

Glutaraldehyde Fixation of Casein Micelles for Electron Microscopy

R. J. CARROLL, M. P. THOMPSON, and G. C. NUTTING

Eastern Regional Research Laboratory¹
Philadelphia, Pennsylvania

Abstract

Fixation of casein micelles from skim-milk (*Bos taurus*) for electron microscopy has been compared using osmium tetroxide, formaldehyde and, for the first time, glutaraldehyde. With the latter, a rapid procedure, coupled with a dispersion method, has been developed. Glutaraldehyde appears to preserve micelle structure better than either osmium tetroxide or formaldehyde and eliminates the need for time-consuming centrifugation. The method is easily adaptable to research on concentrated milks and artificial milk systems as well as on fresh skim milk.

Glutaraldehyde-fixed micelles show a range of sizes from 500 to 2,500 Å. Most of the micelles fall in the range of 1,000 to 1,700 Å in diameter, although this range will vary from milk to milk.

In an attempt to make more reliable size measurements of casein micelles for size distribution studies, an improved and rapid procedure has been developed to prepare casein micelles for electron microscopy. In this investigation we have evaluated and compared previous methods with newly developed techniques. Electron microscopic investigators of casein micelles have usually employed formaldehyde or osmium tetroxide as fixing agents. One of the earliest studies was that of Nitschmann (3), who used 0.4 to 0.8% formaldehyde for 24 hr to preserve micelle structure. Similar fixation steps were employed by Hostettler and Imhof (2), as well as Peters and Dietrich (4). Hostettler and Imhof also reported on the use of osmium tetroxide and phosphotungstic acid to preserve micelle structure, but concluded that formaldehyde treatment was preferable. Shimmin and Hill (7) obtained excellent preservation of micelle structure with 2% osmium tetroxide, followed by centrifugation at 96,000 *g* to remove excess osmium. In 1966, Rose and Colvin (5) reported on size dis-

tribution of micelles in the milk system. In their studies stabilization of the micelles was obtained by dilution of the skim milk in 0.01 M CaCl₂ as advocated by Nitschmann (3). In addition, Rose and Colvin obtained excellent dispersion of the micelles by a slide-dipping technique. The micelles were replicated in formvar film and removed by 10% aqueous ammonium hydroxide solution. Their electron micrographs showed micelles which lacked the three-dimensional form shown by Shimmin and Hill and by Nitschmann.

In this paper we report our attempts to stabilize casein micelles for electron microscopy by a rapid, simplified procedure. Glutaraldehyde, first introduced by Sabatini et al. (6) as a fixative for cytochemical studies by electron microscopy, is now reported as a fixative for casein micelles. Also presented are our observations on short-time osmium tetroxide and formaldehyde fixation, plus our modifications for dispersing casein micelles. Size distributions of casein micelles are compared with those of Nitschmann (3) and Rose and Colvin (5).

Experimental Procedures

Freshly drawn Guernsey milk, separated to remove cream, was used in all experiments. Unbuffered 2% osmium tetroxide, 4% glutaraldehyde, 1% and 0.1% formaldehyde were compared for stabilization of micelle structure. Skim milk (0.2 ml) was added to 2 ml of fixative at room temperature and reacted for 30 min with occasional stirring. The final pH of the skim milk-fixative mixture was 5.90, 5.88, 6.58, and 6.74, respectively, for the reagents. The fixed skim milk was diluted to 10 ml with distilled water and centrifuged at 96,000 *g* for 30 min. The pellet was suspended in distilled water and recentrifuged. The pellet was again dispersed, with difficulty, in distilled water to the desired concentration and a drop placed on a collodion film to air-dry overnight.

Following the procedure of Rose and Colvin (5), skim milk was diluted 1:100 in 0.01 M CaCl₂ and deposited on a glass slide by dipping. The specimen was shadowed with platinum, carbon deposited vertically, and mounted on

Received for publication June 7, 1968.

¹ Eastern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture.

grids for examination under the electron microscope. This procedure departs from the technique of Rose and Colvin, who replicated the specimen in formvar film, removed the specimen with dilute ammonium hydroxide, and finally shadowed the replica.

