# THE LOCALIZATION BY ELECTRON MICROSCOPY OF HeLa CELL SURFACE ENZYMES SPLITTING ADENOSINE TRIPHOSPHATE

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## ABSTRACT

Cultures of normally proliferating Hela cells have been examined in thin sections by electron microscopy following glutaraldehyde fixation, staining in Wachstein and Meisel's adenosine triphosphate containing medium, postosmication, and embedding in an epoxy resin. The cells were stained in suspension in order to ensure uniform accessibility to reagents. Discrete localization of the enzyme reaction product (lead phosphate) was found at the plasma membranes of about half the cells, but nowhere else. It appeared in the form of an intensely electron-opaque deposit lying close against the outer surface of the cells and varying in amount from a chain of small particles to a dense band about 30 m$\mu$ in width. This opaque reaction product was present over microvilli when absent elsewhere on a cell, was heaviest where microvilli and processes were profuse, and was minimal or lacking where cell surfaces were smooth. These observations are discussed in relation to both the idea that surface enzyme activity varies with the physiological phase of individual cells in a population, and the problem of how such enzyme activity becomes manifest at a given site on a morphologically changing membrane system.

## INTRODUCTION

During the last decade, biochemical observations have led to the suggestion that nucleosidephosphatases such as adenosine triphosphatase (ATPase) are involved in processes of active transport across the plasma membrane of cells. Cytochemical staining methods for locating these enzymes have provided direct evidence for their presence in the plasma membrane of numerous cell types showing specialized features, such as the formation of pinocytotic vesicles, or surface activity characterized by microvilli or infoldings, as well as in a variety of intracellular membranes. These findings are consistent with the view that the enzymes provide the energy requirements for various forms of cell surface activity (1, 2) as well as mediating the transport and exchange of ions and metabolites across those membranes which provide large dynamic surfaces for such purposes. A comprehensive review of this latter membrane activity has recently appeared (3) and includes discussions of both the functions of membrane enzymes and their substrate specificity from the viewpoint of cytochemical staining.

Now HeLa cells (4, 5), in common with many other undifferentiated cells, show considerable evidence *in vitro* of surface activity, for their plasma membranes are often folded and organized into microvilli (6, 7), chains of pinocytotic vesicles extend from the surface into the peripheral cytoplasm (7), and both colloidal gold particles (8)

and minute crystals (7, 9) are readily engulfed and ingested. It is thus not surprising that light microscopy staining methods have shown that an enzyme hydrolysing adenosine triphosphate (ATP) is associated with the cell membrane of HeLa cells, especially where there are fine folds and processes (10).

It was therefore considered of interest to extend these earlier observations to the fine structural level, particularly since the cell membrane is known to play an important part in the maturation and release of particles by budding in HeLa cells infected with herpes virus (11), a function shared with the membranes of smooth intracytoplasmic vacuoles apparently belonging to the endoplasmic reticulum (11). Findings regarding the presence or absence of ATP-splitting enzymes in these two types of membrane could have considerable significance in relation to the budding mechanism if the cytochemical reaction product were to provide a distinguishing marker for the different membranes involved (12).

A staining method for this purpose was established in which ordinary HeLa cells of well defined fine structure (7) from normal cultures were fixed with glutaraldehyde and prepared for electron microscopy after staining in Wachstein and Meisel's ATP-containing medium (13) slightly modified as described by Pearse (14). The localization of the electron-opaque enzyme reaction product was studied in thin sections with the electron microscope, and the present communication reports the results of the observations made on this normal material and on appropriate control preparations. The findings of similar investigations on herpes-infected HeLa cells are presented in the following paper (15).

## MATERIALS AND METHODS

### *Chemicals*

Glutaraldehyde was obtained as a 25 per cent aqueous solution from L. Light and Co., Poyle, Bucks, England.

Crystalline disodium ATP was supplied by the Sigma Chemical Co., St. Louis, Missouri.

All other chemicals were of analytical reagent grade.

### *Maintenance and Collection of HeLa Cells*

HeLa cells were cultured on glass in growth medium by methods already described (16, 17). When the cell sheets became confluent, the medium was removed, except for 2 ml in which the cells were collected in suspension by shaking the culture bottles.

