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ROLE OF CELLULAR COMPONENTS OF MOSQUITO CELLS IN VIRAL REPLICATION AND TRANSMISSION

ANNUAL SUMMARY REPORT

Robert H. Schloemer

February, 1978

Supported by

U.S. Army Medical Research and Development Command
Fort Detrick, Frederick, Maryland 21701

Contract No. DAMD 17-77-C-7023

Indiana University School of Medicine
Indianapolis, Indiana 46202

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SECURITY CLASSIFICATION OF THIS PAGE *(When Data Entered)*

REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER

2. GOVT ACCESSION NO.

3. RECIPIENT'S CATALOG NUMBER

4. TITLE *(and Subtitle)*

(4) Role of Cellular Components of Mosquito Cells in Viral Replication and Transmission.

5. TYPE OF REPORT & PERIOD COVERED
Annual Summary Report
May 1, 1977 - Feb. 20, 1978

6. PERFORMING ORG. REPORT NUMBER

7. AUTHOR(s)

(10) Robert H. Schloemer

8. CONTRACT OR GRANT NUMBER(s)

(15) DAMD 17-77-C-7023

9. PERFORMING ORGANIZATION NAME AND ADDRESS

Indiana University School of Medicine
Indianapolis, Indiana 46202

10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS

61102A
(16) 3M161102BS03.00.047 (17) 00

11. CONTROLLING OFFICE NAME AND ADDRESS

U.S. Army Medical Research and Development Command Fort Detrick, Feterick, Maryland 21701

12. REPORT DATE
February 24, 1978

13. NUMBER OF PAGES
28

14. MONITORING AGENCY NAME & ADDRESS *(if different from Controlling Office)*

U.S. Army Medical Research Institute of Infectious Diseases Frederick, Maryland 21701

15. SECURITY CLASS. *(of this report)*
Unclassified

15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT *(of this Report)*

Approved for public release (12) 27 p.

17. DISTRIBUTION STATEMENT *(of the abstract entered in Block 20, if different from Report)*

(11) 24 Feb 78

18. SUPPLEMENTARY NOTES

(9) Annual summary rept. 1 May 77-20 Feb 78,

19. KEY WORDS *(Continue on reverse side if necessary and identify by block number)*

Virus, Mosquito, protein, vaccination.

20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

SECURITY CLASSIFICATION OF THIS PAGE *(When Data Entered)*

20. Abstract

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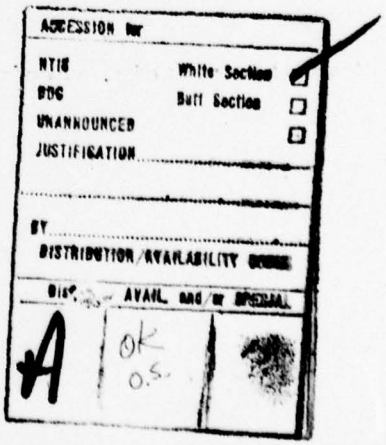
Immunization of mice with uninfected mosquito cells protects against infection by banzivirus grown in mosquito cells. Thus protection is not due to any media components and is due, in part, to the humoral response of the mouse as shown by the fact that antisera to uninfected mosquito cells neutralizes infectivity of mosquito cell-grown banzivirus.



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FOREWORD:

In conducting the research described in this report, the investigator adhered to the "Guide for Laboratory Animal Faculties and Care" as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences - National Research Council.

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PROGRESS REPORT

This report covers the period from May 1, 1977 to February 20, 1978.

A. Background and Statement of Problem

In nature, arboviruses are transmitted to vertebrates via arthropod vectors. Replication of flaviviruses in invertebrate hosts is poorly understood and differs remarkedly from growth of flavivirus in mammalian cells. In particular, infection of mosquito cell lines with flaviviruses generally results in a low-titered, non-cytolytic, persistent infection which is in marked contrast to the destructive effects that these viruses have on vertebrate cell cultures. It is of interest that this response closely resembles the in vivo infections of mosquitoes in which arboviral infections are generally asymptomatic and life-long.

Replication of flaviviruses in A. albopictus cells differs from its replication in vertebrate cells in the following known ways. Progeny virus released from infected mosquito cells consists of only complete virus (RHA), a fact which is in contrast to the formation of both RHA and SHA in mammalian cells. In addition, progeny virions from infected mosquito cells are altered antigenically, probably as a result of host-cell mediated modification of the viral envelope, so that the hemagglutination (HA) activity of the albopictus cell-grown virus is blocked by antisera to uninfected albopictus cells. This result suggests that as flaviviruses mature and are released from mosquito cells, a host cell component becomes associated with the virus. A further observation which tends to support this hypothesis is that antisera against A. aegypti mosquitoes neutralizes infectivity of Sindbis virus which were propegated in mosquitoes.

The primary aim of this research project is to define the role of the viral envelope and of cellular membranes in determining the transmission and expression of viruses which are capable of growing in both arthropod and mammalian cells. Specifically, the following questions were posed:

1) Do arboviruses incorporate a host cell component into the virion as they replicate in mosquito cells?

