

CHANGES IN SURFACE MORPHOLOGY OF CHINESE HAMSTER OVARY CELLS DURING THE CELL CYCLE

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ABSTRACT

Synchronized populations of Chinese hamster ovary (CHO) cells in confluent culture have been examined by scanning electron microscopy and their surface changes noted as the cells progress through the cycle. During G_1 it is characteristic for cells to show large numbers of microvilli, blebs, and ruffles. Except for the ruffles, these tend to diminish in prominence during S and the cells become relatively smooth as they spread thinly over the substrate. During G_2 microvilli increase in number and the cells thicken in anticipation of rounding up for mitosis. It appears that the changes observed here reflect the changing capacity of CHO cells during the cycle to respond to contact with other cells in the population, because, as noted in the succeeding paper (Rubin and Everhart), CHO cells in sparse nonconfluent cultures do not show the same wide range of changes during the cell cycle. Normal, non-transformed cells of equivalent type in confluent culture are essentially devoid of microvilli, blebs, and ruffles. The relation of these surface configurations to the internal structure of the cell is discussed.

INTRODUCTION

Progress of a cell through its life cycle must be based on a continuum of causally related cellular events. This continuum is reflected in two major steps, DNA replication and nuclear division, and these steps allow the cell cycle to be subdivided in the four, well-known periods, G_1 , S, G_2 , and D. The G_1 period is generally assumed to contain those events that lead to and prepare the cell for DNA replication (S period). The G_2 period is believed to contain preparatory steps for mitosis (M period). In addition, a variety of observations have made it clear that events of the G_1 period are important for the regulation of the cell cycle, since cells that cease to proliferate, either *in vitro* or *in vivo*, ordinarily do so because of a block to their progress through the G_1 period.

The present study on the surface morphology of cultured cells with the scanning electron microscope is intended to add another view to the changing character of the cell through the course of its life cycle. In particular, we have attempted to relate changes in the surface structure of cells to known stages in the cycle. For example, the "bubbling" of the cell surface during mitosis has been described many times from observations of living cells by means of time-lapse photography. Recently Fox et al. (1971) discovered that sites on the cell surface that bind certain agglutinins are transiently exposed during mitosis. One would hope eventually to discover the causal connection between surface changes and specific processes going on inside the cell. In this regard, an es-

pecially important problem is the apparent relationship between the cell surface and the regulation of cell reproduction in density-dependent inhibition of cell reproduction and the alteration of this relationship in transformed cells. The extent to which contact inhibition is reflected in relatively gross changes in the surface morphology should emerge from these studies with greater clarity than heretofore.

Furthermore, it has become obvious that for the detailed exploration of changes in surface morphology and cell shape associated with the cell cycle, the scanning electron microscope is a most valuable tool.

METHODS

All observations were made on a line of Chinese hamster ovary (CHO) cells obtained from Dr. Donald Petersen who got them originally from T. T. Puck. The cells were cultured in Ham's F-12 medium (North American Biologicals, Inc., Rockville, Md.) supplemented with 10% fetal calf serum (Flow Laboratories, Inc., Rockville, Md.). Cells were grown in glass vessels and subcultured weekly, using a solution of 0.5% trypsin and 0.1% EDTA to strip cell monolayers from the glass surface. Regular checks for pleuropneumonia-like organisms have always been negative.

Synchronous populations were obtained by the mitotic shake-off method (Tobey et al., 1967). The mitotic index in the shake-off populations used in these experiments was particularly high, ranging from 98 to 99.5%.

The mitotic cells were planted on cover glasses coated with a thin (100 Å) carbon film. Cells were fixed at 1, 3, and 5 h after shake-off to provide early, middle, and late G₁ cells. Samples of these cells were exposed to [³H]thymidine for 15 min before fixing and then autoradiographed. These samples allowed us to assess, by light microscopy, whether cells had completed G₁ and entered the S period.

Because of the variability in the length of G₁ phase in populations of cultured cells, the mitotic cells used to provide cells in the S phase were first resynchronized by a single thymidine block (Galavazi et al., 1966). Mitotic populations on cover glasses were exposed to 5 mM thymidine for 10 h after planting. This is sufficient time for about 95% of the cells to complete the G₁ period and reach the beginning of the S period. The blocked cells were released by removal of the thymidine with a change of the medium.

Samples were fixed at 1, 3, and 4 h after release of the block to provide cells in early, middle, and late S. A sample was fixed at 5 h after release of the block

to provide cells in the G₂ period.¹ Again, at each fixing time samples of cells were exposed to [³H]thymidine for the final 15 min before fixing in order to assess how many cells were in the S period in any of the samples. The fixations were timed on the basis of previously published values (Bostock et al., 1971) for the length of the cycle subsections for this same line of cells.

Scanning Microscopy—Methods

The experiments were repeated twice and observations on populations from both experiments were essentially identical.

Cells for microscopy were rinsed (twice, 2 min each) free of culture media with warm balanced salt solution (Puck's saline G) and then exposed to 2% glutaraldehyde in 0.1 M *s*-collidine buffer at pH 7.1. After 0.5 h in this they were rinsed twice with warm Puck's saline G for a total of 4 min and postfixed in 1% OsO₄ in 0.3 M *s*-collidine for 10 min. A brief rinse (5 min) in distilled, deionized H₂O was followed by dehydration with ethyl alcohol of increasing concentration up to 95%. From here the preparations were carried through a graded series of amyl acetate in alcohol up to 100% amyl acetate.² Thence they were transferred into liquid CO₂ in a critical-point drying apparatus and, after several rinses in CO₂, the temperature of the closed system was raised to 45°C according to the critical-point method of T. F. Anderson (1951).

The resulting dry specimens were lightly coated with carbon and then with gold-palladium and were ready for microscopy.

A Kent Cambridge S-4 scanning electron microscope, operated at 20 kV, was used in all of these studies.

OBSERVATIONS

Assessment of Cell Cycle Synchrony

The mitotic index of the populations used for the electron microscope observations ranged from 98 to 99.5%, and the initial synchrony was accordingly excellent. The decay in synchrony through the ensuing G₁ period was measured by

¹ The loss of synchrony in the 5 h sample was substantial and interpretations of the results with the scanning microscope are therefore somewhat less clear. In this sample 58% of the cells were still in DNA replication, 10% were in mitosis, and the remaining 32% were in G₂.

² It has since been found that cells dehydrated in acetone can be taken directly into CO₂ thus avoiding the amyl acetate step in the above procedure.

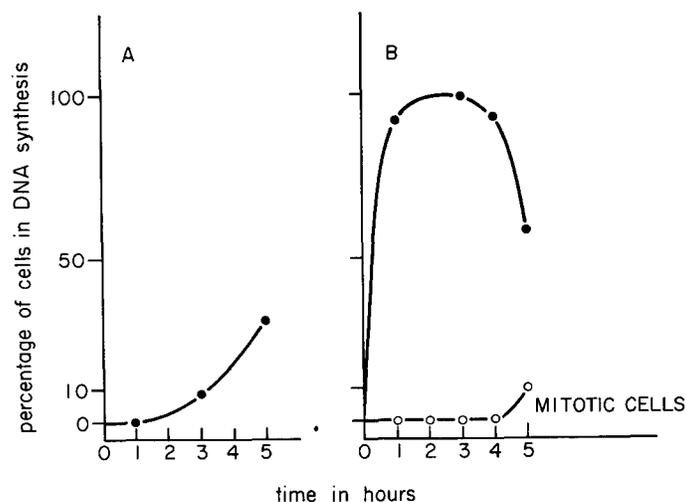


FIGURE 1 A Mitotic cells were collected by the shake-off method and then fixed at 1, 3, or 5 h to provide cells in early, middle, and late G_1 . 9% of the cells had entered S by 3 h, and 30% had entered S by 5 h.

FIGURE 1 B Mitotic cells were collected and cultured for 10 h in the presence of 5 mM of thymidine to block DNA synthesis. The cells were released from the thymidine block by washing, and samples were fixed at 1, 3, and 4 h to provide cells in early, mid-late, and late S phase. The sample fixed at 5 h after release from thymidine was composed of 58% late S, 32% G_2 , and 10% mitotic cells.

following with time the percentage of cells that entered DNA replication, as shown in Fig. 1 A. All of the cells fixed at 1 h were in G_1 , and essentially all of these could be safely assumed to be in early G_1 . The population fixed at 3 h consisted of 9% S cells and 91% G_1 cells and was accordingly accepted as a mixture of middle and late G_1 stages. The population at 5 h was assumed to consist of equal parts of middle G_1 , late G_1 , and early S-phase cells.

The resynchronization of cells at the G_1 -to-S transition by blocking with 5 mM thymidine for 10 h after shake-off led to the cell populations described in Fig. 1 B. 92% of the cells fixed at 1 h after removal of the thymidine block were in the early part of the S phase. 99% of the cells fixed at 3 h after release of the block were in the middle to late S phase, and the population fixed at 4 h represented a slightly later stage of S. The sample fixed at 5 h after the block consisted of 32% G_2 cells, 58% late S-phase cells, and 10% mitotic cells.

In summary, we have highly uniform populations of cells in early G_1 , and in early, middle, and late S. The populations intended to represent middle and late G_1 are less uniform, while the G_2

population is in fact a mixture of late S cells, G_2 cells, and mitotic stages.

Microscopy

In other studies of cultured cells with the scanning microscope we have established to our satisfaction that critical-point drying of these specimens after glutaraldehyde-osmium fixation provides the observer with a specimen that is faithful to the original (Porter et al., 1972). The cells are far less distorted than by drying from water or even from solvents with lower surface tensions. Nitrous oxide or freon (Freon 13) used in the same way as CO_2 gives results that are similar but not better for most purposes.

