAN, R.L., HADAWAY, H. and JAMES, A.T. : (1961) Lancet 1 : 86.

NEWMAN, H.A.I., McCANDLESS, E.L. and ZILVERSMIT, D.B. : (1961) J. Biol. Chem. 236 : 1264

NEWMAN, H.A.I. and ZILVERSMIT, D.B. : (1962) J. Biol. Chem 237 : 2078.

NEWMAN, H.A.I., DAY, A.J., and ZILVERSMIT, D.B. : (1966) Circulation Res., 19 : 132.

NEWMAN, H.A.I. and ZILVERSMIT, D.B. : (1966) Circulation Res., 18 : 293.

NEWMAN, H.A.I., GRAY, G.W. and ZILVERSMIT, D.B. : (1968) J. Atheroscler. Res., 8 : 745.

NOBLE, N.L., BOUCEK, R.J., KAO, K.Y.T. and PARTIN, H.C. : (1956) Fed. Proc., 15 : 463.

NOBLE, N.L., BOUCEK, R.J. and KAO, K.Y.T. : (1957) Circulation, 15 : 366.

OKISHIO, T. : (1961) Med. J. Osaka Univ. 11 : 367.

OLIVER, M.F. : (1967) Circulation, 36 : 337.

OSBORN, G.R. : (1968) "Stages in Development of Coronary Disease observed from 1,500 young subjects. Relationship of hypotension and infant feeding to aetiology." p. 93 _in_ Le Rôle De La Paroi Artérielle Dans L'Athérogénèse, edited by M.J. Lenègre, L. Scebat, and J. Renais, Centre National de la Recherche Scientifique, Paris.

OTT, H., LOHSS, F. and GERGELY, J.: (1958) Klin. Wschr. 36 : 383

PACKHAM, M.A., ROWSELL, H.C., JØRGENSEN, L. and MUSTARD, J.F. : (1967) Exptl. Molec. Path., 7 : 214.

PAGE, I.H. : (1954) Circulation, 10 : 1

PAGE, I.H. : (1941) Annals of Int. Med., 14 : 1741.

PAGE, I.H. : (1968) Circulation, 38 : 1164.

PANGANAMALA, R.V., GEER, J.C. and CORNWELL, D.G. : (1969) J. Lipid Res., 10 : 445.

PAOLETTI, P., PAOLETTI, R. and SAVI, C. : (1958) Boll. Soc. Ital. Biol. Sper. 34 : 1416.

PARKER, F., ODLAND, G.F., ORMSBY, J.W. AND WILLIAMS, R.H. : (1963) p. 35 in Evolution of the Atherosclerotic Plaque, edited by R.J. Jones, The University of Chicago Press, Chicago

PARKER, F., SCHIMMELBUSCH, W. and WILLIAMS, R.H. : (1964) Diabetes, 13 : 182.

PARKER, F., ORMSBY, J.W., PETERSON, N.F., ODLAND, G.F. and WILLIAMS, R.H. : (1966) Circulation Res., 19 : 700.

PATELSKI, J., WALIGORA, Z., and SZULC, S. : (1967) J. Atheroscler. Res., 7 : 453.

PATELSKI, J., BOWYER, D.E., HOWARD, A.N. and GRESHAM, G.A. : (1968) J. Atheroscler. Res., 8 : 221.

PATERSON, J.C., MOFFATT, T. and MILLS, J. : (1956) Arch. Path., 61 : 496.

PATTERSON, M.S. and GREENE, R.C. : (1965) Analyt. Chem., 37 : 854.

PAULUS, H. and KENNEDY, E.P. (1960) J. Biol. Chem., 235 : 1303.

PINTER, K.G., HAMILTON, J.G., and MULDREY, J.E. : (1964) J. Lipid Res., 5 : 273.

POOLE, J.C.F. and FLOREY, H.W. : (1958) J. Path. Bact. 75 : 245.

PORTMAN, O.W. and SUGANO, M. : (1964) Arch. Biochem. Biophys., 105 : 532.

PORTMAN, O.W. : (1967) J. Atheroscler. Res., 7 : 617

PORTMAN, O.W., ALEXANDER, M. and MARUFFO, C.A. : (1967) Circulation, 35 and 36, Suppl. II : 32.

PORTMAN, O.W., BEHRMAN, R.E. and SOLTYS, P. : (1969) Am. J. Physiol., 216 : 143.

PORTMAN, O.W. and ALEXANDER, M. : (1969) J. Lipid Res., 10 : 158.

PORTMAN, O.W., ALEXANDER, M. and OSUGA, T. : (1969) Biochim. Biophys. Acta. 187 : 435.

PRITCHARD, E.T., ARMSTRONG, W.D., and WILT, J.C. : (1968) Am. J. Obst. and Gynaecol., 100 : 289.

