CHARACTERIZATION OF VIRUS SPECIFIC POLYPEPTIDES DURING PICHINDE VIRUS INFECTION IN CELL CULTURES

By

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ABSTRACT

Several aspects of the replication of Pichinde virus, a member of the arenavirus group, were studied in in vitro cell culture. The major emphasis in the study was the identification and characterization of Pichinde virus encoded polypeptides expressed in infected BHK-21 cells. Polypeptides were identified by immunoprecipitation of lysates of infected cells with hamster anti-Pichinde virus immune serum. Several polypeptides exhibited a temporal and multiplicity of infection dependent appearance. Polypeptides with approximate molecular weights of 200,000, 79,000, 64,000, 48,000, 38,000 and 28,000 were identified by SDS-polyacrylamide gel electrophoresis after a 1 hr pulse label of infected cells with L-[^{35}S] methionine. The same polypeptides and five more of 52,000, 36,000, 17,000, 16,500 and 14,000 daltons were identified in pulse-chase experiments. The 79,000, 52,000 and 36,000 dalton polypeptides incorporated ^{3}H-glucosamine. The larger glycoprotein was identified as a precursor to the smaller structural glycoproteins (GP1 and GP2) and has been designated GPC. In the presence of tunicamycin, an inhibitor of glycosylation, GPC was synthesized as a 42,000 dalton precursor (pGPC). The nucleoprotein (NP, 64,000 daltons) was the predominant immunoprecipitable polypeptide.

Polypeptide interrelationships were determined by two dimensional L-[^{35}S] methionine tryptic peptide mapping. The 200,000

dalton polypeptide (L), GPC and NP were distinct primary products of translation. Polypeptides of 48,000, 38,000, 28,000, 17,000, 16,500, and 14,000 daltons were all related to the nucleoprotein. NP17, 16.5 and 14 were derived proteolytically but the larger molecules (NP48, NP38 and NP28) may be distinct premature termination products of translation.

L, NP, GP1, GP2, NP48 and NP38 were found in immunoprecipitates of infected cell extracts and in purified virus preparations. GPC, NP28, NP17, NP16.5 and NP14 were evident only in immunoprecipitates of infected cell extracts. The function, if any, of the NP derivatives is not known. L was postulated to be the viral encoded RNA-dependent RNA polymerase.

The affect of amino acid analogues, actinomycin D, and α amanitin and amphotercerin B, on viral protein synthesis and processing was examined. α-ananitin and amphotercerin B did not affect the SDS-PAGE polypeptide profile. Actinomycin D did not alter the profile of immunoprecipitable polypeptides after a 1 hr pulse-label, however, after a 6 hr chase period NP17, NP16.5 and NP14 were not apparent.

The coding sequences for the primary products of translation (L, NP, GPC) were assigned to either L or S viral RNAs. To do so, prototype Pichinde and Muchinque viruses and a reassortant virus (RE-2) were employed. The reassortant virus contains the L RNA of Pichinde virus and the S RNA of Munchique virus. NP and GPC were shown to be encoded by S RNA. The two-dimensional L-[^{35}S] methionine tryptic peptide maps of these molecules in Munchique

virus and RE-2 virus were identical. L polypeptide tryptic peptide profiles were identical in Pichinde and RE-2 viruses which suggested that L is encoded by L RNA.

A series of very preliminary experiments were performed to characterize properties of Pichinde virus replication, primarily in BHK-21 cells, but also in MDCK and Vero cells grown in vitro. Infection of all three cell lines resulted in a peak of infectious virus released into the medium 2 to 3 days later. This was followed by a rapid decline in viral release to 10% of peak levels by days 8 to 10. Similar curves were observed for synthesis of viral macromolecules in the cell, however, immunofluorescence during this period remained high. We attempted to correlate the immunofluorescence observations with proteolytic products of NP using monoclonal antibodies directed against these molecules.

A model for regulation of Pichinde virus replication in infected cells is presented. The model proposes that the nucleoprotein or its proteolytic products are integral factors in the decline of viral synthesis.

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LIST OF ABBREVIATIONS

MW	molecular weight
PAGE	polyacrylamide gel electrophoresis
c	centi, 10^{-2}
m	milli, 10^{-3}
μ	micro, 10^{-6}
l	liter
g	gram(s)
mol	mole
M	molar
N	normal
g	gravity
^{o}C	degree celsius
V	volt(s)
min	minute(s)
hr(s)	hour(s)
Ci	Curie(s)
rpm	revolutions per minute
(w/v)	weight/volume
(v/v)	volume/volume
U	unit(s)
^{3}H	tritium
^{35}S	sulfur-35
RNA	ribonucleic acid

ABBREVIATIONS (Continued)

rRNA	ribosomal ribonucleic acid
mRNA	messenger ribonucleic acid
HCl	hydrochloric acid
NaCl	sodium chloride
Tris	Tris(hydroxymethyl)aminomethane
TCA	trichloroacetic acid
PMSF	phenylmethylsulfonylfluoride
SDS	sodium docecyl sulfate
PPO	diphenyloxazole
DOC	sodium deoxycholate
BHK	baby hamster kidney
MDCK	canine kidney cells
Vero	African green monkey kidney
PFU	plaque forming unit
MOI	multiplicity of infection
CPM	counts per minute
PBS	phosphate buffered saline
EDTA	ethylenediamine tetracetic acid

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1 Introduction

1.1 Historical perspective

The arenaviruses are a relatively new group of viruses which share two main features that have made them particularly interesting. Members of the group characteristically produce persistent infections in their natural host, and four members of the group cause diseases in man; lymphocyte choriomeningitis, Argentine hemorrhagic fever, Lassa fever, and Bolivian hemorrhagic fever. These diseases can be severe and sometimes fatal (reviewed by Casals, 1975; Oldstone and Peters, 1978). Viral persistence has perhaps provided the main impetus for study of the group because the limited geographic distribution of Argentine and Bolivian hemorrhagic fevers and Lassa fever has discouraged intensive investigation.

The prototype virus for the group is lymphocytic choriomeningitis virus (LCM) which was first isolated from a fatal human case of encephalitis thought at the time to be St. Louis encephalitis (Armstrong and Lillie, 1934). In 1935, Rivers and Scott (1935) reported recovery of the virus from five human cases of aseptic meningitis and at the same time Traub (1935) reported discovery of the virus in a mouse colony. It was later recognized that the house mouse, Mus musculus was the natural host and principal reservoir of LCM virus (Lepine et al., 1937; Armstrong and Sweet, 1939). Although LCM is perhaps the most studied of the arenaviruses and much is known concerning the virus-host interaction (for reviews see Lehmann-Grube, 1971, 1973; Hotchin, 1971, 1974; Cole and Nathanson, 1974; and Lehmann-Grube et al., 1981), the

virus is of limited significance with respect to aseptic meningitis in man since nearly all individuals having LCM infections recover (Murphy, 1977)

Tacaribe virus, the only arenavirus not isolated from rodents, was found in the tissues of fruit eating bats in Trinidad in 1956-1958 (Downs et al., 1963) and Junin virus was isolated from human cases of Argentine hemorrhagic fever (Parodi et al., 1958). A new virus complex was formed when Machupo virus was isolated in Bolivia in 1963 from a fatal case of Bolivian hemorrhagic fever, and was found to be serologically related to Tacaribe and Junin but not to any known arboviruses (Johnson et al., 1965 a). The frequency of isolations increased with the identification of Amapari (Pinheiro et al., 1966), Tamiami (Calisher et al., 1970), Parana (Webb et al., 1970), Pichinde (Trapido and Sanmartin, 1971), and Latino (Johnson et al., 1973). These latter five viruses have not been associated with human disease under natural conditions, but Buchmeier et al. (1974) have demonstrated infection of laboratory workers by Pichinde virus.

The eleven current members of the arenavirus group, their principal vertebrate hosts, and known distribution are listed in Table 1. Junin, Machupo, Amapari, Tacaribe, Pichinde, Parana, Latino, and Tamiami have been organized into what is commonly known as the Tacaribe complex. Several investigations led to the inclusion of LCM in the arenavirus group. The pattern of infection of Machupo virus in neonatal Calomys callosus (Johnson et al., 1965b) resembled that of LCM in the murine system (Lehmann-Grube, 1971) and there appeared to be a morphological similarity between the two viruses (Murphy et al., 1969; Dalton

Table 1

Members of the Arenavirus Group

Virus	Vertebrate Host	Distribution	Reference
Lymphocyte Choriomeningitis	*Mus musculus*	Worldwide	Armstrong and Lillie (1934)
Mozambique	*Mastomys natalensis*	Mozambique	Wulff *et al.* (1977)
Lassa	*Mastomys natalensis*	West Africa	Monath *et al.* (1974)
Junin	*Calomys laucha* *Calomys musculinus* *Akodon azarae*	Argentina	Parodi *et al.* (1958)
Machupo	*Calomys callosus*	Bolivia	Johnson *et al.* (1965a)
Amapari	*Oryzomys goeldie* *Neacomys guianae*	Brazil	Pinheiro *et al.* (1966)
Tacaribe	*Antibesis liturates* *Antibesis jamaicensis*	Trinidad	Downs *et al.* (1963)
Pichinde	*Oryzomys albigularis*	Colombia	Trapido and Sanmartin (1971)
Parana	*Oryzomys buccinatus*	Paraguay	Webb *et al.* (1970)
Latino	*Calomys callosus*	Bolivia	Johnson *et al.* (1973)
Tamiami	*Sigmodon hispideis*	Florida	Calisher *et al.* (1970)

et al., 1968) as well as a demonstrable one-way cross reaction of LCM antigens with antisera to Tacaribe complex viruses (Rowe et al., 1970a).

A new virus group was defined in 1970 (Rowe et al., 1970b) and was given the generic designation arenoviruses (from Arenosus, Latin: sandy) since a granular appearance in virions was characteristic (Dalton et al., 1968). The name was changed shortly thereafter to arenaviruses to avoid possible confusion with adenoviruses. Lassa virus, the etiological agent of Lassa fever (Buckley and Casals, 1970), was included in the group since it was morphologically identical and was found distantly related to LCM on a serological basis (Speir et al., 1970). A 1974 report of the Study Group on Arenaviruses, Vertebrate Virus Subcommittee, International Committee on Taxonomy of Viruses, contains the accepted taxonomy and properties of the family Arenaviridae (Pfau et al., 1974). Mozambique is a relatively new member of the group which was isolated from the same rodent species as Lassa virus (Wulff et al., 1977).

1.2 Biophysical properties and purification

The arenaviruses were originally defined as enveloped RNA viruses which shared a common group specific antigen and which as a group, exhibited a unique structure in thin section electron micrographs (Rowe et al., 1970a, b). The virions were pleomorphic, ranging from 50-300 nm in diameter and averaging 110-130 nm. A dense envelope containing closely spaced spike-like projections, lack of an electron dense core, and variable numbers of 20-30 nm electron dense particles resembling ribosomes, were characteristic of arenavirus particles. This general description hints of the difficulties and

heterogeneity in results which might be expected in attempts to define infectious virus or interfering particles by standard biophysical techniques or to purify either to homogeneity.

Several groups have reported sedimentation coefficients for various arenaviruses. Values for LCM varied from 76S (Pfau, C.J.,; cited in Rawls and Buchmeier, 1975) to 360S (Pederson, 1979) and from 470 to 500S (Pederson, 1970). Pichinde virus values were found between 300 and 325S (Ramos et al., 1972). Buoyant density values displayed less heterogeneity. These have been measured in sucrose, cesium chloride, metrizamide, urografin, and conray, and are summarized in Table 2.

Arenavirus stability and purification have been reviewed in detail elsewhere (Rawls and Buchmeier, 1975). Stability, as expected, varies with salt concentration, temperature and pH, where these parameters have been examined (Parodi et al., 1966; Pfau and Camyre, 1967; Webb et al., 1967; Mifune et al., 1971; Carter, 1972). Results have been similar to those found for Pichinde virus (Carter, 1972). Virus was stable at $4^{\circ}C$, $25^{\circ}C$ and $37^{\circ}C$ but infectivity was lost rapidly at $56^{\circ}C$. Virus was also stable between pH values of 6.0 and 9.0 with infectivity being lost rapidly below pH 6.0. Although stability may be affected by storage conditions and virus concentration, it has been reported that stability increases with the addition of protein to suspensions of Machupo and LCM viruses (Webb et al., 1967; Pederson, 1970).

Concentration and purification of arenaviruses has been accomplished by a number of methods. The method of concentration which has replaced many of the initial protocols (reviewed by Rawls and Buchmeier, 1975) is precipitation with 6 to 10 percent polyethy-

Table 2

Arenavirus Buoyant Density Values

Virus	Buoyant density (gm/cm^3) Sucrose	CsCl	Metrizamide Urografin Conray	Reference
LCM	1.18	1.2	1.12-1.14	Pederson (1973a) Gschwender (1975) Buchmeier et al. (1978)
Junin	1.17 1.12	1.19-1.20		Johnson et al. (1973) Coto et al. (1972)
Machupo	1.17			Johnson et al. (1973)
Amapari	1.18			Johnson et al. (1973)
Tacaribe	1.17	1.20		Johnson et al. (1973) Palmer et al. (1977)
Pichinde	1.18 1.16-1.18	1.18-1.20		Mifune et al. (1971) Carter et al. (1972)
Tamiami	1.15			Gard et al. (1977)

lene glycol 6000 in the presence of 0.4 M sodium chloride. LCM virus (Buchmeier et al., 1978), Pichinde virus (Ramos et al., 1972; Vezza et al., 1977), and Tacaribe and Tamiami viruses (Gard et al., 1977), have all been concentrated this way with good recovery of virus. Concentration 100 fold by molecular filtration has been used successfully with Machupo, Pichinde and Tacaribe virus (Gangemi et al., 1977) and good recoveries of LCM virus have been obtained using precipitation with cold methanol (Welsh et al., 1976), ammonium sulfate (Pederson, 1970) and zinc-acetate (Gschwender et al., 1975).

Purification of concentrated virus with the aim of reducing contaminating protein and at the same time retaining infectivity has usually been accomplished in two stages. LCM virus (Pederson, 1970; Buchmeier et al., 1978) and Pichinde virus (Ramos et al., 1972; Carter et al., 1973a)have been partially purified by centrifugation through sucrose onto a heavy sucrose cushion. Pichinde virus has also been purified initially on glycerol-tartrate gradients (Vezza et al., 1977). Chromatography through glass beads of controlled pore size, after cultivation of infected cells in polyethylene glycol treated fetal calf serum, has been used for LCM virus (Gschwender et al., 1975).

The partially purified arenavirus preparations have then been purified further by equilibrium centrifugation in sucrose gradients (Pederson, 1970; Carter et al., 1973a; Farber and Rawls, 1975; Vezza et al., 1977; Buchmeier et al., 1978), amidotrizoate gradients (Gschwender et al., 1975) or Renografin (Dimock et al., 1982).

1.3 Morphology

A consistent view of arenavirus morphology by electron microscopy has emerged from several investigations which began with the first description of the LCM virus particle (Dalton et al., 1968). As a group, the arenaviruses are usually round, oval or pleomorphic particles ranging in diameter between 50 and 300 nm with a mean diameter of 110 to 130 nm (Murphy et al., 1969, 1970, 1973; Speir et al., 1970; Murphy and Whitfield, 1975). Preparations which are negatively stained exhibit a membrane (virion envelope) which contains variable numbers of closely spaced club-shaped surface projections or spikes (Murphy et al., 1970). The five to ten nm long spikes represent external projections of viral glycoproteins. Spikeless particles of Tacaribe and Tamiami virions (Gard et al., 1977) and Pichinde virus (Vezza et al., 1977), treated with chymotrypsin in the former case, and with pronase and bromelain in the latter, lose their surface projections, coincident with the loss of glycoproteins from polyacrylamide gel resolved preparations. The interior of arenavirus particles is characterized by variable numbers of 20 to 25 nm electron dense granules embedded in an amorphous matrix. Average sized particles contain ten to fifteen of the granules which in some cases appear to have linear structures connecting them (Murphy and Whitfield, 1975). Granules have been identified as host-cell derived ribosomes (Carter et al., 1973a; Pederson, 1973b; Farber and Rawls, 1975). Distinct nucleocapsid structures have not been visualized within the arenavirus particle by thin section or negative staining electron microscopy. LCM virus (Walker et al., 1975), Tamiami virus

(Murphy et al., 1976), and Pichinde virus (Murphy et al., 1977) infected animal tissues contain particles which are morphologically identical to those observed in tissue culture.

The heterogeneity in arenavirus size and number of internal ribosomes has led to some disagreement over the nature of the infectious unit. Mannweiler and Lehmann-Grube (1973) suggest that the well described ribosome-containing particle may not be the infectious unit of LCM virus, but rather point to a 50 to 65 nm structure with a dense core in virus infected cells which has also been observed in the tissue of individuals with Argentine hemorrhagic fever (Maiztegui et al., 1975). However in the case of Tacaribe and Tamiami viruses (Gard et al., 1977) and Pichinde virus (Vezza et al., 1977), infectivity and radioactive label were coincident on gradients with the classic arenavirus morphology and larger particles. These differences have not been clearly resolved at the present time.

Although a distinct nucleocapsid has not been observed in intact viral particles, several investigators have described the morphology of ribonucleoprotein structures derived from virions solubilized with nonidet P-40 or triton X-100 (Palmer et al., 1977; Gard et al., 1977; Vezza et al., 1977; Vezza et al., 1978; Howard and Simpson 1980). The structures, isolated from Tacaribe, Tamiami, and Pichinde viruses, when examined by electron microscopy, were filamentous and contained globular subunits reminiscent of "beads on a string". Circular and linear molecules of RNA were apparent after removal of protein, and ranged in length from 1.3 to 1.7 μm (Vezza et al., 1978). Diameters of 3 to 4 nm were noted for Tacaribe and Tamiami ribonucleo-

proteins with a 4.5 nm spacing of globular subunits (Gard et al., 1977). Ribosomes were not associated with these structures in any case examined.

Maturation of arenaviruses occurs by budding from the plasma membrane, and electron microscopic observations suggest that the membrane becomes more dense at the site of budding (Murphy and Whitfield, 1975). Ribosomes appear to be contained within nascent buds, but earlier in infection they form very prominent intracytoplasmic inclusion bodies which appear to be imbedded in a matrix of viral protein (Abelson et al., 1969).

1.4 Viral macromolecules: nucleic acid

The arenaviruses are classified as enveloped RNA viruses in which the genome is segmented and of negative polarity (complementary to mRNA) (Pfau et al., 1974), but the viruses as a group are unique in that a portion of virion RNA is host cell derived. An initial report (Pedersen, 1970) suggested that detergent lysis of LCM virions released RNase sensitive components which separated into three populations (28S, 21S, 18S) in high salt sucrose gradients. PAGE analysis later revealed the presence of four size classes of RNA designated 31S, 28S, 21S and 18S. Two of these, 28S and 18S, comigrated with cellular ribosomal RNA (Pedersen, 1971). At levels of actinomycin D which inhibited rRNA synthesis, radiolabel incorporation into the 28S and 18S RNA species was reduced, suggestive of a host cell origin for these molecules.

Subsequent investigations consistently yielded at least five virion RNA species from LCM, Pichinde, Junin, Tacaribe, Tamiami, and

Parana viruses (Pedersen, 1973b;Buchmeier et al., 1980b;Carter et al., 1973a;Vezza et al., 1977, 1978; Ramsingh et al., 1980; Anon et al., 1976; Gimenez and Compans, 1980; Vezza et al., 1978; Dutko et al., 1978). The results are summarized in Table 3. Since sedimentation values have only been determined for Pichinde virus (Carter et al., 1973a) and LCM virus RNAs (Pedersen, 1970), the genomic RNAs are referred to simply as L (large) and S (small) with molecular weights as indicated. Molecular weight determinations for total genomic RNA have varied between 3.2×10^6 and 4.8×10^6. A more rigorous determination was attempted for Pichinde virus L and S RNAs, however mean estimates were dependent on the gel system employed (methylmercury hydroxide or glyoxal). Values ranged between 2.5 to 3.1×10^6 daltons for L RNA, and 1.12 to 1.45×10^6 daltons for S RNA (Ramsingh et al., 1980). In general there has been a smaller range in values determined for the smaller S RNA than for L RNA. Intervirus differences seem to reflect real differences in RNA size since co-electrophoresis experiments have been performed with Tacaribe, Tamiami, and Pichinde virus RNAs (Vezza et al., 1978; Gimenez and Compans, 1981), however it is not clear whether intravirus differences reflect, in part, real differences in viral stocks or experimental differences. A 15S RNA species reported in some Pichinde virus preparations (Farber and Rawls, 1975; Dutko et al., 1976) may not be viral in origin (Dimock et al., 1982).

The nature of the electron dense particles characteristic of arenavirus morphology in thin section electron microscopy has been resolved by several investigators. Initial experiments demonstrated that incorporation of radiolabel into 28S and 18S RNAs was inhibited,

Table 3

Arenavirus RNA Species

Virus	Genomic RNA[a]	rRNA[b]	Other RNA	Reference
LCM	L($2.1x10^6$)	28S	4S	Pedersen
	S($1.1x10^6$)	18S	5S	(1971, 1973b)
			5-5S	
	L($3.1x10^6$)			Buchmeier
	S($1.3x10^6$)			et al. (1980b)
Pichinde	L($2.1x10^6$)	28S	4-6S	Carter et al.
	S($1.1x10^6$)	18S		(1973a)
	L($3.2x10^6$)	28S		Vezza et al.
	S($1.6x10^6$)	18S		(1977, 1978)
	L($3x10^6$)	28S		Chinault et al.
	S($1.3x10^6$)	18S		(1981)
	L($2.63x10^6$)	28S		Ramsingh
	S($1.26x10^6$)	18S		et al. (1980)
Junin	L($2.4x10^6$)	28S	4S	Anon et al.
	S($1.34x10^6$)	18S	5S	(1976)
			5-5S	
Tacaribe	L($2.8x10^6$)			Gimenez and Compans
	S($1.3x10^6$)			(1980)
Tamiami[c]				Vezza et al. (1978)
Parana[c]				Dutko et al. (1978)

[a] L and S refer to the large and small viral RNA species respectively. Numbers in parentheses are estimated molecular weights.

[b] These components are highly variable in arenavirus preparations.

Table 3 (Footnotes cont'd)

(c) L and S genomic RNAs have been identified but molecular weights were not estimated.

relative to L and S RNA, by a concentration of actinomycin D which inhibits rRNA synthesis (Pedersen 1971; Carter et al., 1973a). Carter and coworkers (1973a) also showed that 28S and 18S RNA extracted from Pichinde virus had a G+C content and methylation pattern characteristic of the host cell rRNA, which was complemented by studies demonstrating identical oligonucleotide fingerprints for viral and host cell RNA (Vezza et al., 1978). Farber and Rawls (1975) were able to isolate 80S monosomes, and 60S and 40S ribosomal subunits, from detergent disrupted virions. These structures had similar physicochemical properties to ribosomes isolated from uninfected BHK-21 cells. The structures were susceptible to dissociation with EDTA and contained 28S and 18S rRNA. Similar results were reported for LCM virus by Pedersen and Konigshofer (1976) who concluded on the basis of densitites that the viral ribosomes were not degraded.

Although these results suggest that the electron dense particles are indeed ribosomes, their role in viral replication has been elusive. Chinault et al. (1981) have shown that ribosomes released from disrupted Pichinde virus are capable of synthesizing polypeptides when exogenous mRNA, aminoacyl-tRNA, eukaryotic elongation factors 1 and 2, GTP, and the appropriate cations are added, however, there was no template RNA association with the particles in virus. Leung and Rawls (1977) grew Pichinde virus in a hamster embryonic lung cell lines which carried a temperature sensitive lesion in the 60S ribosomal subunit. Virus passaged twice in this cell line and then grown at permissive and nonpermissive temperatures in wild type cells did not show any effect on growth due to temperature sensitive ribosomes. It

was concluded that the virion ribosomes were not necessary for virus replication during the initial phase, however these results do not preclude a role during some other process such as virus assembly. Speculation that ribosomes are inadvertantly packaged during the virus budding process is supported by observations that total rRNA, and the proportion of each species packaged, varies greatly, (Vezza and Bishop, 1977; Vezza et al., 1977; Vezza et al., 1978; Leung et al., 1979) although 28S rRNA appears to be present in all cases.

An origin similar to 28S and 18S rRNA has been suggested for the 4 to 6S RNA species, which can be resolved into 4S, 5S and 5.5S RNAs (Pedersen, 1973). These RNAs represent approximately 7 percent of radiolabeled material incorporated into Junin virus (Anon et al., 1976) and 6 percent of the RNA in LCM virus (Pedersen, 1973). The 5.5S RNA was associated with 28S rRNA and was released by heating (Pedersen, 1973). Pichinde virus 4 to 6S RNA was found to be methylated and had a similar base composition to host cell RNAs (Carter et al., 1973a).

Viral genetic information is encoded by L and S RNA species which are not methylated (Carter et al., 1973a), do not have a 3' terminal polyadenylic acid sequence, or a 5' terminal methylated cap structure, and cannot direct the synthesis of protein in a wheat germ in vitro protein synthesizing system (Leung et al., 1977; Leung, 1978a). Viral RNA annealed with polyadenylic acid-containing polysomal RNA and conferred approximately 60 percent ribonuclease resistance, which confirms that L and S RNAs are of negative polarity (anticomplementary to messenger RNA) (Leung et al., 1977). L and S RNAs appear

to encode separate information. Cross hybridization experiments demonstrated that DNA complementary to approximately 90 percent of L and S RNA did not protect S and L RNA, respectively, from S1 nuclease digestion, whereas homologous hybridizations provided nearly complete protection (Leung et al., 1981). This supports the observation that eight temperature sensitive Pichinde virus mutants could be divided into two groups on the basis of reassortment of L and S RNAs (Vezza and Bishop, 1977).

Vezza et al. (1978) originally arrived at the same conclusion by different means. Oligonucleotides obtained by RNase T_1 digestion of L and S RNAs could be assigned as either L or S specific, suggesting that at least a portion of each RNA carried unique nucleotide sequences. Similar results were obtained for Tacaribe virus L and S RNA and it was also demonstrated that the RNA segments of Tacaribe virus exhibited oligonucleotide fingerprints which were quite different from those of Pichinde virus RNA (Gimenez and Compans, 1980). Vezza and coworkers (1980), Kirk et al. (1980), and Auperin et al. (1982) have also shown RNA fingerprints for arenaviruses of a single type which differ substantially, however it has been suggested that sequence comparisons on the basis of oligonucleotide fingerprints require 90 percent homology (Young et al., 1981). The latter arenavirus study (Auperin et al., 1982) also reported partial nucleotide sequences of the 3'-terminus of viral L and S RNAs in Pichinde virus and a virulent derivative of Pichinde-Munchique virus. Although RNase T_1 oligonucleotide fingerprints were easily distinguishable, Pichinde and Munchique viruses S RNA only differed by 12 of the first 120 3'-

terminal nucleotides while the L RNAs differed by 1 out of 50 nucleotides. S and L RNA 3'-termini differed by 19 of 50, and 20 of 50 nucleotides for Pichinde and Munchique respectively, although of the first nineteen nucleotides only two differences were noted. The first initiation codon was at nucleotides 84 to 86 for S RNA and nucleotides 31 to 33 for L RNA but it was not known if these were utilized.

A further finding has suggested that the 5'-termini of L and S RNAs may be complementary to the 3'-termini (K. Dimock, personal communication). Such an observation would in part account for hairpin structures which have been observed by electron microscopy of Tacaribe, Pichinde, and Tamiami virion RNAs and in part for approximately 20 percent RNase resistance associated with the RNAs (Vezza et al., 1978). Circular molecules and forms with intermediate electrophoretic mobilities have also been observed, but it is not clear how these arise (Vezza et al., 1978; Dutko et al., 1981).

The question of the number of genome copies in the infectious virion was approached by examining the effects of ultraviolet and ionizing radiation on Pichinde virus (Carter, 1972; Carter et al., 1973b). The kinetics of viral inactivation with ultraviolet light were single-hit, and suggested that the virus carried only one copy of the genome per virion. Genome size, estimated from gamma irradiation inactivation kinetics, was approximately 6×10^6 to 8×10^6 daltons. This value exceeds all biochemical estimates of genome size and has not yet been accounted for, although it was suggested that the difference may be due to a yet unknown function of rRNA.

1.5 Viral macromolecules: protein

1.5.1 Virion polypeptides

Most investigators agree that arenavirus virions are composed of a viral encoded nucleoprotein and at least one identifiable glycoprotein, but in most cases, two glycoproteins have been observed. Since arenaviruses mature by budding at the plasma membrane (Murphy and Whitfield, 1975), and in the process incorporate host cell ribosomes (section 1.4), some confusion has understandably arisen over the identity and number of viral specific polypeptides in virions and in cells. Host cell protein is clearly incorporated into the virion envelope, since antibodies reactive against the host cell can inactivate LCM virus (Welsh, 1977).

Virion proteins have been characterized using combinations of physical properties, antigenic properties and when possible, functional properties. Although efforts have been made to correlate these, much remains to be assessed. Polypeptides have primarily been examined by denaturing polyacrylamide gel electrophoresis of disrupted purified virions. Investigations of antigenicity have recently been aided by monoclonal antibody technology and functional properties have been for the most part, limited to enzymatic activities associated with virion polypeptides.

Eight arenaviruses have now been characterized in terms of structural components of the virion and the results are summarized in Table 4. Although there is agreement on the identity of the major structural components, it should be borne in mind that differences in minor proteins are more difficult to compare because of differences in host cell, time of radiolabeling of protein, time of viral harvest,

Table 4

Arenavirus Polypeptides Identified by PAGE of Purified Virus

Virus	Nucleoprotein Polypeptide	M.W.[a]	Glycoprotein Polypeptide	M.W.	Unknown minor[b] Polypeptide	M.W.	Reference
LCM	N	67	G1	55		91	Pedersen (1973b)
			G2	39		75	
						25	
						12	
LCM	NP	63	GP1	44	L	200	Martinez Peralta et al. (1981)
			GP2	35			
LCM	NP	63	GP1	54			Buchmeier et al. (1978)
			GP2	35			
Pichinde	VP1	72	VP2	72	VP4	12	Ramos et al. (1972)
			VP3	34			
Pichinde	N	66	G1	64	P	77	Vezza et al. (1977)
			G2	38		12	
Pichinde	N	68	G1	65		77	Gangemi et al. (1978)
			G2	38		15	
Pichinde	N	62	G1	54		200,95	Young et al. (1981)
			G2	34		72,55	
						47,36,22	Howard and Simpson (1981)
						16,15	
						13,12	

Table 4 (cont'd)

Junin	3	64	5	38	1 2 4 6	91(g) 71(g) 52(g) 25	Martinez-Segovia and de Mitri (1977)
Junin[d]	3	60	G G	39 34		44(g)	Grau et al. (1981)
Tacaribe	N	68	G	42	P	79	Gard et al. (1977)
Tacaribe	N	68	G	38		79,50 15	Gangemi et al. (1978)
Tamiami	N	66	G	44	P	77	Gard et al. (1977)
Machupo	N	68	G1 G2	50 41		84,74 15	Gangemi et al. (1978)
Lassa	N	72	G1 G2	52 39		115(g)	Kiley et al. (1981)
Mozambique	N	72	G1 G2	54 40		115(g) 84(g)	Kiley et al. (1981)

(a) molecular weight estimations ($\times 10^{-3}$)

(b) functions for minor polypeptides have not been determined

(c) analyzed by protein stain. P95, 55, 16, 13, 12 did not radiolabel. P22 was radiolabeled but did not stain

(d) G34 and G39 were sometimes seen as a broad G34-39 band

(g) carbohydrate associated with these minor polypeptides

and purification techniques employed. The viruses which have not yet been examined are Parana, Amapari and Latino.

The major polypeptide constituent of virions is the nucleoprotein which has been variously termed VP1, N, protein 3 or NP (Ramos et al., 1972; Pedersen, 1973b; Martinez-Segovia and de Mitri, 1977; Buchmeier et al., 1978). Molecular weight estimations range between 60,000 and 70,000 for the arenavirus nucleoproteins as a group, with estimates for Pichinde virus having the greatest variation for one virus type (62,000 to 72,000) (Table 4). Some of the variation arises from the limitations of molecular weight determinations in polyacrylamide gels (Weber and Osborne, 1969) but differences in virus stocks from different laboratories also occur (Chapter 4, Figure 22). Attempts to demonstrate association of carbohydrate, phosphate, or sulfate with the nucleoprotein of Pichinde virus have been unsuccessful (Vezza et al., 1977; Chapter 3, sections 2.6 and 2.8). The proportion of the total radiolabel in virus which was present in the nucleoprotein has been calculated as 66.5 to 71 percent (Vezza et al., 1977; Buchmeier et al., 1977). Based on these figures, Vezza et al. (1977) calculate that there are approximately 1530 molecules of nucleoprotein per virion.

The number of glycoproteins found in purified arenavirus preparations varies. A common finding is a glycosylated polypeptide with an estimated molecular weight of 34,000 to 44,000, however two major glycoproteins have been identified in all arenaviruses except Tacaribe and Tamiami (Table 4). It is not clear to which category Junin virus belongs since one group reports one major glycoprotein of

38,000 daltons (Martinez-Segovia and de Mitri, 1977), while another reports two glycoproteins of 34,000 and 39,000 daltons, which are sometimes seen as a broad 34,000 to 39,000 dalton region in PAGE (Grau et al., 1981). The smaller glycoprotein, GP2, which has also been termed VP3, G2, and protein 5 (Ramos et al., 1972; Pederson, 1973b; Martinez-Segovia and de Mitri, 1977), has been estimated to represent 11 to 13.8 percent of total radiolabeled protein in the virion of Pichinde virus or about 440 molecules per virion (Vezza et al., 1977; Buchmeier et al., 1977). GP1, which has also been designated VP2, and G1 (Ramos et al., 1972; Pedersen, 1973b) constituted 10.4 to 10.5 percent of virion protein which had been radiolabeled (Vezza et al., 1977; Buchmeier et al., 1977). The glycoproteins have mainly been identified by carbohydrate specific stains (Gangemi et al., 1978) and radiolabeling in the presence of ^{14}C or ^{3}H-glucosamine hydrochloride (Buchmeier et al., 1977; Gard et al., 1977; Martinez-Segovia and de Mitri, 1977; Vezza et al., 1977; Buchmeier and Oldstone, 1979; Cresta et al., 1980; Kiley et al., 1981; Grau et al., 1981) however one group reported that LCM virus GP-1 contained glucosamine, fucose, and galactose, while GP-2 contained glucosamine and fucose (Buchmeier and Oldstone, 1979). Purification of glycoprotein and quantitation of carbohydrate has not been undertaken.

A variable number of minor polypeptides have been observed in most arenavirus preparations. Differences in polyacrylamide gel electrophoresis systems and molecular weight standards make it particularly difficult to compare the polypeptides between viruses (see

Table 4). None of the minor components were consistently observed in the arenaviruses as a group, however many gel systems employed have been inadequate to resolve both large (greater than 150,000 daltons) and small polypeptides (smaller than 20,000 daltons) at the same time (Howard and Simpson, 1980; Martinez-Segovia and de Mitri, 1977; Gangemi et al., 1978; Grau et al., 1981). Three groups of investigators have analyzed more than one arenavirus at the same time. Although co-electrophoresis experiments were not performed, a minor glycosylated 115,000 dalton polypeptide was observed in both Lassa and Mozambique virus preparations (Kiley et al., 1981). Gard et al. (1977) compared virion polypeptides of Pichinde, Tacaribe, and Tamiami viruses. A 77,000 dalton polypeptide was common to Pichinde and Tamiami viruses, but it was unclear whether a 79,000 dalton polypeptide did or did not comigrate with the 77,000 dalton molecule in samples which were co-electrophoresed. A 12,000 to 15,000 dalton polypeptide has been a consistent finding in Pichinde virions (Ramos et al., 1972; Vezza et al., 1977; Gangemi et al., 1973; Young, P.R. et al., 1981; Howard and Simpson, 1980). None of the minor polypeptides in arenaviruses have been shown to be encoded by viral genetic information.

Polypeptides have been localized in virions after nonionic detergent solubilization followed by separation of the components in sucrose, cesium chloride or metrizamide gradients (Ramos et al., 1972; Buchmeier et al., 1977; Vezza et al., 1977; Gard et al., 1977; Martinez-Segovia and de Mitri, 1977; Buchmeier et al., 1978). Pichinde and Tacaribe virus preparations treated with 2 percent triton X-100, separated into a soluble phase and ribonucleoprotein structures on cesium chloride gradients. The ribonucleoproteins had densities of

1.31 and 1.33 gm per ml respectively, were sensitive to ribonuclease and were composed predominantly of the nucleoprotein, although in the case of Pichinde virus a 77,000 dalton polypeptide was also associated with the ribonucleoprotein structures (Vezza et al., 1977; Gard et al., 1977). In the case of Junin virus minor proteins 1, 2, 4 and 6 as well as the nucleoprotein were found in association after 2 percent triton X-100 treatment (Martinez-Segovia and de Mitri, 1977). Low concentrations of NP-40 (0.1 to 0.2 percent) released structures from Pichinde virus which were composed of nucleoprotein (VP1) and one glycoprotein (VP2) as well as RNA. The structures had a density of 1.32 to 1.34 gm per ml (Ramos et al., 1972). Increased concentrations of NP40 (1 percent) released Pichinde ribonucleoprotein containing only RNA, nucleoprotein (VP1) and a 12,000 dalton polypeptide (VP4) (Buchmeier et al., 1977). Slightly less dense (1.16 to 1.17 gm per cm^3) ribonucleoproteins containing RNA and NP only were isolated on metrizamide gradients after treatment of LCM virions with 2 percent NP40 (Buchmeier et al., 1978).

