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THE UNIVERSITY OF ALBERTA

BIOCHEMICAL AND BIOPHYSICAL STUDIES OF THREE VARIANTS OF MENGO ENCEPHALOMYELITIS VIRUS

by

© TAK WAH MAK

A THESIS SUBMITTED TO THE
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THE UNIVERSITY OF ALBERTA

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled BIOCHEMICAL AND BIOPHYSICAL STUDIES OF THREE VARIANTS OF MENGO ENCEPHALOMYELITIS VIRUS submitted by TAK WAH MAK in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

The controlled degradation at slightly acid pH's, and the early events of the replicative cycle of three plaque variants of Mengo encephalomyelitis virus have been studied.

It has been shown that the three variants of Mengo virus (designated L-, M-, and S-Mengo) are rapidly inactivated when incubated at slightly acidic pH's. The rate of inactivation is maximal at pH 6.2 in the presence of 0.1 - 0.2 M chloride or bromide ions, for which the reaction has a rigorous requirement. The rate of inactivation is temperature dependent, being approximately doubled for each 10° rise in temperature over the range of 7-37°C. At 17° (but not 37°), the M variant may be shown to be inactivated slightly more rapidly than are the other two. The inactivation of all three variants results in the dissociation of the viral capsids into protein subunits with the release of the intact viral genome.

The physico-chemical properties of the isolated protein subunits produced by pH inactivation of all three variants were examined and found to be virtually identical. The experimentally determined molecular weight and intrinsic sedimentation coefficient were found to be 407,000 ± 11,000 and 13.4 ± .13 S, respectively. SDS-polyacrylamide gel electrophoretic analyses revealed that the subunits contain only two (VPI and VPII) of the three major structural polypeptides present in intact virions. The circular dichroic spectra of the subunits of all three variants exhibit comparable ellipticity bands at 208 and 225 nm with amplitudes of -4000° and -2500°,

respectively, suggesting that the proteins possess an apparent α-helical content of approximately 5-10%. The virtual equivalence of the CD spectra for the subunits obtained from the three variants suggests that the general orientation and internal arrangement of their polypeptide chains are similar if not identical.

Electron microscopic studies have shown that the subunit has a well defined, slightly ellipsoidal shape, with dimensions of $16.8 \pm 0.3 \times 14.2 \pm 0.2$ nm. It has been concluded from these studies that the virion contains twelve of these 13.4S subunits arranged in an icosahedral symmetry. It was also shown that each of the 13.4S subunits could be further dissociated in the presence of 2M urea into five 4.7 S protein fragments with a molecular weight of 77,000, and a diameter of 6.8 ± 0.2 nm.

The early events in the replicative cycle of the three Mengo variants in L cells have also been studied. Of the three variants, S-Mengo was shown to attach most rapidly, and L-Mengo least rapidly. The kinetics of attachment of each variant, as calculated from measurements of adsorption of both infectivity (PFU) and radioactivity ($^{32}PO_4$-labeled virus), were similar at 0° and 37°. In a suspended cell system containing 2×10^6 cells per ml and an input multiplicity of 20 PFU/cell, 20%, 85% and 95% of L-, M- and S-virions, respectively, were shown to become tightly bound to cells by 20 minutes. Virus penetration, measured as the rate at which attached virus became resistant to neutralization by Mengo antiserum, was found to occur quite rapidly (more than 70% of adsorbed virus penetrates by 40 min) and the kinetics of penetration for the three variants were shown to

be identical. Studies of the uncoating of the three variants revealed that M-virions are uncoated more rapidly than are L- and S-virions. This observation is consistent with the earlier finding that the eclipse phase of the M-variant is 1.5 hours shorter than that of either the L- or S-variant. Inhibition of protein synthesis does not affect either the rate or the extent of uncoating of any of the variants. The number of L cell receptor sites was determined for each of L-, M- and S-Mengo and was found to be 27,000, 90,000 and 95,000 per cell respectively.

The data obtained show that both infectious and non-infectious particles of all three variants attach to, penetrate, and are uncoated in the L cell. Thus, the high particle per PFU ratios of the variants cannot be explained by an inability of non-infectious virions to complete the early events of the replicative cycle.

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LIST OF ABBREVIATIONS

c.d.	-	circular dichroism
Ci	-	Curie
DTT	-	dithiothreitol
ME	-	mercaptoethanol
nm	-	nanometer
PBS	-	phosphate buffered saline
PFU	-	plaque forming unit(s)
RNA	-	ribonucleic acid
SDS	-	sodium dodecyl sulfate
$\bar{v}$	-	partial specific volume
µg	-	micrograms
[θ]	-	specific ellipticity
$S^{\circ}_{20,w}$	-	intrinsic sedimentation coefficient

All temperatures are in Centigrade degrees.

se non è vero, è molto ben trovato

– Giordano Bruno (1585)

INTRODUCTION

Mengo encephalomyelitis virus, the subject of the investigations described herein, is a picornavirus belonging to the Columbia SK group. It was first isolated by Dick and coworkers in 1946 from a paralyzed rhesus monkey in the Mengo district of Buganda, Uganda. It was also subsequently isolated from mosquitos, mongoose, baboon, squirrel, and man (Dick himself), and was shown to produce lesions in the brain and spinal cord of infected animals and encephalitic symptoms in man (Dick, 1948; Dick et al., 1948 a, b; and Kissling et al., 1956). For some years, the virus was maintained in hamsters and mice by intracerebral passage, but was subsequently adapted to propogate in several lines of cultured mammalian cells.

Serological studies have shown that Mengo virus is very closely related to the Columbia SK (Jungeblut and Sanders, 1940), MM (Jungeblut and Dalldorf, 1943), encephalomyocarditis (Helwig and Schmidt, 1945) and ME (Franklin et al., 1959; Hausen and Schäfer, 1962) viruses (Dick, 1949; Warren et al., 1949), but that it is immunologically distinct from other picornaviruses, such as those of the enterovirus and rhinovirus groups (Jungeblut, 1958; Wenner et al., 1963; and Taylor, 1963). In view of the close antigenic relationship between them, Mengo, Columbia SK, MM, ME and encephalomyocarditis (EMC) viruses are known collectively as the "encephalomyocarditis viruses" or "cardioviruses" (Warren, 1965; Andrews and Pereira, 1967).

Although members of the encephalomyocarditis subgroup were discovered in widely separated areas, and isolated from several different

species, they are now considered to be strains of a single virus, and, not surprisingly, have many physical, chemical and biological properties in common. Like other picornaviruses, they are spherical in shape and are composed of protein and RNA only. Electron microscope and X-ray diffraction studies have indicated that they have diameters in the range 24-30 nm (Faulkner et al., 1961; Dales and Franklin, 1962; Hausen and Schäfer, 1962).

Information regarding the molecular structure of the encephalomyocarditis viruses is limited. However, on the assumption that the construction of regular isometric particles is governed by the same physical principles as have been shown to apply to tobacco mosaic virus (TMV), it has been hypothesized that there is only one kind of efficient design for the protein shell of spherical viruses, namely, one possessing icosahedral symmetry (Casper and Klug, 1962). The first experimental evidence for icosahedral symmetry in a spherical virus came from the X-ray diffraction studies on tomato bushy stunt virus (Casper, 1956) and turnip yellow mosaic virus (TYMV) (Klug et al., 1957). Shortly after these studies, X-ray diffraction studies (Finch and Klug, 1959) and electron microscopic observations (Horne and Nagington, 1959) showed that poliovirus, a member of the picornavirus group, also possesses icosahedral symmetry. Thus, it is highly probable that the encephalomyocarditis viruses also possess this simple, efficient, minimum-energy-requiring structural arrangement.

There is some controversy regarding the particle weight of virions of the encephalomyocarditis group. Virus molecular weight may be calculated from the Svedberg equation if the sedimentation

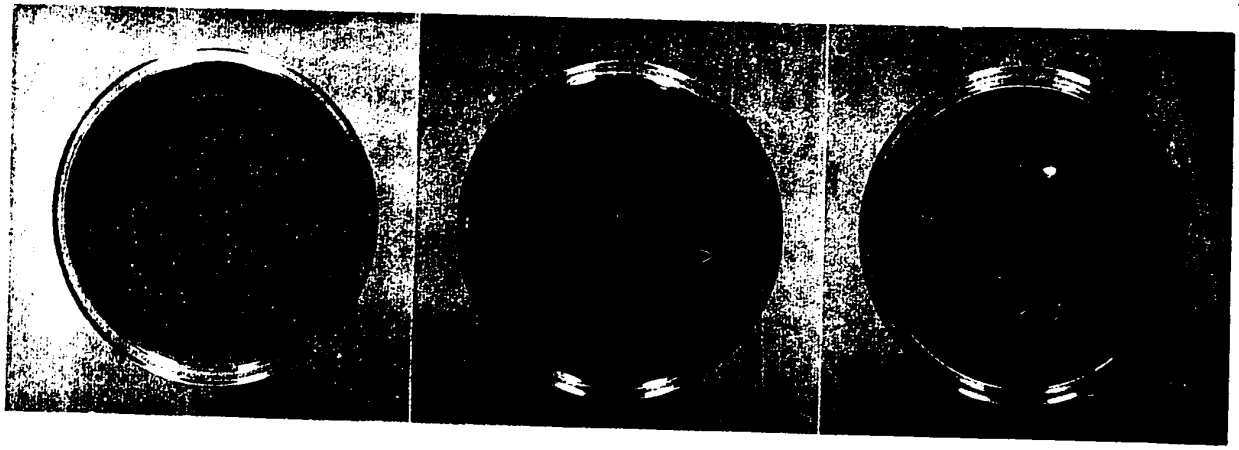
coefficient ($S^{\circ}_{20,w}$), diffusion coefficient ($D^{\circ}_{20,w}$) and partial specific volume ($\bar{v}$) are known. Early estimates of molecular weights based on experimentally determined sedimentation coefficients ranged from 10 x 10^6 daltons for EMC virus (Faulkner et al., 1961) to 5.7 x 10^6 daltons for ME virus (Hausen and Schäfer, 1962). More recently, Scraba et al. (1967), on the basis of measurements of all the above-mentioned parameters, have calculated that the molecular weight of Mengo virus is 8.3 x 10^6 daltons, and Burness (1970) and Burness and Clothier (1970) have reported a molecular weight of 8.5 x 10^6 daltons for EMC virus. It seems likely that these latter values are closer to the true value than are the earlier ones.

That RNA is the genetic material in these viruses has been demonstrated by the isolation of infectious RNA from Mengo virus (Colter et al., 1957), from EMC virus (Huppert and Sanders, 1958) and from ME virus (Franklin et al., 1959). The infectious RNA isolated by phenol extraction is single stranded (Hausen and Schäfer, 1962), and estimates of its sedimentation coefficient have provided values in the range 28-37 svedbergs (Hausen and Schäfer, 1962; Burness et al., 1963). Burness et al. (1963) estimated the molecular weight of EMC-RNA to be 3 x 10^6 daltons, while Hausen and Schäfer reported a value of 2 x 10^6 daltons for the molecular weight of ME-RNA. However, Granboulan and Girard (1969), on the basis of measurements of contour lengths from electron micrographs, have calculated that EMC-RNA has a molecular weight of 2.7 x 10^6 daltons, and Burness (1970) has arrived at a similar value based on the molecular weight and the percent RNA and protein composition of the virion. Finally, Tannock et al. (1970)

have estimated the molecular weight of poliovirus RNA to be 2.57×10^6 daltons from polyacrylamide gel electrophoresis studies.

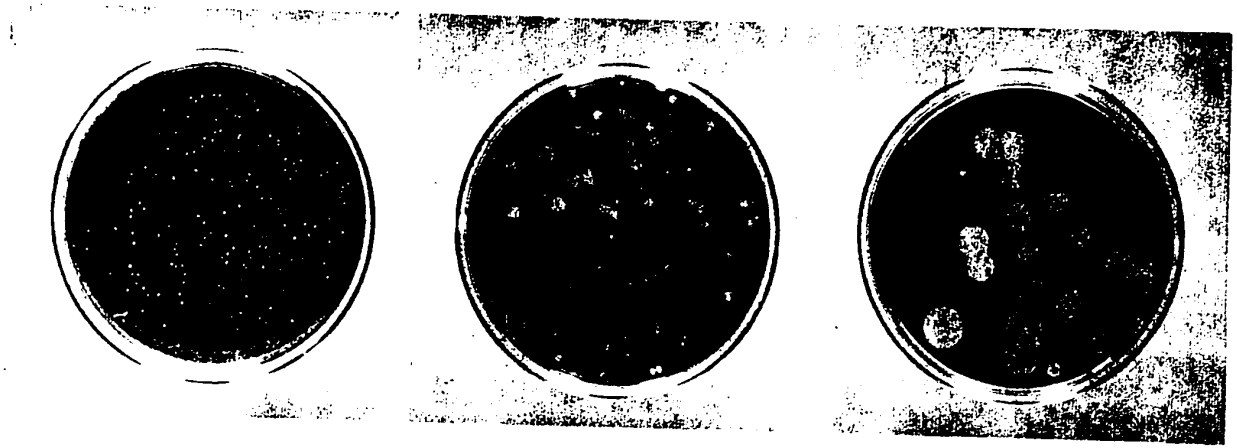
Considerable information concerning the nature and properties of the proteins which comprise the encephalomyocarditis viruses is available. The amino acid compositions of the proteins of EMC (Faulkner et al., 1961) and ME (Rueckert and Schäfer, 1965) viruses have been determined and found to be very similar not only to each other but to that of poliovirus (Munyon and Salzman, 1962). Information regarding the number and molecular weights of polypeptides present in the picornavirus virions has been provided recently. The studies of Maizel and Summers (1968) established that poliovirus contains three major structural polypeptides having molecular weights of 35,000, 28,000 and 24,000, as well as one minor structural polypeptide with a molecular weight of approximately 6,000. Recently, ME virus has also been shown to contain three major and one minor structural polypeptides, similar in size to those found in polio virus (Rueckert et al., 1969).

The intracellular development of encephalomyocarditis viruses has been actively studied in recent years, and significant findings have been made with respect to the events of attachment (Henry and Franklin, 1959; Brownstein and Graham, 1961; Holland, 1962), eclipse of infectivity and cell penetration (Joklick, 1965; Dales and Franklin, 1962), inhibition of host cell RNA and protein synthesis (Franklin and Baltimore, 1962), synthesis of viral RNA and protein (Baltimore and Franklin, 1963; Horton et al., 1966; Roberts et al., 1966), sites of viral synthesis (Hinz et al., 1962; Goodheart, 1967), and viral maturation and release (Franklin, 1962; Dales and Franklin, 1962).



S-MENGO M-MENGO L-MENGO

FIGURE A.1. Plaques produced by the three variants of Mengo encephalomyelitis virus in monolayers of L-929 mouse fibroblasts under nutrient agar overlay. The plaques were visualized by staining with neutral red after 72 hours' incubation at 37° in an atmosphere of 5% CO_2 in air.



S-MENGO M-MENGO L-MENGO

FIGURE A.1. Plaques produced by the three variants of Mengo encephalomyelitis virus in monolayers of L-929 mouse fibroblasts under nutrient agar overlay. The plaques were visualized by staining with neutral red after 72 hours' incubation at 37° in an atmosphere of 5% CO_2 in air.

It has been shown that encephalomyocarditis viruses attach to host cells at an exponential rate, which is influenced by pH, salt concentration, nature of the medium, and (very slightly) by temperature. It has been suggested that they probably attach by complementary interaction with specific receptors on the cell surface (Colter et al., 1964b), an idea that is reinforced by the demonstration that normally insusceptible cells, such as cultured primate cells, can be infected with RNA's derived from purified encephalomyocarditis viruses (Holland et al., 1959a, b; Tovell et al., 1970).

Within 3 hours after infection, synthesis of viral RNA begins, and is followed closely by the production of viral specific proteins (Franklin and Baltimore, 1962; Roberts et al., 1966). Although cytopathogenic effects do occur within the nuclei of infected cells, there is decisive evidence that the synthesis and assembly of viral components occur in the cytoplasm. Particle formation appears to be localized in the cytoplasm, and crystalline arrays of particles of viral size have been observed only in the cytoplasm of EMC or Mengo infected mouse fibroblasts (Dales and Franklin, 1962).

In 1961, Ellem and Colter isolated three plaque variants of Mengo encephalomyelitis virus. They were designated L-, M- and S-Mengo on the basis of the large-, medium-, and small-sized plaques they produced in monolayers of mouse fibroblast cells (strain L929) (Fig. A.1). It was later shown that the agar used in the overlay contains at least two fractions inhibiting S- and M-Mengo plaque development, and that this inhibition may be reversed by the addition of protamine (Campbell and Colter, 1965).

Subsequent studies, conducted to define a broad spectrum of the biological properties of the three variants, revealed a number of differences among them. They were found to differ with respect to the length of the eclipse period in suspended cell culture: the eclipse period for M-Mengo in L cells being only 4 to 4.5 hrs as compared to 5.5 to 6 hrs for the other two variants. This suggested that the intracellular uncoating of the viral genome may be more rapid for M- than for L- and S-Mengo virions (Campbell, 1965). It was also found that the variants differ in their rates of attachment to L cells. In both monolayer and suspension cultures, L-Mengo was shown to infect cells at a much slower rate than does either M- or S-Mengo (Colter et al., 1964a). It was also found that pH has a marked effect on the infectivities of the M- and S-variants for L cells, in both monolayer and suspended cultures. Increasing the pH at which cells and virus interact during the attachment period from 6.8 to 7.8 was shown to increase the apparent titer (as determined by plaque assay) of the L-, S- and M-variant by factors of 2, 10, and 3000, respectively. The effect of pH was shown to be due, in part, to the instability of the variants at pH's below 7.2 (Colter et al., 1964a).

Perhaps the most striking difference among the three variants is in their relative virulence for mice. The LD_{50}'s for 14-16 gm mice of these variants when injected by the intraperitoneal route were found to be 1, 1-5 x 10^4, and $>10^7$ PFU for the L-, M- and S-variants respectively, whereas the intracerebral LD_{50}'s were essentially the same (1-5 PFU) for all three variants. No completely satisfactory explanation for the high I.P. virulence of L-Mengo was

found. L-Mengo was found to have a lower affinity for mouse tissues - including those of the central nervous system - than do either S- or M-Mengo. Furthermore, it was shown to induce the production of at least as much interferon in mice, and to be equally sensitive to inhibition by an exogenous interferon as S- and M-Mengo (Campbell and Colter, 1967 a, b). All three variants have the same optimal growth temperature (36-37°)(Campbell, 1965), and although S-Mengo does not replicate at 40°, L-Mengo has no selective growth advantage over M-Mengo at that temperature. However, L-Mengo (unlike the other two variants) was shown to produce a marked viremia in mice after I.P. challenge, and to be cleared from the circulation after intravenous injection much more slowly than are M- and S-Mengo. These observations prompted the suggestion that - perhaps due to its low affinity for mouse tissues and its slow rate of clearance from blood - L-Mengo can more readily reach the central nervous system (and cause death) than can the other two variants.

The physical and chemical characteristics of these variants and their constituent ribonucleates were also examined. To date, studies employing the techniques of electron microscopy, ultracentrifugation, optical rotary dispersion, circular dichroism, and a variety of chemical methods have failed to reveal any marked differences among them.

The L-, M- and S-Mengo virions have identical morphologies when examined by electron microscopy, being spherical in shape with diameters of 26.2 - 27.2 nm. Analytical ultracentrifugation studies have also established that the molecular weight of each of the Mengo

variants is $8.3 \pm 0.7 \times 10^6$ daltons, and that each has a sedimentation coefficient of 150S (Scraba et al., 1967). Isolated RNA's from L-, M- and S-Mengo were also studied. They were found to be virtually identical in base composition, in "melting" behaviour, and in sedimentation and diffusion coefficients.

Studies on the structural proteins of the three variants have yielded some interesting results. The amino acid composition of the proteins of three variants were found to be essentially identical (Scraba et al., 1969). Analyses by polyacrylamide gel electrophoresis demonstrated that the three variants each contain three major structural polypeptides, designated VPI, VPII and VPIII (mol.wt. 31,000, 28,000 and 20,000, respectively)(O'Callaghan et al., 1970). M-Mengo, however, was found to contain an additional, minor structural component (VPIV, mol.wt. 10,000) which was not detected in any preparations of S- or L-Mengo virions. Immunological studies employing specific Mengo antisera showed that the major structural polypeptides of all three variants are immunologically identical and that all three components (VPI, II and III) contribute to the antigenic composition of the virion (O'Callaghan et al., 1970).

Optical rotatory dispersion and circular dichroic studies of the three variants of Mengo virus and their constituent ribonucleates also suggest that the manner in which the proteins are arranged around the RNA chain in the virus is precise and similar in all three cases, and that there are no significant differences in the density of packing among the virions (Kay et al., 1970).

Differences were found to exist among the Mengo variants with respect to net surface charge and surface distribution of charged groups. These differences make it possible to resolve L-Mengo from either S- or M-Mengo by chromatography on hydroxylapatite. Presumably they also provide the basis for the observations that L-Mengo sediments more rapidly through pre-formed dextran sulfate density gradients and migrates more slowly during electrophoresis in agarose gel than do the other two variants. Each of the variants was found to exhibit a reproducible and characteristic thermal denaturation (i.e., "melting") curve when heated in 0.1 M potassium phosphate (pH 7.1). The curves differ with respect to both the mid-point of the hyperchromic change (Tm), and the temperature range over which 90% of the hyperchromic change occurs.

This thesis summarizes the data arising from: (1) an investigation of the pH inactivation of the three variants of Mengo virus, including the determination of pH optimum, ion requirements, kinetics, and an analysis of the products of the reaction, (2) the biophysical characterization of the viral protein subunits produced by the pH-catalyzed disassembly of the Mengo virion, (3) an electron-microscopic study of the pH-inactivation reaction and of the products thereof, and (4) a detailed analysis of the early events - attachment, penetration and uncoating - in the replicative cycle of the Mengo virus variants.

ROUTINE MATERIALS AND METHODS

Mengo Encephalomyelitis Virus

The three plaque variants (L-, M- and S-Mengo) used throughout these studies were isolated originally by Ellem and Colter (1961), and maintained by periodic passage in cultured L cells.

Tissue Culture: Cells and Media

Cells

Earle's L-929 strain of mouse fibroblast cells (Sanford et al., 1948) was used throughout the studies. These cells were obtained from the American Type Culture Collection, Rockville, Md.

Tissue Culture Media

Growth medium. A medium containing Earle's salts (Earle, 1943) and Eagle's nutrients (Eagle, 1959) was obtained in powdered form from the Grand Island Biological Co., Grand Island, N.Y. (minimum essential medium F-11). The powder was dissolved in deionized distilled water, and sterilized by filtration through a model 2800 Seitz Filter (F.R. Hormann Co., Midale, Conn.). This "Basal medium" was then supplemented by the addition of the following materials to the final concentrations indicated.

