65. The Acetone-Soluble Lipid of the Atheromatous Aorta*

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(Received 15 June 1942)

Aschoff [1906] first drew attention to the high content of cholesterol in atheromatous aortas. Since that time many quantitative micro-analyses have been performed on the lipids of whole aortas showing varying degrees of atheroma. Schoenheimer [1926; 1928] concluded that the total extractable lipid of the aorta increased with the age of the individual and the severity of atherosclerosis. Furthermore, in the early stages the relative amounts of phospholipid and cholesteryl esters increased with increasing total extractable lipid. A stage was reached, however, at which the percentage of phospholipid and cholesteryl ester in the total lipid became constant. He explained these results on the basis of infiltration and deposition of a lipid mixture elaborated elsewhere. These and other early investigations have been reviewed by Wells [1933].

Later studies [Meeker & Jobling, 1934; Zeek, 1936] have dealt with the changing ratios between total cholesterol, free cholesterol and phospholipids in isolated plaques classified according to the degree of atheroma. The results of these more extensive analytical investigations were at variance with those of Schoenheimer. They indicated that the values of the ratio free cholesterol/bound cholesterol in early plaques were lower than those for normal intimal tissues, while in the advanced lesions they were normal or above normal. Total cholesterol, cholesteryl esters, fatty acids and lecithin were found to increase with the progress of atheroma. Weinhouse & Hirsch [1940] and Page [1941] determined free cholesterol, cholesteryl esters and phospholipids (neutral fat by difference) in the lipid mixtures of normal human plasma and atheromatous plaques, and drew attention to the quantitative similarity in the compositions of these lipids.

Comparatively little work has been done on the isolation or identification of the individual constituents of the various fractions of the lipid from atheromatous aortas. Schoenheimer [1928] isolated free cholesterol, the cholesteryl esters of oleic, palmitic and stearic acids, and a galactoside, by fractional crystallization of the acetone, light petroleum (l.p.) and alcoholic extracts of the adventitia-free atheromatous aortas. He also found indications of the presence of 'oxycholesterol' and fatty acids having more than one double bond, but these were not identified. He failed to find glycerides in the mixture. Schoenheimer, von Behring & Hummel [1930] investigated the saturated sterol content of the lipids from various animal sources. Of the many tissues examined, atheromatous aortas yielded unsaponifiable lipid having the highest values for saturated sterol content (5.1–5.3 % of the total sterol). The suggestion was made that such saturated sterols might have been formed by intermediary hydrogenation of cholesterol. However, it is worthy of note that Gardner & Gainsborough [1934] found Schoenheimer's method for separating saturated from unsaturated sterols unsatisfactory from a quantitative point of view. They were not successful in obtaining the same high yields of β -cholestanol from gall-stone cholesterol as were reported by Schoenheimer.

The results of the quantitative studies mentioned above suggest that the plasma is the source of the lipids deposited in the intima. To test this suggestion, further identification of the fatty acids and the substances with which they are combined in the original fat will be required. The present investigation was undertaken to ascertain the nature of the

* Aided by grants from the Josiah Macy Jr. Foundation, New York.

individual components of the 'phospholipid-free'* fraction of the fat extracted from atheromatous intimas, in order that this might be compared with similar lipid fractions from other sources in the body, particularly the plasma.

EXPERIMENTAL

Preparation and extraction of the intimal tissue

Thirty-three complete aortas and parts of six others, all showing atheromatous plaques, were collected at autopsy. Immediately after removal from the cadaver each aorta was slit up the anterior side and the adventitial tissue was stripped off, care being taken to keep the intima uncontaminated by fatty substances from other tissues. The remaining media and intimal tissue were dropped into 30–40% ethanol to prevent decomposition during the 1–2 days which elapsed before the tissues became available for further treatment. On removal from the dilute alcohol the specimens were pinned on a board with the intimal surface down, while the media was stripped off as cleanly as possible. The remaining intima was ground finely in a food-chopper while still moist, regrinding being carried out if necessary. The minced material was spread out in evaporating dishes in a vacuum desiccator over anhydrous CaCl_2 . This procedure removed most of the water in about 2 days. On being reground when dry the tissue was reduced to a fine powder suitable for extraction in the Soxhlet apparatus. The extraction was made with ether which had been freed from peroxide by distillation over SnCl_2 . After removal of the ether from the extract by distillation under reduced pressure, and of moisture by subsequent distillation of benzene from the residue under reduced pressure, the lipid mixture remaining was transferred in l.p. solution from the distillation flask to a weighed round-bottom flask. The solvent was then removed by vacuum distillation and the residue weighed. The 39 aortas yielded 143 g. of dry, fat-free intimal tissue and 35.58 g. of ether-soluble lipid.

