5. The rate of synthesis of 2-naphthyl sulphamate by rat-liver preparations is increased by about 400 % by 17-oxo steroids in concentrations of approximately 10^{-5} M.

6. The rate of synthesis of sulphamate by guinea-pig preparations is not increased by 17-oxo steroids.

7. It is suggested that the mechanism of this activation involves the formation of steroid 17-enol sulphates and that these enol sulphates would be a form of 'active sulphate', having a high sulphate-group potential.

8. The rates of synthesis of certain other sulphate esters by the enzyme preparations are described.

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The Cholesterol Esters Circulating in Human Blood in Health

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The free and esterified cholesterol circulating in human blood has frequently been measured but, because of the experimental difficulties involved, scant attention has been paid to the fatty acids combined with cholesterol. Kelsey & Longnecker (1941), using selective enzymic hydrolysis of the esters (Kelsey, 1939*a*, *b*, *c*), identified some of the esters in the serum of lactating cows and, with chromatographic methods, Lough & Garton (1957) have been able to demonstrate the presence of many more. It seemed possible that knowledge of the identity of the fatty acid component of these esters would throw some light on the metabolism of cholesterol in the human.

In the present work, chromatographic methods were employed to isolate the cholesterol esters from the lipids of normal human sera, to establish their identity and estimate the amount of each fatty acid. The results obtained indicate that over 70 % of the acids are unsaturated and more than half of these are polyethenoid.

* Some of this work formed part of a thesis submitted to the University of Edinburgh for the degree of M.D.

EXPERIMENTAL

Materials

Most of the fatty acids used in examining the characteristics of the columns were prepared from commercial specimens by fractional distillation of their methyl esters at pressures of about 0·1 mm. Hg. The commercial samples of stearic acid were, however, found to be heavily contaminated with palmitic acid. A sample of high purity was eventually prepared by hydrogenation of commercial elaidic acid (L. Light and Co. Ltd.). A sample of pure linoleic acid was kindly provided by Unilever Research Laboratories, Port Sunlight, Cheshire. All solvents were of reagent grade except for the ether, which was of micro-analytical reagent grade. The acetone was slightly acid and the resulting small titration blank was allowed for in the determination of the fatty acids.

Blood samples. Two samples of serum and two of plasma were analysed. Sample I consisted of 100 ml. of pooled sera from 25 patients, none of whom was suffering from liver disease. Sample II was 25 ml. of serum from a healthy male aged 39 years. Sample III was 400 ml. of plasma from two separate pints of transfusion-bank blood; this sample was stored at 0° for 6 weeks before it was extracted. Sample IV was 200 ml. of plasma obtained from another

pint of transfusion-bank blood; it was stored at 0° for 2 days before it was extracted. Nothing was known of the donors of the plasma but it may be safely assumed that they were healthy. Samples III and IV had both been collected into disodium citrate–dextrose solution.

Methods

Lipid-extraction procedure. Each sample of serum was poured into ten times its volume of ethanol and allowed to stand overnight at room temperature. It was then filtered and the protein residues were dispersed in chloroform–methanol (1:1, v/v) equal in amount to the original volume of serum, refluxed for 3 hr. and then filtered. The extraction with chloroform–methanol was repeated and after the final filtration the residues were washed twice with chloroform. All extracts were pooled and evaporated to small volume under reduced pressure, the temperature being kept below 60°. The concentrated liquor was extracted three times with light petroleum (40–60°) and the combined extracts were dried over anhydrous sodium sulphate.

Sample III was poured into its own volume of ethanol and allowed to stand overnight. After filtration, the protein residues were refluxed with ethanol–ether (1:1, v/v) equal in amount to the original volume of plasma. After filtering the mixture again, the protein residues were refluxed with chloroform–ethanol–methylal (1:1:1, by vol.) for 4 hr. A subsequent extract contained no cholesterol and was discarded.

