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Sookja Park for the M. S. in Foods and Nutrition
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Title NON-ESTERIFIED FATTY ACIDS IN HUMAN SERUM AND ERYTHROCYTES

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Two methods for the estimation of non-esterified fatty acids in plasma were evaluated. An attempt was made to obtain a reliable micro-technique to estimate these acids in blood fractions.

The titrimetric method of Trout et al. was satisfactory in the analyses of pure fatty acids and fatty acids added to serum. In the analyses of palmitic and stearic acid solutions, the mean values ranged from 95 to 115 percent of actual values. The mean recovery of palmitic acid added to a serum was 110 percent. The reproductibility of the method was fair in replicate analyses of three standard sera on two to four different days. The mean values determined for Serum A were 645, 653, 641, and 605; for Serum B, 587 and 587; and for Serum C, 565, 571 and 533 μEq per liter of serum.

The colorimetric micro-method of Mendelsohn was also satisfactory in the analyses of pure fatty acids, fatty acids added to a serum, and a fatty acid as a component of standard lipid mixtures.

In the analyses of palmitic, stearic, and oleic acid solutions, the mean values ranged from 97 to 118 percent of actual values. The recoveries of palmitic and oleic acids added to a serum ranged from 81 to 111 percent in four different experiments. In the determination of oleic acid from mixtures of several lipids, the recovery ranged from 95 to 107 percent. The reproductibility of the method was also fair in replicate analyses of a standard serum on 11 different days. The individual determinations ranged from 768 to 1046 μEq per liter of serum with a mean of 917. The mean values of 11 different experiments ranged from 830 to 1013 μEq per liter of serum.

A comparison of methods was made by analyzing four standard sera in replicate by both methods. The values of the non-esterified fatty acids by the method of Mendelsohn were approximately twice those by the method of Trout et al. The difference was attributed to the degrees of extraction of non-esterified fatty acids and/or other acids from the serum by the two methods.

The concentrations of the non-esterified fatty acids in the sera and the erythrocytes of 16 subjects of both sexes, ranging in age from 23 to 66 years, were determined by an adaptation of the colorimetric method of Mendelsohn. The concentrations in the sera ranged from 380 to 1057 μEq per liter with a mean of 800. The concentrations in the erythrocytes were consistently higher than those in the serum for all the subjects and ranged from 552 to 1919 μEq per liter with a mean of 1362.

NON-ESTERIFIED FATTY ACIDS IN HUMAN SERUM AND ERYTHROCYTES

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In Charge of Major

Head of Department of Foods and Nutrition

Dean of Graduate School

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NON-ESTERIFIED FATTY ACIDS IN HUMAN SERUM AND ERYTHROCYTES

INTRODUCTION

Non-esterified fatty acids comprise one class of lipids found in mammalian blood (22, p. 585). The terms unesterified fatty acids and free fatty acids also are frequently used to describe this lipid fraction. As implied, the carboxyl group of these acids is not linked in an ester bond, yet they are not in a completely free state as they occur associated with proteins in blood. Although these acids usually represent five percent or less of the total lipids of any tissue, their role in metabolism is of increasing interest.

Several methods for the determination of non-esterified fatty acids in plasma and serum have been published in recent years, but most require a relatively large sample for analysis. In our laboratory a study is underway comparing the lipids found in serum, erythrocytes, platelets and leukocytes from the blood of healthy humans. It was my aim to modify a published method so as to be able to analyze the small amounts of non-esterified fatty acids found in these fractions.

Two methods were selected for investigation: the titrimetric method of Trout and co-workers (61) and the colorimetric method of Mendelsohn (39). The method of Trout et al. was a recent modification which was reported to increase the specificity of the Dole method that

has been widely used. The colorimetric method of Mendelsohn required the least amount of sample of any published method.

An adaptation of the Mendelsohn method, using one-tenth milliliter of sample, was used to analyze the non-esterified fatty acids in the serum and erythrocytes from 16 subjects.

REVIEW OF LITERATURE

Non-Esterified Fatty Acids in Serum and Plasma

The presence in blood of small amounts of non-esterified fatty acids was first reported by Szent-Györgyi and Tominaga in 1924 (42). For a long time these acids were looked upon only as artifacts formed by autolysis in drawn blood or excised tissues or by hydrolysis of fatty acid esters during the course of lipid extraction and analysis. Fairbairn (19), for example, demonstrated in an experiment with mice and cats that the autolysis of liver phospholipids proceeded rapidly upon the removal of the organ from the body and suggested that phospholipids and glycerides might be the source of the free fatty acids found in the blood of the animals. The effects of temperature and solvents in increasing the activities of certain lipid-hydrolyzing enzymes during extraction have been well recognized in lipid chemistry (30, p. 14).

In 1956 Gordon and Cherkes (25) and Dole (17) independently verified the occurrence of the non-esterified fatty acids as normal constituents in human blood. Through the development of more rapid methods of chemical analysis and demonstrations of marked differences in concentrations under different metabolic conditions, they substantiated the fact that these acids were not laboratory artifacts. The normal concentration of non-esterified fatty acids in man is generally

agreed to be 8-31 mg per 100 ml of plasma, which is comparable to 280-1100 μEq per liter of plasma assuming an average molecular weight of these acids of 280 (9, p. 97; 12, 16, 17, 25, 33; 40; 61). Although they are non-esterified, it is well established that these fatty acids do not exist in the free state; rather they are always bound to plasma proteins (6, p. 147; 9, p. 97; 32). It is estimated that two-thirds of the total non-esterified fatty acids of plasma are attached to albumin and one-third to high density lipoproteins (9, p. 97; 22, p. 587-592; 50). These fatty acids consist mostly of those of longer chain lengths, for example, oleic, palmitic, stearic, and linoleic acids (6, p. 150; 9, p. 97; 13; 22, p. 587; 34).

The main source of the non-esterified fatty acids of plasma is adipose tissue. The production of these fatty acids from adipose tissues which were incubated in vitro was demonstrated by several investigators in animal experiments (47, 51, 54). In in vivo studies in humans, Gordon and Cherkes (26) showed by the measurement of arterio-venous differences in the concentrations of non-esterified fatty acids that these acids were released from adipose tissue into serum. In fasting subjects, strikingly negative arterio-venous differences were found in the saphenous area which is an area rich in adipose tissue. Borchgrevink and Havel (8) also studied arterio-venous differences in human subjects and found that glycerol as well as fatty acids were released from the tissue area drained by the saphenous vein. About three moles

of fatty acids were released from adipose tissue for each mole of glycerol released.

The hydrolysis of chylomicrons also has been reported to be a source of the circulating non-esterified fatty acids. Havel and Fredrickson (31), in a study using radioisotopes, demonstrated that part of the non-esterified fatty acids of plasma resulted from the metabolism of chylomicrons. However, quantitatively the contribution was considered to be small.

