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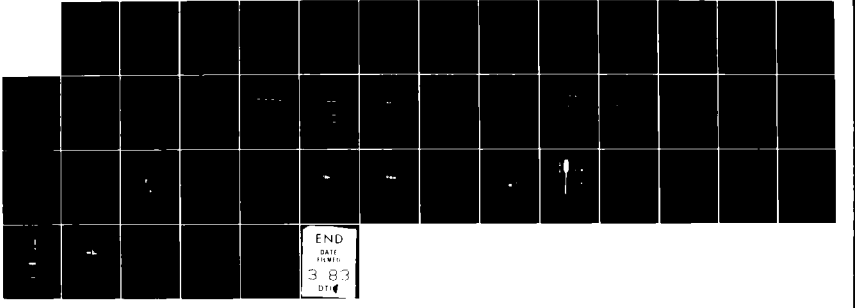

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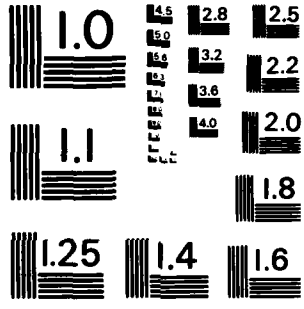

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MORPHOGENESIS OF DENGUE VIRUS:
Molecular Biology and Molecular Organization of Proteins

ANNUAL REPORT

August 1979

(For the period September 1978 - September 1979)

Principal Investigator

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The molecular composition and organization of dengue virus (DV) and Japanese encephalitis virus (JEV) components released from infected cells is described in this report. Substantial progress has been made and is summarized below.

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2. JEV and DV infected cells were found to release considerable amounts of viral polypeptides into cell culture medium. Radioimmune precipitation was used to "pick out" viral polypeptides from a large background of labeled material. Nonimmune separation of polypeptides was attempted using column chromatography.

3. Bifunctional crosslinking reagents have been used to study the molecular organization of flaviviruses. Otpimization of crosslinking conditions has lead to the detection of a number of crosslinked polypeptides. Suggested assignments of monomers constituting the crosslinked products is presented. Preliminary results of polypeptide composition analysis of uninfected and infected cell lysates and plasma membrane fractions are presented.

FOREWORD

In addition to the principal investigator the following personnel contributed to the studies reported here:

Barbara Phelps, Biochemistry
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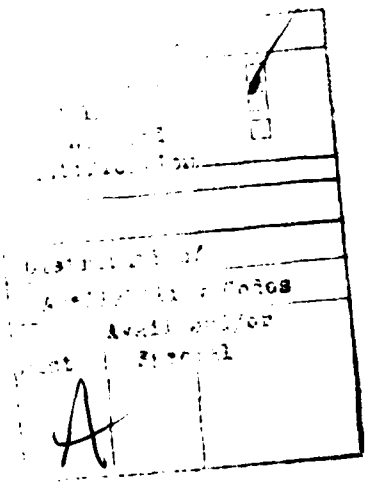
Nancy Wilkison, Tissue culture



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I. SUMMARY

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II. INTRODUCTION

Among arthropod-borne diseases dengue remains the most common known to affect man. The outbreak of dengue reported in the Caribbean a few years ago is still represented by large scale infections in the area (1, 2). Dengue has in recent years been progressing up the Polynesian island chain and epidemic dengue hemorraghic fever (DHF) has recently been reported as occurring in rural Indonesia (3). Although cases of dengue originating in the continental United States have not been reported since the 19th century, substantial numbers of persons returning from dengue endemic areas have developed the disease. Serodiagnoses have confirmed the illness to be due to dengue virus (DV).

Development of DHF with or without DSS has been proposed to be due to immunopathological mechanisms (4, 5), or alternatively, to the virulence of the virus (6). There is considerable evidence to indicate that severe forms of dengue illness arise when immunological sensitization to a given dengue serotype is followed by infection with heterotype DV, although primary infection by DV may, in some instances, lead to DHF or DSS development. The existence of antibody reactive with antigens common to all dengue serotypes, but which do not elicit neutralizing antibody, are believed to confer a state of hypersensitivity without protective effect.

Following virus infection, viral antigens appear on the surface of infected cells and in the extracellular fluid (7-15). The presence of these antigens together with antiviral antibodies and complement results in the fixation of complement (5). Complement fixation in turn can be related to the disease syndrome in at least two ways. Complement fixation leads to the appearance of C3a and C5a anaphyltoxins which can potentiate increased permeability of the vascular endothelium. Another way is by direct complement and/or cell-mediated immunolysis of the antigen-bearing cells. Although relatively little is known of the chemical and physical properties of dengue antigens, knowledge of those properties is essential if we are to understand the immunological mechanisms involved in dengue shock syndrome and in the abrogation of disease through vaccination.

The objective of our contract is the determination of the molecular and biological properties of the dengue antigens. We have forumulated two general hypotheses regarding the distribution of dengue antigens on the surface of the infected cells. The first hypothesis is that there exists two or more dengue antigens on the surface of infected cells. The second is that the proteins comprising these antigens are organized in a predictable and biologically important manner. The first hypothesis was recently shown to be correct. Cardiff and Lund (16) using specific antiserum to dengue antigens clearly demonstrated the cell surface possesses both viral structural and nonstructural antigens. Fractionation and analysis of flavivirus infected-cell membranes strongly suggests that a number of viral proteins are either integral or peripheral to the plasma membrane (17-20).

In this report we describe the progress made during the current contract year year on our study of flavivirus infection of mammalian cells in vitro. Japanese encephalitis virus (JEV) has been used as a model for subsequent studies on dengue virus (DV) since JEV replicates to higher titer and concomitantly has a shorter latent period. Detailed characterization of flavivirus-specified polypeptides which comrpise infectious and noninfectious particles released from cells is essential to our understanding of the immune mechanisms operable in dengue illnesses. It has become clear that antigens (polypeptides) on the surface of infected cells are also of importance in dengue disease (21), therefore knowledge of antigens associated with components released from cells as well as with the plasma membrane are equally important. While studies to control the disease by vaccination continue at the Walter Reed Army Institute of Research, we have continued our studies on the morphogenesis of viral proteins and antigens.

Section III of this report describes the molecular composition of infectious particles isolated from JEV and DV infected culture fluids. Two forms of infectious particles with different molecular compositions were detected. Polypeptides associated with the two forms are tightly bound to the particles and appear to be integral envelope proteins. A method developed for the isolation of envelope glycoproteins from purified virions is described.

Section IV describes the detection of viral polypeptides shed into the medium by infected cells. Radioimmune precipitation was used to "pick out" viral polypeptides from a large background of labeled material. Nonimmune separation of polypeptides was attempted using column chromatography. Sequential chromatography on G-75 Sephadex and concanavalin A-Sepharose was used to isolate putative soluble complement fixing antigen (SCF).

Section V reports on the nearest neighbor analysis of polypeptides comprising JEV and DV. Optimization of crosslinking conditions has lead to the detection of a number of crosslinked polypeptides. Suggested assignments of monomers constituting the crosslinked products is presented. Preliminary results of polypeptide composition analysis of uninfected and infected cell lysates and plasma membrane fractions are presented.

In summary, our studies have been directed toward a characterization of the diversity, both biophysically and biochemically, of infectious DV and JEV as well as viral specified proteins associated with these particles, infected cells or released as soluble components. Results of our studies and those of others indicate that the infected host immunologically reacts to a variety of antigens on the virion, infected cells and in soluble form. We have developed methodologies to isolate flavivirus polypeptides and are currently preparing antibodies to flavivirus components.

The following sections detail the results of our studies on DV and JEV carried out from August 1978 to August 1979.

III. BIOPHYSICAL AND BIOCHEMICAL DIVERSITY OF INFECTIOUS JAPANESE ENCEPHALITIS VIRUS AND DENGUE VIRUS

Biochemical and biophysical studies of flaviviruses generally rely on virus released from infected cells in culture or extracts of infected suckling mouse brain (7-15). In either case the virus is exhaustively purified by sequential ultracentrifugation usually involving rate zonal and/or isopycnic zonal sedimentation. Virus sedimenting as a single zone in such gradients is usually considered "purified" and ready for use in biophysical or biochemical studies. The necessity of obtaining a uniform particle preparation is of importance in conducting biochemical studies, however, from the overall biological point of view, the study of a single class of sedimenting particles may represent only one type of infectious agent present.

Preliminary results indicated that particles of differing molecular composition were released from flavivirus infected cells (1978 Annual Report, Section V, Figs. 4 and 5). In these studies we observed that infectious rapidly sedimenting hemagglutinin (RHA) of JEV and DV had as a polypeptide composition either V3, NV2, V2, and V1 or NV5, NV4, V3, V2, and V1. By modifying methods used for virus isolation we have identified two zones of infectious particles which have distinct sedimentation rates and different polypeptide compositions.

METHODS

Cell culture fluid containing JEV or DV were obtained from acutely infected BHK-21 or VERO cells at 26 or 60 hours postinfection, respectively. JEV or Type-2 DV was used throughout the studies. Viruses were grown in HEPES buffered Eagle's minimum. Essential medium containing 10% fetal calf serum (FCS), penicillin and streptomycin. For reasons described in the results section no inhibitors were used to eliminate host cell protein synthesis. In addition, during the 16 hours prior to harvest cell cultures were fluid changed to FCS-free, methionine-free medium with was supplemented with 10 μci/ml ^{35}S methionine.

Virus-containing cell culture fluid was cleared of cells and large debris by centrifugation at 2000 x g for 10 min prior to a high speed clearing run at 10,000 x g for 60 min. Supernatant fluid was then layered onto a 30% (w/w) cushion of sucrose and centrifuged at 110,000 x g for 120 min. The pellet was resuspended in Tris buffered saline, pH 7.4 and layered onto a 5-30% sucrose (w/w) continuous gradient formed over a 50% (w/w) sucrose cushion. After centrifugation at 196,000 x g for 90 min in an SW41 rotor the gradient was fractionated by bottom puncture and each fraction assayed for acid precipitable radioactivity. For some experiments, selected fractions were examined for infectivity by plaque assay on VERO cells.

Aliquots from fractions containing significant amounts of radioactivity were diluted with buffer and pelleted by centrifugation of 480,000 x g for 30 min. Alternatively, material was precipitated with cold 10% TCA and the TCA then removed by washing the pellet with acetone. Pelleted or precipitated material was resuspended in electrophoresis

sample buffer containing 1% SDS and 0.5% β mercaptoethanol and then subjected to electrophoresis in SDS-containing 10 or 12% polyacrylamide Laemmli-type gel. Following electrophoresis, usually at 12 ma per gel for 4 hours, gels were stained for protein using the method of Fairbanks (22), dried onto blotting paper and radioautographed. Hemagglutination was performed as described by Clark and Casals (23) as modified by Brandt (W. Brandt, personal communication).