Except for some shrinkage caused by dehydration in alcohol, the cells showed little evidence of distortion. Comparison made by phase-contrast microscopy and Nomarski optics of cells before and after fixation failed to reveal any other alterations.

CHANGES IN MORPHOLOGY WITH PROGRESS THROUGH THE G_1 PHASE OF THE CELL CYCLE

EARLY G_1 : Cells fixed 1 h after shake-off. As expected, the majority of the cells in these

preparations are in very late stages of cytokinesis. Nearly all of them are therefore still rounded up and associated in pairs (Fig. 2). They are quite uniform in diameter at 12–13 μm .

It is evident in the lateral view in Fig. 9 that they have attached to the carbon-coated cover glass and have begun to spread over this as substrate. It is apparent also in this same micrograph that the cell surfaces are covered with numerous microvilli and other small projections.

These same preparations contained a number of smooth spheres (2–4 μm) which presumably represent fragments of the cells that have broken away during mitosis. Their significance, if any, is not clear. A residue of these spheres persists into the middle G_1 preparations where they appear in a somewhat shriveled form (Fig. 3).

MIDDLE G_1 : Cells fixed 3 h after shake-off.

By this time the cells have obviously lost their spherical form and some appear almost fully extended (Fig. 3). The visible (upper) surface is covered with small spherical blebs intermingled with numerous microvilli (see below). The majority of the cells is still associated in pairs (see also Fig. 11 *a*) with adjacent edges representing the plane of earlier cleavage. There is some evidence of ruffling at the free edges, but in this early phase it is not striking.

LATE G_1 : Cells fixed 5 h after shake-off.

The cells here (Fig. 4) show a substantially greater variation in apparent size and form than was evident earlier in G_1 . A few units are thinly spread and, judging from observations on cells known to be in S, these have probably entered the S phase of the cell cycle. Other cells are thicker and less extended over the substrate as though still in middle G_1 . Sister cells, still closely associated as pairs, are common. Ruffling has increased in prominence but is confined to margins not in contact with other cells. The surface blebs persist on many cells, and of these some are in intimate contact with sister units.

SUMMARY OF SURFACE CHANGES DURING G_1 :

At the end of cytokinesis the cells are typically covered with microvilli and other equally small protuberances (Fig. 9). The surface blebs mentioned above are not prominent or even clearly evident, the greater number appearing later.

3 h after shake-off the exposed surfaces are covered with microvilli and blebs (Figs. 3, 10 *a*, and 10 *b*). The latter tend to be concentrated over the central thicker parts of the cell whereas the microvilli are more widely distributed. The blebs, which occasionally appear in clusters (Figs. 10 *b*, 11 *b*, and 11 *c*), vary in size, some being as small as 0.2 μm in diameter and others as large as 1.5 μm .

The microvilli are small by comparison measuring only about 0.1 μm in diameter. Their lengths vary greatly, but the majority are in the vicinity of 1.5 μm (Figs. 11 *b* and 11 *c*). Many of the microvilli emerge from the edges of broad flat excrescences in the surface (Figs. 10 *b*, 11 *b*, and 11 *c*).

By the end of G_1 (5 h after shake-off) the cells begin to show ruffles (or lamellipodia) at their free edges, i.e., along those parts of the margin not in contact with other cells (Fig. 4). To a large extent this restriction in the distribution of these extraordinary structures is constant.

Ruffles adopt a variety of appearances. Most commonly they comprise thin folds extending above the surface as much as 6 μm and having a thickness equal to the diameter of the microvilli (0.1 μm). In the living cell they have been observed to surround and engulf small quanta of the medium (Gey, 1955) (Figs. 13 *a-c*).

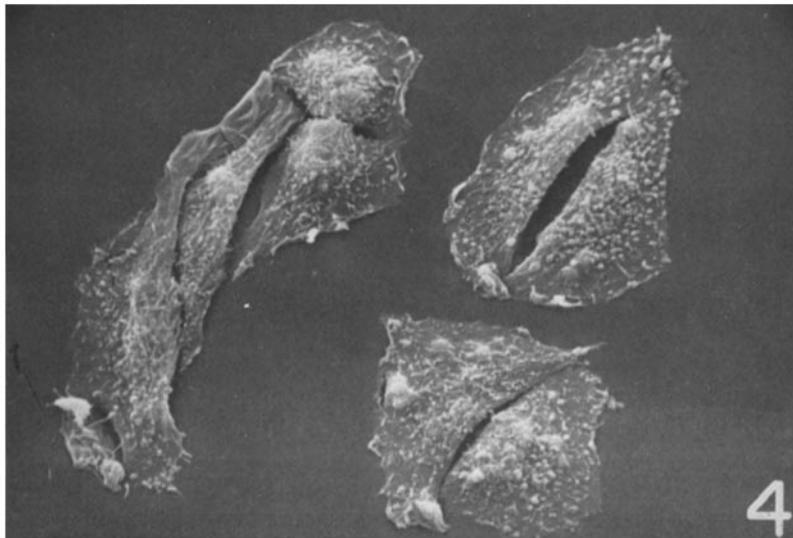
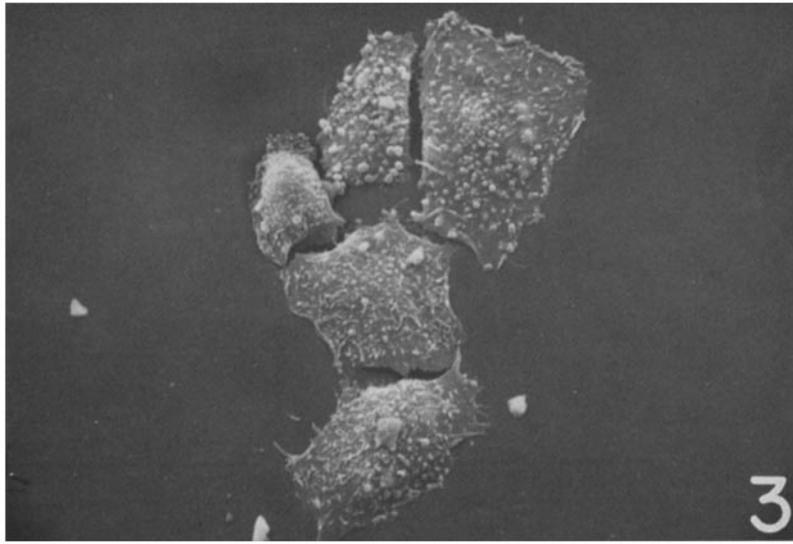
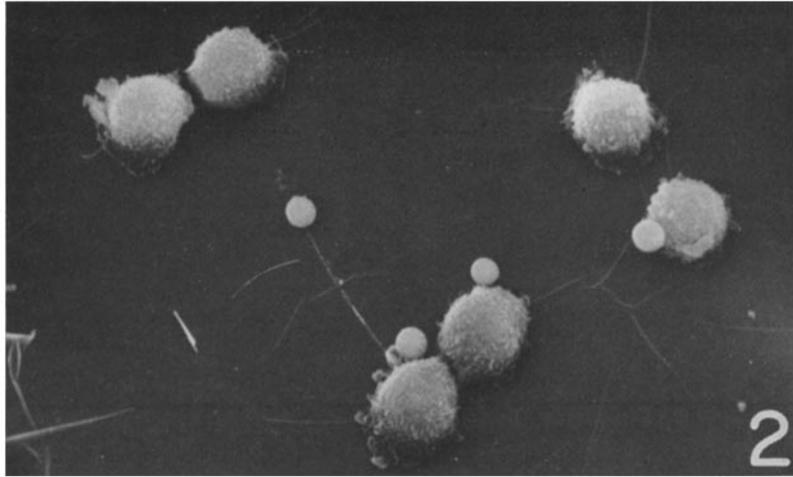
Toward the end of G_1 the cells show a wider variation in their surface characteristics than had been apparent earlier. Some, for example, are totally devoid of blebs whereas others show almost as many as at any previous time in G_1 (Figs. 10 *a* and 11 *a*). The number of microvilli tends

All illustrations are scanning electron micrographs of CHO cells except Fig. 15.

FIGURE 2 Cells fixed 1 h after mitotic shake-off (early G_1). The majority are in the last stages of cytokinesis and are just beginning to spread over the substrate surface. $\times 1,000$.

FIGURE 3 A group of cells fixed 3 h after shake-off (mid- G_1). Spreading is obviously more nearly complete. The surfaces of the cells show many microvilli and a number of zeiotic blebs. $\times 1,000$.

FIGURE 4 At 5 h after shake-off the cells, representing late G_1 , are more completely spread but are still associated in sister cell pairs. Blebs and microvilli cover the exposed cell surface, and ruffles are present in small numbers along the cell margins. $\times 1,000$.



to vary inversely with the number of blebs. Since the number and prominence of blebs tends to diminish in S and G₂, it is assumed that those cells showing few, if any, of these structures have moved into the S phase of the cycle. This assumption is supported by the autoradiographic data.

The relationship of blebs to microvilli, if any, is difficult at this point to define. There is, however, another type of surface extension, a very long and slender filopodium which seems frequently to extend from a bleb (Figs. 10 *a* and 10 *b*). These filopodia appear usually to link cell with cell or, cell with substrate. They are 50–100 nm in diameter and may be several micrometers in length. They, or very similar cell extensions, have been observed, in light microscopy of living cultured cells, to grow quickly, wave around, and finally make contact as indicated (Gey, 1955; Taylor, 1966).

