Anal. Calcd. for $C_{23}H_{32}O_6$: C, 68.29; H, 7.97. Found: C, 67.64; H, 7.61.

2β,4b-Dimethyl-1β-methoxy-2-(γ-propionoxy-β-hydroxy-β-cyanopropyl)-7-ethylenedioxy-1,2,3,4,4aα,4b,5,6,7,8,10,-10aβ-dodecahydrophenanthrene-4-one (XI).—A solution of 500 mg. of IX in 4 ml. of ethylene chloride was treated with 0.07 ml. of triethylamine, 3 ml. of ether and 0.6 ml. of hydrogen cyanide. After 5 minutes, crystals of product began to separate. The mixture was kept at 0° overnight and then diluted with petroleum ether to complete precipitation of the product which was collected, washed with petroleum ether and dried; 510 mg. (96%), m.p. 174–180° dec.

2β,4b-Dimethyl-1β-methoxy-2-(γ-propionoxy-β-cyano-1-propenyl)-7-ethylenedioxy-1,2,3,4,4aα,4b,5,6,7,8,10,10aβ-dodecahydrophenanthrene-4-one (XII).—Cyanohydrin XI, 500 mg., was dissolved in 2.5 ml. of pyridine and treated with 0.25 ml. of phosphorus oxychloride. The reaction solution was kept at room temperature overnight, ice and dilute sodium bicarbonate were added, and the product was extracted with ether. The ether solution was washed with water, dried and concentrated to provide 408 mg. of crude crystalline residue. Recrystallization from ether–petroleum ether gave 260 mg. (54%) of unsaturated nitrile XII, m.p. 125–130°. Ether recrystallization provided pure XII, m.p. 134–136°; λ_{max} 4.59, 5.77 and 5.86 μ.

Anal. Calcd. for $C_{26}H_{35}O_6N$: C, 68.25; H, 7.71; N, 3.06. Found: C, 67.97; H, 7.54; N, 3.20.

2β,4b-Dimethyl-1β-methoxy-2-(γ-propionoxy-α-hydroxyacetonyl)-7-ethylenedioxy-1,2,3,4,4aα,4b,5,6,7,8,10,10aβ-dodecahydrophenanthrene-4-one (XIII).—A solution of 120 mg. of XII in 3.5 ml. of acetone and 0.1 ml. of piperidine was cooled to 0° and treated with 90 mg. of powdered potassium permanganate. The mixture was stirred cold for 1 hour, allowed to warm to room temperature and treated with 0.2 ml. of acetone containing 0.002 ml. of acetic acid. After stirring another hour at room temperature, the reaction mixture was rinsed into a separatory funnel with chloroform and the manganese dioxide was reduced with limited acidified sodium bisulfite solution. The chloroform layer was separated and the aqueous part extracted with chloroform. To the combined chloroform was added 12 ml. of 5% potassium carbonate solution and the total volume was reduced to *ca.* 20 ml. *in vacuo*. After 0.75 hr. stirring at room temperature, the chloroform was separated, washed, dried and concentrated giving 97 mg. of a gum. Chromatography over 5 g. of acid-washed alumina provided from the ether–chloroform fractions 54 mg. of crude crystalline XIII. Recrystallization from ether–petroleum ether gave 29 mg. (24%), m.p. 181–184°, positive tetrazolium test; λ_{max} 2.9–2.95, 5.74, 5.78 and 5.85 μ.

2β,4b-Dimethyl-1β-methoxy-2-(γ-propionoxy-α-hydroxyacetonyl)-1,2,3,4,4aα,4b,5,6,7,9,10,10aβ-dodecahydrophenanthrene-4,7-dione (XIV).—Eighteen milligrams of XIII was treated with a small amount of *p*-toluenesulfonic acid in acetone at room temperature overnight. Recovery of the product as described previously gave 11 mg. of pure XIV, m.p. 184–186° after recrystallization from ether. It reduced tetrazolium reagent and showed λ_{max} 2.9–2.95, 5.74, 5.78, 5.84, 6.08, 6.19, 8.12 and 9.14 μ.