RESULTS

Using the methods described above DV and JEV infected cell cultures yielded two prominent zones of radioactivity after rate zonal centrifugation on 5-30% sucrose gradients. This is seen in Fig. 1 where one band, designated IF, was collected at the 30%-50% sucrose interface. The second and usually more predominant band, designated RHA, was typically collected in fractions 8, 9 and 10. The designation RHA was made on the basis of sedimentation rate, relative to sindbis virus, and positive hemagglutination (fraction 18 and 19 were negative for hemagglutination). The IF zone, like the RHA, was hemagglutinating. Both IF and RHA regions were the only zones to show radioactivity when cell culture fluids from ^{3}H uridine labeled cultures were prepared in a manner exactly as described for ^{35}S methionine labeled preparations. Plaque assays of gradient fractions showed infectious particles to be confined to the IF and RHA zones. The ratios of RHA to IF infectivity was 4:1, however, the specific activity is about the same when consideration is given to the relative amounts of material in the two regions (based on cpm).

Results from a preliminary experiment suggested that incubation of virus infected cells in the presence of actinomycin D followed by a short pules of cyclohexamide during the prelabeling period and then labeling in the presence of actinomycin D reduced the yield of virus by several fold. A repeat of that experiment confirmed this initial finding. As seen in Fig. 2 cell culture fluid for cultures in which inhibitors were not used produced the same amount of virus as twice the volume of inhibitor treated cultures. Polyacrylamide gel electrophoresis of IF and RHA regions yielded similar band patterns for respective regions (Fig. 3). The two IF regions (lanes 1 and 3) show polypeptides NV5, NV4, V3, V2 and V1, while the RHA region (lanes 2 and 4) lack NV5 and NV4 but contain NV2. The absence or dramatically reduced amount of V1 in RHA relative to IF particles is a common observation. The similarity of the bands for the respective gradient regions, as shown in Fig. 3, demonstrated that the presence or absence of drug does not alter the polypeptide composition of IF and RHA particles. In the absence of drug, however, the yield of labeled particles is at least 2-fold greater than when drugs are used therefore drug treatment was eliminated. Occasions where drugs were again used in our experiments will be specifically noted.

As was suggested in last year's report (Section V, Figs. 7 and 8) NV2 may actually be comprised of two polypeptides which are not normally resolved and appear as a single band. That NV2 may be two polypeptides is also suggested in Fig. 2, lane 4. Resolution of two polypeptides in the NV2 region was obtained by thin layer slab gel electrophoresis in a 12% polyacrylamide gel matrix (Fig. 4). As seen the particles

sedimenting to the IF region lack NV2 and in this experiment lack NV5 and NV4 as well (an atypical observation). The NV2 region of the RHA particles show two distinct polypeptides which we have designated NV2 and NV2-1/2.

Since NV2 and NV2-1/2 could be adventitious contaminants adhering to the virus experiments were performed to determine if either NV2 or NV1/2 could be removed by salt treatment or sonication. RHA was pelleted and resuspended in 0.15 M, 0.5 M, or 1 M NaCl and incubated at 25° for 30 min prior to repelleting and polyacrylamide gel electrophoresis. Results of this study (Fig. 5) show that either 0.5 M nor 1 M NaCl treatments lead to the preferential release of NV2 or NV2-1/2, although exposure to 1 M NaCl resulted in significant degradation as judged by the poor recovery. Similarly, sonication was without effect (Fig. 6). Sonication for a total period of one minute did not alter the polypeptide composition of the RHA. In addition, the exposure of RHA to reducing agent did not result in the removal of NV2 or NV2-1/2.

During our studies the question arose as to whether or not infectious particles banding at the interface region were not simply aggregates of RHA. The consistent observation that the polypeptide composition IF and RHA particles were distinct argued against the aggregation hypothesis. In addition, similar attempts to those described above to dislodge peripherally attached NV5 and NV4 from IF particles was without success. Sonication of an admixture of RHA and IF particles for a total of one minute did not lead to a change in the rate zonal density gradient profile (Fig. 7A). Pelleting and resuspension of RHA particles does not lead to the formation of material sedimenting to the IF region. Furthermore, storage of RHA-IF admixture at 4° for 10 days does not result in the spontaneous aggregation of RHA (Fig. 7B).

The concentration of virus is important in our analysis. To determine the relative effectiveness of different methods, equal aliquots of IF particles were precipitated with ammonium sulfate, 10% TCA, acetone, ethanol, and high speed pelleting and the recovered pellets prepared for and analyzed by polyacrylamide gel electrophoresis. Results of the ammonium sulfate, TCA and high speed pelleting procedures are shown in Fig. 8, lanes 1, 2, and 3, respectively. Ammonium sulfate precipitation was the least efficient. The loss of virus was probably the result of dialysis required to remove the salt prior to electrophoretic analysis. Pelleting and TCA precipitation were equally effective as were acetone and ethanol precipitation.

A comparison between JEV and DV RHA has been included to show the substantial differences in molecular weight between the polypeptides of the two viruses (Fig. 8, lanes 4 and 5). Both JEV and DV RHA samples were disrupted in SDS sample buffer containing reducing agent. The estimated molecular weights of the polypeptides are as follows: for JEV, V3 53,000, NV2 22-25,000, V2 13,000, and V1 10,000; for DV, V3 58,000, NV2 18,000, V2 15,000, V1 9,000. Molecular weight standards used as in all our studies are phosphorylase A (94,000), BSA (68,000), ovalbumin (44,000), carbonic anhydrase (29,000), soybean trypsin inhibitor (14,400).

Disruption of either JEV or DV RHA with reducing agent-free sample buffer results in a slightly higher electrophoretic mobility for NV2. Electrophoresis of DV RHA polypeptides run under non-reducing conditions is shown in Fig. 8, lane 6. Since electrophoretic mobility in SDS gels is predominantly related to the configurational size of the macromolecule in question, these results indicate that only NV2 has intramolecular disulfide bonds which significantly affect the configuration of the molecule.

All of the above experiments were carried out using JEV or DV RHA obtained from Vero cell cultures. When DV RHA from BHK cell cultures (Fig. 8, lane 7) was compared with DV RHA from Vero cultures (Fig. 8, lane 5) under identical electrophoresis conditions, RHA polypeptides were essentially of identical molecular weight. However, RHA preparations obtained from BHK cell cultures contained less NV2 and V2 when normalized to the amount of V3 present. In addition, both V3 and NV2 from BHK cell RHA show more electrophoretic heterogeneity than RHA from Vero.

The slowly sedimenting hemagglutinin (SHA) detected in flavivirus infections in very low amounts has been tentatively identified in fractions 14 and 15 of rate zonal gradients (Fig. 7, B). The fraction shows hemagglutinating activity and contains polypeptides V3, NV2 and V1 but no V2 (Fig. 8, lane 8).

One major objective of our research is the production of specific antisera. Rabbits have been inoculated with twice banded RHA. Inoculation has been made with RHA in adjuvant (50 µg RHA protein per inoculation) directly into the area immediately adjacent to the popliteal lymph node. It has been possible by careful examination to palpate and thereby locate the unstimulated node prior to inoculation. The first booster inoculation has been given and rabbits will be given a second booster inoculation.

To prepare antisera against the surface glycoproteins V3 and NV2, twice purified RHA was disrupted with 1% TX-100 and chromatographed on a Sephacryl S200 column. The radioactivity eluted as a single broad peak. Polyacrylamide gel electrophoresis of sequential fractions across the peak demonstrated that all fractions contained V3 and NV2 although V3 was in considerably higher concentration (Fig. 9). Proteins were ethanol precipitated from the fractions, resuspended in balanced salt solution and inoculated (with adjuvant) into rabbits using the route described above. Ten days following initial inoculation all rabbits showed enlargement of popliteal nodes and were reinoculated. Following the 2nd booster inoculation test bleeds will be performed and sera from these rabbits as well as those inoculated with RHA will be examined for precipitating antibody to RHA and uninfected cell components using the Staphylococcus aureus immune precipitation procedure (30, 31), counter current immunoelectrophoresis (39) as well as plaque reduction neutralization.

DISCUSSION

The results presented above show that both infectious JEV and DV particles are released from infected cells in culture in two biophysically and biochemically distinct forms. One form which appears to spontaneously aggregate and sediments to the region of the interface during rate zonal purification runs. These particles incorporate uridine, agglutinate goose red blood cells and are infectious. Polyacrylamide gel electrophoresis of polypeptides from these particles show the presence of the two viral specified nonstructural polypeptides, NV5 and NV4, in addition to those previously reported to comprise JEV or DV (7, 10-12, 24). NV5 and NV4 have been detected in cytoplasmic and plasma membrane fractions isolated from JEV and DV infected cells (9, 17, 19). The results of salt wash, sonication, and reducing agent experiments presented here together with the trypsin sensitivity of JEV polypeptides in cell membrane fractions described by Shapiro et al. (17), strongly suggest that NV5 and NV4 can exist as integral or tightly bound peripheral membrane proteins. Since NV5 and NV4 are glycoslyated (17) and since viral envelope, NV5 and NV4 are most likely located on the outer surface of the IF virion.

Similar reasoning suggests that NV2, a glycoprotein (17, 24), is located on the outer surface of the RHA particle. NV2 has been detected in purified RHA preparations of JEV (10) and Kunjin virus (20, 40), however the localization and significance of the polypeptide has not been reported. The second polypeptide detected as migrating slightly more slowly than NV2 has been tentatively designated as "NV2-1/2". If the polypeptide is analogous to NV2-1/2 of flavivirus Kunjin virus, which is not a glycoprotein (40), then "NV2-1/2" may be an "internal" polypeptide. Further studies including iodination with lactoperoxidase or proteolytic digestion will be necessary to firmly establish the location of NV5 and NV4 on IF virions and NV2 and "NV2-1/2" on RHA virions.

The biological significance of these so-called nonstructural proteins in host-virus interaction could be considerable. Antibody capable of neutralizing the viron has generally been considered to react with glycoprotein V3. In addition to type specific reactivity such as shown by neutralization, V3 is known to possess both complex-reactive and group reactive determinants (41). Although the antigenic properties of NV4 and NV2 are unknown, NV5 has only type specific antigenic reactivity (25). One might expect antibody directed against NV5, being type specific, to neutralize infectious virus. Although NV5 is immunogenic in persons infected with JEV or St. Louis encephalitis (SLE) virus in that antibody reactive to NV5 has been detected in sera of patients (25 and K. Eckels, personal communication), failed to absorb anti-SLE neutralizing antibodies (25).

The presence of more than a single type of infectious particle and an increase in polypeptide diversity associated with these particles complicates our earlier conception of JEV and DV structure and immunology. Elimination of virus by the infected host most probably involves humoral antibody-virus interaction (3, 26), however, the view that only a single type specific antigenic site is involved may likely be incorrect. The importance of antigens on NV5, NV4 and NV2 (maybe NV2-1/2 depending on its location) as well as V3 will have to be evaluated.

One approach we are taking toward evaluating the role of these polypeptides in the neutralization of virus is to prepare specific antisera. As described in our results we currently are generating antisera to RHA and the V3-NV2 fractions from TX-100 disrupted-RHA chromatographed on Sephacryl S200. We are now experimenting with other detergents in order to develop a method which will allow resolution of the individual polypeptides. We are very interested in the IF particle and in obtaining antisera to the envelope polypeptides which comprise it.

