SOME FACTORS AFFECTING THE PLASMA LIPIDS

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by

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SUMMARY

This thesis deals with some factors affecting the plasma lipids in apparently healthy persons, in patients with atherosclerotic arterial disease, and in two groups of subjects predisposed to atherosclerosis; namely, habitual cigarette-smokers and obese persons. It is in five main parts:-

1. A comparison of fasting plasma lipid concentrations in healthy and atherosclerotic subjects.
2. A comparison of fat tolerance tests in healthy and atherosclerotic subjects.
3. An examination of several aspects of gastric function alleged to influence fat tolerance; namely, the basal secretion, motility, and lipolytic ability of the stomach.
4. An investigation of the acute effects of cigarette-smoking on the plasma lipids and thrombotic tendency of the blood.
5. A study of the plasma lipid and other metabolic changes induced by therapeutic starvation in obese subjects.

Chapter I. INTRODUCTION

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Chapter I INTRODUCTION

THE PLASMA LIPIDS

Lipids have been defined by Bloor (1943) as "a group of naturally occurring substances consisting of the higher fatty acids, their naturally occurring compounds, and substances found naturally in chemical association with them". As long ago as 1774 Hewson (see Gage and Fish, 1924) demonstrated that the milky appearance of the plasma after a meal was due to fat particles, and later it was realised that fasting plasma, although optically clear, also contained lipid present in appreciable amounts in an extremely fine dispersion. Subsequently the main chemical classes of plasma lipids were identified as cholesterol, triglycerides, phospholipids and, most recently, the non-esterified fatty acids.

The physical state in which these lipids circulate in the blood is of considerable importance. It is now appreciated that lipids in biological systems exist in the form of protein-lipid complexes known as lipoproteins, thus circumventing the poor solubility of most lipids in aqueous solutions. The plasma lipoproteins have been characterised according to their physical properties, such as density and electrophoretic mobility. The lipoproteins containing cholesterol, phospholipid, and

triglyceride migrate with the globulins on starch granule electrophoresis, and have been classified into three main groups, as illustrated in Table I. Although cholesterol is transported mainly by the low-density lipoproteins, and triglyceride by the very-low-density lipoproteins, each of the three lipoproteins contains cholesterol, phospholipid, and triglyceride in varying proportions (Eder, 1957). The non-esterified fatty acids, on the other hand, are almost entirely bound to albumin (Olson and Vester, 1960).

The objective of this thesis is to consider some of the factors causing alterations in the plasma lipid levels. A description of the chemical structure and metabolism of each of the four main chemical classes of plasma lipids is given below.

CHOLESTEROL

(a) CHEMICAL STRUCTURE OF CHOLESTEROL

Cholesterol was first identified in human blood by Lecanu in 1838, and since then it has been estimated in almost every disease condition of man. Its chemical formula was established finally in 1932 by Rosenheim and King, and is shown in Fig. 1.

Table I. Distribution of Plasma Lipids in the Lipoprotein Classes (from Eder, 1965)

CLASS	DENSITY	ELECTRO-PHORETIC MOBILITY	TOTAL CHOLESTEROL	PHOSPHO-LIPID	TRI-GLYCERIDE
			(mg. per 100 ml plasma)		
Very-low-density lipoproteins	<1.019	$\alpha 2$	25	30	85
Low-density lipoproteins	1.019-1.063	$\beta 1$	125	90	10
High-density lipoproteins	1.063-1.21	$\alpha 1$	50	100	5
Whole serum			200	220	100

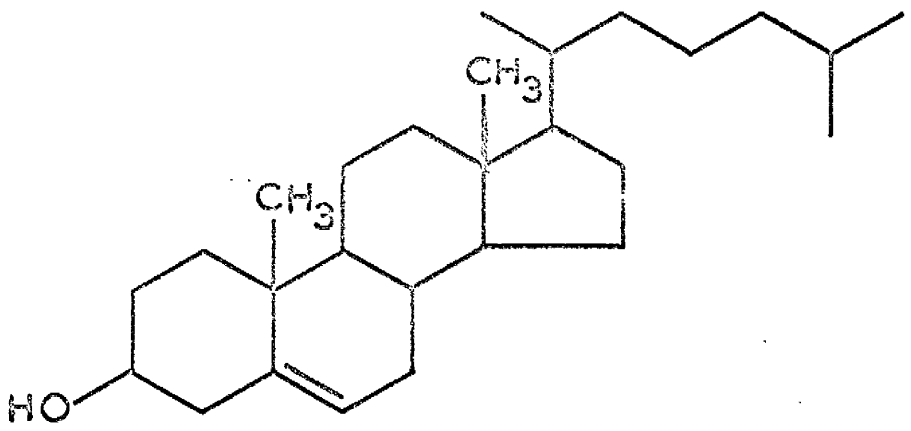


Fig. 1. Structure of Cholesterol (cholest-5-ene-3β-ol)

Plasma cholesterol exists mainly in the form of fatty acid esters. The proportion of free cholesterol in the total plasma cholesterol is remarkably constant within narrow limits at 27 per cent, with the exception of certain liver diseases and lipidoses (Sperry, 1936).

(b) FUNCTIONS OF PLASMA CHOLESTEROL

Cholesterol is a precursor of other steroids, such as the bile acids, adrenocortical hormones, and sex hormones (Kritchevsky, 1958a), and some evidence has been produced to show that the cholesterol found in the testes, kidneys, adrenals, spleen, and lungs may be derived from the circulating

cholesterol (Landon and Greenberg, 1954). It is generally accepted that cholesterol esters are involved in the metabolism and transport of fatty acids. Kritchevsky (1960) has suggested that the plasma cholesterol may also, in several ways, serve a structural purpose. There is a rapid interchange between plasma free cholesterol and red blood cell cholesterol (Hagerman and Gould, 1951), indicating that the plasma cholesterol may be necessary for the integrity of the red blood cell structure. The fact that cholesterol, both esterified and free, is present in all the serum lipoprotein fractions suggests that it may be implicated in maintenance of the stability of the lipoproteins also.

(c) METABOLISM OF CHOLESTEROL

In recent years a number of reviews of human cholesterol metabolism have been published (Kritchevsky, 1958; Whitehouse, 1964; Van Belle, 1965; Taylor and Ho, 1967), and a brief account of the salient points is given here.

Origin of Plasma Cholesterol

The plasma cholesterol is derived from both endogenous and exogenous sources. Almost all tissues in the body are capable of synthesising cholesterol from two-carbon fragments

(Srere et al., 1950), but plasma cholesterol originates mainly from the liver (Kaplan et al., 1963), and probably also from cholesterol synthesis in the intestinal mucosa (Wilson and Dietschy, 1966). The contribution of dietary cholesterol to the plasma cholesterol is usually small, since the intestinal capacity for absorption of cholesterol is limited (Karvinen et al., 1957).

Catabolism of Cholesterol

Plasma cholesterol is in equilibrium with hepatic cholesterol, and the main catabolic pathway of cholesterol is conversion to bile acids in the liver (Gould and Cook, 1958). Some hepatic cholesterol is also excreted unchanged into the bile and thence into the intestine, where it mixes with cholesterol of intestinal cell origin resulting from desquamation and possibly also from direct excretion (Whitehouse, 1964). Faecal excretion of cholesterol and its catabolites occurs, therefore, in the form of bile acids, and of neutral steroids derived from unabsorbed dietary cholesterol and cholesterol of hepatic and intestinal origin.

Regulation of the Plasma Cholesterol Level

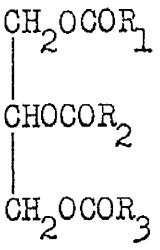
Regulation of the plasma cholesterol concentration therefore

depends on a balance between factors tending to increase the cholesterol level (increased cholesterol synthesis in hepatic and extrahepatic tissues, and intestinal absorption of cholesterol), and factors tending to remove cholesterol from the circulation (increased catabolism to bile acids or excretion as cholesterol, metabolism to other substances such as the steroid hormones, and possibly sequestration in tissue compartments such as muscle or connective tissue). Man appears to have little capacity for increased faecal bile acid or cholesterol output to compensate for increased amounts of absorbed dietary cholesterol (Chevallier, 1961). A negative feedback system whereby increased intestinal absorption of cholesterol reduces hepatic cholesterol synthesis is of considerable importance in the control of the plasma cholesterol level in animals. In man this mechanism appears to function in a less effective manner (Taylor and Ho, 1967).

TRIGLYCERIDE

(a) CHEMICAL STRUCTURE OF TRIGLYCERIDE

Triglycerides are esters formed by glycerol and fatty acids, and may be represented by the following structural formula:



where R_1, R_2, and R_3 are the long-chain, hydrocarbon units of fatty acids, which may or may not be identical. The tendency in all natural fats is towards maximum heterogeneity in the composition of the constituent triglycerides (Diem, 1962). Plasma triglyceride consists of two forms:- lipoprotein triglyceride, which is present in fasting as well as in post-prandial blood; and chylomicron triglyceride - the particulate fat which appears during absorption of a fatty meal. Diglycerides and monoglycerides are believed to be undetectable or present only in trace amounts in plasma under normal circumstances (Hirsch and Ahrens, 1958), although Carlson and Wadström (1956) reported that they might contribute up to 5 to 10 per cent of the total plasma glycerides.

(b) FUNCTIONS OF PLASMA TRIGLYCERIDE

The bulk of dietary fat is transported from the intestine to the liver and other organs in the form of chylomicron triglyceride. The lipoprotein triglyceride is the mode of transport of fat, both exogenous and endogenous in origin, from

the liver to other organs. The plasma triglyceride is utilised in the body either directly to provide energy, or as a building material of cells, or stored in the fat depots to form a source of easily mobilised energy (Borgström, 1960).

(c) METABOLISM OF PLASMA TRIGLYCERIDE

Origin of Plasma Triglyceride

The triglyceride of fasting blood is synthesised in the liver. Its fatty acids are of complex origin, and appear to be derived from at least three sources:- chylomicron glycerides, plasma non-esterified fatty acids, and *de novo* synthesis in the liver from acetate, and thus indirectly from carbohydrate (Borgström, 1960). Similarly, the glycerol moiety may be the product either of fat metabolism, when the triglyceride is synthesised from monoglyceride, or of carbohydrate metabolism, when the starting-point of hepatic triglyceride synthesis is αglycerophosphate. Although triglyceride is also synthesised in adipose tissue by these two routes (Steinberg *et al.*, 1961), there is no evidence that this triglyceride is released into the circulation.

The chylomicron triglyceride is derived mainly from dietary triglyceride. This is hydrolysed in the intestinal lumen to form monoglyceride, which is absorbed into the

intestinal cell where it is resynthesised to triglyceride (Senior, 1964). The chylomicron then formed is composed of a triglyceride droplet with a coating of surfactant material (phospholipid, cholesterol, and traces of fatty acids) and an outer zone of adsorbed protein (Dole and Hamlin, 1962). The chylomicrons are transported via the lymph to the blood stream.

Removal of Plasma Triglyceride

Circulating chylomicrons are, for the most part, removed by the liver and secreted into the plasma again as very-low-density lipoprotein, which is taken up by adipose tissue. A variable proportion of the chylomicron triglyceride, depending on the nutritional state, is removed by adipose tissue (Shapiro, 1965). Some of the chylomicron triglyceride may be utilised as a source of energy by the myocardium (Crass and Meng, 1966) and liver (Shapiro, 1967).

The uptake and subsequent metabolism of chylomicron and lipoprotein triglyceride by adipose tissue and heart muscle requires the activity of an enzyme, lipoprotein lipase (Rodbell, 1960), which is present in high concentrations in both these tissues (Korn and Quigley, 1955). The amount of lipoprotein lipase activity in adipose tissue is related to the rate of glucose utilisation in the tissue, and is enhanced by insulin

(Hollenberg, 1959; Kessler, 1962). The role of this enzyme has recently been reviewed (Rodbell and Scow, 1965). It does not appear to cause intravascular hydrolysis of triglyceride to any appreciable extent, but is thought to act by converting fat particles which have entered capillary endothelial cells into a form which can be transported across the basement membrane into the adipose tissue cells. In contrast to adipose tissue and heart muscle, the liver is deficient in lipoprotein lipase, and appears to possess some mechanism which permits the passage of esterified fatty acids, or perhaps even complete lipoprotein entities, through the cell wall without breakdown (Olivecrona, 1962).

It is generally agreed that during assimilation by the tissues the triglyceride molecule undergoes considerable and rapid degradation, and its constituent fatty acids are used in the synthesis of new triglycerides and phospholipids and also for combustion to carbon dioxide (Shapiro, 1967). In the liver both glycerol and fatty acids are incorporated into fat. In adipose tissue and heart muscle, however, the glycerol appears to be lost during uptake of the fatty acids, which are then esterified with α-glycerophosphate to form triglyceride (Olivecrona, 1962).

PHOSPHOLIPID

(a) CHEMICAL STRUCTURE OF PHOSPHOLIPIDS

Phospholipids are complex esters which contain, in addition to an alcohol and long-chain fatty acid moieties, a phosphoric acid residue and a nitrogen-containing base (Hanahan, 1960). The plasma phospholipids consist predominantly of three glycerophosphatides (phosphatidyl choline, lysophosphatidyl choline, and phosphatidyl ethanolamine), and sphingomyelin. Their chemical formulae are shown in Fig. 2. Chromatography of human plasma lipoproteins has shown that the phospholipids are present in the following proportions (Phillips, 1958):-

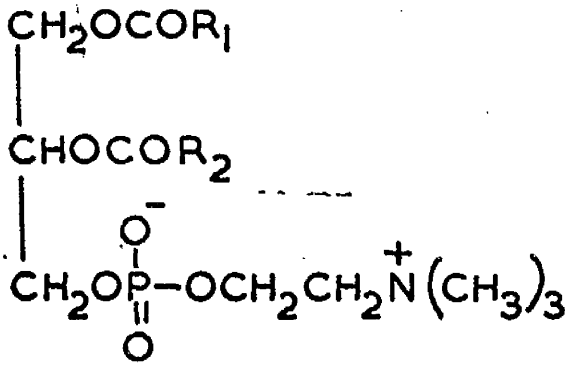
phosphatidyl choline	69.1 per cent
sphingomyelin	19.0 per cent
lysophosphatidyl choline	7.1 per cent
phosphatidyl ethanolamine	4.6 per cent

(b) FUNCTIONS OF THE PLASMA PHOSPHOLIPIDS

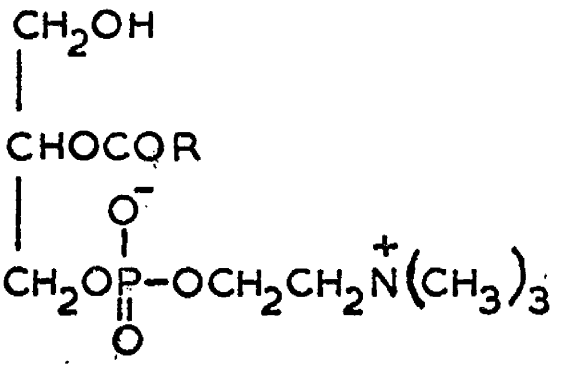
The various functions of phospholipids in the blood and tissues have been reviewed by Rossiter and Strickland (1960). It is now well-accepted that plasma phospholipids exist mainly as components of lipoproteins and chylomicrons. Phospholipids are believed to play an important part in fat transport in

Fig. 2. Chemical Formulae of Plasma Phospholipids

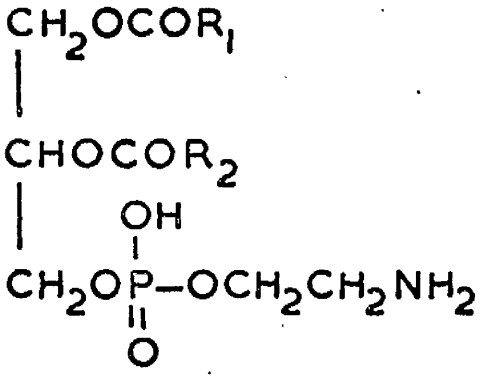
(a) Glycerophosphatides



phosphatidyl choline

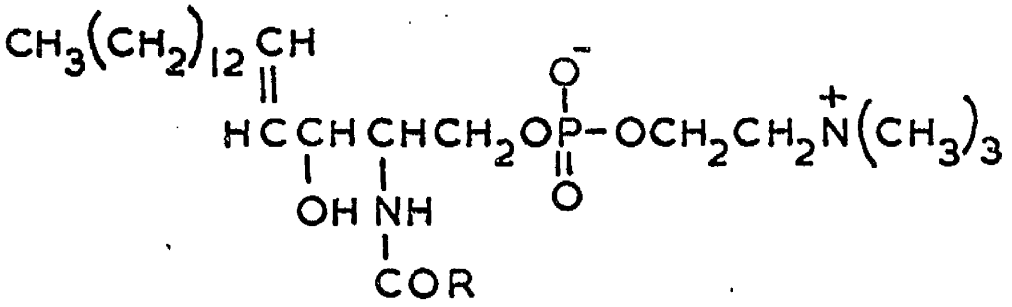


lysophosphatidyl choline



phosphatidyl ethanolamine

(b) Sphingolipids



sphingomyelin

contributing to the structure and stability of these lipid-protein complexes. Certain phospholipids also participate in thromboplastin generation, and may be required for normal blood coagulation. In the tissues, phospholipids are intimately concerned with the structure of certain cellular components, and with the metabolic processes related to these structures, such as ion transport and protein synthesis.

(c) METABOLISM OF PLASMA PHOSPHOLIPIDS

Origin of Plasma Phospholipid

The plasma phospholipids, although present in chylomicrons as well as lipoproteins, are almost entirely of endogenous origin. Experiments with tissue slices *in vitro* have shown that in most tissues the phospholipids are formed *in situ* from the appropriate isotopically-labelled precursors, such as inorganic phosphorus, fatty acids, glycerol, or nitrogen-containing bases (Rossiter and Strickland, 1960). The primary source of the plasma phospholipids appears to be the liver, since in hepatectomised dogs the labelling of phospholipids with P^{32} is negligible in plasma, but normal in the kidneys and intestine (Fishler *et al.*, 1943). The chylomicron phospholipid is not derived from dietary phospholipid, which is broken down almost completely in the lumen of the gut during digestion (Wilson, 1962), but is formed by

synthesis in the intestinal mucosal cell (Senior, 1964).

Removal of Plasma Phospholipid

The removal of plasma phospholipids appears to be carried out mainly by the liver, since the rate of removal of labelled phospholipids from the plasma of dogs is markedly reduced by evisceration (Entenman et al., 1946), or hepatectomy (Harper et al., 1953).

PLASMA NON-ESTERIFIED FATTY ACIDS (NEFA)

(a) CHEMICAL STRUCTURE OF NEFA

The non-esterified fatty acids are straight-chain saturated or unsaturated fatty acids containing an even number of carbon atoms. In addition to the term non-esterified fatty acids or NEFA, first proposed by Dole (1956) and now achieving widespread acceptance, they have been designated as unesterified fatty acids or UFA (Gordon and Cherkes, 1956), and as free fatty acids or FFA (Bragdon, 1959). Since most of the NEFA in plasma is tightly bound to albumin the term free fatty acid is somewhat misleading, and should probably be reserved for the small fraction which exists in free solution and is not measurable by present techniques. The term unesterified fatty acid, introduced shortly

after the term NEFA, is less satisfactory, since it may be confused with unsaturated fatty acid, also abbreviated to UFA.

The principal fatty acids in the NEFA fraction have been found to be present in the following proportions in venous blood (from Nakamura et al., 1967):-

Myristic acid (C14:0)	2.1 per cent
Palmitic acid (C16:0)	32.4 per cent
Palmitoleic acid (C16:1)	6.9 per cent
Stearic acid (C18:0)	10.3 per cent
Oleic acid (C18:1)	37.1 per cent
Linoleic acid (C18:2)	11.1 per cent

The chemical formulae of these fatty acids are shown in Fig. 3.

(b) FUNCTIONS OF PLASMA NEFA

Although small quantities of NEFA had previously been found in lipid extracts from plasma, they were thought to be laboratory artefacts, until in 1947 Cohn et al. and Davis reported that fresh human plasma contained NEFA. Gordon and Cherkes (1957) were the first to postulate that NEFA had an important function as the transport form of fat from the depots to different tissues for oxidation, and this has since been confirmed by numerous other workers. NEFA are extracted from the blood and utilised for energy by the liver, striated muscle, the heart, and other organs (Rothlin et al., 1962). In the

Fig. 3. Chemical formulae of plasma non-esterified fatty acids.

(a) saturated fatty acids

$CH_3 (CH_2)_{12} COOH$ Myristic acid (C14:0)

$CH_3 (CH_2)_{14} COOH$ Palmitic acid (C16:0)

$CH_3 (CH_2)_{16} COOH$ Stearic acid (C18:0)

(b) monounsaturated fatty acids

$CH_3(CH_2)_5CH = CH(CH_2)_7COOH$ Palmitoleic acid (C16:1)

$CH_3(CH_2)_7CH = CH(CH_2)_7COOH$ Oleic acid (C18:1)

(c) polyunsaturated fatty acids

$CH_3(CH_2)_4CH = CH\ CH_2\ CH = CH(CH_2)_7COOH$ Linoleic acid (C18:2)

fasting state the human heart uses NEFA as its major source of energy (Gordon and Cherkes, 1956). The very rapid turnover rate of plasma NEFA underlines their metabolic significance; the half-life for injected NEFA is reported to be approximately two minutes, both in animals (Havel and Fredrickson, 1956; Bierman et al., 1957b) and in humans (Fredrickson and Gordon, 1957 and 1958a; Laurell, 1957).

(c) METABOLISM OF PLASMA NEFA

Origin of NEFA

The main source of the plasma NEFA has been shown to be the adipose tissue (Gordon and Cherkes, 1956; Rothlin et al., 1962). The NEFA carried to the circulation in the thoracic duct lymph has been found to be not of exogenous origin. It is thought to represent fatty acids mobilised from mesenteric adipose tissue, and to be quantitatively unimportant, accounting for only 1 to 7 per cent of the total lymph fatty acids in humans (Blomstrand and Ahrens, 1958). The contribution to plasma NEFA of fatty acids liberated by intravascular lipolysis of newly-absorbed lipids is believed to be small (Borgström, 1960).

Release of NEFA from Fat Depots

The rate of release of NEFA from the fat depots is dependent on the state of carbohydrate metabolism, being increased in fasting, and decreasing sharply after glucose and insulin administration (Dole, 1956; Gordon and Cherkes, 1956). Aminoacid administration has been reported to have an effect similar to glucose, although less marked (Borgström, 1960). Bierman et al. (1957b) have shown that the decreased NEFA level seen after insulin injection is due to decreased outflow of fatty acids from adipose tissue, rather than to increased utilisation of plasma NEFA. The specific activity of plasma NEFA, previously labelled by injection of C^{14} palmitic acid, increases after insulin administration. In vitro studies by Engel and White (1960) have demonstrated that the action of insulin in retarding net NEFA release is mediated through a primary action on carbohydrate metabolism, and not direct inhibition of lipolysis.

Administration of sympathomimetic amines, notably epinephrine, norepinephrine, and isoprenaline, results in rapid release of NEFA from fat depots. That the release of NEFA from fat depots is mediated by the activity of sympathetic nerves is shown by the fact that it can be blocked by adrenergic blocking drugs of either the α or the β type (Innes and Nickerson,

1965). Elevations of the plasma NEFA level occur in many conditions associated with increased sympathetic nervous system activity, such as mental stress (Bogdonoff et al., 1960), long-term exercise (Basu et al., 1960), and hypoglycaemia (Armstrong et al., 1961; Werk et al., 1961; Fröberg et al., 1964).

Removal of Plasma NEFA

By injection of C^{14}-labelled NEFA, the rate at which NEFA disappear from the plasma has been followed (Fredrickson and Gordon, 1958a) and has been found to be dependent on the NEFA concentration in the blood up to a concentration of about 1 meq. per litre. Above this concentration a constant removal of about 30 per cent of the NEFA fraction takes place per minute. The NEFA have been shown to recycle in the plasma several times before oxidation, partly in the free state and partly after incorporation into triglyceride (Fredrickson and Gordon, 1958a). Injected labelled NEFA are distributed extensively to different tissues. Ten minutes after injection liver, lung, and heart are most heavily labelled, while 200 minutes after injection the muscles contain the largest percentage activity (Bragdon and Gordon, 1958). Adipose tissue is also capable of assimilating NEFA (Stern and Shapiro, 1954;

Laurell, 1959). The largest fraction of the labelled acids after injection of labelled NEFA is found in the triglyceride, phospholipid, and cholesterol esters of the different tissues (Dole, 1958).

The utilisation of plasma NEFA has been shown to be increased in starvation, and diminished after carbohydrate-feeding (Lossow and Chaikoff, 1955). Removal of NEFA from the circulation is increased also by muscular exercise. During short-term vigorous exercise the muscular demand may temporarily exceed the supply from adipose tissue, resulting in a fall in the plasma NEFA level (Wood et al., 1965).

INTERDEPENDENCE OF PLASMA LIPIDS

Although in the preceding four sections the metabolism of each of the four main chemical groups of plasma lipids has been considered separately, it will be apparent that this separation is somewhat artificial. The physical association of cholesterol, phospholipid, and triglyceride in each of the plasma lipoprotein fractions and in the chylomicrons (Eder, 1957) has already been mentioned, and it might be anticipated for this reason alone that alterations in the plasma concentration of one lipid might affect other plasma lipid levels. In addition, the complex metabolic processes of the individual lipids are

related in several ways. The central position of the fatty acids in lipid interrelationships has been discussed by Cook (1958). As their coenzyme A compounds, the fatty acids may be transferred to cholesterol esters, to phospholipids, and to triglycerides, and the synthesis of these lipids must be dependent on an adequate supply of fatty acids. There is a constant interchange between circulating plasma NEFA and the fatty acids of other plasma lipids, in particular triglyceride, and the fatty acids of triglyceride incorporated in adipose tissue rapidly reappear as plasma NEFA. Both NEFA and triglyceride may be derived from products of carbohydrate metabolism, and the rates of production and utilisation of both these lipids may be markedly influenced by the state of carbohydrate metabolism (Borgström, 1960). The synthesis of phospholipid may also be connected with that of triglyceride, since the substance α-glycerophosphate is a precursor common to triglyceride and the glycerophosphatides (Diem, 1962). In addition, fatty acids from dietary triglyceride are rapidly transferred to phospholipids in the liver (Olivecrona, 1962).

In confirmation of these theoretical considerations, it has repeatedly been observed that alteration of one plasma lipid may be associated with alteration of one or more of the other plasma lipids. The evidence that dietary factors, in particular the type of fatty acid in the diet, affect several of the plasma

lipids has been reviewed by Hilditch and Jasperson (1959). In general, the feeding of saturated fatty acids increases the plasma levels of cholesterol, triglyceride and phospholipid, whereas unsaturated fatty acids have the opposite effect. A prolonged increase in plasma NEFA concentration has been shown to stimulate increased lipoprotein synthesis in the liver, and result in increased plasma levels of both triglyceride and cholesterol (Nestel and Steinberg, 1963). In experimental animals, infusions of phospholipid which produce sustained elevation of the plasma phospholipids lead to hypercholesterolaemia, but infusions of cholesterol fail to cause a secondary rise in plasma phospholipid. Similarly infusions of triglyceride quickly induce raised plasma concentrations of both cholesterol and phospholipid. Friedman et al. (1965) have described various clinical conditions, characterised by disorders of more than one plasma lipid, which they consider to be analogous to these experimental situations.

FACTORS AFFECTING PLASMA LIPID LEVELS

Plasma lipid levels are influenced by a wide variety of factors, both physiological and pathological (Boyd and Oliver, 1958). They include:- age; sex; race; social class; heredity; body weight; dietary constituents such as the type of fat,

protein, or carbohydrate, or certain vitamins; exercise; cigarette smoking; hormones of the thyroid, pituitary, adrenal, pancreatic, and sex glands; various diseases including wasting diseases, malabsorption, and the nephroses; environmental factors such as the season of the year; and a wide range of drugs. Any investigation of the plasma lipids must, therefore, of necessity be highly selective.

EVIDENCE LINKING ELEVATED PLASMA LIPIDS WITH ATHEROSCLEROSIS

The current widespread interest in plasma lipids arose mainly from evidence linking blood lipid abnormalities with atherosclerosis, and in particular with ischaemic heart disease. This evidence was obtained both from animal experiments and from human clinical and epidemiological studies. The observation that cholesterol was a constituent of the atherosclerotic plaque was made more than a century ago (Vogel, 1843). A number of early investigators were able to induce atherosclerosis in rabbits by feeding cholesterol-rich diets (Saltykow, 1908), and the lipid infiltration of the intima was given the name atherosclerosis by Marchand (1904). Shortly thereafter it was established that cholesterol was the atherogenic stimulus contained in these diets (Anitschkow and Chalatow, 1913). This initiated a series of investigations which involved cholesterol-

feeding in various experimental animals, and which produced gross hypercholesterolaemia and lesions resembling atherosclerosis. The application of these experiments to the problem of atherosclerosis in man, however, has been disputed for several reasons: atherosclerosis does not occur spontaneously in these animals under normal circumstances; the experiments were of short duration; and despite the gross atherosclerotic lesions produced by these animal-feeding experiments myocardial infarction did not occur.

In man, it has been noted repeatedly that atherosclerosis is prominent in clinical states accompanied by hypercholesterolaemia, such as diabetes mellitus (Liebow and Hellerstein, 1949), nephrosis (Steiner and Domanski, 1942) and familial hypercholesterolaemic xanthomatosis (Adlersberg et al., 1949). In 1937 Davis et al. observed that many patients with coronary artery disease had raised serum cholesterol levels, and later workers showed that such patients might in addition have elevated levels of low-density lipoproteins (Gofman et al., 1950) and triglyceride (Albrink and Man, 1959). More recent studies have suggested that peripheral arterial disease also may be associated with high serum lipid concentrations (Juergens et al., 1960; McPherson et al., 1963; Begg, 1965). Numerous epidemiological studies have indicated that people of countries or races with a high incidence of ischaemic heart disease tend to have higher

serum cholesterol levels than those belonging to races with a low incidence of ischaemic heart disease (Keys, 1956). More direct evidence linking elevated serum lipids with ischaemic heart disease has been supplied by large-scale prospective studies such as that in Framingham, Massachusetts, in which there is strong evidence that the risk of developing ischaemic disease is related to the serum cholesterol concentration (Kannel et al., 1964).

PATHOGENESIS OF ATHEROSCLEROSIS AND ITS RELATION TO PLASMA LIPIDS

Although there is a considerable weight of evidence relating elevation of plasma lipids to increased risk of atherosclerotic disease, this does not necessarily imply a causal relationship. The various ways in which elevated plasma lipids might influence either the development of the atherosclerotic process itself, or the severity of the clinical syndrome resulting from it, are still the subject of controversy, and have been discussed in detail in recent reviews (Bajusz, 1956; Fulton, 1965; Van Belle, 1965). Only three of the main theories of atherogenesis will be mentioned here.

The Filtration Theory

The filtration theory (Page, 1954) suggests that the lipid of the atherosclerotic plaque has resulted from infiltration of plasma lipids from the lumen of the blood vessel through the intima. The lipid deposit accumulates over the years, and the process may be accelerated by various factors: (a) the presence of blood lipid in excess amount, or possibly in abnormal form; (b) an increase in the filtration pressure; and (c) abnormal permeability of the vascular endothelium resulting from trauma. The filtration theory affords a plausible explanation of the increased risk of atherosclerosis in the presence of elevated plasma lipids or hypertension, and also of the localisation of plaques at sites of turbulence or velocity change in the arterial system. The encroachment of the plaque on the lumen of the vessel then favours superadded thrombosis from circulatory stasis.

The Thrombogenic Theory

Another theory advanced to explain the relationship between elevated plasma lipids and atherogenesis is based on the observation that increased plasma lipid levels may be associated with an increased thrombotic tendency, whether measured by clotting time (Duncan and Waldron, 1949), platelet survival (Mustard

and Murphy, 1962), platelet adhesiveness or thromboplastin generation (McDonald and Edgill, 1957). This alleged increased liability to thrombosis would tend to aggravate pre-existing atherosclerotic lesions, regardless of the mechanism by which these are formed. Alternatively, the increased thrombotic tendency may lead to formation of mural thrombus or fibrin encrustation on the arterial wall. According to the thrombogenic hypothesis revived by Duguid (1954), these mural lesions become organised and vascularised. Over a period of time lipid accumulates in the lesions as a result of blood destruction from small haemorrhages recurring over long periods, and in this way atherosclerotic plaques are formed.

The Chylomicron Theory

The chylomicron theory of atherogenesis was proposed by Moreton (1947), following Hueper's (1942) demonstration that macromolecular substances injected intravenously in animals were deposited in the arterial intima. This theory has recently been restated by Gordon (1963). He has suggested that the circulating chylomicron, a carrier of triglyceride, phospholipid, and cholesterol, may be pumped into the normally clear "rand-zone" of the stream periphery, and enter the intima, causing the lesion of atherosclerosis. The process may be

facilitated by lessened blood velocity, and by increased chylomicron content of the blood. Plaque formation may thus be the result of a forced filtration of the chylomicron lipids.

In one or several of these ways, therefore, atherosclerotic disease may be caused, or aggravated, by plasma lipid abnormalities. However, since the mechanism of formation of the atherosclerotic plaque is still unknown, in our present state of knowledge the exact role of the plasma lipids must remain a matter for speculation.

RATIONALE OF THE PRESENT STUDY

Plasma cholesterol levels have been found to vary considerably from country to country, and have been shown to correlate with the various national mortalities from coronary artery disease (Boyd and Oliver, 1958). Other plasma lipids have been recorded in only a few of these surveys, mainly on account of their greater technical difficulty. An association has now been established between the risk of ischaemic heart disease and the plasma level of cholesterol (Kannel *et al.*, 1964), and possibly of other lipids (Albrink and Man, 1959; Carlson, 1960b). The male death rate in Scotland from arteriosclerotic and degenerative heart disease is the third highest in the world, being exceeded only by those of

the South African European population and Finland (World Health Organisation, 1962). Within Scotland itself there are marked regional differences in mortality from ischaemic heart disease, the highest death rate being recorded in south-west Scotland (Bronte-Stewart, 1965). It was therefore considered of interest to determine fasting plasma lipid levels in a group of subjects with ischaemic heart disease, and to compare them with a group of apparently healthy controls in an area with a very high incidence of ischaemic heart disease (Glasgow). Little information is available on plasma lipid levels in subjects with forms of atherosclerosis other than ischaemic heart disease. A group of patients with peripheral arterial disease has therefore also been studied to determine whether or not they showed elevated plasma lipid concentrations.

Abnormalities in the plasma lipid response to a fat load have been reported in ischaemic heart disease, particularly in patients who had sustained a recent myocardial infarction (Schwartz et al., 1952; Barritt, 1956). These abnormalities in fat tolerance have been stated by some investigators to give better separation of coronary from healthy subjects than fasting plasma lipids (Brown et al., 1961; Stutman et al., 1961) but this claim has been disputed (Horlick, 1956 and 1957). Much of the apparently contradictory evidence may be due to the manner of selection of the subjects, and the relatively crude

methods of plasma lipid measurement used in earlier studies. In this study fat tolerance tests have been carried out in carefully selected groups of ischaemic heart disease patients and healthy controls in an attempt to determine whether the fat tolerance curve has any advantages over fasting plasma lipid measurement as an indicator of clinical ischaemic heart disease. Fat tolerance tests have been performed also in subjects with peripheral arterial disease to determine if fat tolerance abnormalities are a feature of atherosclerotic disease other than ischaemic heart disease.

Fat tolerance is reported to be influenced by a number of factors related to the upper gastro-intestinal tract. These include gastric acid secretion (Marks et al., 1962; Oliver, 1962), gastric lipolytic activity (Frazer, 1943; Becker et al., 1950), and gastric motility (Turner et al., 1960). A comparison of each of these factors has been carried out in atherosclerotic patients and controls, to determine whether differences in these factors might account for any differences in fat tolerance in the two groups.

In recent years numerous reports have confirmed an association between cigarette smoking and atherosclerotic disease of the coronary and peripheral arteries (Royal College of Physicians of London, 1962). The reasons for this association

are not known, but several possibilities have been raised. One suggestion is that the harmful effects of cigarette-smoking might be mediated by its effects on the plasma lipids, and another by its alleged production of an increased thrombotic tendency of the blood (Ashby et al., 1965). Cigarette-smoking has been shown to be associated with elevated plasma lipids, both in long-term (Gofman et al., 1955; Karvonen et al., 1959) and in short-term studies (Kershbaum et al., 1961 and 1963). An investigation has been undertaken of the short-term effects of cigarette-smoking on plasma lipids and the thrombotic tendency of the blood, as measured by platelet adhesiveness.

Obesity is yet another factor associated both with increased liability to atherosclerotic disease (Keys, 1953; Dawber et al., 1962), and with elevated plasma lipid levels (Waxler and Craig, 1964; Montoye et al., 1966). In the last few years, total starvation has been used to achieve weight reduction, in an effort to alleviate some of the complications of obesity. It has been suggested, however, that rapid weight loss may cause an increased liability to thrombotic complications (Beckett and Lewis, 1960). A study has been undertaken of the lipid changes induced in obese patients by total fasting, and any simultaneous alterations in thrombotic tendency, as measured by platelet adhesiveness, have also been investigated.

The objectives of this study, therefore, are four-fold:-

to compare the plasma lipids before and after fat-loading in atherosclerotic and healthy subjects; to examine several gastro-intestinal factors that may alter the response of these subjects to fat-loading; to investigate acute plasma lipid changes in cigarette-smokers, a group predisposed to atherosclerotic disease; and to follow plasma lipid fluctuations during starvation in the obese, another group with an increased liability to atherosclerotic disease.

Chapter II. METHODS FOR THE ANALYSIS OF PLASMA LIPIDS

CONTENTS

Chapter II. METHODS FOR THE ANALYSIS OF PLASMA LIPIDS

This chapter describes the procedures and chemical methods used throughout this study for the determination of lipids in plasma or serum. Other methods employed only in certain sections of the work are dealt with in the appropriate section.

COLLECTION OF BLOOD SAMPLES

All subjects were admitted to a general medical ward, and fasting blood samples were taken after an overnight fast of approximately twelve hours. Venous blood samples were withdrawn from an antecubital vein using a standard no. 1 needle and a plastic syringe. Care was taken to avoid prolonged venous occlusion, which may result in increased concentrations of the non-filterable blood constituents (Koerselman et al., 1961) or fist-clenching, which may alter non-esterified fatty acid and glucose levels (Rabinowitz and Zierler, 1962). Because of the effect of changes in posture on the concentration of the non-filterable blood constituents (Stoker and Wynn, 1966), subjects remained in bed until the fasting blood sample had been taken, and assumed the recumbent position for at least 30 minutes before any further venepunctures.

PROCESSING OF BLOOD SAMPLES

Plasma for lipid estimations was obtained from heparinised blood (50 units heparin* per ml.) which immediately after collection had been centrifuged for 30 minutes at 1,500 r.p.m. Blood samples for serum were placed for one hour in a heating block at 37°C to allow clot retraction before centrifugation and separation of the serum. Plasma and serum samples for estimation of total or individual non-esterified fatty acids (NEFA) were extracted not later than two hours after venepuncture, since beyond this time the NEFA level tends to rise as a result of triglyceride hydrolysis. The extracts might then be stored in a refrigerator for 24 to 48 hours without alteration in the NEFA concentration (Forbes and Gamlin, 1959). Plasma and serum samples for the other lipid estimations were stored at -15 to -20°C in a deep freeze until analysed.

ESTIMATION OF TOTAL CHOLESTEROL

The quantitative determination of total cholesterol in plasma or serum was performed by the Technicon autoanalyzer

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*Heparin injection B.P. 5,000 units per ml.

method N-24P, with several minor modifications. This method, which is based on that described by Zak et al. (1954), depends on the reaction of ferric chloride in acetic acid and concentrated sulphuric acid with steroids having the 5-ene,3β-ol grouping.

The modifications of method N-24P are as follows:-

(a) A modified aspirator head consisting of a double sampling crook was designed so that after aspiration of a plasma sample a wash of isopropyl alcohol (90%) was sucked up and carried through the system. This gave a steadier baseline.

(b) An extra acidflex tube was incorporated into the manifold to increase the number of acid-carrying tubes from three to four. This allowed a larger volume of acid to react with the sample and resulted in faster colour development.

(c) Isopropyl alcohol (90%) was used in the preparation of standards to approximate more closely to the concentration of isopropyl alcohol in the extracted plasma sample.

A description of the materials and procedure used is given below.

MATERIALS

Isopropanol: Analar (B.D.H.)

Glacial acetic acid: Analar (B.D.H.)

Sulphuric acid (concentrated): Analar (B.D.H.)

Ferric chloride - hydrated ($FeCl_3.6H_2O$): Analar (B.D.H.)

Standard serum: Hyland Labs., Los Angeles, U.S.A.

Cholesterol (B.D.H.)

METHOD

(a) Preparation of Cholesterol Colour Reagent

Ferric chloride (1 g.) was dissolved in 100 ml. distilled water, and glacial acetic acid added to make the solution up to 2 litres. Sulphuric acid (860 ml.) was then added and mixed. The reagent was stored in an amber-coloured bottle in the dark, and was stable for approximately one month.

(b) Determination of Cholesterol

Isopropanol (9.5 ml.) was added to 0.5 ml. of plasma or serum, mixed, and centrifuged. The supernatant was then analysed for total cholesterol. The standard serum was extracted in a similar manner. A flow diagram of the method is shown in Fig. 4. The optical density of the colour produced by the cholesterol and the ferric chloride colour reagent was measured at 520 mμ in a flow cuvette, and a double wash of 90% isopropanol in water was carried out after every sample to prevent cross-contamination. A standard serum sample and a series of cholesterol standards (100 to 500 mg. per 100 ml.)

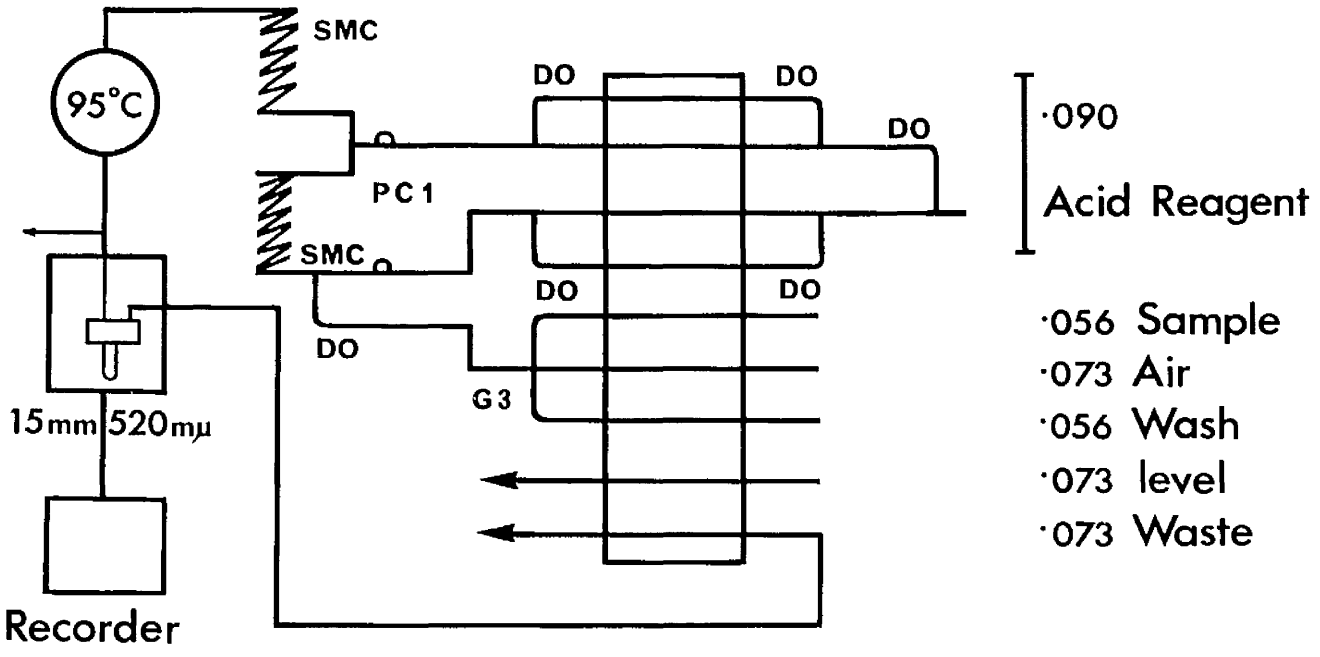

Fig. 4. Flow diagram of method for cholesterol estimation.

in 90% isopropanol were run with each batch of 12 samples.

(c) Calculation

The optical density of the colour reaction was recorded as a tracing on paper calibrated in optical density units. When the peak heights of the standards were plotted against concentration a linear relationship was observed (Fig. 5). The peak heights of the test samples were then converted to concentrations using this graph. The standard serum was used as a correction factor A

$$\left(A = \frac{\text{known concentration of standard serum}}{\text{measured concentration of standard serum}}\right).$$

This factor, when multiplied by the concentrations of the test samples, eliminated errors due to factors such as slight variations in temperature during a day's run.

A comparison of this method with that of Abell et al. (1952) on 388 duplicate serum samples showed a correlation coefficient of 0.80. The regression equation was

$$Y = 0.6861X + 85.33,$$

where Y is the cholesterol value in mg. per 100 ml. obtained by the autoanalyzer method, and X is the corresponding value by the method of Abell et al.

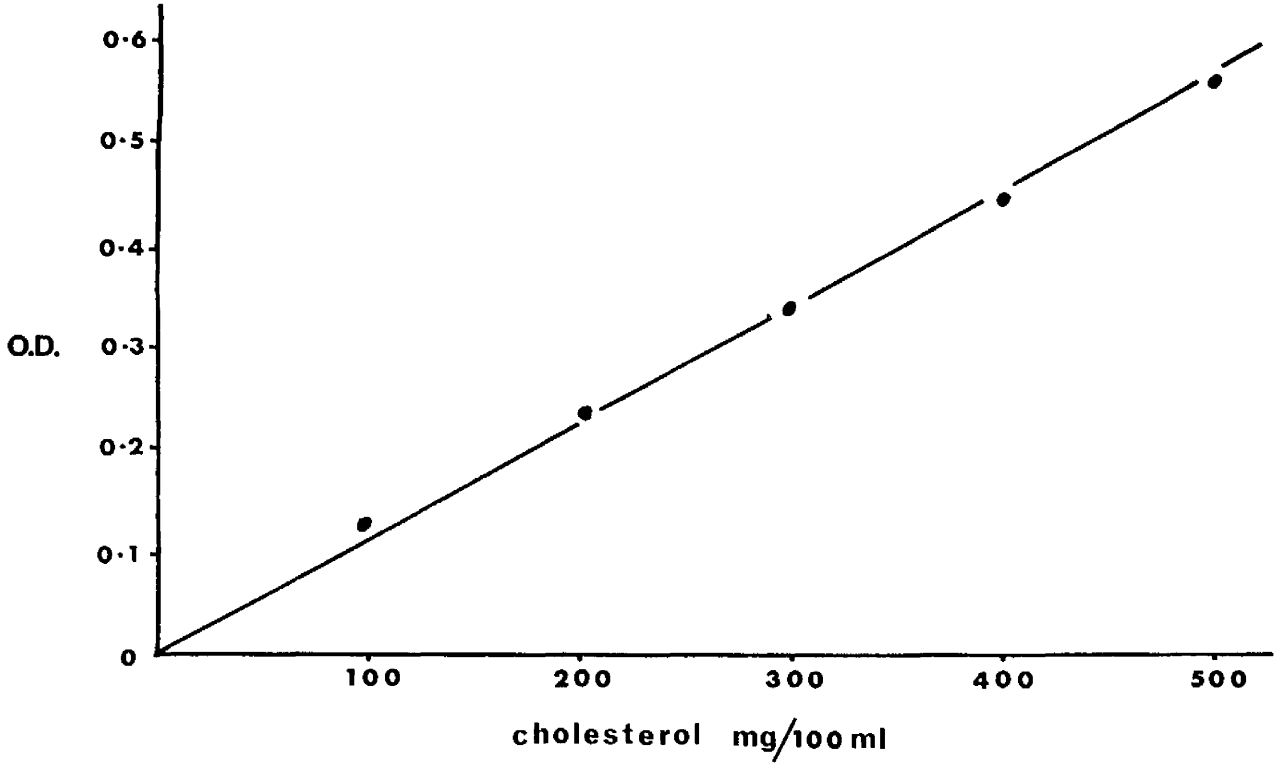

Fig. 5. Cholesterol standard graph.

ESTIMATION OF TRIGLYCERIDE

The quantitative determination of plasma or serum triglyceride was carried out by the method of Van Handel and Zilversmit (1957). This micromethod is based on the measurement of the glycerol component of the glyceride molecule.

The procedure consists essentially of four steps:-

(1) the quantitative extraction of plasma lipids and separation of triglyceride from other glycerol-containing lipids; (2) the complete saponification of triglyceride; (3) the oxidation of glycerol to formic acid and formaldehyde; and (4) the colorimetric estimation of formaldehyde. The extraction procedure used differed from that of Van Handel and Zilversmit, and the method is described below.

MATERIALS

Ethyl alcohol: redistilled.

Bloor's solution: (3:1 ethanol:diethyl ether)

Sulphuric acid: Analar (B.D.H.)

Silicic acid: Mallinckrodt, 72-350 mesh.

Sodium periodate: Analar (B.D.H.)

Sodium arsenite: Analar (B.D.H.)

Chromatropic acid (4,5-dihydroxy-2,7-napthalene-disulphonic acid disodium salt)*.

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*Eastman Organic Chemicals, Rochester, 3, New York.

Triolein: (B.D.H.)

METHOD

(a) Preparation of Chromatropic Acid Reagent

Chromatropic acid (1 g.) was dissolved in 100 ml. water. Immediately before use 9 parts of sulphuric acid (66%) were mixed with 2 parts of chromatropic acid solution to form the chromatropic acid reagent.

(b) Extraction and Separation of Triglyceride

3 ml. of plasma were extracted with Bloor's solution. The precipitated protein was removed by filtration and the filtrate made up to 50 ml. in a volumetric flask. Duplicate 2 ml. aliquots were evaporated to dryness in Quickfit test tubes (20 ml. capacity) and approximately 200 mg. silicic acid added. 5 ml. of chloroform was then added to each tube which was then shaken and centrifuged. The chloroform layer was carefully drawn off without disturbing the silicic acid. The chloroform layer contained the triglyceride, while the phospholipids were retained on the silicic acid.

(c) Determination of Triglyceride

Duplicate 3 ml. aliquots of the chloroform solution were evaporated to dryness in 20 ml. Quickfit test tubes. Ethyl alcohol (1 ml.) and one drop of 5.0% potassium hydroxide

in ethyl alcohol were added, and the tubes heated at 60°C for 30 minutes to ensure complete saponification of the triglyceride. The solutions were then acidified by addition of 4 drops of 6.0% acetic acid in methanol before being taken to dryness in a boiling water bath. 1 ml. of 0.67 M sulphuric acid was then added, and the tubes were gently shaken.

Sodium periodate 0.02 M (0.3 ml.) was added to each tube which was then shaken and allowed to stand for 10 minutes before the addition of 0.3 ml. of 0.2M sodium arsenite. This neutralised any excess of periodate present. At this stage a transient yellow colour appeared which was due to the liberation of iodine. The chromatropic acid reagent was not added until this colour had faded.

Addition of 8.4 ml. of chromatropic acid reagent to each tube was carried out in a shaded room, since the reagent is light-sensitive. The tubes were then stoppered, shaken, and placed in a boiling water bath for 30 minutes. The tubes were then allowed to cool and the optical densities of the solutions were read at 570 mμ in a spectrophotometer (Unicam S.P. 600) in 1 cm. cuvettes, using the blanks as zero. Duplicate triolein standards (25 μg to 100 μg) and chloroform blanks (2 ml.) were treated in the same manner as the plasma extracts.

(d) Calculation

A straight-line graph was obtained by plotting the optical density against the concentration of the standards (Fig. 6), and plasma triglyceride concentrations were calculated from the following formula.

Plasma triglyceride concentration (mg. per 100 ml.) =

$$\text{mg. triolein (from graph)} \times \frac{5}{3} \times \frac{50}{X} \times \frac{100}{Y}$$

where $\frac{50}{X}$ represents $\frac{\text{vol. of Bloor's extract}}{\text{vol. of aliquot taken}}$

$\frac{100}{Y}$ represents $\frac{100}{\text{vol. of plasma extracted}}$

and $\frac{5}{3}$ represents the 3 ml. aliquot taken of the 5 ml. chloroform added.

