

Immunolectron Microscope Studies of Membrane-Microfilament Interactions: Distributions of α -Actinin, Tropomyosin, and Vinculin in Intestinal Epithelial Brush Border and Chicken Gizzard Smooth Muscle Cells

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ABSTRACT The ultrastructural localization of three cytoskeletal proteins, α -actinin, tropomyosin, and vinculin, in the brush border of epithelial cells of chicken small intestine and the smooth muscle cells of chicken gizzard was studied by immunofluorescence and immunolectron microscope labeling of frozen sections of lightly fixed, intact tissues. In the immunolectron microscope studies, a recently described new type of electron-dense antibody conjugate, Imposil-antibody, has been successfully used, along with ferritin-antibody conjugates, in single and double immunolabeling experiments. In the intestinal brush border the results show that vinculin is sharply confined to the junctional complex close to the membrane region of the zonula adherens, in distinct contrast to the more diffuse distributions of the other two proteins. In the smooth muscle cells, the labeling patterns show that vinculin is sharply confined to the membrane-associated dense plaques, closer to the membrane than the α -actinin which is also present in these dense plaques. α -Actinin is also present in the cytoplasmic dense bodies, from which vinculin is absent. Tropomyosin is present diffusely distributed in the cytoplasm, but absent from both dense plaques and dense bodies. These findings with the muscle cells demonstrate, therefore, that the dense plaques and dense bodies are chemically and structurally distinct entities. The results with both tissues, along with those in previous papers (Geiger, 1979, *Cell*. 18:193-205.; Geiger et al., 1980, *Proc. Natl. Acad. Sci. U. S. A.* 77:4127-4131), suggest that vinculin may play an important and widespread role in the linkage of actin-containing microfilament bundles to membranes.

The interactions of microfilaments with membranes are involved in a number of important cell physiological functions, including cell adhesion and contractility. There is as yet, however, little information concerning the molecular components involved in these interactions. To investigate these and other ultrastructural problems, we developed in this laboratory a set of techniques to permit the immunolectron microscopic localization of specific macromolecules inside fixed cells at a resolution of ~ 30 nm. These techniques include: ultrathin frozen sectioning of lightly fixed cells and tissues in a manner that maximally preserves their ultrastructure while permitting ac-

cessibility to the antigen and specific immunochemical labeling of the sections (38, 39); and the preparation of electron-dense antibody conjugates, using either the ferritin (31, 32) or the recently introduced iron-dextran (Imposil) method (11), or both together in simultaneous double-labeling experiments. Imposil is a commercially available preparation consisting of water-soluble particles with an inner core of iron oxyhydroxide surrounded by a shell of alkali-modified dextran (23). The particles have a narrow distribution of sizes, with average outer dimensions of 12×21 nm. The inner core, which is the part that is visualized in transmission electron microscopy, is ani-

sometric with average dimensions of 3×11 nm. A stable covalent conjugate of Imposil and antibody has been prepared (11) in which the main conjugated species contains one Imposil particle bound to one antibody molecule. The anisometric core of an Imposil particle is readily distinguished from the isometric core of a ferritin molecule in electron micrographs. This therefore permits ferritin and Imposil conjugates to be used simultaneously in double-labeling experiments.

These methods are applied here to the comparative ultrastructural localization of three proteins, α -actinin, tropomyosin, and vinculin, within the brush border of intestinal epithelium and in chicken gizzard smooth muscle. The intestinal brush border is of interest in the present context because both in the apical microvilli and at the zonula adherens in the junctional complex, associations of microfilaments with membranes occur. In smooth muscle cells, the membrane-associated dense plaques are also sites of microfilament-membrane interactions (see Discussion). α -Actinin (27) and vinculin (12, 15) have been proposed to play a role in the linkage of microfilaments to membranes, and their comparative ultrastructural distributions in these and other tissues are therefore important to establish. The results show that at specialized sites of membrane-microfilament attachments in both types of cells vinculin is more closely associated with the membrane than is α -actinin or tropomyosin, supporting the suggestion (12, 15) that vinculin may function as a peripheral protein in the attachment of microfilament bundles to membranes in different types of cells.

MATERIALS AND METHODS

Immunochemical Reagents

Rabbit and guinea pig antibodies to chicken gizzard α -actinin (13) and tropomyosin (17), and rabbit antibodies to chicken gizzard vinculin (12), were prepared and characterized as monospecific antibodies, as described. Each of these primary antibodies was affinity-purified on the relevant antigen immunoabsorbent prepared using Ultrogel AcA-22 (LKB Instruments, Inc., Rockville, Md.) (37). Goat antibodies against rabbit and guinea pig IgG were raised and affinity-purified as above, first on immunoabsorbents made with the homologous IgG and then on ones made with the heterologous IgG to remove any cross-reactive antibodies. The affinity-purified antibodies were stored at 4°C in phosphate-buffered saline (PBS) in the presence of 0.01% NaN_3 .

For the immunofluorescence studies, the affinity-purified goat antibodies were labeled with rhodamine-lissamine sulfonylchloride according to Brandtzaeg (2). The modified antibodies were fractionated on a DEAE-cellulose column and fractions containing three to four fluorophore molecules per molecule of antibody were used. Ferritin conjugates of the goat antibodies were prepared by the method of Kishida et al. (19).

Preparation of Imposil-Antibody Conjugates

The method used for conjugation involves the partial oxidation of the dextran outer shell of the Imposil particle with periodate, the reaction of the aldehyde functions of the oxidized Imposil with amino groups on the antibody molecule, and the reduction of the Schiff base linkages so-formed to secondary amines by reaction with borohydride. The scheme is similar to that used by Sanderson and Wilson (29). The detailed conjugation procedure has been described (11), and since then has been modified in only two minor respects: after the oxidation reaction with the periodate, the oxidized Imposil was dialyzed for 12–24 h at 4°C against 0.15 M NaCl, to remove unreacted periodate, instead of being separated by chromatography through a Biogel P-6 column (this has given more reproducible results); also, in the conjugation step, we now use 4.5 instead of 3.0 mg of affinity-purified antibody.

The reaction conditions that are employed for the entire procedure were selected by examination of several reaction variables. A concentration of 0.05 M sodium periodate is used because it is the lowest concentration that produced optimal oxidation of Imposil for subsequent conjugation to antibody. For the conjugation reaction itself, a minimum of pH of 8 was required to effect the conjugation within 24 h at room temperature. Longer reaction times led to the formation of increasing amounts of higher-molecular-weight Imposil-antibody complexes.

After the Imposil is conjugated to antibody, the resultant mixture is separated from unconjugated antibody, and also fractionated, by passage through a Sepharose 4B column (2.4×90 cm) that is equilibrated with 0.1 M sodium phosphate buffer, pH 7.5, at an elution rate of 45 ml/h. Each 3-ml column fraction is analyzed for its total Imposil content by its optical absorbance at 430 nm and for its total antibody content by the Lowry method, or from its radioactivity in those experiments in which a small amount of ^{125}I -labeled IgG was added to the antibody at the beginning of the conjugation reaction. The Sepharose column was first calibrated by gel filtration of different dextran preparations of known average molecular weights in the range between 2.5×10^5 and 2×10^6 (Pharmacia Fine Chemicals, Uppsala, Sweden). Imposil itself was found to elute at approximately the same volume as a dextran of average molecular weight 5×10^5 . Fractions from the Imposil-antibody conjugation mixture are collected and pooled which elute at volumes corresponding to dextran molecular weights between 4×10^5 and 8×10^5 , as indicated in Fig. 1. This pooled material is concentrated by ultracentrifugation for 4–6 h at 340,000 g; the supernate is discarded, and the pellet is resuspended in the phosphate buffer.

Under such conditions, the final preparation, consisting of a mixture of Imposil-antibody conjugates (mostly 1:1 and 2:1 species) and free Imposil, contained ~ 500 – 700 μg protein/ml and 3–4 mg Fe/ml. Approximately 25% of the antibody was recovered in the preparation. We estimate from the protein/Fe ratio that three to four Imposil particles were present per antibody molecule, some considerable (but not measured) fraction of the Imposil being free. There was no indication that the free Imposil interfered in any way with the labeling by the Imposil-antibody conjugates, and so the mixture was used as such.

To test the retention of antibody binding activity in the Imposil-antibody conjugate preparation, a passive hemagglutination assay was carried out. Human erythrocytes were coated with a rabbit antiserum (previously heated at 56°C) directed to whole human erythrocytes, at a level below that which agglutinated the cells. The hemagglutination of these treated cells by serial dilutions of an Imposil-conjugated goat antibody to rabbit IgG, a ferritin-conjugate of the same antibody, and of the unconjugated antibody, was then determined. By this assay, the Imposil-conjugates retained approximately half of the agglutinin activity per milligram of antibody of either the ferritin-conjugate or the original antibody. Imposil itself did not agglutinate the coated cells.

Effects of Fixation Treatments on the Antibody-binding Activities of Soluble Antigens

Each of the antigens, α -actinin, tropomyosin, and vinculin, was radioiodinated by the lactoperoxidase method (22). Each labeled protein, at 2 $\mu\text{g}/20$ μl (2×10^6

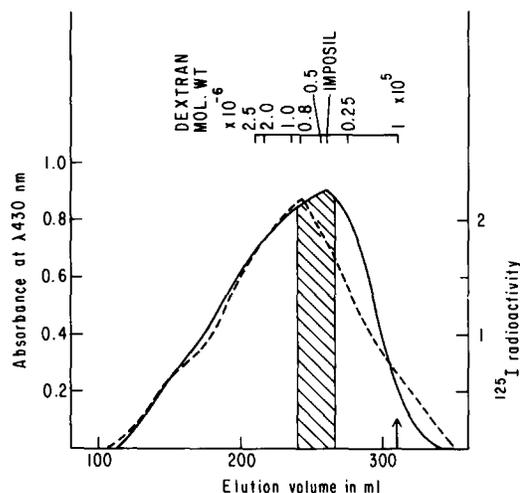


FIGURE 1 The gel filtration of an Imposil-antibody conjugation mixture on Sepharose 4B. Fractions were analyzed by optical absorbance at λ 430 nm (left ordinate, solid line) to measure their total Imposil content, and by radioactivity (right ordinate, dashed line) to measure (in arbitrary units) their total antibody content. The upper calibration scale gives the elution volumes corresponding to the indicated average molecular weights. The arrow on the abscissa corresponds to the peak position observed during filtration of unconjugated antibody. The striped area indicates the fractions that were pooled for use as the final Imposil-antibody conjugate, representing $\sim 25\%$ of the total antibody in the mixture.

cpm), was mixed with a fivefold molar excess of the specific rabbit antibodies followed by a suspension of *Staphylococcus aureus*. (100 μ l of a 10% suspension was sufficient to absorb 10 μ g of antibodies). The suspension medium was PBS containing 0.1% gelatin. After 1 h at 4°C, the suspensions were centrifuged in a Beckman Microfuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) and the radioactivity remaining in the supernate was determined.

