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Virus Replication, Cytopathology, and Lysosomal Enzyme Response of Mitotic and Interphase Hep-2 Cells Infected with Poliovirus

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Mitotic Hep-2 cells, selected by the PEL (colloidal silica) density gradient method and held in mitosis with Colcemid, are readily infected by poliovirus type I (Mahoney). They produce and release the same amount of virus as interphase, random-growing cells. In contrast to interphase cells, mitotic cells show no detectable virus-induced cytopathic effect at the light microscopy level and only slight alterations, consisting of small clusters of vacuoles, at the electron microscopy level. Mitotic cells contain the same total amount of lysosomal enzymes per cell as interphase cells, but they display no redistribution of lysosomal enzymes during the virus infection as interphase cells do. This supports the view that lysosomal enzyme redistribution is associated with the cytopathic effect in poliovirus infection but shows that virus synthesis and release is not dependent on either the cytopathic effect or lysosomal enzyme release. The possible reasons for the lack of cytopathic effect in mitotic cells are discussed.

Papers dealing with virus-infected cells in specific phases of the growth cycle are somewhat controversial. Johnson and Holland (30) report normal poliovirus yield from cells arrested in the mitotic phase by vinblastine, in contrast to the findings of Marcus (36), who showed that poliovirus does not multiply in vinblastine-arrested HeLa cells, as measured by ^{3}H-uridine incorporation. Lake et al. (34, 35) have shown that the cytopathic effect (CPE), as measured by the dye exclusion test, is delayed in Mengo virus-infected mitotic L cells for at least 16 h, although a normal virus replication takes place. Other studies by Eremenko et al. (17, 18) demonstrated a decrease in poliovirus RNA synthesis in HeLa cells during the mitotic phase. The present paper correlates the study of poliovirus-induced CPE, as measured by different methods, and virus synthesis in Colcemid-blocked, mitotic Hep-2 cells, as compared to interphase cells.

For measurement of the CPE, some of the various methods available include: (i) light microscopy (LM) appearance of stained or unstained infected cells, which readily reveals nuclear changes (4); (ii) the dye exclusion test (41), used as a measure of the permeability of the cell wall and therefore as a marker for CPE; (iii) electron microscopy (EM) examinations, which are described in some detail in the case of enterovirus-infected cultured cells by several authors (3, 15, 16, 25, 28, 29, 37, 46); (iv) the redistribution of lysosomal enzymes in infected cells, as a biochemical measure of CPE (21, 24, 50). This method represents a quantitative approach and measures the redistribution of hydrolases which may induce cellular damage. Light microscopy, using cytochemical measurement of specific lysosomal enzymes, can also demonstrate the degree of permeability of the lysosomal membrane in virus-infected cells (2; S. K. Dusing and D. A. Wolff, Bacteriol. Proc., p. 187, 1969).

In the present investigation, we utilized morphological and biochemical methods to examine cytopathology in mitotic and interphase, infected and noninfected, Hep-2 cells. There are several methods described for synchronizing cells or preparing more or less pure cultures of mitotic cells (for review see 38, 39, 49). For accumulating the large number of pure mitotic cells necessary for biochemical and morphological studies and virus titration at the same time, the PEL density gradient as described by Wolff and Pertoft (51) proved to be particularly suitable, especially if used in conjunction with the Colcemid treatment of cells.

MATERIALS AND METHODS

Preparation of mitotic cells. Hep-2 cells were grown in Eagle minimum essential medium with Spinner salts (MEM-S, Grand Island Biologicals, Grand Island, N.Y.), and supplemented with 10% calf serum in 500-ml suspension cultures on the gyratory shaker at 36 C, at cell concentrations

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between 0.5×10^6 and 1.2×10^6 cells/ml. Five hours prior to collecting mitotic cells, 0.05 μg of Colcemid per ml (a gift from Ciba Industria Chimica, S.p.A., Milano) was added. The culture usually showed a mitotic index of 18% after that amount and time of treatment. Mitotic cells were separated from the interphase cells by means of the PEL gradient as described by Wolff and Pertoft (51). The PEL gradient medium was prepared as previously described (51) by mixing 17.5 ml of dialyzed Ludox HS 40 (a gift from Dupont de Nemours & Co., Wilmington, Del.) (43.3%, wt/vol), 26.8 ml of dialyzed 19.6% (wt/vol) polyvinyl-pyrrolidone (40,000 mol wt, Sigma Chemical Co., St. Louis, Mo.), 42 ml of double-strength MEM-S, enough 1 N HCl to adjust the pH to 7.2, and water up to 100 ml. Colcemid was added to a final concentration of 0.05 μg/ml. The density of the final mixture was 1.059 gm/cm³. The gradient was formed in a no. 42 rotor (Beckman Instruments, Palo Alto, Calif.) at $53,664 \times g$ for 45 min using either a Beckman L3-40 or L2-65B ultracentrifuge. For separation and collection of mitotic-phase cells, 3.5×10^8 cells previously exposed to 0.05 μg of Colcemid per ml for 5 h were pelleted and carefully layered on top of one preformed gradient. The tubes were then centrifuged for 25 min at $1,500 \times g$ in the International model CS centrifuge (International Equip. Co., Needham Heights, Mass.), and the mitotic cells were harvested from the top layers (density 1.040 to 1.052 gm/cm³) of the gradient. Usually 5×10^6 mitotic cells per gradient were obtained, with 80 to 90% of the cells in mitosis. Mitotic indices were counted after staining the cells with a 0.022% solution of crystal violet in acetate buffer, pH 5 (45, 51).