In order to remove the bulk of the phospholipid, the lipid mixture (35.58 g.) was taken up in 360 ml. pure, dry acetone and the mixture allowed to stand overnight at a temperature ranging between 5° and 9°. The acetone solution was filtered and the precipitate (P_1) (see scheme) well washed with ice-cold acetone. The combined washings and filtrate (S_1) were set aside. The precipitate (P_1) dissolved in l.p. was added to a small amount of cold acetone with vigorous stirring. This procedure brought about the precipitation of 1.52 g. of phospholipid (P_2) which was not further investigated. Evaporation of the filtrate (S_2) left a residue weighing 1.87 g. which proved to be steryl ester.

Acetone-insoluble steryl ester

The mixture (S_2) did not contain free cholesterol since it gave no digitonide. To determine its nature 400 mg. were saponified by refluxing for 8 hr. with 4% KOH in methanol and enough benzene to keep the lipid in solution. The unsaponifiable matter was extracted with l.p. in a continuous extractor. After removal of petroleum the residue was washed into a 50 ml. volumetric flask with 95% ethanol and diluted to volume. To 10 ml. in a 100 ml. Erlenmeyer flask 30 ml. of 1% digitonin in 90% ethanol were added in a thin stream while the reaction mixture was kept at 70°. After standing for 48 hr. at room temperature the digitonide was filtered on a sintered glass crucible, washed with 90% ethanol, ethyl ether and finally l.p. The precipitate, dried at 90° and cooled in a desiccator, weighed 0.1873 g., representing 0.0456 g. sterol (calculated as cholesterol). Therefore the original material (1.87 g.) contained 1.065 g. sterol equivalent to 1.723 g. (92.1%) steryl ester (calculated as cholesteryl palmitate).

* The acetone-soluble lipid is referred to as phospholipid-free fat although it contained small amounts of phospholipid.

The remainder (40 ml.) of the alcoholic solution of the unsaponifiable matter was concentrated and decolorized with activated carbon. The residue after recrystallization from dilute alcohol and from l.p. melted at 148.4° (corr.); M.P. of cholesterol 148° , 148.5°

[Sobotka, 1938]. Acetylation with acetic anhydride in the presence of anhydrous sodium acetate and recrystallization of the resulting product from dilute alcohol, yielded a compound melting at 114.4° ; M.P. of cholesteryl acetate 115° [Page & Rudy, 1930].

A part of the remaining impure ester was used to characterize the fatty acid constituents. It was redissolved in a small volume of warm acetone, filtered from a small quantity of insoluble matter and taken to dryness. The residue was then saponified, extracted with l.p. to remove the unsaponifiable matter, acidified and extracted with l.p. to yield fatty acids having sap. equiv. 257.5, 258 (palmitic, 256.3). A small quantity of this fatty acid treated with activated carbon, recrystallized from dilute alcohol and dried over P_2O_5 melted at 62° (corr.); m.p. of palmitic acid is 62.6° . The acetone-insoluble ester fraction was therefore almost pure cholesteryl palmitate.

The acetone-soluble lipid fraction

Removal of the solvent from the acetone liquor (S_1) left a residue of acetone-soluble lipid weighing 31.82 g. 1.67 g. were reserved for analysis. One portion weighing 0.663 g. yielded directly 0.699 g. sterol digitonide, representing 0.170 g. sterol (calculated as cholesterol). Therefore the acetone-soluble lipid contained 25.6% free sterol. Two other portions weighing 0.5435 and 0.1199 g. were saponified, the unsaponifiable matter was extracted quantitatively and an excess of digitonin was added in the usual manner. The yields of sterol digitonide were 1.309 and 0.2892 g. respectively, equivalent to 0.3183 and 0.0703 g. cholesterol or 58.6% of the total lipid. The acetone-soluble lipid contained therefore 25.6% free sterol, 58.6% total sterol and by difference 33.0% combined sterol.

The remainder of the 1.67 g. sample was dissolved in ether and washed with aqueous Na_2CO_3 . After removal of the ether the neutral lipid mixture contained 0.015% P, the equivalent of 0.39% phospholipid calculated as lecithin.

The total sterol in the phospholipid-free lipid (33.54 g.) may be calculated by summation of the sterol contents of fractions S_1 and S_2 . In fraction S_2 containing 1.723 g. cholesteryl palmitate there were 1.065 g. bound sterol. Adding this to the values found for S_1 the total sterol content of the phospholipid-free fat becomes 19.72 g. and the free sterol 8.15 g. In other words the phospholipid-free lipid contains 58.8% total sterol and 24.3% free sterol (by difference bound sterol amounts to 34.5%).

The acetone-soluble lipid mixture (30.15 g.) was dissolved in 500 ml. peroxide-free ether and washed with 3 l. 1% aqueous Na_2CO_3 to remove acidic substances. Ether extraction of the acidified aqueous solution yielded 1.73 g. acidic material of very high equivalent weight (491) and containing 3.6% P. The nature of this material has not been determined although its high equivalent weight and P content suggest that it might contain phosphatidic acids. After removal of the solvent from the ether phase the neutral fraction weighed 27.94 g.; therefore the washing process was attended by a loss of 0.48 g.