Sample IV was refluxed for 2 hr. with 6 vol. of acetone–methanol (5:7, v/v) and, after filtering, this process was repeated on the protein residues. The residues were once more extracted with methanol–methylal–chloroform (2:1:1, by vol.) equal to twice the original volume of plasma and finally with twice its volume of ethanol–chloroform–light petroleum (1:1:1, by vol.). The cholesterol recovered in the last fraction amounted to 0·3 mg. (by the Lieberman–Burchard estimation). All fractions were combined and evaporated to dryness under reduced pressure.

To ensure that no cholesterol remained entrapped in the protein residues from samples I, II and III they were separately incubated overnight with 25% (w/v) potassium hydroxide to hydrolyse the protein. The excess of alkali was neutralized with dilute hydrochloric acid and the mixture was extracted repeatedly with light petroleum (40–60°). Quantitative Lieberman–Burchard estimations on the residues from the combined light petroleum extracts showed only traces of cholesterol, ranging from 0·05 to 0·60 mg.

Chromatographic separation of the cholesterol esters. Columns of silicic acid were used (Fillerup & Mead, 1953, 1954). Silica gel (chromatographic grade; 200–300 mesh) gave good separation of an artificial mixture of lipids consisting of cholesterol, cholesterol palmitate, cholesterol oleate and cholesterol stearate, triolein, tristearin and tripalmitin, and egg lecithin (British Drug Houses Ltd.). Satisfactory flow rates were obtained without the admixture of filter aids. In the actual separation of blood lipids, 30 g. of silicic acid was used, giving columns of about 1·8 cm. bore by 30 cm. long. Previous experiments with the artificial mixtures showed that these columns separated satisfactorily lipid mixtures of up to 900 mg., i.e. 30 mg./g. of silica gel. The silicic acid was transferred to the column as a slurry in light petroleum (40–60°) and packed by mechanical vibration. Lipid mixtures from the plasma and serum extracts were transferred to the columns in the minimum volume of light petroleum (40–60°) and washed in with a further small volume of the same solvent. This was followed by 300 ml. of the same solvent, after which 1% (v/v) diethyl ether–light petroleum (40–60°) was used to elute the cholesterol esters. Fractions of 50 ml. were collected and 1 ml. samples were examined for cholesterol by the Lieberman–Burchard test, elution being continued until the result of this test became negative. In sample I the triglycerides, free cholesterol and phospholipids were separated and three 1 ml. samples were removed from each 50 ml. fraction and examined for cholesterol, glycerol and phosphorus. In the other three samples, phosphorus and glycerol analyses were carried out only on a portion of the combined Lieberman–Burchard-positive 1% ether–light petroleum fractions.

Reversed-phase chromatography of fatty acids. Columns were first prepared in the manner described by Howard & Martin (1950) but proved unsatisfactory; the stationary phase was flocculent and difficult to pack and the columns had exceedingly slow flow rates; furthermore, when fatty acids in acetone solution were added to the columns the stationary phase collapsed and shed liquid paraffin. This behaviour appeared to be due to the small particle size of the kieselguhr that was used at this stage. Columns were therefore prepared as described by Silk & Hahn (1954), with preliminary removal of the fine particles by suspension in water. These columns gave satisfactory separation of artificial mixtures of fatty acids. Unsaturated acids were added to the columns in solution in acetone–water, but saturated acids were generally too insoluble and were made up into a small quantity of the mull as recommended by Silk & Hahn (1954). Acetone–water mixtures equilibrated with liquid paraffin were used to elute the fatty acids: the mixtures were stored at 37° when not in use. For sample I the concentration of the acetone was increased by steps of 2·5% between 45 and 80% (v/v), but 5% increments were used in the subsequent work without affecting the sharpness of the separations. Fractions (5 ml.) of the eluate were collected and titrated with 0·02 N-tetramethylammonium hydroxide in aqueous 50% (v/v) ethanol in a Conway burette, 0·1% bromothymol blue being used as indicator; the alkali was standardized daily against 5 mN-benzoic acid in ethanol. Columns were of 1·8 cm. bore and were packed with 25–35 g. of stationary phase. Firm packing was found to be important and was effected by lowering a pad of cotton wool on to the surface of the kieselguhr and ramming with a stainless-steel plunger. The columns were enclosed in a tinplate jacket of 3 in. diameter which was heated by a 50 w heating element (Isotape) wrapped round the outer surface. The temperature was controlled by an oven-type bimetallic thermostat to between 33° and 36°.