To examine the effects of the fixatives on these antigens, each radioiodinated protein at a concentration of 100 μ g/ml in PBS was treated at room temperature with 20 mM ethylacetimidate for 10 min, or 3% paraformaldehyde for 1 h, or 0.1% glutaraldehyde for 1 h, or various combinations as listed in Table I. After these treatments, each sample was dialyzed for 24 h against PBS containing 0.2 M glycine to quench any remaining aldehyde or imidate groups. The dialyzed samples were then centrifuged in a Beckman Microfuge to remove aggregated material (<20% of the original protein). Each treated protein was then tested for its capacity to be immunoprecipitated by its specific antibodies. The antibody concentration in these experiments was chosen in slight antibody excess to produce ~70% of the maximum specific immunoprecipitation of the untreated radiolabeled antigen.

Specimen Preparation and Immunofluorescence Labeling

Semi-thin frozen sections of ~0.5- μ m thickness were prepared from chicken small intestine by the sucrose-infusion technique as previously described (14, 40), except that fixation of the tissue was carried out with 3% formaldehyde without any glutaraldehyde. Postfixation treatment with NaBH₄ was therefore not required. The affinity-purified rabbit antibodies to each of the three antigens, at 50–100 μ g/ml, were individually applied to the sections, followed by the rhodamine-conjugated goat antibodies to rabbit IgG, at 5–20 μ g/ml. The fluorescent-labeled sections were examined with a Zeiss Photomicroscope III instrument using epillumination and the CZ487714 filter combination.

Specimen Preparation and Immunoelectron Microscopic Labeling

Chicken small intestine or chicken gizzard was dissected to blocks of 1-mm dimension or smaller in one of two fixative solutions: 3% paraformaldehyde plus 0.1% glutaraldehyde in PBS (for the single immunolabeling of α -actinin or tropomyosin and for the double immunolabeling of α -actinin and tropomyosin); or 3% paraformaldehyde plus 20 mM ethylacetimidate in PBS (for single or double immunolabeling involving vinculin) (39). In the latter case, after 2- to 10-min incubation, the blocks were transferred into 3% paraformaldehyde plus 0.1% glutaraldehyde. After 1 h in the formaldehyde-glutaraldehyde fixative solution, all of the specimens were rinsed and infused with 0.5–0.6 M sucrose, rapidly frozen in liquid N₂ and ultrathin frozen sectioned as described (38, 39).

In single immunolabeling experiments, the primary antibodies were applied to the sections at concentrations of 50–100 μ g/ml for 10 min at room temperature. After thorough rinsing, the sections were then treated with either the ferritin- or Imposil-conjugated secondary antibody at a concentration of 50–200 μ g antibody protein/ml. In double immunolabeling experiments, the rabbit and guinea pig primary antibodies were mixed and applied together to the section; after thorough rinsing, the two cross-absorbed secondary antibody conjugates were then applied to the section. Positive staining of the immunolabeled section was carried out as described (14). Sections were examined in a Philips Model 300 transmission electron microscope operated at 60 kV.

RESULTS

Immunofluorescence Studies of Frozen Sections of Chicken Intestine

As a preliminary to the immunoelectron microscope investigations, we examined the comparative distributions of the three proteins in intestinal epithelium by immunofluorescence methods at the light microscopic level of resolution. For this purpose, frozen sections of 3% formaldehyde-fixed tissue of 0.5- μ m average thickness were prepared by the sucrose-infusion method (40) for use in immunofluorescent staining. The indirect immunofluorescence procedure was used.

No immunofluorescent staining for any of the three proteins was seen within the microvilli (Fig. 2A, D, and E), but all three were specifically stained within the terminal web. Vinculin staining (Fig. 2A and B) was highly localized to the terminal

bars where two adjacent epithelial cells make contact, with little if any staining visible along the rest of the lateral membranes of the cell. There was also intense vinculin staining in the lamina propria (Fig. 2A), which, not being of central interest to us here, we will not discuss further. Staining for α -actinin was more widespread than for vinculin. While there was a concentration of α -actinin staining in the terminal bars (Fig. 2D), there was also specific staining in other regions of the terminal web, as well as light staining along the lateral membranes, as we had observed earlier (14). Staining for tropomyosin was apparently still more broadly distributed than for α -actinin. There was a more generally diffuse labeling of the terminal web, and that labeling which was concentrated at the terminal bar showed a distinct doublet character (Fig. 2E). In addition, the regions along the lateral membranes of the epithelial cells (as well as the lamina propria) showed substantial staining.

As one type of control for these immunofluorescence experiments, the substitution of rabbit normal IgG for rabbit antibodies in the first stage of the indirect staining technique resulted in no subsequent staining with the fluorescent secondary antibody (Fig. 2C).

Fixation for Immunoelectron Microscopy

Fixation with 3% formaldehyde, as employed for the thicker frozen sections used in immunofluorescence, did not satisfactorily preserve the ultrastructure of the thinner frozen sections (~50–100 nm) used in electron microscopy. In a previous study of ours (14), 3% formaldehyde + 0.1% glutaraldehyde proved to be a satisfactory fixative for the immunoelectron microscopic labeling of α -actinin; but, when it was used for vinculin, no labeling was observed. It was then shown by the immunoelectron methods described in Materials and Methods that treatment of vinculin in solution with 3% formaldehyde + 0.1% glutaraldehyde essentially completely abolished its capacity to react with antibody to the native protein, but did not significantly affect the antigenicity of either α -actinin or tropomyosin (Table I). A two-stage procedure, involving treatment of the tissue with 3% formaldehyde containing 20 mM ethylacetimidate for 2–10 min at room temperature followed by fixation with 3% formaldehyde + 0.1% glutaraldehyde (39), however, was found to permit satisfactory immunofluorescent and immunoelectron microscopic labeling of vinculin, and, in solution experiments, to have essentially no effect on the antigenicity of vinculin, α -actinin, or tropomyosin. On the other hand, when these fixation conditions were employed for the immunoelec-

TABLE I
Effects of Fixation Treatments on Protein Antigen Activities

Antigen	Fixation treatment*			% Activity \ddagger
	EAI	FA	GA	
Vinculin	–	–	–	100
	+	–	–	96
	–	+	–	88
	–	–	+	5
	+	+	–	91
α -Actinin	–	+	+	2
	+	+	+	81
	–	–	–	100
	–	+	+	84
	+	+	+	90
Tropomyosin	–	–	–	100
	–	+	+	84
	+	+	+	100

* Abbreviations: EAI, ethylacetimidate; FA, formaldehyde; and GA, glutaraldehyde. Conditions as described in Materials and Methods.

\ddagger As measured by radioimmune assays described in Materials and Methods.

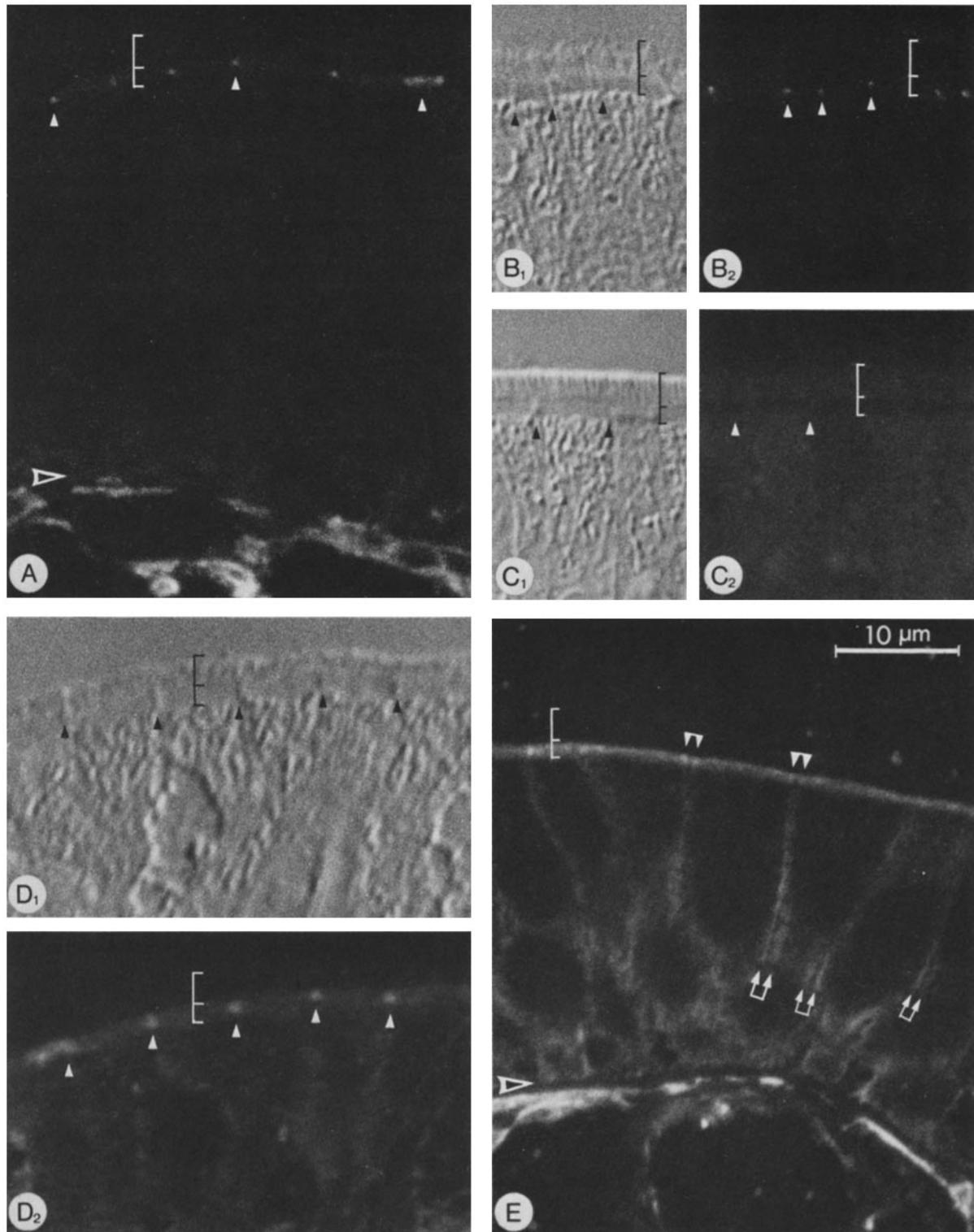


FIGURE 2 Indirect immunofluorescence labeling of chicken intestine for vinculin in *A* and *B*, α -actinin in *D*, and tropomyosin in *E*, using rabbit antibodies as primary reagents, and rhodamine-conjugated goat antibodies to rabbit IgG as the secondary reagent. The control in *C* was stained with normal rabbit IgG in the first stage. Upper and lower parts of the double brackets in these figures indicate the layer of microvilli and the terminal web, respectively. In *A*, vinculin is localized in discrete spots in the terminal web (small arrowheads). Comparison of the Nomarski, *B*₁, and the immunofluorescence, *B*₂, micrographs of the same field reveals that such spots correspond to midpoints of the terminal bars (compare arrowheads in *B*₁ and *B*₂). One of such spots in *A* (arrowhead at the right upper corner) is laterally elongated, which is believed to represent an obliquely sectioned part of the terminal bar. In *A*, certain vinculin-positive structures are also found in the lamina propria below the basal border of the epithelium (large arrowhead). In the Nomarski (*C*₁) and the immunofluorescence (*C*₂) micrographs of a control section, terminal bars show no recognizable fluorescence (compare arrowhead in *C*₁ and *C*₂). In the Nomarski, *D*₁, and the immunofluorescence, *D*₂, micrographs of a section, the terminal bars as well as the terminal web are found to be positive for α -actinin (compare arrowheads in *D*₁ and *D*₂). α -Actinin-positive spots in *D*₂ are significantly wider than vinculin-positive spots in *A* and *B*₂. In *E*, it is seen that the terminal web is positive for tropomyosin but not the microvilli layer. Pairs of tropomyosin-positive sites (paired arrowheads) are localized at the terminal bars and pairs of tropomyosin-positive lines (paired arrows) along cross-sectioned lateral cell borders. Bar, 10 μ m. \times 2,000.

tron microscopic labeling of α -actinin in intestinal epithelium, the distribution of label appeared more diffuse (not shown) than when the first stage was omitted. No such effect was observed with tropomyosin. (In our previous study (14), artifactual displacement of α -actinin was also a subject of concern.) In the following experiments, therefore, immunoelectron microscopic labeling involving vinculin was carried out on sections of tissue that had been fixed by the two-stage procedure, whereas labeling involving α -actinin or tropomyosin was performed after fixation with only the 3% formaldehyde + 0.1% glutaraldehyde step. In chicken intestine, double labeling experiments using the two-stage fixation are shown only for the pair vinculin and tropomyosin, and, in chicken gizzard, double labeling of all three pairs of proteins is shown.