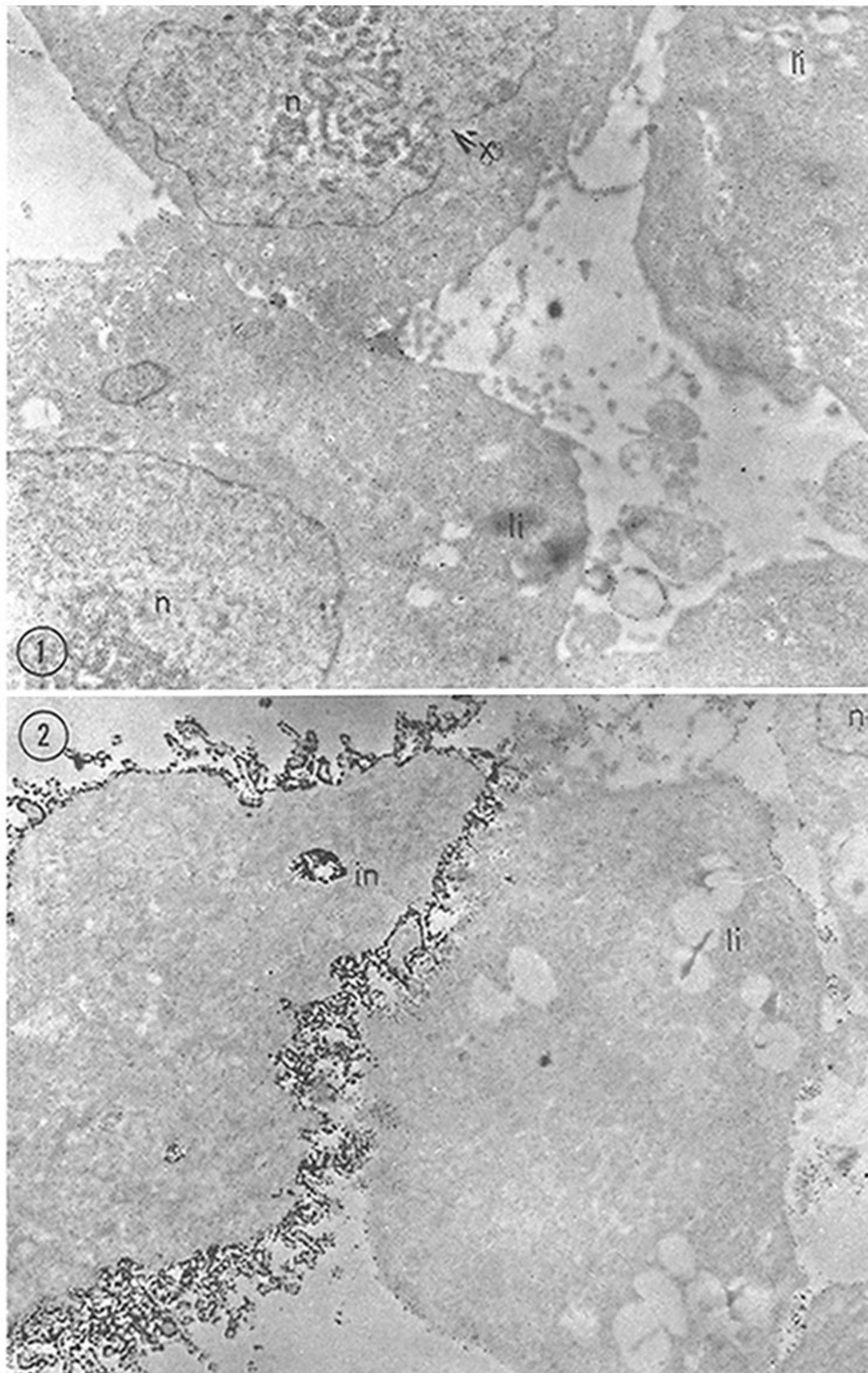
### *Fixation and Cytochemical Staining*

Preliminary light and electron microscopic investigations were made to establish optimal fixation and staining conditions, using, respectively, cells grown on coverslips or shaken into suspension. The cells were fixed at 0°C for up to 30 minutes in phosphate-buffered formol–sucrose (18) or in 5 per cent glutaraldehyde (19) in 0.067 M cacodylate buffer (20) at pH 7.2, and were then stained in the modified Wachstein-Meisel medium (14) at room temperature for various periods. Under comparable conditions, the glutaraldehyde-fixed cells consistently showed more intense staining of cell membranes and the following procedure was finally adopted:—

All the figures are electron micrographs of thin sections of glutaraldehyde-fixed HeLa cells which were stained for the presence of surface ATP-splitting enzymes or exposed to control procedures, and then postfixed in osmium tetroxide.