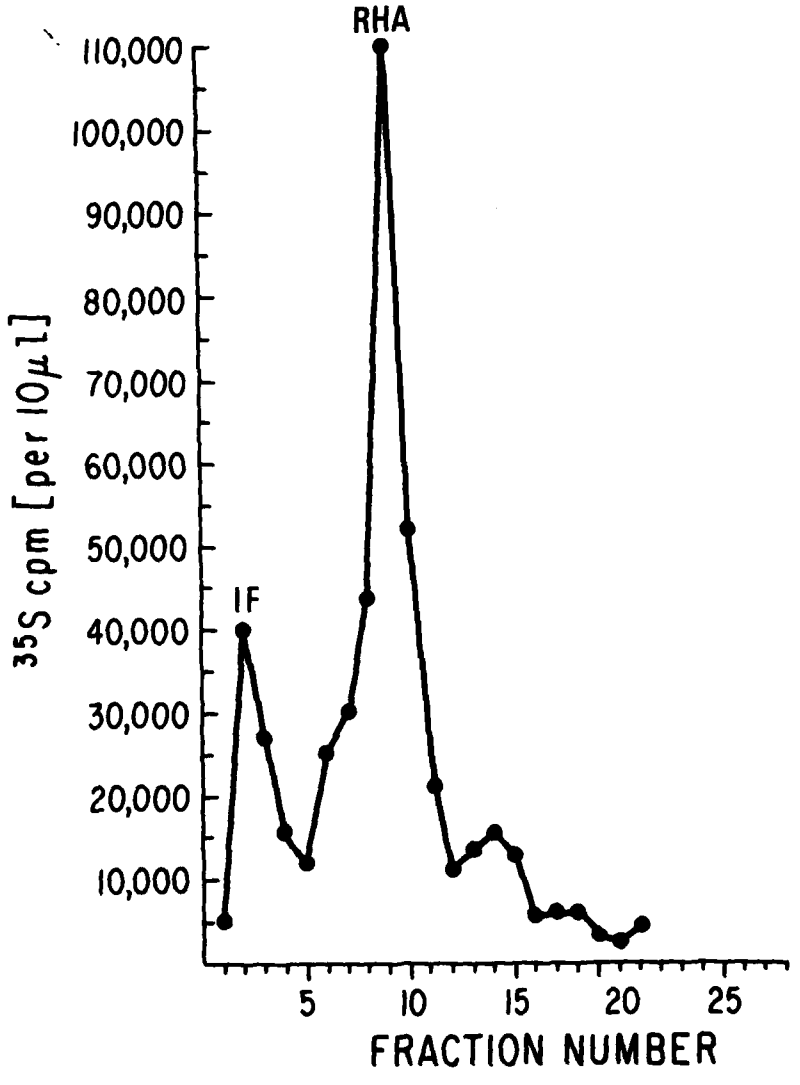

Fig. 1 . Rate zonal centrifugation of JEV in a 5-30% sucrose density gradient containing a 50% sucrose cushion. Centrifugation was for 90 min in a SW41 rotor at 34,000 RPM. Virus-containing tissue culture fluid was cleared at 2000xg for 10 min and then at 10,000xg for 60 min before rate zonal centrifugation.

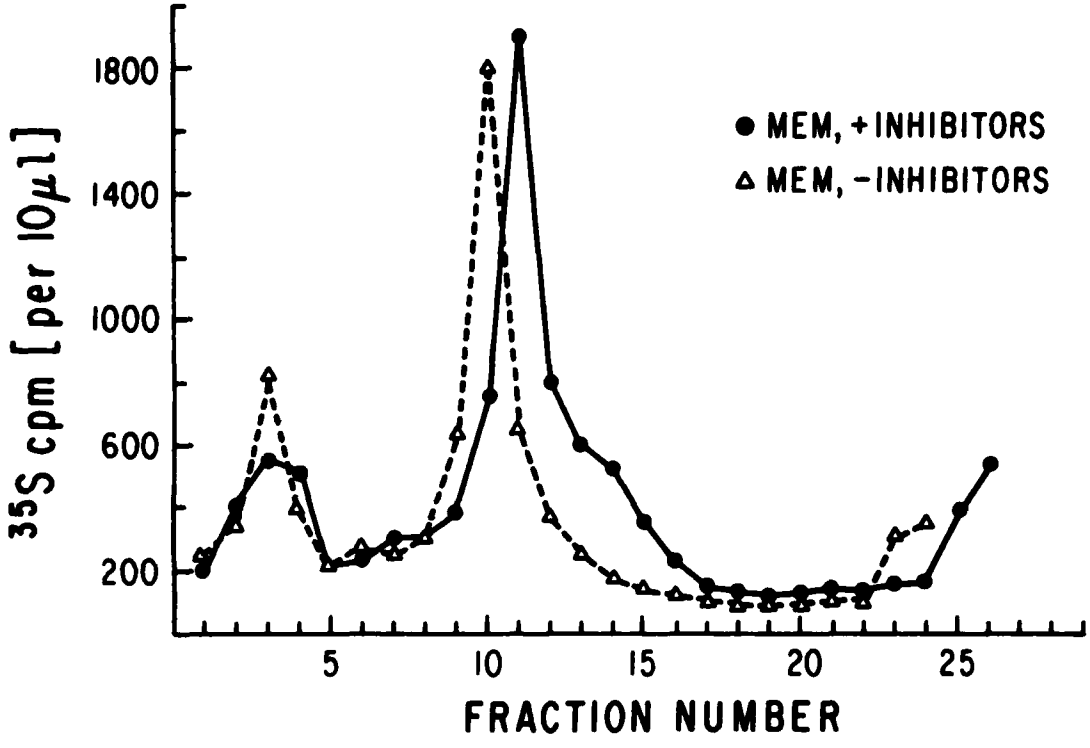

Fig. 2. Rate Zonal centifugation of JEV grown in Vero cells. Cells were grown in MEM with or without exposure to Actinomycin D and cyclohexamide (see text for conditions). The virus yield from 8 75cm^2 T flasks in which cells were treated with inhibitors was compared with the yield from 4 75cm^2 T flasks in which no inhibitors were used.

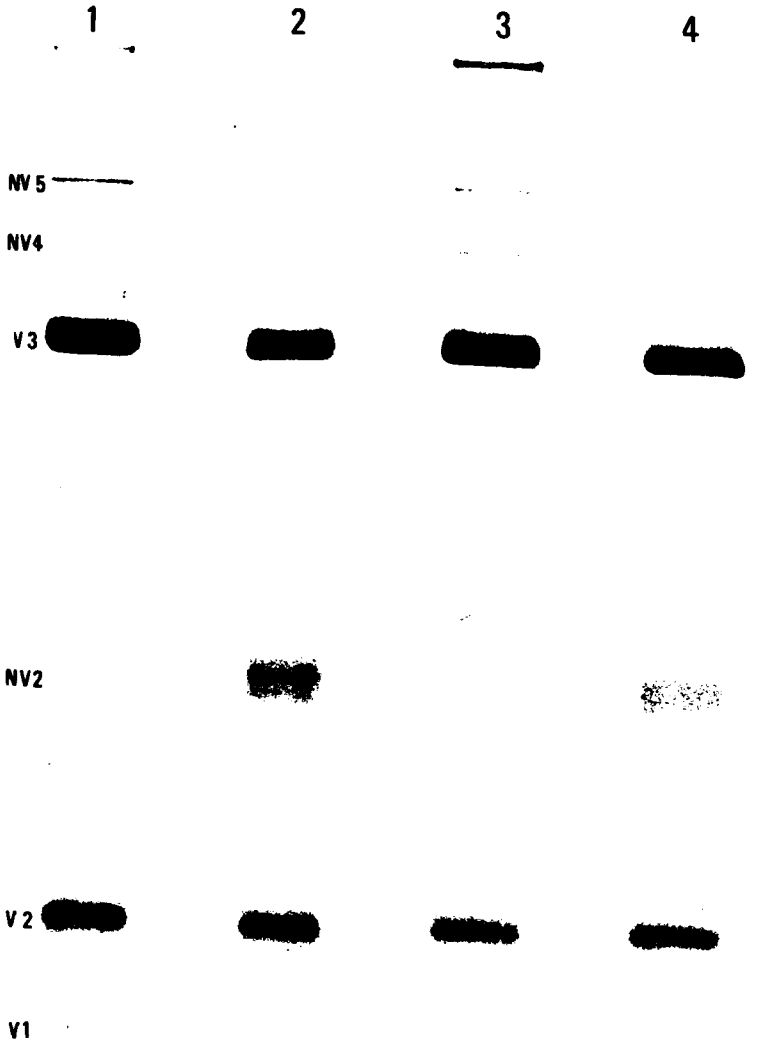

Fig. 3 . Comparison of JEV polypeptides from virus obtained from inhibitor treated and untreated Vero cell cultures. Rate zonal gradient interface region from drug treated (lane 1), RHA from drug treated (lane 2), interface from untreated (lane 3), RHA from untreated (lane 4) cultures.

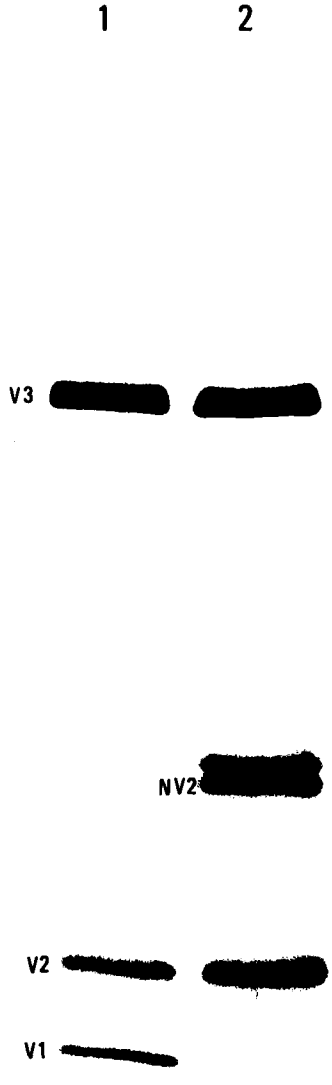

Fig.4 . NV2 region contains two polypeptides. JEV from interface, and RHA regions of rate zonal gradient analyzed by polyacrylamide gel electrophoresis; interface region (lane 1), RHA region (lane 2).

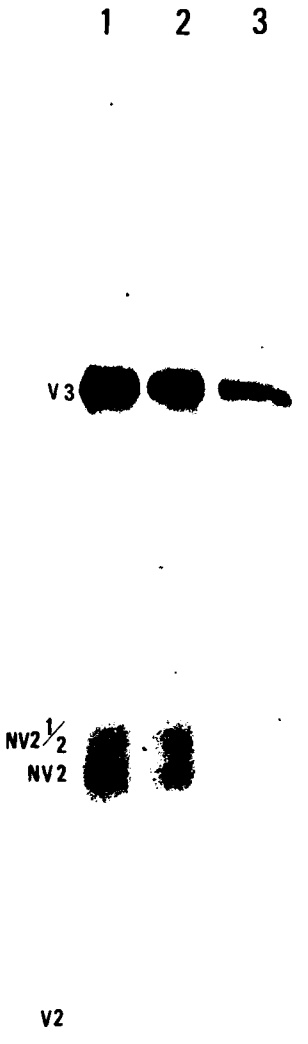

Fig. 5 . Salt wash of JEV RHA. Purified JEV RHA was pelleted and resuspended in buffer containing 0.15 M NaCl (lane 1), 0.5 M NaCl (lane 2), or 1 M NaCl (lane 3). Samples were incubated at 25° for 30 min, concentrated by pelleting and analyzed by polyacrylamide gel electrophoresis.