2) If a host cell component is incorporated into virus, is there any biological significance as related to transmission and replication of the virus?

3) What is the identity of this host cell component? Where is it located in the virus and in the mosquito cell?

B. Results

1. Mosquito cell cultures. In order for any of the following results to be valid, the mosquito cell cultures used must be free of any contaminating, or endogenous virus. Contamination of mosquito cell cultures has proven to be a serious problem for investigators and any results obtained from studies must be considered with this contamination problem in mind.

The mosquito cell cultures used in these experiments do not contain any viral contaminants as determined by the following procedures: (1) supernatants from uninfected A. albopictus cells do not contain an agent which agglutinates goose erythrocytes or which replicates in Vero, LLC-MK_2, BHK-21 or A. albopictus cells as judged by plaque assays. (2) co-cultivation of albopictus cells with either Vero or BHK cells did not result in induction or formation of viruses which could be plaqued or grown in either mammalian cells or albopictus cells. (3) Injection of supernatants from uninfected A. albopictus cells, as well as, of disrupted albopictus cells into suckling mice did not result in death. (4) No syncytium formation in uninfected albopictus cells could be demonstrated. (5) Electron microscopic observation of uninfected albopictus cells, -intact and disrupted- and of the growth media which was pelleted failed to reveal the presence of any virus-like structures.

These assays are performed on a monthly basis and if any contaminants were detected by any one of the above procedures, then data obtained since the previous contamination studies are disregarded and not included in this report. By following this procedure, we have lost two months of data, but we feel that the remaining data is valid since these data were obtained using mosquito cells which were free of any viral contaminants.

2. Protection experiments. As stated in our original grant proposal, immunization of mice with albopictus cells was found to confer resistance to 100 LD_{50} of banzivirus grown in albopictus cells, yet this type of immunization had no effect on mice challenged with mammalian cell-grown banzivirus (Table 1). The possibility that a component of the media adheres to the surface of the uninfected cells used as the immunogen, as well as to the surface of banzivirus employed as the challenge was investigated. Mice were immunized with complete media employed in growing A. albopictus cells and then challenged with mosquito cell-grown virus. These immunized mice exhibited the same sensitivity to challenge as did immunized mice, an observation which tends to eliminate the possibility that a component of the media is involved in the observed protection (Table 1).

A recent experiment was performed to determine whether the host cell protein is present in highly purified virus. The rationale for this experiment was that if this host cell protein was merely a contaminant, loosely associated with the virus, then extensive purification of the virus should remove it. Therefore, when this virus was used to challenge mice-control and immunized with mosquito cells- no protection should be observed. Therefore, radiolabeled banzivirus grown in A. albopictus cells were extensively purified by a procedure detailed later in the report. This virus was examined for its peptide profile by gel electrophoresis and used as the challenge virus in the protection experiment. Also, unpurified virus was included in this experiment for comparison. The results are shown in Table 1, Exp. 3. Surprisingly, no protection was observed in the experimental group as compared to the unimmunized group when purified virus was used as the challenge. Gel electrophoresis of the virus revealed the presence of A. albopictus proteins. Also, when unpurified virus was used no protection was observed. It is important to compare the LD_{50} of this experiment with those of the previous experiments. The average LD_{50} for the control group in experiments 1 & 2 was 5×10^2 PFU/mouse, whereas in experiment 3, the LD_{50} was 4-6 PFU/mouse. This suggests that since a new stock of virus was used in the last experiment, the virus has become more virulent for mice, presumably due to repeated passages through suckling mice.

However protection can be observed if the number of surviving mice are plotted as a function of time after challenge (Table 2). At a dilution of 10^{-5} which is the LD_{50}, 100% of the immunized mice survive at day 7 as compared to 60% of the control group. At a LD_{50} of 10 (10^{-4} dilution) at day 7, all of the immunized mice survived as compared to the control; yet at day 14 almost all of the immunized mice are dead. These results do suggest that protection is being demonstrated at earlier times. At later time points there appears no significant differences between the immunized mice and the control group. A reasonable and testable explanation for this result is that not all of the challenged virus are being destroyed by the mouse immune system. The viruses that do escape replicate in the mouse and are now mouse-derived, viruses are now devoid of the A. albopictus host protein. Therefore antibodies against the mosquito proteins are useless in combating this infection at this time. Death ultimately results because of the high degree of virulence this virus has (LD_{50} of 4-6 PFU/mouse), which is in contrast to the LD_{50} of the virus used earlier.

If this explanation is correct, then this highly virulent strain of virus can be attenuated by multiple passages in a non-murine cell line.

3. Antiserum to Uninfected A. albopictus cells: Uninfected mosquito cells were suspended to M & M media with fetal calf serum which was used to prevent extensive clumping of cells and injected into rabbits. The antisera thus obtained, contained antibodies to M & M media, to fetal calf serum, to A. albopictus cells as well as to banzivirus grown in A. albopictus cells as determined by immunodiffusion assays. We have absorbed out antibodies to M & M media and to fetal calf serum in attempts to better define the remaining antibodies. The resultant sera formed precipitin lines, however faint, when reacted against disrupted A. albopictus cells and against banzivirus grown in A. albopictus cells. However no antibodies were directed against BHK-grown banzivirus. Since this antibody preparation was weak, we have started to produce a higher titered antisera by using A. albopictus cells which now can be resuspended in PBS without clumping.

