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MAYARO VIRUS PROTEINS

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Mayaro virus was grown in BHK-21 cells and purified by centrifugation in a potassium-tartrate gradient (5-50%). The electron microscopy analyses of the purified virus showed an homogeneous population of enveloped particles with 69 ± 2.3 nm in diameter. Three structural virus proteins were identified and designated p1, p2 and p3. Their average molecular weight were p1, 54 KDa; p2, 50 KDa and p3, 34 KDa. In Mayaro virus infected Aedes albopictus *cells and in BHK-21 infected cells we detected six viral proteins, in which three of them are the structural virus proteins and the other three were products from processing of precursors of viral proteins, whose molecular weights are 62 KDa, 64 KDa and 110 KDa. The 34 KDa protein was the first viral protein sinthesized at 5 hours post-infection in both cell lines studied.*

Key words: arbovirus – protein synthesis – Mayaro virus

Mayaro virus is a relatively simple enveloped virus (Saturno, 1963; Mezencio et al., 1989, 1990) and belongs to the Alphavirus genus, Togaviridae family (Casals & Whitman, 1957). Mayaro virus, as other Togavirus, is perpetuated in nature by its ability to infect and replicate in vertebrate and invertebrate cells (Brown & Condreay, 1968). In Brazil, this virus has been isolated from human and other mammalian species, mainly at borders of colonization in the Amazon Region (Causey & Maroja, 1957). The nucleocapsid of the alphaviruses contains a positive single strand RNA (4×10^6 Da) and a single protein (3×10^4 Da). The envelope has been described to contain two (in Sindbis virus) or three (in Semliki forest virus) glycoproteins E1, E2 (both 50 KDa), and E3 (10 KDa) (Garoff et al., 1982).

The intracellular events involved in the processing, transport, and assembly, of viral proteins of alphavirus have been extensively studied and are reviewed by Schlesinger & Schlesinger (1986). The viral RNA function as a mRNA molecule for the synthesis of a RNA-dependent RNA polymerase wich transcribes the viral genome as well as a subgenomic 26S RNA molecule at its 3' end (Garoff et al., 1982).

This smaller RNA molecules serves as a messenger RNA for all structural proteins of the virus particles, which are synthesized sequentially in the following order: capsid protein, p62, and E_1. The p62 protein is an intracellular precursor protein for E_2 and E_3 (Garoff et al., 1982).

In this paper we reported the results of studies about the Mayaro virus structural proteins and the virus-specific proteins synthesized in *Aedes albopictus* and in BHK-21 cells.

MATERIALS AND METHODS

Cells and virus – *Aedes albopictus* (clone $C_6/36$) and BHK-21 cells were maintained and propagated as monolayers in 60 cm^2 bottles at 28 °C and 37 °C, respectively. *A. albopictus* cells and BHK-21 cells were cultivated as described (Mezencio et al., 1989, 1990). Mayaro virus was obtained from the American Type Culture Collection, Rockville, MD, USA, propagated in BHK-21 cells and stored at –70 °C.

Growth, concentration and purification of the virus – Monolayers of BHK-21 cells were

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infected with Mayaro virus at a multiplicity of 5 PFU/cell and allowed to attach for 60 min at 37 °C. Following the attachment period the inocula were removed, growth medium was added, and the cultures were returned to 37 °C. For labelling virus with ^{35}S-methionine (50 uCi/ml), the medium was deficient in methionine. Labeled compound were added 6hr after the adsortion period. Culture fluids were harvested 24 hr after infection when cytophatic effect were distinctly visible. Cellular debris were removed by centrifugation at 600 g for 10 min and the supernatant fluid was clarified at 10,000 g for 20 min. The virus particles were collected by centrifugation at 90,000 g for 90 min and resuspended in PBS pH 7.2 containing 0.4% of bovine serum albumin. Concentrated virus was purified in a linear 5-50% (w/v) tartrate PBS-albumin (0.4%) by centrifugation at 100,000 g for 90 min. After centrifugation, samples of 0.2 ml were collected and assayed for trichloroacetic acid precipitable racioactivity and infectivity. Infectivity titrations of Mayaro virus were performed by plaque assay in L-A9 cells as described previously (Volkmer et al., 1983).

