ELECTRON MICROSCOPE OBSERVATIONS ON THE SURFACE ADENOSINE TRIPHOSPHATASE-LIKE ENZYMES OF HeLa CELLS INFECTED WITH HERPES VIRUS

M. A. EPSTEIN, M.D., and S. J. HOLT, Ph.D.

From The Bland-Sutton Institute of Pathology and The Courtauld Institute of Biochemistry, The Middlesex Hospital Medical School, London, England

ABSTRACT

HeLa cells infected with herpes simplex virus have been examined in thin sections by electron microscopy after cytochemical staining for the presence of surface enzymes splitting adenosine triphosphate. As with uninfected HeLa cultures (18), the opaque enzyme reaction product was localized at the plasma membranes of about half the cells, tending to be present where there were microvilli and absent on smooth surfaces. Where mature extracellular herpes particles were found in association with cell membranes showing the enzyme activity, they were invariably likewise stained, and conversely, those mature particles which lay close against cells without reaction product at the surface were themselves free of it. Particles found budding into cytoplasmic vacuoles were also always without opaque deposit since this was never seen at vacuolar membranes, even in cells having the activity at the surface. The enzyme reaction product thus provided a marker indicating the manner in which the particles escape from cells and mature by budding out through cellular membranes, carrying, in the process, a portion of the latter on to themselves to form the outer viral limiting membrane. In some instances, virus particles were observed with more opaque material covering them than was present at the cell membrane with which they were associated. This finding has been taken as evidence for a physiological waxing and waning of surface enzyme activity of adenosine triphosphatase type. The fine structure of the mature extracellular virus as prepared here, using glutaraldehyde fixation, is also recorded. The observations and interpretations are discussed in full.

INTRODUCTION

The investigation by electron microscopy of the release mechanism of herpes simplex virus from infected cells has shown that immature particles bud out at two types of cellular membrane and escape by becoming enclosed in an outer covering derived from these membranes as they pass through (1). The process takes place at the cell surface and at the smooth membranes limiting cytoplasmic vacuoles, and explains both the way in which the particle acquires the morphological attributes of maturity, and the reason for the close correspondence in structure between the triple-layered outer limiting membrane of the mature virus and the three-layered membranes of cells (2).

Viral release by budding occurs with a number of different agents (3–12), and, besides being im-

plicated in determining the final morphology of herpes virus (1, 2), clearly has a much wider general importance for the structure of certain types of virus (13, 14). It seemed therefore that further investigation of herpes virus budding at various kinds of cell membrane would be of value, were an appropriated marker available for the membranes involved.

Now in the case of another virus released from infected cells by budding, the BAI strain A avian myeloblastosis virus, a marker of this kind has recently been reported. For, using cytochemical staining methods, it was found that where particles budded out through cell membranes carrying adenosine triphosphatase (ATPase) activity, the outer viral membranes likewise showed the presence of this type of enzyme (15, 16). The dense enzyme reaction product thus provided a means of tracing a functional activity in the outer viral membrane back to the cell membrane, and so of confirming the origin of this component of the virus.

ATPase and related enzymes have been found at a variety of different cell surfaces engaged in ionic transport and movement, and are currently believed to be concerned in the supply of energy for these functions (17, 18). Many cultured cells, including HeLa cells, frequently show surface activity and, in the case of the latter cell strain, the associated ATPase-like enzymes have been demonstrated at the plasma membrane by electron microscopy (19). Since this type of cell was used for the original work on herpes virus budding (1, 2), and since ATPase activity was only ever present at one of the two types of membrane where budding can occur (19), it was considered that the study of ATPase in HeLa cells infected with herpes virus might provide a useful method for the further elucidation of the budding process.

Accordingly, herpes-infected HeLa cells have been examined in thin sections with the electron microscope after glutaraldehyde fixation and staining for the presence of adenosine triphosphate (ATP)-splitting enzymes. The distribution of the dense enzyme reaction product was investigated with particular reference to virus maturation and escape by budding, and the results of these experiments are now presented and discussed.

MATERIALS AND METHODS

Culture and Infection of HeLa Cells

The HeLa cultures were grown in flat glass bottles in growth medium as described previously (20, 21) and were infected as monolayers with a heavy dose of the HFEM strain of herpes virus in the same way as in earlier work (2).

Collection, Fixation, and Cytochemical Staining

Two days after infection, the cells were collected by shaking into suspension, fixed in glutaraldehyde (22), and stained cytochemically in the ATP-containing medium of Wachstein and Meisel (23, 24); the methods used for this and for appropriate control procedures are reported in detail in the preceding paper (19).

Preparation of Cells for Electron Microscopy

Cytochemically stained and control cells were prepared for electron microscopy and were examined both with and without uranyl acetate counterstaining, just as was done with uninfected HeLa cells (19).

