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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

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Mengovirus infection of Ehrlich ascites tumor cells caused a change of the intracellular ATP concentration. It increased by 35% within the first 3 h postinfection and then declined to zero within the next 5 h. The decrease in the ATP concentration was due, at least in part, to leakage of ATP into the medium, where it could be demonstrated by the luciferin-luciferase assay. Gross leakage of ATP was observed at 4.5 h postinfection, concomitant with the production of the first intracellular, infectious virus particles. A similar concentration decrease was detected for Mg^{2+}, the polyamines, and K^+, whereas an increase in the Na^+ concentration was observed. The intracellular Mg^{2+} concentration varied synchronously with the ATP level, rising by 16% during the first 3 h postinfection and then progressively falling to lower values in the late period of the infectious cycle. After an initial slight enhancement, the putrescine, spermidine, and spermine concentrations declined at about 1.5 h postinfection. Wherease the intracellular K^+ concentration increased by 17% during the first hour postinfection, the Na^+ concentration diminished by the same value within the same time period, leaving the internal ionic strength unchanged early in infection. Three hours after the beginning of virus infection, there was a rapid decline of K^+ and enhancement of Na^+ within the cell. These alterations of the intracellular energetic and ionic conditions seem to be, at least in part, responsible for the cessation of virus-specific protein synthesis in mengovirus-infected Ehrlich ascites tumor cells commencing 3 to 3.5 h postinfection.

In two recent studies we have compared the protein-synthesizing capacities of mengovirus-infected Ehrlich ascites tumor (EAT) cells with those of their corresponding postnuclear supernatants as a function of time postinfection (9; Egberts et al., submitted for publication). We have shown that mengovirus infection induces the shutoff of amino acid incorporation into host protein in vivo and in vitro and that this arrest is followed by a burst of viral protein synthesis. However, the synthesis of viral proteins can be prolonged in vitro; this leads to a peak of protein synthesis that occurs 1 to 1.5 h later in infection. Moreover, toward the end of the infectious cycle, the postnuclear supernatants exhibit a substantially higher relative protein-synthesizing activity than the intact cells from which they were derived. We have hypothesized that these differences are the result of a virus-induced unbalance of the intracellular optimal energetic and ionic conditions that have been restored in vitro by the addition of adequate amounts of energy and electrolytes to the incubation assays. The disturbance of these intracellular optimal conditions would affect such processes as initiation and elongation of polypeptide chains and, consequently, the ribosome distribution in the virus-infected cells. Indeed, polysomes gradually disappear from the cytoplasm of mengovirus-infected EAT cells late in the infectious cycle (9), and likewise in poliovirus-infected HeLa cells the rate of elongation of polypeptide chains as well as the size of the remaining polysomes decrease at that time (22).

In this report we present our results on the investigation of the intracellular energetic and ionic conditions in mengovirus-infected EAT cells and discuss their relevance to the regulation of protein synthesis in the middle and late phases of mengovirus infection.

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MATERIALS AND METHODS

Growth of EAT cells and mengovirus. Cells and virus were propagated as described previously (9).

Infection of cells. EAT cells were infected with mengovirus at a multiplicity of infection of 30 PFU/cell as described previously (9). At least 97% of the cells were infected as evidenced by trypan blue uptake (19) at 11 h postinfection. Mock-infected cells were used as a control.

Sucrose gradient centrifugation of postnuclear supernatants. Postnuclear supernatants of mengovirus-infected EAT cells were prepared in the presence of 1% Triton X-100 and subjected to sucrose gradient analysis in 0.2 M NaCl and 10^{-2} M EDTA (25) as described previously (9). The absorbancy at 260 nm sedimentation profiles were evaluated by the cut-and-weigh method. The area under the ribosomal subunit peaks was taken as a measure of the total ribosome content, whereas the area of the meniscus fraction, which amounted to 15% of the gradient volume, was taken as a measure of material sedimenting slower than 5S.

