A rapid method for staining large chylomicrons

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Summary In this report, we present a rapid method for producing high-quality micrographs suitable for determining the size distributions of particles in concentrated samples of postprandial chylomicrons and chylomicron remnants. The procedure consists of mixing particles with osmium tetroxide in water to stabilize the lipids of the particles. These fixed and positively stained particles are then negatively stained with phosphotungstate in the presence of dilute sucrose. This dual staining procedure prevents the fusion and clustering of chylomicrons during processing for electron microscopy and is effective with particles of different lipid compositions. In addition, this procedure is simple and rapid, adding only one mixing step and 5 min to the preparation time required for conventional negative stains. —**Anderson, L. J., J. K. Boyles, and M. M. Hussain.** A rapid method for staining large chylomicrons. *J. Lipid Res.* 1989. **30:** 1819–1824.

Supplementary key words negative stain • positive stain • chylomicron remnants • chylomicrons

The negative staining of lipoprotein particles with phosphotungstate (PTA) has long been a widely accepted and routine technique for determining lipoprotein particle size by electron microscopy (1, 2). However, the staining process produces a number of artifacts. The most widely recognized artifact is flattening of the particle by surface tension during drying of the stain (1, 3).

We have identified an even more serious problem in working with concentrated samples of large chylomicrons rich in polyunsaturated fat. These large particles tend to fuse during conventional negative staining. In overcoming this problem, we developed a rapid negative-stain protocol that uses osmium tetroxide (OsO_4), a cross-linker of unsaturated fatty acids, to fix and stabilize the lipids of these particles. This protocol prevents fusion and distortion of triglyceride-rich particles containing primarily either saturated or unsaturated fats. One major advantage of our protocol over previously published procedures using OsO_4 is the rapidity with which samples can be processed. In addition, our technique routinely results in the visualization of a large number of individual particles per field.

MATERIALS AND METHODS

Chylomicrons and chylomicron remnants

Thoracic duct lymph chylomicrons were collected from mongrel dogs as previously described (4). The dogs were fed either Mocha Mix (Presto Food Products, Inc., Industry, CA) (polyunsaturated and monounsaturated fat) or half-milk, half-cream with egg yolks (primarily saturated fat). The collected lymph was centrifuged (SW 28 rotor, 28,000 rpm, 90 min at 20°C) to isolate $S_f > 400$ particles. The creamy top layer was resuspended and filtered through 0.45-μm filters. Some samples of these particles were then washed by recentrifugation in saline EDTA. The chylomicrons (0.1–0.2 ml) were washed by overlaying them with 0.8–0.9 ml of saline and centrifuging them (100,000 rpm, 8 h at 20°C) in a TLA rotor used with a Beckman tabletop ultracentrifuge (4).

Remnants were obtained by injecting chylomicrons into a hepatectomized rabbit at 200 mg of triglyceride/kg body weight (4) and allowing them to circulate for 30 min. Chylomicron remnants were isolated from the plasma by ultracentrifugation (SW 28 rotor, 28,000 rpm, 2 h 45 min at 4°C).

Negative staining

Chylomicrons in saline EDTA were diluted with saline EDTA or with 1% neutral phosphotungstic acid and 0.1% sucrose to achieve a triglyceride concentration of ~200 mg/ml. Unwashed chylomicrons in lymph were diluted as necessary with saline EDTA to lower the protein content but maintain a useful concentration of particles on the grid surface. This generally required a one- to fivefold dilution or a triglyceride concentration of ~50–100 mg/ml. Carbon films prepared within the previous 30 days were used as a support for the stain (5). (Carbon films older than 30 days are more likely to become hydrophobic.) These films were made by evaporating carbon onto freshly cleaved mica sheets in a clean vacuum (Balzers turbo-pumped vacuum system vented with ultrapure nitrogen [Balzers, Hudson, NH]). A small piece (~2 to 3 mm^2) was cut from the carbon-mica sheet, and 5 μl of the chylomicron sample was applied to the interface of the carbon and mica. The floating carbon film and adherent sample were then picked up with a grid. To improve the adhesion of the carbon film to the grid, the grid bars were coated by first dipping the grid in dilute Formvar (1% w/v in 1,2-dichloroethane) and removing excess Formvar with blotting paper. Grids of samples not previously mixed with stain were stained with a drop of 1% PTA to which 0.1% sucrose had been added; care was taken not to let the sample on the grid dry before it was stained. Excess stain and sample were removed with blotting paper, and the grid was air-dried for viewing and micrography at 80 kV in a JEOL 100 CXII electron microscope. In addition to diluting the sample, we could

Abbreviations: LDL, low density lipoproteins; VLDL, very low density lipoproteins; PTA, phosphotungstate.

control particle concentration on the grid surface by manipulating the amount of sample left to dry on the grid and by washing the grid with a drop of stain.

