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ANTIGENTIC RELATIONSHIP BETWEEN
VIRUS ENVELOPE AND HOST CELL MEMBRANE

BY

ANTHONY A. FRER, D.D.S.

A THESIS SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL

OF LOYOLA UNIVERSITY IN PARTIAL FULFULLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

JUNE

1970

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MY WIFE

DENISE

FOR HER LASTING PATIENCE

AND GREAT SACRIFICES

MADE IN ORDER TO FURTHER

MY EDUCATION.

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Anthony A. Frer was born in Chicago, Illinois on February 16, 1942. He graduated from Saint George High School in 1959 and then attended one year of college at Saint Ambrose in Davenport, Iowa. In 1960, he transferred to Loyola University and received a Bachelor of Science degree in 1964. He attended Loyola Dental School from 1964 to 1968, and received the degree of Doctor of Dental Surgery. Since June of 1968, he has been a post graduate student in the Loyola Oral Surgery Department and has been enrolled in the Department of Oral Biology of the Loyola Graduate School working for a Master of Science degree in Oral Biology.

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CHAPTER I

INTRODUCTION

Viruses are the smallest infective agents known; however, the diseases they produce are by no means minor. In the late 1950's the use of monolayer tissue culture cells in virology experimentation became perfected and since that time data concerning viral infectivity has been compiled.

Herpes simplex viruses are unique in that they are the only group of cubically symmetrical viruses known which possesses an envelope derived from and consisting mainly of host cell material. Determining the mechanism by which such an envelope can contribute so markedly to the infective potential of the herpes virus naked particle seems to be a matter of considerable importance in understanding the biology of the virus.

It is the specific intent of this paper to demonstrate and evaluate the antigenic relationship between the envelope of herpes simplex virus and its host cell membrane. I hope to contribute significant information which will be of value in understanding the biology of infection of this virus.

CHAPTER II

REVIEW OF THE LITERATURE

A. Morphology.

The most classic and detailed work regarding the morphology of herpes simplex virus was reported by Wildy, Russel, and Horne in 1960.[1] Herpes virus, strain HFEM, was inoculated into tissue culture HeLa cells and the virus produced within these cells was studied with the electron microscope, using the phosphotungstate method for negative contrast.

The phosphotungstate method for negative contrast was described by Brenner and Horne in 1959.[2] Their technique employed a 2 per cent phosphotungstate acid solution with a pH of 7. The solution was prepared by dissolving phosphotungstic acid in distilled water and adjusting the pH by the addition of 1N KOH. Concentrated virus suspensions were mixed with equal volumes of phosphotungstate and then sprayed on carbon-coated electron microscope grids.

Wildy, Russel, and Horne observed that the viral particles consisted of three main parts: the core, the capsid, and the envelope.

The core occupies a central position, is roughly hexagonal in shape and has an average diameter of 775±5Å.

The capsid which surrounds the centrally located core has an average diameter of 1050±10Å. The capsid is composed of capsomeres; capsomeres being hollow elongated structures which at high magnification

appear polygonal in cross section. When the capsomeres are viewed along their longitudinal axes, the exact shape of the sections can be determined, and in most instances they appear to be hexagonal prisms. The average diameter of the capsomere is 95±1Å.

Upon further inspection, the capsomeres were found to have a variety of arrangements. Many capsomeres were found to be surrounded by six and in some instances five neighboring capsomeres. This suggests that they are situated on axes of five-fold symmetry. Between these points of symmetry, three capsomeres, each surrounded by six others, are found. Spaces are found between each capsomere and when it is possible to count the number of capsomeres on the equator of the capsid, 24 can be identified.

The outer envelope appears as a halo surrounding the capsid and has an average diameter of 1800Å. The periphery of this layer appears more dense than the remainder, giving the appearance of a membrane which at its thickest is 40-50Å thick and which in places appears as much as 100Å thick. On the surface of the envelope are seen periodic projections about 80-100Å long; frequently these projections are spaced at intervals of about 50Å.[3]

B. Cytology.

The mode of development of herpes simplex virus has long been the object of extensive study and controversy. As early as 1921,

investigators studied the cells of tissues infected with herpes virus and reported the presence of intranuclear inclusion bodies. Investigators began to speculate as to the nature of these intranuclear inclusions. Some believed the inclusions were a stage in viral multiplication and composed of viral material, while others believed them to be products of cellular metabolism.

In 1925, Goodposture[4] induced herpetic lesions on rabbit corneas. Upon examination of individual cells, he concluded that there were no intranuclear inclusions, but rather cytoplasmic inclusions were produced.

In 1946, Lepine and Sautter[5] presented their findings regarding the nature of herpetic intranuclear inclusions. These investigators said herpetic inclusions were not virus. They based their findings on the following facts: viruses have been analyzed chemically and have been found to contain nucleoprotein; animal viruses contain nucleoprotein of the desoxyribonucleic acid type; nucleoprotein in tissue stains with toludine blue; nucleoprotein of the desoxyribonucleic acid type gives a positive Feulgen reaction. Lepine and Sautter reported negative Feulgen and toludine blue reactions when staining their tissue sections.

In 1950, Francis and Kurtz[6] employed ultracentrifugation to determine whether herpes virus has a selective affinity for the

nuclei of susceptible cells and to ascertain whether the virus is intimately related to the nucleoprotein of the nucleus. Suspensions of nuclei from embryonic chicken liver cells and herpes simplex virus, J. R. Smith strain, were prepared. The suspensions were allowed to set for 24 hours and were then removed and centrifuged. The supernatant fluids were then titrated intracerebrally with four-fold dilutions to determine whether virus was removed from the fluid, presumably by attachment to available cellular material. If virus was selectively absorbed to nuclei, it should be present in reduced amounts in the remaining supernatant fluid.

The experimental data reported showed that nuclei did not unite with virus to remove it in significant amounts from the supernatant fluid. Francis and Kurtz concluded the inclusions produced by herpes virus are nuclear in location, but intranuclear inclusions are not herpes virus.

With the use of the electron microscope, Morgan, Ellison, Rose, and Moore in 1953[7] were confident that they could end the long-standing controversy regarding the nature of herpetic intranuclear inclusions. They studied sections of chicken embryo chorioallantoic membranes infected with the HRE strain of herpes simplex virus. The nuclei of these cells appear to be altered and contained many particles ranging in size from 100 to 130 mu in diameter. They assumed these particles to be virus.

Upon review of their work reported in 1953, Morgan and his associates stated that their conclusions regarding the particles which they found within the nuclei of the forementioned cells may have been presumptive. In 1954, Morgan, Ellison, Rose, and Moore[8] felt that a detailed study of the changes in herpes virus infected cells was necessary to determine the exact nature of intranuclear inclusions.

The HRE strain of herpes simplex virus was inoculated onto the chorioallantoic membranes of chicken embryos and the ensuing cytological changes were noted.

Margination of fine, dense, reticular material constituted the first apparent change within the infected nuclei. Within the reticulum small, dense primary or central bodies, 30 to 40 mu in diameter differentiated. This reticular material appeared to contribute in some manner to the development of the virus. A membrane, 60 to 80 mu in thickness and 70 to 100 mu in diameter formed and enclosed the central body. Some nuclei contained multiple foci of development where large numbers of particles or central bodies formed as aggregates. The nuclear membrane appeared to disrupt at any stage of recognizable infection with liberation of virus into the cytoplasm. Within the cytoplasm a second membrane was formed having a diameter of 120 to 130 mu.

From these cytological changes, Morgan and his associates stated

that the so-called inclusion bodies are, in fact, altered cell nuclei in which chromatin was marginated. Viral particles were present in addition to intranuclear inclusions. These structures, small viral particles, are stages in the development of the virus. The work of Crouse, Coriell, Blank and Scott[9] supports the findings of Morgan and his associates. Crouse and his associates call these developmental stages of the virus within the nucleus, viral elementary bodies.

In the late 1950's the use of monolayer tissue culture cells in virology experimentation became increasingly more popular. The relative ease of obtaining and maintaining established cell lines attracted many investigators. A review of the literature disclosed the fact that the cell line most often used was the HeLa cell line. My experimentation employed the Hep^{-2} cell line.[10] Due to the basic similarity exhibited by all monolayer tissue culture cells, investigators feel that the data obtained when studying HeLa cells can be considered to apply to Hep^{-2} cells and other monolayer tissue culture cells.

