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Mechanism of Poliovirus Inactivation by Ammonia

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Poliovirus inactivation by ammonia causes a slight reduction in the sedimentation coefficients of viral particles, but has no detectable effect on either the electrophoretic pattern of viral capsid proteins or the isoelectric points of inactivated particles. These virions still attach to cells, but are unable to repress host translation or stimulate the synthesis of detectable amounts of viral RNA. Although ammonia has no detectable effect on naked poliovirus RNA, it causes cleavage of this RNA when still within viral particles. Therefore, the RNA genome appears to be the only component of poliovirus significantly affected by ammonia.

During the course of an investigation on the effects of wastewater sludge on poliovirus, it was found that viral inactivation rates are considerably increased in anaerobically digested but not raw sludge (16, 18). Subsequently, it was shown that the sludge agent responsible for this effect is ammonia (15, 17). This compound is not virucidal as ammonium ion, and the pK for its conversion into virucidal ammonia is 9.2. Because the pH of digested sludge is typically about 2 pH units higher than that of raw sludge, a far greater percentage of indigenous ammonia is present in the virucidal state in digested sludge. This explains why poliovirus inactivation is accelerated in digested, but not raw, sludge.

Studies on the inactivation rates of other enteric viruses with ammonia indicated that sensitivity to this agent appears to be a general property of viruses belonging to the enterovirus group (17). Reovirus, an enteric virus of a different group, is insensitive to ammonia. The physical or chemical dissimilarity between enteroviruses and reoviruses that causes this very different response to ammonia is not immediately evident from structural considerations. A determination of the particular property of enteroviruses that makes them sensitive to ammonia should reveal why reovirus is insensitive, and may allow one to predict the susceptibilities of other viruses to this compound as well. Therefore, the mechanism by which ammonia causes inactivation of poliovirus was determined.

MATERIALS AND METHODS

Virus and cells. Poliovirus type 1 strain CHAT obtained from the American Type Culture Collection was used throughout the course of this investigation. This virus was both produced and assayed for biological activity in monolayer cultures of HeLa cells grown in Eagle minimal essential medium containing 5% newborn calf serum. Radioactively labeled virus preparations made with either [^{3}H]uridine or ^{14}C-reconstituted protein hydrolysate (Schwarz mixture) were grown and purified as described in an earlier publication (16).

Inactivation of poliovirus with ammonia. Purified preparations of poliovirus were diluted into 0.1 M Tris at pH 9.5 either with or without (control) 0.5 M NH_4Cl. After incubation at 4°C for the times specified, loss of biological activity was determined by plaque assay (16).

Adsorption of inactivated poliovirions to HeLa cells. After 24 h of incubation at 4°C in either the Tris or Tris plus NH_4Cl described above, radioactively labeled viral particles were diluted 10-fold with 0.1 M Tris (pH 7.0) and assayed for biological activity and recoverable radioactivity. Samples of each preparation were then adsorbed at 37°C on monolayer cultures of HeLa cells and, after 30 min., unadsorbed virus was removed with a Pasteur pipette. The cells were washed twice and suspended in phosphate-buffered saline (PBS) before measuring cell-associated radioactivity. The results were corrected for loss of radioactivity due to quenching.

Analysis of poliovirions and subviral components. Sedimentation analysis of viral particles, phenol extraction of viral RNA, and sedimentation studies and infectivity analysis of poliovirus RNA were carried out as previously described (16). Isoelectric focusing of poliovirions was performed by the method of Korant and Lonberg-Holm (8). In short, 0.2 ml of the virus preparation to be analyzed was mixed with 0.12 ml of 40% (wt/vol) sucrose containing 1% ampholine (pH 3.5 to 10). These samples were added at the position of 15% sucrose during the generation of 10 to 40% sucrose gradients also containing 1% ampholine. All sucrose gradients were made in glass tubes (13 by 0.6 cm) stoppered with a small piece of dialysis tubing. For electrophoresis, the bottom reservoir contained 1% sulfuric acid (vol/vol) in 40% sucrose and the top reservoir contained 2% 2-aminoethanol (vol/vol). After electrophoresis at a constant voltage of 500 V at 4°C for 6 h, fractions were collected from the bottom of the tubes after perforation of the dialysis mem-

brane. Each fraction was diluted with a small volume of distilled water before determining the pH and total counts per minute.

