THE COMPOSITION OF CERTAIN LIPIDS IN MAN

A comparative study of lipids in total serum, in the serum β-lipoprotein fraction and in arterial atheromatous plaques in certain groups of people.

by

Gerardine Olga Young. B.Sc.

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I N T R O D U C T I O N

In recent years biochemical studies have played an important role in research into the pathogenesis of ischaemic heart disease. A large number of these studies have been concerned with the relationship between serum lipids and ischaemic heart disease (I.H.D.), which has been defined by the World Health Organization (1957) as "the cardiac disability, acute and chronic, arising from reduction or arrest of blood supply to the myocardium in association with disease processes in the coronary arterial system".

Serum lipid concentrations.

Epidemiological surveys in several countries have shown a striking correlation between the prevalence of I.H.D. and the level of serum cholesterol (Keys et al 1952, 1954, 1958; Keys and Keys 1954; Bronte-Stewart et al 1955; Mann et al 1955; Toor et al 1957; Walker and Arvidsson 1954). Several authors have reported that the concentrations of total lipid, cholesterol, triglyceride and phospholipid in the serum are generally higher for subjects with I.H.D. than for those apparently free from this disease (Carlson 1960b; Cohen et al 1960; Gertler et al 1950; Albrink and Man 1959; Schrade et al 1960; Steiner et al 1952). However, although as a group the values for subjects with I.H.D. are higher than for control groups, a considerable degree of overlap exists.

Serum β-lipoprotein concentration.

The term β-lipoprotein is used to denote the serum lipid-protein complex which has an electrophoretic mobility similar to that of β-globulin. This serum fraction corresponds closely to the low density (Sf0-400) lipoproteins separated by ultracentrifugation (Pezold et al 1957).

The possible importance of the serum β-lipoprotein concentration in providing separation between subjects with I.H.D. and healthy subjects was first stressed by Gofman and his co-workers (Gofman et al 1950a, 1950b, 1954, 1956). They determined the concentration of low density lipoprotein fractions by analytical ultracentrifugation and observed an increase in the concentration of the Sf 5-8 fraction and the appearance of a new fraction, Sf 10-30, in the serum of rabbits with atherosclerosis. They noted that the degree of atherosclerosis could be correlated with the increase in the concentration of Sf 10-30 lipoproteins. Examination of human serum revealed a high degree of correlation between the incidence of I.H.D. and the presence of Sf 10-20 lipoproteins, (Gofman et al 1950b). Furthermore, subjects with overt I.H.D. had significantly higher levels of the low-density lipoprotein fractions Sf 0-12 and Sf 12-400 than did apparently healthy people in the same age range of 40 to 50 years. The concentration of the triglyceride-rich Sf 12-400 lipoproteins was more strongly correlated with the incidence of I.H.D. than was that of the cholesterol-rich Sf 0-12 lipoproteins (Gofman et al. 1954). Jones et al (1951) showed distinctly greater separation between groups with I.H.D. and control groups by Sf 12-20 lipoprotein measurements than by total serum cholesterol measurements. In addition, by matching patients and controls at identical serum cholesterol levels, separation between them could still be achieved by the Sf 12-20 lipoprotein measurements.

Other authors (Barr et al 1951; Nikkila 1953; Oliver and Boyd 1955; Brunner and Lobl 1958; Chapin and Proger 1959; Joubert et al 1962) have found that there is a greater concentration of serum β-lipoprotein lipid in subjects with I.H.D. than in apparently healthy controls. Bronte-Stewart et al (1955) reported that differences in total serum cholesterol levels between races with different susceptibility to I.H.D. were due entirely to differences in the concentration of cholesterol in β-lipoprotein. It would seem, therefore, that differences in lipid levels between groups with varying suscep-

tibility to I.H.D. are more marked in the β-lipoprotein fraction than in total serum.

The relationship between diet and serum lipids.

The influence of dietary factors on serum lipids has aroused considerable interest. While attention has been focussed primarily on dietary fat, more recently the possible importance of dietary carbohydrate has been suggested (Albrink and Man 1959).

The effect of dietary fat on the concentration and composition of lipids has been related to both the quantity and the type of fat present in the diet. In several epidemiological studies it was found that in groups where the percentage of dietary calories consumed as fat was about 40%, the prevalence of I.H.D. was high, serum cholesterol levels were high and rose with age. Conversely, in groups eating little fat the incidence of I.H.D. was low, serum cholesterol levels were low and the rise with age was insignificant (Keys 1956; Keys et al 1952, 1954, 1958; Keys and Keys 1954; Walker and Arvidsson 1954; Bronte-Stewart et al 1955; Toor et al 1957). In addition, it was noted that serum cholesterol trends could be correlated with the consumption of animal fat but not with that of vegetable fat (Bronte-Stewart et al 1955; Hardinge and Stare 1954).

Arising from these observations, it was shown in numerous controlled feeding experiments that serum cholesterol, triglyceride and phospholipid levels were increased by the consumption of saturated fat and decreased by the consumption of unsaturated fats, particularly those rich in linoleic acid (Kinsell et al 1952;1953; Kinsell 1954; Beveridge et al 1955;1956; Bronte-Stewart et al 1956; Malmros and Wigand 1957; Hegsted et al 1957; Ahrens et al 1957). The changes in serum lipid concentrations were usually paralleled

by changes in the concentration of lipid in the β-lipoprotein (Bronte-Stewart et al. 1956; Keys et al. 1957). In the course of various feeding experiments it was shown that the fatty acid compositions of the serum lipid fractions changed markedly and tended to reflect the fatty acid compositions of the fat consumed (Kinsell et al. 1957; Ahrens et al. 1959; Bohle et al. 1961; Kingsbury et al. 1962). Ahrens et al. (1959) noted, however, that the phospholipid fatty acid composition was least affected by such dietary manipulations. It is, thus, apparent that dietary fat may influence both the concentration and the fatty acid composition of serum lipids.

The relationship between dietary carbohydrate and serum lipids has not been studied to any great extent. It is, however, known that breakdown of carbohydrate to acetate occurs and that cholesterol, triglyceride and saturated and mono-unsaturated fatty acids can be synthesized from acetate (Cantarow and Schepartz 1957; Stetten and Schoenheimer 1940; Zabin 1951; Anker 1952; Dauben et al. 1953; Lipsky et al. 1957). Several authors (Albrink and Man 1959; Hatch et al.1955; Ahrens et al. 1957) have shown that consumption of a low-fat, high-carbohydrate diet produced a marked rise in serum triglyceride concentration. Antonis and Bersohn (1961) found that on feeding high-carbohydrate low-fat diets, there was a temporary rise in serum triglyceride levels. With continued feeding of this diet the serum triglyceride levels fell to the initial levels within 32 weeks. A high-carbohydrate, low-fat diet thus does not appear to give rise after habituation to increased serum triglyceride levels.

Fairhurst and Waterhouse (1963) have suggested that lipogenesis resulting from a high carbohydrate diet would produce predominantly oleic and saturated fatty acids, but not linoleic acid. It is apparent, therefore, that dietary carbohydrate may influence serum lipid levels and the fatty acid composition of serum lipids.

Essential fatty Acids.

The term "essential fatty acid" (E.F.A.) is applied to those fatty acids which apparently cannot be synthesized by the animal organism and this term, by definition, is limited to "linoleic and arachidonic acids and to such other acids as may be derived metabolically from them" (Holman 1958).

The observations that consumption of fats rich in linoleic acid decreased serum lipid levels in man, together with the data showing deposition of saturated cholesterol esters in the organs of E.F.A.-deficient animals (Alfin-Slater et al 1954) aroused interest in the possible relationship between essential fatty acids and I.H.D. It has been postulated that a relative deficiency of E.F.A. might lead to the presence in the serum of saturated cholesterol esters which would tend to be deposited and thus give rise to the formation of atheroma (Sinclair 1956). Although only an hypothesis, this aroused considerable interest in the content of E.F.A. in the serum of people with differences in their susceptibility to I.H.D.

While E.F.A. deficiency in animals has clearly defined pathological manifestations (Burr and Burr 1929), no such features have as yet been observed in man. On the basis of the changes in fatty acid composition in E.F.A.-deficient animals, Holman (1960) has suggested that a relative deficiency of E.F.A. may be assessed by the ratio of trienoic to tetraenoic fatty acids, a value of less than 0.4 for this ratio indicating that the minimum requirement of linoleate has been met. Several authors have compared the percentages of linoleic acid and of arachidonic acid in the serum lipids of subjects with I.H.D. and of apparently healthy controls. Some authors (Lewis 1958; Schrade et al 1961a; Kingsbury et al 1962) have found that the proportions of linoleic (dienoic) acid and of arachidonic (tetraenoic) acid in the serum lipids were lower for subjects with I.H.D. than for healthy controls.

Kingsbury et al (1962) also noted that the ratio of trienoic to tetraenoic acids was lower for subjects with I.H.D. than for controls. In contrast, other authors (James et al 1957; Caren and Corbo 1958; Smith 1962) found no difference between the fatty acid patterns of healthy subjects and those of subjects with I.H.D. There is thus conflicting evidence regarding the possible association between a relative deficiency of E.F.A. and susceptibility to I.H.D.

Choice of Controls.

In comparative studies between subjects with I.H.D. and healthy controls, the latter subjects are usually people who have no overt manifestations of I.H.D. Autopsy data have, however, shown that in populations where the prevalence of I.H.D. is high, virtually every adult shows some degree of atherosclerosis (Lande and Sperry 1936; Peck et al 1951). Thus, since there is no simple diagnostic procedure by which to assess the presence of coronary arterial disease prior to its clinical manifestations, it is possible that, in high prevalence areas, control subjects may have subclinical I.H.D. This might, in part, account for the conflicting evidence regarding the percentages of E.F.A. in serum lipids and for the overlap found in other serum lipid parameters between subjects with I.H.D. and such control groups.

In South Africa, the incidence of I.H.D. in the white population is one of the highest in the world (World Health Organization, 1956). In contrast, the incidence of I.H.D. in the South African Bantu population is extremely low (Becker 1946; Elliott 1953; Higginson and Pepler 1954; Vogelpoel and Schrire 1955; Sacks 1960; Walker 1963). For the purpose of comparative studies the Bantu may, therefore, be regarded as an ideal control group in assessing the relation between various lipid parameters and the susceptibility to I.H.D. or its established presence.

The relationship between serum lipids and the formation of atheroma.

The role of lipids in the formation of atheroma is a problem which has challenged research workers for the last half century. Early studies drew attention to the fatty nature and the high content of cholesterol in atherosclerotic lesions (Virchow 1856; Gazert 1899; Baldauf 1906; Aschoff 1906), but it was not until 1910 that Windaus performed the first quantitative analysis of cholesterol in atherosclerotic aortas. The now classical experiments of Anitschkow (1933), demonstrating that the feeding of cholesterol to rabbits produced serum hypercholesterolemia and gave rise to the deposition of cholesterol in the arterial wall, stimulated great interest in the relationship between serum lipids and the lipid deposits in the arterial wall in man.

There are two major concepts as to the mechanism whereby circulating lipid might be incorporated into atheromatous plaques. According to the filtration theory (Page 1954), lipid material, in the form of lipoprotein, filters by lateral pressure, from the serum into the arterial wall and is deposited in the intima. The thrombogenic theory (Duguid 1954) favours the concept of initial formation on the intimal surface of a thrombus which becomes covered with endothelium and is thus incorporated into the vessel wall. Subsequent organization of the thrombus, accompanied by fatty degeneration, results in thickening of the intima and the accumulation of lipid.

There is evidence which suggests that β-lipoprotein might be specifically involved in the formation of atheromatous plaques. In several studies, the presence in normal and in atherosclerotic aortic intima of lipoproteins immunologically indistinguishable from β-lipoprotein, has been demonstrated by immuno-electrophoretic techniques (Ott et al 1958; Tracy et al 1961; Hanig et al 1956; Kayden et al 1959; Gero et al 1961).

On the basis of the hypothesis that lipid in arterial atheromatous plaques is derived from lipid in serum or from the β-lipoprotein fraction of serum, one may anticipate that the composition of lipid in atheromatous plaques would resemble that of lipid in serum or the β-lipoprotein fraction of serum.

In the present study atheromatous plaque material has been obtained from the aorto-iliac artery by endarterectomy. The composition of lipid in this material has been compared with that in total serum and serum β-lipoprotein from Bantu, apparently healthy white subjects and white subjects with I.H.D. Since atherosclerosis is a general disease of the vascular system and involvement of the coronary arteries but one of its manifestations, the question arises as to whether it is valid to compare the composition of lipid in plaques from the aorto-iliac artery with that in serum from people with I.H.D. The diagnosis of I.H.D. is unequivocal and easily established, and for this reason subjects with proven I.H.D. were chosen as being representative of subjects with atherosclerosis. It may be argued that it would have been better to analyse lipid from the coronary arteries. However, samples of coronary arteries have to be obtained post-mortem and the lipid and fatty acid composition of these samples might be altered by post-mortem autolysis. For example, triglyceride determinations, as performed here, are based on the estimation of glycerol which may be formed as the result of autolysis of phospholipids. It was, therefore, considered preferable to analyse surgical specimens.

THE PURPOSE OF THIS STUDY

The purpose of this study is :

1. To determine the composition of lipid in total serum and in the serum β-lipoprotein fraction in white subjects with I.H.D., in healthy white subjects and in healthy Bantu subjects.

2. To compare these findings in the three groups of subjects in order to determine whether any lipid parameter differentiates between subjects with I.H.D. and all healthy subjects.

3. To determine the composition of lipid in arterial atheromatous plaques.

4. To compare the composition of lipid in arterial atheromatous plaques with that in the total serum and in the serum β-lipoprotein fraction in order to assess which, if either, of these might be related to the formation of atheroma.

ABBREVIATIONS

Certain abbreviations will be used throughout this study. They are -

I.H.D. Ischaemic heart disease.

E.F.A. Essential fatty acid.

G.L.C. Gas-liquid chromatography.

Nomenclature of fatty acids

The classification proposed by Dole et al (1959) has been adopted. The fatty acids will, in general, be designated by a number referable to the number of Carbon atoms in the molecule. This will be followed by a colon and the number of double bonds in the molecule, also indicated by a number. Thus myristic acid is C14:0, palmitic acid is C16:0, palmitoleic acid is C16:1, stearic acid is C18:0, oleic acid is C18:1, linoleic acid is C18:2, arachidonic acid is C20:4, etc.

In some instances in the text reference will be made to di-, tri-, tetra-, penta-, and hexaenoic fatty acids. This refers to fatty acids having two, three, four, five and six double bonds respectively.

Certain statistical abbreviations used are defined in the chapter on statistical methods (Part I, Chapter 3).

PART I

MATERIAL AND ANALYTICAL METHODS

CHAPTER I

I. CLINICAL MATERIAL

(a) CHOICE OF SUBJECTS

It has been shown in several studies that the incidence of Ischaemic Heart Disease is considerably lower in the Bantu than in the White population of South Africa (Becker 1946; Elliott 1953; Higginson and Pepler 1954; Vogelpoel and Schrire 1955; Sacks 1960). The incidence of I.H.D. is higher in males than in females of comparable age and race (Bronte-Stewart 1959). In populations with different susceptibility to I.H.D., the differences in serum cholesterol levels are most marked in the fifth decade of life (Keys 1956). In this study the subjects, whose serum lipids were analysed, were chosen so as to include males between the ages of forty and fifty years, drawn from the Bantu population, the healthy White population and from a White group with proven I.H.D.

i) The Bantu group

This group consisted of 16 volunteers who are employed as heavy manual labourers at a local quarry and who recently migrated from a rural area. The average age of men in this group was 43 years and their ages ranged from 40 - 45 years. These subjects lived in a compound and ate a diet similar in its composition and in its method of preparation to that of rural Bantu.

ii) The control group

This group consisted of 12 White volunteers drawn from the office staff of a large insurance company. The average age of the men was 42 years and their ages ranged from 40 - 46 years.

iii) Criteria for selection

All the Bantu and control subjects chosen were apparently in a good state of health and had not recently gained or lost weight. It was ascertained by means of interrogation that they had no history of coronary or other vascular disease, no family history of I.H.D. and that they were free from any endocrine disorders, particularly diabetes mellitus. It was, however, not possible to perform electrocardiographic examinations on these subjects. All the subjects had maintained a reasonably constant dietary pattern for several years prior to this study.

Since each of these groups was drawn from a single source it may be argued that neither is representative of the population from which they were sampled. However, the Bantu who migrate to Cape Town are not selected and the group studied is probably a representative sample. The White control subjects, while all clerical workers, are also an unselected group although drawn from one section of the local White population. It is noteworthy that the serum lipid levels found for each of these groups are similar to those found for considerably larger groups drawn at random from the same populations (Bronte-Stewart et al, 1955). These groups may thus be regarded as being representative of the populations in so far as such representivity can be assessed from these serum lipid parameters.

iv) The patients with I.H.D.

These subjects, whose socio-economic background was similar to that of the White controls, were drawn from about 700 White patients who attend the anti-coagulant clinic at Groote Schuur Hospital. The hospital records were examined and those subjects with unequivocal evidence, both clinical and electrocardiographic, of I.H.D. were selected. Subjects who had experienced a myocardial

infarction less than one year prior to this study were excluded, since it has been shown that the serum lipid pattern may be radically altered for as long as six months after myocardial infarction (Cramer 1961). The subjects who fulfilled the abovementioned requirements were contacted and the first twelve who volunteered for this study made up the sample. The average age of the men was 43 years and their ages ranged from 40 to 49 years. All these subjects were taking Dindevan (phenindione) at a dosage that maintained their prothrombin index at an optimal level of between 50 and 60% of normal, with a possible range of 40 - 70%. There is some evidence that Dindevan may influence serum lipid levels in man and in rabbits (Krut and Young, unpublished data; Merskey et al 1959), but this effect is not fully established. While these subjects had maintained a fairly constant dietary pattern for one year prior to this study, it was established that they had, without exception, altered their dietary regime after experiencing a myocardial infarction. Prior to this they had consumed a diet containing a higher proportion of fat, particularly of animal fat. Their diet was modified by reducing the total fat intake and by increasing the consumption of unsaturated fat.

It may therefore be argued that they are not representative of subjects with I.H.D. There is no defence against this criticism. It is, however, extremely difficult to find patients with I.H.D. who have not altered their diet since, in this area, White men with a history of I.H.D. are very conscious of the view that fat in excess quantity and animal fat in particular may be detrimental to their health.

(b) PLAQUE MATERIAL

Intimal material from atheromatous arteries was obtained from twelve male subjects in the fifth decade who were undergoing aorto-iliac endarterectomy. Their average age was 44 years and ranged from 41 to 48 years. Detailed dietary histories were not obtained from these subjects, but it was established that

they were not on special diets. It has been assumed that they were consuming the typical diet of White South Africans which would correspond closely to that of the control group.

II. DIETARY ANALYSIS

A dietary history was obtained from all the subjects whose serum lipids were studied. The details of each subject's diet were obtained by recall. The average quantity and specific type of food, including that eaten at and in between the three main meals, was noted for each day of a typical week. The subjects were questioned regarding the method of preparation and the amount of fat or oil used in preparing the food. The intake of each foodstuff was estimated on the basis of average portions unless there were obvious deviations. The data thus obtained were considered to be representative of the subject's diet.

For each subject the composition of the diet as regards calories, protein, fat and carbohydrate was calculated from food tables (Fox and Goldberg 1944; McCance and Widdowson 1946). The proportion of linoleic acid in the dietary fat was estimated by reference to standard tables (FOOD). These tables were prepared in the U.S.A. and the fatty acid compositions need not accurately reflect the composition of local foods.

It is recognised that dietary analysis based on diet histories gives, at best, a close approximation of the actual intake and composition of the diet.

CHAPTER 2

CHEMICAL METHODS

The chemical methods used in the analysis of lipids in total serum, β-lipoprotein and atheromatous plaques will be described and discussed in three main sections, viz:-

I: QUANTITATIVE ESTIMATION OF LIPIDS

II: THE ISOLATION AND EXTRACTION OF LIPIDS

III: ANALYSIS OF FATTY ACIDS

I: QUANTITATIVE ESTIMATION OF LIPIDS

The concentration of lipid components was determined chemically on aliquots of the lipid extracts obtained from total serum, β-lipoprotein and plaques (Section II). Duplicate determinations were performed on all samples. For total serum and β-lipoprotein the results for each lipid component were expressed as mg./100 ml. of serum.

In calculating the results of lipid determinations in the plaque the problem arose of finding a suitable standard of reference. In some studies comparing the content of lipid in arteries with different degrees of atherosclerosis, the weight of lipid has been related to the total weight of tissue (Buck and Rossiter 1951; Bjorntorp et al 1962; Smith 1960; Böttcher et al 1960a).

In the present study all plaque material was obtained from arteries with a similar degree of atherosclerosis and interest was centred only on the lipid composition and not the amount of lipid in the tissue. The results were obtained as the concentration of lipid (in mg.) in the total extract from the plaques, and the relative proportions of lipid components were calculated. Expressing the lipid constituents as relative proportions of the total lipid provided the basis of comparison with the serum lipids.

i) Total and free cholesterol was estimated by the method of Schoenheimer and Sperry (1934) as modified by Sperry and Webb (1950). The concentration of esterified cholesterol was obtained by subtraction of the concentration of free cholesterol from that of total cholesterol.

ii) Triglyceride was determined by the method of Young and Eastman (1963). The results are expressed as mg. of triolein/100 ml. serum. This method in fact measures tri-, di- and mono-glycerides, but since the latter two components

are present only in small amounts (Eastman and Bronte-Stewart 1962) reference is made to 'triglycerides'.

iii) Phospholipid was determined by the method of Fiske and SubbaRow (1925) as modified for micro-analysis by Bartlett (1959). The standard solution was prepared according to the method of Marinetti et al (1959). The results are obtained as mg. of phosphorus and are expressed as mg. of lecithin/100 ml. serum (mg.lecithin = mg.phosphorus x 25).

The degree of reproducibility of estimation of these methods was examined by calculating the standard error of measurement (S.E.M.) between duplicate readings for 150 samples including all those analysed in this study. The values are shown in Table 1.

	Total Cholesterol	Free Cholesterol	Triglyceride	Phospholipid
Mean (mg./100 ml.)	125.9	37.4	102.1	175.0
Range (mg./100 ml.)	54.0-374.1	12.2-134.0	29.0-254.7	45.8-339.6
S.E.M. (mg./100 ml.)	3.7	1.8	4.6	6.5
S.E.M.(%)	3.0	4.9	4.5	3.7

TABLE 1 : The reproducibility of estimation of total and free cholesterol, triglyceride and phospholipid.

The data in Table 1 show that, over a wide range of lipid concentrations, the standard error of measurement for each component was less than 5%. The

reproducibility could not be calculated for esterified cholesterol since it was not directly estimated. Since the standard error of measurement for both total and free cholesterol is less than 5%, the error in calculating the concentration of esterified cholesterol must be of a similar order.

iv) Cholesterol ester. This term will be used frequently in the text and refers to the cholesterol plus the fatty acids with which it is esterified. This is in contra-distinction to the term "esterified cholesterol" which refers only to the cholesterol moiety. The concentration of cholesterol ester has been calculated according to the following formula :-

$$\text{Cholesterol ester (mg/100ml)} = \text{Esterified cholesterol (mg/100 ml)} \times \frac{\text{M.Wt.Chol.} + \text{M.Wt.F.A.}^{\text{M}} - \text{M.Wt.}H_2O}{\text{M.Wt. cholesterol}}$$

$$= \text{Esterified cholesterol (mg/100 ml)} \times \frac{(386.7 + 278.0 - 18.0)}{386.7}$$

$$= \text{Esterified cholesterol (mg/100 ml)} \times 1.67$$

M.Wt = Molecular Weight

Chol.- Cholesterol

M Mean molecular weight of the cholesterol ester fatty acids calculated from the cholesterol ester fatty acid patterns obtained by G.L.C. analysis of all samples.

The concentration of cholesterol ester, calculated in this manner, has been used in the calculation of total lipid concentrations (see below). Conversion factors of 1.68 and 1.69 have been reported by Buck and Rossiter (1951) and by Page et al (1935) respectively.

v) Calculation of total lipid concentration

The concentration of total lipid was calculated as being the sum of the chemically determined concentrations of free cholesterol, cholesterol ester,

triglyceride and phospholipid. The value thus obtained is not strictly accurate. The triglyceride has been expressed as the triglyceride of oleic acid which has a molecular weight of 282.4, whereas the mean molecular weight of the triglyceride fatty acids, calculated from the fatty acid patterns obtained by G.L.C. was theoretically 271.6. The phospholipid concentration has been expressed as lecithin (mg.lecithin = mg.phosphorus x 25). The factor for converting the concentration of phosphorus to phospholipids is not strictly accurate, since the molar ratio of phosphorus varies for each different phosphatide. However, since lecithin constitutes approximately 75% of the total phospholipid in serum (Nelson and Freeman 1960) the error in such a conversion is relatively small. In addition, the concentration of non-esterified fatty acids has not been included. The average concentration of this component in a fasting serum is approximately 0.05 m.Eq/100 ml. (Rothlin and Bing 1961), i.e. approximately 14 mg./100 ml. Non-esterified fatty acids therefore constitute only about 2 - 3% of the total lipid.

vi) Gravimetric determination of total lipid concentration

At the commencement of this study total lipid present in the extracts was determined gravimetrically.

Aliquots of the lipid extracts were taken to dryness under a stream of nitrogen, re-extracted with chloroform and filtered. The filtered extracts were transferred to weighed planchettes fashioned from light aluminium foil, evaporated to dryness under a stream of nitrogen and dried to constant weight over $CaCl_2$ in a vacuum desiccator. The average lipid weight in the aliquots varied from 0.5 to 1.5 mg. From these weights the total lipid concentration in the extracts was calculated and compared with that calculated as described above (Table 2).

Method	1	2	3	4	5	6	7	8	9	10
Grav.	517	227	592	208	808	566	308	608	333	850
Chem.	398	222	308	164	554	352	171	403	217	709
Grav./Chem.	1.3	1.0	1.9	1.3	1.5	1.6	1.8	1.5	1.5	1.2

Table 2: The concentration of total lipid (mg./100 ml.) as determined gravimetrically (Grav.) and by calculation from chemical estimations (Chem.)

It is apparent from the data in Table 2 that the gravimetric value was consistently higher than the chemical value and the ratio of the one to the other was not constant. The possible error in the values obtained by calculation from chemical estimations has been discussed and found to be relatively small. It is unlikely that this would account for the wide differences found between the two methods. It is more likely that the differences are due to inaccuracies in the gravimetric method. While the balance used is calibrated to weigh to 0.01 mg., a certain measure of error must intrude in weighing such small amounts of lipid, but this could not account for the marked differences found.

It is highly probable that the lipid weights were erroneously high due to contamination with non-lipids. It has been shown that 3:1 ethanol:ether extracts non-lipids from serum (Bjorntorp 1960). These are only partially removed on re-extraction with chloroform (Bjorntorp 1960) and it has been demonstrated that, in the presence of phospholipids, glucose is soluble in chloroform (Krut and Wilkens 1964). In addition, despite the fact that the chloroform extract was filtered through very fine filter paper, the dried lipid residue contained

a fine white precipitate which might have been protein. There are thus several variable factors in this technique which could give rise to erroneously high lipid weights. In view of this, the gravimetric method was discarded and total lipid concentrations have been determined by using the method of calculation based on chemical estimation of the component lipid fractions.

II. THE ISOLATION AND EXTRACTION OF LIPIDS

Venous blood samples were taken from subjects who had fasted overnight. The blood samples were allowed to stand at room temperature(about 20°C) for one hour. The samples were centrifuged for 15 minutes at 3,000 r.p.m. and the serum transferred.

(a) EXTRACTION OF LIPID FROM TOTAL SERUM

The lipid from a known volume of serum (2.0 ml.) was quantitatively extracted in twenty volumes of 3:1 ethanol:diethyl ether according to the method of Bloor (1914). The extract was brought to a final volume of 50 ml. in a volumetric flask.

i) Recovery of lipid in the extraction procedure.

Aliquots of serum were added to the extracting medium. To these were then added known amounts of standard solutions containing cholesterol, cholesterol ester, (cholesterol oleate), triglyceride (glycerol trioleate) and phospholipid (lecithin). Similar aliquots of the same serum, to which no other lipid was added, were also extracted. The concentrations of free and esterified cholesterol (Sperry and Webb 1950), triglyceride (Young and Eastman 1963) and phospholipid (Bartlett 1959) were determined in both extracts and the percentage recovery calculated (Table 3).

Lipid Fraction	mg. in original extract	mg. added	mg. recovered	% recovery
Free cholesterol	0.95	3.00	2.99	99.7
	0.95	2.00	2.04	102.0
	0.95	1.00	0.95	95.0
Esterified cholesterol	4.95	2.96	2.93	99.0
	4.95	2.37	2.39	100.8
	4.95	1.78	1.77	99.4
Triglyceride	2.37	1.00	0.99 (6)	99.6
	2.37	2.00	2.00 (5)	100.3
	2.37	2.50	2.47	98.8
Phospholipid	5.25	1.00	1.00	100.0
	5.25	1.50	1.44	95.9
	5.25	2.00	1.87	100.7

Table 3: The percentage recovery of lipid fractions during the extraction of serum lipids in 3:1 ethanol:ether.

The percentage recovery for a range of lipid concentrations varied from 95.0 to 102.0% for free cholesterol, from 99.0 to 100.8% for esterified cholesterol, from 98.8 to 100.3% for triglyceride and from 95.9 to 100.7% for phospholipid. Satisfactory extraction of these lipids was thus obtained with 3:1 ethanol:ether. This is in agreement with the findings of other authors (Bjorntorp 1960; Sperry 1955). Bjorntorp (1960) has shown that a 3 :1 ethanol:ether extract contains non-lipid components which are removed partly by re-extraction with chloroform. A similar effect was noted here since the total lipid determined by weight invariably exceeded that based on chemical determination of the constituent lipid components (lvi). The only non-lipid component which may interfere with the chemical estimations is non-lipid phosphorus. While certain methods, e.g. that of Folch et al (1951) involve removal of non-lipid components by washing the extract with water, this procedure was not favoured here in view of the solubility in water of certain phospholipids, notably lysolecithin and inositol phosphatide (Hanahan et al 1954, 1958). Methods with and without washing with water both have limitations. However, no significant difference in phospholipid concentration was found when comparing serum extracted with 3:1 ethanol:ether and according to the method of Folch et al (1951).

ii) <u>The reproducibility of the extraction procedure</u>.

The reproducibility of the extraction procedure was examined by extracting ten aliquots of a serum sample in 3:1 ethanol:ether and determining the concentration of free and esterified cholesterol, triglyceride and phospholipid in each extract. The results are shown in Table 4.

	Free Cholesterol	Esterified Cholesterol	Triglyceride	Phospholipid
Mean	47.3	143.6	79.2	173.2
S.D.	1.9	4.5	2.8	5.8
C.V.%	4.1	3.1	3.5	3.3

Table 4: The concentration (mg./100 ml. serum) of free cholesterol, esterified cholesterol, triglyceride and phospholipid as determined on ten aliquots of a single serum sample.

The extraction procedure is seen to give a satisfactory degree of reproducibility. Since it gave good recoveries of the lipid fractions analysed and a high degree of reproducibility this extraction procedure was used throughout the study.

(b) ISOLATION AND EXTRACTION OF β-LIPOPROTEIN IN SERUM

i) Definition of term 'lipoprotein'

The presence of lipoprotein in serum was first demonstrated in 1929 by Macheboeuf (1929) who isolated a conjugated protein which appeared, upon repeated precipitation, to retain a constant ratio of lipid to protein. Using the technique of electrophoresis developed by Tiselius (1939), Blix and his co-workers (Blix 1941; Blix et al 1941) found that most of the serum lipids were found in combination with the α- or β-globulin fractions, hence the term α- and β-lipoprotein. Today the term lipoprotein is used to denote a complex formation of lipid and protein. The exact form in which lipid is bound to protein is not clearly established. However, on the basis of work by Gould (1951) Oliver and Boyd (1958) have suggested that the lipids are held to the peptide by secondary valence bonds of the van der Waals form.

ii) Methods available for isolation of β -lipoprotein.

The isolation and characterization of lipoprotein depends on exploiting various properties of the protein moiety of the complex. These properties include electrophoretic mobility, solubility and density and form the basis of separation of lipoproteins by electrophoresis on paper (Kunkel and Tiselius 1951; Fasoli 1953; Boyd 1954; Jencks et al 1955; Rosenberg 1952), and starch (Kunkel and Slater 1952; Ackerman et al 1954) fractionation with cold ethanol (Cohn fractionation) (Cohn 1950) and ultra-centrifugation (Gofman et al 1949; Havel et al 1955; Green et al 1951) respectively. More recently it has been shown that under certain conditions determined by pH, ionic strength or the presence of metal ions such as Ca or Mg, serum β -lipoproteins react with sulphated polysaccharides such as heparin and dextran sulphate to form insoluble complexes (Burstein and Samaille 1956; 1958a; Bernfield and Nisselbaum 1956; Oncley et al 1957; Antoniades et al 1958; Boyle et al 1959). The mechanism underlying this reaction, thought to involve the formation of a lipoprotein-polyanion complex through the action of electrostatic forces has been studied by Bernfield and his co-workers (Bernfield et al 1957, 1960). The nature of and the specificity of formation of lipoprotein-sulphated polysaccharide complexes has been reviewed by Cornwell and Kruger (1961).

There are thus four main methods of isolating β -lipoprotein viz.: electrophoresis, Cohn fractionation (Cohn et al 1950) ultracentrifugation and precipitation with sulphated polysaccharides. In deciding on the method for use in this study certain factors had to be considered. Ultracentrifugation was not possible since at the commencement of this study an ultracentrifuge was not available. Cohn fractionation, which can be used to separate the lipoproteins in large volumes of serum, requires facilities for operation at $-5^{\circ}C$, which again were not readily available.

Using electrophoretic techniques, the distribution of lipids in the lipoprotein fractions can be determined either by staining techniques (Flynn and de Mayo 1951; Durrum et al 1952; Swahn 1952; Rosenberg et al 1954) or by chemical analysis of eluates (Boyd 1954; Ackerman et al 1954; Langan et al 1955), but these methods are subject to fairly appreciable error, and are suitable only for small volumes (up to 0.2 ml. of serum). Moreover, even using a starch block, the volume of serum which could be separated into lipoprotein fractions by electrophoresis is not large and would not readily allow for detailed quantitative estimations and fatty acid analysis. The method adopted was the precipitation of β-lipoprotein, using the sulphated polysaccharide, heparin, in the presence of $CaCl_2$ (Burstein and Samaille 1956). Of the available methods, this was chosen as it did not require the use of special apparatus and was suitable for large volumes of serum.

iii) β-lipoprotein precipitation technique.

The method of isolation of β-lipoprotein from serum, adapted from that described by Burstein and Samaille (1956) was performed in the following manner:-

30.0 ml. of 0.025 M $CaCl_2$ was added to 3.0 ml. of serum in a centrifuge tube. To this was added 0.6 ml. of 1% heparin (1.0 ml. of heparin containing 25,000 I.U. per ml. diluted to 25.0 ml. with distilled water). A cloudy precipitate formed immediately. After standing for one hour this was centrifuged at 3,000 r.p.m. for 30 minutes. The supernatant was removed by suction using an Emich filter stick. A small "button" of precipitate remained in the centrifuge tube.

The lipid in the precipitated β-lipoprotein was extracted using 3:1 ethanol:ether. Fifty ml. of 3:1 ethanol:ether was added to the precipitate in the tube and the Emich filter stick rinsed several times into the same tube,

using the extraction mixture. The extract was warmed in a water bath (60°C), with constant stirring, filtered and the final volume adjusted to 50 ml. in a volumetric flask.

iv) Comparison with paper electrophoresis.

In order to ascertain that the method used is specific for β-lipoprotein, it has been compared with results obtained using a paper electrophoretic technique.

The paper electrophoretic procedure was identical to that described by Anderson and Keys (1956), using a Veronal buffer (pH 8,6) and applying a D.C. potential of 185 V for 15 hours. 0.1 ml. of serum was electrophoresed in duplicate and a blank strip and marker strip for staining were included in each run. The marker strip was stained with Sudan Black B (Swahn 1953) the position of β-lipoprotein determined and the papers from sample and blank runs cut as described by Anderson and Keys (1956). A typical strip stained with Sudan Black B is shown in Figure 1. The extraction of lipid from the β-lipoprotein on the paper was performed in the following way :-
The section of paper (approximately 2 inches square) was cut out and placed in a 30 ml. capacity test tube and extracted three times by refluxing with 8 ml. of 3:1 ethanol:ether for 15 minutes. The extracts were combined and evaporated to dryness in a test tube fitted with a B14 ground glass joint, prior to chemical estimation of cholesterol, phospholipid and triglyceride. The paper blank was extracted in the same way. Further extraction of the paper by refluxing for one hour with 20 ml. 3:1 ethanol:ether in a Soxhlet apparatus yielded no further lipid as determined chemically, indicating that complete extraction was obtained with the method used. Other authors have reported complete extraction of lipid from paper using similar volumes of 2:1 chloro-

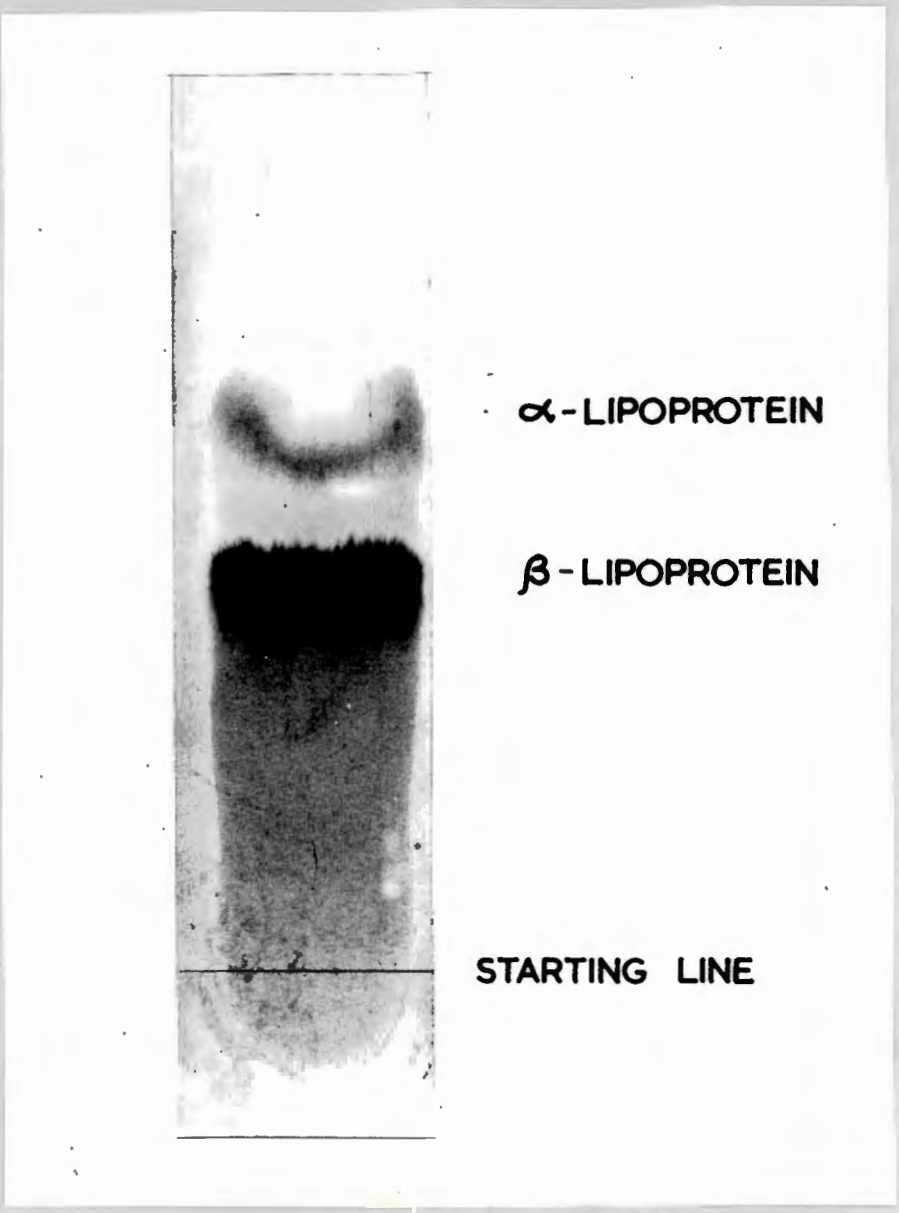


Figure 1. The separation of α- and β-lipoprotein by electrophoresis. A typical electrophoresis paper stained with Sudan Black B.

form : methanol (Langan et al 1955) and 1:1 acetone:alcohol (Boyd 1954). The concentration of total and free cholesterol (Sperry and Webb 1950) and phospholipid (Bartlett 1959) was determined in β-lipoprotein isolated from twenty different sera using the paper electrophoretic technique and the heparin-$CaCl_2$ precipitation technique. The results obtained by the two methods were compared by calculation of the correlation coefficient (Table 5 and Figure 2).

Lipid	r	p	Regression equation	Range of Values (mg/100 ml. serum)
Total Cholesterol	0.9914	<0.001	Y=0.9956x + 0.33	39 - 286
Free Cholesterol	0.9927	<0.001	Y=0.9940x + 0.13	15 - 94
Phospholipid	0.9515	<0.001	Y=0.8545x + 12.10	79 - 209

Table 5: Comparison of the concentration of cholesterol and of phospholipid in β-lipoprotein isolated from serum by paper electrophoresis (y) and heparin precipitation (x).
r = correlation coefficient.

As seen from the data in Table 5 and Figure 2, there was a very high degree of correlation between the two methods. Both total and free cholesterol and phospholipid values were very similar although there was a tendency for the phospholipid values from electrophoretically separated lipoproteins to be slightly lower. This trend was, however, neither very consistent, nor very marked (Figure 2).

In attempting to correlate the triglyceride concentration in β-lipoprotein isolated by paper electrophoresis and heparin precipitation, technical difficulties were encountered. In the triglyceride method (Young and Eastman 1963) the electrophoresis paper blanks gave very high readings (up to twice

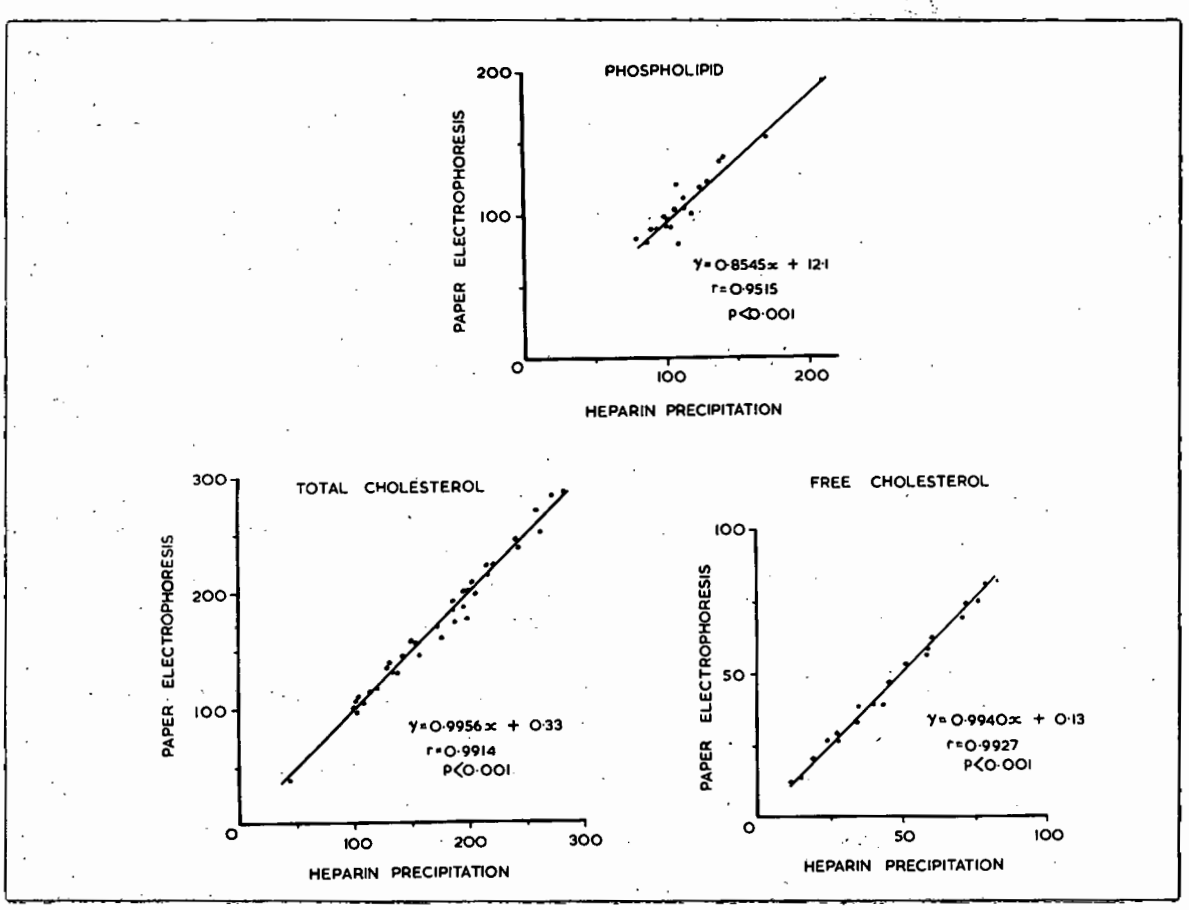


Figure 2: Correlation of the concentrations of total cholesterol, free cholesterol and phospholipid in β-lipoprotein isolated by paper electrophoresis and by the heparin precipitation technique.

that found for 50 μgm. of triolein) and the readings for samples were also extremely high. Moreover, the values for the paper blanks, although always high, were not consistently of the same order and it was felt that subtraction of such blank values from sample readings would introduce considerable error. It was thought that these high blank readings could be due either to the presence of lipid in the paper or to contamination in handling. However, washing the paper with 3:1 ethanol:ether prior to electrophoresis and exercising great care in handling the papers only with solvent-washed instruments did not reduce the blank values to any extent. In addition, it was found that the veronal buffer, containing sodium acetate and sodium barbiturate also gave high blank readings in this triglyceride method. The 3:1 ethanol:ether extract of lipid from the papers contained appreciable amounts of buffer. This could be removed by adding distilled water to the dried extract and re-extracting with petroleum ether. In this way the buffer remained in the water phase. While the blank values decreased somewhat after applying this step, they were still high and the number of transfers involved in this procedure greatly increased the error. It was thus not possible to verify the precipitation technique with respect to triglyceride, using this method of comparison.

The implications of the failure to correlate the two methods with regard to triglyceride concentration were considered. The possibility existed that the β-lipoprotein isolated by precipitation could be contaminated either with α-lipoprotein or chylomicrons. α-lipoprotein lipid consists of approximately 15% triglyceride, 45% phospholipid and 40% cholesterol (Olson and Vester 1960). If, therefore, there was contamination with α-lipoprotein, the concentrations of cholesterol and phospholipid would be considerably higher in β-lipoprotein isolated by precipitation than in that isolated by paper electrophoresis. This

was not the case. In addition, electrophoresis of the β-lipoprotein precipitate, with subsequent staining of the paper for lipid, showed no trace of α-lipoprotein. Thus for cholesterol and phospholipid the precipitation technique is an effective method of separating α- from β-lipoprotein. This is therefore probably true for triglyceride as well. These methods, particularly the staining technique, may not be sufficiently sensitive to detect very small quantities of α-lipoprotein lipid. However, in view of the low percentage of triglyceride in α-lipoprotein, any contamination which might occur would result in only a very small increase in β-lipoprotein triglyceride concentration. Burstein and Samaille (1957, 1958b) have shown that this method is specific for β-lipoprotein and there is no evidence in this study to suggest that the isolated β-lipoprotein is contaminated with α-lipoprotein.