When herpes simplex virus enters susceptible cells, such as HeLa or Hep^{-2} cells, it passes through characteristic stages:

Stage 1 - The virus is fixed to the susceptible cell, but can be neutralized by antiserum.

Stage 2 - The viral particle penetrates the susceptible cell and is no longer susceptible to antiserum.

Stage 3 - The viruses are taken apart by the host cell, following which new viruses are formed.

Stage 4 - The period during which viruses are released from the host cell.

The period of viral attachment to the membrane of a susceptible cell has been demonstrated by Stroker and Ross.[11] Using the HFEM strain of herpes simplex virus, with a known pock-forming ability, HeLa cells were inoculated and herpes antiserum added. It was noted that less than one tenth of the pock-forming particles were able to initiate an infection in the HeLa cells. The pock-forming virus had attached to the susceptible cells, but was neutralized in the presence of the antiserum and presumably did not penetrate the cell membrane.

It is evident that the viral particle is no longer susceptible to antiserum after it has penetrated the membrane of the susceptible cell. Shortly after penetration the viral particle is disassembled by the host cell and new virus begins to develop in the nucleus of the host cell.

The development of the new virus has a profound effect upon the host cell. In 1959, Stroker and Newton[12] showed that infection with HFEM strain of herpes virus prevented HeLa cell multiplication. They based their conclusion on the fact that DNA was present in the cells in amounts too great to be accounted for by the number of virus particles present. The extra DNA present was in fact normal cellular

DNA which had not been distributed to daughter cells, owing to failure in cell division.

In 1961, Wildy, Smith, Newton, and Dendy[13] substantiated the work of Stroker and Newton. They observed in HeLa cells infected with herpes virus two types of cellular lesions; the well known intranuclear lesion of herpes virus and a lesion of mitosis. They described the lesion of mitosis as the appearance of abnormal mitotic cells resulting from spindle damage. It was this spindle damage which prevented HeLa cell division. The intranuclear lesion was described as margination of chromatic material.

In 1967, Shipley, Erlandson, Bailey, Babcock, and Southam[14] inoculated HeLa cells with the HF strain of herpes simplex virus and found that there was no evidence of cytopathology on days one or two. By day four, cell damage was definite but no frequent, and by day seven it was extensive. The overall effect was cell destruction.

The principle cytologic abnormality was diminished staining of the nucleoplasm with margination of chromatin. Nuceoli were swollen. No inclusion bodies were found in nuclei or cytoplasm. The persistence of nucleoli in an otherwise empty-appearing nucleus gave a superficial resemblance to an intranuclear inclusion body, but their identity as nucleoli was evident by their basophilic staining. In many of the altered cells, small clusters of dense granules were observed in the nucleoplasm. The granules had a diameter of 30-60 mu. These granules

were elementary bodies, or developmental stages of the herpes virus.

In 1959, Morgan, Rose, Holden, and Jones[15] infected HeLa cells with the HR strain of herpes simplex virus and studied its development with the electron microscope. The viral particles or elementary bodies, developing within the nucleus of the HeLa cells, were randomly dispersed and possessed a single limiting membrane.[16] At this stage the viral particle is termed a naked particle and possesses a central core and a single limiting membrane, the capsid. As the viral particle leaves the nucleus and passes through the cytoplasmic membrane, a second membrane is acquired. The second membrane is the envelope of the virus.[17]

The mode of release of developing herpes virus in HeLa cells has been the object of much study. It is evident that virus may enter the cytoplasm of an infected cell upon disruption of the cell nucleus, but nuclear disruption does not always occur. Virus can also exit from the cytoplasm of an infected cell if the cell is ruptured, but cell rupture is not common.

In 1959, Morgan, Rose, Holden, and Jones studied viral release mechanisms in HeLa cells infected with the HR strain of herpes simplex virus. Viral egress from intact nuclei is made possible by nuclear membrane reduplication. They observed with the electron microscope a process whereby new nuclear membranes were laid down behind the virus as it passes into the cytoplasm. When the virus had passed into the

cytoplasm, it became lodged in cytoplasmic vacuoles. The vacuoles moved toward the cell membrane of the infected cell and upon union both vacuole and cell membrane open to release virus into the extracellular space. These findings were verified in 1962 by Epstein.[19]

With the release of herpes virus into the extracellular space, infection of surrounding cells takes place without difficulty. It is a known fact that antibody added one-half hour after the addition of herpes simplex virus to tissue culture does not interfer with virus reproduction.

In 1955, Black and Melnick[20] working with herpes virus infected tissue cultures showed that herpes virus can spread through direct contact from cell to cell even when antibody is present in the extra-cellular pathway.[21]

C. Cytochemistry.

Herpes simplex virus is composed of nucleic acid, DNA, and an outer protein coat. The base composition of the DNA of herpes virus is:[22]

Adenylic acid	-	27.6±.01%
Thymidylic acid	-	28.1±0.4%
Guanylic acid	-	21.9±0.3%
Cytidylic acid	-	22.4±0.3%

When DNA of this composition is found in cells infected with herpes virus it can be termed, viral antigen.

In 1956, Lebrun[23] by means of fluorescent antibody staining studied the localization of viral antigens in cells infected with herpes virus.

Lebrun used Hep^{-2} cells infected with the HF strain of herpes simplex virus. According to Lebrun, viral antigen made its appearance as a small spherical dot or spot in the nucleus and increased slowly in amount there, and later appeared in the cytoplasm. Other investigators working with the same strain of herpes virus and different susceptible cells reported the same findings.[24 25]

In 1958, Allison, Newton, and Stroker[26] performed further studies on nucleic acid changes in HeLa cells infected with herpes virus. Using the HFEM strain of herpes simplex virus, they reported that there was a significant change in the DNA content of the infected cells. Nine hours after initial infection, the infected cells showed a 40 per cent increase over the control cells in DNA content. The DNA content of the infected cells rose slightly at six hours and significantly at nine hours. New virus was not detectable in the cells until 12 hours after infection, five hours after a significant rise in the DNA content of the cells was noted.

In 1966, Keir, Subar-Sharpe, Sheddan, Watson, and Wildy[27] using a system of Hep^{-2} cells infected with herpes simplex virus demonstrated the activity of DNA-polymerase within the infected cells which was not normally present within the cells. They concluded that the synthesis of this new DNA-polymerase was directed by the DNA of the invading herpes virus.

In 1968, Ross, Watson, and Wildy[28] who had been studying Hep^{-2}

and HeLa cells infected with herpes virus, began study of a similar tissue culture cell. The new cell studied was the BHK-21 cell. The cells were grown in the same manner as Hep-2 and HeLa cells and were infected with the same virus, HFEM strain of herpes simplex. Using fluorescent staining, it was seen that two hours after infection herpes virus antigens were present as fluorescent dots at the cell membrane and in the cytoplasm of the infected cell. During the third hour post-infection, bright fluorescent patches were seen within the nucleus of most cells and at six hours post-infection the observations were the same except for a significant increase in intensity. At nine hours post-infection there was bright fluorescence seen throughout most of the cells.

D. Envelope and Infectivity.

In 1962, Epstein[29] using an electron microscope and HeLa cells infected with the HFEM strain of herpes simplex virus reported that the host cell membrane is morphologically similar to the envelope of the herpes virus grown within the host cell.

In 1963, Watson and Wildy[30] using the HFEM strain of herpes simplex virus infected HeLa cells and recovered the virus produced within these cells. They then prepared anti-herpes virus serum according to the procedure outlined by Vantsis and Wildy in 1962.[31] Anti-HeLa cell serum was also prepared. It was made by inoculating rabbits intramuscularly with three weekly doses of whole washed HeLa

cells. Normal sera was obtained from rabbits before other inoculation procedures.

Virus, both naked and enveloped particles, which were obtained from the infected HeLa cells were mixed with normal sera, anti-herpes serum, and anti-HeLa cell serum. The degree of virus particle clumping was observed with an electron microscope.