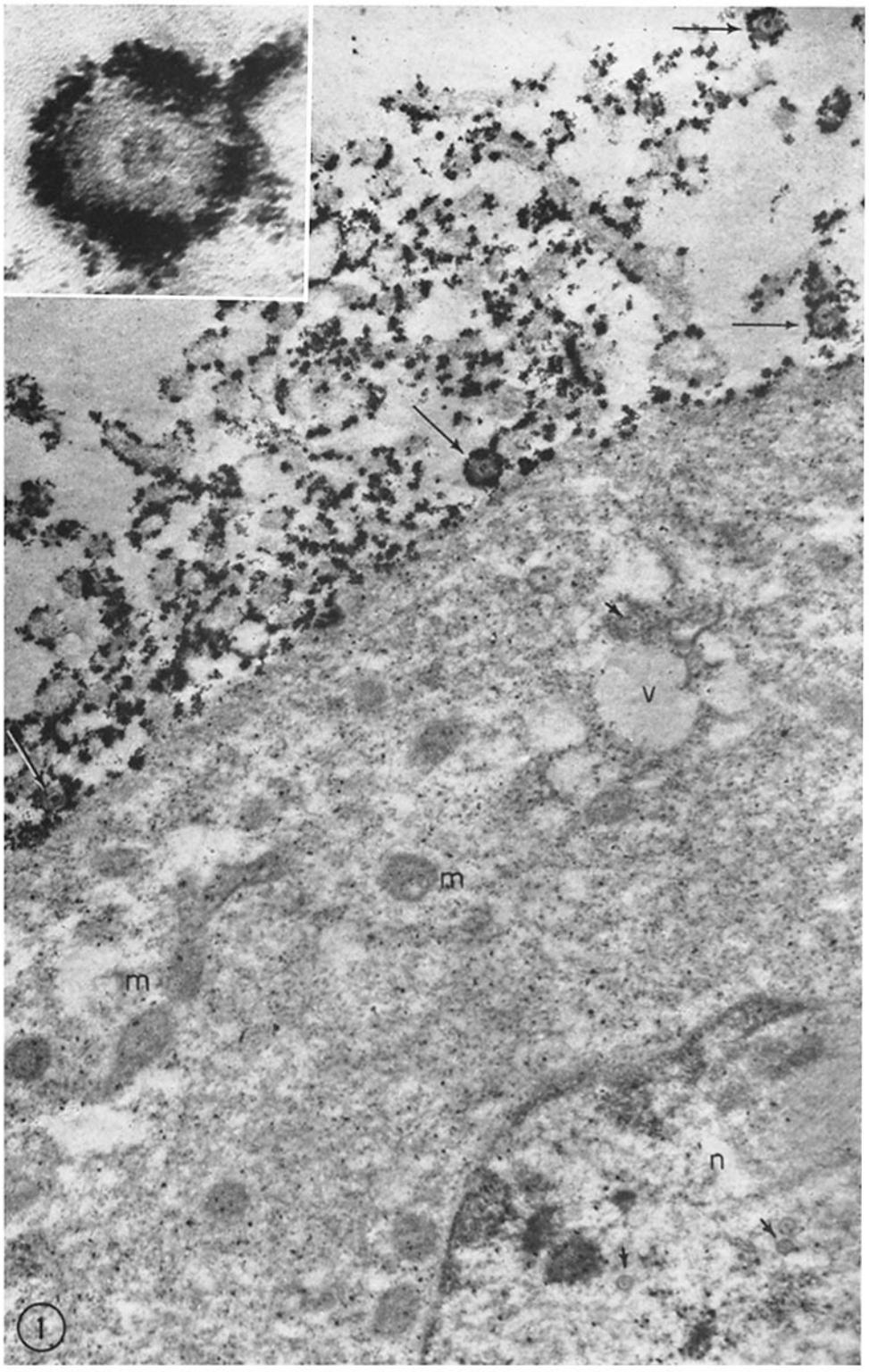
OBSERVATIONS

General Features of the Cultures

The infected cultures of the present experiments resembled similarly prepared uninfected HeLa

All the figures are electron micrographs of thin sections of herpes-infected HeLa cells fixed with glutaraldehyde and stained for surface enzymes splitting ATP.

FIGURE 1 Part of a cell with considerable surface activity. The cell membrane crosses the upper part of the field and is thrown up into profuse tangled microvilli; a moderately heavy deposit of enzyme reaction product (lead phosphate) is localized on the outer aspect of the membrane and follows it as a chain of electron-opaque segments. Mature virus particles (long arrows) associated with this membrane are surrounded by similar opaque reaction product. Immature particles (short arrows) can be seen in the nucleus (*n*) and in the cytoplasm, and the latter also contains mitochondria (*m*) and smooth vacuoles (*v*). × 30,000. Inset, detail of a single mature particle showing the enzyme reaction product at the outer limiting membrane. × 160,000.


v
m
m
n
1

cultures (19) in several ways; the preparations consisted largely of intact cells with little debris, contrast was poor in the absence of counterstaining, and where this was applied, fine structural detail was readily evident. Furthermore, about half the cells incubated in the full ATP-containing medium were surrounded by a dense band of enzyme reaction product which, though varying in amount from cell to cell, was found only in relation to the surface membranes. It was not present after the control treatments. The fine, randomly distributed precipitate sometimes observed over both cell nuclei and cytoplasm (Figs. 1 and 3) was, just as in the case of uninfected cultures (19), also encountered in control preparations and is therefore considered a non-specific effect of the staining medium.