(1) Sodium bicarbonate, 0.33%.

(2) Horse serum (Baltimore Biological Laboratory, Baltimore, Md.), either 5% (for growth of cells) or 1% (for production of virus).

(3) Aureomycin (Lederle Laboratories, Pearl River, N.Y.) 50 μg/ml; penicillin G (Ayerst Laboratories, Montreal, Quebec) 100 I.U./ml; and Streptomycin sulphate (Glaxo-Allenburys Ltd., Weston, Ontario) 100 μg/ml.

Spinner medium. Identical to growth medium, except that the basic powdered medium used lacked calcium -- "minimum essential medium (Eagle) with spinner salts" (Grand Island Biological Co., Grand Island, N.Y.).

Maintenance of cells. The continuous cell line used in this study - the L-929 strain of mouse fibroblasts - was grown in suspension at 37° in spinner medium in 1-liter spinner flasks (Bellco Biological Glassware, Vineland, N.J.). Fresh suspended cell cultures were prepared twice a week by trypsinization of monolayer cultures propagated in growth medium in Blake bottles. The cell monolayers were detached by replacing the growth medium with 0.25% trypsin in Hanks' balanced salt solution (Hanks and Wallace, 1949) and incubating at room temperature for 10-15 minutes. Cells were then collected by centrifugation, resuspended in spinner medium, and transferred to spinner flasks at a concentration of 200,000 cells/ml.

Titration of Virus

Preparation of L cell monolayers. Exponentially growing L cells in suspension culture were collected by centrifugation (5 min at 600 x g) and were resuspended in growth medium to give a suspension containing 2.5×10^6 cells/6 ml. Six ml aliquots of this suspension were

introduced into 60 mm plastic Petri dishes (Falcon Plastics, Los Angeles, Calif.). After incubation at 37° for 24 hrs in a humidified atmosphere of 5% CO_2 in air, confluent monolayers were formed.

Virus diluent. The buffered balanced salt solution (PBS) described by Dulbecco and Vogt (1954) was supplemented by the addition of bovine plasma albumin, fraction V, to a final concentration of 0.2%. Also added were phenol red, penicillin and streptomycin to final concentrations of 0.02%, 100 I.U./ml and 100 μg/ml, respectively. The pH of the solution was adjusted to 7.6 with NaOH.

Overlay diluent. This solution contained three times the normal concentration of Hanks' salts, six times the normal concentration of Eagle's nutrients, 0.75% sodium bicarbonate, 30% heat-inactivated (56° for 45 min) calf serum, and 0.02% phenol red.

Agar overlay. Two volumes of a 1.5% distilled water solution of special Noble agar (Difco Laboratories, Detroit, Mich.) were mixed, at 45°, with one volume of overlay diluent.

Special agar overlay. Identical to regular overlay, except that protamine sulphate (Calbiochem, Los Angeles, Calif.) was added to give a final concentration of 0.025%. Protamine was added in order to increase the size of plaques produced by S-Mengo for easier counting (Colter et al., 1964b).

Plaque assay. Virus samples were serially diluted to appropriate concentrations in virus diluent. Growth medium was removed from the pre-formed L cell monolayers and replaced with 0.1 ml of diluted

virus. After an incubation period of 1 hour at 37° to allow for attachment of virus particles to cells in the monolayers, 4.5 ml of agar overlay were applied. Following an additional 48 hour incubation at 37° in a humidified atmosphere of 5% CO_2 in air, the monolayers were stained by the addition of 3 ml of agar overlay containing .01% neutral red (Fisher Scientific Co., Fair Lawn, N.J.). Virus plaques became visible, and were counted within a few hours.

Growth of the Virus in Roller Bottles

Confluent L cell monolayers were prepared in large cylindrical bottles (Bellco roller bottle, 490 mm (ℓ) x 110 mm (d)) in the following manner. The bottles were first coated with fetal calf serum to facilitate attachment of cells, after which 150 ml of growth medium were added. Cells harvested from suspension culture were then added to give a final concentration of about 10^6 cells/ml and the bottles were rotated on a Bellco roller apparatus at 0.2 rpm for 3 hrs, after which the speed was increased to 1 rpm. The cells grew to confluency during incubation at 37° for 48 hours. The growth medium was then replaced by 30 ml of a suspension of virus in growth medium containing 1% horse serum, the suspension containing approximately 10^8 PFU/ml. The bottles were then rotated at 0.2 rpm for 2 hrs, and at 1 rpm for an additional 20-24 hrs, after which the cell mass was dislodged from the glass by gentle shaking.

Purification of Virus

Virus was purified by the method described by Scraba et al. (1969).

Lysates from infected roller bottles were pooled and centrifuged at 850 x g for 15 minutes. The pellet was resuspended in a small volume of distilled water, the suspension frozen and thawed to release the virus, and centrifuged at 850 x g for 20 minutes. The pooled supernatants were cooled in ice, and the virus was precipitated therefrom by adding cold methanol (-20°) to a final concentration of 20% by volume and allowing the solution to stand for at least 3 hrs at 4°. The precipitate was collected by centrifugation at 8000 x g for 30 min and was resuspended in 0.1M Na phosphate buffer, pH 8.0 (36 ml per 20 roller bottles).

The suspension of methanol-insoluble material was subjected to sonic oscillation for 60 sec. at maximum output in a Raytheon Model DF101 in order to disrupt large aggregates. A solution of α-chymotrypsin (three times crystallized, Worthington Biochemicals, Freehold, N.J.) was added to give a final enzyme concentration of 0.8 mg/ml and the mixture was incubated at 37° for 45 minutes. An equal volume of 0.2 M sodium pyrophosphate (pH 8.0) was then added, followed by ribonuclease (3x crystallized, salt-free, Worthington Biochemicals) to give a final concentration of 0.8 mg/ml, and incubation was continued for an additional 30 minutes. The mixture was then chilled and clarified by centrifugation at 7000 x g for 30 minutes. Virus was sedimented from the supernatant by centrifugation at

100,000 x g for 60 min, and resuspended in a few ml of 0.02 M potassium phosphate buffer (pH 7.1 for the S- and M-variants, pH 6.6 for the L-variant).

Final purification of Mengo virus was achieved by chromatography on hydroxylapatite. Material was eluted from the column (1.6 cm x 22 cm) by means of a concave K phosphate buffer gradient (pH 7.1 for S- and M-Mengo, pH 6.6 for L-Mengo) produced by using 3 chambers in a Varigrad mixing device (Buchler Instruments Inc., Fort Lee, N.J.). The starting phosphate concentration was 0.13 M and the limiting concentration was 0.8 M. A flow rate of 0.5 ml/min was maintained by a peristaltic pump.

Preparation of Radioactively Labeled Virus

$^{32}PO_4$ labeled virus was prepared by infecting (10 PFU/cell) L cell monolayers (grown in large cylindrical bottles) maintained in growth medium containing actinomycin D (2 μg/ml) for 2 hrs prior to infection. After a 1 hr incubation period to allow for virus attachment, the medium was replaced with a phosphate-deficient growth medium supplemented with 10 - 15 μCi/ml of $^{32}PO_4$ Na phosphate (Charles E. Frosst & Co., Montreal, Quebec) and actinomycin D (2 μg/ml). After a 24 hr incubation period, virus was harvested and purified according to the method outlined above.

H^3-uridine labeled virus was prepared in a similar manner, except that during the period of virus replication, the cells were maintained in growth medium containing 5 μCi/ml of H^3-uridine (New England Nuclear, Montreal, Quebec).

C^{14}-amino acid labeled virus was prepared by growing virus in the presence of "amino-acid deficient medium", consisting of Eagle's nutrients in Earle's medium from which the amino acids had been omitted, and supplemented with 1% horse serum and a mixture of C^{14}-labeled amino acids (New England Nuclear) at a level of 2 μc/ml.

CHAPTER I

STUDIES OF THE pH INACTIVATION OF THREE VARIANTS OF MENGO ENCEPHALOMYELITIS VIRUS

Introduction

Several reports describing the effect of pH on the infectivity and/or stability of mammalian viruses have been published. For example, it has been shown that several strains of coxsackie and poliomyelitis viruses are quite stable between pH's 3.0 and 9.5, and that the Lansing strain of poliovirus type 2 retains its infectivity after incubation at a pH as low as 1.5. However, foot and mouth disease virus (FMDV), like poliovirus a member of the picornavirus group, was found to lose its infectivity very rapidly at pH 6.5 (Bachrach and Schwerdt, 1952; Bachrach et al., 1957; Pinheiro and Hsiung, 1963).

It was shown earlier in these laboratories that pH has a dramatic effect on the infectivities of the M- and S-variants of Mengo virus for L cells, in both monolayer and suspension cultures. Increasing the pH at which cells and virus interacted from 6.8 to 7.8 was shown to increase the apparent titer (as determined by plaque assay) of the L-, S- and M-variants by factors of 2, 10 and 3000 respectively. No effect of pH - over the same range - was observed on the infectivity titers (determined by plaque assay on HeLa cell monolayers) of six strains of poliovirus. The effect of pH on the infectivities of the Mengo variants was shown to be due, in part, to

the instability of the M- and S-variants at pH's less than 7.0 (M-Mengo was found to be extensively, and S-Mengo less extensively but still significantly, inactivated at pH 6.8) (Colter et al., 1964a). A similar observation had been made even earlier by Speir (1962).

This chapter summarizes the data arising from an investigation of the kinetics of inactivation of the three Mengo variants at slightly acid pH's, of the ion requirements of the inactivation, and of the products produced from the virions by the inactivation.

Materials and Methods

Reaction Buffer

All solutions in which the inactivation of the Mengo virions was examined were buffered (at pH's from 5.2 to 7.4) with 0.02 M sodium phosphate.

pH Inactivation Studies

In those studies in which the kinetics and/or ion requirements of the inactivation were under examination, the reaction was started by the addition of 0.2 ml of viral suspension (in 0.02 M Na phosphate buffer, pH 7.2) to 4.8 ml of reaction medium equilibrated to the desired temperature. At the desired time intervals, 0.1 ml aliquots of the reaction mixture were removed, added to 4.9 ml volumes of cold (0°) virus diluent, and the number of PFU of virus therein determined by plaque assay.

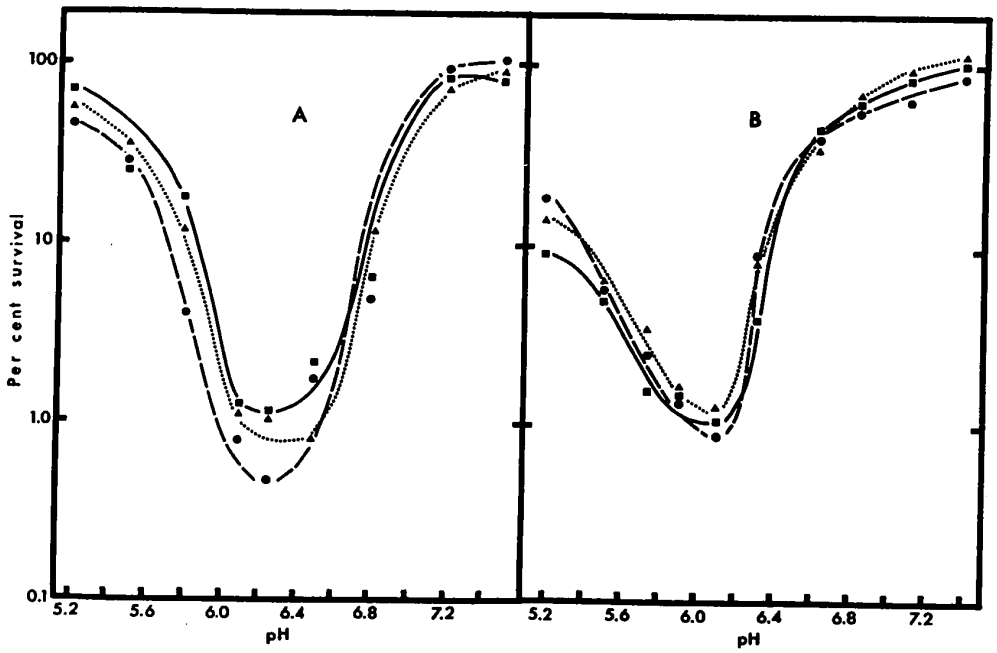


FIGURE 1.1. pH inactivation of Mengo virus variants in sodium halide solutions. Incubation was for 30 mins at 37° in (A) 0.14 M NaBr and (B) 0.14 M NaCl, buffered with 0.02 M Na phosphate at the indicated pH's.

(●) = S-Mengo; (▲) = M-Mengo; (■) = L-Mengo

In those studies designed to provide information regarding breakdown products produced during pH inactivation, highly purified, ^{14}C-amino acid or ^{3}H-uridine labelled virus was inactivated by dialysis (usually for 3 hrs) against a large volume of 0.14 M NaCl - 0.02 Na phosphate, pH 6.2, at 37°.

Sucrose Density Gradient Analyses

Sucrose density gradient analyses were carried out in a Spinco Model L preparative ultracentrifuge using an SW 50 rotor, the volume of the gradients being 4.5 ml in all cases. After centrifugation, fractions (0.15 - 0.2 ml) were collected directly onto filter paper discs from a hole punctured in the bottom of each tube. The filter paper discs were air dried, then washed 1x in cold 10% TCA, 4x in cold 5% TCA, 1x in ethanol-ether (1:1), and finally in ether. The dry discs were then placed in vials containing 5 ml of toluene-based scintillation fluid, and the radioactivity in each was monitored in a Beckman (Model CPM-200) liquid scintillation spectrometer.

Results

Effect of pH on the Stability of the Mengo Variants in Sodium Halide Solutions

Aliquots of pools of L-, M- and S-Mengo were incubated for 30 min at 37° in solutions of NaCl, NaBr, NaI and NaF (0.14 M) buffered with 0.02 M Na phosphate at pH's covering the range from 5.2 to 7.4, as

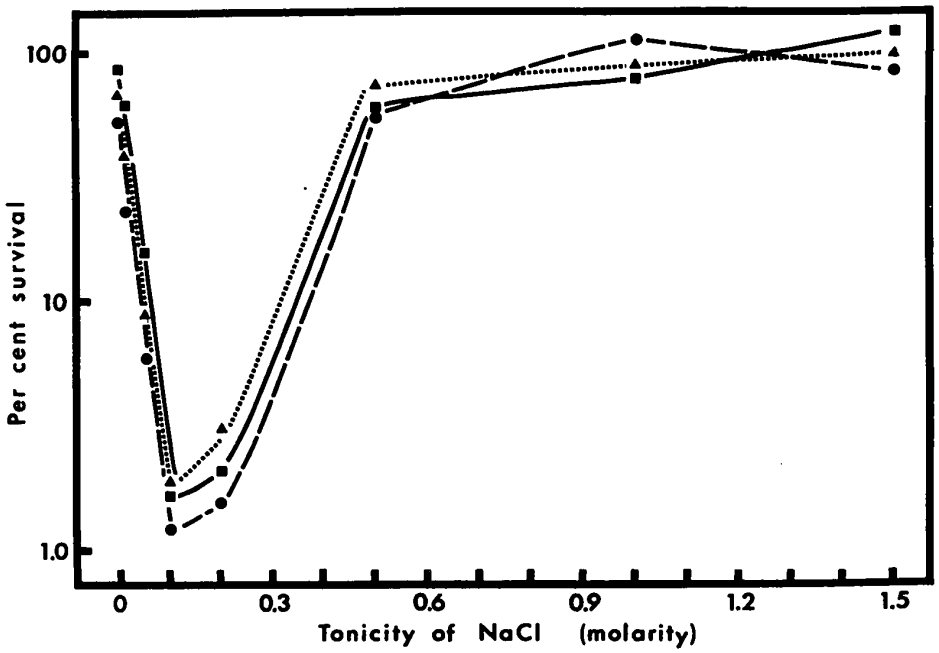


FIGURE 1.2. Effect of salt concentration on the inactivation of Mengo virus variants at pH 6.2. Incubation was for 30 mins at 37°.

(●) = S-Mengo, (▲) = M-Mengo, (■) = L-Mengo

described in Materials and Methods. The number of PFU of virus remaining in each incubation mixture was determined by plaque assay, and the data obtained from the NaCl and NaBr series, expressed as per cent survival of input PFU at each pH value, are shown in Figure 1.1. It is clear that, in solutions of either NaCl or NaBr, all three variants are extensively inactivated at pH's close to 6.0, the pH at which maximum inactivation occurs in both salts and with all 3 variants being 6.2. The data suggest that in the pH range, 5.2 - 5.8, the variants are more stable in NaBr than in NaCl, but otherwise the two sets of curves are remarkably similar. The pH survival curves obtained from the NaI and NaF series are not shown, since in neither salt was any significant inactivation observed at any pH with any of the 3 variants.

Effect of Tonicity on the Inactivation of the Mengo Variants at pH 6.2

The concentration of NaCl in the solution in which the variants are incubated was found to have a profound effect on the extent to which they are inactivated. This finding is illustrated in Figure 1.2, obtained by determining the number of PFU of virus remaining after incubating (30 min, 37°) aliquots of pools of L-, M- and S-Mengo in solutions containing concentrations of NaCl ranging from 0 to 1.5 M, and buffered with 0.02 M Na phosphate at pH 6.2. Little or no inactivation was found to occur either in the absence of NaCl or at salt concentrations of 0.5 M or higher. As the salt concentration was either increased from 0 or decreased from 0.5 M, the extent of the inactivation was found to increase sharply, with maximum inactivation occurring at NaCl concentrations of 0.1 - 0.2 M.

TABLE 1.1

STABILITY OF MENGO VIRUS (M-VARIANT) IN SOLUTIONS OF VARIOUS INORGANIC SALTS

Salt Solution[a]	% Survival of Input PFU's After Incubation[b]	
	at pH 7.2	at pH 6.2
NaCl	100	0.55
NaBr	100	0.60
LiCl	100	0.69
KCl	85	0.44
$CaCl_2$	83	0.64
$MgCl_2$	85	0.25
NaF	100	98
NaI	100	96
$NaNO_2$	95	86
$NaNO_3$	96	89
Na_2SO_4	97	89

[a] All solutions had an ionic strength of 0.16, and all were buffered (at either pH 7.2 or 6.2) with 0.02 M Na phosphate.

[b] Incubation was for 30 mins at 37°.

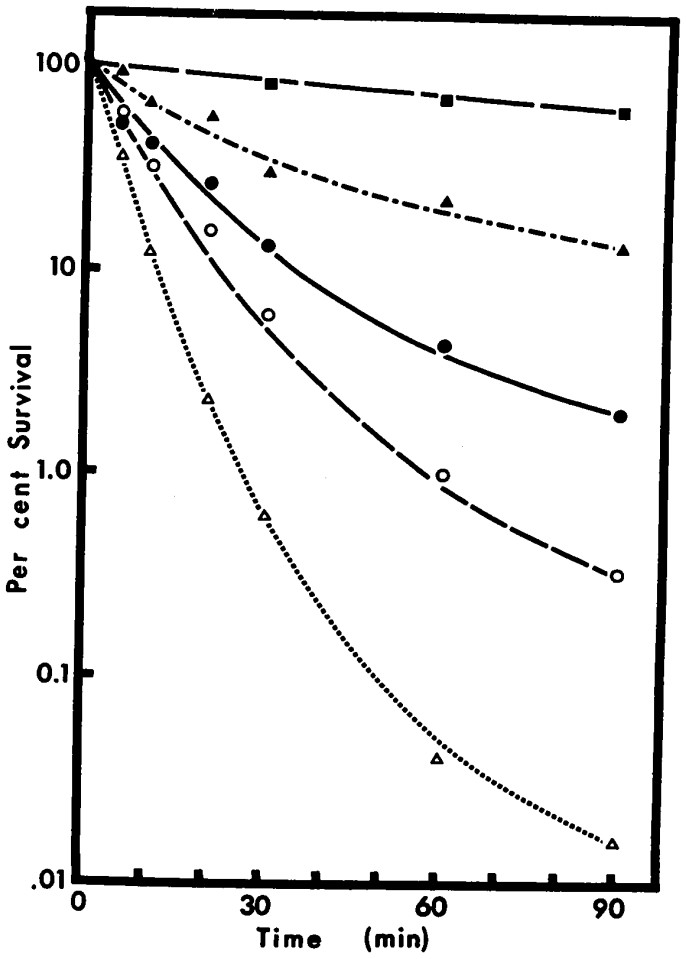


FIGURE 1.3. Effect of temperature on the rate of inactivation of M-Mengo in 0.14 M NaCl - 0.02 M Na phosphate, pH 6.2.

(▲) = 7°, (●) = 17°, (o) = 27°, (△) = 37°
(■) = control; virus incubated at 37° in 0.14 M NaCl - 0.02 M Na phosphate, pH 7.6.

Specificity of the Ionic Requirement for pH Inactivation

The foregoing results suggested that the pH inactivation of Mengo virus has a specific requirement for chloride (or bromide) ions. In order to establish this point more firmly, the stability of the M-variant in solutions of several sodium salts and in solutions of various cationic chlorides was examined. The ionic strength of all solutions was 0.16, and in all cases the number of surviving PFU was determined after incubation at 37° for 30 min in solutions of pH 7.2 and 6.2. The results are summarized in Table 1.1. It may be seen that (a) of the limited number of sodium salts examined, inactivation was found to occur only in NaCl and NaBr, and (b) inactivation proceeded to about the same extent in all cationic chloride solutions examined. In view of these results and those described above, the conclusion that the inactivation is chloride (or bromide) ion dependent seems justified.

