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Photometric adaptation of Dole's microdetermination of free fatty acids

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SUMMARY The method is based on the measurement of color changes in phenol red barbital buffer in heptane–ethanol. It has been applied to human serum and to incubation media containing fatty acids liberated from rat epididymal adipose tissue. The values obtained were found to agree with those using the titrimetric method, within the limit of experimental error.

A PROCEDURE FOR the determination of long-chain fatty acids based on measurement of the optical density of copper or cobalt soaps extracted into chloroform has been devised by Ayers (1). Even if the sensitivity of this procedure is increased by means of triethanolamine buffer (2) and particularly by the use of diethyldithiocarbamate for copper detection (3–5), some circumstances limit the use of such methods for rapid serial analyses of biological material. For instance, there is appreciable interference by phospholipids, a decrease of selectivity with increasing concentration of triglyceride (4), the necessity for prolonged and vigorous extraction on a mechanical shaker if albumin is present,[1] the necessity of a somewhat cumbersome separation of the chloroform phase from the water–copper nitrate–triethanolamine mixture, and incomplete recovery of fatty acids added to human serum.[2, 3]

In the method to be described, fatty acids are extracted first into heptane in a biphasic system as recommended by Dole (6) and then determined photometrically using a simple principle described by Croxatto et al. (7) and suitable modifications for work in a nonaqueous medium. The fatty acids are added to a solution of sodium barbital and phenol red in heptane–ethanol and the optical density is measured. Liberated free diethylbarbituric acid converts the alkaline phenol red into its yellow acid form in almost proportionate amounts since the dissociation constants of phenol red and barbital are within 0.1 unit of each other.

Reagents. The stock buffer indicator consisted of 1% phenol red (Lachema, Brno, Czechoslovakia) in 0.12 M sodium barbital (0.25 g per 10 ml). This solution was diluted before use: 99 ml of absolute ethanol and then 200 ml of heptane were added to 1 ml of the buffer and placed in the automatic burette. Similarly the heptane, distilled water, and extraction mixture (isopropyl alcohol–heptane–N sulphuric acid 40:10:1) were stored in and added from an automatic burette. The changes during storing of these solutions was negligible and without influence on the final analytical values.

Procedure. One milliliter of fatty acids dissolved in heptane, and heptane alone (blank), were placed in clean, dry, matched cuvettes. In this study tubes of 1 cm diameter were used. Heptane was measured out from a 1 ml pipette operated by an injection syringe connected to the pipette by means of plastic tubing. In some cases

[1] M. Vaughan, personal communication.
[2] F. Mosinger, unpublished observations.
[3] See, however, method of K. Itaya and M. Ui in this issue of *J. Lipid Res.* ED.

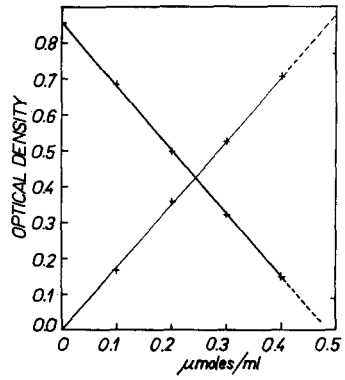

FIG. 1. Relationship between optical density and concentration of palmitic acid in heptane. To 1 ml of heptane containing 0 – 0.5 μmole of palmitic acid, 1.5 ml of barbital–phenol red buffer was added. Descending line: directly read values of optical density. Ascending line: differences between blank value and other optical density values. Each point is the mean of duplicate determinations.

nitrogen was passed through the blank, the standard, and heptane extracts (see *fatty acid determination in serum*, below). Thereafter 1.5 ml of barbital–phenol red reagent was added from an automatic burette to a final volume of 2.5 ml. To avoid evaporation and interference from atmospheric carbon dioxide, the cuvettes must be closed with polyethylene stoppers after addition of the reagent. The optical density values, which reach equilibrium quickly, were found to be stable for 12 hr. They were read at 560 mμ against pure heptane–ethanol.

