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Biochemistry of Theiler's Murine Encephalomyelitis Virus Isolated from Acutely Infected Mouse Brain: Identification of a Previously Unreported Polypeptide

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The WW strain of Theiler's murine encephalomyelitis virus (WW-TMEV) was purified from homogenates of acutely infected mouse brain. Infectious WW-TMEV was found to have an estimated sedimentation coefficient of 156 ($s_{20,w}$) and a density of 1.35 g/cm^3 in CsCl. Electron microscopy revealed a homogeneous population of 26-nm nonenveloped particles. Iodination of sodium dodecyl sulfate (SDS)-disrupted virions revealed four major capsid proteins with molecular weights of 58,000, 37,000, 34,000, and 27,000. A 6,000-dalton polypeptide was observed after long exposures of autoradiograms. The 37,000-, 34,000-, 27,000-, and 6,000-dalton polypeptides corresponded to picornaviral VP1, VP2, VP3, and VP4 capsid polypeptides, respectively. Comparison of autoradiograms of virions radiolabeled before and after SDS disruption indicated that the 58,000-dalton protein, VP2, and VP3 preferentially bound ^{125}I under the labeling conditions used. Direct evidence was obtained that VP2 and VP3 were derived from the 58,000-dalton polypeptide by isolation of the 58,000-dalton polypeptide from polyacrylamide gels run under nonreducing conditions and subjecting it to reelectrophoresis under reducing conditions. The effect of trypsin on purified virions and their polypeptides was also investigated. Trypsin-sensitive sites were found in the 58,000-dalton protein, VP1, and VP2. Our results indicate that, in addition to the four typical picornaviral capsid polypeptides, there is a 58,000-dalton polypeptide present in WW-TMEV, which is sensitive to trypsin and can be reduced into two of the capsid proteins, VP2 and VP3.

Theiler's murine encephalomyelitis viruses (TMEV) are a group of murine enteric viruses which cause an acute encephalitis accompanied by hind limb paralysis after intracranial (i.c.) inoculation into weanling mice (31). Acute TMEV disease in mice appears to be histologically identical to poliovirus infection in monkeys (2, 26, 27) and has been reported to be due to lytic infection of neurons (15). Mice inoculated with certain strains of TMEV survive the acute disease and develop a chronic central nervous system (CNS) infection, characterized by focal, apparently immune-mediated demyelinating lesions in the spinal cord (7, 8, 11). TMEV has been isolated from the intestinal contents of normal mice (23, 24, 33) and has a pH stability similar to that of human enteroviruses (30). Complement fixation assays suggest that TMEV, the cardioviruses, and Vilyusik virus may share antigenic determinants (4, 9). These observations suggest that the polypeptide composition, size, sedimentation coefficient, and density in CsCl of TMEV may be similar to those of other picornaviruses.

The present study was undertaken to determine some of the physical characteristics and polypeptide composition of the WW strain of TMEV (WW-TMEV) (35). Tissue culture-adapted WW-TMEV was not used, since studies with other strains of TMEV (i.e., DA strain) have shown that with tissue culture adaptation the encephalitogenic potential of the virus is completely attenuated (14, 16). The attenuated progeny, having pathogenic effects different from those of the parent stock virus, may possess altered physical properties or polypeptide composition. Furthermore, recent studies have shown that the tryptic peptide maps of certain polypeptides of attenuated poliovirus differ from that of its parent neurovirulent strain (10). Therefore, TMEV was isolated directly from acutely infected mouse brain, purified, and compared with picornaviruses. Echovirus-12, a human enterovirus, was used in these studies as a comparative control.

During the preparation of this manuscript, Lipton and Friedmann published a report on the physical characteristics and polypeptides of several tissue culture-adapted strains of TMEV (17). Their results largely parallel ours in terms

of the physical properties of TMEV and the major capsid proteins. However, it is of interest that they did not describe one protein in the tissue culture-adapted strain which we consistently find in the virus isolated from CNS tissues.

(A preliminary report of this study was presented at the 79th Annual Meeting of the American Society for Microbiology [Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, S91, p. 255.])

MATERIALS AND METHODS

Virus and cell culture. WW-TMEV was a gift of D. Gilden (Philadelphia, Pa.). In vivo neutralization tests using commercially prepared GDVII-TMEV antiserum (Microbiological Associates, Bethesda, Md.) confirmed the identity of the seed virus as TMEV by significantly reducing mortality in suckling mice during the acute disease phase. The original seed virus was passaged twice in suckling mouse brain to produce a stock which contained 10^8 50% lethal doses/ml in clarified 10% (wt/vol) brain homogenate. Human echovirus-12 was prepared by inoculating confluent 75-cm^2 culture flasks of BSC-1 cells at a multiplicity of infection of 0.01 50% tissue culture infectious dose per cell. Infected cultures were maintained in a 5% CO_2 humidified incubator in Earle minimal essential medium (MEM) supplemented with 2% fetal calf serum and 100 U of penicillin and streptomycin per ml. When maximum cytopathic effect developed, culture supernatants were aspirated, clarified of cell debris by centrifugation for 10 min at 400 × *g*, and frozen at −70°C.

