JOURNAL OF VIROLOGY, Jan. 1981, p. 268-273
0022-538X/81/010268-06$02.00/0

Vol. 37, No. 1

Monovalent Cation Metabolism and Cytopathic Effects of Poliovirus-Infected HeLa Cells

C. N. NAIR

Department of Cell and Molecular Biology, Medical College of Georgia, Augusta, Georgia 30912

To better understand the significance of $^{22}Na^+$ accumulation by poliovirus-infected HeLa cells (C. N. Nair, J. W. Stowers, and B. Singfield, J. Virol. **31**:184, 1979), measurements of cellular Na^+, K^+, and Cl^- contents, volume, and density were carried out at intervals after infection. In addition, the rates of $^{22}Na^+$ washout from infected and control cells were determined. Starting at around 3 h postinfection, the Na^+ content of infected cells increased, whereas the K^+ content decreased progressively, resulting in a net loss in the monovalent cation content per cell. The loss in cellular chloride content exceeded that in monovalent cation content. The kinetics of $^{22}Na^+$ washout from infected and control cells revealed the presence of an extra Na^+ compartment in infected cells. A net loss in the monovalent cation activity of infected cells was indicated by the loss of cell water as reflected in a decrease in cell volume and an increase in cell density. In spite of a net loss in monovalent cation content per cell, Na^+ accumulation coupled with cell shrinkage resulted in substantial increases in the concentrations of not only Na^+ but also K^+. The results suggested a possible role for tonicity change in the morphological lesions of poliovirus cytotoxicity.

Cell killing by viruses is associated with inhibition of cellular macromolecular synthesis and changes in cellular morphology. The morphological changes are not direct consequences of viral inhibition of host macromolecular synthesis (3, 12). The disorganization of cellular cytoskeletal framework appears to be involved in the morphological changes induced by poliovirus (13), Newcastle disease virus, and vesicular stomatitis virus (VSV) (17). There have been a few reports documenting changes in cation metabolism of virus-infected cells (5, 7-11, 14) and implicating cation alterations in virus cytotoxicity (7, 11, 14). In Sindbis virus-infected chicken embryo fibroblasts, the changes in monovalent cation concentration correlate well with virus shutoff of cellular protein synthesis and presumably facilitate selective synthesis of viral proteins (11). On the other hand, in VSV-infected L-cells (9), mengovirus-infected ascites tumor cells (7), and poliovirus-infected HeLa cells (14), the cation changes occur only after virus inhibition of host protein synthesis.

We have been interested in the possibility that cation alterations are involved in the morphological lesions associated with poliovirus cytotoxicity. This suspicion is based on our previous observation that poliovirus-infected HeLa cells accumulate $^{22}Na^+$ during the late phase of virus replication, after virus shutoff of host protein synthesis and in coincidence with the appearance of cytopathic effects (14). To evaluate this possibility, it was first necessary to determine whether $^{22}Na^+$ accumulation by virus-infected cells was a reflection of increased Na^+ content and whether it was accompanied by changes in cellular tonicity. The results of experiments bearing on these questions are presented in this communication.

MATERIALS AND METHODS

Virus and cells. Poliovirus type 2 and monolayer cultures of HeLa Ohio cells were used in these studies. Growth of cells and virus was in minimal essential medium containing 5% calf serum and antibiotics. The absence of mycoplasma contamination of cells was determined periodically (14). Unless otherwise stated, replicate monolayer cultures in 35-mm Corning plastic dishes containing 2×10^6 to 3×10^6 cells per dish were used in these experiments. The cultures were infected, at an input multiplicity of ~100 PFU/cell, by incubation with virus for 30 min at 37°C.

Percoll was purchased from Pharmacia Fine Chemicals, and isotone was purchased from Scientific Products. $^{22}NaCl$ was purchased from New England Nuclear Corp. *carboxyl*-^{14}C-inulin was a generous gift of Frederick Leibach.

