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Substrate-attached Membranes of Cultured Cells Isolation and Characterization of Ventral Cell Membranes and the Associated Cytoskeleton

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We describe here an approach for the isolation and characterization of substrate-attached membranes of cultured cells. The procedure for ventral membrane preparation is based on a short incubation with $ZnCl_2$, followed by shearing with a stream of buffer. By varying the intensity of shearing it was possible to obtain reproducibly either entire ventral membranes or highly enriched focal contacts. The contacts with the substrate were retained in these preparations in an apparently intact state as determined by interference-reflection microscopy as well as by scanning and transmission electron microscopy. The formation of close contacts by the cells and by the isolated membranes was sensitive to changes of pH value. Thus in buffers at pH 7·0 to 7·2 the attachment was mediated predominantly by focal contacts, whereas at pH 6·0 the membranes reversibly formed extensive close contacts with substrate. The mechanical shearing removed most of the cytoskeleton, leaving attached only those components which were most tightly associated with the ventral membranes. Microtubules were easily removed, together with most of the intermediate filaments, whereas a considerable portion of the microfilament system was retained even after extensive shearing. Immunofluorescent labeling with antibodies to several microfilament-associated proteins, including actin, vinculin, α-actinin, filamin and tropomyosin, pointed to the specific interaction of each of these proteins with the isolated ventral membranes and focal contacts.

1. Introduction

The capability of forming stable contacts with a variety of extracellular substances is one of the characteristics of most eukaryotic cells. In multicellular organisms cells are primarily associated with each other or with the intercellular matrix, while under culture conditions, they may adhere to the artificial substrate of the tissue culture dish (Vasiliev & Gelfand, 1977; Grinnell, 1978; Trinkaus, 1976; Staehelin, 1974). Such contacts and junctions are probably involved in most of the dynamic cellular activities such as cell spreading, locomotion and cytokinesis, and contribute to the morphogenesis of cells and to their mechanical stability (Pollard & Weihing, 1974; Trinkaus, 1976; Vasiliev & Gelfand, 1977). Moreover, it has been shown in recent years that processes such as growth control and differentiation, as well as

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DNA and protein synthesis, in a variety of cells are strictly dependent upon the formation of cell-to-cell and cell-to-substrate contacts (Stoker *et al.*, 1968; Folkman & Moscona, 1978; Ben-Zeev *et al.*, 1980). In spite of the broad interest in cellular adhesiveness very little is presently known on the detailed molecular aspects of contact formation (both cell-to-cell and cell-to-substrate). Most of the presently available information is either structural in nature or related to adhesive matrix proteins.

Ultrastructural studies have indicated that the closest, and probably the strongest contacts between cultured fibroblasts and the tissue culture substrate occur in small areas over the ventral cell membrane, denoted focal contacts or focal adhesion plaques (Curtis, 1964; Abercrombie & Dunn, 1975; Izzard & Lochner, 1976,1980). In these areas the cell membrane is separated from the substrate by a gap of about 15 nm, as determined by electron microscopy or by interference-reflection microscopy (Cornell, 1969; Abercrombie *et al.*, 1971; Izzard & Lochner, 1980). In addition to its association with the substrate or substrate-attached matrix, the cell membrane at the focal contacts is associated intracellularly with actin-containing filament bundles (Abercrombie *et al.*, 1971; Heath & Dunn, 1978; Geiger, 1979; Wehland *et al.*, 1979). Attempts to characterize this transmembrane linkage focused mainly on those actin-associated proteins that may be involved in the linkage of microfilament bundles to the cell membrane at the focal contacts. α-Actinin, for example, has been shown to be enriched along stress fibers and in particular at their membrane-associated termini (Lazarides & Burridge, 1975; Lazarides, 1976). In closer proximity to the cell membrane we have recently localized a new intracellular protein, vinculin (M_r 130,000), which was specifically associated with the cell membrane at focal contact areas (Geiger, 1979; Burridge & Feramisco, 1980) as well as with other sites of microfilament–membrane association including the *zonula adherens* of intestinal epithelium, dense plaques of smooth muscle and the intercalated discs of cardiac muscle (Geiger *et al.*, 1980).

We describe here procedures for the isolation of whole ventral membranes or individual focal contacts from cultured cells. The isolated membranes retain their cell–substrate focal contacts in an apparently intact form, as well as parts of their cytoskeleton. The various components of the microfilament system were tightly bound to the isolated membranes, while microtubules were removed by this treatment, together with most of the desmin-containing intermediate filaments. The potential use of the isolated ventral membrane preparations for the molecular characterization of cell attachment and membrane–cytoskeleton interactions is discussed.

2. Materials and Methods

(a) *Cells*

Cultures of embryonic chicken gizzard cells were prepared from 10-day embryos and maintained in culture in Dulbecco's Modified Eagle's (DME) medium (GIBCO, U.S.A.) containing 10% fetal calf serum (FCS). For the experiments described below we used cells of passages 2–8. Human foreskin fibroblasts were obtained from Dr D. Rotman from this Institute and PtK_2 cells from Dr W. Franke from the German Cancer Research Center, Heidelberg. Human intestinal epithelial line Henle 407 was obtained from the American

Tissue Culture Collection. Cells used for microscopic observation were plated on 18 or 22 mm square glass coverslips for at least 24 h before use.

(b) *Immunochemical reagents*

Rabbit antibodies to vinculin, α-actinin, tropomyosin, filamin, tubulin and desmin were prepared as described earlier (Geiger & Singer, 1979; Geiger, 1979; Geiger & Singer, 1980). These antibodies were purified on an immunoadsorbent of the respective antigen immobilized on glutaraldehyde-activated Ultrogel AcA22 (IBF, France), according to Ternynck & Avrameas (1976). Purified antibodies to actin were kindly provided by Dr V. Small, Austrian Academy of Science, Salzburg.

Affinity purified goat antibodies to rabbit IgG were coupled to lissamine rhodamine B sulfonyl chloride according to Brandtzaeg (1973). The fluorophore-conjugated antibodies were further chromatographed on DEAE cellulose (Brandtzaeg, 1973) and the fraction containing 3 to 4 fluorophores per IgG molecule was used.

(c) *Microscopy*

Fluorescence microscopy was performed with a Zeiss Photomicroscope III equipped with epi-illuminator and filter sets for selective observation of fluorescein and rhodamine. An objective planapochromat X63/1·4 was routinely used for fluorescence–Nomarski (Differential Interference Contrast; DIC) observations and Neofluar X63/1·3 for fluorescence–phase contrast observations. Interference-reflection microscopy was performed with the same microscope equipped with an extra polarizer and DIC slider together with an Antiflex, Neofluar X63/1·25 objective. Scanning electron microscopy was carried out in a Jeol JSM 35C scanning electron microscope operated at 20 kV. The specimens were observed at an angle of 45°.

Cells for fluorescence microscopy on glass coverslips were treated with detergent (0·5% Triton X-100 in 50 mM-MES (*N*-morpholinoethansulfonic acid, Serva, W. Germany) buffer, 3 mM-EGTA (ethyleneglycol-bis-(β-aminoethyl ether)-N,N′-tetraacetic acid), 5 mM-$MgCl_2$, pH 6·0) for 2 min and then fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min. Immunofluorescent labeling of the preparations was carried out as previously described (Geiger & Singer, 1979). Cells for scanning electron microscopy were seeded on glass coverslips, fixed with 1% glutaraldehyde in 0·1 M-Na-phosphate buffer, pH 7·0, and post-fixed with 1% OsO_4, dehydrated with ethanol and then critical point dried.

3. Results

(a) *Isolation of substrate-attached membranes of cultured fibroblasts: the $ZnCl_2$ method*

Cultured chicken gizzard cells on glass coverslips were rinsed briefly with buffer A (50 mM-MES, 5 mM-$MgCl_2$, 3 mM-EGTA, pH 6·0), then incubated at room temperature for two minutes with buffer A containing 1 mM-$ZnCl_2$. The cultures were rinsed with phosphate-buffered saline, pH 7·2 and subjected to 20 to 40 jets of the same buffer using a Pasteur pipette.