Contamination of the β-lipoprotein precipitate with chylomicrons is likely to occur. While Burstein and Samaille (1958a) have shown that the precipitation technique is specific for β-lipoprotein in non-lipaemic serum, they have reported that, in chylomicron rich serum, chylomicrons may also be precipitated (Burstein and Samaille 1960). In this study all blood samples were taken from subjects who had fasted overnight. All sera would, therefore, presumably be non-lipaemic and contain few, if any, chylomicrons. Contamination of the β-lipoprotein precipitate with chylomicrons would thus be unlikely to occur to any extent. While this reasoning had for the moment to suffice, it did not provide proof.

v) Comparison with ultracentrifugation.

Fortunately at this stage an ultracentrifuge was made available for a limited period. Using this apparatus it was possible to determine whether any chylomicrons in a non-lipaemic serum would be precipitated using heparin and $CaCl_2$.

This was done in the following way :-

An aliquot of fasting serum was centrifuged in a Spinco Model L ultracentrifuge using a 40.3 rotor at 10,000 r.p.m. (7,000 x g.) for 30 minutes. Under these conditions chylomicrons from lipaemic serum were found to be removed as judged by dark-field microscopy. The conditions chosen for ultracentrifugation were deliberately mild in order to avoid any separation of low density lipoproteins. After ultracentrifugation any chylomicrons present formed a greasy film at the surface of the serum. The tube was punctured at the bottom and the clear subnatant withdrawn, taking care that the chylomicron film remained in the tube. The heparin precipitation technique was used to isolate the β-lipoprotein from an aliquot of the subnatant and from the unaltered parent serum. Triglyceride was chemically estimated on the two lipid extracts and the values compared. Eleven fasting sera were examined in this way. The values are shown in Table 6.

	1	2	3	4	5	6	7	8	9	10	11
Serum	31.1	166.9	71.8	72.5	75.2	21.5	30.1	92.1	32.7	97.3	71.5
Subnatant	35.9	170.1	76.2	70.9	79.2	23.7	34.8	89.7	37.3	103.8	68.9

Table 6: The concentration of triglyceride (mg./100 ml. serum) in β-lipoprotein isolated by the heparin precipitation technique from fasting serum and from serum from which the chylomicrons had been removed by ultracentrifugation (Subnatant).

The data in Table 6 show that within the limits of experimental error, the triglyceride concentrations agree well for β-lipoprotein isolated from fasting serum and from the same serum with the chylomicrons removed by ultracentrifugation. This was true for β-lipoprotein triglyceride concentrations

ranging from 21.5 to 170.1 mg./100 ml. which almost covers the range found for subjects included in this study. Several lipaemic sera were also analysed in this way. The triglyceride values were consistently higher in the unaltered lipaemic serum, indicating that chylomicrons were precipitated in addition to β-lipoprotein. This demonstrated that it is only in non-lipaemic serum that chylomicrons are not precipitated with the β-lipopreteins. All sera analysed in this study were non-lipaemic. On the basis of the ultracentrifugal data and the correlation found between the paper electrophoretic and precipitation techniques for cholesterol and phospholipid estimations, (Table 5, Figure 2) it may be concluded that the β-lipoprotein triglyceride isolated in this study was not significantly contaminated either by α-lipoprotein or by chylomicrons.

Several authors have compared the method of separation of β-lipoprotein by precipitation using various sulphated polysaccharides with the methods of ultracentrifugation (Scanu et al 1958; Oncley et al 1957; Antoniades et al 1958; Florsheim and Gonzales 1960; Kritchevsky et al 1963), Cohn fractionation (Oncley et al 1957) and electrophoresis on paper (Oncley et al 1957; Antoniades et al 1958) and on starch (Oncley et al 1957). They have reported good agreement for lipid determinations on β-lipoprotein separated by the precipitation technique and by the other methods. The β-lipoprotein isolated by precipitation with sulphated polysaccharides (e.g. heparin) is generally accepted as corresponding closely to the electrophoretically separated β-lipoprotein, to the Cohn method fractions I and III_0 and to the ultracentrifugally separated fraction of density less than 1.063 and Standard flotation rate $S_f 0\text{-}400$ (Cornwell and Kruger 1961; Pesold et al 1957; Burstein 1961).

vi) <u>Reproducibility of the precipitation technique</u>.

The β-lipoprotein from ten aliquots of a serum sample was isolated by precipitation with heparin and $CaCl_2$. The lipids were extracted with 3:1

ethanol:ether and the concentration of free and esterified cholesterol, triglyceride and phospholipid in each extract was determined chemically. The mean concentration, one standard deviation (S.D.) and coefficient of variation as a percentage (C.V.%) were calculated for each component (Table 7).

	Free Cholesterol	Esterified Cholesterol	Triglyceride	Phospholipid
Mean	40.7	120.3	65.4	110.6
S.D.	1.5	3.8	2.4	3.5
C.V.%	3.8	3.2	3.6	3.2

Table 7: The concentration (mg./100 ml.serum) of the free cholesterol, esterified cholesterol, triglyceride, and phospholipid as determined on β-lipoprotein isolated from ten aliquots of a single serum sample.

A high degree of reproducibility was found, the coefficient of variation for each component not exceeding 4%. The coefficient of variation for each component was similar to that found for lipid extracted from total serum (Table 4) and the results for total serum and β-lipoprotein can thus be compared.

(c) EXTRACTION OF LIPID FROM ARTERIAL ATHEROMATOUS PLAQUES

i) Characterization of plaque material.

Fresh samples of atheromatous artery material were obtained from subjects who had undergone aorto-iliac endarterectomy.

The degree and distribution of atherosclerosis varies considerably between individuals and even within the same vessel of one individual. Various authors

(Hirsch and Weinhouse 1943; Buck and Rossiter 1951; Böttcher et al 1958; Smith 1960) have classified the degree of atherosclerosis according to the pathological appearance of the intimal surface. Böttcher and his colleagues (Böttcher et al 1959, 1960b, 1961, 1962) have adopted the following system of classification of lesions, advocated by the World Health Organization (1958) :-

Stage 0 : No atherosclerotic lesions visible at a magnification of 10.

Stage I : Fatty streaks and/or spots present.

Stage II : Fibrous plaques and/or atheroma present.

Stage III : Lesions as above with additional complications, e.g. ulceration, necrosis, haemorrhage, thrombosis.

Microscopic examination of sections stained with eosin and haematoxylin and Verhoef's Stain for elastic tissue showed that the material analysed included the intima and little of the media of the arterial wall (Figure 3). From appearance to the naked eye all the intimal material analysed in this study would correspond to Stage III as described above.

ii) Extraction of lipid.

The fresh sample of atheromatous artery material was rinsed with normal saline and any adherent blood removed. All atheromatous material visible to the naked eye, was dissected from the surrounding tissue excluding as much media as possible. The excised plaque material was cut into small pieces, transferred to a mortar containing approximately 5 ml. of 3:1 ethanol:ether and ground finely with a pestle. The 3:1 ethanol:ether extract was transferred to a test tube and a further 5 ml. of extracting solution added to the residue in the mortar. The process of grinding and extraction was repeated five times, and the extracts combined. The finely ground residue in the mortar was transferred to a test tube and further extracted by refluxing for 15 minutes with

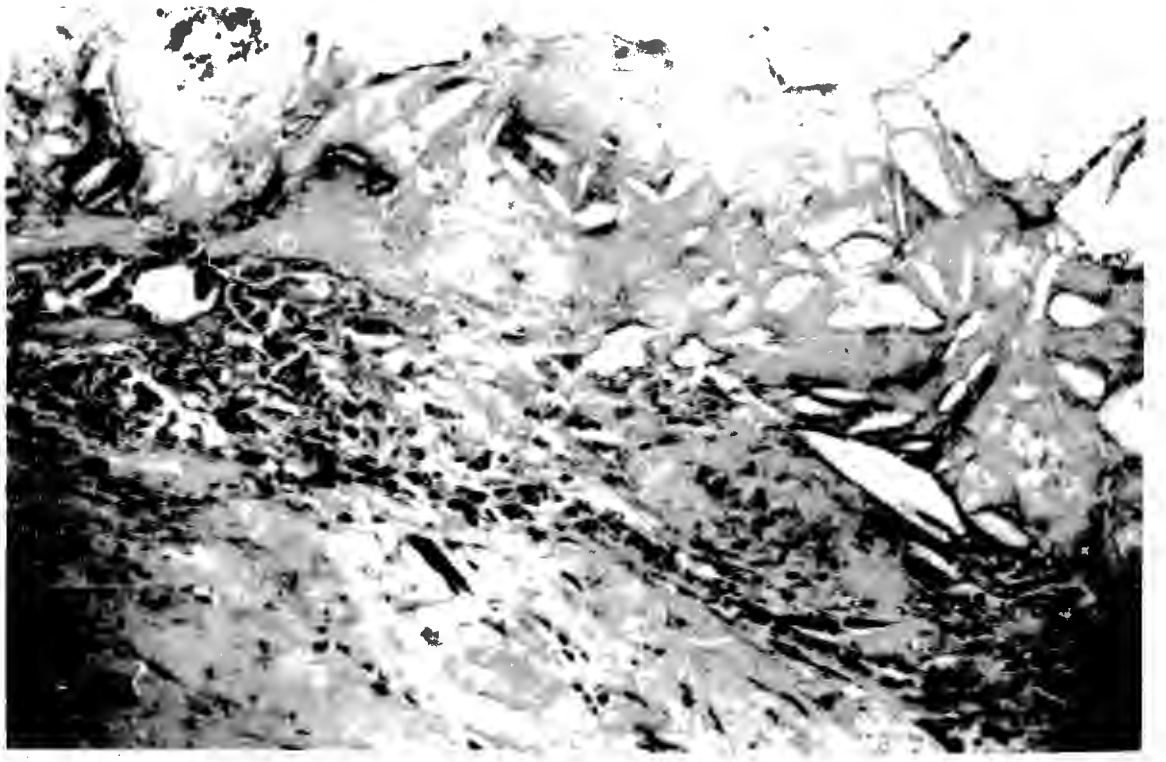


Figure 3. Section of intima of iliac artery showing extensive atheromatous debris with clear spaces where lipid has dissolved out in the preparation of the tissue. There is no attached media.
(Section stained with haematoxylin and eosin.
Magnification X 460).

20 ml. 3:1 ethanol:ether. All extracts were combined, filtered, and the final volume adjusted to 100 ml. in a volumetric flask.

The completeness of this extraction procedure was examined by further extracting the residue on the filter paper by refluxing in a Soxhlet apparatus with 30 ml. 3:1 ethanol:ether for one hour and then with 30 ml. 2:1 chloroform: methanol for one hour. Neither the 3:1 ethanol:ether nor the 2:1 chloroform: methanol extract contained any chemically detectable lipid. Complete extraction of lipid was thus effected by the method used. Since it is not possible to obtain two segments of plaque material with an identical content of lipids, recovery of added lipids could not be formally tested.

III. THE ANALYSIS OF FATTY ACIDS

In order to examine the fatty acid patterns associated with esterified lipid fractions, viz. cholesterol ester, triglyceride and phospholipid, the separation of these lipid classes must first be effected. This was performed by the technique of silicic acid column chromatography.

(a) SILICIC ACID COLUMN CHROMATOGRAPHY

Aliquots from total lipid extracts of total serum, β-lipoprotein and plaques, containing 5 - 10 mg. of total lipid were fractionated using the silicic acid column chromatography method of Macdonald (1962) which is based on the method of Barron and Hanahan (1958). On the advice of Antonis (1961) the solvent used for elution of phospholipids was methanol containing 4% water instead of 20% (v/v) chloroform in methanol used by Macdonald. The presence of the water ensures the elution of water soluble phospholipids. The elution system applied is shown in Table 8.

Fraction	Lipid	Eluant	Volume of eluant
I	Cholesterol ester	15% (v/v benzene in petroleum ether (B.Pt. 40 - 60°C)	75 ml.
II	Triglyceride (and di- and mono-glycerides, non-esterified fatty acids, free cholesterol).	Chloroform	100 ml.
III	Phospholipid	4% (v/v) distilled water in methanol	75 ml.

Table 8: The elution system used for separation of lipid fractions on a silicic acid column.

Fraction II, in addition to triglyceride, contains free cholesterol, mono- and di-glycerides and non-esterified fatty acids (N.E.F.A.) (MacDonald 1962). In fasting serum, mono- and di-glycerides constitute about 10% of the total glycerides (Eastman and Bronte-Stewart 1962). The fatty acid compositions of these fractions are similar to that of triglycerides (Young 1964). The concentration of N.E.F.A. in fasting serum is about 0.05 m Eq/100 ml. (Rothlin and Bing 1961) which is equivalent to about 14 mg./100 ml. Thus, in fraction II, between about 10 and 20% of the total fatty acids are derived from the N.E.F.A. fraction; these non-esterified fatty acids therefore contribute in some measure to the fatty acid composition of fraction II. The fatty acid composition of N.E.F.A. is, however, not very different from that of isolated glycerides (Young 1964). While reference will be made to 'triglyceride' fatty acid composition, the complex nature of this fraction must be borne in mind. Free cholesterol does not give a peak under the conditions used for fatty acid analysis by G.L.C. in this study.

i) Separation of fractions.

In order to determine whether any overlap of the esterified fractions occurred, the definition of the three fractions separated on the silicic acid column was examined. Using a fraction collector, 5 ml. fractions of the eluates were collected and the concentrations of esterified cholesterol, triglyceride and phospholipid were determined on aliquots of these fractions as described in Section I. The results obtained on fractionating an extract of serum lipid in this way are shown in Figure 4. Three well-defined peaks corresponding to esterified cholesterol, triglyceride and phospholipid are evident. In addition, no overlap of fractions occurred.

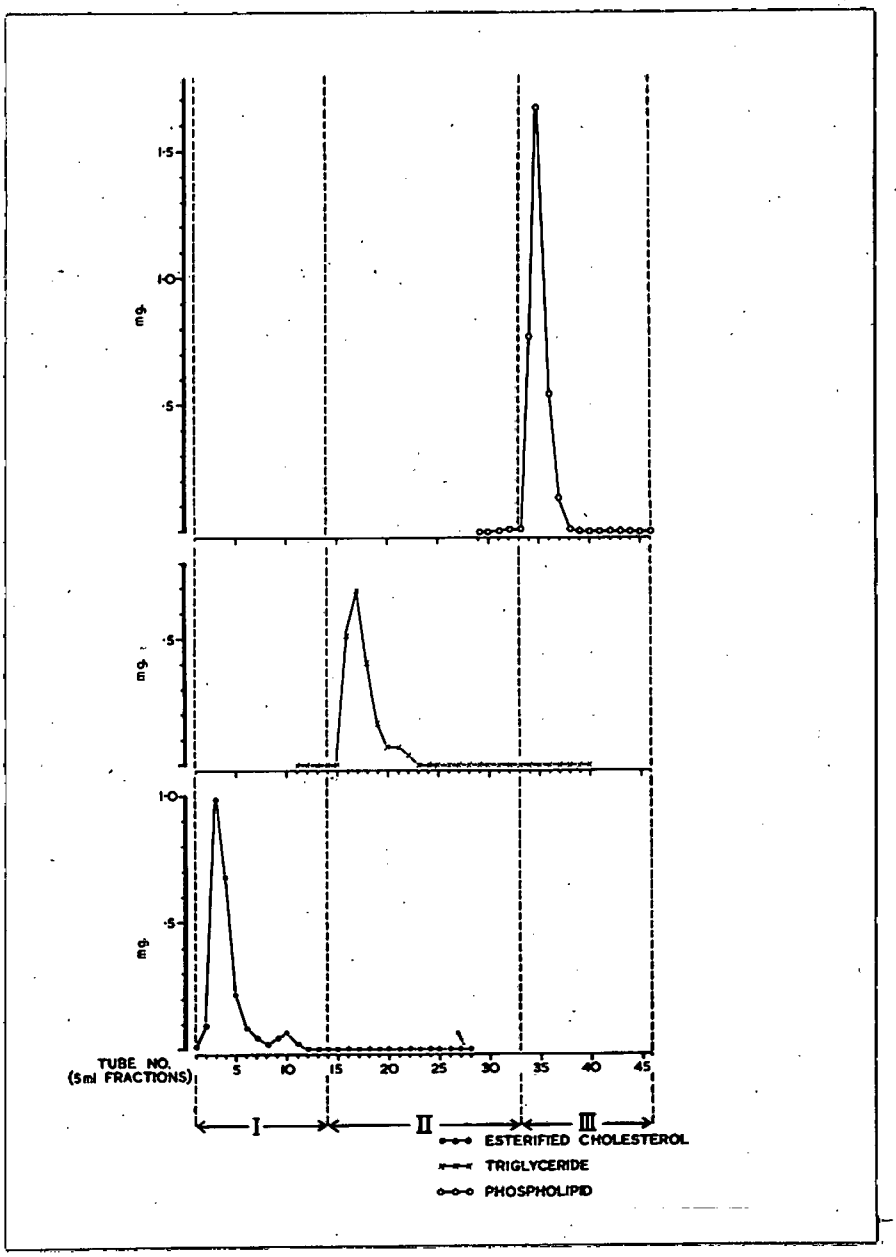


Figure 4: The separation of lipids by silicic acid column chromatography.

There is no overlap of the esterified fractions.

ii) Recovery of lipids.

In order to establish that all the lipid was eluted from the column, the recovery was examined in the following manner:

The concentrations of esterified cholesterol, triglyceride and phospholipid were chemically determined on aliquots of three serum lipid extracts prior to fractionation on the silicic acid column. Similar chemical determinations were performed on the appropriate eluates from the columns and the recovery calculated (Table 9). Aliquots of the three serum lipid extracts examined contained approximately 5 mg., 7 mg., and 9 mg. of total lipid respectively, which is within the range of the lipid loads applied in this study.

Lipid	Concentration in unfractionated aliquot	Concentration in eluted fraction		Recovery
	mg.	Fraction	mg.	%
Esterified cholesterol	1.200	I	1.215	101.3
	1.725	I	1.715	99.4
	2.012	I	2.020	100.4
Triglyceride	0.800	II	0.798	99.8
	1.264	II	1.287	101.8
	1.738	II	1.728	99.4
Phospholipid	2.002	III	2.016	100.7
	2.333	III	2.317	99.3
	3.151	III	3.159	100.3

Table 9: The percentage recovery of esterified cholesterol, triglyceride and phospholipid from silicic acid colums.

The data in Table 9 show that the recovery from the silicic acid columns varied from 99.4 to 101.3% for esterified cholesterol, from 99.4 to 101.8% for triglyceride and from 99.3 to 100.7% for the phospholipid. Satisfactory recovery of lipid from the silicic acid columns was thus achieved.

(b) METHYLATION

The technique used, based on a micromethod described by Stoffel et al (1959) was performed as follows:

The fraction eluted from the silicic acid column was concentrated by evaporating the solvent at 40°C under reduced pressure, using a rotary evaporator. The concentrated lipid extract was transferred to a test tube fitted with a B19 ground glass joint and the final traces of solvent removed by evaporation under nitrogen. 4.0 ml. of 5% (v/v) sulphuric acid in superdry methanol and 0.5 ml. benzene (dried over sodium) were added to the lipid. The mixture was refluxed in a silicone bath at 110 - 115°C for two hours. After cooling to room temperature two volumes of distilled water were added and the methyl esters extracted three times with 5.0 ml. of petroleum ether (B.Pt. 40 - 60°C). The combined extracts were simultaneously dried and neutralized over a sufficient quantity of 4:1 (w/w) sodium sulphate : sodium bicarbonate. The extract containing methyl esters was then transferred to a small conical tube and the solvent evaporated at room temperature under a stream of nitrogen, immediately prior to analysis by G.L.C.

During all the procedures involved in preparing samples for G.L.C., great care was exercised to avoid any possibility of oxidation occurring. Samples were not left to stand in a dried condition, but always contained solvent and air was expelled by nitrogen. All samples not being processed were stored at 4°C under nitrogen.

(c) THE TECHNIQUE OF G.L.C.

Analysis of fatty acids was performed using the technique of gas-liquid chromatography which was developed by James and Martin in 1952 and modified by them in 1956.

i) Apparatus

The apparatus used in this study was the Pye Argon chromatograph which has been modified to give an uninterrupted gas flow during the loading of a sample. This apparatus incorporates the Argon beta-ray ionisation detector developed by Lovelock (1958) and contains a Radium-D source. The detector signals are amplified by an electronic circuit and recorded by a Sunvic recorder which responds linearly with mass.

ii) Preparation of stationary (liquid) phase

The stationary (liquid) phase used throughout was the polyester of ethylene glycol adipate (E.G.A.) prepared according to the method of James (1959). The E.G.A. was coated on to a solid support, in this instance Chromosorb W, acid washed and size graded to 80 -100 mesh. Twenty percent (w/w) E.G.A. was coated on to the Chromosorb W by dissolving the E.G.A. in an excess of chloroform, adding the proportional amount of Chromosorb W with thorough mixing and evaporating the chloroform on a rotary evaporator. The E.G.A.-coated Chromosorb W was packed into a glass column which is a standard fitting for the chromatograph used (Column length 4 feet; internal diameter 4 mm; external diameter 6 mm.). The constricted end of the column was plugged with a small quantity of teased-out glass fibre and the coated Chromosorb W poured into the column and packed with gentle tapping to a height of about 3 ft. 8 ins. About 1 inch of uncoated Chromosorb W was packed above this and a plug of glass fibre placed on top of it. The column was conditioned by "baking" for 24 - 48 hours at 200°C in an auxiliary heating jacket with argon (gas phase) flowing under a pressure of 20 pounds/square inch. This conditioning process ensures the removal of any remaining traces of chloroform and also allows any unadsorbed liquid phase to "bleed"off. Since this "bleeding" of the stationary phase on initial heating

causes temporary base line instability, it is essential that the conditioning process continues until the base line of the recorder settles to a constant level before starting an analytical run.

iii) Operating conditions

The conditioned column was used at an operating temperature of 184°C with an argon pressure of from 10 - 15 lbs./sq.in. giving gas flow rates of 40 to 60 ml. per minute. The detector voltage was maintained constant at 1000 volts. The degree of amplification (which is controlled by means of a built-in sensitivity switch with a ratio of 1:3:10, corresponding to the positions X10, X3 and X1 respectively on the instrument panel) was adjusted according to the time of elution of the components. In general, from the time of injection to elution of C18:2 was recorded on the lowest sensitivity (X10), from elution of C18:2 to elution of C20:4 on higher sensitivity (X3) and beyond elution of C20:4 on the highest sensitivity (X1). The speed of the chart in the recorder was 15 inches per hour. The operating temperature of 184°C was chosen since it is similar to that reported in another study using the same stationary phase (Farquhar et al 1959). It was thus possible to compare relative retention times, which proved helpful in the identification of unknown peaks.

The gas pressure was adjusted so as to give good separation between components. The detector voltage (1000 V) gave a satisfactory response with a minimum of base line "noise". The sensitivity was adjusted to give maximum peak height which makes for greater accuracy in calculating peak areas. Similarly, it was found that the chart speed used resulted in narrower peaks than those obtained with a faster chart speed. This was particularly important for calculation of the long chain fatty acids present in the phospholipid fraction.

(d) STANDARDISATION AND CALIBRATION OF APPARATUS

i) Linearity of response

While the detector and amplifier-recorder systems in the apparatus are designed to give linearity of response, it is essential to test whether the ionization current recorded on the moving chart is directly proportional to the amount of fatty acid methyl ester applied to and eluted from the column under the conditions of operation. Certain mixtures of pure methyl esters of fatty acids have been devised especially for assessing this parameter of apparatus performance. These standards measure the linearity of response when there is a wide variation in peak heights. Before commencing the analysis and at frequent intervals during the course of this study standard mixtures were run on the apparatus to verify that linearity of response was being maintained. It was also checked by means of a suitable standard mixture that the calculated proportions of the fatty acid components in the mixture reflect its composition by weight. All the above fatty acid methyl ester standards were obtained from the Metabolism Study Section, National Institute of Health, U.S.A.

The results of repeated analysis of a standard mixture are shown in Table 10.

	% by weight	% by G.L.C.	
		Mean	S.D.
C14:0	11.82 (9)	11.89	(0.35)
C16:0	23.61 (6)	22.96	(0.49)
C16:1	6.84 (3)	6.85	(0.23)
C18:0	13.08 (9)	13.00	(0.42)
C18:1	44.62 (3)	45.29	(0.81)

Table 10: The percentage composition of a standard mixture as given by weight and determined by G.L.C. The mean and standard deviation for 10 gas chromatographic analyses are shown.

The response of the apparatus under the operating condition can thus be accepted as being linear to the concentration, by weight, of the fatty acid methyl esters.

ii) Resolving power of column

The efficiency of separation can be determined on the basis of the number of theoretical plates in the columns. This is calculated according to the formula (Farquhar et al 1959):-

$$n = 16\left(\frac{tR}{W}\right)^2$$ where

n = number of theoretical plates

tR = retention time (or distance)

W = base width of component in the same units as tR.

In this study, theoretical plates were measured at C18:0 and the average value was approximately 1600 theoretical plates, which is somewhat lower than the value of 2000 advocated for maximum efficiency (Farquhar et al 1959). However, it has been suggested that it may be more meaningful in evaluating the efficiency of polyester columns, to measure their resolution of C18:0 from C18:1 and of C16:0 from C16:1, rather than to calculate only the theoretical plates of the column (Metabolism Study Section). Resolution is calculated according to the formula (Farquhar et al 1959) :-

$$\text{Component resolution} = \frac{2\triangle Y}{Ya + Yb}$$

where $\triangle Y$ = distance between any two peak maxima

Ya and Yb = base widths of components a and b.

A value greater than 1 indicates complete resolution (Metabolism Study Section). The average value for C18:0/C18:1 was 1.05 and for C16:0/C16:1, 1.07, indicating that satisfactory resolution was obtained (Figure 5). When the degree of separation between these peaks fell below 1 the column was replaced.

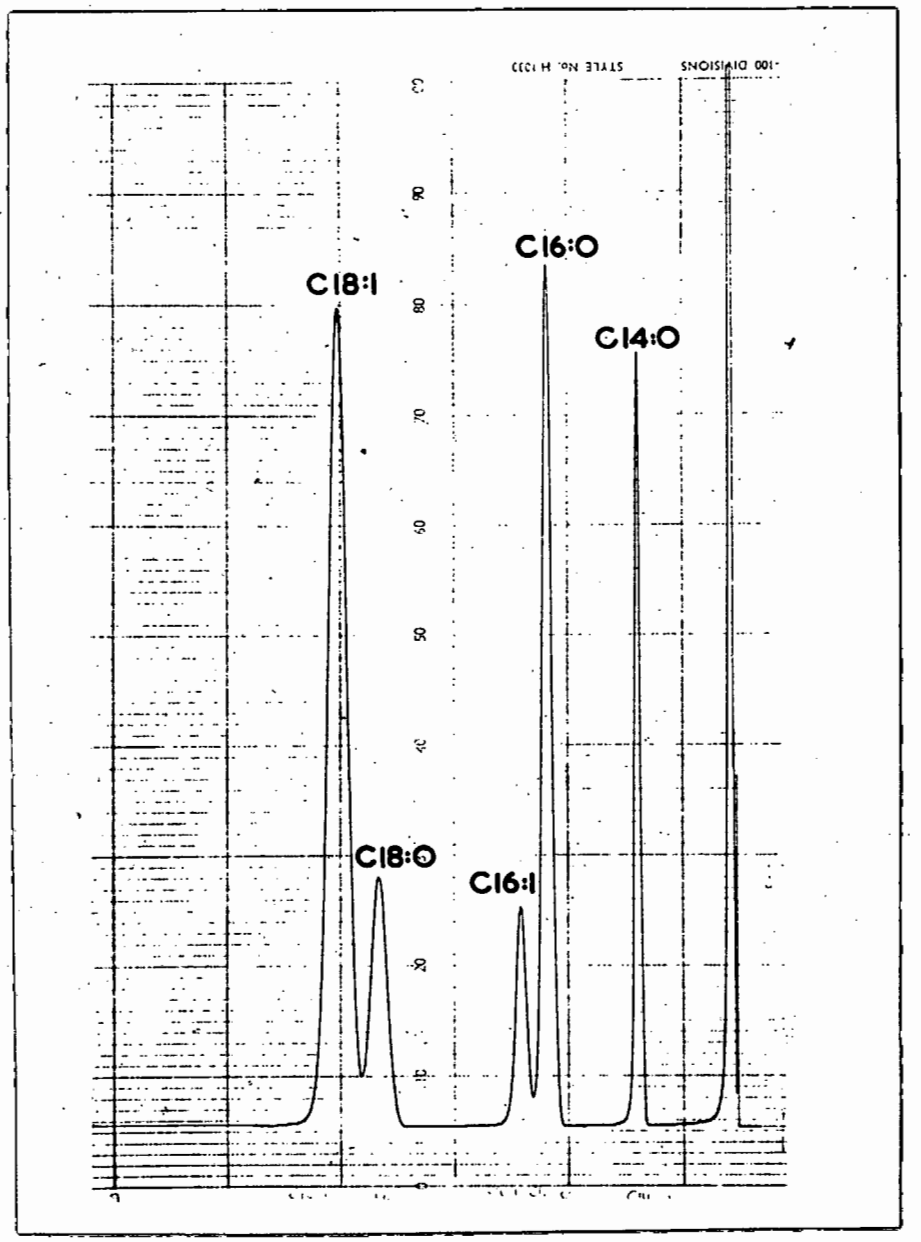


Figure 5: A typical chromatogram of a standard mixture of fatty acid methyl esters showing the degree of separation between components.

(e) LOADING OF SAMPLE

The fatty acid methyl esters, dissolved in a small volume ($\pm$ 0.1 ml.) of petroleum ether (boiling point 40 - 60°C) were contained in a tapered test tube fitted with a B10 ground glass joint. From 0.1 µl. to 0.5 µl., depending on the concentration of the sample, was taken up in a Hamilton micro-syringe and injected through the silicone rubber seal at the top of the glass column.

(f) CALCULATION OF PERCENTAGE COMPOSITION OF FATTY ACIDS

Since the recording system used draws a differential curve, the areas of the peaks represent the relative proportions of the component fatty acid methyl esters. Areas under peaks can be measured by planimetry, by cutting out the curves and weighing the papers, by triangulation, or by automatic integration. The method of triangulation as described by Farquhar et al (1959) was applied here. The area under each peak was calculated (height X width at half height). The sum of the peak areas was obtained and each component expressed as a percentage of the total peak areas.

(g) IDENTIFICATION OF FATTY ACIDS

Identification of fatty acids in G.L.C. is based on the principle that a particular liquid phase will have specific retentive properties for each fatty acid methyl ester under given conditions of temperature and pressure. Factors which determine the degree of retention are the chain length (number of carbon atoms) of the fatty acid and whether the fatty acid is saturated or unsaturated. The degree of retention increases with increasing chain length of the saturated components. When a polar phase such as the polyester of E.G.A. is used, the methyl esters of unsaturated fatty acids emerge later than those of saturated fatty acids with the same chain length and increasing unsaturation causes a progressive increase in retention. In addition, it has been shown that

plotting of either log retention volume or log retention volume relative to a standard substance against the number of carbon atoms in the molecule, gives a straight line for members of an homologous series (James and Martin 1952). This applies to saturated and mono-, di-, tri-, etc. unsaturated fatty acids of an homologous series. Retention volume is obtained by multiplying the flow rate by the retention time. Since in any run the flow rate is constant, the retention volume is directly proportional to the retention time. The log retention time can thus be directly related to the number of carbon atoms in the molecule. In practice, the retention time is measured as the distance from the point of injection to the centre of the relevant peak (Farquhar et al 1959). In different runs the absolute retention times will vary with differences in gas flow rate. However, at a given temperature, the retention times of fatty acid methyl esters relative to one another are constant. Thus the relative retention time can be calculated for each component. This is commonly expressed as the retention time of the component relative to that of C18:0 (stearic acid) (Farquhar et al 1959).

Applying these principles it is possible to identify components by :

(1) Comparing their absolute and relative retention times under certain conditions with those of pure methyl esters of known fatty acids under the same conditions.

(2) Plotting log retention time or log relative retention time against the number of carbon atoms in the molecule for members of an homologous series.

Identification of the unsaturated components can, in addition, be verified by:

(3) bromination, and

(4) hydrogenation of the sample of natural methyl esters.

i) Bromination

During bromination, bromine is bound at the double bonds of the unsaturated fatty acid esters. This increases their molecular weight and they become virtually non-volatile at the temperature of operation (James and Martin 1956). The chromatogram of the brominated sample will thus only show the saturated components of the original sample. The method used in this study was that described by Farquhar et al (1959).

ii) Hydrogenation

On hydrogenating a sample of methyl esters, the unsaturated fatty acid esters are converted to their respective saturated compounds. By comparing the chromatograms before and after hydrogenation it can be established which components of the original sample were unsaturated. Moreover, the relative increase in the proportions of saturated components in the hydrogenated sample is helpful in establishing the chain length of the unsaturated components in the original sample. Samples of methyl esters were hydrogenated according to the method of Farquhar et al (1959).

In this study it was found that, with the exception of two components, all the fatty acids present in the cholesterol ester and triglyceride fractions were present in the phospholipid fraction, which in addition contained several longer chain fatty acids. Identification of the fatty acids in all three lipid fractions was, therefore, based primarily on the identification of these components in the phospholipid fraction, using the techniques mentioned.

(h) APPLICATION OF METHODS FOR IDENTIFICATION OF FATTY ACIDS

i) Comparison of absolute and relative retention times.

The absolute and relative retention times of the fatty acid components of the phospholipid fraction have been compared with those of pure fatty acid methyl esters mixed in known proportions by weight. In addition, the relative retention

times have been compared with the data of Farquhar et al (1959) who identified fatty acids separated on E.G.A. polyester at 184.5°C. The values are shown in Table 11 and the chromatograms of the standard fatty acid mixture and of the phospholipid fraction are shown in Figure 6.

Standard Fatty Acids			Phospholipid Fatty Acids				Data of Farquhar et al (1959)	
Fatty Acid	d	d/C18:0	Peak	d	d/Peak 9	Identification	d/C18:0	Identification
C14:0	2.16	0.280	1	2.15	0.279	C14:0	0.272	C14:0
			2	2.60	0.337	C14:1	0.316	C14:1
			3	3.00	0.390	C15:0	0.372	C15:0
			4	3.52	0.457	?	0.445	Unknown
C16:0	4.13	0.535	5	4.10	0.532	C16:0	0.521	C16:0
C16:1	4.73	0.612	6	4.70	0.610	C16:1	0.590	C16:1
			7	5.60	0.727	C17:0	0.715	C17:0
			8	6.40	0.831	?	0.805	Unknown
C18:0	7.72	1.00	9	7.70	1.00	C18:0	1.00	C18:0
C18:1	8.60	1.11	10	8.55	1.11	C18:1	1.12	C18:1
C18:2	10.36	1.34	11	10.28	1.34	C18:2	1.35	C18:2
			12	14.09	1.83	C20:0	1.89	C20:0
			13	20.00	2.59	C21:0	2.56	C21:0
			14	21.56	2.80	C20:3	2.88	C20:3
C20:4	23.80	3.08	15	23.75	3.08	C20:4	3.17	C20:4
			16	27.10	3.52	?	-	-
			17	30.09	3.91	C20:5	4.08	C20:5
			18	36.79	4.78	?	-	-
C24:0	50.40	6.53	19	50.28	6.53	C24:0	-	-
			20	54.65	7.18	?	-	-
			21	62.32	8.09	C22:6	8.59	C22:6

Table 11. Comparison of absolute and relative retention times for fatty acid components in the phospholipid fraction with those found for standard fatty acids and those given by Farquhar et al (1959).

d = absolute retention time measured as distance in cm.

d/C18:0 = relative retention time i.e. retention time (in cm.) relative to retention time of C18:0.

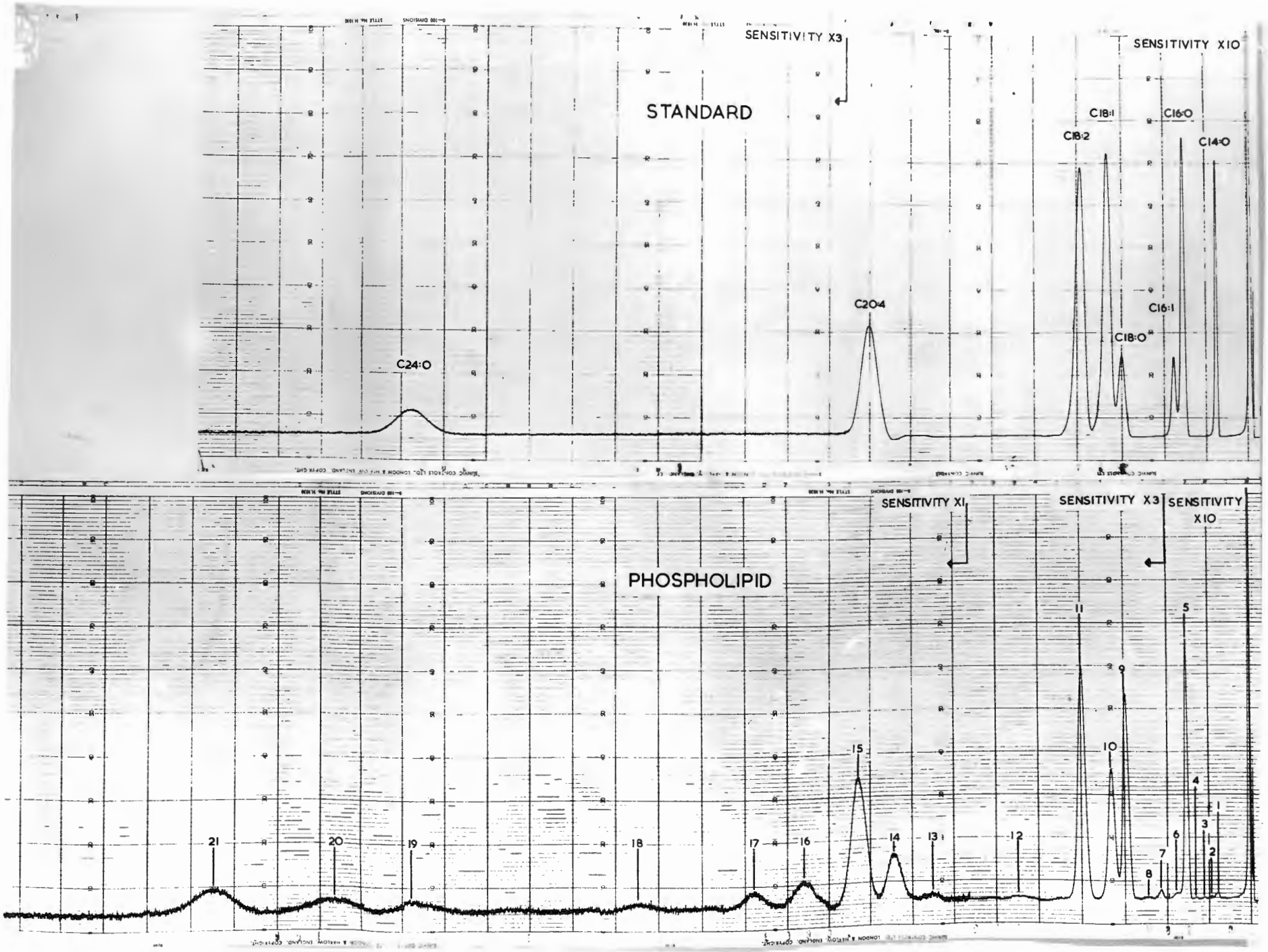


Figure 6: Typical chromatograms of a standard fatty acid methyl ester mixture and of the phospholipid fatty acid methyl esters from serum.

The data in Table 11 and Figure 6 show that the absolute and relative retention times of peaks 1,5,6,9,10,11,15 and 19 agree very closely with the values found for C14:0, C16:0, C16:1, C18:0, C18:1, C18:2, C20:4 and C24:0 respectively in the standard mixture and these peaks are thus identified as shown in Table 11.

The relative times shown by Farquhar et al (1959) are somewhat different from those found here. This may be due to the slight difference in operating temperature in the two studies. However, on the basis of the fairly close similarity in the values for the positively identified C14:0, C16:0, C16:1, C18:1, C18:2 and C20:4 fatty acids, the relative retention times for peaks 2,3,12,13,14,17 and 21 have been matched with those most closely approximating them in the data of Farquhar et al (1959) and these peaks have been tentatively identified as shown in Table 11. Since standards for these components were not available, they have been identified by the methods described below.

ii) Plotting of log retention time.

The log retention time expressed as the log distance (in cm.) has been plotted against the number of carbon atoms in the molecule (Figure 7). It is apparent from Figure 7 that the values for the peaks 1,5,9 and 19, identified as C14:0, C16:0, C18:0 and C24:0 respectively, fall on a straight line. In addition, the values for peaks 3,7,12,13,16 and 18 also fall on this straight line. These peaks thus correspond to the fatty acids C15:0, C17:0, C20:0, C21:0, C22:0 and C23:0 respectively and have been identified accordingly. The identification of C15:0, C17:0, C20:0 and C21:0 by this method confirms that proposed on the basis of comparison with the data of Farquhar et al (1959).

On plotting the distances of peaks 6 and 10, it was found that the values for peaks 2,4,8 and 20 also fell on this straight line (Figure 7). Peaks 6 and 10

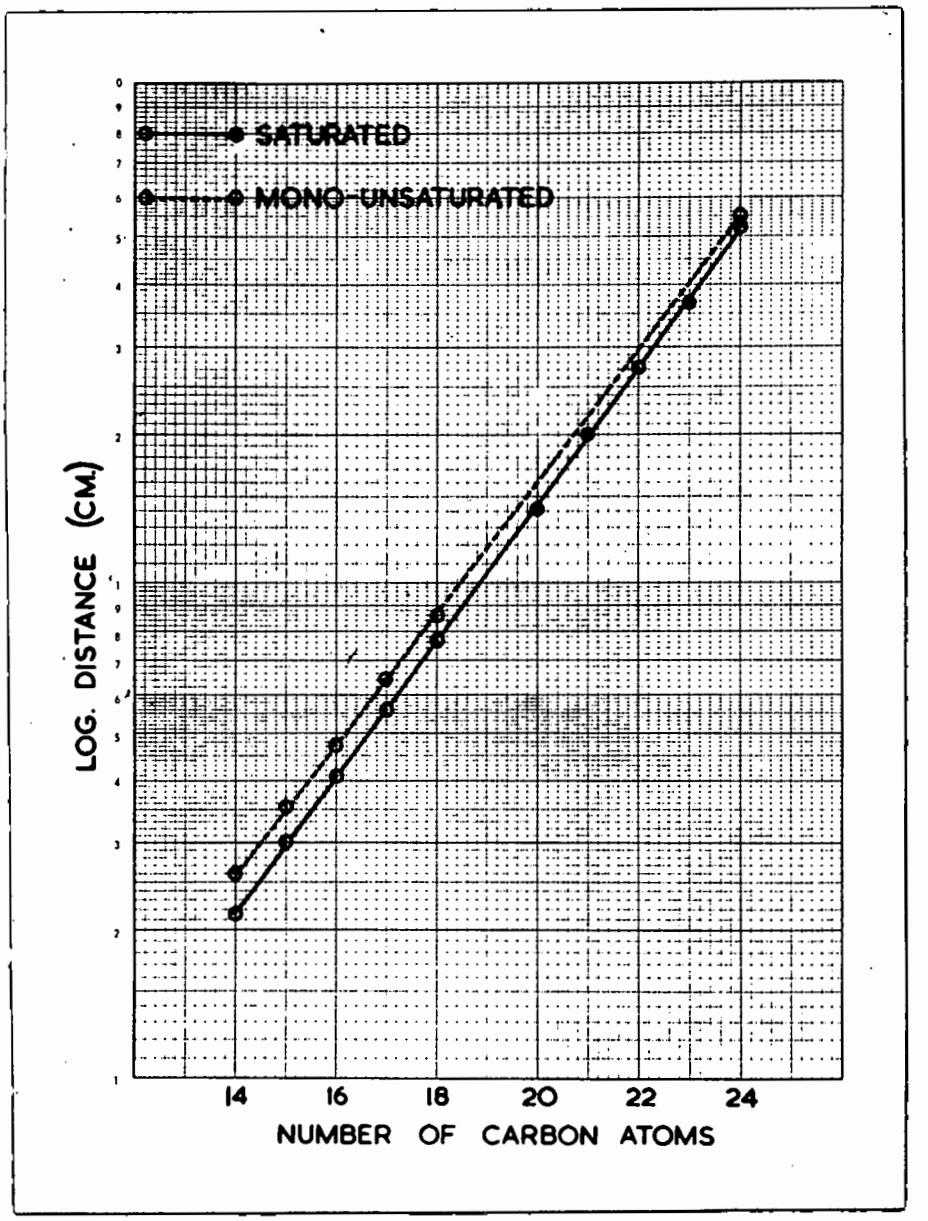


Figure 7: Plot of the log distance of the peaks shown in the chromatogram of serum phospholipid fatty acids (Figure 6) against the chain length of the components.

Note that the saturated fatty acids fall onto a straight line and the mono-unsaturated fatty acids fall onto a separate straight line.

have been identified as C16:1 and C18:1 respectively. The peaks 2,4,8 and 20 which thus fall on the mono-unsaturated fatty acid line have therefore been identified as C14:1, C15:1, C17:1 and C24:1 respectively. The polyunsaturated fatty acids which have been tentatively identified include only one trienoic acid (C20:3), one pentaenoic acid (C20:5) and one hexaenoic acid (C22:6). There are therefore not sufficient values to plot the log retention time against carbon chain length for any of these components.

iii) Bromination

The chromatograms of natural and brominated phospholipid fatty acid methyl esters are shown in Figure 8. While the absolute retention times differ slightly, due to differences in gas pressure, the relative retention times are similar since the column temperature remained constant. It can be seen that all the fatty acids which were identified in the natural sample as being unsaturated are no longer present in the brominated sample. Those fatty acids identified as being saturated are present in both samples. When the percentage composition of the natural sample is calculated so as to include only the saturated fatty acids, it shows close agreement with the percentage composition of the brominated sample (Table 12).

	C14:0	C15:0	C16:0	C17:0	C18:0	C20:0	C21:0	C22:0	C23:0	C24:0
Natural	0.78	0.43	59.34	0.70	27.05	1.60	0.30	4.35	2.33	3.13
Brominated	0.59	0.42	59.26	0.80	27.30	1.70	0.25	4.32	2.38	2.98

Table 12: Comparison of the percentage composition of a natural sample (including only saturated fatty acids) with that of a brominated sample.