Analysis of proteins by polyacrylamide gel electrophoresis (PAGE) – For PAGE, virus in pooled peak fractions were diluted in PBS-albumin (0.4%) and centrifuged at 90,000 g for 90 min. The pelleted virus was treated with loading buffer (62.5 mM tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoetanol and 0.001% bromophenol blue), and the samples were heated at 100 °C for 2 min and then subjected to electrophoresis in 12.5% polyacrylamide gels by using the continuous SDS buffer system of Laemmli (1970), at room temperature in gel slabs. The gels were stained with comassie blue, dried and exposed to Kodak X-Omat (YAR-S).

Electron microscopy – Samples from the purified virus, were pelleted at 90,000 g for 90 min and resuspended in PBS-albumin (0.4%). A drop of the virus suspension was applied to a copper grid covered with carbon-reinforced formvar. The drop was removed with a filter paper and the especimen was immediately replaced by negative stain. A 2% solution of neutral potassium phosphotungstate followed by 1% aqueous solution of uranyl acetate was used as a negative stain. The stain was allowed to settle for only a few seconds before draining with filter paper. The specimen were examined in a JEOL 100-CX electron microscope operating at 80KV.

Metabolic labelling and electrophoresis of virus proteins from infected Aedes albopictus *and BHK-21 cells* – Monolayers of *A. albopictus* and BHK-21 cells were infected with Mayaro virus at 10 PFU/cell. After adsortion period, the inoculum was aspirated, and growth medium was added to each culture. The cells were incubated at 28 °C or 37 °C, respectively. At different times post-infection, the infected cultures were starved for 1 h with Eagle's Minimal Essential Medium in the absence of serum and methionine. After this period the medium was supplemented with 30 uCi/ml of ^{35}S-methionine, and the incubation was continued. One hour later, the medium was removed, and monolayers suspended in 80 ul of loading buffer.

RESULTS

Figure 1 shows a growth curve for Mayaro virus in BHK-21 cells and *A. albopictus* cells. In BHK-21 cells the maximum virus production were obtained after 18 hours post infection and in *A. albopictus* cells after 45 hr post-infection.