Anal. Calcd. for $C_{23}H_{32}O_7$: C, 65.69; H, 7.67. Found: C, 65.67; H, 7.55.

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[CONTRIBUTION FROM THE DONNER LABORATORY, DIVISION OF MEDICAL PHYSICS, AND THE CHEMICAL LABORATORY, UNIVERSITY OF CALIFORNIA]

Composition of Fatty Acids from Certain Fractions of Blood Lipoproteins[1]

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The fatty acids from fractions S_f0-20 and S_f20-400 of the blood lipoproteins have been analyzed. Data on the mixed acids were combined with information obtained by chromatography on charcoal to yield an approximate composition for each lot of acids. Two significant differences in composition were noted: (a) more than 20% of C_{16} monounsaturated acid (palmitoleic) is present in Fraction S_f20-400, whereas this acid is either absent from Fraction S_f0-20 or is present at very low abundance; (b) poly-unsaturated acids are essentially absent from Fraction S_f20-400, whereas Fraction S_f0-20 contains about 4% tetra-unsaturated acid (arachidonic) and 18% di-unsaturated acid (linoleic).

Human sera contain fatty acids of a wide variety, both with respect to chain length and degree of unsaturation. Since nearly all the fatty acids found in the blood stream are present in the form of lipoproteins, it is of interest to study the fatty acids present in some of the different classes of lipoproteins. Selected for the present study were the S_f0–20 and S_f20–400 class[2] lipoproteins. These classes are of particular interest for several reasons. First, lipoproteins of the S_f12–400 class have been shown[3] to be more atherogenic than those of the S_f0–12 class. (The fractionation split at S_f20 rather than at S_f12 would not substantially alter this consideration.) Second, the chemical compositions of the two classes of lipoproteins contrast sharply. On the one hand, the dominant lipid component of the S_f20–400 class lipoproteins is triglyceride, whereas cholesterol esters and phospholipid comprise most of the lipid present in the S_f0–20 class molecules. Further, a relationship between these classes of molecules has been shown by *in vivo* and *in vitro* heparin transformation studies[4,5] which suggest that molecules of the S_f20–400 class normally may be converted to S_f0–20 class lipoproteins during the process of fat absorption.

Since the quantities of fatty acids available for investigation were rather small (170 mg. in the case of frac. S_f0–20), analysis has been based primarily on a partial separation accomplished by chromatography on charcoal, employing the methods described in detail in another journal.[6] Analytical data on the mixed acids were combined with the data obtained on the fractions from chromatography in order to arrive at the approximate compositions shown in Table I. It may be noted that a significant difference in composition of the two fractions concerns the content of poly-un-

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(2) The customary unit of migration rate in the ultracentrifuge is the Svedberg unit. A molecule which undergoes flotation at a rate of 20 × 10^{-13} cm. per sec. per unit field of force has an S_f value of 20 or is a molecule of the S_f20 class.

(3) J. W. Gofman, B. Strisower, O. de Lalla, A. Tamplin, H. B. Jones and F. T. Lindgren, *Modern Medicine* (June 15, 1953).

(4) D. Graham, T. Lyon, J. W. Gofman, H. B. Jones, A. Yankley, J. Simonton and S. White, *Circulation*, **4**, 66 (1951).

(5) F. T. Lindgren, N. K. Freeman and D. M. Graham, *ibid.*, **6**, 174 (1952).

(6) J. Cason and G. A. Gillies, *J. Org. Chem.*, **20**, 419 (1955).

saturated acids. Whereas the poly-unsaturated acids are essentially absent from frac. S_f20–400, about 60% of the unsaturated acid content of frac. S_f0–20 consists of poly-unsaturated acids. The C_{16} unsaturated acid in frac. S_f20–400 is replaced in frac. S_f0–20 by the C_{18} di-unsaturated acid and C_{20} tetra-unsaturated acid.

