

NUCLEUS-ASSOCIATED INTERMEDIATE FILAMENTS FROM CHICKEN ERYTHROCYTES

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ABSTRACT

Chicken erythrocyte nuclei prepared by isolation in isotonic KCl and Nonidet P-40 detergent were found to contain numerous attached filaments with a mean diameter of 11.0 nm. In polypeptide content and solubility properties, they resembled the vimentin type of intermediate filament found in cells of mesenchymal origin. Examination of their association with the nucleus suggests that more than a simple membrane attachment is involved.

Cytoplasmic intermediate filaments in the 10-nm diameter range are widespread in eukaryotic cells (1-17, 20, 22-24, 26, 28-41) and have been classified into four or five major groups (1, 12, 26) according to the type of cell in which they are found, their reaction to colcemid, their polypeptide content, their solubility characteristics, and their immunological properties. The intermediate filaments from various cell types are indistinguishable in ultrastructure and have other features in common including amino acid composition and high α -helix content (40). In this communication, the ultrastructure of a type of intermediate filament found tightly associated with chicken erythrocyte nuclei is described, and the properties of these filaments are compared with those of other systems. On the basis of their solubility and polypeptide content, it is suggested that the erythrocyte intermediate filaments are of the mesenchymal "vimentin" (12) type. The possible role of such filaments in erythrocytes is discussed, and the nature of the attachment to the nucleus is considered.

MATERIALS AND METHODS

Isolation of Nuclei

Freshly obtained chicken blood was diluted into ice-cold PK buffer (5 mM PIPES, 150 mM KCl, pH 7.5), and erythrocytes

were prepared by three low-speed centrifugations, and washes with the same buffer. For nuclear isolation, erythrocytes were resuspended in PK buffer (~50 ml/10 ml of whole blood) and stirred gently while Nonidet-P40 (NP-40) detergent was added to a final concentration of 0.4%. In most experiments, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) was included in this and all subsequent steps. After stirring for 10 min at room temperature, nuclei were centrifuged from the NP-40 lysate (~1,000 g for 10 min), after which they were washed three times in PK buffer without detergent. In some cases, nuclei were further purified by pelleting through PK buffer containing 2.0 M sucrose.

Preparation of Filaments

Nuclei were resuspended in PK buffer and homogenized for 45 s with a VirTis homogenizer (VirTis Co., Inc., Gardiner, N. Y.; 80 on speed setting). Most nuclei remained intact and were pelleted (5,000 g for 15 min), leaving an opalescent supernate which was enriched in 10-nm filaments. This supernate was made to 1.5 M KCl, 2% NP-40, 5 mM PIPES, and centrifuged at 70,000 g for 90 min (11). In some cases, the pellet was resuspended in KCl-NP40 and centrifuged a second time. The final pellets constituted the filament-enriched material that was used for SDS gel electrophoresis and electron microscopy. Filament-enriched preparations made 1.5 M KCl, 2% NP-40 were also separated on 5-30% sucrose gradients containing 1% NP-40, 1.5 M KCl. Centrifugation was carried out in an SW41 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 40,000 rpm for 2 h.

Samples were resuspended in SDS sample buffer and heated at 100°C for 2 min before applying to 10 or 12.5% SDS polyacrylamide gels (20). Standards used were bovine serum albumen (BSA), rabbit immunoglobulin, ovalbumin, chymotrypsinogen, and cross-linked hemoglobin (Sigma Chemical Co., St. Louis, Mo.).

Electron Microscopy

Nuclei were suspended in PK buffer and rapidly frozen by spraying the solution with an atomizer onto a polished metal surface cooled with liquid nitrogen. The frozen material was scraped from the surface, transferred to a liquid nitrogen-cooled porcelain mortar, and finely powdered by hand. In some experiments, a slurry of liquid nitrogen and frozen nuclei was maintained throughout the grinding operation to ensure that the sample remained at liquid nitrogen temperature. The powdered sample was then rapidly thawed in the solution of choice, or stored at -90°C for future examination.

For negative staining of frozen-thawed samples and other filament preparations, a drop of the suspension was placed on a glow-discharged carbon-coated grid, several drops of 2% uranyl acetate (aqueous) were allowed to drip over the surface of the grid, and the excess stain removed with filter paper.