ESTIMATION OF PHOSPHOLIPID

Plasma total phospholipid was estimated by Bartlett's (1959) modification of the method of Fiske and Subbarow (1925), which measures lipid phosphorus. The following steps are involved:- (1) the extraction of the plasma lipids; (2) the destruction of organic materials by wet digestion; and (3) the colorimetric determination of phosphorus in the residue. The colorimetric estimation is based on the observation that certain agents reduce the molybdenum of phosphomolybdic acid

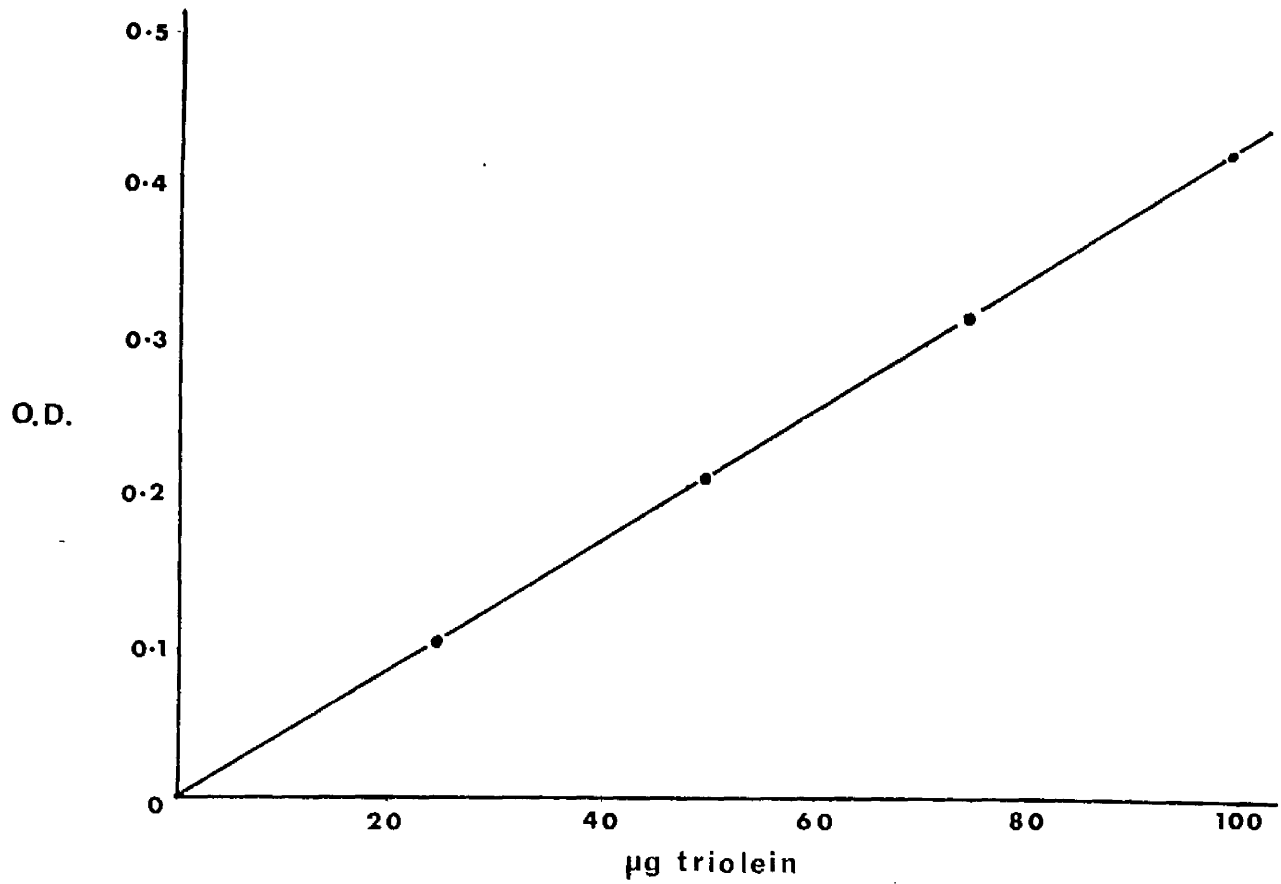

Fig. 6. Triglyceride standard graph.

but have only a negligible effect on the molybdenum of uncombined molybdic acid in the same solution. A description of the method is given below.

MATERIALS

Potassium dihydrogen phosphate: Analar (B.D.H.)

Ammonium molybdate: Analar (B.D.H.)

Sodium metabisulphite: Analar (B.D.H.)

Sodium sulphite (anhydrous): Analar (B.D.H.)

1-amino-2-naphthol-4-sulphonic acid: (B.D.H.)

Sulphuric acid: Analar (B.D.H.)

Hydrogen peroxide (100 vols.) (phosphorus-free): Analar (B.D.H.)

Bloor's solution (3:1 ethanol:diethyl ether)

METHOD

(a) Preparation of Fiske-Subbarow Reagent

1-amino-2-naphthol-4-sulphonic acid (0.5 g.) was dissolved in 195 ml. of 15% sodium bisulphite, and 5 ml. of 20% sodium sulphite was added. The filtered solution was stored in a dark bottle and was stable for two weeks.

(b) Measurement of Phospholipid

Plasma (3 ml.) was extracted into 50 ml. Bloor's solution, as described for the triglyceride estimation. Duplicate 2 ml.

aliquots of the extract were evaporated to dryness and 0.5 ml. of 10 N sulphuric acid added to each tube. The tubes were then placed in a heating-block at 170-180°C for 1½ hours, after which time 0.2 ml. of hydrogen peroxide was added while the tubes were still hot. The tubes were left in the heating-block for a further 1½ hours, in order to ensure complete combustion and to decompose any residual hydrogen peroxide. They were then removed from the heating block and 4.4 ml. of distilled water was added immediately, followed by 0.2 ml. of 5% ammonium molybdate. Fiske-Subbarow reagent (0.2 ml.) was then added, and the contents of the tubes mixed before being placed in a boiling water bath for 7 minutes. Each sample was then diluted to 50 ml. with distilled water and the optical density read in a spectrophotometer (Unicam S.P. 600) at 830 mμ using a red filter. Phosphorus standards (potassium dihydrogen phosphate solution of concentration 5 μg. phosphorus per ml.; standards 5 to 20 μg. phosphorus) and sulphuric acid blanks were carried throughout the procedure.

(c) Calculation

By plotting the optical density of the phosphorus standards against concentration, a linear relationship was obtained (Fig. 7) from which the phosphorus content of the plasma samples were derived. The plasma phospholipid concentration was then

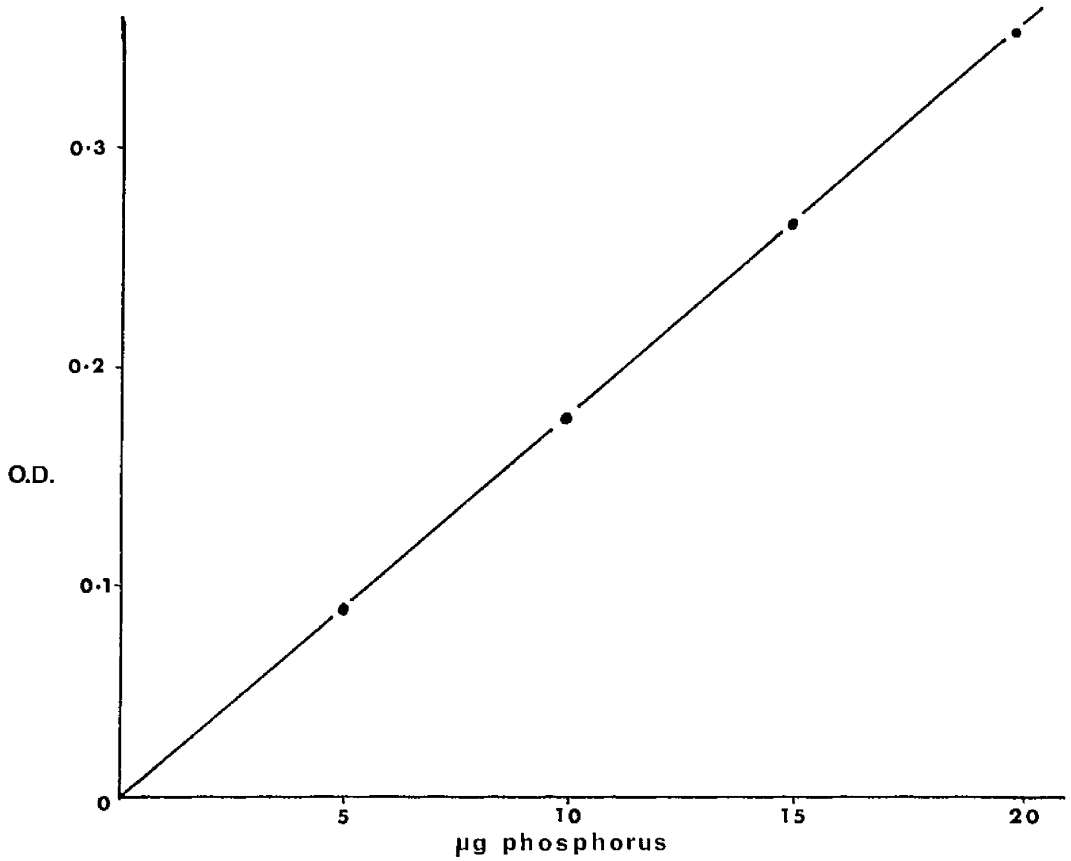

Fig. 7. Phospholipid standard graph.

calculated as mg. lecithin per 100 ml. from the following formula:-

Plasma phospholipid (mg. per 100 ml.) =

$$X \times \frac{50}{2} \times \frac{1}{3} \times \frac{100}{1000} \times 25$$

where X is the phosphorus content of the samples expressed as µg. per ml., and 25 is the factor for conversion of inorganic phosphorus to phospholipid.

PLASMA NON-ESTERIFIED FATTY ACIDS (NEFA)

Total plasma NEFA concentration was measured by the method of Dole and Meinertz (1960). The principles of this procedure are as follows:- plasma lipids are extracted into a one-phase ternary mixture (heptane-isopropyl alcohol-sulphuric acid) which, after the addition of water and heptane, separates into a two-phase system; the upper non-polar phase contains the long-chain fatty acids (NEFA), while other more polar acids remain in the lower phase; the NEFA in an aliquot of the upper phase are then titrated in a second two-phase system. Details of this method are given below.

MATERIALS

n-Heptane: redistilled (May and Baker)

Isopropyl alcohol: redistilled (B.D.H.)

Ethyl alcohol: redistilled.

Nile blue: (B.D.H.)

Sodium hydroxide: Analar (B.D.H.)

Sulphuric acid: Analar (B.D.H.)

Palmitic acid: recrystallised (B.D.H.)

Nitrogen gas

Carbon dioxide-free water

METHOD

(1) Single Extraction Procedure

(a) Preparation of Nile Blue Indicator

Nile blue (0.02 g.) was dissolved in 100 ml. of ethyl alcohol, washed 6 times with hexane, and diluted 1 in 10 with ethyl alcohol.

(b) Extraction

One tube containing 10 ml. extraction mixture, isopropyl alcohol:n-heptane:1 N sulphuric acid (40:10:1), was set up for each sample and also for 3 standards and one blank. Plasma (2 ml.) was pipetted into each sample tube, and carbon dioxide-

free water (2 ml.) into the tubes for standards and blanks. The contents of the tubes were mixed and allowed to stand for 5 minutes. Carbon dioxide-free water (4 ml.) in n-heptane (6 ml.) were added to the sample and blank tubes, the contents mixed, and allowed to separate into two layers. To each of the three standard tubes were added 4 ml. of carbon dioxide-free water and 6 ml. of standard palmitic acid solutions in n-heptane, of concentrations 2.5 mg., 5.0 mg., and 7.5 mg. per 100 ml. respectively.

(c) Titration

An aliquot (3 ml.) from the heptane layer was pipetted into a conical centrifuge tube together with 1 ml. of Nile blue indicator. Dry, carbon dioxide-free nitrogen (passed through dilute alkali and a calcium chloride tower for 30 minutes before use) was then gently bubbled through the mixture while titration of the NEFA was carried out using 0.018 N sodium hydroxide in a Conway burette. The end-point was reached when the infranatent turned pink. Standards and blanks were titrated in the same manner.

(d) Calculation

By plotting the titre against the concentration of the standards a straight line graph was obtained from which the

NEFA concentration of the plasma samples was calculated (Fig. 8). Plasma NEFA (expressed as mg. of palmitic acid per 100 ml. plasma)

$$= X \times \frac{8.6}{3} \times \frac{100}{2} \times \frac{3}{1}$$

where X is the reading obtained from the graph, 8.6 is the volume in ml. of heptane supernatent obtained, and 3:1 is the ratio of volumes of standard and plasma samples titrated.
Plasma NEFA (μeq. per l.)

$$= X \times \frac{8.6}{3} \times \frac{100}{2} \times \frac{3}{1} \times 39.00$$

(2) Double Extraction Procedure

This method was used in the starvation studies described in Chapter VII, in order to eliminate interference by increased plasma concentrations of acetoacetic acid and β-hydroxybutyric acid.

Plasma (4 ml.) was extracted with double the quantity of reagents used in the single extraction procedure, and a 3 ml. aliquot of upper phase was taken for titration of single extraction acidity. A further aliquot (8 ml.) of upper phase was transferred to another extraction tube. After addition of 14 ml. of blank lower phase (the lower phase taken from a blank extraction), the contents of the tube were mixed by inverting about 25 times. The two phases were allowed to separate, and

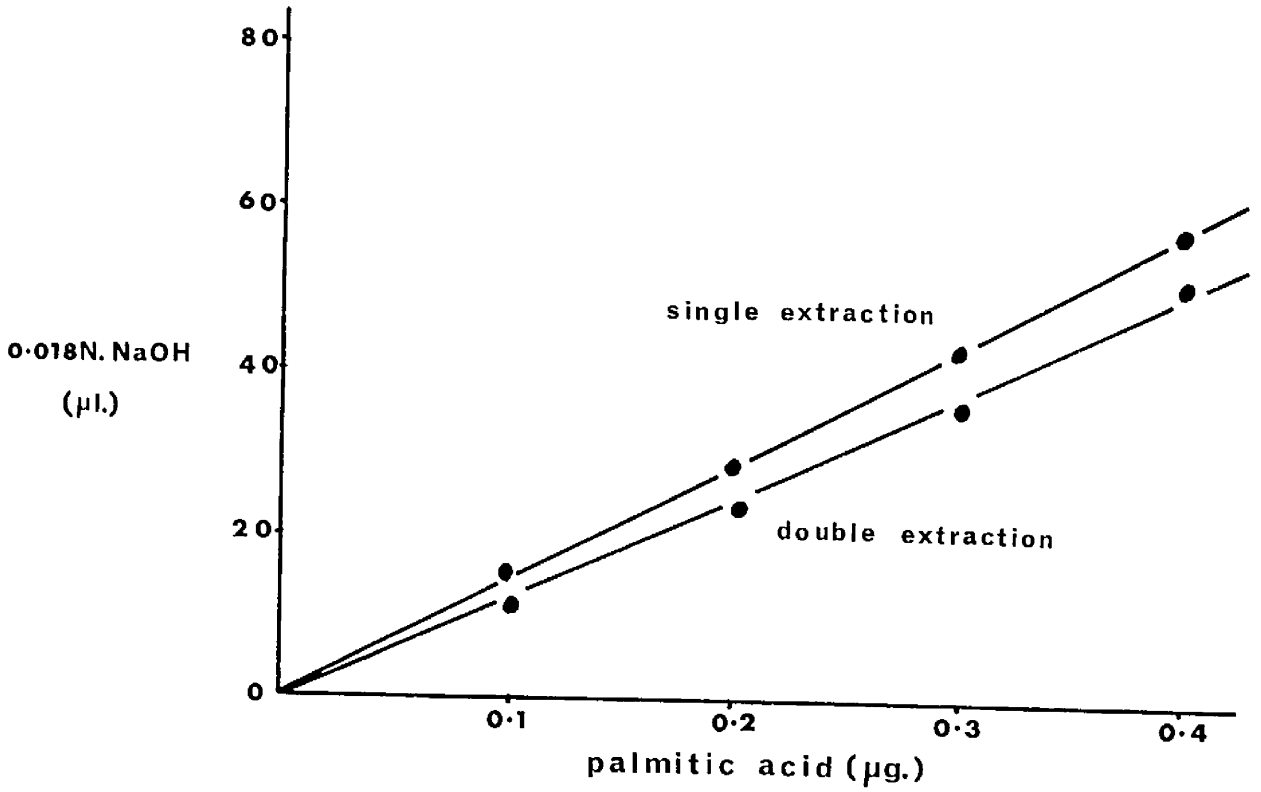

Fig. 8. NEFA standard graph.

a 3 ml. aliquot of upper phase was taken for titration. Palmitic acid standards were treated similarly, and a straight-line graph was obtained for the double extraction procedure (Fig. 8). The plasma NEFA concentrations given by single and double extraction methods were then calculated.

INDIVIDUAL NON-ESTERIFIED FATTY ACIDS

The proportions of the individual non-esterified fatty acids present in serum were measured by a combination of thin layer chromatography and gas liquid chromatography. Details of the procedure are given below.

METHOD

(a) Extraction of Serum Lipids

This was carried out within two hours of venepuncture. Serum (3 ml.) was pipetted into a tube containing approximately 20 ml. of Bloor's solution and the tube placed in a boiling water bath for 2 minutes. The precipitated protein was filtered off, and washed twice with fresh Bloor's solution, and the combined extracts evaporated to dryness with a stream of nitrogen. The residue was dissolved in the minimum volume of diethyl ether and then subjected to thin layer chromatography.

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

A glass plate (20 cm. x 20 cm.) was coated with a layer (0.25 mm. thick) of Adsorbosil impregnated with Rhodamine B. The plate, following application of the lipid extract and standards, was developed in hexane/diethyl ether/methanol/ acetic acid (90:20:3:2 v/v) (Brown and Johnson, 1962) and the separated lipids visualised under ultraviolet light (3650 Å). The band with the same Rf as the fatty acid standard (Fig. 9) was accurately outlined and scraped from the plate into a test-tube. The fatty acids were eluted from the silica gel with petroleum ether (40-60°).

(c) Gas Liquid Chromatography (G.L.C.)

The fatty acids obtained from the T.L.C. plate were methylated, using in an early section of the study (Chapter VI) the methanol:sulphuric acid method (Morrison and Smith, 1964). In later phases of the study (Chapter VII) the more rapid boron trifluoride: methanol method was employed (Metcalfe and Schmitz, 1961).

The fatty acid methyl esters were then separated by gas liquid chromatography on a U-shaped glass column (length 5 ft., internal diameter 4 mm.), packed with 15% polyethylene glycol adipate (P.E.G.A.) on acid-washed, siliconised Gas Chrom P

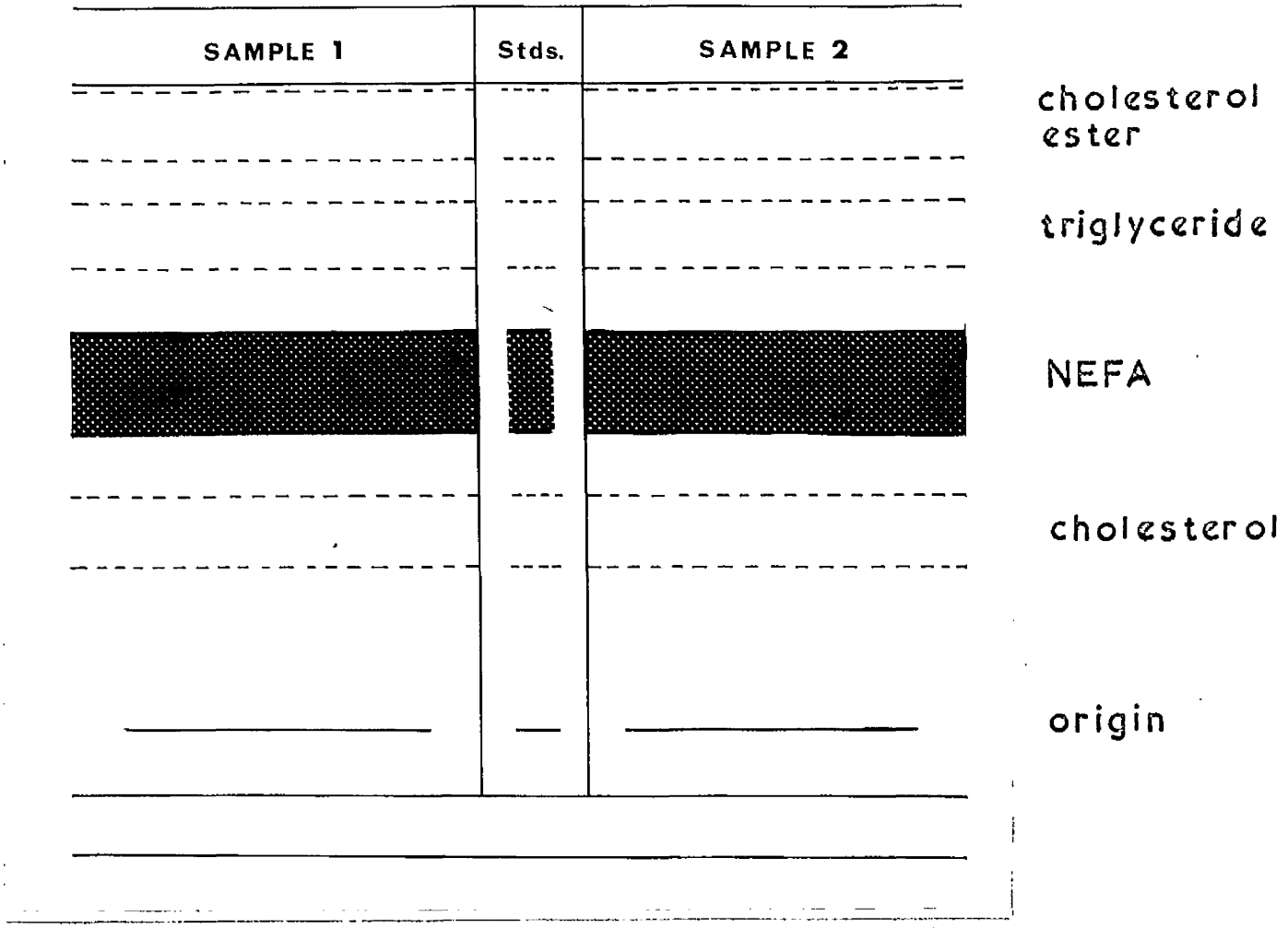

Fig. 9. Diagram of thin layer chromatogram showing separation of NEFA band.

(100-120 mesh). The column temperature was 180°C and the argon flow-rate 30 ml. per minute. Under these conditions fatty acids of chain length C12 to C18 were well separated from each other (Fig. 10).

Calculation

The areas of the fatty acid peaks obtained were calculated by multiplication of the peak height by the width at half the height (Horning, 1964). The proportions of the individual fatty acids were expressed as percentages of the whole.

STATISTICAL ANALYSIS

Differences in mean values between two groups were assessed by the Student 't' test, and the significance of changes from a baseline level by the Student 't' test for paired values. Correlations were performed by linear regression analysis, except in the case of cholesterol, phospholipid, and triglyceride values in male controls (Chapter III) where second order polynomial equations were used. In the latter case, additional lipid values for the eight male subjects described on page 161 were included, in order to extend the age range. Triglyceride, total plasma NEFA, and skinfold thickness values were found to have a skew population distribution, and logarithmic

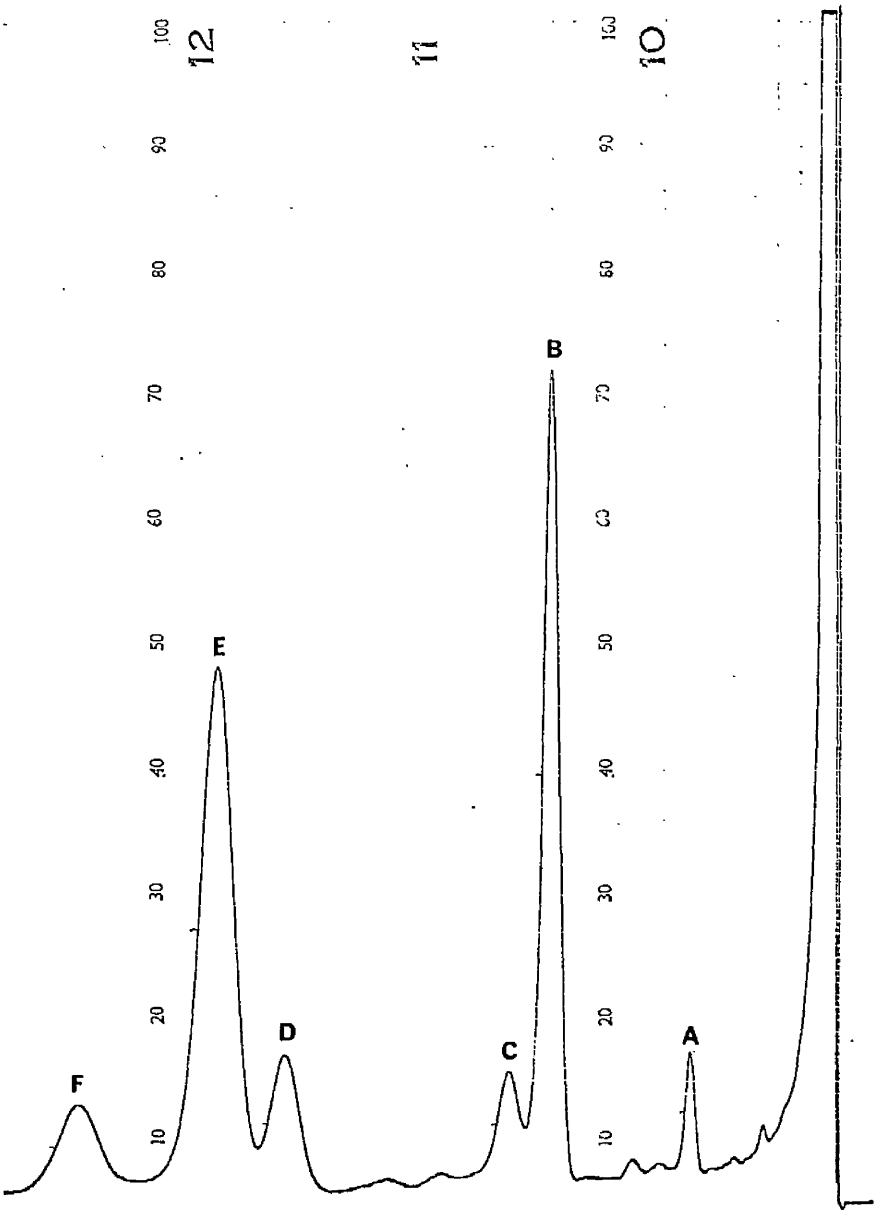

Fig. 10. G.L.C. chart showing separation of individual NEFA (A-myristic acid, B-palmitic acid, C-palmitoleic acid, D-stearic acid, E-oleic acid, F-linoleic acid).

transformation of these values was therefore carried out prior to statistical analysis.

Chapter III. FASTING PLASMA LIPIDS IN HEALTHY AND ATHEROSCLEROTIC SUBJECTS

CONTENTS

Chapter III. FASTING PLASMA LIPIDS

INTRODUCTION

Reports of plasma lipid levels in normal subjects in Great Britain are few in number and, in particular, the literature on plasma triglyceride and NEFA concentrations is scanty. More information is available on plasma lipids in atherosclerotic subjects. In this Chapter the results of fasting plasma lipid estimations in healthy controls and in atherosclerotic subjects are presented. These were, in fact, the basal blood samples taken as a preliminary to the fat-loading test described in Chapter IV.

The influence of several factors which might affect plasma lipid levels is considered - namely age, sex, weight, habitual cigarette-smoking, and a family history of ischaemic heart disease. A comparison is then made between plasma lipids in healthy subjects and patients with atherosclerotic arterial disease.

SUBJECTS

The subjects studied were divided into two main groups:- (a) controls, and (b) arterial disease patients. The clinical

details of these subjects are presented in Table II.

(a) Control Subjects

There were 72 control subjects, of whom 50 were males, with a mean age of 44.3 ± 11.2 years (mean ± standard deviation), and 22 were females, whose mean age was 44.6 ± 13.7 years. The age distribution of these subjects is shown in Fig. 11.

Selection of Control Subjects

The controls were healthy subjects who required minor surgical operations for conditions such as varicose veins and hernias, and who were invited to volunteer as subjects for this study. Those with clinical or electrocardiographic evidence of atherosclerosis were eliminated. The controls were admitted to a medical ward for the purpose of this investigation for 24 to 48 hours prior to operation.

(b) Arterial Disease Subjects

These were 86 subjects with clinical atherosclerotic disease. They consisted of 72 males with a mean age of 53.0 ± 7.9 years, and 14 females of mean age 59.4 ± 6.2 years. The age distribution of these subjects is shown in Fig. 11. Of the 72 male arterial disease subjects, 27 had sustained

TABLE II: Clinical Details of Subjects (mean ± S.D.)

Subjects	Height (cm.)	Relative[/] Weight %	Family History of I.H.D.[*] %	Cigarett Smokers %
Controls - male all ages	170.7 ± 8.4 (n = 34)	111.9 ± 12.9 (n = 34)	11 (n = 36)	57 (n = 47)
Controls - male aged 40 and over	169.9 ± 9.1 (n = 21)	116.0 ± 14.1 (n = 21)	12 (n = 26)	52 (n = 31)
Arterial Disease male	168.7 ± 6.6 (n = 61)	117.9 ± 12.2 (n = 60)	50 (n = 66)	85 (n = 68)
Myocardial Infarct male	169.7 ± 5.8 (n = 24)	116.0 ± 8.3 (n = 24)	58 (n = 26)	84 (n = 25)
Angina Pectoris male	168.1 ± 5.6 (n = 16)	125.9 ± 12.6 (n = 16)	37 (n = 19)	71 (n = 17)
Peripheral Art. Disease - male	168.1 ± 8.1 (n = 21)	113.8 ± 13.3 (n = 20)	52 (n = 21)	96 (n = 26)
Controls - female all ages	161.5 ± 4.3 (n = 11)	120.4 ± 27.4 (n = 11)	25 (n = 20)	36 (n = 22)
Controls - female aged 50 and over	160.0 ± 4.6 (n = 4)	118.0 ± 23.4 (n = 4)	25 (n = 8)	56 (n = 9)
Arterial Disease female	156.2 ± 4.8 (n = 11)	114.3 ± 12.2 (n = 11)	29 (n = 14)	71 (n = 14)

[/] Actual weight as percentage of standard weight (Kemsley et al., 1962)

[*] I.H.D. = ischaemic heart disease

n = no. of observations

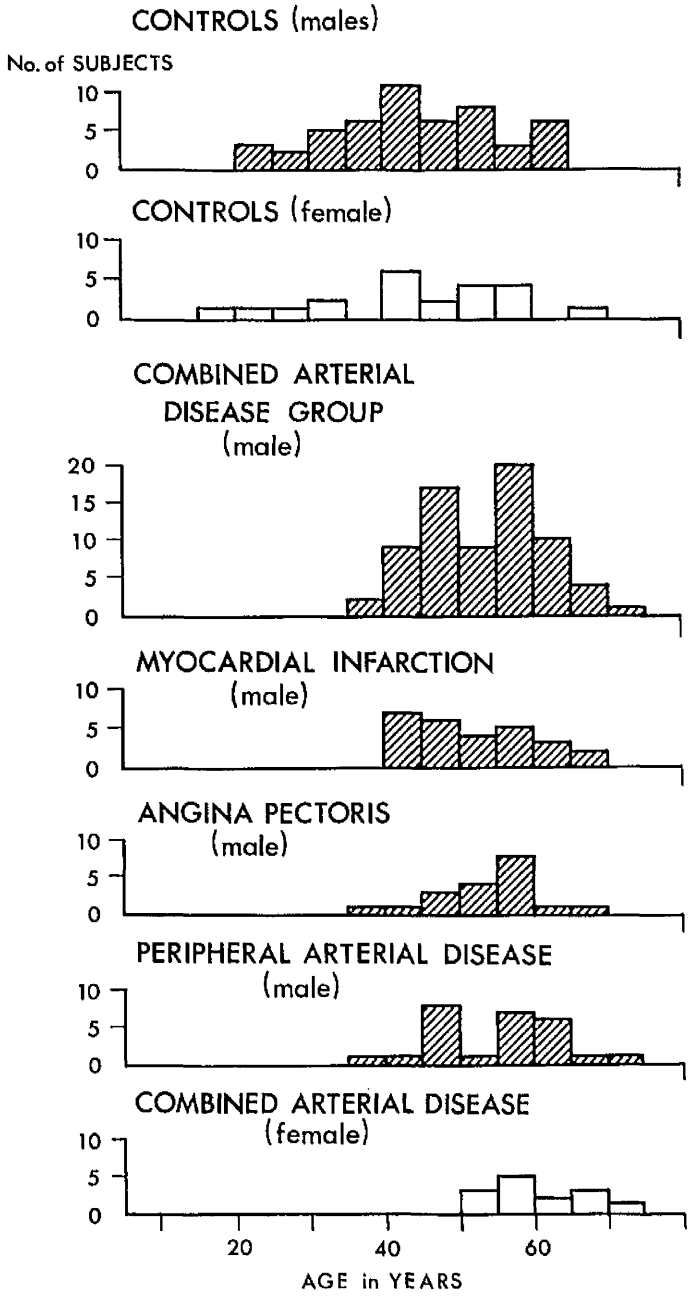

Fig. 11. Age distribution of subjects.

a myocardial infarction, 19 had angina pectoris, and 26 had peripheral arterial disease. Further particulars of these three groups are given below. In the female group there were 7 with myocardial infarction, 2 with angina pectoris, and 5 with peripheral arterial disease. Because of their small numbers, the results of the female arterial disease group are considered as a whole.

Selection of Patients with Myocardial Infarction

These were selected from patients attending a medical outpatient clinic who had sustained a myocardial infarction, confirmed by electrocardiography and/or serum glutamic-oxaloacetic transaminase estimations, at least 6 months previously. All were taking a normal, unrestricted diet. Twelve patients were receiving longterm anticoagulant therapy - ten with phenindione and two with warfarin. Only one subject was taking thiazide diuretics. The 27 male subjects had a mean age of 51.4 ± 8.1 years. Of these 27 subjects, 7 also showed evidence of peripheral arterial disease, and one had both peripheral and cerebral atherosclerotic disease.

Selection of Patients with Angina Pectoris

This group consisted of subjects with a definite history of retrosternal pain induced by exertion, but with no history

of prolonged episodes of chest pain (longer than 30 minutes) or electrocardiographic signs of myocardial infarction. Of the 19 male and 2 female subjects, definite evidence of myocardial ischaemia was present in the resting electrocardiographic tracing in 12, and in a post-exercise tracing in 6 subjects. In two instances the post-exercise tracing showed suggestive, but not diagnostic changes, and one was completely normal. The 19 male subjects had a mean age of 53.3 ± 7.2 years. One had peripheral arterial disease in addition to ischaemic heart disease.

Selection of Patients with Peripheral Arterial Disease

These were subjects with moderate to severe symptoms of peripheral arterial disease who were admitted to hospital for arteriography and assessment for surgical treatment. None had symptoms or electrocardiographic signs of ischaemic heart disease, nor did they have gangrene or infections of the lower limbs. There were 26 male subjects with a mean age of 54.8 ± 8.1 years.

PROCEDURE

The subjects were admitted to the ward on the day prior to the test. A standard meal of known fat content (30 g. fat,

contained in a meal of two eggs, bread and butter, and tea) was given at 5 p.m. and a light supper (a milk drink and biscuits) at 9 p.m. Cigarette-smoking was not permitted from 10 p.m. until completion of the test on the following day. The subjects remained at rest in bed until the fasting blood sample was taken at 9 a.m.

Blood samples were taken for plasma lipid analyses (total cholesterol, triglyceride, phospholipid, and total NEFA), and processed as described in Chapter II.

RESULTS

It was found useful in analysis of the plasma lipid results to divide the subjects into four main groups - male controls, female controls, male arterial disease subjects, and female arterial disease subjects. In these four groups the effects on each of the plasma lipids of the following factors were considered:- age, sex, body weight, habitual cigarette-smoking, and the presence of a family history of ischaemic heart disease. Having taken into account the influence of these factors, a comparison was then made between the plasma lipid levels in healthy controls and arterial disease subjects and, in males, between the different clinical types of arterial disease. Because of the differing age structures of the control

and arterial disease groups (Fig. 11) the results were analysed also in male controls of 40 years and over, and in female controls of 50 years and over, for comparison with those of the arterial disease subjects.

FASTING PLASMA CHOLESTEROL

The mean plasma total cholesterol values for the different groups are given in Table III.

I. EFFECT OF AGE

Male Controls

The plasma cholesterol was found to be relatively low in young male subjects, and to increase with age to a maximum level about the age of 45 years. Thereafter it showed a tendency to decline progressively up to the age of 65. This parabolic relationship of age and plasma cholesterol level was statistically significant (Fig. 12).

Female Controls

The plasma cholesterol was low in the youngest subjects. There was a highly significant progressive increase with age up to the age of 70 (Fig. 13).

TABLE III. Fasting Plasma Cholesterol (mean ± S.D.)

Subjects	No. of observations	Fasting Plasma Cholesterol mg. per 100 ml.
Controls male - all ages	50	221.9 ± 39.1
Controls male - aged 40 and over	34	227.1 ± 38.5
Arterial Disease male	72	252.4 ± 46.2
Myocardial Infarction male	27	256.0 ± 50.8
Angina Pectoris male	19	255.6 ± 40.2
Peripheral Art. Disease male	26	246.2 ± 46.5
Controls female - all ages	22	214.0 ± 47.0
Controls female - aged 50 and over	9	239.4 ± 33.1
Arterial Disease female	14	261.8 ± 41.9

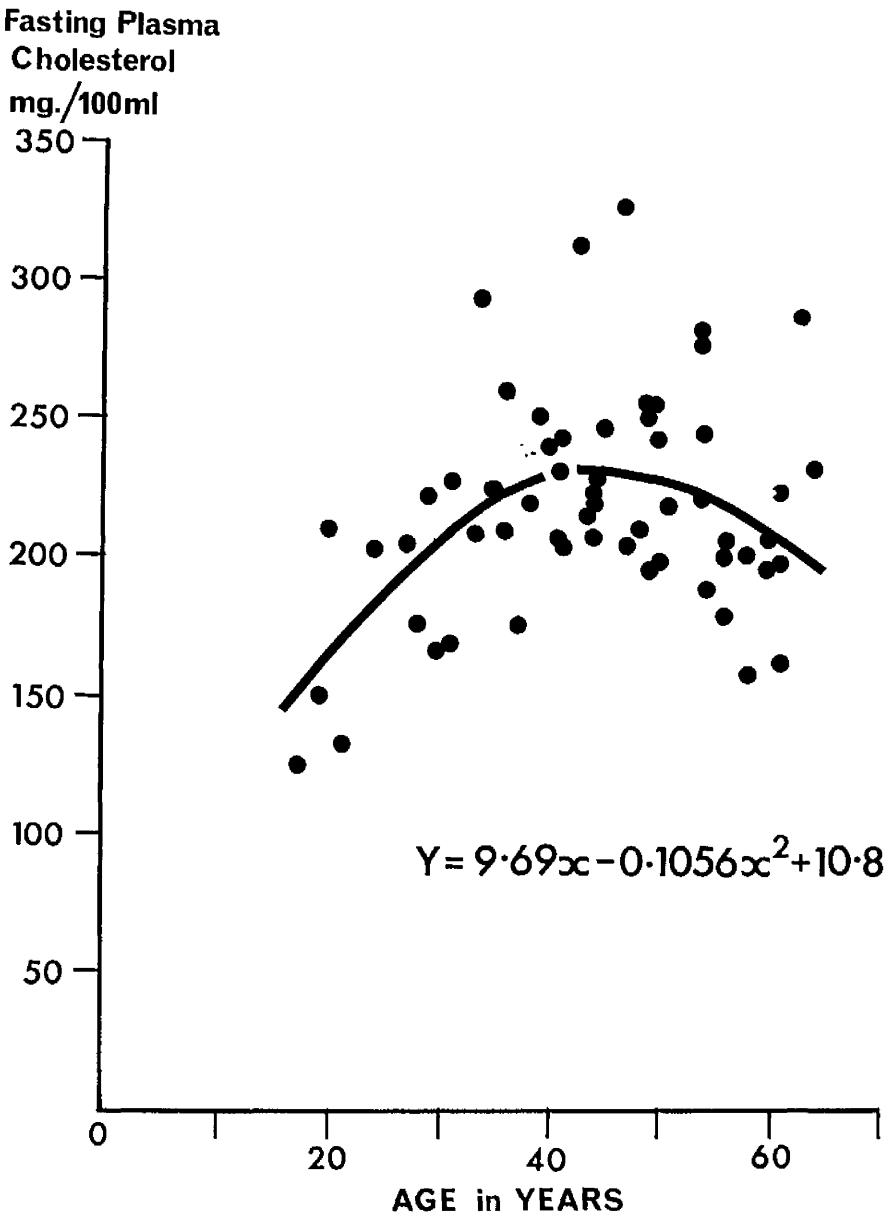

Fig. 12. Effect of age on plasma cholesterol concentration in male controls ($t=3.63$, $P<0.001$).

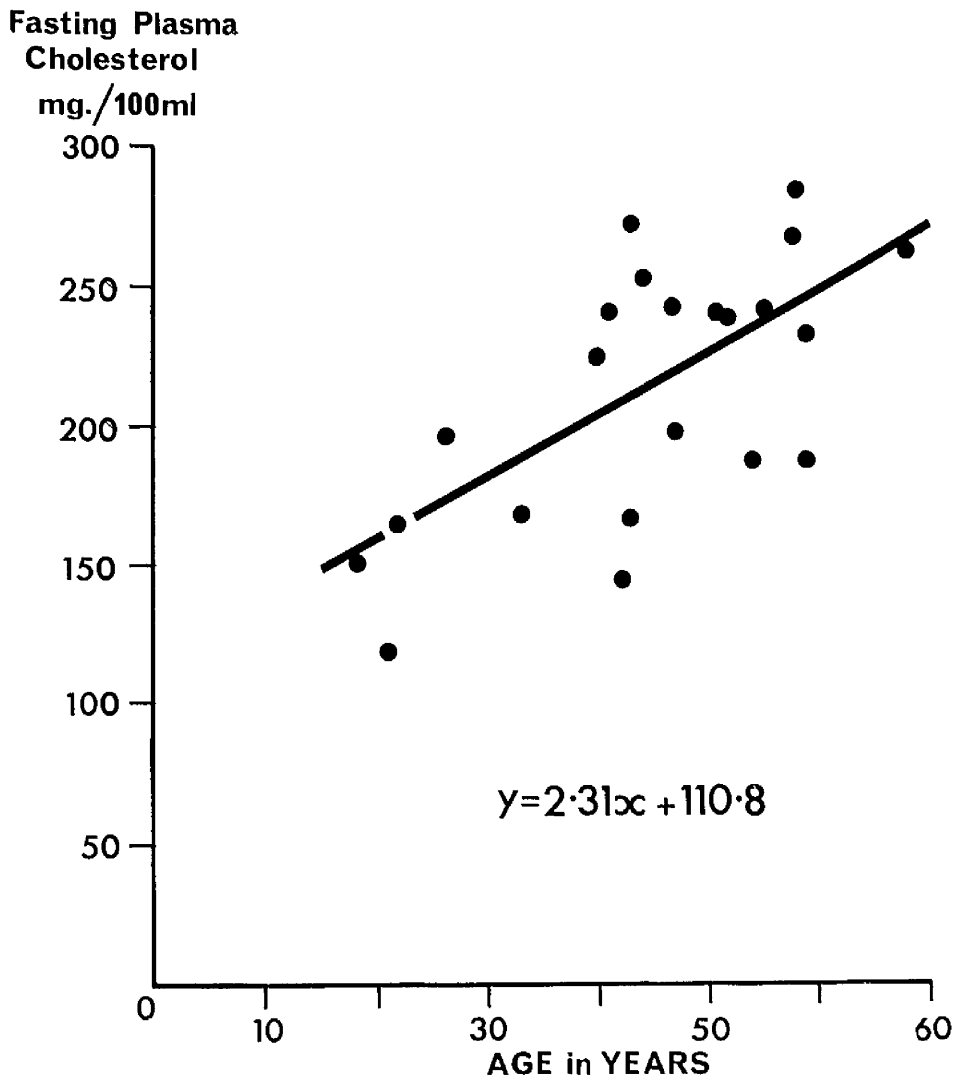

Fig. 13. Effect of age on plasma cholesterol concentration in female controls ($r = +0.67$, $P < 0.001$).

Male Arterial Disease Subjects

The plasma cholesterol was highest in the younger subjects, and decreased significantly with age (Fig. 14). This trend was more marked in the ischaemic heart disease subjects than in those with peripheral arterial disease, in whom it was not statistically significant (Table IV).

Female Arterial Disease Subjects

No significant effect of age was seen in these subjects, who were all aged 50 years and over (Table IV).

II. EFFECT OF SEX

Comparison of Male and Female Controls

Although the mean plasma cholesterol level for the whole group of male controls was higher than that of the female group (Table III) the difference was not statistically significant. On subdivision of the groups according to age, it was found that under the age of 40 years, the mean plasma cholesterol level of males (210.8 $\pm$ 39.3 mg. per 100 ml.) was significantly higher than that of females (160.4 $\pm$ 28.4 mg. per 100 ml., $P < 0.02$). Males continued to have a higher mean cholesterol level than females up to the age of 54 years. In the 55 to 64 year age group, however, the mean value for

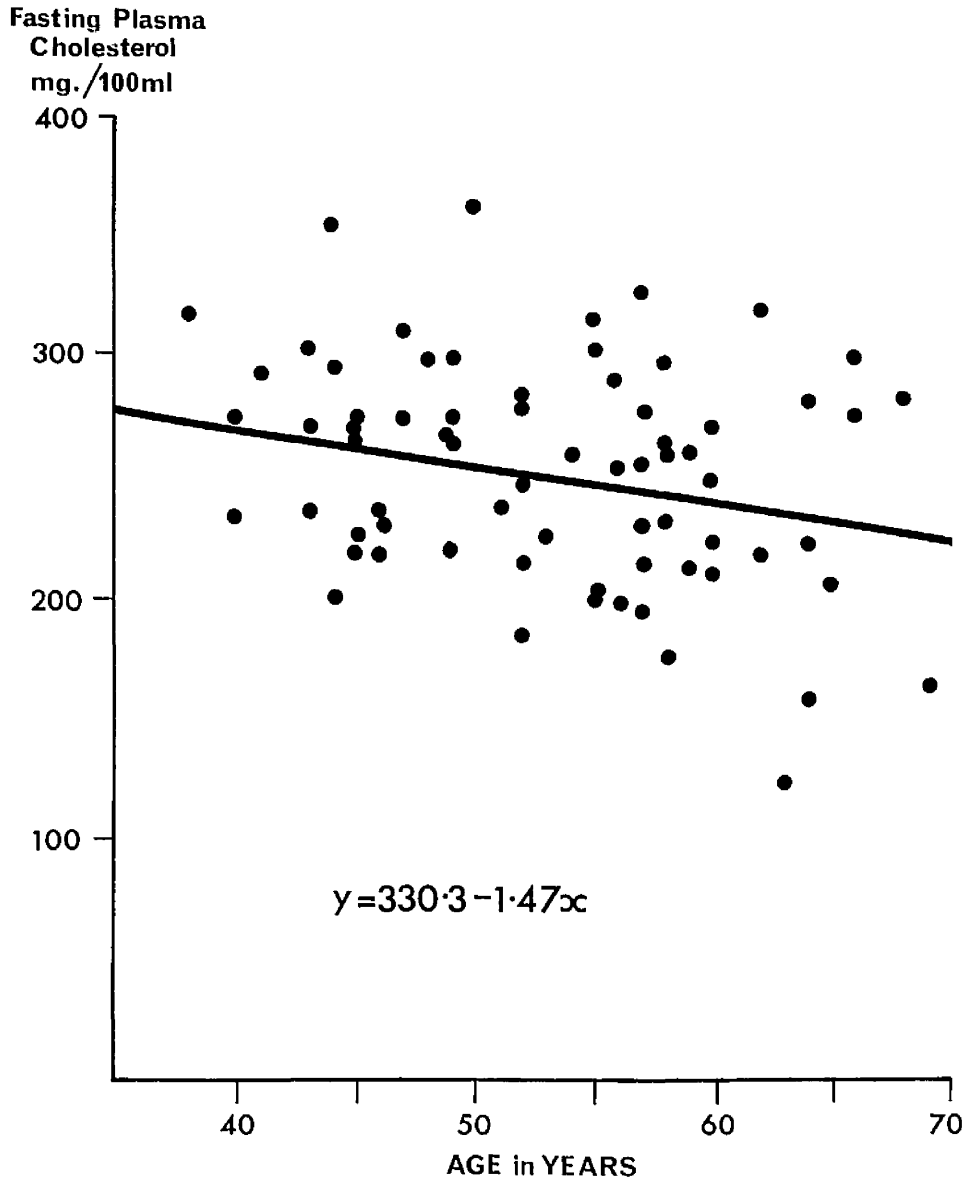

Fig. 14. Effect of age on plasma cholesterol concentration in male arterial disease patients ($r = -0.25$, $P<0.05$).

TABLE IV. Effect of Age on Fasting Plasma Lipids

(correlation coefficients)

Subjects	Cholesterol	Phospholipid	Triglyceride	NEFA
Controls - male	parabolic (n = 57)	parabolic (n = 28)	parabolic (n = 57)	-0.07 (n = 50)
Controls - female	+0.67*** (n = 22)	+0.16 (n = 16)	+0.20 (n = 22)	+0.28 (n = 22)
Arterial Disease - male	-0.25* (n = 72)	-0.05 (n = 50)	-0.31** (n = 72)	-0.11 (n = 72)
Myocardial Infarct - male	-0.24 (n = 27)	0 (n = 19)	-0.41* (n = 27)	-0.16 (n = 27)
Angina Pectoris - male	-0.41 (n = 19)	-0.50 (n = 12)	-0.49* (n = 19)	+0.14 (n = 19)
Peripheral Art. Disease - male	-0.15 (n = 26)	+0.10 (n = 19)	-0.20 (n = 26)	-0.30 (n = 26)
Arterial Disease female	+0.10 (n = 14)	+0.14 (n = 5)	+0.03 (n = 14)	+0.17 (n = 13)

* $P < 0.05$,

** $P < 0.01$,

*** $P < 0.001$

n = no. of observations

females (238.0 ± 39.3 mg. per 100 ml.) was higher than that for males of corresponding age (204.0 ± 40.0 mg. per 100 ml.), although the difference was not statistically significant.

Comparison of Male and Female Arterial Disease Subjects

Comparison of the plasma cholesterol in arterial disease females with that in males of corresponding age (50 years and over) showed that the mean level in females (261.8 ± 41.9 mg. per 100 ml.) was higher than that of the males (244.7 ± 49.9). The difference was not statistically significant.

III. EFFECT OF BODY WEIGHT

No significant association was observed in any of the groups between plasma cholesterol level and body weight (measured either as actual weight or relative weight). Correlation of actual weight with plasma cholesterol gave coefficients of +0.15 in male controls and +0.06 in male arterial disease patients.

IV. EFFECT OF HABITUAL CIGARETTE-SMOKING

Male Controls

The mean plasma cholesterol level in the 27 cigarette-

smokers (223.7 ± 34.3 mg. per 100 ml.) was similar to that in the 20 non-smokers (219.9 ± 47.6 mg. per 100 ml.). The cholesterol level in the 9 heavy cigarette-smokers (20 or more cigarettes daily) was 239.8 ± 42.8 mg. per 100 ml. (mean age of group 41.2 years) which was not significantly higher than that of the non-smokers whose mean age was 46.3 ($t = 1.08$).

Male Arterial Disease Subjects

Comparison of cigarette-smokers with non-smokers was carried out only in the subjects with ischaemic heart disease. There were no non-smokers in the peripheral arterial disease group, the only patient who did not smoke cigarettes being a pipe-smoker.

