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Guanidine-Sensitive Na^+ Accumulation by Poliovirus-Infected HeLa Cells

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The Na^+ content of poliovirus-infected HeLa S3 cells increased during the late phase of virus replication, after virus inhibition of host cell protein synthesis and in coincidence with late viral functions. Guanidine hydrochloride blocked the rise in Na^+ content, whereas the antiguanidine agent choline fully reversed the guanidine block. Expression of one or more late viral functions was essential for Na^+ accumulation to occur because accumulation was inhibited by cycloheximide or guanidine added to the infected culture during the late phase. Increased permeability of infected cell membrane rather than inhibition of cellular Na^+-K^+ adenosine triphosphatase activity appears to be primarily responsible for Na^+ accumulation by virus-infected cells.

According to several published reports, viral mRNA translation is either resistant to (9, 21) or stimulated by (5) Na^+ concentrations which inhibit synthesis of most cellular proteins. It appears that those cellular proteins, which are resistant to hypertonicity, are also resistant to virus inhibition (20). Furthermore, the inhibition of cellular protein synthesis caused by hypertonicity (25) as well as by virus infection (28) occurs at the level of polypeptide chain initiation. These observations point to the possible involvement of hypertonicity due to Na^+ in virus inhibition of host cell protein synthesis. In vitro translation of encephalomyocarditis virus mRNA is stimulated, whereas cellular mRNA translation is inhibited by high concentrations of Na^+. This observation led Carrasco and Smith to hypothesize that picornavirus infection of cells may lead to an influx of Na^+ and that the increased Na^+ concentration may be responsible for viral inhibition of cellular protein synthesis (5). A subsequent study by Egberts and co-workers documented a late increase in the Na^+ content of mengovirus-infected mouse ascites tumor cells, but did not indicate that this phenomenon caused virus inhibition of cellular protein synthesis (8). We have been studying these questions in poliovirus-infected HeLa cells. Our results confirm that Na^+ accumulation in this system follows inhibition of host protein synthesis and coincides with late viral functions. The reason for the late accumulation of Na^+ during infection appears to be increased permeability of the plasma membranes of infected cells rather than inhibition of cellular Na^+-K^+ pump activity. One or more late viral functions are implicated in the enhancement of membrane permeability.

MATERIALS AND METHODS

Chemicals. $^{22}NaCl$, (carrier-free) was purchased from New England Nuclear Corp. [^3H]leucine (specific activity, 6,000 mCi/mmol) and guanidine hydrochloride were purchased from Schwarz/Mann, and cycloheximide, firefly lantern extract, phosphorus standard solution, acid molybdate solution, Fiske-Subbarow reagent, and choline chloride were purchased from Sigma Chemical Co.

Virus and cells. All experiments were carried out with poliovirus type 2 and monolayer cultures of HeLa S3 cells. Growth of cells and virus was in minimal essential medium (MEM) containing 5% calf serum and antibiotics. Absence of mycoplasma contamination of the cultures was determined by the Hoechst 33258 fluorescent stain technique (6).

Virus infection. Replicate monolayer cultures containing 2×10^6 to 3×10^6 cells per 35-mm Corning plastic petri dish were incubated with virus at a ratio of viruses to cells of ~100 at 37°C for 30 min for virus adsorption. Unadsorbed virus was removed, and the cultures were washed twice with phosphate-buffered saline without Ca^{2+} or Mg^{2+} (PBS) (1 ml/culture per wash) before further incubation under experimental conditions.

Assay of Na^+ uptake. The procedure used for measurement of Na^+ uptake by cultures was developed by us and involved incubation of replicate infected or control cultures with MEM containing 0.5 to 1 μCi of $^{22}NaCl$ per ml for the periods indicated in different experiments, removal of radioactive medium, four rapid washes with PBS (2.5 ml/culture per wash) at 4°C, solubilization of the monolayer with 0.33 M KOH, and assay of the radioactivity of the neutralized solution by scintillation counting in a Beckman LS-230 scintillation spectrometer, using ^{32}P settings and

a Triton X100-toluene-based scintillation cocktail. To obtain background, radioactive medium added to one of the cultures was immediately removed, and the culture was washed and processed as described above. The relatively small intercellular volume of monolayer cultures and the washing and background correction steps in the procedure insured that the contribution of externally trapped $^{22}Na^+$ to the measurements of radioactivity was negligible, if any. The adequacy of the procedure to detect changes in intracellular Na^+ concentration was evidenced by its ability to demonstrate ouabain-induced enhancement of Na^+ uptake by cells.

