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Trypsin Sensitivity of the Sabin Strain of Type 1 Poliovirus: Cleavage Sites in Virions and Related Particles†

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Treatment of the Sabin strain of type 1 poliovirus with trypsin produced two stable fragments of capsid protein VP1 which remained associated with the virions. Trypsinized virus was fully infectious and was neutralized by type-specific antisera. The susceptible site in the Sabin 1 strain was between the lysine at position 99 and the asparagine at position 100. A similar tryptic cleavage occurred in the Leon and Sabin strains of type 3 poliovirus, probably at the arginine at position 100, but not in the type 1 Mahoney strain, which lacks a basic residue at either position 99 or position 100. Tryptic treatment of heat-treated virus and 14S assembly intermediates produced unique stable fragments which were different from those produced in virions. The implications of our results for future characterization of the surface structures of these particles and structural rearrangements in the poliovirus capsid are discussed.

Historically, poliovirus has been considered to be resistant to proteolysis by trypsin (9). In this paper we present a detailed characterization of a single tryptic cleavage site in the Sabin strain of type 1 poliovirus and describe tryptic cleavages that occur in several Sabin 1-related particles and in other poliovirus strains.

Treatment of the Sabin strain of type 1 poliovirus with trypsin (0.5 mg of virus per ml; 0.01 mg of trypsin per ml) resulted in the cleavage of VP1 into two stable fragments, VP1T1 and VP1T2, having apparent molecular weights on sodium dodecyl sulfate (SDS)-polyacrylamide gels of 24,000 and 12,000, respectively (Fig. 1, lanes A and B). At room temperature cleavage was detectable after 1 h and complete in 8 to 10 h. Identical treatment of the Mahoney strain of type 1 poliovirus did not result in any cleavage (Fig. 1, lanes C and D). Treatment of poliovirus type 3 Sabin also resulted in cleavage of VP1, yielding fragments similar in size to those from poliovirus type 1 Sabin (Fig. 1, lanes E and F). The tryptic sensitivity of VP1 from the neurovirulent type 3 Leon strain was similar to the tryptic sensitivities of the VP1s from the Sabin 1 and Sabin 3 strains (data not shown). Thus, insensitivity of virions to trypsin is not necessary for neurovirulence.

The physical and biological properties of trypsin-treated Sabin 1 virions were not detectably different from those of uncleaved virions. The fragments of VP1 remained associated with the virions on both sucrose and CsCl gradients (Fig. 1, lanes G and H), indicating that they were tightly bound even at high ionic strength. In addition, sucrose and CsCl gradients in which we used differentially labeled type 1 Sabin and trypsin-treated type 1 Sabin showed that cleavage of VP1 resulted in no change in sedimentation rate (±8S) or in buoyant density (±0.005 g/ml) (data not shown). The infectivity of trypsin-treated virus was indistinguishable from that of the untreated virus (Table 1). Infectivity was also maintained in 0.1% SDS and at pH 3. This stability of trypsin-treated virus was significant as empty capsids isolated from poliovirus-infected cells are labile in 0.1% SDS (15), and rhinoviruses and foot and mouth disease virus (FMDV) are unstable at pH 3 (22). Table 1 also shows that treatment with trypsin did not alter the ability of antisera to Sabin 1 to neutralize infectivity.

Several factors may explain the failure of previous studies to detect this tryptic sensitivity. In early experiments workers monitored the infectivity and physical properties of virions which are not changed by tryptic cleavage (9). Furthermore, cleavage requires high ratios of enzyme to virus and prolonged incubation times and does not occur in the commonly used Mahoney strain.

Western blots in which antisera to synthetic peptides of VP1 were used (1) showed that VP1T1 reacted only with antisera to C-terminal peptides (including peptides representing amino acids 161 through 181 and 245 through 265), whereas VP1T2 reacted with antisera to a peptide corresponding to amino acids 24 through 40 (data not shown). An examination of amino acid sequences for potential tryptic sites in the region from amino acid 24 to amino acid 181 which are present in Sabin 1 and not in the Mahoney strain suggested that cleavage is after the lysine at position 99 in VP1 (14, 17, 20).