The 33 cigarette-smokers, of mean age 52.4 years, had a slightly higher mean cholesterol level (261.0 ± 50.1 mg. per 100 ml.) than the 8 non-smokers of mean age 47.9 years (244.6 ± 27.3 mg. per 100 ml.). This slight difference was accentuated when the cholesterol concentration in the 19 heavy cigarette-smokers (278.8 ± 44.3 mg. per 100 ml.) was compared with that of the non-smokers ($P < 0.10 > 0.05$). The difference, however, reached conventional levels of significance only in the 50 to 59 year age group, where heavy smokers had a mean plasma cholesterol of 298.7 ± 35.4 mg. per 100 ml., as

compared with 238.7 ± 20.3 mg. per 100 ml. ($P < 0.05$) in the non-smokers.

V. EFFECT OF FAMILY HISTORY OF ISCHAEMIC HEART DISEASE

The 4 male controls with a positive family history of ischaemic heart disease had higher plasma cholesterol levels (262.8 ± 42.1 mg. per 100 ml.) than the 23 controls in the same age group with a negative family history (225.0 ± 31.1 mg. per 100 ml., $P < 0.05$). No significant differences in plasma cholesterol concentration were demonstrated between the 33 male arterial disease subjects with a positive family history (258.5 ± 50.1 mg. per 100 ml.) and the 33 with a negative family history of ischaemic heart disease (250.6 ± 39.9 mg. per 100 ml.).

VI. EFFECT OF ATHEROSCLEROTIC ARTERIAL DISEASE

(a) Comparison of Controls and Arterial Disease Subjects

Male controls of 40 years and over had a significantly lower mean cholesterol concentration (227.1 ± 38.5 mg. per 100 ml.) than arterial disease subjects of corresponding age (251.9 ± 46.0 mg. per 100 ml., $P < 0.01$). The difference between the two groups was greatest in the 40 to 44 year age group where the control level was 229.6 ± 30.7 mg. per

100 ml. as compared with the arterial disease level of 273.9 ± 45.9 mg. per 100 ml. ($P<0.02$).

Similarly, the mean cholesterol concentration in the 9 female controls of 50 years and over (239.4 ± 33.1 mg. per 100 ml.) was lower than that in the 14 female arterial disease subjects (261.8 ± 41.9 mg. per 100 ml.), although in this case the difference was not statistically significant.

(b) Comparison of Different Types of Arterial Disease

No significant differences were observed in the mean plasma cholesterol concentrations in the three clinical groups of arterial disease - myocardial infarction, angina pectoris, and peripheral arterial disease (Table III).

SUMMARY OF FACTORS AFFECTING PLASMA CHOLESTEROL

The main factors found to influence the plasma cholesterol level were the age and sex of the subjects, and the presence of atherosclerotic arterial disease. Male controls with a family history of ischaemic heart disease had significantly higher plasma cholesterol levels than others in the same age group, but in arterial disease patients similar plasma cholesterol levels were recorded in those with and without such a family history. Only minor differences in plasma cholesterol concentration

were observed between habitual cigarette-smokers and non-smokers. The plasma cholesterol was not related to body weight.

Male control subjects showed a parabolic effect of age, with a maximum cholesterol level at about 45 years, whereas in females there was a progressive increase with age. The cholesterol level was significantly higher in young males than in young females, but by the sixth decade cholesterol levels in females were slightly greater than those of males.

In male arterial disease subjects the highest levels were seen in the younger subjects, and there was a significant decrease in cholesterol with age. This effect was more marked in the myocardial infarction and angina pectoris patients than in those with peripheral arterial disease. Female arterial disease subjects showed no significant age trends, and tended to have higher cholesterol levels than male arterial disease subjects of corresponding age. Arterial disease patients had higher cholesterol levels than controls, but this difference was significant only in the males. Cholesterol levels were similar in the myocardial infarction, angina pectoris, and peripheral arterial disease subjects.

FASTING PLASMA PHOSPHOLIPID

The mean plasma phospholipid levels for the various groups are shown in Table V.

I. EFFECT OF AGE

Male Controls

A parabolic effect of age on plasma phospholipid concentration was observed, similar to that recorded for the plasma cholesterol (Fig. 15). The maximum mean phospholipid level was attained at about the age of 45 years.

Female Controls

No figures were available in this series for plasma phospholipid estimations below the age of 40 years. In the 16 female subjects aged 40 years and over, only a slight, insignificant tendency for the phospholipid level to increase with age was observed (Table IV).

Male Arterial Disease Subjects

No significant effect of age on fasting plasma phospholipid level was observed (Table IV).

TABLE V. Fasting Plasma Phospholipid (mean ± S.D.)

Subjects	No. of observations	Fasting Plasma Phospholipid mg. per 100 ml.
Controls male - all ages	28	203.7 ± 33.1
Controls male - aged 40 and over	18	210.8 ± 33.9
Arterial Disease male	50	219.8 ± 32.1
Myocardial Infarction male	19	215.9 ± 29.7
Angina Pectoris male	12	215.8 ± 32.1
Peripheral Art. Disease male	19	226.1 ± 34.9
Controls female - all ages	16	228.2 ± 24.9
Controls female - aged 50 and over	8	229.6 ± 24.2
Arterial Disease female	5	259.8 ± 28.8

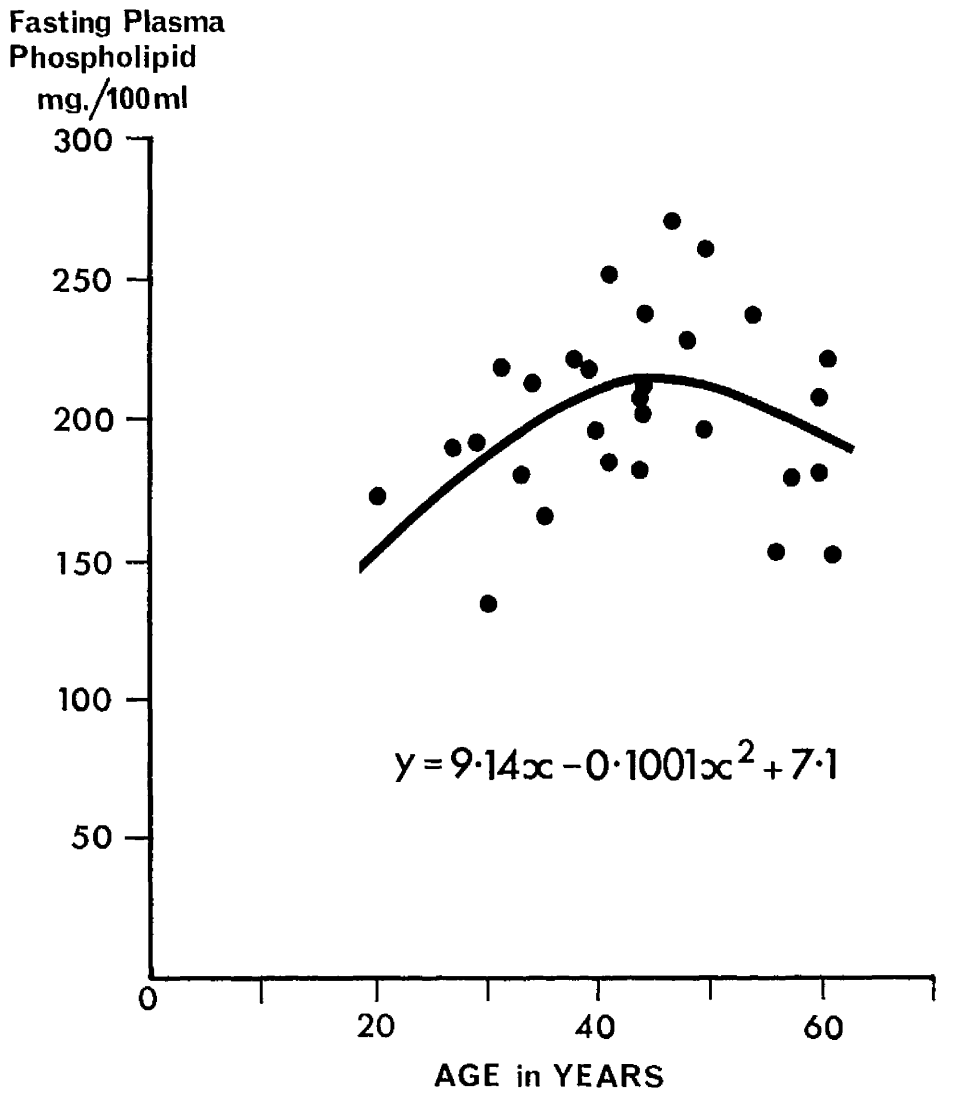

Fig. 15. Effect of age on plasma phospholipid concentration in male controls ($t = 2.35$, $P < 0.05$).

Female Arterial Disease Subjects

No age trend was observed in the very small number of estimations (5) made in this group (Table IV).

II. EFFECT OF SEX

Comparison of Male and Female Controls

Comparison of the plasma phospholipid levels was possible only in subjects over 40 years. The 16 female controls had a slightly higher fasting level (228.2 ± 24.9 mg. per 100 ml.) than the 18 males (210.8 ± 33.9 mg. per 100 ml.). This difference was due almost entirely to the fact that the females in the 55 to 64 year age group had levels of 223.8 ± 26.7 mg. per 100 ml. as compared with 183.6 ± 31.6 mg. per 100 ml. in the 5 males of the same age ($P < 0.10 > 0.05$).

Comparison of Male and Female Arterial Disease Subjects

The 5 female patients had significantly higher fasting plasma phospholipid levels (259.8 ± 28.8 mg. per 100 ml.) than the 32 males of corresponding age (219.5 ± 33.6 mg. per 100 ml. $P < 0.02$).

III. EFFECT OF BODY WEIGHT

No significant association was observed in any of the groups between plasma phospholipid level and body weight (actual weight or relative weight). Correlation of the plasma phospholipid with actual weight gave correlation coefficients of +0.15 in male controls and +0.02 in male arterial disease patients.

IV. EFFECT OF HABITUAL CIGARETTE-SMOKING

Male Controls

No significant difference was observed in plasma phospholipid level between the 14 cigarette-smokers (193.4 ± 27.4 mg. per 100 ml.) and the 11 non smokers (214.6 ± 39.4 mg. per 100 ml.). The 5 heavy cigarette-smokers had a mean phospholipid concentration of 206.2 ± 17.9 mg. per 100 ml.

Male Arterial Disease Subjects

Similar plasma phospholipid levels were recorded in the 23 cigarette-smokers (216.8 ± 33.8 mg. per 100 ml.) and the 5 non-smokers (216.0 ± 13.2 mg. per 100 ml.). The 13 heavy cigarette-smokers had a mean phospholipid level of 225.8 ± 31.9 mg. per 100 ml.

V. EFFECT OF FAMILY HISTORY OF ISCHAEMIC HEART DISEASE

Plasma phospholipid levels in the 24 male arterial disease patients with a positive family history of ischaemic heart disease (219.2 ± 35.2 mg. per 100 ml.) were similar to those in the 22 patients with no family history of ischaemic heart disease (223.9 ± 29.5 mg. per 100 ml.). Insufficient phospholipid estimations were carried out in male controls with a positive family history of ischaemic heart disease to allow comparison with the rest of the group.

VI. EFFECT OF ATHEROSCLEROTIC ARTERIAL DISEASE

(a) Comparison of Controls and Arterial Disease Subjects

Although the mean phospholipid level in male controls was somewhat lower than that in the male arterial disease subjects (Table V), a significant difference between the groups was observed only in the older subjects. In the 55 to 64 year age group, the mean phospholipid concentration in the 5 controls was 183.6 ± 31.6 mg. per 100 ml., while in the 25 arterial disease patients a higher level was recorded (219.2 ± 35.1 mg. per 100 ml. $P < 0.05$). Similarly, the level in the 8 female controls of 50 years and over (229.6 ± 24.2 mg. per 100 ml.) was lower than that in the 5 arterial disease

patients (259.8 ± 28.8 mg. per 100 ml. $P < 0.10 > 0.05$), although owing to the smallness of the numbers this difference does not achieve statistical significance.

(b) Comparison of Different Types of Arterial Disease

No significant differences in mean plasma phospholipid concentrations were observed in the three types of arterial disease patients (Table V).

SUMMARY OF FACTORS AFFECTING PLASMA PHOSPHOLIPID

The main factors found to influence the plasma phospholipid level were the age and sex of the subjects, and the presence of atherosclerotic arterial disease. No correlation was found between plasma phospholipid concentration and body weight, cigarette-smoking, or the presence of a family history of ischaemic heart disease.

In male controls a parabolic effect of age on plasma phospholipid was observed. No age effect was discernible in the male arterial disease patients, nor was any age effect observed in the female control or arterial disease subjects, who were few in number. In both control and arterial disease groups, older females had higher levels than males of corresponding age. Subjects with atherosclerotic arterial disease tended to have higher phospholipid concentrations than controls.

Phospholipid levels were similar in the myocardial infarction, angina pectoris, and peripheral arterial disease subjects.

FASTING PLASMA TRIGLYCERIDE

The mean fasting triglyceride levels for the various groups are shown in Table VI.

I. EFFECT OF AGE

Male Controls

As in the case of the plasma cholesterol and phospholipid, a parabolic effect of age on fasting plasma triglyceride concentration was observed, although there was considerably more scatter in the results (Fig. 16). The maximum triglyceride level was seen in subjects aged between 40 and 50 years.

Female Controls

There was a slight, statistically insignificant, tendency for plasma triglyceride levels to increase with age (Table IV).

Male Arterial Disease Subjects

Plasma triglyceride concentrations were highest in the younger subjects, and decreased slightly but significantly with age (Fig. 17). As in the case of the plasma cholesterol,

TABLE VI. Fasting Plasma Triglyceride (mean ± S.D.)

Subjects	No. of observations	Fasting Plasma Triglyceride mg. per 100 ml.
Controls male - all ages	50	89.4 ± 32.0
Controls male - aged 40 and over	34	93.4 ± 31.7
Arterial Disease male	72	108.4 ± 42.0
Myocardial Infarction male	27	102.8 ± 41.6
Angina Pectoris male	19	105.1 ± 36.0
Peripheral Art. Disease male	26	116.7 ± 46.4
Controls female - all ages	22	80.3 ± 16.6
Controls female - aged 50 and over	9	87.6 ± 19.8
Arterial Disease female	14	85.7 ± 18.3

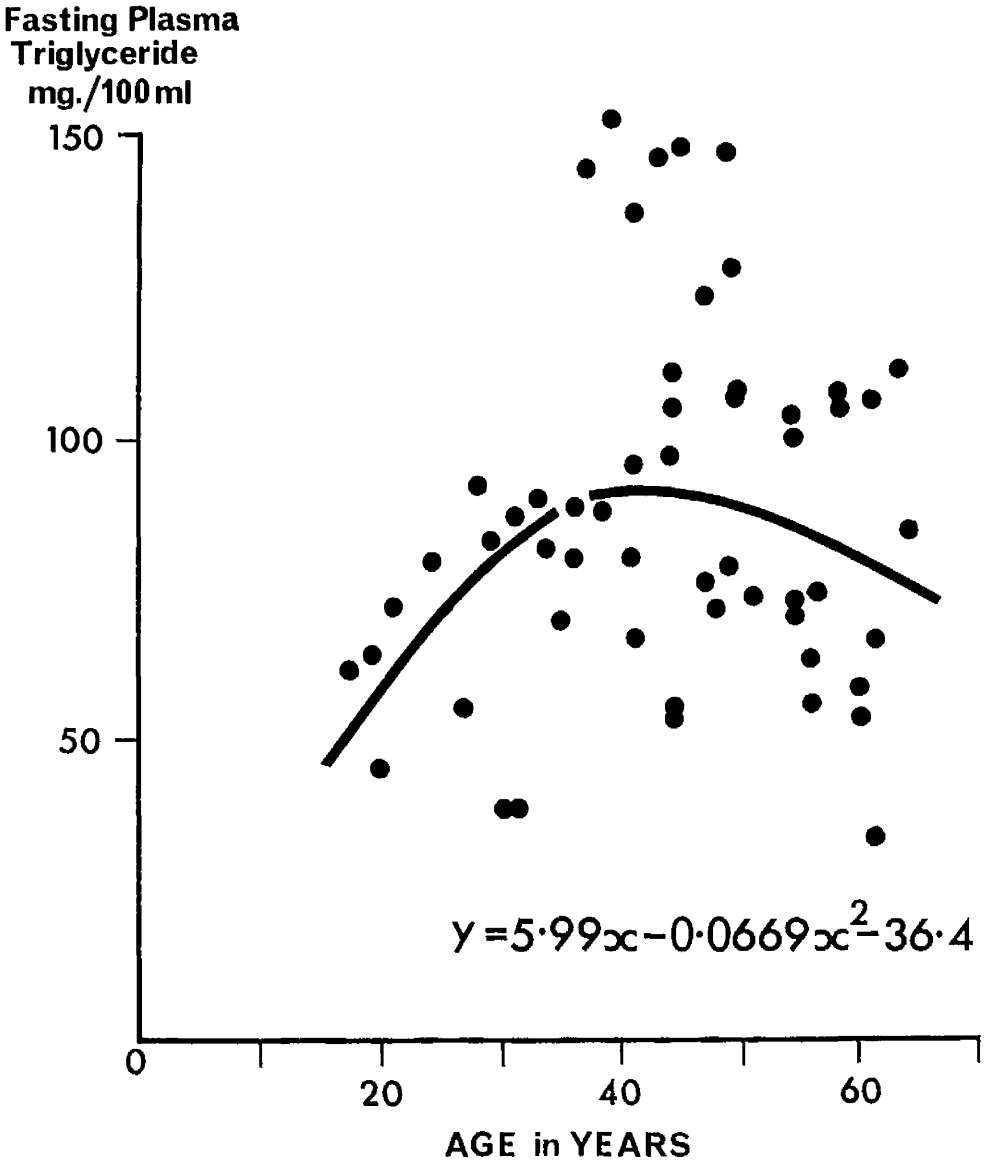

Fig. 16. Effect of age on plasma triglyceride concentration in male controls ($t = 2.84$, $P<0.01$).

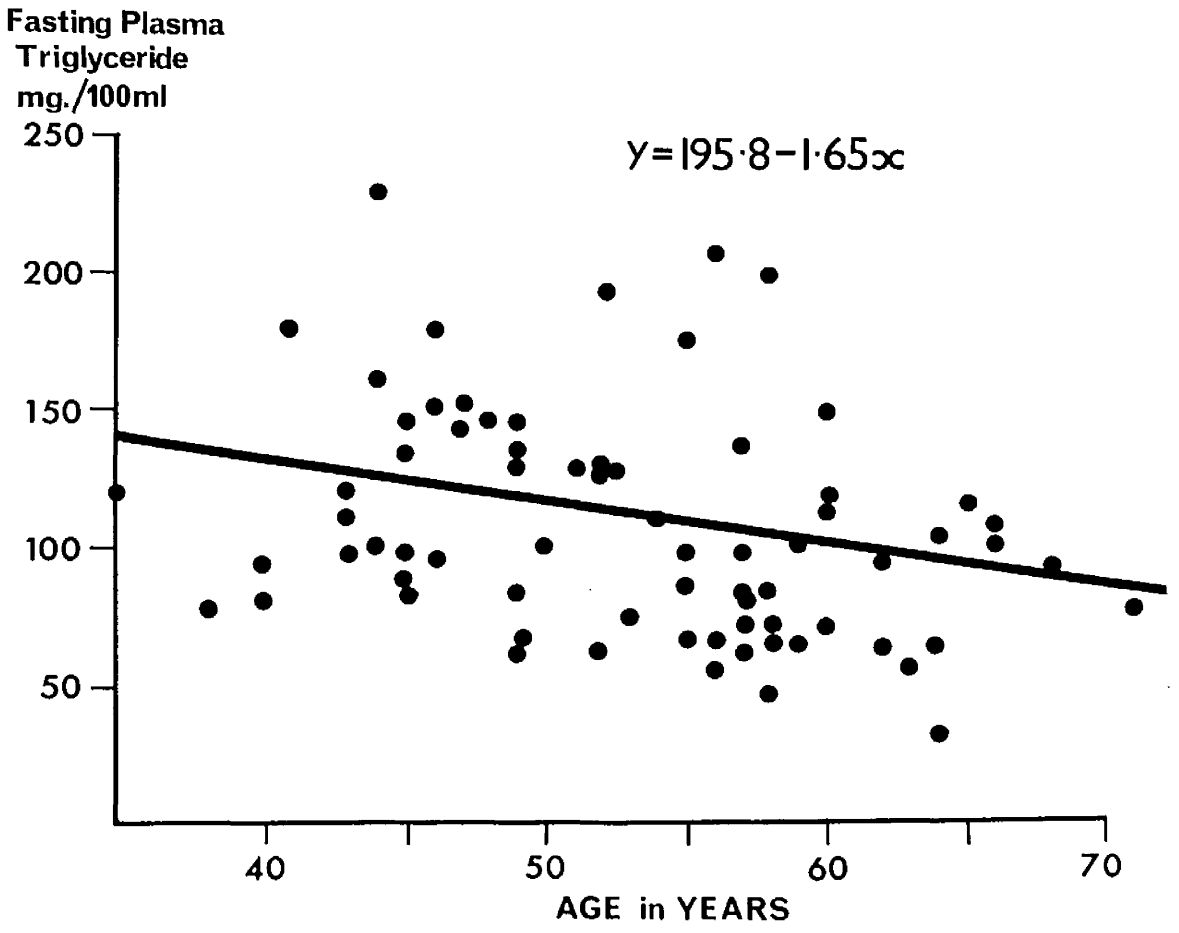

Fig. 17. Effect of age on plasma triglyceride concentration in male arterial disease patients ($r = -0.31$, $P<0.01$).

this trend was more marked in the myocardial infarction and angina pectoris groups, and was statistically insignificant in the peripheral arterial disease group (Table IV).

Female Arterial Disease Subjects

No variation of fasting plasma triglyceride with age was observed in female arterial disease patients (Table IV).

II. EFFECT OF SEX

(a) Comparison of Male and Female Controls

In the younger subjects, plasma triglyceride levels in males tended to be higher than in females, although the differences were not statistically significant. In the 40 to 44 year age group, for example, the mean for the 11 males was 94.1 $\pm$ 30.5 mg. per 100 ml. as compared with 67.2 $\pm$ 10.9 mg. per 100 ml. in the 6 females ($P < 0.10 > 0.05$). As in the case of the plasma cholesterol, the increase in triglyceride with age in females, and the corresponding decrease in males, led to slightly higher levels in the 5 females in the 55 to 64 year age group (86.8 $\pm$ 22.7 mg. per 100 ml.) than in the 9 males (76.7 $\pm$ 28.1 mg. per 100 ml.).

(b) Comparison of Male and Female Arterial Disease Subjects

No significant difference was observed between the plasma triglyceride concentrations in the 14 female patients (85.7 ± 18.3 mg. per 100 ml.) and in the 44 male patients aged 50 and over (99.3 ± 41.2 mg. per 100 ml.).

III. EFFECT OF BODY WEIGHT

(a) Controls

No significant correlation of plasma triglyceride levels with either actual weight or relative weight was observed in male controls (correlation coefficients -0.06 and +0.11 respectively). In the female controls the corresponding correlation coefficients were +0.01 and +0.19.

(b) Arterial Disease Subjects

The male arterial disease patients showed a slight, statistically insignificant, tendency for the fasting plasma triglyceride to increase with increasing relative weight. A relationship between weight and plasma triglyceride was more readily demonstrated when plasma triglyceride was correlated with actual weight. In the male arterial disease group as a whole the correlation, although low-grade, was statistically significant ($r = +0.27$, $P<0.05$). Of the three

groups of arterial disease patients, a significant relationship was demonstrated only in the peripheral arterial disease subjects ($r = +0.49$, $P < 0.05$). Female arterial disease patients showed no such correlation between weight and plasma triglyceride ($r = -0.12$).

IV. EFFECT OF HABITUAL CIGARETTE-SMOKING

No significant difference in plasma triglyceride concentration was observed between smokers and nonsmokers in male controls (93.0 ± 32.4 and 83.4 ± 29.4 mg. per 100 ml. respectively). In the male ischaemic heart disease group, the mean fasting plasma triglyceride was 100.2 ± 37.9 mg. per 100 ml. in the cigarette-smokers, and 109.8 ± 46.0 mg. per 100 ml. in the nonsmokers.

V. EFFECT OF FAMILY HISTORY OF ISCHAEMIC HEART DISEASE

In the four male controls with a positive family history of ischaemic heart disease the fasting plasma triglyceride level (98.0 ± 38.7 mg. per 100 ml.) was similar to those of corresponding age in the rest of the control group (94.7 ± 30.1 mg. per 100 ml.). In the 33 arterial disease patients with a positive family history of ischaemic heart disease it was 105.3 ± 43.0 mg. per 100 ml. as compared with 114.4 ±

41.9 mg. per 100 ml. in the 33 subjects with a negative history.

VI. EFFECT OF ATHEROSCLEROTIC ARTERIAL DISEASE

(a) Comparison of Controls and Arterial Disease Subjects

At all ages, male arterial disease subjects had higher fasting triglyceride levels than controls. The difference, however, was statistically significant only in the 40 to 44 year age group. The 11 controls had a mean level of 94.1 ± 30.5 mg. per 100 ml., whereas the 9 arterial disease subjects had a mean fasting triglyceride concentration of 131.0 ± 50.1 mg. per 100 ml. ($P < 0.05$). In the female subjects, however, similar fasting triglyceride concentrations were recorded in arterial disease and controls (Table VI).

(b) Comparison of Different Types of Arterial Disease

No significant differences were observed in mean fasting triglyceride levels in the three groups (Table VI).

SUMMARY OF FACTORS AFFECTING PLASMA TRIGLYCERIDE

Plasma triglyceride levels were influenced by the age, sex, and weight of the individuals, and by the presence of atherosclerotic arterial disease. They appeared not to be affected by cigarette-smoking, or by the presence of a family

history of ischaemic heart disease.

Male controls showed a parabolic effect of age on plasma triglyceride level. Female controls, on the other hand, showed only an insignificant trend towards increasing plasma triglyceride concentrations with increasing age. In male arterial disease patients, the highest levels were recorded in the younger patients, and there was a progressive decrease in triglyceride level with advancing age. Female arterial disease subjects showed no significant effect of age on plasma triglyceride. Male arterial disease subjects were found to have a low-grade, but statistically significant, correlation between increased body weight and increased plasma triglyceride concentration.

Plasma triglyceride levels tended to be lower in young females than in young males, but in the older age groups females had higher triglyceride levels. Young male arterial disease subjects (aged 40 to 44 years) had significantly higher triglyceride levels than male controls of the same age, but no significant differences were observed between the males of the other age groups, nor between female arterial disease and control subjects.

FASTING PLASMA TOTAL NEFA

The mean fasting plasma total NEFA concentration for the various groups are shown in Table VII.

TABLE VII. Fasting Plasma Total NEFA (mean ± S.D.)

Subjects	No. of observations	Fasting Plasma NEFA microeq. per l.
Controls male - all ages	50	507 ± 150
Controls male - aged 40 and over	34	491 ± 156
Arterial Disease male	72	481 ± 235
Myocardial Infarction male	27	437 ± 240
Angina Pectoris male	19	515 ± 239
Peripheral Art. Disease male	26	503 ± 228
Controls female - all ages	22	539 ± 175
Controls female - aged 50 and over	9	579 ± 204
Arterial Disease female	13	490 ± 162

I. EFFECT OF AGE

No significant influence of age on plasma NEFA concentration was seen in any of the groups (Table IV).

II. EFFECT OF SEX

(a) Comparison of Male and Female Controls

Fasting plasma NEFA levels were similar in the 50 male controls and 22 female controls (Table VII).

(b) Comparison of Male and Female Arterial Disease Subjects

There was no significant difference in fasting plasma NEFA concentration between the 44 male arterial disease subjects of 50 years and over (456 $\pm$ 191 microeq. per l.) and the 13 female arterial disease subjects (490 $\pm$ 162 microeq. per l.).

III. EFFECT OF BODY WEIGHT

No correlation between fasting plasma NEFA and weight (actual weight or relative weight) was observed in any of the groups. Correlation of the plasma NEFA with actual weight gave correlation coefficients of +0.09 in male controls and +0.19 in male arterial disease patients.

IV. EFFECT OF HABITUAL CIGARETTE-SMOKING

(a) Male Controls

The 27 cigarette-smokers had significantly lower fasting plasma NEFA levels (450 ± 128 microeq. per l.) than the 20 non-smokers (569 ± 159 microeq. per l., $P < 0.01$). This difference was even greater when NEFA levels in the 9 subjects who smoked 20 or more cigarettes per day (414 ± 128 microeq./l.) were compared with those of the non-smokers ($P < 0.01$).

(b) Male Arterial Disease Patients

In these subjects also the 33 cigarette smokers had lower fasting plasma NEFA levels (436 ± 214 microeq. per l.) than the 8 non-smokers (606 ± 362 microeq. per l. $P < 0.10 > 0.05$), but in this case the difference did not attain conventional levels of statistical significance. The 19 patients who smoked 20 or more cigarettes daily had a mean fasting plasma NEFA level of 444 ± 157 microeq. per l.

V. EFFECT OF FAMILY HISTORY OF ISCHAEMIC HEART DISEASE

No significant differences were observed in fasting plasma NEFA between subjects with and without a family history of ischaemic heart disease. Male controls with a positive family history had a mean level of 544 ± 73 microeq. per l.,

and those without a family history of ischaemic heart disease a mean level of 486 ± 145 microeq. per l. The corresponding values in male arterial disease patients were 462 ± 151 microeq. per l. and 489 ± 250 microeq. per l.

VI. EFFECT OF ATHEROSCLEROTIC ARTERIAL DISEASE

(a) Comparison of Controls and Arterial Disease Subjects

Controls and arterial disease patients, both male and female, had similar fasting plasma NEFA levels (Table VII).

(b) Comparison of Different Types of Arterial Disease

The plasma NEFA levels did not differ significantly in the three groups of male arterial disease subjects (Table VII).

SUMMARY OF FACTORS AFFECTING FASTING PLASMA NEFA

Of the various factors examined, the only one which appeared to influence the fasting plasma NEFA level was habitual cigarette-smoking. Cigarette-smokers, after an interval of approximately 12 hours since their last cigarette, had significantly lower plasma NEFA levels than non-smokers.

No significant effect on fasting plasma NEFA levels of age, sex, body weight, family history of ischaemic heart disease, or the presence of atherosclerotic arterial disease

could be detected.

VARIATION IN FASTING PLASMA LIPID ESTIMATIONS

In the majority of subjects two fasting plasma lipid estimations were carried out at an interval of 24 to 48 hours. The two results available for each of the plasma lipids were correlated separately for the controls (male and female) and the arterial disease patients (male and female).

Results

The correlation coefficients are shown in Table VIII. The plasma cholesterol showed the least day-to-day variation, but a good correlation was obtained also in triglyceride, phospholipid and NEFA estimations.

CORRELATION OF PLASMA LIPID LEVELS

In all subjects, the results of cholesterol, triglyceride and NEFA estimations were available. In addition, phospholipid measurements were performed in the majority of subjects. An analysis was carried out of the correlations between pairs of lipid estimations on the same blood samples.

TABLE VIII. Variability of Fasting Plasma Lipid Estimations on Two Occasions (correlation coefficients)

Plasma Lipid	Control Subjects	Arterial Disease Subjects
Plasma cholesterol	+0.87*** (n = 40)	+0.91*** (n = 51)
Plasma phospholipid	+0.77*** (n = 27)	+0.73*** (n = 37)
Plasma triglyceride	+0.84*** (n = 40)	+0.82*** (n = 52)
Plasma NEFA	+0.69*** (n = 39)	+0.85*** (n = 52)

***$P < 0.001$

n = no. of pairs of observations

Results

The correlations between fasting plasma lipid levels for male and female controls and male and female arterial disease patients are shown in Table IX.

The strongest correlation was that between cholesterol and phospholipid, a fairly close correlation being obtained in each of the four groups. The correlation between phospholipid and triglyceride was next in order of magnitude, followed by that between cholesterol and triglyceride, which was statistically significant only in male controls and male arterial disease patients.

No evidence was found of any relationship between the plasma NEFA level and plasma concentrations of the other lipids.

SUMMARY OF RESULTS

An assessment was made of the effect on the fasting plasma lipids of five factors:- age, sex, weight, habitual cigarette-smoking, and a family history of ischaemic heart disease. A comparison was then made of plasma lipid levels in healthy control subjects and patients with atherosclerotic arterial disease, both male and female.

TABLE IX. Correlations between Pairs of Plasma Lipids

(correlation coefficients)

	Controls male	Controls female	Arterial disease male	Arterial disease female
Cholesterol and Phospholipid	+0.58*** (n = 28)	+0.67** (n = 15)	+0.73*** (n = 50)	+0.83* (n = 5)
Cholesterol and Triglyceride	+0.50*** (n = 50)	+0.08 (n = 22)	+0.25* (n = 72)	-0.11 (n = 14)
Cholesterol and NEFA	+0.01 (n = 50)	+0.35 (n = 22)	+0.11 (n = 72)	-0.02 (n = 13)
Phospholipid and Triglyceride	+0.54** (n = 28)	+0.48* (n = 15)	+0.41** (n = 50)	+0.41 (n = 5)
Phospholipid and NEFA	+0.14 (n = 28)	+0.13 (n = 15)	-0.20 (n = 50)	+0.62 (n = 4)
Triglyceride and NEFA	-0.05 (n = 50)	-0.20 (n = 22)	-0.02 (n = 72)	+0.24 (n = 13)

n = no. of observations

* $P < 0.05$,

** $P < 0.01$,

*** $P < 0.001$

Effect of Age and Sex

The age and sex of the individuals appeared to exert a marked influence on plasma lipid levels, as summarised in Table IV.

In male controls a parabolic effect of age on cholesterol, phospholipid, and triglyceride was observed. The plasma concentrations of these lipids increased with age up to between 40 and 50 years, and then decreased. Female controls showed a similar increase up to middle age, but thereafter the plasma lipid levels, instead of declining, continued to increase. This trend was statistically significant only in the case of cholesterol. In male arterial disease patients cholesterol and triglyceride levels were highest in the younger subjects, and decreased with age. Female subjects with arterial disease, who were all aged 50 years or over, showed no significant age trends. In subjects under 40 years of age, plasma concentrations of these lipids were higher in males than in females. Over 40 years, however, the concentrations in female control and arterial disease patients increased progressively to reach levels higher than those of males of the same age.

No effect of age or sex on plasma NEFA levels was observed.

Effect of Weight

The fasting plasma triglyceride showed a low-grade, but significant, correlation with body weight in male patients with arterial disease. This effect was not seen in the other groups, nor in relation to other plasma lipids.

Effect of Cigarette-Smoking

Habitual cigarette-smokers had lower fasting NEFA levels, after overnight abstention from smoking, than non-smokers in male control and arterial disease groups. There was also evidence to suggest that cigarette-smokers, particularly those with moderate or heavy cigarette consumption, had higher cholesterol levels than non-smokers.

Effect of Family History of Ischaemic Heart Disease

Subjects with a family history of ischaemic heart disease did not have any definite plasma lipid abnormality. Although cholesterol levels were higher in male controls with a positive family history than in others of the same age group, the numbers were too small to allow any firm conclusions to be drawn. Plasma lipid concentrations were similar in male arterial disease patients with and without a family history of ischaemic heart disease.

Comparison of Controls and Arterial Disease Patients

Subjects with atherosclerotic arterial disease, both male and female, tended to have higher fasting levels of cholesterol, phospholipid, and triglyceride than controls. This effect was more marked when young arterial disease patients were compared with controls of the same age. Plasma NEFA levels did not differ significantly in controls and arterial disease patients.

Effect of Type of Arterial Disease

Plasma lipid concentrations were similar in the three clinical types of arterial disease - myocardial infarction, angina pectoris, and peripheral arterial disease.

Variability of Plasma Lipid Estimations

Fasting lipid estimations on two occasions in the same individuals showed that the least variable of the plasma lipids was cholesterol. Good correlations between the two estimations of phospholipid, triglyceride, and NEFA were obtained.

Correlation of Plasma Lipid Levels

In descending order of magnitude the following correlations between fasting plasma lipids were demonstrated:- cholesterol and phospholipid, phospholipid and triglyceride, cholesterol

and triglyceride. No correlation was found between the plasma NEFA concentration and the plasma levels of other lipids.

DISCUSSION

Fasting Plasma Lipids in Healthy Subjects

The present study has shown comparatively low cholesterol levels in young men, similar to those in other series (Kritchevsky, 1958b), with progressively increasing levels up to middle age. In the past two decades a considerable number of publications from Western countries have reported higher cholesterol levels in middle aged than in young men (Gertler *et al.*, 1950; Keys *et al.*, 1950; Jones *et al.*, 1951; Malmros *et al.*, 1956; Lawry *et al.*, 1957; Lewis *et al.*, 1957; Thomas and Eisenberg, 1957; Carlson, 1960a). There have been very few reports, however, of serial observations on individuals followed through the period of early adulthood although Clark *et al.* in 1967 published a twelve-year follow-up study on four hundred men, aged 17 to 22 years at time of entry to the study, and demonstrated a rapid, progressive rise in cholesterol with age in these subjects.

In the present study, healthy males showed a falling-off in plasma cholesterol levels beyond the age of 45 to 50 years. The majority of published reports have indicated that the mean

cholesterol concentration is lower in elderly than in middle aged men (Keys et al., 1950; Lawry et al., 1957; Searcy et al., 1960). In a co-operative study in approximately ten thousand men (Lewis et al., 1957) the data were subjected to a curvilinear regression analysis, and a maximum cholesterol level was observed about the age of 50 years. Some Scandinavian workers, however, have shown a progressive increase in serum cholesterol up to the age of 60 or 70 years (Lund et al., 1961; Berge and Nicolaysen, 1963).

As reported in the results section (page 64), healthy young females had significantly lower cholesterol levels than young males. The effect of age on cholesterol in females differed from that in males, in that the increase in cholesterol continued beyond middle age. This resulted in higher plasma cholesterol levels in healthy females than in males in the fifth and sixth decades. Similar observations have been made in several other studies. Jones et al. (1951) and Adlersberg et al. (1956) found that the rise in serum cholesterol in the third decade in females was less than that seen in males. These authors, in addition to others (Lawry et al., 1957; Searcy et al., 1960), reported higher cholesterol levels in males between 30 and 50 years than in females in the same age group. They also stated that in females the cholesterol concentration continued to increase in the fourth to sixth

decades, while those of males had levelled off. This resulted in lipid levels in postmenopausal females which were equal to, or greater than, those of males of corresponding age. Several other investigations (Keys et al., 1950; Block et al., 1951; Barr et al., 1952) did not find sex differences in cholesterol level prior to the fortieth year, but were in agreement with the above-mentioned authors in recording higher cholesterol levels in postmenopausal women than in men of similar age.

The influence of age and sex on plasma phospholipid levels in the control series (page 69) was similar to that in the case of cholesterol, although no data were available on phospholipid levels in females under 40 years of age. The information in the literature on plasma phospholipids is much less abundant than that on plasma cholesterol. Carlson (1960a) found that the serum phospholipid concentration increased rapidly up to the age of 40 years, with a much smaller and more gradual increase with age in older individuals. Cramér (1962) reported significantly higher phospholipid levels in normal males in the 55 to 65 year age group than in those aged 20 to 40 years.

Conflicting reports have been published on phospholipid concentrations in females. Barr (1953) found higher phospholipid levels in females than in males under 35 years of age, while

Havel et al. (1955) found no significant difference in phospholipid between the sexes in this age group. Adlersberg et al. (1956) reported that females had higher serum phospholipid levels than males during early adulthood as well as beyond the fifth decade; during the third and fourth decades, however, the reverse held true. Cramér (1962) found similar phospholipid levels in normal females and males below the age of 40, but in the 55 to 65 year age group females had higher levels (274 ± 8 mg. per 100 ml.) than males (236 ± 10 mg. per 100 ml.). Hallberg et al. (1966) showed a steady phospholipid increase in healthy females up to the age of 50 years, with a plateau thereafter. Subsequently Hallberg and Svanborg (1967) demonstrated that serum concentrations of phospholipid, as well as of cholesterol and triglyceride, were higher in postmenopausal women aged 50 years than in premenopausal females of the same age.

In the present series a curvilinear relationship of age with fasting triglyceride was seen in male controls, whereas in females there was a small, statistically insignificant, tendency for triglyceride to increase with age. Earlier studies in males (Page et al., 1935, Peters and Man, 1943; Man and Peters, 1953) were reported to show no variation in serum neutral fat with age. Antonis and Bersohn (1960), however, found a steady

increase in triglyceride up to the fifth and sixth decades in healthy white males, but no significant change in their group of Bantu controls. A rapid increase with age up to 40 years, with a much more gradual increase thereafter, was reported by Carlson (1960a) in a group of 151 healthy males aged 26 to 73 years. An age effect similar to that in the present male control series was reported by Albrink et al. (1961). They found an increase in serum triglyceride levels up to the fifth or sixth decades, with lower levels in older healthy males, with the result that figures obtained for subjects aged 70 and over were similar to those for their 20 to 29 year age group.

The reported age trends for plasma triglyceride levels in females differ from those in males, and are similar to those in the present study. Antonis and Bersohn (1960) found no significant increase with age in premenopausal European females up to the age of 46 years, but considerably higher triglyceride levels were recorded in three postmenopausal females (150 to 158 mg. per 100 ml., as compared with a mean triglyceride value of 68 $\pm$ 20 mg. per 100 ml. for the younger subjects). Cramér (1962) found higher triglyceride levels in eleven females aged 55 to 65 years (mean 84 mg. per 100 ml.) than in eight females aged 20 to 40 years (mean 68 mg. per 100 ml.). In ninety-nine

females aged 17 to 90 years, Feldman et al. (1963) found a stepwise rather than a continuous increase in serum triglyceride with age. Hallberg et al. (1966) reported a very similar stepwise increase in their group of 360 women selected at random from the general population. In a further study, Hallberg and Svanborg (1967) showed that postmenopausal women aged 50 years had higher levels of triglyceride as well as of other plasma lipids than premenopausal women of the same age.

As in this series (page 75), comparison of healthy males and females by Antonis and Bersohn (1960) and Albrink et al. (1961) showed that under the age of 40 years triglyceride levels were slightly but not significantly, higher in males, whereas the reverse was the case in older age groups.

In contrast to the marked effect of age on cholesterol, phospholipid, and triglyceride, plasma NEFA levels did not vary with age in the subjects in this series, whose ages ranged from 18 to 68 years (page 80). Information in the literature on this subject is sparse. Children, however, appear to have higher fasting NEFA levels than adults (Corvilain et al., 1961; Heald et al., 1965). No difference in fasting NEFA levels between males and females was detected in this study. This is in agreement with the majority of the small number of relevant publications. Although Moorhouse et al. (1963) reported that fasting NEFA levels were higher in female than in male

diabetics, others have found no sex difference in NEFA levels in normal subjects (Svanborg and Svennerholm, 1961; Shah et al., 1963; Glennon et al., 1967).

This comparison of plasma lipids in our healthy subjects revealed a large measure of agreement with those reported in the literature from other Western countries, some of which were from very large co-operative studies. This would suggest that our control series is representative of the general population, and that their plasma lipid figures form a valid basis for comparison with those of subjects with atherosclerotic arterial disease.

Reason for Age-Associated Lipid Changes

A marked variation with age in plasma lipids, with the exception of plasma NEFA, has been described in our subjects. The most striking of these was the increase in plasma lipids with age in young adults, both male and female. These age trends in serum cholesterol have been attributed to a high dietary fat intake in Western communities. In economically underdeveloped racial groups, who eat little fat and in whom atherosclerosis is uncommon, the rise in cholesterol with age is reported to be insignificant (Bronte-Stewart, 1958). Further evidence in support of this theory has been provided by the large number of experiments, reviewed by Hilditch and Jasperson (1959), showing that certain types of fat (mainly saturated and of

animal origin) may raise serum cholesterol levels, while other fats (mainly unsaturated and vegetable in origin) have a hypocholesterolaemic effect. Serial studies in young adult males by Clark et al. (1967) showed that the increase in cholesterol, phospholipid, and triglyceride in their subjects was accompanied by weight gain. It appears likely, therefore, that the age-associated rise in plasma lipids in young adults is due to environmental factors such as diet, and possibly lack of exercise and cigarette smoking.

The lower levels seen in older control males may be due to the loss from the group of subjects with high plasma lipid levels and consequent high susceptibility to ischaemic heart disease in middle age. In females, the continued rise in lipids beyond middle age has been investigated by Hallberg and Svanborg (1967), who concluded that it was due mainly to hormonal changes associated with the menopause, but also contributed to by weight gain.

Effect of Atherosclerotic Arterial Disease

More than thirty years ago, it was shown that patients with angina pectoris had higher serum cholesterol levels than healthy subjects (Davis et al., 1937). Subsequent investigations confirmed the presence of raised cholesterol levels in survivors

of myocardial infarction as well as in patients with angina pectoris, although most investigators emphasised the considerable overlap between patients and apparently healthy controls, (Gertler et al., 1950; Oliver and Boyd, 1953, Lawry et al., 1957; Carlson, 1960b; Nicolaysen and Westlund, 1963). More recently, lipid estimations in peripheral arterial disease have suggested elevated serum cholesterol concentrations in these patients also (Juergens et al., 1960; McPherson et al., 1963; Begg, 1965). This study has again demonstrated a higher mean plasma cholesterol level in arterial disease patients than in healthy controls and, in addition, has shown that the lipid abnormality is similar in the three types of arterial disease studied - myocardial infarction, angina pectoris, and peripheral arterial disease (Table III). This study also showed that the elevation of plasma cholesterol was greatest in the younger subjects. This was the case particularly in those with myocardial infarction and angina pectoris, but was much less marked in peripheral arterial disease subjects. Several other workers have noted that younger patients with ischaemic heart disease appear to have higher serum cholesterol concentrations than older subjects (Little et al., 1956; Lawry et al., 1957; Albrink et al., 1961).

The observation that myocardial infarction in itself may produce alterations in plasma lipid levels (Biörck et al.,

1957; Dodds and Mills, 1959) has emphasised the importance of carrying out prospective studies to establish whether or not plasma lipid elevations are causally related to atherosclerosis. A large-scale co-operative study organised by the National Advisory Heart Council in the United States has shown for the first time, that coronary heart disease is associated with antecedent elevation of the serum cholesterol level (Gofman et al., 1956). Another study, commenced by the United States Public Health Service in 1948, has shown that the risk of developing ischaemic heart disease increases with increasing serum cholesterol concentration, as well as with increasing blood pressure level (Kannel et al., 1964).

Arterial disease patients in this study showed, in addition to an elevated plasma cholesterol, a tendency to higher phospholipid levels than controls (page 72). Approximately twenty years ago it was thought that the important lipid disorder in atherosclerosis was an increase in the cholesterol:phospholipid ratio. Ahrens and Kunkel (1949) considered that phospholipids played an important part in maintaining cholesterol in solution since they found that, in sera with a high lipid content, those which were optically clear had a high phospholipid concentration, whereas those which appeared turbid tended to be low in phospholipid. Steiner et al. (1952) reported an increased serum

cholesterol:phospholipid ratio in coronary patients, and suggested that the concentration of circulating phospholipid in these subjects might be insufficient to maintain all the cholesterol in solution, with resultant deposition of cholesterol in the arterial intima. Oliver and Boyd (1953) also found elevations of cholesterol, phospholipid, and cholesterol:phospholipid ratios in two hundred patients with ischaemic heart disease as compared with a control series. Although in the majority of investigations, patients with ischaemic heart disease have been shown to have elevated cholesterol and phospholipid levels, it is now appreciated that an elevated cholesterol:phospholipid ratio merely reflects an increased concentration of low-density lipoproteins (Bragdon et al., 1956). As shown in Table I, these low-density lipoproteins have a much higher content of cholesterol than of phospholipid.

Male arterial disease patients in this study had not only higher cholesterol and phospholipid levels but also higher fasting triglyceride concentrations than controls (page 78). This difference was most pronounced in the younger patients (40 to 44 year age group). Female arterial disease patients, who were all aged 50 years or over, did not have higher triglyceride levels than female controls of the same age. As in the case of cholesterol and phospholipid, this study

showed that similar fasting triglyceride concentrations were present in patients with myocardial infarction, angina pectoris, and peripheral arterial disease (Table VI). That elevated serum triglyceride levels were common in coronary heart disease was first pointed out by Hauss and Böhle (1955). Other workers subsequently suggested that serum triglyceride levels gave a more clear-cut separation between patients with ischaemic heart disease and controls than serum cholesterol (Albrink and Man, 1959; Schrade et al., 1959, 1960, and 1961; Albrink et al., 1961). In a Swedish series of myocardial infarction survivors, Carlson (1960b) found that hypertriglyceridaemia was more common than hypercholesterolaemia in patients under 50 years, while over 50 years the reverse was the case. Nicolaysen and Westlund (1963), however, pointed out that plasma triglycerides may be temporarily elevated after recent myocardial infarction. In their group of patients with old infarction, triglycerides were not more frequently elevated than the serum cholesterol. In the present series, in both males and females, the plasma cholesterol gave better separation at all ages of arterial disease patients from controls than did the fasting plasma triglyceride. In male arterial disease patients, hypertriglyceridaemia was more common in young than in older subjects. Final evaluation of the relative merits of triglyceride and cholesterol measurements

in atherosclerosis must, however, await the findings of a prospective study such as that in Framingham, Massachusetts (Kannel et al., 1964) or the study commenced more recently in Stockholm (Havel and Carlson, 1962).

In contrast to the other plasma lipids, NEFA levels were similar in arterial disease patients and controls (page 82). The three groups of arterial disease patients also showed similar plasma NEFA concentrations (Table VII). In the past ten years, since accurate methods for the measurement of NEFA levels were described (Dole, 1956; Gordon, 1957) a number of reports on fasting NEFA levels in patients with atherosclerosis have appeared. Several investigations on small numbers of subjects have shown no difference in NEFA levels between patients with clinical atherosclerotic disease and controls (Havel and Peterson, 1958; Waddell and Field, 1960; Schrade et al., 1961; Aleksandrow et al., 1962). On the other hand, Kershbaum and Bellet (1964) found slightly higher NEFA levels in seventeen patients with old myocardial infarction than in ten normal subjects, and Rifkind (1966) reported significantly higher values in forty males with a history of myocardial infarction than in forty healthy male controls. The apparent discrepancy in these findings might be due to the conditions under which the tests were carried out. In

view of the well-known lability of the plasma NEFA level (Hollister and Overall, 1964), and its elevation by stress (Bogdonoff et al., 1960), the taking of blood samples at an outpatient visit, as was the case in most of Rifkind's (1966) subjects, might result in higher NEFA levels than those obtained under basal conditions. If patients with a previous myocardial infarction react more strongly to stress than controls, as suggested by Horwitz and Bronte-Stewart (1962) and Kershbaum et al. (1963), this might be sufficient to cause a significant difference in NEFA levels in the two groups.

The results of plasma lipid estimations in our arterial disease patients, therefore, appear to be in accord with the majority of those published. Patients with ischaemic heart disease had higher mean cholesterol, phospholipid, and triglyceride concentrations than controls, and the highest lipid levels occurred in the younger patients. Elevated plasma cholesterol, phospholipid, and triglyceride levels also occurred in patients with peripheral arterial disease. There was, however, a considerable overlap in lipid levels between arterial disease patients and apparently healthy controls. Plasma NEFA levels were similar in controls and patients with arterial disease.

As shown in Table II, the arterial disease patients in this series differed from the controls in certain characteristics

which might affect plasma lipid levels. These characteristics were body weight, the frequency of a family history of ischaemic heart disease, and the proportion of cigarette-smokers. An analysis of their relation to plasma lipid concentrations was therefore carried out.

Effect of Body Weight

In this study, the only lipid which showed an association with body weight was the plasma triglyceride. A lowgrade positive correlation was demonstrated in male arterial disease patients between fasting triglyceride and actual weight. The correlation with relative weight, a better index of obesity than actual weight, was smaller and statistically insignificant. Evidence has accumulated in recent years of an association between plasma triglyceride levels and various measures of body fatness, including relative weight, in both arterial disease and healthy subjects (Schrade et al., 1960; Feldman et al., 1963; Waxler and Craig, 1964; Rifkind and Begg, 1966; Sailer et al., 1966; Hollister et al., 1967). Plasma cholesterol has been shown to have a very low-grade association with body weight, which is as a rule demonstrable only in very large series such as that of Montoye et al. (1966). No evidence has been found of a correlation between body weight and plasma phospholipid (Rifkind and Begg, 1966; Sailer et al., 1966;

Hallberg and Svanborg, 1967), or between body weight and plasma NEFA (Sailer et al., 1966) except in the very obese.

