JOURNAL OF VIROLOGY, Oct. 1974, p. 821-833

Vol. 14, No. 4
Printed in U.S.A.

Polypeptide Composition of Urea- and Heat-Resistant Mutants of Poliovirus Types 1 and 2

ROBERT FENNELL[1] AND BRUCE A. PHILLIPS

Department of Microbiology, University of Pittsburgh, School of Medicine, Pittsburgh, Pennsylvania 15261

Received for publication 16 May 1974

Five urea-resistant and two heat-resistant mutants of poliovirus types 1 and 2 were isolated and their structural and nonstructural polypeptides compared to those of their wild-type, parental strains in an attempt to correlate mutant phenotypes with alterations in specific capsid polypeptides. Four of the seven mutants were found to contain polypeptides which differed in molecular weight from their respective parental viruses. However, resistance of virions to heat- or urea-inactivation could not be attributed to changes in particular capsid polypeptides because alterations were detected in all but one of the capsid components. For two of the urea-resistant mutants and one heat-resistant mutant, no differences were found in the molecular weights of the capsid and noncapsid polypeptides. These results, and the fact that at least 12 selective treatments were required to obtain stable mutants, indicate that: (i) such phenotypes probably can be expressed by mutations affecting one or more of the larger capsid polypeptides, and (ii) such phenotypes reflect multiple mutational steps.

Urea-resistant and heat-resistant mutants of poliovirus can be isolated by the repeated inactivation (with heat or urea) and propagation of the surviving virus population (6, 16). The resistance to inactivation is a genotypic character of virus (2, 3, 6, 10, 12, 17). A comparison of the inactivation kinetics of the RNA obtained from the resistant mutants and the sensitive parental viruses showed that the resistance to inactivation could be attributed to an alteration in the capsid structure and not to an increased stability of the nucleic acid (3, 6). This study was undertaken to investigate (i) whether the capsid polypeptides from urea-resistant and heat-resistant mutants could be differentiated from their parental viruses by polyacrylamide gel electrophoresis; and (ii) whether alterations in specific viral polypeptide could be correlated with resistance to urea or heat inactivation.

MATERIALS AND METHODS

Cell culture. S-3 HeLa cells were used exclusively. Suspension cultures were maintained in Eagle minimal essential medium (MEM) made with spinner salts (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 5% calf serum (Flow Laboratories, Rockville, Md.) and 2.0 mM glutamine. AA/50 MEM is minimal essential medium (Eagle) with spinner salts containing 1/50th the normal amount of each amino acid.

Viruses. The wild-type poliovirus strains used in these experiments were type 1, LSc; type 1, Mahoney (Mah); type 2, MEF_1; and type 2, Sabin (2-Sab). The type 1 viruses were laboratory stocks. The type 1, LSc strain and its urea-resistant mutant, LSc-U^R (see below), were obtained from J. Hallum and J. S. Youngner. The type 2, Sabin vaccine strain was obtained from Lederle Laboratories, Pearl River, N.Y. In all cases, uncloned virus stocks were used. Urea-resistant (U^R) and heat-resistant (H^R) mutants of these virus stocks were isolated as described below.

Propagation of viruses. HeLa cells in suspension culture (4×10^6 per ml) were infected with virus with an input multiplicity of 10 PFU per cell. Thirty minutes after infection, 5% calf serum was added. Six and one-half hours after infection, the cells were centrifuged and the cell pellet was resuspended in a small volume of 0.02 M phosphate buffer, pH 7.0 (PB). After repeated (three to four times) freezing and thawing to rupture the cells, the cell debris was removed by centrifugation and the supernatant fluid was collected. The virus was pelleted (70,000 × *g* for 3 h), sonically treated in a Raytheon sonic oscillator for 2 min, resuspended in PB, and assayed for infectivity.

Isolation of urea- and heat-resistant mutants. Urea-resistant and heat-resistant mutants of the four wild-type (nonresistant) strains of poliovirus were obtained by the method described by Hallum and Youngner (6). The wild-type viruses were propagated to titers of approximately 10^9 PFU per ml. Prior to urea-inactivation, the virus stock was diluted 10^{-1} in PB and sonically treated for 60 s in a Raytheon sonic

[1] Present address: Microbiological Associates, Inc., Walkersville, Md. 21793.

oscillator. The virus then was added to an equal volume of 6 M urea in PB and the mixture was incubated for 2 h at 37 C with occasional mixing. The virus was diluted 10^{-2} in 20 ml of MEM and added to monolayer cultures. The procedure was repeated and after each treatment and passage, the virus population was tested for urea sensitivity. Approximately 12 passages were required to obtain a stable population of virus which showed no significant drop in titer when tested for urea sensitivity.

The same experimental design was used for the isolation of the heat-resistant mutants. Virus stocks were sonically treated, diluted 10^{-2} in PB, and exposed to 50 C for 2 h. The virus then was diluted 10-fold in 20 ml of MEM and passaged in cell monolayers as above. The isolation of heat-resistant viruses likewise required about 12 passages of treated virus populations.

Test of urea sensitivity. Virus in PB (37 C) was added to an equal volume of 6 M urea in PB, pH 7.0, at 37 C. At designated times, 0.1-ml samples were diluted 10^{-2} in cold MEM and assayed for infectivity. The results of virus inactivations were expressed as the $\log_{10}$ of the surviving infectivity, P/Po, where P equals the titer of survivors at time, t, and Po is the titer of virus at zero time. The infectivity was measured by the agar cell-suspension technique using HeLa cells (1).

Test of heat sensitivity. Virus, which was preincubated at 22 C, was diluted 10^{-2} in 9.9 ml of PB at 50 C ($\pm$ 0.25 C). At the designated times, 0.1-ml samples were diluted 10^{-2} in cold MEM and assayed for infectivity. The results of heat-inactivation experiments were expressed also as the $\log_{10}$ of the surviving infectivity.

Preparation of cytoplasmic extracts containing radioactively labeled virus-specific proteins. Cells in suspension culture (4×10^6/ml) containing 4 μg of actinomycin D per ml and 3 mM guanidine were infected with an input multiplicity of 50 PFU per cell. Two hours after infection the cells were washed free of guanidine (reversal) and resuspended in amino acid-deficient medium (AA/50 MEM). Radioactive amino acids were added 90 to 120 min after guanidine reversal. Seven hours after infection the cells were centrifuged into a pellet (800 $\times$ g for 10 min), resuspended in reticulocyte standard buffer (RSB; 0.01 M NaCl, 0.0015 M $MgCl_2$, 0.01 M Tris, pH 7.4) to a concentration of 10^7 cells per ml, and disrupted in a glass Dounce homogenizer. Nuclei and intact cells were removed by centrifugation (1,000 $\times$ g for 4 min). The supernatant fraction was used as a cytoplasmic extract.

Preparation of purified virus labeled with radioactive amino acids. Suspension cultures of cells (4×10^7 per ml) were infected at a multiplicity of 50 PFU per cell. Calf serum was added 45 min after infection. Four hours after infection, the cells were centrifuged and resuspended to 8×10^6 cells per ml in AA/50 MEM containing 4 mM glutamine, 5% calf serum, and labeled amino acid mixture (10 μCi/ml). Six and one-half hours after infection the cells were centrifuged into a pellet and resuspended in PB to a concentration of 4×10^7 cells per ml. The cells were disrupted by freeze-thawing. Cellular debris was removed by centrifugation and the supernatant fluid was layered on an 11 ml, 10 to 30% sucrose gradient containing PB and 10 mM EDTA. The gradient was centrifuged in an SW-41 rotor, 52,000 $\times$ g for 18 h. After centrifugation the gradient was continuously scanned at 260 nm by pumping the gradient through a continuous flow cell in a Gilford recording spectrophotometer. Usually, about 20 fractions were collected. A portion of each fraction was assayed for radioactivity. The virus and empty capsid bands were located as peaks of radioactivity, optical density, and, in the case of virions, infectivity.