The same techniques have been employed to demonstrate that the virus glycoproteins are externally localized, however more definitive techniques have also been employed (Vezza et al., 1977; Gard et al., 1977; Buchmeier et al., 1978). Spikeless particles, as defined by electron microscopy, produced by treatment of Pichinde virus with pronase and bromelain (Vezza et al., 1977) or Tacaribe and Tamiami viruses with chymotrypsin (Gard et al., 1977), exhibited less than 30 percent intact glycoprotein by SDS-PAGE. Treatment of LCM virus with bromelain has led to a similar conclusion (Buchmeier et al., 1978). GP1 and GP2 of Pichinde virus has also been localized

to external regions of the virion by labeling with galactose oxidase-tritiated borohydride according to the technique of Sheffield and Daley (1976) (S. Sengupta and W.E. Rawls, unpublished observations).

Three enzymatic activities have been described in purified preparations of Pichinde virus, however these have not been assigned specifically to the polypeptides observed by SDS-PAGE (Carter et al., 1974; Leung et al., 1979). An RNA-dependent RNA polymerase which required both Mg^{2+} and Mn^{2+} for maximal activity, was detected in virus preparations disrupted with nonionic detergent (Carter et al., 1974). The products of the reaction sedimented as a broad 22 to 26S peak and a 4 to 6S peak. The larger products exhibited RNase resistance after hybridization with viral RNA. Subsequent analysis revealed that the transcriptase activity sedimented in sucrose gradients with ribonucleoprotein complexes (Leung et al., 1979). The latter study also revealed a Mg^{2+} dependent polyuridylic acid polymerase and a Mn^{2+} dependent polyadenylic acid polymerase associated with viral ribosomes. It is not known whether these are host cell or viral encoded enzyme activities.

1.5.2 Synthesis of viral polypeptides

Investigations on the synthesis of viral polypeptides in cell culture have been hampered by observations which suggest that arenaviruses do not interfere appreciably with host cell protein synthesis, however such studies have been undertaken with LCM virus and Tacaribe virus (Buchmeier et al., 1978 ; Saleh et al., 1979).

The nucleoprotein of LCM virus was the only viral protein which could be detected in lysates of infected BHK-21 cells by SDS-PAGE of total cell extracts (Buchmeier et al., 1978). The nucleoprotein was detected as early as 4 to 6 hrs after infection, but identification

of other viral proteins required immune precipitation with sera derived from infected animals. A 75,000 dalton glycoprotein was identified which was not evident in purified virus, and subsequent studies confirmed that this molecule, designated GPC, was a precursor to the two viral glycoproteins (GP1 and GP2) (Buchmeier and Oldstone, 1979). GPC was rich in glucosamine and mannose and labeled poorly with fucose and galactose. In contrast GP1 labeled with galactose, and both GP1 and GP2 contained glucosamine and fucose. Changes in carbohydrate were postulated to occur with proteolytic cleavage of the precursor.

Similar results were obtained during Tacaribe virus infection in BHK-21 cells (Saleh et al., 1979). The immune sera in this case was derived from rabbits immunized with intact virus. A mannose rich 70,000 dalton glycoprotein was identified and was postulated to be a precursor to the single virion glycoprotein which has been identified (see Table 4). It was not clear whether the structural glycoprotein identified by SDS-PAGE represented two approximately equal cleavage products of the 70,000 dalton molecule or whether portions of the polypeptide chain were lost. A minor nonglycosylated polypeptide (79,000 daltons) was also identified but its origin was not determined.

1.5.3 Viral antigens and serological relationships

Arenavirus proteins were initially examined with reference to their antigenicity. Early reports suggested that tissue derived from LCM virus infected guinea pigs contained a soluble complement-fixing (CF) antigen which was separable from infectious virus by ultracentrifugation (Smadel et al., 1939, 1940). The antigen was subsequently

characterized in more detail, (Bro-Jorgensen, 1971) as were CF antigens detected in BHK-21 cells infected with Pichinde virus (Buchmeier 1975; Buchmeier et al., 1977). In both cases, two antigens were identified by immunodiffusion; one was thermolabile, while the other was thermostabile and resistent to protease activity. Although CF antigen did not comigrate with viral polypeptides (Buchmeier et al., 1977), subsequent studies revealed that the antigenic determinant(s) was carried by the viral nucleoprotein (Buchmeier et al., 1977; Buchmeier and Oldstone, 1978). CF antigen appears to account, at least in part, for a course immunofluorescence observed in the cytoplasm of infected cells (Buchmeier et al., 1977).

It should be noted that although the CF antigen is related to the nucleoprotein, other antigens may exhibit CF antigenicity as well. This is supported by observations that LCM virus was susceptible to lysis in the presence of antibody and complement (Welsh et al., 1976).

Several groups have investigated serological relationships of arenaviruses by complement fixation and immunofluorescence (Rowe et al., 1970a; Webb et al., 1970; Casals et al., 1975; Wulff et al., 1978). The arenaviruses were divided into 'New World' (Tacaribe complex) and 'Old World' (LCM, Lassa, Mozambique) on the basis of these tests. Within the Tacaribe complex, Pichinde and Tamiami are more closely related to each other than they are to a cluster consisting of Tacaribe, Machupo, Amapari, Junin, Parana and Latino. LCM and consequently, Lassa and Mozambique viruses, were only distantly related to this group (Rowe et al., 1970a).

A more refined technique has recently been employed to examine arenavirus relationships. The method is dependent on monoclonal antibodies to viral antigens, which are available now for LCM virus and Pichinde virus (Buchmeier et al., 1980a; Kiley et al., 1981; Buchmeier and Oldstone, 1981). Monoclonal antibody to LCM virus antigenic sites precipitates [^{35}S]-methionine labeled nucleoprotein from both Lassa and Mozambique viruses. A second monoclonal antibody reacts with Mozambique virus glycoprotein (G2) but not with Lassa virus (Kiley et al., 1981). LCM virus monoclonal antibody directed against nucleoprotein also fails to recognize either Lassa or Mozambique (Buchmeier and Oldstone, 1981). Indirect immunofluorescence titres indicated that Mozambique and LCM virus were more closely related to each other than to Lassa virus (Buchmeier et al., 1980a).

Similar experiments performed with monoclonal antibody to Pichinde virus suggested that Pichinde virus was more closely related to Tamiami virus than to Junin, Parana, Amapari, Latino, Machupo, Tacaribe, and 'Old World' arenaviruses. A LCM virus nucleoprotein directed monoclone, which was highly cross reactive with 'Old World' arenaviruses did not recognize the nucleoprotein of the Tacaribe complex viruses which were examined (Buchmeier and Oldstone, 1981).

The power of the technique was illustrated by observations that monoclonal antibody directed against LCM virus distinguished between different strains of LCM virus, which were indistinguishable when conventional sera was employed (Buchmeier et al., 1980b). Rigorous determinations of serological interrelationships must await development of monoclonal antibodies reactive against a large variety

of antigenic determinants of a number of arenaviruses.

1.6 Replication in vitro

An understanding of the characteristic persistent infection of cells by arenaviruses necessarily requires a complete biochemical characterization of the arenavirus growth cyclus during acute infection. Most studies have been undertaken in BHK-21, Vero, and L cells but other cell types have been employed with some success. The characteristics of arenavirus replication in cell culture have been extensively reviewed (Hotchin, 1971; Lehmann-Grube, 1971; Pfau, 1974; Rawls and Leung, 1979; Pedersen, 1979; Buchmeier et al., 1980b).

Arenaviruses adsorb to cells in approximately 60 to 120 minutes (Pfau, 1974). The process is complete within 90 minutes for Pichinde virus and the efficiency of adsorption can be increased with DEAE-dextran (Carter, 1972). Release of progeny virus into the medium is detectable 7 to 8 hrs after infection (Carter, 1972; Lehmann-Grube et al., 1975; Buchmeier et al., 1978) but may be a few hours earlier (Dutko and Pfau, 1978) or later (Vezza et al., 1977). At multiplicities of infection of 1 PFU per cell, a rapid rise in virus production occurs to a maximum level at 24 to 36 hrs after infection. These general observations are all dependent upon temperature, multiplicity of infection and defective interfering virus concentration (Carter, 1972; Welsh and Pfau, 1972; Vezza et al., 1977). Although yields of infectious virus are similar, a decrease in temperature from 38°C to 35°C approximately doubles the time required to reach maximum virus titres (Vezza et al., 1977) and higher yields are generally reached with higher input multiplicities (Carter, 1972; Vezza et al., 1977).

Viral antigen has been measured by immunofluorescence and the time of appearance of cytoplasmic and surface antigen correlates with infectious virus production. CF antigen was first seen at 8 hrs after infection in LCM virus infected cells (Lehmann-Grube et al., 1975). The nucleoprotein of LCM virus was apparent in autoradiograms of SDS-PAGE immediately prior to early log phase of viral replication (6 hrs) and surface antigen measured by immunofluorescence was detected at 7.5 hrs (Buchmeier et al., 1977).

Biochemical events occurring during viral replication are not well studied as yet. Viral RNA was of negative polarity (Section 1.4) and an RNA-dependent RNA polymerase was associated with the virion (Section 1.5.1). Messenger RNA transcribed by this enzyme has been found associated with polysomes and appears to be polyadenylated (Leung et al., 1977; Leung, 1978a). The nucleoprotein has been translated from polysomal RNA in in vitro protein synthesizing systems (Rawls, 1977), but other viral proteins have not been identified in this way as yet. The extremely low levels of viral RNAs and protein in infected cells have hampered progress in this area. Leung and co-workers (1977) estimated that less than 1 percent of polyadenylated RNA in infected cells hybridized with ^{32}P-labeled Pichinde virus RNA.

More recently Dimock and co-workers (1982) have examined the replication of Pichinde virus in BHK-21 cells over the course of a 10 day period. Co-ordinate synthesis of the known viral polypeptides preceded a peak in release of infectious virus by less than 24 hrs. The peak in virus production occurred 48 to 72 hrs after infection and was followed by a 90 percent reduction in infectious virus within 8 to

9 days. Protein synthesis, infectious centers and intracellular viral RNA synthesis decreased in a co-ordinate manner although viral antigen detected by immunofluorescence remained extremely high (ninety percent of cells were immunofluorescent). The authors suggested that viral replication was a regulated phenomenon which was independent of the host cell. This will be discussed in more detail in chapter 5.

Several studies, however, have suggested that the host cell play more than a passive role in arenavirus replication. The simplest evidence for this came from studies with Pichinde virus which suggested that the yield of infectious virus per cell in the stationary phase of growth, was only 1 percent of yields obtained with cells growing exponentially (Rawls et al., 1976). Actinomycin D, at concentrations which inhibit host cell RNA synthesis, can cause a reduction of 90 percent in the 24 hr yield of infectious viral progeny for LCM virus (Buck and Pfau, 1969; Stanwick and Kirk, 1971) and Pichinde virus (Rawls et al., 1976) but not for Junin virus (Coto and Vombergar; cited in Rawls et al., 1976). Nearly normal levels of Pichinde virus antigen were synthesized, but an increase in virus surface antigen suggested that inhibition may be mediated at the maturation phase (Rawls et al., 1976).

α-amanitin, which interferes with the function of RNA polymerase II, has also been reported to interfere with Pichinde virus replication (Leung, 1978b) and Junin virus replication (Mersich et al., 1979). Replication was not inhibited in α-amanitin resistant cells but was in α-amanitin sensitive cells of Chinese hamster ovary origin. The results indicated that a host cell mRNA function was required (Leung, 1978b).

Although host cell transcription appeared necessary for arenavirus replication, DNA replication was not since arabinosyl cytosine, 5-bromodeoxyuridine and 5-iododeoxyuridine have no effect (reviewed by Pfau, 1974). Enucleation of cells with cytochalasin B prevents antigen synthesis in Pichinde virus infected cells and the effect seems to be mediated during the latent phase (Banerjee et al., 1976). This contrasts with the somewhat later effect exerted by actinomycin D (Rawls et al., 1976) and may be suggestive of more than one temporally separated host cell function.

1.7 Defective interfering arenaviruses

Much of the work on arenavirus interfering phenomena has been undertaken with LCM virus and Pichinde virus, although Parana and Junin virus have also been employed. Several comprehensive reviews have recently been published on the subject (Rawls and Leung, 1979; Pedersen, 1979; Buchmeier et al., 1980b; Lehmann-Grube et al., 1981).

Early investigations demonstrated that L-929 cells persistently infected with the Armstrong strain of LCM virus were resist nt to superinfection with either Armstrong or WE strains of LCMV (Lehmann-Grube et al., 1969). Staneck and Pfau (1974) later demonstrated homotypic and heterotypic interference in the absence of heterologous interference.

Arenavirus defective interfering particles (DI) are not well defined at the present time mainly due to difficulties which have been encountered in designing sensitive assays for DI particles, and in separating DI from standard virus by physical means. Popescu et al. (1976) developed a sensitive assay which relied on the ability

of DI particles to protect L cells against cytolysis by standard LCM virus. This was reportedly more sensitive than early assays based on interference with infectious center formation (Welsh and Pfau, 1972). A sensitive assay was mandatory since DI particles have never been obtained free of standard virus and viral stocks can now be characterized in terms of a DI to standard virus ratio (Martinez-Peralta et al., 1981).

Some separation of DI virus has now been obtained by physical means, at least for LCM virus, however the almost universally employed technique was to obtain DI virus stocks from persistently infected cell lines. LCM virus DI was obtained from persistently infected L cells (Dutko and Pfau, 1978; Welsh and Buchmeier, 1979; Jacobson et al., 1979) and BHK-21/13S cells (Staneck et al., 1972); Pichinde virus DI from persistently infected BHK-21 cells (Dutko et al., 1976); Parana virus DI from persistently infected BHK-21 cells (Staneck et al., 1972); and Tacaribe virus DI from BHK-21 and Vero cells which were persistently infected (Gimenez and Compans, 1980). Martinez-Peralta and co-workers (1981), on the other hand, obtained LCM virus DI particles after one multiplication cycle from cultures acutely infected with 10 PFU per cell and have managed to physically separate these particles from infectious standard virus.

Where assessed, the density of DI particles was slightly less than standard virus. The density of LCM virus DI was 1.15 to 1.18 gm per cm^3 in sucrose, 1.1385 gm per cm^3 in urografin and 1.13 gm per cm^3 in renografin, while standard virus had densities of 1.18, 1.1395, and 1.14 gm per cm^3 in the three media respectively (Oldstone

et al., 1977; Martinez-Peralta et al., 1981; Welsh and Buchmeier, 1979). DI virus also differs in ultraviolet radiation sensitivity (low compared to standard virus (Welsh et al., 1972; Popescu et al., 1976) and temperature sensitivity (Lehmann-Grube et al., 1981).

Some controversy exists over the biochemical properties of DI particles. Initial reports suggested that LCM DI particles contained normal L and S viral RNA as well as rRNA (Welsh et al., 1975), however Pedersen (1979) did not find S or rRNA in DI particles, and L vRNA was smaller than in standard virus. Martinez-Peralta et al. (1981) also reported the absence of S RNA in LCM virus DI particles, however the mobility of L RNA was not changed and rRNA quantities appeared normal. S RNA was also absent in Parana virus DI particles but L RNA was not altered (Dutko et al., 1978). Differences in Pichinde virus DI particle RNAs were noticed in harvests obtained at different passage times (Dutko et al., 1976). S RNA was absent, however a 15S RNA species was apparent. DI particles harvested at later passages had lost L RNA and eventually gained a new 20S RNA of unknown origin. Parana virus DI particles obtained from persistently infected cells contained neither L nor S viral RNAs, however three new smaller RNA species were observed in addition to rRNA (Gimenez and Compans, 1980). It was not known in any of these investigations whether the new RNA species were in fact viral in origin, and if so, the nature of the deletions.

As might be expected, the polypeptide composition of arenavirus DI particles has not been extensively studied to date. LCM virus DI particles examined by Welsh and Buchmeier (1979) did not differ from standard virus in polypeptide composition. Both glycoproteins

were present and the nucleoprotein was the major viral polypeptide synthesized in persistently infected cells. DI particles isolated during acute infection with LCM virus apparently did differ in polypeptide composition from standard virus (Martinez-Peralta et al., 1981). Fractions of urografin gradients enriched to 99 percent interfering particle contained the nucleoprotein and 200,000 dalton polypeptide as did standard virus, but GP-1 was absent and GP-2 quantities were substantially reduced. Electron microscopic analysis of Tacaribe virus DI particles revealed that the morphology was similar to standard virus (Gimenez and Compans, 1980). The polypeptide composition of DI and standard virus was similar in that all of the previously documented viral polypeptides were apparent (Section 1.5.1), but the glycoprotein was reduced in quantity and the nucleoprotein had a slightly different mobility in DI particles obtained from persistently infected BHK cells. The mobility of the nucleoprotein was not changed in DI particles isolated from persistently infected Vero cells.

1.8 Persistent infection by arenaviruses

The mechanisms responsible for the establishment and maintenance of persistent infections with arenaviruses are not known at the present time. Persistence has been demonstrated in the animal host of known arenaviruses and in cell lines grown in culture. The former phenomenon has been reviewed comprehensively by several authors and will not be discussed here (Rawls et al., 1981; Lehmann-Grube et al., 1981; Buchmeier et al., 1980b).

Persistently infected cell lines have been readily established with LCM virus, Pichinde virus, Tacaribe virus, Parana virus and Junin

virus (Lehmann-Grube, 1971; Boxaca, cited in Damonte and Coto, 1979; Staneck et al., 1972; Staneck and Pfau, 1974; Hotchin et al., 1975; Welsh et al., 1975; Dutko et al., 1976; Gimenez and Compans, 1980). In some cases these were derived from surviving cells after an acute infection (Coto et al., 1981), but arenaviruses were not cytolytic in many cell lines (reviewed by Rawls and Leung, 1979; Pedersen, 1979). Acute infections resulted in the generation of DI particles which may play a role during establishment and maintenance of persistence (Lehmann-Grube et al., 1981) however, in the case of Pichinde virus, an increase in DI particles was not apparent during acute infection (Dimock et al., 1982)

Cell lines persistently infected with arenaviruses generally displayed a cyclic variation in both infectious virus and DI virus activity which lasted from 20 to 78 or more cell pasages (reviewed by Pedersen, 1979; Gimenez and Compans, 1980). The ratio of DI particles to infectious virus appeared to increase dramatically after this however, in the case of Pichinde virus, cultures followed up to 150 generations were still producing infectious virus (Dutko et al., 1976). A cyclic variation was also apparent in viral antigen as detected by immunofluorescence, but the quantities of antigen in the cytoplasm and at the cell surface were considerably lower (Welsh and Oldstone, 1977; Welsh and Buchmeier, 1979).

Persistently infected culture cells had a similar cloning efficiency as uninfected cells (Popescu et al., 1976; Stanwick and Kirk, 1976) which has been taken as evidence of similar growth properties. In the case of LCM virus only minor differences in the synthesis and degradation of acetycholine have been documented in

persistently infected cells (Oldstone et al., 1977). Persistently infected cells were generally resistant to challenge with the same arenavirus or another arenavirus, but not to challenge with a heterologous virus such as vesicular stomatitis virus (Welsh and Pfau, 1972; Staneck et al., 1972; Staneck and Pfau, 1974; Gimenez and Compans, 1980). The proportion of cells expressing cytoplasmic antigen varied but was as high as 100 percent (Gimenez and Compans, 1980). Antigen negative cells have been cloned from persistently infected cultures and under these conditions infection with an arenavirus was demonstrated (Hotchin et al., 1975; Stanwick and Kirk, 1976). Finally, antibody was not required for maintenance of the carrier state.

Rawls et al., (1981) have consolidated several theories proposed to explain viral persistence at the cellular level into two basic concepts. The first concept was that arenaviruses were capable of autoregulation such that replication could occur without destruction of the host cell. The second concept stated that defective interfering particles were produced and were capable of regulating infection by interfering with the replication of standard virus. Neither theory could be favored on the basis of the available information, however, the two concepts should not be viewed as mutually exclusive.

1.9 Purpose of the study

Arenaviruses characteristically produce persistent infections in their animal hosts and in cells grown in vitro. Two concepts have been proposed to explain the properties of the carrier state (Section 1.8). The first is dependent on autoregulation of arenavirus replication, and the second attributes the establishment and maintenance of

the carrier state to the genesis of DI particles. Insufficient information is available to define the carrier state in terms of either concept.

The first concept depends on an intricate assessment of the molecular events surrounding the primary or acute infection which precedes persistence. The present investigations were undertaken in order to obtain basic information concerning the replication of Pichinde virus in tissue culture cells. I embarked on an effort to define and characterize viral polypeptides synthesized in cells grown in vitro. I also attempted to establish the universality of the properties of Pichinde virus replication during acute infection by comparing replication in a number of cell lines. This information contributes to a basic understanding of the replicative process, an understanding which is necessary to interpret broader biological experiments in the in vivo situation. These types of studies can potentially yield a general model of arenavirus persistence and contribute to knowledge of human disease with a putative viral etiology.

2 Materials and Methods

2.1 Tissue culture

Baby hamster kidney cells (BHK-21), clone 13, were obtained from Dr. C.R. Howard (London School of Hygiene and Tropical Medicine, London, England), and were grown as monolayers at 37°C in Dulbecco-modified Eagle medium supplemented with 6 percent calf serum (Grand Island Biological Compnay, Grand Island, New York), 100 U per ml penicillin, 100 U per ml streptomycin sulfate, 60 µg per ml Tylocine (all from Gibco, Grand Island, New York) and 0.075 percent (w/v) $NaHCO_3$. Cultures from which virus was harvested were grown in the same medium supplemented with 6 percent foetal bovine serum instead of calf serum. No differences in cell growth or viral polypeptides were noticed with either serum supplement.

Canine kidney cells (MDCK, NBL-2) and African Green Monkey Kidney Cells (Vero) were obtained from Flow Laboratories (Mississauga, Ontario). α-amanitin sensitive and resistent lines of Chinese Hamster ovary cells (CHO ama^s, CHO ama^r) were obtained from Dr. W.C. Leung (McMaster University, Hamilton, Ontario). All of these cell lines were propagated under the same conditions as BHK-21 cells.

2.2 Viruses

Pichinde virus, strain AN3739 was obtained from Dr. Carlos Sanmartin after passage twelve in baby hamster brain (Trapido and Sanmartin, 1971). The strain was originally isolated from the cricitine rodent Oryzomys albigularis, captured in the Pichinde valley of Colombia, South America. Virus stocks (B8C, 1.6×10^8 PFU per ml) were

obtained after eight passages of virus in BHK-21 cells in this laboratory. Studies in Chapter 5 were conducted with doubly plaque purified stock (2.8×10^8 PFU per ml) prepared in the following manner by Dr. Ken Dimock. A virus plaque in Vero cells was isolated, along with infected cells, by aspiration with a Pasteur pipette. A BHK-21 cell monolayer was infected with plaque material and virus was harvested between 48 and 72 hrs after infection. Plaque purification was repeated and a virus stock was obtained between 30 and 70 hrs after infection of a BHK-21 cell monolayer at a MOI of 0.1 PFU per cell.

Gene mapping studies (Chapter 4) were performed with virus strains obtained from Dr. D.H.L. Bishop (University of Alabama, Birmingham, Alabama). The original source of prototype Pichinde virus (wild type, strain AN3739) has been described (Trapido and Sanmartin, 1971). Munchique virus (wild type) is another Pichinde isolate (strain 4763) from the same rodent species as strain AN3739, but trapped in the Munchique mountains of Columbia, South America, by Dr. Carolos Sanmartin. Munchique virus has undergone two passages in suckling hamster brains, one passage in diploid rhesus fetal lung cells, and has been plaque cloned in Vero cells by Vezza et al. (1980). RE-2 is an arenavirus reassortant between wild type Pichinde virus (strain AN3739) and wild type Munchique virus (strain 4763) isolated by Vezza et al. (1980).

2.3 Virus assay

Infectious virus titres were assayed by plaque formation on monolayers of Vero cells grown in 60mm Corning Petri dishes (Corning, Corning, New York). Virus was serially diluted in culture medium and duplicate 0.3 ml aliquots were pipetted onto subconfluent Vero cell

monolayers. Following a 1.5 h incubation at 37°C, cultures were overlaid with Eagle medium (GIBCO) containing 0.9 percent Bacto-agar (Difco), 10 percent (v/v) feotal bovine serum, 100 U per ml Penicillin, 100 U per ml streptomycin sulfate, 60 µg per ml Tylocine, and 0.15 percent (w/v) $NaHCO_3$. After three days at 37°C, a second agar overlay which contained 0.1 mg per ml neutral red (GIBCO) was added. Unstained areas (plaques) were counted 24 hrs later. All cultures were maintained in a humidified atmosphere containing 5 percent CO_2.

2.4 Virus purification

All procedures were carried out at 4°C. Infected cell culture fluid was clarified of cellular debris by centrifugation at 7000x*g* for 20 min. Polyethylene glycol (average molecular weight 6,000 to 7,500) and NaCl were added to 7 percent and 0.5 *M*, respectively. Mixtures were stirred for 3 hrs, the suspension was centrifuged (7,000x*g*, 20 min) and pellets were resuspended in 4 ml of TEN (10 m*M* Tris-HCl, pH 7.5 containing 100 m*M* NaCl, 1 m*M* EDTA). Suspensions were usually stored at -70°C before layering on discontinuous Renografin (Squibb, Montreal, Quebec) gradients consisting of 5 ml of 10 percent (w/v) Renografin and 2 ml 40 percent (w/v) Renografin in TEN. Following centrifugation at 160,000x*g* (1 hr) in a Beckman SW 40 Ti rotor, the virus band on the 40 percent Renografin cushion was diluted with an equal volume of TEN and layered on a continuous (10 ml) 10 to 40 percent Renografin gradient. Centrifugation of continuous gradients at 160,000x*g* was performed for a minimum of 3 hrs. The virus band was collected and dissociated by heating (100°C, 5 min) in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis sample

buffer (0.37 M Tris-HCl, pH 6.8, containing 10 percent (v/v) glycerol, 10 percent (w/v) SDS and 5 percent (v/v) 2-mercaptoethanol).

2.5 Immune serum

Immune serum to Pichinde virus proteins was raised in inbred MHA and LSH strains of Syrian hamsters (Charles River Breeding Laboratories, Newfield, New Jersey). Animals were initially injected with 2,000 PFU of virus in each hind footpad. Second injections (2,000 PFU) were administered intraperitoneally 4 to 6 weeks later. In some cases animals received a further 2,000 PFU boost two weeks later. Seven days following the final inoculation hamsters were bled by cardiac puncture. Blood was permitted to clot for 30 min at room temperature and overnight at $4^{o}C$. Sera was removed, pooled from ten different animals and stored at $-20^{o}C$ in 1 ml aliquots. Longer immunization schedules increased reactivity to viral glycoproteins but did not alter the number of immunoprecipitable polypeptides.

2.6 Hybridoma antibodies

Hybridoma antibodies were generously provided by Dr. M.J. Buchmeier (Scripps Clinic and Research Foundation, La Jolla, California). Antibodies were supplied as lyophilized ascitic fluid obtained from adult Balb/c mice, primed with 2,6,10,14-tetramethyl pentadecane and injected with $5x10^{6}$ viable hybridoma cells. Lyophilates were redissolved in distilled H_2O and in some cases antibody concentrations were estimated by endpoint titration in indirect immunofluorescence (Section 2.7). PV1-1-3, PV2-14-19 and PV2-14-21 had titres of 1/51,200, 1/1,280 and 1/640, respectively. PV designates that the antibody reacts with protein components of Pichinde virus; the first number is the fusion number, the second number is the clone number,

and the last number represents the subclone. Monoclones PV3A-23 and PV3B-3 were not titred.

2.7 Immunofluorescence

Glass coverslips were seeded with 2 to $5x10^5$ Pichinde virus infected or uninfected BHK-21 cells. Cells were permitted to settle and spread (2 hrs) at 37°C, and were then rinsed 3 times with phosphage buffered saline (PBS), air dried, fixed in acetone (10 min at -20°C) and allowed to dry again. At this stage cells were stored at -20°C. Before use coverslips were rinsed 3 times with PBS. Cells were then incubated for 30 min at 37°C in 0.1 ml of the appropriate dilution of monoclonal antibody. Following this cells were rinsed 9 times with PBS and incubated for 30 min at 37°C in 0.1 ml of a 1:60 dilution of fluorescein isothiocyanate conjugated goat anti-mouse immunoglobulin (Cappel Laboratories, Downington, Pennsylvania). Cells were rinsed 9 times with PBS and were mounted in Tris-buffered glycerol (pH 8) for microscopic examination using a Wild/Leitz ultraviolet microscope.

2.8 Infection of cells

In order to examine viral polypeptides in infected cells, subconfluent monolayers of BHK-21, Vero or MDCK cells, grown in 75 cm^2 tissue culture flasks (Corning, Corning, New York), were infected with various strains of Pichinde virus. Multiplicities of infection were determined from cell counts of duplicate cultures. Cells were dispersed in 20 mls of maintenance medium after treatment with trypsin-EDTA (GIBCO). An aliquot of each culture was counted with a hemocytometer. MOIs are expressed in plaque forming units per cell. Viral infectious titres were determined by plaque forma-

tion on Vero cells (Section 2.3).

2.9 Radiolabeling of virus and viral intracellular protein

In order to label viral proteins, cell monolayers grown in 75 cm^2 tissue culture flasks, were washed three times with Hanks' balanced salt solution and 2 ml of methionine free medium (Hanks' balanced salt solution containing amino acids minus methionine, 6 percent dialyzed calf or foetal bovine serum, 10 mM glutamine, 2 percent $NaHCO_3$, 100 U of penicillin per ml, and 100 µg of streptomycin sulfate per ml) containing 50 µCi of L-[^{35}S]methionine per ml (900 to 1200 Ci per mmol; New England Nuclear Corporation, Dorval Quebec) was added for 1 hr. Protein was also radiolabeled with a mixture of ^{3}H-amino acids (L-[2,3-^{3}H]alanine, 43 Ci per mmol; L-[5-^{3}H]arginine, 20 Ci per mmol; L-[2,3-^{3}H]aspartic acid, 15 Ci per mmol; L-[G-^{3}H]glutamic acid, 27 Ci per mmol; [2-^{3}H]glycine, 23 Ci per mmol; L-[2,5-^{3}H]histidine, 42 Ci per mmol; L-[4,5-^{3}H]isoleucine, 40 Ci per mmol; L-[4,5-^{3}H]leucine, 46 Ci per mmol; L-[4,5-^{3}H]lysine, 40 Ci per mmol; L-[4-^{3}H]phenylalanine, 29 Ci per mmol; L-[3,4(n)-^{3}H]proline, 65 Ci per mmol; L-[3-^{3}H]serine, 11 Ci per mmol; L-[G-^{3}H]threonine, 0.3 Ci per mmol; L-[3,5-^{3}H]tyrosine, 52 Ci per mmol; L-[3,4(n)-^{3}H]valine, 15.8 Ci per mmol; Amersham Corporation) in amino acid free medium. Glycoproteins were labelled for 1 hr with D-[6-^{3}H] glucosamine hydrochloride (100 µCi per ml; 38 Ci per mmol; Amersham Corporation) after a 3 hr preincubation of monolayers in glucose free medium. Labeled proteins were chased, for various time intervals, in the presence of at least a 100 fold excess of unlabeled methionine, amino acids, or glucose, respectively. Radiolabeled virus was purif-

fied from extracellular fluid (section 2.4) after a 12 hr chase of radiolabel in Dulbecco-modified Eagle medium (25 ml containing 6 percent foetal bovine serum)

2.10 Treatment of cells with tunicamycin

Tunicamycin (Lot 361-26E-250-A) was a gift from Dr. R.L. Hamill (Lilly Research Laboratories, Indianapolis, Indiana) and was stored ($4^{o}C$) at a concentration of 0.2 mg per ml in 0.1 N NaOH. Cell monolayers were preincubated in medium containing tunicamycin (5 to 10 µg per ml) for 3 hrs prior to labeling with L-[^{35}S]methionine (50 µCi per ml) for 1 hr in methionine free medium containing tunicamycin. An equal volume of 0.1 N NaOH was added to mock-infected cell monolayers before labeling.

2.11 Treatment of cells with amino acid analogues and metabolic inhibitors

BHK-21 cells grown in 75 cm^2 Corning tissue culture flasks were infected with Pichinde virus at a MOI of 1 PFU per cell. Twenty-four hours after infection, monolayers were preincubated for 0.5 hr in 5 ml of methionine free medium containing 0.1 mM TLCK (N-α-p-tosyl-L-lysine chloromethylketone HCl) 0.1 mM TPCK (L-1-tosylamide-2-phenyl-ethyl chloromethyl ketone) 3 mM canavanine or 3 mM acetidine 2-carboxylic acid (all from Sigma Chemical Co., St. Louis, Missouri). Two ml of fresh medium containing the appropriate inhibitor and 50 µCi per ml L-[^{35}S]methionine was then added for 1 hr. Radiolabel was chased by incubation of cultures in Dulbecco-modified Eagle medium (200 µM methionine) for 6 hrs. Cells were harvested and polypeptides were immunoprecipitated as described (Section 2.12).

To assess the effects of α amanitin, amphotercerin B and actinomycin D (actinomycin C_1) (Sigma Chemical Company) on viral polypeptides, cells were infected at an MOI of 1 PFU per cell and 10 ml of Dulbecco-modified Eagle medium containing either α amanitin (10 µg per ml) and amphotercerin B (10 µg per ml) or actinomycin D (1 µg per ml) was immediately added. Twenty-four hours after infection cultures were incubated for 1 hr in 2 ml of methionine free medium containg 50 µCi per ml L-[^{35}S]methionine and the appropriate inhibitor. At this stage cells were either harvested and polypeptides immunoprecipitated, or radiolabel was chased for 6 hrs before harvest. Cultures containing actinomycin D were maintained in the dark.

2.12 Immunoprecipitation

After the appropriate radiolabeling period (Section 2.9) cells were harvested in PBS by scraping with a rubber policeman. The cell suspension was pelleted (1000 x g, 10 min) and washed three times with 50 volumes of PBS. Pellets (approximately 10^7 cells) were lysed in 3 ml of RIPA buffer (50 mM Tris-hydrochloride, pH 7.2, containing 0.15 M NaCl, 0.1 percent (w/v) sodium dodecyl sulfate, 1 percent (v/v) Triton X-100, 1 percent (w/v) sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine hydrochloride), and cellular debris and nuclei were removed by centrifugation (10,000 x g, 20 min). To each 1 ml aliquot of the supernatent, 5 µl of hamster anti-Pichinde virus serum or 5 µl of normal hamster serum was added. One hundred microliters of a 1:2 (v/v) dilution of protein A-Sepharose CL-4B (Pharmacia Fine Chemicals Incorporated, Montreal, Quebec) was added at the same time. Sepharose beads had been preswollen and washed 3 time with RIPA buffer. After mixing for 2 hrs at 4°C on a rotary

mixer, beads were pelleted (10,000 x g, 1 min) and washed 3 times with RIPA buffer (1 ml per wash). The supernatent was removed in each case with the aid of a fine tip pasteur pipette. Antigen-antibody complexes were eluted by suspending and heating (100 µl, 100°C, 5 min) the beads in SDS-polyacrylamide gel sample buffer (0.37 M Tris-hydrochloride, pH 6.8, containing 10 percent (v/v) glycerol, 10 percent (w/v) SDS, and 5 percent (v/v) 2-mercaptoethanol).

2.13 SDS-polyacrylamide gel electrophoresis

Immunoprecipitable polypeptides were resolved by electrophoretic separation in denaturing polyacrylamide gels prepared according to the method of Laemmli (1970). Gradient SDS-polyacrylamide gels (7.5 to 15 percent polyacrylamide) were 30 cm long, 15 cm wide and 1.5 mm thick. Samples (up to 100 µl) were electrophoresed at a constant voltage of 120 V for 30 hrs in a Tris-glycine buffer (50 mM Tris, 0.38 M glycine, pH 8.6 and 0.1 percent (w/v) SDS). Protein was stained in 0.1 percent (w/v) Coomassie brilliant blue R-250 in methanol:acetic acid:water (1:5:5) for 6 hrs. Gels were destained in 7 percent (v/v) acetic acid and radiolabel was visualized using a film detection method (Bonner and Laskey, 1974). Films (Kodak type BB1, XRP or XRP1) were processed in a Kodak X-omat^{R} automatic processor.

2.14 Quantitation of radiolabel

SDS-slab gels were dried and exposed to X-ray film as described (Section 2.13). Bands were localized, exised with a razor blade, and digested with 400 µl of 30 percent H_2O_2 (8 hrs, 60°C). Five ml of aqueous scintillation counting fluid (ACS, New England

Nuclear, Dorval, Quebec) was added and samples were counted in open tritium and ^{14}C channels of a Beckman liquid scintillation counter.

2.15 Molecular weight determinations

Molecular weights of polypeptides were determined according to the method of Weber and Osborn (1969). Electrophoretic mobilities were compared with known molecular weight standards which included, thyroglobulin (330,000 daltons), phosphorylase b (94,000 daltons), bovine serum albumin (67,000 daltons), catalase (60,000 daltons), ovalbumin (43,000 daltons), lactate dehydrogenase (36,000 daltons), carbonic anhydrase (30,000 daltons), soybean trypsin inhibitor (20,100 daltons), l-lactalbumin (14,400 daltons) (all from Pharmacia, Montreal, Quebec), ferritin (220,000 daltons), myosin (212,000 daltons), and β-galactosidase (130,000 daltons) (all from Biorad Laboratories, Richmond, California). Measurements are averages of at least four determinations and were made from the furthest point of migration of polypeptides in gels.