It has been stated that subjects with ischaemic heart disease are frequently overweight (Stamler et al., 1960; Brown, 1962; Dawber et al., 1962). Men with peripheral arterial disease on the other hand, have been found not to be obese, using relative weight and skinfold thicknesses as criteria (Begg, 1965). In our series, the heaviest subjects occurred in the angina pectoris group (Table II), whose mean relative weight was significantly greater than that of the myocardial infarction, peripheral arterial disease, and male control groups of the same age. Deapite the low-grade association already mentioned between weight and plasma triglyceride, levels of triglyceride or of other plasma lipids were not higher in the angina pectoris patients than in myocardial infarction or peripheral arterial disease patients. There was therefore no evidence in this study that the higher plasma lipid levels in the arterial disease groups were due to a greater proportion of overweight subjects in that group. In elucidation of the connection between overweight and ischaemic heart disease the most recently published findings of the Framingham Study might be of interest (Kannel et al., 1967). They explored the interrelationships between weight change, serum cholesterol, blood pressure levels, and the risk of developing ischaemic heart disease in 5,127 men

and women followed over twelve years for signs of initial development of ischaemic heart disease. Antecedent relative weight and weight gain after the age of 24 years proved to be strongly related to risk of angina pectoris and sudden death, but were unassociated with development of myocardial infarction. An excess risk of angina pectoris and sudden death appeared to exist in obese men both with and without elevations of blood pressure and serum cholesterol, indicating an independent contribution of obesity to the rate of development of these manifestations of coronary heart disease. Unless accompanied by an increase in blood pressure and serum cholesterol level, obesity appeared to play a negligible role in women.

Effect of Family History of Ischaemic Heart Disease

Heredity has been stated to be an important factor predisposing to ischaemic heart disease, and a correlation between myocardial infarction in parents and children has been shown (Boyd, 1953). In our series (Table II) there was a markedly greater proportion of subjects with a family history of ischaemic heart disease in the male arterial disease group than in the controls. The proportion of positive family histories was high in patients with peripheral arterial disease as well as in the myocardial infarction and angina pectoris patients. The difference between the female control and arterial disease patients in

this respect was much less striking. In subjects with a positive family history the increased risk of ischaemic heart disease, whether determined by genetic or environmental factors, might be mediated by plasma lipid abnormalities. The significantly higher plasma cholesterol levels seen in male controls with a positive family history (page 66) suggest that this is the case, although a large series of subjects in the same age group would be required to give a definite answer to this question. Since plasma lipid concentrations were elevated equally in arterial disease patients with and without such a family history, it would appear that environmental factors are at least as important as genetic factors in the determination of plasma lipid abnormalities.

Effect of Cigarette-Smoking

A higher proportion of cigarette smokers was found in the arterial disease group than in the controls (Table II). This was most striking in the peripheral arterial disease group which contained no non-smokers, the only patient who did not smoke cigarettes being a pipe smoker. In several population studies, the serum cholesterol has been observed to be higher in smokers than in non-smokers (Gofman et al., 1955; Thomas, 1958 and 1960; Miller et al., 1958; Karvonen et al., 1959; Dawber et al., 1959; Blackburn et al., 1960; Bronte-Stewart,

1961). The reason for this association is not known, and short-term smoking tests have shown no significant effect on the cholesterol level (Page et al., 1959; Kershbaum et al., 1961). The results of the present study showed no difference in plasma cholesterol between cigarette smokers and non-smokers in male controls. In male arterial disease patients cholesterol levels, particularly in older subjects, appeared to be higher in smokers than in non-smokers. This association between habitual cigarette-smoking and raised plasma cholesterol concentration might account for some of the difference between plasma cholesterol levels in older arterial disease patients and controls. As already mentioned, however, the most striking differences in cholesterol level between arterial disease and control subjects were seen in the younger age groups. There was no correlation between smoking and plasma triglyceride or phospholipid levels.

In both control and arterial disease subjects after overnight abstention from smoking, habitual cigarette-smokers had lower fasting NEFA levels than non-smokers (page 71). This unexpected finding has not been reported previously. Cigarette-smoking in the fasting state has been shown to cause elevation of plasma NEFA levels due to catecholamine release (Kershbaum et al., 1963). A possible explanation of the lower fasting plasma NEFA levels recorded in habitual cigarette-smokers in

this study is withdrawal of the repetitive nicotine-induced stimulation of the adrenal glands to which these subjects have become adapted, with reduction in circulating catecholamines, and consequent fall in plasma NEFA level.

Variability of Plasma Lipid Levels

There was a fairly high correlation between two fasting plasma cholesterol estimations in the same individuals at an interval of 24 to 48 hours (Table VIII). Large fluctuations in plasma cholesterol level from day to day, and even from hour to hour, have been reported in certain individuals, whereas others appear to have a stable cholesterol level (Thomas and Eisenberg, 1957; Peterson et al., 1960). Some observers (Steiner and Domanski, 1943; Morrison et al., 1949) have suggested that the serum cholesterol of patients with ischaemic heart disease is inconstant and fluctuates widely, in contrast to normal persons in whom the level is relatively stable. This has not been borne out by the findings in this study of a rather closer correlation between plasma cholesterol levels in arterial disease patients than in controls.

Day to day variations in plasma levels of other lipids have received little attention. Our figures showed a fairly good reproducibility of phospholipid and triglyceride levels in individuals. Repeated estimations of fasting plasma NEFA

levels in our subjects also showed a fairly high correlation. Hollister and Overall (1964) reported a marked variability in fasting NEFA estimations in the same individuals on several outpatient visits. The higher correlation in our subjects is almost certainly due to the controlled basal conditions under which the blood samples were taken for estimation of this very labile plasma constituent.

Interrelation of Fasting Plasma Lipids

Correlation of the different plasma lipid concentrations in the same blood samples showed relationships between cholesterol, phospholipid, and triglyceride levels, while plasma NEFA levels appeared totally unrelated (Table IX). The closest relationship observed was that between cholesterol and phospholipid, and the next in magnitude that between phospholipid and triglyceride. These results might be explained by consideration of the mechanisms for transport of lipid in fasting blood. Cholesterol, phospholipid, and triglyceride are distributed among the lipoproteins in the globulin fraction (Table I) and it might be anticipated that alterations in one lipid would be associated with alterations in the others. An increase in low-density lipoprotein would be shown by an increase in cholesterol and a rather lesser increase in phospholipid, but would have little effect on

triglyceride. An increase in very low-density lipoprotein, on the other hand, would be reflected by an increase in triglyceride, a considerably smaller increase in phospholipid, and a still smaller increase in cholesterol. Plasma NEFA, on the other hand, are transported by albumin, and plasma levels may therefore fluctuate widely independently of the other lipid levels.

SUMMARY AND CONCLUSIONS

1. Plasma cholesterol, phospholipid, and triglyceride concentrations showed marked changes with age in healthy male and female subjects. Young males had higher levels of these lipids than females, whereas in older subjects the situation was reversed. Plasma non-esterified fatty acids showed no variation with age or sex.

2. Young male subjects with arterial disease had higher plasma levels of cholesterol, phospholipid, and triglyceride than controls of the same age. Differences in plasma lipid levels between older male and female arterial disease patients and controls were small in magnitude. Plasma non-esterified fatty acid levels did not differ significantly in arterial disease patients and controls.

3. Similar elevations of plasma cholesterol, phospholipid, and triglyceride were present in patients with three different clinical types of atherosclerotic arterial disease - myocardial infarction, angina pectoris, and peripheral arterial disease.

4. A low-grade association was demonstrated between body weight and plasma triglyceride concentration in arterial disease patients only. There was no association between body weight and the other plasma lipids.

5. Cigarette-smokers showed significantly lower plasma NEFA levels than non-smokers after overnight abstention from smoking. There was some evidence that cigarette-smokers, particularly those with moderate or heavy cigarette consumption, had higher cholesterol levels than non-smokers, but the results were inconclusive.

6. Plasma lipid concentrations were similar in arterial disease patients with and without a family history of ischaemic heart disease. The small number of male controls with a positive family history had higher plasma cholesterol levels than others in the same age group.

7. Variations in plasma cholesterol and plasma triglyceride concentration were each associated with a similar change in plasma phospholipid level.

Chapter IV. FAT TOLERANCE TESTS IN HEALTHY AND ATHEROSCLEROTIC SUBJECTS

CONTENTS

Chapter IV. FAT TOLERANCE TESTS

INTRODUCTION

Alimentary lipaemia is caused by the presence in the blood of newly-absorbed neutral fat in the form of fairly large particles known as chylomicrons. This phenomenon has been the subject of intensive study in the past sixty years. Earlier workers (Gage and Fish, 1924; Frazer and Stewart, 1937a) used it to make fundamental observations on the mechanisms of fat digestion and absorption, while more recently it has aroused interest because of its alleged role in the causation of atherosclerosis. Following Hueper's (1942) experiments demonstrating that macromolecular substances injected intravenously in animals were deposited in lesions in the arterial intima, Moreton (1947) advanced his hyper- and macrochylomicronaemic theory of atherosclerosis. He found that in clinically healthy subjects there were considerable differences in the height and duration of chylomicron counts following a fat meal. He suggested that prolongation of alimentary lipaemia following repeated ingestion of fatty meals over a period of years was an important atherogenic factor. The finding that chylomicron counts were higher and more prolonged in elderly than in young subjects (Marder et al., 1952) was considered to support this theory, as was

the observation by Zinn and Griffith (1950) of an increased proportion of large chylomicrons in fasting and postprandial blood samples in atherosclerotic individuals. These findings stimulated further investigations of fat tolerance in atherosclerotic subjects, most often the survivors of myocardial infarction. In general, atherosclerotic subjects were found to have more intense and prolonged alimentary lipaemia than persons with no clinical evidence of atherosclerosis (Schwartz et al., 1952; Pomeranze et al., 1954; Barritt, 1956).

In this section an outline of the development of knowledge of alimentary lipaemia will be given, followed by a description of the various methods of quantitating lipaemia and of the fat load used. The results of a standard fat tolerance test in healthy and atherosclerotic subjects will then be presented, and compared with those reported in the literature.

EARLY INVESTIGATIONS OF ALIMENTARY LIPAEMIA

For more than three hundred years it has been known that the serum may become lactescent after ingestion of food. The earliest studies of this phenomenon were reviewed by Gage and Fish (1924). Asellius in 1622 observed that, when food was present in the intestine, the mesenteric lymphatics were rendered visible by the milky appearance of the absorbed food.

In 1650 Pecquet traced the lymphatic channels to the great veins of the neck, and thus showed that the milky fluid absorbed from food in the intestine travelled directly to the blood vessels, and not to the liver as had been thought by Asellius. It was Boyle, however, in 1665 who first discovered that the serum itself may become lactescent as a result of the abundance of absorbed food poured into it through the lacteals and the thoracic duct. In 1774 Hewson observed that the milky serum when examined under the microscope showed numerous fine particles. He also demonstrated that it contained much fat, since it left a grease spot when dried upon paper, and recognised that there were two possible sources of these fat particles: namely the fat in the food, and that remobilised from the fat reservoirs of the body. Hewson's observations were later confirmed and amplified by Gulliver (1840), who showed that the small particles in the lacteal fluid were the same as those in the blood stream. Edmunds in 1877 used the darkfield microscope to study the particles. The first attempt to quantitate the blood lipid changes following a fat load by counting the particles appears to have been made by Neumann in 1907. In 1920 Gage suggested the term chylomicron to designate these fat particles. In collaboration with Fish (1924), he described a technique for chylomicron counts, which they used to obtain information about

the digestibility and absorption of fat in animals and man. Although they presented evidence that the particles were composed of fat, this appears to have been disputed for some years. In 1939 Elkes et al. concluded as a result of further experiments that they consisted of fat, with possibly a layer of adsorbed protein at the oil-water interface. It is now well established that the chylomicron contains protein as an integral part of the particle and is, therefore, a lipoprotein (Borgström, 1960).

METHODS OF QUANTITATING ALIMENTARY LIPAEMIA

Until Van Handel and Zilversmit (1957) described their technique of triglyceride estimation there was no simple, reliable, direct method available for measuring neutral fat. A wide variety of methods were introduced for the quantitation of alimentary lipaemia, and an outline of some of these methods is given below.

(a) Chylomicron Counts

A semiquantitative measurement of lipaemia may be obtained by diluting plasma in some standard way, and counting the chylomicrons in a shallow chamber. This technique, pioneered by Neumann (1907), was developed further by Gage and Fish (1924),

and Frazer and Stewart (1936), and remained in use until the 1950's (Marder et al., 1952; Grüner and Hilden, 1953). It had the advantages over the somewhat crude chemical methods for blood lipid estimations available thirty years ago of being rapid, and of requiring only small volumes of blood. When done carefully, the counts were sufficiently reproducible to reflect changes in particle content of blood during alimentary lipaemia, parallelism being obtained between curves from chylomicrographs and quantitative determination of blood neutral fat by other means (Frazer and Stewart, 1937b; Burr et al., 1954). This technique was later superseded, when it was recognised that the counts were at best only relative values on an arbitrary scale. It proved impossible to obtain absolute values, as in the counting of blood cells, for a number of reasons: the difficulty of counting particles in Brownian motion (which caused particles to fluctuate in and out of focus): the indeterminancy of the lower size limit (making the number of particles that could be counted depend on conditions of lighting and focus): and an apparent increase in the number of particles with dilution (Dole and Hamlin, 1962).

(b) Measurements of Turbidity

The clarity or turbidity of plasma or serum is determined by the size of the fat particles, since particles of more than

one quarter the wavelength of visible light (or 0.1 μ) interrupt light rays, thus giving the serum the appearance of turbidity or lipaemia (Ahrens and Kunkel, 1949). Albrink et al. (1955) showed by ultracentrifugation studies that neutral fat is the only serum lipid consistently associated with lactescence. In order to measure the size as well as the number of fat particles in lipaemic serum, Moreton described a nephelometric method which measured reflected light (Moreton, 1947). A somewhat simpler method introduced later was measurement of the optical density by spectrophotometry; that is, the estimation of the degree of light absorption by lipaemic serum, usually at a wavelength of 650 mμ (range 610-700 mμ). The latter method was the one more commonly used (Barritt, 1956; Bronte-Stewart and Blackburn, 1958; Denborough, 1963). There was general agreement that optical density measurements provided a fairly reliable indication of postprandial changes in serum neutral fat content (Barritt, 1956; Osmon et al., 1957; Jones and Dobrilovic, 1963).

(c) Chemical Estimations

The direct chemical estimation of triglyceride in alimentary lipaemia has been carried out only in recent years (Nikkilä and Konttinen, 1962; Denborough, 1963). Earlier chemical means used were measurement of total plasma lipid by

gravimetric or colorimetric methods (Elkes et al., 1939; Barritt, 1956), or plasma total fatty acids (Chaikoff et al., 1934; Brown et al., 1961). Albrink (1959) described an indirect method of triglyceride estimation by subtraction of cholesterol and phospholipid fatty acids from total plasma fatty acids, and this method was used by a number of investigators (Sullivan, 1962; Talbott and Keating, 1962).

(d) Radioactive Fat Tolerance Tests

Thannhauser and Stanley (1949) introduced the use of I^{131} labelled fat in fat tolerance tests, using I^{131} olive oil. This method had the advantages that the amount of labelled fat required was small and well tolerated, and the radioactive iodine that split off the fatty acid molecule could be determined and information gained as to the speed of catabolism of the labelled fat. The first systematic study of alimentary lipaemia in coronary heart disease using I^{131} labelled triolein was carried out by Likoff and co-workers (1958), and subsequent reports have been published by investigators using this method (Seller et al., 1959; Berkowitz and Croll, 1962). The lipid-bound radioactivity curve has been found to have a close relationship to the fat tolerance curve obtained by other methods, such as optical density and total fatty acids (Brown et al., 1961).

(e) Vitamin A and E Loading Tests

Although the fat soluble vitamin A has been employed as an indicator in the study of fat absorption since the 1930's (Chesney and McCoord, 1934), it has been used in only a few investigations of fat metabolism in hyperlipaemia. Beaumont and his colleagues carried out extensive studies of vitamin A metabolism in patients with ischaemic heart disease, and attributed the frequent occurrence of high plasma vitamin A curves in these subjects to faulty metabolism of chylomicrons (Beaumont and Ardaillou, 1959; Beaumont and Lenègre, 1959). Pelkonen (1963) pointed out that, since vitamins A and E were purely exogenous lipids, they might be used to facilitate interpretation of alimentary lipaemia by eliminating disturbances in endogenous synthesis of lipids. From his findings of high and prolonged plasma vitamin A and E curves in coronary heart disease patients he concluded that the disappearance rate of exogenous lipids was impaired in these subjects.

(f) Additional Lipid Analyses

A number of other methods of lipid analysis have been used in the study of plasma neutral fat in alimentary lipaemia: for example, lipoprotein separation by paper electrophoresis

(Talbott and Keating, 1962), starch electrophoresis (Swahn, 1953: Bierman et al., 1962a) ultracentrifugation (Havel, 1957; Pelkonen and Nikkilä, 1965), and differential flocculation in polyvinylpyrrolidone density gradient columns (Bierman et al., 1962b). These methods have been used to characterise the heterogeneous fat particles which, as described by Gage and Fish (1924), are present in the blood during alimentary lipaemia. The chylomicrons are small (about 0.5 μ) at the beginning and end of the fat tolerance curve, but larger (about 1 μ) at maximal lipaemia.

Numerous investigators of alimentary lipaemia have measured, in addition to neutral fat, the other blood lipids such as cholesterol (e.g. Osmon et al., 1957; Kingsbury et al., 1960), phospholipids (Man and Gildea, 1932; Pomeranze et al., 1954), and, more recently, the plasma nonesterified fatty acids (Dole, 1956; Albrink and Neuwirth, 1960).

TECHNIQUE OF THE FAT TOLERANCE TEST

No single method of performing oral fat tolerance tests has yet achieved widespread acceptance, and almost every investigator has developed his own technique. In most cases this has taken the form of the feeding, after an overnight fast, of 50 to 100 g. of fat, either as a fixed standard load

or on a body weight basis. The fat has usually been of animal origin (eggs, bacon, or cream), and in most cases has been part of a mixed meal, with or without standardisation of the amounts of protein and carbohydrate. When fat alone has been given it has usually been in the form of cream (Brown et al., 1961; Pelkonen and Nikkilä, 1965) or a vegetable oil (Eggstein and Schettler, 1958; Likoff et al., 1958).

Effect of Size of Fat Load on Lipaemia

As might be anticipated, the degree of lipaemia was found to increase with an increase in the amount of the fat load (Gage and Fish, 1924; Marder et al., 1952). Although a fat load of 20 to 35 g. was suitable for the study of alimentary lipaemia by the technique of chylomicron counting, (Gage and Fish, 1924; Marder et al., 1952), it was much less satisfactory for chemical lipid estimations. Kingsbury et al. (1960) stated that plasma lipid changes after fat meals of less than 30 g. were often small and inconsistent, and they recommended a fat load of 50 g. Brown et al. (1963), comparing the effect of meals of 10, 30, 50, and 70 g. in healthy young males, found no significant change in lipids after the 10 g. meal, but consistent elevation after meals of 30 g. fat or more. A few workers used much larger fat loads. Talbott and

Keating (1962) used 177 g. and Pomeranze et al. (1954) 204 g. of fat, while Man and Gildea (1932) gave their subjects 3.5 to 4 g. per kg. of body weight. It was subsequently pointed out by Kingsbury et al. (1960) that fat loads much greater than 70 g. did not cause a proportionate increase in plasma lipids and had the disadvantage of frequently producing nausea.

Effect of Type of Fat

Bang (1918) pointed out that the degree of alimentary lipaemia was affected by the type of fat fed. Eggstein and Schettler (1958) found that lipaemia reached its peak earlier and declined more quickly when liquid oils were fed instead of hard fats. On the other hand Jones and Dobrilovic (1963) reported a delay in peak rise of optical density when corn oil replaced dairy fat. Kingsbury and Morgan (1960) found similar rises in triglyceride but markedly differing effects on cholesterol and phospholipid on feeding different types of oil (arachis oil, heated and crude tobacco seed oils). Emulsification of the lipid in the study of Brown et al. (1961) gave an earlier peak in the plasma lipid concentration curve. Different fats have been shown to give rise to specific types of chylomicrons (Swank and Levy, 1952). Chylomicrons seen after feeding of tristearin and cream varied in shape and were small, whereas triolein gave large, distinctive,

spherical chylomicrons.

Effect of Carbohydrate

There is general agreement that ingestion of carbohydrate along with fat reduces lipaemia. Bang (1918), in his experiment on fat-feeding in dogs, noted that when bread was substituted for meat in the fat meals, the degree of lipaemia was sometimes reduced. Albrink and her co-workers demonstrated that alimentary lipaemia in humans could be reduced by simultaneous oral or parenteral administration of glucose (Albrink and Man, 1956; Man and Albrink, 1956; Albrink et al., 1958). Berkowitz et al. (1959) found that a lower triglyceride curve was obtained when glucose was given with I^{131} labelled triolein than when triolein alone was given. Sullivan (1962) confirmed that the lipaemia occurring after ingestion of a standard fat meal was depressed or eliminated by the addition of glucose to the meal.

Effect of Protein

Bang (1918) in his experiments on dogs found that, although meat alone did not cause lipaemia, the addition of meat to fat meals frequently produced lipaemia greater than that resulting from fat meals alone. Sullivan (1962) also produced prolongation and intensification of alimentary

lipaemia in 4 of his 6 subjects by the addition of protein to a fatty meal.

Effect of Previous Diet on Fat Tolerance

Alimentary lipaemia has been shown to be influenced by the composition of the diet prior to fat loading. Total starvation was found by Gage and Fish (1924) and Albrink and Neuwirth (1960) to cause a lowering in postprandial lipaemia. Reduction of the dietary fat intake had a similar effect in the subjects studied by Pomeranze et al. (1954) and Bronte-Stewart and Blackburn (1958). The substitution of vegetable oils for animal fats in the diet for several weeks was also found to lower fat tolerance curves (Bronte-Stewart and Blackburn, 1958; Denborough, 1963). On the other hand, an increase in the amount of fat in the diet was found by Blix (1926) and Havel (1957) to cause an exaggeration of lipaemia following a fat load, although Horlick (1957) found no change following a smaller dietary fat increase, and Bouchier and Bronte-Stewart (1961) demonstrated no fat tolerance differences in three racial groups with widely differing fat intakes.

REPRODUCIBILITY OF FAT TOLERANCE CURVES IN INDIVIDUALS

A number of investigators have tested the repeatability of the fat tolerance curve in individuals at varying intervals of time. The consensus of opinion appears to be that, although the reproducibility is as a rule fairly close, considerable unexplained variations may occur. Osmon *et al.* (1957) found good reproducibility in tests performed one week apart in two normal young males. Repeated tests in fifteen coronary patients and ten controls by Bronte-Stewart and Blackburn (1958) showed considerable individual variations but the mean curves for the two groups were unchanged. Jones and Dobrilovic (1963) carried out 62 fat tolerance tests in five patients on diets of different fat content. They found that the shape of the fat tolerance curve was dependent on, among other factors, the previous dietary fat intake, and also commented on the fact that unexplained variations occurred in the curves. Denborough (1963) repeated fat tolerance tests in twelve subjects after six weeks to nine months. In ten subjects the curves were very similar to those obtained previously, whereas in the other two subjects the second curve was much lower. The explanation in one case was thought to be marked loss of weight in the intervening period, while no reason for the difference was evident in the other case. Hollister (1963) carried out duplicate

fat tolerance tests one week apart in 12 subjects. He found that, though the pattern of lipaemic response tended to be repeated from one trial to another, the replication was poor at 2 and 4 hours after the meal. A good correlation was obtained between the increases in triglyceride at 6 hours postprandially in the two tests.

PREVIOUS INVESTIGATIONS OF FAT TOLERANCE IN ATHEROSCLEROSIS

In this section are reviewed the reports of fat tolerance tests in atherosclerosis published before the present study was commenced in 1964. Most of these investigations were carried out before a relatively simple, reliable method of triglyceride estimation came into general use, and so indirect methods of measuring neutral fat such as optical density or serum fatty acids were used. In most cases it was found that the lipaemia produced by a fat load in survivors of myocardial infarction, or in patients with angina pectoris, was greater than that produced in persons with no clinical evidence of ischaemic heart disease.

Schwartz et al. (1952) compared 24 male and female controls (aged 17 to 79 years) with 23 patients who had recently sustained an acute myocardial infarction, and found in the coronary group higher optical density readings in the fasting state and 3

and 5 hours postprandially. There was, however, a considerable overlap between the curves obtained in the two groups. They suggested that the lipid abnormality might have been related to the recent myocardial infarction, or to the diet taken by the infarct patients. Woldow et al. (1954) also found increased and prolonged plasma optical density levels in 21 patients with coronary disease, although the ages of the coronary group (mean 55 years) and the control group (mean 31 years) were not comparable. In the same year Pomeranze et al. (1954) reported increased lipaemia, measured as serum fatty acids, in an elderly atherosclerotic group as compared with a younger control group. Barritt (1956) confirmed the presence of increased alimentary lipaemia as measured by serum total lipids in 35 coronary patients four to eight weeks after a myocardial infarction, with again a wide overlap between the fat tolerance curves of the coronary and control subjects. He also drew attention to the fact that intense, prolonged lipaemia occurred in those subjects who already had high fasting lipid levels. Delayed lipaemic peaks were recorded by Kuo et al. (1956) in the majority of their coronary heart disease patients, as well as in hyperlipaemic and hypercholesterolaemic individuals. Bronte-Stewart and Blackburn (1958) also found, despite considerable overlap in the results, significant

differences in optical density between twenty-three patients six months to two years after a myocardial infarction and thirteen controls. They commented on the absence of any correlation between the serum cholesterol level and the degree of lipaemia as measured by optical density, and emphasised that the differences between the coronary and control groups were more prominent in the latter part (or 'clearing phase') of the fat tolerance test than in the earlier, primarily absorptive, phase. These optical density differences were confirmed in subsequent investigations (Bouchier and Bronte-Stewart, 1961; Marks et al., 1962). Although the total fatty acids in cases of coronary heart disease remained elevated longer than in healthy young male controls, Horlick (1956 and 1957) found that in his series the high fat test meal failed to uncover or exaggerate a lipid metabolic defect in individuals with atherosclerosis. Stutman et al. (1961) demonstrated a delay in return to normal of optical density values after fat loading in thirteen coronary patients under 40 years, and suggested that the 9 hour serum optical density offered a simple, practical, and effective tool for separating atherosclerotic from healthy subjects in large populations. Brown et al. (1961) observed that the 9-hour total fatty acid levels gave better separation of their coronary patients from healthy subjects

than either the 9-hour optical density values, the cholesterol, or the fasting total fatty acid levels. Denborough (1963) confirmed by optical density and triglyceride estimations that fat tolerance curves were elevated in some post-myocardial infarction patients and emphasised that high postprandial triglyceride levels were related to high fasting triglyceride levels, and that reduction of the fasting triglyceride level resulted in a reduction in alimentary lipaemia.

Increased alimentary lipaemia in ischaemic heart disease patients was also observed when the lipid-bound blood radioactivity level was used as an index of lipaemic response after ingestion of I^{131}-labelled fat (Likoff et al., 1958; Seller et al., 1959; Brown et al., 1961).

METHODS

Subjects

The subjects for the fat tolerance tests were the same as those whose fasting plasma lipid levels were described in Chapter III: namely, 50 healthy male controls aged 20 to 64 years; 22 healthy female controls aged 18 to 68 years; 72 male patients with atherosclerotic arterial disease (myocardial infarction 27, angina pectoris 19, peripheral arterial disease

26) aged 36 to 70 years; and 14 female patients with atherosclerotic arterial disease aged 50 to 70 years. Further details of these subjects were given on page 57.

Composition of Test Meal

This was a semiliquid meal of the following composition:

Fat	75 g.	- as cream (Nestlé's Sterilised Cream* 325 g.)
Carbohydrate	35 g.	- as glucose (dextrose)
Protein	15 g.	- as milk protein (Casilan).

In addition to the 35 g. of glucose and 15 g. Casilan, a further 12 g. (approx.) of carbohydrate and 9 g. (approx.) of protein were present in the cream.

The ingredients were weighed and mixed with water in a blender on the day before use. The mixture was stored overnight in a refrigerator, and made up to a volume of 750 ml. with water immediately before use.

Procedure

The subjects, having fasted overnight, had a blood sample taken for estimation of fasting lipids, as described in Chapter III. They were then asked to drink the test meal within a period of 30 minutes. Blood samples were taken at 2, 4, 6, and

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*Nestlé Company Ltd., St. George's House, Wood St., London, E.C.2.

8 hours from commencement of the meal. In some cases an additional blood sample was taken at one hour. No further food or drink was taken, and cigarette-smoking was not permitted until the test was completed. Light activity in the ward was allowed during the test, but subjects returned to bed for 30 minutes before each venepuncture.

Estimations

Blood samples for estimation of plasma triglyceride, total cholesterol, phospholipid, and total NEFA were processed as described in Chapter II. In a number of cases the haematocrit was measured by centrifugation of blood, anti-coagulated with sodium EDTA, in haematocrit tubes at 3,000 r.p.m. for 30 minutes in a centrifuge with swing-out head of 15 cm. radius.

RESULTS

CHANGES IN PLASMA TRIGLYCERIDE DURING FAT TOLERANCE TESTS

(a) Male and Female Controls

The mean triglyceride values obtained in male and female controls of all ages during the fat tolerance tests are shown in Table X and Fig. 18. The peak triglyceride level was

TABLE X. Fat Tolerance Tests in Control and Arterial Disease Subjects (mean ± S.D.)

Time of Sample	Plasma Triglyceride mg. per 100 ml.			
	Controls male all ages	Controls female all ages	Arterial Disease male	Arterial Disease female
Fasting	89.4 ± 32.0 (n = 50)	80.3 ± 16.6 (n = 22)	108.4 ± 42.0 (n = 72)	85.7 ± 18.3 (n = 14)
1 hour	106.6 ± 33.4 (n = 24)	83.7 ± 16.4 (n = 6)	129.2 ± 54.8 (n = 33)	100.7 ± 17.8 (n = 10)
2 hour	131.2 ± 48.5 (n = 50)	107.3 ± 20.9 (n = 22)	151.2 ± 63.2 (n = 72)	125.9 ± 26.8 (n = 14)
4 hour	172.6 ± 81.2 (n = 50)	139.0 ± 48.1 (n = 22)	194.2 ± 90.0 (n = 72)	143.9 ± 36.2 (n = 14)
6 hour	157.5 ± 88.5 (n = 50)	126.8 ± 60.4 (n = 22)	181.4 ± 92.0 (n = 72)	136.2 ± 36.9 (n = 14)
8 hour	103.7 ± 63.1 (n = 50)	94.0 ± 42.1 (n = 21)	134.7 ± 80.8 (n = 72)	107.1 ± 42.6 (n = 14)

n = no. of observations

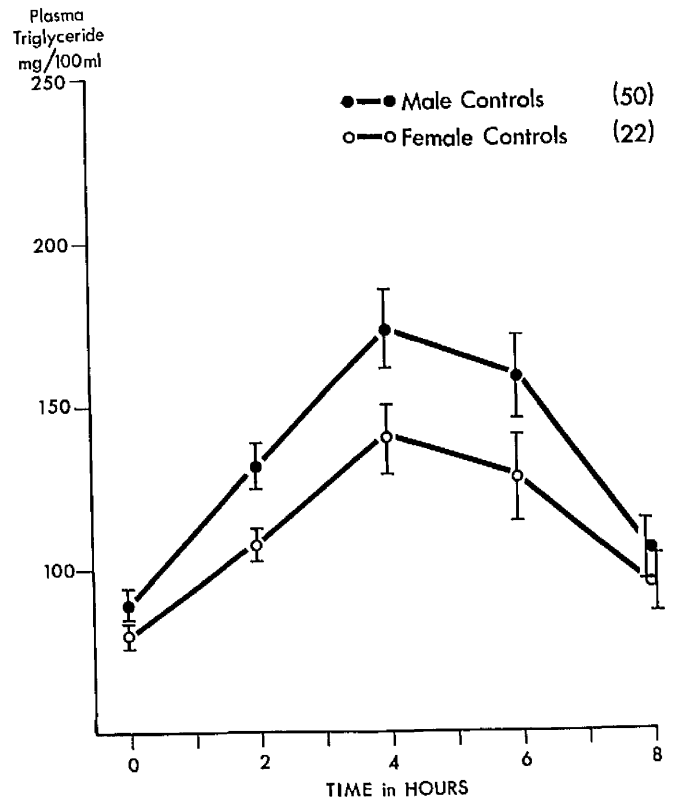


Fig. 18

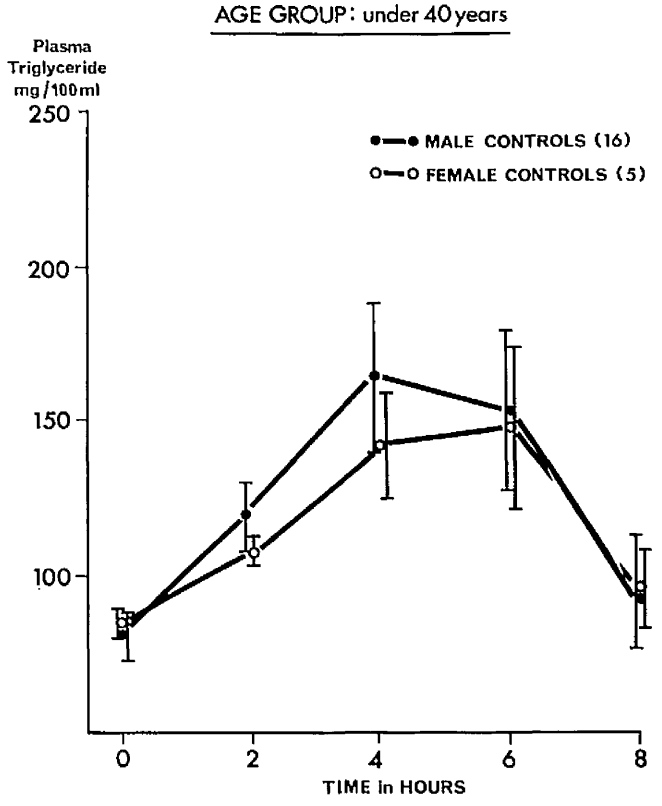


Fig. 19

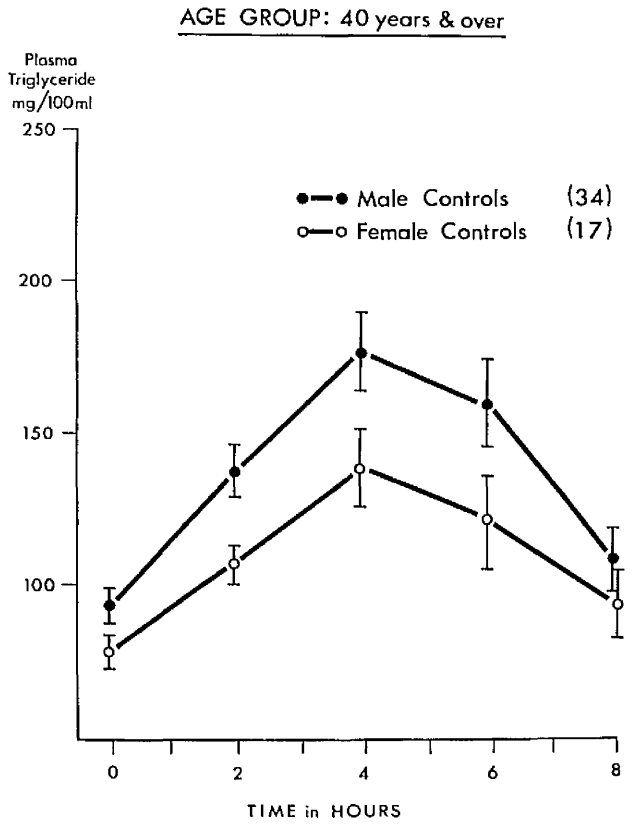


Fig. 20

Figs. 18 to 20. Comparison of fat tolerance tests in male and female controls (mean ± S.E.M.)

attained 4 hours after ingestion of the fat load. On comparison of male and female controls of all ages, the fasting triglyceride level was higher in the males. The differences in mean triglyceride level between males and females became more marked at 2, 4 and 6 hours after the meal, but at no time were the differences statistically significant. On subdivision of the controls into the different age groups, it was evident that the greatest differences in fat tolerance curves between males and females were present in the 40 to 44 year age group (Fig. 21), in which the males had a significantly higher 4-hour level, and in the 45 to 54 year age group (Fig. 22) when the 2-hour level in the males was significantly higher. In subjects under the age of 40 years, the fat tolerance curves tended to overlap (Fig. 19), while in the 55 to 64 year age group both fasting and postprandial triglyceride levels were slightly higher in the females (Fig. 23).

(b) Male and Female Arterial Disease Patients

The mean triglyceride values obtained during fat tolerance tests in male and female arterial disease patients are shown in Table X. Comparison of the fat tolerance tests of arterial disease females with those of males of corresponding age (Fig. 24) revealed slightly higher fasting levels in males. During

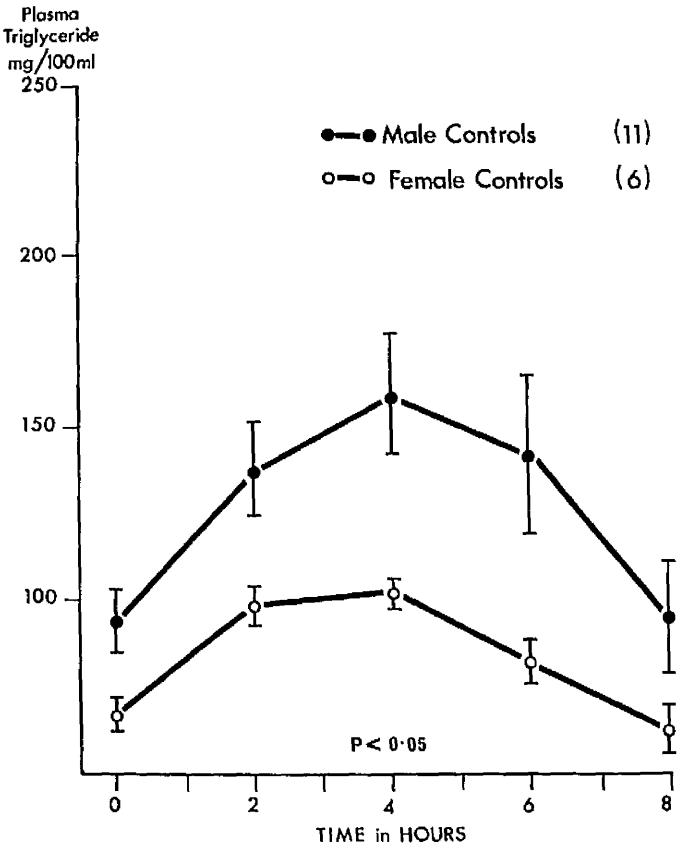


Fig. 21

AGE GROUP: 45 to 54 years

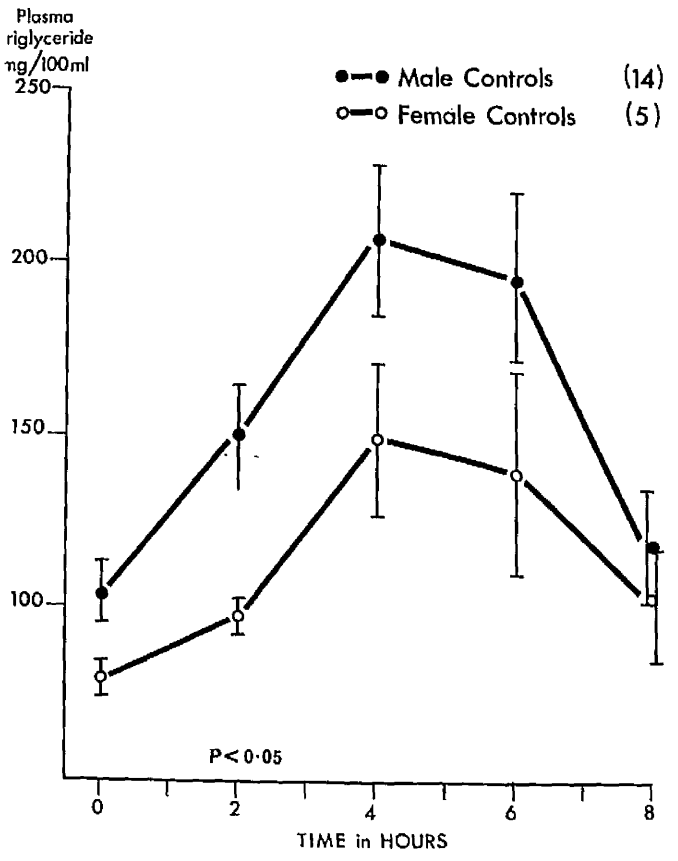


Fig. 22

AGE GROUP: 55 to 64 years

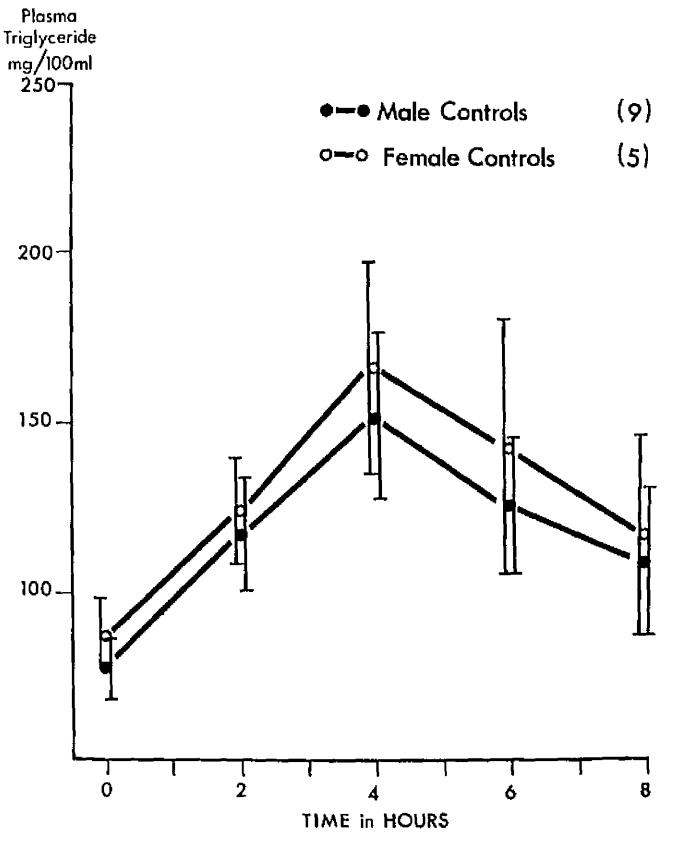


Fig. 23

Figs. 21 to 23. Comparison of fat tolerance tests in male and female controls (mean ± S.E.M.).

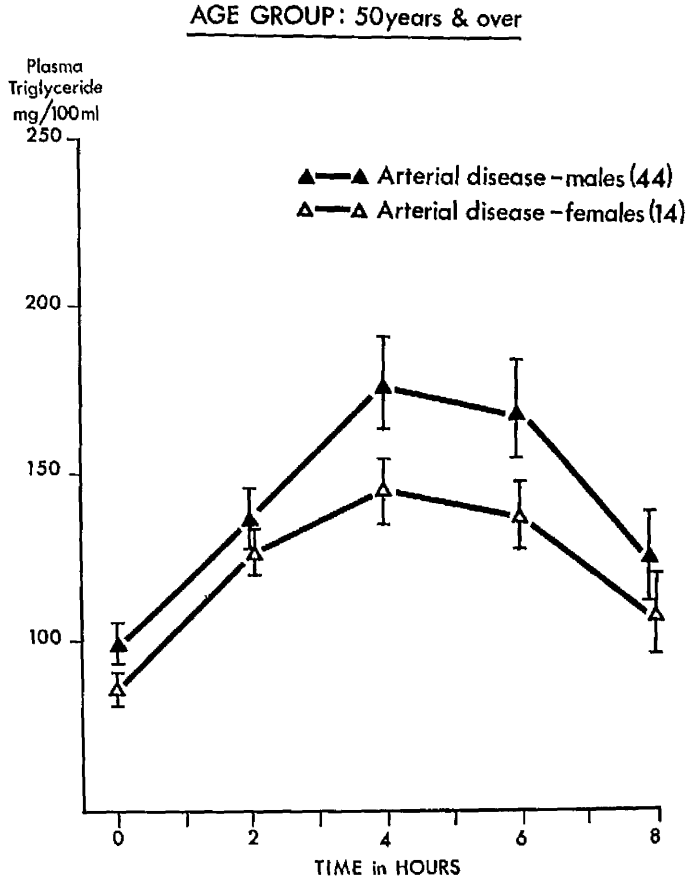


Fig. 24. Comparison of fat tolerance tests in male and female arterial disease patients (mean ± S.E.M.).

the fat tolerance test the differences between males and females were increased at 4 and 6 hours, but at no time did they attain levels of statistical significance.

Comparison of fat tolerance tests in male patients with myocardial infarction, angina pectoris, and peripheral arterial disease revealed no significant differences in the three groups (Table XI).

(c) Comparison of Controls and Arterial Disease Subjects

Comparison of fat tolerance tests in 70 male arterial disease patients with those of the 34 male controls aged 40 years and over (Fig. 25) revealed similar plasma triglyceride curves in the two groups. Both fasting and postprandial triglyceride levels were slightly higher in the arterial disease patients than in controls, but the differences were not statistically significant. The 8-hour triglyceride level gave a separation of arterial disease patients from controls that was only marginally better than that given by the fasting triglyceride level.

On subdivision into age groups, it became evident that the distinction in fat tolerance tests between controls and arterial disease patients was greater in the 40 to 44 year age group (Fig. 26). Although there was still some overlap in

TABLE XI. Fat Tolerance Tests in the Three Groups of Male Arterial Disease Patients (mean ± S.D.)

Time of sample	Plasma Triglyceride mg. per 100 ml.		
	Myocardial Infarction	Angina Pectoris	Peripheral Arterial Disease
Fasting	102.8 ± 41.6 (n = 27)	105.1 ± 36.0 (n = 19)	116.7 ± 46.4 (n = 26)
1 hour	124.1 ± 56.2 (n = 16)	134.0 ± 55.8 (n = 7)	134.0 ± 57.1 (n = 10)
2 hour	137.6 ± 61.8 (n = 27)	149.5 ± 53.3 (n = 19)	166.5 ± 69.8 (n = 26)
4 hour	173.8 ± 91.3 (n = 27)	212.2 ± 85.9 (n = 19)	202.3 ± 90.9 (n = 26)
6 hour	163.2 ± 89.8 (n = 27)	192.3 ± 82.7 (n = 19)	192.5 ± 100.8 (n = 26)
8 hour	128.3 ± 70.4 (n = 27)	118.3 ± 56.3 (n = 19)	153.3 ± 102.5 (n = 26)

n = no. of observations

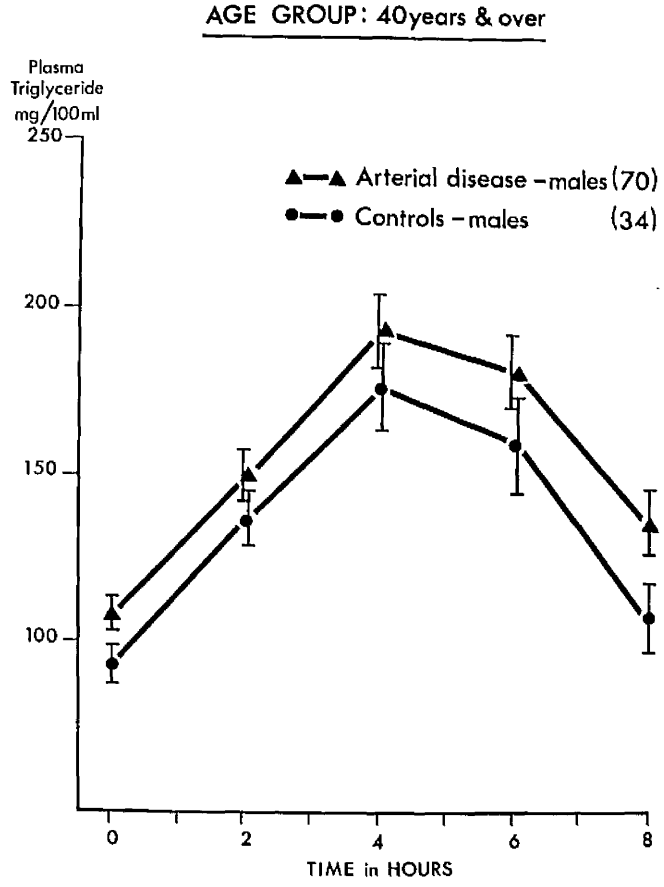


Fig. 25

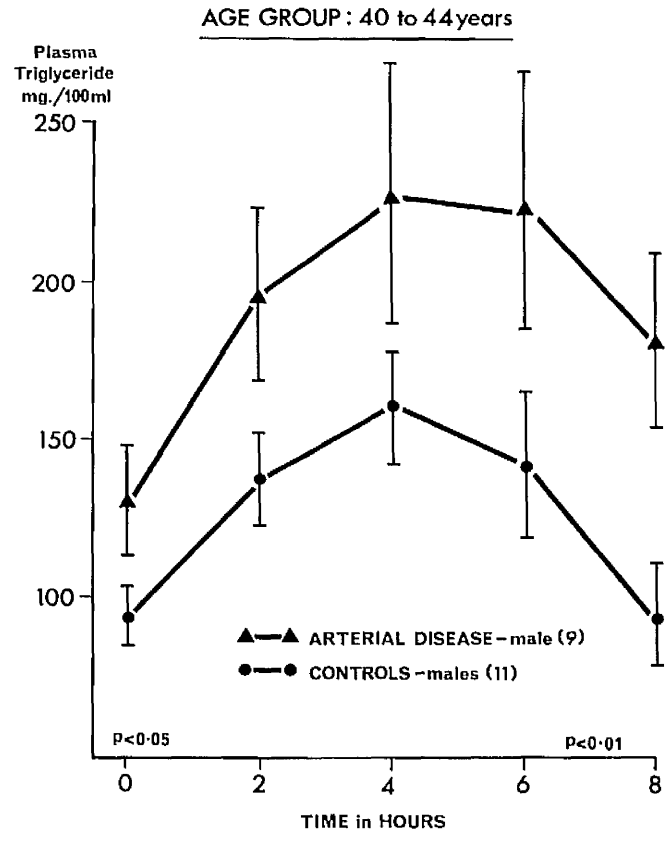


Fig. 26

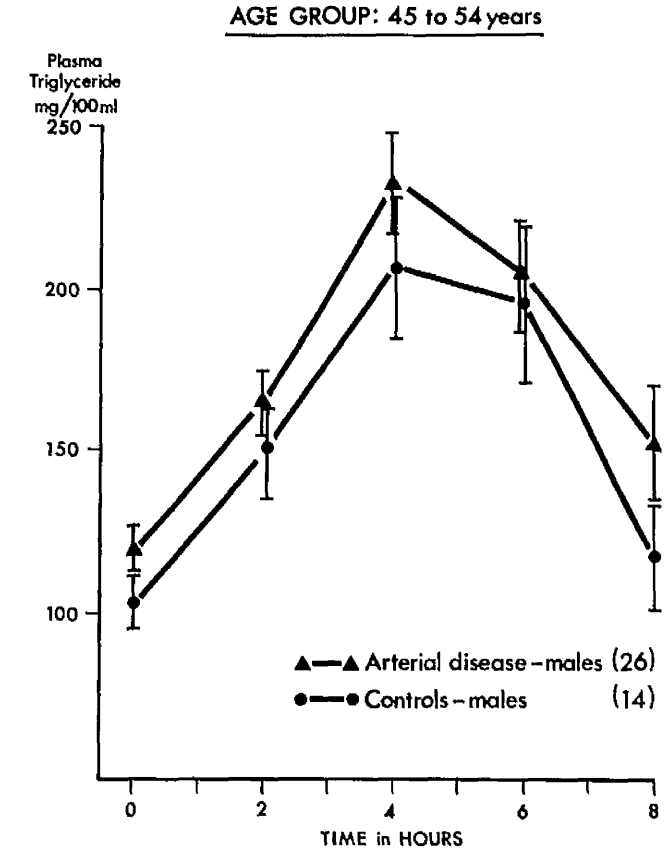


Fig. 27

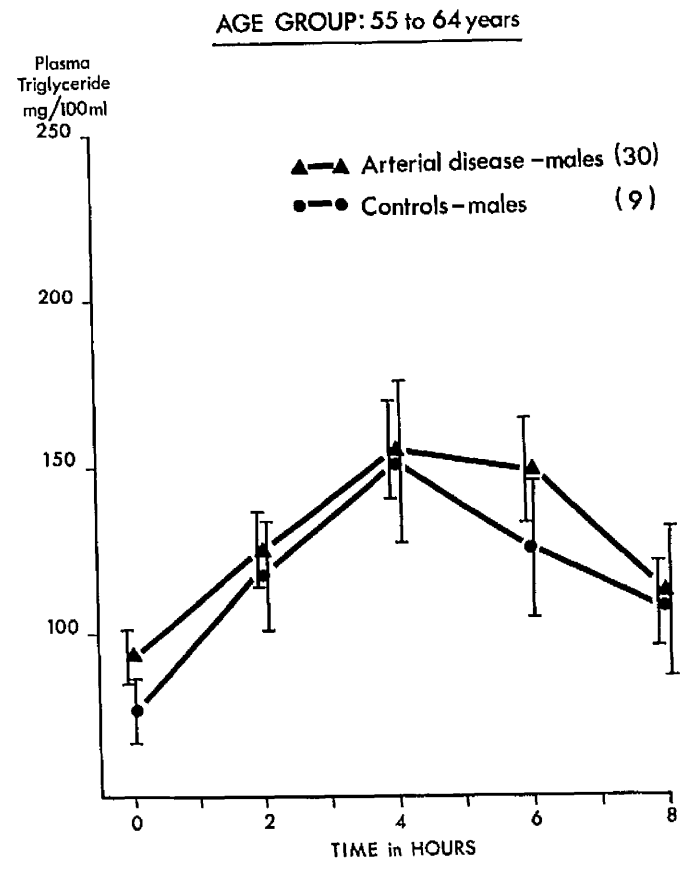


Fig. 28

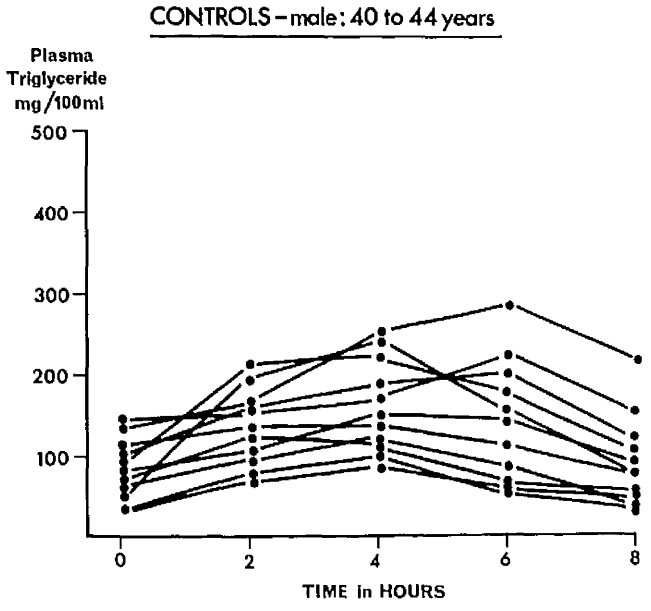
Figs. 25 to 28. Comparison of fat tolerance tests in male controls and arterial disease patients (mean ± S.E.M.)

fasting and postprandial triglyceride levels between controls and arterial disease patients (Figs. 29 and 30), the mean fat tolerance curve in patients was higher than that in healthy controls. In patients the 4- and 6-hour triglyceride levels were similar, whereas in controls there was a considerable fall in triglyceride between 4 and 6 hours. The differences in triglyceride level between patients and controls were statistically significant in fasting blood, and in the 8-hour sample, and the 8-hour level gave a rather better separation of patients from controls than did the fasting level. In the 45 to 54 year age group (Fig. 27) triglyceride concentrations in patients were only slightly higher than those in controls, and the difference was even smaller in the 55 to 64 year age group (Fig. 28).