However, we have done some preliminary experiments using this weak antisera, the results of which provide suggestive evidence that the protection afforded to mice by immunization with A. albopictus cells against virus challenge is, at least, partially due to the humoral response. Anti-albopictus serum was assayed for its ability to inhibit viral hemagglutination and to neutralize viral infectivity (Table 3). Low, but detectable, hemagglutination-inhibition (HAI) activity was present as well as neutralizing activity. While these results are suggestive of the fact that, anti-albopictus antibodies can neutralize the infectivity of the mosquito cell grown virus, a high-titer antibody is necessary to demonstrate this fact conclusively. It is of interest to note that the antibodies to A. albopictus cells do not have any HAI or neutralizing activity when virus grown in mammalian cells were used in these assays.

4. Purification of virus. In order to ascertain whether the mosquito cell derived proteins found in banzivirus is a structural component of the virus, or merely a contaminant, a purification procedure was developed. This procedure which is outlined in Table 4 includes polyethylene glycol precipitation, DEAE-cellulose column chromatography, rate zonal and equilibrium gradient centrifugations. Such a series of steps resulted in 170 fold purification; however a very poor recovery of virus was achieved. Loss of virus has occurred

Table 1. Resistance to challenge with banzivirus induced by vaccination of mice with uninfected A cells.

	Condition of Mice	Source of Banzivirus used as challenge	LD_{50} PFU/mouse
Exp. 1	Unimmunized	A. albopictus	5×10^2
		Vero	4.7×10^2
	Immunized with A. albopictus cells	A. albopictus	4.6×10^4
		Vero	4.5×10^2
Exp. 2	Unimmunized	A. albopictus	5.1×10^2
	Immunized with media	A. albopictus	5.0×10^2
	Immunized with A. albopictus cells	A. albopictus	6.2×10^4
Exp. 3	Unimmunized	A. albopictus	4.1
		A. albopictus, purified	6.0
	Immunized with A. albopictus cells	A. albopictus	4.0
		A. albopictus, purified	5.9

* LD_{50} was calculated from the mortality among groups of 7 to 10 mice injected with 10-fold dilutions of virus titered by plaque assay at 37°C. LD_{50} was determined by the Reed-Muench method.

Table 2. Resistance to challenge with banzivirus induced by vaccination of mice with A. albopictus cells.

Dilution of virus	Condition of Mice	Number of Mice Surviving Day 7	Day 9	Day 14
10^{-5}	Immunized	10	9	7
	Unimmunized	6	5	5
10^{-4}	Immunized	10	9	2
	Unimmunized	3	1	0
10^{-3}	Immunized	10	5	0
	Unimmunized	1	0	0

Groups of 10 mice - control and immunized-were injected with dilutions of purified banzivirus. Mice were immunized with uninfected A. albopictus cells.

Table 3. Effect of anti-mosquito sera on Hemagglutination and Infectivity of Banzivirus.

Serum	Source of Virus	HAI	Neutralization
Control	A. albopictus	<1:4	<1:4
	BHK	<1:4	<1:4
Anti-albopictus	A. albopictus	1:16	1:8
	BHK	<1:4	<1:4

HAI Units. The dilution of a serum required to inhibit 8 hemagglutinating units of banzivirus

Neutralization: Dilution of serum required to cause a 50% reduction in the number of plaques.

mainly in two steps - centrifugation of the virus and in the isopynic sucrose gradient centrifugation. In all experiments that were performed, banzivirus is very labile if present in sucrose for greater than 3 hours and is also very labile when it is being pelleted by ultracentrifugation. Nevertheless this experiment was necessary to determine whether the host cell proteins found in virus preparation is a component of the virus. The efficacy of these procedures in purifying radiolabelled banzivirus can be seen in figures 1 and 2. Figure 1 represents the distribution of virus as assayed by hemagglutination assays and of radioactivity in a rate zonal sucrose gradient. The majority of the virus is present in 3 fractions as is a peak of radioactivity and a large amount of cellular proteins is removed from the virus. Results of the isopynic sucrose gradient centrifugation (Fig. 2) shows even further removal of radioactivity from the peak of virus. The density of this peak of HA and radioactivity is 1.175-1.18 which is consistent with the known density of other flaviviruses.

When the virus recovered from the equilibrium density gradient was subjected to gel electrophoresis, three viral proteins and a protein derived from mosquito cells were present (Figure 3). This virus was also used in the protection experiments (Table 1, experiment 3).