For thin sectioning, suspensions of nuclei or erythrocytes were fixed with 2% glutaraldehyde in PK buffer for 1 h at 0°C , washed with several changes of buffer, and pelleted at 10,000 g for 15 min. The pellets were then cut into small fragments for subsequent handling. Post-fixation was done for 90 min at 0°C in 1% OsO_4 in PK buffer and, after several more washes, some samples were mordanted with 0.5% tannic acid in 0.5 M sodium cacodylate buffer, pH 7.0, for 30 min at 0°C (27, 30). All samples were subsequently washed in PK buffer, dehydrated with ethanol, and embedded in an Epon-Araldite mixture. Thin sections were stained with uranyl acetate and lead citrate, or with 1% aqueous KMnO_4 (21), and examined with a Siemens 102 electron microscope operated at 60 kV. Magnifications were calibrated with a diffraction grating replica (2,160 lines/mm) or with negatively stained catalase crystals (42).

RESULTS

(a) Conditions under Which Intermediate Filaments Were Observed in Negatively Stained Preparations

After rapid freezing, grinding, thawing in isotonic media, and negative staining, erythrocyte nuclei were frequently associated with smooth, ~ 10 -nm diameter filaments which appeared to be attached to irregular masses of chromatin (Figs. 1–6). The extent and organization of these filaments could be most clearly seen in preparations that were lightly stained and then observed with dark-field illumination (Fig. 1). Smooth filaments were most abundant after isolation in 0.15 M KCl, 5 mM PIPES, using NP-40 detergent to lyse the plasma membrane. Substitution of sodium ions, Tris buffer, and Triton X-100 all resulted in reduced numbers of filaments. This could be due partly to the physical removal of more filaments from the nuclei, but it was also evident that partial breakdown of filaments was occurring under these conditions. If nuclei were frozen and ground as usual, but thawed in water to disperse the chromatin, intermediate filaments were never seen.

The behavior of the filaments in solutions of intermediate salt concentration is considered below (section *d*).

(b) Location of Intermediate Filaments in Avian Erythrocytes

To determine the cellular location of the filaments, whole erythrocyte and nuclei were fixed under a variety of conditions, embedded in Epon-Araldite, and sectioned. Filaments 7–10 nm in diameter were seen in isolated nuclei but only after a tannic acid mordanting step was included in the glutaraldehyde- OsO_4 protocol (27, 30). Under these conditions, nuclei showed adhering filaments (Fig. 9), but their contrast was very low unless 1% KMnO_4 (21) was used to stain the sections (Fig. 10). With KMnO_4 staining, filaments were seen most readily in tangential sections of nuclei, and it was clear that they were attached to the chromatin mass (Fig. 9). Residual membrane material present in these preparations was derived from the plasma membrane, the nuclear envelope of erythrocytes being very fragile (18), and completely dispersed in low concentrations of nonionic detergents. Filaments could not be seen in sections of intact erythrocytes (Fig. 11), presumably because of the dense texture of the cytoplasm of these cells. These sections also illustrated the unusual nature of the erythrocyte nuclear membrane; nuclear pores were few and the usual double membrane was often discontinuous (Fig. 11). The normal morphology of membranes in adjacent lymphocytes indicated that fixation was adequate.

These findings suggested that the filaments were of cytoplasmic origin, but it was not clear whether they were binding adventitiously to nuclei during isolation or were really attached to the nuclei.

(c) Isolation of Fibers

To further characterize the filaments and obtain more information concerning their location, a number of previously reported purification schemes for intermediate filaments were tried. As in the case of other intermediate filaments, they proved resistant to extraction with high salt (2.0 M KCl) and nonionic detergents, especially NP-40 (22). It became clear, however, that methods involving salt and detergent extraction of whole cells or nuclei (12, 37, 38) were unsatisfactory in this case because the filaments constituted such a minor component of the cell, that the extracts were dominated by residual nuclear protein. To over-