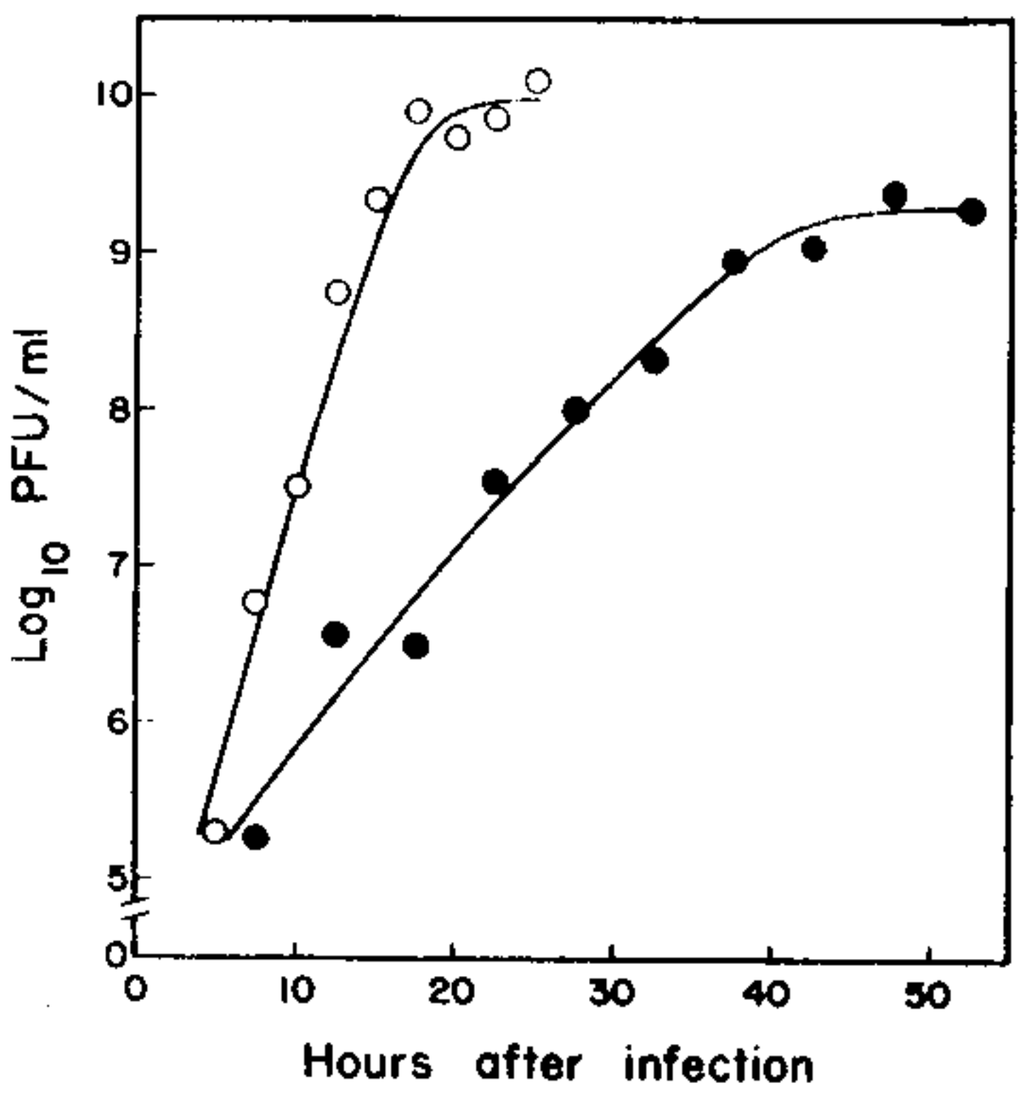

Fig. 1: growth curve of Mayaro virus. Monolayers were inoculated at a multiplicity of 10 PFU/cell and adsorbed for 1 hr at 37 °C. Points represent the yield from duplicates assayed for plaque formation in L-A9 cells, *Aedes albopictus* cells (●), BHK-21 cells (○).