As an indication of the effectiveness of this purification procedure in removing cellular contaminants, labeled, disrupted, uninfected A. albopictus cells was mixed with supernatant fluids of infected mosquito cells. This mixture was then subjected to this purification procedure. The final purified virus preparation was assayed for the presence of radioactivity due to cellular components and none was detected. This result not only suggests that we are removing most, if not all, cellular components from banzivirus but also suggests that the host cell protein found in mosquito cell-grown banzivirus is not just associated with the virus. For if indeed this was the case, then in the mixing experiment, labeled cellular components should have been observed with the purified virus. None was detected.

As an additional proof of the efficiency of this purification procedure, in removing cellular contaminants, unlabeled virus grown in mosquito cells was purified and subjected to iodination using the chloramine T procedure. Figure 4 shows the electropherogram of this experiment. It is noteworthy to point out that in addition to the three viral proteins, at least two other proteins are present - one having a molecular weight of 23,000 Daltons and the other which appears to be heterogeneous having a molecular weight of 35,000 to 45,000 Daltons. A possible explanation why this protein (MW of 35,000-45,000) was not detected previously may be related to the means by which the virus was radiolabeled - i.e., hypertonic saline. This protein may not be labeled under the hypertonic saline regimen. By recent improvement in the radiolabeling procedure, this protein (MW 35,000-45,000) has been consistently detected in purified virus grown in A. albopictus cells.

5. Radiolabeling procedure. Although the use of hypertonic saline reduced host cell protein synthesis by 75% and allowed viral proteins to be labeled, we were dissatisfied with this procedure since large amounts of virus as well as large quantities of isotopes were required to show the

Table 4. Purification of Banzivirus.

Material	Virus Recovery		PFU/mg protein
	PFU	%	
Crude medium	70×10^{10}	100	1.2×10^{8}
Clarified medium 1,000 xg 15 min.	69×10^{10}	99	1.6×10^{8}
Resuspended PEG precipitate	67×10^{10}	96	9.6×10^{8}
DEAE cellulose pooled fractions	5.2×10^{10}	74	10.7×10^{8}
Pelleted virus through sucrose	2.2×10^{10}	3	65×10^{8}
Rate zonal centrifugation pooled fraction	1.8×10^{10}	2.5	118×10^{8}
Equilibrium gradient centrifugation pooled fractions	0.1×10^{10}	.15	180×10^{8}

Figure 1. Distribution of banzivirus hemagglutination and radioactivity following rate zonal centrifugation

Banzivirus were grown in A. albopictus cells in the presence of 75 mM excess NaCl and ^{3}H amino acids. After 24 hours of incubation, the supernatant fluids were removed and clarified by centrifugation at 1,000 xg for 15 minutes. After precipitation of the virus with polyethylene glycol (PEG) 6000 (8%) and 2.3 g/l of NaCl, the virus were chromographed on DEAE-cellulose. The pooled fractions containing virus were pelleted by centrifugation and layered onto a 5-40% sucrose gradient and centrifugation for 3.5 hours in a SW 25.1 rotor at 25,000 rpm. The gradient was fractionated into 1 ml aliquots and each aliquot was assayed for hemagglutination activity as well as radioactivity.

