Visualization of a 21-nm Axial Periodicity in Shadowed Keratin Filaments and Neurofilaments

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ABSTRACT   Unidirectional and rotary shadowing techniques have been applied in studying the surface structure of two types of intermediate filaments. Keratin filaments and neurofilaments demonstrate a ~21-nm axial periodicity which probably indicates the helical pitch of the outer shell of the filament. Analysis of unidirectionally shadowed keratin showed that the helix is left-handed. The observation of a left-handed helix of 21-nm pitch supports the three-stranded protofilament model of Fraser, Macrae, and Suzuki (1976, *J. Mol. Biol.* 108:435-452), and indicates that keratin filaments probably consist of 10 three-stranded protofilaments surrounding a core of three such protofilaments, as predicted by models based on x-ray diffraction of hard keratin filaments. Neurofilaments do not demonstrate an easily identifiable hand, so their consistency with the model is, as yet, uncertain.

Electron microscopy of intermediate filaments has previously failed to detect any significant structural details such as subunit dimensions or a periodicity reflecting helical symmetry. Negatively stained keratin filaments appear smooth, except for an irregular fibrillar lattice (Fig. 1). Neurofilaments also seem fibrillar and are often decorated by knobs distributed irregularly along their lengths (Fig. 1).

Improved methods for preparation of shadowed specimens have recently given reliable and detailed images of isolated macromolecules at a resolution of 3-5 nm (1, 2, 3). The most useful innovation has been to include glycerol in which the macromolecules are sprayed. Glycerol appears to improve spreading and to prevent nonspecific aggregation of the protein molecules. We have applied these techniques to keratin filaments and neurofilaments with significant results. In both cases the shadowed filaments demonstrate an axial periodicity of ~21 nm. These results have been reported briefly (4). Also, in a recent independent study in another lab (5), a similar periodicity was observed in filaments reconstituted from keratin, desmin, and neurofilament protein.

MATERIALS AND METHODS
Preparation of Intermediate Filaments

Keratin filaments were prepared from fresh bovine snout by the procedure of Steinert and Idler (6). The entire epidermal layer was shaved off and minced. The tissue was then extracted overnight at 4°C or for 4-5 h at room temperature in 50 mM Tris, pH 9, 1 mM EGTA, 8 M urea, and 1 mM DTT. The preparation was centrifuged at 15,000 rpm for 15 min in a Beckman 35 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA) at 4°C to remove tissue debris. The resulting supernatants were centrifuged at 35,000 rpm for 1 h to remove smaller contaminants and insoluble aggregates. Supernatants were dialyzed against 5 mM Tris, pH 7.6, 1 mM EDTA, and 1 mM DTT to reassemble the keratin filaments. The filaments that then formed could be recycled several times by disassembly in 8 M urea, centrifugation, and dialysis.

Neurofilaments prepared by the method of Delacourte et al. (7) from bovine spinal cord were the generous gift of M. Lifsics and R. C. Williams, Jr., Department of Molecular Biology, Vanderbilt University. They were kept frozen in 10% glycerol until ready for use, then incubated at 37°C.

Continuous glycerol gradients, 10 to 50% in glycerol and 12 ml in total volume, were poured at room temperature. For keratin filaments the buffer contained 5 mM Tris, pH 7.6, 1 mM EGTA, and 1 mM DTT, as well as the glycerol. Neurofilament buffers contained 100 mM MES, pH 6.5, 0.5 mM $MgCl_2$, 1 mM EGTA, and 1 mM DTT. After they were poured, the gradients were cooled to 4°C, then layered with sample. Approximately 1 ml of protein solution at 1-3 mg/ml (determined by the Bio-Rad Protein Assay, Bio-Rad Laboratories, Richmond, CA) was layered on top of the gradient. The samples were centrifuged in a Beckman swinging bucket rotor (SW41, Beckman Instruments, Inc.) at 4°C, 35,000 rpm for 3.5 h (neurofilaments) or 4 h (keratin). Gradients were eluted into 0.75-ml fractions that were assayed by negative-stain electron microscopy and Bio-Rad Protein Assay (Bio-Rad Laboratories). SDS PAGE was performed on several gradients using the method of Laemmli (8), with 7.5% acrylamide.