Mitotic cells were used for the experiment after being washed twice with MEM-S and resuspended in fresh MEM-S containing 0.05 μg of Colcemid per ml. Interphase cells were taken from random-growing suspension cultures containing 2 to 4% mitotic cells.

Virus infection. Mitotic or interphase cells were infected with poliovirus type I (Mahoney) and a multiplicity of 100 plaque-forming units (PFU) per cell for 30 min at 36 C, washed twice, and resuspended in MEM-S containing 0.05 μg of Colcemid per ml at a concentration of 2.5×10^5 cells/ml in small, 20-ml cultures in 125-ml tubes agitated on the gyratory shaker at 36 C.

Harvest and examination of cells. At different intervals after poliovirus infection, cells were harvested by centrifugation, and samples were examined for the distribution of the lysosomal enzymes beta-glucuronidase (EC 3.2.1.31) (1) and beta-glucosaminidase (EC 3.2.1.29, chitobiase) (10). This distribution was measured by determining the percent content of lysosomal and extralysosomal (cytoplasmic) fractions of the cells after Dounce "T" homogenization and separation as described previously (24). Other cell samples were used for Giemsa staining, mitotic index determination, and virus titration. For electron microscopy, cells were fixed in 6% glutaraldehyde and postfixed in 2% osmium tetroxide, followed by embedding in Epon 812. Sections were cut on a Porter-Blum-MT-1-ultramicrotome (Ivan Sorvall, Norwalk, Conn.) and examined in a Hitachi HS 8-S electron microscope.

RESULTS

Light microscopy. Cultures of interphase, as well as mitotic-phase Hep-2 cells were infected with a multiplicity of 100 PFU/cell, as described above, and stained with Giemsa. Figure 1 shows interphase Hep-2 cells from an uninfected suspension culture. This appearance is not changed by Colcemid treatment (0.05 μg/ml) for 13 h, as shown by the cell in the lower left corner of Fig. 2 (which is not in metaphase arrest, as are the remaining cells) 8 h after selection of the mitotic cells with the PEL gradient. Chromosomes are not readily visible in the mitotic cells because prolonged Colcemid treatment causes clumping of chromosomes. Figure 3 shows the poliovirus-induced CPE (8 h postinfection [PI]) in interphase Hep-2 cells. The nucleus is somewhat smaller, apparently shrunken, and shows broader (blue stained) threads of chromatin than do the uninfected cells. This corresponds to the "condensed chromatin" seen in electron micrographs of similarly infected cells. The cytoplasm contains a well-defined, light (blue) stained region which is always adjacent to the nucleus. In Fig. 4, the cell marked "M" is a mitotic cell, and, although poliovirus-infected for 8 h (as it will be proved later), does not show any sign of a CPE on examination by light microscopy. The cell marked "I" shows the characteristic poliovirus-induced CPE. This cell is an interphase cell in a culture with a mitotic index of 90%. The picture shows that Colcemid treatment does not affect the formation of CPE in interphase cells. It was consistently found that all interphase cells in such cultures showed CPE, but there was never any sign of CPE in a mitotic cell. Also, the total cell count of the mitotic cell culture remained constant up to 12 h PI.

Electron microscopy. Since the poliovirus infection of metaphase-arrested cells does not result in CPE observable at the light microscopy level, a series of electron microscopy investigations was undertaken to look for ultrastructural changes. Figure 5 shows the ultrastructure of an interphase Hep-2 cell 8 h after infection with poliovirus. Very similar or identical findings have been reported in a variety of cells infected with different enteroviruses (3, 15, 16, 25, 28, 29, 37, 46). The cytopathic changes include margination

