ACTIN FILAMENT DESTRUCTION BY OSMIUM TETROXIDE

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ABSTRACT

We have studied the destruction of purified muscle actin filaments by osmium tetroxide (OsO_4) to develop methods to preserve actin filaments during preparation for electron microscopy. Actin filaments are fragmented during exposure to OsO_4. This causes the viscosity of solutions of actin filaments to decrease, ultimately to zero, and provides a convenient quantitative assay to analyze the reaction. The rate of filament destruction is determined by the OsO_4 concentration, temperature, buffer type and concentration, and pH. Filament destruction is minimized by treatment with a low concentration of OsO_4 in sodium phosphate buffer, pH 6.0, at 0°C. Under these conditions, the viscosity of actin filament solutions is stable and actin filaments retain their straight, unbranched structure, even after dehydration and embedding. Under more severe conditions, the straight actin filaments are converted into what look like the microfilament networks commonly observed in cells fixed with OsO_4. Destruction of actin filaments can be inhibited by binding tropomyosin to the actin. Cross-linking the actin molecules within a filament with glutaraldehyde does not prevent their destruction by OsO_4. The viscosity decrease requires the continued presence of free OsO_4. During the time of the viscosity change, OsO_4 is reduced and the sulfur-containing amino acids of actin are oxidized, but little of the osmium is bound to the actin. Over a much longer time span, the actin molecules are split into discrete peptides.

KEY WORDS actin · fixation · microfilament network · osmium tetroxide

An unstated assumption in many electron microscope studies of cytoplasmic actin filaments is that these filaments are adequately preserved by the fixation procedures employed. We questioned the validity of this assumption after discovering that osmium tetroxide (OsO_4) destroys the birefringence of pellets of actin filaments (37). We found that this change in birefringence is accompanied ultrastructurally by the conversion of long, straight, and unbranched actin filaments into branching meshworks of fibrous material, similar in appearance to the "microfilament networks" commonly observed in fixed cells (15, 32, 48, 58).

A similar change in actin filament structure may occur during fixation of cells with OsO_4. For example, whereas filamentous networks are observed in the cortex of amebas fixed with glutaraldehyde and then OsO_4 (39), straight, unbranched actin filaments are seen in the cortex of similar cells by negative staining (11). The same is true of fixed, sectioned (58) vs. negatively stained (26) nerve growth cone and fixed, sectioned (1) vs. negatively stained (7) sea urchin egg cortex. This is a cause for concern because virtually all that we know about the ultrastructure of actin filaments in non-muscle cells comes from electron micrographs of thin sections of cells fixed with OsO_4. If the microfilament networks observed inside cells are a fixation artifact, we are far from

understanding how actin filaments participate in cellular structure and movement.

Clearly, not all actin-containing filaments are destroyed during preparation for electron microscopy. The actin-containing filaments in skeletal muscle (20), the intestinal brush border (35), sperm acrosomal processes (52, 54), and the stress fibers of cultured cells (14) all appear straight and unbranched like negatively stained actin filaments (21). However, in all of these filaments actin may be associated with other proteins, such as tropomyosin and/or α-actinin, and tropomyosin, at least, stabilizes actin filaments during treatment with OsO_4 (37, 51). Another possible explanation for the excellent preservation of actin filaments in the studies cited above is simply that the filaments were well-fixed by the particular methods employed.

In an effort to understand and avoid the deleterious effects of OsO_4 on actin filaments during fixation, we have examined in detail the interaction of OsO_4 with purified actin. These quantitative studies have revealed that, under proper conditions of pH, buffer type and concentration, temperature, and OsO_4 concentration, it is possible to preserve the structure of purified actin filaments during preparation for electron microscopy. Brief accounts of some of this work have appeared in abstract form (33, 34).

MATERIALS AND METHODS

Materials

We obtained chemicals from the following sources: OsO_4 (Stevens Metallurgical Corp., New York); "pure, EM grade" glutaraldehyde, 10% solution sealed in ampules under inert gas (Electron Microscopy Sciences, Fort Washington, Pa.); "EM grade" glutaraldehyde, 25% solution (TAAB Laboratories, Reading, England); embedding resins and "ultrapure" glutaraldehyde, 8% solution sealed in ampules under inert gas (Tousimis Research Corp., Rockville, Md.); reagent grade thiourea (Fisher Scientific Co., Pittsburgh, Pa.); sodium cacodylate, grade 1 imidazole, piperazine-*N*,*N*-bis(2-ethane sulfonic acid) (PIPES), Tris base, ATP, dithiothreitol (Sigma Chemical Co., St. Louis, Mo.); Nessler's reagent (BDH Chemicals Ltd., Poole, England).

Protein Preparation

We prepared actin from rabbit or chicken muscle by a minor modification of the method of Spudich and Watt (49), employing one sedimentation of the filaments from 0.8 M KCl. The actin was >98% pure judging from electrophoresis in sodium dodecyl sulfate. Monomeric actin was stored at 4°C in 2 mM Tris-Cl, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM dithiothreitol, and 0.02% sodium azide (pH 8). Rabbit muscle tropomyosin was purified by Bailey's method (5), using a single ammonium sulfate precipitation. Tropomyosin was stored frozen in water.

Cell Culture

Acanthamoeba castellanii was grown in liquid culture (38).

Biochemical Methods

We measured viscosity in Cannon-Manning semi-micro-viscometers (Cannon Instruments, State College, Pa.), size 150, which use 1-ml samples and have buffer flow times of about 30 s at 20°C. Constant temperature was maintained by immersion in a water bath. Viscosity is expressed as the specific viscosity

$$\eta_{sp} = \frac{\text{sample flow time}}{\text{buffer flow time}} - 1.$$

Gel electrophoresis was carried out in 10% polyacrylamide gels with a buffer consisting of 25 mM Tris-glycine (pH 8.6) and 0.1% sodium dodecyl sulfate (50). Samples were prepared by boiling in 2.5 mM Tris-glycine, 1% sodium dodecyl sulfate, and 1% β-mercaptoethanol.

For amino acid analyses, samples were dissolved in 6 N HCl, flushed with N_2, evacuated, sealed, and hydrolyzed at 110°C for 18 h. The analysis was performed at the Harvard University Biological Laboratories Protein Chemistry Laboratory, on a Beckman 121 M amino acid analyzer with automatic integrator (Beckman Spinco, Palo Alto, Calif.). The columns were overloaded slightly to obtain accurate data on cysteine. Consequently, the concentrations of several of the amino acids were hand calculated from the absorbance at 570 nm.

To rapidly separate actin from free osmium compounds before amino acid analysis and gel electrophoresis, we used gel filtration on a 1.5 × 22-cm column of Sephadex G-25 medium, equilibrated with 50 mM sodium phosphate, 50 mM KCl, and 5 mM $MgCl_2$ (pH 7.0).

The concentrations of actin solutions were measured by their absorbances, using extinction coefficients of 1.09 ml/mg per cm at 280 nm and 0.65 ml/mg per cm at 290 nm (43). Tropomyosin concentration was measured at 280 nm, using an extinction coefficient of 0.3 ml/mg per cm (57). The concentration of protein in suspensions of intact amebas was estimated by the method of Hartree (17), using bovine serum albumin as a standard. Nitrogen was measured using Nessler's reagent by the method of Lang (27).

Preparation of Samples for Electron Microscopy

NEGATIVE STAINING: We diluted actin to 0.06 mg/ml with 50 mM sodium phosphate and 2 mM $MgCl_2$ (pH 7.0); immediately applied samples to Formvar- and

carbon-coated grids rendered hydrophilic by glow discharge; washed the grid with one drop of 100 mM KCl and 2 mM $MgCl_2$; and then stained with 1% uranyl acetate in water.