We have used three criteria to evaluate the nature of the material left attached to the substrate: (a) Nomarski (or phase) optics, (b) interference-reflection microscopy, and (c) immunofluorescent labeling for vinculin without any permeabilization step. The results are shown in Figure 1. Whole cells, permeabilized with Triton X-100, fixed and immunolabeled for vinculin, exhibited the typical labeling pattern consisting of elongated arrowhead-shaped patches and

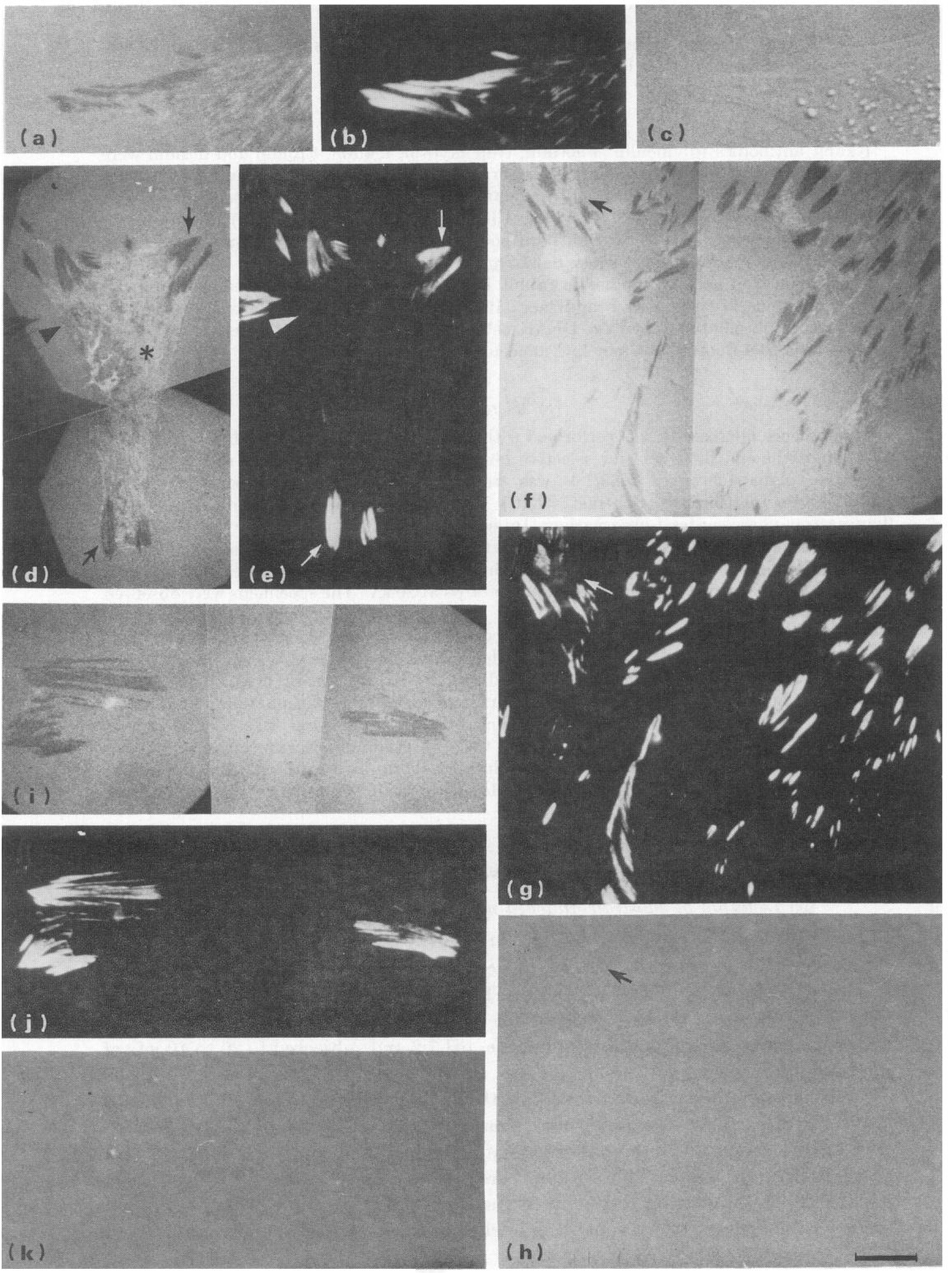

FIG. 1.

streaks (Fig. 1(b)). Analysis of the same cells by interference-reflection microscopy indicated that the vinculin-rich areas coincided with interference-reflection dark focal contacts (Fig. 1(a)) in line with previous studies on vinculin distribution (Geiger, 1979; Burridge & Feramisco, 1980). The cells could be easily visualized by DIC (Fig. 1(c)) or by phase contrast microscopies.

The incubation in the presence of $ZnCl_2$ followed by shearing resulted in an almost complete disappearance of intact cells from the coverslips, as demonstrated by the DIC photomicrographs in Figure 1(h) and (k). We were often able to detect some residual membrane material (arrow in (Fig. 1(h)), especially in moderately sheared preparations. Examination of the same cultures with interference-reflection optics indicated that the typical pattern of cell–substrate contacts was retained by the membranes. We were thus able to detect focal contacts (interference-reflection dark areas, arrows in Fig. 1(d)), close contacts (interference-reflection grey areas, arrowhead) as well as elevated areas of the cell membrane which appeared bright (marked with an asterisk; for further details see legend to Fig. 1). The amount of membrane material left on the coverslips was inversely related to the intensity of shearing (number of buffer jets) in any given cell type. Thus, in gently sheared cultures (about 20 buffer jets) most of the ventral membrane was retained (Fig. 1(d)), whereas in intensely sheared cultures most of the membrane in non-focal contact areas was removed (Fig. 1, (f) and (i)).

The other assay for the integrity of the focal contacts and the exposure of their cytoplasmic faces was immunofluorescent labeling of the purified substrate-attached material with vinculin antibodies. The results shown in Fig. 1(e), (g) and (j) indicated that vinculin labeling was retained on the purified membranes in focal contacts only. The fluorescent staining in these areas was very intense, essentially identical to that obtained in whole cells after permeabilization and fixation.

The procedure outlined here was successfully applied for several additional cell types, including human foreskin fibroblasts, epithelial PtK_2 cells, Henle 407 and epidermal carcinoma A-431. Examples of the preparation of ventral membranes from Henle 407 and PtK_2 cells (including phase-contrast photomicrographs of whole cells and isolated membranes, as well as interference-reflection

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FIG. 1. Isolation of ventral membranes of chicken gizzard cells. (a) to (c): Whole cells, permeabilized, fixed and immunolabeled for vinculin. (a) Interference reflection image; (b) the same area indirectly immunolabeled for vinculin using 10 μg pure rabbit anti-chicken vinculin/ml and 7 μg rhodamine-conjugated goat anti-rabbit Ig/ml; (c) the same area observed with Nomarski optics. (d) to (e): Ventral membranes prepared by the $ZnCl_2$ method followed by gentle shearing. (d) Interference-reflection image. Notice focal contacts (arrows) and areas of close contact (arrowhead). Loosely associated areas along the membrane appear bright as indicated by the asterisk. (e) Immunofluorescent labeling of the same area for vinculin; notice the exclusive association of the label with focal contacts. (f) to (h): Ventral membrane preparation, moderate shearing. (f) Interference-reflection image indicates that loosely attached membranes were partly removed while focal contacts were retained in an apparently intact form. (g) Immunofluorescent labeling of the same area for vinculin. (h) The same field observed with Normarski optics. Some residual material is marked with an arrow in (h) and in corresponding sites in (g) and (f). (i) to (k): Ventral membrane preparation, extensive shearing. (i) Interference-reflection image indicating the complete removal of non-focal contact membranes and the preservation of focal contacts in an apparently intact state; (j) the same area immunolabeled for vinculin; (k) the same area, observed with Nomarski optics.