The virus or empty capsid fractions were pooled and dialyzed overnight against 1,000 volumes of PB. The dialyzed material was brought up to 4.0 ml with PB. Two grams of CsCl was added and the mixture was centrifuged to equilibrium in a SW 39 rotor (37,000 RPM for 18–24 hours at 5 C). Five drop fractions were collected from the bottom of the tube. A portion of each fraction was assayed for radioactivity. The virus or noninfectious empty capsids were located as peaks of infectivity and radiactivity, respectively.

Polyacrylamide gel electrophoresis of viral polypeptides. Purified virus, empty capsids, or cytoplasmic extracts were dissociated by the addition of one-tenth volumes of glacial acetic acid, 5 M urea, and 10% sodium dodecyl sulfate (SDS). After 30 min of incubation at 37 C, the dissociated polypeptides were dialyzed overnight against 6,000 volumes of 0.01 M phosphate buffer, pH 7.0, containing 0.1% SDS, 0.5 M urea, and 0.01 M 2-mercaptoethanol (14). Immediately prior to electrophoresing, the samples were placed in boiling water for approximately 5 min.

Samples containing the dissociated radioactive viral polypeptides in 5% sucrose were layered on 10% acrylamide gels (20 cm by 5 nm) containing 0.5 M urea, 0.1% SDS, and 0.1 M phosphate buffer, pH 7.0. After electrophoresis, the gels were fractionated and collected automatically into scintillation vials by a Savant Autogeldivider and fraction collector. Approximately 100 fractions were collected for each gel.

The gel fractions were dried and 0.3 ml of hydrogen peroxide (30%) was added to each vial. All vials were incubated at 37 C overnight. Three drops of Soluene-100 (Packard Instrument Co, Downers Grove, Ill.) or NCS reagent (Amersham-Searle, Arlington Heights, Ill.) were added and the sealed vials incubated overnight at 37 C. One drop of saturated ascorbic acid and 12.5 ml of Aquasol scintillation fluid (New England Nuclear Corp., Boston, Mass.) were added and the fractions were counted in a Packard model 3375 scintillation spectrometer. Discrimination settings were adjusted to give 10% spillover of [^{14}C]CPM into the tritium channel and 0.1% spillover of [^{3}H]CPM into the ^{14}C-channel. With these discrimination settings the counting efficiency was approximatey 45 and 65% for ^{3}H and ^{14}C, respectively, when compared to the radioactivity determined when the channels were set for optimal, nondiscriminating counting. The recovery of radioactivity from the fractionated gels varied between 70 and 95%.

Reagents and isotopes. Radioactive amino acid

mixtures (^{14}C and ^{3}H) were obtained from New England Nuclear Corp., Boston, Mass. Actinomycin D was obtained from Merck Sharp & Dohme, West Point, Pa. Guanidine-hydrochloride was purchased from Eastman Organic Chemicals, Rochester, N.Y. The urea used for the inactivation kinetics was an ultra-pure grade purchased from Schwartz-Mann, Orangeburg, N.Y.

RESULTS

Inactivation kinetics. The exposure of wild-type strains of poliovirus type 1 to 3 M urea at 37 C resulted in a multi-hit type of inactivation kinetics (Fig. 1). The maximum drop in titer was obtained by 15 min of inactivation, after which time the surviving fraction of virus remained unchanged. Type 2 strains were significantly more resistant to urea-inactivation (Fig. 1), an interesting finding because most type 2 strains appear to be more heat resistant than type 1 strains (16). Their inactivation kinetics more closely resembled single-hit kinetics. Despite the greater initial resistance of the type 2 strains, as compared to type 1 viruses, the same number of urea exposures and passages was required before a stable population of resistant mutants was obtained. It was found that if a virus population exhibited, for example, a 0.5 $\log_{10}$ drop in titer in 15 min, such a virus population, upon further passage in the absence of the selection teatment, would revert rapidly to a less resistant state.

In contrast, all of the urea-resistant mutants exhibited no significant inactivation after 15 min in 3 M urea at 37 C. The inactivation kinetics of the mutants were determined after at least two passages of the virus without urea treatment and reflect the kinetics of the populations used in subsequent experiments.

Two strains of viruses were used to obtain heat-resistant mutants. Heat inactivation of these viruses appeared to follow single-hit kinetics and again the wild-type 2 strain was more resistant than the wild-type 1 strain (Fig. 2). As

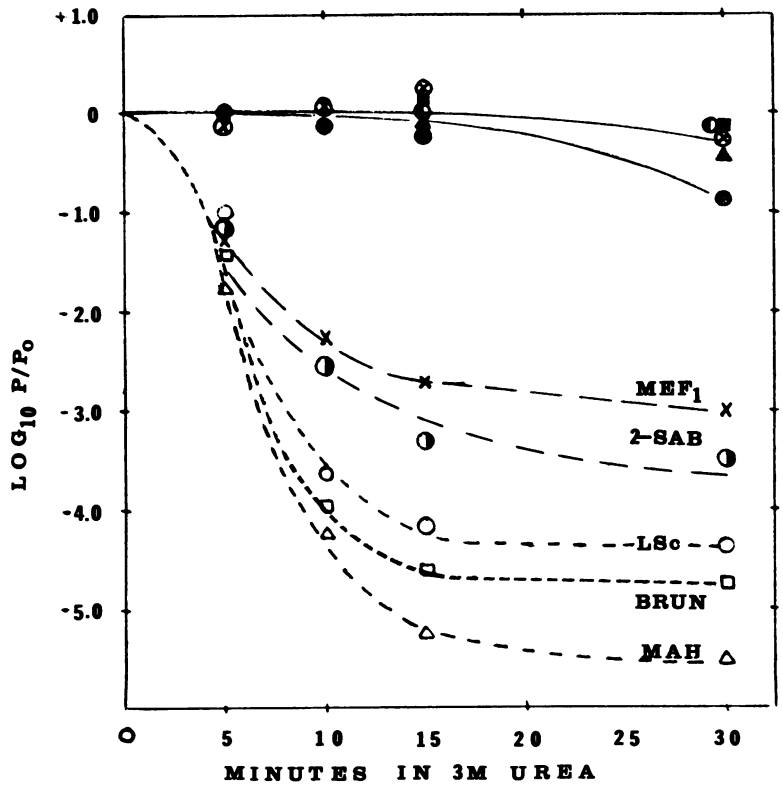


FIG. 1. *Urea-inactivation kinetics of the urea-resistant mutants and their wild-type viruses. Virus in PB, at 37 C, was added to an equal volume of 6 M urea in PB and incubated at 37 C. Samples were diluted in MEM for a plaque assay of the surviving infectivity. P = PFU/ml at the sample time; P_o = PFU/ml at zero time. Symbols: LSc, ○; LSc-U^R, ●; MEF_1, ×; MEF_1-U^R, ⊗; Brun, □; Brun-U^R, ■; Mah, △; Mah-U^R, ▲; 2-Sab, ◑; 2-Sab-U^R, ◐.*

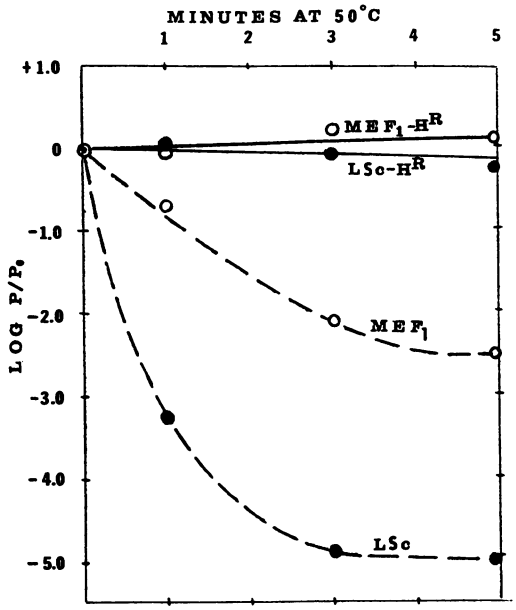


FIG. 2. *Heat-inactivation kinetics of the heat-resistant mutants and their wild-type viruses. A 1-ml amount of virus (at 22 C) was added to 9.0 ml of PB preincubated at 50 C. At various times, samples were diluted in MEM (at 22 C) and assayed for the surviving virus.*

noted above, about 12 passages of virus, each following heat exposure, were necessary in order to get stable mutants.