FIGURE 1 Survey picture of parts of four cells from a control preparation. Uranyl acetate counterstaining was not applied and little cytoplasmic detail can be distinguished apart from distorted lipid bodies (*li*) and ingested crystals (7) at *x*. The over-all contrast of the cells is low, but the nuclei (*n*) of those on the left of the field are evident. There are no deposits of electron-opaque material. × 11,000.

FIGURE 2 Survey picture of parts of four cells stained for ATPase-like activity, but with no counterstain. The low contrast resembles that of the control preparation shown in Fig. 1, and only distorted lipid bodies (*li*) and a nucleus (*n*) can be recognized within the cells. The cell on the left is covered by a profusion of microvilli and is surrounded by an intensely electron-opaque band of enzyme reaction product (lead phosphate) which follows the plasmalemma with close precision; an invagination of this stained surface has been cut across at *in*. The surfaces of the other three cells are smooth and devoid of dense material. × 11,000.


n
x
li
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2 ml of cell suspension was drawn into a syringe warmed to 37°C and squirted (21) into 2 ml of the iced glutaraldehyde fixative. The cells were at once deposited from the fluid mixture by centrifuging in an horizontal centrifuge for 1 minute at 100 *g*, the fluid was poured off, fresh iced fixative substituted, and the lightly packed cells resuspended in it. Fixation in suspension was continued for 30 minutes and all subsequent changes of reagents were made by depositing the cells, decanting the fluid, replacing it, and resuspending in new fluid.

In this way the cells were washed twice for 2 to 3 minutes in 5 ml of ice cold 0.25 M sucrose buffered at pH 7.2 with 0.1 M cacodylate buffer, exposed for 10 minutes at room temperature to 5 ml of the staining medium, washed twice for 2 to 3 minutes in 5 ml of 0.25 M sucrose buffered at pH 7.2 with 0.1 M Tris buffer, and finally resuspended in 2 ml of this last fluid at 0°C. In each case, control experiments to determine the extent of non-specific adsorption of lead were made by repeating the above procedure, except that the ATP was omitted from the staining solution.

### *Preparation of Cells for Electron Microscopy*

Cytochemically stained or control cells in suspension in Tris buffer were drawn into a syringe, squirted into phosphate-buffered osmium tetroxide with glucose (22), pelleted while osmicating, and the pellets were cut into 1 mm cubes in 50 per cent alcohol excatly as in earlier work (7, 21). The glutaraldehyde showed a further advantage over formaldehyde at this stage, in that it gave better cohesion of cells during pelleting by centrifugation and imparted a brownish colour to them, making them easier to see and handle than those of the paler formalin-fixed preparations.

The 1 mm cubes of pelleted cells were dehydrated in a series of graded alcohols and were embedded in Epikote 812 (of Shell Chemical Company Ltd., London, and stated to be identical with American-made Epon 812) essentially as described by Luft (23) but using 2 per cent benzyldimethylamine as the accelerator. Sections were cut with glass knives on a Porter Blum microtome and were mounted on carbon-coated grids (24) for examination in a Siemens Elmiskop I electron microscope. Material from each batch of cytochemically stained or control cells was examined both directly and after counterstaining in the section for 30 minutes at 37°C on the surface of a fresh saturated solution of uranyl acetate (25) in 50 per cent alcohol.