To verify this identification, the N-terminal sequences of VP1T1 and VP1T2 were determined after electroelution of the fragments from preparative gels (10). The N-terminal sequence of VP1T1 is N-K-D-K-L (IUPAC-IUB standard single-letter code [see reference 11a]), confirming the identification of the cleavage site as the lysine at position 99 (Fig. 2). Interestingly, the potential tryptic sites at the lysine at position 101 and the lysine at position 103 were not cleaved even after the initial cleavage between residues 99 and 100. The N-terminal sequence of VP1T2 is G-L-G-Q-M, which is identical to the N-terminal sequence of intact VP1. Amino acid analysis showed that there was 1 mole-equivalent of cysteine in VP1T2. Since residues 1 through 99 contain only one cysteine (residue 86) and have no potential tryptic sites between residues 86 and 99 (Fig. 2), it is unlikely that additional tryptic cleavages occur near the C terminus of VP1T2.

A number of lines of evidence suggest that the cleavage site of the VP1 of Sabin 3 is similar to the cleavage site of the VP1 of Sabin 1. Both intact VP1 and the small fragment of Sabin 3 VP1 migrate considerably slower than the corresponding polypeptides from Sabin 1, whereas the larger VP1

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fragments from the two strains comigrate (Fig. 1). Since the N termini of the VP1 molecules of Sabin 1 and Sabin 3 differ in 22 of the first 26 residues (17, 25), this migration pattern suggests that the small fragment from Sabin 3 is derived from the N terminus. In addition, Western blots in which antisera to synthetic peptides from VP1 were used showed that the large fragment of Sabin 3 VP1 contains amino acids near the C terminus (data not shown). An examination of the Sabin 3 sequence suggested that the arginine at position 100 is the tryptic site in Sabin 3. The lysine at position 103 is less probable since this lysine is also present in Sabin 1 and is not cleaved.