Comparison of female arterial disease patients with female controls of the same age (Fig. 31) revealed similar fat tolerance curves in the two groups. Triglyceride levels in the controls tended, for the most part, to be higher than those in arterial disease patients.

(d) Correlation of Fasting and Postprandial Triglyceride Levels

On examination of the fat tolerance curves, it was evident that the highest postprandial triglyceride levels occurred in



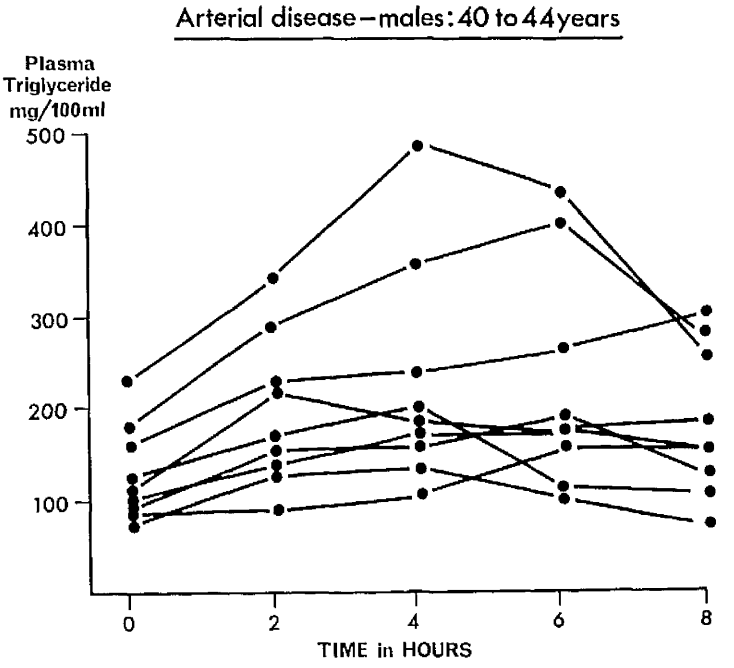


Fig. 29

Fig. 30

Figs. 29 and 30. Individual fat tolerance curves in male controls and arterial disease patients aged 40 to 44 years.

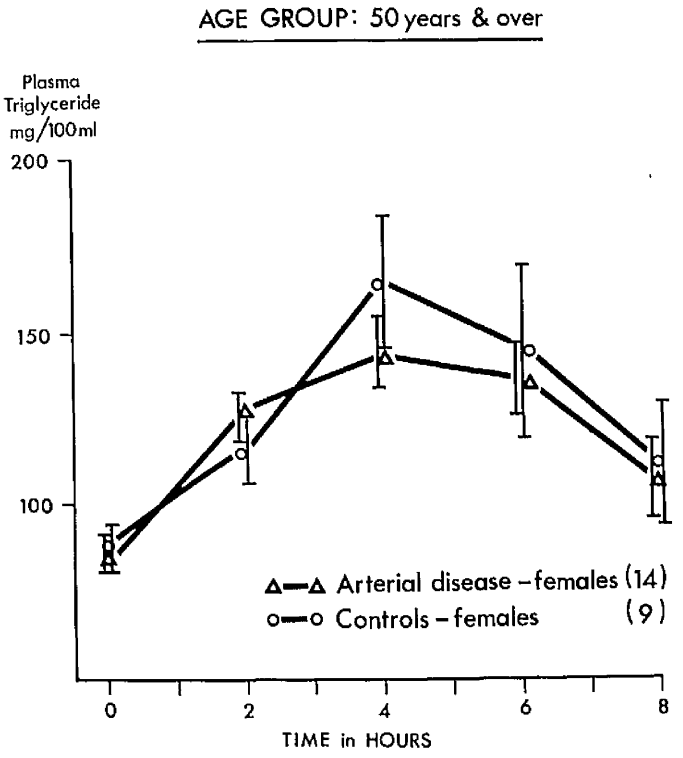


Fig. 31. Comparison of fat tolerance tests in female controls and arterial disease patients (mean ± S.E.M.).

subjects with initially high fasting triglyceride concentrations. This was confirmed by correlating postprandial with fasting triglyceride levels (Table XII). A similar close correlation between fasting and postprandial triglyceride levels was seen in controls and arterial disease patients at all times after the fat load.

SUMMARY OF PLASMA TRIGLYCERIDE CHANGES

In healthy controls postprandial triglyceride concentrations during fat tolerance tests tended to be higher in males than in females, but statistically significant differences were observed only in the 40 to 44 and 45 to 54 year age groups. The differences in the 8-hour levels were smaller than those in the other postprandial levels. In the 55 to 64 year age group, plasma triglyceride concentrations in the females were at all times slightly higher than in the males. No statistically significant differences in fat tolerance tests were seen between male and female arterial disease patients, or in the three clinical types of male arterial disease patients.

Comparison of arterial disease and control subjects revealed that young male arterial disease patients had significantly higher fat tolerance curves than healthy males of corresponding age, and the greatest differences were seen

TABLE XII. Correlation of Postprandial with Fasting Triglyceride Levels (correlation coefficients)

	Controls male and female	Arterial Disease Patients male and female
1 hour and fasting triglyceride	+0.89*** (n = 30)	+0.93*** (n = 43)
2 hour and fasting triglyceride	+0.76*** (n = 72)	+0.88*** (n = 86)
4 hour and fasting triglyceride	+0.73*** (n = 72)	+0.86*** (n = 86)
6 hour and fasting triglyceride	+0.73*** (n = 72)	+0.83*** (n = 86)
8 hour and fasting triglyceride	+0.75*** (n = 71)	+0.77*** (n = 86)

*** $P < 0.001$

n = no. of pairs of observations

in the 8-hour sample. The differences in fat tolerance between arterial disease and control subjects became progressively less with age. There were no significant differences in fat tolerance between female controls and female arterial disease patients, who were all aged 50 years or over.

The highest postprandial triglyceride levels occurred in subjects with the highest fasting triglyceride levels. Both in controls and in arterial disease patients there was a close correlation between fasting and postprandial triglyceride levels.

CHANGES IN PLASMA CHOLESTEROL DURING FAT TOLERANCE TESTS

All four groups of subjects showed a small increase in plasma cholesterol concentration one hour after ingestion of the fat load (Table XIII). In male controls the cholesterol concentration at other times during the fat tolerance test was not significantly different from the fasting level, but in the other subjects it remained slightly elevated.

CHANGES IN PLASMA PHOSPHOLIPID DURING FAT TOLERANCE TESTS

In all four groups of subjects there was a progressive increase in plasma phospholipid concentration during the fat tolerance test up to the time of the 6-hour estimation (Table XIV,

TABLE XIII. Changes in Plasma Cholesterol during Fat Tolerance Tests

(mean ± S.D.)

Subjects	Fasting Plasma Cholesterol mg. per 100 ml.	Mean Increase from Fasting Level				
		1 hour	2 hour	4 hour	6 hour	8 hour
Controls - male all ages	221.9 ± 39.1 (n = 50)	9.6** ± 15.7 (n = 23)	1.9 ± 18.2 (n = 50)	-0.5 ± 18.7 (n = 50)	2.2 ± 18.8 (n = 50)	1.3 ± 20.2 (n = 50)
Controls - female all ages	214.0 ± 47.0 (n = 22)	12.3 ± 15.6 (n = 6)	6.1† ± 10.5 (n = 22)	12.1*** ± 14.7 (n = 22)	11.4† ± 13.9 (n = 22)	10.4* ± 20.8 (n = 22)
Arterial Disease male	252.4 ± 46.2 (n = 72)	12.5** ± 19.9 (n = 33)	7.2** ± 20.3 (n = 72)	3.5 ± 18.6 (n = 72)	5.0* ± 18.4 (n = 72)	6.1* ± 22.5 (n = 72)
Arterial Disease female	261.8 ± 41.9 (n = 14)	10.5 ± 12.4 (n = 10)	4.9 ± 13.3 (n = 14)	6.3 ± 10.8 (n = 13)	13.9** ± 14.7 (n = 14)	8.0 ± 15.3 (n = 14)

*P < 0.05, †P < 0.02, **P < 0.01, ***P < 0.001

n = no. of observations

TABLE XIV. Changes in Plasma Phospholipid during Fat Tolerance Tests (mean ± S.D.)

Subjects	Fasting Plasma Phospholipid mg. per 100 ml.	Mean Increase from Fasting Level				
		1 hour	2 hour	4 hour	6 hour	8 hour
Controls - male all ages	203.7 ± 33.1 (n = 28)	9.3 ± 22.3 (n = 4)	6.8 ± 19.9 (n = 28)	16.5** ± 24.3 (n = 28)	20.9*** ± 27.5 (n = 28)	12.8*** ± 14.7 (n = 28)
Controls - female all ages	228.2 ± 24.9 (n = 16)	--	11.0 ± 21.9 (n = 16)	22.1** ± 20.9 (n = 16)	31.3*** ± 26.9 (n = 16)	29.7* ± 43.5 (n = 15)
Arterial Disease male	219.8 ± 32.1 (n = 50)	2.5 ± 21.4 (n = 11)	9.6** ± 22.9 (n = 50)	15.6*** ± 21.5 (n = 50)	23.5*** ± 24.2 (n = 50)	19.3*** ± 25.8 (n = 50)
Arterial Disease female	259.8 ± 28.8 (n = 5)	--	8.0 ± 17.1 (n = 5)	17.4 ± 20.8 (n = 5)	33.0 ± 38.3 (n = 5)	20.4 ± 32.6 (n = 5)

*$P < 0.02$, **$P < 0.01$, ***$P < 0.001$

n = no. of observations

Fig. 32). The plasma phospholipid level had decreased by 8 hours, but still remained well above the fasting level.

There was no significant correlation between the fasting and the 6-hour phospholipid level. In all subjects there was a weak positive correlation between the increases in phospholipid and triglyceride at the 6-hour estimation. This correlation was statistically significant only in the group of 28 male controls ($r = +50$, $P < 0.01$).

CHANGES IN PLASMA NEFA DURING FAT TOLERANCE TESTS

Ingestion of the test meal was followed by a reduction in plasma NEFA level. This was obvious at one hour after the meal, but in most cases the plasma NEFA concentration reached its lowest level at two hours (Table XV, Fig. 33). By the time of the 4-hour estimation a rebound rise in NEFA to levels above the fasting concentration had occurred, and the 6-hour estimation showed a continued rise. By the 8th hour the NEFA concentration had commenced to fall again, but remained significantly above fasting levels. The plasma NEFA curves obtained in the course of the fat tolerance tests showed no significant differences between males and females, or between controls and arterial disease patients (Fig. 33).

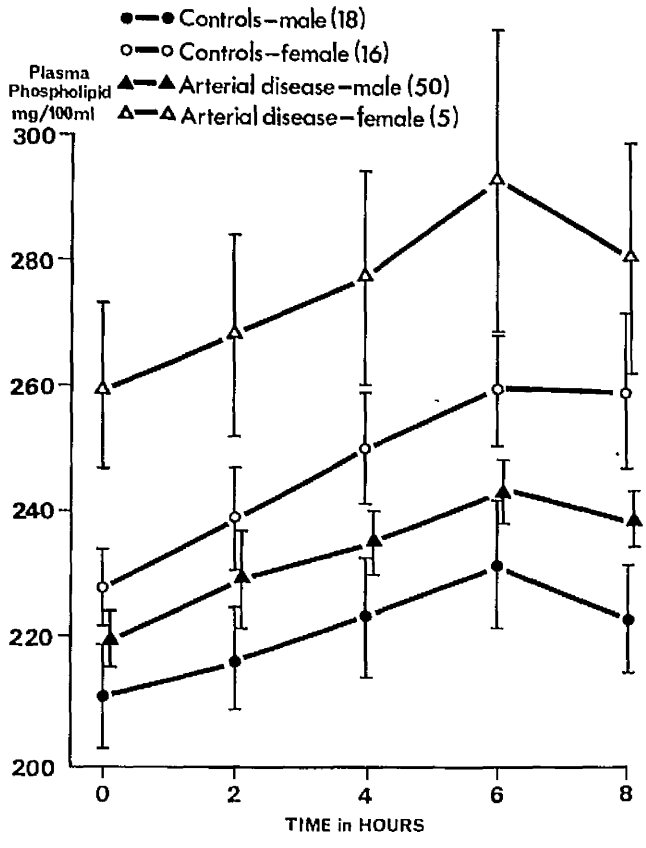


Fig. 32. Plasma phospholipid changes during fat tolerance tests in subjects aged 40 years and over (mean ± S.E.M.).

TABLE XV. Changes in Plasma NEFA during Fat Tolerance Tests (mean ± S.D.)

Subjects	Fasting Plasma NEFA microeq./l.	Mean Increase from Fasting Level				
		1 hour	2 hour	4 hour	6 hour	8 hour
Controls - male all ages	507 ± 150 (n = 50)	-127*** ± 137 (n = 24)	-101*** ± 145 (n = 50)	61/ ± 166 (n = 50)	133*** ± 192 (n = 50)	86*** ± 152 (n = 48)
Controls - female all ages	539 ± 175 (n = 22)	-60 ±69 (n = 6)	-106** ±155 (n = 22)	43 ± 170 (n = 22)	91/ ± 161 (n = 22)	70* ± 153 (n = 22)
Arterial Disease male	481 ± 235 (n = 72)	-61** ± 105 (n = 33)	-72*** ± 104 (n = 72)	79*** ± 133 (n = 71)	195*** ± 176 (n = 72)	149*** ± 163 (n = 72)
Arterial Disease female	490 ± 162 (n = 13)	-57 ± 178 (n = 10)	-170** ± 145 (n = 13)	43 ± 165 (n = 13)	186*** ± 101 (n = 13)	109 ± 198 (n = 13)

*P<0.05, /P<0.02, **P<0.01, ***P<0.001

n = no. of observations

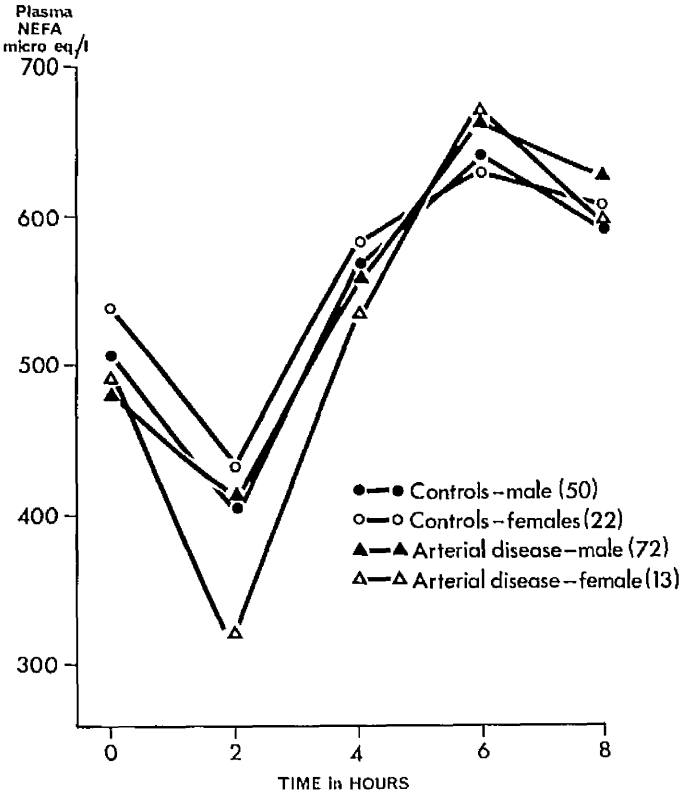


Fig. 33. Plasma NEFA changes during fat tolerance tests.

CHANGES IN HAEMATOCRIT DURING FAT TOLERANCE TESTS

The changes in haematocrit in the course of the fat tolerance tests are shown in Fig. 34. The only significant changes in all 34 subjects in whom the haematocrit was recorded was a transient mean increase of 1.3 ± 2.0 per cent at one hour ($P < 0.001$). The male arterial disease patients showed a further slight increase at 2 hours, but in the other subjects the haematocrit had returned to fasting levels by two hours, and showed no significant change thereafter.

The magnitude of the increase in haematocrit at one hour correlated positively with the increase in plasma cholesterol concentration which occurred at the same time ($r = +0.49$, $P < 0.01$).

SUMMARY OF RESULTS OF FAT TOLERANCE TESTS

I. Changes in Triglyceride

(a) Differences between Arterial Disease Patients and Controls

The fat tolerance curves of young male arterial disease patients (40 to 44 years) were significantly higher than those of male controls of the same age. Both fasting and 8-hour triglyceride concentrations were significantly higher in the arterial disease patients, but the difference between the

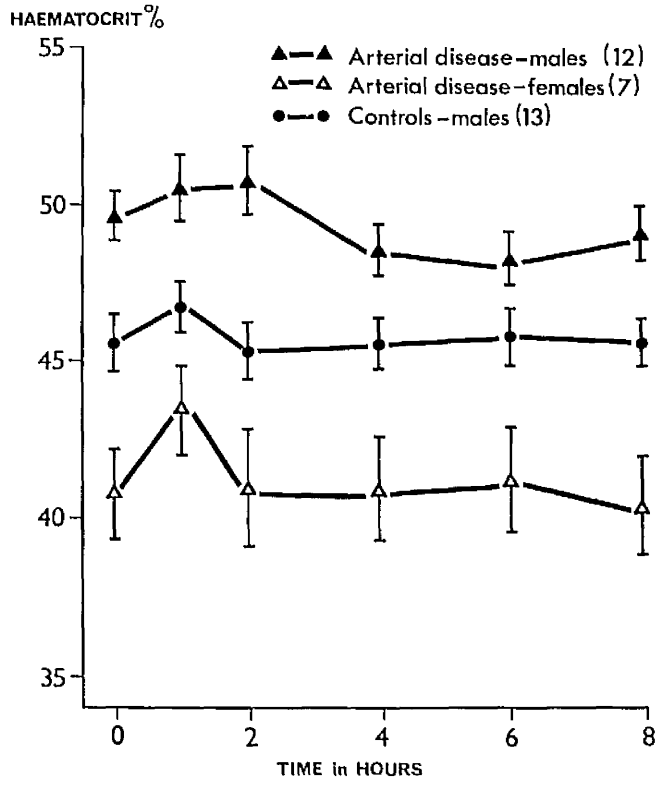


Fig. 34. Changes in haematocrit during fat tolerance tests (mean ± S.E.M.).

two groups in 8-hour triglyceride levels was greater than that in fasting triglyceride. In older male subjects, there was no significant difference in fat tolerance between arterial disease patients and controls.

No differences in fat tolerance were found between female arterial disease patients and female controls. Fat tolerance curves were similar in myocardial infarction, angina pectoris, and peripheral arterial disease patients.

(b) Sex Differences

Male controls tended to have higher fat tolerance curves than females in the 5th and 6th decades. In older subjects, however, triglyceride levels in females were slightly higher than in males.

No significant differences were found in fat tolerance between male and female arterial disease patients.

(c) Relationship between Fasting and Postprandial Triglyceride Levels

Subjects with elevated fasting triglyceride levels had the highest postprandial levels. There was a close correlation between fasting and postprandial triglyceride concentrations.

II. Changes in Other Plasma Lipids and Haematocrit

(a) Plasma Cholesterol

All groups of subjects showed a small increase in plasma cholesterol at one hour. In male controls the cholesterol then returned to fasting levels, while in other subjects it remained slightly elevated.

(b) Plasma Phospholipid

In both arterial disease and control subjects there was a progressive increase in plasma phospholipid concentration up to six hours, with a slight fall thereafter.

(c) Plasma NEFA

All subjects showed a similar fall in NEFA, maximal at 2 hours, with a subsequent rise to concentrations approximately 40 per cent above the fasting level at 6 hours, followed by a small decrease.

(d) Haematocrit

There was a small but significant increase in haematocrit at one hour following ingestion of the meal. This increase correlated positively with the increase in plasma cholesterol concentration which occurred at this time.

DISCUSSION

Many observers have reported coronary artery disease to be associated with an exaggerated and prolonged postprandial rise in blood neutral fat. It has been suggested that a prolonged response to a fat meal may provide a better indication of the fat defect in coronary artery disease than the fasting triglyceride level or other lipid parameters (Seller *et al.*, 1959; Brown *et al.*, 1961; Albrink, 1962). In this study, only young male subjects with arterial disease were found to have fat tolerance tests significantly different from those in apparently healthy subjects (Fig. 26). Fat tolerance curves in older male arterial disease patients were lower than in young patients, and indistinguishable from those in healthy controls. Similarly, fat tolerance curves in female arterial disease patients, who in the present series were all aged 50 years or more, did not differ from those obtained in healthy females of the same age (Fig. 31). Even in the group of young male subjects in whom significant differences were found in the mean plasma triglyceride curves there was no clear demarcation between the lipaemic responses of patients and controls. Several patients had very high plasma triglyceride levels and several controls very low plasma triglyceride levels throughout the test, but in the majority the curves appeared similar in the two groups (Figs. 29 and 30).

There have been few attempts to define a normal fat tolerance curve. Sklarin et al. (1961) stated that the most useful criterion for a normal response was the return of the serum triglyceride to fasting levels by nine hours after a fat meal (109 g. fat). Brown et al. (1963) suggested that, in normal young men, a fasting serum triglyceride level of more than 5.2 meq. per l. or a postprandial level exceeding 9.0 meq. per l. after a 70 g. fat meal were probably abnormal. Hatch et al. (1966) adopted a modification of Sklarin's criteria. They suggested that a fat tolerance curve should be considered abnormal if the triglyceride level had not returned to within 80 mg. per 100 ml. of the fasting level by nine hours after the 109 g. fat meal. If Sklarin's standards were applied to our 40 to 44 year old subjects, making no allowances for the differences in size of fat load and in timing of the last blood sample, seven of the nine arterial disease patients and three of the eleven controls were abnormal. By Hatch's standards, only three patients and one control had abnormal fat tolerance curves. These criteria were unsatisfactory, however, in their assumption that all subjects with elevated plasma triglyceride concentrations showed a delayed return to fasting levels. In our series, the subjects with the greatest alimentary lipaemia in both arterial disease and control groups had normal fat tolerance curves by these criteria

since their 8-hour levels were similar to their high fasting triglyceride levels.

The close correlation in the subjects in this study between fasting and postprandial triglyceride concentrations at all stages of the fat tolerance test was in accord with Denborough's (1963) observation that the fasting and maximal postprandial triglyceride levels correlated well. More recently Brown et al. (1966) suggested that the prolonged alimentary lipaemia frequently seen in ischaemic heart disease patients was a function of an elevated fasting triglyceride concentration in these subjects. Subjects with low or normal fasting triglyceride levels would therefore be found to have low plasma triglyceride concentrations throughout the course of a fat tolerance test, whereas an exaggerated lipaemic response to a fat load would be anticipated in persons with already elevated fasting triglyceride levels. These observations cast doubt on the value of fat tolerance tests. Denborough (1963) stated that measurements of either plasma optical density or triglyceride concentration after a meal had no advantage over the estimation of fasting plasma triglyceride. Pelkonen and Nikkila (1965), who carried out a large series of fat tolerance tests in 95 coronary patients and 78 healthy persons, were of the opinion that basal lipid

levels separated coronary from healthy individuals as well as did the fat tolerance test. Our results showed that the 8-hour triglyceride gave wider separation of arterial disease patients from controls, but did not produce significant differences in triglyceride levels between patients and controls where they did not already exist in the fasting state. The fat tolerance test therefore did not uncover any latent lipid abnormality. In view of the wide overlap between patients and controls, the fat tolerance test appears to be of no value in detection of lipid abnormalities in the individual. It has only a minor advantage over the fasting triglyceride estimation in the comparison of groups of arterial disease patients with healthy subjects, and this advantage must be outweighed by the time-consuming nature of the test.

The finding in this study of increased alimentary lipaemia in only the younger subjects with atherosclerotic arterial disease is in keeping with other observations suggesting that the lipid abnormalities in ischaemic heart disease are greater in the younger patients. This was noted with respect to serum cholesterol concentration (Little et al., 1956; Lawry et al., 1957; Albrink et al., 1961) and possibly also with respect to fasting triglyceride (Oliver, 1966). The data on fasting plasma lipids reported in Chapter III showed higher fasting plasma levels of triglyceride as well as cholesterol

in younger patients. The fat tolerance results in the present study do not confirm the suggestion (Seller et al., 1959; Brown et al., 1961; Albrink, 1962) that the fat tolerance test is a better indicator of abnormal lipid metabolism in atherosclerosis than the other plasma lipid parameters. In all age groups, the fasting plasma cholesterol level permitted distinction between arterial disease patients and controls as well as, and in most cases better than, the fasting or 8-hour triglyceride level. It has been suggested that apparently healthy persons with high fat tolerance curves have a high risk of ischaemic heart disease (Brown et al., 1963). Definite conclusions on these points can be reached only after analysis of the results of large scale prospective studies, such as are already in progress (Kannel et al., 1964; Havel and Carlson, 1962), of the influence of plasma cholesterol and triglyceride levels on the development of ischaemic heart disease.

The differences in fat tolerance between arterial disease patients and controls in this series was less marked than that reported in some other studies. This might be due to several factors. In a considerable number of publications, arterial disease patients were compared with a control series of much younger persons (Schwartz et al., 1952; Pomeranze et al., 1954; Woldow et al., 1954; Horlick, 1956 and 1957;

Likoff et al., 1958). By virtue of their age, plasma lipids in these 'controls' would be lower than those of persons of similar age to the ischaemic heart disease patients. Other investigators used as their control series convalescing hospital inpatients (Mitchell and Bronte-Stewart, 1959) or longterm inmates of mental hospitals (Bronte-Stewart and Blackburn, 1958), whose plasma lipids could not be assumed to be the same as those of the healthy population. In this series, as in some others (Barritt, 1956; Pelkonen and Nikkilä, 1965), controls were selected from healthy active individuals prior to their admission for minor surgical operations. An additional factor reducing the differences in plasma lipids between arterial disease patients and controls in this series might be the fact that in a population such as this with a very high prevalence of atherosclerotic disease, a considerable proportion of apparently healthy controls must have clinically undetected atherosclerosis.

The small increase in plasma cholesterol found in all groups in the present series one hour after ingestion of the fat load was presumably a haemoconcentration effect, since the magnitude of the cholesterol increase correlated positively with the magnitude of the haematocrit increase. With the exception of the male controls, the subjects in this study showed smaller increases in cholesterol in the later stages

of the fat tolerance test. Reports in the literature of the effect of fat loading on plasma cholesterol levels were conflicting. Nevertheless it was generally agreed that changes in plasma cholesterol after a fat meal were small in comparison with those in other plasma lipids, and could not be used as an index of fat absorption and removal.

A large number of investigators found that plasma cholesterol levels were unchanged following fat meals varying in amount from 20 g. to 204 g. fat (Blix, 1926; Pomeranze et al., 1954; Albrink et al., 1958; Hollister, 1963). Others reported that fat loading caused small but significant increases in plasma cholesterol level (Talbott and Keating, 1962; Brown et al. 1963). Havel (1957) found no significant change in cholesterol in normal males, but in seven out of eight test meals in two normal females the serum cholesterol rose 15 mg. per 100 ml. or more. Yet other workers recorded a significant fall in serum cholesterol during fat tolerance tests (Nikkilä and Konttinen, 1962; Angervall, 1964; Kallio, 1967).

Some of the apparently contradictory results might be accounted for by the fact that some of the series are small in number, and others by the differing compositions of the test meals used. Kingsbury and Morgan (1960) administered various types of oils to healthy subjects and showed that some types of oils produced a consistent rise in serum cholesterol

levels, while others produced a consistent fall. That the contradictions could not all be resolved on this basis, however, was evident from the fact that in the various experimental series quoted above, meals of cream or butterfat were shown to cause a rise, a fall, or no change in plasma cholesterol levels. Recent studies of cholesterol absorption in man showed intestinal absorption of cholesterol to be slower than that of triglyceride, the peak appearance of absorbed cholesterol in thoracic duct lymph being approximately eight hours after a meal (Hellman et al., 1960). The small but significant increases in cholesterol seen during the latter phases of fat tolerance tests in most of the subjects in this series might be due in part to cholesterol absorbed from the test meal. It might, however, be a reflection of the increased plasma concentrations of lipoproteins concerned in triglyceride transport, which contain small amounts of cholesterol in addition to triglyceride and phospholipid.

The plasma phospholipids in all groups of subjects in this study showed a progressive increase in concentration up to the time of the 6-hour estimation, and had only decreased slightly at the time of the 8-hour estimation. Man and Gildea (1932) reported considerable increases in phospholipid after a fat load of 3.5 to 4 g. per kg. of body weight, but little or no change in phospholipid following a standard 60 g. fat

meal. Several other workers recorded increased phospholipid concentrations following meals of 177 g. fat (Talbott and Keating, 1962) and 80 g. fat (Angervall, 1964), while Albrink and Man (1956), Osmon et al. (1957) and Stutman et al. (1961) stated that serum phospholipids were unchanged after fat loads of 60 to 70 g. In the present series the maximum phospholipid concentration occurred later than the lipaemic peak, and phospholipid levels remained considerably elevated at 8 hours when the triglyceride concentration in most cases had returned to fasting levels. Havel (1957) also noted that raised phospholipid levels persisted longer than the lipaemia, and Kallio (1967) found elevations of serum phospholipids as long as 10 hours after the fat meal. There was some evidence in the present series of a weak correlation between the magnitudes of the phospholipid and triglyceride increases, and Angervall (1964) found a similar correlation in his subjects. The increase in plasma phospholipids during alimentary lipaemia was presumably due partly to the phospholipid content of chylomicrons, and partly to the proportionately greater phospholipid component of the very-low-density lipoproteins which transported triglyceride (derived from chylomicrons) from the liver to the fat depots. It might therefore be anticipated, as was found in this study, that the correlation

between plasma phospholipid and triglyceride would be less close during alimentary lipaemia than in the fasting state, when these lipids were present only in the form of lipoprotein.

Ingestion of the test meal in all subjects in this study resulted in an initial fall in plasma NEFA lasting one to two hours, with a subsequent rise above fasting levels. A similar pattern of NEFA response to mixed meals has been reported by a number of other workers (Dole, 1956; Albrink et al., 1958; Shah et al., 1963; Konttinen and Rajasalmi, 1963; Hollister, 1963). The administration of carbohydrate alone causes an abrupt fall in plasma NEFA within half-an-hour and lasting for several hours (Dole, 1956; Gordon and Cherkes, 1956; Bierman et al., 1957a; Rothlin et al., 1962; Castelli et al., 1966). A similar, though less marked, fall in NEFA concentration follows ingestion of protein (Castelli et al., 1966). On the other hand, after administration of fat alone NEFA levels are unchanged or show a gradual rise similar to that seen when fasting is continued for the same length of time (Dole, 1956; Gordon and Cherkes, 1956; Albrink and Neuwirth, 1960). The initial fall in NEFA level after ingestion of a mixed meal appears, therefore, to be due to its carbohydrate content. The decrease in plasma NEFA level has been shown to be due to decreased output of NEFA from fat depots (Carlson and Orö, 1963), the mechanism probably being inhibition of

lipolysis, or promotion of re-esterification of NEFA within the fat depots.

The origin of the plasma NEFA which cause the delayed rise in alimentary lipaemia is disputed. In the fasting state, changes in plasma NEFA concentration are believed to be due almost entirely to changes in the rate of fatty acid liberation from adipose tissue. During alimentary lipaemia, however, NEFA may be released by intravascular hydrolysis of chylomicron triglyceride. It is known that fatty acids are liberated by lipolysis of chylomicrons during the *in vitro* clearing of lipaemic serum (Robinson and French, 1953), and that plasma NEFA levels are elevated during the enhanced clearing of lipaemia induced by heparin injection *in vivo* (McDaniel and Grossman, 1955; Fredrickson and Gordon, 1958b). Intravenous infusions of triglyceride emulsions have been followed by a prompt and marked rise in NEFA levels (Forbes, 1965). The relevance of these observations to the normal mechanisms of clearing of alimentary lipaemia is doubtful. However, since Dole *et al.* (1959) have shown that the arterial NEFA pattern during the lipaemia induced by a fat load may vary according to the fatty acid composition of the ingested fat, it is probable that some of the NEFA are derived from freshly absorbed fat. On the other hand, Dole (1956) found that the NEFA rise during lipaemia induced by fat alone was no greater

than that of continued fasting, and that an abrupt decrease in plasma NEFA level could be produced by glucose administration during alimentary lipaemia without delaying clearance of the lipaemia. These findings suggest that the NEFA level in the lipaemic as well as in the fasting state is dependent mainly on mobilisation of endogenous fatty acids. It would appear, therefore, that the elevated NEFA levels after a mixed meal are due to NEFA derived partly from fat depots and partly from hydrolysis of newly-absorbed triglyceride. There is some evidence, also, that the liver may play a major part in the clearing of alimentary lipaemia, and may release NEFA into the circulation (Dole and Hamlin, 1962).

In the present study the inhibition of NEFA release produced by the test meal was similar in arterial disease patients and controls, and in the myocardial infarction, angina pectoris, and peripheral arterial disease patients. The rebound increases in NEFA demonstrated in the latter half of the fat tolerance tests were also similar in arterial disease patients and controls, and did not vary with the degree of alimentary lipaemia. NEFA release has been reported to be much greater in ischaemic heart disease patients than in controls in response to the stimulus of cigarette-smoking (Kershbaum et al., 1962). No such difference in NEFA release between patients with atherosclerotic arterial disease and

controls has been demonstrated during alimentary lipaemia.

SUMMARY AND CONCLUSIONS

1. The fat tolerance curves of male arterial disease patients aged 40 to 44 years were significantly higher than those of healthy men of the same age. Patients with myocardial infarction, angina pectoris, and peripheral arterial disease showed similar abnormalities in alimentary lipaemia. Fat tolerance tests in older male and female patients did not differ from those of controls of corresponding age.

2. There was a close correlation between fasting and post-prandial triglyceride levels, both in controls and in arterial disease patients. High fat tolerance curves occurred in persons with elevated fasting triglyceride levels.

3. In arterial disease patients of all ages, elevation of the plasma cholesterol was more common than elevation of the fasting triglyceride or 8-hour triglyceride levels.

4. An increase in plasma cholesterol in all groups of subjects one hour after ingestion of the test meal was associated with an increase in haematocrit. In male controls the plasma cholesterol then returned to fasting levels, but male and female patients and female controls showed a small but

significant increase in cholesterol in the later stages of the fat tolerance test.

6. In all subjects the plasma phospholipid increased during the fat tolerance test, reaching its maximum at six hours, and decreased slightly thereafter. The increase in phospholipid was much smaller than the increase in triglyceride, but persisted for longer.

7. Ingestion of the test meal caused a fall in plasma NEFA for one to two hours, followed by a rise to above the fasting level, maximal at six hours. Plasma NEFA curves were similar in arterial disease patients and controls.

Chapter V. GASTRIC FUNCTION IN RELATION TO FAT TOLERANCE AND ATHEROSCLEROSIS

CONTENTS

Chapter V. GASTRIC FUNCTION

INTRODUCTION

The observation of increased and prolonged alimentary lipaemia in patients with ischaemic heart disease led to much speculation as to the cause of this abnormality. Although defective heparin-activated clearing of plasma lipids was reported in some studies (Block et al., 1951; Herzstein et al., 1954), this was not confirmed by others (Hood et al., 1954; Baker, 1957), and in a four-way crossover experiment Mitchell and Bronte-Stewart (1959) detected no differences in plasma clearing activity or substrate between patients and controls. A number of investigators showed that artificial fat emulsions administered intravenously were cleared at the same rates in patients and controls, in contrast to the greater lipaemia found in patients when the same fat emulsions were given orally (Berkowitz et al., 1961; Bouchier and Bronte-Stewart, 1961; Feinberg et al., 1961; Mayfield et al., 1962). It was realised, however, that intravenously administered fat might be cleared by a mechanism different to that for orally fed fat, and Balodimos et al. (1962) suggested that the fat emulsion particles were treated as foreign bodies and removed by the reticulo-endothelial system. This difficulty was overcome

by Nestel and his colleagues (1962), who injected C^{14}-labelled chylomicrons intravenously, and found similar clearance in two hyperlipaemic subjects.

These findings were considered by some investigators to indicate that impairment of fat tolerance was due to factors governing the entry of fat into the circulation, rather than to impaired fat clearing. It was postulated that increased alimentary lipaemia occurred in subjects whose intestinal lipolysis was defective, with the result that more fat entered the circulation in a particulate form which persisted for longer than normal (Frazer, 1943; Becker et al., 1950; Marder et al., 1952; Tietz et al., 1960). Becker et al. (1950) found prolonged chylomicronaemia as well as diminished gastric and pancreatic secretions in elderly subjects, and suggested that the diminished gastric acidity in these subjects was an index of inefficient gastro-intestinal function. It was suggested also that diminished secretion of acid by the stomach might be accompanied by diminished secretion of gastric lipase, leading to inadequate emulsification of fat prior to its encounter with pancreatic lipase, and thus to greater alimentary lipaemia (Bronte-Stewart and Krut, 1962). Delayed gastric emptying was considered to be an additional factor contributing to prolongation of alimentary lipaemia (Frazer and Stewart, 1939; Marder et al., 1952).

The main aspects of gastric function which have been implicated in abnormal fat tolerance in atherosclerosis, therefore, are deficient gastric secretion, delayed gastric emptying, and inefficient gastric lipolysis. In the present study an investigation has been made of each of these factors in controls and arterial disease patients. Since if these factors are important, an exaggeration of their effects might be anticipated in states with failure of gastric secretion as in Addisonian pernicious anaemia, or with gastric hypersecretion as in duodenal ulceration, subjects in these categories were also studied.

METHODS

An account of the methods used throughout this Chapter is given below.

SUBJECTS

The studies of basal gastric secretion, gastric emptying by the Hunt technique, and gastric lipolysis were carried out in the majority of the subjects described in Chapter III. These were as follows:- 32 male controls aged 27 to 61 years (mean 45.2 years), of whom 23 were aged 40 years or more (mean 50.3 years), 51 male arterial disease patients of mean age

51.6 years (23 with myocardial infarction of mean age 50.0 years, 14 with angina pectoris of mean age 52.8 years, and 14 with peripheral arterial disease of mean age 52.9 years). Studies were carried out in addition on 5 male arterial disease patients with duodenal ulcer demonstrated by barium meal examination (aged 41 to 55 years, mean 48.4 years), and on 5 male patients with Addisonian pernicious anaemia, proven by Schilling test, and treated for at least one year prior to the gastric studies (aged 50 to 69 years, mean 60.0 years).

A further 10 patients (8 males and 2 females) acted as subjects for the radiological determination of gastric emptying. Their ages ranged from 17 to 61 years (mean 46.4 years). They had no clinical evidence of gastrointestinal disease, and their diagnoses were as follows:- hypertension (3), chronic bronchitis (3), epilepsy, hydronephrosis, gravitational ulcer, and atrial fibrillation.

PROCEDURES

(a) Basal Secretion

After an overnight fast, subjects were intubated by the nasal route using a Willi-Rusch radio-opaque gastric tube (no. 16, or in a few cases no. 14). The position of the tube was checked using an image intensifier with the patient in

the erect posture, and adjusted if necessary until the terminal portion of the tube lay along the greater curvature with the tip in the most dependent part of the stomach. Subjects remained sitting throughout the remainder of the procedure and were instructed to eject saliva into the carton provided. The stomach was emptied by aspiration of the fasting juice using a 50 ml. syringe, and the fasting juice was kept. The basal gastric secretion was then collected for 30 minutes, by continuous aspiration with a syringe. The volumes of the fasting and basal gastric juice were measured, and the following estimations carried out:-

pH. The pH was determined by use of a Radiometer pH meter.

Acid concentration. The acid content was measured by titration of 5 ml. of gastric juice with $N/_{10}$ sodium hydroxide to a pH of 7, using phenol red as indicator.

Chloride concentration. This was determined by potentiometric titration of the chloride with silver nitrate as described by Sanderson (1952), using a Radiometer titrator.

(b) Gastric Emptying

After completion of the 30-minute collection of basal gastric secretion, a modification of the Hunt test meal technique (Hunt, 1954) was carried out, as described below.

Composition of the test meal

The test meal consisted of a combination of the phenol red solution described by Hunt with the semi-liquid test meal used for determination of fat tolerance in the same subjects.

Phenol red stock solution

Phenol red powder (3 g.) was dissolved in 25 ml. of N sodium hydroxide by allowing to stand, and shaking the solution occasionally. The solution was diluted to approximately a litre, filtered, and made up to two litres with water.

Test meal

On the day before use, the test meal was made up as follows:-

80 g. fat (Nestlé's sterilised cream 348 g.)

37 g. carbohydrate (dextrose)

16 g. protein (Casilan)

These constituents were homogenised with water in an electric mixer, and allowed to stand in a refrigerator overnight to allow frothing to subside. Immediately before use it was warmed to approximately 37°C, phenol red stock solution (28 ml.) was added, and the mixture made up to 800 ml. with water. The test meal consisted of 750 ml. of this mixture, and the remaining 50 ml. was reserved for comparison with the recovered

gastric contents.

Determination of rate of gastric emptying

Subjects remained seated throughout the procedure, with a no. 16 Willi-Rusch radio-opaque tube *in situ* in the stomach. The 750 ml. test meal was poured down the tube by means of a glass funnel. Sixty minutes later, aspiration of the remaining gastric contents into a container surrounded by crushed ice was carried out, using a 50 ml. syringe. Three gastric washouts, each of 250 ml. warm tap water, were then carried out, and the tube was withdrawn.

The volumes of the recovered gastric contents and washouts were measured. The pH, acid concentration, and chloride concentration of the original test meal and recovered gastric contents were determined as described on page 162. In 18 instances the glyceride-glycerol concentrations of extracts (1 ml. in 250 ml. Bloor's solution) of the initial watery phase (page 190) and the rest of the recovered test meal were measured, as described on page 42.

Determination of phenol red concentration

Measurement of the phenol red concentration of the original test meal, recovered test meal, and washouts was carried out as follows. Aliquots (10 ml.) of original test

meal, gastric contents, and the three washouts were each mixed with 50 ml. of trisodium phosphate buffer (trisodium phosphate 27.5 g. in 1 litre water), and made up to 250 ml. with distilled water in volumetric flasks. Duplicate 6 ml. aliquots of each of the solutions were pipetted into cellulose nitrate tubes and centrifuged for 1 hour at 40,000 r.p.m. in a Spinco, model L, preparative ultracentrifuge. This separated the solutions into three layers - an upper, cream-like layer of fat particles, a middle zone consisting of a clear purple solution, and a protein precipitate at the bottom of the tube. The tubes were divided horizontally below the fat layer, using a tube slicer, and the clear solution pipetted into 1 cm. cuvettes, taking care to avoid disturbance of the protein precipitate. The optical density of the purple colour was read at 570 mμ in a spectrophotometer (Unicam S.P.600), and the concentration of phenol red obtained from a standard graph.

The phenol red concentration of the washouts was used to estimate their content of the test meal recovered at one hour. The volume of gastric contents present in the stomach at one hour, and the volume which had passed the pylorus during that hour were then calculated, as described by Hunt (1954).

(c) Gastric Emptying of Fat

Determination of the fat content of the original test meal, recovered gastric contents, and washouts was carried out by the Gerber method. This technique is used for the measurement of total fat in milk and milk products, and the apparatus and procedure are described in detail in British standards 696 (1955). An outline of the method is given below.

Reagents

1. Sulphuric acid for milk testing (density 1.812 - 1.817)
2. Amyl alcohol.

Apparatus

1. Standard Gerber butyrometer for milk-testing.
2. Standard double-ended stopper, or lock stopper.
3. Standard milk pipette to deliver 10.94 ml.
4. Standard pipette to deliver 10.0 ml. sulphuric acid.
5. Standard pipette to deliver 1.0 ml. amyl alcohol.
6. Centrifuge with spinning platform.
7. Water bath 65°C.

Procedure

Sulphuric acid (10 ml.) was pipetted into the butyrometer, and 10.94 ml. freshly mixed test meal (or recovered gastric

contents, or washout) added slowly. Amyl alcohol (1.0 ml.) was then added, and the tube stoppered and shaken in a covered rack. The tubes were then centrifuged at approximately 1100 r.p.m. for 4 minutes. After centrifugation the tubes were placed stopper downwards in a waterbath at 65°C for at least 3 minutes.

When the tubes were removed from the waterbath the fat column level (colourless liquid) was adjusted so that it lay between the calibration marks. The difference between the lowest point of the fat meniscus and the point of separation of the fat from the acid solution (dark-brown to violet in colour) gave the percentage by weight of the fat in the test liquid. A small correction factor, which varied according to the length of the fat column, was applied to the butyrometer reading. By this method, the fat content of the 750 ml. test meal in 16 instances was found to be 72.8 $\pm$ 3.5 g., as compared with the expected value of approximately 75 g. calculated from the manufacturer's figures for the composition of the cream used in the test meal.

(d) Gastric Lipolysis

The degree of lipolysis which occurred during the 60 minutes the 75 g. fat meal remained in the stomach was measured

by estimation of NEFA release. The subjects were those controls and arterial disease patients whose gastric emptying was measured by the Hunt method.

As described in the previous section, the recovered test meal was cooled rapidly by collection in a container surrounded by crushed ice, in order to prevent further enzymatic lipolysis after removal from the stomach. Aliquots of the recovered gastric contents and of the original test meal were taken for estimation of NEFA, and processed as follows:- An aliquot of the original test meal (3 ml.) was pipetted into 5 ml. methanol and mixed on a Vortex mixer. The NEFA were then extracted by addition of 10 ml. petroleum ether (60° - 80°). An aliquot (3 ml.) of the petroleum ether extract was titrated for NEFA content as described in Chapter II. In the case of the recovered gastric contents, because of its very high NEFA content only 1 ml. was added to 5 ml. methanol, petroleum ether (20 ml.) was used to extract the NEFA, and 1 ml. of the petroleum ether extract was made up to a volume of 3 ml. with petroleum ether before titration.

(e) Radiological Determination of Gastric Emptying

Determination of gastric emptying was carried out using enteric-coated barium granules[1], as described by Horton et al.

[1] Paines and Byrne Ltd., Pabyrn Laboratories, Greenford, Middlesex, England.

(1965). These barium granules were devised to avoid any possibility of irritation of the gastric mucosa by the barium sulphate, and to give a compound similar in specific gravity to food.

A standard breakfast, containing 75 g. fat, was given to all subjects. Its composition was as follows:- porridge (4 tablespoons), eggs (2), butter, cream, milk, 1 cup tea. Barium granules (2 teaspoonfuls) were mixed with the porridge. The subjects were asked to swallow the porridge at intervals during the meal in order to ensure even distribution of the granules throughout the food in the stomach. All food and drink was then withheld until the test was completed, but the patients' activities were otherwise unrestricted.

Patients were screened in the erect posture, using an image intensifier and commencing three hours after the meal. The outline of the greater curvature of the stomach was readily detected. Screening was carried out briefly at hourly intervals until emptying of the stomach was completed. If the stomach was seen to be almost empty at the hourly screening, the examination was repeated half-an-hour later. The gastric emptying time was taken as the time which elapsed from commencement of the meal until the stomach was first seen to be empty. As recommended by Horton et al. (1965), the presence of a few

residual granules (approximately 10) in the stomach was ignored, since they were assumed to be trapped in gastric mucosal folds.

Hourly venepunctures were carried out for 8 hours, and plasma triglyceride estimated as described in Chapter II.

I. GASTRIC SECRETION

Since gastric acid is easily measured, it has been the component of gastric juice studied most extensively. In addition to hydrochloric acid, however, gastric secretions are known to contain various inorganic ions, pepsinogens, other proteins and polypeptides, mucus, and intrinsic factor (Farrar and Bower, 1967). The measurements of gastric acid secretion most frequently employed are those of basal acid output (that is, acid secretion without identifiable stimulation other than that provided by the presence of the tube), and the maximal (histamine-stimulated) acid output of the stomach. The basal acid secretion fluctuates to a greater extent than the maximal acid secretion and, of the measurements made on basal secretion, the volume of gastric juice shows the greatest reproducibility and the amount of free acid the least (Baron, 1963). Hunt and Kay (1954) and Murray *et al.* (1957)

have found that the basal acid secretion occupies about one-fifth of the maximal power.

GASTRIC SECRETION IN RELATION TO ALIMENTARY LIPAEMIA AND ATHEROSCLEROSIS

There is some evidence suggesting diminished gastric secretion in subjects with prolonged alimentary lipaemia and atherosclerosis. Becker et al. (1950) reported decreased acid and pepsin secretion, in addition to low pancreatic and blood lipase levels, in elderly subjects who also showed prolonged chylomicronaemia following fat loads. Patients with ischaemic heart disease were found to have lower basal and maximal acid outputs than age-matched controls, and an inverse correlation was demonstrated between mean basal acid secretion and the degree of lipaemia following 75 g. fat tolerance tests (Marks et al., 1962). Oliver (1962) also found evidence of reduced gastric secretion in ischaemic heart disease. In his series, 44 per cent of patients and only 16 per cent of controls failed to secrete free acid in response to a small dose of histamine.

