HIGH RESOLUTION SCANNING ELECTRON MICROSCOPY OF ISOLATED AND *IN SITU* CYTOSKELETAL ELEMENTS

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ABSTRACT

Evidence is presented that cytoskeletal structures (actin filaments, intermediate filaments, and microtubules) can be resolved by scanning electron microscopy after osmium impregnation of biological material, using thiocarbohydrazide as a ligand, followed by critical-point drying. These different classes of filaments or tubules can be identified both as purified protein polymers and as structured organelles within cryofractured or detergent-extracted cells.

KEY WORDS microfilaments · intermediate (10-nm) filaments · microtubules · cytoskeleton · scanning electron microscopy · myogenesis

The three-dimensional organization of cytoskeletal elements has become a topic of wide interest in the field of cell biology. Structural studies in this field have relied principally on three approaches: (*a*) high-voltage transmission electron microscopy (2, 26); (*b*) conventional transmission electron microscopy (TEM) with serial sectioning (3) or negative staining (1, 3, 22); (*c*) immunohistochemical techniques, mainly at the light microscopic level (5, 6, 16). Although three-dimensional information is readily obtained by secondary emission, scanning electron microscopy (SEM), this method has not been exploited for studies of cytoskeletal structure, largely because of inadequate resolution. Several commercial instruments achieve resolutions of 3–5 nm, yet routine specimen coating techniques generally add a 15–25 nm layer of metal, which limits the use of this approach for high resolution of most subcellular materials.

As part of an investigation relating cytoskeletal elements to the assembly of myofibrils within embryonic chick muscle, we have compared several coating techniques used in preparing specimens for SEM. In this paper, we present evidence that at least one of these methods permits the resolution by SEM of microtubules, intermediate filaments, and microfilaments, both as purified polymers and as structured organelles within cells. When used in combination with either cryofracture or detergent extraction, it is now feasible to visualize cytofilamentous structures by SEM and to take advantage of the great depth of field intrinsic to the instrument.

MATERIALS AND METHODS

Preparation of Proteins

Rabbit skeletal muscle F-actin was a gift from Dr. Roger Lucas, and was used in a buffer containing 0.1 M KCl, 2 mM $MgCl_2$, 2 mM EGTA, 10 mM Tris-maleate, pH 7.0. The preparation exhibited a single band of ~43,000 daltons when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (15).

Porcine brain microtubule proteins were a gift from Dr. David Soifer; microtubules were obtained from this preparation by two cycles of polymerization-depolymerization (20) and finally assembled in 0.5 mM $MgCl_2$, 1 mM EGTA, 1 mM GTP, 4 M glycerol, 50 mM morpholinoethane sulfonic acid (MES), pH 6.9. This sample consisted primarily of α- and β-tubulins with a small amount of several microtubule-associated proteins.

Intermediate filament protein ("skeletin") was purified from chicken gizzard smooth muscle according to the method of Small and Sobieszek (22) and polymerized into filaments by dialysis against 10 mM sodium acetate, 1 mM cysteine, pH 4.0. By SDS-PAGE, it consisted of primarily a 55,000-dalton peptide with a small amount of actin.

Preparation of Samples for Scanning Electron Microscopy

After appropriate dilution, a drop of the filament preparation was placed on a gelatin-coated Thermanox

plastic coverslip (Lux Scientific Corp., Newbury Park, Calif.), allowed to adhere for ~30 s, and thoroughly rinsed with the sample buffer. Each coverslip was then immersed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4, for 15 min, rinsed, and further immersed in 1% OsO_4 in the same buffer for 15 min, at ambient temperature.

Further osmium impregnation was carried out according to the Malick and Wilson modification (18) of the method of Kelley et al. (12), with further adjustments to accommodate thin specimens and cell monolayers on coverslips. The filament preparations on coverslips were rinsed for 15 min with five to seven changes of distilled water, and then treated with a freshly prepared and filtered, saturated solution of thiocarbohydrazide (TCH, Polysciences, Inc., Warrington, Penn.) in water. After rinsing in water as described above, samples were reacted again for 15 min with 1% OsO_4 in water. The TCH-osmium cycle was repeated once more, and the samples were then dehydrated in a series of acetone (25, 50, 70, 90, and 100%) and critical-point dried from liquid CO_2.