EMBEDDED ACTIN PELLETS: 0.5-mg actin filament samples were sedimented into hard pellets by centrifugation at 144,000 *g* for 45 min in a Ti-50 rotor. These pellets were ~ 8 mm in diameter and ~ 1.5 mm thick at their centers. The pellets were covered with 1–2.5% glutaraldehyde in various buffers for various lengths of time as described in Results. After twice rinsing the pellets for 2–5 min with 2 ml of buffer, we covered the pellets with 2 ml of buffered OsO_4 under conditions described in detail in Results. The pellets were then washed twice with 3 ml of water for 2–5 min at 0–4°C and rapidly dehydrated in a graded series of either acetone (30, 50, 75, 95, 100, and 100%) or ethanol (30, 50, 70, 95, 100, and 100%). The pellets were pried from the centrifuge tubes, transferred to glass tubes, and warmed to room temperature in the 100% solutions. After 100% ethanol, the pellets were treated twice with propylene oxide for 10 min. Then the pellets were infiltrated by immersion in a 1:1 solvent:Epon mixture for 3 h at room temperature and then in pure Epon mixture for at least 4 h. Infiltrated material was cured at 60°C for ~40 h. Thin sections were stained with 1% uranyl acetate and Reynolds lead citrate. We examined the grids in a Zeiss EM-10 kindly loaned to the Woods Hole Marine Biological Laboratory Physiology Course or a Siemens Elmiskop 1A.

OsO_4 Assays

OsO_4 concentration was determined by a colorimetric thiourea assay (2), using a freshly made solution of OsO_4 as a standard. 0.2-ml samples were mixed with 0.8 ml of 4.2 M HCl, and then centrifuged at 1,100 *g* for 1 min to remove any protein precipitate. An aliquot of the supernate containing from 2 to 350 nmol of OsO_4 was diluted to 0.9 ml with 4.2 M HCl and mixed with 0.1 ml of aqueous 10% thiourea. Pink color development was completed by incubation in a boiling water bath for 2 min. In protein-free samples, the absorbance at 480 nm was a linear function of OsO_4 concentration with an extinction coefficient of 3.18/μmol per cm. In samples containing protein, an orange-brown color developed which interfered with the measurement at 480 nm. In these cases, we measured the absorption spectrum of the sample between 350 and 750 nm with a Cary model 14 spectrophotometer (Cary Instruments, Fairfield, N. J.) and drew a corrected base line between A_{350} and A_{750}. The absorbance at 480 nm above this baseline was a linear function of OsO_4 concentration with an extinction coefficient of 2.74/μmol per cm. To measure osmium bound to protein, we followed the procedure of Beer et al. (6), which involves oxidation of reduced osmium to OsO_4 with sodium peroxide and colorimetric determination of OsO_4 with thiourea.

RESULTS

We examined the interaction of OsO_4 with purified actin filaments in three ways: first, we used viscometry to evaluate quantitatively the factors influencing the destruction of actin filaments by OsO_4. Second, we studied the reaction by measuring the amount of OsO_4 reduced by actin, the amount of OsO_4 bound to actin, and the chemical changes in actin exposed to OsO_4. Finally, we used this information to design a fixation procedure which includes OsO_4 but which employs conditions where actin filaments are not damaged. Because actin is destroyed very rapidly by OsO_4 under the conditions usually used to fix cells, we carried out our experiments with much milder conditions, to slow the reaction enough to collect good data. Generally, this was accomplished by using low concentrations of OsO_4.

Quantitative Analysis of Actin Filament Destruction by OsO_4

Viscometry proved to be a convenient quantitative method to measure the rate and extent of actin filament destruction by OsO_4. The viscosity of actin filament solutions decreases when exposed to OsO_4, following a biphasic time-course (Fig. 1). The initial phase of viscosity decrease is slow. It is followed by a rapid first-order decay in viscosity. Solution conditions influence the progress of both phases of the reaction but in general, the duration of the slow phase is influenced to a greater extent than the rate of either phase. The conditions tested are described in detail later.

Electron microscopy of negatively stained samples show that the decrease in viscosity of actin filament solutions in the presence of OsO_4 is due to the progressive fragmentation of the filaments (Fig. 2). Untreated actin filaments (0 min) are long, unbranched, and gently curved fibers whose solution has a high viscosity. Under the conditions of the experiment illustrated in Fig. 2, the slow phase of viscosity change lasts for 13 min, during which the integrity of the actin filaments is maintained. By 15 min, the viscosity is decreased by 36% and the filaments are shorter and contain sharp bends. After 45 min, the viscosity is decreased by 93% and only short fragments of filaments are present.

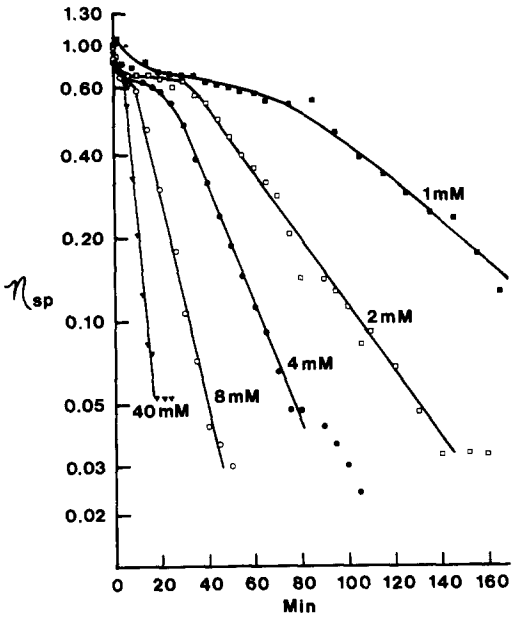

FIGURE 1 Influence of OsO_4 concentration on the time-course of viscosity changes of actin filament solutions. Actin filaments at a concentration of 0.5 mg/ml were reacted with 1, 2, 4, 8, or 40 mM OsO_4 in 50 mM sodium phosphate, 50 mM KCl, and 5 mM $MgCl_2$ (pH 7.0) at 20°C.

To test whether the destruction of actin filaments by OsO_4 requires the continued presence of free OsO_4, we rapidly separated the reactants near the onset of the fast phase of reduction in viscosity and found that the viscosity of the damaged actin filaments was relatively stable thereafter (Fig. 3). A parallel sample left in the presence of OsO_4 continued to decrease in viscosity. As discussed later, there are a few osmium molecules bound to each actin molecule by the time the reactants were separated in this experiment, but the presence of this bound osmium does not lead to further changes in viscosity.

Factors Influencing the Rate of Actin Filament Destruction by OsO_4

OSO_4 CONCENTRATION: The time-course of actin filament destruction is determined by the OsO_4 concentration (Fig. 1). The duration of the slow phase varies inversely with the OsO_4 concentration. At the high concentrations of OsO_4 commonly used to fix biological specimens (1% = 40 mM), there is a rapid, immediate, first-order decay in actin solution viscosity. At lower concentrations, actin filaments are stable for some time.

TEMPERATURE: The rate of loss of viscosity of actin solutions in the presence of OsO_4 is dependent upon the temperature (Fig. 4). In the experiment illustrated in Fig. 4, a high concentration of OsO_4 was used so that the viscosity measurements at low temperature could be made within a convenient period of time. As shown here and in a published experiment employing glutaraldehyde-fixed actin filaments (36), the time required to reach half the starting viscosity is about five to six times longer at 2°C than at 20°C.

