THE SYNTHESIS AND FUNCTION OF THE NONSTRUCTURAL POLYPEPTIDES OF SINDBIS VIRUS

by

Henry Brzeski B.Sc. (Warwick)

Submitted for the degree of Doctor of Philosophy at the University of Warwick

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The madman shouted in the market place. No-one stopped to answer him.

Thus it was confirmed that his theses were incontrovertible...

Dag Hammarskjold

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Acknowledgements

I would like to thank my supervisor, Dr. Kennedy, for his help and enthusiasm throughout the period of this work; I am especially grateful for his example of leading from the front. I would also like to thank the various members of the alphavirus group, past and present, for the many useful criticisms and discussions of the last three years.

I am grateful to my wife for the moral support she has provided during this time, especially in the last few months when the outlook was bleakest.

I declare that this thesis has been composed entirely by myself and that it has not been used in any previous application for any degree. All work reported herein is my own unless specifically acknowledged.

SUMMARY

Sindbis virus an alphavirus, has a single stranded RNA genome with a molecular weight of 4.2 million (42S RNA). Inside infected cells two RNA species are found. One, 42S RNA, is assumed to code for the RNA polymerase(s) and is also destined to enter mature virus, the second, 26S RNA, has a molecular weight of 1.8 million and is a subgenomic fragment representing the 3' end of 42S RNA. The 26S RNA functions as a messenger for the structural proteins of the virus. The aim of this work was to discover the mechanism by which Sindbis virus, in particular, regulated the rate at which it synthesised the two RNA species such that the rate of structural protein and virion RNA synthesis were co-ordinated for efficient virus assembly.

It was considered that the regulatory ability would probably lie with the nonstructural polypeptides i.e. those comprising the RNA polymerase. For this reason it was first necessary to characterise the nonstructural polypeptides synthesised by Sindbis virus as no such polypeptides had been previously described. Pulse and pulse-chase experiments conducted early in the infectious cycle indicated the existence of three new stable polypeptides nsp60, nsp82 and nsp89 along with three other polypeptides p215, p150 and p76 which were unstable under chase conditions. Inhibition of proteolytic enzymes resulted in the accumulation of p215 and a larger polypeptide p230 along with a concomitant reduction in the amount of smaller polypeptides synthesised, suggesting that p230 was the entire translation product of the nonstructural region of 42S RNA. This precursor-product relationship was confirmed by tryptic peptide mapping. The gene order was established as 5' -nsp60-nsp89-nsp82-3' and the order of cleavage of the precursor was also determined.

Having characterised the nonstructural polypeptides it became necessary to determine the mechanism by which 26S RNA was transcribed from a 42S negative strand RNA. This was achieved by determining the relative sensitivities of the synthesis of each RNA species to UV inactivation. The rate of inactivation of 26S RNA was consistent with its transcription from a template with a molecular weight of 1.8 million (which is the size of 26S RNA) and so, since there is no negative strand of the same size as 26S RNA, it was concluded that 26S RNA was synthesised by internal initiation of transcription on the 42S negative strand RNA. Further analysis of the UV inactivation data indicated that, on average, a single template RNA supported the synthesis of one molecule of 42S and three molecules of 26S RNA.

In an attempt to characterise the polymerase(s) responsible for the synthesis of 26S and 42S RNA, 15 RNA -ve temperature-sensitive mutants were examined with respect to the rate at which they synthesised the two RNA species. These mutants are unable to synthesise RNA when incubated continuously at the restrictive temperature and hence are assumed to have a lesion in the gene(s) coding for the polymerase(s). Under shift-up conditions no mutant suffered a loss of 42S RNA synthetic ability but all mutants showed a relative decrease in the rate of 26S RNA synthesis and in the case of three mutants this was marked. Examinations of the polypeptide phenotypes of these mutants suggested that there was a basic polymerase activity responsible for the transcription of both 42S and 26S RNA but that a virus specified polypeptide was required for the initiation of 26S RNA synthesis. Experimental evidence was consistent with this regulatory polypeptide being nsp89.

Finally, a number of experiments performed in this laboratory suggested that one of the structural polypeptides could also regulate the 42S to 26S RNA ratio. These results are summarised and further results are presented which indicate that this ability resides in the core protein. The data are assembled into a model which suggests a mechanism whereby the rate of synthesis of virion (42S) RNA and the structural proteins can be closely monitored and regulated such that they are produced in optimal amounts for the assembly of mature virus.

ABBREVIATIONS

BHK	baby hamster kidney
CEF	chicken embryo fibroblasts
c.p.e.	cytopathic effect
c.p.m.	counts per minute
DI	defective interfering
DMSO	dimethyl sulphoxide
DNA(s)	deoxyribonucleic acid(s)
DNAse	deoxyribonuclease
EEE	Eastern Equine Encephalitis
EMC	Encephalomyocarditis
GMEM	Glasgow modified Eagle's medium
HEPES	N - 2 - hydroxyethylpiperazine - N' - 2 - ethanesulphonic acid
JEV	Japanese Encephalitis Virus
k_i	first order inactivation constant for nucleic acids
ln	logarithm to base e
mRNA(s)	messenger ribonucleic acid(s)
mRNP(s)	messenger ribonucleoprotein(s)
NDS	naphthalene - 1,5 - disulphonic acid (disodium salt)
PFU	plaque forming unit
p.i.	post infection
PPO	2,5 - diphenyloxazole
RNA(s)	ribonucleic acid(s)
RNAse	ribonuclease
SDS	Sodium dodecyl sylphate
SFV	Semliki Forest virus
$t_{37\%}$	time required to reduce nucleic acid synthesis to 37% of its original value
TCA	trichloroacetic acid
UV	ultra-violet
VEE	Venezuelan Equine Encephalitis
VSV	Vesicular Stomatitis virus
WEE	Western Equine Encephalitis

INTRODUCTION

Distribution and Classification

Sindbis virus was originally isolated from a member of the Culex species of mosquito in Egypt by Taylor et al. and has since been found in Uganda, South Africa, India, Malaya, the Philipines and Northern Australia in such diverse species as mosquitoes, mites, wild birds and man. Except for a small number of cases (cited in Horsfall and Tamm) Sindbis virus rarely appears to produce any symptoms of infection in man although antibodies to Sindbis virus have been detected in a number of cases without any symptoms being apparent. For this reason both Sindbis and another closely related virus, Semliki Forest Virus (SFV), have been used extensively as model systems for biochemical investigations in preference to such related viruses as Eastern (EEE), Western (WEE), and Venezuelan (VEE) Equine Encephalitis viruses which, because of their pathogenicity, are more interesting clinically but more dangerous to work with in the laboratory. All these viruses are classified as alphaviruses; the type member, Sindbis virus, has the cryptogram R/1;4/5;Se/S; I, V/Ve/Di. Alphaviruses are also distantly related to a second group of viruses, the flaviviruses, by serological criteria. Members of this genus include Yellow Fever, Dengue, and Japanese Encephalitis viruses (Fenner). It would now appear that alphaviruses and flaviviruses are biochemically distinct (Westaway and Shew). These two groups were originally known as A and B arboviruses (arbo from arthropod borne) respectively because they were all originally isolated from mosquitoes, mites or ticks. In turn both groups are classified as part of the family Togaviridae because all the viruses are surrounded by a lipid membrane (toga is latin for cloak). On the other hand, there are a number of viruses which are classified as arboviruses or togaviruses which appear to have no resemblance to the alphaviruses at the biochemical level. For instance, Bunyamwera virus, originally isolated from a mosquito (Smithburn et al.), rubella virus (Holmes and Warburton) and lactic dehydrogenase virus possess lipid envelopes and yet they all differ from alphaviruses with regard to the biochemical aspects of their structure and growth. In order to avoid these inconsistencies Baltimore (1971) has suggested an

alternative classification scheme based on the genome structure of the various viruses. In this case alphaviruses are classified as members of Group IV, along with the picornaviruses, as they both have a single stranded messenger RNA as their genome.

Cell Susceptibility

As mentioned above the original isolate of Sindbis virus came from a mosquito (Taylor et al.) and hence it is not surprising to find that cell cultures derived from these insects support alphavirus growth well. The interesting observations to emerge from these experiments are that cultures of mosquito cells produce similar yields of Sindbis virus to those found in vertebrate cell lines but that the mosquito cells do not die. At the end of the growth cycle, virus production decreases dramatically but never ceases, the cultures become persistently infected and are resistant to super-infection by the same virus (Peleg; Stevens; Stollar and Shenk). This is in contrast to observations made in all other cell cultures where infection with Sindbis virus or SFV has resulted in the death of the cells. This has been shown in HEp-2 cells (Erlandson et al.), human lymphoblastoid cells (Hilfenhaus), chick embryo fibroblasts (CEF), Pekin duck embryo and kidney cells, rhesus monkey kidney cells (Henderson and Taylor), baby hamster kidney cells (BHK), Vero and rabbit kidney cells (Bergold and Mozzali). It has also been reported as being lethal to embryos in fertile eggs (Frothingham) and to suckling but not adult mice (Johnson) although the strain used in this work was avirulent for mice of all ages (N. Dimmock, personal communication). However, Sindbis virus does not give rise to any symptoms when inoculated into either monkeys or birds (Taylor et al.)

It is to be hoped that a good understanding of the growth of both Sindbis virus and SFV during a normal lytic infection of BHK or CEF cells will provide the necessary basic knowledge with which to answer more difficult questions, for instance, the mechanisms of persistence in the mosquito and virulence in the animal. In addition, these viruses will hopefully act as models for the other alphaviruses so reducing the amount of more dangerous work which would otherwise be necessary in order to characterise them.

Structure of the Sindbis Virus Particle

During the course of the introductions most references, where relevant, will be concerned with aspects of Sindbis virus replication. However, because of the high degree of similarity between Sindbis and SFV, it is invariably true that an observation made with one of these viruses will prove to be true for the other. For this reason, only occasional reference will be made to SFV either to confirm isolated observations made with Sindbis virus or to describe experiments which have not yet been repeated with Sindbis virus.

Examination of negatively stained thin sections of the Sindbis virus particle, using the electron microscope, indicate that the virus is spherical and consists of three different regions; a central region, the nucleocapsid, surrounded by a lipid bilayer which, in turn, appears to contain a number of spikes (Compans). Measurement of the particle under the electron microscope gives a value of 60-70nm for the diameter of the virus (Simpson and Hauser, 1968a; Compans; Brown et al.; Horzinek and Mussgay) and its S value has been calculated to be 237 (Horzinek and Mussgay).

The virus contains three structural proteins; the core protein and two envelope proteins, E1 and E2, all of which are present in equimolar amounts (Schlesinger et al., 1972). Two of these proteins, the envelope proteins, can be labelled using the iodine-lactoperoxidase system (Sefton et al.). Experiments with the closely related virus, SFV, have shown that the two equivalent proteins are also labelled when the virus is treated with [^{35}S] -formyl methionyl sulfone methyl phosphate (Gahmberg et al.). Both of these techniques are used to label external proteins, as the reagents are unable to penetrate the lipid membrane. For this reason it was concluded that the two envelope proteins are exposed on the surface of the virus. Similarly, these two proteins are degraded by treating either Sindbis virus (Compans) or SFV (Kennedy, 1974; Utermann and Simons) with proteolytic enzymes. This is again indicative of these two proteins being exposed on the surface while the third protein, which is not affected by any of these treatments, is assumed to be located inside the virus particle. This core protein is present as a complex with the genetic material

of the virus, single stranded RNA, and together they form the nucleocapsid which is the central portion of the virus as seen in the electron micrographs (Simpson and Hauser, 1968b). All three proteins appear to be phosphorylated to the small extent of 0.03 - 0.1 moles of phosphate per mole of each structural protein (Waite et al.). In addition, it has been suggested that there is some protein kinase activity associated with the virus but that this is present only at very low levels i.e. at approximately 1% of the corresponding vesicular stomatitis virus (VSV) controls (Tan and Sokol). The VSV protein kinase is thought to be of host cell origin (Imblum and Wagner; Moyer and Summers).

The RNA of the virus is found as a complex with the core protein (Yin and Lockart). It accounts for 6% of the total weight of the virus and 30% of the weight of the nucleocapsid (Laine et al.). The RNA has a sedimentation coefficient of 42S (Pfefferkorn et al.) in sucrose gradients and hence is usually referred to as 42S RNA. Its molecular weight has been estimated to be 4.2×10^6 by comparison with ribosomal RNAs on polyacrylamide gels and on sucrose gradients (Simmons and Strauss, 1972a) and has a base composition of 29.6% adenine, 25.8% guanine, 24.9% cytosine and 19.7% uracil (Pfefferkorn and Hunter, 1963a). In common with the majority of cellular and viral messenger RNAs it has a poly (A) tract at the 3' end (Johnston and Bose; Eaton and Faulkner, Eaton et al.). Visualization of these RNA molecules under the electron microscope has shown the presence of circular RNAs formed by hybridization of the 3' and 5' ends of the RNA forming what have been graphically described as pan handles (Hsu et al.). This would suggest that there is some complementarity between the sequences at the 3' and 5' ends. The 5' end of Sindbis virus RNA is capped and methylated in a similar manner to other viral and cellular messenger RNAs. This cap takes the form of a guanosine linked by its 5' hydroxyl group to the 5' terminal triphosphate of the viral RNA. It has been suggested that the terminal sequence of Sindbis virus 42S RNA is $m^7G^{5'}$ pppApUpG... (Hefti et al.). The purpose of these modifications at the 3' and 5' ends of the 42S RNA in particular and cellular messenger RNAs in general has not yet been established. However, in the case

of poliovirus, a group IV virus like Sindbis virus, the 5' terminal is not capped but is instead blocked by a small peptide (Lee et al.).

The RNA, when complexed with approximately 230 core proteins, gives rise to a nucleocapsid (Laine et al.) which has a diameter of 32 - 40 nm (Brown et al.; Horzinek and Mussgay; Simpson and Hauser, 1968b) and a sedimentation coefficient of 140S (Yin and Lockart). The core protein from SFV has a high proportion of hydrophilic amino acids and is rich in lysine (Kennedy, 1974; Simons and Kaariainen). The core protein in Sindbis virus has a molecular weight of 30,000 (Strauss et al. 1969). The arrangement of the RNA inside the nucleocapsids is not known, however, treatment of nucleocapsids from SFV with increasing concentrations of ribonuclease (RNAse) led to only slight changes in the size and morphology of the nucleocapsid even though the RNA had been extensively degraded (Kääriäinen and Söderlund); the pattern of degradation led the authors to propose that the RNA was regularly folded inside the nucleocapsid and that only those folds which protruded from the nucleocapsid were digested by the RNAse. The nucleocapsid appears to have icosahedral symmetry when viewed under the electron microscope (Horzinek and Mussgay).

The nucleocapsid is surrounded by a lipid bilayer (Compans) which is centred at a radius of 23nm from the middle of the virus particle (Harrison et al.). The membrane is acquired as the virus buds through the host cell membrane (Brown et al.; Pfefferkorn and Hunter 1963b) and is derived from previously synthesised lipid molecules and is not the result of de novo lipid synthesis (Pfefferkorn and Hunter, 1963b). David found that the lipid composition did not vary much between virus grown in CEF or BHK cells but that the composition of the lipids did not resemble that of the plasma membrane of either cell type. This led him to suggest that the lipid composition of the virus reflected the lipid affinities of the various virus structural proteins.

The two envelope proteins, E1 and E2 are embedded in the membrane and are the spikes seen in the electron microscope (Compans). These two polypeptides have molecular weights of 53,000 and 45,000 respectively and

are glycosylated (Schlesinger et al., 1972; Sefton and Keegstra). The carbohydrate accounts for approximately 8% of the weight of each of the proteins and consists mainly of mannose and glucosamine with smaller amounts of galactose, glucose, fucose and sialic acid (Sefton and Keegstra). The composition of the carbohydrate varies slightly depending on the cell type in which the virus was grown but this does not affect infectivity (Keegstra et al.; Burke and Keegstra). By comparison, the carbohydrates of Sindbis virus grown in A. albopictus cells are completely lacking the terminal sialic acid residues which are present in virus grown in vertebrate cells but the particle/PFU ratio did not differ significantly from virus grown in BHK or CEF cells (Stollar et al.). Removal of the envelope proteins by digestion with proteases causes clumping of the virus and reduces the infectivity (Compans) and so they are presumably present to render the virus water soluble by providing it with a hydrophilic surface. The glycosylation of envelope glycoproteins seems to be a common mechanism employed by a number of viruses, for instance herpesvirus (Keller et al.), vaccinia virus (Garon and Moss), Newcastle Disease Virus and Sendai virus (Mountcastle et al.).

Addition of non-ionic detergents to a suspension of Sindbis virus solubilises the lipid bilayer and at the same time removes the envelope glycoproteins indicating that they are buried to some extent in the lipid membrane. Digestion of the glycoproteins of SFV with thermolysin or subtilisin leaves small hydrophobic segments of the envelope proteins behind in the membrane. These peptides have a molecular weight of approximately 5,000 and it has been suggested that they act as anchors for the envelope proteins (Utermann and Simons). It has proved possible to cross link the envelope proteins to the core protein using dimethyl suberimidate which will cross link amino groups which are not more than 1.1nm apart. Since this corresponds to approximately $\frac{1}{4}$ of the width of the lipid membrane, the hydrophobic peptide must extend through $\frac{3}{4}$ of, and perhaps all of, the membran and then might act as a recognition site for the core protein when the virus matures by budding through the cell membrane (Garoff and Simons).

One of the envelope proteins, E2, is derived from a precursor, pE2, and this processing appears to be necessary before the virus can bud through the membrane (Jones et al., 1974). In the case of SFV the fragment cleaved off is also found in the virus as E3, a small peptide with a molecular weight of approximately 10,000 (Garoff et al.) but no such peptide has been found in the Sindbis virus particle.

The virus also has haemagglutinating activity associated with it (Mussgay and Rott). By solubilising the envelope glycoproteins with Triton X 100 and separating them by isoelectric focussing it was possible to assign the haemagglutinating activity to E1 (Dalrymple et al.).

The various aspects of Sindbis virus growth will be considered in the relevant sections that follow; virus entry, translation of 42S RNA and the alphavirus replicase will be reviewed in Section I, the various RNA species will be described in Section II while the late phases of the virus growth and maturation will be considered in Section IV.

SECTION I

CHARACTERISATION OF THE NONSTRUCTURAL POLYPEPTIDES SYNTHESISED IN CEF CELLS INFECTED WITH SINDBIS VIRUS

INTRODUCTION

The Primary Stages of Infection

(i) Entry of Virus into the Cell

Very little is known concerning the actual infection process, which could conceivably take place by two methods. Firstly, the virus might fuse with the host cell membrane leading to release of the nucleocapsid into the cell or secondly, the virus particle might be taken up by pinocytosis followed by fusion of the virus with the resulting vacuolar membrane or degradation of the structural proteins resulting in the release of the virion RNA into the cell cytoplasm. Available evidence to date is consistent with the fusion of the virus and cell membranes as being the preferred method of virus entry but confirmation will require further experimentation. Investigations of the interaction between the virus and synthetic lipid vesicles, liposomes, derived from sheep red blood cells, gave results which were consistent with membrane fusion being the method of attachment. The liposomes were protein free, which argued against a specific receptor being necessary for fusion, and the pH dependence for adsorption of virus to the protein free liposomes was very similar to that seen for agglutination of sheep red blood cells by virus. The fusion did, however, appear to be dependent on the lipid composition of the liposomes (Mooney et al). Similarly, examination of freeze etched cells under the electron microscope immediately after the cells had been infected with Sindbis virus suggested that the virus simply fused with the cell membrane, as virus was distributed fairly evenly over the cell surface. This study suggested that there were approximately 10^5 binding sites for Sindbis virus on each CEF cell (Birdwell and Strauss).

