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Resolution of the Major Poliovirus Capsid Polypeptides into Doublets

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Using the pH gradient electrophoretic technique of Vrijsen and Boeyé, the coat protein of poliovirus types 1 and 2 was resolved into six components, C1 to C6, instead of the classical VP1-2-3 (the much smaller polypeptide VP4 was excluded from this study). The multiple components ran true upon reelectrophoresis, and there were no technical artifacts. Their resolution did not depend on a particular method for the preparation or disruption of the virion. The C1–C6 components of pH gradient electrophoresis and the classical VP1–VP3 polypeptides were examined with regard to (i) their migration in normal and pH gradient reelectrophoresis and (ii) their kinetics of leucine incorporation in emetine-stopped pulses. It is concluded that C1 and C2 were both derived from VP1, C3 and C4 from VP2, and C5 and C6 from VP3.

INTRODUCTION

The VP1–VP3 group of poliovirion polypeptides can be resolved into multiple components by normal polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) (Vanden Berghe and Boeyé, 1972); however, resolution into six components is achieved more easily and reproducibly in the presence of a pH gradient. In this paper, "normal electrophoresis" will refer to SDS–PAGE in a system entirely buffered at pH 7.2; "pH gradient electrophoresis" means that the catholyte was maintained at pH 11.0 and the anolyte was maintained at pH 6.5. Under the latter conditions, the gel (initially at pH 7.2) is rapidly invaded by an alkaline zone (Vrijsen and Boeyé, 1978).

The converging results of reelectrophoresis and emetine-stopped pulse-labeling experiments are presented which show that the "classical" VP1, VP2, and VP3 each yield a doublet in pH gradient electrophoresis. The two polypeptides in each doublet appear to be nonidentical, though encoded by similar regions of the viral genome. A preliminary account of these findings appeared elsewhere (Boeyé, 1976).

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MATERIALS AND METHODS

Virus. Poliovirus of strains 1a/S3 (type 1) and MEF-1 (type 2) was grown in suspended HeLa cells, labeled, and purified as described (Boeyé, 1965; Vanden Berghe and Boeyé, 1973).

Extraction of capsid polypeptides. A suspension of poliovirus in 0.01 *M* sodium phosphate buffer (pH 7.2) supplemented with 1% SDS and 1% mercaptoethanol was incubated for 1 hr at 60°. After cooling, sucrose was added to a final concentration of 10% and the mixture was electrophoresed immediately.

Gels and electrophoresis. All procedures were as described by Vrijsen and Boeyé (1978). The length of the gels was 9 cm and the potential was 25 V.

Radioactivity profiles. Gels were cut into 1-mm slices, and these were either counted after adding soluene and a toluene-based scintillation mixture or eluted for 24 hr at room temperature in 0.3 to 0.5 ml of 0.1% SDS. Eluate samples of 20 μl were counted in Bray's mixture. Normal counting time was 10 min, but this was increased to 200 min as needed. When required, eluate pools were concentrated by ultrafiltration in a Minicon B-15 (Amicon) apparatus.

For each series of radioactive profiles, one gel loaded with cold 1a/S3 protein was

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run simultaneously and stained with Coomassie brilliant blue as a help with the interpretation of the radioactive band pattern.

Incorporation studies using emetine (modified from Rekosh, 1972). HeLa cells were collected 3 hr after high-multiplicity infection and brought to a density of 4×10^6 cells/ml at 37° in Eagle's medium devoid of amino acids. [^{3}H]Leucine was added 15 min later, and its incorporation was completely stopped after a short time (to be specified) by adding 200 μg/ml of emetine (Sigma) and a 1000-fold excess of unlabeled leucine. After 6 hr of incubation, the cells were collected by centrifugation and frozen to −70°. The supernatant was again centrifuged for 90 min at 40,000 rpm. The pellet, which contained the extracellular virus, was resuspended in phosphate buffer. To prepare cytoplasmic extracts, the thawed cells were resuspended for 15 min in RSB supplemented with excess leucine, disrupted in a Dounce homogenizer, and centrifuged to remove the nuclei. The extracellular virus and 120 μg of carrier virus were added to the cytoplasmic extract. The mixture was dialyzed against 0.01 *M* phosphate buffer (pH 7.2) containing 0.5% SDS and layered onto a 15 to 30% sucrose gradient. The sucrose solutions contained 0.01 *M* phosphate (pH 7.2) and 0.5% SDS. The virus was collected as the 160 S peak after centrifugation for 75 min at 29,000 rpm.