## OBSERVATIONS

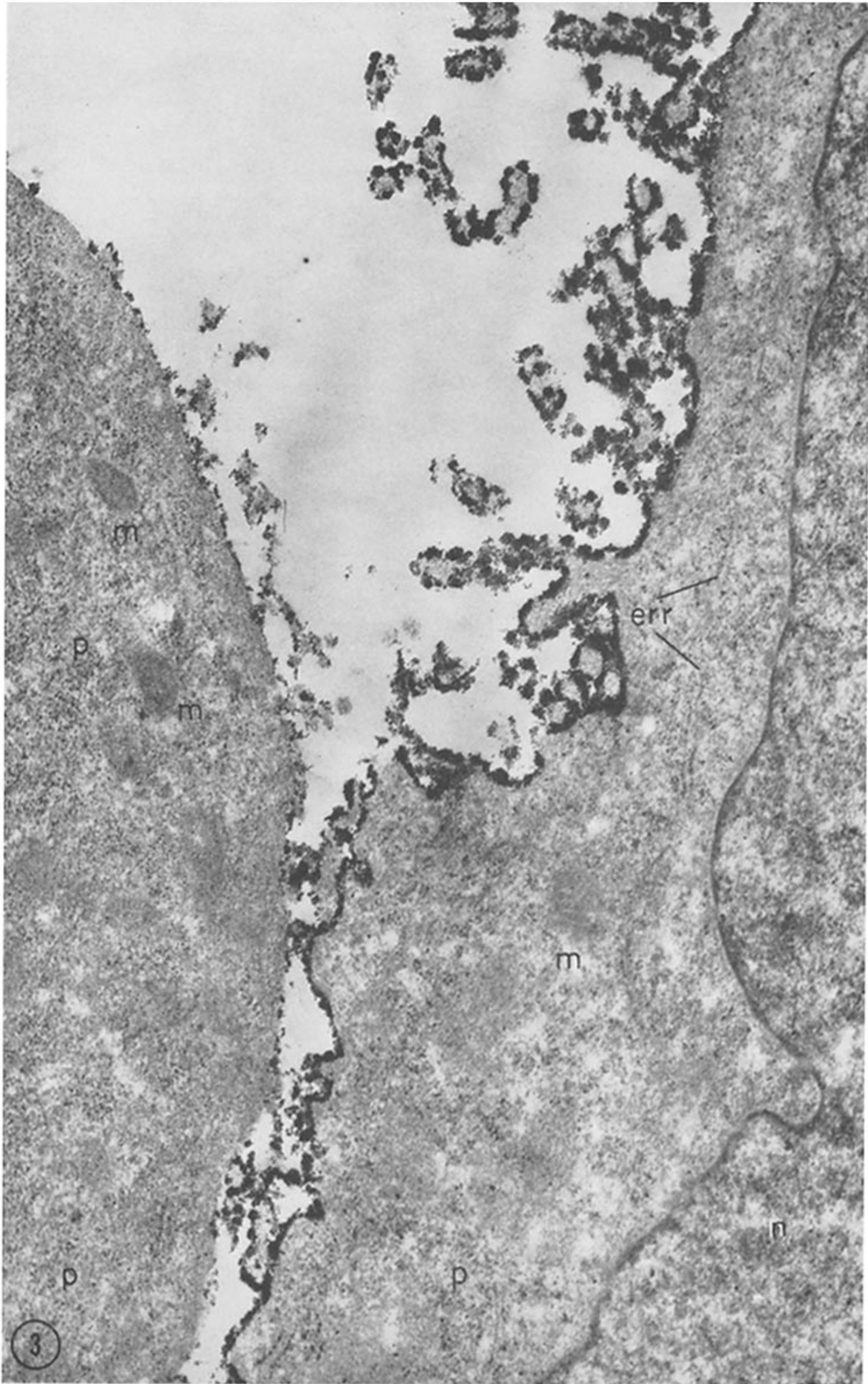
### *Without Uranyl Acetate Counter-staining*

The general shape and the nuclei of cells in the control preparations could be distinguished without counterstaining, but the density of these cells did not differ greatly from that of the surrounding embedding resin, and cytoplasmic detail was not evident (Fig. 1) apart from distorted lipid bodies. Although the cells exposed to the ATP-containing medium presented a morphological appearance exactly similar to that of control cells, a proportion of them also possessed a surrounding band of intensely electron-opaque reaction product (lead phosphate). This lay against the outer surface of the plasma membrane and followed it with close precision, even over folds, microvilli, and invaginations (Fig. 2). In rare instances, cells with the dense external deposit were found to have exactly similar material located on the inner aspect of the membrane limiting larger intracytoplasmic spaces (Fig. 2). Opaque material of this sort was never observed at any other sites, nor in any of the control preparations (Figs. 1 to 5), although a very fine precipitate, presumably containing lead, could be seen in many cells. This tended to be associated with nuclear structures (Figs. 1 and 2) and, since the effect was comparable in both control and cytochemically stained preparations, it is regarded as a non-specific direct action of the lead-containing medium on the tissue.

### *After Uranyl Acetate Counter-Staining*

Counterstaining of sectioned material with uranyl acetate did not influence the results of cytochemical staining in any way (Figs. 3 to 5), but did serve to make many features of cell struc-

FIGURE 3 Portions of two cells stained for ATPase and counterstained with uranyl acetate. The cytoplasm appears dense, is filled with large numbers of free particles as at *p*, and both mitochondria (*m*) and rough endoplasmic reticulum (*err*) can be seen. Part of the nucleus (*n*) of the cell on the right is included in the field, and the double nature of its envelope is clear. The surface of this cell is thrown up into numerous microvilli and covered with a band of opaque enzyme reaction product; in contrast, the smooth plasmalemma of the other cell carries only a few tiny dense particles. × 30,000.


m
p
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err
m
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3

ture visible (Figs. 3 to 5). Despite the cytochemical staining procedures to which the cells were submitted, fine structural detail was quite well preserved although there was a tendency in some cases for cytoplasmic membranes to be indistinct (Fig. 4). However, mitochondria (Fig. 3), smooth vacuoles, endoplasmic reticulum (Figs. 3 and 4), cytoplasmic particles (Figs. 3 and 4) and nuclear envelope (Figs. 3 and 4) could be seen, after counterstaining, in the cells of both cytochemically stained and parallel control preparations.