Measurement of Na^+, K^+, and Cl^- contents. Replicate, infected, and control cultures in 60-mm Corning plastic dishes (5×10^6 to 8×10^6 cells per dish) were incubated with growth medium at 37°C. At intervals thereafter, one each of infected and control cultures was rapidly washed twice with warm isotonic sucrose (2.5 ml per dish per wash). The cells were harvested by scraping with a rubber policeman, suspended in water, and homogenized by forcing through

a syringe needle. The homogenate was lyophilized, and the residue was extracted by overnight incubation with 0.1 N HNO_3. The Na^+ and K^+ contents of the clarified extracts were measured with a flame photometer, and the Cl^- content was measured with a chloridometer by using standard procedures. On replicate cultures, the cell counts were determined with a Coulter Counter.

Determination of $^{22}Na^+$ washout rates. Replicate, infected, and control cultures were incubated with growth medium containing $^{22}NaCl$ (1 μCi/ml). At 4 h postinfection (p.i.) the radioactive medium in the dishes was replaced with nonradioactive warm medium (2.5 ml/35-mm dish), and the incubation was continued. At intervals, the rinse medium was removed from pairs of cultures, and the radioactivities of the rinse medium and the monolayers were assayed by scintillation counting (14). The background radioactivity due to radioactive medium trapped on cell surfaces, etc., was obtained as follows. Growth medium containing a known concentration of ^{14}C-inulin was added to replicate cultures (incubated in parallel with the experimental dishes) and immediately removed from them (there was no uptake of ^{14}C-inulin by infected or control cells under these conditions). The residual ^{14}C radioactivity in the monolayers was assayed by scintillation counting after alkaline (0.33 M KOH) solubilization of the cell contents and neutralization. From the radioactivity measurements, the volume of medium trapped by the monolayers was calculated. $^{22}Na^+$ radioactivity contained in this volume of rinse medium was subtracted from cell-associated $^{22}Na^+$ radioactivity.

Percoll-gradient sedimentation of cells. Discontinuous Percoll gradients were prepared by successively layering in cellulose nitrate tubes 54, 45, 36, and 27% (vol/vol) Percoll in Hanks balanced salt solution. Infected cultures at different stages of infection (see Fig. 3) and control cultures (~7.5×10^6 cells per 60-mm dish) were gently trypsinized and well dispersed in warm 18% (vol/vol) Percoll in Hanks balanced salt solution. The cell suspensions were layered on top of the gradients, and the gradients were centrifuged at 10,000 rpm in a Beckman SW50.1 rotor at 25°C for 15 min. The cell bands were visually located.

Measurement of cellular volume. The cells were harvested by scraping with a rubber policeman, suspended in warm medium, and well dispersed by gentle pipetting. Cell volume was determined electronically by using a Coulter Counter (Model ZB) connected to an automatic cell size analyzer. The instrument was calibrated with 18.12-μm-diameter polystyrene beads. Before volume measurements, the cell suspensions were diluted with isotone, which is buffered saline containing Na^+ (180 meq/liter), K^+ (5.1 meq/liter), EDTA (1.0 mmol/liter), and 2-phenoxyethanol (preservative). Volume analysis was carried out on cell suspensions in isotone containing approximately 10,000 cells per ml.

RESULTS

Na^+ and K^+ contents of infected cells. To determine whether the amount of Na^+ or K^+ per cell varied during the course of infection, cultures were extracted with 0.1 ml of 0.1 N HNO_3 at intervals after infection, and the concentrations of Na^+ and K^+ in these extracts were assayed by flame photometry. The results, expressed as concentrations per 0.1 ml of extracts, (Fig. 1) indicate that starting around 3 h p.i. there was an increase in the relative Na^+ content and a reciprocal decrease in the relative K^+ content of infected cells. There was no significant variation in the monovalent cation contents of uninfected cells incubated in parallel with the infected cells (unpublished data). The above results confirm that $^{22}Na^+$ accumulation by poliovirus-infected HeLa cells observed in an earlier study (14) was a reflection of a true increase in the Na^+ content of these cells.