The ^{14}C-virus used for co-electrophoresis in these experiments was separately labeled with [^{14}C]leucine, purified as above, and added immediately before protein extraction.

RESULTS

Fine Resolution of Poliovirus Polypeptides in pH Gradient Electrophoresis

Figure 1 shows the six-band pattern (excluding VP4) into which the protein of 1a/S3 poliovirions was resolved by pH gradient electrophoresis and the proposed designation of the bands. Normal SDS-PAGE of the same virion protein (Fig. 2A) yielded only the classical VP1-2-3 pattern and a trace of VP0.

Samples of uniformly ^{14}C-labeled 1a/S3 protein were submitted to either normal or pH gradient electrophoresis. The resulting radioactivity profiles (not shown) confirmed the results illustrated in Figs. 1 and 2A and were in excellent agreement with

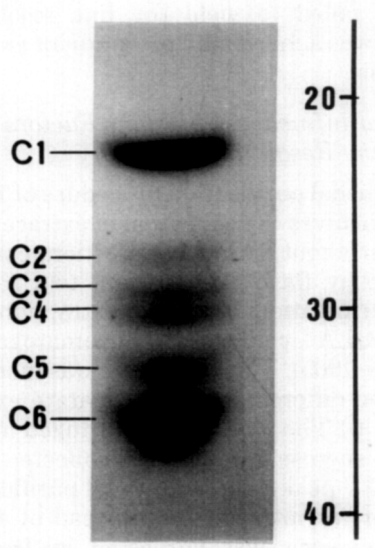

FIG. 1. pH Gradient SDS-PAGE of virion polypeptides (strain 1a/S3). Duration of electrophoresis, 24 hr; catholyte at pH 11.0 and anolyte at pH 6.5. Scale, distance in millimeters from cathodic end. C1-C6, designation of bands.

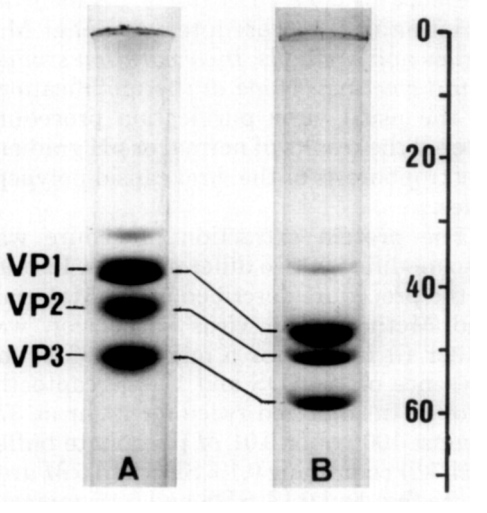

FIG. 2. SDS-PAGE of virion polypeptides (strain 1a/S3): normal and discontinuous (Laemmli) technique. (A) Normal electrophoresis (catholyte and anolyte at pH 7.2), 26 hr. (B) Electrophoresis according to Laemmli (1970) (3 mA/gel, 5.5 hr); the acrylamide and bisacrylamide concentrations in the separation gel were increased to 12.5 and 0.333%, respectively. Scale, distance in millimeters from cathodic end.

the densitometric scans of duplicate gels stained with Coomassie blue.

As illustrated in Fig. 2B, discontinuous electrophoresis according to Laemmli (1970) failed to yield the fine resolution which was achieved by pH gradient electrophoresis.

Lack of Influence of Various Factors upon Fine Resolution

Our usual purification procedure of 1a/S3 virus involves the collection of extracellular virus at about 28 hr postinfection, concentration by flash evaporation, two rounds of centrifugation, a butanol extraction, and ECTEOLA - cellulose chromatography (Boeyé, 1965). This procedure was modified in three different ways in separate experiments: (i) The virus was centrifuged in a 15 to 30% sucrose gradient and collected from the 160 S peak as an additional purification step; or (ii) intracellular (instead of extracellular) virus was harvested by freezing and thawing of the cells, the other steps of the procedure remaining unchanged; or (iii) the virus was harvested prematurely (9 hr p.i.) by homogenizing the cells and purified by sucrose gradient centrifugation in the presence of 0.5% SDS (for a detailed description of the procedure, see under Materials and Methods, *Incorporation studies using emetine*). None of the modifications to the usual virus purification procedure altered the results of normal or pH gradient electrophoresis of the viral capsid polypeptides.