Actin Filaments

Rabbit skeletal muscle actin filaments were prepared from acetone powder by a modification of the procedure of Spudich and Watt (9). They were given to us by Drs. Michael Reedy and Kenneth Taylor, Department of Anatomy, Duke University Medical Center. The regulated actin filaments were fixed in 1% glutaraldehyde (10) and diluted into a buffer containing 1 mM Tris, pH 7.6, 10 mM KCl, 0.5 mM DTT, and 45% glycerol (3).

Electron Microscopy

Shadowed specimens were prepared for electron microscopy using the methods developed by Fowler and Erickson (1) for visualizing fibrinogen (see also Shotton et al. [2] and Tyler and Branton [3]). Samples from glycerol gradients were adjusted to 200-300 μg/ml (10 times the concentration used for individual
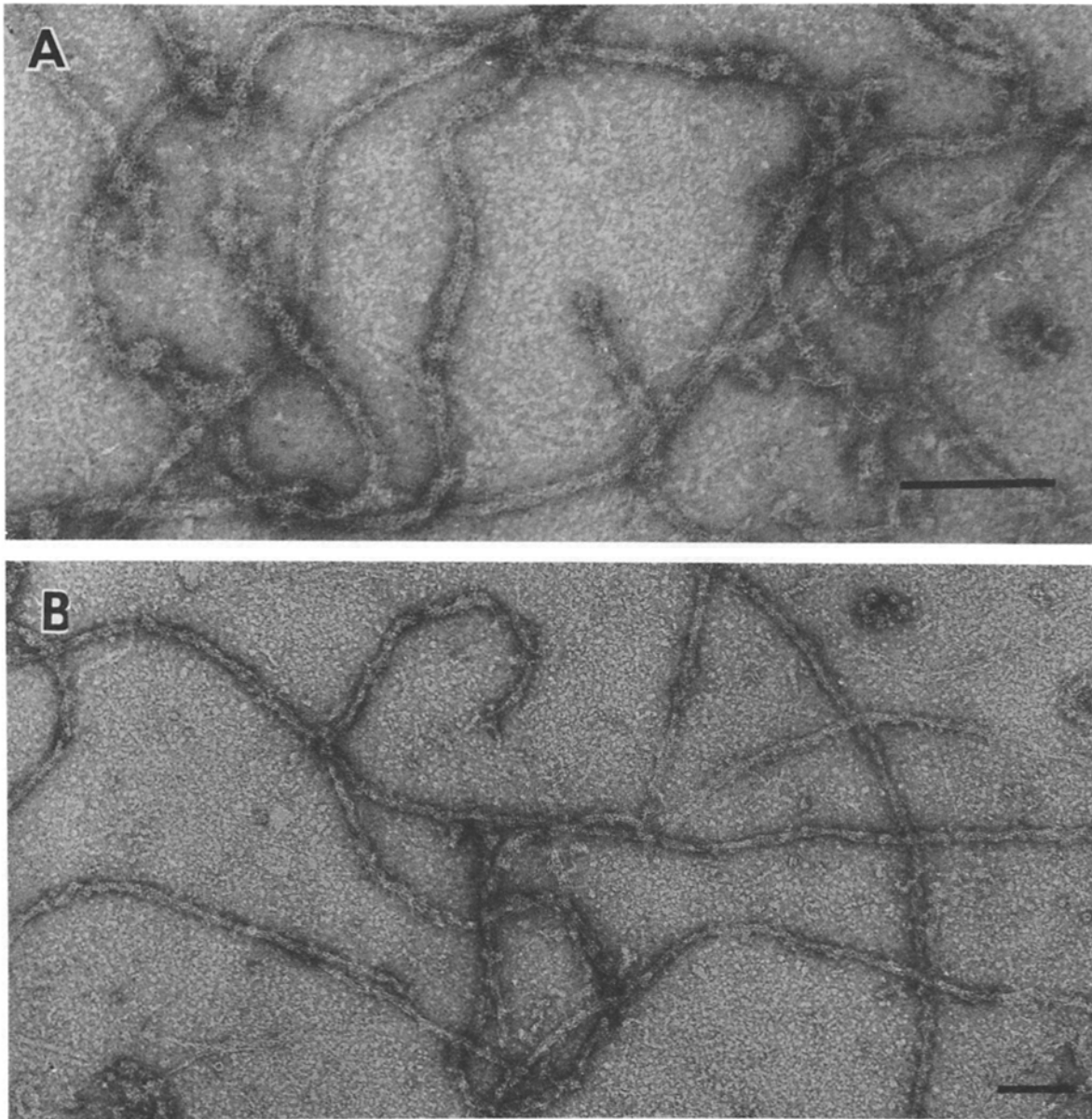
FIGURE 1   Negative stain of keratin filaments and neurofilaments shows no regular substructure, even at high magnification. Panel A is a micrograph of negatively stained keratin filaments showing slight fibrillar details within the filaments. Panel B is a micrograph of negatively stained neurofilaments, showing similar fibrillar structure but no measurable periodicity. Bars, 100 nm. A, × 230,000; B, × 117,000.