Positive staining

Chylomicrons in saline EDTA were mixed 1:1 (v/v) with 4% OsO_4 in water and fixed for 2–5 min before application to a carbon film on mica. A Formvar-treated grid was touched to the carbon surface, and excess sample was removed from the film with one or two drops of distilled water. The grid was then air-dried and viewed in the electron microscope at 80 kV.

Dual staining

Chylomicrons or chylomicron remnants in saline EDTA or in diluted lymph were mixed 1:1 (v/v) with 4% OsO_4 and fixed for 2–5 min. The fixed sample was then applied to a carbon film on mica, and the film (with adherent sample) was picked up with a Formvar-treated grid. Excess sample was removed, but the grid was not allowed to dry before being stained with 1% PTA with 0.1% sucrose or 0.5% PTA with 0.05% sucrose. The grid was air-dried and viewed in the electron microscope at 80–120 kV. Samples with an excess of protein (such as unwashed chylomicrons) were diluted first with saline EDTA, mixed with OsO_4, and stained.

Histograms

For quantification, two preparations with different particle size distributions were chosen from 10 separate chylomicron samples. Both preparations contained washed, dual-stained chylomicrons of $S_f > 400$. Five micrographs of each preparation were taken at random from areas with appropriate particle density (areas with large numbers of individual particles) and were printed at a final magnification of 50,000 ×. Micrographs representing small and large chylomicrons, obtained at different times after Mocha Mix feeding, were used for quantification. The first 500 particles encountered were measured. When 500 particles were not observed in a single micrograph, an additional micrograph was used to achieve the total of 500.

RESULTS AND DISCUSSION

Negative staining

Initial attempts at conventional negative staining of lymphatic postprandial chylomicrons were unsatisfactory. These large particles tended to cluster on the grid and could not be dispersed, making accurate measurements impossible. We therefore tried spreading agents, such as proteins (particularly albumin) and sucrose, and found sucrose to be the most effective. It is best used at a very dilute concentration, 0.05 to 0.1%. (This concentration of sucrose also worked as a spreading agent with smaller very low density lipoprotein (VLDL) particles or low density lipoprotein (LDL) particles.) More dilute concentrations of sucrose were not effective as a spreading agent, and concentrations above this range caused defects in the stain; concentrated sucrose decomposed under electron bombardment in the thick layers of stain surrounding the particles, producing holes and irregularities.

In the well-spread preparation (**Fig. 1A**), it was evident that an additional problem existed. Although some round particles of various sizes were preserved, many very large and irregular particles were also present. These appeared to result from the fusion of smaller particles. The large, irregular particles were present whether or not the chylomicrons were mixed with stain before or after being applied to the carbon. The presence of large amounts of albumin had little effect; unwashed particles in lymph fused as readily as washed particles in saline EDTA. Although the extent of fusion varied from area to area on the grid, we felt that measurements from such samples were not reliable indicators of particle size.

Positive staining

As a negatively stained sample dries, the chylomicrons trapped in the stain are subjected to surface tension. The tension can flatten the lipid cores of particles (*1*, 3) and apparently can cause the particles to fuse. Large chylomicrons, particularly those rich in unsaturated fats and thus having liquid core lipids at room temperature, are particularly susceptible to distortion by surface tension. One means of preventing these distortions is to use OsO_4, a chemical capable of cross-linking the fatty acids of both the surface phospholipids and the triglyceride cores of these particles. Stabilization, or solidification, of the lipids might be expected to prevent fusion and flattening. Fixation with OsO_4 has been used previously to stabilize triglyceride-rich particles against flattening during drying for negative staining (6), for heavy metal shadowing (1, 7), and even for direct positive staining of particles (1, 3, 7). However, these investigators used buffered osmium solutions or osmium vapor fixation, which require either further sample processing or considerable time for osmication.