The protein extraction procedure was also modified in two different ways. Instead of the procedure described under Materials and Methods, the virus suspension was either (i) heated for 5 min at 100° in the presence of 1% SDS and 1% mercaptoethanol or (ii) dialyzed twice for 24 hr at 37° against 100 vol of 0.01 *M* phosphate buffer (pH 7.2) containing 0.1% SDS and 5 *M* urea in the first and 0.1% SDS and 0.1% mercaptoethanol in the second dialysis (after Vanden Berghe and Boeyé, 1972). Again, the results of both normal and pH gradient electrophoresis of the viral protein were unchanged.

In the last step of our usual purification procedure, the virus is eluted from ECTEOLA-cellulose using 0.02 *M* phosphate buffer of pH 7.2. Reelution of the column using the same buffer supplemented with 0.5 *M* NaCl yielded a mixture of virions and empty particles from which the latter were purified as the 80 S peak in a 15 to 30% sucrose gradient. These empty particles were found by the normal electrophoresis to contain VP0, VP1, and VP3 but no VP2 or VP4, which is in agreement with the known composition of procapsids (Maizel *et al.*, 1967; Phillips and Fennell, 1973). The components resolved by pH gradient electrophoresis were VP0, C1, C2, and a C5–C6 group (Fig. 3). Thus, the protein of procapsids could be further resolved by pH gradient electrophoresis, just like that of mature virions.

Tritiated MEF-1 (type 2) virion protein was co-electrophoresed in the pH gradient technique with ^{14}C-labeled 1a/S3 (type 1) protein, and the results are shown in Fig. 4. Both virus strains yielded essentially the same six-peak pattern with minor differences: (i) all MEF-1 components migrated slightly slower than their 1a/S3 homologs, and (ii) C2 was more abundant in MEF-1 than in 1a/S3 protein.

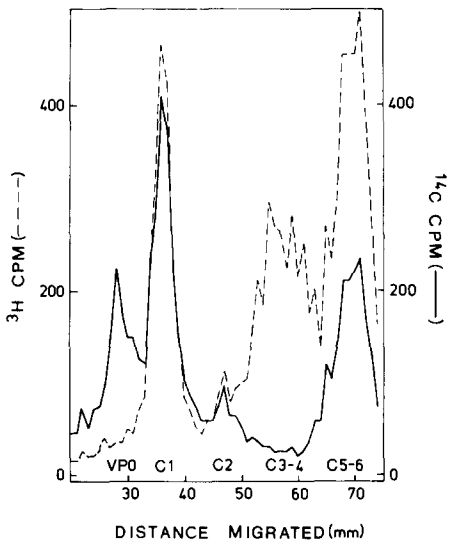

FIG. 3. pH Gradient Co-electrophoresis of Procapsid and Virion Polypeptides (strain 1a/S3). Duration of electrophoresis, 40 hr. Solid line, polypeptides of ^{14}C-labeled procapsids. Dashed line, polypeptides of ^{3}H-labeled virions. In this and all following figures, migration was from left (cathode) to right (anode).

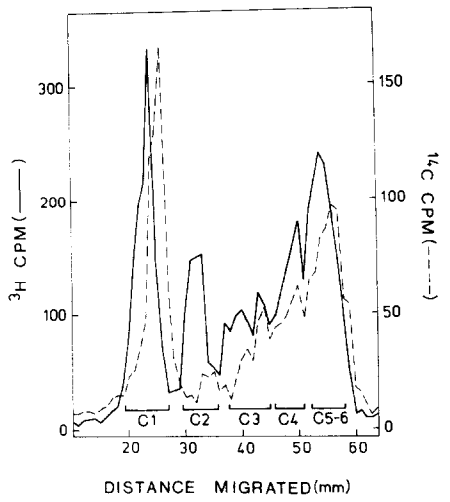

FIG. 4. pH Gradient Co-electrophoresis of 1a/S3 and MEF-1 Virion Polypeptides. Duration of electrophoresis, 38 hr. Solid line, strain MEF-1, ^{3}H-labeled virions. Dashed line, strain 1a/S3, ^{14}C-labeled virions.

In summary, fine resolution of the poliovirus capsid polypeptides was achieved by pH gradient electrophoresis and the results were independent of (i) the virus purification or (ii) protein extraction procedures, (iii) the state of maturation (procapsid or mature virion), and (iv) the serotype (type 1 or 2).

