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STRUCTURAL COMPONENTS OF THE MENGO VIRION

by

© BARRY R. ZIOLA

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

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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 STRUCTURAL COMPONENTS OF THE MENGO VIRION submitted by BARRY R. ZIOLA in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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Date August 25, 1975

To Lorraine

for continuous love and encouragement

during the pursuit of this work.

ABSTRACT

The single-stranded RNA genome of Mengo virus was found to have a molecular weight of $2.44 \pm 0.08 \times 10^6$. This value has been determined from studies using the methods of polyacrylamide gel electrophoresis, sedimentation in DMSO, sedimentation after reaction with formaldehyde, and electron microscope measurements of the viral replicative-form RNA.

SDS-polyacrylamide gel electrophoresis of solubilized purified virus has revealed the presence of four major polypeptides, designated α, β, γ, and δ, having estimated molecular weights of 32,500, 29,600, 23,700, and 10,600, respectively. In addition, two minor capsid polypeptides were detected. The first (D2) has a molecular weight of 57,700 and the second (ε), a molecular weight of 39,000.

Purification of the four major capsid polypeptides has been achieved by chromatography of solubilized virus on SDS-hydroxylapatite columns. With linear gradients of sodium phosphate (pH 6.0 or 6.4) containing 0.1% SDS and 1 mM DTT, the proteins eluted as separate peaks between 0.25 and 0.45 M phosphate. Amino acid composition analysis has been carried out on each of the purified polypeptides. The δ polypeptide is the most interesting in this regard because it contains no histidine, arginine, tryptophan or cysteine. The amino acid composition data also confirmed the earlier SDS-polyacrylamide gel estimated molecular weights of α, β, and γ, and led to reevaluation of the molecular weight of δ from 10,600 to 7,350.

From calculations based on the molecular weights of the capsid polypeptides and the distribution of radioactivity among the capsid components, it has been concluded that an average Mengo virion can be

represented by the structural formula: $RNA \cdot (\alpha,\beta,\gamma,\delta)_{58-59} \cdot D2_{1-2} \cdot \epsilon_{1-2}$.

Amino- and carboxyl-terminal amino acid sequence analysis has been conducted on each of the four major capsid components. The δ protein does not possess a free N-terminal amino acid. For the α, β, and γ proteins, the primary amino acid sequences of the ten residues at the N-terminals have been obtained. The C-terminal peptide bond of the γ protein was found to be resistant to carboxypeptidase hydrolysis. Carboxypeptidase analysis of α, β, and δ, however, has revealed the C-terminal and penultimate amino acid residues. This data has been interpreted in light of the proteolytic cleavages which generate the four major capsid proteins from larger precursor proteins.

Finally, during the course of these studies, a method was developed for the purification of proteins in quantities necessary for physicochemical characterization. The protein to be purified is first separated from contaminants by preparative SDS-polyacrylamide gel electrophoresis. The protein is then electrophoresed from gel slices into small columns of SDS-hydroxylapatite, and subsequently recovered by elution of the SDS-hydroxylapatite with 0.5 M sodium phosphate (pH 6.4) containing 0.1% SDS and 1 mM DTT.

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LIST OF ABBREVIATIONS

EMC virus	- encephalomyocarditis virus
ME virus	- Maus-Elberfeld virus
FMDV	- foot and mouth disease virus
TMV	- tobacco mosaic virus
PFU	- plaque forming unit(s)
RNA	- ribonucleate
rRNA	- ribosomal ribonucleate
RF RNA	- replicative-form ribonucleate
VP	- viral protein
NCVP	- non capsid viral protein
α-chtgn A	- α-chymotrypsinogen A
α-cht	- α-chymotrypsin
LDH	- lactic dehydrogenase
CBP A	- carboxypeptidase A
CBP B	- carboxypeptidase B
myo	- myoglobin
DMSO	- dimethyl sulfoxide
SDS	- sodium dodecyl sulfate
TRIS	- tris(hydroxymethyl)aminomethane
EDTA	- ethylenediaminetetraacetic acid
DTT	- dithiothreitol
RBBR	- Remazol Brilliant Blue R
IAA	- iodoacetamide
TPCK	- tosylsulfonyl-phenylalanyl chloromethyl ketone
DFP	- diisopropylfluorophosphate (diisopropylphosphofluoridate)

LIST OF ABBREVIATIONS (Continued)

rpm	- revolutions per minute
uv	- ultraviolet
nm	- nanometers
μCi	- microcurie
cpm	- counts per minute
g	- centrifugal force relative to gravity
nmoles	- nanomoles

All temperatures are in degrees Celsius.

I. GENERAL INTRODUCTION

Picornavirus Classification

The term picornavirus (pico meaning small and rna signifying the type of nucleic acid in the virion) was adopted for a class of small, icosahedral, non-enveloped RNA viruses by the 1963 International Enterovirus Study Group. By definition, the viruses included are 150 to 300 Å in diameter, are insensitive to inactivation by ether, and contain (single-stranded) RNA. The Study Group dealt only with the small RNA viruses of animals, leaving out those bacterial and plant viruses which are clearly picornaviruses by these criteria.

Accumulated data currently favours classification of picornaviruses according to a scheme such as that shown in Table 1 (Andrewes and Pereira, 1972; Newman et al., 1973; Scraba and Colter, 1974). Although the calciviruses are included as a subgroup, their larger size and characteristic morphology (Zwillenberg and Bürki, 1966; Almeida et al., 1968; Wawrzkiewicz et al., 1968) clearly distinguishes them from other viruses in Table 1. The distinctive base composition of the calcivirus RNA (Newman et al., 1973; Burroughs and Brown, 1974) and the finding that the caliciviruses possess only one major capsid polypeptide (Bachrach and Hess, 1973; Burroughs and Brown, 1974), further emphasizes the uniqueness of these viruses. Based on this information, Burroughs and Brown (1974) have suggested that the caliciviruses be grouped together in a separate family called the Caliciviridae. Since the caliciviruses are not "typical" picornaviruses, subsequent usage of the term picornavirus will be restricted to include only those viruses comprising the first

Subgroup	Viruses	Distinguishing Characteristics
Enterovirus	Polio (3 serotypes) Coxsackie A (24 serotypes) Coxsackie B (6 serotypes) Echo (~ 35 serotypes) Enteroviruses of various animals	Buoyant density (in CsCl) = 1.33-1.35 g/ml Virions stable pH 3-10 Empty capsids produced *in vivo*
Cardiovirus	EMC (Encephalomyocarditis) MM (Maus-Elberfeld) Mengo Columbia-SK	All are antigenically related. Buoyant density = 1.34 g/ml Virions stable at low pH, but dissociate at $5 < pH < 7$ in the presence of Cl^- or Br^- ions No stable empty capsids produced *in vivo*
Rhinovirus	Human rhinovirus (~ 90 serotypes) Equine and bovine rhinoviruses	Buoyant density = 1.38-1.45 g/ml Virions dissociate at pH < 5 Some varieties produce empty capsids *in vivo*
Aphthovirus	Foot-and-mouth disease virus (7 serotypes)	Buoyant density = 1.43 g/ml Virions dissociate at pH < 6.5 Empty capsids produced *in vivo*
Calcivirus	Vesicular exanthema (pigs) Feline calcivirus San Miguel sea lion virus	Buoyant density = 1.37 g/ml Virions dissociate at pH < 4 Slightly larger (35-40 nm diam.) than the above viruses; show cup-like capsomeres when negatively stained

Table 1. Picornavirus classification scheme (modified from Scraba and Colter, 1974). The various picornavirus subgroups also have distinctive pathological properties. These are discussed in detail by Andrewes and Pereira (1972). Echo is an abbreviation for "enteric cytopathic human orphan."

four subgroups in Table 1.

In addition, recent reports in the literature have suggested that human hemmorrhagic conjunctivitis (Yamazaki et al., 1974; Esposito et al., 1974), human hepatitis type A (Provost et al., 1975), and Aleutian disease of mink (Yoon et al., 1975) may be caused by picornaviruses. Since the viruses associated with these diseases have thus far been identified as picornaviruses only on the basis of limited biophysical and morphological characterization of the virion and its RNA component, they have not been included in Table 1.

Properties of the Virion

The physical and hydrodynamic properties of intact picornavirus virions have been well studied. The results of such studies on the Mengo virion (Scraba et al., 1967) can be considered as representative of picornaviruses in general. In the electron microscope, negatively stained Mengo virions appeared as isometric particles with a dry diameter of approximately 27 nm. In solution, the virus particles behaved as spheroids (frictional ratio of 1.10) having a diameter of about 30 nm and containing some 0.23 g water/g dry virus. The sedimentation coefficient ($S_{20,w}$) of the Mengo virion was found to be 151 S; the diffusion coefficient ($D_{20,w}$), 1.47 X 10^{-7} cm^2/sec; and the partial specific volume ($\bar{v}$), 0.70 ml/g. Substituting these values in the Svedberg equation, a particle weight of 8.3 ± 0.7 X 10^6 was calculated for the Mengo virion.

The RNA Component

There is no evidence for the presence of lipid as an integral component of the picornavirus virion, or for the essential presence of any carbohydrate other than the ribose moeity of the RNA (Rueckert, 1971; Burness et al., 1973). Consequently, picornaviruses are comprised only of an RNA molecule enclosed in a protein coat. Infectious RNA has been isolated from Mengo virus (Colter et al., 1957), from poliovirus (Alexander et al., 1958), from EMC virus (Huppert and Saunders, 1958) and from ME virus (Franklin et al., 1959), clearly establishing RNA as the picornavirus genetic material. The infectious RNA was subsequently shown to be single-stranded (Hausen and Schäfer, 1962). Base composition analyses have found no unusual nucleotides in picornavirus RNAs. As well, with the possible exception of some human rhinovirus genomes, picornavirus RNAs have been found to contain approximately equimolar amounts of adenylate, cytidylate, guanylate, and uridylate (Newman et al., 1973).

Estimates of the picornavirus RNA molecular weight have ranged from 1.2 to 3.0 X 10^6. Based mainly on sedimentation rates in the ultracentrifuge, earlier studies found the poliovirus RNA molecular weight to be 1.2 to 2.0 X 10^6 (for summary, see Cooper, 1969). Similarly, Hausen and Schäfer (1962) estimated a molecular weight of 2.0 X 10^6 for ME virus RNA, while Burness et al. (1963) reported a value of 3.0 X 10^6 for EMC virus RNA. Scraba et al. (1967) calculated a molecular weight of 1.74 X 10^6 for Mengo virus RNA by inserting the RNA sedimentation and diffusion coefficients into the Svedberg equation. Shortly thereafter, Fenwick (1968) reported a molecular weight of

2.4 X 10^6 for EMC virus RNA based on the sedimentation rate of the RNA in buffered formaldehyde. Granboulan and Girard (1969) subsequently estimated the molecular weight of poliovirus RNA to be 2.7 X 10^6 from contour length measurements of the RNA in electron micrographs. Utilizing the virus particle weight and the percent RNA (and protein) composition of the virion, Burness (1970) calculated the same molecular weight for EMC virus RNA. Finally, Tannock et al. (1970) confirmed that the molecular weight of poliovirus RNA is larger than earlier estimates by reporting a value of 2.57 X 10^6 on the basis of polyacrylamide gel electrophoresis studies. Summarizing the more recently reported values, it is most likely that the molecular weight of the picornavirus RNA is 2.4 to 2.7 X 10^6.

The Protein Component

Amino acid composition analyses of the total protein from several different picornaviruses have been reported. The results, which have been tabulated by Rueckert (1971), show remarkable similarity. With respect to the composition analysis of the total Mengo virus capsid protein (Scraba et al., 1969), there is an excess of acidic residues (glx, asx) compared to basic residues (his, arg, lys). The content of sulfur-containing residues (1/2 cys, met) is low while the content of non-polar residues is high (45 mole %). There is also a high content of non-α-helix forming residues (val, ile, ser, cys, thr, gly, pro; Fasman, 1963), with the proline content being exceptionally high (8 mole %). With regard to α-helix content of the Mengo virus capsid protein, Scraba et al. (1967) and Kay et al. (1970) have calculated from optical

rotatory dispersion and circular dichroism measurements that the protein of the Mengo virion *in situ* possesses less than 5% α-helical conformation.

Sedimentation of total picornavirus capsid protein in sucrose density gradients or in the ultracentrifuge has indicated that the polypeptides of poliovirus (Maizel, 1963), ME virus (Rueckert, 1965), and EMC virus (Burness and Walter, 1967) were each single species of molecular weight 26,000 to 30,000. Polyacrylamide gel electrophoresis analyses, however, revealed that the picornavirus capsid protein is comprised of several types of non-identical polypeptide chains (Maizel, 1963; Summers *et al.*, 1965; Rueckert, 1965; Rueckert and Duesberg, 1966). With the discovery that the electrophoretic mobility of a polypeptide in SDS-containing polyacrylamide gels is (to a first approximation) directly proportional to the logarithm of the polypeptide molecular weight (Shapiro *et al.*, 1967; Weber and Osborn, 1969; Dunker and Rueckert, 1969), it became possible to determine simultaneously both the number of different polypeptides comprising the picornavirus capsids and their molecular weights. For example, in the case of poliovirus (Maizel and Summers, 1968) and ME virus (Rueckert *et al.*, 1969), four major capsid polypeptides have been found with molecular weights approximately 33,000, 29,000, 25,000 and 6,000 to 10,000. Similar studies on the polypeptide compositions of three Mengo virus variants (O'Callaghan *et al.*, 1970), however, found only three major capsid proteins of molecular weights 31,000, 28,000 and 20,000. Of the three virus variants studied, only M-Mengo was found to contain a fourth polypeptide of molecular weight approximately 10,000.

Morphology and Architecture of the Virion[1] .

Although the morphological unit or capsomere (Caspar et al., 1962) organization of many different icosahedral viruses has been determined from electron microscopy of negatively stained particles (Madeley, 1972), this has not been the case for picornaviruses. Their capsids are extremely compact and are not penetrated by the heavy salts commonly used as negative stains. It is for this reason that the virus particle surface detail is not readily distinguishable in electron micrographs. It is not surprising, therefore, that on the basis of examination of one or two "favourably oriented" particles in a large group, it has been reported that the poliovirus capsid is composed of 32 (Mayor, 1964) or 42 (Agrawal, 1966) or 60 (Horne and Nagington, 1959) capsomeres.

From earlier X-ray diffraction studies of poliovirus crystals, Finch and Klug (1959) had concluded that the picornavirus virion possesses icosahedral (532) symmetry. From the observed regular modulation in intensity of the diffracted X-rays, they calculated that the virus capsid is composed of 60 structurally equivalent asymmetric units, each 60 to 65 Å in diameter. It was not possible, however, to define the precise arrangement of these subunits in the virion.

The break-through in the problem of picornavirus architecture was provided largely by Rueckert and collaborators (Rueckert et al., 1969; Dunker and Rueckert, 1971) who, instead of examining intact virions, studied the virus dissociation products formed under controlled conditions. It has been shown that the cardioviruses, MC (Dunker and

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[1] For further information, see review by Rueckert (1971).

Rueckert, 1971), EMC (McGregor et al., 1975), and Mengo (Mak et al., 1970) are inactivated by incubation at pH 5.5 to 7.0 in the presence of 0.1 to 0.2 M chloride or bromide ions. This inactivation is accompanied by the dissociation of the viral capsid into protein subunits which have a sedimentation coefficient of 13 to 14 S and a molecular weight of 400,000 to 450,000. Rueckert et al. (1969) found that the ME virus 14 S subunit contains equimolar amounts of three polypeptides, designated α, β, and γ (molecular weights 33,000, 30,500, and 25,000, respectively). Incubation in 2 M urea caused each subunit to dissociate further into five 5 S fragments having an approximate molecular weight of 86,000 and the same polypeptide composition as that of the 14 S subunit. This data was incorporated into a model for the ME virus capsid (Rueckert et al., 1969) wherein the basic structural unit is an aggregate of one molecule of each of the α, β, and γ polypeptides (i.e., the 5 S fragments). These structural units are then bonded together in clusters of five to form the 14 S subunits, one of which is centered at each vertex of the icosahedral virus capsid.

Re-examination of the Mengo virus polypeptide composition has shown that the Mengo virion possesses four major polypeptides of approximately the same molecular weights as those of polio or ME virus (Ziola and Scraba, 1974; see Chapter IV.). Mak et al. (1974) subsequently examined the pH and urea dissociation products of Mengo virus (i.e., the 13.4 S subunit and the 4.7 S fragment) as was done for ME virus (see above). From SDS-polyacrylamide gel electrophoresis studies, it was determined that the polypeptide compositions of the Mengo 13.4 S subunit and 4.7 S fragment are the same as the compositions of the analogous ME virus dissociation products. Electron microscope studies

demonstrated that the Mengo 13.4 S subunit has a well defined, slightly ellipsoidal shape with surface dimensions of 16.8 X 14.2 nm. The 4.7 S fragment was found to have an approximately spherical shape of diameter 6.8 nm. Upon dividing the surface area of the 13.4 S subunit by the surface area of the 4.7 S fragment, a value of 5.2 was obtained, which strongly suggests that there are five 4.7 S fragments per 13.4 S subunit. Similarly, by dividing the surface area of the Mengo virion by the surface area of the 13.4 S subunit, a value of 12.0 was obtained. Consequently, the Mengo virus capsid can also be represented by the model proposed by Rueckert et al. (1969) for the ME virus capsid. The morphological unit or capsomere can be considered to be the 13.4 S subunit (12 per virion), while the structure unit is the 4.7 S fragment (60 per virion, corresponding to the 60 to 65 Å asymmetric structure unit originally proposed by Finch and Klug, 1959).

Although a better understanding of the picornavirus capsid architecture is now available, the elucidation of the total capsid structure is not complete. Two major problems remain. First, the exact nature of the interaction of the three non-identical polypeptides comprising the fundamental structural unit is not known. The polypeptides may be located at specific sites in the 4.7 S fragments or they may exist as a single interwoven "trimeric" complex. It is also possible that the actual arrangement is somewhere inbetween; for example, an interwoven complex of the α and γ polypeptides could occupy a discrete part of the 4.7 S structural unit as would also the remaining protein, β. Second, the exact location of the smallest capsid polypeptide (molecular weight 6,000 to 10,000) is unknown. Mak et al. (1974) have proposed that this protein is clustered in trimers, one trimer being located at the center

of each of the Mengo virus icosahedral facets. In the case of bovine enterovirus, however, Johnston and Martin (1971) have proposed an internal location; while for Coxsackie B3 virions, Philipson et al. (1973) have proposed that the smallest capsid polypeptide is grouped in pentamers at the vertices of the icosahedron.

Biosynthesis of Virus-Specific Polypeptides and Assembly of the Virion[1]

In early studies of the poliovirus replicative cycle, some fourteen different virus-specific polypeptides were detected in infected HeLa cell cytoplasmic extracts (Summers at al., 1965). Four of these were shown to correspond to the virus capsid proteins, designated VP 1 to 4, while the remainder were non-capsid viral proteins (NCVP) 1 to 10. The sum of the molecular weights of the fourteen polypeptides, as estimated by SDS-polyacrylamide gel electrophoresis, was approximately 450,000. This is nearly twice the theoretical coding capacity of the poliovirus RNA (i.e., an RNA molecule of molecular weight approximately 2.6×10^6 contains some 7,500 nucleotides and can code for a maximum protein mass of about 275,000 daltons). This inconsistency was resolved when a study of the kinetics of formation of the virus-specific polypeptides revealed that the high molecular weight proteins produced early in infection were cleaved at later times to yield the smaller capsid and non-capsid polypeptides (Summers and Maizel, 1968). Similar posttranslational cleavages have been shown to occur during the replication of

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[1] A comprehensive treatment of this subject is found in reviews by Casjens and King (1975), and Rueckko and Fry (1975).

other picornaviruses, including a coxsackievirus (Kiehn and Holland, 1970), a rhinovirus (McLean and Rueckert, 1973), and the cardioviruses EMC (Butterworth et al., 1971) and Mengo (Kiehn and Holland, 1970; Paucha et al., 1974).

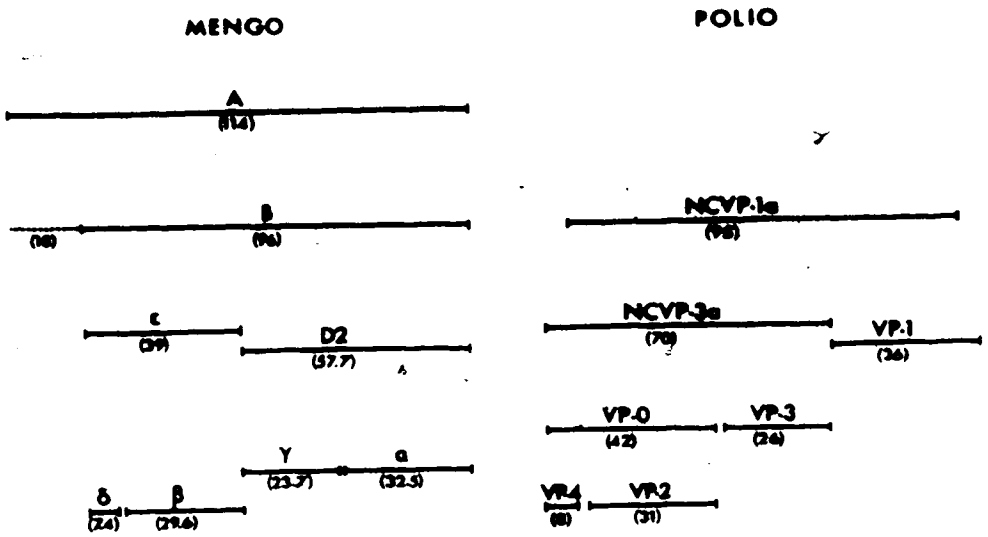
A number of studies have strongly suggested that the entire picornavirus RNA genome is translated in the infected cell into a single "polyprotein" molecule of molecular weight approximately 260,000. Giant polypeptides of greater than 200,000 molecular weight have been observed in picornavirus-infected cells in the presence of amino acid analogues (Jacobson et al., 1970; Paucha et al., 1974), in the presence of zinc ions (Butterworth and Korant, 1974), or in the presence of protease inhibitors (Korant, 1972; Summers et al., 1972). Giant polypeptides have also been observed during the replication cycle of temperature-sensitive poliovirus mutants at the restrictive temperature (Garfinkle and Tershak, 1971). Under normal infection conditions, however, it appears that this "polyprotein" is unstable and undergoes specific proteolytic cleavages while it is still in the process of being synthesized. For example, in both polio and EMC virus-infected cells, the largest polysome bound nascent polypeptides found had molecular weights of approximately 100,000 to 130,000 (Jacobson et al., 1970; Butterworth and Rueckert, 1972a).