Chloride content versus Na^+ plus K^+ content. As infection progressed, it appeared that the K^+ loss exceeded the Na^+ gain (Fig. 1). It was of interest to find out whether these changes in the cation contents caused alterations in the concentration of "free" cations and thereby affected cellular tonicity. To look at this possibility, the Cl^- and Na^+ plus K^+ contents of infected and control cells were assayed at intervals during incubation. The rationale was that if the Cl^- content changed along with the Na^+ plus K^+

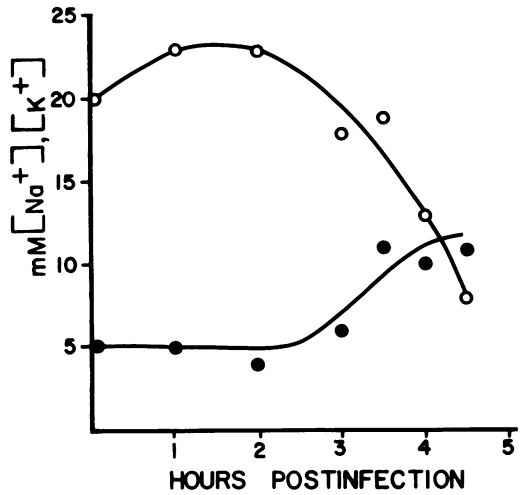

FIG. 1. *Na^+ and K^+ contents of poliovirus-infected HeLa cells. To measure Na^+ and K^+ contents, replicate infected cultures were incubated with growth medium. At the intervals indicated, cultures were rapidly washed with isotonic sucrose and lysed with water. The lysates were lyophilized and extracted with dilute HNO_3. The Na^+ and K^+ contents of the extracts were assayed by flame photometry. Symbols: K^+ concentration, ○; Na^+ concentration, ●. Notice that the values are not expressed as concentrations per cell volume but rather as concentrations in 0.1 ml of HNO_3 with which 7.5×10^6 cells were extracted at each time interval.*

content, this was indicative of a change in the concentration of free Na^+ plus K^+. The results (Table 1) demonstrate that the Cl^-/Na^+ plus K^+ ratio remained unchanged for control cells but dropped progressively for infected cells. In other words, as infection progressed, there was a disproportionate loss of Cl^- from the cells.

Kinetics of $^{22}Na^+$ washout from infected and control cells. As an additional check on the possibility of increased cation "binding" in infected cells, the rates of $^{22}Na^+$ washout from infected and control cells were compared on the assumption that bound cation will wash out less readily than free cation. The results show that during the first 4 min or so the rate of $^{22}Na^+$ washout from infected cells (Fig. 2A) was slower than that from control cells (Fig. 2B). Similar results were obtained in three separate experiments. These results suggest the presence in infected cells of an extra Na^+ compartment which is clearly absent from control cells. The bottom line in Fig. 2A, representing this compartment, was obtained by subtracting, from the counts remaining after 1- and 2-min rinses, the corresponding points on the middle line, which indicates the very slowly exchanging Na^+ compartment.

Evidence for tonicity changes in infected cells. The above results suggested that the variations in the monovalent cation contents of infected cells may actually affect intracellular tonicity. Osmolarity changes will be immediately corrected for by prompt water movement across the plasma membrane. Specifically, hypotonicity would induce efflux of water, an increase in cell density, and a decrease in cell volume. Similarly, hypertonicity would effect influx of water, a decrease in cell density, and an increase in cell volume. Therefore, changes in cellular density and volume were monitored as parameters of tonicity changes.

Changes in infected-cell density. To monitor density changes, infected cells at different stages of infection and control cells were sedimented through discontinuous Percoll gradients. The results (Fig. 3) show that the control cells characteristically banded at the interface of 45 and 36% Percoll. The infected cells, on the other hand, contained populations having different densities. At 2.5 and 3 h p.i., the infected cells contained three populations, one similar in density to control cells and two lighter-density pop-

TABLE 1. *Ratio of Cl^- content to Na^+ plus K^+ content of poliovirus-infected and control HeLa cells*[a]

Time after infection (h)	Cl^-/Na^+ plus K^+ ratio	
	Control cells	Infected cells
1	0.59	0.56
2	0.55	0.48
3	0.57	0.47
4	0.56	0.44
5	0.54	0.36

[a] To obtain Cl^-/Na^+ plus K^+ ratios, HNO_3 extracts (0.1 ml) of cultures (8×10^6 cells per culture) were prepared, and the Na^+ plus K^+ and Cl^- concentrations in each extract were assayed as described in the text.

