Microdetermination of the Fatty Acids in Blood Serum

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Herb & Riemenschneider (1953) and Evans, Waldron, Oleksyshyn & Riemenschneider (1956) have described methods for the spectrophotometric determination of polyethenoid fatty acids in blood depending upon the change in optical density at five different wavelengths due to isomerization with potassium hydroxide in ethylene glycol at 180°. In the present paper this technique and that of O'Connell, Lipscomb & Daubert (1952) have been modified, making it possible to perform duplicate determinations on 2 ml. of serum. The method of van de Kamer, Pikaar, Bolssens-Frankena, Couvée-Ploeg & van Ginkel (1955) for the determination of higher saturated fatty acids has also been modified to permit such analyses with only 4 ml. of serum. This method depends on reverse-phase chromatography on a rubber column (Boldingh, 1953). A determination of the total fatty acid content can easily be included, which allows the calculation of the total unsaturated fatty acid content. The amount of total unsaturated and total polyethenoid fatty acids being known, the monoethenoid fatty acids can be calculated by subtraction.

METHODS

Reagents

Potassium hydroxide (21%, w/w) in ethylene glycol. This was prepared according to Herb & Riemenschneider (1952). After heating to 190°, it is preferable to cool the ethylene glycol to room temperature before adding the KOH and to flush well with nitrogen before heating again to 190°. The reagent, prepared in this way, and stored under nitrogen at approx. -20°, remained essentially colourless for at least one year.

Absolute methanol. This was prepared with aluminium amalgam (Pestemer, 1951).

Purified nitrogen. Purest cylinder nitrogen is washed twice with 0.1M-vanadyl sulphate according to Meites & Meites (1948), and afterwards twice with 20% NaOH and twice with water.

Rubber for chromatography. This is purified by percolation in a column (90 cm. × 4 cm.) with 3 l. of a mixture of acetone and 25% (w/w) HCl (9:1) for 9 hr. instead of boiling with acetone-HCl (van de Kamer *et al.* 1955).

Thymol blue solution. 0.1% in 96% ethanol, not neutralized.

Other reagents. See van de Kamer *et al.* (1955).

As the amounts of fat which are handled are very small, it is important to use thoroughly cleaned solvents and glassware.

Preparation of extract of fatty acids from serum

Serum (2 ml.) is added dropwise and under agitation, to a 100 ml. volumetric flask, with ground-glass stopper, containing about 60 ml. of ethanol-ether (3:1, v/v; Bloor, 1914). After adding some carborundum chips, the solution is boiled for 2 min., cooled to room temperature, filled up to the mark and filtered through a fat-free filter. A volume (80 ml.) of the filtrate is pipetted into a 100 ml. Erlenmeyer flask, and 2 ml. of N-sodium ethoxide, 0.1 ml. of 0.1% ethanolic quinol solution (Wilson & Hansen, 1935) and some carborundum chips are added. The solvent is distilled off completely on a water bath, the last traces being blown off with nitrogen. The soap is transferred to a 100 ml. separating funnel (stopcock and stopper lubricated with glycerol) with 36 ml. of 75% (v/v) ethanol. The solution is shaken three times with 10 ml. of light petroleum (b.p. 40-60°), the combined extracts are shaken once with 8 ml. of 75% ethanol and the latter is added to the soap solution. The soap solution is transferred to the separating funnel with 36 ml. of water, and after acidifying with 2 ml. of 4N-HCl, extracted three times with 10 ml. of light petroleum. The combined extract is dried with anhydrous Na₂SO₄, filtered and made up to 50 ml. with light petroleum.

Determination of polyethenoid fatty acids

Isomerization. Fatty acid solution (15 ml.) in light petroleum is transferred in duplicate to the reaction tubes (Fig. 1) and some carborundum chips are added. The solvent is evaporated on a water bath under nitrogen. The fatty acids are concentrated in the bottom of the tube by washing down with light petroleum. To the residue, 0.5 ml. of KOH in ethylene glycol is added by means of a measuring pipette, connected with rubber tubing to a syringe. Nitrogen is flushed over for 15 min. and the tubes are placed, at intervals of 1 min., in the bath, kept at 180° ± 0.3; each tube is shaken after 30, 60 and 90 sec. After being heated for exactly 15 min., the tubes are cooled under tap water and the reaction mixture is diluted with methanol to 10 ml. in volumetric flasks with ground-glass stoppers. A blank of 0.5 ml. of reagent is run through the whole procedure. Optical densities are read against the blank on a Beckman DU spectrophotometer at 268, 315, 346 and 374 mμ, with 1 cm. quartz cells. A methanol dilution (1:5) is made for the density reading at 233 mμ.