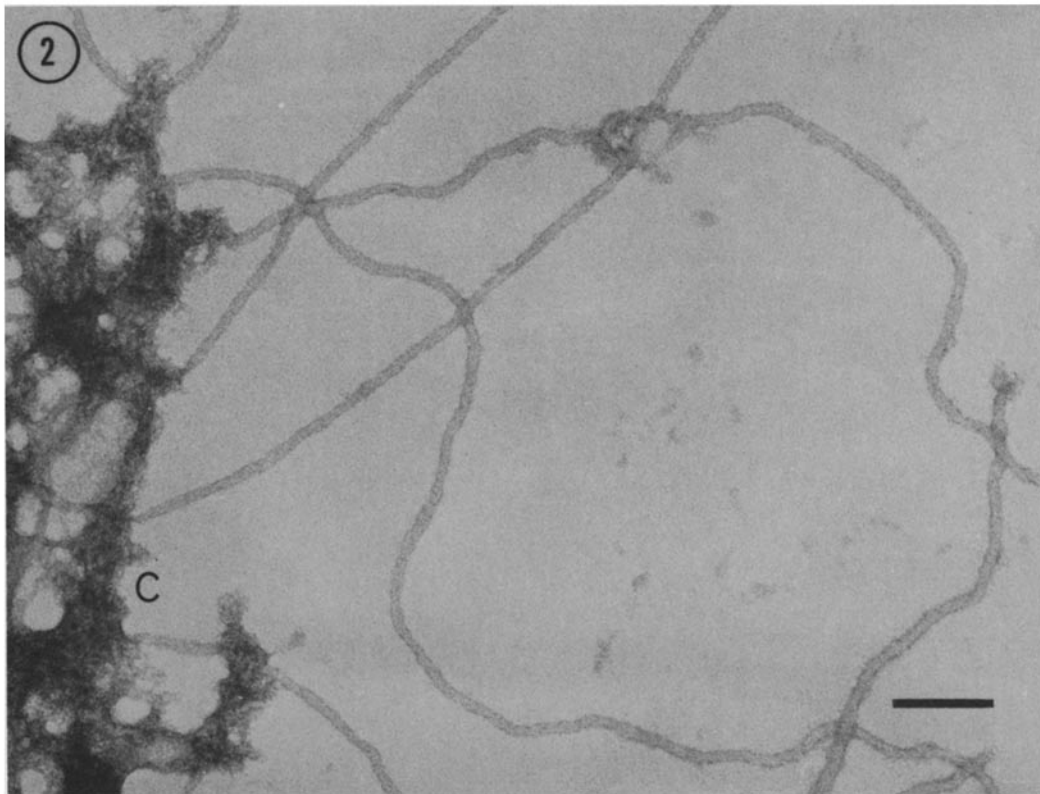
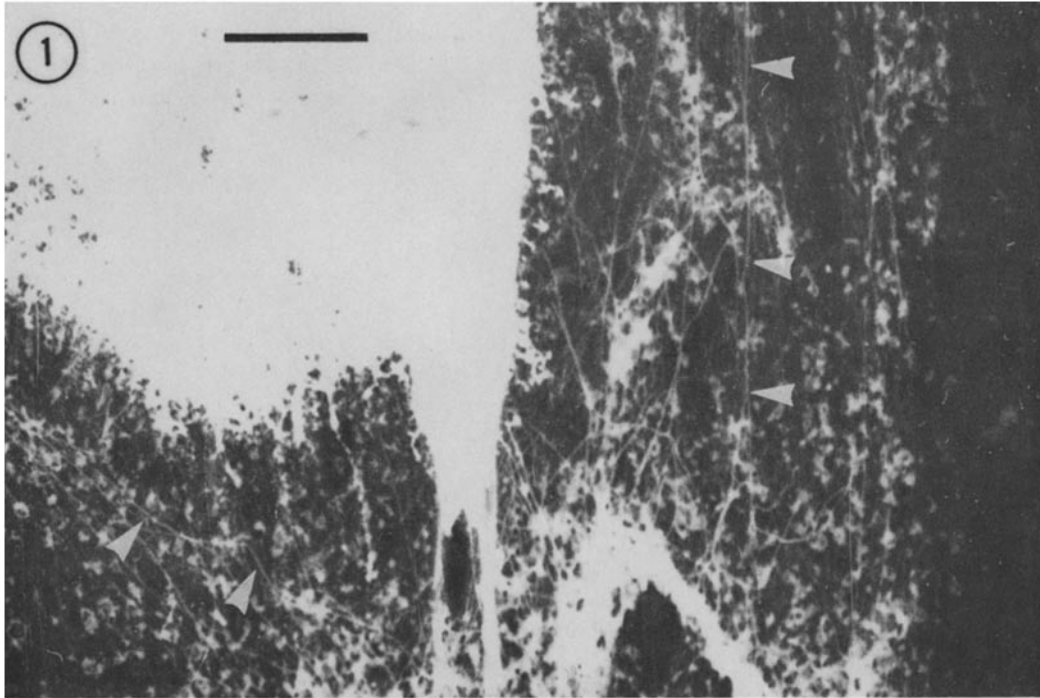
