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ADSORPTION AND AGGREGATION OF GAMMA GLOBULIN ONTO CHYLOMICRONS IN SERUM OF LOWERED IONIC STRENGTH†

In the course of experiments involving centrifugation of fresh postprandial human serum diluted with isotonic sucrose, an unexpected phenomenon was noted; the usual opaque fat layer at the surface was missing, and a white pellet was deposited at the bottom of the tube. Microscopic and chemical examinations revealed the deposit to be composed of chylomicrons. Further study of this phenomenon demonstrated that lowering the ionic strength of serum led to adsorption of gamma globulin onto chylomicrons, with resulting increase in their density. This adsorbed gamma globulin was, at least in part, aggregated as evidenced by its biological activity.

METHODS

Venous blood was obtained from healthy donors approximately four hours after a high-fat meal. After clotting at room temperature, the serum was separated by centrifuging at 1,000 x *g* for 15 minutes. Serum was respun under the same conditions to insure removal of platelets and cells. All subsequent procedures for separation of chylomicrons were performed at 0-5° C.

Sucrose was dissolved in distilled water to give a 0.25M solution. Buffered sucrose, used for washing sedimented chylomicrons, was prepared by mixing one volume of phosphate-buffered saline (0.015M mixed phosphate pH 7.4 in isotonic saline) with four volumes of 0.25M sucrose. These sucrose solutions were passed through Seitz filters to remove small particles which might interfere with dark-field microscopic examination.

In a typical experiment sedimented chylomicrons were prepared as follows: 2 ml. of fresh serum was mixed with 8 ml. of 0.25M sucrose and centrifuged for 40 minutes at 15,000 x *g* (Lourdes model LRA, with swinging cup rotor). The pellet was washed repeatedly by suspending in buffered sucrose and spinning for 20 minutes at 10,000 x *g*. Floated chylomicrons were prepared by layering 2 ml. of isotonic saline over 8 ml. of

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serum, or by mixing 2 ml. of fresh serum with 8 ml. of saline and centrifuging under the same conditions. The surface layer of chylomicrons was conveniently collected by scooping it off with a dry stainless steel spatula. When desired these floated chylomicrons were washed by suspending them in saline and spinning them up again. The concentration of chylomicrons in the washed suspensions was estimated by measuring optical density at 650 mμ in a Coleman Jr. spectrophotometer.

Darkfield microscopic examinations were made in a Zeiss Ultraphot II, employing an oil immersion planachromat objective with iris diaphragm and darkground ultracondenser Z. Specimens were made into thin preparations between clean standard slides and No. 0 coverslips.

Lipid analyses were performed by previously described methods.[1] Protein was estimated by a modified Lowry Procedure.[2] Immunoelectrophoresis was done by the method of Scheidegger.[3]

Anticomplementary activity was determined by incubating for 30′ at 38° serial dilutions of the preparation to be assayed with a predetermined concentration (4 hemolytic units) of complement in the form of fresh human serum or lyophilized pooled guinea pig serum (Cappel Laboratories, West Chester, Pa.). Residual complement was then observed following addition of sensitized sheep red cells.

The capacity of sedimented chylomicrons to cause increased capillary permeability was studied by observing the extent of blue discoloration about skin injection sites in guinea pigs previously given 1 ml. of 0.5% Evans Blue solution, as described by Ishizaka and Campbell.[4]

RESULTS

SEDIMENTATION OF CHYLOMICRONS ON CENTRIFUGATION OF SERUM DILUTED WITH ISOTONIC SUCROSE

When fresh human serum was diluted with two to four volumes of isotonic sucrose and centrifuged for 40 minutes at 15,000 x *g*, a white pellet was deposited at the bottom of the tube. This pellet gave a milky appearance when suspended in saline or sucrose, and consited of innumerable bright spherical particles, approximately 0.2 to 0.5 microns in size, on darkfield microscopy. The appearance of these particles was identical with that of chylomicrons in whole serum, or with suspensions of chylomicrons which had been floated up through a layer of saline by centrifuging serum.