Animals. ICR mice (Flow Laboratories, Inc., Dublin, Va.), less than 24 h old, were inoculated i.c. with 20 μl of clarified 10% (wt/vol) brain homogenate containing 10^5 50% lethal doses of WW-TMEV. Nine days postinoculation, brains were aseptically removed and homogenized in Hanks balanced salt solution with Ten Broeck tissue grinders to yield a 10% (wt/vol) homogenate. The homogenate was then frozen at −70°C until used, whereupon it was thawed and centrifuged for 10 min at 400 × *g* to remove cell debris. Control animals were sham inoculated with uninfected 10% (wt/vol) brain homogenate.

Virus purification. Supernatants of clarified brain homogenates were mixed with an equal volume of fluorocarbon (Freon-TF; E. I. du Pont de Nemours & Co., Inc. Palo Alto, Calif.), blended for 10 min, and centrifuged for 10 min at 400 × *g*. Brain lipids were extracted into the fluorocarbon (lower) phase; proteins and virus remained in the aqueous (top) phase.

The aqueous phase was carefully aspirated and subjected to fluorocarbon extraction three more times. The extracted virus was concentrated by pelleting for 1 h at 280,000 × *g* in a Beckman ultracentrifuge with an SW41 rotor (Beckman Instruments, Inc., Palo Alto, Calif.), suspended in 0.02 M Trizma-HCl [tris(hydroxymethyl)aminomethane (Tris)-hydrochloride], pH 7.2, layered onto a preformed 15 to 35% (wt/wt) sucrose gradient, and centrifuged for 2 h at 280,000 × *g*. Approximate 0.4-ml fractions were collected, and the absorbancy at 260/280 nm was determined. Two peaks were obtained, the faster of which contained the maximum infectivity. The faster-sedimenting peak fractions were pooled, adjusted to contain less than 10% (wt/wt) sucrose, pelleted for 2 h at 280,000 × *g*, suspended in CsCl with a density of 1.34 g/cm^3 in 0.02 M Tris-hydrochloride, pH 7.2, and centrifuged for 36 h at 250,000 × *g* in an SW50.1 rotor at 5°C. Approximate 0.15-ml fractions were collected, and those fractions containing virus were pooled, adjusted to contain less than 7% CsCl, and pelleted for 2 h at 250,000 × *g*. The final pellet was resuspended in 0.02 M Tris-hydrochloride, pH 7.2, and stored at −70°C.

Sedimentation analysis. Sedimentation coefficients were calculated by the method of McEwen (21) from rate-zonal sucrose gradient centrifugations, assuming a particle density of 1.30 g/cm^3. Sedimentation values were derived for virus particles from CsCl gradients after removal of bound CsCl by repeated pelleting and washing steps for 2 h at 280,000 × *g* in 0.02 M Tris-hydrochloride, pH 7.2, before rate-zonal centrifugation in 5 to 15% (wt/wt) sucrose gradients. A portion of the viral pellet was checked for infectivity before centrifugation.

EM. Purified preparations of virus were examined by electron microscopy (EM), using phosphotungstic acid (PTA), pH 6.0, as the negative stain. Two-microliter samples of virus were placed on polyvinyl formal filmed grids onto which was added a volume of PTA sufficient to cover the grid. Excess PTA was aspirated, and the grid was allowed to dry at room temperature. EM was performed on virus preparations immediately before electrophoresis (see below).

Radiolabeling of virus. Purified infectious virus was labeled in vitro by addition of 200 μCi (7.4 megabecquerels) of ^{125}I (Amersham/Searle, Arlington Heights, Ill.) per μg of whole virus, using a modification of the chloramine T method of Carthew and Martin (3). In other experiments, purified infectious virus was disrupted in 5% sodium dodecyl sulfate (SDS) before radiolabeling by addition of an equal volume of 10% SDS to the virus preparation and boiling for 1 min. Labeled whole virus or disrupted virus was separated from unbound ^{125}I by gel filtration on a 3-ml Sephadex G-10 column equilibrated in 0.02 M Tris-hydrochloride, pH 7.2, which had been previously exhaustively washed with 5% bovine serum albumin followed by 0.02 M Tris-hydrochloride, pH 7.2, or 5% SDS in 0.02 M Tris-hydrochloride, pH 7.2. Fifty-microliter fractions were collected. For some experiments, echovirus-12 was labeled in tissue culture. Four hours before infection, confluent 75-cm^2 flasks of BSC-1 cells were treated with MEM deficient in methionine. The cells were then infected (see above), and at the end of the 1-h adsorption period the medium was changed to MEM containing 3 μCi of [^{35}S]methionine per ml (Amersham/Searle).

