A GENETIC STUDY OF THE BUNYAMWERA COMPLEX OF THE GENUS BUNYAVIRUS (FAMILY: BUNYAVIRIDAE)

by

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A thesis presented to

The Faculty Of Science

University Of Glasgow

for

The degree of Doctor of Philosophy

April, 1981

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D E D I C A T I O N

To the memory of my dear father, Mr Samuel Iroegbu Oriala, who died on 16th May, 1978, while I was studying in Scotland.

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ACKNOWLEDGEMENT:

I wish to thank Professor J. H. Subak-Sharpe for generously providing the facilities used in this research. I am sincerely grateful to Dr. C. R. Pringle, not only for diligently supervising this work and critically reviewing the manuscript, but also for his friendship which encouraged me at the most difficult times. Thanks to Mrs. Valerie Devine and Mrs. Margaret Wilkie for their technical assistance; and to Mr. John Parry, for the electron microscopy. I am equally grateful to Mr. Shadrack Okere who sacrificed his valuable time and typed this thesis. I wish, also, to thank Miss Elena Notarianni and Dr. S. U. Ighedosa for assisting in the proof-reading of the typed scripts. My gratitude, also, goes to Miss Elizabeth McKay for her kindness and for showing me parts of Scotland; and to the staff of the various service units of the Institute of Virology, Glasgow - Media, Cytology, Washroom, and the Office - whose assistance enabled me to overcome certain problems at various stages in this work. I will remember the entire staff and students of the Institute of Virology, past and present, for their much cherished friendliness.

I must not forget my sponsors, the University Of Nigeria, Nsukka, who supported me, financially, in the course of my study. My sincere gratitude, also, goes to Professor Nduka Okafor, for encouraging me to study Virology; and to my friends in Nigeria, especially, Mr. G. A. Nwankwo, for helping my family while I was away. To Grace, my wife, I must say that I am deeply impressed by your remarkable understanding.

C. Ukwuoma Iroegbu Glasgow, 1981.

ABBREVIATIONS.

5-AZA	5-azacytidine
BAT.	Batai virus
BHK-21	Baby hamster kidney cells, clone 13.
BS-C-1	African green monkey kidney cells.
BUN.	Bunyamwera virus.
CI	complementation index.
Ci	Curie
C.P.E.	cytopathologic effect.
c.p.m.	counts per minute.
EMS	ethyl methane sulphonate.
E.O.P.	efficiency of plating.
5-Fu	5-fluorouracil.
GRO	Guaroa virus.
MAG.	Maguari virus.
m.o.i.	multiplicity of infection.
mol. wt.	molecular weight.
NTG	N-methyl-N'-nitro-N-nitrosoguanidine.
PAGE	polyacrylamide gel electrophoresis.
PEG.	polyethylene glycol.
PBS	phosphate buffered saline.
p.f.u.	plaque-forming unit.
p.i.	post infection
RF.	recombination (reassortment) frequency.
r.p.m.	revolutions per minute.
SDS	sodium dodecyl sulphate.
TCA	trichloroacetic acid.
$\underline{ts}$	temperature-sensitive.
ts^+	wild type

S U M M A R Y

The Bunyamwera group of arthropod-borne viruses is one of the 13 serological groups that make up the genus, Bunyavirus of the family Bunyaviridae. There are 19 serologically distinct viruses in the Bunyamwera group and these have been subdivided into five, on the basis of greater antigenic similarity, as follows; the Bunyamwera Complex, the Wyeomyia Complex, Main Drain virus, Kairi virus and Guaróa virus. The Bunyamwera Complex contains 12 of these 19 viruses and the three viruses used in the genetic studies reported in this thesis represent the span of antigenic variation in the complex.

The bunyaviruses, in general, are negative-strand RNA viruses with three unique single-stranded genome segments (L, M and S) of total molecular weight, $4\text{-}6\times10^6$ daltons. There are four virus structural proteins two of which are nonglycosylated and are designated L (large) and N (nucleocapsid). The other two, G1 and G2, are glycosylated proteins located in the viral envelope.

This thesis consists of a description of genetic interaction between three members of the Bunyamwera Complex and demonstrates that recombination by reassortment of genome segments occurs in the Bunyamwera Complex. The three viruses studied, Batai virus, Bunyamwera virus and Maguari virus, originated from three different continents - Asia, Africa and South America respectively - with no recognised overlap in their distribution.

Sixty-three (63) stable temperature-sensitive (ts) mutants - 5 of Batai virus, 8 of Bunyamwera virus and 50 of Maguari virus - were isolated. The permissive and restrictive temperatures adopted were 31° C and 38° C respectively. With the exception of three spontaneous ts mutants of Maguari virus, the rest (60 mutants) were induced by 5-Fluorouracil (5-Fu). The frequency of isolation of mutants was dependent on the concentration of 5-Fu used. The frequency of isolation of the Maguari virus spontaneous ts mutants was 2.7%.

The ts mutants were used to investigate the homologous and heterologous recombination potential of these three viruses of the Bunyamwera Complex. Complementation between non-recombining ts mutants i.e. mutants belonging to the same recombination group was also investigated.

Recombination experiments enabled classification of the Batai virus and Bunyamwera virus ts mutants into two recombination groups (I and II) and Maguari virus ts mutants into three recombination groups (I, II and III) with two confirmed double mutants of groups I and II and five unclassified ts mutants. Temperature-sensitive mutants belonging to different recombination groups recombined at high frequencies (RF ranging from 1.1% to 98.4%), whereas, no recombination was detected between ts mutants belonging to the same recombination group (RF <1%). This indicated that the mechanism of recombination was reassortment of the genome subunits. Recombination was an early event in the multiplication

cycle and was detected first at 4 hours post infection during the eclipse period.

Complementation was detected among group I ts mutants of Maguari virus and was interpreted as intracistronic complementation because of it s sporadic pattern. No complementation was detected between Maguari virus group II mutants.

Recombination (reassortment) was also observed in the heterologous crosses of all three viruses. Similar to the homologous crosses, heterologous reassortment was restricted to ts mutants belonging to different recombination groups. For example, it only occurred between Batai group I and Bunyamwera group II ts mutants and was never detected in crosses of Batai group I and Bunyamwera group I or Batai group II and Bunyamwera group II mutants. This indicated that groups I and II of each virus were equivalent and that they probably represented mutation affecting the same two subunits of the viral genome. The heterologous reassortment was bi-directional such that it occurred in the Batai virus group I and Bunyamwera virus group II cross as well as in the reciprocal cross of Batai virus group II and Bunyamwera virus group I. Similar bi-directional recombinations were observed between ts mutants of Batai and Maguari viruses; and Bunyamwera and Maguari viruses. These results contrast with the predominantly unidirectional heterologous recombination reported for the California encephalitis group of bunyaviruses.

In a preliminary characterisation of the wild type viruses by SDS-polyacrylamide gel electrophoresis of their polypeptides, it was observed that the G1 and N polypeptides of all three viruses were distinguishable. With exception of one Bunyamwera virus ts mutant, BUNts7(II), which expressed a G1 polypeptide that migrated faster than the parental wild type virus, thirty ts mutants analysed expressed polypeptide profiles similar to their respective parental wild type viruses. Comparative analyses of the polypeptides of 77 non-ts clones isolated from the progeny of eleven different heterologous crosses showed that 97-98% of these were true recombinants and derived the G1 protein from their group I parents and the N protein from their group II parents. It was inferred from these observations that group I consisted of N gene mutants whereas group II consisted of the G gene mutants. The G gene assignment was confirmed by data obtained from analyses of neutralisation specificity of the recombinant viruses.

The results obtained in this work indicate that there is no genetic barrier to exchange of genetic material between the three viruses of the Bunyamwera Complex despite their geographical isolation and varying degree of antigenic relatedness. Potentially, therefore, the Bunyamwera Complex could be regarded as a single gene pool. Presumably the integrity of these viruses in nature is preserved by geographical and ecological factors.

The occurrence of complementation between *ts* mutants of the same recombination group (group I) indicate that one of the genome subunits (in addition to the G1/G2 protein determination) codes for more than one protein, or that the product of that segment (N or L protein) is a multifunctional or multimeric protein.

The isolation of three recombination groups of *ts* mutants (of Maguari virus) provides, for the first time, genetic markers for the three genome segments of the bunyavirus genome.

INTRODUCTION

I. GENERAL INTRODUCTION

I.1 TAXONOMIC GROUPING

The family Bunyaviridae is the largest and most diverse taxonomic grouping of the arthropod-borne viruses (arboviruses). At present 105 serologically distinct viruses are included in the genus Bunyavirus. These viruses have been classified into 13 serological groups with four viruses ungrouped. The serological groups (See Table 1) are Anopheles A, Bunyamwera, Bwamba, C, California encephalitis, Capim, Guama, Koongol, Mirim, Olifantsvlei, Patois, Simbu and Tete groups. The four ungrouped viruses are Kaeng Khoi, Gamboa, Guaratuba and Jurona viruses. The genus presents a complex pattern of antigenicity characterised by a network of immunological cross-reactions involving certain viruses belonging to different serogroups in addition to gradations in antigenicity within each group.

The assay methods commonly used to identify bunyaviruses include neutralisation test, complement fixation (CF) test and hemagglutination inhibition (HI) test (wherever possible) (Berge, 1975; Berge et al 1970, 1971; Casals, 1963a,b; 1971; Casals and Whitman, 1960; Theiler and Downs, 1973). Neutralisation reactions have mostly been used in classification of bunyaviruses, but the CF test is often used in situations where neutralisation or HI reactions prove less definitive in assigning viruses to groups (Bishop and Shope, 1979).

In certain serogroups, evaluation of neutralisation and CF test data reveals a complex antigenic relatedness among member viruses. For example, among the group C viruses, the relationships by neutralisation reactions are clearly divergent from the relationships by CF reactions. Six group C viruses isolated from the same locality, a single forest near Belem, Brazil, segregate in pairs according to their antigenic relatedness by neutralisation reactions as follows; Caraparu and Apeu, Marituba and Murutucu; Oriboca and Itaqui (Berge, 1975; Casals and Whitman, 1961; Shope and Whitman, 1966; Shope et al, 1961). Using the CF reactions, the relationships are reshuffled and the pairing is as follows: Caraparu and Itaqui; Apeu and Marituba; and Murutucu and Oriboca (Shope and Causey, 1962). Presuming that the neutralisation antigen(s) and the CF antigen(s) are expressed by separate genome subunits (See Gentsch and Bishop, 1978; Gentsch et al, 1979, 1980), the divergence in the reactivities of the two antigens could be explained by reassortment of the genome subunits at some remote time (Bishop and Shope, 1979).

All the 13 serogroups of the genus bunyavirus are antigenically linked at one point or the other by intergroup immunological cross-reactions between member viruses. Calisher et al (1973) reported HI cross-reactions between CoAn57389 virus of the Anopheles A group and La Crosse virus of the California encephalitis group; and Tensaw virus of the Bunyamwera group. It was on this basis that Anopheles A group was proposed

a member of the genus Bunyavirus. Cross-reactions have also been observed in HI and neutralisation tests between Bunyamwera group viruses such as Batai, Ilesha and Bunyamwera and other groups such as Simbu (Sathuperi and Manzanilla viruses), Bwamba (Bwamba virus), California ancephalitis (Tahyna and California ancephalitis viruses) and Koongol (Koongol virus) (Casals, 1963b). Several other intergroup cross-reactions have been demonstrated (See Bishop and Shope, 1979 and Theiler and Downs, 1973).

The following categories of taxonomic units have been adopted (Berge, 1975), to reflect the varying degrees of antigenic relatedness among bunyaviruses, (a) groups (b) complexes, (c) virus (serotype) (d)subtype (e) variety.

The serologic inter-relationship exhibited by Guaroa virus is worth mentioning separately. Groot et al (1959) first showed that Guaroa virus and California encephalitis virus were related by HI reaction. This was the only relationship known at the time. Subsequently it was shown that Guaroa virus was related to members of the Bunyamwera group (Bunyamwera, Cache Valley, Kairi and Wyeomyia viruses) by CF test (Theiler and Downs, 1973) but not by neutralisation or HI tests. Guaroa has been assigned to the Bunyamwera group on the strength of the above CF test reactions. Genetic experiments have failed to detect genetic recombination between Guaroa virus and California encephalitis group viruses (Gentsch et al, 1980) or Bunyamwera Complex viruses

(Pringle, C. R. and Iroegbu, C. U., unpublished data).

There are sixty three other viruses included in the family Bunyaviridae (See Table 2). These are serologically unrelated but morphologically and morphogenetically similar to the bunyaviruses (Fenner, 1975/76; Porterfield et al, 1975/76). They were not classified as separate genera in the family but retained as "bunyaviruslike viruses" or "possible members of the genus Bunyavirus" (Porterfield et al, 1975/76) pending further characterisation. As will become evident later, biochemical analyses indicate certain structural details which could easily distinguish these viruses. They have been subdivided into 11 serological groups with 11 viruses ungrouped (Table 2). Unlike the bunyaviruses, not many intergroup immunological cross-reactions have been detected among the various groups. In recognition of the differences between these viruses and the bunyaviruses and the uniqueness of some of the serogroups, new genera comprising the bunyaviruslike viruses are being proposed (Bishop D.H.L, 1980 personal communication). However, they will be referred to in this text as bunya-like viruses.

I.2 GEOGRAPHICAL DISTRIBUTION

Members of the genus Bunyavirus are widely distributed. They have been isolated in every continent except Antarctica (See Table 1). Although bunyaviruses are widely distributed in nature, most serotypes tend to be localised. For example, Bunyamwera virus has only

been isolated in Africa and nowhere else, whereas California encephalitis virus seems to be restricted to the North American continent (Bishop and Shope, 1979). Serological studies sometimes suggest wider distribution. For instance, presence of Bunyamwera virus has been reported in North Borneo , Colombia and the Amazon delta of Brazil on the basis of serological evidence (Berge, 1975; Smithburn, 1954). However, since Bunyamwera virus has only been isolated in Africa, the antibodies found in Asia and South America probably represent infections by other, closely related bunya-viruses (Bishop and Shope, 1979).

As shown in Table 2, the bunya-like viruses have a similar pattern of distribution.

I.3 HOST RANGE, DISEASE AND TRANSMISSION

The host range of bunyaviruses is as wide and varied as their geographical distribution (Table 1). They variously infect insects, rodents, wild and domestic animals, and man. Twenty two bunyaviruses have been isolated from man including members of the Anopheles A, Bunyamwera, C, California encephalitis, Guama and Simbu groups; and many more have been implicated from serological data in overt and clinical infections of humans (See Table 1); (Berge, 1975; Bishop and Shope, 1979; Theiler and Downs, 1973). Some members of the genus have been associated with serious and sometimes fatal human infections. For example, the first isolate of La Crosse virus (California encephalitis group) came

TABLE 1: Geographical distribution and host range of Bunyaviruses.

ABBREVIATIONS: (Berge, 1975)

BUN	Bunyamwera virus
BWA	Bwamba virus
TAC	Tacaiuma virus
GER	Germiston virus
ILE	Ilesha virus
GRO	Guaroa virus
WYO	Wyeomyia virus
ITQ	Itaqui virus
MAD	Madrid virus
MTB	Marituba virus
MUR	Murutucu virus
NEP	Nepuyo virus
ORI	Oriboca virus
RES	Restan virus
INK	Inkoo virus
LAC	La Crosse virus
GMA	Guama virus
SHU	Shuni virus
ORO	Oropouche virus

TABLE 1: *Geographical distribution and host range of Bunyaviruses

BUNYAVIRUS GROUP	GEOGRAPHICAL DISTRIBUTION		HOST RANGE	
	CONTINENT	VIRUS TYPE	ISOLATION	ANTIBODY
Anopheles A	Central and South America and the Carribbean countries	Anopheles A Lukuni Tacaiuma	Mosquitoes Primates man (TAC)	Man, pig, horse, bat, birds, rodents
BUNYAMWERA	Europe and Asia	Batai and its variants, Calovo and chittoor	mosquitoes	man, horses, cattle, sheep, pigs
	Africa	Birao, Bunyamwera, (Beliefe)' Germiston, Ilesha and Shokwe	mosquitoes man (BUN; GER, ILE)	man, goats, cattle, sheep, pigs bats.
	North America	Cache Valley, Lokern, Main Drain, Northway, Tensaw	mosquitoes, midges, hares rabbits, dog, rats	man mammals birds rodents
	Central and South America and the Carribbean countries	Anhembi, Guaroa Kairi, Maguari, Santa Rosa, Sororoca[2], Taiassui[2], Tlacotalpan[2] Wyeomyia, Tucunduba	mosquitoes man (GRO, WYO) primates horses, rodents other insects	man, birds mammals, rodents
BWAMBA	Africa	Bwamba, Pongola	mosquitoes man (BWA)	man, primates domestic mammals and birds

TABLE 1 (CONTD.)

Group C	Central and South America, and the Carribbean countries	Apeu, Caraparu, Itaqui, Madrid, Marituba, Murutucu, Nepuyo, Ossa, Restan, Oriboca	mosquitoes man (APEU, ITQ, MAD, MTB, MUR, NEP, OSSA, ORI RES), primates, marsupials rodents, other insects	man, rodents marsupials, primates
CALIFORNIA	Europe	Tahyna[3], Inkoo[2], other varieties of Tahyna	mosquitoes man (TAH) vertebrates (not INK)*	man, domestic and wild mammals
	Africa	Lumbo	mosquitoes	
	South America	Melao[2] Serra do Navio	mosquitoes	
	North America	California encephalitis[3]	mosquitoes,	man
		Jamestown Canyon	other insects	veterbrates
		Jerry Slough, La Crosse[3]	man (LAC)	
		Snow shoe hare[3], Keystone[3], San Angelo[3] Trivittatus[3]	other mammals (rodents only rarely)	
CAPIM	Central and South America and the Carribbean countries	Capim, Acara Bushbush, Guajara, Juan Diaz, Moriche	mosquitoes rodents (commonly) marsupials other animals	rodents mostly

TABLE 1 (CONTD.)

GUAMA	Central and South America	Guama, Catu, Bertioga[4], Bimiti, Moju	mosquitoes man (GMA, CATU) primates, rodents marsupials, bats other animals	man rodents, other mammals
	North America	Mahogany Hammock	mosquitoes cotton rat	
Koongol group	Australia	Koongol Wongal	mosquitoes	cattle
Mirim	Central and South America	Mirim, Minatitlan	mosquitoes and other insects, sentinel animals - monkey, hamster	vertebrates
Olifantsvlei	Africa	Olifantsuvei Bobia, Botambi	mosquitoes, other insects	
PATOIS	Central or North America	Patois, Pahayokee, Shark River and Zegla[4]	mosquitoes and other insects wild and sentinel rodents	rodents, man, pig
SIMBU	Africa	Akabane, Ingwavuma, Nola, Sabo, Sango, Sathuperi, Shamonda, Shuni, Simbu, Thimiri and Yaba-7	mosquitoes, ticks, midges, sheep, goat, cattle, man (SHU)	man, birds, domestic and farm animals

TABLE 1 (CONTD.)

	Asia	Aino, Akabone, Ingwavuma, Kaikalur, Sathuperi, Thimiri	mosquitoes, ticks, midges sheep, cattle birds	man, domestic animals, birds
	Australia	Akabone, Aino (Samford), Facey's Paddock, Peaton, Thimiri	mosquitoes midges	domestic animals
	South America	Inini, Manzanilla, Oropauche and Utinga	mosquitoes and midges, man (ORO) monkey, sloth and bird	man, birds, primates
	North America	Buttonwillow, Mermet	rabbit, bird midges	sheep, rabbit, rodents, birds
TETE	Africa	Bahig, Matruh, Batama, Tsuruse and Tete	birds and their parasitising ticks (mostly)	birds
	Europe	Bahig[3], Matruh	birds and ticks	birds
UNASSIGNED	Asia	Kaeng Khoi	bats, bedbugs	man, rats
	Central and South America	Gamboa[3], Guaratuba, Jurona	mosquitoes, rodent, birds	birds, rodents

TABLE 1 (CONTD.)

FOOTNOTES:

1. Not registered
2. No verterbrate isolates
3. Transovarially transmitted
4. No arthropod isolate

* Most of the information contained in this table will be found in Berge (1975); Bishop and Shope (1979) and Theiler and Downs (1973). The contributions of various workers who provided these information and who are not mentioned in this text, are highly appreciated.

from brain tissue of a 4 year old girl who died from meningo-encephalitis after symptoms of fever, headache, vomiting convulsions and pleocytosis (Thompson et al, 1965). Akabane and the related Aino viruses (Simbu group; Bunyavirus) are of veterinary importance. They have been associated with serious epizootics causing congenital defects in cattle, sheep and goats in Japan and Australia (Blood, 1956; Coverdale et al, 1978; Hartley et al, 1975; Inaba et al, 1975, Kurogi et al, 1975; 1976; 1977; and Parsonson et al, 1975; 1977).

Most isolations of bunyaviruses have been from arthropods, principally mosquitoes, but frequently also from midges and ticks (Berge, 1975). Different bunyaviruses appear to have specific vectors. It has been shown experimentally that mosquitoes can transmit bunyaviruses such as Bunyamwera virus (Berge, 1975; Ogunbi, 1968; Smithburn et al, 1946), Cache Valley virus (Yuill and Thompson, 1970), and Maguari virus (the Trinidadian strain, Tr20659)(Aitken and Spence, 1963; Theiler and Downs, 1973) to vertebrates. Other examples of experimental transmission of bunyaviruses have been given by Berge (1975) and Bishop and Shope (1979). Bunyaviruses can be transmitted from female arthropods to their offspring via the egg and between adults by venereal transmission. Transovarial transmission has been inferred by isolation of virus from infected eggs, larvae or pupae. For example, La Crosse virus has been recovered from mosquito eggs and larvae collected in the field (Balfour et al, 1975; 1976; Berry et al, 1974;

TABLE 2: * Geographical Distribution and host range of Bunyaviruslike viruses**

VIRUS GROUP	GEOGRAPHICAL DISTRIBUTION		HOST RANGE	
	CONTINENT	VIRUS TYPE	ISOLATION	ANTIBODY
Anopheles B	South America	Anopheles B; Boraceia	mosquitoes	man
Bakau	Asia	Bakau; Ketapang	mosquitoes ticks primates	man
Crimean Hemorrhagic Fever-Congo[1]	Asia	Crimean hemorrhagic fever[2,3]	ticks man	man man
		Hazara	ticks	
	Africa	Congo[3]	man cattle, sheep goats, pigs ticks	man goats, cattle sheep, pigs hares
	Europe and Middle-East	Crimean hemorrhagic fever, Congo	ticks, man	man cattle horses sheep goats, pigs hares
Kaisodi	Asia (India and Malaya)	Kasiodi, Lanjan	ticks	rodents (rats)
	North America	Silver water[4] (Canada)	ticks snow shoe hares	snow shoe hare rodents rabbits cattle

TABLE 2 (CONTD.)

Mapputta	Australasia (Australia and the neighbouring Pacific Islands)	Gan Gan Mapputta Maprik Trubanaman	mosquitoes	Antibodies to Trubanaman virus in man, horses, wallabies and Kangaroos
Nairobi sheep Disease	Africa	Dugbe[2,3]	ticks, man goats, cattle, sheep, pigs, giant pouched rat, midges and mosquitoes	cattle, goat sheep
		Nairobi sheep disease[2]	ticks,. sheep, goats	man sheep, goats
	Asia	Ganjam	ticks mosquitoes man	
Phlebotomus Fever	Africa	Arumowot	mosquitoes insects, rodents	
		Gordil	mouse	
		Saint floris	gerbil	
		COAr3319[5], Sud \n754-61[5]	hedgehogs, rodents monkey	

TABLE 2 (CONTD.)

Asia, Europe Medittarenean region (including Southern Europe, Middle-East and Northern Africa)	Sililian sandfly fever[2,3] Naples sandfly fever[2,3] ISS. Phl.3[5]	Phlebotomus flies man	rodents cattle, sheep man
	Krimabad and Salehabad	Phlebotomine insects	man (both) birds (Karimabad) sheep (Salehabad)
North America	Rio Grande[6]	rats	rodents opossums tortoises horses, sheep birds, horned toad
South and Central America	Candiru[2,3], Chagres[2,3] and Punta Toro[2,3]	Phlebotomine insects, man, rodent	man
	Aguacate	Phlebotomine insects	
	Anhanga[6]	sloth	
	Bujaru[6]	rodent	man, rodent
	Cacao	Lutzomyia insects	man
	Caimito, Chilibre	Lutzomyia insects	
	Frijoles and Nique	mosquitoes	rodents
	Icoaraci	Lutzomyia insects rodents	marsupials

TABLE 2 (CONTD.)

		Itaporanga	mosquitoes	man, birds
			mice, marsupials	marsupials
			bird	bats
		Pacui	Phlebotomine	rodents
			flies, rodents	marsupials
		Urucuri[6]	rodents	rodents primates birds
SAKHALIN[7]	Asia (Islands off the Pacific coast of USSR around sea Okhotsk) (Eastern USSR)	Sakhalin	ticks	sea birds
	North America (Coastal Oregon, USA)	Sakhalin	ticks	
	(Great Island, Newfoundland USA)	Avalon	ticks	sea birds
	Europe (Clo Mor, Cape Wrath, Scotland)	Clor Mor	ticks	
	Southwest New Zealand (Macquarie Island)	Taggert	ticks	penguins

TABLE 2 (CONTD.)

Thogoto	Africa	Thogoto[2,3]	ticks man trade animals camel	camels sheep goat
	Europe (Sicily)	Si Ar 126[8]	ticks	
Turlock	Africa and Europe	M'poko	mosquitoes	man
	Asia	Umbre	mosquitoes bird	birds sentinel chicken
	Australia	Barma Forest Marweh	mosquitoes	
	North and South America	Turlock	mosquitoes wild birds sentinel mice chicks	wild animals and birds horses
Uukuniemi	Africa (Egypt)	Eg An 1825-61[5]	southward-migrating palearctic bird	

TABLE 2 (CONTD.)

	Asia (including Pacific coast sea of Okhotsk USSR)	Manawa Zaliv Terpeniya	ticks ticks	--
	America (Pacific coast of USA)	Oceanside	ticks	
	Europe	Grand Arbaud and Ponteves[4]	ticks (including nymphs and larvae)	
		Uukuniemi	ticks birds mouse	man cattle birds
UNASSIGNED	Africa	Rift Valley fever[2,3,9]	mosquitoes sheep, cattle man	man vertebrates
		Bhanja[9]	tick, hedgehogs squirell, cattle sheep	
		Tataguine[2,3]	mosquitoes man	man rodents domestic animals monkey
		Witwatersrand[2]	mosquitoes sentinel hamsters rodents	man rodents

TABLE 2 (CONTD.)

Eurasia	Bhanja	ticks, goat man (laboratory acquired infection)	domestic animals man
	Khasan	tick	
	Razdan	ticks	
	Tamdy	ticks	
America (North)	Lone Star	ticks	man racoons
	Sunday Canyon	ticks	
Australia	Belmont	Culex mosquitoes	domestic fowl, horse
	Kowanyama	Anopheles mosquitoes	kangaroo, cattle, sheep, pigs, wallabies bandicoots, rats, wild birds

FOOTNOTES TO TABLE 2

1. Crimean hemorrhagic fever virus (isolated from Crimean regions of USSR) and Congo virus (from Zaire, Africa) are not serologically distinguishable and they are considered together as Crimean hemorrhagic fever-Congo virus (Casals, 1969; Chumakov et al, 1970)
2. Associated with human disease
3. Human isolates reported
4. Transovarially transmitted
5. Unregistered isolate
6. No arthropod isolates
7. Sea-birds may be important in disermination of viruses of the group
8. Closely related to and may be subtype of Thogoto virus
9. Laboratory-acquired infection

* Most of the information contained in this Table will be found in Berge (1975); Bishop and Shope (1979) and Theiler and Downs (1973). The contributions of the various workers who provided the information and who are not mentioned in this text, are highly appreciated.

**Three new genera comprising of the bunya-like viruses have been established recently. They are Phlebovirus, Nairovirus and Uukuvirus. This information was only available after this thesis had been completed hence it could not be included in the main text.

FOOTNOTES TO TABLE 2 (CONTD.)

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Bunyaviridae

Intervirology 14: 125-143 (1980)

Pantuwatana et al, 1974). Danielova and Ryba (1979) experimentally demonstrated transovarial transmission of Tahyna virus by Aedes vexans, following observation of a relationship between onset of Tahyna virus circulation and the increased emergence of Aedes vexans in summer months (Danielova, 1972; Danielova et al, 1972). This was consistent with the recovery of Tahyna virus from first generation larvae of Culiseta annulata mosquitoes in Southern Moravia (Bardos et al, 1975). Transovarial transmission has, therefore, been suggested to be a mechanism of overwintering of some bunyaviruses (Danielova and Ryba, 1979; Watts et al, 1974; see also Watts et al 1973 and Thompson, 1977).

The bunya-like viruses also exhibit a wide host range (Table 2). They infect a variety of domestic and wild animals as well as birds and man. Unlike bunyaviruses, they have been more frequently isolated from ticks and different flies (Phlebotomine and sand flies) and less frequently from mosquitoes and midges. Two bunya-like viruses, Gordil and Saint Floris viruses (Phlebotomus fever group), were isolated simultaneously from the same animal, the stripped grass mouse (Lemnyscomys striatus), in 1971 in the Central Africa Republic (Annual Report, Pasteur Institut de Bengui, 1971; cited by Bishop and Shope, 1979). This is significant in view of the potentiality of bunyaviruses to hybridise (See Section V.2). Certain bunya-like viruses have been associated with serious and sometimes fatal human infections, particularly the Crimean hemorrhagic

fever virus, a tick transmitted virus, which has been responsible for epidemics of hemorrhagic fever in the Crimean regions of the USSR and adjacent parts of Europe, Iran and Pakistan (Chumakov, 1963; 1969a, 1973). Sicilian and Naples sandfly viruses and the Phlebotomus fever group viruses, Candiru, Chagres and Punta Toro (Berge, 1975; Gaidamovich et al, 1974; George, 1971; Goverdhan et al, 1976; Peralta et al, 1965; Sabin, 1951; Tesh et al 1976; Woodall, 1967) have also been associated with human diseases. Rift Valley fever virus which causes serious disease in man with complications resulting in gastrointestinal hemorrhages, encephalitis and blindness, also affects sheep and cattle (Berge, 1975).

Apart from Rift Valley fever virus, another bunya-like virus of veterinary importance is Nairobi sheep disease virus. It was first isolated by Montgomery (1917) from diseased sheep suffering from severe gastroenteritis. It has also been reported to cause up to 73% mortality in sheep flock with disease characterised by glomerulonephritis, marked leukopenia and a drop in total serum protein (Weinbren et al, 1958).

A role of migratory birds in dissemination of these viruses is indicated by the isolation of bunya-like viruses from ticks taken from the body of birds, as well as animals. It has been suggested that the occurrence of Crimean hemorrhagic fever-Congo viruses in widely separated regions of Africa, Europe and Eurasia may involve dissemination of viruses by intercontinental

passage of infected migratory birds or ticks carried by such birds (Chumakov, 1969b; Kaiser et al, 1974).

II. VIRAL STRUCTURAL COMPONENTS

II.1 THE STRUCTURAL PROTEINS

There are three major structural proteins in a bunyavirus particle, two glycosylated proteins (G1 and G2) located on the surface and a non-glycosylated nucleoprotein (N). The molecular weight estimates for the G1, G2 and N proteins of various viruses are given in Table 3. In a number of cases a large molecular weight non-glycosylated protein (L) has been detected but in small amounts. The proportion of each of these proteins in a La Crosse virion, expressed as a percentage of the total protein content of the virion, has been estimated at 3-5% (L), 50-51% (G1), 14%(G2) and 31-32% (N) (Obijeski et al, 1976a). The number of molecules of L, G1, G2 and N proteins per La Crosse virion has also been estimated at 25, 650, 629 and 2126, respectively, from this data (Obijeski et al, 1976a). This indicates that G1 and G2 are present in approximately equimolar amounts.

II.1(i) THE GLYCOPROTEINS

The bunyavirus proteins designated G1 and G2 were identified as glycoproteins following preferential labelling by [^{3}H]glucosamine (Obijeski et al, 1976a; Rosato et al, 1974a; White, 1975). It had been reported earlier that Bunyamwera, California encephalitis, La Crosse, Lumbo, Murutucu, Oriboca and Tahyna viruses

TABLE 3: *Molecular weight estimates of the virion polypeptides of Bunyaviruses and Bunya-like viruses

VIRUS	VIRAL PROTEINS (M.WT.x10^3)					Reference
	L[b]	G1	G2	N[b]	Other	
BUNYAVIRUSES						
Bunyamwera		115	38	19		5
	200	128	31	23		10
	145	104	32	22		7
		85-83	33-30	23-20		15
Germiston[a]	185	125	27	18		9
Main Drain		115	38	21		5
Guaroa		115	32	21		3
Murutucu		85-83	33-30	23-20		15
Oriboca		85-83	33-30	23-20		16
		125	30	22		17
California encephalitis		115	39	21		3
		85-83	33-30	23-20		15
		82	38	18	30	19
Tahyna		115	38	21		3
		85-83	33-30	23-20		15
Lumbo		85	35	25		1
		115	38	22		3
La Crosse	180	120	35	25		8
		110	38	24		5
		85	45	26		6
Snow shoe hare		115	38	21		4,5
Melao		117	38	24		3
South River		120	38	22		3
Jamestown Canyon		118	38	21		3
Trivittatus		118	38	23		3
Keystone		118	38	23		3
Capim		125	30	32		17
Guama		125	30	22		17
Patois		125	30	22		17
Tete		125	30	22		17

TABLE 3 (CONTD.)

BUNYA-LIKE VIRUSES						
Belmont	145	104	32[6]	22		7
Karimabad		62	50	21		14
Qalyub[a]		85	75	40	115	2
Rift Valley fever[a]		65	56	25	100	13
Uukuniemi	170	75	65	25		11,12,18

TABLE 3 (CONTD.)

REFERENCES:

1. Bouloy and Hannoun (1976b)
2. Clerx and Bishop (1981)
3. El Said et al (1979)
4. Gentsch and Bishop (1976)
5. Gentsch et al (1977)
6. McLerran and Arlinghaus (1973)
7. McPhee, D. A. and E. G. Westaway (cited by Bishop and Shope, (1979)
8. Obijeski et al (1976a)
9. Ozden and Hannoun (1980)
10. Pennington et al (1977)
11. Pettersson et al (1971)
12. Pettersson et al (1977)
13. Rice et al (1980)
14. Robeson et al (1979)
15. Rosato et al (1974a)
16. Rosato et al (1974b)
17. Trent, D. W. (cited by Bishop and Shope,)
18. von Bonsdorff and Pettersson (1975)
19. White, (1975)

a) additions to the original table (by Bishop and Shope, 1979)

b) not glycosylated

* This Table is a modification and expansion of a Table by Bishop and Shope (1979); Bunyaviridae; Comp. Virol. Vol. 4 pp. 94/95.

possessed G1 and G2 proteins of sizes 82-85x10^3 daltons and 30-45x10^3 daltons, respectively, (Bouloy and Hannoun, 1976b; McLerran and Arlinghaus, 1973; Rosato et al, 1974a,b; White, 1975). Subsequent molecular weight determinations, however, indicated that the sizes of G1 proteins of different bunyaviruses ranged from 110x10^3 daltons to 130x10^3 daltons (El Said et al, 1979; Gentsch and Bishop, 1976, Gentsch et al 1977a; Obijeski et al, 1976a; Ozden and Hannoun, 1980; Pennington et al, 1977). White (1975) described four California encephalitis virus structural proteins, three of which were glycoproteins. The third glycoprotein had a molecular weight 30x10^3 but it has not been observed by other authors.

Treatment of La Crosse virus particles with proteolytic enzymes removed the spikes from the virus envelope concomitantly with the G1 and G2 proteins (Obijeski et al, 1976a). This indicates that the glycoproteins constitute the spikes and are located on the surface of the viral envelope. Additional evidence that the G1 and G2 proteins of La Crosse virus are located on the surface comes from an observation that <u>in vitro</u> labelling of the intact virus particles with iodine by the lactoperoxidase method, labelled only these two proteins (Obijeski et al, 1976a). Furthermore it has been demonstrated that disruption of bunyavirus particles with non-ionic detergent yields a nucleocapsid core structure containing no G1 or G2 (see next section) (Obijeski et al, 1976a; Rosato et al, 1974a, b; White, 1975).

Analysis of the oligosaccharides of the glycoproteins of certain California encephalitis group viruses indicated that they contained only A type oligosaccharide (Vorndam and Trent, 1979) similar to the type "A" serum glycoprotein (Johnston and Clamp, 1971). The large glycoprotein (G1) yielded two oligosaccharide species of 2600 and 1900 m.wt. respectively, each with at least one sialylated form. The G2 yielded only the 1900 m.wt. species and two sialylated forms. The number of carbohydrate attachment sites on each glycoprotein was determined by analysing the tryptic glycopeptides of glucosamine-labelled virions. It was deduced from this analysis that snow shoe hare and Trivittatus viruses differed in the number of attachment sites recognisable by glycosylation enzymes. Vorndam and Trent have suggested that this difference might involve differences in the primary and perhaps tertiary structures of the protein molecules. The G1 of snow shoe hare virus contained four attachment sites, whereas, the Trivittatus virus G1 contained two. The ratio of glucosamine in the two core species (2,600 and 1900) of G1 protein also differed in the two viruses. The ratio of glucosamine in the 2600 m.wt. species to that in the 1900 m.wt. species of snow shoe hare virus was 1 : 4 whereas the ratio of glucosamine in the two core species of Trivittatus virus G1 was approximately 1 : 1. This indicated that the larger core species of snow shoe hare virus G1 was either attached less frequently than the smaller core species or it was restricted to one of the four possible

attachment sites (Vorndam and Trent, 1979). The G2 protein of both viruses yielded a major and a minor tryptic glycopeptide each. This could indicate that there is a single site on this protein which is commonly glycosylated and a second site which is less frequently occupied (Vorndam and Trent, 1979). Gentsch and Bishop (1979) have reported that the tryptic peptides of the G1 and G2 proteins of La Crosse virus are distinguishable. This indicates that the two glycoproteins are unique polypeptides.

With respect to glycoprotein content, the only point of similarity that is apparent between bunyaviruses and bunya-like viruses is that the latter also contain two glycoproteins designated G1 and G2. It has been confirmed in the case of Uukuniemi virus that these polypeptides are located in the surface of the virion (Pettersson et al, 1971; von Bonsdorff and Pettersson, 1975). The sizes of the bunya-like virus glycoproteins differ from the sizes of G1 and G2 of bunyaviruses with the exception of Belmont virus (see Table 3). The bunya-like virus G1 proteins are in the range 62-85x10^3 daltons and G2, in the range 50-75x10^3 daltons (Clerx and Bishop, 1981; Pettersson et al, 1971; 1977; Rice et al, 1980; Robeson et al, 1979; von Bonsdorff and Pettersson, 1975), compared with the 110-130x10^3 daltons and 30-45x10^3 daltons, respectively, of the bunyavirus proteins.

(ii) THE NUCLEOCAPSID PROTEINS, N and L

As was indicated earlier, non-ionic detergents rupture the bunyavirus envelope and release a core structure containing protein and nucleic acid (Obijeski et al, 1976a; Rosato et al, 1974b; White, 1975). The protein components are the major nucleocapsid protein, N (Molecular weight 18-26x10^3 daltons) and the minor protein of large molecular weight, L (Molecular weight, 145-200x10^3 daltons). Not all investigators have detected the L protein. It has been detected in Bunyamwera virus infected cells (Pennington et al, 1977) as well as in preparations of Germiston virus (Ozden and Hannoun, 1980) and La Crosse virus (Obijeski et al, 1976a).

The bunya-like viruses have N proteins of size 21-40x10^3 daltons (Clerx and Bishop, 1981; Pettersson et al, 1971; 1977; Rice et al, 1980; Robeson et al, 1979; von Bonsdorff and Pettersson, 1975). The L protein has been detected in Uukuniemi (molecular weight, 170x10^3 daltons; Pettersson et al, 1971; 1977; von Bonsdorff and Pettersson, 1975) and in Belmont virus (Mol.wt. 145x10^3 daltons; see Table 3).

II.2 THE GENOME STRUCTURE OF BUNYAVIRUSES

Growth experiments with California encephalitis virus have shown that replication of the virus is neither inhibited by 5-bromo-2-deoxyuridine (Whitney et al, 1969) nor by actinomycin-D (Goldman et al, 1977). Similarly, Kascsak and Lyons (1977) reported that in the presence of 0.1 and 0.5 μg of actinomycin-D per ml of incubation

medium, Bunyamwera virus yields from infected BHK-21 cells were 85% and 75%, respectively, of the yield of untreated controls. They also observed that $5x10^{-6}$M 5-Fluoro-2-deoxyuridine and 10 µg/ml cytosine arabinose, drug levels which reduced vaccinia virus (DNA virus) yield by more than 95%, had no significant effect on Bunyamwera virus. These observations indicated that the genomes of the bunyaviruses were RNA and not DNA. McLerran and Arlinghaus (1973) demonstrated that the purified nucleic acid of La Crosse virus genome was sensitive to alkaline hydrolysis and was digested by pancreatic ribonuclease in the presence of 0.3M potassium chloride. DNA is resistant to these treatments and pancreatic ribonuclease is known to digest single-stranded RNA but not double-stranded RNA at salt concentration above 0.15M (Arlinghaus et al, 1968). These indicate that La Crosse virus genome RNA was single stranded. Other methods, including base composition, density in Cesium sulphate and radiotracer incorporation have been used to characterise the genome nucleic acid of different bunyaviruses. All the results obtained are consistent with the bunyaviruses genome being single stranded RNA (Bouloy et al, 1973/74 Clewey et al, 1977, Obijeski et al, 1976b).

Sucrose gradient analysis of the genome RNA of Bunyamwera (Kascsak and Lyons, 1977), La Crosse (Obijeski et al, 1976b) and Lumbo (Bouloy et al, 1974) viruses resolved them into three size classes designated L (large; 30S), M (medium; 25S) and S (small; 15S).

Bouloy et al(1973/74), co-extracted Lumbo virus RNA with the RNA of Sindbis virus which is a linear unsegmented molecule with a sedimentation coefficient of 42S. The Lumbo virus RNA was recovered in the same three size classes as before and the Sindbis virus RNA remained intact. This confirmed the segmented nature of the bunyavirus genome. Three RNA species with similar sedimentation coefficients have been extracted from other bunyaviruses also, including Main Drain, Guaroa, snow shoe hare, Tahyna and Trivittatus viruses (L. El Said, J. Gentsch, R. Klimas and D.H.L. Bishop cited by Bishop and Shope, 1979). Polyacrylamide agarose or polyacrylamide gel electrophoresis has also been used to demonstrate three RNA subunits in La Crosse, Lumbo, Melao and snow shoe hare viruses (Bouloy et al, 1973/74; Clewey et al, 1977; Gentsch and Bishop, 1976; Gentsch et al, 1977a; Obijeski et al, 1976b). Only in a few cases have different results been reported. For example, McLerran and Arlinghaus (1973) reported six genome subunits (50-60S, 35S, 27S, 20S, 16S and 4S) of La Crosse virus resolved by sucrose density gradient analysis. Kascsack and Lyons (1977) resolved four sizes of Bunyamwera virus RNA by polyacrylamide-agarose gel electrophoresis, two of which (0.8 and 0.5×10^6-dalton segments) were obtained from a 16S peak (corresponding to the SRNA) in a sucrose density gradient. Obijeski et al (1980) also reported four RNA segments of 5' and 3'- end-labelled La Crosse genome resolved by 2.2% polyacrylamide gel electrophoresis. Analysis of the

3' - and 5' - terminal nucleotide sequences of the fourth segmented (designated X) showed that the 5' -terminal sequence of the X RNA was similar to that of the M RNA but additional nucleotides were also detected in the X RNA. The origin of the fourth segment, is not known. Obijeski et al (1976b), however, observed that the L, M and S RNAs were present in equimolar amounts. This strongly supports a tripartite structure for bunyavirus genome RNA.

Table 4 shows the molecular weight estimates for the genome RNAs of different bunyaviruses (and some bunyalike viruses). Clewey et al (1977) have carried out an independent determination of the molecular sizes of snow shoe hare virus RNA species involving pancreatic ribonuclease and nuclease T1 digestion of 32p-labelled RNA species followed by DEAE-cellulose column chromatography of the digests. This method gave molecular weight values of 2.8×10^6 (L), 1.6×10^6 (M) and 0.4×10^6 (S) which are within, but on the lowerside of, the molecular weight ranges given on Table 4. Although Obijeski reported that the RNA subunits of La Crosse virus were present in molar equivalent, others have observed that the molar ratio of L, M, and S RNA species varies from one bunyavirus to another and even for the same virus it varies in different preparations (Bishop and Shope, 1979). In most preparations the S RNA species appeared in higher proportions. It has been suggested that the imbalance of the RNA species may be as a result of interference by defective virus particles (Kascsak and Lyons, 1978).

TABLE: 4: Molecular weights of Bunyavirus and Bunya-like virus RNA species estimated by gel electrophoresis.

REFERENCE

1. Bouloy et al (1973/74)
2. Clerx and Bishop (1981)
3. Gentsch and Bishop (1976)
4. Gentsch et al (1977)
5. Goldman et al (1977)
6. Kascsak and Lyons (1977)
7. McPhee, D.A. and E. G. Westaway (cited by Bishop and Shope, 1979)
8. Obijeski et al (1976b)
9. Ozden and Hannoun (1980)
10. Pettersson and Kaariainen (1973)
11. Pettersson et al (1977)
12. Rice et al (1980)
13. Robeson et al (1979)

a. polyacrylamide-agarose gel electrophoresis
b. Formamide gel electrophoresis
c. polyacrylamide gels
d. additions to the original table by Bishop and Shope (1979)

This table is a modification and expansion of a table by Bishop and Shope (1979); Bunyaviridae. Comp. Virol. Vol. 14 p.88.

TABLE 4: Molecular weights of Bunyavirus and Bunya-like virus RNA species estimated by gel electropheresis

VIRUS	Molecular weights ($x10^6$) L	M	S	Reference
BUNYAVIRUSES				
Bunyamwera (c)	3.0	1.9	0.34	4
(a)	3.8	2.4	(0.8)0.5	6
(c)	2.9	1.8	0.3	7
Germiston (c)(d)	3.8	2.5	0.5	9
Main Drain (c)	3.1	2.0	0.4	4
California encephalitis (a) (BFS-283)	(6.7)4.1	2.2	0.4	5
La Crosse (a)	2.9	1.8	0.4	8
Lumbo (c)	2.9	2.0	0.5	1
Snow shoe hare (c)	3.0	1.9	0.45	4
(c)	2.9	1.9	0.45	3
BUNYA-LIKE VIRUSES				
Belmont (a)	3.2	2.4	0.3	7
Karimabad (c)	2.6	2.2	0.8	13
Punta Toro(c)	2.8	1.8	0.75	13
Qalyub (c)(d)	5.0	2.0	0.7	2
Rift Valley fever(d)	2.7	1.7	0.6	12
Uukuniemi (b)	4.1	1.0	(0.88)0.78	10
(c)	2.9	1.3	0.6	11
	2.6	1.2	0.5	11

The oligonucleotides derived from the L, M and S RNA species of some bunyaviruses by ribonuclease digestion have been resolved by two diamensional gel electrophoresis. Comparison of the resulting fingerprints indicated that the L, M and S RNAs of La Crosse virus, snow shoe hare virus, Lumbo virus, Tahyna virus, Trivittatus virus or Guaroa virus are unique segments (Clewey et al, 1977; Gentsch et al, 1977b; 1979; 1980; El Said et al, 1979). The fingerprint patterns obtained for the various bunyaviruses were also distinguishable. A comparison of eleven La Crosse virus isolates from different localities in U.S.A. indicated that La Crosse virus from different ecological niches could be distinguished also by their nucleotide fingerprints (El Said et al, 1979).

Obijeski et al (1980) have analysed the 5'- and 3'-terminal sequences of La Crosse virus genome RNA species. The 3'-termini were labelled with cytidine 3', 5'-(5'-^{32}P)-biphosphate and the 5'-termini with (γ-^{32}P)-ATP and polynucleotide kinase after dephosphorylation with calf intestinal alkaline phosphatase. They observed that the first eleven nucleotides at the 3'-end of all the RNA species (L,M and S) were identical. The first eleven residues of the 5'-end of the M and S RNAs were also identical and complementary to the eleven conserved residues of the 3'-end. They further observed that for each RNA species, the 40-50 nucleotides at both termini adjacent to the eleven conserved residues may be extensively base paired. The significance of these observations will be discussed later in

relation to the configuration of the ribonucleoprotein structure. Similar conservation of eleven 3'-terminal nucleotides have also been reported for the RNA species of La Crosse and snow shoe hare viruses by Clerx-van Haaster and Bishop (1980).