Analysis of the products of cell-free protein synthesis directed by EMC or poliovirus RNA indicates there is only a single translation initiation site (Öberg and Shatkin, 1972; Boime and Leder, 1972). Subsequent to translation initiation, it appears that a short "lead-in" peptide is cleaved off (Öberg and Shatkin, 1972; Smith, 1973). It has been suggested that a single initiation site requires equimolar

synthesis of all virus-specified proteins (Jacobson and Baltimore, 1968a; Butterworth, 1973). This appears to be the case during EMC virus replication (Butterworth and Rueckert, 1972a; Butterworth, 1973). Lucas-Lenard (1974) and Paucha et al. (1974), however, have shown that at late times in the Mengo virus replication cycle (6 to 7 hr post-infection), there is an increase in the amount of capsid protein synthesized relative to non-capsid protein. Since the capsid protein is translated from the 5' end of the RNA, it has been suggested that the translation of the Mengo virus genome is under some kind of control and a model has been proposed in which a virus-specified protein acts as a specific termination factor (Paucha et al., 1974).

A comparison of the synthesis of virus-specified proteins in cells infected with polio, EMC, and a human rhinovirus has shown that the pattern of polypeptide cleavages among these representative members of three picornavirus subgroups (see Table 1) is very similar (Butterworth, 1973); and it has been shown that the Mengo virus-specific polypeptides arise by a series of posttranslational cleavages nearly identical to those observed with the other picornaviruses (Paucha et al., 1974). Summarizing the results obtained in the Mengo virus replication studies: (a) there are three large, primary gene products, designated A, F, and C, resulting from cleavages of the nascent "polyprotein" molecule; (b) two smaller, stable non-capsid viral polypeptides, G and H (of molecular weights 16,400 and 14,000), also appear to be primary cleavage products; (c) polypeptide F is a stable end product of molecular weight 38,000; (d) polypeptide C undergoes further proteolytic cleavages to produce the stable non-capsid polypeptides I and E (of molecular weights 12,600 and 56,000); and (e) polypeptide A is the

precursor of the capsid polypeptides, which are believed to be produced by the following sequence of cleavages (SDS-polyacrylamide gel electrophoresis estimated molecular weights X 10^{-3} are given in brackets). The poliovirus cleavage scheme according to Butterworth (1973) is included for comparison.



Using pactamycin to inhibit protein synthesis initiation in infected cells (Summers and Maizel, 1971; Butterworth and Rueckert, 1972b), it was established that the gene-order (from the 5'- to the 3'-terminus of the RNA) for the stable Mengo virus-specified proteins is: δ, β, γ, α, G, H, F, I, and E (Pancha et al., 1974).

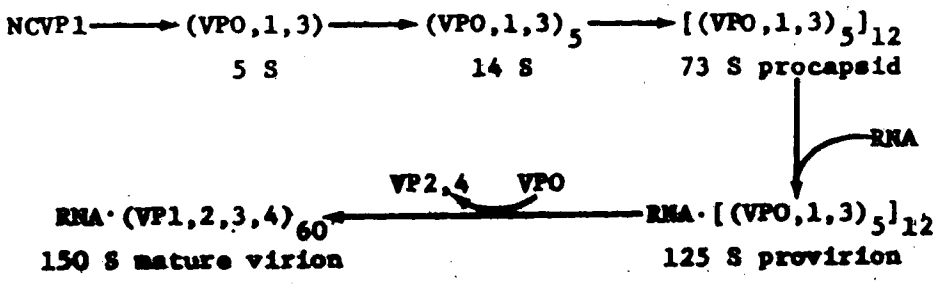
Very little is known about the mechanisms involved in the cleavage of the picornavirus precursor polypeptides. Korant (1972) has suggested that there are two distinct types of proteases involved in the cleavages which lead to the four poliovirus capsid polypeptides.

From *in vitro* experiments he has shown that uninfected cell extracts can carry out the primary cleavages of the nascent poliovirus "polyprotein" and that these cleavages are inhibited by inhibitors of serine proteases. The specificity of the primary cleavages was found to reside in the conformation of the "polyprotein" since the action of α-chymotrypsin on the native molecule yielded products similar to those obtained with infected cell extracts. When α-chymotrypsin acted upon denatured "polyprotein", however, only low molecular weight fragments resulted. In addition, infected cell extracts were found to cleave the "polyprotein" to the proteins found in poliovirus empty capsids (see below). Consequently, it appears that both host and virus-specified (or induced) proteases are required for the processing of picornavirus polypeptides.

Considerable experimental work has been done in an attempt to elucidate the pathway by which picornaviruses are assembled. Jacobson and Baltimore (1968b) found that 73 S empty capsids accumulated in HeLa cells which were infected with poliovirus in the presence of 3 mM guanidine hydrochloride. Upon removal of the guanidine, the radioactively-labeled protein material of the empty capsids was converted into mature poliovirions. From these observations, it appeared that the 73 S particle is an intermediate in the virus assembly process; consequently, it was termed the "procapsid". It had previously been shown that the poliovirus empty capsids which occur during the course of a normal infection contain the polypeptides VP0 (this was originally termed NCVP6), VP1, and VP3; whereas the capsid of the mature virion contains VP1, VP2, VP3, VP4, and only a very small amount of VP0 (Maizel *et al.*, 1967). Fernandes-Tomas and Baltimore (1973) subsequently reported finding a 125 S particle in poliovirus-infected cells which is

composed of VPO, VP1, and VP3 plus an single-stranded 35 S viral RNA molecule. This particle was termed the "provirion" and from its existence, it appears that during the morphogenesis of poliovirus, the RNA is inserted into the 73 S procapsid prior to the final proteolytic cleavage which yields the mature virion (i.e., VPO → VP2 + VP4).

Studies by Phillips et al. (1968) had shown that poliovirus infected cells also contain 5 S and 14 S particles which possess the same polypeptide composition as the 73 S procapsids. Subsequent experiments demonstrated that the 14 S particles could be assembled in vitro into 73 S structures (Phillips, 1969, 1971). Addition of the rough cytoplasmic fraction from infected cells greatly enhanced the 14 S particle self assembly (Perlin and Phillips, 1973), suggesting that in vivo the 14 S particles are assembled into procapsids on rough cytoplasmic membranes which have been modified by a virus-specified or virus-induced protein. On the basis of these and the above data, the following scheme has been proposed for poliovirus morphogenesis (Phillips, 1972; Casjens and King, 1975):



The major criticism of the experiments on which the proposed assembly pathway is based is that precursor-product relationships between the various particles cannot be unambiguously defined by pulse-chase experiments due to the numerous and rather large pool sizes of the

intermediary structures. It must also be considered that some of the particles may be the product of abortive assembly rather than structural intermediates. In this regard, Ghendon et al. (1972) have shown that poliovirus-infected MiO cells (from rhesus monkey tonsils) do not accumulate 73 S empty capsids in the presence of guanidine; instead, 14 S particles accumulate. Upon removal of the guanidine, the 14 S particles appear to convert directly to mature virions. No 73 S procapsids are formed, suggesting that these particles may not play an obligatory role in poliovirus morphogenesis.

Although procapsids (and presumably, provirions) are generally associated with poliovirus infection, these particles are not produced in all picornavirus infections (Table 1). The cardioviruses (the most notable exceptions in this regard) do, however, form other sub-viral particles during their replication cycles. Upon examination of cytoplasmic extracts of EMC virus-infected HeLa cells, McGregor et al. (1975) found two capsid precursor particles which sedimented in glycerol gradients at 13 S and 14 S, respectively. The 13 S particle was detected following a 10 min pulse with radioactively-labeled amino acids at 3.5 hr post-infection. When the pulse was followed by a 20 min chase, the 13 S particle was replaced by the 14 S particle. Electrophoretic analysis revealed that the 13 S particle contained only the capsid precursor protein A (molecular weight approximately 100,000). The 14 S particle, however, was found to contain equimolar amounts of three polypeptides: ε, α, and γ. From comparison with the acidic pH (+ 0.1 M Cl^-) dissociation product of the EMC virion, it was concluded that these two particles can be represented by the structural formulas: 13 S = $(A)_5$ and 14 S = $(\varepsilon,\alpha,\gamma)_5$. Based on this data, McGregor et al. (1975) proposed the

following scheme for the assembly of the EMC virion:

$$A \longrightarrow \underset{13\ S}{(A)_5} \longrightarrow \underset{14\ S}{(\epsilon,\alpha,\gamma)_5} \longrightarrow \underset{150\ S\ mature\ virion}{RNA \cdot (\alpha,\beta,\gamma,\delta)_{57-58} \cdot (\epsilon,\alpha,\gamma)_{2-3}}$$

From this scheme it would appear that formation of the cardiovirus capsid from the precursor 14 S particles, encapsidation of the viral RNA, and the final proteolytic cleavage ($\epsilon \rightarrow \beta + \delta$) must occur in a highly concerted fashion. However, examination of cytoplasmic extracts of Mengo virus-infected L cells has revealed the presence of a sub-viral particle having a sedimentation coefficient of approximately 50 S (E. Rueckert, personal communication). This 50 S particle may be a "condensation" product of viral RNA and viral capsid protein since it can be radioactively-labeled with both ^{3}H-uridine and ^{14}C-amino acids. Electrophoretic analysis of the protein component has revealed the presence of approximately equimolar amounts of three polypeptides - ϵ, α, and γ. Consequently, it is possible that the Mengo viral RNA may associate with capsid protein prior to the final morphogenetic cleavage of the ϵ precursor protein to the capsid polypeptides β and δ. In summary, while neither of the virus assembly schemes outlined appears to encompass all available information, each provides a good starting point for further experiments designed to elucidate the picornavirus assembly process. It must be remembered also, that the observed differences between the enterovirus (i.e., poliovirus) and the cardiovirus (i.e., EMC and Mengo virus) assembly processes may be a true reflection of fundamental differences in inter-molecular interactions which exist in the virions of the different picornavirus subgroups.

With this information as background, experiments were undertaken to further characterize the Mengo virus structural components. This thesis summarizes data obtained regarding: (1) the molecular weight of the viral RNA genome; (2) the number of non-identical polypeptides in the virus capsid, their individual molecular weights, and the quantities of each present per virus particle; (3) the isolation of the individual capsid polypeptides; and (4) the chemical characterization of the purified capsid components by amino acid analysis and by N- and C-terminal sequence analysis. The results of these studies are discussed with respect to the information obtained about the structure of the virion and its mode of assembly.

II. ROUTINE MATERIALS AND METHODS

Tissue Culture Media

Growth medium. Minimum essential medium F-11 containing Earle's salts (Earle, 1943) and Eagle's nutrients (Eagle, 1959) was purchased in powdered form from the Grand Island Biological Co., Grand Island, N.Y. The powder was dissolved in deionized distilled water and sodium bicarbonate added to a final concentration of 0.12%. After sterilization by Millipore filtration (0.22 μm pore size - Millipore Corp., Bedford, Ma.), the "basal medium" was supplemented by the addition of the following materials:

(1) Horse serum (Flow Laboratories, Rockville, Md.), either 5% for the growth of cells or 1% for the production of virus.

(2) Penicillin G (Glaxo-Allenburys Ltd., Toronto, Ont.) and Streptomycin sulfate (Sigma Chemical Co., St. Louis, Mo.) at final concentrations of 100 I.U./ml and 50 μg/ml, respectively.

Spinner medium. The composition of this medium is identical to growth medium except for the omission of calcium from the starting powder (minimum essential Eagle's medium F-14, containing spinner salts - Grand Island Biological Co.).

Mengo Encephalomyelitis Virus

The L, M, and S Mengo-type variants of Mengo encephalomyelitis virus used throughout these studies were originally isolated by Ellem and Colter (1962), and have been maintained by passage in cultured L cells.

Cultured L Cells

Earle's L-929 strain of mouse fibroblast cells (Sanford et al., 1948) was used for the propagation of the three Mengo virus variants. The cells were obtained from the American Type Culture Collection, Rockville, Md.

L cells in growth medium were added to 1-litre Blake bottles (Kimble Products, Owens-Illinois Co., Toledo, Ohio) and incubated at 37°. Upon growing to confluent monolayers, the cells were detached by replacing the growth medium with 0.25% trypsin (Difco Laboratories, Detroit, Mich.) in 10 mM sodium phosphate, pH 7.4, containing 142.8 mM NaCl and 2.8 mM KCl. The trypsin solution was removed after an incubation at room temperature of approximately 15 seconds. A portion of the trypsinized cells was then suspended in fresh growth medium and used to reseed new Blake bottles. The remaining cells were suspended in spinner medium and transferred to 1- or 2-litre spinner flasks (Bellco Biological Glassware, Vineland, N.J.) at a concentration of 2 X 10^5 cells/ml. The cells were kept in suspension by means of a magnetic stirring device and incubated at 37° to allow further growth to take place.

Virus Growth in Roller Bottles

Confluent L cell monolayers were prepared in large cylindrical bottles (490 mm X 110 mm diameter - Bellco Biological Glassware). The bottles were first coated with fetal calf serum (Flow Laboratories) to facilitate the attachment of cells, after which 150 ml of [illegible] were added. L cells harvested from suspension culture [illegible] to give a final concentration of approximately 10^6 cells/ml. The roller bottles were rotated on a Bellco roller apparatus at [illegible]

after which the speed was increased to 1 rpm. Cell growth was confluent after incubation at 37° for approximately 48 hr. The growth medium (5% horse serum) was then replaced by 20 ml of growth medium (1% horse serum) containing the virus inoculum (approximately 10^8 PFU/ml - equivalent to a multiplicity of infection of 10). The bottles were then rotated at 0.2 rpm for 3 hr and at 1 rpm for an additional 20 to 24 hr. At the end of this time, the cell mass was dislodged from the glass by gentle shaking.

Virus Purification

The procedure described by Ziola and Scraba (1974) was used for the purification of Mengo virions.

Lysates of infected L cell cultures were pooled and centrifuged at 1,000 g for 15 min. The pellet was resuspended in a small volume of distilled water, frozen and thawed twice to release trapped virus, and recentrifuged. The combined supernatants were chilled in ice and the virus was precipitated therefrom by adding cold methanol (-20°) to a final concentration of 20% and allowing the solution to stand overnight at 4°. The precipitate was collected by centrifugation at 5,000 g for 30 min, resuspended in 0.2 M sodium phosphate buffer, pH 7.8, and homogenized by hand in a tissue homogenizer (Arthur H. Thomas Co., Philadelphia, Pa.) to disrupt large aggregates. A solution of α-chymotrypsin (3X crystallized - Worthington Biochemicals, Freehold, N.J.) was added to give a final enzyme concentration of 0.8 mg/ml, and the mixture incubated with stirring at 37° for 20 min. An equal volume of 0.2 M sodium pyrophosphate, pH 8.0, was added and the incubation continued for an additional 20 min. The mixture was then chilled and clarified by

centrifugation at 20,000 g for 10 min. Virus was sedimented from the supernatant by centrifugation at 120,000 g for 60 min and resuspended by homogenization in a small volume of 0.1 M sodium phosphate buffer, pH 7.4.

The partially purified virus was sedimented through a discontinuous sucrose gradient (13 ml of 15% sucrose layered on 13 ml of 30% sucrose) by centrifugation for 20 hr at 20,000 rpm in a Spinco SW 27 rotor (4°). The pellet was resuspended in 0.1 M sodium phosphate, pH 7.4, again with homogenization, and mixed with an aqueous solution of Cs_2SO_4 (final density = 1.31 g/cm^3). After centrifugation for 20 hr at 40,000 rpm in a Spinco SW 50.1 rotor (4°) the virus, which appeared as a white band in the middle of the gradient, was collected through the side of the tube with a syringe. Salt was removed either by gel filtration on a G25 Sephadex column (3 cm^2 X 20 cm) or by dialysis against 0.1 M sodium phosphate buffer, pH 7.4.

Preparation of Radioactively Labeled Virus

^{3}H-uridine labeled virus. Individual roller bottles of L cell monolayers were infected with virus (10 PFU/cell) suspended in 15 ml of growth medium containing 2 µg actinomycin D/ml (Schwarz/Mann Biochemicals, Orangeburg, N.Y.). After incubating at 37° for 2 hr, ^{3}H-uridine (New England Nuclear, Montreal, Que.) in 5 ml of growth medium was added to each bottle such that the final isotope concentration was 1.5 µCi/ml. After a 20 to 24 hr incubation at 37°, the virus was harvested and purified as described.

^{3}H- or ^{14}C-amino acid labeled virus. L cell monolayers were infected with virus suspended in a medium consisting of Eagle's

nutrients in Earle's medium from which the amino acids were omitted, and supplemented with 1% horse serum and a mixture of ^{3}H- or ^{14}C-labeled amino acids (at final concentrations of 2.0 and 0.4 μCi/ml, respectively - New England Nuclear).

III. MOLECULAR WEIGHT OF THE MENGO VIRUS RNA GENOME

Introduction

The molecular weight of a virus genome, and directly therefrom its maximum protein coding capacity, must be known before undertaking studies to determine the number of virus-specified proteins synthesized during the replication cycle of the virus in an infected cell. In the case of the picornaviral RNA, recent reports in the literature have supported a molecular weight of 2.5 to 2.7 X 10^6 for its sodium salt. (Penwick, 1968; Granboulan and Girard, 1969; Tannock et al., 1970). Assuming a mean nucleotide residue weight of 345, an RNA molecule of this size would contain approximately 7,500 nucleotides. Based on a mean amino acid residue weight of 110, such an RNA molecule could specify a maximum protein mass of 280,000 daltons. The Mengo RNA molecular weight of 1.74 X 10^6 reported earlier by Scraba et al. (1967) could, on this basis, code for a maximum protein mass of only 185,000 daltons.

Experiments were undertaken therefore, to determine if the reported size difference between the Mengo RNA and the RNA of the other picornaviruses is in fact real. The results of these studies are reported in this chapter.

Materials and Methods

Preparation of viral ribonucleoprotein. The method outlined for the purification of Mengo virions was utilized in obtaining milligram quantities of poliovirus, Wistar Chat type I, from lysates of infected

HeLa cells. The HeLa cells were obtained from the American Type Culture Collection and maintained in tissue culture as described for L cells with the exception that fetal calf serum was used in place of horse serum. Bacteriophage R17 was a gift from Dr. W. Paranchych, Department of Biochemistry, University of Alberta, and TMV was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. RNA was extracted from these viruses, as well as from Mengo virus, by the phenol-macaloid-SDS procedure of Scraba et al. (1967).

Extraction of cellular ribonucleotes. L cell rRNA was isolated by a modification of the method of Scherrer and Darnell (1962). L cells growing in suspension culture were collected by centrifugation and re-suspended in "RNA buffer" (0.06 M NaCl, 0.01 M TRIS, 0.001 M EDTA, pH 7.4) at approximately 20 X their original concentration. The cell suspension was made 0.5% and 0.2% with respect to SDS and sodium deoxycholate, and shaken mechanically for 10 min with an equal volume of RNA buffer-saturated redistilled phenol. The upper aqueous layer and the material at the interface were recovered after centrifugation (1,500 g for 15 min), shaken again with a fresh aliquot of phenol, incubated at 55° for 5 min and then chilled in ice. After centrifugation, the aqueous layer was collected and residual phenol removed by extraction with ether. The RNA was precipitated by adding three volumes of 95% ethanol and stored at -20°.

When radioactively labelled 18 S rRNA was required, monolayers of L cells were grown to confluency in growth medium containing 1.5 µCi ^{3}H-uridine/ml (New England Nuclear). The 18 S rRNA was isolated by rate zonal centrifugation of the extracted cellular RNA using linear

gradients of 5 to 30% glycerol in "RNA buffer". Sedimentation was for 20 hr at 25,000 rpm in a Spinco SW 27 rotor (4°).

Radioactively labeled 23 S and 16 S bacterial rRNA were prepared by growing E. coli in complete broth medium containing 1 μCi ^{14}C-uracil/ml (Schwarz/Mann Biochemicals). After harvesting, the cells were converted to protoplasts by a 7-min incubation at 4° in "RNA buffer" made 0.45 M in sucrose and containing 80 μg lysozyme/ml. The protoplasts were then collected and the rRNA extracted as described above.

Gel electrophoresis of ribonucleates. Recrystallization of acrylamide and N, N'-methylenebisacrylamide (Eastman Organic Chemicals, Rochester, N.Y.) was done according to the method of Loening (1967). Gels (7.5 cm X 0.5 cm) having acrylamide concentrations of 2.2, 2.4, and 2.6% (with the bisacrylamide concentration always 5% that of the acrylamide) were made in electrophoresis buffer (RNA buffer containing 0.2% SDS). Polymerization was catalyzed by N,N,N',N'-tetramethylethylenediamine and ammonium persulfate, both at final concentrations of 0.08%. After a preelectrophoresis period of at least 30 min, samples containing 5 to 25 μg of RNA (in 10 to 20 μl of "RNA buffer" to which SDS and sucrose had been added to final concentrations of 0.2% and 10%, respectively) were layered onto the gels directly beneath the electrophoresis buffer. R17 RNA was used as an internal standard for all gels and was mixed with the other RNA samples immediately prior to layering. Electrophoresis was for 5 to 8 hr at 5 to 7 mA/gel, after which the gels were chilled in ice, removed from the tubes, and rinsed in distilled water. The positions of the RNA species were then determined by scanning the gels at a wavelength of 260 nm in a Gilford Model 240

recording spectrophotometer (Gilford Instrument Laboratories Incorp., Oberlin, Ohio). Gels to be photographed were stained overnight in 40% methyl Cellosolve containing 0.01% toluidine blue O (Fisher Scientific Co.) and destained by washing with 30% methyl Cellosolve.