The bar represents 10 μm.

photomicrographs of ventral membrane preparations) are shown in Fig. 2 (a) to (c) and (d) to (f), respectively.

It is important to point out here the significance of the various steps in the purification procedure. The addition of zinc ions at concentrations of 1 to 10 mM was essential for membrane preparation. In the absence of $ZnCl_2$ many cells detached completely from the substrate while others remained intact on its surface. The short treatment with 1 mM-$ZnCl_2$ had no apparent effect on focal contact structure, nor did it alter the cytoskeletal organization as determined by

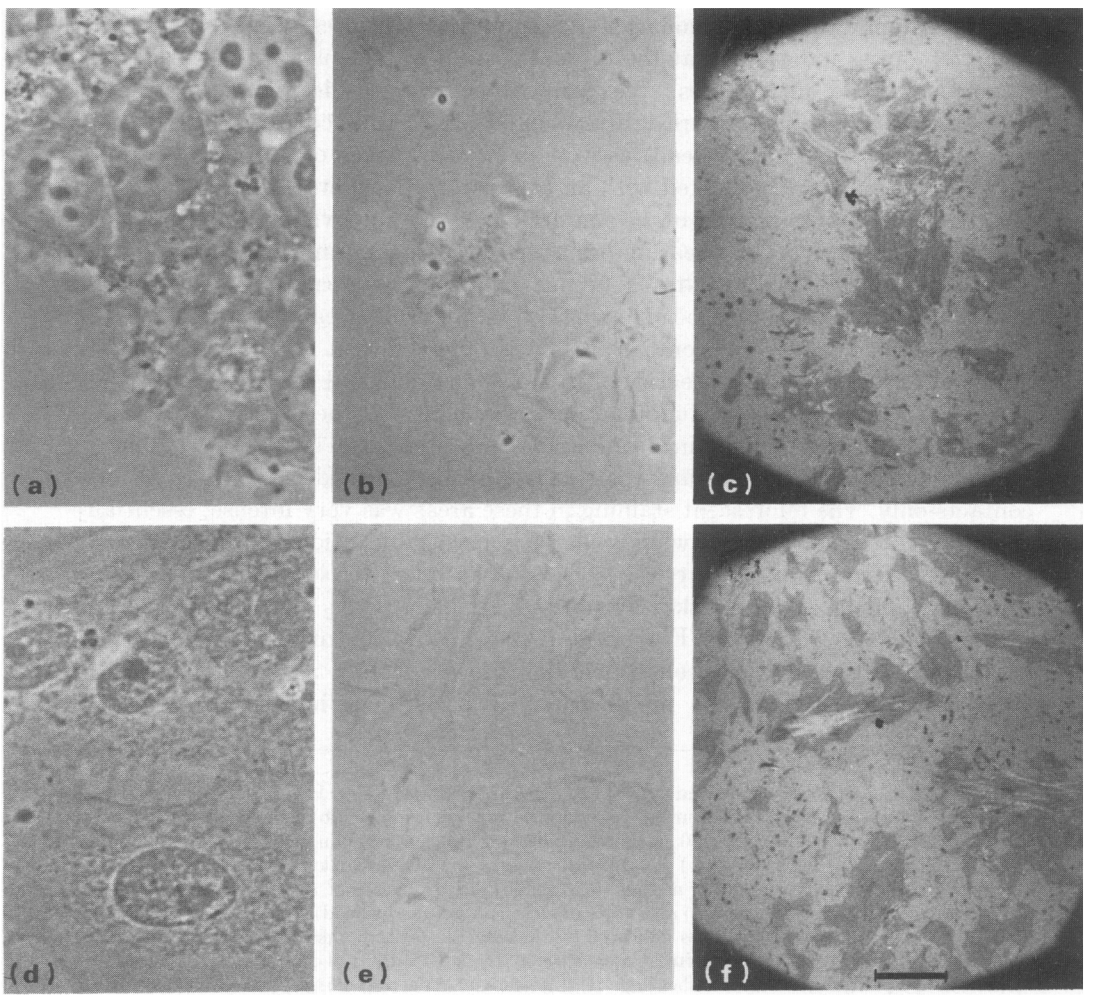

FIG. 2. Preparation of ventral membranes from Henle 407 cells ((a) to (c)) and from PtK_2 cells ((d) to (f)). (a) Phase photomicrograph for intact Henle 407 cells, (b) Phase photomicrograph of isolated ventral membranes of Henle 407 cells. (c) Interference-reflection image of isolated membranes from Henle 407 cells. Notice the extensive close contact between the membrane and the substrate. (d) Phase photomicrograph of PtK_2 cells. (e) Phase photomicrograph of ventral membranes of PtK_2 cells. (f) Interference-reflection image of isolated membranes of PtK_2 cells.

The bar represents 10 μm.

immunofluorescent labeling of permeabilized cells with antibodies against microfilament components, tubulin and desmin (not shown). Effects on the microtubular system and the microfilament system occurred only after much longer incubation (over 10 min) with higher $ZnCl_2$ concentrations. Attempts to remove the $ZnCl_2$-treated cells from the substrate with a stream of MES buffer at pH 6·0 (instead of PBS at pH 7·2) were not successful and most of the cells were retained, apparently intact, on the substrate. When the entire treatment (both the preincubation with $ZnCl_2$ and the shearing) was carried out at pH 7·0 the focal contacts and the associated vinculin appeared slightly deteriorated, and the yield of purified membranes was quite low.

Another important factor in ventral membrane isolation is the time allowed for cell attachment and spreading. Chicken gizzard cells form early stable attachments with glass surfaces as soon as 30 minutes after plating in serum-containing medium. By two to three hours most of the cells are firmly attached to the substrate and form focal contacts with it. Nevertheless, when the $ZnCl_2$ procedure for isolation of focal contacts was applied to cultures earlier than five to six hours after plating, the yield of isolated membranes was very low. It thus appears that a stage of focal contact maturation is necessary to render the cell membrane stable and strengthen the cell–substrate contacts in these areas.

(b) *Ultrastructural analysis of isolated ventral membranes*

Scanning electron microscopy provided further details on the integrity of the isolated substrate-attached membranes and the structural details of their cytoplasmic faces. In Figure 3(a) intact chicken gizzard cells are shown exhibiting smooth surfaces both at the cell center and along the peripheral lamellipodium. In the ventral membrane preparation the entire dorsal part of the cell was absent and the residual membrane was flatly associated with the substrate (Fig. 3(b) and (c). Often the attached membranes were of small size, only a few (3 to 5) micrometres (Fig. 3(d)). These membrane fragments are most likely isolated focal contacts, though a direct immunocytochemical analysis will be necessary to assess this statement.

The cytoplasmic aspect of the isolated membranes (especially when observed with high magnification) was associated with parallel arrays of filaments and filament bundles (Fig. 3(c) and (d)). Examination of ultra-thin cross sections of epon-embedded chicken gizzard cells as well as purified focal contacts with a transmission electron microscope indicated that the average distance measured between the isolated membranes and the substrate surface was similar to that found in intact cells, in the range of 15 to 20 nm (not shown).

