Synthesis of Virus Macromolecules in L-929 Cells Infected with Mayaro Virus

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SUMMARY

Two electrophoretically distinct protein subunits were found in Mayaro virus, a group A arbovirus. In addition, the cytoplasm of L-929 cells infected with Mayaro virus contained five new proteins, two of which were identified as virus structural proteins. All five new proteins were present by 3 hr after infection. The slower migrating virus structural protein stimulated the production of virus-neutralizing antibody.

INTRODUCTION

All of the arboviruses which have been analysed contain an RNA genome which appears homogeneous in the ultracentrifuge and has a molecular weight of $2 \times 10^6$ (Mussgay & Rott, 1964). Accordingly, the RNA would be expected to contain enough genetic information to code for proteins of a total molecular weight of approximately $2 \times 10^6$ (Wecker, 1959). The protein component of the arboviruses appears to be composed of two or three subunits. Yin & Lockart (1968) analysed Sindbis virus by acrylamide gel electrophoresis and detected two protein components, the faster of which could be associated with the nucleocapsid. Strauss et al. (1968) also detected two structural proteins from Sindbis virus. The slower migrating protein was associated with the lipoprotein envelope, and the faster with the nucleocapsid. Friedman (1968) demonstrated that Semliki Forest virus may be composed of three proteins, two of which could be associated with the nucleocapsid. In addition, Friedman examined infected chick embryo cells and detected five virus specific proteins in the cell cytoplasm. Three of the proteins were virus structural proteins, whereas two of the proteins could not be detected in purified virus. Late in infection only the virus structural proteins could be detected in the cell cytoplasm. Hay, Skehel & Burke (1968) analysed Semliki Forest virus by acrylamide gel electrophoresis and detected two protein components. These authors also detected six new proteins in the cytoplasm of infected cells, two of which could be identified as the structural components of the virus particle.

Our investigation was concerned with the structural and non-structural proteins of Mayaro virus, a group A arbovirus closely related to Semliki Forest virus.

METHODS

Tissue cultures. L-929 cells, obtained from Dr W. K. Joklik, Duke University School of Medicine, were grown either as monolayers in medium 199 with 10% calf serum or as

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suspending cultures in Eagle's minimal essential medium modified for suspension cultures (S-MEM) (Eagle, 1959) supplemented with 10% calf serum.

**Virus.** Mayaro virus, a group A arbovirus, was obtained from the American Type Culture Collection, Washington, D.C., as the fifth passage in mouse brain. The virus was adapted to grow in L-929 cells by five serial passages in monolayer cultures and was propagated in monolayers of L-929 cells by infecting $5 \times 10^7$ cells at an m.o.i. of 2 p.f.u./cell. Ten ml. of maintenance medium (medium 199 with 5% foetal calf serum) was added and virus was harvested 18 hr after infection by removing the maintenance medium from the cells, followed by centrifugation at 820g to clarify the virus suspension. Such virus suspensions were utilized for purification of virus and for production of antiserum against Mayaro virus. Virus infectivity was assayed under agar in L-929 cell monolayers in 60 mm. plastic Petri dishes (Falcon Plastics). The overlay consisted of 5 ml. of medium 199 containing 5% foetal calf serum and 1% agar. On the third day after inoculation, an additional 5 ml. of overlay medium containing 1/8000 neutral red was added to the cultures. Plaques were counted 6 hr later.

**Labeling and purification of virus.** L-929 monolayer cultures were partially depleted of amino acids by incubation overnight in amino acid deficient medium which was prepared by mixing five parts complete Eagle's MEM without serum with 95 parts Eagle's MEM which contained no amino acids. Foetal calf serum, which had been dialysed against 0.15 M-NaCl, was added to give a final concentration of 10%. The cells were infected at an m.o.i. of 2, and adsorption was allowed to continue for 30 min. at 37°, after which 10 ml. of the amino acid deficient medium containing actinomycin D (30 µg/ml) was added. Three hr after infection, either 100 µC tritiated amino acids (reconstituted protein hydrolysate, approximately 3 Ci/mm, Schwartz Bioresearch) or 30 µC [14C]amino acid mixture (Schwartz Bioresearch) were added to each flask. The virus was harvested 18 hr after infection.