TABLE I

COMPOSITION OF FATTY ACIDS FROM BLOOD LIPOPROTEINS

Acids	Per cent. in mixture from Fraction S_f0–20	Fraction S_f20-400
Stearic	24	22
Palmitic	38	34
Arachidonic[a]	4	1
Oleic[a]	16[b]	21
Linoleic[a]	18	Nil
Palmitoleic[a]	[b]	22

[a] The unsaturated acids were not isolated and characterized; only number of double bonds was determined. These names are used because these are the unsaturated acids occurring in the human fat depot. [b] All the mono-unsaturated acid is indicated as oleic acid because the chromatogram, Fig. 1, indicates no significant amount of material adsorbed much less strongly than palmitic acid, in contrast to the chromatogram in Fig. 2.

The distribution between C_{16} and C_{18} unsaturated acids in frac. S_f20–400 is based on the equivalent weight determined for the mixed acids. A possible source of error in such a calculation is the presence in the acids of neutral material, which would cause the apparent equivalent weight to be higher than the actual value. If any neutral material were present in the acids from frac. S_f20–400, this would mean that the content of palmitoleic acid is even higher than indicated in Table I. No other of the indicated values is dependent on equivalent weight of the mixture. Presence of the C_{16} unsaturated acid is strongly indicated by the pattern of the chromatogram in Fig. 2, which shows a large amount of material considerably less strongly adsorbed than palmitic acid (maximum concentration in Cut 12). The contrast with the chromatogram in Fig. 1 may be noted (Cut 4 is rich in palmitic acid). The iodine values of these early fractions in Fig. 2 (*cf.* Table IV) were much higher than those of later fractions, and the small amount of solid acid in cut 5 appeared to be palmitic acid, not myristic acid.

The differences in the amount and degree of unsaturated fatty acids present in the two lipoprotein groups appear to be related to the different forms in which the fatty acids are combined. In the S_f20–400 class, for instance, fatty acids are present primarily as triglyceride esters, a form characterized by a low iodine number. In the S_f0–20 class, however, the fatty acids are present primarily as esters of cholesterol and as phospholipid, both forms of which have for a long time been recognized for their high iodine number, and for their exclusive content of the most highly unsaturated fatty acids present in serum.

Since some phospholipid and cholesteryl esters are present in the S_f20–400 class, it is of interest to note that no linoleic acid was found in the fatty acids of this lipoprotein class. This suggests that there may exist differences between the cholesteryl esters and phospholipids present in the two groups; however, definitive proof of such a difference would require fractionation of the total lipid from each lipoprotein class into triglyceride, fatty acid, cholesterol ester and phospholipid, followed by analysis of the fatty acids present in these particular lipids of each class. This would require very large amounts of lipoproteins.

Experimental

Isolation of the Fatty Acids from Liproproteins.—Lipoprotein fractions were prepared from a single blood bank plasma sample. The unaltered plasma was ultracentrifuged at 81,000 × *g* for 24 hours to yield a top fraction composed of lipoprotein molecules of the S_f 20–400 class. This top fraction was removed with a capillary pipet. To the remainder of the preparative tubes of this run was added an appropriate amount of a concentrated NaCl solution sufficient to bring the solvent density to 1.063 g./ml. at 26°. Another similar 24-hour preparative run was made and this yielded a top fraction, consisting this time of S_f0–20 class lipoproteins.

Each aqueous lipoprotein sample was treated with $^3/_5$ volume of methanol and extracted four times with four volumes of diethyl ether. (Shortly before the extraction the ether was shaken with a dilute, weakly acidic solution of ferrous sulfate to destroy any peroxides that may have been present.) After the ether had been removed from each sample with a stream of nitrogen, the sample was treated with 50% aqueous potassium hydroxide at 50° for 24 hours. The saponified sample was acidified with 6 *N* hydrochloric acid, and the fatty acids were extracted with three portions of diethyl ether. Finally, the ether was removed from each sample with a nitrogen stream, then benzene was added and distilled at reduced pressure in order to remove last traces of water. The residue was dried to constant weight in a vacuum desiccator; wt. of acids from frac. S_f0–20, 170 mg.; from frac. S_f20–400, 380 mg.

