

SEPARATION AND CHARACTERIZATION OF HUMAN SERUM CHYLOMICRONS*

BY ANGELO SCANU, M.D., AND IRVINE H. PAGE, M.D.

(From the Research Division of the Cleveland Clinic Foundation and the Frank E. Bunts Educational Institute, Cleveland)

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During the early phase of transport of exogenous fats, lymph and blood assume a milky appearance due to an increase of large fat particles, known as chylomicrons. Separation can be achieved by chemical means; protamine sulfate (1), toluidine blue (2), heparin and calcium chloride (3, 5), polyvinylpyrrolidone (4); however, ultracentrifugation (6) is the method commonly used. Chemical characterization of lymph and serum chylomicrons has not been satisfactorily achieved; this may be owing to: (a) variation of laboratory procedures, which do not yield a product of constant composition; (b) variation in source: human lymph (7-14), human serum (12), rat lymph (2, 15, 16), dog lymph (17, 18), and dog plasma (19); (c) limited number of reports on chylomicron composition and lack of comparative studies between chylomicrons derived from lymph and blood.

Another problem is the relationship between chylomicrons and serum lipoproteins. Robinson (15) found no protein in chylomicrons separated from rat chyle; other reports (2, 9, 11, 14, 16, 17) however, show small percentages present in human and rat chyle chylomicrons. The nature of this protein is unknown. Middleton (20) reported the presence in human serum chylomicrons, of a protein antigenically related to serum beta lipoproteins. Recently, Robdell (21) found that serine, threonine, and aspartic acid are the N-terminals of the chylomicron protein from human lymph serum.

Our study concerns: (a) Separation, purification, and chemical composition of chylomicrons from serum of people in absorptive phase; (b) separation and characterization of their protein moiety; (c) participation of the chylomicron protein in a clearing factor system "in vitro"; (d) comparison of the chylomicrons with ultracentrifugally separated creamy top fractions from pre-incubated mixtures of serum and coconut oil.

Materials and Methods

Fasted normal human subjects received a fat breakfast containing approximately 1.5 gm. of animal fat per kilo body weight. 500 ml. of blood was withdrawn 3 hours after the

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meal and serum separated from clotted blood by centrifugation. Milky serum was stored at 3°C. no longer than 24 hours before use. Occasionally, analyses were performed on hyperlipemic plasmas received from the blood bank. During the course of our experiments over 15 liters of milky serum or plasma were studied.

Separation of Chylomicrons.—*Step 1.* In 10 ml. plastic tubes, 5 ml. samples of serum or plasma were layered under 4 ml. 0.15 M solution of sodium chloride and centrifuged at 9,000 g for 10 minutes in a Spinco 30.2 rotor at 15°C. Top fractions were layered under 5 volumes of 0.055 M phosphate pH 7 and recentrifuged at 35,000 g for 10 minutes. This process was then repeated up to 4 times to remove very low density lipoproteins (*i.e.* —S 70 to 400). *Step 2.* Final top fractions from step 1, layered under phosphate buffer, were spun for additional 18 hours at 105,000 g in a Spinco 40 rotor. After separation of the top fractions, bottom fractions were collected, concentrated by pervaporation, and the turbidity related to residual lipides was removed by ultracentrifugation. *Step 3.* Top fractions from step 2, layered under phosphate buffer, were treated as in step 2. Top and bottom fractions were collected.

Aliquots of top fractions from steps 1 and 2, bottom fractions from steps 2 and 3, and final chylomicron preparations (top step 3) were dialyzed against 0.15 M solutions of sodium chloride, and then stored at 3°C. until used.

Preparation of Emulsions of Serum and Coconut Oil.—Aliquots of one liter of serum, from which chylomicrons had been removed by ultracentrifugation, were mixed with an equal volume of 1 per cent ediol,¹ a coconut oil emulsion, in phosphate buffer pH 7. The mixture was incubated for 2 hours at 37°C., then kept at 3°C. for no longer than 24 hours until used. Separation and purification of the fat particles from the creamy emulsions were performed by applying the same procedure as for chylomicrons.

Delipidation Procedures.—Extraction of chylomicrons by a cold mixture 3:1 of ethanol-ethyl ether (22) or by a freezing and thawing procedure (23) yielded a protein residue insoluble in water. Only partial delipidation procedures were, therefore, applied. The following was the one commonly used: A sample of chylomicron (2 to 3 ml.) was gently shaken with ethyl ether at 3°C. and the mixture placed in a continuous liquid-liquid extractor to which a water jacket was applied around the extraction chamber so as to insure a continuous flow of tap water, cooled to a temperature of 3°C. Ether extraction was conducted continuously for 12 hours until the chylomicron solution did not show visible turbidity. At the end of this process the ether phase was aspirated and traces of ether in the water phase removed by a stream of nitrogen.

In some instances, in which prolonged delipidation was not required, as for immunological studies, chylomicrons, after being shaken with cold ether, were spun at 30,000 R.P.M. in the International refrigerated centrifuge. After separation of the ether phase a fairly clear sample was obtained.

Studies on the delipidized protein residue had to be performed immediately after lipide removal, because of the tendency of the protein to precipitate. Lyophilization was attempted; this procedure, however, reduced the solubility of the chylomicron protein.

Chemical Composition.—Protein concentration was determined by the Lowry method (24). When lipemic samples were analyzed, however, turbidity had to be removed by shaking the solution with ether after the blue color had fully developed. Accuracy of this method was checked by Pregl's modification of the Kjeldahl method (25).