**Virus concentration and purification.** Virus was concentrated by ammonium sulphate precipitation at neutral pH (Sreevalsen et al. 1968). After dialysis against 0.05 M-borate buffer (pH 9.0) containing 0.12 M-NaCl (borate saline), the concentrated virus was sedimented through 15% (w/v) sucrose in borate saline on to a 60% (w/v) sucrose pad by centrifugation at 51,000g for 2 hr. The visible virus band was withdrawn with a Pasteur pipette and dialysed overnight against phosphate saline to remove excess sucrose. The concentrated virus was purified further by banding in an exponential 5 to 30% (w/v) sucrose gradient in phosphate saline and centrifuged at 51,000g for 2 hr. Three-drop fractions were collected from the bottom of the tube and analysed for infectivity and radioactivity.

**Disruption of Mayaro virus and isolation of virus protein.** The purified radioactive virus was disrupted by treatment with sodium dodecyl sulphate (0.5% final concentration). The proteins were extracted at room temperature by shaking with an equal volume of phenol saturated with borate saline. The phenol phase was washed once in cold borate saline, after which the proteins were precipitated from the phenol phase with 6 vol. cold ethanol and a few drops of 2 M-ammonium acetate. The protein precipitate was obtained by centrifugation and dissolved in 0.5 ml. of an aqueous solution of 0.1% sodium dodecyl sulphate and 0.1% 2-mercaptoethanol. The resulting protein solution was dialysed against 0.1 M-tris + HCl, pH 8.2, containing 0.1% sodium dodecyl sulphate, 0.1% 2-mercaptoethanol and 0.5 M-urea. Mayaro virus also was disrupted by the method of Summers, Maizel & Darnell (1965) and analysed by acrylamide gel electrophoresis without prior extraction of the protein in phenol. In order to determine the purity of the virus preparation employed, samples of disrupted virus were analysed by immunodiffusion against antiserum prepared against stock virus suspension.
Synthesis of Mayaro virus

Radioactive labelling of cytoplasmic virus proteins. L-929 suspension cultures were partially depleted of amino acids by overnight incubation in the amino acid deficient medium. Before infection, the suspension cultures were exposed to actinomycin D (3·0 μg./ml.) for 3 hr. After treatment with actinomycin D, the cells were infected with 2 p.f.u. Mayaro virus/cell. Simultaneously, tritiated amino acids (10 μc/ml.) were added to the suspension cultures. When proteins produced late in the infective cycle were to be labelled, the same procedure was followed except that the radioactive amino acids were added 6 hr after inoculation of the cultures with virus.

Preparation of cytoplasmic extract. Cytoplasmic extract was prepared according to the method of Yin & Lockart (1968), and the extract was processed for electrophoresis according to the techniques of Summers et al. (1965).

Polyacrylamide gel electrophoresis. Analytical acrylamide gel electrophoresis was performed in ethylenediacylate cross-linked gels, which are soluble in piperidine (Choules & Zimm, 1965). Gels were composed of 7·5 % (w/v) acrylamide+0·5 % ethylenediacylate (Borden Chemical Co.)+0·5 M-urea+0·1 % sodium dodecyl sulphate in 0·1 M-tris+HCl, pH 8·2. The gels were polymerized with ammonium persulphate and N,N,N’-tetramethyl-ethylenediamine in 80×5 mm. tubes. The reservoir buffers consisted of 0·1 % sodium dodecyl sulphate and 0·5 M-urea in 0·1 M-tris+HCl, pH 8·2. The gels were subjected to electrophoresis at 5·5 mA/gel 2 hr to remove excess catalyst. Sucrose was added to the samples to a concentration of 6 % and phenol red was added as a tracking dye. Samples were layered on top of the gels and run at 5·5 mA/gel until the phenol red had migrated through 70 mm. of the gel. Preparative acrylamide gel electrophoresis was accomplished by employing a modification of the device described by Duesberg & Rueckert (1965). Electrophoresis was performed on acrylamide gels cross-linked with N,N’-bismethyleneacrylamide (Eastman Organic Chemical Co.) and consisted of 7·5 % (w/v) acrylamide+0·2 % bismethyleneacrylamide+0·5 M-urea+0·1 % sodium dodecyl sulphate in 0·1 M-phosphate buffer, pH 7·2. The reservoir buffer consisted of 0·5 M-urea+0·1 % sodium dodecyl sulphate in 0·1 M-phosphate buffer, pH 7·2.