Possible Artifactual Origin of the Multiple Components in pH Gradient Electrophoresis

Since the migration of the poliovirus polypeptides in pH gradient electrophoresis takes place at about pH 10.5 to 11 (Vrijsen and Boeyé, 1978), one may well wonder whether this alkaline pH does not cause partial degradation of the polypeptides. The following experiments were performed to detect possible alkaline degradation: (i) poliovirion protein was dialyzed for 24 hr at room temperature against a mixture of 1% mercaptoethanol and 1% SDS in 0.01 *M* phosphate buffer at pH 11.0; the pH was then lowered to 7.2, the extraction procedure was repeated, and the polypeptides were electrophoresed using the normal technique. The polypeptide pattern obtained after this alkaline pretreatment was similar to that of Fig. 2A and indistinguishable from that of a control where all operations had been carried out at pH 7.2; (ii) after a 24-h pH gradient electrophoresis, the gel segment containing components C1 to C6 was eluted. The eluate was concentrated, resubmitted to the original protein extraction procedure, and reelectrophoresed in the normal system; the results were similar to those shown in Fig. 2A and identical to those of controls in which the first electrophoresis was omitted. Thus, pretreatment of the SDS complexes of poliovirion polypeptides at pH 11, either in free solution or in an acrylamide gel, failed to produce detectable alterations in their migration patterns. The experiments to be presented below, in which single components were resolved by pH gradient electrophoresis and rerun in the same system, allow the same conclusion. In addition, (i) incipient resolution of VP2 and VP3 into doublets was also achieved by electrophoresis at a lower pH, including the normal (pH 7.2) technique, and (ii) nonviral test proteins failed to yield multiple components in pH gradient electrophoresis (Vrijsen and Boeyé, 1978). It is concluded that pH gradient electrophoresis does not cause the observed multiplicity of the poliovirus capsid polypeptides but merely resolves preexisting components.

Origin of the Components Resolved in pH Gradient Electrophoresis

Two kinds of reelectrophoresis experiments were performed. First, tritiated poliovirion protein was electrophoresed using the normal technique, the gel being sliced and eluted. Aliquots of the slice eluates were used to determine the radioactivity profile (Fig. 5O) and the remainder was combined into pools corresponding to peaks VP1, VP2, and VP3 and to the regions in between these peaks. ^{14}C-Labeled virus was added to each pool, and the mixture was extracted and reelectrophoresed using both the normal and pH gradient technique. As shown in Fig. 5A–5C, the pools corresponding to VP1, VP2, and VP3 each yielded a single peak under normal reelectrophoresis; the intermediate fractions, as expected, yielded two peaks (results not shown). The VP1 material in pH gradient reelectrophoresis yielded mainly C1 (Fig. 5D); the VP2