BUFFER TYPE AND CONCENTRATION: The rate of decay of viscosity of actin filament solutions exposed to OsO_4 depends on the buffer type and concentration (Fig. 5). The most striking finding is that, in comparison with an unbuffered solution, sodium phosphate buffer inhibits filament destruction in a concentration-dependent fashion, whereas some other buffers, including imidazole, favor destruction. In additional experiments, which are not illustrated in Fig. 5, we found that the time-course of the viscosity change in 50 mM s-collidine (pH 7.0) is similar to that in 10 mM imidazole (pH 7.0) and that the reaction in 50 mM PIPES (pH 7.1) is similar to that in 50 mM cacodylate (pH 7.3). Although the duration of the slow phase varies from 0 min in imidazole to more than 30 min in 100 mM sodium phosphate, the rate of the fast phase is similar in most of the buffers tested. Addition of 0.5 mM dithiothreitol to 50 mM sodium phosphate (pH 7.0) does not change the time-course of the viscosity change.

HYDROGEN ION CONCENTRATION: The rate of actin filament destruction is affected by the pH of the solution (Fig. 6). Although the rate of the fast phase of the viscometric change is similar at each pH shown in Fig. 6, the slow phase is longer at neutral or acid pH's.

CROSS-LINKING WITH GLUTARALDEHYDE: Although glutaraldehyde covalently cross-links the actin molecules within an actin filament (28), actin filaments pretreated with glutaraldehyde are also destroyed by OsO_4. The time-course of the viscosity change of glutaraldehyde-pretreated actin filaments (Fig. 7) differs from that of native actin filaments in three ways: (*a*) there is a transient initial rise in viscosity, (*b*) the rapid first-order decay in viscosity begins sooner, and (*c*) at the end of the fast phase the viscosity plateaus at ~6% of the starting viscosity, a value substantially

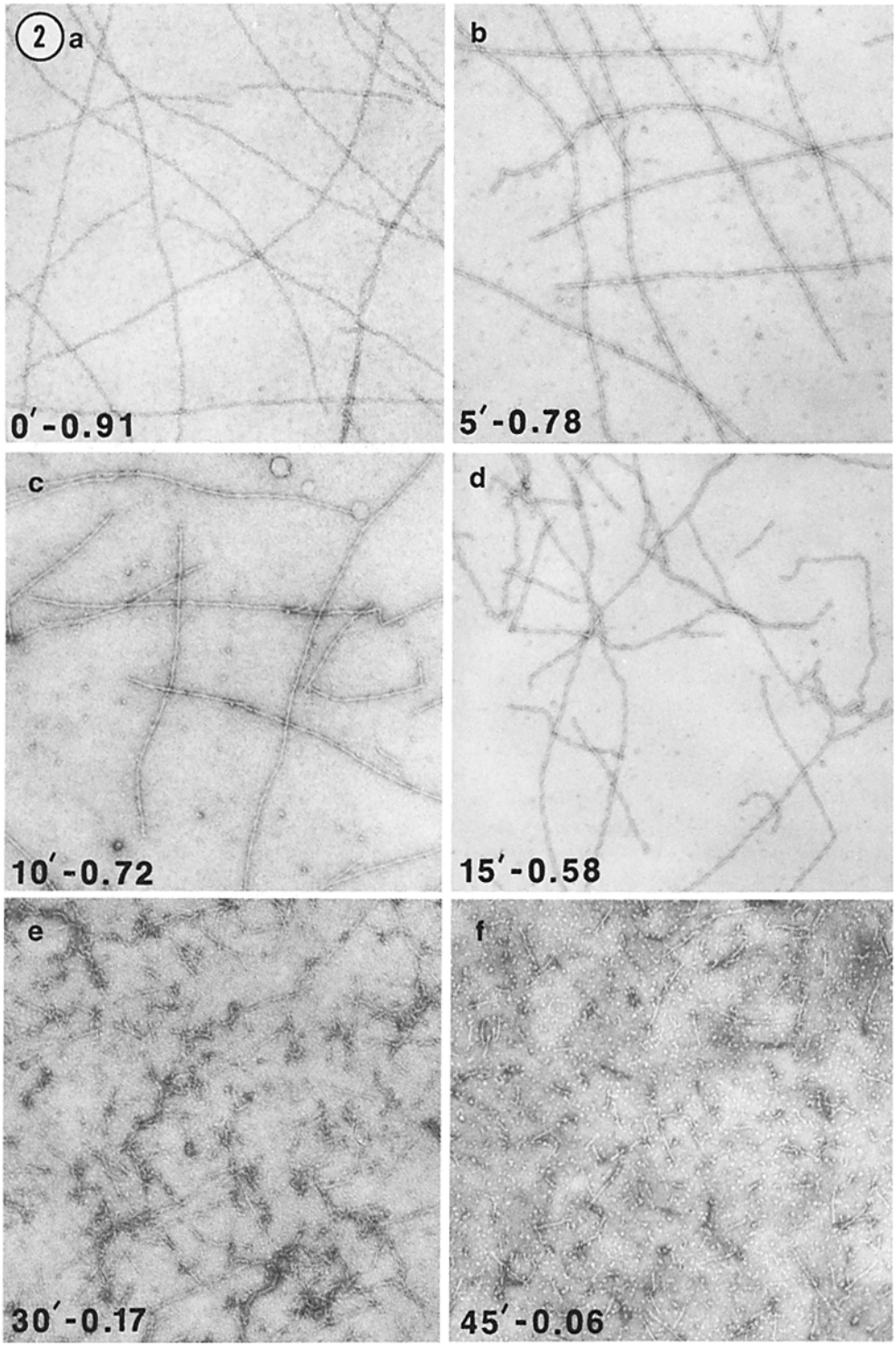

FIGURE 2 Comparison of the viscosity and ultrastructure of actin filaments exposed to 8 mM OsO_4. The reaction was carried out with 0.5 mg/ml actin in 50 mM sodium phosphate, 50 mM KCl, and 5 mM $MgCl_2$ (pH 7.0) at 20°C. The length of OsO_4 treatment and the specific viscosity of a parallel sample at that time are indicated in each lower left corner. × 52,000.

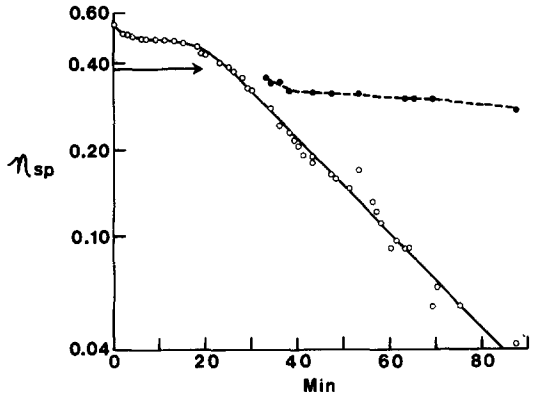

FIGURE 3 The effect of separating free OsO_4 from actin filaments after 20 min of reaction. A control sample consisting of 0.5 mg/ml of actin filaments in 50 mM KCl, 50 mM sodium phosphate, and 5 mM $MgCl_2$ (pH 7.0) was reacted with 4 mM OsO_4 at 20°C (○). A second sample consisting of 1.5 mg/ml of actin filaments in the same buffer was reacted with 4 mM OsO_4 for 20 min at 20°C, then it was passed through a 1.5 × 9-cm column of Sephadex G-25 medium to separate actin from free OsO_4. After the actin concentration was adjusted to 0.5 mg/ml, the viscosity was measured (●).