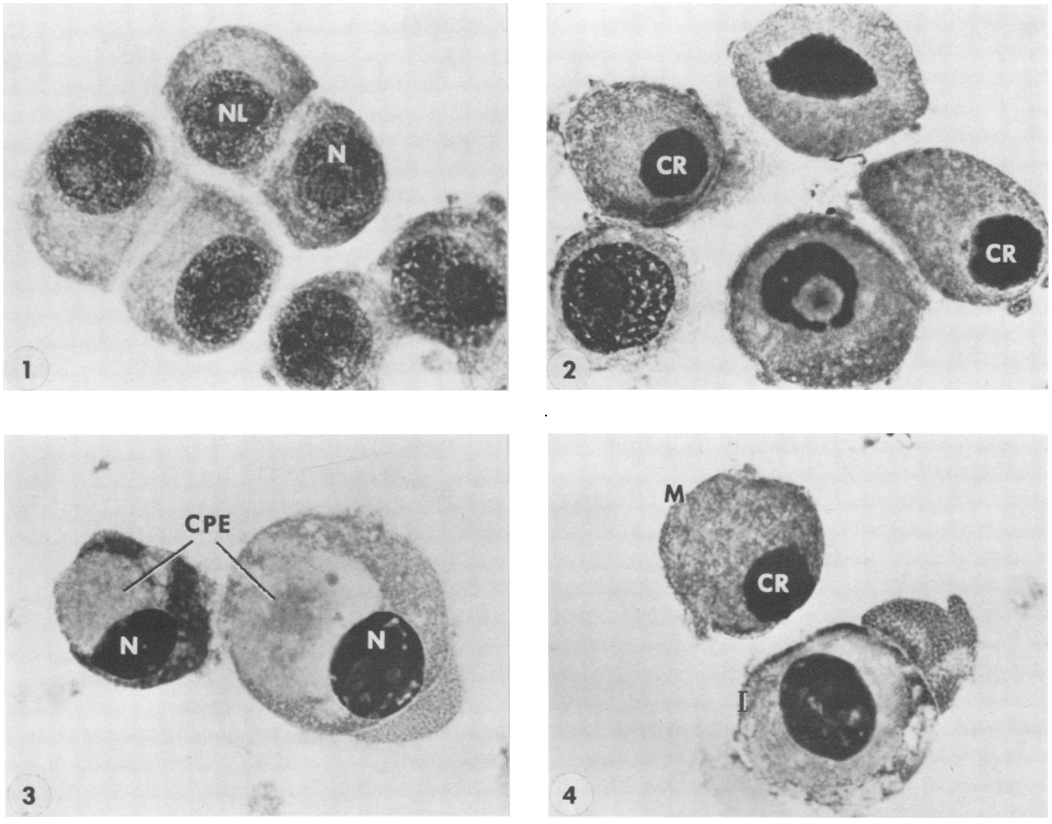


FIG. 1. *Normal, control Hep-2 cells from a suspension culture. Note the round nucleus (N) with well developed nucleolus (NL). Cytoplasm homogenously stained. Giemsa stain, ×960.*

FIG. 2. *Uninfected Colcemid-treated cells accumulated by the PEL gradient method. Mitotic cells show condensed chromosomes (CR) and homogeneous cytoplasm. The culture contained 90% mitotic cells. Lower left corner: interphase cell. Giemsa stain, ×960.*

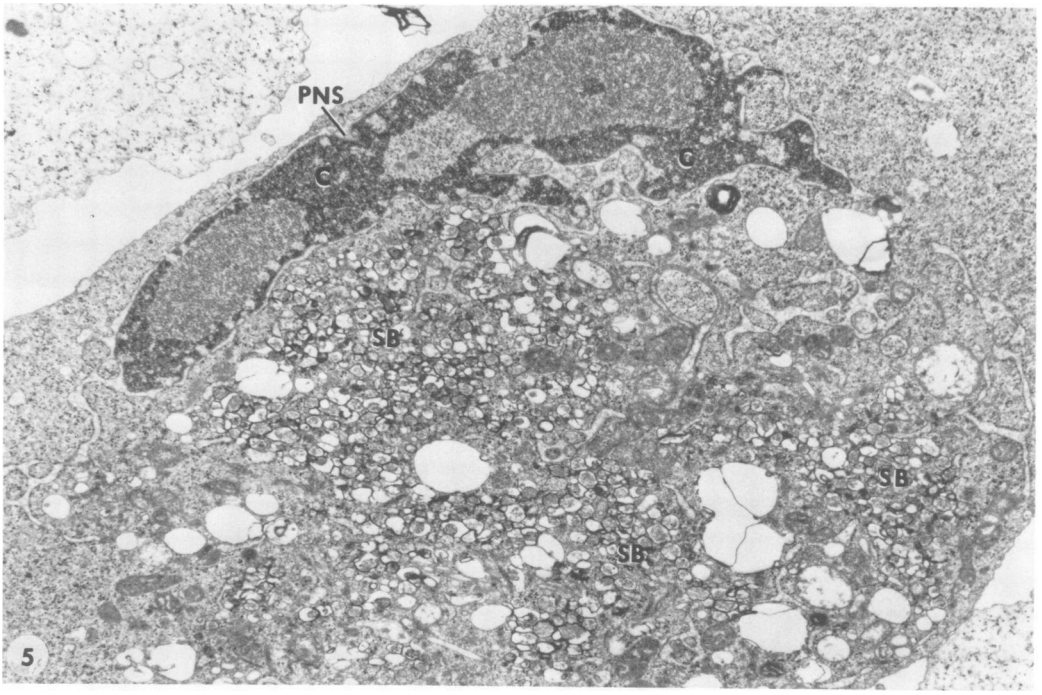
FIG. 3. *Poliovirus-infected interphase cells 8 h postinfection. Note the condensed chromatin in shrunken nucleus (N). The cytoplasm shows a lighter (blue) stained area adjacent to the nucleus. This area represents the pathologically altered part of the cell (CPE) (compare with Fig. 5). Giemsa stain, ×960.*