2.16 Two-dimensional peptide mapping

Tryptic digestion of L-[^{35}S]methionine (200 μCi per ml) labeled polypeptides immunoprecipitated from BHK-21 cells infected with Pichinde virus was performed essentially by the method of Morrison and Lodish (1975). Polypeptides were resolved on 7.5 to 15 percent gradient SDS-polyacrylamide gels and were localized by autoradiography of fixed dried gels (Section 2.13). Radiolabeled bands were exised and swollen in 8 ml of 0.02 M ammonium bicarbonate (pH 8.0) containing 50 μg of bovine trypsin (Sigma Chemical Company, St. Louis, Missouri) which had been treated with diphenyl carbamyl chloride. The mixture was incubated at 37°C for 12 hrs with gentle

agitation. Fresh ammonium bicarbonate (5 mls) and trypsin (50 µg) were added, and incubation was continued for a further 12 hrs. The two incubation solutions were pooled, fresh trypsin (50 µg) was added, and the digestion was continued for 12 hrs. Digests were concentrated by lyophilization (3 times) and stored in 10 to 20 µl of glass distilled water. L-[^{35}S]methionine labeled tryptic peptides were separated by electrophoresis at pH 3.5 in pyridine:acetic acid: water (1:10:100) and by chromatography in n-butanol:pyridine:acetic acid:water (5:4:1:5) on thin layer sheets of silica gel (20 by 20 cm, 0.2 mm thick; polygram Sil N-HR; Brinkmann Instruments Incorporated, Westbury, New York) as described by Dobos and Rowe (1977). Peptides were visualized by fluorography either with a second chromatography step (20 percent (w/v) PPO in acetone) or with En^{3}Hance (New England Nuclear, Dorval, Quebec). Thin layer sheets were exposed to Kodak X-ray film (Kodak RP-1 or XRP-1) for various times up to 3 months.

3 Identification and Characterization of Pichinde Virus Cell-Associated Polypeptides

3.1 Introduction

Pichinde virus contains five distinct size classes of RNA. Three of these are of host cell origin (28S, 18S, 4 to 6S) and the remaining two (L and S) encode the viral genetic information (Carter et al., 1973a; Leung et al., 1977; Vezza et al., 1977). The genetic information contained in the two RNA species is unique (Vezza et al., 1977; Leung et al., 1981) and can potentially code for 280,000 and 130,000 daltons of protein for L and S, respectively.

Pichinde virion structural proteins have been described by several investigators (Ramos et al., 1972; Buchmeier et al., 1977; Vezza et al., 1977). The predominant polypeptide is a nonglycosylated nucleoprotein of 66,000 to 72,000 daltons. Two glycopeptides, GP1 (64,000 to 72,000 daltons) and GP2 (34,000 to 38,000 daltons), are located on the surface of the virion. A cell associated complement fixing antigen appears to be a degradation product of viral structural proteins. A 77,000 dalton polypeptide which is a minor virion component is of undetermined origin at the moment. These polypeptides account for approximately 152,000 daltons of protein (NP, GP1, GP2) or 37 percent of the estimated coding capacity of the viral genome.

I initially undertook to characterize viral specific polypeptides during acute infection of BHK-21 cells. To do so, hamster anti-Pichinde virus antibody and immunoprecipitation were employed. An

understanding of the viral replicative cycle cannot be obtained until all viral gene products are identified and assigned functions.

3.2 Results

3.2.1 Time course of radiolabel incorporation

In order to assess the number and kinetics of synthesis of viral polypeptides in Pichinde virus infected BHK-21 cells it was necessary to assess the kinetics of incorporation of radiolabel into protein. Subconfluent cell monolayers were incubated in methionine-free medium in the presence of 50 µCi per ml of L-[^{35}S]methionine. Acid precipitable intracellular radioactivity was measured at times up to 2.5 hrs. Incorporation was linear for 1 hr (Fig. 1). Subsequent labeling periods were restricted to 1 hr or less.

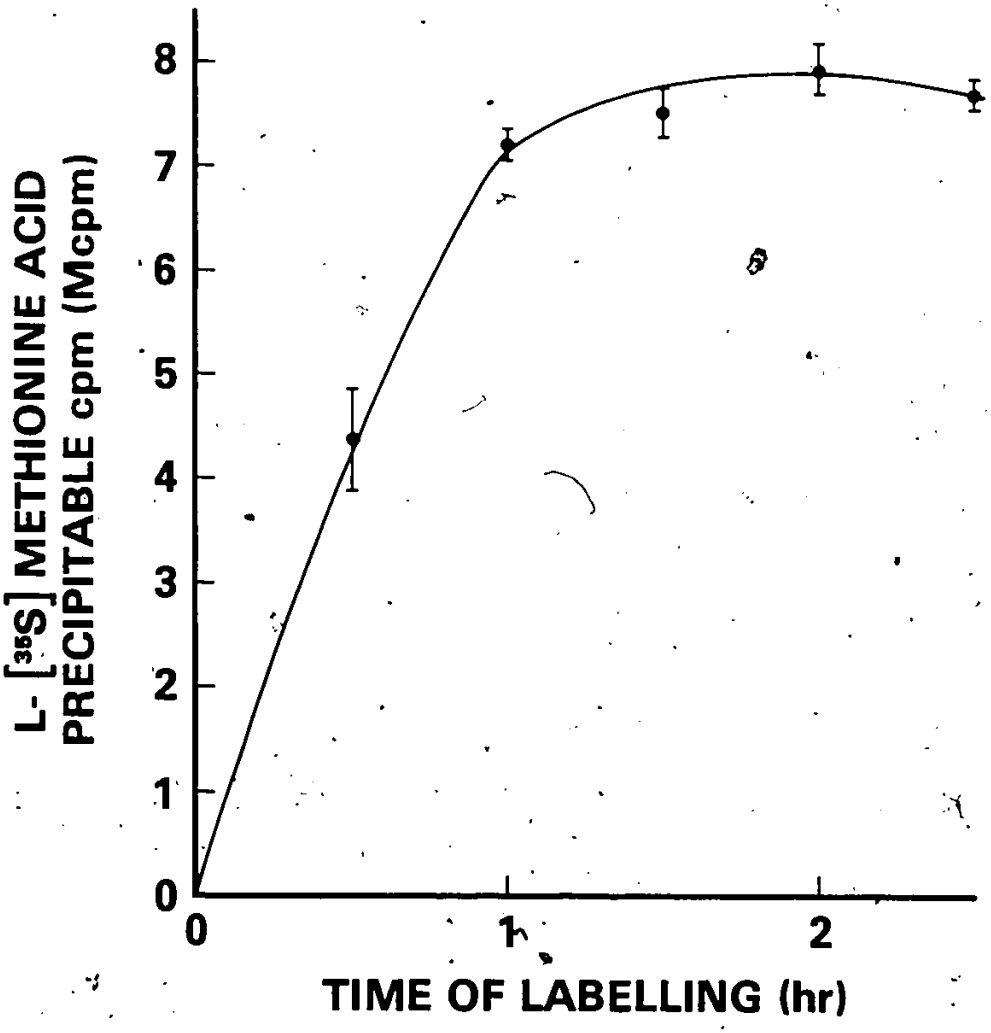
3.2.2 Conditions for immune specificity

In order to identify hamster anti-Pichinde virus sera specific polypeptides in infected BHK-21 cells, three protocols for cell lysis and immunoprecipitation were examined. Ideally, a suitable technique should result in immunoprecipitation and/or entrapment of very small quantities of radiolabeled polypeptides which are common to infected and uninfected cell lysates. Reduced background levels increase the probability of detection of immune sera specific polypeptides. Pichinde virus encoded or induced polypeptides should be evident in infected cell lysates treated with immune sera and not in infected cell lysates treated with normal sera, or in mock-infected cell lysates treated with either sera.

Table 5 summarizes the three protocols which were compared using these criteria. Utilization of either the TNE (Buchmeier et

Figure 1

Kinetics of incorporation of radiolabel into acid precipitable material. BHK-21 subconfluent monolayers were infected with Pichinde virus at a MOI of 1. Twenty-four hours after infection cultures were incubated with 50 μCi per ml L-[^{35}S]methionine in methionine free medium. Cells were disrupted with RIPA and incorporation into trichloroacetic acid (10%) precipitable material was measured. Each point represents the mean and standard error of the mean for four determinations.


L- [35S] METHIONINE ACID
PRECIPITABLE cpm (Mcpm)
0
1
2
3
4
5
6
7
8
0
1
2
TIME OF LABELLING (hr)

al., 1979) or the LiCl (Schaffhausen et al., 1978) techniques resulted in high levels of radiolabelled polypeptides which were common to uninfected and infected cell lysates treated with normal hamster sera or immune hamster sera. RIPA treatment greatly reduced backgrounds, since greater than 80% of the radiolabel was immune sera specific, as estimated from fluorographs of material resolved by SDS-PAGE. Although BHK-21 cells utilized in these experiments were persistently infected with Pichinde virus low levels of 64,000, 17,000 and 14,000 dalton polypeptides were detectable. The three polypeptides are characterized in sections 3.2.5 and 3.2.9.

3.2.3 Effect of MOI and time

Polypeptides which could be immunoprecipitated from infected BHK-21 cells were examined during a 48 hr period at MOIs of 0, 0.1, 1,0, 10, and 50 PFU per cell. Twelve hours after infection small quantities of nucleoprotein (NP) were evident at a MOI of 0.1. At this time substantially greater synthesis of NP was evident at a MOI of 1.0, 10, or 50. A 79,000 dalton protein (GPC) was evident at a MOI of 1, 10 or 50 but was not apparent at lower MOIs. Minor components of 52,000, 38,000, and 28,000 daltons were evident only at MOIs of 10 and 50. One other immune sera specific polypeptide (L) having a molecular weight of approximately 200,000 was detectable at MOIs of 10 and 50 (Fig. 2).

Following a 1 hr label with L-[^{35}S]methionine at 24 hrs after infection the quantities of L, GPC and NP had increased at the four MOIs examined. Although the three polypeptides were evident at a MOI of 0.1, the quantities of L and GPC appeared low relative to the quant-

Table 5

Conditions for Cell Lysis and Immunoprecipitation of Pichinde Virus Polypeptides[a]

Technique	TNE	LiCl	RIPA
Reference	Buchmeier et al. (1976)	Schaffhausen et al. (1978)	-
Cee Lysis	TNE (10 mM Tris-HCl, 0.1 M NaCl, 1 mM EDTA, 2% Nonidet P-40, pH 7.4)	20 mM Tris-HCl, 0.137 M NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10% glycerol, 1% Nonidet P-40, pH 8.0.	RIPA (50 mM Tris-HCl, 0.15M NaCl, 0.1% SDS, 1% DOC, 1% Triton X-100, pH 7.2)
Immunoprecipitate washing	3x with TNE	3x with (0.1 M Tris-HCl, 0.2 M LiCl, 0.1% 2-mercaptoethanol, pH 8:0) 1x with (1 mM Tris-HCl, 0.15 M NaCL, pH 7)	3x with RIPA
Immune Sera Specific Polypeptides (MX X 10^{-3})	64, 17, 14	64	64, 17, 14
Proportion of Total[b]	<10%	<5%	>80%
Nonspecific Polypeptides[c]	26 minor 1 major	20 minor 7 major	5 minor

Table 5 (Footnotes cont'd)

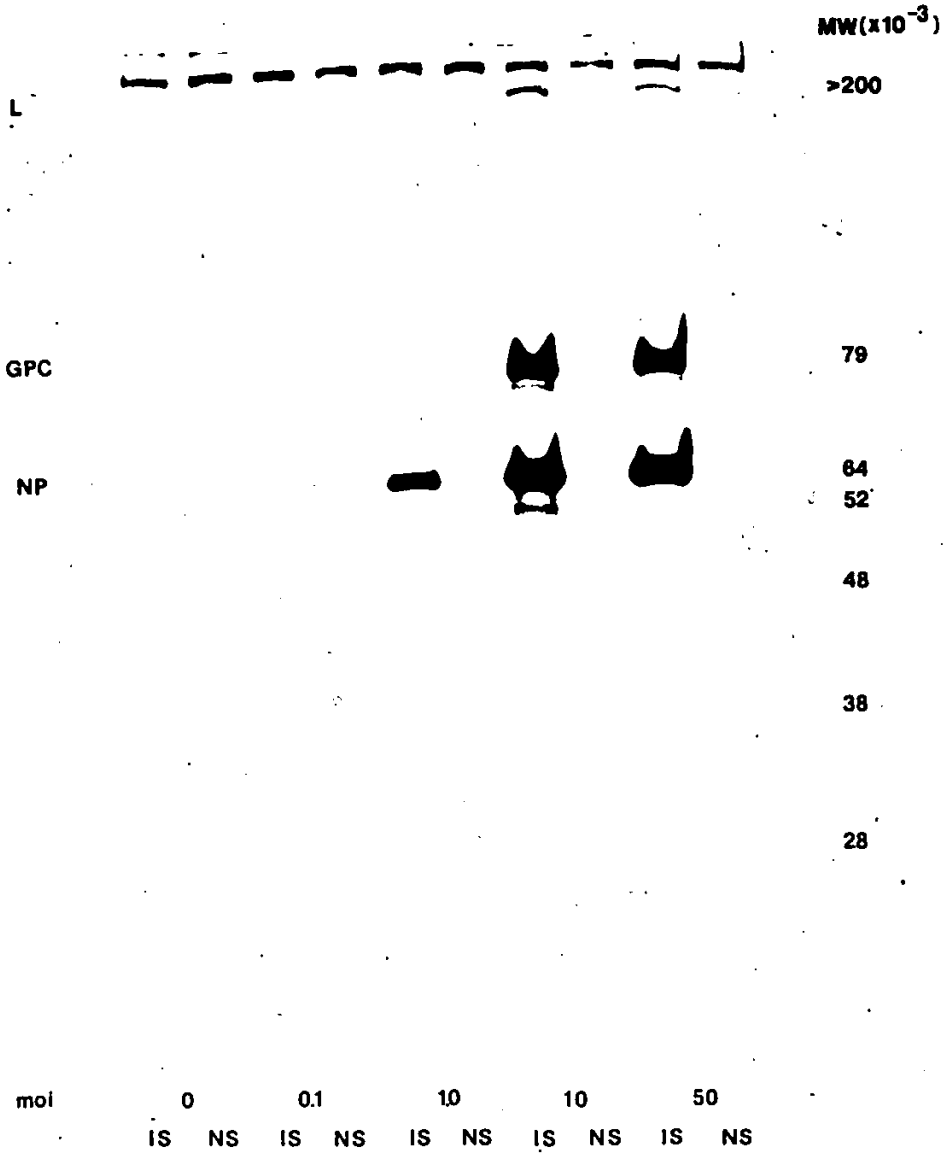
(a) BHK-21 cells were persistently infected.

(b) estimate of proportion of radiolabel in immune sera specific polypeptides relative to total radiolabel.

(c) polypeptides evident in normal sera and immune sera reactions. Minor polypeptides were not as evident as the nucleoprotein (64,000 daltons) and major polypeptides were more evident than the nucleoprotein.

Figure 2

Immunoprecipitable polypeptides in BHK-21 cells at 12 hrs after infection. Mock-infected and Pichinde virus infected cell polypeptides were labeled with L-[^{35}S]methionine as described in Materials and Methods. Aliquots of lysate (1 ml) were treated with normal hamster serum (NS), or hamster anti-Pichinde virus serum (IS), and protein A-Sepharose CL-4B. Immune complexes were eluted in SDS sample buffer and resolved on 7.5 to 15 percent gradient SDS-polyacrylamide slab gels. Molecular weight markers were included in the gel and polypeptides were visualized by fluorography.


MW(x10^{-3})
>200
L
GPC
NP
79
64
52
48
38
28
moi
0
0.1
1.0
10
50
IS NS IS NS IS NS IS NS IS NS

ities at higher MOIs. Polypeptides of 52,000, 48,000, 38,000, and 28,000 daltons were clearly visible at MOIs of 1, 10, and 50. Mock infected cells did not synthesize any of these immunoprecipitable polypeptides (Fig. 3).

The quantities of L, GPC, and NP as well as polypeptides which had molecular weights of 52,000, 48,000, 38,000 and 28,000 had increased substantially by 48 hrs after infection at a MOI of 0.1. The intensities of all polypeptides were increasingly reduced with increasing MOI (Fig. 4). The seven polypeptides were the only ones consistently observed in immunoprecipitates of infected cell extracts following a 1 hr labelling period.

3.2.4 Prozone and independent sera effects

In order to ensure that immune sera specific polypeptides were not overlooked when small quantities of immune sera were employed, and that the polypeptide profile was constant with independent sera the following experiment was performed. BHK-21 cells were infected at a MOI of 1 with Pichinde virus stocks obtained in this laboratory. Twenty-four hours after infection cells were incubated in medium containing L-[^{35}S]methionine for 1 hr. Lysates were treated with various amounts of sera from hamsters which had been injected as described in Materials and Methods, with Pichinde virus from this laboratory and Pichinde and Munchique viruses obtained from Dr. D.H.L. Bishop (University of Alabama). Immune sera specific polypeptides had molecular weights of approximately 200,000, 79,000, 64,000, 52,000, 48,000, 38,000, 36,000, 30,000 and 28,000 (Fig. 5). Sera obtained independently for the three virus stocks, and various concentrations of the sera resulted in identical polypeptide profiles.

Figure 3

Immunoprecipitable polypeptides in BHK-21 cells at 24 hrs after infection. Mock infected and Pichinde virus infected cell polypeptides were analyzed as in figure 2.

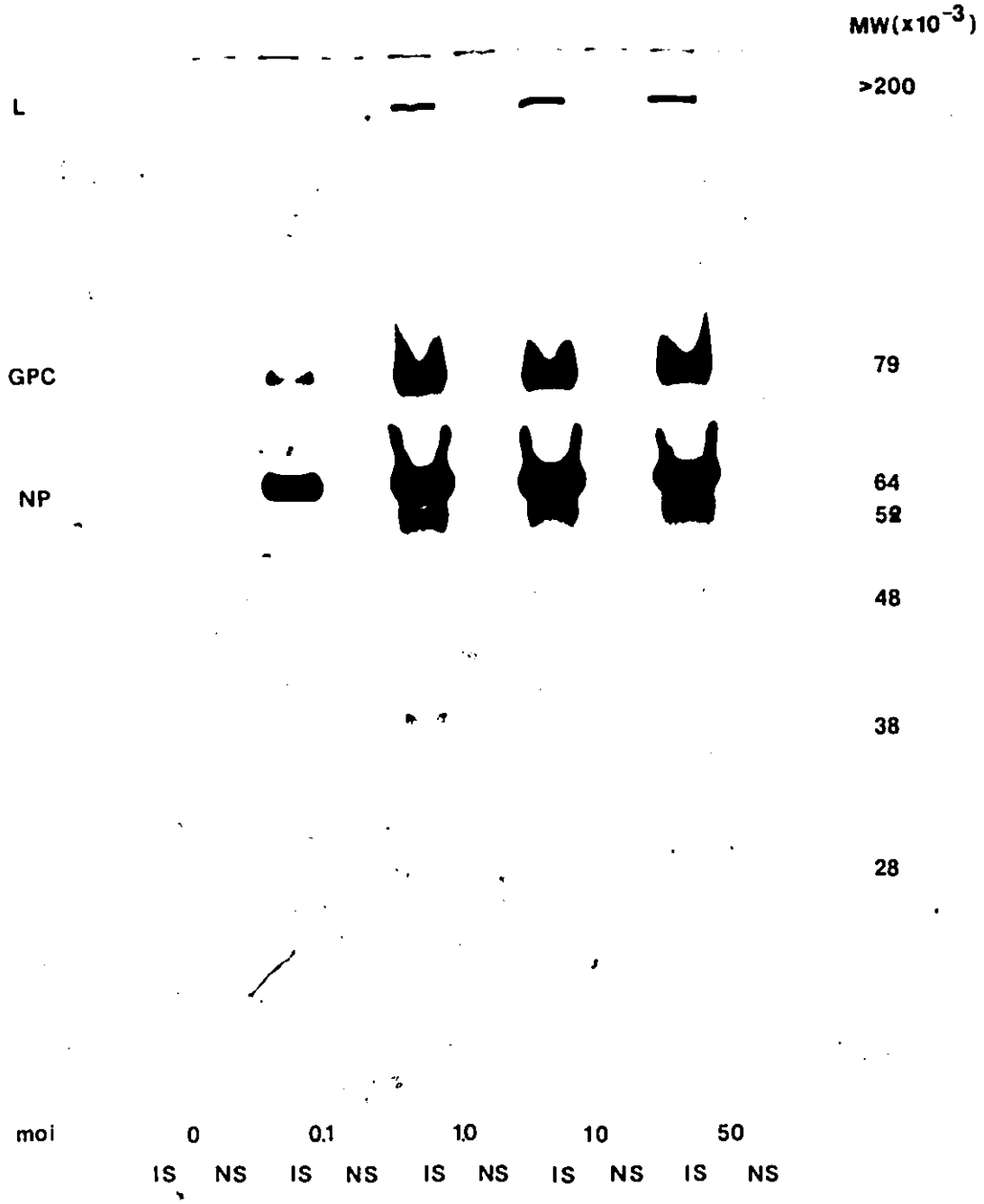

MW(x10^{-3})
>200
L
GPC
NP
79
64
52
48
38
28
moi
0
0.1
1.0
10
50
IS NS IS NS IS NS IS NS IS NS

Figure 4

Immunoprecipitable polypeptides in BHK-21 cells at 48 hrs after infection. Mock infected and Pichinde virus infected cell polypeptides were analyzed as in figure 2.

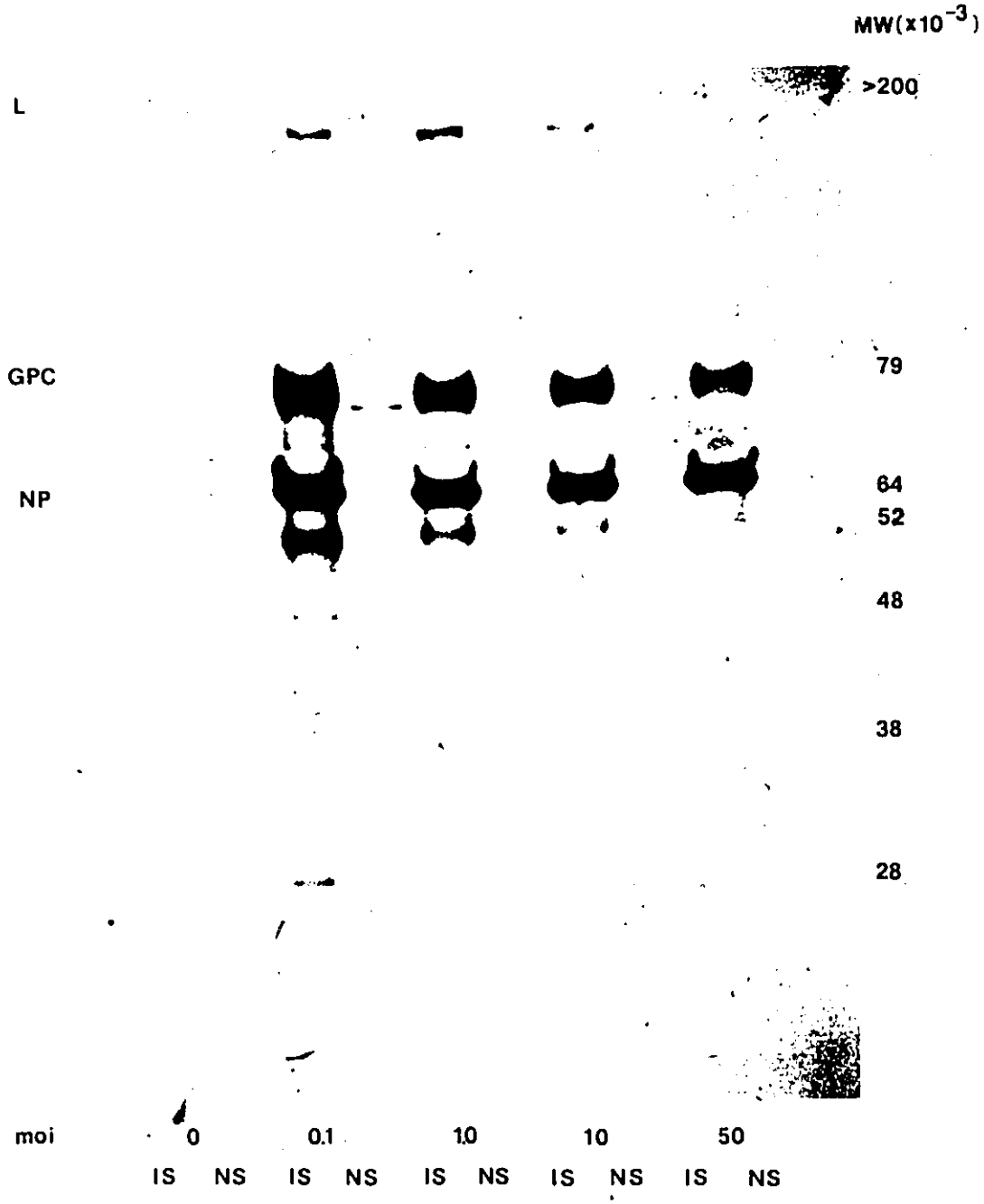
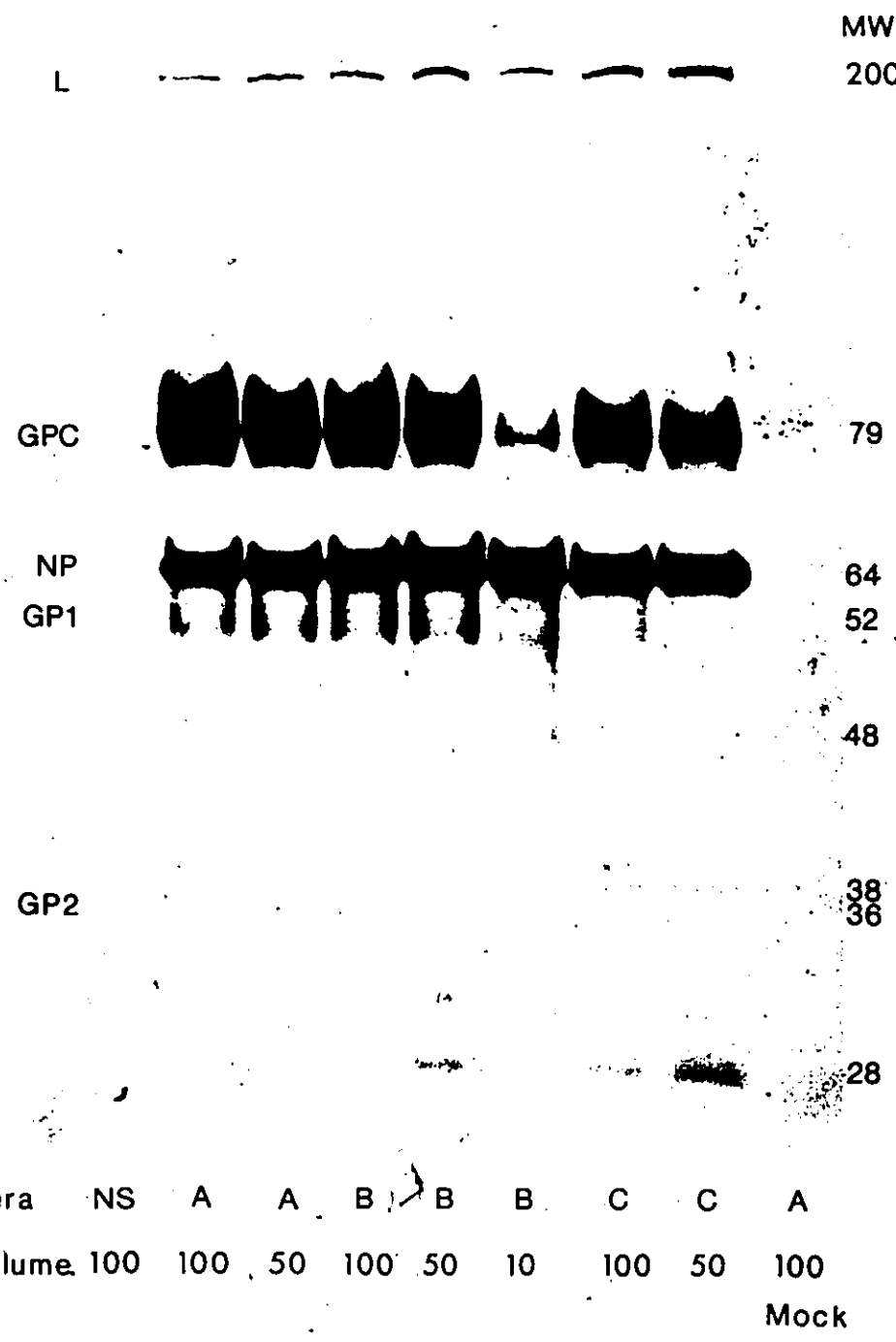

MW(x10^{-3})
>200
L
GPC
NP
79
64
52
48
38
28
moi
0
0.1
1.0
10
50
IS NS IS NS IS NS IS NS IS NS

Figure 5

Sera type and concentration effects. BHK-21 cells were infected at a MOI of 1 with Pichinde virus from this laboratory. Twenty-four hours after infection cultures were incubated for 1 hr in methionine free medium containing 50 µCi per ml L-[^{35}S]methionine. One ml aliquots of lysate were immunoprecipitated with different concentrations of three independently obtained sera. Sera A is hamster anti-Pichinde virus sera (passage 8 of strain AN3739 from this laboratory). Sera B is hamster anti-Pichinde virus sera (strain AN3739 from Dr. D.H.L. Bishop, University of Alabama) and sera C is hamster Anti-Munchique virus sera (virus provided by Dr. D.H.L. Bishop). NS is normal hamster sera. Volumes used in each case are indicated. Polypeptides were resolved by 7.5-15% gradient SDS-PAGE and were visualized by fluorography.


MW(x10^{-3})
L
200
GPC
79
NP
64
GP1
52
48
GP2
38
36
28
Sera NS A A B B B C C A
(µl)Volume 100 100 50 100 50 10 100 50 100
Mock

Polypeptides are fully characterized in following sections. however the quantity of 200,000, 79,000 and 64,000 dalton polypeptides in each region of the electrophoretogram was determined by scintilation counting of hydrogen peroxide digested acrylamide gel slices (Table 6). Each sera reacted strongly with the nucleoprotein and L while reactivities towards GPC varied at equivalent serum dilutions.

3.2.5 Precursor-product relationships of immunoprecipitable polypeptides

To assess the kinetics of synthesis and post-translational processing of immunoprecipitable polypeptides, pulse and pulse-chase experiments were performed. The polypeptide profile obtained after a 1 hr labeling period with L-[^{35}S]methionine was compared to the profile of immunoprecipitates obtained from a 1 hr preincubation in methionine free medium, followed by a 10 min labeling period. The profiles of immune sera specific polypeptides were identical, which suggested that the polypeptides which were evident did not result from enzymatic cleavage of a larger precursor molecule (Fig. 6a-f). Radiolabel was chased in the presence of 100 fold excess unlabelled methionine. No change in the polypeptide profile occurred during a 30 min chase (Fig. 6g,h), however, following a 90 min chase, label appeared in polypeptides of 52,000 and 36,000 daltons which had mobilities consistent with their identification as GP1 and GP2. Label appeared to decrease in the 48,000, 38,000, and 28,000 dalton polypeptides and in a 15,000 dalton polypeptide which was not consistently observed (Fig. 6i,j).

Table 6

Effect of Sera Concentration and Sera Isolate on the Immunoprecipitable Polypeptide Profile[a]

Polypeptide	L-[^{35}S]methionine (counts per minute)		
Sera	L	GPC	NP
NS(100)[b]			434
A(100)[c]	532	I9,819	14,866
A(50)	513	14,481	15,629
B(100)[d]	530	13,575	15,260
B(50)	570	9,660	17,495
B(10)	504	1,535	18,978
C(100)[e]	546	6,537	15,721
C(50)	588	4,256	16,848

[a] BHK-21 cells were infected with Pichinde virus stocks from this laboratory, 1 ml of lysate was immunoprecipitated in each case.

[b] 100 µl of normal hamster sera

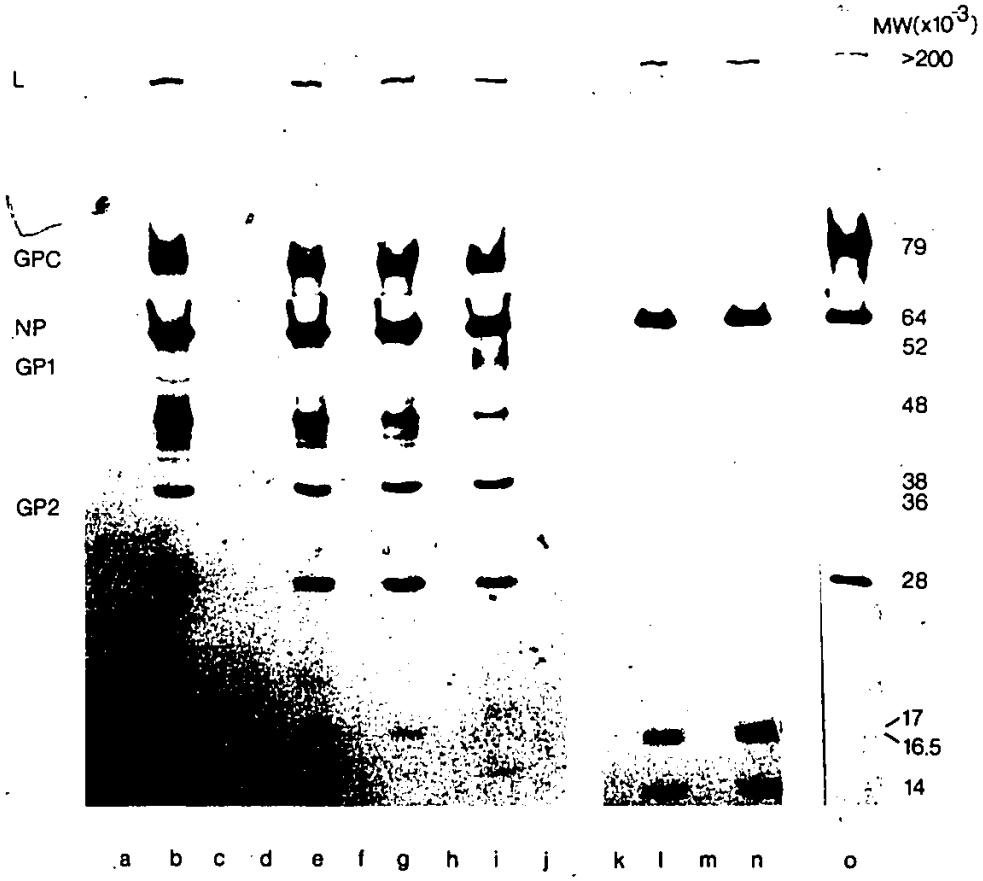
[c] 100 µl of hamster anti-Pichinde virus (this laboratory) sera

[d] 100 µl of hamster anti-Pichinde virus (Dr. D.H.L. Bishop) sera

[e] 100 µl of hamster anti-Munchique virus sera.

Figure 6

Processing of immunoprecipitable polypeptides. BHK-21 cells were infected at a MOI of 1. Twenty-four hours after infection cells were lysed on plates and analyzed as described in Materials and Methods. Mock-infected (a) and Pichinde virus infected (b) cell polypeptides were treated with hamster anti-Pichinde virus serum after a 1 hr preincubation in methionine-free medium and a 10 min pulse-label with L-[^{35}S]methionine. An equal portion of the lysate from virus-infected cells was treated with normal hamster serum (c). Mock-infected (d) and Pichinde virus infected (e-n) cell polypeptides were also labelled for 1 hr without prior incubation in methionine-free medium and were then treated with normal hamster serum (f, h, j, k, m) or immune hamster serum (d, e, g, i, l, n). Radiolabel was chased in the presence of at least a 100-fold excess of unlabeled methionine for 30 min (g, h), 90 min (i, j), 6 hrs (k, l), or 9 hrs (m, n). The polypeptide profile obtained after a 1 hr label with L-[^{35}S]methionine (e) was compared with that of polypeptides labeled for 1 hr with a mixture of 18 ^{3}H-labeled amino acids (o). Immune complexes were analyzed by 7.5-15 percent gradient SDS-PAGE and polypeptides were visualized by fluorography.