Analysis of the Mixed Acids.—Titration was carried out in alcohol, with phenolphthalein as indicator. Iodine number was determined by the micro method of Chargaff,[7] which was found to give reproducible values within 1% of those obtained by the usual macro method.[8] Determination of poly-unsaturated acids was by alkali isomerization to conjugated acids and spectrophotometric determination of the isomerized acids. The experimental method used was that described by O'Connor and co-workers[9] and the method of calculation elaborated by Brice and co-workers[10] was employed. Application of the method to known mixtures of 50 mg. of linoleic and linolenic acids gave accuracy as good as that previously claimed. Mono-unsaturated acid was determined by difference, from iodine number and content of poly-unsaturated acids. Results of the analyses are assembled in Table II.

TABLE II

ANALYSIS OF MIXED ACIDS

	Frac. S_f0-20	Frac. S_f20-400
Neutral equiv.	294[a]	268
Iodine number	61.5	44.0
% Oleic and palmitoleic acid	16	43
% Linoleic acid	18.4	0.01[b]
% Linolenic acid	0.4[b]	0.5[b]
% Arachidonic acid	3.8	1.0

[a] This dark-colored fraction of acids obviously contained some neutral material; about 5% of the mixture failed to dissolve in the 95% ethanol used for chromatography. The molecular weight of the mixture shown in Table I is 274. [b] The accuracy of the method of analysis is not sufficient to attach significance to values less than 1%.

(7) E. Chargaff, *Z. physiol. Chem.*, **199**, 221 (1931).

(8) "Official and Tentative Methods of Analysis of the Association of Agricultural Chemists," 6th Ed., 1945, p. 494.

(9) R. T. O'Connor, D. C. Heinzelman and F. G. Dollear, *Oil and Soap*, **22**, 257 (1945).

(10) B. A. Brice and M. L. Swain, *J. Optical Soc. Am.*, **35**, 532 (1945); B. A. Brice, M. L. Swain, B. B. Schaeffer and W. C. Ault, *Oil and Soap*, **22**, 219 (1945).

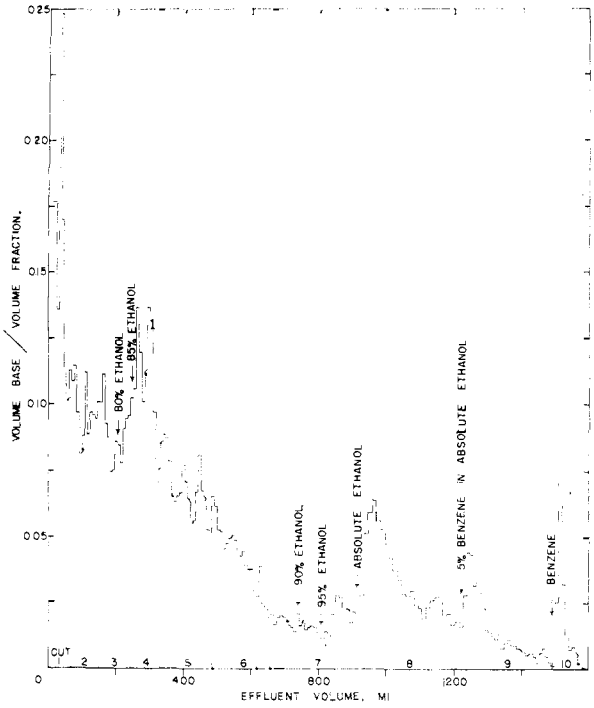

Fig. 1.—Chromatography of 128 mg. of mixed acids from frac. S_f0–20. Adsorbent was 1.35 g. of Darco G-60 charcoal mixed with 2.7 g. of Celite 521; diameter of column of adsorbent, 10 mm.; acid applied to column in 50 ml. of 95% ethanol; initial solvent for elution, 75% ethanol; normality of base for titration of eluted fractions, 0.007346; base equivalent to acid applied, 60 ml. Width of steps indicates volumes titrated; "cuts" are the fractions combined for further investigation. For additional data on cuts shown in the chromatogram, refer to Table III.