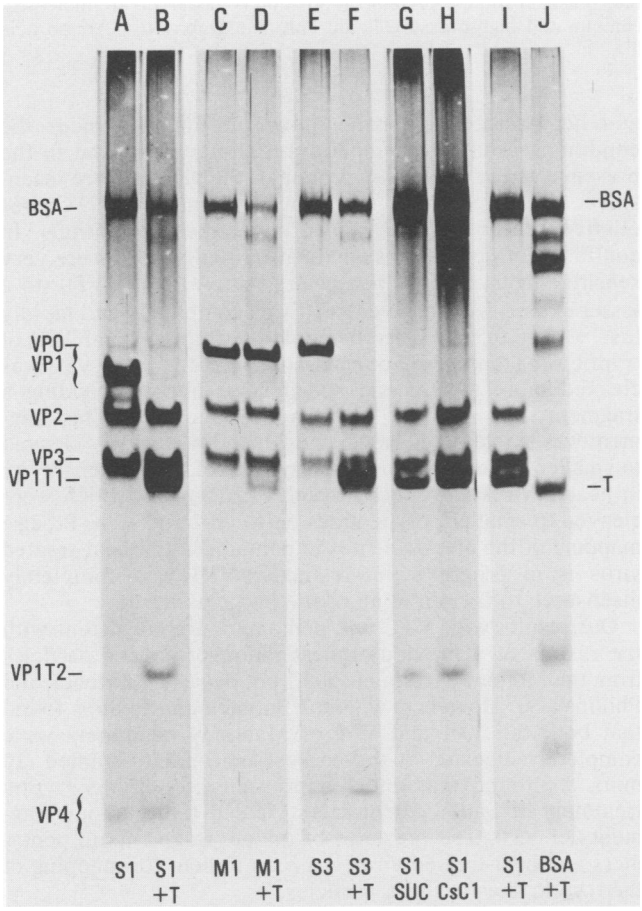

FIG. 1. Trypsin cleaved VP1s of poliovirus type 1 Sabin and type 3 Sabin, but not type 1 Mahoney. Viruses were purified by differential centrifugation and CsCl gradient banding (and in the case of Sabin 3, sucrose gradient centrifugation before CsCl gradient banding). All samples contained bovine serum albumin (BSA) as a carrier protein. Tryptic digestions were carried out with twice-crystallized trypsin (0.5 mg of virus per ml; 0.01 mg of trypsin [catalog no. T-8253; Sigma Chemical Co., St. Louis, Mo.] per ml) for at least 12 h at room temperature in 250 mM NaCl–5 mM Tris-acetate (pH 7.4). Samples were subjected to SDS-polyacrylamide gel electrophoresis in the presence of 3.5 M urea (13, 18) and visualized by silver staining (27). The positions of bovine serum albumin, trypsin (T), the Sabin 1 virion proteins (VP0 [the uncleaved precursor of VP2 and VP4], VP1, VP2, VP3, and VP4), and the cleavage fragments of VP1 (VP1T1 and VP1T2) are indicated. Different strains of poliovirus have VP1s which differ markedly in electrophoretic mobility (13). Mahoney 1 and Sabin 3 VP1s migrated near the position of VP0 from Sabin 1. Lane A, Sabin 1 (S1); lane B, Sabin 1 treated with trypsin; lane C, Mahoney 1 (M1); lane D, Mahoney 1 treated with trypsin (the light band migrating just below Mahoney VP3 is trypsin [see lanes I and J]; note that this band is present in lanes B and F and was removed by sedimentation or CsCl banding [see lanes G and H]); lane E, Sabin 3 (S3); lane F, Sabin 3 treated with trypsin (VP1T2 from Sabin 3 is the faint band migrating just above VP1T2 from Sabin 1); lane G, trypsin-treated Sabin 1 purified by sucrose gradient centrifugation (suc); lane H, trypsin-treated Sabin 1 purified by CsCl gradient centrifugation; lane I, trypsin-treated Sabin 1 (compare with lane J); lane J, trypsin-treated bovine serum albumin. Each lane contained about 1.5 μg of virus, 0.15 μg of bovine serum albumin, and 0.03 μg of trypsin, except lane J, which contained about 3 μg of bovine serum albumin and 0.06 μg of trypsin.

TABLE 1. Infectivity of trypsin-treated Sabin 1

Treatment[a]	log % of infectivity[b]	
	Virus	Trypsin-treated virus[c]
PBSA, 1 h, room temp[d]	2.0	2.0
PBSA, 1 h, 37°C	2.0	2.1
PBS–0.1% SDS, 1 h, room temp[e]	2.0	2.0
PBS–0.1% SDS, 1 h, 37°C	1.9	2.1
pH 3 buffer, 1 h, room temp[f]	1.9	2.0
pH 3 buffer, 1 h, 37°C	1.9	1.9
0.01 μl of antiserum to type 1 Sabin[g]	<0.3	<0.3
0.001 μl of antiserum to type 1 Sabin[g]	1.3	1.1

[a] Approximately 10^8 PFU of [^{3}H]leucine-labeled virus was treated as indicated and assayed for remaining infectivity by a plaque assay (23).
[b] Infectivity was normalized to the amount of [^{3}H]leucine-labeled virus by scintillation counting.
[c] Virus was treated for approximately 15 h at room temperature with trypsin, resulting in more than 90% cleavage of VP1 into VP1T1 and VP1T2.
[d] PBSA, Dulbecco phosphate-buffered saline containing 1.0 mg of bovine serum albumin per ml.
[e] PBS, Dulbecco phosphate-buffered saline.
[f] The pH 3 buffer used was 0.1 M sodium phosphate (pH 3).
[g] Approximately 500 PFU of virus in 1 ml was mixed with the indicated volume of antiserum; this preparation was incubated at 37°C for 1 h and then overnight at 4°C and assayed for infectivity (23).