Kinetics of Inactivation at Different Temperatures

As would be anticipated, the rate at which Mengo virus is inactivated at pH 6.2 is temperature dependent. This fact is illustrated by the data summarized in Figure 1.3, obtained from an examination of the rates of inactivation of M-Mengo at temperatures of 7°, 17°, 27°, and 37°. The control in this case consisted of virus incubated at 37° in phosphate buffered physiological saline of pH 7.6. The rate of inactivation was found to approximately double with each 10° increase in reaction temperature over the range examined. Half-lives were calculated to be 24, 13, 7.4, and 3.3 minutes for M-Mengo

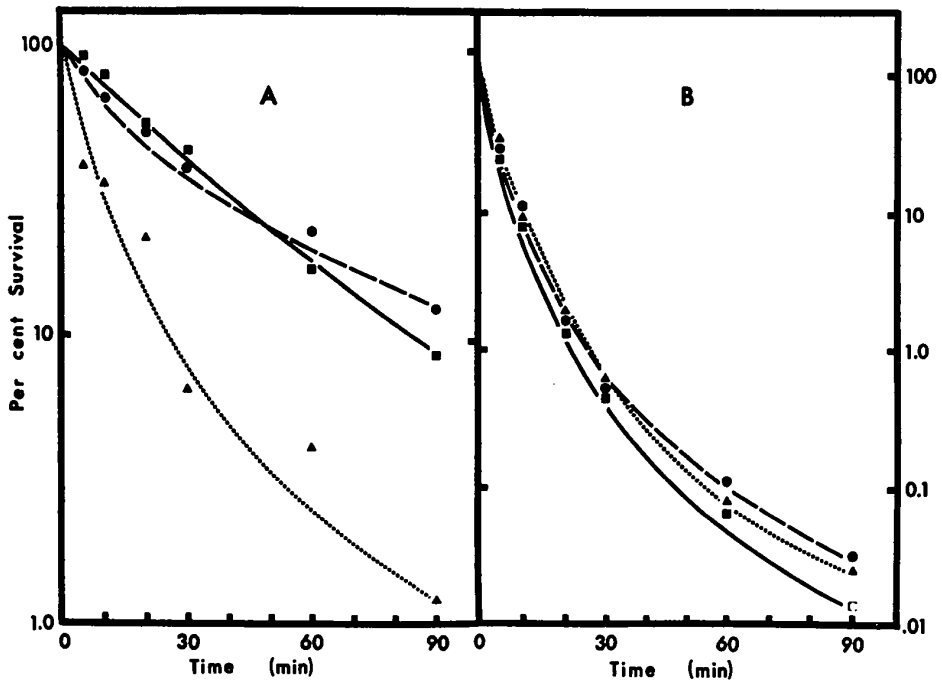


FIGURE 1.4. Rates of inactivation of L-, M- and S-Mengo in 0.14 M NaCl - 0.02 M Na phosphate, pH 6.2, at (a) 17° and (B) 37°.

(●) = S-Mengo, (▲) = M-Mengo, (■) = L-Mengo

samples incubated at 7°, 17°, 27°, and 37°, respectively. (The half-life of the control sample - a measure not of the rate of pH inactivation but of thermal inactivation at 37° - was 90 minutes).

The rates of inactivation of L-, M- and S-Mengo at pH 6.2 and at a temperature of 37° were compared, and were found to be essentially identical (Figure 1.4B). This was rather unexpected since the earlier studies carried out in this laboratory had suggested that, of the three variants, M-Mengo is most sensitive to pH-inactivation (Colter et al., 1964a). For this reason, and since the earlier studies had been carried out at room temperature, the rates of inactivation of the three variants at 17° were compared. The results, summarized in Figure 1.4A, show that at the lower temperature, M-Mengo is inactivated more rapidly than are the two variants, and tend to substantiate the earlier conclusion.

Breakdown Products of pH Inactivated Virions

In order to determine whether or not pH inactivation destroys the physical integrity of Mengo virions, highly purified preparations of C^{14}-amino acid-labeled L-, M- and S-Mengo, inactivated by dialysis for 3 hours at 37° against 0.14 M NaCl buffered at pH 6.2, were examined by sucrose gradient analysis. Aliquots (0.2 ml) of the treated preparations (residual infectivity was <0.5% input) were layered on 5-40% linear sucrose gradients and centrifuged for 80 min at 35,000 rpm in an SW50 rotor. Samples of untreated (control) virus suspensions were subjected to centrifugation in identical gradients at the same time.

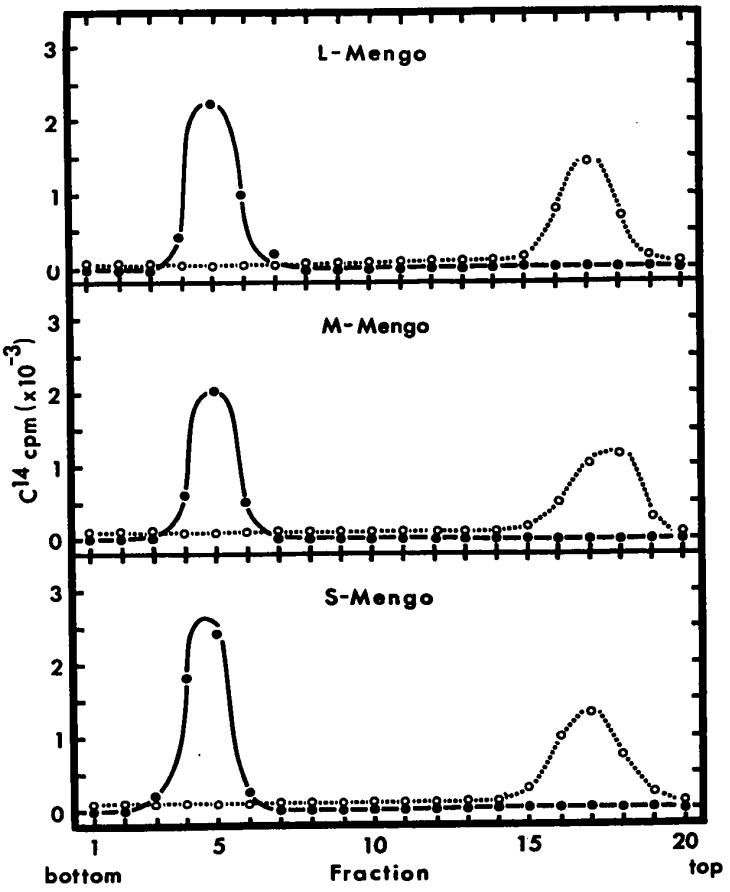


FIGURE 1.5. Sucrose density gradient sedimentation analysis of ^{14}C-amino acid labelled Mengo virions. (●) control samples, (○) inactivated by dialysis against 0.14 M NaCl - 0.02 M Na phosphate, pH 6.2, for 3 hrs at 37°. Centrifugation was for 80 mins at 35,000 rpm, in a Spinco SW50 rotor, through a 5-40% linear sucrose density gradient.

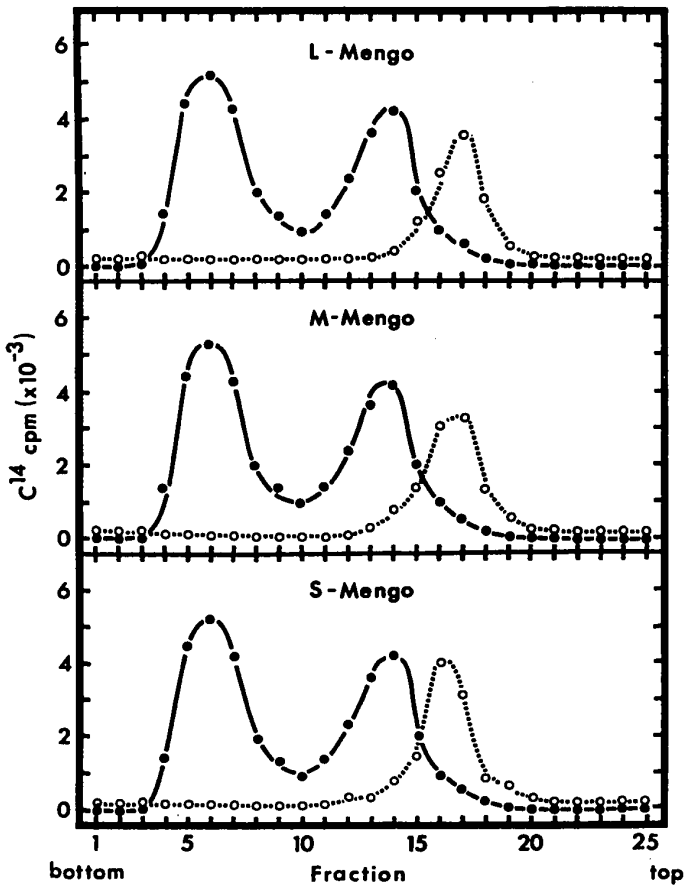


FIGURE 1.6. Sucrose density gradient sedimentation analysis of ^{14}C-amino acid labelled Mengo virions inactivated by dialysis as described in Figure 1.5. (o) pH inactivated virus preparations; (•) O.D.$_{260m\mu}$ profile given by L cell ribosomal (18 and 28S) RNA's. Centrifugation was for 3 hrs at 35,000 rpm, in a Spinco SW50 rotor, through a 5-20% linear sucrose gradient.

The results, summarized in Figure 1.5, show that pH inactivated L-, M- and S-virions were completely degraded into fragments which banded near the top of the gradients, whereas the untreated virions sedimented to the expected position in the gradients (Mengo virus, as shown by Scraba *et al*. (1967) has a sedimentation coefficient of 150S). In order to obtain an estimate of the size of these fragments and to assess their homogeneity, aliquots of pH-inactivated virions were layered, together with a mixture of 18 and 28S ribosomal RNA's as an optical density marker, on 5-20% linear sucrose gradients and centrifuged for 3 hours at 35,000 rpm. The $OD_{260\ m\mu}$ of, and the radioactivity in, each fraction was measured, and the results are illustrated in Figure 1.6. It is clear that pH-inactivation of all three variants results in the breakdown of the protein capsids to fragments that sediment in a single-discreet band. The symmetry of the peaks suggest that the fragments are homogenous with respect to molecular size, and their position relative to those of the 18 and 28S ribosomal RNA's indicate that the fragments have a sedimentation coefficient of the order of 15S.

The fate of the viral genome during pH inactivation was also examined. Highly purified uridine-^{3}H labelled M-Mengo virus was inactivated by incubation at pH 6.2 for 60 min at 37°, after which the RNA was isolated by the modified sodium dodecyl sulfate-phenol technique described by Scraba *et al*. (1967). The isolated viral RNA was then examined by sucrose density gradient centrifugation, and the result is shown in the upper panel of Figure 1.7. It is clear that under

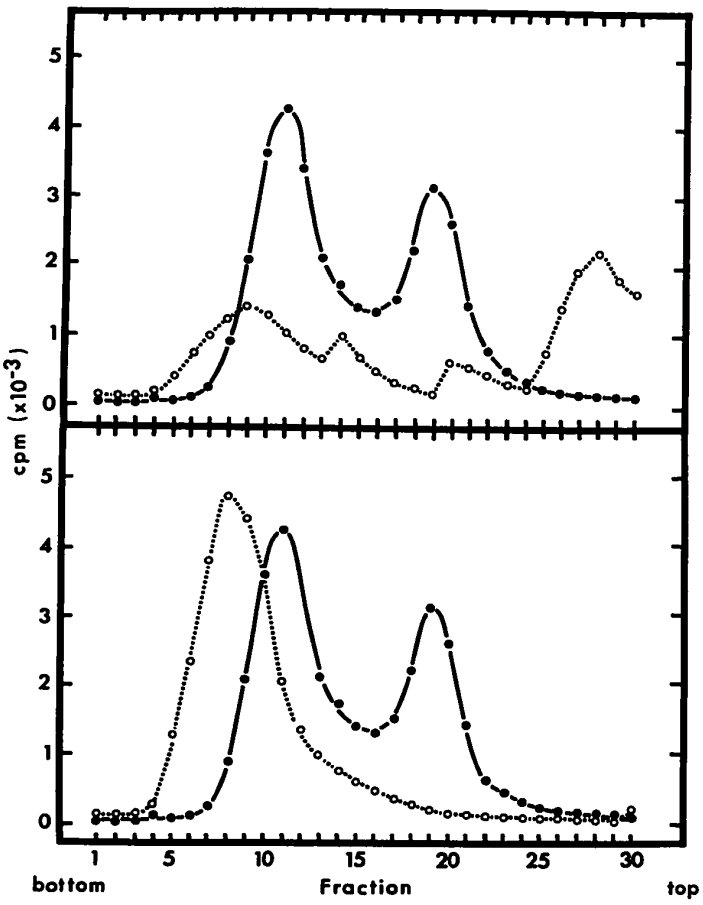


FIGURE 1.7. Sucrose density gradient sedimentation analysis of ^{3}H-labelled RNA isolated from highly purified virus inactivated by incubation for 60 mins at 37° in (upper panel) 0.14 M NaCl - 0.02 M Na phosphate, pH 6.2, and (lower panel) 0.14 M NaCl - 0.02 M Na phosphate, pH 6.2, containing 0.02% Macaloid and 0.1% dextran sulfate. Centrifugation was for 3 hrs at 35,000 rpm in a Spinco SW 50 rotor, through a 5-20% linear sucrose density gradient.

(o) = viral RNA; (●) = L cell ribosomal RNA added as an optical density marker.

these conditions of pH inactivation, the viral genome is extensively degraded, probably due to the presence of traces of ribonuclease, perhaps introduced during the purification of the virus. When the experiment was repeated, with the inactivation carried out in the presence of 0.02% Macaloid (Baroid Division, National Lead Co., Houston, Texas) and 0.1% dextran sulfate (mol.wt. 60,000 - 90,000; Sigma Chemical Co., St. Louis, Missouri) to inhibit any ribonucleases present, intact, viral RNA (sedimentation coefficient of 35S, Scraba et al. (1967)) was recovered (lower panel, Figure 1.7). Quite clearly, pH inactivation and dissociation of the Mengo virions can proceed without degradation of the viral genome.

Discussion

The rapid and essentially quantitative breakdown of Mengo virions to fragments of uniform size (approximately 15S) with the release of the intact viral genome when incubated at slightly acid pH's in the presence of chloride or bromide ions is a striking phenomenon. Several characteristics of the inactivation reaction seem rather surprising: (1) the sharpness of the pH optimum; (2) the specificity with respect to anion requirements (which suggests that the diameter of the anion may be the critical factor); (3) the sharpness of the optimum with respect to the concentration of chloride ions; and (4) the fact that the viral capsid breaks down in such a precise manner.

A comparison of the pH inactivation of the three variants shows that they are essentially identical with respect to the pH optimum, ionic requirement, the products produced from the virus by the inactivation, and the kinetics of inactivation at 37°. The last finding was rather unexpected since earlier studies (Colter et al., 1964a) carried out in these laboratories had suggested that, of the three variants, M-Mengo is most sensitive to pH inactivation. However, the earlier studies were carried out at pH 6.8, and an examination of the kinetics of inactivation at 17° indicated that, at that temperature, M-Mengo is inactivated somewhat more rapidly than are the other two variants.

To attempt to draw conclusions - on the basis of the data presented here - regarding the architecture of the Mengo virion, the forces responsible for maintaining its integrity under normal conditions, and the mechanism by which it is disassembled at slightly acid pH's, would be wildly (and unjustifiably) speculative. Nonetheless, from an examination of the pH-survival profiles (Figure 1.1), it is tempting to suggest that protonation of imidazole rings of histidine residues as the pH is lowered from 7.0 to 6.2 might create ionic interactions leading to decreased stability of the virions. However, to assign responsibility for the breakdown to one or two specific amino acid residues in the virion would be naive. It seems much more likely that the mechanism of disassembly is a highly complex one, involving not only protein-protein interactions, but protein-ribonucleate interactions as well.

The observations described here raise some interesting questions. Does the subviral fragment produced by disassembly of the virion at pH 6.2 have any biological significance,- that is, is it produced during either the intracellular uncoating of the virus or the assembly of progeny virions? What is its polypeptide composition, and how does it compare with that of the intact virion? How does it fit into the architecture of the virion? Studies designed to answer some of these questions are described in the following chapter.

CHAPTER II

STUDIES OF THE PROTEIN SUBUNITS OF pH INACTIVATED MENGO VIRUS VARIANTS

Introduction

In the previous chapter, it was shown that the three variants of Mengo virus are rapidly inactivated at slightly acid pH's in the presence of 0.1 - 0.2 M chloride or bromide ions. The inactivation was shown to be accompanied by the complete dissociation of the viral capsids into protein subunits of uniform size, having a sedimentation coefficient of the order of 15S as estimated from sucrose density gradient analysis. The investigations described in this chapter were undertaken to elucidate the polypeptide composition of the subunits produced by pH-inactivation of the three variants, and to characterize the subunits using a variety of physicochemical techniques including sedimentation velocity, sedimentation equilibrium, circular dichroism and electron microscopy.

Materials and Methods

Inactivation of the Virus and Separation of Breakdown Products

Highly purified, C^{14}-amino acid-labeled virus was inactivated either by dialysis at 37° (usually for 3 hours) against a large volume

of 0.14 M NaCl-0.02 M Na phosphate (PBS), pH 6.2, or (after being sedimented by centrifugation for 1.5 hours at 100,000 x g) by incubation for 30 min at 37° in a small volume of PBS, pH 6.2. Aliquots of these preparations (residual infectivity was <0.5% of input) were then layered on 10-30% linear sucrose gradients (in PBS, pH 6.2) and centrifuged for 16-18 hours at 25,000 rpm in an SW 25 rotor (Spinco Model L preparative ultracentrifuge). Fractions (1.0 - 1.3 ml) were collected from a hole punctured in the bottom of the centrifuge tube, and the radioactivity and absorbancy (at 280 mμ) of each was measured.

Solubilization of Intact Virions and Viral Breakdown Products for Gel Electrophoresis

Intact virions (^{14}C-labeled), and fractions isolated by sucrose density gradient centrifugation of pH-inactivated virions, were solubilized by incubation (2-3 hours at 37°) with 2% sodium dodecylsulfate (SDS; recrystallized from 50% ethanol), 1% 2-mercapto-ethanol (ME), and 8M urea. The samples were then dialyzed overnight against 1,000 - 2,000 volumes of 0.01 M sodium phosphate buffer, pH 7.1, containing 0.1% SDS and 0.1% ME.

Polyacrylamide-Gel Electrophoresis

Gels were prepared as described by Maizel (1966) and consisted of 5% acrylamide, 0.13% N, N'-methylene-bisacrylamide, 0.1 M sodium phosphate buffer (pH 7.1) and 0.1% SDS. Polymerization was catalyzed by N,N,N',N'-tetramethylethylene diamine and ammonium per-sulfate at final concentrations of 0.05 and 0.075%, respectively.

Samples containing 100 to 300 μg of viral protein, or suitable levels of radioactivity, in 0.10 to 0.15 ml of electrophoresis buffer (0.1% SDS in 0.10 M sodium phosphate buffer, pH 7.1) were made 12% with respect to sucrose, applied to the gels by layering beneath a layer of buffer, and electrophoresed for either 4-6 hrs (10 cm x 0.6 cm gels) or 8-16 hrs (20 cm x 0.6 cm gels) at 4 mamp per gel at 20°. Following electrophoresis, the gels were chilled in ice and sliced into 1.5 mm fractions by employing a mechanical gel slicer (Canalco, Rockville, Md.). Gel fractions were then incubated overnight with 12% ammonium hydroxide to elute radioactive proteins and the radioactivity in each was counted after the addition of 10 ml of dioxane-based scintillation fluid.

Preparation of Viral Subunits for Physicochemical Analysis

After sucrose gradient analysis of the inactivated virus as described above, the fractions containing the viral subunit were pooled and dialyzed (three changes of buffer at five-hour intervals) against a large volume of PBS, pH 6.2, at 0°. Samples were then concentrated using a Sartorius-membrane filter apparatus (BDH Laboratory, Chemical Division, Toronto, Canada) to concentrations of 1-2 mg/ml and 3-4 mg/ml for sedimentation equilibrium and sedimentation velocity studies, respectively. Protein concentration was estimated from the absorbancy measured in a Gilford spectrophotometer (Model #240), using the extinction coefficient of Mengo protein ($E^{1\%}_{280nm}$ = 17.0; Scraba et al., 1969).

Dissociation of Viral Subunits by Urea and Sucrose Density Gradient Sedimentation Analysis

Highly purified, ^{14}C-amino acid labeled viral subunits were further dissociated by incubation for 1-2 hr at 0° in the presence of 2-3 M urea. Aliquots of these preparations were then layered on 5-20% sucrose gradients (in PBS, pH 6.2, containing 3 M urea) and centrifuged for 6-1/2 hours at 45,000 rpm in an SW50 rotor. Fractions (10 drops each) were collected from a hole punctured in the bottom of the centrifuge tube and the amount of radioactivity in each was measured.

Analytical Ultracentrifugation Studies

Analytical ultracentrifugation studies were carried out in a Spinco Model E ultracentrifuge equipped with an electronic speed control and an RTIC temperature control unit. Sedimentation velocity studies were performed at 40,000 rpm at 7° in a standard single sector cell (analytical D rotor) utilizing the schlieren optical system. The intrinsic sedimentation coefficients ($S^{\circ}_{20,w}$) were calculated by the method of Schachman (1959) and were corrected for solvent viscosity, temperature and density, using Timmermann's tables (Timmermann, 1960). In calculations of both intrinsic sedimentation velocity and molecular weight from sedimentation equilibrium studies, the previously determined value of 0.731 ml/gm for the partial specific volume ($\bar{v}$) of the viral protein was used (Scraba et al., 1969).

High speed sedimentation equilibrium studies (Yphantis, 1964) were performed in a 12 mm double sector synthetic cell at 5° and at rotor speeds of 10,000 and 23,000 rpm for the "15S" viral subunit

and "5S" fragment respectively. Photographs were taken on Kodak spectroscopic metallographic plates (type II G) at the time of equilibrium (~24 hr), and the fringes were measured with a Nikon 6C comparator. Molecular weights were calculated using the meniscus depletion method by evaluating the limiting slope of a plot of lny vs x^2 and employing the equation:

$$M = \left(\frac{2RT}{(1-\bar{v}\rho)\omega^2}\right)\left(\frac{d\ln y}{d(x^2)}\right)$$

where M = molecular weight, R = ideal gas constant, T = temperature in degrees Kelvin, $\bar{v}$ = partial specific volume of the solute, ρ = density of the solvent, ω = angular velocity of the rotor in radians per second, lny = natural logrithm of the fringe displacement, and x = distance in centimeters from the axis of rotation.

Circular Dichroism Studies

The ultraviolet circular dichroic (c.d.) spectra of the viral protein subunit was examined in a Cary Model 60 recording spectropolarimeter equipped with a Model 6001 circular dichroism attachment and a thermostable cell housing maintained at 10°C. Measurements of viral protein (0.1 - 0.2 mg/ml) were made over the wave-length range of 202 to 250 nm in a 1 cm cell of special design (Opticell, Beetsville, Maryland). The instrument was calibrated using an aqueous solution of d-10-camphor-sulfonic acid (Eastman Organic Chemicals, recrystallized) which has a difference in molecular extinction coefficient (E_L-E_R) of 2.16.

The results were recorded in terms of specific ellipticity [θ] - which is given by the relation $[\theta] = \theta M/100\ell c$, where M is the mean residue weight (a value of 112 for Mengo viral protein was employed; Scraba, 1968), θ is the observed ellipticity in degrees, ℓ is the path length in decimeters, and c is the concentration of viral protein in g/ml. The units of [θ] are degrees cm^2 per decimole. The errors in the spectral zone investigated, in terms of specific ellipticities, were ±500 degrees $cm^2 dmole^{-1}$.