In order to support the suspicion that these sedimented particles were chylomicrons, their lipid content was compared with that of floated chylomicrons from samples of the same serum. The pellet resulting from centrifugation of serum diluted with four parts of sucrose was washed repeatedly with buffered sucrose solution. Floated chylomicrons were collected and washed in standard fashion by spinning them up through saline. As is shown in Table 1, both floated and sedimented preparations had

similar ratios of total cholesterol, phospholipid and triglyceride, and the analyses were in both cases typical of chylomicrons.[5]

ADSORBTION OF GAMMA GLOBULIN ONTO CHYLOMICRONS ON DILUTION OF SERUM WITH ISOTONIC SUCROSE

Thus by morphological and chemical criteria the particles which sedimented from serum diluted with sucrose solution were indeed chylomicrons. Obviously they must have been altered in some way so as to increase markedly their density, for sedimentation through the serum-sucrose mixture could not otherwise have occurred. Studies were next done to determine whether serum proteins might be adsorbed onto chylomicrons on dilution of serum with the non-ionic solution.

TABLE 1. LIPID COMPOSITION OF FLOATED AND SEDIMENTED CHYLOMICRONS

	Percent of total lipid as		
Washed suspension of	*Total cholesterol*	*Phospholipid*	*Triglyceride*
Floated chylomicrons	7.6	4.2	88.2
Sedimented chylomicrons	4.5	2.7	92.8

Washed particles, prepared by flotation and by sedimentation from serum as described above, were compared for their protein content. The sedimented chylomicrons had associated with them 0.45 per cent of the total serum protein, as contrasted with only 0.07 per cent for the floated chylomicrons.

The next series of studies indicated that the protein adsorbed to chylomicrons on dilution of serum with isotonic sucrose was largely, if not entirely, gamma globulin. The washed sedimented chylomicrons suspended in saline gave a strongly positive precipitin reaction on mixing with rabbit anti-human gamma globulin serum; semi-quantitative precipitin studies suggested that approximately two per cent of the total serum gamma globulin was associated with the chylomicrons. Figure 1 shows precipitin lines obtained following immunoelectrophoresis of floated chylomicrons (top well), whole serum (middle well), and sedimented chylomicrons (lower well), all prepared from a single serum specimen. After electrophoretic separation the slots were filled with a potent rabbit antiserum to whole human serum. No detectable gamma globulin or other serum protein was associated with the washed floated chylomicrons. In contrast, a strong arc corresponding to the gamma globulin arc of whole serum, was