For the density readings before isomerization, 15 ml. of extract in light petroleum is evaporated under nitrogen in a reaction tube, overheating being avoided (the optical

densities are not influenced by this treatment). The residue is dissolved in 10 ml. of methanol and optical densities are read at the wavelengths mentioned above.

Calculation. From the increases in density (i.e. the difference between the density before and after isomerization), determined at the appropriate wavelengths, and the total fatty acid concentration (determined separately) the values of k_{233} , k_{268} , k_{315} , k_{346} and k_{374} are calculated. These are the increases in density that could be obtained if the density measurements were carried out at a concentration of 1 g. of total blood fatty acids/l. of methanol. The content of each of the polyethenoid fatty acids (as a percentage of the total blood fatty acids) may then be calculated from the following equations (Herb & Riemenschneider, 1953; Hammond & Lundberg, 1953):

$$\text{Linoleic acid (\%)} = 1.093 k_{233} - 0.573 k_{268} - 0.259 k_{315} - 0.033 k_{346} - 0.279 k_{374}$$

$$\text{Linolenic acid (\%)} = 1.105 k_{268} - 0.879 k_{315} + 0.190 k_{346} - 1.245 k_{374}$$

$$\text{Arachidonic acid (\%)} = 1.650 k_{315} - 1.667 k_{346} - 0.079 k_{374}$$

$$\text{Pentaenoic acid (\%)} = 1.449 k_{346} - 1.351 k_{374}$$

$$\text{Hexaenoic acid (\%)} = 3.413 k_{374}$$

Fig. 1. Tube of Pyrex glass for the isomerization of the polyethenoid fatty acids.

Effect of the saponification on the polyethenoid fatty acids

As it might be expected that the saponification causes an undesirable premature isomerization of the polyethenoid fatty acids, due to the heating with concentrated alkali, the absorption spectra of the serum extracts after single, double and no saponification were determined.

To resaponify, the completely dry soap is dissolved in 2 ml. of *N*-sodium ethoxide and 0.1 ml. of 0.1% ethanolic quinol solution, the mixture homogenized thoroughly while warming in the water bath and the solvent distilled off again under nitrogen. The soap is extracted with light petroleum in the described manner.

To prepare a serum extract without saponification, the solvent of the Bloor's extract is distilled off under nitrogen and the residue is extracted several times with light petroleum (b.p. 40–60°).

For the determination of the absorption spectra, the solution in light petroleum is evaporated under nitrogen, the residue is dissolved in 10 ml. of methanol and the optical densities are read at intervals of 2 m μ . The absorption spectra are shown in Fig. 2.

The absorption curve before isomerization is lowest after single saponification and shows weakly the characteristic peaks of conjugated polyethenoid fatty acids. As these peaks are increased after double saponification and are

Fig. 2. Absorption spectra of the fat of pooled serum B before and after isomerization: (a), (b) and (c) after a single, double and no saponification respectively and without isomerization; (d) after a single saponification and isomerization.

completely absent without saponification, it appears that the conjugation is caused by the saponification. Consequently, no conjugated polyethenoid fatty acids are present in human serum.

Determination of total and saturated fatty acids

The method for the determination of the saturated fatty acids (van de Kamer *et al.* 1955) is modified as follows.