It has been postulated that the non-esterified fatty acids might be the transport form of fat from adipose to other tissues for oxidation (26). Several investigators have demonstrated that these acids were extracted from the circulating blood. Using an arterio-venous measurement with cardiac catheters, Gordon and co-workers (24, 26) observed that the human myocardium in vivo extracted fatty acids from the perfusing blood. By calculating the rate of oxygen extraction, these investigators concluded that the non-esterified fatty acid was a major source of energy for the heart muscle when the subject was in the fasting state. Ballard and co-workers (2), using similar measurements of arterio-venous differences, confirmed that a sufficient amount of non-esterified fatty acids of blood was taken up by the heart in fasting humans to account for much of the oxidative metabolism provided they were all oxidized. Gordon and co-workers (26) indicated that hepatic and skeletal tissues also utilized the circulating fatty acids.

Corroborating evidence from animal experiments that hepatic tissues extracted fatty acids from plasma was reported by Spitzer and Miller (58). They observed that when dogs were poisoned with carbon tetrachloride, the level of non-esterified fatty acids in the plasma increased while no appreciable increase in the level of esterified fatty acids occurred. That non-esterified fatty acids were the major fuel delivered to working muscles in fasting humans was demonstrated by Havel and co-workers in 1963 (33).

The quantitative significance in metabolism of the non-esterified fatty acids of blood was emphasized when it was demonstrated that their rate of turnover in the blood was rapid (4; 20; 21; 31). The half-life of these acids was found to be only about two minutes in dogs, rabbits, or humans. Fredrickson and Gordon investigated the plasma turn-over rate in man by injecting intravenously labeled fatty acids bound to albumin and measuring the disappearance of radioactivity from plasma and its appearance in the expired carbon dioxide. From the turn-over rate of the fatty acids the oxidation of circulating non-esterified fatty acids was calculated to be the source of 35 to 50 percent of the energy in man in the fasting state at rest. During exercise the oxidation of non-esterified fatty acids was calculated to provide 41 to 49 percent of the energy in fasting humans by Havel and co-workers (33), who also used labeled fatty acids.

Several dietary and metabolic factors which might influence the

concentration of non-esterified fatty acids in plasma have been investigated. The level fluctuates inversely with the rate of carbohydrate metabolism (17; 25). Factors which enhance the rate of carbohydrate metabolism reduce the concentration of non-esterified fatty acids in the plasma. Dole (17) observed in normal human subjects that these acids increased during fasting and decreased sharply after a mixed meal, feeding of glucose, or injection of insulin. In diabetic subjects, in which utilization of carbohydrate is defective, the feeding of glucose or injection of insulin did not reduce the concentration of the non-esterified fatty acids in plasma. Injection of glucagon, which mobilizes endogenous glucose, did decrease the concentration of these fatty acids, however (5).

The feeding of amino acids to fasting human subjects was reported also to cause a decrease in the concentration of circulating non-esterified fatty acids (25). The decrease was less striking than that produced by the injection of glucose, but had the same duration. Munkner (45) observed that feeding of intact proteins likewise decreased the level of non-esterified fatty acids in humans.

The effect of the ingestion of fat on the concentration of non-esterified fatty acids in plasma or serum is not as well defined. Some investigators have reported that the concentration increased with the feeding of fat in humans (28; 49), whereas others reported that there was no demonstrable effect on the level of circulating free fatty acids

even when fat was fed in sufficient quantity to produce gross lipemia (17, 25). Even the decrease of these fatty acids in human plasma for a short time after the oral administration of oleic acid and vegetable and animal fats has been reported (45). With parenteral feeding in animals, however, Havel and Fredrickson (22, p. 606) observed a clear-cut increase in the concentration of circulating non-esterified fatty acids when fat was administered abruptly and in a large quantity as a fat emulsion or triglyceride-rich lipemic plasma.

That the absolute concentration of non-esterified fatty acids in plasma may increase without concurrent increase in the proportion of fatty acids of total lipid was emphasized by Grossman and co-workers (27). They observed no increase in the proportion of the non-esterified fatty acids in total blood lipids of rats during fat absorption, although the absolute concentration of the fatty acids in the plasma increased. In fact, after the feeding of corn oil, the amount of non-esterified fatty acids represented a smaller portion of the total lipid than it did at fasting.

A variety of hormonal agents have been shown to affect the level of non-esterified fatty acids in the blood. An increase in the concentration of fatty acids resulted from the administration of epinephrine (18, 25, 29, 48), norepinephrine (44, 51, 59), or growth hormones (1, 48). These increases were attributed by the investigators to the effects the hormones have in stimulating the lipolytic enzymes

of the adipose tissues which accelerated the release of the fatty acids (1; 48, 51).

Interrelationships among hormones in the mobilization of fatty acids from tissues have also been demonstrated. Shafrir and co-workers (53; 54) reported that the effect of epinephrine on the concentration of free fatty acids in plasma was abolished in adrenalectomized or hypophysectomized rats. Schotz and co-workers (51) reported that increased release of free fatty acids from adipose tissues of rats due to the addition of adrenocorticotropic hormone, epinephrine, or norepinephrine was inhibited by the addition of adrenergic blocking agents. These investigators also suggested that the mobilization of non-esterified fatty acids from adipose tissue was at least partly under the control of vasomotor nerves.

The level of non-esterified fatty acids in serum has been reported to be affected by cigarette smoking. Kershbaum and co-workers (35, 36, 37) observed that smoking in humans and nicotine administered to dogs caused a rapid and consistent rise in the concentration of non-esterified fatty acids and that these rises were accompanied by simultaneous increases in the level of circulating catecholamines. The investigators postulated that these effects were probably due to sympathetic and adrenal stimulation by nicotine.

Various stresses have been reported to affect the mobilization of non-esterified fatty acids. Bogdonoff and co-workers (7) observed

that acute psychological anxiety resulted in a significant increase of plasma fatty acids in humans. The effect of physiological stress on their concentration was extensively studied by Mallov (38). He reported that stress induced in rats by electric shock, ethanol intoxication, or cold exposure resulted in an increase in the level of plasma non-esterified fatty acids. Mallov proposed that these stresses increased the mobilization of the fatty acids from adipose tissue as a result of enhanced lipolytic activity due to lipase activation by catecholamines released from the adrenal and sympathetic nerve endings during the stress.

Exercise is known to affect the concentration of the non-esterified fatty acids of plasma (10, 11, 23). During exercise the concentration of these acids initially decreased and then increased immediately after exercise. The results were interpreted by the investigators as an indication that exercise accelerated the efflux of the fatty acids from the plasma and increased the mobilization of the fatty acids from adipose tissues.

From the studies to date, it is apparent that the non-esterified fatty acids of plasma are the form in which depot fats are transported to other tissues for oxidation. The mechanism by which these fatty acids are released from the depots is not elucidated at present. However, the opinion of the several investigators is that this mechanism is under the control of the endocrine system.