BEHAVIOR OF CELLS AFTER RESYNCHRONIZATION WITH THYMIDINE

EARLY S: Cells fixed 1 h after release from a 10 h block with thymidine.

By this time the cells have enlarged noticeably over those harvested in G₁. To some extent this may reflect the fact that some of them have spread more thinly, but in part it probably results from growth and increase in cell volume (Fig. 5). The cells adopt highly variable shapes and seem not to show any preferred form. The more thinly spread cells have noticeably fewer microvilli (Figs. 5 and 12).

MID-LATE S: Cells fixed 3 h after release from 10 h block.

As the cycle progresses toward G₂ the cells spread more thinly and become essentially epithelioid. Frequently, as illustrated in Fig. 6, they appear in micro colonies and reach confluency. Generally, cell margins in contact show no ruffling (Figs. 6 and 7), but one exception to this rule is evident at the arrow in Fig. 6 (see also Fig. 12). The exposed surfaces of the cells show only a few blebs and the majority of the cells are completely devoid of these structures. It is worthy of note that the cell at *A* in Fig. 6 is in contact with adjacent cells and is exceptional in showing a number of blebs.

LATE S: Cells fixed 4 h after release from 10 h block.

A comparison of these cells with those earlier in S reveals three well-defined changes. Here the cells have thickened and are withdrawing their margins to some extent (Fig. 7). There is increasing evidence of ruffling at their edges as the cells adopt a more independent existence. The surface blebs so evident in G₁ have essentially disappeared.

G₂: Cells fixed 5 h after release from 10 h block.

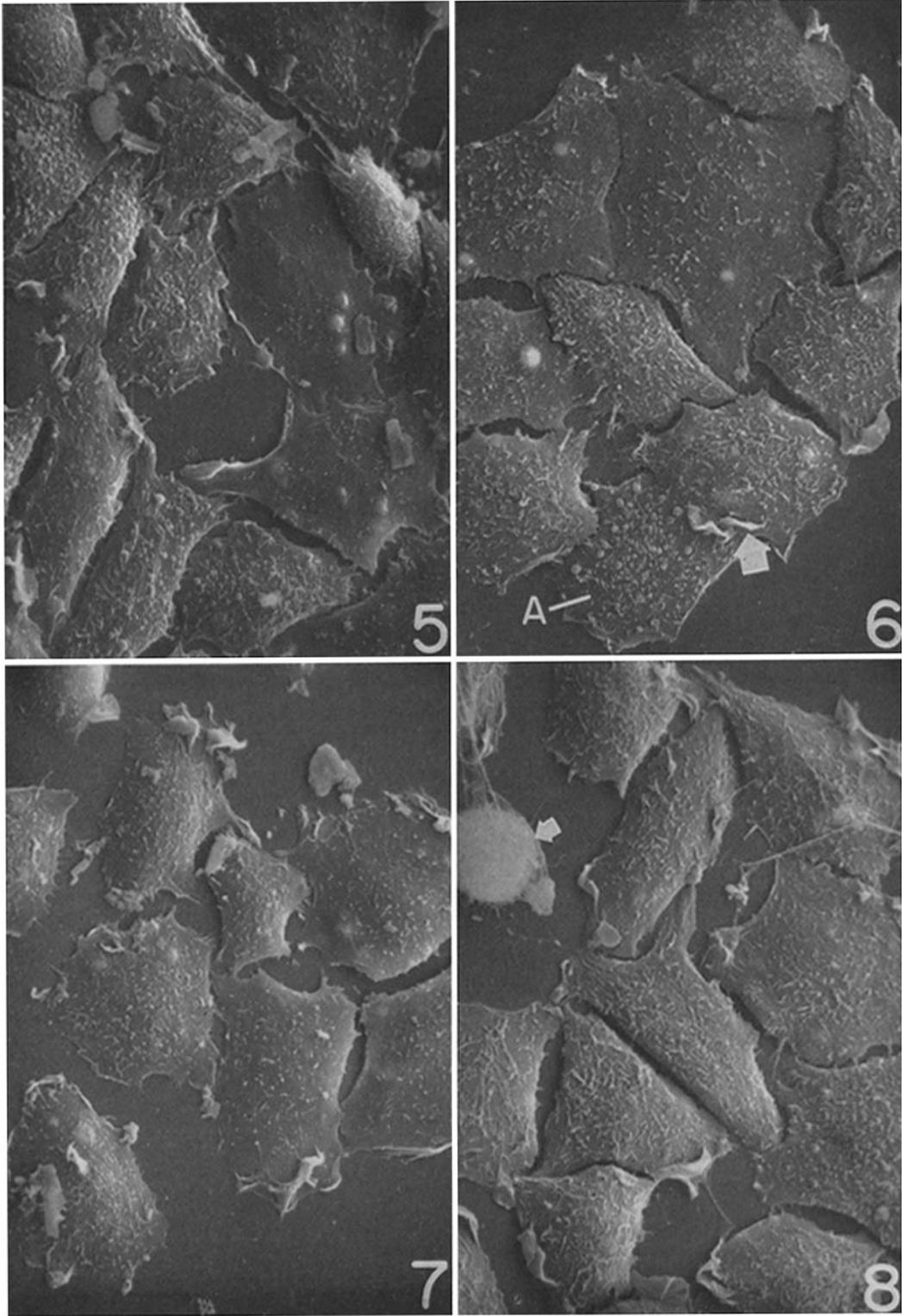
By this time a few of the cells have rounded up and entered mitosis (at arrow, Fig. 8). Others in the population are thickening and sending out slender filopodia which apparently anticipate the large number found on the dividing cell (Fig. 14). Ruffling is prominent along the free, uncontacted edges and, rarely, a few blebs are

FIGURE 5 The cells here are predominantly in early S. Some are extensively spread and are relatively free of microvilli and other surface features. Ruffling at the cell margins is present but not to the extent displayed by cells in Figs. 7 and 8. Other cells, possibly still in G₁, show an abundance of microvilli and blebs. $\times 1,000$.

FIGURE 6 The population here is in the S phase of the cycle. The surface blebs are on the wane in frequency though microvilli are still fairly prominent. Contact between the cells may have reduced the ruffling except at the edges of the colony. $\times 1,000$.

FIGURE 7 Here the cells are in late S or early G₂. They seem to be adopting an independent existence though some of the separation between them may be a result of shrinkage during dehydration. The cell margins are extraordinarily active in terms of ruffling. It is also evident that the cells are thickening in comparison with those shown in Figs. 5 and 6. $\times 1,000$.

FIGURE 8 The majority of the cells are beginning to pull in their thinner margins in preparation for the rounding up characteristic of mitosis. One cell at the upper left has already gone into prophase. The rest are in late G₂. Microvilli and ruffles are common. $\times 1,000$.



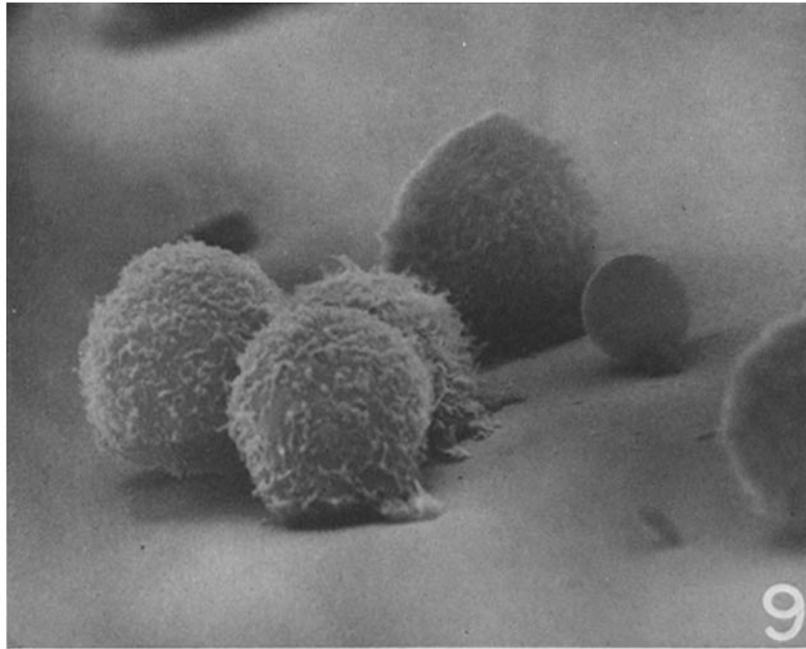


FIGURE 9 A lateral view of cells in early G_1 , fixed 1 h after shake-off. They have obviously made contact with the substrate and are beginning to spread. The surfaces are covered with small microvilli, of which some may be remnants of the filopodia which hold the cell to the substrate during mitosis and cytokinesis. $\times 2,300$.

evident among increasing numbers of microvilli. The individual cells are obviously larger than those in the populations fixed during G_1 .

