THE STABILIZATION OF SERUM LIPID EMULSIONS BY SERUM PHOSPHOLIPIDS

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Certain sera with marked elevations in total lipid concentration are completely clear, whereas other sera with small or only moderately elevated lipid content are grossly milky or lipemic. Since lipids exist in the serum as colloidal particles, the clarity or turbidity of serum will be determined by the size of these particles. Particles with diameters less than $\frac{1}{4}$ of the wave length of visible light (or 0.1μ) will not be seen in the visible light range and the containing serum will be clear, whereas larger particles by interrupting light rays give the serum the appearance of turbidity or lipemia. The purpose of the present study is to analyze the inherent qualities which make for invisible particle size of lipid droplets in clear sera and visible droplets in lipemic sera and to define the possible relationship of this problem to that of atherosclerosis.

Macheboeuf (1) in 1928 isolated a protein fraction rich in phospholipids and cholesterol esters which even in high concentration dissolved completely and transparently in water. He suggested that a lipid-protein combination might explain the manner of existence of lipid in clear serum, and his later work (2) has tended to implicate the protein, rather than the lipids, as the more fundamental determinant of lipid "solubility." However, Boyd (3) in 1937 observed a quantitative relationship between low serum phospholipid levels and lipemia. The present study demonstrates that sera with high total lipid concentration which contain predominant amounts of phospholipid are invariably clear and that in all sera tested the enzymatic removal of serum lecithin produces or increases lipemia. While the present data do not deny the importance of the lecithin-cholesterol-protein complex of Macheboeuf, they support the thesis that the concentration of serum phospholipids may be the limiting factor in determining the clarity of serum.

Materials and Methods

Patients whose sera provided the basis for this work were under study at the Hospital of The Rockefeller Institute for Medical Research. Clear high lipid sera were obtained from 16 patients with chronic partial intrahepatic biliary obstruction and cirrhosis, 13 of whom developed xanthomatous skin lesions (4) (hereafter referred to as "primary biliary cirrhosis"). Lipemic sera were for the most part from patients with the nephrotic syndrome.

Blood was drawn at least 12 hours after the last meal, allowed to clot, and the serum separated. Chemically clean glassware was used throughout; sterile precautions were not taken. Sera were analyzed chemically and enzymatically immediately, or after variable periods of

storage at 4°C. when it was found that storage for as long as 1 year did not alter materially the analyses discussed herein.

Total serum lipids were measured by a recently modified manometric lipid carbon method (5). Lipid phosphorus was determined by a modification of the Fiske and SubbaRow method (6), and lipid P was converted to phospholipid by the factor of 25. Free and total cholesterol were measured by the Schoenheimer-Sperry method (7). More recently, all major lipid components have been determined by these methods on a single alcohol-ether extract of a tungstic acid precipitate of 2 cc. of serum with neutral fat calculated by subtracting the lipid carbon of cholesterol and phospholipid from the total lipid carbon, as described in reference 5.

The lecithinase used in these experiments was the alpha-toxin of *Cl. welchii*. MacFarlane and Knight (8) in 1941 identified *Cl. welchii* alpha-toxin as an enzyme splitting lecithin into phosphorylcholine and diglyceride with pH optimum of 7.0 to 7.6, activated by Ca^{++} and inhibited by Ca^{++} precipitants and *Cl. welchii* antitoxin. The enzyme kinetics were further characterized by Zamecnik, Brewster, and Lipmann (9) in 1947, who concluded that lecithin was the specific substrate for the enzyme. In 1948 MacFarlane (10) brought forward evidence for slow enzymatic degradation of sphingomyelin as well as lecithin, but cephalins are stated by Zamecnik and coworkers (9) and by MacFarlane (10) to be unaffected. The enzyme has not been crystallized nor obtained in a pure form.

The concentrate of the clostridial culture used in these studies contained 200 M.L.D. of alpha-toxin per cc. as determined by intravenous injection in 13 to 14 gm. Rockefeller Swiss mice (11). Hereafter, for convenience, various "M.L.D.'s of toxin" will be understood to refer to appropriate dilutions of this concentrate. Moreover, no lipase activity was found in this concentrate when measured by hydrolysis of Tween 20 (12), or by titration of free fatty acids in ether extracts of serum after incubation with alpha-toxin. There were 570 units of hyaluronidase activity per cc., as measured viscosimetrically (13). For experimental use the enzyme concentrate was diluted with a calcium-rich gelatin solution buffered at pH 7.2 with borate as suggested by Adams (14). Lipid P was determined in such mixtures after trichloracetic acid precipitation and extraction with Bloor's alcohol-ether mixture. Acid-soluble P was measured on the trichloracetic acid supernate.

RESULTS

1. Lipid Patterns in Clear and Lipemic Sera.—Fig. 1 presents four pairs of abnormal serum lipid patterns with various lipid concentrations as contrasted to the pattern and lipid content in normal serum. The major component in each clear serum is seen to be phospholipid, whereas in lipemic nephrotic sera neutral fat predominates. Cholesterol fractions did not differ significantly in sera 2 and 3 or in sera 4 and 5, and in the other pairs the total cholesterol was similar. As an illustration of the lack of correlation between total lipid content and its physical state in the serum, it is seen that clear serum 8 contains almost three times as much lipid as milky serum 3. A larger series of data is presented in Table I.