RESULTS

As reported earlier, a reduction in the infectivity of poliovirus of 99% during 10 days at 4°C in anaerobically digested sludge has little effect on the sedimentation coefficients of viral particles (16). A further examination of these particles, however, revealed that their RNA genomes had been nicked and some cleavage of viral proteins had occurred. Although the compound responsible for viral inactivation was later shown to be ammonia (17), sludge contains an assortment of enzymes and other substances that may cause breakdown of viral RNA and proteins after the particles have been inactivated by ammonia. To determine which component of the virus is the target of ammonia, the effects of extraneous substances had to be eliminated. Therefore, the mechanism of inactivation of poliovirus by ammonia was determined by using highly purified poliovirus in a solution containing ammonium chloride buffered with Tris. Possible breakdown of inactivated virions by heat was minimized by performing the reaction at 4°C. In the absence of NH_4Cl, little if any decrease in the specific infectivity of purified poliovirus labeled with ^{14}C-protein hydrolysate occurred in 0.1 M Tris buffer at pH 9.5 after 64 h (Fig. 1). Incubation with 0.5 M NH_4Cl under these same conditions caused the specific infectivity of poliovirus to decrease more than five orders of magnitude.

Sedimentation analysis of inactivated virions. The first feature of poliovirions inactivated by ammonia to be examined was their sedimentation coefficients. Viral particles were found to sediment at slightly reduced rates after inactivation by ammonia (Fig. 2). This result indicates that ammonia causes only a slight structural alteration of the poliovirion.

Analysis of the capsid proteins of inactivated poliovirus particles. Because some cleavage of viral proteins occurs during inactivation of poliovirus by ammonia in sludge (16), possible breakdown of viral proteins in particles inactivated by ammonia in the absence of sludge was determined. No significant difference was found in the proteins of infectious and inactivated particles by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 3). Therefore, ammonia does not appear to cause cleavage of viral proteins under the conditions of this experiment.

It has been reported that poliovirus (11) and other picornaviruses (3, 9) have two predominant isoelectric points. In the case of poliovirus, these isoelectric points are at pH values of about

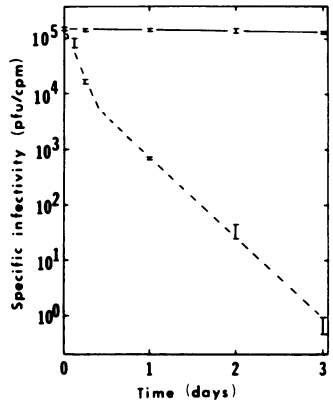

FIG. 1. *Rate of poliovirus inactivation by ammonia. Samples of purified ^{14}C-labeled poliovirus were diluted 10-fold into 0.1 M Tris, pH 9.5 (——), or Tris plus 0.5 M NH_4Cl, pH 9.5 (- - -), incubated at 4°C for the times specified, and assayed for recoverable PFU and radioactivity. The specific infectivity of each sample was then calculated. The results shown here represent two separate experiments.*

