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STUDIES OF THE STRUCTURE AND REPLICATION OF MENGO VIRUS

by

© GARRY A. LUND

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled STUDIES OF THE STRUCTURE AND REPLICATION OF MENGO VIRUS submitted by GARRY A. LUND in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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To my parents

for their constant

encouragement and support.

[redacted]

[redacted] to lactoperoxidase-catalyzed iodination and their reactivities in immunological tests with specific antisera. When intact virions were subjected to iodination for a brief period of time (1 min), radioactive iodine was incorporated predominantly into the α polypeptides and to a lesser extent into β polypeptides. Only with longer incubation times (15 min or more) did label appear in the γ and δ polypeptides; and this coincided with a progressive loosening and ultimate collapse of the viral capsid. Antisera specific for each of the capsid polypeptide species were produced in rabbits using isolated proteins as antigens. Reaction of virions with these antisera in plaque-neutralization and hemagglutination-inhibition tests showed that only the anti-α serum was capable of blocking virus-cell interactions. Complement-fixation and immunodiffusion tests confirmed the observations that the α polypeptides occupy most of the external surface of the virus particle and that the β polypeptides are partially exposed. The γ and δ polypeptides apparently occupy internal locations in the capsid of the Mengo virion.

Isolation of the Mengo virus stable non-capsid polypeptides has been achieved. Capsid polypeptides were

removed from infected-cell lysates using ultracentrifugation and affinity chromatography techniques. Chromatography on SDS-hydroxylapatite columns resolved the denatured non-capsid polypeptides into three main peaks. Components of the two [redacted] containing polypeptides F plus G and E plus I, respectively, were separated by chromatography on G-100 Sephadex columns in the presence of 0.1% SDS.

Chromatography on Bio-Gel A-5m columns resolved the undenatured non-capsid polypeptides into two peaks. Selected fractions containing isolated E and F polypeptides were assayed for potential protease and polymerase activities. A preliminary assay using the precursor polypeptides A, B, C and D as substrates failed to detect any protease activity associated with the F polypeptide. Assay mixtures containing polypeptide E demonstrated an RNA polymerase activity which was dependent upon exogenous viral RNA template and oligo(rU) primer. This activity was not stimulated by the addition of viral capsid polypeptides. Cosedimentation of the isolated polypeptide E and viral RNA in sucrose density gradients demonstrated that this polypeptide had an affinity for the viral RNA. Polypeptide E was also capable of binding to poly(A) segments covalently bound to sepharose beads.

The association of a small polypeptide called VPg with purified Mengo virus RNA has been demonstrated. Analysis on 0.1% SDS - 10% polyacrylamide gels revealed that VPg

migrated between capsid polypeptide δ and the bromophenol blue marker and that it was sensitive to treatment with proteinase K.

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TABLE OF CONTENTS

		Page
LIST OF TABLES	.....	xiii
LIST OF ILLUSTRATIONS	.....	xiv
LIST OF ABBREVIATIONS	.....	xviii
CHAPTER I.	GENERAL INTRODUCTION	1
	Picornavirus Classification	1
	Properties of the Virion	3
	Virion RNA	4
	Virion Proteins	8
	Morphology of the Virion	10
	Virus Replication: Attachment and Uncoating	12
	Alterations of Cellular Metabolism .	17
	Biosynthesis of Viral Polypeptides .	21
	Replication of Viral RNA	26
	Virion Assembly	30
CHAPTER II.	ROUTINE MATERIALS AND METHODS	34
	Tissue Culture Media	34
	Sterilization procedures	34
	Growth medium	34
	Spinner medium	34
	Amino acid deficient medium ...	35
	Virus diluent	35

		Page
CHAPTER II.	(Continued)	
	Overlay diluent	35
	Agar overlay	35
	Cultured L Cells	36
	Virus	37
	Virus growth in roller bottles .	37
	Virus purification	37
	Preparation of radioactively labeled virus	37
	Plaque assay of infectious virus	38
CHAPTER III.	SURFACE STRUCTURE OF THE MENGO VIRION	39
	Introduction	39
	Materials and Methods	40
	Lactoperoxidase-catalyzed iodination of intact and disrupted virions	40
	Antisera	42
	Immunodiffusion	44
	Complement-fixation (CF) tests .	44
	Plaque-neutralization (PN) tests	45
	Plaque-neutralization enhancement (PNE) tests	46
	Hemagglutination inhibition (HI) tests	46

Results 47

Iodination of Mengo Virions ... 47

Immunodiffusion 51

CF tests 51

PN, PNR and HI tests 54

Discussion 55

CHAPTER IV. ISOLATION AND PARTIAL CHARACTERIZATION OF MENGO VIRUS-SPECIFIC POLYPEPTIDES E, P AND VPg 61

Introduction 61

Materials and Methods 62

Preparation of infected cell extracts 62

Affinity chromatography of cell lysates 64

SDS-hydroxylapatite chromatography 65

SDS-Sephadex gel filtration chromatography 66

Bio-Gel A-5m filtration chromatography 67

Assay of polymerase activity ... 68

Poly(A) Sepharose column chromatography 69

Isolation of VPg 70

TABLE OF CONTENTS (Continued)

		Page
CHAPTER IV.	(Continued)	
	Results	71
	Separation of Mengo virus non-capsid proteins from capsid proteins	71
	Separation of viral non-capsid polypeptides using denaturing conditions	75
	Separation of undenatured viral noncapsid polypeptides	87
	Characterisation of the polymerase	96
	Isolation of VPg	109
	Discussion	113
CHAPTER V.	BIBLIOGRAPHY	119

LIST OF TABLES

Table		Page
1.	Picornavirus classification scheme	2
2	Lactoperoxidase-catalyzed iodination of intact and disrupted Mengo virions	49
3	Complement-fixation tests	53
4	PN, PNE and HI titers of antisera against Mengo capsid polypeptides	56

LIST OF ILLUSTRATIONS

Figure		Page
1	Cleavage schemes for Mengo and Polio virus-specific polypeptides	24
2	Polypeptides of Mengo virions labeled *in vitro* by lactoperoxidase-catalyzed iodination	48
3	Immunodiffusion analyses of whole and dissociated Mengo virions	52
4	Electrophoretic profile of an L cell lysate at the initial stage of purification (before the 37,000 rpm centrifugation step)	72
5	Electrophoretic profile of the pellet from the 37,000 rpm centrifugation step	73
6	Electrophoretic profile of the S_{37}	74
7	Affinity chromatography of the S_{37} supernatent	76
8	Electrophoretic profile of the noncapsid polypeptides present in Pool 1 from the affinity chromatography column	77
9	SDS-hydroxylapatite chromatography of noncapsid polypeptides from Pool 1 of the affinity chromatography column	78
10	Electrophoretic profile of the noncapsid polypeptides found in Pool 1 from the SDS-hydroxylapatite column	80
11	Electrophoretic profile of the noncapsid polypeptides found in Pool 2 from the SDS-hydroxylapatite column	81
12	Electrophoretic profile of the noncapsid polypeptides found in Pool 3 from the SDS-hydroxylapatite column	82
13	SDS-Sephadex chromatography of the noncapsid polypeptides from Pool 2 of the SDS-hydroxylapatite column	83

(Continued)

LIST OF ILLUSTRATIONS (Continued)

Figure		Page
14	SDS-Sephadex chromatography of the noncapsid polypeptides from Pool 3 of the SDS-hydroxylapatite column	84
15	Electrophoretic profile of the noncapsid polypeptides following fractionation by G-100 Sephadex chromatography	85
16	Electrophoretic profile of the noncapsid polypeptides following fractionation by G-100 Sephadex chromatography	86
17	Bio-Gel A-5m chromatography of the noncapsid polypeptides from Pool 1 of the affinity chromatography column using PBS (minus Ca^{+2} and Mg^{+2} salts) containing 1% NP-40 as the buffer	88
18	Electrophoretic profile of the polypeptides found in Pool 1 from the Bio-Gel A-5m column	89
19	Electrophoretic profile of the polypeptides found in Pool 2 from the Bio-Gel A-5m column	90
20	Electrophoretic profile of the polypeptides found in Pool 3 from the Bio-Gel A-5m column	91
21	Electrophoretic profile of the polypeptides found in Pool 4 from the Bio-Gel A-5m column	93
22	Bio-Gel A-5m chromatography of the noncapsid polypeptides from Pool 1 of the affinity chromatography column using 0.05 M TRIS-HCl (pH 8.0) containing 0.05 M KCl and 1% NP-40 as the buffer	94
23	Bio-Gel A-5m chromatography of the noncapsid polypeptides from Pool 1 of the affinity chromatography column using 0.05 M TRIS-HCl (pH 8.0) containing 0.05 M sodium phosphate, 0.05 M KCl and 1% NP-40 as the buffer	95

(Continued)

LIST OF ILLUSTRATIONS (Continued)

Figure		Page
24	Glycerol gradient centrifugation of the noncapsid polypeptides	97
25	Effect of magnesium concentration on polymerase activity	98
26	Template and primer requirements for polymerase activity of polypeptide E	99
27	Assay of polymerase activity found in cell lysates during varying stages of purification	101
28	Effect of capsid polypeptides on polymerase activity in Pool 1 from the affinity chromatography column	102
29	Sucrose density gradient co-sedimentation of viral RNA and polypeptide E	104
30	Sucrose density gradient co-sedimentation of viral RNA and polypeptides F, G, H and I	105
31	Poly(A) sepharose chromatography of the S37 lysate	106
32	Electrophoretic profile of the polypeptides present in fraction number 29 from the poly(A) sepharose column	107
33	Electrophoretic profile of the polypeptides present in fraction number 9 from the poly(A) sepharose column	108
34	Electrophoretic profile of the precursor polypeptides used as substrate in the protease assay	110
35	Electrophoretic profile of the polypeptides present in the incubated protease assay mixture	111
36	SDS-hydroxylapatite chromatography of virus capsid polypeptides	112

(Continued)

LIST OF ILLUSTRATIONS (Continued)

Figure		Page
37	Electrophoretic profile of VPg	114
38	Electrophoretic profile of VPg treated with proteinase K	115

LIST OF ABBREVIATIONS

nm	- nanometers
SDS	- sodium dodecyl sulfate
mRNA	- messenger RNA
EMC virus	- encephalomyocarditis virus
ME virus	- Maus-Elberfeld virus
FMDV	- food and mouth disease virus
Å	- Angstrom units
M.W.	- molecular weight
TPCK	- tolylsulfonyl-phenylalanyl-chloromethyl ketone
TLCK	- tolylsulfonyl-lysyl-chloromethyl ketone
cRNA	- complementary RNA
RI-RNA	- replicative intermediate RNA
RF-RNA	- replicative form RNA
pfu	- plaque-forming units
moi	- multiplicity of infection
µCi	- microcurie
mg	- milligram
g	- gram
ml	- milliliter
mm	- millimeter
PN	- plaque neutralization
PNE	- plaque neutralization enhancement
HI	- hemagglutination inhibition
CF	- complement fixation
p.i.	- post infection

LIST OF ABBREVIATIONS (Continued)

vRNA	- viral RNA
HEPES	- [illegible]-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
μM	- micromolar
[illegible]	- [illegible] per minute
rpm	- revolutions per minute
g	- centrifugal force relative to gravity
VP	- viral protein
NCVP	- non-capsid viral protein
NP-40	- Nonidet P-40
IgG	- immunoglobulin G
DTT	- dithiothreitol
TCA	- trichloroacetic acid
BSA	- bovine serum albumin
EDTA	- ethylenediaminetetraacetic acid
TRIS	- tris(hydroxymethyl)amino methane
RBBR	- Remazol Brilliant Blue R
mA	- milliampere
PBS	- phosphate buffered saline
BME	- Eagle's basal medium

All temperatures are in degrees Celsius.

I. GENERAL INTRODUCTION

Picornavirus Classification

The word picornavirus (from pico - small and rna - designating a ribonucleate genome) was developed in 1963 by the International Enterovirus Study Group to describe small (15 to 30 nm in diameter), icosahedral, non-enveloped RNA-containing viruses of animal origin. There are also plant, insect and bacterial viruses which conform to this description but the term has been formally applied only to those viruses of animals.

The picornaviruses have been classified according to their differences in pH stability and buoyant densities in cesium salt gradients (Andrewes and Pereira, 1972; Newman et al., 1973; Scraba and Colter, 1974).

A recent classification scheme (Melnick et al., 1974) includes two genera, Enterovirus and Rhinovirus, which comprise the family Picornaviridae. This scheme is shown in Table 1. The caliciviruses had previously been included in the picornavirus classification scheme. However, their larger size and distinctive morphology (Zwillenberg and Bücki, 1966; Almeida et al., 1968; Wawrzkiewica et al., 1968) are atypical of picornaviruses. As well, it has been shown that calicivirus capsids have only one major structural polypeptide species (Bachrach and Hess, 1973; Burroughs and Brown, 1974) while all the picornavirus capsids are composed

TABLE 1

Picornavirus Classification Scheme

[illegible] PICORNAVIRUSES

Genus Enterovirus

A. Enteroviruses:	Polio (3 Serotypes) Coxsackie A (23) Coxsackie B (6) Echo (31) Enteroviruses of mice, swine, cattle	Sedimentation coefficient ~155 S Buoyant density ($CsCl$) ~1.34 g/ml Virions stable pH 3-10 Empty capsids produced *in vivo*
B. Cardioviruses:	EMC MM Mengo Columbia-SK MM (Serologically related)	Sedimentation coefficient ~155 S Buoyant density ~1.34 g/ml Virions labile 3<pH<7 in the presence of 0.1 M Cl^- or Br^- No empty capsids produced *in vivo*

Genus Rhinovirus

A. Human Rhinovirus (>113)	Sedimentation coefficient ~155 S Buoyant density ~1.40 g/ml Virions labile pH <5
B. Foot-and-Mouth Disease Virus (7)	Sedimentation coefficient ~145 S Buoyant density ~1.43 g/ml Virions labile pH<6.5
C. Equine Rhinovirus (2)	Sedimentation coefficient ~150 S Buoyant density ~1.45 g/ml Virions labile pH<5

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".

of four major polypeptide species (Rueckert, 1971; Penner et al., 1974).

Properties of the Virion

The picornavirion is an isometric particle composed of one molecule of single-stranded RNA (30% by weight) enclosed in a protein capsid (70% by weight). Carbohydrate (other than the ribose moiety of the RNA) or lipid has not been detected in the virion (Rueckert, 1971; Burness et al., 1973; Drzenek and Bilello, 1974; Mak and Rueckert, 1975). The physical and hydrodynamic properties of Mengo virions can be extended to picornaviruses in general. In the electron microscope, negatively stained Mengo virions have an anhydrous diameter of 27 nm. In solution, the virion behaves as a spheroid with a diameter of approximately 30 nm containing about 0.25 gram of water per gram of dry virion. The sedimentation coefficient ($S^{\circ}_{20,w}$) of 151 S, diffusion coefficient ($D^{\circ}_{20,w}$) of 1.47×10^{-7} cm^2/sec, and partial specific volume ($\bar{v}$) of 0.70 ml/g give a particle weight for the Mengo virion of $8.3 \pm 0.7 \times 10^6$ when substituted into the Svedberg equation (Scraba et al., 1967).

Virion RNA

Infectious RNA has been isolated from a number of different picornaviruses, including several serotypes of rhinovirus (Nair and Lonberg-Holm, 1971; Fiola and Saltsman, 1969; Dimmock, 1966), Mengo virus (Colter et al., 1957), polio virus (Alexander et al., 1958), Coxsackie virus (Mattern, 1962), EMC virus (Huppert and Sanders, 1958), ME virus (Franklin et al., 1959) and FMDV (Bachrach et al., 1964). The fact that the infectious RNA was single-stranded was demonstrated by Hausen and Schäfer (1962).

RNAs from several picornaviruses have been translated in cell-free systems; among them poliovirus (Rekosh et al., 1970), EMC virus (Dobos et al., 1971), FMDV (Chatterjee, 1976) and Mengo virus (Abreu and Lucas-Lenard, 1976). Comparison of the translation of EMC, ME and Mengo virus RNAs in cell-free systems from animal cells indicated that *in vitro* translation of picornavirus RNA starts at a single initiation site and that the *in vitro* products from the three viruses are very similar to each other and to the *in vivo* precursor and coat proteins (Eggen and Shatkin, 1972; Oberg and Shatkin, 1972). This is in contrast to other studies on the *in vitro* translation of poliovirus RNA which seem to demonstrate two different initiation sites on the viral RNA genome (Celma and Ehrenfeld, 1975). However, it still remains to be proven that two initiation sites are utilized *in vivo*. It is generally accepted that only one

initiation site is present on picornaviral RNA. The number of termination sites present on the viral RNA is somewhat uncertain. The existence of a weak internal termination site which gives rise to an overproduction of capsid protein late in the infectious cycle has been proposed (Lucas-Lenard, 1974; Paucha et al., 1974).

Early estimates of the molecular weight of picornavirus RNA ranged from 1.2×10^6 for poliovirus (Cooper, 1969) to 3.0×10^6 for EMC virus (Barness et al., 1963). More recent studies have shown that the molecular weight of the RNA is between $2.4 - 2.8 \times 10^6$; 2.6×10^6 for poliovirus (Tannock et al., 1970), 2.8×10^6 for FMDV (Wild and Brown, 1970), 2.4×10^6 for type 2 rhinovirus (Nair and Lonberg-Holm, 1971), 2.5×10^6 for type 14 rhinovirus (Nair and Lonberg-Holm, 1971) and 2.44×10^6 for Mengo virus (Ziola and Scraba, 1974).

Most picornavirus RNAs contain essentially equimolar amounts of adenylate, cytidylate, guanylate and uridylate residues (Newman et al., 1973). The only exception to this appears to be the human rhinoviruses which have a high adenylate content (Brown et al., 1970; McGregor and Mayor, 1971). No evidence has been found for the presence of methylated bases in the RNA (Grado et al., 1968). The nucleotides of picornavirus RNA are not, however, equally distributed along the length of the RNA genome. It has been shown that a polyadenylic acid sequence is present at the 3' end of such picornavirus RNA genomes as poliovirus (Armstrong

al al., 1972; Yogo and Wimmer, 1972; Spector and Baltimore, 1974), EMC virus (Hruby and Roberts, 1976; Goldstein al al., 1976), Columbia S-K virus (Johnston and Rose, 1972), Mengo virus (Miller and Plagemann, 1972; Spector and Baltimore, 1975a) and rhinovirus (Nair and Owens, 1974). There is some controversy as to the exact length of the poly(A) sequence, with estimates ranging from 15 to 17 nucleotides (Miller and Plagemann, 1972) to 50 to 125 nucleotides (Spector and Baltimore, 1975b). It has recently been demonstrated that the reason for this difference may be an overestimation of the length of the poly(A) segment in certain instances (Burness et al., 1975). Based on these observations, a size range of 16 to 75 nucleotides is probably a more accurate figure for the size of the poly(A) segment of picornavirus capsid RNA in general.

The poly(A) tract appears to be required for infectivity of purified viral RNA (Goldstein et al., 1976; Spector and Baltimore, 1974). The presence of the poly(A) segments has been correlated to messenger function (Johnston and Rose 1972; Hruby and Roberts, 1977), as well as to a possible involvement in encapsidation of the RNA (Spector and Baltimore, 1975a). The genomic poly(A) segment seems to be genetically coded (Dorsch-Häsler et al., 1975), in contrast to considerable evidence which suggests that the poly(A) of cellular mRNA is synthesized by post-transcriptional addition, presumably involving terminal adenylate transferases (Braurerman, 1974).

In addition to poly(A), the RNAs of Mengo, EMC and FMD viruses contain a tract of poly(C) 100 to 200 nucleotides long (Brown et al., 1974; Perez-Bercoff and Gander, 1977; Porter et al., 1974). In FMDV, the poly(C) tract is located 400 nucleotide residues in from the 5' end of the RNA (Newlands et al., 1978) and is near the 5' end of both Mengo and FMDV RNAs (Chumakov and Agol, 1976; Perez-Bercoff and Gander, 1977). The poly(C) tract may have some role in replication of the viral RNA since partially purified EMC RNA polymerase will synthesise poly(G) using exogenous poly(C) as a template (Rosenberg et al., 1972). This activity is similar to one reported for the replicase of phage Q β, the discovery of which lead to the suggestion that the poly(C) tract may be a recognition site for the replicase (Käppers and Sumper, 1975). However, the RNAs of entero- and rhinovirus subgroups seem not to contain extensive poly(C) tracts (Brown et al., 1975). Since the mechanism of replication of all picornaviruses appears similar, this proposed recognition function seems unlikely.

It has recently been shown that a small protein called VPg, of molecular weight about 4,000, is covalently attached to the 5' terminus of the virion RNA of EMC, polio and FMD viruses (Hruby and Roberts, 1978; Lee et al., 1976; Sanger et al., 1977). The protein is also present on the 5' end of the nascent strands of the polio replicative intermediate structure and attached to the poly(U) of polio minus strands (Nomoto et al., 1977). Poliovirus mRNA, however, does not

contain VPg (Nomoto et al., 1976). Also absent is the "capping group" (Lee et al., 1976), a structure which has been found at the 5' end of most eukaryotic mRNAs. The 5' terminal nucleotide sequences of poliovirus mRNA and genome RNA are identical (Pettersson et al., 1977). Since available evidence suggests that poliovirus mRNA is not encapsidated into progeny virus (Levintow, 1975), the above information suggests that VPg may have some regulatory role in deciding which RNA molecules become encapsidated during morphogenesis of the virion. It has also been suggested that VPg may play a role in initiation of poliovirus RNA synthesis, possibly by acting as a primer (Flanegan et al., 1977; Nomoto et al., 1977).

Virion Proteins

Early ultracentrifugal studies on the total extracted protein from poliovirus (Maizel, 1963), ME virus (Rueckert, 1965) and EMC virus (Burness and Walter, 1967) indicated the presence of a single, relatively homogeneous polypeptide species of molecular weight 26,000 to 30,000. Subsequent studies employing polyacrylamide gel electrophoresis demonstrated that the protein of poliovirus (Maizel, 1963) and of ME-, EMC- and Mengo-viruses (Rueckert, 1965; Rueckert and Duesberg, 1966) was composed of several different polypeptide species. With the advent of polyacrylamide gel

electrophoresis in the presence of SDS (Maizel, 1964; Shapiro et al., 1967; Weber and Osborn, 1969; Dunker and Rueckert, 1969, it became possible to simultaneously determine the number, molecular weight and relative amounts of each polypeptide chain in the whole virion. Such studies have shown that the picornavirus protein capsid is composed of four major polypeptide species, designated VP1 or α, VP2 or β, VP3 or γ and VP4 or δ, of average molecular weights 34,000, 30,000, 25,000 and 5,000 to 8,000 respectively. Furthermore, there appears to be 60 copies of each in a complete capsid (Rueckert, 1976). Also present in 1 to 2 copies per virion is the uncleaved precursor of δ and β, called VP0 or ϵ.

The amino acid composition of whole protein from several picornaviruses has been tabulated (Rueckert, 1971). Notable features common to the proteins are a low content of sulfur-containing residues (2 - 3 moles %), a high content of apolar residues (50 moles %), a high content of proline (6 - 8 moles %) and a relatively large amount of residues which do not form α-helices (35 - 40 moles %). These last two points are reflected by the low α-helical content of Mengo capsid proteins in situ (5 - 10%), as measured by optical rotatory dispersion and circular dichroism techniques (Scraba et al., 1967; Kay et al., 1970).

Individual capsid polypeptides have been purified from a number of picornaviruses and their amino- and carboxyl-terminal amino acid residues determined. With one exception, the amino-terminal residues of the three larger polypeptide

species of the reported picornaviruses are gly, asx and ser or thr for α (VP1), β (VP2) and γ (VP3) respectively (Rueckert, 1976). It has been shown that the N-terminal of the δ or VP4 polypeptide of Mengo virus is blocked (Ziola and Scraba, 1976). Similarly, only three free N-termini could be detected in intact poliovirus (Burrell and Cooper, 1973), and these were identical to the three reported for Mengo virus. Thus it seems evident that a blocked N-terminal of δ (VP4) could be a common structural feature of picornaviruses.

Morphology of the Virion

Analysis of the picornaviral capsid structural organization was first made possible by the development of techniques for purification and crystallization of poliovirus (Schaffer and Schwerdt, 1955, 1959). Finch and Klug (1959) concluded from X-ray diffraction patterns of these crystals that the poliovirus capsid possesses 5:3:2 symmetry. By extrapolating from their data, they suggested that the virion was composed of 60 identical asymmetric structure units, each with a diameter of 60 to 65 Å and a mass of 80,000 daltons (the latter value calculated using the then accepted but incorrect molecular masses of 2×10^6 daltons for the RNA and 6.7×10^6 daltons for the virion).

Subsequently, data on turnip yellow mosaic virus revealed that its capsid was composed of 180 polypeptide

chains and not the 60 chains deduced from X-ray diffraction patterns which were virtually identical to those obtained for poliovirus (Klug et al., 1957). In addition, electron microscopy revealed that the 180 polypeptide chains were arranged into 32 morphological units (capsomers) rather than 60 (Klug et al., 1966; Finch and Klug, 1966). This illustrated that 5:3:2 symmetry and repeat periods in X-ray diffraction patterns are insufficient evidence to conclude that a virion is composed of 60 subunits and, as a result, cast doubt on the poliovirus model.

Attempts to elucidate the capsid structure employing negative staining in the electron microscope added further confusion. The picornaviruses have unusually compact capsids which are essentially impermeable to the electron-dense salts used as negative stains. Thus very little surface detail is evident in electron micrographs, and microscopists have variously suggested 32- (Mayor, 1964), 42- (Agrawal, 1966) and 60- (Horne and Nagington, 1959) capsomer structures.

The key to resolving the capsid architecture came from an examination, not of intact virus, but of its dissociation products. Cardioviruses, when incubated in physiological saline at pH 5 to 6.5, dissociate into RNA, homogeneous 13 to 14 S subunits (molecular weight approximately 425,000) and an insoluble precipitate of α and ε chains (Rueckert et al., 1969; Dunker and Rueckert, 1971; McGregor et al., 1975; Mak et al., 1970). The 13 - 14 S subunits contain equimolar amounts of α, β and γ and, by treatment with 2M urea, can be

dissociated further into 5 S particles of molecular weight 86,000. These 5 S particles also contain equimolar amounts of α, β and γ. On this basis, Dunker and Rueckert (1971) proposed that the 5 S particle was the basic structure unit and that five of these were clustered to form the 13 - 14 S pentamer subunit, one of which is centered at each of the 12 vertices of the icosahedral particle. Similar studies with Mengo virus yielded identical results and in addition electron microscopy showed that the dimensions of the 5 S and 13 - 14 S subunits were consistent with the proposal that the 5 S subunit is the fundamental structure unit of the capsid and that the 13 - 14 S subunit was a pentameric cluster of 5 S subunits (Mak et al., 1974). The observed 68 Å diameter of the 5 S subunit is in close agreement with the value originally predicted by Finch and Klug (1959).

Virus Replication: Attachment and Uncoating

The initial step of viral infection involves the attachment of virus particles to specific receptors in the plasma membrane of susceptible cells. The receptors for a given virus are limited in number (10^4 to 10^5 per cell) and thus can be saturated with excess virus (Crowell, 1966; Lonberg-Holm and Korant, 1972; Lonberg-Holm and Philipson, 1974). The presence on membrane surfaces of specific "receptor families" has been demonstrated for a number of enteroviruses

and human rhinoviruses (Lonberg-Holm et al., 1976). A picornavirus belonging to one family can block attachment of other viruses of that same family (e.g. poliovirus type 1 can block attachment of poliovirus type 2). It cannot, however, block attachment of viruses specific for other receptor families. Receptors for poliovirus and coxsackie-virus B have characteristic sensitivities to inactivation by proteolytic enzymes (Zajac and Crowell, 1965). However, these receptors were not inactivated by neuraminidase, while receptors for EMC virus were inactivated by similar treatment (Angel and Burness, 1977).

The initial attachment of virus to cell receptors is reversible, and infectious virus can be removed from cells by washing with physiological saline. This loose attachment is subsequently converted to an irreversible virus-receptor complex, the conversion in some cases being temperature dependent (Lonberg-Holm and Philipson, 1974). Infectious virus can be recovered from such complexes by treatment with SDS (Maizel, 1962). Lonberg-Holm and Philipson (1974) suggest that the transition from a weak to strong interaction may be accomplished by the diffusion of additional receptors in the plane of the membrane to the initial site of virus-attachment. At low temperatures the fluidity of the membrane lipids would be decreased, and this would restrict the mobility of the receptors and hinder the establishment of the irreversible complex. Receptors for Mengo virus (Mak et al., 1970), poliovirus (Bachtold et al., 1957) and FMDV (Brown

et al., 1962), whose attachment is essentially temperature-independent, may already be clustered on the cell surface; whereas receptors for rhinovirus, whose attachment is temperature-dependent (Lonberg-Holm and Korant, 1972) may be distributed throughout the membrane.

Interaction of picornaviruses with susceptible cells produces a rapid loss of infectivity of the virus, a process called eclipse. A large proportion of poliovirus attached to cells in the cold could be subsequently eluted by raising the temperature (Fenwick and Cooper, 1962; Joklik and Darnell, 1961). The eluted particles, which are unable to reattach to cells, contain infective RNA which is resistant to ribonuclease (Joklik and Darnell, 1961; Mandel, 1967). These "A-particles" sediment at approximately 90% of the rate of infectious virions (Fenwick and Cooper, 1962). Coxsackieviruses B3 and A13 also undergo these specific alterations and it has been demonstrated that the eluted particles lack the polypeptide VP4 (Crowell and Philipson, 1971; Cords et al., 1975). The loss of infectivity of poliovirions has also been equated with a loss of the polypeptide VP4 (Breindl, 1971). Virions which have been treated in vitro with acid, alkali, heat, UV radiation or 2M urea also produce particles which lack VP4 and are unable to attach to cells (Lonberg-Holm and Yin, 1973; Breindl, 1971a,b; Katagiri et al., 1971; Roizman et al., 1959). The artificially produced particles are immunologically similar to the eluted A-particles, both

of which are said to possess "C" or "H" antigenicity. The intact virus particle is said to have "D" or "N" antigenicity (Mayer et al., 1957; Hummeler et al., 1962). The antigenic shift accompanying the formation of empty particles occurs whether or not the RNA is released from the virion (Breindl, 1971a,b; Katagiri et al., 1968, 1971).