Almost all the infected cells were associated with various stages of virus growth and maturation, immature particles being seen in the nuclei and cytoplasm (Figs. 1 and 3) and mature forms close around the plasma membranes or within cytoplasmic vacuoles (Figs. 1 to 5), just as in earlier work (25, 26, 1). Also, as in previous experiments (1), such cytoplasmic vacuoles were found to be frequently present in the infected cells, but enzyme reaction product was never associated with them (Figs. 1 and 4).

Relation of Virus to Enzyme Distribution

The immature particles, being always confined to the nucleus or cytoplasmic matrix (Figs. 1, 3, and 4), were not observed in relation to ATPase reaction product at the cell surface.

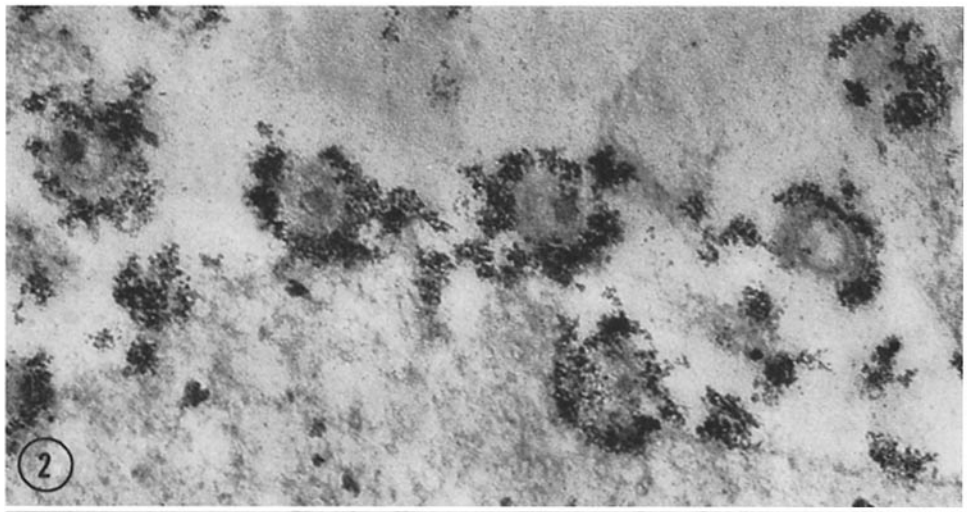
In contrast, the association of the mature form of the virus with the enzyme was variable and depended directly on the site. Where mature extracellular particles were found lying close to cells with a deposit of electron-opaque material at their plasma membranes, the particles were surrounded by similar material (Figs. 1, 2, and 4). The amounts of the deposit at the cell and viral membranes usually corresponded closely, but in a number of instances there was very considerably more around the virus (Fig. 2). On the other hand, mature virus derived from cells without evidence of surface ATPase-like activity and lying close to surface membranes free of opaque reaction product was itself invariably also unstained (Figs. 3 and 5). Similarly, fully formed particles within cytoplasmic vacuoles were never found with dense material, even in cells carrying a band of deposit at the plasmalemma (Fig. 4) and even where the vacuoles lay very near to such an active surface (Fig. 4). Thus, particles with and without ATPase activity on their outer limiting membranes could be observed in the closest proximity in relation to a single individual cell (Fig. 4), the presence or lack of the enzyme in the virus corresponding with the type of membrane at which it formed.

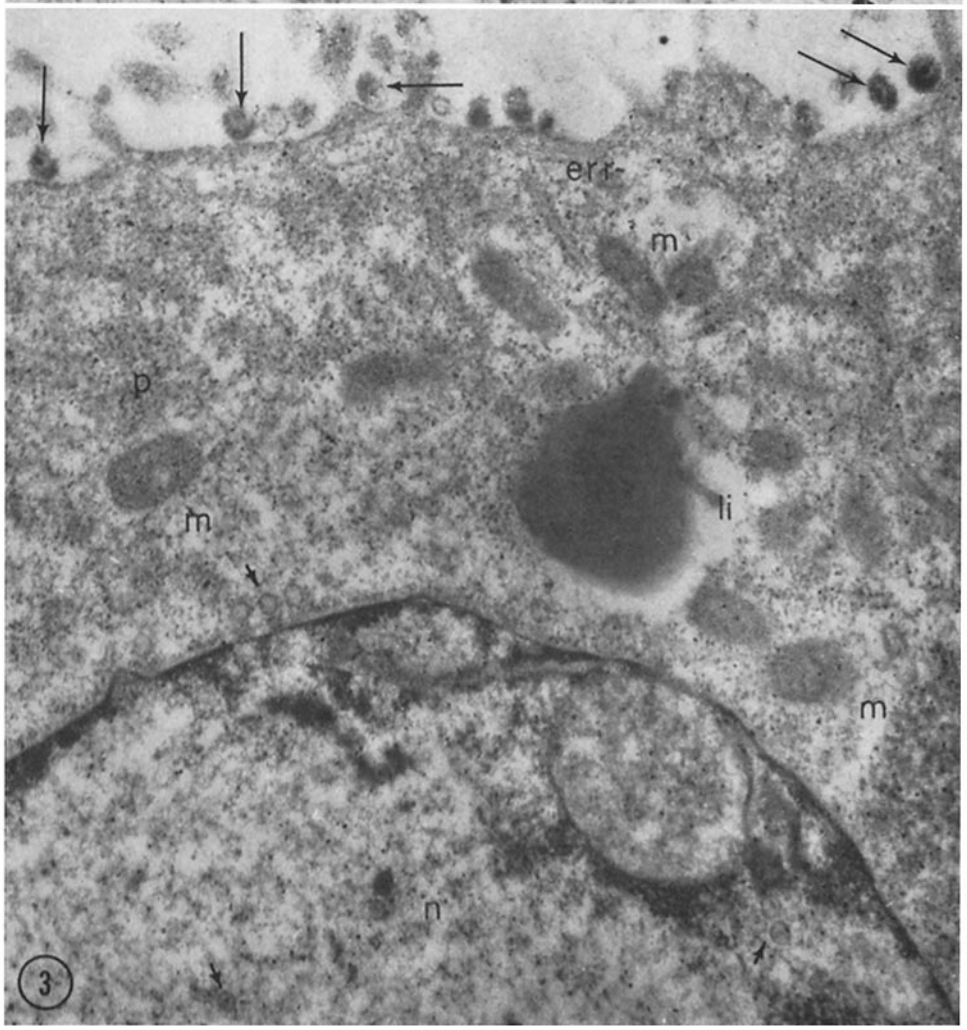
Where mature herpes particles were associated with electron-opaque reaction product this dense material lay against the outer surface of the viral limiting membrane (Figs. 1 and 2) in exactly the same manner as it occurred at the surface of both uninfected HeLa cells (19) and those in which the virus was multiplying (Figs. 1 and 4).