Electron Microscopic Studies

Samples of virus and viral subunit were prepared for electron microscopy by the negative staining method described by Huxley and Zubay (1960). Copper grids (200 mesh) carrying a parlodion-carbon film were pretreated by a brief (20 sec) glow discharge in a Balzers Micro BA3 unit under low vacuum (10^{-2} Torr) in order to produce a hydrophilic surface. A drop of the virus or viral subunit suspension was placed on this surface and 60 sec was allowed for attachment of the particles. Excess solution was removed by touching the edge of the grid with filter paper, the surface was washed with three successive drops of distilled water, and then with three drops of the negative stain. The following aqueous solutions were employed as electron-dense negative stains: 2% sodium phosphotungstate (pH 7.1); 3% sodium silicotungstate (pH 7.2); 1% uranyl acetate (pH 4.0); and 1% uranyl formate (pH 4.5).

The recipes for the preparation of viral subunits for electron microscopic examination were as follows: (1) "15S" subunits - 0.2 ml of a suspension of highly purified virus (approximately 5×10^{13} particles/ml

in 0.1 M potassium phosphate, pH 7.4) was diluted with 0.7 ml water, and 0.1 ml of 1.0 M NaCl and 0.04 ml of 0.1 N HCl were added. The resulting solution was then incubated at 37° for 30 sec, and immediately thereafter, samples were applied to glow-treated grids; (2) "5S" fragments - the same procedure was followed except that the virus suspension was diluted with 0.5 ml water, and 0.2 ml of 10 M urea were added prior to incubation at 37°. Such preparations always contained a mixture of intact virions and subunits (and fragments).

Grids were examined in a Philips EM300 electron microscope equipped with an anti-contamination device and operated at an accelerating voltage of 80 KV. Double condenser illumination was used, and the objective lens aperture was of 50μ diameter. Photographs were taken on Kodak electron microscope cut film (Type 4489) or Kodalith LR film (Type 2572) at nominal magnifications of 60,000 X to 110,000 X. The true magnification was calibrated with a diffraction grating replica (Ernest F. Fullam, Inc., Schenectady, N.Y.); and whenever possible the diameter of the Mengovirus particle (26.7 ± 0.5 nm; Scraba et al., 1967) was measured on photographic negatives or prints as an internal standard.

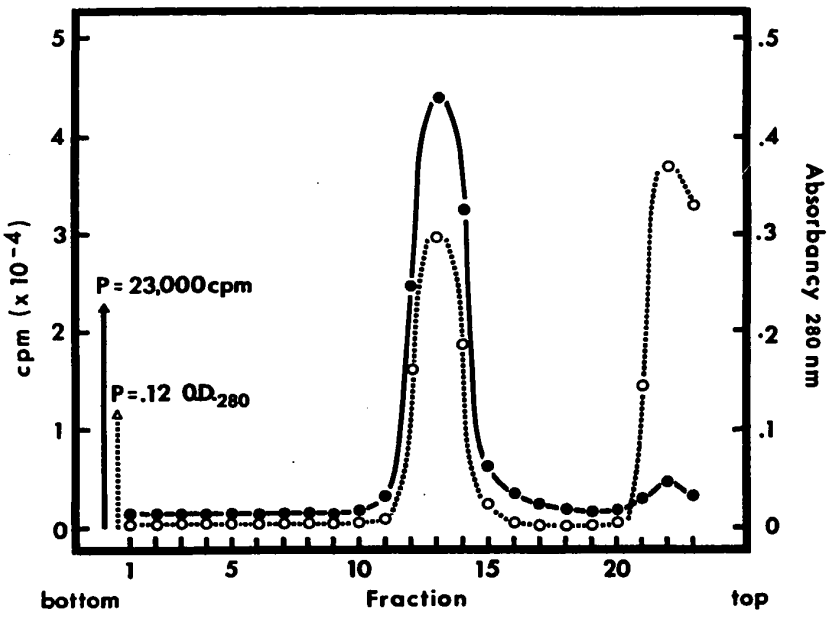


FIGURE 2.1. Sucrose density gradient sedimentation analysis of pH-inactivated virus. Purified ^{14}C-amino acid labeled virions, inactivated by incubation in 0.14 M NaCl - 0.02 M Na phosphate, pH 6.2, for 3 hours, were layered on 10-30% sucrose gradients and centrifuged for 18 hours at 20,000 rpm.

(●——●) radioactivity; (O---O) $O.D._{280}$

P = pellet

Results

Sucrose Density Gradient Centrifugation of pH-Inactivated Virions and Isolated Subunits

Highly purified preparations of L-, M- and S-Mengo were inactivated by incubation at pH 6.2 and then subjected to centrifugation as described in Materials and Methods. The sedimentation profile obtained from such an experiment is illustrated in Figure 2.1.

With each of the three variants, the bulk of the radioactivity (70 - 80%) was found in a well-defined, relatively symmetrical peak corresponding to fractions 11-14 of the gradient. Lesser amounts of radioactivity were found in a pellet and in the top two or three fractions of the gradient. The pellet and top fractions were found to contain of the order of 20% and 5% respectively, of the total radioactivity. However, when subunits were prepared from C^{14}-amino acid labeled virions, and the pooled fractions dialyzed to remove sucrose and concentrated to give a solution containing 2-4 mg of protein per ml for physico-chemical studies (see Materials and Methods), a different sedimentation profile was observed upon recentrifugation in sucrose density gradients (Figure 2.2, upper panel). The material in the concentrated solution sedimented in three discreet peaks instead of in a single homogenous peak corresponding to a sedimentation coefficient of about 15S. The sedimentation coefficients of the material in these peaks were estimated to be approximately 14, 22, and 29S, as judged by their positions relative to those of 18 and 28S marker mammalian ribosomal RNA's. This was a somewhat disturbing

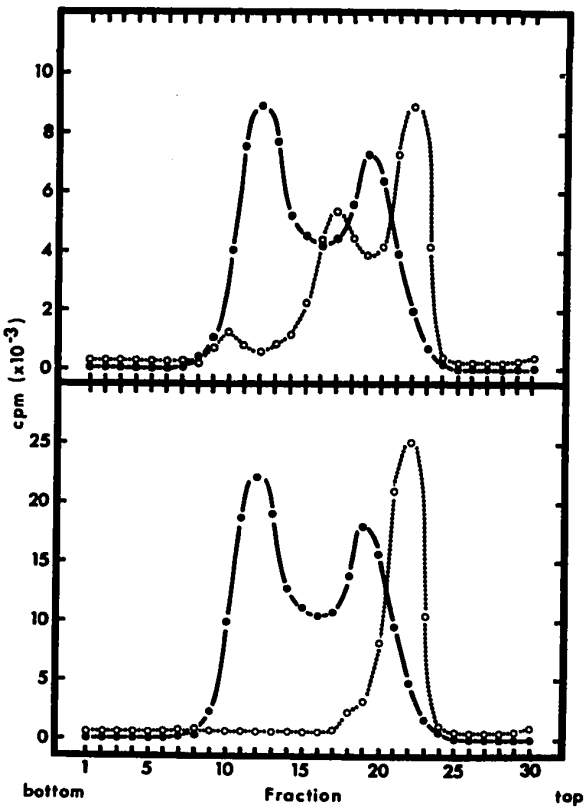


FIGURE 2.2. Effect of DTT (dithiothreitol) incubation on the sedimentation profile of purified, highly concentrated preparations of viral protein subunit. Preparations of ^{14}C-amino acid labeled protein subunit, purified as shown in Figure 2.1, were concentrated to 2-4 mg/ml of protein subunit as described in Materials and Methods. One half of the sample was incubated with 1 mM DTT for 1 hour at 0° and the other half was untreated. The samples were then layered onto 5-20% linear sucrose density gradients (PBS, pH 6.2) and centrifuged for 3 hr at 35,000 rpm in an SW 50 rotor. Untreated sample, upper panel; DTT-treated sample, bottom panel. o---o, ^{14}C-radioactivity; •—•, sedimentation profile of ^{3}H-uridine-labeled 18S and 28S marker ribosomal RNA's.

observation since precise measurements of the physicochemical parameters of the subunit could not be carried out using preparations that contained a mixture of several molecular species. However, subsequent experiments showed that incubation of the purified, concentrated subunit preparations with 1 mM dithiothreitol (DTT) for 1 hr at 0° prevented, and reversed, the aggregation. The sedimentation profile of DTT-treated preparations of viral subunits showed a single, uniform peak of the order of 14S as shown in Figure 2.2, lower panel. These results indicate that the viral protein subunits polymerize by the formation of disulphide cross linkages during isolation and concentration. In view of these observations, all preparations of viral subunits employed in subsequent ultracentrifugation studies were incubated with DTT (1 mM; 1 hr; 0°) prior to further analysis.

Composition Analysis of Fractions Isolated from pH Inactivated Virions

The identity and relative concentrations of the polypeptides present in the three fractions (main peak, pellet and top) obtained by sucrose density gradient centrifugation of pH inactivated virions were determined by acrylamide gel electrophoretic analysis. The pellet, "15S" and top fractions isolated from all three variants, as well as preparations of ^{14}C-labeled L-, M- and S-Mengo virions, were disrupted by treatment with SDS, ME and urea, and the resulting mixtures of solubilized polypeptides were examined by electrophoresis in 5% polyacrylamide gels containing 0.1% SDS. Characteristic electrophoretic patterns given by the polypeptides of the "15S", pellet and

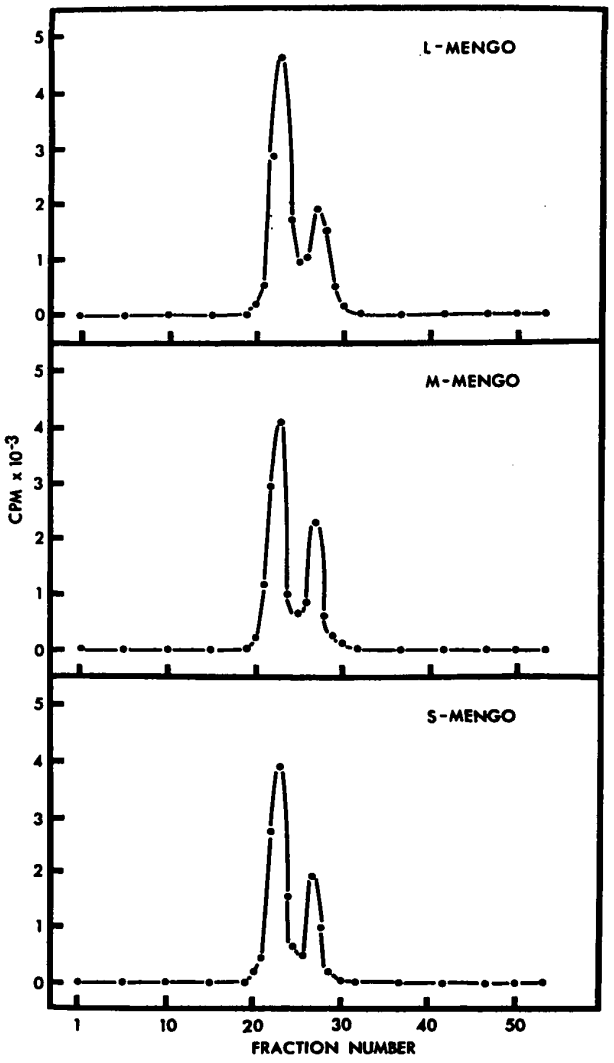


FIGURE 2.3. Polyacrylamide gel electrophoretic analysis of 15S fragments isolated from pH-inactivated Mengo variants. Fractions 10-14 of sucrose density gradients (Figure 2.1) were pooled, solubilized in SDS, ME, and urea, and applied to 5% SDS-gels. Electrophoresis was for 4 hr at 4 m amp per gel.

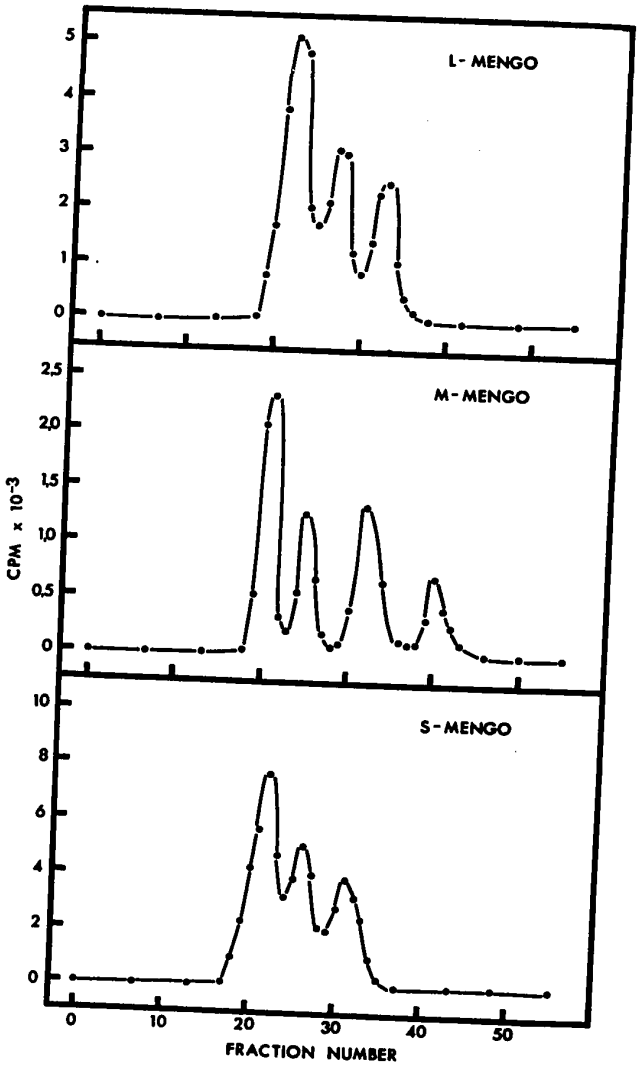


FIGURE 2.4. Polyacrylamide gel electrophoretic analysis of the pellet fractions of pH-inactivated Mengo variants. The pellet fractions of sucrose density gradients described in Figure 2.1 were resuspended in PBS buffer, pH 7.8, solubilized, and applied to 5% SDS-gels.

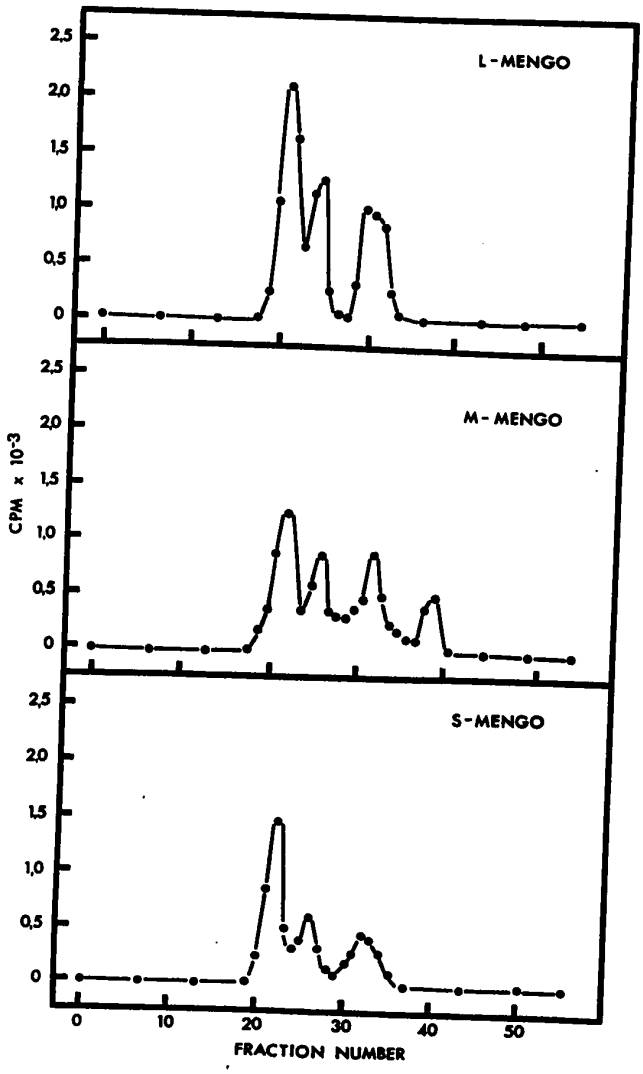


FIGURE 2.5. Polyacrylamide gel electrophoretic analysis of the top fractions of pH-inactivated Mengo variants. Fractions 18-20 of sucrose density gradients described in Figure 2.1 were pooled, solubilized, and applied to 5% SDS-gels. Electrophoresis was carried out for 4 hr at 4 m amp per gel.

top fractions isolated from pH-inactivated suspensions of the three variants are shown in Figures 2.3, 2.4 and 2.5 respectively.

It is clear from Figure 2.3 that the "15S" subunit derived from all three variants contains only two of the three major structural polypeptides that have been shown to be present in the intact virions (O'Callaghan et al., 1970). These two components were identified as structural proteins VPI and VPII from their mobilities relative to those of marker VPI, VPII and VPIII extracted from intact L-, M- and S-Mengo virions. No VPIII was detected in any "15S" preparation from any of the variants; and none of the "15S" fractions isolated from M-Mengo contained any trace of VPIV, the fourth and minor structural polypeptide present only in M-Mengo virions.

All three major structural polypeptides - VPI, VPII and VPIII - were found in the pellets obtained by sucrose density gradient centrifugation of pH-inactivated L-, M- and S-Mengo. The polypeptide VPIV was found in all pellets derived from M-Mengo virions. These findings are illustrated in Figure 2.4. However, the amounts of VPIII (and, in the case of M-Mengo, VPIV) in the pellets relative to the amounts of VPI and VPII therein, were found to be much larger than those present in the intact virions.

Electrophoretic patterns obtained from the top fractions (see Figure 2.5) were very similar to those given by the solubilized pellets. All three major structural polypeptides were found in all top fractions, and the minor component, VPIV, was present in those top fractions derived from M-Mengo.

TABLE 2.1

POLYPEPTIDE COMPOSITION OF INTACT MENGO VARIANTS AND OF FRACTIONS ISOLATED FROM pH-INACTIVATED VIRIONS

Variant	Preparation[b]	Percentage Composition[b]							
		VPI		VPII		VPIII		VPIV	
		c	d	c	d	c	d	c	d
L	Virion	62	-	27	-	9	-	-	-
	"15S"	68	48	32	22	-	-	-	-
	Pellet	49	12	25	6	25	6	-	-
	Top	53	3	27	1.3	20	1	-	-
M	Virion	59	-	26	-	8	-	5	-
	"15S"	68	48	32	22	-	-	-	-
	Pellet	40	10	21	5	27	6.8	11	3
	Top	47	2	25	1	22	1	10	0.5
S	Virion	60	-	28	-	9	-	-	-
	"15S"	69	48	31	21	-	-	-	-
	Pellet	48	12	24	6	27	6.8	-	-
	Top	54	2.7	22	1.1	25	1.3	-	-

[a]Values given for "virion" are for intact virions, and are taken, in part, from data previously reported (O'Callaghan et al., 1970). "15S", "pellet" and "top" preparations refer to fractions isolated from pH-inactivated virions by sucrose density gradient centrifugation.

[b]Values shown are the arithmetic means of those obtained from several experiments. The amount of radioactivity in each peak was expressed as a percentage of the total amount recovered.

[c]Expressed as a percentage of total counts recovered in the gel.

[d]Expressed as a percentage of total counts recovered in the sucrose gradient as described in Figure 2.1.

If one makes the reasonable assumption that - in the gradient fractions as well as in intact virions - the amount of radioactivity in each polypeptide is proportional to the relative amount of that molecular species present, it is possible to calculate the percentage composition of each fraction in terms of the polypeptides it contains. These calculations have been made and the results are summarized in Table 2.1. It seems clear that the composition of the "15S" subunit is the same regardless of whether it is derived from L-, M- or S-Mengo. It contains only VPI and VPII, and its percentage composition with respect to these two polypeptides is 67.9 - 69.2% and 30.8 - 32.1%, respectively (mean values are shown in Table 2.1). The polypeptide compositions of the pellet and top fraction were found to be similar for all three variants. The entire virion complement of VPIII (and of VPIV in the case of M-Mengo) could be accounted for in these two fractions. Approximately 90-95% of the VPIII present in L-, M- and S-Mengo virions, and 60-70% of the VPIV found in M-Mengo was found in the pellet.

Ultracentrifugation Studies of "15S" Protein Subunits

Sedimentation velocity analyses of purified "15S" protein subunits derived from all three variants were carried out as described in Materials and Methods. The schlieren patterns revealed that viral subunits treated with DTT sedimented as a single homogenous peak. The intrinsic sedimentation coefficients ($S^{\circ}_{20,w}$) of the subunits derived from all three variants were found to be essentially identical, with values obtained from a number of runs ranging from 12.9 to 14.3 S,

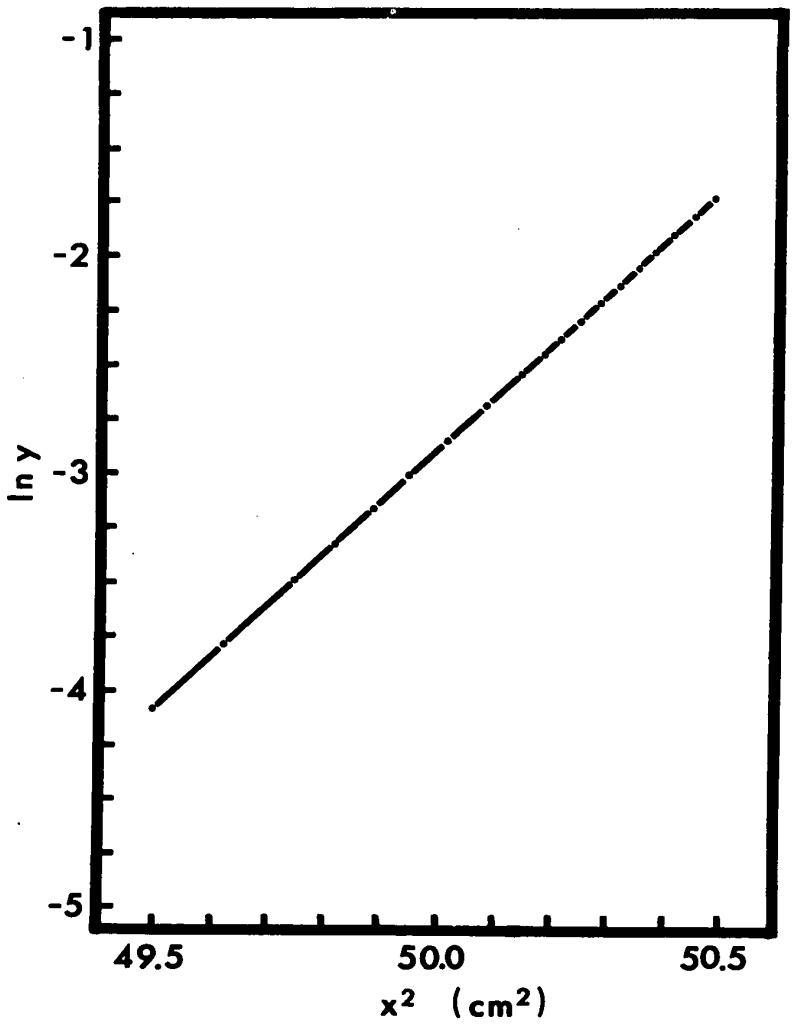


FIGURE 2.6. Plot of the natural log of the concentration (y; fringe displacement) as a function of the square of the distance from the axis of rotation (x^2) for Mengo protein subunit in PBS buffer, pH 6.2.