Sedimentation of ribonucleates under denaturing conditions. Glycerol gradients containing deuterated DMSO (d_6-DMSO, 99.5 atom %, BioRad Laboratories, Richmond, Ca.) were prepared according to the method described by Sedat et al. (1969). Linear 5-ml gradients were established between d_6-DMSO (containing 15% glycerol and 0.001 M EDTA, pH 7.0) and a mixture of 0.1 volume fraction d_6-DMSO with 0.9 volume fraction of DMSO (containing 0.001 M EDTA, pH 7.0). Onto each of these was layered a 15-µl sample of radioactive RNA (in "RNA buffer" containing 0.2% SDS) to which 100 µl DMSO and 20 µl dimethyl formamide had been added. Centrifugation was at 45,000 rpm for 16 hr in a Spinco SW 50.1 rotor (24°). Fractions were collected onto filter paper disks which were then dried, washed with trichloroacetic acid, and assayed for radioactivity in a toluene fluor.

The sedimentation behavior of Mengo RNA in buffered formaldehyde was studied using the ultracentrifugal method described by Boedtker (1968). Mengo RNA, stored in ethanol (-20°), was collected and dissolved in a solution of 1.1 M formaldehyde (Matheson, Coleman, and Bell), 0.09 M Na_2HPO_4 and 0.01 M NaH_2PO_4. The RNA solution was heated at 63° for 15 min, cooled in ice water and used immediately. The sedimentation coefficient of the RNA was determined using a Spinco Model E ultracentrifuge equipped with an ultraviolet photoelectric scanning device.

The sedimentation behavior of Mengo RNA in formaldehyde-SDS-sucrose gradients (Simmons and Strauss, 1972) was also examined. A 15-µl sample of radioactive RNA (in a solution containing 1.0 M formaldehyde, 0.2% SDS, 0.001 M EDTA, and 0.1 M sodium phosphate, pH 7.1) was added to 150 µl of the same solution. After heating at 63° for 10 min, the sample was layered onto a 13-ml gradient of 15 to 30% sucrose (in the formaldehyde-SDS buffer). Centrifugation was at 34,000 rpm for 22 hr in an International SB 283 rotor (23°). Fractions were collected and assayed for radioactivity as described above.

Electron microscopy of Mengo virus replicative form (RF) RNA. A suspension of mouse L cells (10^7 cells/ml) was infected with M-Mengo virus at a multiplicity of 10 to 20 PFU/cell. After 30 min at room temperature, the infected culture was diluted 5-fold with warm medium and incubated at 37° for 5 hr. The cells were then collected by centrifugation, resuspended in a small volume of 0.01 M sodium acetate, pH 5.2, and disrupted by a combination of homogenization and freeze-thawing. RNA was isolated by extraction with SDS-phenol at room temperature and precipitated with ethanol. The Mengo RF RNA was isolated from the other cellular and viral RNA species by the method of Agol et al. (1972), which involves LiCl fractionation, DNase treatment, and sucrose gradient centrifugation. The putative Mengo RF RNA was found in a rather broad peak in the sucrose gradient with an average sedimentation coefficient of approximately 20 S.

Peak fractions were pooled and prepared for electron microscopy by the "aqueous technique" described by Davis et al. (1971). After staining with uranyl acetate in 90% ethanol and rotary shadowing with

95% Pt-5% carbon at an angle of 8°, the molecules were visualized in a Philips EM 300 electron microscope operated at 60 kV. Photographs were taken at nominal magnifications of 16,000 and 20,000 X, and actual magnifications were determined from photographs of a diffraction grating replica (Ernest F. Fullam, Inc., Schenectady, N.Y.). Lengths of RNA molecules were measured on positive prints with a map measure.

RESULTS

Polyacrylamide gel electrophoresis. Since the electrophoretic mobility of an RNA molecule in an acrylamide gel has been found to be directly proportional to the logarithm of its molecular weight, the technique of polyacrylamide gel electrophoresis may be used to estimate the molecular weights of single-stranded RNA molecules (Bishop et al., 1967; Beacock and Dingman, 1968; Loening, 1969). Figure 1 illustrates the results of an experiment in which this method was used to estimate the molecular weight of M-Mengo virus RNA. The RNAs (with their molecular weights in brackets) that were used to calibrate the gels were as follows: TMV RNA (2.0×10^6; Boedtker, 1960), R17 RNA (1.1×10^6; Gesteland and Boedtker, 1964), and the 28 S and 18 S L cell rRNAs (1.71 and 0.70×10^6, respectively; Loening, 1968). A plot of log molecular weight versus distance migrated for these markers gave a straight line as shown in Figure 2; and from an extrapolation of this line, the molecular weight of the M-Mengo RNA was calculated to be 2.36×10^6.

Table 2 lists the molecular weights of the RNAs isolated from the three variants of Mengo virus, as determined from measurements of their electrophoretic mobilities in 2.2, 2.4, and 2.6% polyacrylamide

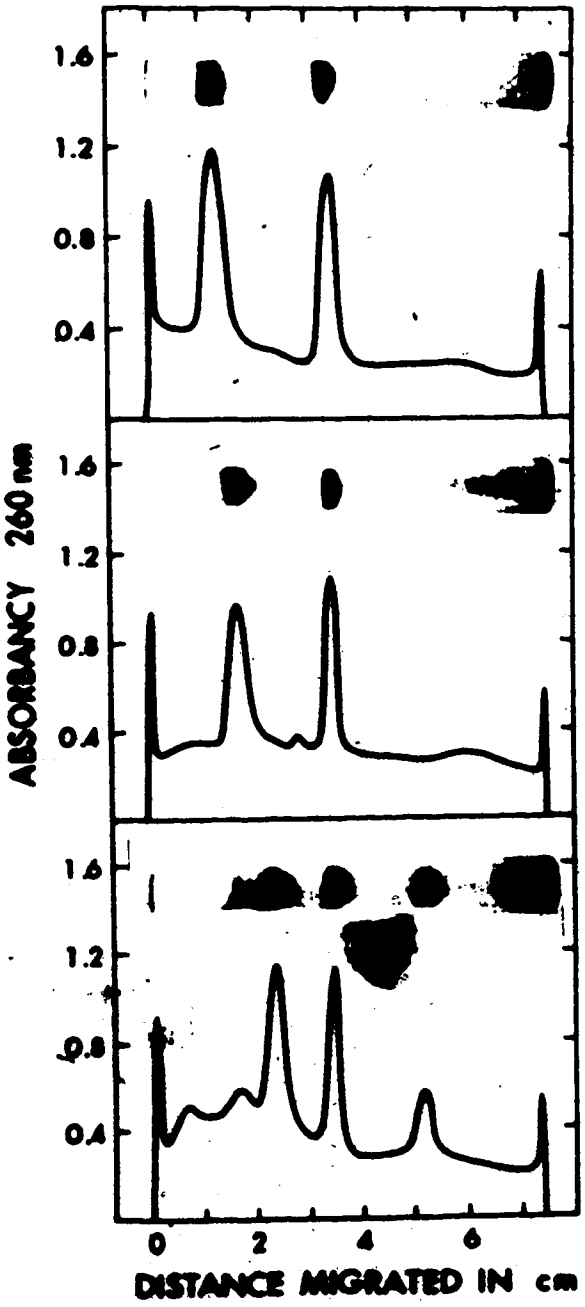


Figure 1. Polyacrylamide gel electrophoresis of M-Mango RNA (upper panel), TMV RNA (middle), and L cell rRNA (bottom); each mixed with R17 RNA prior to being layered onto the gel. Electrophoresis was in 2.4% acrylamide gels for 6 hr at 6 mA/gel. Superimposed above the 260 nm scan of each gel is a photograph of the same gel after staining of the RNA with toluidine blue O.

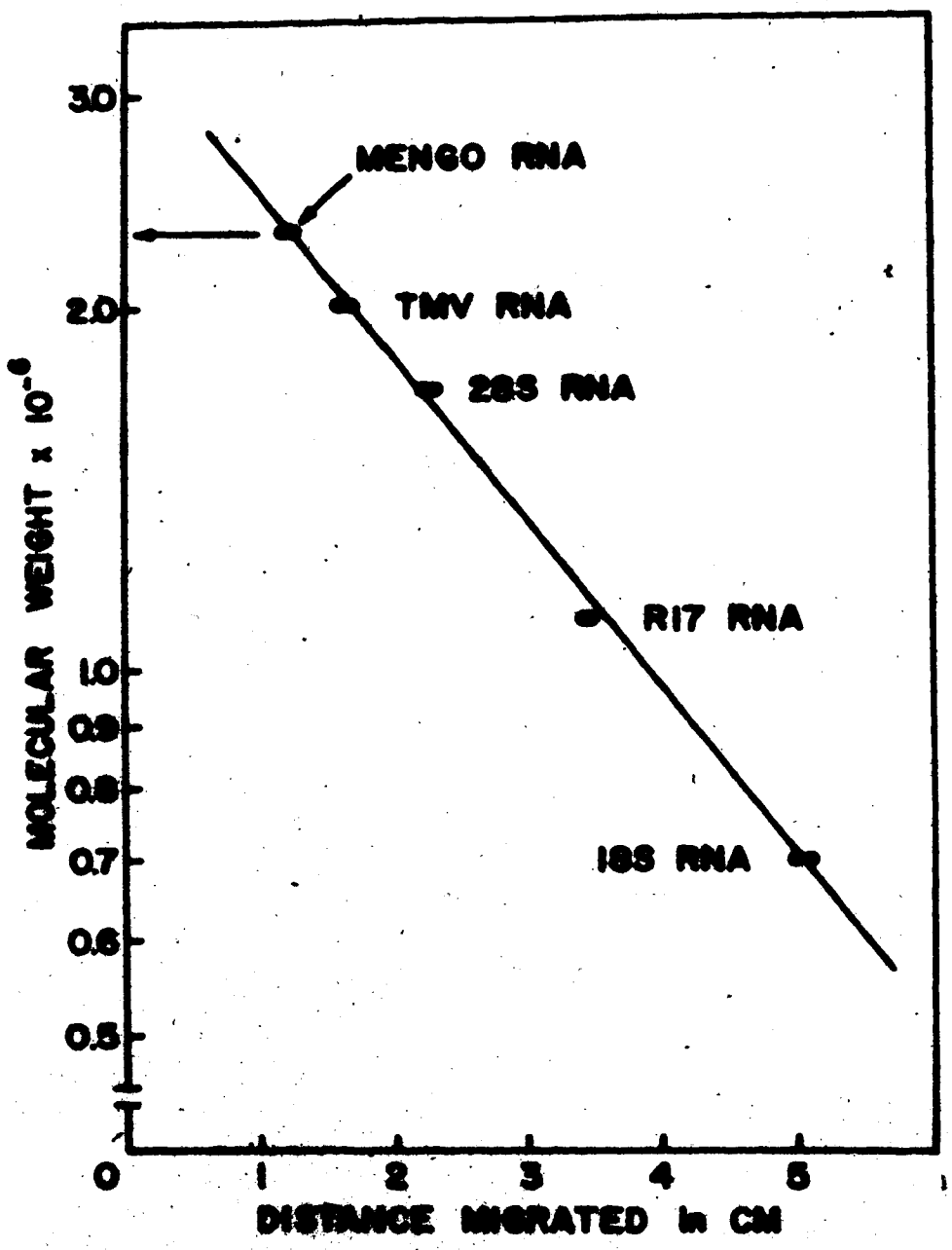


Figure 2. Estimation of the molecular weight of M-Mengo RNA. Data from Figure 1 and a duplicate set of gels electrophoresed concurrently were used to construct the graph. The molecular weights assigned the marker RNAs are given in the text. From extrapolation of the line, the M-Mengo RNA migration distance corresponded to a molecular weight of 2.36×10^6.

Gel acrylamide concentration (%)	Viral RNA			
	S-Mengo	M-Mengo	L-Mengo	Polio (W. Chat I)
2.2	2.44 ± 0.03 (6)	2.46 ± 0.03 (6)	2.47 ± 0.02 (4)	2.37 ± 0.03 (4)
2.4	2.32 ± 0.08 (9)	2.32 ± 0.09 (9)	2.36 ± 0.09 (6)	2.27 ± 0.06 (8)
2.6	2.37 ± 0.13 (9)	2.36 ± 0.10 (9)	2.31 ± 0.05 (6)	2.32 ± 0.09 (4)
Average	2.38 ± 0.06	2.38 ± 0.07	2.38 ± 0.08	2.32 ± 0.05

Table 2. Molecular weights of Mengo and poliovirus ribonucleates as determined by polyacrylamide gel electrophoresis. The values shown are molecular weights X 10^{-6}. Two separate RNA preparations were used for the L-Mengo variant and the poliovirus. Three preparations were used for the S- and M-Mengo variants. The numbers in parentheses indicate the number of molecular weight determinations done at that acrylamide concentration.

gels. The values for the S-, M-, and L-Mengo RNAs were found to be essentially identical at each gel concentration and the average molecular weight of all three - based on all estimations at all gel concentrations - was calculated to be $2.38 \pm 0.06 \times 10^6$. Also shown in Table 2 are the comparable experimental values for the molecular weight of poliovirus RNA. The difference between the estimated molecular weight of poliovirus RNA (2.32×10^6) and that of Mengo virus RNA is not significant, a conclusion reinforced by the obeservation that polio and Mengo RNAs could not be resolved when co-electrophoresed.

Sedimentation under denaturing conditions. For these studies only the M-Mengo RNA was used since the acrylamide gel electrophoresis experiments had shown that the molecular weights of the RNA of the three Mengo variants are identical (Table 2).

It has been shown by Strauss et al. (1968) that DMSO is an effective denaturant of both single- and double-stranded RNA and that in DMSO the sedimentation coefficients of single-stranded RNA molecules are proportional to the 0.31 power of their molecular weights. With the DMSO-glycerol gradients employed here, Mengo RNA was co-sedimented with marker RNAs in order to establish the relationship between sedimentation rate and molecular weight. Data obtained from one such rate-zonal centrifugation is illustrated in Figure 3. The RNA markers used were the 18 S L cell rRNA (0.70×10^6; Leaming, 1968) and the 23 S and 16 S E. coli rRNA (1.07 and 0.56×10^6, respectively; Stanley and Bock, 1965). When the logarithms of the molecular weights of the three marker RNAs were plotted against the logarithms of the distances they sedimented in the DMSO-glycerol gradient, a straight line was obtained

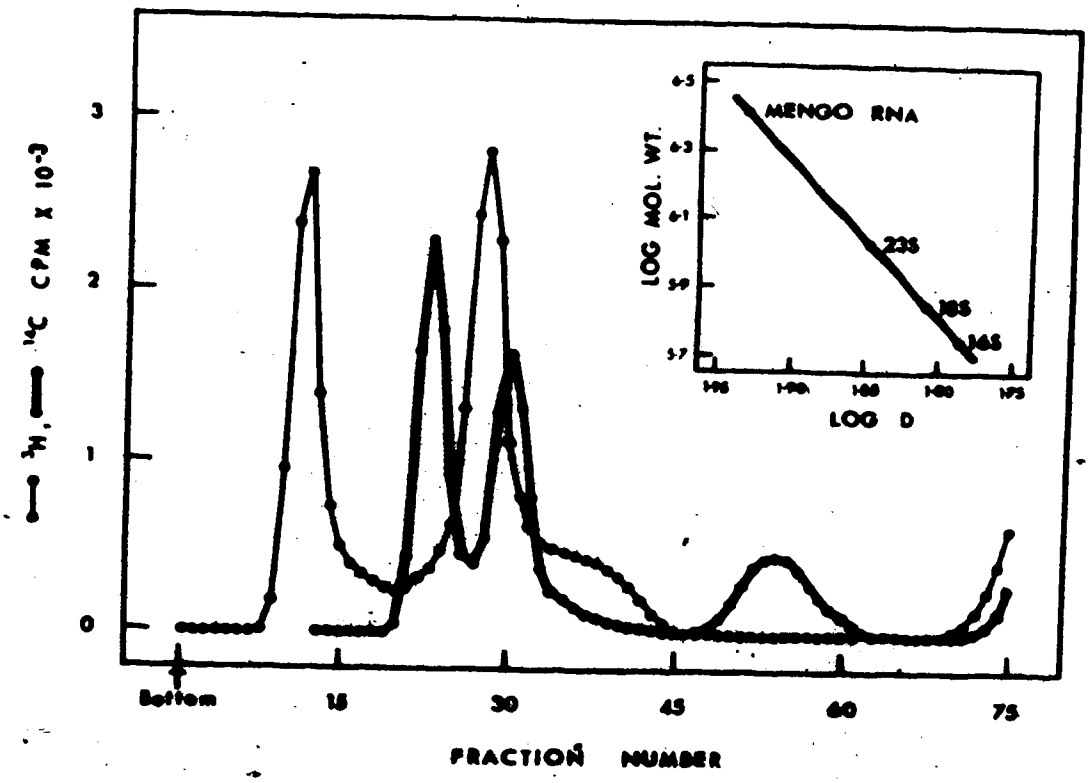


Figure 3. Coedimentation of M-Mango RNA and marker RNAs in deuterated DMSO gradients. Five microliters of ^{3}H-labeled M-Mango RNA (in "RNA buffer" containing 0.2% SDS), 5 µl ^{3}H-labeled 18 S L cell rRNA, and 5 µl ^{14}C-labeled E. coli rRNA were mixed with 100 µl of DMSO and 20 µl of dimethyl formamide prior to layering on the gradient. Sedimentation was for 16 hr at 45,000 rpm. Inset: Plot of the logarithm of the molecular weight versus logarithm of the distance migrated (log D).

(see insert, Figure 3). This straight line was extrapolated, and from it, the distance that the M-Mengo RNA had sedimented was found to correspond to a molecular weight of $2.57 \pm 0.02 \times 10^6$ (average of six determinations).

Boedtker (1968) found that the molecular weights of single-stranded RNAs could be obtained from measurements of their sedimentation coefficients in the presence of formaldehyde. The sedimentation rate ($S_{20,w}$) was found to be related to the molecular weight (M) by the equation: $S_{20,w} = 0.05(M)^{0.4}$. Upon inserting the observed M-Mengo RNA sedimentation coefficient into the equation, a molecular weight value of $2.38 \pm 0.03 \times 10^6$ was calculated (average of three determinations).

The modification of the Boedtker method described by Simmons and Strauss (1972), i.e., measurements of sedimentation velocity in formaldehyde-SDS-sucrose gradients, was also utilized to obtain an estimate of the molecular weight of M-Mengo RNA. The same marker RNAs used with the DMSO-glycerol gradients were employed here as well. The distribution of radioactively labeled RNAs following centrifugation was similar to that illustrated in Figure 3. The molecular weight of the M-Mengo RNA, calculated by the same procedure used in the case of the DMSO-glycerol gradient analysis, was found to be $2.41 \pm 0.03 \times 10^6$ (average of nine determinations). This value is in good agreement with both the value calculated from measurements of sedimentation rates in buffered formaldehyde and the value obtained from the polyacrylamide gel electrophoresis studies. In this regard, it is relevant to note that Kaper and Waterworth (1973) have reported that estimates of the molecular weights of single-stranded RNAs of several plant viruses, based on measurements of sedimentation rates in formaldehyde and on migration

rates in acrylamide gels, were in close agreement.

Electron microscopy. Figure 4 is an electron micrograph of double-stranded RNA molecules isolated from M-Mengo virus-infected L cells. All molecules seen in the electron microscope were linear (as reported for poliovirus RF RNA; Granboulan and Girard, 1969), with no circular (as reported for EMC virus RF RNA; Agol et al., 1972) or branched molecules being detected. It was observed that, in two separate preparations of Mengo RF RNA examined, there was a rather broad distribution of lengths from 1.50 to 3.00 μm. The mean length of the 264 molecules measured was 2.25 ± 0.3 μm. Using a value of 3.17 Å (Granboulan and Franklin, 1966) for the internucleotide distance in single- or double-stranded RNA molecules prepared for electron microscope examinations, and the average nucleotide residue weight (Na^+ form) in Mengo RNA of 343 (Scraba et al., 1969), it can be calculated that a single-stranded RNA molecule, 2.25 μm in length, would have a molecular weight of 2.44×10^6. Although this value agrees well with those obtained by other methods, the observed variation in the lengths of the RF molecules leads us to conclude that the estimate of the Mengo RNA molecular weight by this method is rather imprecise (e.g., the calculated standard deviation for the M-Mengo RNA molecular weight is 0.3×10^6).

Discussion

Estimates of the molecular weight of Mengo virus RNA have been made from polyacrylamide gel electrophoresis analysis, from three different ultracentrifugal methods and from measurements of the length of

Figure 4. Electron micrograph showing molecules of double-stranded, replicative-form Mengo DNA. Stained with uranyl acetate and rotary shadowed with Pt-C.

Mengo RF RNA in electron micrographs. The values obtained are in relatively close agreement, with the average value for the molecular weight being $2.44 \pm 0.08 \times 10^6$. The particle weight of the Mengo virion has been estimated to be 8.32×10^6 (Scraba et al., 1967), which means that the viral genome constitutes approximately 29.5% of the total virion weight. Both values, i.e., the molecular weight of the RNA and the RNA content of the virion, are in good agreement with values reported previously for other picornaviruses (Rueckert, 1971). The molecular weight estimation differs markedly from the value of 1.74×10^6 reported earlier (Scraba et al., 1967). The most plausible explanation for this discrepancy is that in the earlier study in which the Mengo RNA molecular weight was calculated from sedimentation and diffusion coefficients by the Svedberg equation, estimates of the diffusion coefficient were too high due to some degradation of the RNA during the long (16 to 20 hr) ultracentrifuge runs required to measure this parameter.