Polyacrylamide gel electrophoresis. Discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed (12) on vertical slabs (0.75 by 100 by 280 or 100 by 100 mm), using sample wells 10 mm wide formed in the stacking gel. The stacking gel, prepared in 0.125 M Tris-hydrochloride, pH 6.8, and 2 mM ethylenediaminetetraacetic (EDTA), consisted of the acrylamide monomer (5%, wt/vol), *N,N'*-methylenebisacrylamide; (0.08%, wt/vol), *N,N,N',N'*-tetramethylenediamine (TEMED; 0.05%, vol/vol), ammonium persulfate (0.1%, wt/vol), and SDS (1.0%, wt/vol). The resolving gel, prepared in 0.375 M Tris-

hydrochloride, pH 8.0, and 2 mM EDTA, consisted of the acrylamide monomer (15%, wt/vol), *N,N*-methylenebisacrylamide (0.4%, wt/vol), TEMED (0.5%, vol/vol), ammonium persulfate (0.1%, wt/vol), and SDS (1.0%, wt/vol). Immediately before electrophoresis, samples of virus were processed by boiling for 1 min in sample buffer containing 67.4 mM Tris-hydrochloride, pH 6.8, 1% SDS, 2 mM EDTA, 2 or 4% 2-mercaptoethanol (2-ME), 2% glycerol, and 0.05% phenol red. For electrophoresis under nonreducing conditions, samples of virus were processed in sample buffer without 2-ME. After preparation in the appropriate sample buffer, 50 μl of sample was loaded onto the gel and electrophoresed at a constant current of a 14 mA, using a model 220 or 221 electrophoresis unit (Bio-Rad Laboratories, Richmond, Calif.). After electrophoresis, the gels were fixed at room temperature for 45 min in 12.5% (wt/wt) trichloroacetic acid, stained for 60 to 90 min in a solution of 0.2% Coomassie brilliant blue R-250 (Sigma Chemical Co., St. Louis, Mo.), and destained at 50°C overnight in 25% methanol and 7% glacial acetic acid except in certain experiments outlined below. Gels were transferred to filter paper, dried under vacuum, and exposed to Kodak XRP-1 X-ray film at 22° or −70°C. An EC transmission densitometer was used in some instances to locate polypeptides in autoradiograms.

Molecular weight determinations were performed by coelectrophoresing protein markers of known molecular weight in an adjacent well of the gel.

Reelectrophoresis of polypeptides. Certain experiments required isolation of viral polypeptides run under nonreducing conditions and reelectrophoresis under reducing conditions. Gels run under nonreducing conditions were lightly fixed in 50% methanol, 10% acetic acid, and 1% Coomassie blue for 30 min, destained for 60 min, and sliced into 0.5-cm slices, and the radioactivity per slice was determined. Slices containing polypeptides of interest were equilibrated for reelectrophoresis in 0.125 M Tris-hydrochloride, pH 6.8, 1 mM EDTA, and 0.1% SDS. Before reelectrophoresis, the slices were boiled for 1 min in reducing sample buffer containing 4% 2-ME. Slices were minced and placed in the sample well, and the spaces around the pieces were filled with 1% Noble agarose (Difco Laboratories, Detroit, Mich.) in 0.125 M Tris-hydrochloride, pH 6.8, 1 mM EDTA, and 0.1% SDS. After addition of the running buffer, the sample well was overlaid with 20 μl of the sample buffer in which the slice had been boiled. After electrophoresis, the gel was processed as usual.

RESULTS

Virus isolation and physical characterization. When crude extracts of virus were pelleted and centrifuged through a linear 15 to 35% (wt/wt) sucrose gradient, two peaks were obtained. Infectious virus was present in the faster-sedimenting peak and was further purified by centrifugation in CsCl. Virus banded at a buoyant density of 1.35 g/cm^3 (Fig. 1). Rate-zonal centrifugation after removal of bound CsCl revealed these particles to have an estimated sedimentation coefficient of 156 ($s_{20,w}$). In comparison, echovirus-12 banded at 1.33 g/cm^3 and had an estimated sedimentation coefficient of 159 ($s_{20,w}$) (data not shown). The differences in calculated sedimentation coefficients were not significant. WW-TMEV from the CsCl gradient was found to be highly infectious when inoculated i.c. into suckling mice and consisted of a homogeneous population of intact 26-nm nonenveloped particles by EM (Fig. 2). It was not possible to appreciate the icosahedral symmetry by these techniques.