Hydrolysis. The combined cholesterol ester fractions were washed with water and evaporated to dryness; the residue was dissolved in 10 ml. of diethyl ether and 10 ml. of 3 M-sodium ethoxide was added. Water (10 ml.) was then added to reduce the risk of isomerization and the mixture was refluxed in an atmosphere of nitrogen. After acidification of the reaction mixture with dilute hydrochloric acid the fatty acids were extracted with light petroleum, the combined extracts were washed with water, dried over sodium sulphate and evaporated to dryness in a stream of nitrogen.

Hydrogenation. Samples of fatty acids (10–20 mg.) from the hydrolysis mixture in ethyl acetate solution were hydrogenated at 1·1 atmosphere pressure with 10% palladized charcoal. Constant agitation for 48 hr. with 50 mg. of catalyst was necessary to ensure complete hydrogenation.

Oxidation. Another 10–20 mg. sample of the fatty acids was treated with alkaline potassium permanganate at room temperature for 18 hr. (Crombie, Comber & Boatman, 1955). At the end of this time the iodine value of the product was still about 20, probably because partial conjugation induced during hydrolysis prevented full hydroxylation of the polyethenoid acids. No further reduction in iodine value was obtained if oxidation was continued for 48 hr. Nevertheless, as a precautionary measure to ensure the fullest possible oxidation, all the samples were oxidized for at least 48 hr. The product was then examined by reversed-phase chromatography. In no case was any acid eluted in 50–60% acetone, indicating that all polyethenoid acids had undergone some degree of oxidation.

Ester and free cholesterol. Ester and free cholesterol were estimated by the method of Obermer & Milton (Milton & Waters, 1955).

Glycerol. Glycerol was estimated by the method of Lambert & Neish (1950) after hydrolysis with 25% potassium hydroxide in 50% ethanol at 100° for 1 hr. The accuracy of the method was tested with tristearin and complete recovery of glycerol was obtained.

Iodine value. Iodine values were determined by the method of Yasuda (1931).

Phosphorus. The method of Fiske & Subbarow (1925) was used for the estimation of phosphorus, after wet ashing with sulphuric acid.

Alkaline isomerization. Alkaline isomerization was carried out by the method of Holman (1957) and the extinctions were measured in a Unicam SP. 500 spectrophotometer.

RESULTS

Separation of blood total lipids. Fig. 1 shows the results of the cholesterol, phosphorus and glycerol analyses on fractions recovered from the silicic acid column for sample I. A small amount of phosphorus-containing material was eluted early, in pure light petroleum. This material gave ammonia on incubation with urease, and glycerol was obtained after prolonged alkaline hydrolysis. This fraction was observed for all samples except the last one; in this, the solvent mixtures used for extraction of the plasma lipids contained a high proportion of acetone. Presumably this lipid is either a glycerophosphatide–urea complex or else urea dissolved in a solution of glycerophosphatides.

Separation of cholesterol ester fatty acids. The cholesterol esters recovered from the silicic acid columns were weighed and hydrolysed and the recovered fatty acids titrated. Table 1 shows the comparison of the yields obtained with the expected recoveries calculated from analysis by digitonin precipitation, carried out on the original plasma samples. A sample of the fatty acids was separated by reversed-phase chromatography. The

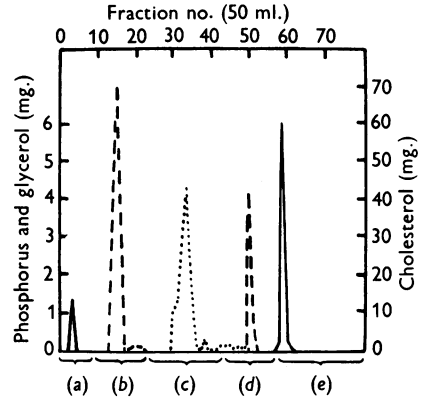

Fig. 1. Elution pattern of mixed serum lipids on a column of silicic acid. (*a*) Light petroleum; (*b*) 1% of ether in light petroleum; (*c*) 3% of ether in light petroleum; (*d*) 10% of ether in light petroleum; (*e*) ether–methanol (1:1, v/v). —, Phosphorus; - - -, cholesterol; ....., glycerides.

peaks obtained were similar in all experiments and a representative picture of their chromatographic pattern is shown in Fig. 2. There are two large clearly defined peaks, *A* and *B*, eluted by 60 and 65% acetone respectively, and a third smaller peak, *C*.