Non-Esterified Fatty Acids in Erythrocytes

The lipids of erythrocytes have been considered to be quite stable since they are a part of the framework of the cells (3, p. 90-109). The total lipid content is appreciably lower than in plasma and is made up of a smaller proportion of neutral fats but more phospholipids and non-esterified fatty acids (3, p. 90-109; 40).

The non-esterified fatty acid fraction of erythrocytes has been investigated to only a very limited extent. In 1961 Mendelsohn (40) reported an investigation in which he found marked changes in the concentration of these acids in human erythrocytes in vivo, contrary to earlier beliefs that all lipids of erythrocytes were quite stable. Following an oral administration of glucose to normal human subjects, the concentration of the non-esterified fatty acids in the erythrocytes increased, whereas their concentration in plasma decreased. In diabetic patients these changes were much less pronounced and in many cases did not occur at all. This defect in diabetic patients could be corrected by the administration of insulin. In another series of studies in the same year, Mendelsohn (41) investigated the metabolism of non-esterified fatty acids in vitro. He observed that the concentration of the fatty acids in normal human erythrocytes increased when they were incubated in a phosphate buffer containing glucose. Very little increase was observed in the erythrocytes of diabetic subjects.

However, the addition of insulin to the erythrocyte suspension of the diabetic subjects increased the concentration of the non-esterified fatty acids. Mendelsohn proposed that normal human erythrocytes synthesize fat from carbohydrate but that this ability is lacking in the erythrocytes of diabetics.

INVESTIGATION OF METHODS FOR DETERMINING NON-ESTERIFIED FATTY ACIDS

Review of Methods

Methods for the estimation of non-esterified fatty acids in serum or plasma have been developed in several laboratories. Most of the methods are similar in that the basic procedure is a titration of the fatty acids with sodium hydroxide after their extraction, but one colorimetric method has been published.

In 1947 Davis (16) published a titrimetic method which has been modified by a number of investigators and has been in wide use. In Davis' method the serum was first buffered to pH 6, since the extraction of acids with short chain lengths, such as acetic acid, was eliminated by this buffering, and a pH of 6 was the highest pH that permitted quantitative extraction of oleic acid. The non-esterified fatty acids were extracted into ether in the presence of sodium dedocyl sulfate. The sodium dedocyl sulfate was found to eliminate the emulsification of serum albumin with water in ether. The ether extract was evaporated and dissolved in ethanol and titrated with sodium hydroxide using thymol blue as an indicator. This method was reported to be sensitive to 0. 1 μ moles of fatty acids and suitable for extracting a small amount from a large volume of a dilute solution.

Grossman and co-workers (27) modified the Davis method by

using petroleum ether in place of ether as the extracting solvent. Selden and Westphal (52) made several modifications: sodium hydroxide and hydrochloric acid rather than sodium dedocyl sulfate were used to reduce the emulsification in the ether extraction; the residue of ether extract was dissolved in methanol instead of ethanol; and Nile blue rather than thymol blue was used as the indicator. Gordon and Cherkes (25) extracted the fatty acids with ether in the presence of sodium dedocyl sulfate but added sodium sulfate and used a pH meter rather than an indicator to determine the end point of the titration.

Shore and co-workers (55) published a titrimetic method which they claimed resulted in a more complete extraction of fatty acids. The serum was first extracted with ether and the residual aqueous phase was then acidified to pH 2 by an hydrochloric acid solution in order to achieve quantitative removal of fatty acids with a minimum hydrolysis of lipids. Petroleum ether was added to the evaporated ether residue; the mixture was washed with hydrochloric acid. The petroleum ether layer was evaporated and the residue was dissolved in ethanol. The ethanol mixture was boiled to expel carbon dioxide, and the hot solution was titrated with sodium hydroxide using phenolphthalein as an indicator. The end point was checked with a pH meter.

Dole (17) published a relatively simple and a more rapid method in 1956. Heptane was used as the extracting solvent in the presence of isopropanol, which the author stated was more efficient than ethanol in

bringing heptane and water together into a single-phase system. The plasma and heptane mixture was divided into a two-phase system by adding water. A measured volume of the heptane layer was titrated with sodium hydroxide using thymol blue as the indicator. The author stated that replicate analyses of the fatty acids in one milliliter of plasma could be determined with only a three percent coefficient of variation.

Among all the various methods and modifications which have been published, those of Gordon and Cherkes (25) and of Dole (17) have been used most widely. The method of Gordon and Cherkes requires rather elaborate apparatus. The Dole method requires only a moderate amount of skill, time, and special apparatus; however, this method is not strictly specific for the fatty acids. It is known that plasma lactate interferes in the Dole method (61). Trout and co-workers in 1960 (61) modified the Dole method to improve its specificity. The heptane layer which contains the acids was first washed with 0.05 percent sulfuric acid and thus freed of lactic acid and of acetone insoluble material which interfere. Trout et al. reported that with this change the Dole procedure yielded results which agreed well with those of Gordon and Cherkes.

All the above mentioned titrimetric methods require a large sample (1 to 15 milliliters), the preparation of a standard sodium hydroxide solution each day, and precautions to insure a carbon

dioxide-free atmosphere for titration.

In 1958 Mendelsohn (39) devised a relatively simple colorimetric method for the determination of micro amounts of non-esterified fatty acids in plasma. In his method only 0. 2 ml of sample was used. The plasma was acidified with dilute sulfuric acid and deproteinized with a methylal-methanol mixture. Methanol was added in order to bring all the liquid into a single phase. Petroleum ether was then added, and the non-esterified fatty acids were extracted into the petroleum ether. Water was added and the system separated into two phases. An aliquot of the upper petroleum ether layer was removed and evaporated, and the residue was dissolved in isopropanol. A red color which could be measured photoelectrically was developed by adding a rosaniline reagent to the isopropanol solution.

Titrimetric Method of Trout and Co-Workers

Modifications of the Method

For the investigation of a titrimetric method in this study, the procedures of the Dole method as modified by Trout et al. (61) were followed with two minor modifications. A 0. 1-ml Farmer and Abt microburet was used instead of the 0. 1-ml Rehberg buret, because the Rehberg buret was not available. The Farmer and Abt microburet is

graduated in units of 0. 002 ml and could be estimated to 0. 0005 ml, whereas the Rehberg buret is graduated in units of 0. 001 ml. It was recognized that the use of the Farmer and Abt microburet might be a limitation in analytical accuracy.

The length of the shaking period during washing of the heptane extract with sulfuric acid was reduced to three minutes from the five minutes suggested in the original procedure. Shaking times of one-and-one-half minutes, three minutes, and five minutes were compared in the analysis of a sample. Three minutes of vigorous shaking gave the most satisfactory results.