Skimmilk fixed in 4% glutaraldehyde (0.2 ml:2 ml) was diluted 1:100 in 0.01 M CaCl_2 or distilled water and applied to a glass slide by dipping. The slide was air-dried, shadow-cast with platinum at a 3:1 angle (18.5°). A heavy layer of carbon was deposited vertically to support the specimen.

From our observations on fixation and dispersion techniques, the following procedure has been developed:

1. Dip a clean glass microscope slide in a 0.1% collodion solution in amyl acetate. Air-dry. Deposit a thin film of carbon on the slide in an evaporator, followed by a 1-min exposure to a high-voltage glow discharge at a $50\text{-}\mu$ vacuum.
2. Fix the skimmilk in a 1.0% glutaraldehyde solution for 15 min. The recommended volumes are 0.2 ml of skimmilk to 2 ml of fixative.
3. Dilute the fixed skimmilk with distilled water (1:10-1:50).
4. Dip the prepared glass slide into the diluted skimmilk and hang vertically to air-dry. Blot off excess solution at bottom of slide with filter paper.
5. Place glass slide with dispersed skimmilk in the evaporator and shadow-cast with platinum from platinum-carbon pellet at desired angle.
6. Remove glass slide, score the film, and float on to a clean water surface. Pick up the film on copper grid for examination in the electron microscope.

The reasons for the slide pretreatment and other details are discussed under Results.

An RCA EMU-3G electron microscope,² operating at 100 kv and using a $50\text{-}\mu$ objective aperture, was employed in this study. All micrographs were made at original magnification of $7,800\times$, photographically enlarged, and are reproduced as negative images. Measurements for micelle size distribution were made on photographic enlargements at $59,500\times$. Particles below 170\AA were not included in the measurements because of the uncertainty of whether or not they were micelles. Some flattening

² Mention of commercial items is for your convenience and does not constitute an endorsement by the U.S. Department of Agriculture over others not mentioned.

of the micelles occurred upon drying, but no correction was made for this.

Results

A control sample of skimmilk, unfixed but otherwise subjected to the same centrifuging steps described under Experimental Procedures, is shown in Fig. 1a. The casein micelles appear swollen, much flattened, and aggregated. Micelles fixed in 2% osmium tetroxide, as shown in Fig. 1b, appear nearly spherical as evidenced from shadow lengths. In addition, a coarse and uneven micelle surface is evident. The micelles fixed in 4% glutaraldehyde, Fig. 1c, present an appearance similar to micelles fixed in osmium tetroxide. Hostettler and Imhoff (2) advocated 0.4-0.8% formaldehyde fixation for 24 hr for preservation of micelle structure. But under the experimental conditions of 30 min fixation, little or no preservation of micelle structure is obtained. Fig. 1d shows micelles fixed in 1% formaldehyde. They are swollen and somewhat flattened, as judged from shadow lengths. When 0.1% formaldehyde is used, Fig. 2a, micelles obtained are almost identical to the unfixed control micelles of Fig. 1a. For short-time fixation, osmium tetroxide and glutaraldehyde fixation retain much more three-dimensional structure than formaldehyde. But centrifuging steps required to remove excess fixative result in a tightly packed pellet most difficult to resuspend. Accurate measurement of the individual micelle for size distribution studies is not possible.

Rose and Colvin (5) have reported on size distribution of casein micelles. Their method for dispersion of casein micelles eliminates the problem of aggregation. Fig. 2b shows casein micelles prepared by their CaCl_2 dipping technique, except the replication procedure was eliminated and the micelles examined directly in the electron microscope. Although little aggregation is found, micelle fine structure is not as apparent as when fixed with osmium tetroxide or glutaraldehyde.