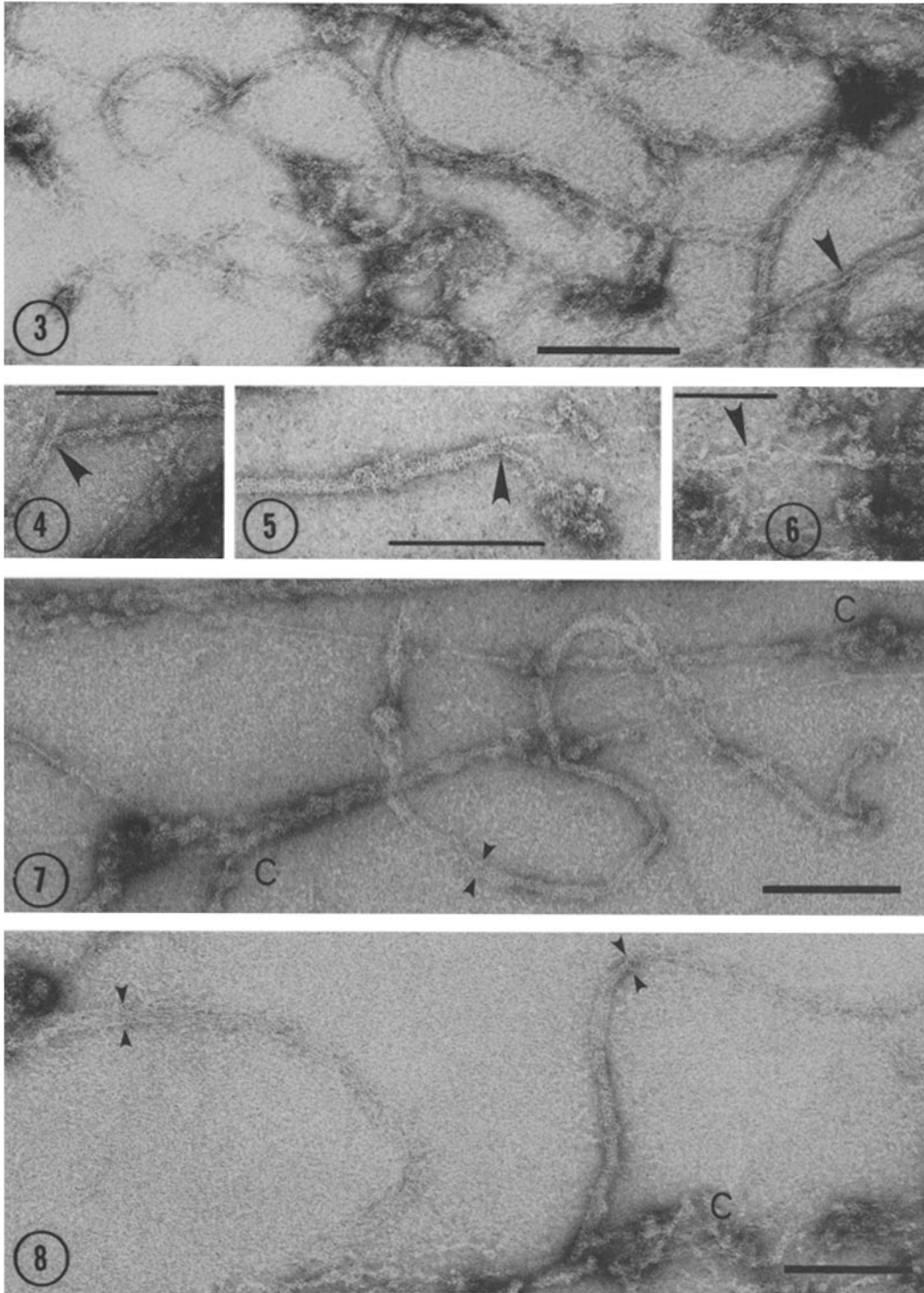