The small peak eluted in 45–50% acetone was not observed in the later experiments. It was present only when the light-petroleum extract was not washed thoroughly with water and it seems probable that it represents a trace of the hydrochloric acid used to convert soaps into free acids.

The acids comprising peak *A* were recovered and hydrogenated and the resulting saturated acids were again examined by reversed-phase chromatography (Fig. 3). There is a small rather irregular peak *D* eluted with 65% acetone corresponding to palmitic acid, a larger well-defined peak *E*, eluted with 75% acetone, and a small ill-defined peak *F* eluted with 80% acetone and corresponding to arachidic acid.

The irregular shape of peak *D* was also observed when the columns were tested with samples of pure acids. Palmitic acid is eluted slowly from these columns with 65% acetone, forming a low plateau rather than a peak. It appears probable that, under these conditions, the 3° fluctuation of temperature permitted by the thermostat in use is sufficient to cause deformation of the elution curve. The acids of peak *B*, after similar treatment, gave the results shown in Fig. 4. There are clearly defined peaks *G* and *H*, corresponding to palmitic acid and stearic acid respectively.

Another portion of the mixed acids obtained by hydrolysis was treated with alkaline potassium

permanganate. The result of reversed-phase chromatography of the oxidation products is shown in Fig. 5. The fraction eluted with 45% acetone contains all the oxidation products, and the small peaks *N*, *O* and *P* represent the unchanged palmitic acid, stearic acid and arachidic acid present in the original mixture.

Figs. 3 and 4 show that peak *A* consists of acids containing 16, 18 and 20 carbon atoms and that peak *B* consists of acids containing 16 and 18 carbon atoms. The C_{18} acid in peak *A* is eluted in 60% acetone, which is the expected behaviour of linoleic acid. Similarly, the C_{18} acid in peak *B* is eluted in 65% acetone, which is characteristic of oleic acid. The C_{20} acid present in peak *A* is even more unsaturated than the linoleic and must therefore be trienoic or tetraenoic. This acid was presumed to be arachidonic since this is the most widely distributed natural C_{20} polyene and, moreover, alkaline isomerization gave evidence of its presence.

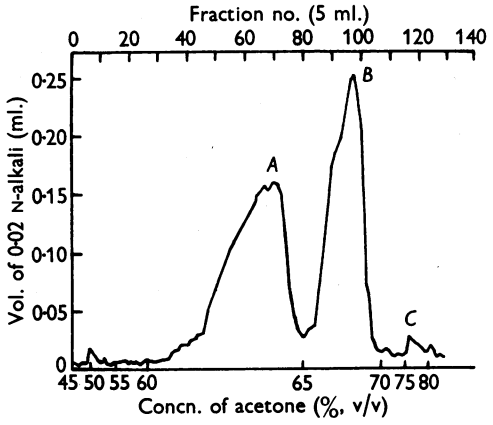

Fig. 2. Reversed-phase chromatography of fatty acids (total fatty acids 59 mg.).

When molar percentages of the various acids comprising each peak were calculated from these results (Table 2) it was found that the palmitic acid remaining after oxidation was approximately equal to the sum of the C_{16} acids present in peaks *A* and *B*. In sample III there was insufficient material on which to carry out the oxidation experiment, so for the purposes of calculation it was assumed that, as was the case in the two previous samples, all the C_{16} acid present was saturated. Since iodine values calculated on this assumption agreed closely with the observed values it seems

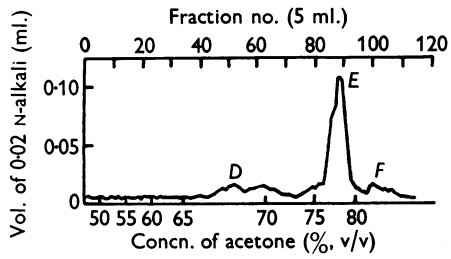

Fig. 3. Reversed-phase chromatography of fatty acids from peak *A* of Fig. 2 after hydrogenation (hydrogenated acids 5·7 mg.).