Whole cell preparations were derived from embryonic chick muscle cultures (4) and processed for SEM by a method developed in this laboratory (Ip and Fischman, manuscript in preparation). Briefly, cultures grown on plastic coverslips were fixed in glutaraldehyde, OsO_4-TCH-treated as described above, and dehydrated through a series of acetone followed by a series of amyl acetate in acetone. A coverslip was then removed from 100% amyl acetate, inverted but not drained, and carefully placed, cell-side down, onto a clean copper plate. The entire "sandwich" was plunged into liquid nitrogen, and allowed to equilibrate for a few minutes. The coverslip was then peeled off under liquid nitrogen and transferred quickly into 100% amyl acetate at room temperature. Critical point drying was then performed conventionally from liquid CO_2.

Scanning Electron Microscopy

Scanning electron microscopy was performed with a JEM 100C instrument equipped with a high resolution scanning device (ASID-4D) and a side-entry goniometer. An accelerating voltage of 40 kV was used. Images were recorded at a specimen tilt of 20°–30° from the horizontal axis, on Kodak PXP-120 film.

Transmission Electron Microscopy

Filament preparations were negatively stained on Parlodion-carbon-coated grids with 1% uranyl acetate without prior fixation and viewed in the JEM 100C instrument at 80 kV. Images were recorded on Kodak 4463 film.

RESULTS

As a prelude to our SEM study of cytoskeletal elements within cryofractured or detergent-extracted cells, we decided to examine samples of purified F-actin, intermediate (10 nm) filaments, and microtubules by negative staining and SEM. The negatively stained TEM images of these preparations have been well-documented (3, 9, 20–23) and would serve as a basis for interpreting our SEM images.

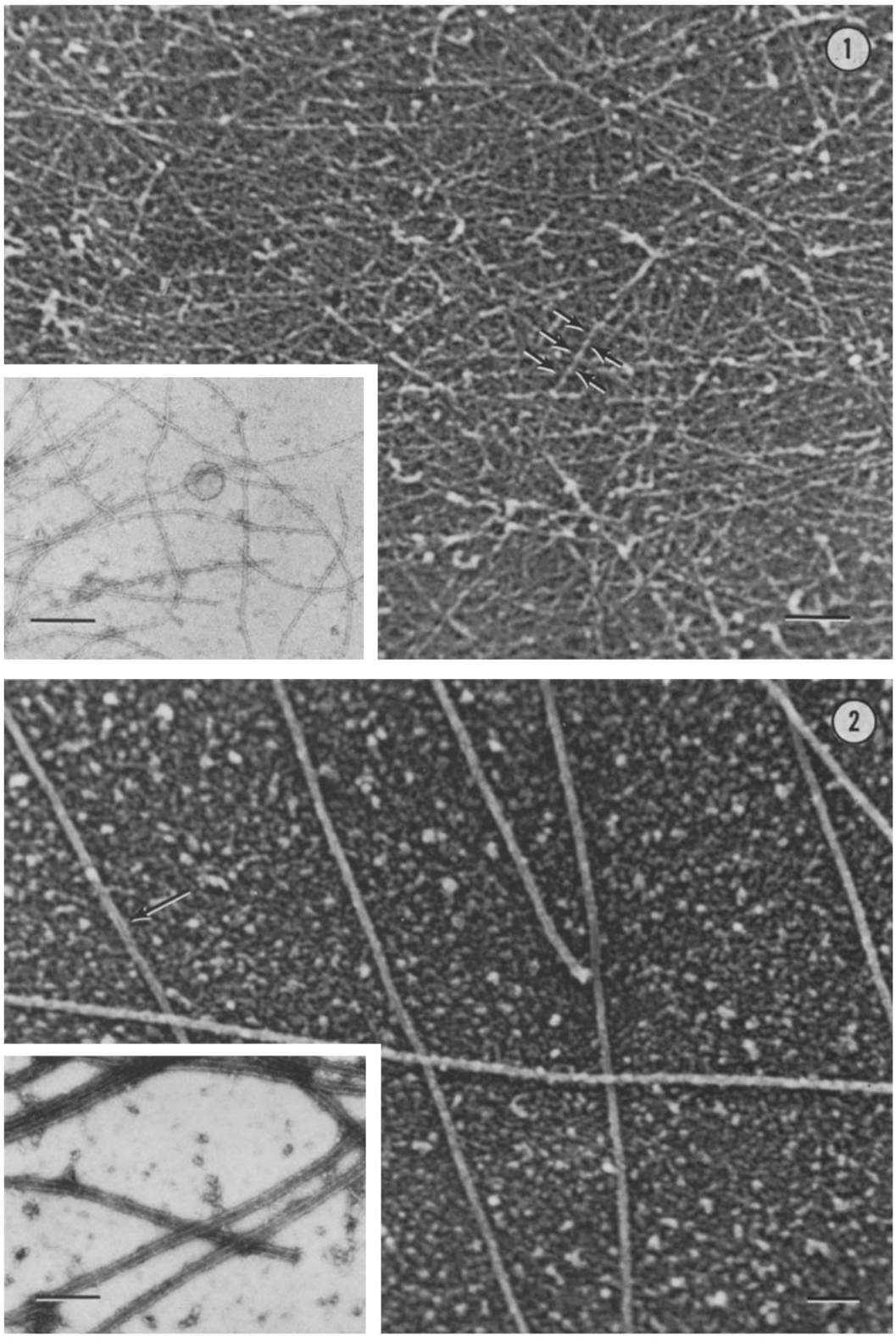
When F-actin is deposited on coverslips, processed by the osmium-TCH procedure, and examined at instrumental magnifications of 20,000–50,000 by SEM (Fig. 1), the filaments exhibit a diameter of 14–16 nm, which is approximately twice the width of actin filaments examined by negative stain TEM (Fig. 1, *inset*). The filaments tend to be straight or gently curved, unbranched, and often appear beaded (arrows, Fig. 1). Studies are in progress to determine whether this beadedness is related to the helical substructure of the F-actin filament.