Since the addition of pancreatic extract to fat meals in these subjects was found to reduce postprandial lipaemia

(Frazer, 1943; Becker et al., 1950), it was suggested that the diminished gastric secretion indicated a reduced ability to digest fat, either in the stomach because of gastric lipase deficiency, or in the small intestine because of pancreatic lipase deficiency (Marder et al., 1952; Bronte-Stewart and Krut, 1962). This was thought to result in absorption of a greater part of the dietary fat in particulateform, thus causing exaggerated alimentary lipaemia.

RESULTS

Collections of fasting and basal gastric juice were carried out in controls, arterial disease patients, pernicious anaemia patients, and duodenal ulcer patients as described on page 160. The results for all subjects are shown in Tables XVI to XIX.

(a) Effect of Age on Fasting and Basal Gastric Secretion

Both fasting and basal gastric secretion showed a tendency to decrease with age. In the case of fasting juice, male arterial disease patients showed a low-grade but significant negative correlation between age and the volume of fasting juice ($r = -0.38$, $P < 0.05$) while in male controls there was a similar, but statistically nonsignificant, trend ($r = -0.18$).

TABLE XVI. Fasting Gastric Secretion in Controls, and in Duodenal Ulcer and Pernicious Anaemia Patients (mean ± S.D.)

	Controls male all ages	Controls male 40 and over	Arterial Disease with D.U.	Pernicious Anaemia
Volume (ml.)	32.5 ±16.6 (n=25)	30.9 ±17.7 (n=17)	65.2 ±38.5 (n=5)	24.5 ±22.7 (n=4)
pH (mean and range)	2.65 1.28-7.80 (n=22)	2.60 1.55-7.60 (n=15)	2.36 1.45-3.65 (n=5)	7.63 7.19-8.20 (n=4)
Acid concentration (meq./l.)	30.1 ±18.6 (n=21)	28.7 ±15.1 (n=15)	22.4 ±20.7 (n=5)	0 (n=4)
Acid output (meq.)	1.27 ±1.03 (n=21)	1.16 ±0.92 (n=15)	1.93 ±2.96 (n=5)	0 (n=4)
Chloride concentration (meq./l.)	107.6 ±25.2 (n=21)	107.2 ±27.9 (n=15)	97.0 ±17.8 (n=5)	77.8 ±15.5 (n=4)
Chloride output (meq.)	3.85 ±1.86 (n=21)	3.53 ±1.90 (n=15)	6.78 ±5.29 (n=5)	1.51 ±1.45 (n=4)

n = no. of observations

TABLE XVII. Fasting Gastric Secretion in Arterial Disease Patients (mean ± S.D.)

	Arterial Disease males	Myocardial Infarction	Angina Pectoris	Peripheral Arterial Disease
Volume (ml.)	34.9 ±22.4 (n=43)	41.4 ±25.5 (n=22)	26.0 ±17.7 (n=11)	30.3 ±16.0 (n=10)
pH (mean and range)	3.65 1.32-8.20 (n=37)	2.96 1.32-8.13 (n=18)	4.30 1.40-8.20 (n=9)	4.30 1.40-7.60 (n=10)
Acid concentration (meq./l.)	32.3 ±25.4 (n=36)	35.2 ±22.5 (n=17)	33.7 ±30.2 (n=9)	26.2 ±27.3 (n=10)
Acid output (meq.)	1.12 ±1.36 (n=36)	1.51 ±1.59 (n=17)	0.68 ±1.09 (n=9)	0.85 ±1.03 (n=10)
Chloride concentration (meq./l.)	109.4 ±34.6 (n=32)	111.2 ±28.9 (n=17)	107.7 ±57.7 (n=7)	107.1 ±22.5 (n=8)
Chloride output (meq.)	3.78 ±3.02 (n=32)	4.59 ±3.35 (n=17)	2.49 ±2.57 (n=7)	3.19 ±2.34 (n=8)

n = no. of observations

TABLE XVIII. Basal Gastric Secretion in Controls, and in Duodenal Ulcer and Pernicious Anaemia Patients (mean ± S.D.)

	Controls male all ages	Controls male 40 and over	Arterial Disease with D.U.	Pernicious Anaemia
Volume (ml./30 mins.)	45.6 ±21.0 (n=32)	45.7 ±23.5 (n=23)	63.0 ±25.3 (n=5)	21.4 ±5.2 (n=5)
pH (mean and range)	2.89 1.17-7.91 (n=32)	3.06 1.24-7.91 (n=23)	2.53 1.24-4.83 (n=5)	7.84 7.18-8.77 (n=5)
Acid concentration (meq./l.)	34.2 ±24.9 (n=32)	31.3 ±22.5 (n=23)	34.0 ±36.2 (n=5)	0 (n=5)
Acid output (meq./30 mins.)	1.71 ±1.44 (n=32)	1.57 ±1.32 (n=23)	2.37 ±2.42 (n=5)	0 (n=5)
Chloride concentration (meq./l.)	110.4 ±23.5 (n=31)	111.7 ±23.6 (n=23)	104.4 ±35.5 (n=5)	63.2 ±24.5 (n=5)
Chloride output (meq./30 mins.)	5.01 ±2.12 (n=30)	4.92 ±2.19 (n=23)	6.67 ±3.38 (n=5)	1.42 ±0.75 (n=5)

n = no. of observations

TABLE XIX. Basal Gastric Secretion in Arterial Disease Patients (mean ± S.D.)

	Arterial Disease males	Myocardial Infarction	Angina Pectoris	Peripheral Arterial Disease
Volume (ml./30 mins)	40.4 ±27.5 (n=51)	44.0 ±29.8 (n=23)	29.1 ±20.6 (n=14)	45.6 ±28.1 (n=14)
pH (mean and range)	3.41 1.16-8.07 (n=51)	3.40 1.30-8.07 (n=23)	3.48 1.38-7.78 (n=14)	3.39 1.16-7.70 (n=14)
Acid concentration (meq./l.)	33.1 ±26.3 (n=51)	31.2 ±25.4 (n=23)	37.1 ±26.2 (n=14)	32.2 ±29.3 (n=14)
Acid output (meq./30 mins)	1.46 ±1.55 (n=51)	1.63 ±1.63 (n=23)	1.20 ±1.48 (n=14)	1.43 ±1.55 (n=14)
Chloride concentration (meq./l.)	111.6 ±28.4 (n=49)	115.0 ±26.7 (n=23)	113.5 ±31.1 (n=13)	103.8 ±29.6 (n=13)
Chloride output (meq./30 mins)	4.53 ±2.93 (n=49)	5.02 ±3.19 (n=23)	3.41 ±2.49 (n=13)	4.78 ±2.75 (n=13)

n = no. of observations

In the case of basal juice, in male controls over the age of 40 years a significant negative correlation between age and volume was demonstrated ($r = -0.42$, $P < 0.05$), but in male patients the falling-off in basal secretion did not reach levels of statistical significance ($r = -0.27$). There was a fairly close relationship between the volumes of fasting juice and basal juice obtained in individuals, both controls ($r = +0.53$, $P < 0.01$) and arterial disease patients ($r = +0.67$, $P < 0.001$).

(b) Comparisons of Gastric Secretion in Cigarette-Smokers and Non-Smokers

There were no significant differences in fasting or basal secretion between cigarette-smokers and non-smokers, although in the ischaemic heart disease group the smokers had a slightly greater volume of basal secretion (40.1 $\pm$ 28.7 ml.) than the non-smokers (30.3 $\pm$ 15.6 ml.). Amongst the controls the differences between the volume of basal secretion in smokers (44.6 $\pm$ 22.2 ml.) and non-smokers (40.8 $\pm$ 14.8 ml.) were even smaller.

(c) Comparisons of Gastric Secretion in Controls and Arterial Disease Patients

The volume and composition of the fasting and basal gastric secretions in arterial disease patients were almost

identical with those obtained in controls in all the characteristics examined, namely:- pH, acid content, and chloride content (Tables XVI to XIX).

(d) Comparisons of Gastric Secretion in Three Clinical Types of Atherosclerotic Arterial Disease

No significant differences were found in myocardial infarction, angina pectoris, and peripheral arterial disease patients in the volume or composition of the fasting and basal gastric secretions (Tables XVII and XIX). There was no difference in gastric secretion in the myocardial infarction patients between those receiving long term treatment with phenindione and the rest of the group.

(e) Comparison with Gastric Secretion in Duodenal Ulcer and Pernicious Anaemia Patients

The five male patients with proven duodenal ulceration produced a greater volume of fasting juice (65.2 ± 38.5 ml.) than the ten male controls (33.0 ± 17.2 ml., $P < 0.05$), and the 27 arterial disease patients aged 40 to 54 years (41.0 ± 24.8 ml., $P < 0.10 > 0.05$). The duodenal ulcer patients also had fasting juice of lower pH, and higher fasting acid and chloride outputs than the other subjects (Tables XVI and XVII),

but these differences did not attain levels of statistical significance. The basal gastric secretion in the duodenal ulcer patients showed similar differences in composition from that of controls and arterial disease patients, but the differences were not statistically significant (Tables XVIII and XIX).

The volumes of fasting and basal juice produced by the five pernicious anaemia patients was less than that produced by controls and arterial disease patients of 50 years and over, but not significantly so. The pH of the gastric secretion was, of course, alkaline, and the acid content zero. The pernicious anaemia patients had a lower chloride concentration in fasting juice, and in the basal juice there was a highly significant difference in chloride concentration between the pernicious anaemia patients (63.2 $\pm$ 24.5 meq. per l.) and the twelve controls of similar age (114.3 $\pm$ 20.3 meq. per l., $P < 0.001$).

DISCUSSION

The present study of basal gastric secretion showed no evidence of reduced secretion in atherosclerotic arterial disease such as was reported by Marks *et al.* (1962) and Oliver (1962). Basal gastric secretion has been found to

show rather marked fluctuations from day to day (Levin et al., 1950). It is likely, however, that if significant differences in gastric secretory capacity between arterial disease patients and controls had existed, they would have been detected by this technique since the expected gastric hypersecretion was found in the small group of arterial disease patients with duodenal ulceration, and gastric hyposecretion in the pernicious anaemia patients (Tables XVI and XVIII). The present study did not show an inverse correlation between basal acid secretion and postprandial lipaemia as reported by Marks et al. (1962). It tended, in fact, to show the reverse effect. The younger arterial disease patients, in whom postprandial lipaemia was abnormally high, had a higher basal acid output than the older patients in whom alimentary lipaemia was less marked. In addition, there was no demonstrable difference in gastric secretion between young arterial disease patients and controls of similar age, although significant differences between these two groups in fat tolerance had been found. There is no obvious explanation for the difference between the results of this study and those of Marks et al. (1962) in patients with ischaemic heart disease. The peripheral arterial disease patients in the present series did not differ from the ischaemic heart disease patients with respect to gastric

secretion (Table XIX) or alimentary lipaemia (Table XI). The majority of patients in Marks' series were receiving long-term treatment with phenindione, whereas only a minority of the subjects in this study were being treated with anti-coagulants. However, Marks et al. (1962) measured gastric secretion before and after commencement of phenindione in three subjects and found it unchanged. Similarly, in the patients in this series on long-term phenindione therapy no effect on gastric secretion could be demonstrated.

Both controls and patients in this series showed a decrease in basal acid secretion with age. A number of investigators (Levin et al., 1951; Vakil and Mulekar, 1965) have found a similar falling-off of both basal and histamine-stimulated acid secretion in older healthy males and females. Vakil and Mulekar (1965) showed that cigarette-smokers had a tendency to greater gastric secretion than non-smokers, although the differences were not statistically significant. A similar trend was seen in the present study (page 173).

II. GASTRIC EMPTYING

HISTORICAL REVIEW

The wellknown observations by Beaumont (1833) on gastric function in Alexis St. Martin, a subject with a traumatic gastric fistula, include an account of intermittent contractions

of the stomach. In 1886 Hofmeister and Schütz published their classic description of gastric peristalsis occurring in excised canine stomachs, and in 1898 Cannon described the same phenomenon in unanaesthetised cats. Following these observations many studies designed to elucidate the mechanisms controlling gastric emptying were carried out by physiologists both in experimental animals and in man. Hirsch (1893) showed that the gastric emptying rate was more rapid in dogs with high than with low duodenal fistulas. Similar experiments on gastric emptying in animals with duodenal fistulas were carried out by Cohnheim and Dreyfus (1908), who found that re-injection of the chyme into the intestine in these animals slowed the rate of gastric emptying. Moritz (1901), who carried out many experiments on himself, noted that beer and thin soup left his stomach more rapidly than thick soup or solid foods. The information obtained from numerous subsequent experiments on the factors controlling gastric emptying will be summarised in a later section.(page 181).

METHODS OF MEASURING GASTRIC EMPTYING

(a) Intubation Techniques

Gastric intubation for the assessment of gastric function was introduced by Leube (1883), and the earliest studies of

gastric emptying in man were carried out by a single recovery technique - the withdrawal by gastric tube of residual gastric contents at a fixed interval of time after ingestion of the liquid test meal (Moritz, 1901). In a modification of this method, some workers carried out repeated aspiration and measurement of the gastric contents at intervals after the same test meal, the whole recovery minus a small sample being returned to the stomach after each withdrawal (Folley and Abbott, 1942; Vendel, 1946). A further elaboration of this method is the serial test meal technique, in which the gastric contents are withdrawn on different days at different times after taking standard meals containing a marker, and the various measurements are then synthesised against time. Several modifications of this method have been used. The most widely-used is that of Hunt (1954), in which a liquid test meal is used containing phenol red as a marker to determine the degree of dilution of the original test meal by gastric secretion.

(b) Radiological Techniques

Cannon (1898, 1904) used the then newly-developed X-ray technique to study gastric emptying of bismuth meals in animals. Radiological methods have also been used to determine gastric emptying rates in humans, the size of the shadow projected on the X-ray plate by radio-opaque material in the stomach being

taken as an index of the volume of the gastric contents, (Hellebrandt and Tepper, 1934). Serial radiological studies have proved to be one of the most popular methods of obtaining information on the rate and pattern of gastric emptying (Hawkins et al., 1953).

The use of barium sulphate has been criticised on the grounds that it may separate from the rest of the food in the stomach (Griffith et al., 1966), and that it is a gastric irritant and promotes early emptying of the stomach (Horton et al., 1965). The first criticism has been met by feeding an ordinary meal of known composition, using only a small quantity of barium emulsion to outline the stomach (Annegers and Ivy, 1947). The use of enteric-coated granules of barium sulphate along with a standard meal, as described by Horton et al. (1965), avoids any possibility of irritation of the gastric mucosa, and also gives a compound similar in specific gravity to food. Another major drawback of these radiological methods is the fact that a two-dimensional image of the stomach on an X-ray plate cannot give an accurate measurement of the volume of gastric contents, and these methods can therefore be used only to determine accurately the time taken by the stomach to empty completely and not to give information about the pattern of gastric emptying. A radiological technique by which the

pattern of gastric emptying could be determined was described by Weir and Card (1965). By introducing known volumes of a radio-opaque meal and measuring the resulting areas from a series of X-ray films, a calibration curve connecting volume and area could be constructed for a given subject. On another day, a radio-opaque meal was given and films were taken at known time intervals thereafter. By reference to the calibration curve, the volume of the meal remaining in the stomach at any given time could be determined.

(c) Radio-isotope Techniques

A more recently-developed method of assessing the rate of gastric emptying involves the use of radio-isotopes (Griffith et al., 1966). Chromium^{51} is incorporated in a standard breakfast, and the stomach is scanned at half-hourly intervals until almost empty. The exponential pattern of gastric emptying shown by the methods described previously has been confirmed by this method.

PHYSIOLOGICAL MECHANISMS CONTROLLING GASTRIC EMPTYING

Gastric emptying occurs for the most part as the result of rhythmic peristaltic waves which force chyme into the duodenum, although occasional emptying of liquids without evident gastric peristalsis has been reported (Shay and

Gershon-Cohen, 1934). The peristaltic waves, which at first are initiated about the incisura angularis and later at a higher level, involve in sequence the pyloric antrum, the pyloric sphincter, and the proximal duodenum. These structures, as the antral peristaltic wave approaches, are relaxed, open, and receptive (Quigley and Louckes, 1962). Peristalsis is initiated primarily by distension of the stomach by ingested food, and is influenced by vagal activity. Since the pattern of gastric peristalsis returns to normal soon after vagotomy, however, the intactness of the myenteric plexus is believed to be a more important factor.

The regulation of gastric emptying is thought to depend on varying degrees of inhibition of gastric motility. This inhibition is mediated by neural and hormonal mechanisms brought into action by cephalic pathways, or by stimulation of the precardial or postcardial receptors in the alimentary tract (Hunt, 1959). The receptors which play the most important part in regulating gastric emptying are found in the duodenum, and have been shown to be sensitive to pH, osmotic pressure, chemical changes, and volume changes (Hunt, 1959). The rate of gastric emptying is regulated by weakening the force of peristalsis or abolishing it altogether (by vagal reflexes or by hormonal action such as that of enterogastrone) rather than

by altering the frequency of peristaltic waves (Jungmann and Venning, 1952).

Pattern of Gastric Emptying

In 1898 Marbaix observed that in the healthy stomach the rapidity of emptying was proportional to the degree of distension of the stomach. This observation has been confirmed on numerous occasions since then (Hawkins et al., 1953; Hunt and McDonald, 1954). By the serial test meal technique, it was shown that the pattern of emptying of liquid test meals is exponential; that is, a constant fraction of test meal and secretions leaves the stomach every minute, so that a plot of the volume of meal remaining in the stomach against time on a linear scale gives a straight line. Because the emptying of the meal is exponential in form, the time required for the volume of the meal in the stomach to fall by half is a constant for any given stomach (Hunt, 1959).

Emptying of Different Foods

It has been shown that test meals fed in a homogeneous form may dissociate within the stomach into different phases which leave the stomach at different rates. In order to demonstrate this phenomenon, Wiggins and Dawson (1961) used finely emulsified test meals containing carbohydrate, fat,

protein, and the water-soluble unabsorbable marker substances phenol red and polyethylene glycol. They found that this meal tended to separate into a solid phase rich in fat and poor in marker, and a liquid phase poor in fat and rich in marker. The liquid phase was preferentially emptied from the stomach, and was also more readily sampled by a small-bore gastric tube. The findings of Borgström et al. (1962) confirmed these results. Harkins et al. (1964) studied gastric emptying of mixed test-meals in rats and demonstrated that carbohydrate emptied from the stomach more rapidly than fat or protein.

Factors affecting Rate of Gastric Emptying

This subject has been reviewed in detail by Thomas (1957). Various properties of the gastric contents are known to affect the rate of gastric emptying: the physical state of the food (large food particles are retained for longer than small particles); the osmotic activity (isotonic solutions leave the stomach more rapidly than either hyper- or hypotonic solutions); the pH of the gastric contents (acid solutions delay gastric emptying, weak alkaline solutions accelerate, whereas strongly alkaline solutions delay gastric emptying); and the presence of fats (numerous observers have found that fat coming into contact with the duodenal or upper intestinal mucosa inhibits gastric tone and motility). Other factors known to influence

the rate of gastric emptying include exercise, emotional disturbances, changes in oxygen tension, environmental temperature, various pathological conditions (such as haemorrhage, certain vitamin deficiencies, gastritis and enteritis and visceral irritations), and certain drugs. Malhotra (1967) has recently produced evidence that admixture of saliva with the test meal slows the rate of gastric emptying.

GASTRIC EMPTYING IN RELATION TO ALIMENTARY LIPAEMIA AND ATHEROSCLEROSIS

It has been suggested by a number of investigators that the degree and duration of alimentary lipaemia are influenced by the rate at which the stomach makes the fat meal available to the intestine for digestion and absorption. Frazer and Stewart (1939) observed a shift of the peak of chylomicron curves to the right in some subjects, and attributed it to delayed emptying of the stomach. Annegers and Ivy (1947) carried out serial radiographs following a radio-opaque fatty meal, and found variations in different persons in the rates of gastric emptying of the meal. These differences were reproduced on repeated examinations. They concluded that such differences could be a factor in determining the time of maximal chylomicronaemia, but that they did not explain why

some persons regularly exhibited chylomicron curves of twice the height and of considerably longer duration than others. Becker et al. (1950) also noted a shift to the right in chylomicron curves in several young persons who had been very apprehensive during the test, and suggested that in these subjects emotional stimuli had caused an inhibition of gastric motility. Marder et al. (1952) found that fat tolerance tests, measured by chylomicron counts and nephelometry, showed a higher curve and a later peak in elderly as compared with young subjects. They demonstrated a delay in gastric emptying of a barium meal in their elderly as compared with their young subjects, and felt that this accounted for the delayed peak in their fat tolerance tests. Basset and Kuo (1963) studied duodenal motility after intraduodenal infusions of fat mixed with barium. They found that, as compared with normal persons, four subjects with hypertriglyceridaemia showed evidence of duodenal stasis and a prolongation of emptying time of the second part of the duodenum.

RESULTS

(1) DETERMINATION OF GASTRIC EMPTYING BY THE HUNT TECHNIQUE

Comparisons of gastric emptying rates were made in controls and arterial disease patients, as well as in small groups of

patients with duodenal ulcer and pernicious anaemia. The degree of gastric emptying in one hour of a 750 ml. semi-liquid test meal was measured by the intubation technique described on page 162. The results for all subjects are shown in Table XX.

Effect of Age and Sex on Subjects

There was some evidence that the rate of gastric emptying was affected by age. Male arterial disease patients showed a lowgrade but statistically significant negative correlation of age with the percentage (by volume) of the meal recovered at 60 minutes ($r = -0.29$, $P < 0.05$). This was due mainly to the high recovery rates in the younger of the twelve peripheral arterial disease patients ($r = -0.67$, $P < 0.05$). In both meal and female controls there was a tendency for gastric emptying to be delayed in older subjects, but the correlation between age and the percentage of test meal recovered was not statistically significant ($r = +0.23$). No differences were observed in gastric emptying rates between males and females in either controls or patients.

Effect of Habitual Cigarette-Smoking

In the arterial disease group, habitual cigarette-smokers appeared to have slower rates of gastric emptying than nonsmokers, but no such effect was demonstrated in the controls.

TABLE XX. Results in All Subjects of Determination of Gastric Emptying at One Hour by Hunt Technique (mean ± S.D.)

Subjects	Volume recovered ml.	Volume passing pylorus ml.	Volume recovered %
Controls - male all ages (n = 31)	625 ± 112	278 ± 171	70.4 ± 15.1
Controls - male aged 40 and over (n = 22)	637 ± 114	273 ± 187	71.4 ± 16.4
Arterial Disease male (n = 47)	632 ± 130	272 ± 163	70.7 ± 15.4
Myocardial Infarct male (n = 23)	663 ± 114	248 ± 133	73.4 ± 13.1
Angina Pectoris male (n = 12)	559 ± 131	319 ± 167	64.3 ± 16.3
Peripheral Arterial Disease - male (n = 12)	645 ± 142	271 ± 210	71.8 ± 18.1
Arterial Disease + Duodenal Ulcer (n = 5)	634 ± 129	265 ± 106	70.4 ± 12.0
Pernicious Anaemia (n = 5)	712 ± 60	140 ± 107	84.2 ± 11.7

n = no. of observations

In the control group the percentage of test meal recovered at one hour was 66.9 ± 12.1 per cent in the 17 cigarette-smokers and 72.6 ± 19.6 per cent in the 14 non-smokers ($t = 1.00$). In the ischaemic heart disease group, however, comparison of the percentage of the test meal recovered in the 27 cigarette-smokers (73.1 ± 14.2 per cent) with that of the 8 non-smokers (60.3 ± 14.2 per cent) revealed significantly higher values in the cigarette-smokers, ($P < 0.05$). This difference was slightly accentuated when the recovery in the 16 smokers of 20 or more cigarettes daily (75.8 ± 9.3 per cent) was compared with that in the non-smokers ($P < 0.01$). In the entire group of arterial disease patients whose cigarette-smoking history was recorded, a gradient in gastric emptying rate was observed accorded to the daily cigarette consumption. In the 23 subjects with a cigarette consumption of 20 or more daily the mean volume of test meal recovered at 60 minutes was 75.9 ± 12.8 per cent; in the 15 cigarette-smokers whose consumption was less than 20 daily it was 67.9 ± 18.3 per cent; and in the nonsmokers 60.3 ± 14.2 per cent. The difference was statistically significant between the first and last groups ($P < 0.01$). In no case were these differences in gastric emptying rates, which were apparently related to differences in habitual cigarette-consumption, due to differing age structures of the groups. Nor were the age effects on

gastric emptying described in the previous section due in fact to differences in cigarette consumption since older arterial disease patients appeared to smoke as heavily as the younger patients.

Effect of Atherosclerotic Arterial Disease

The rates of gastric emptying were found to be very similar in controls and arterial disease patients, and in the myocardial infarction, angina pectoris, and peripheral arterial disease patients (Table XX). On subdivision into different age groups no significant differences were observed, although gastric emptying was rather slower in the 21 male arterial disease patients aged 40 to 49 years than in the 10 controls of the same age (volume recovered 75.5 $\pm$ 15.0 per cent as compared with 66.9 $\pm$ 16.1 per cent).

Comparison with Duodenal Ulcer and Pernicious Anaemia Patients

Gastric emptying in the 5 patients with duodenal ulceration was similar to that in controls and other patients of the same age. Although in the 5 pernicious anaemia patients gastric emptying was slower than in the other groups, the volume recovered at 1 hour (84.2 $\pm$ 11.7 per cent) was not significantly greater than that in male controls of 50 years and over (75.2 $\pm$ 16.2 per cent).

Acid and Chloride Secretion during Test Meal

The pH, acid concentration, and chloride concentration of the recovered gastric contents are shown in Table XXI. No significant differences were found between the controls and the arterial disease patients, nor in patients belonging to the myocardial infarction, angina pectoris, and peripheral arterial disease groups. As anticipated, the pH was significantly higher and the acid and chloride concentrations lower, but not significantly so, in the pernicious anaemia subjects. The reverse was the case in the duodenal ulcer patients, but the differences were not statistically significant.

Comparison of Watery Phase with Rest of Recovered Test Meal

During recovery of the test meal it became evident that the test meal did not remain homogeneous in the stomach. In a number of subjects, if gentle suction was applied at the commencement of aspiration, it was possible to obtain approximately 10 to 30 ml. of non-turbid or only slightly turbid yellowish fluid before the rest of the semiliquid test meal appeared. The composition of this watery phase was analysed in 18 instances and is shown in Table XXII. On comparison with the rest of the recovered gastric contents it was found to have a markedly lower glyceride and NEFA content, an

TABLE XXI. Composition of Original Test Meal and of Gastric Contents recovered at One Hour (mean ± S.D.)

Subjects	pH	Acid Concentration meq./l.	Chloride Concentration meq./l.
Original test meal			
All Subjects	6.45 ± 0.13	4.5 ± 1.7	11.7 ± 1.9
Recovered gastric contents			
Controls - male all ages	4.70 ± 0.48 (n = 31)	33.2 ± 15.5 (n = 31)	32.8 ± 11.6 (n = 29)
Controls - male aged 40+	4.72 ± 0.49 (n = 22)	32.5 ± 15.4 (n = 22)	33.2 ± 13.0 (n = 22)
Arterial Disease male	4.93 ± 0.65 (n = 48)	31.1 ± 12.5 (n = 46)	32.8 ± 11.1 (n = 46)
Myocardial Infarct male	4.91 ± 0.78 (n = 23)	30.7 ± 13.8 (n = 22)	31.7 ± 12.3 (n = 23)
Angina Pectoris male	5.16 ± 0.28 (n = 13)	29.8 ± 11.8 (n = 12)	32.8 ± 8.3 (n = 12)
Peripheral Arterial Disease - male	4.70 ± 0.61 (n = 12)	33.3 ± 11.5 (n = 12)	34.9 ± 11.6 (n = 11)
Arterial Disease + Duodenal Ulcer	4.70 ± 0.53 (n = 5)	34.8 ± 16.7 (n = 5)	28.2 ± 6.8 (n = 5)
Pernicious Anaemia	5.46 ± 0.18 (n = 5)	20.8 ± 5.6 (n = 5)	25.3 ± 7.6 (n = 4)

n = no. of observations

TABLE XXII. Composition of Watery Phase in 18 Subjects compared with Rest of Recovered Test Meal (mean ± S.D.)

Estimation	Watery Phase	Recovered Test Meal
pH	4.68 ± 0.96	4.91 ± 0.41
Acid meq./l.	20.9 ± 11.0	28.3 ± 12.3
Chloride meq./l.	37.8 ± 14.9	35.1 ± 12.5
Phenol red ug/ml.	1.38*** ± 0.29	1.83 ± 0.32
Glycerides g./100 ml.	2.30*** ± 1.93	6.04 ± 1.73
NEFA meq./l.	5.81*** ± 3.20	19.62 ± 9.13

*** Significantly lower than corresponding value for recovered test meal $P < 0.001$.

appreciably lower phenol red concentration, and to be only slightly lower in pH and higher in chloride content.

Gastric Emptying of Fat

Because of this evidence that the test meal might separate into phases of different composition, and that the different components of the meal might therefore leave the stomach at different rates, estimation of the gastric emptying of fat was carried out in 16 subjects. The total fat concentrations of the original test meal and the recovered gastric contents and washouts were measured, and the percentage of fat remaining in the stomach at one hour was calculated. No significant difference was found between the percentage of fat recovered in 7 controls (76.7 ± 9.2 per cent) and that in 9 arterial disease patients (71.9 ± 10.2 per cent). The somewhat higher mean value seen in the controls in this small series might have been due to the fact that they were rather older (53.9 ± 10.8 years) than the arterial disease patients (47.4 ± 6.6 years).

Relationship of Gastric Emptying to Peak of Fat Tolerance Test

Despite the fact that the gastric emptying rate was not measured on the same day as the fat tolerance test, there was

some evidence of a relationship between rate of gastric emptying and the time of maximal lipaemia during the fat tolerance test. The mean volumes of test meal recovered at 60 minutes was calculated for all subjects whose fat tolerance peak occurred at two hours, four hours, six hours and eight hours respectively (Table XXIII). This showed that gastric emptying tended to be most rapid in those with a 2-hour peak, intermediate in those with 4-hour and 6-hour peaks, and slowest in those with an 8-hour peak.

(2) RADIOLOGICAL DETERMINATION OF GASTRIC EMPTYING TIME

An attempt was made to demonstrate the relationship between gastric emptying and the time of the fat tolerance peak by a more direct method than that described above. A 75 g. fat tolerance test was carried out simultaneously with determination of the gastric emptying time by the use of enteric-coated barium granules, as described on page 168.

RESULTS

The relationship of the gastric emptying time to the plasma fat tolerance curve in the ten subjects is shown in Fig. 35. In order to eliminate differences in the fasting triglyceride concentrations, the plasma fat tolerance curves

TABLE XXIII. Relationship between Time of Fat Tolerance Peak and Rate of Gastric Emptying (mean ± S.D.)

Time of maximal lipaemia during fat tolerance tests	Volume of test meal recovered at 60 minutes %
2 hours	65.7 ± 23.2 (n = 5)
4 hours	71.9 ± 14.0 (n = 47)
6 hours	73.9 ± 14.0 (n = 22)
8 hours	80.5 ± 13.7 (n = 5)

n = no. of observations

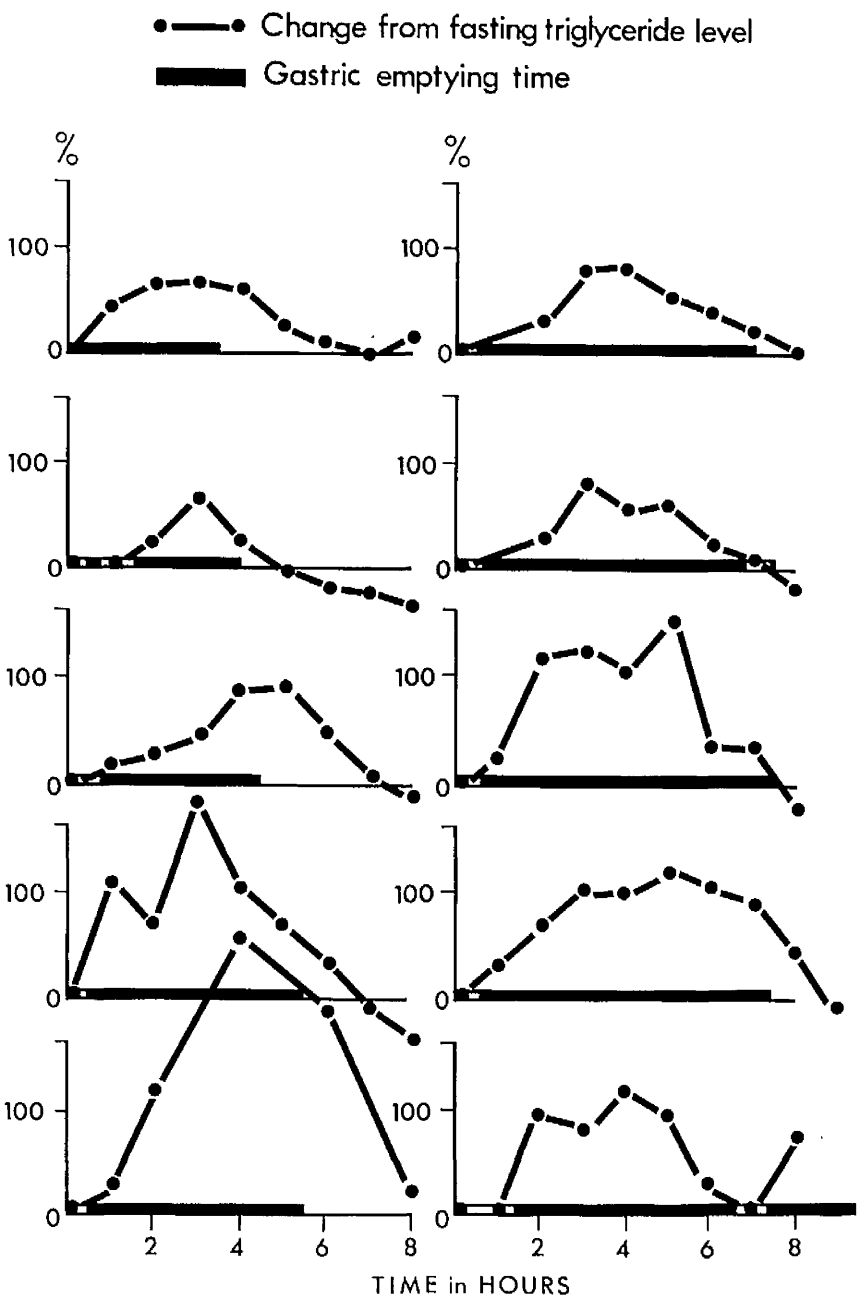


Fig. 35. Relation of plasma triglyceride changes during fat tolerance tests to time required for completion of gastric emptying.

are presented as percentage changes from the fasting level. The figure shows that the fat tolerance curves appeared to return to fasting levels more rapidly in the subjects whose stomachs emptied rapidly. During screening it was evident that in some subjects gastric emptying did not occur at a uniform rate. The size of the gastric shadow might remain apparently unchanged for several hours, and then rapid emptying might occur. The hourly plasma triglyceride determination showed that in a number of subjects the rise and fall in lipaemia were not smooth, but showed more than one peak, probably due to erratic gastric emptying. This phenomenon was most marked in the subject with the slowest gastric emptying time of 9½ hours, when the 8-hour sample showed a considerable rise in triglyceride for the third time.

GASTRIC EMPTYING - SUMMARY OF RESULTS

(1) Hunt Test Meal (a) Volume

Gastric emptying rates were similar in controls and arterial disease patients, in the groups of patients with myocardial infarction, angina pectoris, and peripheral arterial disease, and in those with duodenal ulceration. Gastric emptying was slower, but not significantly so, in the pernicious anaemia patients.

The younger arterial disease patients had slower gastric emptying rates than the older patients, but the reverse was the case in the controls. Habitual cigarette-smokers had slower gastric emptying rates than non-smokers.

(b) Gastric Secretion during Meal

No differences were found between controls and arterial disease patients in the pH and acid and chloride content of the recovered test meal. In pernicious anaemia patients, the pH of the recovered test meal was significantly higher and the acid and chloride concentrations lower than in controls of corresponding age. The patients with duodenal ulceration showed evidence of greater gastric secretion during the time the meal was in the stomach, but the differences were not statistically significant.

(c) Gastric Emptying of Fat

Evidence was found of separation of the test meal in the stomach into a low-fat watery phase and a high-fat phase. No difference between controls and arterial disease patients in gastric emptying of fat were seen in a small series of subjects.

(d) Relation of Gastric Emptying to Fat Tolerance Peak

Comparisons of rates of gastric emptying with the fat tolerance tests carried out in the same subjects on a different day indicated that the time of the fat tolerance peak was related to the rate of gastric emptying.

(2) Radiological Determination of Gastric Emptying Time

Simultaneous determinations were carried out of fat tolerance and of the time required for complete gastric emptying, using a 75 g. fat breakfast incorporating enteric-coated barium granules. In subjects with rapid gastric emptying the plasma triglyceride peak occurred earlier, and there was a more rapid return towards fasting triglyceride levels.

DISCUSSION

The studies of gastric emptying showed no evidence of a difference between controls and arterial disease patients in gastric motility, at least in the first hour after ingestion of the meal. This does not, of course, completely rule out such a difference. Emotional stimuli are well-known to have a marked influence on gastric motility (Thomas, 1957), and a technique which involves the trauma of swallowing a gastric

tube cannot be regarded as physiological. It is probable that in a number of subjects who showed little evidence of gastric emptying in the hour during which the test meal was in the stomach there was inhibition of gastric motility due to apprehension or nausea.

Previous investigations have suggested a slower rate of gastric emptying in elderly than in young subjects (Marder et al., 1952), and in the present study older male controls also showed a tendency to delay in gastric emptying. The reverse effect of age on gastric emptying seen in the arterial disease patients in this series is difficult to explain. Because of the higher cigarette consumption in the patients, a comparison of gastric emptying in smokers and non-smokers was carried out to determine if this accounted for the differences between the two groups. Although cigarette-smokers were found to have a significantly slower rate of gastric emptying than non-smokers in the ischaemic heart disease group (page 187) there was little evidence of a reduction in cigarette-smoking with age to account for the increase in gastric motility with age in these patients. Nor is there an obvious explanation of why gastric emptying should be delayed in patients who were habitual cigarette-smokers. The effects of nicotine on the gastrointestinal tract are complex, being mediated by its action on the central

nervous system, and by its stimulatory and inhibitory effects on autonomic ganglia. The effects of cigarette-smoking are even more complex, but Carlson (1916) and Schnedorf and Ivy (1939) found that a few puffs of cigarette-smoke inhibited gastric hunger contractions in man and dogs for up to an hour, although subcutaneous injection of nicotine (1 mg.) had no effect. Subsequent experiments designed to demonstrate an acute effect of cigarette-smoking on gastric motility in humans yielded inconclusive results (U.S. Public Health Service, 1964). Inhalation of tobacco smoke in dogs with gastric and duodenal fistulae, however, resulted in a brief period of excitation followed by a long period of inhibition of gastric emptying (Chvoles and Skulov, 1936). The results in the present series suggest a reduction of gastric motility in habitual cigarette-smokers, even after overnight abstention from smoking. Smoking has long been believed to reduce hunger (Murray, 1793), and has been shown to interfere with weight gain (Brožek and Keys, 1957), and it may be that the mechanism of action is inhibition of the motility and hunger contractions of the stomach.

The dissociation of the homogeneous test meal into a fat-poor liquid phase and a more solid fat-rich phase, previously shown by Wiggins and Dawson (1961) and Borgström et al. (1962) is also demonstrable in some subjects in this study. This

suggested the importance of studying the rate of gastric emptying of fat as well as of the test meal as a whole, and in the small series available no significant differences between controls and arterial disease patients were demonstrated.

Although there is considerable variability in individual subjects, a relationship has been shown between the gastric emptying rate and the time of maximum postprandial lipaemia (Table XXIII, Fig. 35). Such a relationship was suggested previously by Annegers and Ivy (1947). There is no evidence that the intensity of alimentary lipaemia is significantly influenced by the rate of gastric emptying, and the study reported earlier (Table XII) has shown that it is a function of the fasting triglyceride concentration.

III. GASTRIC LIPOLYSIS

HISTORICAL REVIEW

Shortly after Claud Bernard's well known observations in 1850 on the emulsifying and digestive action of pancreatic juice on neutral fat in the small intestine, Marcet (1858) reported that digestion of neutral fat occurred in the stomach also. This finding was confirmed by Cash (1880), who demonstrated in addition that extracts of the gastric mucosa could bring

about fat digestion in the test tube. Volhard (1900) also observed the *in vitro* lipolytic activity of gastric juice and extracts of gastric mucous membrane, and proposed that a specific gastric lipase was present. The properties and kinetics of the lipase in gastric juice were the subject of a series of experiments by Stade (1903). Several of these workers had suggested that a specific gastric lipase was present, but experiments *in vivo* to confirm this hypothesis yielded inconclusive results, owing to the difficulty of excluding the possibility of reflux of duodenal juice (Oppenheimer, 1936; Borgström *et al.*, 1957) and the existence of a true gastric lipase has often been disputed. Studies on gastric juice obtained from Pavlov pouches in dogs (Hull and Keeton, 1917; Takata, 1921) showed the existence of a heat-labile, lipolytic factor, the activity of which was greatest in gastric juice of low acidity or where the acid was rapidly neutralised. Schønheyder and Volqvartz (1946) investigated gastric lipase obtained either from glycerol extracts of dried mucosa or from gastric juice, and found that under the conditions of their experiments it was active mainly against short-chain triglycerides. Siurala (1956), using the histochemical technique described by Gomori (1946), demonstrated distinct lipase activity in the zymogenic and mucous neck cells in the body of the human

stomach, as well as in the gastric mucosa in several other species. Bank et al. (1964) investigated the lipolytic activity of gastric juice on a triglyceride emulsion, and reported that, though some of the lipolysis was caused by gastric hydrochloric acid, about 60% of the activity was due to a heat-labile factor.

It appears therefore to be established that an appreciable degree of lipolysis occurs in the stomach, and that the greater part of this lipolysis is brought about by an enzyme rather that by gastric acid. It has not yet been established whether the enzymatic acitivy is due to a lipase originating in the stomach, or is due to regurgitation into the stomach of duodenal contents containing pancreatic lipase. There would appear, however, to be a considerable amount of evidence of the existence of a true gastric lipase, since, as already mentioned, lipolytic activity in the mucosa as opposed to on the surface has been demonstrated in extracts of washed gastric mucosa, and by histochemical techniques.

PROPERTIES OF GASTRIC LIPASE

The lipase obtained from human gastric mucosa has been stated to be remarkably stable (Gyotoku, 1928). Schønheyder and Volqvartz (1946) reported that in a pH range of 3 to 7 the half-time decomposition rate of the lipase from human gastric

juice was more than seven hours. Early investigations suggested that its optimum pH was in the region of pH5 (Davidsohn, 1912; Lichtenberg, 1932), but later work showed that it may vary, depending on the substrate and on the species investigated. The optimum pH for hydrolysis of tripropionin or tributyrin by gastric lipase was found to be 5.5 to 5.8, whereas the optimum for trilaurin or tristearin was 7.2 to 7.9 (Schønheyder and Volqvartz, 1946). Popiela et al. (1965) demonstrated that 78 per cent inhibition of gastric lipase was produced by p-chloromercuribenzoic acid, but that, like pancreatic lipase, it was unaffected by di-isopropyl-fluorophosphate.

FACTORS AFFECTING GASTRIC LIPOLYTIC ACTIVITY

Gastric Acid Secretion

Hull and Keeton (1917) and Takata (1921), in their investigations of gastric juice obtained from Pavlov pouches in dogs, found that lipolytic activity was greater in specimens which were of low acidity, or which were neutralised rapidly after collection. Bank et al. (1964), although they showed no direct correlation between the degree of acidity of human gastric juice and its lipolytic activity, reported that when acidity was low lipolytic activity tended to be high, and

vice versa. In contrast to this, Popiela et al. (1965) found a positive correlation between free acidity and the lipolytic activity of human gastric juice, and concluded that the production or secretion of the enzyme was in some way dependent on the secretion of hydrochloric acid.

Type of Fat

The extent of lipolysis in the stomach may be affected by the degree of emulsification of the fat (Volhard, 1900), and by the fatty acid chain length, being greatest in the case of short-chain fatty acids (Schønheyder and Volqvartz, 1946). It may also be affected by the degree of saturation of the fatty acids. Herting and Ames (1955) found in rats greater lipolysis of saturated than unsaturated fats, whereas Bank et al. (1964) showed that human gastric juice was more active against unsaturated fats.

Effect of Age

Schønheyder and Volqvartz (1946) found no significant differences in the hydrolysis of triglyceride by gastric juice in vivo between children and adults.

GASTRIC LIPOLYSIS IN RELATION TO ALIMENTARY LIPAEMIA AND ATHEROSCLEROSIS

Frazer (1943) showed that, following ingestion of a pancreatic lipase preparation along with a 30 g. fat meal, the chylomicron curve was lower than that obtained following the 30 g. fat meal alone. If the pancreatic extract was inactivated by heating before addition to the meal, it caused no such change in the lipaemic curve. Becker et al. (1950) also showed depression of chylomicron counts following pancreatic lipase administration in older subjects with high curves. In young people with low curves, on the other hand, addition of lipase resulted in a shift of the curve to the right, with a delayed return of chylomicron counts to the basal level. Tietz et al. (1960) found a definite improvement in fat tolerance in 4 out of 14 individuals when pancreatic extract was fed along with a fat meal. Reports have appeared also of improvement in fat tolerance of patients with atherosclerosis after feeding of an aminopolysaccharide extracted from hog gastric mucosa (Rossi and Rulli, 1958; Cantone et al., 1959). It has been suggested (Marks et al., 1962) that the greater alimentary lipaemia seen in patients with ischaemic heart disease may result from deficient lipolysis in the gastrointestinal tract, and that lipolysis of fat in the stomach may be an important

step in the preparation of fat for complete digestion by the more powerful pancreatic lipase.

RESULTS

The gastric lipolytic activity was compared in controls and arterial disease patients, as well as in small groups of patients with duodenal ulcer and pernicious anaemia. The degree of gastric lipolysis was determined by measurement of the NEFA concentration of the gastric contents recovered at one hour, as described on page 167. The results for all subjects are shown in Table XXIV.

All subjects showed evidence of lipolysis of the triglyceride in the test meal. The NEFA concentration of the recovered gastric contents showed a marked variation, the lowest recorded level being 1.83 meq. per l. and the highest 93.60 meq. per l. (Fig. 36). The degree of gastric lipolysis was not affected by the age of the individuals. The mean NEFA concentration of the recovered gastric contents was similar in controls and arterial disease patients, and in the three clinical groups of arterial disease patients (Table XXIV). There was no evidence of any relationship between the basal gastric secretory capacity and the amount of fat-splitting of the test-meal, nor could any characteristic be discerned

TABLE XXIV. NEFA Concentration of Recovered Test Meal in All Subjects (mean ± S.D.)

Subjects	NEFA Concentration meq. per l.
Original test meal	
All Subjects	0.48 ± 0.20
Recovered gastric contents	
Controls - male all ages	18.23 ± 16.12 (n = 32)
Controls - male aged 40+	19.07 ± 18.44 (n = 23)
Arterial Disease male	17.15 ± 10.18 (n = 48)
Myocardial Infarct male	17.35 ± 7.70 (n = 23)
Angina Pectoris male	19.18 ± 15.40 (n = 13)
Peripheral Arterial Disease - male	15.21 ± 7.44 (n = 12)
Arterial Disease + Duodenal Ulcer	26.24 ± 10.69 (n = 4)
Pernicious Anaemia	28.13 ± 23.14 (n = 5)

n = no. of observations

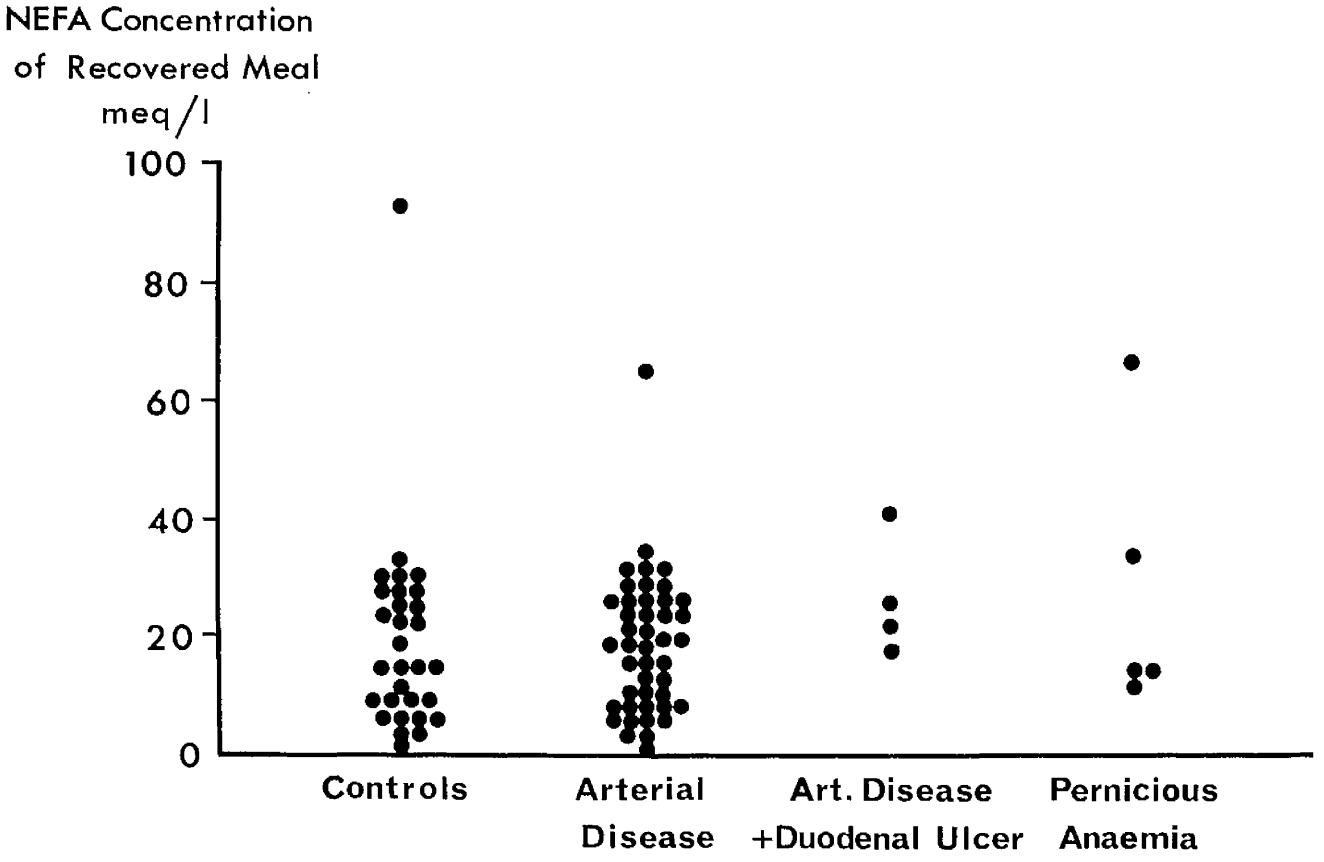


Fig. 36. NEFA concentration of recovered test meal in all subjects.

which distinguished subjects with marked degrees of lipolysis from others in the same group. The subjects with the highest NEFA concentrations in the control and arterial disease groups (Fig. 36) had basal acid outputs in thirty minutes of 2.70 meq. and 0 meq. respectively. The mean NEFA level was higher in patients with duodenal ulcer than in controls, and an even higher level was seen in the pernicious anaemia patients. Owing to the marked variability in all groups, however, the differences were not statistically significant.

DISCUSSION

Our studies of NEFA release showed that gastric lipolysis took place in all subjects. The degree of lipolysis varied very markedly from subject to subject, and appeared to be entirely haphazard. No relationship could be demonstrated between gastric secretory capacity and lipolytic ability. Gastric lipolysis took place to a similar extent in healthy controls and in patients with arterial disease.

Fat-splitting by gastric juice has been shown to represent the effects of hydrolysis both by acid and by a heat-labile factor, presumably a lipase (Bank et al., 1964). This may explain some of the contradictory reports in the literature, some workers stating that lipolytic activity was greater when

gastric acidity was low (Hull and Keeton, 1917; Takata, 1921), while others found that gastric juice of high acid content had greater lipolytic activity (Popiela et al., 1965). The greater mean NEFA release in our duodenal ulcer patients than in our controls is probably the result of greater acid hydrolysis, while the high levels in the patients with pernicious anaemia must be presumed to be due to enzymatic hydrolysis.