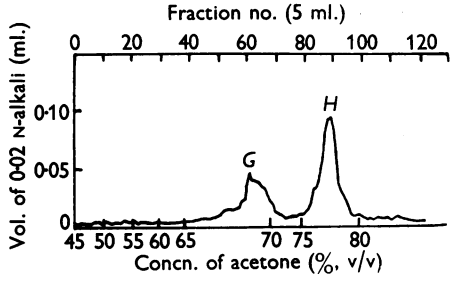

Fig. 4. Reversed-phase chromatography of fatty acids from peak *B* of Fig. 2 after hydrogenation (hydrogenated acids 9·6 mg.).

Table 1. *Comparison of yields of cholesterol ester fatty acids from silica-gel chromatography with expected yields calculated from analysis by digitonin precipitation*

		Sample no. I	II	III	IV
Cholesterol levels (mg./100 ml. of serum) calc. as free cholesterol from analysis by digitonin precipitation	Total	147	105	156	312
	Free	56·2	35	62	96
	Ester	90·8	70	94	216
Total cholesterol ester (mg.) calc. as cholesterol oleate, from analysis by digitonin precipitation		153	529	39·6	366
Wt. of ester (mg.) recovered from silicic acid columns		230	544	46	396
Theoretical wt. of fatty acid (mg.) calculated as oleic acid from analysis by digitonin precipitation		66	229·5	17·1	159
Acid (as oleic) recovered from hydrolysis of cholesterol esters from silicic acid columns	By titration	65·9	240	18	140
	By wt.	—	236	—	—

probable that this assumption is essentially correct. In sample IV peak *A* was larger and contained a larger amount of C_{16} acid. After hydrogenation the amount of saturated C_{16} acid was much greater than in the other three samples. On the other hand, after oxidation, the amount of saturated C_{16} acid remaining was substantially less than the total found after hydrogenation; therefore one must conclude that this sample contained an unsaturated C_{16} acid. Moreover, the close agreement between the observed iodine values and the theoretical values calculated on the basis that this acid is monoethenoid indicates that this sample, unlike the previous ones, contained palmitoleic acid.

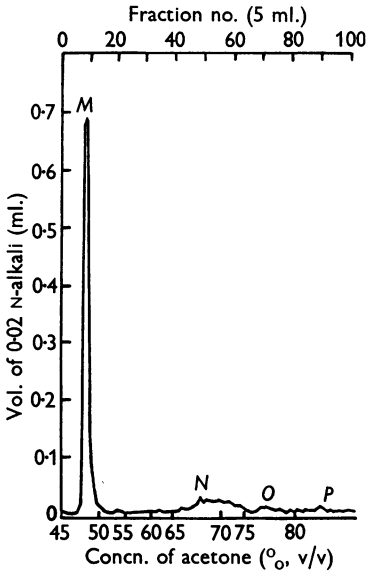

Fig. 5. Reversed-phase chromatography of total acids after oxidation (24 mg.).

Table 2. *Relative proportions of cholesterol ester fatty acids deduced from reversed-phase chromatography (molar percentages)*

Acid	Sample no. I	II	III	IV
Palmitoleic	—	—	—	19·1
Oleic	33·7	29·6	36	28·7
Linoleic	25·3	39	31·6	41·7
Arachidonic	7·1	3·5	7·3	6·1
Other C_{20} unsaturated acids	9·3	—	—	—
Palmitic	18·4	23·5	17	2·2
Stearic	7·8	3	4	2·3
Arachidic	1·6	1	—	—
Total	103·2	99·6	95·9	100·1

Iodine values were determined on the total acids recovered from hydrolysis and on the acids recovered from peaks *A* and *B* (Fig. 2). Comparison between the observed values and theoretical values calculated from the chromatographic data showed close agreement within the limits imposed by the small amounts of material available. In addition, alkaline isomerization was carried out on the acids recovered from samples III and IV and figures for total polyethenoid acids agreed closely with those obtained by chromatography. Alkaline isomerization also showed evidence of small amounts of linolenic acid, eicosapentaenoic acid and docosahexaenoic acid.