Figure 1

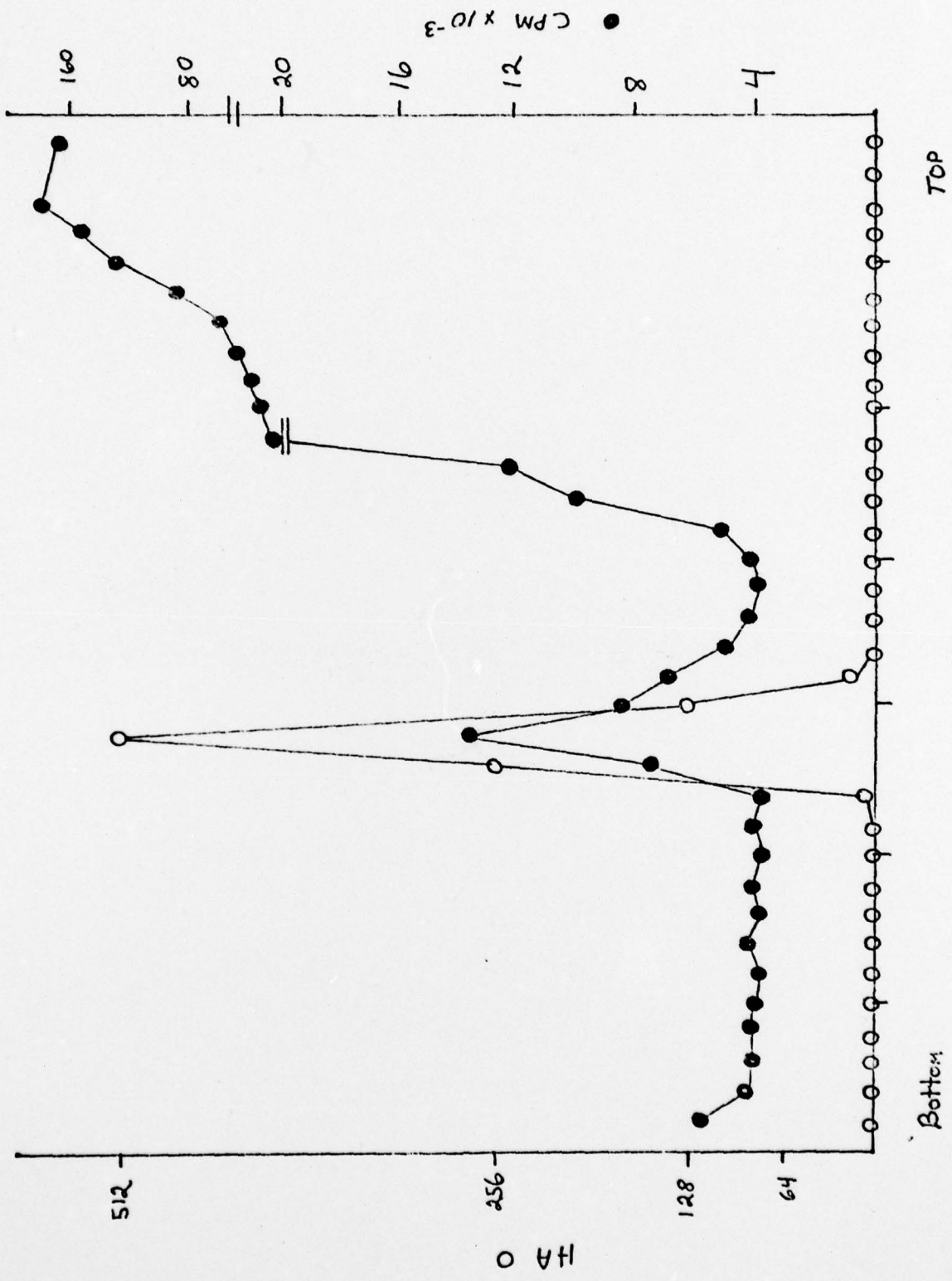


Figure 2. Distribution of banzivirus hemagglutination and radioactivity following isopynic gradient centrifugation

Fractions of the rate zonal gradient (Figure 1) which contain virus were pooled and layered on top of performed 28-50% sucrose gradients and then were centrifuged in a SW 25.1 rotor for 24 hours at 25,000 rpm. The gradient was fractionated into 1 ml aliquots and each aliquot was assayed for hemagglutination activity as well as radioactivity. In addition, the specific gravity of each fraction was determined (data not shown).