FIG. 4. *Poliovirus-infected cells at 8 h postinfection from a culture with 90% mitotic cells, same treatment as in Fig. 2. Upper left: mitotic cell (M), no CPE visible, condensed chromosomes (CR). Lower right: interphase cell (I), showing the same typical CPE as the interphase cells in Fig. 3. Giemsa stain, ×960.*

and condensation of the chromatin in the nucleus and an enlargement of the perinuclear space. The most obvious change is the formation of numerous vacuoles (small bodies; reference 16). The well-defined region of the small bodies is always adjacent to the nucleus and corresponds to the region marked CPE in Fig. 3.

Changes in mitotic cells after poliovirus infection are far less extensive than in interphase cells (Fig. 6). The changes in ultrastructure due to the poliovirus infection consist of rather small clusters of vacuoles, canaliculi, and membrane-bounded bodies, but are located throughout the cytoplasm rather than in one juxtanuclear area as with the interphase cell. The prominent endoplasmic reticulum-like structures observed in Fig. 6 are also found in uninfected, nonarrested mitotic HeLa cells (19). This picture was taken at 8 h PI, and no further ultrastructural changes were observed at 12 h PI. After 17 h of exposure (12 h PI) to the Colcemid at 0.05 μg/ml, all cells began to die (both infected and uninfected), as measured by dye exclusion, thus preventing further observation.

Figure 7 shows a mitotic, uninfected cell treated with Colcemid for 13 h. The cell shows the same long and concentrically arranged tubules of endoplasmic reticulum (ER) as the cell in Fig. 6 but no clusters of small bodies. The chromosomes are less condensed than those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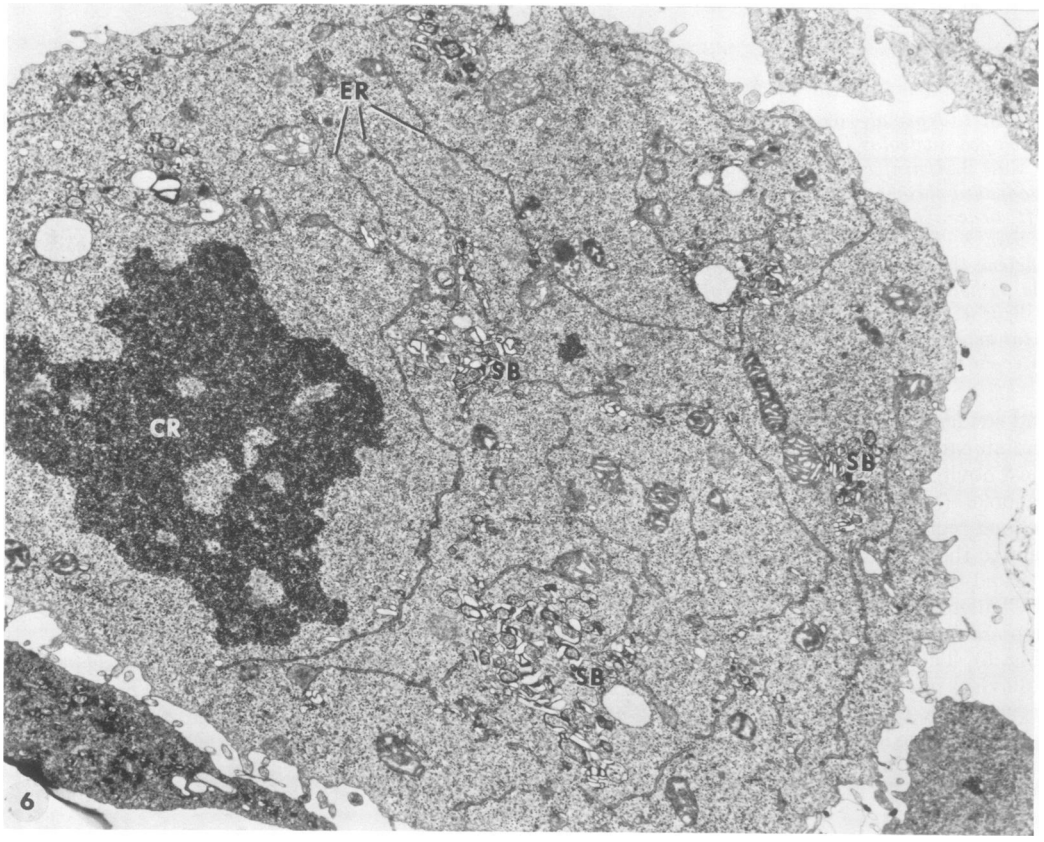
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observed in Fig. 6, but the degree of chromosomal condensation may vary from cell to cell, infected or not, and is due to the Colcemid treatment.