DISCUSSION

The high degree of unsaturation of the blood-cholesterol fatty acids was the most noteworthy finding in this work. Previous workers in this field (Bloor, Blake & Bullen, 1937; Clément, Clément & Louedec, 1954) have drawn attention to the relationship of the plasma-cholesterol esters to the phospholipids and triglycerides, the former being the most unsaturated and the latter the least.

The character of the fatty acids identified in this work appears to throw some light on the question of the site of esterification. It has long been known that some degree of esterification occurs in the intestine (Mueller, 1915, 1916) and the esterases of pancreatic juice have recently been studied in detail (Swell & Treadwell, 1955); work by Glover, Green & Stainer (1959) suggests that esterification occurs in the connective tissues of the gut wall. The cholesterol which reaches the gut is derived either from the biliary secretion or from the diet, the former being entirely free and the latter, being almost completely of intracellular origin, is also almost entirely free. Some 60–80% of the cholesterol reaching the blood via the thoracic duct is esterified (Bollman & Flock, 1951). The predominant unsaturated fatty acid in the human diet is oleic acid and saturated acids form at least 50% of the total. On the other hand, linoleic acid forms only a small proportion of the dietary fat. In the plasma samples examined in this work the linoleic acid of the cholesterol esters is approximately equal in amount to the oleic acid whereas the saturated acids formed only 4·5–27·8% of the total. It appears therefore that the greater the number of double bonds that a dietary fatty acid contains, the greater the likelihood of it being esterified with cholesterol. This view is supported by the fact that the rate of esterification by the intestinal cholesterol esterases is greater the longer the chain length of the acid esterified, and is still greater when unsaturation is present; oleic acid, for example, is esterified at almost twice the rate of stearic acid (Swell & Treadwell, 1955).

In this work no fatty acid of chain length shorter than C_{16} was found, although lauric acid and myristic acid are present in significant amounts in the diet and, according to Swell & Treadwell (1955), are esterified with cholesterol at almost the same rates as stearic acid and palmitic acid. This raises doubt whether the saturated acids are esterified with cholesterol in the gut or in other sites such as the liver and blood. Cholesterol esterase activity does not appear to have been demonstrated in human liver although such activity has been shown in animals (Byron, Wood & Treadwell, 1953). On the other hand, cholesterol esterase activity has been demonstrated in human blood (Sperry, 1935, 1936; Tayeau & Nivet, 1956). It seems possible therefore that whereas the unsaturated acids are probably esterified with cholesterol in the gut, esterification of the saturated acids may take place in the blood. If saturated and unsaturated acids are esterified at different sites it may well be that the saturated and unsaturated esters of cholesterol have different functions.

SUMMARY

1. The cholesterol esters of two samples of human serum and two samples of human plasma have been separated from other lipids, by chromatography on silicic acid, and their component acids have been characterized by reversed-phase partition chromatography.

2. Unsaturated acids formed a high proportion of the total acids present.

3. The relationship between the pattern of the dietary fatty acids and the plasma-cholesterol ester fatty acids is discussed.

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A Study of the Protein Impurities in Gelatins with Ion-Exchange Resins

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From his work on mammalian collagens and gelatins, Eastoe (1955) concluded that the amino acid composition of a gelatin may be regarded as representative of a highly purified form of the collagen from which it was prepared. The amino acid composition of the gelatins derived from various mammalian sources is remarkably constant, which suggests that the proportion of impurity proteins is small. Purification of the gelatin by ethanolic coacervation (Stainsby, 1955), followed by deionization with mixed-bed resins (Janus, Kenchington & Ward, 1951), was shown to produce only very small changes in composition (see Table 6). Certain of the amino acids present in large