The neutral lipid mixture (27.94 g.) was transferred with 25 ml. benzene into 140 ml. 6% methanolic KOH in a round-bottom flask. The mixture was refluxed for 3 hr. in a stream of O_2 -free N_2 . After cooling, the saponification mixture was poured into 700 ml. 30% ethanol and extracted five times with 200 ml. portions of peroxide-free ether, sufficient time being allowed after each shaking for sharp separation of the two layers. The ether solution was then washed once with 500 ml. and a second time with 250 ml. of 20% ethanol containing 0.5 g. KOH. The dilute alcoholic solutions were combined and set aside for the removal of the fatty acids.

The unsaponifiable matter from the acetone-soluble fraction

On removal of the solvent from the ether solution there remained 18.19 g. unsaponifiable matter, which is 65.1% of the neutral acetone-soluble lipid.

Three samples of the unsaponifiable matter (dried over P_2O_5 *in vacuo*) weighing 0.8034, 0.8224 and 0.2119 g. gave digitonides weighing respectively 3.054, 3.132 and 0.8041 g. These represent sterol percentages of 92.4, 92.6 and 92.3 (calculated as cholesterol), thus indicating that the unsaponifiable matter contained 92.4% (av.) or 16.81 g. of sterol.

Assuming that the losses were mechanical and therefore proportionally distributed between the components, it may be calculated from the data shown in the scheme that the amount of sterol to be expected at this point was 17.39 g. The amount of sterol lost during saponification and subsequent extraction of the products of hydrolysis was therefore 0.58 g.

The saturated sterol content of the unsaponifiable matter precipitated as digitonide was investigated by the method of Schoenheimer [1930]. The values found (11.7 and 12.9%) were rather more than double those reported by Schoenheimer, v. Behring & Hummel [1930] (5.1–5.3%) for the sterol mixture obtained from atheromatous aortas.

Further work is being done on the saturated sterols and their distribution amongst the components of atheromatous fat.

The total fatty acids from the acetone-soluble fraction

The dilute soap solution remaining after extraction of the unsaponifiable matter was acidified to Congo red with dilute HCl. This was followed by several extractions with peroxide-free ether to remove the free fatty acids. The aqueous alcoholic solution was set aside to be examined for glycerol content. The solvent was removed from the ethereal extract by distillation under reduced pressure and the residue dried by distilling benzene from it. A yield of 8.33 g. mixed fatty acids was thus obtained.

100 mg. of this fatty acid mixture dissolved in 25 ml. alcohol-ether mixture took 3.58 ml. of 0.0993N NaOH for neutralization, indicating equiv. wt. 281.3. The remainder of the acid mixture was extracted twice with small volumes of l.p. in which 0.5144 g. was insoluble. The petroleum-insoluble fatty acid had i.v. 58.5 (by the micro-method of Yasuda [1931]), but attempts to recrystallize its Pb and Ba salts were unsuccessful. After removal of the petroleum from the filtered extract, the residue had i.v. 104.

In view of the technical difficulties of separating such a small quantity of mixed fatty acids into 'liquid' and 'solid' fractions by the lead salt and ether or alcohol processes and the desirability of keeping the concentration of oleic acid in the unsaturated fraction low, the mixed fatty acids (7.00 g.) were crystallized from 300 ml. pure dry acetone at -70° in a special recrystallization and filtration chamber which was cooled with alcohol and solid CO₂. This procedure yielded 3.66 g. of a solid fraction having i.v. 74. On removal of the acetone from the filtrate the residual liquid acid (3.34 g.) had i.v. 136.

The liquid fraction of the fatty acids

Repeated bromination and preliminary fractionation of the insoluble brominated acids. 3.137 g. of the liquid fraction of the fatty acids dissolved in 32 ml. pure anhydrous ether were brominated at -10° by the addition of 1.7 g. dry Br over a period of 60 min. After standing 5 hr. at about -15° the precipitate was filtered off in a sintered glass crucible and washed five times with 5 ml. portions of cold ether. This precipitate of ether-insoluble polybromide, dried at 80° for 3 hr., weighed 0.4165 g. It was reserved for later examination.

The ethereal filtrate was shaken with aqueous sodium thiosulphate to remove the excess of Br. After removal of the ether *in vacuo*, benzene was distilled from the residue under reduced pressure to remove moisture. Extraction of the residue with two 100 ml. portions of boiling l.p. left 0.504 g. dark-coloured insoluble gum.

The petroleum extract, on concentration to 100 ml. and standing overnight in a refrigerator, deposited a precipitate which when filtered off, washed with l.p. and dried, weighed 0.972 g. The filtrate and washings were concentrated and allowed to stand in the refrigerator for several weeks, when a further 0.389 g. of the petroleum-insoluble material precipitated. Thus the first bromination of the 'liquid' fraction of the fatty acids yielded 1.361 g. of ether-soluble petroleum-insoluble brominated fatty acids.

