Freely Extractable Lipid of Human Blood Plasma

I. Methodology and Observations in Normal and Abnormal Subjects

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LIPID solvents in use in biologic laboratories may be divided generally into those that cause a complete breakdown in lipoprotein complexes, and those which extract only a small portion of the plasma lipids with resultant minimal disruption of the basic lipoprotein complexes. The latter group includes pentane, hexane, benzene, chloroform and a number of others.

Forbes[1] extracted some cholesterol from lyophilized plasma with cold chloroform and called this fraction "unbound cholesterol." Byers,[2] using a different solvent system (10 per cent ethanol and 90 per cent ether) extracted a cholesterol fraction which he called "readily extractable cholesterol."

Over a period of some years sporadic attempts were made in this laboratory to develop a chemical technic for extracting, in a reasonably quantitative fashion, chylomicronous fat. To date, such efforts have been of no avail, but in the course of this work it was found that extraction of plasma with benzene and with some of the other solvents noted above, under specific conditions, resulted in an entity which, in plasma from normal subjects, had the composition shown in Table I. Repeated extraction of the plasma yielded no further lipid. Because of the constancy of level of this material in a given individual, the relatively small range in a group of young normal subjects and the relative simplicity of the method, it was decided to carry out the determinations in normal and abnormal subjects under various conditions.

MATERIALS AND METHODS

The materials are as follows: (1) solvent: reagent grade benzene; (2) a 0.2 M phosphate buffer of pH 6.0 is prepared by adding 12 ml. of 0.2 M disodium phosphate to 88 ml. of 0.2 M monopotassium phosphate. The 0.2 M disodium phosphate may be prepared by dissolving 2.84 gm. of anhydrous reagent grade in CO_2-free distilled water and making up to 100 ml. volume. The 0.2 M monopotassium phosphate may be prepared by dissolving 2.72 gm. in CO_2-free distilled water and making up to a volume of 100 ml.; (3) mechanical shaker; and (4) screw cap tubes (50 ml.), with polyethylene cone caps.

In a 50 ml. screw cap tube are placed 2 ml. of plasma and 2 ml. of phosphate buffer, and in a second tube are placed 2 ml. of water and 2 ml. of phosphate buffer; 20 ml. of solvent is then added to each tube. The tubes are tightly capped, using washed polyethylene cone caps, and shaken in a mechanical shaker for

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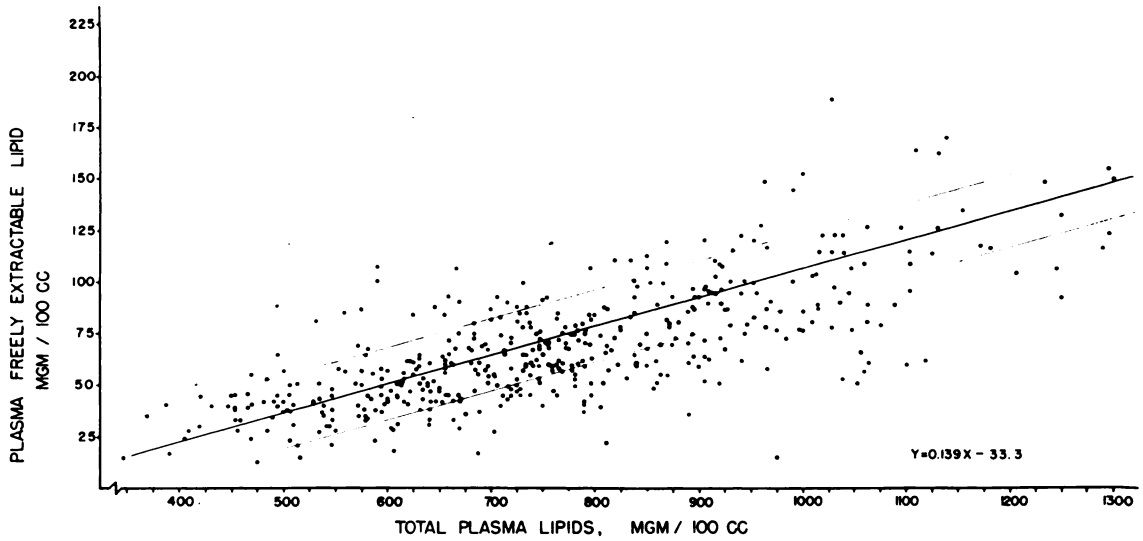