Contrary to an earlier report by McLerran and Arlinghaus (1973) that the La Crosse virus RNA was infectious, Obijeski et al (1976b) could not isolate infectious La Crosse virus RNA under experimental conditions which allowed isolation of infectious Sindbis RNA. S. Krams-Ozden (cited by Bouloy and Hannoun, 1976a), also could not extract infectious RNA from Lumbo virus preparations. Furthermore, Bouloy and Hannoun (1976b) isolated messenger RNAs from infected cell polysomes and demonstrated, by annealing experiments, that majority of these were complementary to the viral genome RNA. This confirmed that the Lumbo virus genome was negative-stranded RNA. This is consistent with the observation that Lumbo virus particles contain an RNA-dependent RNA polymerase (Bouloy and Hannoun, 1976a). To sum up, bunyavirus genome is made up of three distinct subunits (L, M and S) of single-stranded RNA (total molecular weight, $4\text{-}6\times10^6$ daltons) which are not infectious and exhibit negative polarity relative to the messenger RNA.

Similarly, the bunyalike viruses also possess a tripartite genome. This has been demonstrated with Karimabad and Punta Toro (Robeson et al, 1979), Qalyub (Clerx and Bishop, 1981), Uukuniemi (Pettersson et al 1977), Belmont (McPhee and Westaway, cited by Bishop and

Shope, 1979) and Rift Valley fever (Rice et al, 1980) viruses (See Table 4).

II.3 STRUCTURE OF THE NUCLEOCAPSID

The nucleocapsid is a composite structure made up of RNA in close association with protein. Saikku et al (1971) first demonstrated that the nucleocapsid released when Inkoo virus (California encephalitis group) preparation was treated with non-ionic detergent contained both RNA and protein labels. RNA and protein were also recovered from nucleocapsids of California encephalitis virus (White, 1975) and La Crosse virus (Obijeski et al, 1976a,b). As was previously indicated, the protein components consisted of the major nucleocapsid protein, N (Obijeski et al, 1976a; White, 1975) and a small quantity of the L protein (Obijeski et al, 1976a). All the RNA and N protein of the La Crosse virion were recovered in the nucleocapsid and the protein-to-RNA ratios were found to be similar for all three RNA species (L, M and S). It was calculated that there were about 1,209 N protein molecules associated with L RNA species, 750 molecules with M RNA and 167 molecules with S RNA (Obijeski et al 1976b). Obijeski et al (1976a) also noted that the N protein had approximately a ten-fold mass excess over the viral RNA. Despite this excess, the RNA of the nucleocapsid was still accessible to digestion by ribonuclease (Obijeski et al, 1976b).

Velocity sedimentation in sucrose gradients resolved three size classes of La Crosse virus nucleocapsid, 115S, 90S and 65S (Obijeski et al, 1976b). Circular nucleocapsids have been reported in preparations of La Crosse virus (Obijeski et al, 1976b) and Lumbo virus (Samso et al, 1975). Negative contrast electron microscopy revealed convoluted and supercoiled La Crosse virus nucleocapsid strands (10-12 nm in diameter) which occasionally appeared as "beaded" strands (2-3 nm diameter). Such convoluted and supercoiled configurations did not permit a comparison of the lengths of the nucleocapsid sizes (Obijeski et al, 1976b). Coiled strands (2-3 nm thick) of Inkoo virus nucleocapsids with regular convolutions as well as supercoiled strands (7-10 nm thick) have also been observed by electron microscopy (Saikku et al, 1971).

In view of the circular configuration of the nucleocapsid, investigations have been carried out to ascertain whether bunyavirus RNAs existed as covalently closed circles. This was done by looking for the presence of 5'-end nucleotides and 3'-end riboses in deproteinised viral RNA. It was reported that pppAp structures were found in the 5'-termini of the three RNA segments of La Crosse virus (Obijeski et al, 1976b) and snow shoe hare virus (Gentsch et al, 1977a). In addition, no capped inverted nucleotides or poly(A) sequences were detected in any of the segments. Three La Crosse virus RNA species each containing a $[^3H]$-labelled 3'-end have been recovered when La Crosse virus

RNA was oxidised with periodate and reduced with tritiated sodium borohydride (Obijeski et al, 1976b). These observations indicate that the RNA of bunyaviruses in the native form do not exist as covalently closed circles, the circular configuration of the nucleocapsid notwithstanding. Consistent with this is the electron microscopic observation of only linear forms of La Crosse RNA species when spread under denaturing conditions with formamide, urea and heating to 53° C (Dahlberg et al, 1977). However, Samso et al (1976) have observed circular RNA in three size classes corresponding to L, M and S RNA in Lumbo virus preparations. As it is evident that bunyavirus RNAs may not exist as covalently closed circles, the circular RNAs observed in Lumbo virus may arise from hydrogen bonding.

The nucleocapsid of the bunya-like virus, Uukuniemi virus, has similarly been isolated in three size classes, 150S, 120S and 90S (Pettersson and von Bonsdorff, 1975). These have also been shown to be circular (Pettersson et al, 1971; Pettersson and von Bonsdorff, 1975; von Bonsdorff et al, 1969). No supercoiled structures were encountered in Uukuniemi virus preparations and so the nucleocapsids could be uncoiled. The lengths of the uncoiled nucleocapsids were 2,800, 1,400 and 700 nm respectively (Pettersson and von Bonsdorff, 1975). When spread under mild denaturing conditions, circular RNA structures in three size classes with regions of secondary structures ("panhandles") were observed in Uukuniemi virus preparations.

These "panhandles" were suggested to represent hydrogen bonding of about 100 inverted complementary base sequences at the 5'- and 3'-ends of the RNA molecules (Hewlett et al, 1977; Pettersson and Hewlett, 1976). Hydrogen bonding was suggested because Pettersson et al (1977) had shown that Uukuniemi virus native RNAs did not exist as covalently closed circles.

The observation that 40-50 nucleotides at both the 5'- and 3'- ends adjacent to the conserved eleven nucleotides in each RNA segment of La Crosse virus may be extensively base paired (Obijeski et al, 1980) could explain the "panhandle" structures of Uukuniemi RNA preparations. It would also suggest that the circular nucleocapsid and RNA structures observed in both the bunyaviruses and the bunya-like virus do not result from covalent bonding but from hydrogen bonding of complementary base pairs at the 5' and 3' ends of the RNA (see model proposed by Obijeski et al, 1980; Appendix II). The actual configuration assumed by the nucleocapsids and the manner, (if), the segments are associated to each other in the intact virus particle, remain undetermined. An understanding of this could elucidate the mechanism of virus assembly as well as genetic recombination by reassortment.

II.4 THE VIRION-ASSOCIATED RNA POLYMERASE ACTIVITY

Virion-associated polymerase activity has been detected in one bunyavirus, Lumbo virus (Bouloy and Hannoun, 1976a) and in one bunya-like virus, Uukuniemi virus (Ranki and Pettersson , 1975). This is consistent with the non-infectious negative strand RNA genome observed in these viruses. In both Lumbo virus and Uukuniemi virus the polymerase activity was low and required all four ribonucleoside phosphates to be present. The Lumbo virus polymerase additionally required Mg^{2+}, Mn^{2+} as well as monovalent cations, whereas the Uukuniemi virus polymerase only required Mn^{2+}. However, purification of Uukuniemi nucleocapsids, free of the components of the viral envelope, resulted in an additional requirement of Mg^{2+} for optimal activity of the polymerase (N. Ranki and R.F. Pettersson cited by Obijeski and Murphy, 1977). Both polymerases showed high temperature optimum (Lumbo virus enzyme, 38°C; Uukuniemi enzyme, 37-40° C). Bouloy and Hannoun (1976a) inferred that the Lumbo virus polymerase was indeed an integral part of the virion on observation that the activity sedimented at the same density as the $[^3H]$-uridine-labelled virus (1.17 g/cm^3). Similarly, Ranki and Pettersson (1975) concluded that the polymerase of Uukuniemi virus was located inside the virus particle because its activity was only expressed after disruption of the virus with a non-ionic detergent. They also observed the the Uukuniemi virus enzyme was inhibited by pancreatic ribonuclease, but not by actinomycin D,

deoxyribonuclease or rifampin, indicating that it was indeed an RNA-dependent RNA polymerase. Annealing experiments showed that the products of both polymerases were complementary to their respective progeny viral RNAs. The efficiency of in vitro transcription catalysed by the Uukuniemi polymerase was low. The products were not full-sized copies of the genome RNA templates but consisted mainly of small RNA fragments. These fragments were true transcripts of the complementary RNA because they annealed to it.

The low efficiency of in vitro transcription by Uukuniemi RNA polymerase (Ranki and Pettersson, 1975) is comparable to the efficiencies of similar enzymes in Newcastle disease virus (Huang et al, 1971), Pichinde virus (Carter et al 1974) and Lumbo virus (Bouloy et al, 1975), whereas, the activity of Vesicular stomatitis virus polymerase (Baltimore et al, 1970) is comparatively higher.

Investigations with La Crosse and snow shoe hare viruses did not show any in vitro polymerase activity in these viruses (D.H.L. Bishop and J.F. Obijeski, cited by Obijeski and Murphy, 1977). The above workers, however, detected complementary RNA synthesis in La Crosse and snow shoe hare virus-infected cells in which synthesis of new protein had been inhibited by treatment with cycloheximide or puromycin from the time of infection. This suggests presence of an RNA polymerase presumably from the virions.

III.1 BUNYAVIRUS REPLICATION

Productive infection of a host cell by an enveloped virus involves:

- adsorption, penetration and uncoating of the infectious virus;
- virus genome-directed synthesis of viral proteins and nucleic acid;
- assembly of virus structural components and eventual release of virions from infected cells.

Little is known about the early events in bunyavirus infection (i.e. adsorption, penetration and uncoating) except that enzymic removal of the surface glycoproteins, G1 and G2, resulted in reduction of La Crosse virus infectivity to about 10^{-5} to 10^{-6} the infectivity of the untreated virus (Obijeski et al, 1976a). This indicated an involvement of the glycoprotein-containing spikes in adsorption. It is not clear whether bunyaviruses replicate and form infectious particles without the involvement of the host cell nucleus. Goldman et al, (1977) reported replication of California encephalitis virus to high titres in enucleated XC (Rous Sarcoma - transformed rat) cells. This result is consistent with the observation that actinomycin D which inhibits DNA replication does not inhibit bunyaviruses, thus indicating that cellular transcription is not required for initiation of bunyavirus infection. It was observed, however, that enucleated BS-C-1 cells did not yield infectious virus even when enucleated as late as 6 hours after

infection with Bunyamwera virus (Pennington and Pringle, 1978). It was suggested that inhibition of viral replication in enucleated BS-C-1 cells was the result of disappearance of cellular structures (e.g. the Golgi apparatus) needed for maturation (Pringle, 1977a).

The growth of bunyaviruses of the California group is characterised by an early eclipse phase lasting 4-6 hours and an exponential phase of about 16-18 hours (Goldman et al, 1977; Lyons and Heyduk, 1973; Obijeski et al, 1976a). Similar determinations have been made with Bunyamwera virus (Kascsak and Lyons, 1978). Generally, bunyaviruses grow to titres between 10^7 and 10^9 pfu per ml of incubation medium in monolayers of established cell lines. A multiplicity dependent auto-interference has been observed in bunyavirus-infected cells. Obijeski et al (1976a) observed that at input multiplicities of infection ranging from 1 to 10 pfu per cell, the maximum La Crosse virus yield in BHK-21 cells was less than 10^7 pfu per ml whereas multiplicities of 0.01 and 0.002 pfu per cell yielded over 10^7 pfu per ml in the same experiment. The highest yield was obtained from cloned virus at a multiplicity of 0.002 pfu per cell. These observations indicated that there was autointer-ference with La Crosse virus replication at higher multiplicities of infection. Similar observations have been made by Kascsak and Lyons (1978) with Bunyamwera virus. They demonstrated that the interference at high multiplicities of Bunyamwera virus infection was due to generation of defective interfering particles. They also

showed that this phenomenon was host cell-dependent in that interference was high in BHK-21 and Vero cells but but less so in Madin-Darby bovine kidney cells (MDBK) and Aedes albopictus cells. Analysis of viral RNA synthesis showed that interference was at the level of viral genome replication resulting in a reduction in synthesis of the larger genome subunits (L and M RNAs), but not of the small subunits (the S RNA) (Kascsak and Lyons, 1978).

The effect of bunyavirus infection on the host cell is, to some extent, host dependent. While bunyaviruses produce extensive CPE in mammalian cells, the effect of virus replication in insect cells is less destructive. There is efficient shut-off of host cell protein synthesis in bunyavirus-infected vertebrate cells. Pennington et al (1977) observed a decline in host protein synthesis in Bunyamwera infected BS-C-1 cells beginning at about 5 hours post infection. By about 7 hours post infection host cell protein synthesis was completely halted. Similar observations have been reported in separate experiments with Trivittatus and Bunyamwera viruses by L. El Said; I. Lazdins and I. Holmes (cited by Bishop and Shope, 1979). Electron microscopic examination of mammalian cells infected by bunyaviruses revealed extensive proliferation of the unit membranes and vesicles of the Golgi complex (Lyons and Heyduk, 1973; Murphy et al, 1968a, b; 1973). The brain and liver tissues of new born mice infected by bunyaviruses showed similar cytopathological changes

characterised by Golgi body proliferation, vesiculation and cytoplasmic rarifaction (Murphy et al, 1973). Bunyavirus infection of mammalian cells and tissues terminate with severe cytonecrosis resulting in disruption of the cell membrane (Lyons and Heyduk, 1973; Murphy et al, 1973).

The effect of bunyavirus infection of insect cells is somewhat different. Lyons and Heyduk (1973) observed electron microscopic changes such as proliferation of the Golgi complex and vesiculation (as above) in a small proportion of California encephalitis virus-infected Aedes albopictus cells. Light microscopy revealed no cytopathic effect in the California encephalitis virus-infected mosquito cells. Infectious centre assays indicated that only a low proportion of the mosquito cells were productively infected (Lyons and Heyduk, 1973). Furthermore, the growth rates of cells from persistently infected and non-infected cultures were comparable. It was suggested, therefore, that the lethally infected cells observed by electron microscopy were replaced with a sufficient number of sensitive cells to establish a steady-state interaction, thus partially explaining the phenomenon of bunyavirus persistence in the A. albopictus cell cultures. Crimean hemorrhagic fever virus, a bunyalike virus of the Crimean hemorrhagic fever - Congo group produced similar cytopathological changes in brain and liver tissues of new born mice as bunyaviruses (Murphy et al, 1973).

III.2 REPLICATION OF BUNYAVIRUSES: SYNTHESIS OF VIRUS STRUCTURAL COMPONENTS

i) SYNTHESIS OF RIBONUCLEIC ACID (TRANSCRIPTION)

It has been mentioned in section II.2 of this introduction that the RNA isolated from bunyaviruses is not infectious and in section II.4, that purified bunyavirus particles contain a RNA-dependent RNA polymerase. It has also been indicated in section II.4 that the products of RNA synthesis mediated by bunyavirus polymerase are complementary to the viral RNA in that they could anneal to give a ribonuclease-resistant duplex.

The transcription of viral genome RNA to complementary or messenger RNA proceeds in two stages termed primary and secondary transcriptions, respectively. Primary transcription is effected solely by the virion-associated RNA polymerase without the involvement of newly synthesized enzymes. Secondary transcription is an amplification of complementary RNA synthesis following availability of newly synthesized enzymes and templates.

That primary transcription occurs and involves only the infecting viral genome RNA as template and the virion-associated RNA polymerase has been demonstrated by inhibiting de novo synthesis of protein and measuring the accumulation of radiotracer-labelled viral complementary RNA. Kascsak and Lyons (1977) reported three classes of virus-specified RNA; 33S, 26S and 16S, in Bunyamwera virus-infected BHK-21 cells treated before and at the time of infection with both cycloheximide and actinomycin-D. These size classes were equivalent

to transcripts of the three subunits of the viral genome RNA. As a control, the authors included reovirus, a double-stranded RNA virus, and Semliki forest virus (a positive single-stranded RNA virus). The radioactivity in Semliki forest virus-infected and mock-infected cultures were lower than those found in reovirus- and Bunyamwera virus-infected cultures. These results show that there was synthesis of Bunyamwera virus complementary RNA in which the parental genome served as the sole RNA template and the virion-associated polymerase the sole catalytic enzyme. Other experiments which showed similar results have been mentioned in section II.4. In the absence of inhibitors of protein synthesis (puromycin or cycloheximide) the synthesis of viral complementary RNA is amplified. It has been estimated that at a multiplicity of infection of 80 pfu per cell, the ratio of primary to secondary RNA synthesis is about 1 : 100 (Bishop and Shope, 1979). Some of the complementary RNA of Lumbo virus have been isolated from infected cell polysomes (Bouloy and Hannoun, 1976b). Bouloy and Hannoun (1976b) have also isolated two types of ribonucleoprotein from Lumbo virus-infected cells, one containing virus progeny (negative strand) RNA and the other containing the complementary RNA. Similar observations have been made in cells infected by influenza virus (Pons, 1971) and VSV (Soria et al, 1974) and suggest that viral progeny RNA synthesis may occur on the complementary RNA template in the RNP form (Bouloy and Hannoun, 1976b). Free cRNA was not detected.

ii) VIRAL PROTEIN SYNTHESIS (TRANSLATION)

The Kinetics of Bunyamwera virus protein synthesis in BS-C-1 cells have been analysed by Pennington et al (1977). All four structural proteins, L, G1, G2, and N, were detected in the cell extracts. The N protein was the first to be detected, at about 2 hours post infection. The rate of synthesis increased, reaching maximum at 4 hours post infection and then declining. The glycoprotein, G1, was first detected at four hours post infection, the rate of its synthesis increased, peaked at 8 hours post infection and also declined. The onset of synthesis of L and G2 could not easily be detected because both proteins comigrated with host cell proteins. The L protein, however, peaked and declined at the same time as N, whereas the kinetics of G2 synthesis were similar to G1. Synthesis of all four Bunyamwera virus structural proteins continued for many hours (up to 22 hours post infection) after the maximum rate of synthesis had been attained (Pennington et al, 1977).

J. F. Obijeski (cited by Obijeski and Murphy, 1977) similarly identified all four bunyavirus proteins (L, G1, G2 and N) in extracts of La Crosse virus -infected cells. The La Crosse virus protein synthesis followed a pattern similar to Bunyamwera virus with early appearance of the N protein (2 hour p.i) and detection of G1 and G2 later in infection (4 hours and 6-8 hours post infection respectively). Protein synthesis was detectable up to 12-15 hours post infection.

Cash et al (1979) detected a single polycistronic m RNA of the M RNA segment, which codes for G1 and G2 (Gentsch and Bishop, 1979), in snow shoe hare virus-infected cells. This could suggest that the G1 and G2 proteins are cleavage products of a precursor protein. However, pulse-chase, short-labelling and amino acid analogue incorporation experiments did not detect any precursor polypeptides of Bunyamwera virus (Pennington et al, 1977).

In the bunya-like virus, Qalyub of the Nairobi sheep disease group, Clerx and Bishop (1981) detected large molecular weight glycoproteins in cell extracts which were not present in purified virus preparations. Similarly, Ulmanen et al (1981) have reported a precursor glycoprotein (molecular weight about 110×10^3 dalton) in an *in vitro* translation of Uukuniemi virus specified RNAs.

III.3 REPLICATION OF BUNYAVIRUSES: MATURATION OF VIRUS (MORPHOGENESIS AND MORPHOLOGY)

Existing knowledge on the maturation of bunyaviruses and bunya-like viruses is confined to electron microscopic studies of infected cells. Generally the bunyaviruses and bunya-like viruses appear to mature by budding from the Golgi complex and endoplasmic reticulum. Virus particles have been observed to accumulate within the cisternae of these cell organelles, particularly in the Golgi complex (Holmes, 1971; Lyons and Heyduk, 1973; Murphy et al 1968a, b; 1973). The extensive proliferation of the Golgi complex and vesicles (Lyons and Heyduk,

1973; Murphy et al, 1973) may be necessary to provide more budding surfaces for the replicated virus. Lyons and Heyduk (1973) observed crescent-shaped segments of thickened membranes and these were presumed to represent the initiation of the budding process. The inhibition of glycosylation of G1 and G2 proteins by tunicamycin and concomitant observation that no snow shoe hare virus particles were detected inside and outside drug-treated cells suggested that the release of intact infectious virus require glycosylation of G1 and G2 proteins (Cash et al, 1980).

Two main mechanisms of release of mature bunyavirus particles from infected cells have been suggested. The first mechanism involves fusion of virus-containing vesicles with the plasma membrane followed by expulsion of the virus particles by exocytosis. Consistent with this mechanism, Murphy et al, (1973) observed vesicles containing virus particles close to the plasma membrane as well as fused vesicles which had opened to the extracellular space in the vicinity of extracellular virus accumulation. Besides, a large number of extracellular virus particles have been observed in association with plasma membranes of intact infected cells at probable sites of release. The second mechanism of virus release which has been frequently reported is the rupture of plasma membrane and discharge of cytoplasmic contents as a final stage in cell lysis. Budding from the plasma membrane has been observed only infrequently (Holmes, 1971; Lyons and Heyduk, 1973; Murphy et al, 1968a, b; 1973).

Holmes (1971) has suggested that there could be a morphological transition between the intracellular and extracellular bunyavirus particles. Intracellular virus found near the Golgi complex appeared to contain a central fine filamentous material. Occasionally, a central dense spot (about 15 nm in diameter) was observed in intracellular virions. By contrast, the virions in the peripheral vesicles were uniformly dense like the extracellular virions. An electron-lucent envelope was present on extracellular virions. Murphy et al (1968a, b; 1973), however, did not observe such differences between intracellular and extracellular bunyavirus particles in infected mouse brain tissue. They rather observed that the extracellular virions appeared ragged and had a broad external layer (probably due to extension of the surface projections) whereas the intracellular virions had a smoother surface, probably due to compaction of the surface projections. Murphy et al (1973) occasionally observed a hexagonal arrangement of the projections on the surface of some bunyaviruses.

Bunyavirus particles are mostly spherical and sometimes oval (probably due to compression) and exhibit external surface projections (spikes; 5-10 nm long). Different size estimates have been obtained by different methods. Smithburn and Bugher (1953) estimated the size of Bunyamwera virus particle, by an ultrafiltration method, as 70-100 nm (diameter). Electron microscopic measurements of virions of various bunyaviruses estimated the sizes between 80 and 120 nm (diameter) with the mean

values ranging from 92 to 105 nm (Holmes, 1971; Lyons and Heyduk, 1973; Murphy et al 1968a, b;1973; Obijeski et al, 1976a; Southam et al, 1964; Stevenson and Holmes, 1972).

The morphology and morphogenesis of bunya-like viruses are essentially similar to bunyaviruses, hence the term bunya-like viruses. Jelinkova et al (1975) observed morphological and size differences in intracellular and extracellular Crimean hemorrhagic fever (CHF) virus particles and, like Holmes (1971), they concluded that there could be a morphological transition during maturation of CHF virus. Hexagonal arrangement of surface projections have frequently been observed in Uukuniemi virus preparations (Polson and Stannard, 1970; Saikku and von Bonsdorff, 1968; Saikku et al, 1970 and von Bonsdorff and Pettersson, 1975). Von Bonsdorff and Pettersson (1975) have also reported an irregularly arranged electron-opaque material (presumably the virus nucleocapsid) in a zone beneath the viral envelope in Uukuniemi virus-infected chick embryo fibroblasts. The size range of the virions of bunya-like viruses is comparable with that of bunyaviruses.

IV GENETICS OF BUNYAVIRUSES

Almost all the information available on bunyavirus genetics at the inception of the work reported in this thesis was obtained from studies with the California encephalitis group viruses. Much of that information will be reviewed later in a comparative discussion of these results with those obtained with viruses of the

Bunyamwera complex. Only a brief review of bunyavirus genetics will be given here.

IV.1 TEMPERATURE-SENSITIVE MUTANTS

The genetics of bunyaviruses have been investigated with temperature-sensitive (ts) mutants. No other types of mutants have been isolated. Only a few of the ts mutants used were spontaneous mutants. The rest were induced by chemical mutagenesis. J. Gentsch and D.H.L. Bishop (cited by Bishop and Shope, 1979) isolated spontaneous ts mutants of snow shoe hare virus at a frequency of 2.3%.

Temperature-sensitive mutants of different bunyaviruses - snow shoe hare, La Crosse, Trivittatus, Tahyna, Guaroa (Gentsch and Bishop, 1976; Gentsch et al, 1977b; 1979; 1980), Lumbo and Germiston (Ozden and Hannoun, 1978; 1980) viruses - have been variously induced with 5-Fluorouracil (5-Fu), 5-azacytidine (5-AZA) or N-methyl-N'- nitrosoguanidine (NTG). The highest percentage of ts mutants of snow shoe hare virus was isolated after 5-azacytidine treatment (Bishop, 1979; Bishop and Shope, 1979). The types of mutants isolated were similar irrespective of the mutagen used in their induction. The ts mutants of Lumbo virus were isolated from a heat-resistant population obtained after three passages at 39.2° C (Ozden and Hannoun, 1978). The other California group viruses were also cloned in BHK-21 cells at the restrictive temperature before mutagenisation (D.H.L. Bishop, personal communication).

The permissive and restrictive temperature of assay of the ts mutants varied. Ozden and Hannoun (1978) used 30° C (permissive temperature) and 38.8° C (restrictive temperature) to screen for Lumbo virus ts mutants, whereas the same authors used 32° C and 39.5° C as permissive and restrictive temperatures, respectively, for Germiston virus ts mutants (Ozden and Hannoun, 1980). The other viruses mentioned above were studied in the laboratories of D.H.L. Bishop in Birmingham, Alabama, U.S.A. and they employed 33° C and 39.8° C as the permissive and restrictive temperatures, respectively (Gentsch and Bishop, 1976; Gentsch et al, 1977b; 1979; 1980).

Generally, the ts mutants were obtained by isolation of clones at random from unselected plaques. Clones which after amplification at the permissive temperature showed an efficiency of plating (EOP; plaques at restrictive temperature/plaques at permissive temperature) of less than 10^{-3} and leak yield (one step growth at restrictive temperature/one step growth at permissive temperature) of less than 10^{-3} were retained for further genetic analysis (Gentsch and Bishop, 1976; Ozden and Hannoun, 1978).

IV.2 GENETIC RECOMBINATION

In a recombination experiment cell monolayers were infected singly and multiply with pairs of ts mutants and incubated for a period at the permissive temperature. The progeny of the culture was assayed at restrictive and permissive temperatures.

Recombination was assessed by determining the percentage of putative non-ts recombinants in the total yield from mixedly infected cultures. The recombination frequency was usually multiplied by 2 to take account of the double ts recombinants presumed to occur in equal proportions to the non-ts recombinants.

High frequency of recombination has been demonstrated in both homologous and heterologous crosses of bunyavirus ts mutants. In the homologous crosses of ts mutants of La Crosse, snow shoe hare, Tahyna, Trivittatus and Guaroa viruses, the recombination frequencies ranged from 1% to 45% (Gentsch and Bishop, 1976; Gentsch et al, 1979; 1980). Ozden and Hannoun (1978, 1980) obtained recombination frequencies of 10-42.1% (Lumbo virus ts mutants) and 1-40% (Germiston virus ts mutants). The high frequencies of recombination suggested reassortment of genome subunits as is the case in orthomyxoviruses, reoviruses, and arenaviruses, other RNA viruses which possess segmented genomes (to be reviewed in section V). Contrary to the expectation that a tripartite genome would yield three groups of recombining ts mutants, the ts mutants of the bunyaviruses cited above belonged to only two recombination groups. A clonal analysis of the non-ts clones (putative recombinants) isolated at the restrictive temperature showed that they were indeed genetically stable wild type recombinants and not heterozygotes or aggregates of both parental ts mutants which complemented to give plaques at the restrictive temperatures (Gentsch and Bishop, 1976; Gentsch et al, 1977b).

Temperature-sensitive progeny obtained from recloning the putative non-ts recombinants occurred at frequencies not more than the spontaneous mutation rate 1.7-2.3% (Gentsch et al, 1977b; see also Bishop, 1979; Bishop and Shope, 1979).

High frequency heterologous recombination was obtained with certain combinations of California encephalitis group viruses, that is; crosses of La Crosse and snow shoe hare, La Crosse and Tahyna, snow shoe hare and Tahyna, La Crosse and Trivittatus and Tahyna and Trivittatus viruses (Gentsch et al, 1977b; 1979; 1980). No heterologous recombination was detected between ts mutants of snow shoe hare virus and Trivittatus virus. Except in the crosses of La Crosse and Tahyna virus ts mutants, heterologous recombinations in the California encephalitis group were generally uni-directional For example, recombinants were obtained in the cross of snow shoe hare virus group I ts mutant (SSH I) and La Crosse virus group II ts mutant (LAC II) but not in the reciprocal cross of snow shoe hare virus group II ts mutant (SSH II) and La Crosse virus group I ts mutant (LAC I). La Crosse virus ts mutants (LAC I and LAC II) recombined bi-directionally with Tahyna virus ts mutants (TAH I and TAH II) in that there was recombination in both LAC I x TAH II and LAC II x TAH I crosses (Gentsch et al, 1980). The fairly high frequencies of heterologous recombination (3-9%), again, indicate reassortment of the genome subunits of different California group viruses. The RNA composition of non-ts clones (putative

heterologous recombinants), isolated at the restrictive temperature, have been analysed by oligonucleotide fingerprinting and shown to be true recombinants. For example, Gentsch et al (1977b) isolated two types of recombinants from the LAC II x SSH I cross and showed that their respective genotypes were SSH/LAC/LAC and SSH/LAC/SSH (in the order, L/M/S RNAs). These results enabled the authors to assign their recombination groups to specific genome subunits. Details, including the merits, of these assignments will be included in the discussion of this thesis.

In addition, a comparison of the genotypes and phenotypes of the recombinants and their parental wild type viruses resulted in assignment of proteins to specific genome subunits. Only the N polypeptides of La Crosse and snow shoe hare virus were distinguishable by SDS-polyacrylamide gel electrophoresis. A comparison of the polypeptides of the recombinants and their parental wild type viruses both by SDS-polyacrylamide gel electrophoresis and by tryptic peptide analysis of their N proteins showed that the SSH/LAC/LAC recombinant exhibited the N protein of La Crosse virus whereas the SSH/LAC/SSH recombinant possessed an N protein derived from snow shoe hare virus. From these observations, Gentsch and Bishop (1978) concluded that the S RNA subunit codes for the N protein. This observation was confirmed by <u>in vitro</u> translation of the S RNA subunit of snow shoe hare virus to N protein (Cash et al, 1979). Gentsch and Bishop (1979) have compared the tryptic peptide digests

of the G1 and G2 of the SSH/LAC/SSH recombinant and their parental wild type viruses (La Crosse and snow shoe hare viruses). Their results showed that the above recombinant possessed G1 and G2 proteins derived from the La Crosse virus parent. Since the only contribution of the La Crosse virus ts mutant to the genotype of the recombinant is the M RNA subunit, it was concluded that the M genome subunit codes for both the G1 and G2 proteins (Gentsch and Bishop, 1979).

IV.3 COMPLEMENTATION

Gentsch and Bishop (1976) were able to demonstrate complementation between snow shoe hare virus ts mutants from different recombination groups. Complementation has also been reported between recombining ts mutants of Lumbo virus (Ozden and Hannoun, 1978). No complementation has been reported to occur among ts mutants belonging to the same recombination groups (apart from the results reported in this thesis; Results Section II.2.2). It would be expected, however, that ts mutants of the recombination group with mutation(s) in the M subunit would complement among themselves since the M subunit codes for the two unique glycoproteins G1 and G2.

V. GENETIC RECOMBINATION IN OTHER RNA (ANIMAL) VIRUSES

Two types of recombination have been described among animal viruses. The first involves reassortment of genome subunits of those viruses which possess segmented genomes. The second is intramolecular recombination which has been reported in DNA viruses as well as

in two positive strand RNA viruses, poliovirus and foot-and-mouth disease virus (picornaviruses).

V.1 RECOMBINATION BY REASSORTMENT

Apart from the bunyaviruses mentioned in section IV.2, high frequency recombination by reassortment of genome subunits has also been observed in other viruses with fragmented RNA genomes such as reoviruses (Fields and Joklik, 1969), orbiviruses (Gorman et al, 1978; Walker et al, 1980), orthomyxoviruses (Simpson and Hirst, 1968), the arenaviruses (Vezza and Bishop, 1977; Vezza et al, 1980). Reovirus will be described as an example of viruses with genomes of segmented double-stranded (ds) RNA, whereas, influenza virus (orthomyxovirus) will be taken as an example of viruses with segmented single-stranded (ss) RNA genomes.

REOVIRUSES: Reovirus possesses a genome made up of ten unique ds RNA segments. The ds RNA segments fall into three size classes designated L (3 segments), M (3 segments) and S (4 segments) with total molecular weight of about 15×10^6, all enclosed in a double-shelled protein capsid (Bellamy et al, 1967; Shatkin et al, 1968; Watanabe et al, 1968). One of the two RNA strands is designated a positive strand because it is of similar polarity as the messenger RNA.

A brief description of the mechanism of reovirus replication is essential to the understanding of the mechanism of genetic reassortment. Replication of reovirus involves partial uncoating of the ds RNA genome,

sufficient to allow the virion-associated transcriptase to transcribe each of the ten genome subunits into single stranded (ss) RNA molecules (Shatkin et al, 1968; Watanabe et al, 1968). It is worth mentioning at this point that the ss RNA is the positive strand and that the negative strands have never been found free in the cytoplasm. They are copied from preformed positive strand transcripts and remain associated with the latter to yield ds RNA molecules (Acs et al, 1971). Thus, the synthesis of the negative strand is the same event as the synthesis of progeny ds RNA molecules and it occurs within particulate structures containing all ten ss RNA species (Zweerink et al, 1972). A greater proportion of the ss RNA is, however, associated with polysomes and functions as m RNA, (Bellamy and Joklik, 1967). Since the ds RNA never exists in a free or naked form (not enclosed in a protein-shelled particulate structure) at any time in the replication cycle, the reassortment of RNA molecules, most probably, occurs at the level of single-stranded RNA (Cross and Fields, 1977).

Genetic interaction between ts mutants of reoviruses have been extensively studied. Fields and Joklik (1969) detected high frequency of recombination (3% to 50%) among certain pairs of reovirus type 3 ts mutants, whereas in other pairs the recombination frequency was zero. In this way, seven (A-G) out of the ten recombination groups presently recognised, were initially defined (Cross and Fields, 1972; Fields and Joklik, 1969). Ramig and

Fields (1979) isolated 28 revertants of the ts mutants of each of the above seven groups and backcrossed them with parental wild type viruses. From this, they were able to distinguish the true revertants (three in number) from pseudorevertants (25 in all) in which the expression of the parental ts lesion had been suppressed by mutation in another gene (extragenic suppressor mutation). The majority of the extragenic suppressor mutations detected were non-ts and were identified only by their effect on the phenotype of the original ts lesion. However, non-parental ts lesions were also rescued by the backcross and probably represented suppressor mutations with ts phenotype. The new, non-parental, ts mutants included those that recombined with ts mutants of all the previously defined recombination groups. These were classified into two new recombination groups designated H and I. The tenth recombination group, J, was defined by Ahmed et al (1980). Members of this group were rescued from pseudorevertants, obtained from L cells which had been persistently infected with ts mutants, by backcrossing the pseudorevertants with the wild type reovirus. The "all-or-none" mode of recombination and the non-detection of any statistical difference between the recombination frequencies obtained (Fields, 1971) indicated that recombination was by reassortment of the genome subunits. Reassortment occurred early in the replication cycle and was multiplicity dependent (Fields, 1971). Although the proportion of noninfectious to infectious particles in the reovirus stocks used by Fields was very high (50-100 : 1), the non-infectious particles did not take

part in genetic recombination. Spandidos and Graham (1976) have observed that although defective virus lacking the L_1 genome RNA subunit could complement some reovirus type 3 ts mutants, they could not recombine with them. High frequency heterologous reassortment has also been reported between reovirus types 1, 2 and 3 and has been used, advantageously, in conjuction with phenotypic differences between the virus types, in mapping the reovirus genome (Sharpe et al, 1978).

ORTHOMYXOVIRUSES: The orthomyxoviruses are the influenza viruses of man and animals (Melnick, 1973). The high rate of genetic recombination detected after co-infection of cells with genetically distinct influenza viruses led Hirst (1962) to postulate that influenza viral genome was segmented and that the recombination was by reassortment of such segments. Subsequently, the genomes of influenza viruses were shown to consist of 8 negative ss RNA segments (Bean and Simpson, 1976; Palese and Schulman, 1976a; 1976b; Pons, 1976; Ritchey et al, 1976). McGeoch et al (1976) showed by oligonucleotide mapping that each of the eight segments was unique. Several workers have demonstrated genetic reassortment in crosses of influenza strains of human origin (Kilbourne et al, 1967) or human and/or animal influenza A viruses (Kilbourne, 1969; Almond et al, 1979). Almond and Barry (1979) have been able to construct genetic maps by comparison of the electrophoretic and phenotypic properties of heterologous recombinants derived from two strains of fowl plague virus (avian

influenza viruses). The most easily detected reassortants of influenza viruses are those bearing the ribonucleoprotein of one parent and either the hemagglutinin or the neuraminidase of the other (Easterday et al, 1969; Laver and Kilbourne, 1966). Webster (1970) has demonstrated that recombinants with desired antigenic characteristics could indeed be "tailored" and selectively isolated. Genetic reassortment between human influenza A viruses, and between human and animal influenza viruses is of epidemiological importance.

Simpson and Hirst (1968) were the first to demonstrate genetic recombination between ts mutants of influenza virus. Like the reoviruses, there was high frequency of recombination which occurred in an "all-or-none" fashion (5-20%). Although Sugiura et al (1975) have only reported seven recombination/reassortment groups of influenza A virus Wisconsin strain, Hirst (1973) reported eight groups. Like reoviruses, reassortment occurs early in the replication cycle but unlike the reoviruses non-infectious (defective) particles of influenza virus partake in genetic recombination (Hirst, 1973; Hirst and Pons, 1973; MacKenzie, 1970; Simpson and Hirst, 1968; Sugiura et al, 1972) hence the disproportionately high recombination frequencies obtainable at very low multiplicities of infection (Hirst, 1973).

V.2 INTRAMOLECULAR RECOMBINATION

Recombination in the DNA viruses is outside the scope of this introduction. Before the brief review of

the evidence for intramolecular recombination in the picornaviruses, poliovirus and foot-and-mouth disease virus which follows, it may be necessary to mention a few properties of the positive-strand RNA viruses in general. In contrast to the negative strand RNA viruses, the genomes of the positive strand RNA viruses are of the same polarity as the messenger RNA and can indeed serve as messengers in a cell-free translation system (Cancedda and Schlesinger, 1974; Simmons and Strauss, 1974, Smith et al, 1970). The RNA is infectious and since the viruses possess no virion-associated RNA polymerase, an RNA-dependent RNA polymerase is synthesized de novo in the cell and is specified by the viral genome RNA (Baltimore and Franklin, 1963; see also review by Pfefferkorn and Shapiro, 1974). There is a translation of polycistronic gene product followed by its cleavage into functional polypeptide units (Burrell et al, 1970; Holland and Kiehn, 1968; Jacobson and Baltimore, 1968; Summers and Maizel, 1968; Waite, 1973). Base-paired, double-stranded replicative forms consisting of the negative strand and positive strands have been reported (Montagnier and Sanders, 1963).

THE PICORNAVIRUSES

The only examples of intramolecular recombination among viruses with unsegmented RNA genomes have been poliovirus (genus Enterovirus) (Cooper, 1968, Cooper et al, 1971; Granoff, 1962; Hirst, 1962; Ledinko, 1963) and foot-and-mouth disease virus (FMDV; genus Aphthovirus) (Lake et al, 1975; MacKenzie et al, 1975; McCahon et al, 1977; Pringle, 1965; 1968). Ledinko (1963) detected

recombination in poliovirus at frequencies up to 0.4%. It was also observed that, with equal input multiplicities, the recombination frequency was time dependent in that it increased from 0.2% at the time of appearance of the first progeny virus to 0.4% at the completion of viral replication cycle. Similarly, Cooper (1968) observed an increase in recombination from 0.28% at 3 hours to 0.42% at 7 hours post infection. Recombination frequencies as high as 3.27% have been detected in foot-and-mouth disease virus, using improved standardisation techniques (McCahon et al, 1977).

The uniqueness of the above intramolecular recombinations among unsegmented RNA genomes makes it desirable to ascertain that they are indeed true recombination by demonstration of reciprocal recombinants in single, mixed-infected cells. Although this has neither been demonstrated in FMDV (Slade and Pringle, 1971) nor in poliovirus (Cooper, 1977), the results obtained over the years have consistently indicated recombination. Besides, the genetic map constructed for poliovirus (Cooper, 1968; Cooper et al, 1971) accommodates information obtained from recombination, physiological and inhibitor studies. King et al (1980) have also established the co-linearity of the genetic map (McCahon et al, 1977) and the physical map of FMDV genome. The exact mechanism of the recombination in picornaviruses is not known but it may be similar to the mechanism of recombination of DNA viruses which involves covalent breakage and enzymatic rejoining of nucleic acid molecules. Appropriate enzymes (similar

to the endonucleases and ligases in DNA virus-infected cells) for analogous processing of RNA have yet to be identified. An alternative means suggested could be the copy-choice mechanism (Cooper, 1977).

VI. EXPERIMENTAL DESIGN

This project was undertaken to determine whether viral genes could be freely exchanged between viruses of the Bunyamwera complex (Bunyamwera group).

Batai virus, Bunyamwera virus and Maguari virus were chosen for the investigation because these three viruses were isolated from, and appeared to be confined to, different continents (see Figure 1).

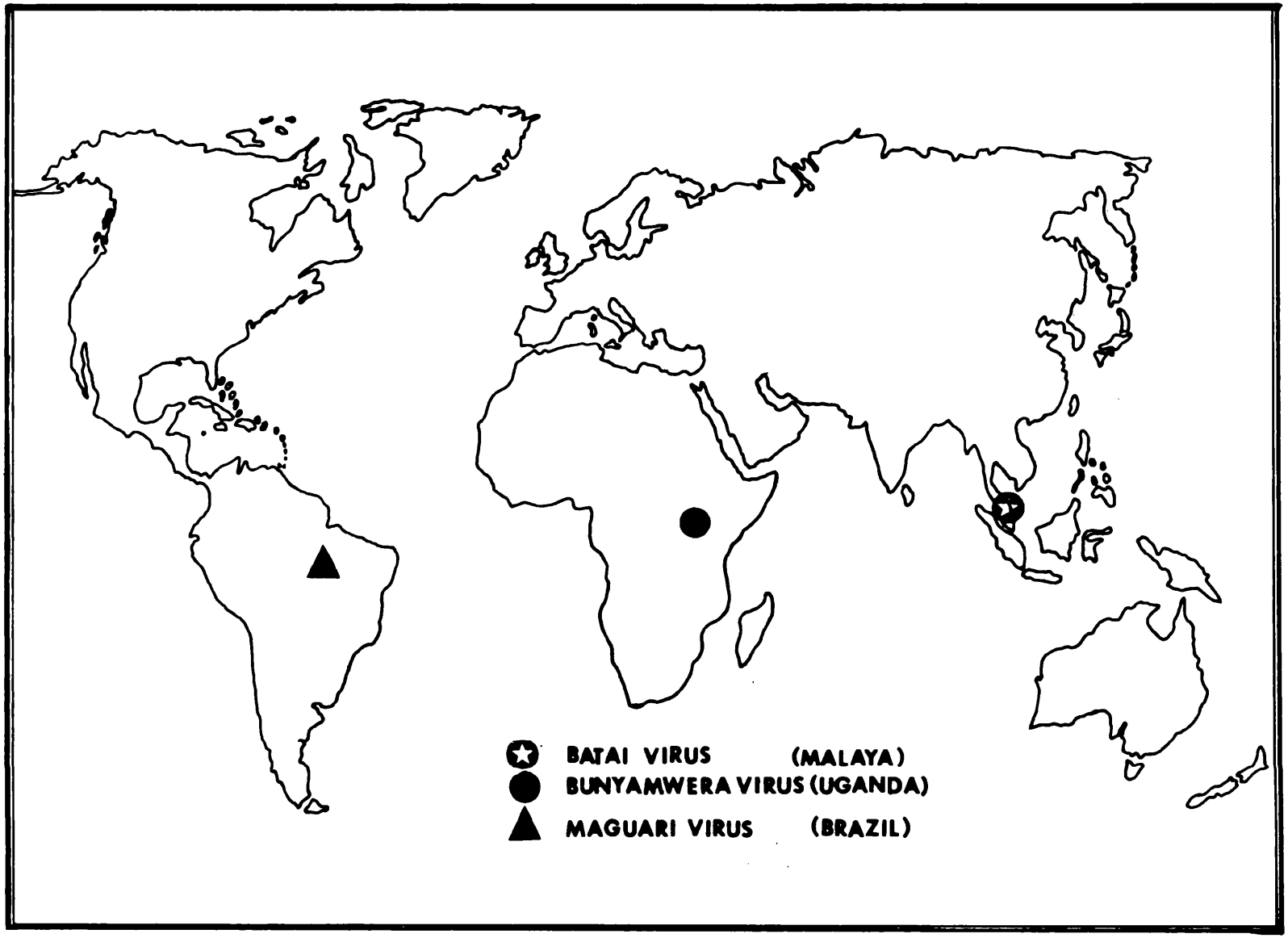
Batai virus was first isolated in Malaya from Culex gelidus mosquito by the US Army Medical Research Unit (Theiler and Downs, 1973). Later, virus strains, serologically indistinguishable from Batai, were isolated in India, Japan and Sarawak (Chittoor Strain), Czechoslovakia, Yugoslavia and the Ukrainiam S.S.R. (Calovo strain) (Berge, 1975; Theiler and Downs, 1973). From serological studies, it appears that Batai virus and its variants are prevalent in human and animal populations throughout its range (Berge, 1975).

Bunyamwera virus was first isolated by Smithburn et al (1946) from a mixed pool of Aedes mosquitoes captured in an uninhabited part of Bwamba County, Uganda, Africa. Subsequent isolations have been made from man and mosquitoes in Uganda, Kenya, Cameroon and Nigeria (Berge, 1975). Serological surveys show that, up to 80% of the inhabitants of certain areas in Africa had

FIGURE 1

Geographical areas in which Batai, Bunyamwera and Maguari viruses were first isolated, respectively.

Fig·1


BATAI VIRUS (MALAYA)
BUNYAMWERA VIRUS (UGANDA)
MAGUARI VIRUS (BRAZIL)

previously been infected by the virus (Berge, 1975).

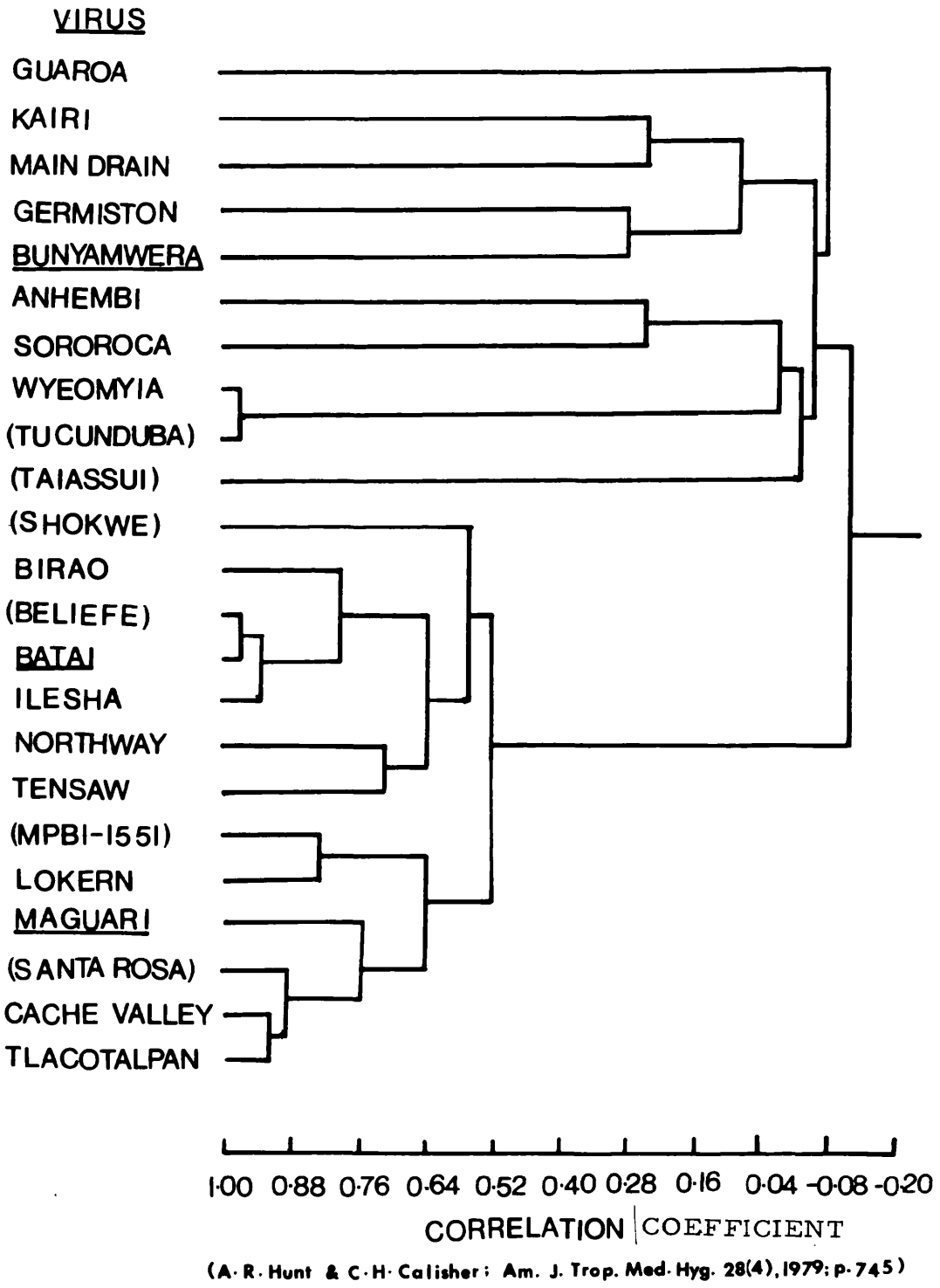
Maguari virus was first isolated from a mixed pool of mosquitoes captured in Utinga Forest, Brazil, in 1957 (Causey et al, 1961). Subsequently, it was isolated from a variety of insects in Brazil, Trinidad, Ecuador, Colombia and Argentina as well as from horses in French Guiana and Colombia and sentinel mice in Brazil (Berge, 1975). Although it has not been isolated from humans, serological surveys indicate that Maguari virus infection of man and animals is wide-spread in South and Central America.