The oily residue (3.356 g.) obtained by evaporation of the petroleum filtrate was mixed with the petroleum-insoluble gum (0.504 g.) and 3.605 g. of the mixture were debrominated in accordance with the directions of Rollet [1909]. The methyl esters thus obtained weighed 1.987 g. and had i.v. 112. Since the i.v. of methyl oleate is 85.6, not all of the acids having more than one double bond had been removed by bromination. The free fatty acids were recovered after cold saponification. This was carried out by allowing 1.974 g. of the ester mixture to stand overnight in 20 ml. 5% methanolic KOH. The saponification mixture was then poured into an equal volume of distilled water and extracted with l.p. to remove traces of ester which might have escaped hydrolysis.

The soap solution was acidified and extracted once with l.p. and once with benzene. Removal of the solvents *in vacuo* left a residue of fatty acids weighing 1.787 g. and with i.v. 119.

1.771 g. of the acid mixture were rebrominated in 20 ml. ether in the same way as before and yielded 0.0555 g. of the ether-insoluble brominated acid and 0.080 g. of the petroleum-insoluble brominated compound. Evaporation of the l.p. filtrate left a residue of soluble brominated acids. On debromination this residue yielded methyl esters having i.v. 94.2; consequently two brominations had failed to remove completely the unsaturated acids having more than one double bond. However, the amount of the highly unsaturated fatty acids which escaped precipitation as bromo-derivatives by virtue of the solubility of their β -forms was so small, that it was considered inadvisable to subject the remainder of the acid mixture to further bromination.

To determine whether any saturated acids had been carried over with the unsaturated fraction, 1.137 g. of the residual methyl ester were treated with hydroxylamine and sodium ethoxide in absolute alcohol, according to the method of Lewis [1926]. For this test, alcoholic hydroxylamine was prepared according to Lewis except that only the clear supernatant liquid pipetted off from the precipitate of NaCl was used. The reaction mixture on standing for 45 days at 10° gave no precipitate, and when concentrated and cooled to -10° gave no evidence of the presence of insoluble hydroxamates. It was concluded that the liquid fraction contained no appreciable quantity of the saturated fatty acids. Furthermore, since the methyl esters, which appeared to contain only very small amounts of fatty acids having more than two double bonds, had i.v. 94.2 (94.0, 94.4), the mixture probably consisted of about 90% methyl oleate and 10% methyl linoleate.

Arachidonic acid. At various stages there had been collected, in all, 0.4720 g. ether-insoluble polybromide, and according to its bromine content (67.3%) this appeared to be almost all octabromo-arachidic acid. The ether-insoluble polybromide was extracted with pure hot dioxan until only a small amount (57.3 mg.) of residue remained. This had a decomposition temperature of 233° (uncorr.) and gave the following analytical figures: C, 25.0, 25.1; H, 3.49, 3.58; Br, 67.5, 67.3. $C_{20}H_{32}O_2Br_8$ requires: C, 25.5; H, 3.42; Br, 67.7; decomp. 240°.

Linoleic acid. The ether-soluble brominated fatty acid (1.441 g.) which crystallized from l.p. was redissolved in boiling petroleum (B.P. 80–100°), treated with activated carbon and filtered while hot. White needles separated from the petroleum on standing overnight in the refrigerator. A second recrystallization yielded a product having M.P. 114.3° (corr.) and a neutral equivalent of 595 (593, 596). The substance was therefore tetrabromostearic acid (M.P. 113–114°; mol. wt. 600) derived from linoleic acid.

By means of two complete bromination procedures, insoluble bromine derivatives of arachidonic and linoleic acids were isolated from 3.137 g. of the liquid fraction of the fatty acids, in quantities equivalent to 0.1583 g. of arachidonic acid and 0.6858 g. of linoleic acid (corrections for aliquots having been made). Therefore the liquid fraction of the fatty acid contained about 5% arachidonic acid, 22% linoleic acid (isolated as the so-called α -forms) and 73% oleic acid (including small amounts of the β -forms of arachidonic and linoleic acids).

The solid fraction of the fatty acids

The usual method of resolving a mixture of saturated fatty acids by fractional distillation of their methyl esters was not attempted since the mixture in hand was largely oleic acid and the amount of saturated acid present was very small.

Lewis [1926] has investigated the relative solubilities of the sodium salts of the corresponding hydroxamic acids of butyric, octoic, lauric, oleic, palmitic and stearic acids in alcohol. The solubilities of the C_{16} and C_{18} saturated acid derivatives were so slight that these could be quantitatively separated from the compounds of butyric, octoic and oleic acids. However, the solubility of the lauric acid compound was such that it was considered impossible to obtain a theoretical separation of this acid from palmitic or stearic acids under the conditions given for the other separation.