Analysis of acrylamide gels. After electrophoresis, the gels were frozen at −70° and sliced into 1 mm. fractions with stacked razor blades. Each gel slice was then hydrolysed in 0·5 ml. of 0·2 M-piperidine by incubation at 37° overnight in liquid scintillation vials. Ten ml. of Beckman Cocktail D scintillation fluid was added to each vial and radioactivity was determined in a Beckman CPM·100 liquid scintillation counter.

Preparation of antibody to purified virus protein. Virus protein was purified from disrupted virus by preparative acrylamide gel electrophoresis. The protein was dialysed against 0·15 M-NaCl and concentrated by pressure dialysis. Protein was emulsified with Freund’s incomplete adjuvant so that 250 μg. was contained in 0·1 ml. of emulsion. Each rabbit was inoculated in each footpad with 250 μg. of protein and, simultaneously 500 μg. of protein was administered intravenously. Two intravenous booster injections of 250 μg. each were given at 5 day intervals and the animal was bled from the marginal ear vein 10 days after the last injection.

RESULTS

Replication of Mayaro virus in L-929 cells

The replication of Mayaro virus in L-929 cells was determined in order to identify the period of maximal virus synthesis. Suspension cultures of L-929 cells (5×10⁵ cells/ml.) were prepared and infected with Mayaro virus at an m.o.i. of 2 p.f.u./cell. Samples were
removed at hourly intervals for 12 hr following infection and assayed in L-929 cells (Fig. 1). A period of exponential release of virus into the medium occurred between 4 and 7 hr after infection, and the virus yield was approximately 200 to 500 p.f.u./cell.

![Fig. 1](image1.png)

**Fig. 1.** Replication of Mayaro virus in L-929 suspension culture cells.

![Fig. 2](image2.png)

**Fig. 2.** Gel-electrophoresis pattern of proteins derived from purified isotopically labelled Mayaro virus. Disrupted virus was subjected to electrophoresis on 8% acrylamide gels at 5.5 mA/gel, pH 8.1. Migration from left to right.

**Structural proteins of Mayaro virus**

Purified Mayaro virus was disrupted with 0.5% sodium dodecyl sulphate and 0.1% 2-mercaptoethanol according to the technique of Summers et al. (1965). When disrupted virus was diffused against antiserum to intact Mayaro virus, one precipitin line was observed, thus indicating the purity of the virus suspension used. After the disrupted virus was dialysed against 0.1 M-tris + HCl, pH 8.2, the proteins were separated by acrylamide gel electrophoresis. Purified Mayaro virus could be disrupted into two protein units which exhibited different electrophoretic mobilities (Fig. 2). The radioactivity of the slower moving virus protein (VP-1) was approximately two and a half times that of the more rapidly migrating virus protein (VP-2). Some radioactive material was present which had a faster electrophoretic migration than VP-2; however, the position and level of this material was not constant and its significance could not be determined. Extraction of virus with phenol also yielded two proteins with the electrophoretic characteristics described above. Immuno-diffusion analysis of the separated proteins revealed that antiserum produced against against intact virus particles was composed of antibody against VP-1 with no detectable antibody VP-2. Purified Mayaro virus, therefore, contained two electrophoretically distinct protein components; however, the possible existence of other minor proteins could not be excluded.
Synthesis of Mayaro virus

Development of nonstructural proteins in L-929 cells infected with Mayaro virus

Since there was evidence indicating the synthesis of virus specific protein before the virus structural proteins were synthesized, the cytoplasm of infected cells was examined for nonstructural virus proteins similar to those described for other viruses (Summers et al. 1965). Cytoplasmic extracts from infected and uninfected L-929 cell cultures, removed at different intervals after inoculation, were analysed by acrylamide gel electrophoresis. Before applying the extract to the electrophoresis column, virus protein labelled with $^{14}$C was added to each sample. The proteins were separated by gel electrophoresis, and the radioactivity of the fractions was determined. Equal amounts (200 μg.) of proteins from both control infected and virus-infected cells were analysed simultaneously in separate gels in the same electrophoresis run. The addition to both samples of virus protein labelled with $^{14}$C permitted the identification of structural proteins and also provided suitable markers for comparing the protein bands of separate gels.