(c) *The pH-induced alterations of cell–substrate contacts in living cells and in isolated ventral membranes*

As mentioned above, the preparation of ventral membranes was sensitive to changes in the specific buffer used. We have therefore tested directly the effect of various buffer conditions on the pattern of cell–substrate and ventral membrane–

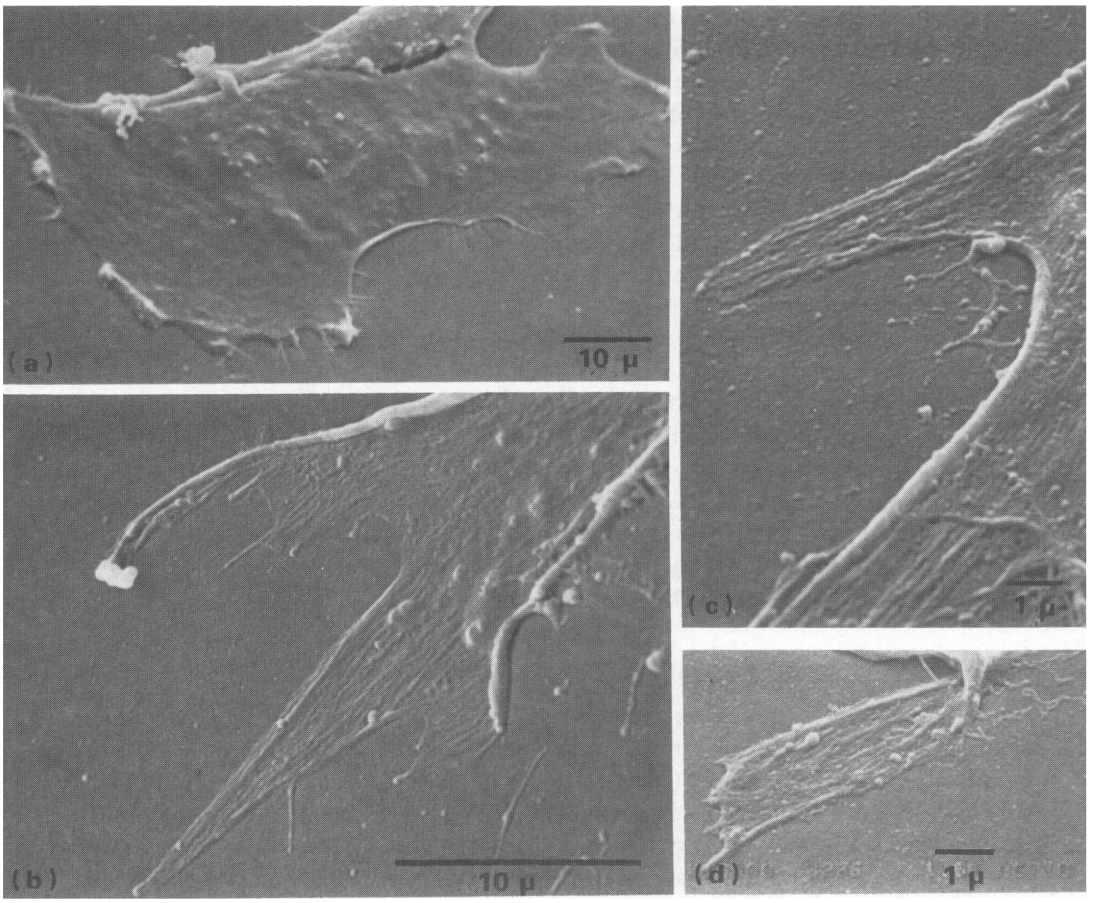

FIG. 3. Scanning electron photomicrographs of cells and isolated ventral membranes of chicken gizzard cells. (a) Whole cells. (b) Preparation of ventral membrane; notice the slightly folded edges of the membranes and the fine fibrillary texture of its cytoplasmic aspect. (c) Higher magnification of ventral membranes; notice the membrane-bound fibers and the presence of extracellular, substrate-attached material (possibly fibronectin). (d) Small substrate-attached membrane fragment which may represent an individual focal contact.

substrate contacts. We have noticed that viable cells maintained in the DME medium were associated with the glass coverslips predominantly through their focal contacts (Fig. 4(a), arrows) with only a few apparent close contacts (Fig. 4(a) arrowhead; grey areas in interference-reflection). A very similar pattern of focal and close contacts was obtained when the DME medium was substituted with other buffers at pH 7·0 to 7·2 including PBS, pH 7·2 (Fig. 4(b)) and 100 mM-HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethane sulfonic acid; Sigma, U.S.A.), pH 7·0 (Fig. 4(c)). However, when the viable cells were incubated with MES buffer, pH 6·0, at concentrations of 50 to 100 mM, the entire ventral membrane of the intact cells formed a broad, continuous close contact with the substrate (Fig. 4(d)). The extensive attachment formed at pH 6·0 was readily reversible. Thus when cells

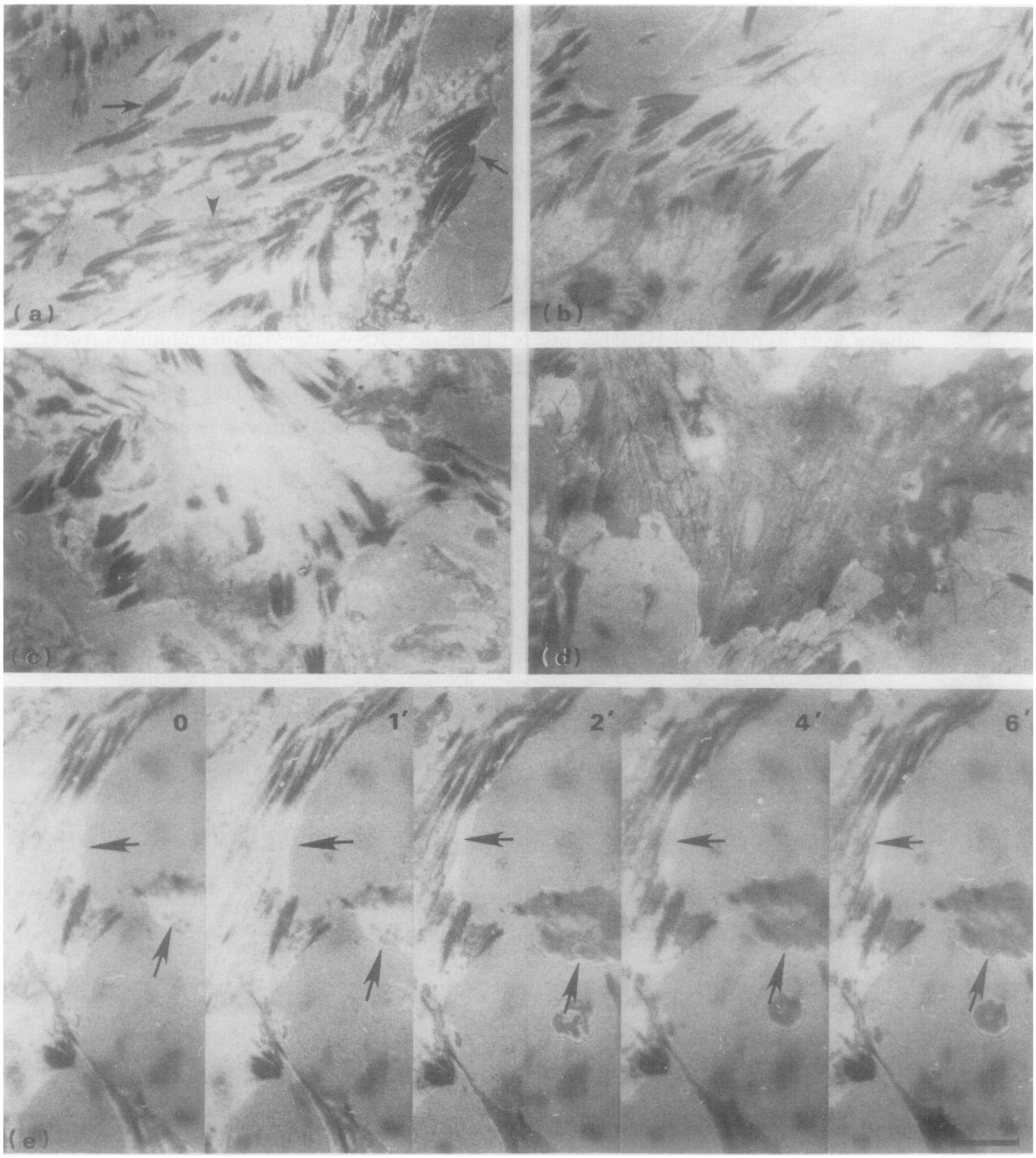

FIG. 4. Effect of pH on the pattern of cell–substrate contact of viable chicken gizzard cells. Cells in a sealed chamber were incubated at 25°C with DME (a), PBS (b), 100 mM-HEPES buffer, pH 7·0 (c), and 100 mM-MES buffer, pH 6·0 (d). Notice that the attachment at pH 7·0 to 7·2 ((a) to (c)) is mediated predominantly by focal contacts (arrows) with only limited areas of close contact (arrowhead). At pH 6·0 (d) extensive close contact is formed over the entire membrane. (e) The kinetics of formation of the pH-induced close contacts. Cells in PBS ((e), 0) were rinsed with 100 mM-MES buffer pH 6·0 and the progressive changes in the pattern of cell-substrate contact recorded after 1, 2, 4 and 6 minutes. Notice the increase in areas of close contact which is apparent as soon as 2 min after the buffer was changed.