Polyacrylamide gel electrophoresis of virion polypeptides. Representative electropherograms are shown in Fig. 3 and 4. Highly purified, radioactively labeled virions were prepared for electrophoresis as described. The polypeptides from the mutant viruses were co-electrophoresed with those of their respective parental strains. Controls consisted of co-electrophoresing ^{3}H- and ^{14}C-labeled preparations of the same virus, as exemplified in Fig. 3A. Differences in the polypeptide patterns were detected in four of the seven pairs of viruses. A split in the VP-2a polypeptide of the Brun-U^R virus was consistently found (Fig. 3D). The VP-1 polypeptide of the LSc-U^R virus was approximately 5,000 daltons smaller than the VP-1 of the parental LSc virus; and the VP-3 polypeptide of the LSc-U^R virus was approximately 4,000 daltons larger than that of the LSc virus (Fig. 3E). The 2-Sab-U^R virus contained both a VP-1 and a VP-2 component smaller than their respective components present in the parental virus, 2-Sab (Fig. 3F). The heat-resistant mutant of the LSc strain, LSc-H^R, contained a BP-3 component of greater molecular weight (migrated slightly slower) than the VP-3 polypeptide of the parental, LSc virus (Fig. 4A). No differences were detected in the polypeptide compositions of the urea-resistant mutant of type 1, Mahoney (Mah-U^R), or either of the mutants derived from type 2, MEF_1 (MEF_1-H^R and MEF_1-U^R). No differences were ever detected in the VP-4 polypeptides when the electrophoresis was carried out for shorter times so as to retain this component on the gel (Fig. 3A).

Polyacrylamide gel electrophoresis of empty capsid polypeptides. The 73*S* empty capsids of the mutants and their respective parental viruses were also examined for differences in polypeptide composition. The electropherograms showed the same alterations of the polypeptides as seen in the virion polypeptides (Fig. 5). The LSc-UR empty capsid polypeptides, VP-1 and VP-3, were, respectively, smaller and larger than the corresponding polypeptides of the parental LSc strain (Fig. 5C). There was some difficulty in the identification of certain polypeptides (see arrow Fig. 5C). When such aberrant polypeptides arose, it was difficult to adhere to the standard definition of poliovirus polypeptides described by Maizel et al. (8). So the question remained as to whether a given polypeptide could be defined as one of the VP-2 or VP-3 polypeptides.

The VP-1 and VP-2 polypeptides of the 2-Sab-U^R empty capsids were smaller than those of the parental 2-Sab virus (Fig. 5E). The 2-Sab-U^R empty capsids, unlike the virions, contained several components migrating the region of its VP-1; one of these polypeptides migrated coincidently with the VP-1 of its parental type (see bracketer region, Fig. 5E). The type 1, Brunhilde empty capsids contained very little VP-2 component; therefore, any possible differences in the VP-2 polypeptides could not be detected (Fig. 5D). The empty capsids of all the strains examined, except type 1, Brunhilde, contained a major VP-2 component(s). The electrophoresis of the polypeptides from the type 2, MEF_1 strain repeatedly showed a single major VP-2 polypeptide (Fig. 5B). None of the empty capsids from the resistant mutants showed any difference from their respective parental types in the mobility of their NCVP-6 polypeptide.

It proved difficult to obtain partially purified empty capsids from the heat-resistant mutants. No significant differences between parental and mutant viruses could be detected; however, the amount of radioactivity in the empty capsid polypeptides was very small (Fig. 6).

Polyacrylamide gel electrophoresis of cytoplasmic polypeptides. Radioactively labeled, virus-specific polypeptides from infected cells