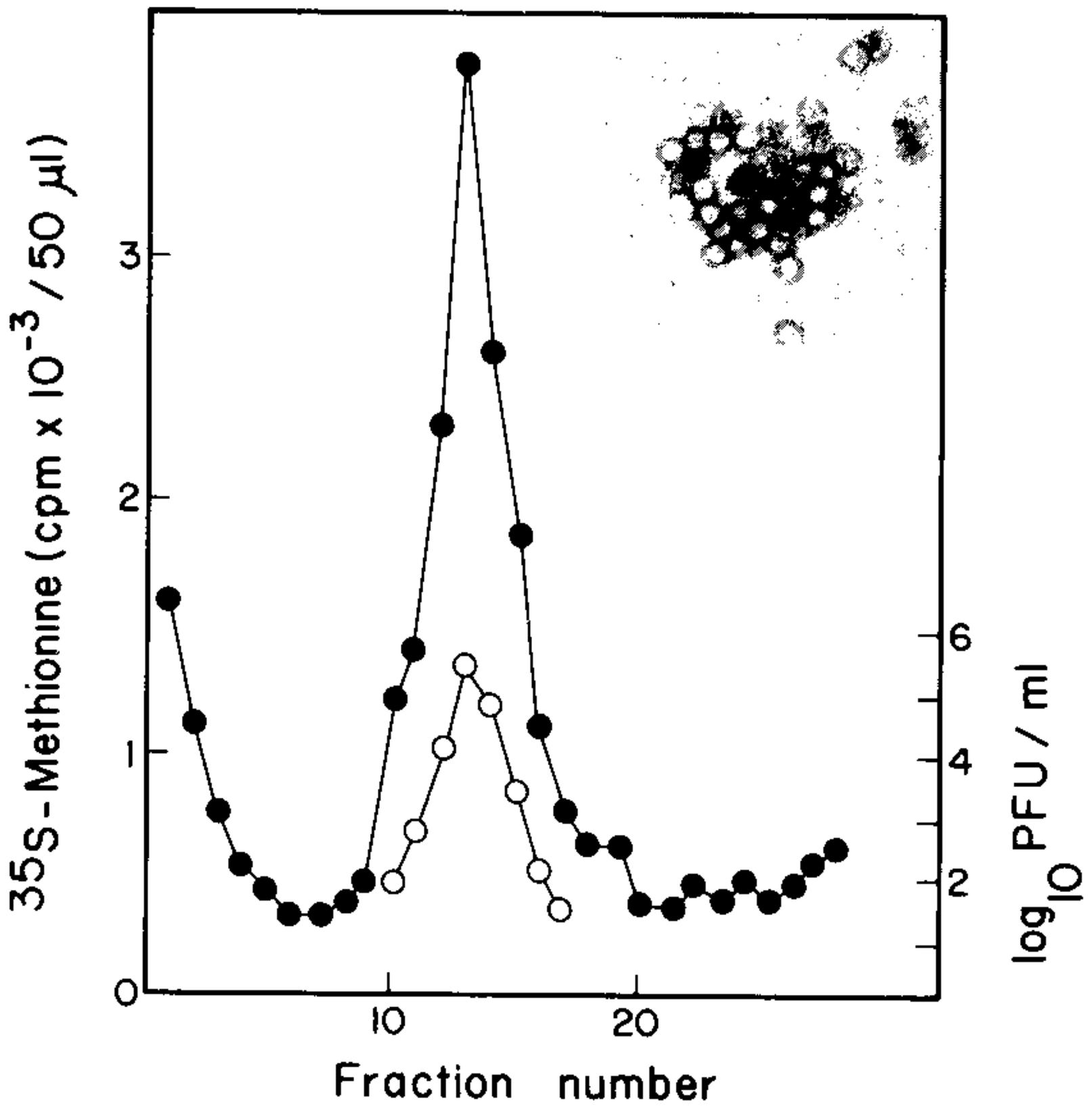

Fig. 2: purification of Mayaro virus by centrifugation in potassium-tartrate gradient (5-50%). Mayaro virus was labeled with ^{35}S-methionine and purified as described in Materials and Methods. Fractions of same volume were collected from the gradient and assayed for acid-precipitable radioactivity (●) and infectivity (○). Insert: electron micrograph of purified Mayaro virus, X 65,000.

^{35}S-methionine labelled Mayaro virus grown in BHK-21 cells were concentrated by centrifugation and purified on tartrate-PBS albumin gradients. The fractions containing purified virus were identified by infectivity and radioactive label (Fig. 2). The electron microscopy of the purified fractions which contained the maximum of radioactivity, showed a homogeneous population of enveloped particles with 69 ± 2.3 nm in diameter (Fig. 2, insert).

To analyze the structural proteins of Mayaro virus the purified virus labelled with ^{35}S-methionine, was dissociated with SDS and beta mercaptoethanol. The labelled virus proteins were resolved by electrophoresis in 12.5% polyacrylamide gels. Three structural polypeptides were identified and designated as p1, p2 and p3 (Fig. 3). The polypeptide P2 is the minor viral component (Fig. 4).