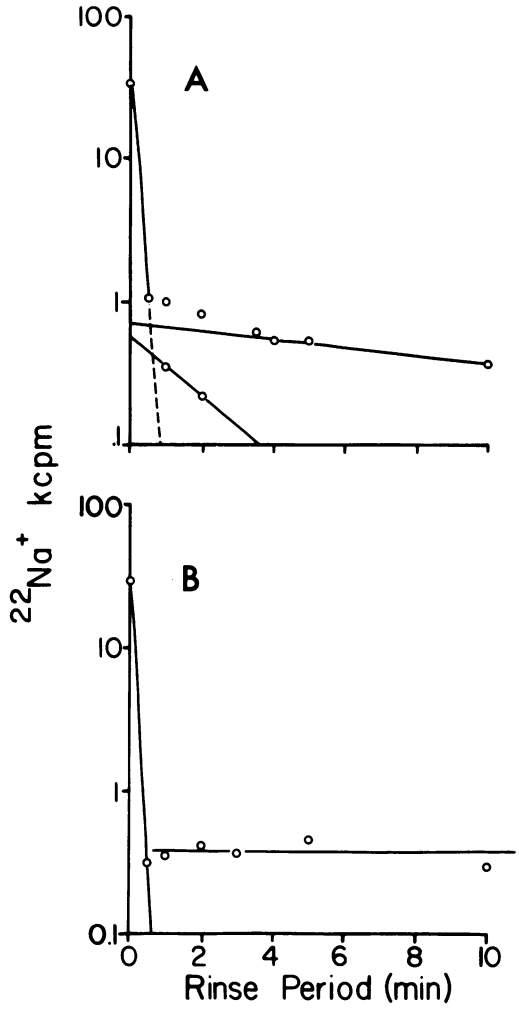

FIG. 2. *Kinetics of $^{22}Na^+$ washout from poliovirus-infected (A) or control (B) HeLa cells. Replicate, infected, or control cultures were incubated with growth medium containing $^{22}NaCl$. At 4 h p.i., the radioactive medium was replaced with fresh warm medium (rinse). At intervals, the rinse medium was removed from pairs of cultures, and the radioactivity of the rinse medium and the cultures was assayed. The background radioactivity was determined and subtracted from cell-associated radioactivity. See the text for details of procedures.*

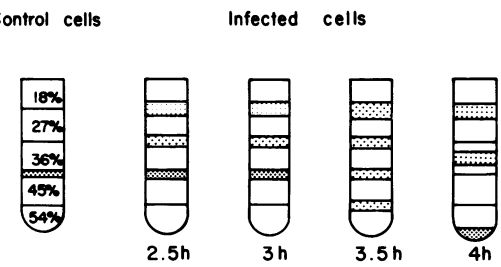

FIG. 3. *Percoll-gradient sedimentation of uninfected or poliovirus-infected HeLa cells. Discontinuous Percoll gradients were prepared by successively layering in cellulose nitrate tubes 54, 45, 36, and 27% (vol/vol) Percoll in Hanks balanced salt solution. Suspensions of virus-infected or control cells in 18% (vol/vol) Percoll were layered on top of the gradients. The gradients were centrifuged at 10,000 rpm for 15 min in a Beckman SW50.1 rotor at 25°C. The cell bands are indicated by dotted areas in the gradients.*

ulations. At 3.5 and 4 h p.i., the concentration of cells banding at normal density decreased (as judged visually), the lighter-density fractions were still present, and new heavier-density fractions appeared. The heaviest cells pelleting to the bottom were present in the 4-h infected cultures. All bands, including the pellet, consisted of intact cells as determined by phase microscopy.