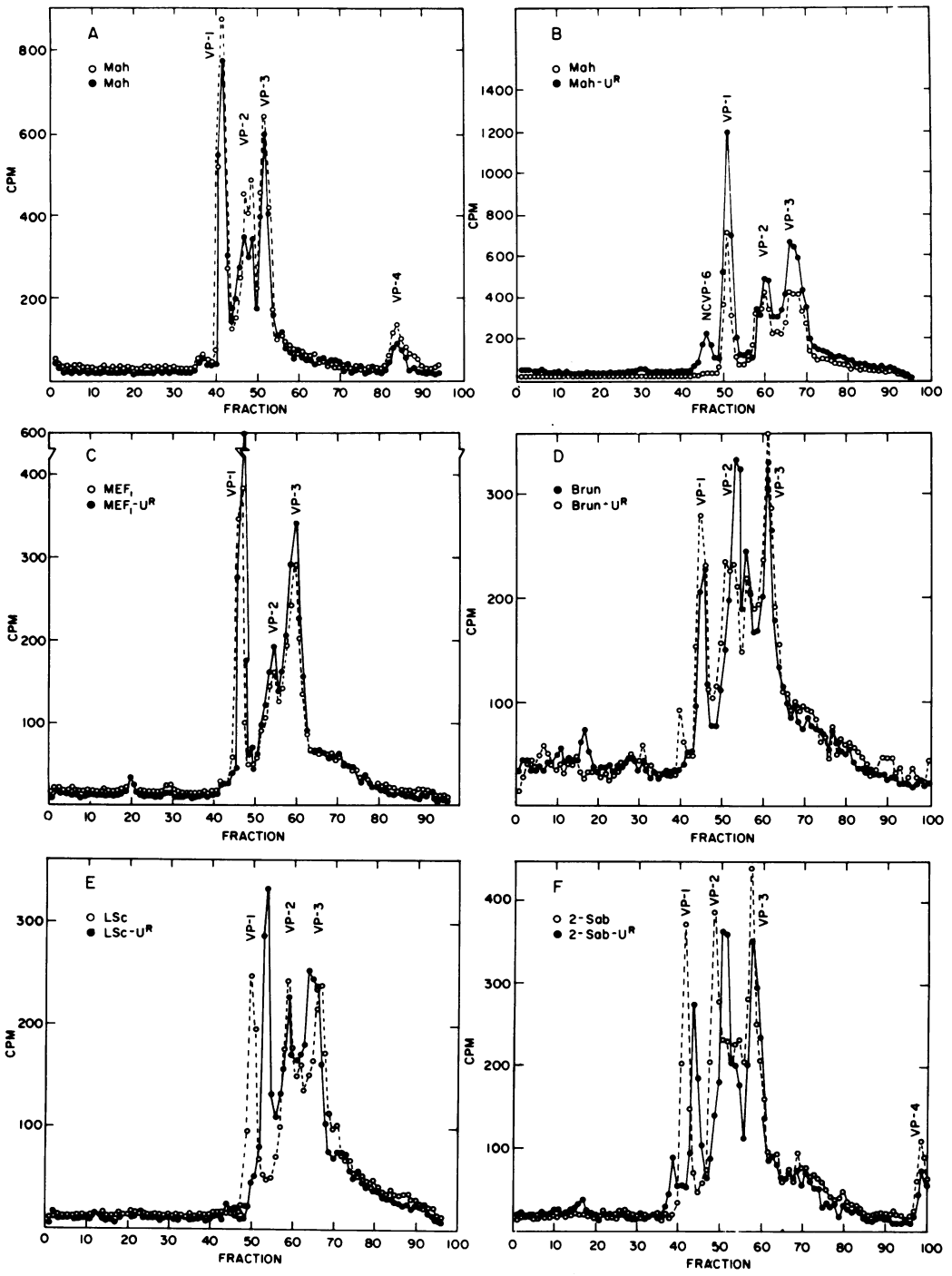


FIG. 3. *Polyacrylamide gel electrophoresis of the dissociated virion polypeptides from the urea-resistant mutants and their wild-type viruses. The radioactively labeled polypeptides from dissociated virions were layered on 10% acrylamide-SDS gels (10 cm by 6 mm), electrophoresed, fractionated into scintillation vials, and counted for radioactivity. Panel A. Co-electrophoresis of two preparations of type 1, Mahoney virion polypeptides. Electrophoresis was for 15 h at 80 V, 8 mA. Symbols:* [^{3}H]*Mah*, ●; [^{14}C]*Mah*, ○. *Panel B. Co-electrophoresis of type 1, Mahoney, and Mah-U^R virion polypeptides. Electrophoresis was for 22 h at 80 V, 8 mA. Symbols:* [^{14}C]*Mah*, ○; [^{3}H]*Mah-U^R*, ●. *Panel C. Co-electrophoresis of type 2, MEF$_1$ and MEF$_1$-U^R virion polypeptides. Electrophoresis was for 22 h at 80 V, 8 mA. Symbols:* [^{14}C]*MEF$_1$*, ○; [^{3}H]*MEF$_1$-U^R*, ●. *Panel D. Co-electrophoresis of type 1, Brunhilde and Brun-U^R virion polypeptides. Electrophoresis was for 16 h at 80 V, 7 mA. Symbols:* [^{14}C]*Brun*, ●; [^{3}H]*Brun-U^R*, ○. *Panel E. Co-electrophoresis of type 1, LSc and LSc-U^R virion polypeptides. Electrophoresis was for 21 h at 80 V, 8 mA. Symbols:* [^{14}C]*LSc*, ○; [^{3}H]*LSc-U^R*, ●. *Panel F. Co-electrophoresis of type 2, Sabin and 2-Sab-U^R virion polypeptides. Electrophoresis was for 15 h at 80 V, 7 mA. Symbols:* [^{14}C]*2-Sab*, ○; [^{3}H]*2-Sab-U^R*, ●.

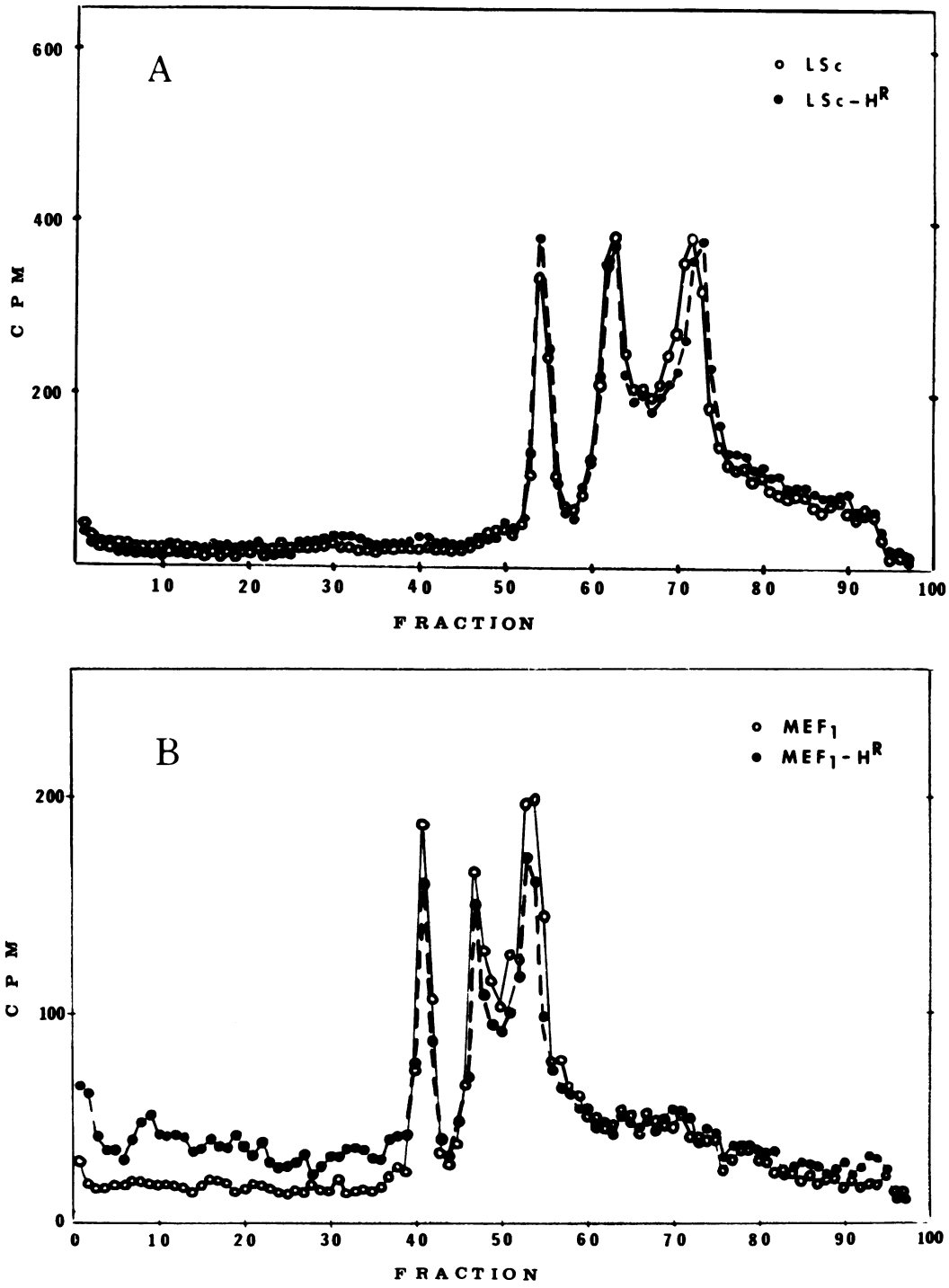


FIG. 4. *Polyacrylamide gel electrophoresis of the dissociated virion polypeptides from the heat-resistant mutants and their wild-type virions. Preparation and electrophoresis of virion polypeptides was the same as in Fig. 6. Panel A. Co-electrophoresis of type 1, LSc and LSc-H^R virion polypeptides. Electrophoresis was for 22 h at 80 V, 8 mA. Symbols: [³H]LSc, O; [¹⁴C]LSc-H^R, ●. Panel B. Co-electrophoresis of type 2, MEF₁ and MEF₁-H^R virion polypeptides. Electrophoresis was for 15 h at 80 V, 7 mA. Symbols: [¹⁴C]MEF₁, O; [³H]MEF₁-H^R, ●.*