Hunt and Calisher, (1979), have analysed 23 viruses of the Bunyamwera group by plaque reduction neutralisation (PRN) tests. Their results reveal a gradation in antigenic relatedness of members of this group as shown in Figure 2. This gradation in antigenicity has warranted the classification of Bunyamwera group viruses into five subdivisions based on their antigenic relatedness. Seventeen of these viruses are included in the Bunyamwera complex, and three in the Wyeomyia Complex. The other three viruses, Main Drain, Kairi and Guaroa stand singly in separate subdivisions (Hunt and Calisher, 1979). At a correlation coefficient of about 0.53, (Figure 2), Batai and Maguari viruses are more closely related to one another than either is to Bunyamwera virus (a negative correlation shown in Figure 2). Although Bunyamwera and Germiston viruses have been included in the Bunyamwera Complex pending more extensive PRN tests with different antisera, both viruses showed limited cross-reactions with other viruses of the group, thus indicating that

FIGURE 2

Dendrogram showing antigenic relationships among 23 Bunyamwera group viruses. (Reproduced from A.R. Hunt and C. H. Calisher; Am. J. Trop. Med. Hyg. 28(4), 1979; p. 745).

Fig. 2



(A. R. Hunt & C. H. Calisher; Am. J. Trop. Med. Hyg. 28(4), 1979; p. 745)

Bunyamwera and Germiston viruses might constitute a separate subdivision (Complex) (Hunt and Calisher, 1979). On ecological grounds, Hunt and Calisher (1979) have also suggested that Batai virus, which is the only member of the group found in both Europe and Asia, may represent an evolutionary and geographical link between the African and American members of the Bunyamwera group.

METHODS

A. CELLS

The BS-C-1 line of African green monkey kidney cells (Hopps et al, 1963) was used by Dr. C. R. Pringle for initial cloning of the bunyaviruses and preparation of the wild type virus stocks. A continuous cell line derived from baby hamster kidney cells, BHK-21 clone 13 (MacPherson and Stoker, 1962) was supplied by the Cytology Unit, Institute of Virology, Glasgow, and used for other purposes including mutagenesis and radiolabelling of virus. All cell cultures were maintained by propagation in roller bottles with Eagle's medium (Glasgow modification) supplemented with 10% calf serum.

Monolayers were prepared as follows : The roller bottle cultures were washed twice with 20 ml amounts of versene and left for 3 minutes with a residual film of versene (about 3-5 ml) at 37° C. Trypsin-versene (5 ml) was then added and used to detach the cells from the walls of the bottle. Cell clumps were dispersed into single cells by repeated pipetting through a fine-bore pipette. The trypsinised cells were resuspended in appropriate volumes of Eagle's medium and used to prepare the following cultures as shown in table 5 .

Nunc disposable Petri dishes and tissue culture flasks as well as the disposable Linbro microwell plates were purchased from Flow Laboratories Ltd., Irvine, Ayrshire, Scotland. All cultures were 90 - 100% Confluent after incubation for 12 - 24 hours at 37° C in an atmosphere of 5% carbon dioxide.

TABLE 5

CULTURES	CELLS	
	BKK-21	BS-C-1
Petri dish (50 mm)	2×10^6 cells/dish	1×10^6 cells/dish
Petri dish (30 mm)	10^6 cells/dish	0.5×10^6 cells/dish
One-ounce (30 ml) bottles	2×10^6 cells/bottle	1×10^6 cells/bottle
20-ounce (500 ml) bottles	9×10^6 cells/bottle	5×10^6 cells/bottle
Microwells (Linbro, 2 cm^2)	1.5×10^5 cells/well	0.75×10^5 cells/well

* The passage history, in suckling mice, of the three viruses investigated.

Virus	Number of passages in suckling mice.
Batai	4
Bunyamwera	10
Maguari	3

B. VIRUSES AND ANTISERA

1. VIRUSES

Batai, Bunyamwera and Maguari viruses were obtained originally as suckling mouse brain homogenates* from Dr. N. Karabatsos, Vector-borne Diseases Laboratory, Centre for Disease Control, Fort Collins, Colorado, U.S.A. Each virus was then cloned by Dr. C. R. Pringle in the Institute of Virology, Glasgow by three sequential plaque isolations from single plaques on BS-C-1 monolayers. The clones were amplified into working virus stock in one-ounce (30 ml) bottle cultures of BS-C-1 cells. The monolayers were infected with cloned virus at a multiplicity of about 0.1 pfu per cell and incubated for 30 minutes at 31° C to absorb the virus. Then the cultures were further incubated at 31° C in 5 mls of Eagle's medium supplemented with 10% Calf serum and harvested 3 - 4 days later when the cytopathic effect (CPE) was complete. All the stocks were assayed for infectivity at 31° C, 34° C, 37° C, 38° C and 39° C to determine their titres and the temperature range for growth of the viruses.

2. ANTISERA

Homologous antisera in the form of mouse ascitic fluids were also provided by Dr. Karabatsos.

C. ASSAY OF INFECTIVITY

Virus-containing fluids were diluted in ten-fold series up to 10^{-6} and 0.2 ml of each dilution was used to infect BHK-21 monolayers in 50 mm tissue culture Petri dishes. After 30 minutes absorption at 31° C, the infected monolayers were overlayed with 0.6% (w/v) agarose in

Eagle's medium containing 2.5% (v/v) calf serum. Incubation was continued for 4 - 5 days in a Co_2-gassed incubator. Then the monolayer was fixed with 1% glutaraldehyde in phosphate buffered saline for 4 - 24 hours. The plaques were counted after removing the agarose overlay and staining the monolayer with Giemsa.

For virus isolation, the monolayers were overlayed with a second agarose overlay containing 0.02% (w/v) neutral red (stain) on the fourth day of incubation. The cultures were returned to the 31° C incubator and left overnight to allow the neutral red to diffuse through the agarose layer and stain the cells underneath. Then the plaques, appearing as unstained circular patches against a red background of stained cells, were picked using Pasteur pipettes. The film of cell debris together with a small plug of agarose was flushed into 2 ml of chilled Eagle's medium containing 10% calf serum and stored at -70°C.

D. MUTAGENISATION OF VIRUS AND ISOLATION OF TEMPERATURE-SENSITIVE (ts) MUTANTS

1. MUTAGENISATION

Monolayer cultures of BHK-21 cells in one-ounce bottles were infected with wild type virus at a multiplicity of about 0.1 pfu per cell and incubated for 30 minutes at 31° to absorb the virus. The excess inoculum was removed by gently washing the monolayer three times with 5 ml amounts of prewarmed Eagle's medium. Then a 5 ml volume of incubation medium

containing either 50, 100, 200 or 400 ug of 5-Fluorouracil (5-Fu (Sigma Chemical Company, London, UK) per ml was added and incubation was contnued at 31° C. The cultures were harvested when complete CPE was observed in control cultures infected as above but incubated in the absence of 5-Fu. All harvests were assayed for infectivity and stored frozen at -70° C.

2. ISOLATION OF ts MUTANTS

The mutagenised virus (or the unmutagenised virus in the case of isolation of spontaneous ts mutants) was appropriately diluted to obtain one to three plaques per plate (estimates for the appropriate dilutions were derived from the results of preliminary infectivity assays). A volume of 0.2 ml of the diluted virus was used to infect BHK-21 monolayer cultures in 50 mm Petri dishes. The monolayers were overlayed with agarose containing medium and plaques were later picked after incubation for 4 days at 31°C as described in Section C. The only plaques picked were apparently single plaques at least 20-30 mm from other plaques. These clones were screened for their ability to form plaques at 31° C (the permissive temperature) and at 38° C (the restrictive temperature). Those clones which produced confluent or numerous plaques at 31° C, and a few or no plaques at 38° C were amplified by a single passage in BHK-21 cells and retested. The clones which showed a relative efficiency of plating (plaques at 38° C/plaques at 31° C) of 10^{-3} or less were accepted as ts mutants.

E. ASSAYS FOR RECOMBINATION (REASSORTMENT)

Monolayers of BHK-21 cells in multiwell plates (2.5×10^5 cells per 2 mm^2 well) were infected in duplicate either with individual ts mutants at a multiplicity of 5 pfu per cell or multiply with pairs of ts mutants at a combined multiplicity of 10 pfu per cell. The inoculum was adsorbed for 45 minutes at $+4^{\circ}$ C and then 1 ml of incubation medium was added to each well. After 4 hours incubation at 31° C this medium was removed and the monolayers washed twice by aspiration to remove the unabsorbed virus. Fresh prewarmed medium was added and the incubation was continued at 31° C for an additional 14 hours. The cultures were harvested by freeze-thawing and assayed for plaque-formation at 31° C and 38° C. The recombination (reassortment) frequency (RF) was calculated as a percentage according to the formula quoted by Gentsch and Bishop (1976).

$$RF = \frac{(AB)^{38^{\circ}} - (A + B)^{38^{\circ}}}{(AB)^{31^{\circ}}} \times 2 \times 100$$

where A and B are any pair of ts mutants, and the superscript is the temperature of assay. The factor 2 was introduced in the formula on the assumption that two types of recombinants were produced; that is, the wild type (non-ts) recombinant and the recombinant with a double ts genotype derived from both parents. These recombinants were assumed to be produced in equal proportions. The assay at 38° C, however, only detects the wild type recombinants.

F. ASSAYS FOR COMPLEMENTATION

Duplicate monolayers of BHK-21 cells in multiwell plates were infected singly or multiply as in section E. Adsorption was carried out at +4° C for 45 minutes after which 1 ml of Eagles medium was added to each well and cultures were incubated for 2 hours at 39° C in a Co_2-gassed incubator. The latter procedure was followed in order to achieve rapid absorption and thorough removal of unabsorbed virus. The unabsorbed virus was removed by three washes with incubation medium which was prewarmed at 39° C. Then 1 ml of prewarmed medium was added to each well and the cultures were sealed with a Linbro cover (sheet) with adhesive back measuring approximately 14.9 x 9.8 cm (Flow Laboratories, Ltd., Irvine, Ayrshire, Scotland). Incubation was continued for further 16 hours with the cultures totally immersed in a precision water bath (Grant Instruments (Cambridge) Ltd., Cambridge, UK) at 38° C or 38.5° C (according to the ts mutants involved). All cultures were harvested by freeze-thawing and assayed for infectivity at both the permissive (31° C) and the restrictive (38° C or 38.5° C) temperatures. The complementation index (CI) was calculated according to the formula used by Pringle (1970a)

$$CI = \frac{(AB)^{31} - (AB)^{38(38.5)}}{(A + B)^{31}}$$

where A and B are any pair of ts mutants and the superscript is the temperature of assay.

G. NEUTRALISATION ASSAY

Each wild type virus and the recombinant clones which were isolated from the heterologous crosses (see results, section IL3) were mixed with homologous and heterologous antisera and held at $+4^{\circ}$ C for 18 hours. The residual infectivity after the above treatment with antisera was measured by plaque assay at 31° C. A control series was included which consisted of virus samples mixed with diluent and held at $+4^{\circ}$ C for 18 hours. All neutralisation assays were carried out in duplicate. The anti-Batai virus and the anti-Bunyamwera virus sera were used at a final dilution of $\frac{1}{50}$ each. The anti-Maguari virus serum was used at a final dilution of $\frac{1}{20}$.

H. RADIOLABELLING OF VIRAL PROTEINS

BHK-21 cell monolayers in 20-oz (500 ml) bottles were infected with virus at a multiplicity of 5 pfu per cell and absorbed for 30 minutes at 31° C. The incubation (Eagle's) medium was added and the culture incubated for 6 hours at 31° C. Then the incubation medium was replaced with 25 ml of Eagle's medium (containing 2.5% calf serum) in which L-methionine had been substituted by 5 uCi $\left[{}^{35}S\right]$ - methionine (Radiochemical Centre, Amersham, UK) per ml. The specific activities of the $\left[{}^{35}S\right]$ - methionine, when freshly supplied, ranged from 1000Ci per m. mol to 1100Ci per m. mol. After incubation for 3 - 4 days post infection the culture fluids were collected when CPE was maximal. The virus containing fluids were clarified by centrifugation at 10,000rpm for 10 minutes. The supernatant

fluids were centrifuged at 20,000 rpm for 1 hour to sediment the virus. All centrifugation was done using a sorvall type SS 34 rotor in a sorvall RC-5B Centrifuge (Dupont Instruments, Newton, Connecticut U.S.A.). The centrifuge tubes were drained carefully and the virus pellet resuspended directly in 75 ul of electrophoresis dissociation buffer (samples destined for polyacrylamide gel electrophoresis) or in 2.5 ml of NTE buffer pH 8.0 (samples for sucrose gradient centrifugation).

I. POLYACRLAMIDE GEL ELECTROPHORESIS

$[^{35}S]$ - methionine-labelled virus preparations were dissociated and the proteins separated on vertical slab gels using the procedure of SDS-polyacrylamide gel electrophoresis described by Marsden et al (1976), except that the same running buffer was used in the upper as well as in the lower reservoirs. Virus pellets were resuspended directly in the electrophoresis dissociation buffer (described in Appendix I, 8.8; see also section H, above) and heated at 100° C for 5 minutes before loading onto the slab gel.

The discontinuous gels used were either single concentration (7.5% or 10%) gels or 6 - 15% linear gradient gels. The most commonly used single concentration gel was made up of 7.5% acrylamide/0.2% bisacrylamide in 0.25 M Tris-HCl, pH 8.9 containing 0.1% SDS and 0.1% ammonium persulphate. Polymerisation was initiated by addition of TEMED (0.4ul per ml of gel solution). The composition of

the alternative single concentration gel was 10% acrylamide/0.25% bisacrylamide, 0.4 M Tris-HCl pH 8.9, 0.1% SDS and 0.1% ammonium persulphate. TEMED was added as above to initiate polymerisation.

The 6 - 15% linear gradient gels were made up with the solutions shown in table 6 below. The gels (gradient gels) were poured using a Gradco peristaltic pump (Gradco International Ltd., Winchester, Hunts UK).

The stacking gel was composed of 5% acrylamide/0.13% bisacrylamide in 0.25 M Tris-HCl buffer, pH 6.7, containing 0.1% SDS and 0.1% ammonium persulphate. TEMED was also added as above to initiate polymerisation. The composition of the electrophoresis (running) buffer is given in Appendix I, 8.7.

All the slab gels were formed and maintained between two parallel glass plates separated by two 2 mm thick perspex spacers (one on each side) and sealed on three sides with Scotch Teflon tapes (3 M Company, St. Paul, Minnesota, U.S.A.). The running (resolving) gel solution (which when set measured approximately 16 x 16 x 0.2 cm) was poured first and allowed to polymerise. Then a twelve-tooth Teflon Comb was inserted between the glass plates and above the limits of the running (resolving) gel. The stacking gel solution was poured to fill the spaces between the combs and about 1 hour later when the gel polymerised, the comb was carefully withdrawn leaving 12 slots into which the samples could be loaded.

Approximately 10 - 30 ul of each sample was loaded into each of the slots beginning from the second slot.

The first slot was always left blank and served as a marker for orientation of the gel. Generally, therefore, eleven out of the twelve slots were loaded with samples. Electrophoresis was for 3 - 4 hours at 40 mA per gel at $+4^{o}$ C. Gels were stained with 0.2% Comassie brilliant blue and dried under vacuum using a BioRad slab gel drier (BioRad Laboratories, Rickmond, California, U.S.A.) for 90 minutes. Radioactive bands were located by autoradiography using Kodirex 40T film (Eastman Kodak Company, Rochester, N.Y. USA).

TABLE 6

Materials (solutions)*		6%	15%
30% acrylamide + bis (29.25% acrylamide + 0.75% bisacrylamide)	(ml)	19.2	48
2.25 M Tris-HCl pH 8.9	(ml)	16	16
20% SDS	(ml)	0.5	0.5
Deionised Water	(ml)	60.8	32
10% Ammonium persulphate (freshly prepared)	(ml)	1	1
TEMED	(µl)	40	40

* The volumes of solutions quoted above were sufficient to prepare two gels.

J. PARTIAL PURIFICATION OF VIRUS

1. CONCENTRATION OF VIRUS BY POLYETHYLENE GLYCOL PRECIPITATION

Four roller bottle cultures of BHK-21 cells were infected with virus at a multiplicity of 0.01 pfu per cell.

After 48 hours incubation at 31° C, the incubation medium was replaced with L-methionine-free Eagle's medium containing 5 uCi $\left[{}^{35}S\right]$ - methionine per ml and 2.5% calf serum. Incubation was continued for an additional 48 hours at 31° C or until CPE was extensive. The culture fluid was clarified by centrifugation at 10,000 rpm for 10 minutes using a sorval SS34 rotor and the RC-5B Centrifuge. The virus in the supernatant was concentrated by an overnight precipitation at +4° C with polyethylene glycol 6000 (PEG) added to a final concentration of 6% (w/v). Precipitation was done in the presence of 0.5 M sodium chloride and the precipitate was sedimented by centrifugation at 10,000 rpm for 10 minutes as above. The virus-containing precipitate was resuspended in 2.5-3 ml of NTE (buffer) solution.

2. GRADIENT PURIFICATION OF VIRUS

Concentrated virus suspensions were partially purified by centrifugation in a 20 -65% continuous sucrose gradient. The gradients were prepared in Beckman Cellulose nitrate centrifuge tubes (No. 302237, Beckman Instruments, Glenrother, Fife, Scotland) as follows.

A 2-ml 65% sucrose cushion was laid down, followed by 8 mls each of 60%, 40%, 30% and 20% sucrose solutions. All solutions were layered, one over the other in the above sequence, by gently running the sucrose solution down the side of the tube to avoid mixing by turbulence at the interface of the solutions. The layered solutions were left overnight at +4° C to allow transformation of the stepwise gradient into a continuous 20 - 65% gradient by diffusion.

A virus suspension (about 2.5 ml) was loaded onto the 20 - 65% continuous sucrose gradient and centrifuged at 22,000 rpm and +4° C for 12 hours using a Beckman AH627 swing-out rotor (Beckman Instruments, Glenrother, Fife, Scotland) in a Sorvall OTD-50 ultracentrifuge (DuPont Instruments, Newton, Connecticutt, U.S.A.). Portions of the gradient containing opaque band(s) were normally collected by piercing through the tube to reach the band with a B-D Iuer-lok disposable syringe needle (18G1½TW, guage) fitted to a 10-ml B-D disposable syringe (Beckton, Dickinson (B-D) and Company Ltd., Dublin, Ireland). The virus-containing portion of the gradient (the virus band) was diluted in NTE buffer and concentrated by centrifugation at 20,000 rpm and +4° C for 60 minutes using a Sorvall SS34 rotor and the RC-5B centrifuge.

The only exception to the above band-collection procedure was during a preliminary characterisation of gradients when fractionation was carried out. Thirty-five 1-ml fractions were collected from one of the gradients (total volume of each gradient including the virus sample being about 36.5 ml) containing Maguari virus sample. Each fraction was analysed to determine the distribution of virus infectivity and radioactivity throughout the gradient.

The absorbance of each fraction was determined at 680 nm (visible light wavelength) in a C595 double beam digital U-V spectrophotometer (Cecil Instruments, Cambridge, UK) in order to locate the fractions corresponding to the observed bands. To measure the radioactivity, 10 ul of

each fraction was dropped onto a 2.5 cm disc of Whatman No. 1 filter paper (W & R Balston Ltd, London, England). The discs were airdried and held in chilled 5% TCA for 15 minutes (with intermittent swirling of the TCA and its contents). This was followed by three seperate 5-minute washes in chilled 5% TCA and two washes (5 minutes each) in ice-cold absolute alcohol. The discs were then dried under a 275-watt infra red reflector lamp. Samples were finally immersed seperately in 5 ml of toluene-PPO mixture (scintillation fluid) and the radioactivity counted in a Beckman LS 330 scintillation counter (Beckman Instruments, Glenrother, Fife, Scotland). The virus infectivity in each fraction was determined by plaque assay technique as described in the methods (section C).

K. PREPARATION OF VIRUS FOR ELECTRON MICROSCOPY

Electron microscopy of virions was carried out with the assistance of Mr J. E. Parry, Institute of Virology, Glasgow.

Virus samples were negative-stained by mixing equal volumes of the virus suspension (in NTE buffer pH8.0) and sodium silicotungstate (EMSCOPE supplies, Ashford, Kent, England). A 5 µl volume of the mixture was placed on a copper grid, 200 mesh size (TAAB supplies, Reading, England) and allowed to stain for 3 minutes after which excess sample was drained by blotting with a Whatman No. 1 filter paper. The grid was viewed in an Elmiskopf 101 electron mioroscope (Siemens and Halske, Karlsruhe) at an accelerating voltage of 80 kV.

RESULTS

R E S U L T S

1.. GROWTH KINETICS OF BATAI, BUNYAMWERA AND MAGUARI VIRUSES

Monolayer cultures of BHK-21 cells in multiwell (Linbro, 2.00 mm^2) plates were infected in duplicate with wild type virus at a multiplicity of 5pfu per cell. After adsorption at $+4^{o}$ C for 30 minutes, the excess inoculum was removed and the monolayers washed twice by aspiration of two changes of fresh medium. Prewarmed medium was added and the cultures were incubated at 31^{o} C in a carbon dioxide-gassed incubator. The entire cultures (culture medium and the cell monolayer together) were harvested at two-hour intervals up to 34 hours by freezing and thawing. The yield of infectious virus was measured by the plaque assay method as described in the Methods (Section C). A multiplicity of 5pfu per cell was used to ensure that every cell was infected by at least one virus particle. Adsorption at $+4^{o}$ C was designed to achieve synchrony of penetration, uncoating and replication.

Figures 3a, b and c show the single-step growth curves of Batai, Bunyamwera and Maguari viruses respectively. After an eclipse phase lasting 4 - 6 hours, the virus titre increased exponentially, reaching maximum titres at about 22 hours post infection.

Lyons and Heyduk (1973) observed that in mosquito cell cultures, the ratio of released to cell-associated California encephalitis virus, 24 hours after infection, was 5.8 : 1 whereas in Vero cells the yield of cell-

FIGURE 3

Growth curves of:

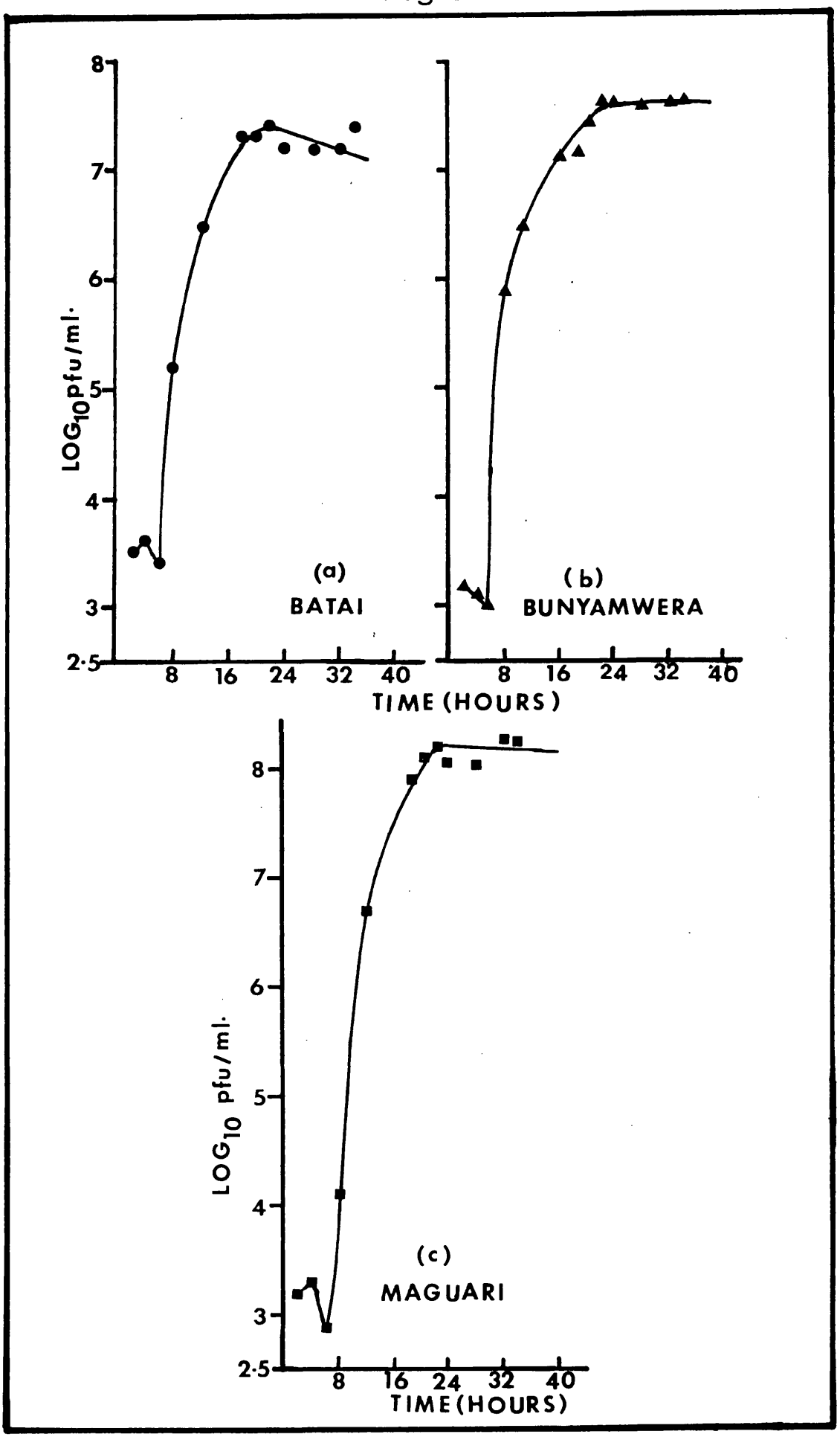
a) Batai virus (●——●)

b) Bunyamwera virus (▲——▲)

c) Maguari virus (■——■)

Monolayer cultures of BHK-21 cells were infected at multiplicity of about 5 pfu of virus per cell and incubated at 31° C. Culture were harvested at 2-hour intervals up to 36 hrs p.i. and assayed at 31° C for infectivity.

Fig·3


8
7
6
5
4
3
2·5
LOG10Pfu/ml·
(a)
BATAI
(b)
BUNYAMWERA
8 16 24 32 40
TIME (HOURS)
8
7
6
5
4
3
2·5
LOG10 pfu/ml·
(c)
MAGUARI
8 16 24 32 40
TIME (HOURS)

associated virus was almost the same as the released virus giving an approximate ratio of unity. Therefore, in order to study the kinetics of release of Maguari virus from mammalian cells, monolayer cultures of BHK-21 cells in multiwell plates were infected as described for the single-cycle growth experiments. The cultures were harvested at two hourly intervals for a period of 34 hours to determine the proportion of released and cell-associated virus. The titre of the released virus was obtained by assay of infectivity in the culture supernatants. The titre of cell-associated virus was obtained by scraping the monolayer into 1ml PBS, following a preliminary wash in 1ml of PBS. The paired samples were assayed immediately after harvest or stored frozen at -70° C. A control culture infected as above was harvested as a unit (i.e. culture fluid and monolayer together) at the same two hour intervals as the released and cell-associated viruses. All samples were assayed for infectious virus by plaque assay at 31° C.

The rate of release of Maguari virus from 2 hours to 34 hours post infection is shown in Figure 4. There was progressive increase of cell released virus between 6 and 32 hours. Figure 5 compares the yield of released and cell-associated virus at various times after infection. For most of the exponential growth phase (6 to 20 hours) the yield of released virus closely paralleled the cell-associated virus. As shown in Figure 6, the sum of the released and

FIGURE 4

Kinetics of virus release from Maguari virus-infected BHK-21 cell monolayers.

Released infective virus is expressed as a percentage of the total yield of infective virus [i.e. (released virus)/(released virus + cell-associated virus) x 100].

Fig. 4

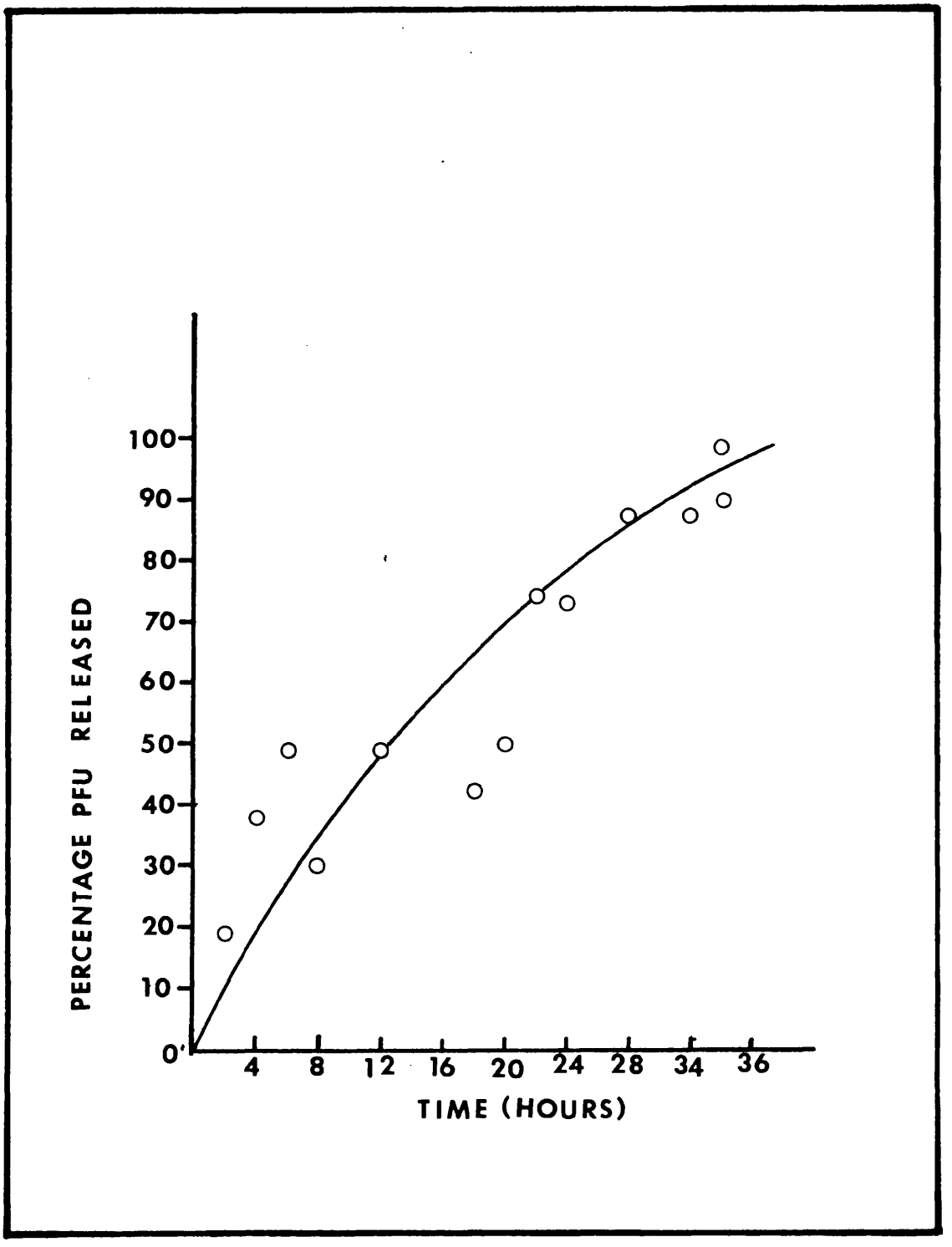

PERCENTAGE PFU RELEASED
100
90
80
70
60
50
40
30
20
10
0
4
8
12
16
20
24
28
34
36
TIME (HOURS)

FIGURE 5

Kinetics of Maguari virus release from infected BHK-21 cell monolayers. Comparison of yields of released virus and cell-associated virus during a single cycle of virus replication.

The yield of cell-released virus (O——O) was determined by harvesting the culture medium and assaying it at 31° C for virus infectivity.

To determine the yield of cell-associated virus (■----■), the monolayer was washed 3 times with PBS after harvesting the culture medium. Subsequently the culture medium was replaced with an equivalent volume of PBS and cell-associated virus was harvested by scraping the monolayer followed by assay of infectivity.

Fig.5

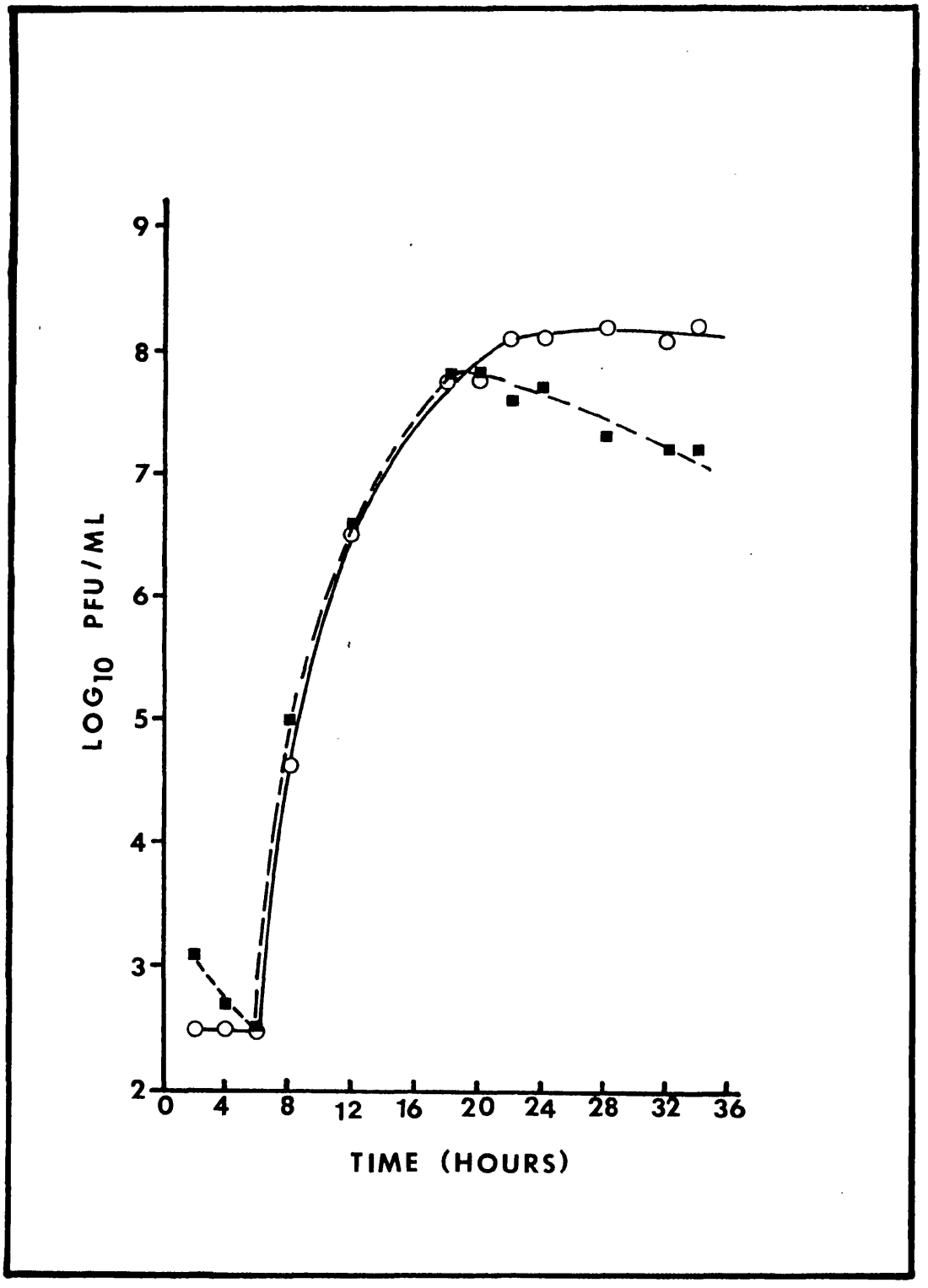


FIGURE 6

Comparison of the total yield of infectious Maguari virus from BHK-21 cell monolayers at different periods of incubation estimated by

i) sum of released virus and cell-associated virus yields (O——O);

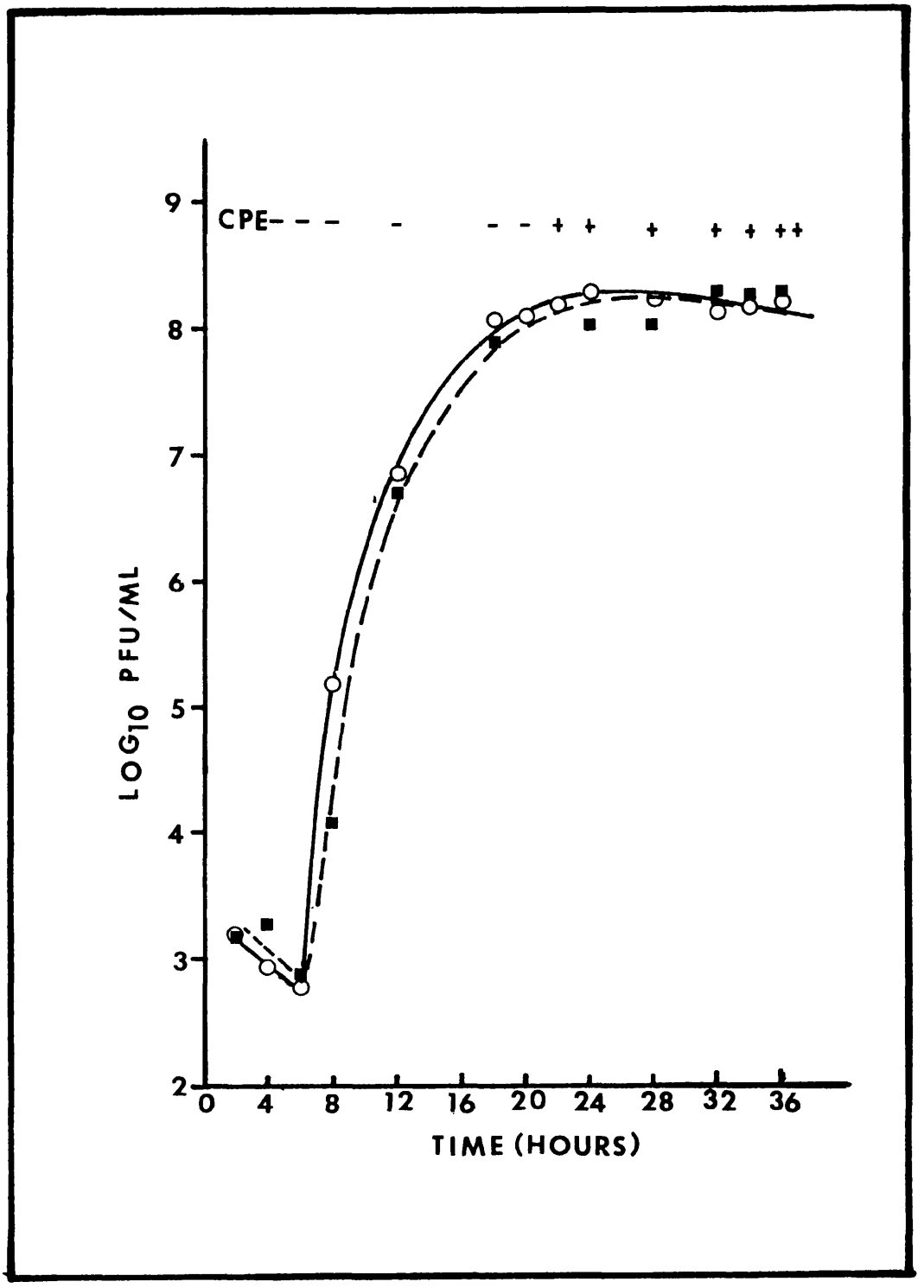
AND

11) yield of infected BHK-21 cell monolayer with culture medium and monolayer harvested together (control culture). (■- - -■)

- absence of CPE

+ presence of CPE

Fig. 6



cell-associated virus (calculated total yield) was about the same as the actual total yield obtained by harvesting the whole culture without fractionation. These showed that there was a fairly rapid release of Maguari virus from the infected BHK-21 cells. The efficiency of release was comparable to that of California encephalitis virus from Vero cells rather than that of CE virus from arthropod (mosquito) cells (Lyons and Heyduk, 1973). There was an apparent decay in the yield of cell-associated virus after 18 hours post infection without a simultaneous increase in the yield of released virus (Figure 5). The cause of this decrease was not determined but can be accounted for by loss of cells dislodged from the monolayer in the process of washing after the onset of cytopathic effect (CPE).

Cultures were also examined at two hourly intervals for CPE. The CPE of Maguari virus produced rounding and progressive detachment of cells from the substrate. No CPE was detected until 22 hours post infection. At this time only a few cells were involved but progressively more cells rounded and detached and by 34 hours post infection about 50% of the cells had become involved. At a multiplicity of 5 pfu per cell, Maguari wild type virus produced approximately 100% CPE at about 60 to 72 hours post infection.

1.2 LOCATION OF THE VIRUS-CONTAINING BAND IN THE CONTINUOUS SUCROSE GRADIENT

The procedure for partial purification of virus by centrifugation in a 20 - 65% continuous sucrose gradient has been described in the Methods (Section J). After centrifugation the gradient was fractionated and the fractions were analysed for virus infectivity and radioactivity as well as absorbance as described in the Methods (Section J.2)..

As shown in Figure 7 three opaque bands were observed; one (band-1) in the upper half of the gradient, the other (band-2) in the lower half and the third band (band-3) below band-2. There were three peaks of absorbance corresponding to the three opaque bands as follows; fractions 15-25 (band-1), fraction 7-11 (band-2) and fraction 3-6 (band-3). The highest peak (fraction 7-11) corresponded to band-2. There was a single infectivity peak (fraction 8-11) and it was in the zone of band-2. The highest radioactivity peak was in fraction 7-11 corresponding to the peak of infectivity and the band-2 zone. One radioactivity peak, fraction 4-6, corresponded with band-3 but there was no radioactivity peak corresponding to band-1 (fractions 15-25). There was a small peak of radioactivity in fraction 13-16 at the leading edge of band-1. This may be due to labelled cellular components or defective virus particles. The high radioactivity in fraction 29 may be due to other labelled cell materials or residual radioactivity from the growth medium.

FIGURE 7

Location of virus-containing band in 20-65% continuous sucrose gradient centrifugation of PEG-precipitated Maguari virus preparation.

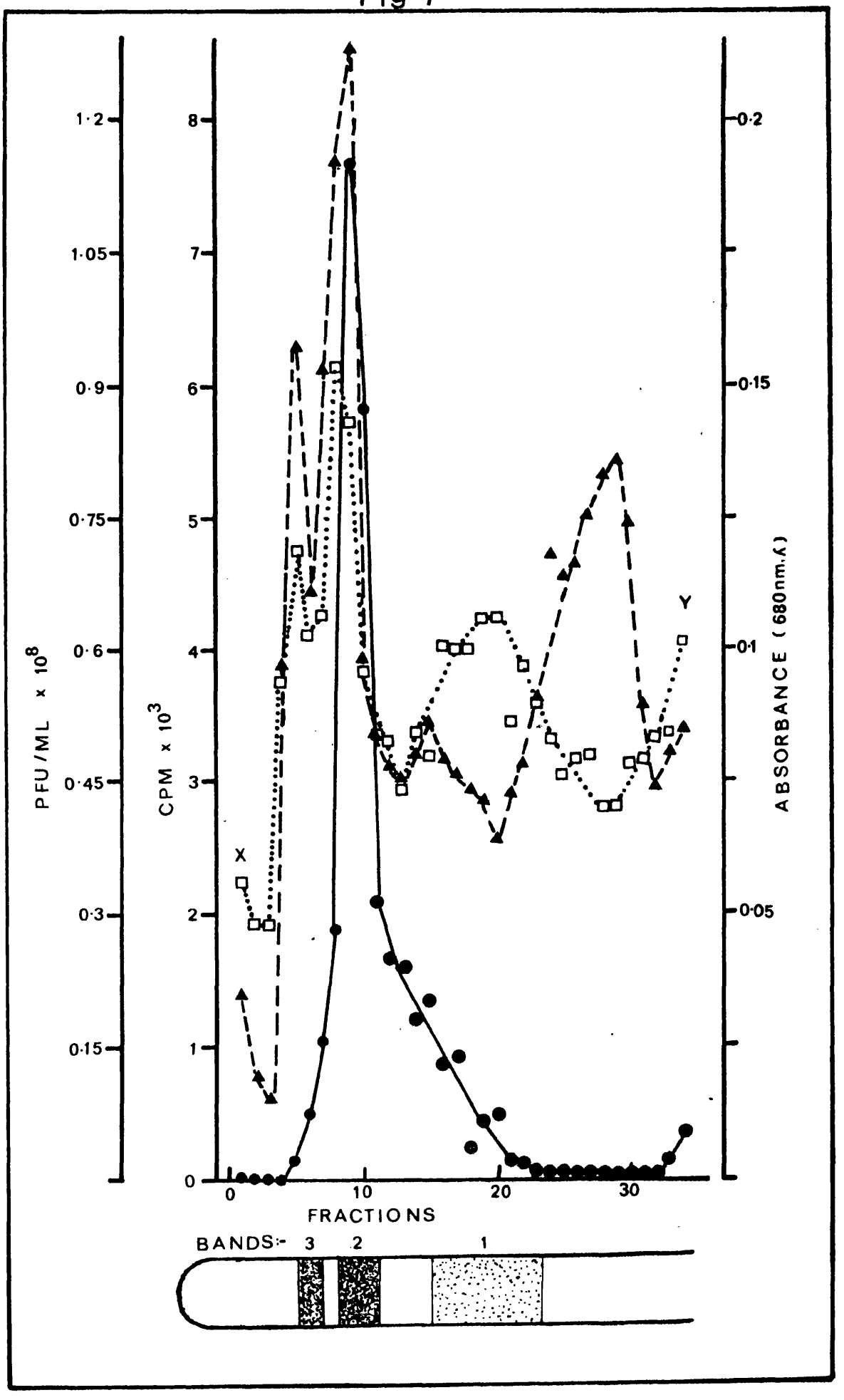
The sucrose gradient was fractionated in 1 ml amounts and each fraction analysed for:

a) absorbance at 680 nm (λ) (□.....□) to locate the fractions containing materials from the three opaque bands (1, 2 and 3) observed.

b) [^{35}S]-methionine-labelled protein (▲-----▲)

c) infectivity (●——●) to locate the band (1, 2 or 3) containing most of the infective virus.

Fig·7


PFU/ML x 10^8
CPM x 10^3
ABSORBANCE (680nm.A)
FRACTIONS
X
Y
BANDS:- 3 2 1

The relatively high absorbance in the regions marked X and Y and the slightly increased infectivity around Y may be due to the sediments at the bottom of the tube, Y occurring at the top as pelleted debris was stirred up during removal of the last few fractions. These results indicated that band-2 contained most of the infectious virus.

There was virtually no loss of infectivity by centrifugation at 10,000 rpm for 10 minutes to clarify the culture fluid (see Table 7a). About 97% of the infectivity in the clarified virus-containing fluid was recovered after PEG-precipitation. Approximately 60% (2.8×10^8 pfu) of the total virus infectivity in sucrose gradient (about 4.6×10^8 pfu) was recovered from band-2 (Table 7b).

The fractions representing each band were pooled (fractions 3-6 for band-3; 7-11 for band-2 and 14-24 for band-1) and concentrated by centrifugation at 20,000 rpm for 60 minutes at $+4^{\circ}$ C. The resulting pellets were resuspended in 20 µl of NTE buffer and samples were examined under the electron microscope as described in the Methods (Section K). Virions were observed in the band-2 sample as well as in the band-1 sample but the latter contained a considerable amount of debris whereas the band-2 sample contained little or no debris (Figures 8a, b and 9a, b). The virions observed by negative staining were mostly spherical in shape and measured approximately 72 nm (average diameter). No virions were observed in the

FIGURE 8:

Electron microscopy of partially purified Maguari virus preparation.

a) Electron micrograph of materials obtained from band-2 (fractions 8-11) of the 20-65% continuous sucrose gradient centrifugation. The arrow shows Maguari virions.

b) A magnified electron miscrograph of two virions from a partially purified Maguari virus preparation.