Preparation of cell extracts. At various times postinfection, 700 ml of cells (6×10^5 cells/ml) was harvested by centrifugation at $225 \times g$ for 5 min without further cooling. A small part of the supernatant was cooled to 0°C and used to resuspend and transfer the pelleted cells into 15-ml centrifuge tubes, in which the cells were again packed at $450 \times g$ for 2 min. After removal of the supernatant by aspiration, the tubes were recentrifuged at $450 \times g$ for 3 min. The remaining amount of medium that had adhered to the wall of the centrifuge tubes was removed by aspiration. The cells were suspended in 4 ml of water. For K^+ determination (see below), 0.5 ml of the cell suspension was diluted with 4 ml of water. The remainder was sonically treated for 20 s at 0°C, mixed with 5 ml of 1 N $HClO_4$, and again sonically treated for 20 s. Insoluble material was removed by centrifugation at $10,000 \times g$ for 15 min. The supernatant was neutralized with 10 N KOH (approximately 0.5 ml) at 0°C and centrifuged at $10,000 \times g$ for 10 min. The supernatant was frozen and stored at −80°C.

Determination of K^+. A 4.5-ml amount of each ninefold-diluted cell suspension in water (see above) was mixed with 0.5 ml of 10% sodium dodecyl sulfate and sonically treated for 20 s. A 100-μl amount of the clear solution was diluted with 9.9 ml of water, and the K^+ concentration was determined in a Perkin-Elmer 403 atomic absorption flame spectrophotometer.

Determination of putrescine, spermidine, and spermine. The polyamines were isolated from virus-infected cells essentially following the method described by Inoue and Mizutani (14). A 2-ml amount of each KOH-neutralized perchloric acid extract (see above) was applied to a Dowex 50 × 8 column (200 to 400 mesh, 2.5 by 0.8 cm) previously washed with 4 N NaOH and then with 6 N HCl and water. After adsorption, the column was sequentially washed with 40 ml of 0.1 M Na_2HPO_4 (pH 8)-0.7 M NaCl and with 10 ml of 1 N HCl. The polyamines were eluted with 15 ml of 6 N HCl, evaporated to dryness in a rotary evaporator at 50°C, dissolved in 0.2 ml of saturated $NaHCO_3$ solution, and shaken with 1 ml of 10^{-2} M dansylchloride in acetone for 3 h at room temperature. The acetone was removed in vacuo, and the remaining solution (suspension) was extracted twice with 1 ml of ethyl acetate. A 20-μl portion of the organic phase was used for thin-layer chromatography on silica gel plates (Schleicher and Schüll, Dassel, Germany) with ethyl acetate-cyclohexane (3:4) as a solvent (7). The chromatograms were densitometrically evaluated in a Farrand Vis-UV-2 chromatogram analyzer (exciter, 340 nm; analyzer, 510 nm).

Determination of intracellular ATP, ADP, and AMP. One milliliter of each KOH-neutralized perchloric acid extract (see above) was frozen and dried over phosphopentoxide in an oil pump vacuum and in the cold. The residue was dissolved in 0.1 ml of water, and a small amount of insoluble $KClO_4$ was removed by low-speed centrifugation. A 10-μl amount of the concentrated solution was used for thin-layer chromatography on polyethyleneimine-impregnated cellulose (Macherey-Nagel & Co., Düren, Germany). ATP, ADP, and AMP were used as references. The chromatograms were sequentially developed with 1 M acetic acid (until the solvent front climbed 5 cm above the origin) and 0.5 M LiCl (13 cm above the origin) without drying the sheets between the two chromatography steps (20). The ATP, ADP, and AMP spots, which were visualized with UV light, were cut out, and the UV-absorbing material was eluted with 1 ml of 0.7 M $Mg(OAc)_2$-0.02 M Tris-acetate, pH 7.5 (16). The absorbancies of the resulting solutions were measured at 260 nm; they were corrected for background absorbance.