Polymerized microtubules are also resolved by SEM after the osmium-TCH procedure (Fig. 2). The diameter of these structures is 32–37 nm as compared to a diameter of 25–28 nm after uranyl acetate negative staining (Fig. 2, *inset*). By SEM, the microtubules exhibit an electron-transparent core, presumably the result of beam penetration through the upper layer of protofilaments (arrow, Fig. 2). Some microtubules appear rough-surfaced but no obvious periodicity has been detected. The granular texture of the background in both SEM and TEM images may correspond to intermediates of microtubule assembly (21) which have attached to the gelatin-coated support.

Intermediate filaments which have been reconstituted from chicken gizzard "skeletin" are shown in Fig. 3. In agreement with previous reports (3, 22), these filaments have a complex, branched appearance in which considerable variability of

FIGURE 1 Scanning electron micrograph of purified rabbit skeletal muscle F-actin, prepared for SEM by the OsO_4-TCH method. Individual filaments, 14–16 nm in diameter, are clearly resolved. Occasionally, beaded structures are seen along the filaments (arrows). Bar, 0.2 μm. × 50,000. (*inset*) Transmission electron micrograph of F-actin, negatively stained with uranyl acetate. Bar, 0.2 μm. × 50,000.

FIGURE 2 Scanning electron micrograph of porcine brain microtubules purified and assembled by two cycles of polymerization-depolymerization. Of interest is the hollow appearance of the microtubules (arrow) presumably due to beam penetration through the upper layer of protofilaments. Bar, 0.2 μm. × 40,000. (*inset*) Microtubules as seen by negative-stain TEM. Bar, 0.2 μm. × 50,000.



filament diameters is evident. Presumably, this variability reflects extensive side-to-side aggregation of individual filaments. In areas where single filaments are resolved, their diameters measure 10–12 nm by negative-stain TEM and 18–22 nm by SEM.

Having satisfied ourselves that the osmium-TCH procedure permitted the visualization of individual cytofilaments in the SEM, we proceeded to examine cultured embryonic chick muscle cells, the interiors of which had been exposed by cryofracturing in liquid nitrogen-frozen amyl acetate following fixation and osmium impregnation. Within myotubes (Fig. 4), it is possible to resolve microfilaments, microtubules and intermediate filaments, based on their orientation and individual diameters. Large vesicular structures (arrowheads, Fig. 4), which we assume to be membrane-enclosed, may represent immature elements of the sarcoplasmic reticulum. Thin-section studies of this material are in progress.

DISCUSSION

We have demonstrated that, with suitable specimen preparation, it is feasible to resolve and identify the various classes of filamentous structures within a cell or cytoskeleton by SEM, and thus obtain direct three-dimensional information regarding their organization *in situ*. Previous studies have pointed to this possibility (13, 14, 17) by showing that isolated organelles are amenable to high-resolution SEM observation. However, except for studies of the erythrocyte ghost (7), there have been few reports on high-resolution SEM analysis of cytoplasmic filament arrays.