Of the cells incubated in the full staining medium, about half in a given section were surrounded by the electron-opaque material. Such cells were indistinguishable from neighbouring cells without the material, except that their surface membranes were thrown up into processes and microvilli (Figs. 2 to 5) and they appeared to be randomly distributed. Cells with an opaque band around them could be found both immediately beside other similar cells, or beside those without such a band (Figs. 2 and 3), and in some cases an individual cell showed the material on one surface but not on another (Fig. 4). The amount of material present was also found to vary from cell to cell, ranging from a chain of tiny electron-opaque particles (Fig. 3) to a zone up to about 30 m$\mu$ in width (Figs. 3 to 5). Although the wider bands appeared continuous at low magnifications (Fig. 3), examination at higher powers revealed that they were composed of fairly discrete lumps and segments (Fig. 5). The heaviest deposits were always associated with areas of cell surface covered by processes and dense microvilli (Figs. 2 to 5), whilst cells having a smooth surface were either free of opaque material or carried only minimal amounts (Figs. 2 to 4). In some cases poorly defined microvilli showed little evidence of the reaction product.

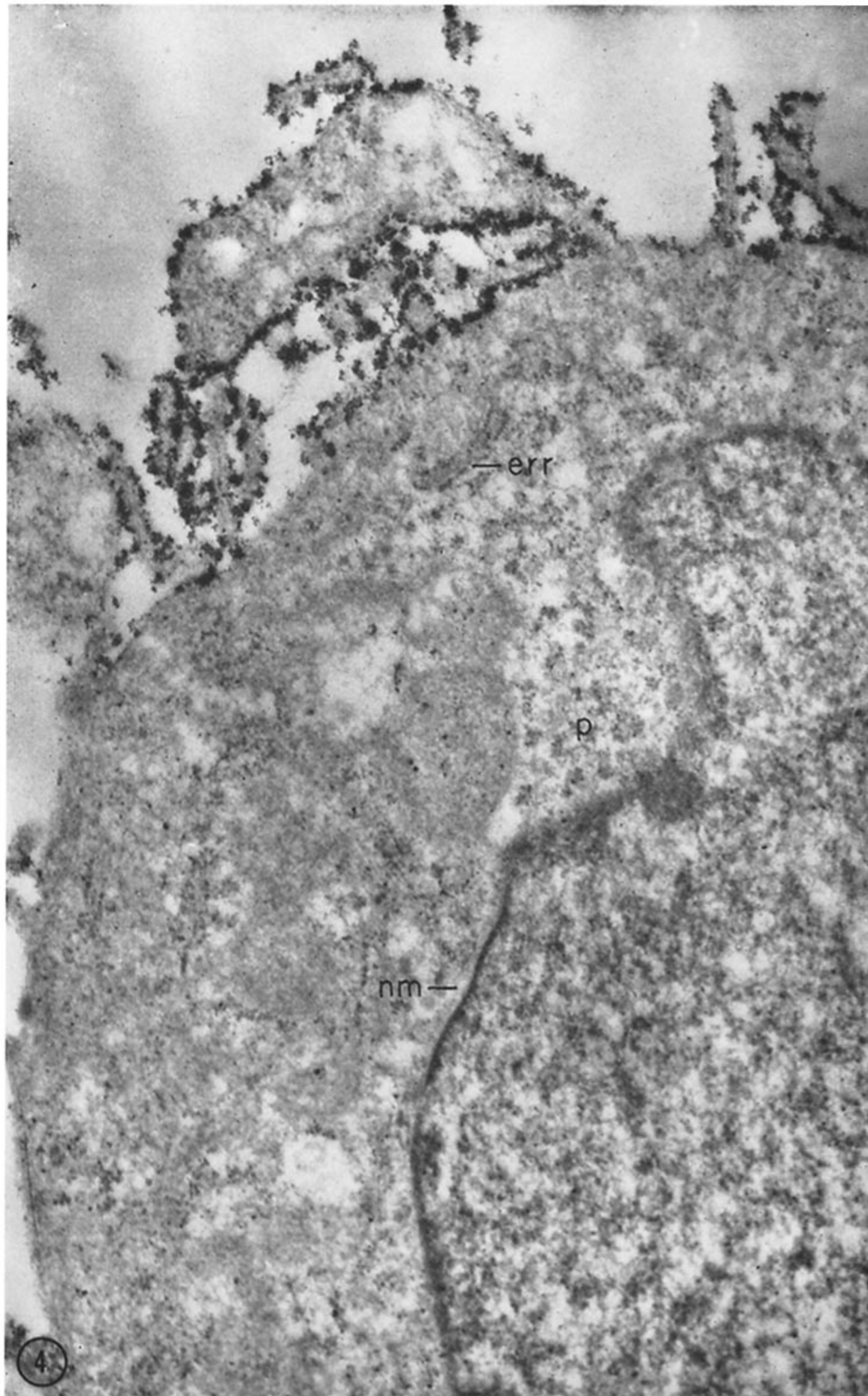
## DISCUSSION

Glutaraldehyde fixation followed by the enzyme staining procedures and postosmication achieved relatively good preservation of the HeLa cells (Figs. 2 to 4), although of not quite the same standard as that obtained with osmium tetroxide alone (7); only lipid bodies showed considerable distortion (Figs. 1 and 2). On the other hand, the failure of glutaraldehyde and osmium tetroxide to show cytoplasmic membranes satisfactorily in all instances (Fig. 4) is not thought to be due to the enzyme staining procedure but to be an intrinsic effect of the fixation, since other similar examples have been encountered both in this laboratory and elsewhere (26).