(ii) Translation of 42S RNA to Give Nonstructural Polypeptides

After entry of the virus into the cell, the next stage in the infectious cycle is the translation of 42S RNA to produce the replicase enzymes. Sindbis virus RNA is infectious (Scheele and Pfefferkorn, 1969b) and hence no virion bound polymerase is necessary as 42S RNA can obviously code for its own replicase. In addition Sindbis virus does not require any nuclear

functions as it will grow in the presence of actinomycin D (Ben-Ishai et al.), a drug which prevents RNA transcription from a DNA template, and also grows well in enucleate cells (Kos et al.). Interestingly, the same study showed that Japanese Encephalitis Virus (JEV), a flavivirus which is thought to be distantly related to Sindbis virus, required nuclear functions for the first four hours of infection. In this respect JEV resembles the orthomyxoviruses which require nuclear functions for the first two hours of their replication cycle (Barry; Rott et al.). Whether 42S RNA is translated after having been released from the nucleocapsid or the nucleocapsid is translated directly as a messenger ribonucleoprotein (mRNP) is not known, It has been suggested that specific proteins attached to HeLa cell mRNA cause the binding of mRNP to 40S ribosomal subunits under conditions in which the naked mRNA is not bound (Liautard; Lebleu et al.) and so it is conceivable that the core protein might act in a similar fashion.

The primary translation product of 42S RNA has not been observed in infected cells and it is unlikely that it ever will be because of very low levels of infecting virion RNA compared with the high levels of host cell mRNA undergoing translation, however the synthesis of the nonstructural polypeptides has been detected later in infection. It is important to point out at this stage some of what is known about the synthesis of the three structural polypeptides which are produced by translation of a polycistronic mRNA, the product of which is cleaved by proteolytic enzymes to give three discrete polypeptides. Inhibition of proteolytic enzymes using zinc ions (Bracha and Schlesinger, 1976a), or incubation of temperature sensitive mutant infected cells at the restrictive temperature (Scheele and Pfefferkorn, 1970; Schlesinger and Schlesinger, 1973) causes the accumulation of larger polypeptides containing the sequences of some or all of the structural polypeptides. In this respect the mechanism of alphavirus protein production resembles that of picornaviruses which also synthesise large precursors to their proteins which are subsequently cleaved (Jacobson et al.; Butterworth and Rueckert). It seemed likely, then, that the nonstructural polypeptides

of alphaviruses would also be synthesised in a similar manner and results to date appear to confirm this.

There have been two sets of reports of nonstructural polypeptides synthesised by SFV infected BHK or CEF cells. In one report two nonstructural polypeptides were detected in wild type SFV infected BHK cells (Clegg, Brzeski and Kennedy); these two polypeptides, nsp63 and nsp90, were shown by tryptic peptide mapping to be unrelated to each other and to the structural polypeptides (the number portion of the name of the polypeptide refers to its molecular weight in thousands). In addition, three larger polypeptides were found, p200 p184 and p150, which were assumed to be precursors to the nonstructural polypeptides on the basis of their instability under pulse-chase conditions and their accumulation in cells treated with inhibitors of proteolytic enzymes. The gene order was shown to be 5' - nsp63 - nsp90 - 3' by salt synchronisation of protein synthesis. Two polypeptides, with similar mobilities to nsp63 and nsp90, were present in an extensively purified preparation of enzymically active RNA dependent RNA polymerase obtained from cells infected with the same strain of SFV (Clewley and Kennedy). Hence, the assumption that at least some of the nonstructural polypeptides synthesised in SFV infected cells are also involved in RNA synthesis appears to be valid. Further evidence for this relationship rests on the susceptibility of RNA synthesis to the addition of inhibitors of protein synthesis. Compounds that inhibit protein synthesis or proteolytic enzymes result in a decrease in the amount of RNA synthesised, compared to untreated controls, if added early in the infectious cycle, when nonstructural polypeptide synthesis is at its maximum but have no effect later when structural protein synthesis predominates and the rate of nonstructural polypeptide synthesis is reduced (Clegg, Brzeski and Kennedy).

A second series of reports described the existence of a total of four nonstructural polypeptides in SFV infected secondary CEF cells. The four polypeptides were, in order from the N terminus, ns70 - ns86 - ns 78 - ns60. It was proposed that ns70 and ns86 were derived from a precursor, p155, while ns78 and ns 60 are derived from p 135. These two polypeptides are in turn thought to be derived from a , as yet undetected, 300,000

molecular weight precursor (Lachmi and Kääriäinen, 1976). Two of the nonstructural polypeptides, ns78 and ns86, have been tryptic peptide mapped and are apparently unrelated to each other or to the structural polypeptides, while p155 and p135 were designated as precursors on the basis of their instability under pulse-chase conditions (Lachmi et al.; Lachmi and Kääriäinen, 1976). Unfortunately the position is complicated somewhat by the use of a temperature sensitive mutant for the majority of this work and also by the tryptic peptide mapping technique, which is in essence one dimensional, and hence does not always provide adequate resolution.

In addition to these two reports, Kaluza has described a polypeptide which has a molecular weight of 78,000 in SFV infected CEF cells. Host cell protein synthesis had been inhibited by a combination of fowl plague virus infection and actinomycin D prior to super infection with SFV. This polypeptide was claimed to be unrelated to the structural proteins but no tryptic peptide maps were presented nor were any other nonstructural polypeptides detected.

Finally, the presence of large polypeptides has been reported in cells infected with RNA -ve temperature sensitive mutants of Sindbis virus; such mutants are assumed to have a defect in the nonstructural polypeptide(s) responsible for RNA synthesis. Waite reported the synthesis of polypeptides with molecular weights of 100,000 - 130,000 in cells infected with ts 11 while recently ts 24 has been shown to synthesise a 200,000 molecular weight polypeptide which did not contain any structural protein sequences (Bracha et al.). This polypeptide might well be analogous to the p200 found in SFV infected cells (Clegg, Brzeski and Kennedy).

The nonstructural proteins are assumed to be, in part at least, components of the polymerase. The location of this polymerase is almost certainly on membranes in both Sindbis virus (Sreevalsan and Yin; Ben-Ishai et al.) and SFV (Clewley and Kennedy; Friedman et al., 1972) infected cells. In the case of SFV the polymerase appears to be associated almost exclusively with membranes which have a density of 1.16 g/cm^3 (Clewley and Kennedy; Friedman et al., 1972). The use of very short labelling periods with RNA

precursors resulted in preferential labelling of these membranes, which was consistent with their being the site of RNA synthesis (Friedman et al., 1972) Examination of thin sections of SFV infected cells using the electron microscope showed the presence of essentially two different types of vacuole not seen in uninfected cells. One type, termed CPV-II, appeared to be involved in the maturation of virus while the second type, CPV-I, contained small spherules inside the vacuole (Grimley et al.). Autoradiography followed by electron microscopic observation of cells labelled for very short periods with $[^3H]$-uridine showed preferential incorporation of label into CPV-I (Grimley et al.). In addition, purification of the membrane fraction which contained polymerase activity, followed by examination of these membranes under the electron microscope showed the presence of CPV-I (Friedman et al., 1972). Hence it seems likely that these vacuoles are in some way connected with RNA synthesis, perhaps by providing the membranes upon which the polymerase enzymes are located. It is not known whether the polymerase is situated on the inside or the outside of these vacuoles. It may be directly associated with the spherules or alternatively the spherules may be a consequence of the presence of the polymerase on the outside of the vacuole.

The enzyme purified from SFV infected BHK cells was shown to contain two nonstructural polypeptides and was found to be membrane bound (Clewley and Kennedy). Unfortunately the activity was not dependent on added RNA and so it was proposed that the polymerase was simply completing previously initiated RNA chains.

It is assumed that the nonstructural proteins are translated from 42S RNA and to support this suggestion it has been found that 42S RNA is associated with polysomes in cells infected with both Sindbis virus (Mowshowitz; Martire et al.) and SFV (Kennedy, 1972). It has recently been suggested (Martire et al.) that the majority of 42S RNA is translated on soluble polysomes. However there are two factors that make such a conclusion unreliable. Firstly, the experiments were performed late in the infectious cycle when very few nonstructural polypeptides were being

synthesised and secondly, the soluble polysome fraction also contained nucleocapsids which co-sedimented in the polysome region of the sucrose gradient thus making it difficult to estimate the amount of 42S RNA associated with polysomes and that which was in nucleocapsids. The estimated percentage of total RNA synthesis which was found in nucleocapsids was given as 40% but the RNA ratio was not given. However, the reported 42S to 26S RNA ratio for Sindbis wild type virus infected cells is never greater than one and so the proportion of total label incorporated into 42S RNA never exceeds 50%. Therefore, in the work reported by Martire et al. the actual amount of labelled 42S RNA on polysomes would have been just 10% of the total incorporation. Some of this labelled 42S RNA appeared to be associated with membranes but the remainder, perhaps 5% of the total incorporation, would not have been detectable in the soluble fraction against the high background of nucleocapsids and hence their interpretation that the majority of 42S RNA was associated with soluble polysomes should be viewed with some caution.

Another approach that might be used to define the nonstructural polypeptides coded for by 42S RNA, namely cell free translation, has met with limited success. In experiments using either SFV (Smith et al.; Glanville et al.) or Sindbis virus 42S RNA (Cancedda et al., 1974) no discrete polypeptides were obtained when the RNA was translated in various cell free systems. In addition tryptic peptide mapping of the products showed that structural polypeptides were being produced even though the sequences coding for the strucural proteins are at the 3' end of the 42S RNA. In one case, using Sindbis virion RNA, discrete polypeptides were seen which were different from those produced by translation of the structural protein messenger RNA (Simmons and Strauss, 1974b), unfortunately they were not tryptic peptide mapped and so it is not possible to state with certainty that they were in fact nonstructural. It has been suggested that this inability to translate 42S RNA correctly is due to the synthesis of hydrophobic peptides in an aqueous environment (Smith et al.). Similar observations have been made for the envelope proteins of SFV (Smith et al.;

synthesised and secondly, the soluble polysome fraction also contained nucleocapsids which co-sedimented in the polysome region of the sucrose gradient thus making it difficult to estimate the amount of 42S RNA associated with polysomes and that which was in nucleocapsids. The estimated percentage of total RNA synthesis which was found in nucleocapsids was given as 40% but the RNA ratio was not given. However, the reported 42S to 26S RNA ratio for Sindbis wild type virus infected cells is never greater than one and so the proportion of total label incorporated into 42S RNA never exceeds 50%. Therefore, in the work reported by Martire et al. the actual amount of labelled 42S RNA on polysomes would have been just 10% of the total incorporation. Some of this labelled 42S RNA appeared to be associated with membranes but the remainder, perhaps 5% of the total incorporation, would not have been detectable in the soluble fraction against the high background of nucleocapsids and hence their interpretation that the majority of 42S RNA was associated with soluble polysomes should be viewed with some caution.

Another approach that might be used to define the nonstructural polypeptides coded for by 42S RNA, namely cell free translation, has met with limited success. In experiments using either SFV (Smith et al.; Glanville et al.) or Sindbis virus 42S RNA (Cancedda et al., 1974) no discrete polypeptides were obtained when the RNA was translated in various cell free systems. In addition tryptic peptide mapping of the products showed that structural polypeptides were being produced even though the sequences coding for the strucural proteins are at the 3' end of the 42S RNA. In one case, using Sindbis virion RNA, discrete polypeptides were seen which were different from those produced by translation of the structural protein messenger RNA (Simmons and Strauss, 1974b), unfortunately they were not tryptic peptide mapped and so it is not possible to state with certainty that they were in fact nonstructural. It has been suggested that this inability to translate 42S RNA correctly is due to the synthesis of hydrophobic peptides in an aqueous environment (Smith et al.). Similar observations have been made for the envelope proteins of SFV (Smith et al.;

Glanville et al.) and Sindbis virus (Cancedda et al., 1974) and the glycoproteins of VSV (Morrison et al.; Grubman and Summers).

It was considered that investigation of the nonstructural polypeptides of Sindbis virus was desirable for two reasons. Firstly, the two contradicting reports of the number of nonstructural polypeptides in SFV infected cells suggested that something might be amiss with the strain of SFV used in this laboratory and so the AR 339 strain of Sindbis virus was used as a comparison by characterising the synthesis of the nonstructural polypeptides in more detail than has been carried out to date with SFV. Secondly, a number of temperature sensitive mutants of the same strain of Sindbis virus had already been isolated (Atkins et al.) and it was hoped to use those mutants with lesions in their RNA synthesising machinery to investigate the manner in which the various RNAs were synthesised. After characterisation of the nonstructural polypeptides it was hoped to assign specific functions to the various polypeptides. For these reasons experiments were devised to find and characterise the nonstructural polypeptides and the manner in which they were synthesised. The results of these experiments are presented in the following pages.

RESULTS

(i) Detection of Sindbis Virus Nonstructural Polypeptides

In order to detect the nonstructural polypeptides it is not sufficient just to label those polypeptides synthesised early in the infectious cycle as host cell protein synthesis masks the small amount of nonstructural polypeptides made. Conversely, at later times in the infectious cycle, when host cell protein synthesis has been largely inhibited, nonstructural polypeptide synthesis is also greatly reduced (Clegg et al.). Therefore, to detect the Sindbis virus specified nonstructural polypeptides, it was necessary to investigate a number of parameters that might affect their production.

Comparison of the polypeptide profiles of Sindbis virus infected BHK and CEF cells showed that host cell polypeptide synthesis in CEF cells could routinely be reduced to 10% of the level of uninfected controls by eight hours post infection whereas, in BHK cells, very little inhibition of host cell protein synthesis could be detected. The inability of Sindbis virus to inhibit BHK cell polypeptide synthesis was not due to inefficient virus replication in BHK cells because RNA synthesis, as measured by incorporation of $[^3H]$-uridine into TCA insoluble c.p.m., was found to be similar in both BHK and CEF cells (Table 1). A similar observation has been made concerning the difference in response of the primary transcription of VSV when grown in BHK or CEF cells (Flamand and Bishop). Therefore, in order to facilitate the detection of the nonstructural polypeptides of Sindbis virus against a background of host cell polypeptides, experiments were carried out in CEF cells

To ensure the provision of a large number of messenger RNAs early in the growth cycle high multiplicities of infection, 100 PFU/cell, were routinely used.

Using the above mentioned conditions the polypeptide profiles of infected and uninfected cells were compared at various times after infection in order to determine the optimal time for detection of the nonstructural polypeptides. This was judged to be at 4 hours post infection and the polypeptide profiles of infected and mock infected cells, labelled for 30

	$[^3H]$ -uridine incorporation (c.p.m.)	
Cell type	Mock infected cells	Virus infected cells
BHK	5571	73235
CEF	3618	58710

Table 1. Incorporation of $[^3H]$ -uridine into TCA insoluble products by BHK or CEF cells infected with wild type Sindbis virus. Duplicate cultures of BHK or CEF cells were infected with wild type virus or mock infected for 1h at 4°C. The virus was then replaced with pre-warmed 199 ADH for 3h after which time the cells were labelled with HEDA containing $[^3H]$ -uridine for 2.5 h. The amount of label incorporated into TCA insoluble products was then determined.

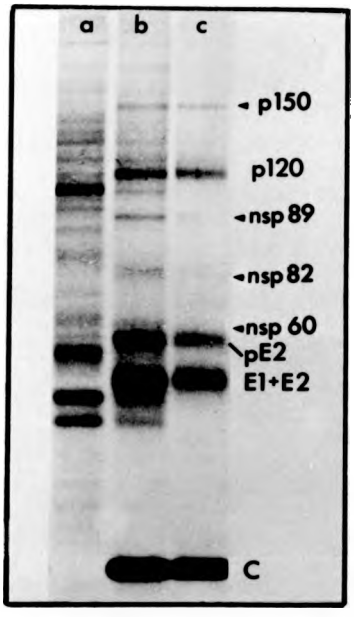

Fig. 1. Polypeptides synthesised by Sindbis virus infected CEF cells during early and late phases of the growth cycle. Infected (b and c), and mock infected (a) CEF cells were incubated in GMEM - met. At 4 (a and b) or 8(c) hours post infection the medium was replaced with labelling medium for 30 min. after which time the monolayers were extracted and the polypeptides prepared for polyacrylamide gel electrophoresis. Electrophoresis is from top to bottom on this and all subsequent electrophoretograms.

minutes with [^{35}S]-methionine at this time are shown in Fig. 1. together with the polypeptide profile of cells labelled 8 hours post infection when structural protein synthesis predominates. Comparison of these three profiles shows a number of virus specified polypeptides synthesised at 4 hours post infection (lane b) which are not seen in mock infected cells (lane a). Some of these polypeptides (C, E1, E2, pE2 and p120) co-migrate with the structural polypeptides or their precursors seen late in the infectious cycle (lane c) while four of these polypeptides, termed p150, nsp89, nsp82 and nsp60, are not seen at all in mock infected cells and only in reduced amounts later in infection and so might possibly be regarded as nonstructural polypeptides.

(ii) The Effect of Pulse and Chase Conditions on the Appearance of Sindbis Virus Nonstructural Polypeptides

The combined molecular weight of the four putative nonstructural polypeptides detected so far totalled approximately 380,000. However, the total expected coding capacity of the nonstructural region of 42S RNA was of the order of 210,000 - 240, 000 (see Discussion). This then suggested that either some of these polypeptides were precursors of others or that there was a second initiation site on the 42S RNA which could give rise to another distinct set of polypeptides as has been found in ØX174 (Barrell et al.). The structural polypeptides of Sindbis virus have been shown to be produced by processing of a larger precursor (Cancedda et al., 1974; Simons and Strauss, 1974b) and evidence suggesting that a similar scheme operates for the nonstructural polypeptides of SFV has also been published (Clegg, Brzeski and Kennedy; Lachmi et al. 1975; Lachmi and Kääriäinen, 1976).

In order to investigate whether this was also true for Sindbis virus, the stability of the various polypeptides seen in Fig. 1 was examined under short pulse and pulse - chase conditions. In such an experiment it would be expected that only the precursors would be labelled during a short pulse. After a period of chase, however, when the pool of radioactive methionine is diluted by excess unlabelled methionine further incorporation essentially stops and the fate, if any, of the various polypeptides synthesised during the pulse can be determined (Fig. 2). Comparison of the polypeptides labelled

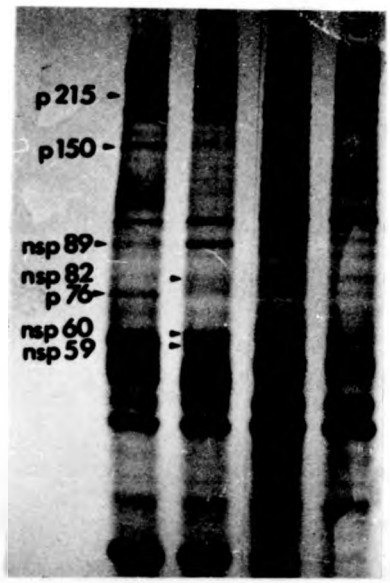

Fig. 2. Polypeptides produced by Sindbis virus infected CEF cells under pulse and pulse-chase conditions. Infected (a and b) and mock infected (c and d) CEF cells were incubated in GMEM -met for 4h after which time the medium was replaced with labelling medium for 10min. At the end of this period one of each culture (a and c) were harvested immediately while the remaining two (b and d) received chase medium for a further hour. The polypeptides were then prepared for polyacrylamide gel electrophoresis.

with [^{35}S] -methionine in infected cells during a 10 minute pulse (lane a) with those present after a chase (lane b) and those present in mock infected cells (lanes c and d) shows the presence of two previously unidentified polypeptides, p215 and p76, together with p 150 and a small amount of nsp 89. After a one hour chase (lane b), however, the intensity of the bands corresponding to p215, p150 and p76 decreases while the bands corresponding to nsp89, nsp82 and nsp60 increase in intensity. A further novel polypeptide can also be seen in lane b. It has an electrophoretic mobility slightly greater than that of pE2 and has been termed nsp59. The instability of p215, p150 and p76 on chasing suggests that they are intermediates or precursors of the stable end products nsp89, nsp82 and nsp60. It is also possible, however, that p215, p150 and p76 are in fact "dead end" products produced by aberrant mechanisms inside the cell and that their disappearance on chasing is simply due to degradation.