Fig. 6. Electrophoresis of JEV polypeptides on 12% polyacrylamide gel. Virus was either sonicated or not before rate zonal purification. Lane 1 virus was not sonicated. Lane 2 virus was sonicated twice for 30'.

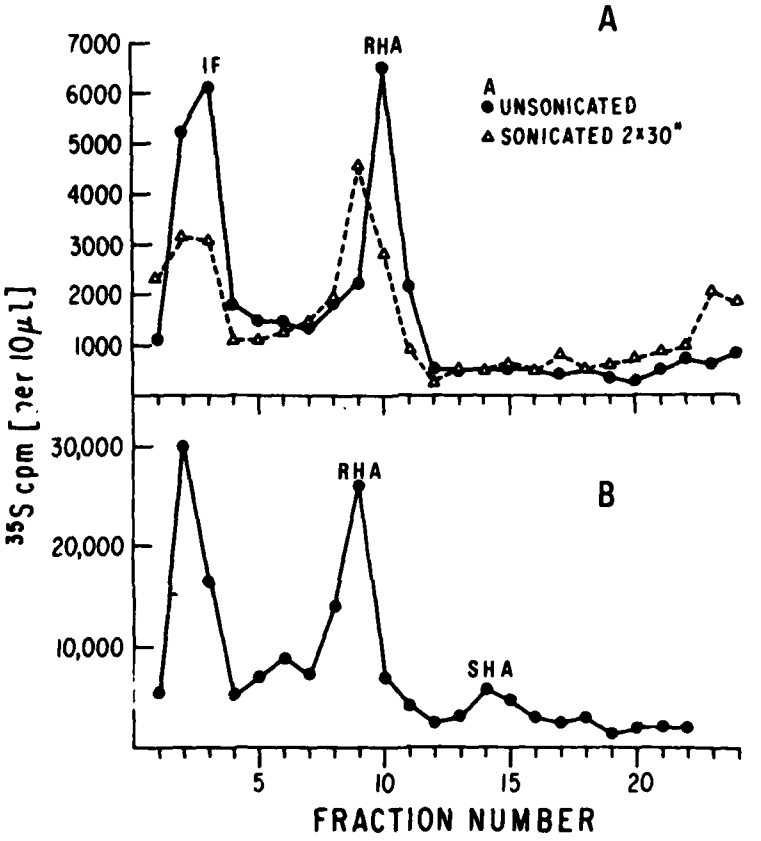

Fig. 7 . Rate zonal centrifugation of JEV in 5-30% sucrose gradient containing a 50% sucrose cushion. Fractions were collected by drop puncture and acid precipitable radioactivity of individual fraction determined. A comparison is shown of gradient profiles before and after sonication (A) and the unsonicated sample stored at 4° for 10 days before centrifugation (B).

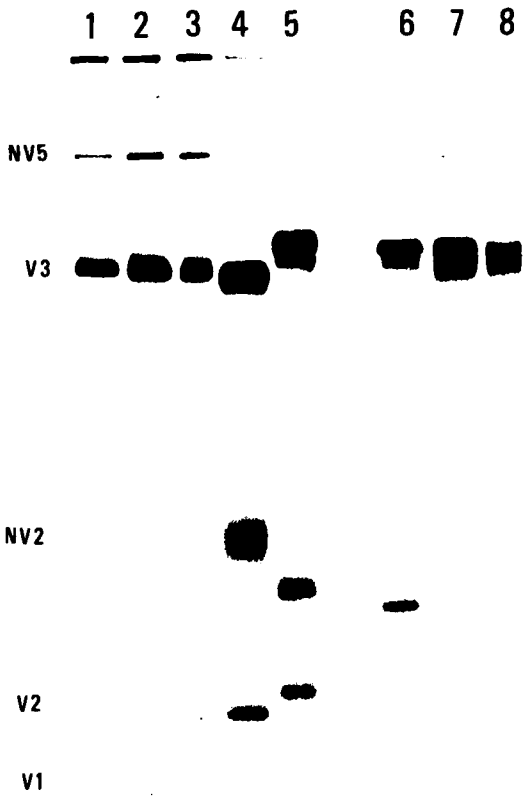

Fig. 8 . Electrophoresis of JEV and DV polypeptides. JEV grown in Vero cells and sedimenting onto the cushion during purification, (interface region), was concentrated prior to electrophoresis by precipitation with ammonium sulfate (lane 1), pelleting at 485,000xg for 30 min (lane 2), TCA precipitation (lane 3).

Polypeptides of purified JEV and DV RHA obtained from Vero cell culture were compared; JEV RHA (lane 4) and DV RHA (lane 5). Virus run in lanes 1 through 5 was disrupted with sample buffer containing reducing agent. DV RHA disrupted with sample buffer free of reducing agent was run in lane 6.

Both RHA and SHA regions from rate zonal gradients of DV which was grown in BHk cells was disrupted in sample buffer containing reducing agent; RHA (lane 7), SHA (lane 8).

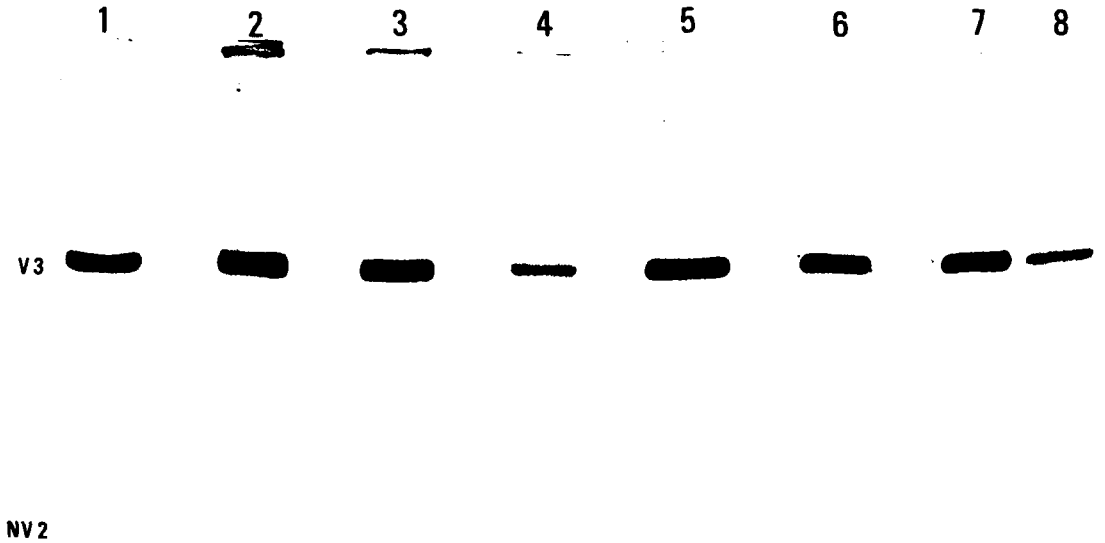

Fig. 9.. Fractionation of JEV RHA polypeptides on a Sephacryl 200 column. JEV was disrupted in TX-100 buffer. Lanes 1 through 8 represent material eluting in sequential fractions in a single broad peak.

IV. SOLUBLE FORMS OF STRUCTURAL AND NONSTRUCTURAL COMPONENTS RELEASED FROM CELLS IN CULTURE

Recently several reports have described the release in soluble form of viral polypeptides from cells in culture (27-29). Cells infected with murine leukemia virus release appreciable amounts of soluble viral envelope glycoprotein and small quantities of nonglycosylated structural proteins (27). Similarly, cells infected with vesicular stomatitis virus (VSV) release appreciable amounts of viral specific proteins (28-29). Results of these latter studies lead to the hypothesis that the major viral glycoprotein exists in three states in the plasma membrane: 1) an unstable association which can lead to passive release of the protein, 2) a stable association brought about by a transmembrane interaction with a cytoplasmic viral matrix protein, and 3) further stabilization through a coalescence of glycoprotein-matrix protein complexes. Promotion of high levels of glycoprotein shedding occurs when a ts mutant of VSV defective in synthesis of stable matrix protein is grown at the nonpermissive temperature.

Cells infected with flavivirus are reported to release a soluble complement fixing antigen (SCF) of molecular weight 40-53,000 (10, 42, 43). The relationship between SCF and the other viral specific polypeptides detected in infected cells and mature virions is unknown however NV3 of JEV is thought to be SCF. In this section we describe the detection of JEV and DV polypeptides which appear as soluble components in the extracellular fluid of virus infected cell cultures. The potential significance of these released or exfoliated components to the development or abrogation of disease is discussed.

METHODS

Tissue culture fluid from ^{35}S methionine labeled virus infected cells was harvested during log phase growth of the virus and prior to cytopathic effects as judged by phase microscopy and sluffing of cells from the substrate. Culture fluid were processed as described in Section III. Briefly, fluid was cleared of any large debris by sequential differential centrifugation. Cleared supernatant fluid was layered over a 30% sucrose cushion and centrifuged to pellet infectious virus. The supernatant fluid was carefully aspirated to avoid collection of the sucrose cushion and centrifuged at 110,000 x g for 16 hours. Supernatant fluid (S16) was again carefully removed with care taken to avoid collecting pelleted material.

Components present in the S16 was precipitated with 40% ammonium sulfate and pelleted at 12,000 x g for 1 hr. The pellet was resuspended in phosphate buffered saline (PBS), pH 7.2 and dialyzed for 15 hr at 4° against PBS and then cleared at 2000 x g for 10 min before applying to a PBS equilibrated Sephadex G-75 column (60 x 0.9 cm). The column was eluted with PBS and 1 ml fractions were collected. Aliquots from each fraction were assayed for acid precipitatble radioactivity.

SDS-polyacrylamide gel electrophoresis was used to analyze column fractions. Aliquots of selected fractions were diluted 10 fold with cold 10% TCA and precipitates washed with acetone prior to disruption with sample buffer. Electrophoresis in 10% or 12% Lammeli-type gel systems was performed the resulting polypeptide distribution and analyzed as described in Section III.

Concannavalin A-Sepharose affinity chromatography was used to isolate glycoprotein(s) in fractions determined to be of interest as determined from result of gel electrophoresis data. Aliquots of G-75 column fractions were adjusted to 0.5% TX100, 0.1% sodium deoxycholate (DOC) 1 mM $CaCl_2$, 0.1 mM $MgCl_2$ in PBS (pH 7.2). The sample was loaded onto the column and washed with PBS containing detergents and salts. Fractions were collected and assayed for radioactivity. After thorough washing and when the radioactivity in the fractions had dropped to background levels, material adhering to these columns was eluted with 0.2 mM α-methyl mannoside (α-mm). Fractions containing α-mm eluted material were TCA precipitated and the precipitates examined by SDS-gel electrophoresis.