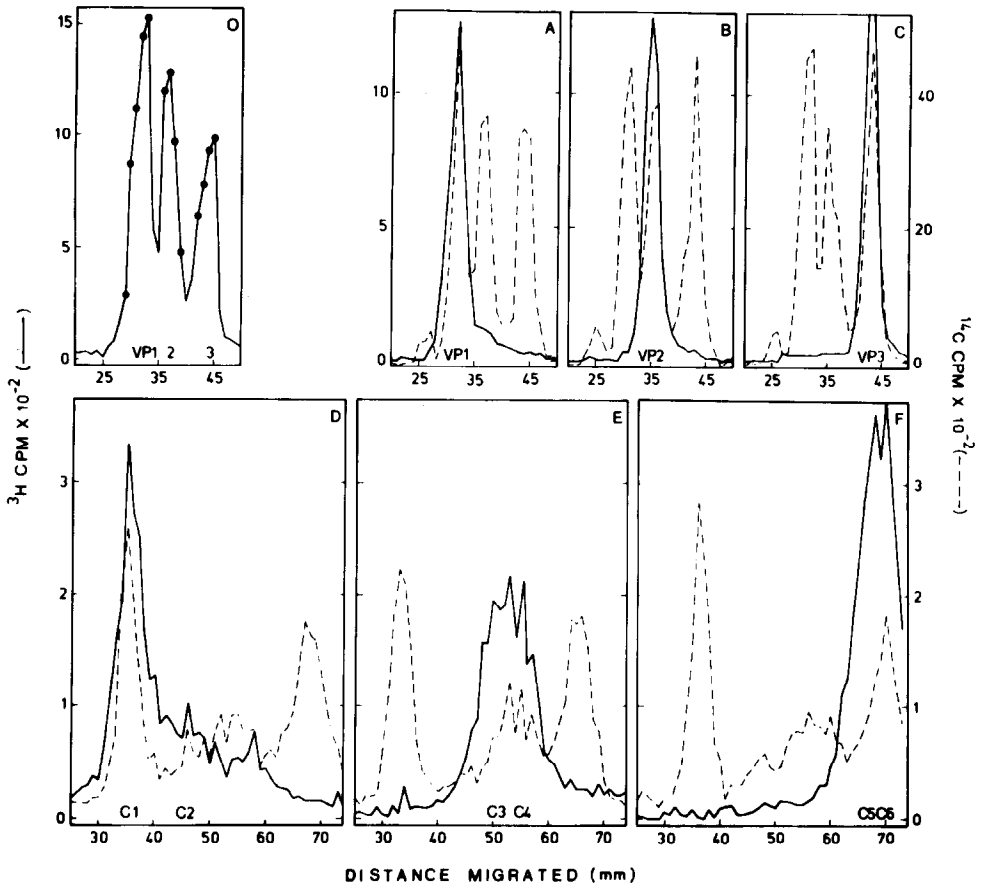

FIG. 5. Normal and pH gradient reelectrophoresis of VP1, VP2, and VP3 (strain 1a/S3). (O) Primary electrophoresis of ^{3}H-labeled 1a/S3 virion protein (normal technique, 24 hr). Slices were eluted in 0.5 ml of 0.1% SDS, of which 20 μl was used to determine the radioactivity profile. Full circles show fractions that were subsequently joined into three pools (representing VP1, VP2, and VP3) for reelectrophoresis; 40- to 60-μl samples of these pools were mixed with ^{14}C-virus, submitted to the protein extraction procedure, and reelectrophoresed simultaneously in the normal and pH gradient system. (A–C) Normal reelectrophoresis, 24 hr. Solid line, pooled eluates; dashed line, ^{14}C-protein. (A) VP1 pool, (B) VP2 pool, (C) VP3 pool. (D–F) pH gradient reelectrophoresis, 40 hr. Solid line, pooled eluates; dashed line, ^{14}C-protein. (D) VP1 pool, (E) VP2 pool, (F) VP3 pool.

material yielded a broad peak covering the whole C3–C4 area (Fig. 5E), and the VP3 material yielded both C5 and C6 (Fig. 5F).

The reverse kind of reelectrophoresis was also performed. In this case, the protein was first submitted to pH gradient electrophoresis. The excised peaks were rerun in the pH gradient system for further purification and finally reextracted and reelectrophoresed in the normal system. The results (not shown) can be summarized as follows: C1 on normal reelectrophoresis comigrated with VP1, C3 with VP2, and both C5 and C6 with VP3 (due to insufficient radioactivity, no conclusive results were obtained with C2 or C4).

Taken together, the reelectrophoresis experiments establish the electrophoretic equivalence of C1 and VP1, which means that the same molecules which migrate as C1 in pH gradient electrophoresis migrate as VP1 using normal electrophoresis. Similarly, C3 appears to be derived from VP2, and both C5 and C6 appear to be derived from VP3. The results concerning C2 and C4 remained inconclusive. It may be recalled, however, that procapsids having VP1 and lacking VP2 yielded C2 but neither

C3 nor C4 (Fig. 3), and this suggests that C2 is derived from VP1 and C4 from VP2. The combined evidence yields three tentative doublets: VP1 = C1 + C2, VP2 = C3 + C4, and VP3 = C5 + C6.

Genetic Relations Between Doublet Polypeptides

Rekosh (1972) used the labeling kinetics of the poliovirus capsid polypeptides in emetine-stopped pulses of [^{3}H]leucine incorporation to determine the order of the RNA regions encoding the polypeptides. As the length of the pulses was increased, VP1, VP3, and VP2 became labeled in turn.

The Rekosh experiment was easily reproduced using our 1a/S3 strain and the normal electrophoretic technique to separate VP1, VP2, and VP3 (not shown). The experiment was repeated using the pH gradient technique, and the most relevant results are shown in Fig. 6. C1 was the first component into which [^{3}H]leucine was incorporated (Fig. 6A), and the next was C6 (this component was exceptionally represented by twin peaks). After 3 min, there was no detectable ^{3}H radioactivity in the C3 to C4 area (Fig. 6A), but these components became progressively labeled as pulse length increased (Figs. 6B–6D). The data did not allow any conclusion about C2, which was present only in trace amounts, nor about C5, which appeared as a shoulder in some of the ^{3}H-profiles (Figs. 6A and 6B). To determine the ^{3}H/^{14}C ratio for C5, this component was further purified by reelectrophoresis. To this end, the relevant fractions (Figs. 6B–6D) were pooled, con-