The fine structure of the mature virus was best seen when deposited material was absent, as in control preparations or outside cytochemically stained cells which were without ATPase activity (Fig. 5). Here, the triple-layered outer membrane, absence of inner membrane, and eccentric nucle-

FIGURE 2 Detail of cell surface. Peripheral cytoplasm limited by the plasmalemma occupies the lower half of the field with five mature extracellular virus particles lying close against it; the particles have been sectioned in various planes, and that on the right is slightly damaged. Each particle is enclosed by a covering of opaque, deposited material which is very much heavier than the scattered segments at the surface of the cell. × 90,000.

FIGURE 3 Portion of a cell lacking apparent surface activity. The top of the field is crossed by the cell membrane which carries few microvilli and is without deposited opaque reaction product. Numerous mature virus particles (long arrows) lie close against this membrane and are likewise free of stain. The nucleus (*n*), below, contains immature virus particles (short arrows) several of which are also present in the cytoplasm (short arrow). In addition, masses of free particles (*p*), a large lipid body (*li*), mitochondria (*m*) and rough endoplasmic reticulum (*err*) can also be recognized in the cytoplasmic matrix. This cell is from the same preparation as that of Fig. 1. × 30,000.


2


err
m
p
m
li
m
n
3

oid known from material fixed and embedded in other ways (2) could all be recognised (Fig. 5).

DISCUSSION

It is of interest that the well preserved infected cells examined in the present work included the same proportion with a dense band of surface enzyme reaction product as was found amongst the cells of uninfected cultures (19). In each case about half the cells showed evidence of enzyme activity presumably associated with normal surface function (17, 18).

That this type of function was not apparently disturbed by herpes infection is not surprising when it is considered that herpes virus can be released from cells over a considerable period of time (27–29) before causing vital damage, and, although some normal cellular activities are known to become distorted under such circumstances (30, 31), many other activities must continue unaltered. The present observations on the ATPase-like surface enzymes of the infected cells (Figs. 1, 2, and 4) provide an example of such a cellular function unchanged by herpes multiplication. A similar persistence of this type of activity occurs in cells producing avian myeloblastosis virus (15, 16), this being the only other known instance of a virus carrying enzymes on to itself along with enveloping portions of cellular membrane during escape and maturation by budding.