In the presence of normal serum only occasional small clumps occurred whose distribution was not informative. In the presence of anti-HeLa cell serum, marked clumping was observed and most of the clumped particles were enveloped; only occasional naked particles were seen in the clumps. In the presence of anti-herpes serum much clumping occurred, all the clumped particles were naked, no enveloped particles whatsoever were included.

Watson and Wildy concluded that the envelope contains host cell antigen and that the viral antigen is restricted to naked particles.

Watson and Wildy also demonstrated that the envelope of herpes simplex virus possesses receptor sites. Using the electron microscope, it was seen that enveloped virus particles attached to influenza virus of the WSE strain.[32]

Many investigators state that the envelope of herpes virus plays an important role in infectivity. In 1963, Smith[33] infected rabbit kidney cells with the VA strain of herpes simplex virus. Smith demonstrated that removal of the virus envelope changed virus

infectivity. Viral envelopes were removed with diethyl ether and infectivity was found to be markedly reduced or completely lost.

In 1964, Smith [34] employed ultracentrifugation to study the role of the viral envelope in infectivity. By means of ultracentrifugation, Smith was able to isolate naked and enveloped virus particles from the HSV-UIII and SOK strains of herpes simplex virus. Plaque titrations, virus staining and virus particle counts were done on all isolated viral fractions after centrifugation. Particles in the electron micrographs were counted and classified as either naked or enveloped.

The results obtained are illustrated in Table I. Table I shows the results of two experiments in which two strains of herpes virus, HSV-UIII and SOK, were fractionated, titrated and the particles counted. In the first experiment, it is seen that HSU-UIII fractions four and five contained less than one per cent enveloped forms, and less than one-thousandth of the total infectious units.

The particle pock forming unit ratios of the two virus fractions were extremely high, over 10^5 and 10^4 respectively. This was in sharp contrast to the next three fractions, which contained 88-92 per cent enveloped forms which were highly infectious and had relatively low particle pock forming unit ratios.

Similar results were obtained with the SOK virus strain and these are shown in the lower part of Table I. Fraction five contained more than 99 per cent naked particles and had a particle pock forming

unit ratio of 8,000. This was in sharp contrast to the highly infectious fractions 8-10, which contained large portions of enveloped particles, 70-82 per cent, and had a particle pock forming unit ratio of 10-12.

Smith concluded from these results that those fractions containing only naked herpes virus particles are practically noninfectious. Fractions containing enveloped particles are highly infectious.

TABLE I

DENSITY GRADIENT FRACTIONATION OF HERPES SIMPLEX VIRUS

	Fraction No.*	No. physical particles/ml ($X10^7$)	No. PFU/ml ($X10^7$)	Particle:PFU ratio	% enveloped forms
	8	170	2.1	81	92
Exp #1	7	180	2.6	69	91
Strain	6	110	1.5	74	88
HSV-VIII	5	60	.005	12,000	<1
	4	110	<.001	>100,000	<1
	3	<1	<.001		
	10	140	14	10	70
	9	220	18	12	72
Exp #2	8	500	41	12	82
Strain SOK	7	250	27	9	18
	6	110	.1	1,100	<1
	5	24	.003	8,000	<1

*Eleven fractions were collected in each experiment; lower numbered fractions were taken from the lower part of the tube by the drip method and were therefore more dense.

*Most of the naked particles in this fraction and those following it were coreless, as judged by both positive and negative staining.

CHAPTER 3

MATERIALS AND METHODS

A. Tissue Cultures.

Monolayer tissue culture cells of the Hep-2, Human Epidermoid Carcinoma, cell line were used. These cells were obtained as an already established cell line growing on the flat surface of 32 oz. media bottles. The line was donated by Dr. Max Rosenbaum of Great Lakes Naval Hospital.

1. Growth Media Preparation. The Hep-2 cells were grown in Eagle's Minimum Essential Medium with 10 per cent calf serum. The media was purchased in premeasured powdered packets.

To prepare the growth medium, two sterile 2,000 ml flasks were filled with 1,200 ml of triple distilled water at 15-20°C. Three packets of premeasured media were opened and poured into the flasks of triple distilled water with a gentle stiring action. Each packet was rinsed with triple distilled water to remove all traces of powdered medium. For each packet of medium used, 2.2 gm of $NaHCO_3$ was carefully weighed on an Ohaus triple beam balance and added to the flasks. The contents of each flask were poured back and forth to insure complete mixing. When mixing was complete, the total volume of both flasks, 2,400 ml, was increased to a final volume of 3,000 ml by adding an additional 600 ml of triple distilled water. The pH of the medium was then adjusted to a final working pH of 7, by the addition of 1N NaOH

or 1N HCL.

The 3,000 ml volume of growth media was then sterilized by membrane filtration with a Millipore filter and collected in 900 ml aliquots. Sterile 1,000 ml media bottles were used to collect each 900 ml aliquot.

To each 1,000 ml media bottle containing 900 ml of media was added 100 ml of inactivated calf serum. The calf serum was stored frozen in 100 ml bottles. The frozen calf serum was thawed in 37°C water bath and then inactivated by transferring the thawed calf serum to a water bath at 56°C for one-half hour.

To each 1,000 ml bottle of growth media was added 10 ml of Antibiotic-Antimycotic solution and 0.5 ml of Fungizone. The Antibiotic-Antimycotic solution contained: 10,000 u/ml of penicillin; 25,000 mcg/ml of Fungizone, and 10,000 mcg/ml of streptomycin. The Fungizone was purchased from Squibb. Each bottle or vial contained 50 mg of dry powdered Fungizone which was reconstituted by adding 10 ml of sterile water.

The growth medium containing 10 per cent calf serum and antibiotics was stored in the refrigerator until needed.

2. STV-Saline, Trypsin, Versene Solution Preparation. STV solution is required to remove Hep^{-2} cells from the glass surface to which they adhere during growth.

STV is prepared aseptically as follows: to one-liter sterile medium bottle add 840 ml of sterile water, 100 ml of 10X saline, 8.3 ml

of 2.5% Hyland trypsin, 20 ml of 1% versene, 10 ml of Antibiotic-Antimycotic solution, and 0.5 ml of Fungizone. Additional sterile water was added to obtain an over-all volume of 1 liter.

a. Trypsin Preparation. Trypsin was purchased from Hyland Laboratories in California. It was premeasured and packed in bottles in a freeze-dry state in 2.5 gm units. To obtain a working solution of 2.5%, 100 ml of sterile water was added to the stock bottle. The stock bottle was stored in the freezer compartment of the refrigerator and removed and thawed in a 37° water bath when needed.

b. 10X Saline Preparation. The following ingredients were weighed carefully on an Ohaus triple beam balance scale and added to one liter of triple distilled water:

NaCL	-	80gm
KCL	-	4 gm
$NAHCO_3$	-	3.5 gm
Dextrose	-	10 gm
Phenol red 5 per cent	-	18 ml

The one liter solution of 10X saline was then sterilized by membrane filtration with a millipore and put into ten 100 ml stock bottles. These stock bottles of 10X saline were stored in the freezer compartment of the refrigerator.

c. Versene Preparation. One per cent versene solution was prepared by dissolving 10 gm of versene in one liter of triple distilled water. The versene solution was then autoclaved at 15 lbs. of pressure for 15 minutes and stored in the refrigerator.

3. Hep^{-2} Cell Line Maintenance. The Hep^{-2} cell line used in this study was obtained growing on the flat surface of 32 oz. tissue culture bottles. The cell line required considerable maintenance in order to continue. Twice a week, approximately every four days, it was necessary to engage in a cell-splitting procedure. The cell-splitting procedure removed the Hep^{-2} cells growing in a solid sheet on the flat surfaces of 32 oz. tissue culture bottles, and enabled them to be transferred to other bottles so that growth could continue.

The procedure was accomplished, using aseptic techniques, as follows:

1) The growth medium within the tissue culture bottles was carefully drained in such a manner as to avoid removal of Hep^{-2} cells growing on the glass surface. The medium was discarded.

2) To each tissue culture bottle, 25 ml of sterile STV was added. The bottles were then placed in the 37°C incubator for 15 minutes. The tissue culture bottles were placed flat side down so that the STV would bathe the Hep^{-2} cells.

3) The tissue culture bottles were removed from the incubator and 25 ml of 10 per cent calf serum growth media was added to each bottle to counteract the action of the trypsin which had now removed the Hep^{-2} cells from the glass surface.