We chose to mix chylomicrons with OsO_4 in water and to apply this mixture to a grid directly. Excess OsO_4 was removed with a drop of water before the grid could dry. Particles prepared in this way appeared as discrete structures stained dark grey to black (Fig. 1B). No fused particles were seen. However, when concentrated samples were used, the particles tended to cluster. These clusters were unstable in the electron beam, making measurements difficult and inaccurate. Previous investigators overcame particle clustering by extensive dilution (1, 7), but this method is not convenient for size determinations

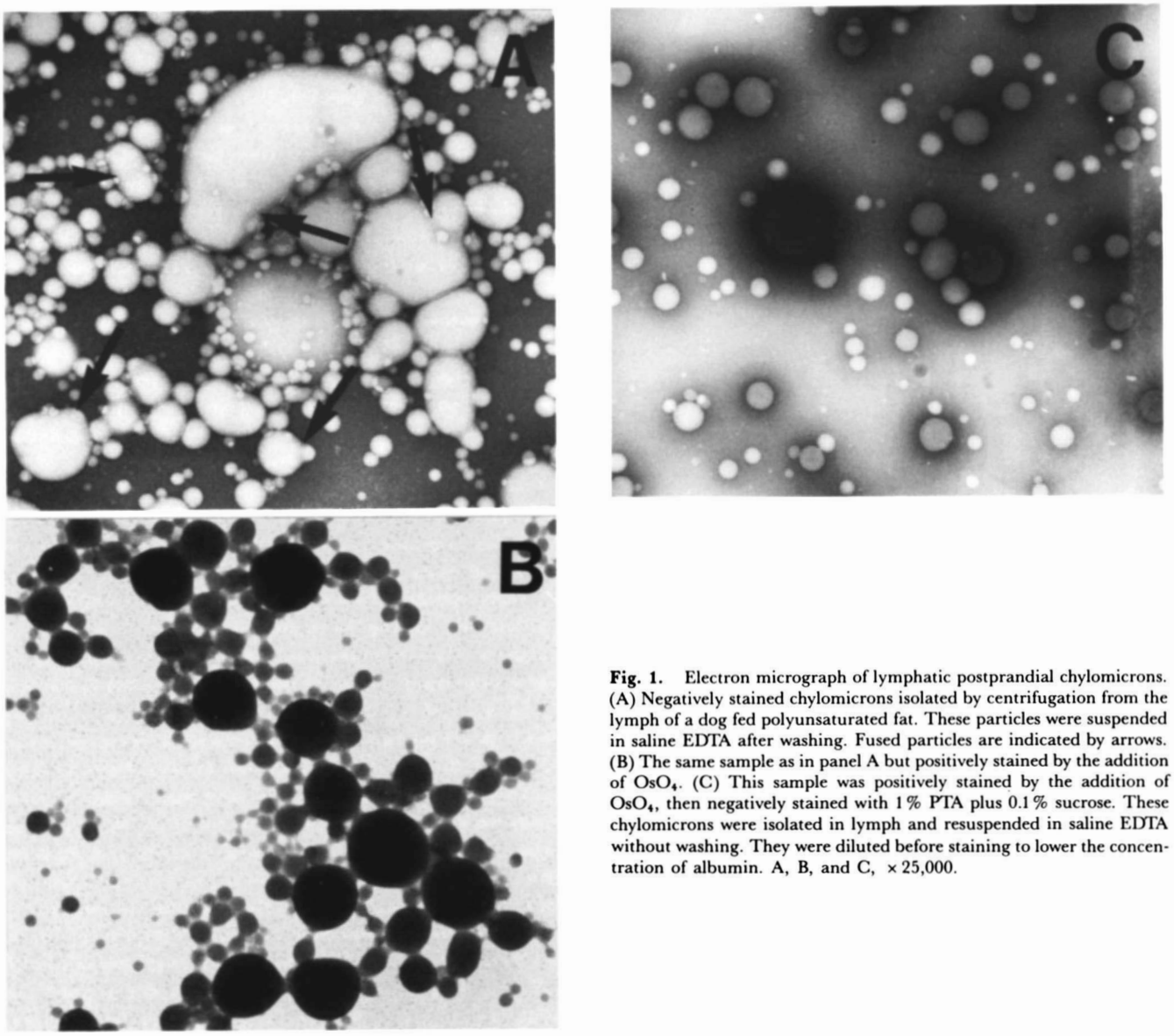


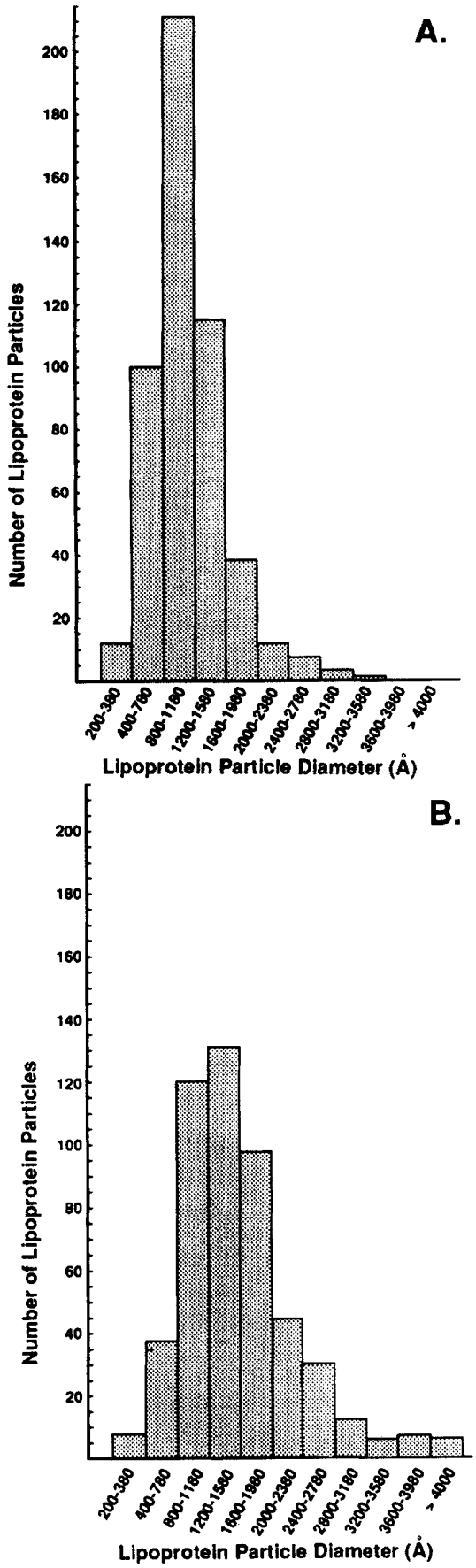
Fig. 1. Electron micrograph of lymphatic postprandial chylomicrons. (A) Negatively stained chylomicrons isolated by centrifugation from the lymph of a dog fed polyunsaturated fat. These particles were suspended in saline EDTA after washing. Fused particles are indicated by arrows. (B) The same sample as in panel A but positively stained by the addition of OsO_4. (C) This sample was positively stained by the addition of OsO_4, then negatively stained with 1% PTA plus 0.1% sucrose. These chylomicrons were isolated in lymph and resuspended in saline EDTA without washing. They were diluted before staining to lower the concentration of albumin. A, B, and C, ×25,000.

because large numbers of micrographs are then required to document the diameters of a reasonable quantity of particles.

Dual staining

We next combined the positive and negative staining procedures so that the enhanced dispersal of particles in PTA and sucrose would enable us to visualize particles that were first stabilized with OsO_4. Fig. 1C is an example of the images produced by first mixing the chylomicrons with 4% OsO_4 in water, followed by staining with 1% PTA and 0.1% sucrose. These fixed particles were dark grey to black, had rounded profiles and discrete edges, and were visible against the light grey to