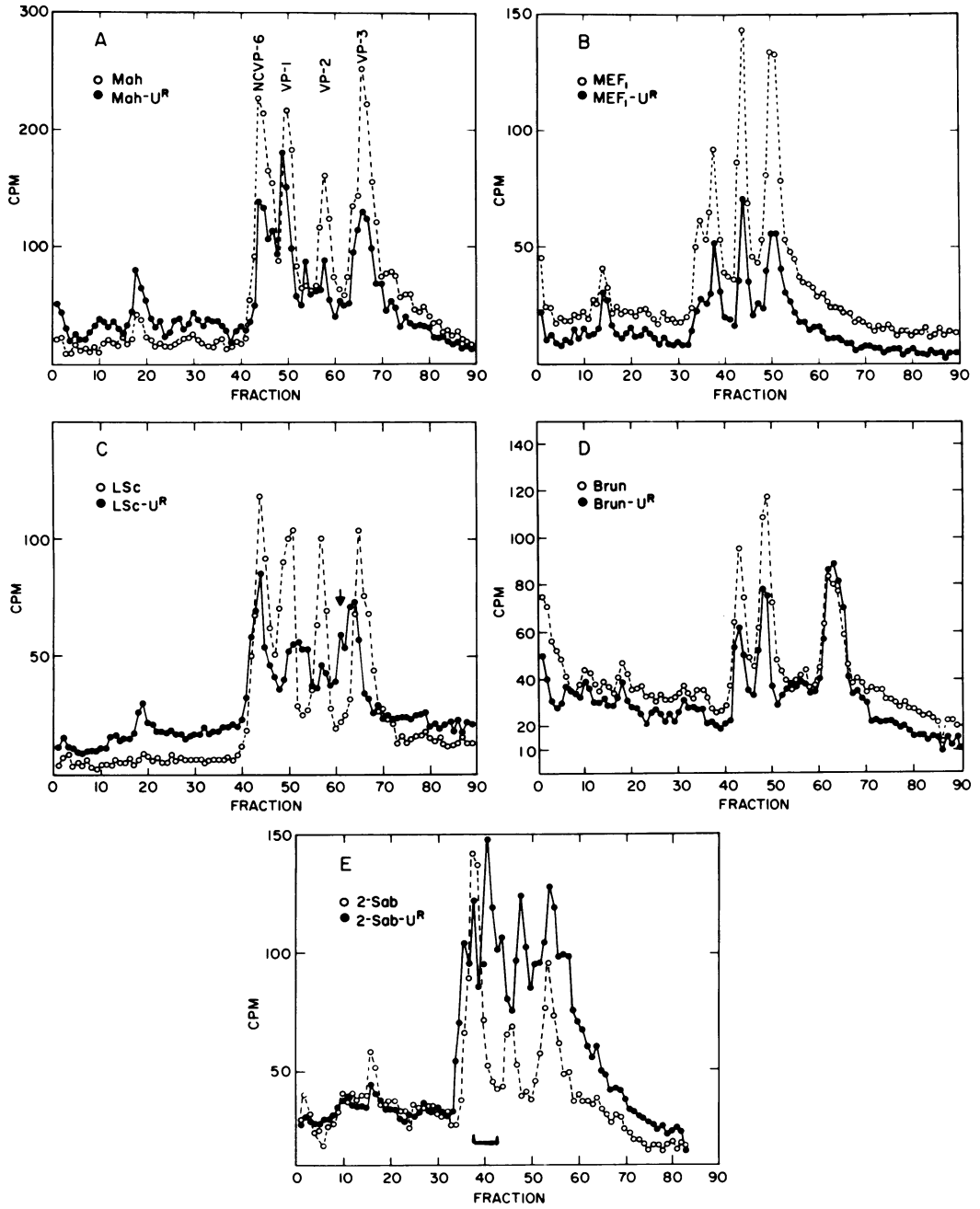


FIG. 5. *Polyacrylamide gel electrophoresis of the dissociated empty capsid polypeptides from the urea-resistant mutants and their wild-type viruses. The purified empty capsids were dissociated and prepared for electrophoresis on 10% acrylamide-SDS gels as described in Materials and Methods. Panel A. Co-electrophoresis of type 1, Mahoney and Mah-U^R empty capsid polypeptides. Electrophoresis was for 20 h at 80 V, 7 mA. Symbols: [^3H]Mah, O; [^14C]Mah-U^R, ●. Panel B. Co-electrophoresis of type 2, MEF_1 and MEF_1-U^R empty capsid polypeptides. Electrophoresis was for 15.5 h at 80 V, 8 mA. Symbols: [^3H]MEF-U^R, ●; [^14C]MEF_1, O. Panel C. Co-electrophoresis of type 1, LSc and LSc-U^R empty capsid polypeptides. Electrophoresis was for 20 h at 80 V, 8 mA. Symbols: [^3H]LSc, O; [^14C]LSc-U^R, ●. Panel D. Co-electrophoresis of type 1, Brunhilde and Brun-U^R empty capsid polypeptides. Electrophoresis was for 20 h at 80 V, 8 mA. Symbols: [^14C]Brun, O; [^3H]Brun-U^R, ●. Panel E. Co-electrophoresis of type 2, Sabin and 2-Sab-U^R empty capsid polypeptides. Electrophoresis was for 20 h at 80 V, 8 mA. Symbols: [^14C]2-Sab, O; [^3H]2-Sab-U^R, ●.*

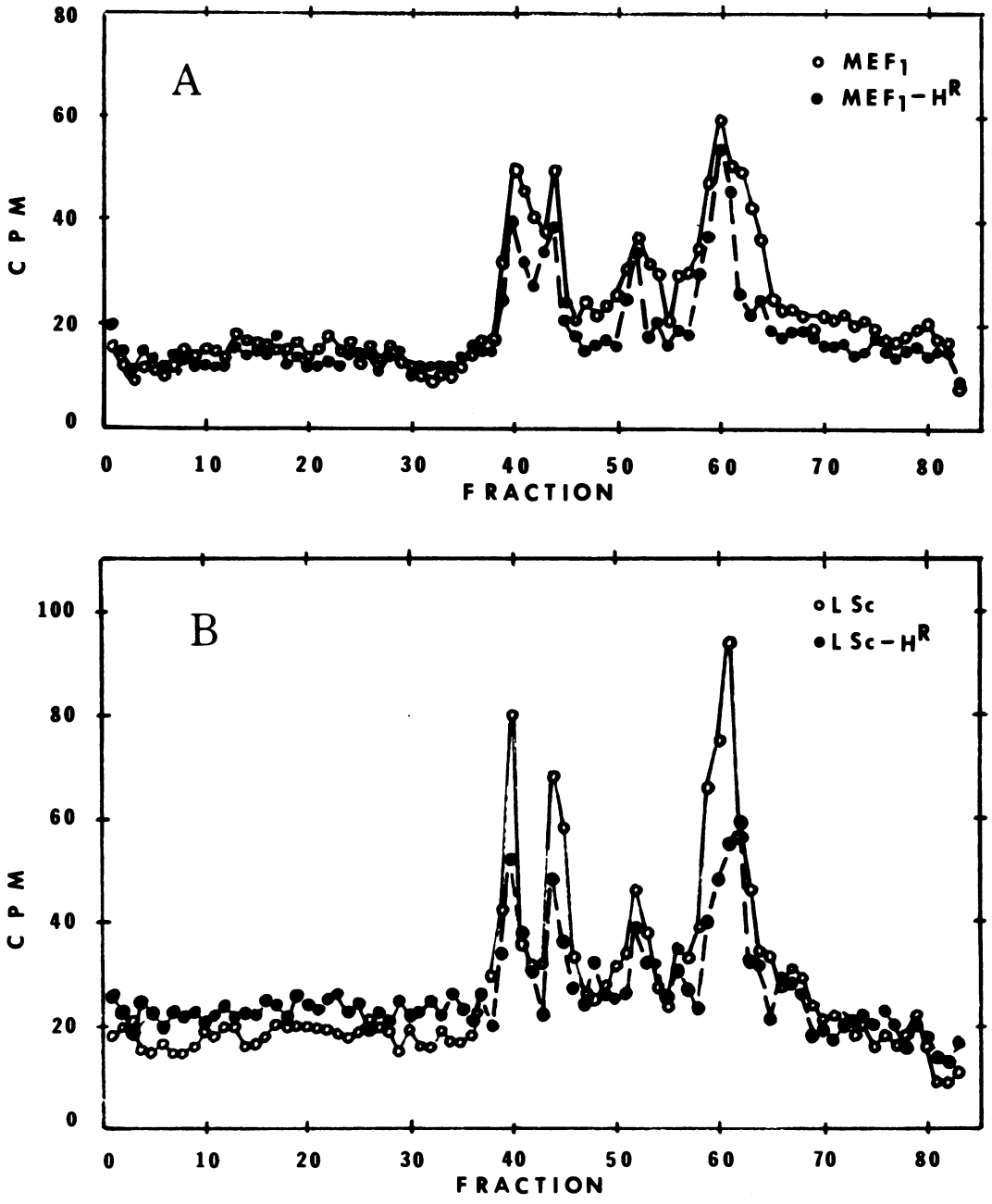


FIG. 6. *Polyacrylamide gel electrophoresis of the dissociated empty capsid polypeptides from the heat-resistant mutants and their wild-type viruses. The purified empty capsids were dissociated and prepared for electrophoresis on 10% acrylamide-SDS gels as described in Materials and Methods. Panel A. Co-electrophoresis of type 2, MEF$_1$ and MEF-H^R empty capsid polypeptides. Electrophoresis was for 22 h at 80 V, 8 mA. Symbols: [^{3}H]MEF$_1$, O; [^{14}C]MEF$_1$H^R, ●. Panel B. Co-electrophoresis of type 1, LSc and LSc-U^R empty capsid polypeptides. Electrophoresis was for 22 h at 80 V, 8 mA. Symbols: [^{14}C]LSc, O; [^{3}H]LSc-H^R, ●.*

were prepared and electrophoresed as described above. Unlike the VP-3 polypeptide seen in the electropherograms of the virion and empty capsid polypeptides, the VP-3 component from the Mah-U^R-infected cells repeatedly migrated slightly faster than the VP-3 polypeptide of the type 1, Mahoney-infected cells (Fig. 7A). A similar disparity was detected in the electro-