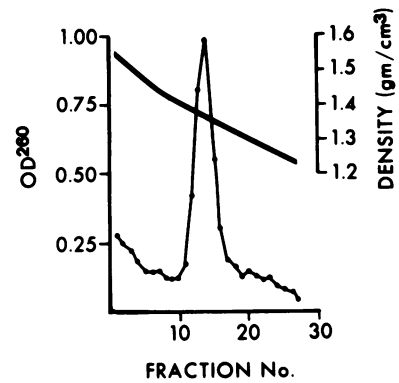

FIG. 1. *CsCl gradients of fluorocarbon-extracted WW-TMEV. After fluorocarbon extraction and sucrose density centrifugation, virus was mixed with CsCl and centrifuged for 36 h at 250,000 × g. Infectious virus banded at 1.35 g/cm^3.*

Polypeptides of whole and disrupted TMEV. SDS-PAGE of whole TMEV revealed four major polypeptides in Coomassie blue-stained gels, with apparent molecular weights of 58,000, 37,000, 34,000, and 27,000 (Fig. 3, lane A) (designated P58, P37, P34, and P27, respectively); a minor 64,000-dalton polypeptide was occasionally observed (P64). To enhance detection of polypeptides, purified whole infectious virus was radiolabeled with ^{125}I and subjected to SDS-PAGE. Autoradiography revealed the same polypeptides seen in stained gels, plus an additional minor one with an apparent molecular weight of 22,000 (P22) (Fig. 3, lane C). Prolonged exposures of autoradiograms revealed an additional 6,000-dalton polypeptide (P6). Discontinuous SDS-PAGE of echovirus-12 revealed four major polypeptides with apparent molecular weights of 40,000, 30,000, 22,000, and 10,000, which closely approximate the published reports for VP1, VP2, VP3, and VP4 of echovirus-12 (11) (data not shown).

P37 appeared as a major constituent in stained gels (Fig. 3, lane A), but was a minor band in ^{125}I-labeled whole-virus preparations (Fig. 3, lane C), suggesting that during the strong oxidation-

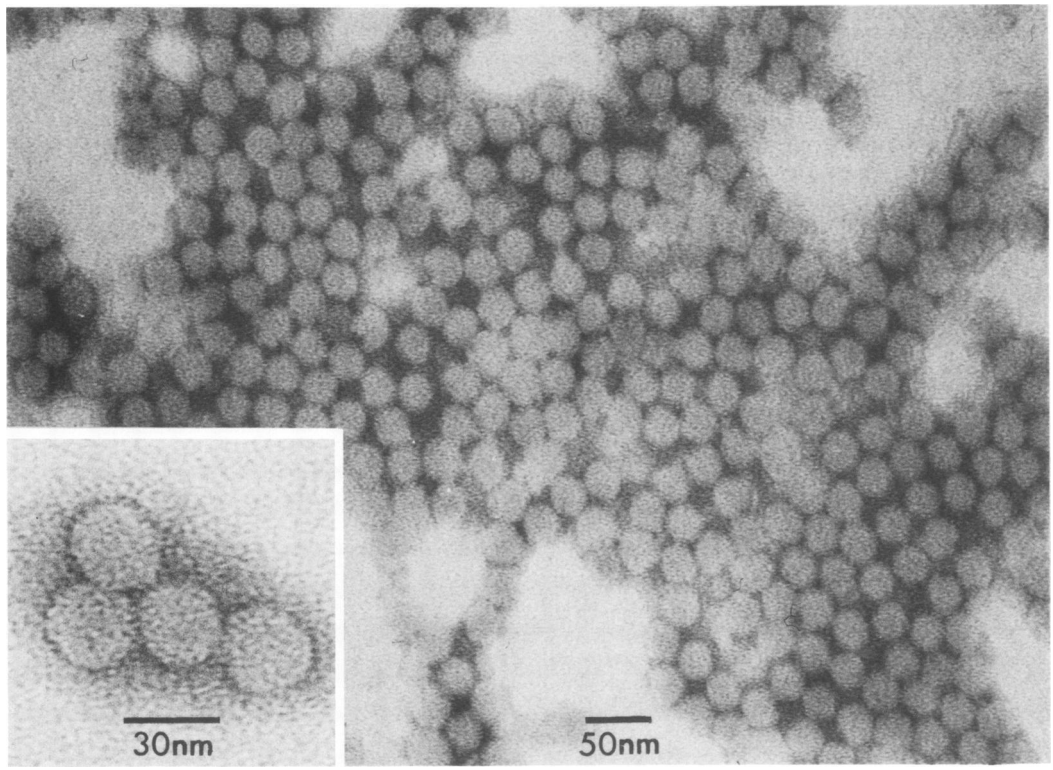

FIG. 2. *Negative stain of purified WW-TMEV. WW-TMEV purified by fluorocarbon extraction and by sucrose and CsCl density gradient centrifugations was negatively stained with 2% PTA and examined by electron microscopy before SDS-PAGE.*