In view of Lewis's findings, the acids of the solid fraction were converted into the corresponding sodium hydroxamates and these were fractionally crystallized. This procedure served to resolve the mixture into simpler fractions whose approximate composition could be calculated from their neutral equivalents and iodine values.

Preparation and fractionation of the hydroxamic acid derivatives

3.634 g. of the solid fatty acid mixture were dissolved in 25 ml. pure anhydrous ether into which diazomethane was passed. After removal of the solvent, the residue of methyl esters was treated with hydroxylamine in the presence of sodium ethoxide and the reaction mixture was left in a flask carrying a $CaCl_2$ tube for 45 hr. at 9° . The precipitate that formed, when filtered off and suspended in dilute acetic acid, yielded 0.9710 g. of free hydroxamic acid (fraction 1). Repeated concentration of the mother liquors followed by cooling, filtration and suspension of the sodium salts in dilute acetic acid, yielded a second and a third fraction while a fourth fraction was obtained by the addition of dilute acetic acid and water to the mother liquor from fraction 3. Fraction 1 was recrystallized from 25 ml. methanol to give four subfractions, 1 (a), 1 (b), 1 (c) and 1 (d). The weights and melting points of all these fractions are recorded in Table 1. Each of them was subjected to acid hydrolysis which liberated fatty acids in the amounts and of the characteristics also shown in Table 1.

Table 1

Fraction	Hydroxamic acid		Free fatty acid					
	Wt., g.	M.P.	Wt., g.	Neutral equivalent	I.V.	Composition		
						g.	%	Fatty acid
1 (a)	0.2360	92–93°	0.4199	264.8	—	0.2914	69.4	Palmitic
1 (b)	0.2074					0.1285	30.6	Stearic
1 (c)	0.1065	90°	0.1010	271.4	10.5	0.0464	45.9	Palmitic
						0.0428	42.4	Stearic
						0.0118	11.7	Oleic
1 (d)	0.4211	Oil	0.3996	278.0	66.9	0.0655	16.4	Palmitic
						0.0364	9.1	Stearic
						0.2977	74.5	Oleic
2	0.4152	Oil	0.3944	278.7	71.5	0.0592	15.0	Palmitic
						0.0213	5.4	Stearic
						0.3139	79.6	Oleic
3	1.423	Oil	1.352	280.0	81.2	0.1298	9.6	Palmitic
						1.222	90.4	Oleic
4	0.9327	Oil	0.8861	279.0	89.2	0.8666	97.8	Oleic + β -form
						0.0177	2.0	Linoleic
						0.0018	0.2	Arachidonic

The fatty acids from sub-fractions 1 (a) and 1 (b) were pooled since they had practically the same properties, and the ratio of palmitic acid to stearic acid was calculated from the

equivalent weight. The composition of the acids derived from the remaining sub-fractions was calculated from the iodine values in conjunction with the corresponding equivalents. Similar calculations were applied to fractions 2, 3 and 4 (see Table 1).

Arachidonic and linoleic acids in the solid fraction. 0.510 g. of the derived fatty acid from fraction 4 of the hydroxamic acids was subjected to bromination, giving 2.7 mg. ether-insoluble polybromide representing 0.9 mg. arachidonic acid and 20.4 mg. petroleum-insoluble tetrabromide, the equivalent of 9.5 mg. linoleic acid. Therefore this fraction contained about 0.2% α -arachidonic acid, 2.0% α -linoleic acid and 97.8% oleic acid with small amounts of the β -forms of linoleic and arachidonic acids.

Summation of the results of these estimations indicated that the solid fraction (3.634 g.) of the fatty acids contained 0.5923 g. palmitic acid, 0.2290 g. stearic acid, 2.712 g. oleic acid plus β -acids, 0.0177 g. linoleic acid and 0.0018 g. arachidonic acid. The fractionation was attended by a loss of 0.080 g. The percentage composition of the solid fraction is given in Table 2.

The approximate composition of the total fatty acids

Table 2 gives also the composition of the mixed fatty acids (free of petroleum-insoluble acids) obtained by summation of the analytical data for the liquid and solid fractions. Adding the amounts of palmitic acid bound in the acetone-insoluble cholesteryl palmitate and petroleum-insoluble acids to the above figures gave the approximate distribution for the total bound fatty acid contained in the phospholipid-free fat (Table 2).

Table 2. *Compositions of fatty-acid fractions (%)*

	Liquid	Solid	Liquid + solid	Liquid + solid + petroleum-insoluble acids + palmitic acid from cholesteryl palmitate
Stearic acid	—	6.4	3.4	2.9
Palmitic acid	—	16.7	8.7	14.6
Oleic + β -acids	73	76.3	74.7	65.2
Linoleic acid	22	0.5	10.8	9.4
Arachidonic acid	5	0.1	2.4	2.1
Petroleum-insoluble acid	—	—	—	5.8

The glycerol fraction

Isolation and identification of glycerol.