TABLE 2.2

PHYSICAL PARAMETERS OF THE PROTEIN SUBUNIT PRODUCED BY pH-INACTIVATION OF THE MENGO VIRUS VARIANTS

Variant	Sedimentation Coefficient ($S^{\circ}_{20,w}$)	Average $S^{\circ}_{20,w}$	Molecular Weight	Average Molecular Weight
L	13.3	13.4 ± .14	396,000	408,000 ± 17,000
	13.5		420,000	
M	13.4	13.4 ± .55	396,000	407,000 ± 8,000
	14.3		406,000	
	12.9		416,000	
	13.0		410,000	
	13.4			
S	13.6	13.4 ± .28	418,000	407,000 ± 16,000
	13.2		396,000	
Mean Values		13.4 ± .13		407,000 ± 11,000

and the average being 13.4 S ± 0.13 (Table 2.2). In the absence of DTT, however, the schlieren patterns showed that the subunit preparations contained (in addition to the major component) two species having sedimentation coefficients of 23 and 30S and probably representing dimers and trimers of the 13.4S subunit. These results are in good agreement with those obtained by sucrose density gradient analysis (Figure 2.2, upper panel).

High speed equilibrium ultracentrifugation studies (Yphantis, 1964) were employed to determine the homogeneity and molecular weight of the viral subunit. Numerous analyses of the subunits derived from all three virus variants were performed as described in Materials and Methods. Representative data obtained from these studies are shown in Figure 2.6 which is a plot of $\ln y$ vs x^2 obtained with a preparation of the subunit from M-Mengo virions. From the constant slope of the plot obtained (with this and all other preparations) it was concluded that preparations of the viral subunit are comprised of a single, remarkably homogenous molecular species which has a molecular weight of approximately 400,000. A compilation of molecular weight values of the protein subunit derived from the three variants is shown in Table 2.2. The average molecular weights of the subunits of the L-, M- and S-variants were shown to be identical - values of 408,000, 407,000 and 407,000 respectively, being obtained. All values were within the range of 396,000 to 420,000; and the mean of all molecular weight results was 407,000 ± 11,000.

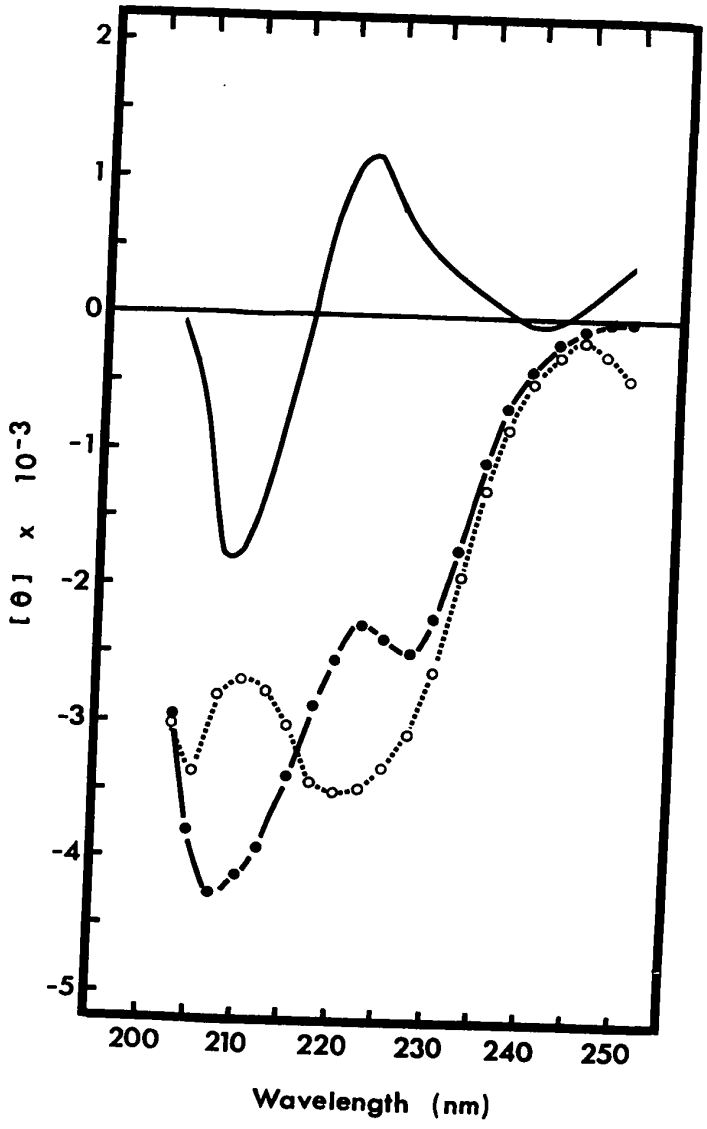


FIGURE 2.7. Circular dichroic spectrum of M-Mengo viral protein subunit (●——●). Also included are a theoretical spectrum of viral protein (o---o)(see Discussion) and a curve describing the difference between the spectra of the experimental and theoretical viral protein (——).

TABLE 2.3

CHARACTERISTICS OF CIRCULAR DICHROIC SPECTRA FOR THE PROTEIN SUBUNITS PRODUCED BY pH-INACTIVATION OF MENGO VIRUS VARIANTS

Variant	Peaks		Troughs	
	λ (nm)	[θ] (degrees)[a]	λ (nm)	[θ] (degrees)[a]
L-Mengo				
Experimental	224	-2300	227 208	-2500 -4000
Calculated[b]	210	-2200	222 205	-3830 -2960
M-Mengo				
Experimental	222	-2250	228 208	-2400 -4300
Calculated[b]	211	-2700	220 205	-3500 -3200
S-Mengo				
Experimental	222	-2550	225 208	-2700 -3800
Calculated[b]	212	-3100	218 205	-3980 -3670

[a]Maximum errors for ellipticity due to c.d. noise level are ±500°.

[b]Calculated values were deduced from difference spectra by subtracting 21% of the c.d. contribution of the isolated RNA from that of the intact virion.

Circular Dichroism Studies

The circular dichroic spectrum of the highly purified viral subunit was examined in the ultraviolet region (202-250 nm), where the contributions of the peptide chromophores are most prominent. A representative scan obtained with a preparation of the subunit derived from the M-Mengo variant is illustrated in Figure 2.7 (closed circles), and the salient features of the data derived from such studies of the subunits of all three variants are summarized in Table 2.3. Examination of the experimental protein curve in Figure 2.7 shows that the protein subunit from the M variant possesses two negative ellipticity bands with minima at 208 and 225-228 nm, an observation that holds true for the subunits produced from the L- and S-variants as well. Furthermore, Table 2.3 indicates that the amplitudes of the minima for the subunits prepared from the three variants are the same within the limits of experimental error, suggesting that they possess similar amounts of periodically ordered conformations.

Analysis of Fragments Obtained from the Dissociation of Viral Subunits by Urea

Recently, Dunker and Rueckert (1971) found that the capsids of ME virus (like Mengo, a cardiovirus) could be dissociated at pH 5.7 in the presence of 0.1 M NaCl into 14S subunits (mol.wt. 420,000); and that these 14S subunits could be further dissociated with 2M urea into 5S fragments (mol.wt. 86,000).

When ^{14}C-labelled, 13.4S Mengo viral subunits were incubated with 2M urea and the suspension then subjected to sucrose density

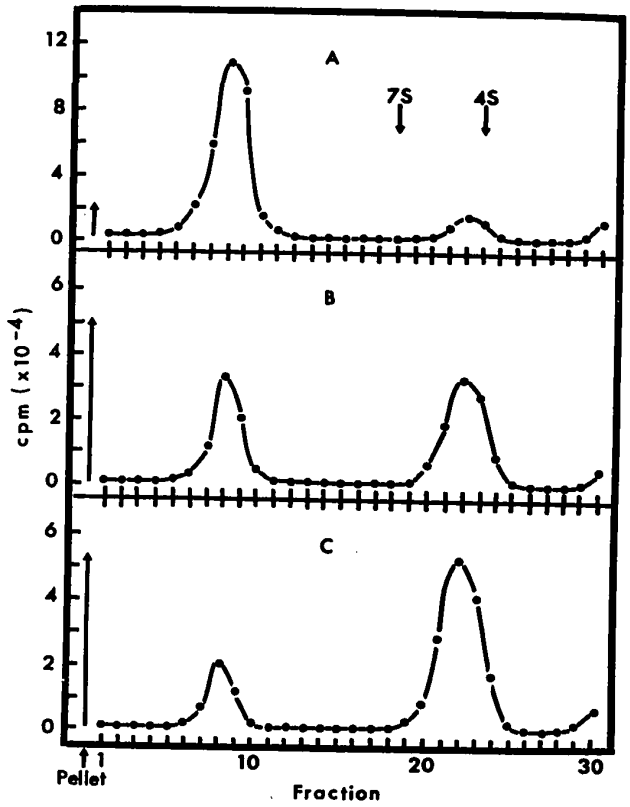


FIGURE 2.8. Sucrose density gradient analysis of fragments dissociated from 13.4S subunits. Highly purified ^{14}C-labeled 13.4S subunits were incubated with 2M urea for 0 hr (A), 1 hr (B), and 2 hr (C) at 0° to promote dissociation. The samples were then centrifuged through sucrose gradients as described in Materials and Methods. Centrifugation was at 45,000 rpm in a Spinco SW 50 rotor for 6-1/2 hr.

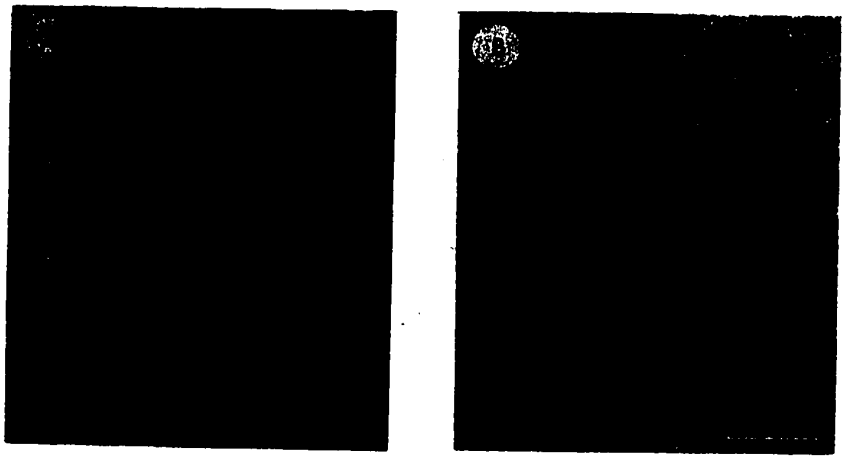


FIGURE 2.9. M-Mengo virus (A) and purified protein subunits (B), negatively stained with 1% uranyl acetate and 1% uranyl formate, respectively.

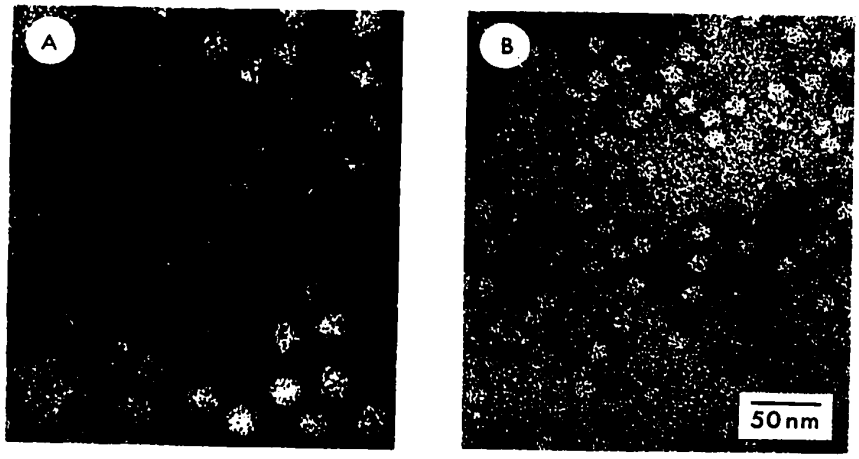


FIGURE 2.9. M-Mengo virus (A) and purified protein subunits (B), negatively stained with 1% uranyl acetate and 1% uranyl formate, respectively.

gradient centrifugation, a peak of radioactivity was found which sedimented more slowly than 13.4S subunits (Figure 2.8). The symmetry of the peak suggested that the fragments contained therein were homogenous with respect to molecular size. The position of the peak relative to those of the 13.4S viral subunits, 7S γ globulin (Mann Research Laboratories, N.Y.) and 4.3S t-RNA (a gift from Dr. C.J. Smith, Department of Biochemistry, University of Alberta), indicated that they had a sedimentation coefficient of approximately 4.7S. Subsequently, the molecular weight of these fragments was determined by sedimentation equilibrium centrifugation in the analytical ultracentrifuge (as described in Materials and Methods), and a molecular weight value of 77,000 was obtained.

Electron Microscopic Studies of the 13.4S Viral Subunit and the 4.7S Fragment

The general appearance of a typical preparation of M-Mengo virus negatively stained with uranyl acetate is shown in Figure 2.9A. It is clear from this photograph that the virions are essentially spherical in shape as reported earlier by Scraba et al. (1967). At high magnification, the negatively stained virions seem to reveal some capsid subunit structures; however, the subunits are not well enough defined by the stain to permit an analysis of their arrangement.

The structure of the viral protein subunits obtained by pH inactivation of whole M-Mengo virions was also examined in the electron microscope. A micrograph of one such preparation is shown in Figure 2.9B. The subunits of all three variants, when negatively stained with uranyl acetate, uranyl formate or sodium phosphotungstate,

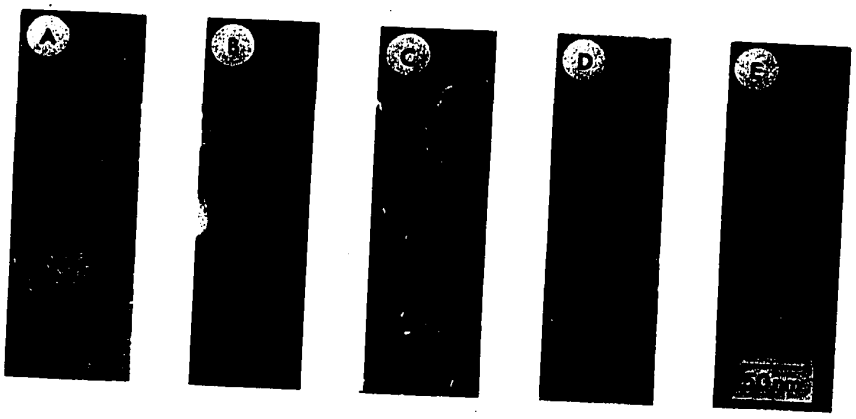


FIGURE 2.10. Electron micrographs of Mengo virus and its pH degradation products. (A) Mengo virus, whole virions, (B) empty capsids, (C) chains of subunits, (D) individual 13.4S subunits, and (E) "hamburgers" (dimers of the subunit).

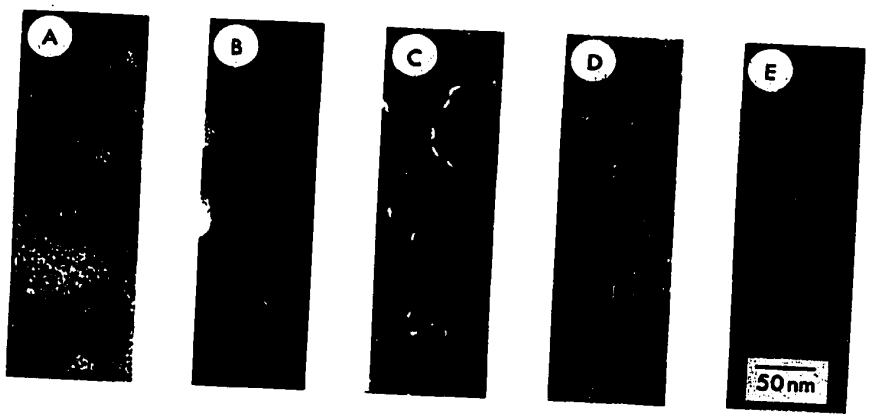


FIGURE 2.10. Electron micrographs of Mengo virus and its pH degradation products. (A) Mengo virus, whole virions, (B) empty capsids, (C) chains of subunits, (D) individual 13.4S subunits, and (E) "hamburgers" (dimers of the subunit).

appear as well-defined approximately spherical entities. Careful measurements of 400 of these established that the subunit has an ellipsoidal shape with average dimensions of 16.8 ± 0.3 x 14.2 ± 0.2 nm (Figure 2.9B). Also present in electron micrographs are particles having a morphology best described as "sausage-like" with average dimensions of 16.4 x 4.2 nm (Figure 2.10C). Given these dimensions and the fact that pH inactivation produces only a single homogenous molecular species, it seems safe to conclude that these "sausage"-shaped particles represent the subunit viewed on its side. Another type of morphological structure that appears in these preparations has the appearance of a "hamburger". These particles are probably face-to-face dimers of the subunits. Two "hamburgers" can be seen in Figure 2.10E.

In an effort to gain some insight into the mechanism of inactivation, the process of degradation was examined directly in the electron microscope. Virus particles (L-, M- or S-Mengo) were pH-inactivated briefly (20-30 seconds) at 37° and samples were prepared immediately for electron microscopic examination by negative staining. Extensive degradation of the viral capsids into the 13.4S subunits was observed in all preparations. However, in addition to surviving virus particles and individual subunits, several intermediate structural forms were observed. Some structures had the appearance of empty capsids like those previously reported for poliovirus (Dimmock et al., 1967; Van Elsen et al., 1968). However, unlike the stable empty capsids of polioviruses which are naturally-occurring (Dree and Borner, 1965; Dree and Demme, 1966) these Mengovirus empty capsids are unstable

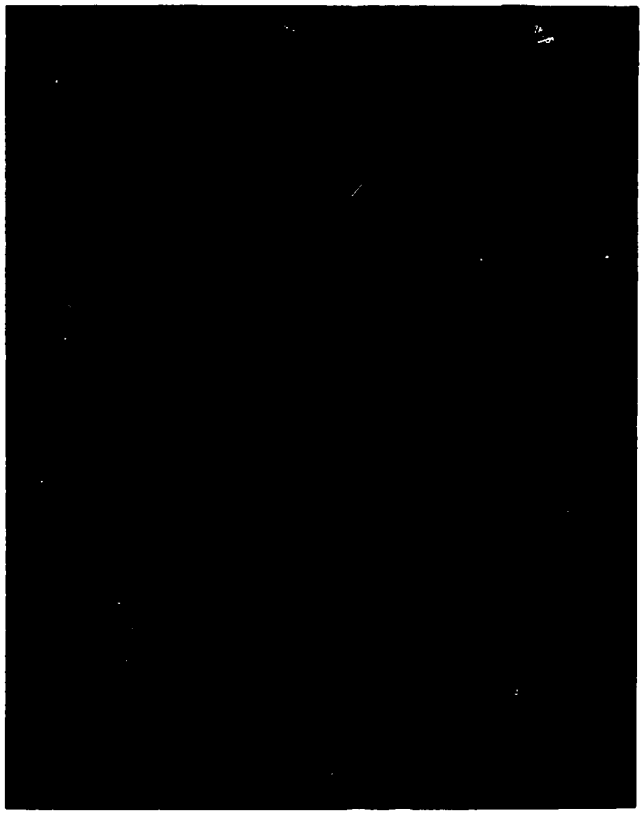
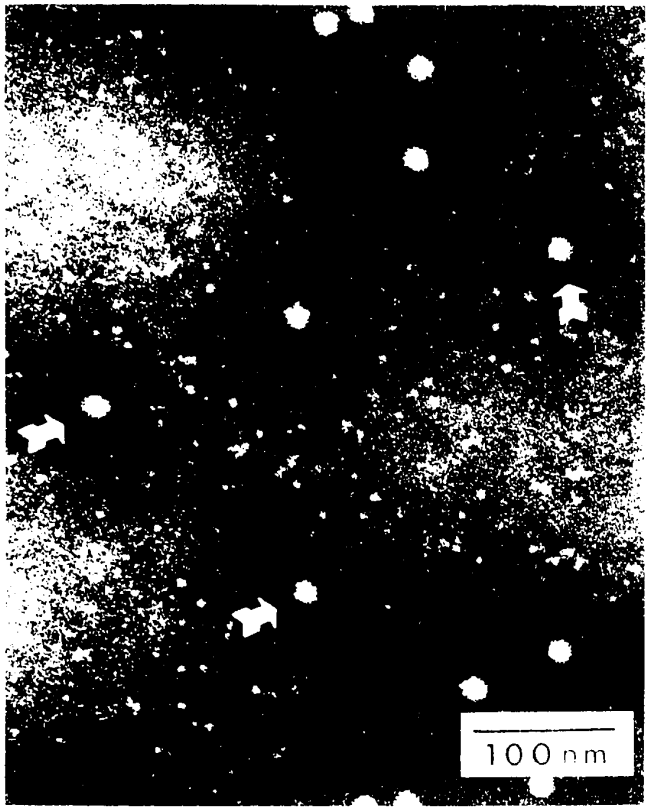


FIGURE 2.11. 4.7S viral protein fragments negatively stained with Na phosphotungstate. Arrows show intact virus particles.


100 nm

and dissociate rapidly into individual subunits. This observation is consistent with the observation by Hall and Rueckert (1971) that empty capsids of ME virus are relatively unstable as compared to those of poliovirus. Also observed after brief periods of inactivation were chains of subunits linked end to end. Several of these are shown in Figure 2.10C. It is quite conceivable that these structures are intermediates arising during the disassembly of the empty capsids into individual viral subunits. An hypothesis of the sequence of degradation based upon the electron microscopic observations is illustrated in Figure 2.10.