FIG. 1. Comparison of 477 freely extractable and total plasma lipid determinations in an unselected group of normal and abnormal subjects. The correlation coefficient between freely extractable and total lipid is 0.749. The least squares and limit of confidence lines apply only to values for the normal group (sixty-six subjects).

fifteen minutes. They are next centrifuged at approximately 2,000 r.p.m. for ten minutes, and 15 ml. of the supernatant is pipetted into 25 ml. volumetric flasks. The solvent is evaporated under partial vacuum at approximately 50°C., to complete dryness, and the total lipids are determined by the colorimetric method of Bragdon.[3]

In the majority of plasmas studied, in addition to freely extractable lipid, the following analyses were carried out: free and total cholesterol,[4] unesterified fatty acids,[5] phospholipids[6] and glycerides.[7]

For reasons that will be noted, if the patient has received heparin, extraction of the blood plasma for the determination of the freely extractable lipid must be carried out immediately or the plasma must be frozen. Otherwise, the blood may be kept in a refrigerator overnight.

RESULTS

Normal Plasma Values

Fasting blood was obtained from twenty-eight young male Air Force Cadets, ranging in age from eighteen to twenty-four years. These men were in "perfect" physical condition. Their diet was essentially "average American" in type. The mean value for freely extractable lipid was 47 mg. per 100 ml., with a S.E. of ±2.40 (Table I). The mean value of total lipids was 596 mg. per 100 ml., with a S.E. of ±13.73. The mean total cholesterol was 161 mg. per 100 ml., with a S.E. of ±4.28.

Plasmas from Patients with Known or Suspected Lipid Abnormalities

Fasting blood samples were obtained from a group of male and female patients. The freely

TABLE I
Fasting Plasma of the Normal Young Man

Lipid Fraction	mg./100 ml. Plasma	Per Cent of Freely Extractable Lipid
Cholesterol ester	31.0	66
Free cholesterol	3.8	8
Unesterified fatty acids	7.5 (as palmitic acid)	16
Glycerides	4.7	10
Phospholipids	0	0
Total	47.0 (±2.40)	100

Mean Total Plasma Cholesterol.....161 mg./100 ml. (±4.28)
Mean Total Plasma Lipid..596 mg./100 ml. (±13.73)

NOTE: Figures in parentheses are standard errors.

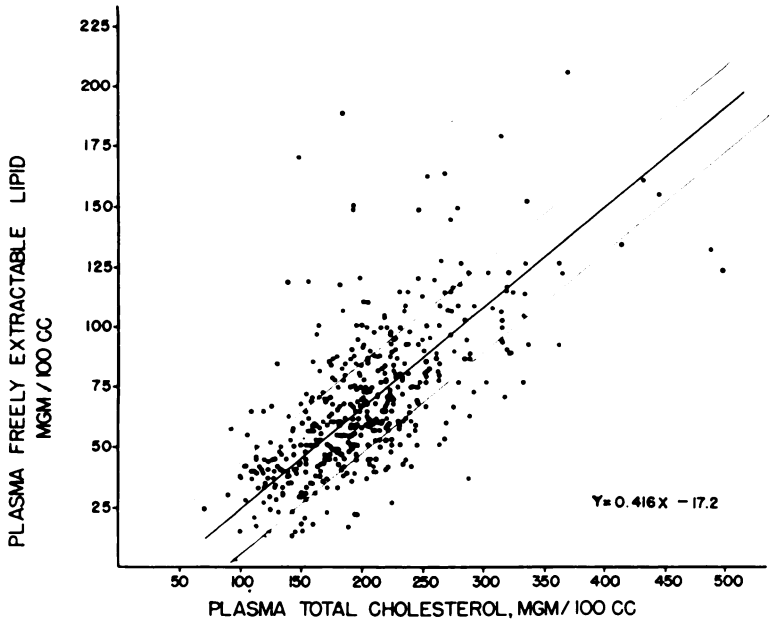

FIG. 2. Comparison of 487 freely extractable lipid and total plasma cholesterol determinations in an unselected group of normal and abnormal subjects. The correlation coefficient between total cholesterol and freely extractable lipid is 0.519. The least squares and limit of confidence lines apply only to values for the normal group (sixty-six subjects).