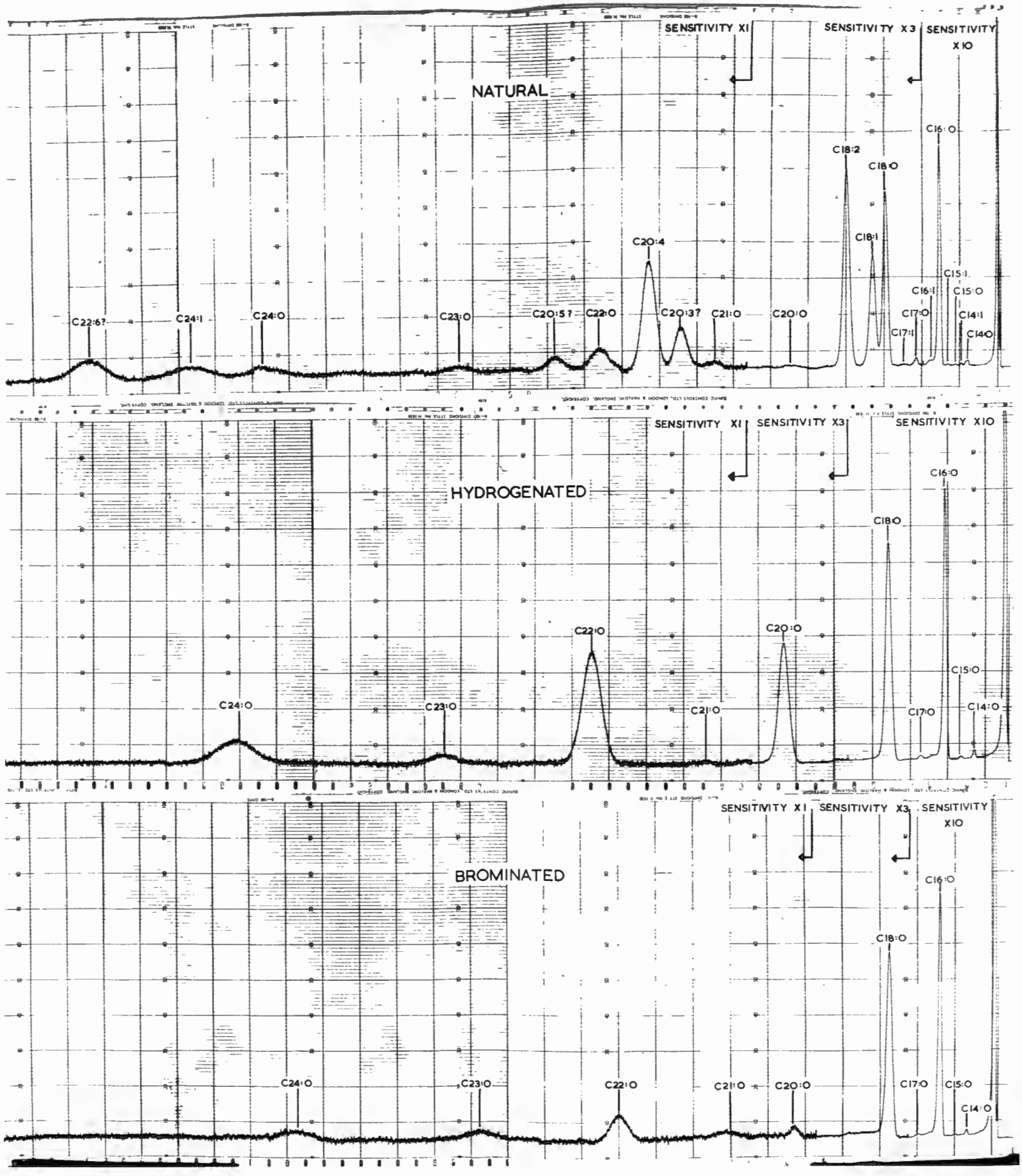


Figure 8:

Typical chromatograms of natural, hydrogenated and brominated fatty acid methyl esters of phospholipid isolated from serum.

Note that the unsaturated components are not present in the brominated or hydrogenated samples.

These data confirm the identification of saturated and unsaturated fatty acids and show that the components tentatively identified as C20:3, C20:5 and C22:6 are indeed unsaturated fatty acids.

iv) Hydrogenation

The chromatograms of the natural and hydrogenated samples are shown in Figure 8. Owing to a decreased flow rate the actual retention times are greater for the hydrogenated than for the natural sample. However, since the column temperature was the same in both runs, relative retention times are identical. Plotting log retention time or log relative retention time against chain length produces a straight line for the components of the hydrogenated sample (Figure 9). As in the brominated sample, all the unsaturated fatty acids have disappeared from the hydrogenated sample. Here, however, there is an increase in the proportions of the saturated fatty acids. Table 13 shows the percentage composition of the natural sample with the peaks identified as described. On the basis of this identification the percentage composition of the hydrogenated sample has been calculated (theoretical composition) and this has been compared with the determined composition of the hydrogenated sample (Table 13).

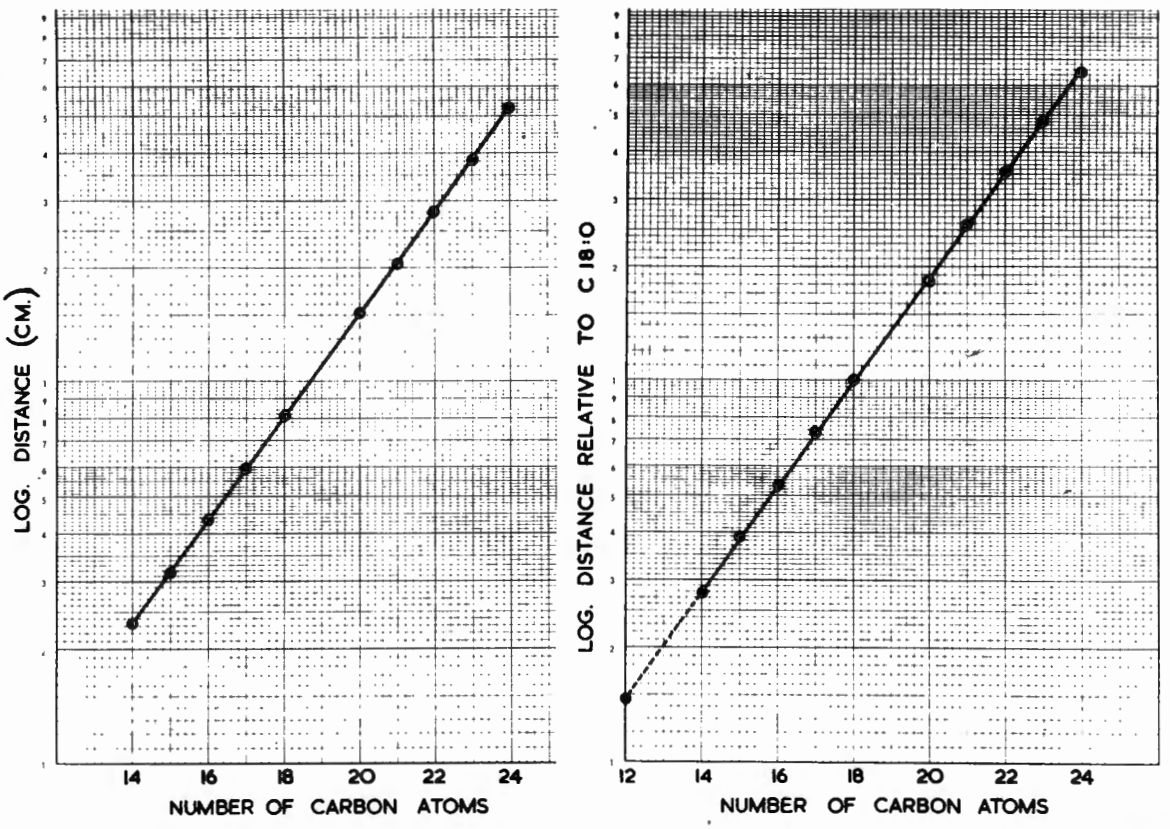


Figure 9: The log distance and the log distance relative to C18:0 plotted against the chain length of the components of the hydrogenated sample.
In each case the points fall on a straight line.
Extrapolation of the relative retention distance line gives the relative retention of C12:0.

Fatty acid	Natural	Fatty acid	Hydrogenated (theoretical)	Hydrogenated (Determined)
C14:0 C14:1	0.38 0.21	C14:0	0.59	0.81
C15:0 C15:1	0.21 0.21	C15:0	0.42	0.95
C16:0 C16:1	28.98 1.20	C16:0	30.18	30.53
C17:0 C17:1	0.34 0.12	C17:0	0.46	0.54
C18:0 C18:1 C18:2	13.11 10.47 21.16	C18:0	44.74	43.00
C20:0 C20:3 C20:4 C20:5	0.78 2.86 7.56 1.60	C20:0	12.80	12.85
C21:0	0.30	C21:0	0.30	0.25
C22:0 C22:6	2.27 3.43	C22:0	5.70	5.71
C23:0	1.14	C23:0	1.14	1.18
C24:0 C24:1	1.75 1.92	C24:0	3.67	4.18

Table 13: The percentage composition of natural phospholipid fatty acids and the theoretical and determined percentage composition of an hydrogenated derivative of the natural sample.

The theoretical percentage composition of the hydrogenated sample, calculated for the fatty acids identified as shown in Table 13, agrees well with the percentage composition found in the hydrogenated sample. There is some discrepancy between the theoretical values and the values found for C14:0, C15:0 and C24:0 but this is probably due to error in calculating the areas of these

small peaks. The close agreement between the theoretical and determined percentages for C20:0 and C22:0 supports the identification of peaks 14,17 and 21 as C20:3, C20:5 and C22:6 respectively. Since pure standards of these fatty acids were not available, it was not possible to establish conclusively the identity of these peaks.

Using the methods described, all the fatty acids present in the phospholipid fraction have been identified as shown in Figure 8. Since the column temperature remained constant at 184°C the fatty acid components of the cholesterol ester and triglyceride fractions have been identified by comparing their relative retention times with those found for identified peaks in the phospholipid fraction. In both cholesterol ester and triglycerides, a peak with a relative retention time of 0.145 was present. This relative retention time corresponds to that of C12:0 as determined by plotting log relative retention time against chain length (Figure 9). In addition, this peak occurred in both brominated and hydrogenated samples of cholesterol esters and triglycerides and it has therefore been identified as C12:0.

The cholesterol ester fraction contained a peak which disappeared on bromination and hydrogenation and had a relative retention time of 1.72. This value agrees closely with the value of 1.73 given by Farquhar et al (1959) as the relative retention time of C18:3. This peak has accordingly been identified as C18:3. Identification of the peaks present in the cholesterol ester and triglyceride fractions is shown in the chromatograms in Figure 10.

(i) <u>REPRODUCIBILITY OF FATTY ACID ANALYSIS</u>

In order to determine the degree of reproducibility of fatty acid estimations by G.L.C., a standard mixture was analysed ten times at random intervals during the course of the study. The percentage of each fatty acid was determined and the coefficient of variation calculated for each component (Table 14).

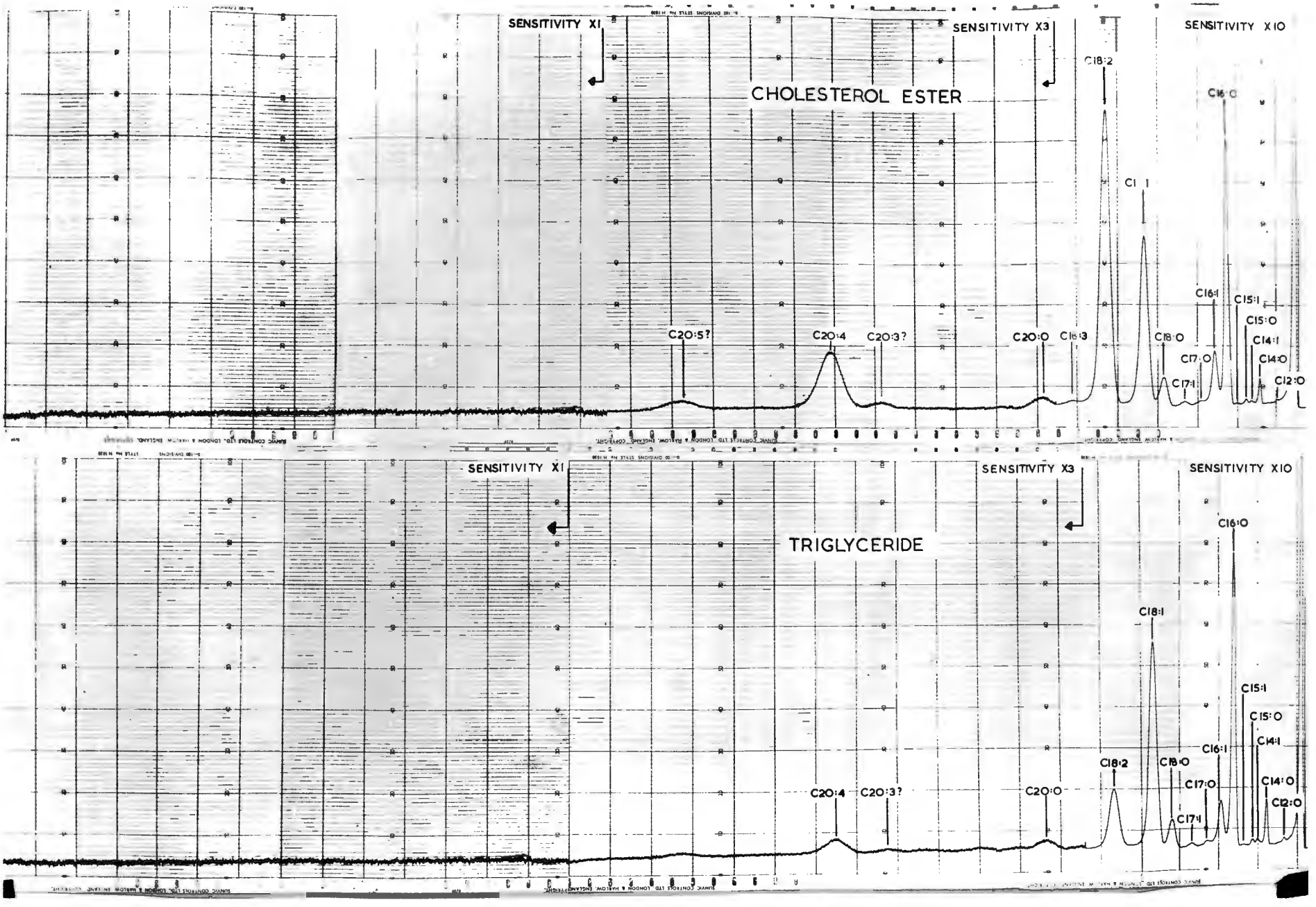


Figure 10: Typical chromatograms showing all the fatty acid components appearing in the cholesterol ester and triglyceride fractions of serum.

	C14:0	C16:0	C16:1	C18:0	C18:1
Mean	11.89	22.96	6.85	13.00	45.29
S.D.	0.35	0.49	0.23	0.42	0.81
C.V.%	2.94	2.14	3.36	3.23	1.79

Table 14: The percentage composition of a standard fatty acid methyl ester mixture as determined by ten analyses by G.L.C.

S.D. = One standard deviation

C.V.% = The coefficient of variation expressed as a percentage.

These data show that there is a high degree of reproducibility in estimating the fatty acid composition by G.L.C. The error is greater for the smaller components and this is probably due to inaccuracies in measuring the areas of the smaller peaks. It has been reported (Farquhar et al 1959) that on repeated analysis of a standard fatty acid methyl ester mixture containing four components, the coefficient of variation for any single component was less than 1.5%. Such a high degree of reproducibility was not achieved here, probably due to the differences in peak heights of the components. However, since the various lipid fractions analysed in this study contain numerous fatty acids in widely differing proportions, the degree of accuracy attained for the standard mixture is probably a fairer reflection of that in actual lipid samples.

The reproducibility of the whole procedure used in determining the fatty acid composition of each lipid class has been examined. Six 1.0 ml. aliquots of a serum sample were extracted as described. The extracted lipids were fractionated into the three lipid classes by silicic acid chromatography, methylated and the methyl esters analysed by G.L.C. The mean percentage, one standard

deviation (S.D.) and the coefficient of variation expressed as a percentage (C.V.%) are shown for each major component (Table 15).

Fatty acid	Cholesterol Ester			Triglyceride			Phospholipid		
	Mean	(S.D.)	C.V.%	Mean	(S.D.)	C.V.%	Mean	(S.D.)	C.V.%
C14:0	1.0	(0.05)	4.7	2.3	(0.01)	5.0	0.8	(0.04)	5.0
C16:0	12.0	(0.45)	3.7	28.0	(0.78)	2.8	32.2	(1.03)	3.2
C16:1	4.8	(0.19)	4.0	5.0	(0.20)	4.1	1.6	(0.08)	4.8
C18:0	1.9	(0.09)	4.5	5.3	(0.20)	3.7	14.6	(0.53)	3.6
C18:1	22.1	(0.60)	2.7	40.6	(0.93)	2.3	10.5	(0.37)	3.5
C18:2	46.0	(0.83)	1.8	13.7	(0.45)	3.3	18.0	(0.54)	3.0
C20:4	6.6	(0.30)	4.6	1.2	(0.06)	5.1	8.6	(0.35)	4.1

Table 15: The percentages of the major fatty acids present in the cholesterol ester, triglyceride and phospholipid fractions, as determined on six separate aliquots of a serum sample.

The degree of reproducibility is high. While the coefficient of variation is lower for the more abundant components, in no instance does it exceed 5% .

The reproducibility of estimation of smaller components was lower than that found for the major components. However, with the exception of certain longer chain fatty acids present in the phospholipid fraction, these fatty acids were not used for statistical comparison between groups. The values found for the longer chain fatty acids of the phospholipid fraction are shown in Table 16.

	C20:3(?)	C22:0	C20:5(?)	C23:0	C24:0	C24:1	C22:6(?)
Mean	2.4	0.7	1.5	0.3	1.0	1.4	2.8
S.D.	0.18	0.08	0.12	0.06	0.12	0.25	0.41
C.V.%	7.3	11.4	8.3	19.4	12.3	17.7	14.6

Table 16: The percentage of the longer chain fatty acids present in the phospholipid fraction, as determined on six separate aliquots of a serum sample.

The data in Table 16 show that the degree of reproducibility of estimation was low for these fatty acids. The error incurred is due largely to the inaccuracy in measuring the areas of these peaks, which because of their relatively small proportions and long retention times tend to be rather low and wide (Figure 8). With the development of temperature programming techniques it is possible to decrease the retention times and consequently increase the peak height to width ratio of these components. The apparatus available for this study does not allow the application of temperature programming techniques. When comparisons are made between groups of subjects for these fatty acids (Table 16) the low degree of reproducibility of estimation must be borne in mind.

(j) LIMITATIONS OF THE G.L.C. TECHNIQUE USED

While peaks 10 and 11 have been identified as C18:1 (oleic acid) and C18:2 (linoleic acid) respectively, certain limitations of this identification must be mentioned.

Using a non-polar stationary phase for separation of fatty acids, Hirsch et al (1960) have shown that isomers of oleic acid are present in human adipose tissue. While this is not necessarily so for serum lipids, it is possible that isomers of oleic acid may be present in serum lipids, particularly in view of the fact that such isomers are commonly produced by incomplete hydrogenation of natural oils in the manufacture of edible fats. A polar stationary phase such as E.G.A. does not separate oleic acid from its isomers. Thus, while reference will be made to C18:1 as oleic acid, it must be borne in mind that the percentages reported may include isomers of C18:1.

Peak 11 has been identified as C18:2 (linoleic acid) since its relative retention time is identical to that of a pure methyl linoleate standard, and also corresponds closely to that given by Farquhar et al (1959) for C18:2

with the double bonds at the 9 and 12 positions. However, linoleic acid can theoretically occur in four different forms, viz: cis-cis, cis-trans, trans-cis and trans-trans (Deuel 1955a). The only form of linoleic acid which occurs in nature is cis-cis 9-12 octadecadienoic acid. It is possible that industrial processes used in hardening oils could give rise to one or other of the isomers of linoleic acid and it has been suggested (Bronte-Stewart 1958) that consumption of fats such as margarine might result in the presence of these isomers in the serum lipids.

Since the stationary phase used in this study does not resolve isomers of oleic acid, it is similarly possible that it does not separate isomers of linoleic acid. It has therefore not been possible to establish that the linoleic acid present in the various lipid fractions is in fact natural or cis-cis linoleic acid. Böttcher (1960) has established by means of ozonolytic degradation and infra-red spectrophotometry that the C18:2 acid in the cholesterol ester fraction from human atheromatous plaques is 98% cis-cis. Although this does not mean that the same holds for serum lipids, it does suggest that unnatural isomers of linoleic acid are rare. While C18:2 will often be referred to as linoleic acid in the text, the possible limitations of this reference must be borne in mind.

CHAPTER 3

STATISTICAL METHODS

STATISTICAL METHODS

The following statistical formulae were employed in this study.

Mean: $\bar{X} = \Sigma X / N$ . . . . . (1)

where X = each observation and N = number of observations

Standard deviation (S.D.) for a variate :

$$S.D. = \sqrt{\Sigma x^2 / N-1} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (2)$$

where $\Sigma x^2 = \Sigma X^2 - (\Sigma X)^2 / N$

The difference between 2 means was analysed by t-test, t being calculated as :

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{\dfrac{\Sigma x_1^2 + \Sigma x_2^2}{\dfrac{(N_1 \times N_2)(N_1 + N_2 - 2)}{(N_1 + N_2)}}}} \quad \ldots\ldots\ldots\ldots \quad (3)$$

The significance of the difference between two means was determined by reference to standard tables* for the distribution of t.

Differences were considered to be highly significant when $p < 0.001$, significant when $p < 0.01$ and of probable significance when $p < 0.05$.

N.S. = Not statistically significant i.e. $p > 0.05$.

The correlation coefficient between two variates X and Y were analysed as

$$r = \frac{N.\Sigma XY - \Sigma X.\Sigma Y}{\sqrt{N.\Sigma X^2 - (\Sigma X)^2}\ \sqrt{N.\Sigma Y^2 - (\Sigma Y)^2}} \quad \ldots\ldots\ldots \quad (4)$$

The significance of r was similarly determined by reference to standard tables* .

Regression lines were calculated by the method of least squares, the correlations having been assumed to be linear, $Y = a + bx$.

$$b_{YX} = \frac{N.\Sigma XY - \Sigma X.\Sigma Y}{N.\Sigma X^2 - (\Sigma X)^2} \qquad \ldots\ldots\ldots\ldots\ldots\ldots (5)$$

$$a_{YX} = \frac{\Sigma Y - b.\Sigma X}{N}$$

To test the variability of repeated analyses the coefficient of variation (C.V.) was calculated as :

$$C.V. = \frac{S.D. \times 100}{\overline{X}} \quad \text{and expressed as a percentage.} \ldots (6)$$

The standard error of measurement (S.E.M.) between duplicate readings was calculated as

$$S.E.M. = \sqrt{\frac{\Sigma \Delta 2}{2N}} \qquad \ldots\ldots\ldots\ldots\ldots\ldots (7)$$

where Δ = difference between two single tests performed on each sample

N = number of duplicate determinations.

[*] Fisher,R.A. and Yates,F. Statistical Tables for Biological Agricultural and Medical Research, 5th Ed. Revised and Enlarged. 1957. Oliver and Boyd, London.

PART II

RESULTS AND DISCUSSION

CHAPTER I

THE COMPOSITION OF LIPID IN TOTAL SERUM AND IN THE SERUM β-LIPOPROTEIN FRACTION

I : THE QUANTITATIVE COMPOSITION OF LIPID IN TOTAL SERUM AND IN THE SERUM β-LIPOPROTEIN FRACTION.

II : THE FATTY ACID COMPOSITION OF LIPID IN TOTAL SERUM AND IN THE SERUM β-LIPOPROTEIN FRACTION

III : THE RELATIONSHIP BETWEEN DIET AND SERUM LIPIDS

I. THE QUANTITATIVE COMPOSITION OF LIPID IN TOTAL SERUM AND IN THE SERUM β-LIPOPROTEIN FRACTION

The concentrations of individual lipid components and of total lipid have been determined in total serum and the serum β-lipoprotein fraction from Bantu, controls and patients with I.H.D. The values are presented and compared in Table 17 and Figures 11 and 12.

COMPONENT	Bantu (B)	Control (C)	I.H.D.	I.H.D. vs. C	I.H.D. vs. B	C. vs. B
	mg./100 ml.	mg./100 ml.	mg./100 ml.			
	Mean (S.D.)	Mean (S.D.)	Mean (S.D.)	p	p	p
		Total Serum				
Total lipid	476.5 (85.0)	737.8 (97.7)	845.1 (183.0)	NS	<0.001	<0.001
Total cholesterol	142.9 (30.1)	247.9 (33.0)	285.5 (62.7)	NS	<0.001	<0.001
Esterified cholesterol	107.0 (22.1)	178.9 (23.4)	201.7 (46.8)	NS	<0.001	<0.001
Free cholesterol	35.9 (8.9)	69.0 (10.4)	83.7 (16.7)	<0.02	<0.001	<0.001
Triglyceride	72.0 (22.2)	116.2 (56.9)	160.1 (52.0)	NS	<0.001	<0.001
Phospholipid	190.0 (28.9)	253.4 (35.9)	264.3 (50.6)	NS	<0.001	<0.001
		β-Lipoprotein				
Total lipid	266.2 (76.4)	560.3 (79.4)	679.6 (151.5)	<0.05	<0.001	<0.001
Total cholesterol	92.1 (25.9)	213.1 (26.7)	245.4 (56.5)	NS	<0.001	<0.001
Esterified cholesterol	65.8 (18.1)	152.0 (19.9)	169.8 (39.1)	NS	<0.001	<0.001
Free cholesterol	26.3 (8.4)	61.1 (9.2)	75.6 (17.9)	<0.05	<0.001	<0.001
Triglyceride	57.1 (18.9)	106.4 (49.1)	149.0 (48.0)	<0.05	<0.001	<0.001
Phospholipid	73.0 (15.7)	139.1 (18.8)	168.6 (38.3)	<0.05	<0.001	<0.001

Table 17: The concentration of total lipid and of each lipid component in total serum and β-lipoprotein in each group. The values for each component are compared between the groups.

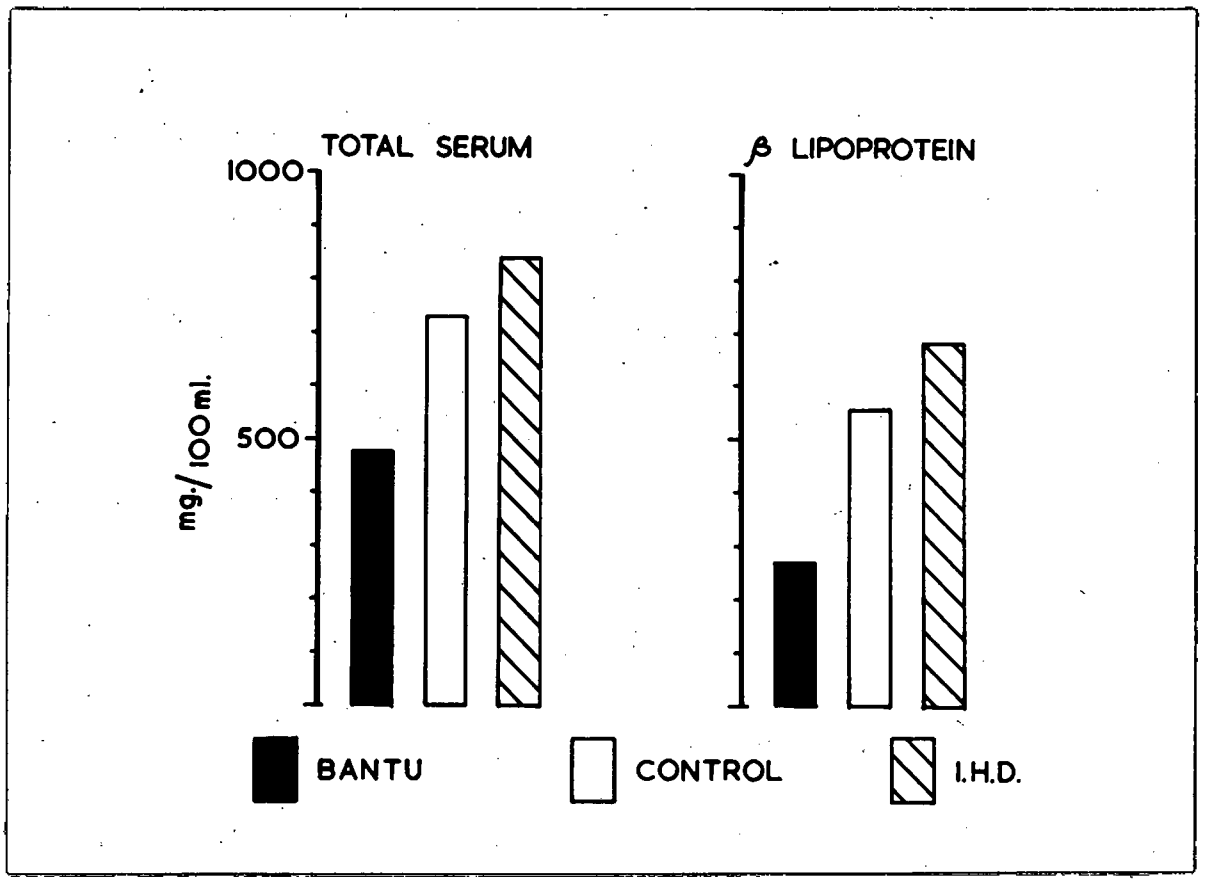
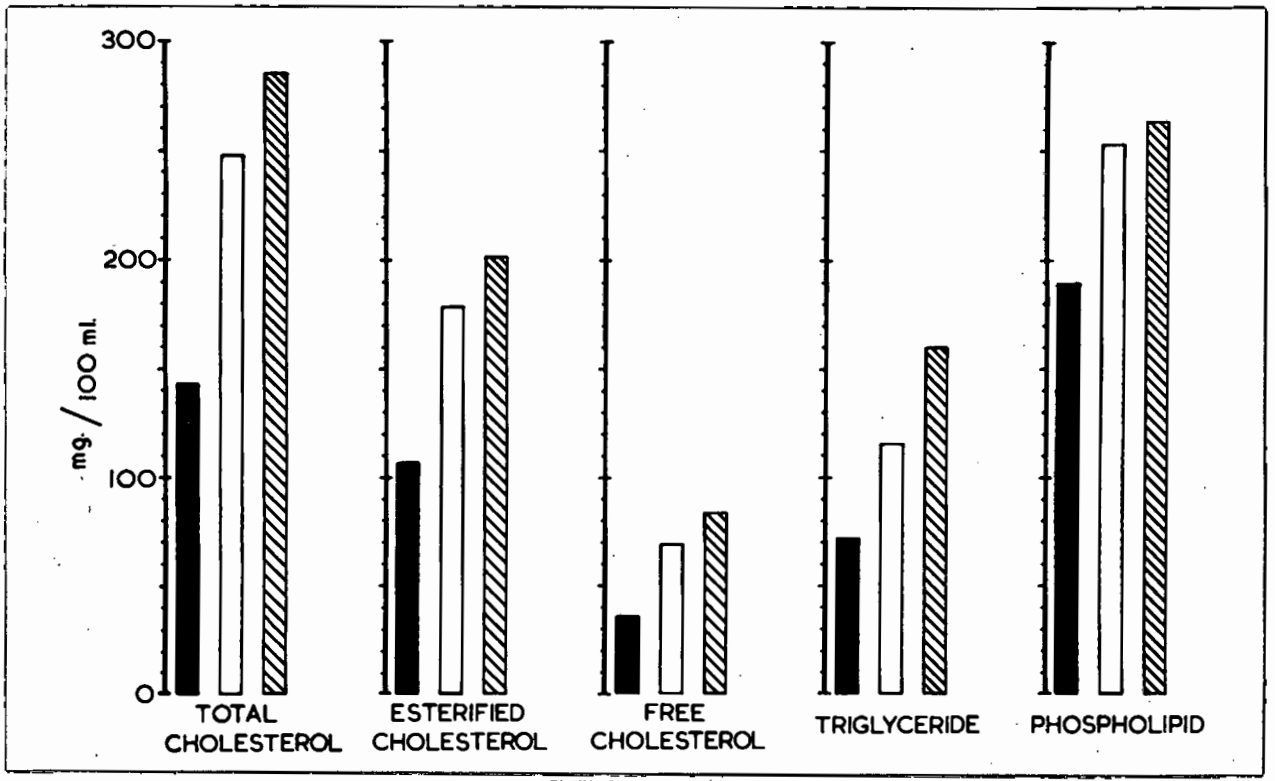
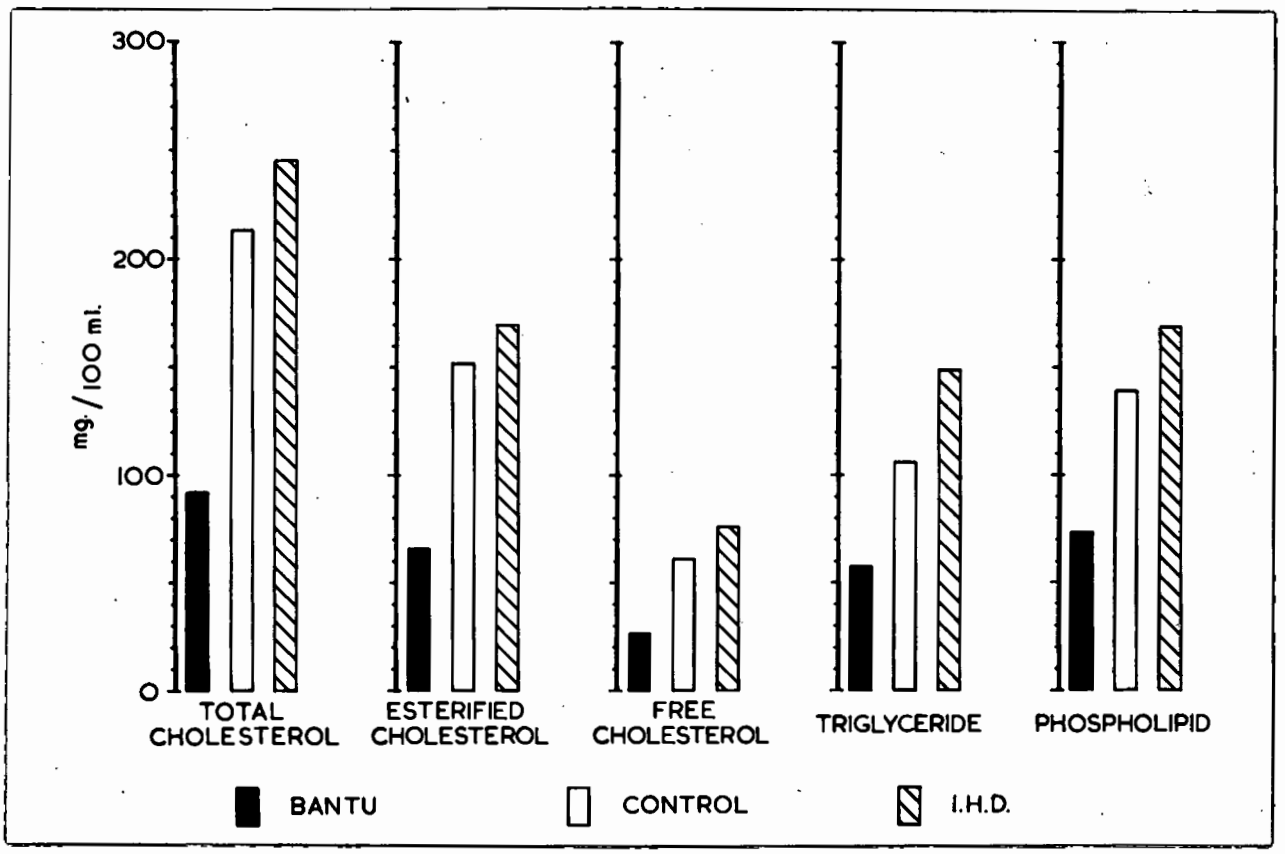


Figure 11: The concentration of total lipid in total serum and in the serum β-lipoprotein fraction.

Note the graded increase in concentration from Bantu through controls to patients with I.H.D. in both total serum and β-lipoprotein.



TOTAL SERUM



β-LIPOPROTEIN

Figure 12. The concentration of each lipid component in total serum and in the serum β-lipoprotein fraction.
For each component there is a graded increase in concentration from Bantu through controls to patients with I.H.D.

For both total serum and β-lipoprotein the concentration of total lipid and of each lipid component shows an increase from Bantu through controls to patients. In all instances the mean values for the Bantu group are strikingly lower than those for either patient or control groups ($p < 0.001$ in all cases). The concentration of total lipid and of the various lipid fractions is generally higher for patients than for controls. In total serum, only the concentration of free cholesterol is significantly higher, while in β-lipoprotein the concentrations of total lipid, free cholesterol, triglyceride and phospholipid are significantly higher for patients than for controls.

In general, for both total serum and β-lipoprotein, the data reveal a graded relationship between the three groups, with a distinct trend towards an increase in the concentration of total lipid and of each lipid component from Bantu through controls to patients.

i) Total lipid.

In total serum the mean values for patients and controls are similar, but the values for Bantu are markedly lower than for both the other groups. In β-lipoprotein the concentration of total lipid is higher for the patients than for the controls or Bantu. The concentration of total lipid in β-lipoprotein thus provides better separation between groups than does that in total serum.

In total serum the values for controls and patients are comparable with those reported in the literature (Page et al 1935; Chapin and Proger 1959; Schrade et al 1960; Hallgren et al 1960; Nelson and Freeman 1960) and those for controls and Bantu are similar to those previously found for comparable groups in Cape Town (Bronte-Stewart et al 1955). In β-lipoprotein the values for patients and controls are similar to those found using an electrophoretic

staining technique (Chapin and Proger 1959). The values for patients and Bantu are similar to those found for atherosclerotic subjects and young white men respectively, using Cohn fractionation for isolation of β-lipoprotein (Böttcher and Woodford 1961).

ii) Total cholesterol.

In both total serum and β-lipoprotein the concentration of total cholesterol increases progressively from Bantu through controls to patients. The mean values for Bantu are lower than those for the other two groups. While the mean concentrations are higher for patients than for controls, the differences are not statistically significant. Thus in both total serum and β-lipoprotein the concentration of total cholesterol does not separate patients from all healthy subjects.

In total serum and β-lipoprotein the values for patients and controls are similar to those reported by other authors (Gertler et al 1950; Albrink and Man 1959; Carlson 1960a, 1960b; Cohen et al 1960), while those for the Bantu are similar to the values found previously in Cape Town (Bronte-Stewart et al 1955).

iii) Esterified and free cholesterol

In both total serum and β-lipoprotein the mean values for esterified cholesterol and free cholesterol show trends similar to those found for total cholesterol (Table 17). Again, the values for Bantu are considerably lower than for the other two groups. While the concentration of esterified cholesterol is similar for patients and controls, in both total serum and β-lipoprotein the concentration of free cholesterol is significantly higher for patients than for either of the other groups. The concentration of free cholesterol thus effectively separates the three groups.

In view of the marked differences in the concentrations of free and esterified cholesterol in each group, the distribution of total cholesterol between the free and esterified forms has been examined. The relative proportions are shown in Table 18.

	Fraction	Bantu (B)	Control (C)	I.H.D.	I.H.D. vs.C	I.H.D. vs.B	C vs.B
		Mean (S.D.)	Mean (S.D.)	Mean (S.D.)	p	p	p
% Esterified cholesterol	Serum	75.0 (2.3)	72.2 (1.5)	70.5 (1.9)	<0.02	<0.001	<0.001
	β	71.6 (3.6)	71.3 (2.5)	69.2 (1.2)	<0.02	<0.02	NS
% Free cholesterol	Serum	25.0 (2.3)	27.8 (1.5)	29.6 (1.9)	<0.02	<0.001	<0.001
	β	28.4 (3.6)	28.7 (2.5)	30.8 (1.2)	<0.02	<0.02	NS
E/F	Serum	3.03 (0.38)	2.61 (0.19)	2.39 (0.21)	<0.02	<0.001	<0.001
	β	2.57 (0.46)	2.51 (0.37)	2.27 (0.14)	<0.05	<0.05	NS

Table 18: The percentage of total cholesterol present in the esterified and free forms, and the ratio of esterified to free cholesterol (E/F) in total serum and β-lipoprotein (β) from Bantu, controls and patients with I.H.D.

The major proportion of total cholesterol is present in the esterified form. In each group, despite the fairly wide variation noted for the absolute concentrations, the relative proportions of free cholesterol and esterified cholesterol are rather constant and are similar for total serum and β-lipoprotein.

When the three groups are compared, for total serum the percentage of free cholesterol shows a graded increase and the percentage of esterified cholesterol and the E/F ratio show a graded decrease from Bantu through controls to patients. These parameters effectively separate each group from the others. In β-lipoprotein, the percentage of esterified cholesterol and the E/F ratio are significantly lower and the percentage of free cholesterol is significantly higher for

the patients than for controls or Bantu, and the values for the last two groups are similar. Thus in both total serum and β-lipoprotein the percentage of free cholesterol is higher and that of esterified cholesterol is lower for the patients than for the other groups.

It will be recalled that the absolute concentration of free cholesterol was higher for patients than for controls or Bantu. These data indicate that the absolute concentration of free Cholesterol and the relative proportions of free and esterified cholesterol in both total serum and β-lipoprotein provide better separation between the groups than does the concentration of total cholesterol.

iv) Triglyceride

It can be seen from the data in Table 17 and in Figure 12, that both in total serum and in β-lipoprotein there is a progressive increase in the concentration of triglyceride from Bantu through controls to patients. The mean levels for the Bantu are much lower than those in both other groups. In total serum, despite an impressive difference in the mean value between patients and controls, this difference is not significant, emphasizing the marked degree of overlap between these groups. In β-lipoprotein the mean value is significantly higher for patients than for controls and the concentration of triglyceride in β-lipoprotein provides a better parameter for separating patients from healthy subjects than does that in total serum.

The mean serum triglyceride values found in Johannesburg (Antonis and Bersohn 1960) for patients with I.H.D., White controls and Bantu, in the 41-50 year age group, are similar to those reported here. The mean values for patients and controls agree with those reported by Carlson (1960a, 1960b) and by Albrink and Man (1959). The values of Schrade et al (1960) are almost double those found here and elsewhere. This discrepancy could be due to their

method of determination. They have apparently calculated the triglyceride as being the arithmetic difference between total lipid, determined gravimetrically, and the sum of the other lipid components, determined chemically. In the present study it was found that gravimetric determination of total lipid gives erroneously high values (Part I, Chapter 2, Section I). If this is so for the total lipids determined by Schrade et al, this could account for the high triglyceride values reported by them.

For β-lipoprotein the triglyceride concentration for controls is similar to that found by Hillyard et al (1955) for normal men in the ultracentrifugally separated fraction of density less than 1.063, which corresponds closely to the β-lipoprotein isolated in the present study. The values for the patients and the Bantu are similar to those reported for atherosclerotic men and healthy young men respectively (Böttcher and Woodford 1961).

It has been suggested that control groups with a socio-economic background similar to that of patient groups may not form a homogenous population with regard to serum triglyceride concentration. Several authors (Albrink and Man 1959; Carlson 1960a, 1960b; Antonis and Bersohn 1960) have reported a tendency for a skewed frequency distribution. In the present study the groups are too small for the assessment of frequency distribution.

v) Phospholipid.

Comparison of the concentration of phospholipid in the three groups shows that the trends in total serum and β-lipoprotein are not the same (Table 17, Figure 12). For β-lipoprotein the concentration increases from Bantu through controls to patients, and the mean value for patients is significantly higher than that for controls. This parameter thus separates the groups. In total serum, while the value for Bantu is lower, the values for patients and controls are identical. It is noteworthy that in total serum,

while the mean cholesterol and triglyceride concentration for patients were both impressively, though not significantly, higher than those for controls, the mean phospholipid concentrations are identical in these groups. The mean phospholipid concentration in the Bantu group is equal to approximately 70% of that found for patients, while the cholesterol and triglyceride values for Bantu are only about 50% of those for patients (Figure 12). The differences between the groups are thus less marked for serum phospholipid than they are for triglyceride and cholesterol.

For patients and controls the mean serum phospholipid concentrations are similar to those reported in the literature (Gertler et al 1950; Steiner et al 1952), and the values in β-lipoprotein agree very closely with those found for comparable groups, using a variety of techniques for separation of β-lipoprotein (Barr et al 1951; Russ et al 1951; Cornwell et al 1961; Cohen et al 1960; Carlson 1960a, 1960b).

vi) <u>The percentage composition of lipid</u>.

Since differences between the groups are more marked for some lipid fractions than for others, this suggests that the proportions in which lipid fractions are present in total serum and β-lipoprotein might be different in the three groups. The proportions of individual lipid components expressed as a percentage of the total lipid, have therefore been calculated and the values are shown in Table 19 and Figure 13.

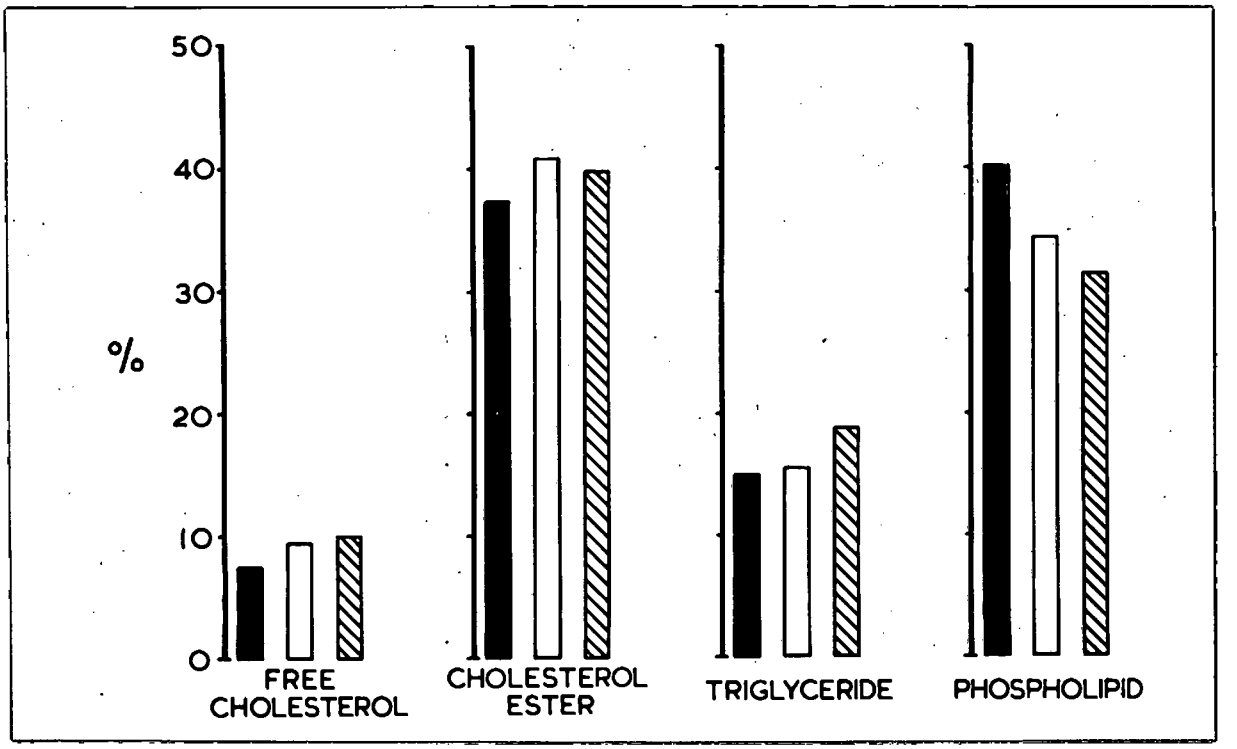
Component	Bantu (B) % Mean (S.D.)	Control(C) % Mean (S.D.)	I.H.D. % Mean(S.D.)	I.H.D. vs. C. p	I.H.D. vs. B. p	C vs. B. p
		Total Serum				
Free cholesterol	7.5 (0.8)	9.4 (0.9)	10.0 (0.8)	NS	<0.001	<0.001
Cholesterol ester	37.4 (2.8)	40.8 (3.9)	39.8 (3.0)	NS	<0.05	<0.05
Triglyceride	15.0 (3.3)	15.5 (6.1)	18.8 (3.7)	NS	<0.01	NS
Phospholipid	40.2 (3.4)	34.4 (2.6)	31.4 (1.5)	<0.01	<0.001	<0.001
		β-Lipoprotein				
Free cholesterol	9.7 (1.1)	11.0 (1.2)	11.1 (0.8)	NS	<0.01	<0.01
Cholesterol ester	41.1 (3.9)	45.6 (5.4)	42.2 (3.8)	NS	NS	<0.02
Triglyceride	21.4 (4.8)	18.5 (6.3)	21.9 (4.8)	NS	NS	NS
Phospholipid	27.8 (2.6)	24.9 (0.8)	24.8 (1.2)	NS	<0.01	<0.01

Table 19: The percentage composition of lipid in total serum and in β-lipoprotein from Bantu, controls and patients with I.H.D.