Figure 8 shows an interphase cell, not yet blocked in metaphase, although treated for 13 h with Colcemid. It demonstrates typical poliovirus-induced CPE with a segregated nucleolus and numerous small bodies. The chromatin appears somewhat less condensed than in Fig. 5, due to the plane of sectioning. The long strands of ER are a consequence of the Colcemid treatment (19). This indicates that the Colcemid was taken up by interphase cells and that the drug did not interfere with the development of CPE.

Lysosomal enzyme redistribution. As shown by several authors (21, 24, 50), there is a redistribution (release) of lysosomal enzymes in the cytoplasm of poliovirus-infected cells. This redistribution of enzymes is thought to be the cause of, or at least associated with, the cytopathic changes observed in those cells during infection. Since the poliovirus-infected mitotic cells showed no CPE by light microscopy and only slight vacuolation in the electron microscope, we investigated the lysosomal enzyme distribution in cultures of mitotic-phase and interphase Hep-2 cells infected with poliovirus. The results are shown in Fig. 9, where the mitotic cells show no significant redistribution of lysosomal enzymes. Interphase cells, however, show a distinct lysosomal enzyme redistribution, parallel to the development of the CPE as observed in either the light or electron microscope. Percent values of enzyme redistribution (release) and the time course were in close agreement with previously reported data (24).

Determinations of the total cellular beta-glucuronidase content showed that mitotic cells, infected or not, contain similar amounts to interphase cells. The beta-glucuronidase values of infected and uninfected mitotic cells are 38.5 ($\pm$7.1) Fishman units per 10^6 cells, whereas uninfected, interphase cells contained 42.6 ($\pm$0.93) units per 10^6 cells. Infected, interphase cells contained 21.7 ($\pm$4.8) units per 10^6 cells, a value which probably reflects leakage of enzyme out of the cell into the culture fluid as a consequence of cellular breakdown.

Virus production and release. Both cell-associated and free virus in cultures of mitotic and interphase cells was measured by plaque titration of samples harvested at various times after infection (Fig. 10). In both mitotic and interphase cell cultures, intracellular virus accumulated in the first 8 h PI. The newly formed viruses were released rather rapidly as shown by the increase in extracellular virus titer.