(iii) Processing of High Molecular Weight Precursors in the Presence of Zinc Ions

Zinc ions have been shown to inhibit the cleavage of the structural proteins involved in the maturation of picornaviruses (Korant et al; Butterworth and Korant). Zinc ions also prevent the processing of the envelope precursor in Sindbis virus infected cells (Bracha and Schlesinger, 1976a) and the processing of the nonstructural polypeptides in SFV infected cells (Clegg, Brzeski and Kennedy). In the latter case, addition of zinc ions later in infection did not affect RNA synthesis but when added earlier, its effects paralleled those of cycloheximide suggesting that, in SFV infected cells, zinc ions in some way affect the synthesis of functional polymerase.

The effect of various concentrations of zinc ions on the polypeptide profile of Sindbis virus infected cells can be seen in Fig. 3. Increasing the concentration of zinc ions from 0.001 mM (lane a) to 0.1 mM (lane d) caused a progressive decrease in the amounts of label incorporated into p150, nsp89, p76 and nsp60 while there was a corresponding increase in the amount of label incorporated into p215 and a new polypeptide p230. This is further strong evidence for the idea that the nonstructural polypeptides are translated as a

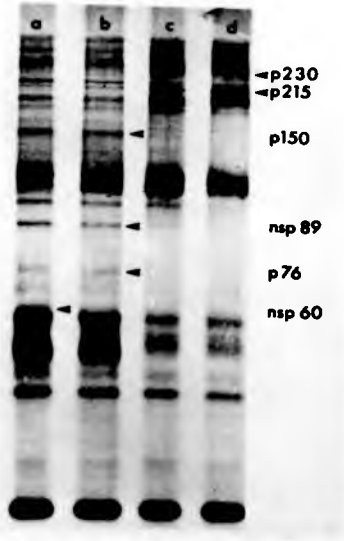

Fig. 3. Polypeptides synthesised by Sindbis virus infected CEF cells treated with zinc ions. Replicate cultures of CEF cells infected with Sindbis virus were incubated in GMEM - met for 3.5h and then the medium was replaced with HEDA containing (a) 0.001mM, (b) 0.01mM, (c) 0.05mM and (d) 0.1mM zinc ions for 30 mins. This was then replaced with labelling medium containing the appropriate concentration of zinc ions for a further 30 mins. after which time the polypeptides were processed for electrophoresis.

large polyprotein which is subsequently cleaved proteolytically to provide the nonstructural polypeptides. The size of p230 is such that it could represent the entire translation product of the nonstructural region of the 42S RNA. The polypeptide profile of zinc treated mock infected cells did not differ appreciably from untreated cells (results not shown).

(iv) Determination of the Molecular Weights of the Nonstructural Polypeptides and Their Precursors

The molecular weights of the polypeptides were determined as described in Materials and Methods (Section V, iv) and the calibration curves are shown in Fig. 4. The discontinuous gel system of Laemmli was found to be unsuitable for molecular weight determinations because a biphasic or triphasic curve was always obtained when the $\log_{10}$ (molecular weight) of the standards was plotted against the distance they had moved (results not shown); for this reason the continuous gel system of Fairbanks et al. was used. Even under these conditions, however, it was noticed that the linear relationship between $\log_{10}$ (molecular weight) and distance moved did not hold over a large range of molecular weights when a 7.5% polyacrylamide gel was used. A similar observation has been made by Abraham and Cooper (1975) when they attempted to measure the molecular weights of polypeptides synthesised by poliovirus infected cells. For this reason polypeptides whose molecular weights were less than 100,000 were electrophoresed on 7.5% gels in order to determine their molecular weights while those larger than 100,000 were determined on 3.5% polyacrylamide gels. Estimation of molecular weights using polyacrylamide gels necessarily involves a number of assumptions but by far the largest error involved is in the assumed value for the molecular weights of the standards which, except for those whose primary sequence is known, are derived from sedimentation data or electrophoretic experiments. The molecular weights obtained from these latter experiments may be affected by the shape of the protein or any residual tertiary structure remaining after treatment with SDS and so the molecular weight determined experimentally may vary by an unknown amount from the true value. These variations become more pronounced with the increasing molecular weight of the polypeptide and so,

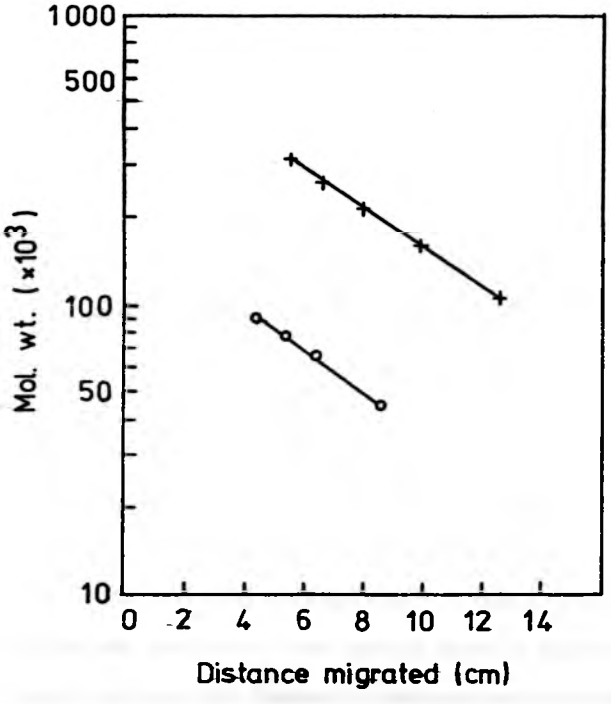

Fig. 4. Calibration curves for determination of the molecular weights of the nonstructural polypeptides and their precursors. The calibration curve for polypeptides with a molecular weight greater then 100,000 was determined by electrophoresing a commercial preparation of oligomers on a 3.5% polyacrylamide gel (+ ——— +). Those with molecular weights of less than 100,000 were calibrated on a 7.5% polyacrylamide gel using commercial preparations of proteins purchased from Sigma (o ——— o), See Section (V,iv).

in order to circumvent some of these objections, a commercial preparation of molecular weight markers was purchased from BDH. These markers consisted of oligomers of a protein with a molecular weight of 53,000 and provided a series of markers from the monomer to the hexamer (molecular weight 318,000) to act as standards on the 3.5% polyacrylamide gel. The graph shown in Fig 4. illustrates the good relationship exhibited between the mobility of the markers and the logarithm of their molecular weight. On the basis of their behaviour under pulse and pulse-chase conditions the polypeptides were prefixed by p, signifying precursor, or nsp, standing for nonstructural polypeptide, followed by the molecular weight of the polypeptide in thousands. The newly detected polypeptides are summarised in Table 2 along with the molecular weights as determined above.

(v) Tryptic Peptide Mapping of the Nonstructural Polypeptides

In order to define the various relationships between the structural polypeptides, the stable putative nonstructural polypeptides and their proposed precursors, the polypeptides were compared using the technique of tryptic peptide mapping, which allowed an indirect comparison of the primary sequence of the various polypeptides. Trypsin digestion results in cleavage of polypeptide chains at those points where a lysine or arginine occurs and this generates peptide fragments characteristic of the polypeptide from which they were derived. Separation of these peptides by two dimensional chromatography followed by autoradiography leads to a pattern of spots, or tryptic peptide map, which is characteristic of the peptides present in the original digest and hence is a reflection of the primary sequence of the polypeptide.

Briefly the experimental procedure was as follows: infected cells were labelled as in Figs. 1, 2 and 3 except that higher concentrations of [^{35}S] methionine were used (200-400 μCi/ml). The polypeptides were separated by polyacrylamide gel electrophoresis and the bands corresponding to the relevant polypeptides were excised and digested with trypsin. The isolated peptides were performic acid oxidised and then separated by two dimensional chromatography. The results of this experiment are shown in Figs. 5, 6 and 7.

Fig. 5 shows the tryptic peptide maps of the structural polypeptides

Designation of polypeptide	Molecular weight
p230	230,000
p215	215,000
p150	150,000
nsp89	89,000
nsp82	82,000
p76	76,000
nsp60	60,000
nsp59	59,000

Table 2. Molecular weights of the nonstructural polypeptides and their precursors.

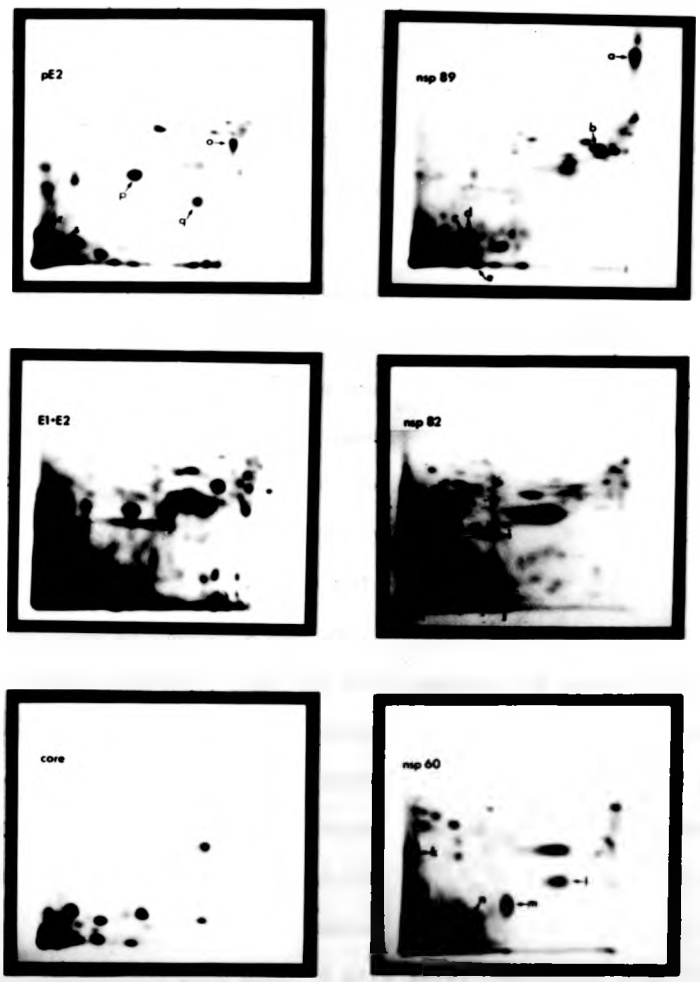

Fig. 5. Tryptic peptide maps of the structural and nonstructural polypeptides. The polypeptides were prepared under identical conditions to those described in the legend to Fig. 2. except that higher concentrations of $[^{35}S]$-methionine were used. The polypeptides were separated by polyacrylamide gel electrophoresis trypsin digested, performic acid oxidised and the resulting peptides separated by two dimensional chromatography as described in Section V, vi. First dimension separation is from left to right, the second is from bottom to top in these and all subsequent separations.

together with nsp89, nsp82 and nsp60. A comparision of the maps shows that nsp89, nsp82 and nsp60 do not show any similarities to any of the structural polypeptides or to each other and hence their primary amino acid sequences differ. It is, therefore, highly probable that nsp89, nsp82 and nsp60 are indeed nonstructural. Fig. 6 shows the tryptic peptide maps of nsp59, pE2 and p76 and nsp82.. Comparision of these maps clearly shows that nsp59 and pE2 are related (spots o, p, q, r and s) as are p76 and nsp82 (spots f, g, h, i and j). It is therefore apparent that nsp59 is a stable portion of pE2 and is not, therefore, nonstructural whereas p76 is closely related to nsp82. The nature of the apparent change in molecular weight of p76, which may be due to some post-translational modification, will be discussed later. The typtic peptide maps of nsp89, nsp82 and nsp60 along with their proposed precursors are shown in Fig. 7. A number of conclusions can be drawn from these maps: firstly, the map of p150 contains the spots characteristic of both nsp89 and nsp60 (spots a, b, c, d, e, k, l, m and n) but lacks those of nsp82. p 150 can, therefore be regarded as a precursor to nsp89 and nsp60; secondly, p230 and p215 contain the spots characteristic of nsp60, nsp82 and nsp89 and so can reasonably be regarded as precursors of all three nonstructural polypeptides; thirdly, the maps of p230 and p215 are very similar except that p230 contains a prominent peptide, labelled x, which although absent from p215, is also present in p150. This peptide x might be characteristic of the fragment of approximately 15,000 molecular weight which is removed from p230 to give p215. The data from the peptide maps allows the various polypeptides to be positioned relatively in p230 as follows; removal of nsp82 from p230 gives rise to p150, while removal of the polypeptide containing fragment x from p230 generates p215, It is therefore possible to deduce that the small 15,000 molecular weight polypeptide and nsp82 are situated at opposite ends of p230 and that, therefore, nsp89 and nsp60 occupy a central position between them. An independent method by which these polypeptides can be aligned is described in the next section.

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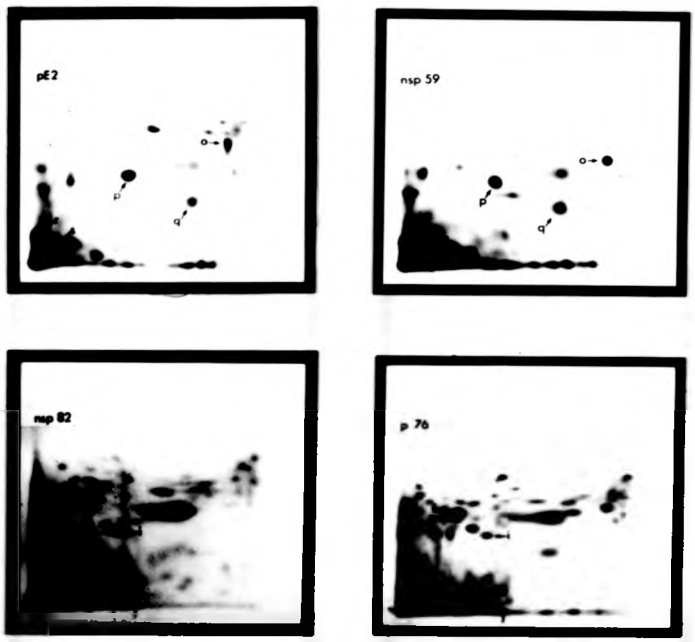


Fig. 6. Tryptic peptide maps of nsp59, p76, pE2 and nsp82. The polypeptides were labelled under the same conditions as described in the legend to Fig.2 except that higher concentrations of $[^{35}S]$ -methionine were used. The two unstable polypeptides pE2 and p76 were labelled under pulse conditions while the stable polypeptides nsp82 and nsp59 were labelled under chase conditions.

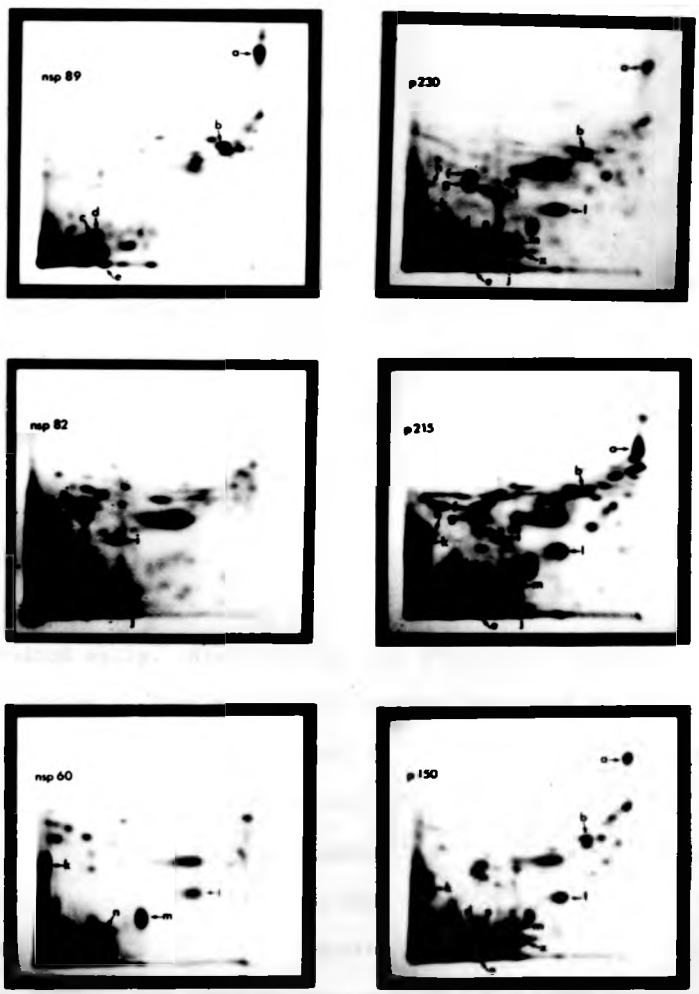


Fig. 7. Tryptic peptide maps of the nonstructural polypeptides and their proposed precursors. The stable and unstable polypeptides were labelled as described in the legend to Fig. 6. with the exception of p215 and p230 which were labelled as described in the legend to Fig. 3. In both cases higher concentrations of label were used.

(vi) Ordering of Genes Coding for the Nonstructural Polypeptides

The genes coding for the nonstructural polypeptides were ordered using the hypertonic salt technique originally described by Saborio et al. who used it to order the genes in poliovirus RNA. Addition of medium, made hyptertonic with NaCl, to infected cells in culture causes inhibition of protein synthesis by virtue of its inhibitory effect on the initiation of protein synthesis. The hypertonic salt does not inhibit elongation of the nascent polypeptides which continues normally until all ribosomes have "run off" and protein synthesis has ceased. Restoration of the isotonicity of the medium, by replacing the hypertonic medium with isotonic medium, results in synchronous initiation of protein synthesis. In the case of SFV structural proteins, where the polypeptides are synthesied from a polycistronic mRNA, sequential translation takes place (Clegg). Firstly, the conditions used by Clegg (1975) were verified for Sindbis virus infected CEF cells when it was found that increasing the concentration of NaCl in Earle's salts solution by 225 mM was sufficient to reduce the protein synthesis of infected cells to less than 1% of control untreated cells. Similarly, it was found that infected cell protein synthesis was reduced to less than 1% of control untreated cells 15 minutes after addition of the hypertonic salt (results not shown) and so the experimental conditions used were essentially those described by Clegg (1975).