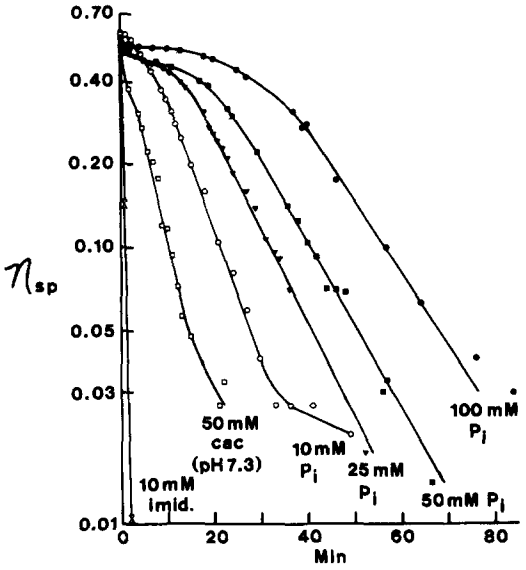

FIGURE 5 Influence of buffer type and concentration on the time-course of viscosity changes of actin filament solutions exposed to OsO_4. Actin filaments at a concentration of 0.5 mg/ml were reacted with 4 mM OsO_4, 50 mM KCl, and 5 mM $MgCl_2$ in the buffers at pH 7.0, except for cacodylate at pH 7.3, at 20°C.

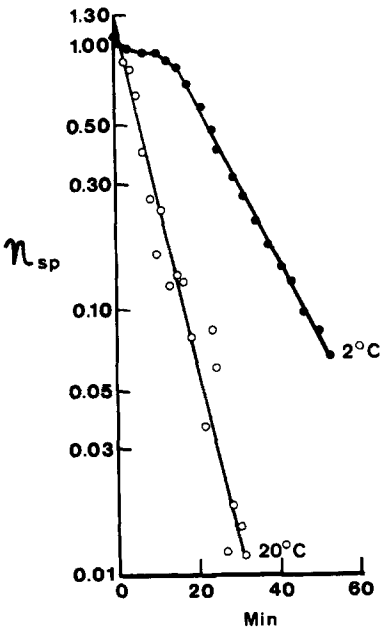

FIGURE 4 Influence of temperature on the time-course of viscosity changes of actin filament solutions exposed to OsO_4. Actin filaments at a concentration of 0.5 mg/ml were reacted with 20 mM OsO_4 in 50 mM KCl, 50 mM sodium phosphate, and 5 mM $MgCl_2$ (pH 7.0) at either 2°C (●) or 20°C (○).

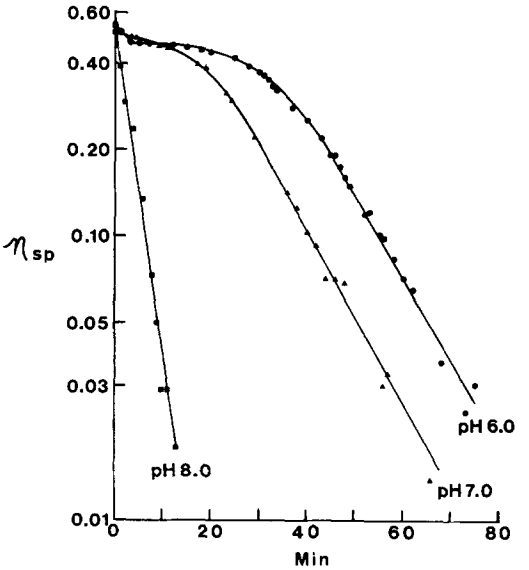

FIGURE 6 Influence of pH on the time-course of viscosity changes of actin filament solutions exposed to OsO_4. Actin filaments at a concentration of 0.5 mg/ml were reacted with 4 mM OsO_4 in 50 mM KCl, 50 mM sodium phosphate, and 5 mM $MgCl_2$ at 20°C at pH 6.0 (●), 7.0 (▲), or 8.0 (■).

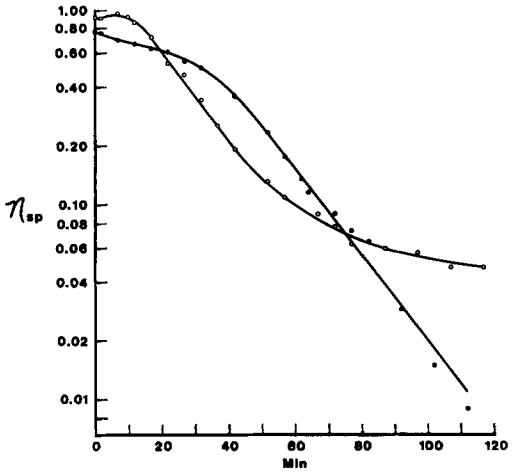

FIGURE 7 Influence of glutaraldehyde pretreatment on the time-course of the viscosity change of actin filament solutions exposed to OsO_4. A sample of polymerized actin was reacted with 1% glutaraldehyde in 50 mM KCl, 100 mM sodium phosphate, and 3 mM $MgCl_2$ (pH 7.0) at room temperature for 15 min. Then it was dialyzed against 200 vol of the same buffer without glutaraldehyde for 20 h at 4°C. The concentration was adjusted to 0.5 mg/ml and a sample was reacted with 4 mM OsO_4 at 20°C (○). A control sample containing untreated actin filaments at a concentration of 0.5 mg/ml was reacted with 4 mM OsO_4 in the same buffer at 20°C (●).

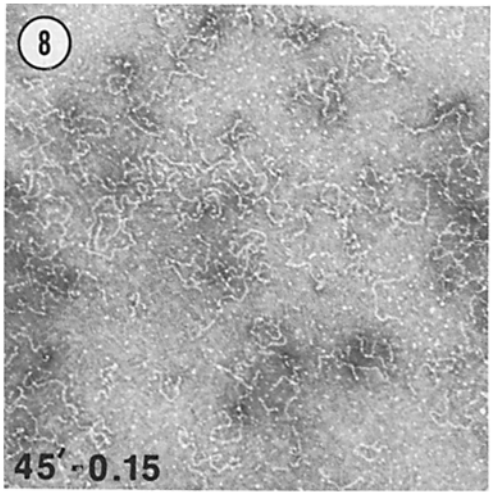

FIGURE 8 Electron micrograph of actin filaments pretreated with glutaraldehyde and then exposed to OsO_4. A sample of actin at 0.5 mg/ml in 50 mM sodium phosphate, 50 mM KCl, and 5 mM $MgCl_2$ (pH 7.0) was treated with 1% glutaraldehyde for 30 min at 20°C, then dialyzed against 200 vol of the same buffer for 14 h at room temperature. This glutaraldehyde-pretreated actin was treated with 8 mM OsO_4 as described in Fig. 2. This negatively stained sample was taken after 45 min when the specific viscosity, 0.15, was 6% of the starting value. This specimen can be compared with that in Fig. 2 *f* which was not pretreated with glutaraldehyde.

higher than that of native actin filaments. Like native actin filaments (Fig. 2), the glutaraldehyde-pretreated filaments are fragmented during exposure to OsO_4 (Fig. 8), but the fragments are longer and each fragment contains many sharp bends. The greater length of these fragments presumably accounts for the higher final viscosity.