Immune precipitates of S16 and RHA using anti-JEV antiserum were analyzed for viral polypeptide content by polyacrylamide gel electrophoresis. Alquots of JEV, S16 and RHA were adjusted to the detergent and salt buffer concentrations described for the ConA-Sepharose fractionation above. Anti-JEV hyperimmune mouse ascites fluid was added and the aliquots incubated at 31° for 90 min. Staphylococcus aureus (Staph A) prepared by the method of Kessler (30, 31) was added. After incubation at 31° for 30' the Staph A was pelleted and washed with buffer twice. The final pellets were resuspended in electrophoresis sample buffer to dissociate the Staph A and disrupt the antibody-antigen interaction. The Staph A was then pelleted and supernatants applied to a 10% SDS-polyacrylamide gel. Electrophoresis and visualization of polypeptides were as described in Section III.

RESULTS

Prior to attempting to detect JEV and DV polypeptides released from infected cells, experiments were performed to determine if our methods would lead to the detection of VSV proteins shed from cells as reported by Little and Huang (28, 29). VSV, New Jersey strain, was used to infect BHK cells. The cells were then labelled with ^{35}S methionine under conditions similar to those used for JEV and DV. Cell culture fluids were harvested and prepared for chromatography on a Sephadex G-75 column. Radioactive material in VSV S16 concentrates eluted as a single major peak immediately behind blue dextran. dextran (data not shown). Polyacrylamide gel electrophoresis of TCA precipitates of fractions comprising the peak revealed that all VSV proteins were present in S16 (Fig. 10). As mentioned in the methods section, care was taken to avoid collecting fluid from cells showing cytopathic effect. The presence of intact virus in the S16 is highly unlikely. Centrifugation used for the preparation of S16, 16 hr at 110,000 x g, has been calculated to clear from solution all particles of sedimentation coefficient greater than 25.

Based upon the VSV results which showed that the methods to be used for JEV and DV were feasible, S16 concentrate from JEV infected cultures was chromatographed on the G-75 Sephadex column. As seen in Fig. 11, radioactive components were detected in one major zone which eluted behind the blue dextran marker. All the radioactivity eluted well ahead of the phenol red indicator. A small peak (fraction 8) superimposed on the trailing edge of the major peak was consistently observed in both JEV and DV S16 preparations.

Several protein standards were run on the G-75 column as a means of calibration. The elution of those standards is shown in Fig. 12. As expected for G-75 Sephadex, BSA (68,000 mw) elutes several fractions behind the blue dextran which marks the excluded volume fractions. In addition to carbonic anhydrase (29,000 mw) and cytochrome C (11,700 mw), horseradish peroxidase (40,000 mw) has also been chromatographed and elutes intermediate to BSA and carbonic anhydrase. Fraction 14 in Fig. 12 corresponds to Fraction 1 on Fig. 11.

Fractions containing radioactivity (Fig. 11) were precipitated with TCA and precipitable material examined by polyacrylamide gel electrophoresis. An autoradiograph of the resulting gel is shown in Fig. 13. The polypeptide pattern reflects the elution as expected on the basis of the profile of standard proteins (Fig. 12). We have tentatively identified NV4, V3, NV3, NVX, NV2, V2 and V1. NV4 (72,500 mw) ran as a relatively tight band (lane 3) as would be expected of a slightly glycosylated protein. The heavily glycosylated V3 (53,000 mw) appeared as a broad band predominately in fractions 5 and 6. NV3 (51,000 mw) overlapped V3 and eluted in fractions 5-7. The multiple of bands in the V3-NV3 region may reflect microheterogeneity in either or both of these two polypeptides. NVX (32,000 mw) detected in fractions 2-6, if existing in the S16 as a monomer, should have eluted in fractions 9 and 10. The elution of NVX at an anomonously high molecular weight indicated that the polypeptide was complexed as a homo- or heterodimer. NV2 (22,000-25,000 mw) migrated as a very diffuse band in fractions 7-9 and as a much tighter band in fractions 10 and 11. Fraction 11 also contains V2 (14,000 mw). V1 (10,500 mw) eluted in fractions 13 and 14).

When S16 preparations of DV cell culture fluids were analyzed by G-75 column chromatography and gel electrophoresis patterns similar to that of JEV were observed. Typical polypeptide profiles for DV S16 column fractions are seen in Fig. 14. As with JEV, DV NV4 was detected in fractions 2-4 and V3 appears in fractions 4-6. NV3 eluted in fractions 5-7; again a similar pattern to JEV NV3. The apparent molecular weight of DV V3 (58,000) and DV NV3 (53,000) are somewhat than the molecular weights for the corresponding JEV polypeptides. The only other DV polypeptide detected in the S16 preparation was NVX which like the JEV preparation appears in fractions 4-6. However, unlike the JEV polypeptide, NVX of DV had a considerably lower estimated molecular weight (25,000).

The molecular weight of the JEV polypeptide tentatively designated as NV3 is the same as that reported for JEV SCF (10). Since NV3 is glycosylated an aliquot of a fraction containing the putative NV3 was loaded onto a ConA-Sepharose column and material adhering to the ConA eluted with α-mm. The G-75 column fraction used had a polypeptide pattern similar to fraction 6 in Fig. 13. Fig. 15 shows the radioactivity profile of the ConA-Sepharose chromatography. A considerable amount of radioactivity material did not adhere to the column and appeared in the wash through fractions 2-6. Continued washing did not release additional material. When buffer containing 0.2 mM α-mm was added, components eluted in a broad peak (fractions 15-26) and continued addition of 0.2 mM or of 0.5 mM α-mm did not result in the release more labeled material.

Fractions containing non-adhering material and α-mm eluted material were separately pooled, TCA precipitated and the precipitates analyzed by gel electrophoresis. Fig. 16 illustrates the polypeptides contained in the wash through (lane 1) and α-mm eluate (lane 2). JEV RHA was run in lane 3. The eluted material contained a major polypeptide band, the diffuseness of which suggests a high degree of glycosylation. In addition to the major band two more discrete bands (a and b) migrated slightly more rapidly, however, these may be cell specified polypeptides. Experiments in progress in which inhibitors of cell RNA synthesis were included will indicate the viral or cell nature of these components. All three bands migrated more rapidly than V3 of RHA (lane 3).

Since S16 preparations contain a variety of viral specified proteins as shown by electrophoretic analysis of G-75 Sephadex fractions, an attempt was made to determine which if any of the soluble viral components were reactive with anti-JEV antiserum. When an aliquot of JEV S16 was immunoprecipitated with the antiserum as described in the methods section and the precipitate run on a SDS-polyacrylamide gel four distinct bands were detected (Fig. 17). From the positions of molecular weight standards the four bands were identified as NV5, NV4, V3 and NV3 (Fig. 17, lane 1). Barely perceptible were bands of NV2 and V2. An aliquot of S16 containing the same amount of radioactivity as used for the immunoprecipitation was TCA precipitated and also subjected to electrophoresis (lane 2). As seen the gel was badly overloaded and only is useful in demonstrating that the high degree of specificity of the immunoprecipitation procedure.

The failure of the antisera to precipitate viral polypeptides smaller than NV3 could have been due to the absence of antibody capable of reacting with the lower molecular weight species. This possibility was anticipated (P. K. Russell, personal communication) and JEV RHA was disrupted with the immunoprecipitate buffer and then immunoprecipitated. Gel electrophoresis of the precipitate revealed that V3 was readily precipitated but that only a very small amount of NV2 was precipitated (lane 3). Other structural polypeptides were not precipitated. TCA precipitated JEV RHA (run in lane 4) served as control and demonstrated the presence and location of the RHA structural polypeptides.

DISCUSSION

These results demonstrate that JEV and DV infected cells release both structural and nonstructural polypeptides in soluble form into cell culture medium. Thus, the flaviviruses appear to be similar in this regard to the murine leukemia virus (MuLV) (27) and VSV (28, 29). Also like MuLV and VSV, both JEV and DV infected cells release major amounts of envelope glycoprotein. The detection of V3 as the major component released from JEV and DV infected cells in consistent with the presence of the polypeptide on the plasma membrane (17-19), although plasma membranes of JEV infected cells contain V3 as a major viral component (17, 18) whereas NV3 is the major component in plasma membrane from DV infected cells (19). However, some caution must be taken in making such a comparison since different cell types and conditions were used in the membrane studies.

VSV glycoprotein stability in plasma membranes is related to the transmembrane association between the glycoprotein and cytoplasmic matrix protein (27, 28). The presence of defective matrix protein which is unable to bind to the glycoprotein results in the release of the glycoprotein from the cell membrane. The appearance of flavivirus glycoprotein and other viral proteins in the extracellular fluid may be related to virus morphogenesis in a similar way. That is, flaviviruses, unlike MuLV and VSV, do not mature by budding at the plasma membrane but rather assemble in the lumen of the endoplasmic reticulum (20, 32), Nevertheless, in flaviviruses infection both cytoplasmic and plasma membranes "incorporate" all viral polypeptides in a qualitatively nondiscriminatory manner (17-19, 20). Since the morphogenesis of flaviviruses is associated with the cytoplasmic membranes, it is possible that the type(s) of protein-protein interaction necessary to stabilize viral polypeptides in the plasma membrane is (are) absent. The lack of a stabilizing interaction could lead to the spontaneous release of polypeptides analogous to that demonstrated for VSV (29).

The presence of JEV and DV polypeptides on the surface of and released from infected cells has significant immunological implications. Humoral antibody complement-mediated immunolysis of DV infected cells has been demonstrated (33). Direct cytotoxic T-cell killing of cells possessing viral antigens could, depending on conditions, bring about onset or abrogation of serious disease. Early and rapid elimination of infected cells by immunolysis or T-cell killing could block or abate illness. Alternatively, if the infection involved capillary entothelial cells then destruction of infected cells could lead to more serious hemorraghic manifestations.

The detection of both structural and nonstructural polypeptides as soluble antigenic forms during JEV and DV infection could also be of considerable importance. We have shown that the soluble polypeptides are antigenic (Fig. 17). Therefore, if as in culture, there is release of viral antigens into the blood of infected hosts these antigens could 1) aid in the stimulation of the immune response, 2) serve to adsorb and render ineffective neutralizing and potentially complement fixing antibodies, and 3) in DV infection serve as a source of soluble complement fixing antigen. As mentioned in the introduction to this report, dengue hemorraghic fever is most probably an immunopathologic disease resulting from heterotypic DV infection (1-4). Until our findings, as reported here, it was thought only the only soluble DV antigen to be released in significant amounts was SCF. The reason for this is principally that the methods used previously for the isolation of SCF simply precluded their detection. Further studies are necessary to establish the significance of our observations. It would be most interesting to know if sera from host infected with flavivirus possess antibody to these soluble polypeptides and also if the soluble components can block neutralizing antibody activity.