Note the fairly clear background compared with Figures 9a, b.

FIG 8

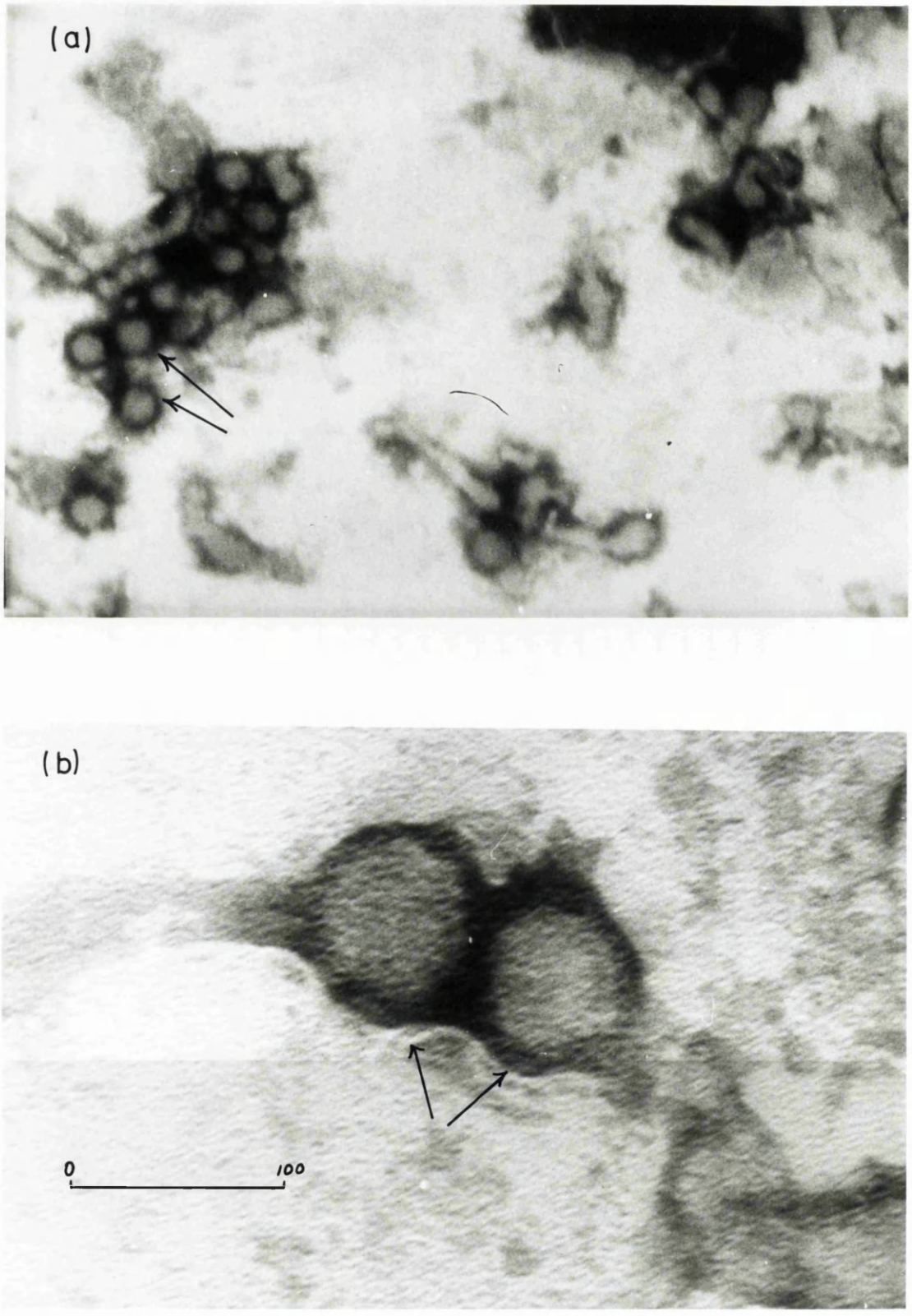


FIGURE 9

Electron microscopy of partially purified Maguari virus preparation.

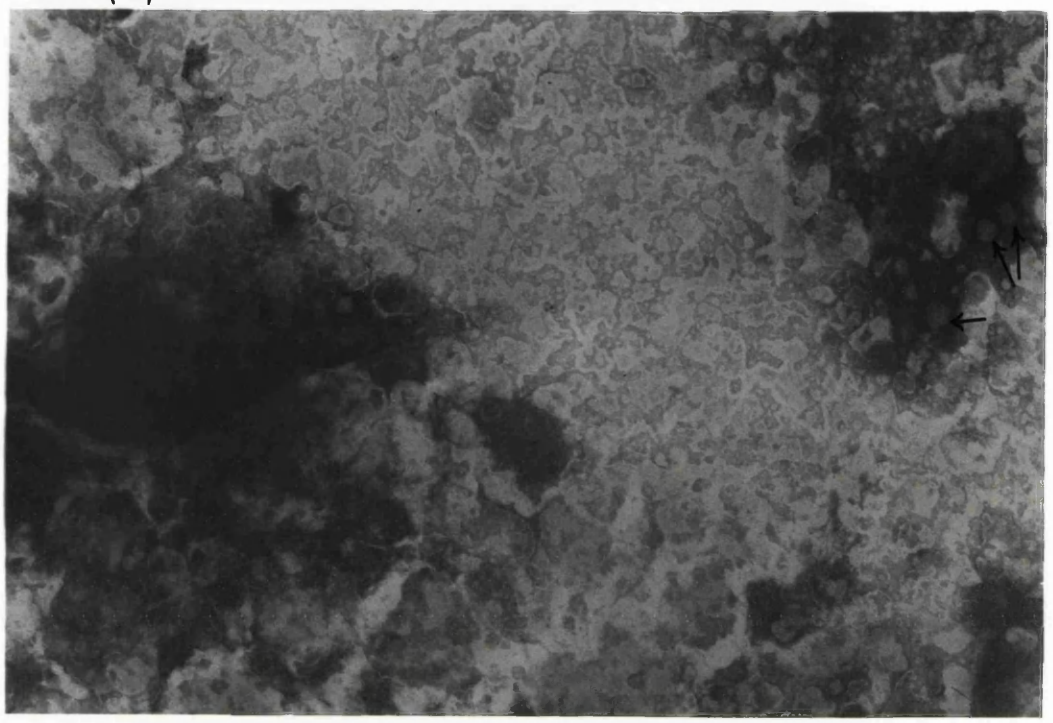
a) Electron micrograph of materials obtained from band-1 (fractions 14-24) of the 20-65% continuous sucrose gradient centrifugation.

b) A magnification of part of (a) above to show the virions more distinctly.

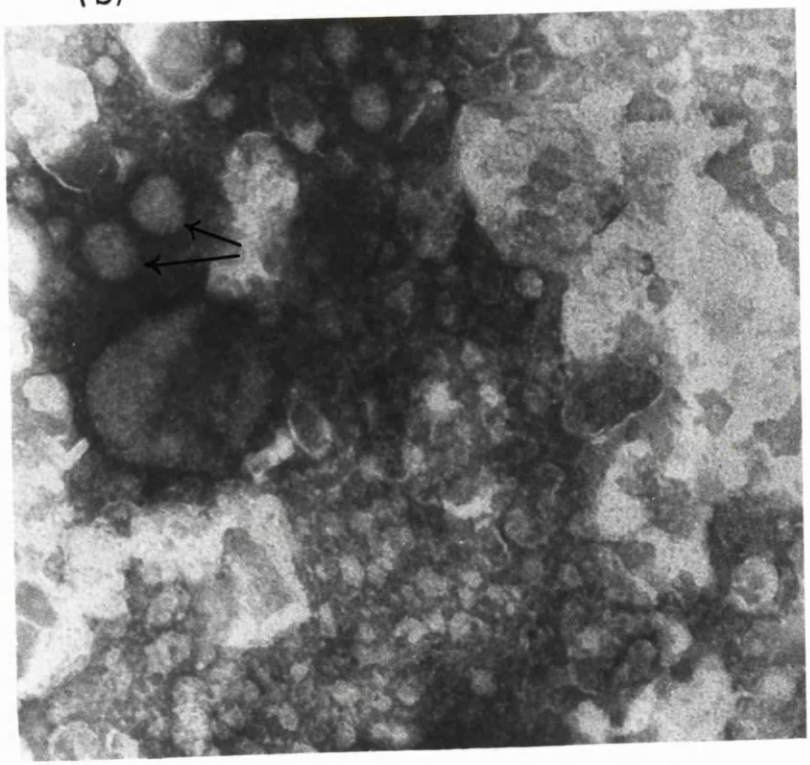
The arrows show the virions:
Note the cellular materials at the background and compare with Figures 8a, b.

FIG. 9

(a)



(b)



TABLES 7a, b.

Recovery of infectious virus at different stages in the partial purification of Maguari virus preparation.

a) Infectious virus recovered after ;

(i) clarification of culture fluid by centrifugation at 10,000 r.p.m for 10 minutes at +4°C.

(ii) overnight PEG-precipitation of virus at +4°C.

b) Proportion of infectious virus recovered from band-2 (the single infectivity peak)*.

* PEG-precipitated virus was resuspended in 2.5 ml. of NTE buffer and sonicated for about one minute to break up the precipitates. Then the suspension was centrifuged at 3,000 r.p.m. to remove unresuspended clumps. 1 ml. of the supernatant was diluted to 2.5 ml and then loaded onto 20 - 65% sucrose gradient.

TABLE 7a

SAMPLE	pfu per ml	Total volume	Estimate of total infectious virus	Percentage recovery
Unclarified culture fluid	5.8×10^7	150	8.7×10^9	--
Clarified culture fluid	6×10^7	150	9×10^9	100
PEG-precipitated virus	3.5×10^9	2.5	8.7×10^9	97

TABLE 7b

SAMPLE	pfu per ml	Total volume	Total infectious virus (approx.)	Percentage recovery
Total virus in gradient*	1.4×10^7	34 ml	4.6×10^8	100
Virus present in band-2 (approx.)	5.7×10^7	5 ml	2.8×10^8	60%

band-3 preparation. These observations indicated that band-2 contained virions and little cellular materials. Therefore, it was considered that one cycle of gradient centrifugation achieved partial purification of these bunyaviruses.

II. GENETICS OF BUNYAMWERA COMPLEX

II.1 MUTAGENISATION AND ISOLATION OF TS MUTANTS

Conditional lethal mutants, temperature-sensitive mutants in particular, have been valuable tools in genetic characterisation of viruses. They are very suitable for genetic studies because a single phenotype (e.g. temperature sensitivity) encompasses mutations affecting any indispensable function of the viral genome.

Ts mutants were obtained by induction by chemical mutagenesis using 5-Fluorouracil (5-Fu). The procedure involved infecting monolayers of BHK-21 cells in one-ounce (30 ml) bottles with wild-type virus and incubating at 31° C in the presence of 5-Fu. The cultures were harvested 3 - 4 days later when the CPE in the infected unmutagenised control cultures were observed to be complete (see Methods, Section D, for details). Different concentrations of 5-Fu (50, 100, 200 or 400 µg/ml) were used. The cultures were assayed for virus infectivity (plaque production) at 31° C as described in the Methods (Section C). All cultures were in duplicate . The depression in the yield of

infective virus in the mutagenised cultures was estimated as follows:

$$\text{Infectivity depression } (\%) = \frac{n - m}{n} \times 100$$

where n and m represent the yields (pfu/ml) of the unmutagenised and the mutagenised cultures respectively. Figures 10a, b and c show that 5-Fu caused considerable reduction in the yield of each virus even at 50 μg per ml concentration.

The permissive temperature used in all experiments was 31° C. Although Batai and Bunyamwera viruses produced plaques at 39° C, a temperature of 38° C was chosen as the restrictive temperature because Maguari virus produced plaques at 38° C but not at 39° C (Table 8).

The procedures for isolation of clones and screening for temperature sensitivity have been outlined in the Methods (Section D.2). A total of 1,883 clones were isolated from the three viruses and screened for temperature sensitivity (ability to produce plaques on BHK-21 cell monolayers at 31° C but not at the restrictive temperature of 38° C). Table 9 shows the number of clones of each virus screened and the respective numbers (and percentages) of ts mutants isolated. Three hundred and twenty two Batai virus clones were screened. Eighteen (5.6%) of these were scored as temperature sensitive in the initial screening but after further testing only 5 (1.6%) were acceptable ts mutants.

FIGURE 10

Depression in the yield of infective virus by different concentrations of the mutagen, 5-Fu (µg/ml).

a) Batai virus (●——●)

b) Bunyamwera virus (■——■)

c) Maguari virus (O——O)

Fig·10

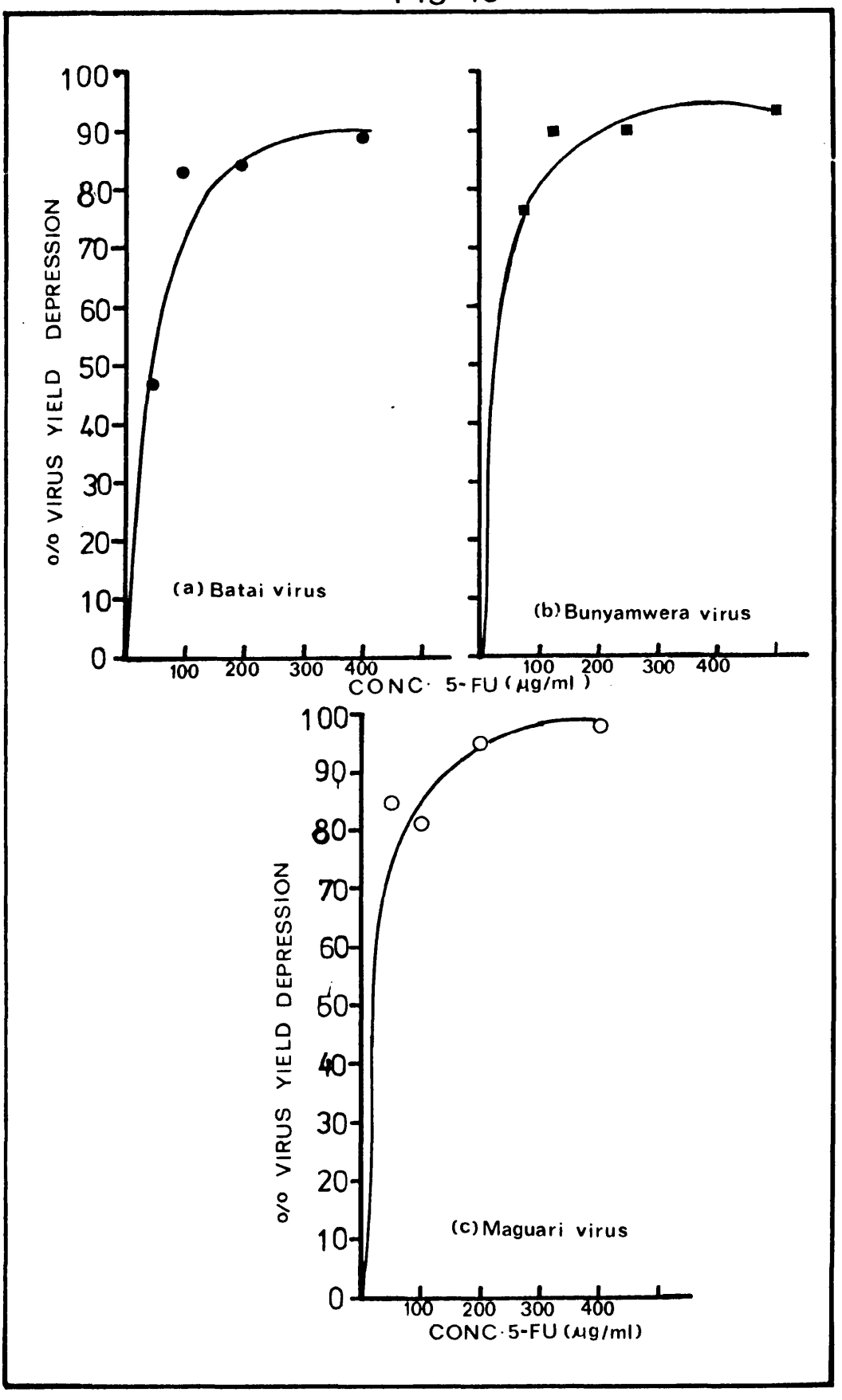

100
90
80
70
60
50
40
30
20
10
0
% VIRUS YIELD DEPRESSION
(a) Batai virus
(b) Bunyamwera virus
100 200 300 400
CONC· 5-FU (μg/ml)
(c) Maguari virus
100 200 300 400
CONC·5-FU (μg/ml)

TABLE 8

Plaque forming ability of Batai, Bunyamwera and Maguari viruses in BHK-21 cell monolayers at 31°, 37°, 38° and 39° C.

VIRUS	TITRE (pfu/ml) ESTIMATED BY PLAQUE ASSAY AT				E.O. P. at 38° C
	31°	37°	38°	39°	
BATAI	7×10^6	6.8×10^6	6.1×10^6	6×10^6	0.9
BUNYAMWERA	3.4×10^7	1.8×10^7	1.5×10^7	1.3×10^7	0.4
MAGUARI	1.08×10^7	8.3×10^6	1.4×10^6	<10	0.13

TABLE 9

Number of clones screened and number of ts mutants isolated

VIRUS	No. of clones screened	Concentration of 5-Fu used (µg/ml)	Number of ts clones isolated in the initial screening	Percentage of ts clones in the initial screening	Number of clones finally accepted as ts mutants	Percentage of stable ts mutants
BATAI	322	50	18	5.6	5	1.6
BUNYAMWERA	188	50	21	11.2	8	4.3
MAGUARI	1,373	0-200	166	12.1	50	3.6

Similarly, out of the 188 Bunyamwera virus clones screened, 21 (11.2%) were temperature-sensitive in the initial screening but only 8 (4.3%) were finally accepted as ts mutants. The 1,373 clones of Maguari virus yielded 166 (12.1%) temperature sensitive clones in the initial screening but only 50 (3.6%) were accepted as ts mutants. Thus in all three viruses only about one-third of the ts clones initially identified were sufficiently stable for further study. Maguari virus was chosen as the reference virus (hence the comparatively large number of clones screened) because it produced larger plaques and replicated to higher titres at 31° C. The ts mutants finally isolated from the three viruses have been listed in Tables 10, 11 and 12 respectively. Only those with an efficiency of plating of 10^{-3} and below were considered suitable for use in these investigations. The efficiency of plating of these viruses ranged from 10^{-4} to 10^{-8} (see Tables 10, 11 and 12). Leaky or reverting mutants were recloned. A good number of the ts clones could not attain the required (working) titres, 10^{7} - 10^{8} pfu per ml, when they were amplified. They were, therefore passaged a few more times to obtain higher titres. The passage levels at which the ts mutants were most frequently used have also been included in Tables 10, 11 and 12.

The 1,373 clones of Maguari virus were isolated from cultures treated with different concentrations of 5-Fu (in the range 50-200 µg/ml) as well as from

TABLE 10

The Maguari virus/ts mutants. Efficiency of plating $\left(\frac{\text{pfu at } 38^{\circ}\text{ C}}{\text{pfu at } 31^{\circ}\text{ C}}\right)$

VIRUS		ASSAY OF INFECTIVITY (TITRE) AT		PASSAGE LEVEL	EFFICIENCY OF PLATING
OLD CODE	NEW CODE	31°	38°		
tsM32	MAGts1(I)	1.8×10^{7}	<10	2	$< 5.6 \times 10^{-7}$
ts484	MAGts2(I)	3.5×10^{7}	3×10^{2}	2	8.57×10^{-6}
ts577	MAGts3(I)	4.5×10^{7}	<10	4	$<2.2 \times 10^{-7}$
tsM848	MAGts4(I)	1.9×10^{8}	2×10^{3}	RECLONED	10^{-5}
tsM1051	MAGts5(I)	3.0×10^{8}	3.1×10^{3}	3	1.042×10^{-5}
tsM1101	MAGts6(I)	5.3×10^{7}	<10	2	$<1.9 \times 10^{-7}$
tsM1370	MAGts7(I)	1.7×10^{8}	<10	RECLONED	$<5.8 \times 10^{-8}$
tsM290	MAGts8(II)	2.5×10^{7}	50	4	2.04×10^{-6}
tsM468	MAGts9(II)	10^{7}	1.1×10^{3}	3	1.05×10^{-3}
tsM935	MAGts10(II)	1.1×10^{7}	50	3	4.65×10^{-6}
tsM1019	MAGts11(II)	5.2×10^{7}	1.1×10^{7}	3	2.1×10^{-4}
tsM1058	MAGts12(II)	5×10^{7}	9×10^{3}	2	1.8×10^{-4}
tsM1122	MAGts13(II)	1.8×10^{8}	1.6×10^{4}	3	8.8×10^{-5}
tsM1156	MAGts14(II)	4.8×10^{7}	3.3×10^{3}	3	7.8×10^{-5}
tsM1168	MAGts15(II)	2.9×10^{7}	2.2×10^{4}	3	7.4×10^{-4}
tsM1288	MAGts16(II)	1.2×10^{8}	2.0×10^{3}	3	1.69×10^{-5}

TABLE 10 (CONTD.)

tsM133	MAGts17(II)	3.8×10^{8}	<10	3	$<2.63 \times 10^{-8}$
tsM1012	MAGts18(II)	2.8×10^{8}	2.5×10^{2}	2	1.09×10^{-6}
tsM842	MATts19(II)	3.6×10^{7}	1.3×10^{4}	2	3.5×10^{-4}
tsM444	MAGts20 (I + II)	5.7×10^{6}	<10	3	$<1.76 \times 10^{-6}$
tsM794	MAGts21(I + II)	3.0×10^{8}	<10	RECLONED	$<3.4 \times 10^{-8}$
tsM866	MAGts22(II)	3.3×10^{8}	$6.x\ 10^{3}$	2	1.82×10^{-5}
tsM904	MAGts23(III)	1.5×10^{7}	<10	3	$<6.9 \times 10^{-7}$
tsM1192	MAGts24(I)	3.8×10^{7}	1.5×10^{3}	3	3.97×10^{-5}
tsM1238	MAGts25(I)	3.3×10^{7}	5×10^{3}	2	9.4×10^{-5}
tsM1300	MAGts26(I)	1.1×10^{7}	<10	2	$<9.2 \times 10^{-7}$
tsM844	MAGts27(UC)	1.4×10^{8}	<10	3	$<7.4 \times 10^{-8}$
tsM523	MAGts28(I)	2.2×10^{8}	<10	2	$<4.65 \times 10^{-8}$
tsM1053	MAGts29(I)	4.7×10^{7}	<10	3	$<2.1 \times 10^{-7}$
tsM1252	MAGts30(II)	2.7×10^{7}	1.5×10^{4}	2	5.5×10^{-4}
tsM702	MAGts31(II)	4.8×10^{7}	3.6×10^{4}	3	7.4×10^{-4}
tsM1351	MAGts32(II)	1.6×10^{8}	2×10^{2}	2	1.3×10^{-6}
tsM1368	MAGts33(UC)	7.5×10^{7}	2.7×10^{3}	2	3.7×10^{-5}
tsM835	MAGts34(II)	2×10^{7}	<10	3	$<5.1 \times 10^{-7}$
tsM822	MAGts35(II)	1.5×10^{7}	<10	1	$<6.9 \times 10^{-7}$
tsM849	MAGts36(II)	7×10^{8}	2.5×10^{5}	2	3.5×10^{-5}
tsM621	MAGts37(II)	3.1×10^{7}	1.7×10^{2}	1	5.63×10^{-6}
tsM520	MAGts38(II)	1.3×10^{8}	4×10^{5}	2	3.2×10^{-3}
tsM1071	MAGts39(II)	2.1×10^{8}	6×10^{2}	3	2.86×10^{-6}

TABLE 10 (CONTD.)

tsM1043	MAGts40(II)	1.1×10^{8}	<10	3	$<9.5 \times 10^{-8}$
tsM1100	MAGts41(I)	5.9×10^{7}	<10	1	$<1.7 \times 10^{7}$
tsM1145	MAGts42(II)	4.6×10^{6}	100	2	2.165×10^{-5}
tsM1159	MAGts43(II)	5.6×10^{7}	5×10^{3}	2	9.01×10^{-5}
tsM319	MAGts44(UC)	9.3×10^{5}	<10	1	$<1.1 \times 10^{-5}$
tsM193	MAGts45(II)	1.5×10^{7}	1.9×10^{2}	1	1.3×10^{-5}
tsM170	MAGts46(UC)	1.7×10^{6}	<10	2	$<6.06 \times 10^{-6}$
tsM914	MAGts47(I)	2.1×10^{7}	<10	2	$<4.8 \times 10^{-7}$
tsM410	MAGts48(II)	5.3×10^{7}	6.1×10^{3}	3	1.2×10^{-5}
tsM1361	MAGts49(I)	4.8×10^{7}	4.9×10^{3}	2	1.03×10^{-4}
tsM228	MAGts50(UC)	1.2×10^{7}	<10	2	$<8.3 \times 10^{-8}$
WILD TYPE	MAGts^{+}	5.2×10^{7}	2×10^{7}	-	0.3

UC = Unclassified

TABLE 11

The Batai virus *ts* mutants.* Efficiency of plating.

VIRUS		INFECTIVITY ASSAY (TITRE) AT		Passage Level	Efficiency of plating (E.O.P.)
OLD CODE	NEW CODE	31^{o}	38^{o}		
*ts*B54	BAT*ts*1(I)	1.2×10^{6}	<10	1	$< 8.3 \times 10^{-6}$
*ts*B7	BAT*ts*2(II)	1.7×10^{6}	65	1	3.8×10^{-5}
*ts*B107	BAT*ts*3(II)	1.2×10^{7}	243	1	2×10^{-5}
*ts*B201	BAT*ts*4(II)	3.9×10^{6}	<10	1	$< 2.6 \times 10^{-6}$
*ts*B185	BAT*ts*5(II)	5.9×10^{7}	6575	1	10^{-4}
B*ts*$^{+}$	BAT*ts*$^{+}$	7×10^{6}	6.1 x 106	-	0.9

* See footnote under Table 12

TABLE 12

The Bunyamwera virus ts mutants.* Efficiency of plating.

VIRUS		INFECTIVITY ASSAY (TITRE) AT		Passage Level	Efficiency of plating (E.O.P.)
OLD CODE	NEW CODE	31°	38°		
tsBN9	BUNts1(I)	5×10^6	50	1	10^{-5}
tsBN61	BUNts2(I)	4.7×10^6	150	1	3.2×10^{-5}
tsBN105	BUNts3(I)	2.6×10^7	300	1	10^{-5}
tsBN111	BUNts4(I)	3.3×10^7	2.5×10^3	1	10^{-5}
tsBN137	BUNts5(I)	1.5×10^8	$<10^2$	3	$<6.7 \times 10^{-7}$
tsBN31	BUNts6(II)	2.6×10^7	$<10^2$	1	$<3.8 \times 10^{-6}$
tsBN39	BUNts7(II)	5.5×10^7	$<10^2$	Recloned	$<1.8 \times 10^{-6}$
tsBN67	BUNts8(II)	3.7×10^7	2.7×10^4	3	0.0007
BNts$^+$	BUNts$^+$	3.4×10^7	1.5×10^7		0.4

* All ts mutants of Batai and Bunyamwera viruses were induced with 50 ug of 5-Fu per ml of culture medium.

untreated cultures. Only a few clones were isolated from a single mutagen treatment to avoid reisolation of clonally related mutants. Table 13 shows the Maguari virus ts mutants and the mutagen concentrations (if known) at which they were isolated. Temperature-sensitive mutants were isolated at higher frequency with increasing concentration of 5-Fu. One hundred and eleven clones were isolated from the unmutagenised (control) cultures and screened for spontaneous mutants. Three of these were temperature-sensitive [(MAGts24(I), MAGts25(I) and MAGts30(II)] giving a spontaneous mutation frequency of 2.7%.

The temperature-sensitive mutants of Batai and Bunyamwera viruses were isolated from cultures treated with 50 µg of 5-Fu per ml of culture medium. The rationale for this was to minimise the probability of obtaining multiple ts mutants.

Initially the ts mutants were numbered chronologically in the order in which the clones were isolated. The numbers were prefixed with the letters B, BN and M to indicate that they were either Batai, Bunyamwera or Maguari ts mutants respectively. The mutants were re-coded sequentially after classification by recombination analysis (see Section II.2) to reflect the recombination grouping. The conventional three-letter code for these viruses (International Catalogue of Arboviruses, Berge, T.O. (ed), 1975), BAT; BUN; and MAG; were substituted for the letters B, BN and M respectively. Thus the ts mutant, once coded tsM290 subsequently became MAGts8(II) and tsM577 was re-coded

TABLE 13: Maguari *ts* mutants.

Frequency of isolation in relation to mutagen concentration and the origin of individual mutants.

Conc. of 5-Fu (μg/ml)	No. of clones screened	*Ts* mutants isolated	No. of *ts* mutants isolated	% *ts* mutants isolated
0	111	MAG*ts*24(I), MAG*ts*25(I) MAG*ts*30(II)	3	2.7
50	240	MAG*ts*7(I), MAG*ts*16(II) MAG*ts*21(I+II), MAG*ts*26(I) MAG*ts*31(II), MAG*ts*32(II) MAG*ts*33(UC), MAG*ts*49(I)	8	3.3
100	330	MAG*ts*2(I), MAG*ts*3(I) MAG*ts*4(I), MAG*ts*10(II) MAG*ts*19(II), MAG*ts*22(II) MAG*ts*23(III), MAG*ts*27(UC) MAG*ts*28(I), MAG*ts*34(II) MAG*ts*35(II), MAG*ts*36(II) MAG*ts*37(II), MAG*ts*38(II) MAG*ts*47(I)	15	4.5
200	203	MAG*ts*5(I), MAG*ts*6(I) MAG*ts*11(II), MAG*ts*12(II) MAG*ts*13(II), MAG*ts*14(II)	14	6.8

TABLE 13 (CONTD.)

		MAGts15(II), MAGts18(II) MAGts29(I), MAGts39(II) MAGts40(II), MAGts41(I) MAGts42(II), MAGts43(II)		
ND	489	MAGts1(I), MAGts8(II) MAGts9(II), MAGts17(II) MAGts20(I+II), MAGts44(UC) MAGts45(II), MAGts46(UC) MAGts48(II), MAGts50(UC)	10	2%
TOTAL	1373	50	50	3.6%

ND = Not determined

UC = Unclassified

MAG*ts*3(I). Both the old and the new codes have been given in tables 10,11 and 12 for reference purposes.

II.2 RECOMBINATION AND COMPLEMENTATION

II.2.1 RECOMBINATION: HOMOLOGOUS CROSSES

The temperature-sensitive (*ts*) mutants isolated from each of the three viruses (Batai, Bunyamwera and Maguari viruses) were crossed in homologous pairs and assayed for recombination as described in the Methods (Section E). They were subsequently classified into recombination groups on the basis of their recombination patterns.

II.2.1(i) PRELIMINARY IDENTIFICATION OF A RECOMBINING PAIR OF *ts* MUTANTS

Classification of the *ts* mutants of each virus was initiated by choosing one *ts* mutant as a reference mutant and crossing it with a few other *ts* mutants. The mutants used for these preliminary crosses were chosen because they showed a low efficiency of plating

(EOP, $\frac{\text{Yield assayed at } 38^{o}\text{ C}}{\text{Yield assayed at } 31^{o}\text{ C}}$, about 10^{-4} - 10^{-6}),

at the restrictive temperature. The progeny of these preliminary crosses was assayed for an increased yield of plaque-forming virus at the restrictive temperature and the recombination frequencies were calculated as shown in the Methods (Section E).

MAG*ts*8 was used as the reference mutants for Maguari virus *ts* mutants. All the mutants used in the preliminary screening were diluted appropriately

to a titre of about 10^7 pfu per ml and 0.2 ml of each ts mutant was mixed with 0.2 ml of MAGts8, containing 2 x 10^6 pfu (mixed inoculum) or with 0.2 ml Eagle's medium (inoculum for singly-infected controls). This was to ensure that the amounts of each ts mutant in both the mixed and the singly infected cultures were similar. BHK-21 cells in multiwell (Linbro) plates were then infected in duplicate with the above inocula. After 45 minutes adsorption at +4° C, 1 ml of Eagle's medium was added to each well and incubation was continued for 2 - 4 hours at 31° C in a CO_2-gassed incubator. The medium was then removed, the monolayer washed twice by aspiration of medium and 1 ml of fresh Eagle's medium, prewarmed to 31° C, was replaced. All cultures were harvested at 22 hours post infection and assayed at 31° C and 38° C for recombinant virus. Twenty two hours incubation was used prior to the determination of an optimal incubation period for a recombination experiment because it was the average period for a single growth cycle of the viruses (see Figures 3a, b and c). From the foregoing experiment MAGts3 and MAGts7 were observed to produce more plaque-forming virus at 38° C in the mixed infections with MAGts8 compared with the singly infected controls. MAGts3 and MAGts7 were then crossed with each other

and assayed for recombination as above. No recombination was observed, indicating that both ts mutations were probably located on the same genome subunit whereas MAGts8 mutation was located on a different subunit (results shown in Table 14). On the basis of the above observations MAGts3 and MAGts7 were classified in group I and MAGts8 in group II. The group numbers were assigned arbitrarily and do not correspond to the groups I and II of the California group viruses previously described by Gentsch and Bishop (1976) and Gentsch et al (1979). From this point the group numbers were attached to each ts mutant (i.e. MAGts3(I), MAGts7(I) and MAGts8(II)) to indicate their recombination groups. The procedure described above for Maguari virus was also applied to the Batai virus and Bunyamwera virus ts mutants. Subsequently, experiments were carried out with the above recombining ts mutants of Maguari virus to determine the optimal conditions (period of incubation and multiplicity of infection) for a standard recombination test for use in the assignment of the rest of the ts mutants to recombination groups.

TABLE 14: Preliminary identification of recombining Maguari virus *ts* mutants.

VIRUS	22-HR. YIELD ASSAYED AT		R.F (%)	RECOMB. GROUPS
	31° C	38°		
MAG*ts*3	---	<10		I
MAG*ts*7	---	<10		I
MAG*ts*8	---	<10		II
MAG*ts*3 x MAG*ts*7	8.8×10^6	$<10^2$	0.0023	
MAG*ts*3 x MAG*ts*8	6.3×10^7	3.6×10^5	1.5	
MAG*ts*7 x MAG*ts*8	2.7×10^7	3.4×10^6	25.2	

II.2.1(ii) RECOMBINATION FREQUENCY IN RELATION TO TIME OF HARVEST

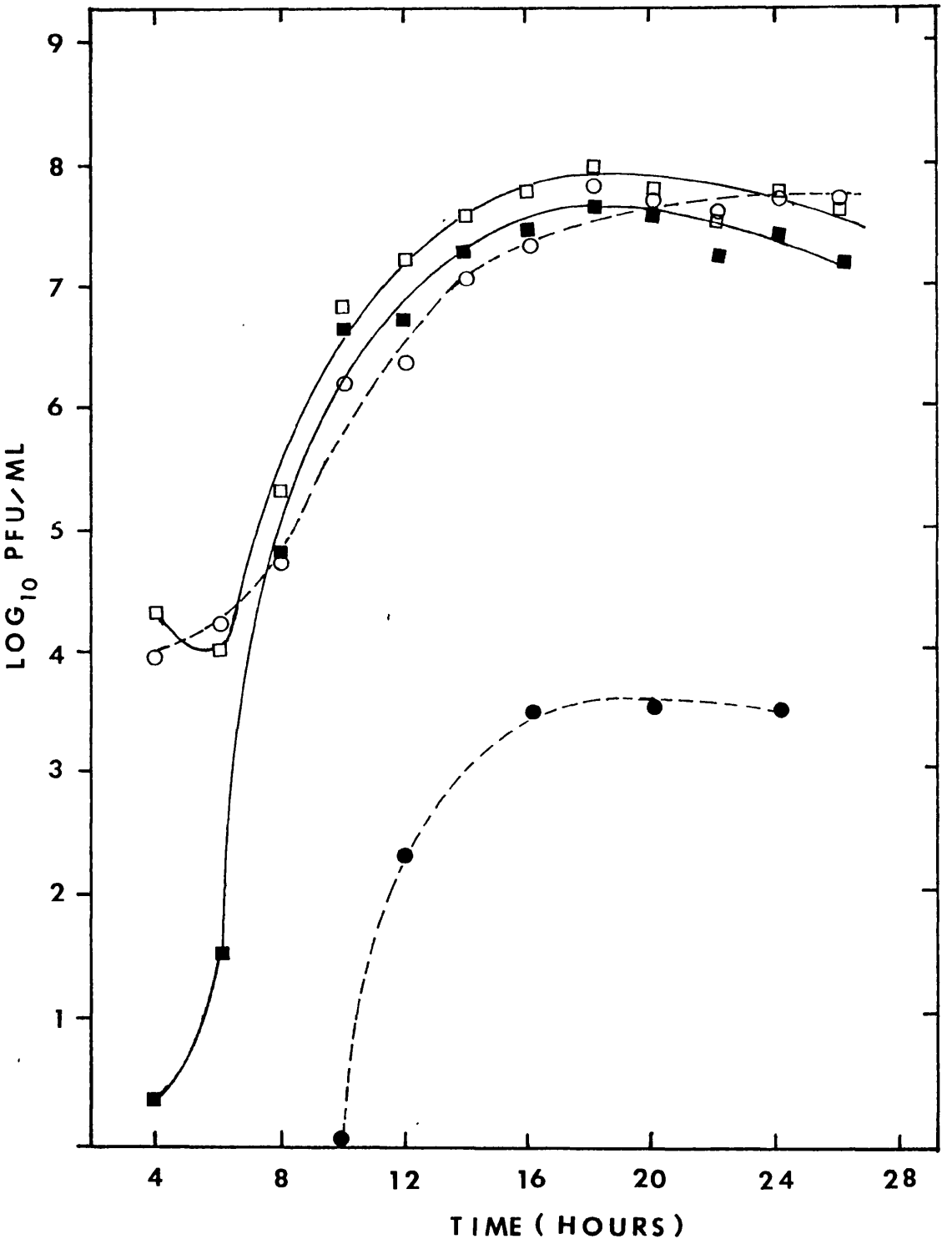
Two of the recombining ts mutants, MAGts3(I) and MAGts8(II), identified in the preliminary experiments, described in Section II.2.1(i), were used to determine the kinetics of release of the presumptive recombinant virus and to establish the optimum time of harvest for a standard recombination assay. These ts mutants were used to infect BHK-21 cells in multiwell (Linbro) plates at a combined multiplicity of 10 pfu per cell in mixed infections and at 5 pfu per cell in single infections. After adsorption at +4° C the monolayers were incubated and washed as described in the Methods (Section E). Cultures were harvested at two-hour intervals up to 26 hours by freezing at -70° C. The yield of plaque-forming virus was measured at 31° C and 38° C using the infectivity assay procedure described in the Methods (Section C). The result, shown in Figure 11, indicated that the putative recombinant virus appeared early in the growth cycle and was detected as early as 4 hours after infection. From 10 hours onwards the proportion of recombinant virus in the progeny was essentially constant. This signified that the recombinational events (i.e. reassortment) occurred early in the multiplication cycle and were virtually complete by 10 hours. The yield of progeny virus appeared to be highest at 18 hours post infection (see Figure 11), so subsequent recombination experiments were harvested at 18 hours post infection.

FIGURE 11

Kinetics of the appearance of recombinant virus. BHK-21 cell monolayer were infected with two recombining mutants of Maguari virus, MAG<u>ts</u>3(I) and MAG<u>ts</u>8(II) and the proportion of recombinant virus in the progeny was determined throughout the growth cycle.

Symbols: (□——□) MAG<u>ts</u>3(I) x MAG<u>ts</u>8(II) at 31° C (total yield); (■——■) MAG<u>ts</u>3(I) x MAG<u>ts</u> 8(II) at 38° C (recombinant yield); (O-----O) sum of the single infection yields at 31° C (self-infection control); (●------●) sum of single infection yields at 38° C (revertant control).

Fig·11



II.2.1(iii) RECOMBINATION FREQUENCY IN RELATION TO MULTIPLICITY OF INFECTION

High titred stocks of MAG*ts*3(I) and MAG*ts*8(II) (about 1.8×10^8 and 3.1×10^8 pfu per ml respectively) were used to determine the optimum multiplicity for a standard recombination test.

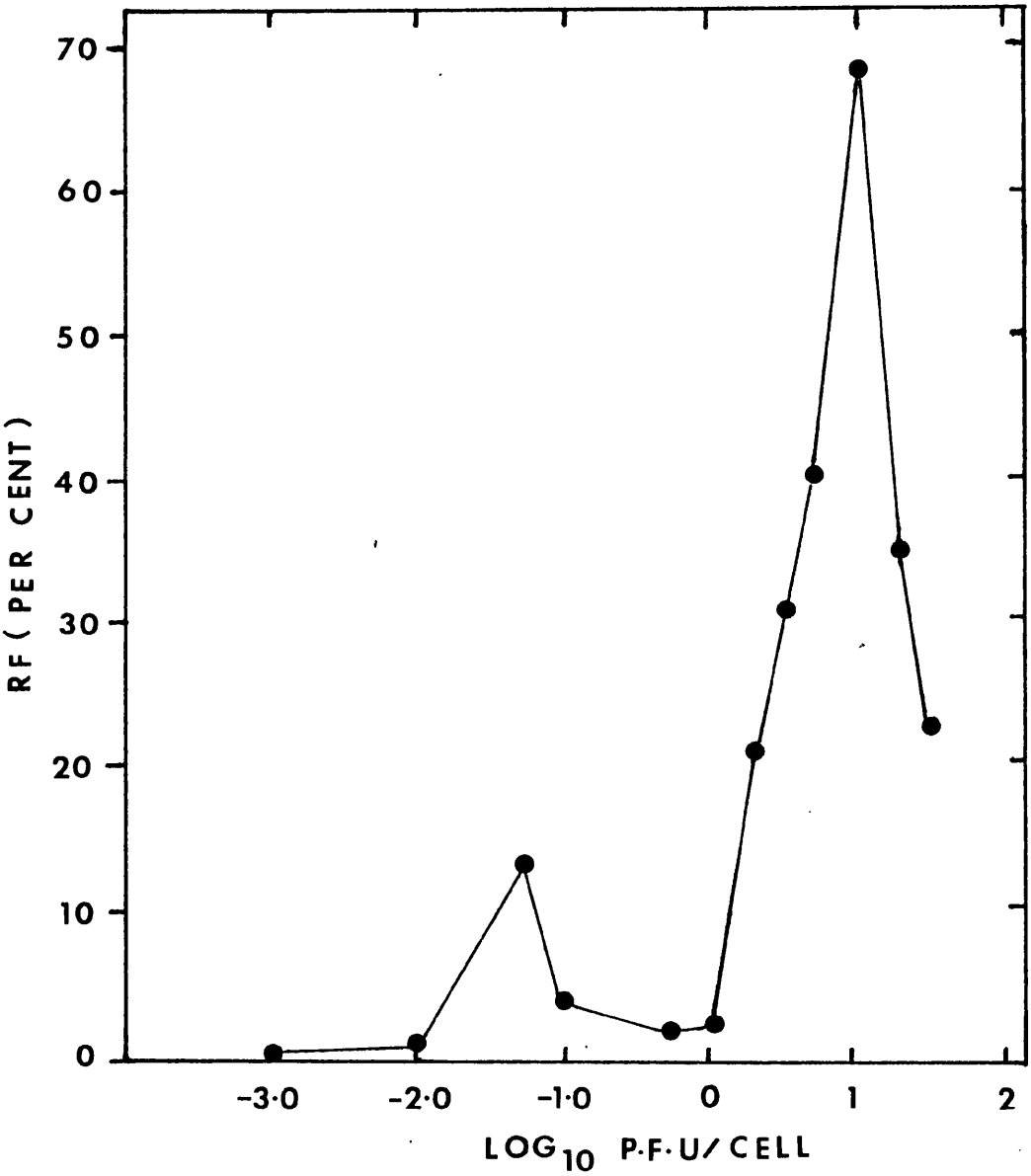
First the number of cells per well in 24-well Linbro plates was estimated by trypsinisation of BHK-21 cells from confluent wells and counting the single cells by means of a hemocytometer. This was estimated at about 2.5×10^5 cells per well. The two *ts* mutants, MAG*ts*3(I) and MAG*ts*8(II) were then diluted proportionally to achieve multiplicities of 30, 20, 10, 3, 2, 1. 0.5, 0.1, 0.05, 0.01, and 0.001 pfu per cell respectively. The inoculum used to infect each monolayer was 0.1 ml and each dilution was such that this volume would deliver the appropriate multiplicity.

The BHK-21 cultures (similar to those used, above, to estimate the number of cells per well) were singly and multiply infected with MAG*ts*3(I) and MAG*ts*8(II) at the appropriate multiplicities and as in the Methods (Section E) except that both singly and multiply infected cultures received the same amount of virus (pfu per cell). After 18 hours incubation at 31° C the cultures were harvested and assayed for recombination. Figure 12 shows the effect of multiplicity on the recombination frequency. At multiplicities below one, the recombination frequencies were low and variable. This could be explained by the low probability of co-infection of cells.

FIGURE 12

Frequency of recombination in relation to multiplicity of infection with mutants MAG*ts*3(I) and MAG*ts*8(II) of Maguari virus.

Fig·12



For example at a multiplicity of 0.05 pfu per cell (i.e. 0.025 pfu per cell of each ts mutant) only one in twenty cells would be infected by a single particle of either mutant and not more than one in sixteen hundred cells would be simultaneously infected by both mutants. The chance of a cell being multiply infected even at such low multiplicities, however, would be increased sometimes by aggregation of virus particles. Aggregation could be responsible, therefore, for the recombination frequency of 12.7% realised when cell monolayers were infected at a multiplicity as low as 0.05 pfu per cell. In Figure 12, there was a linear increase in recombination frequency with increasing multiplicity of infection at multiplicities above unity. The maximum recombination frequency was achieved at 10 pfu per cell, beyond which it diminished rapidly. The reason for the decrease is not certain. It could be due to an increase in the leak yield of the singly infected controls, a multiplicity dependent interference (e.g. by antointerfering defective virions) or a combination of both. In all subsequent recombination experiments, a multiplicity of 10 pfu per cell was used.

II.2.1(iv) CLASSIFICATION OF ts MUTANTS INTO RECOMBINATION GROUPS

MAGts7(I) and MAGts8(II), the previously identified recombining pair of ts mutants, were crossed against the rest of the Maguari ts mutants. These crosses were carried out in a series of separate experiments and in

each experiment the MAG*ts*7(I) x MAG*ts*8(II) cross was included as a control. The progeny of the crosses was harvested after incubation at 31° C for 18 hours and was assayed for recombination as previously described (Methods Section E). In this way 42 out of the 50 *ts* mutants of Maguari virus were initially classified into two recombination groups, 15 in group I and 27 in group II. The remaining eight mutants were unclassified and included some mutants which were low titred (10^5 - 10^6 pfu per ml).

Twenty of the grouped mutants and two of the ungrouped mutants (MAG*ts*20 and MAG*ts*21) were crossed in all combinations in order to establish the homogeneity of the two recombination groups. Table 15 shows that *ts* mutants belonging to different groups recombined at high frequency (RF ranging between 1.1% and 98.4%) whereas there was no appreciable recombination ($\ll$1%) in crosses of *ts* mutants belonging to the same group. In situations where some mutants became very leaky or showed reversion, the number of plaques formed at 38° C by the progeny of the mixed infected culture was enhanced and false recombination could be detected within the existing groups. This was corrected by repeating the assays at a slightly raised restrictive temperature (38.3° C or 38.5° C). This operation eliminated the leak yield but not the revertants. Reverting *ts* mutants were recloned and the progeny *ts* clones used in subsequent recombination experiments

TABLE 15: Homologous Recombination;

Maguari virus ts mutants.

The values represent recombination frequencies of all combinations of 20 ts mutants of Maguari virus.

In these results and others which have been represented in Tables 13, 14, 15, 16, 17 and 19, RF values less than 1% were generally interpreted as lack of recombination between the paired mutants.

The figures given under the self crosses are percentage EOPs $\left(\frac{\text{pfu } 38^{\circ}\text{ C}}{\text{pfu } 31^{\circ}\text{ C}}\right)$ of the progeny of the singly infected controls multiplied by 2 to bring them to the same terms as the RF.

ND = Not determined.

