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## HETEROGENEITY OF FAT PARTICLES IN PLASMA DURING ALIMENTARY LIPEMIA \*

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Particles of fat large enough to scatter light and cause turbidity appear in plasma after a fatty meal. Gage and Fish described the appearance of these particles ("chylomicrons") in the dark-field microscope and appreciated that they could be heterogeneous (1). They observed small particles circulating during early and late stages of fat absorption, and a predominance of larger particles during peak lipemia. Further attempts, however, to classify these particles by using dark-field microscopy have yielded inconsistent results (2, 3). Centrifugation of lipemic plasma also has not proved useful for sharply fractionating fat particles. There appears to be an array of fat particles of different sizes and flotation rates that can be spun up by varying the force and duration of centrifugation in a saline ( $d < 1.006$ ) or plasma medium (4). Arbitrary separations can be made based on the choice of centrifugal conditions (5, 6).

Properties other than size and density characterize these fat particles. In lymph and plasma, they are negatively charged and move toward the anode on free electrophoresis (7). With this technique, Swahn found that in a variety of lipemic samples, turbidity associated with larger fat particles migrated with  $\alpha_2$  globulin while small-particle turbidity migrated with beta globulin (8). Carlson and Olhagen, using starch column electrophoresis, observed two turbid peaks in plasma from a patient with essential hyperlipemia (9). In the present study, zone electrophoresis on starch granules has been used to separate two distinct groups of fat particles appearing in plasma during alimentary lipemia. This separation also

can be accomplished by differential flocculation in polyvinylpyrrolidone (PVP) density gradient columns (10).

### METHODS

Normal male medical students, aged 21 to 25, were used as test subjects. After an overnight fast, they were fed a liquid formula containing 250 g of either butter fat or corn oil, blended with 200 ml skim milk. The formula was ingested within 15 minutes, and the subjects received no other food during the remainder of the test. Samples of venous blood were collected in Vacutainer tubes containing EDTA<sup>1</sup> before, and approximately 2, 6, and 10 hours after the fat meal. Samples of buttock adipose tissue were aspirated by the method of Hirsch and colleagues (11).

C<sup>14</sup>-labeled human lymph was obtained through an indwelling thoracic duct cannula after the feeding of palmitic acid-1-C<sup>14</sup> in corn oil to donor subjects with neoplastic disease.<sup>2</sup> The lymph was allowed to clot in sterile collection bottles and was then stored at 4° C for several days. Lymph samples were warmed to room temperature before use.

Zone electrophoresis on starch granule blocks was performed according to the method of Kunkel and Trautman (12) by using barbital buffer at pH 8.6 and ionic strength 0.1  $\mu$ . A constant voltage of 6 volts per cm<sup>2</sup> was applied for 18 to 24 hours. Environmental temperature was kept at 18 to 20° C (block temperature was approximately 25° C under these conditions), since it was noted that cold increased reversible agglutination of fat particles (13) and hence resulted in the retention of some turbid particles at the origin. Aging of plasma, regardless of temperature, had a similar effect; hence electrophoresis was performed on the day of collection of plasma samples whenever possible.

Filter paper imprints were made of the fractionated plasma on the starch block, dried in air, and stained for protein with bromphenol blue and for lipid with Fat Red 7 B or Oil Red 0. These imprints were used to rapidly trace migration distances of plasma protein and lipid fractions. Starch segments  $\frac{1}{2}$ -inch wide were cut, placed in test tubes, and eluted with 3 ml 0.9 per cent NaCl con-

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<sup>1</sup> Disodium ethylenediamine tetraacetate, Becton, Dickinson and Co.