Monolayer cultures of *A. albopictus* cells were mock-infected or infected with Mayaro virus with m.o.i. of 10. At 1 hr intervals throughout 14 hr and in 24, 30, 45 and 50 hr period, cells were radiollabeled with ^{35}S-methionine for 1 hr and then cells extracts were prepared. Analysis by SDS-PAGE and autoradiography of the cell extracts revealled a total of six proteins unique to the infected cell extracts. These proteins had relative molecular weights corresponding to 34 KDa, 50 KDa, 54 KDa, 62 KDa, 64 KDa and 110 KDa (Fig. 5A). The first viral protein 34 KDa was apparent at 5 hr post-infection, and identified as the 34 KDa protein. Most of the Mayaro associated proteins were apparent in the extracts

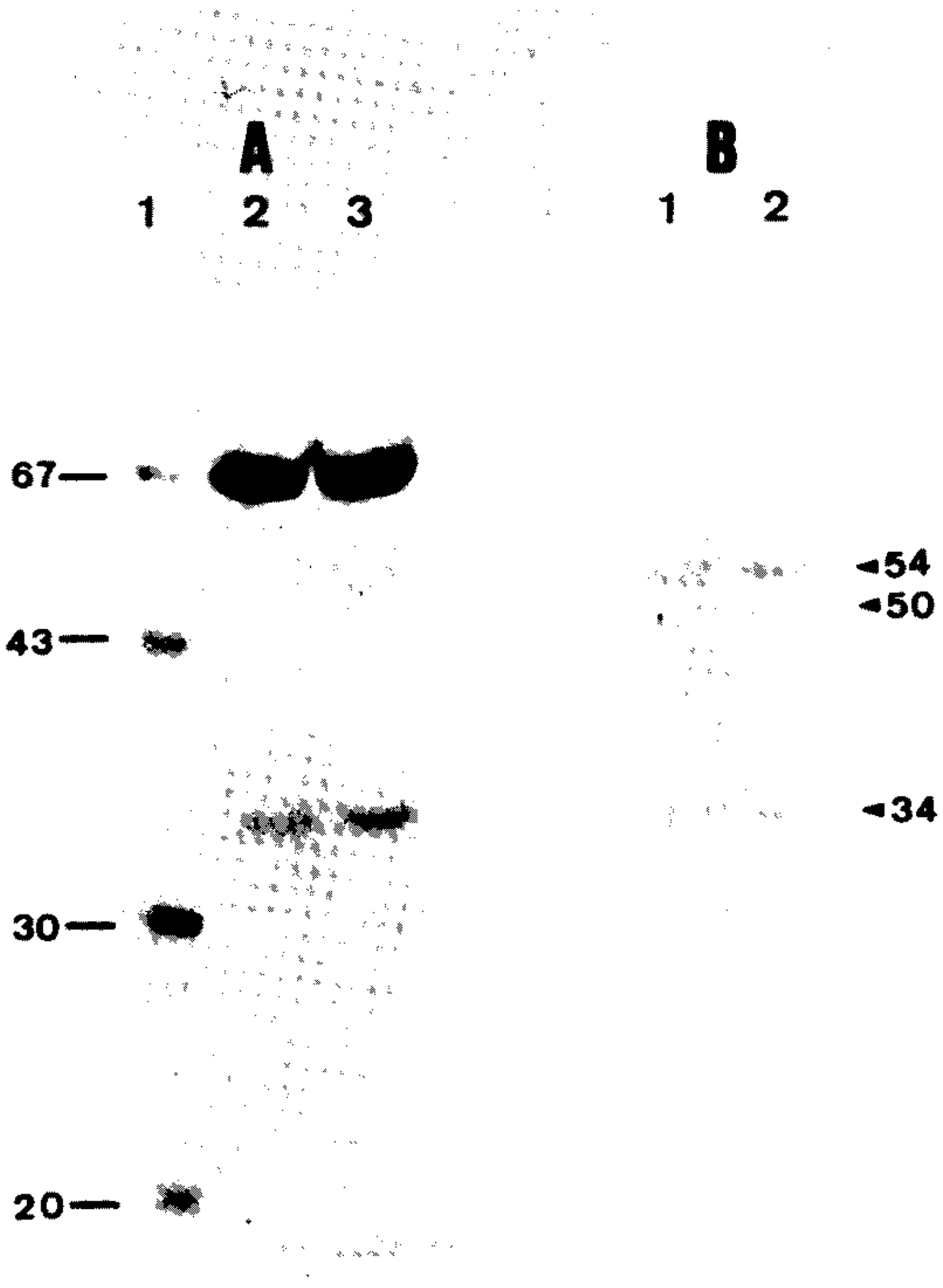

Fig. 3: PAGE of purified Mayaro virus. A – Stained with comassie blue. Mobilities of standard proteins (slot 1); purified Mayaro virus proteins (10 ul slot 2, 20 ul slot 3). B-Labelled purified Mayaro virus (10 ul slot 1, 20 ul slot 2).