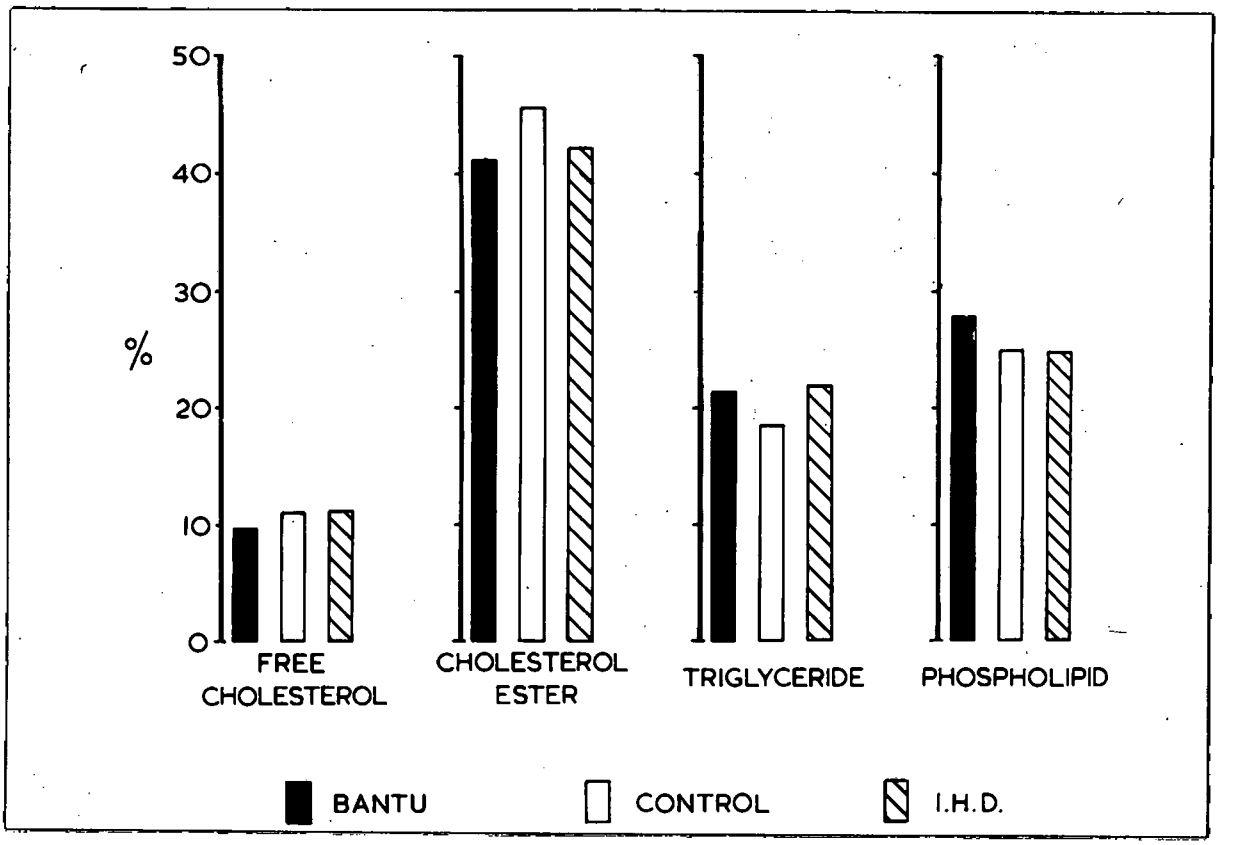
It is apparent from the data in Table 19 that for both total serum and β-lipoprotein the percentage composition of lipid is relatively constant in each group. This constancy of composition is noteworthy in view of the wide variation in the absolute concentrations of lipid fractions within each group (Table 17).

On comparing the percentage composition of lipid in total serum and in β-lipoprotein in each group, it is apparent that, while the percentages of cholesterol ester and triglyceride are similar, the total serum has a lower percentage of free cholesterol and a higher percentage of phospholipid than β-lipoprotein (Table 19).

When the three groups of subjects are compared, the percentage composition of lipid shows different trends in total serum and β-lipoprotein and



TOTAL SERUM



β-LIPOPROTEIN

Figure 13: The relative proportions of lipids in total serum and in the serum β-lipoprotein fraction, Note the reversal in the trends for phospholipid which shows a decrease from Bantu through controls to patients with I.H.D.

differences between groups are generally more marked in total serum than in β-lipoprotein.

In β-lipoprotein the percentage composition of lipid is identical for patients and controls. Thus despite differences in the concentrations of total lipid, free cholesterol, triglyceride and phospholipid, the relative proportions of lipid are similar for these two groups. The percentages of cholesterol ester and triglyceride tend to be similar in all groups, although the percentage of cholesterol ester is greater for the controls than for the Bantu. The Bantu differ markedly from the other two groups by having a lower percentage of free cholesterol and a higher percentage of phospholipid.

In total serum the percentages of free cholesterol and cholesterol ester are lower for the Bantu than for the patients and controls, and for these two groups the values are similar. The percentage of triglyceride is higher for the patients than for the Bantu, but the values for the Bantu and the controls are similar. These parameters do not separate the three groups. In contrast, the percentage of phospholipid shows a striking graded decrease from Bantu through controls to patients and the value for each group differs significantly from those of the other groups. Thus, while the absolute concentration of phospholipid was similar for patients and controls the relative proportion of phospholipid is greater for controls, and the Bantu who have the lowest absolute concentration have the highest relative proportion of phospholipid. The percentage of phospholipid in total serum thus differentiates between patients and apparently healthy subjects.

The decrease in the percentage of phospholipid in total serum, from Bantu through controls to patients is interesting in view of the suggestion by several authors that phospholipids may play an important role in the stabilization and solubilization of lipids (Ahrens and Kunkel 1949; Ahrens 1950; Dixon 1958;

Wilkens and Krut 1963). These data could suggest that a relative decrease of phospholipid in relation to other lipids may produce a lesser degree of stability of the latter lipids. In view of this and the striking differences in the percentage of phospholipid in the serum of the groups in this study, the proportions of lipid relative to one another and particularly in relation to phospholipid have been calculated. The various lipid ratios are shown in Table 20 and Figure 14.

RATIO	Bantu (B)	Control (C)	I.H.D.	I.H.D. vs.C	I.H.D. vs.B	C. vs.B
	Mean (S.D.)	Mean (S.D.)	Mean (S.D.)	p	p	p
		Total Serum				
$C/_T$	2.09 (0.53)	2.48 (0.86)	1.89 (0.54)	NS	NS	NS
$T/_P$	0.38 (0.11)	0.46 (0.23)	0.60 (0.13)	NS	<0.001	NS
$EC/_P$	0.56 (0.07)	0.71 (0.07)	0.76 (0.07)	NS	<0.001	<0.001
$FC/_P$	0.19 (0.03)	0.27 (0.03)	0.31 (0.03)	<0.01	<0.001	<0.001
$C/_P$	0.75 (0.09)	0.98 (0.09)	1.08 (0.09)	<0.01	<0.001	<0.001
$(FC+CE+T)/_P$	1.51 (0.19)	1.92 (0.23)	2.19 (0.15)	<0.01	<0.001	<0.001
		β-Lipoprotein.				
$C/_T$	1.69 (0.44)	2.29 (0.74)	1.76 (0.57)	NS	NS	<0.02
$T/_P$	0.79 (0.23)	0.75 (0.27)	0.89 (0.22)	NS	NS	NS
$EC/_P$	0.89 (0.11)	1.10 (0.12)	1.01 (0.09)	NS	<0.001	<0.001
$FC/_P$	0.35 (0.06)	0.45 (0.06)	0.45 (0.03)	NS	<0.001	<0.001
$C/_P$	1.25 (0.15)	1.51 (0.17)	1.46 (0.11)	NS	<0.001	<0.001
$(FC+CE+T)/_P$	2.63 (0.33)	3.03 (0.13)	3.04 (0.20)	NS	<0.001	<0.001

Table 20: Lipid ratios in total serum and β-lipoprotein from Bantu, controls and patients with I.H.D.

T = Triglyceride
P = Phospholipid
C = Total Cholesterol
FC = Free Cholesterol
CE = Cholesterol Ester
EC = Esterified Cholesterol

The relative proportions of lipids expressed as lipid ratios, are different in total serum and β-lipoprotein in each group of subjects and thus reflect the differences in composition previously noted (Table 19). When the three groups

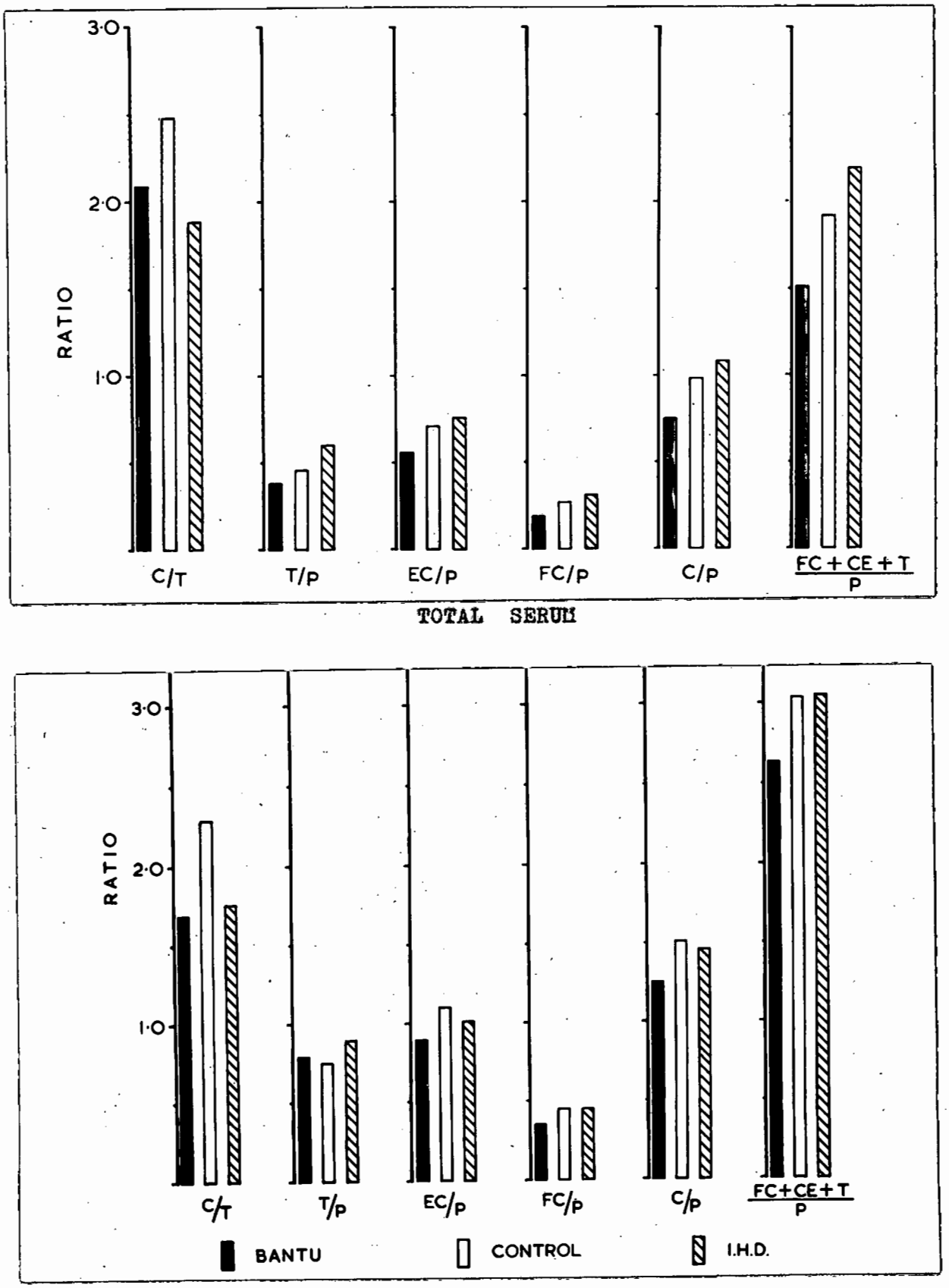


Figure 14: Lipid ratios in total serum and in the serum β-lipoprotein fraction.
In total serum there is a graded increase from Bantu through controls to patients with I.H.D. whenever phospholipid forms the denominator.

of subjects are compared, different trends are found in total serum and in β-lipoprotein.

In β-lipoprotein the ratios show no grading between the groups. The ratio of cholesterol to triglyceride ($C/_T$) is higher for controls than for Bantu, but otherwise the values for the groups are similar. Despite marked differences in the concentrations of these lipid fractions (Table 17), the ratio of triglyceride to phospholipid ($T/_P$) is similar for all groups. The ratios of free cholesterol to phospholipid ($FC/_P$), of total cholesterol to phospholipid ($C/_P$) and of 'neutral lipid' to phospholipid ($\frac{FC+CE+T}{P}$) are similar for patients and controls, while the values for Bantu are lower. None of these ratios separate the three groups.

In total serum the $C/_T$, $T/_P$ and $EC/_P$ ratios show no marked grading between the groups. The ratio of cholesterol to triglyceride ($C/_T$) is similar for all groups. The relative proportion of triglyceride to phospholipid ($T/_P$) is higher for the patients than for the Bantu, but this is the only difference between groups. The ratio of esterified cholesterol to phospholipid ($EC/_P$) is lower for the Bantu than for the patient and control groups where the mean values are similar. In contrast, the $FC/_P$, $C/_P$ and $\frac{FC+CE+T}{P}$ ratios all show a marked graded increase from Bantu through controls to patients. For these ratios the mean values for each group are significantly different from those of the other groups. These parameters thus separate patients from healthy subjects. Other authors have reported that the $C/_P$ ratio is higher in subjects with I.H.D. than in healthy subjects (Morrison et al 1950, 1952; Steiner et al 1952).

For each of these ratios phospholipid forms the denominator. If, as has been suggested, the relative proportion of phospholipid is important in determining the 'stability' of lipids, one may speculate that the marked increase in the $FC/_P$, $C/_P$ and $\frac{FC+CE+T}{P}$ ratios reflects progressively decreasing

stability of free cholesterol, total cholesterol and of neutral lipid in general from Bantu through controls to patients with I.H.D.

Whatever the possible implications of these findings, these data indicate that the relative proportions of lipid in total serum provide better separation between the three groups than does any single lipid parameter.

vii) The relationship between lipid concentrations in total serum and β-lipoprotein.

In many respects the concentrations of lipid fractions showed similar trends in total serum and in β-lipoprotein. It would, therefore, be of interest to determine what proportion of serum lipid is present in the form of β-lipoprotein. In addition, the concentrations of total lipid, free cholesterol, triglyceride and phospholipid in β-lipoprotein provided separation between the groups. The question arises as to whether not only the concentrations but also the relative proportions of lipids present in the form of β-lipoprotein are different in the three groups. In order to examine these points, the percentages of total serum lipids present as β-lipoprotein have been calculated. The values are shown in Table 21 and Figure 15.

	Bantu (B)	Control (C)	I.H.D.	I.H.D. vs.C.	I.H.D. vs.B.	C. vs.B.
	%	%	%			
	Mean (S.D.)	Mean (S.D.)	Mean (S.D.)	p	p	p
Total lipid	55.5 (6.5)	76.0 (4.1)	80.5 (5.9)	<0.05	<0.001	<0.001
Total cholesterol	63.8 (7.6)	86.2 (4.1)	86.1 (7.7)	NS	<0.001	<0.001
Esterified cholesterol	61.0 (8.4)	84.8 (3.8)	86.2 (8.6)	NS	<0.001	<0.001
Free cholesterol	72.4 (9.4)	89.1 (8.9)	90.1 (8.2)	NS	<0.001	<0.001
Triglyceride	79.1 (7.8)	92.5 (6.2)	93.2 (4.4)	NS	<0.001	<0.001
Phospholipid	38.5 (5.9)	55.2 (6.6)	63.6 (5.7)	<0.01	<0.001	<0.001

Table 21: The percentage of total serum lipid present in the form of β-lipoprotein.

$$\% = \frac{\beta\text{-lipoprotein concentration (mg/100 ml)}}{\text{Total serum concentration (mg/100 ml)}} \times 100$$

In all groups the major portion of total lipid is carried in β-lipoprotein, confirming that this fraction is the chief vehicle for the transport of lipid in serum. In each group the percentages of total cholesterol, free and esterified cholesterol and triglyceride carried in β-lipoprotein are high, while the percentages of phospholipid are considerably lower. These data are in agreement with ultracentrifugal data showing that low-density lipoproteins carry the major proportion of serum cholesterol and triglyceride but that serum phospholipid is distributed fairly evenly between low density (β) and high density (α) lipoproteins, with a tendency for the high density lipoproteins to carry a greater proportion of phospholipid (Jones et al 1951; Hillyard et al 1955; Olson and Vester 1960).

When the values for the three groups are compared, it is seen that for total lipid and for each lipid fraction, the percentage carried in β-lipoprotein is very significantly lower for the Bantu than for either of the other groups. With the exception of the percentages of serum total lipid and phospholipid carried in β-lipoprotein, the mean values for patients and controls are similar. The percentage of serum total lipid carried in β-lipoprotein is probably significantly higher for patients than for controls ($p < 0.05$). It will be recalled that the concentration of total lipid in β-lipoprotein was higher for patients than controls (Table 17). Thus both the concentration and the relative proportion of total lipid carried in β-lipoprotein are higher for patients than for either controls or Bantu and both these parameters provide separation between the groups.

The percentage of phospholipid shows a progressive increase from Bantu through controls to patients and the mean value for each group is significantly different from those of the other groups. This parameter clearly separates patients from healthy subjects. In Table 19 it was seen that phospholipid constitutes approximately 25% of the total lipid in β-lipoprotein. The

increase in the percentage of total serum phospholipid carried in β-lipoprotein from Bantu through controls to patients can therefore be interpreted as reflecting the increase in the absolute concentration of β-lipoprotein lipid in these groups.

The percentage of total serum cholesterol carried in β-lipoprotein in the patient group is similar to the values reported in several studies (Barr et al 1951; Oliver and Boyd 1955; Carlson 1960b; Cohen et al 1960). The value for the control group is similar to the values reported by some authors (Bronte-Stewart et al 1955; Cramer 1962; Cornwell et al 1961), but is somewhat higher than that found by others (Cohen et al 1960; Russ et al 1951; Carlson 1960a; Nikkilä 1953). The value for the Bantu group is similar to that previously found in Cape Town (Bronte-Stewart et al 1955). The percentages of total serum phospholipid carried in β-lipoprotein for the controls and patients agree very closely with the values calculated from the data of several authors, who used a variety of techniques for separation of β-lipoprotein (Barr et al 1951; Carlson 1960a, 1960b; Cohen et al 1960; Cramer 1962; Cornwell et al 1961; Nikkilä 1953; Russ et al 1951). Values for the percentage of total serum triglyceride found in β-lipoprotein have not, to our knowledge, been reported.

The data in this and other studies indicate that the major portion of serum lipid is associated with β-lipoprotein. In order to assess whether variations in the concentration of lipid in total serum are related to variations in the β-lipoprotein concentration, the correlation coefficient and the regression equation have been calculated for total lipid and for each lipid component in all cases studied (Table 22, Figures 16 and 17).

Component	r	p	Regression Equation
Total lipid	0.9666	<0.001	y = 0.9462x + 212.8
Total cholesterol	0.9712	<0.001	y = 0.9356x + 53.5
Free cholesterol	0.9779	<0.001	y = 0.9505x + 11.1
Esterified cholesterol	0.9599	<0.001	y = 0.9217x + 43.2
Triglyceride	0.9887	<0.001	y = 1.0330x + 9.05
Phospholipid	0.8853	<0.001	y = 0.9465x + 115.3

Table 22: The relationship between the concentration of lipid in total serum and in β-lipoprotein in all subjects (n = 40).

r = Correlation coefficient
y = Concentration in total serum
x = Concentration in β-lipoprotein

The high degree of correlation between the concentration of lipid in total serum and that in β-lipoprotein is most striking ($p < 0.001$ in each instance). The concentration of total lipid and each lipid component in total serum is thus directly related to the concentration in β-lipoprotein. Furthermore since in every instance the regression line has approximately a 1:1 slope, these data indicate that any increase in the concentration of lipid in total serum beyond a certain level is due almost entirely to an increase in the concentration in β-lipoprotein. This suggests that there is a certain maximum level of lipid carried in α-lipoprotein and that any lipid in excess of this is carried in β-lipoprotein. One may speculate that, regardless of differences in total serum lipid concentrations, the α-lipoprotein lipid concentration would remain rather constant.

Similar observations have been made by other authors. In an inter-racial study, despite great differences in total serum concentration, the α-lipoprotein cholesterol concentration was similar in different racial groups (Bronte-Stewart et al 1955). Cramer (1962) reported a high degree of correlation between the

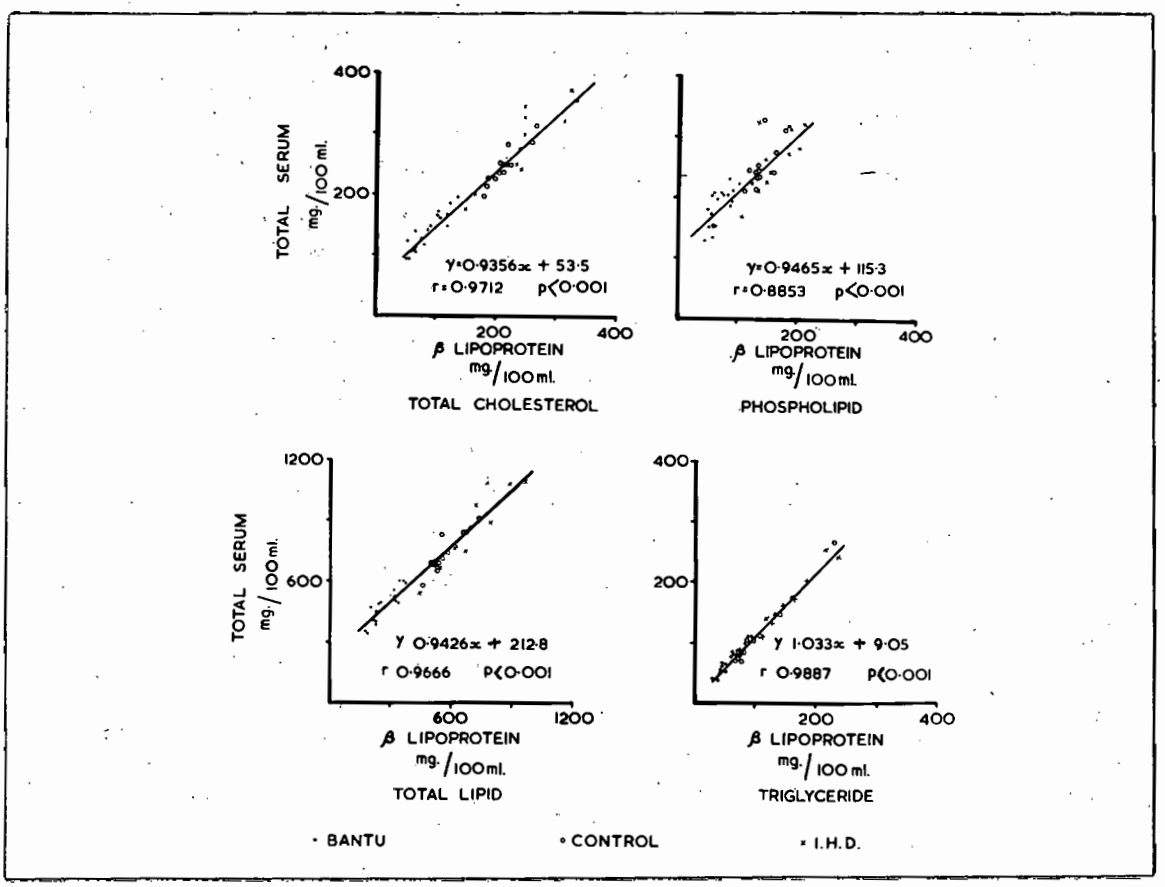


Figure 16: The correlation between the concentration of lipid in total serum and in β-lipoprotein.

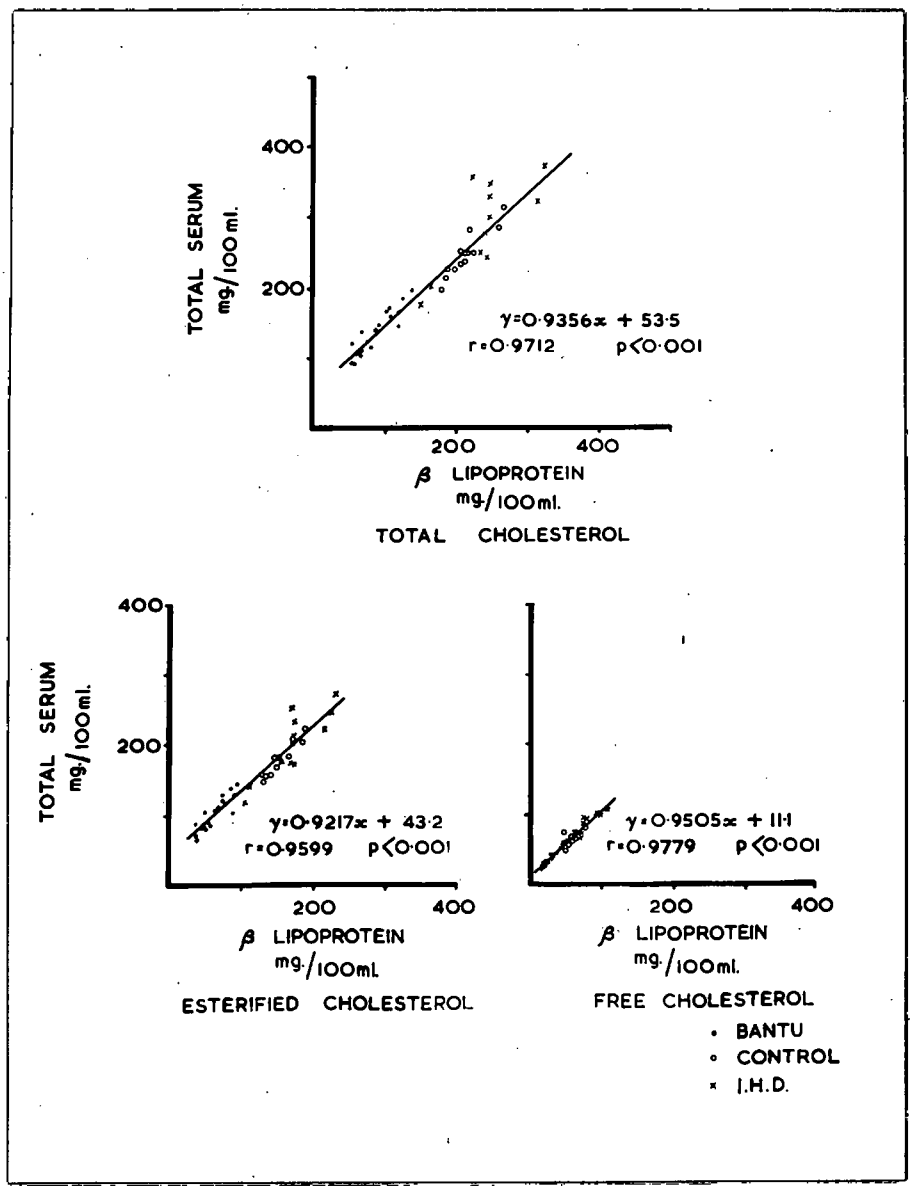


Figure 17: The correlation between the concentration of lipid in total serum and in β-lipoprotein.

glyceride concentration in total serum and in β-lipoprotein. The data of Chapin and Proger (1959) show a high degree of correlation between total serum and β-lipoprotein phospholipid concentrations in the serum of young men and women and patients with I.H.D. Havel et al (1955) noted a distinct positive correlation between the concentration of cholesterol in total serum and in the ultracentrifugally separated fraction of density 1.019 - 1.063. This latter correlation applied only in the case of young males and females and did not hold in patients with excessively high serum lipid levels (essential hyperlipidaemia). In the present study, for cholesterol and for other lipid components, the correlations are valid over the whole range of concentrations observed.

viii) <u>The influence of β-lipoprotein lipid on the relative proportions of lipids in total serum.</u>

It will be recalled from Table 19 that on comparing the relative proportions of lipid components in β-lipoprotein, the values for patients and for controls were similar, while the Bantu had a lower proportion of free cholesterol and a higher proportion of phospholipid (Table 19). Similarly, for the lipid ratios in β-lipoprotein, there was no graded relationship between the three groups (Table 20). In contrast, in total serum the percentage of phospholipid showed a marked graded decrease from Bantu through controls to patients (Table 19). This graded decrease in the percentage of phospholipid was reflected in a graded increase in the FC/P, C/P, and FC+CE+T/P ratios from Bantu through controls to patients (Table 20). The relative proportions of lipids in total serum thus provided better separation between the groups than did those in β-lipoprotein. An explanation for this finding becomes apparent when the composition and relative proportion of β-lipoprotein lipid is examined.

In each group of subjects the proportion of phospholipid is lower in β-lipoprotein than in total serum (Table 19). This indicates that

(high density) lipoprotein has a higher proportion of phospholipid than β (low density) lipoprotein, which is in agreement with data in other studies (Barr et al. 1951; Havel et al. 1955; Chapin and Proger 1959). The percentage of phospholipid in total serum is therefore a reflection of the α to β-lipoprotein ratio. The data in Table 22 can be regarded as suggesting that the concentration of α-lipoprotein lipid is similar in all groups (see page 76). Any increase in the concentration and relative proportion of β-lipoprotein lipid would, in that case, produce a decrease in the percentage of phospholipid in total serum. The data in Tables 17 and 21 show that the concentration as well as the relative proportion of β-lipoprotein lipid increased from Bantu through controls to patients. The decrease in the proportion of phospholipid and the increase in the C/P, FC/P, FC+CE+T/P ratios in total serum, from Bantu through controls to patients, are thus a reflection of the increasing concentrations of β-lipoprotein lipid in the three groups. This finding is in agreement with the observation made by Havel and Carlson (1962) that an increase in the serum C/P ratio could be related to an increase in the concentration of low-density lipoproteins.

CONCLUSIONS

In every subject and group the major proportion of lipid was carried in the form of β-lipoprotein.

Beyond a certain minimum level any increase in the total serum concentration of each lipid fraction could be ascribed to an increase in the concentration of that component in the β-lipoprotein fraction.

In total serum there was a graded increase from Bantu through controls to patients in the concentration of each lipid component. While the levels in the Bantu group were consistently lower than in the other two groups, the difference between patients and controls was significant only for free cholesterol. In the β-lipoprotein fraction a graded increase from Bantu through controls to patients was also evident in the concentration of each lipid component. Here too, the Bantu group consistently showed levels significantly lower than in the other two groups, while the concentration in the controls was significantly lower than in the patients for the sum of all lipids (total lipid), free cholesterol, triglyceride and phospholipid. There is thus better separation between the patients and the controls in the concentration of lipids in the β-lipoprotein fraction than in the total serum.

The concentration of each lipid component was expressed as a percentage of all the lipids (total lipid). In total serum there was a significant graded decrease in the percentage of phospholipid from Bantu through controls to patients. In the β-lipoprotein there were no differences between the patient and control groups in the relative proportions of the various lipid components. The Bantu, however, differed from both other groups for a number of parameters.

The sum of all the lipids (total lipid) and the phospholipid present in β-lipoprotein, expressed as a percentage of the total serum lipids, showed a significant graded increase from Bantu through controls to patients.

In total serum, the ratios of esterified to free cholesterol, of total cholesterol to phospholipid, of free cholesterol to phospholipid and of neutral lipid to phospholipid provided good separation between the three groups. In β-lipoprotein the values for these ratios were similar for controls and patients, while the values for the Bantu differed from those in both the other groups. The differences in the relative proportions of lipid in relation to phospholipid in the total serum could be related to the differences in the concentration of β-lipoprotein lipids in the three groups.

II. THE FATTY ACID COMPOSITION OF LIPID IN TOTAL SERUM AND IN THE SERUM β-LIPOPROTEIN FRACTION

Using the technique of gas-liquid chromatography, the fatty acid compositionsof the cholesterol ester, triglyceride and phospholipid fractions have been determined in total serum and in the β-lipoprotein fraction from Bantu, controls and patients with I.H.D. The results which are presented give a qualitative and quantitative description of the fatty acids present in these fractions. All fatty acids have been identified as described in Part I, Chapter 2, Section III. The major fatty acids are defined as those fatty acids which have been positively identified, which can be estimated with a satisfactory degree of reproducibility and which, on the average, constitute more than 1% of the total fatty acids. In each lipid fraction the percentages of the major fatty acids have been compared statistically between the groups. In the phospholipid fraction certain other fatty acids have also been compared statistically between the groups.

(a) CHOLESTEROL ESTER FATTY ACID COMPOSITION

The results are shown in Tables 23 and 24 and in Figure 18.

	BANTU		CONTROL		I.H.D.	
	%		%		%	
Fatty Acid	Mean (S.D.)	Range	Mean (S.D.)	Range	Mean (S.D.)	Range
C12:0	0.2 (0.2)	tr- 0.5	0.3 (0.2)	tr- 0.6	0.3 (0.2)	tr- 0.5
C14:0	1.0 (0.9)	tr- 4.1	0.9 (0.2)	0.6- 1.2	0.9 (0.3)	tr- 1.4
C14:1	0.4 (0.3)	tr- 0.8	0.3 (0.2)	tr- 0.6	0.3 (0.3)	tr- 0.7
C15:0	0.4 (0.3)	tr- 1.4	0.2 (0.1)	tr- 0.4	0.4 (0.1)	tr- 0.6
C15:1	0.3 (0.2)	tr- 0.6	0.2 (0.1)	tr- 0.5	0.2 (0.2)	tr- 0.5
C16:0	14.1 (2.0)	11.5-19.5	11.8 (1.3)	9.4-13.6	12.2 (1.9)	8.9-14.9
C16:1	7.3 (2.3)	4.4-11.3	4.2 (0.6)	3.3- 5.8	4.4 (1.0)	2.9- 6.1
C17:0	0.6 (0.2)	tr- 1.1	0.2 (0.2)	tr- 0.7	0.4 (0.1)	tr- 0.6
C17:1	0.4 (0.3)	tr- 0.9	0.4 (0.2)	tr- 0.7	0.2 (0.2)	tr- 0.6
C18:0	2.1 (0.8)	1.2- 3.8	2.0 (0.6)	1.4- 3.4	1.4 (0.5)	0.7- 2.5
C18:1	27.7 (3.0)	23.8-34.0	21.7 (3.6)	15.7-30.5	18.8 (2.4)	15.2-23.1
C18:2	35.6 (3.9)	30.1-43.0	46.8 (4.3)	35.8-52.9	52.2 (5.2)	43.5-63.0
C18:3	0.5 (0.3)	tr- 1.3	0.8 (0.4)	tr- 1.3	0.7 (0.7)	tr- 2.4
C20:0	0.8 (0.7)	tr- 2.0	0.3 (0.2)	tr- 0.8	0.3 (0.3)	tr- 1.2
C20:3?	1.1 (0.8)	tr- 3.0	0.7 (0.3)	tr- 1.3	0.7 (0.5)	tr- 2.0
C20:4	7.5 (1.2)	5.6- 9.4	6.9 (1.3)	5.3- 8.9	5.8 (1.6)	4.0- 9.0
C20:5?	0.9 (0.5)	tr- 2.4	2.2 (0.8)	1.2- 4.1	1.5 (1.0)	0.6- 3.8

Table 23: The fatty acid composition of the cholesterol ester fraction of total serum in Bantu, controls and patients with I.H.D.

tr = trace amount, i.e. less than 0.5%.

The major fatty acids are underlined.

	BANTU			CONTROL			I.H.D.		
	%			%			%		
Fatty Acid	Mean	(S.D.)	Range	Mean	(S.D.)	Range	Mean	(S.D.)	Range
C12:0	0.2	(0.2)	tr- 0.6	0.3	(0.2)	tr- 0.6	0.2	(0.2)	tr- 0.5
C14:0	0.7	(0.4)	tr- 2.2	1.0	(0.1)	0.8- 1.1	0.7	(0.3)	tr- 1.3
C14:1	0.3	(0.2)	tr- 0.5	0.4	(0.3)	tr- 0.6	0.4	(0.3)	tr- 0.6
C15:0	0.3	(0.3)	tr- 1.2	0.2	(0.1)	tr- 0.4	0.3	(0.1)	tr- 0.6
C15:1	0.4	(0.3)	tr- 0.5	0.4	(0.2)	tr- 0.6	0.3	(0.2)	tr- 0.5
<u>C16:0</u>	13.8	(1.1)	11.8-15.8	12.1	(2.4)	9.5-18.5	12.1	(1.6)	8.7-14.4
<u>C16:1</u>	7.7	(2.1)	5.0-12.6	4.2	(0.6)	3.3- 5.1	4.4	(1.2)	3.0- 6.0
C17:0	0.5	(0.2)	tr- 0.8	0.3	(0.1)	tr- 0.5	0.3	(0.2)	tr- 0.7
C17:1	0.3	(0.2)	tr- 0.7	0.4	(0.2)	tr- 0.8	0.3	(0.1)	tr- 0.5
<u>C18:0</u>	1.9	(0.6)	1.2- 3.0	2.2	(1.4)	1.2- 6.1	1.3	(0.3)	0.9- 1.8
<u>C18:1</u>	28.2	(4.0)	23.0-35.1	21.3	(3.3)	15.6-27.1	18.2	(2.1)	14.7-21.7
<u>C18:2</u>	35.7	(4.4)	28.2-42.1	47.0	(5.5)	33.5-54.9	52.0	(4.6)	44.1-60.9
C18:3	0.5	(0.4)	tr- 1.6	1.0	(0.4)	0.5- 1.6	0.7	(0.7)	tr- 1.6
C20:0	0.5	(0.3)	tr- 1.3	0.4	(0.2)	tr- 0.7	0.4	(0.3)	tr- 0.8
C20:3?	1.0	(0.5)	tr- 2.2	0.6	(0.3)	tr- 1.1	0.6	(0.3)	tr- 0.9
<u>C20:4</u>	7.8	(1.5)	5.2-10.0	6.5	(1.3)	4.5- 8.4	6.3	(1.3)	4.4- 9.1
C20:5?	0.8	(0.4)	tr- 1.6	2.1	(1.3)	0.5- 5.0	1.4	(1.0)	tr- 2.9

Table 24: The fatty acid composition of the cholesterol ester fraction of β-lipoprotein in Bantu, controls and patients with I.H.D.

tr = trace amount, i.e. less than 0.5%.

The major fatty acids are underlined.

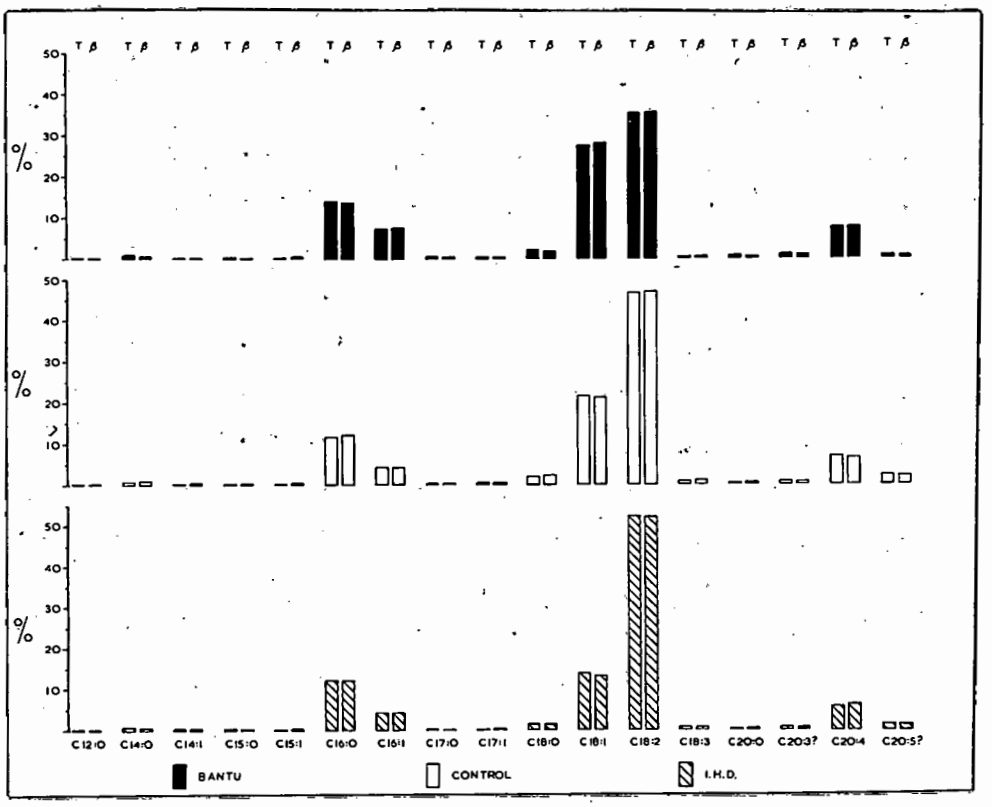


Figure 18. The fatty acid composition of the cholesterol ester fraction in total serum (T) and in the serum β-lipoprotein fraction (β) in each group.
The values shown are means.
Note the striking similarity in the fatty acid composition in total serum and in β-lipoprotein in each group.

The data in Tables 23 and 24 show that in both total serum and in β-lipoprotein the cholesterol ester fraction has a characteristic fatty acid pattern. In every sample analysed the same fatty acids appeared consistently and, despite differences between groups, in each group there is a predominance of unsaturated fatty acids, particularly C16:1, C18:1, C18:2 and C20:4. The proportion of saturated fatty acids, notably C16:0 and C18:0, is considerably lower and in all groups the sum of all saturated fatty acids seldom exceeds 20% of the total fatty acids (Table 25).

		Bantu	Control	I.H.D.
		%	%	%
		Mean (S.D.)	Mean (S.D.)	Mean (S.D.)
Saturated Fatty Acids	Total	19.1 (2.5)	15.5 (1.9)	15.2 (2.1)
	B	17.7 (1.6)	16.1 (3.7)	15.1 (1.7)
Unsaturated Fatty Acids	Total	80.6 (2.7)	83.3 (2.0)	84.1 (2.1)
	B	81.8 (1.7)	82.6 (3.8)	84.6 (1.6)

Table 25: The percentage of saturated and of unsaturated fatty acids in the cholesterol ester fraction in total serum (total) and in β-lipoprotein (B).

This high degree of unsaturation and particularly the high proportion of polyunsaturated fatty acids is characteristic of the cholesterol ester fatty acids and distinguishes them from triglyceride and phospholipid fatty acids. These findings are in agreement with early observations that cholesterol esters show a high degree of unsaturation (Bloor 1924; Channon and Collison 1929; Schaible 1932; Kelsey and Longenecker 1941; Bloor et al 1938).

In general the local data are remarkably similar to those found in studies on human serum using the techniques of alkali isomerization (Evans et al 1956;

Luddy et al 1958; Wright et al 1959; Kingsbury et al 1962a, 1962b; Bjorntorp et al 1962; Riley and Hunn 1960) and G.L-C. (Tuna et al 1958; Swell et al 1960a, 1960b; Hallgren et al 1960; Schrade et al 1961; Lawrie et al 1961) for analysis of fatty acids.

i) Comparison between total serum and β-lipoprotein

The data in Tables 23 and 24 and Figure 18 show that the same fatty acids appear consistently in total serum and in β-lipoprotein. Furthermore, in each group of subjects the proportion of each fatty acid is remarkably similar in total serum and in β-lipoprotein. This similarity was particularly marked when comparing the fatty acid patterns for each serum sample. The remarkable similarity between total serum and β-lipoprotein suggests that the α-lipoprotein fatty acid pattern is very similar to that of β-lipoprotein. This has been shown to be true for lipoproteins separated by Cohn fractionation (Böttcher and Woodford 1961) and by ultracentrifugation (Green et al 1960; Lindgren et al 1961; Cornwell et al 1962).

ii) Comparison between groups

Despite the general picture of a characteristic fatty acid pattern for cholesterol esters, differences are present between the three groups. The major fatty acids, viz. C16:0, C16:1, C18:0, C18:1, C18:2 and C20:4 together account for more than 95% of the total fatty acids (Table 26).

	Bantu	Control	I.H.D.
	Mean (S.D.)	Mean (S.D.)	Mean (S.D.)
Total serum	95.2 (1.9)	95.6 (0.8)	96.2 (1.2)
β-lipoprotein	95.9 (1.0)	95.4 (1.2)	96.7 (1.3)

Table 26: The percentage of the total fatty acids represented by the sum of the six major components.

The percentages of each of these fatty acids are compared between the groups in Table 27 and Figure 19.

Fatty Acid	Fraction	Bantu (B) % Mean (S.D.)	Control (C) % Mean (S.D.)	I.H.D. % Mean (S.D.)	I.H.D. vs.C p	I.H.D. vs.B p	C vs.B p
C16:0	Total	14.1 (2.0)	11.8 (1.3)	12.2 (1.9)	NS	<0.02	<0.01
	B	13.8 (1.1)	12.1 (2.4)	12.1 (1.6)	NS	<0.01	<0.05
C16:1	Total	7.3 (2.3)	4.2 (0.6)	4.4 (1.0)	NS	<0.001	<0.001
	B	7.7 (2.1)	4.2 (0.6)	4.4 (1.2)	NS	<0.001	<0.001
C18:0	Total	2.1 (0.8)	2.0 (0.6)	1.4 (0.5)	<0.02	<0.02	NS
	B	1.9 (0.6)	2.2 (1.4)	1.3 (0.3)	<0.05	<0.01	NS
C18:1	Total	27.7 (3.0)	21.7 (3.6)	18.8 (2.4)	<0.05	<0.001	<0.001
	B	28.2 (4.0)	21.3 (3.3)	18.2 (2.1)	<0.02	<0.001	<0.001
C18:2	Total	35.6 (3.9)	46.8 (4.3)	52.2 (5.2)	<0.01	<0.001	<0.001
	B	35.7 (4.4)	47.0 (5.5)	52.0 (4.6)	<0.01	<0.001	<0.001
C20:4	Total	7.5 (1.2)	6.9 (1.3)	5.8 (1.6)	NS	<0.01	NS
	B	7.8 (1.5)	6.5 (1.3)	6.3 (1.3)	NS	<0.02	<0.05

Table 27: Comparison of the percentages of the major fatty acids of the cholesterol ester fraction in total serum (total) and β-lipoprotein (B) in Bantu, controls and patients with I.H.D.

The percentages of C16:0, C16:1 and C20:4 are significantly higher for the Bantu than for both the other groups, but the values for patients and controls are similar. Although the percentage of C18:0 is lower for patients than for controls or Bantu, there is no grading between the groups. In contrast there is a graded decrease in the percentage of C18:1 and a graded increase in the percentage of C18:2 from Bantu through controls to patients. For both these fatty acids the value for each group differs from those of the other groups.

