THE CYTOCHEMICAL EXAMINATION OF POLIOVIRUS-INDUCED CELL DAMAGE

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INTRODUCTION

It was suggested by Defendi (1962) that the activation of lysosomal enzymes may precede and contribute to cell destruction during viral infection. Both biochemical procedures and cytochemical techniques at the light microscope level have been employed to demonstrate the cytoplasmic location of lysosomal enzymes, before and during viral infection (Allison and Sandelin, 1963; Allison and Mallucci, 1965; Macieira-Coelho et al., 1965; Dusing and Wolff, 1969). It was shown that as a result of certain virus infections, lysosomal enzymes diffused from their localized position into the surrounding cytoplasm. Other studies on virus-cell interactions indicated that only cytolytic viruses are capable of producing these changes (Wolff and Bubel, 1964; Flanagan, 1966). Viruses which were slowly liberated from cells, or which did not initiate cytolysis, such as vaccinia virus, vesicular stomatitis virus, or herpes simplex, caused little alteration of lysosomal enzyme distributions.

Poliovirus-induced cytopathic morphological alterations have been extensively examined by electron microscopy (Mattern and Daniel, 1965; Dales et al., 1965; Anzai and Ozaki, 1969; Levinthal et al., 1969; Bienz et al., 1973). In the present experimental work, enzyme cytochemistry at the electron microscope level was employed as a new approach to investigate the fate of lysosomal enzymes during cytolytic infections of the poliovirus HEp-2 cell system.

MATERIALS AND METHODS

Cell Culture

HEp-2 cells in suspension cultures were propagated in Eagle's Minimal Essential Medium with Spinner Salts (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% (vol/vol) inactivated calf serum (North American Biologicals, Inc., North Miami, Fla.) and 4% (wt/vol) Methocel (Dow Chemical Co., Midland, Mich.) (Guskey et al., 1970).

Virus

Poliovirus, type 1, Mahoney strain, was supplied by Dr. H. C. Bubel (Department of Microbiology, University of Cincinnati Medical School).

Electron Microscope Cytochemistry

For cytochemical examination, one 30-ml HEp-2 cell sample was infected each hour, for 8 consecutive hours, at a multiplicity of 75 plaque-forming units (PFU) per cell. The virus was allowed to adsorb for 30 min at 36°C after which the cells were again centrifuged and resuspended in 30 ml of culture medium in a 125-ml Erlenmeyer flask and incubated in the gyrotory incubator shaker.

All samples were fixed simultaneously by adding 2.0% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.0) containing 7.5% (wt/vol) sucrose to the suspended cells in growth medium. After 45 min, the cells were pelleted and further fixation in 2.0% glutaraldehyde with sucrose alone was carried out for another 45 min. The cells were then washed in two changes of sucrose-cacodylate buffer. Finally, two 10-min rinsings in 0.1 M sodium acetate buffer, pH 5.0, with 7.5% sucrose were used to lower the pH in preparation for cytochemical enzyme staining. A modification of the Gomori method (Gomori, 1952), as formulated by Dales and Kajioka (1964), was employed for the detection of acid phosphatase. The reaction mixture, which consisted of one part 3% (wt/vol) sodium β-glycerophosphate (Sigma Chemical Co., St. Louis, Mo.), five parts 0.1 M sodium acetate buffer, pH 5.0, and five parts 0.12% (wt/vol) lead nitrate, was used at 36°C for 45 min. The cells were washed, resuspended in agar and after dehydration, embedding, and sectioning, were stained in lead citrate and uranyl acetate (Reynolds, 1963).

RESULTS

The specificity for the staining reaction was determined by comparing two uninfected controls which were fixed simultaneously with the infected cells. Fig. 1 is an example of an uninfected control cell incubated in a Gomori medium with no β-glycerophosphate substrate, and Fig. 2 is an uninfected cell incubated in the complete reaction solution. The reaction product in Fig. 2 is a granular electron-opaque precipitate within confined areas in the central portion of the cytoplasm. In the development of a technique for demonstrating the lysosomal enzyme, we found that the omission of the commonly used osmium tetroxide postfixation resulted in some loss of detail, but eliminated the dark osmiophilic bodies which could be confused with lysosomes.