Preparation and Characterization of Imposil-antibody Conjugates

The preparation and properties of these conjugates, which have been partially described elsewhere (11), are presented here in some detail (Materials and Methods). The final pooled preparation contains Imposil-antibody conjugates (probably mostly 1:1 and 2:1 species) along with free Imposil, from which any unconjugated antibody is removed by the fractionation on the Sepharose 4B column. Substantial antibody activity is retained in the Imposil-antibody conjugates, ~50% as measured by a hemagglutination assay (Materials and Methods).

Immunoelectron Microscopy of Vinculin in Intestinal Epithelium

Ultrathin frozen sections of tissue fixed by the two-stage procedure were first treated with rabbit antibodies to vinculin, and then with goat antibodies to rabbit IgG that were conjugated either to ferritin or to Imposil. Closely similar results were obtained with Imposil-antibody conjugates (Fig. 3A) as had previously been obtained with ferritin-antibody (15). Vinculin labeling of the epithelial cells was sharply confined to the region of the junctional complex close to the zonula adherens (region 2 in Fig. 3A). The tight junctions and spot desmosomes (region 1 and 3, respectively) were not labeled, nor was the remainder of the terminal web or the microvilli. Labeling fell off sharply beyond 60 nm from the membrane at the zonula adherens. Controls using rabbit normal IgG in place of the rabbit antivinculin antibodies in the first stage of the labeling reactions were free of Imposil (not shown).

Immunoelectron Microscopy of α -Actinin or Tropomyosin in Intestinal Epithelium

As previously recorded with ferritin-antibody labeling (14), in the junctional complex Imposil-antibody labeling for α -actinin was concentrated in the region around the zonula adherens, with some labeling also observed near the tight junctions (Fig. 3B). Very little label was found around the spot desmosomes. There was also considerable labeling elsewhere in the terminal web, particularly on the "roots" of the core microfilaments extending out of the microvilli. Few Imposil particles were observed within a distance of ~30 nm from the cell membrane at the zonula adherens (Fig. 3B) corresponding to the ferritin results shown in Fig. 3B of Geiger et al. (14).

Ferritin-antibody indirect labeling for tropomyosin is shown in Fig. 3C. Labeling in the terminal web was broadly diffuse (see Fig. 4B), but was to some extent more intense in the

vicinity of the junctional complex, particularly near the zonula adherens. It was clearly noticeable, however, that tropomyosin labeling was markedly diminished within a distance of ~100 nm from the cell membrane at the zonula adherens.

Double Immunoelectron Microscopy for Vinculin and Tropomyosin in Intestinal Epithelium

For these experiments, a mixture of rabbit antibodies to vinculin and guinea pig antibodies to tropomyosin was applied to the frozen sections in the first step of the indirect double-labeling reaction, and then a mixture of Imposil-conjugated goat antibodies to rabbit IgG and ferritin conjugated goat antibodies to guinea pig IgG (the two antibodies having been first cross-absorbed against the heterologous IgG) was used in the second step. The section shown in Fig. 4A was cut nearly parallel to the plane of the terminal web, and different portions of this section are shown enlarged in Fig. 4B and C. In the regions near the microvilli (Fig. 4C) there was no Imposil labeling for vinculin, and the ferritin labeling for tropomyosin was absent within the roots of microvilli but present in the regions between the microvilli in the terminal web. In the regions close to the zonula adherens (Fig. 4B), there was a predominance of Imposil over ferritin particles, whereas further into the terminal web only ferritin particles were observed. These results closely correspond to those obtained in the single-labeling experiments shown in Fig. 3A and C, which show that vinculin labeling in the brush border is largely confined to the zonula adherens, whereas tropomyosin labeling is excluded from that region but is extensive elsewhere in the terminal web. A control for the distinct specificities seen in the double-labeling experiments is shown in Fig. 4D, in which the specimen was treated exactly as above except that guinea pig normal IgG was substituted for the guinea pig antibodies to tropomyosin. Only Imposil labeling (for vinculin) was observed.

Quantitative Analysis of the Immunoelectron Microscopic Label Distributions in Intestinal Epithelium

The results of experiments such as those exemplified in Figs. 3 and 4 suggested qualitatively that although the three proteins vinculin, α -actinin, and tropomyosin were all located around the zonula adherens, there was a differential distribution of these proteins in this region, with relative proximity to the cell membrane of the zonula adherens decreasing in the order vinculin/ α -actinin/tropomyosin. To obtain a more quantitative picture of these relative distributions, a large number of micrographs from single-labeling experiments with each of the three proteins with both ferritin and Imposil conjugates were analyzed. The region abutting the zonula adherens was divided into six 50-nm-wide zones, starting from the cell membrane, and the ferritin or Imposil particles in each zone were counted. The results are plotted in Fig. 5 as the percent of the total label counted, that is, the numbers of ferritin or Imposil particles found in each 50-nm zone, divided by the total number found in the entire 300-nm region, multiplied by 100. The distributions obtained with ferritin conjugates and with Imposil conjugates for the same antigen were indistinguishable and are taken together. The bar diagrams for vinculin (Fig. 5A), α -actinin (Fig. 5B), and tropomyosin (Fig. 5C) are plotted together in Fig. 5D. The results show clearly that the mean

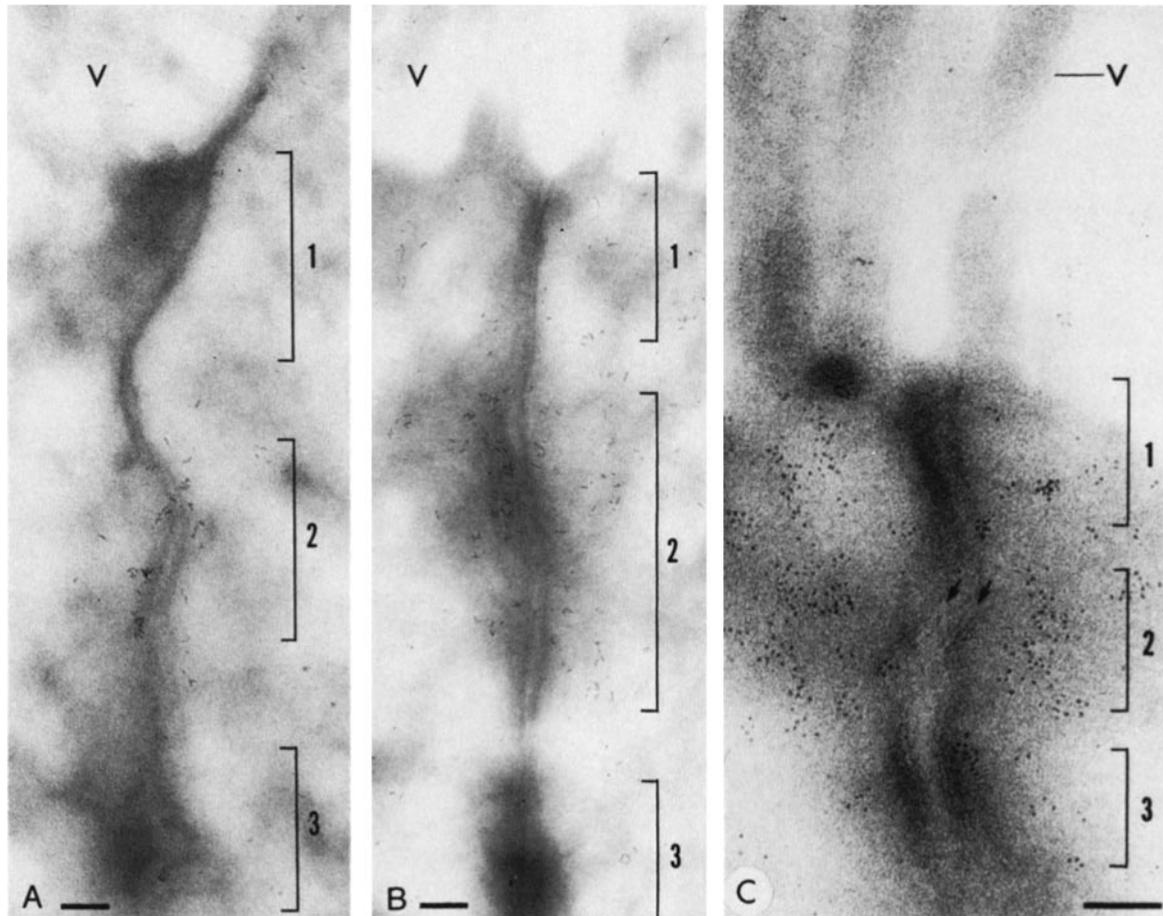


FIGURE 3 Indirect single immunoelectron microscope labeling of the brush border of chicken intestinal epithelium for vinculin (A), α -actinin (B), and tropomyosin (C). In A and B, primary rabbit antibodies and then Imposil-conjugated goat anti-rabbit IgG were used. In C, the primary rabbit anti-tropomyosin was followed by ferritin-conjugated goat anti-rabbit IgG. Brackets 1, 2, and 3 indicate the tight junctions, zonula adherens, and spot desmosomes, respectively, and V the microvilli. In A, Imposil labeling for vinculin is localized close to the zonula adherens and is not seen at the tight junction or the spot desmosome, or in the microvilli. In B, Imposil labeling for α -actinin is mainly situated near the zonula adherens, but some sparse labeling is observed near the tight junction. In C, the ferritin labeling for tropomyosin is most abundant at the level of the zonula adherens, less abundant near the tight junction and near the spot desmosome, and is absent in the microvilli. At the zonula adherens, only few ferritin particles are found in wide central areas of 50- to 100-nm width adjacent to the cell membranes (arrows, C). Bars, 0.1 μ m. A and B: \times 60,000; and C: \times 100,000.

distance of the labels from the membrane decreased in the order vinculin/ α -actinin/tropomyosin, confirming the qualitative conclusions discussed above.