Determination of extracellular ATP. Various times after virus infection, 20-ml samples of suspension culture (5.5×10^5 cells/ml) were cooled in an ice water bath and centrifuged at $450 \times g$ for 5 min at 0°C. One milliliter of the supernatant was used for ATP measurement by the luciferin-luciferase assay (23). It was mixed with 3 ml of 0.05 M $MgCl_2$, 0.1 M sodium arsenate, pH 7.4, and 0.2 ml of luciferase-solution (Boehringer Mannheim, Mannheim, Germany; 1 mg of enzyme protein per ml of water) in a scintillation vial. Immediately after mixing, the vial was lowered into the counting chamber of a Packard liquid scintillation counter, and the luminescence was measured during 10-s intervals. To obtain accurate values for the ATP concentration of the samples, the linearly declining parts of the luminescence intensity curves were extrapolated to zero time. The amounts of ATP in all samples analyzed were within the linear range of the assay.

RESULTS

Retention of cellular macromolecules. Though postnuclear supernatants from mengovirus-infected EAT cells, prepared under our conditions (9), are unable to reinitiate protein synthesis, they nevertheless reflect the cells' protein-synthesizing potentials with respect to chain elongation. The prolonged synthesis of

viral proteins in these postnuclear supernatants relative to that in intact cells (insert of Fig. 1) suggested to us that some compound(s) essential for protein synthesis may have been partially lost in vivo but restored in the cell-free system. Since the in vitro protein-synthesizing activities of extracts prepared from cells late in infection also decrease, alterations in the translational machinery must occur that cannot be compensated for in vitro. To ascertain that the declining activities of the postnuclear supernatants are not due to cellular loss of the macromolecular constituents, other than mRNA, of the protein-synthesizing apparatus during the infectious cycle, we measured the amount of ribosomes in mengovirus-infected cells as a function of time postinfection. The extracts were subjected to sucrose gradient analysis at 0.2 M NaCl and in the presence of EDTA, according to the method described by Warner (25). Under these conditions all ribosomes are dissociated into partially unfolded subunits. Their amounts in the cell, which were determined by planimetric evaluation of the sedimentation profiles, were taken as a measure of the leakage of cytoplasmic macromolecular compounds into the medium. Figure 1 shows a fairly constant amount of ribosomes in mengovirus-infected EAT cells throughout infection, except at the end, when the cells start to lyse and lose cytoplasmic material. The isolation of total RNA (5) from the various postnuclear supernatants with phenol gave the same result (Fig. 1).

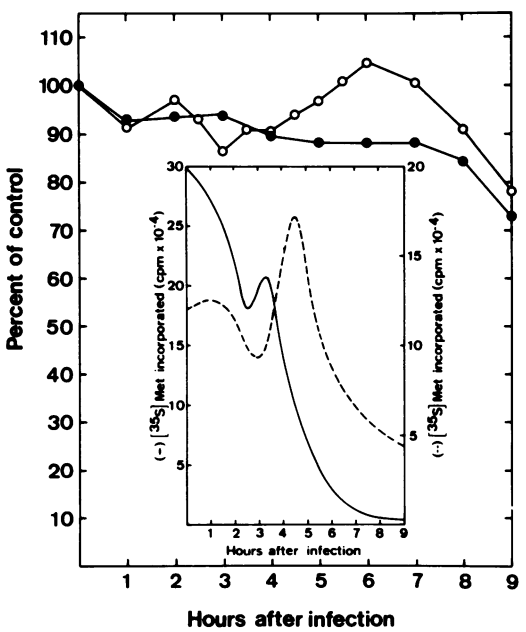

FIG. 1. *Ribosome and RNA content of mengovirus-infected EAT cells as a function of time postinfection. The ribosome contents* (○) *were determined by sucrose gradient centrifugation of postnuclear supernatants in 0.2 M NaCl and 10^{-2} M EDTA (25). Total RNA* (●) *was isolated from postnuclear supernatants according to the method described by Brawerman (5) and measured by UV absorption at 260 nm. In the insert, the amino acid-incorporating capacities of mengovirus-infected EAT cells* (—), *as a function of time postinfection, are compared with those of their corresponding postnuclear supernatants* (----); *the curves of the insert are taken from a previous publication (9).*