medium dark grey background. Furthermore, because these well-dispersed round particles showed none of the fusion artifacts seen without the use of prior OsO_4 fixation, they could be measured accurately and easily. The only difference between this image and conventional negative stains is the variable electron density of the particles caused by the presence of OsO_4 in the lipid core.

Washed samples of chylomicrons in saline EDTA, as well as unwashed chylomicrons in lymph, were readily stained with this procedure. However, the samples in lymph required prior dilution with saline EDTA to prevent the precipitation of protein during osmication. The chylomicrons in Fig. 1C are an example of chylomicrons isolated from dog lymph and diluted for staining. Osmium tetroxide in water is completely compatible with PTA and sucrose; therefore, the order of adding either stain or



OsO_4 to the sample is not important. Osmium tetroxide and stain can even be mixed with the sample before it is applied to a carbon film.

The lipoprotein particles found in any given preparation of chylomicrons were of diverse sizes; individual preparations also exhibited great differences in the distribution of particle sizes. **Fig. 2** shows histograms of particle diameters in two preparations of chylomicrons. The two examples were chosen to illustrate the extremes of particle size distribution. However, preparations resembling one or the other profile were not uncommon. In these chylomicron preparations, the presence of large numbers of particles smaller than 1000 Å (many actually of LDL size, 200–300 Å) was not expected. These small particles were seen in preparations stained by conventional negative staining as well as in dual-stained preparations and thus are not likely to be an artifact of the staining procedure. The isolation and washing of $S_f > 400$ particles does not remove the smaller particles, which presumably originate from the lymphatic drainage of the viscera and musculature.