molecules) in buffers containing at least 20% glycerol. For keratin filaments the buffer was 5 mM Tris, pH 7.6, and for neurofilaments the buffer was 100 mM MES, pH 6.5.

A small quantity of the final solution was sprayed onto pieces of freshly cleaved mica. The samples were then shadowed with platinum–carbon (90:10; Pt:C) at an angle of ~5° for unidirectional shadowing and ~8-10° for rotary shadowing.

Micrographs were taken on a Philips EM-300 at a magnification of 39,000 times. Magnifications were calibrated from micrographs of negatively stained catalase crystals, assuming a periodicity of 17.7 nm (11).

For observations of handedness, the specimen preparation, microscopy, and photographic reproduction were controlled to make the final print (Fig. 5 only) of a view looking down on the replicated surface. Other figures are printed as views looking through the replica from the mica side.

Measuring Periodicity

Filaments were selected for measurement if the periodicity could be seen for 4 or 5 consecutive repeats. The average repeat for a given filament was equal to the total distance divided by the number of repeats. The average and standard deviation for each of the three types of filaments were determined from measurements of 20–25 individual filaments.

RESULTS

The surface structure of both types of intermediate filaments was best demonstrated by unidirectional shadowing. Keratin filaments prepared by unidirectional shadowing showed an axial periodicity, as seen in Fig. 2. The periodicity was manifested by globular deposits of metal, usually oriented at an angle to the axis of the filament. In addition, prominent sawtooth-shaped shadows appeared along some of the filaments, as in panel A of Fig. 2. In many cases the shadows were rounded. The shape of the shadows probably depended on the relative angles of the filament and metal source. The periodicity was the same for both extremes of shadow shape. The axial periodicity of keratin filaments averaged 20.8 nm ± 1.2 nm.

Keratin filaments were also rotary shadowed. In these specimens the periodicity appeared as small spots of metal in the center of the filament, but it was seen only with difficulty and could not be measured accurately.