4) The Hep^{-2} cell-growth media mixture contained within the tissue culture bottles was poured into 50 ml centrifuge tubes. The 50 ml

centrifuge tubes were placed in an International Clinical Centrifuge, Model CL, and centrifuged for 10 minutes at 5,000 rpm.

5) The 50 ml tubes were removed from the centrifuge and the supernatant fluid discarded, leaving the Hep^{-2} cells on the bottom of the 50 ml centrifuge tubes.

6) The Hep^{-2} cells on the bottom of each 50 ml centrifuge tube were collected into one tube to which was added 10 ml of 10% calf serum growth media. This tube was then gently shaken to insure mixture of the Hep^{-2} cells in the growth medium.

7) From this tube containing the Hep^{-2} cell growth media mixture, 1/10 ml was removed and placed in a small test tube. To this sample was added 1/10 ml of trypan blue.

8) One drop of the Hep^{-2} cell-trypan blue mixture from the small test tube was placed on a hemacytometer slide and covered with a glass coverslip. The Hep^{-2} cells were then counted.

9) Dilution equation

$$\text{ave. \#cells counted} \times 2 \times 10^5 = \text{cells}/10\text{ ml}$$

$$\frac{\#\text{cells}/10\text{ ml}}{\#\text{cells}/\text{ml}} = \text{volume to Q.S. 10 ml to}$$

$$\#\text{cells}/\text{ml} = 1 \times 10^5$$

10) Using the above equation, it was possible to determine how much additional growth media had to be added to the initial 10 ml Hep^{-2} cell-growth medium mixture. This amount of growth medium was added. The final volume obtained determined how many 32 oz. tissue

culture bottles could be made.

To one 32 oz. tissue culture bottle, 50 ml of Hep^{-2} cell-growth media mixture was added.

11) The newly seeded 32 oz. tissue culture bottles remained in the incubator for four days, the time needed for the Hep^{-2} cells to form a solid sheet on the glass, and the cell splitting procedure was again performed.

The 32 oz. tissue culture bottles were prepared in order to maintain the Hep^{-2} cell line. Plastic tissue culture flasks containing 5 ml of the Hep^{-2} cell-growth media mixture were also prepared in great numbers for the actual viral experimentation. The small flasks were prepared by adding 5 ml of Hep^{-2} cell-growth media mixture to each plastic flask with a Cornwall syringe. The plastic flasks were then placed in the 37°C incubator for four days at which time the Hep^{-2} cells were seen to be fully sheeted out. At this time, the tissue culture flasks were removed from the incubator and the 10 per cent calf serum growth medium drained and discarded. Fresh medium containing 2 per cent calf serum was added to each plastic tissue culture flask. The 2% calf serum maintenance medium kept the Hep^{-2} cells viable for one week or until needed for experimentation.

B. Hep^{-2} Cell Membrane Isolation.

Hep^{-2} cell membranes, plasma membranes in a pure state, were isolated free of all other cellular components by means of ultracentri-

fugation. The basic technique used was developed by Bosmann, Hagopian, and Eylar in 1968.[35] The tissue culture cells which they studied were HeLa cells. In my study I used Hep-2 cells and verified the presence of pure cell membranes by means of electron micrographic studies.

1. Preparation of Reagents for Ultracentrifugation. Three basic stock reagents were required for ultracentrifugation procedure. The reagents required were: .05m Tris buffer, pH 7; .01 m EDTA in .02m Tris buffer pH 7; and 45% sucrose solution.

a. .05M Tris Buffer. Tris Buffer (500 ml of .05M) was prepared by adding 3.025 gm of Tris buffer to 500 ml of triple distilled water. The pH was adjusted to 7 by the addition of 1N HCL or NaOH as needed.

b. .01M EDTA in .02M Tris Buffer. EDTA (250 ml of .01M EDTA in .02M Tris buffer) was prepared by diluting 100 ml of the original 500 ml .05 M Tris buffer solution to 250 ml with triple distilled water and adding .7306 gm of EDTA. The pH was adjusted to 7 by the addition of 1N HCL or 1N NaOH as needed.

c. 45 per cent Sucrose Solution. One liter of 45 per cent sucrose solution was prepared by dissolving 450 gm sucrose in 1,000 ml of triple distilled water.

2. Ultracentrifugation Technique. Eight 32 oz. tissue culture bottles containing fully sheeted out Hep-2 cells were prepared for ultracentrifugation. The 10 per cent calf serum growth medium was drained from each bottle and discarded. To each bottle was added

25 ml of prewarmed STV solution. The tissue culture bottles were then placed in the 37^0C incubator for 15 minutes flat side down in order to bathe the Hep^{-2} cells in STV and thereby free them from the glass. When the Hep^{-2} cells were free of the glass surface, 25 ml of prewarmed 10 per cent calf serum growth media were added. The contents of the tissue culture bottles were drained into eight 50 ml plastic centrifuge tubes and tightly capped. The 50 ml tubes were placed in an International Clinical Centrifuge, Model C1, and centrifuged for 10 minutes at 5,000 rpm to obtain packed cells. Upon removal from the centrifuge, the packed Hep^{-2} cells were seen adhering to the bottom of the 50 ml centrifuge tubes. The supernatant fluid from each tube was discarded and the Hep^{-2} cells collected into one 50 ml centrifuge tube.

The Hep^{-2} cells were then washed three times with Hanks balanced salt solution. This was accomplished by adding 50 ml of cold Hanks solution to the centrifuge tube, gently shaking the tube so as to free the Hep^{-2} cells and again bring them into solution, and centrifuging for 10 minutes at 5,000 rpm. The supernatant was discarded after each centrifugation procedure.

After the third washing procedure, the packed Hep^{-2} cells, approximately 2 ml in volume, were transferred to a 10 ml Dounce homogenizer tube. To the homogenizer tube was added 3 ml .01M EDTA in .02M Tris solution. The homogenizer tube was placed in an ice bath and using a glass rod plunger the Hep^{-2} cells were homogenized. A

total of 15 homogenizing strokes was found to produce sufficient cell rupture. The degree of cell rupture was ascertained after each five strokes using a microscope.

The homogenate was suspended in 50 ml of .01M EDTA in .02M Tris buffer. This mixture was placed into a 50 ml centrifuge tube and placed into the angle head rotor of the International Preparative Ultracentrifuge, Model B-35, and centrifuged at 6,000 rpm and 4°C for 10 minutes. This procedure effectively removed whole cells, nuclei, mitochondria and other cellular contaminants from the cellular or plasma membranes contained in the supernatant fluid. The supernatant fluid was retained and the pellet discarded.

The supernatant fluid, 50 ml, was adjusted to 45 per cent sucrose by adding 22.5gm of pure sucrose. Six 50 ml angle head rotor centrifuge tubes were rinsed in triple distilled water and made ready for sucrose solution layering. Each of the six tubes was numbered according to the position it would occupy in the rotor head.

Tube No. 1 occupied position 1 in the rotor head and was opposed in the rotor head by tube No. 2, tube No. 5 was opposed by tube No. 6 and Tube No. 3 by tube No. 4.

To tube No. 1 and No. 3 were added 18 ml of supernatant-sucrose solution. Layered over this were 13 ml of 35% sucrose solution, and on top of this 10 ml of 30% sucrose solution was placed. Over this 5 ml of 25% sucrose solution was added. The remainder of the tube was

filled with .05M Tris buffer. To tube No. 5 were added 14 ml of supernatant-sucrose solution. Layered over this were 13 ml of 35% sucrose solution, above this was placed 10 ml of 30% sucrose solution. Over this 5 ml of 25% sucrose solution were placed. The remainder of the tube was filled with .05M Tris buffer.

Tube Nos. 1, 3, and 5 were carefully weighed on a triple beam balance scale and the weight of each tube recorded. The opposing 50 ml centrifuge tube Nos. 2, 4 and 6 were layered with sucrose in the same manner as were tube Nos. 1, 3 and 5. Tube Nos. 2, 4 and 6 contained no supernatant sucrose solution; they served as blanks. Tube Nos. 2, 4 and 6 were also weighed on the triple beam balance scale to insure that there weight was exactly the same as there opposing tubes in the angle head rotor.