Chromatographic separations were carried out by the method described in detail elsewhere.[6] The chromatogram for frac. S_f0–20 is shown in Fig. 1, and that for frac. S_f20–400 in Fig. 2. Analytical data for the cuts of eluent are shown in Tables III and IV, and chemical investigation of certain fractions is described below.

TABLE III

DATA ON CHROMATOGRAM IN FIG. 1

Cut no.[a]	Appearance of recovered acid	Base,[b] ml.	% of total[c]
1	Mostly charcoal acid	(9.65)	
2	*ca.* 70% colorless liquid	8.52	13.0
3		8.24	12.6
4	Mostly white solid	9.86	15.1
5		10.38	15.9
6		7.64	11.7
7		4.78	7.3
8	Pale yellow solid	9.36	14.3
9	Pale yellow, crystalline	5.06	7.7
10	Yellow and gummy	1.52	2.3

[a] Quantity of acids in each cut was not sufficient to permit determination of the iodine number. [b] Titration not corrected for the trace of inorganic acid ("charcoal acid") eluted continuously from the adsorbent. After the first few hundred ml., this acid is equivalent to about 0.4 ml. of base for each 100 ml. of eluent. Since fatty acid appeared in the eluent soon after elution began in this run, enough charcoal acid was eluted with the fatty acids to give total titration greater than the equivalency of fatty acid applied. [c] These percentages are based on total titration of eluted fractions containing fatty acid, 65.36 ml. of the 0.007346 *N* base, although this value is greater than the equivalency of acids applied to the column. Since the earlier fractions contain more charcoal acid, the percentage of fatty acids credited to these fractions on the basis of titration is slightly too large. This factor has no significant effect on the composition estimated for the mixture (Table I).

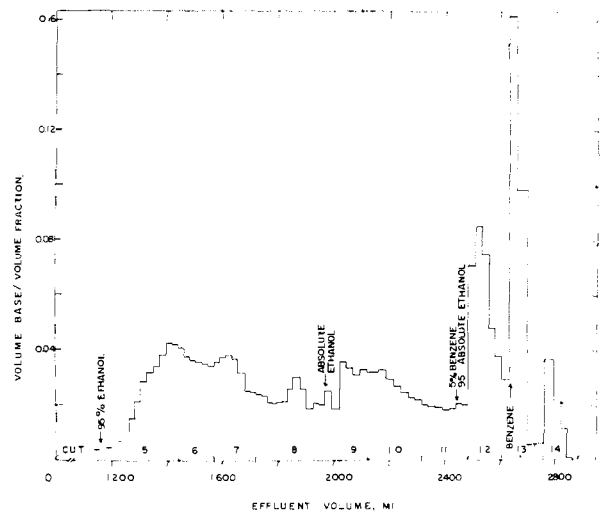

Fig. 2.—Chromatography of 315 mg. of mixed acids from frac. S_f20–400. Adsorbent was 6.7 g. of Darco G-60 charcoal mixed with 13.4 g. of Celite 521; diameter of column of adsorbent, 20 mm.; acid applied to column in 100 ml. of 75% ethanol; initial solvent for elution, 75% ethanol; normality of base used for titration of eluted fractions, 0.02015; base equivalent to acid applied, 58 ml. Width of steps indicates volumes titrated; "cuts" are the fractions combined for further investigation. For additional data on cuts shown in the chromatogram, refer to Table IV.