Since osmium tetroxide is so highly toxic, glutaraldehyde fixation followed by the slide-dipping technique was tried. The skimmilk was fixed in 4% glutaraldehyde diluted in 0.01 M CaCl_2 and dispersed by slide dipping. Excellent results were obtained with good dispersion and retention of micelle structure. Since the micelle structure is already stabilized by glutaraldehyde fixation, the need for the CaCl_2 became questionable. In Fig. 2c are shown glutaraldehyde-fixed micelles, diluted in dis-

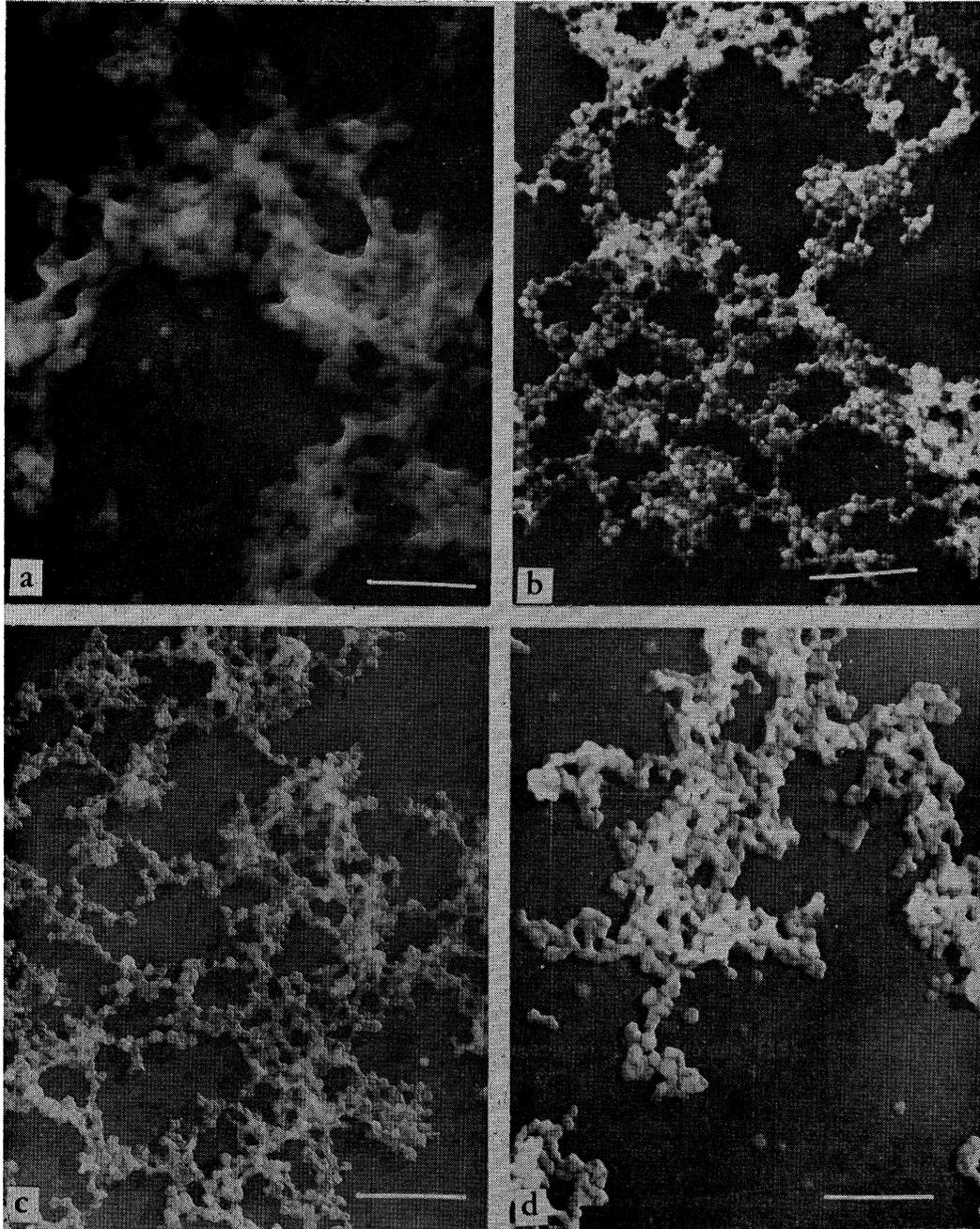


FIG. 1. Casein micelles. a. Unfixed. b. Fixed in 2% osmium tetroxide. c. Fixed in 4% glutaraldehyde. d. Fixed in 1% formaldehyde.