Measurement of protein synthesis. Protein synthesis by cultures was determined as acid-insoluble [^{3}H]leucine incorporation. At different times after infection, cultures were incubated for 35-min intervals with MEM containing 1 μCi of [^{3}H]leucine per ml. After incubation, the radioactive medium was removed, and the cultures were washed with PBS as described above. Acid-soluble radioactivity was removed from cultures by two 5-min rounds of extraction with 1 ml of warm trichloroacetic acid. The acid-insoluble ^{3}H radioactivity of cultures was solubilized and counted. To obtain background radioactivity, radioactive medium added to one culture was immediately removed, and the culture was processed for assay of acid-insoluble radioactivity as described above.

ATP determinations. Replicate infected cultures were incubated with MEM. Cultures removed at intervals were washed four times with PBS (1 ml/wash per culture) at 4°C. To extract acid-soluble material, 1 ml of cold 5% trichloroacetic acid was added to the cultures, which were then chilled for 5 min on crushed ice. This step was repeated with 0.5 ml of trichloroacetic acid, and the two extracts were combined and neutralized with 1 M KOH. ATP content of cell extracts was determined by the bioluminescence assay procedure described by Stanley and Williams (26). The assay mixture consisted of 29 parts of 0.1 M glycyl glycine buffer, pH 7.45, 2 parts of 0.1 M $MgSO_4$, and 1 part of reconstituted firefly lantern extract in a total volume of 1.9 ml. To the assay mixture, 25 μl of a 1:100 dilution of the neutralized cell extract was added. After very rapid mixing, the light emission during the first 10 s was counted in a scintillation spectrometer with the coincidence circuit cut off. ATP standards were included in each assay. Counts obtained with the reaction mixture not containing cell extract were considered background and subtracted from all other values.

Assay of Na^+-K^+ ATPase activity. Replicate infected cultures were incubated with MEM. Pairs of cultures (~6 × 10^6 cells) were removed at intervals and washed with reticulocyte buffer at 4°C. The cells harvested by scraping were suspended in reticulocyte buffer containing 10 mM dithiothreitol for 5 min at 4°C. Cell extracts were prepared by Dounce homogenization. The adenosine triphosphatase (ATPase) activity of each extract was assayed in the presence and absence of 3 mM ouabain. The difference in activity was attributed to Na^+-K^+ ATPase. The reaction mixture contained, in a 3.1-ml total volume, 0.03 M Tris-hydrochloride, pH 7.5, 0.1 M NaCl, 0.0145 M KCl, 3 mM $MgCl_2$, 5 mM disodium ATP, and 0.2 ml of cell extract or the above ingredients plus 3 mM ouabain. The mixture was incubated at 37°C for 10 min. The reaction was stopped by adding to the reaction mixture 1 ml of cold 25% trichloroacetic acid. Inorganic phosphate content of the reaction mixture was determined by the method of Fiske and Subbarow (10). Acid-insoluble materials were pelleted by low-speed centrifugation. A 1-ml amount of the supernatant was mixed with 3 ml of glass-distilled water, 1 ml of acid molybdate solution, and 0.25 ml of Fiske-Subbarow reagent. After 10 min at room temperature for color development, adsorption at 660 nm was measured.

RESULTS

Late accumulation of Na^+ versus protein synthesis by infected cells. In Fig. 1 the cumulative uptake of $^{22}Na^+$ and the rate of incorporation of [^{3}H]leucine into acid-insoluble material by infected cells are plotted as functions of time after infection. Viral inhibition of cellular protein synthesis started about 1 h postinfection (p.i.) and became quite pronounced by 2 h p.i. In contrast, the amount of $^{22}Na^+$ taken up by the cells remained unchanged until 2.5 h p.i. and then rose steadily. Late viral protein synthesis paralleled this rise in $^{22}Na^+$ uptake and peaked at about 3 h p.i. Uninfected control cultures