The Mengo RNA genome of molecular weight 2.44×10^6 and having a mean nucleotide residue weight of 343 (Scraba et al., 1967) would contain approximately 7,100 nucleotide residues. Assuming a mean amino acid residue weight of 110, the RNA could code for a maximum protein mass of 260,000 daltons. In this regard, Paucha et al. (1974) have reported that the primary translation products of the Mengo virus genome in vivo have a total molecular weight of approximately 270,000.

IV. MENGO VIRUS STRUCTURAL POLYPEPTIDES

Introduction

In view of the physical and chemical similarity exhibited by Mengo, ME, EMC, and polio viruses (Rueckert, 1971), it is reasonable to expect that their structural components would be much alike. This is true for the genetic material of these viruses since a molecular weight of approximately 2.5×10^6 has been found for the single-stranded RNA genome (Na^+ form) of each virus (see Chapter III.). With respect to the protein components of the virus capsids, however, substantial differences have been reported between the polypeptide composition of Mengo virions (O'Callaghan et al., 1970) and that of either poliovirus (Summers et al., 1965) or ME virus (Rueckert et al., 1969). First, of three Mengo variants studied, only one was found to contain a polypeptide in the molecular weight range 6,000 to 10,000, while both polio and ME virions contain such a polypeptide. Second, whereas both polio and ME virions contain two polypeptides in the molecular weight range 29,000 to 35,000, only one was found in the case of Mengo virus. Finally, Mengo virus was reported to contain a polypeptide of molecular weight about 20,000, although no comparable species was found in either polio or ME virions. A re-examination of the Mengo virus capsid components was undertaken to determine if the polypeptides comprising the Mengo virion do indeed differ from those of the polio or ME virions; and more important, to establish both the molecular weights of the structural polypeptides and the number of molecules of each that are present in the Mengo capsid.

Materials and Methods

Purified ^{14}C-amino acid labeled Mengo virus was pelleted by centrifugation at 120,000 g for 60 min, resuspended in 0.01 M sodium phosphate, pH 7.2, and the suspension made 4 M, 1%, and 5% in urea, SDS, and β-mercaptoethanol, respectively. The solution was then heated in boiling water for 3.5 min to solubilize the viral polypeptides. Samples (25 to 75 µl), containing 20 to 40 µg of viral protein and suitable levels of radioactivity, were layered onto the gels directly beneath the electrophoresis buffer (0.1 M sodium phosphate, pH 7.2, containing 0.1% SDS). Gels consisted of 10% acrylamide, 0.2% N,N'-methylenebisacrylamide, 0.1% SDS, 0.5 M urea, and 0.1 M sodium phosphate, pH 7.2. Polymerization was catalyzed by N,N,N',N'-tetramethylethylenediamine and ammonium persulfate at final concentrations of 0.075 and 0.038%, respectively. Electrophoresis was for 8 to 10 hours (0.5 cm X 14 cm gels) at 5 to 7 mA/gel.

For molecular weight determinations of the viral polypeptides, proteins of known molecular weights were used as markers (Shapiro et al., 1967). Those employed in the present study were bovine serum albumin (BSA), ovalbumin, pepsin, and myoglobin (Schwarz/Mann Biochemicals), and α-chymotrypsinogen (Sigma Chemical Co.), and lysozyme (Worthington Biochemicals). Aliquots of the mixture of marker proteins were treated as described above and 25 to 50 µl samples, containing 7 to 10 µg of each marker, were layered on separate gels and subjected to electrophoresis at the same time as were the virus samples. In addition, lysozyme and ovalbumin were used as internal standards in each virus gel (i.e., were added to the virus samples prior to the 3.5 min incubation in boiling

water).

Following electrophoresis, the gels were stained with Coomassie blue according to the method of Weber and Osborn (1969). After destaining, the positions of the protein peaks were determined by scanning the gels at a wavelength of 540 nm in a Gilford Model 240 recording spectrophotometer. Radioactive gels were then sliced into 1-mm fractions using a template and the fractions incubated at 37° overnight with 0.3 ml of 95% NCS tissue solubilizer (Amersham/Searle Corp., Oakville, Ont.). After elution, each fraction was assayed for radioactivity by adding 5 ml of toluene fluor and counting in a Beckman Model LS-230 liquid scintillation counter.

Results

The polypeptide compositions of highly purified preparations of S-, M-, and L-Mengo were examined. Figure 5 illustrates the data obtained from the SDS-polyacrylamide gel electrophoresis of solubilized ^{14}C-amino acid labeled M-Mengo to which had been added the marker proteins ovalbumin and lysozyme. The upper panel shows the pattern obtained by scanning the stained (Coomassie blue) gel at a wavelength of 540 nm and the lower panel shows the distribution of radiolabel in the same gel. As can be seen, M-Mengo contains a total of six polypeptides. Four of these are major constituents and have been designated α, β, γ, and δ to conform with the nomenclature employed by Dunker and Rueckert (1969) to describe the major structural polypeptides of ME virus.

The molecular weights of the viral polypeptides were determined on the basis of their electrophoretic mobilities relative to those of

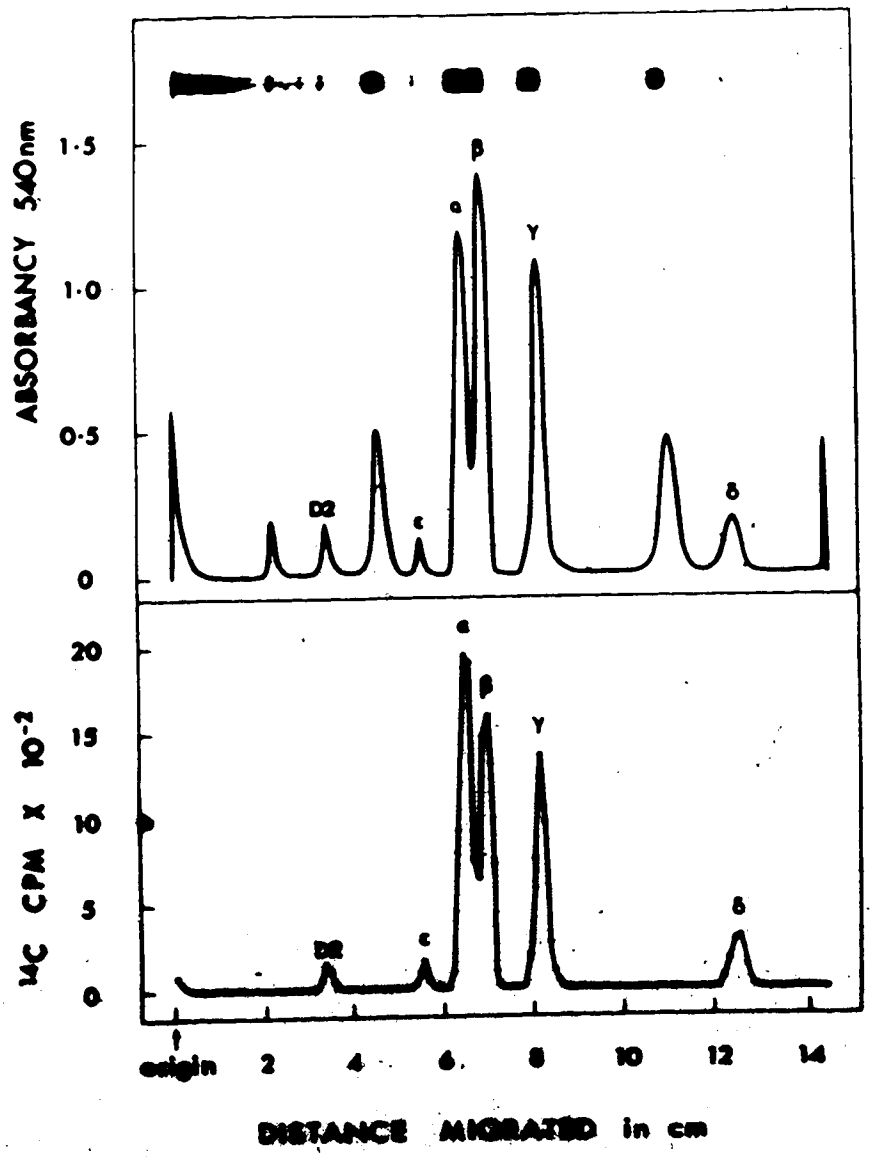


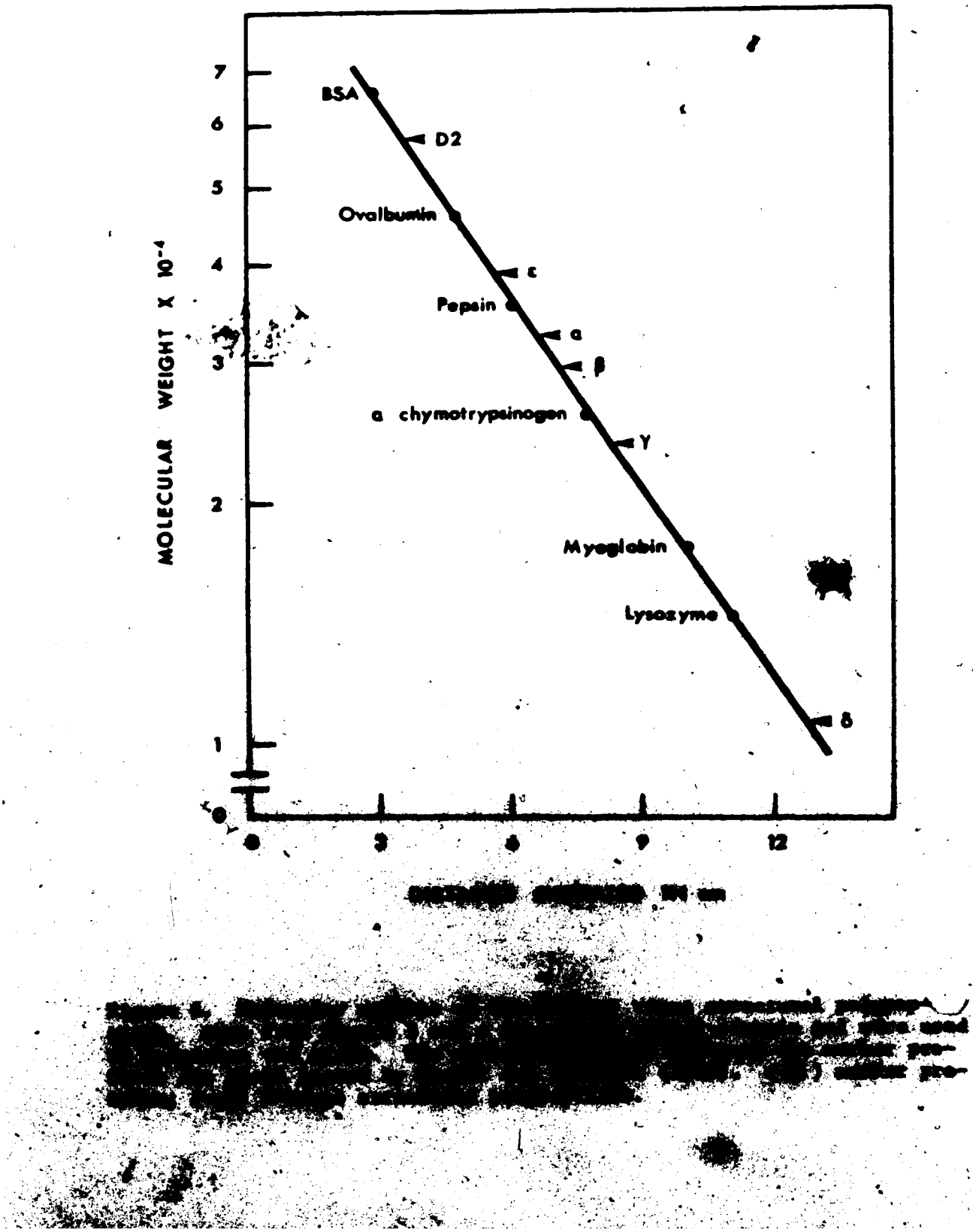
Figure 5. [illegible] gel electrophoresis of purified ^{14}C-amino acid labeled filange virus. Ovalbumin and lysozyme were added to the virus solution prior to solubilization. Electrophoresis was for 10 hr at 7 ma per gel, after which the gel was stained with Coomassie blue and scanned at a wavelength of 340 nm. In the upper panel, the viral polypeptide peaks are marked; the unmarked peaks, from left to right, are a minor component of the envelope preparation, ovalbumin, and lysozyme. [illegible] Superimposed above the graph is a photograph of the stained gel. The radioactive profile of the gel (lower) was obtained to verify the positions of the viral polypeptides. The gel was fractionated and radioactivity counted as described in Materials and Methods.

known molecular weights (Shapiro et al., 1967). The mobilities of the marker and viral proteins were measured both from gels in which they were coelectrophoresed (as in Figure 5), and from gels in which they were electrophoresed separately but concurrently. The standard curve from which the molecular weights of the viral polypeptides were estimated is shown in Figure 6. Also shown in this figure are the positions of the viral polypeptides relative to those of the marker proteins.

Data obtained from eight analyses of each of the three Mengo variants are summarized in Table 3. It is clear that the three variants are identical with respect to both the number and molecular weights of the polypeptides that they contain - the estimated molecular weights being 57,700 (D2), 39,000 (ε), 32,500 (α), 29,600 (β), 23,700 (γ), and 10,600 (δ). The amount of radioactivity found in each of the six polypeptide bands following electrophoresis of virus labeled with a mixture of ^{14}C-amino acids is expressed, in column 6, as a fraction of the total radioactivity recovered from the gel. From these values and the estimated molecular weights of the polypeptides, and assuming uniform incorporation of radioactivity into each polypeptide, it was calculated that an average Mengo virus particle contains approximately 60 molecules of each of the α, β, and γ chains, about 35 of the δ chain, and 1 or 2 of each of the D2 and ε chains.

Discussion

The [illegible] virus polypeptide composition differs markedly from that [illegible] by [illegible] et al. (1970), but the differences and the difficulty in reconciling the data obtained by these


MOLECULAR WEIGHT X 10^{-4}
7
6
5
4
3
2
1
BSA
D2
Ovalbumin
c
Pepsin
a
β
α chymotrypsinogen
γ
Myoglobin
Lysozyme
δ
3
6
9
12

[illegible]	Molecular weight			Fraction of total ^{14}C cpm	Number of chains/ virion
	M-Mengo	L-Mengo	Average		
[illegible]	57,700	57,800	57,700 ± 300	0.007	0.7
[illegible]	39,000	38,900	39,000 ± 300	0.013	2.0
[illegible]	32,500	32,400	32,500 ± 300	0.345	62
[illegible]	29,600	29,600	29,600 ± 400	0.311	62
[illegible]	23,700	23,700	23,700 ± 200	0.265	66
[illegible]	10,600	10,600	10,600 ± 400	0.059	33

[illegible] of the Mengo virion as analyzed by SDS-polyacrylamide gel electrophoresis. [illegible] of column 5 are the average of twenty-four determinations; eight for each variant, [illegible] to the nearest hundred. The values of column 6 were calculated using the [illegible] each polypeptide peak following electrophoresis of virus labeled with ^{14}C-[illegible] are an average of eighteen determinations; six for each variant. The [illegible] were calculated using the relation: $n = (WF)/M$, where n is the number of chains, [illegible] capsid proteins (5.88×10^6), F is the fraction of total ^{14}C cpm (column 6), [illegible] of the polypeptide in question. W was obtained by subtracting the Mengo RNA [illegible] from the virion particle weight (8.32×10^6; Scraba et al., 1967).

workers has been found to consist of 2 species, α and β, which have been resolved by using longer gels of higher acrylamide concentration. It seems clear, too, that the VPIII (molecular weight approximately 20,000) was, in fact, an aggregate of δ polypeptides which survived the less vigorous solubilising conditions used by these investigators. The data presented here, except for the presence of polypeptide D2, resemble closely those reported by Rueckert et al. (1969) regarding the structural polypeptides of ME virus.

The minor components found in all virus preparations studied, have been tentatively identified as viral polypeptides D2 and ε in accordance with the model for post-translational cleavage proposed by Butterworth and Rueckert (1972a) for EMC virus. The assignment was made primarily on the basis of the molecular weights of the two species. Butterworth and Rueckert (1972a) have suggested that polypeptide D2 is the precursor of the α and γ chains, and that polypeptide ε is the precursor of the δ and β chains. This hypothesis is compatible not only with the estimated molecular weights of the various Mengo capsid polypeptides (Table 3), but also with data obtained by Pancha et al. (1974) from comparable studies on the biosynthesis of Mengo-specific polypeptides in L cells.

[redacted]

Considering only the major structural polypeptides (α, β, γ, and δ), Mak et al. (1974) have proposed a model for the construction of the Mengo virus capsid on the basis of electron microscopy and physicochemical analyses of intact and degraded virions. They suggest that the asymmetric structure unit is composed of one chain each of the α, β, and γ polypeptides, and that five structure units associate to form a capsomere. There are 12 such capsomeres in the virus capsid, one located at each of the twelve vertices of an icosahedral surface. Thus, the virus capsid would contain a total of 60 copies each of the α, β, and γ polypeptides. Composition analysis of Mengo virus structural proteins reported above supports the model in this regard. Both from the standpoint of precursor-product relationships during post-translational cleavage of the virus-specific polypeptides (Butterworth et al., 1971; Pancha et al., 1974) and of icosahedral symmetry (Mak et al., 1974), one would expect that the Mengo virus capsid would also contain 60 copies of the δ polypeptide. Composition analysis, however, reveals the presence of only about half this number. Assuming equivalent labeling of all of the structural polypeptides, there are two possible explanations for this apparent discrepancy: (1) the δ polypeptides, once cleaved from their [illegible] bound to the other capsid proteins and [illegible]; or (2) the molecular [illegible] by polyacrylamide gel electro- [illegible], resulting in a [illegible].

that although Dunker and Rueckert (1969) reported an SDS-polyacrylamide gel electrophoresis molecular weight of 10,500 for the δ polypeptide of ME virus, the molecular weight of this protein was found to be only 7,300 by a gel filtration method (Stoltzfus and Rueckert, 1972).

From the data presented, it is concluded that the Mengo virion can be represented by the structural formula $(\alpha, \beta, \gamma, \delta)_{58-59} \cdot D2_{1-2} \cdot C_{1-2} \cdot RNA$. Summing the molecular weights for all of these components gives a value of 8.04×10^6 for the virion particle weight. This is in good agreement with the value of 8.32×10^6 obtained for the Mengo virus particle directly by sedimentation-diffusion experiments (Scraba et al., 1967).

V. PURIFICATION AND AMINO ACID COMPOSITIONS OF THE MAJOR CAPSID POLYPEPTIDES

Introduction

It is now generally agreed that the capsids of the mammalian cardioviruses Mengo, MM, and EMC each contain four major and one or two minor polypeptide components (Ziola and Scraba, 1974; Rueckert et al., 1969; Burness et al., 1974). There are approximately sixty copies of each of the major polypeptides and one or two copies of each of the minor polypeptides per virion (Scraba and Colter, 1974; McGregor et al., 1975). In order that the structure and assembly of these viruses be better understood, it is essential that their individual capsid polypeptides be purified and characterized.

One of the problems encountered during initial attempts to purify the major Mengo virus capsid polypeptides was their insolubility in common aqueous solvent systems. Preliminary experiments indicated that these proteins were soluble only in strongly denaturing solvents such as 8 M urea, 6 M guanidine hydrochloride, 0.1% SDS or 90% formic acid. Also, the [illegible] molecular weights of the three capsid polypeptides α, β, and γ ([illegible], [illegible], and [illegible], respectively; see Chapter IV.) [illegible] of [illegible] molecular sieve chromatographic [illegible]. [illegible] and the individual polypeptides [illegible] in the [illegible] be-[illegible]

major capsid components.

Materials and Methods

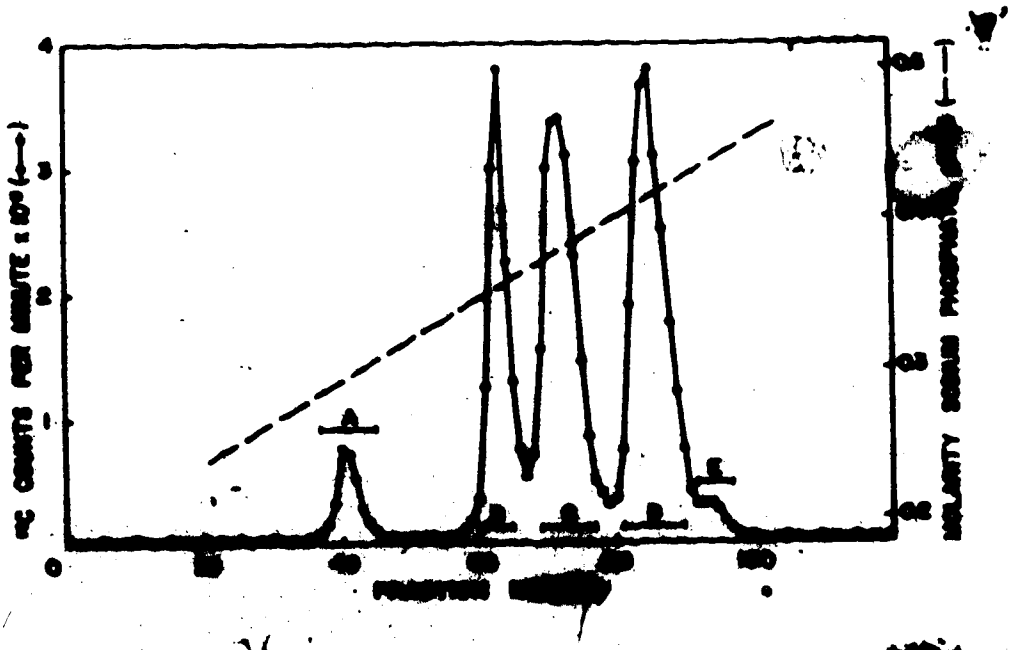
Mengo virus. The M plaque-type variant was used exclusively in these studies.