Protein synthesis in Sindbis virus infected cells was inhibited by incubating them in hypertonic medium for 40 minutes. The cells were then allowed to initiate protein synthesis synchronously by replacing the hypertonic medium with isotonic medium containing $[^{35}S]$-methionine and labelling for increasing intervals of time. This was followed by a chase period to allow stable end products to accumulate (Fig. 8). The first nonstructural polypeptide to appear labelled was nsp60 (lane b) after 4 minutes in isotonic medium, the next labelled nonstructural polypeptide was nsp89 (lane c) and finally nsp82 appeared (lane d). It can be seen, then that increasing the time for which the cells are in labelled medium leads to sequential labelling of the polypeptides, with those polypeptides further from the point of initiation of translation (the 5' end of the RNA) requiring longer periods

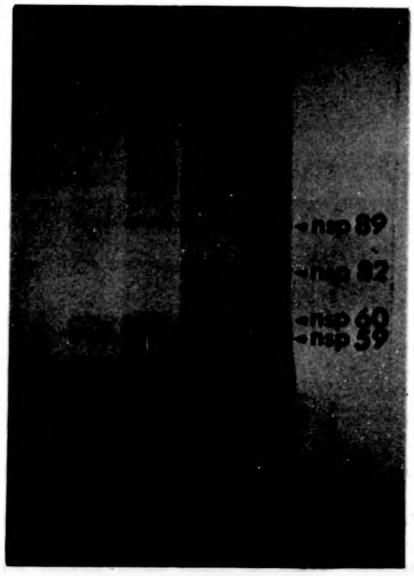


Fig. 8. Synthesis of polypeptides in Sindbis virus infected CEF cells under short pulse-chase conditions after synchronous initiation of protein synthesis. Replicate cultures of infected CEF cells were treated with HEDA containing an extra 225mM NaCl at 4h post infection for 40 mins. The hypertonic medium was then replaced with isotonic labelling medium for (a) 2 min, (b) 4 min, (c) 6 min, (d) 8 min and (e) 10 min. At the end of the labelling period the medium was replaced with chase medium for one hour and the cells were then prepared for polyacrylamide gel electrophoresis. Approximately equal amounts of extract (30 µg) were analysed.

time in isotonic medium before they were synthesised and hence labelled. This experiment, therefore allows the gene order to be defined as 5'-nsp60 - nsp89 - nsp82 - 3'. This is in complete agreement with the relative order of the polypeptides in p230 as determined by tryptic peptide mapping and extends it further, as it is now possible to define the order from the N - terminus. It is also possible to place the small unidentified polypeptide (x) at the N terminus, by default, because both nsp82 and polypeptide x are terminal (by tryptic peptide mapping) and nsp82 occupies the C terminal (by salt synchronisation). Lachmi and Kääriäinen have recently reported the existence of a fourth nonstructural polypeptide in SFV infected CEF cells. It was considered that the hypertonic salt technique would prove advantageous to investigate the possibility of the existence of a fourth polypeptide in Sindbis virus infected cells for two reasons; firstly, the fourth polypeptide must be synthesised after nsp82 because all three products of p230 have been accounted for and have been synthesised in the first ten minutes after release from hypertonic salt inhibition, secondly, it was consistently noted that recovery of viral protein synthesis was considerably more rapid than host protein synthesis in infected cells therefore the background would be reduced and so facilitate the detection of any new polypeptides. The results of such an experiment, where labelling was continued until twenty two minutes after release of hypertonic salt inhibition are shown in Fig.9. It can be seen that no new polypeptide was detected, even twenty two minutes after release of inhibition; conditions under which the majority of p230 is produced in approximately ten minutes.

(vii) Order of Cleavage of Precursors

The results obtained in Fig. 2 suggested that the cleavage of the various precursors might follow a rigid sequence because although p76 was detectable after a ten minute pulse, the cleavage of p 150 to give nsp60 and nsp89 seemed to take longer. To investigate whether the processing of p230 must follow a rigid sequence or whether it can be cleaved nascently the hypertonic salt inhibition technique was used in an experiment slightly

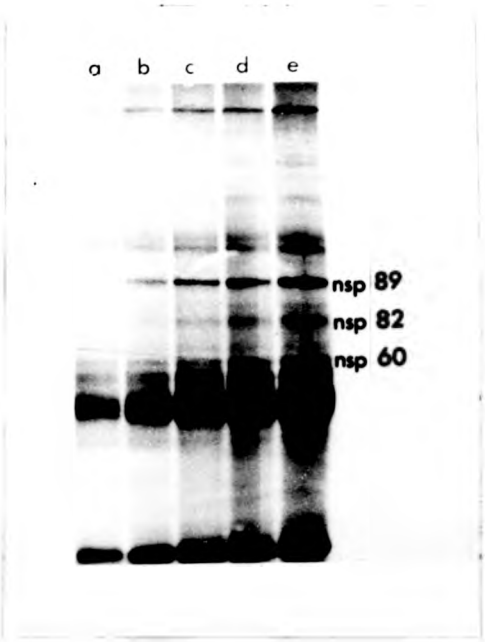
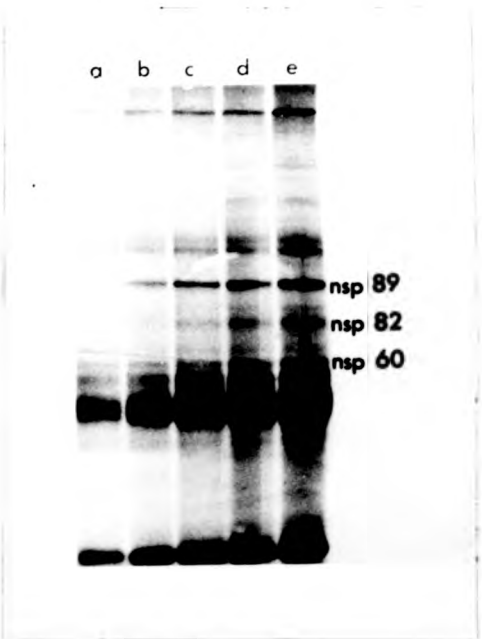


Fig. 9. Synthesis of virus specified polypeptides under long pulse-chase conditions after synchronous initiation of protein synthesis. Replicate cultures of virus infected cells were incubated in GMEM - met for 4h after which time the medium was replaced with HEDA containing excess 225mM NaCl for 40 min. The hypertonic medium was then replaced with isotonic labelling medium for (a) 6 min., (b) 10 min., (c) 14 min.,(d) 18 min. and (e) 22 min. and then replaced with chase medium for a further hour. The cells were then prepared for polyacrylamide gel electrophoresis. Approximately equal amounts of each sample (30ug) were analysed.



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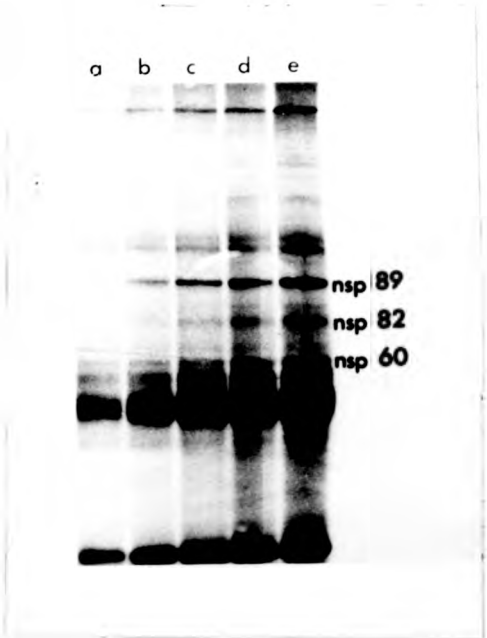


Fig. 9. Synthesis of virus specified polypeptides under long pulse-chase conditions after synchronous initiation of protein synthesis. Replicate cultures of virus infected cells were incubated in GMEM - met for 4h after which time the medium was replaced with HEDA containing excess 225mM NaCl for 40 min. The hypertonic medium was then replaced with isotonic labelling medium for (a) 6 min., (b) 10 min., (c) 14 min.,(d) 18 min. and (e) 22 min. and then replaced with chase medium for a further hour. The cells were then prepared for polyacrylamide gel electrophoresis. Approximately equal amounts of each sample (30ug) were analysed.

modified from that shown in Fig. 8. The infected cells were labelled with [^{35}S] -methionine for increasing lengths of time as above except that, in this case, the samples were processed for polyacrylamide gel electrophoresis immediately after the end of the pulse. The results of this experiment are shown in Fig. 10. The result shows that no stable nonstructural polypeptides have been produced after twelve minutes incubation in medium containing isotonic salt even though all the nonstructural polypeptides have been translated by this time (Fig. 8). This should be contrasted with the structural polypeptides where the core protein is cleaved from the nascent polypeptide chain (Clegg) and can be seen in Fig. 10, four minutes after release of the hypertonic salt inhibition (lane b). However, ten minutes after release of the block p150 and p215 can be seen (lane e) and are more prominent after twelve minutes labelling (lane f). The fact that no stable nonstructural polypeptides can be seen after twelve minutes labelling, even though this is sufficient time for their synthesis, together with the simultaneous appearance of p150 and p215 at such a time when synthesis of p230 would have been completed (nsp82 has been synthesised by ten minutes; Fig. 8) strongly suggests that p230 must be synthesised in its entirety before processing can occur and that the first stage in processing is the removal of the C terminal polypeptide as p76. The other product, p150, appears to be cleaved more slowly.

(viii) Do any of the Nonstructural Polypeptides Undergo Post-translational Modification?

The apparent change in molecular weight of p76 to 82,000 could involve post-translational modification. This modification was considered most likely to involve glycosylation considering the dramatic apparent change in molecular weight but phosphorylation was also examined to see whether any other of the nonstructural polypeptides were also being modified in this way.

Sindbis virus infected and mock infected cells were labelled with [^{3}H]-methionine or [^{3}H]-glucosamine at four hours post infection. At the end of the labelling period the cultures were processed and the polypeptides

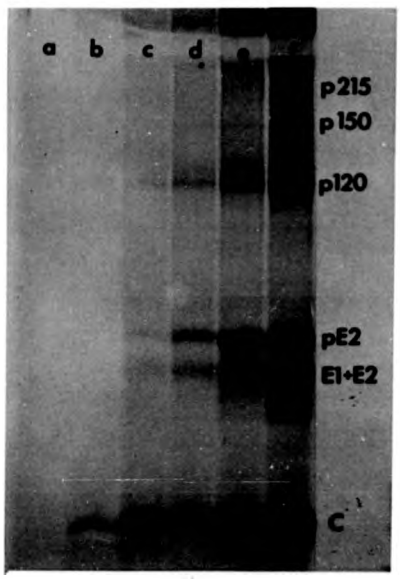


Fig. 10. Synthesis of polypeptides in Sindbis virus infected CEF cells under short pulse conditions after synchronous initiation of protein synthesis. Replicate cultures of infected CEF cells were incubated in GMEM - met for 4 hours after which time the medium was replaced with HEDA containing an extra 225mM NaCl for 40 min. The hypertonic medium was then replaced with isotonic labelling medium for (a) 2 min., (b) 4 min., (c) 6 min., (d) 8 min., (e) 10 min and (f) 12 min. At the end of the labelling period the polypeptides were prepared for polyacrylamide gel electrophoresis.

electrophoresed on polyacrylamide gels. In order to detect the [^{3}H] labelled polypeptides the polyacrylamide gel was then processed for fluorography as originally described by Bonner and Laskey. Briefly, this involves removal of water from the gel by successive washings of the gel in DMSO, followed by impregnation with a solution of PPO in DMSO. The PPO was then precipitated in situ by washing with water and the dried gel exposed to pre-flashed film at -70°C (Laskey and Mills). The process is described in detail in Materials and Methods (Section V,v). Polypeptides labelled with [^{3}H] -methionine could be clearly seen after four days exposure but no bands could be detected in those lanes containing samples which had been labelled with [^{3}H]-glucosamine, even after an exposure of fourteen days (results not shown). The experiment was repeated with an increased labelling time and a higher concentration of the isotope with no more success. It is thought that the experiment failed due to a combination of poor incorporation of [^{3}H]-glucosamine and a relatively inefficient method of detection as it was not possible to detect the envelope polypeptides which are glycosylated (Schlesinger et al., 1972; Schlesinger et al., 1976; Duda and Schlesinger). The glycosylation of nsp82 is, therefore, an open question.

The nonstructural polypeptides were also examined to see if any of them could be labelled with [^{32}P]-orthophosphate. It had been previously reported that purified Sindbis virus contained structural polypeptides all of which had been phosphorylated to a small extent (3-10 mole%) (Waite et al.). The labelled polypeptides were processed for gel electrophoresis by a modification of the technique described by O'Farrell (Section V, ii). Briefly, this involved removal of the cells from the monolayer followed by disruption by sonication. Any [^{32}P] labelled nucleic acids which might give rise to a high background in some parts of the gel were digested by a combination of nucleases and the resulting polypeptide mixture was processed for electrophoresis as usual. The results in Fig. 11 show very little difference between the mock infected and infected cells labelled with [^{32}P] -orthophosphate (lanes c and d) and so it was concluded that nsp89,

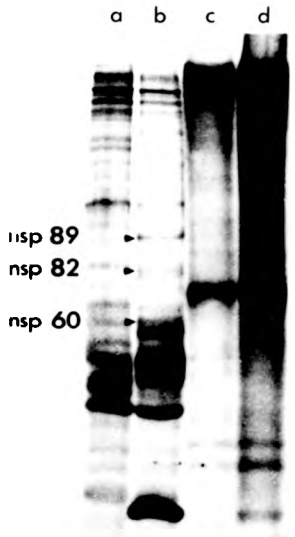


Fig. 11. Comparison of polypeptides labelled with [^{32}P] -orthophosphate and [^{35}S] -methionine synthesised by virus infected cells. Duplicate cultures of infected (lanes b and d) or mock infected (lanes a and c) cells were incubated in GMEM - met for 4 hours after which time the medium was replaced with labelling medium (lanes a and b) or PFEDA containing [^{32}P] -orthophosphate (1mCi/ml; lanes c and d) for 30 min. and then the labelling medium was replaced with chase medium while the PFEDA containing [^{32}P] -orthophosphate was replaced with HEDA containing 10 times the normal concentration of phosphate and all cultures were chased for 1h. The samples were then prepared for polyacrylamide gel electrophoresis as described in Section V.ii.

nsp82 and nsp60 are not phosphorylated to any large extent.

(ix) Is the Small N - terminal Polypeptide Stable?

A number of different gel concentrations and configurations were constructed in order to resolve a polypeptide with a molecular weight of 10,000 - 15,000. These included a gradient gel of 5% - 25% polyacrylamide and a fixed concentration 15% polyacrylamide gel all of which proved unsuccessful. The lack of success in detecting this polypeptide was not thought to be due to its lacking methionine as a consquence of its small size because it appeared to contain a peptide labelled with $[^{35}S]$ - methionine which was present in the tryptic peptide map of p230. In order to find the optimal concentration of polyacrylamide that would resolve this polypeptide (assuming it actually existed) Dr. Kennedy suggested electrophoresing the samples on a transverse gradient gel i.e. a gradient gel in which the gradient is constructed from left to right instead of from bottom to top as is normally used. Samples were prepared by labelling Sindbis virus infected cells with $[^{35}S]$-methionine under various conditions which might be expected to label this missing polypeptide and then they were handed to Dr. Kennedy who kindly constructed and ran the transverse gradient gels. The results of the experiment (Fig. 12) show two things; firstly, there are no new virus specified polypeptides detectable with molecular weights greater than that of core; secondly, a small polypeptide, termed z1, is present in the pulse labelled, infected culture (a) and is still present after chasing for three hours (b) but is absent in mock infected cells under all conditions (c and d). The same host polypeptide (h) has been labelled in all four figures to act as a reference point and so aid comparisons.

To investigate whether this was in fact the missing N- terminal polypeptide it was necessary to tryptic peptide map it. However, in order to avoid constructing gradient gels from which to isolate this polypeptide a rough estimate was made of the gel concentrations that gave optimal separation (18%), a fixed concentration gel was made up and infected and mock infected $[^{35}S]$-methionine labelled polypeptides, prepared

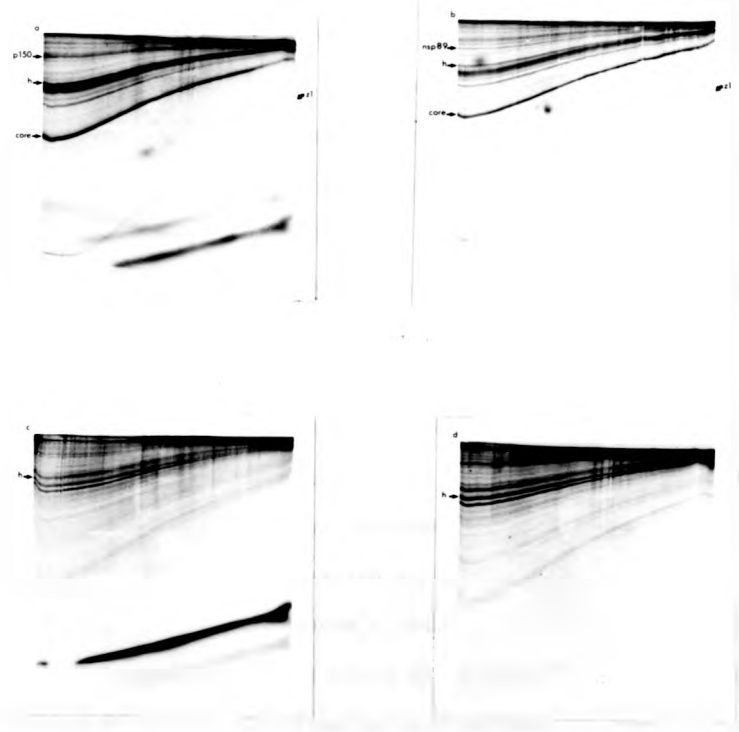


Fig. 12. Separation of Sindbis virus infected cell polypeptides on transverse polyacrylamide gels. Duplicate cultures of infected (a and b) or mock infected (c and d) cells were incubated for 4h in GMEM - met which was then replaced with labelling medium for 10 min. After this time one of each culture (a and c) were processed immediately while the other two (b and d) were incubated in chase medium for a further 3h. The samples were electrophoresed on linear 10% - 25% gradient polyacrylamide gels constructed by Dr. Kennedy. The gradient runs from left (10%) to right (25%).

under identical conditions to those in Fig. 12, were extracted and electrophoresed. The resulting polypeptide profile is shown in Fig. 13. This indicates the existence of not only z1, as seen in the gradient gels, but also of two other polypeptides z2 and z3 specific to virus infected cells. c marks the position of cytochrome c run as a marker in an adjacent lane of the slab gel. Using the known viral polypeptides and cytochrome c as markers it was possible to estimate molecular weights for z1, z2 and z3 which were 7,000 26,000 and 29,000 respectively but these must be regarded as very crude estimates as the gel system used was discontinuous (see Section I, iv) and also, in the case of z1, the molecular weight was obtained by extrapolation.

Tryptic peptide mapping of z1, z2 and z3 were performed as described above with labelling conditions as in Fig. 13 except that the concentration of $[^{35}S]$ -methionine was increased to 250 μCi/ml. Standards of pE2, p230 p215 and core were prepared as described in Section I (v). The tryptic peptide maps are shown in Fig. 14. These maps clearly demonstrate that z1 is not related to pE2 and therefore, does not represent the fragment cleaved from it on maturation to E2 (Jones et al., 1974) the single prominent spot (arrowed) could be present in core or in p230 (peptide x) and so it is not possible to state definitely from these maps alone what the origin of z1 is. It is quite obvious however, that z2 and z3 are both related to core in some way. To see if it were possible to resolve the polypeptides more effectively, different methods for the separation of peptides in the first and second dimensions were investigated. It was finally decided that electrophoresis in the first dimension at pH 2.1 followed by chromatography in the second dimension with a butanol/ pyridine/water solvent gave good separations under conditions that were sufficiently different from those used in the previous method. The maps of core and z1 generated under these new conditions are shown in Fig. 15 and clearly demonstrate that z1 is a fragment of the core polypeptide. With the benefit of hindsight and comparison of the maps of z1, z2 and core in Fig. 14 it is possible to imagine that z1 and z2 are generated by a

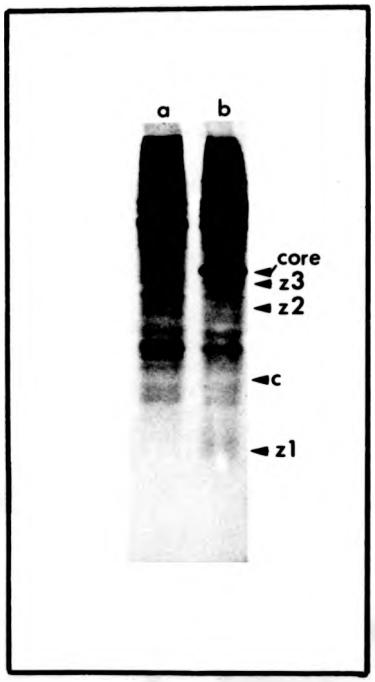


Fig. 13. Separation of virus specified polypeptides using an 18% polyacrylamide gel. Infected (lane b) or mock infected (lane a) cells were labelled as described in Fig. 12. and then chased for 3h, the samples were processed as usual and electrophoresed on an 18% polyacrylamide gel. c represents the position of a band corresponding to cytochrome c which was electrophoresed in an adjacent lane.