MW(x10-3)
>200
L
GPC
NP
GP1
GP2
79
64
52
48
38
36
28
17
16.5
14
a b c d e f g h i j k l m n o

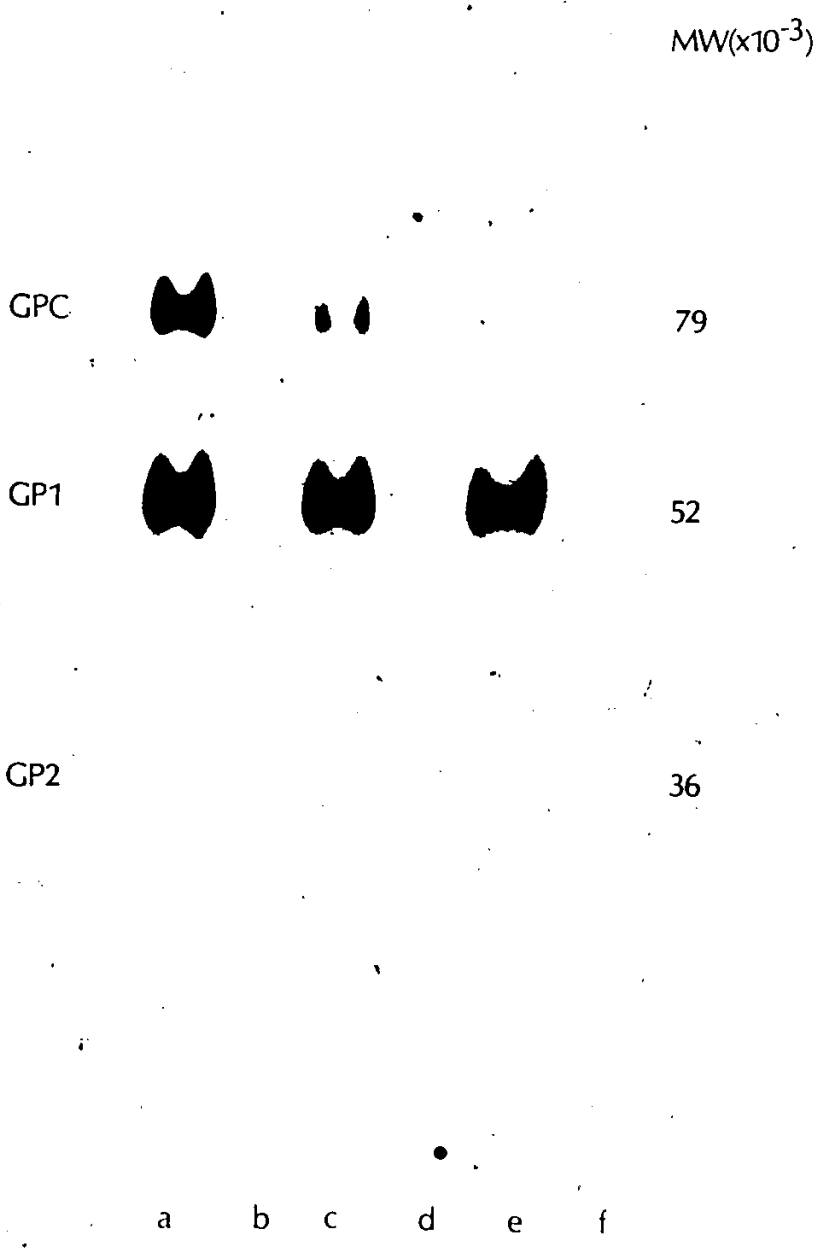
During longer chase periods radiolabel shifted from GPC into GP1 and GP2, however GP2 labeled poorly with L-[^{35}S]methionine. After 6 and 9 hr chase periods most labeled polypeptides decreased substantially in intensity. L, NP, GP1, GP2 and the 38,000 and 28,000 dalton polypeptides were still apparent. Three additional polypeptides with apparent molecular weights of 17,000, 16,500 and 14,000 were visible at this time (Fig. 6k-n). Since radiolabeling with L-[^{35}S]methionine can exclude detection of polypeptides which have a low or negligible methionine content, infected cell polypeptides were also labeled with a mixture of sixteen ^{3}H-amino acids. The profile obtained after a 1 hr pulse label was identical to that obtained with L-[^{35}S]methionine (Fig. 6o).

3.2.6 Identification of glycoproteins

In order to identify viral glycoproteins, BHK-21 cells infected with a MOI of 1 were labeled with D-[6-^{3}H]glucosamine at 24 hrs post infection. During a 3 hr pulse, label was evident in three polypeptides with molecular weights of 79,000, 52,000 and 36,000. Shorter labeling periods of 1 hr did not result in significant D-[6^{-3}H]glucosamine incorporation. The mobilities of the 52,000 dalton and 36,000 dalton polypeptides were consistent with their identification as GP-1 and GP-2, respectively. After 6 and 9 hr chase periods, radiolabel decreased in the 79,000 dalton polypeptide but was still apparent in GP-1 and GP-2. The 79,000 dalton polypeptide is a cell associated precursor to GP-1 and GP-2 and was designated GPC. GP-2 labeled poorly with D-[6^{-3}H]glucosamine. The total radiolabel in glycoproteins declined by 6 and 9 hrs (Fig. 7).

Figure 7

Identification of immunoprecipitable glycoproteins. BHK-21 cells, infected at a MOI of 1, were labeled with D-[6-^{3}H] glucosamine hydrochloride at 24 hrs after infection. Cultures labeled for 3 hrs were lysed in RIPA and aliquots were treated with hamster anti-Pichinde serum (a) or normal hamster serum (b). Radiolabel incorporated during 3 hrs was chased for 6 hrs (c) and 9 hrs (e). Normal hamster serum treated lysates of 6 hrs and 9 hr chases are shown in tracks (d) and (f) respectively. Polypeptides were separated by SDS-PAGE and visualized by fluorography. Molecular weights averaged from three determinations are indicated.


MW(x10-3)
GPC
79
GP1
52
GP2
36
a
b
c
d
e
f

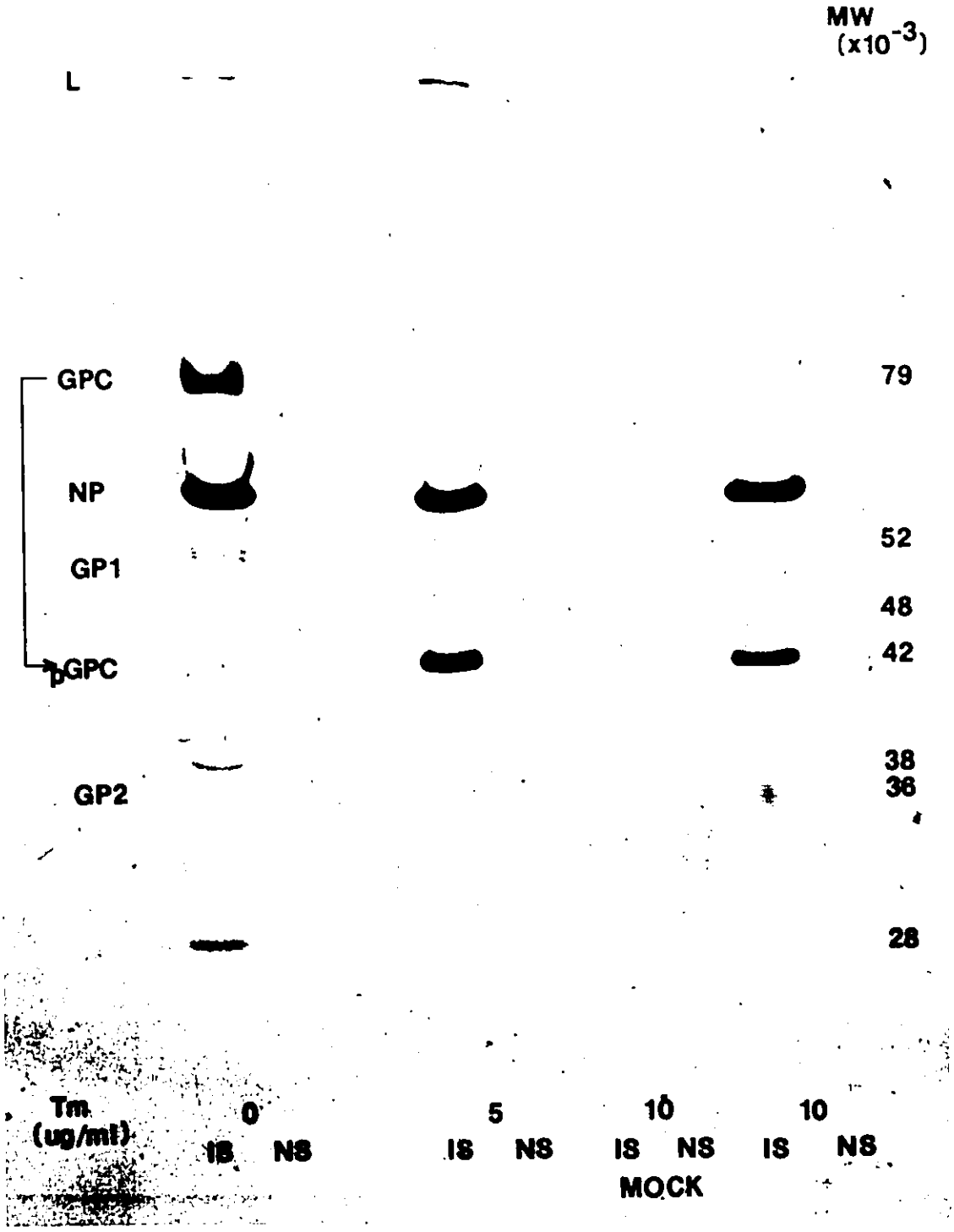
3.2.7 Effect of tunicamycin on glycoprotein

The cell associated glycoprotein precursor (GPC) to GP1 and GP2 had a molecular weight of 79,000 which included an undetermined proportion of carbohydrate. The molecular weight of the polypeptide synthesized in the absence of the prosthetic group was assessed with infected cells which had or had not been incubated for three hours in medium containing tunicamycin. In the absence of tunicamycin, immunoprecipitates of L-[^{35}S]methionine labeled cell lysates contained GPC, which migrated with an apparent molecular weight of 79,000. Small quantities of GP1 and GP2 were apparent and indicated a limited degree of processing. Immunoprecipitates of Pichinde virus infected cells labeled in the presence of 5 and 10 µg per ml tunicamycin did not contain a polypeptide with the mobility of GPC, however, a new polypeptide of 42,000 daltons was observed (Fig. 8). Immunoprecipitates of mock-infected cells did not contain the 42,000 dalton polypeptide. The mobility of all other immune sera specific polypeptides was not altered.

In order to determine the relationship of the 42,000 dalton polypeptide to GPC, infected cells were labeled with 200 µCi per ml L-[^{35}S]methionine, with and without tunicamycin in the medium. The polypeptides were excised from denaturing acrylamide gels after electrophoretic separation and digested with trypsin. Tryptic peptides containing L-[^{35}S]methionine were electrophoretically and chromatographically separated in parallel. The profiles of GPC synthesized in the absence of tunicamycin and the 42,000 dalton polypeptide synthesized in the presence of tunicamycin were similar (Fig. 9).

Figure 8

Effect of tunicamycin on synthesis of GPC in Pichinde virus-infected cells. BHK-21 cells infected with Pichinde virus at a MOI of 1, were labeled with L-[^{35}S]methionine for 1 hr in the presence and absence of tunicamycin (Tm), at 24 hrs after infection. Polypeptides were immunoprecipitated and analyzed by SDS-PAGE and fluorography. Equal portions of lysates were treated with normal hamster serum (NS) and hamster anti-Pichinde serum (IS).


MW
(x10-3)
L
GPC
NP
GP1
pGPC
GP2
79
52
48
42
38
36
28
Tm
(ug/ml)
0
5
10
10
IS
NS
IS
NS
IS
NS
IS
NS
MOCK

The 42,000 dalton polypeptide has been designated pGPC to indicate its precursor relationship to GPC.

3.2.8 Phosphate and sulfate associated with immunoprecipitable polypeptides

To assess sulfation and phosphorylation of immunoprecipitable polypeptides, BHK-21 cells were infected with Pichinde virus at a MOI of 1. Twenty-four hours post infection infected and mock-infected cells were incubated in either sulfate or phosphate free medium for 3 hrs. Macromolecules were then labeled in the presence of 250 µCi/ml of [^{35}S]sodium sulfate or [^{32}P]sodium orthophosphate for 3 hrs. Neither phosphate nor sulfate label could be detected in association with any of the immunoprecipitable polypeptides (data not shown).

3.2.9 Relationships of immunoprecipitable polypeptides

Although several polypeptides were consistently observed in immunoprecipitates of virus infected cells, these may or may not be derived from individual mRNA species. To determine the number of primary products of translation, polypeptides were analyzed by two dimensional tryptic peptide mapping of L-[^{35}S]methionine labeled material. Each sample was compared to a nucleoprotein standard, which was prepared at the same time and included in the same chromatogram. The peptide map profiles of L, GPC and NP were distinct from each other which suggested that these polypeptides were not derived from each other or a common precursor (Fig. 10).

On the other hand, the profile of the tryptic L-[^{35}S]methionine containing peptides of the 48,000 dalton polypeptide shared many features with that of the 64,000 dalton nucleoprotein. One peptide

Figure 9

Comparison of GPC and a 42,000 dalton polypeptide. GPC, synthesized in the absence of tunicamycin (-Tm) and a 42,000 dalton polypeptide synthesized in the presence of tunicamycin (+Tm, 5 µg per ml), were labeled with L-[^{35}S]methionine (200 µCi per ml) and digested with trypsin as described in Materials and Methods. Each digest was separated on one half of a silica gel chromatography sheet (20 by 20 cm). Electrophoretic separation (E) was performed in pyridine-acetic acid-water (1:10:100) in the direction indicated from the origin (O). Chromatography (C) was performed in n-butanol-pyridine-acetic acid-water (5:4:1:5). Radiolabel was visualized by fluorography.

GPC

– Tm

+ Tm

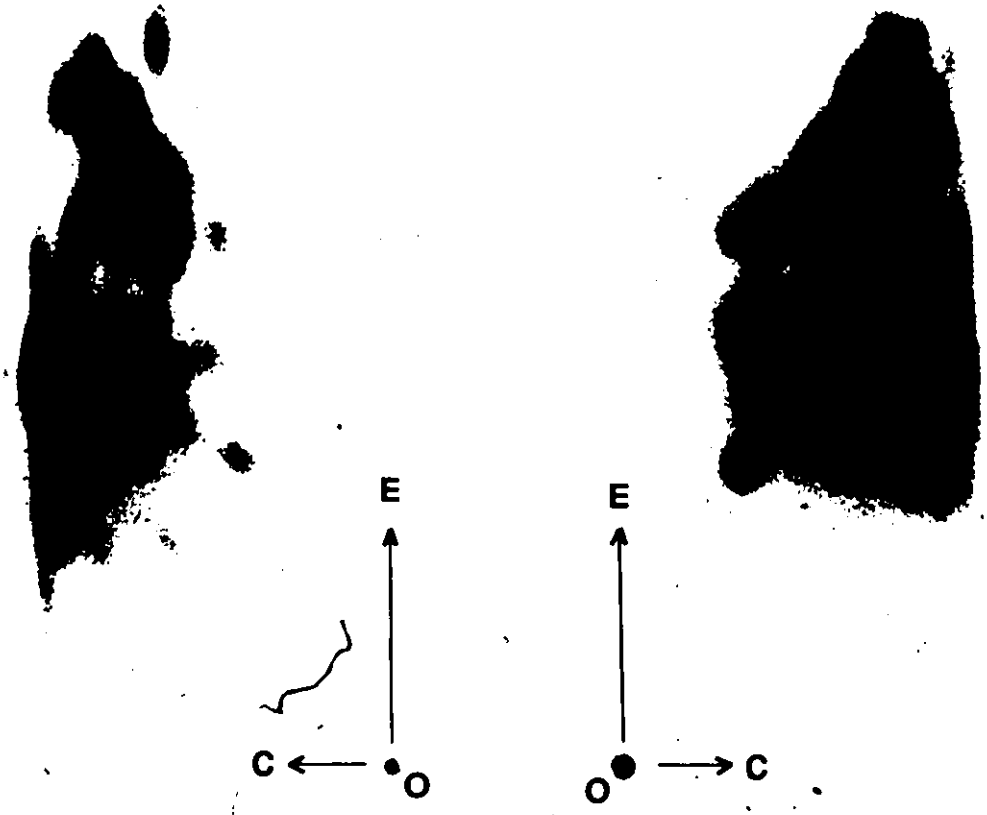
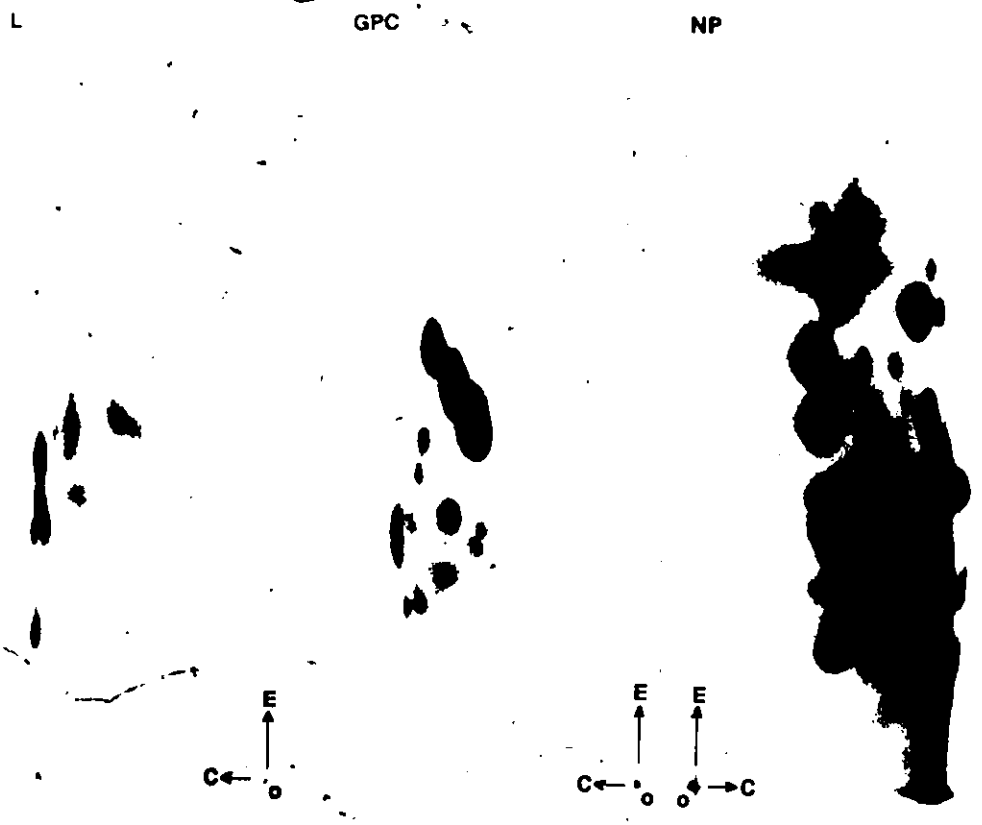


Figure 10

Two dimensional tryptic peptide mapping of L, GPC, and NP. The 200,000 (L), 79,000 (GPC) and 64,000 (NP) dalton polypeptides were labeled with L-[^{35}S]methionine (200 μCi/ml) and digested with trypsin as described in Materials and Methods. Each tryptic digest was separated on one half of a 20x20 cm silica gel chromatography sheet. Digests of NP were separated on the remaining half so that each digest could be compared directly with a standard. Electrophoretic separation (E) was performed in pyridine:acetic acid:water (1:10:100), in the direction indicated from the origin (O). Chromatography (C) was performed in n-butanol:pyridine:acetic acid:water (5:4:1:5). Chromatograms were coated with 2,5-diphenyloxazole (20% w/v in acetone) and peptides were visualized with Kodak X-ray film (type BB1).


L
GPC
NP
E
C
o
E
E
C
o
o
C

was lost in NP and four new ones were apparent in the 48,000 dalton polypeptide. This suggested that the smaller polypeptide may have been derived from NP by proteolytic cleavage and justified designation of the smaller polypeptide as NP48 to denote this (Fig. 11). The 38,000 dalton polypeptide was also found to be related to NP, however, in this case, twelve tryptic peptides were lost and two new ones were apparent in the methionine containing tryptic peptide profiles (Fig. 12). In the case of the 28,000 dalton polypeptide thirteen of the tryptic peptides comigrated with those of NP, four peptides were lost from NP and one new peptide was evident in the smaller molecule (Fig. 13). The latter two polypeptides have been designated NP38 and NP28 respectively.

Although NP48, NP38 and NP28 were evident during very short labeling periods, polypeptides of 17,000, 16,500 and 14,000 daltons were not apparent, except during long labelling times or long radiolabel chase times following a short labelling period (section 3.2.5). Since the 17,000 and 16,500 dalton polypeptides could not always be clearly separated from each other, it was necessary to compare them with NP as a single tryptic digest. The tryptic peptide profile was very similar to that of NP. Ten tryptic peptides were lost and one new peptide was apparent in the mixture of NP17 and NP16.5 (Fig. 14). The profile of methionine containing tryptic peptides of the 14,000 dalton polypeptide was similar to NP but was distinct from NP17 and NP16.5. Twelve tryptic peptides were lost from NP and no new peptides were apparent in NP14 (Fig. 15).

Figure 11

Tryptic peptide map comparison of NP and a 48,000 dalton immunoprecipitable polypeptide. L-[^{35}S]methionine containing tryptic peptides were analyzed as in figure 10. The lower half of the figure represents peptides evident in the chromatogram (upper). Shaded areas in NP represent peptides which have been lost in proteolytic processing. Shaded areas in the 48,000 dalton polypeptide tryptic digest represent peptides which are not evident in NP. Approximately equal amounts of radioactivity were compared.

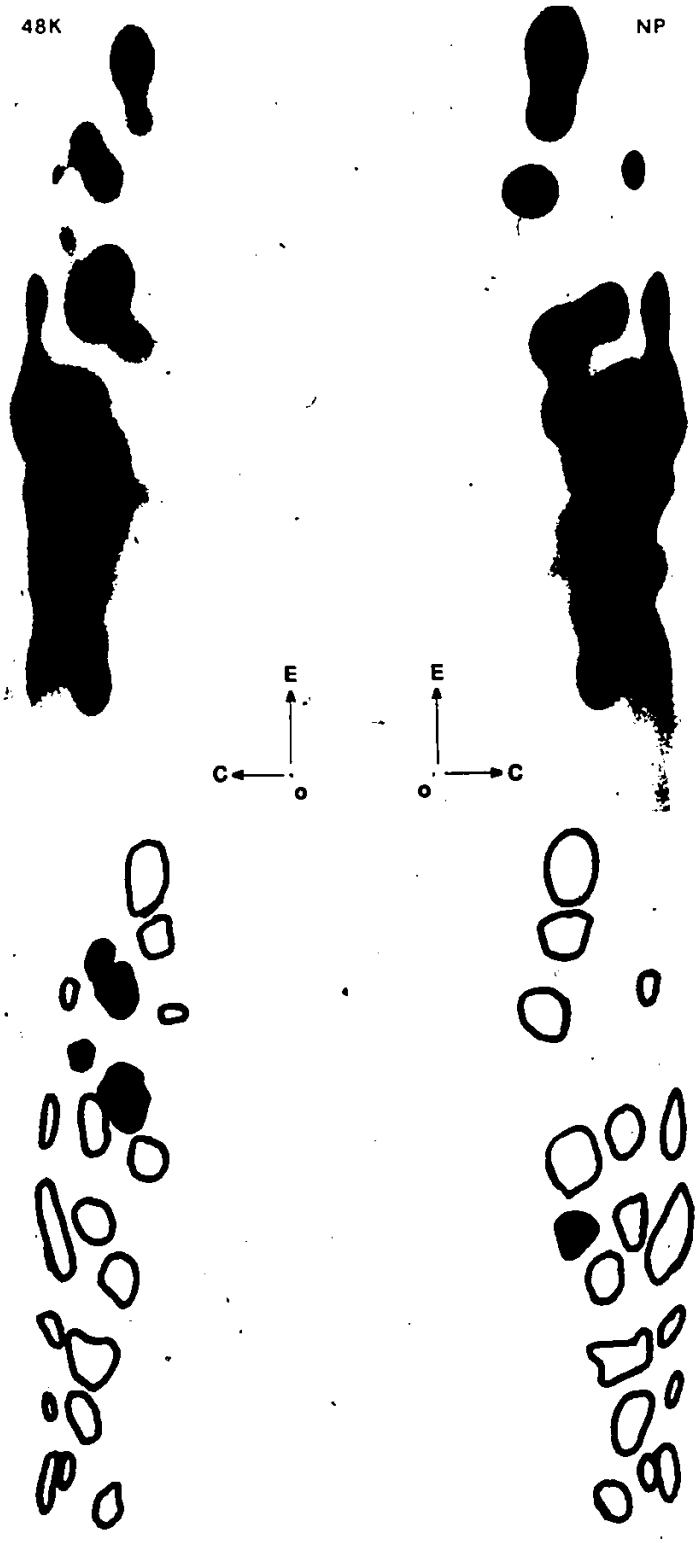

48K
NP
E
C
o
E
o
C

Figure 12

Tryptic peptide map comparison of NP and a 38,000 dalton immunoprecipitable polypeptide. L-[^{35}S]methionine containing tryptic peptides were analyzed as in Figure 10. The lower half of the figure represents tryptic peptides evident in the upper chromatogram. Shaded areas in NP represent peptides which are not present in the 38,000 dalton polypeptide. Shaded areas in the 38,000 dalton polypeptide tryptic digest represent peptides not evident in NP. Approximately equal amounts of radioactivity were compared.

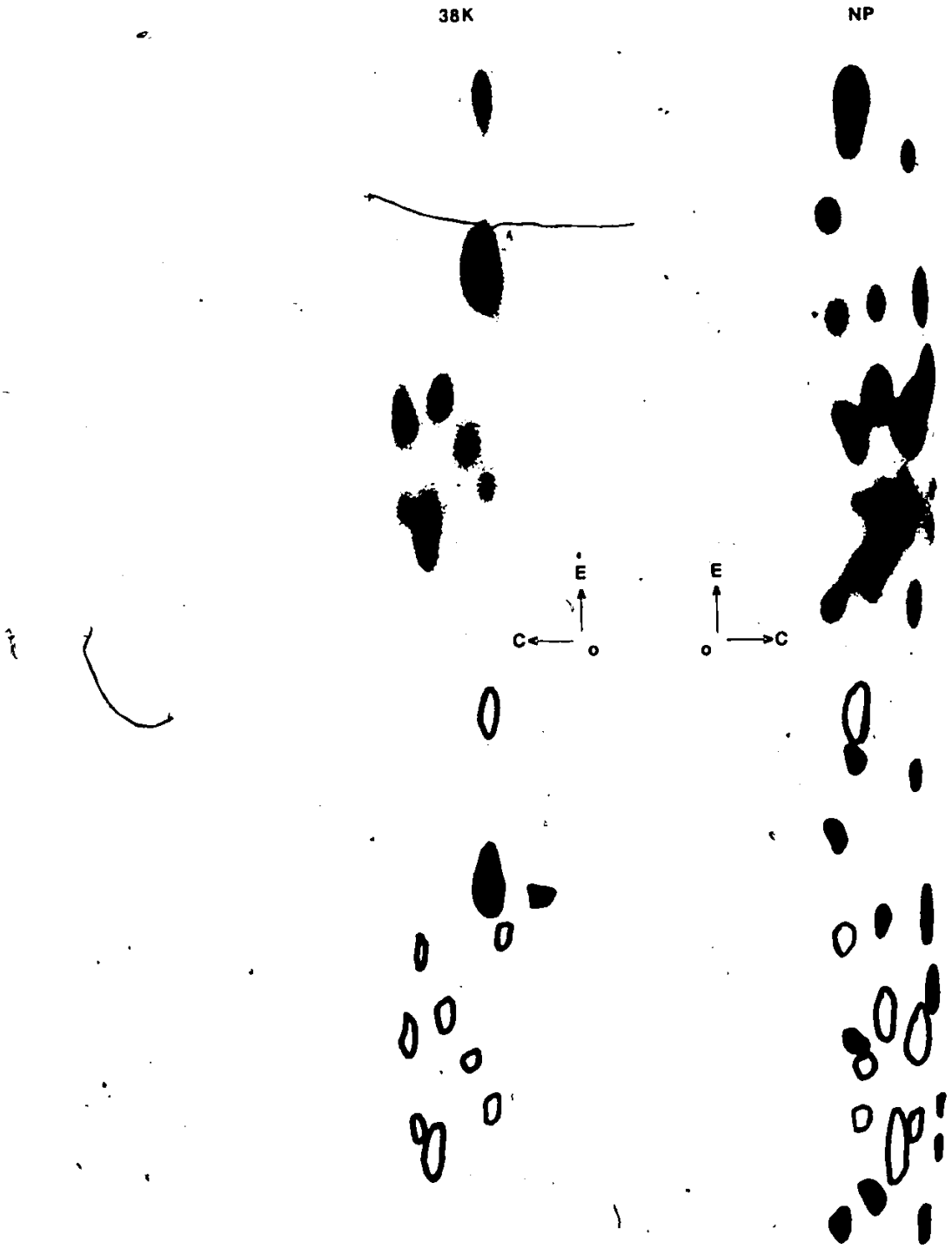

38K
NP
E
C
o
E
o
C

Figure 13

Tryptic peptide map comparison of NP and a 28,000 dalton immunoprecipitable polypeptide. L-[^{35}S]methionine containing tryptic peptides were analyzed as in Figure 10. The lower half of the figure represents the peptides evident in the upper chromatogram. Shaded areas in NP represent tryptic peptides not evident in the 28,000 dalton polypeptide. Shaded areas in the 28,000 dalton polypeptide are tryptic peptides not evident in NP. Approximately equal amounts of radioactivity were compared.

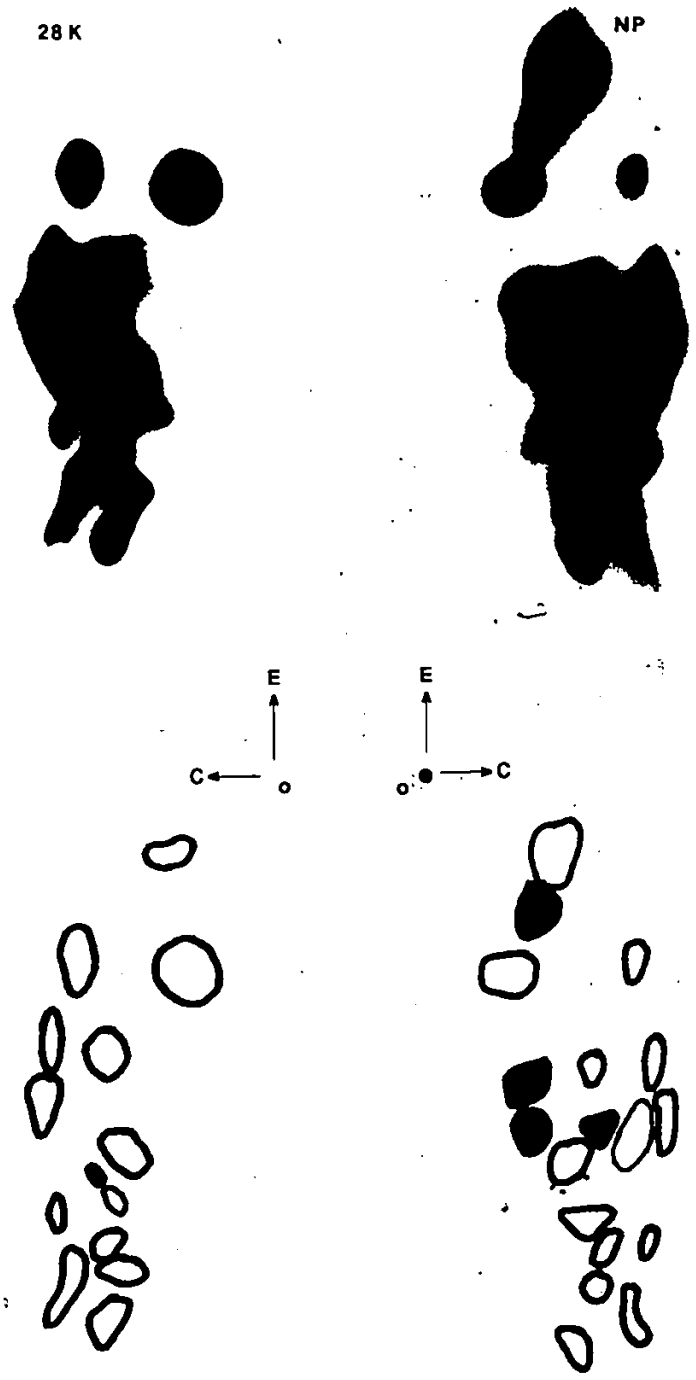
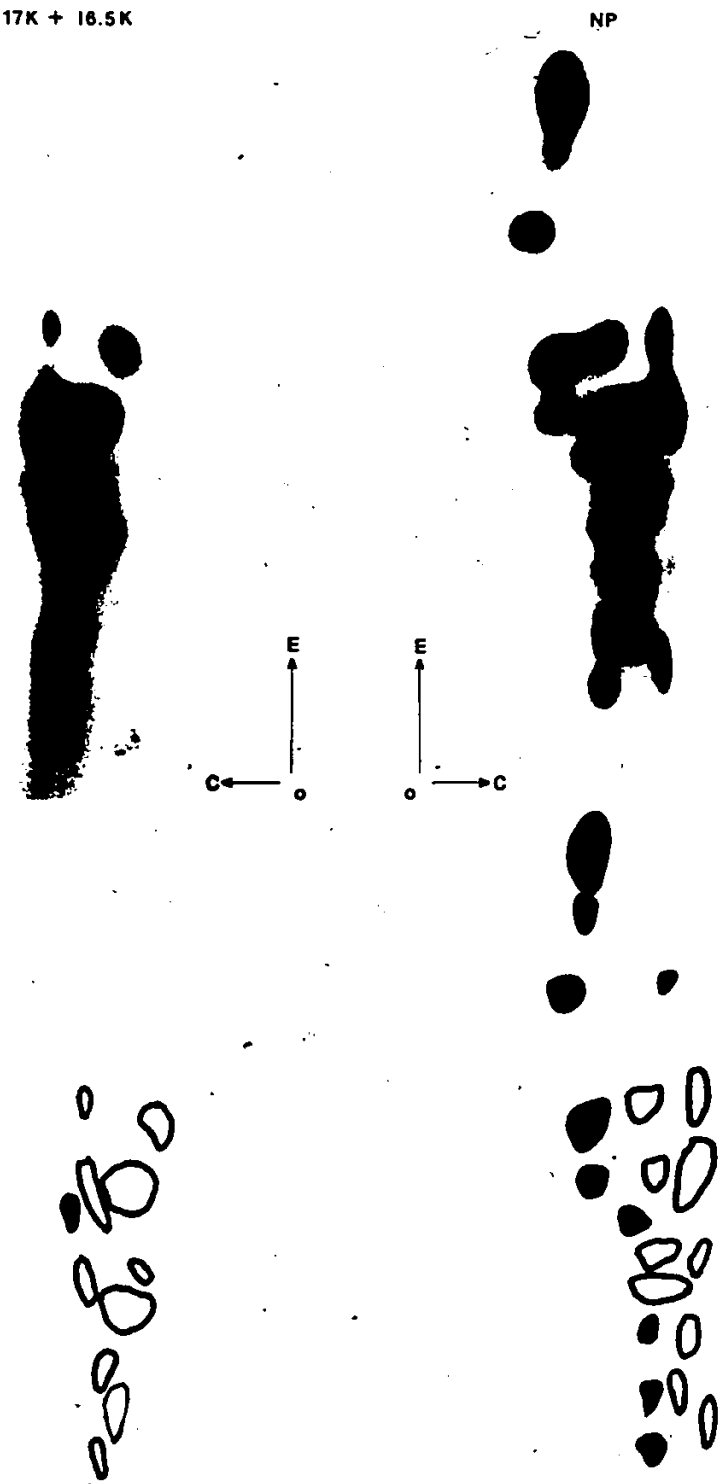

28 K
NP
E
C
E
C

Figure 14

Tryptic peptide map comparison of NP and immunoprecipitable polypeptides of 17,000 and 16,500 daltons. L-[^{35}S]methionine containing tryptic peptides were analyzed as in Figure 10. Since the 17,000 and 16,500 dalton polypeptides were not always clearly resolved they were isolated and treated as a single digest. The lower half of the figure represents the peptides evident in the upper chromatogram. Shaded areas in NP are tryptic peptides which are not evident in the smaller polypeptides. Shaded areas in the 17,000 and 16,500 dalton polypeptide digest are tryptic peptides which are not in NP. Approximately equal amounts of radioactivity were compared.


17K + 16.5K
NP
E
C
O
E
O
C

Although these are the only polypeptides which were consistently observed in immunoprecipitates, it was possible to analyze the tryptic maps of polypeptides having molecular weights of 40,000 and 30,000 which were not always evident. Both molecules shared tryptic peptides with NP. Two new peptides were evident in NP40 and no additional peptides could be distinguished in NP30 (Figs. 16 and 17). The methionine containing tryptic peptide profiles were very similar to those of NP38 and NP28 respectively.

3.2.10 Comparison of virus with polypeptides immunoprecipitable from infected cells

Radiolabeled Pichinde virus was prepared by labeling infected cells with 200 μCi per ml [^{3}H]amino acids or L-[^{35}S]methionine for two hours, followed by a twelve hour chase in the presence of at least 100 fold excess unlabeled amino acid. Virus was purified by density gradient centrifugation and the [^{3}H]amino acid and L-[^{35}S] methionine polypeptide profiles were compared to polypeptides which were immunoprecipitable from the infected cell. L, NP, GP1 and GP2 were common to all three preparations. GPC was evident only in the infected cell polypeptide profile. Small quantities of NP48 and NP38 were detected in virus and cell isolates (Fig. 18).