Figure 2

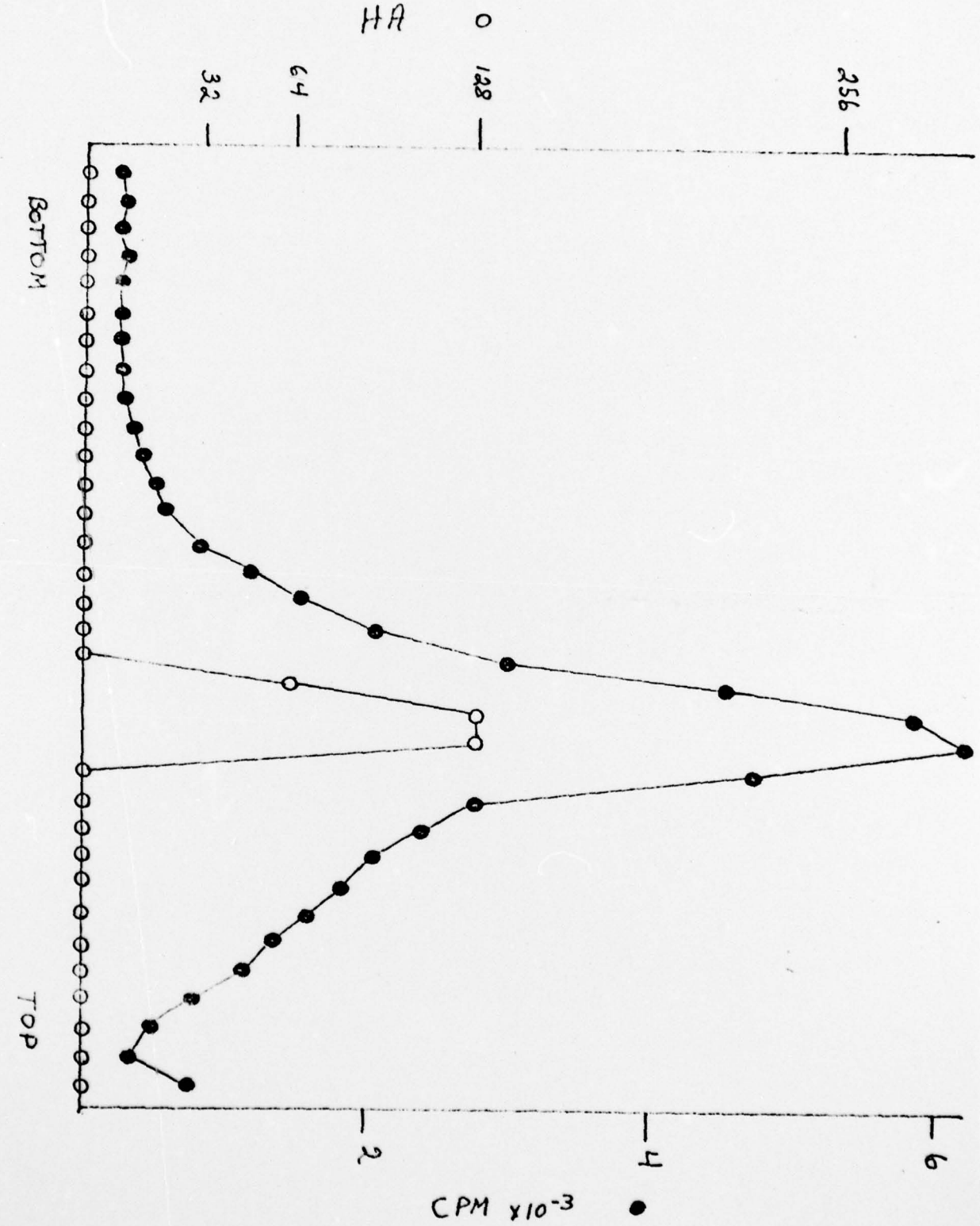


Figure 3. Electropherogram of virus isolated from the isopynic sucrose gradients. Aliquots of the isopynic sucrose gradient which contained virus were pooled and precipitated with trichloroacetic acid. The virions were then subjected to electrophoresis on a 5-12.5% polyacrylamide gels. The gel was then sliced and radioactivity was determined by scintillation counting.