Single Immunoelectron Microscopic Labeling in Gizzard Smooth Muscle

As discussed above, fixation conditions that we have found to allow the successful immunoelectron microscopic labeling of vinculin in ultrathin frozen sections involve the two-stage procedure. Specimens of chicken gizzard fixed in this manner showed a sharp localization of vinculin labeling to the dense plaques that are associated with the plasma membranes of the smooth muscle cells, whether these are next to other smooth muscle cells (Fig. 6A) or to connective tissue (see Figs. 7C and 8C). This localization was the same whether Imposil-antibody (Fig. 6A) or ferritin-antibody (15) conjugates were used as the secondary reagent. There was no labeling above background in the cytoplasm and, of particular interest, the cytoplasmic dense bodies were not labeled (Fig. 6B).

For the single labeling of α -actinin and tropomyosin, fixation

with 3% formaldehyde and 0.1% glutaraldehyde was employed because it is generally more effective than the two-stage procedure in ultrastructural fixation. The labeling of α -actinin was largely localized to two intracellular sites in the smooth muscle cells, the membrane-associated dense plaques, and the cytoplasmic dense bodies. This was observed with either ferritin-antibody (Fig. 6C, D, and E) or Imposil-antibody (Fig. 6F) conjugates. The results shown in Fig. 6E are also of technical interest in connection with certain problems that can arise in immunoelectron microscopic labeling experiments. The cytoplasmic dense body marked by an arrowhead in Fig. 6E is intensely labeled for α -actinin only at one end, while other dense bodies in the same field are more uniformly labeled. We suggest that this dense body has been obliquely cut and that most of it lies unexposed within the surfaces of the section. If the penetrability of the antibody labeling reagents into the section is limited to a short depth below the section surface, the labeling pattern that is observed could thereby be accounted for. An alternative explanation, that α -actinin is confined to one end of the dense body, is ruled out in view of the more uniform distributions of label observed within most of the

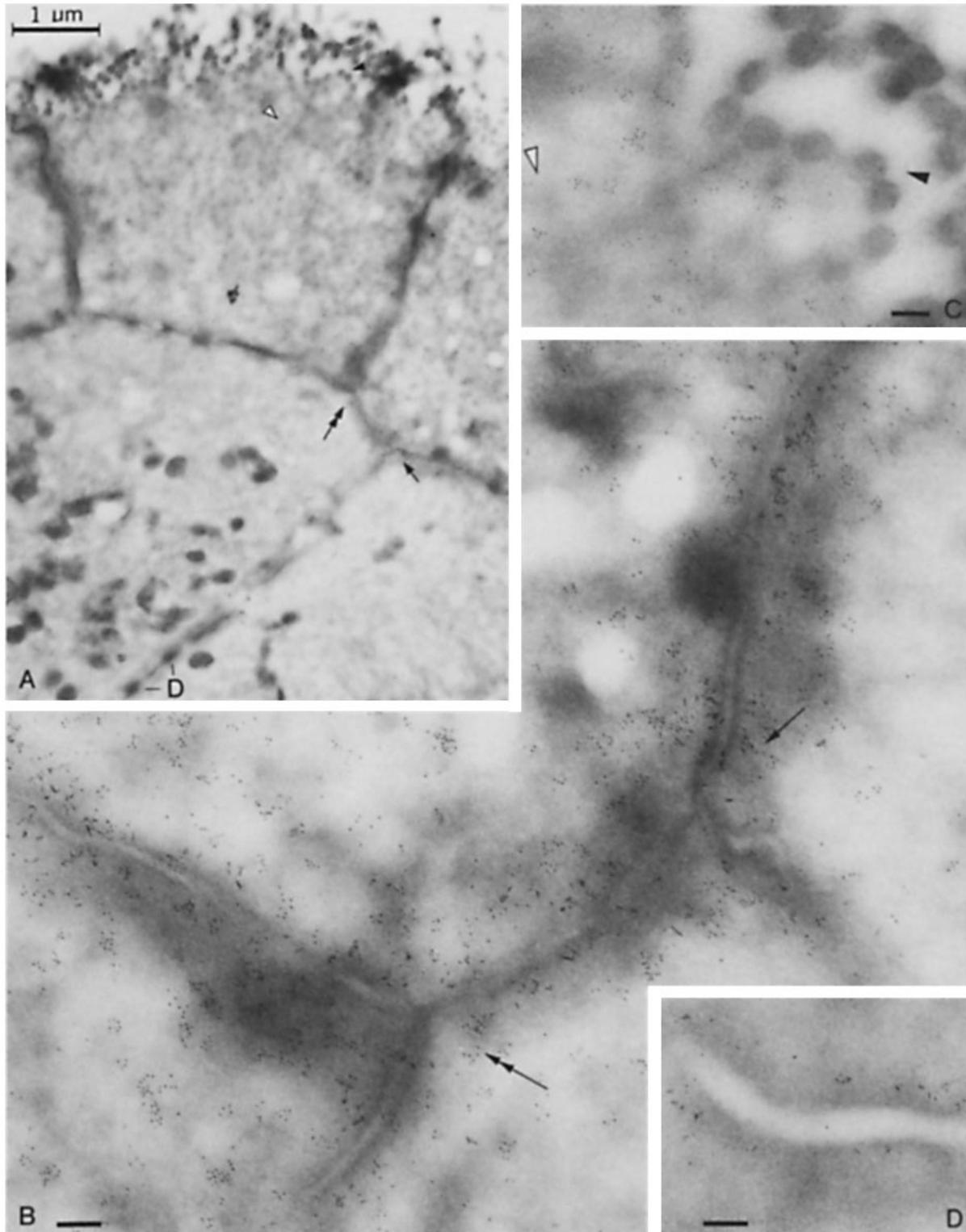


FIGURE 4 Indirect double immunoelectron microscopic labeling of the intestinal brush border for vinculin (rabbit anti-vinculin and then Imposil-conjugated goat anti-rabbit IgG) and tropomyosin (guinea pig anti-tropomyosin and then ferritin-conjugated goat anti-guinea pig IgG). *A* is a section nearly parallel to the epithelium surface. *B* and *C* are enlarged portions of *A*. The positions the the directions of single- and double-headed arrows in *A* correspond to those of the arrows in *B*, and the locations and the directions of arrowheads in *A* correspond to those of the arrowheads in *C*. In *B*, a portion of a section slicing through the level of zonula adherens, Imposil particles representing vinculin and ferritin particles representing tropomyosin occupy mutually exclusive areas; the former the narrow inner areas closer to the membrane and the latter the wide outer areas. In *C*, there is ferritin staining for tropomyosin exclusively, which appears to be localized in the interspaces between roots of microvilli (white arrowhead) but not in the cross sections of microvilli (dark arrowhead). The section in *D* is a control which was stained first with a mixture of rabbit anti-vinculin antibodies and normal guinea pig IgG, and then with a mixture of Imposil-conjugated goat anti-rabbit IgG and ferritin-conjugated goat anti-guinea pig IgG. Only Imposil particles are observed near zonula adherens. Bars, 0.1 μm . *A*: $\times 14,000$; *B* and *D*: $\times 70,000$; and *C*: $\times 60,000$.

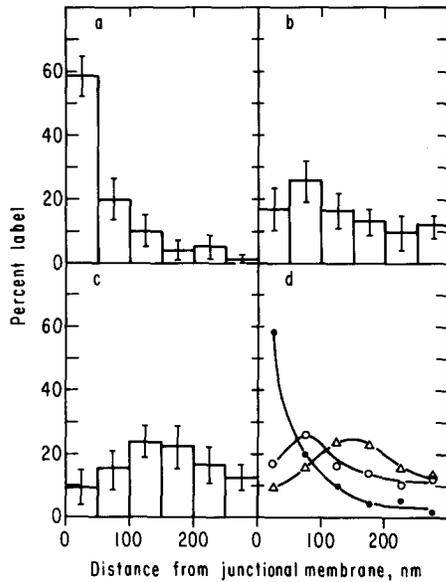


FIGURE 5 Distribution of ferritin or Imposil particles close to the zonula adherens after indirect immunolabeling of vinculin (a), α -actinin (b), and tropomyosin (c). Starting from the middle of the junctional membrane of an adherens as zero, the perpendicular distance from the membrane into the cell was divided into six 50-nm lengths, and particle counts in equal rectangular areas were made. The percent of the total counts in the six areas that were found in each area is plotted in the bar graphs. The results are summarized in d, where \bullet , \circ , and Δ represent the data for vinculin, α -actinin, and tropomyosin, respectively.

other dense bodies in the same field.

The labeling of tropomyosin in the cytoplasm of the smooth muscle cell was intense, but, quite remarkably, was uniformly absent from both the membrane-associated dense plaques and the cytoplasmic dense bodies. This was observed with both ferritin-antibody (Fig. 6G and H) and Imposil-antibody (Fig. 6I) conjugates. There is a suggestion in Fig. 6H that the cytoplasmic labeling of tropomyosin is more concentrated close to the dense plaques, within a region of ~ 100 nm from the edge of the plaque, than it is farther away.

Double Immunoelectron Microscopic Labeling in Gizzard Smooth Muscle

In double-labeling experiments in which vinculin was one of the antigens to be labeled, the two-stage fixation procedure was used in the specimen preparation. Unlike the case with the intestinal epithelium brush border, with chicken gizzard specimens the distribution of α -actinin labeling was not much different whether the two-stage procedure or the 3% formaldehyde plus 0.1% glutaraldehyde fixation was employed.

The double-labeling of vinculin (with an Imposil conjugate) and α -actinin (with a ferritin-conjugate) demonstrated (Fig. 7) that vinculin labeling was confined to the membrane-associated dense plaques, whereas the labeling for α -actinin was found on both the dense plaques and the cytoplasmic dense bodies in the same fields. Of particular interest, however, are the results shown in Fig. 7C. In a dense plaque that was sectioned obliquely, it is evident that within the plaque there was a segregation of Imposil and ferritin labeling: vinculin labeling was more concentrated than α -actinin labeling near the membrane that was associated with the plaque.

Double-labeling experiments for vinculin and tropomyosin

are shown in Fig. 8. In Fig. 8A, vinculin has been labeled indirectly with an Imposil-antibody and tropomyosin with a ferritin-antibody conjugate, whereas in Fig. 8B and C the labeling was reversed. The results were closely parallel. Tropomyosin labeling was observed throughout the cytoplasm, but not in either the dense plaques or dense bodies, and vinculin labeling was largely confined to the dense plaques. In Fig. 8C, there is a dense plaque where a smooth muscle cell is in contact with connective tissue, and this plaque is also heavily labeled for vinculin but not for tropomyosin.