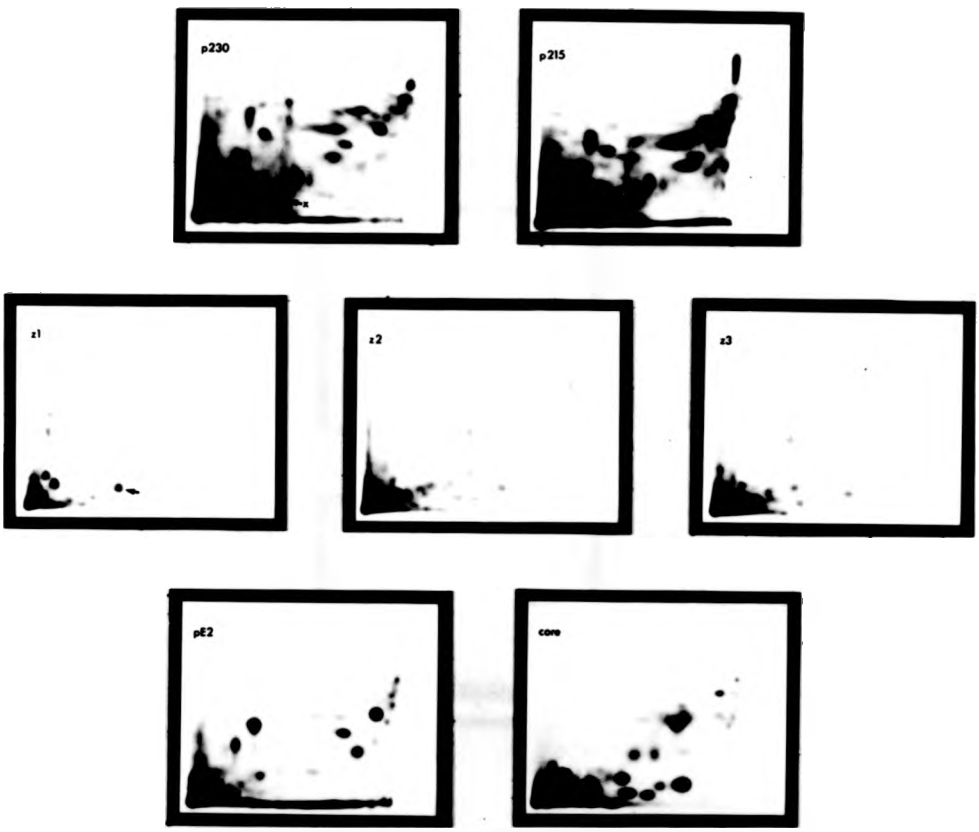


Fig. 14 Tryptic peptide mapping of z1, z2 and z3. Peptide mapping was carried out as described in Section V,vi. Polypeptides were prepared by incubating 10 vial cultures with labelling medium in which the concentration of $[^{35}S]$ -methionine had been increased to 250μCi/ml. The polypeptides were labelled as described in Fig. 2, pE2; Fig. 3, p230 and p215; Fig. 12, core, z1, z2 and z3. The first dimension is from left to right and the second from bottom to top. The significance of the arrow is explained in the text.

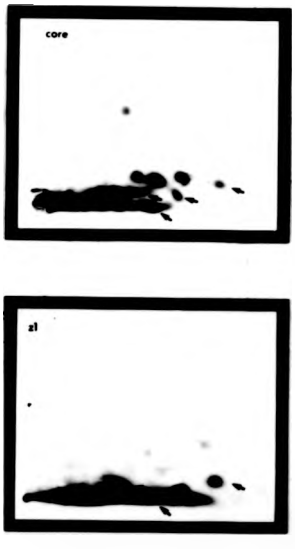


Fig. 15. Separation of tryptic peptides of core and z1 by an alternative procedure. The same preparation of peptides used in Fig. 14 were separated by the alternative procedure described in Section V,vi. Electrophoresis was from left to right and chromatography from bottom to top.

single specific cleavage of core and that superimposition of the maps of z1 and z2 would generate a pattern which would closely resemble that of core. It therefore, seems possible that there might exist a smaller, still undetected peptide, corresponding to that removed from core in the process of generating z3. Since z1, which has a molecular weight of less than 10,000 was resolved under these conditions, it would seem reasonable to conclude that the N terminal polypeptide x, which was not detected, is unstable and is broken down into fragments which are too small to be resolved under these conditions.

A portion of this work has already been published and a reprint is included in the Appendix.

DISCUSSION

The results presented above have shown the existence of a number of previously undescribed polypeptides, not all of which could be strictly defined as nonstructural in its narrower sense of describing those polypeptides which are unrelated to the structural polypeptides. The small polypeptides z1, z2 and z3 are, in all probability, artefacts although the possibility that z1 and z2 were generated by a single cleavage of the core polypeptide appears likely judging by the peptide maps shown in Fig. 14 and this might, in turn, suggest that this cleavage had been performed with enzymic specificity. It must be borne in mind, however, that firstly, there appears to be little difference in the amount of z1 after a ten minute pulse or after a three hour chase (Fig. 12 a and b); secondly, that the process for the preparation of samples for polyacrylamide gel electrophoresis involves washing the polypeptides with trichloroacetic acid which could lead to a specific cleavage at some acid sensitive site and thirdly, that the core polypeptide is the most intense band observed on an autoradiograph and that z1, z2 and z3 are easily detected because they are found in areas of the gel where there are few, if any, host specified polypeptides. In this case a small amount of acid hydrolysis of the core protein would be more readily detectable than that of any other polypeptide. The evidence, therefore, suggests that z1, z2 and z3 are artefacts of the procedure for preparation of samples for polyacrylamide gel electrophoresis and so they will not be considered further.

The origin of nsp59 is a little more amenable to explanation. Its tryptic peptide map shows it to be very closely related to pE2. However, under pulse - chase conditions nsp59 is stable whereas pE2 undergoes proteolytic cleavage to E2 (Schlesinger and Schlesinger, 1972), The electrophoretic mobility of nsp59 also differs from that of pE2 in that the mobility of nsp59 is greater. Kaluza recently reported the existence of a polypeptide in SFV infected cells that is stable and has an electrophoretic mobility slightly larger than that of pE2. It also appeared to be antigenically related to pE2 and could be labelled with

proposal that there is only a single initiation site for nonstructural polypeptide synthesis on Sindbis virus 42S RNA (Cancedda et al., 1975). However, it does seem reasonable to consider whether the larger polypeptides, p150, p215 and p230 are truly precursors i.e. can be cleaved productively, or alternatively, that they are aberrant polypeptides which are not processed and so are detectable on polyacrylamide gels but which are slowly degraded and hence give the appearance of chasing. This dilemma is illustrated by observation of p144, the "precursor" of the structural polypeptides. This can occasionally be detected in wild type infected cells but is processed only slowly, if at all (results not shown). The polypeptides contained in p144 consist of a soluble polypeptide, core, at the N terminal end followed by two hydrophobic envelope polypeptides (Clegg). Wirth et al. have described a possible mechanism for the translation of 26S RNA which necessitates nascent removal of core in order that the following nascent envelope polypeptides may be inserted into the membrane.. If this is correct, it is easy to see why a polyprotein consisting of one soluble and two hydrophobic polypeptides would be difficult to cleave productively after it had been synthesised in its entirety. Although this is true for the products of translation of 26S RNA, the products of the nonstructural region differ considerably from this. At least two of these polypeptides, nsp60 and nsp89, have been identified in an enzymatically active, purified RNA polymerase preparation from Sindbis virus infected cells (S.I.T. Kennedy, personal communication). Two polypeptides, nsp63 and nsp 90, with similar molecular weights were found in a highly purified preparation of SFV RNA dependent RNA polymerase (Clewley and Kennedy). It has been reported that the RNA polymerase in cells infected with either Sindbis virus (Sreevalsan and Yin) or SFV (Michel and Gomatos, Clewley and Kennedy; Friedman et al., 1972) is membrane bound and hence, because nsp60 and nsp89 are at the N terminal end of p230 it could be inserted directly into the membrane as it is translated. Additionally, the first cleavage of p230 involves

the C terminal polypeptide and so nascent cleavage need not occur (Section I, vii). It seems, then, that in the case of the nonstructural polypeptides p150, at least, can act as a precursor to nsp89 and nsp60 and that p215 and p230 may also be true precursors even though their existence may be transient.

It is quite possible that translation of poliovirus RNA involves the same sort of problem experienced by the alphavirus 26S RNA. Poliovirus has a single initiation site for protein synthesis near the 5' end (Oberg and Shatkin; Boime and Leder) and produces its polypeptides by sequential translation (Saborio et al.) synthesising capsid protein before the nonstructural proteins (Butterworth; Taber et al.) some of which are membrane bound (Lundquist et al.). Experiments have shown that the precursor to the capsid proteins in both polio (Jacobson et al.) and encephalomyocarditis (EMC; Butterworth and Rueckert) virus infected cells is cleaved nascently, as is a small polypeptide of molecular weight around 30,000 (NCVPX). The remainder of the cleavages of the structural and nonstructural polypeptides are carried out after completion of translation (Butterworth; Abraham and Cooper, 1975). These nascent cleavages may be a consequence of the presence of hydrophobic and hydrophilic proteins in a single polyprotein and so, for this reason, resembles the nascent cleavage of the alphavirus structural polyprotein. In this respect, patterns obtained on polyacrylamide gels after pulse and pulse-chase experiments with picornaviruses and alphaviruses are very similar representing a change from large polypeptides, seen in pulse experiments, to small products in chase experiments. However, whereas proteolytic cleavage of the precursors in alphavirus infected cells is apparently precise, this does not appear to be the case in poliovirus infected cells (Abraham and Cooper, 1975; Beckman et al.).

Addition of inhibitors of proteolytic cleavage to cells infected with picornaviruses (Korant; Summers et al; Abraham and Cooper, 1975) and alphaviruses (Morser and Burke; Clegg, Brzeski and Kennedy, Bracha and Schlesinger, 1976a) prevents processing of most, if not all, of the

precursors resulting in production of primary translation products, the single exception being the removal of the core polypeptide of alphaviruses from the structural protein precursor (Bracha and Schlesinger, 1976a). It is still not known whether these proteolytic enzymes are host or viral specified. The observation that zinc ions interact with the capsid peptide of rhinovirus (Korant and Butterworth) together with the report that one of the capsid peptides of EMC aids cleavage of EMC polypeptides synthesised in cell free systems (Lawrence and Thach), has led to the suggestion that at least one protease in picornavirus infected cells is coded for by the virion RNA. In alphavirus infected cells, release of core is not prevented by zinc ions (Bracha and Schlesinger, 1976a); this, together with the observation that its removal from the nascent polypeptide chain is rapid has led to the suggestion that it might also carry protease activity (Clegg and Kennedy). This possibility can be considered unlikely if the following two pieces of evidence are considered; firstly, removal of zinc from inhibited cells did not lead to restoration of proteolytic activity even though fresh core was sythesised (Bracha and Schlesinger, 1976a) and secondly, temperature sensitive mutations which cause accumulation of a high molecular weight precursor to either the structural or non-structural polypeptides, and so might represent a lesion in a virus specified protease, do not cause the accumulation of both percursors (See Sections II and IV). These observations together with the reports that zinc ions can inhibit certain host cell specified proteases (Danø and Reich; Bosmann) seem to suggest that processing of most alphavirus polypeptides is under host control. It is possible, however, that core has proteolytic activity which is specific for its own removal and is not inhibited by zinc ions, while the remainder of the polypeptides are cleaved by host cell proteases.

The three nonstructural polypeptides of Sindbis virus are translated sequentially resulting in the synthesis of p230, whose existence may be transient. It is important to determine whether p230 is the entire translation product or whether there are other polypeptides synthesised after

nsp82. Attempts to demonstrate a fourth polypeptide by increasing the labelling period after removal of a hypertonic salt block did not reveal any new virus specified polypeptides (Fig. 9) but it could be argued that the new polypeptide(s) have a similar molecular weight to one of the pre-existing viral structural polypeptides and hence remain undetected, as has been suggested for SFV (Lachmi and Kääriäinen, 1976). It has been reported (Strauss et al., 1976) that Sindbis virus RNA -ve mutants i.e. those mutants with lesions in the nonstructural polypeptides, form four complementation groups which could be interpreted to mean that there are four nonstructural polypeptides. As the authors point out however, production of uncleaved precursors by these mutants could make assignment of each complementation group to a single polypeptide unreliable and would, presumably, overestimate the number of genes (This is discussed further in Section III). In addition, no precursor larger than p230 has been detected in wild type virus infected cells either in the presence or absence of zinc ions, or in cells infected with temperature-sensitive mutants (Section III).

It is possible to calculate the approximate size of the nonstructural region of the 42S RNA using the molecular weights of $4.2 \pm 0.2 \times 10^6$ and $1.8 \pm 0.1 \times 10^6$ for 42S and 26S RNA respectively, these values being the average of three reported determinations (Simmons and Strauss, 1972a; Martin and Burke; Levin and Friedman). This gives a value for the size of the non structural region of 42S RNA of $(4.2 - 1.8) \times 10^6$ or $2.4 \pm 0.3 \times 10^6$. Since this figure is accurately known it is possible to estimate the coding capacity of the nonstructural region in three ways:-

(a) the structural genes produce a polypeptide with a molecular weight of 144,000 from an RNA with a molecular weight of 1.8×10^6; in proportion then, it would be expected that the nonstructural region of 42S RNA would code for a polypeptide with a molecular weight of 192,000.

(b) assuming that the ratio of weight of polypeptide coded for by a given weight of RNA is approximately 1 to 10, then a RNA with a molecular weight of 2.4×10^6 would produce a polypeptide with a molecular weight of 240,000.

(c) poliovirus RNA, which has a molecular weight of 2.5×10^6, codes for a polypeptide with a molecular weight of 210,000 (Abraham and Cooper, 1975).

Taking into account the fact that eucaryotic messenger RNAs appear to have non-translated sequences which precede (Baralle; Brawerman) and follow (Forget et al.; Proudfoot) the actual translated sequences and also that 42S RNA must have a polymerase recognition site and possibly a nucleation site for nucleocapsid formation at the 5' end then (b) could be an overestimate while (a) may be an underestimate because these sequences are presumably present in 26S RNA and will therefore be over-estimated in (a). Taking this into account, a reasonable estimate for the total coding capacity for the nonstructural region of the 42S RNA would appear to be 210,000 - 240,000 which would suggest that p230 is probably the entire translation product.

These results are in accord with those published by Clegg, Brzeski and Kennedy for the nonstructural polypeptides of SFV where two polypeptides, nsp90 and nsp63, were found to be synthesised via precursors with molecular weights of 200,000, 184,000 and 150,000. No equivalent to nsp82 was detected in infected cell extracts or in a preparation of highly purified SFV RNA dependent RNA polymerase which was found to contain two viral polypeptides, nsp90 and nsp63, and a host polypeptide (Clewley and Kennedy).

These observations on the synthesis of the nonstructural polypeptides reported here differ from those reported for SFV by Lachmi et al. and Lachmi and Kääriäinen. They have suggested the existence of four nonstructural polypeptides in their SFV infected cells which are produced pair - wise from two precursors. They suggest that these two precursors are cleaved from a still larger polypeptide with a molecular weight of 300,000 but have found no viral specified polypeptide with a molecular weight larger than 155,000. The peptide maps that have been presented so far seem to suggest that the putative nonstructural precursors do not contain any sequences in common with the structural proteins but no maps have yet been presented which demonstrate any sequence relationships between their

nonstructural polypeptides and the proposed precursors. The situation has been complicated by their use of an RNA +ve mutant for the majority of this work; when infected cells are incubated at the restrictive temperature it is not unusual for these mutants to synthesise aberrant polypeptides (Keränen and Kääriäinen). The reason for these discrepancies is unclear. There are two possible, but unlikely, reasons for the differences in behaviour; firstly, the strain of SFV used by Kääriäinen is in some way different from those of SFV and Sindbis used in this laboratory and secondly, the experiments performed by Kääriäinen et al. were carried out in secondary CEF cells while primary CEF or BHK cells were used in the experiments in this laboratory. Perhaps the differences will resolve themselves when the nonstructural polypeptides and precursors synthesised by the strain of SFV used by Kääriäinen et al. have been characterised more completely.

On the other hand, it must be borne in mind that ØX 174 has been shown to produce more protein than can be coded for by the amount of DNA it contains. It achieves this by using one DNA sequence to code for two separate protein sequences using different reading frames i.e. it has overlapping genes (Barrell et al.). No evidence has yet been obtained for such an occurrence in animal virus infected cells but the possibility of its existence cannot be ignored, especially in those viruses with small genomes where genetic space is at a premium.

A summary of the results obtained for Sindbis virus is shown in Fig. 16 which is a schematic representation of the production of p230 and its subsequent processing to give the nonstructural polypeptides. It is proposed that synthesis of the nonstructural polypeptides is initiated at or near the 5' end of 42 S RNA and the genes are translated sequentially in the order nsp60 - nsp89 - nsp82 in order to produce a polyprotein p230. When translation of this product is complete, or almost complete, the first cleavage takes place at the C terminal end, point C, (c.f. the structural precursor where the first cleavage is N terminal) to release nsp82, as p76, and generate p150. The cleavage of p150, at point B, to generate nsp60 and nsp89 appears to take place more slowly than the first cleavage.

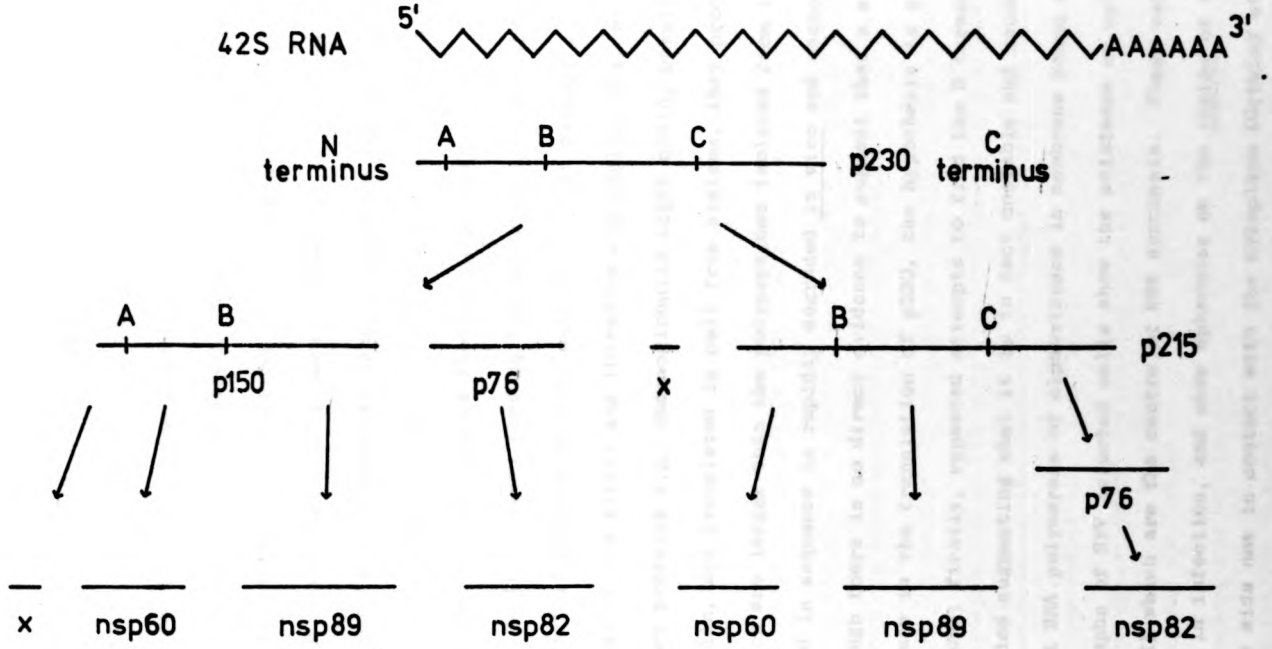


Fig. 16 Proposed mechanism of Sindbis virus nonstructural polypeptide synthesis and processing.