Each tube was placed in its proper position in the angle head rotor and centrifuged for 16 hrs. at 23,500 rpm and 3°C. After 16 hours of centrifugation tube Nos. 1, 3, and 5 were recovered and the sugar solutions discarded. The membrane pellet on the bottom of each tube was retained. To each centrifuge tube was added 50 ml of .05M Tris buffer and the tubes recentrifuged for 1 hr. at 23,500 rpm and 3°C. The supernatant from each tube was again discarded and the membrane pellets on the bottom of each tube collected and suspended in 10 ml of Hanks balanced salt solution. The membranes now suspended in Hanks were stored in the ultra-low deepfreeze, -90°C.

3. Electron Micrographic Study. In order to verify that the ultracentrifugation technique employed did isolate Hep^{-2} cell membranes, free of all other cellular components, electron micrographs were made.

One drop of the membrane solution was placed on each of two carbon-coated grids. The grids were then placed in the refrigerator for two minutes. The grids were removed from the refrigerator and floated on the surface of triple-distilled water, specimen-side down, for 10 seconds. The grids were lifted from the triple-distilled water with a tweezer and bathed in 2 per cent euryl acetate solution for two minutes. The excess euryl acetate solution was drained and the grids replaced in the refrigerator for 10 minutes to dry.

The dried grids were viewed with an electron microscope. The electron microscope used was an RCA, Model EMU-3H. A total of 26 exposures were taken from both grids. (Table II)

TABLE II

Electron Microscope: RCA EMA-3H

Exp. - Exposures

Tap. - Magnification

Exp. No.	Tap. No.
1	3
2	3
3	6
4	6
5	7
6	8
7	8
8	8
9	8
10	8
11	8
12	8
13	3
14	7
15	7
16	7
17	7
18	7
19	8
20	8
21	6
22	7
23	8
24	8
25	8
26	8

C. Herpes Simplex Virus Isolation.

The stock solution of herpes simplex virus used in this study was the MacIntyre strain, Vr. 539, ATCC.

1. Infection of Hep^{-2} Cell Monolayer. One 32 oz. tissue culture bottle containing Hep^{-2} cells fully sheeted out was carefully drained of 10 per cent calf serum growth medium. The stock solution of herpes virus was thawed and .5 ml added to the 32 oz. tissue culture bottle. The tissue culture bottle was gently rotated so that the virus solution would coat all of the cells in the Hep^{-2} cell monolayer. The tissue culture bottle was then placed in the 37°C incubator for one hour. Every 15 minutes the tissue culture bottle was again gently rotated to insure infection of all Hep^{-2} cells in the monolayer. At the end of one hour, the tissue culture bottle was removed from the incubator and 50 ml of 2 per cent calf serum maintenance media was added. The tissue culture bottle was returned to the incubator for an additional 48 hrs.

Upon completion of the 48 hr. incubation period, the 32 oz. tissue culture bottle was removed from the 37°C incubator and placed in the ultra-low deepfreeze until its contents were frozen. The 32 oz. tissue culture bottle was removed when frozen and placed directly into a warm water bath to thaw. This rapid freeze-thaw procedure was repeated three times. After the third thaw, the 50 ml volume of Hep^{-2} cells and virus was placed in a 50 ml plastic centrifuge tube. The contents of the tube was centrifuged for 10 min. at 6,000 rpms, and 4°C. The supernatant containing the virus was retained and

the Hep^{-2} cell pellet at the bottom of the tube discarded. The supernatant was immediately placed in the ultra-low deepfreeze for storage.

2. Preparation of Reagent for Ultracentrifugation of Virus. Only one reagent, cesium chloride, was required for the ultracentrifugation procedure. The cesium chloride was purchased in crystal form. A cesium chloride solution was prepared by mixing 45 grams of crystalline cesium chloride with 100 ml of sterile water. The resulting solution had a density of 1.34.

3. Ultracentrifuation Technique. The basic ultracentrifugation procedure employed in this study was reported by Smith in 1964.[36]

Six 15 ml plastic centrifuge tubes were washed and thoroughly rinsed three times in triple distilled water. Using a volumetric flask and triple distilled water, the plastic centrifuge tubes were accurately calibrated into three equal 5 ml fractions. Each 5 ml fracion was clearly marked on the outside of each plastic centrifuge tube with a grease pencil. Fraction I was designated as that 5 ml fraction at the bottom of the plastic centrifuge tube, fraction II the middle of the plastic centrifuge tube, and fraction III the top of the plastic centrifuge tube.

The virus supernatant was removed from the ultra-low deepfreeze and quickly thawed in a warm water bath. To tube Nos. 1 through 4 were added 4 ml of cesium chloride over which were layered 11 ml of virus supernatant. To tube Nos. 5 and 6 were added 4 ml of cesium chloride

over which were layered 11 ml of Hanks balanced salt solution. Tube Nos. 5 and 6 did not contain any virus supernatant and served as blanks.

The six plastic centrifuge tubes were placed into their respective tube holders and tightly capped. The tube holders were inserted into the swinging-bucket rotor head of the International Preparative Ultracentrifuge, Model B-35, and centrifuged for 28 hrs. at 35,000 rpms and 4°C.

4. Recovery of Viral Fractions. Upon completion of the 28 hr. centrifugation period. The centrifuge tube holders were removed from the swinging-bucket rotor head and six plastic centrifuge tubes recovered. Tube Nos. 1 through 4 were immediately placed in the cold room at 4°C.

In the cold room tube Nos. 1 through 4 were pricked with a 25g needle. Using the bottom-drip method, viral fractions I, II and III were collected from each plastic centrifuge tube. The fractions were collected in glass vials and tightly stopped with rubber caps. The viral fractions were immediately placed in the ultra-low deepfreeze, -90°C for storage.

D. Preparation and Mixing of the Hep^{-2} Cell Membranes and Viral Fractions.

The Hep^{-2} cell membranes which were suspended in 10 ml of Hanks balanced salt solution were removed from the ultra-low deepfreeze

and quickly thawed in a warm water bath. The membrane solution was transferred to a small sterile blender flask and mixed for 3 min. with a Virtis "23" blender to break up membrane aggregates.

Viral fractions I, II, and III were removed from the ultra-low deepfreeze and quickly thawed in a warm water bath. Three sterile test tubes were placed in a test tube rack and marked I, II, and III; these test tubes correspond to viral fractions I, II, and III. To each sterile test tube was added 1.5 ml of its respective stock viral fraction and 1.5 ml of Hep^{-2} membrane solution. The test tubes were placed in the 37°C incubator for 2 hrs.

E. Viral Dilutions and Plaque Assay.

In this study plaquing to determine viral infectivity was accomplished by means of methyl cellulose overlay media. This plaquing technique was introduced by Tytell and Neuman in 1963.[37]

1. Virus Solution before Ultracentrifugation. The virus solution obtained after passage of the stock herpes simplex virus through Hep^{-2} cells and before ultracentrifugation to obtain viral fractions I, II, and III was serially diluted and plaqued.

A dilution series was prepared ranging from 10^{-1} through 10^{-7} in the following manner: In a test tube rack was arranged three series of sterile test tubes with seven test tubes in each series. To each of the 21 test tubes was added 1.8 ml of sterile Hanks balanced salt solution. Strict aseptic technique was employed. The test tubes

in each series were labeled Nos. 1 through 7. From the stock viral solution .2 ml was removed with a sterile pipette and added to each tube labeled No. 1 in each dilution series. Using a different sterile pipette for each dilution series .2 ml of the contents of tube No. 1 was transferred to tube No. 2; .2 ml of the contents of tube No. 2 was transferred to tube No. 3, and consecutive transfer continued throughout each tube in each dilution series.

Twenty-four plastic tissue culture flasks containing Hep-2 cell monolayers which were fully sheeted out were removed from the 37°C incubator. Each plastic tissue culture flask was carefully drained of medium. Three plastic tissue culture flasks were labeled C to designate them as controls; the remaining 21 were labeled so as to correspond with each tube, Nos. 1 through 7, in each dilution series. Beginning with test tube No. 7 and proceeding through the series to test tube No. 1, to each plastic tissue culture flask was added .5 ml of viral solution from its respective test tube in the dilution series. The plastic tissue culture flasks were placed in the 37°C incubator for two hours and on each one-half hour of the two-hour period, gently rotated to insure that all cells in the monolayer were coated with .5 ml of viral solution.