<sup>2</sup> Courtesy of Dr. Allen E. Dumont, Department of Surgery, New York University College of Medicine.

taining 0.1 per cent EDTA<sup>3</sup> by stirring 1 to 2 minutes in a Vortex mixer.<sup>4</sup> The starch was allowed to settle, and samples of each fraction were removed for protein determination (14) and nephelometry. Before determination of turbidity, the samples were centrifuged briefly at 2,000 G to separate the last traces of starch as sediment. Turbidity measurements were then made on the supernatant fluid at 20° C by using a locally made nephelometer designed to record wide-angle light scattering in the visible spectrum. Turbidity measurements were expressed in arbitrary units in a linear range of the instrument, as determined by analysis of serial dilutions of standard latex particles. Differential flocculation of plasma particulate fat in PVP density gradient columns was performed by a method described in detail elsewhere (10).

To determine particulate triglyceride fatty acid composition, particles in the turbid peaks were isolated, their triglycerides separated by thin-layer silicic acid chromatography, and methyl esters of the triglyceride fatty acids analyzed by gas-liquid chromatography. For this purpose, starch block fractions containing the turbid peaks were pooled, and 1 to 2 ml of the combined eluate was poured into 30 per cent sucrose as a layer under a 2- to 3-cm column of 0.9 per cent NaCl containing 0.1 per cent EDTA in 2 × 12 inch Lusteroid tubes. The tubes were centrifuged for  $2.7 \times 10^6$  G-min (90,000 G for 30 minutes) in a Spinco SW 39 swinging bucket rotor. Under these conditions, all the fat particles were packed into a narrow band at the surface of the saline layer and were removed by suction. The particulate fat was then extracted in chloroform-methanol (2:1 vol:vol)

and the extracts were washed once with one-fifth volume of 0.9 per cent NaCl containing 0.1 per cent EDTA. The chloroform layer was evaporated to dryness, and the lipid was then taken up in a small volume of petroleum ether (bp 30 to 60° C) and applied to thin-layer silicic acid plates (15). The plates were developed in a solvent system of petroleum ether-ethyl ether (90:10 vol:vol) at room temperature for 45 minutes. Under these conditions, a translucent triglyceride spot in the center of the plate appeared clearly separated from other lipid classes. The scrapings from this area were refluxed in methanol-HCl at 100 to 110° C for 2 hours (16). Gas-liquid chromatography of the fatty acid methyl esters was performed on ethylene glycol succinate polyester columns operating at 185° C with argon as the carrier gas and with a strontium ionization detector (17). Samples of buttock adipose tissue and dietary fat were extracted in chloroform-methanol and processed in a similar manner.

To determine the lipid composition of fat particles in the turbid peaks, appropriate starch block fractions were pooled, either from individual electrophoretic separations with large plasma samples of 10 to 15 ml, or from several separations with 2-ml samples, and were centrifuged twice in the manner above. Samples of chloroform-methanol extracts of isolated fat particles were analyzed for cholesterol by the ultramicro method of Searcy, Bergquist, and Jung (18), for phospholipid by a modification of the Bartlett procedure (19), and for total fatty acids by the single-extraction and titration procedure of Dole and Meinertz (20) after saponification with 0.5 N KOH in ethanol for 2 hours at 80° C. Additional fat particle extracts, obtained from three individual electrophoretic separations, were fractionated on silicic acid columns to isolate phospholipids (21). Phospholipid classes were resolved by thin-layer silicic acid chromatography with a solvent system of chloroform-

<sup>3</sup> Whenever possible, solutions contained 0.1 per cent EDTA as an inhibitor of trace-metal-catalyzed unsaturated fatty acid oxidation.

<sup>4</sup> Scientific Industries, Inc., Springfield, Mass.