TABLE 15

Homologous recombination: Maguari virus ts mutants

Group	I							II											
ts	MAGts 1(I)	MAGts 2(I)	MAGts 3(I)	MAGts 4(I)	MAGts 5(I)	MAGts 6(I)	MAGts 7(I)	MAGts 8(II)	MAGts 9(II)	MAGts 10(II)	MAGts 11(II)	MAGts 12(II)	MAGts 13(II)	MAGts 14(II)	MAGts 15(II)	MAGts 16(II)	MAGts 17(II)	MAGts 18(II)	MAGts 19(II)	MAGts 22
MAGts1(I)	<0.01	<0.003	<0.003	<0.007	<0.013	<0.002	0.27	14.7	6.8	ND	2.9	44.4	20.8	40.3	59.1	1.8	2.5	98.4	40.7	8.1
MAGts2(I)		<0.013	<0.002	<0.05	<0.006	0.004	<0.006	84.7	4.4	3.5	3.3	15.1	7.5	6.3	40.4	2.5	3.6	4.8	12.2	11.5
MAGts3(I)			<0.004	<0.001	0.002	0.006	<0.023	14.2	16.0	4.7	20.8	32.9	41.1	16.2	15.8	1.9	16.6	47.3	17.1	15.4
MAGts4(I)				0.033	<0.001	0.008	<0.04	6.6	10.5	1.4	4.1	2.1	12.6	9.5	6.1	3.3	6.9	9.2	2.4	4.1
MAGts5(I)					<0.04	0.005	0.44	5.2	7.2	ND	1.7	2.6	5.3	8.6	2.3	1.7	1.2	3.5	1.5	12.1
MAGts6(I)						0.003	<0.0001	22.3	1.1	2.7	5.1	3.5	1.2	22.9	13.6	1.6	2.8	13.1	1.4	6.7
MAGts7(I)							0.06	27.1	14.95	5.7	12.1	41.6	20.0	38.2	11.8	16.2	24.4	13.6	13.2	33.7
MAGts8(II)								0.002	<0.00002	<0.002	<0.13	0.55	0.11	0.184	0.003	0.005	0.04	<0.00003	<0.182	0.23
MAGts9(II)									<0.016	<0.06	<0.06	<0.002	<0.004	<0.003	0.14	<0.002	<0.6	0.005	<0.004	0.14
MAGts10(II)										0.003	<0.001	0.4	0.0042	<0.002	<0.005	<0.003	0.002	<0.002	0.032	0.9
MAGts11(II)											0.28	0.18	0.016	<0.026	<0.12	<0.005	0.06	0.002	0.54	0.02
MAGts12(II)												0.53	0.302	0.025	0.18	0.009	0.21	<0.01	<0.25	0.6
MAGts13(II)													0.022	0.005	<0.001	0.011	0.05	0.012	<0.023	0.013
MAGts14(II)														<0.003	0.3	<0.003	0.665	0.017	0.116	<0.003
MAGts15(II)															0.048	<0.016	<0.001	0.014	<0.28	<0.05
MAGts16(II)																0.146	<0.052	0.064	<0.016	0.15
MAGts17(II)																	0.003	<0.009	0.049	0.002
MAGts18(II)																		0.052	0.3	ND
MAGts19(II)																			0.8	0.73
MAGts22(II)																				0.014

ND - not determined

Underlined figures represent average EOP (x2)% of ts mutants determined from the infectivity assay of progeny of singly infected cultures at 31° and 38° C simultaneously.

(see Table 10). Increasing the restrictive temperature did reduce the numerical value of the RF but did not completely eliminate recombination. The two unclassified ts mutants MAGts20 and MAGts21, did not recombine with any of the other 20 (results shown elsewhere in Table 19), so they could neither be included in group I or II, nor, in fact could they be considered possible candidates for a third recombination group. They were assumed to be double ts mutants. The classification of the other (Twenty two) Maguari virus ts mutants is shown in Table 16.

The six unclassified ts mutants (excluding MAGts20 and MAGts21) were investigated in separate experiments in a continuing search for the third recombination group expected from the tripartite structure of the bunyavirus genome (See Section II.2.1(v)).

Similarly, the Batai virus ts mutants were classified into two groups. One out of the 5 Batai virus ts mutants isolated was assigned to one group (group I) and four were classified in another group (group II). These results are shown in Table 17.

Table 18 shows the classification of the Bunyamwera virus ts mutants. The eight ts mutants isolated also fell into two recombination groups, 5 in group I and 3 in group II.

The group I and II nomenclature in the Maguari virus ts mutant classification above was extended to Batai and Bunyamwera viruses only after the heterologous recombinations had been detected (see Section II.3) and it had become evident that the two recombination

TABLE 16: Homologous Recombination;

Maguari ts mutants.

Representative group I [MAGts7(I)] and group II [MAGts8(II)] ts mutants were crossed with other Maguari virus ts mutants and the RF values were determined as described in the methods (Section E).

The data presented here were obtained from more than one experiment. Therefore, the values quoted for the efficiency of plating of MAGts7(I) and MAGts8(II) are not necessarily those used to determine all the RF values in Column 10.

TABLE 16 Homologous Recombination; Maguari virus ts mutants.

	X SELF			X MAGts7(I)			X MAGts8(II)			Recombination
	18-HR YIELD ASSAYED AT		%E.O.P.	18-HR YIELD ASSAYED AT			18-HR YIELD ASSAYED AT			Group
VIRUS	31°	38°	X2	31°	38°	RF	31°	38°	RF	
MAGts24	1.2×10^{8}	10^{3}	0.1002	2.2×10^{7}	250	<0.0002	4.4×10^{7}	1.2×10^{6}	5.6	I
MAGts25	--	1.2×10^{4}	--	3.3×10^{7}	9.2×10^{3}	<0.0087	2.4×10^{7}	1.8×10^{6}	14.6	I
MAGts26	--	<10	--	8.3×10^{6}	50	0.0012	7.3×10^{6}	2.6×10^{5}	7.03	I
MAGts28	1.7×10^{8}	<10	<0.00001	3.6×10^{7}	1.5×10^{4}	0.08	9.1×10^{7}	9.9×10^{5}	2.2	I
MAGts29	3.4×10^{8}	1.02×10^{3}	0.0006	2.6×10^{8}	2.10^{4}	<0.002	2.4×10^{8}	1.7×10^{6}	1.5	I
MAGts30	2.6×10^{6}	1.8×10^{4}	--	2.3×10^{7}	1.5×10^{6}	12.6	5.5×10^{7}	2×10^{4}	<0.005	II
MAGts31	--	100	--	1.5×10^{7}	4.7×10^{5}	6.5	4.7×10^{7}	300	0.00057	II
MAGts32	--	1.2×10^{3}	--	2.4×10^{7}	1.1×10^{6}	9.3	6.8×10^{7}	4.2×10^{3}	0.0087	II
MAGts34	--	<10	--	9.25×10^{6}	1.4×10^{5}	3.0	4.8×10^{7}	450	0.002	II
MAGts35	3.9×10^{6}	125	--	2.5×10^{7}	6.8×10^{5}	3.9	8.8×10^{7}	1.7×10^{3}	<0.002	II
MAGts36	5.6×10^{8}	1.9×10^{5}	0.07	4.7×10^{8}	5.2×10^{7}	22	8.5×10^{7}	3.4×10^{4}	<0.07	II
MAGts37	4.5×10^{6}	<10	$<2.2\times10^{-4}$	4.0×10^{7}	1.6×10^{6}	6.4	1.2×10^{8}	7.6×10^{3}	0.01	II
MAGts38	4×10^{6}	2.3×10^{3}	0.11	3.9×10^{7}	9.5×10^{5}	4.9	1.6×10^{7}	7.8×10^{3}	<0.09	II

TABLE 16 (CONTD.)

MAGts39	--	2.8×10^{4}	--	2.0×10^{7}	6.9×10^{6}	6.5	2.4×10^{7}	1.2×10^{3}	<0.00008	II
MAGts40	--	2.7×10^{3}	--	1.2×10^{7}	1.3×10^{6}	21.6	2.9×10^{7}	2.9×10^{3}	0.00027	II
MAGts41	1.3×10^{5}	<10	<0.02	3.8×10^{5}	$<10^{2}$	<0.003	1.3×10^{6}	3.7×10^{4}	4.2	I
MAGts42	4.1×10^{7}	10^{4}	0.025	3.9×10^{7}	1.1×10^{6}	4.9	4.4×10^{7}	2.9×10^{3}	<0.025	II
MAGts43	--	4.3×10^{3}	--	2.4×10^{7}	6.8×10^{5}	5.6	4.4×10^{7}	9.8×10^{3}	0.025	II
MAGts45	--	150	--	5.2×10^{6}	8.7×10^{4}	3.34	4.1×10^{7}	2.5×10^{3}	0.012	II
MAGts47	5.5×10^{7}	7.5×10^{3}	0.03	9×10^{7}	2.5×10^{3}	<0.03	3.3×10^{7}	8.10^{5}	4.8	I
MAGts48	2.7×10^{6}	10^{3}	0.07	3.4×10^{7}	10^{6}	6.0	1.1×10^{7}	1.1×10^{4}	0.007	II
MAGts49	--	2×10^{4}	--	2.7×10^{7}	2.5×10^{4}	0.04	6.0×10^{6}	2.8×10^{5}	8.5	I
MAGts7(I)	2.4×10^{7}	7.5×10^{3}	0.06	--	--	--	2.7×10^{7}	3.4×10^{6}	25.2	I
MAGts8(II)	3.1×10^{7}	4.3×10^{2}	0.002	2.7×10^{7}	3.4×10^{6}	25.2	--	--	--	II

TABLE 17

Homologous recombination; Batai virus ts mutants.

Group	I	II			
ts mutants	BATts1(I)	BATts2(II)	BATts3(II)	BATts4(II)	BATts5(II)
BATts1(I)	0.08	54.5	7.2	23.10	2.2
BATts2(II)		0.001	0.001	0.03	0.04
BATts3(II)			0.028	0.006	0.014
BATts4(II)				0.0008	0.0007
BATts5(II)					0.01

Underlined figures represent EOP (x2)% of the ts mutants as in Table 16.

* See legend to Table 16, paragraph 2.

TABLE 18

Homologous recombination*; Bunyamwera virus ts mutants.

Group	I					II		
ts	BUN ts1(I)	BUN ts2(I)	BUN ts3(I)	BUN ts4(I)	BUN ts5(I)	BUN ts6(II)	BUN ts7(II)	BUN ts8(II)
BUNts1(I)	0.982	0.006	0.04	0.09	0.0031	10.4	10.98	24.7
BUNts2(I)		0.012	0.14	0.248	0.122	14.95	3.75	49.4
BUNts3(I)			0.013	0.095	0.0006	27.5	2.8	7.1
BUNts4(I)				0.1	0.0031	17.42	14.8	31.1
BUNts5(I)					0.006	4.25	19.84	27.9
BUNts6(II)						0.7	0.009	0.67
BUNts7(II)							0.28	0.002
BUNts8(II)								0.004

Underlined figuures represent EOP (x2)% of the ts mutants as in Table 16.

* See legend to Table 16, paragraph 2.

groups in all three viruses were equivalent.

II.2.1(v) MAGUARI VIRUS RECOMBINATION GROUP III

In a virus whose genome consists of three subunits and in which recombination occurs by reassortment of the genome subunits, the ts mutants would be expected to fall into three recombination groups on the assumption that:

(i) ts mutation could occur in every gene in the viral genome;

(ii) the viral genome subunits reassort independently;

(iii) all three subunits contain unique genetic information.

If any of the above conditions is not fulfilled, it would be unlikely that three recombination groups would be observed. During the greater part of this investigation, mutants belonging to the putative third group eluded detection. The reason for this is not clear. However, one ts mutant, MAGts23 [henceforth to be referred to as MAGts23(III)] was assigned to the missing group. When MAGts23(III) was first observed to recombine with both MAGts3(I) and MAGts8(II) as well as with MAGts7(I), the prediction that MAGts20 and MAGts21 could be double ts mutants of the predominant groups I and II was tested. It would be recalled that MAGts20 and MAGts21 did not recombine with any of the other 20 ts mutants previously crossed in all

combinations (see SectionII.2.1(iv) and also the results on Table 19). As shown in Table 20 , both ts mutants (MAGts20 and MAGts21) recombined with MAGts23(III) at frequencies of 9.2% and 2.4% respectively. This confirmed that MAGts20 and MAGts21 were double ts mutants of groups I and II. These mutants were, therefore, designated MAGts20(I + II) and MAGts21(I + II) to reflect their mutational status. The MAGts20(I + II) x MAGts21(I + II) cross did not show any detectable recombination, again reinforcing the conclusion that they were double ts mutants of groups I and II (see Table 20).

MAGts23(III) recombined with the other 20 ts mutants except one [MAGts18(II)] at frequencies ranging from 1.5% to 43.1% (Table 19). These are comparable with the high frequencies of recombination observed in the crosses between mutants of groups I and II (cf Table 15). In a number of cases the putative recombinant clones from the progeny of crosses involving MAGts23(III) produced rather atypical plaques at the restrictive temperature (38^{o} C). They resembled leak-plaques in that they were small, poorly cytolytic and in most cases appeared mainly as clumps of dying (or dead) cells. In contrast to leak-yields, however, the putative recombinant progeny plaqued in proportion to the dilution of the culture. Leaky mutants, on the other hand, have often been observed to produce confluent CPE or numerous plaques in one dilution (frequently lower dilutions, 10^{-1}, 10^{-2}) and few or no plaques in the succeeding ten-fold dilution. Besides,

virus

TABLE 19: Homologous Recombination; Maguari/ts mutants.

Crosses involving the group III mutant, MAGts23(III), the two double ts mutants of groups I and II, MAGts20(I+II) and MAGts21(I+II), and the 20 ts mutants which were compared in all combinations in Table 12.

* Obtained from yield of singly infected controls.

TABLE 19 **Homologous Recombination, Maguari virus ts mutants.**

	X SELF*			X MAGts20(I+II)			X MAGts21(I+II)			X MAGts23(III)		
	18-HR YIELD ASSAYED AT			18-HR YIELD ASSAYED AT			18-HR YIELD ASSAYED AT			18-HR YIELD ASSAYED AT		
VIRUS	31°	38°	%EOP X2	31°	38°	RF	31°	38°	RF	31°	38°	RF
MAGts1(I)	$2x10^5$	<10	<0.01	$8.5x10^5$	$3.8x10^2$	0.88	$2.7x10^7$	$1.4x10^3$	0.004	$7.5x10^4$	$1.6x10^3$	4.3
MAGts2(I)	$1.8x10^5$	<10	<0.11	$6x10^5$	<10	<0.013	$2.9x10^7$	$5.9x10^3$	0.134	$1.1x10^6$	$2.4x10^4$	4.2
MAGts3(I)	$5.2x10^6$	100	0.004	$8.3x10^5$	50	<0.005	$2.6x10^7$	$1.1x10^4$	0.08	$2.8x10^7$	$4.9x10^5$	3.6
MAGts4(I)	$1.7x10^7$	$1.3x10^3$	0.015	$4.3x10^5$	<10	<0.013	$2x10^7$	$4.9x10^3$	0.04	$5.5x10^6$	$5.3x10^4$	1.9
MAGts5(I)	$3.4x10^7$	$7.0x10^3$	0.04	$6.3x10^5$	$5.0x10^2$	0.16	$2.5x10^7$	$4.1x10^3$	0.024	$2.3x10^7$	$3.4x10^5$	2.7
MAGts6(I)	$1.5x10^7$	$1.9x10^3$	0.02	$3.1x10^7$	$6.3x10^3$	0.0286	$3.5x10^7$	$2.0x10^4$	0.1	$7.5x10^6$	$1.8x10^5$	4.7
MAGts7(I)	$8.5x10^6$	10^3	0.024	$4.3x10^7$	50	<0.0001	$1.4x10^7$	$2.9x10^4$	0.40	$3.0x10^7$	$1.3x10^6$	6.3
MAGts8(II)	$1.3x10^6$	$2x10^2$	0.03	$5.5x10^7$	10	<0.0001	$1.1x10^7$	$1.4x10^3$	0.02	10^6	$2.8x10^4$	5.7
MAGts9(II)	$4.8x10^5$	<10	<0.004	$3.5x10^6$	$4x10^2$	0.023	$1.1x10^7$	$2.3x10^2$	<0.013	$9.9x10^6$	$1.6x10^6$	31.6
MAGts10(II)	$1.30x10^8$	$3.3x10^3$	0.005	$3.6x10^7$	$6.8x10^2$	<0.003	$2.2x10^7$	$1.5x10^2$	0.0014	$9.5x10^6$	$3.4x10^5$	6.8
MAGts11(II)	$2.3x10^7$	$7.3x10^3$	0.06	$1.2x10^8$	$6.9x10^4$	<0.003	$1.2x10^7$	$3.5x10^4$	0.27	$2.1x10^7$	$2.3x10^5$	2.2
MAGts12(II)	$8.5x10^7$	$2.6x10^4$	0.06	$5.6x10^7$	$1.5x10^4$	<0.0001	$8.5x10^6$	$1.8x10^4$	0.15	$1.0x10^7$	$2.4x10^6$	41.2

TABLE 19 (CONTD.)

MAG<u>ts</u>13(II)	1.7×10^{7}	2×10^{4}	0.23	8.6×10^{5}	3.8×10^{2}	<0.0025	2.4×10^{7}	10^{2}	<0.00004	5.0×10^{7}	9×10^{6}	36.0
MAG<u>ts</u>14(II)	3.1×10^{7}	2.3×10^{4}	0.15	1.5×10^{8}	6.5×10^{2}	<0.0001	2.7×10^{7}	7.4×10^{3}	0.04	2.0×10^{6}	1.8×10^{5}	15.6
MAG<u>ts</u>15(II)	9.5×10^{6}	1.3×10^{4}	0.3	5.7×10^{6}	2.9×10^{3}	<0.004	2.1×10^{7}	8.5×10^{2}	0.0065	3.4×10^{6}	7.2×10^{5}	31.9
MAG<u>ts</u>16(II)	1.1×10^{6}	5.3×10^{2}	0.05	2.5×10^{6}	2.5×10^{2}	<0.0016	1.7×10^{7}	8.3×10^{2}	<0.0018	2.2×10^{6}	1.7×10^{4}	1.5
MAG<u>ts</u>17(II)	8.9×10^{5}	<10	<0.001	2.2×10^{6}	50	<0.003	1.3×10^{7}	10	<0.0012	8.5×10^{5}	1.8×10^{4}	4.2
MAG<u>ts</u>18(II)	1.6×10^{8}	1.0×10^{4}	0.013	1.1×10^{8}	4.7×10^{3}	<0.0065	1.2×10^{8}	2.8×10^{2}	<0.0015	10^{7}	1.2×10^{4}	<u>0.04</u>
MAG<u>ts</u>19(II)	3.5×10^{7}	6.3×10^{4}	0.4	8.1×10^{6}	4×10^{4}	<0.0025	1.4×10^{7}	1.2×10^{3}	<0.00004	2.7×10^{6}	6.8×10^{5}	43.1
MAG<u>ts</u>20(I+II)	1.7×10^{6}	50	0.006	SEE TABLE 20			SEE TABLE 20			SEE TABLE 20		
MAG<u>ts</u>21(I+II)	4.1×10^{7}	8.5×10^{2}	0.004									
MAG<u>ts</u>22(II)	2.4×10^{8}	2.4×10^{4}	0.02	7.3×10^{5}	1.9×10^{4}	0.0025	1.4×10^{7}	2.8×10^{3}	0.038	3.10^{7}	2.0×10^{5}	1.2
MAG<u>ts</u>23(III)	2.7×10^{6}	<10	$<7.44\times10^{-4}$	SEE TABLE 20			SEE TABLE 20			--	--	--

Underlined figures, Column 13: No recombination

TABLE 20: Homologous Recombination; Maguari virus ts mutants.

Crosses involving MAGts23(III), MAGts20(I+II)* and MAGts21(I+II)*

The underlined figures are %EOPs of progeny of singly infected controls multiplied by 2.

VIRUS	18-HR YIELD ASSAYED AT		%EOPx2 / R.F.
	31° C	38° C	
MAGts20	2.9×10^5	<10	<0.003
MAGts21	1.4×10^7	1.9×10^3	0.013
MAGts23(III)	2.03×10^5	<10	<0.01
MAGts20 x MAGts21	3.2×10^6	<10	<0.013
MAGts20 x MAGts23(III)	3.8×10^5	1.7×10^4	9.2
MAGts21 x MAGts23(III)	8×10^6	1.8×10^5	2.4

MAGts20 and MAGts21 could unequivocally be confirmed double-ts mutants of groups I and II if, when crossed with Maguari virus wild type, each yields ts mutants which would recombine with either group I or group II as well as group III ts mutants.

this plaque morphology was observed even when MAGts23(III) was crossed with stable, non-leaky ts mutants which gave no detectable yield at the restrictive temperature.

Cloning of leaky ts mutants often produced ts mutants with the same leaky characteristics as the parents. The progeny from cultures multiply infected with MAGts23(III) and other ts mutants were, therefore, cloned at 31° C and 38° C. Each clone isolated was qualitatively screened at 31° C and 38° C to confirm a non-ts phenotype. This was to ascertain that the progeny of the mixedly infected cultures at 38° C were indeed recombinants. Table 21 shows the results of the clonal analysis in selected crosses involving MAGts23(III). All the clones picked at 38° C showed the non-ts phenotype expected of recombinant virus. Putative non-ts recombinant clones were also isolated at 31° C and at frequencies even higher than the RF estimated from standard recombination assays. These observations confirm that recombination occurred between MAGts23(III) and ts mutants belonging to groups I and II, including the two mutants with ts lesions in both groups I and II.

Another ts mutant, MAGts33 which showed recombination with MAGts4(I), MAGts7(I) and MAGts8(II) (Table 22) was further tested with more ts mutants from both group I and group II in order to include it in group III. This effort was frustrated by the high reversion rate of this ts mutant. The progeny of the MAGts33 x MAGts23(III) cross even exhibited an enhanced plaque

TABLE 21

CLONAL ANALYSIS OF PROGENY OF SOME OF THE CROSSES INVOLVING MAG*ts*23(III)

VIRUS	TEMP. OF ISOLATION	NUMBER OF CLONES ISOLATED	NO. OF CLONES SHOWING PLAQUES AT 38° C	% *ts*$^+$ CLONES	R.F. (STANDARD SELECTION ASSAY FOR *ts*$^+$)
MAG*ts*3(I)xMAG*ts*23(III)	31° 38°	12 49	5 49	41.7 100	3.6
MAG*ts*8(II) x MAG*ts*23(III)	31° 38°	30 15	12 15	40 100	5.7
MAG*ts*23(III) x MAG*ts*20(I+II)	31° 38°	52 10	17 10	32.7 100	9.2

TABLE 22: Homologous Recombination; Maguari virus ts mutants.

Crosses involving groups I, II, III and (I+II) mutants and the unclassified Maguari ts mutants.

VIRUS	18-HR YIELD ASSAYED AT 31°C	18-HR YIELD ASSAYED AT 38°C	RF/(EOPx2) %
MAGts3(I)	3×10^7	250	0.002
MAGts6(I)	1.9×10^7	<10	<0.0001
MAGts8(II)	2.1×10^7	9.8×10^3	0.09
MAGts14(II)	4.4×10^7	1.05×10^4	0.05
MAGts16(II)	2.3×10^7	<10	$<8.8 \times 10^{-5}$
MAGts48(II)	2.7×10^6	<10	<0.0007
MAGts20(I+II)	5.8×10^6	<10	<0.0003
MAGts21(I+II)	1.2×10^7	<10	<0.0002
MAGts23(III)	1.5×10^5	<10	<0.013
MAGts27	9.5×10^6	<10	<0.0002
MAGts33	1.8×10^8	5.6×10^6	6.2*
MAGts44	8.8×10^8	9.5×10^3	0.001
MAGts46	2.1×10^4	<10	<0.09
MAGts7(I)	2.4×10^7	7.5×10^3	0.06
MAGts50	3×10^6	<10	0.0007
MAGts33 x MAGts3(I)	1.4×10^8	8.6×10^5	<0.004
MAGts33 x MAGts6(I)	6.7×10^7	4.3×10^6	<0.0001
MAGts33 x MAGts8(II)	9.5×10^7	1.4×10^7	18
MAGts33 x MAGts14(II)	1.5×10^8	7.8×10^6	2.9
MAGts33 x MAGts16(II)	1.23×10^8	7.7×10^6	3.4
MAGts33 x MAGts48(II)	1.4×10^8	9.7×10^6	5.9
MAGts33 x MAGts 7(I)	3.3×10^7	3.8×10^5	2.1
MAGts33 x MAGts 4(I)	1.7×10^8	9.3×10^5	1.1

TABLE 22 (CONTD.)

MAGts33 x MAGts20(I+II)	1.03×10^8	3.1×10^6	<0.0003
MAGts33 x MAGts21(I+II)	3.8×10^7	3.0×10^5	<0.0002
MAGts33 x MAGts23(III) MAGts 4(I)	1.2×10^8	1.2×10^7 <10	11.3
MAGts27 x MAGts3(I)	2.3×10^7	8×10^3	0.07
MAGts27 x MAGts8(II)	2.9×10^7	8.3×10^3	<0.09
MAGts44 x MAGts7(I)	1.3×10^7	1.025×10^4	0.14
MAGts44 x MAGts8(II)	3.4×10^7	9.8×10^3	0.04
MAGts46 x MAGts3(I)	1.44×10^7	<10	<0.0001
MAGts46 x MAGts8(II)	2.5×10^7	1.1×10^4	0.007
MAGts50 x MAGts7(I)	3.9×10^6	250	0.00006
MAGts50 x MAGts8(II)	7.3×10^6	100	0.003

production at the restrictive temperature. As a result of the above observations MAG*ts*33 was left unclassified pending recloning and repeat of the tests with a more stable clone. Other ungrouped *ts* mutants include MAG*ts*27, MAG*ts*44, MAG*ts*46, and MAG*ts*50 (see Table 22). Two of these (MAG*ts*27 and MAG*ts*50), may be multiple *ts* mutants since they failed to recombine with mutants from either groups (I or II) even though their titres (pfu per ml) were reasonably high (10^7 - 10^8). They have not yet been crossed with the group III mutant [MAG*ts*23(III)]. The status of MAG*ts*44 or MAG*ts*46, on the other hand, can not be determined until stocks of higher titre (up to 10^7 pfu per ml) can be obtained.

A summary of the classification of the *ts* mutants isolated from all three bunyaviruses investigated is outlined in Table 23. In group I there was one Batai *ts* mutant, 5 mutants of Bunyamwera virus and 15 of Maguari virus. In group II there were 4 Batai *ts* mutants, 3 of Bunyamwera virus and 27 of Maguari virus. One Maguari *ts* mutant, MAG*ts*23(III) was the only representative of group III. Two Maguari virus *ts* mutants, MAG*ts*20(I + II) and MAG*ts*21(I+II), and none of Batai virus or Bunyamwera virus were double *ts* mutants of groups I and II. Five Maguari *ts* mutants have still to be classified.

II.2.2 COMPLEMENTATION

Assays of complementation between recombining *ts* mutants were discontinued because recombination occurred at such high frequencies and so early in the

TABLE 23

SUMMARY CLASSIFICATION OF *ts* MUTANTS

VIRUS	GROUPS					TOTAL
	I	II	III	I+II	UNCLASSIFIED	
BATAI	1	4	--	--	--	5
BUNYAMWERA	5	3	--	--	--	8
MAGUARI	15	27	1	2	5	50

multiplication cycle that any complementation was obscured. Furthermore, recombination analysis alone was capable of classifying the mutants into discrete groups revealing the organisation of the genome.

Attention was turned, therefore, to complementation within recombination groups because bunyaviruses have been shown to possess four structural proteins, L, G1, G2 and N (Obijeski et al 1976a, Pennington et al 1977) and possibly one or more non-structural proteins (D.H.L. Bishop, personal communication). This would mean at least one structural protein in excess of the three genome subunits, suggesting that at least one genome subunit may be coding for more than one protein. In recombination by reassortment, two genes carried on the same subunit would segregate together and so would not be distinguished in a recombination test. Complementation could, however, identify such genes.

Monolayers of BHK-21 cells were coinfected with pairwise combinations of Maguari group I or group II ts mutants and assayed for complementation. All complementation assays were carried out as described in the Methods (Section F). The restrictive temperature was sometimes raised to 38.5° C to eliminate or reduce the leak-yield background of some of the ts mutants. The first complementing pair of mutants identified in group I, MAGts6(I) and MAGts7(I), were subsequently used to determine the effect of multiplicity on bunyavirus complementation. The ts mutants were propagated in 20-ounce (500 ml) bottle cultures of BHK-21 cells. On harvest, 3 - 4 days post- infection

at complete CPE, the cultures were clarified of cell debris by centrifugation at 10,000 rpm for 10 minutes and virus was concentrated by pelleting at 20,000 rpm for one hour through a 30% glycerol cushion. Centrifugation was carried out with a Sorvall RC-5B centrifuge. The pellet was resuspended in 0.5 ml of Eagle's medium (containing 10% calf serum) and diluted in ten-fold series down to 10^{-7}. Dilutions 10^{-4} - 10^{-7} were assayed for infectivity at 31° C while dilutions 10^{0} - 10^{-3} were used for the complementation experiment. The titres of both ts mutants were found to be similar (about 10^{8} pfu/ml). Similar dilutions (10^{0} - 10^{-3}) of both ts mutants were used to infect BHK-21 monolayers in multiwell (Linbro) plates both multiply and singly. After incubation at 38° C for 18 hours the cultures were assayed for complementation as previously described (Methods, Section F). The results of the assays (summarised in Table 24) indicate that the ts mutants complemented more at higher concentrations (multiplicities). The highest complementation index (35) was realised at the dilution 10^{-1} (approximate multiplicity of infection of 40 pfu per cell). At dilution 10^{0} (approximately 400 pfu per cell), the complementation index was depressed (1.6). The reason for this decrease is not certain. One explanation could be an increase in the leak yield of the singly infected cultures. Alternatively it could be explained by multiplicity dependent interference (by defective virus particles). Clonal analysis of 37 clones isolated from

TABLE 24

Complementation between MAG*ts*6(I) and MAG*ts*7(I) (group I mutants) at different multiplicities of infection.

VIRUS	DILUTION	18-HR YIELD ASSAYED AT		CI
		31°C	38.5°C	
MAG*ts*6(I)	10^{0}	100	<10	--
MAG*ts*7(I)		225	<10	--
MAG*ts*6(I) + MAG*ts*7(I)		525	<10	1.6
MAG*ts*6(I)	10^{-1}	25	<10	--
MAG*ts*7(I)		<10	<10	--
MAG*ts*6(I) + MAG*ts*7(I)		1225	<10	35
MAG*ts*6(I)	10^{-2}	<10	<10	--
MAG*ts*7(I)		<10	<10	--
MAG*ts*6(I) + MAG*ts*7(I)		200	<10	>10
MAG*ts*6(I)	10^{-3}	<10	<10	--
MAG*ts*7(I)		<10	<10	--
MAG*ts*6(I) + MAG*ts*7(I)		<10	<10	<1.0

the above mixed infections at 31° C showed that all were temperature-sensitive. This confirmed that MAG*ts*6(I) and MAG*ts*7(I) indeed complemented each other at 38° C but did not recombine. Subsequently, 7 *ts* mutants from the group, previously used for examining the homogeneity of the recombination group were tested for complementation in all combinations and the results are shown in Table 25. The pairs, MAG*ts*1(I) and MAG*ts*3(I), MAG*ts*2(I) and MAG*ts*6(I), MAG*ts*3(I), and MAG*ts*6(I) and MAG*ts*6(I) and MAG*ts*7(I) showed significant complementation. The *ts* mutants did not fall into definite non-overlapping complementation groups. This pattern has been observed, however, in the rhabdovirus, Chandipura virus and interpreted as intracistronic complementation (Gadkari and Pringle, 1980a). The above observations indicate that the *ts* mutants of group I are not all identical and that the group I genome subunit may be coding for more than one protein.

In a similar screening of some group II *ts* mutants, using MAG*ts*8(II) as a standard mutant, complementation was not unambigously detected (Table 26). Though the duplicate cultures which were simultaneously infected with MAG*ts*8(II) and MAG*ts*45(II) and incubated at the restrictive temperature gave a complementation index of 7.3 in one assay, this was considered to be due to virus released as a result of "leakage" and/or reversion.

TABLE 25

Complementation among Maguari group I *ts* mutants.

	MAG*ts*1	MAG*ts*2	MAG*ts*3	MAG*ts*4	MAG*ts*5	MAG*ts*6	MAG*ts*7
MAG*ts*1	--	2	167.5	4.6	0.9	5.08	3.3
MAG*ts*2		--	0.4	3.85	4.6	15	0.8
MAG*ts*3			--	1.3	0.143	19.4	1.75
MAG*ts*4				--	4.4	6.7	1.93
MAG*ts*5					--	1.0	1.8
MAG*ts*6						--	33
MAG*ts*7							--

TABLE 26

COMPLEMENTATION AMONG MAGUARI GROUP II *ts* MUTANTS

	+ SELF		+ MAG*ts*8(II)		
	18-HR YIELD ASSAYED AT		18-HR HIELD ASSAYED AT		CI
VIRUS	31°C	38°/38.5°C	31°C	38°/38.5°C	
MAG*ts*8(II)	735	514	--	--	--
MAG*ts*9(II)	<10	<10	223	30	0.3
MAG*ts*10(II)	<10	<10	430	283	0.2
MAG*ts*11(II)	323	173	365	320	0.04
MAG*ts*12(II)	1.04×10^5	6.9×10^4	4.13×10^5	3.33×10^5	0.8
MAG*ts*13(II)	1.1×10^4	1.3×10^4	3.2×10^3	2.2×10^3	0.09
MAG*ts*14(II)	<10	<10	138	118	0.03
MAG*ts*15(II)	2700	--	1300	<10	0.4
MAG*ts*16(II)	<10	<10	335	105	0.3
MAG*ts*17(II)	28	<10	497	622	<0.01
MAG*ts*18(II)	83.3	--	766	150	0.8
MAG*ts*31(II)	125	--	1325	<10	1.5
MAG*ts*32(II)	50	--	1825	500	1.7
MAG*ts*40(II)	2.9×10^4	--	3310	50	0.1
MAG*ts*43(II)	2075	--	2375	150	0.8
MAG*ts*45(II)	100	--	7833	1750	7.3

II.3 HETEROLOGOUS RECOMBINATION

Temperature-sensitive mutants representing groups I and II of Batai, Bunyamwera and Maguari viruses were crossed in homologous and heterologous pairwise combinations and assayed for recombination. The results (Table 27) showed that in all the homologous crosses and the heterologous crosses of Batai virus and Bunyamwera virus ts mutants, recombination occurred at high frequencies (RF ranging from 6.5% to 54.5%). In the heterologous crosses of Maguari virus ts mutants and Batai virus or Bunyamwera virus ts mutants the recombination frequencies were low (0.2% - 0.79%). Heterologous recombination was restricted, in that, group I mutants of one virus only recombined with group II mutants of the other virus and vice versa. There was no detectable recombination in pairwise combinations of group I mutants, or in cases of group II mutants inter-se. The heterologous recombination was bi-directional, in that, recombination occurred between BAT(I) and BUN(II) as well as in the reciprocal cross of BAT(II) and BUN(I). Similarly, bi-directional recombination was detected in crosses of BAT and MAG ts mutants and BUN and MAG ts mutants (Table 27). These results indicated that the recombination groups I and II of all three viruses are homologous and represent mutations affecting the same two subunits of the viral genome.

The group III mutant, MAGts23(III) was defined so late in the investigation that comprehensive heterologous crosses have not been completed. However, the results

TABLE 27: Heterologous recombination between group I and group II ts mutants of Batai virus, Bunyamwera virus and Maguari virus.

In the heterologous crosses recombination was initially assessed from the proportion of the yield of non-ts clones at 38° C in multiply infected cultures relative to the singly infected controls. Subsequently clones from crosses giving RF values below 1% were tested by SDS-polyacrylamide gel electrophoresis to verify their recombinant status. The underlined RF values included non-ts clones confirmed as recombinants by this procedure.

TABLE 27

VIRUS		BATAI		BUNYAMWERA		MAGUARI	
		GROUP I BATts1(I)	GROUP II BATts2(II)	GROUP I BUNts5(I)	GROUP II BUNts8(II)	GROUP I MAGts7(I)	GROUP II MAGts8(II)
BATAI	GROUP I BATts1(I)	0.07	54.5	*0.002	53.6	0.006	0.2
	GROUP II BATts2(II)		0.05	6.5	0.05	0.3	0.0002
BUNYAMWERA	GROUP I BUNts5(I)			0.003	27.3	0.05	0.66
	GROUP II BUNts8(II)				0.005	0.79	0.003
MAGUARI	GROUP I MAGts7(I)					0.06	27.1
	GROUP II MAGts8(II)						0.001

Underlined figures show recombination.

* Result from a cross of BUNts2(I) x BATts1(I).

of the recombination experiments shown in Table 28 indicated that MAG*ts*23(III) recombined with both group I and group II *ts* mutants of Bunyamwera virus [(BUN*ts*5(I) and BUN*ts*8(II), respectively)]. Clones were isolated at 38° C from the progeny of these crosses and have been shown to have the non-*ts* phenotype expected of recombinant virus. These results need further confirmation, however, in view of the fairly high leak yields of the Bunyamwera virus *ts* mutants in this particular experiment.

TABLE 28: Heterologous recombination;

MAG*ts*23(III) x BUN*ts*5(I) x BUN*ts*8(II).

VIRUS	18-HR YIELD ASSAYED AT		RF/%EOPx2
	31° C	38° C	
BUN*ts*5(I)	1.6×10^7	3.7×10^4	0.5
BUN*ts*8(II)	1.6×10^8	6.3×10^5	0.8
MAG*ts*23(III)	1.1×10^6	<10	<0.002
BUN*ts*5(I)xMAG*ts*23(III)	1.14×10^7	1.1×10^5	1.2
BUN*ts*8(II)xMAG*ts*23(III)	1.8×10^7	1.7×10^6	12.5

III. PHENOTYPIC CHARACTERISTICS OF THE HETEROLOGOUS RECOMBINANTS

The putative recombinant clones were phenotypically characterised in the first instance to ascertain that they were indeed recombinants. This was necessary especially in the case of those heterologous pairs of mutants which recombined at low frequencies. Secondly, there was the need to determine the contribution of the respective parental mutants to the genotype of the recombinants. This was done by comparing the recombinants with either the parental wild type viruses or their ts mutant parents wherever there were phenotypically distinguishable differences between the strains.

The phenotypic characteristics examined included temperature-resistance, virion polypeptide electrophoretic mobility, plaque morphology and surface antigens.

III.1 CLONAL ANALYSIS

Clones were generally isolated at 38° C from the progeny of the heterologous crosses and were tested for ability to form plaques at both 31° C and 38° C. In cases where either of the parental mutants exhibited leakiness, the clones were isolated at 38.5° C. Raising the restrictive temperature effectively reduced the chances of isolating non-recombinant leaky clones rather than true wild type recombinants. All of 120 clones isolated from ten different crosses produced plaques at both permissive and restrictive temperatures. Ninety of these clones (each cross being represented by at least four randomly selected clones) were amplified by propagation in one-ounce (30 ml) bottle cultures of BHK-21 cells. The progeny were tested for infectivity at both

restrictive and permissive temperatures by the plaque assay method. The EOP's of the selected clones have been compared with those of their ts parents in Table 29. The results indicated that the clones isolated at 38° C or 38.5° C from the progeny of the heterologous crosses exhibited the wild type phonotype expected of recombinant viruses (efficiency of plating ranging from 0.14 to 3.6).

III.2.(i) COMPARISON OF VIRUS STRUCTURAL PROTEINS BY POLYACRYLAMIDE GEL ELECTROPHORESIS

$[^{35}S]$-methionine-labelled polypeptides (see Methods Section H) of Batai virus, Bunyamwera virus and Maguari virus were compared by SDS-polyacrilamide slab gel electrophoresis (see Methods Section I). The polypeptides of vesicular stomatitis virus, Indiana serotypes (VSV-Indiana) and Chandipura virus, also labelled with (^{35}S) methionine, were included in one of the gels as molecular weight markers.

Figure 13 shows the electrophoretic separation of the virion polypeptides. Tracks 1 and 2 are polypeptides of VSV-Indiana and Chandipura virus respectively. Tracks 3 and 5 are polypeptides of Batai virus and Maguari virus respectively, while tracks 4 and 7 are polypeptides of Bunyamwera virus, wild type. Track 6 is Bunyamwera virus ts mutant, BUNts7(II) and track 8 represents $[^{35}S]$-methionine-labelled polypeptides of mock-infected BHK-21 cells. In this gel the L polypeptides of both the rhabdoviruses (VSV-Indiana and Chandipura virus) and the bunyaviruses were not resolved. The other polypeptides, G, N, NS and M of VSV-Indiana and Chandipura virus

TABLE 29: The efficience of plating $\left[\frac{\text{pfu at } 38^{\circ} \text{ C}}{\text{pfu at } 31^{\circ} \text{ C}}\right]$

of putative recombinant clones.

All the clones were isolated at the restrictive temperature (38° C/38.5° C). They have been recoded to correspond with the new codes of their parental ts mutants (see Section II.1, Tables 10, 11 and 12) except that the original designations of the virus types, B (Batai), BN (Bunyamwera) and M (Maguari) have been retained. The symbols and code numbers of the clones show the group I parent first, followed by the group II parent and thirdly the serial number of the clones. For example the code BBN1/8/1 is a clone obtained from the progeny of a cross of Batai (B) group I ts mutant, BATts1(I) and Bunyamwera (BN) group II ts mutant, BUNts8(II).

* The efficiency of plating of the parental ts mutants.

**The efficiency of plating of the parental wild type viruses.

TABLE 29 : Efficiency of plating (EOP) of some of the non-ts clones isolated from the heterologous crosses compared with the EOP of their ts parents and the parental wild type viruses.

Virus clone/ts mutant		Virus litre (pfu/ml) Assayed at		
Old code	New code	31°C	38°C/38.5°C	EOP
tsB54	BATts1(I)	$1.2x10^6$	10	* $8.3x10^{-6}$
tsBN67	BUNts8(II)	$3.7x10^7$	$2.7x10^4$	$7x10^{-4}$
BNB67/54/1	BBN1/8/1	$2.5x10^6$	$2.6x10^6$	1.00
BNB67/54/2	BBN1/8/2	$1.6x10^7$	$4.3x10^7$	2.7
BNB67/54/3	BBN1/8/3	$3.5x10^6$	$1.2x10^7$	3.4
BNB67/54/4	BBN1/8/4	$1.4x10^6$	$7.5x10^5$	.6
BNB67/54/5	BBN1/8/5	$6x10^6$	$9.8x10^6$	1.6
BNB67/54/6	BBN1/8/6	$7.3x10^5$	$1.1x10^6$	1.5
BNB67/54/7	BBN1/8/7	$1.6x10^7$	$1.4x10^7$	.9
BNB67/54/8	BBN1/8/8	$5x10^7$	$2.1x10^7$	.4
BNB67/54/9	BBN1/8/9	$4.9x10^6$	$8x10^6$	1.6
BNB67/54/10	BBN1/8/10	$1.1x10^6$	$1.5x10^6$	1.4
tsB7	BATts2(II)	$1.7x10^6$	65	*$3.8x10^{-5}$
tsBN137	BUNts5(I)	$1.5x10^8$	10^2	* $6.7x0^{-7}$
BNB137/7/1	BNB5/2/1	$1.1x10^7$	$1.3x10^7$	1.2
BNB137/7/2	BNB5/2/2	$2.9x10^7$	$1.4x10^7$	.5
BNB137/7/3	BNB5/2/3	$6.3x10^6$	$1.1x10^7$	1.8
BNB137/7/4	BNB5/2/4	$1.2x10^7$	$9.5x10^6$	.8
BNB137/7/5	BNB5/2/5	$1.3x10^7$	$1.1x10^7$	.9
BNB137/7/6	BNB5/2/6	$9x10^6$	$1.4x10^7$	1.5

TABLE 29 : (CONTD.)

tsB54	BATts1(I)	1.2×10^{6}	10	$*\ 8.3\times10^{-6}$
tsM290	MAGts8(II)	2.5×10^{7}	50	$*2.04\times10^{-6}$
MB290/54/2	BM1/8/2	6.1×10^{7}	3.6×10^{7}	0.6
MB290/54/3	BM1/8/3	3.3×10^{7}	3.7×10^{7}	1.1
MB290/54/5	BM1/8/5	1.8×10^{7}	1.8×10^{7}	1.0
MB290/54/6	BM1/8/6	3.8×10^{7}	4.6×10^{7}	1.2
MB290/54/7	BM1/8/6	7×10^{7}	3.3×10^{7}	0.5
MB290/54/9	BM1/8/9	1.4×10^{7}	3.1×10^{7}	2.3
MB290/54/16	BM1/8/16	8.5×10^{7}	3.7×10^{7}	0.4
MB290/54/17	BM1/8/17	5.5×10^{8}	3.2×10^{8}	0.6
MB290/54/19	BM1/8/19	1×10^{8}	6.5×10^{7}	0.7
tsB7	BATts2(II)	1.7×10^{6}	65	$*3.8\times10^{-5}$
tsM1370	MAGts7(I)	1.7×10^{8}	10	$*\ 5.8\times10^{-8}$
MB1370/7/4	MB7/2/4	3.2×10^{6}	1.6×10^{6}	0.5
MB1370/7/5	MB7/2/5	1.1×10^{6}	1.6×10^{6}	1.4
MB1370/7/6	MB7/2/6	2×10^{8}	2×10^{8}	1.0
MB1370/7/8	MB7/2/8	2.4×10^{8}	7×10^{7}	0.3
MB1370/7/15	MB7/2/15	1.2×10^{7}	6.3×10^{6}	0.5
MB1370/7/16	MB7/2/16	2.8×10^{8}	1.4×10^{8}	0.5
MB1370/7/17	MB7/2/17	5.9×10^{6}	8×10^{5}	0.1
MB1370/7/18	MB7/2/18	7.4×10^{8}	5.4×10^{8}	0.7
tsBN9	BUNts1(I)	5×10^{6}	50	$*10^{-5}$
tsM290	MAGts8(II)	2.5×10^{7}	50	$*2.0\times10^{-6}$
MBN290/9/1	BNM1/8/1	1.5×10^{8}	4.1×10^{7}	0.3
MBN290/9/2	BNM1/8/2	7.5×10^{7}	5.6×10^{7}	0.7
MBN290/9/3	BNM1/8/3	3.2×10^{7}	2.8×10^{7}	0.9
MBN290/9/4	BNM1/8/4	8×10^{7}	4.4×10^{7}	0.6

TABLE 29 : (CONTD.)

tsBN61	BUNts2(I)	4.7×10^{6}	1.5×10^{2}	$*3.2\times10^{-5}$
MBN290/61/1	BNM2/8/1	2.5×10^{6}	1.3×10^{6}	0.5
MBN290/61/2	BNM2/8/2	2.2×10^{6}	1.7×10^{6}	0.8
MBN290/61/3	BNM2/8/3	2.5×10^{6}	6.8×10^{5}	0.3
MBN290/61/4	BNM2/8/4	8.5×10^{7}	5.3×10^{7}	0.6
MBN290/61/5	BNM2/8/5	1.1×10^{8}	5.5×10^{7}	0.5
MBN290/61/6	BNM2/8/6	2.5×10^{7}	1×10^{7}	0.4
tsBUN105	BUNts3(I)	2.6×10^{7}	3×10^{2}	$*10^{-5}$
MBN290/105/1	BNM3/8/1	5.2×10^{7}	2.4×10^{7}	0.5
MBN290/105/2	BNM3/8/2	1.3×10^{8}	4.1×10^{7}	0.3
MBN290/105/3	BNM3/8/3	4.2×10^{7}	2×10^{7}	0.5
MBN290/105/4	BNM3/8/4	6.6×10^{7}	1.4×10^{7}	0.2
MBN290/105/5	BNM3/8/5	3.2×10^{7}	1.4×10^{7}	0.4
MBN290/105/6	BNM3/8/6	1.1×10^{8}	3.1×10^{7}	0.3
MBN290/105/7	BNM3/8/7	5.9×10^{7}	3.1×10^{7}	0.5
MBN290/105/8	BNM3/8/8	1.1×10^{7}	10^{7}	0.95
MBN290/105/9	BNM3/8/9	2.3×10^{7}	2.1×10^{7}	0.9
MBN290/105/10	BNM3/8/10	3×10^{7}	2.0×10^{7}	0.7
MBN290/105/11	BNM3/8/11	3×10^{7}	3.1×10^{7}	1.0
MBN290/105/12	BNM3/8/12	2×10^{6}	3×10^{7}	0.2
MBN290/105/13	BNM3/8/13	3.3×10^{7}	2.1×10^{7}	0.6
MBN290/105/14	BNM13/8/14	3.3×10^{7}	2.1×10^{7}	0.6
MBN290/105/15	BNM3/8/15	10^{7}	3.2×10^{6}	0.3
tsBN111	BUNts4(I)	3.3×10^{7}	2.5×10^{3}	$*7.6\times10^{-5}$
MBN290/111/1	BNM4/8/1	3×10^{6}	5×10^{5}	0.2
MBN290/111/2	BNM4/8/2	1.6×10^{6}	5.5×10^{5}	0.3

TABLE 29 : (CONTD.)