Total lipides were determined gravimetrically by Sperry's method (26). Cholesterol was determined by the method of Abell *et al.* (27). Phospholipides were calculated by multiplying lipide phosphorus values, obtained by the method of Fiske and Subbarow (28) by 25. Triglyceride values were obtained by the method of Van Handel and Zilversmit (29). Hexosamine content was estimated by the method of Neuhaus and Letzring (30).

¹ Ediol, Schenley Laboratories, Inc. New York.

Electrophoresis.—Moving boundary electrophoresis was determined by the Longworth modification of the Tiselius method employing barbital buffer at pH 8.6, ionic strength 0.1μ .

Paper electrophoresis was performed using a Durrum (31) cell with a barbital buffer of pH 8.6, ionic strength 0.05μ . Bromophenol was used for protein staining; Sudan black for lipide staining.

Agar electrophoresis was performed according to the technique previously described (22).

Sedimentation diagrams were obtained in Spinco analytical ultracentrifuge at 52,640 r.p.m. at a temperature of $+20^{\circ}\text{C}$.

Immunological Studies.—Specific antisera were obtained by immunizing rabbits against one of the following antigenic solutions: human crystalline serum albumin, human serum alpha₁ lipoprotein or fasting normal human serum. Immunizing solutions were precipitated with alum hydroxide before injection. Each rabbit received an intraperitoneal injection at the beginning of the week and one intravenous injection on each of the following 3 days. The procedure, increasing the amount of antigen each week, was repeated 4 weeks for a total amount of 100 mg. of antigen injected. Rabbits were bled on the 7th day after the final injection.

Commercial equine anti-normal human serum lot 581, obtained from Pasteur Institute, was also used.

Specific absorbed antisera were obtained by adding increasing amounts of albumin or alpha₁ or beta lipoprotein (ether extracted) to anti-normal human serum. Specific antisera against beta lipoprotein were prepared according to Burstein's method (32).

Immunochemical analyses were performed by the previously described techniques of agar immunoelectrophoresis and agar double diffusion (21).

Assays for "Clearing Factor."—A partially purified preparation of enzyme was obtained from post-heparin human plasma according to the method of Nikkilä (33). To avoid presence of alpha lipoproteins in the precipitate, the procedure was carried out at pH 6.5. Assays of clearing activity were determined by fall in optical density production of non-esterified fatty acids (NEFA) according to techniques previously described (34).

An assay of clearing activity in agar plates was also investigated. 30 gm. of agar (Bacto Difco) was dissolved in 400 ml. of hot distilled water. After cooling, the solidified agar, cut in small pieces, was washed for several days with distilled water. The agar was then added to hot phosphate buffer pH 7 to obtain a 1.5 per cent solution. 20 ml. aliquots were poured in a Petri dish and mixed with 2.5 ml. of 1 per cent solution of bovine serum albumin, both solutions made in phosphate buffer pH 7. This mixture yielded a uniformly opaque substrate, in which round wells, 7 mm. in diameter, were cut to contain the enzyme preparation. The plates were incubated at 37°C ., usually for 6 hours; enzymatic activity was indicated by the appearance of clear rings around the wells.

"Finger Printing" Technique.—A modification of Ingram's technique was used (35). Heat-denatured protein samples (1 mg.) were digested by $25 \mu\text{g}$. of trypsin for 2 hours in ammonium carbonate buffer at pH 8.2. Electrophoretic and chromatographic separation of the partial hydrolysate was performed in a large sheet (31 x 29 cm.) of Whatman paper 3 MM. Electrophoretic separation was obtained in 1 hour in a Durrum cell with an acetate buffer (pH 5.5, μ 0.025), a potential gradient of 11 V/cm., and a current of 50 milliamperes. Chromatographic separation was achieved by a solvent system 3:1:1 of *n*-butanol-acetic acid-water. Spots were revealed by ninhydrin spray.

RESULTS

Studies on Chylomicrons.—Top fractions of *Step 1*, extracted with ether, gave a protein solution of a concentration ranging from 1 to 1.5 per cent.