Micelles in Fig. 1a-d and 2a were centrifuged at 96,000 *g* for 30 minutes, washed, recentrifuged, dispersed in water, and air-dried on collodion support grids. The marker represents one micron.

tilled water and dispersed by slide dipping. Glutaraldehyde is not electron-dense and apparently does not contribute to the background of the micrographs. Hence, the removal of unreacted glutaraldehyde is unnecessary.

While the results are gratifying, close inspection of Fig. 2c reveals a prominent dense ring or halo effect around each micelle. This artifact was traced to the heavy layer of carbon deposited after shadow casting to stabilize

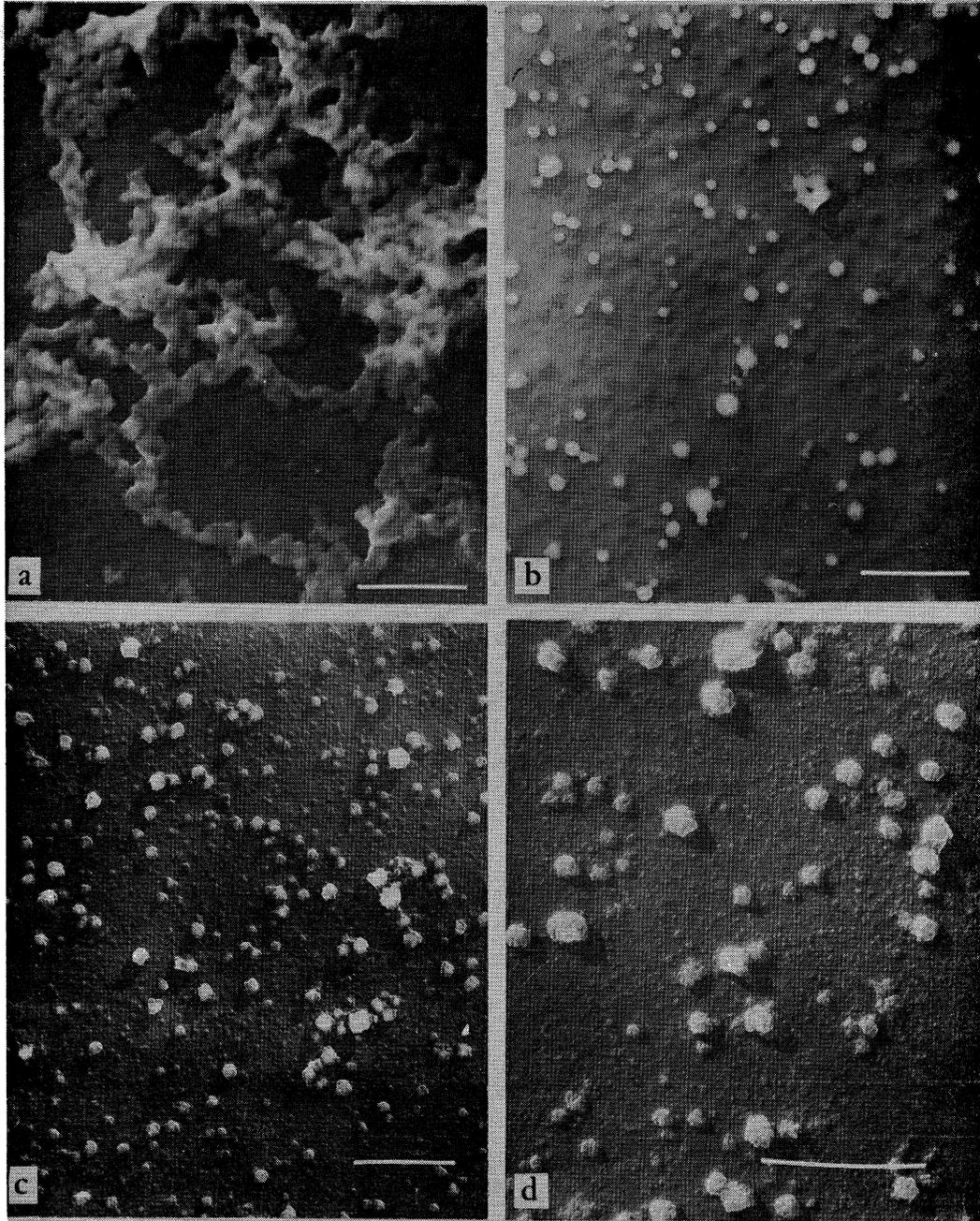


FIG. 2. Casein micelles. a. Fixed in 0.1% formaldehyde. b. Unfixed, dispersed in 0.01 M CaCl_2 on glass slide, carbon support. c. Fixed in 4% glutaraldehyde, dispersed in distilled water on glass slide, carbon support. d. Fixed in 1% glutaraldehyde, dispersed in distilled water on collodion-carbon-pre-coated glass slide.

The marker represents one micron.

the support film. In addition, it was difficult to separate the carbon film from the glass slide. Evidently the CaCl_2 aids in the stripping procedure. The difficulty in stripping the car-

bon film was eliminated by first coating the glass slide with a thin film of collodion (0.1% collodion in amyl acetate). The halo effect, due to heavy carbon, was eliminated by laying

down a thin film of carbon on the collodion-coated slide before slide dipping and then using platinum-carbon pellets for shadow casting. Enough carbon is deposited to stabilize the film in the electron microscope. Using a carbon-collodion-coated slide for dipping created another problem. The hydrophobic character of the carbon film prevented adequate dispersion of the micelles. This problem was solved by subjecting the carbon-collodion slide to a glow discharge for 1 min at pressure of 50μ in the evaporator (8), which changes the character of the film to hydrophilic. Results of these steps are shown in Fig. 2d, at somewhat higher magnification. The individual micelles are well preserved, well dispersed with no aggregation, and show considerable fine surface structure.