FIGURE 1 Dark-field electron micrograph of erythrocyte nucleus after freezing, grinding, thawing in PK buffer, and applying to a carbon-coated grid. Long, smooth filaments are seen near the periphery of the nucleus (arrowheads). Uranyl acetate stain. Bar, 1 μ m.

FIGURE 2 Negatively stained 10-nm filaments associated with a fragment of nucleus prepared as in Fig. 1. C, chromatin. Bar, 100 nm.



FIGURES 3-6 Higher magnifications of nucleus-associated 10-nm filaments showing the tendency to split longitudinally (arrowheads). In several places, longitudinal electron-opaque lines are evident. Preparation and staining as in Fig. 1. Bars, 100 nm.

FIGURES 7 and 8 If nuclear fragments are thawed in half-strength PK buffer, the filaments tend to disperse into subfilaments (at arrowheads). Chromatin fibers and aggregates (C) are distinguishable from the 10-nm filaments and subfilaments. Uranyl acetate negative stain. Bars, 100 nm.

come this problem, a fraction enriched in filaments was prepared from nuclei by displacing the filaments with a brief homogenization and repelleting the nuclei. After this treatment, nuclei remained intact, while the supernate contained numerous "10-nm" filaments, together with fragments of membrane, chromatin, and other debris, as monitored by electron microscopy. The filament-enriched supernate was then made to 2 M KCl, 5 mM PIPES, 2% NP-40, and the filaments were pelleted by centrifugation at 70,000 *g* for 2 h (11). In other experiments, the filament-enriched supernate was separated on a 5–30% sucrose gradient containing 1.5 M KCl, 5 mM PIPES, 1% NP-40, and fractions were collected. In all cases, the final pellets or gradient fractions were examined in the electron microscope after negative staining, and aliquots were heated with SDS sample buffer and run on 10% SDS polyacrylamide gels.

Both crude and 2.0 M sucrose-purified nuclei contained filaments which could be detached by a brief VirTis homogenization and were resistant to high salt and nonionic detergents. Some filaments were, however, stripped from the nuclei during pelleting through 2.0 M sucrose, as shown by their recovery from the supernate. Intermediate filaments were also found in fractions of a nuclear homogenate that had been separated on a 10–30% sucrose gradient containing 1.5 M KCl, 5 mM PIPES, 2% NP-40 (Fig. 12). No filaments were obtained from the initial NP-40 lysate of the nuclei, indicating that they were not free in the cytoplasm.