Alteration of the intracellular ATP concentration. In the course of this analysis, we noticed that the amount of UV-absorbing material in the meniscus fractions of the sucrose gradients continuously increased by approximately 20% during the first 3 h postinfection and then dropped to 60% of the control value by the end of the infectious cycle (data not shown). Using perchloric acid extracts of the infected cells, we were able to correlate this alteration with a change in the intracellular ATP concentration (Fig. 2). ATP was determined by thin-layer chromatography on polyethyleneimine-impregnated cellulose (20). Whereas the intracellular concentration of ATP increased by approximately 30% within the first 3 h postinfection, the concentration of ADP and AMP stayed low throughout the infectious cycle (Fig. 2). Figure 2 also shows the adenylate energy charge, defined as $\frac{1}{2}([ATP] + 2[ATP])/([AMP] + [ADP] + [ATP])$ (3) as a function of time postinfection. This quantity, which can be used to express the energy status of the cells and which has a strong, regulatory influence on the cell's protein-synthesizing potential, decreases at 3.5 h after the beginning of virus infection. The mono-, di-, and triphosphates of the other nuclear bases were barely detectable and were therefore not determined.

Alteration of the intracellular Mg^{2+}, K^+, Na^+, and polyamine concentrations. Parallel with the variation of the intracellular ATP concentration, we observed an initial 16% enhancement of the Mg^{2+} concentration within the first 3 h postinfection. Then it continuously dropped to lower values (Fig. 3). The relative K^+ concentration behaved similarly, except that it rose by 17% during the first hour postinfection, stayed constant for 1.5 h, and then declined progressively (Fig. 3). Exactly the opposite concentration change was detected for Na^+, except during the late period of the infectious cycle. The initial reduction during the first hour postinfection was 17% of the control. Two hours later the Na^+ concentration increased and reached a con-

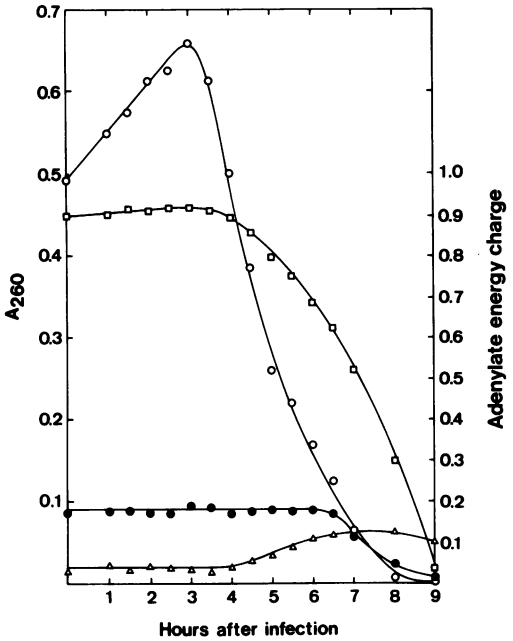

FIG. 2. *Adenylate contents and adenylate energy charge of mengovirus-infected EAT cells as a function of time postinfection. The ATP (○), ADP (●), and AMP (△) contents were determined by thin-layer chromatography of perchloric acid extracts of the virus-infected cells on polyethyleneimine-impregnated cellulose (20) as described in Materials and Methods. (□) Adenylate energy charge.*

stant value at 5 h postinfection. Between 3.5 and 4 h after the beginning of virus infection, the K^+ and Na^+ concentrations had attained their original levels. Since polyamines can partially substitute for Mg^{2+} in several reactions of protein synthesis (2, 13, 15, 24), we examined whether the virus-induced reduction of the intracellular Mg^{2+} concentration was compensated for by a corresponding augmentation of the polyamine concentration. The polyamines were isolated from perchloric acid extracts of the postnuclear supernatants by Dowex 50 chromatography (14), dansylated, and subjected to thin-layer chromatography on silica gel sheets (7). The quantitative evaluation of the chromatograms resulted in three curves which show the variation in the putrescine, spermidine, and spermine concentrations (Fig. 4). After an initial increase of between 5 and 20% of the control values, the concentrations of putrescine, spermidine, and spermine declined progressively in the middle and late phases of the infectious cycle.