MBN290/111/3	BNM4/8/3	3.2×10^{7}	2.1×10^{7}	.7
MBN290/111/4	BNM4/8/4	3.8×10^{7}	1.5×10^{7}	.4
MBN290/111/5	BNM4/8/5	3×10^{7}	7.5×10^{6}	.3
MBN290/111/6	BNM4/8/6	2.2×10^{7}	9.5×10^{6}	.4
MBN290/111/7	BNM4/8/7	4.5×10^{7}	1.3×10^{7}	0.3
tsBN137	BUNts5(I)	1.5×10^{8}	10^{2}	* 6.7×10^{-7}
MBN290/137/1	BNM5/8/1	3.1×10^{7}	1.2×10^{7}	0.4
MBN290/137/2	BNM5/8/2	1.3×10^{7}	3.1×10^{6}	0.2
MBN290/137/3	BNM5/8/3	2.7×10^{7}	1.2×10^{7}	0.4
MBN290/137/4	BNM5/8/4	1.3×10^{6}	4.5×10^{5}	0.4
MBN290/137/5	BNM5/8/5	3.2×10^{7}	1.6×10^{7}	0.5
MBN290/137/6	BNM5/8/6	6×10^{6}	2.0×10^{6}	.5
MBN290/137/7	BNM5/8/7	2.3×10^{7}	1.3×10^{7}	.6
MBN290/137/8	BNM5/8/8	2.5×10^{7}	1.5×10^{7}	.6
MBN290/137/9	BNM5/8/9	3.3×10^{7}	3.1×10^{7}	.9
MBN290/137/10	BNM5/8/10	8.5×10^{6}	1.2×10^{7}	1.4
MBN290/137/11	BNM5/8/11	2.6×10^{7}	1.3×10^{7}	.5
MBN290/137/12	BNM5/8/12	8×10^{6}	1.4×10^{7}	1.8
MBN290/137/13	BNM5/8/13	2.6×10^{7}	2.4×10^{7}	.9
MBN290/137/14	BNM5/8/14	1.8×10^{6}	5.5×10^{5}	0.3
MBN290/137/15	BNM5/8/15	1.9×10^{7}	1.4×10^{7}	0.8
MBN290/137/16	BNM5/8/16	2×10^{7}	1.5×10^{7}	0.8
MBN290/137/17	BNM5/8/17	3.8×10^{7}	2.0×10^{7}	0.5
MBN290/137/18	BNM5/8/18	1.9×10^{7}	6.6×10^{6}	0.4
MBN290/137/19	BNM5/8/19	8.5×10^{7}	3.0×10^{7}	0.4
tsBUN67	BUNts8(II)	3.7×10^{7}	2.7×10^{4}	*7×10^{-4}
tsM1370	MAGts7(I)	1.7×10^{8}	10	* 5.8×10^{-8}

TABLE 29 : (CONTD.)

MBN1370/67/1	MBN7/8/1	2.5×10^7	9×10^7	3.6
MBN1370/67/2	MBN7/8/2	1.4×10^8	3×10^7	.2
MBN1370/67/3	MBN7/8/3	2.5×10^7	3×10^6	.13
MBN1370/67/4	MBN7/8/4	10^8	3.3×10^7	.3
	BATts+	7×10^6	6.1×10^6	**.9
	BUNts+	3.4×10^7	1.5×10^7	**.4
	MAGts+	1.1×10^7	1.4×10^6	**.13

FIGURE 13.

Electrophoresis in 7.5% polyacrylamide gel of [^{35}S]-methionine-labelled polypeptides of Batai, Bunyamwera, and Maguari viruses in comparison with the rhabdoviruses, vesicular stomatitis virus and Chandipura virus.

Track 1, vesicular stomatitis virus Indiana serotype, ts mutant (ts G114);

Track 2, Chandipura virus, wild type;

Track 3, Batai virus, wild type;

Tracks 4 and 7, Bunyamwera virus, wild type;

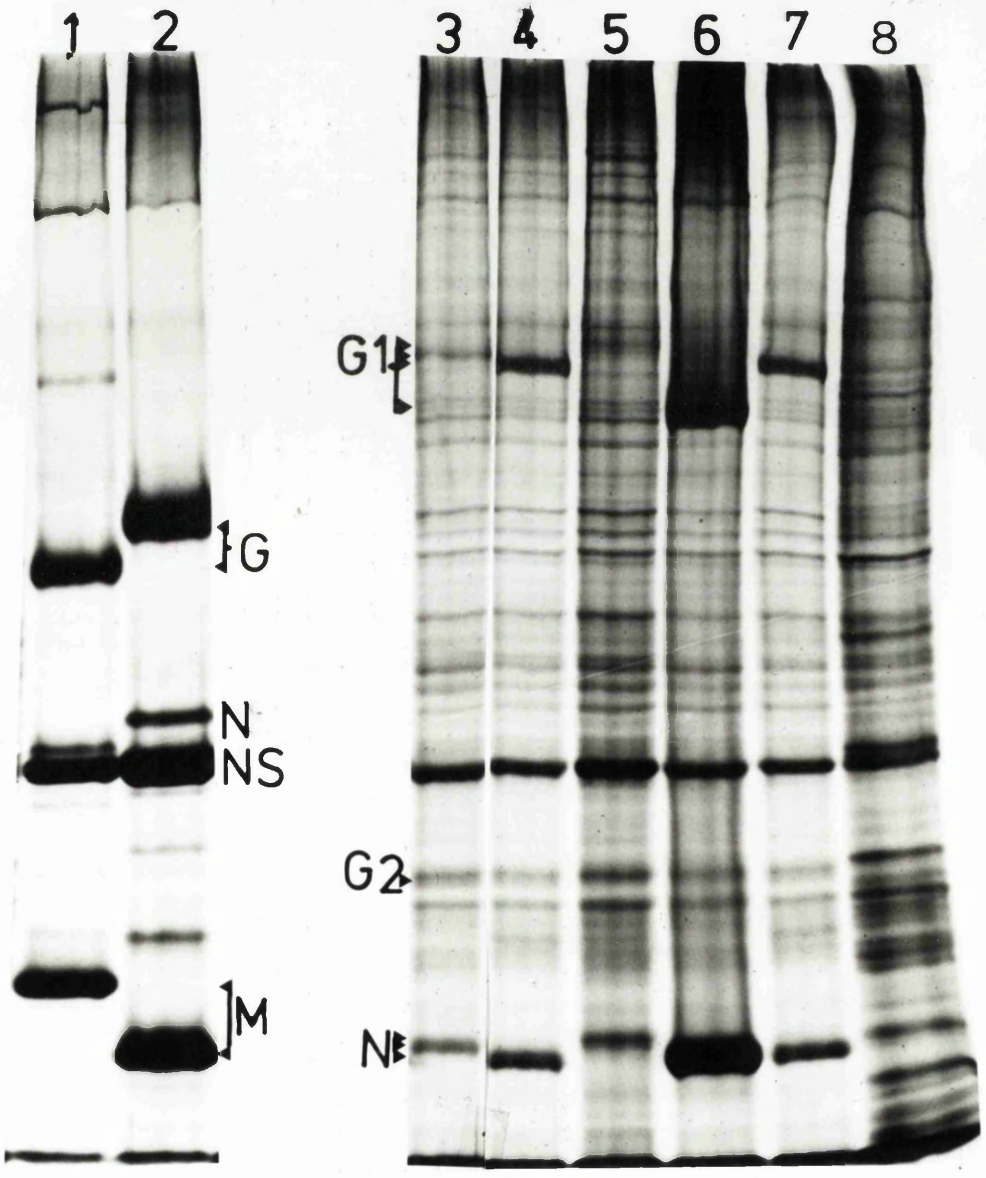
Track 5, Maguari virus, wild type;

Track 6, Bunyamwera virus ts mutant, BUNts 7(II);

Track 8, mock-infected BHK-21 cell extract.

All the samples (tracks 1 - 8) were run in a single gel slab. Tracks 1 and 2 were cut out of the auto-radiogram and separated from the rest for clarity in labelling of the bands.

Fig.13



respectively and the G1, G2 and N polypeptides of Batai, Bunyamwera and Maguari viruses were identified. The estimated molecular weights of the marker polypeptides are as follows:

VSV-Indiana:- VP II (G polypeptide), $61x10^3$; VP III (N polypeptide), $45.6x10^3$; VP IV (NS polypeptide), $37.8x10^3$; and VP V (M polypeptide), $28.3x10^3$ (Wunner and Pringle, 1972); Chandipura virus:- L, $151-162x10^3$; G, $69 \pm 2x10^3$; N, $50 \pm 4 \times 10^3$; NS, $42 \pm 4 \times 10^3$ and M, $24x10^3$ (Bishop and Smith, 1977).

Comparison of the migration rates of the polypeptides of Batai, Bunyamwera and Maguari viruses and the marker polypeptides showed that the polypeptides of the three viruses of the Bunyamwera complex were similar to the polypeptides detected by Pennington et al (1977) in Bunyamwera virus-infected BS-C-1 cells and designated L($200x10^3$), G1 ($128x10^3$), G2($31x10^3$) and N, ($23x10^3$). In subsequent experiments the L polypeptide was easily detected whereas the G2 polypeptide was rarely resolved. In this experiment it was observed that the G1 and N polypeptides of Batai, Bunyamwera and Maguari viruses could be distinguished whereas the L and G2 (wherever resolved) were indistinguishable (see also Figures 14(A) and (B)).

As shown in Figure 13, track 6, the Bunyamwera virus ts mutant, BUNts7(II) possessed a G1 polypeptide which migrated faster than the G1 of the parental wild type virus (cf tracks 4 and 7). This ts mutant has been investigated further and the observations are reported in Section III.2(ii).

The above experiment was repeated using partially purified virus in order to determine whether any additional minor proteins could be resolved. The partial purification process was as described in the Methods (Section J) except that virus was concentrated by centrifugation at 20,000 rpm for 1 hour, using a Sorvall type SS34 rotor and an RC-5B centrifuge, instead of PEG-precipitation. Figure 14(A) shows [^{35}S]-methionine-labelled polypeptides of mock-infected BHK-21 cells in track 1, and similarly-labelled polypeptides of partially purified Batai virus, Bunyamwera virus and Maguari virus preparations in tracks 2, 3 and 4 respectively. All four bunyavirus structural polypeptides, L, G1, G2 and N were detected. The G1 and N polypeptides were again distinguishable in all three viruses whereas the L and G2 polypeptides were not. No additional polypeptides were unambiguously identified. The polypeptide band marked X appeared to run faster in the Bunyamwera virus and Maguari virus preparations compared to a similar polypeptide in the Batai virus preparation or the host cell polypeptide closest to them. The nature of this polypeptide was not known but it was presumed to be the host cell actin which is marked Y in Figure 14(B) and shown in Figure 13 to co-migrate with the Chandipura virus NS polypeptides (see Gadkari, 1979). The reason for the slight alteration in the mobility of X polypeptide (presumed to be actin) is not certain. It may be caused by traces of sucrose from the gradient in the virus samples.

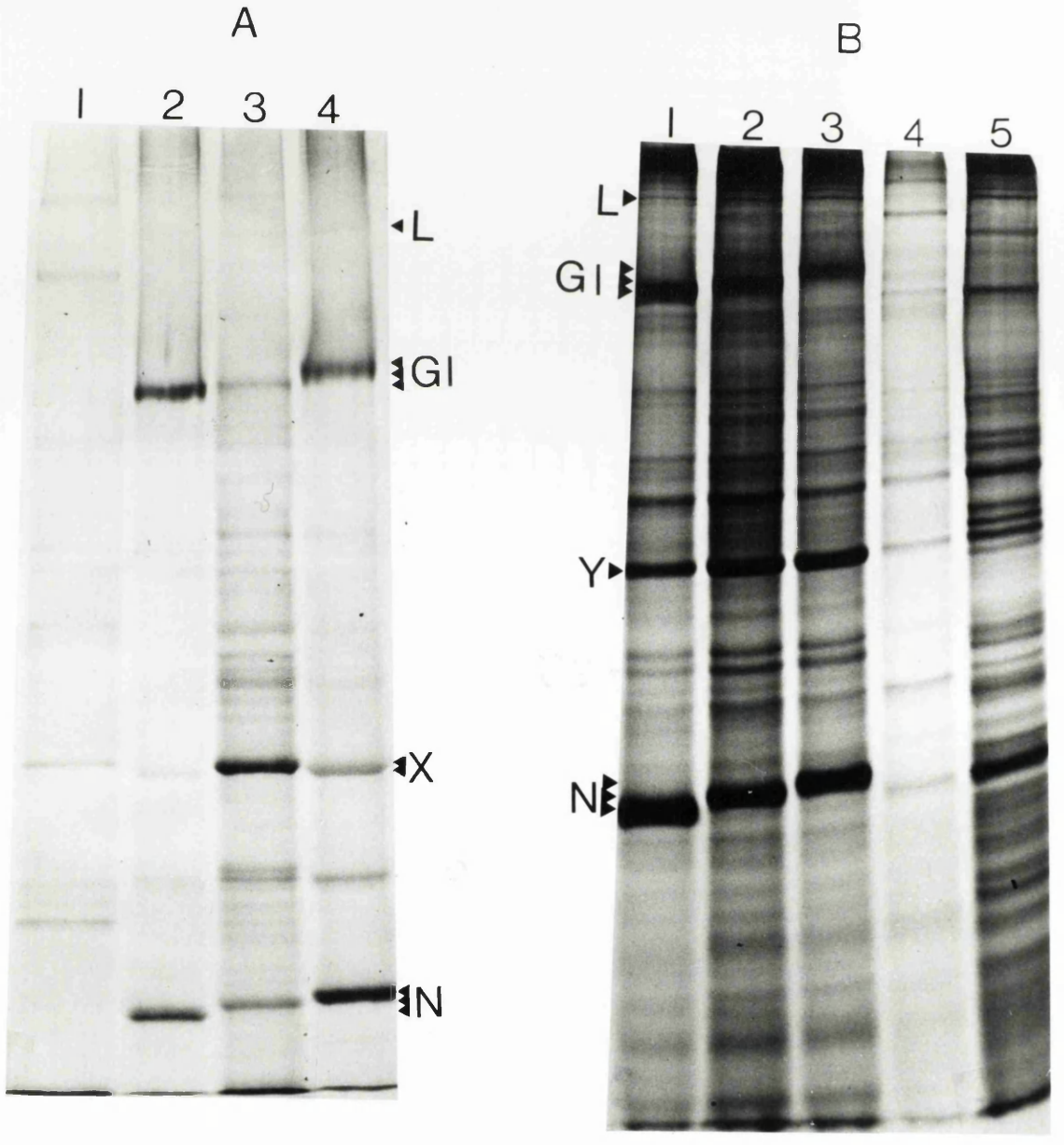
FIGURE 14

Comparison of [^{35}S]-methionine-labelled polypeptides of partially purified and unpurified preparations of Batai virus, Bunyamwera virus and Maguari virus.

A. Electrophoresis in 7.5% polyacrylamide gel of ^{35}S methionine-labelled polypeptides of partially purified Batai virus, Bunyamwera virus and Maguari virus. Track 1, mock infected BHK-21 cell extract
Track 2, Batai virus, wild type;
Track 3, Bunyamwera virus, wild type;
Track 4, Maguari virus, wild type.

B. Electrophoresis in 10% polyacrylamide gel of (^{35}S)methionine-labelled polypeptides of unpurified Batai virus, Bunyamwera virus, Maguari virus and mock-infected BHK-21 cells.
Track 1, Batai virus, wild type;
Track 2, Bunyamwera virus, wild type
Track 3, Maguari virus, wild type
Track 4, mock-infected BHK-21 cell culture supernatant
Track 5, mock-infected BHK-21 cell extract obtained after freezing and thawing cell monolayer in alcohol/dry-ice.

Fig·14



III.2(ii) FURTHER CHARACTERISATION OF BUNts7(II)

Figure 13, track 6 (Section III.2(i) shows that the G1 polypeptide of BUNts7(II) migrated faster than the G1 of wild type Bunyamwera virus. Non-ts revertants of the above mutant were examined by polyacrylamide gel electrophoresis to determine whether the altered mobility of G1 polypeptide was related to the ts phenotype. The revertants were isolated at 38.5° C and tested for their ability to produce plaques at 31° C and 38.5° C respectively. The isolation and assay were carried out at 38.5° C because BUNts7(II) showed a high degree of leak at 38° C but much less so at 38.5° C. As shown in Table 30, all the clones isolated plated at efficiencies ranging from 0.2 to 1.00. This indicated that they were wild-type revertants.

The polypeptide profiles of the revertant clones, their ts parents, BUNts7(II), and the Bunyamwera virus wild-type, were compared in 7.5% polyacrylamide slab gel as shown in Figure 15. Track 5 shows the polypeptides of BUNts7(II) and track 6 the wild type Bunyamwera virus while track 11 is a mixture of BUNts7(II) and the wild type parent (Bunyamwera virus). The rest of the tracks (1 - 4 and 7 - 10) show the polypeptides of 8 revertant clones. In track 6, there are two polypeptide bands below the G1 polypeptide of the wild type virus coinciding with the position of the G1 of BUNts7(II). These two bands may be host cell polypeptides co-migrating with the G1 of BUNts7(II). The revertant clones expressed G1 polypeptides

TABLE 30

Efficiency of Plating of Revertant Clones isolated from BUN*ts*7(II) at 38.5° C.

Clone/Virus	Infectivity of virus assayed at		E. O. P.
	31° C	38.5° C	
Bunyamwera *ts*$^{+}$	3.35×10^{7}	1.5×10^{7}	0.45
BUN*ts*7(II)	7.05×10^{7}	1.53×10^{4}*	2.2×10^{-4}
Revertant Clones			
1	5.5×10^{6}	2.3×10^{6}	0.4
2	2.7×10^{6}	1.7×10^{6}	0.6
3	4.7×10^{6}	8.5×10^{5}	0.2
4	3.3×10^{6}	8×10^{5}	0.2
5	2.2×10^{6}	10^{6}	0.5
6	2.6×10^{6}	2.6×10^{6}	1.0
7	3.5×10^{6}	1.93×10^{6}	0.6
8	2×10^{6}	1.4×10^{6}	0.7

* assay done at 38° C.

FIGURE 15

Electrophoresis in 7.5% polyacrylamide gel of [^{35}S]methionine-labelled polypeptides of revertants of Bunyamwera virus, BUN*ts*7(II) compared with the parent mutant BUN*ts*7(II) and the parental wild type, Bunyamwera virus

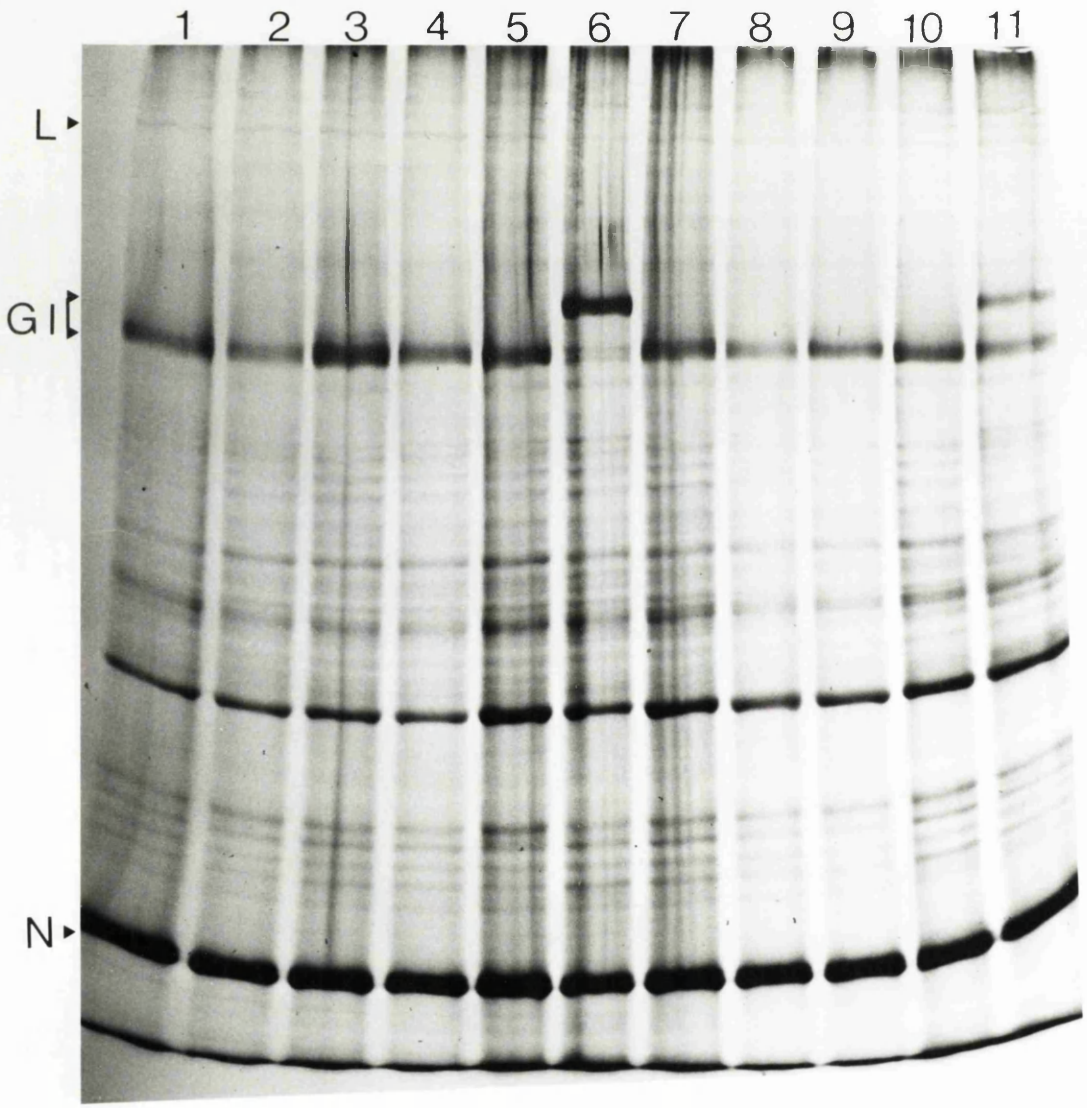
Tracks 1, 2, 3, 4, 7, 8, 9 and 10, revertants;

Track 5, BUN*ts*7(II);

Tract 6, Bunyamwera virus, wild type

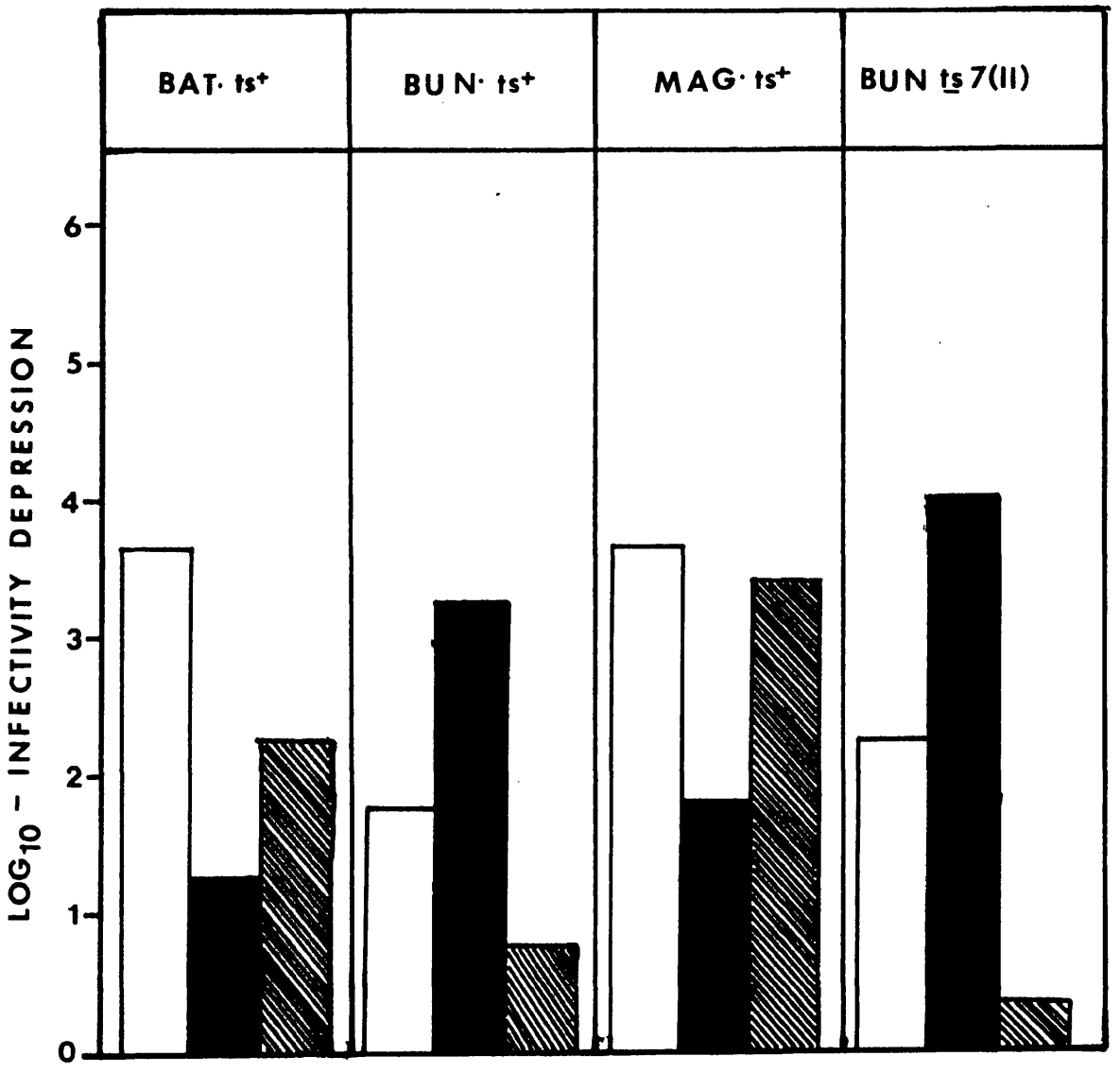
Track 11, mixture of BUN*ts*7(II) and Bunyamwera virus wild type.

Fig·15



with electrophoretic mobilities similar to their parental ts mutant, BUNts7(II). This confirmed that the altered electrophoretic mobility of the G1 polypeptide of BUNts7(II) was due to an independent non-temperature- sensitive mutation affecting this glycoprotein. It is not known whether the enhanced mobility of the G1 was due to a defect in glycosylation or not. As shown in Figure 16, mutant BUNts7(II) retained the neutralisation specificity of Bunyamwera virus and the mutation did not appear to have any gross effect on the antigenic properties of the virus.

Fig·16



Fig·16: Comparison of the neutralisation characteristics of wild type Batai virus, Bunyamwera virus, Maguari virus and BUN ts 7(II).

□-anti-Batai virus serum; ■-anti-Bunyamwera virus serum; ▨-anti-Maguari virus serum.

III.3.1 COMPARISON OF THE PROTEINS OF THE RECOMBINANTS AND THEIR PARENT VIRUSES BY POLYACRYLAMIDE GEL ELECTROPHORESIS

The differences in the electrophoretic mobilities of the G1 and N polypeptides of Batai, Bunyamwera and Maguari viruses made it possible to confirm the recombinant nature of the non-ts clones isolated from the heterologous crosses, and the homology of groups I and II of all three viruses.

Temperature-sensitive mutants of all three viruses, including those used in the heterologous crosses were compared with their wild type parents by SDS-polyacrylamide slab gel electrophoresis of their polypeptides These comparisons were necessary because the recombinants were generated from interactions between the ts mutants rather than the wild type viruses. Out of 31 ts mutants compared (listed in Table 31), including those done in collaboration with Dr C.R. Pringle, only one -BUNts7(II)- differed from the wild type parent (as described in the preceding section; see Figures 13, 15, and 17(B)). The rest exhibited polypeptide phenotypes similar to their respective wild type parents. Figures 17(A), 17(B) and 17(C) show comparisons of ts mutants of Batai virus, Bunyamwera virus, and Maguari virus with their respective wild type parents. Figure 17(A) shows Batai virus wild type (track 1), BATts1(I) (track 2) and BATts2(II) (track 3). Figure 17(B), shows Bunyamwera virus wild type (track 1), BUNts5(I) (track 2), BUNts8(II) (track 3) and BUNts7(II) (track 4). Maguari virus wild type is compared in Figure 17(C) (track 1) with

TABLE 31: List of the 31 ts mutants compared by polyacrylamide gel electrophoresis with their parental wild type viruses.

VIRUS	ts MUTANTS		
	Group I	Group II	Group III
BATAI	BATts1(I)	BATts2(II) BATts3(II) BATts4(II) BATts5(II)	--
BUNYAMWERA	BUNts1(I) BUNts2(I) BUNts3(I) BUNts5(I)	BUNts6(II) BUNts7(II)* BUNts8(II)	--
MAGUARI	MAGts1(I) MAGts2(I) MAGts3(I) MAGts4(I) MAGts5(I) MAGts7(I) MAGts24(I) MAGts41(I)	MAGts8(II) MAGts9(II) MAGts10(II) MAGts11(II) MAGts12(II) MAGts17(II) MAGts15(II) MAGts16(II) MAGts18(II) MAGts19(II)	MAGts23(III)

FIGURE 17(A)

Electrophoresis in 7.5% polyacrylamide gel of [^{35}S]methionine-labelled polypeptides of Batai virus wild type and ts mutants

Track 1, Batai virus, wild type;

Track 2, BATts1(I)

Track 3, BATts2(II)

Fig·17 (A)

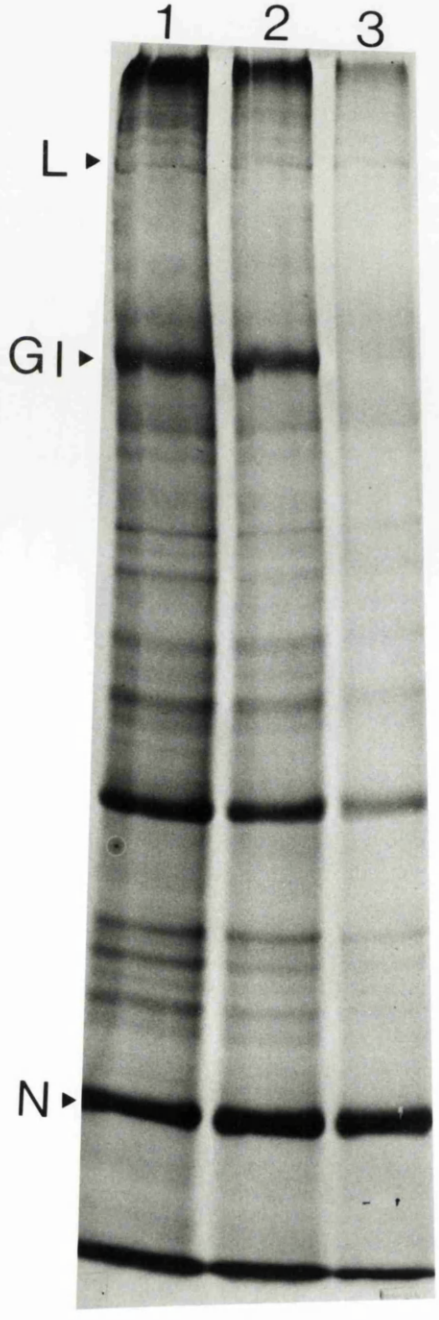


FIGURE 17(B)

Electrophoresis in 7.5% polyacrylamide gel of $[^{35}S]$ methionine-labelled polypeptides of Bunyamwera virus wild type and *ts* mutants.

Track 1, Bunyamwera, wild type

Track 2, BUN*ts*5(I)

Track 3, BUN*ts*8(II)

Track 4, BUN*ts*7(II)

Fig.17(B)

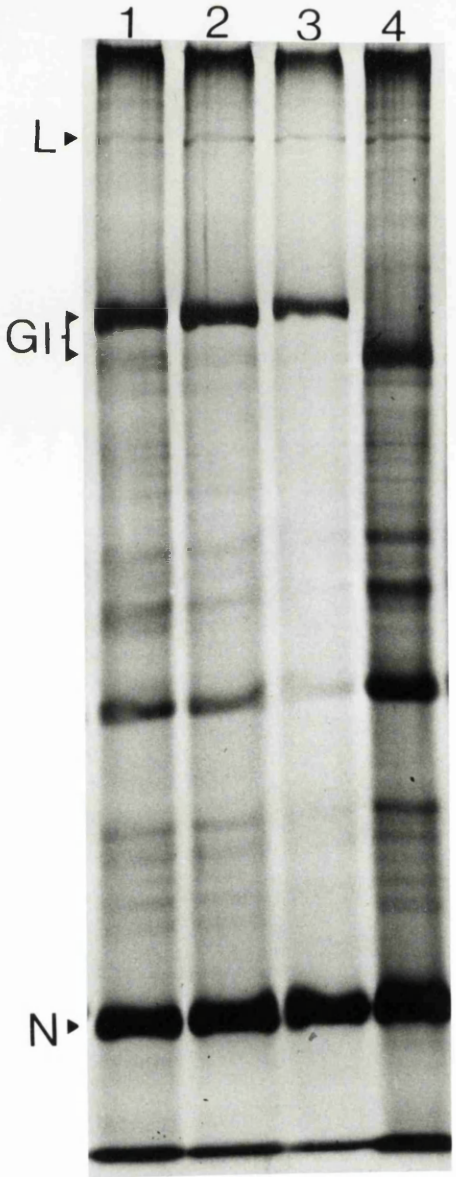


FIGURE 17(C)

Electrophoresis in 7.5% polyacrylamide gel of [^{35}S] methionine-labelled polypeptides of Maguari virus wild type and *ts* mutants.

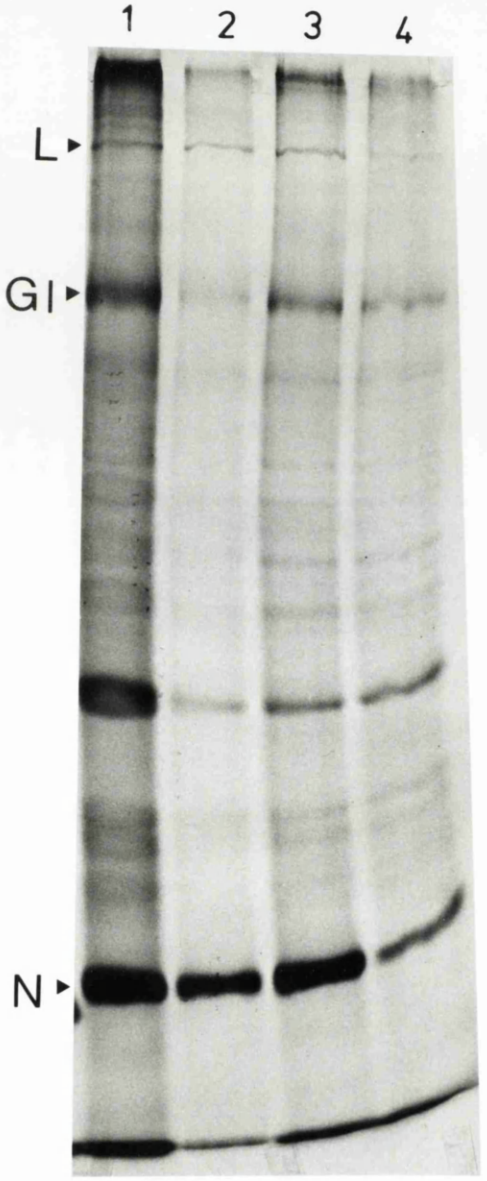
Track 1, Maguari virus, wild type

Track 2, MAG*ts*8(II);

Track 3, MAG*ts*7(I);

Track 4, MAG*ts*23(III)

Fig. 17(C)



MAG*ts*8(II) (track 2), MAG*ts*7(I) (track 3) and MAG*ts*23(III) (track 4). Although the G1 polypeptide of BAT*ts*2(II) did not appear distinctly in Figure 17(A) (track 3), previous experiments had shown that this *ts* mutant possessed a G1 polypeptide similar to its wild type parent.

In subsequent experiments, the heterologous recombinants were compared with their *ts* mutant parents or their parental wild type viruses. In Figure 18, the polypeptides of Bunyamwera virus wild type (tracks 1 and 7), Batai virus wild type (tracks 2 and 8) and the non-*ts* recombinants derived from the heterologous crosses of their groups I and II mutants have been compared. The recombinants obtained from the BUN*ts*5(I)xBAT*ts*2(II) cross (tracks 3 and 5) possessed Bunyamwera virus G1 protein derived from the BUN*ts*5(I) parent and Batai virus N protein from the BAT*ts*2(II) parent. In the reciprocal cross, BUN*ts*8(II)xBAT*ts*1(I), the recombinants (tracks 4 and 6) exhibited a Batai virus G1 polypeptide from the group I parent, BAT*ts*1(I), and a Bunyamwera virus N polypeptide from the group II parent, BUN*ts*8(II).

In Figure 19, tracks 1 and 5 represent Bunyamwera virus wild type, track 2, Batai virus wild type, track 3, a non-*ts* recombinant obtained from a BAT*ts*1(I)xBUN*ts*7(II) cross, and track 4, the BUN*ts*7(II) parent (the *ts* mutant with the additional mutation affecting the electrophoretic mobility of the G1 polypeptide. The recombinant possessed Batai virus G1 protein [from BAT*ts*1(I)] and a Bunyamwera virus N protein [from BUN*ts*7(II)].

FIGURE 18

Electrophoresis in 7.5% polyacrylamide gel of [^{35}S]-methionine-labelled polypeptides of Batai virus and Bunyamwera virus and four recombinant clones*.

Tracks 1 and 7, Bunyamwera virus, wild type;

Tracks 2 and 8, Batai virus, wild type;

Track 3, BNB5/2/3)progeny of BUN(I)xBAT(II cross);

Track 4, BBN1/8/3 (progeny of BAT(II)xBUN(1) cross);

Track 5, BNB5/2/4 (progeny of BUN(I)xBAT(II) cross);

Track 6 BBN1/8/5 (progeny of BAT(I)xBUN(II) cross).

* The recombinants expressed the G1 polypeptides of their respective group I parents and the N polypeptide of their group II parents.

Fig. 18

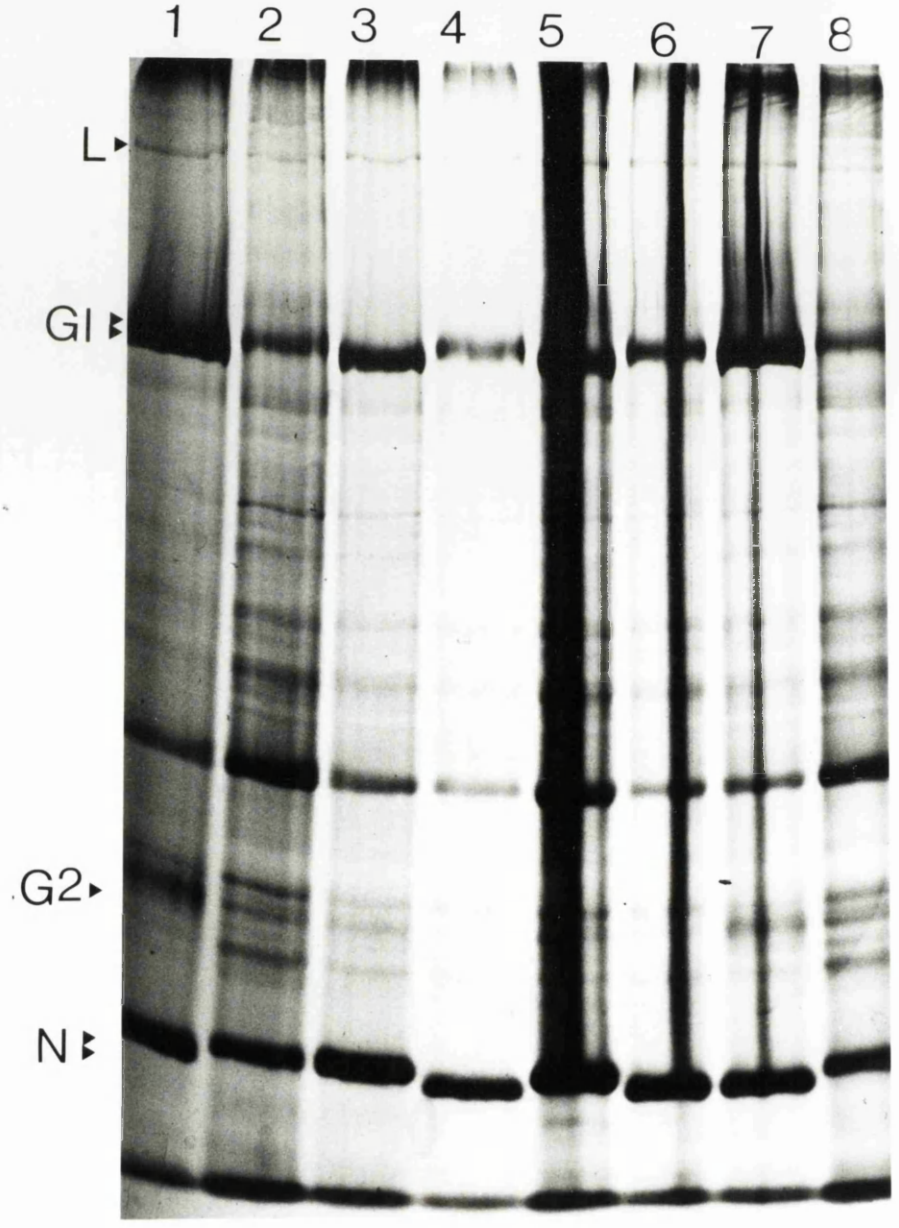


FIGURE 19

Electrophoresis in 7.5% polyacrylamide gel of [^{35}S] methionine-labelled polypeptides of Batai virus; Bunyamwera virus; a mutant, BUN<u>ts</u>7(II), and a recombinant*.

Tracks 1 and 5, Bunyamwera virus, wild type;

Track 2, Batai virus, wild type;

Track 3, BBN1/7/4 (progeny of BAT<u>ts</u>1(I) x BUN<u>ts</u>7(II) cross);

Track 4, BUN<u>ts</u>7(II).

* BBN1/7/4 (recombinant) derived its G1 polypeptide from BAT<u>ts</u>1(I), the Batai virus group I parent and its N polypeptide from BUN<u>ts</u>7(II), the Bunyamwera virus group II parent.

Fig. 19

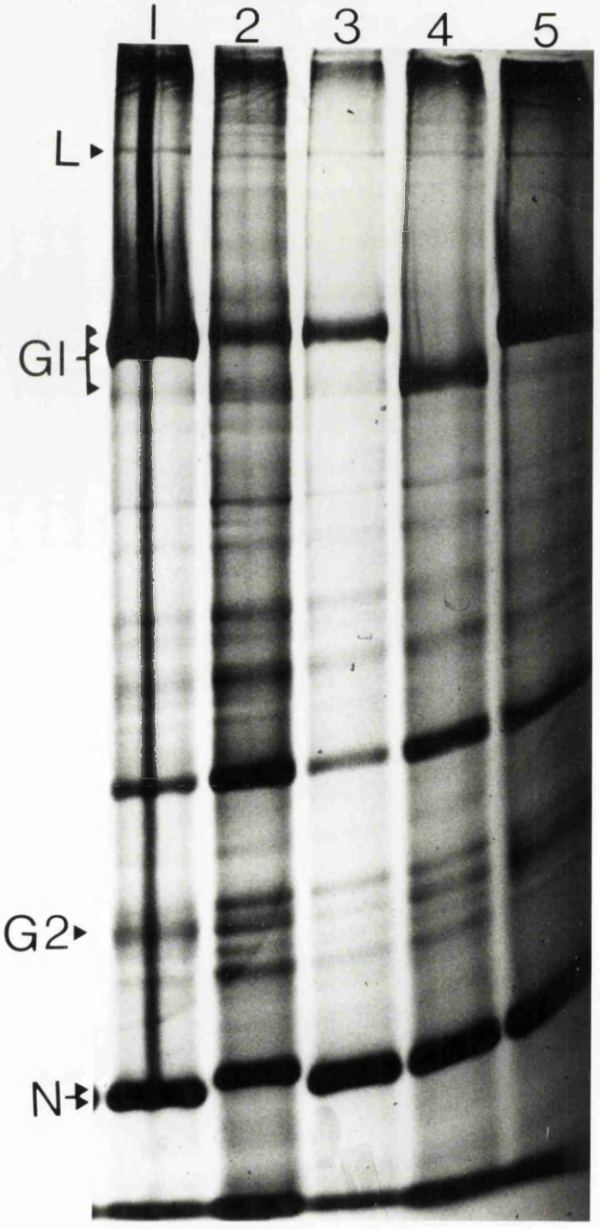


Figure 20(A) shows the polypeptides of Batai virus wild type (track 3) and Maguari virus wild type (track 2) compared with a recombinant (track 1) obtained from a cross of BATts1(I) and MAGts8(II). The recombinant possessed a G1 protein derived from BATts1(I), the group I parent, and an N protein from MAGts8(II), the group II parent. In the reciprocal cross, BATts2(II)x MAGts7(I), the recombinants (Figure 20(B), tracks 1 and 3), compared with their parental wild type viruses, Batai virus (tracks 2 and 5) and Maguari virus (track 4) possessed a G1 protein derived from MAGts7(I) and an N protein from BATts2(II).

Figure 21 shows a similar comparison of Bunyamwera and Maguari viruses and the non-ts recombinants derived from the crosses of their ts mutants. Tracks 1 and 9 represent Maguari virus wild type; tracks 3,4 and 5, recombinants obtained from a BUNts5(I)xMAGts8(II) cross; track 6, a recombinant from a BUNts8(II)xMAGts7(I) cross and tracks 7 and 8, recombinants obtained from a BUNts1(I)xMAGts8(II) cross. The results of these experiments again showed that the G1 protein was derived from the group I parent in each cross whereas the group II parent contributed the N protein. The inference drawn from these observations was that the group I ts mutants had defects in the N gene and not in the G1/G2 genes whereas the group II mutants had defects in the G1/G2 genes. In other words group I consisted of N gene ts mutants and group II consisted of G1/G2 gene ts mutants. Assignment of the G1/G2 proteins is supported by other data (see Section III.4). However, the N gene

FIGURE 20

Electrophoresis in 7.5% polyacrylamide gel of [^{35}S] methionine-labelled polypeptides of Batai virus and Maguari virus and three recombinant clones*.

A. Track 1, BM1/8/7 (recombinant progeny of BAT(I) x MAG(II) cross);

Track 2, Maguari virus wild type;

Track 3, Batai virus wild type;

B. Track 1, MB7/2/1, (recombinant progeny of MAG(I) x BAT(II) cross);

Tracks 2 and 5, Batai virus, wild type

Track 3, MB7/2/5 (recombinant progeny of MAG(I) x BAT(II) cross);

Track 4, Maguari virus, wild type.

*Each recombinant expressed the G1 polypeptide of the group I parent and the N polypeptide of the group II parent.

Fig. 20

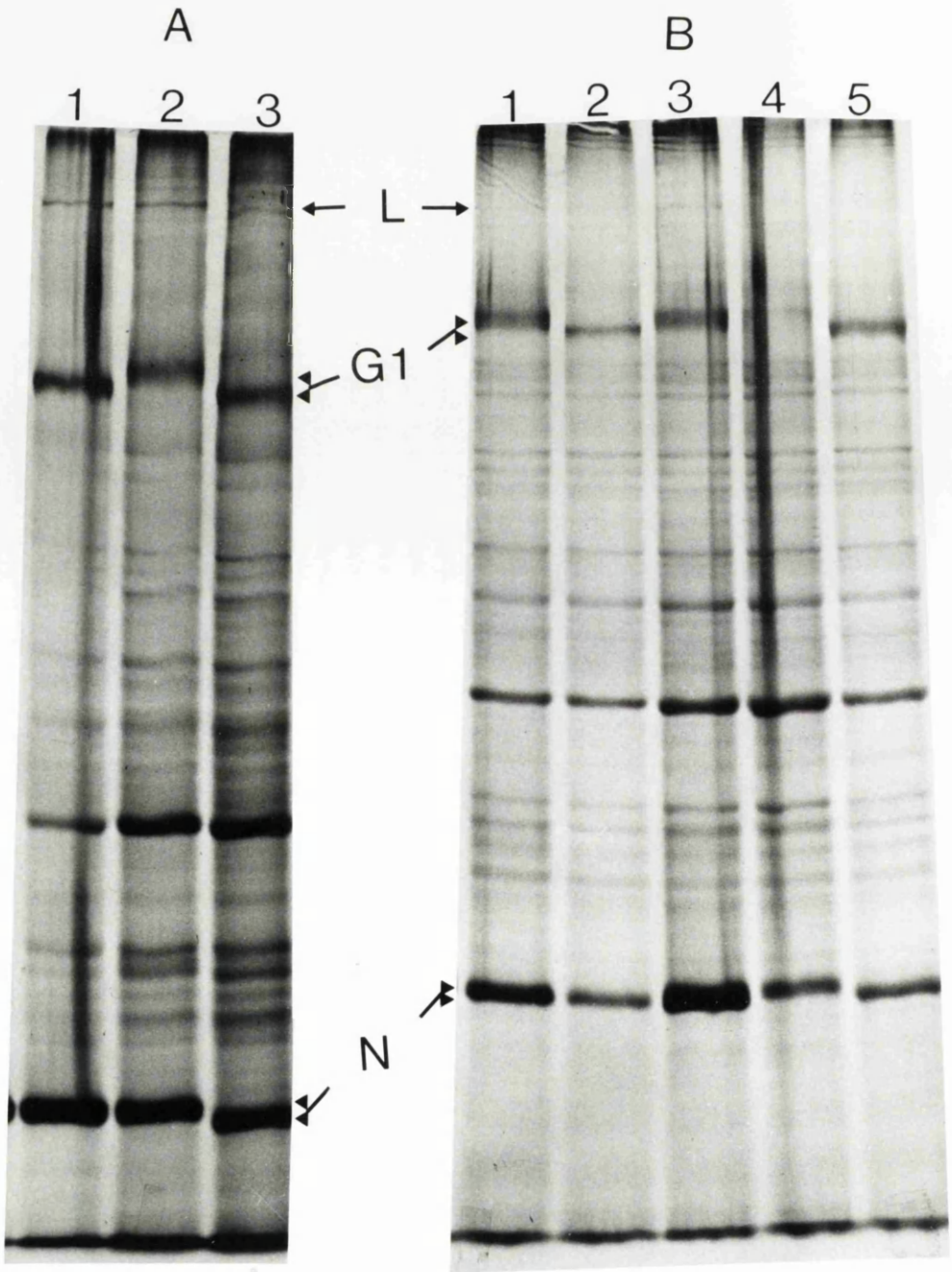


FIGURE 21

Electrophoresis in 6-15% gradient polyacrylamide gel of [^{35}S]-methionine-labelled polypeptides of non-ts (recombinant) progeny* from crosses of Bunyamwera virus and Maguari virus ts mutants.