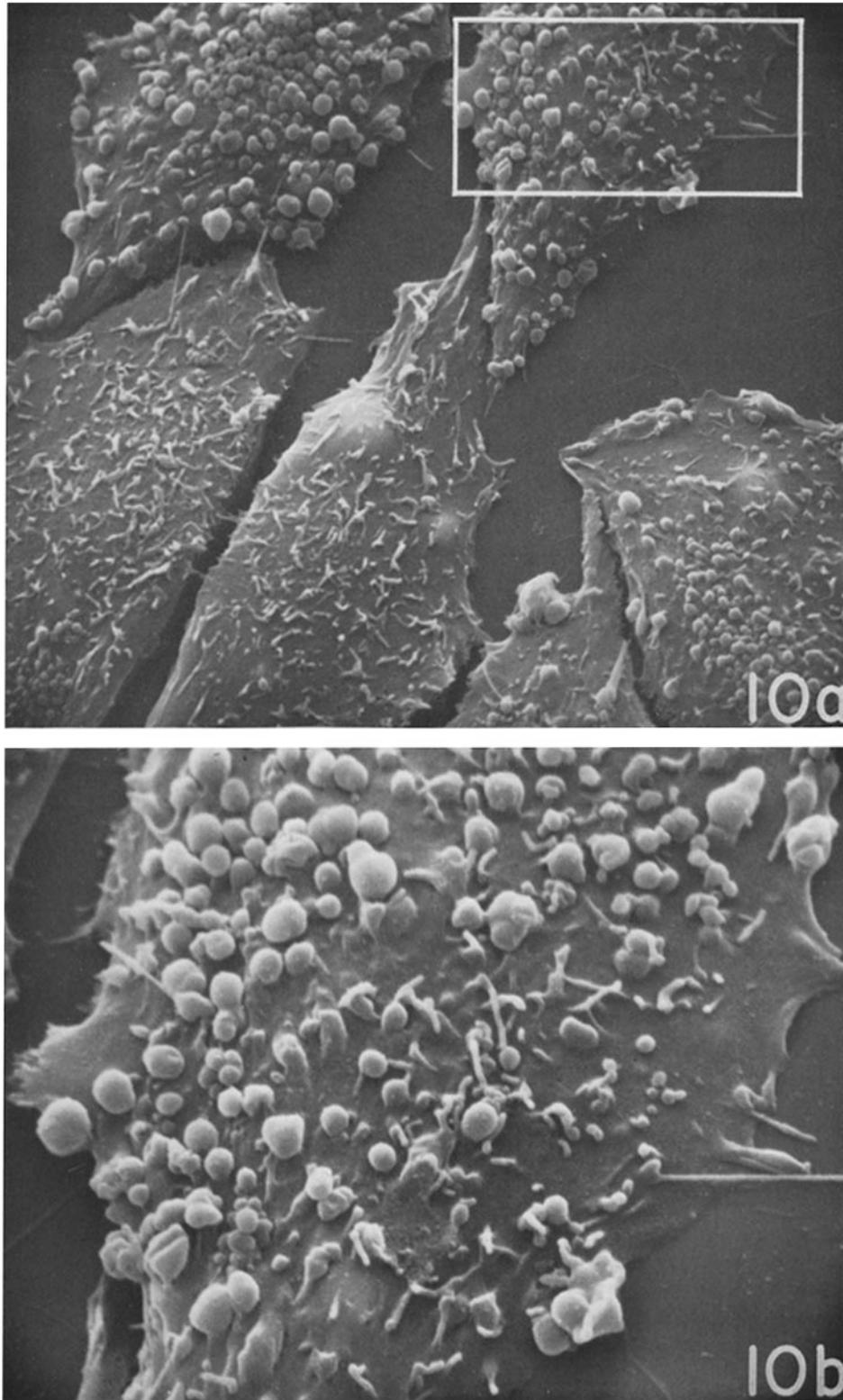
SUMMARY OF SURFACE CHANGES DURING S AND G_2 : A number of early S cells in any one preparation appear relatively smooth and free of both blebs and microvilli (Figs. 5-7 and 12). Whether at some time during S all cells possess these characteristics has not been demon-

strated by this study (but see Rubin and Everhart, the succeeding paper).

The changes from this well-spread condition to the spherical form of the cell in metaphase are dramatic. By G_2 the blebs have largely disappeared and the population of microvilli begins to increase. Marginal ruffling, present but not especially striking in S, becomes a more dominant feature in G_2 (Figs. 7 and 13 *a-c*). As the cells

FIGURE 10 *a* A group of cells from late G_1 fixed 5 h after shake-off. The surfaces are dominated by blebs and microvilli. A little ruffling is appearing at the cell margins. Blebs range in size all the way from 0.2 to 1.5 μm . Several cells have established contacts with other cells, contacts which have been disrupted during shrinkage induced by dehydration. A few filopodia are evident and the majority seem to arise from blebs. A part of the cell at the upper right (rectangle) is shown at higher magnification in Fig. 10 *b*. $\times 2,600$.

FIGURE 10 *b* Scanning micrograph depicting details of blebs and microvilli. The former can be seen to occur in clusters of small units which could be remnants of the larger ones that largely cover this cell surface. The microvilli are relatively short here and seem to be in a stage of formation from small hillocks on the surface. Two filopodia (much longer than microvilli) are depicted as emerging from small blebs. Whether this is a constant feature of filopodia has not been determined. $\times 6,500$.



achieve their maximum size and begin to thicken in G₂, the filopodia which characterize the dividing cell first appear and they increase greatly in number as the cell enters mitosis (Figs. 8 and 14).

Ruffles are present on free edges of the cells but seem to be excluded from margins in intimate (or even less than intimate) contact with other cells. Occasionally, as in Fig. 12, they encroach on the zone of contact, or even invade it (arrow, Fig. 6), but these occurrences are rare.

Some of the contacting cell margins in these preparations have been pulled part by shrinkage of the cell bodies which probably takes place during dehydration (e.g., in Figs. 4, 7, and 11 *a*). Where this has not happened one is able to examine the contacts in some detail. In some instances they show the involvement of small extensions of one cell slightly overlapping the surface of the other (Figs. 6 and 12), but this is not a feature in all instances.

There is likewise no easily recognized uniformity in the ruffles except that they tend to have minimal thickness consisting of two plasma membranes and two internally opposed cytoplasmic cortices (Fig. 13 *a*) (Abercrombie et al., 1971). In stereo images (Figs. 13 *b* and 13 *c*) they can be seen to extend more or less erectly from the surface to heights measured at 6 μ m.

The filopodia which attach the dividing cell to the substrate during mitosis are unique in several respects (Fig. 14). They may be very long, as much as two to three times the diameter of the cell and they frequently arborize at their tip

ends.³ In these respects these particular extensions of the surface are distinctive and dissimilar to filopodia that appear in fewer numbers at other phases of the cycle.

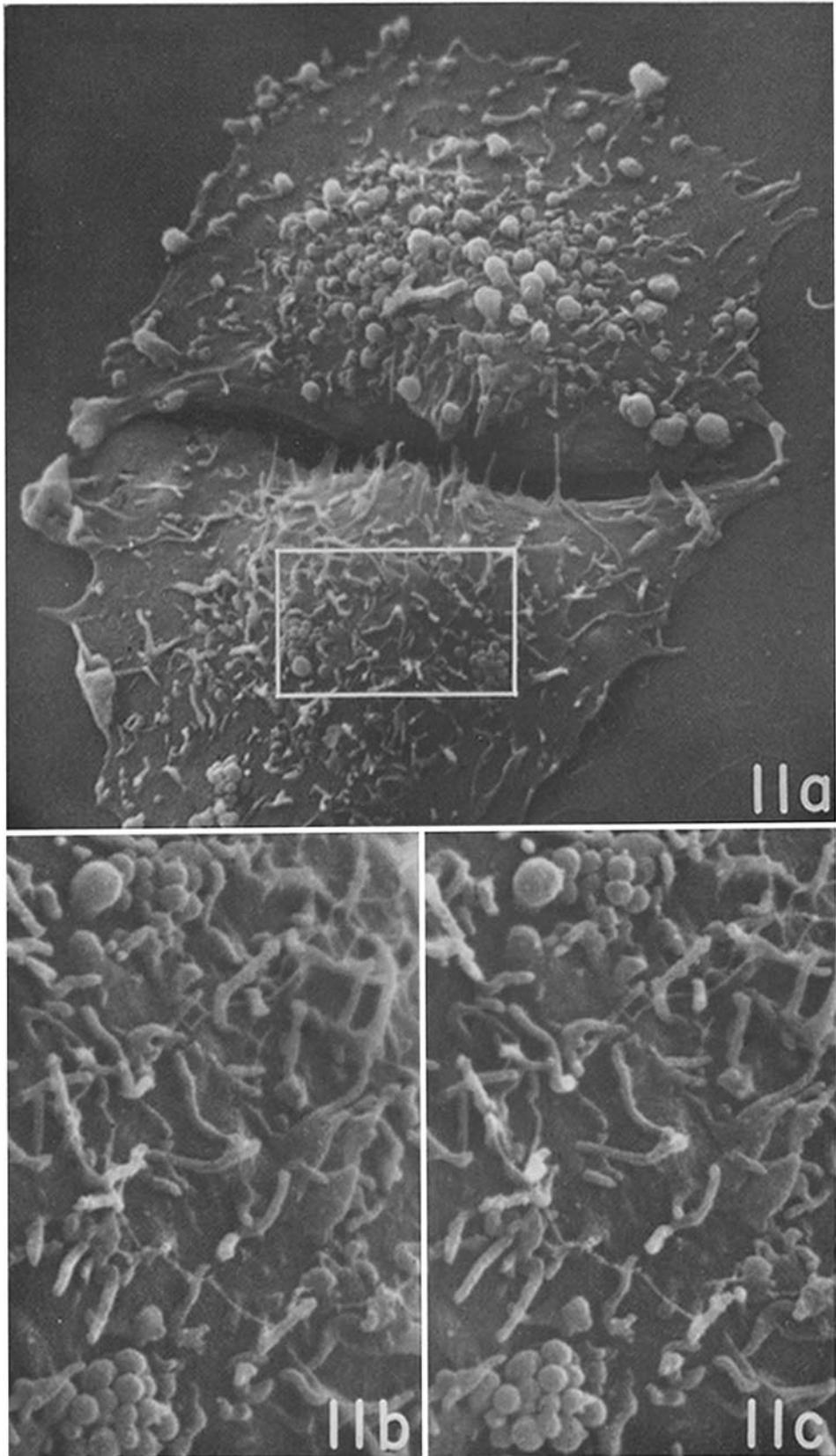
THE SURFACES OF CELLS FROM PRIMARY EXPLANTS OF CHINESE HAMSTER LUNG