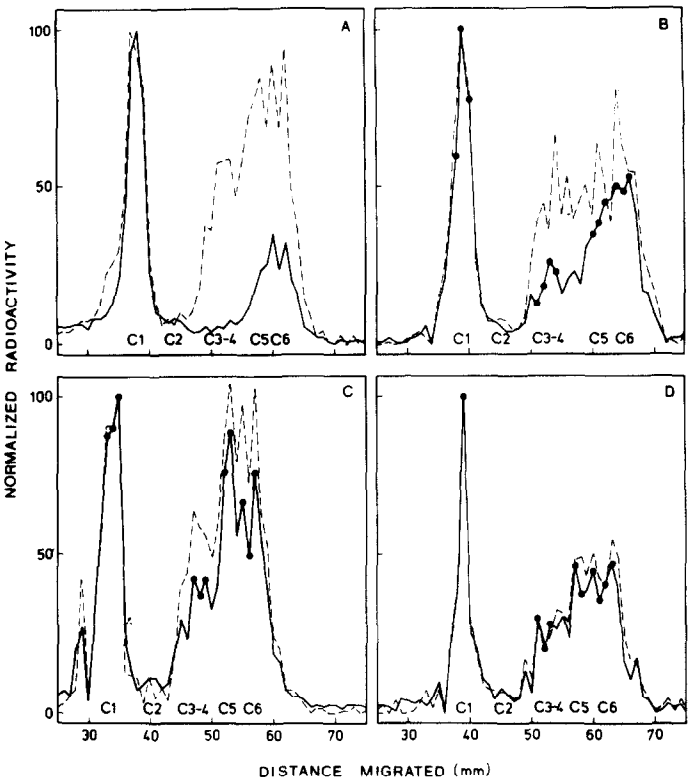

FIG. 6. Emetine-stopped incorporation of [^{3}H]leucine into 1a/S3 virion polypeptides. pH gradient electrophoresis, 38.5 hr. Solid line, [^{3}H]leucine incorporation during pulse; dashed line, protracted [^{14}C]leucine incorporation. Radioactivity normalized to 100 for Cl top fraction. (A) Pulse length, 3 min. Profile determined from radioactivity of whole gel slices. (B–D) Profiles determined using 25-μl samples of 0.25-ml slice eluates. Filled circles, fractions to be pooled and reelectrophoresed (fractions pooled as C5: (B) 60–62; (C) 52–53; (D) 57–58; fractions pooled as C6: (B) 64–66; (C) 55–57; (D) 60–63). Duration of pulse: (B) 5 min , (C) 7 min (D) 30 min.

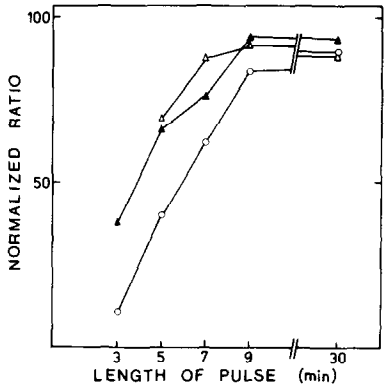

FIG. 7. Kinetics of [^{3}H]leucine incorporation into 1a/S3 virion polypeptides. The ^{3}H/^{14}C ratio was computed for C1, C3-C4, C5, and C6 (see Fig. 6). The values for the 3-min pulse were taken directly from data shown in Fig. 6A, and the other values were determined after pH gradient reelectrophoresis (see text). The normalized ratio of a component was obtained (after Rekosh, 1972) by dividing the ^{3}H/^{14}C ratio of this component by the ^{3}H/^{14}C ratio of C1 × 100. Open circles, C3-C4; open triangles, C5; filled triangles, C6.

centrated, reextracted, and reelectrophoresed as before (pH gradient technique, 38 hr). For purposes of comparison, the same procedure was also applied to pools of fractions representing C1, the C3-C4 area, and C6. The ^{3}H/^{14}C ratios were computed from the radioactivity profiles (not shown), normalized, and plotted against the duration of the pulse (Fig. 7). The data show the existence of three different labeling kinetics represented by C1, and C5-C6 group, and the C3-C4 group.