The distribution pattern of the electron-opaque enzyme reaction product found associated with the HeLa cells is considered significant for several reasons. It was completely absent in the control preparations, it was precisely localized at a particular site, the cell membrane, and it was never found at any other site; the occasional cytoplasmic spaces having reaction product within and against their limiting membranes (Fig. 2) clearly differed from smooth-surfaced vacuoles and would seem to be invaginations of cell membrane sectioned in such a plane as not to include their connections with the cell surface.

The question of the enzymic specificity of the reaction leading to the deposition of lead phosphate in the present case cannot, of course, be answered by using only ATP as substrate, for it has been demonstrated that this compound may be hydrolysed in aldehyde-fixed tissues under cytochemical staining conditions by combinations of specific ATPase with enzymes like adenosine diphosphatase or by less specific nucleoside phosphatases such as apyrase (27, 3). However, the intimate association of the reaction product with what appears to be one type of membrane whether at the cell surface or deeply invaginated into the cytoplasm (Fig. 2) is strongly suggestive of a topographical specificity of whatever enzymes are concerned. If alternative sites were responsible for hydrolysis of the substrate, it is unlikely that lead phosphate would migrate exclusively to within 30 m$\mu$ or less of the membrane and that staining would be confined to the plasmalemma alone.

FIGURE 4 Part of a cell treated with ATP medium; uranyl acetate was applied to the section. Cytoplasmic particles (*p*) are evident, and a rough cisterna (*err*) and part of the outer nuclear envelope (*nm*) can just be recognized. ATPase activity is restricted to that part of the cell membrane which is thrown up into microvilli and a large cytoplasmic process. The smooth regions of the surface, on the left, are free of reaction product. × 41,000.