These observations led some workers to postulate that the capsid polypeptide VP4 was responsible for attachment of virus to cell receptors (Breindl, 1971a; Philipson et al., 1973). An alternate hypothesis put forward by Butterworth et al. (1975) explains the inability of eluted virus to reattach as a conformational rearrangement of the capsid polypeptides, the loss of VP4 being incidental. The hypothesis is supported by the observation that human rhinovirus type 2 can be resolved into two conformationally isomeric populations by isoelectric focussing (Korant et al., 1975; Butterworth et al., 1975). Virions isoelectric at pH 6.4 had a full complement of RNA and polypeptides, and were fully infectious, while the population isoelectric at pH 4.5 was noninfectious even though it had a full complement of RNA and polypeptides (including VP4). Isoelectric focussing data on poliovirus also supports the [illegible]hesis of Butterworth (Mandel, 1971). Virions isoelectri[illegible] at [illegible] 7.0 and at pH 4.5 were detected. Treatments w[illegible]h i[illegible]ted infectivity (heat, adsorption-elution from c[illegible]eutralization by specific antibodies) irreversibly stabilized the capsid in the conformation with isoelectric point pH 4.5.

These treatments were also shown to change the antigenicity of the virion from D to C. Poliovirus can be stabilized in its native conformation by the methylthiopyrimidine S-7 (Lonberg-Holm et al., 1975) and by glutathione (Fenwick and Cooper, 1962), as measured by increased heat stability. Human rhinovirus type 2 is similarly stabilized against pH and heat inactivation by SDS. These agents also inhibit infection of cells and cell-mediated eclipse, suggesting that conformational rearrangement of the viral capsid is required for the infection of cells by virions.

The observation that isolated cellular membranes can cause eclipse of virus (Chan and Black, 1970; Roesing et al. 1975; De Sena and Mandel, 1976) suggests that modification of the adsorbed virion is attributable to a component of the plasma membrane, perhaps the receptor. The process by which the RNA genome enters the cell subsequent to the initial capsid modification remains obscure. Lonberg-Holm and Whiteley (1976) proposed that eclipse of loosely attached poliovirus results in elution of the A-particle and that eclipse of tightly bound virus results in intercalation of the lipophilic A-particle (Lonberg-Holm et al., 1976) into the cell membrane, followed by uncoating of the RNA. Alternatively, De Sena and Mandel (1977) proposed that the A-particles of poliovirus progress through a series of modification steps, ultimately leading to uncoating. The RNA present in A-particles becomes sensitive to ribonuclease following treatment with chymotrypsin or detergents (De Sena

and Mandel, 1976) and these authors suggested that similar factors may play a role in the processing of A-particles through a series of unstable intermediates to a final state of uncoating.

Little morphological evidence concerning penetration and uncoating of picornaviruses is present. Engulfment of the virions into a vacuole has been proposed (Dales, 1973) but micrographs seeming to show a direct penetration of the plasma membrane have also been published (Dunnebacke et al., 1969).

Alterations of Cellular Metabolism

Picornavirus infection of cells produces a characteristic early inhibition of cellular protein, RNA and DNA synthesis. The rate and extent of inhibition depends on strain of virus and cells and multiplicity of infection used (McCormack and Penman, 1967). In most systems analyzed, shutoff of host cell protein synthesis is complete by 3 to 4 hours after infection. Shutoff is paralleled by a gradual disaggregation of the heterogeneous host cell polyribosomes (average sedimentation rate = 200 S) and the formation of larger virus-specific polyribosomes, sedimenting more homogeneously at approximately 350 S (Baltimore, 1969). The host cell mRNAs have a relatively long half-life (Greenberg, 1972) and it has been shown that the viral

inhibition of host protein synthesis is not due to extensive degradation of the cellular mRNAs, although very small changes in size (which are difficult to detect) may be adequate to inactivate these messengers (Colby et al., 1974). This implies that inhibition occurs at the level of initiation and it has been suggested that a virus-specified protein selectively interferes with attachment of cellular mRNA to ribosomes (Penman and Summers, 1965; Willems and Penman, 1966; Baltimore, 1969). Poliovirus mutants unable to shut off host protein synthesis map in the region of the genome which codes for structural proteins (Steiner-Pryor and Cooper, 1973). This evidence led to the proposal that a structural protein is involved in shutoff, perhaps by acting as a new initiation factor (Cooper et al., 1973; Wright and Cooper, 1974). However, deletion mutants which do not produce capsid proteins behave normally with respect to the inhibition of host protein synthesis (Cole and Baltimore, 1973).

An alternative model to account for shutoff has been proposed by Ehrenfeld and Hunt (1971). In vitro studies showed that viral double-stranded RNA could inhibit globin synthesis in rabbit reticulocyte cell-free systems, this may have been accomplished by the sequestration of initiation factor eIF-3 (Kaempfer and Kaufman, 1973). Also, addition of double-stranded RNA to cultured cells produced an in vivo inhibition of protein synthesis (Cordell-Stewart and Taylor, 1973). However, it was later shown that both

viral and cellular protein synthesis are inhibited by double-stranded RNA *in vitro* (Robertson and Mathews, 1973; Celma and Ehrenfeld, 1974). In addition, shutoff occurs early in infection when little, if any, double-stranded RNA is present and can also occur if viral RNA replication is absent (Baltimore, 1969).

Leibowitz and Penman (1971) proposed that shutoff is due to viral RNA being more efficient than cellular mRNA in initiating translation. *In vitro* studies show that uninfected and EMC virus-infected mouse cell extracts are equally active in translating mRNAs of either viral or cellular origin, but that only the viral message is translated when viral and cellular mRNA are simultaneously added to the infected cell extract (Lawrence and Thach, 1974; Thach *et al.*, 1975). Also, a hypertonic medium selectively blocked initiation of cellular protein synthesis in infected cells early in the infectious cycle, while viral protein synthesis continued unimpaired (Saborio *et al.*, 1974; Nuss *et al.*, 1975). This model does not account for the fact that inhibition of cellular protein synthesis occurs before the synthesis of significant levels of viral RNA (Baltimore, 1969; Nuss *et al.*, 1975). Nuss *et al.* (1975) suggested, therefore, that a virus-specified product alters a cellular factor, thereby affecting the rate of initiation complex formation for all messengers. Viral RNA with a high affinity for initiation would still be translated, but cellular mRNAs with a low affinity for initiation would

not be translated. *In vitro* systems, because of their low rate of initiation, would not be able to detect this phenomenon.

Inhibition of cellular RNA synthesis also occurs early after picornavirus infection (Baltimore, 1969) but the mechanism remains obscure. It would appear to be mediated by a virus-specified protein, since irradiation of the infecting virus (Franklin and Baltimore, 1962) or inhibitors of protein synthesis (Baltimore *et al*., 1963) prevent shutoff of cellular RNA synthesis. Recently, Schwartz *et al*., (1974) and Miller and Penhoet (1972) showed that nuclei isolated from Mengo- or EMC-virus-infected cells demonstrated an inhibition of RNA polymerase II activity appearing before inhibition of polymerases I and III, suggesting two separate mechanisms. However, when extracts were made from the nuclei these differences were no longer observed and polymerase activity was fully recovered.

Inhibition of DNA synthesis in infected cells appears to be a consequence of protein synthesis inhibition. This is supported by the observation that inhibitors of protein synthesis in uninfected cells mimic the shutoff of DNA synthesis produced in infected cells (Hand *et al*., 1971; Hand and Tamm, 1972).

Biosynthesis of Viral Polypeptides

Early studies of the replication of poliovirus revealed the presence of about 14 different virus-specified polypeptides in cytoplasmic extracts of infected HeLa cells (Summers et al., 1965). Of the 14 polypeptides, 4 were shown to correspond to the virus capsid proteins (designated VP1 to 4) while the remaining ones were non-capsid polypeptides (designated NCVP1 to 10). Summation of the molecular weights of the 14 viral polypeptides gave a value approximately twice that of the theoretical coding capacity of the poliovirus genome. This puzzle was resolved when it was recognized that the higher molecular weight polypeptides were cleaved after translation to yield the smaller stable capsid and non-capsid polypeptides (Summers and Maizel, 1968; Jacobson and Baltimore, 1968).

This process has also been shown to take place in cells infected with enteroviruses (Holland and Kiehn, 1968), cardioviruses (Butterworth et al., 1971; Dubos and Martin, 1972; Lucas-Lenard, 1974; Paucha et al., 1974), human rhinoviruses 1A and 2 (McLean and Rueckert, 1973; Butterworth, 1973) and [redacted] (Vande Woude et al., 1972; La Porte and Lenoir, 1972; Black, 1975). It was proposed by Jacobson and Baltimore (1968) that the entire viral genome was translated into one giant polypeptide (called "polyprotein") and that all the viral proteins were produced by subsequent cleavages.

Polyprotein is not normally observed in labeled cell extracts. One exception is coxsackie virus-infected cells in which a polypeptide of MW 200,000 has been detected after a short pulse label early in infection (Kiehn and Holland, 1970). Polyprotein can be detected if proteolytic cleavages in infected cells are inhibited by incorporation of amino acid analogues (Jacobson et al., 1970; Paucha et al., 1974), by addition of protease inhibitors such as TPCK or TLCK (Korant, 1972; Summers et al., 1972), or by zinc ions (Butterworth and Korant, 1974). Some temperature-sensitive mutants of poliovirus also accumulate high molecular weight precursors at the restrictive temperature (Garfinkle and Tershak, 1971). In the absence of inhibitors the largest polypeptide normally found in infected cell extracts has a molecular weight of 100,000 to 130,000 (Jacobson et al., 1970; Butterworth and Rueckert, 1972a), indicating that cleavages occur on nascent polypeptide chains.

Studies on the in vitro translation of picornavirus RNA revealed only one initiation site at or near the 5' end (Öberg and Shatkin, 1972; Boime and Leder, 1972; Smith, 1973), a result which is compatible with the idea of polyprotein being equivalent to the uninterrupted translation of the entire genome. Also revealed was the presence of a short "lead-in" polypeptide which was rapidly cleaved from the 5' end of the capsid precursor following initiation of translation. This polypeptide was then rapidly degraded (Smith, 1973). The presence of a unique initiation site

made possible the genetic mapping of the polypeptides using pactamycin, a drug which specifically inhibits initiation of protein synthesis (Taber et al., 1971; Summers and Maizel, 1971). Using this technique, it has been shown that the pattern of post-translational cleavages leading to the generation of the stable end products is very similar for EMC virus (Butterworth and Rueckert, 1972b), Mengo virus (Lucas-Lenard, 1974; Paucha et al., 1974), poliovirus (Butterworth, 1973), rhinovirus 1A (Butterworth, 1973; McLean and Rueckert, 1973) and FMDV (Sanger et al., 1977). The models of polypeptide synthesis and processing for Mengo virus and poliovirus are presented in Figure 1.

In Mengo virus-infected cells, three large polypeptides designated A, F and C and two smaller polypeptides designated G and H are produced by primary cleavages of the nascent polyprotein. Polypeptide A, translated from the 5′ region of the genome, is the precursor for the virion capsid proteins and is processed by a series of secondary cleavages to give ϵ, α and γ. The cleavage of ϵ to δ and β is termed a morphogenetic cleavage and occurs during encapsidation of the viral RNA (Jacobson and Baltimore, 1968b). Polypeptide C, translated from the 3′ region of the viral RNA, also undergoes secondary cleavages to yield D and then E, which is stable. Polypeptide F, produced during the primary cleavages, is also stable.

The nature of the enzyme(s) involved in these cleavages is unknown. The primary cleavages, which can be inhibited

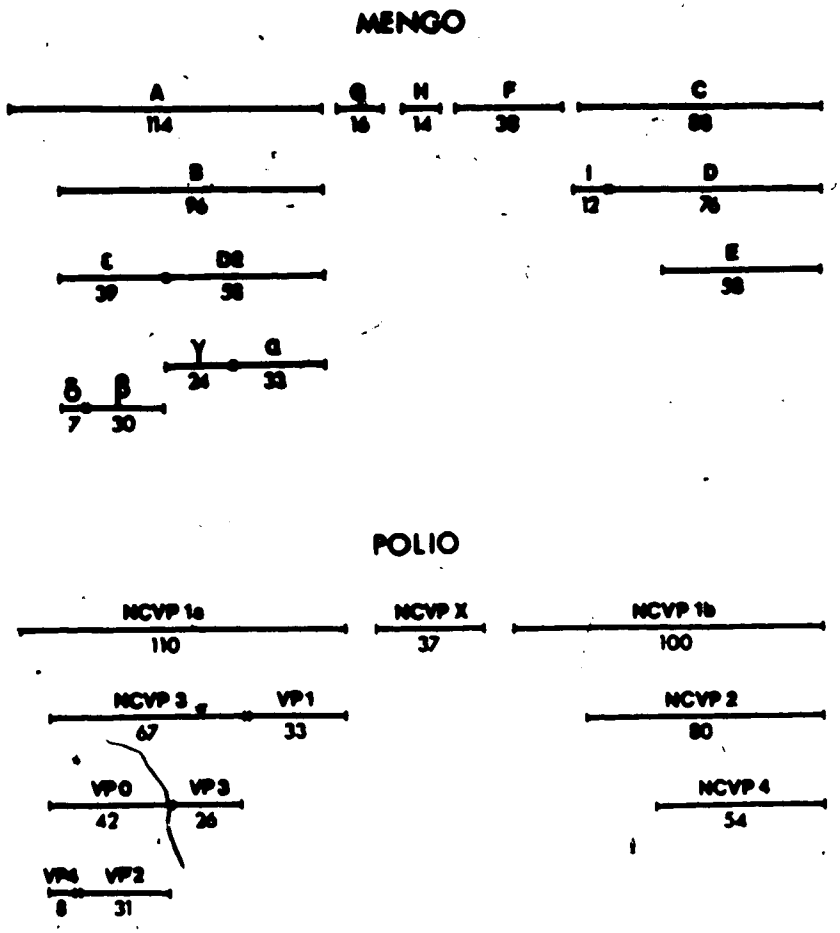


Figure 1. Cleavage schemes for Mengo and Polio virus-specific polypeptides. Numbers below the lines refer to molecular weight, in thousands, of each polypeptide.

by TPCK or TLCK, appear to be mediated by cellular proteases. Korant (1972) showed that poliovirus polyprotein, isolated from monkey-kidney cells infected in the presence of TPCK, could be cleaved by uninfected cell extracts into products identical to those produced by *in vivo* primary cleavages. Conformation of the polyprotein also appeared to be important since α-chymotrypsin could cleave native polyprotein into products similar to those produced with infected cell extracts but cleaved denatured polyprotein into small fragments. Korant (1973) also demonstrated that the secondary cleavages of capsid precursors are probably mediated by a viral specific enzyme. Partially purified polypeptide 1a of poliovirus (analogous to polypeptide A of Mengo) could be cleaved by extracts of infected cells to yield the capsid proteins. Uninfected cell extracts did not contain this activ[illegible].

Another distinct viral-coded protease appears to be present in infected cells. EMC RNA translated in an *in vitro* cell-free system from uninfected cells, yields as one of its products a polypeptide slightly longer than polypeptide A (Lawrence and Thach, 1975). This polypeptide was identical to A except for an extra 12,500 daltons of protein at the amino terminal end. Infected cell extracts did not produce this larger version of A, but an additional small protein of MW 12,500 was present. The activity responsible for this cleavage copurified with the viral capsid protein γ. Since the γ sequence is contained in A,

this process may be autocatalytic.

Replication of Viral RNA

In addition to its role as messenger, infecting picornaviral RNA acts as a template for RNA replication. Kinetic analysis shows that replication of viral RNA begins within half an hour of the initiation of infection and proceeds exponentially until approximately 3 to 4 hours post-infection when the rate of synthesis becomes linear (Baltimore, 1969; Darnell et al., 1967). The rate remains linear for about 1 hour and then declines gradually until replication ceases at 7 to 8 hours (Baltimore et al., 1966; Darnell et al., 1967). Once formed, a new RNA molecule becomes available for replication, translation or ultimately, encapsidation. The control processes which decide the fate of a newly synthesized RNA molecule remain to be clarified. It has been proposed that encapsidation of newly synthesized viral RNA, which would limit the availability of replicative template, is responsible for the alteration of RNA synthesis kinetics from the exponential to the linear form (Baltimore, 1969).

Replication of the viral RNA takes place exclusively in the cytoplasm on smooth membranes which make up a structure referred to as the replication complex (Caliguiri and Tamm, 1969; Caliguiri et al., 1973). This complex, sediment-

ing at 250 S, contains all of the viral RNA polymerase activity and replicating structures, referred to as replicative intermediates (RI). RI consists of a single strand of template RNA hydrogen-bonded to the growing 3' ends of progeny molecules whose completed 5' ends are free (Thach et al., 1974; Bishop and Levintow, 1971; Levintow, 1974). The RI itself sediments at 20 - 70 S in sucrose gradients, indicating heterogeneity in size. Evidence that the RI is a functional intermediate in the synthesis of viral RNA was obtained by in vitro pulse-chase experiments which showed flow of radioactive label from RI structures into single stranded progeny molecules (Girard, 1969; McDonnell and Levintow, 1970).

The infecting viral RNA initially acts as a template for the simultaneous formation of six to seven strands of complementary RNA (cRNA; Baltimore, 1968), each one being synthesized by a separate polymerase molecule, in the RI structure. The cRNA then serves as a template for the synthesis of viral plus strand RNA molecules, also in an RI structure. Most of the RIs extracted from infected cells contain a complete single-stranded template of cRNA. From 5 to 10 times as much vRNA is produced than cRNA (Baltimore, 1969). Genetic evidence suggests that different polymerases may be involved in the synthesis of cRNA and viral (plus) RNA. Temperature sensitive mutants of poliovirus synthesize either no RNA or only cRNA, and these mutations map in slightly different places within the

genome (Cooper, 1970).

In addition to the 250 S replicative complex which makes mostly viral RNA, a minor fraction sedimenting at 70 S has also been identified (Caliguiri, 1974). This smaller complex produces mostly cRNA, suggesting that it is the primary site for cRNA synthesis. Thus, there may be different cellular locations for the two replication complexes.

Also present in infected cells is a fully hydrogen-bonded double-stranded RNA called the replicative form (RF). It accumulates only late in infection (Baltimore and Girard, 1966) and this fact, along with pulse-chase experiments (Baltimore, 1968; Girard, 1969) suggests that it is a byproduct of the RI (Bishop and Levintow, 1971).

Data which showed that picornavirus RNA synthesis does not occur in the nucleus (Franklin and Baltimore, 1962) together with the observation that these viruses replicate in the presence of Actinomycin D (Reich et al., 1962) implied the presence of a virus-induced RNA dependent RNA polymerase (replicase). The first evidence for such a replicase was obtained with Mengo virus-infected cells (Baltimore and Franklin, 1962, 1963) and poliovirus-infected cells (Baltimore et al., 1963b). When isolated from cytoplasmic extracts, the replicase activity was found to be associated with a replication complex which included protein, nucleic acid and smooth cytoplasmic membranes. Activity of the replication complex in vitro was limited

to completion of previously initiated viral RNA strands (Girard, 1969; Dietzschold and Ahl, 1970). Dissociation of the replicase from membranes with detergents resulted in a rapid, irreversible loss of activity (Girard et al., 1967; Plagemann and Swim, 1968). The instability of the replicase has made definitive studies of its composition and enzymatic properties very difficult; nevertheless some progress has been made. A partially purified replicase complex from EMC virus-infected cells was shown to contain five polypeptides, one of which has a molecular weight identical to the viral non-structural protein E (Rosenberg et al., 1972). This replicase was able to polymerize guanylate residues using GTP and a polycytidylate template. Analysis of the replicase extracted from poliovirus-infected cells showed the presence of predominantly one virus-specific polypeptide which co-migrated with the poliovirus equivalent of E (i.e. NCVP4; Lundquist et al., 1974). Recent studies with Mengo virus (Roesch and Arlinghaus, 1975), EMC virus (Traub et al., 1976) and FMDV (Polatnick et al., 1967) have also indicated a role for polypeptide E in the replicase activity. A primer-dependent RNA polymerase able to copy a poly(A) template has recently been isolated from poliovirus-infected cells (Flanegan and Baltimore, 1977). The identity of the viral-specific polypeptide(s) responsible for this activity was, however, not established.

Together, these observations suggest that the picorna-

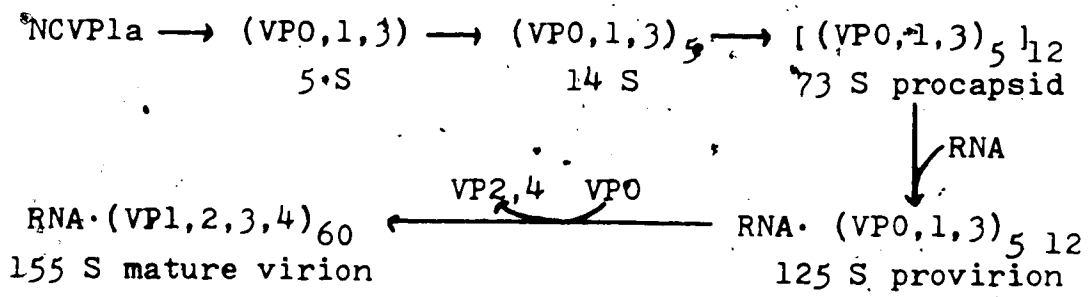
virus replicase is composed of a viral polypeptide (polypeptide E) in association with yet to be characterized cellular polypeptides.

Virion Assembly

Assembly of the picornavirion occurs in a series of steps in which individual polypeptides aggregate in equimolar proportions into structures of increasing size, which combine with RNA and ultimately form a complete virion. Caliguiri and Compans (1973) suggested that assembly occurs on the smooth cytoplasmic membranes, in conjunction with viral RNA replication.

Much of the assembly process has been clarified by an examination of sub-viral particles isolated from infected cells. Early studies on poliovirus assembly demonstrated the presence of a 5 S particle composed of one molecule each of the polypeptides VP0, VP1 and VP3 (Phillips et al., 1968). It was proposed that five of these 5 S particles aggregated to form the 14 S structure also isolated from infected cells (Phillips and Fennel, 1973; Phillips, 1971). In 1973, Maizel et al. examined the "empty capsids" extracted from poliovirus-infected cells and showed that they were composed of equimolar amounts of VP0, VP1 and VP3. Jacobson and Baltimore (1968b) subsequently demonstrated that the 73 S empty capsids accumulated in poliovirus-

infected HeLa cells to which 3mM guanidine hydrochloride had been added. Removal of guanidine resulted in a flow of radioactive label from empty capsids into mature poliovirions, suggesting that this structure, which they termed the "procapsid", was an assembly intermediate. Also found in poliovirus-infected cells was an entity, sedimenting at 125 S, called the "provirion" (Fernandez-Tomas and Baltimore, 1973). The provirion was identical to the 73 S procapsid except that it also contained a 35 S single-stranded viral RNA molecule. This data implies that during the assembly of poliovirus, RNA is inserted into a 73 S procapsid before the final proteolytic (morphogenetic) cleavage of VP0 to VP2 + VP4 which produces the mature virion. With the demonstration that 14 S particles could assemble into 73 S procapsids *in vitro* (Phillips, 1969, 1971), and the observation that this process was stimulated by addition of a rough cytoplasmic membrane fraction from infected cells (Perlin and Phillips, 1973), it appeared that the complete scheme for poliovirus morphogenesis had been resolved. This assembly sequence (Phillips, 1972; Casjens and King, 1975) is shown below.



However, some doubt exists as to the role of the 73 S procapsid in virion assembly. Poliovirus-infected MiO cells accumulated 14 S rather than 73 S particles in the presence of guanidine (Ghendon et al., 1972). Removal of the inhibitor resulted in a direct flow of radioactive label from the 14 S particles into mature 155 S virions without the appearance of 73 S procapsids. Thus, the procapsid may be a product of abortive assembly or a storage form of excess 14 S particles rather than an obligatory intermediate in assembly. Also, a direct kinetic precursor-product relationship between the 5 S and 14 S particles of poliovirus has yet to be demonstrated.

Stages in the assembly of cardioviruses appear to be somewhat different from those of poliovirus. McGregor et al. (1975) have shown that EMC virus-infected HeLa cells contain two capsid precursor particles sedimenting at 13 S and 14 S. Kinetic analysis showed that the 13 S particle (a pentamer of the capsid precursor polypeptide A) was converted into the 14 S particle (a pentamer of ϵ, α and γ polypeptides), demonstrating that in apparent contrast to poliovirus assembly, the precursor polypeptides aggregate before proteolytic cleavages occur. The model proposed by McGregor et al. (1975) for EMC virus assembly is shown below.

$$(A) \longrightarrow \underset{13\ S}{(A)_5} \longrightarrow \underset{14\ S}{(\epsilon\alpha\gamma)_5} \xrightarrow[\epsilon \quad \delta+\beta]{RNA} \underset{150\ S\ virion}{RNA\cdot\ (\delta\beta\gamma\alpha)_{60-n}(\epsilon\alpha\gamma)_n}$$

Cardiovirus-infected cells do not produce stable 73 S empty capsids. Also, *in vitro* treatments which produce empty capsids from purified enteroviruses do not form empty capsids from cardioviruses (Rueckert, 1976; Scraba and Colter, 1974).

More recently, however, additional subviral particles have been detected in cardiovirus-infected cells. A particle composed of five B polypeptide chains has been identified in cells infected with EMC or rhinoviruses (McGregor and Rueckert, 1977) and a 50 S particle composed of ϵ, α and γ polypeptide chains has been identified in Mengo virus-infected cells (Lee *et al.*, 1978).

Using this information as background, experiments described in this thesis were undertaken to characterize more fully the structure of the intact Mengo virion and its RNA. The disposition of the capsid polypeptides with respect to the external surface of the intact Mengo capsid have been examined by immunological and iodination techniques and the results compared to the data obtained with other picornaviruses. The non-capsid polypeptides E and F have been isolated and partially characterized in an attempt to assign enzymatic activities to these polypeptides.

II. ROUTINE MATERIALS AND METHODS

Tissue Culture Media

Sterilization procedures. All media were sterilized by filtration through nitrocellulose filters with a pore size of 0.22 microns (Millipore Corporation, Bedford, Ma.).

Growth medium. Eagle's basal medium (BME diploid) with Earle's salts and glutamine (catalogue number G-13) was obtained in powdered form from the Grand Island Biological Company, Grand Island, New York. The medium was dissolved in distilled, deionized water and sodium bicarbonate was added to a final concentration of 0.12% before filtration. Before use this medium was supplemented with:

(1) Horse serum (Flow Laboratories, Rockville, Md.) to a final concentration of 5% for growth of cells or 1% for production of virus;

(2) Penicillin G (Glaxco-Allenburys Ltd., Toronto, Ont.) and streptomycin sulfate (Sigma Chemical Co., St. Louis, Mo.) to final concentrations of 100 IU and 50 µg/ml respectively.

This medium was used for growth of cells in roller bottles, Blake bottles and tissue culture flasks.

Spinner medium. This medium, used for growth of cells in spinners, was identical to Eagle's basal 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.).

Amino acid deficient medium. Composition of the medium was similar to Eagle's basal medium (diploid) except that it contained twice the normal amount of calcium chloride (400 mg/l) and no amino acids other than glutamine. Sodium bicarbonate was added to a final concentration of 0.06% before filtration. Sterile medium was supplemented with 1% horse serum and antibiotics as described above.

Virus diluent. Phosphate-buffered saline (PBS) of Dulbecco and Vogt (1954) was supplemented with 0.2% bovine serum albumin fraction V (Gallard-Schlesinger Chemical Manufacturing Corp., Carle Place, N.Y.), 0.002% phenol red (J.T. Baker Chemical Co., Phillipsburg, N.J.) and penicillin and streptomycin sulfate at 200 IU and 100 µg/ml respectively.

Overlay diluent. This solution contained three times the normal concentration of Hank's salts, six times normal concentrations of both Basal Eagle's amino acids (Baltimore Biological Laboratory, division of Becton Dickinson and Company, Cockeysville, Md.) and MEM vitamin solution (Gibco), five times the usual concentrations of penicillin and streptomycin (i.e. 500 IU and 250 µg/ml respectively), 0.78% sodium bicarbonate and 30% inactivated (56° for 45 min.), calf serum.

Agar overlay. This was prepared by mixing 1 volume of overlay diluent with 2 volumes of a 1.6% solution of Noble agar (Difco Laboratories, Detroit, Mich.) in distilled water at 45°.

Cultured L Cells

Earle's L-929 strain of mouse fibroblasts (Sanford et al., 1948) were used for growth and plaque assay of Mengo virus. They were originally obtained from the American Type Culture Collection, Rockville, Md. Cells were maintained in monolayer culture in 1-liter Blake bottles (Kimble Products, Owens-Illinois Co., Toledo, Ohio). When the cultures had reached confluence the growth media was removed and the monolayers rinsed with a solution of 0.25% trypsin (Difco) in buffer containing 10 mM phosphate pH 7.4, 142.8 mM sodium chloride and 2.8 mM potassium chloride and incubated at room temperature until the cells began to detach (1 to 2 minutes). Cells from one bottle were resuspended in BME-5% H.S. and used to maintain the Blake bottle stock. The remaining cells were resuspended in spinner medium and transferred to 1- or 2-liter spinner flasks (Bellco Biological Glassware, Vineland, N.J.) at a concentration of 2×10^5 cells per milliliter. Cells were kept in suspension by means of a magnetic stirring device while growing at 37°. L cell monolayers were also grown in large cylindrical bottles (490 mm x 110 mm in diameter - Bellco Biological Glassware), coated with fetal calf serum (Flow Laboratories) prior to seeding in order to facilitate the attachment of cells. The roller bottles were either seeded directly with cells from Blake bottles (one Blake bottle of cells per roller bottle) or by cells harvested from spinner

cultures. In both cases approximately 10^8 cells in a volume of 100 ml fresh growth medium were added per roller bottle. The bottles were rotated at 0.5 rpm overnight after which the speed was increased to 1 rpm. Monolayers reached confluence after 48 hours of growth at 37°.