In contrast to the CHO transformed cells, these normal cells in culture possess a relatively simple surface fine structure (Fig. 15). Blebs, microvilli, and ruffles are absent, and the cell surface is smooth. The cells are very flat, irregular in outline with many extensions, and with many contacts between them. The particular group of cells in Fig. 15 is very likely contact inhibited, and probably the surface features are strongly dependent upon this arrested state of growth. It would be interesting to examine surfaces of normal cells over the cell cycle, but current methods for synchronization of normal cells would be marginally satisfactory for this. For present purposes it is clear that normal cells in dense cultures are strikingly different in surface

³ Our attention has been directed to the fact that slender cell extensions (microextensions) similar to these filopodia have been interpreted as retraction fibrils which form as the cell pulls in from its extended form in rounding up for mitosis (Dalen and Scheie, 1969). We have reasons to doubt this interpretation because some of these filopodia reach from the top center of the dividing cell to the substrate and because some are observed in late G₂ cells to extend from the cell center even before retraction. Obviously the question cannot be settled until more observations are made of these and similar cells, using critical-point drying in specimen preparation.

FIGURE 11 *a* Two sister cells in mid-G₁, fixed at 3 h after shake-off. One has developed a large number of blebs; the other shows predominantly microvilli although there are clusters of small blebs which possibly represent an early stage in bleb formation. The two cells were connected by a number of slender strands (filopodia?) until these were broken in preparation for microscopy. The area in the rectangle is shown at greater magnification and in stereo in Figs. 11 *b* and 11 *c*. $\times 2,000$.

FIGURES 11 *b* and 11 *c* These represent stereo views of a part of the lower cell in Fig. 11 *a*. When studied with a stereo viewer (folding pocket CF-8 stereoscope, manufactured by Abrams Instrument Corp., Lansing, Mich., and available from Ernest F. Fullam, Inc., Schenectady, N. Y.) they provide information on the microvilli not otherwise available. It becomes evident, for example, that many of the microvilli stand up erectly from the cell surface; that they are uniform in diameter along their length, and from one to another; and that some of them arise from small flat excrescences of the surface. The small filament which crosses the field appears in stereo to be supported by microvilli and held above the cell surface. It is probably a strand of mucoprotein, a remnant of a cell exudate. $\times 8,000$.



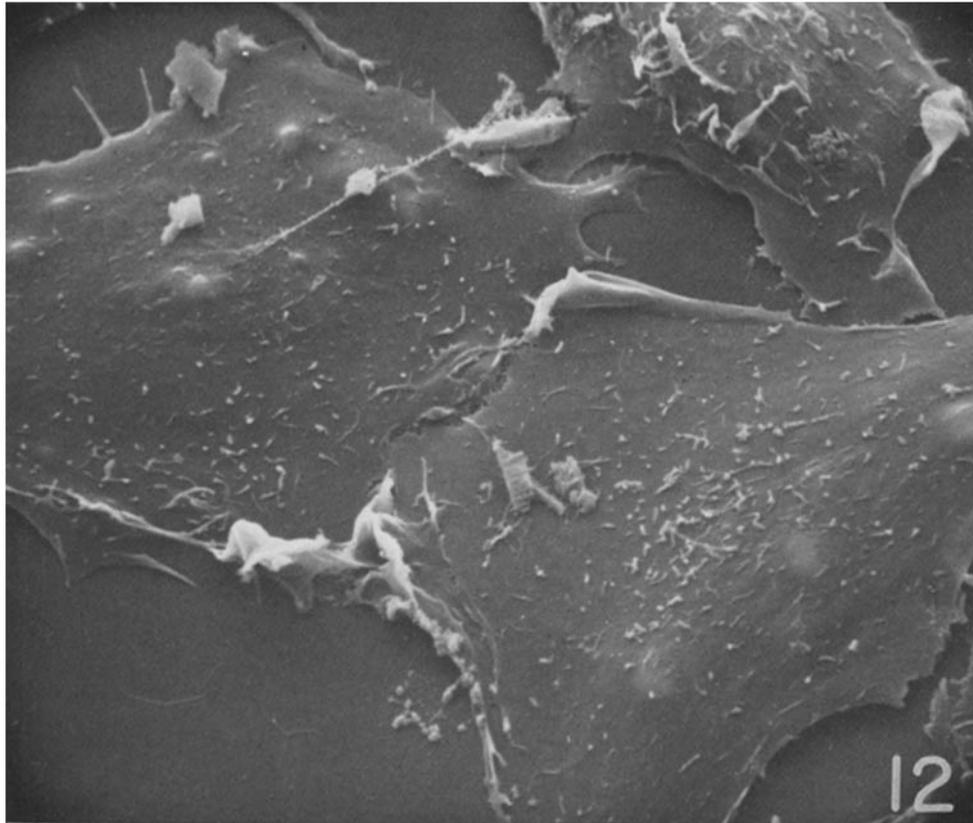


FIGURE 12 Two cells typical of the S phase. They were fixed 1 h after release from the second thymidine block. The surfaces are free of blebs and show only microvilli and ruffles. A few fragments of debris contaminate the surface. In the upper right cell, shown only in part, a few ruffles have moved toward the cell center. For the most part, however, these structures are associated with the edge of the cell. Some are actively forming adjacent to the zone of contact between the two central cells. Presumably an established junction between cells will not tolerate ruffle formation. $\times 2,600$.

structure from the transformed cells described here.

DISCUSSION AND CONCLUSIONS

These observations add one more dimension to the study of the progression of the cell through its life cycle. There are several distinct changes in surface morphology and form of the cell, and these changes correlate with the four major subsections of the cycle. G_1 begins with the cells still in a generally spherical form, and the cell surface is covered with many microvilli. By mid- G_1 the cell has flattened and its surface contains groups of blebs that may almost completely replace the microvilli over the central body of the cell. About the time of transition from G_1 to S

the blebs disappear from the cell and ruffles become more common. During S the surface still shows microvilli, but these are less numerous than in G_1 . In the most extremely flattened cells the microvilli are almost completely absent, and the cell surface is quite smooth. During G_2 the number of microvilli appears to increase along with marginal ruffling, and finally in late G_2 the long filopodia that dominate the surface of the mitotic cell begin to appear.

This succession of changes in surface morphology takes place in parallel with a succession of changes within the cell, but the causal connections between the two sets of changes are completely unknown. A main part of the difficulty is that the functional significance of the