RACHMILEWITZ, D., EISENBERG, S., STEIN, Y., and STEIN, O. : (1967) Biochim. Biophys. Acta 144 : 624

READER, R. and WYNN, A. : (1966) Med. J. Aust. 2 : 740.

RENKONEN, O. : (1966) Ann. Med. Exp. Biol. Fenn. 44, Suppl. 10. p. 1.

ROBERTS, J.C. and STRAUS, R. : (1965) Editors, "Comparative Atherosclerosis" Harper (Hoeber), New York.

ROBERTSON, A. and SPRECHER, H. : (1966) Pediatrics 38 : 1028.

ROBERTSON, A.F. and SPRECHER, H. :(1968) "A Review of Human Placental Lipid Metabolism and Transport" Acta Paediatrica Scandinavica, Supplement 183.

ROBERTSON, A., SPRECHER, H. and WILCOX, J. : (1968) Nature, 217 : 378.

ROBERTSON, A.L. Jr. : (1965) Cleveland Clinic Quarterly, 32 : 99.

ROBERTSON, A.L. Jr. and INSULL, W. Jr. : (1967) Nature 214 : 821.

ROBERTSON, A.L. Jr., and PAGE, I.H. : (1967) Circulation 35 and 36, Suppl. II : 34.

ROHEIM, P.S., HAFT, D.E., GIDEZ, L.I., WHITE, A. and EDER, H.A. : (1963) J. Clin. Invest., 42 : 1277.

ROSSITER, H., TUME, R.K. and DAY, A.J.: Unpublished Observations.

ROSSITER, R.J. : (1968) "Metabolism of Phosphatides" in Metabolic Pathways, vol. 2., Lipids, Steroids, and Carotenoids, 3rd edition, edited by D.M. Greenberg, Academic Press, New York.

RUFFER, M.A. : (1911) J. Path. Bact., 15 : 453.

SACHS, B. : (1968) Am. Heart J., 75 : 707.

ST CLAIR, R.W., LOFLAND, H.B. Jr. and CLARKSON, T.B. : (1968) J. Lipid Res., 9 : 739.

ST CLAIR, R.W., LOFLAND, H.B. Jr., PRICHARD, R.W. and CLARKSON, T.B. : (1968) Exptl. Molec. Path., 8 : 201.

SANDBERG, L.B. and CLEARY, E.G. : (1968) Biochim. Biophys. Acta, 154 : 411.

SANDISON, A.T. : (1967) Degenerative Vascular Disease p. 474 in "Diseases in Antiquity" Edited by D. Brothwell, and A.T. Sandison, Charles C. Thomas, Springfield, Illinois.

SANDLER, M. and BOURNE, G.H. : (1963) Editors "Atherosclerosis and Its Origin" Academic Press, New York.

SCHETTLER, G., KAHLKE, W. and SCHLIERF, G. : (1967) "Essential Hypercholesterolemia" p. 412 in Lipids and Lipidoses, edited by G. Schettler, Springer- Verlag, Berlin, Heidelberg and New York.

SCHETTLER, G. : (1968) Editor, "Lipids and Lipidoses", Springer-Verlag, Berlin, Heidelberg and New York.

SCHETTLER, F.G. and BOYD, G.S. : (1969) Editors, "Atherosclerosis", American Elsevier.

SCHOENHEIMER, R. : (1926) Ztschr. f. physiol. Chem., 160 : 61.

SCHOENHEIMER, R. : (1928) Ztschr. f. physiol. Chem., 177 : 143.

SCOTT, R.F., FLORENTIN, R.A., DAOUD, A.S., MORRISON, E.S., JONES, R.M. and HUTT, M.S.R. : (1966) Exptl. Molec. Path., 5 : 12.

SCHRADE, W., BIEGLER, R. and BÖHLE, E.: (1961) J. Atheroscler. Res. 1 : 47

SCHWARTZ, C.J., ARDLIE, N.G., CARTER, R.F. and PATERSON, J.C. :(1967) Arch. Path., 83 : 325.

SCHWENK, E. and WERTHESSEN, N.T. : (1952) Arch. Biochem. Biophys., 40 : 334.

SCHWENK, E. and STEVENS, D.F. : (1960) Proc. Soc. Exp. Biol. Med., 103 : 614.

SHAFRIR, E. : (1968) Israel J. Med. Sci., 4 : 277.

SHORE, M.L., ZILVERSMIT, D.B. and ACKERMAN, R.F. : (1955) Am. J. Physiol., 181 : 527.

SHYAMALA, G., NICHOLS, C.W. Jr. and CHAIKOFF, I.L. : (1966) Life Sciences, 5 : 1191.

SIMONTON, J.H. and GOFMAN, J.W. : (1951) Circulation, 4 : 557.