The contrast of dual-stained preparations could be manipulated by varying the density of the electron micrograph negative, the concentration of PTA used for staining, or the accelerating voltage of the microscope. We found that using less OsO_4 (either a more dilute solution or mixing less with the particles) in an attempt to decrease the density of the chylomicrons was not satisfactory. Particles fixed in lower OsO_4 concentrations were less stable, as were those fixed as usual but for shorter times. With these dual-stained particles, however, the negatives most suitable for printed images were obtained by using a shorter exposure than is appropriate for most other electron microscopy. In addition, lowering the concentration of PTA from 1% to 0.5% made it possible to lighten the background. This resulted in an image similar to a positively stained one (**Fig. 3A**). These lightly stained preparations sometimes showed particle clustering; however, even in a cluster of particles the individual particles remained discrete. The density of the lipid core of particles could also be lowered by using higher accelerating voltages, such as 120 kV (Fig. 3B). The extent of OsO_4 bound within the core of chylomicrons was dependent upon the degree of saturation of the fatty acids. Chylomicrons from a dog that had been fed half-milk, half-cream and egg yolks bound little OsO_4 (Fig. 3C) in comparison with chylomicrons from a dog that had been fed unsaturated fat (Fig. 3A).

Fig. 2. Histograms of the diameters of 500 particles in two preparations of washed $S_f > 400$ chylomicrons isolated from the lymph of two different dogs fed a meal of polyunsaturated and monounsaturated fat (Mocha Mix). (A) For this preparation, the lymph was collected for 1–3 h after Mocha Mix feeding, and the chylomicrons were isolated as described in Materials and Methods. (B) For this preparation, the chylomicrons were isolated from the lymph 5–7 h after Mocha Mix feeding.