SDS-hydroxylapatite chromatography. Hydroxylapatite in powder form was obtained from BioRad Laboratories; technical grade SDS was purchased from Matheson, Coleman, and Bell; and DTT was from Sigma Chemical Co. All other chemicals used were reagent grade.

Initially, chromatography on SDS-hydroxylapatite was carried out according to Moss and Rosenblum (1972). Unlabeled virus (1 to 4 mg) was mixed with suitable amounts of virus labeled with ^{14}C-amino acids. The virus was pelleted at 120,000 g for 60 min and resuspended at a concentration of 2 to 4 mg/ml in 0.01 M sodium phosphate, pH 6.4, containing 1% SDS and 1% β-[illegible]. This solution was heated at 100° for 5 min to dissociate the virions, diluted 10-fold with 0.1 M sodium phosphate, pH 6.4, and [illegible] on SDS-hydroxylapatite column (0.95 cm^2 X 10 cm). After washing with 1 to 2 bed volumes of "column buffer" (0.01 M sodium phosphate, pH 6.8, containing 0.1% SDS and 1 mM DTT), the column was eluted at 4 ml/hr with a linear gradient of 0.2 to 0.5 M sodium phosphate, pH 6.4, containing 0.1% SDS and 1 mM DTT. [illegible] collected. Aliquots of each fraction were diluted in water, precipitated with trichloroacetic acid, collected on glass fiber filters, and dried in a vacuum oven. [illegible]

Following completion of the amino acid composition studies reported in this chapter, the SDS-hydroxylapatite chromatography procedure was modified in order to improve column reproducibility regarding the complete separation of the γ and α polypeptides (see Results). The altered procedure is described in detail since it represents a considerable modification of the method originally described by Moss and Rosenblum (1972).

Only small particle size (DNA grade) hydroxylapatite powder from BioRad Laboratories was used. The powder was suspended in "column buffer", fine material removed, and the slurry thoroughly degassed. SDS-hydroxylapatite columns (0.46 cm^2 X 18 cm) were then prepared as described by Moss and Rosenblum (1972). Up to 5 mg of virus (see above) was suspended at 4 mg/ml in 0.01 M sodium phosphate, pH 6.4. The suspension was made 2% with respect to both SDS and β-mercaptoethanol, and heated at 100° for 8 min. The polypeptide solution was then diluted 20-fold with 0.01 M sodium phosphate, pH 6.4, cooled to room temperature as quickly as possible by holding under cold running water, and run into an SDS-hydroxylapatite column. After washing with 1 to 2 bed volumes of "column buffer", the column was eluted at 3 to 4 ml/hr with a linear gradient consisting of 0.01 M sodium phosphate, pH 6.4, and 0.5 M sodium phosphate, pH 6.8, both solutions containing 0.2% SDS and 1 mM EDTA. Fractions containing the eluted [illegible] were pooled, concentrated by ultrafiltration and phosphate removed by [illegible] suitable for [illegible].



[illegible] chromatography of ^{14}C-[illegible] acid labeled [illegible]

After dialysis, the solutions were lyophilized and the resultant SDS-protein residues dissolved in 0.01 M sodium phosphate, pH 7.4, containing 5% β-mercaptoethanol and 4 M urea such that the final SDS concentration was 2%. After heating at 100° for 5 min, the polypeptide composition of each peak in Figure 7 was determined by electrophoresis in 10% SDS-polyacrylamide gels as described in Chapter IV.

Polypeptide recovery. The pooled fractions indicated in Figure 7 were mixed with 5 volumes of acetone containing 0.1 N hydrochloric acid. After 1 to 2 days at room temperature, the protein precipitates were recovered by low speed centrifugation, washed once with ethanol-ether (1:1) and once with ether. The dried precipitates were then dissolved in 90% formic acid, stored frozen at -70° and lyophilized as required. Recovery of protein was in excess of 80%.

Amino acid analysis. The proteins were hydrolyzed in redistilled, constant-boiling 6 N hydrochloric acid containing 0.1% phenol. Hydrolysis was for 24 and 72 hr at 110° in sealed evacuated tubes; after which the acid was removed [illegible] (Moore and Stein, 1963). The residues were dissolved in [illegible] and analyzed [illegible]

calculated from the composition of 22 hr hydrolysates assuming a loss of 10% during hydrolysis. Initially, an analysis of lysozyme was completed in order to establish the accuracy of the technique in our hands. The recovery of tryptophan from a 22 hr lysozyme hydrolysate was 90.0%, identical to the value reported by Liu and Chang (1971).

Results

From electrophoresis in 10% SDS-polyacrylamide gels, it is known that the Mengo virus capsid contains four major and two minor structural polypeptides (see Chapter IV.). These polypeptides were completely adsorbed when ^{14}C-amino acid labeled Mengo virus was solubilized and applied to an SDS-hydroxylapatite column. Upon elution with a 0.2 to 0.5 M phosphate gradient, pH 6.4, containing SDS and DTT, five peaks were resolved as indicated by the letters A to E in Figure 7. Dialysis of a portion of each peak against 0.01 M sodium phosphate, pH 6.4, containing 0.1% SDS and 0.1% β-mercaptoethanol, followed by rechromatography on new SDS-hydroxylapatite columns resulted in the same order of elution depicted in Figure 7.

The polypeptide compositions of the pooled peak fractions were determined by SDS-polyacrylamide gel electrophoresis. The upper portions of Figure 8 illustrate the migration positions of each of the Mengo virus structural polypeptides. It is apparent from the densitometer tracings shown in Figure 8 that the pooled regions A, B, C, D, and E contain [illegible] polypeptides, respectively. [illegible] the major structural polypeptide 4 as well as [illegible] polypeptide 4. Peak B contained all of

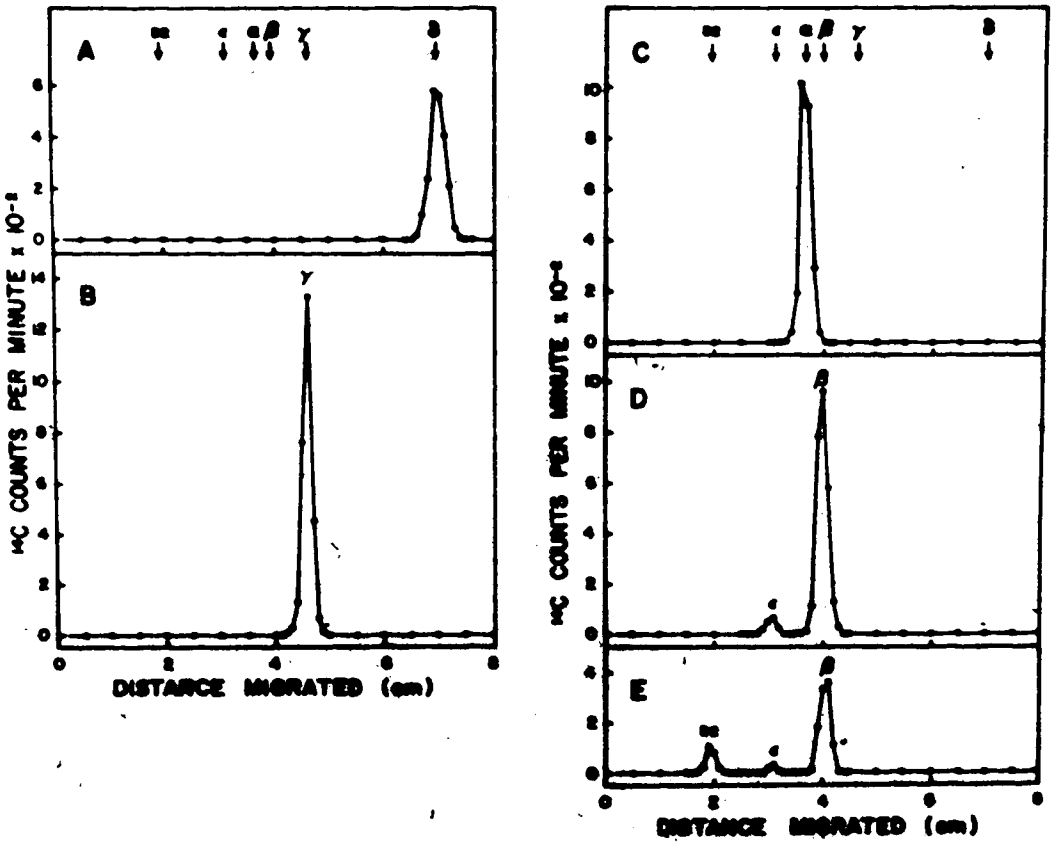


Figure 8. SDS-polyacrylamide gel electrophoresis of ^{14}C-amino acid labeled Mengo virus structural polypeptides following fractionation by SDS-hydroxylapatite chromatography. The letters A to E refer to the pooled fractions in Figure 7. Samples of each pooled region were prepared for electrophoresis as described in Materials and Methods. Electrophoresis was in 10% SDS-polyacrylamide gels for 10 hr at 5 mA/gel, after which the gels were sliced and counted as described in Chapter IV.

the minor polypeptide D2, a trace of ε, and some of the major polypeptide β (from the trailing edge of peak D). Consequently, in terms of increasing phosphate concentration, the order of elution is δ, γ, α, and β followed by the two minor capsid polypeptides ε and D2.

Although the δ and β [redacted]tides were consistently purified using the SDS-hydroxylapatite chromatography protocol of Hoss and Rosenblum (1972), some variability was observed in the column elution pattern of the γ and α polypeptides. The problem was found to be two-fold. First, using a given batch of hydroxylapatite, an extra peak occasionally appeared between the true γ and α peaks (i.e., peaks B and C, Figure 7). Not unexpectedly, this peak was found to contain varying amounts of the γ and α polypeptides. Second, it was noted that some variability exists in the commercially available hydroxylapatite itself. Although the original batch of hydroxylapatite powder (Bio-Gel HTP; BioRad Laboratories) gave excellent resolution of γ and α, a subsequent batch would not completely resolve these two proteins even though the overall order of polypeptide elution was the same as that of Figure 7. This altered elution behavior was attributed to variability in the hydroxylapatite since it was observed under identical chromatographic conditions and with the same virus preparation used initially. When an "aggregate" peak of γα was observed or when γ and α were incompletely resolved, the column fractions containing these two proteins were pooled, the protein was recovered by acidic acetone precipitation, and γ and α were purified to homogeneity using a combined SDS-polyacrylamide gel electrophoresis, SDS-hydroxylapatite chromatography method. This method is described in the Appendix of this thesis.

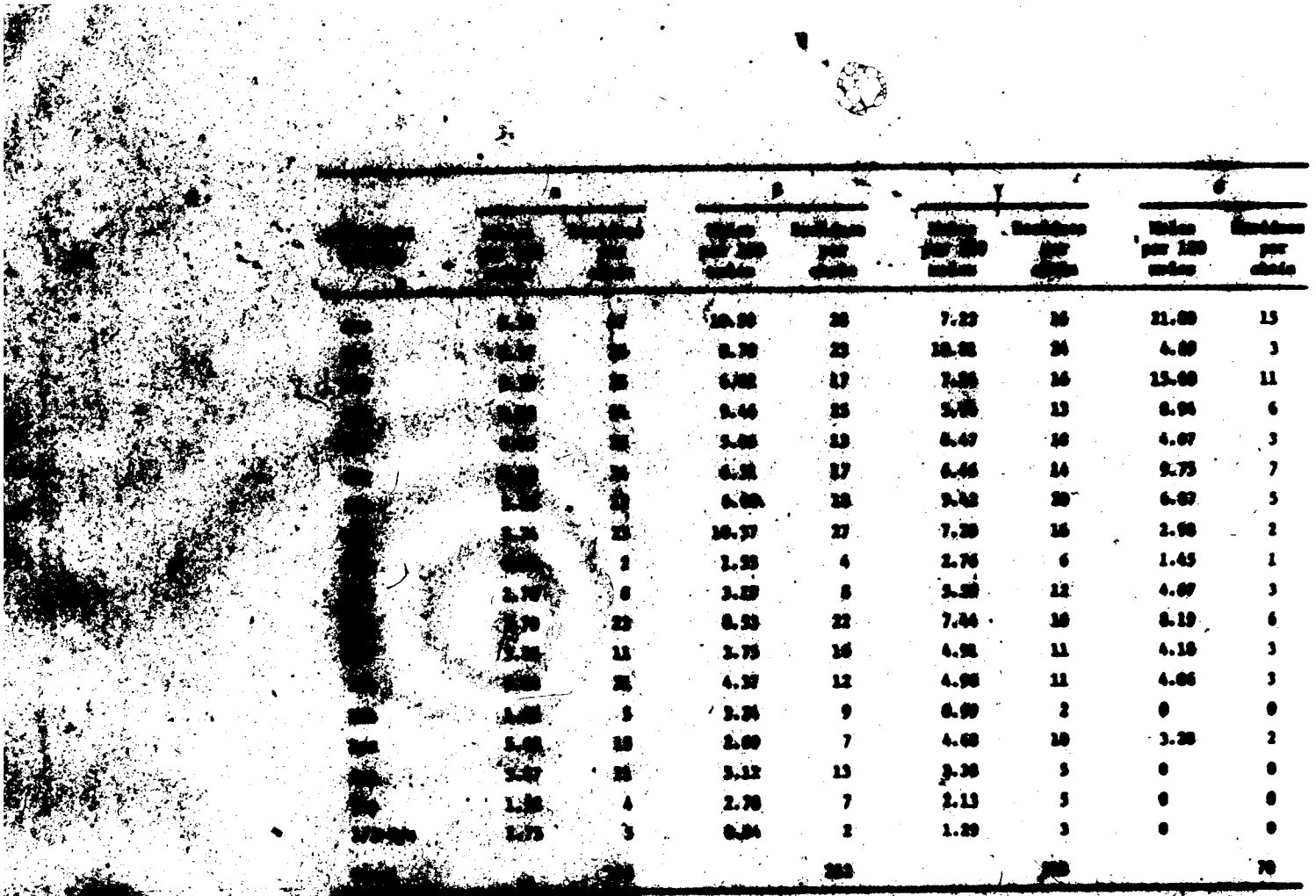
The [redacted] hydroxylapatite chromatography procedure

described in Materials and Methods was subsequently developed by varying the individual parameters of the original procedure of Moss and Rosenblum (1972). The γα "aggregation" problem was solved by employing more vigorous dissociation conditions followed by rapid cooling of the virus polypeptide solution to room temperature. Overcoming the γ and α "resolution" problem required modification of two parameters: first, the utilization of small particle size hydroxylapatite (coupled with thorough degassing of the hydroxylapatite slurry before pouring into columns); and second, the imposition of a decreasing pH gradient upon the increasing phosphate gradient. The latter modification was derived from the elution behavior of the Mengo virus structural polypeptides when chromatographed on SDS-hydroxylapatite columns at different pH's. At pH 8.5, no virus protein eluted with a 0.2 to 0.5 M phosphate gradient. At pH 7.4, the virus polypeptides began to elute only at a phosphate molarity of approximately 0.45. At pH 6.4 (see Figure 7), all the virus polypeptides eluted between 0.25 and 0.45 M phosphate. At this pH, the eluted γ peak was sharp while the α and β peaks were successively less so. At pH 6.0, the virus proteins eluted over the same phosphate molarity range and in the same order as at pH 6.4. Here, however, the γ peak was diffuse while the α and β peaks were extremely sharp. Based on the information obtained from columns run at pH 6.4 and 6.0, a pH gradient of 6.4 to 6.0 was superimposed on a 0.2 to 0.5 M phosphate gradient. Using this modified SDS-hydroxylapatite chromatography procedure, in conjunction with the [illegible] described above, has consistently resulted in [illegible] between γ and α peak resolution at least as good as that shown in Figure 7.

Amino acid composition analysis of the Mengo virus structural polypeptides. The amino acid compositions of each of the four major structural polypeptides is summarized in Table 4. The value for each amino acid residue represents the average of two determinations; these being obtained using polypeptides purified from separate preparations of virus. The small amount of ε protein contaminating the β protein was disregarded in these determinations since the majority of the ε molecule is composed of amino acid residues analogous to those of the β molecule (ε is the precursor of the β and δ polypeptides; Pauche et al., 1974).

The amino acid composition of each protein was found to differ significantly from each of the others. For example, the amino acid analyses of Table 4 indicates that the relative ratio of cysteine in the four Mengo structural proteins is 5:2:3:0. This ratio was confirmed by the relative radioactive content of the separated proteins (5.0:1.9:3.0:0) obtained from SDS-polyacrylamide gel electrophoresis of ^{3}H-cystine labeled Mengo virus. The δ protein is the most unusual of the four Mengo capsid proteins with respect to amino acid composition. This particular protein was found to contain no histidine, arginine, tryptophan or cysteine, while being exceptionally rich in serine and aspartate + asparagine residues.

Table 5 presents the amino acid composition of the total Mengo virus capsid protein. This was accomplished in two ways: first, by [illegible] the amino acid compositions of the capsid proteins (Dunker et al., [illegible]). For example, [illegible] the amino acid [illegible] polypeptides. A comparison [illegible] with the complete analysis [illegible] respectively, the other amino acid



	α		β		γ		δ	
			Moles per 100 moles	Residues per chain	Moles per 100 moles	Residues per chain	Moles per 100 moles	Residues per chain
	6.38	47	10.50	28	7.22	16	21.00	15
	4.34	32	3.39	23	10.81	24	4.69	3
	8.07	59	6.02	17	7.65	16	15.00	11
	4.38	32	5.46	15	5.85	13	8.34	6
	4.38	32	5.08	13	6.47	14	4.17	3
	6.38	47	6.21	17	6.46	14	9.75	7
	3.83	28	6.80	18	9.42	20	6.67	5
	3.26	24	10.37	27	7.20	16	2.98	2
	0.55	4	1.51	4	2.76	6	1.45	1
	2.707	8	3.17	8	5.58	12	4.17	3
	3.70	27	8.53	22	7.44	16	8.19	6
	1.36	10	3.75	10	4.91	11	4.18	3
	1.63	12	4.37	12	4.98	11	4.06	3
	1.23	9	3.34	9	0.99	2	0	0
	1.91	14	2.69	7	4.68	10	3.28	2
	1.37	10	3.12	13	2.36	5	0	0
	1.18	8	2.70	7	2.13	5	0	0
	0.75	5	0.84	2	1.29	3	0	0
				283		230		70

[illegible] virus capsid polypeptides. The abbreviations for the [illegible] Commission on Biochemical Nomenclature (1966). No attempt [illegible] residues; Asx, Glx, that were present in the amide [illegible] were obtained by extrapolation to zero hydrolysis-time. The [illegible] were calculated from the amino acid composition of each protein using the electro- [illegible] molecular weight of the α, β, and γ chains (see Chapter IV.) and the methionine- [illegible] molecular weight of the δ chain (see Results).

Amino acid residue	Total residues (α + β + γ + δ)	Moles per 100 moles amino acid	
		After [illegible] oxidation	Before [illegible] oxidation
Asx	86	10.2	10.4
Thr	75	8.9	9.5
Ser	69	8.1	7.2
Glx	68	8.1	8.5
Pro	59	7.0	8.0
Gly	62	7.3	7.2
Ala	60	7.1	6.9
Val	68	8.1	8.4
Met	13	1.5	1.0
Ile	31	3.7	3.4
Leu	67	7.9	7.5
Tyr	35	4.1	3.9
Phe	[illegible]	5.6	5.3
His	[illegible]	1.9	2.2
Lys	[illegible]	4.0	4.2
Arg	[illegible]	3.4	3.7
Trp	16	1.9	1.6
1/2-Cys	[illegible]	2.1	1.7
[illegible]	[illegible]		

[illegible]

composition of the unfractionated Mengo virus capsid protein confirms the amino acid compositions of the individual capsid polypeptides.

Methionine is the only amino acid of those present in low frequency that is common to all four polypeptides. The minimum molecular weights calculated from the content of this amino acid in each polypeptide are shown in Table 6. The molecular weight of each polypeptide as determined from SDS-polyacrylamide gel electrophoresis studies is also shown. If the gel electrophoresis molecular weights and the methionine minimum molecular weights of the capsid proteins are accurate, the calculated number of methionine residues per chain should be integral. This is true for α, β, and γ but not for δ. Since the SDS-polyacrylamide gel electrophoresis determined molecular weight for δ (10,600) is near the critical inflection point in 10% SDS-polyacrylamide gels (Bamber and Butchart, 1969), the value of 1.44 strongly suggests that the gel-estimated molecular weight is too high. Consequently, the methionine minimum molecular weight of 7,356 has been taken as the true molecular weight of the δ polypeptide.

[redacted]

	α	β	γ	[redacted]
[redacted]	32,500	29,600	23,700	10,600
[redacted]	15,760	7,370	3,960	7,350
[redacted]	2.06	4.02	5.98	1.44
[redacted]	31,500	29,500	23,800	7,350

[redacted] molecular weights of the three native capsid polypeptides. The SDS-polyacrylamide gel electro- [redacted] Fig. 17. The molecular weights based on amino acid sequence were [redacted] number of methionine residues per polypeptide. In this regard, it [redacted] of the polypeptides and number of methionines.

limited in capacity to microgram quantities of each capsid component. In contrast, SDS-hydroxylapatite chromatography permits rapid purification of milligram amounts of the individual Mengo virus capsid polypeptides. Burness et al. (1974) have reported using calcium phosphate (brushite) chromatography in the presence of SDS to separate the structural polypeptides of EMC virus. The polypeptide elution profile obtained (see Figure 4, panel 4; Burness et al., 1974), however, does not exhibit the peak sharpness (i.e., resolution) of the profile for SDS-hydroxylapatite chromatography of the Mengo polypeptides (Figure 7). This suggests that the hydroxylation of calcium phosphate (to form hydroxylapatite) produces a more efficient chromatographic system.