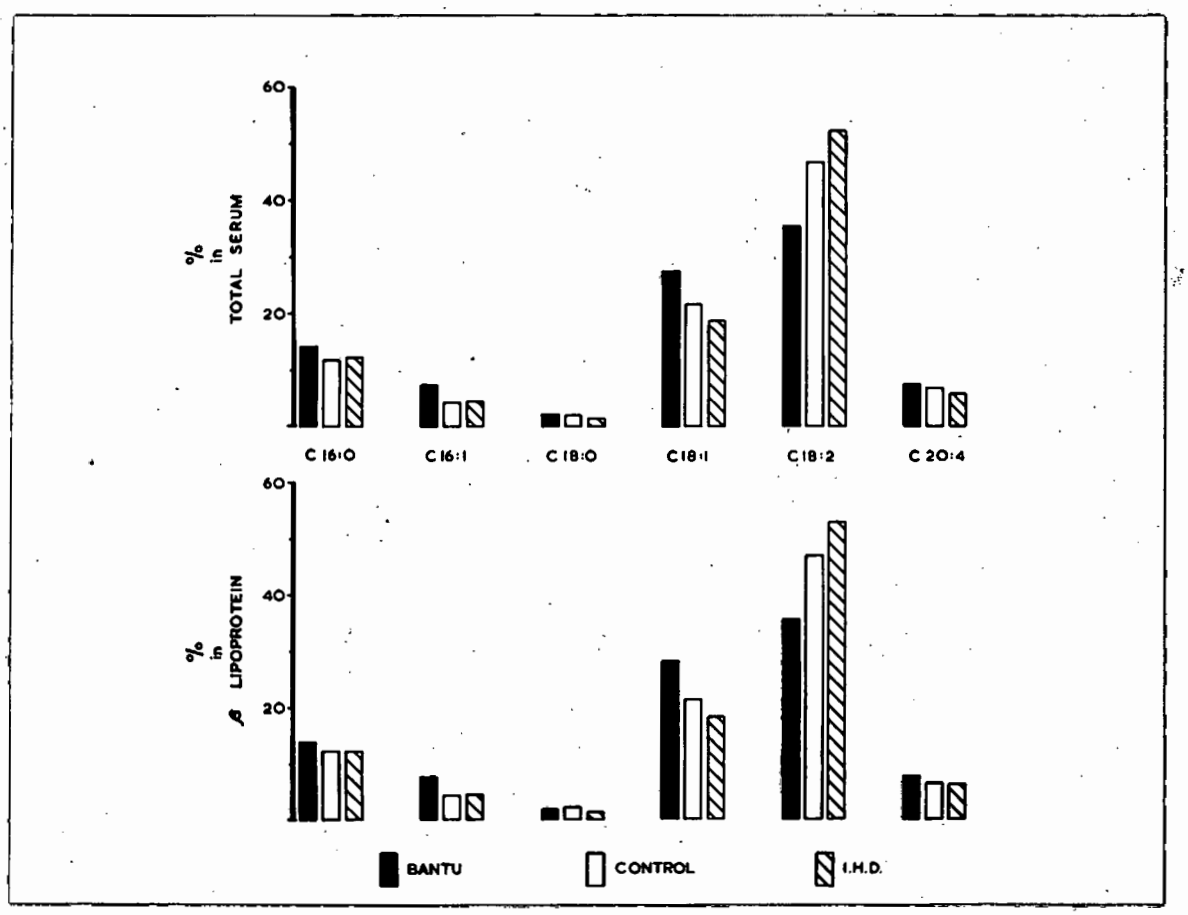


Figure 19. The percentages of the major fatty acids in the cholesterol ester fraction.
Note the reciprocal relationship between the percentages of C18:1 and C18:2 and the higher percentage of C16:1 in the Bantu group.

These data suggested that an increase in linoleic acid (C18:2) may be associated with a decrease in oleic acid (C18:1). This has been examined in the three groups by calculation of the correlation coefficient (Table 28, Figure 20).

Comparison	Fraction	Bantu		Control		I.H.D.	
		r	p	r	p	r	p
C18:1 ▼ C18:2	Total	-0.7142	<0.01	-0.9391	<0.001	-0.9209	<0.001
	β	-0.9014	<0.001	-0.7808	<0.01	-0.8249	<0.001
C16:1 ▼ C18:2	Total	-0.7139	<0.01	-0.6547	<0.02	-0.6054	<0.05
	β	-0.8039	<0.001	-0.5930	<0.05	-0.6281	<0.05
C20:4 ▼ C18:2	Total	0.2856	NS	0.1997	NS	0.4040	NS
	β	0.4435	NS	0.3842	NS	0.5174	NS
C16:0 ▼ C18:2	Total	-0.0037	NS	-0.2825	NS	-0.5602	NS
	β	-0.1140	NS	-0.6085	NS	-0.4503	NS
C18:0 ▼ C18:2	Total	0.0958	NS	-0.1506	NS	0.3919	NS
	β	0.3927	NS	-0.5847	NS	0.0943	NS

Table 28: The relationship between C18:2 and C18:1, C16:1, C20:4, C16:0 and C18:0 respectively.
r = Correlation coefficient.

It is apparent that there is a striking negative correlation between C18:2 and C18:1 in each group of subjects. However, since they are expressed as a percentage of the total, the proportions of all the fatty acids must be dependent on each other. In other words, if there is an increase in one fatty acid, one should expect that this would, arithmetically, give rise to

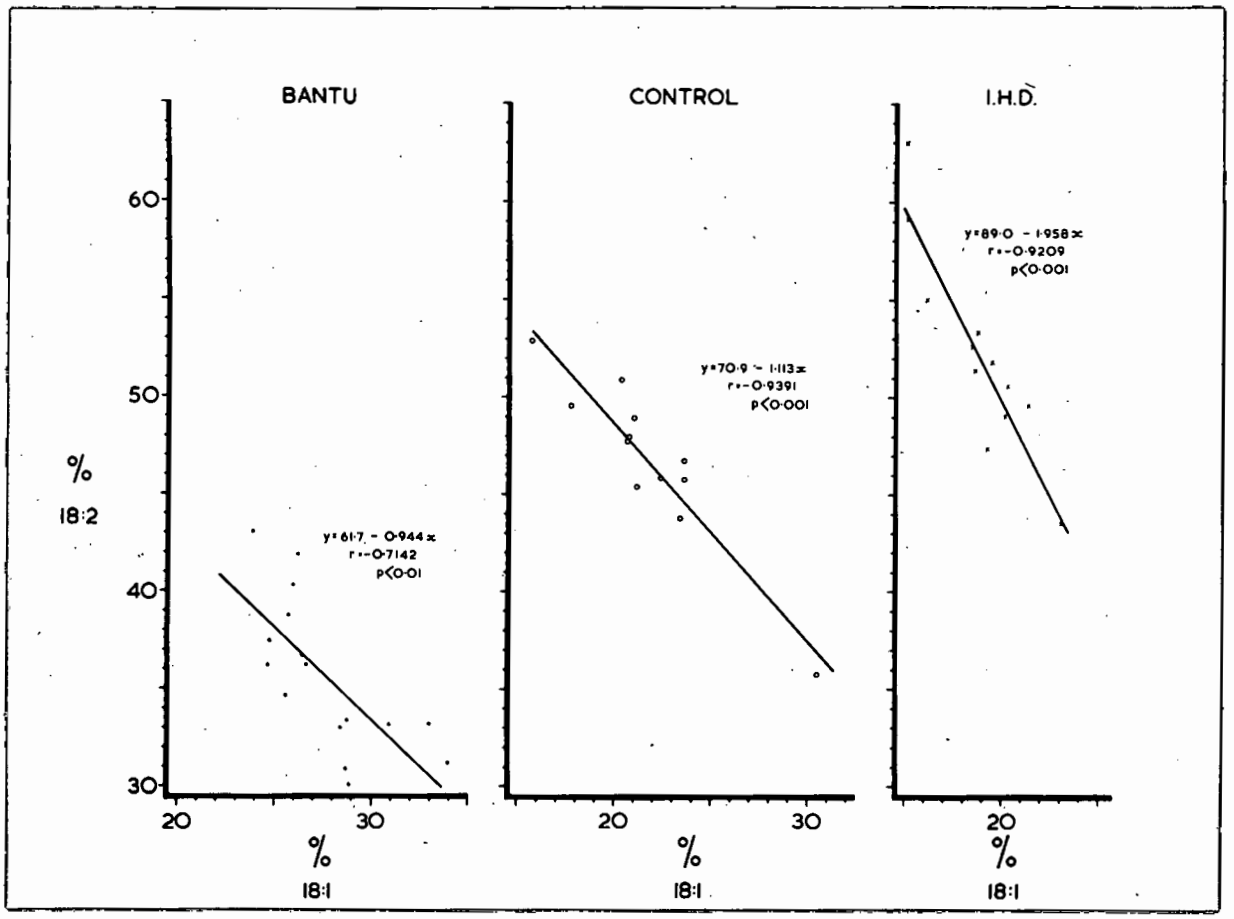


Figure 20. **The correlation between the percentages of C18:2 and C18:1 in the cholesterol ester fraction of total serum in each group.**

a decrease in the others. If, however, there is a preferential decrease in certain fatty acids, one would expect to find a more marked negative correlation for such fatty acids.

In order to examine whether the negative correlation between C18:2 and C18:1 is a true phenomenon or due merely to arithmetic, the correlation coefficients have been calculated between C18:2 and the four other major fatty acids (Table 28). The data in Table 28 show that there is no correlation in any group between the percentage of C18:2 and that of C16:0, C18:0 or C20:4 respectively. This suggests that any negative correlation is probably not due only to arithmetic. In addition to the negative correlation between C18:2 and C18:1 there is, however, a marked negative correlation in each group between the percentage of C18:2 and C16:1 (Figure 21). It appears that a decrease in the proportion of C18:2 is related to a selective increase in the proportions of C18:1 and C16:1.

It is tempting to offer some explanation for this observation. One might speculate that this reciprocal relationship between the proportions of C18:2 and both C18:1 and C16:1 reflects an attempt on the part of the organism to maintain the optimal degree of fluidity of cholesterol esters. There is evidence to show that in plants and animals the fatty acid pattern is influenced by environmental temperature (Dean and Hilditch 1933; Henriques and Hansen 1901; Cuthbertson and Tompsett 1933). Thus oil obtained from fish in tropical waters tends to be more saturated than that from fish in cold waters (Lovern 1935). Similarly, sunflower seed oil obtained from plants grown in cold climates yields a higher percentage of C18:2 than that obtained from plants in warmer climates (Deuel 1955a). It would seem that these plants and animal organisms attempt to maintain the degree of fluidity of their component esters at an optimal level by balancing the proportion of saturated and unsaturated fatty acids in response to environmental temperature.

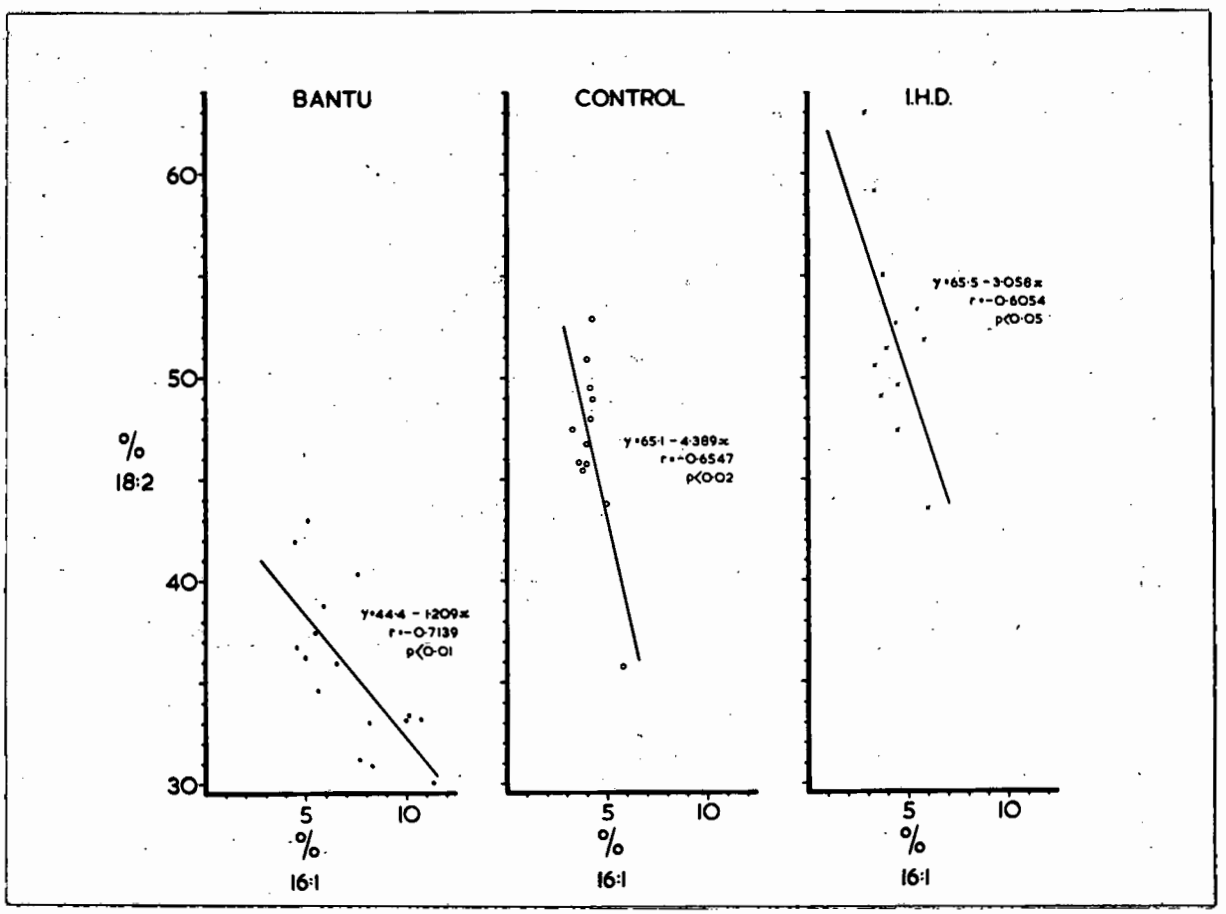


Figure 21. The correlation between the percentages of C18:2 and C16:1 in the cholesterol ester fraction of total serum in each group.

In humans, body temperature is 37°C. Cholesterol palmitate (melting point 81°C) and cholesterol stearate (melting point 80°C) are solids at body temperature, while the two cholesterol esters which liquefy at temperatures approaching that of the human body are cholesterol linoleate (melting point 43°C) and cholesterol oleate (melting point 49°C) (Cook 1958). The last two esters are the most abundant in the cholesterol ester fraction of serum. It would seem, therefore, that the human body may also attempt to regulate the degree of fluidity of its cholesterol esters. However, since the human body does not appear to be able to synthesize C18:2 (Lipsky et al 1957), the amount of linoleic acid available is dependent on the dietary factors. Both C18:1 and C16:1 (no melting point appears to have been determined for C16:1 but on the basis of its chain length it probably has a melting point lower than that of C18:1) can be synthesized (Mead 1958). It is possible, therefore, that since the amount of C18:2 is the limiting factor, the body may compensate for an unfavourable cholesterol ester fatty acid composition by synthesizing more or less C18:1 and C16:1 according to the amount of C18:2 available.

The reciprocal relationship observed between C18:2 and C18:1 and C18:2 and C16:1 may therefore be related to differences in diet in the three groups. This is in keeping with similar negative correlations between C18:2 and both C18:1 and C16:1 noted in the depot fat of males and females drawn from White and Bantu populations similar to those sampled here (Krut 1961).

The relationship between diet and serum fatty acid patterns will be discussed in Section III.

iii) <u>The relationship between the percentage of C18:2 and the concentration of cholesterol</u>

In discussing the increase in the percentage of C18:2 from Bantu through controls to patients, the possible role of C18:2 in the transport of cholesterol must be considered. There is evidence to show that conditions which

cause an increase in serum cholesterol level create an increased demand for essential fatty acids, chiefly linoleic acid (Holman and Peifer 1955; Peifer and Holman 1956; Holman 1960). On the basis of human and animal studies, several authors (Alfin-Slater et al 1954; Deuel 1955b; Peifer and Holman 1956; Aaes-Jørgensen 1961) have found support for the concept that essential fatty acids, chiefly linoleic acid, are required for the normal transport of cholesterol. In addition it has been postulated from experimental evidence in animals and man that cholesterol is removed from the plasma more easily if it is in the form of cholesterol linoleate (Boyd 1963). It would, on the basis of the above evidence, be reasonable to anticipate that, provided there is no limitation in the availability of linoleic acid in the diet, an increase in serum cholesterol level would be associated with an increase in the percentage of C18:2 in the cholesterol ester fraction. The relationship between the percentage of C18:2 and the concentration of esterified cholesterol provides correlative evidence in favour of this concept (Table 29, Figure 22).

		Bantu		Control		I.H.D.		All groups	
		r	p	r	p	r	p	r	p
% C18:2 vs Esterified cholesterol	Total	0.5301	<0.05	0.4297	NS	0.5504	NS	0.7845	<0.001
	β	0.3496	NS	0.5655	NS	0.5390	NS	0.7984	<0.001

Table 29: The relationship between the percentage of C18:2 and the concentration of esterified cholesterol in total serum and β-lipoprotein.

It can be seen that although there is no significant correlation in the control or patient groups, and a low degree of correlation for the Bantu, when the values for all subjects are considered there is a significant positive correlation between the percentage of C18:2 and the concentration of esterified

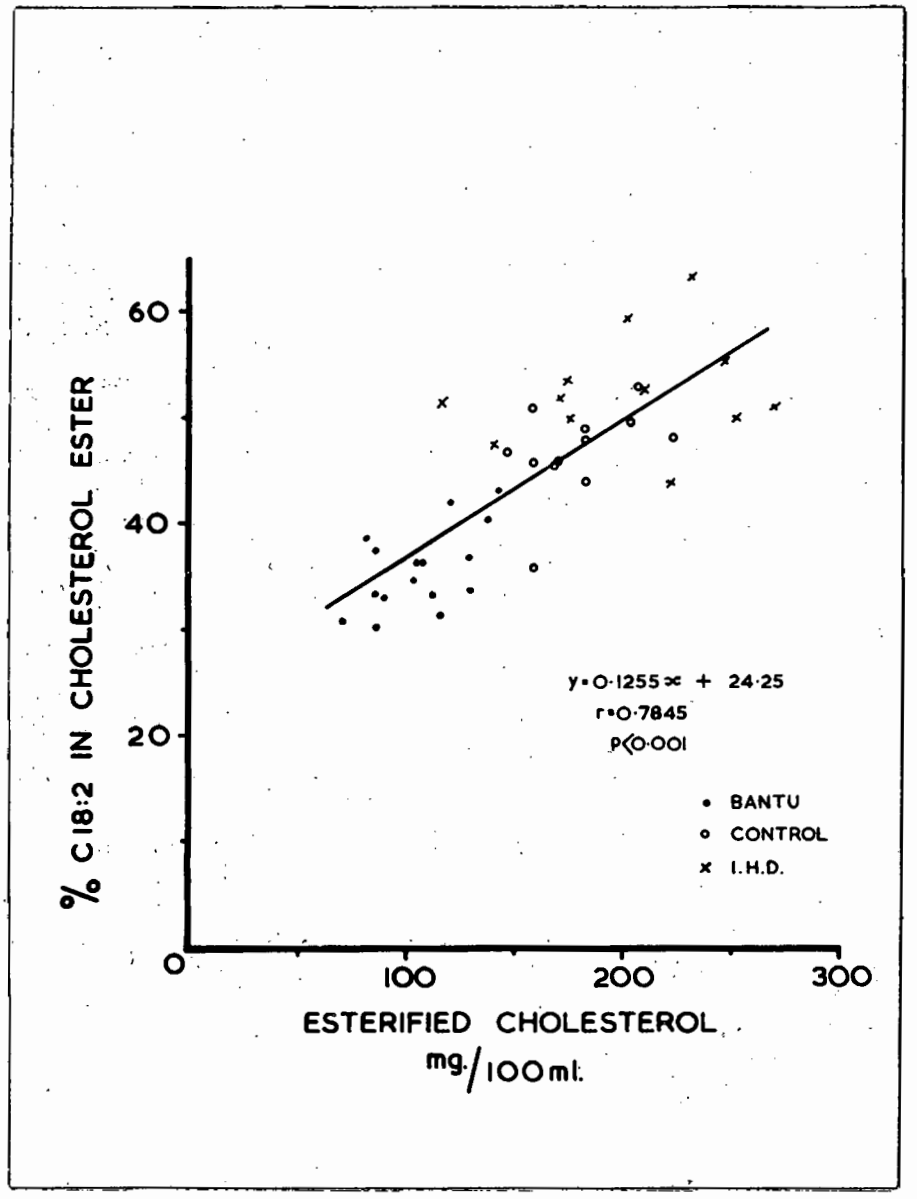


Figure 22: The correlation between the concentration of esterified cholesterol and the percentage of C18:2 in the cholesterol ester fraction of total serum in all subjects.

cholesterol. This suggests that where differences in cholesterol concentration are great, the increase in the concentration of cholesterol is associated with an increase in the percentage of C18:2.

This suggestion is partly supported by the data of Scott et al (1963). They found that New Yorkers with a mean serum cholesterol level of 212 mg.% had a mean linoleic acid percentage of 57.5% in the cholesterol ester fraction, whereas poor East Africans with a mean serum cholesterol level of 153 mg.% had a mean linoleic acid percentage of 41.0. However, in contradistinction to this they found that,despite similar cholesterol concentrations in New Yorkers and upper class East Africans (212 mg.% and 211 mg.% respectively), the percentage of C18:2 was considerably lower in the upper class East Africans than in the New Yorkers (57.5%). Also, despite marked differences in serum cholesterol concentration between poor and upper class East Africans (153 mg.% and 211 mg.% respectively) the mean levels of linoleic acid, 41.0% and 42.2%, were remarkably similar in these two groups. They suggested that these findings may reflect differences in the diet of the groups, in that the higher cholesterol linoleate value for New Yorkers may simply be in response to higher amounts of this fatty acid in the American diet. Their findings could therefore be regarded as illustrating the limitations imposed by the dietary level of linoleate in determining the proportions of C18:2 available for esterification with cholesterol. They did not, however, give any dietary data.

Fairhurst and Waterhouse (1963) showed a direct relationship between changes in cholesterol ester concentration and changes in cholesterol linoleate. Popjak (1946) noted an increase in the Iodine Value of the non-phospholipid fraction associated with an increase in the serum cholesterol concentration in rabbits. Contrary to these findings, an inverse relationship between the percentage of C18:2 in the cholesterol ester fraction and the concentration of serum cholesterol has been found by Swell et al (1960b), who compared these

parameters in children and older people, both White and Negro. They also did not report any dietary data. In a metabolism ward study we have noted (Krut and Young, unpublished observations) that feeding sunflower seed oil causes a decrease in serum cholesterol concentration with a concurrent increase in the proportion of linoleic acid in the cholesterol ester fraction. Feeding of butter causes an increase in serum cholesterol concentration and a decrease in the proportion of linoleic acid in the cholesterol ester fraction.

It would seem, therefore, that the relationship between the percentage of C18:2 in the cholesterol ester fraction and the serum cholesterol concentration is complex and may be influenced by several factors.

iv) Assessment of E.F.A. deficiency

The increase in the percentage of linoleic acid (C18:2) from Bantu through controls to patients has been stressed. It will be recalled that the mean level of arachidonic acid (C20:4) was lower for the patients than for either the controls or the Bantu (Table 27, Figure 19). While it is possible that the decreased proportion of C20:4 in the patient group may reflect a relative deficiency of E.F.A., in view of the high level of linoleic acid found for patients, this is highly improbable. The absence of grading for the percentage of C20:4 in the three groups is noteworthy in view of the very marked grading evident for C18:2, which has been shown to be the metabolic precursor of C20:4 (Mead 1960). The lower percentage of C20:4 for the patient group, which had the highest percentage of C18:2, could suggest that there may be some fault in the mechanism for converting C18:2 to C20:4. Another possibility is that in the patient group, more C18:2 may be converted to C20:4, but the metabolism and degradation of C20:4 may be greater than in the other groups. There is no evidence to support these suggestions which must therefore remain speculative.

It has been suggested that the minimum requirement of E.F.A. has been satisfied when the value for the ratio of trienoic to tetraenoic fatty acids is less than 0.4 (Holman 1960a). In the present study this ratio has been calculated as C18:3 + C20:3/C20:4. While both C18:3 and C20:4 have been positively identified, C20:3 could not be identified with absolute certainty. For the calculation of this ratio it has been assumed that the fatty acid tentatively identified as C20:3 is in fact this fatty acid. The mean values for the trienoic : tetraenoic fatty acid ratio in the Bantu, control and patient groups respectively are 0.22 ($\pm$ 0.13), 0.22 ($\pm$ 0.07) and 0.21 ($\pm$ 0.07) in total serum and 0.20 ($\pm$ 0.07), 0.24 ($\pm$ 0.13) and 0.21 ($\pm$ 0.15) in β-lipoprotein. These values are similar in the three groups and are considerably lower than 0.4. Using this parameter there is no evidence of E.F.A. deficiency in any group.

v) Comparison with other data

The data of other authors provides conflicting views on the proportions of C18:2 and C20:4 in the cholesterol ester fatty acids of different groups of people. Lewis (1958) reported that the percentage of dienes was lower for subjects with I.H.D. than for either healthy White subjects or Bantu, and the percentage of tetraenes showed a graded decrease from Bantu through White subjects to patients. Kingsbury et al (1962a) found that the percentages of C18:2 and C20:4 were lower and the trienoic : tetraenoic acid ratio was higher in the serum of atherosclerotic subjects when compared with that of controls. Similar findings were reported by Schrade et al (1961a).

In contrast to these findings other authors found no difference in the proportions of C18:2 and C20:4 when comparing patients with I.H.D. and controls (James et al 1957; Caren and Corbo 1958; Smith 1962). Similarly Scott et al (1963) reported that New Yorkers had a higher percentage of C18:2 in the cholesterol

ester fraction than did poor East Africans, who have a lower susceptibility to I.H.D. than the New Yorkers. The inconsistency of the findings in various studies suggests that the differences between groups may reflect other influences, such as that of the diet, and that these parameters have no relationship to differences in susceptibility to I.H.D. is reflected by the data in the present study.

(b) TRIGLYCERIDE FATTY ACID COMPOSITION

The results are shown in Tables 30 and 31 and in Figure 23.

	BANTU			CONTROL			I.H.D.		
	%			%			%		
Fatty Acid	Mean	(S.D.)	Range	Mean	(S.D.)	Range	Mean	(S.D.)	Range
C12:0	0.3	(0.2)	tr- 0.6	0.2	(0.1)	tr- 0.5	0.3	(0.2)	tr- 0.7
C14:0	2.2	(0.5)	1.4- 3.0	2.1	(0.6)	1.4- 3.2	3.2	(1.5)	1.3- 5.2
C14:1	0.2	(0.1)	tr- 0.6	0.3	(0.1)	tr- 0.7	0.3	(0.2)	tr- 0.6
C15:0	0.6	(0.3)	tr- 1.2	1.0	(0.5)	tr- 1.7	0.6	(0.2)	tr- 0.9
C15:1	0.3	(0.2)	tr- 0.5	0.4	(0.3)	tr- 0.7	0.3	(0.2)	tr- 0.6
C16:0	28.5	(2.9)	23.1-32.8	28.4	(3.9)	22.7-35.8	28.9	(2.5)	24.4-32.2
C16:1	7.2	(1.3)	4.9-10.2	4.9	(0.9)	2.9- 6.4	6.1	(1.1)	4.6- 8.1
C17:0	0.8	(0.3)	tr- 1.3	0.8	(0.3)	tr- 1.3	0.7	(0.2)	tr- 1.1
C17:1	0.5	(0.2)	tr- 1.0	0.5	(0.2)	tr- 0.8	0.4	(0.1)	tr- 0.6
C18:0	6.7	(1.6)	4.0-10.9	5.7	(1.2)	3.7- 7.5	5.1	(1.5)	3.6- 9.1
C18:1	39.5	(4.8)	30.7-46.7	40.4	(3.1)	35.9-44.6	35.8	(3.6)	29.0-44.1
C18:2	10.1	(2.6)	5.6-14.0	13.4	(4.0)	5.9-21.0	15.3	(5.6)	8.0-24.3
C20:0	0.7	(0.2)	tr- 1.1	1.1	(0.4)	0.5- 1.7	0.8	(0.3)	tr- 1.4
C20:4	2.1	(1.1)	1.0- 4.1	1.3	(0.4)	0.6- 1.7	1.4	(0.5)	0.8- 2.5

Table 30: The fatty acid composition of the triglyceride fraction of total serum in Bantu, controls and in patients with I.H.D.

tr = trace amount, i.e. less than 0.5%.

The major fatty acids are underlined.

Fatty Acid	BANTU % Mean (S.D.)	BANTU % Range	CONTROL % Mean (S.D.)	CONTROL % Range	I.H.D. % Mean (S.D.)	I.H.D. % Range
C12:0	0.3 (0.2)	tr- 0.5	0.2 (0.1)	tr- 0.6	0.2 (0.1)	tr- 0.6
C14:0	1.7 (0.6)	0.8- 2.9	2.2 (0.2)	0.6- 3.2	2.9 (1.4)	1.2- 5.3
C14:1	0.2 (0.1)	tr- 0.6	0.3 (0.1)	tr- 0.6	0.3 (0.2)	tr- 0.7
C15:0	0.5 (0.2)	tr- 0.9	1.2 (0.6)	tr- 2.2	0.6 (0.2)	tr- 0.9
C15:1	0.2 (0.1)	tr- 0.6	0.3 (0.2)	tr- 0.7	0.4 (0.2)	tr- 0.6
C16:0	28.4 (3.1)	23.3-35.8	26.7 (3.7)	21.2-35.6	29.2 (3.3)	24.0-34.9
C16:1	7.0 (1.7)	4.8-11.4	5.3 (1.2)	3.6- 6.9	6.4 (1.1)	4.1- 8.3
C17:0	0.8 (0.3)	tr- 1.1	0.9 (0.3)	tr- 1.6	0.8 (0.4)	tr- 1.7
C17:1	0.6 (0.1)	tr- 0.8	0.5 (0.2)	tr- 1.0	0.4 (0.2)	tr- 0.7
C18:0	5.6 (1.3)	3.8- 8.4	5.6 (1.4)	3.8- 8.6	5.0 (1.2)	3.4- 7.4
C18:1	40.9 (4.2)	35.5-47.6	39.8 (3.0)	34.7-46.3	35.1 (3.4)	30.7-44.2
C18:2	10.5 (3.3)	4.7-15.6	14.5 (4.9)	6.7-21.4	15.7 (5.1)	9.0-24.2
C20:0	0.7 (0.3)	tr- 1.7	1.1 (0.2)	0.8- 1.5	0.8 (0.2)	tr- 1.2
C20:4	2.1 (0.8)	1.1- 3.9	1.5 (0.6)	0.8- 2.5	1.4 (0.4)	0.9- 2.3

Table 31: The fatty acid composition of the triglyceride fraction of β-lipoprotein in Bantu, controls and in patients with I.H.D.
tr = trace amount, i.e. less than 0.5%.
The major fatty acids are underlined.

The data in Tables 30 and 31 show clearly that there is a characteristic fatty acid pattern for the triglyceride fraction in both total serum and β-lipoprotein. In all samples analysed, the same fatty acids appeared consistently, although some were present in trace amounts only. While differences are apparent between the groups, in every subject from each group the most abundant fatty acids are C16:0 and C18:1. Other fatty acids present in relatively large proportions are C16:1, C18:0 and C18:2. Palmitic acid and oleic acid are commonly found in abundance in many plant and animal triglycerides, and palmitoleic, stearic and linoleic acids are likewise present in many natural triglycerides (Hilditch 1956). The fatty acids of human serum triglyceride are thus not unlike those found in other naturally occurring triglycerides.

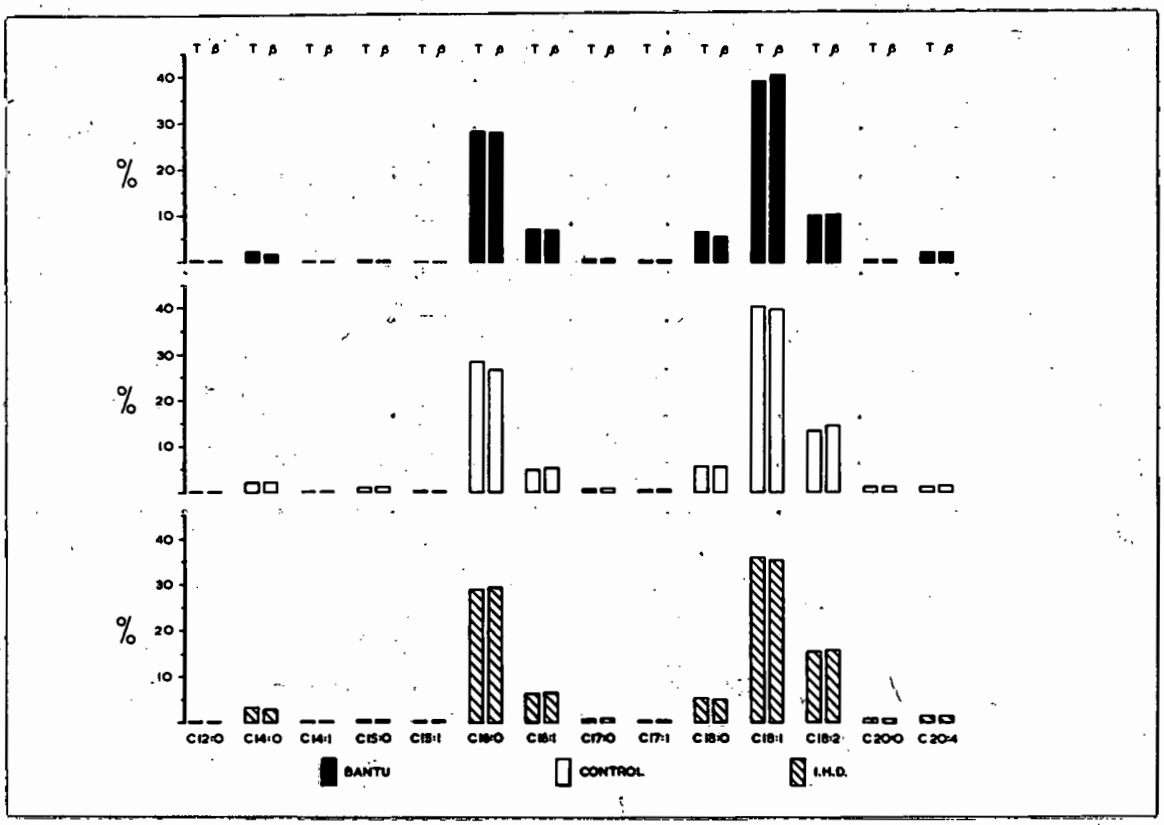


Figure 23. The fatty acid composition of the triglyceride fraction in total serum (T) and β-lipoprotein (β) in each group.

The values shown are means.

Note the striking similarity in the fatty acid composition in total serum and in β-lipoprotein in each group.

The proportion of saturated fatty acids is lower than that of unsaturated fatty acids, of which the mono-unsaturated fatty acids constitute the major portion (Table 32).

		BANTU %	CONTROL %	I.H.D. %
	Fraction	Mean (S.D.)	Mean (S.D.)	Mean (S.D.)
Saturated Fatty Acids	Total	38.7 (3.2)	38.1 (4.2)	39.1 (5.1)
	B	37.2 (3.3)	36.7 (4.3)	39.0 (5.3)
Unsaturated Fatty Acids	Total	59.8 (3.4)	61.2 (4.3)	60.1 (5.5)
	B	61.5 (3.3)	62.3 (4.7)	60.0 (5.5)
Mono-unsaturated fatty acids	Total	46.6 (5.3)	45.3 (2.8)	41.9 (3.5)
	B	47.9 (4.3)	45.2 (2.7)	41.5 (3.2)
Poly-unsaturated fatty acids	Total	13.2 (3.3)	16.0 (4.5)	18.2 (5.7)
	B	13.5 (3.8)	17.2 (4.5)	18.6 (5.4)

Table 32: The percentage of saturated, unsaturated, mono-unsaturated and poly-unsaturated fatty acids in the triglyceride fraction in total serum (Total) and β-lipoprotein (B).

This high content of mono-unsaturated fatty acids is characteristic of the triglyceride fraction and distinguishes it from the cholesterol esters and the phospholipids (Sections (a) and (c)). In general, the local data are remarkably similar to those obtained by other authors who analysed the triglyceride fatty acids by alkali isomerization (Luddy et al 1958; Schrade et al 1959; Bjorntorp 1960) and G.L.C. (Lawrie et al 1961; Schrade et al 1961a, 1961b; Hallgren et al 1960; Lindgren et al 1961).

i) Comparison between total serum and β-lipoprotein

There is again a striking similarity between the fatty acid patterns of total serum and β-lipoprotein. Not only are the component fatty acids identical in both, but the proportions of each fatty acid are remarkably similar within each group of subjects (Figure 23). Since the major portion of total serum triglyceride is associated with β-lipoprotein (Chapter 1, Section I, Table 21), one would have anticipated that the fatty acid pattern of total serum and β-lipoprotein would be fairly similar. The almost identical results obtained suggest that the fatty acid pattern of triglyceride in serum ∝-lipoprotein is very similar to that of β-lipoportein. This is confirmed by the work of Lindgren et al (1961) who found very similar triglyceride fatty acid patterns in the ultracentrifugally separated Sf10-20 and high density lipoproteins, which correspond closely to β-lipoproteins and ∝-lipoproteins respectively. Böttcher and Woodford (1961) reported that the triglyceride fatty acid pattern was virtually identical in ∝- and β-lipoproteins separated by Cohn fractionation although they noted a tendency for the oleic acid content to be slightly higher in the β-lipoprotein. There is no support for this latter observation in the local data.

ii) Comparison between groups

While there is a characteristic fatty acid pattern for triglycerides, certain differences are present between the groups. The major fatty acids, viz. C14:0, C16:0, C16:1, C18:0, C18:1, C18:2 and C20:4 together account for more than 95% of the total fatty acids (Table 33).

	Bantu	Control	I.H.D.
	Mean (S.D.)	Mean (S.D.)	Mean (S.D.)
Total serum	96.3 (0.9)	96.3 (0.9)	95.8 (1.8)
β-lipoprotein	96.2 (0.9)	95.5 (1.7)	95.8 (1.4)

Table 33: The percentage of the total fatty acids represented by the sum of the seven major components.

Comparison of the percentages of each of these fatty acids in the three groups is shown in Table 34 and Figure 24.

Fatty acid	Fraction	Bantu (B) % Mean (S.D.)	Control (C) % Mean (S.D.)	I.H.D. % Mean (S.D.)	I.H.D vs.C p	I.H.D. vs.B p	C. vs.B p
C14:0	Total	2.2 (0.5)	2.1 (0.6)	3.2 (1.5)	<0.05	<0.05	NS
	B	1.7 (0.6)	2.2 (0.2)	2.9 (1.4)	NS	<0.01	NS
C16:0	Total	28.5 (2.9)	28.4 (3.9)	28.9 (2.5)	NS	NS	NS
	B	28.4 (3.1)	26.7 (3.7)	29.2 (3.3)	NS	NS	NS
C16:1	Total	7.2 (1.3)	4.9 (0.9)	6.1 (1.1)	<0.01	<0.05	<0.001
	B	7.0 (1.7)	5.3 (1.2)	6.4 (1.1)	<0.05	NS	<0.01
C18:0	Total	6.7 (1.6)	5.7 (1.2)	5.1 (1.5)	NS	<0.02	NS
	B	5.6 (1.3)	5.6 (1.4)	5.0 (1.2)	NS	NS	NS
C18:1	Total	39.5 (4.8)	40.4 (3.1)	35.8 (3.6)	<0.01	<0.05	NS
	B	40.9 (4.2)	39.8 (3.0)	35.1 (3.4)	<0.01	<0.001	NS
C18:2	Total	10.1 (2.6)	13.4 (4.0)	15.3 (5.6)	NS	<0.01	<0.02
	B	10.5 (3.3)	14.5 (4.9)	15.7 (5.1)	NS	<0.01	<0.02
C20:4	Total	2.1 (1.1)	1.3 (0.4)	1.4 (0.5)	NS	<0.05	<0.05
	B	2.1 (0.8)	1.5 (0.6)	1.4 (0.4)	NS	<0.05	NS

Table 34: Comparison of the percentages of the major fatty acids of the triglyceride fraction in total serum (Total) and β-lipoprotein (B) in Bantu, controls and patients with I.H.D.

While the percentage of C14:0 tends to be higher for the patient group than for the other two groups, the percentages of C16:0 and C18:0 are generally similar in all groups. The content of saturated fatty acids is thus rather constant. The percentage of C16:1 is not markedly different for patients and Bantu, but the value for controls is lower than in the other two groups. For oleic acid the mean value is lower for patients than for either Bantu or

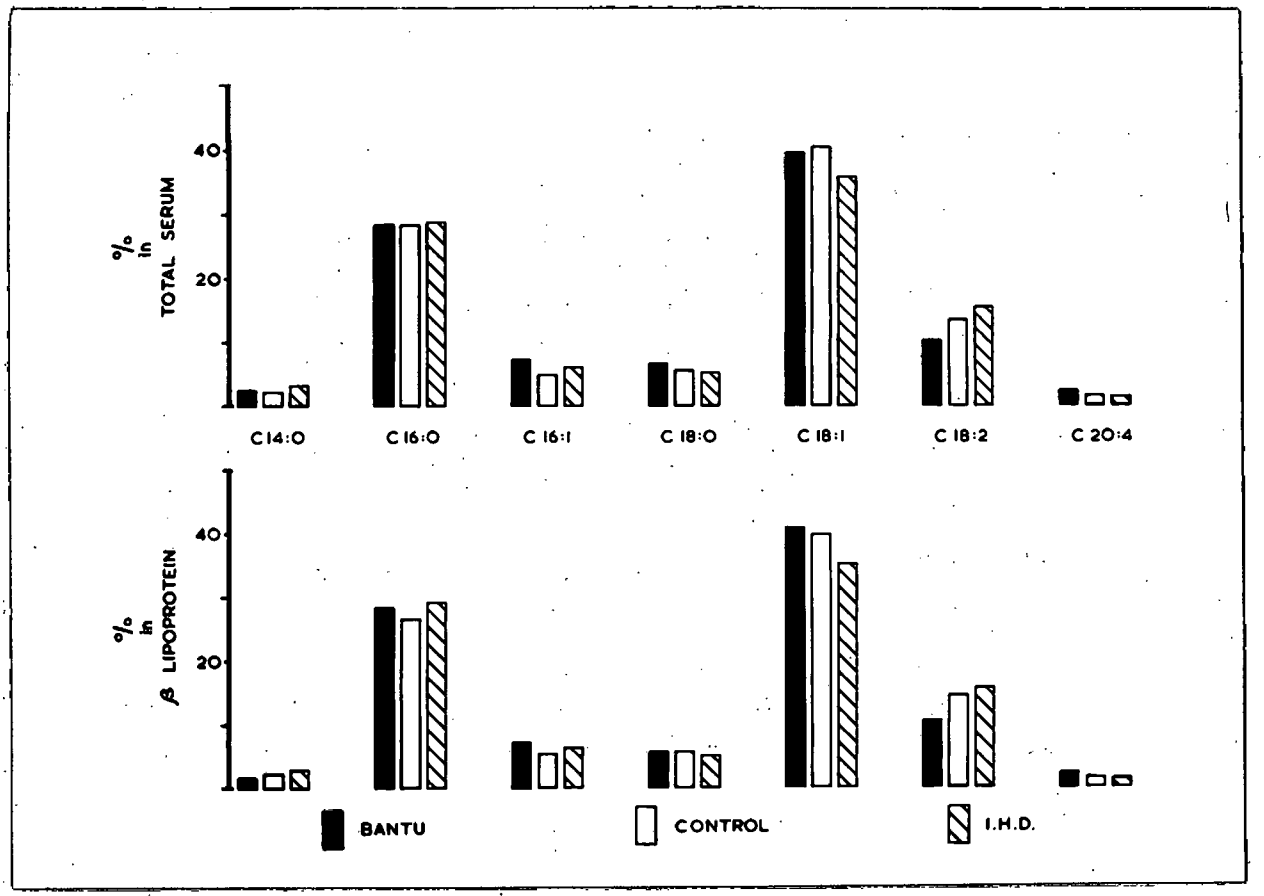


Figure 24. **The percentages of the major fatty acids in the triglyceride fraction.**
There is a trend for the percentage of C18:2 to increase from Bantu through controls to patients with I.H.D.

controls , but in the latter groups the mean values are similar. Neither of these fatty acids show any grading between the groups. Similarly, the Bantu have a higher percentage of C20:4 than the other groups but the values for patients and controls are similar. There is a tendency for a graded increase in the percentage of C18:2 from Bantu through controls to patients, but while the mean value for controls is lower than that for patients, the difference is not significant. The only fatty acid which separates patients from both other groups is C18:1. It is possible that the lower percentage of C18:1 for the patients may be due to their increased level of C18:2. This, together with the tendency towards an increase in linoleic acid percentage from Bantu through controls to patients may be related to dietary factors and will be discussed in Section III. There is no significant graded relationship between the three groups and, in general, the similarity between the groups is more noteworthy than the difference.

iii) <u>The relationship between the percentage of C18:2 and the percentages of C16:1 and C18:1</u>

It will be recalled that in the cholesterol ester fraction the percentage of C18:2 was inversely related to that of C16:1 and of C18:1 in each group of subjects. In the triglyceride these fatty acids again showed some differences between the groups. The relationship between these fatty acids has been examined by calculation of the correlation coefficient (Table 35).

Comparison	Fraction	Bantu		Control		I.H.D.	
		r	p	r	p	r	p
C16:1 ▼ C18:2	Total	-0.2366	NS	0.1485	NS	-0.5546	NS
	B	-0.1378	NS	-0.1803	NS	-0.4207	NS
C18:1 ▼ C18:2	Total	-0.7956	<0.001	-0.3640	NS	-0.1187	NS
	B	-0.6712	<0.01	-0.4062	NS	-0.0110	NS

Table 35: The relationship between the percentages of C18:2 and C18:1 and C18:2 and C16:1 in total serum (Total) and β-lipoprotein(B).
r = Correlation coefficient.

The percentages of C16:1 and C18:2 are not related in any group. There is a reciprocal relationship between the percentages of C18:1 and C18:2 in the Bantu group only. This may be related to dietary factors and will be discussed in Section III.

iv) Comparison with other data

Schrade and his co-workers (1959, 1961a) compared the serum triglyceride fatty acid composition of healthy subjects with that of patients with atherosclerosis and found no significant differences between them. Böttcher and Woodford (1961) reported that the triglyceride fatty acid composition of α - and β-lipoprotein was similar in healthy young men and in patients with atherosclerosis. In contrast to the findings of the present study, the data of Antonis and Bersohn (1960) showed that subjects with I.H.D. had a lower proportion of polyenoic fatty acids than either healthy White controls or Bantu and that there was a tendency for a graded decrease in the percentage of dienoic acids from Bantu through White controls to subjects with I.H.D. Moreover, the mean values for dienoic and tetraenoic acids in all groups were considerably higher than those found here and elsewhere. The groups studied by Antonis and Bersohn were drawn from populations similar to those of the present study, and the concentration of triglyceride in the various groups was remarkably similar in the two studies. In view of these similarities, the differences in the triglyceride fatty acid compositions in the two studies are puzzling. Differences in the techniques of alkali isomerization and G.L.C. are unlikely to account for the marked differences observed. Differences in diet may also be responsible for the different results obtained, since it has been shown that the serum triglyceride fatty acid composition is markedly affected by the composition of the dietary fat (Ahrens et al 1959). It is, however, noteworthy that there is very good agreement between the local data

and that for comparable groups reported by authors in European countries (Böttcher and Woodford 1961; Schrade et al 1961; Hallgren et al 1960) and in the U.S.A. (Lindgren et al 1961). Dietary differences between subjects in Cape Town and Johannesburg are therefore unlikely to account for the findings of Antonis and Bersohn (1960), which differ from all other data reported to date.

The data of Scott et al (1963) who compared the serum triglyceride fatty acid composition in New Yorkers with a high susceptibility to I.H.D. and upper class and poor East Africans with a low susceptibility to the disease, are similar to those reported here. They found that the only significant difference between these groups was in the percentage of C18:2, which was significantly higher in the New Yorkers. They suggest that these findings may be related to differences in dietary habits and this may also be the case in the present study.

v) Comparison with depot fat triglyceride

Depot fat lipid is more than 99% triglyceride (Hirsch et al 1960) and a large proportion of this triglyceride pool is derived from exogenous fatty acids (Jeanrenaud 1961; Vaughan 1961). It is noteworthy that the triglyceride fatty acid pattern in the serum and β-lipoprotein of the three groups studied here is essentially similar to that in depot fat in comparable groups of subjects (Krut 1961). This is in agreement with the opinion expressed by Cornwell et al (1962) who compared depot fat and serum triglyceride fatty acid compositions from various laboratories.