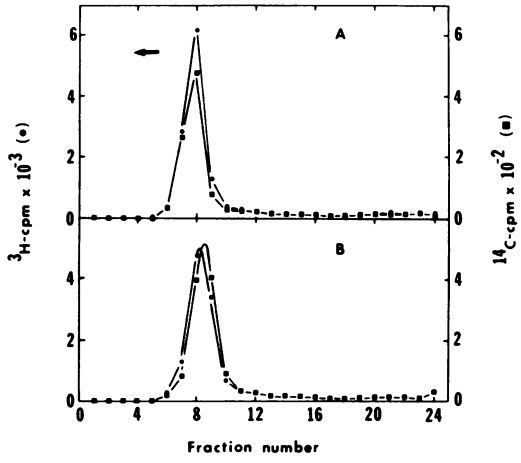

FIG. 2. *Effect of ammonia on the sedimentation coefficients of poliovirus particles. Purified poliovirus labeled with ^{14}C-protein hydrolysate was diluted 10-fold into 0.1 M Tris, pH 9.5 (A), or Tris plus NH_4Cl, pH 9.5 (B), and incubated for 24 h at 4°C. After treatment, the samples were assayed for recoverable infectivity, mixed with a small volume of purified poliovirus labeled with [^{3}H]uridine, and analyzed by density-gradient centrifugation (15 to 30% glycerol, 0.1 M NaCl, 0.01 M Tris, pH 7.5, 0.001 M EDTA, SW27.1 rotor, 27,000 rpm, 4 h, 4°C). The arrow shows the direction of sedimentation.*

7 and 4.5. Because interconversion of infectious poliovirus particles occurs between the two isoelectric forms, the conformational alteration associated with the change in isoelectric point is reversible. When inactivation of poliovirus results in an alteration of the capsid, however, the

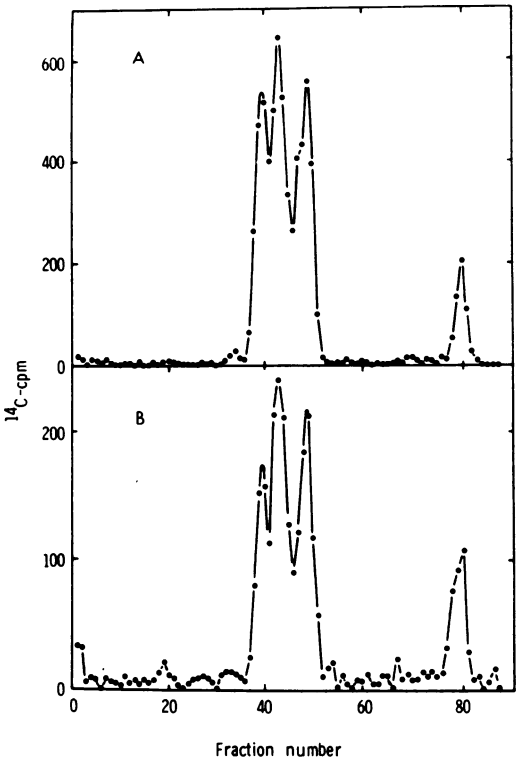

FIG. 3. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns of poliovirus proteins extracted from infectious and ammonia-inactivated viral particles. Purified poliovirus labeled with ^{14}C-protein hydrolysate was diluted 10-fold into either 0.1 M Tris, pH 9.5 (A), or Tris plus 0.5 M NH_4Cl, pH 9.5 (B), and incubated for 24 h at 4°C. The proteins of these particles were then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as previously described (16).*

particles are normally irreversibly stabilized in the lower pH state. Therefore, if the capsid is the component of the poliovirus particle that is modified during viral inactivation by ammonia, irreversible stabilization of the viral particles in the lower pH state is likely to result.

The strain of poliovirus type 1 used in these experiments was found to have two predominant isoelectric points at pH values of 7.5 and 4.5 (Fig. 4A). Heat inactivation of these particles during 5 min at 45°C caused about a 3-log reduction in viral infectivity and the conversion of most virions into subviral particles that sedimented at about 80S (results not shown). The isoelectric point of these heat-inactivated particles appears to be stabilized at a pH of about 4.4 (Fig. 4B). However, the isoelectric points of virions inactivated almost five orders of magnitude by ammonia are not significantly different than those of native virions (Fig. 4C). From this result, it appears that ammonia does not cause a major conformational alteration in the capsids of inactivated particles. This suggestion is supported by the finding that virions inactivated by ammonia have only a slightly reduced ability to attach to cells during adsorption studies (Table 1).