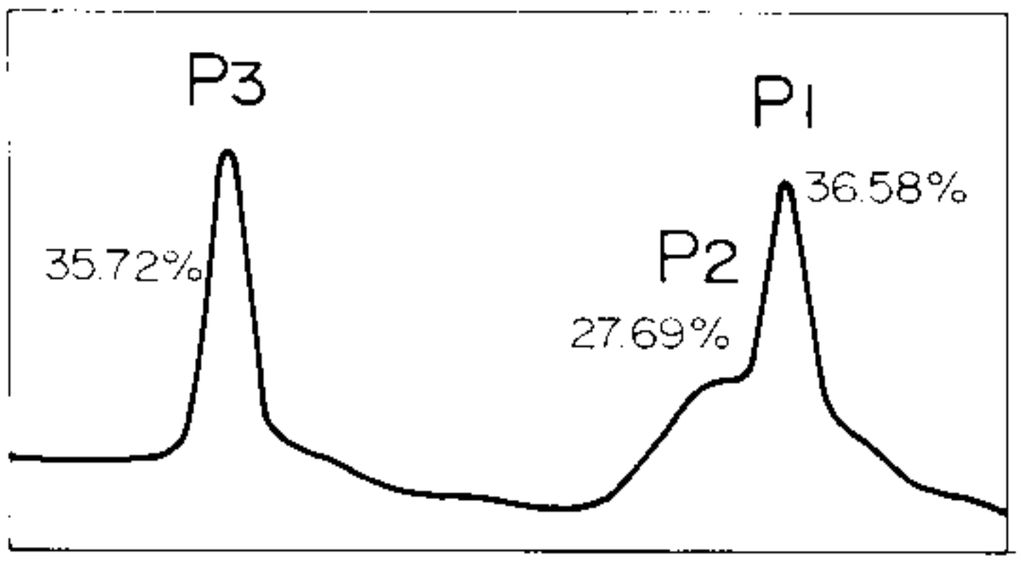

Fig. 4: densitometer tracings of the autoradiograms of purified Mayaro virus.

prepared at 8 hr post-infection. A discret decline in the host protein synthesis in infected *A. albopictus* cells was apparent at 12 h after infection (Fig. 5A).

In BHK-21 infected cells, m.o.i. of 10, it was possible to detect the same six proteins found in *A. albopictus* infected cells (Fig. 5B). In both cultures the 34 KDa viral protein was first sinthesized at 5 hr post-infection for *A. albopictus* cells and at 6 hr post-infection for BHK-21 cells (Fig. 5).