The bar represents 10 μm.

incubated at pH 6·0 were washed with PBS or HEPES buffer the extensive close contacts disappeared and the attachment was mediated mainly by focal contacts, and *vice versa*. Such pH-induced changes in the apparent cell–substrate contact were quite rapid as demonstrated in the series of photomicrographs in Figure 4(e) (0 to 6 min). These cells were initially incubated with PBS (Fig. 4(e) to (o)) then the buffer was changed to MES buffer and the same area was photographed at one to two-minute intervals. It is apparent that many of the loosely attached areas of the ventral cell membrane became progressively associated with close contacts as pointed out by the arrows on the plate.

When isolated ventral membranes prepared by the $ZnCl_2$ method as described above were exposed to HEPES buffer, PBS or MES buffer, similar changes were observed. At pH 7·0 to 7·2 (HEPES buffer or PBS), the adhesion of the isolated membranes to the glass coverslips was confined to focal contacts (Fig. 5(a)) while at pH 6·0 (MES buffer) an extensive formation of close contacts over the entire

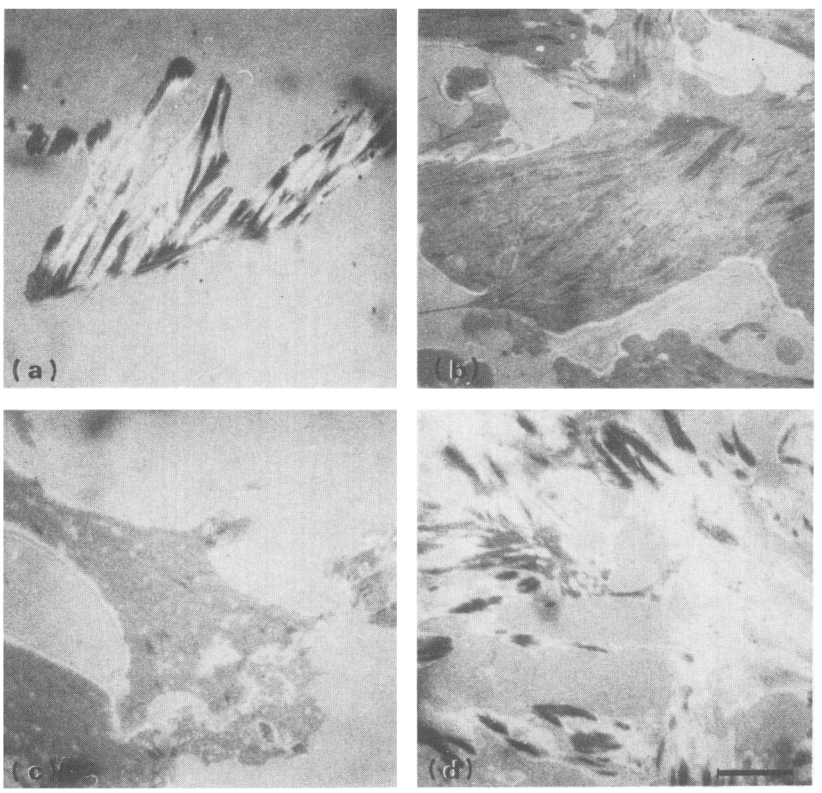

FIG. 5. Effect of pH on the pattern of contacts between isolated ventral membranes of chicken gizzard cells and the substrate. (a) Isolated ventral membranes prepared by the $ZnCl_2$ method, and maintained in PBS. (b) Ventral membranes maintained in 100 mM-MES buffer, pH 6·0. (c) Isolated ventral membranes maintained in PBS and then the buffer replaced with MES buffer, pH 6·0. Notice the formation of extensive close contact over most of the area of the isolated membrane. (d) Isolated ventral membranes maintained in MES buffer, pH 6·0 and then the buffer replaced with PBS. Notice the disappearance of the extensive close contacts.

The bar represents 10 μm.

ventral membrane was observed (Fig. 5(b)). The process was readily reversible, namely when membranes maintained at pH 7·0 were incubated with MES buffer at pH 6·0 a large close contact was formed as shown in Figure 5(c). Similarly, the extensive close contact formed at pH 6·0 was reversed when the buffer was replaced with PBS at pH 7·2 (Fig. 5(d)).

(d) *Cytoskeletal associations with isolated, substrate-attached membranes of chicken gizzard cells*

The cytoplasmic faces of the purified ventral membranes became exposed and accessible for antibody reagents. It was thus possible to fluorescently label these areas with antibodies to a variety of cytoskeletal components without the requirement for permeabilization with detergent or solvents.

Besides vinculin (see Fig. 1 above) the most prominent cytoskeletal protein which was retained on the isolated ventral membranes was actin. In whole chicken gizzard cells actin was packed into tight bundles localized along both the ventral and the dorsal cell membrane as can be appreciated from Figure 6(a). These actin-containing stress fibers terminated in areas of focal contacts, usually at the

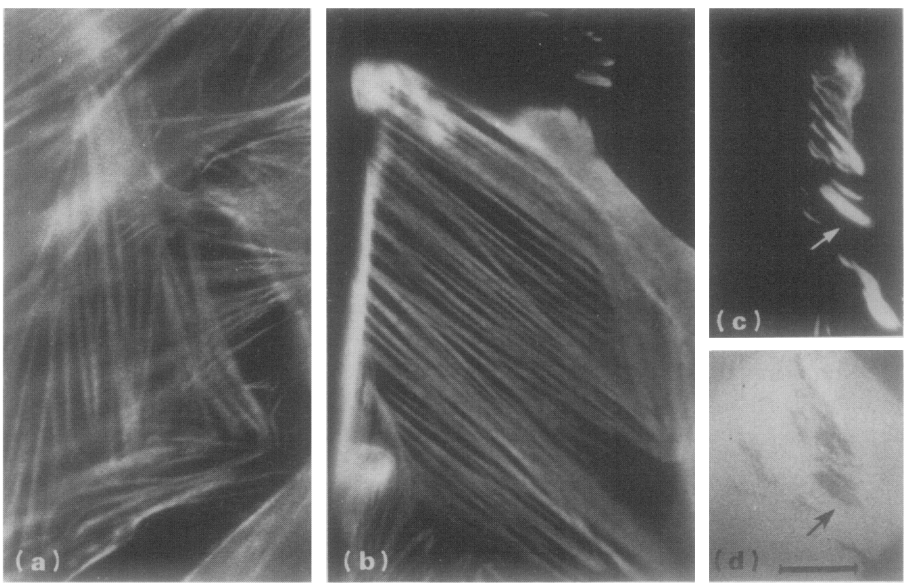

FIG. 6. The distribution of actin in whole chicken gizzard cells (a) and on isolated substrate-attached membranes ((b) and (c)). (a) Indirect immunofluorescent labeling for actin of whole cells (15 μg of rabbit anti-actin/ml and then 10 μg of rhodamine conjugated goat anti-rabbit IgG/ml). Notice that both the dorsal and ventral stress fibers can be seen. (b) Ventral membranes prepared by the $ZnCl_2$ method (gentle treatment), indirectly immunolabeled for actin. Notice that only one layer of stress fibers is still associated with the ventral membrane. The edges of the isolated ventral membrane are slightly folded (for comparison see Fig. 3(b). (c) Isolated ventral membranes exposed to extensive shearing, immunolabled for actin. Most of the stress fibers were removed while actin associated with the termini of these fibers was retained. (d) Interference reflection pattern of the same area. The residual actin labeling found after extensive shearing is apparently associated with focal contacts. Arrows in (c) and (d) point to the same area.