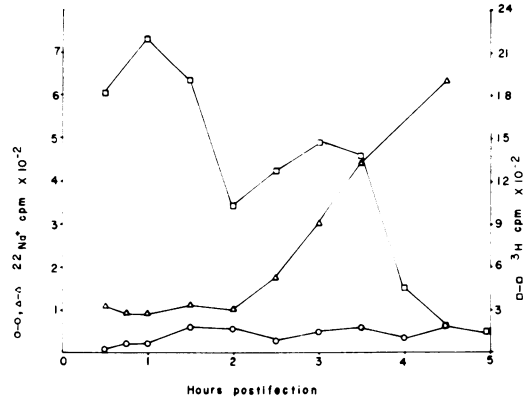

FIG. 1. *Time course of Na^+ accumulation and of the rate of protein synthesis by infected cells. To measure cumulative Na^+ uptake, replicate infected cultures were incubated with MEM containing $^{22}NaCl$ only or $^{22}NaCl$ plus 2 mM guanidine hydrochloride. Thereafter, at the intervals indicated, one culture from each group was removed from incubation, processed, and assayed for $^{22}Na^+$ radioactivity. To monitor the rate of protein synthesis, replicate infected cultures were incubated with MEM. At 30-min intervals acid-insoluble [^{3}H]leucine incorporation by one culture was determined. See text for the details of the procedure. Symbols: □, [^{3}H]leucine incorporated into acid-insoluble material; cumulative $^{22}Na^+$ uptake in the absence (△) or presence (○) of 2 mM guanidine hydrochloride.*

incubated and assayed in parallel with the infected cultures did not show any increase in $^{22}Na^+$ uptake (unpublished data). Similarly, no accumulation of $^{22}Na^+$ occurred when the infected cultures were incubated with medium containing 2 mM guanidine hydrochloride (Fig. 1 and see below).

Effect of guanidine, choline, and guanidine plus choline on Na^+ accumulation. Certain amino acids and methylated or ethylated amino alcohols or amines have been shown to reverse guanidine inhibition of poliovirus replication (16, 17). It was of interest to determine the effect of an antiguanidine agent on guanidine inhibition of Na^+ accumulation by infected cells. Therefore, the time course of $^{22}Na^+$ uptake by infected cells in the presence of guanidine, the antiguanidine agent choline, or guanidine plus choline was compared with the time course of uptake by infected, control cells. The results (Fig. 2) show that the inhibition of Na^+ uptake by 0.4 mM guanidine was indeed reversed by the simultaneous presence of 20 mM choline. The reversal was paralleled by the appearance of signs of cell injury and restoration of progeny production (unpublished data). These results indicate that Na^+ accumulation and late viral functions are intimately connected.

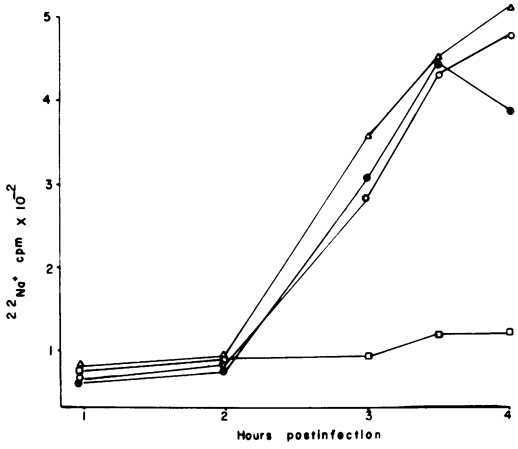

FIG. 2. *Reversal by choline of guanidine inhibition of Na^+ accumulation by infected cells. Replicate infected cultures were incubated with MEM containing $^{22}NaCl$ ($^{22}Na^+$-MEM) or with $^{22}Na^+$-MEM containing guanidine, choline, or guanidine plus choline. Thereafter, at the intervals indicated, the $^{22}Na^+$ radioactivity of one culture from each group was assayed. Symbols: radioactivity of cultures incubated with $^{22}Na^+$-MEM alone (△), $^{22}Na^+$-MEM plus 0.4 mM guanidine (□), $^{22}Na^+$-MEM plus 20 mM choline (○), or $^{22}Na^+$-MEM plus 0.4 mM guanidine plus 20 mM choline (●). See text for other details.*

Sensitivity of Na^+ accumulation to inhibition of late viral functions. Cycloheximide or guanidine, when added to poliovirus-infected cultures during the late phase of replication, inhibits virus progeny production by arresting late viral functions (1). To further understand the relation between late functions and Na^+ accumulation, these inhibitors were added to infected cultures at 2.0, 2.5, or 3.0 h p.i., and at 0.5-h intervals thereafter Na^+ uptake was determined (Fig. 3A and B). The addition of either agent as late as 3 h p.i. inhibited further accumulation of Na^+. Since both agents promptly arrest viral RNA and protein synthesis, these results implicate expression of late viral functions in Na^+ accumulation by infected cells.