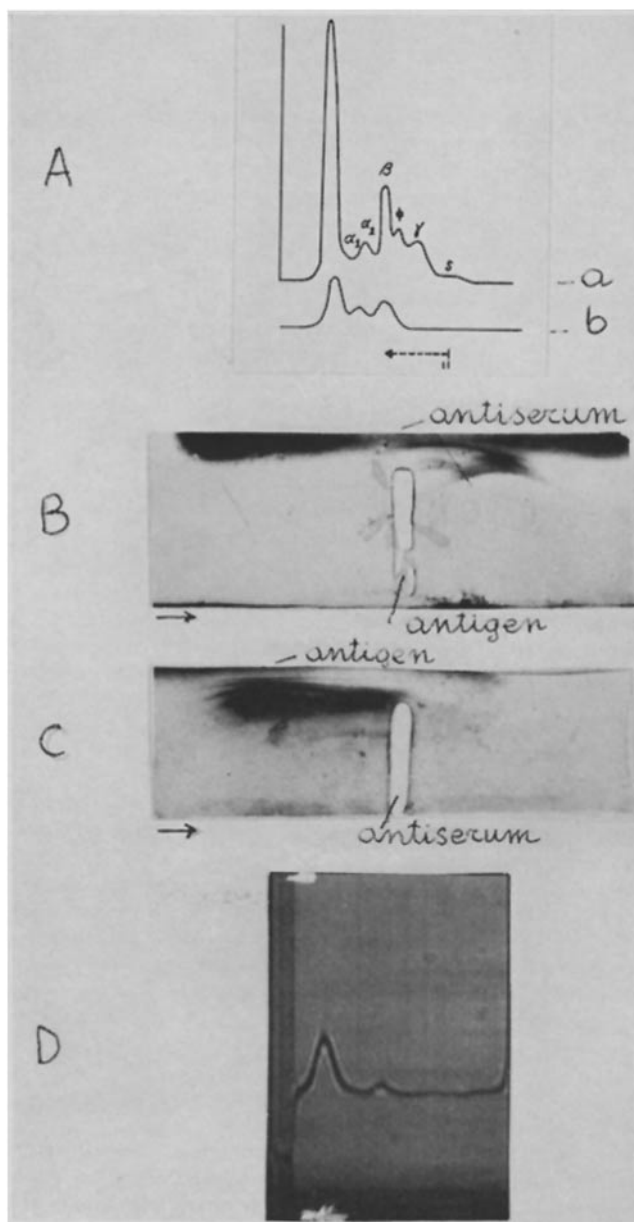


FIG. 1. Moving boundary electrophoresis, immunoelectrophoresis and diagram of sedimentation of a preparation of top fraction step 1, partially delipidized by ethyl ether. A: *Tiselius pattern*. (a) normal serum. (b) Top fraction step 1, delipidized. B: *Agar immunoelectrophoretic pattern*. Antigen, top fraction step 1, delipidized, 70 μg . N. Antiserum, equine anti-normal human serum, 700 μg . N. The antigen was separated electrophoretically; the antiserum was then added. Time of diffusion, 8 days. Stain, azocarmine. C: *Agar immunoelectrophoretic pattern*. Antigen and antiserum, same as B. The antiserum was separated electrophoretically; the antigen was then added. Time of diffusion and stain, same as B. D: *Diagram of sedimentation*. 0.2 per cent partially delipidized top fraction, step 1 in 0.9 per cent sodium chloride at $+20^{\circ}\text{C}$.; 52,460 R.P.M.; time, 32 minutes.

Because of the instability of this solution on standing, analysis had to be performed in the first 3 to 4 hours after partial removal of the lipides. The results are shown in Fig. 1. Tiselius pattern (Fig. 1 A *b*) shows the presence of three main components with the mobility of albumin, alpha and beta globulins. A greater heterogeneity is, however, shown by the two immunoelectrophoretic patterns (Figs. 1 B and 1 C) which indicate at least 5 different antigenic determinants. Similar results were obtained with the double diffusion technique of Ouchterlony. The sedimentation diagram (Fig. 1 D) corroborated the findings.

The results of the analysis of the bottom fractions of *Steps 2 and 3* are summarized in Fig. 2. Several serum components (albumin, α_1 , α_2 , and beta globulin) were detected by agar electrophoresis (Fig. 2 A) in the bottom fractions of step 2, which showed a protein concentration of approximately 2 per cent. Immunoelectrophoresis (Fig. 2 B and 2 C) gave several lines of precipitation.

The maximum protein concentration observed in bottom fractions of step 3 was between 0.06 and 0.08 per cent. Agar electrophoresis (Fig. 2 D) showed only two boundaries stainable with amido Schwartz or Sudan black. With these two serum components, identified as α_1 and beta lipoproteins, immunoelectrophoresis (Figs. 2 E and 2 F) and the agar double diffusion technique (Fig. 2 G) revealed a third line of precipitation, albumin.

The results of the analysis of the chylomicron fractions after the final washing (top fraction of step 3) are shown in Fig. 3. After delipidation with ether, a maximum protein concentration of 0.08 per cent was obtained. This protein was more unstable than the chylomicron protein from step 1. Because of the low protein level a Tiselius pattern could not be obtained. A sedimentation diagram (Fig. 3 D) revealed a small, flat peak, almost identical in position to the peak of a delipidized α_1 lipoprotein sample (22). Agar electrophoresis (Fig. 3 A) showed two faint boundaries stainable with amido Schwartz: densitometry evaluation gave an approximate ratio of the slow to the fast moving component of 1:3. Immunoelectrophoresis (Figs. 3 B and 3 C) gave two lines of precipitation, one for each boundary. Position of the boundaries and shape of the lines of precipitation were identical with those exhibited by samples of serum beta and α_1 lipoprotein, extracted with ether. The data obtained by the double diffusion technique corroborated these findings (Fig. 3 E). Furthermore, absorption experiments showed disappearance of one or the other line of precipitation when equine anti-human serum was absorbed with either α_1 or beta serum lipoprotein.

Attempts were made to obtain a chylomicron preparation completely free of protein. Further washings, however, did not remove appreciable amounts of protein from the purified chylomicrons. At this stage a clumping phenomenon may also occur.