Duplicate portions (2 ml.) of serum are extracted and saponified as described and the light petroleum extracts are titrated, after addition of 20 ml. of ethanol, under nitrogen with approx. 4 mN-sodium ethoxide. (Alternatively, one fatty acid extract in light petroleum can be prepared, taking appropriate quantities of serum and reagents, serving for the determination of both the polyethenoid fatty acids and the total and saturated fatty acids.) After titration, which yields the total fatty acid content, taking an average molecular weight for the fatty acids of 277, the two solutions are combined and the solvent is distilled off completely. The soap is dissolved in 27 ml. of water and 1 ml. of 6N-KOH, and 2 ml. of 4.7% KMnO_4 solution is

added. The solution is mixed and allowed to stand for 1 hr. After adding 2 ml. of 6N- H_2SO_4 and solid NaHSO_4 to remove the excess of KMnO_4 , the solution is extracted three times with 10 ml. of light petroleum (b.p. 40–60°). The combined extracts are dried with anhydrous Na_2SO_4 and the solvent is distilled off. The residue is concentrated in the bottom of a test tube and can easily be added to the rubber column in three transfers by means of 1 ml. of acetone-neutral arachis oil mixture (4:1) with a Pasteur pipette. During the treatment with KMnO_4 , no saturated fatty acids are attacked; however, the acids C_8 and C_{10} are not quantitatively extracted from the mixture.

The iodine value was measured by a modification of the method of Wijs (1898). To the soap solution, remaining after a determination of the total fatty acid content (or to a portion of the fatty acid extract in light petroleum, corresponding to 2 ml. of serum), 0.1 ml. of 0.1% ethanolic quinol solution is added and the solvent is distilled off completely. To the residue, 10 ml. of 0.02N-Wijs's solution is added and well mixed. After standing for 1 hr. in the dark, 50 ml. of water and 10 ml. of 0.05N-KI solution are added and the mixture is titrated with 0.02N-sodium thiosulphate. A blank is treated in the same manner.

Table 1. *Increases in optical density ($E_{1\text{ cm.}}^{1\%}$) produced by isomerization of cod-liver oil by different methods*

For the density readings before isomerization, 200 mg. of cod-liver oil was dissolved in 50 ml. of 2:2:4-trimethylpentane. From this solution a 1:5 dilution was made for the reading at 233 μ .

(a) 70–80 mg. of cod-liver oil isomerized according to Herb & Riemenschneider (1952). The reaction mixture was diluted with methanol to 100 ml. and from this solution a 1:20 dilution was made with methanol.

(b) 40 mg. of lauryl alcohol, containing approx. 4 mg. of cod-liver oil, isomerized according to Herb & Riemenschneider (1953). The reaction mixture is diluted to 100 ml. with methanol.

(c) 10 ml. of light petroleum (b.p. 40–60°), containing approx. 0.4 mg. of cod-liver oil, is evaporated and the residue isomerized as described under Methods. The reaction mixture is diluted to 10 ml. with methanol.

(d) Same as (c), but with 15.5 min. reaction time, instead of 15 min.

Wavelength (m μ)	...	...	...	233	268	315	346	374
Macro method (a)				15.9 $\pm$ 0.9	15.7 $\pm$ 1.3	14.6 $\pm$ 0.3	10.0 $\pm$ 0.7	3.5 $\pm$ 0.3
Micro method (b)				13.6 $\pm$ 0.8	12.7 $\pm$ 0.6	14.5 $\pm$ 0.7	11.2 $\pm$ 0.6	4.0 $\pm$ 0.3
Modified micro method (c)				13.7 $\pm$ 0.4	12.5 $\pm$ 0.2	13.8 $\pm$ 0.3	10.6 $\pm$ 0.4	3.8 $\pm$ 0.1
Modified micro method with 15.5 min. reaction time (d)				13.5 $\pm$ 0.3	12.3 $\pm$ 0.3	13.5 $\pm$ 0.3	10.4 $\pm$ 0.3	3.6 $\pm$ 0.2

Table 2. *Determination in replicate of the fatty acids in two pooled sera*

Figures given are mean values, the number of determinations being given in parentheses.