Upon completion of the two-hour incubation period, the plastic tissue culture flasks were removed from the incubator and drained of excess viral solution. To each plastic tissue culture flask was

added 6-7 ml of methyl cellulose overlay media. Great care was taken to insure even coverage of the monolayer with methyl cellulose. The plastic tissue culture flasks, containing methyl cellulose were returned to the 37^{0}C incubator for five days. After five days of incubation, the plastic tissue culture flasks were removed from the incubator and placed in the refrigerator for 30 min. to soften the methyl cellulose overlay. The methyl cellulose was drained and the inside of each plastic tissue culture flask rinsed with 2 ml of Hanks balanced salt solution. To each plastic tissue culture flask was added 5 ml of neutral red dye; the flasks were allowed to stand for 30-45 min. Each plastic tissue culture flask was examined for the presence of plaques and the plaques observed were recorded.

2. Viral Fractions I, II, and III. Viral fractions I, II, and III were removed from the ultra-low deepfreeze and quickly thawed in a warm water bath. A dilution series was prepared and plaque titrations performed in exactly the same manner as discussed in E_1 of the materials and methods. Plastic tissue culture flasks were examined for the presence of plaques and the plaques observed were recorded.

3. Viral Fractions I, II, and III with Hep^{-2} Membranes. The test tubes containing viral fractions I, II, and III with Hep^{-2} membranes were removed from the 37°C incubator after two hrs. A dilution series was prepared and plaque titrations performed in exactly the same manner as discussed in E_1 of the materials and methods.

Plastic tissue culture flasks were examined for the presence of plaques and the plaques observed were recorded.

CHAPTER IV

FINDINGS

A. Tissue Cultures.

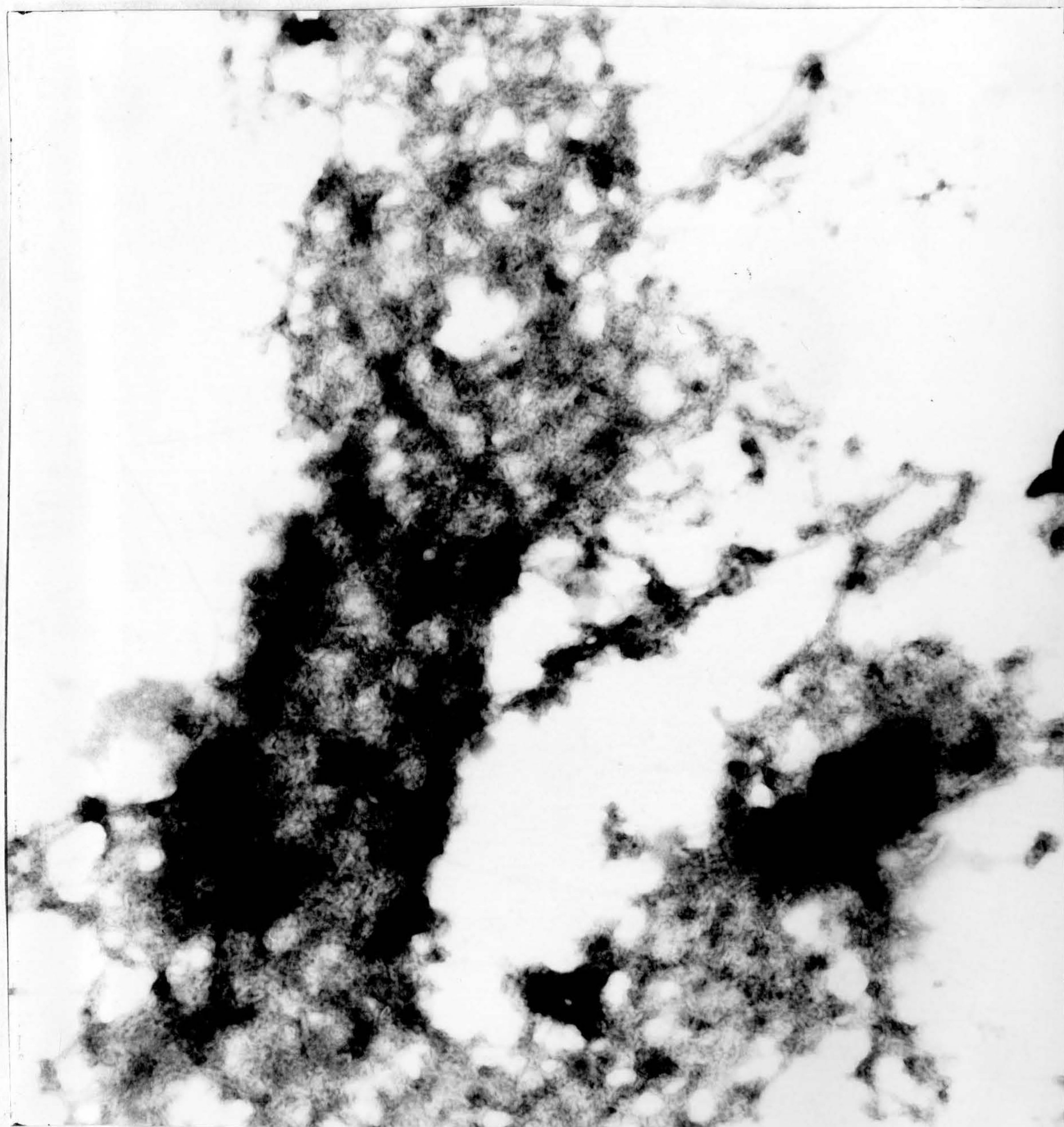
Hep^{-2} cell monolayer tissue cultures were grown on the flat surface of 32 oz. tissue culture bottles and small plastic tissue culture flasks. Eagle's Minimum Essential Medium with 10 per cent calf serum proved to be an effective growth medium. When the Hep^{-2} cell monolayers were fully sheeted out, Eagle's Minimum Essential Medium with 2 per cent calf serum maintained the monolayer cultures for 5-7 days. After the seventh day, the Hep^{-2} cells became nonviable and began to detach from the surface upon which they were growing and float freely in the media.

B. Hep^{-2} Cell Membrane Isolation.

Hep^{-2} cells were subjected to the ultracentrifugation procedure and upon removal from the ultracentrifuge small white-brown pellets were observed on the bottom of each centrifuge tube. The white-brown pellets were suspended in 10 ml of Hanks balanced salt solution and prepared for electron microscopic viewing. The electron micrographs disclosed that the white-brown pellets obtained were pure Hep^{-2} cell membranes. The membranes appeared to be aggregated or clumped together.