PRESENCE OF TROPOMYOSIN: When treated with OsO_4, actin-tropomyosin filaments are more stable than bare actin filaments, providing the buffer used favors actin filament destruction (Fig. 9). In 50 mM PIPES, pH 7.1 (data not shown), or in 50 mM cacodylate, pH 7.2 (Fig. 9), the presence of tropomyosin delays the onset of the fast phase of viscosity decrease. In contrast, when the same experiment is carried out in sodium phosphate buffer, which inhibits the rate of filament destruction by OsO_4, the time-course of the viscosity change is not influenced by the presence or absence of tropomyosin.

Chemistry of the Reaction between OsO_4 and Actin

OsO_4 REDUCTION: OsO_4 is reduced in the

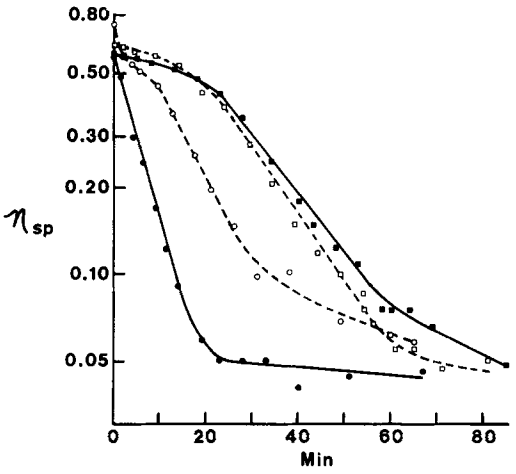

FIGURE 9 Influence of tropomyosin on the time-course of viscosity changes of actin filament solutions exposed to OsO_4. Actin filaments at a concentration of 0.5 mg/ml with (dashed line) or without (solid line) 0.12 mg/ml of tropomyosin were reacted with 4 mM OsO_4 in 50 mM KCl and 5 mM $MgCl_2$, with either 50 mM cacodylate, pH 7.2 (●, ○) or 50 mM sodium phosphate, pH 7.0 (■, □).

presence of purified actin (Fig. 10). After 30 min of reaction, the amount of OsO_4 reduced is proportional to the concentration of actin. 17 mol of OsO_4 are reduced per mole of actin. With longer times of reaction the amount of OsO_4 reduced is not proportional to the actin concentration. This is most pronounced at high protein concentrations, presumably because the rate of the reaction decreases as the OsO_4 is depleted. After 24 h most of the OsO_4 is reduced. At the lowest actin concentration there are 850 mol of OsO_4 reduced per mole of actin.

The time-course of the OsO_4 reduction by actin is biphasic (Fig. 11). After a rapid initial reduction of ~8–20 mol of OsO_4 per mole of actin, there is a slow reduction of the remaining OsO_4 at a constant rate. In the concentration range below 2.3 mM OsO_4, both the amount of OsO_4 reduced during the initial rapid phase and the rate of the slow phase depend on the OsO_4 concentration. Between 2.3 and 4.4 mM OsO_4 the reaction follows essentially the same time-course. Higher concentrations were not tested because it is difficult to measure the small changes in OsO_4 concentration which result.

The initial rapid reduction of 8–20 mol of OsO_4 per mole of actin (Fig. 11) takes place during the initial slow phase of the viscosity change (Fig. 1) during which the filaments remain largely intact. The secondary rapid phase of the viscosity decrease takes place during the slow phase of the OsO_4 reduction.

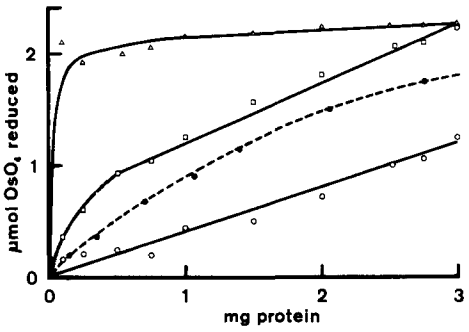

FIGURE 10 OsO_4 reduction by actin and whole amebas. Various concentrations of actin in 50 mM KCl, 50 mM sodium phosphate, and 3 mM $MgCl_2$, pH 7.0 (solid line) and of *Acanthamoeba castellanii* in 50 mM sodium phosphate, pH 7.0 (dashed line) were reacted with 2.4 mM OsO_4 at 20°C. The amount of OsO_4 reduced at 30 min (○, ●), 4 h (□), and 24 h (△) was determined by the difference between the initial concentration and the concentration of OsO_4 remaining at each time-point.

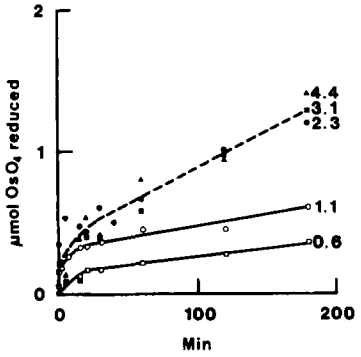

FIGURE 11 Influence of OsO_4 concentration on the time-course of OsO_4 reduction by actin. Solutions containing 1 mg/ml (25 nmol/ml) of actin filaments in 50 mM KCl, 50 mM sodium phosphate, and 3 mM $MgCl_2$ (pH 7.0) were reacted with 0.6, 1.1, 2.3, 3.1, and 4.4 mM OsO_4 at 20°C. The amount of OsO_4 reduced per milliliter was determined by the difference between the initial concentration and the concentration of OsO_4 remaining at each time-point.

We have included in Fig. 10 the amount of OsO_4 reduced by whole cells for comparison. As expected for a cell containing lipid and nucleic acids, which also reduce OsO_4 (6, 24), the amount of OsO_4 reduced per milligram of protein is about twice that for pure actin after 30 min of reaction.

SPECTROSCOPIC DETECTION OF REACTION PRODUCTS: The reaction of OsO_4 and actin yields a product with a light brown color. This is manifested by the development of a broad absorption peak with a maximum at 315 nm which extends into the visible part of the spectrum (Fig. 12). This peak at 315 nm develops within 2 min, during which time there is little change in absorbance at 280 nm. At least part of the color change is due to a modification of the protein or to binding of a colored derivative of OsO_4 to the protein, because the protein is brown and has an absorption peak at 315 nm after it is separated from lower molecular weight compounds by gel filtration. With very long reaction times, there is a modest increase in absorbance at 280 nm (Fig. 12).

OSMIUM BINDING TO ACTIN: Most of the products of OsO_4 reduction in the presence of actin are *not* bound to the protein. We reacted 1 mg/ml of actin with 3.8 mM OsO_4 in 50 mM sodium phosphate (pH 7.0), 50 mM KCl, and 3 mM $MgCl_2$ for various times and then separated the protein from small molecules by gel filtration. The amount of osmium bound to the protein was

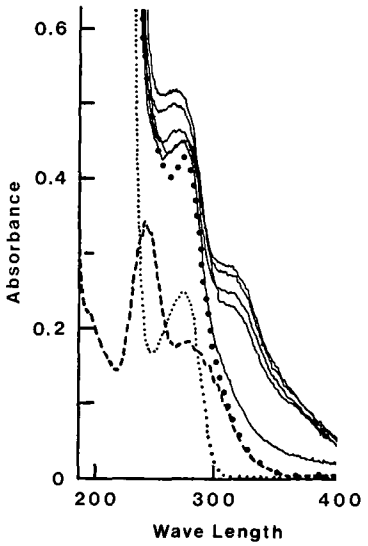

FIGURE 12 Ultraviolet absorption spectra of actin, OsO_4, and the products of their reaction. Actin was 0.2 mg/ml in water (fine dotted line); 0.1 mM OsO_4 in water (dashed line); the sum of the spectra of actin and OsO_4 (large dotted line); 0.2 mg/ml actin reacted with 0.1 mM OsO_4 in water at room temperature for various times: five curves (solid line) were recorded at 2 min (the lowest curve), 3.5, 7, 23, and 52 h (the highest curve).