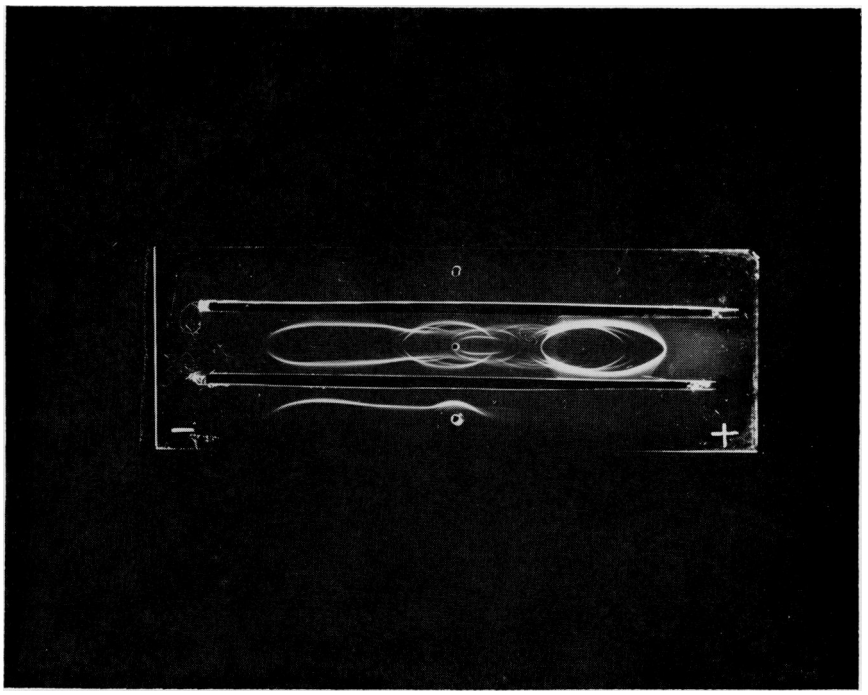

FIG. 1. Immunoelectrophoretic study of serum proteins associated with washed floated and sedimented chylomicrons. The upper well contained washed floated chylomicrons, the center well whole human serum, and the lower well washed sedimented chylomicrons. After electrophoresis, the troughs were filled with a potent rabbit antiserum to whole human serum. As is seen, no detectable serum protein was associated with the floated chylomicrons. In contrast, the washed sedimented chylomicron specimen gave a precipitin arc corresponding to gamma globulin in the whole serum, and in addition gave another strong precipitin line situated at the origin and apparently fusing with the globulin arc, for which there was no counterpart in the whole serum specimen.

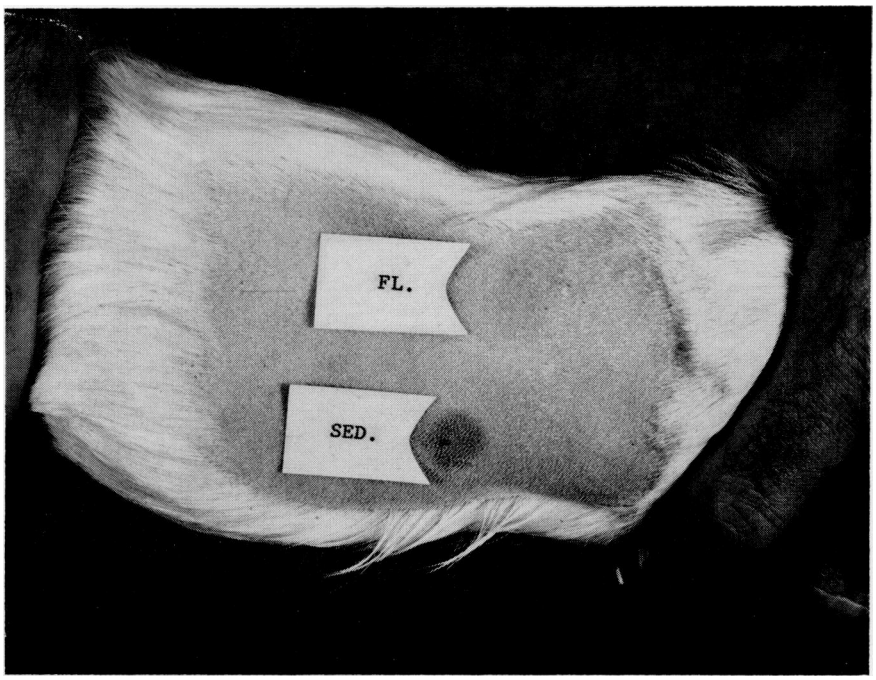

FIG. 2. Skin reaction to the injection of sedimented chylomicrons. Figure 2 shows the reaction in a guinea pig injected intracutaneously 10-15 minutes previously with equivalent amounts of saline suspensions of floated (FL) and sedimented (SED) chylomicrons. The guinea pig had been prepared by administration of Evans blue so as to permit ready detection of changes in capillary permeability. The positive reaction to injection of sedimented chylomicrons contrasts sharply with the lack of response to floated chylomicrons.

present in the washed preparation of sedimented chylomicrons. In the sedimented chylomicron specimen there was another precipitin line, apparently continuous with the gamma globulin arc but extending towards the origin, for which no counterpart was seen in the whole serum sample. The nature of this second precipitin line is not known, but it might represent gamma globulin altered by dilution of serum in sucrose or aggregated or otherwise changed as a result of adsorption to chylomicrons. In any event, the immunoelectrophoresis established that association of serum protein with chylomicrons on dilution of serum with sucrose was a specific reaction involving primarily gamma globulin, rather than a non-specific adsorption of serum proteins in general.