TABLE IV

DATA ON CHROMATOGRAM IN FIG. 2

Cut no.	Appearance of recovered acid	Iodine no.	Base,[a] ml.	% of total[b]
1-4	Charcoal acid		(12.4)	
5	Semi-solid	48.4	6.04	11.2
6	White solid with decreasing amounts of adhering liquid	47.5	4.75	8.8
7		34.9	4.53	8.4
8			7.32	13.5
9		24.2	4.20	7.8
10			4.30	8.0
11		12.8	3.33	6.2
12			7.72	14.3
13	White solid, trace of orange impurity		9.91	18.3
14			1.97	3.5

[a] Titration of cuts 5–14 is not corrected for the trace of inorganic acid ("charcoal acid") eluted continuously from the adsorbent. After the first few hundred ml., this acid is equivalent to about 0.2 ml. of base for each 100 ml. of eluent. [b] These values are based on total eluted acids titrated, equivalent to 54.07 ml. of the 0.02015 *N* base. The appreciable amount of fatty acid retained in the column reflects the particularly large amount of charcoal used in this run. Less than half this ratio of charcoal was used in chromatographing Frac. S_f0–20 (Fig. 1).

Recovery of Fatty Acids.—Each cut of combined titrated fractions was made slightly alkaline (to avoid any possibility of esterification) and boiled down to a small volume, then diluted with four parts of water, acidified with sulfuric acid, and extracted with purified hexane in a continuous extractor until nothing more was extracted. Both phenolphthalein and the charcoal acid were left in the aqueous phase quantitatively. Hexane was purified by stirring with two or three lots of fuming sulfuric acid, after which it was washed with carbonate, dried, and distilled. This hexane was used for extraction only if it showed no absorption in the

ultraviolet above 200 mμ. Fatty acids, obtained by evaporation of the hexane, were dried in a vacuum desiccator.

Purification and Identification of Fatty Acids.—The solid acids were purified by crystallization from acetone. They were identified by means of the mixed melting point technique using known samples of the acids and their *p*-bromoanilides.

Estimation of Composition of Fractions.—The estimated composition, summarized in Table I, of the fatty acids in the two blood lipoprotein fractions is outlined in detail below.

Fraction S_f0–20 (*cf.* **Table III**).—Cuts 8–10 are considered to represent the stearic acid content, 24%. Analysis of the mixed acids (*cf.* Table II) shows the presence of about 38% of unsaturated acids. The pattern in the chromatogram together with investigations of the cuts indicates that the remaining 38% is palmitic acid. The calculated molecular weight for this mixture is 274 (*cf.* Table II).

Cut 4.—One crystallization from acetone yielded white crystals, m.p. 57–59.3° (softening at 53°); mixed m.p. with myristic acid, 45–47°; mixed m.p. with palmitic acid, 60.0–61.4° (softening at 56°).

Cut 5.—One crystallization from acetone yielded white crystals, m.p. 61.3–62.4° (softening at 55°); mixed m.p. with palmitic acid, 62.2–62.4° (softening at 57°). One crystallization of the *p*-bromoanilide (for preparation, *cf.* below) from acetone gave material of m.p. 111.4–112.6°; mixed m.p. with *p*-bromoanilide of palmitic acid, 111.6–112.6°.

Cut 8.—One crystallization from acetone yielded white crystals, m.p. 66.4–68.9°; mixed m.p. with stearic acid, 66.0–69.8°.

Cut 9.—Two crystallizations from acetone gave white crystals, m.p. 68.1–69.4°. Three crystallizations of the *p*-bromoanilide from acetone gave white crystals, m.p. 113.9–115.0°; mixed m.p. with *p*-bromostearanilide, 114.2–115.2°.