Fate of intracellular ATP. The gradual disappearance of ATP from the cytoplasm of mengovirus-infected EAT cells 3 h after infection, without any concomitant intracellular accumulation of ADP and/or AMP, poses the question as to the fate of the triphosphate. We observed a slight but significant augmentation of the UV absorbancy of the culture medium in the first

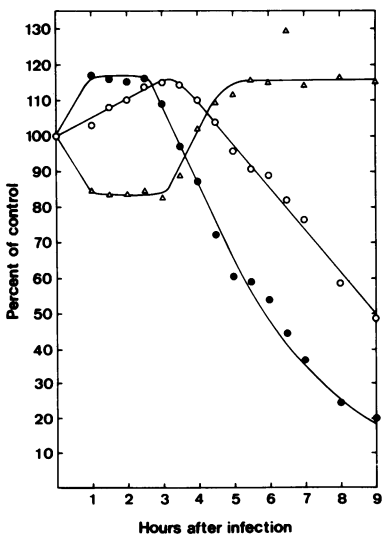

FIG. 3. *Relative Mg^{2+} (○), K^+ (●), and Na^+ (△) contents of mengovirus-infected EAT cells as a function of time postinfection. The contents were determined by flame spectrophotometry and are expressed as percentages of the values from uninfected cells (0 h).*

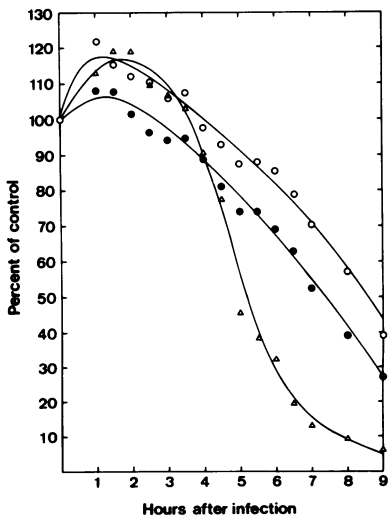

FIG. 4. *Relative putrescine (△), spermidine (●), and spermine (○) contents of mengovirus-infected EAT cells as a function of time postinfection. The polyamine contents were determined by thin-layer chromatography of the dansylated polyamines on silica gel sheets (7); they are expressed as percentages of the values obtained from uninfected cells (0 h).*

hours of the infectious cycle (Fig. 5). However, when we used the extremely sensitive and specific luciferin-luciferase assay (23), we could detect a distinct enhancement of the extracellular ATP concentration starting at 3 h postinfection. This initial ATP leakage into the medium (insert of Fig. 5) turned into a gross discharge of ATP 1.5 to 2 h later. The ATP release was synchronous with the appearance of the first infectious virus particles inside of the infected cell (Fig. 6). The ATP concentration of the medium increased up to 7 h postinfection and then dropped rapidly (Fig. 5). In the early and middle phases of the infectious cycle, no additional ATP could be detected when the contents of the medium were phosphorylated with creatine phosphate in the presence of myokinase and creatine kinase, indicating that ATP that had leaked into the medium was not hydrolyzed to ADP and/or AMP (data not shown). Figure 6 also shows the uptake of trypan blue by mengovirus-infected EAT cells as a function of time postinfection.