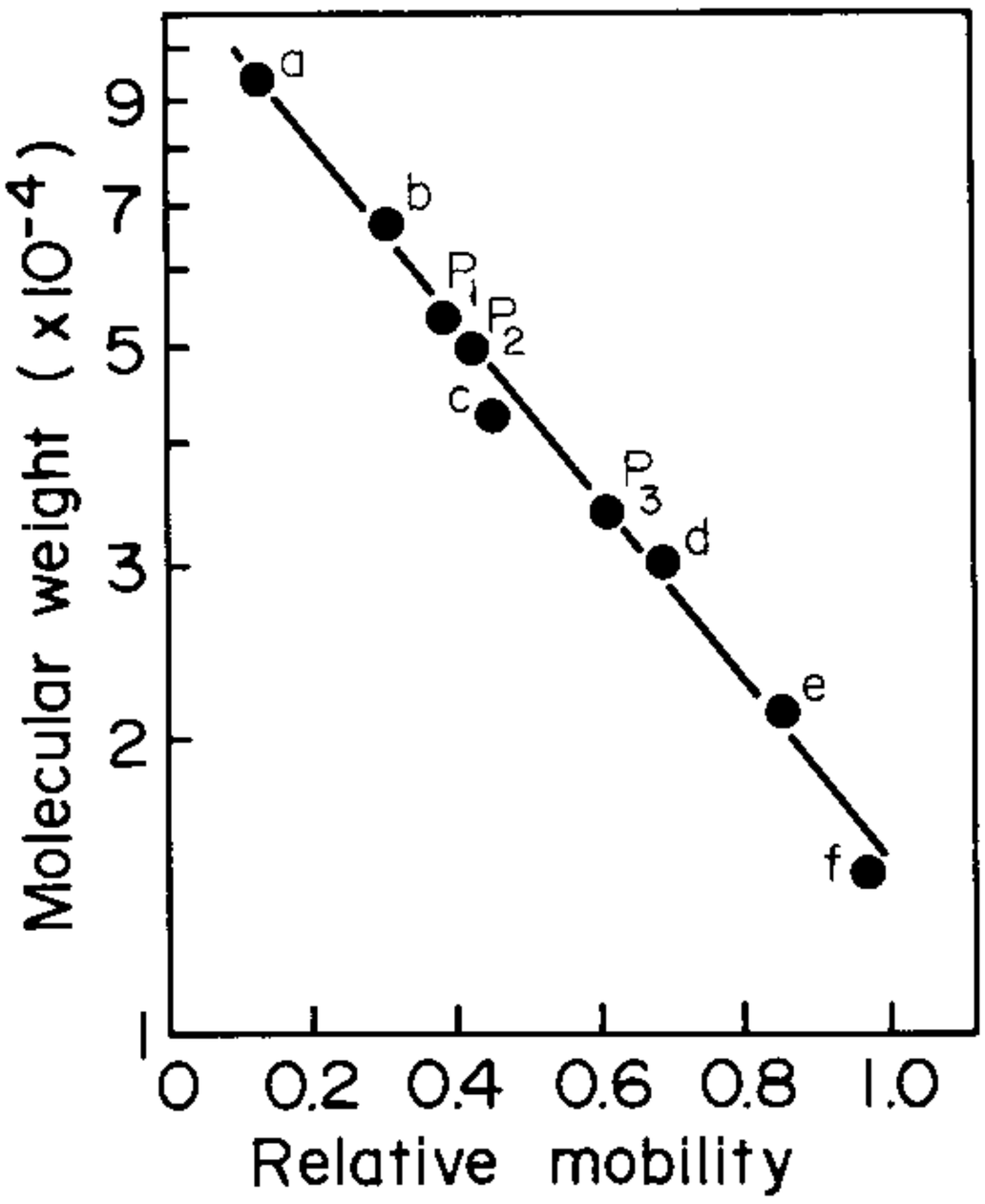

Fig. 5: time course of Mayaro virus protein synthesis. The cells were pulse-labelled for 1 hr with 30 uCi/ml of 35S-methionine at different times. The cells were lysed with sample buffer, SDS-polyacrylamide electrophoresis was performed, and dried gels were autorradiographed. A – *Aedes albopictus* cells: lane 1, mock infected cells; lane 2 to 10, respectively 1, 3, 5, 8, 12, 14, 30, 45 and 50 hr post-infection. B – BHK-21 cells; lane 1, mock infected cells; lane 2 to 8, respectively, 1, 3, 6, 8, 10, 25 and 46 hr post-infection.

DISCUSSION

This study provides the first evidence of the characterization of Mayaro virus specific proteins.