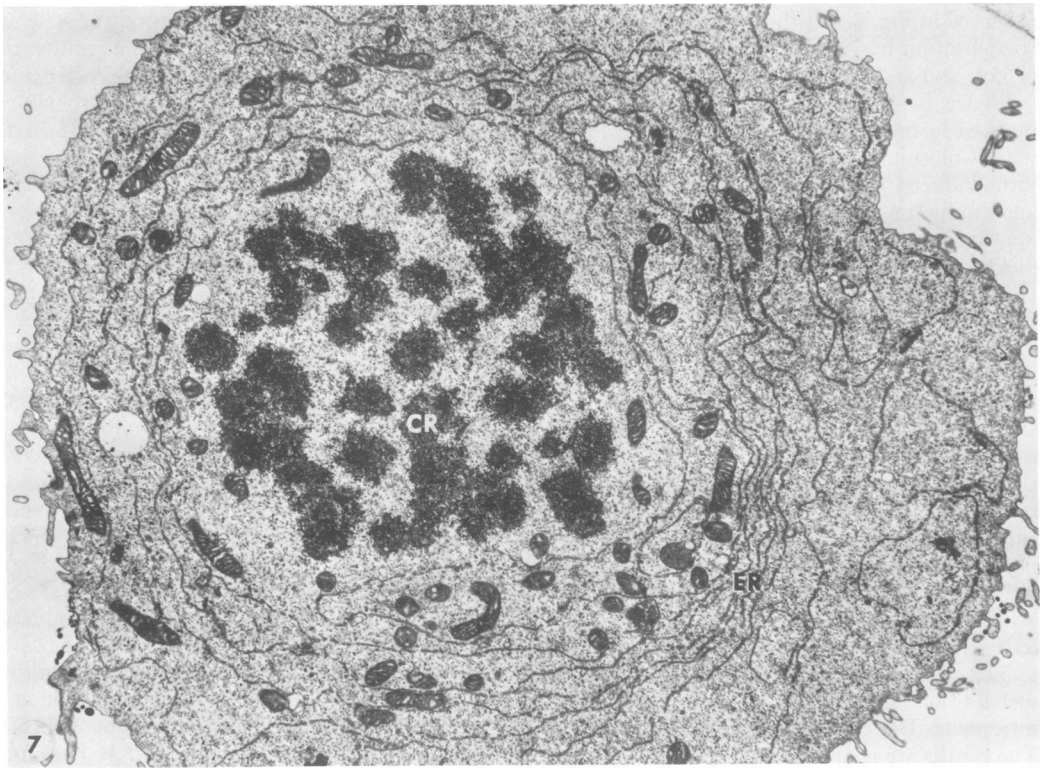
Controls. Control experiments were performed to elucidate possible effects of Colcemid or the PEL gradient mixture on virus production, virus release, and enzyme release. The influence of Colcemid was tested by adding 0.05 μg/ml to interphase Hep-2 Spinner cultures 2 and 6 h before infection with poliovirus and by determining virus production at 8 h PI (Table 1). The influence of the PEL mixture was tested by adding interphase cells to this mixture in the same proportion as used for a gradient and holding the mixture at room temperature for 2 h. The cells were then washed twice and infected with poliovirus and harvested at 8 h PI. Beta-glucuronidase redistribution and virus production were measured in the infected cells, as well as the Colcemid-treated and untreated control cultures (Table 1). No differences were observed in total virus production per cell in these different cultures or in the CPE of individual cells, as monitored with the Giemsa stain. Also, no significant differences could be detected in total content of the lysosomal enzyme, beta-glucuronidase. However, Table 1 shows that Colcemid slightly depresses the virus-induced enzyme release, but at the same time somewhat enhances virus release. The PEL-gradient mixture, on the other hand, has no significant effect on virus release but slightly enhances virus-induced enzyme redistribution.

DISCUSSION

This study shows that mitotic (metaphase-arrested) Hep-2 cells are readily infected by poliovirus and produce and release essentially the same amount of virus as cultures of interphase cells, similar to the finding of Johnson and Holland (30). Our results with mitotic-phase Hep-2 cells show no lysosomal enzyme redistribution and no CPE, at least at the light microscope level, during the period of poliovirus synthesis. This absence of CPE is similar to the results of

FIG. 5. *Electon micrograph of a poliovirus-infected interphase cell at 8 h postinfection. Shrunken nucleus with condensed chromatin (C), enlarged perinuclear space (PNS), juxtanuclear region (corresponding to region "CPE" in Fig. 3) with many small vacuoles (small bodies [SB]).* $\times$*10,000.*