Proteolytic sensitivity has been shown to be a useful tool for investigating structure and function of virions (5) and related particles (2, 19, 24). Therefore, our observation of a tryptic site in Sabin 1 virions prompted an investigation of the tryptic sensitivity of several Sabin 1-related particles, including heat-treated virus and two putative assembly intermediates. The heat-treated particles were prepared by incubation of virus at 56°C for 10 min. Under these conditions the particles are known to lose one of the capsid proteins, VP4, and most of the viral RNA, and to undergo a change in antigenicity (11). Assembly intermediates were prepared by propagating Sabin 1 in HeLa cells in the presence of low levels of guanidine, which causes the accumulation of capsids (12). Empty capsids were then isolated as the 80S fractions from a sucrose gradient. Provided that capsids were maintained at pH 6 and at 4°C, they reacted with virion-specific antiserum but not with heat-treated virus-specific serum and dissociated to 14S material when they were treated at pH 8.5. These properties are expected for normal capsids (NTC) (15, 21). Pentamers (14S subunits) were obtained by dissociating NTC at pH 8.5.

Direct comparison of the tryptic sensitivities of Sabin 1 and related particles was complicated by three factors. (i) The pH-dependent equilibrium between NTC and pentamers precluded direct comparison of the tryptic sensitivities of these two types of particles at the same pH; therefore, the tryptic sensitivities of the virus, the heat-treated virus, and NTC were examined at pH 6.2, and the tryptic sensitivities

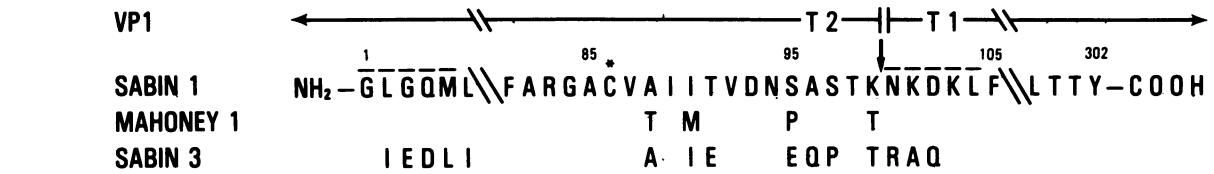

FIG. 2. Trypsin cleaved VP1 of poliovirus type 1 Sabin between the lysine at position 99 and the asparagine at position 100. The cleavage site determined by amino acid composition and sequencing is indicated on the sequence of Sabin 1 VP1 (17). The Mahoney and Sabin 3 sequences are given for comparison (14, 20, 25). The N-terminal amino acids (dashes) and the cysteine at position 86 (asterisk) are indicated. Five N-terminal amino acids were determined by using the Edman technique and high-pressure liquid chromatography (6). Amino acid sequences are shown by the IUPAC-IUB standard single-letter code (11a).

of the virus and pentamers were examined at pH 8.5. (ii) NTC must be kept cold to prevent an irreversible change in their antigenicity (21); therefore, all digestions were done at 4°C. (iii) Although NTC and pentamers were the only ^{3}H-labeled species present, they in fact represented only approximately 10% of the total protein present; therefore, ^{3}H-labeled virus and heat-treated virus were diluted into unlabeled NTC preparations to control for potential sequestering of trypsin by contaminating proteins.

The time courses of the digestions of NTC, pentamers, and heat-treated virus are shown in Fig. 3. Under the conditions used (low temperature, short times, and in the presence of contaminating proteins), the virions were insensitive to trypsin (data not shown). Similarly, NTC was relatively insensitive to trypsin under these conditions. In contrast, both pentamers and heat-treated virions were very sensitive to trypsin. In pentamers both VP1 and VP0 (to a lesser extent) were cleaved. VP1 was completely and rapidly lost within 0.5 h. This demonstrated the availability of tryptic sites that were not accessible in the virions. VP0 was cleaved more slowly and specifically, initially yielding a fragment that migrated between VP0 and VP1. This fragment was stable between 0.5 and 2 h and was slowly cleaved to smaller fragments. The primary sites of tryptic sensitivity in heated virus were in VP1 and VP2, both of which were cleaved to smaller polypeptides between 2 and 10 h. Precise mapping of the cleavage sites in pentamers and heat-treated virus is in progress. Interestingly, VP3 was completely insensitive to trypsin in all of the particles tested.