The molecular biology of the alphaviruses is almost entirely based on studies with two members of this virus group, namely, the Semliki Forest virus and the Sindbis virus. The structural proteins of these viruses have been exaustively analyzed (Garoff et al., 1982). The subgenomic 26S RNA molecule is used as a messenger RNA for all structural proteins of the virus particle, that is, the capsid protein (30 KDa), and the three membrane proteins: E3, 10 KDa; E2 and E1 50 KDa. Semlike Forest virus contain four structural polypeptides, whereas Sindbis virus lack E3 (Strauss et al., 1968; Simons & Karaaianen, 1970; Schllesinger et al., 1972). The proteins are present in an equimolar ratio in the virus particle and they are all specified by the virus genome. In contrast to the protein composition, the oligosaccharide units of the spike glycoproteins and the lipids of the bilayer appear to be specified by the host cell (Garoff, 1982). The growth curve shows that the maximum virus production in BHK-21 cells is obtained at 18 hours post-infection (Fig. 1). Similar results was verified in the growth of Sindbis virus in VERO cells (Westaway, 1973), in BHK-21 cells (Grimley et al., 1972) and in *A. albopictus* cells (Gliedman et al., 1975). Acheson & Tamm (1967), shows the same results for Semliki Forest virus growth in chick embryo cells (m.o.i. 50). Grimley et al. (1972), found that when the Semliki Forest virus was grown in BHK-21 cells (m.o.i 10), the maximum virus production occurs at 8 hours post infection. In *A. albopictus* cells the maximum virus production is obtained at 45 hours post-infection.

The envelope from mature virus particles detected by electron microscopy has 69 ± 2.3 nm in diameter (Fig. 2, insert). This is in concordance to that described for Mayaro virus grown in *A. albopictus* cells (Mezencio et al., 1989) and in BHK-21 cells (Mezencio et al., 1990). However, Saturno (1963), studying the replication of Mayaro virus in KB cells, human heart cells, and mouse brain cells, found virus-like particles with 40 ± 5 nm in diameter.

A comparison of our results with those reported for others alphaviruses suggested that three structural Mayaro virus proteins p1 (54 KDa), p2 (50 KDa) and p3 (34 KDa) correspond to the alphavirus structural proteins generally recognized. By analogy with others alphaviruses, the structural Mayaro virus proteins p1 and p2, correspond to the glycoproteins E1 and E2, and the p3 protein corresponds to the C nucleoprotein.

The densitometry of the autoradiogram revealled that the polypeptides p1 and p3 (major viral components) are present in equimolar ratio. Comparing the concentration of polypeptides p1 and p3 by Coomassie blue staining and by autoradiography, we can say that probably the presence of methionine in the polypeptide p1 is more abundant than in polypeptide p3.

Studies with alphavirus has been described that the proteins are present in an equimolar ratio in the virus particle (Garoff, 1982). We

found, however, an equimolar ratio only for p1 and p3 proteins of Mayaro virus. The protein p2 is a minor viral component in virus particles.

Similar to Sindbis virus, Mayaro virus lacks the glycoprotein of smallest molecular weight (E3). However the total molecular weight of structural Mayaro virus proteins is 138 KDa wich is very close to the value found for Sindbis virus (130 KDa) and Semliki Forest virus (140 KDa). It is possible that in Mayaro virus, like Sindbis virus and Semliki Forest virus, the subgenomic 26S RNA is responsible for codifying the structural proteins.

Six Mayaro virus proteins were detected by radiollabelling techniques in *A. albopictus* and in BHK-21 infected cells. The BHK-21 infected cells show a more severe inhibition of host protein synthesis, and at 25 hr post-infection very little host protein synthesis was detected (Fig. 5B). Analysis of the kinectic of synthesis of the Mayaro virus polypeptides in BHK-21 cells showed that a clear division into early and late categories could be made.

The two proteins p62 and p64 are probably break down products of the 110 KDa precursor polyprotein. As in Semliki Forest virus and in Sindbis virus (Garoff, 1982), the processing of virus proteins probably take place in the same way. The p64 protein is the precursor of the 54 KDa (E_1) and 6 KDa proteins, and the p62 protein is the precursor of the 50 KDa (E_2) and 10 KDa (E_3) proteins. The fact that the 34 KDa protein was the first protein observed in infected cells is an evidence that the protein capsid is the first product of the 26S RNA translation.

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