Bottom fraction—step 2

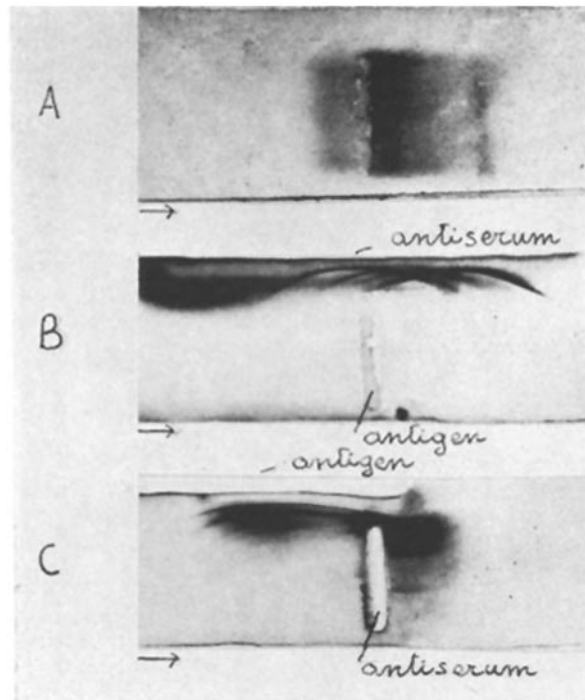


FIG. 2. Agar electrophoresis and immunochemical analyses of undelipidized bottom fractions of steps 2 and 3. A: *Agar electrophoretic pattern*. Bottom fraction, step 2, 70 μg . N. Stain, amido Schwartz. B: *Agar immunoelectrophoretic pattern*. Antigen, bottom fraction step 2, 70 μg . N. Antiserum, equine anti-normal human serum, 700 μg . N. The antigen was separated electrophoretically; the antiserum was then added. Time of diffusion, 8 days. Stain, azocarmine. C: *Agar immunoelectrophoretic pattern*. Antigen and antiserum, same as B. The antiserum was separated electrophoretically; the antigen was then added. Time of diffusion and stain, same as B. D: *Agar electrophoretic pattern*. Bottom fraction, step 3, 70 μg . N., E: *Agar immunoelectrophoretic pattern*. Bottom fraction, step 3. Same as B. F: *Agar immunoelectrophoretic pattern*. Bottom fraction, step 3. Same as C. G: *Agar double diffusion technique*. Central well, equine anti-normal human serum. (1) α_1 lipoprotein preparation containing trace amounts of albumin. (2) and (4) undelipidized bottom fraction, step 3. (3) Mixture of α_1 and β lipoprotein containing trace amounts of albumin.