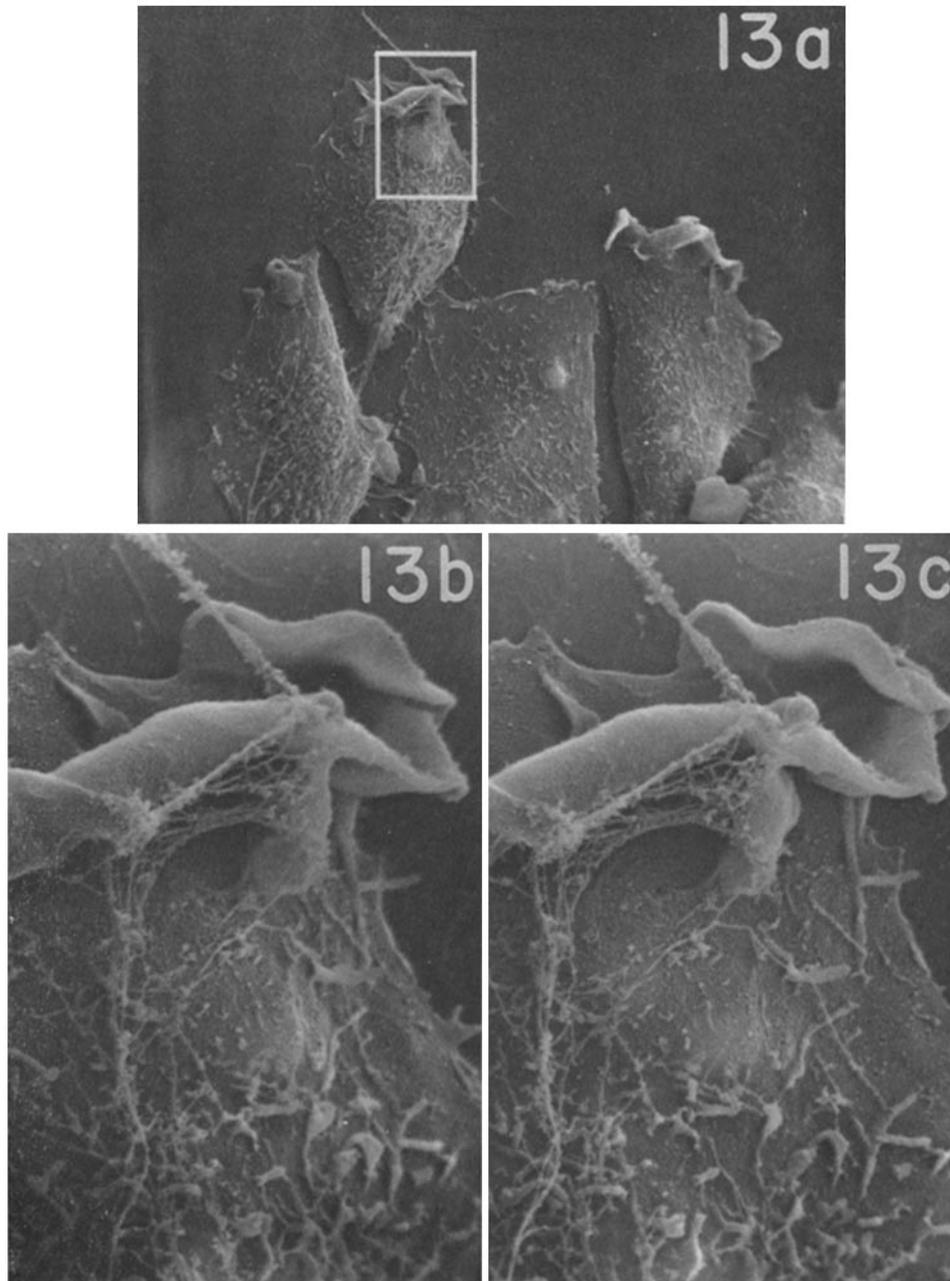


FIGURE 13 *a* The margin of a small colony of cells in late G_2 . The individual cell is covered with microvilli and in one instance a few blebs. Ruffles are especially prominent along the free edges of cells at the outside of the colony. A small amount of fibrous material, probably cell exudate, adheres to some of the cells. $\times 1,000$.

FIGURES 13 *b* and 13 *c* Stereo images of the leading edge of a cell in Fig. 13 *a* (area enclosed in rectangle). Stereo viewing makes it clear that three major lamellipodia comprise this ruffling. One (upper right) is still continuous with the edge of the cell and the other two have moved somewhat in from the edge. All lamellipodia show undulating forms presumably reflective of activity at the time of fixation. Remnants of cell exudate are evident on the cell surface and over the ruffles. $\times 5,500$.

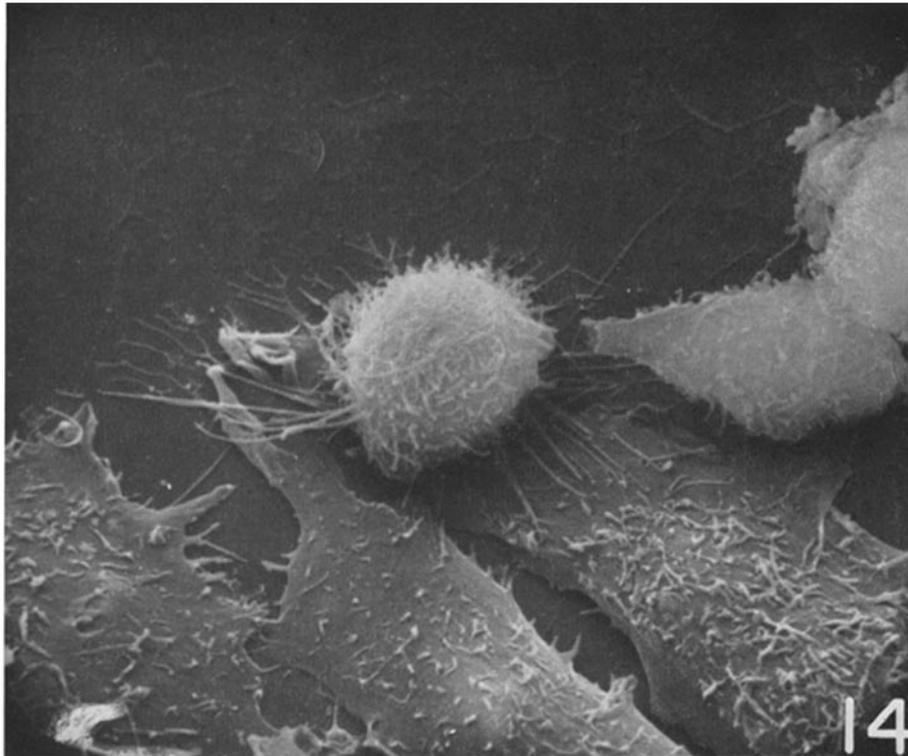


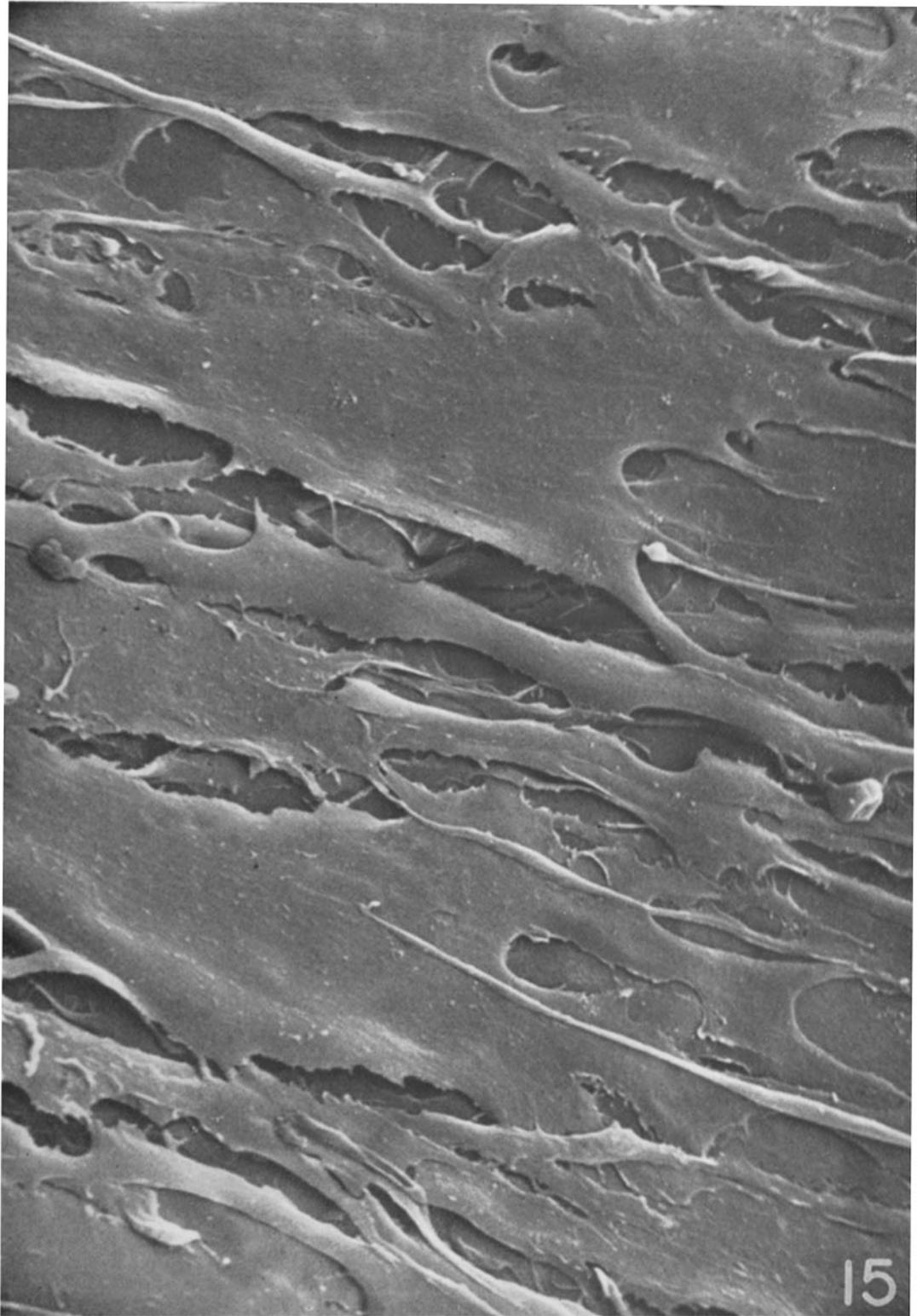
FIGURE 14 Cells fixed in late G_2 , 5 h after release from the first thymidine block. A few are in stages of mitosis, the central one probably in late prophase or even metaphase. It is attached to the substrate and adjacent cells by numerous filopodia which show aborizations at their tips. Other cells in the micrograph have separated from adjacent units preparatory for mitosis. Microvilli dominate their surface topology. $\times 2,600$.

microvilli and blebs and ruffles is not entirely understood. The observations, nevertheless, affirm that the cell surface is in some way involved with other changes occurring within the cell as it moves through its cycle.

These phenomena duplicate in large part the behavior of normal, nontransformed cells, to the extent that the details have been observed with the light microscope. As noted many times and recorded cinematographically (P. Weiss, 1972,

personal communication), normal tissue cells, grown under *in vitro* conditions regarded as optimal, round up during mitosis and attach to the substrate by a complex system of filopodia (as in Fig. 14). During the G_1 phase which follows, they reextend over the culture surface, and develop the several surface excrescences mentioned above, including blebs, microvilli, filopodia (or microspikes), and ruffles (or lamellipodia) (Abercrombie et al., 1970). Eventually

FIGURE 15 A population of cells from an explant of Chinese hamster lung. Mostly in monolayer, the cells have achieved confluency and presumably their morphology reflects some degree of contact inhibition. They are obviously thinly spread, are highly asymmetric (bipolar) in form, are essentially without microvilli, blebs, or ruffles. They show a surface topography that is typical of normal cells grown under these culture conditions. The picture is different only during mitosis and early G_1 when the surfaces are studded with microvilli and blebs. $\times 1,000$.