radiolabeling reaction, ^{125}I was attaching preferentially to P34 and P27. To ensure a more complete reaction, virus was disrupted in 5% SDS before iodination. SDS disruption allowed for a substantial increase in the labeling of P37 (Fig. 4). These results indicated that the major capsid proteins were P37 (VP1), P34 (VP2), P27 (VP3), and P6 (VP4), and that P34 (VP2) and P27 (VP3) were preferentially labeled in whole virus. Less intense iodination conditions resulted in both P34 (VP2) and P27 (VP3) simultaneously labeling to relatively equal degrees (data not shown).

Electrophoresis under reducing and nonreducing conditions. The relationship of P58 to the capsid polypeptides was investigated. A preparation of purified TMEV was divided into two portions; one was iodinated as whole virus, and the second was disrupted in 5% SDS followed by iodination. These preparations were then subjected to coelectrophoresis under reducing and nonreducing conditions.

When virus was disrupted in SDS before iodination and reduced in 2-ME, little if any P58 was detectable (Fig. 5, lanes E and F). P34 (VP2) and P27 (VP3) appeared to be slightly enhanced relative to P37 (VP1). In contrast, P58 was detectable in virus preparations electrophoresed under nonreducing conditions (Fig. 5, lane D). P64 was not detectable under any condition in which virus was disrupted before radiolabeling.

When identical experiments were performed with whole radiolabeled virus, similar results were obtained (Fig. 5, lanes A through C). P58 was present in such preponderance under nonreducing conditions that it overloaded this region of the gel (Fig. 5, lane C). The relative quantity of P58 decreased as the percentage of 2-ME increased (compare lanes A and B, Fig. 5). P40 was detected in these gels and was presumably the VP0 of WW-TMEV, since it was only occasionally seen (see Discussion). P22 was present only in the lanes of whole radiolabeled virus (compare lanes A–C with D–F, Fig. 5).

P34 (VP2) and P27 (VP3) are derived from P58. P58 appeared to be a major constituent in gels of both whole and disrupted, radiolabeled virus preparations. Since the results obtained in the above experiments indicated that P58 seemed to decrease in quantity as the

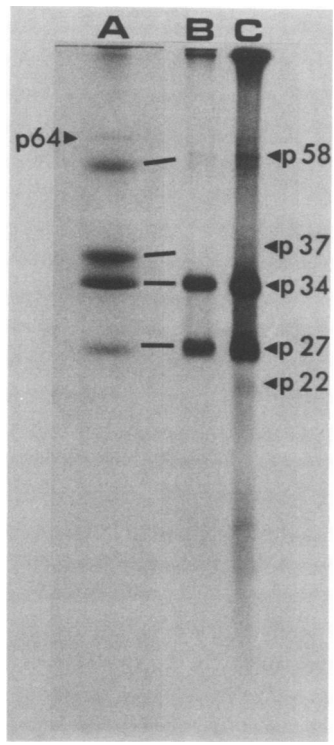

FIG. 3. *Discontinuous SDS-PAGE of purified WW-TMEV. Purified virus was subjected to electrophoresis as described in Materials and Methods. The polypeptides were detected by staining with Coomassie blue (lane A) or autoradiography (lanes B and C) of whole virus labeled with ^{125}I. Lane C was loaded with 10 times more counts per minute than lane B and is overexposed to enhance detection of the 58,000-dalton polypeptide. Numbers indicate the apparent molecular weights ($\times 10^3$) of the viral polypeptides determined by comparison to coelectrophoresed marker proteins run in an adjacent well (phosphorylase a, 95,000; bovine serum albumin, 68,000; ovalbumin, 45,000; carbonic anhydrase, 32,000; cytochrome c, 13,500; insulin, 5,750). A 6,000-dalton polypeptide was visible only after prolonged exposure of the autoradiograms. The figure is a composite; lane A is from another electrophoretic run than lanes B and C.*

strength of the reducing conditions increased, a means of isolating P58, and characterization of its reduced products was developed. The experimental approach was to electrophorese whole radiolabeled virus under nonreducing conditions, locate P58, remove it from the gel, and subject it to reelectrophoresis under reducing conditions (Fig. 6). These results demonstrate that P34 (VP2) and P27 (VP3) were derived from P58 by reduction. It is of interest that not all of the P58 was reducible under these conditions, which was in agreement with the results shown in Fig. 5, lanes A and B. Prolonged exposures of these autoradiograms failed to show any other reduction products.