It should be noted also that a dilution of 1000 times of a saturated sodium hydroxide solution is necessary in order to obtain an approximately 0. 0020 N solution. A dilution of 100 times is suggested in the original Dole method. It is assumed that the direction was a typographical error.

Reagents

1. Isopropanol (Baker), redistilled.
2. Heptane (Eastman) bp 98. 5°C, redistilled.
3. Thymol blue (Baker).
4. Ethanol, 100 percent, redistilled.
5. Sulfuric acid, 1 N.

6. Sodium hydroxide solution, saturated. 110 g of reagent grade sodium hydroxide was added to 100 ml of redistilled water in a 600-ml Erlenmeyer flask. The contents of the flask were mixed well by shaking, and the flask was kept tightly stoppered for at least two days before use. The flask was fitted with a soda lime column and a glass delivery tube.
7. Dilute sodium hydroxide. Approximately 0.0010 or 0.0020 N solutions were made by a dilution of 2000 or 1000 times of the saturated sodium hydroxide solution with redistilled water.
8. Extraction mixture: isopropanol, 40 parts; heptane, 10 parts; and 1 N sulfuric acid, 1 part.
9. Titration mixture: 0.01 percent solution of thymol blue and 90 percent of ethanol in water. One part of 0.1 percent thymol blue solution in redistilled water was mixed with 90 parts of redistilled alcohol.
10. Standard palmitic acid solution, 0.0003 N. 0.0192 gm of palmitic acid (Applied Science Laboratories) was made up to 250 ml with heptane. The standard solution was divided into 25-ml volumetric flasks and stored at $-5^{\circ}C$ until the time of use. Each day of analysis the standard solution was allowed to resume its volume before the aliquots were measured.

Equipment

1. Pyrex test tubes, with stoppers, 22 x 150 mm.
2. Maisel-Gerson reaction vessels.
3. Pyrex centrifuge tubes, 12 ml.
4. Volumetric pipets and flasks.
5. Farmer and Abt microburet, 0.1 ml.
6. Compressed nitrogen gas.
7. Polyethylene capillary tubing.
8. Centrifuge, International Centrifuge, Size 1, Type SB.

Standardization of Alkali

The approximately 0.0010 or 0.0020 N sodium hydroxide solution was made each day of analysis. Three-milliliter aliquots of 0.0003 N palmitic acid were measured into five 12-ml centrifuge tubes. One milliliter of titration mixture was added to each tube. The acids in the heptane layer were titrated with freshly made dilute sodium hydroxide. Nitrogen gas was bubbled through the contents using a fine polyethylene capillary tube to expel carbon dioxide and to keep the water and heptane phases mixed during the titration. As the green-yellow end point was approached the nitrogen stream was interrupted from time to time to examine the indicator color in the heptane layer. A fluorescent lamp was used to aid in discerning the end point.

The reagent blank consisted of three milliliters of heptane and one milliliter of titration mixture titrated in the same way as were the standard palmitic acid solutions.

Procedure for Sera

Extraction and Washing. One milliliter of serum and 10 ml of extraction mixture were mixed by shaking in a glass stoppered test tube. Then 6 ml of heptane and 4 ml of water were added, and the mixture was vigorously shaken for two minutes. A 5-ml aliquot of the upper heptane layer was transferred to a Maisel-Gerson reaction vessel and 5 ml of 0.05 percent sulfuric acid was added. The vessel was vigorously shaken for three minutes and then centrifuged for five minutes at 1300 rpm.

Titration. Three milliliters of the washed heptane layer were transferred to a 12-ml centrifuge tube containing 1 ml of the titration mixture. The acids in the heptane layer were titrated with 0.0010 N sodium hydroxide using the same procedures as in the standardization of alkali.

Procedure for Standard Solutions

One milliliter of heptane containing a known amount of a fatty acid was extracted, washed, and titrated the same as the serum

samples, except 0.0020 N sodium hydroxide was used.

Calculations

The normality of the sodium hydroxide solution was calculated as follows:

$$\underline{N}\ NaOH = \frac{3\ (ml.\ acid) \times 0.0003\ (\underline{N}\ acid)}{ml\ NaOH}$$

The amount of non-esterified fatty acids, expressed as μEq per liter of sample, was calculated as follows:

$$\mu Eq/l = \frac{\underline{N}\ NaOH \times ml\ NaOH}{ml\ original\ solution \times heptane\ dilution\ factor} \times 10^6$$

For serum,

$$\mu Eq/l = \frac{\underline{N}\ NaOH \times ml\ NaOH}{(1\ ml)\ (3/8)} \times 10^6$$

For standard solutions,

$$\mu Eq/l = \frac{\underline{N}\ NaOH \times ml\ NaOH}{(1\ ml)\ (3/9)} \times 10^6$$

Analyses of Standards and Test Sera

Validity of the method of Trout and co-workers, with the minor modifications introduced in this laboratory, was investigated by analyses of standard fatty acid solutions and recoveries of fatty acids added to a serum. Standard solutions of palmitic acid and stearic acid in different concentrations and a serum with a known amount of palmitic

acid added were analyzed. The results are shown in Table 1.

Table 1. Analyses of Pure Fatty Acids and a Fatty Acid Added to Serum by the Method of Trout and Co-Workers

Experiment	Sample	Amount of Fatty Acid in Sample	Amount of Fatty Acid Determined	Recovery	
				Aliquot	Mean
		μEq/l	μEq/l	%	%
I	Palmitic acid	400	504	126.0	114.7
			504	126.0	
			432	108.0	
			396	99.0	
		800	791	98.9	101.2
			863	107.9	
			791	98.9	
			791	98.9	
		1600	1726	107.9	107.9
			1726	107.9	
			1726	107.9	
II	Stearic acid	400	414	103.5	103.5
			414	103.5	
		800	689	86.1	94.8
			827	103.4	
			758	94.8	
		1600	1516	94.8	100.7
			1689	105.6	
			1654	103.4	
			1585	99.1	
III	Serum + Palmitic acid (400 μEq/l)	983	1099	111.8	109.7
			1066	108.4	
			1082	110.0	
			1066	108.4	

The concentrations measured were in general slightly higher than the actual amounts of the fatty acids, mean values ranging from 95 to 115 percent of predicted values. The recoveries from serum were about as much higher than predicted values as those obtained by Trout et al. were below, 110 percent compared to their mean recoveries of 93 percent. The tendency to high values may be due to one or more of the following: errors in preparation of the fatty acid solutions, errors in standardization of the sodium hydroxide, or errors in the titration. From the variation which occurred among aliquots, the greatest source of error appears to be in accurate control in the addition of the sodium hydroxide using the Farmer and Abt buret.