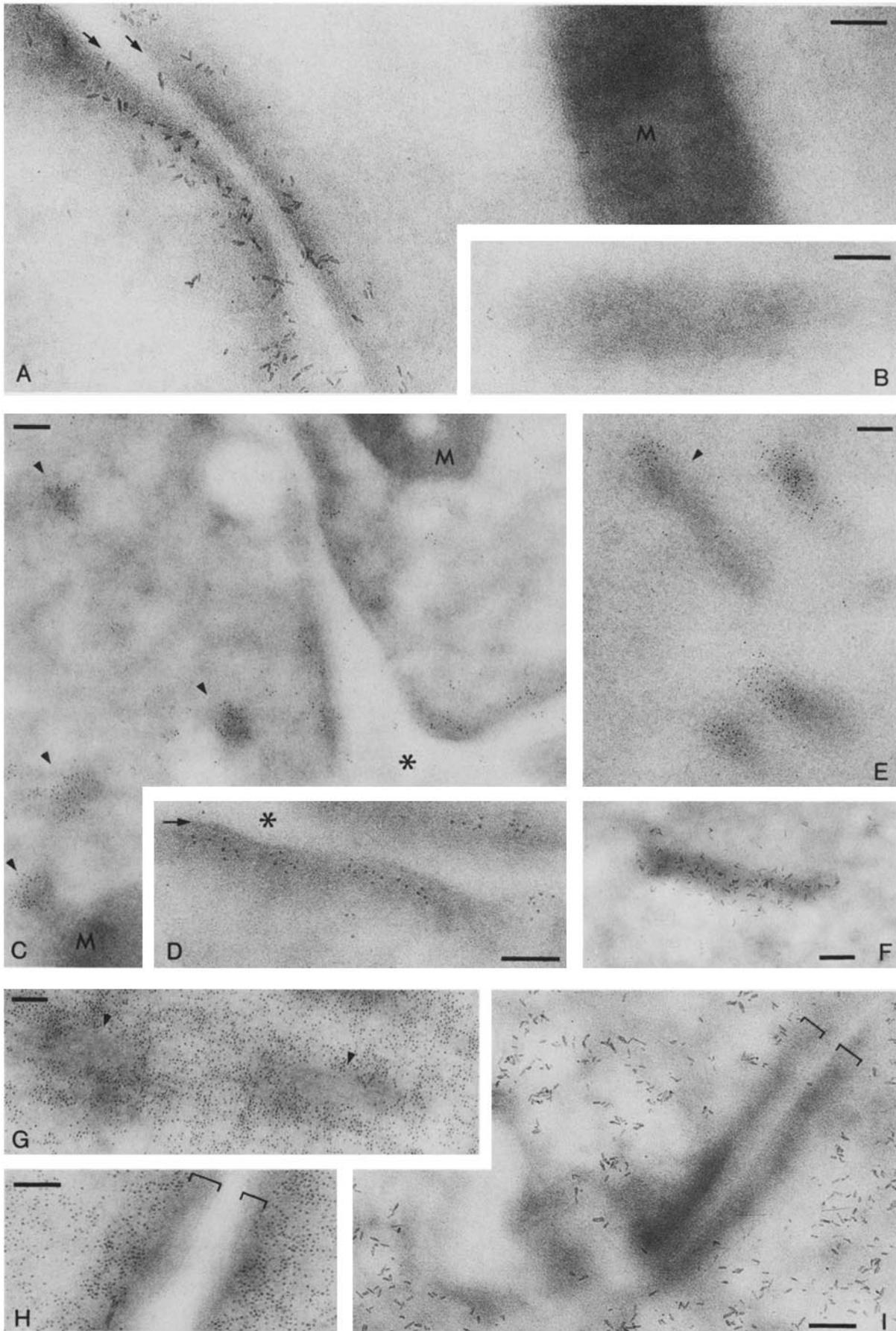
The double labeling for α -actinin and tropomyosin (Fig. 9) showed that the α -actinin labeling (with an Imposil-antibody conjugate) was largely localized to the dense plaques and cytoplasmic dense bodies, while tropomyosin labeling (with a ferritin conjugate) was essentially absent from these structures but was distributed throughout the cytoplasm. On rare occasions, a cytoplasmic dense body appeared to be simultaneously labeled for both α -actinin and tropomyosin, but with the two labels largely confined to different ends of the body (Fig. 9C, white arrowhead; contrast this with the dense body marked by the black arrowhead in the same figure). We attribute this to a rare situation where an obliquely sectioned dense body was only partially cut through at one end, the other end lying under the surface of the section with a layer of cytoplasm intervening. It is very likely this intervening cytoplasm which was labeled for tropomyosin and not the underlying end of the dense body itself. The labeling reagents for α -actinin were presumably unable to penetrate this thickness of cytoplasm to reach the α -actinin within the dense body below. The other end of the same dense body that was cut through by sectioning was thus exposed to the labeling reagents for α -actinin but was not labeled for tropomyosin because no cytoplasm was exposed. In the more general case, entire dense bodies were cut through by the ultrathin sectioning process and were directly exposed without intervening cytoplasm at the surface of the section. Hence, no tropomyosin labeling was seen.

An example of one additional set of controls for the double-labeling experiments is shown in Fig. 9D and E. In this experiment with sections of chicken gizzard, the first stage of the labeling process was carried out with a mixture of guinea pig antibody to α -actinin and rabbit normal IgG (Fig. 9D), or with a mixture of rabbit antibody to tropomyosin and guinea pig normal IgG (Fig. 9E). Both samples were then treated in the second stage with the same mixture of ferritin-conjugated goat antibodies to guinea pig IgG and Imposil-conjugated goat antibodies to rabbit IgG as was used in the double-labeling experiments. It is clear that the sample in Fig. 9D is exclusively labeled with ferritin particles on the dense plaques and cytoplasmic dense bodies as expected for the α -actinin distribution, whereas the sample in Fig. 9E is exclusively labeled with Imposil particles in the distribution expected for tropomyosin. This demonstrates that the two primary labeling reagents were completely specific for their two respective antigens in the section.

DISCUSSION

Immunoelectron Microscope Technique

It is not our purpose here to discuss the technique extensively. Some of its features, however, as revealed by the experiments in this paper should be considered briefly. The first feature is the use of Imposil-antibody conjugates for the immunoelectron microscopic labeling of antigens on ultrathin frozen sections.



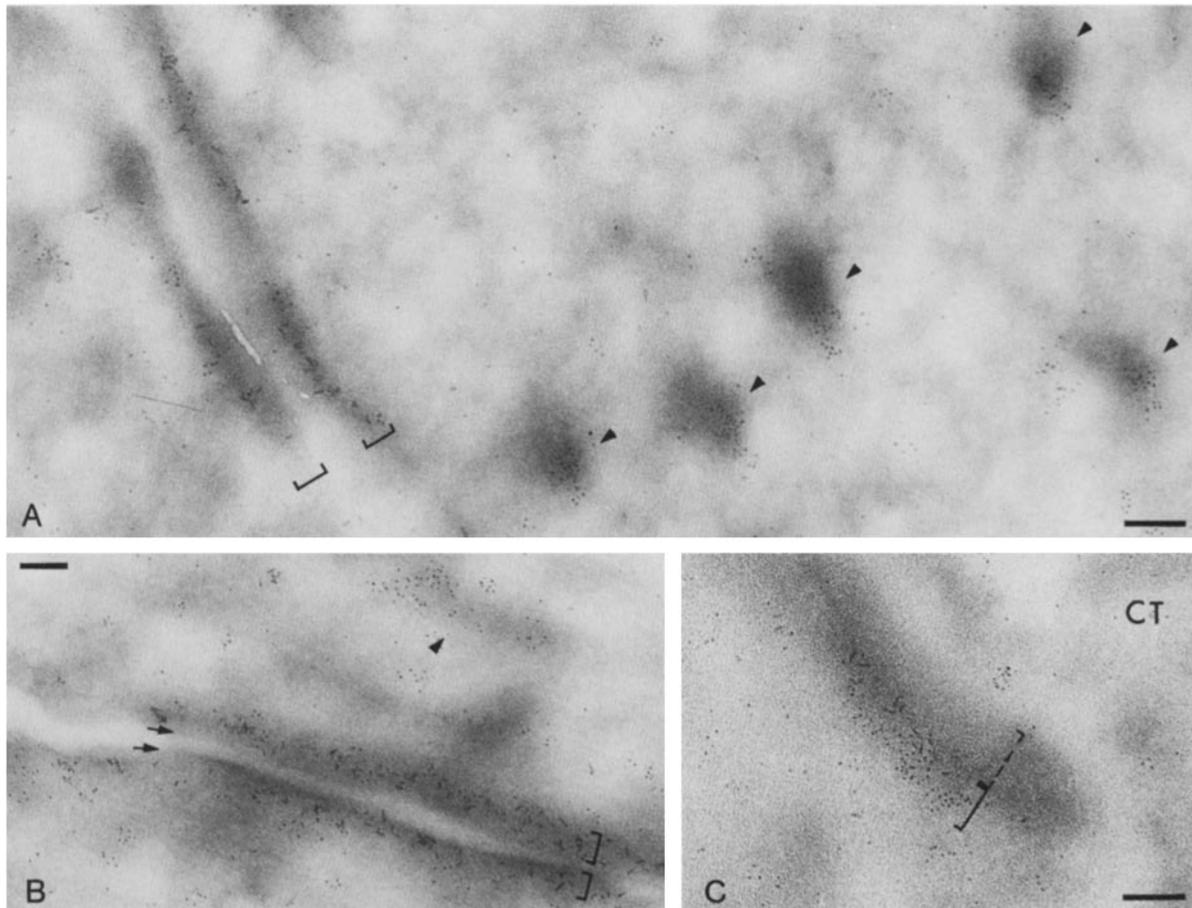


FIGURE 7 Indirect double immunoelectron microscopic labeling for vinculin (Imposil conjugates) and for α -actinin (ferritin conjugates) on the same specimens. The primary reagent used was a mixture of rabbit antibodies to vinculin and guinea pig antibodies to α -actinin, while the secondary reagent was a mixture of an Imposil conjugate of goat antibodies to rabbit IgG and a ferritin conjugate of goat antibodies to guinea pig IgG (see Materials and Methods). Vinculin labeling (Imposil) is exclusively on dense plaques (brackets in A and B) associated with the cell membrane (arrows in B) and is absent from cytoplasmic dense bodies (arrowheads in A and B), whereas α -actinin labeling (ferritin) is found on both dense plaques and dense bodies. In C, a region of a cell that is adjacent to connective tissue (CT) rather than another cell, a dense plaque exhibits both vinculin and α -actinin labeling. In this obliquely-cut section, the cell membrane shows a wide profile (broken-line bracket) outside the plaque (solid-line bracket). The labeling of this dense plaque for vinculin (Imposil) is clearly closer to the cell membrane than the labeling for α -actinin (ferritin). Bar $0.1 \mu\text{m}$. A and C: $\times 80,000$; and B: $\times 60,000$.