Clear serum with elevated total lipid content has been found only in obstructive jaundice, whether intrahepatic or extrahepatic in origin (4, 15). The correlation between clarity and phospholipid predominance is seen in Fig. 2, where typical patterns of high lipid sera in a variety of abnormal conditions are presented. Only the high phospholipid serum of obstructive jaundice is clear; all other high lipid sera are milky.

In 25 normal sera, in which total lipids ranged from 440 to 766 mg. per cent, the ratio of phospholipid to total lipid (PL/TL) was found to have a maximum range of 0.31 to 0.40. In Fig. 3 the PL/TL of 19 nephrotic and 24 liver disease sera are plotted against their respective total lipid concentrations. All lipemic sera (solid symbols), regardless of total lipid content, fell below the normal range of PL/TL, whereas 21 of 24 clear sera (open symbols) were in the normal or high PL/TL range. The difference between the mean ratios of these two groups was statistically significant. Other serum lipid ratios are compared in Table II.

Comment.—On the basis of a large number of lipid patterns determined in a variety of diseases associated with elevated total lipids, the high phospholipid proportion in clear sera contrasted strikingly with the low phospholipid

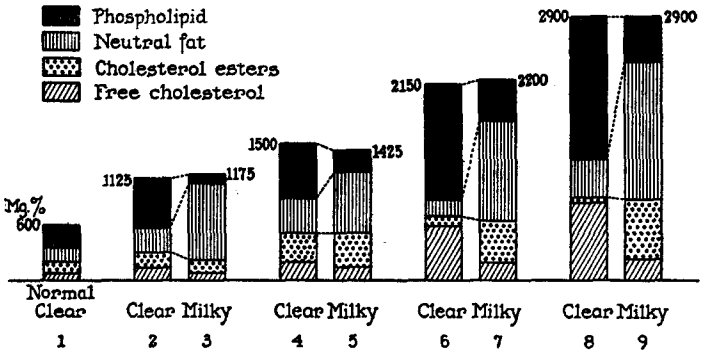

FIG. 1. Serum lipid patterns in normal and in selected pairs of sera with equally elevated total lipid concentration. Clear sera from patients with primary biliary cirrhosis; milky sera from nephrotic patients.

proportion in lipemic sera. Pertinent to this point is the report of Boyd and Connell (17) of a serum with total lipid of only 447 mg. per cent occurring in a patient with severe hepatitis in which the phospholipid content was only 52 mg. per cent; the serum was grossly lipemic.

2. Unsuccessful in Vitro Attempts at Clarification of Lipemic Serum.—If the PL/TL ratio is of importance in maintaining the clarity of serum, the addition of clear high phospholipid serum to lipemic low phospholipid serum might result in clarification.

In four experiments aliquots of clear high lipid serum and clear normal lipid serum were mixed under sterile conditions with aliquots of lipemic nephrotic serum in various proportions from 1:1 to 10:1, and held for 24 to 48 hours at 25 and 37°C. There was no change in turbidity of these mixtures as measured in a Coleman, Jr., spectrophotometer at 650 mμ.

Comment.—In regard to these unsuccessful attempts to clarify lipemic serum, it is of interest that a number of laboratories have recently produced triglyc-

TABLE I

Serum Lipid Patterns of 36 Lipemic and Clear Sera Paired According to Total Lipid Content

Serum No.	Lipemic or clear	Total lipid	Total phospholipid	Neutral fat	Total cholesterol	Free cholesterol	Free/total* cholesterol ratio	Phospholipid* / Total lipid ratio	Phospholipid* / Total cholesterol ratio
		mg. per cent	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*			
Normal mean ±S.D.‡	Clear	636 ±171	219 ±55	238 ±71	185 ±48	56 ±15	0.30 ±0.09	0.31–0.40	1.0–1.3
1	Lipemic	1128	312	298	518	136	0.26	0.28	0.60
2	Clear	1165	394	416	385	150	0.39	0.34	1.02
3	Lipemic	1144	240	521	383	111	0.29	0.21	0.63
4	Clear	1138	325	529	312	100	0.32	0.29	1.04
5	Lipemic	1230	342	458	430	121	0.28	0.28	0.56
6	Clear	1236	635	164	437	304	0.70	0.51	1.45
7	Lipemic	1235	294	541	400	119	0.30	0.24	0.74
8	Clear	1259	356	624	279	132	0.47	0.28	1.28
9	Lipemic	1377	216	636	525	131	0.25	0.16	0.41
10	Clear	1366	431	500	435	141	0.33	0.32	0.99
11	Lipemic	1497	354	552	591	164	0.28	0.24	0.60
12	Clear	1514	688	424	402	284	0.71	0.45	1.71
13	Lipemic	1618	351	670	591	186	0.31	0.22	0.59
14	Clear	1632	744	429	459	343	0.75	0.46	1.62
15	Lipemic	1618	270	751	591	133	0.23	0.17	0.46
16	Clear	1601	547	571	483	313	0.65	0.34	1.13
17	Lipemic	1695	414	431	850	289	0.34	0.24	0.49
18	Clear	1686	769	346	571	311	0.54	0.46	1.34
19	Lipemic	1700	480	595	625	171	0.27	0.28	0.77
20	Clear	1738	863	250	625	493	0.79	0.50	1.38
21	Lipemic	1729	462	542	725	201	0.28	0.27	0.64
22	Clear	1752	869	283	600	478	0.80	0.50	1.45

* Ratio on weight basis.