The structures of the viral protein fragments obtained by dissociating 13.4S subunits with urea were also examined in the electron microscope. A micrograph of a preparation of M-Mengo at an intermediate stage of disassembly is shown in Figure 2.11. The putative 4.7S fragments appear as spherical entities with average diameters of 6.8 ± 0.2 nm.

Discussion

Polypeptide Composition of 13.4S Viral Protein Subunits

The results of the polypeptide composition analyses of the subunit show that the protein subunit of all three variants contains only two (VPI and VPII) of the three major structural polypeptides present in intact virions. These two polypeptides were found to be present in the same relative amounts in the subunits of all three

TABLE 2.4

PHYSICAL AND HYDRODYNAMIC PROPERTIES OF THE PROTEIN SUBUNIT OF MENGO VIRUS VARIANTS

Property	Value
"Molecular weight" (i.e., molar mass in grams)	407,000[a]
Sedimentation coefficient ($S^{\circ}_{20,w} \times 10^{13}$ sec)	13.4[a]
Frictional coefficient ($f \times 10^{7}$)	1.36[b]
Frictional ratio (f/f_o)	1.45[c]
Axial ratio (a/b)	8/1[d]
Effective hydrodynamic volume ($V_e \times 10^{18}$ ml)	0.514[e]

[a]Values taken from Table 2.2.

[b]Calculated from the equation: $f = M(1-\bar{v}\rho)/NS$ (Schachman, 1959).

[c]Calculated from the equation: $f/f_o = 1.19 \times 10^{-15} \left(\frac{M^{2/3}(1-\bar{v}\rho)}{S\bar{v}^{1/3}} \right)$ (Svedberg and Pederson, 1940).

[d]Value taken from Table IX in Schachman (1959).

[e]Calculated from the equation: $f = 6\pi\eta \left(\frac{3V_e}{4\pi} \right)^{1/3} \frac{f}{f_o}$ (Schachman, 1959).

virions - VPI and VPII comprising 68% and 32% of the subunit respectively. VPIII, which accounts for approximately 10% of the total protein of intact virions, was found in a pellet at the bottom of the tube after sucrose density gradient centrifugation of pH-inactivated virus.

Hydrodynamic Properties of the 13.4S Viral Subunit

Certain hydrodynamic parameters - frictional coefficient (f), frictional ratio (f/f_o), axial ratio (a/b), and effective hydrodynamic volume (V_e) - of the Mengo protein subunit may be computed from the sedimentation-molecular weight data reported in this study by the use of equations given by Svedberg and Pederson (1940) and Schachman (1959). Since the subunits of all three variants exhibit the same sedimentation coefficient and molecular weight (M) values within the limits of experimental error, mean values for $S^{\circ}_{20,w}$ and M (see Table 2.2) were inserted into these equations to calculate the hydrodynamic characteristics shown in Table 2.4. The computed frictional ratio of 1.45 indicates that the protein subunit displays hydrodynamic behavior characteristic of a structure which is ellipsoidal in shape and which has an approximate axial ratio (a/b) of eight (Schachman, 1959). However, this axial ratio must be considered to be a maximal one, since hydration - which together with molecular shape determines the value of f/f_o - was considered to be zero.

Circular Dichroism Studies

The positions of the c.d. ellipticity bands at 208 and 225 nm are characteristic of the presence of some α-helical segments in the subunit moieties, although the patterns are reminiscent of those of

proteins with very low helix content such as ribonuclease (Jirgensons, 1970). It should be noted that both transitions are somewhat red-shifted with respect to synthetic polypeptides, where they occur at 206 and 222 nm, respectively (Holzworth and Doty, 1965). Such a displacement in the position of these bands could be the result of the influence of optically active, aromatic side-chain chromophores on the ellipticity in the peptide region (Beychok, 1968). The specific ellipticities ([θ] values) at 208 and 225 nm are -4,000° and -2,500°, respectively, which when compared with literature estimates of -40,000° for fully helical polypeptides (Townend et al., 1966; Greenfield and Fasman, 1969), indicate that the viral protein subunits of all three variants possess an apparent α-helical content of only 5-10%. This value is in line with estimates made from earlier difference optical rotatory dispersion and c.d. studies of the Mengo system (Scraba et al., 1967; Kay et al., 1970). This finding of low helix content correlates well with the amino acid composition of the viral proteins (Scraba et al., 1969), which were shown to be composed of large amounts (45 moles %) of non α-helix forming amino acids as well as 8 moles % of proline.

The specific ellipticities for the protein subunits were also calculated from difference spectra by subtracting 21% of the c.d. contributions of the isolated RNA from that of the intact virion as follows:

$$[\theta]_{protein} = \frac{[\theta]_{virus} - 0.21\ [\theta]_{RNA}}{0.79}$$

The c.d. data for the intact virion and its isolated RNA were taken from an earlier publication (Kay et al., 1970), and the resulting computed pattern of the M protein subunit is presented in Figure 2.7 (open circles) for comparison with the experimental curve. The pertinent ellipticity characteristics calculated for all three subunits are presented in Table 2.3 along with the experimental data. It is to be noted that while the calculated c.d. spectrum for the subunit resembles that of the experimental curve in that it possesses the two transitions characteristic of the α-helix, and that these (like those of the experimental curve) are of small magnitude, there are clearly differences in the positions of these bands in that they are blue-shifted relative to those of the experimental curve. These differences are highlighted when one examines the profile of the difference spectrum generated by subtracting the c.d. of the calculated curve from that of the experimental one (solid line of Figure 2.7). In particular, this pattern suggests that the difference between the experimental and calculated c.d. curves appears to be largely the result of a positive c.d. contribution at 222 nm and a negative one at 209 nm.

In an effort to explain the deviations noted between calculated and experimental curves, it is of interest to recall that isolated Mengo RNA shows a positive contribution at 223 nm, as well as a negative trough at 209 nm (Kay et al., 1970). It is thus conceivable that while the c.d. of the protein component is mainly due to interactions within the protein coat, it may also include terms from protein-nucleic acid interactions. If such interactions exist, the

transition moments due to the RNA may be interfering with those of the peptide chromophores.

In speculating as to the basis for the differences between the experimental and calculated c.d. curves for the viral subunit, two other possibilities must be considered. First, the RNA may have to undergo a localized melting of secondary structure to allow folding of the molecule into the tightly compact form found in the virus. Additionally, in this highly compacted state, the RNA molecule may find itself in an environment in which the activity of water is considerably less than that in free solution. This would alter the stacking structure of the bases and hence their optical properties relative to RNA in a free state (Maestre and Tinoco, 1967). This explanation as well as the former one imply that the ellipticity of the virion may not be a simple summation of those of the fragments obtained by dissociating the virus.

Still another reason for the lack of correspondence in the two cases may reside in the fact that the experimental curve pertains to only two (VPI and VPII) of the three structural proteins which comprise the intact virion, while the calculated curve also includes a term for the additional structural protein, VPIII. If the latter protein were to have an unusual amino acid composition relative to the other proteins and hence a different secondary and tertiary structure, its exclusion may well have an important bearing on the resulting c.d. spectrum.

Morphology of the Mengo Virion

More than a decade ago, Finch and Klug (1959) examined single crystals of poliovirus by X-ray diffraction and concluded that the virion had icosahedral (5:3:2) symmetry, and that the capsid was therefore likely to be composed of 60 n asymmetric structural units ("capsomers"). Further analysis of the precession photographs revealed an intensity modulation in the diffracted X-rays which was thought to correspond to a center-to-center subunit spacing of about 60 Å. Poliovirus was believed at that time to have a particle weight of 6.7×10^6 daltons and to contain 25% RNA. From these figures it was calculated that the poliovirus capsid was composed of 60 identical subunits, each of 60 Å diameter and having a molecular weight of 80,000 daltons.

However, subsequent work on small, RNA-containing, icosahedral plant viruses made it clear that 5:3:2 symmetry and a 60 Å periodicity did not exclusively specify a 60 n subunit particle. For example, turnip yellow mosaic virus (TYMV) was shown by electron microscopy (Huxley and Zubay, 1960; Finch and Klug, 1966) to be composed of 32 rather than 60 capsomeres, and the total capsid protein was shown by chemical analysis (Harris and Hindley, 1960) to consist of 180 identical polypeptides. The TYMV capsid is assembled from these 180 polypeptides quasi-equivalently spaced in 12 clusters of 5 chains and 20 clusters of 6 chains; the 60 Å periodicity in this case corresponded to the spacings between the 32 capsomers. These results and the revised particle weight and RNA composition values for the animal picornaviruses (Rueckert, 1971) left the X-ray data open to

question; and new models were proposed which had 42 (Agrawal, 1966) or 32 (Mayor, 1964) capsomers, with the 32-capsomer configuration being the one which has appeared most frequently in the literature.

Resolution of this controversy about the capsid architecture has been delayed because of two sources of difficulty. The first stems from the fact that the capsids of picornaviruses are extremely compact, and are not penetrated by the negative stains which have proved so useful in elucidating the capsomer arrangement in plant viruses such as TYMV and animal viruses of the adeno- (Valentine and Periera, 1965), papova- (Mattern et al., 1967), and herpes- (Wildy et al., 1960) virus groups. About all that it has been possible to conclude from electron microscopic examination of picornaviruses is that they are approximately spherical in shape with anhydrous particle diameters of approximately 27 nm (see Figure 2.9A which shows the appearance of Mengo virions negatively stained with uranyl acetate). A second complication has been introduced by the observation that the capsids of picornaviruses contain non-identical polypeptides (Rueckert et al., 1969; Jacobson et al., 1970; and O'Callaghan et al., 1970). Theories of virus structure have generally dealt with capsids containing a single type of polypeptide. It has therefore been postulated that the picornavirus capsid might be composed of pentamers of one type of polypeptide and hexamers or tetramers or trimers of other types.

One approach to the elucidation of structure of these virions is to degrade them under controlled conditions and to examine the products of degradation by physicochemical, chemical and electron

microscopic techniques. In what follows, the results obtained by using this approach with the Mengo virion are discussed.

Results described in this and the previous chapter show that Mengo virus capsids can be readily dissociated at slightly acid pH's into protein subunits having a molecular weight of about 407,000. SDS-polyacrylamide gel electrophoresis also revealed that the subunits contain only two (VPI and VPII - which together comprise approximately 90% of the total virus protein) of the three major structural polypeptides in the intact virions. Since the virion is composed of protein and ribonucleate only, the number of protein subunits (VPI + VPII) per virion can be calculated as follows:

$$\text{Number of subunits per virion} = \frac{.9(M.W._{Virus} - M.W._{RNA})}{M.W._{Subunit}}$$

Using a molecular weight of 8.3×10^6 (Scraba et al., 1967) for the Mengo virus virion and 2.6×10^6 (Tannock et al., 1970; Granboulan and Girard, 1969; and Ziola, unpublished results) for that of the viral genome, a value of 12.3 subunits per virion is obtained.

Electron microscope studies indicate that the individual viral subunit has a slightly ellipsoidal shape with dimensions of 16.8 x 14.2 nm. Assuming that the subunits join edge to edge to form the surface of the viral capsid, the number of protein subunits per virion can also be calculated from the following relationship:

$$\frac{\text{Surface area of virus}}{\text{Surface area of one face of subunit}} = \frac{4\pi r^2}{\pi ab}$$

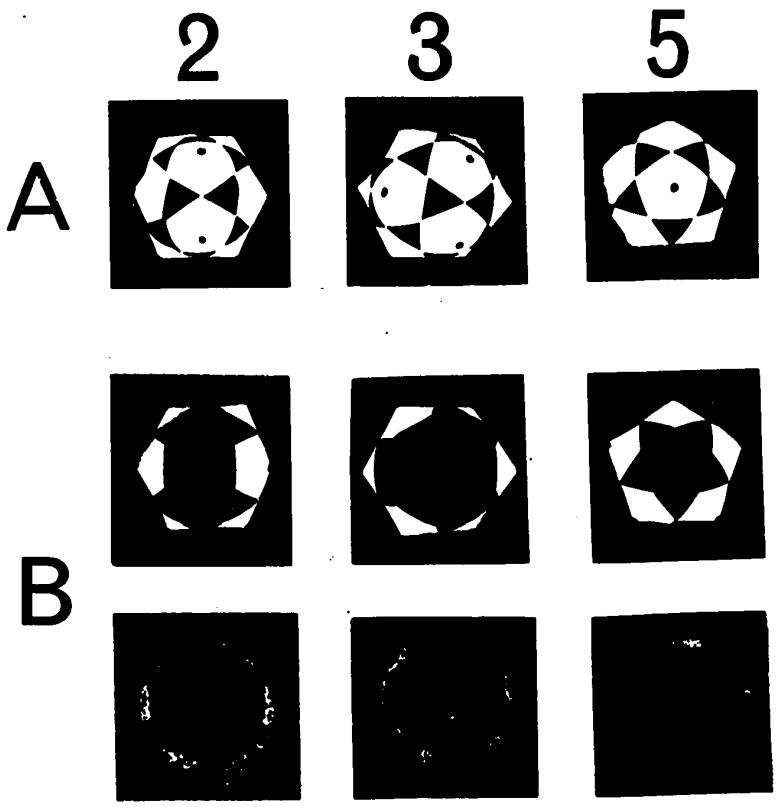


FIGURE 2.12. A model of the Mengo virus capsid consisting of 12 identical cpasomers.

A. Photographs of the model viewed along the 2-, 3-, and 5-fold axes of symmetry.

B. Empty capsids. Comparison of the model (selected capsomers removed) with actual micrographs of Mengo virus empty capsids.

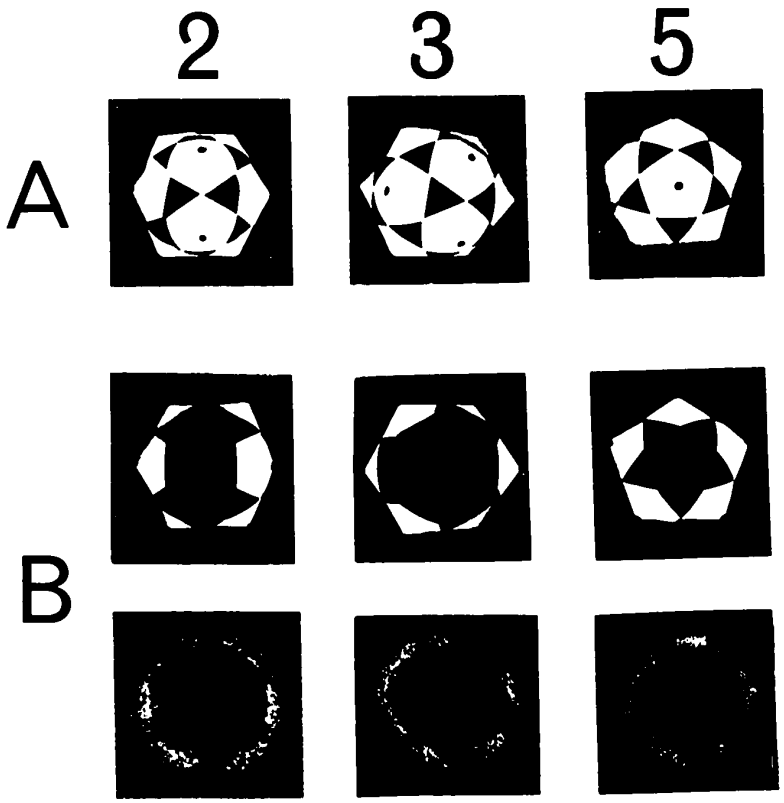


FIGURE 2.12. A model of the Mengo virus capsid consisting of 12 identical cpasomers.

A. Photographs of the model viewed along the 2-, 3-, and 5-fold axes of symmetry.

B. Empty capsids. Comparison of the model (selected capsomers removed) with actual micrographs of Mengo virus empty capsids.

where r is the radius of the virion, and a and b are the half length and width of the viral subunit, respectively. Using 26.7 nm for the diameter of the virion (Scraba et al., 1967), a value of 12.0 subunits per virion is obtained.

One arrangement of the protein subunits in the virion that is compatible with the icosahedral symmetry and the data presented above is that in which each asymmetric structural subunit is located with its center at one of the 12 vertices of the icosahedron. It is quite possible that the twelve 13.4S subunits are so arranged in the complete Mengo virion. A model has been constructed along these lines, and photographs appear in Figure 2.12A.

The structural arrangements observed in electron micrographs of empty capsids produced by limited pH-degradation of the Mengo virion are compatible with this model. Electron micrographs showing the arrangement of subunits in favorably oriented empty capsids are shown in Figure 2.12B. As can be seen, the subunits in the empty capsids appear to be arranged in manners similar to those of the 2-, 3- and 5-fold symmetries of a twelve capsomer model icosahedron from which one or more capsomers have been removed. Such an arrangement represents the simplest icosahedral surface lattice group, T = 1 (Casper and Klug, 1962).

Dunker and Rueckert (1971) reported that the capsids of ME virus can be dissociated at pH 5.7 in the presence of 0.1 M NaCl into 14S subunits with molecular weight of 420,000 and that these 14S subunits could be further dissociated by treatment with 2M urea into 5S fragments which have a molecular weight of approximately 86,000.

When the 13.4S Mengo viral subunits were treated with 2-3 M urea, they were similarly dissociated into 4.7S fragments which were found to have a molecular weight of approximately 77,000. Assuming that the 13.4S subunits dissociate quantitatively into 4.7S fragments, a simple calculation ($M.W._{13.4S}/M.W._{4.7S}$) indicates that each 13.4S subunit is constructed of 5.3 such fragments. An estimate of the number of 4.7S fragments per 13.4S subunit can also be made from the dimensions obtained from electron microscopic studies from the following relationship:

$$\frac{\text{Surface area of each face of 13.4S subunit}}{\text{Surface area of each face of 4.7S fragment}} = \frac{\pi ab}{\pi r^2}$$

where a and b are the half length and width of the 13.4S subunits respectively, and r is the radius of the 4.7S fragment. Given that the dimensions of the 13.4S subunit are 16.8 x 14.2 nm and that the radius of the 4.7S fragment is 3.4 nm, a value of 5.2 is obtained. Thus, there are 5 of the 4.7S fragments per 13.4S subunit, or a total of sixty 4.7S fragments per virion. This 4.7S fragment is reminiscent of the structural unit postulated by Finch and Klug (1959) from the X-ray diffraction studies on poliovirus.

It is therefore probable that the capsid structures of cardioviruses are represented by the 60 identical subunit model of Finch and Klug, with the added information that the subunit is composed of three physically associated, non-identical polypeptide chains. The proposed model for the Mengovirion may be criticized in that the location of the VPIII in the capsid structure has not been ascertained. Since

there are of the order of 40-60 molecules of VPIII in a single virion[1], our working hypothesis at present is that these proteins are clustered in dimers or trimers at the center of each icosahedral facet, providing the binding between 13.4S subunits. It is hoped that the location of VPIII can be determined by using immunological methods to prepare virus samples for electron microscopy (e.g., Brown and Smale, 1970).

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[1]Calculated from the relationship:

$$\text{Number of VPIII molecules per virion} = \frac{\text{M.W.}_{\text{virus}} - (\text{M.W.}_{\text{RNA}} + 12\ \text{M.W.}_{13.4\text{S}})}{\text{M.W.}_{\text{VPIII}}}$$

CHAPTER III

STUDIES OF THE EARLY EVENTS OF THE REPLICATIVE CYCLE OF THREE VARIANTS OF MENGO ENCEPHALOMYELITIS VIRUS IN MOUSE FIBROBLAST CELLS

Introduction

The full cycle of viral replication involves a complex sequence of events culminating in the formation of a large number of progeny virus particles. The early events of this cycle,- namely attachment of virus particles to the host cell, penetration of the cellular membrane by adsorbed virions, and the intracellular release of the viral nucleic acid - must occur before the biosynthesis of viral-specific macromolecules (structural and non-structural polypeptides, multiple copies of the viral genome) can be initiated.

The process of attachment of a virus to its host cell is generally considered to be electrostatic in nature, and there is evidence that stable adsorption depends on complementarity between specific molecules on the two interacting surfaces (Tolmach, 1957; Philipson, 1963; Colter *et al.*, 1964a). Under suitable conditions of geometrical and electrostatic complementarity the two particles are held together by ionic interactions (and perhaps by van der Waal's forces and hydrogen bonds as well) of sufficient strength to counteract dispersive forces. To date, attempts to identify the specific chemical groups involved in the attachment of any virus agent to its host cell

have met with limited success at best. On the basis of a series of physical and chemical measurements, Allison and Valentine (1960) hypothesized that negatively charged phosphate groups on cell surfaces probably interact with positively charged amino groups on the surface of virions. The observation that -SH blocking agents such as p-chloromercuribenzoate (PCMB) abolish both the hemagglutinating activity and infectivity of certain enteroviruses prompted Choppin and Philipson (1961) to suggest that sulfhydryl groups in the capsid protein of these viruses may be involved in their attachment to host cells. However, subsequent studies by Philipson (1964), which showed that poliovirus is degraded by PCMB, cast serious doubt on the validity of the earlier conclusion.

The initial attachment of a virus to its host cell is a reversible process. In the case of poliovirus, the cell-virus complex (formed at 0°) has been shown to be dissociated by treatment with 6 M LiCl, 8 M urea or pH 2.6 (Holland, 1962), and by exposure to 0.05% sodium dodecyl sulfate (SDS) (Mandel, 1962),- observations that are not altogether surprising. Cell-virus complexes involving entero-viruses (Philipson and Bengtsson, 1962) and vaccinia virus (Postlethwaite and Maitland, 1960) have been reported to be disrupted by sonication, and EDTA has been shown to release foot-and-mouth disease virus (FMDV) from association with its host cell (Brown et al., 1962). However, in the normal situation, virus particles, attached to cells at 0°, penetrate into the cell and enter into an "irreversible eclipse" phase relatively rapidly when the cell-virus complex is incubated at 25°-37°. The eclipse phase is the period between the intracellular uncoating of

the virions (after which no - or a greatly reduced number - of intact virions can be recovered from the infected cell) and the appearance of progeny virus particles.

The uptake (penetration) of virus particles into the cell has been studied in detail in a number of virus-cell systems. As early as 1948, Fazekas de St. Groth suggested that adsorbed viruses are transported into the interior of the cell by an active process of ingestion, that he termed viroplexis. However, following the conclusive demonstration by Hershy and Chase (1952) that the T2 bacteriophage injects its genome into the host E. coli cells while the remainder of the virus particle remains extracellular, it was widely believed for some time that animal viruses infect cells by the same mechanism. However, it is now clear from studies of myxo-, adeno-, herpes-, picorna- and pox viruses that these (and probably all) mammalian viruses, are taken up by a mechanism comparable to that proposed by Fazekas de St. Groth.