measured in two ways: by direct chemical assay and by the change in absorbance at 280 nm. Both assays produced considerable scatter in the data, but both indicated that very few osmium molecules are bound to each actin molecule. By direct chemical determination of OsO_4 following oxidation of the protein with sodium peroxide, we found <2 mol of osmium bound per mole of actin after 2 h of reaction. The spectroscopic estimation involved calculation of the A_{280} expected from the nitrogen content of the sample, measurement of the A_{280} of the reacted protein, and calculation of the osmium content from the difference between these two values. We made two assumptions in this calculation: (*a*) the osmium bound to the protein had the same extinction coefficient as free OsO_4, and (*b*) any absorbance at 280 nm greater than that due to protein was due solely to osmium, not to some other absorbing reaction product. For the short periods of time examined here, the latter assumption seems to be valid because, as shown in Fig. 12, the A_{280} of the reaction mixture is approximately the same as the sum of the absorption of the separate reactants. By this spectrophotometric assay, ~8 mol of osmium were bound per mole of actin after 80 min of reaction.

CHANGES IN ACTIN PRIMARY STRUCTURE: During the reaction with OsO_4 the sulfur-containing amino acids of actin are rapidly oxidized and the polypeptide backbone is slowly cleaved at discrete positions to yield smaller peptides (Fig. 13). All five cysteine residues are oxidized to cysteic acid, and 15 of the 16 methionine residues are oxidized to methionine sulfone (Fig. 13 *a*). A trace of methionine sulfoxide was also detected. There was a concomitant rapid loss of two or three lysine residues, but the reaction product was not identified. All of the other amino acids were recovered quantitatively. The half time for cysteine oxidation was about 10 min whereas that of methionine was about 50 min. In this experiment, the duration of the slow phase of viscosity change was about 20 min, and the viscosity had reached half the starting value after 34 min, indicating that the destruction of the actin filaments takes place after the cysteine oxidation and on a similar time-course as the methionine oxidation. Furthermore, it seems likely that the oxidation of these amino acids accounts for the bulk of the rapidly reduced OsO_4.

During the exposure to OsO_4 the peptide backbone of actin is cleaved in a number of places (Fig. 13 *b*). The size and order of appearance of the peptides in this experiment was typical of a number of other experiments performed under different conditions, indicating that the sites of cleavage are specific. Most of the peptide bond cleavages take place well after the filaments are destroyed, as judged by viscosity measurements (Fig. 13 *b*) and after the sulfur-containing amino acids are oxidized (Fig. 13 *a*). The size of the peptide products is not consistent with the molecule splitting exclusively at cysteine and/or methionine residues. Other proteins vary in their susceptibility to cleavage by osmium (30).

Electron Microscopy of Embedded Material

Electron microscopy of thin sections of embedded actin filament pellets fixed in various ways allowed us to test independently the influence of various methods of glutaraldehyde cross-linking, OsO_4 treatment, and dehydration on actin filament structure. Of these variables, the method of OsO_4 treatment was found to be the most critical. The ultrastructural observations corroborated the results of the viscosity measurements on actin filament solutions described above.

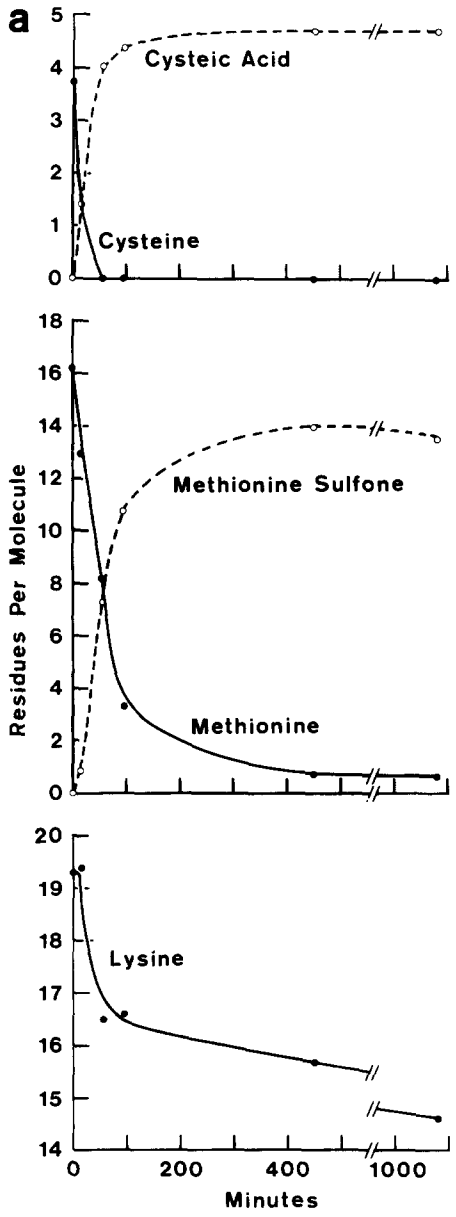

FIGURE 13 Comparison of changes in actin primary structure and viscosity during exposure to OsO_4. Actin at 1 mg/ml was reacted with 4 mM OsO_4 in 50 mM sodium phosphate and 2 mM $MgCl_2$ (pH 7.0) at 20°C. The time-course of the viscosity change was measured. At intervals, samples were passed through a 0.9 × 20-cm column of Sephadex G-25 fine, equilibrated and eluted with 100 mM sodium phosphate (pH 7.0) to separate protein from free osmium. A portion of each desalted protein sample was boiled in 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol and analyzed by gel electrophoresis in sodium dodecyl sulfate. Another portion of each sample was hydrolyzed for amino acid analysis. The graphs show the time-course of the conversion of cysteine to cysteic acid and the conversion of methionine to methionine sulfone. There was also a partial loss of lysine, but the product was not identified. All of the other amino acids were recovered quantitatively.

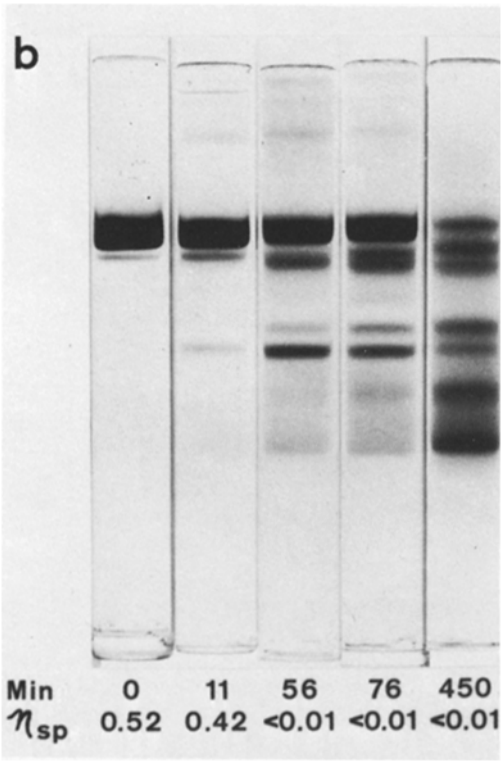

FIGURE 13*b*

In combination with a constant regimen of OsO_4 treatment and dehydration, variations of the glutaraldehyde primary fixation did not influence the appearance of embedded actin filaments. We tested glutaraldehyde from three different suppliers at concentrations between 100 and 250 mM (1.0 and 2.5%) for periods of time from 30 min at room temperature to 24 h at 4°C. Failing to find any differences, we arbitrarily chose for primary fixation in the following experiments 100 mM glutaraldehyde (Electron Microscopy Sciences) in 50 mM KCl, 50 mM sodium phosphate, and 5 mM $MgCl_2$ at pH 7.0 for 30 min at 22°C.