‡ Ahrens, Eder, and Van Slyke (5).

TABLE I—*Concluded*

Serum No.	Lipemic or clear	Total lipid	Total phospholipid	Neutral fat	Total cholesterol	Free cholesterol	Free/total* cholesterol ratio	$\frac{\text{Phospholipid*}}{\text{Total lipid ratio}}$	$\frac{\text{Phospholipid*}}{\text{Total cholesterol ratio}}$
		mg. per cent	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*			
23	Lipemic	1752	336	808	608	168	0.28	0.19	0.55
24	Clear	1743	688	539	516	357	0.69	0.40	1.33
25	Lipemic	1997	330	1100	567	211	0.37	0.17	0.58
26	Clear	2034	1288	100	646	442	0.69	0.63	2.00
27	Lipemic	2198	444	1101	653	189	0.29	0.20	0.68
28	Clear	2232	1350	132	650	524	0.81	0.61	2.08
29	Lipemic	2270	345	1125	800	254	0.32	0.15	0.43
30	Clear	2248	1088	527	625	493	0.79	0.48	1.74
31	Lipemic	2332	480	1142	710	212	0.30	0.21	0.68
32	Clear	2384	1230	294	860	685	0.80	0.52	1.43
33	Lipemic	2712	430	1683	616	196	0.32	0.16	0.70
34	Clear	2680	1331	599	750	604	0.85	0.50	1.77
35	Lipemic	2910	501	518	891	243	0.27	0.17	0.56
36	Clear	2969	1400	731	838	679	0.81	0.47	1.67

eride emulsions for intravenous alimentation of patients using a variety of agents as stabilizers, such as human serum albumin (18), gelatin (18), soy bean phosphatides (19), and surface-active agents like Span 20 (20) and monoglycerides (21). Although creamy emulsions with particle size not exceeding 1.5 μ have been nade, a completely transparent emulsion of hydrophobic lipids such as is seen in the serum of obstructive jaundice has never been duplicated.

3. In Vitro Production of Lipemia in Clear Serum.—If the PL/TL ratio is of importance in maintaining the clarity of serum, the removal of phospholipids from clear serum might be expected to result in lipemia. This has been accomplished successfully by enzymatic degradation of serum lecithin by *Cl. welchii* alpha-toxin. This enzymatic cleavage was of particular suitability to the present investigation since the strongly polar and hydrophilic phosphorylcholine group which might act as a dispersing agent for the hydrophobic lipids was split from the lecithin molecule. Although there is evidence that sphingomyelin as well as lecithin is hydrolyzed by this enzyme (10), the rate of hydrolysis of these two choline-containing phospholipids in serum has not been

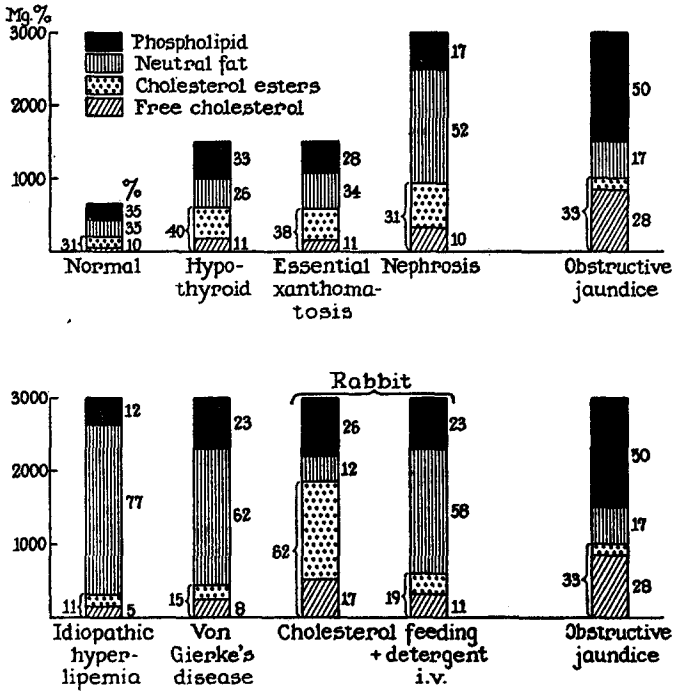

FIG. 2. Representative serum lipid patterns in a variety of conditions associated with elevated serum lipids. All sera are lipemic except that found in obstructive jaundice (acute or chronic intra- or extrahepatic obstruction). Hypothyroid pattern from data of Gildea, Man, and Peters (16).