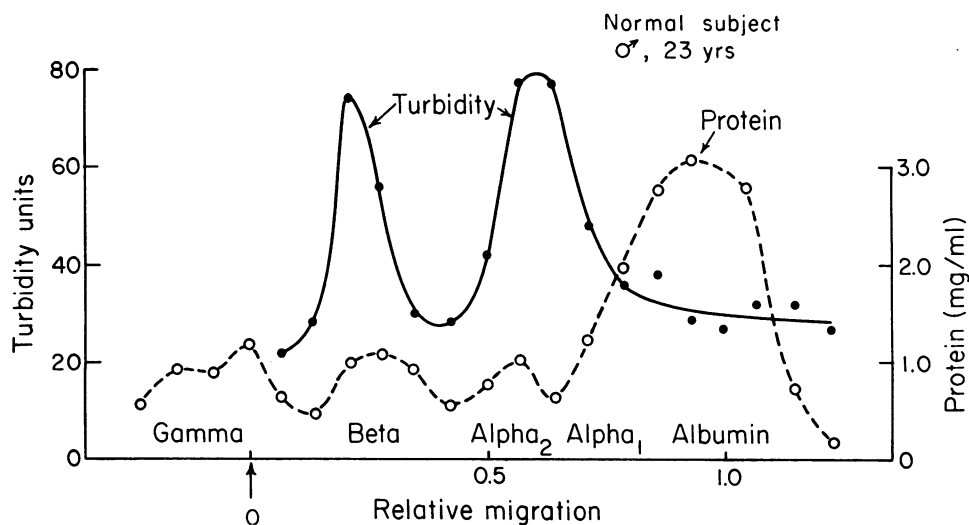


FIG. 1. PLASMA DURING ALIMENTARY LIPEMIA 10 HOURS AFTER 250 g BUTTER FAT P.O. (STARCH BLOCK ELECTROPHORESIS).

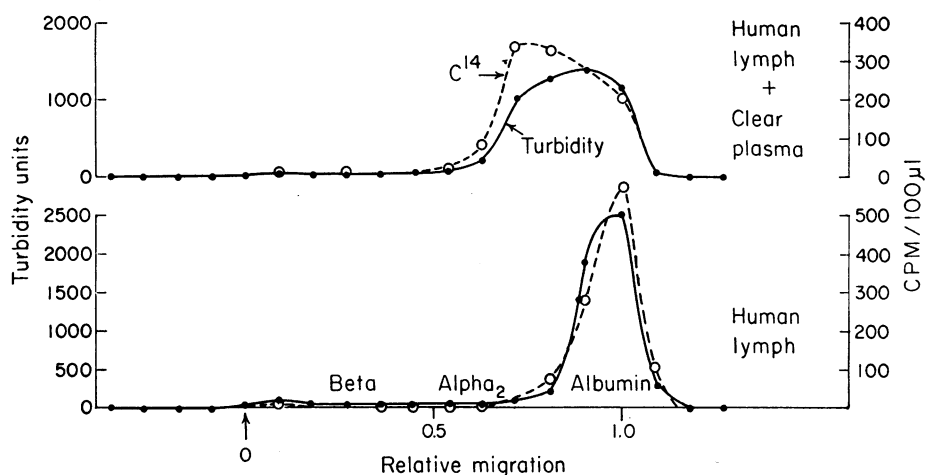


FIG. 2.  $C^{14}$ -LABELED HUMAN LYMPH (STARCH BLOCK ELECTROPHORESIS).

methanol- $H_2O$ - $NH_4OH$  (75:25:3:1 vol:vol). The fatty acid composition of the phospholipids was determined by the methods above.

#### RESULTS

Electrophoresis of lipemic plasma on granular starch separated the fat particles into two distinct turbid areas (Figure 1). One group, "primary" particles, migrated in the region of  $\alpha_2$

globulin (migration distance =  $0.60 \pm 0.10$ ; <sup>5</sup> albumin = 1.00), while the other, "secondary" group migrated in the region of beta globulin (migration distance =  $0.23 \pm 0.08$ ). Filtration of plasma through the starch block by continuous buffer flow failed to separate these two populations.

On the other hand, fat particles in thoracic duct lymph migrated as a single turbid zone, usually between  $\alpha_2$  globulin and albumin (Figure 2). Lymph particles incubated with clear fasting plasma *in vitro* at  $37^\circ C$  still migrated as a single turbid zone, but at a slightly slower rate, with  $\alpha_2$  globulin. Radioactivity and turbidity moved together when  $C^{14}$ -labeled, human, thoracic-duct lymph was subjected to electrophoresis (Figure 2).