SIPERSTEIN, M.D., CHAIKOFF, I.L. and CHERNICK, S.S. : (1951) Science, 113 : 747.

SIPERSTEIN, M.D. and CHAIKOFF, I.L. : (1952) J. Biol. Chem., 198 : 105.

SKIPSKI, V.P., PETERSON, R.F. and BARCLAY, M. : (1964) Biochem. J. 90 : 374.

SMITH, E.B. : (1960) Lancet, I : 799.

SMITH, E.B. : (1962) Lancet, II : 530.

SMITH, E.B. : (1965) J. Atheorscler. Res., 5 : 224.

SMITH, E.B., EVANS, P.H. and DOWNHAM, M.D. : (1967) J. Atheroscler. Res., 7 : 171.

SMITH, E.B. : (1968) Editorial, J. Atheroscler. Res., 8 : 197.

SMITH, E.B., SLATER, R.S. and CHU, P.K. : (1968) J. Atheroscler. Res., 8 : 399.

SMITH, G. ELLIOT : (1912) The Royal Mummies. Cairo, Musee du Caire. Quoted by Sandison.

SNYDER, F. : (1964) Ann Biochem., 9 : 183.

SOLOMON, D. : (1967) "The Biology and Pathogenesis of Vascular Disease" p. 285 in Advances in Gerontological Research vol. 2 Academic Press, New York

SOMLYO, A.P. and SOMLYO, A.V. : (1968) "Vascular Smooth Muscle I Normal Structure, pathology, Biochemistry, and Biophysics" p. 197 in Pharmacological Reviews vol. 20, The Williams and Williams Co., U.S.A.

STEIN, O., SELINGER, Z. and STEIN, Y. : (1963) J. Atheroscler. Res., 3 : 189.

STEIN, Y., and STEIN, O. : (1962) J. Atheroscler. Res., 2 : 400.

STEIN, Y., STEIN, O. and SHAPIRO, B. : (1963) Biochim. Biophys, Acta, 70 : 33

STILL, W.J.S., MARTIN, J.M. and GREGOR, W.H. : (1964) Exptl. Molec. Path. 3 : 141.

STOUT, R.W. : (1968) Lancet II : 702.

STOUT, R.W. : (1969) Lancet, II : 467.

STOUT, R.W. and VALLANCE-OWEN, J. : (1969) Lancet, I : 1078.

STRONG, J.P. and McGILL, H.C. Jr. : (1962) Amer. J. Path., 40 : 37.

STRONG, J.P. and McGILL, H.C. Jr. : (1969) J. Atheroscler. Res., 9 : 251.

SWELL, L., FIELD, H. Jr. SCHOOLS, P.E. Jr. and TREADWELL, C.R. : (1960a) Proc. Soc. Exptl. Biol. Med., 103 : 651.

SWELL, L., FIELD, H. Jr., SCHOOLS, P.E. Jr., and TREADWELL, C.R. : (1960b) Proc. Soc. Exptl. Biol. Med., 105 : 662.

SWELL, L., FIELD, H. Jr., TREADWELL, C.R. : (1960) Proc. Soc. Exptl. Biol. Med., 105 : 129.

SWELL, L., LAW, M.D., SCHOOLS, P.E. Jr., and TREADWELL, C.R. : (1961) J. Nutrition, 75 : 181.

SWELL, L., SCHOOLS, P.E. Jr., and TREADWELL, C.R. : (1962) Proc. Soc. Exptl. Biol. Med., 109 : 682.

SWELL, L., LAW, M.D. and TREADWELL, C.R. (1963) J. Nutrition 81 : 263

SZABO, A.J., GRIMALDI, R.D. and JUNG, W.F. : (1969) Metabolism, 18 : 406.

SUZUKI, M. and O'NEAL, R.M. : (1964) J. Lipid Res. 5 : 624.

TAYLOR, H.E. : (1953) Am. J. Path., 29 : 871.

TEAL, S.W. and GAMBLE, W. : (1965) Biochem. Pharmacol. 14 : 896.

THORP, J.M. and WARING, W.S. : (1962) Nature, 194 : 948.

TRACY, R.E., MERCHANT, E.B., and KAO, V.C. : (1961) Circulation Res., 9 : 472.

TUME, R.K., BRADLEY, T.R., DAY, A.J. : (1969) J. Atheroscler. Res., 9 : 151

TUNA, N. and MANGOLD, H.K.: (1963) "Fatty Acids of the Atheromatous Plaque" p. 85 in Evolution of the Atherosclerotic Plaque, edited by R.J. Jones, The University of Chicago Press, Chicago and London

UEMURA, K., STERNBY, N., VANECEK, R., VIHERT, A., and KAGAN, A. : (1964) Bull. Wld. Hlth. Org., 31 : 297.