Virus

The M plaque-type variant of Mengo encephalomyocarditis virus, originally isolated by Ellem and Colter (1961) was used throughout these studies.

Virus growth in roller bottles. Growth medium was poured off confluent monolayers and was replaced by 20 ml of growth medium (supplemented with 1% horse serum) containing the virus inoculum (about 10^8 pfu per milliliter giving a moi of about 10). The bottles were rotated at 1 rpm for 20 to 24 hours by which time most cells had lysed; any remaining cells were dislodged from the glass by shaking, and the infected lysates were pooled.

Virus purification. The procedure used for virus purification has been described by Ziola and Scraba (1975) and involved methanol precipitation, treatment with α-chymotrypsin, differential centrifugation, sedimentation through sucrose and equilibrium centrifugation in Cs_2SO_4.

Preparation of radioactively labelled virus. Confluent monolayers of L cells in roller bottles were infected with

Mengo virus in the amino acid deficient medium described previously. Ninety minutes to 2 hours post infection, a mixture of ^{3}H- or ^{14}C-labeled amino acids (New England Nuclear, Montreal, Que., catalogue numbers NET-250 and NEC-445 respectively) were added to a final concentration of 2 µCi per milliliter and 0.2 µCi per milliliter respectively. The cells were rotated for an additional 20 to 24 hours before virus was harvested and purified.

Plaque assay of infectious virus. The procedure used was that described by Campbell and Colter (1965).

III. SURFACE STRUCTURE OF THE MENGO VIRION

Introduction

The arrangement of the polypeptides within the picornavirus capsid and the individual roles of each polypeptide have only recently begun to be examined and understood. It has been proposed that VP4 serves as a carrier of D (native) antigenicity on the surface of polio virions (Breindl, 1971b) and that it is the viral component which recognizes cellular receptors during attachment (Breindl, 1971a). Data obtained with eluted coxsackievirus B3 was also interpreted as indicating that VP4 was located on the surface of the native virion (Crowell and Philipson, 1971). Although this model was accepted by the Study Group on Picornaviridae (1975), recent work has challenged this interpretation. Talbot et al. (1973) showed by complement fixation that VP4 was not located on the surface of the native FMD virion. In addition, Lonberg-Holm and Butterworth (1976) and Beneke et al. (1977) have shown that iodination of native polio virions with ^{125}I labeled the VP1 polypeptide predominantly, with no labeling of VP4. Iodination of native bovine enterovirus particles labeled only VP1 (Carthew and Martin, 1974) while reaction with pyridoxal phosphate followed by reduction with ^{3}H sodium borohydride labeled VP1, VP2 and VP3 (Carthew, 1976). In no instance was any labeling of VP4 observed, indicating that none of its tyrosine, hystidine or lysine

residue are exposed on the external surface of the virus particle.

The identity of the asymmetric structure unit ($\alpha\beta\gamma$) and its disposition in the Mengo virus capsid (60 copies; T=1 icosahedral lattice) have been determined (Mak et al. 1974), but the detailed spatial relationships among the α, β, γ and δ polypeptides remain to be described. Results detailed in this chapter show that the surface of the intact Mengo virion is occupied primarily by the α polypeptides, and to a lesser extent by β. Both enzymatic iodination and immunological tests demonstrated this polypeptide distribution, and the immunological data indicates that the α polypeptides are primarily responsible for attachment of the virion to susceptible cells.

Materials and Methods

Lactoperoxidase-catalyzed iodination of intact and disrupted virions

These experiments were performed by B.R. Ziola. They are included in this thesis because the results are complementary to the immunological data.

Solid-state bovine lactoperoxidase was prepared by coupling the enzyme (Sigma Chemical Co.) to CNBr-activated Sepharose 4B (Pharmacia) by the procedure of David and Reisfield (1974). The preparation, having a final enzyme

concentration of 0.5 mg/ml of settled beads, was stored at 4° in PBS (Dulbecco and Vogt, 1954) containing 2×10^{-5} M Merthiolate. Aliquots of the enzyme preparation were washed twice with PBS prior to use.

Iodination of intact virions was accomplished by the addition, in sequence, of: 75 µl of PBS (or 75 µl PBS containing 4×10^{-5} M "carrier" NaI), 10 µl of solid-state lactoperoxidase suspension, 10 µl of ^{125}I (150 µCi; Amersham) and 6 µl of 0.15% H_2O_2 in PBS to 100 µg of purified virions in 200 µl of PBS. Reactions were allowed to proceed at room temperature for 1, 15 or 30 minutes; additional aliquots of H_2O_2 being added at 7.5 minute intervals for the latter two incubation times. During the reaction period, the mixtures were continuously agitated in order to keep the solid-state enzyme in suspension. Reactions were terminated by the addition of 50 µl of 0.5 M NaI and 0.2 M NaN_3 in PBS, followed by low speed centrifugation to remove the lactoperoxidase-sepharose beads. Iodinated virus was separated from free ^{125}I by centrifugation through a solution of 15% sucrose in PBS using a Spinco Type 50 rotor (45,000 rpm, 4 hours, 4°). The pelleted virus was resuspended in 0.01 M sodium phosphate (pH 7.4) containing 2% SDS, and aliquots containing 50 µg of protein were made 5% in β-mercaptoethanol, heated at 100° for 5 minutes and loaded onto 10% polyacrylamide -0.1% SDS gels prepared as described by Ziola and Scraba (1974).

Disrupted virions were prepared by heating 50 µg of

purified virus in 100 µl of 0.2% SDS in PBS at 100° for 7 minutes. The constituent polypeptides were iodinated as above following dilution to 200 µl with PBS. The iodinated polypeptides were separated from free ^{125}I by chromatography on a Sephadex G-25 (fine) column which was equilibrated with PBS containing 0.1% SDS. The polypeptide-containing fractions were dialyzed against 0.1% SDS in distilled water, then lyophilized. The iodinated polypeptides were resuspended in buffer, heated, and subjected to SDS-gel electrophoresis as described above.

Antisera

Antiserum specific for the 13.4 S viral capsid subunits [$(\alpha\beta\gamma)_5$] (Mak et al., 1974) was prepared by injecting rabbits with the subunits purified as described by Mak et al., 1971. The inoculation schedule consisted of an intramuscular injection of 500 µg of capsid subunits in Freund's complete adjuvant followed by an intravenous injection of 500 µg of subunits in PBS five weeks later. Blood was collected before the immunization procedure (for production of control serum) and two weeks after the intravenous injection.

Antiserum specific for an α + β polypeptide mixture was also prepared. The antigen was purified by heat-disrupting virus in the presence of 2% SDS and 5% β-mercaptoethanol followed by the separation of these two species from the other capsid polypeptides by electrophoresis in SDS-polyacrylamide gels. Remazol brilliant blue (RBBR)-myoglobin

and RBBR-lysozyme (Griffith, 1972) were used as markers to locate the region of the gels containing the α and β polypeptides. These gel segments were excised and emulsified mechanically in a 1:1 mixture of 0.1% SDS in 0.1 M sodium phosphate (pH 7.4) and Freund's complete adjuvant. A rabbit was innoculated intramuscularly and subcutaneously every 14 days with a total of approximately 400 μg of antigen. Blood was collected before immunization (for production of control serum) and one week following the sixth pair of injections.

Antisera specific for the individual α, β, γ and δ viral capsid polypeptides were prepared using the purified polypeptides (Ziola and Scraba, 1975, 1976) as antigens. Fractions from a SDS-hydroxylapatite column containing the individual polypeptides were dialyzed against 0.1% SDS in 0.01 M sodium phosphate (pH 7.4), and assayed for purity by analytical electrophoresis on SDS-polyacrylamide gels. The immunization schedule consisted of intravenous injection into rabbits of 100 μg aliquots of each polypeptide preparation. Concurrently, 100 μg aliquots of each polypeptide (20 μg in the case of δ) were emulsified with an equal volume of Freund's complete adjuvant and injected intramuscularly. The intramuscular injections were repeated six times at two-week intervals. Blood was collected before immunization (for control serum production) and one week after the last intramuscular injection.

In all cases, the blood was allowed to clot at room

temperature for one hour and then stored overnight at 4°. The clot was collected by centrifugation at 1000 g for 10 minutes and the serum supernatents removed. Serum was divided into 5-ml portions and stored at -20° in the presence of 0.01% sodium azide.

Immunodiffusion

The procedure used was a microadaptation of the double diffusion method of Ouchterlony (1948). A plexiglass template (1.5 x 1.5 inches with wells 3 mm in diameter containing stainless steel ball bearings) was supported on a 2 x 2 inches glass slide by short pieces of nylon fishing line. Melted agarose (1% in PBS or 0.1 M sodium phosphate, pH 7.2) was poured between the templates and slides and allowed to solidify. The ball bearings were then removed, the wells filled with the appropriate dilutions of antigen and antisera, and the slides incubated for 2 to 3 days at room temperature in humidified chambers. Following incubation, the templates were removed and the slides soaked overnight in PBS. The slides were stained with 0.25% Coomassie brilliant blue R (Sigma Chemical Co.) in 9% acetic acid-45% methanol and destained in 7.5% acetic acid-5% methanol.

Complement-fixation (CF) tests

A standardized diagnostic complement fixation procedure was used throughout these studies (Casey, 1965). Hemolysin and guinea pig complement were obtained from Flow Laboratories

(Inglewood, Ca.). Sheep blood was collected in an equal volume of Alsever's solution, and the red blood cells allowed to stabilize at 4° for 3 to 5 days before using. The antigens employed in the assays were freshly purified intact virions in 0.1 M sodium phosphate (pH 7.4) or virions which had been disrupted by heating at 56° for 10 minutes in 0.14 M sodium chloride, 0.02 M sodium phosphate (pH 6.2). The concentrations of the viral protein antigens were adjusted to approximately 230 µg/ml prior to making serial dilutions. The sera were diluted 10-fold with Veronal buffer and the endogenous complement activity inactivated by heating at 56° for 30 minutes. Serial dilutions of the inactivated sera were then made and used in the assays. Complement-serum controls indicated that the anti-β, -γ and -δ sera were anticomplementary at dilutions less than 1/80 to 1/160. This activity was removed by pretreatment with guinea pig complement (Schmidt, 1969).

Plaque-neutralization (PN) tests

A standard virus suspension was prepared by diluting purified virus to a concentration of 1200 PFU/ml, using virus diluent. Twofold serial dilutions of sera were made using virus diluent and 0.2 ml samples mixed with 0.2 ml aliquots of standard virus suspension. The mixtures were incubated at 37° for 1 hour and then assayed for their ability to form plaques in L cell monolayers (Campbell and Colter, 1965).

Plaque-neutralization enhancement (PNE) tests

In certain cases, viral infectivity is not neutralized by formation of a simple virus-antibody complex. In such instances, heterologous anti-globulin must be added to produce "neutralization enhancement" (Wadell, 1972). To test for such a phenomenon in this system, plaque neutralization mixtures were set up and incubated for 1 hour as described above. A further 0.2 ml of virus diluent containing a 200-fold dilution of goat anti-rabbit IgG serum (27.9 mg of antibody protein per milliliter of antiserum; Miles-Yeda Laboratories) was then added to each test serum dilution and these were then incubated at 37° for an additional hour. Aliquots were then assayed for plaque-forming ability as described above.

Hemagglutination-inhibition (HI) tests

The microtechnique of HI titration described by Sever (1962) was employed. The buffer used for making dilutions was 0.05 M H_3BO_3, 0.12 M KCl, pH 8.0. Sera were kaolin-adsorbed (Spence, 1968) prior to making the twofold serial dilutions. A virus suspension containing 4 hemagglutination units was added to the serial dilutions of sera and incubated at room temperature for 2 hours. A 0.5% (v/v) suspension of human type O erythrocytes in borate buffer (made from a standardized 20% suspension of erythrocytes in Alsever's solution) was added to the dilutions and incubated for an additional hour before reading the results.

Results

Iodination of Mengo Virions

Iodination of intact Mengo virions with ^{125}I results in the incorporation of label primarily into the α polypeptides (Figure 2A). The β polypeptides are labeled to a lesser extent, and only trace amounts of label are found in the γ and δ polypeptides. These labeling conditions (i.e., 1-minute labeling without carrier NaI) produce minimal capsid alterations so the labeling pattern observed probably reflects the native capsid conformation. Lactoperoxidase-catalyzed iodination labels the aromatic ring of tyrosine residues almost exclusively (Phillips and Morris, 1970). Mengo virus α, β, γ and δ polypeptides contain 11, 10, 11 and 3 tyrosine residues, respectively (Ziola and Scraba, 1975). Using these two observations, it is possible to calculate the relative accessibility of the tyrosine residues of each polypeptide to radioiodination (Table 2). If a random distribution of tyrosine residues in each polypeptide is assumed, it can be estimated that the α polypeptides occupy approximately 75% of the external surface of the virion and the β polypeptides, 25%. The tyrosine residues of the γ and δ polypeptides are apparently not exposed on the exterior of the virus capsid.

Addition of 1×10^{-5} M carrier NaI to the 1-minute enzyme-catalyzed reaction resulted in an increased labeling of the β polypeptides relative to the α polypeptides (approx-

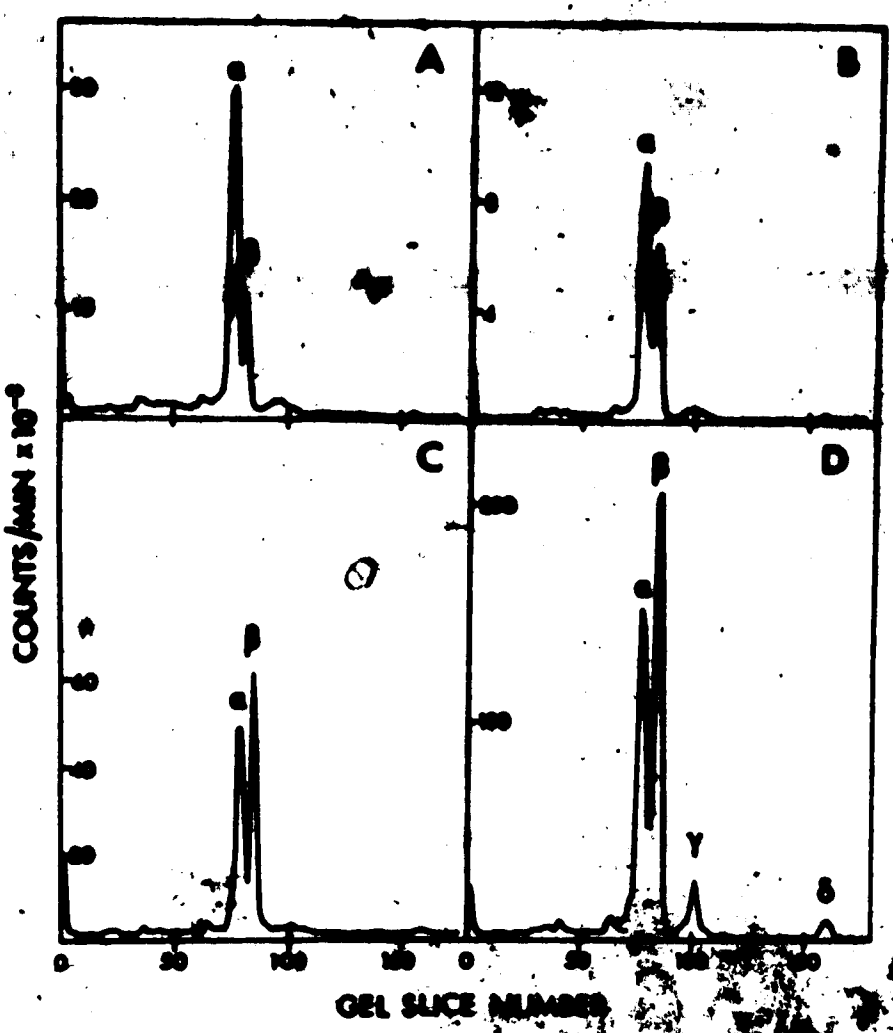


Figure 2. The polypeptides of Mengo virions labeled in vitro by lactoperoxidase-catalyzed iodination. The procedures and conditions for ^{125}I iodination are described in Materials and Methods. The recovered virions (50-μg aliquots) were disrupted by boiling in a solution containing 2% SDS and 5% β-mercaptoethanol, then layered onto 0.6 x 20 cm gels of 10% polyacrylamide-0.1% SDS and electrophoresed for 18 hr at a current of 6 mA per gel (Ziola and Scraba, 1974). Following electrophoresis, the gels were stained with Coomassie brilliant blue R (Weber and Osborn, 1969). Each gel was cut between the α and β polypeptide bands before being fractioned into 1 mm slices. The radioactivity in the gel slices was determined by counting in an LKB Wallac gamma counter. These experiments were all done on the same day, with the same batch of virus in order to standardize conditions and permit comparisons. Panel A: Iodination for 1 min; no carrier NaI present; panel B: Iodination for 1 min; 1 x 10^{-5} M NaI present; panel C: Iodination for 3 min; no carrier NaI present; panel D: Iodination for 3 min; 1 x 10^{-5} NaI present.

TABLE 2

LACTOPEROXIDASE-CATALYZED IODINATION OF INTACT AND DISRUPTED MENGO VIRIONS

Incubation Conditions	^{125}I Incorporation per tyrosine residue for polypeptide[a] α	β	γ	δ
1 min, minus carrier NaI	1.00	0.32	trace	trace
1 min, plus carrier NaI	1.00	0.37	trace	trace
15 min, minus carrier NaI	1.00	0.97	trace	trace
15 min, plus carrier NaI	1.00	1.13	0.18	0.23
Disrupted virus, 15 min, plus carrier NaI	1.00	0.89	1.00	0.26
Theoretical relative ^{125}I incorporation[b]	1.00	0.91	1.00	0.27

[a] Amino acid composition analyses have shown that the α, β, γ and δ polypeptides contain 11, 10, 11 and 3 tyrosine residues respectively (Ziola and Scraba, 1975). For each incubation condition, the actual radioactive counts have been normalized to a value of 1.00 per tyrosine residue for the α polypeptide. The values shown for the other polypeptides are counts/min/tyrosine residue relative to α.

[b] These are the expected relative iodination values for the capsid polypeptides from disrupted virions. Since α, β, γ and δ are present in equimolar proportions in the capsid (Ziola and Scraba, 1974, 1975), the theoretical relative ^{125}I incorporation was calculated directly from the number of tyrosine residues in each polypeptide.

imately 60% that of α; Figure 2B; Table 2). The increased labeling is probably due to a conformational rearrangement of the capsid polypeptides induced by the incorporation of iodine atoms which resulted in additional tyrosine residues becoming accessible.

The iodination-induced conformational rearrangement was also demonstrated using a longer incubation time. After a 15 minute reaction period, in the absence of carrier NaI, the β polypeptides were labeled to nearly the same extent as the α polypeptide (Figure 2C, Table 2). Upon addition of carrier NaI, the labeling of the γ and δ polypeptides also began to occur (Figure 2D, Table 2). In spite of the conformational rearrangements, the virions were still largely intact since they could be recovered following sedimentation through 15% sucrose. However, iodination for 30 minutes (with or without carrier NaI present) produced a degradation of the virus capsid such that intact virions could not be recovered following sedimentation through 15% sucrose. This phenomenon has also been observed in the case of bovine enterovirus (Carthew and Martin, 1974).

Iodination of virus disrupted by heating in SDS resulted in a labeling of all four capsid polypeptides in the relative amounts expected from their tyrosine contents (Table 2). Thus, the observed differences in labeling of intact virions probably reflects the exposure of tyrosine residues of the individual capsid polypeptides to the external environment rather than an intrinsic difficulty in

iodination of individual tyrosine residues.

Immunodiffusion

The results of [redacted]no diffusion tests using whole and disrupted Mengo virions as antigens are shown in Figures 3A and B respectively. They demonstrate that all the specific antisera have activity against disrupted virions, but that only the anti-α, anti-β and anti-αβ sera react with native virions. Thus, the antigenic determinants of the α and β polypeptides are located on the external surface of the virion, while those of the γ and δ polypeptides are not.

CF tests

The serum and antigen titers obtained using the specific sera are shown in Table 3. Table 3A shows that, with the exception of the anti-δ serum, all sera tested had complement-fixing titers of approximately the same order of magnitude. This suggests that the differing sera reactivities measured by the other immunological techniques (see below) were not due to widely varying antibody titers or to ar absence of specific antibodies.

Results shown in Table 3B indicate that the surface of the intact Mengo virion is occupied primarily by the α and β polypeptides. Disruption of the viral capsid produced a significant increase in the antigen titers for the β and δ polypeptides. Thus, while at least one part of each of the β polypeptides is exposed to the external environment in the

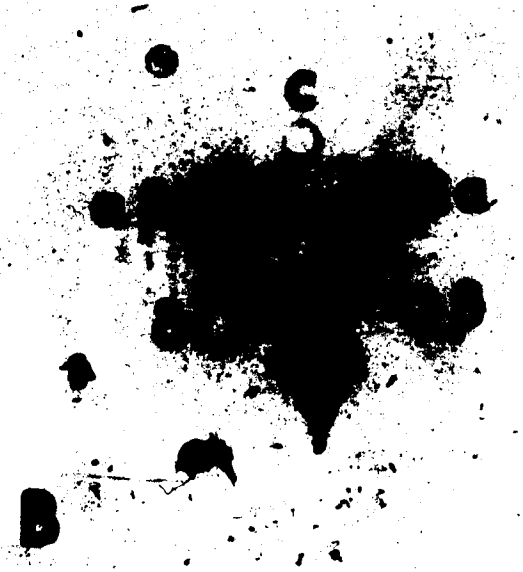


Figure 3. Immunodiffusion analyses of whole and dissociated Mengo virions. Panel A: The center well contained intact virions in 0.1 M sodium phosphate, pH 7.2. Specific antisera (in the outer wells) were dialyzed against this buffer before being applied. "C" indicates control (preimmunization) rabbit serum. Panel B: The buffer used in this case was PBS, pH 6.2. After extensive dialysis at room temperature against this buffer, the Mengo virion capsids had largely dissociated into $(\alpha\beta\gamma)_5$ subunits, as assayed by electron microscopy. The dissociated virus suspension was added to the center well, and specific antisera (also dialyzed against PBS) to the outer wells. "C" indicates control serum.

TABLE 3

COMPLEMENT-FIXATION TESTS

(A) SERUM TITERS[a] VS. DISRUPTED VIRUS

Serum	Titer
Anti-αβγ	1280
Anti-α	640
Anti-β	1280
Anti-β	1280
Anti-γ	320
Anti-δ	<20
Control[c]	<20

(B) "ANTIGEN TITERS"[b] VS. INTACT AND DISRUPTED VIRUS

Serum	Intact Virus	Disrupted Virus
Anti-α	8	16
Anti-β	32	256
Anti-γ	<4	16
Anti-δ	<4	<4
Control[c]	<4	<4

[a] Reciprocal of the highest dilution of serum producing 50% hemolysis

[b] Reciprocal of the highest dilution of antigen producing 50% hemolysis at maximum serum titer

[c] Control titers are those of pre-immunization rabbit serum

intact virion, a second part of each must remain buried in the interior of the capsid and thus be inaccessible to specific antibodies. The antigenic determinants of the γ polypeptides appear not to be exposed to the external environment since anti-γ serum produces a positive CF result only when the virus is disrupted. The fact that the antigen titer for the anti-α serum remains about the same whether the virion is intact or disrupted suggests that most of the α determinants are exposed on the surface of the native virion.

No conclusions about the location of the δ polypeptide could be drawn from these studies, since no CF titer was observed for the anti-δ serum. Attempts to demonstrate CF activity at antigen dilutions less than 1:4 were not successful because of the anti-complementarity of the concentrated virus in the antigen controls (which was not alleviated by pretreatment with guinea pig complement). The anti-δ serum did, however, exhibit activity in the immunodiffusion test using disrupted virus. This difference is probably explained by the high sensitivity of the immunodiffusion test compared to the CF test (Schmidt, 1969).

PN, PNE and HI tests

Data obtained from plaque-neutralization, plaque-neutralization enhancement and hemagglutination-inhibition experiments indicate that only antibodies specific for the α polypeptides were able to block the attachment of virus

to susceptible cells (Table 4). Antibodies specific for the β polypeptide were not able to do so, even though they were able to produce positive results in the immunodiffusion and CF tests.

The very high relative titers obtained with the anti-αβγ serum are probably due to the fact that the antigen used to produce this serum was in an undenatured form, while all the other sera were produced using SDS-denatured polypeptide antigens. The low titers of the anti-αβ and anti-α sera were probably not due to the respective omission of γ and βγ polypeptides from the immunizing antigen mixture since the addition of anti-β and/or anti-γ serum to the anti-α serum (or, similarly, the addition of anti-γ serum to the anti-αβ serum) did not produce an increase in the PN, PNE or HI titers. Also, the similarities in CF titer for the anti-α, -β and -γ sera indicate that the inability of the β and/or γ polypeptides to produce neutralizing antibodies was not due to their denaturation by SDS, since the α polypeptide was in a similar state. Thus, the PN, PNE and HI tests demonstrate that the α polypeptides alone are responsible for the initial attachment of Mengo virions to receptors on L cells and human erythrocytes.

Discussion

Results of the solid state lactoperoxidase-catalyzed

TABLE 4

PLAQUE-NEUTRALIZATION (PN), PLAQUE-NEUTRALIZATION ENHANCEMENT (PNE) AND HEMAGGLUTINATION-INHIBITION (HI) TITERS OF ANTISERA[a] AGAINST MENGO CAPSID POLYPEPTIDES

Serum	PN titer[b]	PNE titer[b]	HI titer[b]
Anti-$\alpha\beta\gamma$	204,800	N.T.[c]	2560
Anti-$\alpha\beta$	12,800	N.T.	160
Anti-α	800	3200	40
Anti-β	100	200	<10
Anti-γ	100	200	<10
Anti-δ	100	200	<10
Control (preimmunization) serum	100	100	<10

[a] Expressed as the reciprocal of the highest dilution of serum resulting in a 50% inhibition of virus plaque formation.

[b] Expressed as the reciprocal of the highest dilution of serum that blocked hemagglutination by 4 HAU of virus. Human type O cells (as 1.5% suspensions in borate buffer) were used.

[c] N.T., not tested.

iodination of intact Mengo virions has demonstrated that the surface characteristics of the virion are contributed mainly by the α and - to a lesser extent - the β polypeptides. This finding is in general agreement with that reported for several other picornaviruses. Iodination of rhinovirus type 2 resulted in incorporation of ^{125}I primarily into VP2 polypeptides, with small amounts of radioactivity being found in the VP1 and VP3 polypeptides (Lonberg-Holm and Butterworth, 1976). Similar experiments with FMDV (Talbot et al., 1973; Sanger et al., 1976), bovine enterovirus (Carthew and Martin, 1974) and poliovirus (Lonberg-Holm and Butterworth, 1976) all demonstrated almost exclusive iodination of VP1. In no instance was ^{125}I incorporated into the VP4 polypeptides of an intact picornavirus. The lack of labeling was not due to a lack of tyrosine residues in this polypeptide species since VP4/VP0 in empty capsid structures did incorporate label (Carthew and Martin, 1974; Lonberg-Holm and Butterworth, 1976). Empty capsids are not produced in vivo by Mengo virus, nor can they be formed in vitro, but each of the δ polypeptides does contain three tyrosine residues (Ziola and Scraba, 1975), and these are readily iodinated when the capsid structure is disrupted (Figure 2D). Thus a general feature of the picornavirus capsid appears to be that one of the three largest polypeptide species contributes most of the external surface characteristics, with one or both of the others making minor contributions.

Some degree of caution is necessary, however, when interpreting the results of iodination experiments. Unless the correct labeling period and reaction conditions are chosen, extensive iodination can cause extensive conformational changes and even degradation of the capsid structure (Carthew and Martin, 1974; Table 2). Thus polypeptides which normally do not have tyrosine residues exposed to the external environment may appear to be part of the external capsid structure. In addition, the assumption that the tyrosine residues are randomly distributed along a polypeptide chain may not be correct, and a polypeptide which is partially exposed on the exterior of the capsid may not be detected if all of its tyrosine residues are buried in the interior.

Reactions of intact Mengo virions with antisera specific for individual polypeptides have confirmed the iodination results. Immunodiffusion and complement fixation experiments showed that only the α and β polypeptides are exposed on the external surface of the capsid (Figure 3A, Table 3B). The γ and δ polypeptides appear to occupy internal locations, and their antigenic determinants are only exposed to the external environment when the capsid is disrupted (Figure 3B, Table 3B). Thus, neither the tyrosine residues nor the antigenic determinants of the γ and δ polypeptide species are located on the external surface of the virion.

Rowlands et al. (1971) showed by studies on the

immunogenicity of trypsin-treated FMDV that VP1 was responsible for the stimulation of neutralizing antibodies to the virus.

Results of the HI, PN and PNE tests reported here demonstrated that the analogous α polypeptides are responsible for the attachment of Mengo virions to susceptible cells (Table 4). Antisera specific for the β, γ or δ polypeptides did not contain neutralizing activity. This is especially interesting in the case of β, which was shown by CF and immunodiffusion tests to be at least partially exposed on the exterior of the intact capsid. Apparently, binding of anti-β antibodies to the surface of the Mengo virion does not inhibit its attachment to cellular receptors.