err
p
nm
4

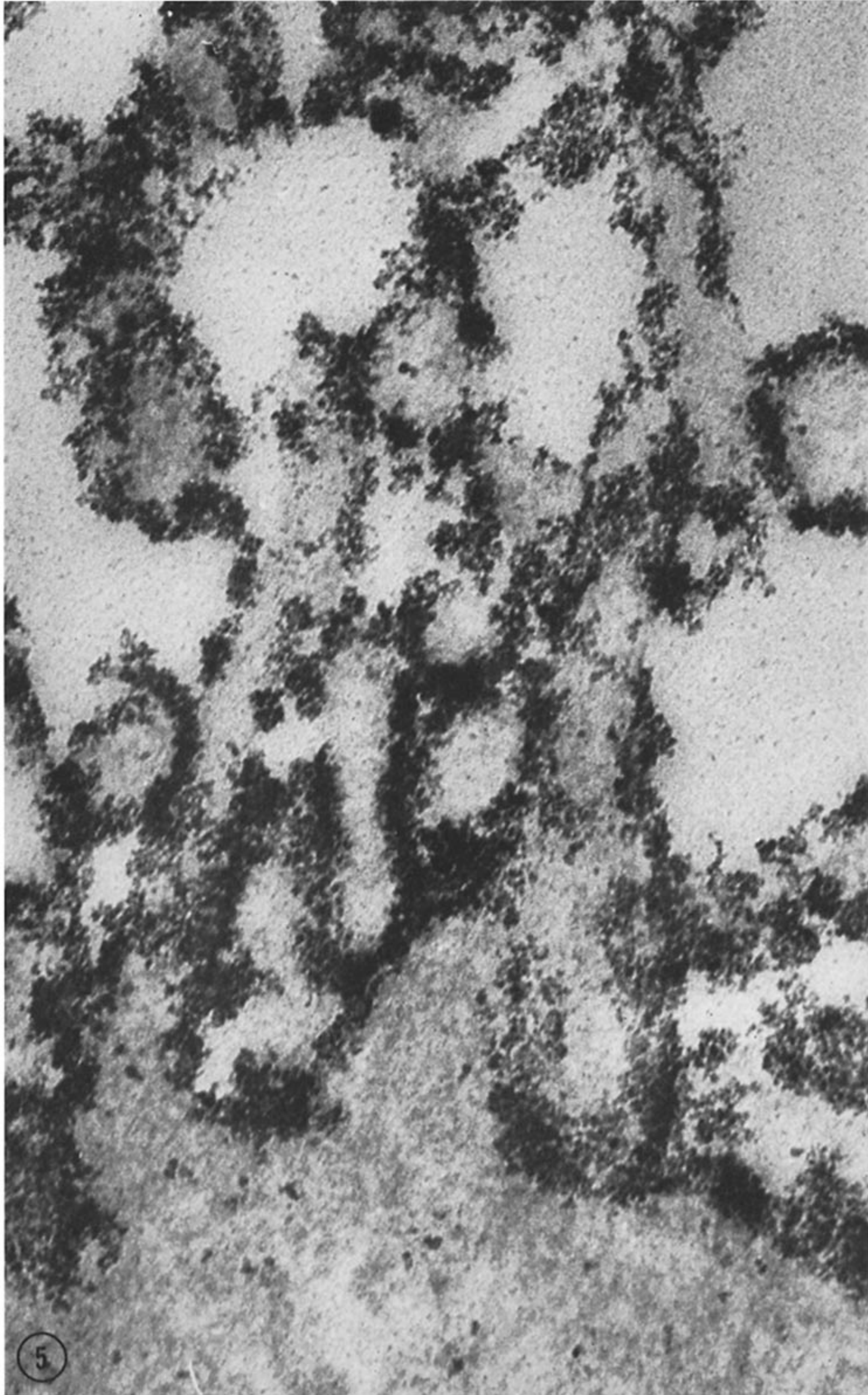
It is, of course, possible to put forward various more complex hypotheses for the localization found, for example, that there are active intracellular phosphatases, and that the plasma membrane is permeable to ATP, but not to lead ions. A surface deposit of lead phosphate might very well be formed under these conditions. It is true that in cytochemical staining procedures, complex factors govern the penetration of reagents through the sample and can affect the accuracy with which the enzyme reaction product is deposited at a given cellular site (29, 30). However, although permeability barriers to certain components of phosphatase-staining media are encountered in intact blocks of fixed tissue and can cause staining artefacts, these have been shown to be absent in tissue preparations less than 60 $\mu$ thick, and in any case are not due to barriers to lead ions, passive diffusion of which appears to be rapid and unrestricted (30). In the present work, fixed cells were treated in suspension and therefore were all equally accessible to reagents up to the stage of pelleting during postosmication. Since the staining procedure was applied to all cells under these same ideal conditions for enzyme localization, the variation in distribution of reaction product noted among individual cells, such as its absence from some, and its restriction to certain regions on the surface of others (Fig. 4), is considered to reflect real differences in enzyme distribution. This, in turn, must spring from differences in physiological activity among the various cells in the cultures. The alternative explanation, *i.e.* that reaction product might have been removed differentially from cells after staining, during dehydration and embedding, is considered less likely, for throughout these processes the cells were in a constant relationship with their neighbours, within pellets, and removal effects would not have affected individual cells, but rather groups in smaller or larger areas.