extractable lipid, total cholesterol and total plasma lipids were determined. A plot which shows the relation of the total lipids to the freely extractable lipid may be seen in Figure 1. The correlation coefficient is 0.749. The relationship between freely extractable lipids, and total cholesterol is shown in Figure 2. The coefficient of correlation is much lower than in the case of the total lipids (0.519). Generally speaking, the freely extractable lipid tended to be proportionately higher than the total cholesterol. In most instances where glycerides were determined, the freely extractable lipid reflected not only the level of total cholesterol but of total glycerides. Many of the patients included in these graphs had atherosclerotic involvement, with values for glycerides proportionately higher than for total cholesterol.

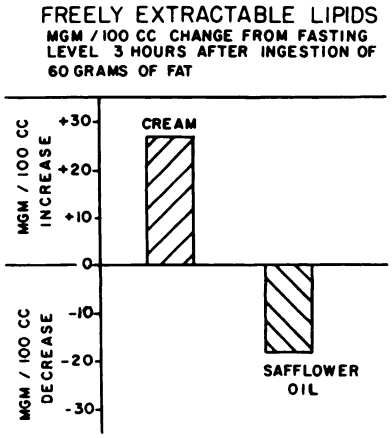

FIG. 3. Butterfat "loading" increased and safflower "loading" decreased the freely extractable lipid in the majority of a group of twenty-one subjects.

Fat Loading

After fasting blood samples were taken, a group of twenty-one patients received an emulsion containing 60 gm. of butterfat, 16 gm. of protein and 22 gm. of lactose. Three hours after the emulsion was given, a second blood sample was taken. A week or two later the same patients received an emulsion containing 60 gm. of safflower oil, 30 gm. of protein and approximately 50 gm. of lactose. Blood samples were obtained at the same intervals as before. With butterfat, the freely extractable lipid increased 27 mg. per 100 ml. above

the fasting level; with safflower oil there was a decrease of about 18 mg. per 100 ml. (Fig. 3).

With butterfat, two patients showed no change of the total plasma lipids and the others had an average increase of 123 mg. per 100 ml. Insignificant changes from the fasting level of total lipids followed ingestion of safflower oil. Plasma total cholesterol did not change significantly with either fat.

After ingestion of either butterfat or safflower oil, approximately 50 per cent of the unesterified fatty acids appears in the freely extractable lipid fraction. There was a major increase in the plasma unesterified fatty acids fifteen minutes after injection of heparin, and essentially all of the unesterified fatty acids appeared in the freely extractable lipid fraction (Fig. 4).

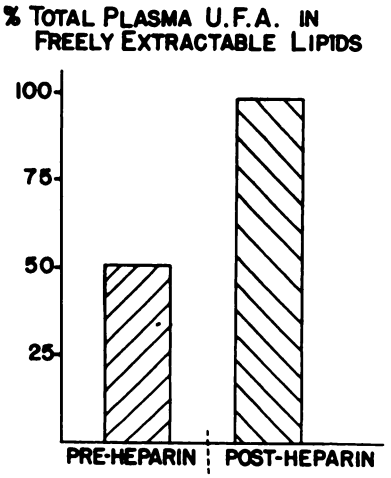

FIG. 4. In fasting plasma and after a fatty meal approximately 50 per cent of the total unesterified fatty acids appear in the freely extractable lipid. After injection of heparin, almost all the unesterified fatty acids are in this fraction. This suggests that the unesterified fatty acids are very loosely bound to protein, particularly after heparin administration.

COMPOSITION OF THE FREELY EXTRACTABLE LIPIDS IN NORMAL AND ABNORMAL PLASMAS

Normal Plasmas

The large amount of cholesterol esters and the lack of phospholipid (Table I) might suggest that the major source of the freely extractable lipid in normal subjects is the low density lipoproteins, since Avigan[8] has shown that cholesterol, but not phospholipid, may be removed from these entities without disrupting their fundamental integrity.