It has been demonstrated that the ability of polio- and rhinovirions to attach to cells is dependent on the native or D conformation of the virus capsid. Alteration of the conformation to the C form results in a loss of infectivity. The location of the site(s) responsible for attachment to cells and for binding of neutralizing antibodies in the D form has been the subject of some controversy. Breindl (1971a,b) proposed that the VP4 polypeptides expressed these activities, since the D to C conformational change was accompanied by the loss of VP4 (Crowell and Philipson, 1971; Korant et al., 1972). An alternative model has been proposed in which the D and C forms reflect different polypeptide conformations on the surface of the virion, with the attachment of virions to cells being

dependent on the presence of the entire intact D surface conformation (Noble and Lonberg-Holm, 1973; Korant et al., 1975; Butterworth et al., 1975). Enzymatic iodination studies of empty capsids (C conformation) provided data which is consistent with the hypothesis. Naturally-occurring empty capsids of bovine enterovirus showed increased labeling of VP0 and VP3; in contrast to the native virion which was labeled in VP1 only (Carthew and Martin, 1974). Artificially produced empty capsids of poliovirus incorporated an increased amount of ^{125}I into the VP2 polypeptides, while intact virus was labeled primarily in the VP1 polypeptides (Lonberg-Holm and Butterworth, 1976; Beneke et al., 1977). In no case was VP4 in an intact virion labeled by surface-active reagents. The presence of two isoelectric forms of picornaviruses (Mandel, 1971; Chlumecka et al., 1973; Korant et al., 1975) is also consistent with the second model.

The inability of cardioviruses to form stable empty capsids has prevented the extension of the experiments described above to the Mengo virion. However, the data presented in this chapter is still compatible with the second model, with the qualification that only one polypeptide, α, possesses the immunogenic and cell attachment sites. The same would appear to be true for FMDV (Cavanagh et al., 1977).

IV. ISOLATION AND PARTIAL CHARACTERIZATION OF MENGOVIRUS-SPECIFIC POLYPEPTIDES E, F AND VPg

Introduction

The discovery of an RNA-dependent RNA polymerase (RNA replicase) activity in picornavirus-infected cells (Baltimore and Franklin, 1962; Baltimore et al., 1963b; Dalgarno and Martin, 1965) has prompted much research in an effort to identify and characterize the viral polypeptide(s) responsible for this activity. Results of such studies have suggested that NCVP4 (E) (Rosenberg et al., 1972; Lundquist et al., 1974; Loesch and Arlinghaus, 1975; Traub et al., 1976; Polatnick et al., 1967), NCVP1 (A) (Röder and Koschel, 1975) or NCVP2 (D) (Korant, 1975) plays some role in RNA replicase activity. However, in only two instances has a template-dependent replicase activity been demonstrated (Traub et al., 1976; Flanegan and Baltimore, 1977).

The covalent linkage of a small protein (termed VPg) of molecular weight about 4,000 to the 5'-end of virion RNA has been reported for EMC, polio and FMD viruses (Hruby and Roberts, 1978; Lee et al., 1976; Sanger et al., 1977). It has been suggested that VPg may play some role in initiation of viral RNA synthesis (Flanegan et al., 1977; Nomoto et al., 1977).

Comparatively little work has been done to examine the proteolytic processes by which the stable capsid and noncapsid

proteins are generated in infected cells. Korant [redacted] that the initial cleavages are probably mediated by cellular proteases (1972) and that the secondary ones are more likely mediated by viral-specific enzyme (1972). The identity of the [redacted] established, although Sinsheimer and Scraba (1976) and Pelham (1978) have suggested that P is a possible candidate for the viral protease.

This chapter is a report of the isolation of the Mengo virus noncapsid polypeptides E and P from infected cell lysates. This was accomplished by chromatographic procedures using denaturing and non-denaturing conditions. Partial characterization of the polypeptides isolated using the non-denaturing conditions is also reported. Results indicate that the Mengo virus polypeptide E possesses RNA polymerase activity that is dependent upon exogenous viral RNA template and primer.

Evidence is also presented that a VPg protein is attached to the RNA of Mengo virus.

Materials and Methods

Preparation of infected cell extracts

Confluent monolayers of L cells in roller bottles were infected at a m.o.i. of 100 pfu/cell with M-Mengo virus suspended in a 5.0 ml of virus diluent. The bottles were

[illegible] for 1 hr to allow virus attachment. They were [illegible] with warm (37°) PBS and overlayed with [illegible] horse serum. After 3.5 hr post-[illegible] was replaced with 10 ml amino acid-[illegible] (Sigma), and the monolayers incubated for one hr to deplete the intracellular pools of amino acids. At 4.5 hr post-infection, the cells were pulse-labeled by removing the amino acid-deficient medium, rinsing the monolayers once with warm (37°) PBS and incubating with 10 ml of amino acid-deficient medium containing 1% horse serum, 25 mM HEPES and 5 μCi/ml ^{14}C-amino acids (NEC 445, New England Nuclear) for 0.5 hr. At 5.0 hr post-infection the labeling medium was removed, the monolayers washed once with warm (37°) PBS and then incubated for 1 hr with BME containing 1% horse serum and 10^{-6} M pactamycin (a gift from the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute, U.S.A.). At 6.0 hr post-infection the medium was removed and the monolayers washed three times with 25 ml cold (4°) PBS. Cells were collected from roller bottles by scraping into a fourth aliquot of cold PBS and pelleting by low speed centrifugation. The washed cells were lysed with either PBS (minus Ca^{+2} and Mg^{+2} salts) containing 1% NP-40 (Shell Oil of Canada, Ltd.) or 0.05 M TRIS base, 0.05 M sodium phosphate (pH 8.0) containing 0.05 M KCl and 1% NP-40 ("lysis buffer"; 1 - 2 ml per roller bottle of cells). Nuclei were removed by low speed centrifugation

(1000 g for 10 min) and the lysate clarified by centrifugation in a SW 50.1 rotor at 37,000 rpm at 4° for 1 hr. The supernatant from this step, denoted S_{37}, was subsequently subjected to affinity chromatography as outlined below.

Affinity chromatography of cell lysates

Antiserum directed against the capsid proteins of Mengo virus was prepared in rabbits using heat-disrupted (56° for 10 min) virions as antigen. Immunization was initiated by injection of 250 µg protein intravenously plus 250 µg intramuscularly; the intramuscular injection aliquot being suspended in an equal volume of Freund's complete adjuvant and PBS. Fourteen days after the first injections, an additional 250 µg of disrupted virus antigen was injected intravenously, followed at 4 day intervals by additional intravenous injections. Blood was collected one week after the fourth intravenous injection, and serum prepared by allowing it to clot at room temperature for 1 hr and then stand overnight at 4° before centrifuging (1000 g for 10 min). The IgG fraction was purified from serum by ammonium sulfate precipitation followed by chromatography on DE-52 cellulose (Whatman Biochemicals Ltd.) and lyophylization of the fractions containing anti-Mengo IgG's.

IgG from 20 ml of immune serum was resuspended in 15 ml of 0.01 M sodium phosphate (pH 7.2) and mixed overnight at 4° with 1 g of Affi-Gel 10 (Bio-Rad Laboratories), a N-hydroxysuccinimide ester of agarose to which the IgG couples

through its free amino groups (Cuatrecasas and Parikh, 1972). Following coupling, the gel-immobilized antibody was washed with PBS and poured into a column with dimensions (diameter x length) of 0.8 x 25 cm. The column was washed with 0.1 M TRIS-HCl containing 3.0 M NaSCN and 1% NP-40 ("elution buffer") and then equilibrated with the appropriate lysis buffer.

S_{37} infected cell lysates (2 - 3 ml) were loaded onto the column at 4° and washed through at a flow rate of 0.5 - 1.0 ml/hr. The protein peak, as monitored by radioactivity, was pooled and used in the procedures detailed below. The column was recycled by washing with elution buffer followed by equilibration with lysis buffer.

SDS-hydroxylapatite chromatography

When used in this procedure, the infected-cell lysate was made in PBS (minus Ca^{+2} and Mg^{+2} salts) containing 1% NP-40, and the affinity column was equilibrated with the same buffer.

A 1 ml aliquot of the noncapsid polypeptide peak from the affinity column was made 2% SDS and 5% β-mercaptoethanol, then heated at 100° for 5 min. The solution was then diluted 10-fold with 0.01 M sodium phosphate (pH 6.2), and loaded onto a 0.8 x 15 cm column of DNA grade hydroxylapatite (Bio-Rad Laboratories) equilibrated with 0.01 M sodium phosphate (pH 6.2) containing 0.1% SDS and 1 mM dithiothreitol ("column buffer"). After washing with 3 to 4 bed volumes of column

buffer, the polypeptides were eluted at a flow rate of 4 ml/hr with a linear gradient derived from 0.1 M sodium phosphate (pH 6.2) and 0.6 M sodium phosphate (pH 6.2), both buffers containing 0.1% SDS and 1 mM dithiothreitol (DTT). Aliquots of fractions were spotted on filter discs which were dried and counted using a toluene-based fluor. Conductivity measurements of selected fractions were converted to molarity of phosphate by using a standard curve. Selected peaks from the column were pooled as indicated in Figure 9, dialyzed against 0.1% SDS in distilled water and lyophilized. Aliquots were resuspended in 0.01 M sodium phosphate (pH 7.2) containing 2% SDS and 5% β-mercaptoethanol, heated at 100° for 5 min and subjected to electrophoretic analysis on 10% polyacrylamide-0.1% SDS gels as described by Ziola and Seraba (1974).

SDS-Sephadex gel filtration chromatography

Lyophylized pools 2 and 3 from the SDS-hydroxylapatite column were each suspended in 0.3 ml distilled water, and SDS and β-mercaptoethanol added to 2% and 5% final concentrations, respectively. The resuspended material was heated at 100° for 5 min and then loaded onto separate 1.5 cm x 80 cm columns of G-100 superfine Sephadex (Pharmacia) equilibrated with 0.1 M sodium phosphate (pH 7.2) containing 0.1% SDS and 1 mM DTT. The columns were operated at a flow rate of 4 ml/hr. and fractions of 1.0 ml were collected and monitored for radioactivity as described above. Aliquots were removed from

each of the pooled regions indicated in Figures 13 and 14, dialyzed against 0.1% SDS in distilled water, and lyophilized. The SDS-protein residues were resuspended in 0.01 M sodium phosphate, (pH 7.2) and the final SDS and β-mercaptoethanol concentrations adjusted to 2% and 5% respectively. Following heating at 100° for 5 min, the polypeptide composition of each peak in Figures 13 and 14 was determined by electrophoresis in 10% polyacrylamide-0.1% SDS gels.

Bio-Gel A-5m filtration chromatography

Infected-cell lysates produced for this procedure were either suspended in PBS (minus Ca^{+2} and Mg^{+2} salts) containing 1% NP-40, or 0.05 M TRIS-HCl (pH 8.0) containing 0.05 M KCl and 1% NP-40, or 0.05 M TRIS-HCl (pH 8.0) containing 0.05 M sodium phosphate, 0.05 M KCl and 1% NP-40. The affinity chromatography column was equilibrated with the same buffer in which the cell lysate was made. A 1.5 x 50 cm column of Bio-Gel A-5 m, 200 - 400 mesh (Bio-Rad Laboratories) was also equilibrated with the same buffer in which the cell lysate was made. A 1.0 ml aliquot of the non-capsid polypeptide peak from the affinity column was chromatographed at 4° on the Bio-Gel column using a flow rate of 6.0 ml/hr. One ml fractions were collected and monitored for radioactivity.

Aliquots from selected areas of the peaks were made 2% SDS and 5% β-mercaptoethanol, heated at 100° for 5 min and then dialysed against 0.01 M sodium phosphate (pH 7.2) con-

taining 2% SDS and 5% β-mercaptoethanol. The dialyzed aliquots were subjected to electrophoretic analysis in 10% polyacrylamide-0.1% SDS gels.

Assay of polymerase activity

In a standard reaction mixture, 100 µl of enzyme preparation was added to 100 µl of assay mixture, the final concentration of reagents being: 0.05 M TRIS-HCl (pH 8.0), 0.05 M KCl, 0.005 M DTT, 0.5 mM GTP, 0.5 mM CTP, 0.5 mM ATP, 20 µCi ^{3}H-UTP/ml, 0.01 M $MgCl_2$, 2 µg actinomycin D/ml, 1.0 mM phosphoenolpyruvic acid (PEP), and 20 µg pyruvate kinase/ml. In those assays in which they were included, the final concentrations of Mengo viral RNA and oligo(rC)$_{11-19}$ were 20 µg/ml and 10 µg/ml, respectively. The reaction mixture was incubated at 37°, and at appropriate intervals an aliquot of 40 µl was removed and spotted onto filter paper which had been soaked with a solution containing 0.1 M sodium EDTA and 25 mM sodium pyrophosphate (pH 7.0), then dried. The filter paper discs were washed for 30 min with cold (0°) 10% trichloroacetic acid (TCA), twice for 10 min with cold 5% TCA and finally with 95% ethanol. The discs were allowed to dry and the TCA-insoluble radioactivity measured using a toluene-based fluor.

The unlabeled nucleotides were obtained from Terochem Laboratories Limited, DTT was from Bio Rad, ^{3}H-UTP (NET-380) was from New England Nuclear, actinomycin D was from Mann Research, PEP and pyruvate kinase were from Sigma, and

oligo$(rU)_{11-19}$ was from Collaborative Research. Mengo viral RNA was prepared from freshly purified Mengo virions as described by Scraba et al., (1967). Sedimentation velocity runs of the viral RNA were carried out in a Spinco model E ultracentrifuge before use in the assay in order to ensure that the viral RNA was intact (35 S) and homogeneous.

Poly(A) Sepharose column chromatography

Infected cell extract was prepared as outlined above, the only difference being that the lysis buffer in this case was 0.05 M [illegible] (pH 8.0) containing 0.05 M KCl and 1% NP-40. A column of poly(A) sepharose (Pharmacia) of dimensions 0.8 [illegible] cm was prepared and washed with 0.1 M TRIS-HCl (pH 8.0) containing 90% formamide. The column was then equilibrated with the lysis buffer. A one-ml aliquot of the S_{37} infected-cell lysate was loaded onto the column, which was then washed with lysis buffer (4°) at a flow rate of 6 ml/hr. After collecting 20 1-ml fractions, the buffer was changed to 0.05 M TRIS-HCl (pH 8.0) containing 1.0 M KCl and 1% NP-40 and an additional 20 fractions were collected. The fractions were monitored for radioactivity, and aliquots of the radioactive peaks were made 2% SDS and 5% β-mercaptoethanol, heated at 100° for 5 min and then dialyzed against 0.01 M sodium phosphate (pH 7.2) containing 1% SDS and 5% β-mercaptoethanol. The polypeptide components of each peak were analysed by electrophoresis in 10% polyacrylamide-0.1% SDS gels.

Isolation of VPx

Purified ^{14}C-amino acid-labelled Mengo virions were disrupted by heating at 100° for 5 min in a buffer consisting of 0.01 M sodium phosphate (pH 6.8) containing 2% SDS and [illegible]. The RNA and polypeptides were separated by SDS-hydroxylapatite chromatography as described by Ziola and Scraba (1975). The RNA peak, as detected by absorbance at 260 nm, was pooled and dialysed extensively against 0.01 M sodium phosphate (pH 7.2) to remove the SDS. RNA was precipitated by the addition of 2½ volumes of cold 95% ethanol and storage overnight at -20°. It was then collected by centrifugation at 15,000 rpm for 30 min in a JA-20 rotor (Beckman) at 4°. The RNA pellet was resuspended in 400 µl of 0.01 M TRIS-HCl (pH 7.0) and divided into two equal aliquots. RNase A (P-L Biochemicals), RNase T1 (P-L Biochemicals) and bovine serum albumin (Sigma) were added to each sample at final concentrations of 50 µg/ml, 100 units/ml and 400 µg/ml, respectively. Both samples were incubated in polypropylene tubes at 37° for 1½ hr. Proteinase K (BDH Chemicals) was then added to one sample to a final concentration of 200 µg/ml, and both samples were incubated for an additional 1½ hr at 37°. Following incubation, the samples were precipitated by the addition of 5 volumes of acetone. The precipitated products were then analyzed by electrophoresis in 10% polyacrylamide-0.1% SDS gels.

Results

Separation of Mengo virus non-capsid proteins from capsid proteins

A representative SDS polyacrylamide gel pattern of an infected cell lysate (labeled with ^{14}C amino acids) before the 37,000 rpm centrifugation step is shown in Figure 4. This pattern is identical to that observed previously for Mengo virus (Paucha et al., 1974) and is very similar to those produced by other picornaviruses (Summers and Maizel, 1968; Butterworth et al., 1971). Pactamycin (10^{-6} M) was added at 5 hr p.i. to prevent any further initiation of viral RNA translation (MacDonald and Goldberg, 1970; Taber et al., 1971; Paucha et al., 1974), and all pre-existing precursor polypeptides were cleaved to stable end-products during the subsequent 1 hr chase period (Figure 4). This procedure was used because the capsid precursors were not totally removed from the lysate during the subsequent affinity chromatography step.

Centrifugation of the lysate at 37,000 rpm for 1 hr removed those cellular structures whose sedimentation coefficient was greater than 60 S (ribosomes, polysomes, mitochondria). Also found in the pellet was a majority of the viral capsid proteins (Figure 5). The supernatant from this step contained primarily the stable non-capsid polypeptides, but varying residual amounts of the capsid polypeptides were also present (Figure 6).

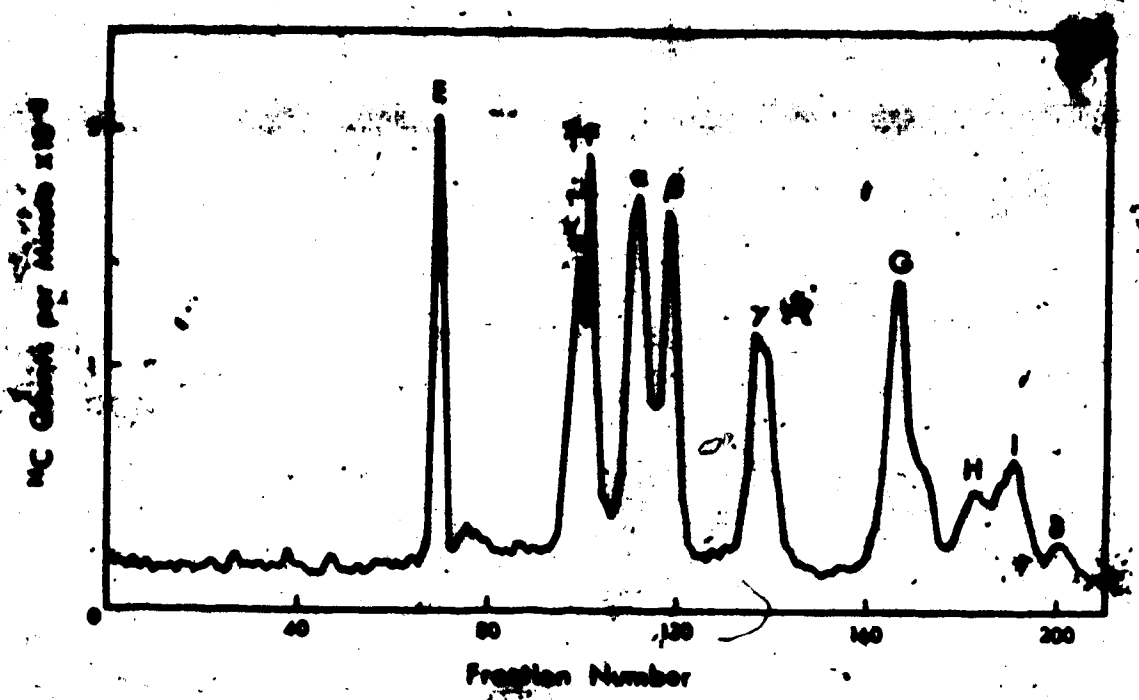


Figure 4. Electrophoresis in 0.1% S[illegible]% polyacrylamide gels of an L cell lysate at the initial stage of purification (i.e. before the 37,000 rpm centrifugation step). Infected cells were pulse-labeled with ^{14}C-amino acids for 30 min at 4.5 hr post-infection, and then incubated for 1 hr in the presence of unlabeled amino acids and 10^{-6} M pactamycin. Following electrophoresis at 8 mA/gel for 20 hr, the gels were frozen on dry ice and sliced into 1 mm fractions using a brass template. The slices were incubated overnight at 50° with 0.3 ml of 5.7% water in NCS tissue solubiliser (Amersham). Radioactivity was measured following the addition of 5 ml of toluene-based fluor to each gel slice.

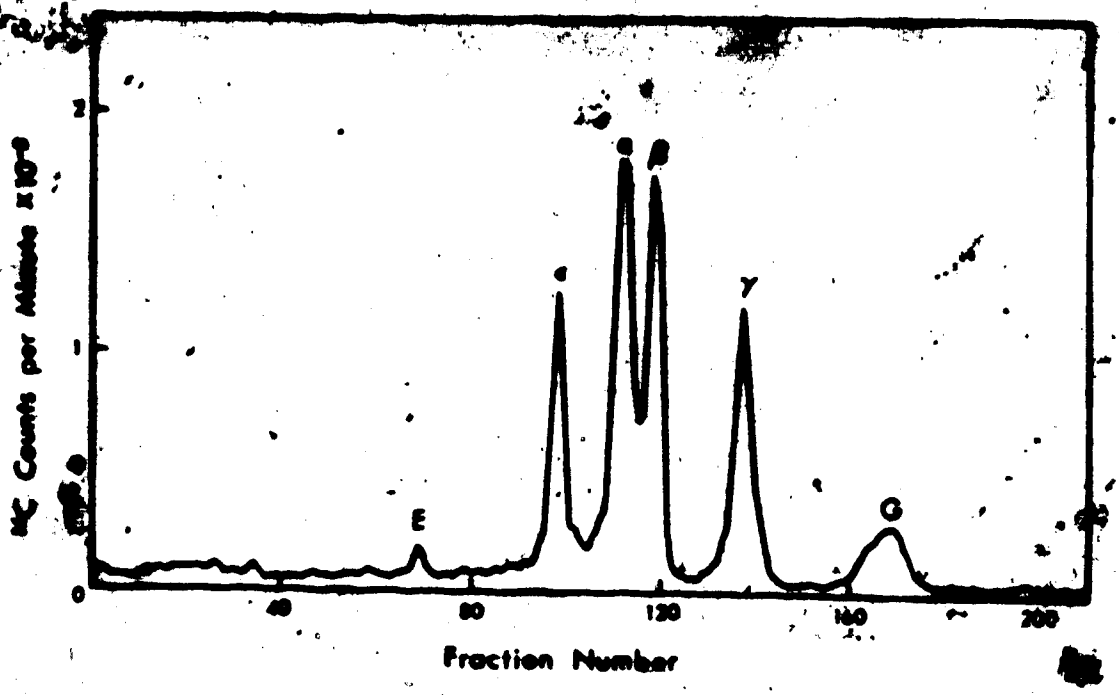


Figure 5. Electrophoretic analysis on 0.1% SDS-10% polyacrylamide gels of the pellet obtained from the 37,000-rpm centrifugation step. Lysates of infected cells (labeled with ^{14}C-amino acids) were centrifuged at 37,000 rpm for 1 hr in a SW 50.1 rotor at 4°. The pellet was resuspended in 0.01 M sodium phosphate (pH 7.2) containing 2% SDS and 5% β-mercaptoethanol and heated at 100° for 5 min before being loaded onto the gels. Electrophoresis was at 8 mA/gel for 20 hr; subsequently 1 mm gel slices were obtained and assayed for radioactivity.

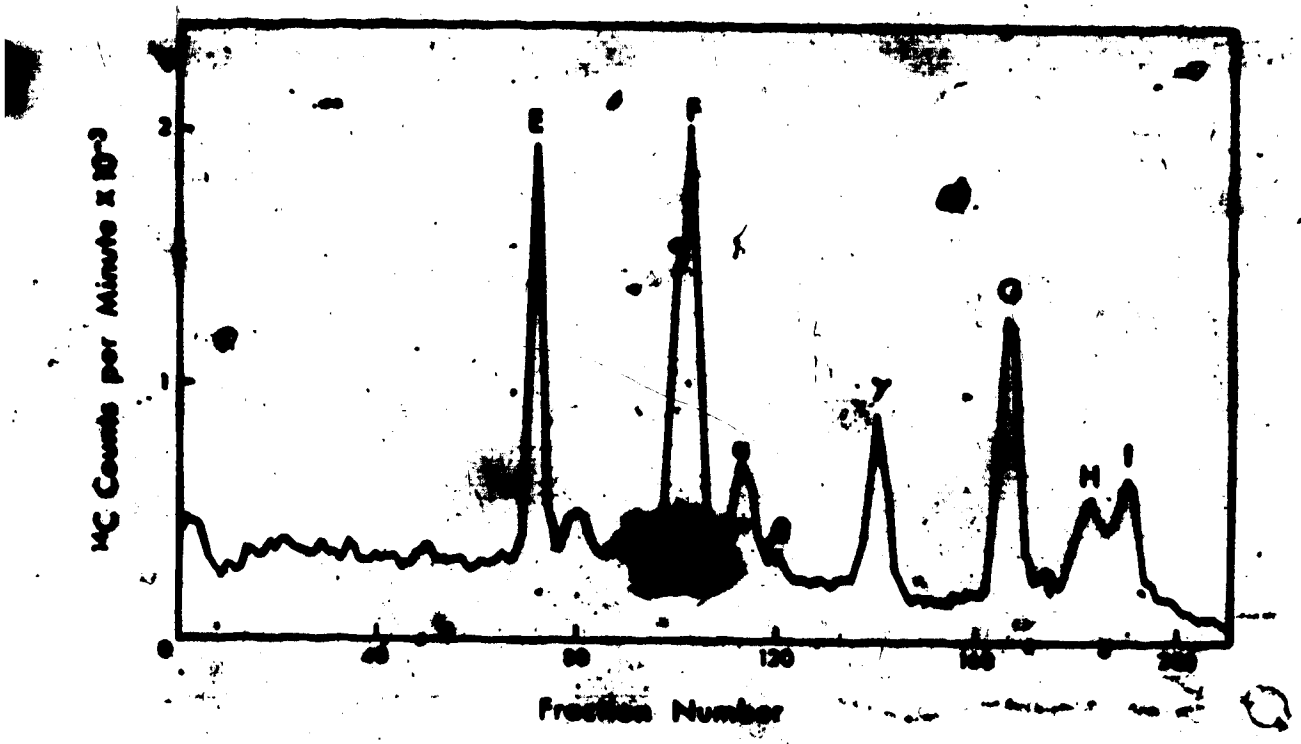


Figure 6. Electrophoretic analysis on 0.1% SDS-10% polyacrylamide gels of the supernatant obtained from the 37,000 rpm centrifugation step ("S$_{37}$"). Following centrifugation of the lysate, an aliquot of the supernatant was analyzed by electrophoresis as described in the legend for Figure 4.

The remaining viral capsid polypeptides were removed from the lysate by passage through an affinity column consisting of viral capsid protein-specific [illegible] coupled to agarose beads (Figure 7). The peak containing the non-capsid polypeptides was pooled as indicated (Pool 1) and used for subsequent procedures. The column was recycled by removing bound capsid polypeptides using a buffer containing 3.0 M NaSCN. Such an affinity column was stable for several weeks at 4° and could be used repeatedly for this adsorption procedure. An SDS-polyacrylamide gel profile of the material found in peak 1 of the affinity column is shown in Figure 8. It is evident that the capsid polypeptides have been completely removed, leaving only the non-capsid and unlabeled host L cell polypeptides in the lysate.

Separation of viral noncapsid polypeptides using denaturing conditions

The elution profile obtained when the affinity column-adsorbed lysate was subjected to chromatography on hydroxylapatite in the presence of SDS is shown in Figure 9. The size of the first major peak, eluting between 0.3 and 0.35 M sodium phosphate, varied considerably from preparation to preparation. Analysis of this peak by SDS-polyacrylamide gel electrophoresis revealed that there was no [illegible] peptide species present; counts were observed throughout the gel and no major amounts of the noncapsid polypeptides were

Figure [illegible] from the [illegible] noted L cells [illegible] to which [illegible] [illegible] in subsequent [illegible] the point at which buffer containing [illegible] to recycle the column. [illegible] rate was 1.0 ml/hr. [illegible] were assayed for [illegible].

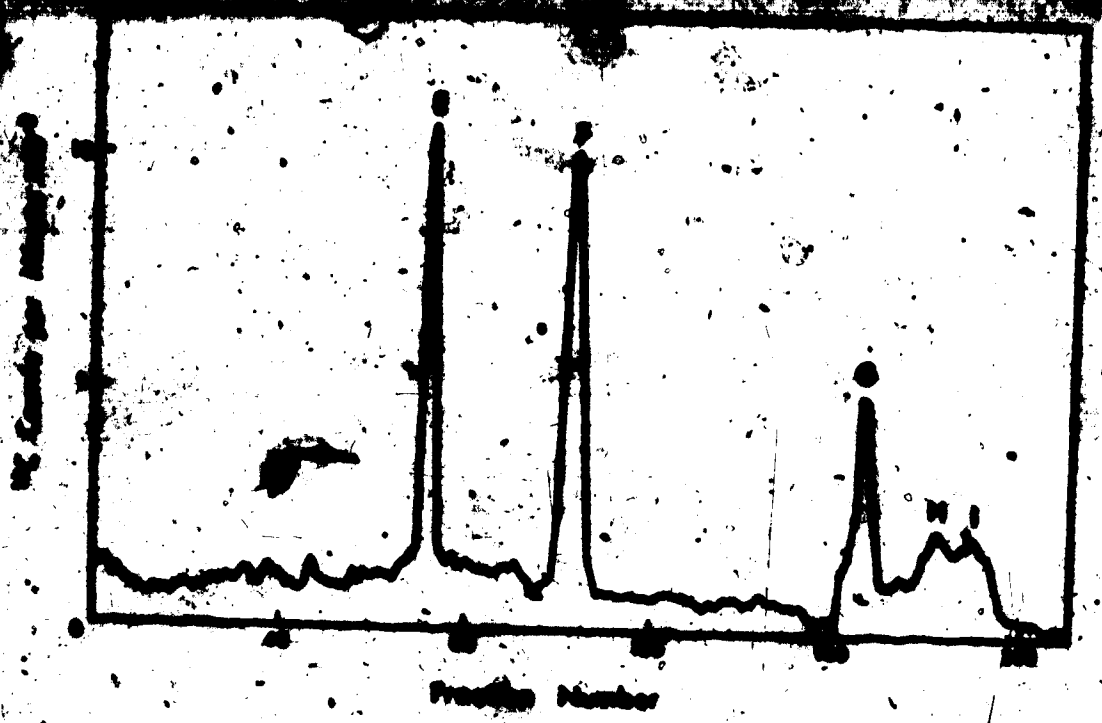


Figure 8. Electrophoretic analysis on 0.1% SDS-10% polyacrylamide gels of the nucleocapsid polypeptides [illegible] in Pool I from the affinity chromatography column (Figure 7). Conditions for electrophoresis, [illegible] activity counting were identical to those described in the legend of Figure 4.