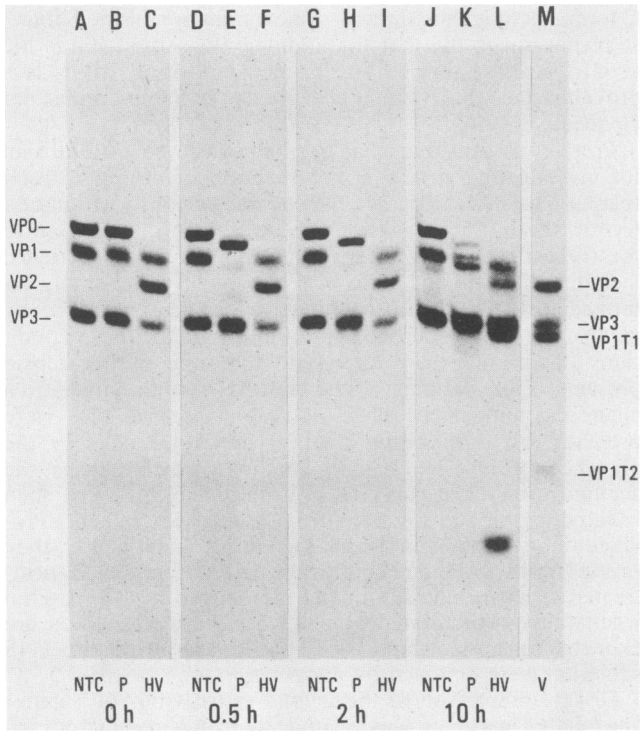

FIG. 3. Comparison of the tryptic sensitivities of various [^{3}H]leucine-labeled poliovirus type 1 Sabin-related particles. All samples were treated at 4°C with trypsin (about 0.5 mg of virus-related particles per ml; 0.01 mg of trypsin per ml) for the times indicated. Digestion was stopped by adding soy bean trypsin inhibitor and immediately boiling the preparation in 1% SDS. The samples were subjected to SDS-polyacrylamide gel electrophoresis as described in the legend to Fig. 1, and the proteins were detected by fluorography. Lanes A through L contained poliovirus capsids (NTC), pentamers (P), and heat-treated virus (HV). NTC and heat-treated virus digests were done in Dulbecco phosphate-buffered saline containing 1.0 mg of bovine serum albumin per ml (pH 6.2), and pentamer digests were done in Dulbecco phosphate-buffered saline containing 1.0 mg of bovine serum albumin per ml and 10 mM Tris (pH 8.5). Lane M is a marker lane that contained ^{3}H-labeled Sabin 1 virions (V) treated with trypsin.

Our results with NTC and pentamers are consistent with the results of a previous investigation of similar particles from the Mahoney strain of type 1 poliovirus by Putnack and Phillips (19). However, whereas Putnack and Phillips found that both the VP1 and VP0 of Mahoney pentamers were completely digested at the earliest time point sampled (10 min), we found that under appropriate conditions tryptic treatment of Sabin 1 pentamers yielded three unique fragments of VP0. The presence of unique fragments in pentamers and heat-treated virus is a requirement for mapping of the tryptic sites in these particles.

A number of conclusions can be drawn from the properties of trypsinized virus. First, consistent with the results of studies in which other methods were used, the tryptic sensitivity which we observed indicates that the region around amino acid 99 is exposed on the viral surface (1, 3, 16). Furthermore, by this criterion the region immediately C terminal to the lysine at position 99 is less accessible since the lysine at position 101 and the lysine at position 103 are not cleaved. Second, a full complement of intact VP1 is not required for the infectivity of the virion. Specifically, the tryptic cleavage which introduces both a positive charge and a negative charge between amino acids 99 and 100 of VP1 does not disrupt any interactions with cellular components required for infectivity. Third, the lack of release of either fragment means that the fragments are anchored to the virion by extensive interactions with one another or with other viral proteins.