Volume changes in virus-infected cells. Whether or not volume changes due to water efflux or influx were involved in the observed density changes of infected cells was tested by direct measurement of cellular volume with a Coulter Counter connected to an automatic size analyzer. The results (Table 2) demonstrate a small increase in the volume of control cells over a 4-h period of incubation. With infected cells, there was no volume change at 1, 2, or 2.5 h p.i. However, by 3 h p.i., the volume of infected cells had decreased by 36%. The volume decrease was 59% at 3.5 h p.i. and 55% at 4 h p.i. Measurement of cellular water content as the difference between wet and dry weights of monolayers, minus the weight of extracellular fluid (determined by the ^{14}C-inulin procedure described above) corroborated the above findings; the water content of infected cells at 4 h p.i. was only 53% of that of control cells (unpublished data). A similar reduction in the volume of infected cells was observed also when the experiment was repeated with a suspension culture of HeLa S3 cells (unpublished data).

From the volume changes observed, it appeared that during the later stages of infection the cellular concentrations of Na^+ and K^+ will not parallel the changes noted earlier in the contents of these cations (Fig. 1). The cellular

TABLE 2. *Volume of poliovirus-infected and control HeLa cells*[a]

Duration of incubation (h)	Cellular vol (μm^3)	
	Control cells	Infected cells
1	2,211	2,211
2	2,312	2,211
2.5	2,212	2,213
3	2,412	1,407
3.5	2,513	905
4	2,513	1,005

[a] Cell volume was measured with a Coulter Counter connected to an automatic size analyzer which plots frequency distribution of cell sizes in the population. The volume in each case is the volume of cells at the peak of the size distribution curve.

contents of Na^+ and K^+ presented in Fig. 1 were related to cell numbers rather than to cell volume since these were expressed as concentrations in the same volume (0.1 ml) of cell extracts derived from the same number of cells. When cation concentrations were calculated from the data shown in Fig. 1 in accordance with the volume changes shown in Table 2 and the results were plotted, the pattern presented in Fig. 4 was obtained. It will be noted that the volume decrease between 3 and 4 h p.i. had the effect of vastly increasing the total cellular concentration of not only Na^+ but also K^+.

DISCUSSION

The results presented here demonstrate that in poliovirus-infected HeLa cells both the contents of Na^+ and K^+ per cell and the cellular concentrations of these cations were profoundly altered, starting at around the midphase of infection (Fig. 1 and 4). These changes were associated with a decrease in cellular volume (Table 2) and an increase in cell density (Fig. 3). Presumably the cell interior became hypotonic due to a net loss of monovalent cation activity. However, the extent of cell shrinkage was such as to vastly increase the cellular concentrations of Na^+ and K^+ (Fig. 4). Apparently this increase did not induce hypertonic conditions since it was not accompanied by an appreciable volume increase (Table 2). An explanation for this seeming anomaly might be that a high proportion of the cations existed in an osmotically inactive state. Indirect evidence to support this possibility was obtained from two types of experiments. First, it was noted that the Cl^-/Na^+ plus K^+ ratio dropped as a function of time after infection (Table 1). This could be explained by a steady increase in the fraction of bound cation even though other explanations are possible. Second, when $^{22}Na^+$ washout rates from infected and

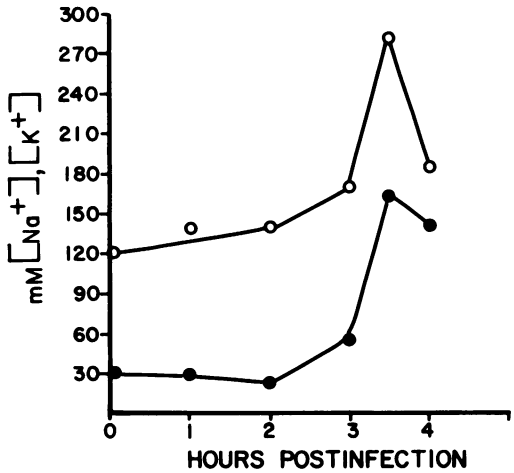

FIG. 4. *Na^+ and K^+ concentrations in poliovirus-infected HeLa cells. Cellular concentrations of Na^+ (●) and K^+ (○) at intervals after infection were calculated from the contents of these cations plotted in Fig. 1 and the volume measurements shown in Table 2.*