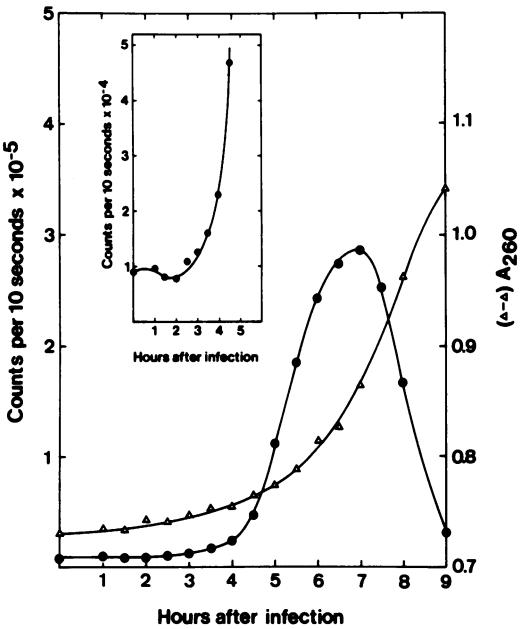

FIG. 5. *Release of UV-absorbing (at 260 nm) material and ATP from mengovirus-infected EAT cells into the medium as a function of time postinfection. Symbols:* (△) *Kinetics of the release of UV-absorbing (at 260 nm), low-molecular-weight material; proteins and nucleic acids were precipitated with perchloric acid in order to reduce the background absorbancy of the culture medium.* (●) *Kinetics of the release of ATP as determined by the luciferin-luciferase assay (23); the medium was analyzed directly without any pretreatment. In the insert, the scale on the ordinate is expanded five times and the time scale on the abscissa is reduced by a factor of 2.*

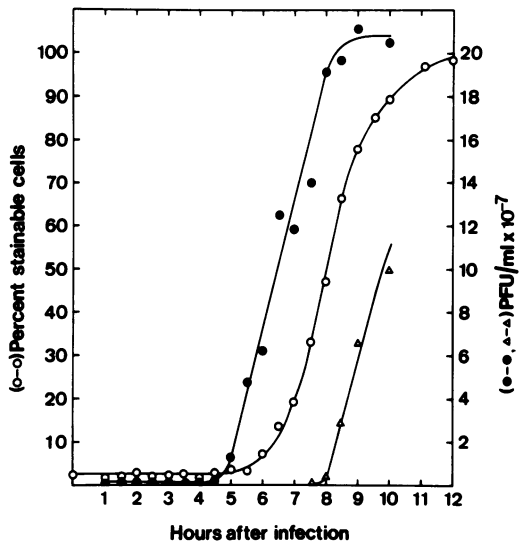

FIG. 6. *One-step growth of mengovirus in EAT cells as followed by the plaque assay (8) and uptake of trypan blue (19) as a function of time postinfection. After virus adsorption, the cells were washed once with fresh medium and then suspended and incubated in their original growth medium. At various times postinfection, 10-ml aliquots of suspension culture were frozen and thawed three times and then assayed for total infectious virus particles* (●). *At the same time the supernatant of a second 10-ml aliquot, which had been cooled to 0°C and centrifuged at 450 × g for 5 min, was analyzed in the same way* (△) *to determine the extracellular virus concentrations. The uptake of trypan blue was measured as described by Phillips (19)* (○).

DISCUSSION

The incorporation of radioactively labeled amino acids into protein by postnuclear supernatants, prepared from mengovirus-infected EAT cells at various times postinfection, results in an activity-time curve similar to that which is obtained when protein synthesis is measured in intact virus-infected cells (9) (see insert of Fig. 1). However, there are also significant differences between the two curves. First, the ratio of the translational capacity early after infection and the maximal activity of viral protein synthesis is considerably lower in vitro than in vivo. Second, maximal synthesis of viral protein in vitro is observed approximately 1 to 1.5 h later than in vivo. Third, in vitro the relative amino acid-incorporating activity in the late period of the infectious cycle is substantially higher than in vivo, where it is virtually nil at that time. The differences between both activity-time curves are significant with respect to the regulation of protein synthesis in mengovirus-infected EAT cells, because they suggest that some essential factor(s) required

for protein synthesis is reduced in vivo but restored in the cell-free system. We examined the intracellular levels of ATP and several cations to determine whether their concentrations varied during the infectious cycle. These compounds were chosen because they are supplied at optimal concentrations in the cell-free system.