	Pooled serum A		Pooled serum B	
	(mg. %)	(% of total fatty acids)	(mg. %)	(% of total fatty acids)
Linoleic acid	66.3 $\pm$ 1.1 (12)	19.4	67.4 $\pm$ 1.2 (12)	19.3
Linolenic acid	6.7 $\pm$ 1.0 (12)	2.0	5.1 $\pm$ 0.5 (12)	1.5
Arachidonic acid	17.4 $\pm$ 0.9 (12)	5.1	17.7 $\pm$ 0.4 (12)	5.1
Pentaenoic acid	4.3 $\pm$ 0.5 (12)	1.3	3.3 $\pm$ 0.5 (12)	0.9
Hexaenoic acid	6.3 $\pm$ 0.1 (12)	1.8	5.8 $\pm$ 0.6 (12)	1.7
Lauric acid	4.5 $\pm$ 1.1 (3)	1.3	3.8 $\pm$ 1.0 (12)	1.1
Myristic acid	7.6 $\pm$ 0.7 (3)	2.2	7.7 $\pm$ 2.4 (12)	2.2
Palmitic acid	75.3 $\pm$ 2.8 (3)	22.0	78.2 $\pm$ 3.4 (12)	22.5
Stearic acid	20.0 $\pm$ 3.0 (3)	5.9	22.3 $\pm$ 2.0 (12)	6.4
Total fatty acids	342 $\pm$ 4.1 (40)	100	348 $\pm$ 6.7 (36)	100
Total saturated fatty acids	107 $\pm$ 2.4 (3)	31.3	112 $\pm$ 5.3 (12)	32.2
Total polyethenoid fatty acids	101 $\pm$ 2.5 (12)	29.6	99 $\pm$ 2.0 (12)	28.5
Monoethenoid fatty acids	134	39.2	137	39.4
Iodine value { Found	—	—	104.8 $\pm$ 2.1 (12)	—
Calcd.	106.2	—	103.0	—
Mean mol. wt. of fatty acids, calcd.	276.8	—	276.7	—

RESULTS

Comparison of different methods

The validity of the new method has been tested by carrying out comparative determinations both with the macro and the micro method of Herb & Riemenschneider (1952, 1953). For this purpose we used a sample of cod-liver oil, since this oil contains polyethenoid fatty acids with two to six double bonds. The results of the determinations in cod-liver oil are shown in Table 1. As fish oils are known to contain polyethenoid fatty acids to which the normal calculation cannot be applied (Herb & Riemenschneider, 1953), the results are given in optical densities only. The modified method and the micro method of Herb & Riemenschneider agree very well, whereas the macro method shows somewhat higher optical densities at 233 and 268 $m\mu$ and lower optical densities at 346 and 374 $m\mu$. Statistical treatment cannot be applied because of the mutual dependance of the measured optical densities at different wavelengths.

Accuracy and normal values

To test the accuracy of the new method, two samples of pooled sera (*A* and *B*) collected from several hundred patients were each analysed 12 times. The results, including the calculated values for the monoethenoid fatty acids, the found and calculated iodine values and the calculated mean molecular weights of the fatty acids, are given in Table 2.

The standard deviations are identical for both sera and do not vary much for small and large quantities. The calculated iodine value for pooled serum *B* is in good agreement with the experimentally determined value. The calculated mean molecular weight of the fatty acids corresponds to the values in the literature, i.e. 277.2 (Smith & Kik, 1933); 281 (Wilson & Hansen, 1935); 277 (Pagé & Michaud, 1951). In Table 3 the values are presented for ten fasted, normal males. The mean composition for these ten persons is identical with that of the two samples of pooled sera.

DISCUSSION

From the experiments with cod-liver oil (Table 1) it follows that the results with the new method are identical with the original micro method of Herb & Riemenschneider (1953). Although an important point of difference is that the reaction tubes with reagent are not warmed before addition of the sample, the experiments under (*d*) indicate that this effect can be neglected. The explanation for the deviation between the two methods of Herb & Riemenschneider remains obscure, nevertheless it seems justifiable to use their constants for the new method.

Table 3. Determination of the fatty acids in serum of fasted, normal male subjects