FIG. 6. *Electron micrograph of a poliovirus-infected mitotic cell at 8 h postinfection. No nuclear membrane, but condensed chromosomes (CR) and prominent endoplasmic reticulum (ER). Poliovirus-induced changes consist only in small peripheral clusters of "small body"-like vacuoles (SB).* $\times$*9,000.*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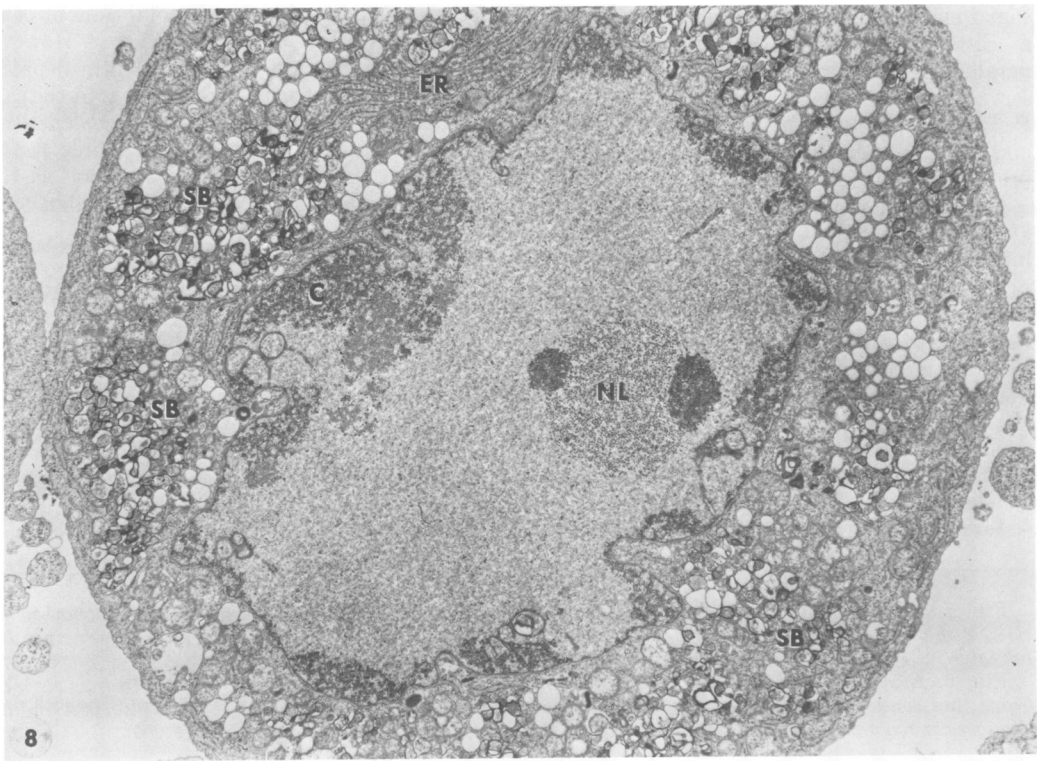
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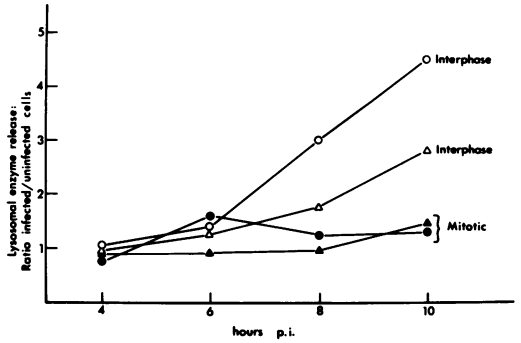

FIG. 9. *Lysosomal enzyme redistribution in poliovirus-infected, interphase cells (open symbols) and mitotic cells (filled symbols). The percent of enzyme release from lysosomes in infected and non-infected cultures was determined as described earlier (24), and is presented as the ratio of the percent redistribution values for infected to uninfected cultures. Symbols;* ○, ●, *beta-glucuronidase;* △, ▲, *beta-glucosaminidase.*

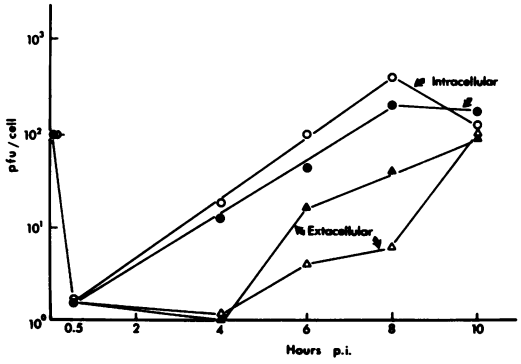

FIG. 10. *Replication of poliovirus in mitotic and interphase Hep-2 cells. Symbols: circles, intracellular virus; triangles, extracellular virus; open symbols, interphase cells; closed symbols, mitotic cells.*

Lake et al. (34, 35) who described an absence of CPE, as measured by dye exclusion, in mitotic-phase L cells infected with Mengo virus. Using the electron microscope, however, certain cytoplasmic changes induced by the virus infection can be detected (Fig. 6). We have interpreted these changes, i.e., the clusters of small vacuoles, as structures involved in virus replication rather than as primarily degenerative cytopathic changes. It has been proposed by several authors that poliovirus replicates within each membranous structure of interphase cells (6, 11, 12, 13, 16, 23, 28, 46), but that this also holds true for mitotic cells remains to be proved.

Our Colcemid-blocked, mitotic cultures showed a limited life span, since after 17 h of Colcemid treatment, infected and noninfected cultures died off rather rapidly, apparently due to the toxicity of Colcemid. It was therefore not possible to observe if a poliovirus infection in mitotic cells leads to severe cytopathic changes and eventually cell death later than about 12 h PI, as Lake et al. (34, 35) showed with Mengo virus in mitotic L cells. We cannot agree with their conclusion that lysosomes are not involved in virus-induced cytolysis, for in our system alterations as observed in interphase cells were temporally correlated with lysosomal enzyme redistribution, whereas the limited vacuolation (and lack of cytolysis) in mitotic cells was not accompanied by such enzyme release. This parallelism of severe cellular alteration and enzyme release indicates, but does not prove, involvement or at least an association of lysosomal enzyme redistribution with poliovirus-induced CPE.