Tracks 1 and 9, Maguari virus, wild type

Tracks 2 and 10, Bunyamwera virus wild type

Track 3, BNM5/8/1 (recombinant from BUNts5(I) x MAGts8(II) cross);

Track 4, BNM5/8/2 (recombinant as above)

Track 5 BNM5/8/3 (recombinant as above)

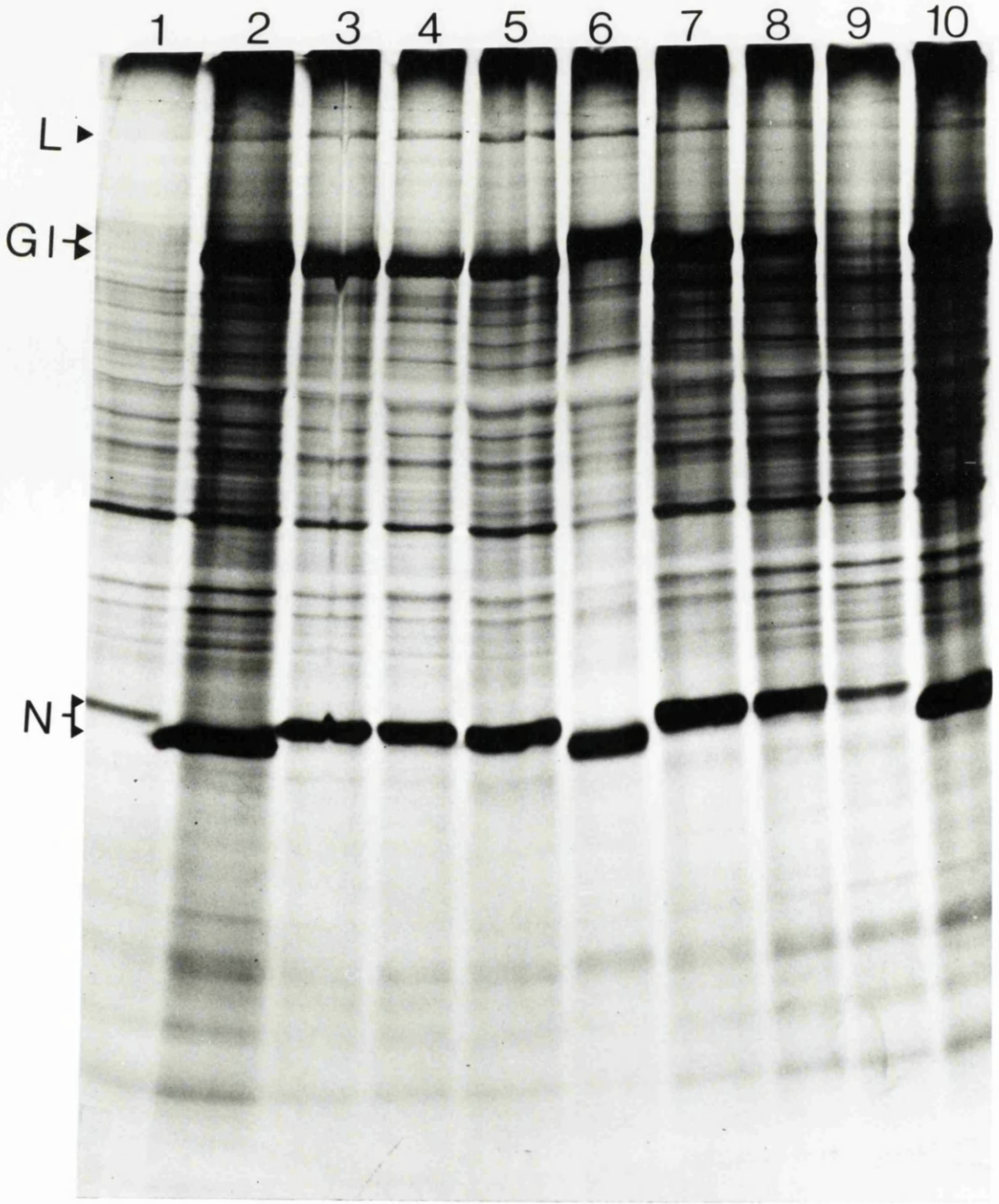
Track 6 MBN7/8/2 (progeny MAG(I) x BUN(II) cross).

Track 7 BNM 1/8/1 (recombinant from BUNts1(I) x MAGts8(II) cross);

Track 8 BNM1/8/2 (recombinant as above)

* The recombinants exhibited the G1 polypeptides of their group I parents and the N polypeptides of their group II parents.

Fig. 21



assignment is less certain and other explanations are possible (see Discussion, Section II.5; Tables 35a, b and Figure 26). Overall, the above assignments are supported by all but two of seventy seven non-ts clones analysed and the two exceptions are ambiguous rather than inconsistent.

Altogether, the polypeptide composition of 84 non-ts clones from the progeny of eleven different heterologous crosses were analysed by SDS-polyacrylamide gel electrophoresis. Six of these 84 clones (7.1%), BNM2/8/2, BNM2/8/3, BNM3/8/12, BNM3/8/3, BNM4/8/1 and BNM4/8/2, isolated from three different BUN(I)xMAG(II). Crosses (see Table 32) exhibited the Maguari virus N polypeptide but the G1 polypeptide was not clearly labelled in each case. Consequently the parental origin of the G1 of these clones was unknown. The clones could be either recombinants with unexpressed BUN G1 or Maguari virus revertants. Whether recombinants or revertants, they do not conflict with the N gene assignment for group I. It is not clear why the G1 polypeptide was unlabelled in these preparations but lack of expression of G1 in released virus may be more indicative of recombinant virus since mutants $\overline{ts}^{MAGts8(II)}$ and wild type of Maguari virus normally expressed the G1 polypeptide. Two other clones, BBN1/7/3 and BNM2/8/1, representing 2.4% of the total clones analysed, exhibited both the G1 and N polypeptides of their Bunyamwera and Maguari (group II) parents respectively. These clones must be revertants because non-ts recombinants with both G1 and N polypeptides of the group II parent cannot be obtained by reassortment

TABLE: 32 : SDS-polyacrylamide gel electrophoretic analysis of the protein phenotype of non-ts clones isolated from the progeny of the heterologous crosses.

VIRUS (CROSS)	No. of clones tested by PAGE	Clones tested*	Virus protein phenotype	
			G1	N
BATts1(I)xBUNts8(II)	6	BBN 1/8/1; BBN1/8/3; BBN1/8/4 BBN1/8/5; BBN1/8/6; BBN1/8/2	BAT	BUN
BATts1(I)xBUNts7(II)	8	BBN1/7/1; BBN1/7/2; BBN1/7/4 BBN1/7/5; BBN1/7/6; BBN1/7/7 BBN1/7/9; BBN1/7/10	BAT	BUN
	1	BBN1/7/3	BUN	BUN
BAts2(II)xBUNts5(I)	4	BNB5/2/1; BNB5/2/3 BNB5/2/4; BNB5/2/	BUN	BAT
BATts1(I)xMAGts8(II)	4	BM1/8/2; BM1/8/17; BM1/8/6 BM1/8/7	BAT	MAG
BATts2(II)xMAGts7(I)	4	MB7/2/5; MB7/2/18; MB7/2/6 MB7/2/16	MAG	BAT
	2	MB7/2/4; MB7/2/8	MAG	MAG

TABLE 32 : (CONTED.)

BUNts1(I)xMAGts8(II)	4	BNM1/8/1; BNM1/8/2; BNM1/8/3 BNM1/8/4	BUN	MAG
BUNts2(I)xMAGts8(II)	3	BNM2/8/4; BNM2/8/5; BNM2/8/6	BUN	MAG
	1	BNM2/8/1	MAG	MAG
	2	BNM2/8/2; BNM2/8/3	ND	MAG
BUNts3(I)xMAGts8(II)	13	BNM3/8/1; BNM3/8/2; BNM3/8/3 BNM3/8/4; BNM3/8/5; BNM3/8/6 BNM3/8/7; BNM3/8/8; BNM3/8/9 BNM3/8/10; BNM3/8/11; BNM3/8/14 BNM3/8/15	BUN	MAG
	2	BNM3/8/12; BNM3/8/13	ND	MAG
BUNts4(I)xMAGts8(II)	5	BNM4/8/3; BNM4/8/4; BNM4/8/5; BNM4/8/6 BNM4/8/7	BUN	MAG
	2	BNM4/8/1; BNM4/8/2	ND	MAG

TABLE 32 : (CONTD.)

BUN<u>ts</u>5(I)xMAG<u>ts</u>8(II)	19	BNM5/8/1; BNM5/8/2 BNM5/8/3; BNM5/8/4 BNM5/8/5; BNM5/8/6 BNM5/8/7; BNM5/8/8 BNM5/8/9; BNM5/8/10 BNM5/8/11; BNM5/8/12 BNM5/8/13; BNM5/8/14 BNM5/8/15; BNM5/8/16 BNM5/8/17; BNM5/8/18 BNM5/8/19	BUN	MAG
BUN<u>ts</u>8(II)xMAG<u>ts</u>7(I)	3	MBN7/8/1; MBN7/8/2 MBN7/8/4	MAG	BUN
	1	MBN7/8/3	MAG	BUN+MAG

ND: Not expressed

* Some of the clones were cross-checked or analysed by Dr C. R. Pringle.

whether group I is assigned to L gene or N gene (see Figure 26).

One of the non-ts clones (1.2%), MBN7/8/3, isolated from the MAGts7(I)xBUNts8(II) cross exhibited an atypical N polypeptide phenotype (Figure 22, track 6). While this clone possessed a G1 polypeptide derived from the Maguari virus parent, MAGts7(I) as expected, it also exhibited a Maguari virus N polypeptide in addition to the Bunyamwera-derived N polypeptide expected of a non-ts recombinant from that cross. This virus was recloned and investigated further as described in Section III.3.2.

Seventy-five (97%) of the remaining 77 non-ts clones, derived their G1 polypeptide from the group I parent and their N polypeptide from the group II parent. This is consistent with the assignment of N gene mutation to group I and G1/G2 gene mutation to group II. The remaining two clones, MB7/2/4 and MB7/2/8, representing 2.6% of the 77 clones, possessed MAG (the group I parent) G1 and N polypeptides. These two clones could be either revertants of MAGts7(I) or recombinants in which the L genome segment was exchanged (see Figure 26, progeny R'2). If they are the latter, they would support the opposite assignment, that is, the assignment of L gene for group I rather than the N gene assignment for group I. Whether they are recombinants or not can only be determined by oligonucleotide fingerprinting since the L polypeptides are not distinguishable. It may be noteworthy that MB7/2/4, and MB7/2/8, the clones with both G1 and N of the group I parent, as well MBN7/8/3,

FIGURE 22

Electrophoresis in 6-15% gradient polyacrylamide gel of [^{35}S] methionine-labelled polypeptides of Bunyamwera virus, Maguari virus and the recombinants* derived from croses of their ts mutants.

Tracks 1 and 8, Maguari virus wild type;

Tracks 2 and 9, Bunyamwera virus wild type;

Track 3 BNM5/8/4 (recombinant from BUNts5(I)x MAGts8(II) cross);

Track 4, BNM5/8/5 (recombinant as above)

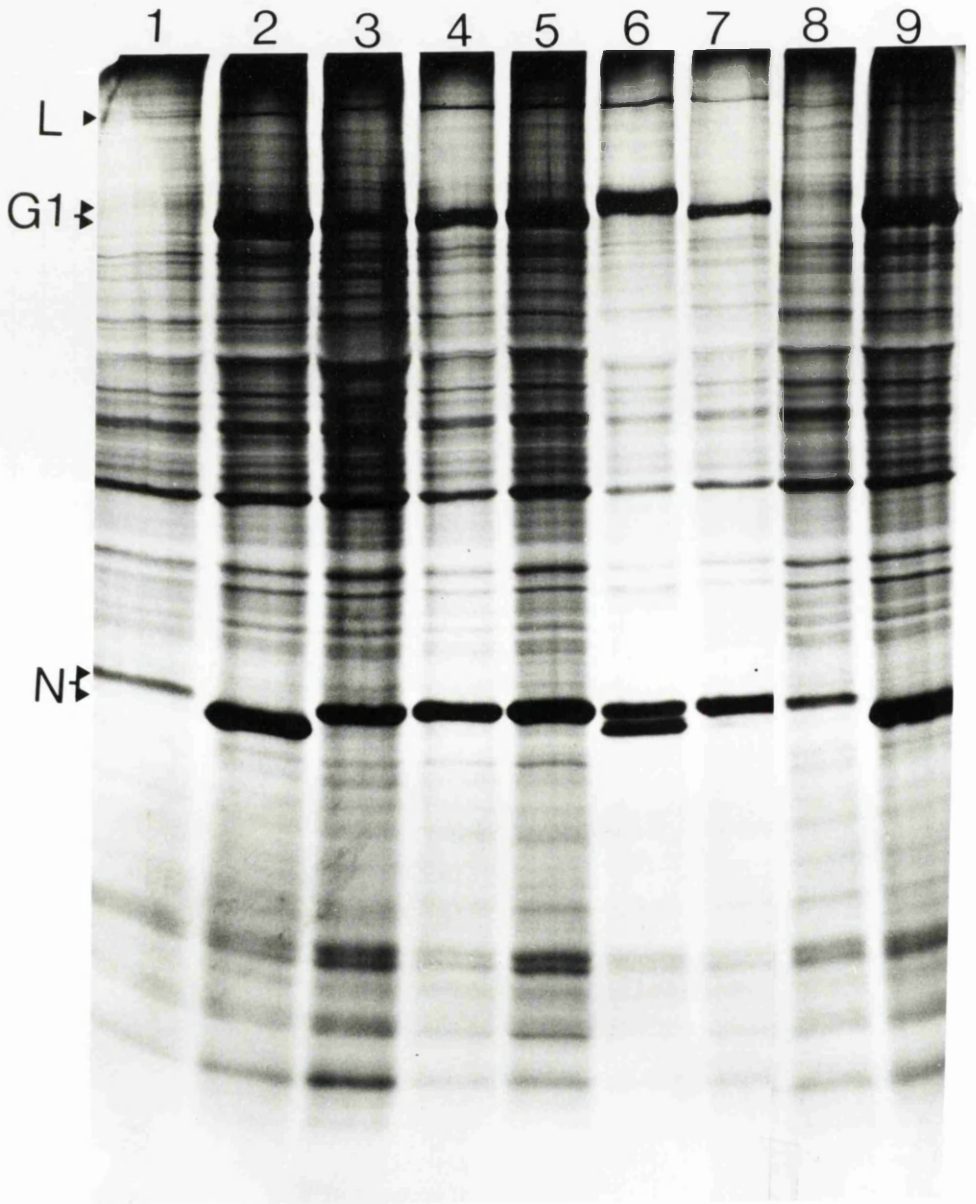
Track 5, BNM5/8/6 (recombinant as above)

Track 6, MBN7/8/3 (progeny of MAGts7(I) x BUNts8(II) cross'; note the double N bands)

Track 7, BNM1/8/4 (recombinant from BUNts1(I) x MAGts8(II) cross)

* The recombinants derived the G1 polypeptide from their group I parent and the N polypeptide from the group II parent (except MBN7/8/3 above which exhibited a mixed-MAGN+BUN N-phenotype).

Fig.22



the clone with atypical N polypeptide phenotype (Figure 22, track 6), all came from heterologous crosses involving MAG*ts*7(I). It is not known whether these clones arose as a result of some undiscovered pecularities of MAG*ts*7(I) since the other mutants involved in the crosses did not give rise to any unpredicted clones in crosses with other mutants.

The main limitation of the group assignment described here is that only the G1 and N polypeptides could be distinguished by polyacrylamide gel electrophoresis. This question is reviewed later in the discussion Section II.5. However, at least 97% (98% if the six partially characterised clones are included) of the non-*ts* clones derived their N polypeptides from the group II parent (indicating mutation of the N protein in group I) whereas only 50% of the non-*ts* clones would be expected to derive their N polypeptides from the group II parent if group I corresponded to the L gene (see Figure 26).

III.3.2 THE NON-TEMPERATURE-SENSITIVE CLONE WITH A MIXED PHENOTYPE (MBN 1/8/3)

As was shown in Figure 22, track 6, one of the 4 non-ts clones isolated from the MAGts7(I) x BUNts8(II) cross (MBN 1/8/3) exhibited a Maguari virus N polypeptide in addition to the Maguari virus G1 and Bunyamwera virus N polypeptide expected of a wild type recombinant from that cross. This virus was recloned and plaques were picked at both 31°C and 38.5° C. Generally three types of clones were isolated. These have been represented in Figure 23 as follows: clones which possessed Maguari virus G1 and N polypeptide (tracks 2 and 7), clones which retained the mixed N protein phenotype like their parent, i.e. clones with MAGG1 + BUN N + MAG N (track 1) and clones with the recombinant phenotype, Maguari virus G1 and Bunyamwera virus N (tracks 3, 8, 9 and 10). Tracks 4, 5 and 6 represent Bunayamwera virus wild type, the original non-ts clone with mixed N phenotype, and Maguari virus wild type respectively.

The numbers of each type of clone isolated at both 31° C and 38.5° C have been given in Table 33. Altogether 27 clones were isolated and analysed, 14 at 31° C and 13 at 38.5° C. Of the 14 clones isolated at 31° C, 4 (28.6%) resembled the Maguari virus parent i.e. both G1 and N proteins were of the Maguari virus type; 8(57.1%) were the expected recombinant clones possessing a G1 derived from the Maguari parent, MAGts7(I) an N protein derived from BUNts8(II), the

FIGURE 23

Electrophoresis in 7.5% polyacrylamide gel of [^{35}S] methionine-labelled polypeptides of recombinant clone MBN7/7/3 and clones derived from it. The autoradiogram shown here is of a gel slab containing all three types clones isolated when MBN7/8/3 was recloned at 31° and 38° C.

Track 1, clone shown polypeptide profile similar to the parent recombinant with MAG G1 and both MAG N and BUN N polypeptides;

Tracks 2 and 7, clones showing MAG G1 and N polypeptides;

Track 3, recombinant with MAG G1 and BUN N polypeptides;

Track 4, Bunyamwera virus wild type (control);

Track 5 MBN7/8/3 (parent control);

Track 6, Maguari virus wild type (control);

Tracks 8, 9 and 10, recombinant clones with MAG G1 and BUN N polypeptides.

Fig·23

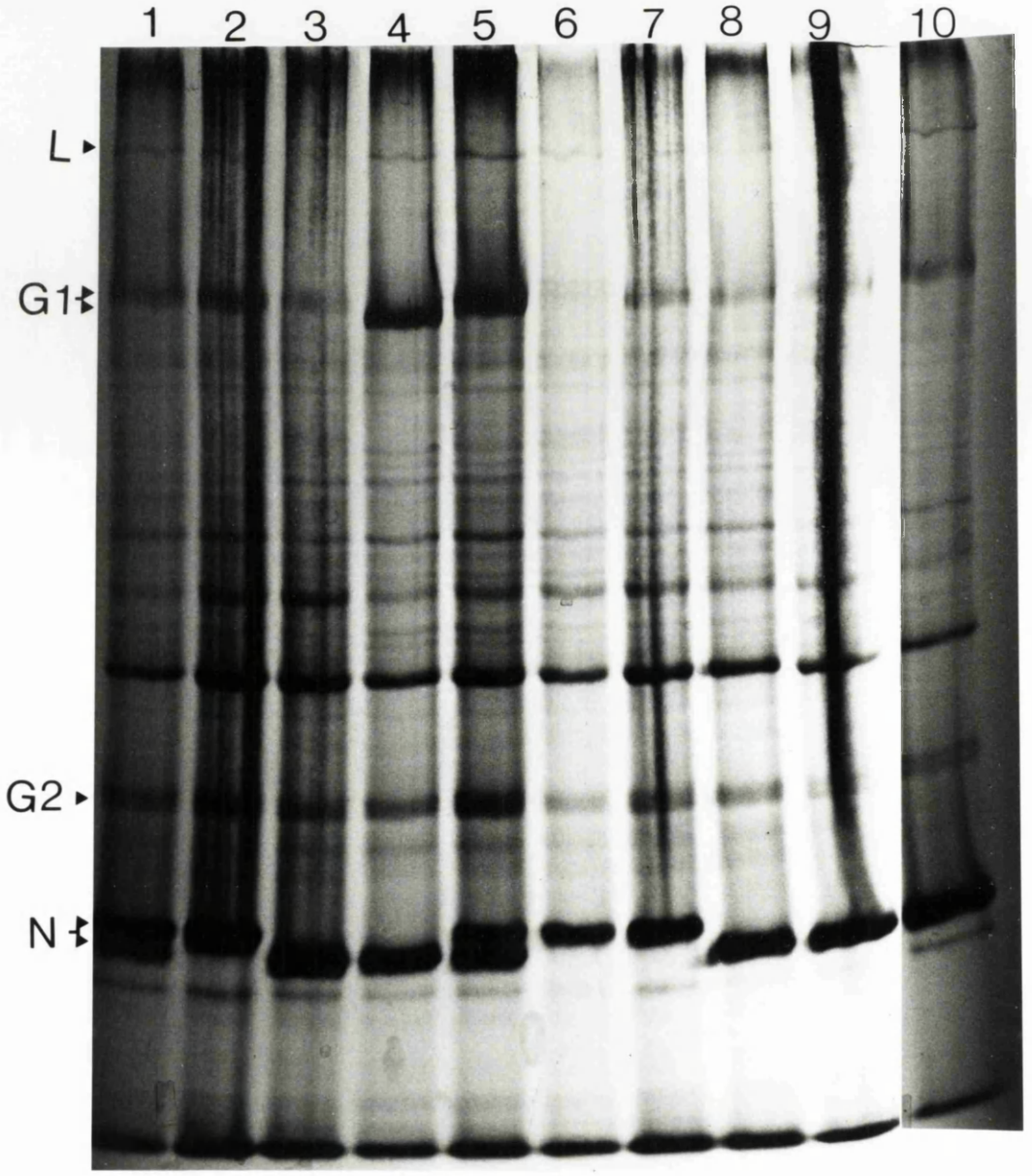


TABLE 33

Summary of clonal analysis of the progeny of the recombinant with mixed N protein phenotype, MBN7/8/3, by SDS-polyacrylamide gel electrophoresis.

Type of clone isolated	Number isolated at 31° C	Percentage isolated at 31° C	Number isolated at 38.5° C	Percentage isolated at 38.5° C
MAG.G1+MAGN	4	28.6	0	0
MAG.G1+MAGN+BUN N	2	14.3	2	15.4
MAG.G1+BUNN	8	57.1	11	84.6
TOTAL CLONES ISOLATED	14		13	

Bunyamwera virus group II parent. Two clones (14.3%) remained phenotypically mixed like their parental recombinant clone, MBN 1/8/3.

Two clones (15.4%) out of the 13 isolated at 38.5° C still had mixed phenotype (two N proteins one each from Bunyamwera and Maguari viruses respectively) and 11 clones (84.6%) exhibited the recombinant phenotype (Maguari virus G1 and Bunyamwera virus N). No clones isolated at both temperatures resembled the Bunyamwera virus wild type parent. Also, no clones isolated at 38.5° C resembled the wild type Maguari virus parent. As shown in Table 34, the four clones isolated at 31° C which were indistinguishable from the Maguari virus parent were not temperature-sensitive. These clones were assumed to be revertants of MAG*ts*7(I). It is not certain whether this mixed clone represents aggregation (due to specific surface alterations), heteroploidy or heterozygocity.

III.4 COMPARISON OF THE HETEROLOGOUS RECOMBINANTS AND THEIR PARENT VIRUSES BY PLAQUE NEUTRALISATION ASSAY

The antigenic characteristics of the heterologous recombinants and their parental wild type viruses were examined by plaque neutralisation test. The neutralisation experiment was carried out as described in the Methods (Section G). Figure 24 illustrates the effect of specific anti-sera produced against the three viruses on the activities of the recombinant

TABLE 34

Efficiency of plating of four clones of the recombinant MBN7/8/3 isolated at 31° C which expressed MAG G1 + N polypeptides.

CLONE/VIRUS	VIRUS INFECTIVITY ASSAYED AT		Efficiency of plating
	31° C	38° C	
3	2.1×10^7	2.63×10^6	0.13
5	1.5×10^7	2.6×10^6	0.17
11	1.5×10^7	4.2×10^5	0.03
12	1.1×10^7	1.3×10^6	0.12
MAG<u>ts</u>7(I)	1.7×10^8	10	5.8×10^{-8}
MAGts$^+$	1.08×10^7	1.4×10^6	0.13

FIGURE 24

The neutralisation characteristics of wild type Batai virus, Bunyamwera virus and Maguari virus and their heterologous recombinants.

Symbols:-  -anti-Batai virus serum

 -anti-Bunyamwera virus serum

 -anti-Maguari virus serum

NB: The results present in this Figure and Figures 16 and 25 were obtained from experiments done in collaboration with Dr C. R. Pringle.

Fig. 24

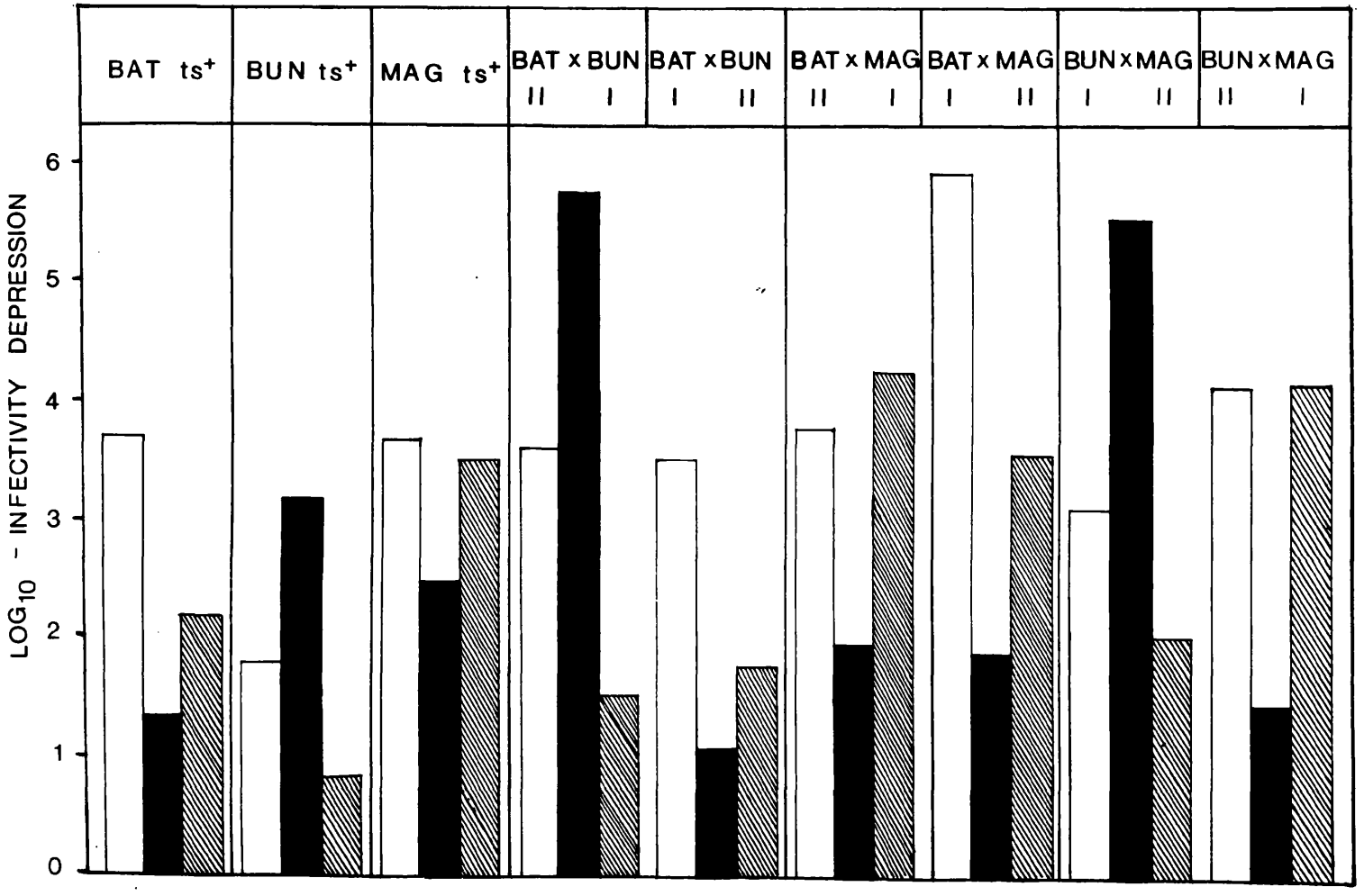


Fig. 24

The neutralisation characteristics of wild type Batai virus, Bunyamwera virus and Maguari virus, and their heterologous recombinants.

□ -anti-Batai virus serum; ■ -anti-Bunyamwera virus serum; ▨ -anti-Maguari virus serum.

clones as well as their parental wild type viruses. The antisera showed good specificity except that Batai virus was neutralised to almost the same degree by the heterologous anti-Maguari virus serum as by the homologous anti-Batai virus serum. In general, the recombinant clones were neutralised more by the antiserum specific to the parental virus from which their G1 protein was derived. In other words, the neutralisation pattern followed the segregation of the G1 (and G2) protein(s).

The G1 and G2 proteins are mentioned together because it is assumed in this work that both proteins are coded by the same genome subunit, the M subunit, as has been unequivocally established for the California group bunyaviruses (Gentsch and Bishop 1979).

The correlation between neutralisation pattern and the segregation of G1 protein can be illustrated with the recombinant obtained from the cross of Batai mutant, BAT*ts*1(I) and Bunyamwera mutant, BUN*ts*8(II). This recombinant (see Figure 24) was neutralised to a greater extent by anti-Batai virus serum than by the anti-Bunyamwera virus and the anti-Maguari virus sera respectively. As was deduced earlier (Results, Section III.3.1) in the cross of BAT*ts*1(I) and BUN*ts*8(II), the Batai virus *ts* mutant contributed the G1 (and G2) protein (see Figure 18, tracks 4 and 6); whereas in the reciprocal cross, BUN*ts*5(I) x BAT*ts*2(II), the Bunyamwera virus *ts* mutant contributed G1 (and G2) (Figure 18, tracks 3 and 5).

A similar segregation of neutralisation specificity together with the G1 (G2) protein was observed in the crosses of Maguari and Batai ts mutants, and Maguari and Bunyamwera ts mutants. These results agree with the observations of Gentsch et al (1980) that the glycoproteins elicit the neutralising antibodies. This further confirms that the group I parents donated the G1 (and G2) proteins of the heterologous recombinants and hence that the group II mutation is located in the genes coding for the glycoproteins.

III.5 COMPARISON OF THE PLAQUE MORPHOLOGIES OF THE HETEROLOGOUS RECOMBINANTS AND THEIR PARENT VIRUSES.

In a comparative analysis of the plaque morphology of the three viruses (Batai, Bunyamwera and Maguari), Maguari virus consistently produced larger but more opaque plaques on monolayers of BS-C-1 cells at 31° C. The difference was less distinct in BHK-21 clone 13 cells or PTK-2 cells.

A comparison of the plaque morphology of the wild type parents and the recombinant progeny derived from crosses of ts mutants of the three viruses showed that the recombinant progeny from the Maguari x Batai crosses produced plaques which resembled the group I parent in size and the group II parent in opacity (Figure 25(i). The same association of plaque size with the group I parent and plaque opacity with the group II parent was also observed with recombinants obtained from crosses of Bunyamwera and Maguari ts mutants (Figure 25).

FIGURE 25.

Plaque characteristics of Batai, Bunyamwera, and Maguari viruses and their recombinants on BS-C-1 monolayers at 31°C.

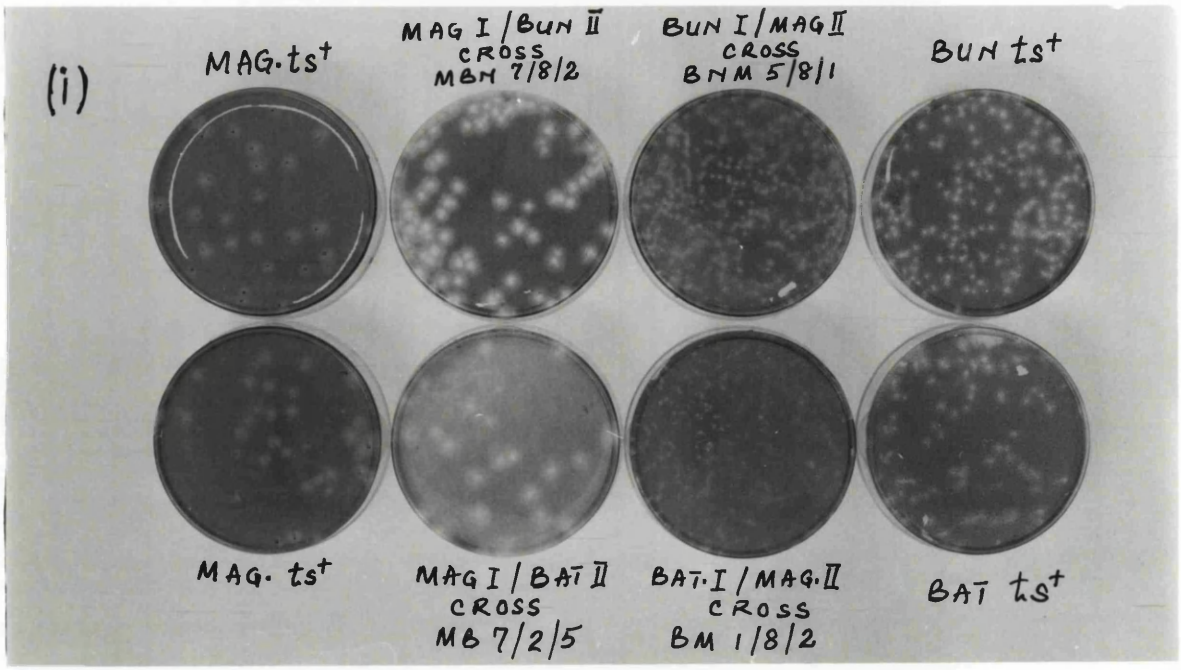
i) Comparison of Batai virus and Maguari virus; and Bunyamwera virus and Maguari virus; and their recombinants - MB7/2/5 and BM1/8/2; and MBN7/8/2 andBNM5/8/1.

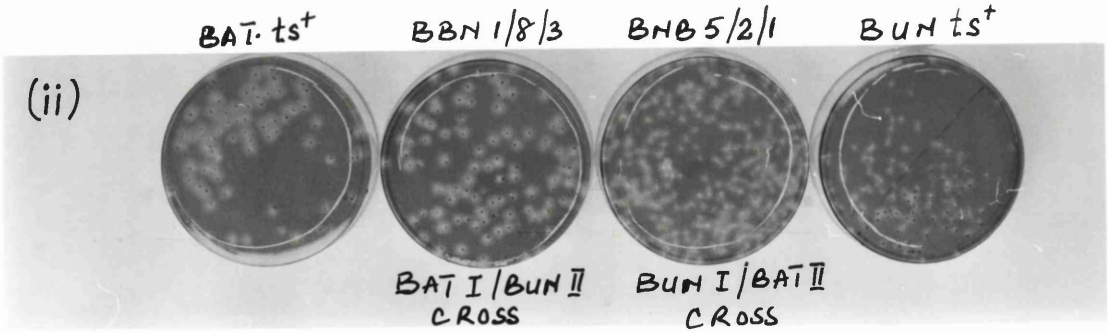
In the recombinants, plaque diameter resembles the group I parent and the plaque opacity is taken from the group II parent.

ii) Comparison of Batai virus wild type and Bunyamwera virus wild type and their recombinants, BBN1/8/3 and BNB5/2/1.

These crosses only show that plaque diameter is determined by G protein (group I parent). There is no difference in opacity between the wild type parent viruses.

FIG. 25


(i)
MAG.ts+
MAG I / BUN II CROSS MBH 7/8/2
BUN I / MAG II CROSS BHM 5/8/1
BUN ts+
MAG. ts+
MAG I / BAI II CROSS MB 7/2/5
BAI. I / MAG. II CROSS BM 1/8/2
BAI ts+


(ii)
BAI. ts+
BBN 1/8/3
BNB 5/2/1
BUN ts+
BAI I / BUN II CROSS
BUN I / BAI II CROSS

The plaques produced by Batai virus and Bunyamwera virus were not sufficiently different to show this association unequivocally; although the same tendency was evident (Figure 25(ii)).

The above observations indicate that the G1 and/or G2) protein may be important in determining the rate of spread (plaque diameter) and the N (or L) protein is important in determining the lytic potential (plaque opacity) of the viruses.

DISCUSSION

D I S C U S S I O N

I. CHARACTERISTICS OF THE WILD TYPE VIRUS

The single cycle of growth lasting about 22 hours observed with Batai virus, Bunyamwera virus or Maguari virus is comparable to the 20 hours estimated by Kascsak and Lyons (1978) for Bunyamwera virus and the 20-24 hour cycle of the California encephalitis group viruses (Bishop and Shope, 1979; Goldman et al, 1977; Lyons and Heyduk, 1973; Obijeski et al, 1976a). The 6-hour eclipse period, during which there was no net output of infectious virus from the cell, coincided with the period of primary transcription (Bouloy and Hannoun, 1976a; 1976b; Kascsak and Lyons, 1977) and onset of synthesis of viral structural proteins (Pennington et al, 1977).

There was efficient release of Maguari virus from infected BHK-21 cells. However, approximately 30% remained cell-associated (see Figure 4) at 20 hours. The CPE of Batai virus, Bunyamwera virus and Maguari virus in BHK-21 or BS-C-1 cells was macroscopically characterised by rounding and detachment of cells from the monolayers as in California encephalitis virus infection of Vero cells (Lyons and Heyduk, 1973). CPE was first detectable at 22 hours post infection with 5 pfu per cell and involved only a small percentage of cells. Progressively, the majority of the cells became involved by 48-72 hours. Generally, Batai, Bunyamwera and Maguari viruses produced maximum CPE in cultures of BHK-21 cells in 3-4 days at 31^{o} C with multiplicities of infection between 0.1 and

* Although the differences in cut-off temperatures (and E.O.P at 38°C) of the three viruses did not appear to affect the type of ts mutants isolated, it is possible that such differences might affect the sensitivity of the assay method in detecting recombination. This would be so, especially in recombination experiments involving Maguari virus ts mutants in which the restrictive temperature was raised to 38.5°C to reduce leak.

2 pfu per cell. The outcome of infection of mosquito (Aedes albopictus) cells with Batai, Bunyamwera or Maguari virus (C.R. Pringle, personal communication) was also similar to that observed by Lyons and Heyduk (1973) in mosquito cells infected by California encephalitis virus. In both cases no CPE was observed under the light microscope, although infectious virus was released and genetic recombination of the viruses of the Bunyamwera complex occurred at normal frequency.

A comparison of the diameter and opacity of plaques produced by these three viruses on BS-C-1 monolayers suggested that Maguari virus spread more rapidly than either Batai virus or Bunyamwera virus, whereas the latter two viruses were more cytolytic. Similar comparison of the plaque morphology of the heterologous recombinants and their wild type parent viruses on BS-C-1 monolayers suggested that the G1 and/or G2 proteins were important in determination of the rapidity of spread (plaque diameter), and the L or N proteins in lytic potential of the viruses.

Table 9 shows that whereas Batai and Bunyamwera viruses could form plaques at 39° C, Maguari virus could not. All three viruses produced plaques at 38° C and for this reason the latter temperature was chosen a common restrictive temperature. At 38° C, the efficiency of plating (pfu at 38° C/pfu at 31° C) of Maguari virus (0.13) was less than that of Batai virus (0.9) or Bunyamwera virus (0.4). However, it did not appear to affect the type of ts mutant recovered since the mutants

belonged predominantly to recombination groups I and II.

Four virus-specified structural proteins L, G1, G2 and N were detected by SDS-polyacrylamide slab gel electrophoresis, although G2 was only rarely resolved. Considering the size of bunyavirus genomes (total molecular weight of about 5.4×10^6; Gentsch et al, 1977a), these viruses would be capable of specifying more than these four structural proteins which account for only about 70% of the coding capacity of the bunyavirus genome. However, no other proteins (structural or non-structural) were unequivocally identified. A band marked X (Figure 14A) was prominent in partially purified samples but it appeared to co-electrophorese with a band marked Y in unpurified samples (Figure 14B) which was presumed to be actin. The variation in migration rate, in partially purified virus, of X relative to the actin band in mock-infected cell preparation or crude virus preparation may result from traces of sucrose in the partially purified virus samples.

II. GENETICS OF BUNYAMWERA COMPLEX VIRUSES

II.1 THE TEMPERATURE-SENSITIVE MUTANTS

With the exception of three Maguari virus ts mutants, which arose spontaneously, all the mutants were induced with 5-fluorouracil (5-Fu). 5-Fu is an analogue of the pyrimidine base uracil and induces mutation by causing mispairing of bases during replication of the nucleic acid (RNA). It is incorporated in place of uracil in

the RNA molecule but behaves like cytosine by pairing with guanine instead of adenine (Gardner, 1972). This mispairing may result in a change in the amino acid sequence of the gene product producing a conformational change in the protein which affects its functional stability at elevated temperature. Pringle (1970b) using 5-Fu, 5-azacytidine (5-AZA) and ethyl methyl sulphonate (EMS) to generate ts mutants of VSV-Indiana observed that the same type of mutants were induced irrespective of the type of mutagen used (see also Pringle, 1975). Similar observation was reported by Bishop and Shope (1979) for ts mutants of snow shoe hare virus (California encephalitis group) using the mutagens 5-Fu, 5-AZA, and N-methyl-N'-nitro-N-nitrosoguanidine (NTG). Generally, 5-Fu has been observed to be more effective than other mutagens for induction of ts mutants of negative-strand RNA viruses (Clark and Koprowski, 1971; Holloway et al, 1970; Pringle, 1970a, b; 1975; 1977b; Rettenmier et al, 1975). Therefore, 5-Fu alone was considered sufficient for induction of the mutants required for genetic study of the viruses of the Bunyamwera Complex. To obviate the likelihood of re-isolating clonally related ts mutants, however, only a few clones were picked from each mutagen treatment. The rate of isolation of ts mutants increased with concentration of 5-Fu (3.3% at 50 ug of 5-Fu per ml of incubation medium to 6.8% at 200 ug per ml). Similar relationships between the frequency of isolation of mutants and mutagen concentration have been reported by Pringle (1970 a, b)

for VSV-Indiana, Sambrook et al (1966) for rabbitpox virus and Bishop and Shope (1979) for several California encephalitis group bunyaviruses. Ghendon (1963) has further observed a dependence of the rate of induction of poliovirus ts mutants on time of treatment with nitrous acid. The latter relationship was not investigated here.

The spontaneous ts mutation rate of 2.7% observed with Maguari virus is comparable to the 2.3% spontaneous mutation rate observed with snow shoe hare virus by J. Gentsch and D.H.L. Bishop (cited by Bishop, 1979).

Many of the clones which were identified by the initial screening as temperature-sensitive mutants proved highly unstable and were discarded. For example, only 50 (30%) of the 166 Maguari virus clones identified as temperature-sensitive by the initial screening at 31° C and 38° C were finally used for genetic investigations. Seventy percent were discarded on account of high reversion rates and leakiness. Batai virus and Bunyamwera virus were similar in this respect. Leakiness and genetic instability have been associated with a high proportion of ts clones of other viruses, also, during the initial screening (Burge and Pfefferkorn, 1966; MacKenzie, 1970; Simpson and Hirst, 1968). Sixty three ts mutants (Batai, 5; Bunyamwera, 8; and Maguari, 50), were sufficiently stable, phenotypically and genotypically, for routine use. In some cases where leakiness was apparently high at 38° C, raising the restrictive temperature by about half a degree (to 38.5° C) proved

remedial. Reverting mutants were often recloned and only those clones which proved to be more temperature-sensitive and exhibited genetic behaviour similar to the parental mutant were retained for further use.

As shown in the Results Section III.2(ii) the altered migration rate of the G1 of BUNts7(II) was not corrected in the wild type revertants. This indicated that the phenotypic change was due to a mutation unrelated to the ts mutation. The nature of the non-ts lesion is unknown, but it is possible that the glycosylation of the protein was affected. The glycosylation of BUNts7(II) will be examined in detail to provide an insight into the nature of the mutation and the mechanism of glycosylation of bunyavirus protein. The non-ts mutation associated with BUNts7(II) is indicative of the existence of other induced mutations in addition to the ts lesions.

II.2. HOMOLOGOUS RECOMBINATION

The assay method used to test for recombination only detected the recombinant with the wild type phenotype. It has been assumed, however, that two types of recombinants are formed in equal proportions, the non-ts recombinant and a recombinant with double ts lesions derived from both mutant parents (Bishop, 1979; Bishop and Shope, 1979; Cross and Fields, 1977; Gentsch and Bishop, 1976). To compensate for the absence of the double-ts recombinant, Gentsch and Bishop (1976) introduced a factor, 2, in the formula for calculating recombination frequency. Although identification of the double-ts recombinants in bunyavirus harvests has been

reported (Bishop, 1979; Bishop and Shope, 1979) it has not been shown that they occur in equal frequency with the non-ts recombinant. However, for comparative purposes, the formula of Gentsch and Bishop has been used to calculate the RF in this work.

The high frequencies of recombination between ts mutants in different groups and the absence of recombination between mutants in the same group indicates reassortment of genome subunits. The early occurrence of recombination in BHK-21 cells co-infected with Maguari virus ts mutants (detected as early as 4 hours post infection) is similar to the observations with influenza virus (MacKenzie, 1970) and reovirus type 3 (Fields, 1971). Recombination between ts mutants of Maguari virus had reached equilibrium by 10 hours post infection, that is, about eight hours before the maximum yield of virus was obtained.

Whereas the low and variable recombination frequencies (RF) obtained at multiplicities of infection below unity (Figure 12) could be explained by low probability of co-infection and the variability of the number of cells which were doubly infected, the decline of the RF at multiplicities beyond 10 pfu per cell was unexpected. A similar decrease was observed in complementation tests when cells were coinfected at very high multiplicities (about 400 pfu per cell) with two complementing ts mutants, MAGts6(I) and MAGts7(I), at 38° C (see Table 24). An increased leak yield of the singly infected controls could bring about such results. However, the leakage

observed in these experiments was not sufficient to explain a decrease of the magnitude observed. An alternative explanation is multiplicity-dependent interference, similar to the interference observed with La Crosse (Obijeski et al, 1976a) and Bunyamwera virus (Kascsak and Lyons, 1978) presumed to be a consequence of the presence of defective interfering particles. This interference has been shown to occur early in replication cycle (about 8 hours post infection) and affected the replication of standard virus RNA, resulting in generation of Bunyamwera virus particles which did not contain the full complement of genome subunits (Kascsak and Lyons, 1978).

Recombination frequencies obtained in the homologous crosses of all three viruses varied from 1.1% to 98.4% with a mean value of 27%. There were variations in values of the RF even in repeat crosses of the same pair of ts mutants (e.g. the standard cross, MAGts3(I)x MAGts8(II)). Assuming random reassortment, the maximum RF value (by the formula of Gentsch and Bishop adopted here) should be 50%. The reason for the wide variation in the RF values is unknown, but it may be due to inequality in input of ts mutants since the multiplicities of infection were not critically controlled in the experiments described in the Results Section II.2.1(iv). Another explanation could be the participation of defective particles in recombination. Defective particles could interact with the ts mutants to give viable non-ts recombinants. Hirst (1973) and Hirst and Pons (1973) have

shown that the participation of defective particles in influenza virus reassortment could account for the disproportionately high frequencies of recombination even at low multiplicities of infection with influenza virus. They further showed that aggregation of virus particles enhanced recombination and could be particularly important in genetic interactions involving the defective particles (Hirst and Pons, 1973). It is possible that aggregation of bunyavirus particles may be one of the factors that influenced the high RF values obtained in certain crosses. It is also likely that the high RF of 98.4% obtained in the cross of MAG*ts*1(I) and MAG*ts*18(II) was an experimental artifact since only one other RF value (84.7%) approached that value (see Table 15).