Although the preparation and some properties of Imposil-antibody conjugates have been described (11), and an application of the conjugates has been made to the structure of the adhesion sites of cultured fibroblasts (7), the work described here represents the first applications of the conjugates to ultrastructural studies of tissues. The labeling characteristics of Imposil-antibody conjugates are closely similar to those of their ferritin counterparts. Similar densities of labeling could usually

be achieved, while nonspecific background staining of the ultrathin frozen sections was low for both conjugates. Our impression from these experiments is, in fact, that the background staining is even lower with the Imposil- than with the ferritin-conjugates. Although Imposil particles are somewhat larger than ferritin molecules, this did not adversely affect the apparent resolution of the technique in the experiments reported. We conclude that Imposil-antibody conjugates are

FIGURE 6 Indirect single immunoelectron microscope labeling of chicken-gizzard smooth muscle for vinculin (A and B), for α -actinin (C-F), and for tropomyosin (G-I). In each case, specific rabbit antibodies were used as the primary reagents, and this was followed in A, B, F, and I with Imposil-conjugated goat antibodies to rabbit IgG, while in C, D, E, G, and H, ferritin-conjugated goat antibodies to rabbit IgG were used. In A, Imposil labeling for vinculin is confined to the dense plaques associated with the muscle cell membranes (the arrows mark the membranes), and, in B, vinculin labeling is absent from a typical cytoplasmic dense body. In C, D, and E, ferritin labeling for α -actinin is specifically associated with both dense plaques near the cell membrane (arrow in D) and dense bodies (arrowheads in C). The asterisks in C and D mark the intercellular space. In E, the arrowhead points to a cytoplasmic dense body which is labeled only at one end (see text for discussion). In F, a cytoplasmic dense body is labeled with Imposil conjugates with a density and specificity comparable to those of the ferritin conjugates in C and E. In G-I, labeling for tropomyosin is densely distributed throughout the cytoplasm, but is specifically absent from the dense bodies (arrowheads in G) and from the dense plaques (brackets in H and I). Bars, $0.1 \mu\text{m}$. A, B, and D: $\times 100,000$; H and I: $\times 80,000$; and C, E, F, and G: $\times 60,000$.

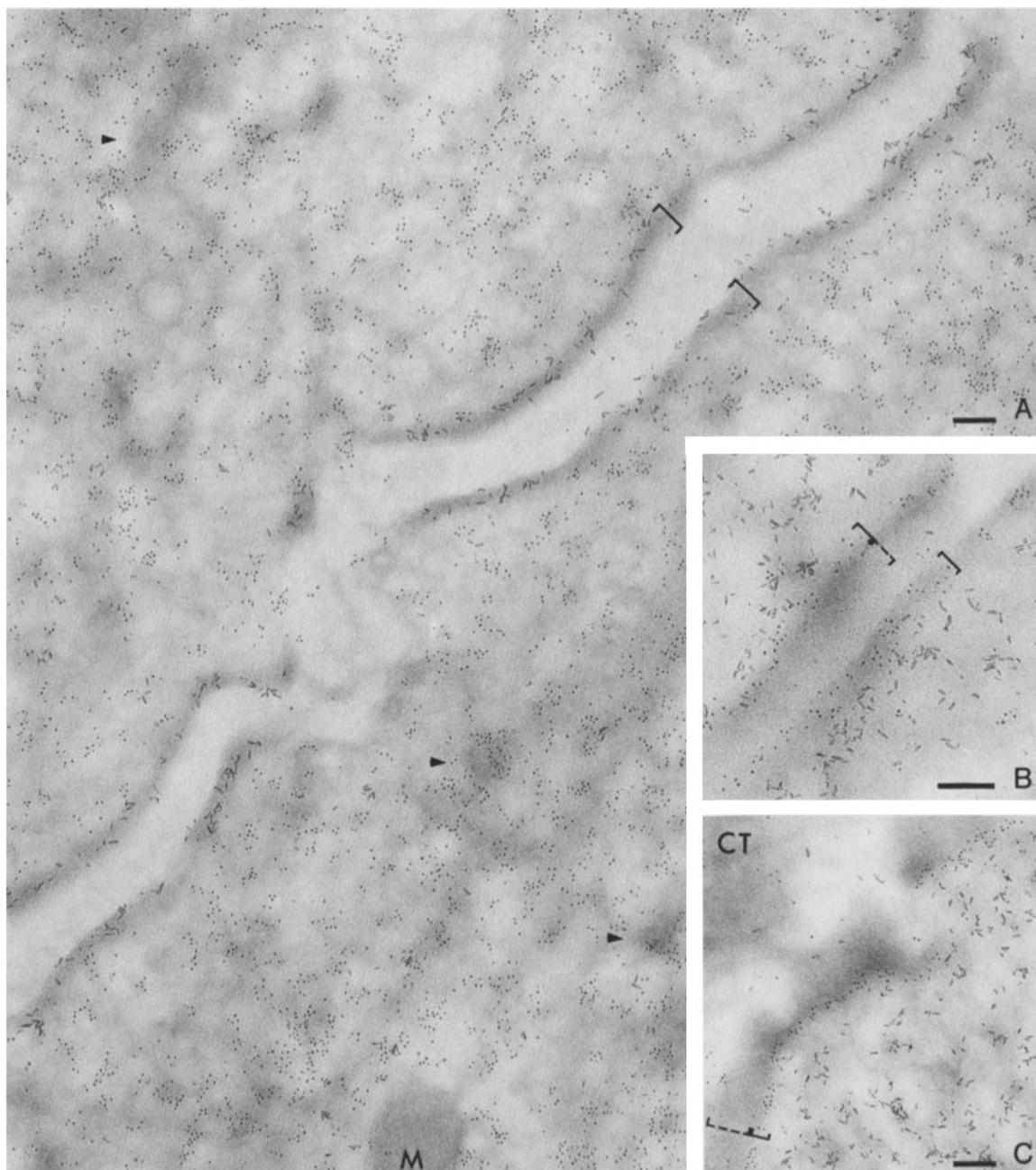


FIGURE 8 Indirect double immunoelectron microscopic labeling for vinculin and for tropomyosin on the same specimens. In *A*, the primary reagent used was a mixture of rabbit antibodies to vinculin and guinea pig antibodies to tropomyosin, while the secondary reagent was a mixture of an Imposil conjugate of goat antibodies to rabbit IgG and a ferritin conjugate of goat antibodies to guinea pig IgG. In *B* and *C*, the primary reagent was the same as in *A*, but the secondary reagent was reversed: a mixture of a ferritin conjugate of goat antibodies to rabbit IgG and an Imposil conjugate of goat antibodies to guinea pig IgG was used. Vinculin labeling (Imposil in *A*, ferritin in *B* and *C*) is exclusively localized to the membrane-associated dense plaques (solid line brackets), whereas tropomyosin labeling (ferritin in *A*, Imposil in *B* and *C*) is dense in cytoplasm but is excluded from cytoplasmic dense bodies (arrowheads in *A*), dense plaques, and a mitochondrion (*M*). Broken-line brackets in *B* and *C* indicate obliquely sectioned cell membranes, that in *C* lying next to connective tissue (*CT*). Bar, 0.1 μm . *A* and *C*: $\times 60,000$; and *B*: $\times 80,000$.

satisfactory immunoelectron microscopic labeling reagents, comparable to ferritin-antibody conjugates in sensitivity, specificity, resolving power, and stability.

A major objective in the development of Imposil-antibody conjugates was to permit the simultaneous double immunoelectron microscopic labeling of two antigens on the same sectioned specimen, in conjunction with ferritin-antibody conjugates (11). The distinctively different profiles shown by the

iron-rich cores of the ferritin and Imposil particles allow the two to be readily distinguished in the same field by transmission electron microscopy. In the course of these studies, we have encountered some problems of specimen preparation that are likely to be of general interest in double-labeling experiments. In particular, the nature of the fixation of a specimen before ultracryotomy is a prime consideration (39). Not only must the fixation procedure allow the ultrastructure of the cell to be

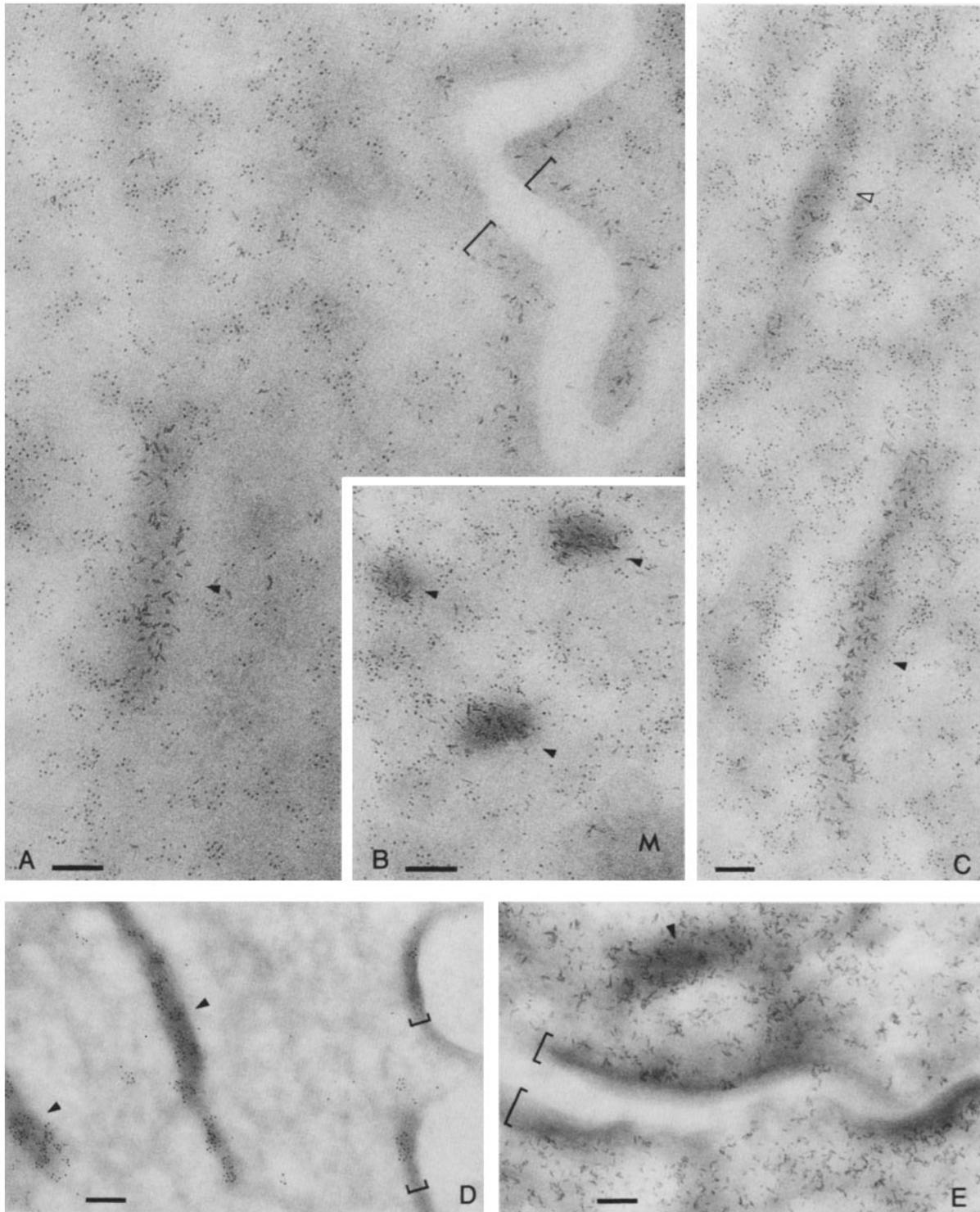


FIGURE 9 A–C: Indirect double immunoelectron microscopic labeling of α -actinin (Imposil conjugates) and tropomyosin (ferritin conjugates) on the same specimens. The primary reagent was a mixture of rabbit antibodies to α -actinin and guinea pig antibodies to tropomyosin, while the secondary reagent was a mixture of an Imposil conjugate of goat antibodies to rabbit IgG and a ferritin conjugate of goat antibodies to guinea pig IgG. Labeling of α -actinin (Imposil) is confined to the dense plaques (brackets in A) and cytoplasmic dense bodies (arrowheads in A–C). Tropomyosin labeling (ferritin) is distributed throughout the cytoplasm but is absent from the dense plaques and generally from the dense bodies. In C, however, ferritin appears to label an area of the dense body indicated by the white arrowhead but not the one indicated by the black arrowhead. This rare event may be due to a labeling of tropomyosin in a layer of cytoplasm that is lying over the dense body that is submerged within the section (see text). M, mitochondrion. D and E: control experiments for the indirect double immunoelectron microscope labeling experiments. In D, the primary reagent used was a mixture of guinea pig antibodies to α -actinin and normal rabbit IgG, while the secondary reagent was a mixture of a ferritin conjugate of goat antibodies to guinea pig IgG and an Imposil conjugate of goat antibodies to rabbit IgG. In E, the primary reagent was a mixture of rabbit antibodies to tropomyosin and normal guinea pig IgG, while the secondary reagent was the same mixture as in D. Note that in D, there is exclusively ferritin labeling (of α -actinin only in the dense plaques [brackets] and cytoplasmic dense bodies [arrowheads]), whereas, in E, there is exclusively Imposil labeling (of the tropomyosin everywhere except in the dense plaques [brackets] and dense bodies [arrowhead]). These controls show that the goat antibodies to rabbit IgG or to guinea pig IgG are absolutely specific. Bar, 0.1 μ m. A and B: $\times 80,000$; C: $\times 60,000$; and D and E: $\times 60,000$.