To determine the labeling kinetics of C2, an emetine experiment was performed using the MEF-1 strain in which C2 is relatively abundant (Fig. 4). The first components to incorporate [^{3}H]leucine were C1 and C2, and their labeling kinetics were indistinguishable; they were followed by C6 (no conclusive data were obtained for C5) and finally by the C3 to C4 group.

Taken together, the emetine experiments establish the identity with regard to translation kinetics of C1 and C2 and of C5 and

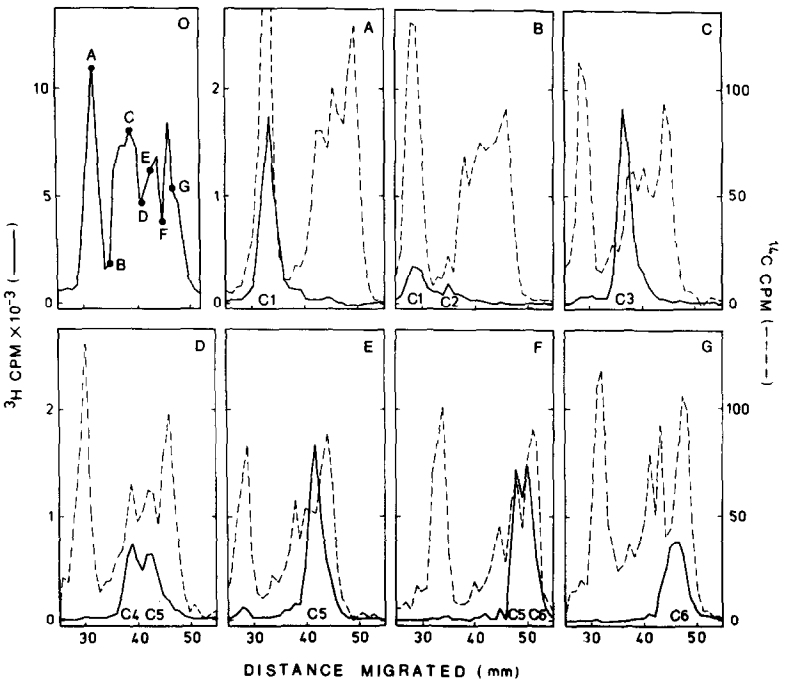

FIG. 8. pH gradient reelectrophoresis of C1 to C6 from 1a/S3 virion protein. (O) pH gradient electrophoresis (24 hr) of ^{3}H-protein. Profile determined using 20-μl samples of 0.5-ml slice eluates. The remainder of the eluates of slices A to G was reelectrophoresed after addition of ^{14}C-virus and reextraction. (A-G) Reelectrophoresis (pH gradient technique, 24 h). Solid line, 100-μl samples of single-slice eluates; dashed line, ^{14}C-protein.

C6. The order of labeling was VP1-VP3-VP2 or (C1, C2)-(C5, C6)-(C3, C4), according to whether the polypeptides were analyzed by normal or by pH gradient electrophoresis. Thus, the six electrophoretic components, C1 to C6, resolved in pH gradient electrophoresis could be attributed to three doublets according to their location on the viral RNA, and these doublets were the same that were independently proposed above as the tentative conclusion of reelectrophoresis experiments.

Lack of Interconversion Between Doublet Polypeptides

In this experiment, 1a/S3 virus of sufficient radioactivity was used to allow reelectrophoresis of single-slice eluates (Fig. 8). The polypeptide pattern after pH gradient electrophoresis is shown in Fig. 8O. The eluates of fifteen different slices were each mixed with ^{14}C-labeled virus and submitted again to an exact repetition of the original protein extraction procedure and to pH gradient electrophoresis. The most relevant radioactivity profiles are shown in Figs. 8A-8G, and the results can be summarized as follows: (i) Material collected at or near the center of peaks C1, C5, or C6 upon reelectrophoresis yielded a single peak (Figs. 8A, 8E, and 8G). This shows the stability of the protein-SDS complexes throughout the extraction procedure and the absence of conversion to the doublet partner; (ii) material taken from the left, center, or right of peak C1 migrated identically upon reelectrophoresis (results partly shown in Figs. 8A-8B), thus showing the original C1 peak to contain a single molecular species. This also held true for peaks C5 (Figs. 8D-8F) and C6 (Figs. 8F-8G); (iii) material collected between two of the original peaks again yielded two peaks (Figs. 8B, 8D, and 8F); (iv) C3 and C4 were not resolved, but the presence of two kinds of molecules was demonstrated by the progressive shift to the right upon reelectrophoresis of successive fractions (Figs. 8C-8D). This single-slice reelectrophoresis experiment allows two conclusions: (i) No interconversion among doublet partners could be demonstrated, and (ii) at least those components which could be clearly resolved in the primary radioactivity profile (i.e., C1, C5, and C6) ran true upon reelectrophoresis.