Effects of trypsin on virus polypeptides. The radiolabeling characteristics of whole virus suggested that P34 (VP2) and P27 (VP3) were oriented on the surface of the virus and that P37 (VP1) was located more internally. Consequently, we investigated the trypsin sensitivity of WW-TMEV polypeptides. Preparations of whole and SDS-disrupted ^{125}I-labeled TMEV were made, and one portion was treated for 20 min at 37°C with 100 μg of trypsin in 0.02 M Tris-hydrochloride buffer, pH 7.2. Control portions containing equivalent counts per minute were incubated with an equivalent amount of buffer without trypsin. Preliminary experiments had indicated that the maximum effect of trypsin treatment was achieved after 20 min of incubation, making longer digestion times unnecessary (data not shown). After digestion, the samples were then subjected to electrophoresis under nonreducing and reducing conditions (Fig. 7).

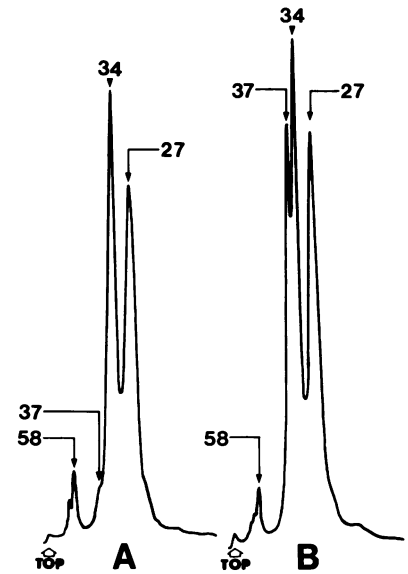

FIG. 4. *Scanning densitometric tracings of TMEV polypeptides labeled before (A) and after (B) capsid disruption. One portion of fluorocarbon-extracted and purified whole WW-TMEV was radiolabeled as described in Materials and Methods. The other portion was disrupted in 5% SDS before radiolabeling. Unbound ^{125}I was removed from both samples by gel filtration, and the samples were coelectrophoresed. P6 is not visible in these densitometric tracings because the long exposures required to detect it resulted in overexposure of P58, P37, P34, and P27. Numbers refer to the molecular weights of the polypeptides ($\times 10^3$).*

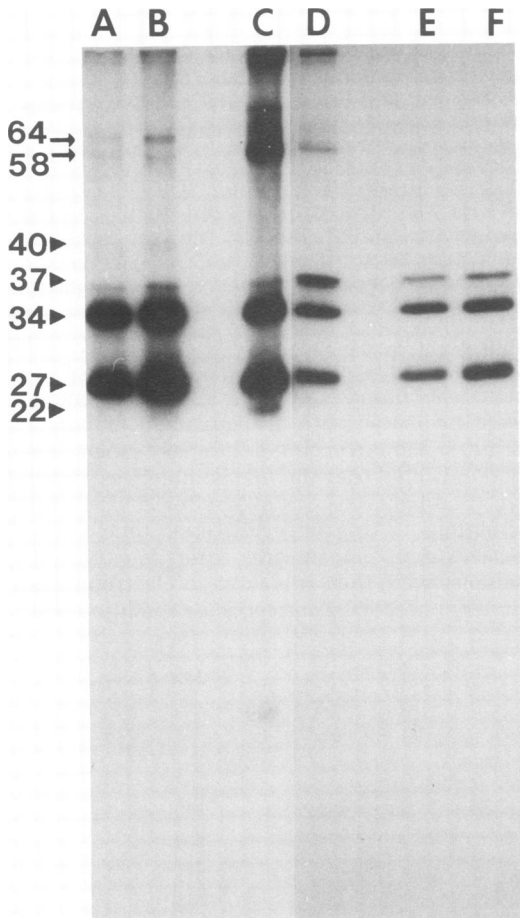

FIG. 5. *Discontinuous SDS-PAGE of WW-TMEV labeled with ^{125}I before (whole virus) or after (disrupted virus) capsid disruption, electrophoresed under reducing and nonreducing conditions. Whole virus (lanes A–C) was prepared for electrophoresis in the presence of 4% 2-ME (lane A), 2% 2-ME (lane B), and without 2-ME (lane C). Disrupted virus (lanes D–F) was prepared by heating whole virus in 5% SDS for 1 min before radiolabeling. Disrupted virus was then prepared for electrophoresis in the presence of 4% 2-ME (lane F), 2% 2-ME (lane E), and without 2-ME (lane D). All samples contained equivalent counts per minute and were coelectrophoresed. The figure is a composite of two different exposures of the same autoradiogram. Numbers refer to the molecular weights of the polypeptides ($\times 10^3$).*