In support of the role of gamma globulins in altering density of chylomicrons upon dilution of serum with sucrose was the following observation. Floated chylomicrons from whole serum were washed by centrifuging them up through saline. When these chylomicrons were suspended in a 1.6 per cent solution in saline of human gamma globulin,* and then diluted with four volumes of isotonic sucrose, they sedimented to the bottom of the tube on centrifugation. In contrast, washed floated chylomicrons suspended in three per cent human albumin** in saline and similarly diluted with sucrose rose to the top after spinning under the same conditions.

To establish that the gamma globulin was adsorbed to chylomicrons, rather than irreversibly incorporated into their structure, the following experiment was done. Sedimented chylomicrons were prepared and washed in the usual manner, and were finally suspended in isotonic saline. Centrifugation of this saline suspension resulted in a surface layer of lipid, not a pellet. The subnatant saline contained gamma globulin as demonstrated by precipitin reactions and by biological activity (see below); therefore at least some of the gamma globulin associated with sedimented chylomicrons came off when they were suspended in physiological saline.

STUDIES ON VARIOUS FACTORS INFLUENCING YIELD OF SEDIMENTED CHYLOMICRONS FROM SERUM

The relationship between the degree of dilution of serum with isotonic sucrose and the yield of sedimented chylomicrons is shown in Table 2. After centrifugation a pellet of chylomicrons was formed when serum was diluted with as little as one volume of sucrose solution. Only a portion

* Human Fraction II, Lot No. H220, Pentex Inc., Kankakee, Ill., and Lederle Poliomyelitis Immune Globulin, Human, Lot No. 2175-451C.

** Crystallized human albumin, Lot 3, Pentex Inc., Kankakee, Ill.

of chylomicrons was sedimented under these conditions, however, as indicated by the presence of a surface fat layer as well as a deposit in these tubes. No surface layer of chylomicrons was present after centrifuging serum diluted with two or with four volumes of 0.25M sucrose.

Dilution of serum with water resulted in sedimentation of chylomicrons similar to that observed on dilution with sucrose solutions. Dilution of serum with 0.25M sucrose dissolved in isotonic saline instead of water led to flotation of the chylomicrons on spinning. These results indicated that the interaction between chylomicrons and gamma globulin leading to change in their density depended on lowered ionic strength of the

TABLE 2. RELATIONSHIP BETWEEN THE DEGREE OF DILUTION OF SERUM AND THE YIELD OF SEDIMENTED CHYLOMICRONS

Serum diluted with	*Yield of sedimented particles as determined after washing by*	
	Optical density	*Protein**
no sucrose added	0	0
1 volume 0.25M sucrose	0.210	120
2 volumes " "	0.310	144
4 volumes " "	0.330	140

* μg per ml, based on a crystalline egg white lysozyme standard.

medium, not on some reaction involving the sucrose *per se*. This could be shown in another manner, namely by dialyzing serum against two volumes of saline or against two volumes of water; on centrifugation chylomicrons in the serum dialyzed against saline spun up, whereas those in serum dialyzed against water sedimented.

It was, of course, possible that dilution of serum with sucrose or water, even though this dilution was limited to four volumes or less, might cause precipitation of some euglobulins which would then contaminate the pellet of chylomicrons. To rule out this possibility serum was overlaid with saline and spun at high speed. The bottom of the tube was then punctured and chylomicron-free serum collected. This serum when diluted with four volumes of 0.25M sucrose gave no precipitate and no pellet was formed on centrifugation.

The pH of the serum specimens ranged from 7.4 to 7.8 and was not appreciably changed after dilution with sucrose solution. In order to determine whether pH influenced the association of chylomicrons and

gamma globulin, serum was adjusted by addition of 0.15 N HCl or NaOH to the levels indicated in Table 3, and the yield of sedimented chylomicrons estimated after dilution with 0.25M sucrose and centrifugation. As is shown in the Table, the yield was essentially the same between pH 6.8 and pH 7.8. When the reaction was more alkaline, chylomicron sedimentation was reduced or blocked.