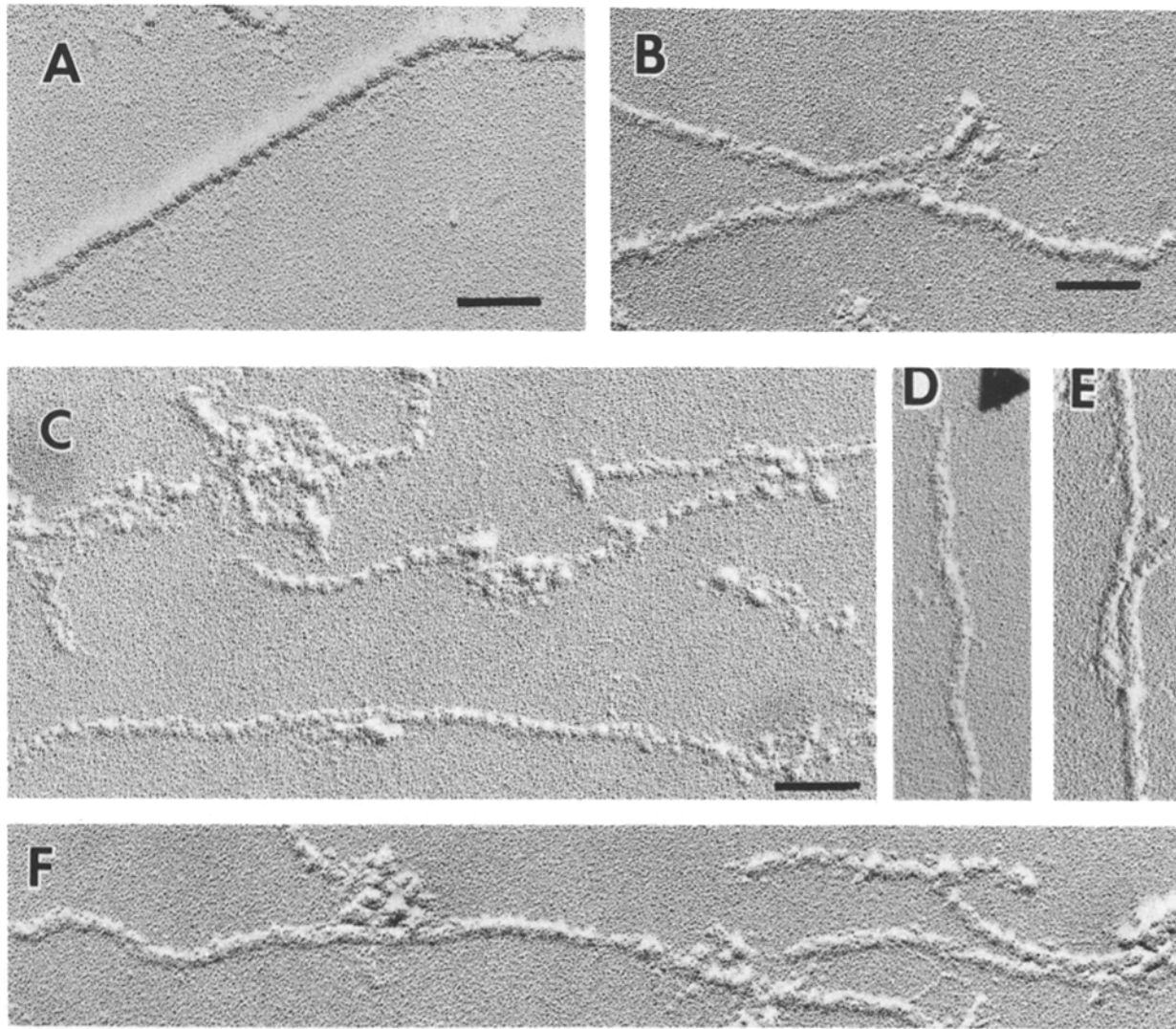The periodicity seen in unidirectionally shadowed neurofil-

FIGURE 2 Unidirectionally shadowed keratin filaments demonstrate a periodicity of ~21 nm. The filament in Panel *A* has sharp sawtooth-shaped shadows. Other filaments have more rounded shadows, as seen in panels *B-E*. Bars, 100 nm. × 17,000.

aments was less regular than that of keratin filaments. The metal deposits were not always of uniform size, and some filaments appeared to have irregular protrusions, either on or between the metal deposits. The axial periodicity was most clearly visualized by way of the shadows, which were as sharp as those from keratin. Panels *A-C* of Fig. 3 show unidirectionally shadowed neurofilaments. Sawtooth-shaped shadows are especially clear in panel *B*. The average periodicity measured from unidirectionally shadowed neurofilaments was 21.7 nm ± 3.6 nm.

Rotary-shadowed neurofilaments (Fig. 3, panels *D-G*) showed a clear periodic structure, in contrast to the relatively structureless surface seen in rotary-shadowed keratin. The periodicity was revealed as a series of cylindrical segments that were slightly tapered at each end. The thin band between the segments is clearly visible on many filaments. An average spacing of 22.9 nm ± 1.7 nm was obtained from several such images. The filaments in panels *D* and *G* show irregularities of unknown origin that appear as large swellings on the filaments.

Actin filaments did not remain intact throughout the shadowing procedure unless they were fixed in 1% glutaraldehyde. The best results were obtained with regulated actin filaments, those with troponin and tropomyosin still present. Unidirectionally shadowed actin filaments, as in Fig. 4, dem-

onstrated an average axial periodicity of 36 nm ± 1.7 nm, which is attributed to the right-handed helical packing of the two strands, as shown previously by Depue and Rice (12). The ~40-nm long rodlike structures (arrows in Fig. 4) are tropomyosin molecules that have dissociated from the actin filaments. Rotary-shadowed actin filaments were also studied, but the periodicity was not seen as clearly.