satisfactorily retained and not result in the loss or artifactual redistribution of either of the two antigens in question, but it must also allow both antigens to retain their capacity to bind to their specific antibodies. Furthermore, the fixation must be such as to permit adequate accessibility of the two antigens in the section to their respective antibody reagents. We have earlier encountered cases where such accessibility on ultrathin frozen sections was markedly different with different fixation procedures (39). Thus, too mild a fixation procedure may allow retention of antigenicity and of accessibility of both antigens to antibody labeling but may result in an inadequate fixation of one or both antigens. On the other hand, too strong a fixation procedure may cause the differential loss of antigenicity or of accessibility of the two antigens in the specimen. In either such case, a distorted view of the relative distributions of the two antigens would then be obtained. At the present time, each specimen-antigen system to be labeled by the methods described in these papers must be separately investigated with respect to the appropriate fixation procedures to be employed. This is illustrated by our results.

Vinculin is a protein whose capacity to bind to its specific antibodies is lost when it is subjected to glutaraldehyde fixation, even a mixture of 3% formaldehyde and 0.1% glutaraldehyde (Table I). This inactivation does not occur with α -actinin or tropomyosin, but is unfortunately not a rare phenomenon (20, 21). A milder fixation procedure, such as the two-stage process using 3% formaldehyde and 20 mM ethylacetimidate in the first step and 3% formaldehyde and 0.1% glutaraldehyde in the second, allows all three proteins to retain their antigenicity. This is presumably due to the reaction of some of the protein amino groups with the monofunctional imidate and their resultant unavailability to subsequent modification by the glutaraldehyde (39). On the other hand, for the same chemical reasons, the two-stage procedure may be less effective in cross-linking and fixing a specimen. It did not seem worth documenting the following point in this paper, but we found that the immunolabeling of α -actinin was somewhat reduced and more diffusely distributed on sections of intestinal epithelium fixed by the two-stage procedure than by treatment with 3% formaldehyde + 0.1% glutaraldehyde. We attributed this to a less satisfactory fixation of α -actinin by the former procedure. As a consequence of these effects, we have not yet found a fixation procedure that we regard as satisfactory for the double immunoelectron microscopic labeling of both vinculin and α -actinin on the same specimen of intestinal epithelium, although, by these criteria, satisfactory double labeling of vinculin and tropomyosin (Fig. 4) in that tissue was carried out. In the smooth muscle system, however, it was possible to carry out successful double immunolabeling of vinculin and α -actinin (Fig. 7).

The object of these remarks is not to develop rigorous criteria here for successful double-labeling experiments but rather to indicate some of the problems that must be taken into account in their prosecution and the methods required to recognize and resolve them. Despite these problems, however, such simultaneous double-labeling experiments can clearly yield important information that is difficult to obtain by two separate single-labeling experiments (see for example Fig. 7C).

Ultrastructure of the Intestinal Epithelium Brush Border

The focus of attention here is on the protein vinculin and its relationship to other proteins at sites where microfilaments

appear to terminate at membranes. Vinculin, a 130,000-dalton protein isolated from chicken gizzard (12), has been shown to be present close to such membrane sites in several different cell types (15) and it has therefore been suggested that it is involved in the linkage of microfilaments to membranes.

Electron microscope morphological studies have revealed a highly organized filamentous ultrastructure and characteristic membrane junctional specializations in the brush border (18, 28). The microvilli at the apical portions of the brush border have within their cores bundles of oriented actin-containing microfilaments attached to the microvilli membranes both at their tips and along their lengths. These microfilament bundles extend out of the microvilli and intermesh with other sets of microfilaments in the terminal web of the brush border. The latter microfilaments appear to associate with the cell membrane at specialized sites of cell-cell contacts, namely, the zonula adherens and possibly the tight junction (18). In addition, there are intermediate filaments which appear to connect with the spot desmosomes on the cell membrane.

The molecules that are present in the various elements of the brush border have come under investigation in recent years. The microvilli are distinct entities in their molecular composition and ultrastructure. The microfilaments in the cores of the microvilli are associated with a unique set of auxiliary proteins (3-6, 14, 25). None of the three proteins investigated in this paper is found within the microvilli, as may be concluded from the absence of specific immunolabeling in the microvilli in Figs. 2-4. Although α -actinin was at one time thought to be present in the microvilli (27), it is now clear that it is not (3, 4, 8, 9, 14, 26).

Immunofluorescence labeling for vinculin (Fig. 2A) showed that it was largely confined to the junctional bar, whereas α -actinin (Fig. 2D) and tropomyosin (Fig. 2E), although showing some concentration near the bar, were also significantly labeled throughout the terminal web. No labeling of any of the proteins was observed in the microvilli. However, at the limited resolution of the light microscope, little further information was attainable. Our previous preliminary studies, by single immunolabeling, of the distribution of α -actinin (14) and of vinculin (15) showed that the association of these proteins with the junctional complex was largely confined to the region near the zonula adherens. An object of our study was a more careful comparative immunoelectron microscope localization of these two proteins and of tropomyosin in this region.

Let us first consider the distribution of tropomyosin in the brush border. Immunoelectron microscopic labeling for tropomyosin was found diffusely throughout the terminal web and showed some increase near the zonula adherens (Fig. 3C) but was largely absent within ~100 nm from the cell membrane. Correspondingly, in double immunolabeling for tropomyosin and vinculin, the labeling patterns for the two proteins were sharply segregated, with tropomyosin largely excluded from a region close to the membrane at the zonula adherens (Fig. 4B), while vinculin was largely confined there (see below). This immunoelectron microscope distribution of tropomyosin is consistent with the lower resolution immunofluorescence observation (Fig. 2E) that tropomyosin labeling around the terminal bar region showed a doublet character (i.e., a decrease of labeling close to the two cell membranes at the junctional bar region).

In single immunoelectron microscopic labeling experiments for vinculin or α -actinin, it appeared that vinculin (Fig. 3A) was more closely confined to the membrane region of the zonula adherens than was α -actinin (Fig. 3B). Because of the

problems discussed in the previous section, we could not find a reliable fixation procedure which would allow double immunoelectron microscopic labeling for α -actinin and vinculin to be carried out in this tissue. However, a quantitative analysis was performed of the single-label distributions of both ferritin and Imposil conjugates for α -actinin and vinculin, as well as tropomyosin. This analysis demonstrated (Fig. 5) that the distributions of the three proteins were quantitatively distinguishable, with the density of vinculin labeling closest to the cell membrane at the zonula adherens, α -actinin labeling next, and finally tropomyosin.

Ultrastructure of Smooth Muscle

Our results add some useful information to the understanding of smooth muscle ultrastructure, which is much less regular than that of striated muscle. The arrangements and interconnections of the filaments within smooth muscle cells are still unsettled. As a result, there is now no clear picture of the relationship between ultrastructure and the molecular mechanisms of contraction for smooth muscle as there is for striated muscle. It is known that smooth muscle cells contain specific structures that are densely stained in conventional electron micrographs, both in the cytoplasm (dense bodies) and along the cell membrane (dense plaques). Actin-containing microfilaments are inserted in a defined orientation into the membrane-bound dense plaques (36) and appear also to enter at least some of the dense bodies (35). Furthermore, Schollmeyer et al. (30) have found with immunoperoxidase labeling of plastic-embedded samples that both dense bodies and dense plaques stain for α -actinin. For these and other reasons the dense bodies and dense plaques have together been thought to bear a structural and functional relationship to the Z-lines of striated muscle sarcomeres.

Our results show that vinculin is immunolabeled exclusively in the membrane-associated dense plaques and not elsewhere in the cell, and particularly not in the cytoplasmic dense bodies (15) (Figs. 6B and 7). We have furthermore shown that α -actinin is in both the dense plaques and the dense bodies (Fig. 6C-F), confirming by a quite different immunolabeling procedure the conclusions of Schollmeyer et al. (30). These single- and double-labeling results therefore demonstrate that dense plaques and dense bodies have different molecular compositions despite their common association with both actin and α -actinin. In chicken cardiac muscle, immunoelectron microscopic labeling experiments have shown that vinculin is present near the fascia adherens of the intercalated disk membrane (where actin filaments terminate) but not at the Z-line (15), whereas α -actinin is present at both the fascia adherens and the Z-line (K. T. Tokuyasu, A. H. Dutton, B. Geiger, and S. J. Singer, manuscript in preparation). The presence of α -actinin at the Z-line is, of course, well known (24). These results strongly suggest that the dense plaques of smooth muscle and the fascia adherens of the intercalated disk membranes of cardiac striated muscle are analogous structures; and, similarly, that the cytoplasmic dense bodies of smooth muscle and the Z-line of striated muscle are analogous structures, but that the two types of structures are distinctly different.

The vinculin and α -actinin that are present in the dense plaques are most likely organized into some distinct molecular arrangement with respect to each other and to the other elements of the plaque (see below). Resolution of the labeling of the two components in the dense plaques was observed in double immunoelectron microscopic labeling experiments with

ferritin- and Imposil-antibody conjugates in favorable specimens (Fig. 7C). In such cases, the vinculin labeling was always more closely apposed to the membrane associated with the dense plaque than was the α -actinin labeling.

Labeling for tropomyosin was strikingly absent from both dense bodies and dense plaques, although it was quite intense in the immediately surrounding cytoplasm (Fig. 6G-I). This result is different from that of Schollmeyer et al. (30) who reported immunoperoxidase labeling for tropomyosin in the dense bodies. That we further generally obtained no tropomyosin labeling of either dense bodies or dense plaques in double immunoelectron microscopic labeling experiments where these bodies were strongly labeled for α -actinin (Fig. 9A-C) eliminates any trivial explanations of our results. The result of Schollmeyer et al. may reflect a labeling of tropomyosin in the cytoplasm surrounding the dense body rather than of the dense body itself (as we have interpreted our rare result of double-labeling of the dense body in the upper half of Fig. 9C, see Results).