Apparently the amounts of certain gamma globulins in serum, or of some cofactor required for their adsorption to chylomicrons were limited. Specimens of very lipemic serum diluted with four volumes of sucrose solution occasionally showed both a pellet and a surface layer after centri-

TABLE 3. EFFECT OF pH OF DILUTED SERUM ON THE YIELD OF SEDIMENTED CHYLOMICRONS

pH of serum adjusted before dilution with 4 volumes of 0.25M sucrose to	*Yield of sedimented particles, as determined after washing by*	
	Optical density	*Protein**
8.8	0	0
8.2	0.260	68
7.8	0.290	136
6.8	0.315	144

* μg per ml, based on a crystalline egg white lysozyme standard.

fugation. The surface chylomicrons from such a specimen were not of a radically different nature from those which sedimented, since they could be transferred to fresh, nonlipemic serum and then spun down after dilution. Another experiment in support of this notion was the following. Serum diluted with sucrose solution was centrifuged to sediment the chylomicrons. When the supernate was then mixed with floated chylomicrons and spun, a surface layer, not a pellet, was usually obtained, indicating that some factor involved in the chylomicron alteration had been consumed during the first separation.

Heat-inactivation of serum (56°C., 30 min.) prevented completely subsequent sedimentation of chylomicrons following dilution with isotonic sucrose. It was of interest to determine which of the reactants, chylomicrons or serum proteins, had been altered by heating. Washed floated chylomicrons and serum free of chylomicrons were prepared as described previously from both heat inactivated and from unheated serum. Results obtained using various mixtures of these are outlined in Table 4. Heated

chylomicrons were fully capable of interacting with the gamma globulin in unheated, particle-free serum on dilution with sucrose. Heated particle-free serum, on the other hand, lost completely its capacity to bring about sedimentation of washed floated chylomicrons after dilution with sucrose. The results indicated that the gamma globulin adsorbed to chylomicrons, or some cofactor required for its adsorption, was heat-labile.

When EDTA, disodium form, was added to serum at 0.01M final concentration, chylomicrons spun to the top after dilution with isotonic sucrose rather than sedimenting as they did in the absence of this chelating agent, suggesting that divalent cations may be required for the interaction

TABLE 4. EVIDENCE THAT HEATING BLOCKS SEDIMENTATION OF CHYLOMICRONS IN DILUTED SERUM BY ALTERING THE SERUM COMPONENTS, NOT THE CHYLOMICRONS

Floated washed chylomicrons from	*Mixed with chylomicron-free serum*	*Yield of sedimented chylomicrons after dilution with 0.25M sucrose (optical density)*
heated (56° C. 30′) serum	heated 56° C. 30′	0
	unheated	0.15
unheated serum	heated 56° C. 30′	0
	unheated	0.15

between gamma globulin and chylomicrons, or for a cofactor necessary in this interaction.

EVIDENCE THAT THE GAMMA GLOBULIN ADSORBED TO CHYLOMICRONS IN DILUTED SERUM IS, AT LEAST IN PART, AGGREGATED

It seemed somewhat surprising that adsorption of small amounts of gamma globulin onto chylomicrons should increase their density sufficiently to account for sedimentation through 0.25M sucrose solutions. This paradox led to consideration of the possibility that the adsorbed globulin was of a particular heavy type, and perhaps aggregated.

Suspensions of washed sedimented chylomicrons were accordingly studied for the ability to fix complement and to increase capillary permeability, two properties known to be developed by gamma globulin aggregated by heating.[6,7]

Table 5 compares the anticomplementary activity of floated and sedimented chylomicrons. Floated chylomicrons displayed no significant

capacity to fix complement, whereas the sedimented chylomicrons displayed this capacity to a high degree, and furthermore blocked both human and guinea pig complement.