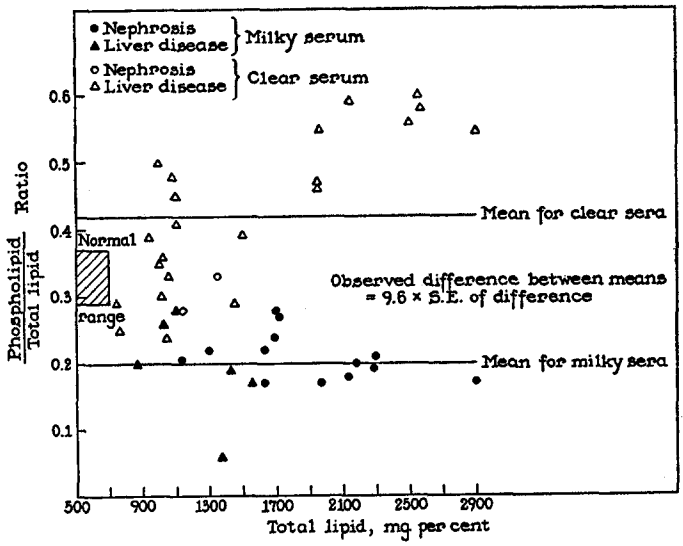

FIG. 3. Correlation between total lipid concentration, lipemia or clarity, and phospholipid/total lipid ratio in primary biliary cirrhosis and nephrosis. The range of normal is found in the hatched area at the left.

compared and is not the subject of this report. Hereafter, for convenience, "lecithin" will signify those phospholipids which are hydrolyzed by *Cl. welchii* alpha-toxin.

TABLE II

Relation of Serum Lipid Components to Serum Milkiness

Ratio	Normal range	Milky sera*	Clear sera‡
Phospholipid: total lipid	0.29–0.37	0.20 ± 0.04	0.42 ± 0.10
" : neutral fat	1.1 –2.0	0.41 ± 0.21	3.44 ± 3.39
" : total cholesterol	1.0 –1.3	0.82	1.25
Cholesterol esters: total lipid	0.17–0.24	0.22	0.17
Free cholesterol: total lipid	0.10–0.16	0.10	0.19

All ratios expressed on basis of weight.
* Mean of 19 sera ± S.D.
‡ Mean of 25 sera ± S.D.

TABLE III

*Effect of Cl. welchii Alpha-Toxin on Soy Bean Lecithin and Soy Bean Cephalin**

Substrate		Enzyme content	Final volume of buffered mixture	Flocculation after 3 hrs. at 37°C.
Concentration	Amount			
mg. per cent	*cc.*	*M.L.D.*	*cc.*	
A. Lecithin				
250	0.1	8	2.6	++
500	"	"	"	+++
1000	"	"	"	++++
2000	"	"	"	++++
B. Cephalin				
250	"	"	"	0
500	"	"	"	0
1000	"	"	"	0
2000	"	"	"	0

* Fractionated from crude soy bean phosphatides (asolectin) according to Levene and Rolf (22).

It was found that, when 0.1 cc. of serum was incubated at 37°C. with 8 M.L.D. of toxin in 2.4 cc. of buffer, the clear mixture became lipemic within 15 minutes with linear increase in optical density which reached a nearly maximal value within 2 hours in all types of sera, with very small increments thereafter for many hours. Since the initial total phospholipid concentration of the sera tested varied from 150 to 2,000 mg. per cent, it is seen that, under the conditions stated, 8 M.L.D. of toxin was exposed to substrate concentrations of only 6 to 80 mg. per cent. (Zamecnik and coworkers (9) have shown that 9 M.L.D. of enzyme is saturated with

substrate at a 2.6 gm. per cent lecithin concentration.) Increases in turbidity were noted in all sera tested regardless of total lipid concentration and whether initially lipemic or clear.

That these changes in turbidity were in fact due to lecithinase action rather than to some unrecognized component in the toxin concentrate is substantiated by the chemical studies detailed below which relate phospholipid breakdown to turbidity change; by the acceleration of the phenomenon by Ca^{++} and Mn^{++} and retardation by Cu^{++} and Fe^{+++}, in line with the kinetic studies of MacFarlane and Knight (8) and Zamecnik, *et al.* (9); and by the production of flocculation in aqueous solutions of soy bean lecithin but not of soy bean cephalin, as detailed in Table III. In regard to the possible action of the hyaluronidase contained in the concentrate, it was found that marked changes in turbidity were not accompanied by changes in viscosity, as measured at 37.0°C. in Ostwald viscosometers, using 3 cc. of high lipid serum and 8 M.L.D. of enzyme in 1 cc. of buffer.

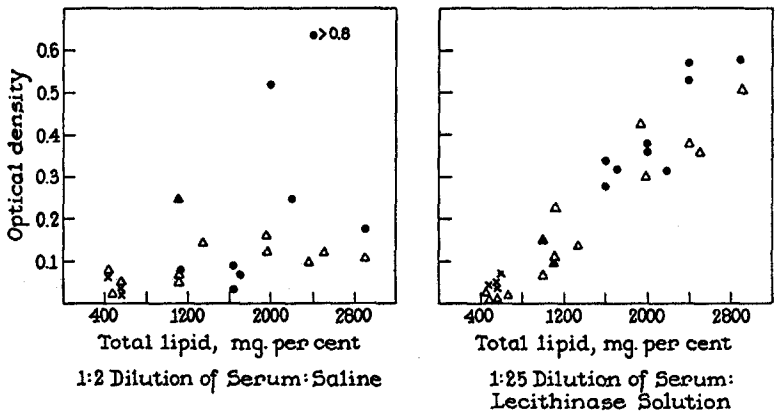

FIG. 4. (Left). Lack of correlation between total lipid content and the optical densities of sera diluted 1:2 with saline. Readings at 650 mμ in small cuvettes, water blank. Symbols as in Fig. 3. x = normal sera.