control cells were compared, it was found that the infected cells in the late phase of infection contained a slowly exchanging Na^+ compartment that was absent from control cells (Fig. 2A and B). The slower washout of $^{22}Na^+$ from infected cells could have resulted either from Na^+ binding to a cellular or a virally-specified moiety or from Na^+ sequestration by the excess smooth membrane material formed in infected cells as a result of membrane proliferation (2, 15, 16). Confirmation of the occurrence of increased cation binding and tonicity changes in infected cells will require parallel measurements of cation activity and cation concentration.

At least some of the morphological changes characterizing poliovirus cytopathic effects could be caused by hypotonicity-induced loss of cell water. This view is not incompatible with the evidence for the involvement of cytoskeletal disorganization in virus-induced morphological changes (13, 17). Tonicity changes may mediate cytoskeletal disorientation. For instance, a hypotonic environment would favor depolymerization of actin filaments (19). Changes in cellular Ca^{2+} concentration could also affect the integrity of the cytoskeleton by promoting the contraction of microfilaments (20) and the disassembly of microtubules (1, 18, 21). We have evidence for alterations in the metabolism of Ca^{2+} by poliovirus-infected HeLa cells (manuscript in preparation).

Studies by others, though not addressed to tonicity changes, have yielded data that are consistent with such changes occurring in cells infected with other viruses. Thus, a moderate but progressive decrease in the Na^+ plus K^+ concentration of Sindbis virus-infected chicken embryo fibroblasts is evident from the results reported by Garry and co-workers (11). Francoeur and Stanners (9) have reported a drop in the K^+ concentration of VSV-infected L-cells during the late phase of infection and a decrease in the volume of infected cells immediately before lysis. During the late phase of infection, VSV-infected cells appear shrunken in phase-contrast micrographs (6) and resemble poliovirus-infected cells in this regard. It will be interesting to determine whether tonicity change is a common denominator of cytopathic effects induced by different viruses.

Density changes of virus-infected cells have not received much attention. Vaccinia virus-infected L-cells reportedly become larger and lighter during the early period (4). In this report it was shown that poliovirus-infected HeLa cells become lighter initially and later heavier than control cells. Although cell shrinkage correlated with an increase in cell density (Table 2 and Fig. 3), there was no evidence that the decrease in cell density was caused by cell swelling. It is likely that membrane hyperplasia (2, 15, 16) and the attendant increase in the lipid content of infected cells were responsible for the density decrease. In support of this explanation were the findings that infected cells in the midphase of infection contained about 30% more lipid than control cells and that the increase in the lighter phospholipid fraction was about 70% (unpublished data). Even infections initiated with a large input multiplicity must progress asynchronously. This may explain the presence in the same infected culture of cell populations separable by their densities (Fig. 3). If so, density banding might provide a simple means of separating infected cells at different stages of infection.

Regardless of the actual mechanism of cell shrinkage, it could have significance for other late viral functions. For instance, cell shrinkage may promote virion assembly by bringing virion components closer together. Virus-specific RNA and protein synthesis might also be affected if, as a result of shrinkage, the cellular concentrations of amino acids, nucleotides, and other requirements for macromolecular synthesis are altered.

ACKNOWLEDGMENTS

I thank Byron Singfield for technical help and Keith Green for use of his chloridometer and many helpful discussions.

This work was supported by Public Health Service grant FR5365 from the Division of Research Facilities and Resources of the National Institutes of Health.