(d) Structure of Intermediate Filaments from Erythrocytes

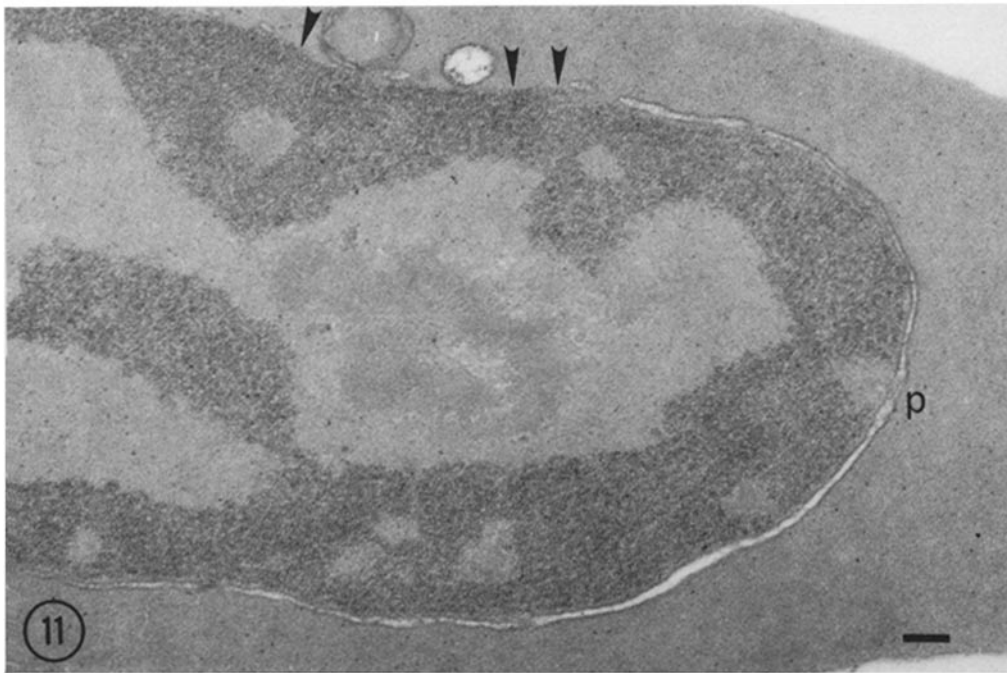
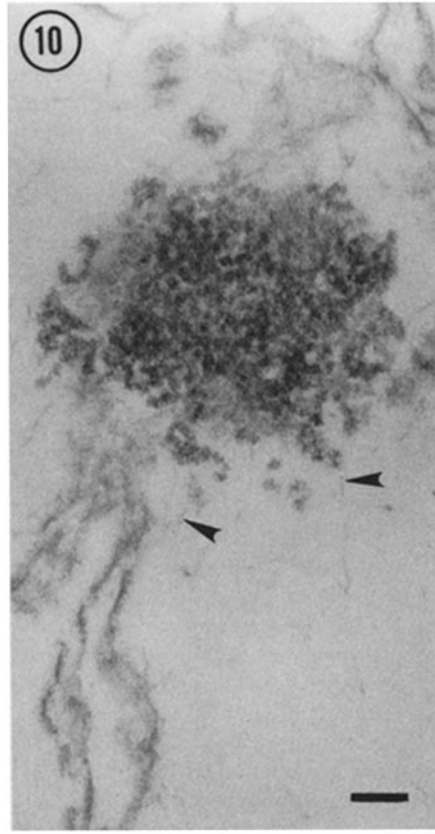
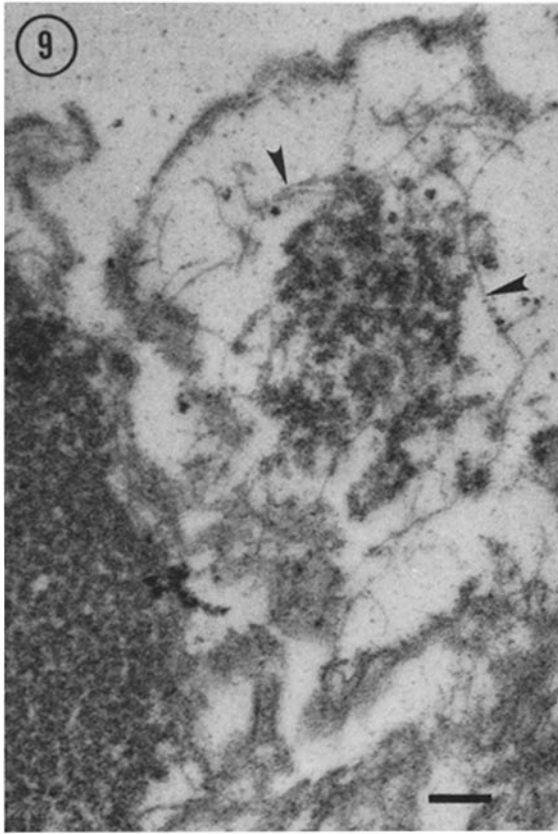
Negative staining of nuclei that had been rapidly frozen in liquid nitrogen, ground to a fine powder under liquid N₂, and thawed in isotonic salt gave the most detailed micrographs of the filaments (Figs. 2–8). They appeared as smooth, flexible, unbranched fibers with a mean diameter of 11 nm and, in many cases, a range of diameters from 10 nm to as high as 14 nm. Regions showing longitudinal striations were interspersed with regions showing substructure, but little or no periodicity. Where striations were present, indicating stain penetration, they took the form of two equally spaced dark lines running parallel to the length of the filament. This is reminiscent of the "fasciate" structure of several classes of 10-nm filaments as noted by Franke et al. (12) but diffi-