Cut 10.—Crystallization gave impure stearic acid. The contaminant appeared to be the neutral material eluted from charcoal with benzene.[11]

Fraction S_f20–400 (*cf.* **Table IV**).—Cuts 13 and 14, calculated as stearic acid, amount to 22% of the total. If this value is added to the 43% of unsaturated acid (*cf.* Table II), this leaves 34% which is regarded as palmitic acid since no myristic acid could be demonstrated in the early fractions from the chromatogram. The distribution of the mono-unsaturated acid between C_{16} and C_{18} may be calculated from the equivalent weight (*cf.* Table II). If x be termed the oleic acid, then $0.43 - x$ is palmitoleic acid, and the following equation may be set up

$$(0.22)(284.5) + 282.5x + (0.43 - x)(254.5) + (0.34)(256.5) + (0.01)(204.5) = 268$$

Solution of this equation gives for x the value of 0.21, or 21% oleic acid and 22% palmitoleic acid. It should be mentioned that any neutral material in the mixed acids, leading to too high a value for equivalent weight, would cause a large change in this ratio of C_{16} to C_{18} unsaturated acids. For example, presence of about 1% neutral material would mean that the true equivalent weight is about 265, and the content of oleic acid calculated on this equivalent weight would be 11%. The low iodine number for cut 11 in the chromatogram (*cf.* Table IV) suggests a very low oleic acid content, for oleic acid is eluted only very slightly faster[6] than palmitic acid (note that cut 3 in Table III, just ahead of the fastest elution of palmitic acid, is mostly liquid).

Cut 5.—One crystallization from acetone yielded a trace of pale yellow solid, m.p. 50–60°; mixed m.p. with myristic acid, 45–48°; mixed m.p. with palmitic acid, 56–61°.

Cut 7.—One crystallization from acetone yielded pale yellow crystals, m.p. 52–61°; mixed m.p. with myristic acid, 45–47°; mixed m.p. with palmitic acid, 58–62°.

Cut 10.—One crystallization from acetone yielded white crystals, m.p. 60.0–61.0°; mixed m.p. with palmitic acid, 60.0–61.5°; mixed m.p. with stearic acid, 55–57.5°.

Cut 12.—One crystallization from acetone yielded white crystals, m.p. 59.5–61.2°; mixed m.p. with palmitic acid, 59.6–61.9°.

Cut 13.—Two crystallizations from acetone yielded white crystals, m.p. 65.6–67.8°; mixed m.p. with palmitic acid, 51.5–60.8°; mixed m.p. with stearic acid, 65–68.6°.

Cut 14.—Because of the nature of the pattern in the chromatogram, this cut of about 10 mg. was examined carefully for presence of acids above stearic in molecular weight (the tetra-unsaturated C_{20} acid, arachidonic, is eluted ahead of stearic acid). The following data indicate that the principal component is stearic acid, probably contaminated with the neutral material from the charcoal.[11] Traces of some higher acid may be present.

TABLE V

MELTING POINTS OF *p*-BROMOANILIDES

p-Bromoanilide	M.p., °C.
Palmitic (C_{16})	112.6–113.3
Stearic (C_{18})	114.2–115.3
Arachidic (C_{20})	116.5–116.9
C_{16} + C_{18}	107.6–108.7
C_{16} + C_{20}	104.4–107.4
C_{18} + C_{20}	107.6–111.8

The cut was crystallized once from 80% ethanol, then twice from acetone, to yield a white solid of m.p. 59–68°; mixed m.p. with stearic acid, 53–68°. The material in the mother liquors was converted to the *p*-bromoanilide, and this was crystallized twice from acetone to yield white crystals, m.p. 113.9–114.3° (softening at 104°); mixed m.p. with *p*-bromostearanilide, 113.7–114.6°.

Preparation of *p*-Bromoanilides.—The *p*-bromoanilides were prepared in high yields from as little as 10 mg. of fatty acid through the corresponding chlorides which in turn were prepared in hexane using phosphorus trichloride.

In Table V are recorded the melting points of pure *p*-bromoanilides and of approximately 1:1 mixtures of them. It may be seen that although the melting points of the pure compounds are quite close together the mixed melting points are definitive for identification. Previous workers[12] reported the melting points of the *p*-bromoanilides of palmitic and stearic acids to be 110 and 114°, respectively. Nitrogen analysis (Kjeldahl) of the *p*-bromoanilide of arachidic acid gave the following. *Anal.* Calcd. for $C_{26}H_{44}NOBr$: N, 3.00. Found: N, 2.93.

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