At 3 to 3.5 h after infection, when in vivo the amino acid incorporation into viral protein is at its maximum, a drastic decrease of the intracellular ATP concentration takes place, without any concomitant change of the intracellular ADP and/or AMP concentration, except toward the very end of the infectious cycle. Whereas the initial augmentation of the ATP concentration probably results from the virus-induced arrest of host-specific DNA and RNA synthesis (data not shown), the following reduction of the ATP level can be the consequence of (i) catabolic degradation of the adenylates, (ii) increasing ATP consumption due to viral RNA synthesis, and (iii) progressive ATP leakage into the extracellular space. We did not examine adenine catabolism. Furthermore, assuming that about 2×10^5 molecules of viral RNA are synthesized per cell (4), the ATP consumption by RNA synthesis would account for no more than 10% of the decrease in the ATP concentration. Rather, the decrease of the intracellular ATP level is the consequence of an increasing permeability of the plasma membrane for ATP. The efflux of ATP into the extracellular space starts at 3 h postinfection (Fig. 5), when in picornavirus-infected mammalian cells in general drastic changes of the cytoplasmic membranes are observed (17, 18). This early and moderate ATP leakage must be distinguished from the extensive outflow of ATP after 4.5 h postinfection which occurs synchronously with the production of the first intracellular, infectious virus particles and which might be caused by the action of lysosomal enzymes on the plasma membrane (1, 10, 11, 26).

Besides the intracellular change of the ATP level, we observed a covariance of the intracellular Mg^{2+}, K^+, Na^+, and polyamine concentration. The initial enhancement of the Mg^{2+} and polyamine concentrations is probably the cellular response to the enlargement of the ATP pool whose increased chelating potential must be neutralized by polyvalent cations in order to maintain optimal conditions for those enzymes requiring bivalent cations as cofactors. Whereas the increase in the Mg^{2+} concentration covered a period of 3 h, the initial enhancement of K^+ and decline of Na^+ were complete within 1 h, if not sooner. Perhaps structural or conformational changes of the plasma membrane, as a result of virus adsorption and penetration, lead to an activation of the Na^+- and K^+-stimulated ATPase and thus to an amplification of the active transport of Na^+ and K^+. Whether such a change of the K^+/Na^+ ratio has any effect on the regulation of cellular processes, especially host-specific RNA and protein synthesis, is still unknown. In any case, the compensation of both concentration changes guarantees a constant and normal, internal ionic strength early in infection. Our data are in contrast to a hypothesis recently advanced by Carrasco and Smith (6), who speculated that the shutoff of host protein synthesis immediately after picornavirus infection of mammalian cells might be the consequence of an increase of the intracellular Na^+ concentration and a concomitant decrease of the K^+/Na^+ ratio.

The leakage of ATP into the medium, of course, has a strong influence on protein synthesis. All reactions of amino acid incorporation are dependent on energy resources. The decline of the intracellular concentrations of electrolytes (K^+, Mg^{2+}), which are absolutely necessary for amino acid incorporation, reinforces the inhibition of translation. Since polyamines in vivo can cause an increase in the efficiency of overall protein synthesis (2, 12, 15, 21, 24), the virus-induced reduction of their intracellular concentrations might also affect the rate of amino acid incorporation. Finally, the competition for ATP by other energy-consuming processes, e.g., viral RNA synthesis, membrane synthesis, etc., all of which begin at about 3 h postinfection, could depress the amino acid-incorporating activity.

On the basis of the above observations, we suggest that the abrupt decline of the protein-synthesizing activity in vivo at 3 h postinfection is primarily caused by leakage into the medium of low-molecular-weight substances essential for amino acid incorporation. As a result, a depression of initiation and elongation of polypeptide chains is observed, which is reflected in a decrease in size and synthetic capacity of virus-specific polysomes late in the infectious cycle (22). When the optimal energetic and ionic conditions of protein synthesis are restored in vitro, the amino acid incorporation into polypeptides is extended, with a maximum at 4.5 to 5 h postinfection (9). Since our postnuclear supernatants are unable to reinitiate protein synthesis (9), this increased amount of translation is due solely to the elongation of polypeptide chains. We therefore postulate a higher sensitivity of chain elongation than chain initiation to energy and ion depletion in vivo. At 4 to 4.5 h postinfection, the formation of polysomes is

suppressed, resulting in a decline of the in vitro protein-synthesizing activity. However, up to the very end of the infectious cycle, a substantial number of polysomes are preserved, though reduced in size (9, 22), which results in a relatively high translational capacity of the postnuclear supernatants late in infection.

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