cult to reconcile with the hollow-tube type of structure seen in transverse sections (8, 40). Occasionally, the filaments were seen to split longitudinally into two subfilaments, and in these cases it was clear that the split occurred at one of the double lines (Figs. 3–6). Further details concerning the mode of dissociation of the filaments was obtained from preparations in which the nuclei had been thawed into lower ionic strength buffer (75 mM KCl, 5 mM PIPES). Under these conditions, dissociation into very thin subfilaments ~2.0 nm in width occurred (Figs. 7 and 8). Because of their small diameter, these subfilaments were poorly resolved, but estimations based on a number of micrographs suggest that each intermediate filament may break down into at least six subfilaments. These preparations were also characterized by structures that appeared to be free subfilaments, having the same size and staining characteristics as those attached to the 10-nm filaments. They were clearly distinguished from associated chromatin and DNA fibers by their small diameter and relative rigidity. Subfilaments have also been observed in other intermediate filament systems: Small and Sobieszek (36) noted their occurrence in smooth muscle filaments, and Steinert (41) studied the structure of reconstituted subfilaments from prekeratin filaments.

When nuclei were briefly homogenized and the low-speed supernate was treated with 2 M KCl, 5 mM PIPES, 2% NP-40, and sedimented on sucrose gradients containing high salt, fibers identical to those seen in isolated nuclei were found throughout the gradient (Fig. 12). The failure to form a distinct band in the gradient was evidently because of the tendency for aggregates to form and sediment faster than individual fibers and smaller fragments. However, apart from the fibers, there was very little other material in the gradient that could be seen in negatively stained preparations, the major contaminants being membranelike fragments (Fig. 12). Optical diffraction analysis of micrographs of the intermediate filaments failed to reveal any underlying structural periodicities.

(e) Polypeptides Associated with Fiber-enriched Fractions

Filament-enriched samples were incubated with SDS sample buffer and subjected to electrophoresis on 10 or 12.5% polyacrylamide SDS gels (Fig. 13). All samples were heterogeneous in that a number of polypeptides were present. However, in



all cases where fibers were seen by EM analysis to be a major constituent of the sample, a major polypeptide with an apparent mol wt in the 54,000–56,000 range was present (Fig. 13). This molecular weight designation was verified by running purified 3T3 cell vimentin (57,000 daltons, reference 12) in adjacent lanes. Even the sucrose gradient fractions, which appeared to have little contaminating material (Fig. 12), showed seven bands in the 40,000–60,000 mol wt region, but again the ~55,000-dalton band was the strongest (Fig. 13). Samples in which filaments were absent did not contain an ~55,000-dalton component.

DISCUSSION

The ultrastructure of avian erythrocytes, nuclei, and chromatin has been described in numerous publications, but with few exceptions (18, 19, 30) intermediate filaments have not been observed. This is evidently because of their lability in low ionic strength media (Figs. 7, 8, and reference 40) and their failure to stain well in sections after conventional treatment (Figs. 9–11, and reference 30).

There seems little doubt from their morphology and resistance to high salt that the structures described above belong to the heterogeneous group of 10-nm intermediate filaments. On the basis of mesenchymal origin, erythrocytes would be expected to contain the vimentin type of intermediate filament (1, 12) rather than the prekeratin/tonofilament type characteristically associated with epidermal cells (8, 11, 14, 15, 39) or any other class of intermediate filament, and several lines of evidence suggest that this is indeed the case. Firstly, the erythrocyte filaments have solubility properties similar to those of vimentin, being soluble at low ionic strength when prekeratin/tonofilament filaments are insoluble (39, 40). Secondly, the major polypeptide band obtained from erythrocyte filament-enriched preparations migrated only slightly faster than 3T3 cell vimentin (Fig. 13). Thirdly, in cases where several prominent bands were obtained from filament-enriched samples, none co-migrated with tonofilament polypeptides. Further characterization of the erythrocyte filaments will require their purification to homogeneity. It will also be useful to test for immuno-



FIGURE 12 Negatively stained preparation of isolated filaments from sucrose gradient, showing that the filaments are relatively free of contaminating material. Bar, 100 nm.