(Right). Correlation between total lipid content and optical densities of 1:25 dilutions of sera incubated with *Cl. welchii* lecithinase for 2 hours at 37°C. Readings at 650 mμ in small cuvettes, blanks of 1:25 dilution of same sera without added enzyme.

Utilizing the testing procedure outlined above, a variety of clear and lipemic sera were incubated with lecithinase. Fig. 4 (right) shows that at 2 hours there was a linear relationship between total lipid concentration and optical density in 28 sera, although the optical densities of these sera prior to enzymatic hydrolysis gave no indication of total lipid content (Fig. 4, left). Also, it will be noted from the serum dilutions used in the two groups of tests that there was more than a tenfold increase in optical density of the enzymatically treated sera as compared to the untreated aliquots. Darkfield microscopic examination during the course of this enzymatic hydrolysis showed the gradual appearance of discrete particles with Brownian movement. At the end of the reaction, and even after standing for 24 hours, the particles remained discrete and no larger than 1 μ in diameter and continued to show Brownian movement.

They were indistinguishable from the chylomicrons of alimentary or nephrotic lipemia.

In attempts to demonstrate possible differences between clear and lipemic sera with regard to their reaction to lecithinase, decreasing amounts of enzyme were added to several aliquots of serum while holding the volume relationships constant. Fig. 5 demonstrates that with decreasing enzyme concentration the onset of turbidity change is progressively delayed, although if sufficient incubation time is allowed, the final optical densities are almost the same. It is of interest that similar families of curves were obtained under these con-

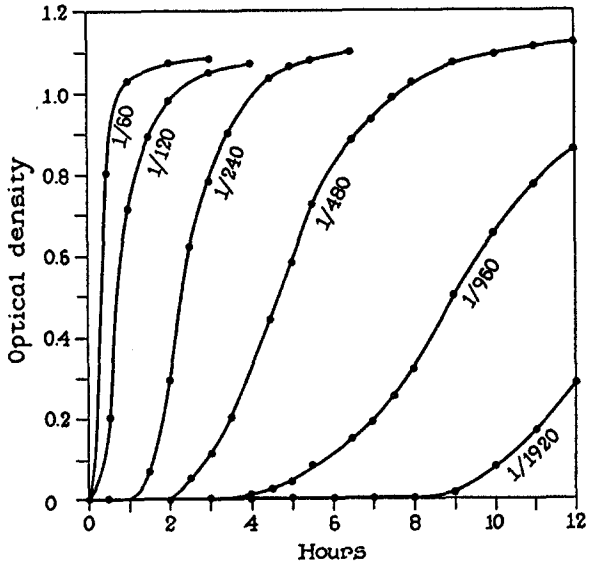

FIG. 5. Serum aliquots incubated with increasing dilutions of concentrated *Cl. welchii* lecithinase preparations at 37°C. (1 cc. serum + 1 cc. buffered enzyme).

ditions regardless of initial serum lipid composition, both in the lipemic sera of nephrotics and in all clear sera, but not in a extremely lipemic sera from a patient with idiopathic familial hyperlipemia (see below).

Comment.—Loss of the hydrophilic group of "lecithin" appears to destroy the stabilizing effect of this phospholipid upon the hydrophobic lipids. Destruction of "lecithin" initiates lipemia in clear sera with PL/TL of 0.29 or more and increases lipemia in turbid sera with PL/TL less than 0.29. "Lecithin" is, therefore, partially effective as a stabilizer even in lipemic sera with relatively small phospholipid fractions.

4. Correlation between Phospholipid Breakdown and Turbidity.—In Fig. 5 it is noted that the onset of change in optical density can be greatly delayed if the enzyme concentration is sufficiently low. The conversion of lipid P to

acid-soluble P during this lag period was determined in order to correlate phospholipid breakdown with optical density changes. Over a wide range of total lipid concentration the quantity of enzyme was determined which would allow an approximately 2 hour lag period before a change in optical density of the serum-enzyme mixture was detectable.

In these experiments several 0.5 cc. aliquots of serum were diluted with 10.0 cc. of buffered enzyme solution in large cuvettes, in order that at intervals the optical densities could be measured spectrophotometrically against a similarly diluted aliquot without enzyme. It was

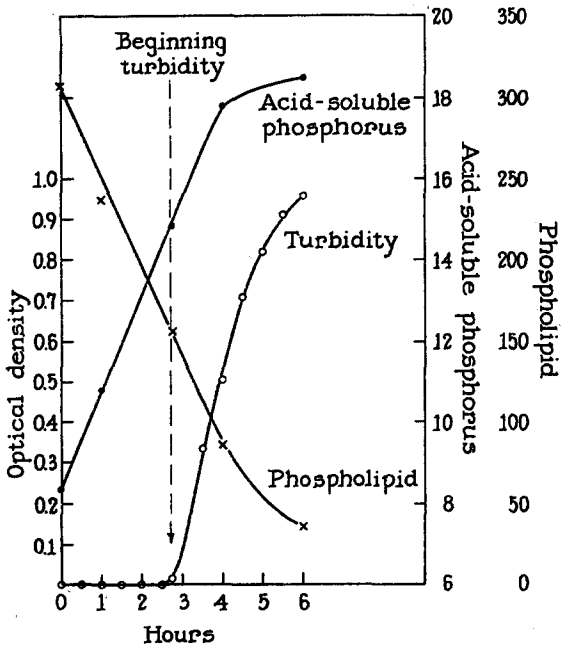

FIG. 6. Conversion of lipid P to acid-soluble P by *Cl. welchii* lecithinase at a linear rate prior to onset of turbidity in a clear serum with 1040 mg. per cent total lipid and 300 mg. per cent phospholipid (1.2 M.L.D. enzyme per 0.5 cc. serum).