Any possible effect of the size of the sample was studied by the analysis of several one- and two-milliliter aliquots of a single test serum. One milliliter aliquots were used by Dole (17) whereas two milliliter aliquots were suggested by Trout et al. (61). The results, which are shown in Table 2, indicate that a one-milliliter sample was as satisfactory as that of two milliliters in the determination of fatty acids by this method.

Reproducibility of the method was investigated by analyzing the non-esterified fatty acids in three different test sera in replicate on two to four different days. The data are presented in Table 3. Although the variation among the aliquots was frequently somewhat large, the mean values on different days for each serum were within

a reasonable range. The mean values determined for Serum A were 645, 653, 641, and 605; for Serum B, 587 and 587 and for Serum C, 565, 571 and 533 μEq per liter.

Table 2. Comparison of One- and Two-Milliliter Samples of Serum as Analyzed by the Method of Trout and Co-Workers

Sample Size of Test Serum	Non-Esterified Fatty Acids	
	Aliquot	Mean
	μEq/l	μEq/l
1 ml	587	580
	529	
	624	
2 ml	572	592
	602	
	602	

Table 3. Repeated Analyses of Three Test Sera by the Method of Trout and Co-Workers

Test Serum	Date of Analysis	Non-Esterified Fatty Acids	
		Aliquot	Mean
		μEq/l	μEq/l
A	10-7-63	626	
		646	
		663	
		646	645
	10-10-63	653	
		653	
		653	653
	10-22-63	657	
		591	
		591	
		723	641
	5-7-64	613	
		613	
		588	605
B	10-15-63	587	
		529	
		646	589
	10-17-63	534	
		614	
		614	
		561	
		614	587
C	10-24-63	538	
		576	
		576	
		576	565

Table 3. Continued

Test Serum	Date of Analysis	Non-Esterified Fatty Acids Aliquot	Mean
		μEq/l	μEq/l
	10-31-63	577	
		545	
		577	
		577	
		577	571
	5-5-64	563	
		504	
		533	533

Colorimetric Method of Mendelsohn

Modifications of the Method

The first effort in investigating the colorimetric method of Mendelsohn was an attempt to modify the method (39) by reducing the size of the sample from 0. 2 to 0. 02 ml of serum. . Analyses using this smaller sample were not satisfactory in several series of experiments. Although the density of the color developed seemed to increase with increasing concentrations of the fatty acids in the standard solutions when observed by eye, corresponding increases in the density could not be consistently measured in the spectrophotometer. The microcuvets, which had to be used for the small volumes of final

colored solution, were markedly stained by the red color of the dye. The color, which could not be satisfactorily removed, obviously interfered with measuring the density of the colored solution in the spectrophotometer.

According to the suggestion of Mendelsohn that 0. 1 rather than 0. 2 ml of sample could be analyzed, a change of the volume of serum into one-half that of the original method was attempted. Then the volume of final solution made it possible to use macrocuvets, which could be cleaned successfully. Analyses using 0. 1 ml of serum and one-half of the volumes of all the reagents except for the final petroleum ether extractions, as suggested by Mendelsohn, were satisfactory. For example, the mean value of the non-esterified fatty acids in a serum using 0. 2 ml of serum was 938 μEq per liter and using 0. 1 ml, was 920 μEq per liter. The mean percent recoveries of a known amount of stearic acid added to the serum were 95. 4 and 95. 2 percent, respectively.

One minor modification in the method of mixing the reagents in the extraction procedure was made. A buzzer, a mechanical agitator, was used instead of stirring with a glass rod and the stirring time was reduced. Stirring the reagents for one minute with a glass rod and mixing the reagents by a buzzer for 30 seconds were compared in an analysis of a serum, and the results by both methods were comparable. The mean value of the non-esterified fatty acids of the serum

determined using the original stirring procedure was 843 μEq per liter and that determined using the buzzer was 851 μEq per liter.

Although Mendelsohn emphasized the reproducibility of analyses of standards, in my experience it was necessary to analyze a series of standards with each experiment. Replication of standard curves could not be obtained satisfactorily even when all experimental conditions were kept as constant as possible. The major cause of variation seemed to be the difficulty in maintaining an absolutely constant temperature of the water bath during the color development.

It should be emphasized that the rosaniline base from the British Drug House, England, as recommended by Mendelsohn, should be used in this method. Due to the unavailability of the dye from England at the beginning of this study, para-rosaniline base purchased from Allied Chemical Corporation was used. The para-rosaniline dye was found to be unsatisfactory in the experiment; the red color which developed on addition of the dye to the fatty acid samples disappeared as soon as the benzene specified in the method was added.

It should also be emphasized that all glass equipment must be completely acid-free since the dye is extremely sensitive to acids.

Reagents

1. Methylal (Matheson, Coleman and Bell), kept refrigerated until used.

2. Methanol (Mallinckrodt), redistilled.

3. Methylal-methanol, 4:1, v/v.

4. Petroleum ether (bp 60°C), redistilled.

5. Isopropanol (Baker), redistilled.

6. Benzene (Baker), redistilled.

7. Sulfuric acid, 1 N.

8. Sulfuric acid, 0.05 percent.

9. Rosaniline reagent.

   About 0.5 g rosaniline base (British Drug House) was added to 50 ml of benzene in a 100-ml round bottom flask equipped with a reflux condenser. The mixture was refluxed for one hour and then allowed to cool to room temperature. The solution was decanted from the undissolved dye and centrifuged for five minutes. The dark-orange fluorescent supernatant was poured into a 500-ml beaker and benzene was added until the transmittance of the diluted reagent was 25 percent measured at 490 mμ in a Beckman spectrophotometer model DU set at 100 percent transmission with redistilled water. The reagent was stored in an amber bottle.

10. Standard stearic acid solution, 20 mg/100 ml.

    The standard stearic acid solution was made by dissolving 10 mg of stearic acid (Applied Science Laboratories) into 50 ml of isopropanol. Exactly two milliliters was

distributed into two-milliliter volumetric tubes and stored at -5°C until used. At the time of preparation of standard curves, the tube was taken out of the freezer and warmed to room temperature. The volume of solution was adjusted exactly to two milliliters, if there was any change in the volume.

Equipment

1. Pyrex centrifuge tubes, 12 ml.
2. Pyrex test tubes, 8 ml (13 x 100 mm).
3. Glass stirring rods with a flattened end.
4. Buzzer.
5. Micro-constriction pipets, (Linderstrom-Lang-Holter): 10, 50, 100, 200, 300 & 400 cmm.
6. Volumetric pipets.
7. Water bath, 46°C.
8. Centrifuge, International Centrifuge Size 1, type SB.
9. Automatic timer.
10. Transfer pipet with constriction and long fine point.
11. Parafilm, 1" x 1" pieces.
12. Racks to hold 12-ml centrifuge tubes.
13. Metal rack to hold 8-ml test tubes.
14. Beckman Spectrophotometer, Model DU.