Na^+-K^+ ATPase activity of infected cells versus Na^+ accumulation. The loss of ATP and other cellular constituents from picornavirus-infected cells in the late phase of infection has been reported (8). Since intracellular ATP is a requirement for the Na^+-K^+ pump activity of cell membranes, depletion of cellular ATP could slow down the Na^+-K^+ pump and thereby increase cellular Na^+ content. To test whether this was happening in our system, we determined the ATP content of infected cultures at intervals during the course of infection. The results (Fig. 4) indicate that no significant loss of ATP had occurred by 4 h p.i., when Na^+ accumulation was well underway.

To determine whether virus infection directly inhibited Na^+-K^+ ATPase activity, we assayed the activity of this enzyme in crude homogenates of infected cells. The results (Table 1) show a small decrease in enzyme activity with time after infection, the activity at 3 and 4.5 h p.i. being only about 87 and 83%, respectively, of that immediately after infection.

The above results did not suggest that inhibition of the cellular Na^+-K^+ pump played a major role in the increase in $^{22}Na^+$ uptake by infected cells. We have further investigated the involvement of Na^+-K^+ ATPase activity in this phenomenon by comparing the effects of ouabain and virus infection, singly and in combination, on Na^+ accumulation. The rationale was that if virus infection caused Na^+ accumulation by inactivating the pump, as ouabain is known to do, an ouabain effect may not be demonstrable in virus-infected cells exhibiting Na^+ accumulation. On the other hand, if each induced Na^+ accumulation by a separate mechanism, an additive effect might be expected. The results of this experiment (Fig. 5) bear out the latter expectation; i.e., ouabain stimulation of Na^+ uptake was as pronounced in late-phase virus-infected cells as in uninfected cells.

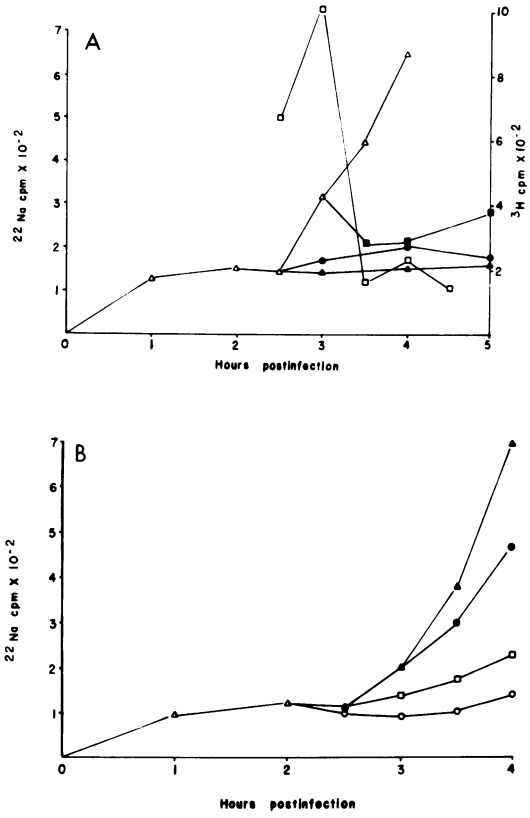

FIG. 3. *(A) Kinetics of cycloheximide inhibition of Na^+ accumulation by infected cells. Replicate infected cultures were divided into four groups and incubated with MEM. Cycloheximide was added to a final concentration of 100 μg/ml to one group of cultures at 2 h p.i., to a second group at 2.5 h p.i., and to a third group at 3 h p.i. At 30-min intervals thereafter, one culture from each cycloheximide group and one culture from the control group were assayed for $^{22}Na^+$ content. See text for details of the procedure. Symbols: $^{22}Na^+$ radioactivity of control cultures (△) and of cultures to which cycloheximide was added at 2 h p.i. (▲), 2.5 h p.i. (●), or 3 h p.i. (■); □, [^{3}H]-leucine incorporation into acid-insoluble material, assayed on a parallel set of cultures as described in the legend to Fig. 1. (B) Kinetics of guanidine inhibition of Na^+ accumulation by infected cells. The experimental details were as described in the legend to Fig. 2, except that 2 mM guanidine was added to the three groups of cultures at the times indicated. Symbols: $^{22}Na^+$ radioactivity of control cultures (△) and of cultures to which guanidine was added at 2 h p.i. (○), 2.5 h p.i. (□), or 3 h p.i. (●).*