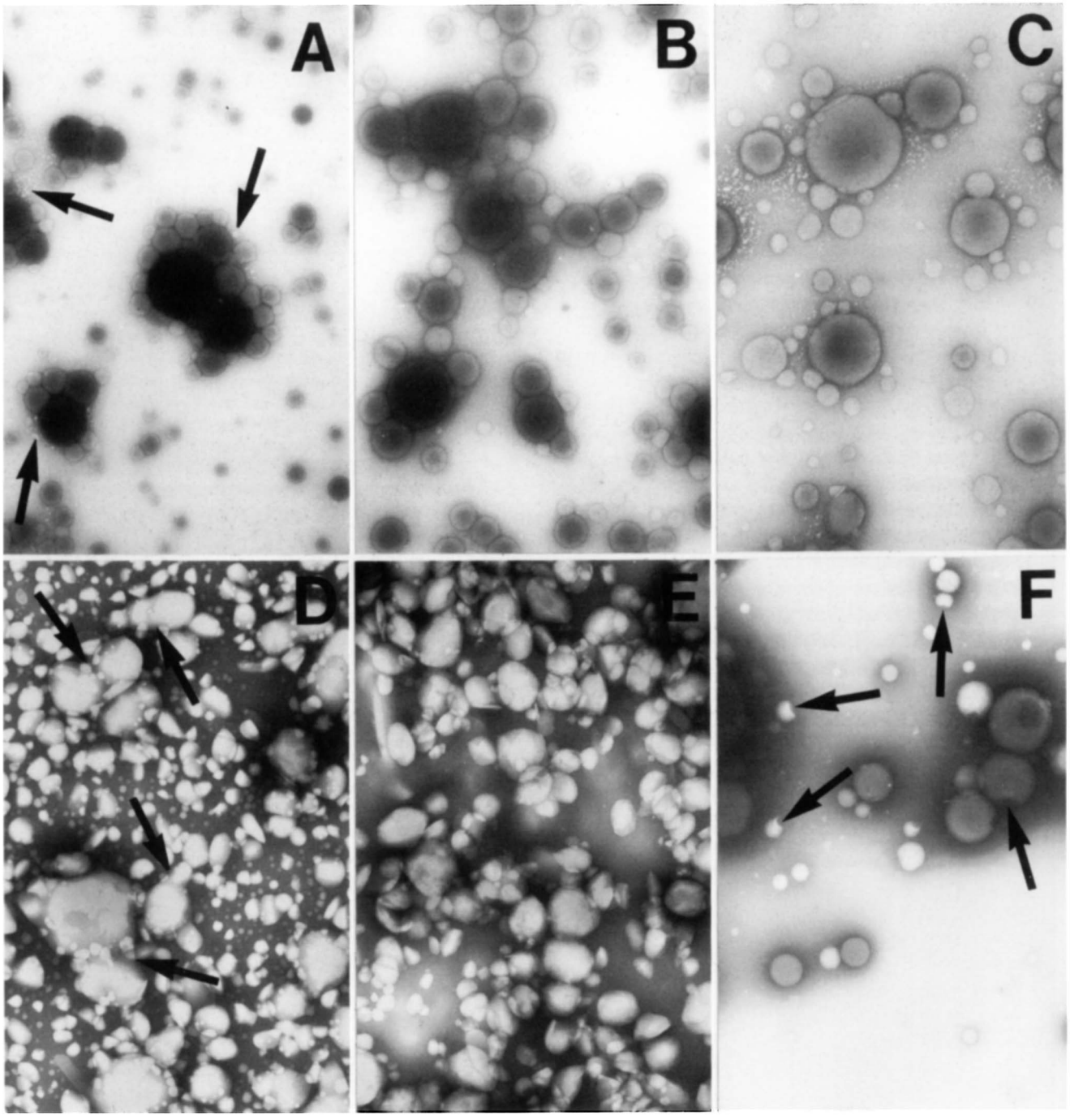


Fig. 3. (A) Washed chylomicrons rich in polyunsaturated fat and stored at 4°C. These dual-stained particles were negatively stained with 0.5% PTA and 0.05% sucrose to lessen the stain density. Artifacts caused by sucrose decomposition are marked by arrows. (B) The same sample as in panel A but photographed at 120 kV to reduce particle-core density. (C) Washed chylomicrons rich in saturated fat that were prepared and stored at room temperature. These particles were dual-stained. (D) The same sample as in panel C but stored at 4°C after collection. This sample was only negatively stained. Occasional fused particles are present (arrows). (E) The same sample as in panel D but dual-stained. (F) Chylomicron remnants isolated from a hepatectomized rabbit. Invagination or dimpling of the surface of particles is present (arrows). All panels, ×25,000.

The dual staining technique was successful in overcoming problems of particle fusion that occurred with both saturated and unsaturated fats. Chylomicrons from dogs fed half-milk, half-cream and egg yolks and stored at 4°C (Fig. 3D) rather than at room temperature (Fig. 3C) tended to have irregular shapes as a result of the crystallization of triglycerides rich in saturated fatty acids at this low temperature (8, 9). Conventional negative staining of

even these particles resulted in some fusion of particles and the production of irregular large particles (Fig. 3D). Far less fusion occurred than with the particles of unsaturated fat, which remained round after storage at 4°C (Figs. 1, 3A, and 3B). A combination of positive and negative stain (Fig. 3E) prevented this fusion artifact, just as it did with particles of unsaturated fat.

Like chylomicrons, large chylomicron remnants were poorly preserved and often fused in conventionally stained samples. Dual staining prevented particle fusion and resulted in an image of remnants different from that obtained with negative staining alone. Using conventional negative-staining procedures, investigators have shown that remnants have small flaps of surface material that extend beyond the particle (10, 11). We saw similar structures in negatively stained canine chylomicron remnants. After dual staining, however, the particles had invaginations or dimples (Fig. 3F). Like a conventional image, Fig. 3F suggests the existence of excess surface material. The only difference is that in samples treated with OsO_4 this material projects into the core of a particle, whereas in untreated samples it projects out beyond the core. Which image is the more accurate reflection of the remnant particle structure as it exists in vivo is open to question. A cross-linking of core and surface fatty acids by OsO_4 may cause the invagination or dimpling seen in these samples. Alternatively, the flaps of surface material seen in negatively stained samples may result from surface-tension effects during drying. The dimpling of lipoprotein particles has been reported in particles isolated after lipolysis and in those found in atherosclerotic lesions (11, 12). The samples in those reports were both osmicated and embedded in plastic. Similar invaginations have also been described in particles isolated from the blood of lecithin:cholesterol acyltransferase-deficient patients and subjected to OsO_4 treatment and negative staining (6).

In summary, the simple, rapid staining procedure reported here appears appropriate for use on a wide variety of triglyceride-rich particles, particularly the large chylomicrons found in lymph after a meal high in polyunsaturated fat. ■

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