During fat absorption in normal subjects, the concentration of these two groups of fat particles in plasma varied independently (Figure 3). Primary particle turbidity reached a maximum in 6 hours, and then fell. This turbidity predominated during the earlier stages of lipemia and reflected the turbidity variation of whole plasma. Secondary particle turbidity, however, gradually increased throughout alimentary lipemia, and was significantly elevated above the 6-hour level at 10 hours ( $p < 0.02$ ). During the later stages of fat absorption, only secondary particle turbidity was detected.

After the ingestion of corn oil, the composition of the triglyceride fatty acids (TGFA) in these

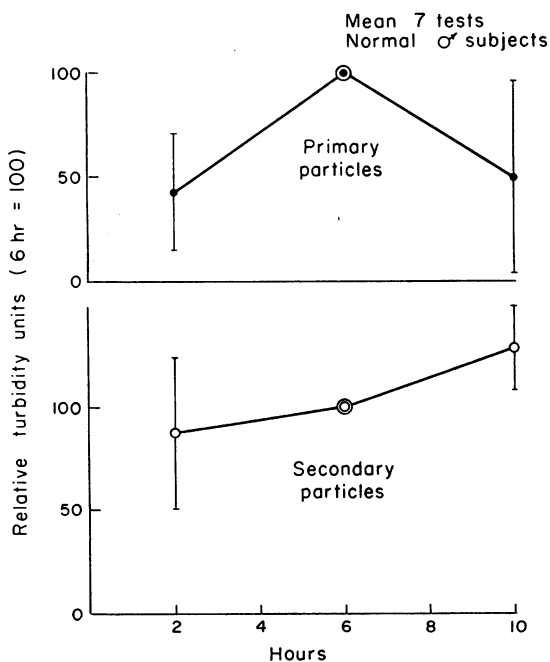


FIG. 3. PARTICULATE FAT TURBIDITY DURING ALIMENTARY LIPEMIA AFTER 250 g CORN OIL OR BUTTER FAT (STARCH BLOCK ELECTROPHORESIS).

<sup>5</sup> Mean  $\pm$  standard deviation for a series of 23 determinations.

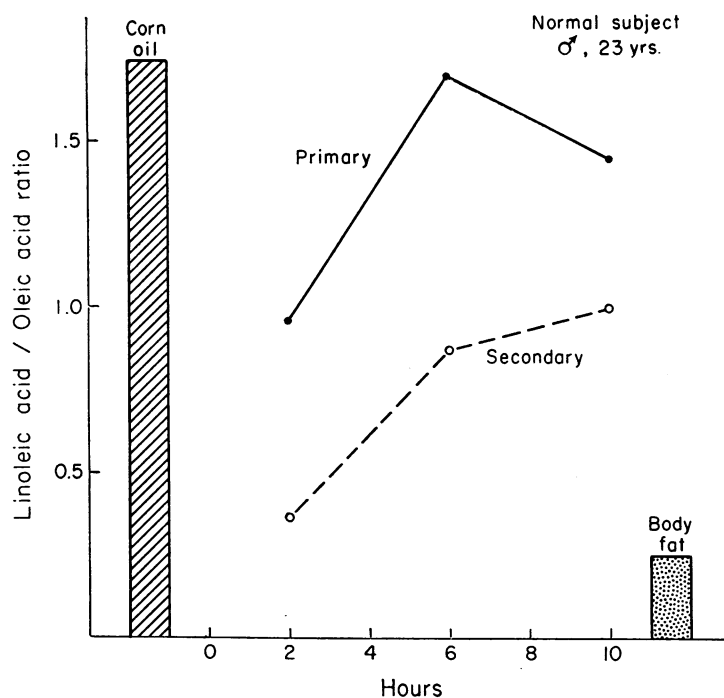


FIG. 4. FATTY ACID COMPOSITION OF PARTICULATE TRIGLYCERIDE DURING ALIMENTARY LIPEMIA AFTER 250 g CORN OIL (STARCH BLOCK ELECTROPHORESIS).

two types of particles differed (Figure 4, Table I). The linoleic acid:oleic acid ratio of the TGFA in primary particles was virtually identical with that of the fed fat during peak lipemia. On the other hand, the TGFA pattern of the secondary particles, initially like that of body fat, shifted toward the pattern of fed fat as absorption proceeded, but never became identical with it.