Bottom fraction—step 3

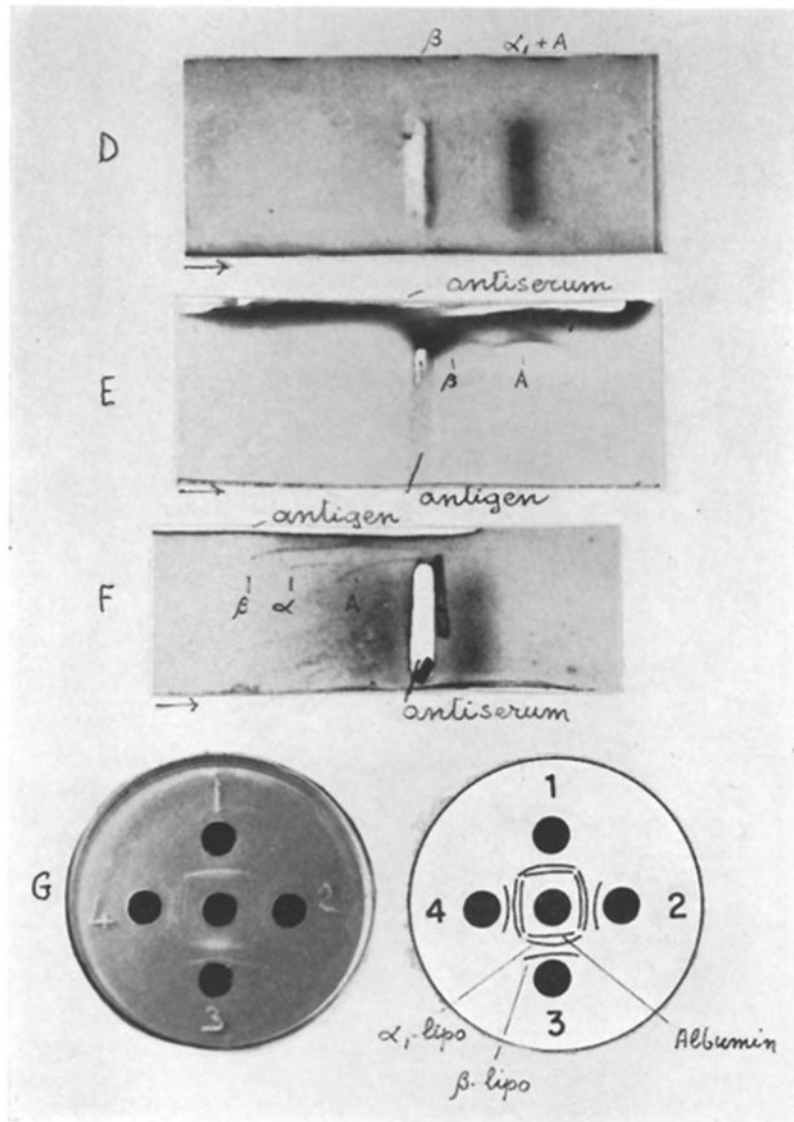


FIG. 2—concluded

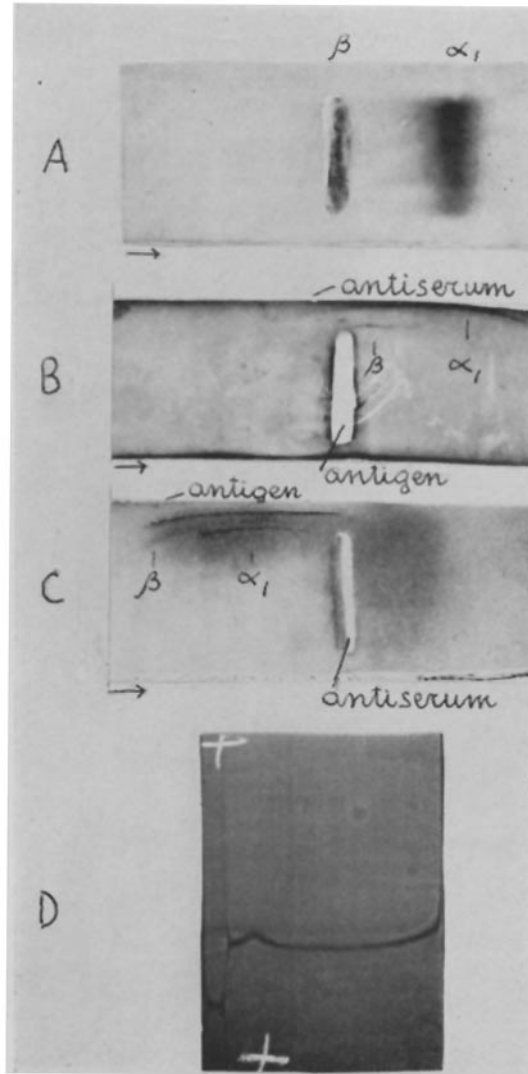


FIG. 3. Agar electrophoresis, immunochemical analyses and diagram of sedimentation of a final chylomicron preparation (top fraction, step 3), partially delipidized by ethyl ether. A: *Agar electrophoresis*. Top fraction, step 3, partially delipidized, 70 μg . N. Stain, amido Schwartz. B: *Agar immunoelectrophoretic pattern*. Antigen, same as A, 70 μg . N. Antiserum, equine anti-normal human serum, 700 μg . N. The antigen was separated electrophoretically; the antiserum was then added. Time of diffusion, 8 days. Stain, azocarmine. B': Detail from

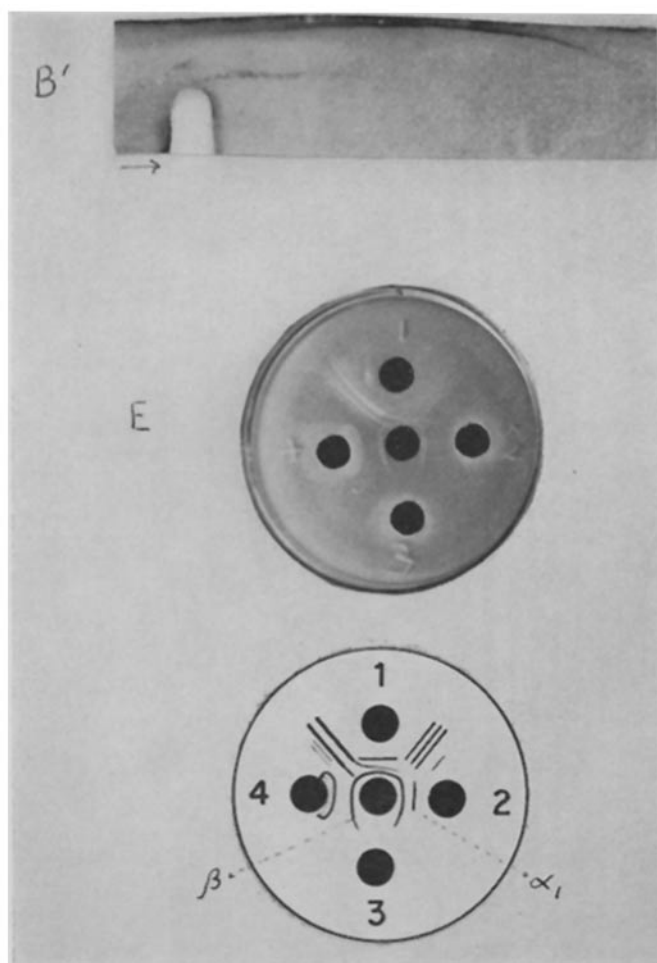


FIG. 3—concluded

B. C: *Agar immunoelectrophoretic pattern*. Antigen and antiserum, same as B. The antiserum was separated electrophoretically; the antigen was then added. Time of diffusion and stain, same as B. D: *Diagram of sedimentation*. 0.08 per cent partially delipidized top fraction, step 3, in 0.9 per cent sodium chloride at $+20^{\circ}\text{C}$.; 52,460 R.P.M.; time 32 minutes. E: *Agar double diffusion technique*. Central well, partially delipidized top fraction, step 3. (1) equine anti-normal human serum. (2) Mixture of anti-human α_1 and anti-human beta lipoprotein sera. (3) Anti-human albumin serum. (4) Anti-human beta lipoprotein serum. The lines of precipitation present between wells 1 and 4 and wells 1 and 2 are due to antigenic determinants in wells 2 and 4, which reacted with the antiserum in well 1.

Studies on Mixtures of Serum and Coconut Oil.—Analyses of top and bottom fractions resulting from the three different preparative steps, showed data very similar to the ones reported for chylomicrons. The final creamy top fractions (step 3) yielded, after delipidation, a protein solution of a maximum concentration of approximately 0.1 per cent. Agar immunoelectrophoresis and agar double diffusion technique revealed presence of beta and alpha lipoproteins in almost the same proportion as in chylomicrons.

Chemical Composition of Chylomicrons and Particles from Serum-Coconut Oil Emulsion.—The results, shown in Table I, indicate a higher percentage of cholesterol and phospholipide in the chylomicron fraction than in the serum-coconut oil emulsion. It must be borne in mind, however, that the factor 25,

TABLE I
Chemical Composition of Serum Chylomicrons and Fat Particles from a Mixture of Serum and Coconut Oil Emulsion

	Chylomicrons	Fat particles from serum-coconut oil emulsion
	<i>per cent*</i>	<i>per cent*</i>
Total cholesterol	4.2-4.6	1.8-2.1
Phospholipides †	8.9-9.6	2.3-2.8
Triglycerides	85.0-88.0	89.0-91.0
Total protein	1.9-2.2	1.5-1.8
Hexosamine	0.1	0.08

* Percentage is based on total lipide weight.