It has been suggested that the region from residue 90 through residue 103 is a major immunodominant site in both type 1 and type 3 poliovirus (4, 16). The lack of qualitative or quantitative differences in the ability of antisera to neutralize Sabin 1 and trypsin-treated Sabin 1 implies either that cleavage of VP1 does not affect the ability of antibodies to bind to this putative immunodominant site or that the

antisera contain a high percentage of antibodies against other sites on the virus surface. These two possibilities could be distinguished most easily by using Sabin 3, since a battery of monoclonal antibodies specific for this region is available (16). Experiments with these antibodies are in progress and will be described elsewhere.

Tryptic sensitivity has also been demonstrated in FMDV (26). However, the tryptic sensitivity of Sabin 1 differs significantly from that of FMDV. Although VP1 is cleaved in both viruses, the site in FMDV is in the C-terminal one-half of the protein, as opposed to the N-terminal one-half in Sabin 1. Moreover, tryptic treatment destroys FMDV infectivity, but has no effect on poliovirus infectivity (26).

Finally, treatment of assembly intermediates and conformationally altered virions (such as heat-treated virions) with trypsin results in unique cleavage profiles. Mapping of the proteolytic fragments in these particles should provide precise information about changes in the surface exposure of capsid proteins during assembly and upon conformational rearrangements of the virion. Such information will be particularly informative when considered in conjunction with the results of the high-resolution crystallographic studies of poliovirus currently in progress in our laboratory (7, 8).

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LITERATURE CITED

1. **Chow, M., J. L. Bittle, J. Hogle, and D. Baltimore.** 1983. Antigenic determinants in the poliovirus capsid protein VP1, p. 257–261. *In* R. M. Chanock and R. A. Lerner (ed.), Modern approaches to vaccines. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
2. **Edwards, J., E. Mann, and D. T. Brown.** 1983. Conformational changes in Sindbis virus envelope proteins accompanying exposure to low pH. J. Virol. **45**:1090–1097.
3. **Emini, E. A., B. A. Jameson, and E. Wimmer.** 1983. Priming for and induction of anti-poliovirus neutralizing antibodies by synthetic peptides. Nature (London) **304**:699–703.
4. **Emini, E. A., B. A. Jameson, and E. Wimmer.** 1984. Identification of a new neutralization antigenic site on poliovirus coat protein VP2. J. Virol. **52**:719–721.
5. **Golden, J. S., and S. C. Harrison.** 1982. Proteolytic dissection of turnip crinkle virus subunit in solution. Biochemistry **21**:3862–3866.
6. **Hewick, R. M., M. W. Hunkapiller, L. E. Hood, and W. J. Dreyer.** 1981. A gas-liquid solid phase peptide and protein sequenator. J. Biol. Chem. **256**:7990–7997.
7. **Hogle, J. M.** 1982. Preliminary studies of crystals of poliovirus type 1. J. Mol. Biol. **160**:663–668.
8. **Hogle, J. M.** 1983. High-resolution structural studies of poliovirus, p. 7–11. *In* R. M. Chanock and R. A. Lerner (ed.), Modern approaches to vaccines. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
9. **Holland, J. J., and B. H. Hoyer.** 1962. Early stages of enterovirus infection. Cold Spring Harbor Symp. Quant. Biol. **27**:101–112.
10. **Hunkapiller, M. W., E. Lujan, F. Ostrander, and L. E. Hood.** 1983. Isolation of microgram quantities of proteins from polyacrylamide gels for amino acid sequence analysis. Methods Enzymol. **91**:227–236.
11. **Icenogle, J., S. F. Gilbert, J. Grieves, J. Andregg, and R. R. Rueckert.** 1981. A neutralizing monoclonal antibody against poliovirus and its reaction with related antigens. Virology **115**:211–215.

11a. **IUPAC-IUB Commission on Biochemical Nomenclature.** 1969. A one-letter notation for amino acid sequences. Biochem. J. **113**:1–4.