Uninfected and infected cells up to 2 h post infection (PI) showed localized acid phosphatase in lysosomes (L) in the perinuclear region of the cell. Fig. 3 clearly demonstrates that no detectable changes in enzyme distribution had occurred in infected cells up to this time. Significant alterations in the enzyme pattern developed between 3 and 5 h PI in a large population of cells. At the end of this period, the majority of cells appeared as in Fig. 4, where the enzyme had diffused from confined sites and was redistributed throughout one large juxtanuclear region of the cytoplasm. This area could be recognized as distinctly separate from the remainder of the seemingly unaffected cell. Within this zone of diffuse enzyme reaction product, there was always a continuous network of vacuolar structures which we will refer to as membranous cisternae (MC) (Amako and Dales, 1967).

Cell samples infected for longer periods of time, up to 8 h PI, revealed that during later stages of the infectious process, acid phosphatase redistribution continued and the enzyme remained associated with the increasingly larger membranous areas which had spread throughout the entire cytoplasm in many cells.

A series of electron micrographs at higher magnification supplied additional information regarding cytoplasmic alterations in relation to redistributed enzyme. Fig. 5 shows normal lysosomes as they are seen in uninfected cells or during the first 2 h after exposure to the virus. It is evident from the observation of a cell infected for 4 h (Fig. 6) that acid phosphatase was initially distributed from each lysosome into an area in the immediate vicinity of the original organelle and in each small area the enzyme is seen to be associated with newly formed membranous cisternae. We recognized these first changes as early as 3 h PI in some cells. Cells infected from 5 to 8 h had enzyme throughout a larger zone of membranous cisternae, as shown in Fig. 7. This micrograph also emphasizes the fact that not all lysosomes of a cell were affected similarly since a portion of the cytoplasm often contained intact lysosomes outside the region containing diffused enzyme and cisternae.

Additional light microscope studies (not shown) were carried out using simultaneous coupling techniques for acid phosphatase and β-glucuronidase (Barka and Anderson, 1962; Hayashi et al., 1964). Results of this work substantiated the electron microscope evidence of poliovirus-induced enzyme redistribution.

DISCUSSION

This investigation shows that in poliovirus-infected cells, lysosomal enzyme redistribution and forma-