Although the genetic reassortment described in the results Section II.2.1 has been referred to as recombination, true intramolecular recombination was not observed. If the latter did occur the frequency could be so low, considering the sizes of the genome subunits, that it would be obscured by the high reassortment frequency as well as the background levels of revertants and leakiness. In spite of the small sizes of the bunyavirus genome segments (see Table 4), intramolecular recombination could be detected in the M (mol. wt; $1.8\text{-}2.4 \times 10^6$) and/or the L (mol. wt; $2.9\text{-}4.1 \times 10^6$) segments, at least. Recombination frequencies up to 2.2% and 3.2% have been observed with poliovirus (Cooper et al, 1975) and foot-and-mouth disease virus (McCahon et al, 1977) respectively. These are picornaviruses with single-stranded RNA genome

of molecular weights in the range $2.3x10^6$ to $2.9x10^6$ (Cooper, 1977). Therefore, more strict standardisation of recombination experiments may be needed to investigate intramolecular recombination in the bunyaviruses.

The ts mutants of Batai virus and Bunyamwera virus were classified into two (I and II) and Maguari virus ts mutants into three (I, II and III) unambiguous recombination groups. In addition there was a fourth class of ts mutants of Maguari virus identified as double-ts mutants of groups I and II. It is not known whether other double-ts mutants belonging to groups I and III or groups II and III exist among the 26 ts mutants which have not been crossed with the group III mutant. For instance the failure to detect recombination in the cross of MAGts18(II) and MAGts23(III) may have been a technical error or MAGts18(II) may represent a double-ts mutant of groups II and III.

Since bunyavirus genome consists of three unique subunits (Gentsch et al, 1977a; Obijeski et al, 1976b; Ozden and Hannoun, 1980), three groups of recombining mutants would be expected from bunyaviruses. However, only two recombination groups were obtained with ts mutants of the California group viruses (Gentsch and Bishop, 1976; Gentsch et al 1977b; 1979; 1980). Ozden and Hannoun (1978; 1980) also obtained two groups of ts mutants of Lumbo virus (California encephalitis group) and Germiston virus (Bunyamwera Complex).

The isolation of a Maguari virus group III ts mutant indicates that there is no form of "linkage"

between two of the three subunits such that the two always reassort together to give two recombination groups only. The high frequency of recombination between all three groups confirms independent reassortment of genome subunits at least in the case of Maguari virus of the Bunyamwera Complex. The infrequency of group III mutants may be due to the nature of the gene product. However, it might also be a consequence of suppressor mutations in view of the high frequency of spontaneous ts mutations. Ramig and Fields (1979) and Ahmed et al (1980) working with reovirus have shown that reversion at a particular locus was frequently due to extragenic suppressor mutations. Such revertants were termed pseudorevertants. In reovirus type 3 new recombination groups were discovered among the ts mutants rescued from the pseudorevertants by crossing with wild type reovirus. The same phenomenon might occur in bunyaviruses and it is being investigated.

II.3 COMPLEMENTATION

In the absence of recombination, complementation tests have been widely used in genetic study of viruses to define the functional units of the genome. In the case of viruses with segmented genome, complementation can be similarly used to define functional units within individual segments of the genome.

Complementation was detected among Maguari virus group I ts mutants but not among the group II ts mutants. Some combinations of group I mutants complemented

efficiently, the highest complementation index (CI) being attained with a combination of MAG*ts*1(I) and MAG*ts*3(I) (CI $\geq$ 168). In other combinations there was either low level complementation or no complementation at all (see Table 25). As shown in table 24, complementation of MAG*ts*6(I) and MAG*ts*7(I) was multiplicity dependent. The decrease in complementation at very high multiplicities is presumably due to a multiplicity-dependent interference. Complementation between group I *ts* mutants indicates that the complementing mutants carry mutations on different sites within the same genome subunit. Either the genome subunit carries two separate genes and therefore specifies two gene products (proteins) or the gene product is a multimeric or multifunctional protein. The Maguari virus group I *ts* mutants could not, however, be subdivided into non-overlapping complementation groups. This suggests that the complementation could be intracistronic. Gadkari and Pringle (1980a) have observed similar extensive intracistronic complementation pattern in group I *ts* mutants of Chandipura virus. While it is desirable to distinguish between intra- and inter-cistronic complementations, complementation index alone does not seem a good criterion for such distinction (Ghendon, 1972). A distinction between the two can only be made if genetic complementation data are supported by information on the physiology of the *ts* mutants.

The data obtained from the analyses of the polypeptides of heterologous recombinants by SDS-polyacrylamide gel electrophoresis (see Results Section III.3.1)

favour assignment of N gene to recombination group I, but an L gene assignment cannot be excluded by the existing data. This question is discussed in greater detail later in Section II.5. If recombination group I corresponds to the N gene, the complementation observed among group I mutants would suggest that the segment coding for N protein (S RNA; Cash et al, 1979; Gentsch and Bishop, 1978) also codes for another polypeptide. Such a gene product has not been identified in the Bunyamwera complex or in the California encephalitis group. Another (nonstructural) polypeptide, apart from L, G1, G2 and N, has, however, been observed in extracts of Uukuniemi virus-infected cells (Ulmanen et al, 1981).

If recombination group I corresponds to the L gene, which is generally assumed to code for the L protein, which may be the virus-specified RNA-dependent RNA polymerase, intracistronic complementation suggests that the viral polymerase may be a multifunctional or multimeric enzyme as appeared to be the case in the rhabdovirus, Chandipura virus (Gadkari and Pringle, 1980 a, b).

The absence of complementation among group II Maguari virus) ts mutants was unexpected since the G1 and G2 proteins are probably (by analogy with the California encephalitis group) both derived from the M genome subunit (Gentsch and Bishop, 1978). These proteins have been assigned to group II in this work (see Section II.5). Group II would, therefore, be expected to consist of G1 ts mutants as well as G2 ts

mutants. One explanation for the lack of complementation among group II ts mutants could be that G1 and G2 are products of a post-translational cleavage of a precursor protein. So far, however, no procursor proteins have been unequivocally identified in bunyavirus-infected cells. On the other hand, it is possible that a precursor protein is processed so rapidly (as soon as it is synthesized) that it escapes detection. Precursor glycoproteins have, however, been reported in the bunya-like viruses, Qalyub virus (Clerx and Bishop, 1981) and Uukuniemi virus (Ulmanen et al, 1981).

Alternatively, one of the presumptive M RNA gene products may be more susceptible to mutation. Therefore, more group II ts mutants may need to be screened before the possibility of complementation within this group can be ruled out.

II.4 HETEROLOGOUS RECOMBINATION

The heterologous recombination experiments indicated that the viruses of the Bunyamwera Complex, Batai (BAT), Bunyamwera (BUN) and Maguari (MAG), could exchange two, at least, of the three genome subunits.

Batai virus and Bunyamwera virus ts mutants recombined at high frequencies giving RF values (6.5% and 53.6%) which were comparable to those obtained in homologous crosses. The Maguari virus groups I and II ts mutants, however, recombined with ts mutants of Batai virus or Bunyamwera virus at low frequencies (below 1%) (see Table 27). The reason for the low RF obtained is not clear. Like the variations in the homologous RF,

it could be explained by inequalities of ts mutants, presence of defective interfering particles, differences in growth rates of the ts mutants or more likely, differences in cut-off temperatures of the ts mutants and hence the viability of recombinants.

High frequency recombination was unambiguously detected between the Maguari virus group III mutant, MAGts23(III) and the Bunyamwera virus group II mutant, BUNts8(II) (see Table 28). Although the RF obtained in the cross of MAGts23(III) and BUNts5(I) suggested positive recombination, this result has been interpreted with caution until progeny with the appropriate recombinant phenotype have been isolated, because of the high leak background of mutant BUNts5(I).

Heterologous recombination was bi-directional, that is, it occured in both BAT(I)xBUN(II) and BAT(II) x BUN(I) crosses. Likewise, heterologous recombination was detected in both BAT(I)xMAG(II) and BAT(II)xMAG(I) crosses as well as in the BUN(I)xMAG(II) and BUN(II) x MAG(I) crosses. These results indicated that groups I and II of the three viruses were homologous. The bi-directional, heterologous recombination detected among viruses of the Bunyamwera Complex contrasts with the outcome of heterologous recombination between members of the California encephalitis group.

In the California encephalitis group recombination was detected between snow shoe hare virus (SSH) group I ts mutants and La Crosse virus (LAC) group II ts mutants but not between group II mutants of SSH and group I mutants of LAC (Gentsch et al, 1977b). Similar

uni-directional heterologous recombinations were observed between ts mutants of Tahyna virus (TAH) and SSH, TAH and Trivittatus virus (TVT), and TVT and LAC (Gentsch et al, 1980). Gentsch et al (1979, 1980) have suggested that the uni-directional recombination they observed between these California encephalitis group viruses reflected inability of certain gene products from different viruses to function together and yield viable progeny. Alternatively, they suggested that it could be because the isolation procedure ensured detection of only phenotypically stable (i.e. cytolytic) virus and discriminated against any recombinants formed which were non-cytolytic or which failed to mature and give infectious virus at the restrictive temperature (Gentsch et al, 1980).

The only exceptions to the uni-directional recombination pattern in the California encephalitis group involved crosses between ts mutants of TAH and LAC. Like the viruses of the Bunyamwera Complex, TAH and LAC ts mutants showed a bi-directional heterologous recombination, that is, recombination occurred in the cross of group I mutant of LAC and group II mutant of TAH; and in the cross of group II mutant of LAC and group I mutant of TAH (Gentsch et al, 1980).

There seems to be a limit to cross hybridisation between bunyaviruses. No heterologous recombination was observed between ts mutants of SSH and TVT or between ts mutants of Guaroa virus (GRO) and any of the California encephalitis group viruses mentioned above (Gentsch et al, 1980). Likewise, heterologous recombination has not

been detected between ts mutants of GRO and the viruses of the Bunyamwera Complex (BAT, BUN or MAG) (Pringle and Iroegbu, unpublished data). It is conceivable that Guaroa virus may fall into a separate group quite distinct from any of the present thirteen serological groups in the genus Bunyavirus. However, its precise status will remain uncertain until the outcome of heterologous recombination experiments between GRO and the other three subdivisions of the Bunyamwera group (Wyeomyia, Main Drain and Kairi) is known. Further work is to be done involving crosses of the three viruses of the Bunyamwera Complex (Batai, Bunyamwera and Maguari viruses) with Wyeomyia Complex viruses, Main Drain virus or Kairi virus.

II.5 PHENOTYPIC CHARACTERISATION OF HETEROLOGOUS RECOMBINANTS AND THE PROBLEM OF GENE ASSIGNMENT

Prior to isolation of group III ts mutant, Iroegbu and Pringle (1981) suggested that group I of Bunyamwera Complex represented the N gene subunit. This assignment was at variance with the California encephalitis group assignment where no N protein mutants had been identified. The present situation is that G gene assignment (group I in the California encephalitis group and group II in the Bunyamwera Complex) is valid but the assignment of the other groups, particularly in the case of Bunyamwera Complex viruses, is less certain for the reasons outlined below (see Tables 35a, b).

The differences in the migration rates in SDS-polyacrylamide gel of the G1 and N polypeptides of the three Bunyamwera Complex viruses were used to establish

TABLE 35

Assignment of recomgination groups I and II of:

(a) California encephalitis group viruses to genome subunits M and L respectively by oligonucleotide fingerprinting (Gentsch et al, 1977b)

(b) Bunyamwera complex viruses to polypeptides N and G1/G2 respectively by analyses of polypeptides by SDS-polyacrylamide gel electrophoresis.

TABLE 35a

	Assignment of recombination groups and proteins to genome segments		
	L	M	S
Recombination groups	II[a]	I[a]	?
Structural polypeptides	L	G1 and G2[c]	N[b,d]

TABLE 35b

	Assignment of recombination groups to proteins		
Recombination groups	I or III	II	I or III
Structural polypeptides	L	G1 (and G2)	N

? = not identified

a = Gentsch et al, 1977b

b = Gentsch and Bishop, 1978

c = Gentsch and Bishop, 1979

d = Cash et al, 1979

the parental origin of G1 and N proteins of the recombinants. Invariably the recombinants derived their G1 protein from the group I parent and their N protein from the group II parent, and it was inferred that group I mutants carried ts defects on the N protein (i.e. they were N mutants) whereas the group II was made up of G1/G2 ts mutants (Table 35b). This assignment is tentative pending confirmation by analysis of the genotype of the recombinants. The weakness of the assignment procedure is that only the G1 and N polypeptides are distinguishable and the origin of the L and G2 proteins could not be determined, although it is presumed that the G2 gene is linked to the G1 gene. This tentative assignment was principally based on the fact that the heterologous recombination was bi-directional and that the group I parent donated G1 protein and group II parent donated the N protein in recombinants obtained from eleven different crosses irrespective of the viruses and/or the ts mutants involved (see Table 32).

It is recognised, however, that this result does not exclude an alternative assignment. Figure 26 shows the progeny expected in group I x group II cross on the hypotheses.

i) that group I $\equiv$ N gene

OR

ii) that group I $\equiv$ L gene (assuming that the L genome subunit codes for L protein)

In hypothesis (i) i.e. group I $\equiv$ N, two types of

FIGURE 26.

Progeny of crosses of recombination group I and recombination group II ts mutants predicted on the hypothesis

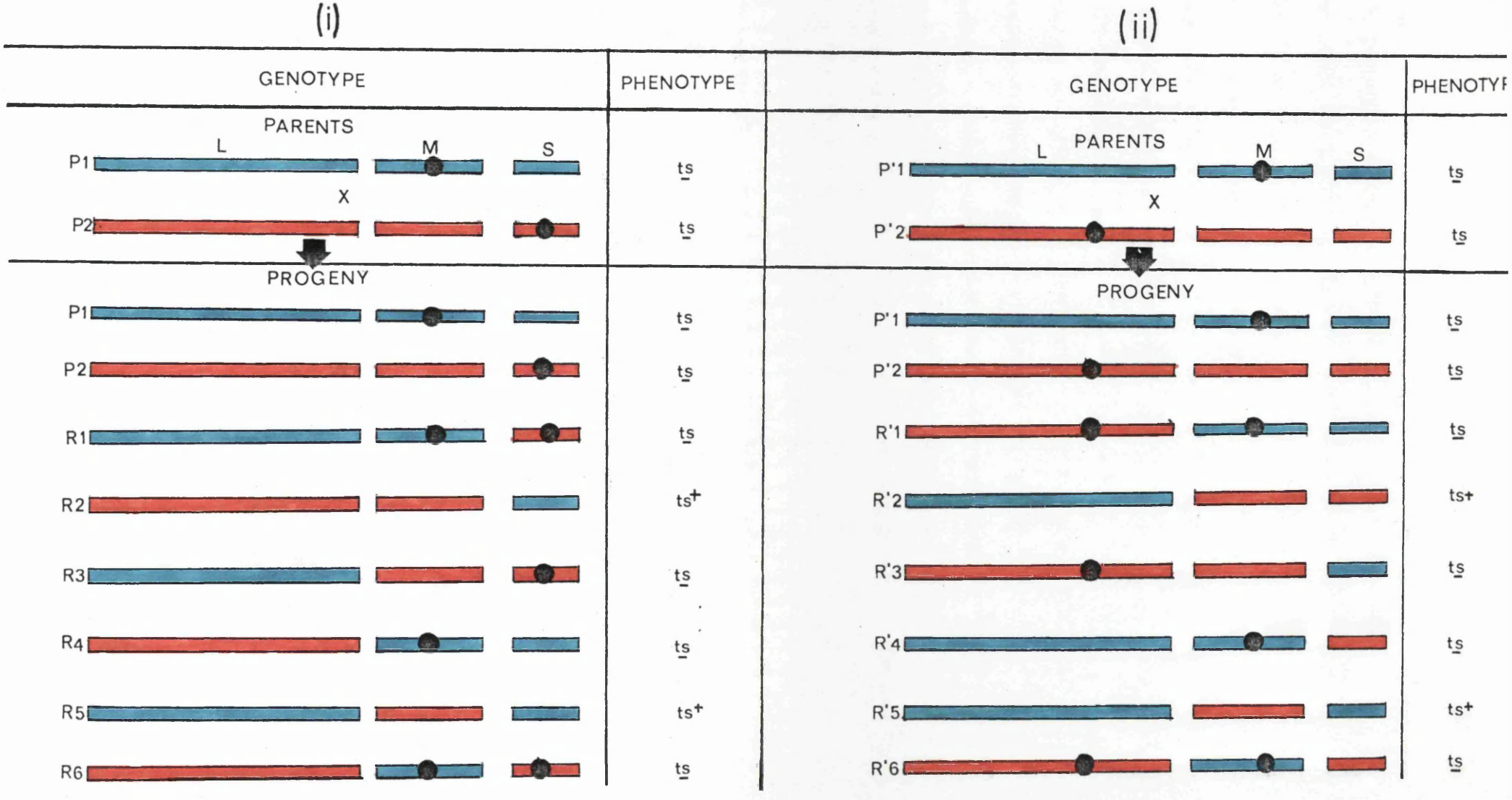
i) that group I mutants carry ts lesions on the S RNA genome segment (i.e. N gene)

OR

ii) that group I mutants carry ts lesions on the L RNA genome segment (presumably L gene)

Symbol:- ● = mutational lesion producing ts phenotype.

Fig 26


(i)
GENOTYPE
PHENOTYPE
PARENTS
L
M
S
P1
X
P2
ts
ts
PROGENY
P1
P2
R1
R2
R3
R4
R5
R6
ts
ts
ts
ts+
ts
ts
ts+
ts
(ii)
GENOTYPE
PHENOTYP
PARENTS
L
M
S
P'1
X
P'2
ts
ts
PROGENY
P'1
P'2
R'1
R'2
R'3
R'4
R'5
R'6
ts
ts
ts
ts+
ts
ts
ts+
ts

non-ts recombinants, R2 and R5 are obtainable. These differ only in their L segments (R2 has an L segment of the P2 parent whereas R5 has an L segment of the P1 parent). Since only the G1 and N proteins can be differentiated, R2 and R5 are not distinguishable because they would exhibit the same phenotype.

In hypothesis (ii), i.e. group I = L, two types of non-ts recombinants, R'2 and R'5 are also obtainable, and these differ in their S genome subunits (i.e. N proteins). An analysis of their polypeptides by SDS-polyacrylamide gel electrophoresis could detect R'5 as a recombinant and not R'2. The latter recombinant is phenotypically indistinguishable from the P'2 parent because both the G1 and N polypeptides are derived from the P'2 parent, and it cannot be distinguishable from a P'2 revertant.

The cross involving mutant BUNts7(II) of group II with the non-ts G1 protein mobility difference confirmed that group II represented the gene specifying the G1 protein, since the non-ts atypical mobility phenotype was substituted by a normal BAT G1 protein donated by BATts1(I) in a heterologous recombination of BUNts7(II) and BATts1(I) (see Figure 19). On the other hand the assignment of group I remains uncertain for the following reasons: Table 36 shows the expected outcome of crosses of mutants of the three Maguari virus recombination groups with mutants of a heterologous virus, in this case Bunyamwera virus, depending on whether (A) the L gene, or (B) the N gene is assigned to group I. It can be

TABLE 36: Recombinant phenotype expected according to gene assignment.

	A. GROUP I ≡ L gene				B. GROUP I ≡ N gene			
BUN PARENT / MAG PARENT	BUN(I)		BUN(II)		BUN(I)		BUN(II)	
	EXPECTED	OBSERVED	EXPECTED	OBSERVED	EXPECTED	OBSERVED	EXPECTED	OBSERVED
MAG(I)	NIL	-	(i)MAG(G) BUN(N) (ii)MAG(G) MAG(N)	3 *	NIL	-	MAG(G) BUN(N)	3
MAG(II)	(i)BUN(G) MAG(N) (ii)BUN(G)BUN(N)	44 0	NIL	- -	BUN(G) MAG(N)	44	NIL	-
MAG(III)	(i)MAG(G) BUN(N) (ii)BUN(G)BUN(N)	12[1] 30[2]	MAG(G) BUN(N)	10	(i)BUN(G)MAG(N) (ii)MAG(G)MAG(N)	0 2	MAG(G)BUN(N) MAG(G)MAG(N)	10 0

- = phenotype not observed

* One clone with MAG(G) and MAG(N) as well as BUN(N) (see Results; Section III.3.2)

** The inserted figures represent the number of clones observed.

1. Clones with MAG(G) and BUN(N) phenotype only observed as ts clones isolated at 31°C. Non-ts clones were not isolated.
2. Out of the 30 non-ts BUN(G):BUN(N) clones isolated, 6 were obtained at 31°C and 24 at 39°C.

NB: The results for the MAG III x BUN I, and MAG III x BUN II crosses were obtained after this thesis had been completed. These results do not alter the interpretations given in the text.

seen that the results obtained are compatible with the N gene assignment but depend on isolation of a single non-unique recombinant type rather than two, one, the same recombinant phenotype and the other, a recombinant with the phenotype of one of the parents. The results of the MAGIIIxBUNII cross, however, favour the alternative hypothesis that group I represents the L gene (Pringle and Iroegbu, data not shown). This assignment, however, relies on failure to recover recombinants with a wild type phenotype in the progeny and not on identification of a unique phenotype. The only truly discriminatory cross is the MAG(III)xBUN(I) cross. Unfortunately, this was the heterologous combination (Table 28) which yielded equivocal evidence of recombination, and the recombinants have still to be identified in the progeny of this cross. Consequently the actual gene assignment remains uncertain. It is unlikely that complete gene assignment can be obtained on the basis of genetic experiments alone in view of the inability to discriminate the L protein. Definitive assignments must await analysis of the genomes of these viruses by oligonucleotide finger-printing.

The gene assignments for the viruses of the California encephalitis group are based on more definitive criteria, principally the oligonucleotide finger-printing of heterologous recombinants. However, for the reasons outlined below they are also not entirely conclusive. In analyses of the clones obtained from the SSH group IxLAC group II cross, Gentsch et al (1977b)

detected two types of recombinants, SSH/LAC/LAC and SSH/LAC/SSH (the nomenclature of the recombinants being in the order, L/M/S of the genome subunits) and deduced that groups I and II ts mutants of SSH and LAC carried mutations on the M and L genome subunits respectively (see Table 35a). Since the M RNA codes for G1/G2 (Gentsch and Bishop, 1979) and the S RNA codes for the N protein (Cash et al, 1979; and Gentsch and Bishop, 1978) it was deduced that SSH group I and LAC group I are G1/G2 mutants, and SSH group II and LAC group II are mutants of the gene product(s) of the L genome subunit, presumably the L protein. The absent group III would presumably be N protein mutants.

However, only two clones were isolated which fortuitously were the two recombinant clones expected. In a subsequent experiment in which the progeny of a mixed infection of the wild type viruses was analysed, it was observed that the same two recombinant genotypes occurred at high frequency and represented 25% of the progeny (see Table 37a). The other four of the expected six recombinant genotypes were not isolated. If the non-ts genotypes of the two clones from the SSH group I x LAC group II cross were a consequence of recombination between revertants rather than ts mutants, an erroneous assignment would have been made since the data obtained from the various subsequent experiments outlined in Table 37b do not conflict with an assignment of either the L or the N gene to recombination group II. The assignment of the G1/G2 gene to recombination group I is unambiguous,

TABLES 37a, b

ANALYSES OF RECOMBINANT/NON-TS CLONES ISOLATED FROM PROGENY OF HETEROLOGOUS CROSSES OF CALIFORNIA ENCEPHALITIS GROUP VIRUSES REPORTED BY GENTSCH ET AL (1977b, 1979, 1980)

LEGEND:

ABBREVIATIONS

LAC	=	La Crosse virus
SSH	=	Snow shoe hare virus
TAH	=	Tahyna virus
TVT	=	Trivittatus virus

a	=	clones isolated from two separate experiments; 11 from one experiment (all the same phenotype TAH G1 and SSH N) and 29 from the second experiment (two phenotypes detected; 23 clones = TAH G1 and SSH N; 6 clones = TAHG1 and TAH N)
nd	=	not distinguishable
?	=	data not provided
+	=	recombination detected
-	=	recombination not detected

TABLE 37 a

VIRUS	Recombination	No. of the non-ts clones isolated and analysed	POLYPEPTIDES OF NON-TS CLONES (IF DISTINGUISHABLE)		No. of each type of recombinant/ non-ts clone analysed	Genotype of non-ts/recombinant clones (L/M/S)	No. of clones fingerprinted	Reference
			G1	N				
SSH IxLACII	+	2	nd	SSH	1	SSH/LAC/SSH	1	Gentsch et al 1977
			nd	LAC	1	SSH/LAC/LAC	1	" "
SSH IIxLACI	-	3	nd	LAC	3	LAC/LAC/LAC	3	Gentsch et al, 1979
SSHts$^+$ x LACts$^+$	+	8	nd	LAC	3	LAC/LAC/LAC	3	" "
			nd	LAC	1	SSH/LAC/LAC	1	" "
			nd	SSH	1	SSH/LAC/SSH	1	" "
			nd	SSH	3	SSH/SSH/SSH	3	" "

TABLE 37b

VIRUS	Recombination	No. of the non-ts clones isolated and analysed	POLYPEPTIDES OF NON-TS CLONES (IF DISTINGUISHABLE)		No. of each type of recombinant/ non-ts clone analysed	Genotype of non-ts/recombinant clones L/M/S	No. of clones fingerprinted	Reference
			G1	N				
SSHIxSSH/LAC/SSH II	+	1	?	?	1	SSH/LAC/SSH	1	Gentsch et al, 1979
LACIx SSH/LAC/SSHII	+	1	?	?	1	LAC/LAC/SSH	1	" "
SSHIxSSH/LAC/LACII	+	?	nd	SSH	?	SSH/LAC/SSH	1	" "
SSHIIxSSH/LAC/LACI	+	?	nd	LAC	1	SSH/SSH/LAC	1	" "
LAC IxTAH II	+	9	TAH	LAC	6	LAC/TAH/LAC	1	Gentsch et al, 1980
			TAH	TAH	1	LAC/TAH/TAH	1	" "
			LAC	TAH	1	TAH/LAC/TAH	1	" "
			LAC	LAC	1	?	-	" "
LAC II x TAH I	+	10	LAC	TAC	8	TAH/LAC/TAH	1	
			LAC	LAC	2	TAH/LAC/LAC	1	
TAH II x SSH I	+	40[a]	TAH	SSH	34	?	0	
			TAH	TAH	1	SSH/TAH/TAH	1	
TAH I x SSH II	-	-	-	-	-	-	-	

TABLE 37b (CONTD.)

TAH II x TVT I	+	3	nd	nd	3	TVT/TAH/TVT	3	
TAH I x TVT II	-	-	-	-	-	-	-	
TVT I x LAC II	+	1	?	?	1	TVT/LAC/TVT	1	
TVT II x LAC I	-	5	?	?	5	LAC/LAC/LAC	5	
TVT IxLAC/LAC/SSH II	+	1	?	?	1	TVT/LAC/TVT	1	
TVT IIxLAC/LAC/SSH I	-	-	-	-	-	-	-	

on the other hand, because of the pattern of segregation of the neutralising antigen in heterologous crosses (Gentsch et al, 1980).

Tables 37a, b are a summary of the analyses of heterologous crosses of California encephalitis group viruses contained in three different reports. One of the features of these results was that the heterologous recombinations were uni-directional, with the exception of the TAH x LAC crosses. However, analyses of the progeny of this solitary example of bi-directional recombination observed in the California encephalitis group heterologous crosses, that is, between TAH and LAC ts mutants, provided anomalous data. In the LAC group I x TAH group II cross, four types of clones were isolated. Two of these (LAC/TAH/LAC and LAC/TAH/TAH) were expected according to the assignments predicted by Gentsch and co-workers. One of the remaining two was a recombinant with TAH/LAC/TAH genotype whereas the other clone (not finger-printed) exhibited LAC G1 and N polypeptides and could be either a LAC revertant or the TAH/LAC/LAC recombinant. The explanation offered by the authors for the presence of the latter two unpredicted recombinants was that either they could be clones from the TAH group II x LAC group I cross which had been mistaken while processing a large number of clones, or double segment revertants of original double ts recombinants. The latter explanation is less likely. If no mistakes were made in the identity of the clones during processing, however, detection of the two unpredicted recombinants could raise questions about the validity of the initial assignments.

Anomalous results could also be obtained as a result of extragenic suppressor mutations similar to the high frequency extragenic suppression observed with revertants of ts mutants of reovirus (Ramig and Fields, 1979; and Ahmed et al, 1980). For example, a suppressor mutation in the L or M segment of the ts recombinant, R3 (Figure 26) would produce a non-ts phenotype which could be mistaken for the non-ts recombinant, R'2 of the alternative hypothesis (i.e. group I ≡ L). Similarly, ts phenotypes of the other recombinants could be extragenically suppressed. Such a situation calls for a re-evaluation of assignments made with data in which some recombinants were observed only at low frequency.

In all crosses where many clones were analysed one type of recombinant appeared at higher frequency than the other. For example in the LAC group II x TAH group I cross (see Table 37b) there were probably 8 recombinants with TAH/LAC/TAH genotype and only 2 with TAH/LAC/LAC. Similar inequalities in the frequencies of the two types of recombinants obtained in the other crosses are apparent in Table 37b. The genotypic analyses of the more frequent recombinants showed that their genotype would fit either assignment of the L or N gene to the California encephalitis group recombination group II.

The number of putative recombinant clones actually fingerprinted were few and could not exclude the occurrence of other types of recombinants, as was the case in the LAC group I x TAH group II cross.

Considering the above points, it is apparent that the data on which the California encephalitis group ts mutants have been assigned to genome subunits (shown on Tables 37a, b) are not quite conclusive, the potential of the oligonucleotide analyses notwithstanding.

One point of correspondence between the Bunyamwera complex ts mutants and the California encephalitis group mutants is that G1/G2 mutants were present in both groups of viruses. In other words, the group II mutants of the Bunyamwera complex are equivalent to the group I of the California group viruses. It is still uncertain which of the remaining two recombination groups (I or III) of the Bunyamwera complex viruses corresponds with the group II of the California encephalitis group. The Maguari group III mutant was isolated late and was not used in the heterologous recombination experiments described here. Data from experiments carried out subsequently has been included in Table 36.

It is not certain whether MBN7/8/3, the clone with both BUN N and MAG N (see Figure 23), represents aggregation, heterozygocity or heteroploidy. Over 100 clones (wild type virus, recombinants and ts mutants) have been characterised by SDS-polyacrylamide gel electrophoresis of their polypeptides. No other clones exhibited a mixed protein phenotype. If MBN7/8/3 were heteroploid or a heterozygote, the low rate ($< 1\%$) at which it was isolated may reflect the mechanism of virus assembly. During virus assembly, structural components would either be selected randomly and packaged, or the selection

is co-ordinated by some unknown mechanism. Random selection of structural components would increase chances of packaging errors and might be expected to produce incomplete virions as well as polyploids (partial or complete) at a rate greater than the frequency of isolation of MBN7/8/3-type clones. Co-ordinated (less random) mechanism of assembly would less frequently permit error in packaging.

III. IMPLICATIONS OF HYBRIDISATION BETWEEN TAXONOMICALLY DISTINCT BUNYAVIRUSES

The heterologous recombination observed between ts mutants of Batai, Bunyamwera and Maguari viruses indicates that the viruses of the Bunyamwera Complex can exchange genome segments. Therefore, any restrictions to hybridisation of these viruses in nature may be geographical or ecological rather than genetical.

The gradations and diversities in antigenicity among members of certain bunyavirus serological groups and the immunological cross-reactions between bunyaviruses belonging to different groups (Berge, 1975; Bishop and Shope, 1979; Theiler and Downs, 1973) suggest evolution of some bunyaviruses by ancestral reassortment of genome subunits. Demonstration of heterologous recombination between viruses of the Bunyamwera complex is consistent with the above postulation. The geographical barrier to natural reassortment might occasionally be surmounted by simultaneous occurrence of a number of bunyaviruses in the same localities.

Like the California encephalitis group viruses (Gentsch et al, 1980), the neutralising antigen of the three viruses of the Bunyamwera complex have been shown to segregate with particular genome subunits during reassortment. This observation calls for a reassessment of serological cross-reactions as a key criterion for defining bunyaviruses and bunyavirus groups. A taxonomic grouping based on antigenic relatedness is unlikely to contain a homogeneous population of bunyaviruses after several cycles of cross-hybridisation between member viruses or between viruses of different serological groups. On the other hand, the potential of genetic interactions, particularly recombination, as tools in taxonomy of bunyaviruses is worth exploration. A classification based on genetic interaction would essentially bring virus taxonomy in line with the taxonomy of eucaryotes and may eventually herald the definition of true virus species.

The epidemiological implications of heterologous exchange of genetic materials is well illustrated with influenza. Evidence for the naturally occurring heterologous recombinants of influenza A viruses of man or influenza A viruses of man and/or animals (see Webster et al, 1975) has led to the hypothesis that recombination among different strains of influenza A virus (both of man and animals) could be a mechanism in the evolution of pandemic and epidemic strains (Laver and Webster, 1973; 1979; and Scholtissek et al, 1978). Likewise hybridisation of bunyaviruses, many of which are associated

with human illness, could have significant epidemiological consequences for man.

So far, heterologous exchange of genome subunits has only been observed between viruses of the Bunyamwera complex or between viruses of the California encephalitis group, but not between viruses of the Bunyamwera complex and the California encephalitis group viruses (C.R. Pringle, personal communication). Similarly, recombination has not been detected between the viruses of the Bunyamwera complex or the California encephalitis group and Guaroa virus which has been suggested a possible link between the two groups. Even within the California encephalitis group there are limitations to heterologous recombination as indicated by the unidirectional recombinations and the absence of recombination between SSH and TVT (Gentsch et al, 1980). It has been suggested that these limitations may be due to incompatibility of gene products of some of these viruses in certain combinations (Gentsch et al, 1979; 1980). That this may be so is indicated by the detection of only two of the expected six recombinant genotypes when wild type SSH and LAC viruses were crossed (Gentsch et al, 1979). The apparent incompatibility of certain combinations of the genome subunits of the California encephalitis group viruses suggests that the number of new California encephalitis group viruses which could evolve by heterologous reassortment is strictly limited. Similar restrictions, presumably genetic, in the compatibility of certain combinations of the hemagglutinin and neuraminidase of influenza A viruses has been observed

(Hinshaw et al, 1981).

It will be necessary in the future to determine the limits of recombination within the genus Bunyavirus more precisely by studying the interactions of the viruses of the Bunyamwera complex with Wyeomia virus, Main Drain virus and Kairi virus which represent the remaining three subdivisions of the Bunyamwera group.

APPENDIX

A P P E N D I X I

MATERIALS

STOCK SOLUTIONS AND CHEMICALS

All chemicals used were obtained from BDH Chemicals Ltd., Poole, England, unless otherwise stated.

1. SOLUTIONS FOR CELL CULTURE

All the solutions for cell cultures as well as the overlay medium were prepared by the staff of the Media Section, Institute of Virology, Glasgow.

1.1 EAGLE'S MEDIUM (GLASGOW MODIFICATION)

This consisted of Eagle's basal medium modified to contain twice the normal concentration of amino acids and vitamins (MacPherson and Stoker, 1962). Two types of Eagle's medium (all modified as above) were used, one for growth and maintenance of cells in culture and the other for use in agarose overlay medium. Each type was prepared and stored in two component solutions designated Eagle's A and Eagle's B. Eagle's A was a solution of salts and hydrochloric acid in distilled water and was sterilised by autoclaving at a pressure of 15 pounds per square inch for 15 minutes. Eagle's B solution contained heat-labile solutes such as amino acids, antibiotics and vitamins as well as salts, all dissolved in distilled water. To preserve the integrity of the heat-labile substances, Eagle's B was sterilised by filtration

through a Millipore GS 0.22 micron membrane (Millipore Filter Corporation, Bedford, Massachusetts, U.S.A.). The major differences between the growth medium and the overlay medium were that in the agarose overlay medium hydrochloric acid and phenol red (indicator) were omitted from the Eagle's A and B solutions respectively.

1.1.1 EAGLE'S A SOLUTION

Eagles A solution contained 0.023% w/v) each of $CaCl_2.2H_2O$ and $MgSO4.7H_2O$ and 0.1 µl/ml concentrated hydrochloric acid.

1.1.2 EAGLE'S B SOLUTION

This was a mixture of three seperately prepared solutions:

(i) Solution of salts and antibiotics

This solution was made up of 10.24% (w/v) NaCl, 0.64% (w/v) KCl, 0.24% (w/v) $NaH_2PO_4.2H_2O$, 7.2% (w/v) Glucose, 0.01% $Fe(NO_3)_3$ (16 µl/ml), 0.47% (w/v) L-Glutamate, Penicillin (1,600,000 units), 0.224% (w/v) streptomycin and 0.02% antimycotic.

(ii) Solution of amino acids

The composition of this solution was L-arginine mono-HCl (0.84 µg/ml), L-cystine (0.48 µg/ml), L-histidine mono-HCl (0.384 µg/ml), L-isoleucine

(1.048 µg/ml), L-leucine (1.048 µg/ml), L-lysine mono-HCl (1.462 µg/ml), L-phenylalanine (0.66 µg/ml), L-threonine (0.952 µg/ml), L-tryptophan (0.16 µg/ml), L-tyrosine (0.724 µg/ml), L-valine 0.936 µg/ml), L-methionine (0.3 µg/ml) and inositol (0.07 µg/ml), and in addition 5.5% (w/v) $NaHCO_3$ and 1% phenol red indicator.

(iii) Solution of vitamins

This solution contained 0.5 µg/ml each of choline chloride, folic acid, nicotinamide, D,L-pantothenic acid (calcium salt), pyridoxal hydrochloride and thiamine hydrochloride as well as 0.05 µg/ml of riboflavin.

The three basic solutions of Eagle's B were mixed in the following proportions:

Salts and antibiotics, (i)	500 ml
Amino Acids, (ii)	400 ml
Vitamins, (iii)	32 ml

The volume was made up to one litre with distilled water and the solution gassed with carbon dioxide to a pH 6.5 before sterilisation.

All the amino acids, vitamins and the antimycotic were obtained from Sigma London Chemical Company,

London, England. The antibiotics, penicillin and streptomycin were obtained from Glaxo Laboratories Limited, Greenford, England.

1.2 VERSENE

0.2 µg/ml of the disodium salt of diaminoethane-tetra-acetic acid (EDTA) and 1.5 µl/ml of 1% phenol red indicator all dissolved in Dulbecco's phosphate buffered saline (calcium and magnesium salt-free). The pH was adjusted to 7.1-7.3 and the solution sterilised by autoclaving.

1.3 TRYPSIN

0.25% (w/v) Difco trypsin (Difco Laboratories, Detroit, Michigan, U.S.A.) was dissolved in tris-saline and finally sterilised by Millipore filtration.

1.3.1 TRYPSIN-VERSENE

A mixture of one part of trypsin and four parts of versene was used for cell transfer.

2 OVERLAY MEDIUM

2.1 EAGLE'S MEDIUM (FOR AGAROSE OVERLAY)

Eagle's A and B solutions were prepared as described above but without hydrochloric acid and phenol red respectively. Each solution was adjusted to pH 6.6 by flushing with carbon dioxide.

2.2 AGAROSE

2.4% (w/v) Agarose (gel electrophoresis quality; LITEX-DENMARK) was dissolved by heating in distilled water; and $CaCl_2.2H_2O$ and $MgSO4.7H_2O$ were added in the same proportion as in Eagle's A solution. Agarose was used in these experiments because plaque formation was inhibited under agar overlay.

3 GLUTARALDEHYDE-FIXATIVE

This was obtained under the trade name, CIDEX (Arbrook Products Ltd, Livingston, Scotland) as a solution containing 2% glutaraldehyde. This was used as a one percent solution prepared by diluting the original solution two-fold with complete Dulbecco's phosphate buffered saline (PBS).

4 GIEMSA STAIN

1.5% (w/v) Giemsa in glycerol was heated at 56° C for 90 - 120 minutes, cooled and added to an equal volume of methanol. The stain was diluted (one part of stain to ten parts of PBS) for use in staining fixed cell monolayers.

5 NEUTRAL RED

0.4% (w/v) neutral red chloride was dissolved by heating in distilled water, filtered and then autoclaved to sterilise. The stain was used at a final concentration of about 0.002% to stain the monolayer.

6 TRIS-SALINE

0.8% (w/v) NaCl, 19% KCl solution (2 µl/ml), 0.01% (w/v) Na_2HPO4, 0.1%(w/v) dextrose, 0.3%(w/v) Sigma-Tris (hydroxymethyl amino methane) (Sigma London Chemical Company Limited, London, England), 1.5 µl/ml of 1% phenol red, penicillin (5,000,000 units) and 0.01% (w/v) streptomycin were dissolved in distilled water and the solution was sterilised by millipore filtration. The p.H was adjusted to 7.4.

7 PHOSPHATE-BUFFERED SALINE (DULBECCO'S)

Complete phosphate-buffered saline (PBS) was obtained by mixing three stock solutions A, B and C in the proportion of 8 parts of A with one part each of B and C. Solution A contained 1% NaCl, 0.25% KCl, 0.14% Na_2PO4, 0.025% KH_2PO4 and the pH was adjusted to 7.2. Solutions B and C were made up of 0.1% each of $CaCl_2.2H_2O$ and $MgCl_2.6H_2O$ respectively in distilled water. All solutions were autoclaved to sterilise.

8 SOLUTIONS FOR POLYACRYLAMIDE GEL ELECTROPHORESIS OF VIRAL PROTEINS

8.1 ACRYLAMIDE AND BISACRYLAMIDE

A stock solution containing 29.25% (w/v) acrylamide and 0.75% (w/v) NN'-methylene bisacrylamide was prepared in deionised water. Both chemicals were purchased either from Koch-Light Laboratories Ltd, Colnbrook, Bucks

England, or from BDH Chemicals Ltd, Poole, England. The solution was filtered through a Whatman No. 1 filter paper (W & R Balston Ltd., London, U.K.) and stored at +4° C.

8.2 RUNNING GEL BUFFER

The running gel buffer consisted of 18.15% tris-HCl, pH 8.9 and 0.4% (w/v) SDS, all dissolved in deionised water.

8.3 SODIUM DODECYL (LAURYL) SULPHATE

A stock solution of 10% (w/v) sodium dodecyl sulphate (SDS) was prepared in deionised water.

8.4 STACKING GEL BUFFER

The stacking gel buffer was made up of 5.9% (w/v) tris-HCl, pH 6.7 and 0.4% (w/v) SDS dissolved in deionised water.

8.5 AMMONIUM PERSULPHATE, 10% (w/v)

This was prepared fresh at each requirement, with deionised water as solvent.

8.6 N,N,N'N'-tetramethylenediamine, TEMED

This is a product of the BioRad Laboratories, Richmond, California, U.S.A. and was used to initiate polymerisation of acrylamide and bisacrylamide.

8.7 ELECTROPHORESIS BUFFER

This solution was prepared in deionised water and contained 0.6% (w/v) tris-HCl, 0.4% (w/v)

glycine and 0.1% (w/v) SDS.

8.8 DISSOCIATION BUFFER (BOILING MIX)

The dissociation buffer consisted of 0.15 M tris-HCl, pH 6.7, 6% (w/v) SDS, 15% v/v) β-mercaptoethanol, 30% (v/v) glycerol and 0.015% (w/v) bromophenol blue.

8.9 COMASSIE BLUE STAIN

A solution composed of 0.2% (w/v) Comassie brilliant blue R-250 (BioRad Laboratories, Richmond, California), water and acetic acid in the ratio 50 : 50 : 7 was prepared for staining proteins in polyacrylamide gel.

8.10 DESTAINING SOLUTION

This solution consisted of5 parts methanol, 7 parts glacial acetic acid and 88 parts water.

9 SOLUTIONS FOR SCINTILLATION COUNTING

9.1 TOLUENE SCINTILLATION FLUID

This was composed of 10 gm 2-5 diphenyloxazole (PPO) and 125 mg 1,4-di [2-(5-phenyloxazole)] benzene (POPOP) in 2.4 litres of toluene, stirred overnight to dissolve at room temperature. All the chemicals used here were obtained from Koch-Light Laboratories Ltd, Colnbrook, Bucks, England).

9.2 TRICHLOROACETIC ACID (TCA)

This chemical was obtained from Koch-Light Laboratories, Colnbrook, Bucks, England and dissolved in water and stored in 10% concentration.

9.3 ABSOLUTE ALCOHOL (ethanol)

The absolute alcohol used was of the analytical grade (ANALAR - BDH).

10 SOLUTIONS FOR VIRUS PURIFICATION

10.1 NTE BUFFER

This was a solution containing 0.1 M NaCl, 20 mM tris-HCl and 1mM EDTA, all dissolved in distilled water. The pH was adjusted to 8.0

10.2 SUCROSE

A stock solution of 65%(w/v) sucrose ultra pure density gradient grade (Schwarz-Mann, a Division of Beckton, Dickinson and Company (B-D), Orangeburg, N.Y., U.S.A.), was prepared in NTE buffer. From this, lower concentration, such as 20%, 30%, 40% or 60%, of sucrose were prepared as required. All dilutions were made by addition of NTE buffer.

10.3 POLYETHYLENE GLYCOL (PEG)

A stock solution of 30% (w/v) polyethylene glycol (molecular weight 6000-7000) was prepared in NTE buffer pH 8.0. For virus concentration, this solution was diluted in the ratio: 1 part

of PEG solution to 5 parts virus-containing fluid, to achieve a final concentration of 6% PEG.

APPENDIX II

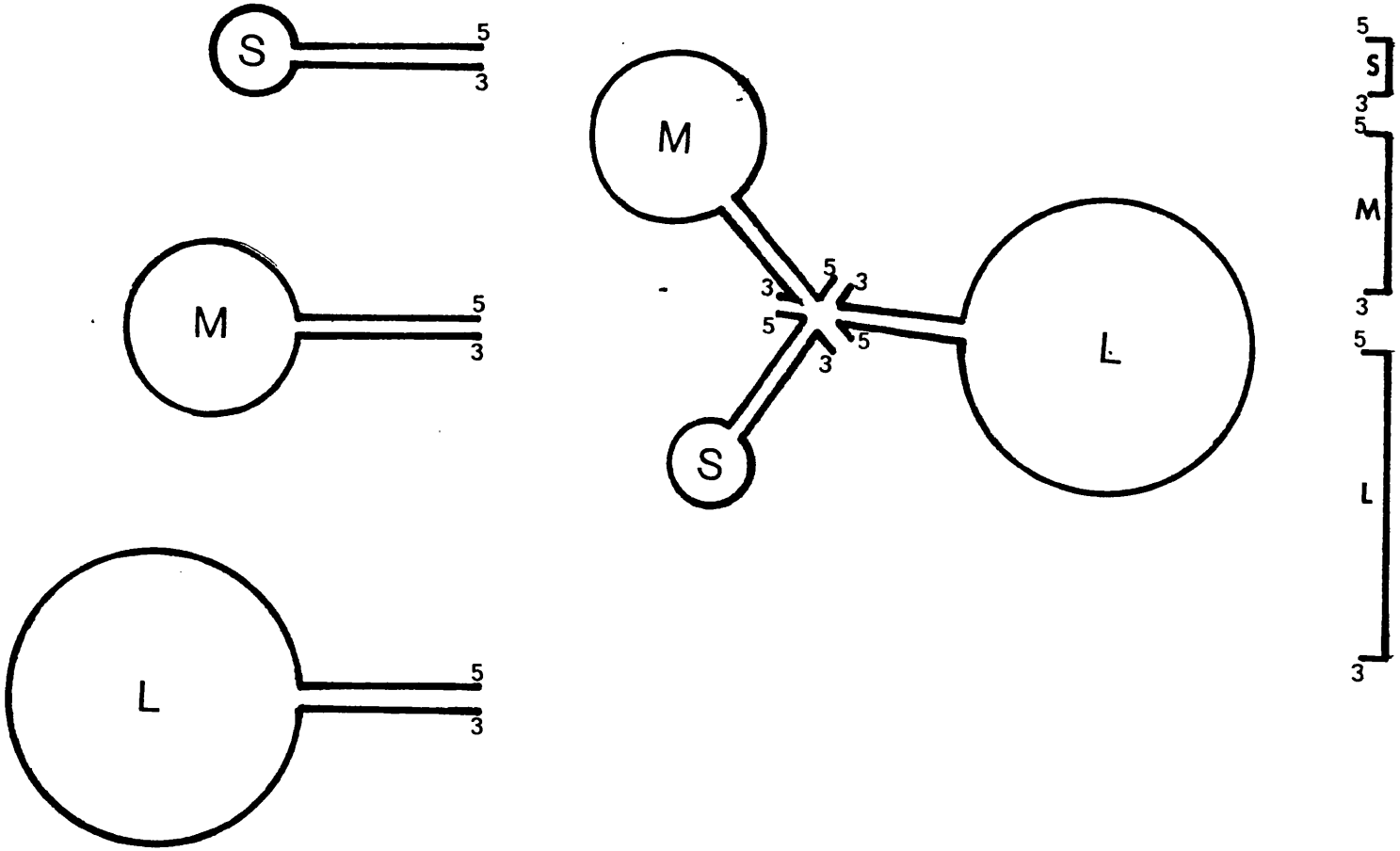


Fig.27 Examples of possible homologous and heterologous interactions of La Crosse virus RNAs.
(J.F. Obijeski, J. McCauley and J.J. Skehel; Nucleic Acids Research, Vol. 8 (11), 1980; p.2436)

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