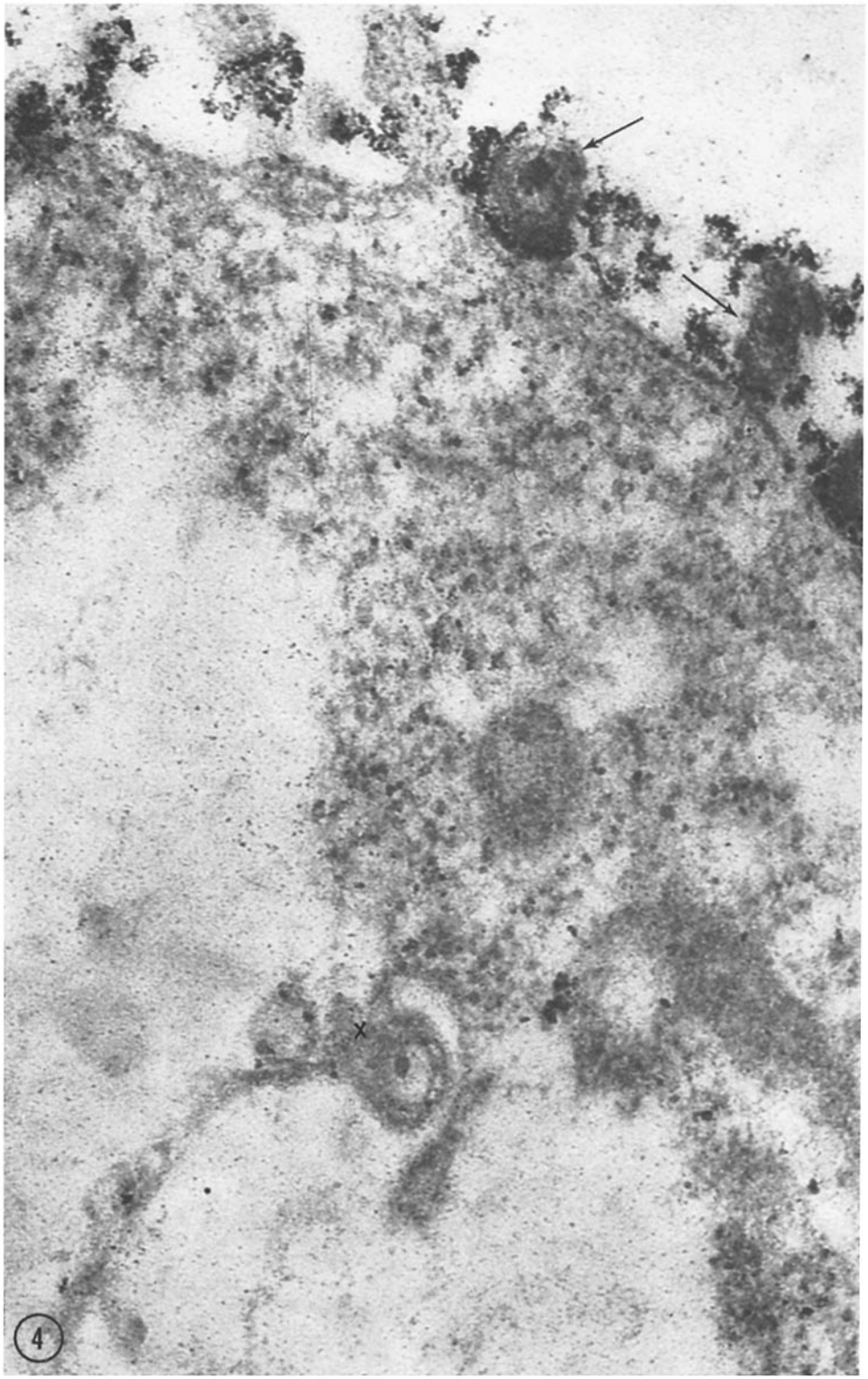
Recognition of mature herpes particles after the preparation methods used here presented no difficulty (Figs. 1 to 5), since they showed all the fine structural features found previously where osmium tetroxide or permanganate fixation was followed by embedding in an epoxy resin (2), and this morphological equivalence (Fig. 5) after the use of several different fixatives suggests that the structural preservation achieved is valid. On the whole, the particles fixed with glutaraldehyde (22) stood up well to the cytochemical procedures applied in the present experiments (Figs. 1 to 5).

The close and invariable correspondence between the presence or absence of ATPase reaction product at the plasma membrane of individual cells and the presence or absence of this product around the mature extracellular virus particles associated with the cells (Figs. 1 to 5), is a clear indication of the manner in which the particles pass through the cell membranes and of the relationship of the latter to the outer viral membranes. It constitutes, in fact, firm functional confirmation for the process of herpes virus release by budding which hitherto has rested on morphological evidence alone (1), and at the same time shows that when the enzyme was present at a cell's surface it was carried passively on to the maturing virus during budding, as an irrelevant coincidence. This is also evident from the finding that the smooth membranes limiting cytoplasmic vacuoles were always lacking in ATPase activity without this seeming to affect the considerable amount of virus budding observed there (Fig. 4) (1).

The functional difference in respect of ATPase between vacuolar membranes and the cell membrane has already been noted and commented upon (19). The present results have a further significance for this, since they show that although these spatially distinct elements of the morphologically continuous membrane system of cells (32–35) may differ in one function, such as the possession of ATPase, they are nevertheless readily able to share an unrelated function, in this case virus budding.

The manner in which the ATPase reaction product present on some of the cells can be used as a marker to relate the cell membrane to the ATPase-carrying membranes of nearby mature virus particles (Figs. 1, 2, and 4) has just been pointed out; but, in addition to this, the dense deposited material can serve as a marker in another way. The suggestion has been made that ATPase activity at the surface of HeLa cells varies with

FIGURE 4 Detail of peripheral cytoplasm with the cell membrane above; microvilli are present at the cell surface which carries some deposit of ATPase reaction product. This opaque material is also associated with the mature extracellular virus lying against the cell (long arrows). Below and to the left, parts of two cytoplasmic vacuoles are included in the field; at x an immature virus particle is in the process of escaping and maturing by budding into the vacuole. Since the vacuolar membrane is without ATPase activity, this particle is being released unaccompanied by enzyme, thus contrasting, in this respect, with those at the surface. × 105,000.