DISCUSSION

Carrasco and Smith predicted Na^+ accumulation by picornavirus-infected cells and suggested that this increase in Na^+ content is the cause of virus inhibition of host protein synthesis (5). In this study we have demonstrated an increase in cumulative $^{22}Na^+$ uptake by poliovirus-infected HeLa cells. However, contrary to the prediction of Carrasco and Smith, the rise in Na^+ uptake occurs after, not before, the inhibition of host protein synthesis becomes evident (Fig. 1). In this respect our results are similar to those of Egberts et al., who reported a late increase in the Na^+ content of mengovirus-infected ascites tumor cells (8). However, there are differences between our results and theirs. In their system there was an initial drop and a later relatively small increase in cellular Na^+ concentration (8). The Na^+ increase coincided with loss of ATP from infected cells and rapidly leveled off. In our system an early drop in $^{22}Na^+$ uptake was absent, the late increase in $^{22}Na^+$ uptake was continuous and large, representing at the highest point a fourfold increase over the early-phase level (Fig. 1), and no significant change in intracellular ATP concentration was observed

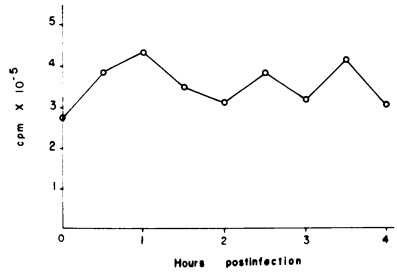

FIG. 4. *ATP concentration of poliovirus-infected cells as a function of time after infection. Replicate infected cultures were incubated with MEM. Acid-soluble material was extracted from cultures removed at intervals. ATP content of the neutralized extracts was assayed by a bioluminescence procedure. See text for details.*

TABLE 1. *Na^+-K^+ ATPase activity of poliovirus-infected cells*[a]

Time p.i. (h)	Amt (μg) of P_i/mg of protein[b]
0	4.08
1	4.88
2	3.64
3	3.56
4.5	3.40

[a] Extracts of infected cells were prepared by Dounce homogenization. ATPase activity of the extracts was assayed in the presence or absence of 3 mM ouabain, as described in the text. The difference between the activity with and without ouabain was attributed to Na^+-K^+ ATPase, which accounted for 14 to 19% of the total activity at different times after infection.

[b] Protein determinations were made by the method described by Lowry et al. (15), using bovine serum albumin as standard.

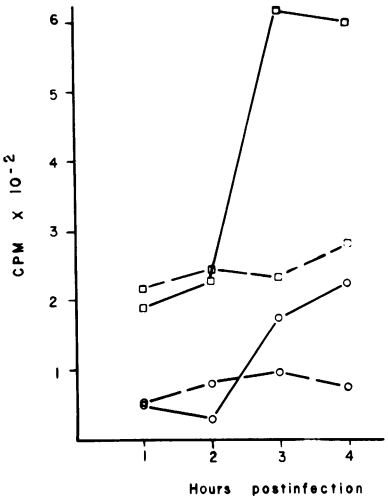

FIG. 5. *Additivity of virus- and ouabain-induced stimulation of Na^+ uptake by cells. Infected or uninfected replicate cultures were incubated with MEM. At 1, 2, 3, and 4 h p.i. one pair of cultures was removed from each group. The medium in one culture of each pair was replaced with medium containing $^{22}NaCl$ (0.5 μCi/ml), and the medium in the other culture was replaced with medium containing $^{22}NaCl$ plus 3 mM ouabain. After incubation for 30 min, all four cultures were processed, and their $^{22}Na^+$ content was assayed as described in the text. Symbols: O- - -O, uninfected, untreated control cultures; O——O, infected untreated cultures; □- - -□, uninfected, ouabain-treated cultures; and □——□, infected, ouabain-treated cultures.*

for 4 h p.i. (Fig. 4), when Na^+ accumulation was well underway (Fig. 1). Egberts and co-workers have concluded that the fluctuations in Na^+-K^+ levels and the various manifestations of cytotoxicity are ultimately caused by changes in cellular ATP concentration (8). Our results do not lead us to this conclusion. We have no explanation for the differences between our results and theirs except that differences in the virus-cell systems and in the procedures used may somehow be involved. We could find only a small reduction in Na^+-K^+ ATPase activity of infected cell homogenates (Table 1), ruling out inhibition of synthesis or activity of this enzyme as the primary cause of Na^+ accumulation. However, a small inhibition of the enzyme activity observed toward the late phase of infection (Table 1) could explain a portion of Na^+ accumulation.