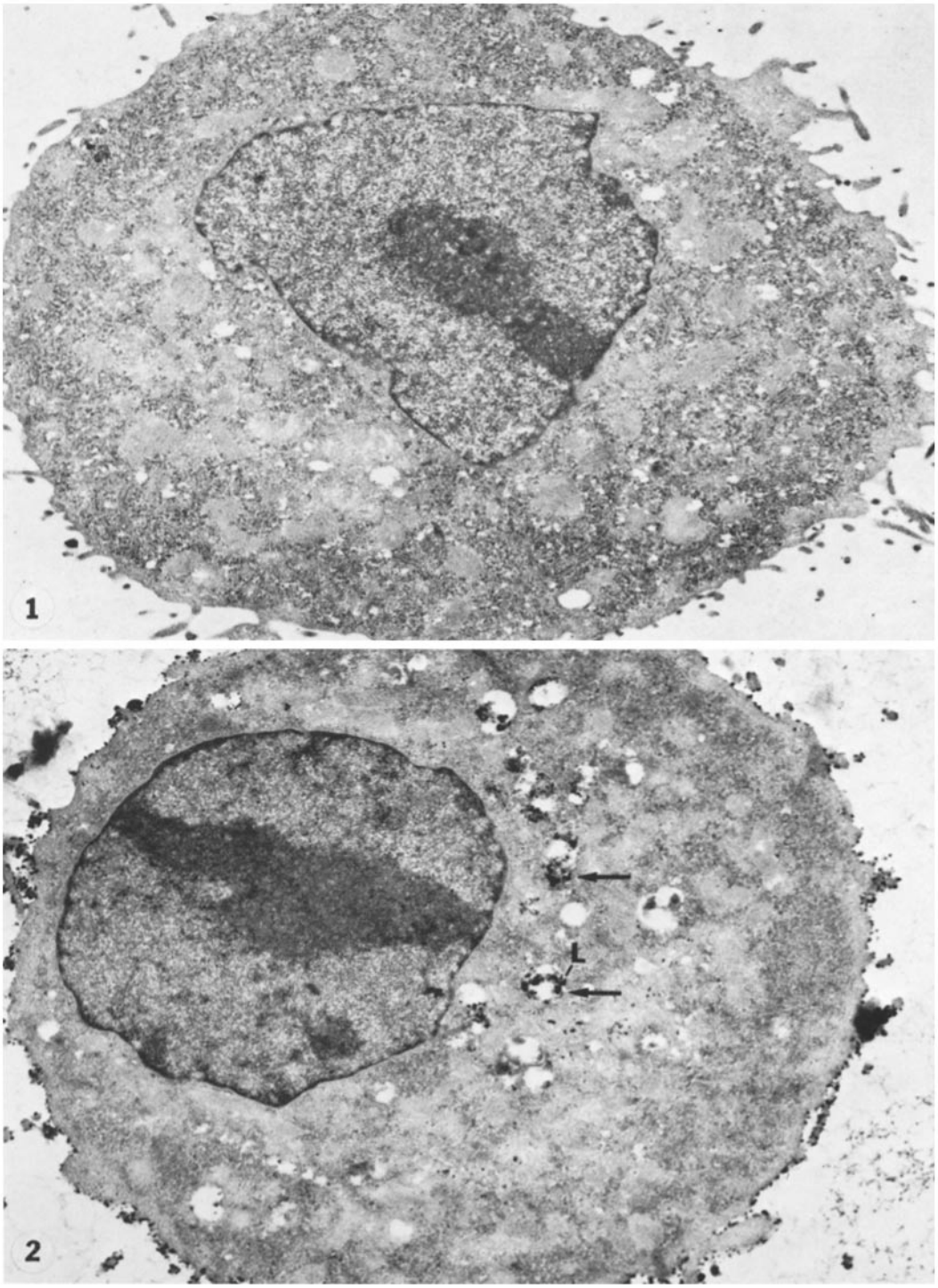

FIGURE 1 Acid phosphatase activity control. The substrate, sodium β-glycerophosphate was omitted from the Gomori (1952) cytochemical staining solution resulting in a lack of dark precipitate in this uninfected HEp-2 cell. × 11,600.

FIGURE 2 Uninfected HEp-2 cell control. This micrograph demonstrates acid phosphatase (indicated by arrows) localized in lysosomes (*L*). × 12,100.