Fig. 3. Electrophoretic patterns of tritium-labelled proteins derived from the cytoplasm of uninfected and infected L-929 cells. Mayaro virus structural protein marked by $^{14}$C label. The cytoplasm of cells was analysed (a) 1 hr after inoculation, (b) 2 hr after inoculation, (c) 3 hr after inoculation, and (d) 12 hr after inoculation. Electrophoresis was performed at 5-5 mA/gel. ○—○, $[^{3}H]$ counts/min. in infected cells/$[^{3}H]$ counts/min. in control; ●—●, $[^{14}C]$ counts/min. in virus structural protein.

No virus specific proteins were detected 1 hr after infection (Fig. 3a). However, proteins could exist so soon after infection in concentrations too low to be detected by this technique. By 2 hr after infection, two non-structural virus proteins exhibiting greater electrophoretic mobility than the virus proteins were found in infected cell cytoplasm (Fig. 3b). Three hr after infection, VP-1 and VP-2 were observed in the electrophoretic profile (Fig. 3c).
Another major non-structural virus protein, (NSVP-3), less mobile than VP-1, was also evident at this time. Four hr after infection, the structural proteins had increased in amount, and NSVP-3 had decreased. This pattern was maintained throughout the experiment with a decrease in the amount of NSVP-3 present. Since each gel had received the same total quantity of protein (200 µg.), the relative amount of protein present in each peak should have been indicated by the total counts per peak. The structural proteins increased in amount from 3 to 6 hr after infection and then decreased slightly (Table I). However, NSVP-3 was present in maximal amount 3 hr after infection, declined throughout the remainder of the experiment and was barely evident 12 hr after infection (Fig. 3d).

Table 1. Radioactivity associated with virus-specific proteins
in cells infected with Mayaro virus

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<tr>
<th>Hr after infection</th>
<th>Radioactivity (counts/min.)</th>
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<tr>
<td></td>
<td>VP-1</td>
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<tr>
<td>3</td>
<td>316</td>
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<tr>
<td>4</td>
<td>721</td>
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<td>9</td>
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Fig. 4. Electrophoretic pattern of proteins derived from the cytoplasm of Mayaro virus infected and uninfected L-929 cells, 7 hr after inoculation. Tritiated amino acid mixture was added to each culture 6 hr after inoculation to label the late proteins in the infected cells. [14C] virus protein was added to each gel. Electrophoresis was performed at 5.5 mA/gel. ○ — ○, [3H] counts/min. in infected/[3H]counts/min. in control; • — •, [14C]counts/min. in virus structural protein.

In order to determine whether NSVP-1, NSVP-2 and NSVP-3 were synthesized continually, cytoplasmic extracts of cells which had been labelled 6 hr after infection were examined for virus non-structural proteins. VP-1 and VP-2 were present 7 hr after infection but were no longer being synthesized late in the replicative cycle. However, NSVP-3 was present at this time, indicating that this protein was synthesized late in the replicative cycle (Fig. 4).
Identification of the immunogen of Mayaro virus

Preliminary data indicated that, although two protein subunits were present in Mayaro virus, only one was active in the induction of precipitating antibody. These data, in conjunction with the observation of Strauss et al. (1968) that the slower migrating component of Sindbis virus was associated with the virus envelope, suggested that VP-1 of Mayaro virus might be an envelope antigen and might therefore be responsible for the induction of neutralizing antibody. To test this possibility, the neutralizing capacity of an antiserum against VP-1 was assayed by plaque-reduction tests which used a challenge of $10^3$ p.f.u. of Mayaro virus. Anti-VP-1 antiserum, diluted 1/16 in Hanks's balanced salt solution, completely neutralized the Mayaro virus, whereas an antiserum dilution of 1/32 resulted in only a 30% reduction in the number of plaques. By analogy with Sindbis virus, one would expect VP-1 to be associated with the virus envelope.