The bar represents 10 μm.

cell periphery. Upon preparation of the ventral membranes using the $ZnCl_2$ technique, most of the dorsal aspects of the microfilament system were removed, leaving behind a single layer of membrane-associated actin bundles which grossly retained their native organization (Fig. 6(c)). Upon further exposure to shear by buffer stream, the greater part of the ventral membrane was removed together with most of the associated actin (Fig. 6(d)).

It is noteworthy that a strong actin labeling was obtained on purified focal contacts even when the stress fibers that were originally associated with these areas were removed (Fig. 6, compare the actin immunolabeling in (c) with the interference-reflection pattern in (d)).

The distribution of several other actin-associated proteins in whole cells and on purified ventral membranes is depicted in Figure 7. α-Actinin labeling in permeabilized whole cells exhibited a dotted distribution along stress fibers (both the dorsal and ventral stress fibers) and was specifically enriched near focal contact areas (Fig. 7(a)). Upon removal of the dorsal portion of the cells, α-actinin remained firmly associated with the residual ventral membrane, both along the stress fibers and at their termini (Fig. 7(b)). Individual focal contacts were also strongly labeled for α-actinin (arrow in Fig. 7(c)). We have often found regularly organized α-actinin labeled dots on loosely attached areas of the cell membrane. These dots apparently corresponded to the vertices of the polygonal microfilament arrays (arrowheads in Fig. 7(c)).

Tropomyosin labeling of whole cells was associated with actin filament bundles, as demonstrated in Figure 7(d). Most of these filamentous arrays were removed after the $ZnCl_2$/buffer jet treatment, leaving only a single layer of cables on the ventral membrane (Fig. 7(e)). The immunolabeling for tropomyosin at the termini of stress fibers or on the focal contacts themselves were markedly reduced.

Filamin labeling was similarly associated with stress fibers and sheets both in whole cells (Fig. 7(f)) and in isolated membranes (Fig. 7(g)) exhibiting a typical dotted distribution. Most of the fibers were removed during membrane preparation and the immunolabeling for filamin was primarily associated with broad sheets and with membrane-associated dots as shown in Figure 7(g). These intensely labeled dots marked with arrowheads corresponded to the vertices of the polygonal actin networks.

FIG. 7. Indirect immunofluorescence labeling of chicken gizzard cells ((a), (d), (f)) or isolated ventral membranes ((b), (c), (e), (g)) for α-actinin ((a) to (c)), tropomyosin ((d), (e)) and filamin ((f), (g)). In whole cells the labeling is primarily associated with filament bundles both at the dorsal and the ventral focal planes of the cells. α-Actinin in isolated ventral membranes was associated with a single layer of filament bundles and sheets in a typical striated pattern and was especially enriched at their termini. Intense labeling was associated with isolated focal contacts (arrow in (c)) and with membrane-bound dots corresponding to the vertices of polygonal microfilament networks (arrowheads in (c), see text). (d) and (e) Immunofluorescent labeling of whole cell (d) and isolated ventral membrane (e) for tropomyosin. Tropomyosin labeling on the isolated membranes was associated with stress fibers exhibiting a slightly dotted distribution. The labeling for tropomyosin at the termini of stress fibers and in focal contact areas was markedly reduced. (f) and (g) Immunofluorescent labeling of whole cells and ventral membranes for filamin. In isolated ventral membranes striated labeling was associated primarily with membrane-bound filament sheets and bundles. Often, intensely labeled dots were detected on the membrane (arrowheads in (g)). These dots correspond to the vertices of polygonal actin meshwork as described above for α-actinin.

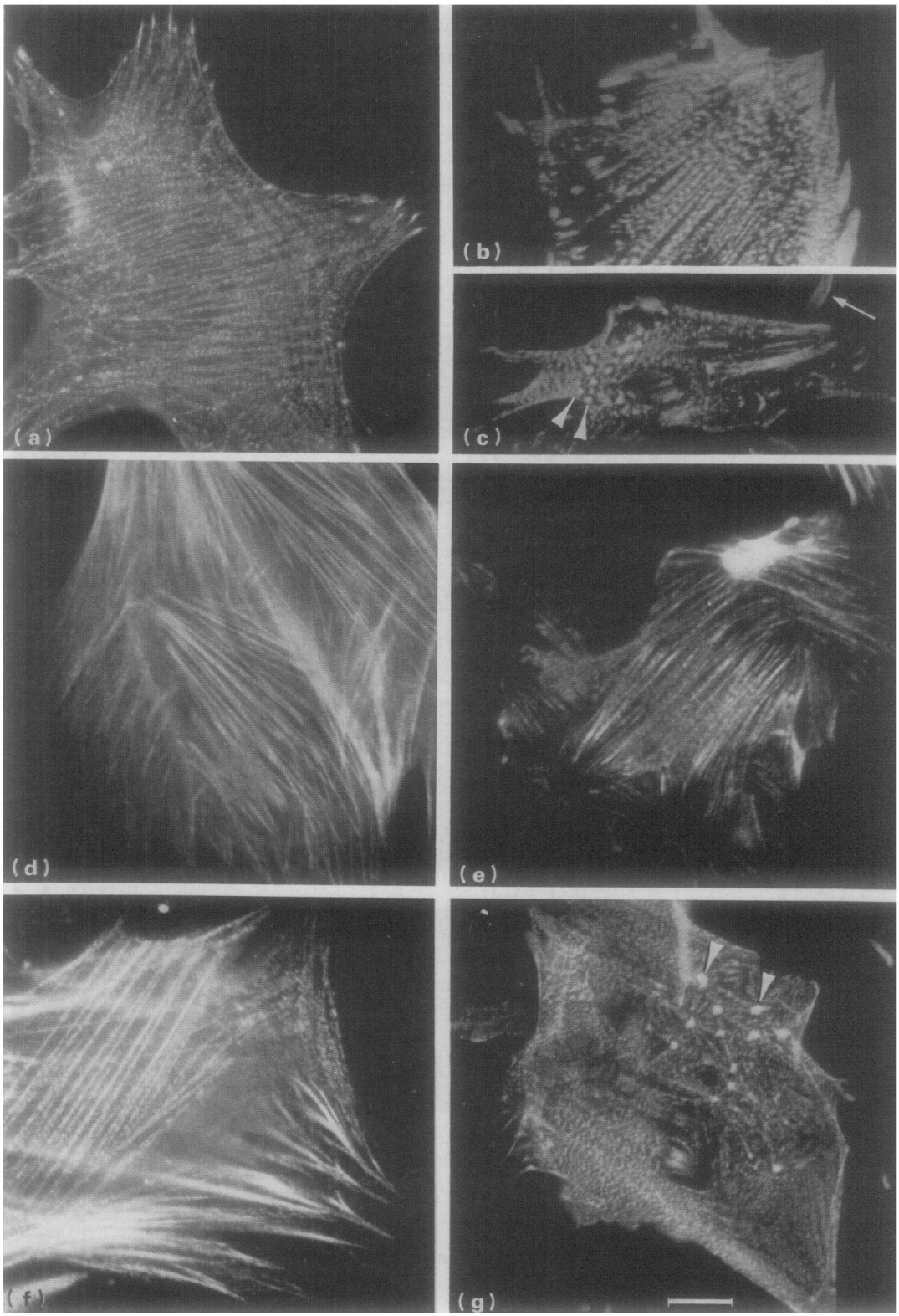

FIG. 7.