In our experience, routine sputter coating severely hampers the use of SEM at magnifications necessary for the resolution of cytoskeletal elements. Indeed, several authors have pointed out that some of the features observed in sputter coated specimens at high magnifications are, in fact, artifactual (8, 10). In this report, we demon-

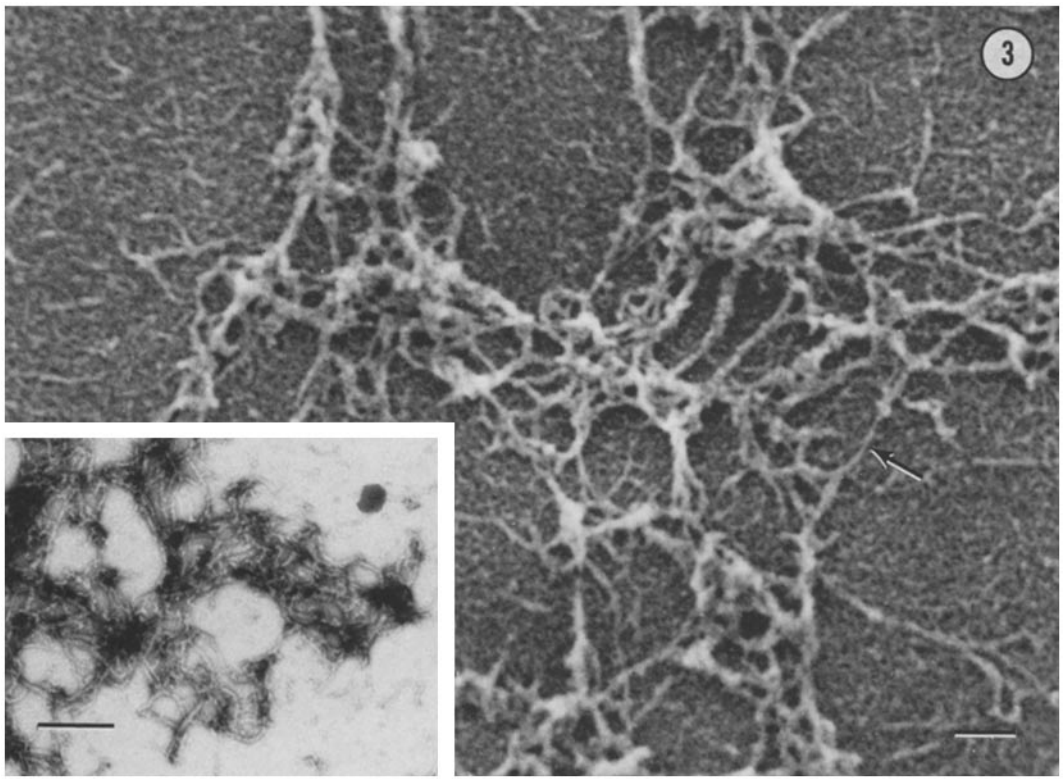


FIGURE 3 Scanning electron micrograph of intermediate filaments assembled from partially purified "skeletin." The filaments tend to be aggregated and branched, making diameter measurement difficult. The apparently single filament shown by the arrow has a diameter of 18.7 nm. Bar, 0.2 μm. × 40,000. (*inset*) In vitro assembled intermediate filaments as seen by negative-stain TEM. Bar, 0.2 μm. × 50,000.