Fat particles were also separated into two distinct turbid bands by differential flocculation in PVP density gradient columns. One group of particles floated to the top of the gradient, and corresponded to the particles migrating in the

$\alpha_2$  globulin region (primary particles); those that remained at the bottom of the gradient corresponded to particles migrating in the beta globulin region (secondary particles). Variations in turbidity and triglyceride fatty acid patterns of the two types of particles in lipemic samples fractionated by both of these methods showed close agreement.

Primary particles appeared to be larger than secondary particles. Centrifugation for  $0.4 \times 10^6$  G-min (35,000 G for 10 minutes) was sufficient to pack them on top of a 2- to 3-cm column of saline ( $d < 1.006$ ) in a swinging bucket rotor, whereas

TABLE I  
*Linoleic acid:oleic acid ratio of particulate triglyceride fatty acids during alimentary lipemia after corn oil in four normal subjects*

Subject	Diet	Adipose tissue	Primary particles			Secondary particles		
			2 hour	6 hour	10 hour	2 hour	6 hour	10 hour
JH	1.74	0.21	1.10	1.44	1.31	0.55	0.52	1.34
MS	1.74	0.25	0.96	1.69	1.44	0.37	0.87	0.99
SP	1.74	0.21	0.49	1.69	1.58	0.34	1.02	1.07
BB	1.74	0.27	1.55	1.70	1.77	0.78	0.93	0.95
Mean	1.74	0.24	1.03	1.63	1.53	0.51	0.84	1.09
SD		0.03	0.44	0.13	0.20	0.20	0.22	0.18

TABLE II  
Fatty acid composition of particulate phospholipids during alimentary lipemia

Subject	Dietary fat	Time	Particles	Phospholipid fraction	Total fatty acids				
					16:0*	18:0	18:1	18:2	Other
		<i>hours</i>			%	%	%	%	%
HK	Corn oil	4-7	Primary	Total	12.4	1.5	31.5	54.8	
			Secondary	Total	40.0	22.1	18.1	12.4	7.4
					37.7	17.4	29.3	4.1	11.5
MS	Corn oil	6	Primary	Lecithin	12.4	1.5	31.5	54.8	
			Secondary	Lecithin	32.7	17.2	21.2	24.5	4.3
					25.9	15.2	41.1	17.8	
JH	Butter	6	Primary	Lecithin	27.5	14.5	31.3	2.0	24.7
			Secondary	Lecithin	32.1	18.7	39.7	9.5	
					17.6	17.4	45.6	15.5	3.9

\* Shorthand designation for fatty acids, identified by chain length and number of double bonds, as described by Dole and colleagues (23).

$3 \times 10^6$  G-min was required to pack secondary particles. From a nomogram (22) based on Stokes's law, the minimal diameter of the primary particles was calculated to be approximately 200  $m\mu$  ( $S_f = 1,000$ ) and of the secondary particles, 70  $m\mu$  ( $S_f = 400$ ). Both groups of particles adhered to the origin on filter paper electrophoresis. They both contained cholesterol and phospholipid in similar proportions. Primary particles, however, appeared to have a greater proportion of dietary fatty acids incorporated into their phospholipids than secondary particles during alimentary lipemia (Table II), as reflected by

differences in linoleic acid—18:2 (23)—composition.

These two types of particles and the nonturbid, very low density lipoproteins that can be spun up after prolonged centrifugation of particle-free plasma in saline ( $d < 1.006$ ) are compared in Table III.