Figure 3

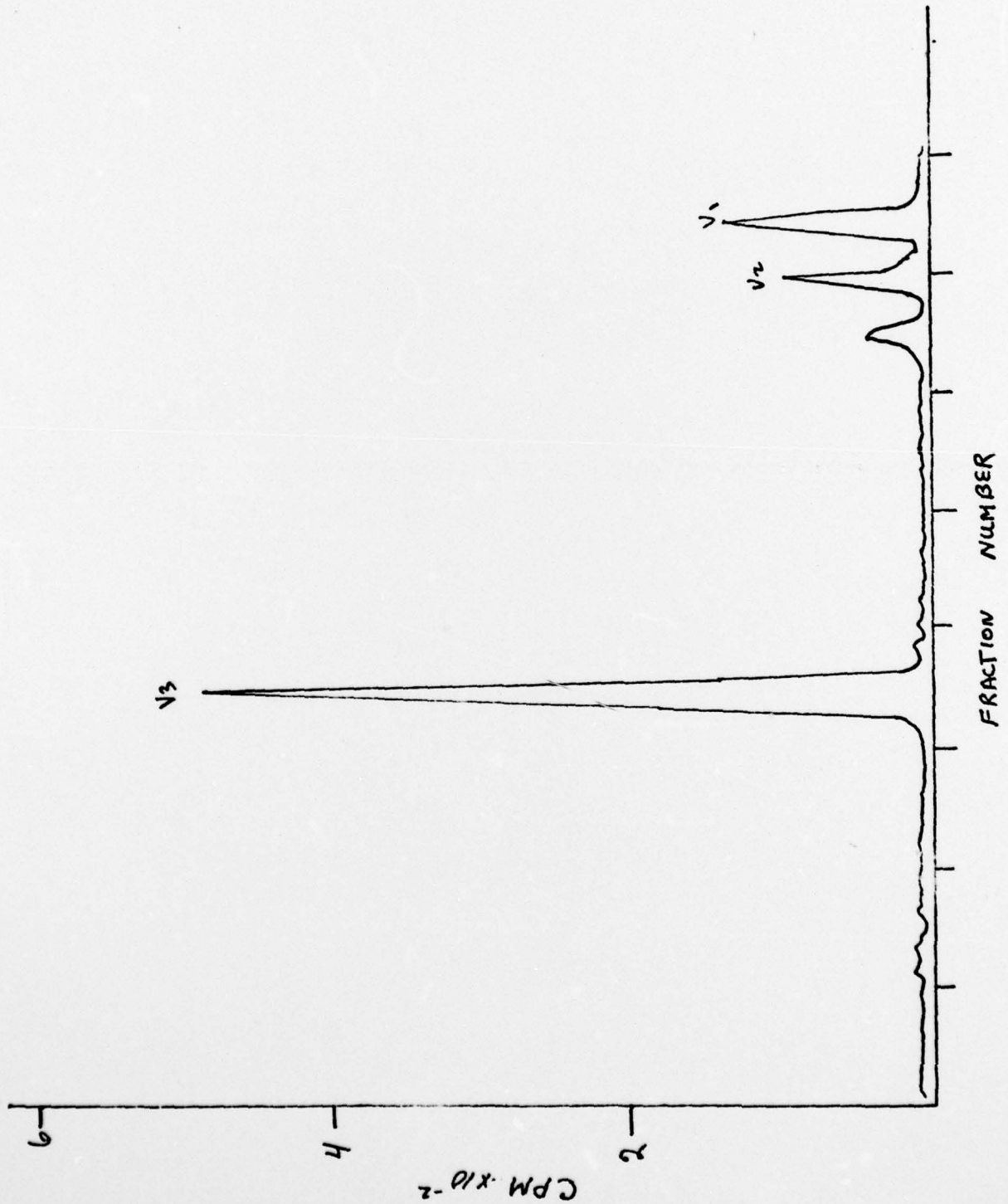
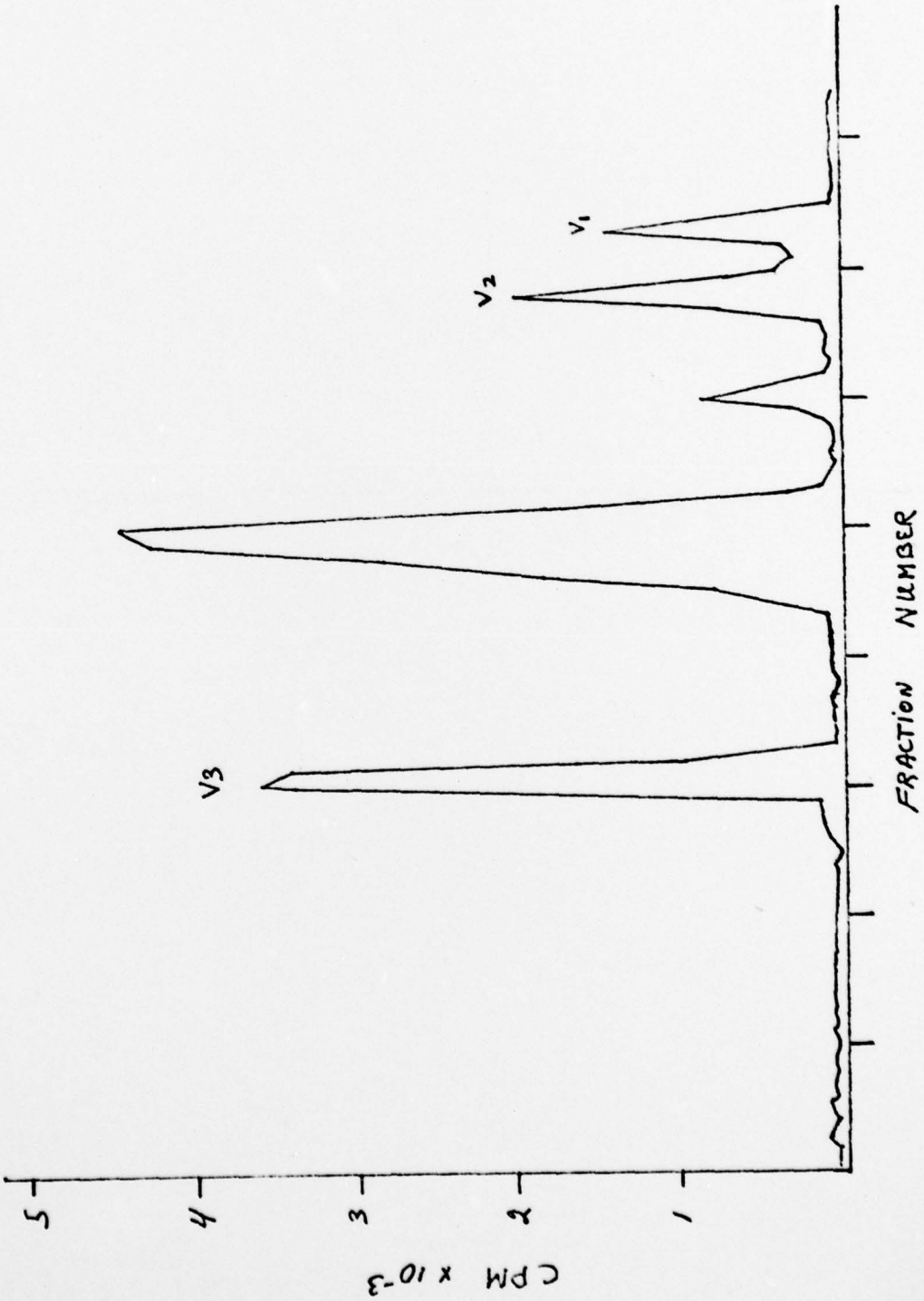


Figure 4. Electropherogram of purified banzivirus iodinated via chloramine T.

Unlabelled banzivirus grown in A. albopictus cells was purified by procedures outlined in table 4. The purified virus was then iodinated using the chloramine T method and then subjected to electrophoresis on a 5-12.5% polyacrylamide gel.

Figure 4.



presence of radiolabeled viral proteins. In addition, we were concerned about the high levels of pools of intracellular amino acids which dilute the radioactive isotope. Therefore, we have investigated alternative means to radiolabel virus. The procedure we are currently using is to starve the cell cultures for amino acids for four-six hours prior to infection so as to reduce intracellular amino acids pools. After adsorption of the virus, hypertonic saline is added to the infected cells along with media devoid of unlabeled amino acids and the appropriate labeled amino acid. Higher levels of radioactivity are present in purified virions and the cost of these experiments have been reduced by 50%. The results of a typical experiment is essentially identical to that shown in figure 4. That is, in addition, to the three viral proteins, two non-viral proteins appear in purified virions We feel that the appearance of the new protein (MW of 35,000-45,000) is due to improved labeling procedures.