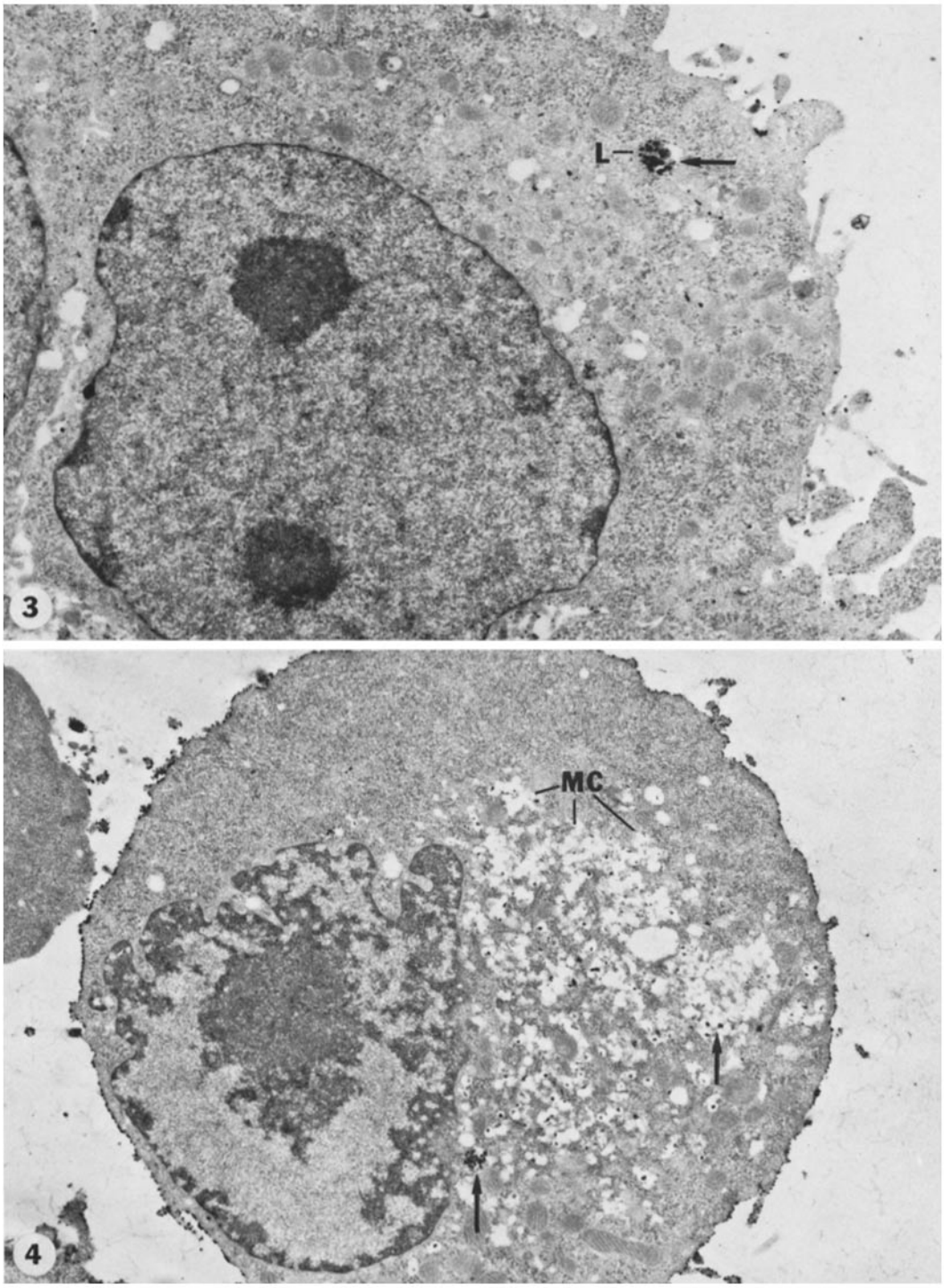

FIGURE 3 HEp-2 cell at 2 h postinfection with poliovirus. Acid phosphatase (indicated by an arrow) is shown to be localized in lysosomes (*L*) at this time as it is in uninfected cells. × 10,500.

FIGURE 4 HEp-2 cell at 5 h postinfection with poliovirus. Acid phosphatase (indicated by arrows) is shown to have been released from lysosomes and redistributed. *MC*, membranous cisternae. × 9,200.