Actin filament preservation varied from excellent to very poor depending upon the OsO_4 treatment. Under conditions where the viscosity of actin filament solutions is stable, the structure of embedded actin filaments is preserved. For example, in pellets treated with 4 mM OsO_4 in 50 mM KCl, 50 mM sodium phosphate, and 5 mM $MgCl_2$ (pH 6.0) for 20 min at 0°C, the actin filaments are long and straight (Fig. 14 *a*). In cross sections, it is seen that the filaments are aggregated laterally. Due to this aggregation and the inevitable superimposition within the thickness of the section, individual filaments are sometimes difficult to see in longitudinal sections. Nonetheless, only intact actin filaments are found throughout these pellets. Tilney (52) also found that OsO_4 treatment in phosphate buffer at pH 6 in the cold

favors filament preservation. In contrast, pellets treated with OsO_4 under conditions where the viscosity of actin filament solutions decays rapidly consist of a meshwork of ill-defined fibrous material (Fig. 14 *b*). This material is predominantly randomly oriented, short, and bent 6-nm wide filaments, which are presumably fragments of actin filaments like those in Fig. 8. In addition, there are always a few regions in each pellet with longer bent fragments. In summary, we found that any of the following factors can lead to the disruption of actin filaments in embedded material: high osmium concentration (e.g., 40 mM = 1%), elevated temperature (22°C), extended exposure to OsO_4 (1 h or longer), alkaline pH (≥7), or nonphosphate buffer (e.g., cacodylate). When all of these deleterious conditions are combined, such as 40 mM OsO_4, 50 mM cacodylate buffer, pH 7.3, for 60 min at 22°C, the whole glutaraldehyde-fixed actin pellet *dissolves*.

We found it advantageous to omit uranyl acetate en bloc staining of these actin filament pellets because even well-preserved specimens fixed with glutaraldehyde and OsO_4 swell and partially dissolve in 1% aqueous uranyl acetate.

Methods of dehydration had little influence on the appearance of embedded actin filaments. Dehydration with ethanol plus propylene oxide or with acetone gave similar results.

DISCUSSION

It is remarkable that osmium tetroxide, used since 1864 (46; referred to in reference 31) as a histological stain, persists as one of the most important fixatives for the preparation of biological materials for electron microscopy. Its ability to preserve membranes, lipids, and lipoproteins and its staining properties have thus far outweighed its disadvantages. Its main disadvantage is its disastrous effect on the cytoplasmic matrix.

Our approach to this problem has been to study a simple model system: the reaction between OsO_4 and actin filaments in vitro. We are optimistic that our data will be useful in designing protocols that will minimize the disruption of actin filaments during fixation of cells with OsO_4, even

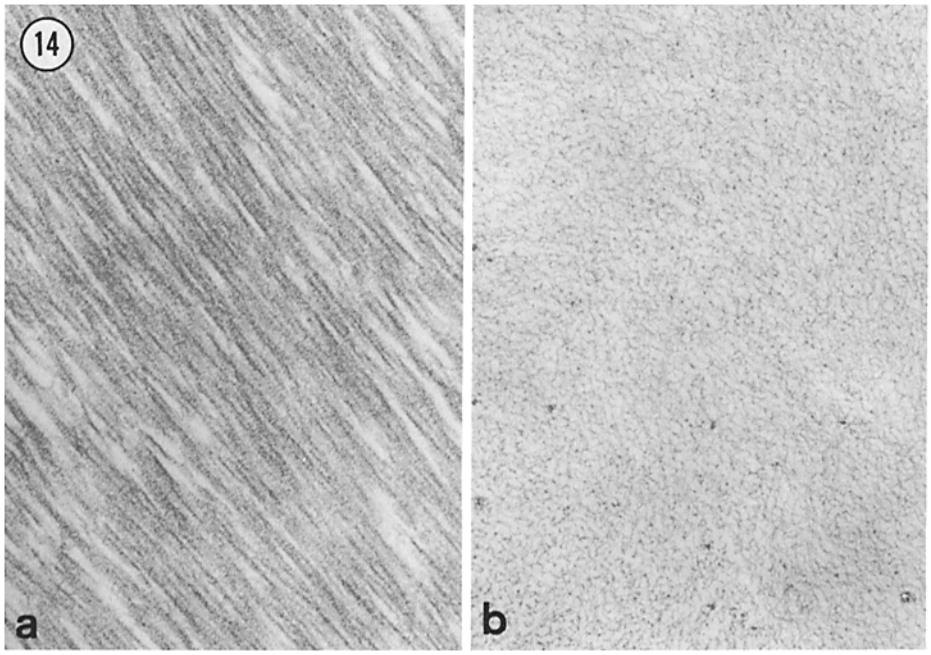

FIGURE 14 Electron micrographs of thin sections of actin filaments fixed with OsO_4 under favorable and unfavorable conditions. Two pellets of 0.5 mg of actin were prepared as described in Materials and Methods. Both (*a*) and (*b*) were treated with 1% glutaraldehyde in 50 mM sodium phosphate (pH 7.0), 50 mM KCl, and 5 mM $MgCl_2$ for 30 min at room temperature. (*a*) was then rinsed in the same buffer at pH 6.0 and treated with 4 mM OsO_4 in the same buffer (pH 6.0) for 10 min at 2°C. (*b*) was rinsed in the same buffer at pH 7.0, then treated with 40 mM OsO_4 in the same buffer (pH 7.0) for 1 h at room temperature. Both were dehydrated in ethanol and propylene oxide. × 49,000.

though the chemistry of the reaction between OsO_4 and proteins is still obscure. In the following paragraphs, we review what is known about the reaction between OsO_4 and proteins and how this relates to our present findings.

Previous Work on the Reaction of OsO_4 with Proteins

Little is known about the reactions of OsO_4 with proteins compared to the well-characterized interactions of OsO_4 with lipids (12, 24, 25, 55) and nucleic acids (9, 10, 44). Before our present study, it was established that OsO_4 binds to (8) and can partially cross-link some proteins (19, 40, 56), but it was also found that OsO_4 oxidizes some amino acids (16), destroys enzyme activity (47), alters protein secondary structure (29), and upon prolonged exposure, liquifies protein gels (40). Each of these reactions will be discussed in turn in relation to the usefulness of OsO_4 as a fixative.

Porter and Kallman (40) found that addition of OsO_4 to concentrated solutions of serum proteins caused gelatin and attributed this to cross-linking of the proteins. Subsequently, Hopwood (19) used gel filtration, viscometry, and polyacrylamide gel electrophoresis of native proteins to confirm that bovine serum albumin is partially aggregated after exposure to OsO_4, and Wood (56) used gel electrophoresis in detergent to show that mitochondrial proteins are also partially aggregated after OsO_4 treatment. Porter and Kallman felt that such cross-linking of proteins in the cytoplasmic matrix was responsible for the "instantaneous cessation of Brownian movement" observed when cells are exposed to OsO_4.