The acidic aqueous-alcoholic solution remaining after extraction of the fatty acids from the saponification mixture was made slightly alkaline by the addition of Na_2CO_3 solution and then brought back to a slightly acidic reaction with acetic acid. The alcohol and water were removed by distillation under reduced pressure. When the residue was almost dry, 95% ethanol was added and refluxed to wash down the walls of the flask. After removal of the alcohol by distillation under reduced pressure, 25 ml. dioxan* were added to the salty residue. The bottom of the flask was heated on a steam bath. A small ball of glass wool was dropped into the flask and the dioxan solution was drawn through this with a pipette for transference to a small distillation flask. This procedure was repeated twice with smaller volumes of dioxan. On removal of the dioxan by distillation under reduced pressure, the small amount of residue in the flask was extracted with pure anhydrous ether to remove any traces of fatty substance that might have been left after extraction of the fatty acids. The residue was transferred in aqueous solution to a weighed test tube which was then placed over anhydrous CaCl_2 in a vacuum desiccator. The dry residue weighed 59 mg.

* The dioxan had been refluxed for 48 hr. with metallic Na and afterwards distilled.

To the residue in the test tube, 0.67 ml. benzoyl chloride and 6 ml. 10% NaOH were added. The tube was stoppered and shaken for 10–15 min. The solid benzoate which formed was removed, dissolved in 10 ml. 95% alcohol and treated with a small amount of active carbon. After filtration, water was added to the filtrate and this was then set aside to crystallize. The crystalline product so obtained when recrystallized from dilute ethanol had m.p. 71°, and an authentic specimen of glyceryl tribenzoate prepared in the same manner melted at 72.5°. The accepted m.p. of glyceryl tribenzoate is 71–72°; the value of 76° quoted in some references is for a metastable form rarely encountered [Huntress & Mulliken, 1941]. A mixture of the product and the authentic glycerol tribenzoate melted at 72° while the mixed m.p. with authentic ethylene glycol dibenzoate (m.p. 71.5–72°) was 45–48°.

Calculation of the glyceride content of the acetone-soluble lipid

The glyceride content of the total phosphatide-free fat was calculated on the basis of the amount (0.059 g.) of glycerol isolated from the neutral fraction (28.42 g.) of the acetone-soluble lipid. Since the equivalent of the fatty acids was 281.3, the amount of glycerol isolated represented 0.565 g. of glyceride. Therefore the total acetone-soluble lipid (31.82 g.) contained 0.596 g. of glyceride and the original phosphatide-free fat (31.82 g. acetone-soluble lipid S_1 + 1.72 g. cholesteryl palmitate from S_2 = 33.54 g.) contained the same amount of glyceride or 1.8%.

The amount of bound fatty acid in combination with substances other than sterol was calculated in the following manner. The acetone-soluble lipid (30.15 g.) was found to contain 58.6% total sterol (i.e. 17.66 g.). During removal of the acidic material (1.73 g.) from the acetone-soluble lipid a loss of 0.48 g. was noted. The loss appeared to be purely mechanical and probably should be distributed proportionately between the fractions, so that the true weight of the neutral material would be 28.39 g. and would include 17.66 g. total sterol. The 27.94 g. taken for saponification should therefore contain 17.38 g. total sterol. Assuming that 56.3% of this is bound and that the average equivalent weight of the fatty acids combined with the sterol is 281.3, then 7.11 g. of fatty acid are combined with sterol. Since the total combined fatty acids found weighed 8.33 g., obviously 1.22 g. were bound by non-sterol alcohol.

On the assumption that all of this non-sterol alcohol was glycerol, the neutral material (27.94 g.) contained 1.27 g. glyceride. Correcting for losses and aliquots the total phospholipid-free fat (33.54 g.) contained 1.34 g. or 4.0% glyceride. On the basis of the glycerol actually isolated the glyceride content was only 44% of that estimated in the above manner. Therefore, 1.8% is the minimal and 4.0% is probably the maximal content of glyceride in the phospholipid-free fat.

DISCUSSION

The results of this study of the acetone-soluble portion of the lipids confirm and extend the data published by Schoenheimer [1928]—the only other investigator to report upon the nature of the individual components of the lipid deposited in the tissues of atherosclerotic aortas. Schoenheimer examined material obtained by acetone-extraction of atherosclerotic aortas and utilized physical methods of separation (repeated fractional crystallization). In the present study only intimal tissue from atheromatous aortas was used; this was extracted with ether and the phospholipids were removed by acetone-precipitation. The methods of examination used (saponification followed by fractionation of the products or of derivatives) proved more suitable for the isolation of minor constituents than did the purely physical procedures used by Schoenheimer, and also permitted a nearly quantitative estimation of the amount of each.