Procedure

Extraction. With a constriction pipet 100 µl of serum was delivered into a 12-ml centrifuge tube, and the serum was acidified by adding 10 µl of 1 N sulfuric acid with shaking. Then 0.5 ml of the methylal-methanol mixture was added, and the contents were mixed well by shaking and by stirring with a glass rod. One and one-half milliliters of methanol and 400 µl of petroleum ether were added and the contents of the tube were mixed by a buzzer until it formed a single phase. Four hundred microliters of redistilled water was added to separate the system into two phases. Two milliliters of petroleum ether was added and the tube was buzzed for 30 seconds. Then the tube was centrifuged at 3,000 rpm for two minutes, and the upper petroleum ether layer was transferred into an 8-ml pyrex test tube using a constriction pipet with a fine bore. The extraction was repeated using another two milliliters of petroleum ether and the upper petroleum ether layer was transferred into the test tube containing the first extraction.

Color Development and Reading of Density. The four milliliters of petroleum ether was evaporated to dryness in a hot water bath under a hood. Five-tenths of a milliliter of isopropanol was added and the residue was dissolved, aided by a slight shaking. Then 0.5 ml of rosaniline reagent was added and the contents of the tube were mixed

by buzzing. The tube was covered with a 1" x 1" square of Parafilm and it was placed in a metal rack in a water bath of 46°C. After 30 minutes the tube was removed, placed into a beaker of cold water for five minutes, and then placed in a rack. Four milliliters of benzene was added, and the contents of the tube were mixed by buzzing. Optical density of the test solution was read within two minutes after the benzene was added. The density of the color of the solution was measured at 520 mμ in a Beckman spectrophotometer, Model DU, against a blank of 0.5 ml isopropanol and 4.5 ml benzene. Macrocuvets were used, and the test solution was directly transferred to the cuvets by pouring. The solution was drawn out of the cuvet with suction and the cuvets were washed between readings with absolute ethanol and redistilled acetone.

The reagent blank consisted of 0.5 ml of isopropanol and 0.5 ml of rosaniline reagent treated exactly as was the test solution.

Preparation of the Standard Curve

With micro-constriction pipets, duplicate aliquots of 50, 100, 150, 200, and 250 μl of the standard solution were delivered into ten 8-ml test tubes. The contents of each test tube were made up to 0.5 ml by adding, respectively, 450, 400, 350, 300, and 250 μl of isopropanol. The reagent blank was 0.5 ml of isopropanol. The color was

developed and the density was measured as above. The standard curve was prepared by plotting the average values of duplicate aliquots against concentration.

Calculations

The weight of non-esterified fatty acids in the sample was read from the standard curve, and the weight was converted into μEq per liter on the assumption that the average molecular weight of the non-esterified fatty acids in the blood was 280. The formula for calculation was:

$$\mu Eq/l = \frac{\mu g \text{ fatty acids}/0.1 \text{ ml}}{280} \times 10^4$$

Analyses of Standards and Test Sera

In investigating the use of the Mendelsohn method, four types of experiments were performed: standards of several different fatty acids of known concentration were determined; recoveries of known amounts of fatty acid added to serum were calculated; fatty acids as components of standard lipid mixtures of known composition were determined; and the reproducibility of the method was checked by analyses of replicates of control serum on 11 different days ranging over a period of several months.

In Table 4 is a summary of analyses of pure palmitic, stearic, and oleic acids from six different experiments. The mean recoveries in four experiments ranged from 97 to 104 percent, but in two experiments the amounts determined were considerably higher than the actual weights, 115 and 117 percent. The ranges of individual aliquots determined in any one experiment were within plus and minus five percent of the mean value.

Table 4. Analyses of Pure Fatty Acids by the Method of Mendelsohn

Experiment	Fatty Acid	Amount of Fatty Acid in Sample	Amount of Fatty Acid Determined	Recovery Aliquot	Recovery Mean
		μg	μg	%	%
I	Palmitic acid	20	24.0	120.0	117.5
			23.0	115.0	
II	Stearic acid	20	22.0	110.5	
			22.5	112.5	104.0
			20.5	100.3	
			18.5	92.5	
III	Stearic acid	20	23.3	116.5	
			24.3	121.5	115.2
			21.5	107.5	
IV	Stearic acid	20	18.5	92.5	
			18.3	91.5	96.7
			21.2	106.0	
V	Oleic acid	31.7	30.2	95.3	
			31.0	97.8	98.9
			32.8	103.5	
VI	Oleic acid	31.7	33.5	105.7	103.3
			32.0	100.9	

The data on the recovery of fatty acids added to serum are shown in Table 5. Analyses of palmitic and stearic acids added to serum in different concentrations were made on four different dates. Except for one low recovery of stearic acid, the mean percent recoveries were similar to those reported by Mendelsohn; mean recoveries ranged from 89 to 111 percent in this study whereas they ranged from 90 to 110 percent in the study of Mendelsohn (39).

Two mixtures of different concentrations of oleic acid and triolein, and one Hormel lipid standard[1] which contained equal amounts of five lipid fractions, i. e., oleic acid, triolein, hydrogenated lecithin, cholesterol, and cholesteryl oleate, were analyzed. The results, which are shown in Table 6, demonstrated that oleic acid could be determined accurately in the presence of triolein and in combination with other lipid classes. Mean recoveries from a mixture of five percent oleic acid and 95 percent triolein in two experiments were 104 and 95 percent, and that from a mixture of 33 percent oleic acid and 67 percent triolein in one experiment was 95 percent. Mean recoveries of oleic acid in two series of analyses of the Hormel lipid standard were 102 and 108 percent.

The results of replicate analyses of the control serum are

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[1] TLC Reference Mixture No. 2, The Hormel Institute, University of Minnesota.

Table 5. Recovery of Fatty Acids Added to Serum as Determined by the Method of Mendelsohn

Experiment	Fatty Acid	Amount of Fatty Acid Added	Recovery Aliquot	Recovery Mean
		μg	%	%
I	Palmitic acid	10	90.2	
			90.0	89.2
			87.4	
	Palmitic acid	20	97.1	96.1
			95.1	
	Palmitic acid	30	91.6	
			89.7	89.4
			86.9	
II	Stearic acid	20	95.9	95.2
			94.4	
III	Stearic acid	20	112.9	111.1
			109.1	
			111.3	
IV	Stearic acid	10	96.4	
			89.6	97.2
			105.7	
	Stearic acid	20	95.6	
			87.9	92.5
			94.3	
	Stearic acid	30	83.8	
			79.3	80.9
			79.6	

Table 6. Determination of Oleic Acid as a Constituent of Lipid Mixtures of Various Composition

Experiment	Lipid Mixture	Amount Determined of Actual Amount	
		Aliquot	Mean
		%	%
I	5% oleic acid + 95% triolein	105.5 101.5 104.1	103.7
II	5% oleic acid + 95% triolein	104.6 88.1 91.8	94.8
III	33% oleic acid + 67% triolein	94.6 96.2	95.4
IV	20% oleic acid + 80% triolein, cholesterol, cholesteryl oleate, and hydrogenated lecithin, all in equal parts*	106.8 107.1	107.0
V	20% oleic acid + 80% triolein, cholesterol, cholesteryl oleate, and hydrogenated lecithin, all in equal parts*	110.6 91.7 104.0	102.1

*TLC Reference Mixture No. 2, The Hormel Institute, University of Minnesota.

summarized in Table 7. The 29 individual determinations ranged from 768 to 1046 μEq per liter of serum with a mean value of 917 ± 50.[2] Although the range of variation of individual determinations was large, the daily mean values ranged somewhat less widely, 830 to 1013 μEq per liter.