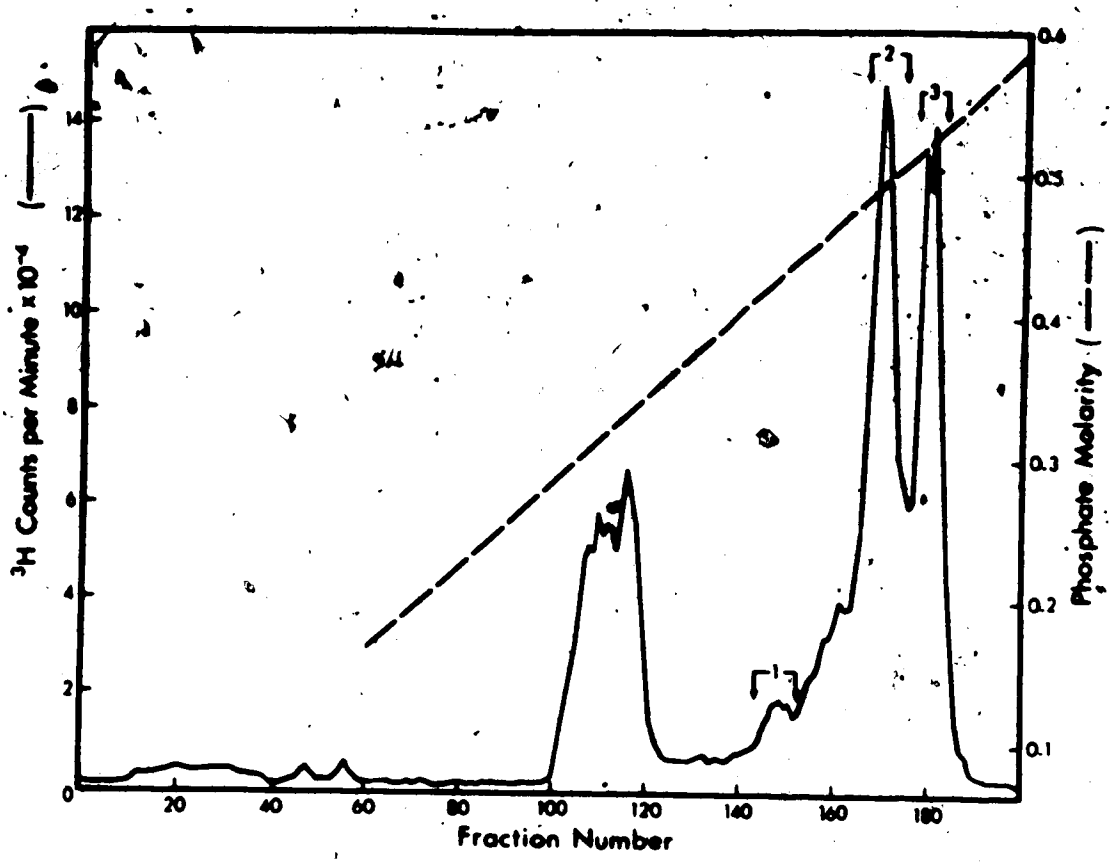


Figure 9. SDS-hydroxylapatite chromatography of ^{3}H-amino acid-labeled noncapsid polypeptides from Pool 1 of the affinity chromatography column (Figure 7). The polypeptides were eluted from the column by a 140 ml phosphate gradient (pH 6.2) constructed from 70 ml of 0.1 M sodium phosphate and 70 ml of 0.6 M sodium phosphate; both buffers containing 0.1% SDS and 1 mM DTT. The numbers 1 to 3 identify the sets of fractions (1.0 ml) which were pooled for subsequent analysis.

present (not shown). The presence of this peak may indicate that some degradation of the non-capsid polypeptides has occurred during the purification procedures. SDS-polyacrylamide gel analysis of pools 1, 2 and 3 from Figure 9 is shown in Figures 10, 11 and 12, respectively. Pool 1 contained noncapsid polypeptide H plus a species migrating slightly faster than polypeptide F. This second species may represent a smaller, degraded form of F but positive identification has not been made. Pool 2 contained the viral noncapsid polypeptides F and G plus several host polypeptides (detected by staining with Coomassie Blue). Pool 3 was composed of the noncapsid polypeptides E and I and several host polypeptides.

Chromatography of the F plus G polypeptide mixture on Sephadex produced the elution pattern shown in Figure 13. A similar pattern was observed when the E plus I polypeptide mixture was chromatographed on Sephadex (Figure 14). Analysis by SDS-polyacrylamide gel electrophoresis revealed that peak 1 in Figure 13 contained polypeptide F plus about 5 host cell polypeptides while peak 2 contained pure polypeptide G (Figure 15). Similarly, Figure 16 shows that peak 1 from Figure 14 contained polypeptide E plus about 4 host cell polypeptides. Peak 2 contained pure I.

Thus, using this two-column procedure, relatively pure preparations of E, F, G and I have been obtained. Although it was not attempted in this study, polypeptide H could probably be separated from the other main polypeptide

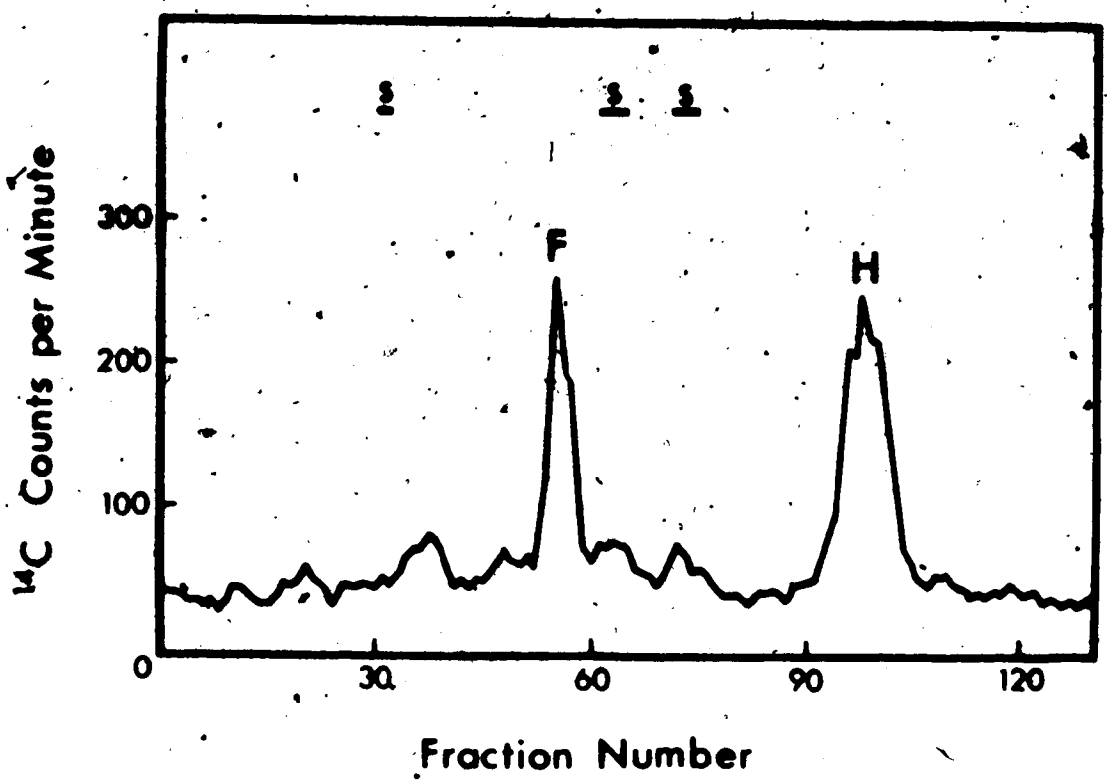


Figure 10. Electrophoretic analysis on 0.1% SDS-10% polyacrylamide gels of the noncapsid polypeptides found in Pool 1 from the SDS-hydroxylapatite column (Figure 9). The Pool 1 region was dialyzed against 0.1% SDS in distilled water, and lyophilized. Aliquots were suspended in 0.01 M sodium phosphate (pH 7.2), and SDS and β-mercaptoethanol were added to 2% and 5% final concentrations. The sample was heated at 100° for 5 min before being subjected to electrophoresis. The bars above the profile (labeled "s") indicate the position of Coomassie Blue-stained bands. Electrophoresis was at 6 mA/gel for 12 hr. Conditions for fractionation of the gels and radioactivity counting were the same as those described in the legend to Figure 4.

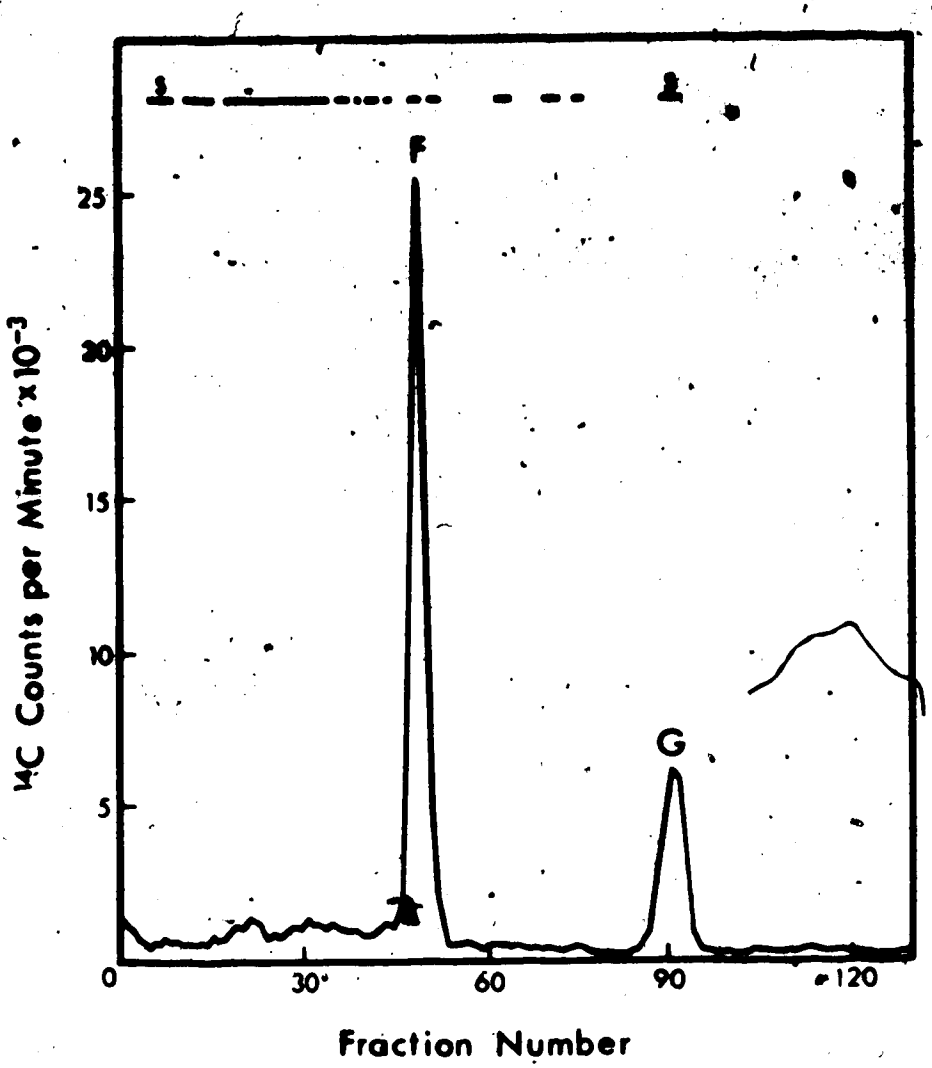


Figure 11. Electrophoretic analysis on 0.1% SDS-10% polyacrylamide gels of the noncapsid polypeptides found in Pool 2 from the SDS-hydroxylapatite column (Figure 9). The sample was prepared and electrophoresed as described in Figure 10. Bars above the profile labeled "s" indicate the position of Coomassie Blue-stained bands.

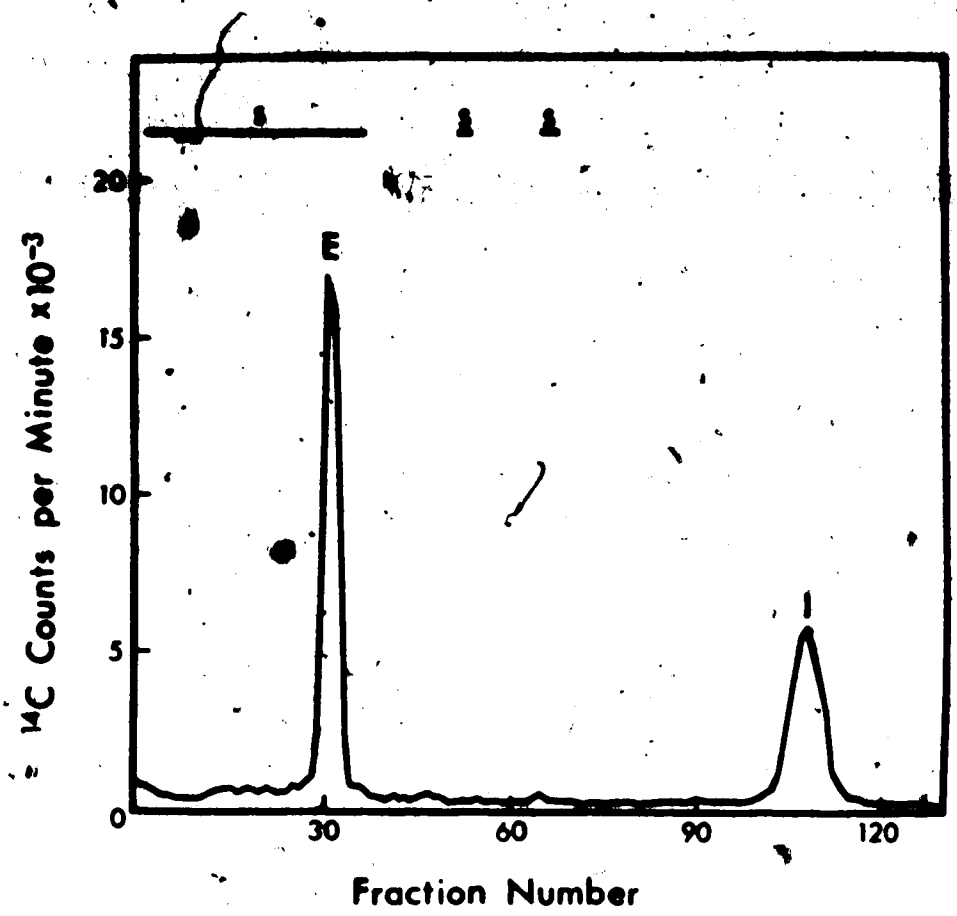


Figure 12. Electrophoretic analysis on 0.1% SDS-10% polyacrylamide gels of the noncapsid polypeptides found in Pool 3 from the SDS-hydroxylapatite column (Figure 9). The sample was prepared and electrophoresed as described in Figure 10. Bars above the profile labeled "s" indicate the position of Coomassie Blue-stained bands.

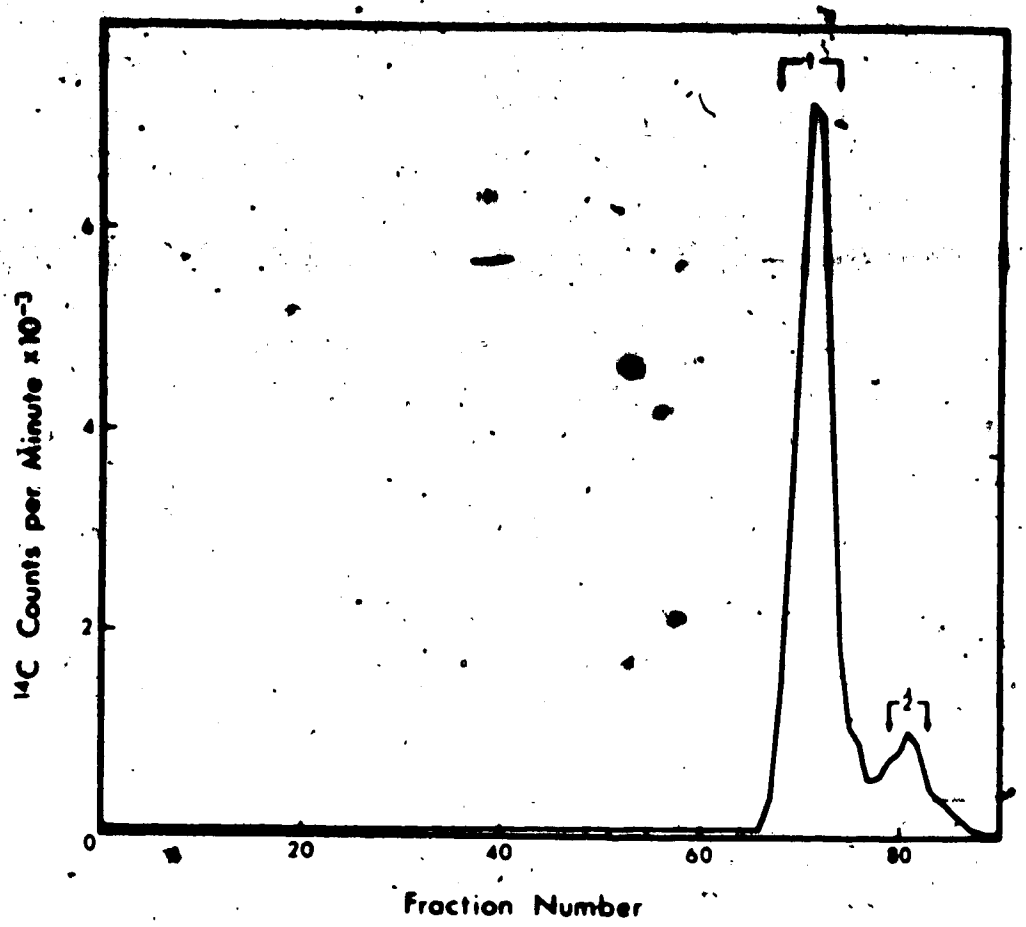


Figure 13. SDS-Sephadex G-100 chromatography of the non-capsid polypeptides from Pool 2 of the SDS-hydroxylapatite column (Figure 9). The dialyzed, lyophilized pooled fractions were resuspended in 0.3 ml distilled water, and the concentrations of SDS and β-mercaptoethanol adjusted to 2% and 5%, respectively. The sample was heated at 100° for 5 min and then chromatographed on the G-100 superfine Sephadex column. The column was eluted with 0.1 M sodium phosphate (pH 7.2) containing 0.1% SDS at a flow rate of 6.0 ml/hr, and 0.5 ml fractions were collected. An aliquot of each fraction was assayed for radioactivity. The numbers 1 and 2 refer to the sets of fractions which were pooled.

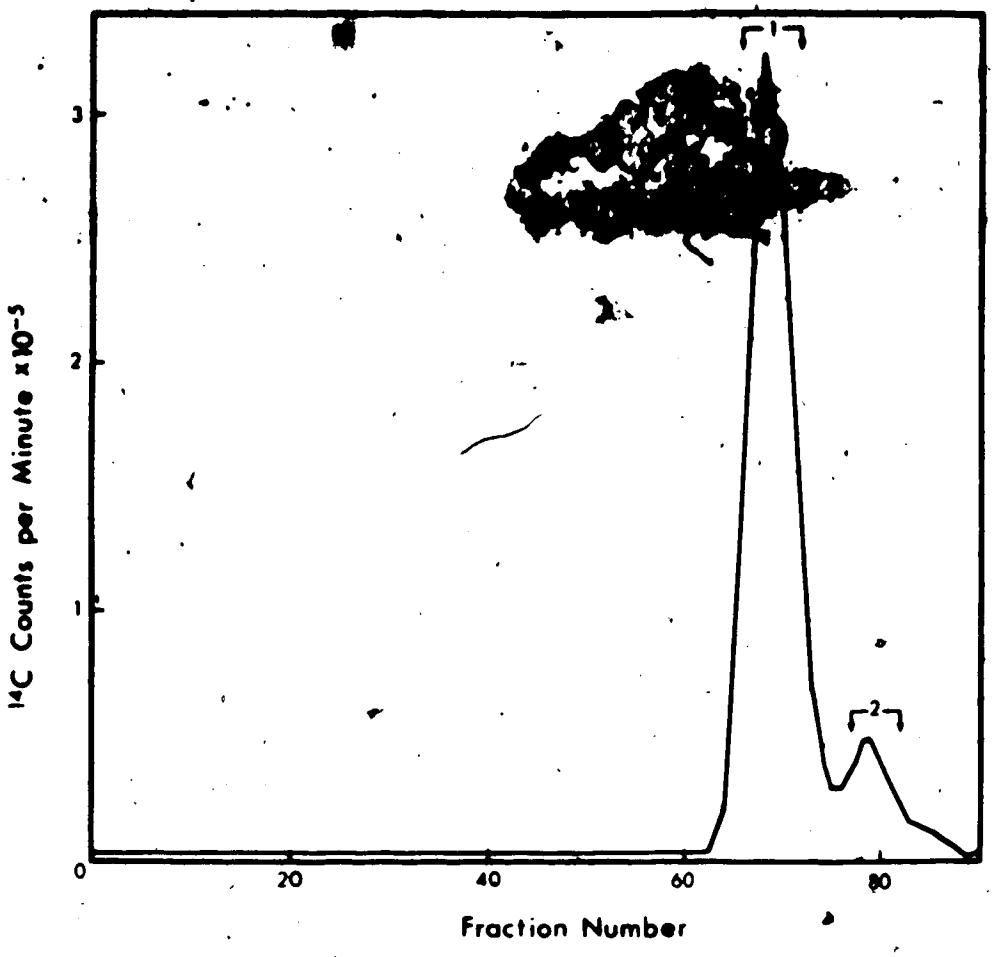


Figure 14. SDS-Sephadex G-100 chromatography of the non-capsid polypeptides from Pool 3 of the SDS-hydroxylapatite column (Figure 9). The material was prepared and chromatographed as described in Figure 13. The numbers 1 and 2 refer to the sets of fractions which were pooled.

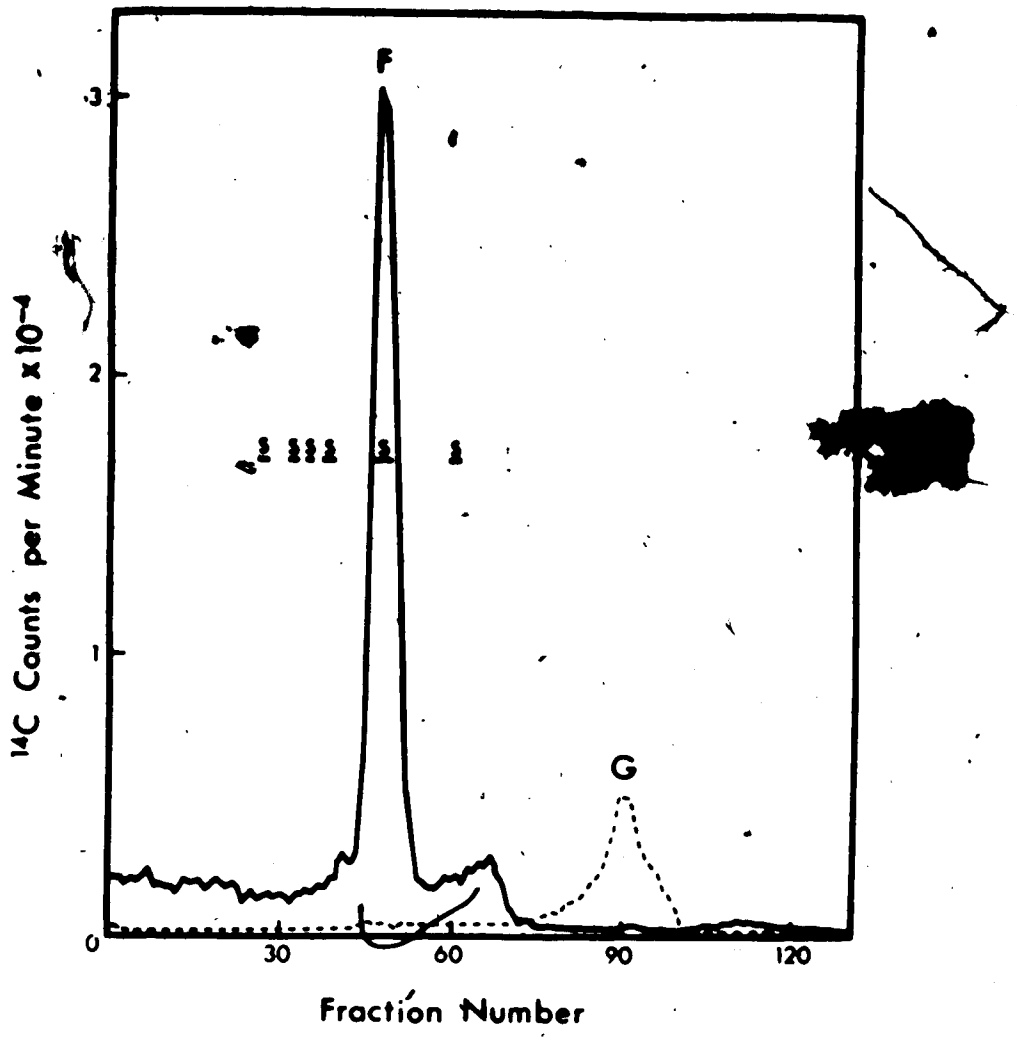


Figure 15. Electrophoretic analysis on 0.1% SDS-10% polyacrylamide gels of the noncapsid polypeptides following fractionation by G-100 Sephadex chromatography (Figure 13). Aliquots of Pools 1 and 2 (Figure 13) were dialyzed against 0.1% SDS in water, lyophilized and prepared for electrophoresis as described in Figure 10. Material present in Pool 1 is represented by the solid line, while the dashed line represents material found in Pool 2. The conditions for electrophoresis, fractionation of gels and radioactivity determination were the same as those described in Figure 10. The bars labeled "s" indicate the position of Coomassie Blue-stained bands in the gel containing polypeptide F. No such bands were observed in the gel containing polypeptide G.

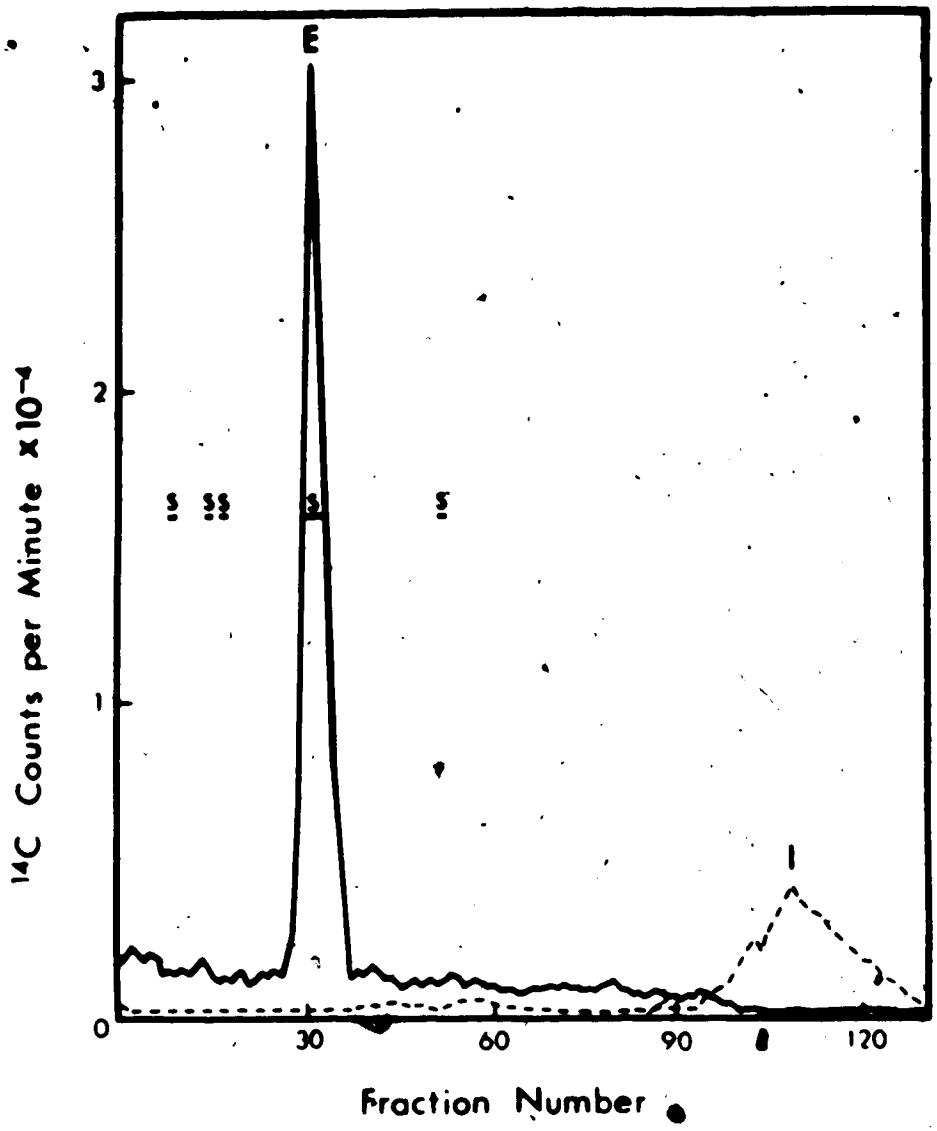


Figure 16. Electrophoretic analysis on 0.1% SDS-10% polyacrylamide gels of the noncapsid polypeptides following fractionation by G-100 Sephadex chromatography (Figure 14). Samples were prepared as in Figure 15. Material present in Pool 1 is represented by the solid line while the dashed line represents material found in Pool 2. The conditions for electrophoresis, fractionation of gels and radioactivity determination were as described in Figure 10. The bars labeled "s" indicate the position of Coomassie Blue-stained bands in the gel containing polypeptide E. No such bands were observed in the gel containing polypeptide I.

present in Pool 1 by chromatography on Sephadex. Preliminary experiments have indicated that these polypeptide preparations are suitable for use as antigens to raise specific antibodies in rabbits. SDS-complexed polypeptides have frequently been used to produce specific antiserum (Johnson et al., 1972; Pederson and Eddy, 1974; Croft et al., 1974; Stumph et al., 1974; Lund et al., 1977). The host cell polypeptides present in the E and F pools should pose no difficulty since L cell proteins are poor antigens in rabbits (Eva Paucha, personal communication).