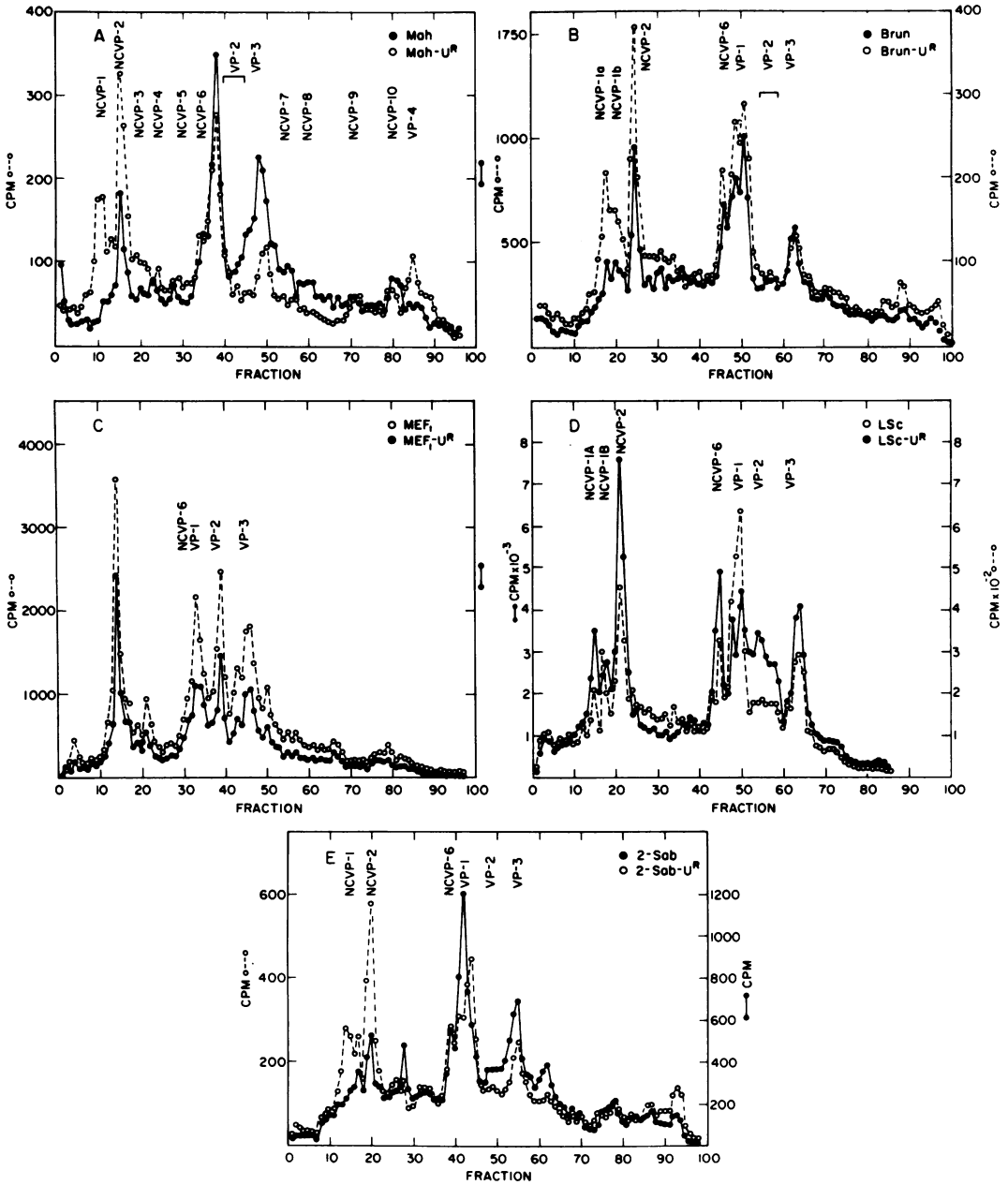
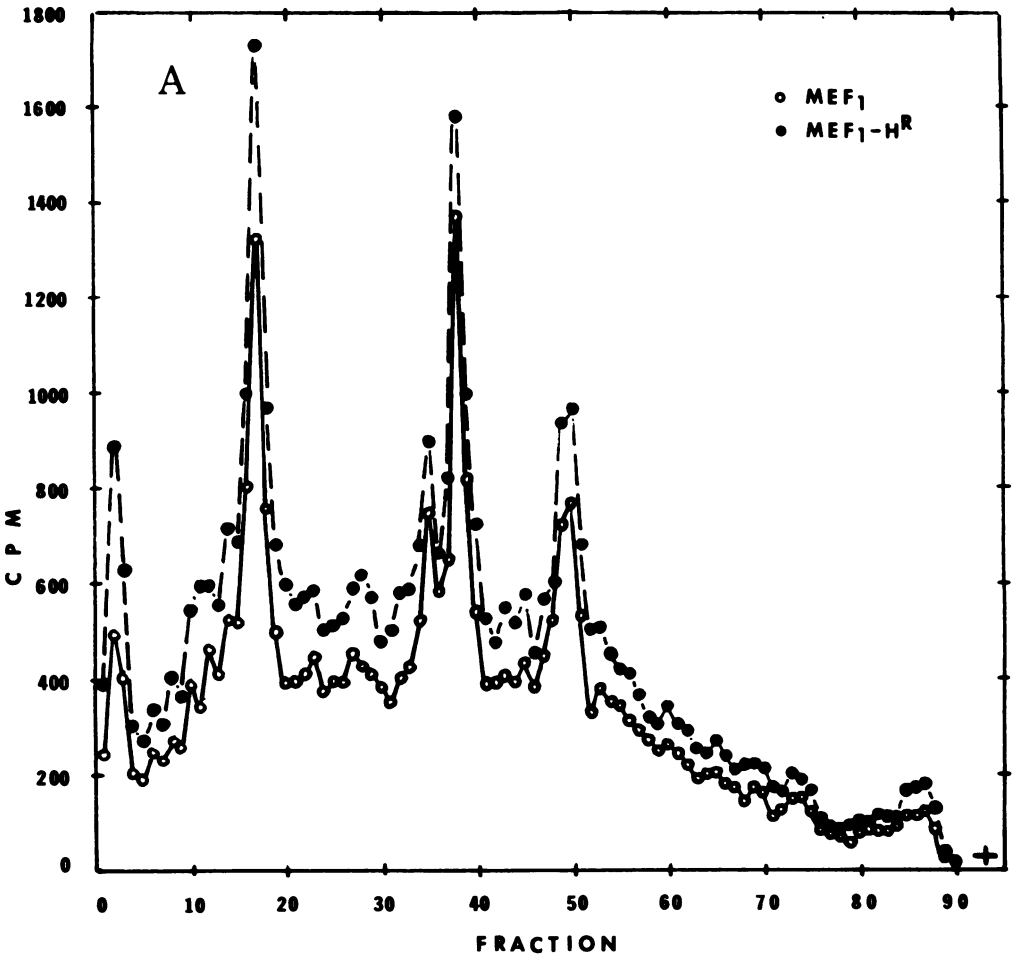
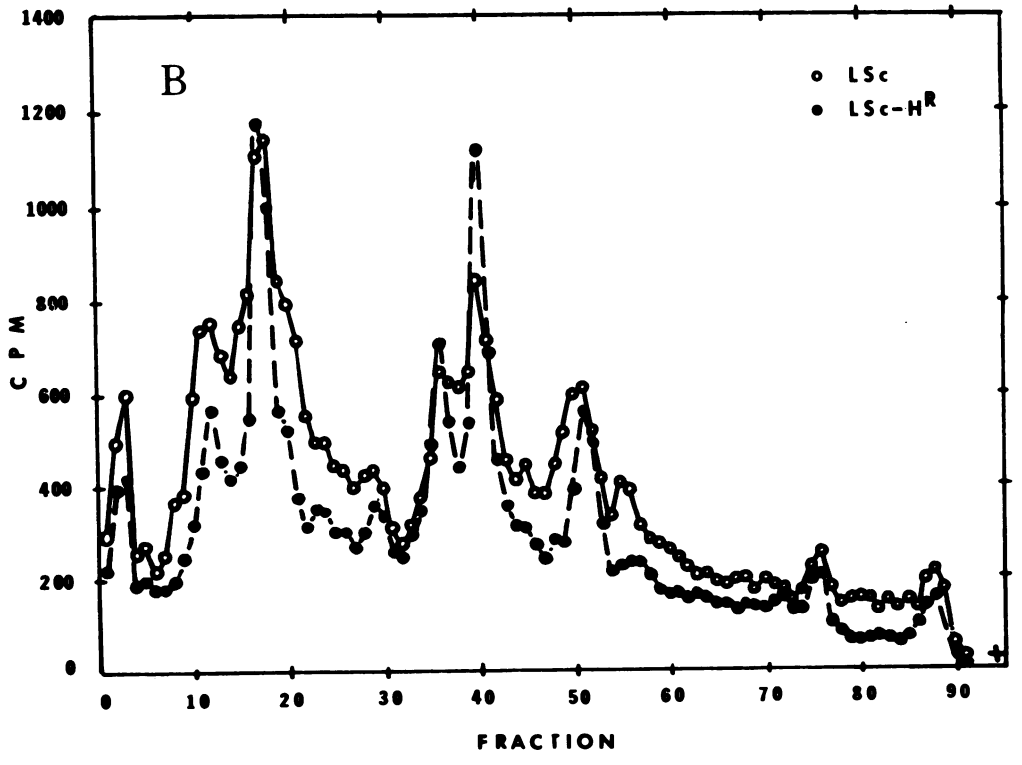