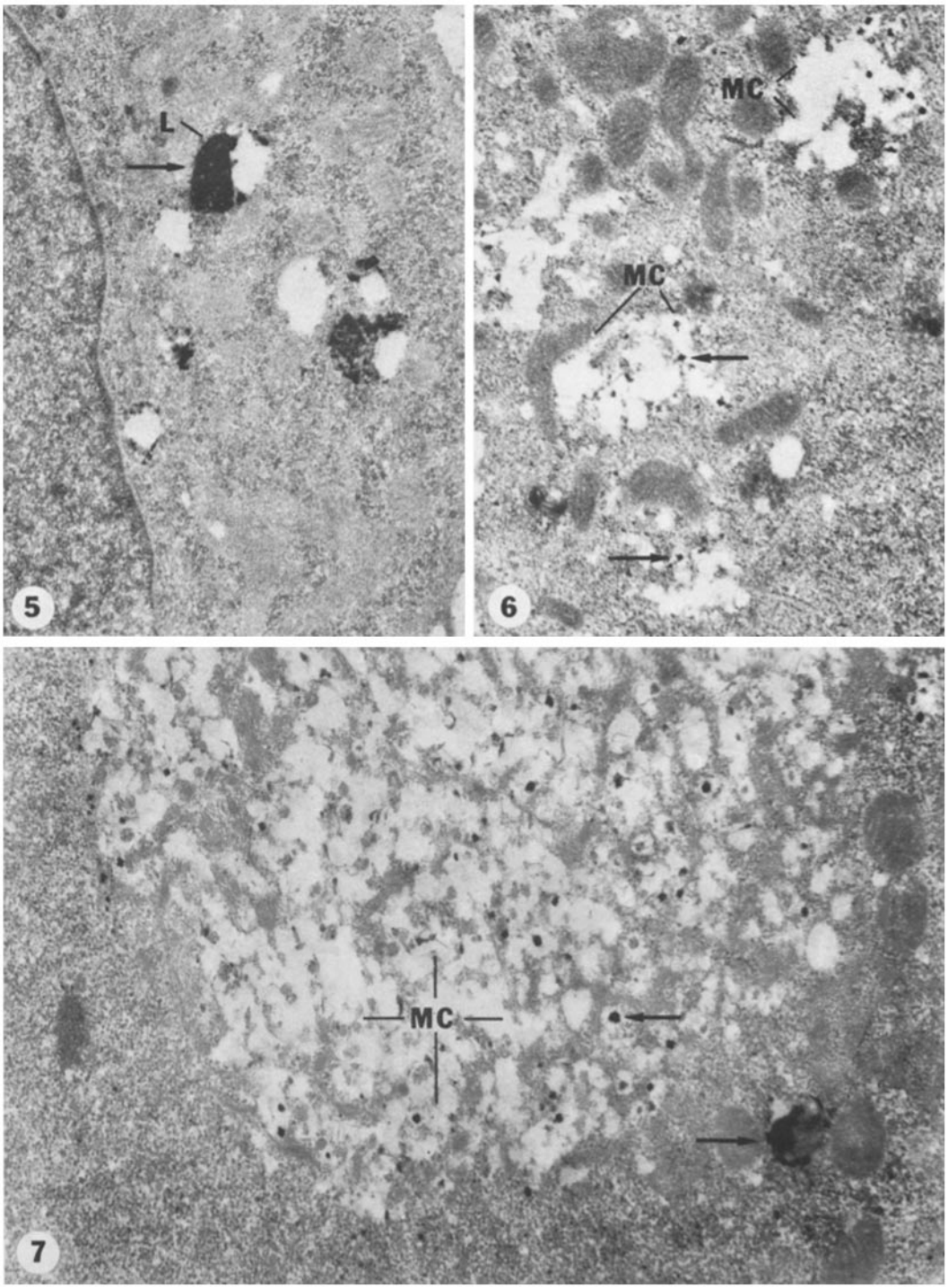

FIGURE 5 Higher magnification of HEp-2 cell cytoplasm showing acid phosphatase (indicated by an arrow) as it appears in lysosomes (*L*) after 1 h of infection with poliovirus. × 20,000.

FIGURE 6 Higher magnification of HEp-2 cell cytoplasm at 4 h postinfection with poliovirus. Note that the acid phosphatase (indicated by arrows) is initially released from each lysosome into the immediate location of the original organelle. *MC*, membranous cisternae. × 21,000.