Actin provided an important control for two aspects of this study. First, actin provided the control for visualizing an axial repeat in a structure known to be helical. The periodicity seen in unidirectionally shadowed actin (Fig. 4) represents the 37-nm pith of the two actin strands forming the filament. Second, observation of the right-handedness of the actin filament helix (12) was confirmed and served as a control for determining the handedness of the intermediate filament helices.

The right-handed helix of actin was demonstrated by slashes of metal deposited at an acute angle to the axis of the filament, as seen from left to right. For the determination of handedness, the view in this image is from above the replica looking down on the shadowed surface. The slashes have the same orientation on all unidirectionally shadowed actin filaments, regardless of the angle of metal deposition (Fig. 5, panels a1 and a2). The metal deposits on the keratin filament in Fig. 5*b* form an obtuse angle with the filament axis, as seen from left to right.
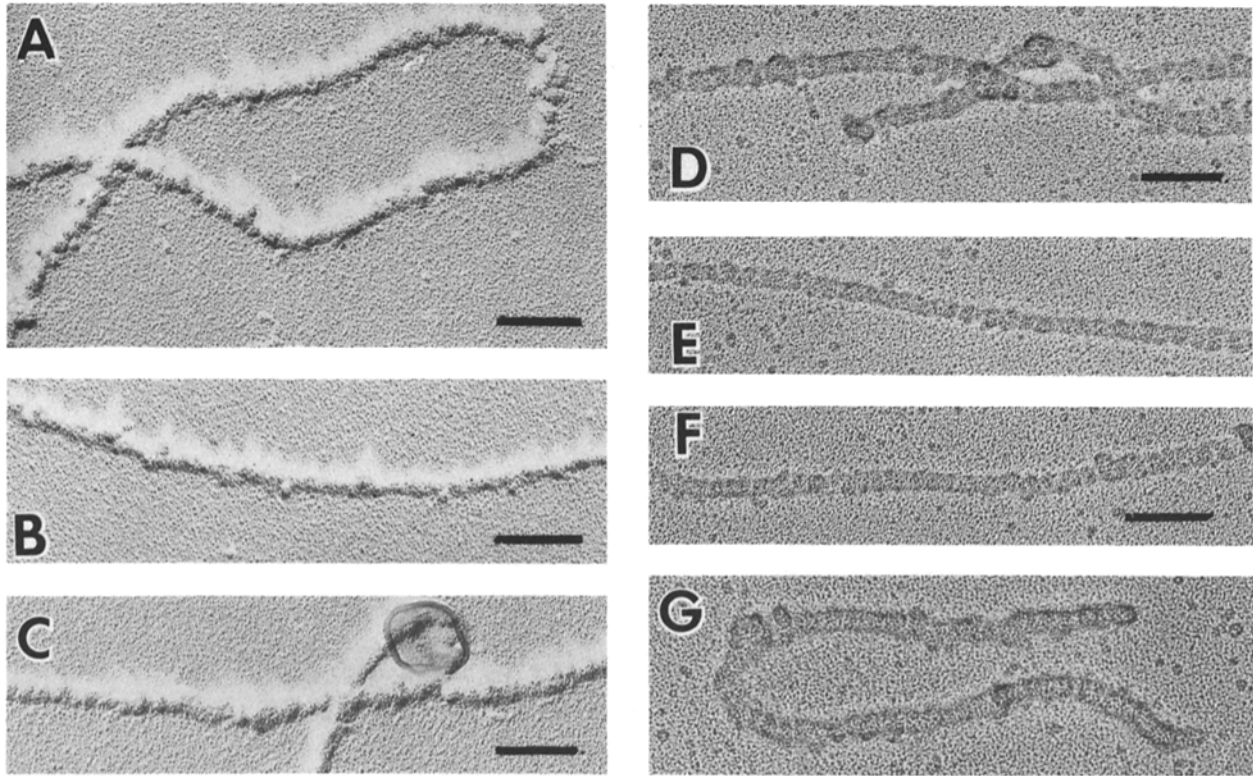
FIGURE 3 Unidirectional and rotary-shadowed neurofilaments show a periodicity of 21–22 nm. Unidirectionally shadowed filaments show irregular sharp shadows in panels A–C. In panels D–F, the repeat is clearly visible and more regular on these rotary-shadowed specimens. Bars, 100 nm. × 117,000.
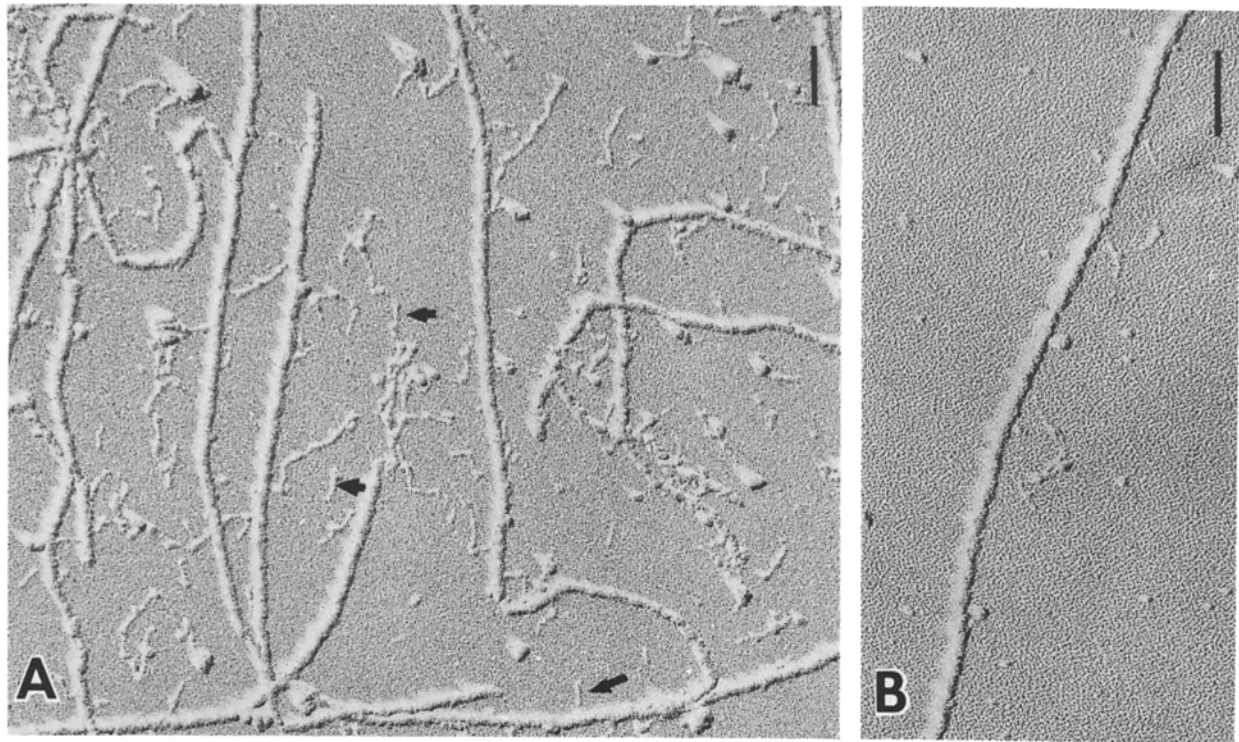