That patients with achylia gastrica were capable of gastric hydrolysis of fat was suggested many years ago (Pesthy, 1906), and has been confirmed in the present study. Adult patients with pernicious anaemia have been described as having total gastric secretory failure, since acid, pepsin, and intrinsic factor are absent from the gastric secretions, and, in addition, gastric atrophy is usually present in biopsy studies of the gastric mucosa (Wenger et al., 1967). It appears unlikely therefore that the gastric mucosa in these patients is capable of producing a lipase, and gastric lipolysis must be due to lipase regurgitated from the duodenum, or possibly to salivary lipase (Scheer, 1928). Despite the precautions taken in the present study (page 162), it is likely that some saliva was swallowed during the time the test meal was in the stomach. In the other subjects also, the marked variability in NEFA release, and its lack of correlation with gastric

secretory capacity, suggest that a chance process such as regurgitation of the powerful pancreatic lipase, rather than gastric secretion of a fat-splitting enzyme, is responsible for most of the lipolysis which takes place in the stomach.

SUMMARY AND CONCLUSIONS

1. Basal gastric secretion was similar in volume and composition in healthy controls and arterial disease patients. Gastric secretion was greater in young than in older subjects, and slightly greater in cigarette-smokers than in non-smokers.

2. The volume of test meal which left the stomach in 60 minutes was similar in healthy controls and in arterial disease patients. Controls showed slowing of gastric emptying with age, whereas the reverse effect was seen in arterial disease patients. Gastric emptying was slower in habitual cigarette-smokers than in non-smokers.

3. Gastric emptying of fat was similar in healthy controls and in arterial disease patients.

4. The peak in postprandial lipaemia occurred earlier in subjects with a rapid gastric emptying rate.

5. Gastric lipolysis occurred to a variable degree in all subjects, but did not differ in controls and arterial disease patients. The extent of gastric lipolysis showed no relation to the degree of postprandial lipaemia, and the evidence was against a significant role of gastric lipase in man.

Chapter VI. EFFECT OF CIGARETTE-SMOKING ON PLASMA LIPIDS, BLOOD GLUCOSE, AND PLATELET ADHESIVENESS

CONTENTS

Chapter VI CIGARETTE-SMOKING

ASSOCIATION BETWEEN CIGARETTE-SMOKING AND ATHEROSCLEROSIS

Cigarette-smoking has long been suspected to have harmful effects. The first convincing reports of an association between cigarette-smoking and ischaemic heart disease, however, were incidental findings in studies designed primarily to investigate the relationship between cigarette-smoking and lung cancer. Some years earlier Pearl (1938) had suggested that total mortality rates increased with increasing numbers of cigarettes smoked, and English et al. (1940) showed a positive association between cigarette-smoking and ischaemic heart disease. Doll and Hill (1954 and 1956) in Great Britain, and Hammond and Horn (1954, 1958a and 1958b) in the U.S.A. carried out large-scale prospective investigations of mortality ratios in men in relation to their smoking habits. The findings of these two independent studies were remarkably similar. The mortality ratios were greater in cigarette-smokers than in non-smokers, and increased with increased cigarette consumption. This excess mortality was due only in part to an increase in bronchial carcinoma; the larger part of the excess death rate was accounted for by deaths due to coronary artery disease. Mortality ratios in cigar- and pipe-smokers were much less than in cigarette-smokers, but greater than in non-smokers. There was a reduction

in mortality from coronary artery disease in those who gave up smoking. Other reports were published subsequently which tended to confirm these findings (Buechley et al., 1958). All the currently available evidence on the association between cigarette-smoking and ischaemic heart disease obtained from these and other investigations was summarised and evaluated in the report of the Royal College of Physicians of London (1962) on "Smoking and Health", and somewhat later in the report by the U.S. Public Health Service (1964). In these reports it was concluded that the evidence strongly suggested that heavy cigarette-smoking contributed to, or accelerated, the development of coronary heart disease or its complications, at least in men under 55 years.

A causal relationship of cigarette-smoking to atherosclerotic arterial disease has been suspected for many years, but has been little investigated. It appears, however, that the association of cigarette-smoking with peripheral arterial disease is even stronger than that shown with ischaemic heart disease. Weinroth and Herzstein (1946) found that male diabetics who smoked had a 50 per cent greater incidence of peripheral arterial disease than non-smokers. Eastcott (1962) reported that all but 2 of 114 new patients with obliterative arterial disease of the lower limbs were regular cigarette-smokers. In a study of 100 consecutive male and female patients

operated on for peripheral atherosclerotic occlusive disease, Lord (1965) found that 94 were heavy smokers, compared with 34 of 100 consecutive men and 19 of 100 consecutive women in the same age group with non-arterial lesions who showed good pedal pulses.

ASSOCIATION BETWEEN CIGARETTE-SMOKING AND PLASMA LIPIDS

The reason for the association between cigarette-smoking and atherosclerotic arterial disease is not known. In several studies, however, smokers have been shown to have increased plasma levels of cholesterol and other lipids, which might be associated with an increased liability to ischaemic heart disease. Gofman and his associates (1955) reported higher serum cholesterol and lipoprotein levels in cigarette-smokers than in non-smokers in men aged 20 to 29 years. High plasma cholesterol concentrations occurred more frequently in smokers than in non-smokers in a group of medical students (Thomas, 1958). In the Framingham study, cholesterol levels were found to be higher in cigarette-smokers, and to remain higher in those who stopped smoking (Dawber et al., 1959). Significantly higher cholesterol concentrations in smokers than in non-smokers were found in subjects in Finland, but not in the United States (Blackburn et al., 1960). Karvonen et al. (1959) also reported higher cholesterol values in cigarette-smokers, especially

in heavy smokers. In South Africa, higher total cholesterol and beta-lipoprotein cholesterol concentrations were found in heavy cigarette-smokers of all ages and races (Bronte-Stewart, 1961).

A few studies have shown no long-term effect of cigarette-smoking on plasma lipids. Konttinen (1962) in young men, and Acheson and Jessop (1961) in elderly men found no differences in serum cholesterol between smokers and nonsmokers.

The acute effects of cigarette-smoking on plasma lipids have also been studied. The main effect described has been a rapid and consistent rise in plasma NEFA concentration (Kershbaum et al., 1961; Soloff and Schwartz, 1964; Milton, 1966). This rise in NEFA level has been shown to be due to stimulation of the sympathetic nervous system and adrenal glands by nicotine (Kershbaum et al., 1963). The NEFA response to cigarette-smoking has been reported to be exaggerated in patients with healed myocardial infarction, the rise being more than double that observed in non-coronary patients and normal subjects (Kershbaum et al., 1962). There has been general agreement that cigarette-smoking results in no acute change in cholesterol, phospholipid, or triglyceride concentrations (Page et al., 1959, Kershbaum et al., 1961).

ASSOCIATION BETWEEN CIGARETTE-SMOKING AND BLOOD CLOTTING MECHANISMS

The link between cigarette-smoking and atherosclerotic arterial disease might be provided by altered blood clotting mechanisms. Reports of the effect of cigarette-smoking on blood coagulation are conflicting. It was found by Blackburn *et al.* (1959) to have no effect on plasma Stypven time, and by Mustard and Murphy (1963) to cause no change in various blood clotting mechanisms. On the other hand Ambrus and Mink (1964) reported hypercoagulability, using various clotting tests, and Engelberg (1965) found an increased thrombotic tendency *in vitro* following cigarette smoking. Platelet studies were reported to show decreased platelet survival times in smokers as compared with nonsmokers (Mustard and Murphy, 1963), and acute increases in platelet adhesiveness following cigarette smoking (Ambrus and Mink, 1964; Ashby *et al.*, 1965).

PURPOSE OF PRESENT INVESTIGATION

The factors responsible for platelet adhesiveness have not been completely established, but some which have been suggested are the blood glucose (Bridges *et al.*, 1965), and various plasma lipid components including triglyceride (Philp and Payling Wright, 1965) and fatty acids (Thompson, 1966). Since these are factors affected by cigarette-smoking, the

present investigation was carried out in order to determine the short term effect of cigarette-smoking on all these factors, and the relationship between them.

This work has been the subject of a previous publication (Murchison and Fyfe, 1966).

METHODS

(a) Patients

Twelve patients (8 males and 4 females) from a general medical ward who habitually smoked ten or more cigarettes daily were investigated. They were aged from 37 to 67 years with a mean age of 55 years. Their diagnoses were: ischaemic heart disease (3), peripheral arterial disease (3), hypertension, idiopathic diarrhoea, simple goitre, rheumatic heart disease, epilepsy, and pneumonia.

(b) Procedure

The patients fasted overnight and remained at rest in bed throughout the experiment. Two basal venous blood samples were taken 20 minutes apart. The patients were then instructed either to smoke two of their usual brand of cigarettes during a 15-minute period, or to sham-smoke by puffing the unlit cigarette for the same length of time. A third blood sample was taken at the end of the 15-minute period, and three further blood samples at 10-minute intervals. Within the next few days the experiment was repeated with sham-smoking substituted

for smoking and vice versâ. Seven patients smoked in the first experiment, and five in the second experiment.

The serum cholesterol and triglyceride, and total and individual NEFA were determined as described in Chapter II. The following estimations were also carried out:-

Blood sugar, by an autoanalyzer method (Technicon method N-9), measuring total reducing substances.

Platelet adhesiveness, by a method based on that of Payling Wright (1941). 2 ml. of citrated blood (1 part 3.8% sodium citrate to 9 parts blood) was rotated in a spherical flask at $3\frac{1}{2}$ r.p.m. for 20 minutes, commencing 5 minutes after venepuncture. Platelet counts were done by direct counting of whole blood using 1% ammonium oxalate and phase-contrast microscopy (Brecher and Cronkite, 1950). The percentage of adhesive platelets was expressed as:

$$\frac{\text{Initial count - final count}}{\text{Initial count}} \times 100$$

RESULTS

Total Plasma NEFA

The mean plasma NEFA levels during the smoking and sham-smoking tests are shown in Table XXV.

There was a significant rise in the mean plasma NEFA

TABLE XXV. Changes in Plasma NEFA during Smoking and Sham-Smoking Tests (mean ± S.D.)

TIME OF SAMPLE		PLASMA NEFA microeq./l.	
		SMOKING	SHAM-SMOKING
BASAL PERIOD	0 min.	395 ± 149	369 ± 76
	20 min.	393 ± 149	359 ± 69
AFTER SMOKING OR SHAM-SMOKING	35 min.	453 ± 168***	381 ± 72
	45 min.	496 ± 176**	403 ± 82
	55 min.	442 ± 98	386 ± 83
	65 min.	399 ± 95	369 ± 89

Significant difference from mean basal level

**P < 0.01, ***P < 0.001

level after smoking, reaching a maximum 10 minutes after the end of the smoking-period, and falling again to the basal level over the succeeding 20 minutes. While the plasma NEFA level increased to a variable extent in every case after smoking, after sham-smoking the plasma NEFA level rose in 5 cases, was unchanged in 3, and fell in 4. The small mean increase after sham-smoking was not statistically significant.

Individual Serum NEFA

The changes in proportions of the individual serum NEFA after smoking are shown in Table XXVI, and after sham-smoking in Table XXVII.

Both smoking and sham-smoking were followed by a significant increase in the concentration of oleic acid in the NEFA fraction, and a significant fall in the concentration of palmitic acid. This effect was more notable after sham-smoking than after smoking, but did not correlate well with the magnitude of the changes in total plasma NEFA. The other fatty acids in the NEFA fraction showed a similar tendency for an increase in unsaturated at the expense of saturated fatty acids, but the individual changes were not significant.

TABLE XXVI. Changes in Proportions of Individual Serum NEFA during Smoking Test (percentage mean ± S.D.)

NEFA	BASAL PERIOD		AFTER SMOKING			
	0 min.	20 min.	35 min.	45 min.	55 min.	65 min.
Myristic C14:0	2.6 ±0.5	2.4 ±0.5	2.7 ±0.6	2.7 ±0.9	2.5 ±0.5	2.4 ±0.4
Palmitic C16:0	29.5 ±2.0	29.8 ±2.4	28.5* ±1.9	27.7* ±1.7	28.4 ±3.5	28.4 ±3.0
Palmitoleic C16:1	6.3 ±1.0	6.6 ±0.9	6.8 ±0.8	6.6 ±0.7	6.4 ±0.8	6.3 ±0.7
Stearic C18:0	12.5 ±2.0	12.5 ±2.0	11.8 ±2.0	11.8 ±1.8	12.6 ±2.4	12.9 ±1.8
Oleic C18:1	39.9 ±3.8	39.7 ±3.6	40.8 ±3.5	41.7* ±3.0	40.2 ±4.9	40.8 ±4.8
Linoleic C18:2	9.2 ±1.5	8.9 ±1.7	9.4 ±1.3	9.4 ±1.6	9.9 ±2.8	9.1 ±1.8

Significant difference from mean basal level * $P < 0.05$

TABLE XXVII. Changes in Proportions of Individual Serum NEFA during Sham-Smoking Test (percentage mean ± S.D.)

NEFA	BASAL PERIOD		AFTER SHAM-SMOKING			
	0 min.	20 min.	35 min.	45 min.	55 min.	65 min.
Myristic C14:0	2.9 ±1.2	2.5 ±0.6	2.6 ±0.8	2.2 ±0.5	2.4 ±0.6	2.6 ±0.8
Palmitic C16:0	30.7 ±3.5	29.8 ±2.5	29.7 ±3.2	27.8* ±2.3	28.8* ±1.7	29.4 ±2.2
Palmitoleic C16:1	6.5 ±0.9	6.4 ±0.6	6.1 ±0.9	6.4 ±0.7	6.5 ±1.1	6.3 ±0.7
Stearic C18:0	12.4 ±2.1	12.4 ±1.8	12.5 ±2.2	12.4 ±1.6	12.4 ±2.4	11.9 ±2.7
Oleic C18:1	37.5 ±3.5	40.0 ±4.6	40.5* ±5.2	41.4* ±4.8	40.5** ±4.7	41.0* ±5.7
Linoleic C18:2	9.9 ±2.6	8.9 ±1.2	8.6 ±1.4	10.2 ±2.9	9.3 ±2.1	8.7 ±1.7

Significant difference from mean basal level

*$P < 0.05$, **$P < 0.01$.

Serum Cholesterol

Serum cholesterol values during smoking and sham-smoking tests are shown in Table XXVIII. No significant changes in serum cholesterol concentration occurred in either group.

Serum Triglyceride

Serum triglyceride values during smoking and sham-smoking tests are shown in Table XXIX. No significant changes in serum triglyceride concentration occurred in either group.

Blood Glucose

The changes in mean blood glucose level following smoking and sham-smoking are shown in Table XXX.

During both smoking and sham-smoking tests there was a small but significant increase in blood glucose between the first and second basal levels. For this reason, the 20-minute level was taken as the baseline in assessing the effects of smoking and sham-smoking on blood glucose.

Smoking was followed by a small but significant increase in blood glucose. This happened in every case and showed no tendency to return to the fasting level. There was no change after sham-smoking.

TABLE XXVIII. Serum Cholesterol Concentrations during Smoking and Sham-Smoking Tests (mean ± S.D.)

TIME OF SAMPLES		CHOLESTEROL mg./100 ml. SMOKING	SHAM-SMOKING
BASAL PERIOD	0 min.	265 ± 44	253 ± 52
	20 min.	266 ± 40	259 ± 52
AFTER SMOKING OR SHAM-SMOKING	35 min.	266 ± 40	260 ± 51
	45 min.	272 ± 47	258 ± 52
	55 min.	270 ± 46	256 ± 47
	65 min.	270 ± 47	265 ± 55

TABLE XXIX. Serum Triglyceride Concentrations during Smoking and Sham-Smoking Tests (mean ± S.D.)

TIME OF SAMPLE		TRIGLYCERIDE mg./100 ml. SMOKING	SHAM-SMOKING
BASAL PERIOD	0 min.	97 ± 29	101 ± 26
	20 min.	98 ± 29	102 ± 32
AFTER SMOKING OR SHAM-SMOKING	35 min.	99 ± 31	98 ± 28
	45 min.	96 ± 30	96 ± 26
	55 min.	94 ± 31	96 ± 27
	65 min.	96 ± 32	98 ± 33

TABLE XXX. Changes in Blood Glucose during Smoking and Sham-Smoking Tests (mean ± S.D.)

TIME OF SAMPLE		BLOOD GLUCOSE mg./100 ml. SMOKING	SHAM-SMOKING
BASAL PERIOD	0 min.	68.2 ± 8.6	68.6 ± 8.4
	20 min.	72.6 ± 9.2	71.3 ± 8.6
AFTER SMOKING OR SHAM-SMOKING	35 min.	73.6 ± 9.2	70.8 ± 9.0
	45 min.	76.7* ± 9.2	70.4 ± 8.4
	55 min.	76.7* ± 8.0	70.1 ± 7.3
	65 min.	76.9* ± 7.1	71.4 ± 7.4

*Significant difference from 20-minute level $P < 0.05$.

Platelet Count

The platelet counts during smoking and sham-smoking tests are shown in Table XXXI. After sham-smoking no change occurred in the platelet count. Following smoking, however, the small increase in total platelet count over the basal figure was significant at the 1% level, as determined by the Student 't' test for paired values (Bradford Hill 1967).

Platelet Adhesiveness

The mean platelet adhesiveness values during smoking and sham-smoking tests are shown in Table XXXII.

There was a mean rise in platelet adhesiveness in both smoking and sham-smoking groups. In the sham-smoking group, 9 of the 12 subjects showed an increase, and in this group the mean rise was statistically significant ($P < 0.05$). The change after smoking was more variable, a rise in 7 cases and a fall in 5 cases, and the mean rise was not statistically significant.

Comparison of Arterial Disease Patients with Other Subjects

There was no significant difference between the 6 patients with arterial disease and the others in basal lipid levels, blood glucose, and platelet adhesiveness, or in the magnitude of the responses to smoking. The maximum NEFA increase after smoking was 127 $\pm$ 66 microeq./l. in the arterial disease patients and 163 $\pm$ 46 microeq./l. in the non-arterial disease

TABLE XXXI. Changes in Platelet Count during Smoking and Sham-Smoking Tests (mean ± S.D.)

TIME OF SAMPLE		PLATELET COUNT/cu.mm. SMOKING	SHAM-SMOKING
BASAL PERIOD	0 min.	157,600 ±32,600	165,000 ±43.800
	20 min.	155,500 ±41,800	153,000 ±38,400
AFTER SMOKING OR SHAM-SMOKING	35 min.	-	-
	45 min.	166,300** ±35,000	161,400 ±47,500
	55 min.	-	-
	65 min.	157,100 ±34,700	160,400 ±50,100

Significant difference from mean basal level, ** $P < 0.01$ as determined by the Student 't' test for paired values.

TABLE XXXII. Changes in Platelet Adhesiveness during Smoking and Sham-Smoking Tests (mean ± S.D.)

TIME OF SAMPLE		PLATELET ADHESIVENESS % SMOKING	SHAM-SMOKING
BASAL PERIOD	0 min.	35.5 ± 14.1	41.0 ± 8.3
	20 min.	36.9 ± 13.3	43.7 ± 7.9
AFTER SMOKING OR SHAM-SMOKING	35 min.	-	-
	45 min.	39.6 ± 12.2	48.3* ± 7.3
	55 min.	-	-
	65 min.	35.1 ± 13.0	47.3 ± 11.1

Significant difference from mean basal level *$P < 0.05$ as determined by the Student 't' test for paired values.

subjects.

Relationships between Changes in NEFA, Glucose and Platelet Adhesiveness

There was no correlation between basal platelet adhesiveness and basal levels of either plasma NEFA or blood glucose.

In the sham-smoking group it was evident that there was a good correlation between the percentage rise in NEFA and the rise in platelet adhesiveness, as shown in Fig. 37.

$$\% \text{ rise} = \frac{\text{45-minute level - Mean basal level}}{\text{Mean basal level}} \times 100$$

This correlation was significant at the 1% level ($r = +0.71$). There was a similar, but less significant, correlation between percentage changes in NEFA and platelet adhesiveness after smoking ($r = +0.58$, $P < 0.05$), as shown in Fig. 38. These correlations of platelet adhesiveness changes with changes in total NEFA were greater than the corresponding correlations with changes in the individual fatty acids. In the smoking group, however, there was a rise in glucose, and Fig. 39 illustrates the negative correlation between changes in blood glucose and platelet adhesiveness ($r = -0.70$, $P < 0.02$).

The relationship between the percentage change in platelet adhesiveness (Y) and the percentage changes in NEFA (X_1) and blood glucose (X_2) after smoking can be expressed as follows:

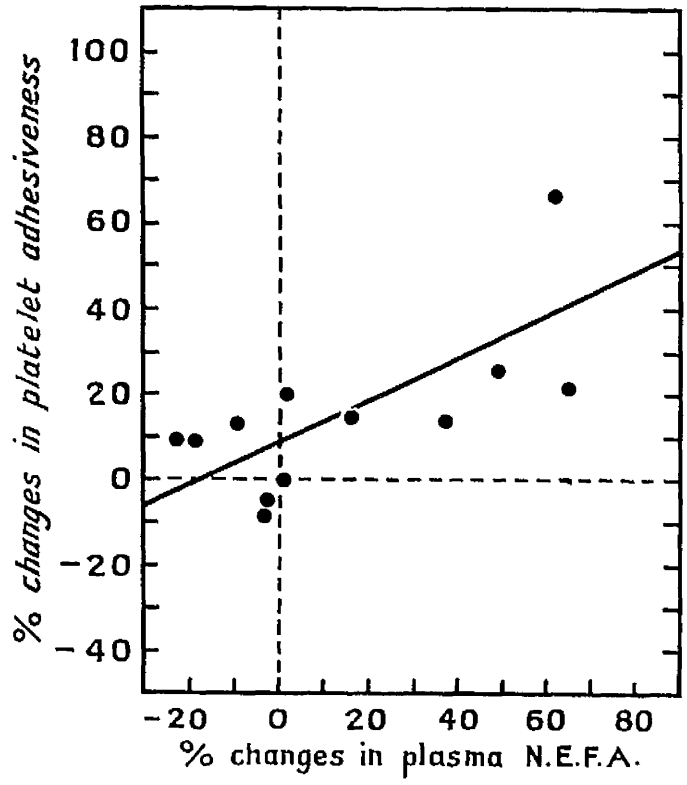


Fig. 37. Correlation between percentage changes in plasma NEFA and platelet adhesiveness after sham smoking ($y = 0.51\ x + 9.19$, $r = +0.71$).

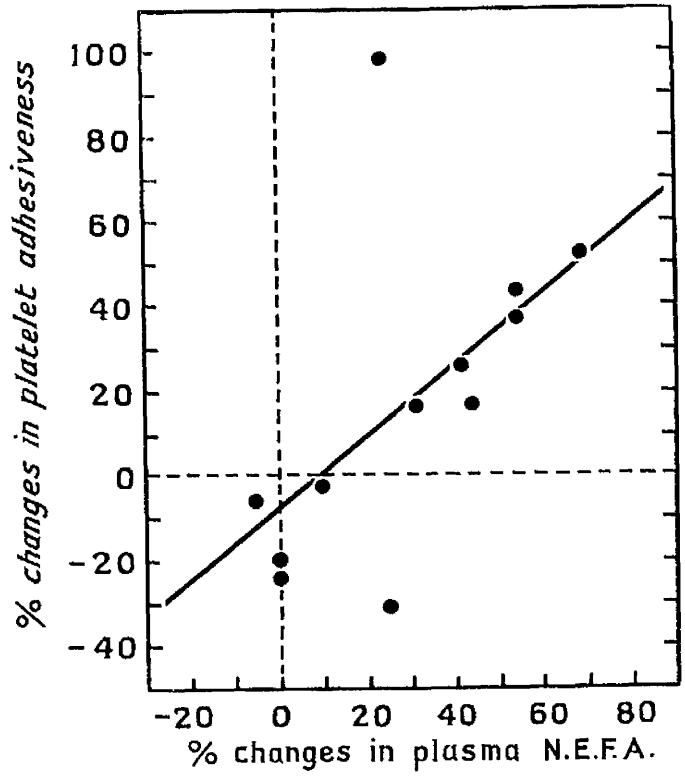


Fig. 38. Correlation between percentage changes in plasma NEFA and platelet adhesiveness after smoking ($y = 0.86\ x - 8.22$, $r = +0.58$).

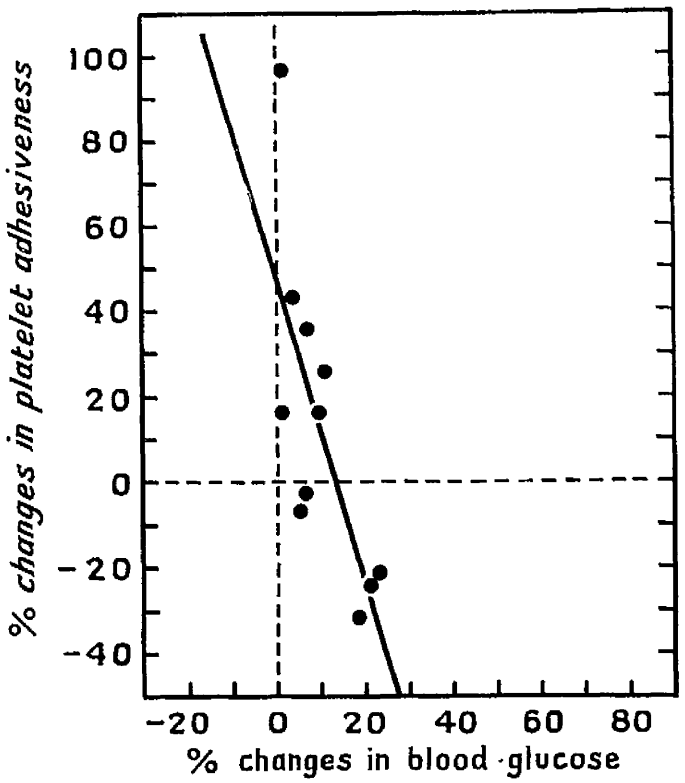


Fig. 39. Correlation between percentage changes in blood glucose and platelet adhesiveness after smoking ($y = 3.6\,x + 46$, $r = -0.70$).

$$Y = 0.43X_1 - 3.0X_2 + 30.5$$

Using this formula, the correlation between the actual and predicted values for platelet adhesiveness is significant at the 1% level ($r = +0.73$).

DISCUSSION.

The significant plasma NEFA increase induced by cigarette-smoking confirmed the findings of previous workers (Kershbaum et al., 1961; Soloff and Schwartz, 1964). Although Kershbaum et al. (1962) reported that patients with healed myocardial infarction showed a markedly exaggerated NEFA response to cigarette-smoking, neither in the present series (page 211) nor in that of Soloff and Schwartz (1964) were there significant differences in this respect between arterial disease patients and controls. It was of interest that sham-smoking also was followed by a plasma NEFA increase in five of the twelve tests. The fact that the rise followed a steady basal period suggested that it was an effect of sham-smoking rather than of repeated venepuncture, and might possibly be due to the embarrassment of puffing an unlit cigarette. Irving and Yamamoto (1963), in their investigation of the effects of cigarette-smoking on cardiac output, recognised that sham-smoking may produce physiological effects.

The increase in plasma NEFA was found to be accompanied by an increase in unsaturated fatty acids at the expense of the saturated fatty acids. Rothlin et al. (1962) observed a similar change in NEFA pattern when an acute increase in plasma NEFA was induced by norepinephrine infusion. Conversely, a rise in saturated and fall in unsaturated fatty acids occurred on lowering of the plasma NEFA level either by inhibition of adipose tissue lipolysis (Nakamura et al., 1967), or by increased NEFA utilisation during muscular exercise (Wood et al., 1965). Increased adipose tissue lipolysis appears, therefore, to be associated with an increased proportion of unsaturated fatty acids in the NEFA fraction, while the reverse change in NEFA pattern is found during inhibition of lipolysis. These changes in NEFA pattern are presumably a consequence of the greater unsaturated to saturated fatty acid ratio found in depot fat than in the plasma NEFA fraction (Insull and Bartsch, 1967). It was somewhat surprising that the change in NEFA pattern after sham-smoking was more striking than that following smoking, and that the changes in individual NEFA did not correlate well with the change in total plasma NEFA level.

The lack of effect of short-term cigarette-smoking on fasting serum cholesterol and triglyceride levels confirmed the findings of earlier workers (Page et al., 1959; Kershbaum

et al., 1961). Because of this lack of effect it was suggested by Mustard and Murphy (1963) that the higher serum cholesterol levels in habitual cigarette-smokers than in nonsmokers might be due to dietary differences in the two groups, or that hypercholesterolaemia and cigarette-smoking might be independent consequences of some common cause. It is possible, however, that habitual cigarette-smoking may itself be the cause of elevation of the plasma cholesterol concentration. Shafrir et al. (1959) showed that sustained or repeated elevation of plasma NEFA levels in dogs by subcutaneous injection of epinephrine in oil was followed after several days by a substantial rise in plasma cholesterol. Kaplan et al. (1957) and Dury (1957) also found an elevation in serum lipids in dogs and rabbits after administration of epinephrine in oil. It has been postulated that the increased circulating NEFA stimulate the production of cholesterol and other lipoprotein lipids by the liver (Shafrir et al., 1959; Shafrir and Steinberg, 1960). This suggests a mechanism by which NEFA elevation by repeated cigarette-smoking, as well as by psychic stress, might result in elevated blood cholesterol levels.

Some earlier workers reported that cigarette-smoking caused increased blood glucose levels (Lundberg and Thyselius-Lundberg, 1931), but Rehder and Roth (1959) suggested that the

rise shown was due to stress rather than an effect of smoking. Since in our subjects blood glucose concentrations increased following smoking but not sham-smoking, the increase can be attributed to smoking *per se*.

The increased platelet count following cigarette-smoking is presumably due to nicotine-induced catecholamine release, since Backman *et al.* (1925) have shown that injections of catecholamines are followed by a significant increase in circulating platelets.

Platelet adhesiveness has been reported to be increased in patients with ischaemic heart disease (McDonald and Edgill, 1957), and also in disseminated sclerosis (Caspary *et al.*, 1965; Payling Wright *et al.*, 1965). Since fatty-acid abnormalities have been described in both these conditions (Colehour and Leonard, 1964; Thompson, 1966), a relationship between platelet-adhesiveness and serum fatty acids has been suggested. The proposal was supported by the *in vitro* studies of Kerr *et al.* (1965), who showed that saturated fatty acids induced greater platelet aggregation than unsaturated fatty acids, some of which seemed to have an inhibitory effect. The results of the present study showed that when the blood glucose remained constant there was a good correlation between the changes in platelet adhesiveness and the changes in NEFA (Fig. 37). When the blood glucose also altered (in the

smoking test) the relationship between the changes in platelet adhesiveness and NEFA was less close (Fig. 38). In this situation, the rise in blood glucose seemed to have a suppressive effect on platelet adhesiveness, since there was a negative correlation between the changes in blood glucose and platelet adhesiveness (Fig. 39).

Bridges et al. (1965) showed that platelet adhesiveness increased after an oral glucose load, but he was unable to show a correlation between the rise in blood glucose and the rise in platelet adhesiveness. This situation, however, was quite different to the present one in which endogenous glucose was being mobilised. Bridges et al. (1965) also showed that the addition of glucose in vitro resulted in an increase in platelet adhesiveness, but this was contrary to the work of Hellem (1960) who was unable to demonstrate any effect within the physiological range for glucose.

A significant increase in platelet adhesiveness following cigarette-smoking in the non-fasting state has been reported by Ashby et al. (1965). Although in the present series the platelet adhesiveness showed a considerable increase after smoking in seven subjects, it decreased in the five other subjects, in whom there was a marked rise in blood glucose. This inhibitory effect of the blood glucose was most evident in two patients who felt nauseated at the end of the smoking

period, and who showed the largest increase in blood glucose in association with a delayed NEFA rise. Since Ashby et al. (1965) measured only platelet adhesiveness it is not possible to explain the discrepancy between his results and those presented here. It may be, however, that in the non-fasting state the blood glucose response to smoking is less striking. The somewhat surprising finding of a significant increase in platelet adhesiveness after sham-smoking is clearly associated with the unopposed effect of the rise in NEFA which has already been discussed.

SUMMARY AND CONCLUSIONS

1. The smoking of two cigarettes was followed by significant increases in plasma total NEFA, blood glucose, and total platelet count. There was a small, but statistically insignificant, increase in platelet adhesiveness.

2. Sham-smoking produced no significant changes in plasma total NEFA, blood glucose, or total platelet count in the group as a whole, although in five of twelve cases an appreciable increase in plasma NEFA occurred. There was a significant increase in platelet adhesiveness following sham-smoking.

3. Changes in plasma total NEFA were found to show a significant positive correlation with changes in platelet adhesiveness, whereas changes in blood glucose correlated negatively with changes in platelet adhesiveness. In both smoking and sham-smoking tests, therefore, increases in plasma total NEFA were associated with increased platelet adhesiveness, but where an increase in blood glucose occurred platelet adhesiveness was inhibited.

4. The increases in plasma total NEFA were associated with an increase in the proportion of unsaturated fatty acids at the expense of the saturated fatty acids. The changes in platelet adhesiveness correlated more closely with changes in plasma total NEFA than with changes in the individual NEFA.

5. Serum cholesterol and triglyceride concentrations did not alter significantly during either smoking or sham-smoking tests.

Chapter VII. THERAPEUTIC STARVATION - PLASMA LIPID AND OTHER METABOLIC CHANGES

CONTENTS

Chapter VII THERAPEUTIC STARVATION

OBESITY AND ATHEROSCLEROSIS

For many years it has been believed that obesity predisposes to ischaemic heart disease. Much of the earlier evidence of this association was obtained from life insurance statistics which showed increased mortality rates from heart disease in overweight subjects (Armstrong et al. 1951; Katz et al., 1958). Most surveys of ischaemic heart disease demonstrated that in relation to height patients were heavier than controls (Gertler and White, 1954; Thomas et al., 1958; Acheson, 1961; Brown, 1962), although others showed no difference (Epstein et al., Keys, 1956). One large prospective study in Framingham, Massachusetts, showed that gross obesity predisposed strongly to ischaemic heart disease (Dawber et al., 1962), whereas in another prospective study (Paul et al., 1963) there was no clearcut relationship of overweight to the development of ischaemic heart disease. Since all prospective studies have shown an association between elevated cholesterol values and ischaemic heart disease, it is possible that obesity might predispose to ischaemic heart disease by its effect on the plasma lipid levels.

OBESITY AND PLASMA LIPIDS

Many investigations of the effect of obesity on plasma lipids have been carried out, and conflicting reports have appeared. In general, plasma lipid levels have been found to be higher in the obese than in persons of average weight, although many obese subjects may have normal or even low plasma lipid concentrations.

A positive correlation between serum cholesterol concentration and body weight has been reported by some investigators (Gofman and Jones, 1952; Lewis et al., 1957), whereas others have found no such relationship (Taylor et al., 1962; Keys et al., 1963), and have been unable to demonstrate any effect of recent weight gain on serum cholesterol (Feldman et al., 1963; Hallberg and Svanborg, 1967). However, Montoye et al. (1966) have shown in a large population study that there is, in fact, a low but statistically significant correlation between serum cholesterol concentrations and various measures of body fatness (relative weight and skinfold thicknesses). No effect has been found of body weight on plasma phospholipid levels (Rifkind and Begg, 1966; Sailer et al., 1966; Hallberg and Svanborg, 1967).

Plasma triglyceride levels, also, have been shown to be higher on average in the obese than in subjects of normal weight (Waxler and Craig, 1964; Hollister et al., 1967)

although it has been pointed out that many obese subjects have normal or low triglyceride levels (Rifkind and Begg, 1966). A positive correlation between plasma triglyceride and overweight, measured by a variety of methods, has been demonstrated by many workers (Schrade et al., 1960; Benedek, 1965; Evans and Ostrander, 1967). Albrink et al. (1962) has reported that elevated triglyceride levels are more closely associated with weight gain than with obesity per se, and Feldman et al. (1963) and Hallberg and Svanborg (1967) have found higher triglyceride levels in women with a history of recent weight gain.

The plasma NEFA values of obese subjects have been shown repeatedly to be higher after an overnight fast than those of subjects of normal weight (Dole, 1956; Corvilain et al., 1961; Glennon et al., 1965). Further fasting has been found to reduce the differences in NEFA level between obese and non-obese subjects (Gordon, 1960; Opie and Walfish, 1963; Beck et al., 1964).

THERAPEUTIC STARVATION IN OBESITY

Obesity has long been recognised as an undesirable state, both for aesthetic reasons and on account of its complications. In addition to the reduced life expectancy and increased risk

of ischaemic heart disease already mentioned, obesity is associated with respiratory, locomotor, and metabolic disorders. Clinicians are constantly faced with the problem of reducing a patient's weight in an effort to alleviate these complications, but the success rate in such endeavours has been very low.

In the last few years total starvation has been used as a means of initiating weight reduction (Bloom, 1959) and also as a long-term measure, either by means of intermittent fasts (Duncan et al., 1962; Stewart et al., 1966), or by prolonged starvation (Drenick et al., 1964; Thomson et al., 1966. Such therapeutic starvation has been shown to be acceptable to most patients, and to be an effective method of reducing weight rapidly.

Rapid weight reduction produces many changes in metabolism, affecting particularly salt and water balance and the metabolism of carbohydrate and fat. It is important to ensure that such sudden changes are entirely safe. It has been suggested, for example, that plasma lipid changes resulting from rapid weight reduction may aggravate atheromatous arterial disease or induce thrombosis (Beckett and Lewis, 1960). An investigation has been carried out, therefore, of the plasma lipid and other metabolic changes induced by short-term therapeutic starvation, together with a study of blood platelet function.

METHODS

(a) Patients

The patients had all presented at medical clinics for advice on weight reduction or with symptoms which were attributable mainly to obesity. Sixteen patients, 13 women and 3 men, completed the 10-day starvation period, and 2 of these patients (E. Gib and M.L.) underwent a second period of starvation after an interval of several months. These 18 episodes of starvation are described in this study, and the initial status of the patients is summarised in Table XXXIII. A further 2 female patients, both with features of a depressive illness, commenced starvation, but declined to continue after 2 or 3 days. They are therefore not considered further in this study.

(b) Procedure

The patients were treated singly or in pairs in a general medical ward. They were ambulant in the ward for most of the day. During an initial control period of 3 to 7 days, they continued on a diet similar to the one they had had prior to admission. In 10 instances this had been an unrestricted diet, and in 8 instances a 600 Calorie diet. They then fasted for 10 days. Unlimited quantities of acaloric fluids, such as

TABLE XXXIII. Initial Status of Patients

Patient	Age	Sex	Height (cm.)	Weight (kg.)	Relative* Weight %	Presenting Symptoms and Signs
J.S	16	F	162.6	77.3	141	Obesity
I.F.	19	F	162.6	72.7	132	Obesity
E.Gib(1)	21	F	168.9	83.3	143	Obesity. Mild depression.
E.Gib(2)	21	F	168.9	79.4	136	Obesity. Mild depression.
B.H.	26	M	165.1	100.7	175	Obesity. Paraplegia.
M.L.(1)	28	F	158.8	88.4	168	Obesity.
M.L.(2)	28	F	158.8	76.8	146	Obesity.
M.W.	30	F	168.9	113.5	194	Obesity.
E.Gr.	37	F	157.5	96.9	187	Obesity.
H.B.	38	F	160.0	84.5	158	Obesity.
M.G.	43	F	160.0	91.3	170	Obesity.
W.H.	48	M	176.5	111.8	172	Obesity.
M.M.	53	F	161.3	99.5	183	Obesity.
C.H.	54	F	147.3	102.2	221	Varicose veins.
I.S.	57	F	160.0	92.4	172	Obesity.
L.H.	58	F	154.9	76.4	151	Dyspnoea. Mitral stenosis.
J.R.	58	F	152.4	88.2	180	Osteoarthritis of knees.
P.M.	64	M	163.8	106.9	188	Obesity.

*Actual weight as percentage of standard weight (Kemsley et al., 1962)

water, black coffee, or unsweetened lemon tea, were allowed during this period. No vitamin supplements or drugs were administered. Following completion of the 10-day starvation period, all patients commenced a 600 Calorie diet, and after a period varying between 1 and 4 days they were allowed home. They returned after 1 to 4 weeks, in most cases after 2 weeks, for follow-up examination.

The following determinations were made in each case:

Weight

While in hospital, the patients were weighed daily, on rising, in bed attire. At the follow-up examination, they were weighed in indoor clothes. In order to make this weight comparable with the others, deductions were made of 2.7 kg. for females and 4.6 kg. for males, as recommended by Kemsley et al. (1962).

Skinfold thickness

The skinfold thicknesses were measured by means of Harpenden calipers at the lower angles of the scapulae, and at the midpoints of the triceps and ulnae. At each site, the mean thickness of the two sides was used. Skinfolds were measured during the control period and at the end of the 10-day fast. In 5 subjects they were also measured during the

refeeding phase, and in 12 subjects at the follow-up examination.

Fluid balance

Fluid intake and urine output were measured for each 24-hour period from 8.00 a.m. throughout the whole stay in hospital.

Ketonuria

Ketonuria was assessed each day using Acetest tablets (Ames).

Timing of blood samples

Blood samples for lipid, glucose, and haematological estimations were withdrawn after an overnight fast on 3 occasions during the control period, and on day 2 (that is, after 60 hours' fasting) day 4 or 5, day 6 or 7, and day 9 of the fast. Further samples were obtained after refeeding for 24 to 48 hours prior to discharge from hospital, and again at the follow-up visit. The following measurements were carried out on each blood sample:- serum cholesterol and triglyceride, and total and individual plasma NEFA, as described in Chapter II; haematocrit (page 132); blood sugar (page 216); and platelet count and platelet adhesiveness (page 216).

RESULTS

I. General Effects of Total Starvation

The fast was tolerated well by all patients, although mild fatigue and lethargy were fairly constant symptoms. One patient had mild diarrhoea lasting for less than 24 hours during the starvation period, but this did not necessitate termination of the fast. No other side-effects were encountered in this series.

Although the patients were purposely not questioned systematically about the presence or absence of hunger, this was not complained of after the first 1 to 2 days. Most patients, however, were not anorexic, and several stated that they would have enjoyed a meal if this had been provided.

II. Changes in Weight

A rapid and marked weight loss occurred during starvation, and a prompt, but smaller, weight increase was seen on resumption of food intake (Fig. 40).

During the basal period, the 10 patients on an unrestricted diet showed a small mean weight loss of 0.25 $\pm$ 0.10 kg. per 24 hours. This occurred in the first 24 to 48 hours after admission, and the weight remained steady thereafter. The

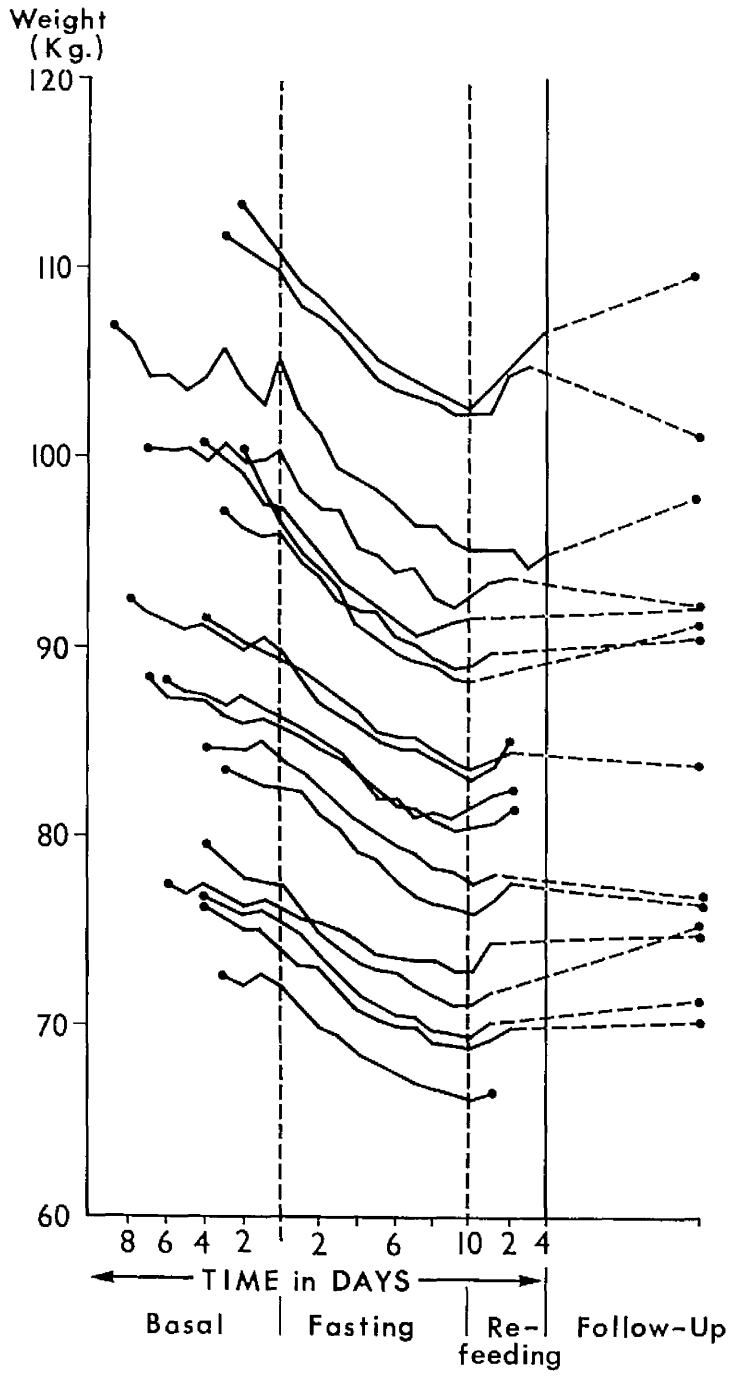


Fig. 40. Individual weight changes during starvation and refeeding.

basal weight loss was significantly greater in the 8 subjects on a 600 Calorie diet (0.74 ± 0.51 kg. per 24 hours), and continued throughout the basal period.

Weight loss during starvation was greatest in the first 24 hours, and decreased progressively thereafter (Fig. 41). The total weight lost during the 10-day fast ranged from 3.9 to 9.4 kg. (mean 6.8 kg), and the largest weight losses occurred in the most obese subjects. Resumption of calorie intake was associated with a prompt weight increase which continued for several days (Figs. 40 and 41).

Follow-up examination in 14 of the patients showed that, at approximately 2 weeks from the end of the fast, they weighed on average 7.1 ± 2.4 kg. less than their initial weight recorded on admission to hospital, and 1.5 ± 2.1 kg. more than at the end of the fast.

III. Skinfold Measurements

Weight loss during starvation was accompanied by significant decreases in thickness of the subscapular and triceps skinfolds, but not of the ulnar skinfold (Table XXXIV). On refeeding, the subscapular skinfold promptly showed a small but significant increase of 1.4 mm. ($P < 0.05$) from the level observed at the end of the fast, while the triceps and ulnar skinfolds were

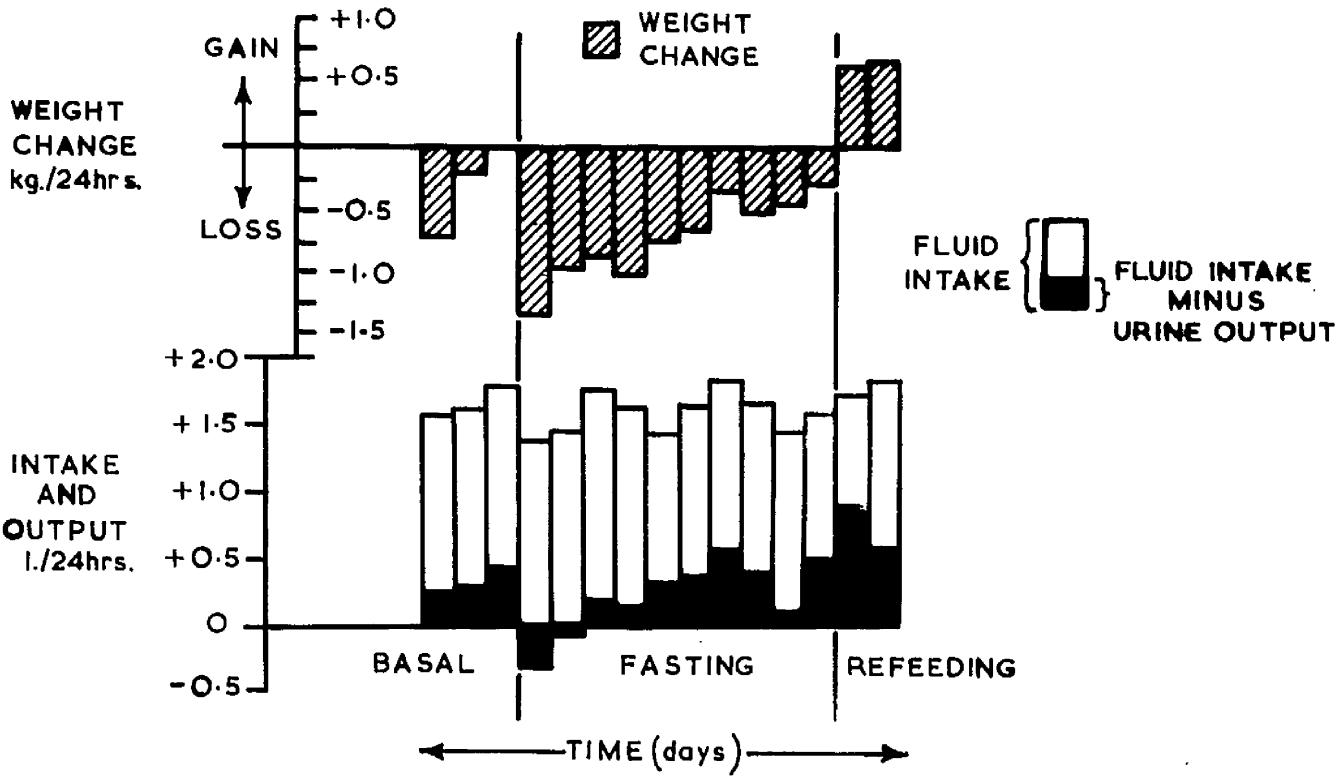


Fig. 41. Mean weight changes and fluid balance during starvation and refeeding.

TABLE XXXIV. Changes in Skinfold Measurements during Starvation and Refeeding (mean ± S.D.)

Skinfold	Skinfold Thickness mm.		
	Before Fast	End of Fast	Follow-up(1)
Subscapular	31.1 ± 6.7	27.1 ± 6.9 ***	29.9 † ± 5.2
Triceps	28.0 ± 6.1	26.3 ** ± 6.4	25.7 *** ± 6.0
Ulnar	12.5 ± 4.6	12.1 ± 4.3	12.2 ± 4.6

Significant differences from prefasting level

† $P < 0.01$, ** $P < 0.01$, *** $P < 0.001$

(1) 12 subjects only

unchanged. Follow-up examination in 12 patients showed that the triceps skinfold thickness had continued to decrease (Table XXXIV). At this time the subscapular skinfold, though significantly less than in the prestarvation period, was greater than at the end of the fast. Although the changes in the individual skinfold thicknesses did not show a close relationship to the amount of weight lost, a significant positive correlation was observed between the decrease in the mean of the subscapular and triceps skinfold thicknesses and the amount of weight lost during the whole period of observation ($r = +0.65$, $P < 0.02$).

In the prestarvation period, the only skinfold measurement which correlated significantly with the degree of obesity as measured by relative weight was the subscapular skinfold ($r = +0.61$, $P < 0.01$). No differences were observed in ulnar skinfold thicknesses between subjects with childhood-onset and adult-onset obesity.

IV. Fluid Balance

The main changes noted in fluid balance were a diuresis in the first few days of starvation, and fluid retention associated with resumption of food intake (Fig. 41). Complete fluid balance figures for the whole period (basal, fasting,

and refeeding) were obtained for 10 patients, and the results are summarised in Table XXXV. Although the fluid intake in individuals fluctuated considerably from day to day, especially during the fasting period, there was no consistent trend from one period to the next (Fig. 41).

Following admission to hospital, there was a diuresis lasting for 24 to 36 hours; for example, in 7 of our 13 patients the urine output exceeded the fluid intake in the first complete 24-hour period after admission. After the diuretic phase the urine output was relatively constant for the rest of the basal period. For this reason the values for the first complete 24-hour period were discarded when the mean basal values were calculated for comparison with the fasting and refeeding periods.