FIG. 6. *Reelectrophoresis under reducing conditions of P58 isolated from a gel electrophoresed under nonreducing conditions. P58 was located in a gel of whole radiolabeled virus run under nonreducing conditions and prepared for reelectrophoresis as described in Materials and Methods. Numbers refer to the molecular weights of the polypeptides ($\times 10^3$).*

When the virus was incubated with trypsin, several cleavages were noted to occur (Fig. 7, lanes A and C). Under reducing and nonreducing conditions, trypsin had the effect of completely digesting P58, P37 (VP1), and nearly all of P34 (VP2). P22, a minor component observed in whole-virus preparations, was markedly enhanced by trypsin treatment. Presumably P22 was a specific cleavage product of either P58, P37 (VP1), or P34 (VP2) and appeared in some autoradiograms due to opportunistic proteases with trypsin-like activity in preparations of purified whole radiolabeled virus. A new polypeptide, P30, was also observed after trypsin treatment. Presumably it too was derived from one of the larger polypeptides. There were numerous other small polypeptides in the lanes containing whole TMEV (Fig. 7); these probably represented specific cleavage products of the major polypeptides, but interpretation of them was not possible with the techniques used. Trypsin treatment of SDS-disrupted virus preparations was unsuccessful due to the high percentage of SDS present in these preparations.

DISCUSSION

The capsid polypeptides of mature picornavirions consist of four species with molecular weights of 32,000 to 35,000 (VP1), 28,000 to 30,000 (VP2), 23,000 to 27,000 (VP3), and about

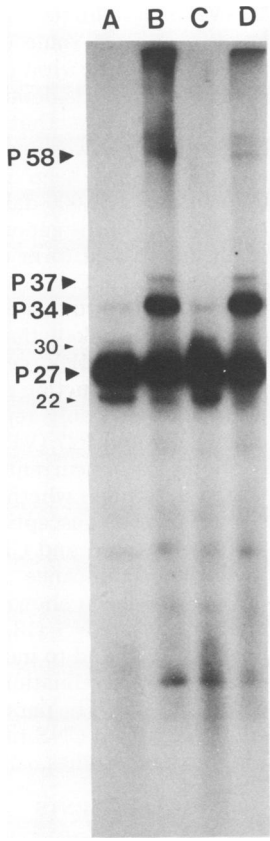

FIG. 7. *Effect of trypsin on ^{125}I-labeled WW-TMEV. Purified whole WW-TMEV was radiolabeled with ^{125}I and incubated with or without trypsin at 37°C for 20 min immediately before coelectrophoresis under reducing (R) and nonreducing (NR) conditions. (A) R + trypsin; (B) NR − trypsin; (C) NR + trypsin; (D) R − trypsin. All lanes were loaded with equal counts per minute. Numbers refer to the molecular weights of the polypeptides (×10³).*

7,000 (VP4) with the exception of foot-and-mouth disease virus (FMDV), which has a VP4 polypeptide of about 14,000 daltons (28). Most picornaviruses probably contain equimolar quantities of the capsid proteins; however, their orientation in the virus particle is still not completely understood. Occasionally, traces of a fifth polypeptide, VP0, with a molecular weight of about 40,000 are also observed. Infectious picornavirions are nonenveloped icosahedral particles with a diameter of about 30 nm, have an average sedimentation coefficient of 156 ($s_{20,w}$), and characteristically band in CsCl at a buoyant density of about 1.34 g/cm^3 (28). During preparation of this manuscript, Lipton and Friedmann described the polypeptide composition of several strains of tissue culture-adapted TMEV (17). Their results and the results reported here are essentially in agreement; the modest differences in the molecular weights of TMEV polypeptides probably reflect slight differences in molecular weight calibration curves and other variations of SDS-PAGE that differ between laboratories. The conventional nomenclature (VP0 through VP4) to designate picornaviral proteins may be premature for TMEV, since the polyprotein cleavage-assembly process of TMEV has not yet been reported. However two lines of experimental evidence strongly suggest that TMEV P37, P34, and P27 are three of the four structural polypeptides of the virus, VP1, VP2, and VP3. First, in experiments where viral polypeptides were detected in polyacrylamide gels by staining, three major bands were detected, P37, P34, and P27 (in addition to P58) (Fig. 3, lane A). Second, when virions were treated with SDS under conditions that ensure complete disruption of picornaviral capsids (20), radiolabeled, and electrophoresed, three predominant species, P37, P34, and P27, were detected in nearly equal quantities. The smallest structural polypeptide, VP4, has been always difficult to demonstrate in picornaviruses, but was reported by Lipton and Friedmann in tissue culture virus (17) and was observed in our studies of brain-derived virus. VP4 may be more difficult to demonstrate, however, when TMEV is radiolabeled in vitro with ^{125}I than when radiolabeled in tissue culture. Taken together, these data indicate that WW-TMEV does contain four typical capsid polypeptides seen in all picornaviruses.