Moss and Rosenblum (1972) reported that although proteins were most often eluted from SDS-hydroxylapatite in order of increasing molecular weight, this was not always so. Consequently, polypeptide elution from SDS-hydroxylapatite need not necessarily be in the same order as the separation obtained by polyacrylamide gel electrophoresis. The polypeptide elution profile [illegible] upon SDS-hydroxylapatite chromatography of the Mengo virus structural proteins (Figure 7) conforms to these observations. The general order of elution was from the smallest to the largest [illegible], the α protein (MW 32,300) eluted at a [illegible] (MW [illegible]).

[illegible] amino acid [illegible] (Table 4), [illegible] do [illegible] the following [illegible] polypeptides: 1) the acid [illegible] residues (his + arg + [illegible] 1/2 cys); 3) there is

a high proline content; and 4) there is a high content of aliphatic residues (leu + ile + val). Since proline, valine, leucine, isoleucine, threonine, and serine are all known to be non α-helix formers in proteins (Fasman, 1963), the high content of these residues in all four Hmagg polypeptides suggests that there can be little α-helix content in the intact virus. This is borne out by optical rotatory dispersion and circular dichroism measurements (Harada et al., 1967; Ray et al., 1970) which indicate that less than 5% of the protein in intact virions is in an α-helical conformation.

The amino acid compositions of the major capsid proteins from the Hmagg and HK viruses are virtually identical (Sarabia et al., 1969; Hunsperger and Schäfer, 1969). However, comparison of the amino acid compositions of the individual HK capsid polypeptides (Stoltzfus and Hunsperger, 1972) with the compositions of the analogous Hmagg polypeptides (Table 4) does reveal significant differences. For example, in terms of residues per molecule, it was found that the Hmagg virus β polypeptide contains five additional [illegible] than HK virus β polypeptide (two [illegible] + [illegible] [illegible] of which are [illegible] glycine, threonine, and [illegible]). [illegible] of

representing 60 chains per virion. Given that α, β, γ and δ are synthesized in equimolar amounts *in vivo* (Pauling *et al.*, 1974) and considering the design principles of icosahedral capsid construction (Caspar and Klug, 1962), it follows that each Mengo virion should contain 50-60 copies of δ (depending upon the number of ε polypeptides in the capsid). If the methionine minimum molecular weight of 7,350 is accepted as the true molecular weight of the δ polypeptide, the calculation referred to previously gives a value of approximately 50 δ chains per virion. That true molecular weight of δ is less than that obtained from SDS-polyacrylamide gels has also been demonstrated recently by Stoltzfus and Rueckert (1972). They estimated the molecular weights of the δ polypeptides from both MK virus and Mengo virus to be 7,200 to 7,300 by gel filtration in the presence of guanidine hydrochloride.

In summary, a column chromatography procedure which permits the isolation of the major Mengo virus structural polypeptides in one step is described. The amino acid compositions of the Mengo capsid proteins have been determined and based on these data, a re-evaluated molecular weight of 7,250 has been obtained for the δ polypeptide.

VI. AMINO- AND CARBOXYL-TERMINAL SEQUENCES OF THE MAJOR CAPSID POLYPEPTIDES

Introduction

It has been established that the capsid polypeptides of picornaviruses arise by posttranslational cleavages of a large protein precursor (for a recent review on this subject, see Hurabbo and Fry, 1975). Since the Mengo virus capsid polypeptides can be readily purified (Ziola and Scraba, 1975; see Chapter V.), N- and C-terminal sequence analysis of the individual proteins was undertaken to determine if any peptide bond specificity is involved in the cleavage reactions which generate these proteins. It was anticipated that this information would lead to a better understanding of the mechanism(s) by which these *in vivo* proteolytic cleavages occur. The results of these studies are reported in this chapter.

Materials and Methods

Polypeptide isolation. The Mengo virus M plaque-type variant was used [illegible] these studies. The four major capsid polypeptides were isolated using [illegible] modified SDS-hydroxylapatite chromatography procedure described in Chapter V. [illegible] capsid protein was required, [illegible] which solubilized virus was first [illegible] containing 0.1% SDS and 1 ml [illegible] virus [illegible]. The [illegible] was then eluted by increasing [illegible] to 0.2 M.

N-terminal dansylation. Dansylation was carried out essentially to the procedure of Gray (1972). 200 to 800 µg of protein were dissolved in 0.1 ml of 1% SDS by briefly heating at 100°. 0.1 ml of N-ethylmorpholine and 0.15 ml of dansylchloride (25 mg/ml in anhydrous dimethylformamide) were then added. After 18 hr at room temperature, the dansylated protein was precipitated and washed with acetone, and dried under vacuum. After hydrolysis in 6 N hydrochloric acid (containing 0.1% phenol) at 110° for 18 hr, the hydrolysate was dried, dissolved in 0.2 ml of water, and extracted twice with 0.1 ml of ethylacetate. Each phase was dried separately, dissolved in 5 to 10 µl of 50% pyridine, and analyzed by thin layer chromatography on 5 cm X 5 cm Cheng Chin polyamide sheets (Pierce Chemical Co., Rockford, Ill.). The sheets were developed in the first dimension with 12% formic acid, in the second dimension with benzene : acetic acid (9 : 1), and again in the same direction with ethylacetate : methanol : acetic acid (20 : 1 : 1).

Automated N-terminal sequence analysis. Automated Edman degradations were performed with a Beckman Sequencer, Model 890B. All reagents were either "Sequenal" grade (Pierce Chemical Co.) or "distilled in glass" grade (Burdick and Jackson Laboratories Inc., Muskegon, Mich.). Samples of protein (25 to 40 nmoles) were dissolved in 0.4 ml of 90% formic acid and dried on the walls of the spinning reaction cup before starting the standard single coupling, double cleavage sequencing procedure of Edman and Begg (1967). Residue identifications were by analysis on a Beckman 120C amino acid analyzer following hydrolysis of portions of the phenylthiohydantoin derivatives with hydriodic acid (ultrapure - Alfa Inorganics Ltd., Ventron, Inc.), and with 0.2 M sodium hydroxide, 0.1 M

sodium dithionite (Smithies et al., 1971). When it was necessary to distinguish between glutamic acid and glutamine or aspartic acid and asparagine, the thiazolinone derivatives were converted to their isomeric phenylthiohydantoins by exposure to 1 N hydrochloric acid at 80° for 10 min (Edman and Begg, 1967). The latter were then identified by thin layer chromatography on pretreated silica gel plates (F254 - Merck and Co. Inc., Rathway, N.J.) using 1,2-dichloroethane : acetic acid (7 : 30) as a solvent system (Edman, 1970).

Carboxypeptidase analysis. DFP treated CBP A was obtained from Worthington Biochemicals and prepared as described by Ambler (1972). A 10 µl suspension (containing 830 µg of enzyme) was added to 1 ml of water with mixing. The enzyme crystals were collected by centrifugation at 2,000 g for 5 min, suspended in 100 µl 1% sodium bicarbonate, and cooled in an ice bath. Sodium hydroxide (0.1 N) was added in 25 µl-portions, with mixing, until the protein dissolved. The pH was then returned to approximately 8.5 with 0.1 N hydrochloric acid. This enzyme solution was diluted to an appropriate volume and used immediately. When CBP B (Worthington Biochemicals) was used, the enzyme was pre-incubated with 0.2 mM DFP for 30 min at room temperature in order to inhibit any residual chymotrypsin activity. In addition, 0.15 mM DFP was present in all reaction mixtures to which CBP B was added.

The purified Mengo virus capsid proteins were maleylated according to the method of [illegible] et al. (1969) in order to increase their solubility in aqueous solvents. The proteins were dissolved at approximately 2 mg/ml in 6 M guanidine hydrochloride, 0.1 M sodium pyrophosphate, pH 8.9. Two 50 µl-aliquots of maleic anhydride (99.5% zone refined -

Aldrich Chemical Co., Milwaukee, Wis.) in redistilled 1,4-dioxane were added at 0.5 min intervals with vigorous stirring. The final maleic anhydride concentration used was 100 times that of the reactive groups (lysines + free amino terminals) in the protein. After a further 60 min at room temperature, the samples were dialyzed against several changes of 0.5% ammonium bicarbonate, and subsequently lyophilized.

The maleylated proteins were dissolved in buffers of appropriate pH and incubated either at room temperature or 37° with CBP A, with CBP A plus CBP B, or with penicillocarboxypeptidase S1 (a gift from Dr. L. B. Smillie, Department of Biochemistry, The University of Alberta). Appropriate enzyme controls were done in each instance. The enzyme digestions were stopped by acidification to pH 1.5 to 2.0, the insoluble material was removed by centrifugation, and the amino acids in the supernatants were identified by chromatography on a Durram D500 amino acid analyzer. Serine, glutamine, and asparagine were found to have the same column elution times; consequently, when a "serine" peak appeared during analysis of the viral protein digests, the amounts of these three amino acids was determined following incubation of a separate sample with asparaginase or glutaminase or both, prior to chromatography. The enzymes were obtained from Worthington Biochemicals and digestions were carried out according to the procedures listed in the Worthington Enzyme Manual (1972).

Hydrazinolysis. Single determinations of the C-terminal amino acid residues of the Mengo virus γ and δ polypeptides were attempted by two procedures. Lysozyme was used as a control protein in each instance. Hydrazine sulfate, 97% anhydrous hydrazine, and benzaldehyde were obtained from [illegible]. The protein [illegible] (less than [illegible]) was [illegible].

Hydrazinolysis was first performed using the conditions of Bradbury (1956). 25 nmoles of each protein and 4 mg of hydrazine sulfate were dried for 6 hr at room temperature under vacuum conditions in glass test tubes which had drawn out constricted necks. Anhydrous hydrazine (0.2 ml) was then added to each tube and the mixtures were frozen in an ethanol - dry ice bath. The tubes were evacuated, sealed, and heated at 60° for 21 hr. Excess hydrazine was removed by lyophilization and the residues were dissolved in 0.4 ml of 1 N hydrochloric acid. After twice extracting with 0.2 ml of benzaldehyde, the aqueous layers were evaporated to dryness. The residues were then dissolved in 0.2 M sodium citrate, pH 2.2, and analyzed on a Beckman Model 121 amino acid analyzer.

The second hydrazinolysis procedure (Braun and Schroeder, 1967) differed only slightly. The resin IRC-50 (4 mg) was used in place of the hydrazine sulfate and incubation was at 80° (for 32 hr) rather than 60°. After excess hydrazine had been removed, the residue of IRC-50, amino acid hydrazides, and free amino acids was suspended in 0.3 ml of water. The resin was removed by centrifugation and washed with 0.3 ml of water. The pH of the combined supernatants was lowered to approximately 1.5 and the free amino acids were extracted and analyzed as described above.

Results

Amino-terminal analysis. Dansylation of unfractionated Mengo virus capsid polypeptides revealed the presence of three N-terminal amino acid residues. From [illegible] test mixture of dansylated amino [illegible] acid for

asparagine), and serine. Dansylchloride treatment of the individual, purified proteins revealed the N-terminal residues for α, β, and γ to be glycine, aspartic acid (or asparagine), and serine, respectively. No free N-terminal amino acid was indicated for the δ protein.

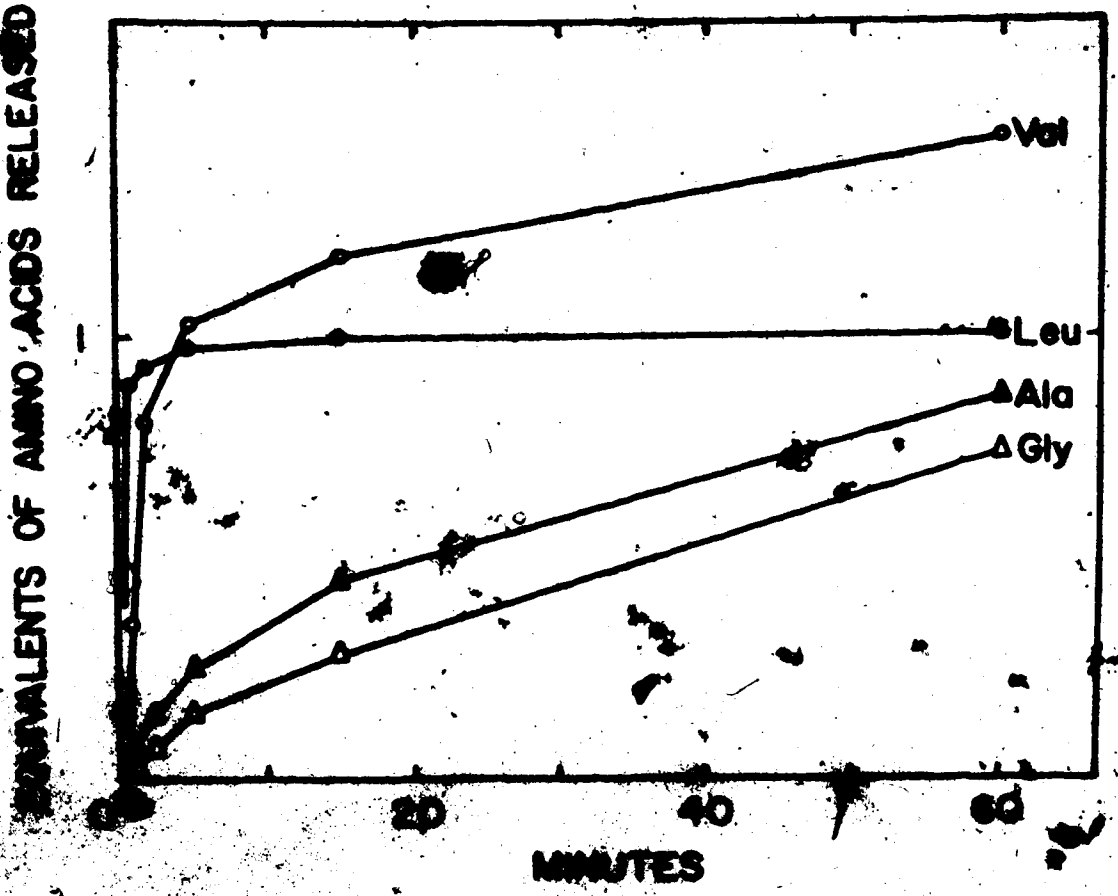
Application of the automatic Edman sequenator to each of the purified capsid polypeptides confirmed the dansylchloride results and, in the case of α, β, and γ, permitted sequence assignments to be made for ten amino acid residues beginning at the N-terminals (Table 7). The automated sequence analysis was arbitrarily stopped after the eleventh residue. It is anticipated, however, that as many as 20 residues could have been sequenced since recovery of the N-terminal residues were 50 to 70% and repetitive yields were greater than 90%.

For each protein, an additional automated Edman degradation was run on a second independent preparation. This was done to confirm the initial sequence assignments; and in addition, to determine which of the "acidic" residues are actually present in the amide form.

Carboxyl-terminal analysis. All carboxypeptidase digestions were carried out on 10 to 25 nmoles of derivatized viral protein. In preliminary experiments, each of the maleylated polypeptides was dissolved in 0.2 M N-ethylmorpholine, pH 8.5, and incubated at room temperature with CBP A at a substrate to enzyme ratio of 40. Under these conditions, leucine, valine, alanine, and glycine were released from the α protein. CBP A [illegible] of α was then [illegible] using a substrate to enzyme ratio of [illegible]. From the [illegible] of amino acids released it can be [illegible] to the [illegible] while the penultimate [illegible] substrate to enzyme

	Amino-Terminal Sequence	Carboxyl-Terminal Sequence
α	Blocked	- Leu - Leu - Ala
β	Asp - Gln - Asn - Thr - Glu - Glu - Met - Glu - Asn - Leu -	(Val,Leu) - Arg - Gln
γ	Ser - Pro - Ile - Pro - Val - Thr - Ile - Arg - Glu - His -	?
δ	Gly - Val - Glu - Asn - Ala - Glu - Lys - Gly - Val - Thr -	(Val,Gly,Ala) - Val - Leu

Table 2. Amino- and carboxyl-terminal amino acid sequences of the four major H-Hango virus capsid polypep-

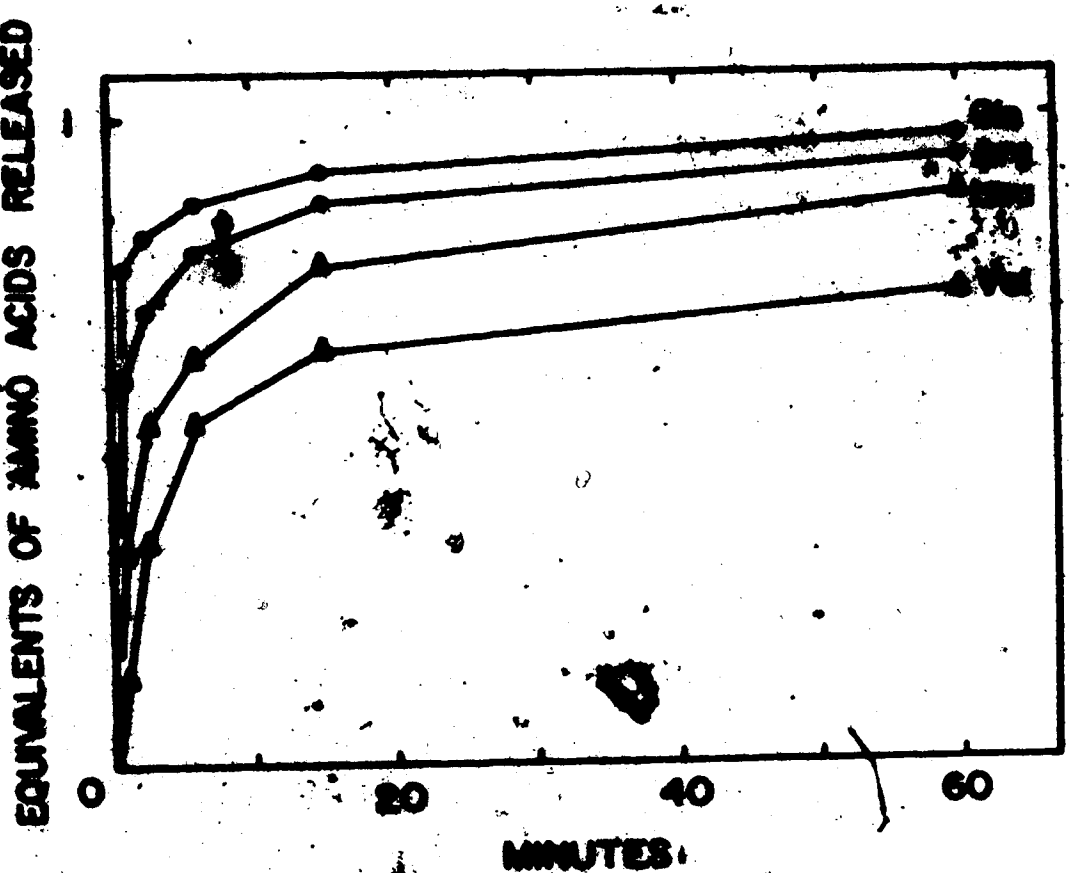

EQUIVALENTS OF AMINO ACIDS RELEASED
Val
Leu
Ala
Gly
20
40
60
MINUTES

ratio of 250, confirming that the C-terminal amino acid sequence of α is (Val,Gly,Ala) - Val - Leu.

Only glutamine was released during the preliminary CBP A digestion of the β protein. Upon addition of CBP B, arginine, leucine, and valine were also released. Digestion of maleylated β using a substrate to CBP A ratio of 100 and including 0.85 enzyme commission units CBP B per 100 moles of substrate resulted in the data depicted in Figure 10. Verification of these results was obtained by suspending maleylated β protein in 0.2 M sodium acetate, pH 5.1, and digesting the fine, silky precipitate at 37° with 6.8 X 10^{-3} OD_{2} [illegible] icillocarboxypeptidase S1. Combining results, the C-termina[illegible] sequence of the β protein has been found to be (Val,Leu) - [illegible].

Leuci[illegible]anine were released from the δ protein during the preliminary [illegible] at pH 8.5. Conducting the reaction at substrate to CBP A ratio [illegible] 100 or 250 gave the same pattern of release ratps for these two amino acids (Figure 11, upper panel). From these data, however, it can not be deduced whether alanine or leucine is the terminal amino acid. Addition of 4 M urea to the reaction mixture modified the CPB A digestion such that alanine clearly appears to be the primary enzyme hydrolysis product (Figure 11, lower panel). Based on this data, it was concluded that the C-terminal amino acid sequence of the δ protein is - Leu - Leu - Ala. That alanine is the terminal residue was subsequently confirmed by hydrazinolysis of the δ polypeptide (see below).