DISCUSSION

The improved resolution of poliovirus capsid polypeptides by pH gradient electrophoresis reported by Vrijsen and Boeyé (1978) was confirmed and shown to be independent of the serotype and of the methods used for virus purification and protein extraction. It is therefore unlikely that the multiple components observed were preparative artifacts, as have been observed with proteins of coronaviruses (Sturman, 1977). The possibility that they were formed by partial degradation of the protein during electrophoresis was also examined and rejected.

Whereas normal SDS-PAGE or discontinuous electrophoresis according to Laemmli (1970) resolved only VP1, VP2, and VP3, pH gradient electrophoresis usually resolves six components (C1-C6). The pattern was not entirely constant, however, as neighboring components sometimes failed to separate (e.g., C3-C4 in Fig. 8O; C5-C6 in Fig. 4); on the other hand, the results occasionally suggested further resolution of a single component (e.g., C3 in Figs. 4 and 5D-5F). Due to these and other technical difficulties, no single experiment yielded all of the information being sought, and a clear picture emerged only by juxtaposition of the results. According to this picture, C1 and C2 formed a doublet corresponding to VP1, C3 and C4 a second doublet corresponding to VP2, and C5 and C6 a third doublet corresponding to VP3. With regard to the C1-C2 doublet, for example, this correspondence means (i) that the molecules which migrate as either C1 or C2 in pH gradient electrophoresis will migrate as VP1 in normal SDS-PAGE, and (ii) that C1 and C2 in emetine-stopped pulses are labeled equally and according to the same kinetics as VP1. Thus, although we demonstrated a higher number of electrophoretic components, our findings are still basically in agreement with the accepted VP1-2-3-4 scheme for the composition of the poliovirus capsid.

Several SDS complexes of the same pro-

tein may coexist, at least when a limited amount of SDS is made available for binding; however, saturation is reached at a SDS/protein weight ratio of about 4.5:1 (Stoklosa and Latz, 1975), and this ratio was largely exceeded in our extraction procedure (SDS/protein ratio ca 30:1) and during electrophoresis (unlimited supply of SDS). Even so, the question may be asked whether the two electrophoretic components in each doublet could not be different dodecyl sulfate complexes of the same polypeptide. Therefore, we looked for possible interconversion to the doublet partner upon isolation of single components and exact recreation of the conditions under which the protein–detergent complexes were originally formed (extraction of capsid protein). Since no interconversion was found and each component ran true upon reelectrophoresis, it may be tentatively concluded that the two polypeptides in each doublet were not identical.

If so, they must differ slightly in charge, molecular weight, or both. Such differences may conceivably originate in the accumulation of mutants as a result of serial passages (Phillips and Fennell, 1973). To prevent any such accumulation, however, each virus population examined in this work was grown from a single plaque by only four passages, and a mutational origin of the observed multiplicity can therefore be excluded.

The observed microheterogeneity probably originates in the posttranslational processing of the proteins. Since the capsid proteins are carved out of a common precursor by successive cleavages (Summers and Maizel, 1968; Jacobson and Baltimore, 1968), microheterogeneity may result from multiplicity of the cleavage sites. Ambiguity of cleavage was already invoked to explain differences in the migration velocity of homologous polypeptides of different poliovirus strains (Cooper *et al.*, 1970) or the appearance of doublets (Beckman *et al.*, 1976). However, no direct proof of ambiguity was offered and the case rests on the unproven assumption that differences in migration velocity in SDS–PAGE necessarily reflect differences in molecular weight.

Ambiguity of cleavage would be expected to reveal itself in multiple N-terminal or C-terminal sequences. The terminal sequences of the major capsid polypeptides of Mengo virus were determined and no mention was made of ambiguities (Ziola and Scraba, 1976). This is also true for foot-and-mouth disease virus (Adam and Strohmaier, 1974; Matheka and Bachrach, 1975), except that in an earlier paper (Bachrach *et al.*, 1973) an ambiguity in the C-terminal sequence of VP2 was reported. Until and unless ambiguity of cleavage is demonstrated by means of sequence analysis, other possible causes of microheterogeneity, such as phosphorylation, deamidation, and glycosylation cannot safely be ruled out.

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