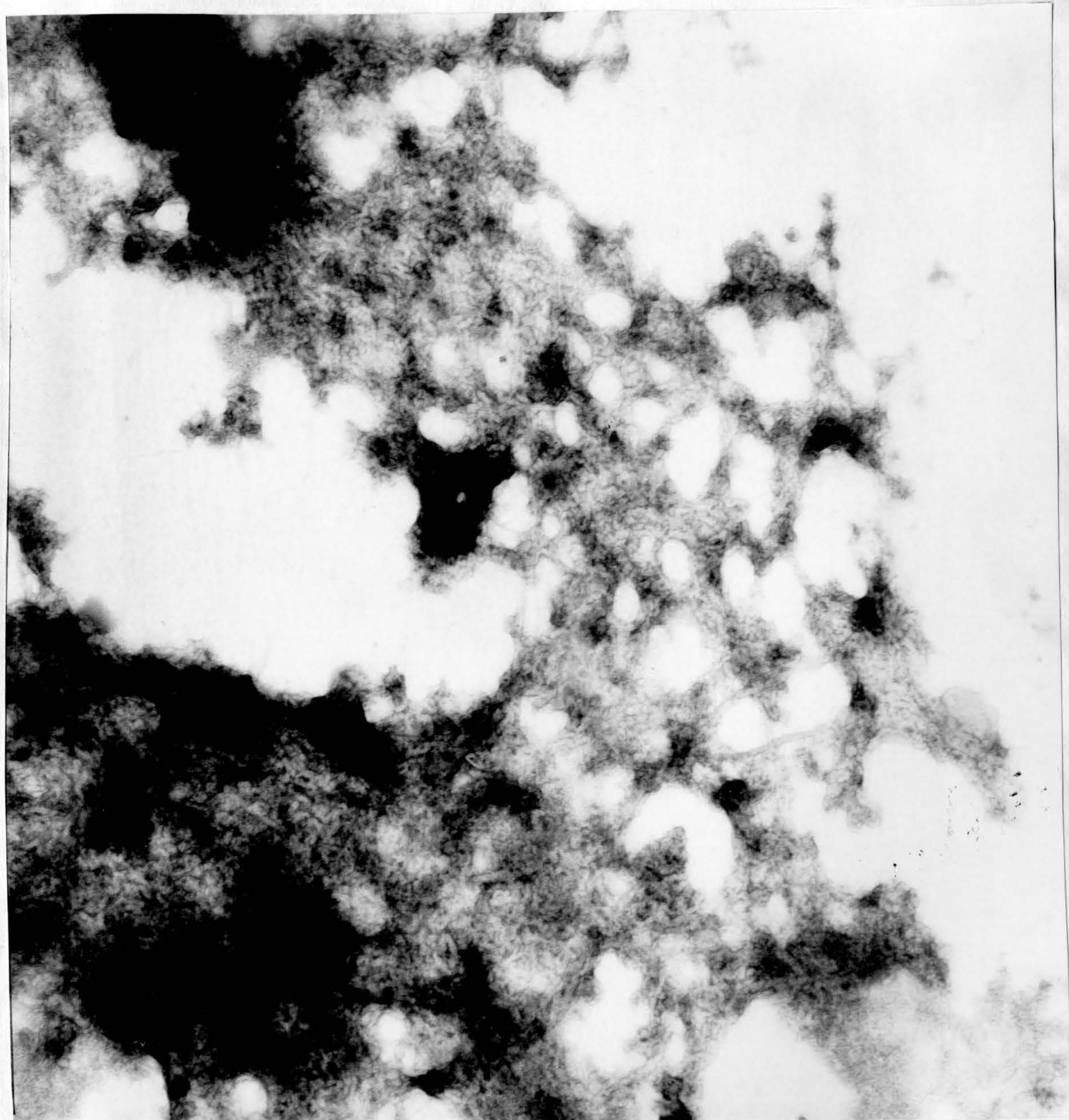
ELECTRON MICROGRAPHS

Hep^{-2} Cell Membranes
Tap. No. 6



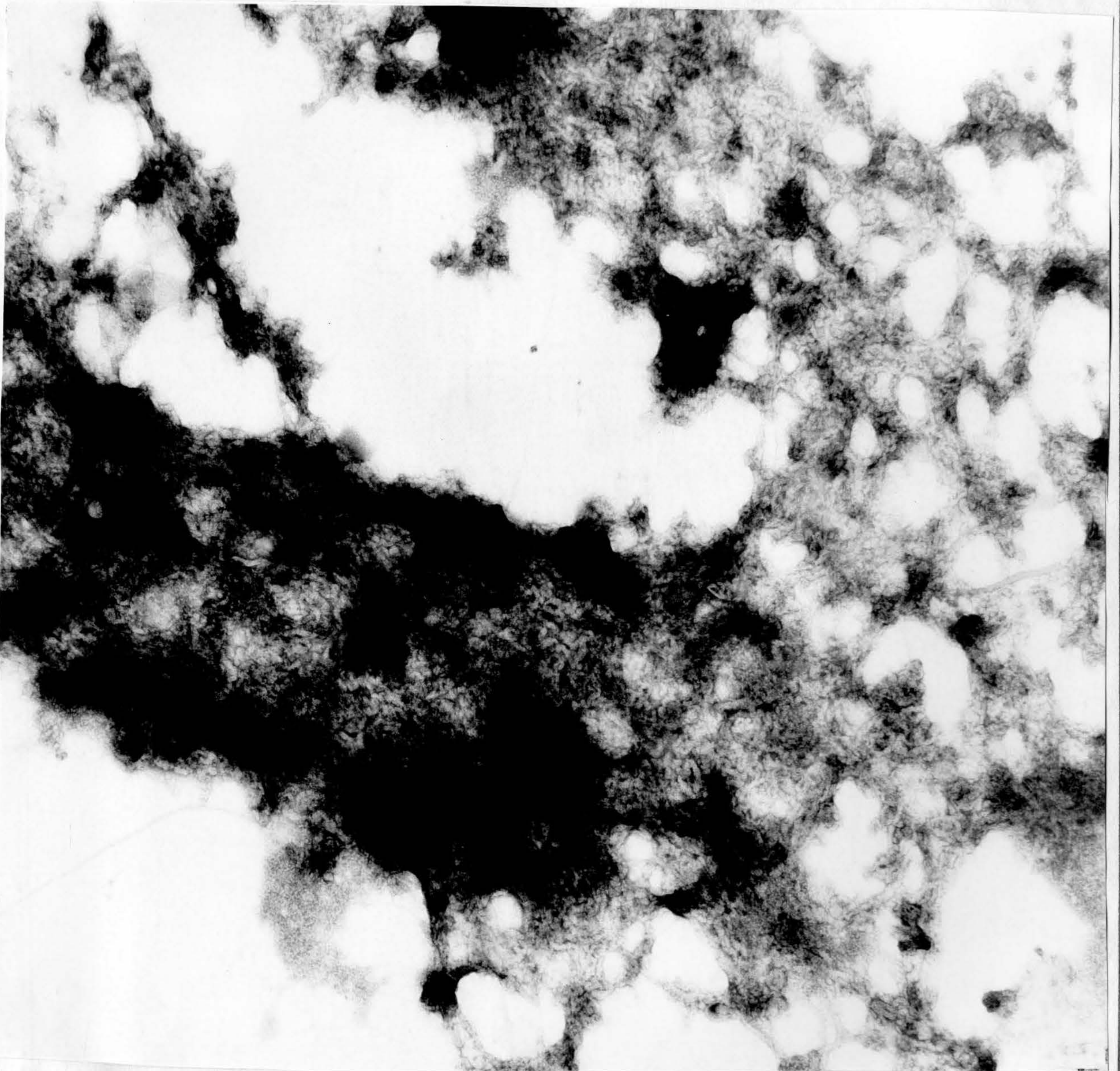
Hep^{-2} Cell Membranes

Tap. No. 7



Hep^{-2} Cell Membranes

Tap. No. 8



C. Herpes Virus Isolation.

One 32 oz. tissue culture bottle containing Hep^{-2} cells, fully sheeted out, was infected with stock herpes simplex virus. There is no doubt that the Hep^{-2} cells became virally infected because within 48 hrs. the Hep^{-2} cells were seen to break away from the glass surface upon which they were growing. The centrifugation procedure as discussed in C_1 of the materials and methods eliminated Hep^{-2} cells and yielded clear virus supernatant.

The virus supernatant was ultracentrifuged according to the procedure set forth in C_2 of the materials and methods. Viral fractions I, II, and III were collected. Examination of the fractions substantiated the data reported by Smith in 1964.[38] Viral fraction I appeared colorless. Viral fraction II was colored. The color observed was light red. Viral fraction III appeared darker in color than viral fraction II. Viral fraction III appeared darker in color than viral fraction II. Viral fraction III was dark red. The color observed in each fraction was due to the phenol red in the growth medium.

D. Preparation and Mixing of Hep^{-2} Cell Membranes and Viral Fractions.

Hep^{-2} cell membranes and viral fractions were prepared and mixed as was discussed in D of the materials and methods. The membrane-viral fraction mixtures appeared homogenous. No percipitation or other visual phenomenon of any type was observed before or after incubation.