The virus release from the infected cell does not parallel the development of CPE and lysosomal enzyme redistribution. Mitotic cells, without CPE, released their newly synthesized virus at about the same rate as interphase cells. From this finding it can be concluded that there must be some kind of a mechanism for the release of newly formed viruses from intact, mitotic cells, which does not depend on cell death and membrane disruption as a mechanism for virus release. We have not yet been able to detect this hypothetical mechanism, but as various other enteroviruses interact with cells in a similar manner, it may well be a similar mechanism as described previously for coxsackievirus in newborn mouse muscle (7).

The mode of action of Colcemid is by an inhibition of spindle fiber formation and function in mitotic-phase cells (8, 9, 47). Colcemid is readily taken up into interphase cells (47) and causes changes in the endoplasmic reticulum (19) and in the location of lysosomes within the cytoplasm (43). Our control experiments with Colcemid-treated interphase cells show clearly that

FIG. 7. *Electron micrograph of an uninfected, Colcemid-arrested mitotic cell. No nuclear membrane, (CR = chromosomes, ER = concentric prominent endoplasmic reticulum).* ×7,000.

FIG. 8. *Electron micrograph of a Colcemid-treated, interphase cell, 8 h after poliovirus infection from a culture with 90% mitotic index. Note the typical poliovirus-induced CPE with condensed chromatin (C), segregated nucleolus (NL), and "small body"-type vacuoles (SB). Prominent endoplasmic reticulum (ER) is a result of Colcemid treatment.* ×7,000.

TABLE 1. *Redistribution of beta-glucuronidase and poliovirus release in Colcemid- and PEL-treated, interphase cells 8 h after poliovirus infection*

Treatment	Poliovirus infection[a]	MI[b]	Beta-glucuronidase redistribution		Virus release (% of total virus produced at 8 h postinfection)
			% of total in extralysosomal spaces of cell	Ratio[c] of infected to uninfected	
Colcemid 2 h before infection	I	6	31.6	2.16	33.2
	N	15	14.6		
Colcemid 6 h before infection	I	12	28.0	1.74	34.8
	N	17	16.1		
PEL mixture 2 h before infection	I		54.2	3.30	21.3
	N		16.4		
None	I		44.2	2.90	17.9
	N		15.2		

[a] I, Infected; N, noninfected.
[b] Mitotic indices (MI) measured after 8 h of poliovirus infection.
[c] This is the ratio of the percentage of total cellular beta-glucuronidase which is found in extralysosomal spaces of infected cells to the percentage measured in uninfected cells.

this compound does not interfere with normal virus production and only slightly inhibits lysosomal enzyme redistribution (Table 1). This effect was somewhat counteracted by the PEL medium which caused a small increase in enzyme redistribution in infected cells. The lack of severe cellular alterations in mitotic cells can therefore be attributed to the mitotic state of the cell and not to the treatment.

The reason for this modified cellular response might be found in an altered metabolism of the mitotic cell as compared to the interphase cell. DNA-dependent RNA synthesis in the mitotic cell is greatly reduced (5, 14, 30, 31, 33, 40, 42) as well as protein synthesis (26, 30, 33, 42) (for review see ref. 38). It is unlikely, however, that reduced DNA-dependent RNA synthesis is the reason for the observed lack of CPE, since actinomycin D has neither an altering effect on poliovirus synthesis, CPE, nor lysosomal enzyme redistribution in infected cells (21, 36; Guskey and Wolff, Bacteriol. Proc., p. 173, 1971).

If cellular protein synthesis is necessary to initiate CPE, it remains to be proved. If so, this cellular synthetic activity must occur early in infection, for, later in the infection, cellular protein synthesis is shut down due to the virus infection itself (21). It has also been proposed that a new protein is formed or made available early in the infection (after 1.5 and before 4 h PI) which initiates CPE (24, 48). This protein might be missing in mitotic cells.

The reduced protein synthesis during mitotis is apparently controlled at the level of translation (20, 27, 44), where the ribosomes are altered in such a way (44) that they are no longer able to combine with mRNA to form polysomes. This ribosomal change would probably affect only the combination with cell mRNA and not with the viral message, for, in our system, a normal amount of viral replication takes place and therefore the ribosomes are able to combine with virus mRNA.

The lack of severe (gross) virus-induced changes in mitotic-phase cells is not due to the absence of lysosomal enzymes, as shown above, but the possibility remains that, in our system, the transport of virus-induced materials to the lysosomes is altered or that the lysosomes themselves are changed in such a way that no lysosomal enzyme redistribution can take place and therefore no CPE develops. Although the biochemical explanation of cytolytic CPE is not yet complete, we feel that the use of mitotic-phase cells offers a new approach to the investigation of the underlying mechanism.

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