Results are expressed as percentage of the total fatty acid content, except the total fatty acid value itself, which is expressed in mg. %. From the mean values, shown in the last column, the calculated mean molecular weight and iodine value for the fatty acids are 276.9 and 106.3 respectively. In subject 3 plasma was used for the determination.									
Subject	...	...	...	...	...	...	...	...	Mean
Total fatty acids	270	635	218	372	315	389	394	275	359±115
Total saturated fatty acids	28.1	31.6	26.4	31.4	32.8	31.0	32.2	31.3	30.8±2.0
Total polyethenoid fatty acids	35.4	23.9	34.4	32.8	31.0	31.3	24.9	24.9	29.6±4.2
Monoethenoid fatty acids	36.6	44.6	39.2	35.8	36.2	37.8	42.9	37.4	39.6±3.4
Linoleic acid	26.3	15.4	23.1	20.2	17.9	22.5	15.2	19.3	19.5±3.7
Linolenic acid	0.6	1.9	1.1	3.0	2.4	2.5	3.0	2.1	2.1±0.8
Arachidonic acid	4.3	3.9	5.7	6.9	6.1	4.0	4.7	6.7	5.1±1.2
Pentanoic acid	1.6	1.2	1.7	1.1	1.3	1.1	0.7	1.2	1.2±0.3
Hexanoic acid	2.6	1.5	2.9	1.4	3.3	1.2	1.2	0.8	1.7±0.9
Lauric acid	0.8	0.8	0.3	1.0	1.2	0.5	0.6	1.5	0.9±0.4
Myristic acid	2.3	3.1	0.9	2.4	2.8	3.2	2.5	2.8	2.5±0.7
Palmitic acid	18.2	22.4	18.2	20.8	22.7	21.4	22.4	20.7	20.8±1.6
Stearic acid	6.8	5.3	7.0	7.0	6.1	5.9	6.7	7.1	6.8±0.7

When the method is applied to serum fat, it is necessary to saponify the fat beforehand, since it contains components which are difficult to saponify and which may therefore give erroneous results. Front & Daubert (1945) showed that unsaponified cholesteryl linoleate gives too low results, possibly because of the insolubility of the ester in the reagent. However, the saponification causes a premature, undesirable conjugation of the polyethenoid fatty acids (Fig. 2), but this proves to be very small, as the results of the analysis of the polyethenoid fatty acids after single and double saponification are identical. In contrast, the results of the analysis without saponification show higher values for pentaenoic and hexaenoic acid, perhaps because of a changed absorption spectrum of the impurities during the treatment with hot alkali. Comparison of the curves (a) and (c) of Fig. 2 makes it clear that the saponification removes most of the impurities of the serum fat. Moreover, other difficulties are encountered when isomerizing the serum fat without saponification. When a Bloor's extract is evaporated, an insoluble precipitate is formed. After extraction of the residue with light petroleum, approx. 5% of the total fatty acids, including polyethenoid fatty acids, remain undissolved. This is demonstrated by saponifying the extracted residue and isomerizing the fatty acids obtained by the described method, the absorption curve showing clearly the peaks of conjugated polyethenoid fatty acids.

The finding that no conjugated polyethenoid fatty acids occur in human serum (Fig. 2, curve c) contradicts the findings of Zirm & Schauenstein (1952), who state that conjugated dienoic, trienoic and tetraenoic acids are present in normal serum. However, these authors did not demonstrate characteristic absorption peaks.

With the micro method of Herb & Riemenschneider (1953) milligram quantities of fat can be determined, weighing of very small quantities being avoided by diluting the sample with lauryl alcohol. However, the amount of reagent (5 ml.) requires a rather large volume of methanol to transfer the reaction mixture quantitatively, which results in low optical-density readings. In the present method 0.5 ml. of reagent is used, making optical density readings possible in 10 ml. of solution. The use of measured fractions of a light-petroleum extract avoids also in this method the weighing of small amounts of fat.

By means of the alkaline-isomerization technique, only the systems of two to six isolated double bonds are determined. However, *iso*-acids are believed to occur only in traces in blood fat, so that with the described method the true composition will be closely approximated. Additional data about the identity and the extinction coefficients of

the pentaenoic and hexaenoic acids in blood serum would be desirable, but as these acids occur only in small quantities the influence on the calculation of the content of other acids will be negligible.

The modifications in the method of van de Kamer *et al.* (1955) allow an accurate determination of small amounts of saturated fatty acids; traces of saturated fatty acids with an uneven carbon chain or with a branched chain, possibly present in blood fat, cannot be detected by this method. With the new purification of the rubber, acid products from the rubber during analysis are reduced to a minimum. In previous work (van de Kamer *et al.*, 1955; Pikaar, 1955) neutralized thymol blue indicator was used for the titration of the 20 ml. fractions, resulting in too low recoveries for the acids C_8 – C_{14} . However, when unneutralized indicator is used, good recoveries are obtained for all acids, also for small quantities. For a good result, blanks have to be taken from a control column.