The point in time at which the N terminal peptide is removed is unknown, nor is it known if there are any temporal constraints on its removal. The fact that repeated attempts to detect this polypeptide have failed suggests that it may be degraded and it is of interest to speculate on what function this peptide might serve. Lead in sequences have been described for the N terminals of polypeptides synthesised in cell free extracts in response to SFV 26S RNA (Clegg and Kennedy) and EMC RNA (Smith) but these are normally only of the order of a few amino acids not the 100 - 150 that precede the synthesis of nsp60. A more convincing explanation is that it acts in an analogous fashion to the "signal sequence" (Milstein et al; Blobel and Dobberstein; Devillers - Thiery et al.). This hypothesis proposes that there is an N terminal lead in sequence for polypeptides which are destined for export from the cell and that this causes the binding of polysomes to membranes as the signal is translated. The nascent polypeptide then passes through the membrane, through special channels, such that it appears on the "outside." This passage involves coincident removal of the signal and therefore can explain why, when the mRNAs of secretery proteins e.g. immunoglobulin light chain, parathyroid hormone and insulin, are translated in cell free systems, they produce polypeptides which are larger than the polypeptides isolated from intact cells. This lead in sequence is rapidly degraded <u>in vivo</u> and cannot be detected. Although there is no direct evidence to suggest that a similar process takes place in the translation of p230, the hypothesis is attractive for two reasons; firstly, repeated attempts to find the N terminal peptide have failed suggesting that it is in fact unstable and secondly, the RNA dependent RNA polymerase of alphaviruses is membrane bound and electron micrographs of SFV infected cells show the existence of cytopathic vacuoles (CPV - I) which are the centre of RNA synthesis. These vacuoles are formed early in infection, and show spherules on the <u>inside</u> of the vacuole i.e. that side not in contact with the cytoplasm (Grimley et al.). The signal sequence would then provide a convenient mechanism by which this insertion could be achieved. A similar mechanism has recently been

proposed for the synthesis of the envelope polypeptides (Wirth et al.). A diagrammatic illustration of how translation and processing of Sindbis virus nonstructural polypeptides might take place is shown in Fig. 17. Translation of 42S RNA generates the N terminal end of p230, which is hydrophobic and results in the attachment of the polysomes to an intracellular membrane (i). Continued translation results in the folding of the nascent polypeptide in the membrane (ii) and (iii) until finally, after termination of translation, the entire molecule of p230 (or perhaps p215) is intact in the membrane (iv). The first proteolytic cleavage takes place generating p76 and p150 (v) which then allows the cleavage of p150 to take place, generating nsp89 and nsp60 (vi). It is considered that the cleavage of p150 cannot take place immediately because either (a) the conformation of the polypeptide does not allow recognition of the cleavage site or (b) that the cleavage site is physically blocked by the rest of the nascent chain. After translation and cleavage of p76 it would be possible for p150 to assume a conformation which would allow it to be cleaved. It is possible that this cleavage generates a single polymerase molecule with three viral subunits and that its conformation is a necessary consequence of the order of cleavage (See Section III). In this respect it would not be unlike the production of the two polypeptide chains of insulin by removal of a central portion of a single polypeptide after it has adopted its three dimensional configuration.

The final point to consider is what happens to the ribomsome after it has completed translation of the nonstructural genes. The structural genes of SFV follow those of the nonstructural genes in 42S RNA i.e. the 26S RNA sequences represent those near the 3' end of 42S RNA (Kennedy, 1976; Wengler and Wengler, 1976) and bearing in mind that no example has yet emerged of internal initiation of protein synthesis on a eucaryotic messenger RNA (Jacobson and Baltimore) it is of interest to speculate whether mechanisms for the internal termination of protein synthesis are present when the mechanisms for internal initiation are not. In this respect, alphaviruses, along perhaps with flaviviruses, are unique,

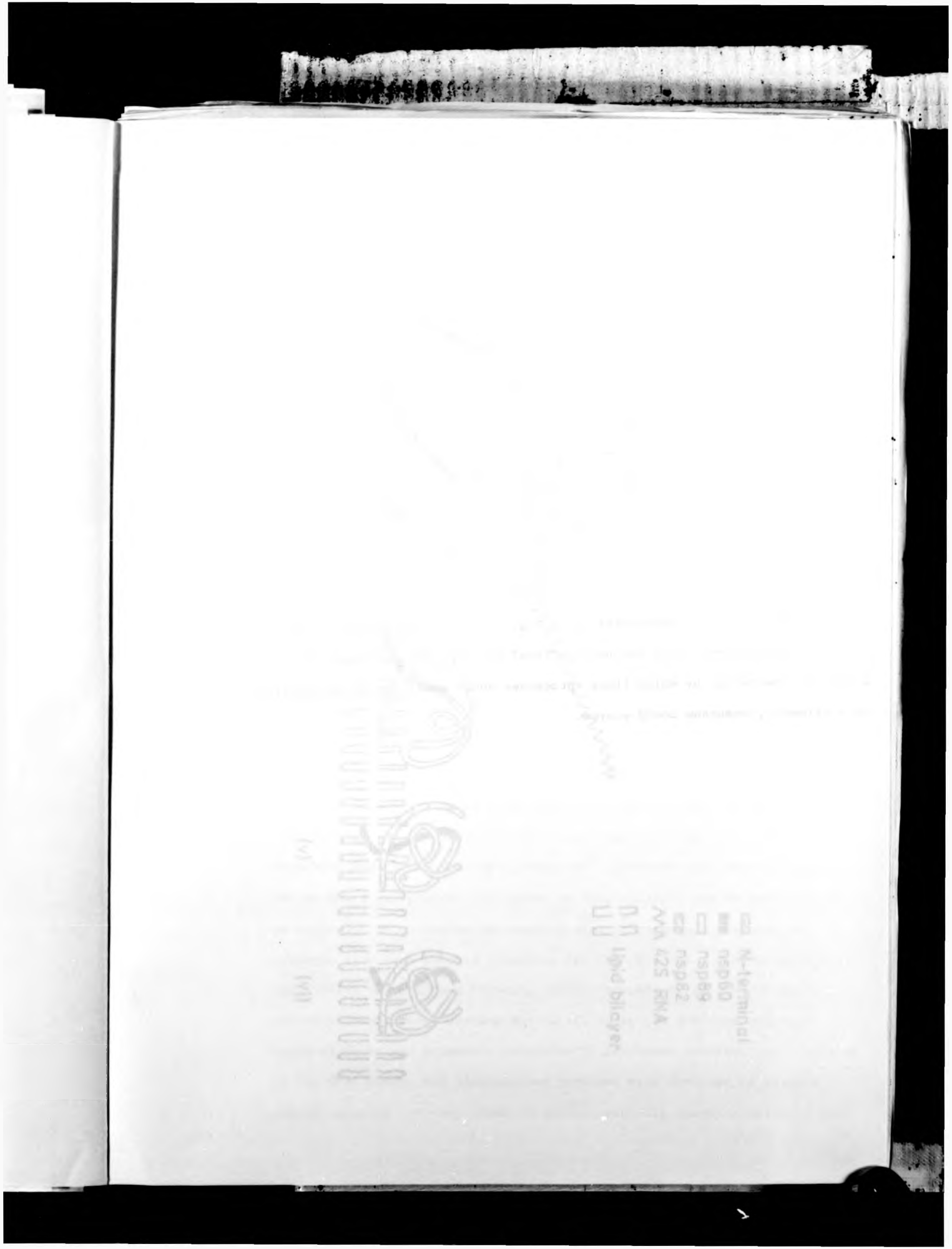
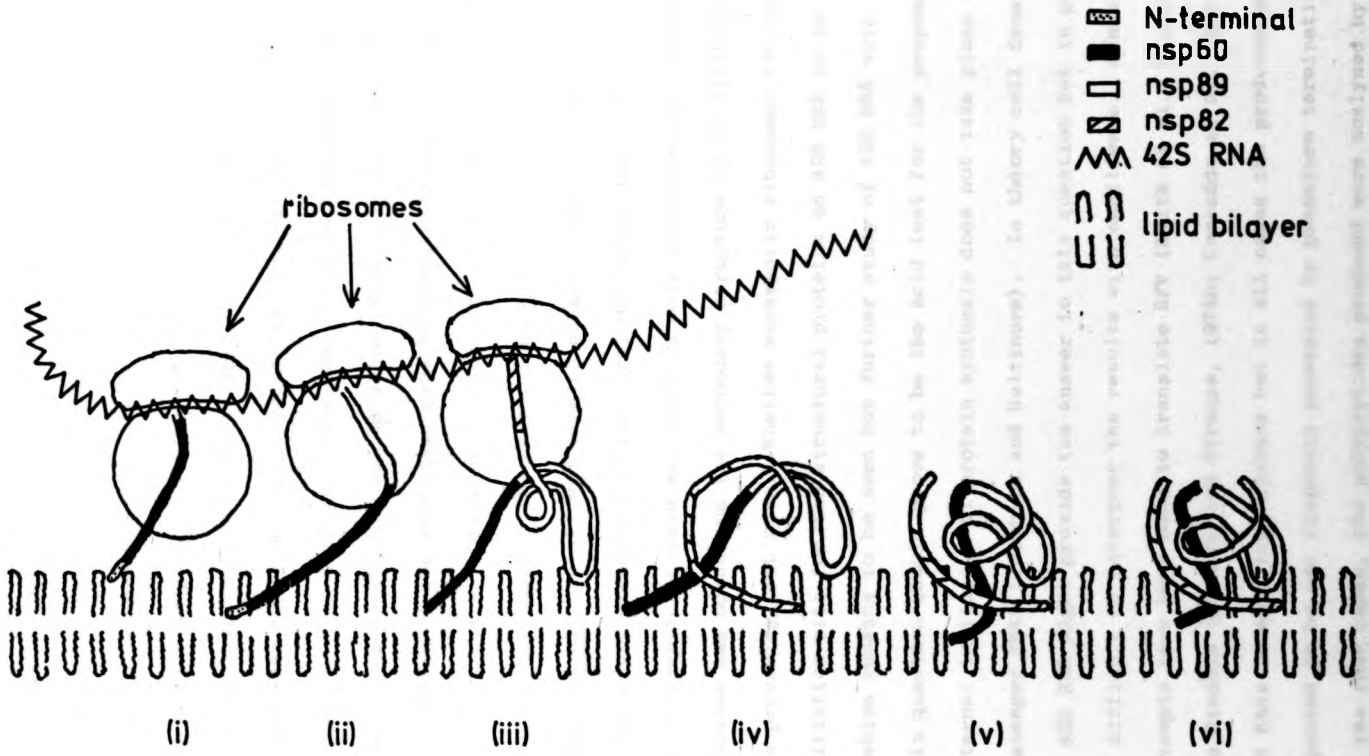
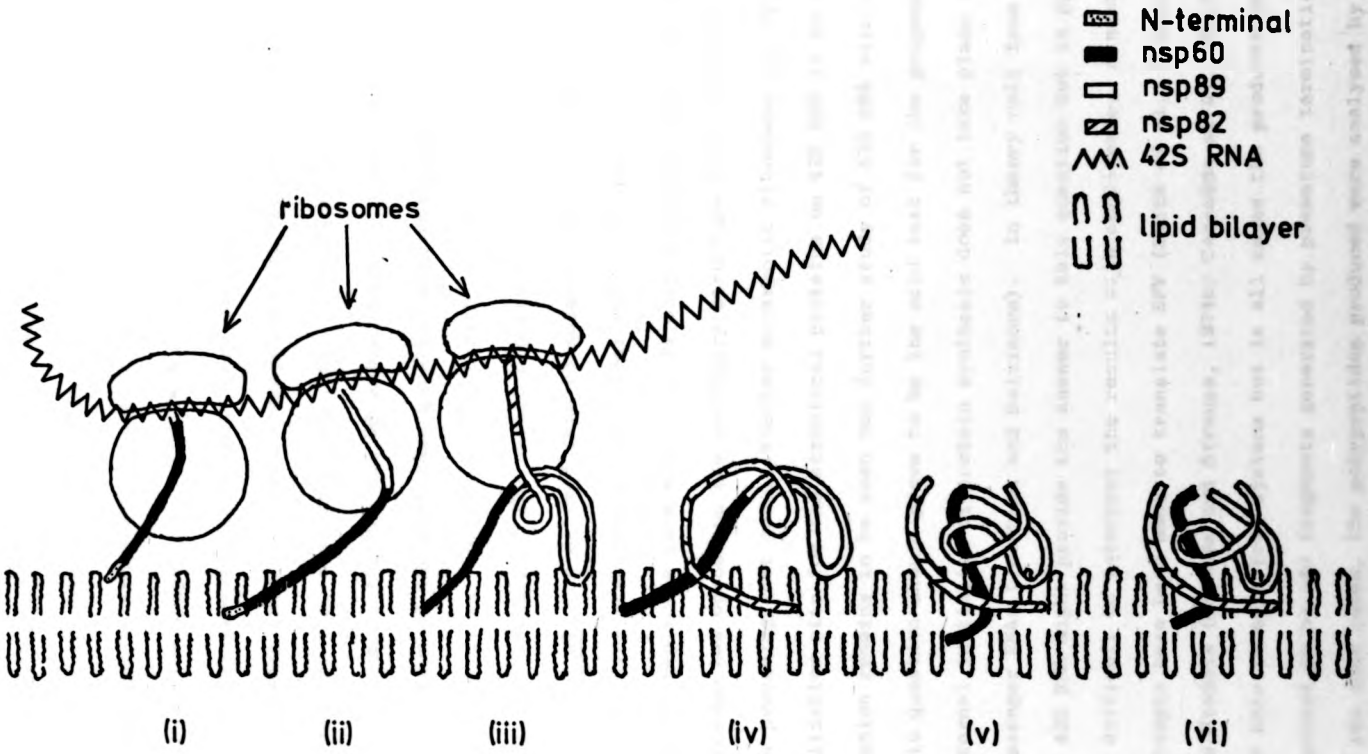


Fig. 17. A figurative representation of Fig. 16. An illustration of the synthesis and processing of the nonstructural polypeptides incorporating a possible mechanism by which these processes could result in the generation of a trimeric, membrane bound enzyme.


N-terminal
nsp60
nsp89
nsp82
42S RNA
lipid bilayer
ribosomes
(i)
(ii)
(iii)
(iv)
(v)
(vi)


N-terminal
nsp60
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42S RNA
lipid bilayer
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because the only other group of viruses, the picornaviruses, known to translate their RNA into a polyprotein do so in one step and so may circumvent this problem. It has been shown that the site for initiation of structural protein synthesis in 42S RNA is cryptic (S.I.T. Kennedy, manuscript in preparation) i.e. fragments from the 3' end of 42S RNA with a size comparable to 26S RNA will direct the synthesis of the structural proteins in a wheat germ cell free extract but fragments larger than this will not, suggesting that the initiation site is obscured perhaps by base pairing of the RNA. This is reminiscent of the RNA phages such as f2, MS2 and Qβ where there is a polarity effect on the translation of the replicase gene by amber mutations in the coat gene which immediately precedes it (Horuichi et al.; Lodish and Robertson; Ball and Kaesberg). The recent sequencing work on the genome of MS2 by Minjou et al. has allowed them to suggest that this polarity is achieved by secondary structure in the viral RNA which obscures the initiation site for the replicase gene and so it is only available for initiation when a ribosome has opened up the secondary structure as it finishes translating the preceding coat gene. Whether eucaryotic ribosomes can open up the initiation site for the structural proteins on 42S RNA in an analogous fashion remains to be seen but further study of 42S RNA with regard to this question should prove to be the acid test for the proposal that internal initiation of protein synthesis does not take place on eucaryotic messenger RNAs (Jacobson and Baltimore). In theory cell free translation of 42S RNA might provide the answer to this question but in practice it is difficult to interpret the results with confidence. A number of attempts have been made to translate SFV (Smith et al.; Glanville et al.) and Sindbis (Simmons and Strauss, 1974b; Cancedda et al., 1974) virion 42S RNAs in cell free systems but in all cases the products obtained appeared to be odd fragments generated by premature termination. In three of the four cases, the polypeptides produced were analysed by tryptic peptide mapping and were found to contain peptides characteristic of the

structural polypeptides. However, it is not possible to tell whether these structural polypeptides were produced by initiation on fragments of 42S RNA or alternatively by internal initiation on 42S RNA which had been denatured during extraction or on 42S RNA in which the internal initiation site had been opened by ribosomes completing the nonstructural genes. It will be difficult to devise an experiment to test this hypothesis, especially without the equivalent of amber mutants which have proved so useful for this purpose in work with the MS2 type of phage.

Having determined the nature of the nonstructural polypeptides of Sindbis virus, the next important question to ask was how were they involved in the synthesis of 26S and 42S RNA? However, before these questions can be considered it is necessary to determine how the small subgenomic 26S RNA is generated in Sindbis virus infected cells. Experiments devised to answer this question are described in the next section.

SECTION II

TRANSCRIPTION OF ALPHAVIRUS RNAS

INTRODUCTION

The RNA Species Found in Alphavirus Infected Cells

When alphavirus infected cells are labelled with an RNA percursor and the purified RNA run on polyacrylamide gels six discrete bands are observed. These are, in order of decreasing mobility, 26S, 33S, 38S and 42S RNAs followed by the RF and finally the RI (Levin and Friedman). The first four RNAs are single stranded; 26S and 42S RNA account for the majority of the single stranded RNA while 33S and 38S RNA are minor components. The RF is a double stranded RNA while the RI, which only just penetrates the gel, is a multistranded species. Each of these species of RNA will now be considered in turn.

(i) The Single Stranded RNAs of Messenger Sense

The 42S RNA has a molecular weight of 4.2×10^6 (Simmons and Strauss 1972a; Martin and Burke). Originally, a number of reports appeared, which suggested that 42S RNA isolated from cells infected with WEE (Screevalsan et al.), SFV (Cartwright and Burke) and Sindbis virus (Dobos and Faulkner 1969; 1970) could be denatured to give 26S RNA using dimethylsulphoxide, heat or formamide. In fact this transformation appears to have been due to nicking of the RNA during isolation, probably in the area of a hairpin loop in the RNA so that, under non denaturing conditions, the RNA appeared to be intact but, under denaturing conditions, the RNA separated into fragments with a size similar to 26S RNA. 42S RNA is now known to be a single continuous polynucleotide chain (Arif and Faulkner, Simmons and Strauss, 1972a). The 42S RNA isolated from Sindbis virus infected cells is infectious (Eaton and Faulkner; Scheele and Pfefferkorn, 1969a) and is virtually indistinguishable from virion RNA, except perhaps in its pattern of methylation (see below). Kennedy (1976), who has compared the intracellular and virion 42S RNA of SFV using the oligonucleotide mapping technique, could find no difference between them. It seems reasonable to assume then, that most of the intracellular 42S RNA is destined to go into progeny virus. As 42S RNA is infectious it must be capable of specifying all the proteins necessary for its replication, which has led to the

suggestion that it is the messenger RNA for the nonstructural polypeptides (see Section I).