4

changes in their physiological state (19) and that it may gradually build up or disappear in an individual cell during the course of other functional alterations. The observation recorded in the present experiments, *i.e.* that mature extracellular herpes virus may sometimes carry more enzyme reaction product than the cell membrane from which it escaped (Fig. 2), lends support to this view. For, where such mature particles are found to carry more ATPase than the cell membrane with which they are associated (Fig. 2), the difference provides an indication of the greater concentration of enzyme present in that membrane when budding actually took place.

For this interpretation to be correct, it must be assumed that once a portion of cell membrane becomes detached and wrapped around a maturing virus particle its functional attributes, such as ATPase activity, cease to undergo normal change and become fixed. It seems likely that this occurs with herpes virus, since in the case of the only other agent known to carry ATPase, the BAI strain A fowl myeloblastosis virus, the enzyme persists on the particles for considerable periods of time (36–38) and measurement of it has in consequence been used for their enumeration (39). Such an arrest of functional change in separated cell membrane fragments is not surprising, once continuity with normal control mechanisms is lost. The mere fact of severance from the cell on incorporation with a virus particle during budding, would be expected to bring about many other sorts of change, and structural alterations in cell membranes doing this have indeed been reported with a variety of different viruses which escape from cells as buds (40–42). It would be of interest to know what happens to a functional capability like ATPase activity in those cell membranes which show such morphological alterations when they are converted into the outer limiting membrane of a virus particle. The use of an enzyme reaction product as a marker, in the manner of the present experiments, could provide a means for investigating this problem were an appropriate cell-virus system to be available.

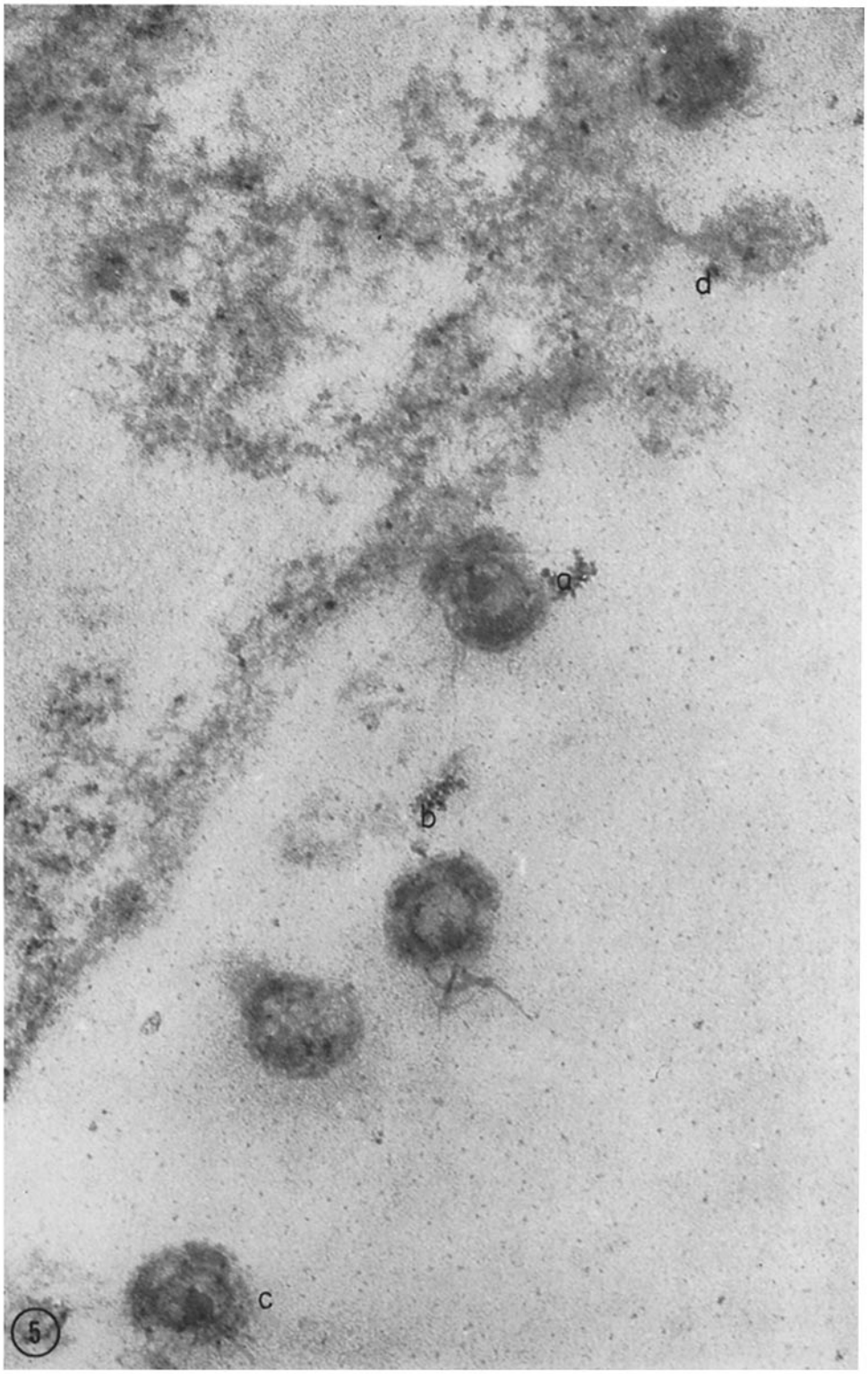
This investigation was supported by the British Empire Cancer Campaign and assisted by grants from the University of London Central Research Fund. The authors gratefully thank Miss B. M. Blake and Mr. G. Ball for their invaluable technical help.