found necessary for these purposes to use from 5 M.L.D. of enzyme per 0.5 cc. of normal sera to 0.6 M.L.D. per 0.5 cc. of sera containing up to 2500 mg. per cent total lipid, a paradoxical relationship explained by the extreme dilution of substrates. At certain intervals relative to turbidity change the reaction was stopped with trichloracetic acid, and measurements made of acid-soluble and lipid P.

Fig. 6 illustrates a representative experiment in which a 2½ hour lag period was produced prior to the onset of turbidity, during which time there was a linear decrease in lipid P and a corresponding increase in acid-soluble P. Similar curves of phospholipid destruction prior to onset or increase in turbidity were demonstrated in this manner in the lipemic sera of 3 nephrotic patients as well as in the clear high lipid sera of 18 patients with biliary cirrhosis.

Similar testing of intensely lipemic sera from a patient with idiopathic familial hyperlipemia (whose lipid pattern is shown in Fig. 2) showed that turbidity change began at zero time even when extremely low enzyme concentrations were used. Thus, no lag period could be produced when 0.5 cc. aliquots of this serum were incubated with 10 cc. of buffered toxin containing from 40 M.L.D. to 0.5 M.L.D. of *Cl. welchii* lecithinase. Fig. 7 shows that phospholipid breakdown resembled that in Fig. 6 but that increases in optical density and phospholipid hydrolysis proceeded hand in hand.

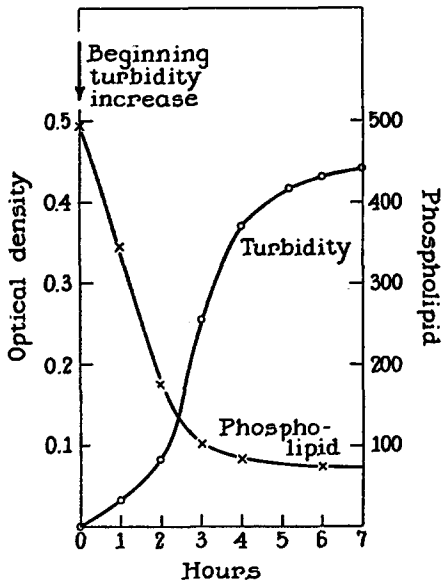

FIG. 7. Hydrolysis of phospholipid by *Cl. welchii* lecithinase with immediate increase in turbidity in serum of idiopathic hyperlipemia with 3000 mg. per cent total lipid and 360 mg. per cent phospholipid (1 M.L.D. per 0.5 cc. serum).

Comment.—Under these conditions significant enzymatic destruction of "lecithin" was demonstrated prior to change in optical density both in clear and in lipemic nephrotic sera. This suggests that, although part of the lipids in nephrotic serum occurs in particles of large enough size to be visible, a significant portion is present as particles of invisible size. As phospholipid is gradually destroyed, these invisible particles gradually coalesce until they reach visible size, with a resulting lipemia proportional to total lipid content. However, in the serum of a patient with idiopathic hyperlipemia, enzymatic removal of "lecithin" resulted in an immediately perceptible increase in optical density, suggesting that the *in vivo* lipid particle size was nearly maximal and that "lecithin" stabilization was relatively unimportant.

DISCUSSION

Boyd (3) in 1937 in a statistical study of 36 non-fasting normal sera found that lipemic sera contained a higher proportion of neutral fats than did clear sera. He suggested that lipemia might be due to a relative insufficiency of phospholipids. The present data on the clarity or milkiness of fasting sera in a variety of disease states lend the weight of experimental evidence to this hypothesis.

Early reports suggested that visible lipid droplets in serum are coated with protein (23, 24). Elkes, Frazer, Schulman, and Stewart (25) in 1945 reported the adsorption of various serum protein fractions at an artificial oil-water interface with flocculation of these coated particles at pH determined by the isoelectric point of the protein used. With 2.5 mg. or more of protein per square meter of interfacial area there was maximum emulsion stability with lipid droplets remaining discrete and non-coalescent. From their data it can be calculated that a lipemic serum with total lipid content of 5000 mg. per cent (about 10 times normal) would require approximately 150 mg. per cent of protein at the oil-water interface, if all lipid was present in droplets of 0.5 μ diameter. Under these conditions, the amount of protein available in serum for forming such a layer would not appear to be the limiting factor in formation of stable lipid emulsions. Indeed, even in nephrosis with severe hypoproteinemia, with elevated serum lipids and severe lipemia, lipid particles remain discrete and do not coalesce, indicating that there is sufficient protein present to form a layer at the interface and therewith a stable lipid emulsion.