FIG. 7. *Polyacrylamide gel electrophoresis of radioactively labeled, viral-specific, cytoplasmic polypeptides from cells infected with the urea-resistant mutants and their wild-type viruses. Cytoplasmic extracts containing radioactively labeled, viral-specific polypeptides were prepared for electrophoresis on 10% acrylamide-SDS gels as described in Materials and Methods. Panel A. Co-electrophoresis of type 1, Mahoney and Mah-U^R cytoplasmic polypeptides. Electrophoresis was for 15 h at 80 V, 8 mA. Symbols: [^{14}C]Mah-U^R, O; [^{3}H]Mah, ●. Panel B. Co-electrophoresis of type 1, Brunhilde and Brun-U^R cytoplasmic polypeptides. Electrophoresis was for 20 h at 80 V, 8 mA. Symbols: [^{14}C]Brun-U^R, O; [^{3}H]Brun, ●. Panel C. Co-electrophoresis of type 2, MEF$_1$ and MEF$_1$-U^R cytoplasmic polypeptides. Electrophoresis was for 16 h at 80 V, 8 mA. Symbols: [^{14}C]MEF$_1$, O; [^{3}H]MEF$_1$-U^R, ●. Panel D. Co-electrophoresis of type 1, LSc and LSc-U^R cytoplasmic polypeptides. Electrophoresis was for 24 h at 80 V, 8 mA. Symbols: [^{3}H]LSc, O; [^{14}C]LSc-U^R, ●. Panel E. Co-electrophoresis of type 2, Sabin and 2-Sab-U^R cytoplasmic polypeptides. Electrophoresis was for 15 h at 80 V, 8 mA. Symbols: [^{3}H]2-Sab, ●; [^{14}C]2-Sab-U^R, O.*


A
MEF1
MEF1-HR
CPM
FRACTION
0
200
400
600
800
1000
1200
1400
1600
1800
10
20
30
40
50
60
70
80
90


B
LSc
LSc-HR
CPM
FRACTION
0
200
400
600
800
1000
1200
1400
10
20
30
40
50
60
70
80
90

phoresis of the LSc-U^R cytoplasmic polypeptides. The electrophoresis of the LSc-U^R virion and empty capsids polypeptides had shown that the VP-3 polypeptide(s) was larger than the VP-3 component of the LSc virus. No difference between the cytoplasmic VP-3 components was detected (Fig. 7D). Three of the resistant viruses had shown alterations in the mobility of the virion VP-1 polypeptide (Brun-U^R, LSc-U^R, 2-Sab-U^R). Examination of the cytoplasmic extracts revealed that cells infected with these viruses contained multiple components which migrated in the VP-1 regions (Fig. 7B, D, and E). In addition, the NCVP-1b polypeptide from the LSc-U^R-infected cells migrated slightly faster than the NCVP-1b polypeptide of the parental LSc virus (Fig. 7D). No difference was detected in the NCVP-1a components and again no difference was detected in the NCVP-6 polypeptides.

A similar analysis of the cytoplasmic polypeptides synthesized in MEF_1-H^R- and LSc-H^R-infected cells showed that there were no detectable differences between the mutants and their parental types (Fig. 8).

TABLE 1. *Alterations in viral polypeptides*

Comparison of strains	Alterations in viral polypeptides detected in:		
	Virion	Empty capsid	Cytoplasmic extract
Mah-U^R vs Mah	None	None	VP-3
MEF_1-U^R vs MEF_1	None	None	None
LSc-U^R vs LSc	VP-1	VP-1	VP-1
	VP-3	VP-3	
Brun-U^R vs Brun	VP-2	None[a]	None
2-Sab-U^R vs 2-Sab	VP-1	VP-1	VP-1
	VP-2	VP-2	
MEF_1-H^R vs MEF_1	None	None	None
LSc-H^R vs LSc	VP-3	VP-3	VP-3

[a] There was insufficient amount of VP-2 in extracts to reliably detect differences between wild-type and mutant viruses.

DISCUSSION

Five urea-resistant and two heat-resistant mutants were isolated from five strains of poliovirus types 1 and 2. The polyacrylamide gel electrophoresis of the polypeptides of these viruses indicated that four of the seven mutants contained a polypeptide(s) which differed in molecular weight from the corresponding polypeptide of their respective parental virions. A summary of these findings is shown in Table 1. The urea-resistant or heat-resistant phenotype of the virion could not be attributed to an alteration in one particular polypeptide because, depending on the virus strain, differences were seen in all but one of the capsid polypeptides (namely, VP-4). No differences were detected in any of the noncapsid viral polypeptides with the exception of the NCVP-1b component of the LSc-U^R virus (Fig. 7D). These findings are not surprising in that there is no covariation with respect to the urea- and heat-resistant markers (6). It should be emphasized that only differences in the molecular weights of the viral polypeptides were detectable in these experiments. Amino acid substitutions in the polypeptides that did not affect cleavage reactions would not be detected.

The electropherograms of the viral polypeptides from empty capsids and cytoplasmic extracts generally supported the similarities or differences found by examination of virion polypeptides. One should note that misleading results might be obtained if only the cytoplasmic extracts from infected cells were examined. For example, the electrophoresis of the cytoplasmic extract of Mah-U^R-infected cells often revealed a slight alteration in the VP-3 polypeptide; purified virions showed no such difference. Similarly, the electrophoresis of the cytoplasmic extracts of LSc-U^R-infected cells did not show the altered VP-3 polypeptide repeatedly detected in the purified virions. Therefore, the examination of the structural polypeptides appears best accomplished by using purified virions and, in addition, other virus-related particles. The use of crude cytoplasmic extracts alone may give incomplete or misleading results.