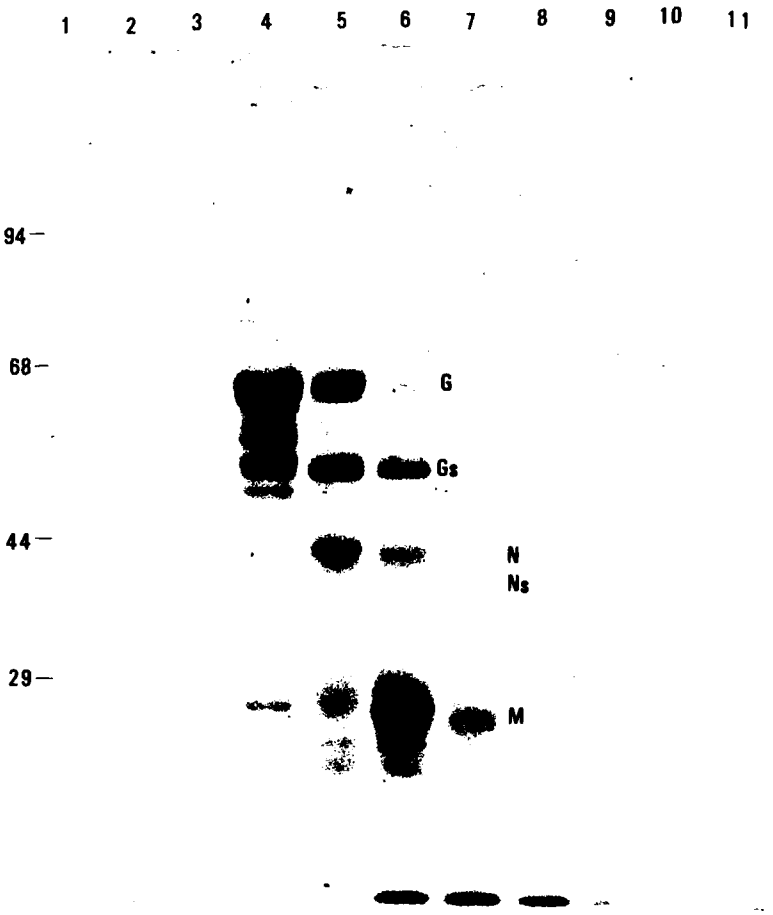

Fig. 10 . Gel filtration of virus-free S16 concentrate from VSV infected cultures as analyzed by polycarylamide gel electrophoresis. Sequential column fractions are shown left to right. Tentative identification of the VSV proteins are designated by G, Gs, N, Ns, and M (see text). Molecular weight standards are shown on left.

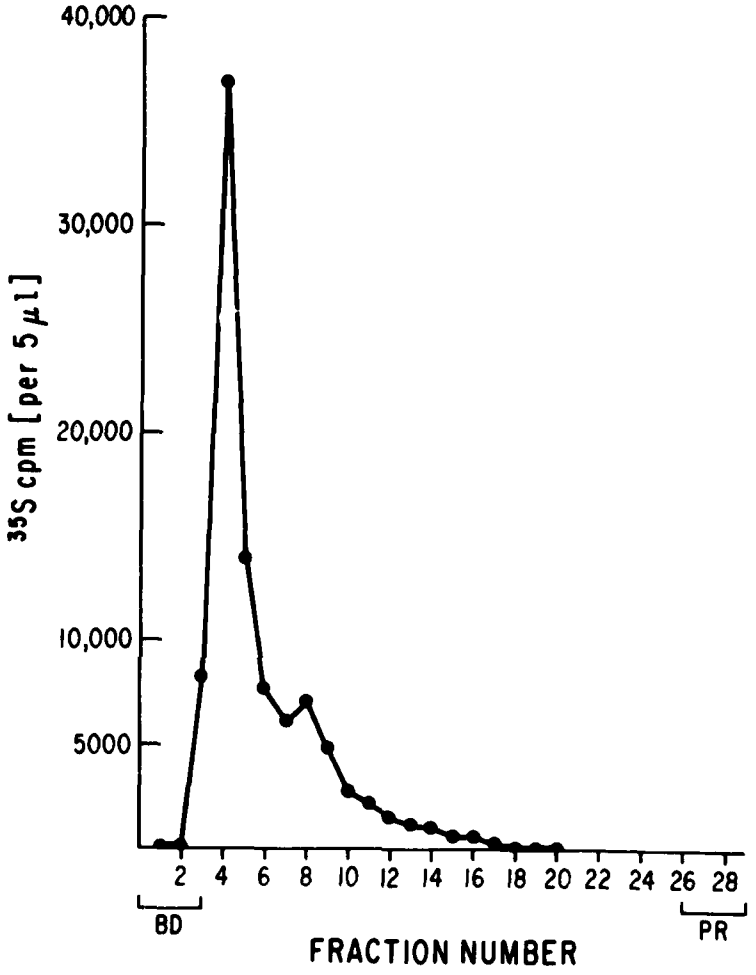

Fig. 11 . Sephadex G-75 column gel filtration of JEV virus-free S16 tissue culture fluid concentrate. Chromatography was performed with PBS, pH 7.4. BD and PR denote blue dextran and phenol red. Virus was grown in the absence of serum during the labeling period.

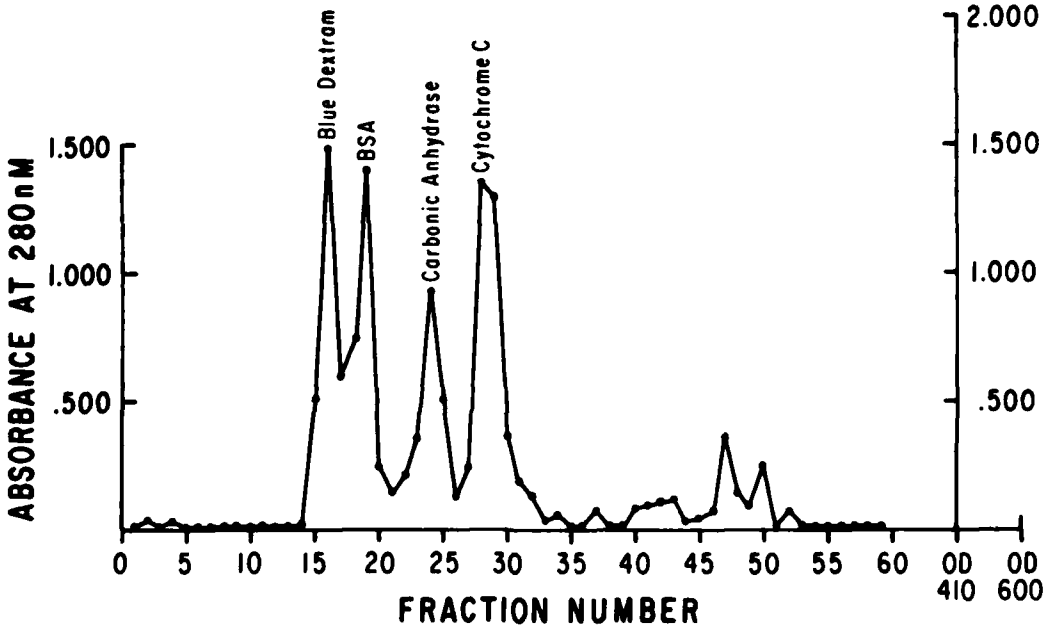

Fig. 12 . Sephadex G-75 gel filtration of protein standards. Conditions used were those also used for flavivirus protein fractionation.

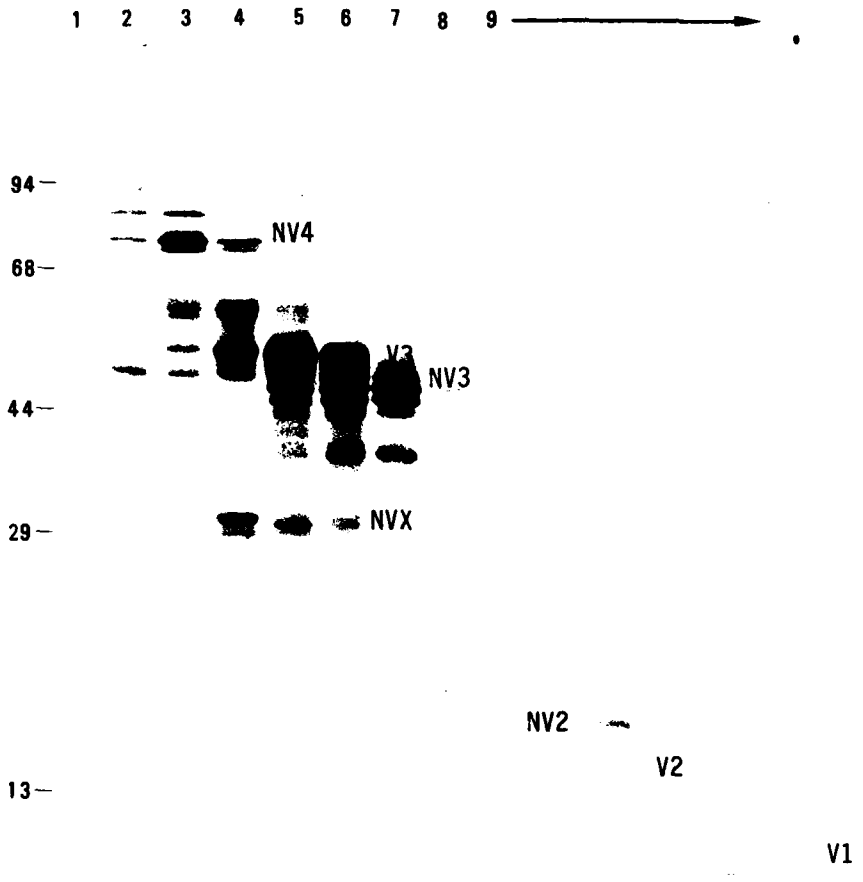

Fig. 13. Polyacrylamide gel electrophoresis of fractions from G-75 Sephadex column. Virus-free tissue culture fluid from JEV infected BHK cell cultures was used. Positions of molecular weight standards are shown on left. Elution fractions are from left to right as indicated.

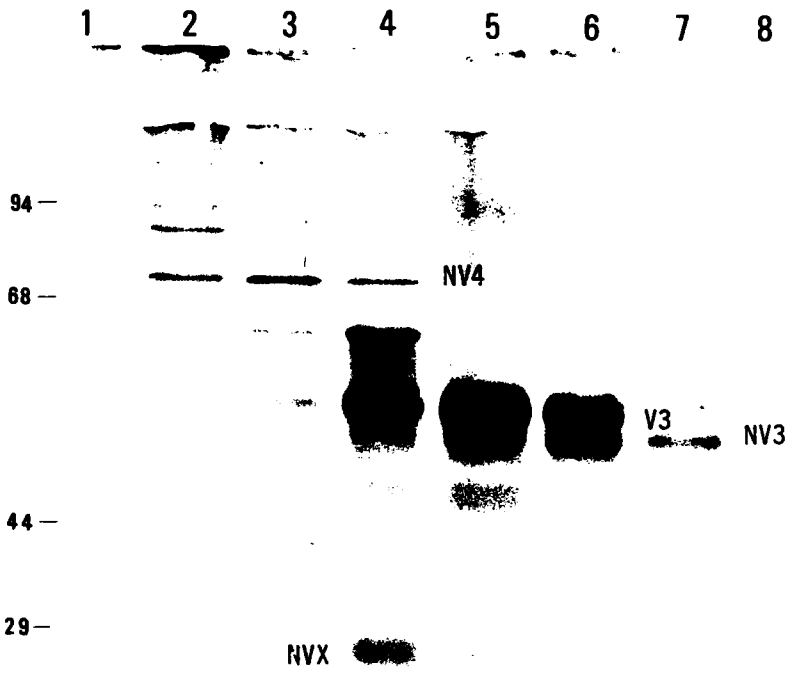

Fig. 14 . G-75 Sephadex gel filtration of DV virus-free S16 concentrate. Sequential fractions 1 through 8 from the peak were analyzed by polyacrylamide gel electrophoresis. Molecular weights of standard proteins are shown on left.