Analysis of viral macromolecular synthesis in cells infected with inactivated virions. The next step after attachment is the uptake of virions by cells. One of the first biochemical events detectable in a cell after infection with viable poliovirus particles is repression of host protein synthesis. It has been suggested that this event requires the synthesis of new viral proteins (2, 7, 13). Therefore, if the RNA genomes of poliovirions taken up by cells are able to stimulate the synthesis of active viral proteins, it is expected that these proteins will cause at least partial repression of cellular protein synthesis. Inactivated particles that are unable to stimulate the synthesis of viral proteins should be unable to repress host protein synthesis.

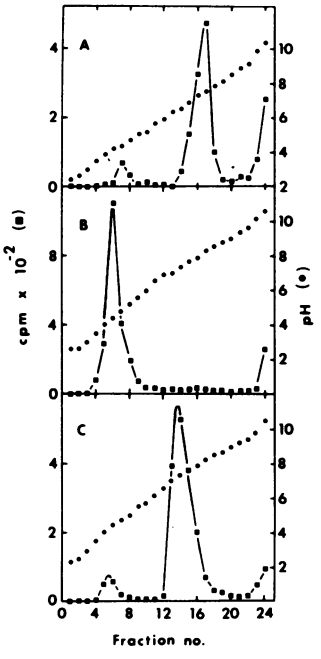

FIG. 4. *Effect of inactivation by heat versus ammonia on the isoelectric points of poliovirus. Purified, ^{14}C-labeled poliovirus was diluted 10-fold into either 0.01 M Tris, pH 7 (B), or 0.1 M Tris, pH 9.5, containing 0.5 M NH_4Cl (C). The former sample was heated for 5 min at 45°C, whereas the latter was incubated for 55 h at 4°C and then dialyzed overnight against 0.01 M Tris, pH 7. Finally, both samples were assayed for loss of infectivity and analyzed by isoelectric focusing along with an untreated control (A).*

TABLE 1. *Effect of inactivation by ammonia on the ability of radioactively labeled poliovirus to adsorb to HeLa cells*[a]

Expt	Label/species	Treatment	Total cpm added	cpm bound	% cpm bound
1	^{3}H/RNA	Tris	15,220	2488	16.3
		Tris-NH_3	14,252	2033	14.3
2	^{3}H/RNA	Tris	11,662	1934	16.6
		Tris-NH_3	12,109	1573	13.0
	^{14}C/protein	Tris	1,188	317	26.7
		Tris-NH_3	1,731	382	22.1

[a] After incubation of purified radioactively labeled poliovirus for 24 h at 4°C in either 0.1 M Tris (pH 9.5) or 0.1 M Tris (pH 9.5) with 0.5 M NH_4Cl, viral particles were adsorbed at 37°C to monolayers of HeLa cells by using a multiplicity of infection of 20 PFU/cell. The total amount of radioactivity added to the cultures and the amount bound after a 30-min adsorption period were then determined.

The rate of protein synthesis is efficiently repressed in cells after infection with viable poliovirus at a multiplicity of infection of 10 PFU per cell (Fig. 5). Infection with the same number of virions inactivated by ammonia had no significant effect on the rate of protein synthesis in these cells. It is probable, therefore, that virions inactivated by ammonia are not able to stimulate the synthesis of active viral proteins under these conditions. Furthermore, it was also shown that these inactivated virions are unable to synthesize detectable amounts of viral RNA (results not shown). These results imply that the normal replication cycle of polioviruses inactivated by ammonia is blocked between the time of viral attachment and the synthesis of viral macromolecules. The most likely cause, but certainly not the only possible cause, of such blockage is that the RNA genomes of inactivated virions are damaged and, as a result, are unable to function normally.

Effect of ammonia on free and virion-associated poliovirus RNA. Before analyzing RNA molecules from inactivated virions, the effect of ammonia on the physical and biological integrity of naked poliovirus RNA was determined. Incubation of poliovirus RNA with NH_4Cl under conditions that reduce viral infectivity about three orders of magnitude had no detectable effect on the sedimentation coefficient (Fig. 6) or infectivity (Table 2) of viral RNA. Therefore, ammonia does not appear to cause inactivation of naked poliovirus RNA.