† Expressed as lecithin ($P \times 25$).

used for phospholipide values, is arbitrary. We are not sure that these phospholipides were lecithin.

Recovery experiments performed during the procedure of lipide extraction revealed that ether removed from the chylomicron fraction 100 per cent of the triglyceride, 80 per cent of the cholesterol, and 50 per cent of the phospholipides. When attempts were made to remove the lipides totally by alcohol-ether, the protein residue was insoluble in water.

One liter of plasma yielded 20 to 25 ml. of packed chylomicrons, containing 15 to 20 mg. of total lipides per milliliter. Therefore, the serum chylomicron concentration of our subjects in the absorptive phase was approximately 0.04 to 0.05 gm. per cent.

Experiments on "Clearing Factor".—The enzyme preparation, precipitated from post-heparin plasma, was inactive when tested against a coconut oil emulsion, used as a substrate. Activity was induced by pre-incubating the substrate with serum alpha₁ lipoprotein. Clearing activity was indicated by a 50 per cent fall in optical density and increased NEFA production (1.6 m.eq./l.).

Agar plate assay showed enzyme activity, by appearance of a clear ring in the opaque gel, when α_1 lipoprotein was present either in the substrate or in the well with the enzyme. Substitution of the α_1 lipoprotein with partially delipidized samples of either chylomicrons or serum-coconut oil mixture, produced a clear ring (see Fig. 4 *b*).

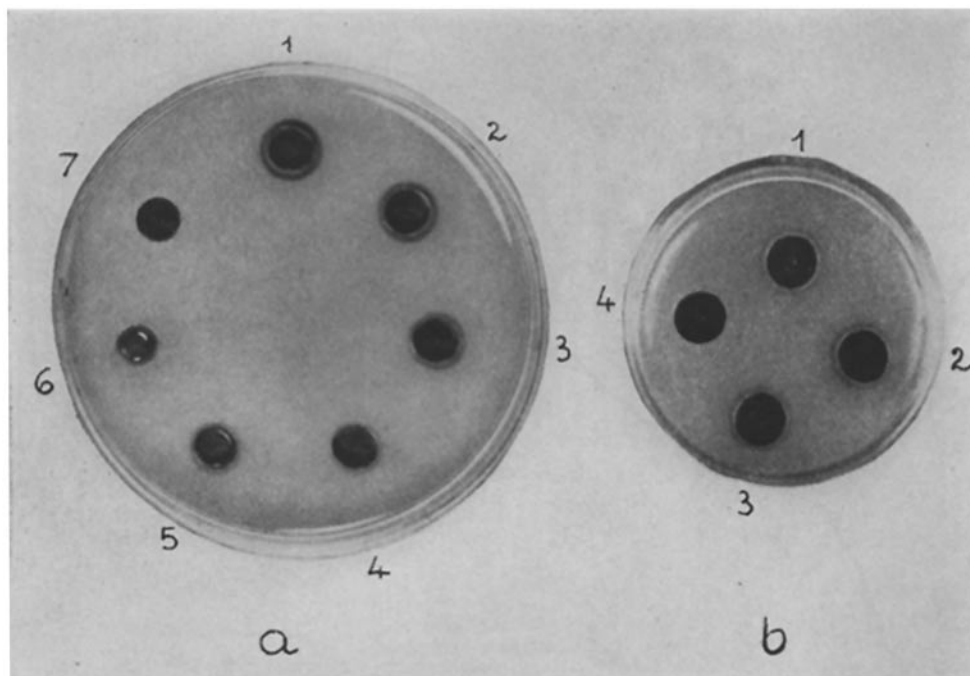


FIG. 4. Determination of "clearing factor" activity in agar plates. (*a*): Bovine albumin and a coconut oil emulsion were mixed in agar to give an opaque gel (see text) in which 7 round wells were cut. An enzyme preparation (post-heparin plasma) was added to wells 1 through 5 in decreasing concentrations. Well 6 contains normal human serum; well 7, saline. Time of incubation: 6 hours. (*b*): Substrate, same as (*a*). The 4 wells contain an enzyme preparation precipitated from post-heparin plasma (see text). In addition the following preparations were added: well 1, serum α_1 lipoprotein; well 2, partially delipidized chylomicrons; well 3, partially delipidized particles from a mixture of serum-coconut oil. Well 4 contains the enzyme preparation only.

Chylomicrons or coconut oil-serum mixture, both undelipidized, were mixed with agar. These substrates tested against the enzyme preparation from post-heparin plasma exhibited a clear ring.

Another series of experiments in agar proved that α_1 lipoprotein, still active when partially delipidized by ether, did not show activity when completely delipidized by alcohol-ether. Beta lipoproteins, when added to either