Since the cultures must clearly have been made up of individual cells in all stages of growth and proliferation, the present experiments provide strong support for the view that the activity of the membrane enzymes is necessary in HeLa cells only for certain definitive aspects of surface membrane function, since opaque enzyme reaction product has been found to be present over microvilli when absent elsewhere on a cell (Fig. 4), to be heaviest where microvilli and cell processes were profuse (Figs. 2, 3, and 5), and to be minimal or lacking where cell surfaces were smooth (Figs. 2 to 4); the occasional finding of poorly defined microvilli with little or no associated reaction product might reflect stages in the formation or resolution of fully functional structures of this type. All these observations suggest that surface enzyme activity waxes and falls away during physiological phases, in parallel with clear-cut morphological changes, and recent studies on the situation in HeLa cells infected with herpes virus (15) have produced evidence that such enzymic variations actually occur in individual cells as their functional state alters over a period of time.

The non-uniform distribution of nucleoside phosphatases throughout the plasma membranes of single cell types revealed by cytochemical staining methods is not restricted to the present case. Differences have been reported in the staining responses of the surface membranes of bile canaliculi and sinusoids in liver (28), between smooth and convoluted areas of the cell membrane and from cell to cell in rat hepatoma (31), and in several similar examples (3), all of which indicate an intimate relationship between cell surface specialization and biochemical function. It is not surprising, therefore, that HeLa cells in tissue culture at all stages of growth, activity and nutrition, and free to adopt an unlimited variety of surface configurations, show the marked heterogeneity in surface enzymic activity reported here.

It thus seems that ATPase or related enzymes probably play a role only during a limited phase of cell membrane function, and if this is so, it has considerable significance in a wider aspect of cell activity. The concepts of membrane flow and of the continuity of cell membrane, endoplasmic reticulum, and nuclear envelope (32–35) imply that although these elements are interconnected morphologically and are perhaps constantly interchanging components, they represent clearly demarcated and distinct functional zones. This is

FIGURE 5 Detail of cell surface after both cytochemical and counterstaining. Profuse filamentous microvilli are present together with a heavy deposit of enzyme reaction product lying against the outer aspect of the cell membrane; the dense material forms a band up to about 30 m$\mu$ in width, which can be seen, at this magnification, to be composed of a series of lumps and segments. × 120,000.



supported by the present finding that ATPase activity is sharply restricted in HeLa cells to a single element (Figs. 2, 3, and 5) or even part of it (Fig. 4), and by several other similar cases (3), but at the same time the formidable question is posed as to how such activity is brought into operation at, and restricted to, those parts of a flowing membrane system which happen, momentarily, to be functioning as a plasma membrane. The rapidity of the interchange of activity seems to preclude *de novo* synthesis of enzyme in a membrane newly occupying a position on an active surface of the cell, or its inactivation, destruction, or physical loss when such a membrane becomes quiescent or internalized in the cell. The possibility that enzymic activity may wax and wane with the configurational state or degree of folding and unfolding of polypeptide chains in membrane systems has been suggested and briefly discussed by Holt (36).

Finally, the absence of staining of mitochondria and of endoplasmic reticulum requires explanation.

In spite of the known high ATPase activity of mitochondria (37), it has been found that, unless particular care is taken during fixation in formaldehyde, mitochondrial ATPase cannot be demonstrated by the Wachstein and Meisel procedure (27, 38). It appears, then, that the newer glutaraldehyde fixative (19, 26), used as described here, has a like inhibitory effect on the mitochondrial enzyme in HeLa cells.

A similar explanation probably accounts for the lack of staining of elements of the endoplasmic reticulum. Since the occurrence of a magnesium-activated ATPase in rat liver microsomes was first shown by Novikoff *et al.* (39), it has been found that not only is it associated with a membranous sub-fraction of liver microsomes (40), but that the membrane enzyme closely resembles the magnesium-activated ATPase of liver mitochondria (41).

In view of this close correspondence between the microsomal enzyme and the aldehyde-inactivated ATPase of mitochondria, it is perhaps not surprising that staining was not seen in any element of the endoplasmic reticulum of HeLa cells fixed in glutaraldehyde or formaldehyde as used here.

The segmented or particulate nature of the reaction product (Fig. 5) is worthy of comment. It is not thought to indicate minute surface differences in enzyme distribution, since the staining method is incapable of resolving with this order of precision, and it is more likely that the segmentation is a consequence of some technical step in the cytochemical or preparative procedures used. A similar fine segmentation of the enzyme reaction product has been observed in other electron microscope studies of ATPase activity at the cell surface (28, 12).

Despite this particulate nature of the reaction product, and the absence of exact information on the nature of the enzyme systems, it seems quite clear that they are restricted to certain cell membranes in a given culture, an observation that has been found to have an important bearing on the results of cytochemical staining experiments on HeLa cells infected with herpes virus (15).

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