Polypeptides identified as NP28, NP17, NP16.5 and NP14 were present in immunoprecipitates of infected cell lysates but were not evident in purified virus. Virus preparations did contain components of 15,000 and 20,000 daltons, and several additional polypeptides which were not consistently observed and were present in low quantities.

Figure 15

Tryptic peptide map comparison of NP and a 14,000 dalton immunoprecipitable polypeptide. L-[^{35}S]methionine containing tryptic peptides were analyzed as in Figure 10. The lower half of the figure represents the peptides evident in the upper chromatogram. Shaded areas in NP are tryptic peptides not evident in the 14,000 dalton polypeptide. Shaded areas in the 14,000 dalton polypeptide tryptic digest are peptides not evident in NP. Approximately equal amounts of radioactivity were compared.

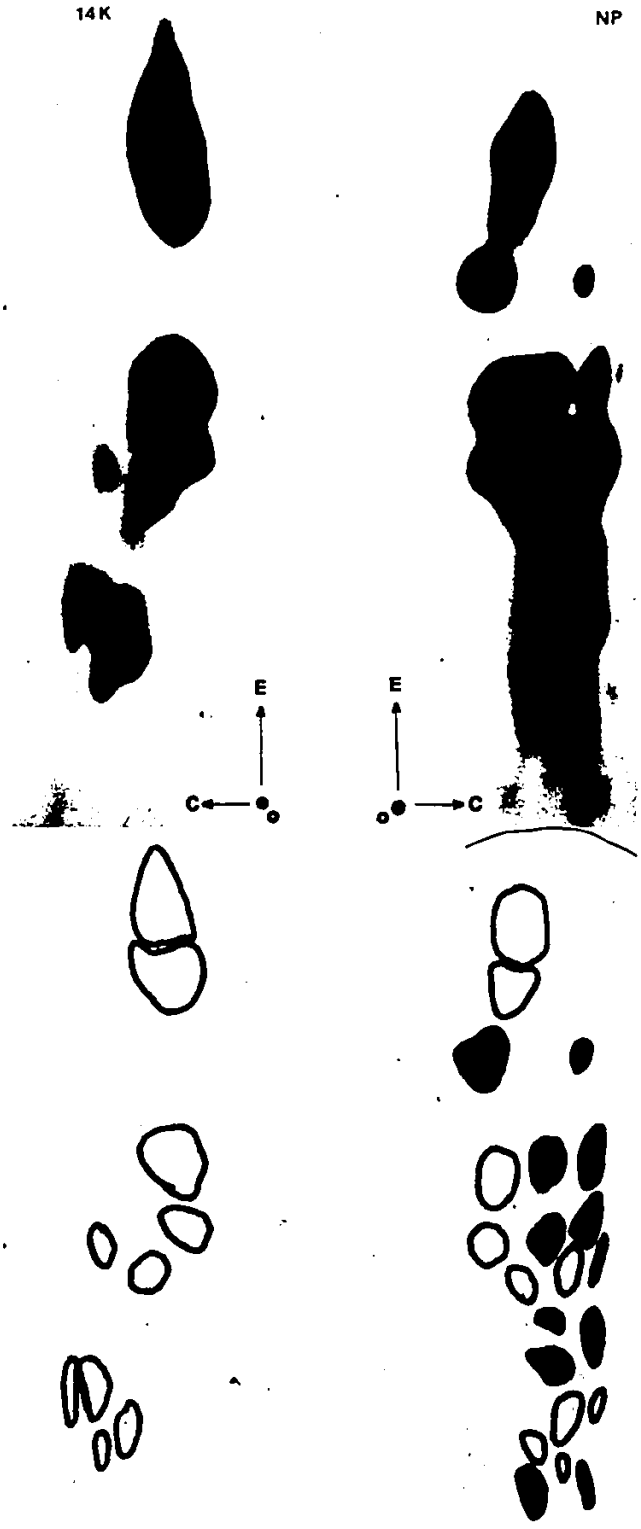

14K
NP
E
C
E
C

Figure 16

Tryptic peptide map comparison of NP and a 40,000 dalton immunoprecipitable polypeptide. L-[^{35}S]methionine containing tryptic peptides were analyzed as in Figure 10. The lower half of the figure represents the peptides evident in the upper chromatogram. Shaded areas in NP are tryptic peptides which are not evident in the 40,000 dalton polypeptide. Shaded areas in the 40,000 dalton polypeptide represent tryptic peptides which are not apparent in NP. Approximately equal amounts of radioactivity were compared.

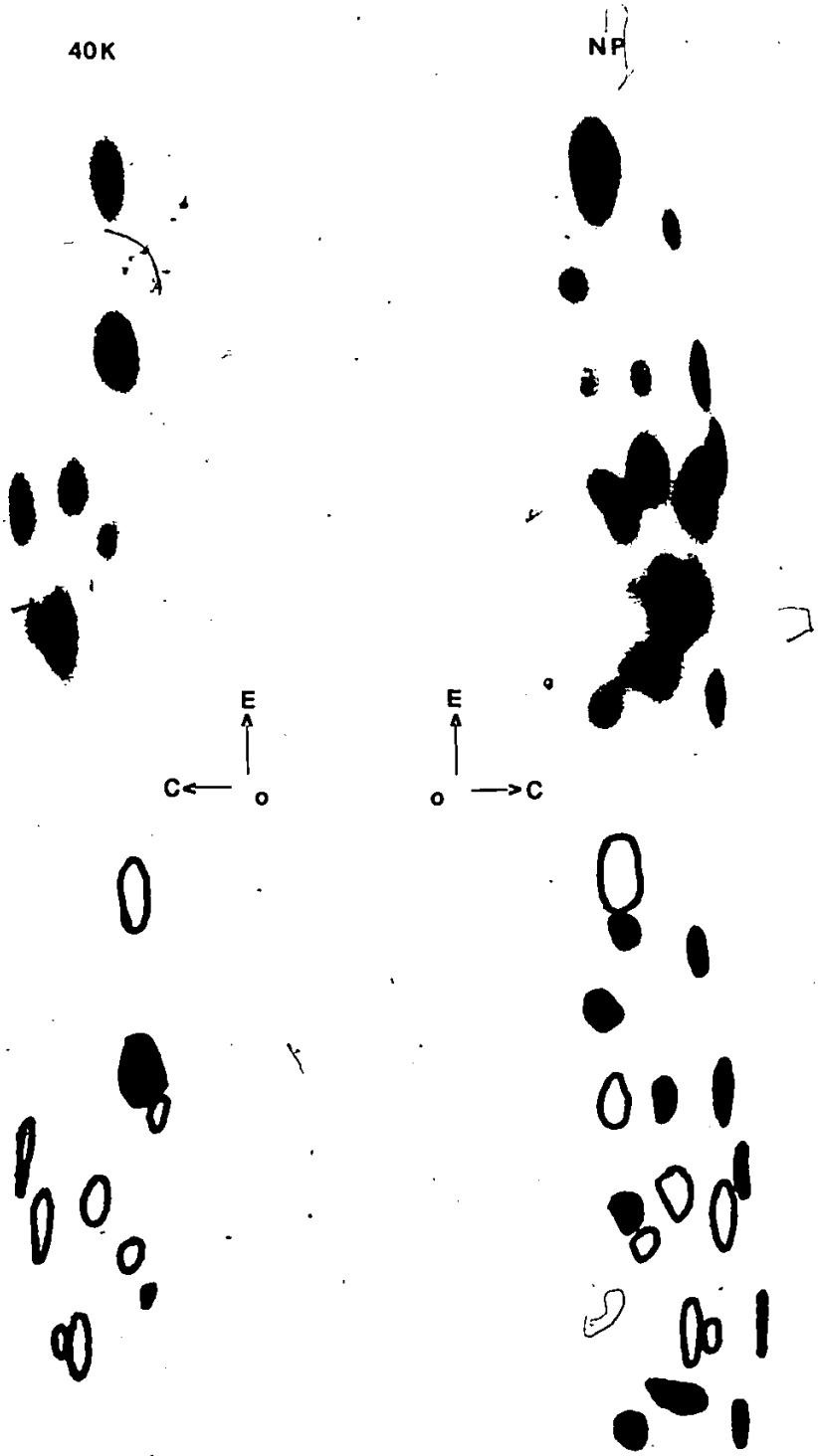

40K
NP
E
C
o
E
o
C

Figure 17

Comparison of NP and a 30,000 dalton immunoprecipitable polypeptide. L-[^{35}S]methionine containing tryptic peptides were analyzed as in Figure 10. The lower half of the figure represents the peptides evident in the upper chromatogram. Shaded areas in NP are tryptic peptides which are not apparent in the 30,000 dalton polypeptide. Shaded areas in the 30,000 dalton polypeptide tryptic digest are peptides not present in NP. Approximately equal amounts of radioactivity were compared.

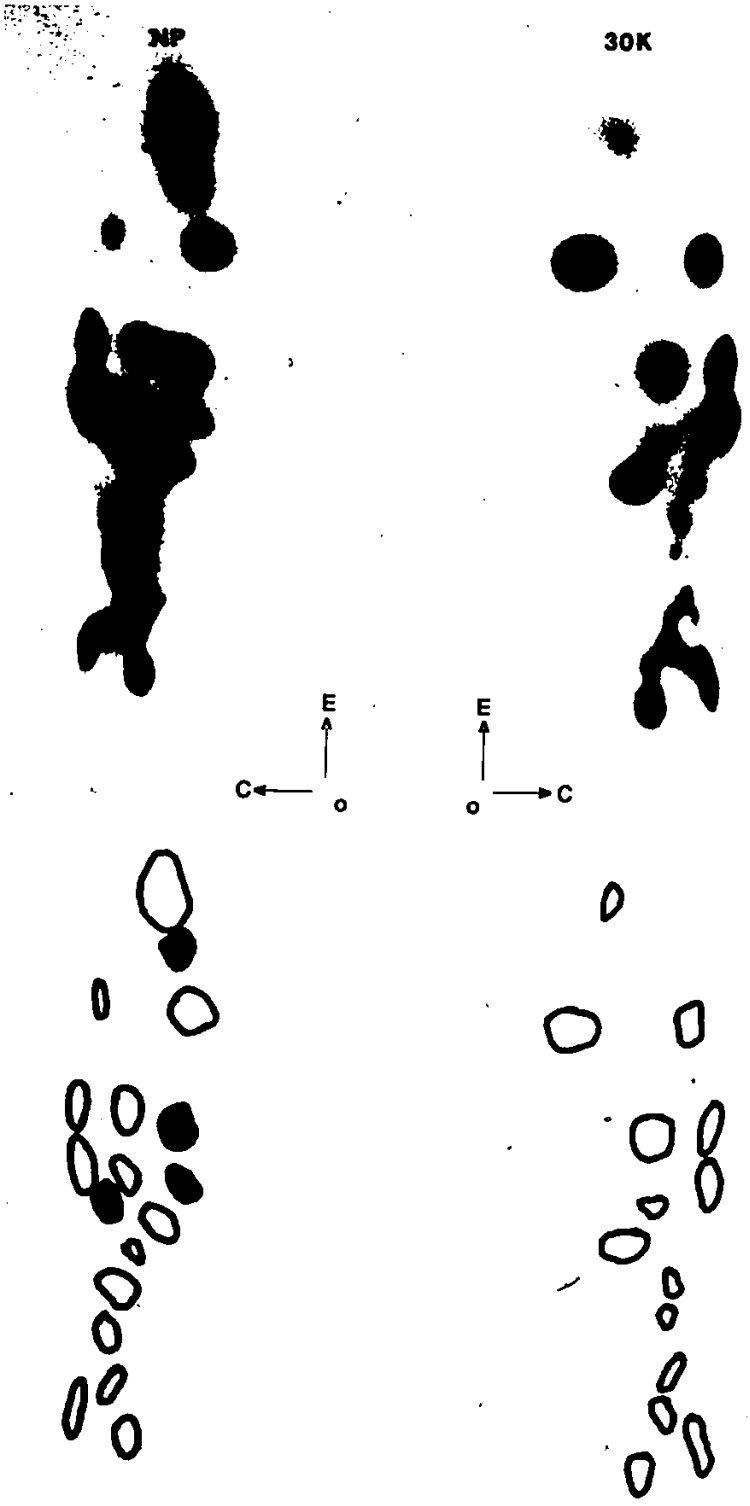
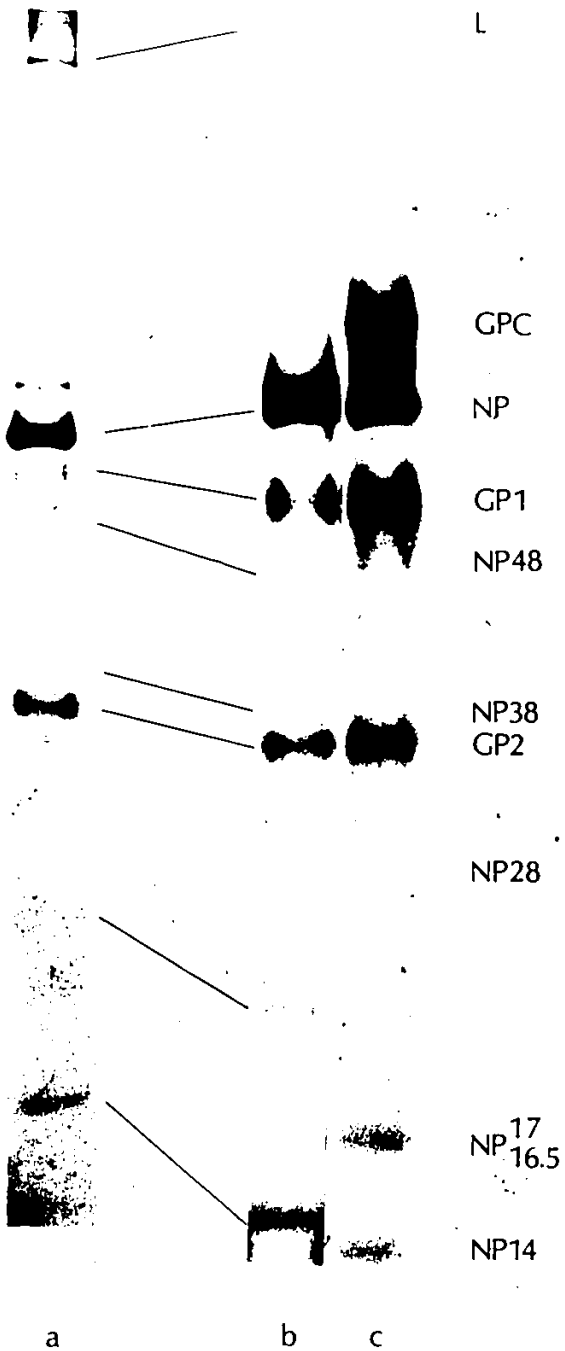

NP
30K
E
C
o
E
o
C

Figure 18

Comparison of immunoprecipitable polypeptides from infected cells with purified virus. Pichinde virus, was labeled with [^{3}H] amino acids (a) or [^{35}S]methionine (b) and was purified as described in Materials and Methods. The viral polypeptides profile was compared with that obtained by immunoprecipitation of infected cell polypeptides labeled with 200 µCi per ml of [^{35}S]methionine for 2 hrs (c). Polypeptides were separated by 7.5-15% gradient SDS-PAGE and visualized by fluorography. Lanes (b) and (c) were electrophoresed on the same gel but were exposed for different times.


L
GPC
NP
GP1
NP48
NP38
GP2
NP28
NP 17 16.5
NP14
a
b
c

3.2.11 Effect of amino acid analogues and metabolic inhibitors on immunoprecipitable polypeptides

We attempted to obtain additional support for earlier observations which suggested that L, pGPC and NP were not derived, in any combination, from a polyprotein. The technique had been used previously with similar intentions for foot and mouth disease virus (Sangar et al., 1977). Pichinde virus infected cells were treated with acetidine-2-carboxylic acid (analogue of proline), canavanine (analogue of arginine) or TLCK and TPCK which are inhibitors of proteolytic enzymes. Following a 1 hr pulse label with L-[^{35}S] methionine and a 6 hr pulse-chase, immunoprecipitable polypeptides were compared to untreated cultures. Acetidine-2-carboxylic acid had no effect on the polypeptide profile (Table 7). Significant alterations in polypeptide mobility were apparent in cultures treated with canavanine and an increase in band width occurred. TLCK and TPCK were cytotoxic even at the low (0.1 mM) concentrations which were employed. Significant proteolysis of the nucleoprotein and lower levels of L-[^{35}S]methionine incorporation were observed. Evidence for a polyprotein precursor was not obtained with these reagents.

Actinomycin D, and α amanitin, in combination with amphotercerin β, were employed to extend previous observations of the negative effect of these inhibitors on Pichinde virus replication (Carter et al., 1973b;Rawls et al., 1976; Leung et al., 1978). BHK-21 cells were infected with Pichinde virus at a MOI of 1. The appropriate inhibitor was added to cultures immediately after infect-

Table 7

Effect of Amino Acid Analogues and Protease Inhibitors[a]

Inhibitor	Alteration(s)
none	none, L, NP, GP1, GP2, NP48, NP38, NP17, NP16.5, NP14 all apparent
acetidine	none (as above), no mobility changes
canavanine	mobility changes in all polypeptides, bands twice as broad, NP17, NP16.5, NP14 not apparent
TLCK	cytotoxic, increased proteolysis
TPCK	as in TLCK

(a) immunoprecipitable polypeptides were examined following a 1 hr pulse-label with L-[^{35}S]methionine and a 6 hr pulse-chase

ion. Twenty-four hours later cells were incubated with inhibitor and L-[^{35}S]methionine for 1 hr. Duplicate cultures were chased in the presence of excess unlabeled methionine for 6 hrs. The results are presented in figure 19. α-amanitin and amphotercerin B had no effect on viral polypeptides synthesized during a 1 hr pulse label when compared to untreated controls (Figure 19 b versus f). An identical polypeptide profile was observed following a 6 hr chase (Figure 19 d versus h). Actinomycin D did not appear to alter the 1 hr pulse-label polypeptide profile (Figure 19j) however NP17, NP16.5, and NP14 were not evident after a 6 hr chase (Figure 19ℓ).

One hour pulse labels were also performed with Pichinde virus infected CHO cells. Two lines were employed. One was sensitive to α amanitin and the other was a resistant variant. The polypeptide profiles obtained were similar to untreated control cultures. Lower levels of L-[^{35}S]methionine incorporation were apparent in cultures treated with inhibitors (Figure 20).

3.3 Discussion

Our findings suggest that the genome of Pichinde virus codes for three primary gene products. These include a large polypeptide (L), a nucleoprotein (NP) and a glycosylated polypeptide (GPC) which is derived from pGPC and is cleaved to yield the two virion glycoproteins. A summary of the polypeptides characterized is presented in Table 8. Several aspects of the study hamper the security with which this conclusion can be reached. Since antisera raised in hamsters infected with Pichinde virus were used to immunoprecipitate the viral polypeptides, putative polypeptides for which antibodies were not represented in the antiserum used would not be detailed. Analysis

Figure 19

Effect of metabolic inhibitors on synthesis of Pichinde virus polypeptides in BHK-21 cells. BHK-21 cells were infected with Pichinde virus at a MOI of 1 and either no inhibitor (lanes a-e), or 10 µg per ml each of α amanitin and amphotercerin B (lanes f-i) or 1 µg per ml of actinomycin D (lanes j-m), was immediately added to cultures. Twenty-four hours after infection cultures were pulse-labeled for 1 hr with 50 µCi per ml L-[^{35}S]methionine in medium containing the appropriate inhibitor. Duplicate cultures were pulse-chased for 6 hrs. Cells were harvested and polypeptides were immunoprecipitated following the pulse-label (lanes b, f, j) or after the pulse chase (d, h, l). An aliquot of lysate was treated, in each case, with normal hamster sera (c, e, g, i, k). Mock infected cells were treated with immune serum (a). Polypeptides were resolved by SDS-PAGE and visualized by fluorography.

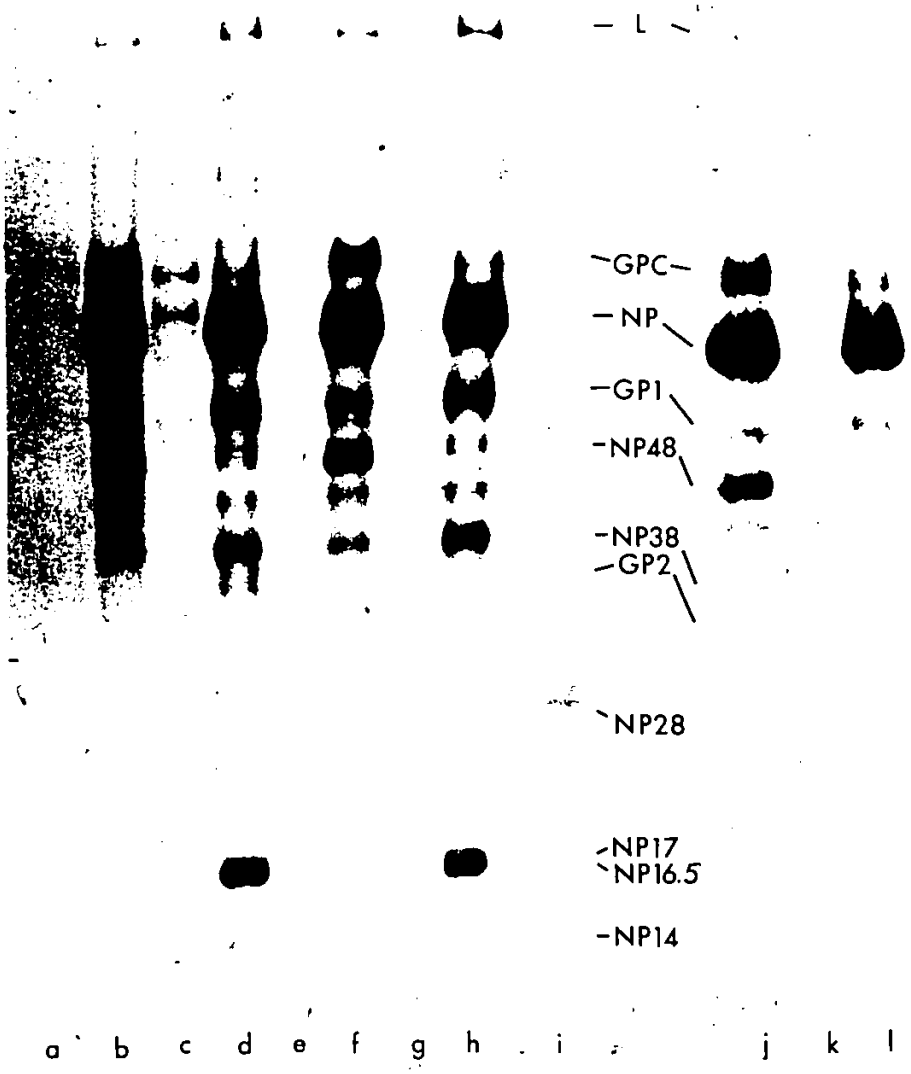

L
GPC
NP
GP1
NP48
NP38
GP2
NP28
NP17
NP16.5
NP14
a b c d e f g h i j k l m

Figure 20

Effect of metabolic inhibitors on the synthesis of polypeptides in CHO cell lines. α amanitin sensitive (ama^s) and resistent (ama^r) cells were infected with Pichinde virus at a MOI of 1. Immediately after infection cells were treated as indicated. At 24 hrs after infection cultures were labeled with L-[^{35}S]methionine for 1 hr. Cells were lysed, polypeptides were immunoprecipitated with normal serum (NS) or hamster anti-Pichinde virus immune serum (IS), and bands were visualized by fluorography of 7.5 to 15 percent gradient SDS-polyacrylamide gels.

(a) Mock, no inhibitor, IS

(b) Pichinde infected, no inhibitor, NS

(c) Pichinde infected, no inhibitor, IS

(d) Mock, α amanitin and amphotercerin B, IS

(e) Pichinde infected, α amanitin and amphotercerin B, NS

(f) Pichinde infected, α amanitin and amphotercerin B, IS

(g) Mock, actinomycin D, IS

(h) Pichinde infected, actinomycin D, NS

(i) Pichinde infected, actinomycin D, IS

(j) Pichinde infected, no inhibitor, NS

(k) Pichinde infected, no inhibitor, IS

(l) Mock, α amanitin and amphotercerin B, IS

(m) Pichinde infected, α amanitin and amphotercerin B, NS

(n) Pichinde infected, α amanitin and amphotercerin B, IS

(o) Mock, actinomycin D, IS

(p) Pichinde infected, actinomycin D, NS

(q) Pichinde infected, actinomycin D, IS

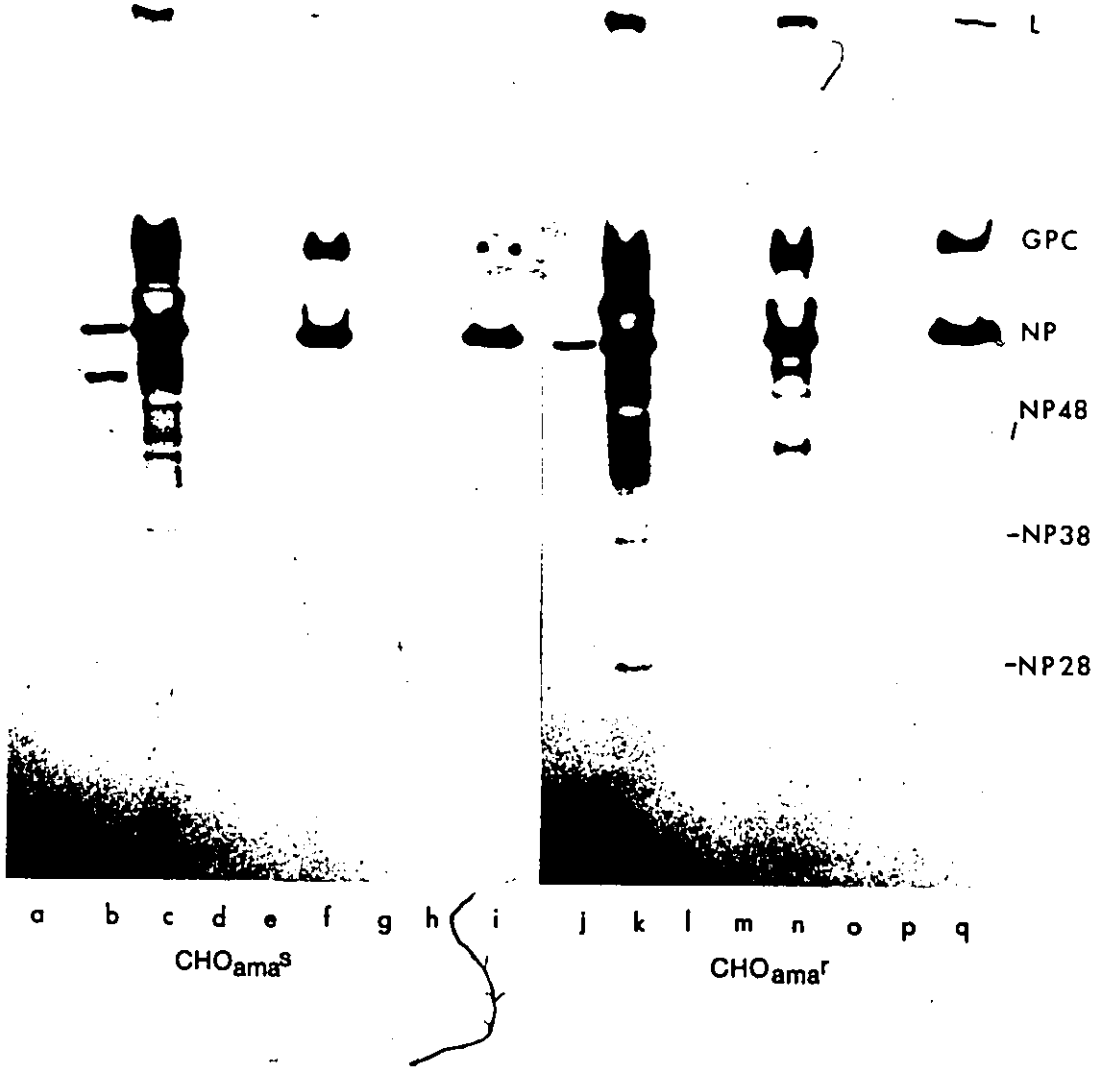

L
GPC
NP
NP48
-NP38
-NP28
a b c d e f g h i j k l m n o p q
CHOama^S
CHOama^r

Table 8

Summary of Pichinde Virus Polypeptides

Polypeptide M.W. ($X10^{-3}$)	Virus (V) or Infected cell (IC)	Presence in Pulse (P) or Pulse-Chase (PC)	Identity
200	V, IC	P, PC	unassigned
79	IC	P	GPC
64	V, IC	P, PC	NP
52	V, IC	PC	GP1
48	V, IC	P	NP derived (NP48)
42	IC	P	pGPC
	(In presence of tunicamycin)		
38	V, IC	P, PC	NP derived (NP38)
36	V, IC	PC	GP2
28	IC	P, PC	NP derived (NP28)
20	V		unassigned
17	IC	PC	NP derived (NP17)
16.5	IC	PC	NP derived (NP16.5)
15	V		possibly NP derived
14	IC	PC	NP derived (NP14)

employing a number of sera obtained after primary immunization and after boosting with three strains of virus failed to demonstrate additional polypeptides suggesting that, if present, the putative polypeptide was nonantigenic for hamsters. Additional polypeptides will have to be identified by in vitro translation of mRNA selected from infected cells with well characterized cDNA sequences. This will be discussed in more detail in chapter 6.

It might also be argued that utilization of L-[^{35}S]methionine to radiolabel polypeptides would exclude from analysis those which have a negligible content of methionine. However, when experiments were performed with a mixture of ^{3}H-amino acids, identical polypeptide profiles were obtained after a 1 hr pulse-label. Finally, the polypeptide profile obtained with a 10 min pulse-label was equivalent to that obtained with a 1 hr pulse-label, which suggests that larger primary translation products were not overlooked.

The polypeptide with an estimated molecular weight of 200,000 was first reported in Pichinde virus (Harnish et al., 1980) and has subsequently been identified by other workers (Young, P.R., et al., 1981). A molecule of similar size has recently been found in LCM virions (Martinez-Peralta et al., 1981; M. Buchmeier, personal communication). In the investigations reported here, the polypeptide was immunoprecipitated from infected cells and lysed purified virus preparations. That this polypeptide, which we have designated L, was virus coded was suggested by its presence in infected BHK-21 cells and Vero cells (Chapter 5) but not in mock infected cells. In addition the polypeptide was synthesized with kinetics which paralleled the synthesis of NP and GPC. Synthesis was dependent upon the multiplicity of virus

used to infect the cells. L appeared not to be glycosylated since its mobility was not altered in the presence of tunicamycin and it did not incorporate radiolabeled glucosamine. The polypeptide was found to be distinct from NP and GPC when tryptic peptide maps were compared, indicating that it was not a precursor of smaller viral polypeptides.

Evidence for the viral specificity of L polypeptide is presented in Chapter 4, however it is tempting to speculate at this point that L represents the viral transcriptase associated with Pichinde virions (Leung et al., 1979). The RNA dependent RNA polymerase has been characterized enzymatically but a viral polypeptide has not been assigned this enzymatic activity. Direct evidence for the suggestion has not been obtained at this point.

As reported for LCM virus (Buchmeier and Oldstone, 1979) and for Tacaribe virus (Saleh et al., 1979), we found a cell associated glycoprotein (GPC) which appeared to be cleaved to yield viral glycoproteins GP1 and GP2. In the case of LCM virus (Buchmeier and Oldstone, 1979) GPC contains most of the L-[^{35}S]methionine tryptic peptides of GP1 and GP2. We have not performed this analysis, however the sum molecular weight of Pichinde virus GP1 and GP2 was 88,000. This was in close agreement with an estimated molecular weight for GPC of 79,000. Molecular weights may not be simply additive for several reasons. Weber and Osborne (1969) suggest that polypeptide mobility in SDS-gels may be influenced by prosthetic groups and molecular weight estimates may not reflect true values. As well, changes in carbohydrate have been observed, in LCM virus and Tacaribe virus, between the cell-associated glycoprotein precursor and the proteolytic products. It was suggested that changes in the oligomannosyl core structure

occurred as happens with the glycoproteins of other enveloped viruses (Hunt et al., 1978; Tabas et al., 1978; Nakamura and Compans, 1979).

We found that radiolabel in GPC chased into GP1 and GP2 but did so at a slow rate. The two glycoproteins were first clearly detectable following a 90 min chase. After a 9 hr chase, GPC was not evident however, the low levels of L-[^{35}S]methionine labeled polypeptides present after 6 and 9 hr chases suggest that much of the radiolabel has been packaged into mature virus. Experiments performed with [^{3}H]glucosamine hydrochloride confirmed the relationship of the three immunoprecipitable glycopeptides. Conclusions concerning kinetic parameters cannot be made because of the large intracellular pools of glucose.

We also attempted to demonstrate association of phosphate and sulfate label with Pichinde virus polypeptides immunoprecipitable from infected cells. Previous studies have suggested that the glycoproteins of enveloped viruses such as influenza are sulfated (Compans and Pinter, 1975; Pinter and Compans, 1975) and that many enveloped RNA viruses contain phosphoproteins (Sokol et al., 1974). As was reported for purified virus (Vezza et al., 1977), we did not find either phosphage or sulfate associated with any of the polypeptides listed in Table 8.

In order to obtain a more accurate estimate of the molecular weight of the protein component of GPC, we examined polypeptides synthesized in the presence of tunicamycin. Tunicamycin has been shown to inhibit the formation of N-acetylglucosamine-lipid intermediates (Takatsuki et al., 1975; Tkacz et al., 1975) which serve as sugar

donors for the synthesis of the oligosaccharide units of glycoproteins (Lennarz, 1975). The polypeptide which was synthesized in the presence of 5 and 10 µg per ml tunicamycin had a molecular weight of 42,000. Comparison of L-[^{35}S]methionine-containing tryptic peptides of GPC with the smaller molecule revealed that the two were very similar. We have therefore designated the 42,000 dalton molecule pGPC to denote a possible precursor relationship.

Non-glycosylated forms of glycoproteins have also been observed in other viruses replicated in the presence of tunicamycin. Faster migrating molecules in SDS-polyacrylamide gels have been observed for both Sindbis and vesicular stomatitis viruses (Leavitt et al., 1977). D-glucosamine and 2-deoxy-D-glucose have also been used by many groups to inhibit glycosylation but these compounds are known to have secondary metabolic effects and in the case of 2-deoxy-D-glucose, incorporation still occurs as a mannose analogue (Bekesi et al., 1969; Duda and Schlesinger, 1975; Kaluza et al., 1973; Scholtissek et al., 1974; Schwarz and Klenk, 1974). Purification of arenavirus glycoprotein, and quantitation of carbohydrate has not been undertaken to date. Pichinde virus GPC may contain as much as 47 percent carbohydrate based on the molecular weight of pGPC and GPC.

Our results suggest that Pichinde virus glycoprotein was synthesized as a 42,000 dalton molecule which may be glycosylated nascently to form a 79,000 dalton GPC. GPC was cleaved, and may undergo carbohydrate changes as in LCM and Tacaribe viruses (Buchmeier and Oldstone, 1979; Saleh et al., 1979), to GP1 and GP2. Similar precursor product relationships have been observed in Sindbis virus (Schlesinger

and Schlesinger, 1972), Newcastle disease virus (Nagai et al., 1976) and Simian Virus 5 (Peluso et al., 1977), but it is not clear whether this phenomenon occurs throughout the arenavirus group. Two glycoproteins have been identified for LCM (Pedersen, 1973b; Buchmeier et al., 1978), Pichinde (Ramos et al., 1972; Vezza et al., 1977), Machupo (Gangemi et al., 1978), Lassa (Kiley et al., 1981) and Mozambique viruses (Kiley et al., 1981). Only one glycoprotein has been observed in Tamiami (Gard et al., 1977), and Tacaribe viruses (Gard et al., 1977; Gangemi et al., 1978). In Junin virus, three to four glycoproteins have been found in virions and it is not entirely clear which of these are viral encoded (Martinez-Segovia and deMitri, 1977; Grau et al., 1981). The single glycoprotein of Tamiami, Tacaribe and perhaps Junin viruses may in fact represent two electrophoretically indistinguishable molecules which were derived from a larger precursor, as has been suggested for Tacaribe virus (Saleh et al., 1979).

The predominant polypeptide evident in immunoprecipitates of Pichinde virus infected cells was a 64,000 dalton, non-glycosylated nucleoprotein (NP). The amount of nucleoprotein synthesized correlated both with the multiplicity of infectious virus and the time of harvest of infected cells. In all cases a peak of synthesis was evident followed by a decline. As has been demonstrated for Tacaribe (Saleh et al., 1979) and LCM viruses (Buchmeier and Oldstone, 1979), this viral protein was detectable in electrophoretograms of cell extracts (data not shown). The mobility of NP immunoprecipitated from infected cells was identical to the mobility of NP packaged

into mature virions.

Other than L, GPC, GP1, GP2 and the nucleoprotein, all of the remaining immunoprecipitable polypeptides which were consistently observed between preparations during a 1 hr pulse label appeared related to NP. NP48, NP38 and NP28 were evident after a 10 min pulse when cells were lysed without removal from the flask suggesting that if they arose from proteolytic activity it occurred at a rapid rate. Three additional polypeptides (17,000, 16,500 and 14,000 daltons) were consistently observed in pulse-chase experiments.