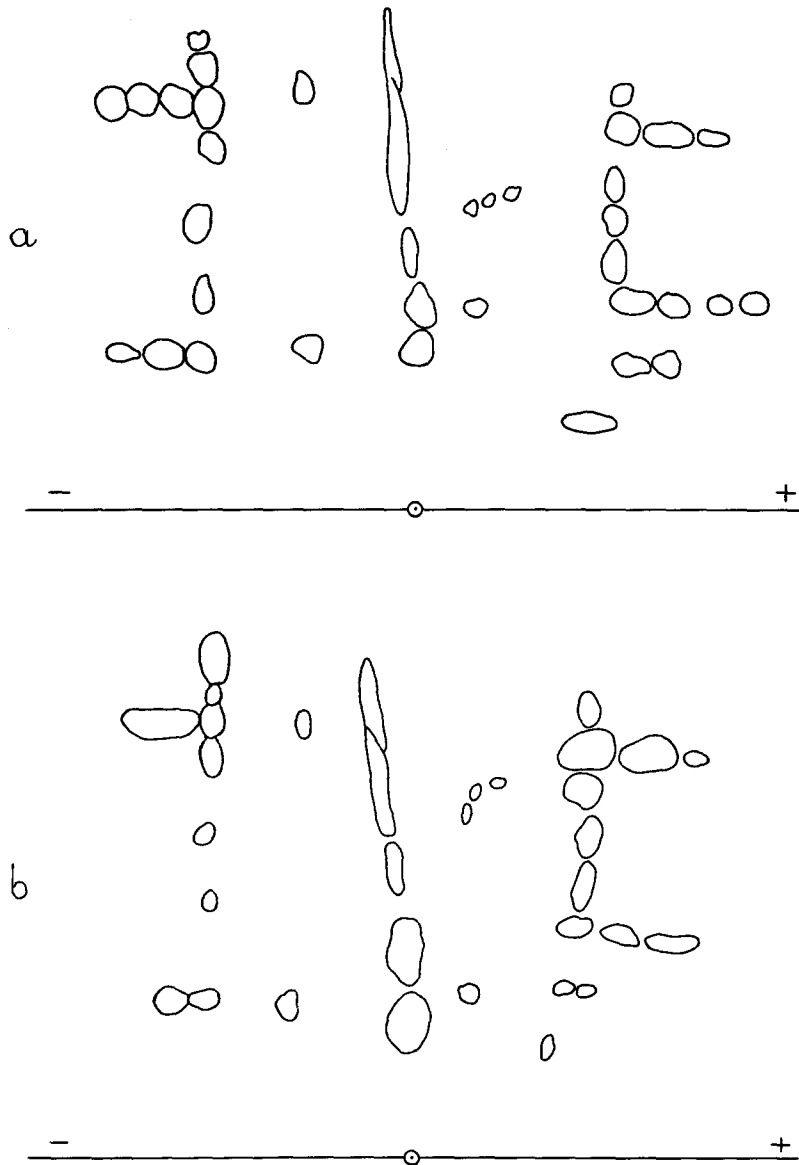


FIG. 5. "Finger prints" of (a) protein from serum chylomicrons, (b) protein from a pre-incubated mixture of serum-coconut oil. These proteins, heat-denatured, were partially digested by trypsin. The components of the hydrolysate were separated, on a large sheet of filter paper by electrophoresis (11 volts/cm., 50 milliamperes, 1 hour) and ascending chromatography (solvent system: *n*-butanol, acetic acid, water 3:1:1). Detection of the spots, ninhydrin spray.

the enzyme or the agar substrate did not induce activity. Activation was, however, induced by a mixture of alpha and beta lipoproteins.

Experiments with the "Finger Printing" Technique.—Our results are limited to a comparative analysis of the proteins obtained by partial delipidation of chylomicrons and of serum-coconut oil mixture. Similar, though not identical, patterns were obtained. A few extra spots were observed in the chylomicron protein pattern (see Fig. 5).

The yield of chylomicron protein was too small to permit separation and comparison of each of its two components with the patterns of either alpha or beta lipoprotein, obtained in preliminary experiments.

DISCUSSION

Our greatest limitation has been the low yield of protein from chylomicrons and from the final washed particles from the serum-coconut oil emulsion. Highly sensitive immunochemical methods were valuable in the characterization of these protein moieties. Other techniques, performed in parallel when sufficient material was available, corroborated these findings.

Analysis of the products of the different steps of chylomicron preparation showed that low speed ultracentrifugation for short periods does not remove many of the serum components. High speed ultracentrifugation for prolonged periods, removed all but a small amount of protein, which constitutes approximately 2 per cent of total lipide weight of the chylomicrons. This yield was fairly constant and did not change with further high speed ultracentrifugation.

Alpha₁ and beta lipoproteins were found to be present in the final washed chylomicron fraction since: (a) two boundaries with the migration rates of intact or partially delipidized alpha₁ and beta lipoproteins were detected by paper and agar electrophoresis; (b) two lines of precipitation similar to those exhibited by intact or delipidized serum alpha₁ and beta lipoproteins were shown by immunoelectrophoresis and agar double diffusion technique; (c) one or the other of the two lines of precipitation disappeared when antisera absorbed with either alpha₁ or beta lipoproteins were used; (d) one specific line of precipitation appeared with immune sera anti-alpha₁ or anti-beta lipoprotein.

Middleton found, on the basis of immunological studies, that S_f 3 to 8 beta lipoproteins are present in chylomicrons (20). We think, however, that his immunochemical methods and the ones used in our experiments cannot classify beta lipoproteins according to ultracentrifugal flotation since low density lipoproteins have a common antigenic determinant (23, 36). The finding that the protein bound to chylomicrons is quite unstable after delipidation, is also indicative of the presence of beta lipoproteins. This class of molecules undergoes rapid denaturation after removal of lipides (23).

Our results have also shown that a coconut oil emulsion added to a large

volume of serum freed of chylomicrons, picks up a minute amount of protein which is not removed by high speed ultracentrifugation. This protein is very likely a constituent of the beta and alpha₁ lipoproteins whose presence in the ultracentrifugally separated particles of coconut oil was detected by the same methods used for chylomicrons. The relative proportion between the two lipoproteins present in the final washed product from serum-coconut oil emulsion was found also very similar to the one in chylomicrons. The data with the "finger printing" technique seem to corroborate these findings, although a few extra spots were found in the chylomicron pattern. These might be attributed to a slight difference in proportion of alpha₁ and beta lipoproteins in the two classes of molecules.