We detected in the empty capsids of most viral strains a significant amount of the VP-2 polypeptide(s). The amount of this component varied from being a major component in type 1, LSc and type 2, MEF_1 empty capsids (Fig. 5B and C) to a very minor component in type 1, Brunhilde empty capsids (Fig. 5D). Early reports on the morphogenesis of type 1, Mahoney poliovirus claimed that the empty capsids did

FIG. 8. *Polyacrylamide gel electrophoresis of radioactively labeled, viral-specific cytoplasmic polypeptides from cells infected with the heat-resistant mutants and their wild-type viruses. Procedure was the same as in Fig. 7. Panel A. Co-electrophoresis of type 2, MEF_1 and MEF_1-H^R cytoplasmic polypeptides. Electrophoresis was for 16 h at 80 V, 8 mA. Symbols: [^{14}C]MEF_1, O; [^3H]MEF_1-H^R, ●. Panel B. Co-electrophoresis of type 1, LSc and LSc-H^R cytoplasmic polypeptides. Electrophoresis was for 16 h at 80 V, 8 mA. Symbols: [^{14}C]LSc, O; [^3H]LSc-H^R, ●.*

not contain the VP-2 polypeptide (7). The VP-2 and VP-4 polypeptides were presumably formed from the cleavage of the NCVP-6 polypeptide during the final association of the empty capsid with the viral RNA to form virions. An intermediate structure, the provirion, was recently characterized by Fernandez-Tomas and Baltimore (5). Evidence presented here indicates that the empty capsids of most poliovirus strains do contain a VP-2 polypeptide (also see ref. 15). It may be that the VP-2 polypeptides found in the empty capsids are not the same as those found in the virions after encapsidation of the viral RNA. Of particular interest was the VP-2 polypeptide found in the empty capsids and virions of the 2-Sab-U^R virus (Figs. 3F and 5E). This VP-2 polypeptide was a major component of the empty capsid particle and had the same molecular weight as the VP-2 polypeptide found in its parental virus. Assuming that empty capsids are precursors to the virion, these data suggest that the empty capsids possess a VP-2 component which may become part of the virion capsid. Vanden Berghe and Boeyé (15) reported that purified poliovirions contained three VP-2 polypeptides. These investigators also reported the isolation of two types of empty capsids containing different amounts of the VP-2 polypeptide. Our experiments also indicated multiple VP-2 components in certain strains of poliovirus. At least one of the VP-2 components may be derived from the empty capsid.

It is clear that the cleavage of the NCVP-6 polypeptide (41,000 daltons) cannot produce two distinct VP-2 polypeptides, each with a molecular weight of 28,000 to 26,000 and a VP-4 polypeptide with a molecular weight of 5,500. One possible explanation is that the two VP-2 polypeptides are formed by the ambiguous cleavage of individual NCVP-6 molecules. Therefore, the VP-2 polypeptides detected in the virions represent a composite of a mixture of particles; some virions contain the smaller VP-2 polypeptide and others contain the larger one. Cooper et al. (4) first suggested that ambiguous cleavage of precursor polypeptides was responsible for the formation of altered copies of the capsid polypeptides.

Rekosh (13) determined that the gene order of the capsid polypeptides was VP-4 - VP-2 -VP-3 - VP-1. If these capsid polypeptides require the entire length of the NCVP-la precursor, then the cleavage of NCVP-la occurs at two points: between NCVP-6 and VP-3, and between VP-3 and VP-1. The VP-4 and VP-2 polypeptides subsequently form from the cleavage of NCVP-6. It was expected, therefore, that a decrease of several thousand daltons in the molecular weight of the VP-1 polypeptide (as in the LSc-U^R and 2-Sab-U^R virions) would produce a corresponding increase in the size of the VP-3 polypeptide. Likewise, a decrease in the size of the VP-3 polypeptide (as in the LSc-H^R virus) should be paralleled by an increase in the size of the VP-1 and/or NCVP-6 polypeptides. The LSc-U^R virus showed both a decrease in the size of its VP-1 polypeptide and an increase in the size of its VP-3 polypeptide (Fig. 3E). The 2-Sab-U^R virus showed no increase in the size of its VP-3 component despite the decreased size of its VP-1 component (Fig. 3F). Likewise, there was no increase in the size of the VP-1 polypeptide of the LSc-H^R virus in spite of the decreased size of its VP-3 component (Fig. 4A). Since no difference was ever detected in the size of the NCVP-la precursor molecules, it seems that there must be portions of the NCVP-la precursor molecule that do not become the capsid polypeptides after cleavage. It is possible, therefore, that there are more cleavage sites than orginally hypothesized. Alternatively, there may exist a mechanism for trimming the initial cleavage products to the proper size. Some of the polypeptides of the mutants may exhibit an altered sensitivity to the trimming mechanism, possibly as a consequenc of conformational changes due to an amino acid substitution, thereby producing polypeptides of altered size.

ACKNOWLEDGMENTS

We gratefully acknowledge the technical assistance of Marie Urban and Navin Patel.

This research was supported by a Public Health Research grant AI 08368 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. Cooper, P. D. 1961. An improved agar cell-suspension plaque assay for poliovirus; some factors affecting efficiency of plating. Virology **15**:153-157.
2. Cooper, P. D. 1962. Studies on the structure and functions of the poliovirion: effect of concentrated urea solutions. Virology **16**:485-495.
3. Cooper, P. D. 1963. Effect of concentrated urea solution on poliovirus strains adapted to different growth temperatures. Virology **21**:322-331.
4. Cooper, P. D., D. F. Summers, and J. V. Maizel. 1970. Evidence for ambiguity in the posttranslational cleavage of poliovirus proteins. Virology **41**:408-418.
5. Fernandez-Tomas, C. B., and D. Baltimore. 1973. Morphogenesis of poliovirus. II. Demonstration of a new intermediate, the provirion. J. Virol. **12**:1122-1130.
6. Hallum, J. V., and J. S. Youngner. 1966. Urea and thermal mutants of poliovirus. J. Bacteriol. **91**:2305-2308.
7. Jacobson, M., and D. Baltimore. 1968. Polypeptide cleavages in the formation of poliovirus. Proc. Nat. Acad. Sci. U.S.A. **61**:77-84.

8. Maizel, J. V., and D. F. Summers. 1968. Evidence for differences in size and composition of the poliovirus-specific polypeptides in infected HeLa cells. Virology **36**:48-54.
9. Maizel, J. V., B. A. Phillips, and D. F. Summers. 1967. Composition of artificially produced and naturally occurring empty capsids of poliovirus Type 1. Virology **32**:692-699.
10. Papaevangelou, G. J., and J. S. Youngner. 1961. Thermal stability of ribonucleic acid from poliovirus mutants. Virology **15**:509-510.
11. Phillips, B. A., and R. H. Fennell. 1973. Polypeptide composition of poliovirions, naturally occurring empty capsids, and 14*S* precursor particles. J. Virol. **12**:291-299.
12. Pohjanpelto, P. 1961. Two different variants of poliovirus. Virology **15**:231-236.
13. Rekosh, D. 1972. Gene order of the poliovirus capsid proteins. J. Virol. **9**:479-487.
14. Summers, D. F., J. V. Maizel, and J. E. Darnell. 1965. Evidence for virus-specific noncapsid proteins in poliovirus infected HeLa cells. Proc. Nat. Acad. Sci. U.S.A. **54**:505-513.
15. Vanden Berghe, D., and A. Boeyé. 1972. New polypeptides of poliovirus. Virology **48**:604-606.
16. Youngner, J. S. 1957. Thermal inactivation studies with different strains of poliovirus. J. Immunol. **78**:282-290.
17. Youngner, J. S., and J. V. Hallum. 1964. Cystine-independent thermoresistant mutants of poliovirus. J. Bacteriol. **88**:265-266.