The structural proteins on the other hand, are coded for by a subgenomi RNA, 26S RNA (Cancedda et al, 1974; Simmons and Strauss 1974b, Cancedda and Schlesinger). It has a molecular weight of 1.8×10^6 and has been shown by competition hybridization to contain approximately one third of the sequences found in 42S RNA (Simmons and Strauss, 1972a). In infected cells, depending on virus strain and conditions, the two types of RNA are synthesised in a ratio of approximately one 42S RNA to four 26S RNA molecules. As the 26S RNA is used solely as a messenger RNA for the synthesis of structural proteins and is not found in virus particles the production of 26S RNA could be considered as a mechanism for gene amplification. Approximately 230 of each of the structural proteins are required to encapsidate one 42S RNA molecule (Laine et al.) so the advantages of such a ploy are apparent. The actual location of the 26S RNA sequences in the 42S RNA of SFV was determined by Kennedy (1976) using the oligonucleotude mapping technique. By comparing the maps of 42S RNA, 26S RNA and fragments of 42S RNA which had progressively lost the 5' or 3' ends he was able to assign groups of spots to relative positions inward from either end. This spot order showed that the sequences corresponding to 26S RNA were located at the 3' end of 42S RNA. Similar results were reported by Wengler and Wengler (1976) using an analogous approach. This has an interesting consequence for the transcription of 26S RNA which will be considered later.

The two minor RNA species, 33S and 38S RNA, appear to be conformational variants of 26S and 42S RNA respectively (Kennedy, 1976). Whether the difference in conformation is due to some difference in physiological function inside the cell or is simply an artefact of extraction is not known but it has been suggested that the incidence of circular RNA molecules is higher in 38S RNA than in 42S RNA (Kennedy, 1976).

(ii) Post-transcriptional Modification of the Single Stranded RNAs

Eucaryotic mRNAs have a number of additions made to the polynucleotide chain after transcription and viral mRNAs are no exception; however, the

purpose of these modifications is not always clear.

In common with the majority of cellular mRNAs those of Sindbis virus have a poly (A) tract at their 3' ends. The number of adenine nucleotides in the poly (A) tract of 26S and 42S RNA has been variously estimated as 30 - 40 (Deborde and Leibowitz) or that there are two populations of 42S RNA, 80% - 90% having 60 - 80 adenines in its poly (A) tract while the remainder have a tract containing 150 - 250 adenines (Eaton and Faulkner). In the second case there did not appear to be any difference in the infectivity of these two different populations of RNA. It is not known whether this variation is due to a difference in the preparation of the RNA or, alternatively, the use of a different strain of virus and cells.

It has recently been reported that the poly (A) tract on the 26S and 42S RNA is transcribed directly from a poly (U) tract on the 5' end of the template used for the synthesis of 26S and 42S RNA (Sawicki and Gomatos). Since the 26S RNA sequences are at the 3' end of 42S RNA, its complementary sequences will correspond to the 5' end of the 42S negative strand RNA, hence termination of both 42S and 26S RNA will take place at the 5' end of the template culminating in the direct transcription of the poly (A) tract from the poly (U) sequence (Sawicki and Gomatos). This is an attractive proposition and is similar to the observation previously made with poliovirus, which also appears to have a poly (U) tract on the negative strand RNA (Yogo and Wimmer, 1973; 1975). The drawback of this hypothesis lies in the production of the 42S negative strand RNA using the 42S positive strand RNA as a template. In order to transcribe a negative strand RNA that will have a poly (U) tract at its 5' end, the polymerase must bind to the 3' end of the 42S positive strand RNA, i.e. at the 3' end of the poly (A) tract. If this is the case it is difficult to see how the polymerase can specifically recognise the 42S positive strand RNA when the 26S RNA and most other cellular RNAs also have poly (A) at their 3' ends. No doubt these inconsistencies will resolve themselves when a cell free RNA synthesising system becomes available.

The other modification which is found on eucaryotic mRNAs is the inverted 5' - 5' linkage, commonly known as the cap, at the 5' end of the mRNA. This takes the form of a guanosine linked by the 5' OH of its ribose to the 5' triphosphate of the RNA such that, unlike the bacterial system, eucaryotic mRNAs do not contain a free 5' terminal triphosphate. In addition, the cap invariably contains a methyl group at the 7 position of the capping guanosine; the 2' OH of the first ribose from the 5' end, excluding the cap, is often methylated as well. These caps have been found in mRNA from mouse myeloma cells (Adams and Cory), HeLa cells (Furuichi et al., 1975b) and reticulocytes (Muthukrishnan et al.) and in viral mRNA in cells infected with reovirus (Furuichi et al., 1975a), VSV (Moyer et al.) and vaccinia virus (Wei and Moss). This list is by no means comprehensive as the cap, along with the poly (A) tract appears to be ubiquitous. In common with the poly (A) tract, the function of the cap is not known although it has been suggested that it aids ribosome binding (Muthukrishnan et al.). However, this hypothesis is based on a comparison of the behaviour of untreated and treated RNA, in which the cap had been removed by reacting the RNA with periodate and sodium borohydride; the ribosome binding ability of the two RNAs were then compared in a cell free protein synthesising system. Nevertheless, it is possible that the RNA was damaged during the chemical manipulations and that the difference in binding ability is due solely to these treatments.

Sindbis virus RNA, in common with the majority of mRNAs is also capped and methylated. The intracellular 42S and 26S RNAs have been shown to contain either $m_2^{2,7}G$ or $m_3^{2,2,7}G$ as their blocking nucleotides (Dubin et al.) while virion 42S RNA has a cap with the structure $m^7G^{5'}$pppApUpGp... This led Dubin et al. to suggest that the extent of methylation of the capping guanosine would provide a useful mechanism by which the virus could regulate the amount of 42S RNA used for translation or encapsidation purposes. This seems unlikely to be the case, however, as the times used for labelling the RNA were late in infection when very little 42S RNA appears to be translated and the majority is due for encapsidation into

virus particles. Why there should be this difference between 42S RNA which is probably in nucleocapsids and which will finally bud out as mature virus, and that which is actually in mature virus, is unclear. The cap structure of the intracellular viral RNAs differs from most other mRNAs in having the di- or tri- methyl derivative of guanosine instead of the more common m^7G; the reason for this is also unknown nor is it known if the methylating and capping enzymes are host or virus specified.

It can be seen then that, whereas the viral RNAs may differ slightly in detail from the majority of host cell mRNAs they are generally of a similar structure and hence are able to serve their necessary function of infiltrating the host cell mRNA population admirably.

(iii) Single Stranded RNAs of Negative Polarity

No such RNA appears to exist free in the cytoplasm, at least in SFV infected cells. Martin and Burke examined the 42S RNA population for the presence of free 42S RNA of negative polarity using hybridization techniques but could not detect any. As negative strand RNAs are a necessary prerequisite to viral RNA replication it seems reasonable to assume they will all be present in those structures associated with RNA synthesis. In fact Bruton and Kennedy (1975) have determined the kinetics of SFV negative strand RNA synthesis by labelling cells and then purifying the RIs. After hybridising excess cold 42S virion RNA to these RIs they were able to determine how many were synthesised during consecutive 15 minute intervals of the growth cycle. This experiment showed that 42S negative strand RNA was first detected 1.5 hours post infection, its rate of synthesis increased rapidly to a maximum at 2.5 hours post infection and then declined to zero by 4 hours post infection. They also showed that RIs consisted of a continuous 42S negative RNA strand to which was hybridised a number of fragments of 42S positive strand RNA; no 26S negative strand RNA was detected.

(iv) Double and Multistranded RNA species

The double stranded 42S RNA, termed RF, is an enigma along with the RF's isolated from other virus infected cells. There is conflicting

evidence as to whether it is an intermediate in RNA replication (Michel and Gomatos; work with SFV replicase in vitro),a by-product of RNA replication (Noble and Levintow; by examining the kinetics of synthesis of RNA in poliovirus infected cells) or an artefact of addition of actinomycin D to the medium (McNaughton et al.;working with rhinovirus infected HeLa cells). In addition, it is relatively easy to generate a "double stranded" 42S RNA as a consequence of RNAse contamination during RNA purification. The RNAse might digest the RI, and so remove the single stranded RNA tails, leaving one continuous RNA strand with a number of smaller fragments hybridised to it. For reasons of doubtful pedigree the RF will not be considered further.

The RI, on the other hand, is assumed to be the structure responsible for RNA transcription. It is envisaged as consisting of a single RNA strand which has attached to it 8-10 polymrease molecules (Martin and Burke). These polymerase molecules are thought to bind at the 3' end of the virus RNA and transcribe a complementary RNA strand. When the polymrease has moved some distance along the RNA a second polymerase can bind, so displacing the previously synthesised RNA strand, as it, also, starts to copy the RNA. This gives rise to a multistranded RNA with a number of copies of RNA of one polarity at various stages of completion, hydrogen bonded for a small part of their length to the complementary RNA strand. It can be seen that treatment of such a structure with RNAse will lead to digestion of the single stranded RNA tails leaving a RNAse resistant core which is essentially double stranded along most of its length.

It has been found that digestion of RIs with RNAse, besides generating the 42S RNA duplex, called RFI, can also cut at a specific site generating two other "double stranded" RNAs, RF II, and RF III (Simmons and Strauss 1972b, Martin and Burke). RF III was shown, by hybridisation, to contain most of the sequences found in 26S RNA while RF II was shown to lack these sequences (Simmons and Strauss 1972b). It was assumed then that this RNAse sensitive site was in that region of the 42S RNA corresponding to the 5' end of the 26S RNA. By labelling the RI under different conditions,

purifying it, treating it with RNAse and separating the three RFs Simmons and Strauss came to a number of conclusions concerning the purpose for which the RI's were used. They decided that 42S RNA synthesis took place on that RI which gave rise to RFI because it was not RNAse sensitive while synthesis of the two smaller RNAs took place on another population of RIs such that the RNAse sensitive site was open to attack. One of these RNAs would be 26S RNA while the second RNA had a molecular weight of 2.4×10^6; initiation of the synthesis of one RNA and termination of the second was thought to take place in the area of this RNAse sensitive site. Under long pulse labelling conditions using a RNA precursor, the molar ratio of RF II to RF III was unity suggesting that all of that RI had been used for RNA synthesis. On the other hand, under short pulse conditions, there was much more label in RF III than RF II on a molar basis which suggested that the rate of synthesis of 26S RNA was faster than that of the larger 2.4×10^6 molecular weight RNA. For this reason the authors suggested that this RNA, which had not yet been detected, was made in far smaller amounts than the other two RNAs. However, it is not now thought to exist and an alternative explanation of these results will be provided later, in the Discussion.

Once the position of the 26S RNA sequences had been identified as those at the 3' end of the 42S RNA it became apparent that this represented a dilemma with respect to the mechanism of transcription of 26S RNA from the 42S negative strand RNA; all attempts to demonstrate the presence of a 26S negative strand RNA had been unsuccessful (Bruton and Kennedy, 1975). Transcription of 26S RNA would necessitate internal initiation of transcription of RNA synthesis on the 42S negative strand RNA but no such mechanism has yet been reported. Poliovirus has no need for such a mechanism, while VSV, which generates a population of smaller mRNAs from a single complementary virion RNA, appears to transcribe these genes sequentially so avoiding the necessity of initiating RNA transcription internally (Ball and White; Abraham and Banerjee). The alternative

mechanism to internal initiation of transcription is processing of 42S RNA to generate a 26S RNA molecule. There is a precedent for such a mechanism in both T7 phage infected E. coli (Dunn and Studier, 1973a; 1973b) and in the production of ribosomal RNAs in eucaryotes (see Attardi and Amaldi) but both these mechanisms involve RNA transcribed from a DNA template. Although this mechanism is more attractive because precedents are known it would be unsatisfactory from another point of view and that is the amount of wastage that would be involved. In wild type infected cells there are 3 42S RNA molecules made for every 10 26S RNA molecules synthesised. If it were assumed that one molecule of 26S RNA is equivalent to one unit of RNA synthesis and that therefore one 42S RNA molecule is equal to 2.33 units of RNA synthesis (the ratio of their molecular weights), then it can be seen that if 26S RNA were produced by processing of a 42S RNA molecule it would require the synthesis of (10 + 3) x 2.33 or 29.9 units of RNA of which only 10 +(3 x 2.33) or 16.9 units would actually be used. The remainder 29.9 - 16.9 or 13 units would be lost in processing, or in other words, 43% of all the RNA synthesised would be degraded again. This goes against the general concensus of opinion which suggests that a virus is an efficient regulator of its growth cycle. For this reason it was of interest to see which of these mechanisms, if either, was actually correct.

The obvious difference between these two alternative mechanisms lies in the size of the template required to generate the primary transcript (Fig. 18 a and b). Since 26S RNA is located at the 3' end of 42S RNA this would necessitate synthesis of the entire 42S RNA molecule before 26S RNA could be rescued from it (Fig. 18a). On the other hand, if 26S RNA were synthesised by internal initiation of transcription, only the 26S RNA need be transcribed (Fig. 18b). It seemed likely that this difference in the size of the template required to produce the primary transcript might be exploited by UV irradiating the infected cell and monitoring the rate of inactivation of the template RNA by measuring the loss of ability to synthesise each species of RNA.

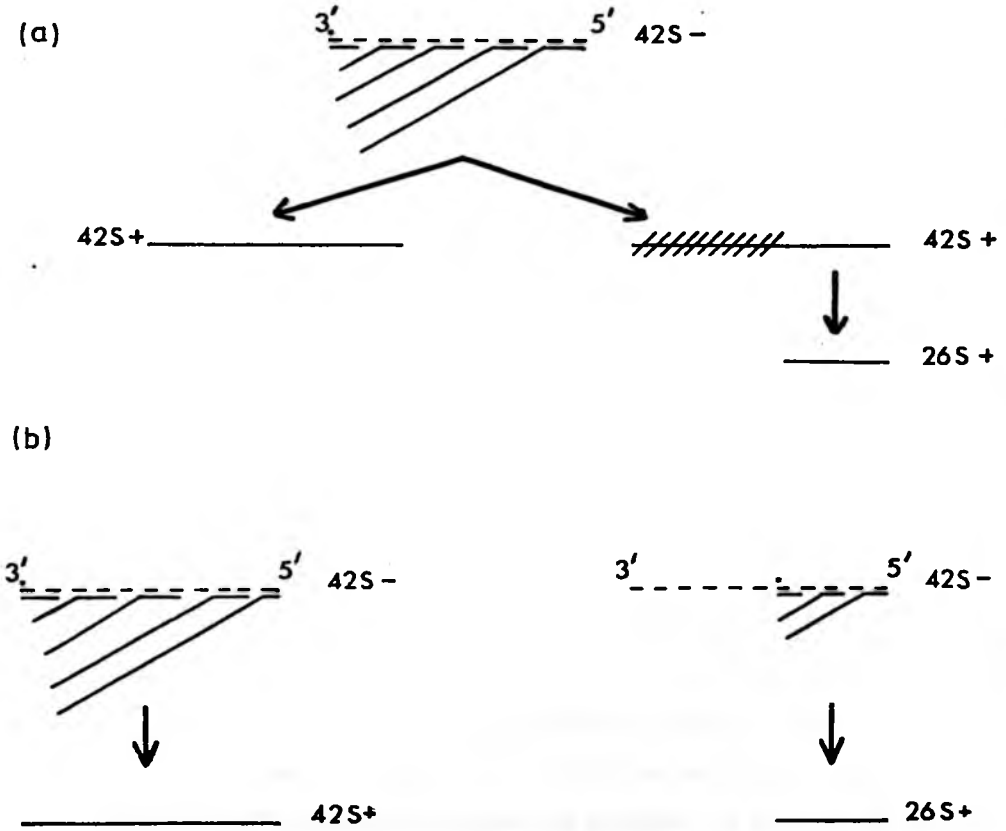

Fig. 18. Possible mechanisms for the generation of a subgenomic RNA species. There are two extreme possibilities for the generation of a subgenomic RNA. Firstly, it can be produced by processing of the 42S RNA (a) or secondly there are two separate RI's one involved in 42S RNA synthesis and the second in synthesis of 26S RNA by a process of internal initiation of transcription (b). --- 42S negative strand RNA; —— positive strand RNA; *transcription initiation site.

UV irradiation of RNA leads to formation of uracil dimers (Miller a Plageman) and these dimers cause premature termination of transcription (Michalke and Bremer; Hackett and Sauerbier). It would seem reasonable to suppose then that the larger the RNA, the more likely it is to be inactivated by UV irradiation because it contains more UV susceptible sites, assuming that the RNAs have a similar base composition. The correlation of the size of the RNA target and the UV sensitivity of the RNA has been demonstrated in E. coli infected with the phage T7 (Brautiga and Sauerbier). In this case, where the RNA is transcribed as a single unit and then processed to give the various discrete mRNAs, it was shown that the target size of a gene, as determined by UV irradiation, represented the sum of the sizes of all the preceding genes up to and including the gene in question i.e. the UV sensitivity of RNA was a direct reflection of its size.

A similar approach was used to show that transcription of the VSV genome involves a single initiation site for transcription at the 3' end of the virion RNA of VSV. A virus suspension was UV irradiated for increasing lengths of time after which the ability of the virus to synthesise RNA in vitro (Abraham and Banerjee) or viral proteins in a coupled transcription-translation system (Ball and White) was examined. In both cases the rate of decay of the gene products were consistent with a single initiation site for transcription, since the rate of inactivation was not dependent on the size of the gene, except for the first gene to be transcribed, but was dependent on the position of the gene in the virion RNA such that the final gene to be transcribed had an apparent size equivalent to the whole of the VSV genome.

It was considered that this approach might be used to determine the size of the template responsible for the synthesis of 26S RNA by irradiating alphavirus infected cells and monitoring the rates at which 26S and 42 S RNA synthesis decayed. It was considered that there could be only one of two possible results. If 26S RNA were produced by processing of the larger 42S RNA (Fig. 18a) then the rate of synthesis of the two RNAs

would decay with similar kinetics because the template responsible for the synthesis of both RNAs would be of the same size, i.e. 4.2×10^6 and would therefore be inactivated at the same rate. On the other hand, if 26S RNA were synthesised by internal initiation of transcription, the rate at which the synthesis of each RNA decayed would correspond to the relative sizes of the 26S and 42S RNAs, or more strictly, the templates on which each RNA was synthesised i.e. 4.2 to 1.8 or 2.3 to 1; this should be compared with a ratio of 1 to 1, which would be the case if the processing mechanism were to operate. It was considered that this difference was sufficiently large to make any interpretation of the experimental results unambiguous. The results of such a series of experiments are described in the following pages.

RESULTS

(i) UV Irradiation of CEF Cells Co-infected with Standard and DI SFV

Preliminary experiments were carried out by infecting CEF cells with Sindbis virus, UV irradiating the cells for various lengths of time and then pulse labelling the cells for one hour with $[^3H]$-uridine. The number of TCA insoluble c.p.m. were determined and that time at which total incorporation had been reduced to 10% of the unirradiated control was found to be approximately ten minutes. The maximum time used in the following experiments was fourteen minutes at an incident energy of 30 ergs/sec/mm^2 (Fig. 19). As the labelling period after irradiation was to be one hour, it was considered that continued translation of 42S RNA might lead to production of more RNA polymerase and that the amount produced would be in inverse proportion to the irradiation time. This would lead to increased RNA synthesis in those cultures receiving little or no irradiation and lead to difficulty in the interpretation of the results so, in order to eliminate this possibility, cycloheximide was added to all cultures during the labelling period to prevent further synthesis of polymerase.

In order to determine the amounts of radioactivity incorporated into each type of RNA, the infected cell RNAs were electrophoresed on polyacrylamide gels and the radioactive RNAs detected by autoradiography. Using radioactive ink as a marker for the autoradiograph, the labelled RNAs were located on the gel, excised and counted in a scintillation counter. A typical RNA gel is shown in Fig. 20 and the areas that would be cut out and counted are indicated.

In order to calculate the actual molecular weights of the RNA templates, instead of the ratios of their molecular weights, it was necessary to include an internal control. This was achieved by co-infecting the cells with both standard and DI SFV. The DI particles can be considered as deletion mutants of SFV where the DI RNA retains the 3' and 5' ends of the standard virus RNA while varying amounts of the internal sequences, depending on the type of DI RNA, have been lost (S.I.T. Kennedy, 1976).