The 66 per cent cholesterol ester (Table I) was calculated using Bragdon's factor of 1.68, which is based on the assumption that the fatty acid in combination with the cholesterol is one with a carbon chain length of 16 to 18.

Abnormal Plasmas

Blood plasma samples from five patients whose total plasma lipids averaged 960 mg. and total cholesterol 296 mg. per 100 ml. were analyzed individually. The mean freely extractable lipid of these patients was 120 mg. per 100 ml., compared with the mean normal of 47 mg. per 100 ml. The glyceride fraction was 20 per cent, as compared to 10 per cent in the normal. Injection of heparin increased the unesterified fatty acids and decreased the glyceride (Table II).

Table III shows the pre- and postheparin composition of the freely extractable lipids in two patients with considerable elevation of glycerides and very moderate elevation of cholesterol.

TABLE II

Composition of the Freely Extractable Lipid of Five Patients with Moderately Elevated Lipids

	Fasting Plasma		15 Min. after Intravenous Administration of 50 mg. of Heparin	
Lipid Fraction	mg./ 100 ml. Plasma	Per Cent of Freely Extractable Lipids	mg./ 100 ml. Plasma	Per Cent of Freely Extractable Lipid
Cholesterol ester	73	61	71	46
Free cholesterol	15	12	14	9
Unesterified fatty acid	8	7	53	34
Glycerides	24	20	17	11
Phospholipids	0	0	0	0
Total	120	100	155	100

Mean Total Plasma Cholesterol . . . 296 mg. per 100 ml.
Mean Total Plasma Lipids . . . . . 960 mg. per 100 ml.

TABLE III

Composition of the Freely Extractable Lipid in Two Patients with Hyperlipemia and Slightly Elevated Cholesterol

Lipid Fraction	Fasting Plasma		15 Min. after Intravenous Administration of 50 mg. Heparin	
	mg./ 100 ml. Plasma	Per Cent Lipid	mg./ 100 ml. Plasma	Per Cent Lipid
Cholesterol ester	126	13	82	14
Free cholesterol	59	6	33	6
Unesterified fatty acids	14	1	68	12
Glycerides	601	60	285	48
Phospholipids	55	5	77	13
Sum of above	855	85	545	93
Total found	1,015	...	589	...
Unaccounted for	160	15	44	7

Mean Total Plasma Cholesterol...275 mg. per 100 ml.
Mean Total Plasma Lipids.....2,850 mg. per 100 ml.
Mean Total Plasma Glycerides..2,192 mg. per 100 ml.
Mean Total Plasma Phospholipids.295 mg. per 100 ml.

In Table IV are the findings in two patients with much greater elevation of cholesterol and considerably greater elevation of total lipids. Percentage composition of the freely extractable lipids in the two groups is not too dissimilar. There are major differences between the two groups in terms of response to heparin. The interpretation of these differences will be considered at another time. The absence of phospholipids in the freely extractable lipids in normal and slightly abnormal plasma, and the presence of phospholipids in the freely extractable lipid of hyperlipemic plasma is of interest.

Blood plasma samples of eight patients without obvious lipid abnormality, who had received oral butterfat emulsion followed by intravenous injection of heparin were analyzed separately. The average values for this group are shown in Table V. The composition of the freely extractable lipid fraction was similar to that reported for the normal fasting subjects,

TABLE IV

Composition of the Freely Extractable Lipid in Two Patients with Hyperlipemia and Hypercholesterolemia

Lipid Fraction	Fasting Plasma		15 Min. after Intravenous Administration of 50 mg. Heparin	
	mg./ 100 ml. Plasma	Per Cent Lipid	mg./ 100 ml. Plasma	Per Cent Lipid
Cholesterol ester	485	14	567	16
Free cholesterol	220	7	179	5
Unesterified fatty acids	31	1	143	4
Glycerides	2,430	70	2,421	70
Phospholipids	140	4	114	3
Sum of above	3,306	96	3,424	98
Total found	3,440	...	3,500	...
Unaccounted for	134	4	76	2

Mean Total Plasma Cholesterol...912 mg. per 100 ml.
Mean Total Plasma Lipids.....4,362 mg. per 100 ml.