TABLE 1 CONCENTRATION OF LONG-CHAIN FATTY ACIDS IN HUMAN SERUM DETERMINED BY TITRIMETRIC AND PHOTOMETRIC METHODS

Serum Sample	Titrimetric Method (T)	Photometric Method (P)	$\frac{P-T}{T} \times 100$
	μmoles/ml		
1	0.78	0.76	−2.56
2	0.88	0.88	0.00
3	1.24	1.24	0.00
4	1.27	1.20	−5.51
5	0.82	0.82	0.00
6	0.85	0.75	−11.76
7	0.28	0.26	−7.14
8	0.92	0.92	0.00
9	0.83	0.85	+2.40
10	0.73	0.77	+5.48
11	0.98	0.95	−3.06
12	0.55	0.59	+7.27
13	1.11	1.17	+5.40
14	0.62	0.64	+3.22
15	0.48	0.55	+14.58
Mean	0.82	0.82	+0.55
±SD	±0.27	±0.26	±6.39

Sera were obtained from 15 patients with various diseases after overnight fasting. The results of the photometric procedure as described in this study were compared with the titrimetric method devised by Dole (6).

Linear relationship between extinction and fatty acid concentration. The relationship between optical density and concentration for several palmitic acid standards (in duplicate) is shown in Fig. 1. The relationship is linear up to 0.4 μmole of palmitic acid in the solution of 1 ml taken initially. The molar extinction coefficient was found to be about 1.5×10^6 at 560 mμ in a 1 cm cell.

Application to fatty acid determination in human serum. The extraction procedure was the same as in the method of Dole (6), but smaller volumes can be used if needed. Serum (1 ml) was added to 5 ml of extraction mixture, followed by 3 ml of heptane, and 2 ml of distilled water. A blank (1 ml of water) and the standard (1 μmole of palmitic acid in 1 ml of heptane) were treated similarly. After 1–2 min shaking and separation of phases, 1 ml of heptane phase was pipetted into the cuvette. Since the extract of serum, as compared with blank or standard extract, contained more volatile acid (probably carbon dioxide), which led to incorrectly high extinctions (about 20–50%), all extracts were blown through with a stream of nitrogen previously passed through a flask containing heptane. For this purpose 5 sec sufficed. Then 1.5 ml of barbital–phenol red reagent was added and the tubes were stoppered.

TABLE 2 ESTIMATION OF LONG-CHAIN FATTY ACIDS LIBERATED INTO THE INCUBATION MEDIUM FROM ADIPOSE TISSUE

Sample of Medium		Titrimetric Method (T)	Photometric Method (P)	$\frac{P-T}{T} \times 100$
		μmoles/ml		
C	1	0.13	0.12	−7.69
	2	0.17	0.17	0.00
	3	0.21	0.17	−19.00
A	4	0.65	0.60	−7.69
	5	1.10	1.23	+11.82
	6	1.41	1.44	+2.13
	7	0.75	0.73	−2.67
	8	1.09	1.08	−0.92
	9	1.05	1.06	+0.95
Mean		0.73	0.73	−2.56
±SD		±0.47	±0.50	±8.46
	10			
Mean ± SD		0.74 ± 0.01 (6)*	0.75 ± 0.04 (5)	
	11	1.12 ± 0.03	1.13 ± 0.01	
Mean ± SD		(7)	(10)	

Krebs-Ringer phosphate buffer (1.5 ml), containing 2% bovine albumin and pieces of epididymal rat adipose tissue (about 120 mg each) were incubated 1 hr at 38° in the air. Thereafter 1 ml of incubation medium was extracted as described for serum by Dole (6) and the acidity in 2 ml of heptane extract was determined by titrimetry or in 1 ml of the same heptane extract photometrically. C = incubation medium without adrenalin; A = incubation medium with adrenalin (5 μg/ml). Samples 1–9, individual values of incubation media; samples 10 and 11, pooled incubation media analyzed for fatty acid contents in replicate.

* Number of determinations.

The results of the photometric method compared with the tritrimetric method of Dole (6) are summarized in Table 1. The mean percentage difference can be seen to be not statistically different from zero.

Determination of fatty acids in incubation media. Table 2 presents results of determinations of fatty acids released from rat adipose tissue into Krebs-Ringer phosphate buffer supplemented with bovine albumin. To obtain a wider range of values the release of fatty acids was stimulated with adrenaline. The incubation medium alone was used as a sample blank in this case.

Comments. The specificity of this photometric method depends on the specificity of the extraction procedure, which is extensively discussed in a paper by Dole and Meinertz (8). No serious limitation is expected even if this method of extraction were to be extended to biological material other than that used in the present work.

Palmitic acid was used as a reference standard and the assumption made that its molar extinction coefficient would not differ greatly from those of other long-chain fatty acids. This assumption is supported indirectly by the similar results obtained by the titrimetric and photometric methods (Tables 1 and 2).

It is recommended that particular attention be directed to the cleaning of the cuvettes. Imperfectly cleaned tubes cause large variations in blank values and imperfectly dried tubes lead to the separation of the single-phase into a two-phase system.

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