DISCUSSION

Two Mayaro virus protein subunits were identified from particles disrupted with sodium dodecyl sulphate, urea and 2-mercaptoethanol. Based on the electrophoretic properties and immunodiffusion analysis of the protein components released from disrupted virus, the virus preparation employed in these experiments appeared to be highly purified. Immunodiffusion analysis of VP-1 and VP-2, which had been purified by preparative acrylamide gel electrophoresis, demonstrated a clear immunological difference between the two proteins. Therefore, the protein component of the Mayaro virus particle apparently consists of at least two distinct polypeptide chains.

Since the Mayaro virus which was analysed for structural proteins was labelled either with [3H]- or [14C]-amino acids, the analysis could be performed with or without prior extraction of the proteins with phenol. Any fragments of nucleic acid which might have migrated into the gel would not be confused with protein due to the lack of radioactive label. The method of Summers et al. (1965), which did not utilize phenol extraction, was employed to analyse the particle and the cytoplasm of infected cells because of its sensitivity.

The identity and function of NSVP-1 and NSVP-2 are not known. By virtue of their rate of migration in polyacrylamide gel, these two proteins must be relatively small compared to the structural proteins of Mayaro virus. Since the cytoplasmic extract was treated with 2-mercaptoethanol, sodium dodecyl sulphate and urea, these two subunits could represent single polypeptide chains dissociated from one or two molecules. It is highly unlikely that NSVP-1 and NSVP-2 resulted from the random breakdown of larger proteins because both proteins were present consistently in the cytoplasmic extract. Although experiments in which the cytoplasmic components were labelled late in infection failed to show NSVP-1 and NSVP-2, these two proteins may nevertheless arise by the cleavage of one virus specific protein synthesized early in the infective cycle.

NSVP-3 is a relatively large protein molecule in comparison with the structural proteins of Mayaro virus. It is not associated with infectious virus, and immunological assays failed to reveal the presence of NSVP-3 in the culture medium. Some poliovirus proteins are synthesized as high molecular-weight components from polycistronic messenger RNA and are subsequently cleaved into the lower molecular-weight form (Summers & Maizel, 1968; Jacobson & Baltimore, 1968; Holland & Kiehn, 1968). The RNA genome of Mayaro virus exhibits a sedimentation coefficient of 33 to 35 S, and therefore could code for proteins of a total molecular weight of $2 \times 10^5$. Since the structural proteins of Mayaro virus have a total molecular weight of $2.8 \times 10^5$, the RNA must also code for NSVP-3.
weight of about $8 \times 10^4$ by analogy with Sindbis virus (personal communication of J. H. Strauss, published by Scheele & Pfefferkorn, 1969), it seems likely that NSVP-3 could be a precursor protein which is subsequently cleaved into smaller virus specific proteins. A second explanation for the presence of NSVP-3 is that this protein may be the result of specific derepression of the host-cell genome by Mayaro virus. Our data are against this hypothesis. Current evidence indicates that actinomycin D inhibits the reading of information in DNA by blocking messenger RNA synthesis. However, NSVP-3 is produced in the presence of actinomycin D after virtually all cellular messenger RNA synthesis has ceased in uninfected cells; therefore, if this protein is the result of the host-cell genome, preformed messenger RNA would have to exist. The presence of such a message is not consistent with the concept of derepression. A third explanation is that NSVP-3 may be an artifact of the system caused by aggregation of two or more virus specific molecules. However, 0.5 M urea and 0.1% sodium dodecyl sulphate were included in the gels to minimize this possibility.

The most plausible explanation for the appearance of NSVP-3 in infected cells is that it is a precursor protein analogous to that found in the poliovirus system. This would explain the decreased amounts of NSVP-3 present late in infection.

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REFERENCES


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