In addition to stearic, palmitic and oleic acids (found by Schoenheimer as cholesteryl esters) we have isolated linoleic and arachidonic acids. No evidence of the presence of

linolenic acid was obtained. The amount of saturated acids was so small that we did not actually isolate pure stearic acid. However, the equivalents of the saturated acid fractions indicated that it was present.

A red viscous petroleum-insoluble acid mixture was also obtained which had i.v. 58.5. Attempts to isolate a pure substance from this were unsuccessful; its insolubility in petroleum suggested that it might be a hydroxy-acid. Several investigators have obtained similar fractions from the fatty acid mixtures of blood and blood plasma. Channon & Collinson [1929] removed this so-called resinous material before determining the constants of the fatty acids from ox blood, but they did not further describe its properties. From the fatty acids of beef, dog, sheep and pig plasma, Bloor [1923] removed acidic substances whose Pb salts were insoluble in hot alcohol. The free 'acid' had i.v. 24. Probably this material was of the same nature as that isolated from the lipids of atheromatous intimas.

Despite the fact that care was taken throughout the fractionation to protect the lipid from unnecessary exposure to air, it is quite possible that a certain amount of oxidation of the unsaturated acids could have taken place and given rise to petroleum-insoluble products. On the other hand the relative constancy of the yield of this fatty acid from individuals of the same species seems to indicate that its presence in the plasma fatty acids is characteristic and that it is not an artefact.

Of some interest is the question whether the highly unsaturated acids (arachidonic and linoleic) were derived from cholesteryl esters, glycerides or phospholipids. The lipid saponified (27.94 g.) actually yielded 175 mg. arachidonic acid and 783 mg. linoleic acid. These values did not represent the total amount of the two acids in the mixture, since it is well known that only a portion of each is precipitated in the α -form by bromination [Green & Hilditch, 1935]. The P content of the lipid saponified indicated that it contained only 0.39% phospholipid (calculated as lecithin) which could have yielded only about 76 mg. of total fatty acid. Therefore a large proportion (if not all) of the highly unsaturated fatty acids was derived from cholesteryl esters and/or glycerides. The latter, it has been shown, may possibly have contributed as much as 1.22 g. fatty acid. However, since glycerides seldom contain a single type of acid [Collin & Hilditch, 1929] it is unlikely that the highly unsaturated acids were derived solely from glycerides. The probability is that they were derived in part at least from cholesteryl esters.

The free acid, removed before saponification of the acetone-soluble lipid, had an equivalent weight of 491 and contained 3.6% P. While not enough of this material was available for fractionation, the above constants suggested that it might be largely phosphatidic acid (oleopalmitophosphatidic acid, mol. wt. 674.9, 4.5% P). This acidic fraction may have been either a precursor or a degradation product of the phospholipids.

A search of the literature failed to reveal a detailed report of a strictly comparable analysis of the lipid of human plasma for comparison with the data obtained from this study of atheromatous aortas. Csonka [1918], working with the total lipid of human plasma, found that the average i.v. of the 'liquid' acids was 87.5 and that this unsaturated fraction constituted about 48.5% of the total acid. Therefore the total acid had an i.v. of about 42. Even although this acid mixture included acids derived from phospholipids which are known to be more saturated than those in the acetone-soluble lipids of the plasma, the value seems to be low. Channon & Collinson [1929] obtained an i.v. of 120 for the fatty acid mixture derived from the acetone-soluble lipid of whole ox blood. The i.v. found here for the corresponding fatty acids of atherosclerotic intimal tissue was 104. On the assumption that the i.v. of Channon & Collinson's 'solid' acids was due to oleic acid and that the 'liquid' acids did not contain saturated acids, the total acid mixture was composed of 83% unsaturated and 17% saturated acids. This composition corresponds very closely with that of the fatty acid mixture from the acetone-soluble lipid of atherosclerotic intimal tissue (88% unsaturated and 12% saturated acids). Further-

more, the acetone-soluble lipids of ox blood and atheromatous intimal tissue yielded the same fatty acids, namely, stearic, palmitic, oleic, linoleic and arachidonic acids. In neither case was linolenic acid identified.

Such a comparison involving two different species may appear open to criticism. However, Bloor [1923] found a remarkable constancy in the ratio of 'solid' to 'liquid' acids in the fasting blood plasma of different species. He also found that the ratio of total fatty acid to unsaponifiable material of plasma lipid is relatively constant in different species, so that the comparisons made appear permissible.