Received for publication, March 11, 1963.

REFERENCES

1. EPSTEIN, M. A., Observations on the mode of release of herpes virus from infected HeLa cells, *J. Cell Biol.*, 1962, **12**, 589.
2. EPSTEIN, M. A., Observations on the fine structure of mature herpes simplex virus and on the composition of its nucleoid, *J. Exp. Med.*, 1962, **115**, 1.
3. MORGAN, C., ROSE, H. M., and MOORE, D. H., Structure and development of viruses observed in the electron microscope, III. Influenza virus, *J. Exp. Med.*, 1956, **104**, 171.
4. MORGAN, C., HSU, K. C., RIFKIND, R. A., KNOX, A. W., AND ROSE, H. M., The application of ferritin-conjugated antibody to electron microscopic studies of influenza virus in infected cells. I. The cellular surface, *J. Exp. Med.*, 1961, **114**, 825.
5. MOORE, D. H., LASFARGUES, E. Y., MURRAY, M. R., HAAGENSEN, C. D., and POLLAND, E. C., Correlation of physical and biological properties of mouse mammary tumor agent, *J. Biophysic. and Biochem. Cytol.*, 1959, **5**, 85.
6. GOLDFEDER, A., GELBER, D., and MOORE, D. H., An electron microscope study of spontaneous mammary carcinomas in a subline of strain DBA mice. *J. Nat. Cancer Inst.*, 1960, **25**, 827.
7. DE HARVEN, E., and FRIEND, C., Electron microscopy of Swiss mouse leukemia virus, *Nat. Cancer Inst. Monographs*, 1960, **4**, 291.
8. DE HARVEN, E., and FRIEND, C., Further electron

FIGURE 5 Small area of cell surface with subplasmalemmal cytoplasm above and to the left. The cell is without microvilli and there is no ATPase-like activity. Mature virus particles similarly without enzyme reaction product lie close to the cell. The triple-layered outer limiting membrane, absence of inner membrane, and eccentric nucleoid known from material fixed and embedded in other ways (2), are well seen in the particles at *a* and *b*. Particle *c* has been damaged. At *d* a thin surface sliver has been sliced off a budding particle which is still attached by a stalk. × 120,000.


d
a
b
c
5

microscope studies of a mouse leukemia induced by cell-free filtrates, *J. Biophysic. and Biochem. Cytol.*, 1960, **7**, 747.