In the first 24 hours of starvation, the urine output again increased and the patients were in negative water balance for the first 48 hours of the fast (Fig. 41). Over the rest of the first week the apparent fluid retention (fluid intake minus urine output) gradually returned to basal levels. During the first 24 hours of refeeding there was a reduction in urine output, with fluid retention significantly greater than that during the basal period. Several patients were observed for 2 to 3 days of refeeding, during which the oliguria and fluid retention persisted.

TABLE XXXV. Fluid Balance in Ten Patients (mean and range)

	Fluid intake ml./24 hrs.	Urine output ml./24 hours	Net Fluid Balance
DAYS BEFORE FAST			
3	1668 (990 - 2660)	1425 (540 - 4560)	
2	1724 (990 - 3480)	1454 (600 - 3800)	+407
1	1889 (1180 - 4070)	1412 (600 - 3090)	
DAYS OF FAST			
1	1455 (720 - 2190)	1745 (500 - 4030)	-290*
2	1570 (600 - 3720)	1627 (600 - 3950)	-57**
3	1839 (630 - 3845)	1628 (800 - 3050)	+211
4	1725 (350 - 2430)	1576 (800 - 3250)	+150
5	1503 (360 - 2440)	1165 (600 - 2000)	+249
6	1733 (730 - 4045)	1358 (600 - 3400)	+375
7	1910 (950 - 3410)	1287 (450 - 3500)	+623
8	1758 (660 - 3290)	1346 (700 - 2090)	+412
9	1518 (450 - 2940)	1405 (500 - 3400)	+113*
10	1694 (950 - 2560)	1168 (450 - 3300)	+527
DAYS OF REFEEDING			
1	1832 (1100 - 3450)	898 (400 - 2050)	+934*
2	1885 (1170 - 2940)	1246 (525 - 3900)	+639

Significant difference from mean basal level * $P < 0.05$, ** $P < 0.01$

V. Ketosis

After one day's fasting, 5 patients had significant ketonuria and another 4 had a trace. By next day the majority of patients had moderate ketonuria, 4 had only a trace, and 2 had negative Acetests. By the fifth day all but one patient were showing heavy ketonuria, and continued to show it until starvation ended. Patient J. S. had negative urine tests for ketones until the sixth day, when she developed a moderate degree of ketonuria which persisted till the end of the 10-day fast. Patients began to show a decline in ketonuria after 24 hours' refeeding, but were not tested in detail thereafter.

VI. Haematocrit

The haematocrit of all subjects in the pre-starvation period was within normal limits at 43.6 ± 3.9% (Table XXXVI). During starvation the haematocrit increased significantly, reaching a maximum between the 4th and 7th days, and subsequently tending to fall (Fig. 42). It returned rapidly to prestarvation levels on refeeding. At follow-up a small but significant reduction of 1.6% below the prestarvation level was noted.

TABLE XXXVI. Changes in Haematocrit, Blood Sugar, and Platelets during Starvation (mean ± S.D.)

Time of estimation	Haematocrit %	Blood sugar mg./100 ml.	Platelet count per cu.mm.	Platelet adhesiveness %
Mean basal level	43.6 ± 3.9	73.5 ± 11.0	188,090 ±43,210	39.2 ±9.3
After 2 days' fast	44.8† ± 4.0	56.0*** ± 8.9	197,560* ±50,210	37.0 ±14.2
After 4 or 5 days' fast	46.1** ±5.1	50.8*** ±11.3	200,190* ±45,080	40.0 ±16.6
After 6 or 7 days' fast	45.5*** ±4.0	54.0*** ± 7.0	208,390 ±47,110	40.3 ±15.4
After 9 days' fast	44.9 ±5.3	59.8*** ± 9.8	191,990 ±39,860	39.1 ±13.3
Refeeding	43.4 ±4.6	77.8 ±12.4	182,920 ±43,760	34.4 ±11.5
Follow-up	42.1* ±4.7	74.9 ±14.3	211,190 ±57,440	41.3 ±14.6

Significant difference from mean basal level.

*P < 0.05, †P < 0.02, **P < 0.01, ***P < 0.001

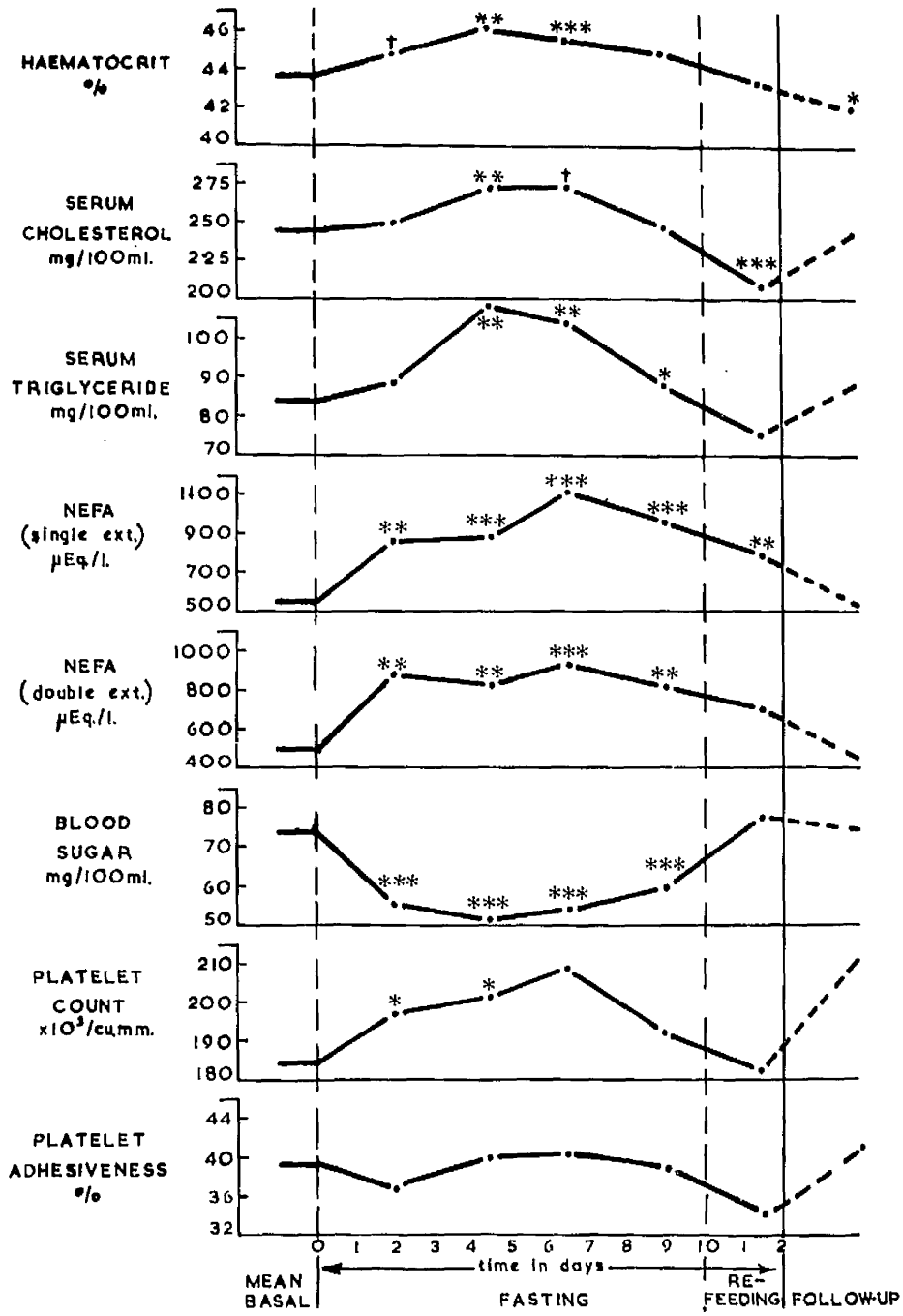


Fig. 42. Haematological and biochemical results.
(Serum triglyceride results for 16 patients only).
(NEFA-double extraction - in 8 patients only).

VII. Blood Sugar

The blood sugar level fell considerably during starvation, reaching its lowest level after 4 or 5 days (Table XXXVI, Fig. 42). It then showed a tendency to rise, but nevertheless remained significantly below the basal level throughout the whole fasting period. On refeeding it rose rapidly to prestarvation levels, and remained at these levels at follow-up.

In the prestarvation period, the fasting blood sugar was not related to the age of the subjects, nor to their degree of obesity as measured by relative weight. There was, however, a significant positive correlation between the mean fasting blood sugar and the triglyceride level ($r = +0.53$, $P < 0.05$).

VIII. Serum Cholesterol

Starvation was associated with a significant increase in serum cholesterol (Table XXXVII, Fig. 42), maximal between the 4th and 7th days, and returning towards the basal level after 9 days. On refeeding there was a rapid and highly significant decrease to 36 mg. per 100 ml. below the basal level. By 2 to 4 weeks after the fast, however, the cholesterol had returned to prefasting levels.

TABLE XXXVII. Plasma Lipid Changes during Starvation

(mean ± S.D.)

Time of estimation	Serum Cholesterol mg./100 ml.	Serum[(1)] triglyceride mg./100 ml.	Plasma NEFA (single ext.) microeq./l.	Plasma NEFA (double ext.) microeq./l.(2)
Mean basal level	245.8 ± 39.1	84.5 ± 19.7	545 ± 126	503 ± 143
After 2 days' fast	250.9 ± 41.7	88.9 ± 18.3	865** ± 416	894** ± 279
After 4 or 5 days' fast	272.6** + 50.2	108.7** ± 18.3	885*** ± 349	836** ± 232
After 6 or 7 days' fast	273.3/ ± 63.8	104.9** ± 21.3	1118*** ± 435	930*** ± 226
After 9 days' fast	248.0 ± 43.8	88.0* ± 40.5	965*** ± 361	823** ± 187
Refeeding	209.5*** ± 37.0	76.3 ± 15.9	799** ± 303	712 ± 246
Follow-up	243.2 ± 45.2	88.2 ± 31.0	538 ± 181	453 ± 176

(1) 16 normoglyceridaemic subjects only.

(2) 8 subjects only.

Significant difference from mean basal level

*P < 0.05, /P < 0.02, **P < 0.01, ***P < 0.001.

The prefasting serum cholesterol levels were within normal limits for the patients' ages with three exceptions - W. H. (48 years) 346 mg. per 100 ml., J. R. (58 years) 319 mg. per 100 ml., J. S. (16 years) 267 mg. per 100 ml. There was no correlation between the serum cholesterol concentration and the degree of obesity.

IX. Serum Triglyceride

The effect of starvation on the serum triglyceride was found to depend on the initial serum triglyceride concentration; in those subjects with initially normal levels small, transient increases occurred, whereas in hypertriglyceridaemic subjects marked decreases were recorded. The initial serum triglyceride levels were not related to the degree of obesity, as measured by relative weight or skinfold thicknesses. There was no correlation between serum triglyceride and serum cholesterol levels, although one subject (W.H.) had elevated serum concentrations of both triglyceride and cholesterol.

The prestarvation triglyceride levels were within the range 58 to 118 mg. per 100 ml. in 16 subjects. In these normoglyceridaemic subjects, starvation resulted in a rise in triglyceride which was maximal after 4 to 5 days, but remained significantly above the initial level throughout the fast (Table XXXVII, Fig. 42). On refeeding there was

a slight fall in triglyceride to levels not significantly lower than prefasting levels. By the time of follow-up examination, it had returned to prefasting levels.

The remaining 2 patients, H. B. and W. H., had grossly elevated initial serum triglyceride levels (328 and 307 mg. per 100 ml. respectively). Both subjects showed rapid and progressive falls in triglyceride throughout the fasting period, but different responses to resumption of food intake (Fig. 43). In H. B., the serum triglyceride continued to fall during refeeding and at follow-up examination 2 weeks later. Two days' refeeding in W. H., however, resulted in doubling of the serum triglyceride, and by 2 weeks later it had returned almost to prestarvation levels.

X. Total Plasma Non-Esterified Fatty Acids (NEFA)

During starvation the "NEFA" level (single extraction technique) rose in all but one patient (J.S.), and after 6 to 7 days was more than double the basal value (Table XXXVII, Fig. 42). The mean "NEFA" level then declined, and on refeeding a further decrease was observed. After 24 hours' refeeding the plasma "NEFA" had fallen in 5 and risen slightly in 3 instances from the level recorded at the end of the fast. After 48 to 96 hours' refeeding it had fallen in 7 out of 8 instances. By the time of follow-up examination the "NEFA"

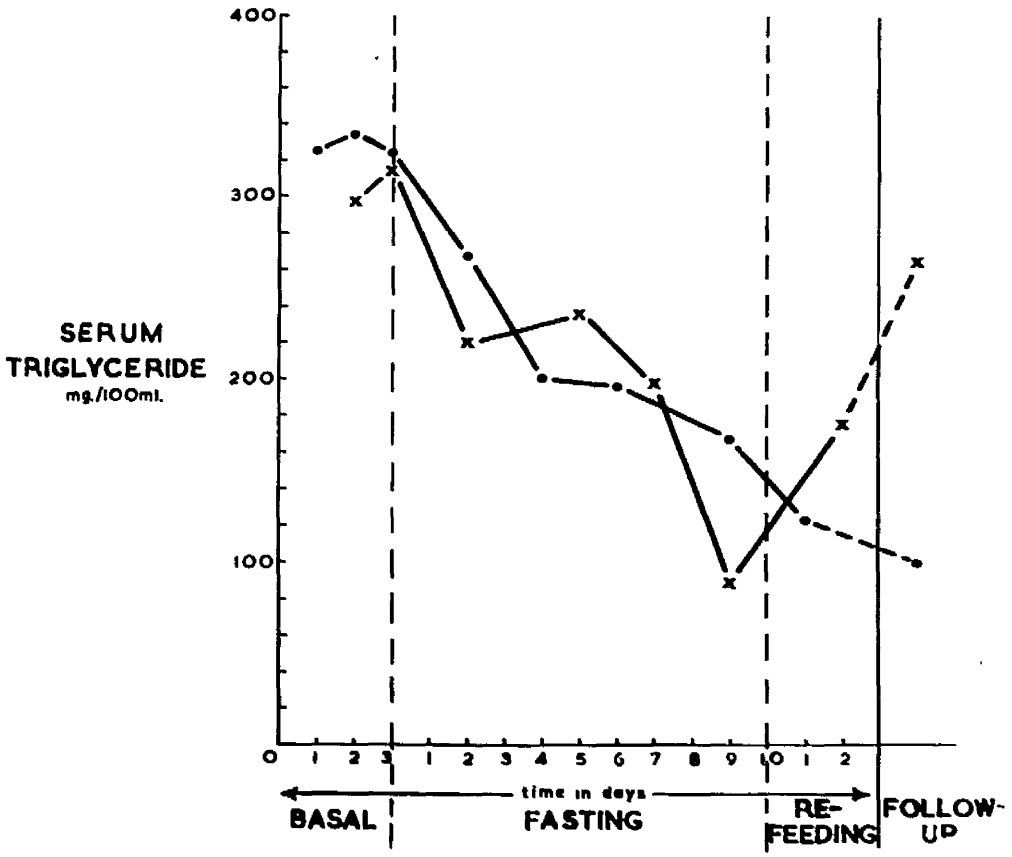


Fig. 43. Plasma triglyceride changes during starvation in two hypertriglyceridaemic subjects.

had returned to prestarvation levels. The changes in plasma NEFA as measured by the more specific double extraction technique showed a similar pattern, although the peak values recorded during starvation were lower (Table XXXVII, Fig. 42). During the basal period 16.0 $\pm$ 2.9% of the apparent "NEFA" value was due to substances other than long-chain, non-esterified fatty acids. This is in agreement with the figure of 10 to 20% quoted by Dole and Meinertz (1960). After 6 to 7 days' starvation this percentage had risen to 28.0 $\pm$ 10.5%, due to the inclusion of ketone bodies.

In the prestarvation period half of the 18 patients had mean "NEFA" levels between 330 and 500 microeq. per litre. The two highest values were 810 and 820 microeq. per litre. The highest "NEFA" levels during the fast were found in these subjects with the highest prestarvation levels; a positive correlation was demonstrated between the mean prestarvation "NEFA" value and the maximum level recorded during starvation ($r = +0.60$, $P < 0.01$). There was also a negative correlation between the mean plasma "NEFA" and the mean blood glucose level for each individual in the basal period ($r = -0.52$, $P < 0.05$). No significant differences were found in "NEFA" level between subjects on a 600 Calorie diet and those on an unrestricted diet, nor between those with childhood-onset

and adult-onset obesity. No relationship was demonstrated between "NEFA" level and age, or degree of obesity.

XI. Individual Non-Esterified Fatty Acids

The elevation of plasma NEFA levels induced by starvation was associated with a change in the proportions of the individual fatty acids (Table XXXVIII). During starvation there was a significant increase in the proportion of oleic acid, with a corresponding decrease in palmitic and myristic acids. The fall in stearic acid did not reach conventional levels of significance. These changes were maximal after 2 to 5 days, and thereafter the pattern showed a tendency to return to the normal prefasting state. Refeeding, however, resulted in a further small increase in oleic and decrease in palmitic acid. At follow-up examination the pattern had reverted to that seen in the basal period.

XII. Platelet Count and Platelet Adhesiveness

As in the case of the haematocrit, the platelet count increased slightly during starvation, and returned to basal values on refeeding (Table XXXVI, Fig. 42). The platelet adhesiveness, however, showed marked variability from day to day and no significant trends were observed during starvation or subsequently (Table XXXVI, Fig. 42).

TABLE XXXVIII. Serum NEFA pattern (Individual fatty acids as percentage mean ± standard deviation of total NEFA)

NEFA	Mean Basal Level	Fasting				Refeeding	Follow-up
		after 2 days	after 4 or 5 days	after 6 or 7 days	after 9 days		
Myristic acid C14:0	2.3 ±0.7	1.7** ±0.4	2.2 ±1.0	2.0 ±0.6	1.9 ±0.7	2.0 ±0.4	2.4 ±0.9
Palmitic acid C16:0	27.5 ±2.4	25.2* ±3.6	24.1† ±3.7	25.1 ±7.0	26.7 ±4.9	24.8† ±2.7	28.4 ±4.1
Palmitoleic acid C16:1	7.0 ±1.4	6.6 ±1.5	6.4 ±1.2	6.0 ±1.7	6.4* ±1.3	6.9 ±1.9	6.3 ±1.2
Stearic acid C18:0	10.0 ±2.5	8.4 ±2.1	7.9 ±2.0	10.3 ±2.9	9.3 ±2.0	8.2 ±1.6	11.0 ±2.5
Oleic acid C18:1	42.7 ±3.8	46.2* ±5.3	48.4** ±4.1	47.2† ±7.7	46.2 ±7.6	47.7* ±4.1	41.2 ±4.2
Linoleic acid C18:2	10.3 ±2.6	11.8 ±4.0	10.9 ±4.0	9.3 ±2.9	9.5 ±3.5	10.2 ±5.2	10.6 ±5.1

Significant difference from mean basal level, * $P < 0.05$, † $P < 0.02$, ** $P < 0.01$

DISCUSSION

Total starvation was tolerated well by the subjects, who made little complaint of hunger after the first one to two days' starvation. The amount of weight lost during the ten-day period of starvation could be regarded as worthwhile from a therapeutic point of view. Despite the early weight gain on refeeding, the nett weight loss of 7.1 ± 2.4 kg. recorded at follow-up examination two to four weeks later was sufficient to convince the patients that an appreciable amount of weight could be lost rapidly and permanently. The serial measurements of skinfold thickness suggested that the central (trunk) depots of adipose tissue were reduced more than the peripheral (limb) depots.

The plasma lipid results showed that the most striking and consistent changes during total starvation were in the NEFA fraction (Table XXXVII), although smaller changes occurred in the other plasma lipids also. The effect of starvation on the plasma lipids will be discussed in some detail. Since these plasma lipid changes are accompanied by, and in some cases secondary to, the starvation-induced alterations in fluid balance and in the glucose and other blood constituents, the mechanisms of these alterations will also be considered.

Total Plasma NEFA

A considerable number of investigations have been reported to show that plasma NEFA levels of obese subjects after an overnight fast are on average higher than those of normal weight (Opie and Walfish, 1963; Glennon et al., 1965) although the reason for this difference is not known. Many of the grossly obese subjects in the present series, however, had plasma NEFA concentrations within normal limits (page 244). It has been suggested that inhibition of the action of insulin by increased plasma NEFA concentrations in some obese subjects leads to tissue resistance to insulin, with compensatory hypersecretion of insulin, and eventually to the glucose intolerance commonly present in longstanding obesity, and in some cases to frank diabetes (Randle et al., 1963). In the subjects presented here a significant negative correlation was demonstrated between fasting blood glucose and plasma NEFA levels in the prestarvation period. The apparently paradoxical finding of lower fasting blood glucose levels in those subjects with higher fasting NEFA levels is compatible with Jenkin's (1967) hypothesis that, when plasma NEFA concentrations are persistently elevated, a stage of increased insulin secretion occurs during which raised plasma NEFA levels and normal or low blood glucose levels are found.

Later pancreatic insufficiency may supervene and fasting blood glucose levels tend to rise.

The estimation of "NEFA" by the single extraction technique of Dole and Meinertz (1960) includes substances such as lactic acid and ketones. During starvation, these were found in our patients to account for approximately 28 per cent (in one case as much as 46 per cent) of the total "NEFA" figure (page 244). A value due almost entirely to true long-chain non-esterified fatty acids is obtained by the double extraction method recommended when exceptional amounts of ketones or short chain acids are believed to be present. The latter technique was followed in eight of the eighteen studies reported here. However, the changes in plasma NEFA during starvation were of such magnitude, and differed so little in pattern after both single and double extraction methods, that useful information was obtained even by the single extraction technique.

Elevations of plasma "NEFA" levels during starvation have been reported by a number of investigators, almost all using techniques of NEFA estimation that included varying amounts of ketones (Laurell, 1956; van Riet et al., 1964). The two-fold rise in plasma "NEFA" in the present study (Table XXXVII) is similar to that observed by Mager and Iampietro (1966) in normal individuals subjected to starvation and cold

exposure, and by Opie and Walfish (1963), and Samaan et al., (1965) in obese subjects. It is considerably smaller than the changes found in normal subjects by Albrink and Neuwirth (1960) and Bloom et al. (1966a). Gordon (1960) and Schwarz et al. (1966) carried out comparisons of the effect of fasting on NEFA levels in obese and non-obese subjects, and found a much higher and more rapid rise in the normal subjects than in the obese. The plasma NEFA concentration is the result of dynamic and rapidly-changing processes in lipid mobilisation, utilisation, and deposition. A very rapid NEFA turnover rate has been demonstrated, about half of the NEFA reaching the plasma being cleared into other tissues within one to three minutes (Fredrickson and Gordon, 1958a). No impairment of NEFA utilisation has been found in obesity (Opie and Walfish, 1963). There is, however, considerable evidence that in some obese subjects the release of NEFA from fat stores is impaired. A decreased rate of NEFA release in vitro from the adipose tissue of obese subjects was found by Laszlo et al. (1961), who were unable to demonstrate any significant qualitative differences on analysis of adipose tissue samples from obese and non-obese subjects. The forearm metabolic studies of Rabinowitz and Zierler (1962) showed that in the basal state NEFA release in the obese was about half that of the non-obese. Diminished or delayed plasma NEFA responses were demonstrated in the obese to lipid-mobilising

stimuli such as catecholamines (Berkowitz, 1964; Heald et al., 1965), exercise (Opie and Walfish, 1963) and sociopsychological stress (Klein et al., 1965).

In the grossly obese subjects in the present series, with one exception (J. S.), total starvation produced a marked and sustained increase in plasma NEFA concentrations. The results reviewed above appear to indicate that NEFA mobilisation in response to a variety of stimuli, including starvation, is rather less in the obese than in lean subjects, despite the tendency of the obese to higher NEFA levels after an overnight fast.

Individual Non-Esterified Fatty Acids

The raised plasma NEFA levels during starvation were associated with an increased proportion of the unsaturated oleic acid, and a decreased proportion ofpalmitic and, to a lesser extent, other saturated fatty acids (Table XXXVIII). A similar change in NEFA pattern has been observed when an acute increase in plasma NEFA is induced by norepinephrine infusion (Rothlin et al., 1962) or, as shown in Chapter VI, by cigarette-smoking. Conversely, a rise in saturated and fall in unsaturated fatty acids occurs during lowering of the plasma NEFA level either by inhibition of adipose tissue lipolysis (Nakamura et al., 1967) or by increased NEFA

utilisation (Wood et al., 1965).

The plasma NEFA pattern is thought to be determined mainly by the rate of release of fatty acids from adipose tissue, but is also influenced by their rate of clearance from plasma. As shown in Table XXXIX, the fatty acid pattern of adipose tissue differs from that of plasma NEFA. In the present study the plasma NEFA pattern shows an approach during starvation towards the fat depot pattern (Table XXXIX), in contrast to the findings of Imaichi et al. (1963). This suggests that the individual fatty acids are not released from adipose tissue in a selective manner, and is consistent with the observation of Hirsch (1965) that marked shrinkage of depot fat in obese subjects by total starvation or prolonged calorie restriction is not associated with changes in the fatty acid composition of adipose tissue.

Serum Cholesterol

Although a low grade positive correlation between serum cholesterol concentration and overweight has been demonstrated in studies of large populations (Montoye et al., 1966), the serum cholesterol has frequently been found to be within normal limits even in the grossly obese. This was the case in the present series, only three of whom had raised serum cholesterol levels (page 242).

TABLE XXXIX. The Pattern of Fatty Acids in Adipose Tissue and Plasma NEFA

Authors	Origin of Fatty Acids	Concentrations of Fatty Acids %					
		Myristic C14:0	Palmitic C16:0	Palmitoleic C16:1	Stearic C18:0	Oleic C18:1	Linoleic C18:2
Imaichi et al., 1963	Depot fat	--	22.8	--	--	52.3	9.0
	Plasma NEFA - before fast	--	29.8	--	--	41.0	9.8
	- after 12-15 day fast	--	30.6	--	--	43.0	9.0
Insull and Bartsch, 1967	Depot fat	2.7	22.7	5.3	7.0	49.9	9.2
Present study	Plasma NEFA -before fast	2.3	27.5	7.0	10.0	42.7	10.3
	-after 4-5 day fast	2.2	24.1	6.4	7.9	48.4	10.9

During starvation a small but significant increase in serum cholesterol occurred in the first four to seven days, and thereafter it tended to return to basal levels. Similar changes were described by other investigators under somewhat different circumstances. Kartin et al. (1944) showed progressive increases in cholesterol and phospholipid levels in normal subjects during starvation combined with water deprivation. Rubin and Aladjem (1954) noted increased concentrations of the cholesterol-rich (Sf 0-12) lipoprotein fraction in healthy subjects who fasted for four to five days. Mager and Iampietro (1966) found that in healthy males subjected to cold and starvation the serum cholesterol rose in the early stages of the fast, but returned to fasting levels by the fourteenth day. Bloom et al. (1966a) showed greater increases in serum cholesterol during four-day fasts in normal females than in normal males.

Reports on serum cholesterol changes during total starvation in obese subjects are contradictory. Ende (1962) found that the serum cholesterol increased during 72-hour fasts in his obese subjects as well as in the non-obese, and Cochran et al. (1964) described a variable slight rise followed by fall in serum cholesterol in twelve obese patients starved for periods of five to twenty-five days. Varying responses

or no change were reported by Drenick et al. (1964) in their male obese subjects, and by van Riet et al. (1964) in their hypercholesterolaemic female subjects. The transient increases in serum cholesterol during starvation in this study were similar to the changes in haematocrit (Fig. 42) and may simply reflect alterations in plasma volume. This effect of total starvation on serum cholesterol concentration is in contrast to that produced by calorie restriction, where little or no change occurs in serum cholesterol level unless the total fat intake is restricted (Galbraith et al., 1966) or the type of dietary fat is altered (Jolliffe et al., 1962).

The sharp decline in serum cholesterol on refeeding was out of proportion to the fluid retention which occurred in this period, as reflected by the fall in haematocrit. Ende (1962) also noted a drop in serum cholesterol in the first 48 hours of refeeding in six of twenty-nine subjects, and commented that it could be only partially accounted for by an increase in plasma volume. A similar decrease in cholesterol to levels significantly lower than that in the prestarvation period was seen in Mager and Iampietro's (1966) data, although they made no comment on it. Although the cause of this fall in serum cholesterol cannot be stated from the present study, it might be contributed to by the unmasking of an actual decrease in serum cholesterol content, obscured during the starvation

period by haemoconcentration. The synthesis of cholesterol and fatty acids has been shown to be markedly reduced during starvation in animals, and on refeeding there is a time-lag before resumption of synthesis of cholesterol, but not of fatty acids (Jansen et al., 1966).

The long-term effect of weight reduction on the serum cholesterol level appears to be small. In this respect the results at two to four weeks are in agreement with those of other investigators (Caldwell et al., 1963; Drenick et al., 1964). Walker et al. (1953) have suggested that those subjects with initially high serum cholesterol levels tend to show a permanent decrease in cholesterol, whereas those whose cholesterol levels are normal or low show no change. No such effect was discernible in the present series.

Serum Triglyceride

The obese subjects in this series could be separated into two distinct categories on the basis of their serum triglyceride concentrations:- two subjects had grossly elevated triglyceride levels, while in the remainder the serum triglyceride was within normal limits (page 242). Serum triglyceride levels have been shown to be, on average, higher in the obese than in subjects of normal weight (Waxler and Craig, 1964; Hollister et al., 1967) despite the fact that many obese subjects have low or normal

triglyceride values (Rifkind and Begg, 1966).

The lowgrade positive correlation between basal glucose and triglyceride levels in the present study is of some interest. This relationship still holds if only those with normal triglyceride levels are considered, and is of the same order as that noted by Albrink and Meigs (1964) in a large series of healthy males. Similarly, Waxler and Craig (1964) demonstrated in their series of chronically obese, but otherwise healthy, females that subjects with a prediabetic glucose tolerance curve have elevated serum triglyceride levels as compared with those with a normal glucose tolerance curve. It has been suggested (Farquhar et al., 1966) that this association between blood levels of glucose and triglyceride results from stimulation of hepatic synthesis of triglyceride by repetitive postprandial elevations of both plasma glucose (a potential substrate for triglyceride synthesis) and insulin (a hormone which enhances the synthetic process).

Partly from conjecture and partly as a result of animal experiments (Bloor, 1914), earlier workers believed that starvation induced marked increases in all blood lipids, but particularly in neutral fat. This "starvation hyperlipaemia" was thought to be due to mobilisation of neutral fat from adipose tissue to meet metabolic requirements (Kartin et al., 1944). Subsequent investigations in obese and non-obese humans have produced conflicting results, some workers reporting significant

increases (Bloom et al., 1966a; Mager and Iampietro, 1966), and others variable responses or no change in plasma total lipids (Drenick et al., 1964; van Reit et al., 1964). This confusion may be due in part to the nonspecificity of the lipid methods employed in most of these investigations. The present study shows that there are, in fact, two types of triglyceride response to starvation:- a small, transient triglyceride increase in those with initially normal levels (Fig. 42); and a marked, progressive decrease in hypertriglyceridaemic subjects (Fig. 43). In the former case, the transient increase in triglyceride, like that in cholesterol, may be the result of haemoconcentration. Withdrawal of carbohydrate is the most likely explanation of the dramatic fall in triglyceride in the two hypertriglyceridaemic patients, since hypertriglyceridaemia has been shown to be most commonly due to excessive carbohydrate intake (Ahrens et al., 1961). One of these patients showed a considerable rise in serum triglyceride levels during the refeeding and follow-up period, despite a diet which contained only 54 g. per day of carbohydrate. The fact that his weight did not rise suggests that he was observing his diet.

Fluid Balance

The diuresis which occurred early in the starvation period, particularly in the first 48 hours, accounted for the

more rapid loss of weight in this phase. This has been noted in all detailed studies of total starvation. Total deprivation of food is not essential to produce the diuresis. Gamble et al. (1923) showed that a reduction in carbohydrate intake alone may be responsible, and Bloom and Azar (1963) found that the substitution for an ordinary mixed diet of an isocaloric diet containing only protein and fat produced changes in water and electrolyte excretion similar to those of total starvation. The diuresis has been shown to cease abruptly on infusion of glucose, or on ingestion of carbohydrate, when fasting is underway (Bloom, 1962). The patients in the present study all retained fluid when dietary intake was recommenced. Even a 300 Calorie diet has been reported to produce such a retention of salt and water (Smith and Drenick, 1966), and it is probably the reintroduction of dietary carbohydrate which is responsible. The mechanism by which carbohydrate controls water balance is not fully understood, but Wright et al. (1963) have suggested that renal concentrating ability is impaired in the presence of low blood sugar levels.

Haematocrit

As might be anticipated from the acute changes in fluid balance the haematocrit was found to increase during starvation. A decrease in plasma volume during the first two weeks of fasting

has been demonstrated by Rapoport et al. (1965a) and Bloom et al. (1966b). A decrease in red cell volume, of lesser degree than that of plasma volume, has also been shown to occur during starvation (Rapoport et al., 1965a), and this may be due to lack of erythropoietin, as suggested by Ito and Reissman (1966) on the basis of their rat experiments.

Blood Sugar

The blood sugar concentration fell considerably during starvation (Table XXXVI). In spite of the low blood sugar levels recorded, the lowest being 36 mg. per 100 ml., no hypoglycaemic symptoms were apparent. Similar findings in obese subjects have been reported by Drenick et al. (1964), and by Rapoport et al. (1965b), but van Riet et al. (1964) found only insignificant decreases in blood sugar during a ten-day fast. The necessity of maintaining adequate blood sugar concentrations during fasting is evident from the fact that, although most tissues readily utilise fatty acids as a source of energy, for certain tissues, such as nerve cells and erythrocytes, glucose is an essential nutrient (Reitsma, 1967). The raised plasma concentrations of NEFA and ketones inhibit utilisation of glucose by muscle during starvation, and gluconeogenesis is enhanced (Randle et al., 1963). The increased production during starvation of hormones with a hyperglycaemic action, such as

glucagon (Unger et al., 1963) and growth hormone (Roth et al., 1963) may also be an important factor in maintaining the blood glucose levels.

Ketones

It is generally accepted that absolute or relative carbohydrate deprivation leads to accumulation of ketone bodies in the blood. The mechanism of ketogenesis is believed to be the stimulation of hepatic production of acetoacetic acid and β-hydroxybutyric acid as a result of the increased plasma NEFA concentrations (Steinberg, 1963). Although obese subjects have been reported to be resistant to the development of ketosis (Kekwick et al., 1959), ketonuria or hyperketonaemia has been a universal finding in studies of therapeutic starvation in obesity (Cochran et al., 1964; Silverstone et al., 1966; Stewart et al., 1966). The patients in the present study usually had ketonuria within two or three days of beginning the fast, and during the latter half of the ten-day period showed heavy ketonuria. The one exception was a girl (J. S.) aged 16 years, who lost only 3.9 kg. in the ten days, had no rise in plasma NEFA, and showed only slight ketonuria, beginning after six days.

Platelet Adhesiveness

In this study starvation was not associated with any significant changes in platelet adhesiveness (Table XXXVI). This is consistent with the fact that there have been no reports of thrombotic episodes in patients subjected to therapeutic starvation.

Various investigators have produced evidence that platelet function is influenced by fatty acids. Long-chain, saturated fatty acids have produced thrombosis following intravenous injection in animals (Connor et al., 1963; Soloff and Wiedeman, 1963), and, by in vitro techniques, have been shown to accelerate thrombus formation (Connor and Poole, 1961), and to cause platelet aggregation (Kerr et al., 1965). In the subjects described in Chapter VI, alteration of plasma NEFA and blood sugar by cigarette-smoking or stress was associated with alterations in platelet adhesiveness. The changes in platelet adhesiveness correlated positively with the changes in plasma NEFA, and negatively with the blood sugar changes. If these alterations in plasma NEFA and blood sugar were directly responsible for the alteration in platelet adhesiveness, a considerable increase in platelet adhesiveness would be anticipated during starvation, in view of the large increase in plasma NEFA and fall in blood sugar in this situation. Since this increase in platelet adhesiveness

did not occur, it appears that changes in blood sugar and plasma NEFA from day to day are not the main factors influencing changes in platelet adhesiveness.

Inter-relationship between Metabolic Changes

Most of the changes described during this study of short-term starvation are believed to result from withdrawal of carbohydrate rather than from deficiency of total calories, and are readily reversed by refeeding with carbohydrates alone or with a mixed diet. This is true in the case of the early diuresis, which leads to reduction of the plasma volume and the consequent increased haematocrit. The reduction in plasma volume is partially the cause also of the small increases in cholesterol and triglyceride which occurred in most of our subjects. An additional or alternative explanation of these serum lipid changes is an increase in hepatic lipoprotein synthesis induced by the increased plasma NEFA levels. Increased delivery of NEFA to the liver has been shown in animal studies to result in increased production and secretion of lipoprotein (Nestel and Steinberg, 1963), but may not have this effect during starvation, when reduction in hepatic synthesis of cholesterol and triglyceride has been described (Jansen et al., 1966).

Carbohydrate deficiency is also the factor in starvation

leading to the rise in blood NEFA level and the associated ketosis. The sharp fall in triglyceride in the two hyperlipaemic subjects in this study is another effect attributable to carbohydrate withdrawal. That metabolic changes similar to those of total starvation can be produced by carbohydrate withdrawal alone has been confirmed by Bloom and Azar (1963). They fed to normal subjects a 2,000 calorie diet consisting of 75 per cent fat and 25 per cent protein, and demonstrated a pattern of weight loss, with increased urinary sodium excretion, and raised blood levels of ketones, NEFA, cholesterol and total lipids, similar to that seen in total starvation.

The evidence that NEFA mobilisation in the obese during starvation is less than that in subjects of normal weight has been discussed earlier (page 249). With one exception, however, the patients in the present series seemed to mobilise fat adequately, and achieved a satisfactory weight reduction. The subject J. S. appeared to have diminished catabolism of adipose tissue; during the ten-day fast there was no rise in plasma "NEFA" concentration, ketonuria was slight and delayed, and the weight loss was only half that achieved by the other subjects. No evidence could be obtained that she was not, in fact, adhering to the zero-calorie regime. It may be that in her case there was accentuation of the adaptive enzymatic changes

during starvation which lead to suppression of fatty acid release, as Kaunitz (1966) has suggested may occur in some obese subjects.

SUMMARY AND CONCLUSIONS

(1) Plasma lipid and other metabolic changes were studied during 18 ten-day periods of starvation in obese subjects. Weight was lost most rapidly early in the starvation period, and the total weight loss during starvation averaged 6.8 kg. Weight loss was accompanied by reduction in the subscapular and triceps skinfold thicknesses. Resumption of food intake resulted in temporary weight gain.

(2) The onset of starvation was associated with a diuresis which accounted for part of the weight loss, and the resumption of food intake with water retention. There was a small but significant increase in haematocrit during starvation.

(3) The most striking change in plasma lipids was in the NEFA fraction which rose to more than double the basal values after six to seven days. NEFA levels fell gradually on refeeding, and by the time of follow-up examination two to four weeks later had returned to prestarvation levels. The changes in plasma NEFA as measured by the more specific double extraction

technique were similar in pattern but peak values were lower. There was an increase in unsaturated at the expense of saturated fatty acids during starvation, maximal after two to five days.

(4) Ketonuria was marked throughout the starvation period in all but one subject, who also failed to show an increase in NEFA levels, and whose weight loss was much less than that shown by the rest of the group.

(5) Starvation resulted in a small but significant increase in serum cholesterol, maximal after four to seven days. On refeeding, there was a rapid fall to levels significantly lower that the prestarvation values. After a further two to four weeks the cholesterol had returned to prestarvation values.

(6) Prestarvation triglyceride levels were within normal limits in sixteen instances, and grossly elevated in two subjects. In normoglyceridaemic subjects, starvation resulted in a small but significant triglyceride increase, maximal after four to five days. By the time of the follow-up examination in these subjects, triglyceride concentrations had returned to prefasting levels. In the two hypertriglyceridaemic subjects starvation resulted in marked reductions in triglyceride. On refeeding, the triglyceride rose in one subject, but continued to fall in the other subject.

(7) The blood sugar decreased during starvation, reaching its lowest level at four to five days, and remained below the prestarvation concentration throughout the starvation period. On refeeding, it returned to prestarvation levels.

(8) The platelet count increased significantly during starvation, but platelet adhesiveness showed no consistent change.

Chapter VIII. GENERAL DISCUSSION AND CONCLUSIONS

Chapter VIII. GENERAL DISCUSSION AND CONCLUSIONS

The findings of the present study have been discussed in detail in the appropriate sections, and an evaluation of the study as a whole will therefore be made in this Chapter.

The main objective at the outset of this study was to investigate the possible role of gastro-intestinal factors in the alimentary hyperlipaemia described in many cases of ischaemic heart disease. It was necessary, therefore, in the first instance to establish the frequency and severity of fat tolerance abnormalities in subjects with and without clinical evidence of atherosclerosis. The need for this was apparent for several reasons:- the wide variation in plasma lipid concentrations from country to country (Boyd and Oliver, 1958), and the lack of information on lipid levels in this area of south-west Scotland; the likelihood in an area such as this with a very high incidence of ischaemic heart disease (Bronte-Stewart, 1965) that a number of the controls had clinically undetectable atherosclerotic disease, so that a simple comparison of gastric function in controls and patients might be uninformative; and the use in many previous studies of inaccurate techniques for lipid estimation (page 118), or unsatisfactory control series (page 146). Standard fat tolerance tests were therefore carried

out on control subjects and atherosclerotic patients (Chapter IV), prior to the gastric function studies (Chapter V), which were then considered in relation to the relevant fat tolerance tests. Since the fasting plasma lipid concentrations were found to have a marked influence on fat tolerance (Table XII), a detailed analysis was made of factors affecting the fasting plasma lipids (Chapter III), including habitual cigarette-smoking. The reason for the close association of cigarette-smoking with atherosclerosis is unknown (page 210), and a further phase of this study was an investigation of the acute effects of cigarette-smoking on plasma lipids and the thrombotic tendency of the blood (Chapter VI). This was followed by an examination of these parameters in another situation producing acute plasma lipid changes in subjects predisposed to atherosclerosis - namely acute starvation in obesity (Chapter VII).

Despite the numerous population studies of plasma cholesterol, and occasionally of other plasma lipids, there have been only a few publications on plasma cholesterol levels in healthy subjects in Great Britain (Oliver and Boyd, 1953; Acheson and Jessop, 1961). The plasma cholesterol levels reported in Chapter III revealed a large measure of agreement with figures published in the United States of America, but differed somewhat from the findings in older males in Scandinavian series (pages 89 and 92). The present study also amplified the rather scanty

information available on triglyceride and NEFA levels in healthy subjects. The finding of a marked overlap in plasma cholesterol, phospholipid, and triglyceride concentrations between arterial disease patients and controls (page 87) emphasises that elevation of the plasma lipids is only one factor in a disease of multifactorial origin. In contrast to the other plasma lipids, plasma NEFA concentrations did not differ in controls and arterial disease patients (Table VII). The finding of similar plasma lipid elevations in ischaemic heart disease and peripheral arterial disease patients (Tables III, V, VI, and VII) indicates that the different clinical types of atherosclerotic disease have a common aetiology, although the factors which determine the localisation of the disease are, as yet, incompletely understood.

The present study (Chapter IV) showed that impaired fat tolerance was not a characteristic of arterial disease patients in general, but occurred in those who developed the disease at an early age (page 139). This was true of patients with peripheral arterial disease as well as of ischaemic heart disease patients (page 140), and suggests that the atherosclerotic process is accelerated in the presence of abnormalities in triglyceride metabolism. It was emphasised that abnormal fat tolerance tests occurred in those with already elevated

fasting triglyceride levels (Table XII). This relationship between fasting and postprandial plasma triglyceride concentrations had previously been obscured by the use of crude techniques for plasma lipid estimation such as optical density measurements. The absence or infrequency of differences in fasting optical density between patients and controls, and subsequent large differences in optical density produced by fat loading suggested that the defect was in the handling of exogenous fat. The present results, however, indicate that the disorder is in endogenous lipid metabolism. Since the 8-hour postprandial triglyceride level gave only marginally better separation of arterial disease patients from controls, and as fat tolerance tests are time-consuming for both patients and staff, it would appear that they are of little value in the detection of lipid abnormalities in atherosclerosis. Furthermore, the plasma cholesterol value was found at all ages to distinguish arterial disease patients from controls better than either fasting or postprandial triglyceride concentrations (page 146). A prospective population study would be required to determine beyond doubt whether antecedent elevation of cholesterol or triglyceride is more closely related to the development of atherosclerotic disease. From the present study, however, it appears that elevation of the plasma cholesterol is the most common plasma lipid abnormality in atherosclerosis, but when

elevation of the plasma triglyceride occurs it may be an important factor in accelerating the disease.

In the past twenty years there have been repeated suggestions that the fat tolerance abnormality is due, not to abnormalities in fat transport or clearing, but to an abnormal mode of fat absorption associated with impaired gastrointestinal function (page 159). The results of the investigations reported in Chapter V, as well as those reviewed in the above paragraph indicate this is not the case. Basal gastric secretion, gastric emptying rates, and gastric lipolysis were found to be similar in arterial disease patients and controls (page 207), even when significant differences were found in alimentary lipaemia (page 176). The rate of gastric emptying was found to influence the shape of the fat tolerance curve (page 198), but not the intensity of the lipaemia, already shown to be dependent on the fasting triglyceride level (Table XII). At the outset of this study the techniques available for determination of gastric emptying could not measure simultaneously gastric emptying and alimentary lipaemia (Method I, p.162), and estimated the time required for completion of gastric emptying rather than the rate of gastric emptying (Method II, p.168). A more recently developed technique (Griffith et al., 1966) may provide a satisfactory method for the simultaneous determination of alimentary lipaemia and gastric emptying rate.

Gastric lipolysis appeared to occur in a random fashion (page 204), and the evidence was against a significant function of gastric lipase.

The recently-publicised association of cigarette-smoking with atherosclerosis (page 210) emerged at several points in this study. A considerably higher incidence of habitual cigarette-smoking was noted in the arterial disease patients, particularly those with peripheral arterial disease, than in the controls (Table II). Some evidence was found of higher cholesterol levels (page 65) and slower gastric emptying (page 187) in cigarette-smokers than in non-smokers. The possibility that the association of cigarette-smoking with atherosclerosis is due to the production of an increased thrombotic tendency was explored in Chapter VI. Evidence was found of a homeostatic mechanism, whereby a rise in NEFA was associated with increased platelet adhesiveness (whether by direct action or as an index of catecholamine release), whereas the rise in blood glucose which frequently accompanied cigarette-smoking inhibited platelet adhesiveness (page 227). Further investigation of these phenomena is indicated to determine whether the increased platelet adhesiveness is due to a rise in NEFA <u>per se</u>, or to the catecholamines released during smoking, and whether or not these changes found in fasting subjects also occur in the nonfasting state. In the platelet studies during starvation (Chapter VII), a situation

in which a marked rise in NEFA was accompanied by a fall in blood glucose, the platelet adhesiveness did not show the anticipated increase (page 265). Interpretation of these findings is difficult, however, since large day-to-day fluctuations occur in platelet adhesiveness due to factors at present unknown (page 260).

The studies of lipid mobilisation during starvation in obese subjects (Chapter VII) emphasises that fat mobilisation occurs readily under these conditions even in subjects with refractory obesity, and that satisfactory short-term weight reduction can be achieved. The possibility of a defect in fat mobilisation in some obese subjects, supported by the findings in one of the patients in this study (page 262), is worthy of further investigation. The role of NEFA as the transport form of fat mobilised to meet energy requirements has been underlined in this study. The older erroneous reports of mobilisation of triglyceride and cholesterol from fat depots during starvation were probably due to failure to appreciate the dehydration effect of total starvation (page 238). The present study also clarifies the changes which occur in plasma triglyceride during starvation, two distinct effects being observed depending on the initial triglyceride level (page 242).

In summary, therefore, this study showed no evidence

that alimentary hyperlipaemia was associated with impaired gastric function. It emphasised the frequency in atherosclerotic disease of elevated plasma cholesterol levels, the lesser frequency of triglyceride disorders, and the interrelationship of plasma cholesterol, phospholipid, and triglyceride concentrations resulting from their common plasma transport mechanism. No differences were found between atherosclerotic patients and controls in plasma NEFA concentrations either in the basal state or following various stimuli, and plasma NEFA levels fluctuated independently of the other plasma lipids.

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SOME FACTORS AFFECTING THE PLASMA LIPIDS

SUMMARY

SUMMARY

Some factors affecting the plasma lipid concentrations were studied in healthy subjects, in patients with atherosclerotic arterial disease, and in two groups of subjects predisposed to atherosclerosis: namely, habitual cigarette-smokers, and obese persons.

1. FASTING PLASMA LIPIDS

In healthy subjects, plasma cholesterol, phospholipid, and triglyceride concentrations were markedly influenced by age and sex. There was a considerable overlap in lipid values between healthy controls and patients with atherosclerotic arterial disease.

At all ages arterial disease patients had significantly higher mean plasma cholesterol levels than controls.

Elevated fasting plasma triglyceride concentrations occurred only in young arterial disease patients. There were no significant differences in triglyceride between patients and controls in older subjects.

Similar plasma lipid abnormalities were seen in the three clinical types of arterial disease studied - myocardial infarction, angina pectoris, and peripheral arterial disease.

Plasma non-esterified fatty acid levels did not differ in patients and controls. The only factor shown to influence plasma non-esterified fatty acids was habitual cigarette-smoking. Significantly lower non-esterified fatty acid levels were found in habitual cigarette-smokers after overnight abstention from smoking.

2. FAT TOLERANCE TESTS

Fat tolerance tests, using a 75 g. fat mixed meal, were carried out in patients and controls.

The fat tolerance curves of male arterial disease patients aged 40 to 44 years were significantly higher than those of healthy men of the same age. Fat tolerance tests in older male and female patients did not differ from those of controls of corresponding age. Patients with myocardial infarction, angina pectoris, and peripheral arterial disease showed similar abnormalities in alimentary lipaemia.

High fat tolerance curves occurred in those subjects whose fasting triglyceride concentrations were already elevated.

In arterial disease patients of all ages elevation of the plasma cholesterol concentration was more common than elevation of the fasting or 8-hour triglyceride value.

3. GASTRIC FUNCTION IN RELATION TO FAT TOLERANCE AND ATHEROSCLEROSIS

Studies of gastric secretion, rates of gastric emptying, and gastric lipolysis were carried out in patients and controls.

Basal gastric secretion was similar in volume and composition in healthy controls and arterial disease patients.

Gastric emptying rates were similar in controls and arterial disease patients.

The peak in postprandial lipaemia occurred earlier in subjects with a rapid gastric emptying rate, determined both by an intubation technique and by a radiological method.

Gastric lipolysis occurred to a variable degree in all subjects, but did not differ in controls and arterial disease patients. The extent of gastric lipolysis showed no relation to the degree of postprandial lipaemia.

4. SHORT-TERM EFFECT OF CIGARETTE-SMOKING ON PLASMA LIPIDS, BLOOD GLUCOSE, AND PLATELET ADHESIVENESS

Plasma lipids, blood glucose, and platelet adhesiveness were measured in six arterial disease patients and six controls before and after smoking and sham-smoking. The changes were similar in patients and controls.

Cigarette-smoking elevated plasma non-esterified fatty acid levels and resulted in an increase in the proportion of unsaturated fatty acids at the expense of saturated fatty acids. Smoking was also followed by a rise in blood glucose and transient increase in the total platelet count. The variable effect of smoking on platelet adhesiveness seemed to be due to opposing actions of changes in non-esterified fatty acid and glucose levels. A rise in plasma non-esterified fatty acid was associated with increased platelet adhesiveness, whereas a rise in glucose inhibited platelet adhesiveness.

5. EFFECT OF THERAPEUTIC STARVATION ON PLASMA LIPIDS

The changes in plasma lipids and other metabolic changes were observed during ten-day periods of total starvation in sixteen obese subjects.

Starvation produced a marked rise in plasma non-esterified fatty acid, and a fall in blood sugar. Small increases in cholesterol and triglyceride occurred in most subjects, but the two patients with hypertriglyceridaemia showed a dramatic fall in triglyceride throughout the fast. The majority of these changes were maximal after four to seven days, but tended to return towards prestarvation levels as fasting continued.

CONCLUSIONS

This study emphasised the frequency of elevated plasma cholesterol values in atherosclerosis, the lesser frequency of triglyceride abnormalities, and the inter-relationship of cholesterol, phospholipid, and triglyceride concentrations resulting from their common plasma transport mechanism. Plasma non-esterified fatty acid levels did not differ in atherosclerotic and healthy subjects, either in the basal state or in response to various stimuli, and showed no correlation with the other plasma lipid values.