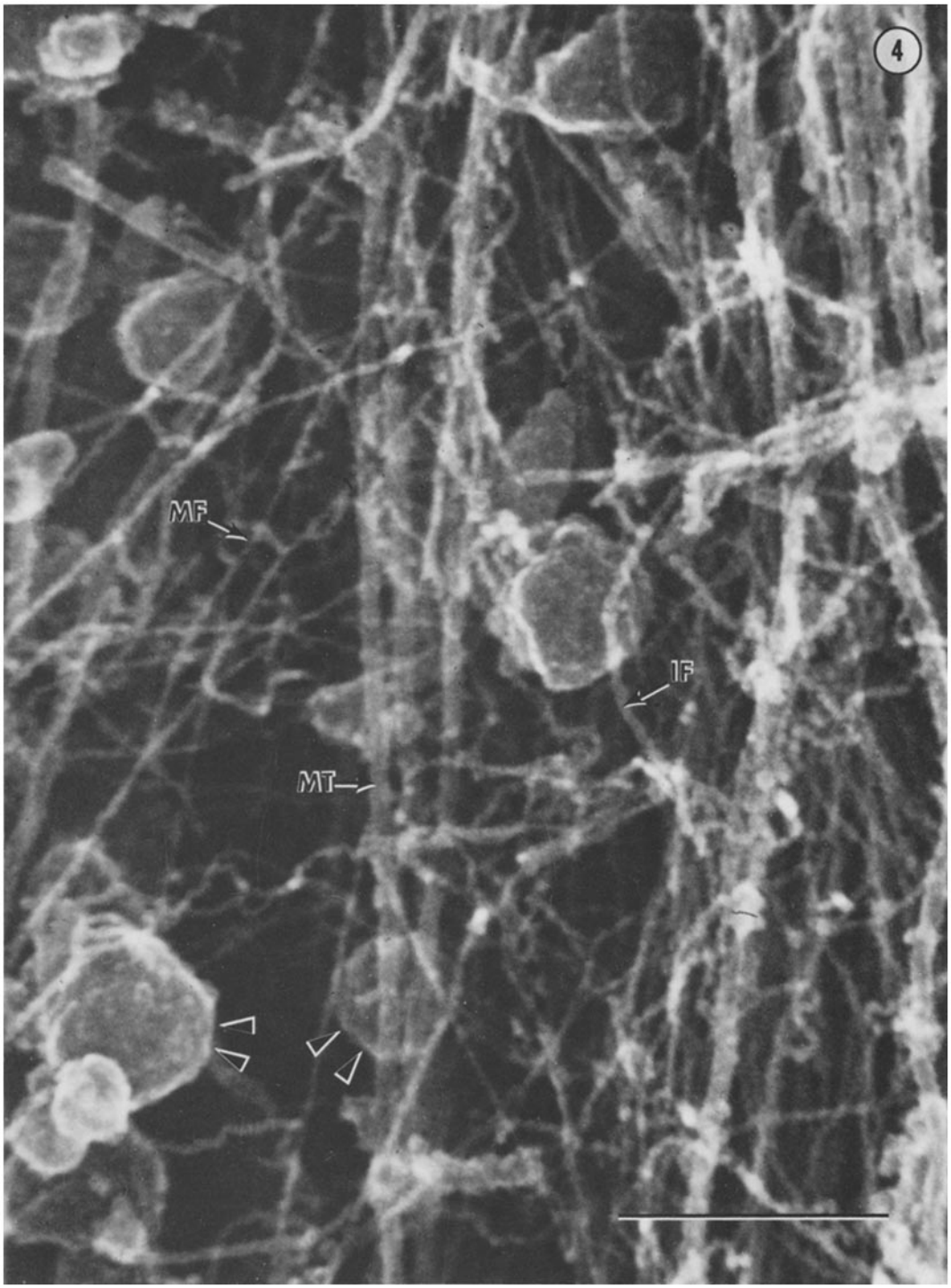


FIGURE 4 Scanning electron micrograph of a myotube from a 1-wk-old chick skeletal muscle culture, processed as described in Materials and Methods. The long axis of the myotube runs from top to bottom in the micrograph. A portion of the myotube was removed during the fracturing procedure, exposing a number of cytoskeletal structures: (*MT*) microtubules; (*IF*) filament 18.7 nm in diameter, presumed to be intermediate filament; (*MF*) filament of 13.8 nm, believed to be actin microfilament. The vesicular bodies (arrowheads) may be elements of the sarcoplasmic reticulum. No myofibrillar components are seen in this particular field. Bar, 0.5 μm. × 80,000.

strate that the osmium-TCH technique provides a reasonable alternative to conventional metal coating. We recognize that a metal thickness of 3–4 nm can also be obtained by high vacuum evaporation. However, we have consistently found it difficult to obtain uniform metal coats by this technique with highly contoured specimens such as cytoskeletons, even with the use of a rotary tilt stage.

Assuming that the "native" diameter of actin filaments, as measured in negatively stained TEM images, is 7 nm (9) and that the osmium-TCH layer is distributed uniformly over the filaments, and further, that there is no significant shrinkage of specimen or coating as a result of dehydration and critical point drying, we calculated the thickness of the coating to be 3–4 nm. If shrinkage is significant (14), this value may have to be revised upward. Transmission electron microscopy of osmium-TCH-coated, dried, re-embedded and thin-sectioned material also confirmed that a coat of this thickness is routinely obtained (reference 11; Ip and Fischman, manuscript in preparation).

Fig. 4 represents our initial attempt at "looking inside the cell" by SEM using the osmium-TCH technique. The results so far have been encouraging. In cultured chick embryo fibroblasts, skeletal, and cardiac muscle cells, we have been able to demonstrate, after various extraction procedures, an insoluble cytoskeletal network composed primarily of actin and 10-nm filaments (11), similar to those seen by others using immunofluorescence and TEM techniques (1, 6, 24, 25). Pudney and Singer (19) have reported similar results. We conclude that the osmium-TCH technique, particularly when used in conjunction with specific localization techniques (e.g., antibody labeling), should provide a valuable alternative to conventional or high-voltage TEM where three-dimensional information is required.

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