The background noted in Fig. 2d (glutaraldehyde-fixed micelles without ultracentrifugation) and absent in Fig. 1a-d and 2a (fixation with ultracentrifugation) reflects the importance of specimen preparation, in this case centrifugation, on the resulting micrographs. Evidently, centrifugation of fixed micelles, while eliminating the background material, does not reflect the complete picture of the existence of the smaller particles in the milk system. Furthermore, while the background is limited by the glass surface, carbon evaporation and glow discharge contribute nothing. However, platinum shadowing contributes to the fine granulation of the background.

With a technique now available to better retain casein micelle form and size, measurements on individual micelles could be undertaken with greater confidence in their validity. As reported by others, it is quite difficult to ascertain whether the small particles are indeed micelles. Therefore, particles less than 170 \AA were not included in our tabulation. Inspection of the graph (Fig. 3) shows a size distribution for Guernsey milk micelles of 500 to $2,500 \text{ \AA}$. In this investigation, 75% of the micelles have diameters of $1,000$ to $1,700 \text{ \AA}$.

Discussion

Brief fixation with osmium tetroxide and formaldehyde has been evaluated and compared with a relatively new fixative, glutaraldehyde. Glutaraldehyde fixation coupled with the slide-dipping technique of Rose and Colvin has resulted in a rapid reproducible method for stabilizing casein micelles for electron microscopy. Much more structure of the casein micelle is observed by direct observation, which eliminates the time-consuming replication steps, as well as possible artifacts introduced by repli-

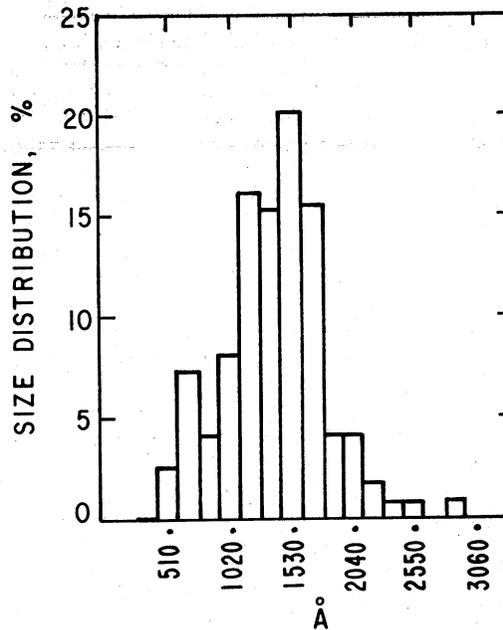


FIG. 3. Size distribution of glutaraldehyde-fixed micelles from fresh skim milk.

cation. The most significant advantages of our procedure are the use of nontoxic glutaraldehyde instead of the highly toxic osmium tetroxide and the elimination of the centrifuging steps of Shimmin and Hill (7).