URRUTIA, G., BEAVAN, D.W. and CAHILL, G.F. Jr., : (1962) Metabolism, 11 : 530.

VIKROT, O. : (1964) Acta Med Scand., 175 : 443.

VIRCHOW, R. : (1856) Phlogose und Thrombose im Gefässystem. p. 458 in Gesammelte Abhandlungen zur Wissenschaftlichen medicin, V. Von Meidinger Sohn und Comp., Frankfurt.

VIRCHOW, R. : (1856) Der atheromatose Prozess der Arterien. Wien. med. Wochschr. 6 : 809.

VOGEL, J. : (1847) "The Pathological Anatomy of the Human Body", translated from the German by G.E. Day, Bailliere, London.

VON ROKITANSKY, C. : (1852) A manual of pathological anatomy Vol. IV (The pathological anatomy of the organs of respiration and circulation) translated from the German by G.E. Day, Sydenham Scoiety, London.

VOST, A. : (1968) J. Atheroscler. Res., 9 : 221.

WALSH, M.R., TEAL, S.W. and GAMBLE, W. : (1969) Arch. Biochem. Biophys. 130 : 7.

WATTS, H.F. : (1963) "Role of Lipoproteins in the formation of atherosclerotic lesions" p. 117 in Evolution of the Atherosclerotic Plaque, edited by R.J. Jones, University of Chicago Press, Chicago, Illinois.

WEINHOUSE, S. and HIRSCH, E.F. : (1940) Arch. Pathol., 29 : 31

WELLER, R.O., CLARK, R.A. and OSWALD, W.B. : (1968) J. Atheroscler. Res., 8 : 249.

WERTHEIMER, H.E. and BENTOR, V. : (1962) Diabetes, 11 : 422.

WERTHESSEN, N.T., MILCH, L.J., REDMOND, R.F., SMITH, L.L. and SMITH, E.C. : (1954) Am. J. Physiol., 178 : 23.

WERTHESSEN, N.T. : (1959) "Control of Aortal Lipid Metabolism and Lipid Movement by Hormones and Vitamins" in Hormones and Atherosclerosis, edited by G. Pincus, Academic Press.

WERTHESSEN, N.T. (1963) p. 439 in I: Biochemical Problems of Lipids, edited by A.C. Fraser. Biochim. Biophys. Acta.

WHEREAT, A.F. and STAPLE, E. : (1960) Arch. Biochem. Biophys. 90 : 224.

WHEREAT, A.F. : (1965) Proc. Soc. Exp. Biol. Med., 118 : 888.

WHEREAT, A.F. : (1966) J. Lipid Res., 7 : 671.

WHEREAT, A.F. (1967) "Recent Advances in Experimental Molecular Pathology. Atherosclerotic and Metabolic Disorder in the Arterial Wall." Exptl. Molec. Path. 7: 233.

WHEREAT, A.F. and ORISHIMO, M.W. : (1968) Exptl. Molec. Path., 9 : 230.

WIESE, H.F., BENNET, M.J., BRAUN, I.H.G., YAMANAKA, W. and COON, E. : (1966) Am. J. Clin. Nutrition, 18 : 155.

WIESE, H.F., COON, E., YAMANAKA, W., BARBER, S., and JOHNSON, P. : (1967) J. Lipid Res., 8 : 312.

WILSON, J.W. and LEDUC, E.H. : (1963) J. Cell. Biol. 16 : 281.

WINDAUS, A. : (1910) Ztschr. f. physiol. Chem., 67 : 174.

WINTERNITZ, M.C., THOMAS, R.M. and LE COMPTE, P.M. : (1938) "The Biology of Arteriosclerosis" Charles C. Thomas, Springfield, Illinois.

WISSLER, R.W. : (1967) Circulation, 36 : 1.

WISSLER, R.W. : (1968) J. Atheroscler. Res., 8 : 201.

WISSLER, R.W. and VESSELINOVITCH, D. : (1968) "Comparative Pathogenetic Patterns in Atherosclerosis" p. 181 in Advances in Lipid Research, vol. 6, edited by R. Paoletti and D. Kritchevsky, Academic Press, New York and London.

WOERNER, C.A. : (1951) J. Gerontol., 6 : 165.

WOODFORD, F.P., BÖTTCHER, C.J.F., OETTE, K. and AHRENS, E.H. Jr. (1965) J. Atheroscler. Res., 5 : 311.

WORLD HEALTH ORGANIZATION : (1958) Technical Report Series No. 143.

YALCIN, S. and WINEGRAD, A.I. : (1963) Am. J. Physiol., 205 : 1253.

YALOW, R.S. and BERSON, S.A. : (1960) Diabetes, 9 : 254.