12. **Jacobson, M. F., and D. Baltimore.** 1968. Morphogenesis of poliovirus. I. association of the viral RNA with coat protein. J. Mol. Biol. **33**:369–378.
13. **Kew, O. M., M. A. Pallansch, D. R. Omilianowski, and R. R. Rueckert.** 1980. Changes in three of four coat proteins of oral polio vaccine strain derived from type 1 poliovirus. J. Virol. **33**:256–263.
14. **Kitamura, N., B. L. Semler, P. G. Rothberg, G. R. Larsen, C. J. Adler, A. J. Dorner, E. A. Emini, R. Hanecak, J. J. Lee, S. van der Werf, C. W. Anderson, and E. Wimmer.** 1981. Primary structure, gene organization and polypeptide expression of poliovirus RNA. Nature (London) **291**:547–553.
15. **Marongiu, M. E., A. Pani, M. V. Corrias, M. Sau, and P. LaColla.** 1981. Poliovirus morphogenesis. I. Identification of 80S dissociable particles and evidence for artifactual production of procapsids. J. Virol. **39**:341–347.
16. **Minor, P. D., G. C. Schild, J. Bootman, D. M. A. Evans, M. Ferguson, P. Reeve, M. Spitz, G. Stanway, A. J. Cann, R. Hauptmann, L. D. Clarke, R. C. Mountford, and J. W. Almond.** 1983. Location and primary structure of a major antigenic site for poliovirus neutralization. Nature (London) **301**:674–679.
17. **Nomoto, A., T. Omata, H. Toyoda, S. Kuge, H. Horie, Y. Kataoka, Y. Genba, Y. Nakano, and N. Imura.** 1982. Complete nucleotide sequence of the attenuated poliovirus Sabin 1 strain genome. Proc. Natl. Acad. Sci. U.S.A. **79**:5793–5797.
18. **Palmenberg, A. C., M. A. Pallansch, and R. R. Rueckert.** 1979. Protease required for processing picornaviral coat protein resides in the viral replicase gene. J. Virol. **32**:770–778.
19. **Putnak, J. R., and B. A. Phillips.** 1982. Poliovirus empty capsid morphogenesis: evidence for conformational differences between self- and extract-assembly empty capsids. J. Virol. **41**:792–800.
20. **Racaniello, V. R., and D. Baltimore.** 1981. Molecular cloning of poliovirus cDNA and determination of the complete nucleotide sequence of the viral genome. Proc. Natl. Acad. Sci. U.S.A. **78**:4887–4891.
21. **Rombaut, B., R. Vrijsen, P. Brioen, and A. Boeye.** 1982. A pH-dependent antigenic conversion of empty capsids of poliovirus studied with the aid of monoclonal antibodies to N and H antigen. Virology **122**:215–218.
22. **Rueckert, R. R.** 1976. On the structure and morphogenesis of picornaviruses, p. 131–213. *In* H. Fraenkel-Conrat and R. R. Wagner (ed.), Comprehensive virology, vol. 6. Plenum Publishing Corp., New York.
23. **Rueckert, R. R., and M. A. Pallansch.** 1981. Preparation and characterization of encephalomyocarditis (EMC) virus. Methods Enzymol. **78**:315–325.
24. **Skehel, J. J., P. M. Bayley, E. B. Brown, S. R. Martin, M. D. Waterfield, J. M. White, I. A. Wilson, and D. C. Wiley.** 1982. Changes in the conformation of influenza virus hemagglutinin at the pH optimum of virus-mediated membrane fusion. Proc. Natl. Acad. Sci. U.S.A. **79**:968–972.
25. **Stanway, G., P. J. Hughes, R. C. Mountford, P. Reeve, P. D. Minor, G. C. Schild, and J. W. Almond.** 1984. Comparison of the complete nucleotide sequences of the genomes of the neurovirulent poliovirus P3/Leon/37 and its attenuated Sabin vaccine derivative P3/Leon 12a(1)b. Proc. Natl. Acad. Sci. U.S.A. **81**:1539–1543.
26. **Strohmaier, K., R. Franze, and K. H. Adam.** 1982. Location and characterization of the antigenic portion of the FMDV immunizing protein. J. Gen. Virol. **59**:295–306.
27. **Wray, W., T. Boulikas, V. P. Wray, and R. Hancock.** 1981. Silver staining of proteins in polyacrylamide gels. Anal. Biochem. **118**:197–203.