TABLE V

Composition of the Freely Extractable Lipid in Eight Patients Three Hours after Oral Administration of an Emulsion Containing 60 Gm. of Butterfat

Lipid Fraction	3 Hours after Fatty Meal		15 Min. after Intravenous Administration of 50 mg. of Heparin	
	mg./ 100 ml. Plasma	Per Cent Lipid	mg./ 100 ml. Plasma	Per Cent Lipid
Cholesterol ester	51.4	56	38.2	29
Free cholesterol	10.8	12	8.6	7
Unesterified fatty acids	15.0	17	74.0	56
Glycerides	14.0	15	10.0	8
Phospholipids	0	0	0	0
Total	91.2	100	130.8	100

Mean Total Plasma Cholesterol...223 mg. per 100 ml.
Mean Total Plasma Lipids.....828 mg per 100 ml.
Mean Total Plasma Glycerides....254 mg. per 100 ml.
Mean Total Plasma Phospholipids 254 mg. per 100 ml.

although the total freely extractable lipid was nearly twice that found in the fasting state. Intravenous administration of heparin in this group caused an actual, as well as a percentual, lowering of esterified cholesterol and of glycerides, and an increase of unesterified fatty acids.

In thirteen patients without gross lipid abnormality who received safflower oil, the composition of the freely extractable lipid before and after injection of heparin was similar to the composition after ingestion of butterfat.

The biochemical and clinical significance of these findings will be the subject of a later report.

SUMMARY AND CONCLUSIONS

The freely extractable lipid is that plasma lipid fraction which can be extracted with benzene under certain specified conditions. The freely extractable lipid level in fasting young normal males averages 47 mg. per 100 ml. The qualitative-quantitative composition of the freely extractable lipid in normal subjects is remarkably constant. Major deviations from normal occur in some patients with atherosclerosis and in all patients in a hyperlipemic state.

In blood plasma with total lipid levels under 1,500 mg. per 100 ml., the bulk of the freely extractable lipid consists of cholesterol esters with a small amount of free cholesterol, glycerides and free fatty acids and no phospholipids. Blood plasma with total lipids in excess of 2,000 mg. per 100 ml. shows a larger proportion of glycerides and some phospholipids in the freely extractable lipid.

Approximately 50 per cent of the unesterified fatty acids of the plasma appear in the freely extractable lipids before heparin is given and nearly all of the unesterified fatty acids appear in the freely extractable lipid after injection of heparin. This suggests that much of the unesterified fatty acid is loosely bound to protein. Administration of heparin also influences other lipid components of the freely extractable lipid.

REFERENCES

1. Forbes, J. C., Dillard, G. H. L., Porter, W. B. and Petterson, O. Fractionation of serum cholesterol. *Proc. Soc. Exper. Biol. & Med.*, 68: 270, 1948.
2. Byers, S. O. and Friedman, M. Fractionation of cholesterol in body fluids by means of solvent extraction. *J. Clin. Invest.*, 35: 405, 1956.
3. Bragdon, J. H. Colorimetric determination of blood lipids. *J. Biol. Chem.*, 190: 513, 1951.
4. Michaels, G. D., Fukayama, G., Chin, H. P. and Wheeler, P. Technics for separation of plasma cholesterol esters for determination of iodine value and of cholesterol. *Proc. Soc. Exper. Biol. & Med.*, 98: 826, 1958.
5. Grossman, M. I., Palm, L., Becker, G. H. and Moeller, H. C. Effect of lipemia and heparin on free fatty acid content of rat plasma. *Proc. Soc. Exper. Biol. & Med.*, 87: 312, 1954.
6. Youngburg, G. E. and Youngburg, M. V. Phosphorus metabolism. I. A system of blood phosphorus analysis. *J. Lab. & Clin. Med.*, 16: 158, 1930.
7. Van Handel, E. and Zilversmit, D. B. Micromethod for the direct determination of serum triglycerides. *J. Lab. & Clin. Med.*, 50: 152, 1957.
8. Avigan, J. Modification of human lipoprotein fractions by lipide extraction. *J. Biol. Chem.*, 226: 957, 1957.