Macheboeuf's extensive studies (2) on lipoproteins have resulted in the separation at acid pH of lipid-protein complexes which go into completely transparent water solution at neutral pH. These complexes, which Macheboeuf has termed "*cênapses*," contain only cholesterol esters and phospholipids, without triglycerides or free cholesterol, and are 50 to 60 per cent protein. His studies raise a question which cannot be satisfactorily answered at present; namely, whether clarity or lipemia of serum may depend not only on the quantity of phospholipid present but also on the character and amount of the protein to which lipids appear to be firmly attached. However, in the present authors' laboratory a procedure has been developed (26) by which lipids can be completely separated from the serum without altering the electrophoretic characteristics of the remaining proteins. Analyses of the lipid pellicle centrifuged to the surface have shown approximately 30 per cent tightly bound protein in normal and nephrotic lipemic sera and only about 15 per cent protein in clear high lipid sera of biliary cirrhosis. Under these conditions the amount of protein tightly bound to lipid was apparently unrelated to clarity or milkiness of serum.

Nagler (27) in 1939 noted that *Cl. welchii* toxin produced an opalescence in

some sera, and used this reaction for the titration of antitoxin. In 1941 MacFarlane and Knight (8) demonstrated that the Nagler reaction was produced by enzymatic hydrolysis of lecithin and that alpha-toxin was in fact lecithinase. The kinetics of the enzyme reaction were further defined for human serum (28) and a preliminary report of the degradation of human serum lipoprotein by a related lecithinase has also appeared (29). In a brief note Elkes and Frazer (30) in 1943, observing the Nagler effect of *Cl. welchii* on serum, suggested that phospholipids may stabilize fat droplets in serum. The present studies demonstrate that, under suitable conditions and in all sera tested, lipid particles coalesce and reach visible size after hydrolysis of "lecithin" by *Cl. welchii* lecithinase, that the resultant turbidity is proportional to total lipid content, and that there is a definite relationship between phospholipid breakdown and production of turbidity. These results seem entirely in keeping with the hypothesis suggested by MacFarlane and Knight in 1941 (8), that fat is liberated from combination with lipoprotein and that "creaming" is due to the decomposition of "lecithin" which acts as a stabilizing agent in lipid-protein emulsions.

The surface-active properties of lecithin have been known for many years. In 1923 Seifriz (31) demonstrated that lecithin stabilizes oil-in-water emulsions. From studies of monomolecular films of lecithin Leathes in 1925 (32) concluded that the hydrophilic phosphorylcholine group reduced the cohesive force which the two fatty acid radicals exerted upon each other, causing each radical to occupy twice its usual area. On the basis of these and the present data, it may be suggested as a working hypothesis that lecithin finds itself at the oil-water interface of the serum lipid droplet where it forms an undetermined type of bond with the protein film, enmeshing cholesterols and triglycerides in the expanded hydrophobic fatty acid portion of its molecule by Van der Waals forces.[1] It may follow that the greater the concentration of lecithin, the larger the total oil-water surface area which it can stabilize; hence, with a given concentration of total serum lipid, the greater the lecithin concentration, the smaller the lipid particle which may exist.

The possible additional effect of bile salts in producing clarity in the high lipid sera encountered in biliary obstruction cannot be assessed at present, since reliable methods for the accurate measurement of bile salts are not yet available. Although the present evidence appears to implicate phospholipids as the key factor in serum lipid emulsification, the possibility that bile salts may play a part cannot be entirely overlooked.

Finally, the implications of differences in serum lipid pattern in the pathogenesis of tissue lipid deposition, especially atherosclerosis, deserve comment.

[1] That this is an oversimplification of a complex colloidal phenomenon can be seen by consulting three recent reviews by surface chemists (33–35).

A strikingly high incidence of premature atherosclerosis has been noted in patients with nephrosis, hypothyroidism, and essential xanthomatosis, where the phospholipid/cholesterol ratio in serum is less than 1 (Fig. 2). On the other hand, atherosclerosis has not been reported in the few autopsied cases of von Gierke's disease or in idiopathic hyperlipemia, where neutral fat levels are greatly elevated but the phospholipid/cholesterol ratio is more than 1. Indeed, the presence of normal aortas in patients who for years have marked lipemia fails to substantiate Moreton's hypothesis (36) that serum lipemia *per se* may initiate atheromatosis. The possible rôle of an intracellular lipemia due to intracellular lecithinase (37) in the genesis of atheromatosis has not been investigated.

In 16 patients with primary biliary cirrhosis and xanthomatous infiltration of the skin (4), clinical, electrocardiographic, and x-ray evidence of atheromatous disease has been lacking. In four of these patients who have been autopsied the degree of atherosclerosis was not greater than that to be expected at their ages, although skin xanthomatosis was severe. In all patients of this group the phospholipid/cholesterol ratio was more than 1 (15). In experimental studies Kellner, Correll, and Ladd (38, 39) have been able to decrease the incidence of atherosclerosis in rabbits by injecting surface-active agents (Tween 80 and Triton A20) intravenously, a procedure which markedly increased the phospholipid/cholesterol ratio of serum (Fig. 2). Thus, on the basis of observations in a variety of disease states and in experimental studies, a relationship appears to exist between the fixation of lipid in intimal cells and decreased phospholipid/cholesterol ratios.