No amino acids were released during the preliminary CBP A digestion of the γ protein, nor were any fragments released during incubations [illegible] and gels revealed [illegible] a substrate to CBP A ratio [illegible] no [illegible] when the γ protein


EQUIVALENTS OF AMINO ACIDS RELEASED
0
20
40
60
MINUTES

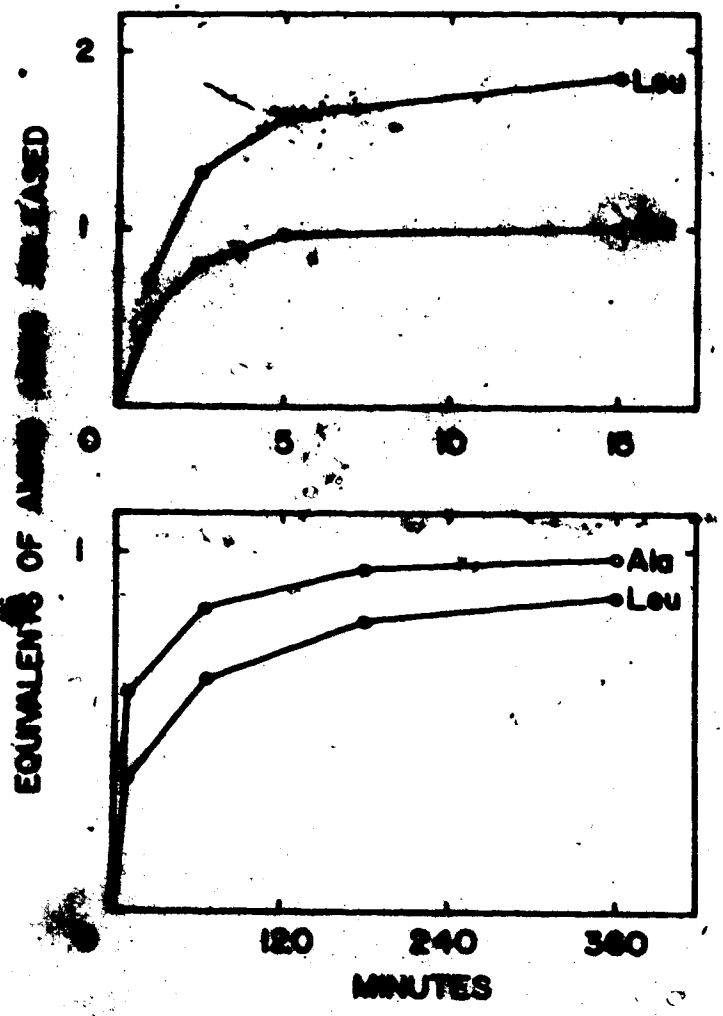


Figure [illegible] hydrolysis of malonylated δ protein. The substrate to [illegible] was 100. The reaction was carried out at [illegible]. Upper panel, incubation in the absence of 4 M urea; lower panel, incubation in the presence of 4 M urea.

was incubated with CBP B. Similarly, penicillocarboxypeptidase S1 (which does release proline; Jones and Hofmann, 1972) proved ineffective when incubated with the γ protein at 37° and pH 5.1 (compare to the results obtained in the case of the β protein). Carboxymethylation of the γ protein according to the method of Hirs (1967) instead of, and in addition to, maleylation did not make the protein more amenable to the action of any of the carboxypeptidases. In summary, the C-terminal peptide bond of the γ protein appears to be totally refractory to carboxypeptidase hydrolysis.

Since the C-terminal amino acid of the γ protein could not be determined by carboxypeptidase analysis, an attempt was made to determine the terminal residue by hydrazinolysis. Comparable quantities of the δ protein and of lysozyme were subjected to hydrazinolysis at the same time. With either of the two experimental approaches used, the C-terminal amino acids determined for the δ protein and for lysozyme were alanine and leucine, respectively (approximately 50% recovery). In the case of the γ protein, however, no free amino acid was found. This suggests that the γ C-terminal may be either asparagine or glutamine which form monohydrazides upon hydrazinolysis and which are consequently removed during the benzaldehyde extraction process. This could not, however, be confirmed.

A complete summary of the C-terminal analysis is included in Table 7.

Discussion

Utilizing a modified Edman degradation technique, Harnell and

Cooper (1973) detected three different N-terminal amino acids in the polypeptides comprising the poliovirion, these being glycine, aspartic acid, and serine. No attempt was made, however, to assign these N-terminals to individual polypeptide species. The same three amino acids have been found as free N-terminal residues in Mengo virus capsid polypeptides: glycine for the α protein N-terminal, aspartic acid for the β protein, and serine for the γ protein (Table 7). The Mengo virus δ polypeptide did not have a free N-terminal amino acid. By inference, it is tempting to suggest that the analogous VP4 polypeptide in the poliovirion would have a blocked N-terminus.

Studies of the N-terminal amino acid residues of isolated capsid polypeptides from FMDV have also been recently published. Three polypeptide species were isolated from disrupted FMDV (serotype O) by ion exchange chromatography in the presence of 8 M urea and their respective N-terminal amino acids identified by a dansylation procedure (Adam and Strohmaier, 1974). The N-terminal residues for the FMDV proteins I, II, and III were found to be glycine, aspartic acid and threonine, respectively. These amino acid assignments have been confirmed for the [illegible] serotype and have been extended to the capsid polypeptides of FMDV serotypes A and C as well (Matheka and Bachrach, 1975). Neither of these groups of authors isolated the smallest FMDV capsid polypeptide for N-terminal analysis. It should be noted here that the nomenclature used to describe the FMDV capsid polypeptides differs from that employed for the cardioviruses (Mengo, EMC, [illegible]) or for the enteroviruses (polio, Coxsackie, etc.). Based upon the SDS-polyacrylamide gel estimated molecular weights, the analogous polypeptides are as follows:

FMDV	cardioviruses	enteroviruses
III or VP_3	α	VP1
I or VP_1	β	VP2
II or VP_2	γ	VP3
VP_4	δ	VP4

Thus, while the N-terminal amino acids of unfractionated Mengo virus and FMDV capsid polypeptides are almost identical (there is only a Ser⟷Thr difference), the terminal residues of the corresponding polypeptides do not match: α = Gly; VP_3 (III) = Thr

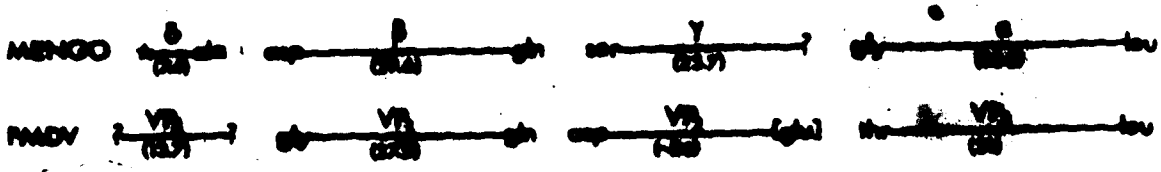
β = Asp; VP_1 (I) = Gly

γ = Ser; VP_2 (II) = Asp.

Bachrach et al. (1973) have reported the results of CBP A analyses of FMDV (serotype A) capsid polypeptides which had been carboxymethylated and maleylated prior to being separated by SDS-polyacrylamide gel electrophoresis. The C-terminal amino [redacted] FMDV VP_1 was found to be glutamine - which is also the C-terminal residue of the analogous Mengo virus β protein (Table 7). Similarly, leucine has been identified as the C-terminal amino acid of both FMDV VP_3 and the Mengo virus α protein. Glutamic acid was tentatively identified as the FMDV VP_2 C-terminal. In the case of the analogous Mengo virus γ protein, however, no C-terminal amino acid was determined using either carboxypeptidase analysis or hydrazinolysis.

A comparative sketch of the N- and C-terminal assignments of the FMDV and [redacted] virus capsid polypeptides follows (molecular weights are given in [redacted]; those for the FMDV capsid polypeptides are from

Vande Woude et al., 1972; and Bachrach et al., 1973).



The similarities of the C-terminal amino acids is not unexpected in view of the structural similarities between various picornaviruses (Rueckert, 1971); however, the non-correspondence of the three almost identical N-terminal residues is both surprising and puzzling.

A hypothetical scheme depicting the events involved in the post-translational processing of the Mengo virus capsid polypeptides is shown in Figure 12. The scheme is based upon the pattern elucidated by Pancha et al. (1974) for the sequence of cleavages involved in the biogenesis of the Mengo virus capsid polypeptides, and incorporates the N- and C-terminal amino acid sequence analyses of these proteins. Also included is information regarding the possible site of action of various cleavage inhibitors which have been studied in other picornavirus replication systems. The scheme will be discussed below under the headings of "nascent", "secondary", and "morphogenetic" cleavages, using these terms in the manner defined by Hershko and Fry (1975).

1.) Nascent cleavages. The entire picornavirus genome is translated in vivo as a single polypeptide chain ("polyprotein") which is cleaved internally as it is being synthesized (Jacobson et al., 1970; Butterworth and Beaudette, 1972a). Such nascent cleavages can be inhibited, or at least significantly retarded, by adding inhibitors of [illegible]

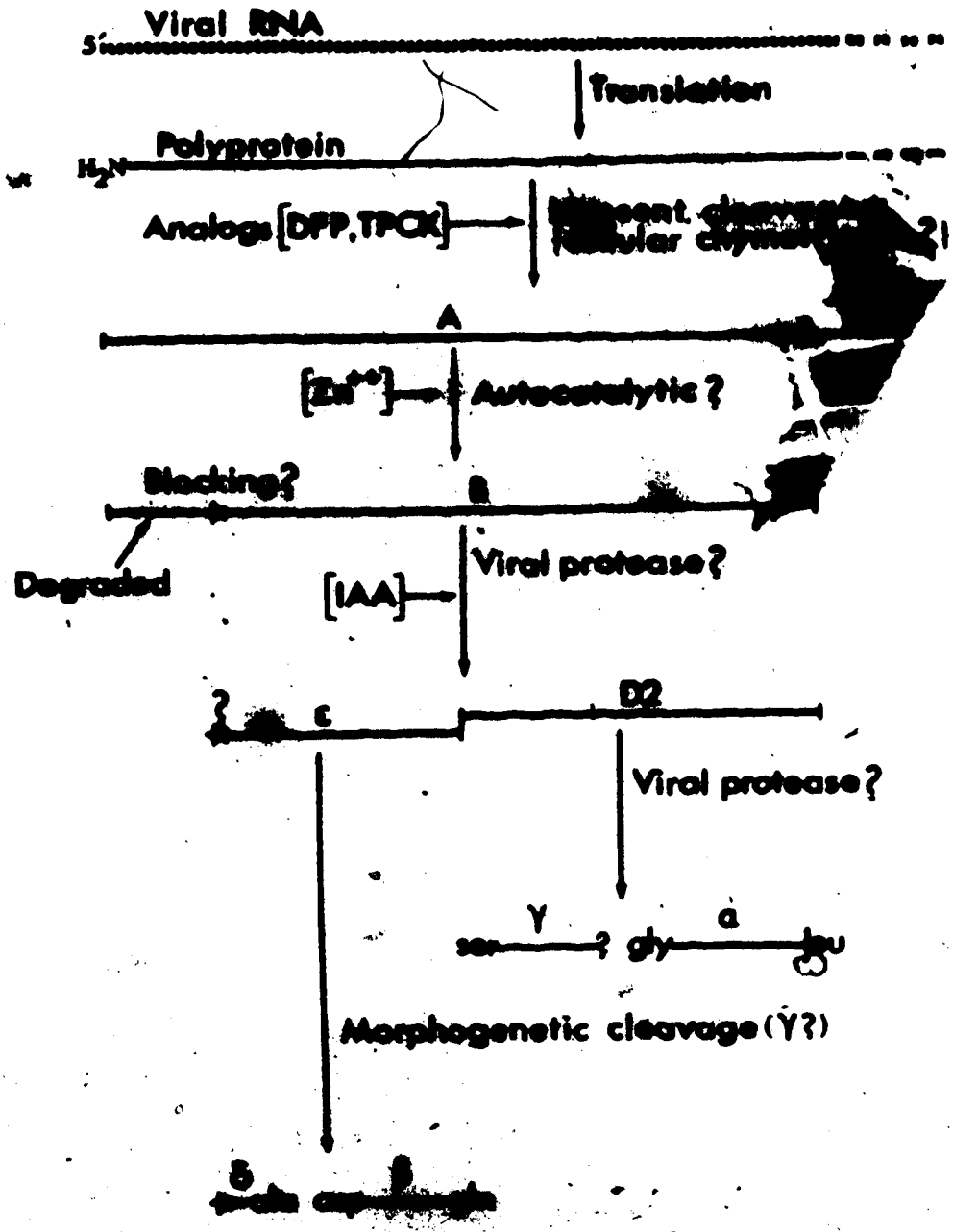


Figure 12. [illegible] involved in the post-translational [illegible] polyproteins.

reported that DFP, which inhibits several proteases including trypsin and α-chymotrypsin, prevents cleavage of the poliovirus "polyprotein" in infected HeLa cells. Summers et al. (1972) subsequently found that in poliovirus-infected HeLa cells, the most effective inhibitors of nascent cleavages were TPCK (tosylsulfonyl-phenylalanyl chloromethyl ketone) and ZPCK (carbobenzyloxy-phenylalanyl chloromethyl ketone). The first of these will inactivate both α-chymotrypsin and sulfhydryl proteases (such as papain), while the second is a specific inhibitor of α-chymotrypsin. Korant (1972), on the other hand, found that the trypsin inhibitor TLCK (tosylsulfonyl-lysyl chloromethyl ketone) was more effective in inhibiting nascent cleavages in poliovirus-infected HeLa cells; whereas TPCK was more effective in monkey kidney cells. Thus the evidence - albeit inconclusive - does favor a role for a chymotrypsin-like activity in the nascent cleavages of the viral "polyprotein". Furthermore, it is highly likely that these cleavages are catalyzed by a resident cellular protease, since Korant (1972) has shown that poliovirus "polyprotein" isolated from monkey kidney cells infected in the presence of TPCK could be converted to the products of in vivo nascent cleavages by in vitro incubation with extracts of uninfected cells (secondary cleavages took place only in the presence of extracts of infected cells - see below).

If it is assumed that the C-terminal portion of the Mengo virus capsid-precursor A (which is a nascent cleavage product) is conserved throughout further processing, then the A protein would have the same C-terminal amino acid residue as the capsid protein e -- namely leucine. In this regard it is relevant to note that the specificity of α-chymo- [illegible] hydrolysis of peptide bonds involving the carboxyl function

of leucine is almost equal to its specificity for peptide bonds involving the carboxyl function of aromatic amino acid residues (Hill, 1965).

Addition of the amino acid analogs p-fluorophenylalanine, canavanine, azetidine-2-carboxylic acid and ethionine (which are analogs of phenylalanine/tyrosine, arginine, proline, and methionine, respectively) to the incubation medium also inhibits or retards nascent cleavages of the viral "polyprotein" (Jacobson et al., 1970; Paucha et al., 1974). Instead of inhibiting the cleavage enzyme, these compounds probably act by modifying the conformation of the "polyprotein" such that it is no longer a suitable substrate for the host cell protease (Jacobson et al., 1970).

2.) Secondary cleavages (A → B). Lawrence and Thach (1975) examined this conversion in vitro by using extracts of EMC virus-infected cells and concluded that the original N-terminal section of A is removed (then rapidly degraded in vivo; Smith, 1973) by the proteolytic action of the capsid polypeptide γ. These authors postulated that the conversion A → B in vivo may be autocatalytic, since the γ sequence is an integral part of A. McGregor et al. (1975) have found $(A)_5$ protomers in EMC virus-infected cells; consequently, it seems reasonable to assume that removal of the N-terminal segment of A by its γ constituent does not occur until a suitable juxtaposition of active site and susceptible peptide bond(s) has been attained by pentamerization. In this regard it is interesting to note that the $(A)_5$ protomers isolated by McGregor et al. (1975) did contain a significant amount of B.

Lawrence and Thach (1975) also demonstrated that the in vitro conversion of A to B could be inhibited by the addition of zinc ions.

Korant et al. (1974) and Butterworth and Korant (1974) had shown previously that Zn^{++} was inhibitory for in vivo posttranslational cleavages in cells infected with rhinoviruses, poliovirus, and EMC virus. The cleavage of the capsid precursor protein (i.e., A or NCVP1a) was very susceptible to inhibition. These authors believe that Zn^{++} acts by binding to the substrate polypeptide (via carboxyl or histidyl groups), thereby preventing protease action. Another possibility is that Zn^{++} binding to A or NCVP1a molecules might prohibit their association into the pentameric structures which may be necessary for autoproteolysis - this could be tested experimentally using the system of McGregor et al. (1975) as a control.

N-terminal analysis has revealed that the Mengo virus δ polypeptide does not have a free amino group at this terminus. The nature of the blocking group and the stage in posttranslation processing at which it is attached (by a cellular enzyme?) remains to be elucidated. A reasonable guess, however, is that this modification occurs at the time when A is converted to B. The function of the blocking group would then be to prevent degradation of B (and subsequently ε) by exopeptidases.

2.) Secondary cleavages (B → ε + D2; D2 → γ + α). While a host enzyme is probably responsible for nascent cleavages of the "polyprotein", and the A → B conversion may be autocatalytic, it is reasonably certain that a virus-specified or virus-induced protease is required for the formation of ε, γ, and α (VP0, VP3, and VP1 in the poliovirus system). Korant (1972) has shown that "polyprotein" isolated from poliovirus-infected, TPCK-treated cells underwent only nascent cleavages when incubated with an extract of uninfected cells. Treatment of the

"polyprotein" with an infected cell extract, however, resulted in the generation of the VP0, VP3, and VP1 proteins.

Furthermore, secondary (but not nascent) cleavages are blocked in poliovirus-infected cells by iodoacetamide (IAA). Korant (1973) has shown that NCVP1a (analogous to protein B, Figure 12) isolated from IAA-treated, poliovirus-infected cells can be cleaved in vitro by infected cell extracts to yield VP0, VP3, and VP1. Because IAA preferentially alkylates sulfhydryl groups, it may be that an -SH group is essential for the catalytic functioning of the putative viral protease in a manner analogous to that of papain (Arnon, 1970).

If the protease activity involved in these secondary cleavages is virus-specified rather than virus-induced, the limited amount of information encoded in the Mengo RNA genome suggests that a single protease species is involved in the conversions B → ε + D2, and D2 → γ + α. A possible candidate for such a Mengo virus-specified protease is the non-capsid polypeptide F. This protein is a stable product of the original "polyprotein" nascent cleavages and has a molecular weight of 38,000 (Paucha et al., 1974). The analogous protein in the poliovirus replication system is NCVPX.

Since the C-terminal amino acid of the ε polypeptide is not known, and because the C-terminal residue of γ could not be determined, it is not possible to make any definitive statement about the peptide bond specificity of the viral protease. It is quite probable, however, that conformation of the substrates is the most important factor in determining which peptide bonds are hydrolyzed.

3.) Morphogenetic cleavage. In EMC virus-infected cells, the conversion ε → δ + β occurs during the concerted process of capsid

formation and RNA encapsidation (McGregor et al., 1975), and may be mediated by the capsid polypeptide γ (Lawrence and Thach, 1975). That there is some protease activity associated with purified Mengo virions has been shown by Holland et al. (1972).

The morphogenetic cleavage probably is not catalyzed by the viral protease referred to earlier because Korant (1973) has shown that the poliovirus capsid precursor NCVP1a isolated from IAA-treated cells could be processed in vitro only to VP0, VP3, and VP1. Thus, without capsid assembly there is no conversion VP0 → VP2 + VP4 (or in the case of Mengo virus, ε → δ + β). It is conceivable that capsid formation by twelve $(\varepsilon,\gamma,\alpha)_5$ units could be accompanied by a conformational change in each ε chain which renders it susceptible to proteolytic attack by a neighbouring γ chain.

Whatever the identity of the enzyme responsible for the morphogenetic cleavage, it may have some specificity for peptide bonds involving the carboxyl function of aliphatic residues, since the C-terminal amino acid of the Mengo virus δ polypeptide is alanine. It is highly likely, however, that the conformational arrangements of the polypeptide substrate plays the dominant role in determining the site of action of this enzyme.

The relatively constant proportions of ε and D2 which are found in purified preparations of virus (1 to 2 chains of each per virion; see Chapter IV.) indicates that the final two cleavages which generate the four capsid proteins are not completed for every precursor molecule. It may be that the residual quantities of ε and D2 polypeptides found in an "average" virus particle are due to the initial condensation of 1 or 2 molecules of each with the viral RNA. This hypothetical RNA·$(\varepsilon, D2)_{1-2}$

entity may then serve as a nucleation center for the $(\epsilon,\gamma,\alpha)_5$ structures during the virus assembly process.

VII. SUMMARY AND PROSPECTS

The experiments described herein have demonstrated that the Mengo virus structural components, both RNA and protein, do not differ significantly from the analogous components of the other picornaviruses which have been studied in this regard. Not unexpectedly, the similarity has been found to extend to the amino acid compositions of the individual Mengo virus capsid polypeptides as well. While the N- and C-terminal analyses of these proteins have not demonstrated that a single peptide bond specificity is involved in the cleavages which generate the capsid polypeptides, these data have provided some information regarding the biogenesis of the structural polypeptides. The observed similarities with the terminal analyses of other picornavirus polypeptides indicates that, evolutionarily, the substrate sites of action for the cleavage enzymes have been well conserved.

The procedures which were developed in order to achieve reproducible purification of the four Mengo capsid polypeptides by SDS-hydroxylapatite chromatography should be directly applicable to the isolation of "insoluble" proteins in other systems, viral or otherwise. The same holds true for the method which has been developed for the quantitative recovery of the Mengo virus γ and α following separation by SDS-polyacrylamide gel electrophoresis (see Appendix).

Stopping short of complete primary sequencing studies (which would be very difficult to undertake due to the large quantities of material required), this thesis has presented a detailed characterization of the individual Mengo virus structural components. With this information as

background, further experimentation regarding certain aspects of Mengo virus replication, assembly and structure can now be undertaken. Those questions which can be readily examined in the near future are briefly discussed below.

Although the C-terminal amino acid residue of the Mengo γ polypeptide could not be obtained by carboxypeptidase digestion or hydrazinolysis, it may be possible to determine the single terminal residue by yet another method. Matsuo et al. (1966) have shown that the C-terminal residues in polypeptides can be cyclized to the oxazolone by the action of acetic anhydride. The active hydrogen in the oxazolone is then replaced with tritium by treatment with ^3H_2O in pyridine. After hydrolysis of the protein in 6 N hydrochloric acid, the tritiated amino acid residue which is present is identified as the C-terminal residue. Although the method has not been widely applied, it is easy to undertake and should be attempted in the case of the γ polypeptide.

Because of the limited quantities of the δ polypeptide which can be readily obtained, it would be difficult to determine the nature of the N-terminal blocking group and the identity of the N-terminal amino acid. It may be possible, however, to isolate sufficient quantities of the ε, B, and A polypeptides to determine by dansylation the stage in posttranslational processing at which the blocking group is added (see Figure 12.). As has been suggested, the blocking may occur at the stage of the A → B conversion.