Electron microscopic studies of several virus-cell systems have established the morphological basis for the process of engulfment (Dales and Franklin, 1962; Dales et al., 1965; Silverstein and Marcus, 1964; Dales et al., 1965). When intimate contact has been established between the virus and cell membrane, the cell membrane "flows over" the adsorbed virus and engulfs it. The invaginated cell membrane then buds off, and the enclosed virion is drawn into the cell (Dales, 1963; Arhelger and Randall, 1964; Dales et al., 1965; Silverstein and Marcus, 1964; Dunnebacke et al., 1969). Evidence that viruses are taken up by cells by this process of phagocytosis has been obtained by the use of

other techniques as well. Boand et al. (1957), Hanson et al. (1957), and Mims (1964) were able to demonstrate, by means of virus-fluorescein labelled antibody conjugates, that influenza and pox viruses are taken up as intact virions into their host cells, and Hirst (1961) and Mandel (1962) showed that enteroviruses retain their morphological integrity, and can, in fact, be recovered from infected cells shortly after infection.

The uncoating of a virus refers to the intracellular release of the viral nucleic acid from its protective coat. Although this process can, and has, been studied by electron microscopy (Dales and Choppin, 1962; Dales et al., 1965; Silverstein and Marcus, 1964), and by following the appearance of naked viral nucleic acid (Sanders, 1960; Brown et al., 1961), a more versatile and definitive method is to study the fate of highly purified, radioactively labeled virus. This method can provide information concerning not only the uncoating of the viral genome, but the fate of other viral components such as protein and phospholipids as well.

The uncoating process has been studied in detail in several virus systems. Joklik and Darnell (1961) and Fenwick and Cooper (1962) investigated the uncoating of poliovirus in HeLa and ERK cells respectively. The observed kinetics of uncoating indicated that over 30% of the virus particles are uncoated within 20 min after adsorption. Their data also indicated that the liberated viral RNA is largely hydrolysed to acid soluble material, with only a very small percentage of the uncoated viral genomes escaping degradation. This latter observation must be viewed with considerable skepticism, particularly in the light of more recent studies of the Mengovirus-L cell system.

Tobey (1964a, b) demonstrated that the parental genome was not degraded to a very large extent after uncoating and in these laboratories it has been shown that it is possible to obtain essentially quantitative recovery of Mengo virus RNA as intact (35S) molecules some 2 hrs after infection of L cells with purified, ^{3}H-uridine labelled M-Mengo (Tovell, 1969). There is some uncertainty regarding the intracellular site of uncoating of picornaviruses. The electron microscopic studies by Dunnebacke et al. (1969) indicated that poliovirions (type I, strain Brunhilde) are uncoated in the cytoplasm, whereas a recent study of the in vitro uncoating of poliovirus (Chan and Black, 1970) suggests that poliovirus Type I strain LSc may be rapidly uncoated at the cell membrane.

The uncoating of poxvirus has been studied by both standard and high resolution radioautographic techniques (Dales, 1963; Dales and Kajioka, 1964) and shown to be a two-step process. Within 20 min after the virus particle is taken up at the cell membrane in phagocytotic vesicles, the outer coat of the vaccinia virus becomes disrupted and the virus core containing the DNA passes into the cytoplasmic matrix. There is a lag of about one hour before the viral core begins to be uncoated, after which naked DNA accumulates, until by 3 hrs after infection, between 60 and 70% of the viral DNA is in a form accessible to DNase.

Recent studies employing a combination of biochemical and electron microscopic techniques to compare the early events in the interaction of different strains of adenovirus (Chardonnet and Dales, 1970a, b) and poliovirus (Dunnebacke et al., 1969; Chan and Black, 1970)

with their host cells have revealed that significant differences exist even among members of a single virus group. Differences among the Mengo variants with respect to their affinities for mouse tissues (Campbell and Colter, 1967a), attachment to L cells (Colter et al., 1964a) and eclipse period (Campbell, 1965) have already been described. As part of a continuing research program aimed at providing a complete understanding of the molecular biology of the three Mengo variants, an investigation of the early events - attachment, penetration and uncoating - in their replicative cycles was undertaken. The results of these studies are summarized in this chapter.

Materials and Methods

Infectious Center Assay

To determine the number of infected cells in an experimental sample, the infectious center technique described by Ellem and Colter (1960) was employed. In brief, cells were sedimented from cell-virus suspension by centrifugation, washed to remove unadsorbed virions, and resuspended in growth medium or virus diluent. The number of infected cells per sample was determined by titrating aliquots of the suspension - or dilutions thereof - on preformed indicator monolayers of L cells.

Attachment Studies

Logarithmically growing L cells were harvested from suspension culture by low speed centrifugation and were resuspended at a

concentration of 2 x 10^6 cells/ml in virus diluent in spinner flasks with magnetic stirring devices at either 37° or 0°. After temperature equilibration, purified $^{32}PO_4$-labeled virus was added at a final concentration of 20 PFU/cell. At 5, 10, 20, 40, 60, 90 and 120 mins after addition of virus, 5 ml samples were removed, and the cells were sedimented by centrifugation at 3,000 rpm for 1 min and washed once with 5 ml of virus diluent. The supernatants were pooled and monitored for radioactivity and infectious virus, while the cells were resuspended in 2 ml of virus diluent and assayed for infectious centers and cell-associated radioactivity.

Estimation of the Number of Receptor Sites per L Cell for the Three Variants of Mengo Virus

Suspensions of highly purified ^{32}P-labelled L-, M- and S-Mengo virions were prepared as described in Routine Materials and Methods, and the concentration of virions therein was determined from measurements of $O.D._{260}$. Aliquots of L cells (harvested from suspension culture) were then incubated (at a concentration of 2 x 10^6 cells/ml) for 1 hour at 0° with increasing input multiplicities of virus (10^2 - 10^6 particles/cell). The cells were then sedimented by centrifugation and washed once with 5 ml of virus diluent. The supernatants were pooled and monitored for both radioactivity and infectious virus, while the cells were resuspended in 2 ml of virus diluent and assayed for cell-associated radioactivity. The amount of radioactivity and the number of PFU of virus bound to the cells were then plotted as a function of input multiplicity. Estimates of the number of virus particles necessary to saturate all receptor sites on the cell were

made from determinations of the input multiplicities at which the curves plateaued.

Penetration Studies

The rates at which the Mengo variants penetrate L cells were measured in monolayer cultures. The experimental design was as follows. Growth medium was removed from preformed monolayers, the plates were held at 4° for 15 mins, after which duplicate sets were infected with 100 and 200 PFU of virus respectively, in 0.2 ml of virus diluent. Following incubation at 4° for 30-45 mins, unattached virus was removed by washing the monolayers twice with 5.0 ml volumes of cold growth medium. Two ml of warm (37°) growth medium were then added to each plate and the monolayers were transferred to an incubator at 37°. At 0, 10, 20, 40, 60, 90, and 120 mins thereafter, 0.5 ml volumes of homologous anti-Mengo rabbit serum of sufficient titer to neutralize more than 99.9% of input virus were added to duplicate plates. Anti-serum to each of the variants was prepared in adult, New Zealand white rabbits by injecting 0.5 ml of purified virus by each of three routes - intravenous, intramuscular and subcutaneous. A booster injection of 0.5 ml was given (intramuscularly) 14 days later, and immune sera was collected after an additional 7 days (O'Callaghan et al., 1970). Pre-immune rabbit serum was added to duplicate, control monolayers at the same time intervals. After the addition of serum (either normal or immune) the plates were incubated for an additional 60 min at 37°. They were then washed twice with 5 ml volumes of growth medium, overlayered with 4.5 ml of nutrient agar, and, after incubation for 48 hrs in a humidified atmosphere of 5% CO_2 in air,

were stained to visualize the plaques. The fraction of input PFU which had been taken up by the cells at the various time intervals was calculated from the equation:

$$\% \text{ penetration} = \frac{\text{PFU (n min)} - \text{PFU (0 min)}}{\text{PFU control (n min)}} \times 100$$

In all experiments, employing all three virus variants, the value obtained for "PFU 0 min" was $\leq 2\%$ of the input PFU of virus.

Uncoating Studies

Exponentially growing L cells were harvested from suspension culture, and resuspended, at a concentration of 20×10^6 cells/ml, in virus diluent containing H^3-uridine-labeled virus at an input multiplicity of 20 PFU/ cell. After an attachment period of 1 hour at 4°, the cells were sedimented by centrifugation, and washed three times with 5 ml volumes of cold virus diluent to remove unattached virus. The cells were then resuspended in 1 ml of cold growth medium and added to a large volume of warm (37°) growth medium to bring the final cell concentration to 1×10^6 cells/ml. After periods of incubation of 0, 10, 20, 40, 60, 90 and 120 minutes, 10 ml samples were removed and chilled rapidly. The cells were pelleted, washed once with 5 ml of cold growth medium, resuspended in 0.4 ml of cold 5×10^{-3} M $MgCl_2$, and sonicated for 4 min at maximum settings in a Raytheon DF101 sonic oscillator (Waltham, Mass.). The sonicates were made 1.0 M with respect to NaCl by the addition of solid NaCl, and were incubated for 30 min at 0°. After removal of cellular debris by a brief centrifugation at 3,000 rpm, the samples were layered on 5-40% sucrose

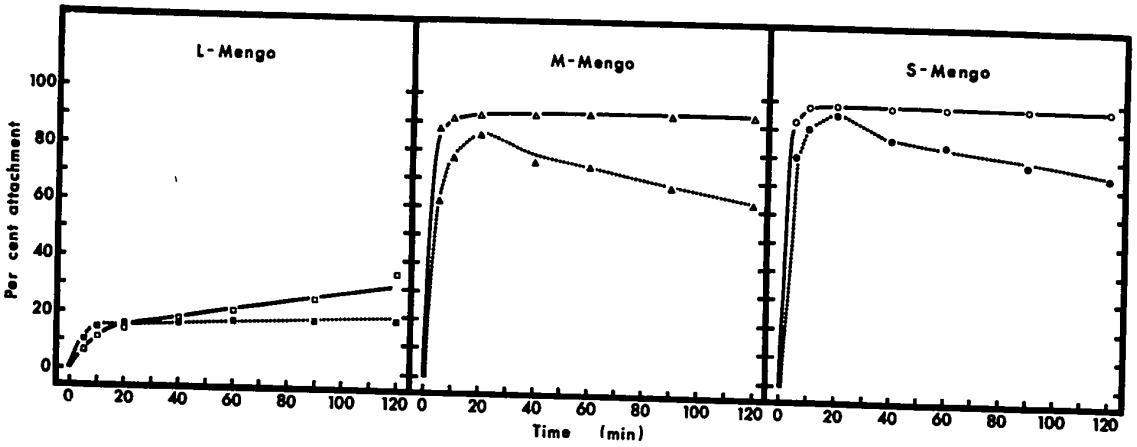

FIGURE 3.1. Kinetics of adsorption of $^{32}PO_4$-labeled Mengo virus variants to L cells at 37°. Exponentially growing L cells at a concentration of 2 x 10^6 cells/ml in virus diluent at 37° were infected with 20 PFU/cell of $^{32}PO_4$-labeled virus. At the times indicated samples were taken, the cells were sedimented and washed, and the supernatants were pooled. Virus attachment was determined by measuring (a) the amount of infectious virus (PFU) removed from the supernatant, L-Mengo □—□, M-Mengo Δ—Δ, S-Mengo o—o, and (b) the amount of radioactivity that became cell associated, L-Mengo ■--■, M-Mengo ▲--▲, and S-Mengo ●--●.

gradients and centrifuged at 35,000 rpm for 30 min at 6° in an SW 50 rotor in a Spinco Model L preparative ultracentrifuge. Fractions were collected on filter paper discs, which were then processed and monitored for radioactivity as described in an earlier section. The rate of uncoating was estimated from measurements of the rate at which radioactivity was lost from the virion band.

Results

Attachment Studies

The rates at which the three Mengo variants attach to L cells were compared by incubating aliquots of L cells in suspension with preparations of highly purified, ^{32}P-labelled L-, M- and S-Mengo virions. After various times of incubation (at either 0° or 37°), the % of input radioactivity and PFU that had become firmly cell associated was measured. In all experiments, the concentration of cells employed was 2×10^6 per ml, and in all experiments the input multiplicity of virus was 2000 total particles/cell (calculated from the $O.D._{260}$ of the viral suspension. $E^{1\%}_{260} = 76.3$; $1\ O.D._{260} = 10^{13}$ virions.) This was equivalent to approximately 20 PFU/cell, since in repeated determinations, the total:infectious particle ratio for all three variants was found to be close to 100. The specific activities of the purified virions varied somewhat from one preparation to another within the range $1.0 - 2.5 \times 10^6$ cpm/$O.D._{260}$ unit.

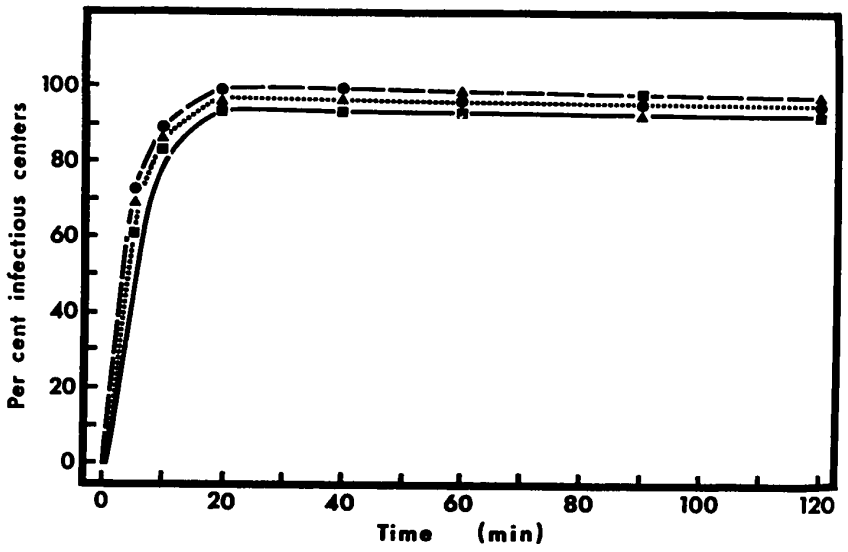

FIGURE 3.2. Rate of formation of infectious centers in L cells infected with Mengo virus variants at 37°. Samples of cell cultures employed in experiments to measure virus attachment, as described in Figure 3.1, were taken at the times indicated and the cells were sedimented and freed from unadsorbed virus by washing. Dilutions of the samples containing a known number of cells were then titrated on indicator monolayers of L cells to determine the number of infected cells per sample. Results are expressed as the percentage of the total cells in each sample that were infected. L-Mengo ■—■; M-Mengo ▲---▲; S-Mengo ●---●.

The pooled results from a number of experiments are summarized in Figure 3.1, in which the percentages of input radioactivity and PFU bound to cells are plotted as a function of time of incubation at 37° for all three variants. The kinetics of adsorption of S- and M-Mengo to L cells are strikingly similar, with 95% and 85% respectively of the input radioactivity (i.e., of the input physical particles) being firmly cell associated after a 20 min incubation period. The picture with L-Mengo is quite different. Not only is the initial rate of attachment slower, but after 20 min, only about 20% of the input radioactivity (and PFU) is bound to cells.

It should be noted that, with the M- and S-variants, the amount of cell-associated radioactivity decreased gradually when the incubation was continued beyond 20 minutes. This observation was found to be quite reproducible, and is reminiscent of the "sloughing" phenomenon described earlier by Joklik and Darnell (1961) and Fenwick and Cooper (1962) in the poliovirus-HeLa cell and poliovirus ERK cell systems respectively. It seems clear that the virions that attach to and then detach from cells lose their infectivity as a result, since the % of cell-bound PFU (calculated from plaque assays of infectious virus in the supernatants) remains constant from 20 to 120 min of incubation. This "sloughing" phenomenon is not observed with L-Mengo at 37°, and does not occur with any of the variants at 0°.

The rates of formation of infectious centers in the same cell-virus mixtures used to provide the data summarized in the preceeding paragraphs are illustrated in Figure 3.2. This data does not, of course, provide any precise information regarding rates of

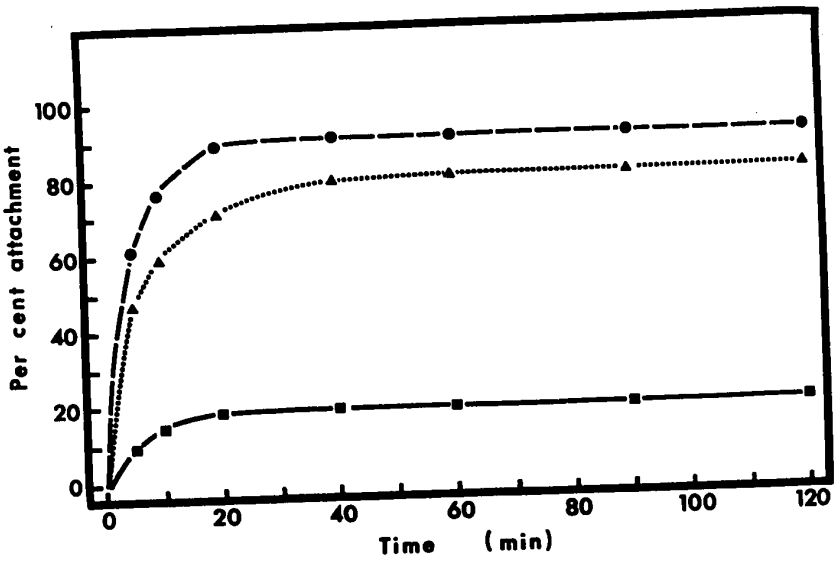

FIGURE 3.3. Rate of attachment of $^{32}PO_4$-labeled Mengo virus variants to L cells at 0°. Exponentially growing L cells were harvested, resuspended at a cell concentration of 2 x 10^6 cells/ml in virus diluent at 0°, and infected with 20 PFU/cell of $^{32}PO_4$-labeled virus. At the times indicated, samples were taken and the cells were collected by sedimentation, washed, and the amount of cell-associated radioactivity was measured. The amount of attachment is expressed as the percentage of total radioactivity in the sample that became cell-associated.

L-Mengo ■——■; M-Mengo ▲---▲; S-Mengo ●---●

attachment, but does show clearly that there was no heterogeneity in the cell populations used with respect to susceptibility to infection with L-, M- and S-Mengo. With all three variants (at an input multiplicity of 20 PFU/cell) all cells were infected by 20 minutes.

The rates of attachment of the three variants to L cells at 0° were also measured, and the data obtained are shown in Figure 3.3. As may be seen from a comparison of this figure and Figure 3.1, the kinetics of attachment of the three variants at 0° and 37° are virtually identical, thus verifying (as expected) that attachment of Mengo virions to L cells is an essentially temperature-independent phenomenon.

The initial rates of attachment of the Mengo variants to L cells were calculated from data obtained from short (5 min) incubation periods and at both 0° and 37°. The initial rate constants (k values) are listed in Table 3.1. With each of the three variants, the three k values (calculated from uptake of radioactivity at 37°, uptake of PFU at 37° and uptake of radioactivity at 0°) are in good agreement. It seems clear that of the three variants, S-Mengo has the highest ($k = 93 \times 10^{-9}$ $cm^3 min^{-1} cell^{-1}$) and L-Mengo the lowest ($k = 7 \times 10^{-9}$ $cm^3 min^{-1} cell^{-1}$) affinity for L cells.

The observation that the rate constants for attachment for each of the variants is the same whether based on uptake of radioactivity or PFU constitutes rather convincing evidence that despite the high total:infectious particle ratios (about 100) characteristic of preparations of all three variants, there is no heterogeneity in the virus populations with respect to ability to attach to L cells.

TABLE 3.1

INITIAL RATE CONSTANTS (k) OF THE ATTACHMENT OF MENGO VIRUS VARIANTS TO L CELLS[a]

Mengo Virus Variant	k (cm^3 min^{-1} $cell^{-1}$)		
	0°C[b]	37°C[b]	37°C[c]
L	8×10^{-9}	10×10^{-9}	7×10^{-9}
M	47×10^{-9}	62×10^{-9}	86×10^{-9}
S	67×10^{-9}	80×10^{-9}	93×10^{-9}

[a]Calculated from the equation $-\frac{\Delta V_5}{\Delta t} = k\,(Vo)(C)$ in which $\frac{\Delta V_5}{\Delta t}$ is the change in the amount of unadsorbed virus (radioactivity or PFU) at 5 minutes after infection, Vo is the amount of virus added at 0 time, and C is the cell concentration in cells per milliliter.

[b]Data obtained by measurement of attachment of radioactivity ($^{32}PO_4$-labeled virus).

[c]Data obtained by measurement of attachment of plaque forming units.

TABLE 3.2

ATTACHMENT OF ^{32}P-LABELLED MENGO VIRIONS TO L CELLS

Variant	Specific Activity of Virus Suspension[a]	Input Multiplicity[b]	Specific Activity of Unadsorbed Virions[a]	Sp.ac.- original / Sp.ac.- after adsorption
L	4.5×10^{-5}	20,000	7×10^{-5}	1.0
		4,000	2.4×10^{-5}	
		2,000	4.2×10^{-5}	
			4.5×10^{-5}	
M	1.5×10^{-5}	40,000	2.6×10^{-5}	0.7
		8,000	1.4×10^{-5}	
		4,000	2.8×10^{-5}	
			2.2×10^{-5}	
S	1.2×10^{-5}	50,000	1.0×10^{-5}	1.3
		10,000	1.1×10^{-5}	
		5,000	0.6×10^{-5}	
			0.9×10^{-5}	

[a] cpm/PFU

[b] Physical particles/cell

Cells were incubated, at a concentration of 2×10^6/ml, with ^{32}P-labelled virions at the input multiplicities shown. After 1 hr at 0°, the cells were sedimented by centrifugation, and the radioactivity and PFU remaining in the supernatant were measured.