CONCLUSIONS

Clarity of high lipid sera is closely correlated with elevated proportions of serum phospholipids, and lipemia (milkiness) with low proportions of phospholipids. Clear high lipid sera occur uniquely in obstructive jaundice, both intra- and extrahepatic in origin.

Destruction of the polar nature of serum "lecithin" by enzymatic hydrolysis, using *Cl. welchii* lecithinase, results in a degree of lipemia which is linearly proportional to total lipid content in clear or lipemic high lipid or normal lipid sera.

Even in grossly lipemic sera, a significant proportion of the serum lipids is masked in particles of invisible size. Enzymatic removal of the stabilizing properties of serum "lecithin" unmasks this hitherto invisible fraction.

The concentration of serum phospholipids available for complex formation with serum proteins appears to be an important factor in determining particle size of serum lipids and hence of their occurrence in serum as masked or as visible particles.

The implications of these findings for studies of the genesis of atheromatosis are discussed.

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BIBLIOGRAPHY

1. Macheboeuf, M., Recherches sur les Lipides, les Stérols et les Protéides du Sérum et du Plasma Sanguins, Paris, Laval, 1928.
2. Macheboeuf, M., Les Cénapses Lipidoprotéidiques, in Exposés Annuels de Biochimie Médicale, Paris, Masson, 1945, **5**, 71.
3. Boyd, E. M., *Tr. Roy. Soc. Canad.*, 1937, **31**, 11.
4. Ahrens, E. H., Jr., Payne, M. A., Eisenmenger, W. J., and Kunkel, H. G., data to be published.
5. Ahrens, E. H., Jr., Eder, H. A., and Van Slyke, D. D., data to be published.
6. Fiske, C. H., and SubbaRow, Y., *J. Biol. Chem.*, 1925, **66**, 375.
7. Shoenheimer, R., and Sperry, W. M., *J. Biol. Chem.*, 1934, **106**, 745.
8. MacFarlane, M. G., and Knight, B. C. J. G., *Biochem. J.*, 1941, **35**, 884.
9. Zamecnik, P. C., Brewster, L. E., and Lipmann, F., *J. Exp. Med.*, 1947, **85**, 381.
10. MacFarlane, M. G., *Biochem. J.*, 1948, **42**, 587.
11. Adams, M. H., and Hendee, E. D., *J. Immunol.*, 1945, **51**, 249.
12. Archibald, R. M., *J. Biol. Chem.*, 1946, **165**, 443.
13. McLean, D., and Hale, C. W., *Biochem. J.*, 1941, **35**, 159.
14. Adams, M. H., *J. Immunol.*, 1947, **56**, 323.
15. Ahrens, E. H., Jr., and Kunkel, H. G., *J. Clin. Inv.*, in press.
16. Gildea, E. F., Man, E. B., and Peters, J. P., *J. Clin. Inv.*, 1939, **18**, 739.
17. Boyd, E. M., and Connell, W. F., *Arch. Int. Med.*, 1938, **61**, 755.
18. Shafiroff, B. G. P., and Frank, C., *Science*, 1947, **106**, 474.
19. McKibbin, J. M., Pope, A., Thayer, S., Ferry, R. M., Jr., and Stare, F. J., *J. Lab. and Clin. Med.*, 1945, **30**, 488.
20. Meng, H. C., and Freeman, S., *J. Lab. and Clin. Med.*, 1948, **33**, 689.
21. Lerner, S. R., Chaikoff, I. L., and Entenman, C., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 388.
22. Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, 1927, **72**, 587.
23. Ludlum, S. DeW., Taft, A. E., and Nugent, R. L., *Colloid. Symp. Ann.*, 1929, **7**, 233.
24. Elkes, J. J., Frazer, A. C., and Stewart, H. C., *J. Physiol.*, 1939, **95**, 68.
25. Elkes, J. J., Frazer, A. C., Schulman, J. A., and Stewart, H. C., *Proc. Roy. Soc. London, Series A*, 1945, **184**, 102.
26. Kunkel, H. G., and Ahrens, E. H., Jr., data to be published.
27. Nagler, F. P. O., *Brit. J. Exp. Path.*, 1939, **20**, 473.

28. Crook, E. M., *Brit. J. Exp. Path.*, 1942, **23,** 37.
29. Petermann, M. L., *J. Biol. Chem.*, 1946, **162,** 37.
30. Elkes, J. J., and Frazer, A. C., *J. Physiol.*, 1943, **102,** 24P.
31. Seifriz, W., *Am. J. Physiol.*, 1923, **66,** 124.
32. Leathes, J. B., *Lancet*, 1925, **1,** 803, 853, 957, 1019.
33. Cockbain, E. G., *Research*, 1947, **1,** 115.
34. Dervichian, D. G., *Tr. Faraday Soc.*, 1946, **42B,** 180.
35. Schulman, J. H., and McRoberts, T. S., *Tr. Faraday Soc.*, 1946, **42B,** 165.
36. Moreton, J. R., *Science*, 1948, **107,** 371.
37. King, E. J., *Biochem. J.*, 1931, **25,** 799.
38. Kellner, A., Correll, J. W., and Ladd, A. T., *Fed. Proc.*, 1949, **8,** 359.
39. Ladd, A. T., Kellner, A., and Correll, J. W., *Fed. Proc.*, 1949, **8,** 360.