The possibility exists that hypertonic salt conditions somehow altered intracellular viral protein synthesis which may result in the formation of "abnormal" viral structural proteins. These "abnormal" viral structural proteins may be incorrectly designated as a host cell protein incorporated into virions. To eliminate this possibility, virus grown in BHK cells were radiolabeled in the absence and in the presence of hypertonic salt and then purified. Gel electrophoresis of virus radiolabeled in the presence of hypertonic saline revealed three viral proteins whose molecular weights are identical to those viral proteins obtained from virus radiolabeled in the absence of hypertonic saline. Therefore, it seems unlikely that the extra proteins found in mosquito cell-grown virus are the result of abnormal viral proteins synthesized in the presence of hypertonic salt.

Methods that totally inhibit mosquito cellular protein synthesis without substantially reducing viral protein synthesis were investigated. It was reasoned that if the extra proteins found in mosquito cell grown virus are of host cell origin then under conditions which inhibited total cellular protein synthesis, virus may be obtained which lacks these extra proteins. If such was found, then it would show that the extra proteins found in the virus are of mosquito cell origin. All methods used to totally inhibit cellular protein synthesis-pretreatment of cells with actinomycin D or with high levels of hypertonic saline-also substantially reduced viral protein synthesis.

To demonstrate that a host cell component was incorporated into banzivirus, mosquito cells were prelabeled for 12 hours with various radiochemicals in media containing 1/100 of the normal amount of amino acids, or of glucose. Prior to infection, external label was removed by extensive washings and incubated for 3 hours. After absorption of the virus, media containing the normal amount of amino acids and/or glucose and hypertonic saline was added. After 24 hours of infection, released virions were harvested, purified by PEG precipitation, and rate zonal centrifugation and examined by gel electrophoresis for presence of radioactivity. As indicated in Table 5, radioactivity was present in two areas of the gel corresponding to proteins having a molecular weight of 23,000 Daltons and 35,000 to 45,000 Daltons when cells were prelabeled with ^{3}H amino acids, ^{35}S methionine and ^{14}C amino acids were used. Significant amounts of ^{3}H glucosamine were found in the 35000 to 45000 molecular weight protein.

Table 5. Distribution in purified various isotopes used to prelabel A. albopictus cells.

Isotope Used in pretreatment of cells	Proteins				
	V3	V2	V1	MW 23,000	MW 35,000-45,000
^{14}C amino acids	-	-	-	+	+
^{3}H amino acids	-	-	-	+	+
^{35}S methionine	-	-	-	+	+
^{3}H-glucosamine	-	-	-	+	+

6. Solubilization of the A. albopictus proteins. Methods were investigated to solubilize the A. albopictus cell proteins from purified virions so that biochemical analyses could be done. Also, this solubilized, purified protein would be used as an immunogen in protection experiments. A non-ionic detergent, Triton X-100, was used so that the proteins would be biologically active after solubilization. Initial experiments utilizing Triton X-100 in the absence or presence of various concentrations of NaCl did not disrupt the virus. We plan to use NP-40, another non-ionic detergent, alone or in combination with Triton X-100 in further experiments.

C. Conclusions:

1. Two mosquito cell proteins have been identified as being associated with banzivirus grown in A. albopictus cells. This fact has been demonstrated by several observations. First, prelabeled cellular components are incorporated into the virus during viral replication in the absence of label. Second, the two mosquito cell proteins are present in virus that have been purified by at least 160 fold. Third exogeneously added cellular proteins when added to the virus prior to purification are not detected in the final purified virus. Fourth, precipitin lines are formed in immunodiffusion assays when anti-albopictus sera reacts with purified banzivirus grown in mosquito cells.

2. Both mosquito cell proteins appear to be glycoproteins in that they contain label derived from ^{3}H-glucosamine.

3. Immunization of mice with uninfected mosquito cells protects against infection by banzivirus grown in mosquito cells. This protection is not due to any media components and is due in part to the humoral response of the mouse, to mosquito cell proteins in that antisera to uninfected mosquito cells neutralize infectivity of mosquito cell grown banzivirus. The extent of protection is dependent upon the degree of virulence of the virus.

D. Research Plans 3/1/78 to 6/30/78.

1. Attenuation of banzivirus by multiple passages through non-murine cell lines.

2. Obtain high titer antibodies to mosquito cells to use in a). HAI assays b). Plaque reduction assays c) identification of mosquito cell proteins in banzivirus.

3. Treatment of purified virus with proteases, iodination and detergents so that location of mosquito cell proteins can be ascertained. Detergents will also be used to solubilize the mosquito cell proteins on the virus so that purified proteins can be biochemically analyzed and can be used as immunogens.