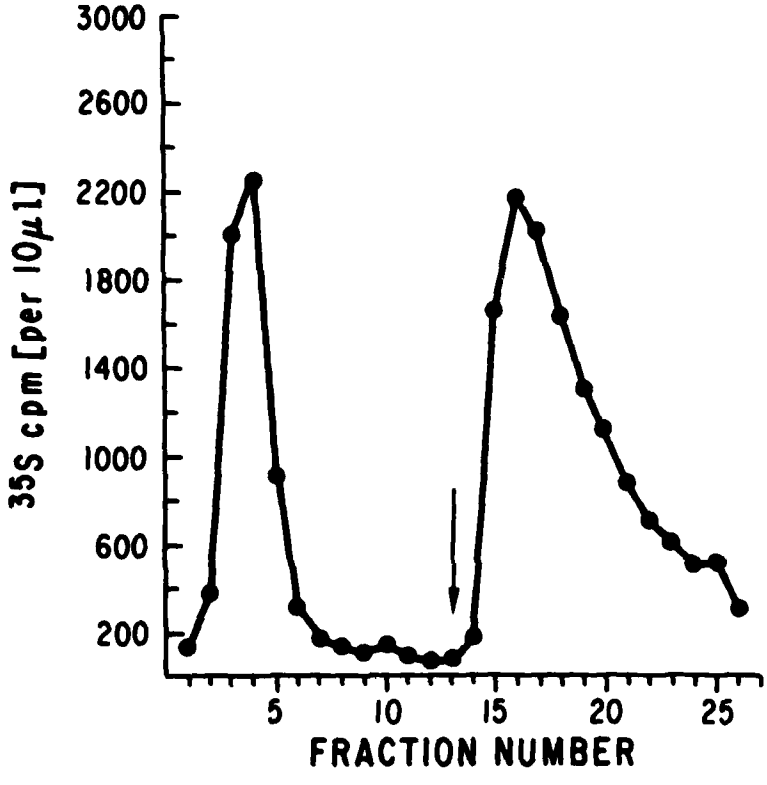

Fig. 15 . Con A-Sepharose rechromatrography. Fraction 6 from G-75 column eluate, adjusted to 0.5% TX-100, was loaded onto a Con A-Sepharose column and washed extensively. The material adhering to the column was then eluted with α-methyl mannoside. The α-mannoside was added at the fraction marked by the arrow.

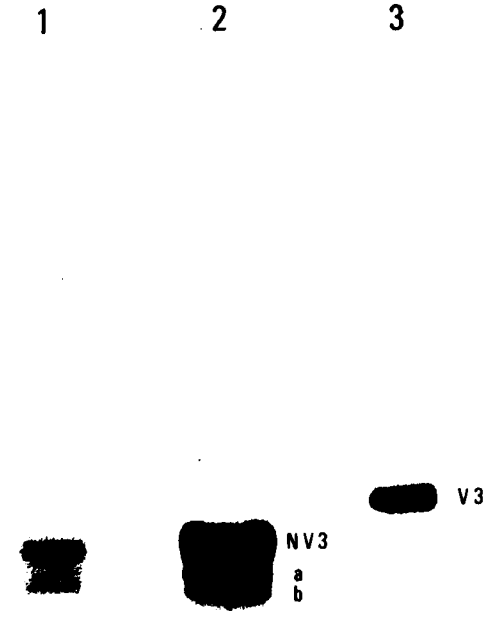

Fig. 16 . Polyacrylamide gel electrophoresis of polypeptides from Con A-Sepharose column. Fraction 6 from G-75 Sephadex column re-chromatographed on Con A-Sepharose. Wash through material (lane 1), α-methyl mannoside eluted material (lane 2), V3 from solubilized from JEV RHA (lane 3).

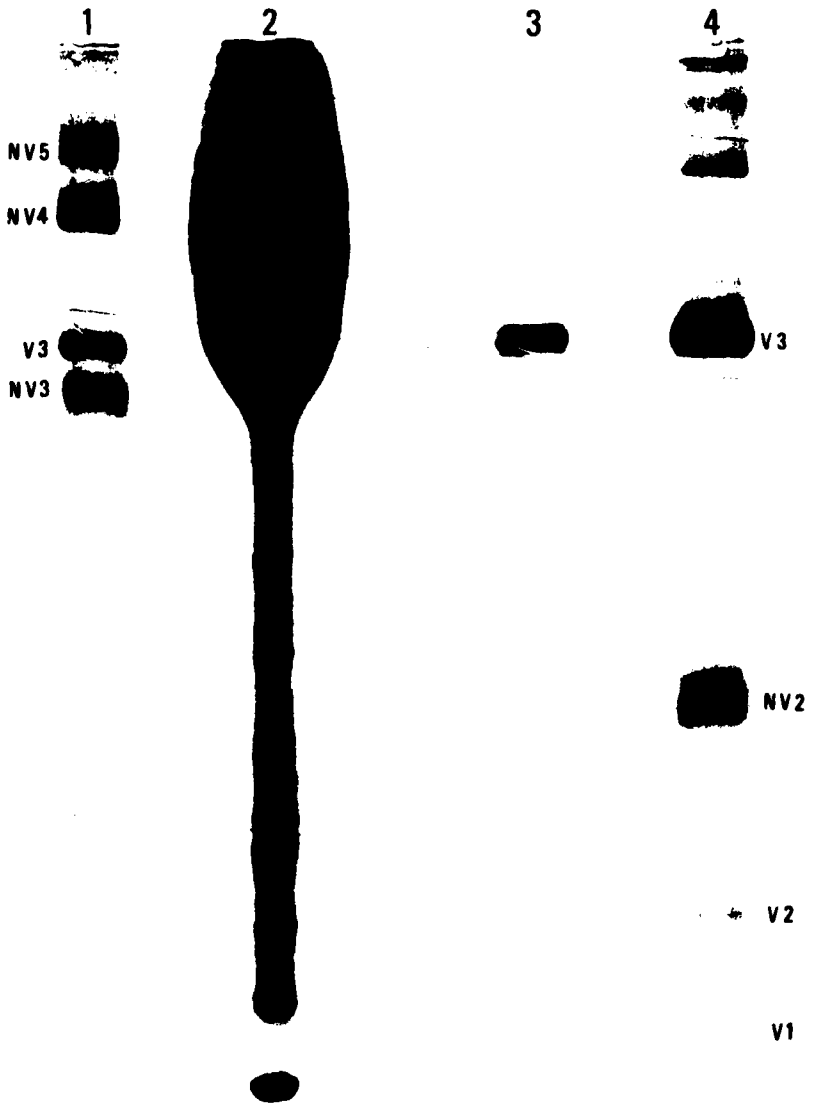

Fig. 17 . Electrophoresis of radioimmune precipitate. Both virus-free S16 and RHA from JEV infected Vero cells was immunoprecipitated with anti-JEV hyperimmune mouse ascites fluid or precipitated with TCA and the precipitates analyzed by polyacrylamide gel electrophoresis. Lane 1, S16 immunoprecipitate; Lane 2, TCA precipitate of S16; Lane 3, RHA immunoprecipitate; Lane 4, TCA precipitate of RHA.

V. MOLECULAR ORGANIZATION OF FLAVIVIRUSES AND CHARACTERIZATION OF POLYPEPTIDES IN INFECTED CELLS.

Electron microscopy of negatively stained preparations of flavivirus RHA show the presence of spike-like projections on the surface of the virions (34, 35). These projections are most likely molecular arrays of envelope glycoprotein(s). The arrangement of the proteins forming such arrays is not known. The basic structural subunits of the alphaviruses, Sindbis virus and Semliki Forest virus appear to be heterodimers of the two envelope glycoproteins (36, 37). These basic subunits may be organized as trimers to yield surface projections seen in the electron microscope (38), although this has yet to be demonstrated.

The organization of polypeptides on and in flavivirus particles can be determined using crosslinking methods similar to those used for alphavirus studies. In addition, once the organization of virion polypeptides is established, the crosslinking of the surface of infected cells will yield important information concerning the organization, morphogenesis, and immunology of flavivirus antigens. In this section we present results of studies on the molecular organization of JEV. Results of initial studies on the detection of structural and nonstructural polypeptides in extracts of JEV infected cells are also presented.

METHODS

JEV RHA was isolated as described in Section III, incubated with 10 mM iodoacetamide for 30 min at 37°, pelleted and resuspended in triethanolamine (TEA) buffer, pH 8.6. An equal volume of dimethylbissuberimidate (DMS) or dithiobispropionimidate (DTBP) in TEA buffer was added and the reaction allowed to proceed at room temperature for 60 min prior to quenching with glycine. The crosslinking reaction for both reagents occurs by a nucleophilic attack upon the ε-amino group of lysine residues by the imidate group on the crosslinking reagent. Once the crosslinking reagent has attached by one end, configuration and rotational freedom conditions dictate that the second attachment site be within an 11Å radius of the first.

Following quenching virus was pelleted and resuspended in electrophoresis sample buffer (free of reducing agent) and samples run on 7.5% or 10% polyacrylamide gels as described in Section III. Gels were stained for positions of protein standard, dried and placed onto X-ray film. Developed films were either examined directly or scanned to yield densitometer presentations.

For the analysis of polypeptides in infected cell lysates, JEV infected BHK cells were exposed to 0.5 μg/ml Actinomycin D from 4 to 16 hr post-infection, pulsed with cyclohexamide from 16 to 16.5 hr post-infection, fluid changed to methionine-free medium containing Actinomycin D and 10 μci/ml ^{35}S methionine, and harvested by scraping into Hank's balanced salt solution (HBSS) at 17 hr post-infection. Cells were washed twice with HBSS and the final pellet resuspended in cold 10 mM Tris-HCl, pH 7.0 containing 10 mM CaCl and 10 mM $MgCl_2$. After allowing the cells to swell for 10 min at ice temperature the cells were disrupted by 15

strokes in a Tenbroeck tissue grinder. Nuclei were pelleted by centrifugation at 600 x g for 10 min. Aliquots of the supernatant were TCA precipitated, acetone washed and analyzed by electrophoresis on 10% or 12% polyacrylamide gels. Uninfected BHK cells labeled in the absence of inhibitors were similarly disrupted and analyzed.

RESULTS

When JEV RHA was exposed to 0.3 mM DMS a crosslinked product was generated whose molecular weight was double that of glycoprotein V3 (1978 Annual Report, Section VI). We have extended these studies by examining the effects of increasing the concentration of crosslinking reagent in the reaction mixture. DTBP at final concentrations of 0.5, 1 and 5 mM were used. Densitometric scans of control and crosslinked JEV RHA components following electrophoresis in nonreducing SDS-polyacrylamide (10%) gels are shown in Fig. 18. Polypeptides V3 and NV2 appeared on the gels; however V2 and V1 had migrated off the gel (Fig. 18, A). Exposure to 0.5 mM DTBP resulted in the formation of a single crosslinked 100,000 molecular weight product as denoted by the numeral 1 (Fig. 18, B). Increasing the concentration of DTBP to 1 mM lead to the generation of two crosslinked products (Fig. 18, B). A concentration of DTBP of 5 mM resulted in the formation of a third band. Molecular weight estimates of bands 2 and 3 could not be determined due to their close proximity to the top of the gel. Nevertheless, increasing the concentration of crosslinking reagent was shown to increase the number of crosslinked products, a predicted result.