On the Structure of Microfilament-Membrane Junctions

The spatial relationships of the three proteins, tropomyosin, α -actinin, and vinculin, that have been revealed in cultured fibroblasts (4), in intestinal epithelium brush border, and chicken gizzard smooth muscle, as well as in chicken cardiac striated muscle (K. T. Tokuyasu et al., manuscript in preparation), suggest that, despite profound differences in the overall ultrastructural organization of these different cell types, certain structural features involving these three proteins, together with actin-containing microfilaments and appropriate membrane sites, may be common to all of these cells. The common feature appears to involve a specialized region of the cell membrane at which actin-containing microfilament bundles terminate. Close to this membrane site, vinculin and α -actinin are concentrated; but tropomyosin is excluded, and is, instead, concentrated in the immediately adjacent region. All of the following appear to share these compositional and structural features: the focal adhesion plaques that are formed where cultured fibroblasts are closely attached to the substratum (12); the region near the zonula adherens in the brush border of intestinal epithelium; the dense plaques in smooth muscle cells; and the fascia adherens of the intercalated disk membrane of cardiac striated muscle cells (K. T. Tokuyasu et al., manuscript in preparation). On the other hand, in another site of microfilament-membrane attachment—the tips of the microvilli of the intestinal brush border—no vinculin or α -actinin is present, and a different type of structure must be involved.

Furthermore, with the dense plaques of smooth muscle (36) and the focal adhesion plaques in cultured fibroblasts (1, 16, 33), it has been shown that the actin-containing filaments that emerge from each of these structures have the same single orientation. Whether this is also the case with the microfilaments emanating from the zonula adherens is not known and may be complicated by the formation of microfilament-membrane associations of the lateral as well as of the end-on type, the former involving the beltlike bundles of microfilaments associated with the zonula adherens (18). In addition, in the smooth muscle dense plaques, the intestinal epithelial zonula adherens, and the cardiac muscle intercalated disk membrane, the immunoelectron microscopic labeling results indicate that the vinculin is situated closer to the membrane of these structures than is α -actinin. It is a possibility, therefore, that in such

structures vinculin may serve to provide a linkage between the termini of actin-containing microfilaments and the cell membrane. Its being closer than α -actinin to the membrane, in any event, makes vinculin a more likely candidate for that role than α -actinin. If vinculin did provide such a linkage it would most likely do so as a peripheral protein of the membrane rather than as an integral protein. Its solubility properties and other characteristics (12) argue against vinculin's being an integral protein. However, these ultrastructural considerations are clearly speculative at this time; they are mainly presented to suggest some future lines of experimental investigation which we intend to pursue.

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REFERENCES

- Begg, D. A., R. Rodewald, and L. I. Rebhun. 1978. The visualization of actin filament polarity in thin sections. Evidence for the uniform polarity of membrane-associated filaments. *J. Cell Biol.* 79:846-852.
- Brandtzaeg, P. 1973. Conjugates of immunoglobulin G with different fluorochromes. I. Characterization by anionic-exchange chromatography. *Scand. J. Immunol.* 2:273-290.
- Bretscher, A., and K. Weber. 1978a. Purification of microvilli and an analysis of the protein components of the microfilament core bundle. *Exp. Cell Res.* 116:397-407.
- Bretscher, A., and K. Weber. 1978b. Localization of actin and microfilament-associated proteins in the microvilli and terminal web of the intestinal brush border by immunofluorescence microscopy. *J. Cell Biol.* 79:839-845.
- Bretscher, A., and K. Weber. 1979. Villin: the major microfilament-associated protein of the intestinal microvillus. *Proc. Natl. Acad. Sci. U. S. A.* 76:2321-2325.
- Bretscher, A., and K. Weber. 1980. Fimbrin, a new microfilament-associated protein present in microvilli and other cell surface structures. *J. Cell Biol.* 86:335-340.
- Chen, W.-T., and Singer, S. J. 1980. Fibronectin is not present in the focal adhesions formed between normal cultured fibroblasts and their substrata. *Proc. Natl. Acad. Sci. U. S. A.* 77:7318-7322.
- Craig, S. W., and C. L. Lancashire. 1980. Comparison of intestinal brush border 95-kdalton polypeptide and α -actinin. *J. Cell Biol.* 84:655-667.
- Craig, S. W., and J. V. Pardo. 1979. α -Actinin localization in the junctional complex of intestinal epithelial cells. *J. Cell Biol.* 80:203-210.
- Driska, S. P., and D. J. Hartshorne. 1975. The contractile proteins of smooth muscle. Properties and components of a Ca^{+2} -sensitive actomyosin from chicken gizzard. *Arch. Biochem. Biophys.* 167:204-212.
- Dutton, A. H., K. T. Tokuyasu, and S. J. Singer. 1979. Iron-dextran antibody conjugates. General method for simultaneous staining of two components in high resolution immunoelectron microscopy. *Proc. Natl. Acad. Sci. U. S. A.* 76:3392-3396.
- Geiger, B. 1979. A 130k protein from chicken gizzard. Its localization at the termini of microfilament bundles in cultured chicken cells. *Cell.* 18:193-205.
- Geiger, B., and S. J. Singer. 1979. The participation of α -actinin in the capping of cell membrane components. *Cell.* 16:213-222.
- Geiger, B., K. T. Tokuyasu, and S. J. Singer. 1979. The immunocytochemical localization of α -actinin in fibroblasts. *Proc. Natl. Acad. Sci. U. S. A.* 76:2833-2837.
- Geiger, B., K. T. Tokuyasu, A. H. Dutton, and S. J. Singer. 1980. Vinculin, an intracellular protein localized at specialized sites where microfilament bundles terminate at cell membranes. *Proc. Natl. Acad. Sci. U. S. A.* 77:4127-4131.
- Goldman, R. D., J. Talian, A. Goldman, and B. Chojnacki. 1979. Adhesion plaques of embryonic fibroblasts are analogous to Z-lines. *J. Cell Biol.* 83(2, pt. 2):324a. (Abstr.)
- Gottlieb, A. I., M. H. Heggeness, J. F. Ash, and S. J. Singer. 1979. Mechanochemical proteins, cell motility, and cell-cell contacts. The localization of mechanochemical proteins inside cultured cells at the edge of an *in vitro* "wound." *J. Cell. Physiol.* 100:563-578.
- Hull, B. E., and L. A. Staehelin. 1979. The terminal web: a reevaluation of its structure and function. *J. Cell Biol.* 81:67-82.
- Kishida, Y., B. R. Olsen, R. A. Berg, and D. J. Prockop. 1975. Two improved methods for preparing ferritin-protein conjugates for electron microscopy. *J. Cell Biol.* 64:331-339.
- Kraehenbuhl, J. P., and J. D. Jamieson. 1972. Solid phase conjugation of ferritin to Fab fragments for use in antigen localization on thin sections. *Proc. Natl. Acad. Sci. U. S. A.* 69:1771-1775.
- Kyte, J. 1976. Immunoferritin determination of the distribution of the distribution of $(\text{Na}^+ + \text{K}^+)$ ATPase over the plasma membranes of renal convoluted tubules. I. Distal segment. *J. Cell Biol.* 68:287-303.
- Marchalonis, J. J. 1969. Enzymic method for the trace iodination of immunoglobulins and other proteins. *Biochem. J.* 113:299-305.
- Marshall, P. R., and D. Rutherford. 1971. Physical investigation on colloidal iron-dextran complexes. *J. Colloid Interface Sci.* 37:390-402.
- Masaki, T., M. Endo, and S. Ebashi. 1967. Localization of 6S component of α -actinin at Z-band. *J. Biochem.* 62:630-632.
- Matsudaira, P. T., and D. R. Burgess. 1979. Identification and organization of the components in the isolated microvillus cytoskeleton. *J. Cell Biol.* 83:667-673.
- Mooseker, M. S., and R. E. Stephens. 1980. Brush border α -actinin? Comparison of two proteins of the microvillus core with α -actinin by two-dimensional peptide mapping. *J. Cell Biol.* 86:466-474.
- Mooseker, M. S., and L. G. Tilney. 1975. Organization of an actin filament-membrane complex. Filament polarity and membrane attachment in the microvilli of intestinal epithelial cells. *J. Cell Biol.* 67:725-743.
- Mukherjee, T. M., and L. A. Staehelin. 1971. The fine-structural organization of the brush border of intestinal epithelial cells. *J. Cell Sci.* 8:573-599.
- Sanderson, C. J., and D. V. Wilson. 1971. A simple method for coupling proteins to insoluble polysaccharides. *Immunology.* 20:1061-1065.
- Schollmeyer, J. E., L. T. Furcht, D. E. Goll, R. M. Robson, and M. H. Stromer. 1976. Localization of contractile proteins in smooth muscle cells and in normal and transformed fibroblasts. *In Cell Motility.* R. Goldman, T. Pollard, J. Rosenbaum, editors. Cold Spring Harbor Laboratory. 361-388.
- Singer, S. J. 1959. Preparation of an electron-dense antibody conjugate. *Nature (Lond.)* 183:1523-1524.
- Singer, S. J., and A. F. Schick. 1961. The properties of specific stains for electron microscopy prepared by the conjugation of antibody molecules with ferritin. *J. Biophys. Biochem. Cytol.* 9:519-537.
- Small, J. V., G. Isenberg, and J. E. Celis. 1978. Polarity of actin at the leading edge of cultured cells. *Nature (Lond.)* 272:638-639.
- Sobieszek, A., and R. D. Bremel. 1975. Preparation and properties of vertebrate smooth-muscle myofibrils and actomyosin. *Eur. J. Biochem.* 55:49-60.
- Somlyo, A. P., A. V. Somlyo, F. Ashton, and J. Vallieres. 1976. Vertebrate smooth muscle: ultrastructure and function. *In Cell Motility.* R. Goldman, T. Pollard, J. Rosenbaum, editors. Cold Spring Harbor Laboratory. 165-183.
- Somlyo, A. V., F. T. Ashton, L. F. Lemanski, J. Vallieres, and A. P. Somlyo. 1976. Filament organization and dense bodies in vertebrate smooth muscle. *In Biochemistry of Smooth Muscle.* N. L. Stephens, editor. University Park Press, Baltimore, Maryland. 445-471.
- Ternynck, T., and S. Avrameus. 1976. Polymerization and immobilization of proteins using ethylchloroformate and glutaraldehyde. *Scand. J. Immunol. Suppl.* 3:29-35.
- Tokuyasu, K. T. 1973. A technique for ultracytometry of cell suspensions and tissues. *J. Cell Biol.* 57:551-565.
- Tokuyasu, K. T., and S. J. Singer. 1976. Improved procedures for immunoferritin labeling of ultrathin frozen sections. *J. Cell Biol.* 71:894-906.
- Tokuyasu, K. T., J. W. Slot, and S. J. Singer. 1978. Simultaneous observation of immunolabeled frozen section in LM and EM. *In Ninth International Congress on Electron Microscopy Vol. 2.* J. Sturgess, editor, Microscopical Society of Canada. Toronto, Canada. 164-165.