E. Viral Dilutions and Plaque Assay.

1. Virus Solution Before Ultracentrifugation. The virus solution before ultracentrifugation to obtain viral fractions I, II, and III was serially diluted and plaqued according to the procedure discussed in E_1 of the materials and methods. The plaque counting procedure disclosed the infectivity of the viral solution before centrifugation, to be 428×10^3 pfu/ml. (Table III)

2. Viral Fractions I, II, and III. Viral fractions I, II, and III were serially diluted and plaqued according to the procedure discussed in E_1 of the materials and methods. Plaque counting procedures on each viral fraction disclosed the infectivity of viral fraction I to be 000 pfu/ml, since no plaques whatsoever were observed. Viral fraction I was not infective. The degree of infectivity of viral fraction II was 70×10^1 pfu/ml and the degree of infectivity of viral fraction III 614×10^1 pfu/ml. (Table III)

3. Viral Fractions I, II, and III with Hep^{-2} Membranes. Viral fractions I, II, and III containing Hep^{-2} membranes were serially diluted and plaqued according to the procedure discussed in E_1 of the materials and methods. Plaque counting procedures on each viral fraction containing membranes disclosed the infectivity of fraction I to be 425×10^1 pfu/ml, and fraction II to be 102×10^1 pfu/ml, and fraction III to be 000 pfu/ml. (Table III) Viral fraction III with Hep^{-2} cell membranes was not infective.

TABLE III

PLAQUE ASSAY OF HERPES SIMPLEX VIRUS

Fraction No.	Dilution Series No.	No. plaques/.5ml	No. PFU/ml	Ave.No. PFU/ml
Virus sol. before ultracentrifugation	1	210	$420X10^3$	
	2	230	$460X10^3$	
	3	202	$404X10^3$	$428X10^3$
I	1	000	000	
	2	000	000	
	3	000	000	000
II	1	26	$52X10^1$	
	2	41	$82X10^1$	
	3	39	$78X10^1$	$70.3X10^1$
III	1	317	$634X10^1$	
	2	323	$646X10^1$	
	3	288	$576X10^1$	$614X10^1$
I $\bar{c}$ Mem	1	235	$470X10^1$	
	2	242	$484X10^1$	
	3	160	$320X10^1$	$425X10^1$
II $\bar{c}$ Mem	1	87	$174X10^1$	
	2	43	$86X10^1$	
	3	23	$46X10^1$	$102X10^1$
III $\bar{c}$ Mem	1	000	000	
	2	000	000	
	3	000	000	000

*Viral fraction I without Hep^{-2} cell membranes produced no plaques.

*Viral fraction III with Hep^{-2} cell membranes produced no plaques.

CHAPTER V

DISCUSSION

Electron micrographs of the Hep^{-2} cell membranes disclosed excessive clumping. The membrane clumping was caused by the ultracentrifugation procedure. Before mixing equal volumes of hep^{-2} cell membranes and viral fractions, it was necessary to disrupt membrane aggregates with the Virtis "23" blender. This procedure was necessary in order to provide greater membrane surface area.

Infection of the 32 oz. tissue culture bottle containing Hep^{-2} cells followed by 48 hrs. of incubation and three rapid freeze-thaw procedures yielded viral particles in varied stages of development. The 48 hr. incubation period allowed viral particles to exit from intact Hep^{-2} cells, acquire an outer viral envelope, and float freely in the 2 per cent calf serum maintenance media bathing the monolayer. The rapid freeze-thaw procedures caused rupture of infected Hep^{-2} cells and liberation of naked viral particles, viral particles without an outer envelope. Centrifugation of the Hep^{-2} cell-virus solution mixture eliminated Hep^{-2} cells and yielded a virus supernatant composed of naked and enveloped viral particles. Serial dilution and plaque titrations were run on the virus supernatant in order to determine its degree of infectivity before ultracentrifugation. The infectivity of the virus supernatant was found to be 428×10^3 pfu/ml.

After ultracentrifugation, viral fractions I, II, and III were

collected. According to Table I of the literature review, viral fraction I should contain only naked viral particles and be noninfective; viral fraction II should contain both naked and enveloped viral particles and be infective; and viral fraction III should be composed almost exclusively of enveloped viral particles and be highly infective. Serial dilutions and plaque titrations of the viral fractions provided data that was in agreement with Table I.

Viral fraction I produced no plaques, 000 pfu/ml, and was therefore composed of noninfective or naked viral particles. Viral fraction II produced plaques, 70.3×10^1 pfu/ml, and therefore did contain both naked and enveloped viral particles. Viral fraction III produced plaques, 614×10^1 pfu/ml. The high degree of infectivity of this fraction suggests that it was composed chiefly of enveloped viral particles.

The ultracentrifugation procedure employed to obtain viral fractions I, II, and III did cause a loss of overall infectivity. The virus supernatant before centrifugation was found to produce 10^3 pfu/ml and fraction II and III produced 10^1 pfu/ml.

Serial dilutions and plaque titrations of viral fractions I, II, and III with and without Hep^{-2} cell membranes showed a significant change in infectivity of the viral fractions. Viral fraction I without Hep^{-2} cell membranes produced 000 pfu/ml; however, viral fraction I with Hep^{-2} cell membranes produced 425×10^1 pfu/ml. In both instances, viral fraction I contained only naked or noninfective viral particles. The

addition of Hep^{-2} cell membranes to this viral fraction produced infectivity. Infectivity was made possible due to the fact that the Hep^{-2} cell membranes added were antigenically similar to the viral envelopes which these naked particles were missing, but was necessary for infection. A possible explanation for this phenomenon is that the membranes in the presence of naked particles acted as a false viral envelope and allowed noninfective viral particles to initiate infection.

Viral fraction II without Hep^{-2} cell membranes produced 70.3×10^1 pfu/ml; however, viral fraction II with Hep^{-2} cell membranes produced 102×10^1 pfu/ml. The increase in infectivity of this viral fraction upon addition of membranes suggests that those viral particles contained in this fraction which were naked acquired a false viral envelope and became infective.

Viral fraction III without Hep^{-2} cell membranes produced 614×10^1 pfu/ml; however, viral fraction III with Hep^{-2} cell membranes produced 000 pfu/ml. Viral fraction III with Hep^{-2} cell membrane became completely noninfective although it contained highly infective particles. The addition of Hep^{-2} cell membranes which were antigenically similar to the viral envelopes of these particles rendered the viral particles noninfective. This data suggests that the infectivity of the enveloped viral particles was lost due to the fact that the membranes blocked the receptor sites on the viral envelope. Blockage of the receptor sites prevented the virus particles from attaching to susceptible Hep^{-2} cells whereby viral infectivity could begin.

CHAPTER VI

SUMMARY

Human Epidermoid Carcinoma, Hep^{-2} cells, were grown in monolayer cultures on the flat surfaces of 32 oz. tissue culture bottles and plastic tissue culture flasks. Hep^{-2} cell membranes were removed from Hep^{-2} cell monolayer tissue cultures by means of ultracentrifugation procedures. Electron micrographs of the membranes were prepared in order to verify that the ultracentrifugation procedure had yielded pure Hep^{-2} cell membranes, free of all other cellular components.

Herpes simplex virus, MacIntyre strain, was inoculated into a Hep^{-2} cell monolayer tissue culture. Following incubation of the virally infected monolayer culture, the culture was rapidly frozen and then thawed in order to produce a virus solution containing both naked and enveloped viral particles. The resultant virus solution was serially diluted and plaqued to determine its degree of infectivity; it was then ultracentrifuged whereby it was divided into three viral fractions. Viral fraction I contained only naked viral particles which were noninfective; viral fraction II contained both naked and enveloped viral particles; and viral fraction III was composed almost exclusively of enveloped particles which were highly infective.

To viral fractions I, II, and III equal volumes of Hep^{-2} cell membranes were added. Viral fractions I, II, and III with and without membranes were serially diluted and plaqued to determine infectivity.

The data obtained suggests that there is definite antigenic similarity between Hep^{-2} cell membranes and the outer viral envelope. It further verifies that the viral envelope is normally required for infectivity; however, in the absence of the viral envelope Hep^{-2} cell membranes can cause naked viral particles to become infective. Enveloped viral particles which are highly infective become noninfective in the presence of Hep^{-2} cell membrane.

CHAPTER VII

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APPROVAL SHEET

This thesis submitted by Dr. Anthony A. Frer has been read and approved by the members of his thesis committee.

The final copies have been examined by the thesis board members, and the signature of the thesis advisor which appears below verifies the fact that any and all necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form, and mechanical accuracy.

This thesis is, therefore, accepted in partial fulfillment of the requirements for the Degree of Master of Science in Oral Biology.

May 21, 1970
Date

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Signature of Advisor