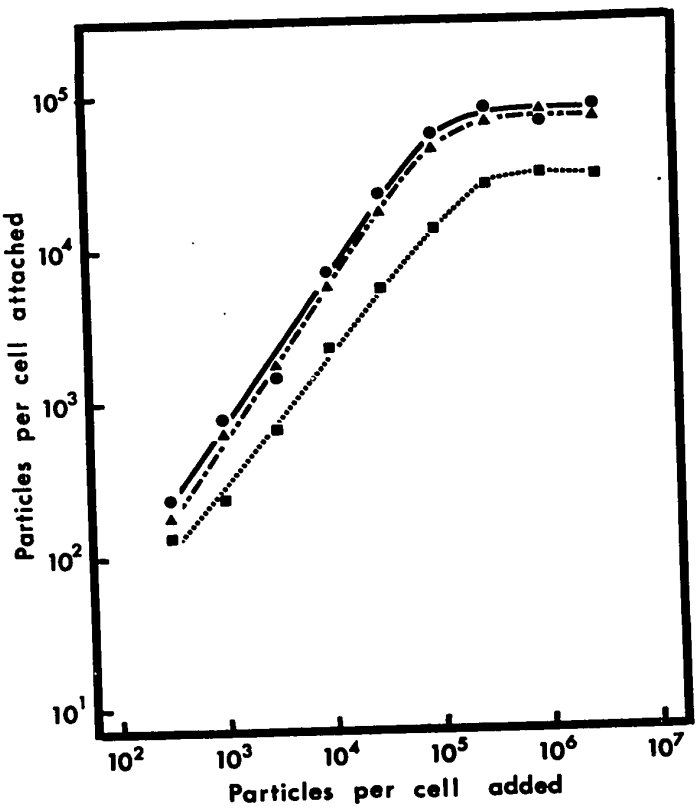

FIGURE 3.4. Titration of the number of Mengo virus receptor sites on L cells. Aliquots of L cells were incubated for 1 hour at 0° with increasing input multiplicities of $^{32}PO_4$-virus. The cells were then sedimented, washed and the radioactivity bound to the cells was measured. The number of virus bound to each cell is ploted as a function of input.

L-Mengo (■---■); M-Mengo (▲---▲); S-Mengo (●——●)

Confirming evidence was obtained from other experiments in which L cells were incubated with purified, ^{32}P-labelled Mengo virions at very high multiplicities, and the ratio of cpm:PFU was determined in both the original virus preparation and in the supernatant after incubation with cells. Illustrative data are shown in Table 3.2. The fact that the ratios are substantially the same before and after incubation with cells shows clearly that all physical particles in the virus preparations attach with equal efficiency to L cells. There is no selection of those virions capable of initiating an infection and registering as PFU.

Number of L Cell Receptor Sites for Mengo Virus

The observation that there are differences among the Mengo variants with respect to both the rate and extent of attachment to L cells prompted an examination of the number of receptor sites per cell for each of the variants.

The experimental approach employed was to incubate aliquots of L cells for 1 hr at 0° with purified, ^{32}P-labelled virions over a wide range of input multiplicities (physical particles/cell; determined from the $O.D._{260}$ of the virus suspension). By plotting the amount of cell-bound radioactivity as a function of input multiplicity, it was possible to estimate the input multiplicity above which no additional radioactivity was bound. That critical input multiplicity is here defined as the number of receptor sites per cell.

Illustrative data obtained from a number of such experiments are presented in Figure 3.4, from which it may be seen that there are significantly fewer receptor sites per cell for L-Mengo than for either

TABLE 3.3

INITIAL RATE CONSTANTS (k) OF THE ATTACHMENT OF MENGO VIRUS VARIANTS TO L CELLS[a]

Mengo Virus Variant	k (cm^3 min^{-1} $receptor^{-1}$)		
	0°C[b]	37°C[b]	37°C[c]
L	3×10^{-13}	3.7×10^{-13}	2.6×10^{-13}
M	5.2×10^{-13}	6.9×10^{-13}	9.6×10^{-13}
S	7.0×10^{-13}	8.4×10^{-13}	9.8×10^{-13}

[a]Calculated from the equation $-\frac{\Delta V_5}{\Delta t} = k\,(Vo)(C)$, using the same data that were used to calculate the rate constants listed in Table 3.1, except that for factor (C), "receptor sites/ml" was used instead of "cells/ml".

[b,c]Same as in Table 3.1.

M- or S-Mengo. There was very considerable variation in the absolute values obtained from one experiment to another, but the observation that there are fewer (by a factor of 3-4) receptor sites for L- than for M- and S-Mengo was completely reproducible. A total of thirteen separate determinations were carried out, and these yielded average values of 27,000-, 90,000- and 95,000-receptor sites/cell for L-, M- and S-Mengo respectively.

In calculating initial rate constants for the attachment of viruses to cells, it is clear that the concentration of receptor sites is a more pertinent parameter than the concentration of cells. It was of interest, then, to recalculate the rate constants listed in Table 3.1, using the same equation shown in the footnote to that table, but substituting "receptor sites per ml" for "cells per ml". The results of this exercise are presented in Table 3.3. Even taking into account the smaller number of receptor sites per cell for L-Mengo, the rate of attachment of this variant to L cells is still slower than those of the other two variants, although the differences are not as large as are suggested by the values in Table 3.1.

Penetration Studies

The penetration of L cells by Mengo virus was studied by measuring the rate at which virions of each variant, adsorbed to cells at 4°, became resistant to neutralization by homologous anti-Mengo rabbit serum after transferring the cells with attached virus to a temperature of 37°. At 4°, virions attach efficiently to cells (see preceeding section), but do not cross the plasma membrane.

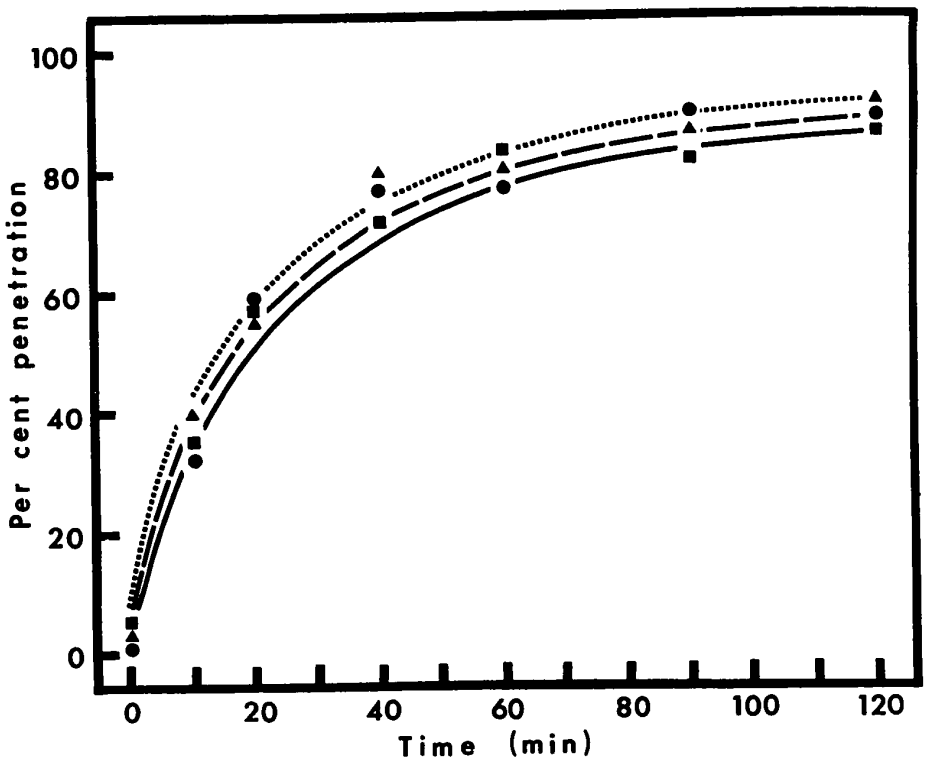

FIGURE 3.5. Kinetics of penetration of L cells by the virus variants - determined by measuring the rate at which adsorbed virus became resistant to neutralization by homologous rabbit anti-serum at 37°. Penetration is expressed as the percentage of infected cells (PFU on control plates) that formed plaques in the anti-Mengo serum treated plates (see details in Materials and Methods).

L-Mengo ■——■; M-Mengo ▲---▲; S-Mengo ●---●

The results of these studies are summarized in Figure 3.5. It is clear from these data that there are no significant differences among the three variants with respect to kinetics of penetration,- once attached to L cells, virions of all three are taken up with equal efficiency by the cells. The initial rate of penetration was found to be quite rapid and to be essentially linear for about 20 min, by which time 55-60% of attached PFU had penetrated the cells (i.e., were no longer vulnerable to inactivation by specific immune serum). After this time, the rate of penetration decreased, although virions continued to enter the cell until by 120 min approximately 88-90% of the PFU that had become attached to the cells at 4° had crossed the cell membrane. The fraction of attached virions that had apparently penetrated the cells at zero time (i.e., the fraction not neutralized by immune serum) was consistently very small, and in no case exceeded 2% of the input PFU,- an observation consistent with the well documented finding that the uptake of virus particles by cells in a temperature-dependent phenomenon.

Uncoating Studies

The rationale underlying the experimental design employed to study the intracellular uncoating of Mengo virions was as follows. It was reasoned that as virions, labelled in their ribonucleic acid moiety, were uncoated in the cell, there would be a progressive decrease in the amount of radiolabel present in the virion band when sonicates of the infected cells - prepared at various times after infection - were subjected to sucrose density gradient analyses. A

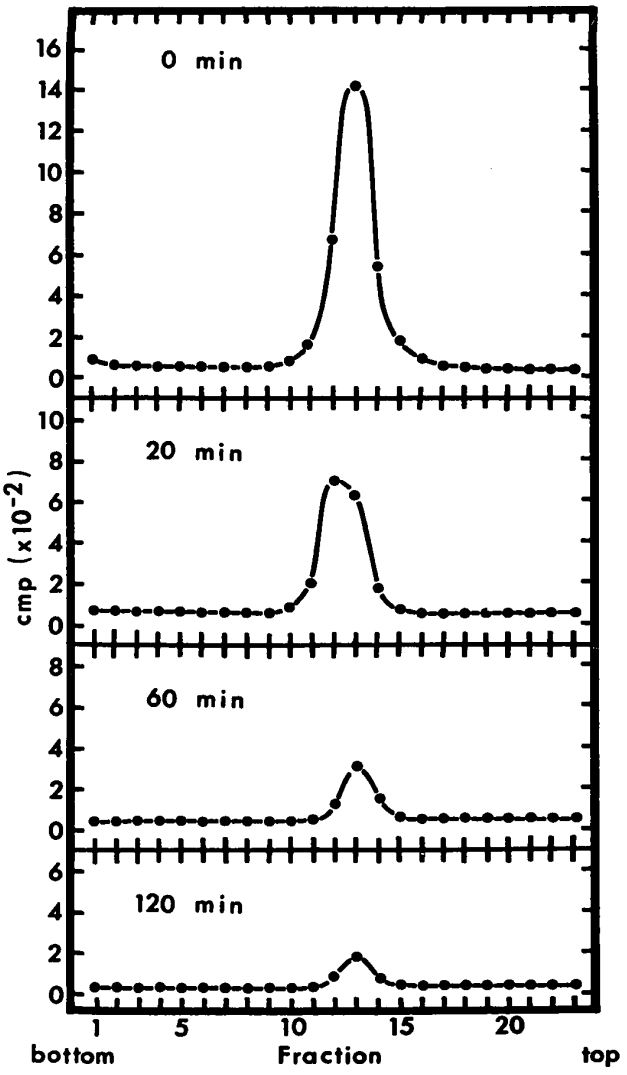

FIGURE 3.6. Sucrose density gradient analysis of uncoating of ^{3}H-UR-labeled M-Mengo virions (for details see Materials and Methods). The rate of uncoating was determined by measuring the progressive decrease of acid insoluble ^{3}H-UR-radioactivity in the 150S (intact virus) region of the gradient.

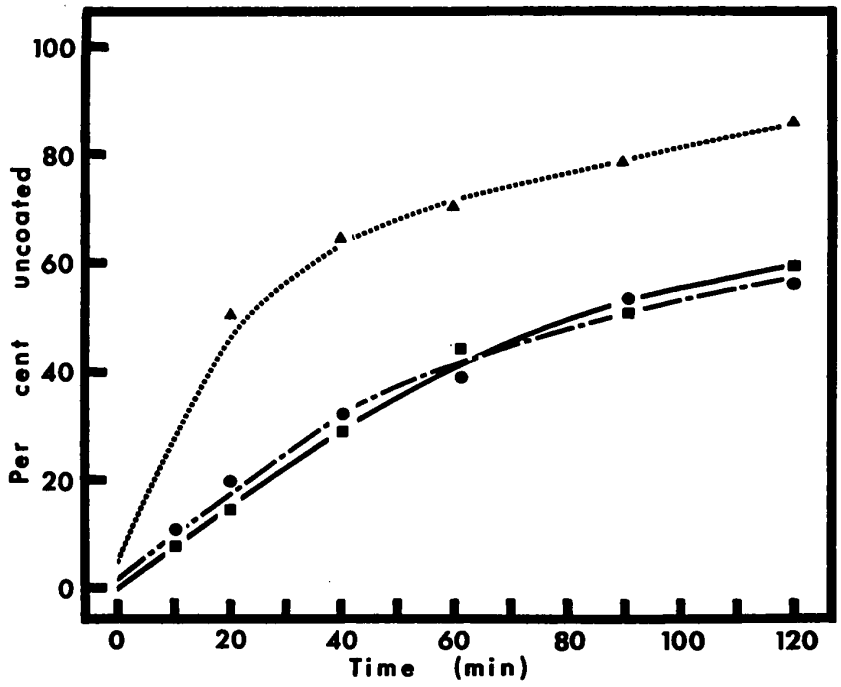

FIGURE 3.7. Kinetics of uncoating of ^{3}H-UR-labeled Mengo virus variants in L cells at 37°. The extent of virus uncoating was determined by measuring the decrease in number of intact virions (^{3}H-UR radioactivity sedimenting at 150 S) at the indicated times. L-Mengo ■——■; M-Mengo ▲---▲; S-Mengo ●---●.

comparison of the rates at which the three variants are uncoated was made possible by (a) the fact that it is possible to get attachment (without penetration) of the virions at 0-4°, and (b) that, once attached to cells, virions of all three variants are taken up by cells (at 37°) at the same rate. It should be pointed out that the data presented here concerning the kinetics of uncoating are really a measure of the rates of the combined processes of penetration and uncoating. That being the case, the times cited as being necessary for the uncoating of a certain % of the virions are longer than the times actually required for the uncoating process itself. However, even if the absolute values must be accepted with a degree of caution, they provide a valid index of the relative rates of uncoating of the three variants, since the latter were shown clearly to penetrate L cells at the same rate (see preceding section).

That the experimental design employed was a valid one is illustrated by Figure 3.6, in which data obtained from sucrose density gradient analyses of sonicates of cells infected with ^{3}H-uridine labelled M-Mengo are presented. It may be seen that the amount of radiolabel present in the virion band (fractions 11-15 under the conditions of centrifugation used) decreased progressively with increasing time of incubation at 37°. The rate of uncoating - in this and all other comparable experiments - was expressed as the rate at which radioactivity was lost from the intact (150S) virion band.

A series of eight experiments - each employing all three variants - was carried out, and the pooled data obtained therefrom are shown in Figure 3.7. The M-variant differs significantly from

L- and S-Mengo with respect to both the rate and extent of uncoating. After 20 min of incubation at 37°, 50% of the M-Mengo virions attached to cells had penetrated the cells and had been uncoated, compared to values of 15-20% in the case of L- and S-Mengo virions. With the latter two variants, an incubation period of 80 min was required before 50% of the input virions were uncoated. The % of input M-Mengo virions uncoated after a 2 hr incubation period was found to be 86% (average of eight estimations), while the % of input L- and S-Mengo uncoated at two hours averaged 55-60%, and never exceeded 65% in any experiment. It is pertinent to point out that in all experiments, and with all three variants, 75-95% of the radioactivity bound to cells as intact virions was recovered from the sucrose density gradients employed to measure uncoating.

The Effect of Inhibition of Protein Synthesis on the Uncoating of Mengo Virus

Although it was considered to be most unlikely that the uncoating of Mengo virions requires the intervention of some factor (viral specified or otherwise) not normally present in L cells, that possibility was examined by measuring the rate of uncoating in cells in which protein synthesis was blocked.

Experiments identical to those summarized in the preceding section were carried out using L cells that had been preincubated for 1 hr, and were maintained during the period of uncoating, in medium containing 10 μg cycloheximide per ml. (It had been established that this concentration of the drug completely blocked

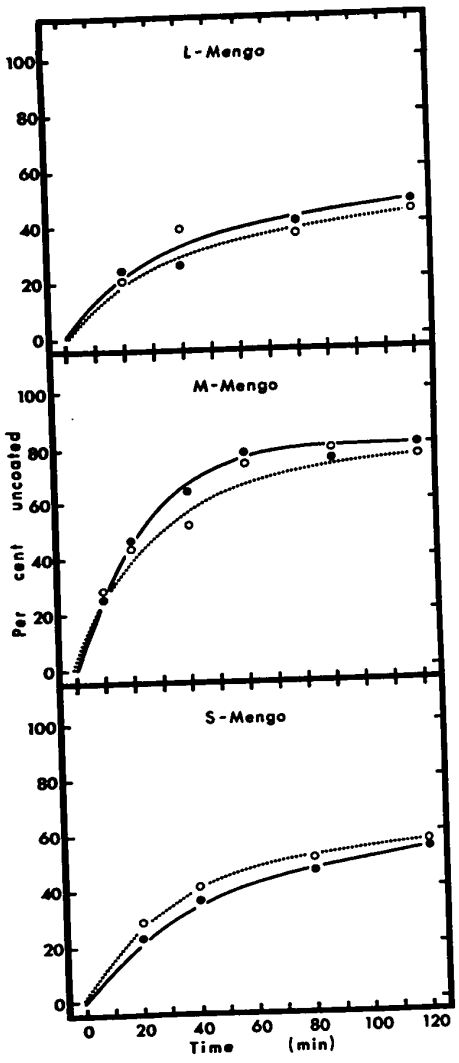

FIGURE 3.8. The rates of uncoating (measured as described in Materials and Methods) of the three variants in L cell suspension cultures in the presence (●—●) and absence (o—o) of cycloheximide.

protein synthesis in our L cells, as judged from measurements of the incorporation of ^{14}C-amino acids into TCA-insoluble products). Data from several such experiments are summarized in Figure 3.8, from which it is clear that inhibition of protein synthesis has no effect on either the rate or extent of uncoating of any of the three variants. This not unexpected finding is in agreement with the observations of Mandel (1967) who found that the uncoating of poliovirus is unaffected by inhibitors of protein synthesis.

Discussion

The results of the experiments described herein show that significant differences do exist among the three Mengo variants with respect to the processes of attachment and uncoating. On the other hand, studies of the rate at which virus particles, once attached to cells, penetrate the cell membrane, indicate quite clearly that virions of all three variants are taken up by cells at the same rate. It must be conceded that, by virtue of the experimental design employed, the penetration studies provided information regarding the infectious particles (PFU) of the virus population only. However, there is no reason to suppose that there is any heterogeneity in the virus population with respect to the penetration step. This point is considered in more detail in a subsequent paragraph.

It was not altogether surprising to find that L-Mengo differs from the other two variants with respect to rate of attachment to L cells.

Earlier studies (Scraba, 1968; Scraba et al., 1969) had shown that L-Mengo may be separated from M- and/or S-Mengo by either chromatography on hydroxylapatite, or by centrifugation through pre-formed density gradients of dextran sulfate. Moreover, it migrates more slowly than do M- and S-Mengo during electrophoresis in agarose gel. These differences almost certainly reflect differences in surface charge characteristics between virions of L-Mengo on the one hand and those of M- and S-Mengo on the other.

It is not easy, however, to reconcile the evidence for these differences in charge characteristics and the amino acid composition of the total proteins of the three variants, which have been shown to be essentially identical (Scraba et al., 1969). It is possible that differences exist among the variants in the fraction of the total acidic amino acid residues (which outnumber the basic amino acid residues by 2:1) that are present in the amide form. Standard methods of amino acid analyses do not discriminate between aspartic and glutamic acids and their amides. It is conceivable too that a single sequence of amino acids in one of the structural proteins of the virion is responsible for binding to hydroxylapatite and to L cells and for the binding of dextran sulfate. Were this the case, one could imagine that a change of one or two amino acid residues at this critical site might provide the basis for the differences cited above. What does seem clear is that a complete understanding of these differences that exist among the variants will come only from the isolation and characterization (e.g., peptide maps, primary sequence analyses) of the individual polypeptides from which their capsids are constructed.

The observation that M-Mengo virions are uncoated more rapidly and more extensively than are those of L- and S-Mengo is compatible with the finding made earlier in these laboratories that the eclipse period during infection with M-Mengo is shorter (by 1.0 - 1.5 hrs) than that seen with either L- or S-Mengo. The reason for the more rapid uncoating of the M-variant is not known. One could speculate that it is uncoated by a different mechanism, or at a different intracellular site than are L- and S-Mengo, but there is no evidence to support such speculation. A more reasonable explanation is simply that virions of M-Mengo are inherently less stable than those of the other two variants. It has been shown that M-Mengo is more rapidly inactivated at pH 6.8 than are either L- or S-Mengo (Colter et al., 1964a), and preliminary studies carried out in these laboratories indicate that in vitro pH inactivation and intracellular uncoating may be related phenomena (the primary protein product of each is the 13.4 S fragment described in earlier chapters). Moreover, a study of the "melting curves" of the three variants revealed that, of the three, M-Mengo has the lowest T_m and dissociates most precipitously when heated. The basis for the instability of M-Mengo virions (relative to those of L- and S-Mengo) is not known, but it is interesting to recall that M-Mengo virions contain a minor structural polypeptide (VPIV, mol.wt. 10,000) that is not present in S- and L-virions.

One of the more interesting conclusions to be drawn from the investigations described in this chapter is that there is no heterogeneity in populations of Mengo virions with respect to any of the three early events in the replicative cycle. It is clear from the

equivalence of rate constants for attachment based on uptake of PFU and of radioactivity, and of cpm/PFU ratios of viral suspensions before and after adsorption with L cells, that all virions in the preparations attach with equal facility to cells. Penetration studies measured the uptake of PFU. However, if there had been discrimination between infectious and non-infectious particles at the level of either penetration or uncoating, the kinetics of uncoating would be dramatically different than those that were observed. Instead of finding that 86% (M-Mengo) and 60% (S- and L-Mengo) of the cell-associated radioactivity was released from intact virions, one might have found values of the order of 1% (total:infectious particle ratios are of the order of 100 with all three variants). In the light of the data presented here, one can only conclude that all virions in purified preparations of the Mengo variants attach to, penetrate, and are uncoated in L cells with equal efficiency. Thus, unfortunately, the present studies have failed to provide an answer to one of the more intriguing puzzles of animal virology,- namely, why such a small percent of the virions in highly purified preparations of animal viruses - judged to be homogenous by the most sensitive physicochemical techniques available - are capable of initiating an infectious cycle in a susceptible cell.

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