Separation of undenatured viral noncapsid polypeptides

Chromatography on Bio-Gel A-5m of the material from the affinity column (Pool 1, Figure 7), employing PBS (minus Ca^{+2} and Mg^{+2} salts) containing 1% NP-40 as the lysis and column buffer, produced the profile shown in Figure 17. Analysis by SDS-polyacrylamide gel electrophoresis of the pools indicated on the graph showed that the leading edge of peak 1 (Pool 1) contained F as the only noncapsid viral polypeptide (Figure 18). Also present were 5 host polypeptides with similar molecular weights. Pool 2 also contained polypeptide F, but large amounts of polypeptides G, H and I were also present (Figure 19). Pool 3, the trailing edge of the first peak, contained primarily polypeptide F but there was some indication of small amounts of higher molecular weight labeled contaminants (Figure 20). Approximately 5 host polypeptides were also present. Pool 4

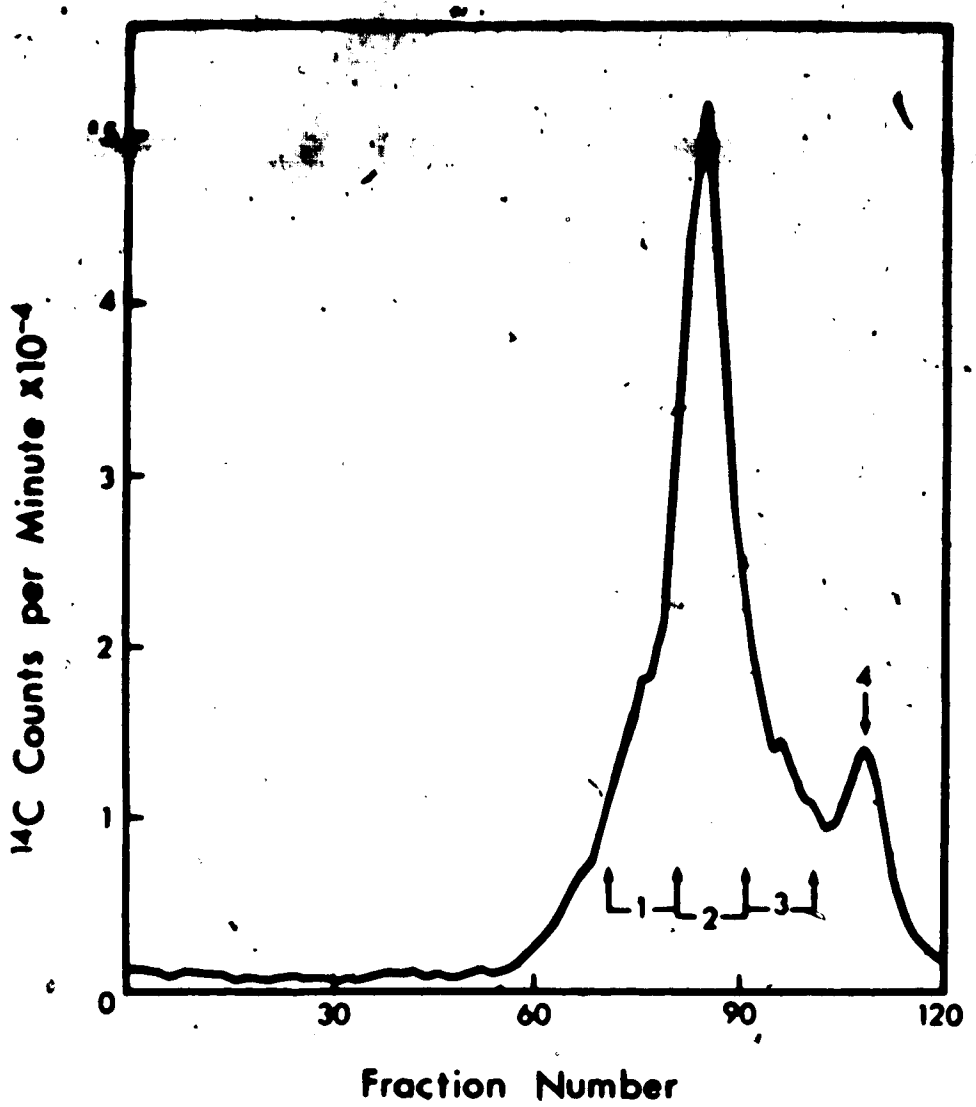


Figure 17. Chromatography of ^{14}C-amino acid-labeled non-capsid polypeptides from Pool 1 of the affinity chromatography column (Figure 7) on Bio-Gel A-5m. Column and lysis buffer were PBS (minus Ca^{+2} and Mg^{+2} salts) containing 1% NP-40. The numbers 1 to 4 refer to the sets of fractions which were pooled. The Bio-Gel column was eluted at a flow rate of 6.0 ml/hr. Fractions of 1 ml were collected, and an aliquot of each assayed for radioactivity.

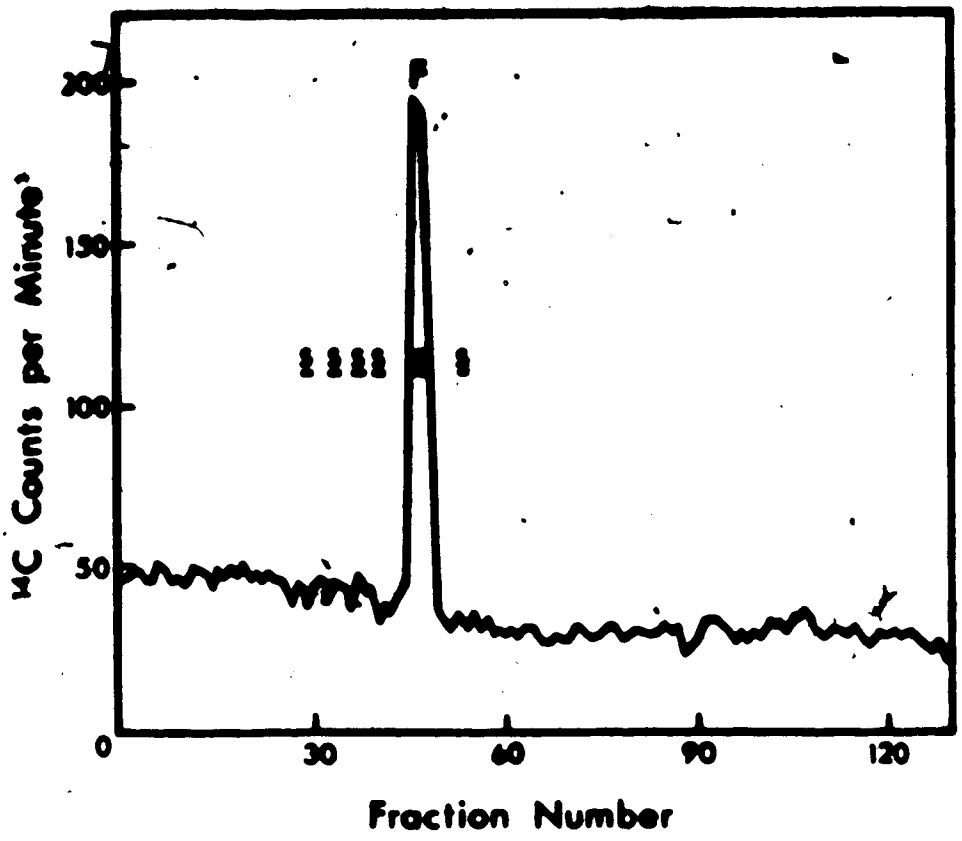


Figure 18. Electrophoretic analysis on 0.1% SDS-10% polyacrylamide gels of the polypeptides found in Pool 1 from the Bio-Gel A-5m column (Figure 17). An aliquot of the pool was made 2% SDS and 5% β-mercaptoethanol, heated at 100° for 5 min and then dialyzed against 0.01 M sodium phosphate (pH 7.2) containing 2% SDS and 5% β-mercaptoethanol. The dialyzed material was subsequently loaded onto the gel, and electrophoresis and analysis carried out as described in Figure 10. The bars labeled "s" indicate the positions of Coomassie Blue-stained bands.

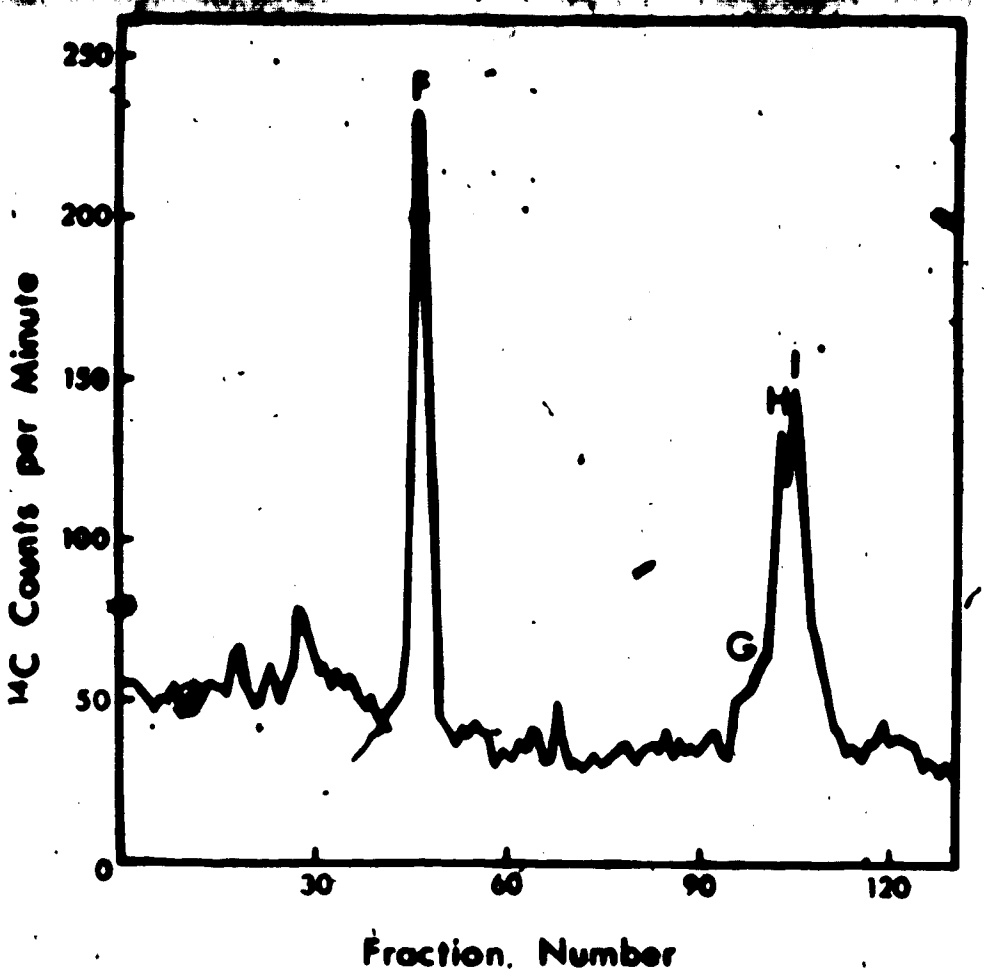


Figure 19. Electrophoretic analysis on 0.1% SDS-10% polyacrylamide gels of the polypeptides found in Pool 2 from the Bio-Gel A-5m column (Figure 17). The sample was prepared for analysis as in Figure 18. Conditions for electrophoresis, fractionation of gels and radioactivity measurement were identical to those described in Figure 10.

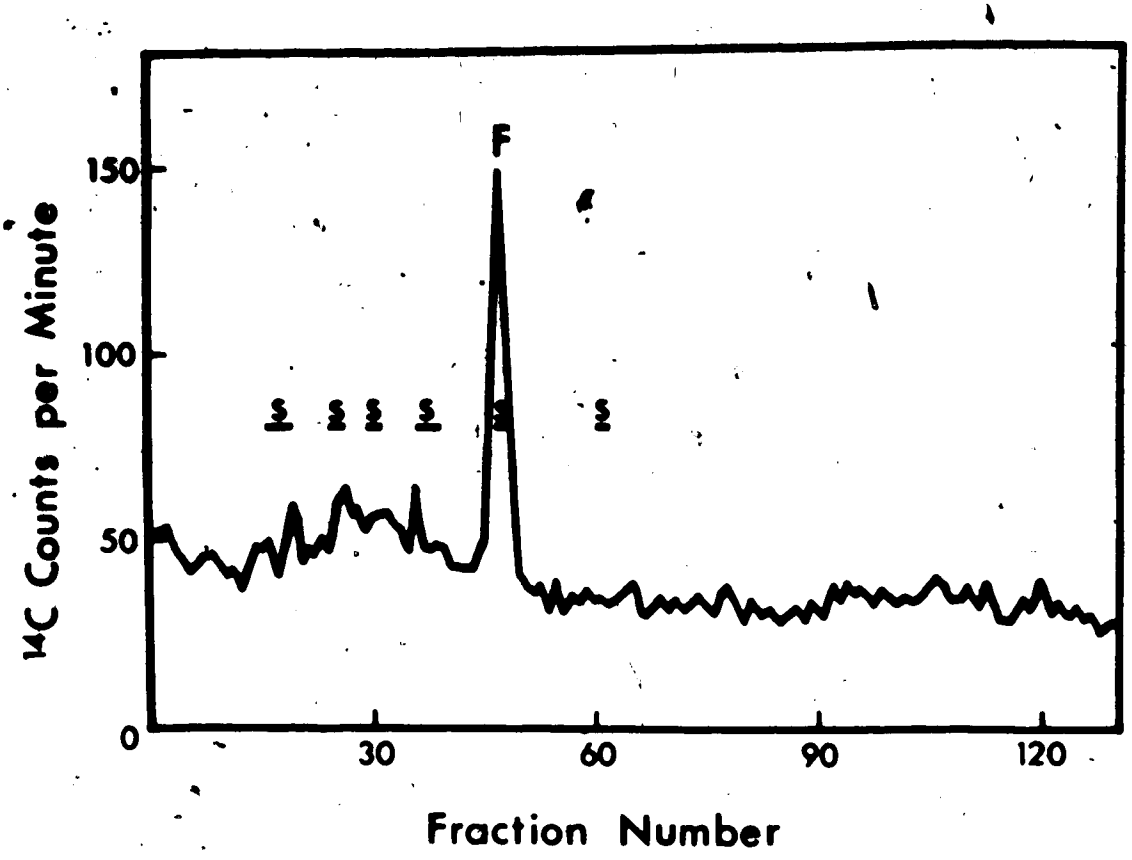


Figure 20. Electrophoretic analysis on 0.1% SDS-10% polyacrylamide gels of the polypeptides found in Pool 3 from the Bio-Gel A-5m column (Figure 17). The sample was prepared for analysis as in Figure 18. The bars labeled "s" identify the Coomassie Blue-stained bands. The conditions for electrophoresis, fractionation of gels and measurement of radioactivity were identical to those described in Figure 10.

from the Bio-Gel column contained E as the sole viral polypeptide plus 4 - 5 host polypeptides (Figure 21). This procedure appeared to be suitable for the purification of E for the purpose of assaying for a potential polymerase activity. However, it was subsequently determined that the viral polymerase activity found in the extracts was very unstable when PBS buffer was used for the purification procedure (not shown). Activity was lost early during the purification scheme. When 0.05 M TRIS-HCl (pH 8.0) containing 0.05 M KCl and 1% NP-40 was used for lysis and column buffer, good recovery of activity was observed (not shown) but the resolution of the E-containing peak from the leading peak was poor (Figure 22). A compromise between these two situations was achieved by employing 0.05 M TRIS + 0.05 M sodium phosphate (pH 8.0) containing 0.05 M KCl and 1% NP-40 as the lysis and column buffer. The resolution of the two peaks was good (Figure 23) and the activity was not lost during the early stages of purification (see below).

The elution profiles observed in these Bio-Gel columns were somewhat puzzling. Normally, one would expect that the largest polypeptide species would elute from the column first, followed in order of decreasing molecular weight, by the smaller ones. The fact that the F, G, H and I polypeptides passed through the column before E suggests that some sort of complex may have formed among these first four polypeptides. Analysis on glycerol gradients of

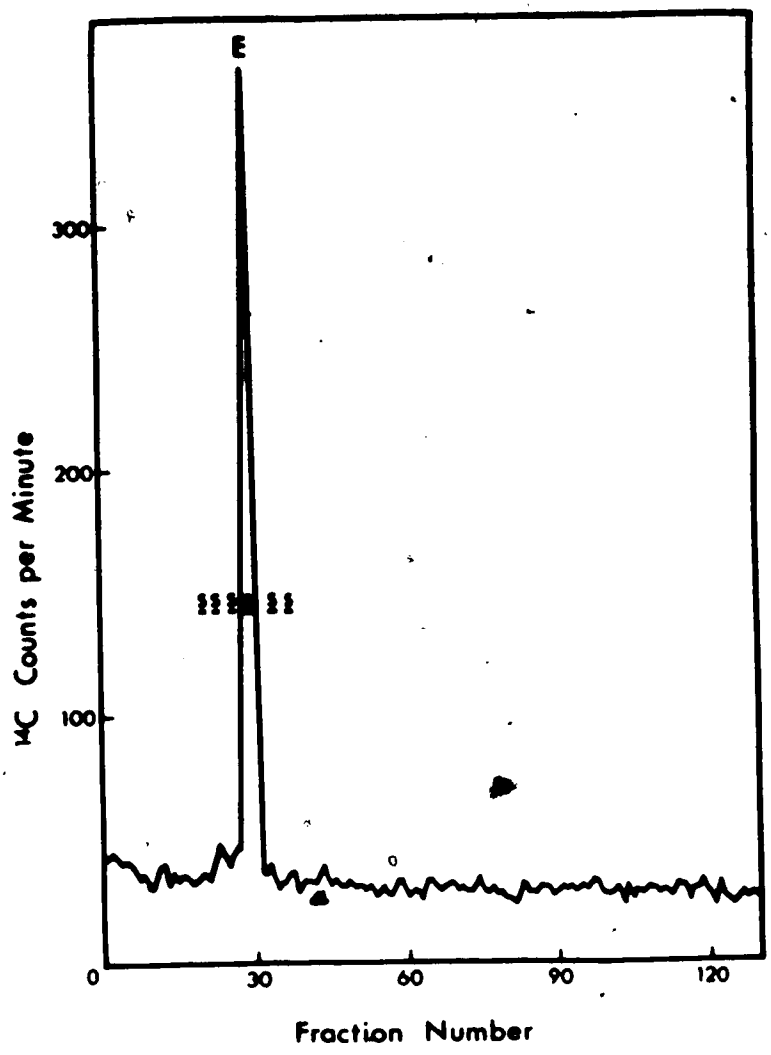


Figure 21: Electrophoretic analysis on 0.1% SDS-10% polyacrylamide gels of the polypeptides found in Pool 4 from the Bio-Gel A-5m column (Figure 17). The sample was prepared for analysis as in Figure 18. The bars labeled "s" identify the Coomassie Blue-staining bands. Conditions for electrophoresis, fractionation of gels and measurement of radioactivity were as described in Figure 10.

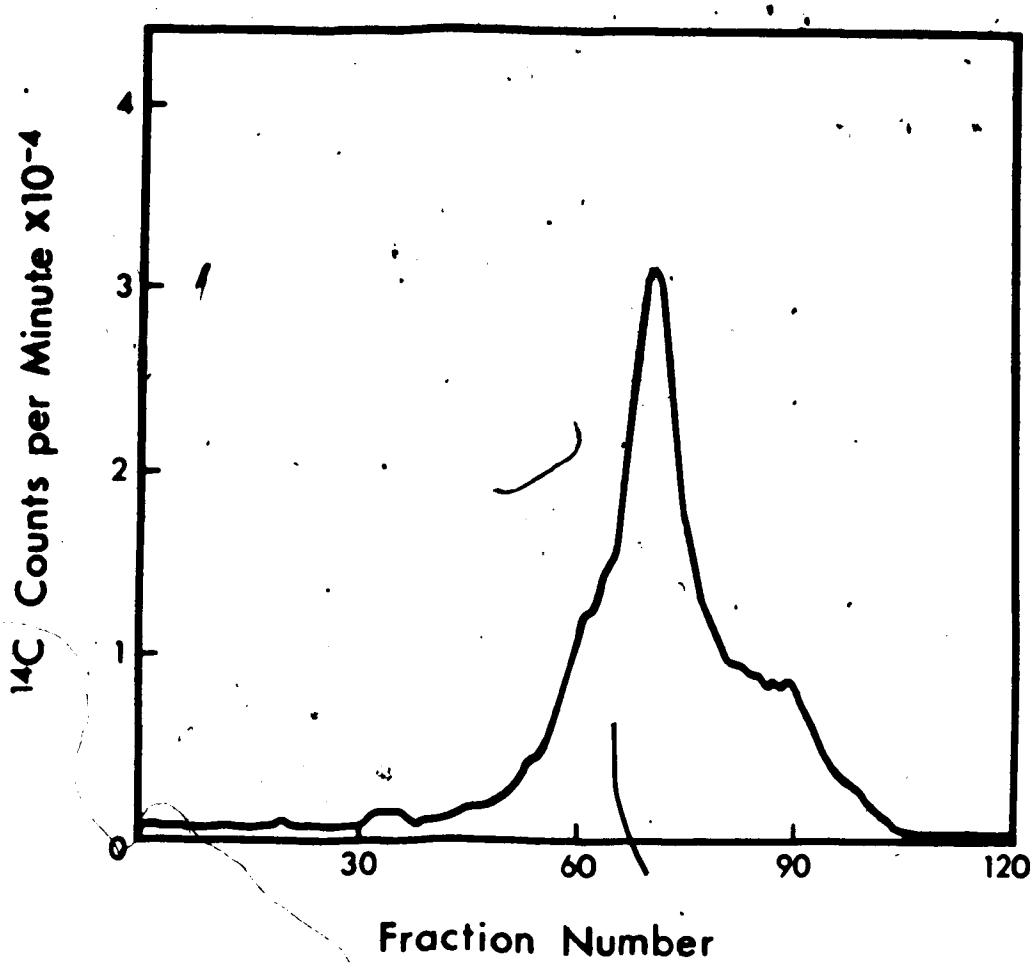


Figure 22. Chromatography of ^{14}C-amino acid-labeled polypeptides from Pool 1 of the affinity chromatography column (Figure 7) on Bio-Gel A-5m. The lysis and column buffer was 0.05 M TRIS-HCl (pH 8.0) containing 0.05 M KCl and 1% NP-40. Elution and radioactivity assay conditions were as described in Figure 17.

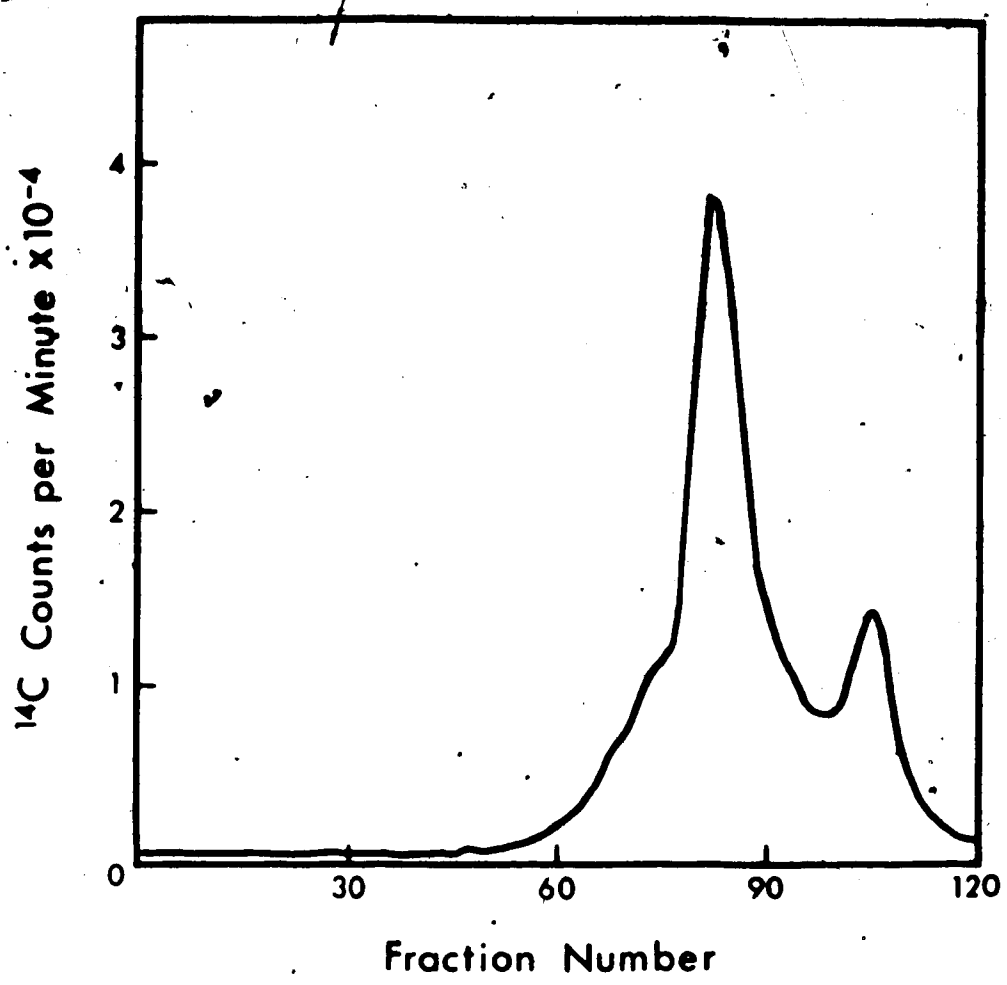


Figure 23. Chromatography of ^{14}C-amino acid-labeled polypeptides from Pool 1 of the affinity chromatography column (Figure 7) on Bio-Gel A-5m. The lysis and column buffer was 0.05 M TRIS-HCl (pH 8.0) containing 0.05 M sodium phosphate, 0.05 M KCl and 1% NP-40. Fractions were pooled in a pattern analogous to that illustrated in Figure 17. Elution and radioactivity assay conditions were as described in Figure 17.

pools 2 and 4 from Figure 17 did not, however, provide any evidence for such an association (Figure 24). The main radioactivity peak associated with F, G, H and I sedimented between the 4.6S BSA marker and the E polypeptide peak.

Characterization of the Polymerase

Assays of polymerase activity found in the crude lysate (before the 37,000 rpm centrifugation step) showed that incorporation of radioactive UTP into a TCA-insoluble form was dependent upon the concentration of magnesium salts used in the assay (Figure 25). Thus, a magnesium concentration of 10 mM was used in all subsequent assays. Assay of the two peak fractions from Figure 23 (equivalent to pools 2 and 4 in Figure 17) revealed a polymerase activity associated with the E polypeptide, the activity being dependent upon the addition of exogenous viral RNA and a primer of oligo(rU) (Figure 26).