Two dimensional polypeptide mapping demonstrated that NP48, NP38, NP28, NP16, NP16.5 and NP14 were all related to NP. Slight differences in resolution were apparent between chromatograms, however, it should be amphasized that two tryptic peptide digests (one standard NP digest and one unknown) were analyzed on each chromatogram to counteract these differences. Since none of the related molecules contained all of the tryptic peptides of NP, no more than two new peptides should be expected in the comparisons. This was the case for every related molecule except NP48 which exhibited four new peptides. Since NP48 migrated in the region of cellular actin some of the peptides may be derived from actin. The sequence of cellular actin has been published; after treatment with trypsin, 13 peptides should be expected, 3 of which contain 2 methionines each (Adelstein and Kuchl, 1970). Although NP17 and NP16.5 were digested with trypsin, as one polypeptide, it is unlikely that one of these was not related to NP since only 1 new peptide out of a total of 11 was apparent. These polypeptides, when resolved, exhibited similar

intensities in SDS gels so that more than one new peptide would be expected.

The peptide maps of NP derived molecules can be used to construct a scheme which orders the products. NP yields NP48, which in turn can be cleaved to NP38 and NP14. NP38 contains all of the polypeptides of NP17 and NP16.5. Alternatively, NP48 can give rise to NP28, which in turn is cleaved to NP17 and NP16.5. Two observations contradict such a scheme. The rapid appearance of NP48, NP38 and NP28 contrasts with the somewhat longer chase times required for the visualization of NP17, NP16.5 and NP14. NP17 and NP16.5 can be derived from NP48, NP39 or NP28 but NP14 can only be derived from NP48 and NP38, yet all three polypeptides exhibit similar intensities. We cannot exclude the possibility that NP48, NP38 and NP28 may represent discreet premature termination products of NP translation or products of smaller mRNA molecules encoded in the same region of the genome as NP. The hypothetical scheme is summarized in Figure 19. If this is the case, then NP17, NP16.5 and NP14 must arise from NP directly. The peptide maps of the three small polypeptides yield all but 3 NP tryptic peptides upon reconstruction.

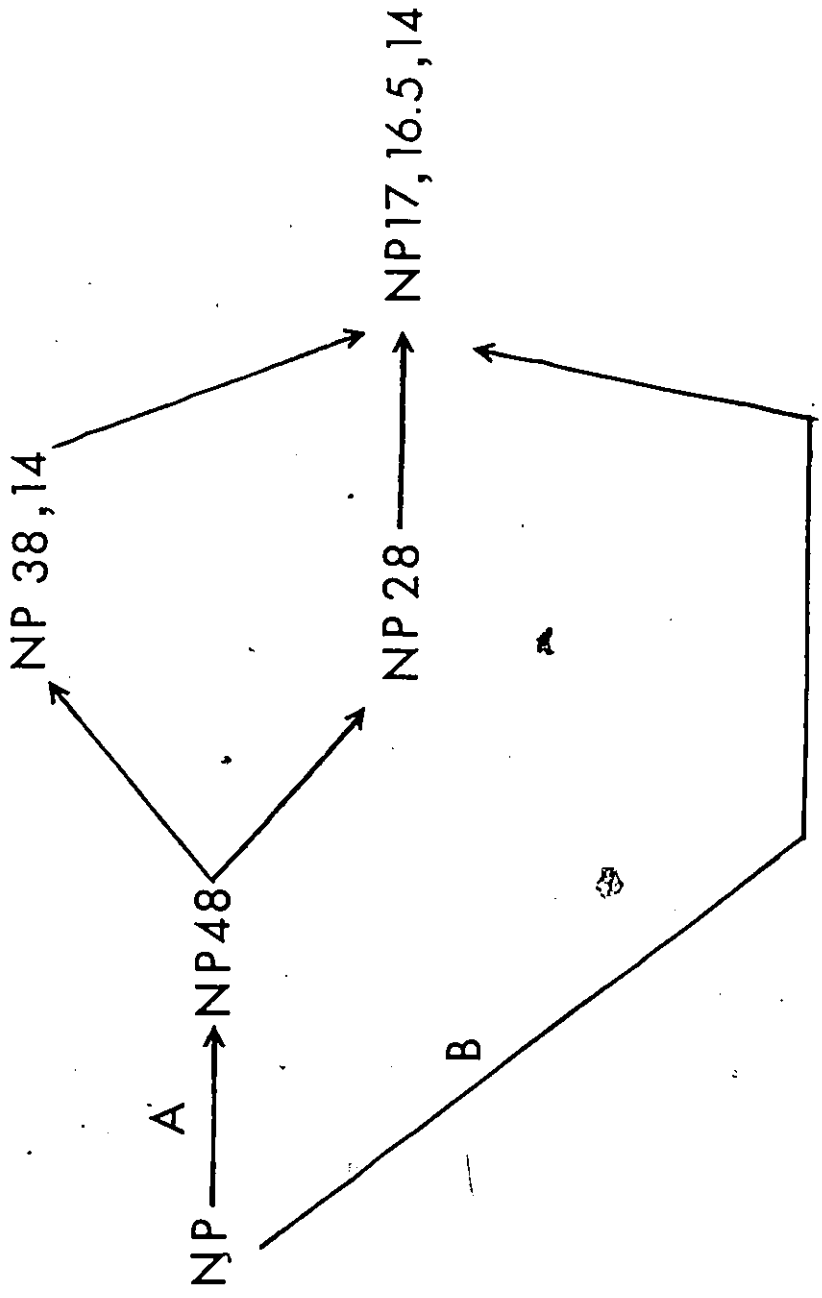
Other minor polypeptides have been inconsistently observed in immunoprecipitates of infected cell lysates. It was possible to analyze the relationship of two of these (30,000 and 40,000 daltons) to the other viral polypeptides. In both cases the tryptic L-[^{35}S] methionine-containing peptide maps were similar to NP and resembled NP28 and NP38 respectively.

Central to the issue of the relative significance of the viral polypeptides, is the question of whether or not they have a function or role in viral replication. At least partial roles have been suggested for viral glycoprotein and nucleoprotein. The glycoproteins are clearly involved in adsorption to the cell (Buchmeier et al., 1980) but other activities such as hemagglutination, hemolysis, neuraminidase, and cell-fusing activities have not been associated with them. The nucleoprotein is the major structural protein associated with the viral nucleic acid (Section 1.5.1) but no other role has been assigned to this molecule. As discussed earlier, a RNA-dependent RNA transcriptase is associated with viral particles (Leung et al., 1979) but has not been assigned to any specific polypeptide (although we have suggested L). Putative roles for NP48, NP38 and NP28 are more difficult to imagine since their quantity is low and they may be premature termination products of translation. On the other hand, NP17, NP16.5, and NP14 are clearly NP proteolytic derivative as evidenced by the kinetics of their appearance. They do not seem to be packaged in virus but they do account for a significant proportion of cellular immunofluorescence after the peak in viral synthesis. The molecules may play a role in shut-off of viral replication which is explored in more detail in Chapter 5.

Polypeptides which were observed in virus labeled with ^{3}H-amino acids or L-[^{35}S]methionine represented primarily those which were immunoprecipitable from the infected cell. GPC was not found in virus. Polypeptides which comigrated with NP28, NP17, NP16.5 and NP14 were not observed in virus, however, 20,000 and 15,000 dalton

Figure 21

Hypothetical cleavage patterns relating the nucleoprotein with smaller related molecules. The nucleoprotein can be proteolytically cleaved to smaller molecules, as indicated, either through a series of intermediate sized polypeptides (route A), or directly to NP17, NP16.5 and NP14 (route B).


NP
A
NP48
B
NP 38,14
NP 28
NP17,16.5,14

polypeptides were observed. These may be related to the cellular NP derived molecules but this could not be examined due to the very low quantities of the polypeptides. Buchmeier et al. (1977) have characterized Pichinde virus complement fixing antigens from infected cells and purified virus. CF antigen in infected cells had molecular weights of 15,000 and 20,000. Neither of these polypeptides comigrated with detectable virion protein, however, CF antigenicity was present in virus preparations.

A survey of the arenavirus literature indicates that minor virion components have been observed in most arenavirus preparations. Vezza et al. (1977) have described a 77,000 dalton polypeptide which was present in Pichinde virions and a similar molecule (79,000 daltons) was immunoprecipitable from Tacaribe virus infected BHK-21 cells (Saleh et al., 1979). Although we observed a similar polypeptide in virus preparations, it did not react specifically with hamster immune sera. A polypeptide of this molecular weight was evident both in cells and virus, but was also evident in mock infected cells and in cell extracts treated with normal hamster sera. Additional minor components in viria (Section 1.5.1, Table 4) of LCM virus (Pedersen, 1973b), Pichinde virus (Howard and Simpson, 1980; Young et al., 1981; and this report), Junin virus (Martinez-Segovia and de Mitri, 1977; Grau et al., 1981) Tacaribe virus (Gard et al., 1977; Gangemi et al., 1978), Tamiami virus (Gard et al., 1977), Machupo virus (Gangemi et al., 1978), and Lassa and Mozambique viruses (Kiley et al., 1981) are of uncertain origin and function. None of the minor components are common to all the arenaviruses. Since the arenaviruses are enveloped

and perhaps do incorporate host cell protein into the virion (Pedersen, 1979), it might be more advantageous to define viral components at the level of mRNA (Chapter 6).

Finally, we attempted to extend early observations on the requirement for a host cell function(s) for efficient replication of Pichinde virus. Initial reports suggested that Pichinde virus replication was inhibited by low concentrations of actinomycin D (Carter et al., 1973b;Rawls et al., 1976), enucleation of cells with cytochalasin B (Banerjee et al., 1976), and α amanitin and amphotercerin B (Leung 1978b). In the case of actinomycin D, the synthesis of virus antigens in the cytoplasm and at the cell surface was not prevented although the yield of virus was reduced (Rawls et al., 1976). The polypeptide profiles obtained from immunoprecipitates of Pichinde virus Infected BHK-21 cells after a 1 hr pulse label and a 1 hr pulse label followed by a 6 hr pulse-chase, were identical in the absence of drug and in the presence of α amanitin and amphotercerin B. When actinomycin D was added to cultures of NP28, NP17, NP16.5, and NP14 were not evident after a 6 hr pulse-chase. All other viral polypeptides remained unchanged. It is tempting to speculate that the smaller nucleoprotein derivatives are involved in viral maturation since actinomycin D blocks a late step in the replicative cycle (Rawls et al., 1976), however, the small molecules are not themselves packaged into mature virions. Similar polypeptide profiles were obtained with α amanitin sensitive and resistant CHO cells although pulse chase experiments were not performed. The levels of L-[^{35}S] methionine incorporation into immunoprecipitable polypeptides was

lower in drug treated cultures, which is perhaps due to the significant cytotoxic effects evident after 24 hrs in the presence of the drug.

Although several polypeptides have been characterized in this study, only three (L, pGPC, NP) of these were unique molecules. It is likely that these arise from individual mRNAs as is the case for rhabdoviruses (Wagner, 1975) and paramyxoviruses (Choppin and Compans, 1975) but this remains to be evaluated. The sum molecular weight of L, pGPC, and NP is 306,000. Viral L and S RNAs can potentially encode 410,000 daltons of protein. These two values agree within theoretical error, but in the absence of amino acid compositional data rigorous calculations are not possible.

4 Assignment of coding function to L and S viral RNAs

4.1 Introduction

Initial experiments have shown that Pichinde virus appeared to encode three primary products of translation. The sum molecular weight of L, pGPC, and NP was 306,000, however, this estimate depended on the assumption that all three polypeptides were viral products. L polypeptide appeared to exhibit the kinetic properties of known viral products (GPC, NP) but a direct demonstration of its viral origin has not been made to this point.

In vitro translation of mRNA in cell free protein synthesizing systems is rapidly becoming the most common means of demonstrating the relationship between a particular gene or portion of a gene and its polypeptide product (Patterson et al., 1977; Hastie and Held, 1978; Woolford and Rosbach, 1979; Ricciardi et al., 1979). In the case of Pichinde virus DNA sequences complementary to genomic RNA are not yet available for selection of mRNA from cell extracts. Al alternative approach was suggested by observations of high frequency genetic recombination in Pichinde virus (Vezza et al., 1977). Vezza et al. (1980) constructed a reassortant virus derived from co-infections with a wild type Pichinde virus and a wild type Munchique virus. I have used the reassortant and parental viruses to assign viral polypeptides to L or S vRNA.

4.2 Results

4.2.1 Immunoprecipitable polypeptides from prototype Pichinde and Munchique viruses and a Pichinde-Munchique reassortant

In order to determine which RNA segment of the viral genome encodes the three primary products of translation which have been designated L, NP and pGPC, BHK-21 cells were infected at a MOI of 1 with wild type Pichinde virus, wild type Munchique virus, or a virus reassortant designated RE-2. Cells infected with a Pichinde virus stock from the author's laboratory were used as a control. Twenty-four hours after infection cells were incubated in methionine free medium, containing 50 µCi per ml L-[^{35}S]methionine, for 1 hr. One ml aliquots of each lysate were used to immunoprecipitate polypeptides. The sera employed in these experiments was derived from hamsters which had been infected with Pichinde virus stocks from the author's laboratory.

In the homologous reaction L, GPC, NP, NP48, NP38 and NP28 were immunoprecipitated as expected. None of the polypeptides were evident in mock infected cells treated with normal hamster sera or immune hamster sera nor in Pichinde virus infected cells treated with normal hamster sera. The same six polypeptides were common to immunoprecipitates of wild type Pichinde, wild type Munchique and RE-2 infected cells. The mobility of the nucleoprotein (NP) and NP38 of wild type Pichinde virus was significantly different from the mobility of NP and NP38 in wild type Munchique and RE-2 reactions. The remaining polypeptides exhibited the same mobilities in all three cases although relative band intensities differed. The intensity of

L in wild type Pichinde and RE-2 reactions was similar but different from the wild type Munchique reaction. The intensities of GPC were most similar in wild type Munchique and RE-2 reactions (Figure 22).

4.2.2 Demonstration that S RNA encodes NP and GPC and L RNA encodes L protein

A determination of which RNA segments encode which proteins can be based on the observation that RE-2 virus contains the L RNA segment of wild type Pichinde virus and the S RNA segment of wild type Munchique virus. We undertook to assign coding function by comparing L, GPC and NP two-dimensional [^{35}S]methionine containing tryptic peptide profiles in each of the three viruses. In this series of experiments homologous hamster antisera were used for immunoprecipitation of wild type Pichinde and wild type Munchique polypeptides. RE-2 polypeptides were immunoprecipitated using a mixture of hamster antisera reactive against the parental viruses.

The [^{35}S]methionine containing tryptic peptides of L, GPC and NP were compared between the parental and RE-2 viruses in pairs on the same chromatography sheet. The mirror image profiles produced in this way eliminate slight differences in mobility of peptides, which may occur between independent separations.

The nucleoprotein of RE-2 and the parental viruses was digested with trypsin and L-[^{35}S]methionine containing peptides were separated in the first dimension by electrophoresis, and is the second dimension by chromatography. The profiles of wild type Munchique and RE-2 nucleoprotein tryptic peptides were essentially identical (Figure 23). On the other hand, the profile of L-[^{35}S] methionine containing tryptic peptides of wild type Pichinde differed

Figure 22

Comparison of immunoprecipitable polypeptides in BHK-21 cells infected with two strains of Pichinde virus, a Munchique virus and a Pichinde-Munchique reassortant. BHK-21 cells were infected at a MOI of 1 with the appropriate virus as indicated. Twenty-four hours after infection cultures were incubated for 1 hr in methionine free medium containing 50 μCi per ml L-[^{35}S]methionine. Cells were harvested, lysed, and 1 ml aliquots of the lysate were treated with normal hamster serum (NS) or hamster anti-Pichinde virus (strain AN3739 from this laboratory) serum (IS) and protein-A-sepharose CL-4B. Immune complexes were eluted and resolved by SDS-PAGE in 7.5 to 15 percent polyacrylamide gradient gels. Polypeptides were visualized by fluorography.

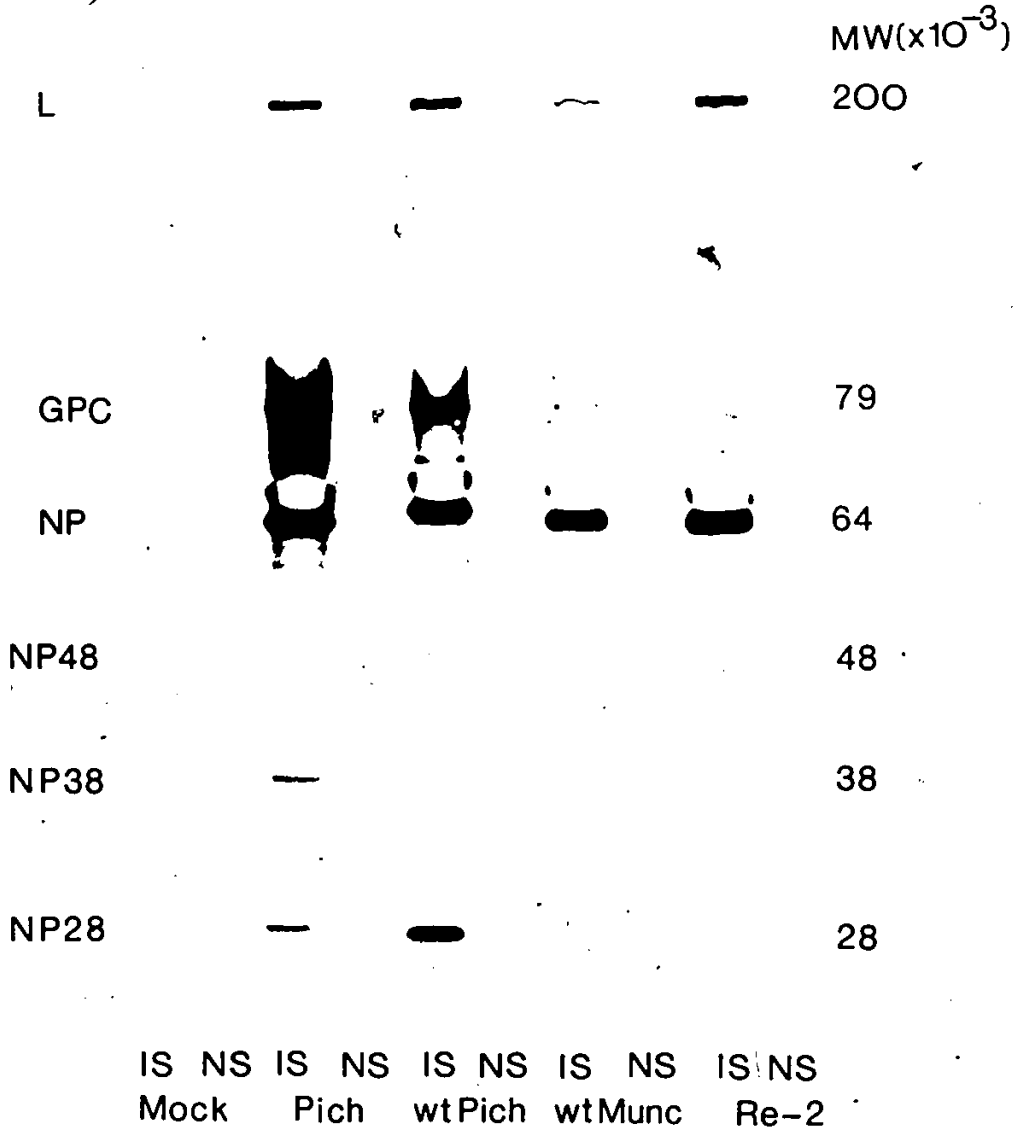
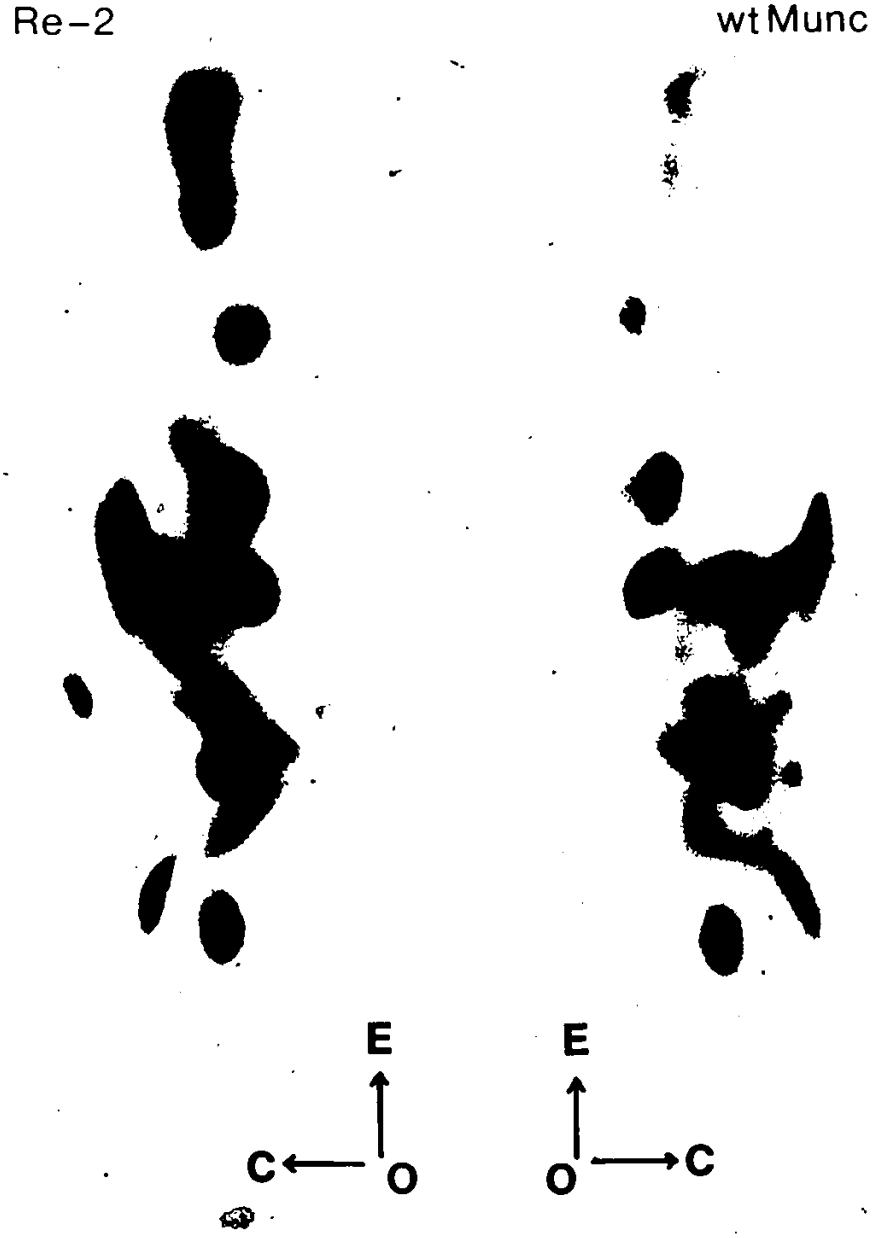

MW(x10^{-3})
L
200
GPC
79
NP
64
NP48
48
NP38
38
NP28
28
IS NS IS NS IS NS IS NS IS NS
Mock Pich wtPich wtMunc Re-2

Figure 23

Comparison of wild type Munchique and RE-2 nucleoproteins. Viral polypeptides were immunoprecipitated from infected cells after labeling in the presence of 200 μCi per ml L-[^{35}S]methionine. Nucleoproteins were isolated and digested with trypsin as described in Materials and Methods. Each digest was separated on one-half of a silica gel chromatography sheet. Electrophoretic separation (E) was performed in pyridine-acetic acid-water (1:10:100) in the direction indicated from the origin (O). Chromatography (C) was performed in n-butanol-pyridine-acetic acid-water (5:4:1:5). Radiolabel was visualized by fluorography.


Re-2
wt Munc
E
C
O
E
O
C

from that of RE-2 (Figure 24). Two major peptides and one minor peptide of this virus were not apparent in the RE-2 or wild type Munchique maps, which suggested that the nucleoprotein was encoded by S RNA. A schematic illustration of the results is presented in figure 25. Common and distinguishing tryptic peptides are shown.

Similar comparisons were performed for GPC and L polypeptides. The two-dimensional L-[^{35}S]methionine-containing tryptic peptide map profile of wild type Pichinde and RE-2 GPCs were different (Figure 26). One very abundant peptide, evident in the RE-2 profile, was very much reduced in the wild type Pichinde virus profile, and a new peptide was evident in the parental virus GPC. In a comparison of wild type Pichinde and wild type Munchique virus profiles, the same differences in GPC tryptic peptides were apparent (Figure 27). The maps of RE-2 and wild type Munchique virus GPCs, although not compared on the same chromatogram, were the same. All three GPCs are schematically shown in figure 28. Common and distinguishing tryptic peptides are indicated. The results have suggested that GPC was also encoded by S RNA.

Comparisons of L polypeptide between the three viruses were more difficult since less than 7 percent of the radiolabel in immunoprecipitable polypeptides was accounted for by L polypeptide. The tryptic peptide profile of wild type Pichinde virus L polypeptide was identical to the profile of RE-2 L polypeptide (Figure 29). Differences were apparent in the L-[^{35}S]methionine-containing tryptic peptides of wild type Munchique virus L polypeptide when compared to the L polypeptide of RE-2 (Figure 30). Two peptides of intermed-

Figure 24

Comparison of wild type Pichinde and RE-2 nucleoproteins. Nucleoproteins were radiolabeled, isolated, digested with trypsin, and resolved electrophoretically and chromatographically as described in figure 23.

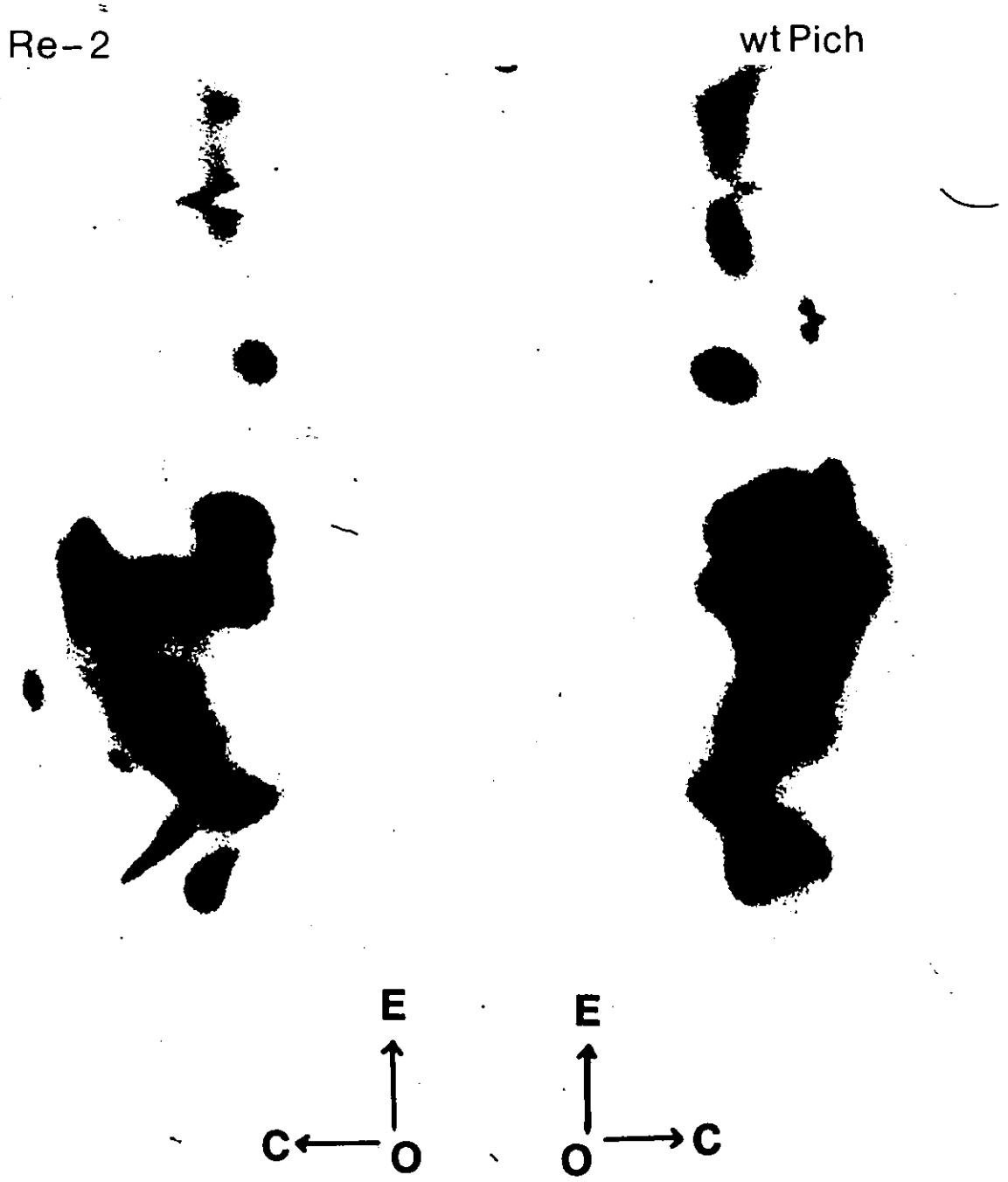

Re-2
wt Pich
E
C
O
E
O
C

Figure 25

Schematic summary of nucleoprotein methionine-containing tryptic peptides in three viruses. Peptides evident in figures 23 and 24 have been illustrated to highlight differences and similarities. Unshaded peptides represent those which were common to all three viruses. Cross hatched peptides were evident only in the wild type Pichinde virus profile. Shaded peptides were common to wild type Munchique and RE-2 tryptic maps, but were not evident in the wild type Pichinde virus profile.

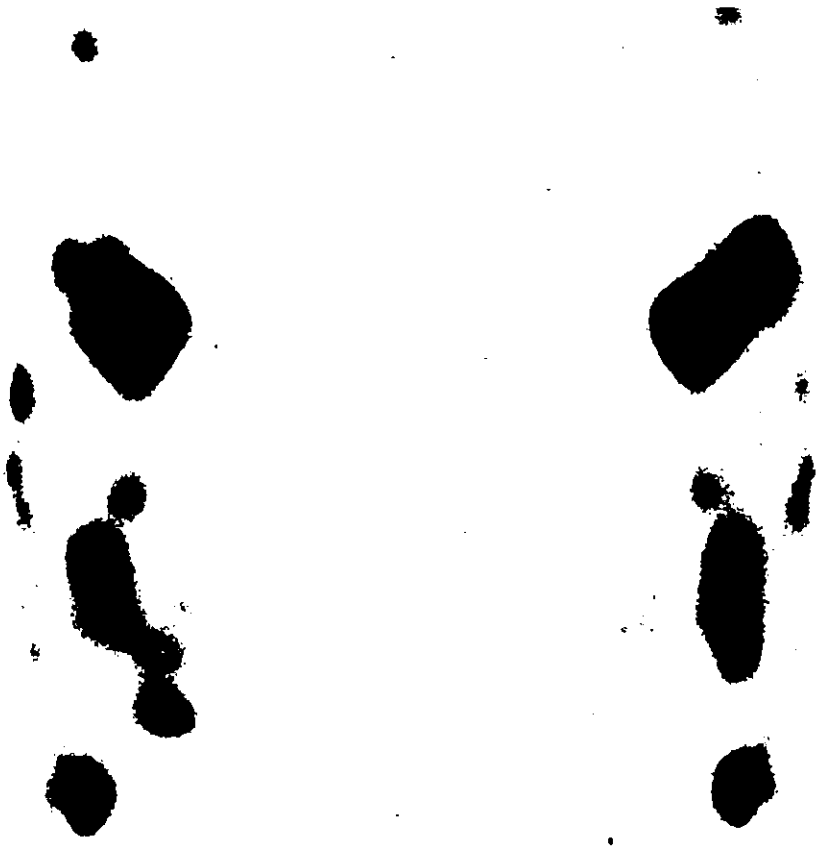
wt PICH
RE-2
wt MUNC

Figure 26

Comparison of wild type Pichinde and RE-2 cell associated glycoprotein (GPC). GPCs were radiolabeled, isolated, digested with trypsin, and resolved electrophoretically and chromatographically as described in figure 23.

wtPich

Re-2



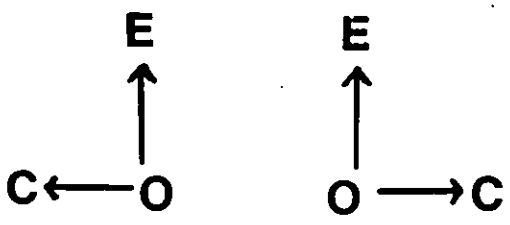


Figure 27

Comparison of wild type Pichinde and wild type Munchique cell associated glycoproteins (GPC). GPCs were radiolabeled, isolated, digested with trypsin, and resolved electrophoretically and chromatographically as described in figure 23.

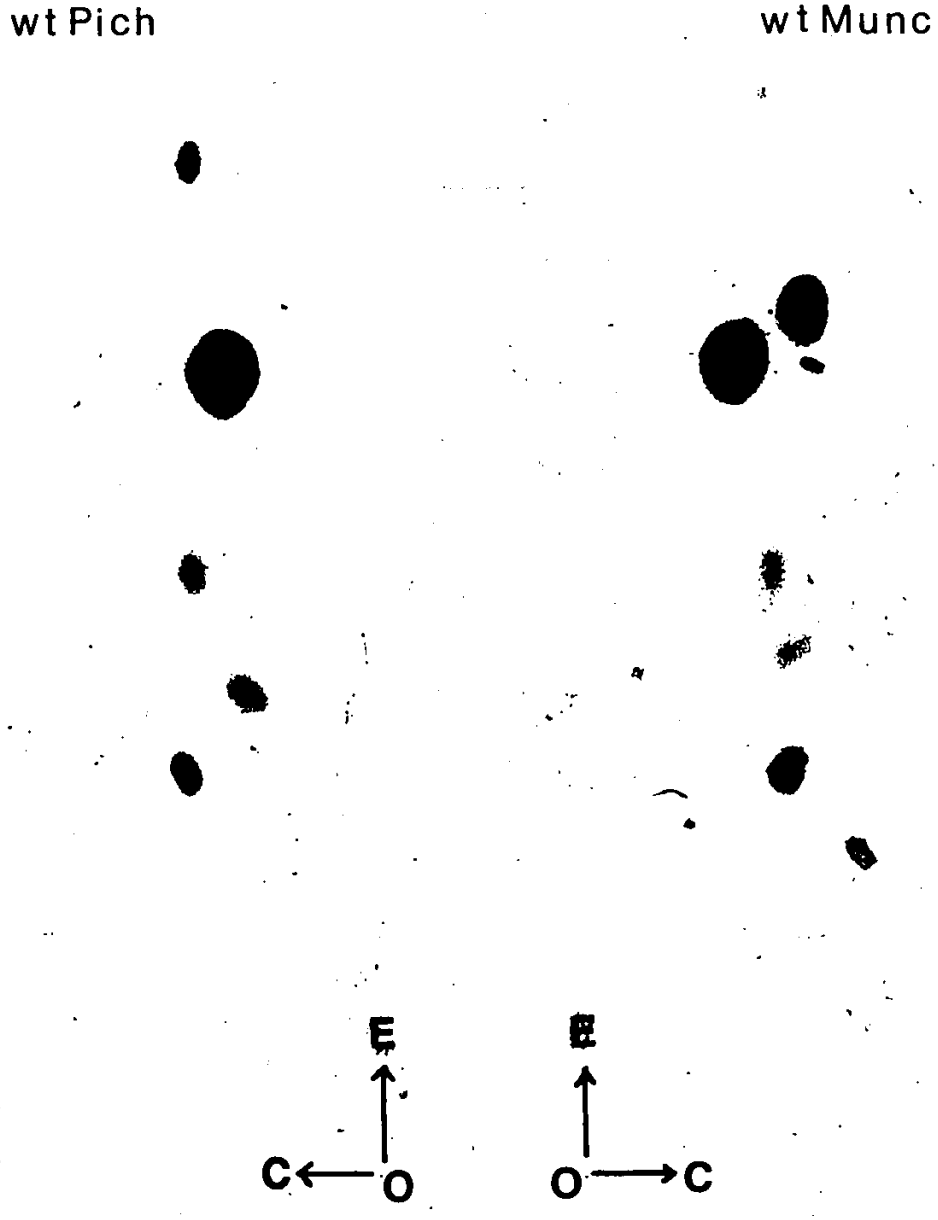

wt Pich
wt Munc
E
C
O
E
O
C

Figure 28

Schematic summary of GPC methionine-containing tryptic peptides in three viruses. Peptides evident in figures 26 and 27 have been illustrated to highlight differences and similarities. Unshaded peptides represent those which were common to all three viruses. Cross hatched peptides were evident only in the wild type Pichinde virus tryptic map. Shaded peptides were common to wild type Munchique and RE-2 tryptic maps of GPC but were very much reduced in the wild type Pichinde virus profile.