FIGURES 9 and 10 Tangential sections of isolated erythrocyte nuclei. In Fig. 9, the central dark chromatin mass is surrounded by smooth filaments (arrowheads). Part of a second nucleus appears at the left of the micrograph. In this case, the nuclear pellet was mordanted with tannic acid and the sections were stained with KMnO_4 (see Materials and Methods for details). Fig. 10 shows a similar section stained with uranyl acetate and lead citrate. Only faint indications of peripheral filaments are seen (arrowheads). Bars, 100 nm.

FIGURE 11 Section through whole erythrocyte showing the unusual features of the nuclear membrane. There are very few nuclear pores (*p*), and the membrane is discontinuous in places (arrowheads). Bar, 100 nm.

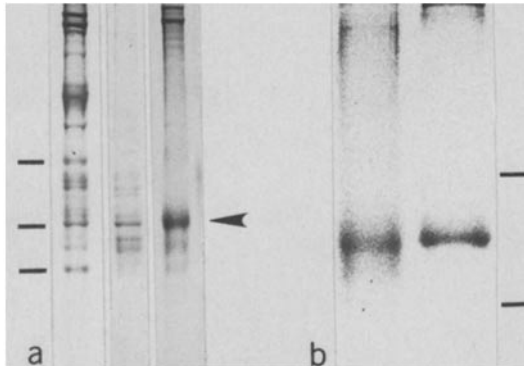


FIGURE 13 SDS gels of fractions enriched in 10-nm filaments. (a) 10% polyacrylamide. Left, whole nuclei; center, sucrose gradient fraction shown in Fig. 12; right, 70,000 g pellet of 1.5-M KCl-treated filament preparation (see Materials and Methods). Arrowhead indicates putative major filament polypeptide. Lines at left indicate positions of molecular weight standards: BSA, 68,000; rabbit IgG (heavy chain), 50,000; ovalbumen, 43,000. (b) 12.5% polyacrylamide. Left, 70,000 g pellet of 1.5-M KCl-treated filament preparation; right, 3T3 cell vimentin (57,000), sample kindly supplied by Dr. W. W. Franke. Lines at right indicate positions of standards: BSA (68,000), ovalbumen (43,000).

logical affinity between the erythrocyte intermediate filaments and other well-characterized types.

The present evidence suggests that the filaments are anchored in the perinuclear region of the cell, probably to the nucleus itself. This would explain their absence from the detergent lysis supernate and their co-purification with nuclei even during centrifugation through heavy sucrose. A perinuclear location for intermediate filaments is common (1, 3, 5, 8, 12, 16), and after colcemid treatment or during spreading of certain cultured cells the vimentin type forms thick cables that appear as nuclear "caps" (1, 12, 37, 40). However, specific attachment to the nucleus is less well documented. If the erythrocyte filaments were simply attached to the outside of the nuclear envelope, it would be expected that as the membrane was solubilized by detergent, the filaments would become detached and appear in the supernate after pelleting the nuclei. Because the erythrocyte filaments remain bound after dissolution of the nuclear envelope, they must be attached in a manner that is independent of membrane integrity, perhaps via structures which extend through the membrane. (An alternative explanation involving, firstly, the release of the filaments from the membrane and, secondly, their attachment to the chromatin mass

seems unlikely in view of the complete lack of filaments in the detergent supernate.) In support of a transmembrane attachment for intermediate filaments, Lehto et al. (30) also noted their retention on nuclei after removal of the envelope, and Franke (7) observed changes in the appearance of the nuclear envelope in places where large numbers of intermediate filaments were attached. These structural changes took the form of an increased density in the space between the two nuclear envelope membranes and a loss of definition in the membranes themselves.

The function of the intermediate filaments in nucleated erythrocytes is unknown, although their association with the nucleus is consistent with an anchoring role (43), and some evidence in support of this has been presented in the case of cultured human fibroblasts (30). Alternatively, they may have no function, merely representing the vestiges of a cytoskeletal system that was needed during erythropoiesis. Because it was not possible to visualize the filaments in fixed, unlysed erythrocytes (Fig. 11), the possibility cannot be excluded that they spontaneously polymerize during nuclear isolation.

A final point that should be mentioned concerns the dramatic changes in the preservation of erythrocyte filaments brought about by changes in ionic composition and buffer type of nuclear isolation media. As the filament polypeptides would contribute to total nuclear proteins and be enriched in many nonhistone chromosomal protein and "nuclear matrix" preparations, this possible source of "contamination" should be considered.

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