#### DISCUSSION

Fractionation of fat particles in plasma by these methods shows that there are two distinct kinds of particles circulating during alimentary lipemia. The larger, primary particles contain predomi-

TABLE III  
Comparison of primary and secondary fat particles and very low density lipoproteins

	Primary particles	Secondary particles	Very low density lipoproteins (24)
Flotation			
Density	1.006	1.006	1.006
G—minutes	$0.4 \times 10^6$	$3 \times 10^6$	$144 \times 10^6$
$S_f$ (calculated)	1,000	400	20-400
Diameter, $m\mu$	200	70	20-70
Electrophoresis			
Free Paper	Alpha <sub>2</sub> -Alb	Beta	Origin to beta
Starch	Origin	Origin	Alpha <sub>2</sub>
Mobility (albumin = 1.00)	Alpha <sub>2</sub>	Beta	
	$0.60 \pm 0.10$	$0.23 \pm 0.08$	
Flocculation			
0 to 5% polyvinylpyrrolidone gradient	Top	Bottom	None
Composition, %*			
Cholesterol	$6 \pm 1$ [3]	$6 \pm 4$ [2]	19
Phospholipid	$10 \pm 3$ [4]	$7 \pm 2$ [2]	20

\* Mean percentage composition by weight  $\pm$  standard deviation for [n] samples.

nantly dietary fat, are the first to be formed, and presumably originate in the intestine and enter plasma through lymph. The smaller, secondary particles appear later, contain a mixture of dietary and body fat, and appear to be derived from sources other than thoracic duct lymph.

Results in the present study indicate that the fat particles in lymph and primary particles in plasma are identical. When lymph was added to clear plasma, lymph particles migrated in the  $\alpha_2$  globulin region, confirming Laurell's results with free electrophoresis (7). Primary particles in plasma showed a similar mobility. During maximal influx of dietary fat, the fatty acid composition of the primary particles resembled that of the fed fat, as in observations made on lymph (25-27) and on large particles isolated from lipemic plasma by centrifugation (6, 28).

It is of interest that significant differences between the dietary and primary particle fatty acid patterns occur during early and late stages of fat absorption. This suggests that dietary fat mixes with endogenous fatty acids during absorption, a phenomenon that may be completely masked by the dietary stream during peak lipemia. Fatty acid patterns in fat particles isolated from human, thoracic duct lymph during fat absorption show similar changes (29). In addition, after the intravenous injection of albumin-bound, labeled fatty acids, incorporation of  $C^{14}$  into lymph particle triglyceride has been observed in rat (30) and man (31).

The liver appears to be an important, but not the only, site of removal of primary particles from circulation. Studies in animals have shown that, after intravenous administration of labeled lymph, a large fraction of the radioactive triglyceride can be found in the liver (32-35). Extrahepatic clearance of lymph particles, however, also has been demonstrated (36, 37).

Presumably, secondary particles can be produced by the liver. Labeled fat particles released by livers isolated from fed rats during perfusion with radioactive triglyceride (38) may be of this type. Other sites of origin of secondary particles cannot be excluded at present; however, it appears unlikely that they are formed in plasma. Incubation of plasma primary particles or lymph with clear plasma *in vitro* fails to produce second-

dary particles. Post-heparin clearing activity results in decreased concentrations of both types of particle (39). In addition, patterns of both triglyceride fatty acids and phospholipid fatty acids are clearly different in the two groups of particles. During alimentary lipemia, they show independent variation of turbidity and fatty acid composition. Thus the evidence supports the hypothesis that the primary particle, after removal from circulation, undergoes partial degradation, and that its components mix with lipid derived from body fat. Resynthesis of particulate fat is followed by re-entry into the circulation as secondary particles. The fate of the secondary particle and its role in the transport of dietary fat to cells remains to be determined.

#### SUMMARY

Two distinct groups of fat particles appearing in plasma during alimentary lipemia have been clearly separated and characterized by zone electrophoresis and differential flocculation with polyvinylpyrrolidone. Both kinds of particle are low enough in density to float in saline ( $d < 1.006$ ).

One type ("primary") appears to originate in the intestine and contains predominantly dietary triglyceride during fat absorption. Another type of particle ("secondary") presumably originates in the liver and contains a mixture of triglycerides derived from both dietary and body fat.

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