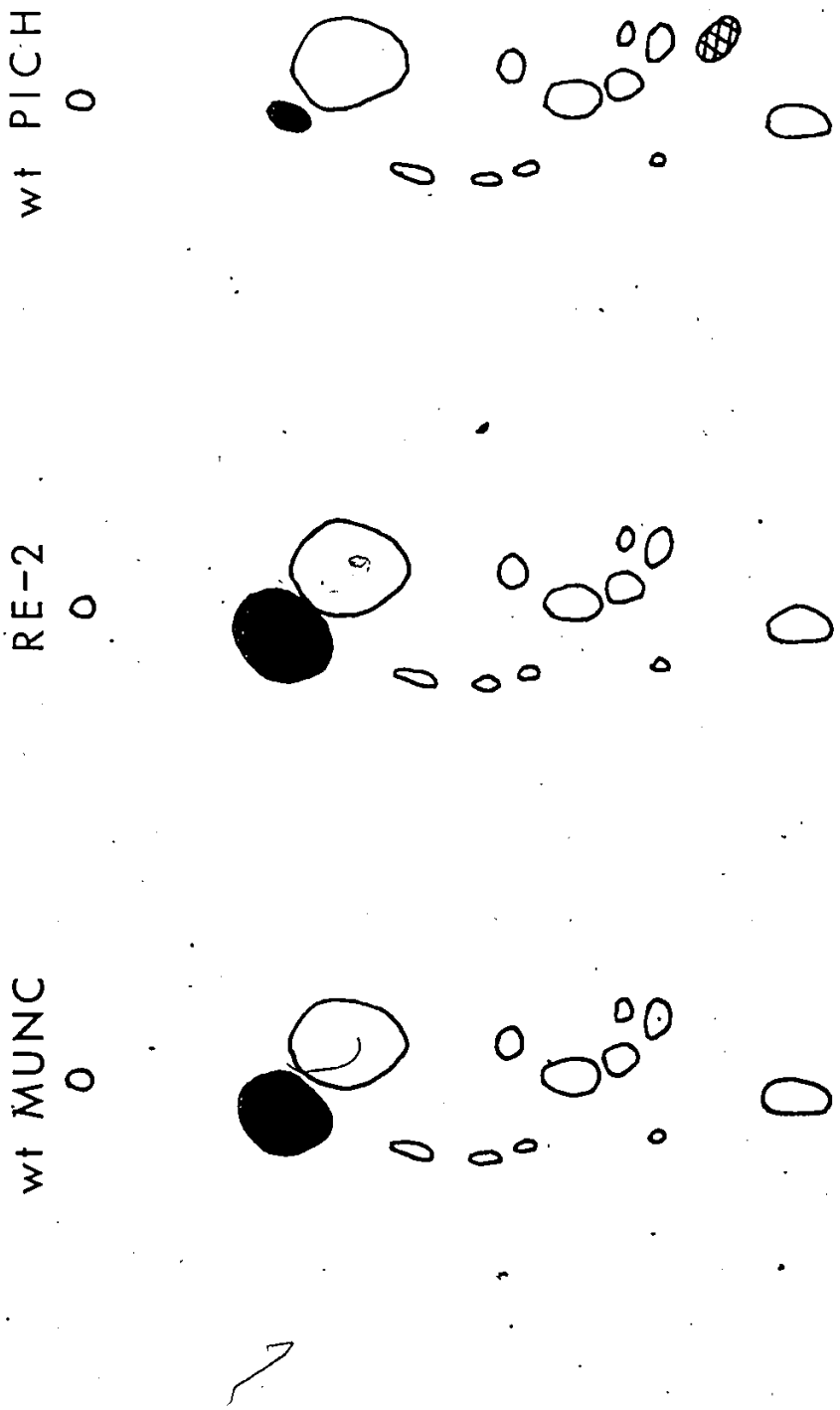

wt PICH
RE-2
wt MUNC

Figure 29

Comparison of wild type Pichinde and RE-2 L polypeptides. Polypeptides were radiolabeled, isolated, digested with trypsin, and resolved electrophoretically and chromatographically as described in figure 23. Approximately equal amounts of radiolabel were compared however exposures of each half of the chromatograph were adjusted.

wt PICH

RE-2

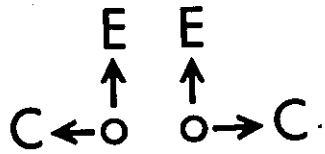
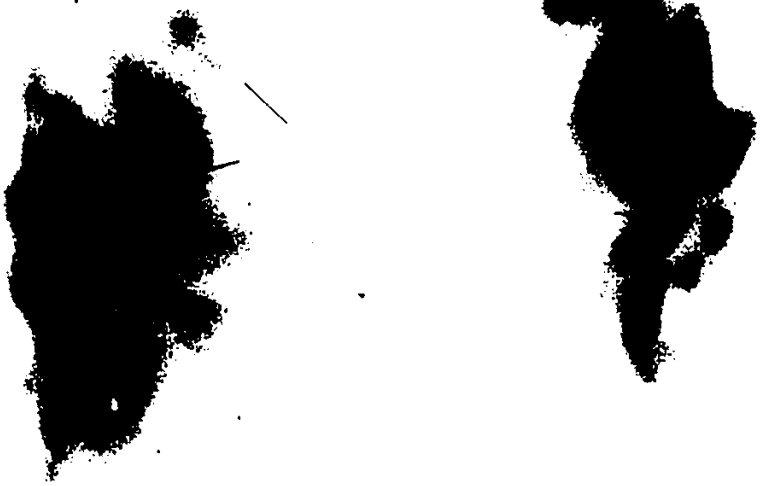


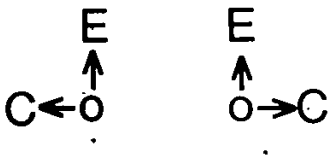
Figure 30

Comparison of wild type Munchique and RE-2 L polypeptides. Polypeptides were radiolabeled, isolated, digested with trypsin, and resolved electrophoretically and chromatographically as described in figure 23. Approximately equal amounts of radiolabel were compared however exposures of each half of the chromatograph were adjusted.

wt MUNC

RE-2





iate intensity and one minor peptide were different in wild type Munchique L polypeptide. One minor peptide was apparent in wild type Pichinde and RE-2 L polypeptide tryptic digests but not in wild type Munchique L polypeptide. The results have been schematically illustrated in figure 31.

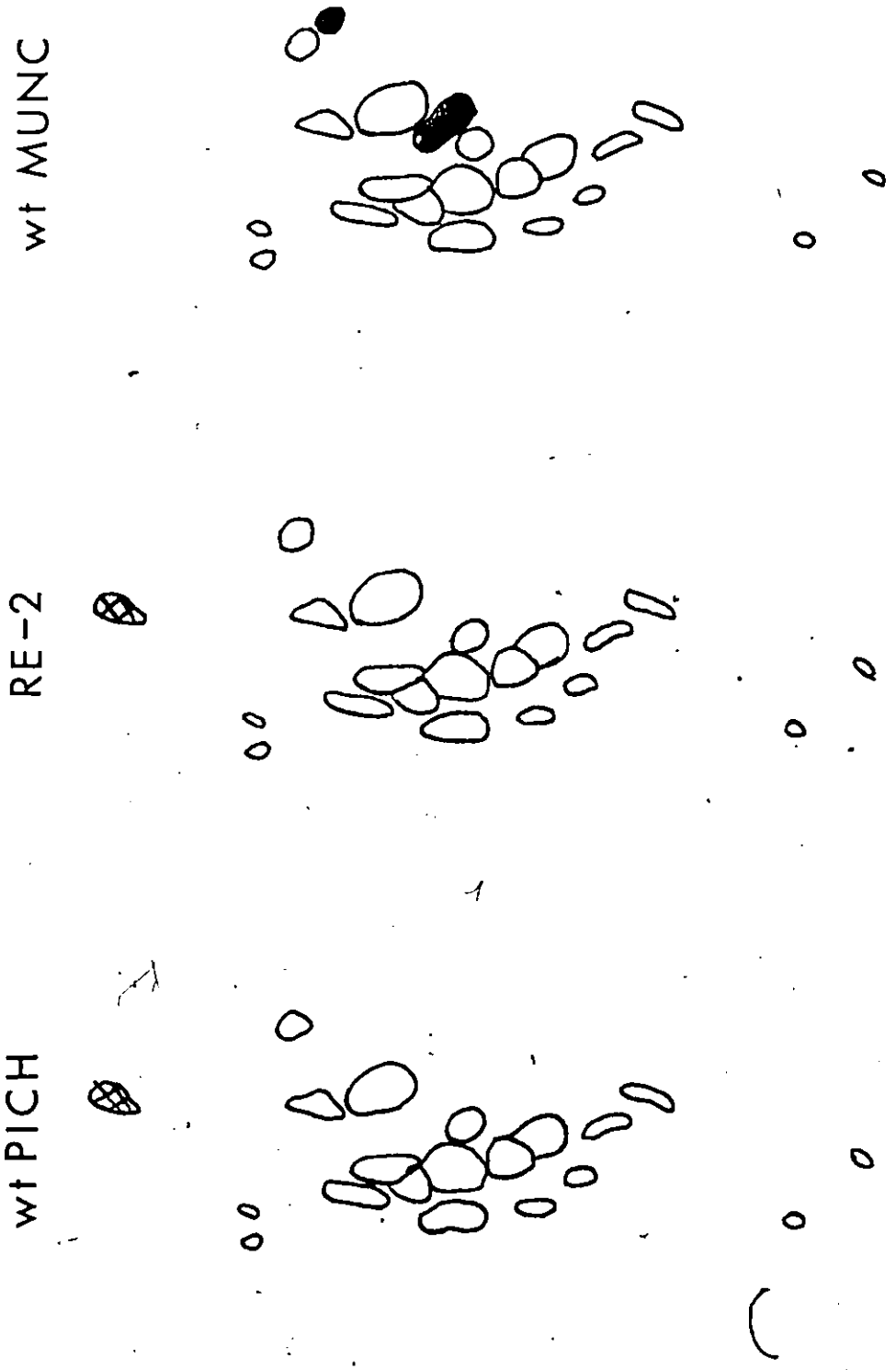
4.3 Discussion

A virus reassortant has been employed to demonstrate that NP and GPC were encoded by viral S RNA and L polypeptide was encoded by viral L RNA. The virus, which has been designated RE-2, contains the L RNA of wild type Pichinde virus and the S RNA of wild type Munchique virus, ad determined by oligonucleotide fingerprint analysis (Vezza et al., 1980).

The profile of immunoprecipitable polypeptides synthesized in BHK-21 cells suggested initially, on the basis of mobility in SDS-PAGE, that NP was encoded by S RNA. The data also confirmed that NP38 was a nucleoprotein-related molecule. Although we used immune sera derived from hamsters infected with our laboratory strain of Pichinde virus for this aspect of the study, we have also employed immune sera specific for each virus strain and identical results were obtained (data not shown). We did observe a correlation between the sera which was employed and the quantity of immunoprecipitable viral polypeptide. Attempts to confirm peptide mapping studies by immunological means (quantitation of each polypeptide with different sera concentrations) were unsuccessful due to prozone effects and the previously documented close relationship of the parental viruses (Vezza et al., 1980).

Figure 31

Schematic summary of L polypeptide methionine-containing tryptic peptides in three viruses. Peptides evident in figures 29 and 30 have been illustrated to highlight differences and similarities. Unshaded peptides represent those which were common to all three viruses. Cross hatched peptides were common only to wild type Pichinde and RE-2 tryptic maps. Shaded peptides were evident only in the wild type Munchique tryptic map.


wt MUNC
RE-2
wt PICH

Comparisons of the nucleoproteins of the parental viruses with the RE-2 virus by two dimensional L-[^{35}S]methionine-containing tryptic peptide mapping revealed that NP was encoded by S RNA. The same result has also been obtained by Vezza et al. (1980) using high pressure ion-exchange column chromatography to resolve tryptic peptides. In their analysis and in mine, two predominant peptides were different in wild type Pichinde nucleoprotein. We have chosen the two-dimensional resolution method for its simplicity, however mirror image comparisons were required to eliminate differences in peptide mobilities between experiments.

The mobilities of L polypeptide and GPC of the various viruses were not different in SDS-PAGE. pGPC was evident in both parental and RE-2 virus in the presence of tunicamycin, but the mobilities did not differ significantly (data not shown). It was necessary to derive gene assignments from comparisons of L-[^{35}S] methionine-containing tryptic peptides for each polypeptide. GPC was mapped to S RNA in this way. L polypeptide, on the other hand, was most similar in wild type Pichinde and RE-2 viruses. The latter comparisons were more difficult due to the low levels of these polypeptides immunoprecipitable from infected cells, however, the L polypeptide tryptic results have been confirmed once.

The gene assignments of L, GPC and NP are illustrated in figure 32. It is not known, at the present time, which viral polypeptide is encoded by 3' and 5' viral S RNA regions, nor whether L polypeptide is the only L RNA encoded molecule. The former question can be examined by N-terminal amino acid sequencing of NP and GPC since the nucleotide sequence of the 3'-end of viral S RNA is known

(Auperin et al., 1982). The latter question must await construction of cDNA clones representative of the entire genome (Chapter 6).

We have suggested that L polypeptide may represent the Pichinde virus transcriptase. If plaque size is in part determined by transcriptase efficiency then plaque size should map to L RNA. Vezza et al. (1980) have determined that plaque phenotype was determined by viral L RNA.

5 Replication of Pichinde virus and studies on regulation of viral replication

5.1 Introduction

Rawls and coworkers (1981) have proposed that viral persistence at the cellular level can perhaps be explained with two basic models. The first proposed that arenavirus were capable of autoregulation of replication and the second concept stated that defective interfering particles played a role in regulation by interfering with the replication of standard virus. Either event should occur during the establishment phase of persistence.

We have undertaken to examine the replication of Pichinde virus in BHK-21 and Vero cells, but have also employed MDCK cells which reportedly do not produce defective interfering Pichinde virus particles (Dutko and Pfau, 1978). To do so, monoclonal antibodies to Pichinde nucleoprotein was employed. Common features of Pichinde virus replication during acute infection of the three cell lines should permit construction of a model of arenavirus replication in the cell.

5.2 Results

5.2.1 Characterization of monoclonal antibody reactivity

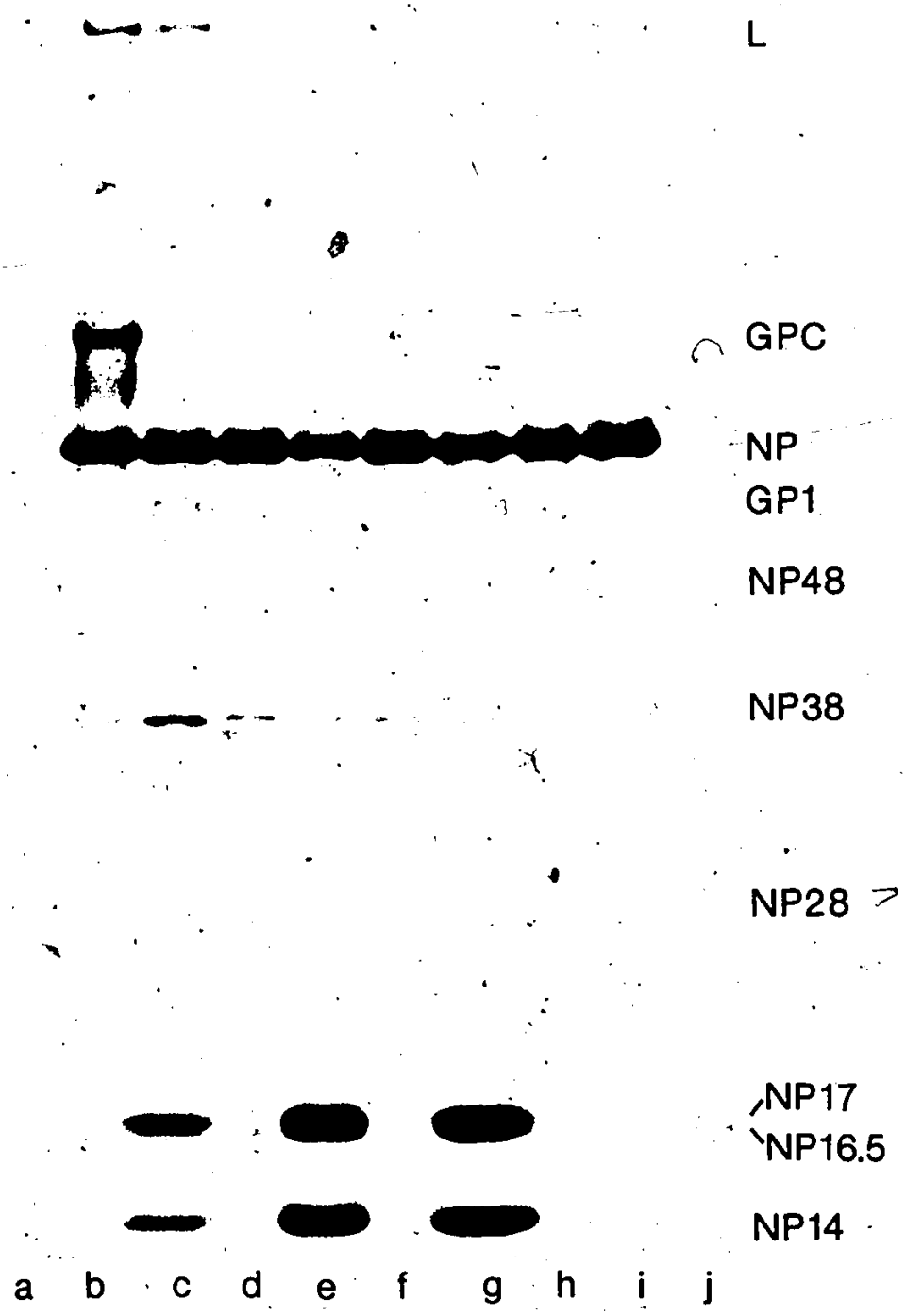
In order to assess the specificity of hybridoma antibody directed against Pichinde virus antigen, BHK-21 cells were infected at a MOI of 1 PFU per cell. Twenty-four hours after infection polypeptides were radiolabeled with L-[^{35}S]methionine for 1 hr, cells were harvested, and aliquots of the lysate were reacted with monoclones

PV1-1-3, PV2-14-19, PV2-14-21, PV3A-23 and PV3B-3. The immunoprecipitable polypeptide profile was also examined following a 6 hr chase of radiolabel in the presence of at least a one hundred fold excess unlabeled methionine. Polypeptide profiles were compared to those obtained when infected cell lysates were reacted with hamster-anti Pichinde virus immune serum.

After a 1 hr labeling period, hamster anti-Pichinde virus serum reacted with L, GPC, NP, NP48, NP38, and NP28 (Figure 32b). These polypeptides were not apparent in reactions of infected cell lysates with hybridoma antibody directed against lymphocyte choriomeningitis virus nucleoprotein, however a very small quantity of Pichinde virus nucleoprotein may have reacted with the heterologous antibody (Figure 32a). Following a 6 hr chase period GPC was processed to GP1 and GP2 (although GP1 is not clearly evident) and some NP was proteolytically cleaved to NP17, NP16.5 and NP14. L, NP, NP48, NP38 and NP28 were still apparent (Figure 32c). Monoclones PV2-14-19 and 2-14-21 reacted primarily with NP, NP48 and NP38 after a 1 hr pulse-label, however small quantities of L were also apparent in autoradiograms (Figure 32d and f respectively). Following a 6 hr chase period both monoclones recognized NP17.5, NP16, and NP14 as well as NP, NP48, NP38 and small quantities of L (Figure 32e and g respectively). Monoclone PV1-1-3 on the other hand, exhibited a somewhat different reactivity. Polypeptides which were apparent after a 1 hr pulse label (NP, NP48, NP38, NP28 and small quantities of L) were identical to those recognized following a 6 hr chase period. Notably, NP17.5, NP16, and NP14 were not recognized by monoclone PV1-1-3 (Figure 32h and i). None of the

Figure 32

Characterization of monoclonal antibody reactivity to Pichinde virus polypeptides. BHK-21 cells were infected with Pichinde virus at a MOI of 1. Twenty-four hours after infection, monolayers were pulse-labeled for 1 hr with 50 μCi per ml L-[^{35}S] methionine (a, b, d, f, h). Duplicate cultures were pulse-chased for 6 hrs before harvesting (c, e, g, i). Viral polypeptides were immunoprecipitated, as described in Materials and Methods, in the presence of hamster anti-Pichinde virus immune serum (b, c), monoclonal antibody to LCM ~~virus~~ nucleoprotein (a), monoclonal antibody PV2-14-19 (d, e), PV2-14-21 (f, g), and PV1-1-3 (h, i). Mock infected cells were treated with hamster immune serum after a 1 hr pulse-label (j). Polypeptides were resolved by SDS-PAGE (4.5 to 15 percent gradient polyacrylamide gels) and visualized by fluorography.


L
GPC
NP
GP1
NP48
NP38
NP28
NP17
NP16.5
NP14
a b c d e f g h i j

hybridoma antibodies reacted with the glycoprotein precursor (GPC) or its proteolytic derivatives (GP1 and GP2). The reactivities of PV3A-23 and PV3B-3 were identical to those of PV2-14-19 and PV1-1-3 respectively (data not shown) and have been summarized in Table 9.

These results suggested that the monoclonal antibodies were directed to antigenic sites of Pichinde virus nucleoprotein and related molecules. If this was the case immunoprecipitation of L should not have been expected. In order to determine the nature of the co-immunoprecipitation, infected cell lysates were reacted with monoclones PV2-14-19 and PV2-14-21 (in equal proportions in the same reaction) and antibody-antigen complexes were removed with protein A-sepharose CL-4B. The reaction was repeated five times in an attempt to remove all of the radiolabeled nucleoprotein. The final supernatant was reacted with two independently prepared hamster anti-Pichinde virus immune sera in an attempt to immunoprecipitate L in the absence of NP. Polypeptides were separated by SDS-PAGE, bands were excised, and the radiolabel in each polypeptide was determined.

Five serial immunoprecipitations with monoclonal antibody did not completely remove the nucleoprotein from infected cell lysates. L and NP were co-immunoprecipitated in each reaction, however, GPC was only apparent in lysate supernatants reacted with hamster anti-Pichinde virus immune sera. The results are summarized in Table 10. Although L and NP were not immunoprecipitated independently, the percent of the total radiolabel present in L remained approximately constant after the first immunoprecipitation.

Table 9

Polypeptides Recognized by Hybridoma Antibody

Hybridoma (monoclone)	Polypeptide Recognized[a] L	GPC	NP	NP48	NP38	NP28	NP17.5	NP16	NP14
PV2-14-19	*		*	*	*		*	*	*
PV2-14-21	*		*	*	*		*	*	*
PV1-1-3	*		*	*	*	*			
PV3A-23	*		*	*	*		*	*	*
PV3B-3	*		*	*	*	*			

[a] assessed by immunoprecipitation of infected cell lysates and visualization of polypeptides by fluorography after SDS-PAGE

Table 10

Serial Immunoprecipitation of Infected BHK-21 Cell Lysates

	monoclonal antibody PV2-14-19 + PV2-14-21[a]					8wk LSH	Bbd
Reaction Number[b]	1	2	3	4	5	6(a)	6(b)
Polypeptide							
L	93[c]	354	158	101	93	200	127
NP	4273	1921	1219	583	359	1215	1007
GPC						671	534
Percent of total (-GPC)	2	16	12	15	21	14	11

[a] monoclonal antibodies PV2-14-19 and PV2-14-21 were mixed in equal proportions based on reactivity

[b] the supernatant of each reaction was used in the next reaction. 6(a) and 6(b) are separate supernatants each of which was treated identically in reactions 1-5

[c] numbers represent means of two determinations and are expressed in counts per minute

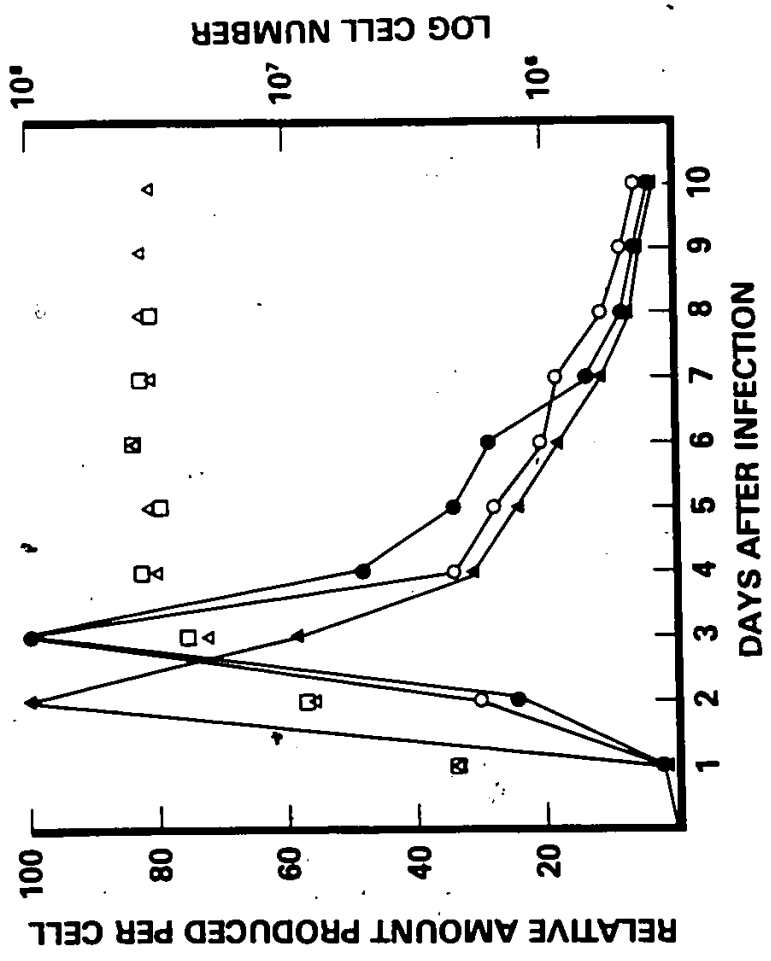
5.2.2 Pichinde virus growth in BHK-21 cells

The growth of Pichinde virus in BHK-21 cells was examined over a ten day period after infection of subconfluent cell monolayers with 0.1 PFU per cell. Viral macromolecules were labeled with either [5,6-^{3}H]uridine (Dimock et al., 1982) or L-[^{35}S]methionine, medium was changed to determine infectious virus titres by plaque assay on Vero cell monolayers, and virus was purified from the remainder by density gradient centrifugation. Infectious virus released into the medium increased rapidly to the maximum which was observed on day 3 after infection. A rapid decrease was observed on day 4 and a steady decline in virus titre occurred from day 4 to day 10 after infection (Figure 33). The release of viral particles containing ^{3}H-uridine labeled RNA closely paralleled the infectious virus release curve (Figure 33) as did the appearance of L-[^{35}S]methionine labeled virus (data not shown).

Incorporation of L-[^{35}S]methionine into immunoprecipitable polypeptides was measured after a 1 hr pulse-label of infected and mock infection cells over the course of the 10 days. Lysates were reacted with excess hamster anti-Pichinde serum to ensure complete removal of viral polypeptides. Duplicate reactions were performed in each case. An aliquot of immunoprecipitable material was counted for radioactivity to determine changes in total incorporation between successive days. The profile of immunoprecipitable polypeptides closely paralleled the other parameters measured, however the peak in incorporation of L-[^{35}S]methionine occurred one day earlier (day 2) (Figure 33). Also shown in the figure are cell numbers for both

Figure 33

Replication of Pichinde virus in BHK-21 cells. BHK-21 cells were infected with Pichinde virus at a MOI of 0.1. Release of infectious virus into the medium was monitored by plaque assay on Vero cell monolayers (●) and medium was changed daily. ^{3}H-uridine labeling of RNA and measurements of incorporation (o) were made by Dr. Ken Dimock (Dimock et al., 1982). L-[^{35}S]methionine incorporation (▲) was measured in immunoprecipitates (antibody excess) of infected cells incubated for 1 hr in radiolabel at the indicated times. Cell numbers of infected (Δ) and uninfected (□) cultures were determined daily.


LOG CELL NUMBER
10⁸
10⁷
10⁶
DAYS AFTER INFECTION
1 2 3 4 5 6 7 8 9 10
RELATIVE AMOUNT PRODUCED PER CELL
100 80 60 40 20

Pichinde virus infected and uninfected cells over the course of the experiment. Virus infection did not appear to alter the growth properties of BHK-21 cells as evidenced by the similarity of the growth curves.

Immunoprecipitable polypeptides were separated by SDS-PAGE and located by fluorography. Individual bands were excised, digested with H_2O_2 and counted for radioactivity. The two primary products of translation of mRNA derived from viral S RNA (NP and GPC), were synthesized on each day after infection in amounts which correlated with the release of a radiolabeled, infectious virus into the medium. The synthesis of L protein also followed a similar curve (Table 11).

5.2.3 Cell associated antigen detected by immunofluorescence

The preceding result suggested that by 10 days after infection only about 1 percent of BHK-21 cells continued to release infectious Pichinde virus. We attempted to correlate these results with measurements of percent of cells producing infectious centers in an infectious assay, and with the percent of cells which exhibited immunofluorescence in an indirect immunofluorescence assay employing hybridoma antibody (PV2-14-19).

Infectious center assays were performed by Dr. Ken Dimock. The peak in infectious centers coincided with the peak in infectious virus production on day 2 after infection, and a similar decrease in both assays was observed up to day 10 after infection. In contrast, the percent of cells which exhibited cytoplasmic immunofluorescence during this time period increased to a maximum on or about day 3 after infection and remained high throughout the course of the experiment (Table 12).

Table 11

Kinetics of Synthesis of Pichinde Virus Specific Polypeptides in BHK Cells

Day After Infection	Radiolabel in Polypeptides: L		GPC		NP	
	CPM in Polypeptide[a]	(Percent of Maximum)	CPM in Polypeptide	(Percent of Maximum)	CPM in Polypeptide	(Percent of Maximum)
1	464	5.9	456	3.5	670	0.5
2	5,176	65	6,557	50	103,075	73
3	7,924	100	13,102	100	136,706	97
4	4,834	61	6,566	49	141,215	100
5	3,906	49	5,679	43	118,991	84
6	6,775	85	8,026	61	89,437	63
7	1,929	24	3,432	26	46,757	33
8	1,389	18	2,631	20	30,650	22
9	2,300	29	5,545	42	22,058	16

[a] Polypeptides were immunoprecipitated and resolved by SDS-PAGE. Individual bands were excised and digested with H_2O_2, as described in Materials and Methods, before liquid scintillation counting.

Table 12

Infectious Center and Immunofluorescence Assay of Pichinde Virus Infected BHK-21 Cells

Day after infection	Relative amount of virus produced per cell	Percent of cells producing infectious centers[a]	Percent of cells immunofluorescent with hybridoma antibody PV2-14-19[b]
Mock	0	0	0
1	2	21	2.5
2	100	68	80
3	40	40	>90
4	30	39	>90
5	18	18	>90
6	3	7	>90
7	2	6	>90
8	1	3	>90
9	1	1	>90
10	1	1	>90

(a) assessed by Dr. K. Dimock

(b) at least 200 BHK-21 cells were examined on each day for cytoplasmic antigen

Since less than 16 percent of nucleoprotein synthesis during peak virus production occurred at day 7 after infection and later, but cytoplasmic immunofluorescence remained high during this time, it was of interest to characterize nucleoprotein antigen in more detail. Further characterization was based on differences in polypeptides immunoprecipitated with monoclones PV2-14-19 and PV1-1-3. Both hybridoma antibodies recognize antigenic determinants of the viral nucleoprotein, however the specific determinants recognized are different. PV2-14-19 reacts mainly with NP (64,000 daltons), NP17, NP16.5 and NP14. In order to assess the nature of cytoplasmic immunofluorescence we attempted to look for differences in infected cells with the two monoclonal antibodies. The high proportion of cells observed which were immunofluorescent at late times during infection may have been due to the three smaller proteolytic NP fragments rather than NP *per se*. If this were the case then infected cells reacted with PV1-1-3 should have had a much reduced fluorescence compared to cells treated with PV2-14-19.

The results are presented in figures 34 and 35. Mock infected cells did not fluoresce when treated with either monoclonal antibody and fluorescein-isothyocyanate conjugated goat anti-mouse immunoglobulin. The percent of cells which were positive for nucleoprotein antigen increased to a maximum on day 3 after infection. Immunofluorescence was most intense at this point and very large granular inclusions which filled the cytoplasm were easily apparent. Granular inclusions became much more pronounced at later time points but the antigen concentration appeared lower. The proportion of cells which

Figure 34

Cytoplasmic immunofluorescence with PV2-14-19 during acute infection of BHK-21 cells with Pichinde virus. Cells were infected with Pichinde virus at a MOI of 0.1. On days 1 to 10 after infection cells were fixed in acetone and treated with monoclonal antibody PV2-14-19. Immunofluorescence was assessed as described in Materials and Methods.

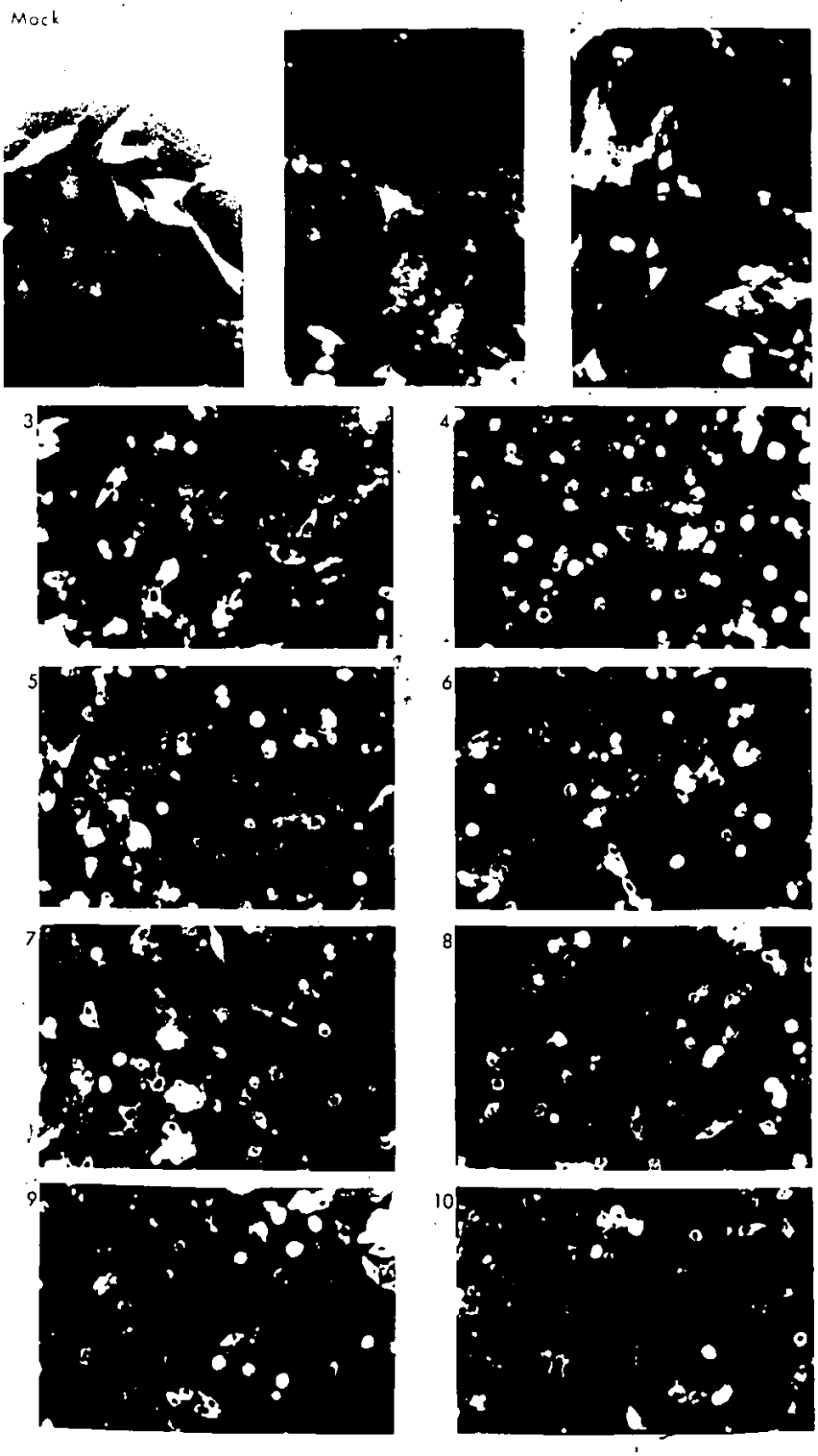
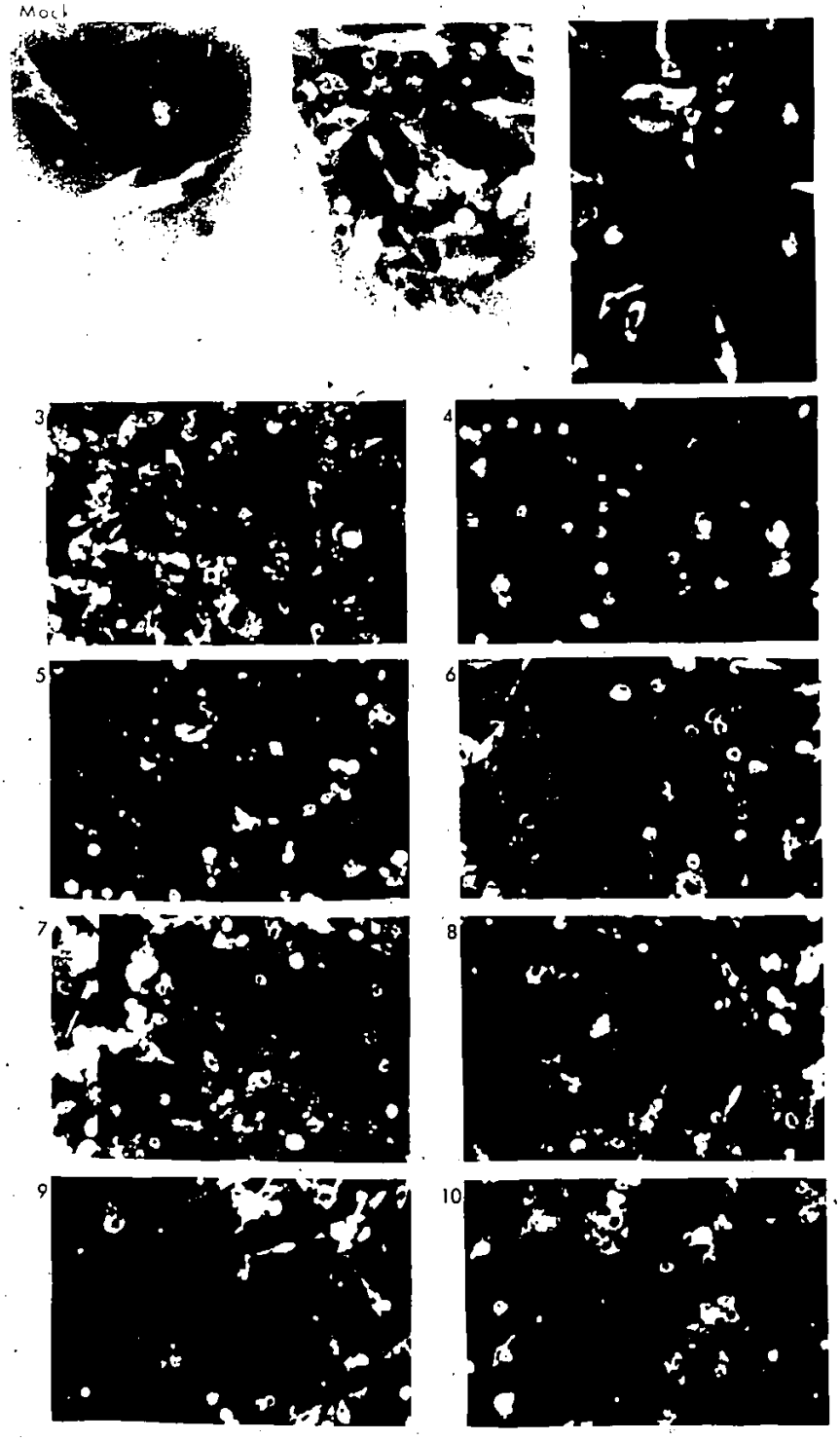

Mock
3
4
5
6
7
8
9
10

Figure 35

Cytoplasmic immunofluorescence with PV1-1-3 during acute infection of BHK-21 cells with Pichinde virus. Cells were infected with Pichinde virus at a MOI of 0.1. On days 1 to 10 after infection cells were fixed in acetone and treated with monoclonal antibody PV1-1-3. Immunofluorescence was assessed as described in Materials and Methods.