Immunofluorescent labeling with antibodies to tubulin on whole cells revealed an elaborate network of microtubules throughout the cytoplasm (Fig. 8(a)). The exposure of the cells to 1 mM-$ZnCl_2$ for two to four minutes did not have any apparent effect on the organization of microtubules (not shown). However, when the buffer jet was applied to the cells, almost the entire microtubular system was removed from the ventral membranes, with only a few tubulin-containing fragments sticking to their surfaces (Fig. 8(b)). The amount of tubulin left on the ventral membranes was inversely related to the intensity of the buffer jet applied to

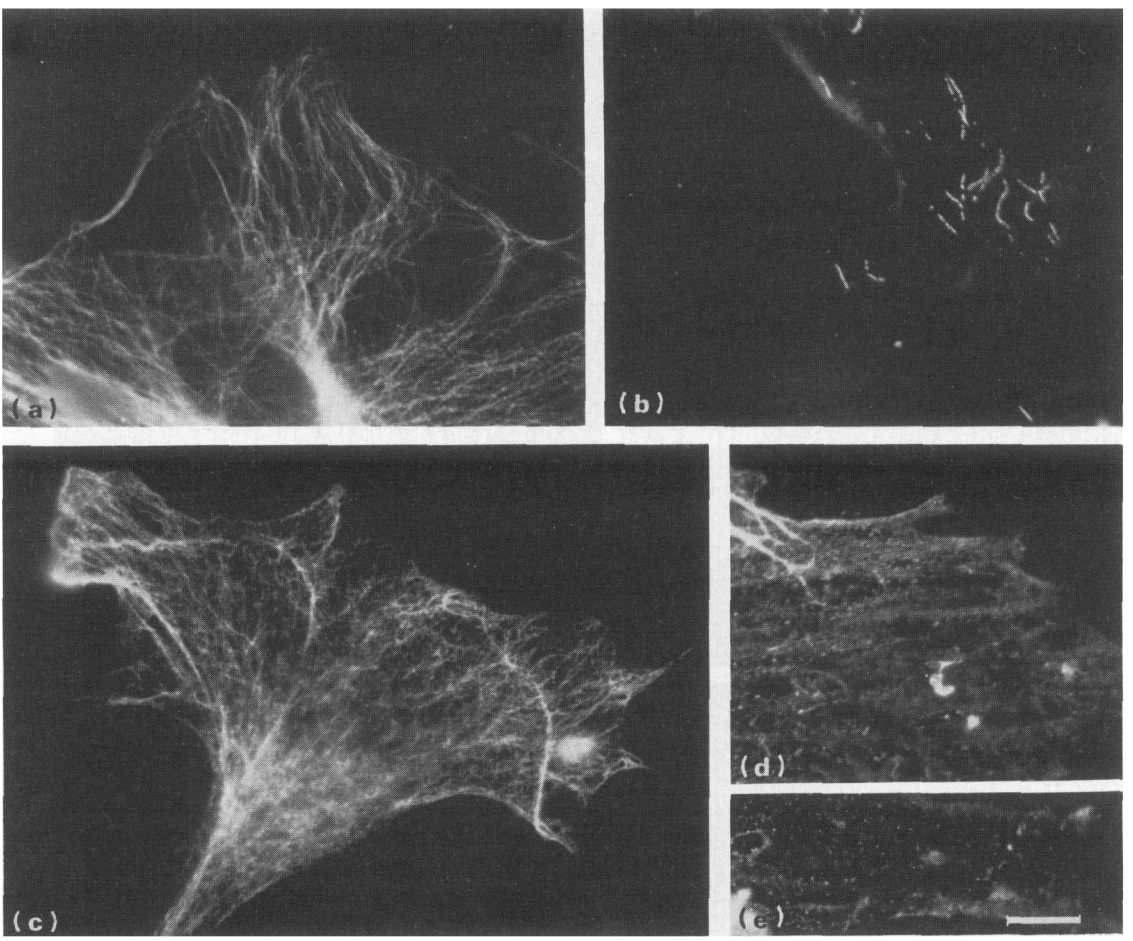

FIG. 8. Immunofluorescent labeling of whole chicken gizzard cells ((a) and (c)) and of isolated ventral membranes ((b), (d) and (e)) for tubulin ((a), (b)) and desmin ((c) to (e)). The fluorescence labeling for both filamentous systems is associated, in whole cells, with elaborate filamentous network. Preparation of the ventral membranes resulted in the removal of nearly all the microtubules (b). This was obtained even after a gentle treatment. The isolated ventral membranes contained only few desmin filaments (upper left corner in (d)) and the majority of label was associated with small dots scattered over the entire cytoplasmic faces of the ventral membranes. After extensive rinsing, only sparse dotted labeling was detected (e).

the cells; in preparations that were exposed to extensive shear no microtubules were found.

Intermediate filaments of the desmin (skeletin) type also formed an elaborate three-dimensional meshwork in whole chicken gizzard cells (Fig. 8(c)). Most of these intermediate filaments were not retained on the purified ventral membranes in a filamentous form and the isolated ventral membranes contained mainly arrays of desmin-labeled dots. We did not observe any relationship between desmin-labeled structures and cell–substrate focal contacts.

4. Discussion

Much effort has been devoted in recent years to the characterization of cellular adhesive processes. The interest in mechanisms of cell contact formation was largely motivated by two major lines of observations. It has been shown that anchorage dependence is a fundamental property of many cultured cells (Stoker & O'Neill, 1968; Hay & Meier, 1976; Folkman & Moscona, 1978; Gospodarowicz *et al.*, 1978; Brouty-Boye *et al.*, 1980). Furthermore, it has been demonstrated that many cellular activities such as regulation of cell growth and differentiation and the induction of a variety of metabolic processes depend upon the formation of cell–cell and cell–substrate contacts.

The second type of observation that directed much attention to studies on the nature of cell contacts was that transformed cells usually have defective adhesive properties that may, to some extent, be related to the "transformed phenotype" of these cells both *in vivo* and *in vitro* (Vasiliev & Gelfand, 1977; Pollack *et al.*, 1975; Ash *et al.*, 1976).

In spite of the wealth of information available on the kinetics of cell attachment, the matrix requirements and the detailed morphology of contact areas (Grinnell, 1978; Yamada & Olden, 1978; Vasiliev & Gelfand, 1977; Abercrombie *et al.*, 1971), very little is presently known of the molecular nature of the various types of cell contacts. This is partly due to the possible molecular heterogeneity of attachment sites and to the difficulties in the isolation of these cellular regions in an intact form and in a sufficient degree of purity. Many attempts have been made in the last few years to isolate cellular components that are involved in cell attachment. In most of these studies the cells were subjected to selective extractions (Lehto *et al.*, 1980) or were induced to detach from the substrate by chelating agents (Culp, 1976; Culp & Buniel, 1976; Culp, 1978). Electrophoretic analysis of the substrate-attached materials indicated that several proteins and glycosaminoglycans were primarily associated with the surface of the substrate. It remained unclear, however, whether these components are associated with specific cell–substrate contact areas. Thus, for example, fibrorectin was usually found in an association with the substrate, although this protein is apparently not associated with focal or close contacts (Geiger, 1981; Chen & Singer, 1980; Birchmeirer *et al.*, 1980; Avnur & Geiger, 1981). In this study we presented an approach for the isolation of a particular class of cell attachment sites, namely the focal contacts.