Recently, a highly purified glutaraldehyde solution³ has become available. The purity of this material has been assessed and its fixing properties determined on a model system by Anderson (1). He found this glutaraldehyde to be the most pure material commercially available. With it, the time of fixation has been reduced to 15 min, and a 1% glutaraldehyde solution, instead of a 4% solution previously used, is adequate for micelle stabilization. Also, when the skim milk-glutaraldehyde ratio is 1:10, the pH of the mixture remains at the pH of milk, 6.6, instead of 5.88 obtained with the 4% glutaraldehyde. Since the casein micelle system is highly dispersed, the time of fixation is quite short as compared with fixation of intact tissue, where penetration may be slow. Consequently, the time to completely process milk samples is 2 hr or less, a considerable improvement over other methods.

The data obtained on micelle size distribution are similar to those of Nitschmann (3), who reported size distributions on micelles using

³ Ladd Research Associates, Inc., Burlington, Vermont 05401.

0.01 M CaCl_2 and formaldehyde as fixing agents. He found a shift toward similar sizes with formaldehyde-fixed micelles as compared with CaCl_2 -dispersed micelles. Both methods show approximately 58% of the micelles with diameters of 800 to 1,000 Å, while we report 75% of the micelles with diameters of 1,000 to 1,700 Å. Nitschmann did not report micelle diameters below 400 Å, whereas Rose and Colvin measured every particle observed in five individual milks. Consequently, their size distributions show a predominance of particles smaller than 250 Å (35 to 64%). Noteworthy is the fact that they report very few micelles larger than 1,500 Å. Our results with 12 to 15% of the micelles larger than 1,600 Å agree with the findings of Nitschmann on the size distribution of casein micelles with 11 to 19% larger than 1,600 Å.

The procedure for stabilizing casein micelle structure has been applied to pooled milks, milks from individual cows, evaporated milks, and artificial micelle systems. Successful preparations for the electron microscope have been obtained in all cases.

References

- (1) Anderson, P. J. 1967. Purification and quantification of glutaraldehyde and its effect on several enzyme activities in skeletal muscle. *J. Histochem. and Cytochem.*, 15: 652.
- (2) Hostettler, H., and K. Imhof. 1952. Electron optische Untersuchungen über die submikroskopische Struktur von Milch und Milch-erzeugnissen. *Landwirtsch. Jahrd. Schweiz* (new series), 1: 307.
- (3) Nitschmann, H. 1949. Elektronenmikroskopische Grossenbestimmung der Calcium-caseinatteilchen in Kuhmilch. *Helv. Chim. Acta*, 32: 1258.
- (4) Peters, I. I., and J. W. Dietrich. 1954. Electron microscope observations on rennet action upon skim milk. *Texas J. Sci.*, 6, no. 4: 442.
- (5) Rose, Dyson, and J. R. Colvin. 1966. Appearance and size of micelles from bovine milk. *J. Dairy Sci.*, 49: 1091.
- (6) Sabatini, D. D., K. Bensch, and R. J. Barnett. 1963. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. *J. Cell Biol.*, 17: 19.
- (7) Shimmin, P. D., and R. D. Hill. 1965. Further studies on the internal structure of the casein micelles of milk. *Australian J. Dairy Technol.*, 20: 119.
- (8) Stephens, R., G. Schidlovsky, and S. Kuzmic. 1967. The use of the "microdiluter" and "ionized" carbon films for negative staining. *Proc. 25th Ann. Meeting Electron Microscopy Soc. of America*, Abstr. 124.