(c) PHOSPHOLIPID FATTY ACID COMPOSITION

The results are shown in Tables 36 and 37 and in Figure 25.

	BANTU			CONTROL			I.H.D.		
	%			%			%		
Fatty Acid	Mean	(S.D.)	Range	Mean	(S.D.)	Range	Mean	(S.D.)	Range
C14:0	0.6	(0.2)	tr- 1.0	0.9	(0.5)	tr- 1.5	0.7	(0.2)	tr- 1.0
C14:1	0.2	(0.1)	tr- 0.5	0.3	(0.2)	tr- 0.6	0.3	(0.2)	tr- 0.5
C15:0	0.3	(0.2)	tr- 0.7	0.6	(0.3)	tr- 1.0	0.3	(0.1)	tr- 0.6
C15:1	0.2	(0.1)	tr- 0.5	0.2	(0.1)	tr- 0.5	0.3	(0.2)	tr- 0.6
C16:0	34.5	(2.5)	27.5-37.9	31.7	(2.4)	29.0-36.1	34.0	(2.2)	31.0-37.1
C16:1	2.1	(0.4)	1.6- 2.9	1.7	(0.4)	1.1- 2.5	1.8	(0.2)	1.4- 2.2
C17:0	0.6	(0.3)	tr- 1.2	0.7	(0.3)	tr- 1.5	0.6	(0.2)	tr- 0.9
C17:1	0.3	(0.1)	tr- 0.5	0.2	(0.1)	tr- 0.5	0.3	(0.1)	tr- 0.6
C18:0	15.0	(1.8)	11.7-17.5	14.3	(0.9)	12.9-16.2	15.1	(1.2)	13.3-17.4
C18:1	15.3	(2.7)	11.4-19.8	10.6	(1.7)	9.0-15.4	11.0	(1.6)	9.1-13.6
C18:2	13.9	(1.9)	9.6-17.2	17.4	(1.2)	15.9-19.2	18.6	(3.2)	12.7-24.3
C20:0	0.7	(0.3)	tr- 1.5	1.0	(0.4)	0.6- 1.7	0.8	(0.3)	0.5- 1.4
C21:0	0.5	(0.2)	tr- 0.9	0.5	(0.3)	tr- 1.0	0.5	(0.4)	tr- 2.0
C20:3 ?	2.5	(0.5)	1.5- 3.4	2.2	(0.7)	1.6- 4.1	1.9	(0.6)	1.0- 2.8
C20:4	8.2	(1.3)	5.1-10.3	9.1	(1.3)	6.6-11.1	7.0	(1.5)	4.5- 9.5
C22:0	0.6	(0.5)	tr- 1.7	0.8	(0.4)	tr- 1.6	0.6	(0.5)	tr- 1.6
C20:5?	0.7	(0.2)	tr- 1.4	1.8	(0.9)	0.8- 4.0	1.3	(0.9)	tr- 3.1
C23:0	0.5	(0.2)	tr- 0.8	0.3	(0.1)	tr- 0.6	0.5	(0.2)	tr- 0.7
C24:0	0.7	(0.3)	tr- 1.0	1.4	(0.4)	0.6- 2.1	0.8	(0.6)	tr- 1.7
C24:1	1.2	(0.6)	tr- 2.7	1.6	(0.3)	0.7- 2.0	0.8	(0.7)	tr- 1.8
C22:6?	1.3	(0.6)	tr- 2.6	3.4	(0.9)	2.4- 5.0	2.4	(1.3)	tr- 4.3

Table 36: The fatty acid composition of the phospholipid fraction of total serum in Bantu, controls and patients with I.H.D.

tr = trace amount, i.e. less than 0.5%

The major fatty acids are underlined.

Fatty Acid	BANTU % Mean	(S.D.)	Range	CONTROL % Mean	(S.D.)	Range	I.H.D. % Mean	(S.D.)	Range
C14:0	0.8	(0.3)	tr- 1.5	1.1	(0.3)	0.8- 1.8	0.7	(0.2)	tr- 0.9
C14:1	0.3	(0.2)	tr- 0.6	0.3	(0.2)	tr- 0.5	0.2	(0.1)	tr- 0.5
C15:0	0.4	(0.2)	tr- 0.9	0.8	(0.5)	tr- 2.0	0.2	(0.1)	tr- 0.5
C15:1	0.2	(0.1)	tr- 0.5	0.2	(0.1)	tr- 0.5	0.3	(0.2)	tr- 0.6
C16:0	35.3	(3.8)	29.7-46.0	32.2	(1.6)	29.8-35.4	33.5	(3.0)	28.2-38.2
C16:1	2.6	(0.7)	1.8- 4.6	1.5	(0.4)	0.9- 2.2	1.8	(0.5)	1.3- 2.8
C17:0	0.7	(0.3)	tr- 1.2	0.9	(0.6)	0.6- 1.3	0.7	(0.4)	tr- 1.9
C17:1	0.4	(0.1)	tr- 0.6	0.3	(0.1)	tr- 0.5	0.2	(0.1)	tr- 0.5
C18:0	14.3	(2.4)	9.0-19.4	14.0	(1.5)	12.5-18.1	14.1	(1.0)	12.9-15.9
C18:1	14.8	(2.1)	11.7-17.9	11.3	(1.7)	9.8-16.1	11.3	(1.5)	8.7-14.3
C18:2	11.8	(2.0)	8.7-16.3	17.4	(1.8)	14.7-19.8	18.3	(3.9)	13.3-26.1
C20:0	1.0	(0.5)	tr- 2.1	1.1	(0.3)	tr- 1.4	0.8	(0.2)	tr- 1.1
C21:0	0.7	(0.4)	tr- 1.4	0.6	(0.2)	tr- 1.2	0.5	(0.4)	tr- 1.2
C20:3?	2.2	(0.7)	1.2- 3.5	2.1	(0.6)	0.9- 3.0	2.1	(0.7)	1.4- 3.2
C20:4	7.1	(1.5)	4.6-10.4	8.3	(1.2)	6.5- 9.7	6.9	(0.9)	5.6- 8.3
C22:0	1.0	(0.9)	tr- 3.7	0.5	(0.5)	tr- 1.3	0.3	(0.3)	tr- 0.9
C20:5?	0.8	(0.2)	tr- 1.1	1.5	(0.7)	tr- 3.8	1.2	(1.1)	tr- 3.8
C23:0	0.6	(0.3)	tr- 0.9	0.4	(0.2)	tr- 0.7	0.4	(0.2)	tr- 0.6
C24:0	1.5	(0.6)	0.7- 2.7	1.8	(0.4)	1.1- 2.9	1.0	(0.7)	tr- 2.3
C24:1	1.8	(1.1)	tr- 4.2	2.2	(0.4)	1.6- 3.1	1.5	(1.1)	tr- 3.9
C22:6?	1.1	(0.6)	tr- 2.5	3.2	(1.3)	1.2- 5.1	2.4	(1.0)	0.9- 4.2

Table 37: The fatty acid composition of the phospholipid fraction of β-lipoprotein in Bantu, controls and patients with I.H.D.

tr = trace amount, i.e. less than 0.5%

The major fatty acids are underlined.

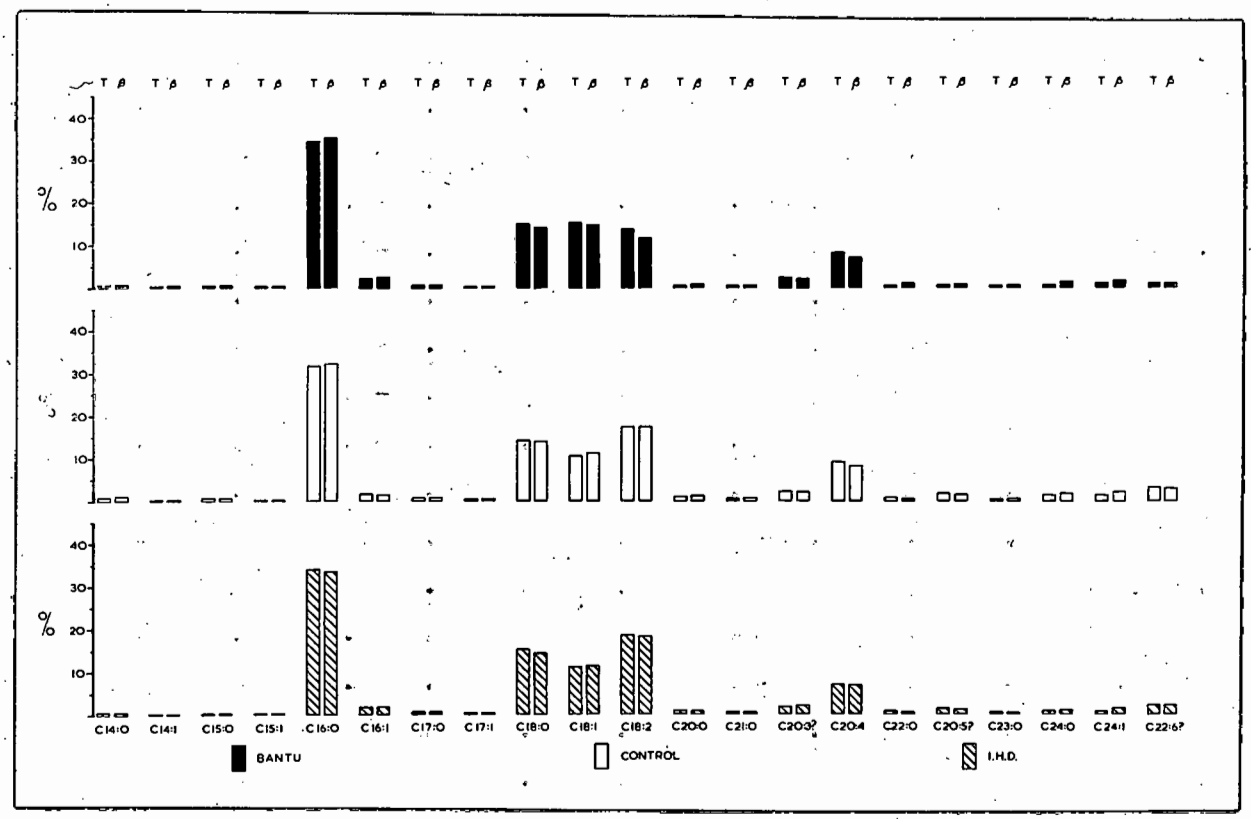


Figure 25. The fatty acid composition of the phospholipid fraction in total serum (T) and in β-lipoprotein (β) in each group. The values shown are means.
Note the striking similarity in the fatty acid composition in total serum and in β-lipoprotein in each group.

The data in Tables 36 and 37 show that the phospholipid fraction of both total serum and β-lipoprotein has a characteristic fatty acid pattern. In every sample analysed the same fatty acids appeared consistently, albeit often in trace amounts. While differences are apparent between groups, in each group the most striking features are the high proportion of C16:0, the high percentage of C18:0 which is present in approximately equal proportions to C18:1 and C18:2, and the presence, in fairly large proportions, of several longer chain fatty acids. The percentage of saturated fatty acids is similar to or somewhat higher than that of unsaturated fatty acids (Table 38).

		Bantu	Control	I.H.D.
		%	%	%
	Fraction	Mean (S.D.)	Mean (S.D.)	Mean (S.D.)
Saturated Fatty Acids	Total	52.3 (2.8)	51.7 (2.2)	52.0 (2.4)
	B	53.6 (3.0)	50.7 (2.8)	50.7 (3.3)
Unsaturated Fatty Acids	Total	46.6 (2.5)	49.9 (2.1)	46.1 (2.4)
	B	44.0 (2.4)	48.8 (2.8)	47.0 (4.1)

Table 38: The percentage of saturated and of unsaturated fatty acids in the phospholipid fraction in total serum (Total) and β-lipoprotein (B).

This high degree of saturation, particularly the high proportion of C18:0, and the presence of very long chain fatty acids is characteristic of the phospholipid fatty acid pattern and distinguishes it from that of the cholesterol ester and triglyceride fatty acids. Similar findings have been reported by other authors (Lindgren et al 1961; Nelson and Freeman 1960; Schrade et al 1960, 1961a, 1961b).

i) Comparison between total serum and β-lipoprotein

On comparing the phospholipid fatty acid patterns of total serum and β-lipoprotein not only are the component fatty acids identical but, for each group, the proportion of each fatty acid is very similar. This similarity between total serum and β-lipoprotein is particularly marked for the more abundant fatty acids viz:- C16:0, C18:0, C18:1 and C18:2, and suggests that the fatty acid patterns of α- and β-lipoprotein are alike.

These findings are in agreement with the data of Böttcher and Woodford (1961) and of Lindgren et al (1961) who separated lipoproteins by Cohn fractionation and ultracentrifugation respectively. While emphasising the overall similarity of the phospholipid fatty acid pattern in different lipoprotein fractions, in both these studies the authors reported that β-lipoprotein tended to have a greater proportion of post C20:4 fatty acids. This was ascribed to the presence in β-lipoprotein of larger proportions of sphyngomyelin, which has been reported by Böttcher and Woodford to have a relatively high content of verylong chain fatty acids. In the present study the percentages of post C20:4 fatty acids are generally similar between the groups although there is a tendency for higher percentages of C24:0 and C24:1 in the β-lipoprotein from Bantu and controls. Since the phospholipids have not been separated into the various phosphatides in this study it is not possible to say whether this tendency reflects an increase in the proportion of sphyngomyelin in the β-lipoprotein fraction. These slight differences do not, however, detract to any extent from what is, in general, a remarkable similarity between the phospholipid fatty acid pattern of total serum and β-lipoprotein.

ii) Comparison between groups

While the existence of a characteristic fatty acid pattern for phospholipids has been established, differences are present between the groups. The

major fatty acids, viz: C16:0, C16:1, C18:0, C18:1, C18:2 and C20:4 together account for somewhat less than 90% of the total fatty acids (Table 39).

	Bantu	Control	I.H.D.
	Mean (S.D.)	Mean (S.D.)	Mean (S.D.)
A Total Serum	89.0 (1.7)	84.8 (1.8)	87.5 (3.2)
β-lipoprotein	86.8 (2.6)	84.6 (1.7)	85.9 (2.8)
B Total Serum	7.2 (1.4)	11.3 (1.7)	7.8 (2.9)
β-lipoprotein	8.4 (1.9)	11.1 (1.9)	8.5 (2.6)

Table 39: The percentage of the total fatty acids in the phospholipid fraction represented by the sum of the six major components (A) and by the sum of the longer chain fatty acids (B).

The percentages of the major fatty acids in the three groups are compared in Table 40 and in Figure 26.

In addition there are certain longer chain fatty acids, including some which have not been identified with absolute certainty, which are characteristic of the phospholipid fraction. These fatty acids, viz: C20:3 (?), C22:0, C20:5 (?), C23:0, C24:0, C24:1 and C22:6 (?) together account for about 8% of the total fatty acids (Table 39). While the degree of reproducibility in estimating these longer chain fatty acids was low (Part I, Table 16), the error in calculation is presumably similar in all groups. In view of the fact that these fatty acids are found in appreciable amounts only in the phospholipid fraction, it was felt that comparison of the mean values in the three groups would be of interest. The mean values for the phospholipid fatty acids are compared in Table 40.

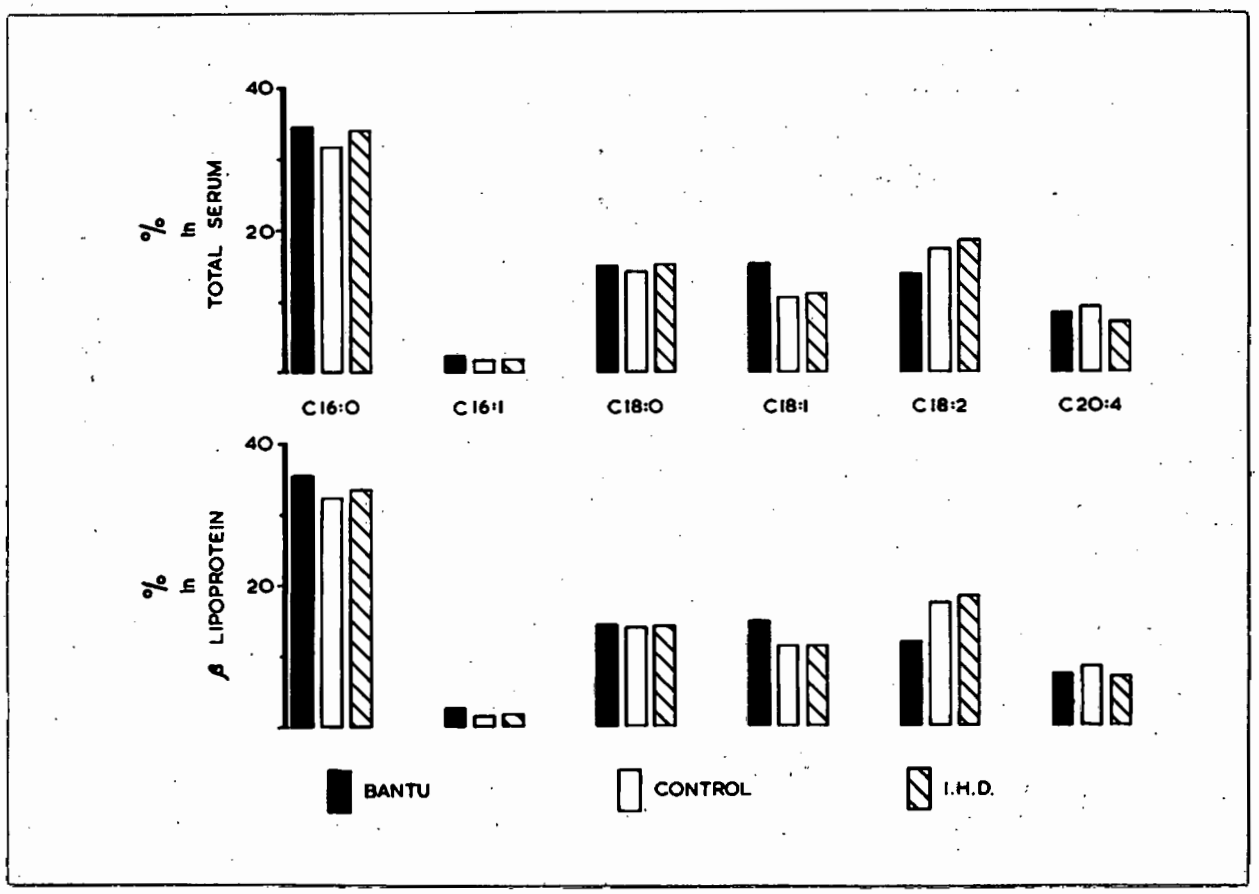


Figure 26. The percentages of the major fatty acids in the phospholipid fraction.

Fatty Acid	Fraction	Bantu (B) % Mean (S.D.)	Control (C) % Mean (S.D.)	I.H.D. % Mean (S.D.)	I.H.D. vs.C p	I.H.D. vs.B p	C vs.B. p
<u>C16:0</u>	Total	34.5 (2.5)	31.7 (2.4)	34.0 (2.2)	<0.05	NS	<0.01
	B	35.3 (3.8)	32.2 (1.6)	33.5 (3.0)	NS	NS	<0.02
<u>C16:1</u>	Total	2.1 (0.4)	1.7 (0.4)	1.8 (0.2)	NS	<0.05	<0.02
	B	2.6 (0.7)	1.5 (0.4)	1.8 (0.5)	<0.05	<0.01	<0.001
<u>C18:0</u>	Total	15.0 (1.8)	14.3 (0.9)	15.1 (1.2)	NS	NS	NS
	B	14.3 (2.4)	14.0 (1.5)	14.1 (1.0)	NS	NS	NS
<u>C18:1</u>	Total	15.3 (2.7)	10.6 (1.7)	11.0 (1.6)	NS	<0.001	<0.001
	B	14.8 (2.1)	11.3 (1.7)	11.3 (1.5)	NS	<0.001	<0.001
<u>C18:2</u>	Total	13.9 (1.9)	17.4 (1.2)	18.6 (3.2)	NS	<0.001	<0.001
	B	11.8 (2.0)	17.4 (1.8)	18.3 (3.9)	NS	<0.001	<0.001
<u>C20:4</u>	Total	8.2 (1.3)	9.1 (1.3)	7.0 (1.5)	<0.01	<0.05	NS
	B	7.1 (1.5)	8.3 (1.2)	6.9 (0.9)	<0.01	NS	<0.05
C20:3 (?)	Total	2.5 (0.5)	2.2 (0.7)	1.9 (0.6)	NS	<0.05	NS
	B	2.2 (0.7)	2.1 (0.6)	2.1 (0.7)	NS	NS	NS
C20:5 (?)	Total	0.7 (0.2)	1.8 (0.9)	1.3 (0.9)	NS	<0.02	<0.001
	B	0.8 (0.2)	1.5 (0.7)	1.2 (1.1)	NS	NS	<0.001
C22:0	Total	0.6 (0.5)	0.8 (0.4)	0.6 (0.5)	NS	NS	NS
	B	1.0 (0.9)	0.5 (0.5)	0.3 (0.3)	NS	<0.05	NS
C23:0	Total	0.5 (0.2)	0.3 (0.1)	0.5 (0.2)	NS	NS	<0.05
	B	0.6 (0.3)	0.4 (0.2)	0.4 (0.2)	NS	NS	NS
C24:0	Total	0.7 (0.3)	1.4 (0.4)	0.8 (0.6)	<0.001	NS	<0.001
	B	1.5 (0.6)	1.8 (0.4)	1.0 (0.7)	<0.01	NS	NS
C24:1	Total	1.2 (0.6)	1.6 (0.3)	0.8 (0.7)	<0.001	NS	NS
	B	1.8 (1.1)	2.2 (0.4)	1.5 (1.1)	<0.05	NS	NS
C22:6 (?)	Total	1.3 (0.6)	3.4 (0.9)	2.4 (1.3)	<0.05	<0.01	<0.001
	B	1.1 (0.6)	3.2 (1.3)	2.4 (1.0)	NS	<0.001	<0.001

Table 40: Comparison of the percentages of the major fatty acids (underlined) and of the longer chain fatty acids of the phospholipid fraction in Bantu, controls and patients with I.H.D.

The values for total serum (Total) and β-lipoprotein (B) are shown.

In general it is apparent that for the major fatty acids the only difference between patients and controls lies in the percentage of C20:4, while the Bantu differ from the other two groups in having higher proportions of C16:1 and C18:1 and a lower proportion of C18:2.

On comparing the percentages of the longer chain fatty acids, it is seen that the proportions of C20:3,(?), C22:0 and C23:0 are similar in all groups. While the proportion of C20:5 (?) is lower for Bantu than for controls, there is no other difference between groups for this component. The percentages of C24:0 and C24:1 are higher for controls than for patients, but here the mean values for patients and Bantu are similar. For C22:6 (?) the Bantu value is lower than for the other groups. When the fatty acids are considered in related groups , e.g. C20:0 polyunsaturated fatty acids (C20:3 (?), C20:4, C20:5 (?)) or saturated fatty acids (C22:0, C23:0, C24:0), it can be seen that the individual fatty acids in any one group do not show the same relationship between Bantu, controls and patients with I.H.D. There is thus no consistent trend.

The metabolism of these very long chain fatty acids has not, to our knowledge, been studied. It is possible that they may be synthesized in the body or be derived from dietary sources. It has been shown in several studies that the fatty acid composition of the serum is influenced by that of the dietary fats (Ahrens et al 1959; Kingsbury et al 1962b; Bohle et al 1961). The presence of very long chain fatty acids, similar to those found here, has been demonstrated in the phospholipids extracted from fish oils (Farquhar et al 1959; de Koning 1964) which are commonly used in the industrial preparation of edible fats. Such fats may thus provide a source of longer chain fatty acids in the serum phospholipid.

iii) The ratio of trienoic to tetraenoic fatty acids

It has been suggested that the minimum requirement of linoleate has been satisfied when the value for the ratio of trienoic to tetraenoic fatty acids is less than 0.4 (Holman 1960a). The mean values for this ratio in the Bantu, control and patient groups respectively are 0.31 ($\pm$ 0.05), 0.25 ($\pm$ 0.08), 0.28 ($\pm$ 0.10) in total serum and 0.32 ($\pm$ 0.08), 0.25 ($\pm$ 0.07) and 0.31 ($\pm$ 0.09) in β-lipoprotein. These values are similar in the three groups and are lower than 0.4. There is, therefore, again no evidence of E.F.A. deficiency in any group.

iv) The relationship between the percentage of C18:2 and the percentages of C16:1 and C18:1

It will be recalled that in the cholesterol ester fraction the percentage of C18:2 was inversely related to the percentages of C16:1 and C18:1 in all groups of subjects. For the triglyceride, there was a reciprocal relationship between the percentage of C18:2 and C18:1 in the Bantu group. Here again, the percentages of C16:1, C18:1 and C18:2 show differences between the groups. The relationship between these fatty acids has been examined by calculation of the correlation coefficient (Table 41).

Comparison	Fraction	Bantu		Control		I.H.D.	
		r	p	r	p	r	p
C16:1 ▼ C18:2	Total	-0.4684	NS	-0.0172	NS	-0.1211	NS
	B	-0.5656	<0.05	0.0952	NS	0.0504	NS
C18:1 ▼ C18:2	Total	-0.6243	<0.01	-0.3520	NS	-0.1827	NS
	B	-0.5026	<0.05	-0.2319	NS	-0.0465	NS

Table 41: The relationship between the percentages of C18:2 and C18:1 and C18:2 and C16:1 in total serum (Total) and β-lipoprotein (B).

r = Correlation coefficient.

There is a negative correlation between the percentage of C16:1 and C18:2 in the β-lipoprotein of the Bantu group, but the percentages of these two fatty acids are not related in any other group. The percentages of C18:1 and C18:2 are inversely related in the Bantu group. This effect may be related to dietary factors and will be discussed in Section III.

v) Comparison with other data

The serum phospholipid fatty acid pattern has been examined in several studies by the techniques of alkali isomerization (Luddy et al 1958; Bjorntorp 1960) and G.L.C. (Lindgren et al 1961; Nelson and Freeman 1960; Schrade et al 1959, 1960, 1961a, 1961b; Böttcher and Woodford 1961; James et al 1957; Dole et al 1959; Hallgren et al 1960; Lawrie et al 1961; Scott et al 1963). The mean values for the major fatty acids in the control group agree closely with those reported for normal healthy subjects by several authors (James et al 1957; Dole et al 1959; Hallgren et al 1960; Lawrie et al 1961). The values for the patients are similar to those found by James et al (1957) and for the Bantu, correspond fairly closely to those for poor East Africans (Scott et al 1963). In many studies the post C20:4 fatty acids have not been individually identified or reported. However, in those studies where values are given for C20:5 (Hallgren et al 1960; Lawrie et al 1961), C22:6 (Schrade et al 1961a; Hallgren et al 1960), C24:0 (Böttcher and Woodford 1961; Hallgren et al 1961) or else for pentaenoic and hexaenoic fatty acids (Luddy et al 1958; Bjorntorp 1960) they agree well with the range of values found for the three groups studied here.

On the basis of the similarity between their data and that of others, Lawrie et al (1961) suggested that "the phospholipid fatty acids are under the control of processes that permit relatively little deviation in their patterns". Ahrens et al (1959) fed menhaden and corn oil to people and found that, while the fatty acids of the serum glycerides and cholesterol esters were markedly

affected and tended to resemble the fed fat, the phospholipid fatty acids were least affected by such extreme dietary manipulations. Similarly, Hallgren et al (1960) found that the difference between a vegetarian and normal subjects as regards C18:2 was less marked in the phospholipid than in the cholesterol ester and triglyceride fatty acids, and the phospholipid tended to have the most constant fatty acid pattern. While these data provide support for the concept that the phospholipid fatty acid pattern is less labile than that of triglyceride and cholesterol ester, nevertheless in the present study differences between groups are found.

Schrade and his co-workers (1959, 1960, 1961a, 1961b) have reported that the percentages of C18:2 and C20:4 were significantly lower and that of C16:0 somewhat higher in the atherosclerotic subjects when compared with the healthy subjects. The data of Scott et al (1963) are in agreement with the local results. They compared the phospholipid fatty acids in New Yorkers and poor and upper class East Africans. The only difference was that New Yorkers, who have a considerably greater susceptibility to I.H.D. than the East Africans, had a significantly greater percentage of C18:2 than the other two groups. In some studies no differences have been found. James et al (1957) showed that the serum phospholipid fatty acid patterns were similar in healthy subjects and in people with coronary artery disease, and the α- and β-lipoprotein phospholipid fatty acid patterns were found to be similar for healthy young males and subjects with atherosclerosis (Böttcher and Woodford 1961).

In considering all the available data from here and other sources, it would appear that the fatty acid composition of the serum phospholipids is relatively constant.

CONCLUSIONS

Each lipid fraction has a distinctive fatty acid pattern; the cholesterol ester fraction is characterized by a high proportion of unsaturated fatty acids, the triglyceride fraction by a high proportion of mono-unsaturated fatty acids and the phospholipid fraction by a relatively high content of saturated fatty acids.

The fatty acid compositions of cholesterol ester, triglyceride and phospholipid were qualitatively identical in total serum and in β-lipoprotein and in each group of subjects the proportions of fatty acids were similar in total serum and in β-lipoprotein.

In all lipid fractions the percentage of C18:2 was lower for the Bantu than for either patients or controls. The percentages of C16:1, C18:1 and C20:4 were higher for the Bantu than for patients or controls except in the triglyceride fraction, where the percentage of C18:1 was similar for Bantu and controls, and in the phospholipid fraction where the percentage of C20:4 was similar for the Bantu and the other two groups. The percentages of C16:0 and C18:0 were generally similar for the three groups, except in the cholesterol ester fraction, where the percentage of C16:0 was higher for the Bantu and the percentage of C18:0 was lower for the patients than for the other groups, and in the phospholipid fraction where the percentage of C16:0 was higher for Bantu than for controls. The patients with I.H.D. differed from both other groups in having a lower percentage of C18:0 and a higher percentage of C18:2 in the cholesterol ester fraction, and lower percentages of C18:1 in both cholesterol ester and triglyceride fractions.

A graded relationship between the values for the three groups was found only for the percentages of C18:1 and C18:2 in the cholesterol ester fraction.

In this lipid fraction there was a reciprocal relationship between the percentages of C18:2 and of C18:1 and C16:1. In the triglyceride and phospholipid fractions there was a reciprocal relationship between the percentages of C18:2 and C18:1 for the Bantu group only.

There was a positive correlation between the concentration of esterified cholesterol and the percentage of C18:2 in the cholesterol ester fraction.

There was no evidence of E.F.A. deficiency in any group as assessed by the ratio of trienoic to tetraenoic fatty acids in the cholesterol ester and phospholipid fractions nor could a relative deficiency of E.F.A. be related to an increased susceptibility to I.H.D. or its established presence in that the percentage of C18:2 was consistently lowest in the Bantu group.

SECTION III

THE RELATIONSHIP BETWEEN DIET AND SERUM LIPIDS

In view of the widely recognized influence of dietary fat and the possible influence of dietary carbohydrate on serum lipid composition, the composition of the diet has been compared in the three groups studied, in order to determine whether the differences in their lipid patterns can in any way be related to differences in their diets.

(a) THE COMPOSITION OF THE DIET IN THE THREE GROUPS

The composition of the diet was determined as described in Part I, Chapter 1. The composition of the average daily diet in each group is shown as a mean in Tables 42 and 43 and in Figures 27 and 28.

	BANTU		CONTROL		I.H.D.	
	Mean	(S.D.)	Mean	(S.D.)	Mean	(S.D.)
Total calories	4023	(330)	2573	(395)	1897	(411)
Fat	8	(1)	52	(5)	34	(8)
Carbohydrate	80	(2)	33	(5)	47	(8)
Protein	12	(2)	16	(2)	19	(4)

Table 42: The total calories consumed and the percentage of calories derived from each of the basic foodstuffs by Bantu, controls and patients with I.H.D.

It is apparent from Table 42 that the differences in the proportion of calories derived from fat and carbohydrate in the three groups are striking. There is also a graded increase in the relative intake of protein from Bantu through controls to patients.

The quantity, proportion and type of fat consumed is shown in Table 43.

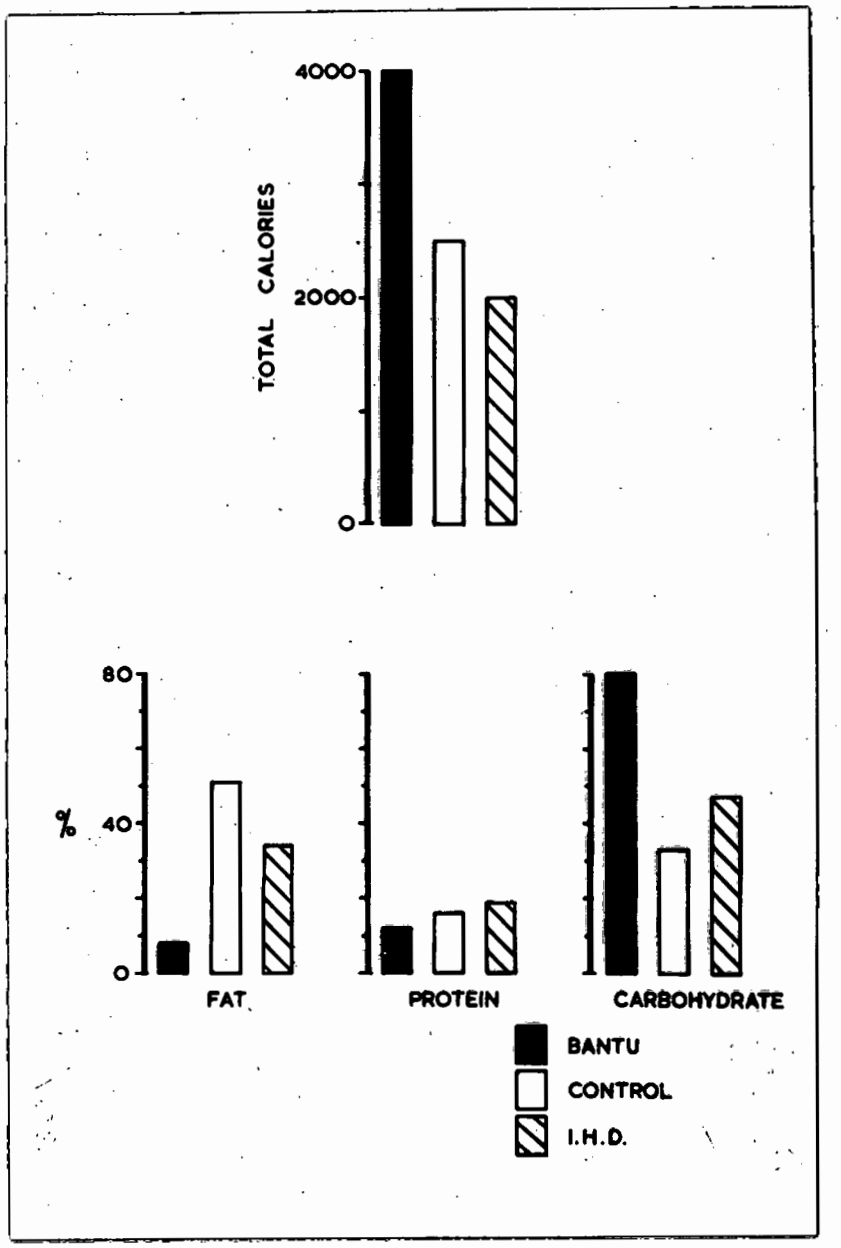


Figure 27. The total daily calorie intake and the percentage of daily calories derived from fat, protein and carbohydrate in each group.

	BANTU		CONTROL		I.H.D.	
	Mean	(S.D.)	Mean	(S.D.)	Mean	(S.D.)
Total fat (gm/day)	37	(4)	148	(29)	70	(21)
Linoleic Acid (gm/day)	7.8	(1.8)	7.3	(2.7)	6.8	(3.0)
% Fat Calories derived from Linoleic Acid	20	(3)	5	(2)	10	(5)
% Total Calories derived from Linoleic Acid	1.8	(0.9)	2.7	(0.9)	3.4	(1.8)

Table 43: The average daily intake of fat and of linoleic acid (gm/day) and the percentage of total calories and of fat calories derived from linoleic acid.

The Bantu group have a very high calorie intake. However, as these men were heavy manual labourers, the excess calories were presumably utilized in the form of energy, since none of the men was obese and all had maintained a fairly constant body weight for at least one year prior to this study. The Bantu diet is characterized by a very high proportion of carbohydrate and a low proportion of fat, of which a fairly large proportion is linoleic acid (Tables 42 and 43).

The calorie intake in the control group was higher than that in the patient group. The diet of the control group contains a relatively low proportion of carbohydrate and a high proportion of fat, of which only a small proportion is linoleic acid.

The diet of the patient group is intermediate between that of the Bantu and controls, and contains a lower proportion of fat and a higher proportion of linoleic acid than that of the control group. It must be pointed out that the diet in this group of subjects with I.H.D. is probably atypical. The reasons for this have been discussed in Part I, Chapter 1.

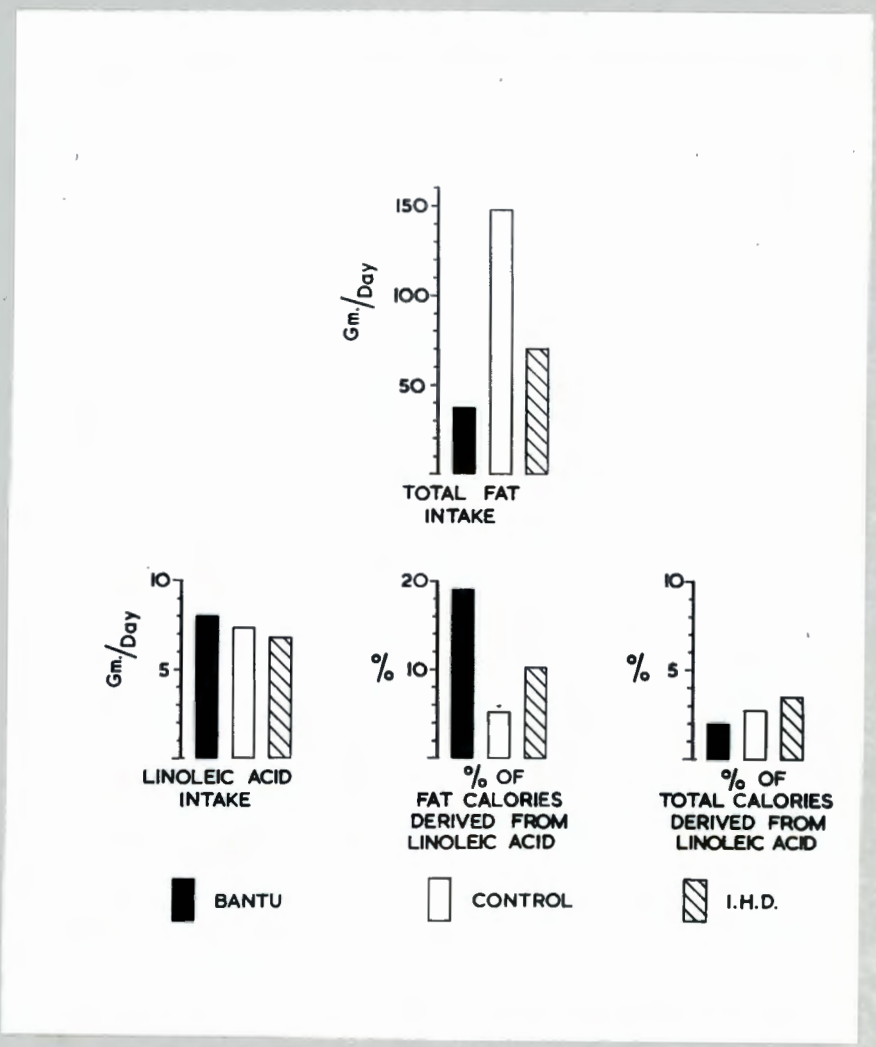


Figure 28. The average daily intake of fat and the amount and relative proportion of linoleic acid consumed by the three groups.
Note the high fat intake of the control group and the graded increase from Bantu through controls to patients in the percentage of total calories derived from linoleic acid.

In each group of subjects the proportion of total calories derived from protein is relatively low.

The absolute intake of linoleic acid is similar in the three groups and in all groups the percentage of total calories derived from linoleic acid exceeds 1%, the value suggested as the minimum allowance for man (Food and Nutrition Board Report 1958). There is, therefore, no evidence that any of the groups subsisted on a diet deficient in E.F.A., and, moreover, the percentage of total calories derived from linoleic acid was highest in the patient group.

(b) THE RELATIONSHIP BETWEEN DIETARY FAT AND SERUM LIPIDS

The relationship between serum lipid concentrations and the quantity, proportion and type of fat in the diet of the three groups has been examined (Table 44).

		BANTU	CONTROL	I.H.D.
Fat intake (gm/day)		37	148	70
% Total calories derived from fat		8	52	34
% Fat calories derived from linoleic acid		20	5	10
Total cholesterol	Total	142.9	247.9	285.5
	B	92.1	213.1	245.4
Triglyceride	Total	72.0	116.2	160.1
	B	57.1	106.4	149.0
Phospholipid	Total	190.0	253.4	264.3
	B	73.0	139.1	168.6

Table 44: The fat intake, the percentage of total calories derived from fat, the percentage of fat calories derived from linoleic acid, and the concentration (in mg/100 ml.serum) of total cholesterol, triglyceride and phospholipid in total serum (Total) and β-lipoprotein (B). The mean values are shown.

The Bantu whose diet has the lowest amount and proportion of fat, containing the highest proportion of fat calories from linoleic acid, have the lowest serum lipid levels. This is in agreement with epidemiological data showing that there is an association between low fat diets, low serum lipids and a low susceptibility to I.H.D. (Keys et al 1952, 1954, 1958; Bronte-Stewart et al 1955).

The diet of the patient group contained a considerably lower amount of fat and a lower proportion of fat calories, of which a greater proportion was linoleic acid, than that of the control group. Despite these findings, the mean serum lipid levels for this group of patients with I.H.D. were generally higher than for the control group. This finding could suggest that elevated serum lipid levels are not related to dietary fat alone, but may also be related to other dietary factors or possibly reflect a more fundamental disorder of lipid metabolism in I.H.D.

(c) THE RELATIONSHIP BETWEEN LINOLEIC ACID IN THE DIET AND IN THE SERUM LIPID FRACTIONS

The relationship between the linoleic acid content of the diet and the percentage of linoleic acid in the esterified lipid fractions of the serum has been examined in the three groups studied. The results are shown in Table 45 and Figure 29.

	BANTU	CONTROL	I.H.D.
C18:2 intake (gm/day)	7.8	7.3	6.8
% Fat Calories derived from C18:2	20	5	10
% Total Calories derived from C18:2	1.8	2.7	3.4
% C18:2 in cholesterol Ester	35.6	46.8	52.2
% C18:2 in triglyceride	10.1	13.4	15.3
% C18:2 in phospholipid	13.9	17.4	18.6

Table 45: The relationship between linoleic acid (C18:2) in the diet and the percentage of linoleic acid in the fatty acids of the cholesterol ester, triglyceride and phospholipid fractions of total serum.

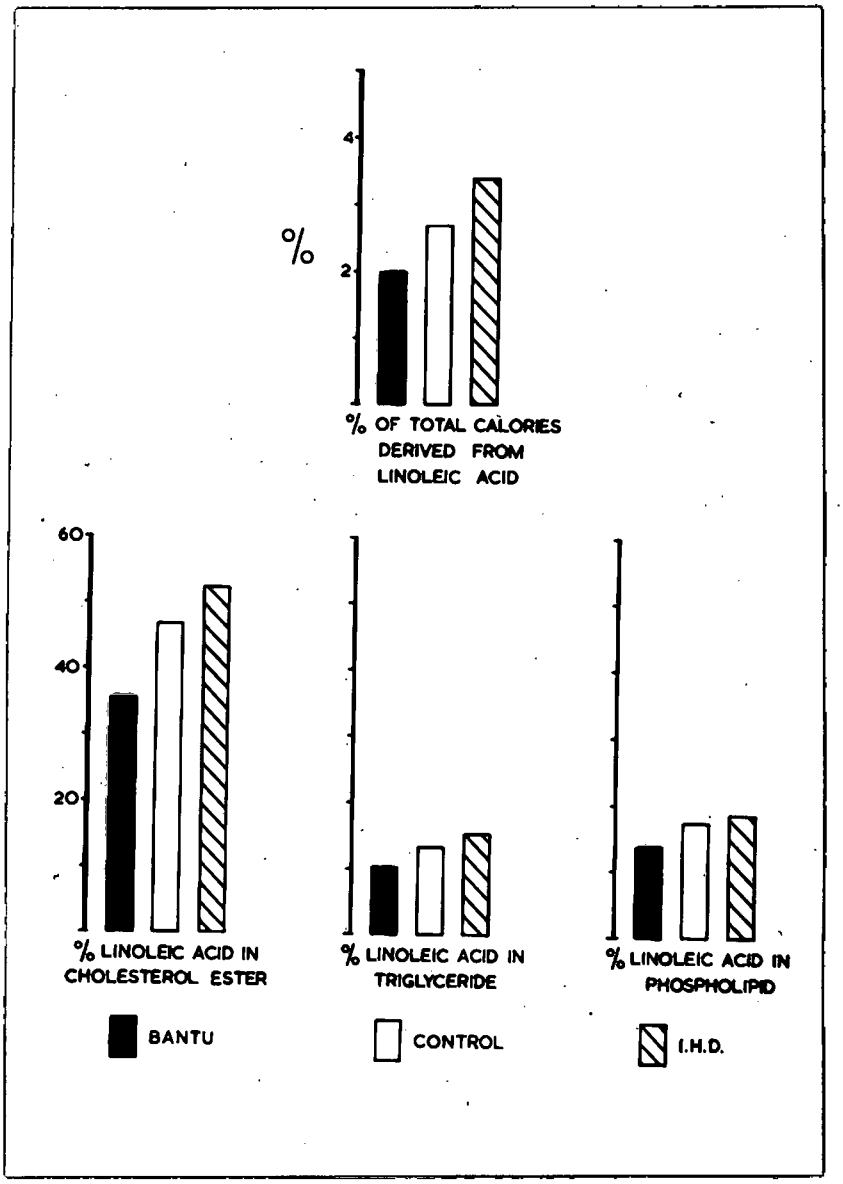


Figure 29. **The relation between dietary linoleic acid and the proportion of linoleic acid in the serum lipid fractions in the three groups.**

It is apparent that the actual intake of linoleic acid is similar in all groups and that the percentage of fat calories derived from linoleic acid bears no relationship to the percentage of this fatty acid present in any serum lipid component. There is, however, a very striking relationship between the percentage of C18:2 in each lipid fraction and the percentage of total calories derived from linoleic acid (Figure 29). It is, thus, the proportion of total calories derived from C18:2 and not the absolute intake of C18:2 or the proportion of C18:2 in the dietary fat which appears to be related to serum lipid C18:2. It will be noted that this trend is less evident in the phospholipid fraction. This is in agreement with the data of Ahrens et al (1959) who showed that the composition of dietary fatty acids had the least influence on the phospholipid fatty acids of the serum.