In these areas, the cell membrane is closely associated with the tissue culture substrate (about 15 nm) as previously reported (Izzard & Lochner, 1976,1980) and

as demonstrated here. An important observation, initially obtained by electron microscopy and later by the use of immunocytochemical approaches, indicated that focal contacts and similar cell-to-cell contacts are specifically associated with actin-containing microfilament bundles (Abercrombie *et al.*, 1971; Heaysman and Pegrum, 1973; Heath and Dunn, 1978). Moreover, remarkable specificity was noted in the apparent associations of several actin-associated proteins with focal contacts and related structures. The most prominent components in these specialized areas were α-actinin (Lazarides & Burridge, 1975; Lazarides, 1976) and vinculin (Geiger, 1979; Geiger *et al.*, 1979; Geiger *et al.*, 1980, Burridge & Feramisco, 1980).

In developing the procedure for the isolation of substrate-attached membranes we have adhered to the following guidelines.

(a) The isolated membrane fragments should retain their substrate attachments (focal and close) in an apparently intact form.

(b) The treatment should remove most of the cellular material while still retaining those cytoskeletal elements most tightly bound to the cell membrane in the contact areas.

(c) Chaotropic agents (detergents or solvents) that may distort membrane structures should be avoided.

(d) The treatment should have minimal effect on the spatial organization of the cytoskeleton and in particular on its interaction with the cell membrane.

The $ZnCl_2$ method described here satisfies most of these criteria. The pattern of close contacts and focal contacts in living cells was not altered by the addition of 1 mM-$ZnCl_2$ for short periods (2 to 5 min), nor had the treatment any apparent effect on the organization of microtubules, intermediate filaments and microfilaments. The amount of residual membranes and cytoskeleton left on the substrate after the $ZnCl_2$/buffer jet treatment seems to be related to the mechanical strength of the substrate attachment itself. Thus, by varying the intensity of the buffer jets we would prepare either whole ventral membranes (gentle treatment) or individual focal contacts (vigorous treatment).

The pretreatment of the cells with Zn^{2+} ions was found to be essential for the efficient removal of the dorsal parts of the cells. When the $ZnCl_2$ treatment was omitted as described by Badley *et al.* (1978) most of the chicken gizzard cells were not affected by the buffer stream, or were completely detached from the substrate. The preservation of both focal contacts (as determined by interference-reflection microscopy) and the various cytoskeletal systems (determined by immunofluorescent microscopy) was poor when $ZnCl_2$ was not used during the isolation of the ventral membranes. The application of Zn^{2+} ions as a general method for the isolation of cell membranes has been described and successfully used before (Warren *et al.*, 1966; Barland *et al.*, 1970; Perdue & Sneider, 1970; Brunette & Till, 1971; Shin & Carraway, 1973). The mechanism of the Zn^{2+} effect on membranes is not completely understood; nevertheless, it seems that the addition of Zn^{2+} ions increases the overall rigidity and fragility of the cell membranes. This was indicated by a variety of experimental approaches including interference-reflection microscopy, scanning and electron microscopy.

An interesting observation described here was the remarkable effect of pH on the

pattern of cell–substrate contact in living cells and in isolated ventral membranes. Thus, chicken gizzard cells maintained in tissue culture medium, in PBS or in HEPES buffer (all pH 7·0 to 7·2) were attached to the substrate predominantly through their focal contacts (Fig. 4). When the cells were incubated with MES buffer, pH 6·0, extensive close (not focal) contacts were formed between the entire ventral membrane of the cells and the substrate. The induction of close contact formation by lowering the pH was achieved not only with intact cells but also with isolated ventral membranes, suggesting that it directly affects the physical interaction of the cell membrane to the substrate.

We do not propose here that the formation of close contacts *in vivo* during cell spreading and locomotion is related to the pH-induced close contact formation. However, this system may prove to be a useful model for studies on the molecular nature of transient cell–substrate attachments that precede focal contact formation (Izzard & Lochner, 1980). Moreover, the formation of extensive close contacts provides a most likely explanation of the high resistance of cells maintained at pH 6·0 to mechanical shearing forces and the difficulty of splitting off their dorsal parts as described above.

The immunofluorescence labeling of the substrate-attached membrane with antibodies to several cytoskeletal components provided us with new data regarding the interaction of the various cytoskeletal networks and the cell membrane. In contrast to most of the existing information which is based on spatial relationships, between various cytoskeletal elements and the cell membrane, the present results reveal those structures and components that are most firmly associated with the ventral cell membranes. The cytoskeletal system that was retained on the isolated membranes in the most intact form was the microfilament system. Microtubules were largely removed, even after moderate shearing, and could be completely removed by additional rinsing. Intermediate filaments of the desmin type were also removed, leaving behind arrays of filaments only in some of the cell margins. Residual immunolabeling with desmin antibodies was associated with numerous small dots over the cytoplasmic faces of the ventral membrane. The significance of these dots and their relationships to the original filamentous meshwork and to the cell membrane are yet to be determined.

Most of the components of the microfilament system were retained on the isolated ventral membranes. This includes actin, α-actinin, tropomyosin, filamin and vinculin. The most prominent component on both the ventral membranes and the individual focal contacts was vinculin. It was retained on the membrane in a tight and extensive association with the focal contacts. Vinculin in that area was resistant toward further detergent extraction (0·5 to 1% Triton X-100 at pH 6·0 to 7·0) although it was partially extracted upon elevation of the pH from 6·0 to 7·5 to 8·0 or the exposure to 1% deoxycholate or 1 M-KI. In these cases the labeling of the residual membrane-associated vinculin was organized in linear arrays of dots over the entire area of the focal contacts. A more detailed analysis of the interaction of vinculin with the ventral cell membrane is now in progress.

Immunofluorescent labeling with actin antibodies pointed to the abundance of this protein on the substrate-bound membrane. In mildly opened cells the ventral sheets of stress fibers were retained in a pattern similar to that seen in whole cells

(cf. Fig. 6(a) and (b)). Occasionally we observed the remains of the polygonal arrays of microfilament bundles (Lazarides, 1976). When a more extensive jet of buffer was used the stress fibers were progressively deteriorated and actin labeling was associated with either broad sheets or with the focal contact themselves (Fig. 6(c)). The association of actin with the focal contact was apparently very tight and was not affected even by extensive shearing.

Each one of the actin-associated proteins had a specific and distinct pattern of distribution over the exposed cytoplasmic faces of the isolated ventral membranes. α-Actinin was primarily associated with focal contacts, and striated filament sheets and cables. In extensively rinsed preparations, however, when the stress fibers were removed, α-actinin association with the focal contacts was not affected. In addition, we found intense α-actinin label in the form of hexagonally organized dots, tightly bound to the ventral membrane. These dots are most likely the residues of the vertices of the polygonal actin arrays, originally described by Lazarides (1976). It therefore indicates that the perinuclear baskets formed by the microfilament system are anchored to the cell membrane *via* their α-actinin-containing vertices. Filamin distribution was similar to that of α-actinin. The labeling was specifically associated with flat membrane-bound sheets exhibiting a typical striated distribution. We often observed intense labeling on the organized dots as described above for α-actinin. However, the labeling of the focal contacts by anti-filamin was considerably less intense than that obtained with α-actinin antibodies, suggesting some segregation between these two actin crosslinking proteins. Tropomyosin was also associated with the substrate-attached membrane fragments but was usually depleted from the focal contacts themselves. It is noteworthy that in addition to the membrane and the associated cytoskeletal structure, a significant amount of extracellular matrix (mainly fibronectin) was associated with the substrate as could be appreciated from immunolabeling with specific fibronectin antibodies (data not shown).

The data presented here suggest that the isolated ventral membranes of fibroblastic cells, as well as of other cell types, may serve as a very useful model for the characterization of the peripheral and integral membrane components which are related to cell contact formation. Studies presently in progress in this laboratory suggest that cytosketetal elements may form specific associations with the exposed cytoplasmic aspect of the substrate-bound membranes. This as well as a detailed biochemical characterization of the ventral membranes and focal contacts may pave the road towards a better understanding of the molecular basis for cellular adhesiveness.

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