FIGURE 4 Unidirectionally shadowed actin filaments show a 36–37 nm repeat. Sharp shadows are visible on many actin filaments in panel A. The small, 40-nm rods are tropomyosin molecules that have dissociated from the filaments (arrows). Panel B shows an isolated actin filament at higher magnification. The repeat is very regular and represents the pitch of the helix. Bars, 100 nm. A, × 81,000; B, × 117,000.
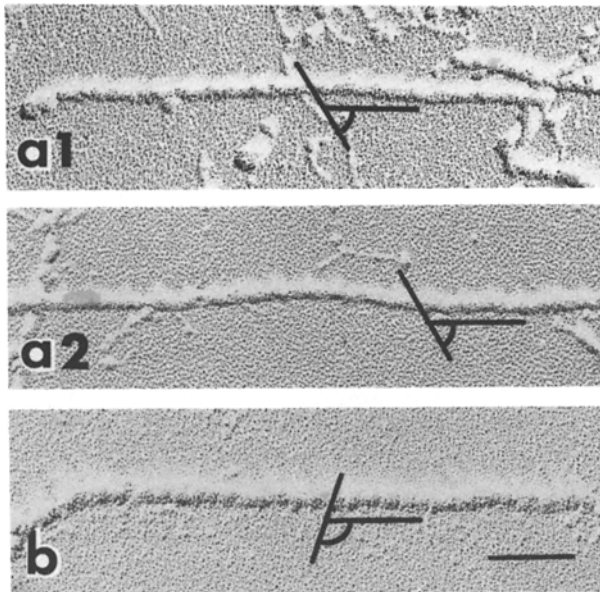
FIGURE 5 Determination of handedness of a helix is possible in unidirectionally shadowed specimens. Panels *a1* and *a2* show actin filaments situated at different angles from metal deposition, both showing helical grooves at an acute angle to the filament axis, indicating a right-handed helix. Panel *b* shows a keratin filament demonstrating a left-handed helix, indicated by the angle >90° from the axis of the filament when seen from the left. × 117,000.