(d) THE RELATIONSHIP BETWEEN DIETARY CARBOHYDRATE AND SERUM LIPIDS

The possible effect of a low-fat high-carbohydrate diet leading to increased triglyceride concentrations has been discussed in the Introduction. The patients with I.H.D. have a higher proportion of carbohydrate in their diet than the controls. It could be argued that this might be responsible for the increased triglyceride levels in the patients. However, the Bantu, who consumed 80% of their total calories in the form of carbohydrate, have the lowest triglyceride levels. This observation indicates that a low-fat, high-carbohydrate diet does not necessarily give rise to increased serum triglyceride levels and is in agreement with the findings of Antonis and Bersohn (1960). Since the serum lipid levels for the Bantu were considerably lower than for both other groups, it would appear that a high carbohydrate diet *per se* does not produce high serum lipid levels.

Fairhurst and Waterhouse (1963) have speculated that lipogenesis resulting from a high carbohydrate diet would produce predominantly saturated and

mono-unsaturated fatty acids. It will be recalled that the percentages of C16:0, C16:1 and C18:1 in the cholesterol ester fraction and of C16:1 and C18:1 in the triglyceride and phospholipid fractions were higher for the Bantu than for both other groups (Part II, Section II, Tables 27, 34 and 40). It is thus possible that there may be preferential synthesis of these fatty acids in the Bantu, whose diet contained a large proportion of carbohydrate.

The data showed that, in the cholesterol ester fraction the percentage of C16:1 and C18:1 were inversely related to that of C18:2 in all the groups and that in the triglyceride and phospholipid fractions there was an inverse relationship between the percentages of C18:1 and C18:2 in the Bantu group only (Part II, Section II, Tables 28, 35 and 41). These inverse relationships may be determined either by increased synthesis of C16:1 and C18:1 or by the proportion of linoleic acid in the diet or by both these factors.

It has been shown that in the patient and control groups the percentage of C18:2 in the serum lipid components can be related to the proportion of total calories derived from linoleic acid (Table 45). However, while the diet of the control group contained a lower proportion of carbohydrate than that of the patient group (Table 42) the proportions of C16:1 and C18:1 in the serum lipids of controls were either similar to or greater than those of the patients (Part II, Section II, Tables 27, 34 and 40). In these two groups the proportions of these fatty acids can thus not be related to the carbohydrate content of the diet. The Bantu group have the lowest proportion of total calories in the form of linoleic acid and the highest proportion of carbohydrate in their diet. The lower level of C18:2, the higher levels of C16:1 and C18:1 and the reciprocal relationship between the percentages of C18:2 and C18:1 and C18:2 and C16:1 in the Bantu group may therefore be related to both the percentage of linoleic acid and the high proportion of carbohydrate in their diet and it is not possible to separate these factors.

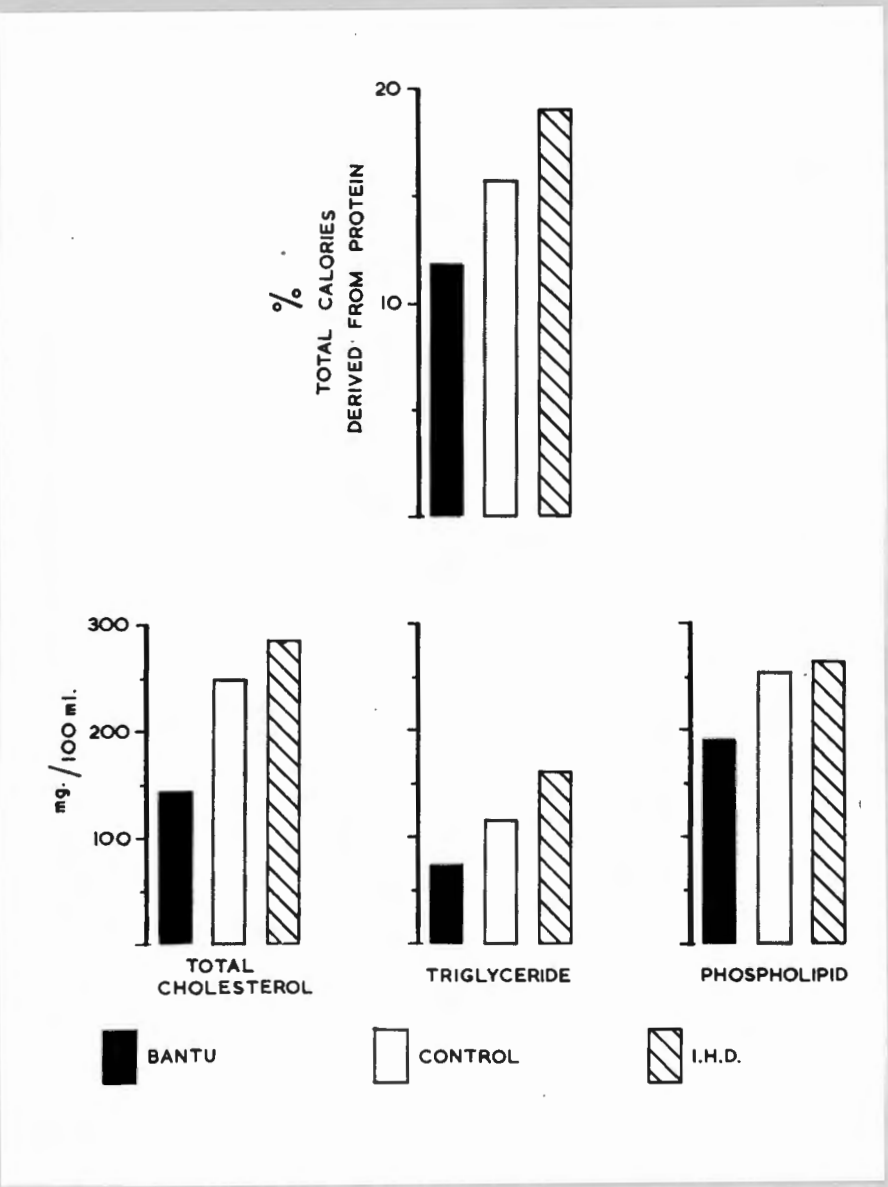


Figure 30. The relation between the percentage of total calories derived from protein and the concentration of lipid components in total serum in the three groups.

(e) THE RELATIONSHIP BETWEEN DIETARY PROTEIN AND SERUM LIPIDS

The influence of dietary protein on serum lipids in adults has not been studied to any great extent. There is, however, evidence to show that a low proportion of protein in the diet can be related to decreased concentrations of serum cholesterol and β-lipoprotein.

Olson et al (1958) fed a control diet containing 15% protein, 30% fat and 54% carbohydrate to middle-aged people. Isocaloric substitution of carbohydrate for protein yielding a diet containing 4% protein, 30% fat and 66% carbohydrate resulted in a decrease in the concentration of serum cholesterol and of low density (β) lipoprotein. Furman et al (1959) reported that isocaloric substitution of carbohydrate for all the protein in formula diets fed to adult men caused a decrease in serum cholesterol and β-lipoprotein. In a survey in Haiti, Sebrell et al (1959) reported that marked hypocholesterolaemia was seen in a population subsisting on a diet deficient in protein but moderate in fat. The data in this study show a progressive increase in the proportion of protein calories from Bantu through controls to patients with I.H.D. (Table 42, Figure 27). It is possible therefore that the increasing concentrations of serum cholesterol and β-lipoprotein lipid from Bantu through controls to patients with I.H.D. may be related to the increasing proportion of protein calories in these groups (Figure 30). If this is so, then the protein content of the diet might effect a much greater influence on serum lipid levels than is generally thought to be the case.

While it is possible that fatty acids may be synthesized from the breakdown products of protein, the only fatty acid which shows a graded increase from Bantu through controls to patients in parallel with the increasing proportions of protein calories is C18:2. Since this apparently cannot be synthesized in the body (Lipsky et al 1957), the intake of protein cannot be simply related to the fatty acid composition of the serum lipid fractions.

CONCLUSIONS

The composition of the diet was found to be markedly different in the three groups. There was no evidence that any group was subsisting on a diet deficient in essential fatty acids.

The serum lipid concentrations could not be simply related to the dietary fat content in the three groups. The proportion of carbohydrate in the diet was not related to the concentration of triglyceride or of any other lipid. The proportion of total calories present as protein showed a graded increase from Bantu through controls to patients with I.H.D. This increase was related to the increase in serum lipid concentrations in the three groups.

The percentages of linoleic acid in the serum lipid fractions are correlated with the proportion of total calories derived from linoleic acid in the three groups. The percentages of certain fatty acids in serum from the Bantu group may be related to the high proportion of carbohydrate in the Bantu diet.

CHAPTER 2

THE COMPOSITION OF LIPID IN ARTERIAL ATHEROMATOUS PLAQUES

(a) THE QUANTITATIVE COMPOSITION OF LIPID IN ATHEROMATOUS PLAQUES.

The values for the concentrations of total lipid and of the lipid fractions extracted from each sample of plaque material are shown in Table 46.

Code number	Free cholesterol	Cholesterol ester	Triglyceride	Phospholipid	Total lipid
	mg.	mg.	mg.	mg.	mg.
A1	22.8	68.5	19.8	23.1	134.2
A2	18.0	50.5	7.4	28.7	104.6
A3	14.7	28.6	10.7	31.9	85.6
A4	46.2	68.7	6.7	37.6	159.2
A5	9.0	29.1	2.8	8.1	49.0
A6	41.7	74.3	8.5	31.4	155.9
A7	43.0	82.2	10.9	34.2	170.3
A8	67.0	139.2	29.6	56.9	292.7
A9	12.7	29.8	5.5	16.9	64.9
A10	18.9	41.0	7.5	22.0	89.4
A11	12.3	30.4	6.6	16.2	65.5
A12	43.0	103.0	20.8	32.5	199.3

Table 46. The concentration of the lipid fractions and of total lipid extracted from each sample of plaque material.

It is evident from the data in Table 46 that the amount of lipid analysed varied enormously. This was due primarily to the fact that the amount of plaque material available for analysis varied greatly. It is possible that another factor contributing to the variation in lipid content was the variation in the amount of lipid present per unit segment of vessel. Despite the wide variation in the amount of lipid extracted it can be seen from Table 47 that the relative proportion of each lipid component in the plaques tends to be fairly constant.

	Cholesterol				Triglyceride %	Phospholipid %
	Total %	Esterified %	Free %	Ester %		
Mean	49.7	28.4	21.2	47.4	8.6	22.8
S.D.	5.3	3.7	4.0	6.0	3.1	5.9
Range	37.0-55.3	19.9-35.7	17.0-29.0	33.3-59.4	4.2-12.5	16.3-37.1

Table 47: The percentage composition of lipid in arterial atheromatous plaques. (Free cholesterol + cholesterol ester + triglyceride + phospholipid equals 100%)

The major constituent of plaque lipid is cholesterol. Free cholesterol and esterified cholesterol are present in rather similar proportions, although the percentage of esterified cholesterol is somewhat higher. This is unlike the ratio of esterified to free cholesterol in the serum lipids (Section II, Table 18) The proportion of phospholipid is about half that of total cholesterol. Triglyceride represents the smallest lipid constituent and seldom exceeded 10% of the total lipid.

Certain lipid ratios will be used for comparing the composition of lipid in plaques with that in total serum and in β-lipoprotein; these ratios have been calculated and the values are shown in Table 48.

$E/_F$	$C/_P$	$FC/_P$	$\frac{FC+CE+T}{P}$
Mean (S.D.)	Mean (S.D.)	Mean (S.D.)	Mean (S.D.)
1.38 (0.31)	2.34 (0.68)	0.99 (0.29)	3.71 (1.13)

Table 48: Lipid ratios in arterial atheromatous plaques.

C = Total Cholesterol
T = Triglyceride
P = Phospholipid
F;FC = Free Cholesterol
E = Esterified Cholesterol
CE = Cholesterol Ester.

i) Comparison with other data

The high proportion of cholesterol in plaque lipid noted in the present stud is in agreement with early observations that cholesterol is the predominant lipid in atherosclerotic lesions (Aschoff 1906; Windaus 1910; Schönheimer 1926,1928; Kimmelstiel 1931) and this has been confirmed in more recent studies (Table 49). The relative proportions of lipid reported here have been compared with the values of other authors (Table 49).

AUTHOR	MATERIAL	Total Cholesterol %	Free Cholesterol %	Cholesterol Ester %	E/F	Triglyceride %	Phospholipid %
Present study		49.7	21.2	47.4	1.38	8.6	22.8
Mead and Gouze (1961)	Aorta Intima and media Stage III	50*	25.1	42.4	1.0*	8.6	15.7
Hirsch and Weinhouse (1943)	Aorta Fibrous plaques	47*	18.1	47.5	1.6*	15.0	14.9
	Calcified tissue	50*	21.9	47.2	1.3*	13.1	13.2
	Atheromatous ulcer	52*	27.2	42.1	0.9*	10.4	16.0
Böttcher et al. (1959; 1960a; 1960b)	Aorta Aorta Stage III	44*	19.8	40.2	1.2*	8.6	33.6
	Intima and inner media	40*	19.3	33.8	1.1*	9.7	38.0
	Aorta Stage II/III	41*	19	36	1.1*	10	33
Björntorp et al (1962)	Femoral artery Intima	52.5				16.3	31.3
Smith (1960)	Aorta Fatty and fibrous nodules	53.3					16.2
	Pearly plaques	42.4					14.0
Buck and Rossiter(1951)	Aorta Intima and media stage III	46.8	29.5	29.0	0.6*	28.2	15.3
Luddy et al (1958)	Aorta Atheromatous plaques	56*	38.9	28.0	0.4*	19.4	13.7
Field et al (1960)	Aorta Thickened intima plaques	39.3				43.6	17.1

Table 49: The composition of lipid in atheromatous material

* Calculated from authors' data

With the exception of the data of Buck and Rossiter (1951) and of Luddy et al (1958) the percentages of total cholesterol, free cholesterol, cholesterol ester and the ratio of esterified to free cholesterol $^{E}/_{F}$ are similar in the different studies. There is, however, some variation in the percentages of triglyceride and phospholipid. It is possible that these latter variations may be due to differences in the techniques of estimation. Field et al (1960) have suggested that the high triglyceride values found by them may be due to differences in the method of estimating triglyceride. The triglyceride value reported by Buck and Rossiter (1951) was calculated by subtraction of all other lipid from total lipid, a method which, it is thought, gives erroneously high values (Part I, Chapter 2, Table 2). Böttcher and Woodford (1962) noted that in their studies the phospholipid was slightly contaminated with non-lipid which would tend to make their results erroneously high.

Another factor which may contribute towards variation in the results is the amount of media included in the sample. Hirsch and Weinhouse (1943) have shown that the composition of lipid is different in the intima and in the media, the latter containing approximately equal proportions of cholesterol, triglyceride and phospholipid. Inclusion of media would thus tend to give higher proportions of triglyceride and phospholipid at the expense of cholesterol.

Differences in the composition of lipid may also be dependent upon the vessel from which plaque material is obtained. Böttcher et al (1959, 1960b) have found that, while the proportion of triglyceride is similar in the aorta and in the arteries of the circle of Willis, in the coronary arteries the proportion of triglyceride is considerably higher and constitutes almost half the total lipid. There are therefore several factors which may influence the lipid composition of atherosclerotic plaque material. It is, however, noteworthy that in most studies the striking features are the predominance of cholesterol, the trend for free and esterified cholesterol to be about equal

in proportion and the relatively low proportion of triglyceride. In these respects the data of other authors are in agreement with the findings in this study.

(b) THE FATTY ACID COMPOSITION OF LIPID IN ATHEROMATOUS PLAQUES

The fatty acid composition of the cholesterol ester, triglyceride and phospholipid fractions of arterial atheromatous plaque lipid are shown in Table 50.

For each lipid fraction the same fatty acids were consistently found, albeit some in trace amounts. In every sample analysed the proportions of the major fatty acids were relatively constant. These data showed that each lipid fraction in the plaque has a characteristic fatty acid pattern. This finding is in agreement with the data of several authors who used the techniques of alkali isomerization (Luddy et al 1958; Wright et al 1959; Björntorp et al 1962) and G.L.C. (Tuna et al 1958; Mead and Gouze 1961; Swell et al 1960a; Smith 1960, 1962a, 1962b), for analysis of the fatty acids in various lipid fractions in atheromatous plaque material.

Fatty Acid	Cholesterol Ester %			Triglyceride %			Phospholipid %		
	Mean	(S.D.)	Range	Mean	(S.D.)	Range	Mean	(S.D.)	Range
C12:0	0.2	(0.2)	tr- 0.5	0.3	(0.2)	tr- 0.5			
C14:0	1.5	(1.1)	0.7- 4.1	2.3	(0.5)	1.3- 3.4	1.2	(0.6)	0.6- 2.1
C14:1	0.3	(0.2)	tr- 0.6	0.3	(0.2)	tr- 0.7	0.3	(0.2)	tr- 0.6
C15:0	0.4	(0.2)	tr- 0.8	0.7	(0.4)	tr- 1.8	0.4	(0.2)	tr- 0.7
C15:1	0.2	(0.2)	tr- 0.5	0.2	(0.1)	tr- 0.5	0.3	(0.2)	tr- 0.6
C16:0	13.3	(2.4)	9.7-18.2	31.3	(5.4)	25.4-44.5	37.7	(6.7)	26.7-48.5
C16:1	5.4	(1.3)	3.4- 7.9	5.4	(1.3)	4.3- 9.1	2.3	(1.0)	0.7- 3.8
C17:0	0.6	(0.4)	tr- 1.7	0.8	(0.4)	tr- 1.8	0.7	(0.4)	tr- 1.2
C17:1	0.2	(0.1)	tr- 0.5	0.5	(0.3)	tr- 1.2	0.4	(0.3)	tr- 1.0
C18:0	1.7	(1.0)	0.8- 4.0	6.6	(0.5)	4.6-10.0	14.6	(5.1)	9.3-23.7
C18:1	27.6	(3.7)	23.0-35.3	37.9	(4.5)	33.1-47.1	11.6	(2.8)	5.8-16.3
C18:2	39.7	(5.0)	29.1-45.2	10.5	(3.4)	6.5-13.7	6.1	(1.3)	4.6- 8.9
C18:3	0.7	(0.6)	tr- 2.4						
C20:0	0.3	(0.2)	tr- 1.3	0.6	(0.4)	tr- 1.1	1.0	(0.3)	0.6- 1.3
C21:0							0.3	(0.2)	tr- 0.6
C20:3?	0.8	(0.8)	tr- 2.5				2.4	(1.3)	1.1- 4.5
C20:4	6.4	(1.3)	4.0- 9.2	2.4	(1.4)	tr- 4.8	7.0	(3.5)	2.7-13.3
C22:0							0.5	(0.3)	tr- 1.1
C20:5?	1.0	(0.9)	tr- 3.5				1.5	(0.5)	0.9- 2.2
C23:0							0.5	(0.3)	tr- 0.9
C24:0							3.9	(1.8)	1.0- 6.6
C24:1							4.9	(2.2)	1.3- 8.3
C22:6?							1.9	(1.1)	tr- 3.6

Table 50: The fatty acid composition of cholesterol ester, triglyceride and phospholipid in arterial atheromatous plaques.

The major fatty acids are underlined.

i) Cholesterol ester fatty acid composition

The cholesterol ester fraction is characterized by a high degree of unsaturation. The unsaturated fatty acids constitute 80.7 (± 5.0) % of the total fatty acids, the polyunsaturated fatty acids constitute 48.5 (± 3.5) %. The saturated fatty acids account for only 17.8 (± 4.0) % of the total fatty acids. The most abundant unsaturated fatty acids are C18:2 (39.7%) and C18:1 (29.6%) while the most abundant saturated fatty acid is C16:0 (13.3%). It is, thus, apparent that the cholesterol ester fraction of plaque lipid has a fatty acid composition similar to that of the cholesterol ester fraction in serum (Section II, Table 23).

Böttcher et al (1960a) compared the cholesterol ester fatty acid composition of lesions with different degrees of atherosclerosis. They found that increasing severity of atherosclerosis was associated with an increase in the proportion of C18:2 and a decrease in the proportion of C18:0. They stressed the high ratio of C18:2 to C18:0 in Stage III lesions and suggested that it may be regarded as being characteristic of the cholesterol ester fatty acids of such lesions. The mean value for the C18:2/C18:0 ratio in the present study is 25.1 (± 2.9) and agrees remarkably closely with the value of 24.2 found by Böttcher et al (1960a).

According to Sinclair's hypothesis (1956), E.F.A. deficient diets may give rise to saturated serum cholesterol esters, and such relatively saturated esters would tend to be deposited and thus give rise to atheroma formation. If this is so, then one would expect to find saturated or relatively saturated cholesterol esters in plaque lipid. The data here show that the proportion of saturated fatty acids is low. Moreover, there is a high percentage of linoleic acid (C18:2) and a fairly large percentage of arachidonic acid (C20:4) both of which are essential fatty acids. Holman (1960a) has suggested

that a trienoic/tetraenoic fatty acid ratio greater than 0.4 is indicative of E.F.A. deficiency. The mean value for this ratio in the plaque cholesterol esters is 0.24 ($\pm$ 0.11). There is, therefore, no evidence to support the concept that the cholesterol esters which have accumulated in the plaques are the result of E.F.A. deficiency.

The local results have been compared with those of other authors who used G.L.C. for analysis of cholesterol ester fatty acids in atheromatous plaque material. In most studies percentages are given only for the major fatty acid components. The mean values for these fatty acids are compared in Table 51.

AUTHOR	C16:0	C16:1	C18:0	C18:1	C18:2	C20:4
Present study	13.3	5.4	1.7	27.6	39.7	6.4
Mead & Gouze (1961)	13.1	5.6		27.4	42.1	7.3
Böttcher et al (1960a)	13.2	5.0	1.6	30.2	36.0	4.7
Tuna et al (1958)	9.1	6.8	1.0	34.5	38.3	9.7
Swell et al (1960a)	25.1	6.7	6.8	37.8	25.1	1.8

Table 51. The percentages of the major fatty acids in cholesterol ester isolated from plaques, as determined by different authors.

The similarity between the local values and those of Mead and Gouze (1961) and of Böttcher et al (1960a) is striking, while the data of Tuna et al (1958) agree fairly closely. The data of Swell et al (1960a) differ from those of the present study and of the other authors. It is possible that this difference may be due to the fact that they analysed "free lying lipid" and not lipid within the arterial intima.

ii) Triglyceride fatty acid composition.

The most abundant fatty acids are palmitic (C16:0) and oleic (C18:1) acids. Other fatty acids present in relatively large proportions are palmitoleic (C16:1), stearic (C18:0) and linoleic (C18:2) acids. The proportion of the saturated fatty acids (40.1 ± 5%) is lower than that of the unsaturated fatty acids (57.2 ± 5.0%). The mono-unsaturated fatty acids constitute 43.2 ± 4.4% of the total fatty acids as compared with 14.0 ± 4.6% for the polyunsaturated fatty acids. The triglyceride fraction is thus characterized by a high content of mono-unsaturated fatty acids, principally C16:1 and C18:1. The triglyceride fatty acid composition in plaques is thus similar to that found in serum (Section II, Table 30).

The local results have been compared with those of other authors. The mean values are shown in Table 52.

AUTHOR	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C20:4
Present study	2.3	31.3	5.4	6.6	37.9	10.0	2.4
Böttcher et al (1960a)	2.8	23.0	5.2	7.3	35.4	12.6	3.9
Luddy et al (1958)						7.03	2.26
Björntorp et al (1962)						7.2	2.0

Table 52. The percentage of the major fatty acids in triglyceride isolated from plaques, as determined by different authors.

The values reported by Luddy et al (1958) and by Björntorp et al (1962) for the percentages of dienoic and tetraenoic acids agree fairly closely with those found here for C18:2 and C20:4 respectively. There is fair agreement between the local data and those of Böttcher et al (1960a), although the local value for C16:0 is somewhat higher and that for C20:4 somewhat lower than those reported by them.

iii) Phospholipid fatty acid composition

The phospholipid fraction is characterized by a high degree of saturation, due largely to the high percentages of C16:0 and C18:0. The saturated fatty acids together account for 59.5 ± 3.3% of the total fatty acids while the unsaturated fatty acids account for only 38.1 ± 3.3%. The percentage of polyunsaturated fatty acids (18.9 ± 4.8%) is similar to that of the mono-unsaturated fatty acids (19.2 ± 3.6%). The percentage of C18:2 is remarkably low and is lower than that of C20:4, which is present in relatively large proportions. In addition there are several very long chain fatty acids, which together constitute 13.2 ± 4.0% of the total fatty acids. Thus the phospholipid fatty acid composition of the plaques also, in general, shows a similarity to that of serum phospholipid.

The phospholipid fatty acid pattern has been analysed by Luddy et al (1958), Björntorp et al (1962) and Böttcher and his co-workers (Böttcher and Van Gent, 1961; Böttcher et al 1958, 1960a, 1960b). Their results are compared with the local data in Table 53.

Reference	C16:0	C16:1	C18:0	C18:1	C18:2	C20:4	C22:0	C24:0	C24:1	III+	V+	VI+
Present study	37.7	2.3	14.6	11.6	6.1	7.0	0.5	3.9	4.9	2.4	1.5	1.9
Böttcher et al (1960a)	29.9	1.1	15.1	9.7	4.2	7.4	4.4	6.4	4.7			
Luddy et al(1958)					7.2x	2.6x				2.3x	0.0x	0.0x
Björntorp et al (1962)					5.1x	5.2x				2.0x	1.4x	0.8x

Table 53. The percentages of fatty acids in the phospholipid from plaques, as determined by several authors.

+ III, V, VI = trienoic, pentaenoic and hexaenoic fatty acids respectively.

x Values given by the authors as dienes, trienes, tetraenes, pentaenes and hexaenes.

The values reported by Böttcher et al (1960a) again agree fairly closely with the local data, although their values are lower for C16:0 and higher for C22:0 and C24:0. Luddy et al(1958) reported percentages of dienoic and trienoic acids similar to those found here, but found a lower percentage of tetraenoic and no penta- or hexa-enoic acids. The data of Björntorp et al (1962) differ from the local data in showing a lower percentage of hexaenoic acids, but are otherwise similar.

In the present study the phospholipids have not been fractionated. However, several authors have shown that the phospholipid in the plaques, as in serum, consists of a mixture of several different phosphatides including 'cephalin', lecithin, sphyngomyelin and lysolecithin and have reported that there is an increase in the proportion of sphyngomyelin associated with increasing severity of atherosclerosis (Weinhouse and Hirsch 1940; Steele and Kayden 1956; Smith 1960; Böttcher and van Gent 1961). Böttcher and van Gent (1961) reported that the phospholipids in atherosclerotic aortas (Stage III) consisted of 9.2% cephalin, 23.4% lecithin, 62.5% sphyngomyelin and 4.9% lysolecithin. The fatty acid pattern of lecithin was characterized by high proportions of C16:0, C18:0 and C18:1 and a low proportion of C18:2, while sphyngomyelin contained high proportions of C16:0, C18:0, C22:0, C24:0 and C24:1 and no C18:2 or C20:4. In view of the high proportions of lecithin and sphyngomyelin reported to be in the plaque, one would expect that the fatty acid pattern of the total phospholipids would bear a resemblance to those of these two phospholipids. In fact, high percentages of C16:0, C18:0, relatively high percentages of C24:0 and C24:1 and a low percentage of C18:2 are found in the present study. This presumably reflects the characteristic patterns reported for lecithin and sphyngomyelin.

CONCLUSIONS.

The lipid present in arterial atheromatous plaques has a fairly constant composition, characterized by a high proportion of cholesterol, a relatively low proportion of phospholipid and a particularly low proportion of triglyceride. Free and esterified cholesterol are present in rather similar proportions.

Each lipid fraction has a characteristic fatty acid composition. The cholesterol ester fraction contains a high proportion of unsaturated fatty acids, the triglyceride fraction a high proportion of mono-unsaturated fatty acids and the phospholipids a high proportion of saturated fatty acids and a relatively high proportion of very long chain fatty acids not present in the other esterified fractions.

CHAPTER 3.

COMPARISON OF THE COMPOSITION OF LIPID IN ARTERIAL ATHEROMATOUS PLAQUES WITH THAT IN TOTAL SERUM AND IN THE SERUM β-LIPOPROTEIN FRACTION

(a) COMPARISON OF THE QUANTITATIVE COMPOSITION OF SERUM AND PLAQUE LIPIDS

The relative proportions of lipid fractions in plaques, in total serum and in β-lipoprotein in the three groups have been compared. The mean values are shown in Table 54 and Figure 31.

	Free Cholesterol		Cholesterol Ester		Triglyceride		Phospholipid	
	%		%		%		%	
GROUP	Total	B	Total	B	Total	B	Total	B
BANTU	7.5	9.7	37.4	41.1	15.0	21.4	40.2	27.8
CONTROL	9.4	11.0	40.8	45.6	15.5	18.5	34.4	24.9
I.H.D.	10.0	11.1	39.8	42.2	18.8	21.9	31.4	24.8
PLAQUES	21.2		47.4		8.6		22.8	

Table 54: The percentage composition of lipid in arterial atheromatous plaques and in total serum (Total) and β-lipoprotein (B) from Bantu, controls and patients with I.H.D.
The values shown are means.

It is apparent from the data in Table 54 and Figure 31 that the percentage composition of lipid in the plaques does not bear a particularly striking resemblance to that in either total serum or in β-lipoprotein for any group of subjects. In total serum, only the percentage of cholesterol ester approaches that in the plaques. In β-lipoprotein, while the percentages of cholesterol ester and of phospholipid approach those of the plaques fairly

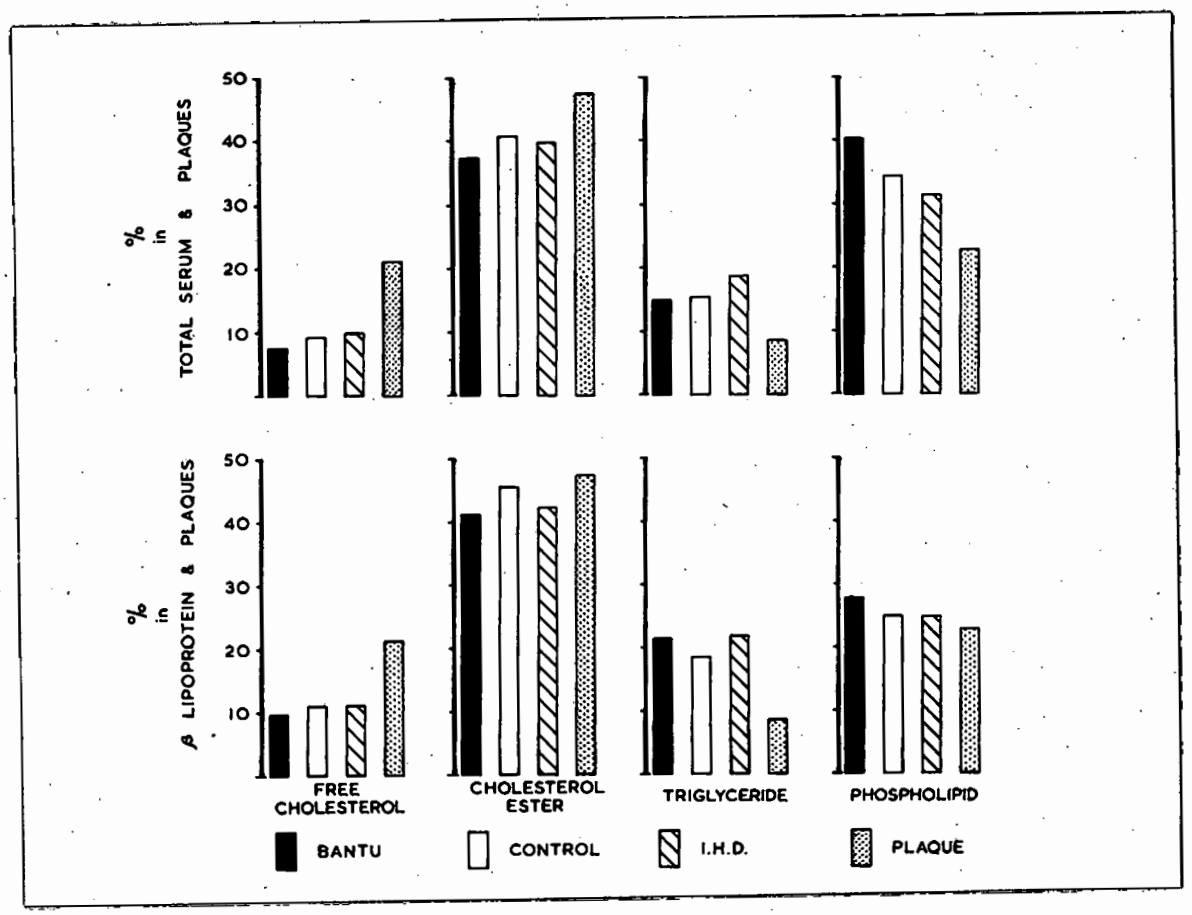


Figure 31. The relative proportions of lipid components in total serum, in the serum β-lipoprotein fraction in the three groups and in arterial atheromatous plaques.

closely, the percentage of triglyceride is considerably higher and that of free cholesterol is considerably lower than in the plaques. The percentage composition of lipid in the β-lipoprotein as such is thus different from that in the plaques. It is perhaps noteworthy that with the exception of triglyceride, the relative proportions of the other lipid components in β-lipoprotein approach those in the plaques more closely than do those in total serum. The trends in the relative proportions of lipid components from Bantu through controls to patients towards the values for the plaques are not particularly remarkable for either total serum or β-lipoprotein.

It will be recalled that certain lipid ratios showed a graded relationship between the three groups of subjects and provided good separation between them (Part II, Chapter I, Table 20). The values for these ratios in total serum and in β-lipoprotein in each group have been compared with those in the plaques (Table 55, Figure 32).

	E/F		C/P		FC/P		FC+CE+T/P	
GROUP	Total	B	Total	B	Total	B	Total	B
BANTU	3.03	2.57	0.75	1.25	0.19	0.36	1.51	2.63
CONTROL	2.61	2.51	0.98	1.51	0.27	0.45	1.92	3.03
I.H.D.	2.39	2.25	1.08	1.46	0.31	0.45	2.19	3.04
PLAQUES	1.38		2.34		0.99		3.71	

Table 55: Lipid ratios in arterial atheromatous plaques and in total serum (Total) and β-lipoprotein (B) from Bantu, controls and patients with I.H.D.

The values shown are means.

F;F.C.= Free cholesterol
E = Esterified cholesterol
CE = Cholesterol Ester
C = Total Cholesterol
T = Triglyceride
P = Phospholipid

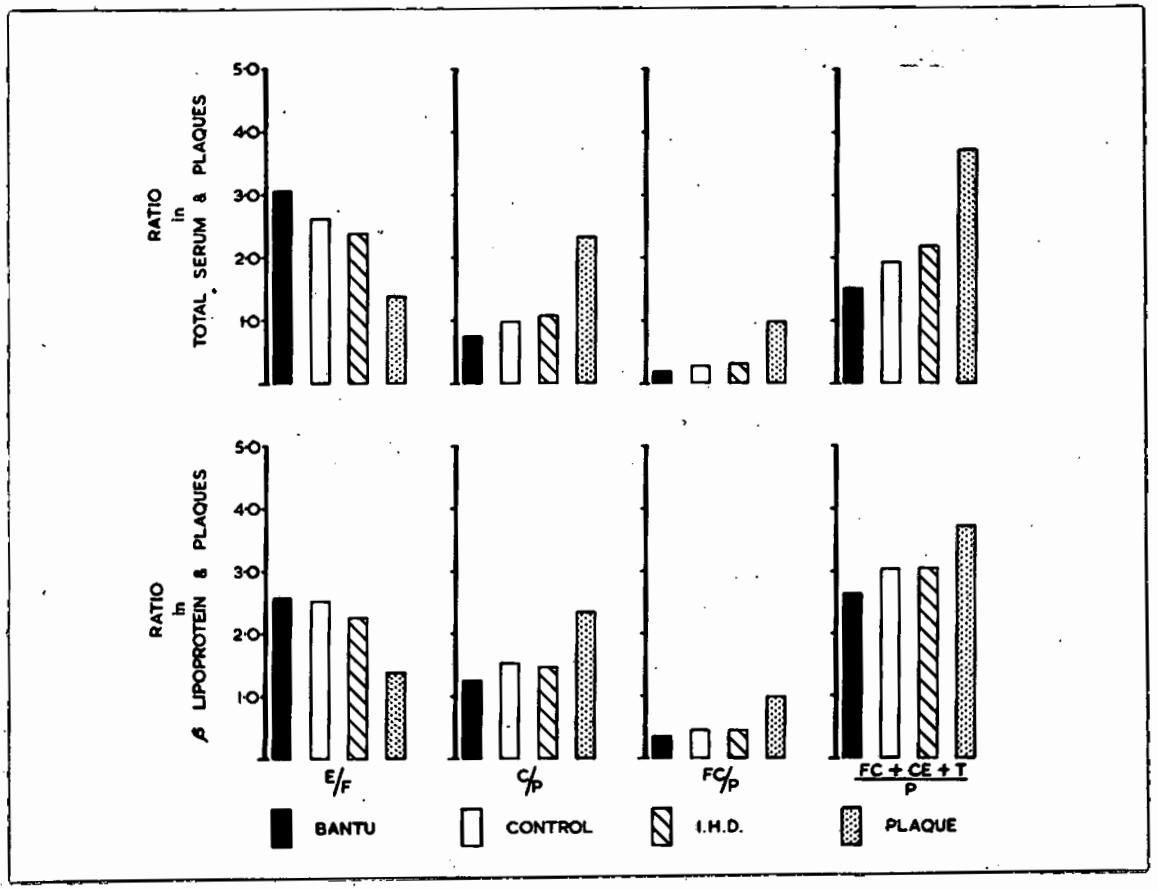


Figure 32. Lipid ratios in arterial atheromatous plaques and in total serum and in the serum β-lipoprotein fraction in the three groups.

For each of these lipid ratios, the mean value in the plaques is considerably different from that in either total serum or β-lipoprotein. The values for β-lipoprotein are again closer to those of the plaques than are those for total serum, but the differences between the values for plaques and those for β-lipoprotein are, with the single exception of the $\frac{FC+CE+T}{P}$ ratio, still considerable.

On comparing the ratios for the three groups of subjects with those in the plaques, certain trends are apparent. In general, the lipid ratios in the serum from patients show a greater resemblance to the lipid ratios in the plaques than is the case for the other two groups.

Comparison with other data. The data of Böttcher and his colleagues (Böttcher et al 1960a; Böttcher and Woodford 1961) show that the atherosclerotic aorta (Stage III) contains a greater proportion of free cholesterol and a smaller proportion of triglyceride than is found in the β-lipoprotein of both healthy young men and older atherosclerotic men. This is in agreement with the findings of the present study. The data of Böttcher and his colleagues show, however, that the proportion of phospholipid was greater in the atherosclerotic aorta than in the β-lipoprotein. This is contrary to the findings of the present study. It has, however, been noted that the phospholipid values found by Böttcher and Woodford (1962) in the atherosclerotic aorta may be erroneously high (Part II, Chapter 2). Buck and Rossiter (1951) and Luddy et al (1958) reported that the plaques contained a higher proportion of free cholesterol and a considerably lower proportion of phospholipid than did serum from normal subjects. These findings are thus in agreement with the local data.

It can be seen from the data in this and in other similar studies that the differences in composition between serum and plaque lipids are greater than any resemblance between them. This would suggest that, if serum lipids do

contribute to plaque formation, this does not occur by any simple process of deposition of serum lipids.

Other processes which could contribute to the lipid composition of the plaques include synthesis of lipid by the arterial wall or possibly preferential deposition of certain serum lipids. It has been shown that the arterial wall in animals is able to synthesise cholesterol, triglyceride and phospholipid. (Siperstein et al 1951; Werthessen et al 1954; Azarnoff 1958; Shore et al 1955; McCandless and Zilversmit 1956, 1959; Zilversmit et al 1954; Newman and Zilversmit 1959; Newman et al 1961). The data of Newman and Zilversmit (1959) and of Loomeijer and van der Veen (1962) indicate that synthesis of triglyceride and phospholipid is quantitatively greater than that of cholesterol. There is also evidence to suggest that the arterial wall in man is able to synthesize cholesterol (Field et al 1960) and phospholipid (Zilversmit et al 1961). The data of Field et al (1960) indicated, however, that while some of the cholesterol in the human intima was derived from synthesis, the major portion was derived from serum. Synthetic processes do therefore not appear to contribute significantly to plaque lipid in man.

The suggestion has also been made (Ahrens and Kunkel 1949; Wilkens and Krut 1963) that preferential deposition of certain serum lipids, particularly cholesterol, may be a mechanism contributing towards the formation of plaques.

Synthesis of lipid in the arterial wall and, or preferential deposition of circulating lipid may, therefore, be important factors in determining the composition of lipid in plaques. The relative role of these factors cannot, however, be evaluated on the basis of the data in this study.

(b) <u>COMPARISON OF THE FATTY ACID COMPOSITION OF SERUM AND PLAQUE LIPIDS</u>

For each group of subjects, in all lipid fractions, the fatty acid compositions of total serum and β-lipoprotein were identical (Part II, Chapter I, Section II). In order to simplify the presentation of results, the qualitative

composition of fatty acids in the plaque material has been compared only with that found in total serum. Statistical comparisons of fatty acid percentages have, however, been made on both total serum and β-lipoprotein values.

i) Cholesterol ester fatty acid composition

The mean values for all the fatty acids present in the cholesterol ester fraction in the total serum of the three groups and in the plaques are shown in Table 56.

Fatty Acid	BANTU	CONTROL	I.H.D.	PLAQUES
	%	%	%	%
	Mean (S.D.)	Mean (S.D.)	Mean (S.D.)	Mean (S.D.)
C12:0	0.2 (0.2)	0.3 (0.2)	0.3 (0.2)	0.2 (0.2)
C14:0	1.0 (0.9)	0.9 (0.2)	0.9 (0.3)	1.5 (1.1)
C14:1	0.4 (0.3)	0.3 (0.2)	0.3 (0.3)	0.3 (0.2)
C15:0	0.4 (0.3)	0.2 (0.1)	0.4 (0.1)	0.4 (0.2)
C15:1	0.3 (0.2)	0.2 (0.1)	0.2 (0.2)	0.2 (0.2)
C16:0	14.1 (2.0)	11.8 (1.3)	12.2 (1.9)	13.3 (2.4)
C16:1	7.3 (2.3)	4.2 (0.6)	4.4 (1.0)	5.4 (1.3)
C17:0	0.6 (0.2)	0.2 (0.2)	0.4 (0.1)	0.6 (0.4)
C17:1	0.4 (0.3)	0.4 (0.2)	0.2 (0.2)	0.2 (0.1)
C18:0	2.1 (0.8)	2.0 (0.6)	1.4 (0.5)	1.7 (1.0)
C18:1	27.7 (3.0)	21.7 (3.6)	18.8 (2.4)	27.6 (3.7)
C18:2	35.6 (3.9)	46.8 (4.3)	52.2 (5.2)	39.7 (5.0)
C18:3	0.5 (0.3)	0.8 (0.4)	0.7 (0.7)	0.7 (0.6)
C20:0	0.8 (0.7)	0.3 (0.2)	0.3 (0.3)	0.3 (0.2)
C20:3 ?	1.1 (0.8)	0.7 (0.3)	0.7 (0.5)	0.8 (0.8)
C20:4	7.5 (1.2)	6.9 (1.3)	5.8 (1.6)	6.4 (1.3)
C20:5 ?	0.9 (0.5)	2.2 (0.8)	1.5 (1.0)	1.0 (0.9)

Table 56: The fatty acid composition of the cholesterol ester fraction in arterial atheromatous plaques and in total serum from Bantu, controls and patients with I.H.D.

Major fatty acids are underlined.

It is apparent that all the cholesterol ester fatty acids present in total serum and in β-lipoprotein are present in the cholesterol ester of the plaques. The percentages of the less abundant fatty acids are very similar in the plaques and in the total serum and β-lipoprotein from all three groups. The percentages of the major fatty acid components are compared in Table 57 and Figure 33.

Fatty Acid	Fraction	BANTU (B)	CONTROL (C)	I.H.D.	PLAQUE (P)	P vs.B	P vs.C	P vs.I.H.D.
		Mean	Mean	Mean	Mean	p	p	p
C16:0	Total	14.1	11.8	12.2	13.3	NS	NS	NS
	B	13.8	12.1	12.1		NS	NS	NS
C16:1	Total	7.3	4.2	4.4	5.4	<0.02	<0.05	NS
	B	7.7	4.2	4.4		<0.01	<0.05	NS
C18:0	Total	2.1	2.0	1.4	1.7	NS	NS	NS
	B	1.9	2.2	1.3		NS	NS	NS
C18:1	Total	27.7	21.7	18.8	27.6	NS	<0.001	<0.001
	B	28.2	21.3	18.2		NS	<0.001	<0.001
C18:2	Total	35.6	46.8	52.2	39.7	NS	<0.01	<0.001
	B	35.7	47.0	53.0		NS	<0.01	<0.001
C20:4	Total	7.5	6.9	5.8	6.4	<0.05	NS	NS
	B	7.8	6.5	6.3		<0.05	NS	NS

Table 57: Comparison of the percentages of the major fatty acids present in cholesterol ester fraction in arterial atheromatous plaques and in total serum (Total) and β-lipoprotein (B) from Bantu, controls and patients with I.H.D. The values shown are means.

While there is undoubtedly a resemblance between the values for the plaques and those for both total serum and β-lipoprotein in all groups, the most striking feature is that the similarity is greatest between the values for the plaques and those for the Bantu group. The slightly lower values for C16:1 and C20:4 in the plaques do not detract to any great extent from this similarity. In contrast, while the values for C16:0, C18:0 and C20:4 are

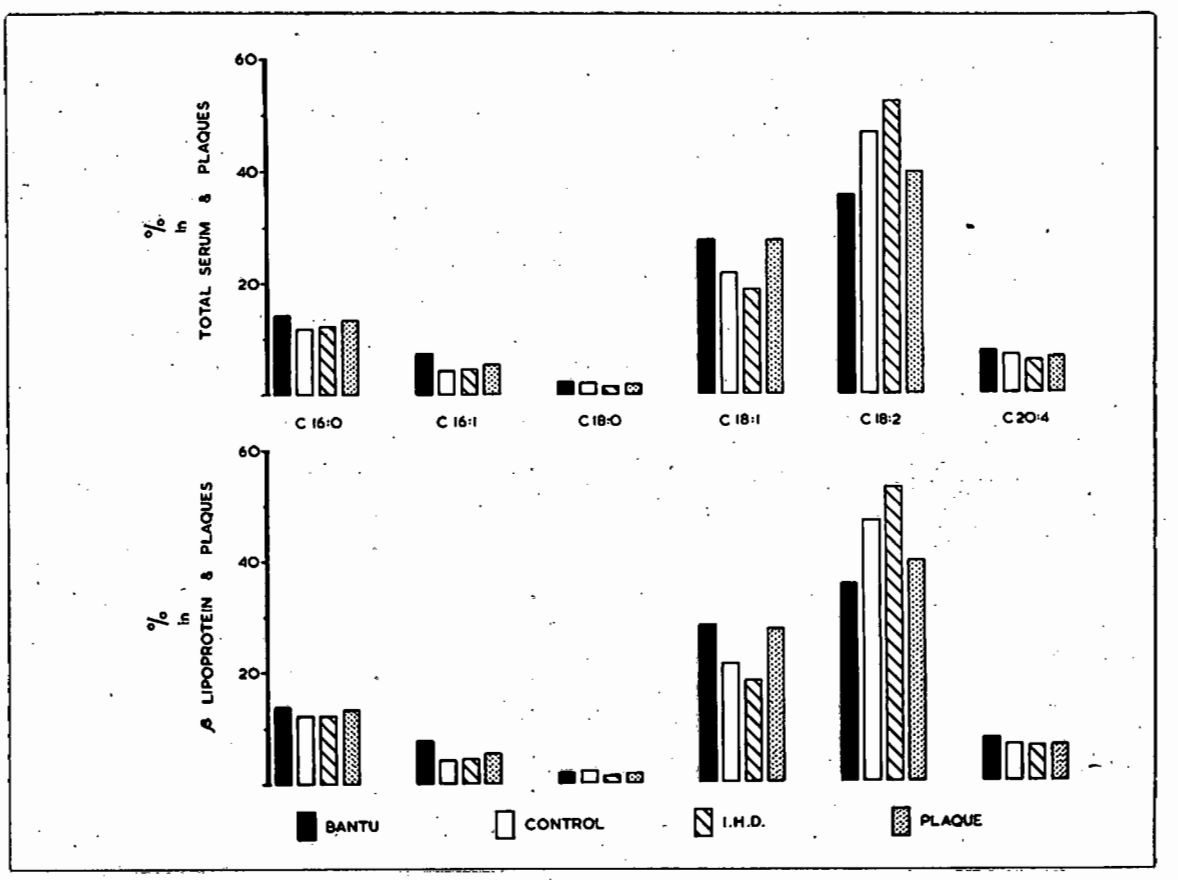


Figure 33. The percentages of the major fatty acids in the cholesterol ester fraction in total serum, the serum β-lipoprotein fraction and in arterial atheromatous plaques.