9. DALTON, A. J., LAW, L. W., MOLONEY, J. B., and MANAKER, R. A., An electron microscopic study of a series of murine lymphoid neoplasms, *J. Nat. Cancer Inst.*, 1961, **27**, 747.
10. HEINE, U., BEAUDREAU, G. S., BECKER, C., BEARD, D., and BEARD, J. W., Virus of avian erythroblastosis. VII. Ultrastructure of erythroblasts from the chicken and from tissue culture, *J. Nat. Cancer Inst.*, 1961, **26**, 359.
11. HEINE, U., DE THÉ, G., ISHIGURO, H., SOMMER, J. R., BEARD, D., and BEARD, J. W., Multiplicity of cell response to the BAI strain A (Myeloblastosis) Avian tumor virus. II. Nephroblastoma (Wilms' Tumor): ultrastructure, *J. Nat. Cancer Inst.*, 1962, **29**, 41.
12. HEINE U., DE THÉ, G., ISHIGURO, H., and BEARD, J. W., Morphologic aspects of Rous sarcoma virus elaboration, *J. Nat. Cancer Inst.*, 1962, **29**, 211.
13. EPSTEIN, M. A., Functional aspects of the structure of some animal viruses, *Brit. Med. Bull.*, 1962, **18**, 183.
14. HUMMELER, K., Relationship of animal virus structures to their immunologic properties as determined by electron microscopy, *Bact. Rev.*, 1963, in press.
15. DE THÉ, G., NOVIKOFF, A. B., HEINE, U., and BEARD, J. W., Preliminary studies of cytochemistry of avian tumor viruses in electron microscopy, *in* Proceedings of the 5th International Congress for Electron Microscopy, (S. S. Breese, editor), New York, Academic Press, Inc., 1962, **2**, PP-2.
16. NOVIKOFF, A. B., DE THÉ, G., BEARD, D., and BEARD, J. W., Electron microscopic study of the ATPase activity of the BAI strain A (myeloblastosis) avian tumor virus, *J. Cell Biol.*, 1962, **15**, 451.
17. NOVIKOFF, A. B., The intracellular localization of chemical constituents, *in* Analytical Cytology, (R. C. Mellors, editor), New York, McGraw-Hill Book Co., 1959, 2nd. edition, 69.
18. NOVIKOFF, A. B., and ESSNER, E., The liver cell, *Am. J. Med.*, 1960, **29**, 102.
19. EPSTEIN, M. A., and HOLT, S. J., The localization by electron microscopy of HeLa cell surface-enzymes splitting adenosine triphosphate, *J. Cell Biol.*, 1963, **19**, 325.
20. PEREIRA, H. G., and KELLY, B., Dose response curves of toxic and infective actions of adenovirus in HeLa cell cultures, *J. Gen. Microbiol.*, 1957, **17**, 517.
21. EPSTEIN, M. A., and POWELL, A. K., The isolation of Type 5 adenovirus using a fluorocarbon; combined morphological and biological studies, *Brit. J. Exp. Path.*, 1960, **41**, 559.
22. SABATINI, D. D., BENTSCH, K. G., and BARRNETT, R. J., New means of fixation for electron microscopy and histochemistry, *Anat. Rec.*, 1962, **142**, 274.
23. WACHSTEIN, M., and MEISEL, E., Histochemistry of hepatic phosphatases at a physiologic pH, *Am. J. Clin. Path.*, 1957, **27**, 13.
24. PEARSE, A. G. E., Histochemistry, London, J. and A. Churchill Ltd., 2nd edition, 877.
25. STOKER, M. G. P., SMITH, K. M., and ROSS, R. W., Electron microscopic studies of HeLa cells infected with herpes virus, *J. Gen. Microbiol.*, 1958, **19**, 244.
26. MORGAN, C., ROSE, H. M., HOLDEN, M., and JONES, E. P., Electron microscopic observations on the development of herpes simplex virus, *J. Exp. Med.*, 1959, **110**, 643.
27. STOKER, M. G. P., and ROSS, R. W., Quantitative studies on the growth of herpes virus in HeLa cells, *J. Gen. Microbiol.*, 1958, **19**, 250.
28. STOKER, M. G. P. and WILDY, P. Release of herpes virus from isolated single cells, *J. Path. and Bact.*, 1958, **75**, 519.
29. WILDY, P., STOKER, M. G. P., and ROSS, R. W., Release of herpes virus from solitary HeLa cells, *J. Gen. Microbiol.*, 1959, **20**, 105.
30. NEWTON, A. A., and STOKER, M. G. P., Changes in nucleic acid content of HeLa cells infected with herpes virus, *Virology*, 1958, **5**, 549.
31. NEWTON, A. A., and MCWILLIAM, S., Incorporation of thymidine by L cells infected with herpes virus, *Biochem. J.*, 1962, **84**, 112 P.
32. PALADE, G. E., The endoplasmic reticulum. *J. Biophysic. and Biochem. Cytol.*, 1956, **2**, No. 4 suppl., 85.
33. BENNETT, H. S., The concepts of membrane flow and membrane vesiculation as mechanisms for active transport and ion pumping, *J. Biophysic. and Biochem. Cytol.*, 1956, **2**, No. 4 suppl., 99.
34. EPSTEIN, M. A., The fine structural organisation of Rous tumour cells, *J. Biophysic. and Biochem. Cytol.*, 1957, **3**, 851.
35. PALAY, S. L., On the appearance of fat droplets in the nuclear envelope, *J. Biophysic. and Biochem. Cytol.*, 1960, **7**, 391.
36. MOMMAERTS, E. B., SHARP, D. G., ECKERT, E. A., BEARD, D., and BEARD, J. W., Virus of avian erythromyeloblastic leukosis. I. Relation of specific plasma particles to the dephosphorylation of adenosine triphosphate, *J. Nat. Cancer Inst.*, 1954, **14**, 1011.

37. SHARP, D. G., MOMMAERTS, E. B., ECKERT, E. A., BEARD, D., and BEARD, J. W., Virus of avian erythromyeloblastic leukosis. II. Electrophoresis and sedimentation of the plasma particles and the enzyme dephosphorylating adenosine triphosphate, *J. Nat. Cancer Inst.*, 1954, **14**, 1027.
38. ECKERT, E. A., SHARP, D. G., MOMMAERTS, E. B., REEVE, R. H., BEARD, D., and BEARD, J. W., Virus of avian erythromyeloblastic leukosis. III. Inter-relations of plasma particles, infectivity, and the enzyme dephosphorylating adenosine triphosphate, *J. Nat. Cancer Inst.*, 1954, **14**, 1039.
39. BEAUDREAU, G. S., and BECKER, C., Virus of avian myeloblastosis. X. Photometric microdetermination of adenosine triphosphatase activity, *J. Nat. Cancer Inst.*, 1958, **20**, 339.
40. HORNE, R. W., WATERSON, A. P., WILDY, P., and FARNHAM, A. E., The structure and composition of the myxoviruses. I. Electron microscope studies of the structure of myxovirus particles by negative staining techniques, *Virology*, 1960, **11**, 79.
41. LYONS, M. L., and MOORE, D. H., Purification of the mouse mammary tumour virus, *Nature*, 1962, **194**, 1141.
42. HOWATSON, A. F., and WHITMORE, G. E., The development and structure of vesicular stomatitis virus, *Virology*, 1962, **16**, 466.