Mock
3
4
5
6
7
8
9
10

were antigen positive did not change. There were no significant differences in immunofluorescence observed between monoclonal antibodies (PV2-14-19 versus PV1-1-3).

Since differences in fluorescence were not readily apparent with the monoclonal antibodies employed we attempted to determine the kinetics of loss of NP, NP17, NP16.5 and NP14 from infected cells. BHK-21 cells were infected with Pichinde virus at a MOI of 0.1 PFU per cell. Seventy-two hours after infection monolayers were incubated for 1 hr in methionine free medium containing 50 µCi per ml L-[^{35}S]methionine. One set of cultures was harvested and polypeptides were immunoprecipitated immediately with PV1-1-3 and PV2-14-19, under conditions of antibody excess. The remaining cultures were harvested at various intervals over the next seven days. Differences in amount of radioactivity immunoprecipitated with PV1-1-3 and PV2-14-19 represent radiolabel associated with NP17, NP16.5 and NP14.

Initially PV1-1-3 and PV2-14-19 precipitated approximately equal amounts of radioactivity (day 0 after radiolabeling) (Table 13). After 24 hrs only about 10 percent of the initial radiolabel reacted with PV1-1-3, however, 40 percent of the initial radiolabel was still available to react with PV2-14-19. The difference in counts per minute immunoprecipitated by the two monoclones represents NP17, NP16.5 and NP14 (final column of Table 13). The identity of NP and it's derivatives at all time points was confirmed by SDS-PAGE. Radiolabel immunoprecipitated with the monoclones remained approximately constant until day 3 after radiolabelling (day 6 after infection). The amount of radiolabel in NP17, NP16.5, and NP14 decreased by approximately 50 percent at each of the next two time points

Table 13

Half Life Determinations for Pichinde Virus

Nucleoprotein and Related Molecules

Days after radiolabel[a]	Days after infection[b]	L-[^{35}S]methionine[c] PV1-1-13	PV2-14-19	(PV2-14-19) -(PV1-1-3)
0	3	4210±447	3706±949	
1	4	444±101	1616±9	1172
2	5	581±18	1784±161	1203
3	6	310±33	1312±70	1002
4	7	N.D.	N.D.	
5[d]	8	132±28	645±29	513
6	9	N.D.	N.D.	
7	10	121±23	358±25	237

[a] Infected BHK-21 cells were incubated for 1 hr in methionine free medium containing 50 µCi per ml L-[^{35}S]methionine.

[b] BHK-21 cells were infected with Pichinde virus at a MOI of 0.1 PFU.

[c] Infected cell lysates were incubated with the appropriate monoclonal antibody and protein A-sepharose Cl-4B for 2 hrs at 4°C. Beads were collected, washed, and an aliquot of released immune complex was counted for radioactivity. Numbers represent mean and Standard Error of the Mean for 3 determinations in antibody excess and have been corrected for background from mocks.

[d] Cell monolayers were confluent at this point.

examined (days 5 and 7 after radiolabeling). Cell monolayers attained confluency at approximately day 5 after radiolabeling.

5.2.4 Comparison of Pichinde virus replication in BHK-21, Vero, and MDCK cell lines

5.2.4.1 Virus growth during a 6 day period

The replication of Pichinde virus in BHK-21 cells was compared to replication in Vero and MDCK cells, in order to determine whether regulation (shut-off) of viral synthesis was unique to BHK-21 cells or not. The three cell lines were seeded into 75 cm^2 Corning tissue culture flasks and cell number and release of infectious virus into the medium was monitored for 6 days following infection of cells at a MOI of 1 PFU per cell. Dulbecco-modified Eagle Medium was changed daily and aliquots were stored at -70°C before assay of infectivity by plaque formation on Vero cell monolayers.

Growth of BHK-21, Vero, and MDCK cells was approximately logarithmic during the first 5 days of the experiment for which cell numbers were obtained (Figure 36). Pichinde virus released from BHK-21 cells was very high on day 2 after infection. At day 5 after infection, infectious virus released per cell was only 16 percent of levels on day 2 (Table 14). These results agree with previous experiments (Sections 5.2.2 and 5.2.3).

Similar results were apparent when Vero and MDCK cells were infected with Pichinde virus. PFU per cell was at a maximum on day 1 to day 2 for Vero cells and on day 2 for MDCK cells. At day 5 after infection PFU released per Vero cell was only 0.5 percent of peak levels, and in the case of MDCK cells approximately 8 percent of PFU

Figure 36

Growth of BHK-21, Vero, and MDCK cell monolayers. Cell lines were seeded into 75 cm^2 tissue culture flasks (Corning) at low cell density. Medium was changed daily and cell numbers were determined in duplicate, for each cell line, at the indicated points.

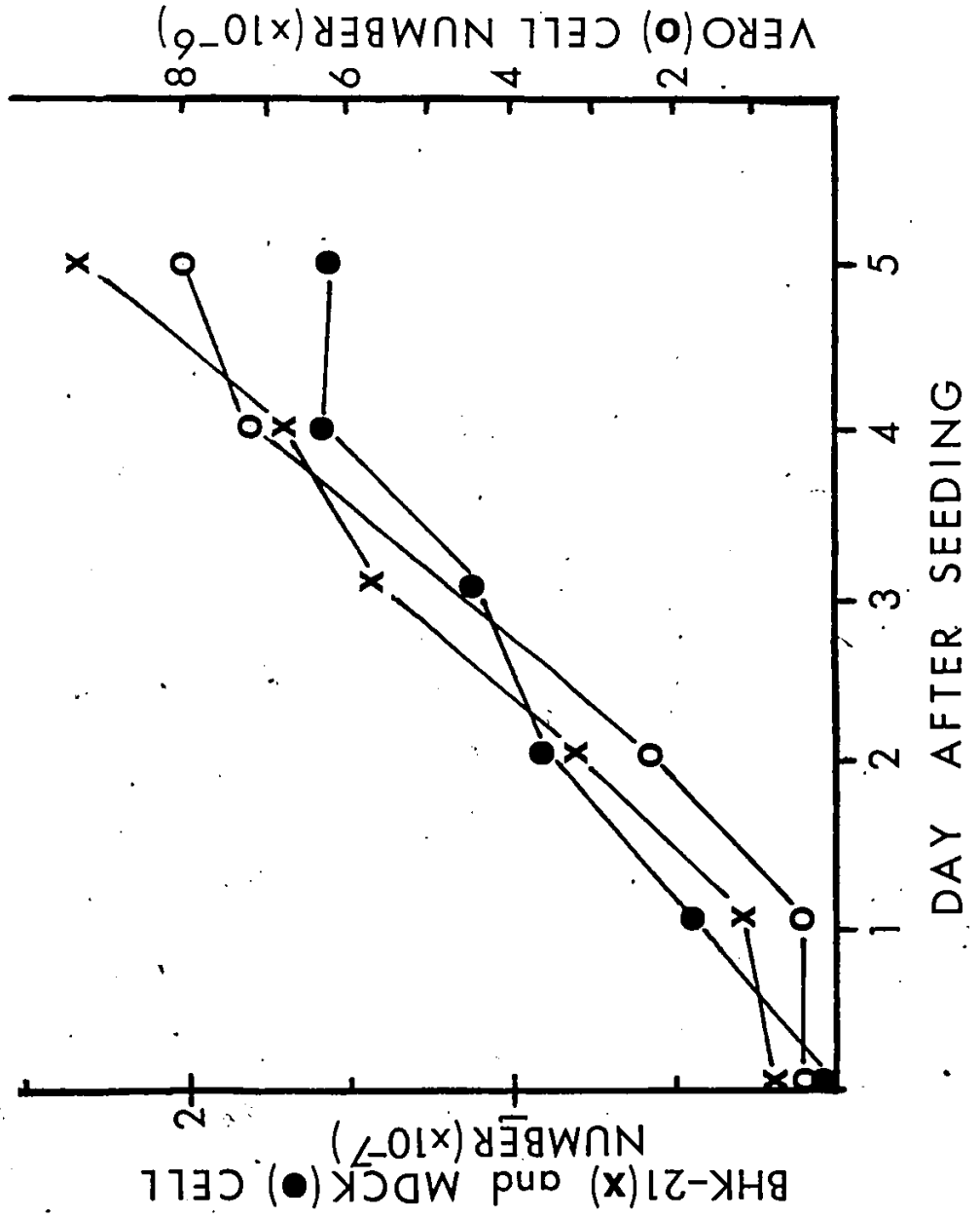

VERO(o) CELL NUMBER(x10⁻⁶)
2
4
6
8
DAY AFTER SEEDING
1
2
3
4
5
BHK-21(x) and MDCK(●) CELL NUMBER(x10⁻⁷)
2

Table 14

Growth of Pichinde Virus in BHK-21, Vero and MDCK Cell Monolayers

Day after infection[a]	BHK-21 PFU per ml[b] ($\times 10^{-7}$)	BHK-21 PFU per cell	Vero PFU per ml ($\times 10^{-5}$)	Vero PFU per cell	MDCK PFU per ml ($\times 10^{-4}$)	MDCK PFU per cell ($\times 10^{-3}$)
1	N.D.	N.D.	0.7	0.2	0.09	0.2
2	13.9	17.8	3.9	0.2	6.6	7
3	10.3	7.2	0.5	0.03	3.6	3
4	6.7	3.9	0.4	0.005	1.3	0.8
5	6.7	2.8	0.1	0.001	1.0	0.6
6	1.7	N.D.	0.07	N.D.	0.7	N.D.

[a] Cell monolayers were infected with Pichinde virus (SG-1) at an MOI of 0.1 PFU per cell.

[b] Infectivity was assayed on Vero cell monolayers as described (Section 2.3).

per cell peak levels was obtained (Table 14). The results also seem to indicate large differences in the three cell lines. Peak levels of PFU released per Vero cell were only 1 percent of levels released from BHK-21 cells and MDCK cells released only about 4 percent of Vero cell levels, on a PFU per cell basis.

5.2.4.2 Synthesis of viral polypeptides in three cell lines

To look for potential polypeptide candidates as regulatory molecules in Pichinde virus replication, we examined polypeptide synthesis using the standard immunoprecipitation protocol. BHK-21, Vero, and MDCK cells were infected with Pichinde virus at a MOI of 1. Polypeptides were radiolabeled at 24 hrs after infection for 1 hr in methionine free medium, containing 50 µCi per ml L-[^{35}S]methionine. Duplicate cultures were pulse-chased for 6 hrs. Immunoprecipitable polypeptides were identical in number in BHK-21 and Vero cells. Increased proteolysis (or premature termination of translation) of the nucleoprotein occurred in Vero cells. Much higher quantities of NP38 and NP28 were evident (Figure 37). In some experiments the quantity of NP38 and NP28 were even higher (data not shown). After a six hour pulse-chase NP17, NP16.5 and NP14 were all present in both Vero and BHK-21 cells, although smaller quantities were present in Vero cells. MDCK cells could not be examined due to insufficient quantities of radiolabelled polypeptides.

It was also noticed that loss of radiolabel from infected cells after a 6 hr pulse-chase was more efficient in BHK-21 cells than Vero cells. In three separate experiments 80 percent of radiolabel in BHK-21 cells was chaseable (Table 15). On the other hand,

Figure 37

Identification of immunoprecipitable polypeptides in Vero and BHK-21 cell monolayers. Cells were infected with Pichinde virus at a MOI of 1 PFU per cell. Twenty-four hours after infection monolayers were incubated for 1 hr in methionine free medium containing 50 µCi per ml L-[^{35}S]methionine. One set of cultures (pulse) was harvested at this point and a second set was harvested following a 6 hr chase period in at least 100 fold excess unlabelled methionine. Polypeptides were immunoprecipitated, resolved by SDS-PAGE, and visualized by fluorography. Polypeptide designations are as in Chapter 3.

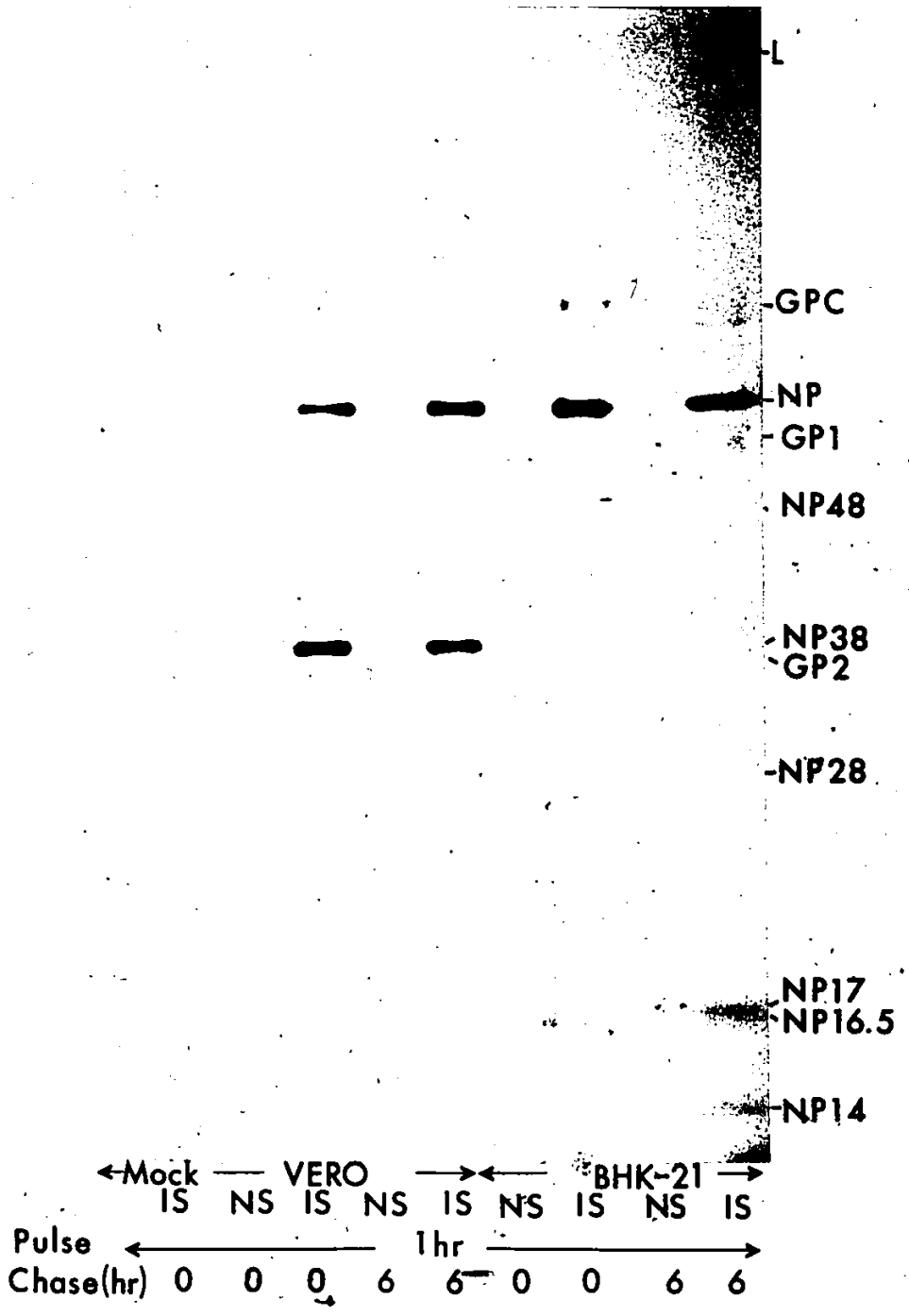

L
GPC
NP
GP1
NP48
NP38
GP2
NP28
NP17
NP16.5
NP14
←Mock — VERO →←— BHK-21 →
IS NS IS NS IS NS IS NS IS
Pulse ← 1hr →
Chase(hr) 0 0 0 6 6 0 0 6 6

Table 15

Synthesis of Viral Polypeptides in BHK-21, Vero, and MDCK Cells

Cell line	MOI	L-[^{35}S]methionine (cpm) Pulse[(a)]	Chase[(b)] (percent of Pulse)
BHK-21	10	5486	1214 (22)
	1	2719	644 (23)
	1	2369	582 (24)
Vero	10	6860	3402 (49)
	1	2375	2137 (90)
	1	2133	2110 (98)
MDCK	10	544	N.D.[(c)]
	1	N.D.	N.D.
	1	N.D.	N.D.

(a) Monolayers were incubated for 1 hr in methionine free medium containing 50 µCi per ml L-[^{35}S]methionine.

(b) Following a 1 hr pulse-label monolayers were incubated in maintenance medium for 6 hrs.

(c) Levels of radiolabel were too low for accurate assessment.

only 10 to 50 percent of radiolabel chased in 6 hr in Vero cells. There was close agreement between values obtained at lower MOI in Vero cells.

5.3 Discussion

The results presented in this chapter provided a very preliminary examination of Pichinde virus replication in cells grown in vitro. Features of viral replication in BHK-21 cells have been more extensively examined (Dimock et al, 1982). The main objective of such studies was to provide the necessary groundwork for investigations designed to determine the molecular events involved in viral growth. It was clear, however, that such investigations could be more appropriately performed with tools not yet fully developed (Chapter 6).

Infection of subconfluent monolayers of BHK-21 cells with a low MOI (0.1 PFU per cell) of Pichinde virus resulted in a rapid increase in the synthesis of immunoprecipitable polypeptides which exhibited a peak 2 days after infection. This was followed, approximately 24 hrs later, by a peak in the release of infectious virus into the medium. Both parameters declined to 10 percent of peak levels by days 8 to 10 after infection. The decline was observed for all three primary viral gene products (L, GPC, NP). The percent of cells producing infectious centers exhibited a similar peak and decline (Dimock et al, 1982) and suggested that virus production was reduced as a consequence of complete shutoff in most of the cells.

Replication of mock-infected and Pichinde virus-infected cells over the course of these experiments appeared similar and did not appear to be responsible for the decline in virus production. The same phenomenon has been observed whether cells were in log phase or stationary phase growth (Dimock et al, 1982).

In contrast to the declines observed in polypeptide synthesis and infectious virus production, the proportion of cells which remained positive for cytoplasmic immunofluorescence remained extremely high (greater than 90 percent of the cells). It was thought that immunofluorescence was not indicative of new polypeptide synthesis. Two monoclonal antibodies were employed to attempt to assess this. PV2-14-19 recognized antigenic sites on NP, NP48, NP38, NP17, NP16.5, and NP14. PV1-1-3 recognized antigenic determinants on NP, NP48, NP38, and NP28. The high levels of NP17, NP16.5, and NP14 in infected cells (Chapter 3) may have produced a strong immunofluorescence pattern in the absence of new NP synthesis, however, the two monoclones reacted equally well with infected cells at early and late stages of viral replication. One might have expected a reduced immunofluorescence intensity with PV1-1-3 if qualitative differences were dramatic.

The observations were extended by examining the time required for radiolabeled polypeptides to either be released from cells or degraded. This type of investigation was complicated by uncertainties in the absolute initial levels of NP and NP17, NP16.5, NP14, however the half life of NP was less than twenty-four hours. Significant quantities of NP17, NP16.5, and NP14 remained in cells

even at 5 days after radiolabelling. The levels at day 7 after radiolabelling were approximately 20 percent of those observed 1 day after radiolabelling. It might be argued then, that immunofluorescence is a crude measure of the small proteolytic products, which are not packaged into virus (Chapter 3), and low levels of nucleoprotein synthesis which maintain the presence of NP17, NP16.5 and NP14 in the cell, but are insufficient for release of infectious virus at high levels. Although infectious centers exhibited a decline coincident with virus production the results cannot be interpreted to mean that polypeptide synthesis was shut down completely in cells.

It should be noted at this point, that all monoclones tested reacted with NP and its related molecules, but L polypeptide was also apparent in autoradiograms. The small quantities of L polypeptide in infected cells probably cannot account for the lack of differences observed in immunofluorescence when PV1-1-3 and PV2-14-19 were compared. It was speculated that L was complexed with NP and was therefore not recognized directly by monoclonal antibody. The approximately constant proportion of L to NP observed after the first immunoprecipitation would support this suggestion, however, it must be argued that monoclonal antibody preferentially reacts with free NP to explain the very low initial ratio.

Dimock et al, (1982) also found that the levels of Pichinde virus L and S RNAs changed during acute infection in a manner which

paralleled the other parameters measured. RNAs were quantitated by blot hybridization employing cDNA probes to individual RNA species. Other arenavirus infections display similar infectious properties and immunofluorescence patterns to those detailed here (reviewed by Rawls and Leung, 1979; Pedersen, 1979; Howard and Simpson, 1980; Buchmeier et al, 1980b).

Preliminary investigations also showed that the shut-off phenomenon was not unique to Pichinde virus infection of BHK-21 cells. Identical increases and declines in viral production were observed in both Vero and MDCK cells, although the levels of infectious virus released into the medium were lower in the latter two cell lines. MDCK cells may be of particular interest because this cell line reportedly does not produce defective particles and may abrogate a role for such particles in the regulated growth cycle of acute infection (Dutko and Pfau, 1978).

It was interesting that the level of synthesis of viral polypeptides in BHK-21 and Vero cells was approximately the same during a 1 hr pulse-label with L-(^{35}S) methionine, when the release of infectious virus was much lower in the latter case. The higher proportion of NP28 in Vero cells may, in part, account for the observed lower levels of virus which mature. The two cell lines appeared also to exhibit differences in the chaseability of the initial radiolabel. It was not clear whether the differences were significant, however the host cell can play a role in maturation after initial polypeptide synthesis.

Observations concerning polypeptides synthesized in MDCK cells could not be made due to the low detectable levels of synthesis in these cells. In part, this may reflect a lower proportion of infected cells, than in the case of BHK-21 and Vero cells. Adsorption efficiency could be increased with DEAE-dextran (Carter, 1972; Dutko and Pfau, 1978), however, at this point, these experiments are not of critical interest.

In summary, Pichinde virus production appeared to be regulated in a manner which was independent of host cell growth cycles. The parameters which have been examined, briefly here and in more detail by Dimock and coworkers (1982), include polypeptide synthesis, genomic RNA replication, and infectious virus production. Quantitation at the level of RNA complementary to L and S genomic RNAs and mRNAs will require development of more sophisticated probes for these molecules (Chapter 6). Although BHK-21 cells have been best characterized, initial indications were that a similar phenomenon occured in Vero and MDCK cells.

It might be argued that the decline in viral production which was observed occured as a consequence of the generation of defective interfering (DI) particles prior to the peak of infectious virus production, and was not due to a proposed autoregulatory effect by viral gene products (Chapter 6). Such particles have been observed for other arenavirus infections (Welsh and Pfau, 1972; Buchmeier et al, 1980b). DI particles do not appear to play a role in the replicative cycle in BHK-21 cells. Although present in doubly plaque purified viral stocks, they did not increase over the course of the

experiments, nor did the ratio of infectious virus to radioactive virus decrease at later times during acute infection (Dimock et al, 1982).

New RNA species have been described in preparations of Pichinde virus DI particles (Dutko et al, 1976). however, evidence for new RNA species containing L and S viral RNA sequences, in cells and in virus, could not be obtained by Dimock et al, (1982). This suggests that at least in the case of acute infection of BHK-21 cells by Pichinde virus, DI particles do not play a significant role. It is clear that such particles are generated in many arenavirus systems (see section 1.7) but their role in both in vivo and in vitro infections remains uncertain.

A model for autoregulation of Pichinde virus infection is presented in Chapter 6. The model is based on the apparent decline in viral synthesis independent of host cell growth and DI particle generation (this thesis and Dimock et al, 1982). Although the results presented here are very preliminary they do permit construction of a model which can be tested with the appropriate tools.

6 Summary, Suggestions for Further Investigation and Model for the Regulation of Viral Replication

The present study has characterized viral polypeptides synthesized after Pichinde virus infection of BHK-21 cells. Three primary products of mRNA translation were identified. A cell associated glycoprotein precursor (GPC) was synthesized initially as a 42,000 dalton molecule (pGPC) in the presence of tunicamycin. The nucleoprotein (64,000 daltons) and pGPC were shown to be encoded by viral S RNA. L protein was synthesized in low quantities in infected cells but was shown to be encoded by viral L RNA. The function of L is not known at the present time although it has been suggested that viral transcriptase activity resides in this molecule. The three molecules account for most of the theoretical coding capacity of the viral genetic information, however, nonimmunogenic polypeptides may have escaped detection. It is therefore necessary to extend the present study by employing techniques not dependent on anti-viral antibodies.

Recent advances in genetic engineering have provided the tools necessary to construct recombinant DNA molecules. We have initiated studies with the objective of obtaining cloned DNA fragments representative of the entire Pichinde virus genome. In collaboration with Dr. Ken Dimock and Dr. Susana Mersich, reverse transcriptase was employed to synthesize DNA complementary to viral RNA. Synthesis

was primed with an eight nucleotide long DNA fragment which was complementary to the 3'-end of viral L and S RNAs (Auperin et al., 1981; Dimock et al., 1982)

$$5' \; G(^{A}_{T})GGATCC \; 3'$$

Complementary second strand DNA was then synthesized with reverse transcriptase, double stranded cDNA was trimmed with S1 nuclease, and Bam H1 linkers were employed to ligate sequences into the Bam H1 site of pBR322. Twenty-two clones containing sequences representative of S RNA were obtained but all inserts were less than 180 nucleotides in length. A number of recombinant molecules remain to be classified.

The average size of cDNA synthesized under the conditions employed (Dimock et al., 1981) was 550 nucleotides. We have been able to increase the average size to 1750 nucleotides in the presence of sodium pyrophosphate but have not yet inserted these molecules into plasmid. Since it is imperative to obtain recombinant molecules representative of the entire Pichinde virus genome we have recently adopted several concerted approaches. Existing clones can be used as primer to extend cDNA synthesis in the presence of reverse transcriptase, although this approach is likely to be tedious if only small inserts are consistently obtained. Alternatively, existing clones can be employed to select mRNA from virus infected cells to be used as template. We also wish to attempt insertion of a cDNA-RNA hybrid directly into pBR 322 as has been successfully done with

Polio virus genetic information (van der Werf et al., 1981).

DNA copies of Pichinde virus genetic information will be employed to select complementary mRNA sequences. mRNA will then be translated in the rabbit reticulocyte cell free protein synthesizing system and the products will be correlated with known protein information. This approach requires DNA copies of the entire Pichinde virus genome in order to conclude that nonimmunogenic polypeptides were not overlooked during experiments described in Chapter 3.

The techniques for identifying the products of specific mRNA species were originally devised as hybrid arrest of translation (Patterson et al., 1977; Hastie and Held, 1978) and later were modified to select only the mRNA of interest by molecular seive chromatography of DNA-mRNA hybrids (Woolford and Rosbach, 1979). We intend to employ another approach. Cloned DNA will be denatured and bound to nitrocellulose filters before hybridization with total infected cell mRNA. Specifically bound mRNA will be eluted and translated in vitro (Ricciardi et al., 1979). Translation of L, NP, and pGPC mRNA should be possible, however, the identity of the protein products must be confirmed either by peptide mapping or 2 two-dimensional gel electrophoresis (O'Farrell, 1975). This is particularly important for any new polypeptides which are identified and for pGPC. pGPC has an estimated molecular weight of 42,000 and can potentially be confused with, or occluded by a 42,000 dalton reticulocyte molecule which is radiolabelled after micrococcal nuclease pretreatment of lysates (Pelham, 1980).

The results presented in Chapter 3 permitted identification of a viral polypeptide which we have designated L. Although it was suggested that this molecule may represent the viral transcriptase there is no direct evidence for this. Several approaches can be employed to determine the validity of this suggestion. The most obvious is purification of L and assessment of enzymatic activity using previously established assay systems (Leung et al., 1979). Since this molecule was present in virus and infected cells in only very low quantities this approach would be facilitated with the use of monoclonal antibody if these become available in the near future.

Although unambiguous proof requires polypeptide purification, there is already evidence which suggests that polymerase activity resides in a L RNA encoded molecule, if not in L protein itself. H. Caussey (personal communication) has demonstrated that temperature sensitive mutants of Pichinde virus (obtained from Dr. D.H.L. Bishop, University of Alabama) which have been assigned to two complementation groups (Vezza et al., 1977), can be divided into protein synthesis positive and negative phenotypes. Protein negative mutants corresponded to Pichinde virus group II temperature sensitive mutants, which carry a lesion in a product of L RNA (Vezza et al., 1980).

On the basis of the viral protein information presented in this thesis it is possible to construct a working model of Pichinde virus replication, with reference to the control of events at the level of transcription. Clearly, most of the elements of such a model remain to be tested, but at the present time the tools required to ask specific questions are in the process of development. Elements

of the model can be summarized as follows:

(1) L polypeptide and NP are both required to transcribe the viral genome;

(2) NP has sites of interaction with other polypeptides. These are (a) site of binding or interaction with L polypeptide, (b) two sites of interaction with other NP molecules, and (c) site of interaction with RNA;

(3) NP concentration in the cell determines accessibility of RNA to polymerase activity;

(4) Binding of nucleoprotein to RNA facilitates binding of subsequent molecules of NP to RNA. The first molecule bound may recognize a specific site or structure such as potential hairpin loops at termini;

(5) NP degradation products retain the L polypeptide recognition site but do not bind to RNA.

Studies described in Chapter 5 and by Dimock and coworkers (1982) suggested that Pichinde virus replication was regulated, such that synthesis of viral macromolecules and release of infectious virus into the medium exhibited a peak, which was followed by a decline to 10% of peak levels. In terms of a model, the first consequence of infection is a reduction in the effective concentration of nucleoprotein such that viral RNA is potentially exposed to polymerase molecules. L and NP interact to form a polymerase complex which initiates transcription at the 3' terminus of viral L and S RNAs.

Although it is clear that complementary RNA, genomic RNA and messenger RNA must be synthesized, the next important step in terms of transcriptional regulation is the buildup of large quantities of nucleoprotein and its degradation products in the cell. This can potentially cause a reduction in the synthesis of mRNA by one of two mechanisms. High concentrations of NP may promote interaction with viral RNA in favour of nucleoprotein complexes. NP may recognize the stem structure of a large hairpin formed by interaction of the 3' and 5' termini. The termini appear to be at least partially complementary as determined by sequence analysis (K. Dimock, personal communication). Alternatively, NP may recognize a specific sequence at the 3'-terminus since this region appears to be conserved (Auperin et al., 1982). Binding of the first nucleoprotein molecule facilitates subsequent NP binding by NP-NP interaction.

The second potential mechanism operating to reduce viral mRNA synthesis depends on observations of proteolysis of the nucleoprotein in infected cells. If these molecules retain polymerase (L polypeptide) recognition sites but lose recognition sites for RNA and nucleoprotein, then decreased mRNA synthesis could be a consequence of reduced available L polypeptide.

Several indirect lines of evidence support the present model. Pichinde virus replication exhibits a peak in viral production and a subsequent decline, which is independent of defective interfering viral particles (Dimock et al., 1982). The nucleoprotein appears to represent most of the viral specific polypeptides evident in infected

cells, although quantitation requires an amino acid analysis of viral proteins (Chapter 3). The regulated growth cycle was apparent in three cell lines tested and it has been reported that one of these (MDCK) does not produce Pichinde virus defective particles (Chapter 5; Dutko and Pfau, 1978). A role for the nucleoprotein proteolytic products may be supported by observations that these molecules (NP 17.5, NP16 and NP14) have a longer half life in infected cells than does the nucleoprotein (Chapter 5). In the case of Vero cells, NP28 was a predominant proteolytic product and may in part account for a lower level of viral release from infected cells compared to BHK-21 cell levels.

Perhaps more importantly, the elements of such a model can be tested experimentally. Since the model depends substantially on protein-protein interactions, these must be clearly demonstrated. Purification of the nucleoprotein and its proteolytic fragments could be accomplished with monoclonal antibody, which is currently available (Chapter 5). Binding studies employing NP and viral RNA can be conducted in vitro. The model predicts that binding of one molecule of NP facilitates subsequent NP-RNA interactions. The initial binding specificity can be assessed with a variety of unrelated RNAs.

Until such time as L specific monoclonal antibody is available, it is unlikely that L polypeptide can be purified to homogeneity for similar studies and for a direct demonstration of NP and L requirement for transcriptase activity. However, other approaches to demonstrate regulatory properties are available. These rely on temperature

sensitive mutants available in this laboratory and the laboratory of Dr. D.H.L. Bishop, and the acquisition of clones of viral genetic information which can be expressed in eukaryotic cells.

It is known that temperature sensitive mutants of Pichinde virus, which have been isolated to date, can be divided into two non-overlapping complementation groups. The two groups correlate with L and S genomic RNA species (Vezza et al., 1977; Vezza et al., 1980). D. Caussey (personal communication) has shown that mutants carrying a ts lesion in L RNA do not synthesize viral protein, however, mutants defective in an S RNA function, at the non-permissive temperature, are protein positive. The two groups of mutants can be used to investigate the Pichinde virus growth cycle. The model predicts that isolation of a mutant which is ts for an S RNA function but does not synthesize viral protein should be possible, since NP maps to S RNA (Chapter 4). Only a limited number of the available mutants have been analyzed to date.

A second prediction of the model is that S RNA ts mutants could be identified which do not exhibit the "shutoff" phenomenon. A ts lesion in NP could potentially permit retention of polymerase activity but interfere with NP-NP interaction necessary for formation of nucleoprotein complexes. This would result in lack of interference with transcription and continued synthesis of viral protein at peak levels. A mutant of this type would probably not release infectious virus at the non-permissive temperature. Alternatively, if NP fragments are involved in "shutoff" these may not be found in

one or more ts mutants and interference with viral transcription would not be observed.

Direct demonstrations of the proposed roles of L and NP in viral replication may be possible if the genetic information encoding the two molecules is cloned and expressed in vitro. Expression and subsequent purification of the molecules may be possible under the control of the lac operon region of plasmid vectors pPCø1, ø2 and ø3 in a strain of Escherichia coli deficient in RNase I and polynucleotide phosphorylase (Hautala et al., 1979). Expression in prokaryotes would only be useful for obtaining large amounts of material for physical studies.

Assessment of regulatory phenomenon would require expression of L and NP in eukaryotic cells. This can be accomplished either with an SV-40 DNA vector (Mulligan et al., 1979) or with a recombinant plasmid containing the required gene in combination with the Herpes Simplex Virus thymidine kinase gene (Lai et al., 1980). Infection of cells with recombinant molecules expressing NP, and wild type virus, may provide clues as to the role of NP in regulation of viral replication.

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