A comparison of the the noncleavable crosslinking reagent, DMS, and the reductively cleavable crosslinking reagent, DTBP, produced surprising results. Although both agents were equally effective in generating crosslinked products, the diversity formed were more numerous than was expected on the basis of the previous studies (Fig. 18). Polypeptides were electrophoresed through 7.5% polyacrylamide gels, a change from conditions used previously; however, this could not account for the many new bands. A more likely possibility was the use in the latter experiments of newly synthesized crosslinking reagents. A test with the older reagents resulted in the formation of only three bands corresponding to the three highest molecular weight crosslinked products seen in Fig. 19. The crosslinked products (XLP) have been assigned numbers corresponding to their estimated molecular weights.

Polypeptides from JEV infected cell lysates were readily detectable after electrophoresis in 12% polyacrylamide gels (Fig. 19, lane 1). Infected cells were exposed to Actinomycin D and cyclohexamide to reduce incorporation of label into host proteins. Uninfected BHK cells were labeled in the absence of inhibitors but were otherwise treated as infected cells. Electrophoresis of control cell lysates showed the pr- ence of numerous polypeptides (Fig. 19, lane 2), however only three of which comigrate with viral polypeptides. The three viral polypeptides are NV5, NV4 and NV3. Although these viral polypeptides could be contaminated with comigrating cell components this is considered unlikely since none of the non-comigrating polypeptides from the BHK cell lysate (lane 2) are found in lysate of inhibitor-treated JEV-infected cell lysate (lane 1).

These data indicate that inhibitor treatment was highly effective in blocking synthesis of cell polypeptides. The results of this initial study indicate that our technology is adequate for studies of the molecular organization of polypeptides on and in infected cells.

DISCUSSION

The crosslinking pattern of JEV RHA polypeptides becomes increasingly complex as the concentration of crosslinking reagent increases. The dramatic increase in complexity of the pattern when newly synthesized reagent is used points to the need for evaluating crosslinking reagent potency. Several enzymes are currently tested to determine if loss in enzyme activity can be correlated with the reactivity of the crosslinking reagent.

Based on the molecular weights of the crosslinked products (Fig. 19), the order of appearance of crosslinked products (Fig. 18), and the known molecular weights of JEV RHA polypeptides (Section IV) the probable monomer composition of the proudcts can be predicted. It should be noted that, with the exception of NV2,, JEV RHA polypeptides migrate with the same mobility in the presence or absence of reducing agent; NV2 mobility is reduced from an estimated 25,000 to 22,000 in the absence of reducing agent (JEV data not shown but results were similar to DV RHA data; Fig. 8, lanes 4 and 5). Since XLP100 has a molecular weight approximately twice that of V3, XLP100 is probably a homodimer of V3.

Similar analyses can be made for the other crosslinked products. For XLP125, the second product to appear (Fig. 18, C) a heterodimer composed of a V3 dimer and an NV2 monomer seems probable. Since XLP130 migrates as a relatively sharp band it probably does not contain NV2 but may be a a trimer of V3 which migrates anomolously due to crosslinking induced configurational changes. Although the analysis could be continued for the other crosslinked products, the composition would only be suggestive. Analysis involving electrophoresis in the second dimension under reducing condition is required to unequivocally demonstrate the composition of these products. These studies are in progress.

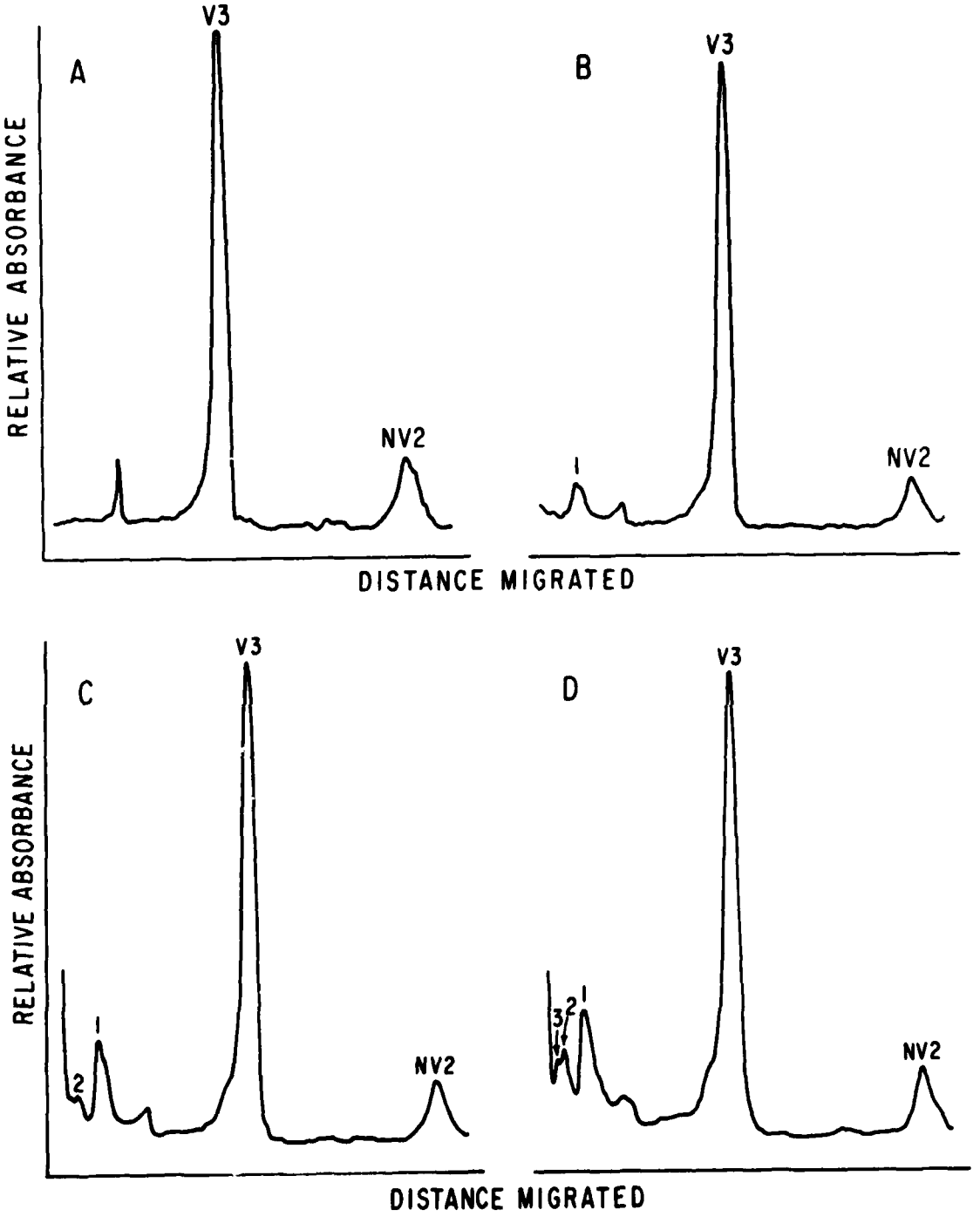

Fig. 18. Electrophoresis of JEV in 10% polyacrylamide gels following crosslinking of virus with different concentrations of dithiobispropionimidate, DTBP; (A) Control, (B) 0.5 mM DTBP, (C) 1 mM DTBP, and (D) 5 mM DTBP. Presentation is of densitometer scans of radioautographs. New bands arise with increasing crosslinker concentration and are denoted by the numbers 1, 2, and 3.

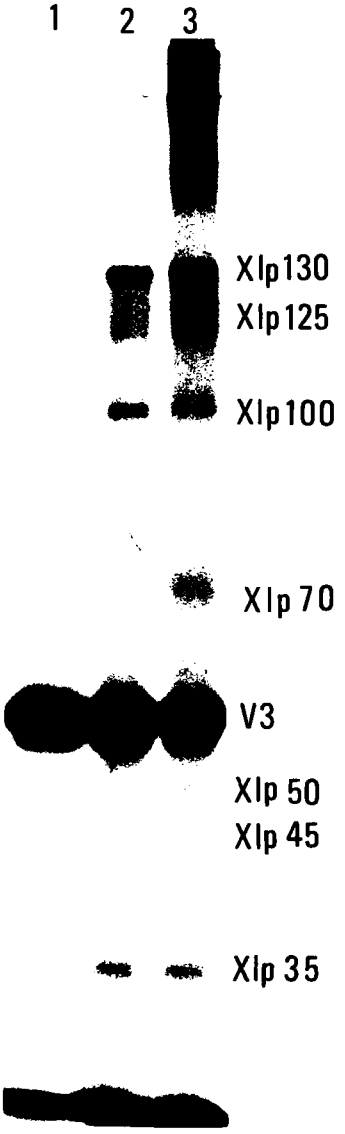

Fig. 19. Polyacrylamide gel electrophoresis of JEV polypeptides following exposure of the virus to crosslinking reagent. Virus was exposed to no crosslinking reagent (lane 1), 2.5 mM DMS (lane 2), or 2.5 mM DTBP (lane 3). Virus was disrupted in sample buffer free of reducing agent and run in a 7.5% polyacrylamide gel.

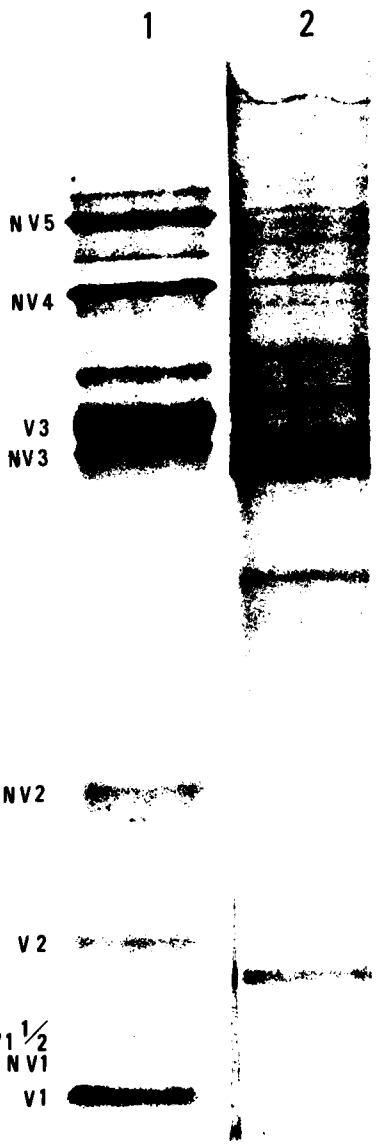

Fig. 20. Labeled polypeptides of cell lysates from JEV infected and uninfected BHK cell cultures. JEV infected cultures were exposed to actinomycin D and cyclohexamide as described in text. Uninfected cultures were not treated with inhibitors.

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