[2] Standard deviation.

Table 7. Repeated Analyses of a Control Serum by the Method of Mendelsohn

Day of Analysis	Non-Esterified Fatty Acids	
	Aliquot	Mean
	μEq/l	μEq/l
1	821	851
	964	
	768	
2	957	920
	954	
	911	
3	939	938
	989	
	885	
4	821	845
	868	
5	1014	998
	982	
6	1000	928
	857	
7	839	870
	900	
8	768	917
	904	
	927	
9	1036	1013
	950	
	1054	
10	804	830
	829	
	857	
11	1046	1009
	1018	
	964	

A Comparison of Analyses by the Methods of Trout and Co-Workers and of Mendelsohn

A comparison of the titrimetic method of Trout and co-workers and the colorimetric method of Mendelsohn was made by analyzing four sera. Each serum was analyzed in replicate by both methods, and the mean values, together with their standard deviations, are tabulated in Table 8. Even though the variation among replicates by both

Table 8. Comparison of Non-Esterified Fatty Acids in Four Sera Analyzed by the Methods of Trout and Co-Workers and of Mendelsohn

Serum	Method of Trout and Co-Workers	Method of Mendelsohn
	μEq/l	μEq/l
A	639 ± 29*	1322 ± 39
B	605 ± 14	1220 ± 42
C	496 ± 14	857 ± 54
D	551 ± 26	917 ± 50

*Mean ± standard deviation.

methods resulted in standard deviations of up to five percent or slightly more of the mean values, the results by the two methods were obviously different. The values of the non-esterified fatty acids determined by the Mendelsohn method were approximately two times those determined by the method of Trout and co-workers.

As previously shown, both the titrimetic and colorimetric methods were satisfactory in analyzing pure fatty acids and fatty acids added to serum. The cause for the difference in values in the analyses of sera is not clear from this study. However, it would seem apparent that the difference is due to the degree of extraction of non-esterified fatty acids and/or other acids from the serum by the two methods. Both Trout et al. (61) and Mendelsohn (39) recognized that other acids in the serum, such as lactic, pyruvic and citric acid, might be extracted with the non-esterified fatty acids. Trout and co-workers claimed that their modification of the Dole method was specific for non-esterified fatty acids, since the washing of the heptane layer with sulfuric acid was demonstrated to remove lactic acid and other acetone-insoluble material such as phospholipids. Trout et al. compared their modification with the original Dole method and with the method of Gordon and Cherkes by analyzing the same samples by the three methods. The values of the non-esterified fatty acids determined by the Trout et al. modification were comparable to those determined by the method of Gordon and Cherkes, whereas the values by the original Dole method were almost 20 percent higher than those by the other two. Mendelsohn justified the specificity of his method by demonstrating that fatty acids of longer chain length were extracted but not those of shorter chain length. However, he did not make any comparison of analyses by his method with those of other methods.

It is recognized that further investigations should be directed toward the problem of specificity in the extraction of the non-esterified fatty acids from serum. However, these experiments were not included in this study. Complete and specific extractions are well recognized problems in analyses of all lipid classes (30, p. 11-41).

DETERMINATION OF NON-ESTERIFIED FATTY ACIDS IN THE SERUM AND THE ERYTHROCYTES OF SIXTEEN SUBJECTS

Description of Subjects

The concentrations of non-esterified fatty acids were determined in the sera and the erythrocytes of nine women and seven men, 23 to 66 years of age. All the subjects were generally healthy and active professionally or as homemakers. Four subjects did, however, have some recognized chronic disorder: EJ had chronic glomerular nephritis which was under control; MB had an under-active thyroid and was taking a thyroid medication; JJ was taking injections for an allergy; and EB had mild arthritis. All of the subjects were non-smokers. Data on the ages, sex, heights, and weights of the subjects are included in Table 9. Leukocyte and erythrocyte counts were made and these data also are presented in Table 9.

Procedures

Blood samples were obtained from the antecubital veins of the subjects after an over-night fasting. Since this work was related to a study underway in this laboratory on lipids in all fractions of the blood, about 30 ml was collected for isolation of serum, erythrocytes, leukocytes and platelets, and for cell counts and blood smears for

Table 9. Non-Esterified Fatty Acids in the Serum and Erythrocytes of Sixteen Subjects

Subjects	Sex	Age	Height	Weight	Erythrocytes	Leukocytes	NEFA* in Serum	NEFA* in Erythrocytes
		years	cm	Kg	No /cmm x 10^6	No/cmm x 10^3	µEq/l	µEq/l
KG	F	23	159	46	5.0	6.9	723	1843
MM	F	26	177	64	4.8	8.9	1024	1364
SP	F	28	163	51	4.3	6.6	715	831
ES	F	29	157	56	5.6	9.0	750	1339
DB	F	42	157	70	5.1	7.8	667	1389
BH	F	43	175	70	5.9	9.6	643	1259
EJ	F	46	157	62	5.5	7.8	1057	1752
MB	F	63	165	74	5.1	7.2	769	1918
MF	F	63	165	69	5.2	8.5	380	552
CG	M	27	185	75	5.3	7.5	754	1532
RM	M	33	184	75	5.6	9.1	907	1518
KP	M	33	183	86	5.8	7.5	911	954
JJ	M	40	168	72	5.8	8.4	742	1340
SB	M	41	178	78	6.1	8.4	714	1661
HH	M	56	183	82	5.1	10.6	738	927
EB	M	66	173	79	---**	---**	1115**	1612

*Non-esterified fatty acids.

**Plasma was analyzed instead of serum and no cell counts were made.

differential counts. The erythrocyte and leukocyte counts were made using the Spencer Bright-Line Hemacytometer.[1]

For the isolation of serum, whole blood was allowed to clot and was centrifuged at 0°C. Serum for the fatty acid determinations was transferred to a 10 x 75 mm tube, which was tightly stoppered and stored at -5°C. At the time of analysis the frozen serum was warmed at 38°C for 15 minutes, cooled to room temperature, and mixed gently using a buzzer. The serum was analyzed in triplicate for the non-esterified fatty acids by the colorimetric method of Mendelsohn as described on pages 26 to 33.