This angle is almost perpendicular to the angle of the actin helix. Thus, the keratin filament helix must be left-handed.

DISCUSSION

Kallman and Wessells (13) observed a 22-nm periodicity in keratin filament bundles from thin sections of epithelial cells. We found that this periodicity is a property of individual keratin filaments and neurofilaments. Henderson et al. (5) have recently reported finding a similar periodicity in these filaments and in desmin. The similarity of the periodicities in the three different types of intermediate filaments provides further evidence that they have a similar structural basis. Our observations further suggest that the periodicity of the keratin filaments is due to a left-handed helix of the surface lattice. This is consistent with the most popular model for this structure, proposed by Fraser, Macrae, and Suzuki (14) for the filaments formed by hard keratins. From their x-ray diffraction studies they concluded that the surface of the filaments should have a 22-nm axial periodicity due to a helix of pitch of 22 nm, but whose handedness was unknown. They presented two models based on principles of packing α-helices into coiled-coils. In one model, based on a two-stranded protofilament, the surface lattice of the complete filament was predicted to be right-handed. In the other model based on a three-stranded coiled-coil protofilament, the surface lattice was predicted to be a left-handed helix. The three-stranded model has received support from the biochemical studies of Steinert (15), which discussed core particles composed of three keratin subunits. The important observation in our analysis is that the 22-nm helix of the keratin filament, as in Fig. 5, is left-handed. This further supports the three-stranded protofilament model. We note that in the model of Fraser et al. (14), the entire filament consists of a core of three protofilaments surrounded by an outer shell of ten protofilaments. The 20–22-nm axial periodicity demonstrated in our micrographs reflects the helical stagger or pitch

of the protofilament rope segments making up the outer shell of the keratin filament.

Krishnan et al. (16) have examined negatively stained neurofilaments at high magnification. They concluded that neurofilaments consist of two strands wound together in a right-handed helix of pitch 100–130 nm. Antibody labeling also detected an unusually long pitch seen on negatively stained filaments (17). The antibodies appeared to form a thick flocculent band that spiraled around the filaments. The pitch of the spiral coat was variable, but averaged ~100 nm.

In our preparation of shadowed neurofilaments there were irregular protrusions that tended to obscure the basic 22-nm repeat in places, but there was no indication of a 100-nm repeat. Filaments reconstituted from the purified 68-kdal neurofilament protein show the 22-nm repeat more clearly than native neurofilaments (reference 5 and Milam and Lifsics, unpublished data). It is possible that the protrusions in our preparations and the 100-nm periodicity in those of Krishnan et al. (16) are due to the 160- and 210-kdal proteins, which may be peripheral to the basic filament structure formed by the 68-kdal protein.

The angular slashes of metal were not found on the neurofilaments with sufficient reliability to specify the hand of this helix, so it may be premature to assume that the model applies to all intermediate filaments. A number of biochemical studies have, however, demonstrated that the different filaments are similar in many respects. These studies and the observation of a similar 21-nm periodicity strongly suggest that the basic structure is very similar for the different types of intermediate filaments.

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