Additional evidence that the gamma globulin associated with sedimented chylomicrons was aggregated, at least in part, was their ability to produce immediate skin reactions on injection into guinea pigs. A comparison of the effect on capillary permeability of floated and sedimented chylomicrons injected into guinea pig skin is shown in Figure 2. The reaction with sedimented chylomicrons appeared immediately, reaching a maximal diameter in approximately 10 minutes and gradually fading,

TABLE 5. ANTICOMPLEMENTARY ACTIVITY OF FLOATED AND SEDIMENTED CHYLOMICRONS

Washed suspensions from 1 ml. of serum of	*Anticomplementary titer* on*	
	Guinea pig serum	*Human serum*
Floated chylomicrons	<10	<10
Sedimented chylomicrons	640	80

* Highest twofold dilution of the chylomicron suspension capable of blocking the action of four units of hemolytic complement.

without necrosis, over the course of 48 hours. An equal quantity of washed floated chylomicrons gave no detectable reaction when similarly injected into guinea pig skin.

DISCUSSION

One practical aspect of the phenomenon described here is its possible usefulness as a technique for harvesting chylomicrons in studies on their structure and composition. The flotation method commonly employed for collecting chylomicrons is time consuming and does not give quantitative recovery; especially during washing in saline, considerable loss of particles and progressive loss in protein occurs.[5] Chylomicrons sedimented from diluted serum are readily manipulated; after washing they may be collected by centrifugation at relatively low speeds (1,000 x g) without loss of particles or of detectable protein. The gamma globulin overcoat which sedimented chylomicrons apparently wear may not interfere with studies on their lipid makeup; lipid analyses on floated and sedimented chylomicrons are, in fact, strikingly similar.

Association between gamma globulin and serum lipids under certain conditions *in vitro* has been noted previously. For example, in 1935 Horsfall and Goodner[8] reported a relationship between lipid content of antiserum and the amount of lipid present in antigen-antibody precipitates. Also, various serological reactions, such as the Wasserman and zinc turbidity tests,[9] appear to be based on interaction between lipid particles and serum globulins. Also to be noted is the recent report of Huggins[10] in which serum gamma globulin apparently adsorbed to erythrocytes in low ionic strength medium.

Little can be said about the precise mechanism which accounts for adsorption of gamma globulin to chylomicrons in normal serum when ionic strength is lowered. "Shielding" ions at potential reactive sites would, of course, be lessened as the ionic strength falls, perhaps allowing electrostatic bonding forces between the chylomicron surface and gamma globulins to come into play.[11] Some of the findings, however, suggest that the interaction may be on a more specific or complicated basis than simple electrostatic attraction. For example heat inactivation at 56°C. prevents the adsorption of globulin to chylomicrons on dilution of serum, and low concentrations of versene exert a similar blocking effect.

Only a small proportion of the total serum gamma globulin is adsorbed to chylomicrons in diluted serum. Perhaps only a small fraction of gamma globulin molecules possess properties necessary for this interaction. Alternatively, most or all gamma globulins may be reactive, but the adsorption might be limited by availability of some unknown cofactor. The heat-lability suggests that complement may play a role in the reaction between chylomicrons and gamma globulins. Against this hypothesis is the ability of washed floated chylomicrons to interact with purified gamma globulin solutions free of complement activity.

The gamma globulin adsorbed to chylomicrons when the ionic strength of serum is lowered is, at least in part, aggregated. Currently there is much interest in the biological activities of aggregated gamma globulin, and the studies reported here provide a method for their production other than by heating or by antigen-antibody interactions.

Could interactions between gamma globulins and chylomicrons occur *in vivo*? No evidence to support such a possibility is now available. If however, this interaction were to take place under some circumstances in the animal, it might play a part in a variety of physiological and pathological phenomena, including chylomicron clearing, reactivity or availability of antibody, and changes in vascular permeability.

SUMMARY

When the ionic strength of human serum is lowered by moderate dilution with sucrose solutions or water, the density of chylomicrons is altered so that they sediment, rather than float, on centrifugation.

Investigation of this phenomenon has revealed adsorption of gamma globulin, but not of other serum proteins, onto chylomicrons under these conditions.

The sedimentation of chylomicrons in diluted serum is essentially unaltered by pH changes in the range 6.8-7.8, but is prevented by heating at 56°C. or by addition of versene.

The gamma globulin adsorbed to chylomicrons on lowering the ionic strength of serum is, at least in part, in the aggregated state, as evidenced by its capacity to fix complement and to cause an immediate reaction in guinea pig skin.

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