In contrast to these results, the specific infectivity of poliovirus RNA extracted with phenol from particles inactivated by ammonia is reduced in proportion to the reduction in the specific infectivity of the virus (Table 3). Furthermore, these RNA molecules have been cleaved, as demonstrated by their decreased sedimentation rates (Fig. 7). From these results it is concluded that poliovirus inactivation by ammonia results in the breakdown and, therefore, inactivation of the viral RNA within the intact particle.

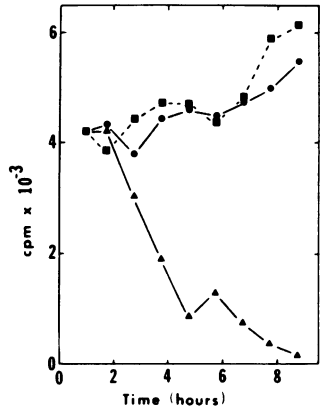

FIG. 5. *Effect of ammonia on the ability of poliovirus to inhibit amino acid incorporation in HeLa cells. Cells were pulse-labeled for 30-min periods at 37°C after mock infection (■), infection with purified ^{14}C-labeled poliovirus at a multiplicity of infection of 10 (▲), or infection with the same number of virus particles inactivated almost five orders of magnitude with ammonia (●). Pulse-labeling was performed with 2 μCi of ^{3}H-labeled reconstituted protein hydrolysate (Schwarz mixture) per ml of medium. Samples were then assayed for total acid-precipitable radioactivity, and the results were plotted after subtracting background incorporation into uninfected cells at 4°C.*

One other explanation for these results is that the virus preparation used for these experiments may not have been totally free of RNase molecules that are not integral parts of the virions. Possibly, such extraneous nuclease molecules could then attack viral RNA within particles whose structures had somehow been altered with ammonia. It is difficult to eliminate this possible explanation, but it is highly unlikely that extraviral nucleases caused cleavage of poliovirus RNA or inactivation of viral particles in these experiments for the following three reasons.

(i) Treatment of purified poliovirus with a RNase inactivator and inhibitor, chymotrypsin and polyvinylsulfate, respectively, did not alter

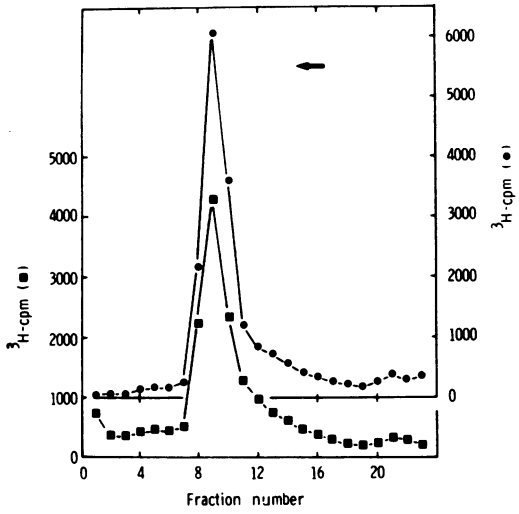

FIG. 6. *Density-gradient analysis of poliovirus RNA after treatment with ammonia. Purified poliovirus labeled with [^{3}H]uridine was extracted with phenol, incubated for 24 h at 4°C in either PBS (●) or 0.1 M Tris (pH 9.5) plus 0.5 M NH_4Cl (■), and analyzed by using glycerol gradient centrifugation (5 to 30% glycerol in 0.1 M NaCl, 0.02 M Tris [pH 8], 0.005 M EDTA, SW50.1 rotor, 45,000 rpm, 2.67 h, 4°C). The arrow shows the direction of sedimentation.*

TABLE 2. *Effect of ammonia on the infectivity of poliovirus RNA*[a]

Sample	Treatment	Recoverable infectivity (PFU)
PBS	−20°C, 24 h	8.2×10^4
PBS	4°C, 24 h	3.5×10^4
0.1 M Tris, pH 9.5 + 0.5 M NH_4Cl	4°C, 24 h	4.5×10^4

[a] Poliovirus RNA extracted with phenol was incubated under the specified conditions and measured for recoverable infectivity.