Table 3 contains values (recalculated from several reports) for the lipids of atheromatous tissues, normal intimal tissue, human plasma, ox blood, human red blood cells and human white blood cells. The micro-analytical results of Boyd [1937], Page *et al.* [1935] and Weinhouse & Hirsch [1940] were recalculated to exclude phospholipids in order that they might be compared with the analytical results obtained in this study of

Table 3. *Percentage composition of the phospholipid-free lipids*

	Atheromatous intimas	Athero- matous aortas Schoen- heimer [1928]	Ulcerated plaques Wein- house & Hirsch [1940]	Early plaques Wein- house & Hirsch [1940]	Normal intimas Wein- house & Hirsch [1940]	Ox blood plasma (calculated) Channon & Collinson [1929]	Human plasma Page <i>et al.</i> * [1935]	Human plasma Boyd [1937]	Human red blood cor- puscles Boyd [1937]	Human white blood cor- puscles Boyd [1937]
Total sterol	58.8	62.0	65.7	51.9	51.7	45.4	41.6	42.0	58.0	54.6
Bound sterol	34.5	35.7	31.4	30.4	31.9	29.3	26.3	29.0	3.7	13.4
Free sterol	24.3	26.3	34.3	21.5	19.8	16.1	14.7	13.0	54.3	41.2
Steryl ester	57.8	60.0	52.8	51.2	53.7	49.2	44.4	48.0	6.2	22.5
N.S.U.S.M.	4.6	3.7				8.3				
Free acid	5.5	—	—	—	—	18.0	—	—	—	—
Glyceride	1.8-4.0	17.9	13.7	13.1	27.3	34.8	41.0	39.0	39.5	36.3
Unaccounted	3.8-6.0	10.0				8.5				
Free sterol/ bound sterol	0.70	0.74	1.09	0.71	0.62	(0.55) fixed	0.55	0.45	14.7	3.1

* Page, Kirk, Lewis, Thompson & Van Slyke.

the acetone-soluble lipids. Channon & Collinson's figures for whole ox blood were adjusted to exclude the free cholesterol from the red blood cells by fixing the ratio free sterol/bound sterol at a value of 0.55 (about the average value found for normal post-absorptive human plasma lipid), while the value given for bound sterol was kept relatively the same. This adjustment was made on the assumption that the red blood cells contained little or no steryl ester.

In the micro-procedures the fraction of the lipid mixture consisting of glycerides, non-sterol unsaponifiable material, free acid and lipid not accounted for was estimated only by difference. Therefore the relative amounts of its components are unknown. On the other hand the large scale analyses gave fairly reliable estimations of these components.

The table shows a close quantitative relationship between the values for the acetone-soluble lipid fractions of human blood plasma and those calculated for ox blood plasma, despite the difference in analytical technique and the assumptions made to render the comparison possible. Also, there is a remarkable similarity in the values for the sterol-containing fractions present in the lipids of atheromatous aortas and plasma, which confirms the observations of Weinhouse & Hirsch [1940] and Page [1941]. The most striking difference between the composition of the lipids from normal or atheromatous intimal tissue and that of the plasma is in the 'neutral' fraction. The acetone-soluble lipids from the intimal tissues contain 13-27% of 'neutral' lipid while the acetone-soluble lipids of the plasma, red blood cells and white blood cells contain 35-41% of this component. Since the non-sterol unsaponifiable material and free fatty acids are minor components of the acetone-soluble fraction, the difference in content of 'neutral' lipid in these two groups of lipids is due most likely to a difference in glyceride content. Direct estimation of the glyceride content of the lipids from atheromatous aortas (2-4%) and

ox blood (18%) gave data enhancing the validity of this supposition. Actually the mean differences are probably greater than is indicated by these figures since the plasma lipid was always obtained from blood in the fasting state, when the glyceride content is at a lower than average value. Therefore it would appear that the glycerides are either not absorbed from the plasma or they undergo subsequent decomposition in the intima.

SUMMARY

1. The composition of the phospholipid-free fat from atheromatous intimal tissue of aortas has been determined.

2. The fatty acid mixture obtained by saponification of this lipid was found to be composed of 2.9% stearic acid, 14.6% palmitic acid, 65.2% oleic acid plus more highly unsaturated acids that escaped precipitation when brominated, 9.4% linoleic acid, 2.1% arachidonic acid and 5.8% petroleum-insoluble acids.

3. The glyceride content of this lipid, as evidenced by the amount of glycerol isolated and the amount of acid found in excess of that required for cholesteryl esters, was 1.8 and 4.0%.

4. A qualitative and quantitative comparison has been made between the phospholipid-free fat of atheromatous aortas and similar fractions from other sources in the body. The differences and similarities in these have been discussed.

The problem was suggested by Prof. G. E. Hall of the Banting and Best Department of Medical Research (now Wing-Commander, R.C.A.F.), who contributed much helpful advice in the early stages of the work. Dr C. C. Lucas of the same department and Prof. A. Hunter of the Department of Pathological Chemistry continued Prof. Hall's interest in the investigation and have given valuable counsel and constant encouragement. The necessary autopsy material was made available by the Department of Pathology (Prof. W. Boyd). The phosphorus determinations were made by Miss M. O'Grady of the Banting and Best Department of Medical Research. To all of these helpers I wish to express my indebtedness.

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