No such activity was found when the polypeptides from pool 2 were assayed (Figure 26), nor was this activity found in unfractionated extracts of uninfected cells (not shown). However, the recovery and stability of activity associated with polypeptide E varied significantly from preparation to preparation. The maximum recovery of activity detected was 5 - 10% of initial values, the dilution of polypeptide E during isolation being taken into account. Because of these factors, the recovery of polymerase activity was assayed following each step of the purification procedure

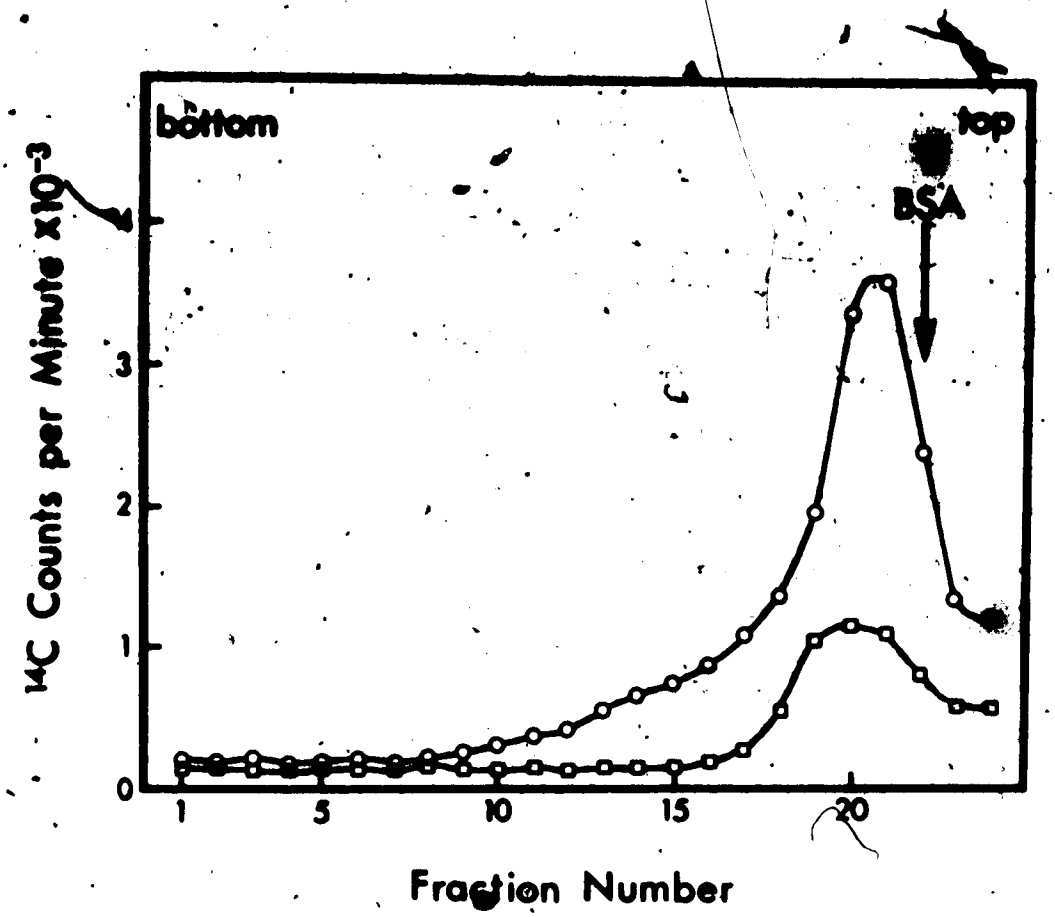


Figure 24. Glycerol gradient centrifugation of the ^{14}C-amino acid-labeled polypeptides from Pools 2 (o) and 4 (□) of the Bio-Gel A-5m column (Figure 17). Polypeptide samples (0.5 ml) were layered onto 12 ml 5% to 20% glycerol gradients in 0.05 M TRIS-HCl (pH 8.0) containing 0.05 M KCl and centrifuged at 35,000 rpm for 19 hr in an SW 41 rotor at 4°. Fractions of 0.5 ml were collected and 100 µl aliquots assayed for radioactivity by standard procedures. The arrow indicates the position to which the 4.6 S BSA marker sedimented in a parallel gradient run under identical conditions.

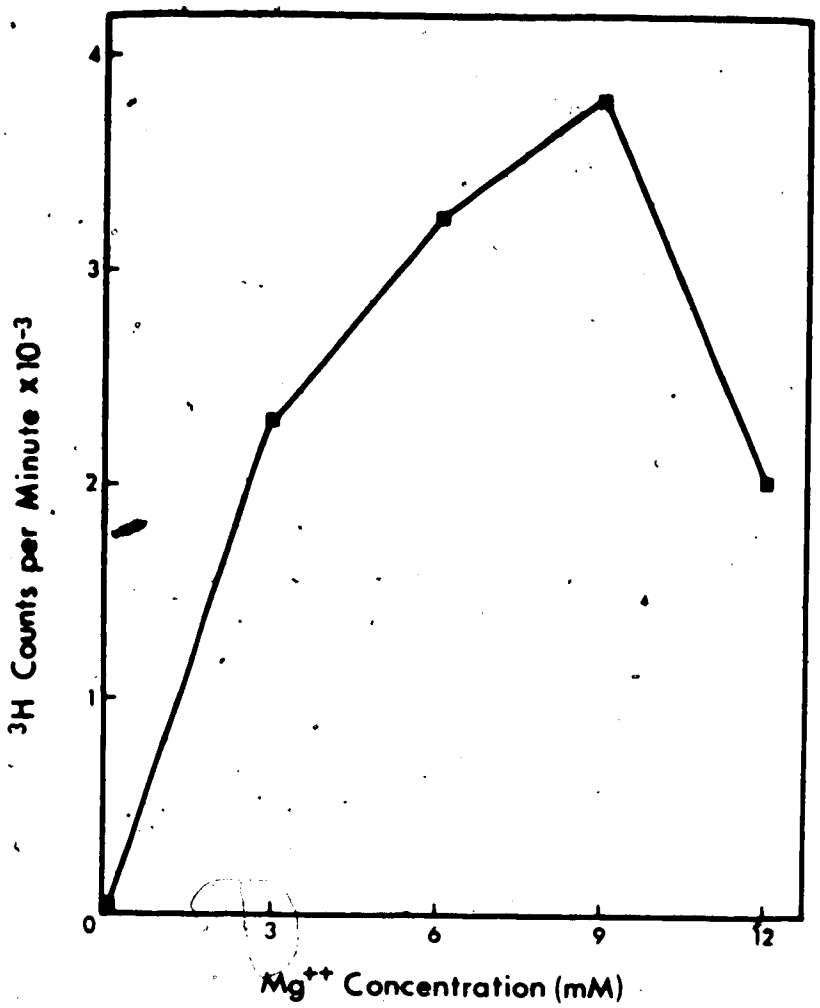


Figure 25. Effect of magnesium concentration on the polymerase activity present in an L cell lysate at the initial stage of purification (i.e. before the 37,000 rpm centrifugation step). A 100 µl aliquot of cell lysate in 0.05 M TRIS-HCl (pH 8.0) containing 0.05 M KCl and 1% NP-40 was added to 100 µl of assay mixture, the final concentrations of reagents being: 0.05 M TRIS-HCl (pH 8.0), 0.05 M KCl, 0.005 M DTT, 0.5 mM GTP, 0.5 mM CTP, 0.5 mM ATP, 20 µCi ^{3}H-UTP/ml, 2 µg actinomycin D/ml, 1.0 mM PEP and 20 µg pyruvate kinase/ml. Magnesium concentrations of 0, 3, 6, 9 and 12 mM were used. The mixture was incubated at 37° and aliquots of 40 µl removed at 0 min and 5 min after addition of the lysate. The aliquots were spotted onto filter papers which had been soaked with a solution containing 0.1 M sodium EDTA and 25 mM sodium pyrophosphate (pH 7.0), then dried. The discs were washed for 30 min with 0° 10% TCA, twice for 10 min with 0° 5% TCA and finally with 95% ethanol. The discs were dried and the TCA-insoluble radioactivity measured using a toluene based fluor. Background levels of radioactivity as measured by the 0 min controls were subtracted from the corresponding 5 min sample to arrive at the values plotted above.

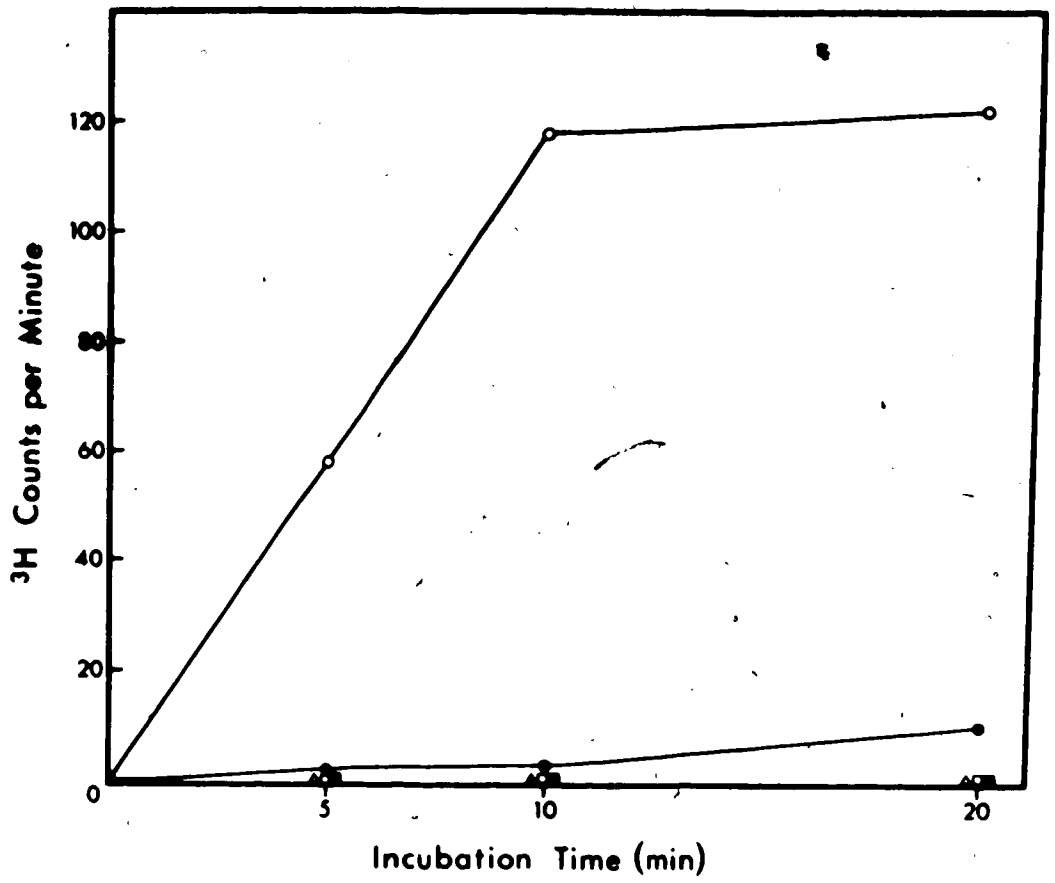


Figure 26. Template and primer requirements for polymerase activity of the isolated noncapsid polypeptides (from Figure 23). The assay conditions used were identical to those described in Figure 25 with the following exceptions: the magnesium concentration used was 10 mM throughout; incubation was extended to 20 min and additional samples taken at 10 and 20 min; where used, the final concentrations of viral RNA and oligo(rU) were 20 µg/ml and 10 µg/ml, respectively. Samples were processed for monitoring of TCA-insoluble incorporation of label as described in Figure 25. □ Assay of polypeptide E (equivalent to Pool 4 in Figure 17) ● Assay of polypeptide E including viral RNA △ Assay of polypeptide E including oligo(rU) ⊙ Assay of polypeptide E including viral RNA and oligo(rU) ■ Assay of polypeptides F, G, H and I (equivalent to Pool 2 in (Figure 17) including viral RNA and oligo(rU).

(Figure 27). After lysis of infected cells, a high level of activity was detected (Figure 27: "Before 37,000 rpm centrifugation"). Polymerase activity in the supernatent from the 37,000 rpm centrifugation step (S_{37}) was greatly reduced, but incorporation of label was stimulated by the addition of viral RNA template and oligo(rU) primer (Figure 27: "S_{37} + RNA + oligo(rU)"). Passage of the S_{37} through the affinity column resulted in an almost total loss of activity (Figure 27: "Pool 1 Affinity column") but addition of viral RNA and oligo(rU) again stimulated polymerization of nucleoside triphosphates (Figure 27: "Pool 1 Affinity column + RNA + oligo(rU)"). However, this stimulation was not as great as that observed in the preceding step. From these observations it appeared that the progressive removal of capsid polypeptides might be at least partially responsible for the ever decreasing levels of polymerase activity recovered. However, attempts to stimulate polymerase activity by the addition of either heat-disrupted purified virus or purified 13.4 S particles to the assay mixture were unsuccessful (Figure 28). Aliquots of protein contained in the fractions eluted from the affinity column by 3.0 M NaSCN (Figure 7) also did not stimulate polymerase activity, nor did addition of uninfected cell lysates to the E preparation. This suggested that the loss of possible host cell-provided cofactors was not responsible for the diminished activity.

Co-sedimentation of purified Mengo virion RNA and

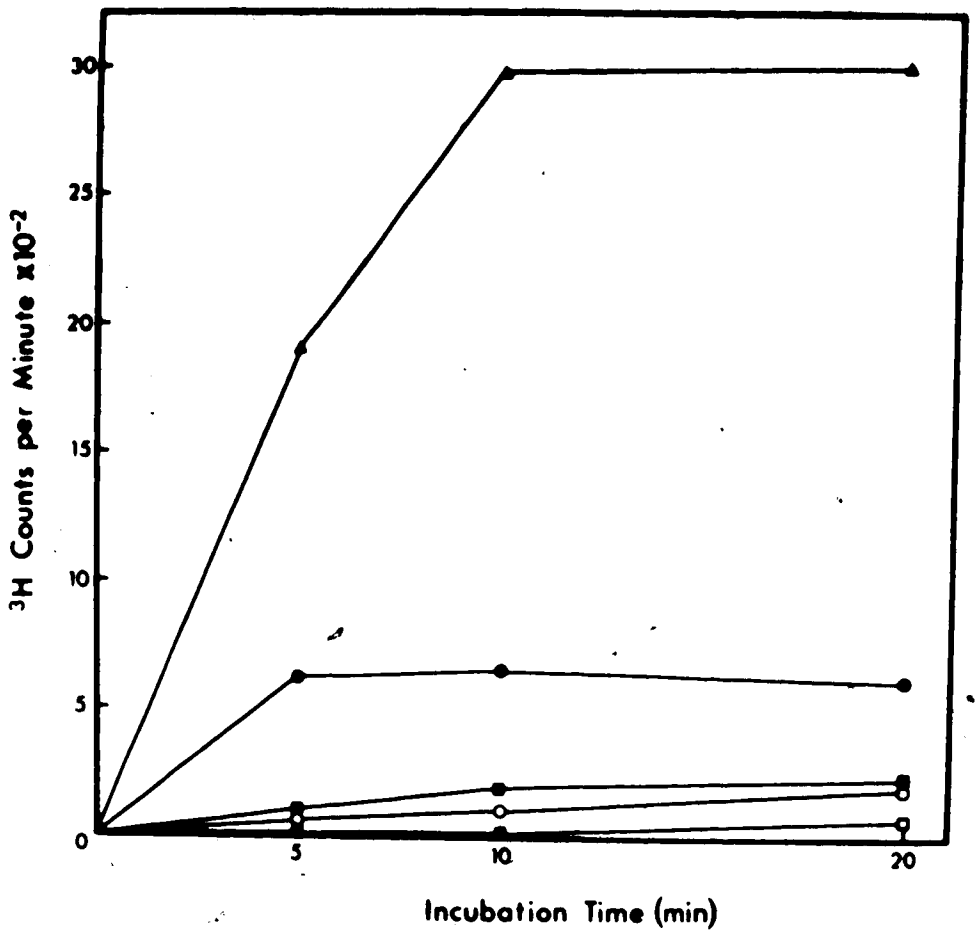


Figure 27. Assay of polymerase activity found in cell lysates during varying stages of purification. Assay conditions and processing of samples were identical to those described in Figure 26. Δ Assay of the lysate before the 37,000 rpm centrifugation step o Assay of the S_{37} lysate
● Assay of the S_{37} lysate including viral RNA and oligo(rU)
□ Assay of Pool 1 from the affinity chromatography column
■ Assay of Pool 1 from the affinity chromatography column including viral RNA and oligo(rU). Passage of the S_{37} lysate through the affinity chromatography column resulted in a two-fold dilution of the polypeptide species present.

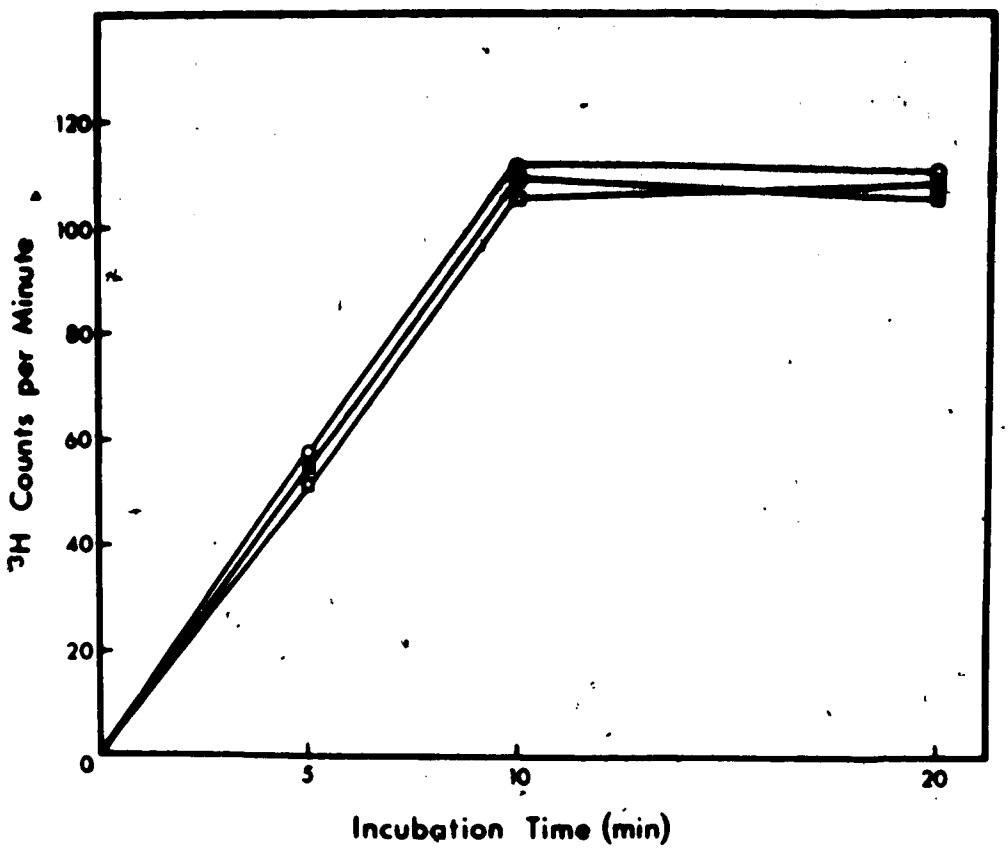


Figure 28. Effect of capsid polypeptides on the polymerase activity detected in Pool 1 from the affinity chromatography column (Figure 7). Assay conditions and processing of samples were identical to those described in Figure 26, with the exception that viral RNA and oligo(rU) were included in all three assays. ■ Assay of Pool 1 from the affinity chromatography column □ Assay of Pool 1 from the affinity chromatography column including heat disrupted virus (56° for 10 min) O Assay of Pool 1 from the affinity chromatography column including 13.4 S particles purified according to the procedure of Mak et al., 1971.

polypeptide E on a linear sucrose gradient indicated that E had some affinity for the RNA (Figure 29). The labeled polypeptide co-migrated with the viral RNA whereas polypeptides F, G, H and I showed no such affinity for the RNA (Figure 30). Polypeptide E by itself on a sucrose gradient remained near the top following centrifugation.

Chromatography of a S_{37} infected-cell lysate on poly (A) sepharose resulted in the adsorption of a peak of radioactivity which could be eluted with buffer containing 1.0 M KCl (Figure 31). Analysis on SDS-polyacrylamide gels of this adsorbed peak showed the presence predominantly of polypeptide E (Figure 32). The material passing straight through the column were polypeptides F, H and I (Figure 33). These results suggest that the affinity of polypeptide E for viral RNA could possibly be due to the presence of the poly (A) sequence on the 3'-end of the RNA.

An attempt was made to demonstrate proteolytic activity associated with polypeptide F. Substrate for this was prepared by pulse-labeling infected cells at 6 hr p.i. with ^{3}H-amino acids for 5 minutes. The cells were harvested and a lysate made with 0.05 M TRIS-HCl (pH 8.0) containing 0.05 M KCl and 1% NP-40. The lysate was centrifuged at low speed to remove nuclei, and then centrifuged at 37,000 rpm for 1 hr in a SW 50.1 rotor.

The labeled precursor polypeptides were pelleted while the unlabeled noncapsid polypeptides remained in the super-

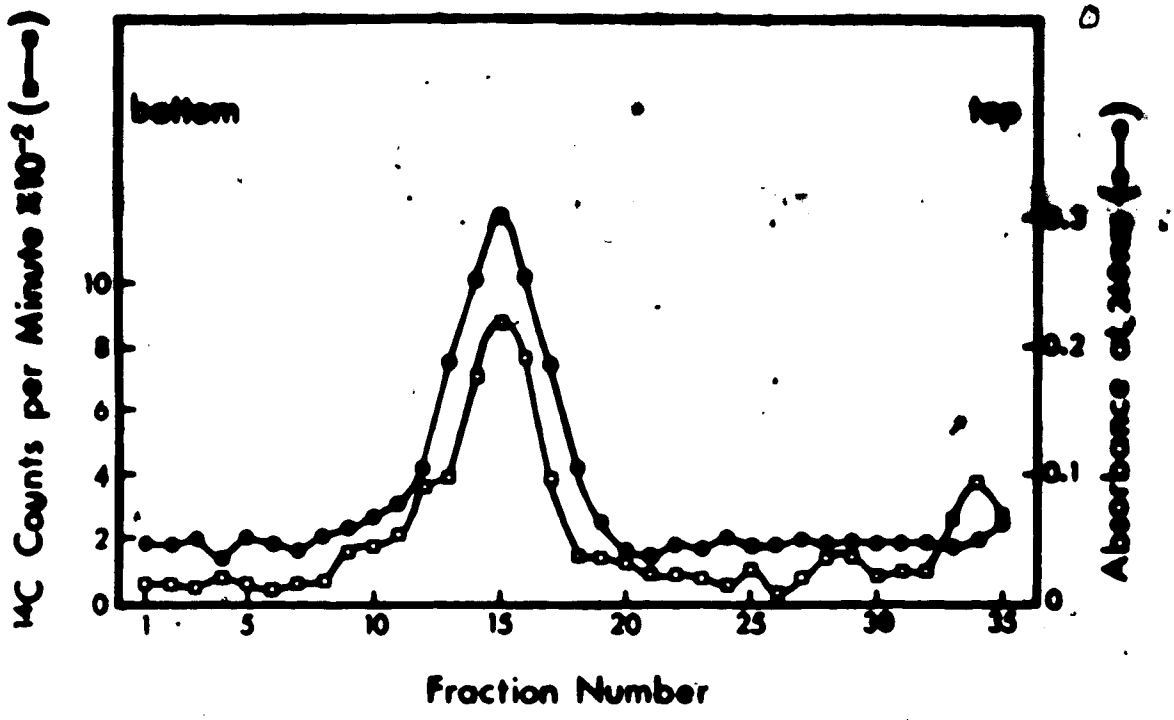


Figure 29. Sucrose density gradient co-sedimentation of purified Mengo virion RNA (●) and ^{14}C-amino acid-labeled polypeptide E (□). Viral RNA was prepared according to the procedure of Scraba et al., (1967). Polypeptide E was isolated by Bio-Gel A-5m chromatography as in Figure 23. Viral RNA and polypeptide E (100 µl of each in 0.05 M TRIS-NCl (pH 8.0) containing 0.05 M KCl and 1% NP-40) were mixed and incubated on ice for 1 hr. The mixture was then layered onto a 4.8 ml 10% to 30% sucrose gradient in 0.05 M TRIS-HCl (pH 8.0) containing 0.05 M KCl and 1% NP-40. The gradient was centrifuged at 35,000 rpm for 5 hr in a SW 50.1 rotor at 4°. Fractions of 5 drops were collected and diluted to a volume of 1.0 ml with distilled water. The absorbance of each diluted fraction at 260 nm was measured. Aliquots of 100 µl were then removed and assayed for radioactivity by standard techniques.

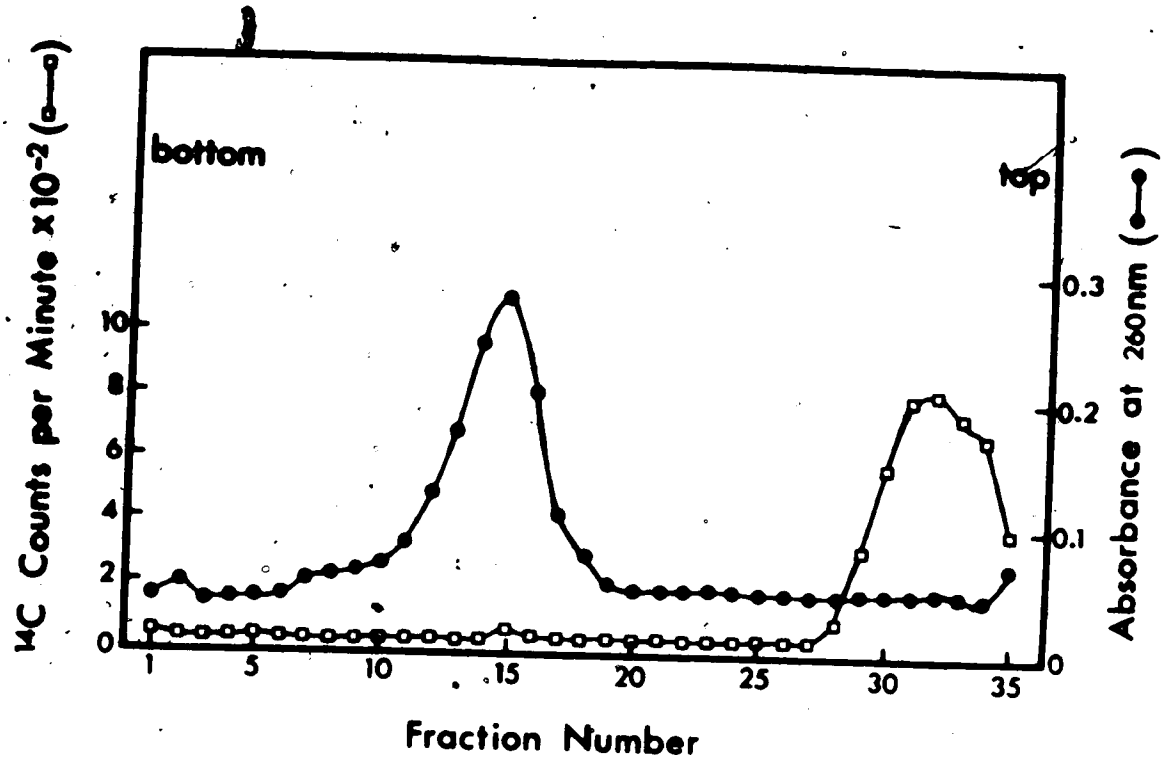


Figure 30. Sucrose density gradient co-sedimentation of purified Mengo virion RNA (●) and a ^{14}C-amino acid-labeled mixture of polypeptides F, G, H and I (□). Viral RNA was prepared according to the procedure of Scraba et al., (1967). The F, G, H and I polypeptide mixture was isolated by Bio-Gel A-5m chromatography as in Figure 23. Centrifugation and assay conditions were identical to those described in Figure 29.

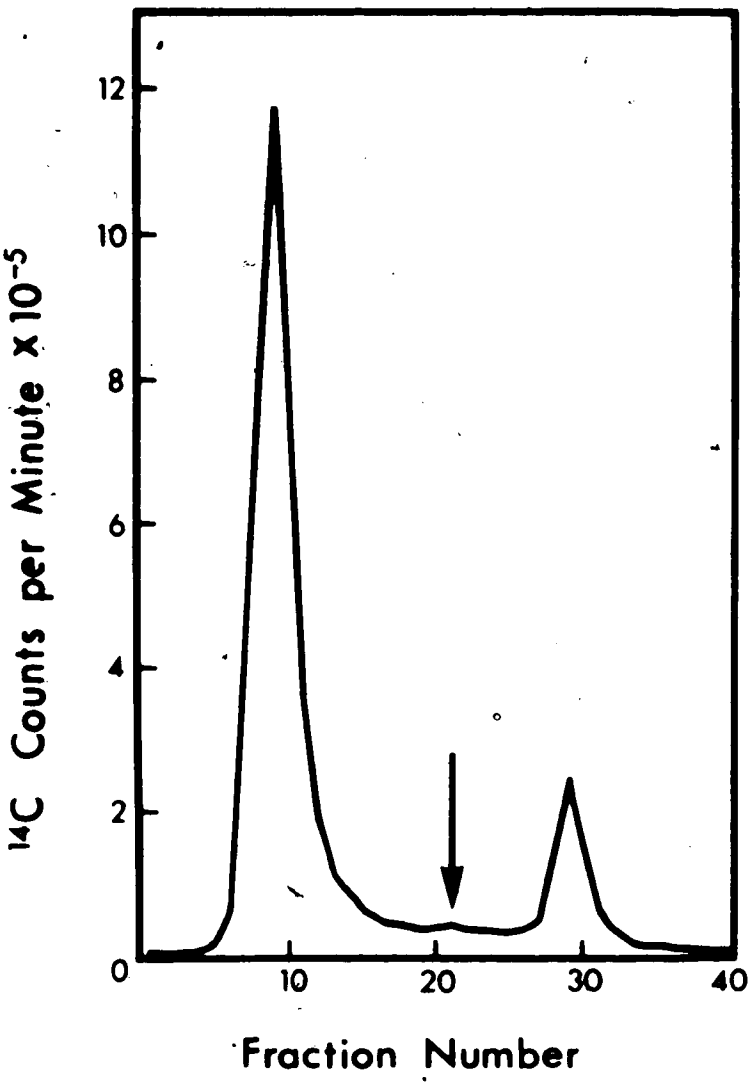


Figure 31. Chromatography on poly(A) sepharose of ^{14}C-amino acid-labeled noncapsid polypeptides present in a S37 lysate. The lysis and column buffer was 0.05 M TRIS-HCl (pH 8.0) containing 0.05 M KCl and 1% NP-40. Elution and radioactivity assay conditions were as described in Figure 17. The arrow indicates the point at which buffer containing 1.0 M KCl was added to the column.