The isolation of the cell fractions was done according to the procedures of Nelken and co-workers which have been modified in this laboratory (57).[2] The isolated erythrocytes were measured and laked in two volumes of redistilled water. The laked erythrocytes for determination of non-esterified fatty acids were transferred into a 10 x 75 mm test tube which was tightly stoppered and stored at -5°C. At the time of analysis the frozen, laked erythrocytes were warmed at 38°C for 15 minutes, cooled to room temperature, and mixed gently using a buzzer. Aliquots of the laked erythrocytes were anlyzed for non-esterified fatty acids in triplicate by the same method used for the analysis of serum.

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[1] American Optical Company.

[2] The isolation of the blood cell fractions was done by E. Smith.

Results and Discussion

The concentrations of non-esterified fatty acids in the serum and the erythrocyte fractions of blood samples obtained from 16 subjects in the fasting state are shown in Table 9. The concentrations in the serum ranged from 380 to 1057 μEq per liter with a mean value of 800. The values determined in these subjects were within ranges reported by several other investigators for non-esterified fatty acids in plasma: 315 to 1210 μEq per liter by Dole (17); 175 to 1373, by Gordon and Cherkes (25); and 490 to 1100, by Bogdonoff et al. (7). The mean value was also similar to reported means: 689 μEq per liter of plasma by Dole (17); 840, by Gordon and Cherkes (25); 765, by Svanborg and Svennerholm (60); and 584, by Trout et al. (61). However, values for these 16 subjects were higher than those reported by Michaels (43), 230 to 740 μEq per liter, and by Mendelsohn whose method was used. Mendelsohn reported a mean value of 306 μEq per liter of plasma with a range of 107 to 410 in 20 subjects in 1958 (39) and a mean value of 506 with a range of 400 to 650 in 15 subjects in 1963 (40).

Because most data reported for non-esterified fatty acids have been in plasma rather than serum, a limited study comparing concentrations in serum and plasma was made. Serum and plasma of capillary blood from two subjects were analyzed on two different days. For subject A, the concentrations of these acids in serum were higher

than those in the plasma on both days; the mean concentrations were 766 compared to 621 μEq per liter on the first day and 954 compared to 806, on the second day. For subject B, however, the serum was higher than the plasma on one day, 830 compared to 659 μEq per liter, but the values were similar on the second day, 741 and 763. The indication that concentrations of non-esterified fatty acids may be generally higher in serum than in plasma is similar to the higher concentration in serum than in plasma of certain other metabolites, e. g., ascorbic acid (15).

The amounts of non-esterified fatty acids in the sera of these subjects did not appear to be related to age, sex or degree of over- or underweight. Higher or lower values were not consistently found among the older or younger subjects of either sex, (Table 9). The lack of any apparent sex difference and the wide range of values among both men and women confirmed the data of Svanborg and Svennerholm (60), who reported means and standard deviations of 781 $\pm$ 174 and 750 $\pm$ 233 μEq per liter for 15 women and 47 men, respectively.

Corvilain and co-workers (14) reported that the concentration of non-esterified fatty acids was higher in the plasma of obese adults than in that of normal adults. But, in these 16 subjects there was no apparent correlation between the concentration of non-esterified fatty acids in the serum and the degree of overweight. For example, the

concentrations in the sera of subjects DB and MB, who were the most overweight, but only moderately so, were 667 and 769 μEq per liter, which were not distinctively higher or lower than those of the other subjects.

No consistent relationship was found between the concentrations of non-esterified fatty acids in the serum and those in the erythrocytes among the subjects in this study. However, the concentration in the erythrocytes of an individual was always higher than that in the serum, ranging up to two-and-one-half times as high.

The concentrations in the erythrocytes ranged from 552 to 1919 μEq per liter with a mean value of 1362 μEq. These values are higher than those reported by Mendelsohn for fasting subjects. Interpreting from a graphic presentation in his paper (40), the mean value for 15 subjects was 720 μEq per liter of erythrocytes with a range of 500 to 900. He stated that most of his erythrocyte data were calculated from whole blood, plasma and hematocrit values. In the limited number of analyses in which non-esterified fatty acids were measured directly on cells, non-laked cells were extracted. Presumably in order to facilitate the extraction, the procedures of his method for plasma were modified by increasing the time of mixing during extraction and adding a second extraction (40). In previous experience in this laboratory (46; 57), extractions of lipids were increased when erythrocytes were laked prior to extraction. It is possible that the extractions of

non-esterified fatty acids were more complete from laked erythrocytes analyzed in this study than from either the non-laked cells or from the whole blood analyzed by Mendelsohn.

Since this study was completed, Ways and Hanahan have published a study on the "Characterization and Quantification of Red Cell Lipids in Normal Man," (62), which includes data on non-esterified fatty acids. They report concentrations ranging from 106 to 380 μEq per liter with a mean of 270. They stated that their values were similar to those of Soloff, Schwartz and Baldwin but much lower than those found by Mendelsohn. Although the method by which Ways and Hanahan determined non-esterified fatty acids is not clear in their paper, they stated that the Mendelsohn method is not specific for non-esterified fatty acids in erythrocytes. They found that appreciable amounts of ethanolamine glycerophosphatides and serine glycerophosphatides which had titratable acidity were extracted from the cells. Specifically, they reported that ethanolamine glycerophosphatide gave five times as much color development, mole for mole, as fatty acids.

Although lecithin did not influence the color development in investigations of the Mendelsohn method performed in this laboratory, the possible influence of other phospholipids was not investigated. It is possible that the values determined for erythrocytes in this study are not indicative of amounts of non-esterified fatty acids only.

SUMMARY AND CONCLUSIONS

The titrimetric method of Trout and co-workers and the colorimetric method of Mendelsohn for the estimation of non-esterified fatty acids in plasma were investigated. Both methods were generally satisfactory for the determination of fatty acids in pure standards, as part of known lipid mixtures, or added to serum. However, the amounts of non-esterified fatty acids in test sera determined by the method of Mendelsohn were approximately twice those determined by the method of Trout et al..

Non-esterified fatty acids in the sera and the erythrocytes of 16 subjects of both sexes, ranging in age from 23 to 66 years, were determined by an adaptation of the colorimetric micromethod of Mendelsohn. The amounts in the serum ranged from 380 to 1057 μEq per liter with a mean value of 800. These values were in the range of those reported by several other investigators. The concentrations in the erythrocytes were always higher than those in the serum for any subject and ranged from 552 to 1919 μEq per liter with a mean of 1362. These values were higher than the few data reported in the literature.

Because of the significance being attributed to the role of the non-esterified fatty acids in metabolism, additional data on comparative values in the several blood fractions would be of value. However, further investigation of procedures for the specific extraction of fatty acids from these samples is needed.

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