FIGURE 7 Higher magnification of HEp-2 cell cytoplasm at 5 h postinfection with poliovirus. Note the acid phosphatase (indicated by arrows) in the region of membranous cisternae formation (*MC*) as well as in lysosomes that have not been affected at this stage of the infectious process. × 30,500.

tion of membranous cisternae are temporally and spacially related. The area of the cell with diffuse enzyme activity always contained vesicular cisternae and the enzyme appeared to be directly associated with these membranes. Probably these structures are the same as the "U bodies" reported by Kallman et al. (1958) and the membrane-bound bodies reported by Dales et al. (1965) in poliovirus-infected cells. Similar bodies were observed by Dales and Franklin (1962) in Mengo virus and encephalomycarditis virus-infected cells. The area of cisternae in Mengo virus-infected cells actually represents the proliferation of new membranes, according to Amako and Dales (1967). In support of this theory, Plagemann et al. (1970) found that in cells infected with Mengo virus there was a two- to threefold increase in the rate of choline incorporation into the phosphatidylcholine of membranes and Mosser et al. (1972 *b*) showed that a similar phenomenon occurs in poliovirus-infected cells. In addition, there is a large increase in the quantity of smooth microsomal membranes with which the synthesis of viral RNA is associated (Mosser et al., 1972 *a*).

Apparently some functional correlation exists between the formation of vesicular bodies and the redistribution of lysosomal enzyme in the poliovirus-infected cell. Amako and Dales (1967) speculated that when the hydrolases are dispelled from lysosomes they become solubilized in the cytoplasm and degrade lipid into a lysoform such as lysolecithin (the result of fatty acid cleavage from lecithin or phosphatidylcholine). The accumulation of lysolipids enhances the incorporation of these lysolipids into new membranes. Later, Dales (1969) presented another theory. He postulated that membrane proliferation after infection with virulent RNA agents is a result of release of lysolecithin which is due to a stimulation by injurious substances of the breakdown and turnover of lipids. Lysolecithin increases enzymatic activity for synthesis of phospholipid and also may act as a surfactant to rupture lysosomes. This hypothesis is supported by the fact that application of chemical and other injurious agents has elicited production of membranes. Use of toxic materials such as silica, carcinogenic dyes, and phenobarbital caused cell injury similar to that produced by small RNA viruses and also provoked proliferation of membranes.

If the latter hypothesis is true, it is probable that in poliovirus-infected cells a protein(s) synthesized under the regulation of the virus genome causes the breakdown and turnover of lipids with the consequence of labilization of lysosomes. Skinner et al. (1968) noted that in cells infected with ECHO virus 12 the development of viral biosynthesis was required for formation of membranous cisternae. In addition, when Guskey et al. (1970) studied the patterns of cytopathology and lysosomal enzyme release in poliovirus-infected HEp-2 cells with the use of inhibitors, it appeared that a virus-elicited protein produced at about 3 h PI, was responsible for lysosomal enzyme release.

Other supporting evidence for the correlation between the formation of membranous structures and lysosomal enzyme redistribution in cells infected with poliovirus was reported recently by Bienz et al. (1973). When mitotic HEp-2 cells (metaphase arrested) were infected, they produced and released the same amount of virus as randomly growing interphase cells, but there were only a few clusters of vesicles, no lytic cytopathology, and no detectable redistribution of lysosomal enzymes.

Our findings demonstrate that in poliovirus-infected cells, lysosomal enzymes are not released suddenly from the organelles into the entire cytoplasm. Instead, there is a controlled release or redistribution which occurs gradually over a period of several hours. The redistribution of enzymes upon release from lysosomes is significant since the effects of these enzymes as a whole are quite broad and may contribute to the cytopathic effect in a number of ways. Also it is clear that the redistribution is tied in closely with the formation and turnover of membranes which is regulated directly or indirectly by the nucleus.

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