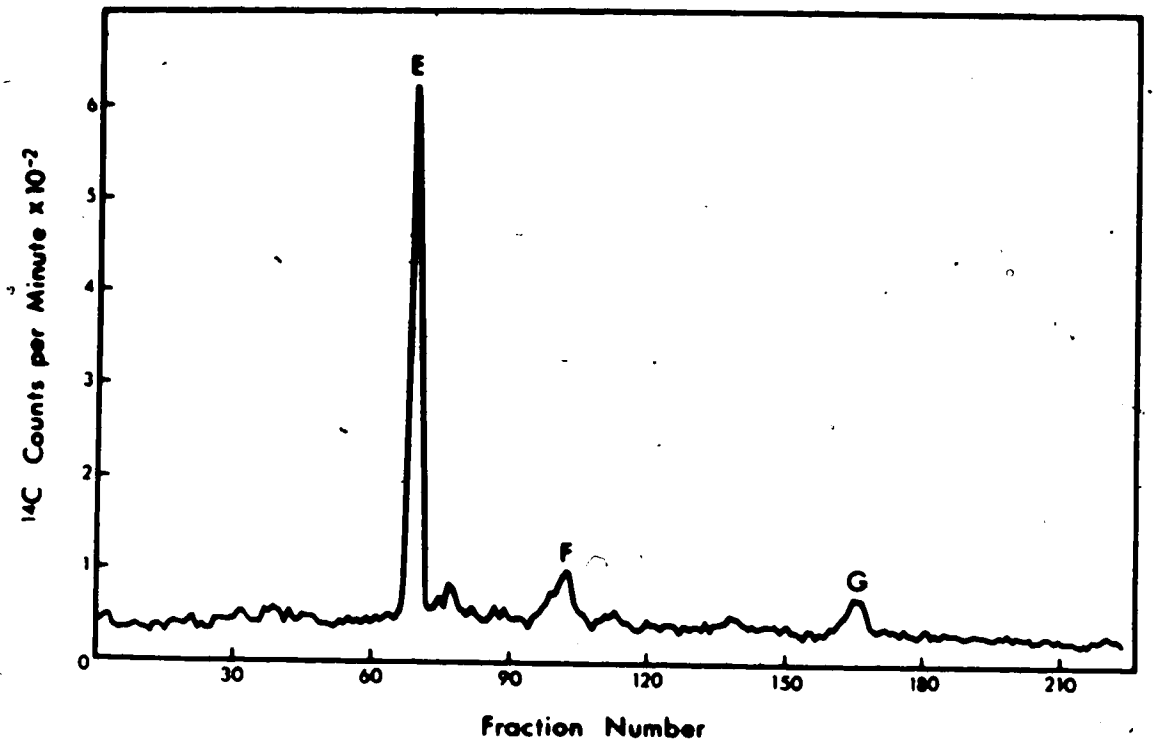


Figure 32. Electrophoretic analysis on 0.1% SDS-10% polyacrylamide gels of the ^{14}C-amino acid-labeled polypeptides present in fraction number 29 from the poly(A) sepharose column. The sample was prepared for electrophoretic analysis as described in Figure 18. Electrophoresis and analysis were carried out as described in Figure 10.

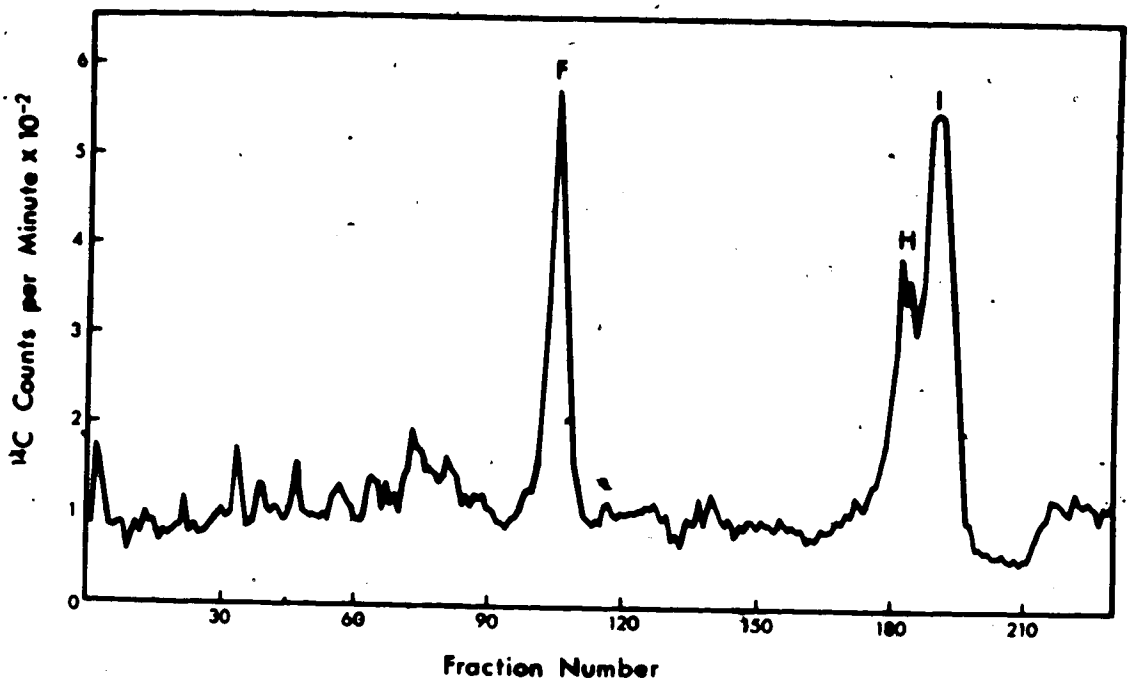


Figure 33. Electrophoretic analysis on 0.1% SDS-10% polyacrylamide gels of the ^{14}C-amino acid-labeled polypeptides present in fraction number 9 from the poly(A) sepharose column. The sample was prepared for electrophoretic analysis as described in Figure 18. Electrophoresis and analysis were carried out as described in Figure 10.

natent (as in Figure 6). An SDS-polyacrylamide gel profile of the pelleted material is shown in Figure 34. It consisted primarily of the precursor polypeptides A, B, C and D. An aliquot of such a preparation was mixed with a sample of polypeptide F (derived from pool 1 Figure 17; see Figure 18) and incubated at 37° for 1 hr. The SDS-polyacrylamide gel of the assay mixture did not differ significantly from the control (Figure 35). It would seem that either F has no protease activity vis à vis these precursors, or the conditions of assay were not conducive to detecting the activity.

Isolation of VPg

Chromatography of radioactively-labeled virus capsid polypeptides on hydroxylapatite revealed that a small but reproducible peak of label was associated with the viral RNA peak, even after the vigorous dissociation conditions employed (heating at 100° for 5 min with 2% SDS and 5% β-mercaptoethanol). This is shown in Figure 36. Radioactivity was also associated with the viral RNA when it was prepared from purified labeled virus using the phenol extraction procedure of Scraba et al., (1967). Reports of a small protein being associated with other picornaviral RNA's (Lee et al., 1976; Sanger et al., 1977; Hruby and Roberts, 1978) suggested that this label was due to a similar polypeptide being associated with Mengo virus RNA. Analysis of the nuclease-treated RNA on 10% polyacrylamide-0.1% SDS gels revealed a small peak of radioactivity migrating between

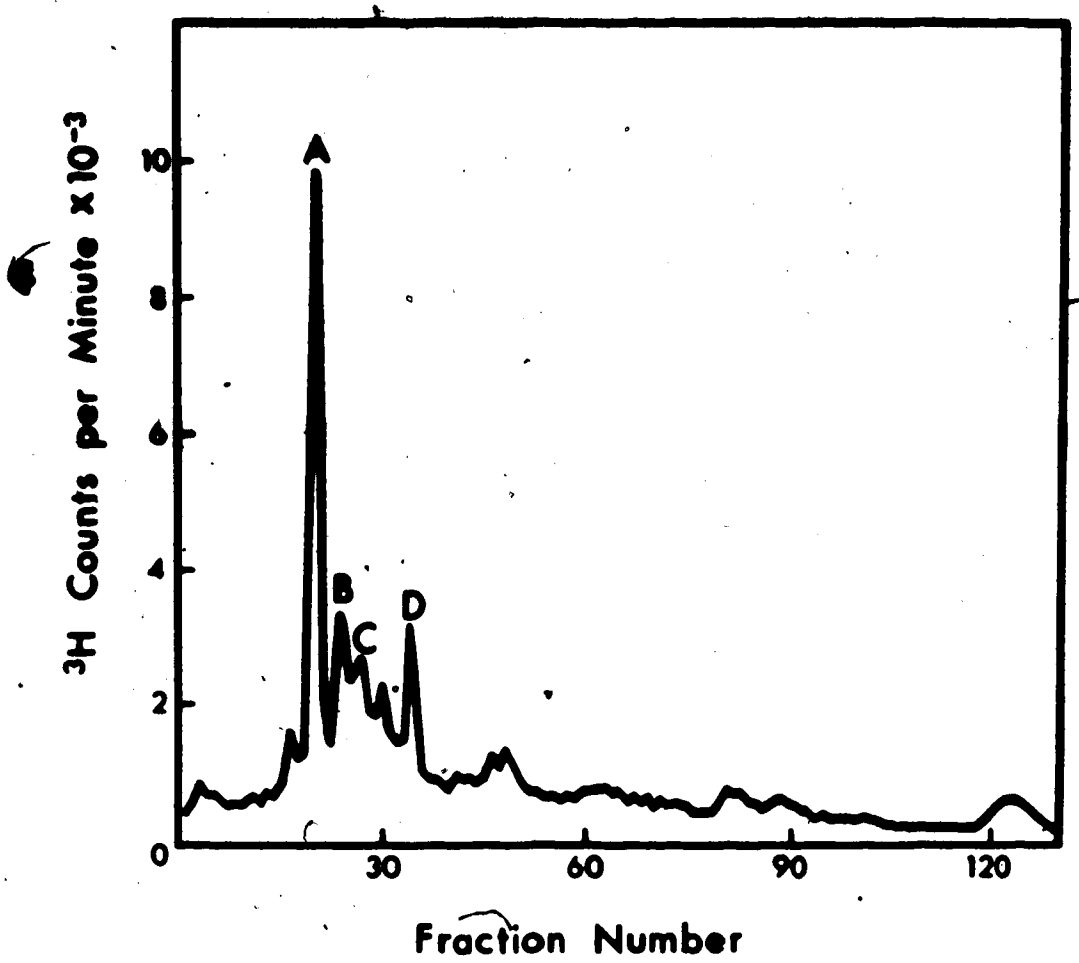


Figure 34. Electrophoretic analysis on 0.1% SDS-7.5% polyacrylamide gels of the ^{3}H-amino acid-labeled precursor polypeptides used as substrate in the protease assay. Infected cells were pulse-labeled for 5 min with ^{3}H-amino acids at 6 hr p.i. A lysate was made with 0.05 M TRIS-HCl (pH 8.0) containing 0.05 M KCl and 1% NP-40 and the nuclei removed by centrifugation at 1000 g for 10 min. The precursor polypeptides were pelleted by centrifugation at 37,000 rpm for 1 hr in a SW 50.1 rotor. Aliquots of the pellet were prepared for electrophoresis as described in Figure 18. Conditions for electrophoresis and analysis were the same as those described in Figure 10.

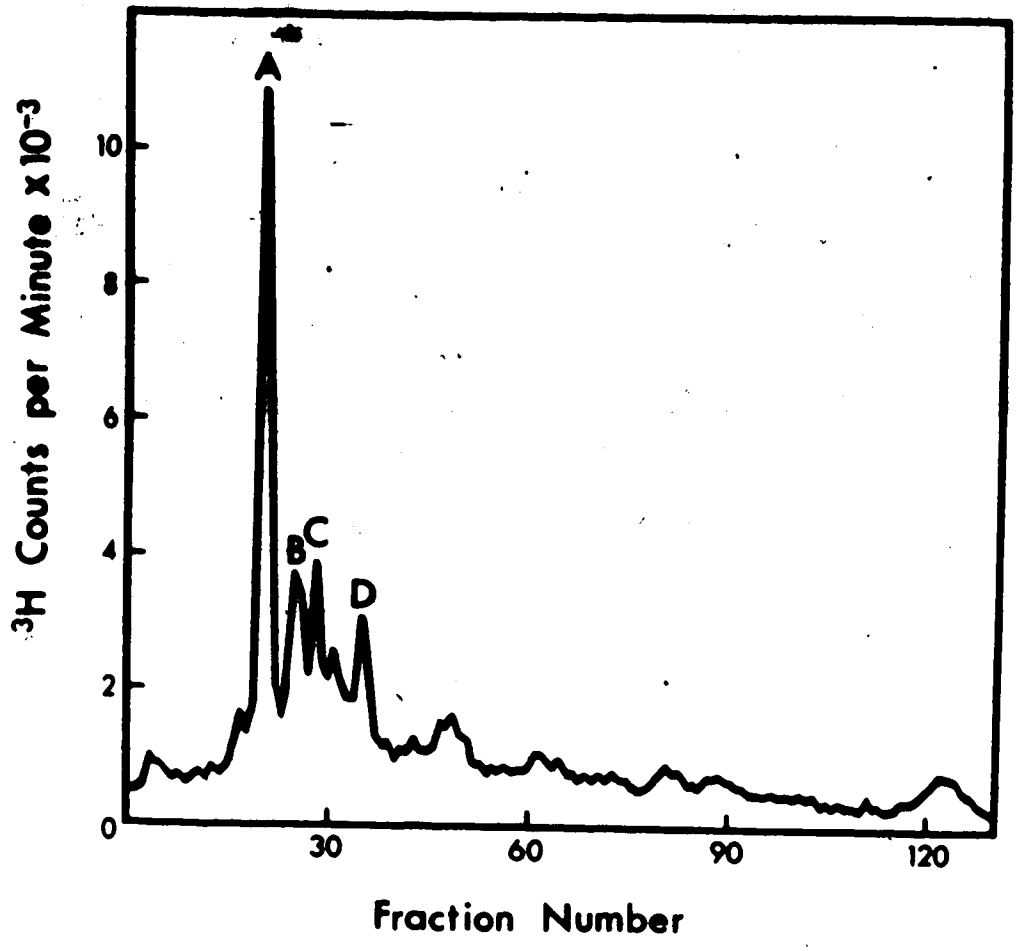


Figure 35. Electrophoretic analysis on 0.1% SDS-7.5% polyacrylamide gels of the ^{3}H-amino acid-labeled polypeptides present in the incubated protease assay mixture. The precursor polypeptide substrates were prepared as outlined in Figure 34. A 50 µl aliquot of polypeptide F (derived from Pool 1 Figure 17; see Figure 18) was mixed with an equal volume of substrate and incubated at 37° for 1 hr. The sample was then prepared for electrophoresis as outlined in Figure 18. Conditions for electrophoresis and analysis were identical to those described in Figure 10.

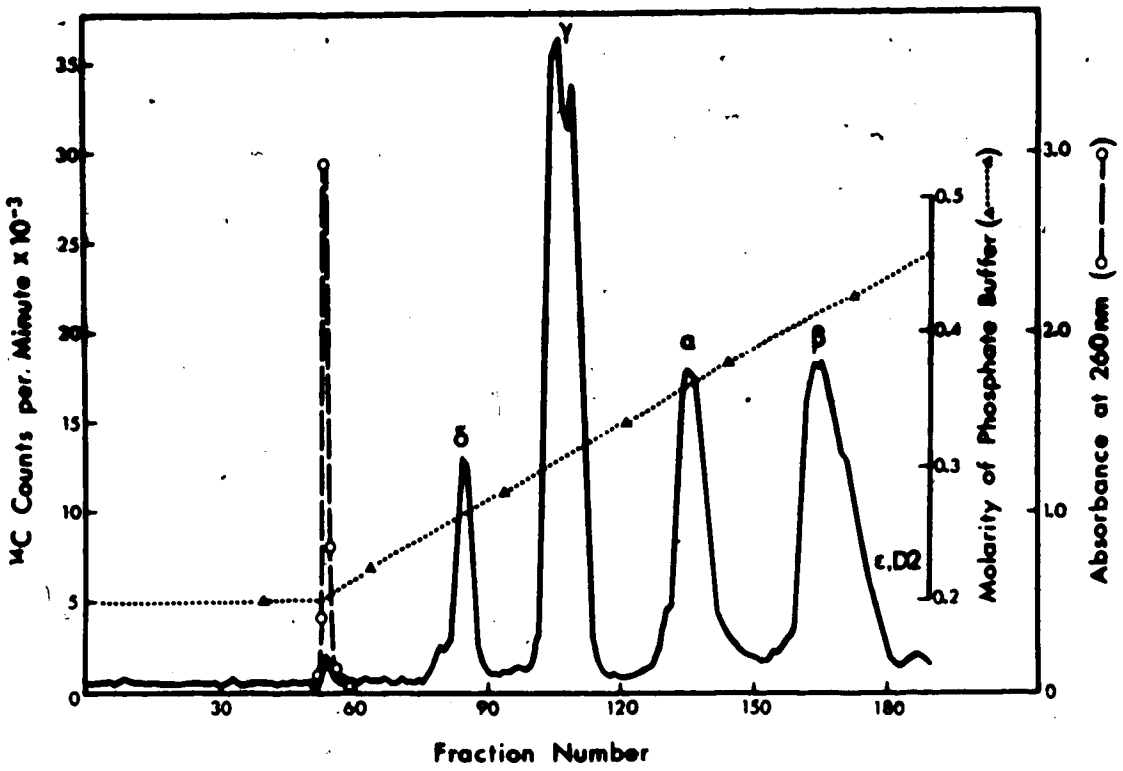


Figure 36. SDS-hydroxylapatite chromatography of ^{14}C-amino acid-labeled virus capsid polypeptides. Purified ^{14}C-amino acid-labeled Mengo virions were disrupted by heating at 100° for 5 min in a buffer consisting of 0.01 M sodium phosphate (pH 6.4) containing 2% SDS and 5% β-mercaptoethanol. The RNA and polypeptides were resolved as described by Ziola and Scraba (1975). The RNA peak (0), as detected by absorbance at 260 nm, was pooled (fractions 55-57) and dialyzed against 0.01 M sodium phosphate (pH 7.2) to remove the SDS. Radioactivity was assayed by standard procedures. Phosphate molarity was determined from conductivity measurements with the use of a standard curve.

the 6 marker polypeptide and the bromophenol blue marker (Figure 37). The size of this peak was diminished by 50 to 60% when the RNA was also incubated with proteinase K (Figure 38), indicating that the labeled material was a protein.

Discussion

As an initial step towards characterizing the functions of Mengo noncapsid proteins, the stable species E, F, G, H and I have been isolated from virus-infected cells. The two larger polypeptides, E and F, were isolated using both denaturing and non-denaturing methods while the smaller polypeptides, G, H and I, have been obtained by the use of denaturing methods.

Previous studies of the picornavirus RNA polymerase (RNA replicase) have involved sequential purification by monitoring the enzymatic activity. Such studies have suggested that polypeptide E (NCVP 4) plays some role in the replicase activity, but the various "purified" replicases were contaminated to varying extents with other viral-specific polypeptides (Lundquist et al., 1974; Loesch and Arlinghaus, 1975; Traub et al., 1976). This chapter has described an alternative approach in studying the replicase: i.e.: isolation of the polypeptide which has been indicated to be responsible for the RNA replicase activity (E) fol-

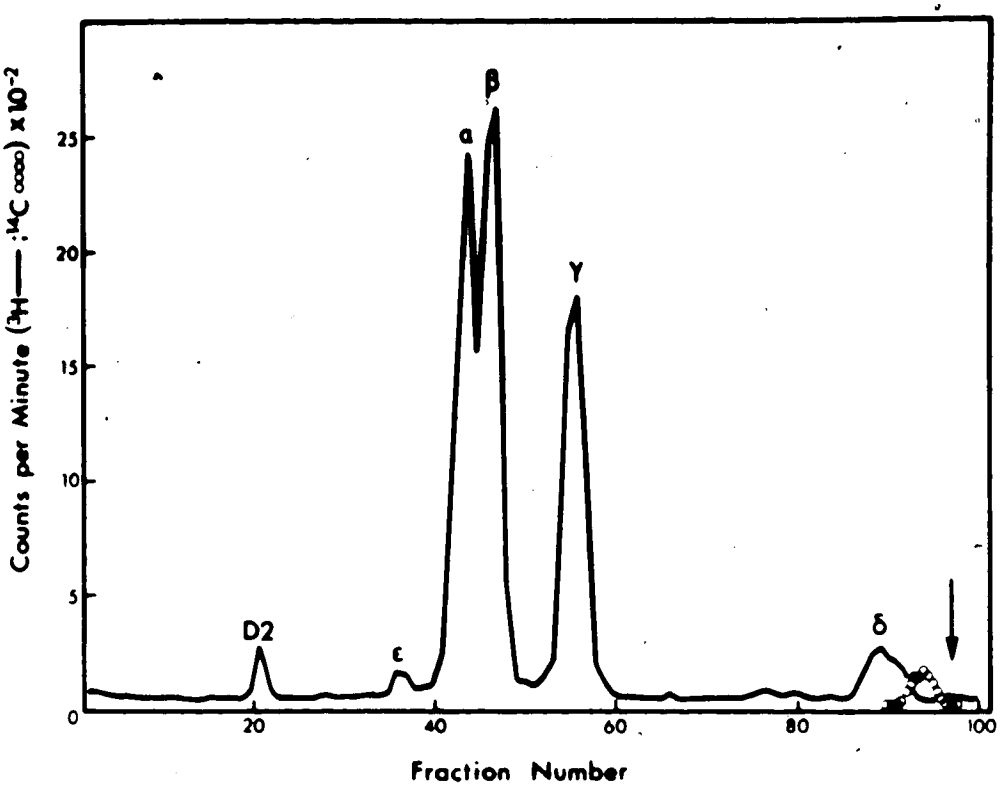


Figure 37. Electrophoretic analysis on 0.1% SDS-10% polyacrylamide gels of ^{14}C-amino acid-labeled VPg. RNA obtained from a SDS-hydroxylapatite column (Figure 36) was precipitated with 2½ volumes of cold 95% ethanol. The pellet was collected by centrifugation at 15,000 rpm for 30 min in a JA-20 rotor. The RNA was resuspended in 400 µl of 0.01 M TRIS-HCl (pH 7.0). RNase A, RNase T1 and BSA were added to 200 µl of the RNA at final concentrations of 50 µg/ml, 100 units/ml and 400 µg/ml, respectively. The sample was incubated at 37° for 3 hr and then precipitated by the addition of 5 volumes of acetone. The precipitate was electrophoresed as described in Figure 10. The open circles show the profile obtained when the gel was fractionated and assayed for radioactivity as described in Figure 4.
The marker capsid polypeptides (indicated by the solid line) were electrophoresed at the same time in a separate gel. arrow indicates the position to which the bromophenol blue marker migrated in both gels.

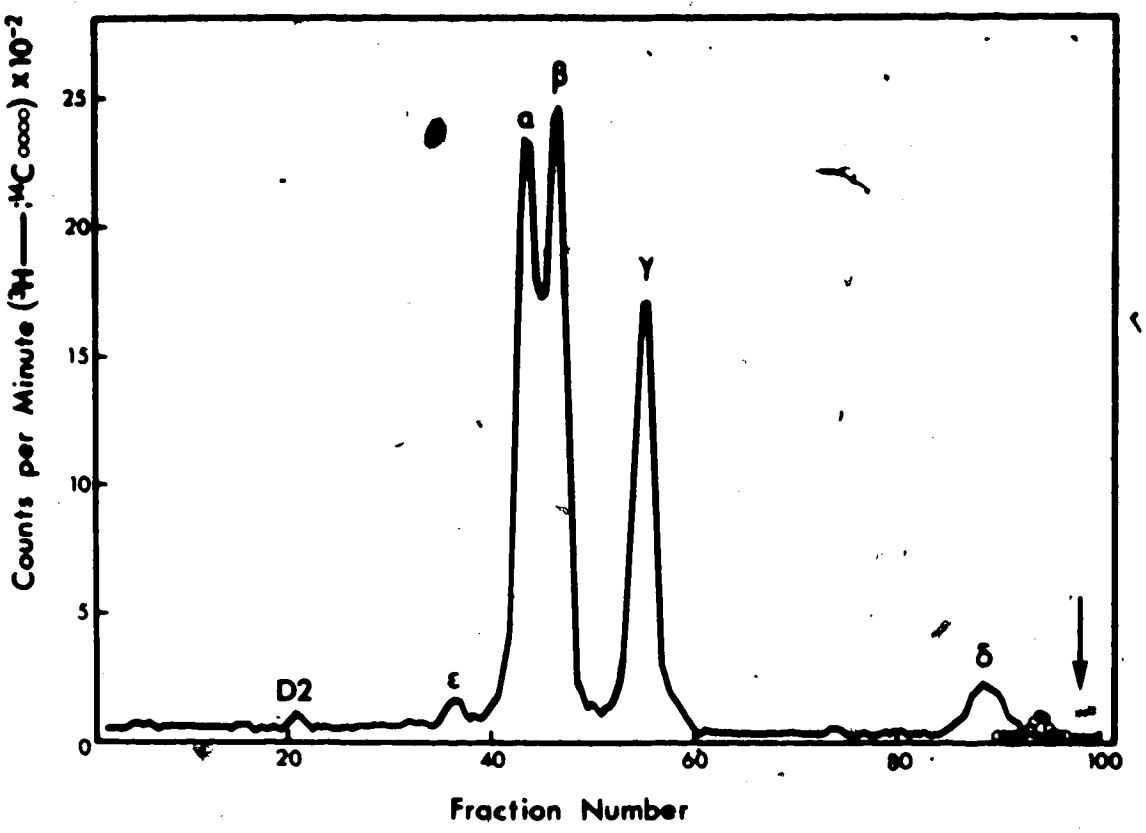


Figure 38. Electrophoretic analysis on 0.1% SDS-10% polyacrylamide gels of ^{14}C-amino acid-labeled VPg which was proteinase K treated. VPg was obtained and treated in an identical manner to that described in Figure 37 with the following exception: proteinase K was added at 1½ hr to the incubation mixture to a final concentration of 200 µg/ml. The sample was then incubated for an additional 1½ hr. The incubation products were assayed electrophoretically as described in Figure 37. The open circles show the profile obtained when the gel was fractionated and assayed for radioactivity as described in Figure 4. The marker capsid polypeptides (indicated by the solid line) were parallelly electrophoresed on a separate gel. The arrow indicates the position to which the bromophenol blue marker migrated in both gels.

lowed by an attempt to assay for that activity. Polypeptide E isolated using nondenaturing conditions (Figure 17) was free from contamination by other viral polypeptides and contained only 5 - 6 host cell-specified polypeptides (Figure 21). Associated with the E polypeptides was an RNA polymerase activity which was dependent on exogenous viral RNA and oligo(rU) primer (Figure 26). The activity, however, was quite unstable and this fact plus the observed varying recoveries of activity suggest that other as yet uncharacterized components are also necessary for a fully functional viral replicase activity. Studies on poliovirus replicase have indicated that the procapsid structure (Yin, 1977) or capsid polypeptides (Ghendon, 1973) play some integral role in the replicase activity. Results reported in this chapter showed that replicase activity was diminished as the capsid polypeptides were progressively removed from the noncapsid polypeptides (Figure 27). However, addition of capsid polypeptides to isolated polypeptide E did not stimulate its observed activity (Figure 28) so the relationship, if any, between these observations and the poliovirus data is unclear.

Another possible explanation for the decreasing recovery of activity is based on a model proposed by Nomoto et al. (1977). These authors suggest that a polymerase core structure combines with a free molecule of VPg (the polypeptide which is also found covalently attached to the 5'-end of the viral RNA) to produce a functional replicase.

The VPg molecule supposedly functions as a primer for RNA replication, during which time it becomes attached to the 5'-end of the RNA. It has been shown in this chapter that Mengo virus RNA also contains a VPg-like polypeptide, and it is conceivable that this polypeptide was lost during the purification procedure outlined for the undenatured E preparation. VPg appears to be relatively hydrophobic when isolated from viral RNA (Hruby and Roberts, 1978), and a large portion of it may have aggregated and been lost during the 37,000 rpm centrifugation step. Affinity chromatography (Figure 7) presumably would have removed any VPg still present in solution (the disrupted virus antigen used to produce antibodies for this column also contained the viral RNA and its covalently-linked VPg). The recovery of a portion of the replicase activity using the primer oligo(rU) is consistent with this possibility. Presumably, however, oligo(rU) is a comparatively poor primer with respect to VPg.

Ziola and Scraba (1976) have suggested that F is a possible candidate for a virus-specified protease. Recent results have shown that when EMC virus RNA is translated in a reticulocyte cell-free system in vitro, an active proteolytic activity, which can cleave pre-A (an uncleaved precursor of A and G) and polypeptide C, is produced (Pelham, 1978). This activity has been attributed either to the non-capsid polypeptides I and/or F. A preliminary assay experiment with isolated polypeptide F related in this chapter was

unsuccessful in demonstrating any associated proteolytic activity. It would seem that further experiments, employing a wider variety of assay conditions, are necessary in order to ascertain whether or not F possesses proteolytic activity. In addition, a possible role of F in binding the replicase to membranes, as reported by Butterworth et al., (1976), should be investigated.

A report of a protease activity associated with a polypeptide of molecular mass 14,000 daltons, produced by granulosis virus (Tweeten et al., 1978) suggests that polypeptides G, H and/or I should also be obtained in undenatured form and assayed for proteolytic activity. Denatured preparations of G, H and I, prepared as reported in this chapter, should be suitable for use as antigens to produce specific antisera which can be used to isolate them from the other viral polypeptides.

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