Frazer has shown that addition of serum to fat emulsions stabilizes the fat particles; when serum is defatted this activity is lost (37). This, and our demonstration that these fat particles pick up lipoproteins from serum upon incubation, lead us to believe that lipoproteins are responsible for the fat stabilizing activity of serum. It is possible that a similar mechanism occurs "in vivo" during the absorptive phase when large fat particles reach the blood stream and come in contact with the serum lipoproteins. This may give an explanation for the findings that lipoproteins are present in chylomicrons and that serum chylomicrons migrate with the alpha (11, 37, 38) and beta globulin fractions (37, 38). Because human lymph was not available for study we do not know whether lipoproteins are also present in chyle chylomicrons. According to Frazer (37) chylomicrons from chyle are different from serum chylomicrons in the sense that they have different electrophoretic mobility and different stability when exposed to the action of the lecithinase of *Clostridium welchii*. Bragdon (16) found that the protein concentration of chylomicrons from rat chyle is significantly lower than that reported for chylomicrons from rat serum.

The demonstration that particles of coconut oil incubated with serum retained lipoproteins, was supported by chemical analyses, which showed the presence of protein, cholesterol, and phospholipide in proportions compatible with a lipoprotein molecule. In chylomicrons the concentrations of cholesterol and phospholipides were higher than would have been expected if only cholesterol and phospholipides of the serum lipoproteins that we assume attached to chylomicrons, were present. It is likely that this "excess" derives from exogenous sources during the process of intestinal absorption of fats and, more precisely, from the cholesterol directly absorbed from the intestinal lumen and from the phospholipides, which, broken down prior to absorption, are resynthesized in the intestinal mucosa. These phospholipides may participate in surface phenomena and contribute to the stability of the chylomicrons.

The hexosamine fraction demonstrated in chylomicrons and particles from coconut oil absorbed with serum, probably came from the attached lipoproteins.

We have shown that hexosamine is one of the components of the serum α_1 lipoprotein (22).

Further supportive evidence for the presence of α_1 lipoprotein in purified preparations of chylomicrons and particles from coconut oil absorbed with serum, is given by our findings on "clearing factor" activity. We showed, in accord with the data of Korn (39) that of the serum components, α_1 lipoproteins activate an otherwise inactive coconut oil substrate. Partial delipidation of the α_1 lipoprotein sample or its addition to a beta lipoprotein solution did not modify its activity. The findings that only serum α_1 lipoprotein activated the enzyme system, and that protein from the partial delipidation of chylomicrons and serum-coconut oil mixture, immunologically identified as serum components, also induced enzyme activation, support the presence of α_1 lipoprotein in chylomicrons. In agreement with these conclusions are our data showing that substitution of the coconut oil, an inactive substrate, with undelipidized chylomicrons or serum-coconut oil mixture, determines appearance of clearing activity in the system.

Rodbell's data (12) which show aspartic acid as one of the three N-terminal amino acids of human serum chylomicrons, are also consistent with our findings.² It has been shown that aspartic acid is the N-terminal residue of human serum α_1 lipoproteins (22, 40, 41).

"Clearing factor" activity can be satisfactorily determined by using a system which contains agar. Our results have shown that agar is a good, inert supportive medium for the coconut oil substrate; the enzyme diffuses in it without apparent denaturation, and shows its activity by clear areas in the opaque gel. Under standard conditions a good correlation was found between concentration of enzyme and the area of clearing—a finding that suggests its use as an accurate and quantitative enzyme assay.

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SUMMARY

Chylomicrons were separated by low and high speed ultracentrifugation from lipemic sera of human subjects in the absorptive phase. The final chylomicron preparation was free from other serum components and contained a small constant amount of protein, approximately 2 per cent of the chylomicron

² At the Annual Meeting of the Federation of the American Societies for Experimental Biology of March, 1958, Philadelphia, Rodbell and Fredrickson presented evidence that α_1 lipoprotein is a component of the human chylomicron protein, by N-terminal analysis and "finger printing" technique. (Rodbell, M., and Fredrickson, D. S., Nature and function of chylomicron proteins, *Fed. Proc.*, 1958, **17**, 298).

fraction. Electrophoresis, immunochemical analysis, and absorption experiments identified the protein component as derived from a mixture of beta and alpha₁ serum lipoproteins.

Large aliquots of an emulsion of serum freed of chylomicrons and coconut oil were incubated at 37°C. for 2 hours and ultracentrifuged as in the preparation of chylomicrons. The fat particles now showed the presence of minute amounts of beta and alpha₁ serum lipoproteins in almost the same proportion as found in chylomicrons. "Finger prints" of delipidized samples of chylomicrons and particles from serum-coconut oil emulsion gave similar, although not identical patterns.

The data on "clearing factor" activity corroborated the finding that serum alpha₁ lipoproteins are contained in chylomicrons and particles from serum-coconut oil emulsion. These two lipide particles, partially delipidized, were both able to activate a "clearing factor" system *in vitro*, a property exhibited only by intact or partially delipidized alpha₁ serum lipoproteins. Clearing activity was satisfactorily determined by using an emulsion of coconut oil mixed in agar as a substrate to give an opaque gel, in which the diffusing enzyme showed its activity by areas of clearing. The results obtained by this technique were in agreement with those based on fall in optical density and non-esterified fatty acid production.

Chemical analysis of serum chylomicrons showed a concentration of cholesterol and phospholipides higher than could be accounted for by the attached beta and alpha₁ serum lipoproteins.

On the basis of these results the assumption is made that in the blood stream small amounts of serum lipoproteins, by a process of adsorption, form a complex with the absorbed triglycerides, cholesterol, and phospholipides, to produce chylomicrons.

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