Note that, in general, the values for the Bantu group resemble those of the atheromatous plaques most closely.

similar for plaques and controls and patients, the percentages of C18:1 and C18:2 are significantly higher in patients and controls when compared with the plaques.

In view of the suggestion (Sinclair 1956) that a relative deficiency of E.F.A. might lead to the deposition in the arterial wall of cholesterol esters and thus give rise to atheroma formation, the ratio of trienoic to tetraenoic acids has been compared in the plaques and in total serum and β-lipoprotein in each group (Table 58)

	BANTU	CONTROL	I.H.D.	PLAQUE
	Mean (S.D.)	Mean (S.D.)	Mean (S.D.)	Mean (S.D.)
Total serum	0.22 (0.13)	0.22 (0.07)	0.21 (0.07	0.24 (0.11)
β-lipoprotein	0.20 (0.07)	0.24 (0.13)	0.21 (0.15)	

Table 58: The ratio of trienoic to tetraenoic acids in the cholesterol ester fraction in arterial atheromatous plaques and in total serum and β-lipoprotein from Bantu, controls and patients with I.H.D.

The mean value for this ratio is similar in the plaques and in total serum and β-lipoprotein from all groups, and is consistently less than 0.4. There is thus no evidence of E.F.A. deficiency, either absolute or relative.

ii) <u>Triglyceride fatty acid composition</u>

The mean values for all the fatty acids present in the triglyceride fraction in total serum and in plaques are shown in Table 59.

Fatty Acid	BANTU % Mean	(S.D.)	CONTROL % Mean	(S.D.)	I.H.D. % Mean	(S.D.)	PLAQUE % Mean	(S.D.)
C12:0	0.3	(0.2)	0.2	(0.1)	0.3	(0.2)	0.3	(0.2)
C14:0	2.2	(0.5)	2.1	(0.6)	3.2	(1.5)	2.3	(0.5)
C14:1	0.2	(0.1)	0.3	(0.1)	0.3	(0.2)	0.3	(0.2)
C15:0	0.6	(0.3)	1.0	(0.5)	0.6	(0.2)	0.7	(0.4)
C15:1	0.3	(0.2)	0.4	(0.3)	0.3	(0.2)	0.2	(0.1)
C16:0	28.5	(2.9)	28.4	(3.9)	28.9	(2.5)	31.3	(5.4)
C16:1	7.2	(1.3)	4.9	(0.9)	6.1	(1.1)	5.4	(1.3)
C17:0	0.8	(0.3)	0.8	(0.3)	0.7	(0.2)	0.8	(0.4)
C17:1	0.5	(0.2)	0.5	(0.2)	0.4	(0.1)	0.5	(0.3)
C18:0	6.7	(1.6)	5.7	(1.2)	5.1	(1.5)	6.6	(0.5)
C18:1	39.5	(4.8)	40.4	(3.1)	35.8	(3.6)	37.9	(4.5)
C18:2	10.1	(2.6)	13.4	(4.0)	15.3	(5.6)	10.5	(3.4)
C20:0	0.7	(0.2)	1.1	(0.4)	0.8	(0.3)	0.6	(0.4)
C20:4	2.1	(1.1)	1.3	(0.4)	1.4	(0.5)	2.4	(1.4)

Table 59: The fatty acid composition of triglyceride in arterial atheromatous plaques and in total serum from Bantu, controls and patients with I.H.D. The major fatty acids are underlined.

All the fatty acids found in total serum and β-lipoprotein are consistently present in the plaques. The percentages of the less abundant fatty acids are remarkably similar in the plaques and in the total serum and β-lipoprotein from all groups. The percentages of the major fatty acid components are compared in Table 60 and Figure 34.

Fatty Acid	Fraction	BANTU (B) Mean	CONTROL (C) Mean	I.H.D. Mean	PLAQUE (P) Mean	P vs.B p	P vs.C p	P vs.I.H.D. p
C14:0	Total	2.2	2.1	3.2	2.3	NS	NS	NS
	B	1.7	2.2	2.9		NS	NS	NS
C16:0	Total	28.5	28.4	28.9	31.3	NS	NS	NS
	B	28.4	26.7	29.2		NS	NS	NS
C16:1	Total	7.2	4.9	6.1	5.4	<0.01	NS	NS
	B	7.0	5.3	6.4		<0.01	NS	NS
C18:0	Total	6.7	5.7	5.1	6.6	NS	NS	<0.05
	B	5.6	5.6	5.0		NS	NS	<0.05
C18:1	Total	39.5	40.4	35.8	37.9	NS	NS	NS
	B	40.9	39.8	35.1		NS	NS	NS
C18:2	Total	10.1	13.4	15.3	10.5	NS	<0.05	<0.01
	B	10.5	14.5	15.7		NS	<0.01	<0.01
C20:4	Total	2.1	1.3	1.4	2.4	NS	<0.02	<0.05
	B	2.1	1.5	1.4		NS	<0.05	<0.05

Table 60: Comparison of the percentages of the major fatty acids present in triglyceride in arterial atheromatous plaques and in total serum (Total) and β-lipoprotein (B) from Bantu, controls and patients with I.H.D.

The overall similarity between the values for the plaque and total serum and β-lipoprotein from all three groups is striking (Figure 34). While the percentage of C16:1 is lower for the plaque than for the Bantu, there is, however, again a greater resemblance between the values for the Bantu group and those for the plaques than is the case for the other two groups. This is particularly marked for C18:2 and C20:4 where, while the Bantu and plaque values are similar, the percentages for both patients and controls are higher and lower respectively.

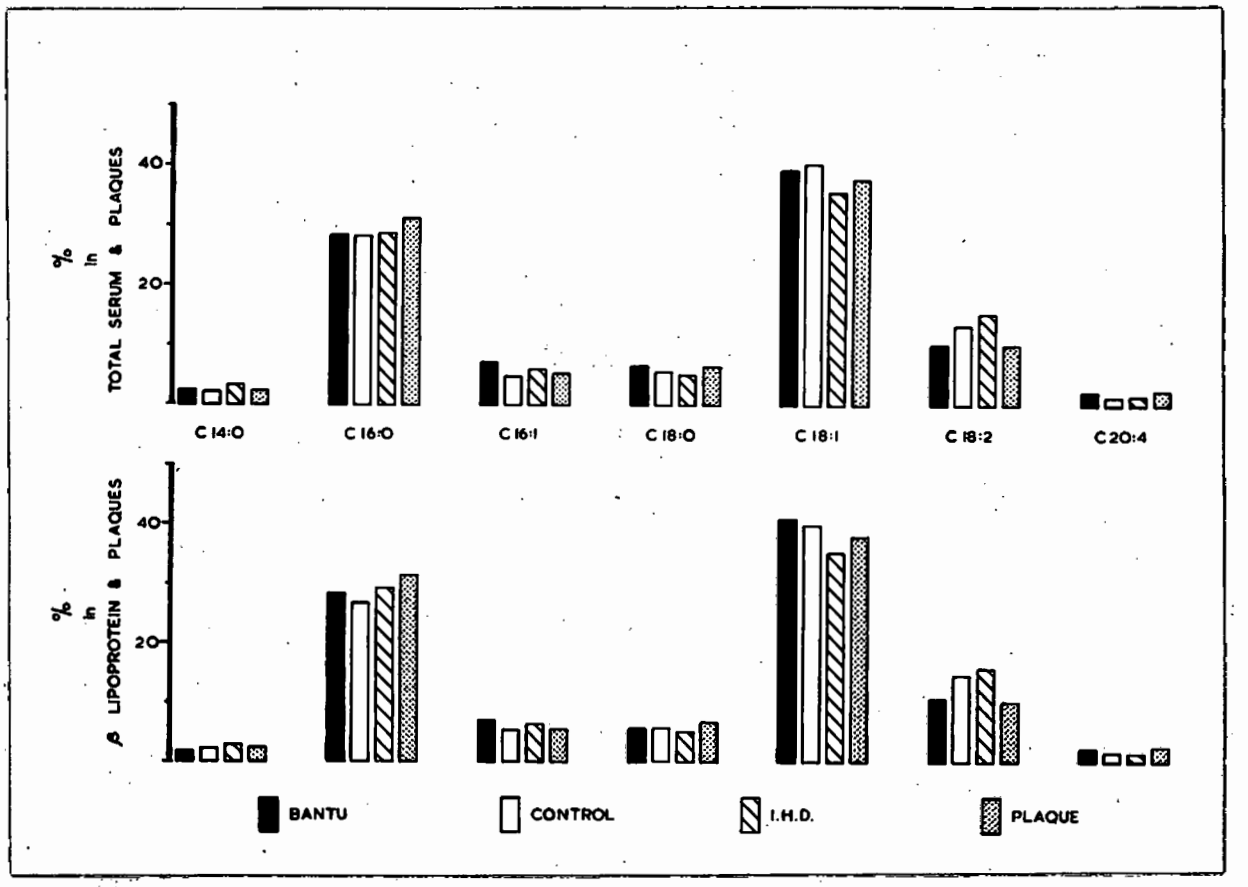


Figure 34. The percentages of the major fatty acids in the triglyceride fraction in total serum, the serum β-lipoprotein fraction and in arterial atheromatous plaques.

Note the similarity between the percentage of C18:2 in the plaques and in the Bantu group.

iii) Phospholipid fatty acid composition

The mean values for all the fatty acids present in the phospholipid fraction are shown in Table 61.

	BANTU		CONTROL		I.H.D.		PLAQUE	
	%		%		%		%	
Fatty Acid	Mean	(S.D.)	Mean	(S.D.)	Mean	(S.D.)	Mean	(S.D.)
C14:0	0.6	(0.2)	0.9	(0.5)	0.7	(0.2)	1.2	(0.6)
C14:1	0.2	(0.1)	0.3	(0.2)	0.3	(0.2)	0.3	(0.2)
C15:0	0.3	(0.2)	0.6	(0.3)	0.3	(0.1)	0.4	(0.2)
C15:1	0.2	(0.1)	0.2	(0.1)	0.3	(0.2)	0.3	(0.2)
C16:0	34.5	(2.5)	31.7	(2.4)	34.0	(2.2)	37.7	(6.7
C16:1	2.1	(0.4)	1.7	(0.4)	1.8	(0.2)	2.3	(1.0)
C17:0	0.6	(0.3)	0.7	(0.3)	0.6	(0.2)	0.7	(0.4)
C17:1	0.3	(0.1)	0.2	(0.1)	0.3	(0.1)	0.4	(0.3)
C18:0	15.0	(1.8)	14.3	(0.9)	15.1	(1.2)	14.6	(1.5)
C18:1	15.3	(2.7)	10.6	(1.7)	11.0	(1.6)	11.6	(2.8)
C18:2	13.9	(1.9)	17.4	(1.2)	18.6	(3.2)	6.1	(1.3)
C20:0	0.7	(0.3)	1.0	(0.4)	0.8	(0.3)	1.0	(0.3)
C21:0	0.5	(0.2)	0.5	(0.3)	0.5	(0.4)	0.3	(0.2)
C20:3 ?	2.5	(0.5)	2.2	(0.7)	1.9	(0.6)	2.4	(1.3)
C20:4	8.2	(1.3)	9.1	(1.3)	7.0	(1.5)	7.0	(3.5)
C22:0	0.6	(0.5)	0.8	(0.4)	0.6	(0.5)	0.5	(0.3)
C20:5 ?	0.7	(0.2)	1.8	(0.9)	1.3	(0.9)	1.5	(0.5)
C23:0	0.5	(0.2)	0.3	(0.1)	0.5	(0.2)	0.5	(0.3)
C24:0	0.7	(0.3)	1.4	(0.4)	0.8	(0.6)	3.9	(1.8)
C24:1	1.2	(0.6)	1.6	(0.3)	0.8	(0.7)	4.9	(2.2)
C22:6 ?	1.3	(0.6)	3.4	(0.9)	2.4	(1.3)	1.9	(1.1)

Table 61: The fatty acid composition of phospholipid in arterial atheromatous plaques and in total serum from Bantu, controls and patients with I.H.D.
The major fatty acids are underlined.

All the fatty acids present in total serum and in β-lipoprotein are again consistently found in the plaque phospholipid. With the exception of certain long chain fatty acids (C24:0 and C24:1) the percentages of the less abundant fatty acids are remarkably similar in the plaque and in the total serum from all groups. The percentages of the major fatty acids and the very long chain fatty acids are compared in Table 62. The values for the major fatty acids are shown in Figure 35.

		BANTU (B)	CONTROL (C)	I.H.D.	PLAQUE (P)	P v. B	P v. C	P v. I.H.D.
		%	%	%	%	p	p	p
Fatty Acid	Fraction	Mean	Mean	Mean	Mean			
C16:0	Total	34.5	31.7	34.0	37.7	NS	NS	NS
	B	35.3	32.2	33.5		NS	NS	NS
C16:1	Total	2.1	1.7	1.8	2.3	NS	NS	NS
	B	2.6	1.5	1.8		NS	NS	NS
C18:0	Total	15.0	14.3	15.1	14.6	NS	NS	NS
	B	14.3	14.0	14.1		NS	NS	NS
C18:1	Total	15.3	10.6	11.0	11.6	<0.01	NS	NS
	B	14.8	11.3	11.3		<0.01	NS	NS
C18:2	Total	13.9	17.4	18.6	6.1	<0.001	<0.001	<0.001
	B	11.8	17.4	18.3		<0.001	<0.001	<0.001
C20:4	Total	8.2	9.1	7.0	7.0	NS	NS	NS
	B	7.1	8.3	6.9		NS	NS	NS
C20:3?	Total	2.5	2.2	1.9	2.4	NS	NS	NS
	B	2.2	2.1	2.1		NS	NS	NS
C20:5?	Total	0.7	1.8	1.3	1.5	<0.001	NS	NS
	B	0.8	1.5	1.2		<0.001	NS	NS
C22:0	Total	0.6	0.8	0.6	0.5	NS	NS	NS
	B	1.0	0.5	0.3		NS	NS	NS
C23:0	Total	0.5	0.3	0.5	0.5	NS	NS	NS
	B	0.6	0.4	0.4		NS	NS	NS
C24:0	Total	0.7	1.4	0.8	3.9	<0.001	<0.001	<0.001
	B	1.5	1.8	1.0		<0.001	<0.001	<0.001
C24:1	Total	1.2	1.6	0.8	4.9	<0.001	<0.001	<0.001
	B	1.8	2.2	1.5		<0.001	<0.001	<0.001
C22:6?	Total	1.3	3.4	2.4	1.9	NS	<0.01	NS
	B	1.1	3.2	2.4		NS	<0.01	NS

Table 62: Comparison of the percentages of major fatty acids (underlined) and of the very long chain fatty acids present in phospholipid in arterial atheromatous plaques and in total serum and β-lipoprotein from Bantu, controls and patients with I.H.D.

Total = total serum
B = β-lipoprotein

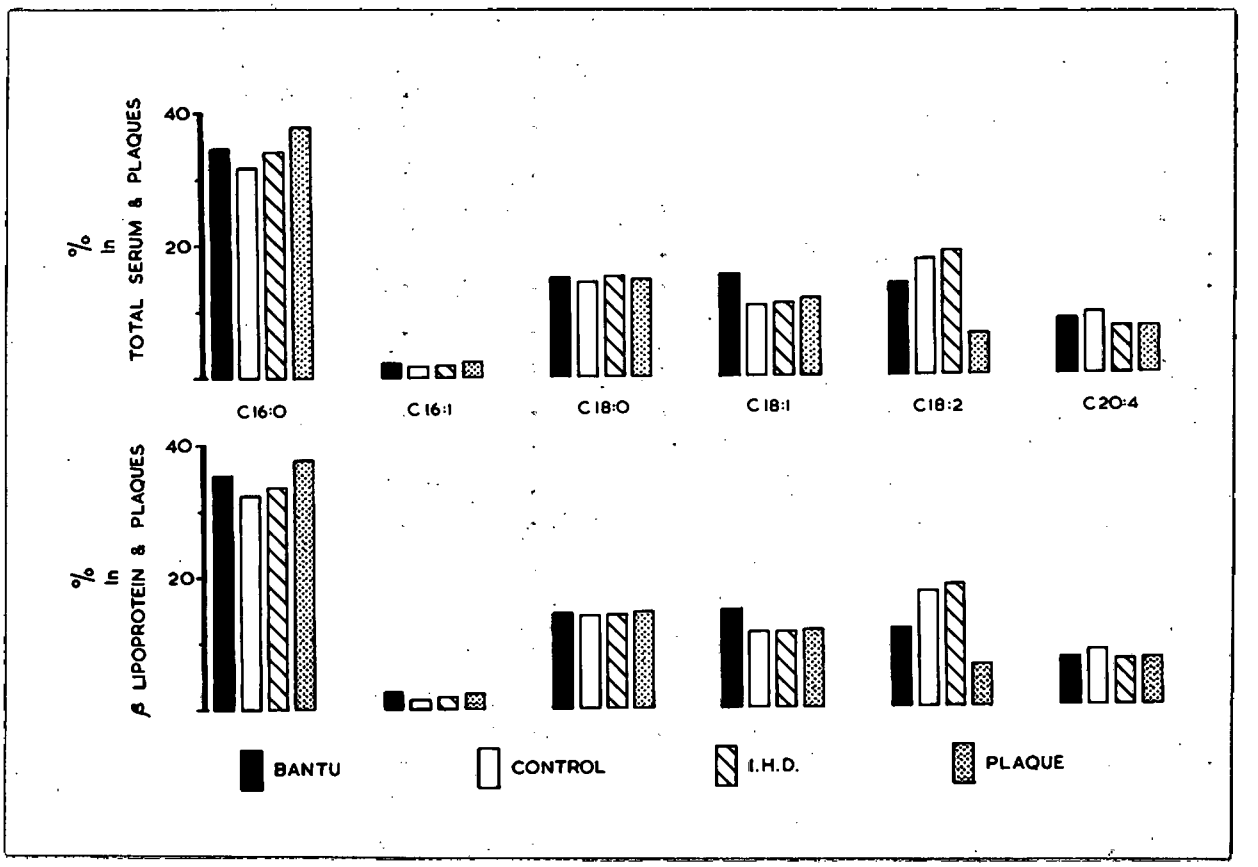


Figure 35. The percentages of the major fatty acids in the phospholipid fraction in total serum, the serum β-lipoprotein fraction and in arterial atheromatous plaques.

The atheromatous plaque phospholipid contains a considerably lower percentage of C18:2 than either total serum or β-lipoprotein in any group.

While the Bantu have a higher percentage of C18:1, the percentages of the other major fatty acids are, with one striking exception, very similar in plaques and in total serum and β-lipoprotein from the three groups. This exception is the percentage of C18:2 which is consistently lower for the plaques than for any of the three groups. The value for the Bantu is, however, much nearer that for the plaques than that for either of the other groups.

When the percentages of the longer chain fatty acids are compared between plaques and the three groups the percentage of C20:5 (?) is lower for the Bantu and that of C22:6 (?) is higher for the controls. It is, however, for C24:0 and C24:1 that a striking difference between plaques and the three groups is again apparent. For these two fatty acids the mean values for the plaques are consistently higher than for any of the groups. It will be recalled that the accuracy in estimating these fatty acids was low (Part I, Table 16). However, the differences between the values for plaques and those for total serum and β-lipoprotein in each group are probably greater than can be accounted for by the error of estimation alone. It is noteworthy that these longer chain fatty acids account for a relatively small proportion of the total fatty acids in serum phospholipids (Part II, Chapter I, Table 39). Their relatively greater abundance in the plaque phospholipids is presumably due to sphyngomyelin (Part II, Chapter 2, p.133)

The ratio of trienoic to tetraenoic fatty acids in total serum and β-lipoprotein from the three groups has been compared with that in the plaques (Table 63).

	BANTU	CONTROL	I.H.D.	PLAQUE
	Mean (S.D.)	Mean (S.D.)	Mean (S.D.)	Mean (S.D.)
Total serum	0.31 (0.05)	0.25 (0.08)	0.28 (0.10)	0.33 (0.06)
β-lipoprotein	0.32 (0.08)	0.25 (0.07)	0.31 (0.09)	

Table 63: The ratio of trienoic to tetraenoic acids in the phospholipid fraction in arterial atheromatous plaques, in total serum and β-lipoprotein from Bantu, controls and patients with I.H.D.

It is apparent that the mean values are similar in the plaques and in total serum and β-lipoprotein for all groups. There is again no evidence of E.F.A. deficiency, either absolute or relative.

Comparison with other data

Several authors have noted a marked resemblance between the cholesterol ester fatty acid pattern in plaques and in serum (Tuna et al 1958; Luddy et al 1958; Bjorntorp et al 1962). The data of Tuna et al (1958), Luddy et al (1958) and Böttcher and his colleagues (Böttcher et al 1960a; Böttcher and Woodford 1961) showed that the cholesterol ester in serum from normal White subjects has a higher percentage of C18:2 and a lower percentage of C18:1 than is found in plaque cholesterol ester. A similar finding is noted here when comparing the cholesterol ester fatty acids in serum from the control group with that in the plaques. In contrast, Lewis (1958) and Swell et al (1960) have reported that the cholesterol ester in plaques contains less dienes (C18:2) and tetraenes (C20:4) and more saturated fatty acids than serum cholesterol ester.

The data of Böttcher and his colleagues (Böttcher et al 1960a; Böttcher and Woodford 1961) and of Bjorntorp et al (1962) show a striking similarity between the triglyceride fatty acid composition of plaques and of serum. Luddy et al (1958) found that the triglyceride in serum from healthy White subjects contained more C18:2 and less C18:1 than that in plaques and this finding is in agreement with the local data.

Bjorntorp et al (1962) reported that the percentage of dienes was considerably lower in plaque phospholipid than in serum phospholipid. Böttcher and his colleagues (Böttcher et al 1960a; Böttcher and van Gent 1961) have described the phospholipid fatty acid composition of atheromatous plaques and of α- and β-lipoprotein from healthy and atherosclerotic subjects (Böttcher and Woodford 1961). When these values are compared, it is apparent that the percentage of C18:2 is lower and the percentage of C24:0 and C24:1 are higher

in the plaques than in either ∝- or β-lipoprotein from both groups of subjects. These findings are in agreement with the local data.

It is apparent that, in general, there is a marked similarity between the fatty acid compositions of lipid fractions in total serum and β-lipoprotein and those in the plaques. The differences found in the percentages of certain fatty acids do not detract to any extent from the overall similarity. These data would be in keeping with the concept that plaque lipids may be derived from serum lipids. However, the most striking feature with regard to the fatty acid composition of lipids in serum and plaques is that, in general, the values for serum from the Bantu group show a greater resemblance to those of the plaques than do those for either of the other two groups. Since the Bantu have a low susceptibility to atherosclerosis, it would seem unlikely that the fatty acid composition of serum lipids <u>per se</u> is related to the deposition of lipid in the arterial wall.

CONCLUSIONS

On comparing the relative proportions of lipid components in total serum with those in the plaques, there is no striking resemblance between the values for any group of subjects and those for the plaques. Only in the case of phospholipid is there a trend from Bantu through controls to patients towards the value in the plaques. In β-lipoprotein the relative proportions of lipid components, with the exception of triglyceride, approach those in the plaques more closely than do those in total serum. This is seen in all the groups. In general, however, the differences in lipid composition between plaques and both total serum and β-lipoprotein are more striking than any resemblance between them.

In total serum, certain lipid ratios show grading between the groups and the values for the patients tend to approach those of the plaques more closely. The values for these lipid ratios in β-lipoprotein again show a greater resemblance to those in the plaques, but there is no graded relationship between the groups. Here again, however, the differences are greater than the similarities. In general, the data suggest that, if plaque lipid is derived from serum, this does not occur by any simple process of deposition of serum lipids.

The fatty acid compositions of lipid fractions in total serum and β-lipoprotein are similar to those of the plaques. The fatty acid composition of lipids in serum from the Bantu, however, showed the greatest resemblance to that of lipid in the plaques. This suggests that the fatty acid composition *per se* need not necessarily indicate the serum origin of plaque lipids.

There is no evidence of E.F.A. deficiency in the serum lipids in any group nor does the fatty acid composition of plaque lipids suggest that E.F.A. deficiency, either absolute or relative, is a contributory factor in the formation of atheromatous plaques.

PART III.

GENERAL CONCLUSIONS

THE COMPOSITION OF SERUM LIPIDS

The relationship between serum lipid levels and the susceptibility to I.H.D. has been examined. The data, in general, support the view that subjects with I.H.D. or groups with increased susceptibility to I.H.D. have elevated serum lipid levels when compared with subjects with low susceptibility to this disease. As in most other studies, there is considerable overlap between lipid concentrations for patients and controls, although certain lipid parameters provided separation between these two groups. In general, the concentration of lipid components in β-lipoprotein provided better separation between the groups than did that in total serum. Any increase in total serum lipid concentration, beyond a certain minimum level, could be attributed almost entirely, if not entirely, to an increase in β-lipoprotein concentration.

On comparing certain lipid ratios, better separation between groups was achieved than by comparing the absolute concentration of lipids. These data would suggest that the relative proportions or "balance of lipids" may be an important factor in determining susceptibility to I.H.D.

In each group of subjects the fatty acid compositions of the esterified lipid fractions were similar in total serum and in β-lipoprotein. These data indicate that each lipid component has a characteristic fatty acid pattern which is not related to the serum fraction in which it is transported.

The fatty acid compositions of the esterified lipids were qualitatively identical in the three groups of subjects. Certain quantitative differences in the fatty acid compositions of these lipid fractions were present between the groups, notably in the percentage of linoleic acid. There was no evidence to suggest that a deficiency of E.F.A., either absolute or relative, might be related to increased susceptibility to I.H.D.

THE RELATIONSHIP BETWEEN DIET AND SERUM LIPIDS

The serum lipid concentration could not be directly related to the dietary fat content, either quantitative or qualitative, in the three groups. It would seem, therefore, that the elevated lipid levels in this patient group are not related to dietary fat alone, but could be related to other dietary factors, e.g. the protein intake, which was directly correlated with serum lipid levels in the three groups, or might reflect a more fundamental disorder of lipid metabolism. There was no evidence that a high carbohydrate diet, as such, gives rise to elevated serum lipid levels.

The percentage of linoleic acid in the serum lipid components was directly related to the percentage of total calories derived from linoleic acid. The differences in the fatty acid composition of lipids in the three groups could be related, at least in part, to differences in their diet.

THE RELATIONSHIP BETWEEN LIPID IN SERUM AND IN ARTERIAL ATHEROMATOUS PLAQUES

The lipid present in the arterial atheromatous plaques was fairly constant in composition. In general the data showed that the fatty acid composition of serum lipid fractions bears a close resemblance to that of plaque lipid. While these data would be in keeping with the concept that the lipid in atheromatous plaques is derived from serum, it is not possible, on the basis of this similarity alone, to conclude that the plaque fatty acids have been derived from serum. In assessing the possible role of a particular fatty acid composition in determining the formation of atheroma, it is noteworthy that the greatest resemblance between the lipid in the plaques and that in serum was found for the Bantu. Since this group is least susceptible to atherosclerosis, it would seem unlikely that the fatty acid composition *per se* is related to the formation of atheroma.

The relative proportions of various lipid fractions in the serum β-lipoprotein fraction showed a closer resemblance to those in the plaques than was the case for total serum. There was some trend for certain lipid ratios in the serum of patients to approach those found in the plaques. In general, however, the differences in composition between serum and plaque lipids were greater than any resemblance. These data suggest that, if plaque lipid is derived from serum, this does not occur by any simple process of deposition of circulating serum lipid.

SUMMARY

1. The composition of lipid in total serum, in the serum β-lipoprotein fraction and in arterial atheromatous plaques has been determined. Blood samples were obtained from 16 Bantu, 12 healthy White subjects (controls) and 12 patients with ischaemic heart disease (I.H.D.). Arterial atheromatous plaque material was obtained by aorto-iliac endarterectomy from 12 other White subjects with I.H.D. All subjects were males between 40 and 50 years of age.

2. The concentration of both free and esterified cholesterol, triglyceride and phospholipid were determined chemically and total lipid concentration obtained from the sum of these determinations. The qualitative and quantitative fatty acid compositions of cholesterol ester, triglyceride and phospholipid were determined by gas-liquid chromatography. Dietary data were obtained by recall.

3. The composition of lipid in total serum and in the serum β-lipoprotein fraction has been compared within each group and between the groups of subjects. The composition of lipid in the atheromatous plaque material has been compared with that in total serum and in the serum β-lipoprotein fraction in each group of subjects.

4. In both total serum and in the serum β-lipoprotein fraction the concentration of total lipid and of each lipid fraction was significantly lower for the Bantu than for the other two groups. Although the mean concentrations of the lipid fractions were generally higher for patients than for controls, these differences were statistically significant only for free cholesterol in total serum and for the total lipid, free cholesterol, triglyceride and phospholipid in the β-lipoprotein. β-lipoprotein lipid concentrations, in general, provided better separation between the groups than did the lipid concentrations in total serum.

5. In all cases the major proportion of serum lipid was present in the β-lipoprotein. There was a highly significant positive correlation between the concentrations of lipid in total serum and in β-lipoprotein. Any increase in the concentration of lipid in the total serum was due almost entirely to an increase in the concentration in β-lipoprotein.

6. The relative proportions of the lipid components were compared between the groups. In total serum only the percentage of phospholipid showed a graded decrease from Bantu through controls to patients. In β-lipoprotein the relative proportions of lipid were similar for patients and controls, but the Bantu showed differences from both other groups for a number of lipid parameters.

7. On examining certain lipid ratios in total serum and in β-lipoprotein, better separation between groups was achieved than by comparing lipid concentrations. This separation was more marked in total serum than in β-lipoprotein.

8. The cholesterol ester, triglyceride and phospholipid fractions each have a characteristic fatty acid composition. The fatty acid composition of these lipid fractions in total serum closely resembled those in the β-lipoprotein in each group of subjects. In each lipid fraction, the percentage of linoleic acid was lower and the percentages of palmitoleic and oleic acids were generally higher in the Bantu group than in either of the other groups.

9. Dietary analysis showed that the concentration of serum lipid could not be directly related to the fat or carbohydrate content of the diet in the three groups. The proportion of protein calories was directly related to serum lipid concentrations. The linoleic acid intake expressed as a percentage of total calories was related to the proportion of linoleic acid in the esterified lipid fractions of serum.

10. Atheromatous plaque lipid was found to have a fairly constant composition, characterised by a high proportion of cholesterol, of which almost half was in the free form.

11. The fatty acid compositions of the esterified lipid fractions in total serum and β-lipoprotein were similar to the corresponding fractions in the plaques. This resemblance was particularly marked for cholesterol ester and triglyceride, but less so for phospholipid. In general, the fatty acid compositions of serum lipid fractions in the Bantu resembled those of the plaques more closely than did those from the other two groups.

On comparing the relative proportions of lipid components in serum and in the plaques, the values for β-lipoprotein approached those of the plaques more closely than did those for total serum. In general, however, differences in lipid composition were more striking than any similarities.

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APPENDIX I

Serum lipid fatty acid data

The fatty acid compositions of the cholesterol ester, triglyceride and phospholipid fractions of human serum reported in other studies are shown together with the findings reported in this study (Tables 1 - 6, pages 183 - 188).

The relevant data reported in the thesis are shown in Tables 23, 24, 30, 31, 36 and 37 (pages 82, 83, 95, 96, 103 and 104), and on pages 84, 85, 93, 97, 101 and 111.

Author	Subjects	C16:0	C16:1	C18:0	C18:1	C18:2	C20:4
		Mean	Mean	Mean	Mean	Mean	Mean
Present study	Bantu	14.1	7.3	2.1	27.7	35.6	7.5
	Control	11.8	4.2	2.0	21.7	46.8	6.9
	I.H.D.	12.2	4.4	1.4	18.8	52.2	5.8
Lawrie et al. 1961	Normals 16-45 years Males and females	13.8	6.0	3.3	21.7	35.2	3.8
Schrade et al. 1961	Normal males 19-42 years	11.7	6.0	2.5	18.7	48.4	5.0
	Normal males 46-71 years	12.0	8.4	3.1	20.7	44.9	3.9
	Atherosclerotic subjects	16.6	10.7	2.9	20.0	38.6	2.8
Lindgren et al. 1961	Normal subjects	10.0	3.2	1.2	17.8	55.3	5.6
Hallgren et al. 1960	Normal males 23-38 years	11.0	4.8	0.8	23.2	46.2	5.9
Scott et al. 1963	New Yorkers 30-45 years	10.3	3.3	1.1	19.2	57.5	8.4
	Poor East Africans 30-45 years	12.6	6.0	2.0	29.1	41.0	8.1
	Upper class East Africans 30-45 years	12.1	5.8	2.1	26.9	42.5	9.2
Böttcher and Woodford 1961	Normal males (β-lipoprotein)	11.2	3.4	1.2	19.4	56.2	4.4
	Atherosclerotic males (β-lipoprotein)	11.8	4.6	1.5	21.8	48.5	5.4
Swell et al. 1960	Atherosclerotic males	13.7	4.1	1.3	25.8	43.3	7.5
Smith 1962	Healthy subjects (Sfo-12 lipoprotein)	14.7			23.3	47.8	5.1
	Subjects with I.H.D. (Sfo-12 lipoprotein)	11.9			24.8	47.9	5.1

Table 1: The percentages of the major fatty acids in serum cholesterol ester, as determined by different authors, using the technique of G.L.C.

Author	Subjects	Dienes	Tetraenes
		Mean	Mean
Present study	Bantu Control I.H.D.	35.6 46.8 52.2	7.5 6.9 5.8
Luddy et al 1958	Normal males	47.5	8.0
Lewis 1958	Healthy Europeans Europeans with I.H.D. Healthy Bantu	53.2 40.6 55.4	9.5 7.6 13.6
Tuna et al 1958	Normal subjects	41.1	8.0
Schrade et al 1959	Healthy subjects Atherosclerotic subjects	47.1 38.0	6.3 5.0
Wright et al 1959	Normal subjects	43.8	6.1
Bjorntorp 1960	Normal subjects 22 - 77 years Normal young males Subjects with essential hypercholesterolemia	42.5 43.8 39.0	5.1 4.6 5.3
Riley and Nunn 1960	Healthy male 39 years	39.0	3.5
Kingsbury et al 1962	Normal subjects 48 - 70 years "Atheromatous" subjects 51 - 74 years	45.1 30.0	8.0 4.7

Table 2: The percentages in serum cholesterol ester of dienes and tetraenes, as determined by different authors, using the technique of alkali isomerization.

Present study Dienes = C18:2
Tetraenes = C20:4

Author	Subjects	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C20:4
		Mean	Mean	Mean	Mean	Mean	Mean	Mean
Present study	Bantu	2.2	28.5	7.2	6.7	39.5	10.1	2.1
	Control	2.1	28.4	4.9	5.7	40.4	13.4	1.3
	I.H.D.	3.2	28.9	6.1	5.1	35.8	15.3	1.4
Lawrie et al. 1961	Normals 16-45 years Males and females	3.8	21.0	7.3	3.7	26.0	11.9	3.4
Schrade et al. 1961	Normal males 19-42 years	1.5	27.8	7.7	3.6	36.4	12.7	3.0
	Normal males 46-71 years	1.7	28.9	7.7	4.3	36.0	11.3	2.4
	Atherosclerotic subjects	2.3	29.5	8.0	3.6	38.3	10.8	2.1
Lindgren et al. 1961	Normal subjects	-	29.8	3.7	4.6	39.1	15.7	1.3
Hallgren et al. 1960	Normal males 23-38 years	3.0	24.9	6.2	4.0	41.4	10.9	0.8
Scott et al. 1963	New Yorkers 30-45 years	2.1	29.4	4.0	4.7	43.1	16.8	
	Poor East Africans 30-45 years	2.7	30.5	5.1	5.6	44.6	11.5	
	Upper class East Africans 30-45 years	3.8	33.3	5.2	6.7	40.5	10.5	
	New Yorkers over 45 years	2.8	29.9	4.7	5.0	43.2	14.5	
	Poor East Africans over 45 years	2.8	30.6	5.3	4.9	44.2	12.1	
	Upper class East Africans over 45 years	3.7	33.1	5.0	6.7	40.7	10.9	
Böttcher and Woodford 1961	Normal males (α-lipoprotein)	2.5	23.6	5.7	5.2	35.8	15.6	2.8
	Atherosclerotic males (α-lipoprotein)	2.6	21.3	7.6	4.9	34.3	14.6	3.1

Table 3: The percentages of the major fatty acids in serum triglyceride, as determined by different authors, using the technique of G.L.C.

Author	Subjects	Dienes	Tetraenes
		Mean	Mean
Present Study	Bantu	10.1	2.1
	Control	13.4	1.3
	I.H.D.	15.3	1.4
Luddy et al 1958	Normal males	14.3	1.3
Schrade et al 1959	Healthy subjects	10.9	0.8
	Atherosclerotic subjects	11.2	1.5
Bjorntorp 1960	Normal subjects 22 - 77 years	8.0	1.6
	Normal young males	9.2	1.2
	Subjects with essential hypercholesterolemia	8.1	1.0
Antonis and Bersohn 1960	Bantu males	30.7	8.3
	European males 18 - 30 years	26.6	5.5
	European males 31 - 40 years	21.7	5.5
	European males 41 - 70 years	20.2	5.8
	Subjects with I.H.D. 40 - 60 years	18.1	4.0

Table 4: The percentage in serum triglyceride of dienes and tetraenes, as determined by different authors, using the technique of alkali isomerization.

Present study Dienes = C18:2
Tetraenes = C20:4

Author	Subjects	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C20:4
		Mean	Mean	Mean	Mean	Mean	Mean	Mean
Present study	Bantu		34.5	2.1	15.0	15.3	13.9	8.2
	Control		31.7	1.7	14.3	10.6	17.4	9.1
	I.H.D.		34.0	1.8	15.1	11.0	18.6	7.0
Lawrie et al. 1961	Normals 16-45 years Males and females	0.8	26.7	2.4	10.3	16.7	17.2	6.2
Schrade et al. 1961	Healthy subjects		30.7	3.6	12.1	15.1	20.7	8.7
	Atherosclerotic subjects		34.9	3.2	11.9	16.7	18.1	5.9
Lindgren et al. 1961	Normal subjects		33.2	1.1	14.3	11.9	21.9	9.3
Hallgren et al. 1960	Normal males 23-38 years	0.5	27.7+		11.9	14.3	20.4	8.8
Scott et al. 1963	New Yorkers 30-45 years	trace	35.2	1.2	13.4	11.8	24.3	14.2
	Poor East Africans 30 - 45 years	0.4	35.6	1.7	15.6	16.7	17.4	12.7
	Upper class East Africans 30 - 45 years	0.4	37.0	1.7	15.3	15.2	17.1	13.4
	New Yorkers over 45 years	0.6	34.5	1.5	15.0	13.4	22.8	12.3
	Poor East Africans over 45 years	trace	34.4	2.0	14.2	15.8	20.0	13.7
	Upper class East Africans over 45 years	0.5	38.0	2.1	14.1	14.4	19.7	11.2
Böttcher and Woodford 1961	Normal males (β-lipoprotein)		30.8	1.0	14.5	11.0	17.0	4.7
	Atherosclerotic males (β-lipoprotein)		28.4	1.3	13.1	13.4	17.6	5.6
James et al. 1957	Normal subjects		26.7		13.3	20.8	18.8	9.7
	Subjects with I.H.D.						19.6	9.6
Dole et al. 1959	Normal males and females	1.1	33.1	2.5	12.8	18.6	16.0	4.3

Table 5: The percentages of the major fatty acids in serum phospholipid, as determined by different authors, using the technique of G.L.C.

+ = C16:0 + C16:1

Author	Subjects	Dienes	Tetraenes
		Mean	Mean
Present study	Bantu Control I.H.D.	13.9 17.4 18.6	8.2 9.1 7.0
Luddy et al 1958	Normal males	14.5	8.2
Schrade et al 1959	Healthy subjects Atherosclerotic subjects	22.2 19.6	6.6 4.5
Green et al 1960	Normal subjects (β-lipoprotein)	14.1	7.4
Bjorntorp 1960	Normal subjects 22 - 77 years	15.3	6.5
	Normal males 22 - 36 years	17.3	6.8
	Xanthomatous subjects	14.5	6.0
	Subjects with essential hypercholesterolemia	13.4	5.7

Table 6: The percentage in serum phospholipid of dienes and tetraenes, as determined by different authors, using the technique of alkali isomerization.

Present study Dienes = C18:2

Tetraenes = C20:4

APPENDIX II

Dietary data for individual subjects

The dietary data for individual subjects sampled in this study are shown in Tables 1 - 3 (pages 191 - 193). The values for each group of subjects reported in this thesis in Tables 42 and 43 (pages 115 and 116) are derived from these individual values.

The method whereby the individual data were obtained is described under "Dietary Analysis" (see page 15) and the questionnaire used in obtaining this information is shown on pages 194 - 197.

Subject	Daily Intake					Percentage of daily calories				
	Total Calories	Carbo-hydrate	Protein	Fat	Linoleic Acid	Carbo-hydrate	Protein	Fat	Linoleic Acid	Fat calories derived from Linoleic Acid
		gm	gm	gm	gm	%	%	%	%	%
1	2031	238	83	83	4.6	47	16	37	2.0	6
2	2541	324	122	84	9.5	51	19	30	3.4	11
3	2136	229	102	90	7.2	43	19	38	3.0	8
4	1492	145	86	63	11.1	39	23	38	7.0	18
5	1850	296	79	39	6.2	64	17	19	3.0	16
6	2242	297	86	79	4.7	53	15	32	1.9	6
7	1370	178	77	39	4.2	52	21	27	2.8	11
8	1537	200	83	45	4.2	52	22	26	2.5	9
9	1609	149	109	64	13.0	37	27	36	7.3	20
10	1858	177	58	102	5.9	38	13	49	2.9	6
11	1526	156	70	69	3.2	41	18	41	1.9	5
12	2568	321	123	88	7.7	50	19	31	2.7	9

Table 1. The composition of the average daily diet of the subjects with I.H.D.

Subject	Daily Intake					Percentage of daily calories				
	Total Calories	Carbo-hydrate	Protein	Fat	Linoleic Acid	Carbo-hydrate	Protein	Fat	Linoleic Acid	Fat calories derived from Linoleic Acid
		gm	gm	gm	gm	%	%	%	%	%
1	2646	261	119	125	5.5	40	18	42	1.9	4
2	2078	159	88	121	5.4	31	17	52	2.3	5
3	2338	164	94	145	5.7	28	16	56	2.2	4
4	2513	155	92	170	6.7	25	15	60	2.4	4
5	2492	205	92	145	6.1	33	15	52	2.2	4
6	2493	255	87	125	6.0	41	14	45	2.2	5
7	2492	192	93	150	3.2	31	15	54	1.1	2
8	2833	207	114	172	11.0	29	16	55	3.5	6
9	3654	301	117	220	6.5	33	13	54	1.6	3
10	2195	187	105	114	8.5	34	19	47	3.5	8
11	2629	224	107	145	11.7	34	16	50	4.0	8
12	2516	220	87	143	11.1	35	14	51	4.0	8

Table 2. The composition of the average daily diet of the control subjects.

Subject	Daily Intake					Percentage of daily calories				
	Total Calories	Carbo-hydrate	Protein	Fat	Linoleic Acid	Carbo-hydrate	Protein	Fat	Linoleic Acid	Fat calories derived from Linoleic Acid
		gm	gm	gm	gm	%	%	%	%	%
1	4547	934	115	39	10.3	82	10	8	2.1	26
2	3531	733	80	31	5.3	83	9	8	1.4	17
3	3848	746	135	36	7.1	78	14	8	1.7	19
4	3804	765	105	36	7.4	81	11	8	1.8	20
5	3871	783	106	35	6.2	81	11	8	1.5	18
6	3922	806	98	34	7.4	82	10	8	1.7	22
7	3648	749	91	32	6.3	82	10	8	1.6	20
8	4147	844	114	35	6.4	82	11	7	1.4	18
9	3772	749	113	36	7.3	79	12	9	1.8	20
10	4375	866	131	43	10.9	79	12	9	2.3	25
11	3738	729	131	34	6.1	78	14	8	1.5	18
12	4505	883	142	45	10.3	78	13	9	2.1	23
13	3965	799	109	37	7.8	81	11	8	1.8	21
14	4603	930	115	47	11.0	81	10	9	2.2	23
15	4001	787	130	37	7.3	79	13	8	1.7	19
16	4095	793	143	39	8.4	77	14	9	1.9	20

Table 3. The composition of the average daily diet of the Bantu subjects.

DIETARY ANALYSIS QUESTIONNAIRE

NAME_____

AGE_____ SEX_____ OCCUPATION_____

A. What kind of bread do you eat, and how much per day?

	number of slices	thickness of slice
White		
Brown		
Whole meal		
Rye		
Other		

B. What do you spread on your bread? Is the spread thick, thin or medium?

	number of slices	thickness of spread
Butter		
Margarine		
Cheese		
Jam		
Honey		
Sandwich Spread		
Peanut Butter		
Other		
Nothing		

C. How much and how often in a week do you eat or drink any of the following?

	times per week	quantity
Milk (including that with cereals, beverages and cooking)		
Skim milk		
Sour milk (Amasi)		
Cheese		
Cream		
Ice-cream		
Milk shakes		

D. How many eggs do you eat in a week, and how are they prepared?

Eggs per week	Boiled	Fried	Scrambled	Poached	Omelette

What fats or oils are used in preparing the eggs?

Butter_____ Margarine_____ Oil_____

E. Do you prefer fat or lean meat? _____

How often and how much of the following do you eat, and how is it prepared?

Type of meat	Times per week	Portion size L(arge) M(edium) S(mall)	Method of Cooking			Cooking fat or oil used
			Fried	Grilled Stewed Boiled	Roasted Baked	
Mutton						
Pork						
Beef						
Sausages						
Pies						
Poultry						
Offal						
Fish(fresh)						
Fish(tinned)						
Bacon						
Tinned meat						
Polony, Ham						

F. How much and how often in a week do you eat any of the following?

	times per week	Size of portion L(arge) M(edium) S(mall)
a) Spaghetti, macaroni, noodles		
b) Rice		
c) Samp		
d) Mealie Rice		
e) Mealie Meal		
f) Beans (dried)		
g) Oats, Kaffir-corn		
h) Breakfast cereals		

G. How much and how often in a week do you eat the following?

Is any cooking fat (e.g. butter) or cooking oil used in preparing them?

	Times per week	Portion L(arge) M(edium) S(mall)	Oil or Fat used
a) Potatoes (boiled)			
(fried)			
(mashed)			
(roasted)			
(chips)			
b) Vegetables (cooked)			
Vegetable salad			
Legumes, (peas, beans, lentils)			

H. How much and how often do you eat (or drink) the following?

	Times per week	Quantity
Cakes		
Biscuits		
Buns		
Rusks		
Pastries		
Sweets		
Chocolate		
Nuts		
Alcohol		
Dried fruit		
Fresh fruit		
Canned fruit		
Sugar (in beverages or on cereals)		

I. How long have you been following the diet described? _____

J. Are you on any special type of diet? _____

_____