LITERATURE CITED

1. **Allison, A. C.** 1973. The role of microfilaments and microtubules in cell movement endocytosis and exocytosis, p. 109–148. *In* Locomotion of tissue cells. Ciba Foundation symposium no. 14. Elsevier/North Holland Publishing Co., Amsterdam.
2. **Amako, K., and S. Dales.** 1967. Cytopathology of mengovirus infection. II. Proliferation of membranous cisternae. Virology **32**:201–205.
3. **Balbanian, R.** 1975. Structural and functional alterations in cultured cells infected with cytocidal viruses. Prog. Med. Virol. **19**:40–83.
4. **Ball, F. R., and E. L. Medzon.** 1973. Sedimentation changes of L-cells in a density gradient early after infection with vaccinia virus. J. Virol. **12**:588–593.
5. **Carrasco, L., and A. E. Smith.** 1976. Sodium ions and the shut off of host cell protein synthesis by picornaviruses. Nature (London) **264**:807–809.
6. **David-West, T. S., and B. O. Osunkoya.** 1971. Cytopathology of vesicular stomatitis with phase microscopy. Arch. Ges. Virusforsch. **35**:126–132.
7. **Egberts, E., P. B. Hackett, and P. Traub.** 1977. Alteration of the intracellular energetic and ionic conditions by mengovirus infection of Ehrlich ascites tumor cells and its influence on protein synthesis in the midphase of infection. J. Virol. **22**:591–597.
8. **Farnham, A. E., and W. Epstein.** 1963. The influence of EMC virus infection on potassium transport in L-cells. Virology **21**:436–447.
9. **Francoeur, A. M., and C. P. Stanners.** 1978. Evidence against the role of K^+ in the shut-off of protein synthesis by vesicular stomatitis virus. J. Gen. Virol. **39**:551–554.
10. **Fuchs, P., and E. Giberman.** 1973. Enhancement of potassium influx in baby hamster kidney cells and chicken erythrocytes during adsorption of parainfluenza (sendai) virus. FEBS Lett. **31**:127–130.
11. **Garry, R. F., J. M. Bishop, S. Parker, K. Westbrook, G. Lewis, and M. R. F. Waite.** 1979. Na^+ and K^+ concentrations and the regulation of protein synthesis in sindbis virus-infected chick cells. Virology **96**:108–120.
12. **Garwes, D. J., P. J. Wright, and P. D. Cooper.** 1975. Poliovirus temperature sensitive mutants defective in cytopathic effects are also defective in synthesis of double stranded RNA. J. Gen. Virol. **27**:45–49.
13. **Lenk, R., and S. Penman.** 1979. Cytoskeletal changes and poliovirus metabolism. Cell **16**:289–301.
14. **Nair, C. N., J. W. Stowers, and B. Singfield.** 1979. Guanidine-sensitive Na^+ accumulation by poliovirus-infected HeLa cells. J. Virol. **31**:184–189.
15. **Penman, S.** 1965. Stimulation of the incorporation of choline in poliovirus-infected cells. Virology **25**:148–152.
16. **Plagemann, P. G. W., P. H. Cleaveland, and M. A. Shea.** 1970. Effect of mengovirus replication on choline metabolism and membrane formation in Novikoff hepatoma cells. J. Virol. **6**:800–812.
17. **Rutter, G., and K. Mannweiler.** 1977. Alterations of actin containing structures in BHK 21 cells infected with Newcastle disease virus and vesicular stomatitis virus. J. Gen. Virol. **37**:233–242.
18. **Sherline, P., K. Schiavone, and S. Brocato.** 1979. Endogenous inhibitor of colchicine-tubulin binding in rat brain. Science **205**:593–595.
19. **Spudich, J. A., and S. Watt.** 1971. The regulation of rabbit skeletal muscle contraction. I. Biochemical studies of the interaction of the tropomyosin-troponin complex with actin and the proteolytic fragments of myosin. J. Biol. Chem. **246**:4866–4871.
20. **Weber, K., P. C. Rathke, M. Osborn, and W. W. Franke.** 1976. Distribution of actin and tubulin in cells and in glycerinated cell models after treatment with cytochalasin B. Exp. Cell Res. **102**:285–297.
21. **Weisenberg, R. C.** 1972. Microtubule formation *in vitro* in solutions containing low calcium concentrations. Science **177**:1104–1105.