the amount of viral inactivation by ammonia or the specific infectivity and sedimentation coefficient of viral RNA extracted from inactivated particles (results not shown). (ii) Inactivation of poliovirions with ammonia in the presence of 20 μg of pancreatic RNase per ml caused no additional loss of viral infectivity, and the RNA extracted from these inactivated particles had the same specific infectivity and amount of cleavage as RNA extracted from particles inactivated in the absence of added RNase (results not shown). (iii) Incubation of ^{3}H-labeled poliovirus RNA with ammonia in the presence of purified, unlabeled poliovirus particles had little effect on the sedimentation coefficient of the labeled RNA (Fig. 8). Because this RNA was not broken down, little or no extraviral nuclease activity was expressed under the conditions of this experiment.

DISCUSSION

Poliovirus inactivation by ammonia reduces the sedimentation coefficient of viral particles by a slight but significant amount, but has no detectable effect on the isoelectric points of these particles or on the pattern of capsid proteins obtained by electrophoresis in sodium dodecyl sulfate-polyacrylamide gels. RNA from inactivated virions sediments more slowly than intact RNA in a density gradient, and its specific infectivity is reduced in proportion to that of the virions from which it was extracted. Neither

TABLE 3. *Effect of ammonia on the infectivity of RNA in poliovirus particles*[a]

Sample	Specific infectivity (PFU/cpm)		
	Virus	RNA	Virus/RNA ratio
Tris	1.0×10^4	1.1×10^0	0.9×10^4
Tris, NH_4Cl	4.0×10^1	3.3×10^{-3}	1.2×10^4

[a] Purified poliovirus labeled with [^{3}H]uridine was incubated for 24 h in 0.1 M Tris buffer at pH 9.5 or in Tris buffer with 0.5 M NH_4Cl at the same pH, and assayed for loss of infectivity and recoverable radioactivity. Viral RNA was then extracted with phenol, and its specific infectivity was determined.

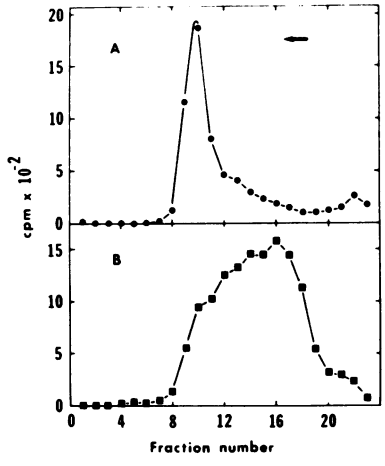

FIG. 7. *Density-gradient analysis of poliovirus RNA extracted after inactivation of virions with ammonia. Samples of viral RNA obtained as described in Table 1 were layered onto 5 to 30% glycerol gradients in 0.1 M NaCl, 0.02 M Tris (pH 8), and 0.005 M EDTA and analyzed for total radioactivity after centrifugation (SW50.1 rotor, 45,000 rpm, 2.67 h, 4°C). The arrow indicates the direction of centrifugation. (A) RNA from sample incubated in 0.1 M Tris, pH 9.5. (B) RNA from sample incubated in 0.1 M Tris, pH 9.5, plus 0.5 M NH_4Cl.*

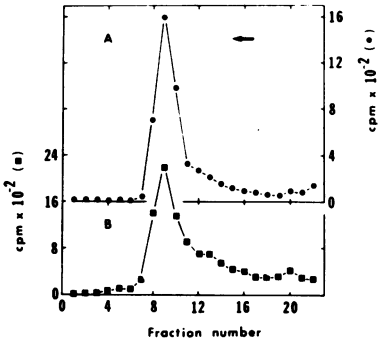

FIG. 8. *Effect of ammonia on the sedimentation coefficient of naked poliovirus RNA incubated in the presence of purified poliovirus. Samples of RNA (2 × 10⁵ PFU) extracted from purified poliovirus labeled with [³H]uridine were suspended in PBS (A) or 0.1 M Tris, pH 9.5, plus 0.5 M NH₄Cl containing 10⁹ PFU of purified, unlabeled poliovirus (B) and incubated for 24 h at 4°C. The sample in Tris-NH₄Cl was reextracted with phenol, and both samples were analyzed by density-gradient centrifugation as described in the legend to Fig. 7.*

extraviral nucleases nor direct attack of the viral genome by ammonia appears to be the cause of RNA inactivation in these experiments. These results suggest that ammonia causes poliovirus inactivation by inducing cleavage of viral RNA within intact virions, possibly by stimulating viral capsid proteins to express nuclease activity.