(and probably in late G₁ and S) the blebs and microvilli disappear and the cells become thinly spread and smooth (Follett and Goldman, 1970). Along margins of contact with other cells the ruffles also vanish (Abercrombie and Ambrose, 1958). Finally, in a growing population, the individual cells in late G₂ detach from adjacent cells and round up again for the next division. Where factors of population density which inhibit growth come into play, this flow of structural change is, of course, altered.

The differences between the transformed CHO cells and normal cells of an equivalent type are differences⁴ in the degree to which the cells spread and lose the various surface structures. In other words, CHO cells, though transformed (and not in the usual sense contact inhibited), do go through a sequence of phenotypic expressions which approach those shown by normal cells.

Two questions of special interest emerge from the above observations. Firstly, one should ask to what extent these changes in cell form and surface morphology are a reflection of contact between cells of the population; and secondly, one should inquire into the relation of the form changes to structural and biochemical phenomena within the cell.

The first of these questions is answered by the results reported in the succeeding paper by Rubin and Everhart. It appears from their study that if the population density is so low that contact between cells is not to any significant extent achieved, the cells continue to show blebs and retain a form characteristic of G₁ cells in a partially confluent culture. When this observation is juxtaposed with those presented here it becomes apparent that CHO cells are susceptible to contact influences, and within the limits of their prescribed capabilities go through structural

changes approaching those displayed by normal cells. This is not an effect of any media borne, diffusible substance; direct cell contact is apparently required as demonstrated also by Schutz and Mora (1968) for 3T3 mouse embryo fibroblasts.

What intracellular changes in morphology are associated with the overall form and surface changes is more difficult to determine. That oriented assemblies of microtubules are involved is suggested by a number of observations on the distribution of microtubules in cultured cells. These are consistent in showing that microtubules are prominent in major cell processes and are oriented parallel to the long axes of the extensions. Colchicine and Colcemid disassemble the microtubules, and the cell simultaneously adopts a polygonal (epithelial-like) form or rounds up as though in preparation for division (Taylor, 1966; Goldman, 1971; Vasiliev et al., 1970; Goldman and Follett, 1969; Freed and Lebowitz, 1970). It has also been observed that nerve cells in the presence of Colcemid fail to extend their processes (Daniels, 1972), from which the investigator concludes that microtubules are "essential to the formation of nerve fibers."

There is some suggestion that the microtubules, influential in the development of pseudopodia and bipolar asymmetry in cells, are focused on and possibly oriented and initiated by elements of the central apparatus (the centrioles and persisting aster). Hence, it is regarded as probable that one intracellular event associated with shape development after early G₁ is the assembly of microtubules in association with the centrioles and centriole-based complex of dense bodies. Their orientation in CHO cells is not bidirectional and may even be random. That they are there in substantial numbers has been determined in another study (Porter et al., 1973). When CHO cells are exposed to dibutyryl-cyclic AMP they acquire a pronounced bipolarity, a response which is inhibited by colchicine (Hsieh and Puck, 1971; Johnson et al., 1971). In these treated cells the microtubules are oriented approximately parallel to the long axis of the cells (Porter et al., 1973). It appears, therefore, that cAMP is effective in inducing assembly and also orientation of microtubules. Whether contact between CHO cells as observed in these studies contributes to a higher intracellular concentration of cAMP has not been shown, but in studies of 3T3 and other cell lines,

⁴ Cells from explants of hamster lung (Fig. 15) are like normal chick fibroblasts⁵ and normal rat fibroblasts⁶ in spreading out thinly during a middle period of interphase and for long periods in late G₁, where density-dependent inhibition blocks continued growth and reproduction.

⁵ Porter, K. R., and G. Weiss. 1973. Chick fibroblasts during collagen production. A scanning electron microscope study. In preparation.

⁶ Porter, K. R., and S. Bandecian. 1973. Comparative study by scanning microscopy of rat sarcoma 4337 and normal equivalent cells. In preparation.

Otten et al. (1971) have found evidence that the cAMP concentration does increase during contact inhibition. It is reasonable to assume therefore that some of the behavior of cells characteristic of contact inhibition is mediated through cAMP (Otten et al., 1971).

It has not been determined whether contact such as observed here between these CHO cells leads to the establishment of tight junctions or gap junctions (nexuses). However, such low resistant pathways (ionic couplings) as gap junctions have been observed between chick fibroblasts (and a number of cell lines) grown under in vitro conditions (Pinto da Silva and Gilula, 1972; Johnson and Sheridan, 1971). Conceivably then, the establishment of contact initiates a membrane propagated event (excitation) which spreads over the cells involved and activates the adenyl cyclase responsible for the synthesis of cAMP and secondarily the initiation of microtubule assembly. Such a possibility deserves investigation. It is interesting in this regard to note that in invasive carcinoma of the cervix where the control of cell form is lost, the number of "gap junctions" between the cells is greatly reduced (McNutt et al., 1971).

Selected Features of the Cell Surfaces

Though the surfaces of cultured cells have been imaged before by scanning microscopy (see e.g., Pugh-Humphreys and Sinclair, 1970; Boyde et al., 1972), the literature provides very little comment on the general occurrence and significance of the observed structures. There is, however, a rapidly developing body of data which relates to some of these structures, and it therefore seems appropriate to attempt an integration even though the scope of this paper is otherwise limited.

Microvilli

This name is generally reserved for small extensions of the cell surface having a uniform diameter of about $0.1 \mu\text{m}$ and variable lengths. They are of course well known on certain differentiated cells such as the adsorptive cells of the small intestine and the mesothelial cells lining the major body cavities. They are less characteristic of cells grown in vitro. Where these latter have been examined with some care (as e.g., by Follett and Goldman, 1970; and by Taylor, 1966), they have been noted to possess

consistently an axial bundle of 40–60 Å filaments. Microvilli in Taylor's terminology are "microspikes," but they are very similar to what have been identified as microvilli. His discovery of small microtubules (150 Å diameter) in microvilli has apparently not been confirmed.

As reported above, microvilli are prominent surface elements on CHO cells after mitosis. They remain numerous during G_1 but seem to diminish in prominence during S. Later, as the cells thicken in G_2 , the microvilli appear again in larger numbers per unit area of cell surface. Observations made by Follett and Goldman (1970) on BHK21/C13 cells over the cell cycle describe a similar fluctuation in numbers of microvilli. The stereo images (Figs. 11 *b* and 11 *c*) depict the microvilli as having uniform diameters, as being without branches, and as standing up erectly from the cell surface, at least in many instances. They seem frequently to emerge from a larger surface excrescence, but our observations to date are insufficient to permit any generalization on this point.

It is therefore a quality of these transformed CHO cells to show microvilli on their surfaces over most of the cell cycle, even when the cells are obviously confluent and presumably responding to some level of contact inhibition. This observation is made more interesting by the fact that normal, first generation cells cultured from explants of Chinese hamster lung tissue and grown to confluence (Fig. 15) are essentially devoid of microvilli except during mitosis. We are not able to decide at this point whether their presence on the transformed cell reflects a physiological difference (related to growth rate) or a constant phenotypic expression of the transformation. It would be valuable to test their response to such environmental variables as pH and glucose concentration.

Microvilli of similar dimensions (but frequently longer) are notably prominent on transformed cells besides CHO and especially on strains of malignant cells. Micrographs made with the scanning microscope are especially impressive in showing them on sarcoma 180 cells (Boyde et al., 1972), on Landschutz cells (Pugh-Humphreys and Sinclair, 1970), on HeLa cells (Porter and Fonte, 1973; see also Pugh-Humphreys and Sinclair, 1970), and on rat sarcoma 4337.⁵ The normal equivalent of the latter sarcoma cell is strikingly free of microvilli during interphase

periods of the cell cycle. It appears, on the other hand, that cells normally free of microvilli acquire them after transformation with an oncogenic virus (Boyde et al., 1972).

There may in fact be some relationship between the presence of microvilli and the agglutinability of cells as though, by providing a greater surface area or by possessing specific receptor sites, the microvilli facilitate the cell's association with agglutinins. In this connection, 3T3 cells, which *in vitro* show few microvilli during G₁, S, and G₂ and are then only weakly agglutinable, normally display a higher concentration of receptor sites for wheat germ agglutinin during mitosis (Burger, 1971) when their surfaces are covered with microvilli (Porter, unpublished observations). The same cells after transformation by polyoma virus show a marked increase in receptor sites during all phases of the cycle (Fox et al., 1971) as well as an increase in numbers of microvilli and other surface excrescences. The surfaces of BHK21 fibroblasts (normally smooth) develop microvilli and blebs after trypsinization (Follett and Goldman, 1970), a treatment which simultaneously "exposes" receptor sites for agglutinins. The occurrence of surface microvilli etc. coincides then with the agglutinability of cells by plant lectins in a number of instances and this leads one to wonder if agglutinability, so typical of cells which have escaped the growth control of contact inhibition, is not a reflection of relatively gross structural features of the cell surface rather than the induction or exposure of sites with specific chemical properties (see also O'Neill and Follett, 1970).

We have noted also that microvilli on these CHO cells serve to hold exudates (presumably glycoproteins) on the cell surface, exudates which might bind agglutinins and which are more readily removed from the relatively smooth surfaces of the normal, nontransformed cell.