If the effect of OsO_4 on cellular proteins were restricted to cross-linking, it might be a good protein fixative, but even in their pioneering studies Porter and Kallman (40) observed deleterious effects of OsO_4 on pure proteins and on the cytoplasmic matrix. They found that prolonged exposure of the gelled proteins to OsO_4 resulted in liquefaction. They also observed a cellular counterpart of this liquefaction: during long fixation with OsO_4 "an amorphous matrix (and its fibrous condensations) is removed from around the formed bodies leaving them clear and available for more detailed study." This was considered to be an advantage in viewing the organelles, but obviously it is a disaster for students of the cytoplasmic matrix. Bahr (4) confirmed that OsO_4 does not fix all cellular proteins in place. After treating liver samples with OsO_4 for 1 h, ~50% of the protein was extracted into saline and water washes. Longer exposure to OsO_4 resulted in *more* (not less) protein being extracted in the washes. Not surprisingly, early electron micrographs of thin sections of cells fixed with OsO_4 alone revealed little in the cytoplasmic matrix but the lucent embedding plastic!

OsO_4 causes changes in both the primary and secondary structures of proteins. Any one of these reactions could alter cellular structure and promote the extraction of proteins.

Several of the amino acids react with OsO_4, judging from color changes in the reaction mixture (3). Cysteine and methionine are the most reactive by this criteria, especially at alkaline pH, but tryptophan, histidine, proline, and arginine also give rapid color changes. Lysine, asparagine and glutamine are slightly reactive, whereas other amino acids commonly found in proteins are not. We confirmed these findings with a spectrophotometric assay (30). Hake (16) showed that OsO_4 oxidatively deaminates all of the amino acids, splits cysteine, oxidizes cysteine to cysteic acid, and oxidizes methionine to methionine sulfone. Several proteins liberated ammonia when treated with OsO_4, suggesting oxidative deamination, but the reaction products could not be identified for technical reasons.

As a result of these oxidative reactions, or other uncharacterized reactions, both soluble and membrane proteins undergo changes in their secondary structure when exposed to OsO_4 (29). Treatment with 2% OsO_4 for 30 min at 4°C in 70 mM phosphate buffer (pH 7.5) caused the loss of 40–60% of the α-helical content. Pretreatment with glutaraldehyde made matters worse; after subsequent treatment with OsO_4 ~70% of the α-helix was destroyed.

The reaction of OsO_4 with proteins converts some of the OsO_4 into a nonvolatile form (18). Much more OsO_4 became nonvolatile when reacted with lipoproteins than when reacted with several nonlipoproteins. No more than 20 mol of osmium were converted to a nonvolatile form per mole of serum albumin or hemoglobin. Binding of the osmium to the protein was suspected but was not demonstrated directly.

Relation of Our Findings to Past Efforts to Fix Cellular Actin Filaments

We found that exposure to OsO_4 destroys actin

filaments by progressive fragmentation, even if the molecules in the filaments are first cross-linked to each other with glutaraldehyde. The reaction requires the presence of free OsO_4 and seems to be related temporally to the oxidation of the sulfur-containing amino acids. Large amounts of OsO_4 are reduced in the presence of actin, but very few osmium molecules bind to each actin molecule. Fortunately, the rate of actin filament destruction by OsO_4 is influenced by many factors, so the reaction rate can be controlled. Under favorable conditions, actin filaments are stable in OsO_4 and appear to be preserved even after embedding and sectioning (Fig. 14 *a*); whereas under unfavorable conditions, including those commonly used to fix cells, microfilament networks are created during fixation (Fig. 14 *b*), or worse yet, the whole pellet of actin dissolves.

These observations suggest that some microfilament networks may be a fixation artifact and that some cellular actin filaments may be damaged beyond recognition during fixation. Since actin is a major structural protein in the cytoplasm, its extraction may be largely responsible for the clarification of the cytoplasmic matrix noted by Porter and Kallman (40).

Electron micrographs of the intestinal brush border fixed in various ways illustrate how fixation methods can alter cellular actin filaments. Mooseker and Tilney (35) succeeded in preserving actin filaments in both the microvilli and the terminal web of isolated brush borders using pretreatment with glutaraldehyde followed by 40 mM OsO_4 in 0.1 M phosphate (pH 6.0) for 45 min at 0–4°C. Previously, these conditions were found by Gibbons and Gibbons (13) to preserve rigor waves of demembranated sperm tails, and they are, with the exception of the high OsO_4 concentration, nearly optimal for slowing the destruction of actin filaments. In contrast, brush-border actin filaments are not preserved by fixation with $KMnO_4$ or with OsO_4 alone under harsh conditions (23). Instead, beautiful microfilament networks are seen within the microvilli (41).

Factors Involved with Actin Filament Preservation during Fixation

It would appear that at least three factors are involved with the successful preservation of actin filaments for electron microscopy: the presence or absence of associated proteins, the success of the initial fixation with a protein cross-linking reagent, and the conditions of treatment with OsO_4. Only the last two factors are under the control of the investigator, so they merit the most careful consideration.

Proteins that bind to actin, such as tropomyosin, can inhibit actin filament destruction (Fig. 9) and their presence probably contributes to the preservation of actin filaments in muscle (20), stress fibers (14), sperm acrosomal processes (52, 54), and the brush border (35). Other actin filaments may lack associated proteins. In these cases, the addition of exogenous tropomyosin (51) or heavy meromyosin (22) may stabilize the filaments during fixation. This is a reasonable explanation for the presence of more decorated filaments in glycerinated cells treated with heavy meromyosin than bare actin filaments in untreated glycerinated cells. Regrettably, the requirement for cell lysis to allow the entry of the exogenous heavy meromyosin or tropomyosin severely limits this approach. In addition, there is concern that the addition of either heavy meromyosin or tropomyosin might alter the state of actin polymerization.

Few actin filaments are preserved in cells fixed with only OsO_4 or $KMnO_4$, so prior cross-linking of cellular proteins with glutaraldehyde or another organic reagent will continue to be important in any fixation procedure employing OsO_4. Glutaraldehyde cross-links actin molecules within actin filaments and cross-links actin and tropomyosin in actin-tropomyosin filaments (28). Some kinds of glutaraldehyde have been found to stabilize brush border and *Thyone* acrosomal actin filaments better than others (35, 53), but the composition of these "good" batches was not compared with that of the "bad" batches, so we do not know what distinguishes a "good" batch. Reedy has developed fixation methods employing secondary cross-linking reactions after glutaraldehyde, which improved the preservation of the filaments in striated muscle (42) and which might be helpful in studies of nonmuscle cells as well.

The final variable is the OsO_4 treatment, which we now understand in some detail. It seems inevitable that we must use OsO_4 in fixing whole cells because without it (or $KMnO_4$, which is an even stronger oxidizing agent), membranes are not preserved (45).

Our work demonstrating that OsO_4 destroys purified actin filaments offers one explanation why it has been difficult to preserve actin filaments

in cells prepared for electron microscopy with fixation procedures including OsO_4. Simple variations in the fixation protocol can cause purified actin filaments to appear as either long, straight filaments or as microfilament networks. Without good actin filament preservation, many important questions remain unanswered: how much of a cell's actin is assembled into filaments, where the actin is concentrated in the cell, whether the actin filaments always exist in highly ordered arrays (like those in microvilli and stress fibers) or whether they can exist in the microfilament network configuration, and how they are associated with membranes, microtubules, and each other. Although we have not solved the problem of how to fix actin filaments inside cells, we are confident that our data on the relation of solution conditions to the rate of actin filament destruction by OsO_4 will aid in the design of fixation protocols that will optimize the preservation of actin filaments.

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