Another possible explanation for the observations made here is that ammonia causes a covalent bond to form between viral capsid proteins and RNA, which blocks the infectivity of the RNA and modifies its sedimentation coefficient. This explanation was eliminated by showing that RNA from poliovirions labeled with ^{14}C-reconstituted protein hydrolysate contained a small but constant amount of radioactive carbon, whether extracted before or after inactivation with ammonia. Because poliovirus RNA has been reported to have a small protein covalently linked to its 5'-terminus (5, 10), the presence of detectable amounts of ^{14}C-label in the viral RNA in this experiment was not unexpected.

Suggestive evidence of RNase activity within particles of another picornavirus, rhinovirus 14, has been previously reported (6). In this case, RNA fragmentation was found to occur in both crude and purified virus preparations simply through incubation of virions at 34.5°C. Dimmock has suggested that "low" temperature inactivation of picornaviruses occurs primarily through inactivation of the viral RNA (4). Perhaps ammonia inactivates poliovirus by accelerating the natural breakdown of viral RNA within intact virions.

The toxicity of ammonia toward a number of organisms displays a similar pH dependence to that found for enteroviruses (1, 12, 14, 19). Although the mechanism of inactivation probably differs from one microbial species to the next, the resistance of these organisms to ammonium ion may have a common cause. This ionized molecule may be unable to penetrate the protective barrier that surrounds the site attacked by ammonia, whereas unionized ammonia may pass this barrier with ease. Differences in the abilities of ionized and unionized ammonia to penetrate cellular membranes are well documented (1, 19), and hydrophobic regions in the inner portion of capsid proteins may prevent passage of ammonium ion into nonmembranous viruses. It is possible that selective penetration of polioviruses by unionized ammonia may cause the internal pH of these virions to increase. If this is the case, cleavage of viral RNA may be due to alkaline hydrolysis.

An explanation for the observed differences in the sensitivities of enteroviruses and reovirus to ammonia (15, 17) is not immediately obvious from the results reported here. The ability of ammonia to penetrate the viral capsids may differ, or the sensitivities of the single-stranded RNA genomes of enteroviruses and the double-stranded RNA genome of reovirus to cleavage by ammonia may be dissimilar. The bacteriophage T4 containing double-stranded DNA is resistant to ammonia (unpublished data). In contrast, the bacteriophage f2 has been found to be inactivated by this compound (W. N. Cramer and W. D. Burge, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, P2, p. 255), a result that has been confirmed in this laboratory with both f2 and the bacteriophage Qβ (unpublished data). As with enteroviruses, the genomes of these phages are single-stranded RNA molecules. Although the rate of inactivation of f2 and Qβ by ammonia was much slower than that found for poliovirus, it would be of interest to determine whether the mechanism of inactivation is the same in each case.

It has been proposed that synthesis of poliovirus proteins is required for the repression of host translation after infection (2, 7, 13). The results reported in this manuscript support this hypothesis. The only portion of the poliovirus particle found to be damaged by ammonia is the viral RNA, but this is sufficient to prevent this virus from repressing the synthesis of host proteins.

Because the isoelectric points of poliovirions are not modified by ammonia, it is assumed that the capsids of virions inactivated by this compound have not undergone a conformational al-

teration. Retention of native capsid conformation is generally believed to be synonomous with retention of native antigenicity. Therefore, it may be feasible to use poliovirus particles inactivated by ammonia to stimulate antibody production against infectious viruses. This possibility is being investigated.

ACKNOWLEDGMENTS

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