Examination of the Tables 2 and 3 shows that 40% of the serum fatty acids are monoethenoid, 30% are polyethenoid, linoleic and arachidonic acid being the greatest components. The remaining 30% consists of saturated fatty acids, of which about three-quarters is palmitic acid, and the rest mainly stearic acid with traces of myristic and lauric acids. Table 3 shows that although the total serum-fatty acid content of fasted subjects shows a wide variance, nevertheless the composition of the fatty acid mixture is rather constant.

Comparison of our results with the data of Evans *et al.* (1956) for the plasma polyethenoid fatty acids on pooled blood from two persons shows a good agreement; separate data, given for five persons, are somewhat different. Their values for average oleic acid (21.1%) and total saturated fatty acids (41.9%), calculated from the iodine value, differ rather from our values (40 and 30% respectively).

SUMMARY

1. A method is described for the determination of the different fatty acids in blood serum.

2. The polyethenoid fatty acids are determined by a combination of the method of Herb & Riemenschneider (1952, 1953) and that of O'Connell *et al.* (1952), in 2 ml. of serum. The results are essentially the same as those obtained with the original method of Herb & Riemenschneider. A saponification is necessary if the method is applied to serum fat.

3. The saturated fatty acids are determined, after removal of the unsaturated fatty acids, by a modification of the method of Boldingh (1953), 4 ml. of serum being required. A determination of the total fatty acid content is included.

4. The monoethenoid fatty acids are calculated by difference.

5. Replicate analyses of two samples of pooled human sera indicate a good reproducibility. Values for normal human blood serum are given.

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The Effect of Soluble Silica on the Metabolism of Rat-Tissue Preparations

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The free silicas (amorphous, quartz, cristobalite and tridymite), in a finely divided form, are strongly fibrogenic in the tissues of man and experimental animals (see, for example, Gardner, 1938; King, Mohanty, Harrison & Nagelschmidt, 1953). Solubilities of about 14 mg. of silica/100 ml. in Ringer solution and blood plasma have been observed with the free silicas. Silicates (e.g. feldspar, mica, sericite) at the same particle size are weakly fibrogenic, if at all, and their silica solubilities are comparatively low, 1 mg. of silica/100 ml. or less. This correlation has been taken as evidence that soluble silica is the toxic agent responsible for the pathogenicity of silica dusts (King, 1950). Other evidence in support of the solubility theory of silicosis is assembled and critically discussed by King (1947, 1950).

The present study concerns the effect of soluble silica on the metabolism of rat-tissue preparations. Many possibilities must be considered for the mode of action of a tissue poison. For example, it might inhibit the activity of one or more enzymes (King, Schmidt, Roman & Kind, 1956, have observed considerable inhibitions of acetylcholine esterase, succinic dehydrogenase, liver esterase and hyaluronidase with 0.025 M-silicic acid), in which case it

would need first to penetrate to the site of activity, or it might change the permeability of cell membranes or protoplasm towards certain substrates. With such considerations in mind the effect of soluble silica on aspects of metabolism of rat-tissue slices has been investigated. The effect on the respiration of tissue homogenates is also described.

Preliminary reports of this work have been published (Rowsell & Leonard, 1957*a, b*).

EXPERIMENTAL

Soluble-silica preparations. Finely ground Madagascar quartz, 99.8% SiO₂, was supplied by the Safety in Mines Research Establishment, Sheffield; 0.75 g. was intimately mixed with 4.5 g. of anhydrous Na₂CO₃ in a platinum crucible. The mixture was heated until a clear melt was obtained, and extracted with water, with heat to facilitate solution, and made up to 100 ml.

A column (45 cm. long, 2.5 cm. in diameter) of the cation-exchange resin Zeo-Karb 225 (The Permutit Co. Ltd., London) was prepared in the H⁺ ion form. After standing with 2N-HCl for 30 min. it was washed with water until neutralization of 100 ml. of effluent required less than 1 ml. of 0.02N-NaOH, with methyl orange as indicator. The sodium silicate solution was poured on the column and silicic acid sols were obtained when the effluent was in the range pH 3.3–3.7. The sols were gassed with N₂ or O₂ to