The results of another experiment suggest that increased permeability of infected cell membrane was responsible for Na^+ accumulation. Thus, the stimulation of Na^+ uptake by ouabain could be readily observed over and above that due to virus infection (Fig. 5), meaning that virus stimulation of Na^+ uptake was due to a mechanism not involving Na^+-K^+ ATPase activity.

The above results seem to suggest that the buildup of Na^+ in poliovirus-infected cells is due to increased influx rather than decreased efflux of this cation. This phenomenon should be added to the list of observations concerning passage of substances across the plasma membranes of virus-infected cells due to a weakening of the permeability barrier (14, 16, 17). Our results do not reveal the mechanism by which virus replication alters membrane permeability. Viral protein synthesis or RNA synthesis or both are essential since both cycloheximide and guanidine inhibit this phenomenon (Fig. 3A and B). However, since in the presence of these inhibitors all subsequent events leading to progeny production are inhibited, it is difficult to know whether a structural component, an assembly intermediate, or virions are actually responsible and whether direct interaction between viral and membrane components is required for the change in membrane permeability. The subviral component or virions may interact with the membrane to open up new Na^+ channels by insertion into the lipid bilayer, or a late viral function may induce a phospholipase activity involved in the weakening of membrane integrity. It may be significant that the various processes of poliovirus replication take place in intimate association with the cellular membranes (3, 22, 24).

The possibility that the initial trigger in the chain of events leading to Na^+ accumulation is Na^+ binding by virions or viral products should also be considered. Presumably the depletion of the cellular free Na^+ pool and the consequent inactivation of the Na^+-K^+ pump would be the mechanism of Na^+ accumulation in this case. As already pointed out, the results from our ouabain experiment do not support this mechanism (Fig. 5). The ouabain results are compatible, however, with the idea that a physically leaky membrane is responsible for the Na^+ influx. Na^+ binding by virions or viral products may still occur, except that the binding will be the result rather than the cause of Na^+ accumulation. Depending on the extent of Na^+ binding relative to Na^+ influx, the free Na^+ concentration of the cells may increase or remain unchanged. Parallel measurements of $^{22}Na^+$ and $^{42}K^+$ uptake by virus-infected cells have indicated that Na^+ accumulation is not matched by K^+ depletion (manuscript in preparation) and therefore might suggest intracellular hypertonicity if there is an increase in free Na^+ concentration. From density measurements of late-infected cells, we have obtained indirect evidence for their hypertonicity (manuscript in preparation). At the moment

we favor the view that Na^+ accumulation by virus-infected cells causes hypertonicity due to an increase in cellular free Na^+ concentration and that this condition is responsible for the late manifestations of virus cytotoxicity. It is obvious that definitive answers to many of these very important questions must await measurement of exchangeable and nonexchangeable Na^+ pools in virus-infected cells. These experiments are currently underway.

Finally, the observation that Na^+ accumulation by infected cultures was absent in the presence of guanidine (Fig. 1), but was restored by the simultaneous presence of choline (Fig. 2), may be significant. Choline is commonly used to replace Na^+ in ion transport experiments. Therefore, it stands to reason that the action of these agents is at the level of a Na^+ (cation)-dependent viral function via their positively charged ions. Guanidine, unlike choline, may not be able to replace the Na^+ (cation) requirement of this function. The correct folding and processing of one or more viral precursor polypeptides could be the cation-dependent viral function with which guanidine and choline might interact. The evidence for guanidine binding to poliovirus capsid precursor polypeptide (12) is consistent with this hypothesis. Such an ionic explanation of guanidine action merits consideration since more conventional approaches have failed to reveal the actual mechanism of guanidine action (2, 7, 11, 13, 18, 23, 27).

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