Earlier work (Ziola and Scraba, 1974; Mak et al., 1974) has revealed that the Mengo virion possesses the same basic architectural design as the ME virion (Rueckert et al., 1969). The exact arrangement of the structural polypeptides in the viral capsid, however, is not

known. In this regard, preliminary iodination and chemical cross-linking experiments with the Mengo virion have provided some tentative answers. It is anticipated, however, that pursuing these particular experimental approaches will provide more definitive information about the structure of the Mengo capsid.

Specific iodination of exposed tyrosine residues on the Mengo virion was catalyzed by bovine lactoperoxidase which had been covalently coupled to Sepharose 4B (David and Reisfeld, 1974). Using short reaction times (less than 5 min), ^{125}I was incorporated only into the largest Mengo capsid polypeptide, α. With longer [illegible] min), ^{125}I incorporation was found in all four capsid proteins. [illegible]thew and Mar[illegible] obtained identical results with bovine enterovirus using soluble r[illegible] than solid-state lactoperoxidase. These data indicate that in the intact picornavirion, the α or VP1 polypeptide occupies much of the surface and only after the capsid starts to disassemble does ^{125}I get incorporated into the other capsid polypeptides. From this information, it was speculated that the α protein is the antigenically dominant protein in the Mengo virion (i.e., induces formation of neutralizing antibodies). Studies with antibodies formed against purified Mengo virus capsid polypeptides have supported this contention (G. Lund, personal communication).

SDS-polyacrylamide gel electrophoresis of Mengo virions which had been solubilized after reaction with dimethyl suberimidate revealed the presence of high molecular weight, cross-linked protein aggregates. From the rates of disappearance of the four capsid proteins as well as the estimated molecular weights of the newly formed cross-linked aggregates, the following (preliminary) observations have been made: 1.) the α protein reacts with the dimethyl suberimidate very rapidly, cross-

linking with itself or with the γ protein; 2.) the γ protein cross-links less rapidly than α, reacts with β in addition to α, and does not cross-link with itself; 3.) the β protein cross-links slowly and does so only with γ, it does not cross-link with itself and is only found associated with α through the formation of an αβγ "artificial" 4.7 S structural unit; and 4.) the δ protein does not react at all. The chemical cross-linking results (in conjunction with the iodination studies) suggest that the α polypeptides occupy the vicinity of the icosahedral vertices in the virion. It appears that the γ protein is "sandwiched" between the α and β polypeptides in the 4.7 S structural units. Finally, the δ polypeptide, because of its non-reactivity, appears to be masked by the other polypeptides; consequently, no indication is presently available as to the location of the δ polypeptide in the virion.

Since the γ capsid polypeptide has been implicated as being the morphogenetic cleavage enzyme (Lawrence and Thach, 1975), it is suggested that preparations of purified Mengo virus be assayed for proteolytic activity. The amino acid sequences of the "matching" ends of the δ and β polypeptides have been obtained (Table 7); consequently, any proteolytic activity in the Mengo virion which catalyzed this reaction should be assayable utilizing synthetic protein substrates which contain an amino acid sequence identical to that of the δ C-terminal and the β N-terminal. If such a proteolytic activity does exist in the Mengo virion, it may require "activation" by a partial loosening of the viral capsid such that the γ (?) protease can interact with the substrate provided. A natural substrate which may be superior to synthetic substrates is the Mengo virus precursor protein A. It may be possible to isolate suitable quantities of this protein from cells infected in the presence of Zn^{++} and/or iodoacetamide.

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APPENDIX

RECOVERY OF PROTEINS FROM POLYACRYLAMIDE GELS BY ELECTROPHORESIS INTO HYDROXYLAPATITE

Introduction

SDS-polyacrylamide gel electrophoresis has gained wide acceptance as an analytical tool for assaying protein at progressive stages of purification. The application of this method to preparative procedures, however, has been hampered by dilution of the protein upon elution or extraction from gels and by contamination of the recovered protein by a gel impurity which is believed to be linear polyacrylate (Chrambach and Rodbard, 1971).

During the course of studies on the Mengo virus capsid proteins (see Chapter V.), difficulty was occasionally encountered in separating two of the four major virus structural polypeptides (i.e., the α and γ chains of molecular weights 32,500 and 23,700, respectively) using the SDS-hydroxylapatite chromatography method of Moss and Rosenblum (1972). As a result, a method was developed for the quantitative recovery of these two proteins following their separation by SDS-polyacrylamide gel electrophoresis. The method overcomes the protein dilution problem of most preparative electrophoresis systems; and in addition, uv absorption spectra of the recovered protein indicate that there is very little contamination by gel impurities. This preparative procedure should prove applicable to any polypeptide isolation where SDS-polyacrylamide gel electrophoresis can be used for separation.

For the purpose of illustration, a description is given of the

isolation of α-chymotrypsinogen A (α-chtgn A) from a mixture of proteins.

Materials and Methods

Lactic dehydrogenase (LDH) and α-chtgn A were obtained from Sigma Chemical Co., carboxypeptidase A (CBP A) and α-chymotrypsin (α-cht) were from Worthington Biochemicals, and Myoglobin (Myo) was from Schwarz/Mann Biochemicals. The molecular weights of these proteins were taken to be 36,000, 25,741, 34,400, 25,330, and 17,600, respectively (Weber and Osborn, 1969; Dunker and Rueckert, 1969). The dye Remazol Brilliant Blue R (RBBR) was purchased from Calbiochem. Acrylamide and N,N'-methylenebisacrylamide were obtained from Eastman Organic Chemicals and recrystallized according to the procedure described by Loening (1967). DTT, technical grade SDS, and hydroxylapatite in powder form (Bio-Gel HTP) were purchased from Sigma Chemical Co.; Matheson, Coleman and Bell; and BioRad Laboratories, respectively. All other chemicals were reagent grade.

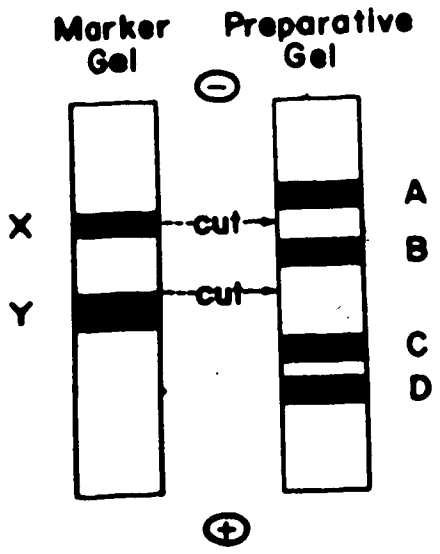
SDS-polyacrylamide gel electrophoresis. All gels consisted of 10% acrylamide, 0.2% N,N'-methylenebisacrylamide, 0.1% SDS, and 0.1 M sodium phosphate, pH 7.2. Polymerization was catalyzed by N,N,N',N'-tetramethylethylenediamine and ammonium persulphate, both at final concentrations of 0.075%. The gels were preelectrophoresed for a minimum of 1 hr.

LDH, α-chtgn A, and α-cht were each dissolved in 0.01 M sodium phosphate, pH 7.2, containing 2% SDS and 10% glycerol. CBP A and Myo were stained by the protein-RBBR coupling procedure of Griffith (1972) and similarly dissolved. A mixture containing 1.5 mg each of LDH and

α-chtgn A, and 3.0 mg of α-cht was made 5% with respect to β-mercapto-ethanol (final volume, 770 µl) and heated at 100° for 5 min. Under these conditions α-cht dissociates to its constituent A, B, and C chains of molecular weights 1,240, 13,930, and 10,160, respectively (Dunker and Rueckert, 1969). 150-µl aliquots were layered onto each of five SDS-polyacrylamide preparative gels (1 cm X 6 cm) directly beneath the electrophoresis buffer (0.1 M sodium phosphate, pH 7.2, containing 0.1% SDS). A 150-µl sample containing 300 µg of each of the prestained marker proteins, CBP A-RBBR and Myo-RBBR, was similarly prepared and layered onto a sixth gel. Electrophoresis was at 9 to 10 mA/gel for 10 hr.

Following electrophoresis, the marker gel was aligned with each preparative gel and the region containing the α-chtgn A cut out. As shown in Figure 13, the preparative gels were cut at the mid-point of the CBP A-RBBR band and at the trailing edge of the Myo-RBBR band. The five gel slices, each 7 to 7.5 mm high, were then stacked in a gel tube having a slightly constricted bottom (Figure 13, upper right). Electrophoresis buffer was used to remove trapped air bubbles from between the slices and along the wall of the tube.

Protein recovery by electrophoresis into hydroxylapatite. As shown in Figure 13 (lower right), an SDS-polyacrylamide gel plug (1.5 cm high) was cast in a second, slightly larger tube (1.35 cm internal diameter). A 3 mm layer of G25 medium Sephadex was then placed on the gel plug. 1 g of hydroxylapatite powder was suspended in electrophoresis buffer and fine material removed twice. The hydroxylapatite slurry was then thoroughly degassed and layered onto the Sephadex. The



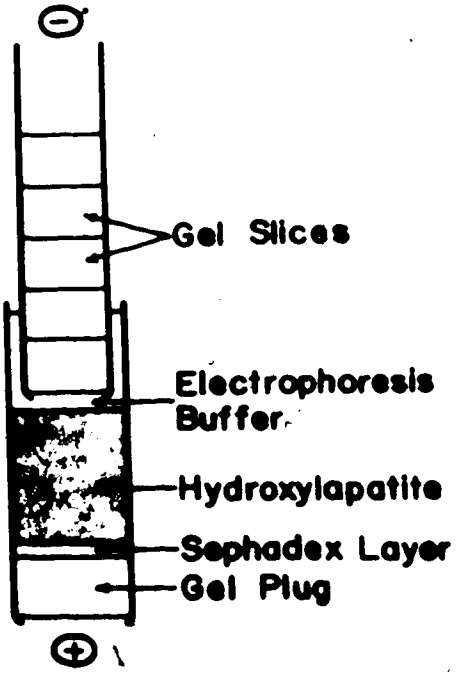


Figure 13. (Left). Separation of α-chtgn A from a mixture of proteins by SDS-polyacrylamide gel electrophoresis. The preparative gel is shown as it would appear if stained with Coomassie blue. The marker gel is shown as it appeared after electrophoresis. The protein bands are: A = LDH, B = α-chtgn A, C = α-cht B chain, D = α-cht C chain, X = CBP A-RBBR, and Y = Myo-RBBR. Slices were cut from the preparative gels as indicated.

Figure 13. (Right). Schematic representation of the apparatus used to recover the α-chtgn A from the preparative gel slices. Dimensions and quantities of the components are given in the Materials and Methods

top and bottom tubes were brought together such that the elution surface of the top tube was just above the surface of the settled hydroxylapatite. After sealing the joint between the two tubes with parafilm to prevent evaporation of the buffer, electrophoresis was carried out at 25 mA for 8 hr. During this time the α-chtgn A migrated from the gel slices into the hydroxylapatite.

Using a Pasteur pipette with a 3 to 3.5 mm orifice, the hydroxylapatite was then carefully transferred to a glass column containing a small bed of G25 medium Sephadex. The hydroxylapatite was washed at 4 to 6 ml/hr with five bed-volumes of 0.12 M sodium phosphate, pH 6.4, containing 1 mM DTT and 0.1% SDS. Increasing the phosphate concentration to 0.5 M eluted the α-chtgn A since it has been shown previously by Moss and Rosenblum (1972) that SDS-complexed proteins are eluted from SDS-hydroxylapatite columns by 0.2 to 0.5 M sodium phosphate, pH 6.4, containing 0.1% SDS and 1 mM DTT. Protein recovery was monitored by measuring the absorbance (at 282 nm) of the eluate.

Analysis of the recovered α-chymotrypsinogen A. Following elution from the hydroxylapatite, the recovered α-chtgn A was divided into two portions. The first was dialyzed against 0.1% SDS in distilled water and lyophilized. The resultant SDS-protein complex was dissolved at a final SDS concentration of 2% using 0.01 M sodium phosphate, pH 7.2, containing 2% β-mercaptoethanol and 10% glycerol. The solution was heated at 100° for 5 min and the purity of the α-chtgn A analyzed by electrophoresis of an aliquot in a 10% SDS-polyacrylamide gel (0.6 cm X 8 cm). For comparison, an aliquot of the starting mixture [illegible] a separate gel (Figure

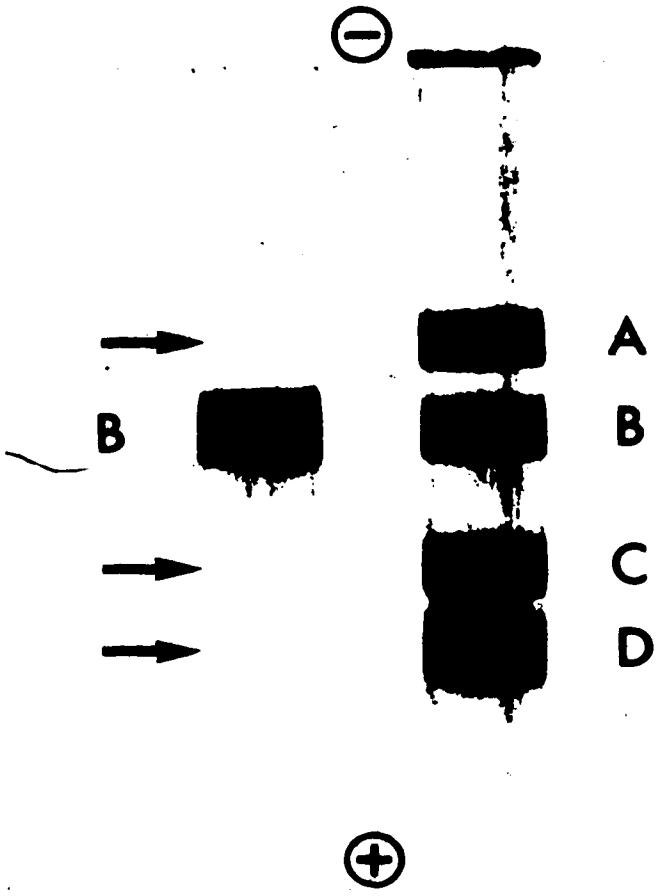


Figure 14. Analytical SDS-polyacrylamide gel electrophoresis of the α-chtgn A purified from a mixture of proteins by the method depicted in Figure 13. For description of the lettering, see Figure 13. The gel on the left shows the recovered α-chtgn A; and the gel on the right, the starting protein mixture. Electrophoresis was for 4 hr at 5 mA/gel after which the gels were stained and destained according to the pro-
(1969).

The second portion of recovered α-chtgn A was set to dialyze against 2,000 volumes of electrophoresis buffer. Dialyzed concurrently was a "standard" solution of α-chtgn A prepared by dissolving 1.5 mg of untreated protein in 2 ml of 0.01 M sodium phosphate, pH 7.2, containing 2% SDS and 5% β-mercaptoethanol, followed by heating at 100° for 5 min. After dialysis at room temperature for 36 hr, the two dialyzates were adjusted to the same absorbance at 282 nm. The uv absorption spectrum of each protein solution was then obtained using a Cary Model 15 recording spectrophotometer (Figure 15).

Results and Discussion

Recovery of α-chtgn A was quantitative (93 ± 5%, average of five determinations) following purification from a mixture of proteins by the method described. Quantitative recoveries of the Mengo virus α and γ proteins have also been routinely achieved (see Chapter V.). Since the purified protein is eluted from the hydroxylapatite in concentrated form (total volume of 3 to 4 ml), protein losses during subsequent reconcentration steps are minimized.

The purity of the recovered α-chtgn A is shown by Figures 14 and 15. Analytical SDS-polyacrylamide gel electrophoresis (Figure 14) revealed that there was no contamination of the α-chtgn A by LDH or the B or C chains of α-cht. Comparison of the uv absorption spectrum of the recovered α-chtgn A with the spectrum of the original α-chtgn A (Figure 15), indicated that there was very little contamination of the recovered protein by gel impurities which strongly absorb uv light of low wave-

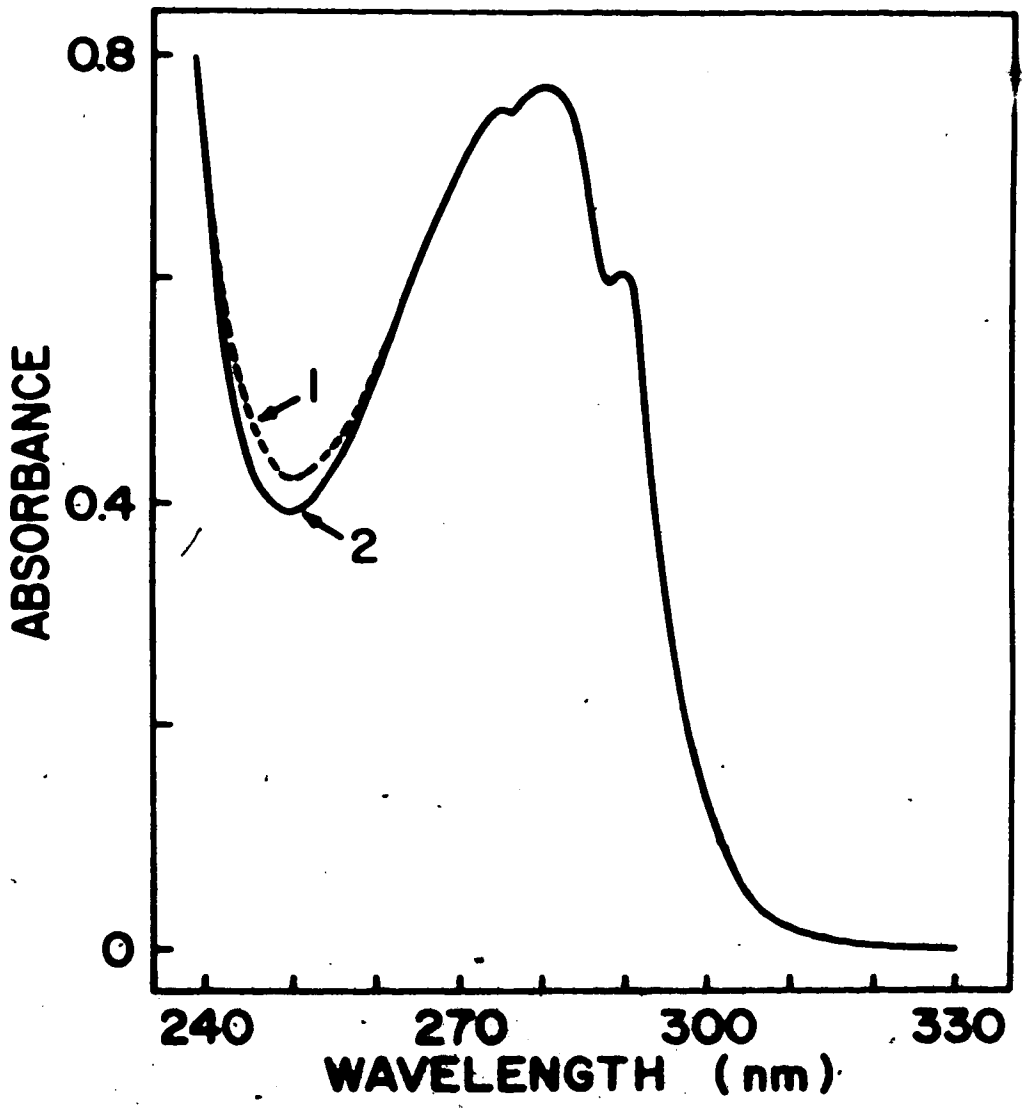


Figure 15. The uv absorption spectrum of α-chtgn A after purification from a mixture of proteins by the method depicted in Figure 13. Curve 1 [illegible] α-chtgn A, and curve 2 corresponds to the [illegible] Materials and Methods for further details.

Any laboratory containing the materials necessary for analytical SDS-polyacrylamide gel electrophoresis can readily use the method described for protein purification. In doing so two aspects must be considered. First, some means must be available for rapidly localizing the region of the preparative gels which contains the protein to be isolated. Concurrent electrophoresis of RBBR-stained marker proteins is one method (Figure 13, left). Should the protein to be purified have a high cysteine content, an alternative procedure is the reversible staining of the protein itself with the dye mercury orange (Sakai, 1968; Stoltzfus and Rueckert, 1972). After the protein is eluted from the hydroxylapatite, the dye can be removed by treatment with acidic acetone. Acidic acetone not only regenerates the protein but also has the added advantage of precipitating the protein while leaving the SDS in solution (Putnam, 1948; Stoltzfus and Rueckert, 1972). In this regard, Mengo virus structural proteins have been routinely recovered from SDS-hydroxylapatite column eluates by acidic acetone precipitation (see Chapter V.).

The second consideration is determining the minimum time necessary for electrophoresis of the protein from the gel slices into the hydroxylapatite (Figure 13, right). In this regard, a RBBR-stained marker protein can be used once the components of the recovery apparatus and the electrophoretic conditions have been standardized. For example, with the protein recovery apparatus shown in Figure 13 (and described above), it required 7 hr at 25 mA to electrophorese CBP A-RBBR from five gel slices into the hydroxylapatite. After electrophoresing for an additional 7 hr, the CBP A-RBBR was still completely contained within the hydroxylapatite even though slow movement through the hydroxylapatite

was evident. Consequently, for the recovery of α-chtgn A (whose molecular weight is approximately 9,000 less than that of CBP A), an electrophoresis clearing time of 8 hr was decided upon. If the protein to be recovered can be stained with mercury orange (see above), the electrophoresis of the protein from the gel slices into the hydroxylpaptite can be followed directly.

In conclusion, a procedure is described for the recovery of proteins following separation from contaminants by SDS-polyacrylamide gel electrophoresis. The method can be undertaken using readily available and inexpensive materials. The new method overcomes the two main problems of preparative polyacrylamide gel electrophoresis systems; namely, recovery of the purified protein in diluted form and extensive contamination of the recovered protein by uv absorbing gel impurities.