The studies reported here also shed light on the orientation of these polypeptides. Most picornaviruses appear to have VP1 as the major capsid protein as assessed by reaction of intact virions with iodine (3, 18), *N*-succinimidyl propionate (34), and acetic anhydride (18). The biological importance of a surface-oriented VP1 as a determinant of virus-cell interaction is further supported by antibody-blocking and protease susceptibility experiments (5, 19). These reports suggest that VP1 is a major contributor to the surface structure of picornaviruses and may be chiefly involved in host cell recognition. However, VP1 is not the exclusive surface protein of picornaviruses. Recent evidence obtained with poliovirus treated with the Bolton-Hunter reagent indicated that VP3 also had a surface orientation (34). When WW-TMEV was disrupted in SDS, labeled, and electrophoresed, nearly uniform labeling of P37 (VP1), P34 (VP2), and P27 (VP3) was achieved, indicating that each protein had nearly equivalent percentages of tyrosine residues. This is in agreement with the fact that the calculated percentages of tyrosine residues per capsid protein for ME virus,

mengovirus, and FMDV are about the same (28). However, when whole virions were radiolabeled and electrophoresed, the majority of the label was found in P34 (VP2) and P27 (VP3). These data suggested either that P34 (VP2) and P27 (VP3) had more of a total surface orientation than P37 (VP1) or that the labeled tyrosine residues of P37 (VP1) were more externally located relative to the tyrosine residues of P34 (VP2) and P27 (VP3). The latter interpretation was supported by the trypsin digestion experiments. Trypsin had the effect of cleaving off all labeled residues of P37 (VP1) and most residues of P27 (VP3), but left the residues of P27 (VP3) apparently unaffected. Our experiments indicate that under all radiolabeling conditions used, P34 (VP2) and P27 (VP3) have more tyrosine residues accessible to the radiolabeling reagents than P37 (VP1), but that of all labeled residues, those of P37 (VP1) are most externally located. More experiments are required to assess whether all of the P37 (VP1) polypeptide is surface oriented.

The trypsin studies reported here contrast with similar studies performed by Lipton and Friedmann (17). When these investigators subjected tissue culture-adapted WW-TMEV to trypsin, they observed an apparent shift of VP1 to the same position as VP2 in SDS-polyacrylamide gels (17). Our studies showed that when whole virions were subjected to trypsin treatment, not only was there an apparent loss of P37 (VP1), but a marked reduction of P34 (VP2) as well, plus the generation of cleavage products, P30 and P22. The data of Lipton and Friedmann (17) could be due to tissue culture adaptation of the virus, a process which has been implicated to cause the VP1 $\rightarrow$ VP2 shift with attenuation of poliovirus (22). This shift, which has not been convincingly shown to correlate with neurovirulence, may be due to relatively minor amino acid substitutions in VP1 which result in altered mobility due to a net change of the hydrophobicity of the polypeptide (10).

Our studies document one notable exception to the structural similarity between other picornaviruses and TMEV, the presence of TMEV P58. This protein appeared to have a surface orientation by its reaction with iodine in whole virions and its susceptibility to trypsin. The observation that P58 was a consistent component of highly purified preparations of TMEV and could be partially reduced into two of the capsid proteins, P34 (VP2) and P27 (VP3), was surprising and strongly argued that P58 was not a host contaminant. Furthermore, preliminary peptide mapping studies have indicated that P58 contains residues in common with P27 (VP3) and P34 (VP2). These data suggested that P34 (VP2) and P27 (VP3) were linked together by a disulfide linkage in at least some of the total amount of P58 present in the viral preparation. We are unaware of any reports detailing a large polypeptide in picornaviruses that can be at least partially reduced into two capsid proteins.

A large polypeptide has been reported in FMDV. FMDV contains a 52,000-dalton protein which maps to the 3′ end of the genome (29) and appears to be an inactivated form of the viral replicase-polymerase (6, 25, 29). Because WW-TMEV P58 was reducible into two of the major capsid proteins, it is very unlikely that P58 maps near the 3′ end of the TMEV genome, and therefore probably has no function as a polymerase or replicase. P58 probably represented a precursor of P34 (VP2) and P27 (VP3) which is present in the virions. We are currently performing experiments to determine whether P58 and the differences in trypsin susceptibilities between tissue culture-adapted and CNS-derived TMEV have biological significance.

Infectious TMEV has been shown to persist for months in the mouse CNS (13, 32; W. G. Stroop, unpublished data) and to induce apparent immune-mediated demyelination primarily of the spinal cord (7, 13, 15). The persistent virus can be isolated directly from CNS tissue by the techniques used for these studies.

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