Because microvilli are so small, they are difficult to observe with the light microscope, and observations on their behavior are neither numerous nor reliable. On HeLa cells the microvilli are especially long, and as Gey showed many years ago (1955), they can be seen in profile with good phase optics. He described them as surprisingly rigid extensions of the surface which moved about their points of attachment as though pivoted there. He further described their rapid emergence from the cell and their equally rapid

disappearance into the cell. They are, in other words, remarkably labile, dynamic structures. In the interval since Gey's observations these HeLa cell microvilli have been better observed by electron microscopy (Pugh-Humphreys and Sinclair, 1970; Porter and Fonte, 1973). They are indeed remarkable in being very long, but in other respects (diameter, etc.) they seem not to differ from other microvilli.

Any literature review will turn up a number of suggestions regarding the function of microvilli. Follett and Goldman (1970), for example, have proposed that the surface membrane is conserved in the form of microvilli to be available for dramatic changes in apparent surface area as when the cell becomes thinly spread. In specialized (differentiated) cell surfaces such as the brush border of intestinal adsorptive cells, microvilli are thought to possess "uphill pumps" for the active transport of sugars. The existence of a similar device on the microvilli of cultured cells has not been demonstrated. Chick fibroblasts transformed by Rous sarcoma virus are, however, known to transport glucose at a higher rate (Hatanaka and Hanafusa, 1970) and these transformed cells have been shown to have many microvilli (Boyde et al., 1972). Furthermore, there is some indication in HeLa cells that surface microvilli fluctuate in number directly with glucose concentration in the medium (Willoch, 1967). So there is a possibility that microvilli are formed to facilitate the diffusion of glucose across the surface of rapidly growing cells. If this is the case, the reduction in number of microvilli during the S phase of CHO cells might imply that sugar (and other diffusible metabolites) uptake is least active at this time, provided of course the surface area is not kept constant by the spreading of the cell which occurs during that period. The alternative suggestion that microvilli are a device the cell has to keep its surface area constant while it goes through several form changes is just as valid.

Blebs

These bulbous excrescences of the cell surface, which are observed to come and go on cells during mitosis and cytokinesis, are familiar to most observers of dividing cells. Less familiar, perhaps, is their presence on cells during interphase. Nevertheless, blebs of a similar nature

have been described on several kinds of cells under a variety of experimental conditions. Costero and Pomerat (1951), for example, observed them along the dendrites of nerve cells grown in tissue culture. Their behavior reminded the authors of boiling of a very viscous fluid, hence the term zeiosis for the phenomena of bleb formation and retraction.

Though such blebs may not all belong to one category of surface structure, it seems very probable that they do. Certainly the observation on the blebs in CHO cells published by Hsie et al. (1971) and by Puck et al. (1972) repeats so closely those of Costero and Pomerat (1951) and of Price (1967) that there is little excuse to regard them as separate structures.

In these scanning images, the blebs dominate the cell surface during early G_1 and persist in a small percentage of cells on into S. Mostly, however, they vanish during the latter half of the cycle. Their shape is spherical when viewed from the top, and from the side they appear to be supported by a stout stalk which has a diameter only slightly less than the bulb above. They obviously vary greatly in size, and we assume that some of the smaller ones represent early stages in the formation of the larger; conceivably also they may be stages in the subsidence of larger ones. There was no clear indication in our preparations that these zeiotic blebs are released from the cell. Only in the earliest preparations (1 h after shake-off) did we recognize anything that could represent a loose bleb, and if cell derived, these may have been released during anaphase or telophase.

These observations on blebs have not extended beyond the recognition of their presence or absence in various phases of the cell cycle. Apparently in cells which are isolated, as in low density cultures (Rubin and Everhart, 1973), they persist much longer, if not through the entire interphase. Their disappearance in S and G_2 in more densely populated cultures would seem to be one result of cell contact. Nothing in our study suggests what they do or why they form. However, transmission microscopy of thin sections of them on some human epithelial cells shows them to contain large numbers of polyribosomes (Price, 1967). It appears that cytoplasmic components other than ribosomes are excluded. Observations by one of us on the CHO cell blebs, incidental

to another study, make it clear that here too they contain an impressive concentration of ribosomes (Porter et al., 1973). That they are observed on normal cells especially during the earliest phases of G_1 should not be overlooked, but their extended and exaggerated presence on the CHO cells is a unique feature of these transformed cells and one deserving further investigation.⁷

Ruffles

As already noted, these extraordinary structures of the cell surface are more prominent in some phases of the cycle than in others. They are not found on cells in mitosis and do not become obvious until late G_1 . Then, during S and G_2 , they seem to increase in number and probably activity. It is also evident from this study that ruffles are preferentially associated with the free edges of cells. This agrees with light microscope observations that they in fact originate at the advancing edges of cells (Abercrombie et al., 1970; Ingram, 1969). Conversely, where the edge of a cell is confluent with that of another, there are generally no ruffles.

The significance of these observations, especially the increase in ruffling in G_2 , is not immediately evident. One might assume that the cell is in greatest need of metabolites for growth at this time and is using all devices to increase the area of contact with the environment including internalization of the medium by pinocytosis. To the extent that diffusion across the cell surface is facilitated by the provision of extra surface, the requirements of the cell are satisfied. There is probably more involved, however, than this simple statement suggests.

The development of ruffles obviously create fairly large areas of membrane. It is easy in stereo images (Figs. 13 *b* and 13 *c*) to see that typical lamellipodia are about $0.1 \mu\text{m}$ thick, a value which agrees with other measurements

⁷ According to recent observations (Harris, A. K., 1972, personal communication) on cells in vitro, blebs develop initially at the cell margin and migrate centripetally (back from the edge) like ruffles. This and their recession into the cell surface with time may account for the accumulation of large numbers of relatively small blebs over the centers of many of the G_1 cells observed here (see Figs. 4, 10 *a*, and 11 *a*).

made on thin sections of ruffles (Abercrombie et al., 1971). Their vertical dimension is highly variable but is of the order of 6–8 μm , and they can extend along the cell margin for 8 or 10 μm . A single ruffle may therefore have a surface area of ca. 100 μm^2 . Since one cell may possess several ruffles they can comprise a substantial fraction of the total cell surface at any one time. Much more accurate measurements could be made from scanning electron microscope images in a study devoted primarily to these structures and their function.

The rate at which lamellipodia form is impressive. Observers of this phenomenon agree that 2–3 min is all it takes for the cell to produce one of these structures, i.e., before it begins its migration back from the free edge (see footnote 7) (Gey, 1955; Ingram, 1969). Abercrombie and co-workers (1970) describe this growth as involving membrane assembly rather than membrane flow. They provide evidence in fact that membrane flow is away from the advancing edge rather than toward it. Just how membrane subunits are fed into the area for assembly becomes a problem. Transmission microscopy of sections of ruffles and the associated leading edge of the cell, done also by Abercrombie and his colleagues (1971), provides little or no clue to the origin of all this membrane. The Golgi complex, demonstrably the source of specialized cell surfaces in differentiated cells, is not represented. Their micrographs show instead a few small vesicles at the base of the ruffle but little else. Perhaps the phenomena involved are comparable to those seen in the rapid elongation of pollen tubes where numerous vesicles are fed into the region of the growing surface (Rosen, 1968). Apart from these vesicles, which conceivably fuse with and contribute to the surface, the only source of membrane subunits would seem to be the cytoplasmic ground substance that floods into the leading edge as the cell extends its margins. A certain amount of this ground substance, which is described as “granular” and as showing “vague and irregular filaments” (Abercrombie et al., 1971; Wagner et al., 1971), is retained between the plasma membranes of the ruffle and is carried back with the lamellipodia to a point where the structure fades into the cell surface again. Possibly membrane components are also returned to the cytoplasm to be fed again (recycled) into the production of more lamellipodia.

Scanning Microscopy

Scanning microscopy of cells and soft tissues has not as yet created an important literature. The major reason is that only very recently have a few laboratories developed adequate preparation procedures and applied them to interesting problems. A study of the lactating mammary gland by Nemanic and Pitelka (1971) illustrates well the values of the approach for soft tissues, and some pioneering work by Boyde and colleagues (1972) has demonstrated its potential for microscopy of cultured cells. One can easily agree with their conclusion that for getting an overall view of the topography of cultured cells quickly and easily there is no equivalent procedure.

Critical-point drying of the specimen is the most significant step in obtaining a relatively undistorted surface (of cell or tissue) for examination. This important procedure, introduced to electron microscopy by T. F. Anderson (1951), is simple to do and requires only a small amount of equipment. In our experience, it is better for most purposes than drying from the frozen state (Porter et al., 1972). This latter procedure is needed only when one wants to avoid fixation with glutaraldehyde or some other reagent. Among the several compounds that may be used for critical-point drying (Freon, N_2O , CO_2), none seems superior to liquid CO_2 . It is certainly the least expensive and least toxic.

In the work reported here and by Rubin and Everhart (1973) attention has been placed on changes in CHO form and surfaces with phases of the cell cycle. The cells were grown under conditions which can of course be varied experimentally and which would doubtless have effects that would be reflected in surface changes. Thus one of the more valuable applications of scanning microscopy will be that of following the response of cells and especially surfaces to environmental factors. But the possibilities do not end there by any means. Features of the surface, apart from microvilli etc., such as the antigenic sites, may require the development of special procedures for their identification, but the prospects are good. It will in some instances be necessary, for correlating internal structure with the surface features, to resort to transmission microscopy of thin sections, but another distinct possibility is to examine whole cells first in a high (1 MeV) voltage microscope, where high energy

electrons should give adequate penetration, and thereafter with the scanning electron microscope for surface characteristics.

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