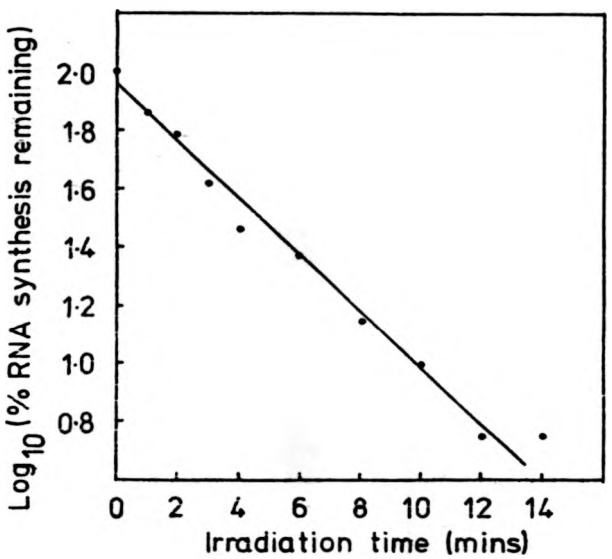

Fig. 19. Effect of UV irradiation on incorporation of $[^3H]$ -uridine into TCA insoluble products. Replicate cultures of CEF cells infected with Sindbis virus were incubated for 5 hours in 199 ADH and then irradiated for 0,1,2,3,4,6,8,10,12 and 14 mins at an incident energy of 30 erg/sec/mm^2. The cells were then labelled for 1h with HEDA containing $[^3H]$ -uridine (2μCi/ml) and cycloheximide (10μg/ml). The monolayers were then washed three times with 5% TCA + 0.1M sodium pyrophosphate and then fixed in the same solution overnight at 4°C. The next day the cells were washed twice with 5% TCA, twice with ethanol/ether (3/1, v/v) and air dried. The monolayers were solubilised by incubation in 0.2ml NaOH for 30 min. at 37°C. An aliquot of each monolayer was then neutralized, triton/toluene scintillator was added and the $[^3H]$-uridine incorporation determined by liquid scintillation counting.

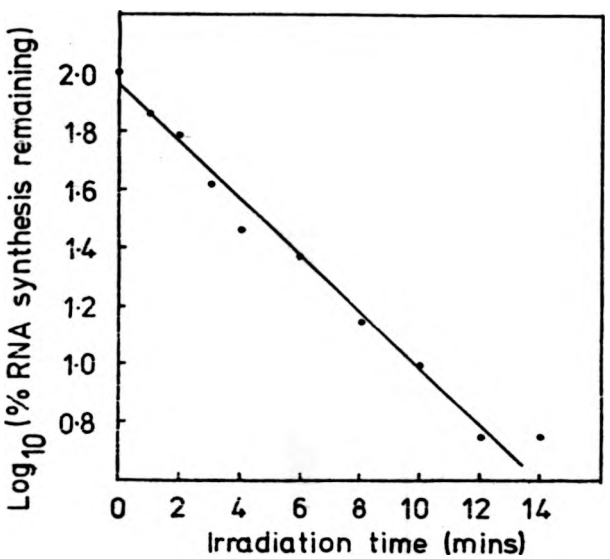

Fig. 19. Effect of UV irradiation on incorporation of [^{3}H] -uridine into TCA insoluble products. Replicate cultures of CEF cells infected with Sindbis virus were incubated for 5 hours in 199 ADH and then irradiated for 0,1,2,3,4,6,8,10,12 and 14 mins at an incident energy of 30 erg/sec/mm^2. The cells were then labelled for 1h with HEDA containing [^{3}H] -uridine (2μCi/ml) and cycloheximide (10μg/ml). The monolayers were then washed three times with 5% TCA + 0.1M sodium pyrophosphate and then fixed in the same solution overnight at 4°C. The next day the cells were washed twice with 5% TCA, twice with ethanol/ether (3/1, v/v) and air dried. The monolayers were solubilised by incubation in 0.2ml NaOH for 30 min. at 37°C. An aliquot of each monolayer was then neutralized, triton/toluene scintillator was added and the [^{3}H]-uridine incorporation determined by liquid scintillation counting.

Fig. 20. Profile of Sindbis virus RNA species separated by polyacrylamide gel electrophoresis. An illustration of a typical separation of virus specified RNAs labelled with [^{32}P] -orthophosphate in the presence of actinomycin D. The black lines designate the areas which would be cut out and counted to determine the incorporation of radioactivity into 42S and 26S RNA.

Cells co-infected with DI and standard SFV contain RFs and RIs of an equivalent size to the DI RNAs (Bruton et al.) hence the replication of the DI RNAs is independent of 42S RNA, except in that the 42S RNA codes for all the necessary proteins. Since internal initiation of 26S RNA synthesis on a negative strand RNA must take place at a point immediately preceding the sequences complementary to the 5' end of 26S RNA then, because the positive strand DI RNAs lack these sequences (Kennedy, 1976) the negative strand DI RNAs cannot contain this putative internal initiation site. The inactivation kinetics of the DI RNA could then be considered to be characteristic for an RNA of this size, which is known (Table 3), and could then act as a reference for the other RNAs inside the infected cell.

UV inactivation follows first order kinetics and hence it is possible to formulate a first order rate equation representing the rate of inactivation of the template with respect to time as follows,

$$-\frac{d(\text{No. of active templates})}{dt} = k_i x \text{ (No. of active templates)} \quad \ldots \text{ Eqn. 1}$$

where k_i is the inactivation constant which will be dependent on the size of the template.

If it is assumed that the rate of transcription of the various RNAs is constant and that this does not change over the labelling period, then the number of c.p.m. incorporated into a particular species of RNA will be proportional to the number of active templates present i.e. c.p.m. in a particular RNA species $\propto$ no. of templates synthesising this RNA and so Eqn. 1 becomes

$$-\frac{d(\text{c.p.m. in specific RNA})}{dt} = k_i \text{ x (c.p.m. in specific RNA)}$$

which on integration gives

$$\ln\left(\frac{\text{c.p.m. in RNA after irradiation for time t}}{\text{c.p.m. in RNA from unirradiated culture}}\right) = k_i \text{ x t}$$

and so a plot of

$$\ln\left(\frac{\text{c.p.m. in RNA after irradiation for time t}}{\text{c.p.m. in RNA from unirradiated culture}}\right) \text{ against t will give a}$$

straight line, if the inactivation follows first order kinetics, and the value of k_i for the various RNAs will be the slopes of the respective lines.

CEF cells were co-infected with DI and standard SFV for four hours, irradiated for various times and then pulse labelled with $[^{32}P]$-ortho-phosphate for one hour. The amounts of label in each type of RNA was quantitated by extraction and separation of the RNAs on polyacrylamide gels. The % RNA synthesis remaining was calculated using the expression

$$\% \text{ RNA synthesis remaining} = \frac{\text{c.p.m. in RNA after irradiation for time t} \times 100}{\text{c.p.m. in RNA from unirradiated culture}}$$

Only those c.p.m. greater than twice the background level were used. The slope of the line,s, was then calculated by the method of least squares; all points were given equal weighting. The graph of $\log_{10}$ (% RNA synthesis remaining) v t (Fig. 21) includes the line of best fit. The inactivation constant k_i, for each RNA was then calculated using the equation

$$k_i = s \times 2.303$$

The fact that incorporation of radioactivity was reduced with first order kinetics suggested that the templates were being inactivated with single hit kinetics and that complicating factors such as direct inactivation of proteins were probably absent.

The k_is of the various RNA templates are summarised in Table 3 along with the calculated size of these templates based on the assumption that DICD. RNA is derived from a template with a size of 0.78×10^6 (Kennedy, 1976). The calculated sizes of the templates for DIY and 26S RNA agree well with the molecular weights of the RNAs derived from these templates (Table 3) suggesting that the assumption of proportionality between the UV sensitivity of a template and its physical size was valid. However, the template for 42S RNA is apparently only 60% of the size of the RNA which is transcribed from it. It was obvious that this paradoxical result could not be correct and so the following hypothesis was devised to explain it. It was proposed that 42S RNA synthesis took place on a large number of template strands but that its level of synthesis was insufficient to

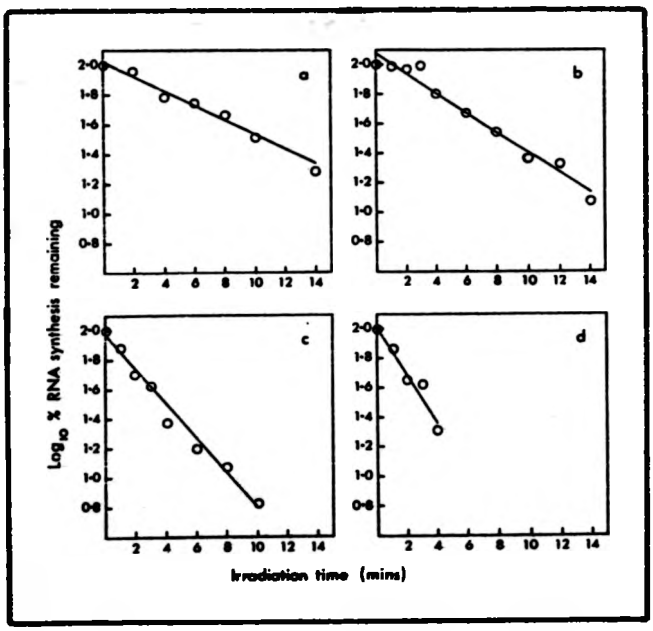

Fig. 21. UV inactivation of RNA synthesis in CEF cells co-infected with standard and DI SFV. Replicate cultures of CEF cells co-infected with standard and DI SFV were irradiated at 4h post infection for O,1,2,3,4, 6,8,10,12 and 14 mins. and then labelled with PFEDA containing [^{32}p] -orthophosphate as described in Section V,x. The radioactivity incorporated into DIC,D (a), DIY (b), 26S (c) and 42S (d) RNA was quantitated and the equation of the line was calculated as described in the text.

	DI SFV RNA species			
	DICD	DIY	26S	42S
k_i (min $^{-1}$)	-0.112	-0.159	-0.270	-0.376
$t_{37\%}$ (min)	8.92	6.29	3.71	2.66
Mol wt x 10^{-6} as determined by inactivation kinetics (DICD = 0.78 x 10^{-6})	(0.78)	1.11	1.88	2.62
Actual mol wt	0.78	1.11	1.8	4.2
Labelling 42S : 26S RNA ratio			1 : 2.84	
Molar 42S : 26S RNA ratio			1 : 6.62	

Table 3. Values of k_i and $t_{37\%}$ for SFV DI, 26S and 42S RNAs. Numerical values for k_i and $t_{37\%}$ were computed as described in the text from the data presented in Fig. 21. The 42S to 26S RNA ratio was determined from that culture which received no irradiation.

necessitate the use of all the templates at any instant in time; however, over the one hour labelling period all of the templates contributed to the synthesis of 42S RNA. For this reason the measured size of the template could be regarded as a time averaged size because the only templates which contribute to the measurements are those actively involved in RNA synthesis at any particular moment while all the templates can be inactivated at all times. Hence, the apparent size of the template represents the true size of the template modified by a factor. This factor is obtained by dividing the average number of templates involved in 42S RNA synthesis at any particular time by the total number of templates used during the hours pulse. It should be relatively easy to test this hypothesis which would predict that, by using conditions under which the 42S to 26S RNA ratio is altered in favour of 42S RNA, the proportion of templates involved in 42S RNA synthesis should also increase and that this would lead to an increase in the apparent size of the template as determined by UV inactivation. Such an experiment is described below.

(ii) UV irradiation of a Sindbis Virus RNA -ve mutant at the Restrictive Temperature

In order to achieve this change in the RNA ratio in favour of 42S RNA a temperature sensitive mutant, N2, was used. When this mutant is shifted from the permissive temperature (30°C) to the restrictive temperature (39°C) it synthesises relatively more 42S RNA than similar cultures maintained at 30°C (see Section III). The experiment was carried out essentially as described above except that incubation was carried out at 30°C for five hours post infection followed by a further ninety minutes at 39°C prior to irradiation; labelling of the RNA with $[^{32}P]$ - orthophosphate was then carried out at 39°C. The results of this experiment, along with those of a similar experiment involving wild type Sindbis virus, are shown in Fig. 22. The inactivation constants calculated from Fig. 22 are shown in Table 4 along with the sizes of the 42S RNA templates which were calculated on the assumption that k_i for 26S RNA represents a template with a size of 1.8×10^6, as found in the previous experiment. The results

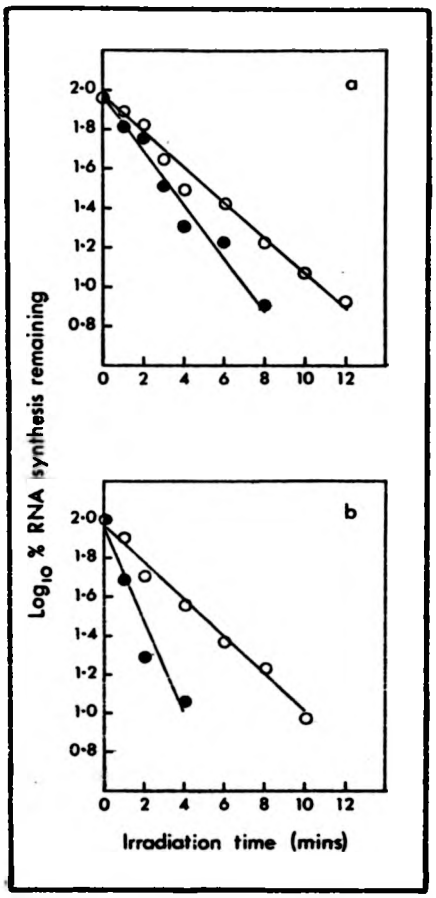

Fig. 22. UV inactivation of RNA synthesis in CEF cells infected with wild type Sindbis virus or the mutant N2. Replicate cultures of CEF cells infected with wild type virus (a) or N2 (b) were incubated at 30°C for 5h and then shifted up to 39°C for a further 90 min. The infected cells were then UV irradiated as described in the legend to Fig. 21 and then labelled with [^{32}P] -orthophosphate at 39°C for 1h. The radioactivity incorporated into 42 (●) and 26S (○) RNA was quantitated and the equation of the line calculated as described in the text.

	Sindbis virus RNA species			
	Wild type		N2	
	26S	42S	26S	42S
k_i (min $^{-1}$)	-0.206	-0.315	-0.221	-0.534
$t_{37\%}$ (min)	4.85	3.18	4.52	1.87
Mol wt x 10^{-6} as determined by inactivation kinetics (26S RNA = 1.8 x 10^{-6})	(1.8)	2.74	(1.8)	4.35
Labelling 42S : 26S RNA ratio	1 : 2.30		1 : 0.83	
Molar 42S : 26S RNA ratio	1 : 5.37		1 : 1.93	

Table 4. Values of k_i and $t_{37\%}$ for Sindbis wild type virus and N2 specified 42S and 26S RNAs. Values for k_i and $t_{37\%}$ were calculated from the experimental results presented in Fig. 22 as described in the text. The 42S to 26S RNA ratios were those expressed by the cultures which received no irradiation.

shown in Table 4 give rise to two important observations. Firstly, the inactivation constants for the 42S RNA template are similar in cells infected with either Sindbis wild type virus or SFV. This corresponds with a similarity in the 42S to 26S RNA ratio determined at the same time (Tables 3 and 4) suggesting that a similar situation might prevail inside cells infected with either of the viruses, leading to a similarly reduced inactivation constant. Secondly, the inactivation constant for the 42S RNA template in N2 infected cells is larger than that of the wild type and its value is such as to give a size for the template of 4.35×10^6 which is in good agreement with the accepted size for 42S RNA of 4.2×10^6 (Simmons and Strauss, 1972a). This increase in the inactivation constant also corresponds with an increase in the relative amount of 42S RNA synthesised in N2 infected cells at the restrictive temperature.

The increase in k_i observed in N2 infected cells, where the 42S to 26S RNA ratio has changed in favour of 42S RNA, is consistent with the hypothesis put forward in II (i). The dependence of k_i on the relative level of 42S RNA synthesis has a number of interesting consequences for the mechanism of transcription of 42S and 26S RNA. These will be elaborated upon in the discussion.

DISCUSSION

The inactivation kinetics for the 26S RNA template calculated from Fig. 21 and shown in Table 3, namely 1.88×10^6, clearly indicates that 26S RNA and its template are both of the same size.
It is thus possible to eliminate any possibility that the 26S RNA is produced by processing of a 42S RNA molecule and so it must, therefore, be synthesised by internal initiation of transcription on a 42S negative strand RNA which acts as a template.

The observation that, in wild type infected cells, 42S RNA is synthesised on a template with an apparent size of 2.62×10^6, whereas in N2 infected cells it appears to have a size of 4.35×10^6, is consistent with the hypothesis put forward in Section II (i) and this will now be considered in a little more detail.

Assuming that the number of template RNAs present in an infected cell is large, it is possible to estimate the time of irradiation required to introduce, on average, one hit per template RNA, using the Poisson distribution as follows,

$$P_r = \frac{z^r}{r!} \cdot e^{-z} \qquad \text{Eqn. 2}$$

where P_r is the probability of a template sustaining r hits when the average number of hits is z/template. The only parameter in equation 2 which can be measured experimentally is the probability of a template receiving no hits, P_o, after irradiation for time t, which is given by

$$P_o = \frac{\text{c.p.m. in RNA after irradiation for time t}}{\text{c.p.m. in RNA from unirradiated cultures}}$$

Therefore, substituting 0 for r and 1 for z, equation 2 becomes

$$P_o = \frac{1^0}{0!} \cdot e^{-1} \qquad \text{Eqn. 3}$$

or, on simplifying and rearranging

$$P_o = \frac{1}{e} = 0.368$$

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In other words, when the number of active templates has been reduced to 37% of its original number or, in this case, when the level of RNA synthesis is reduced to 37% of the unirradiated control then there is, on average, 1 hit per RNA template (Ball and White). The time at which this occurs for the various RNA species was calculated using the data from Figs. 21 and 22 and is shown in Tables 3 and 4; they are expressed as $t_{37\%}$, being the time of irradiation required to reduce the level of a specific RNA synthesised in one hour to 37% of the unirradiated control level.

Having calculated $t_{37\%}$ it is now possible to determine the number of hits per minute/unit length of RNA template. For the sake of convenience the unit length will be taken as having a molecular weight of 10^6. This is quite simply calculated as follows; the number of hits per minute/RNA template species is simply the reciprocal of $t_{37\%}$ and so the number of hits per minute/unit length of RNA template will be found by dividing the reciprocal of $t_{37\%}$ by the molecular weight of the template in millions i.e. number of hits per minute/RNA template species =

$$\frac{1}{t_{37\%} \times \text{mol. wt. of RNA template} \times 10^{-6}}$$

The result of this calculation for the various RNA templates listed in Table 3 is summarised in Table 5. From this it can be seen that the number of hits per minute/unit length of RNA template is fairly constant for the templates of DICD, DIY and 26S RNA, while the value obtained for the 42S RNA template in SFV infected cells is considerably less than that for the other template RNAs. Averaging the inactivation rates for the DICD, DIY and 26S RNA templates gives a value of 0.146 hits per minute/unit length of RNA template or a predicted value of 0.615 hits per minute/42S RNA template compared with an experimentally derived value of 0.376. There is no apparent reason why the 42S RNA template should be hit any less frequently than are the other templates and so it is probable that there are in fact 0.615 hits per minute/42S RNA template and the reason for the low experimental value is as follows. Assuming that there are n

	DI SFV RNA species			
	DICD	DIY	26S	42S
Number of hits/ minute/unit length of RNA	0.144	0.143	0.150	0.089

Table 5. Number of hits/minute/unit length of SFV DI, 42S and 26S RNAs. These values were computed as described in the text.

	DI SFV RNA species			
	DICD	DIY	26S	42S
Number of hits/ minute/unit length of RNA	0.144	0.143	0.150	0.089

Table 5. Number of hits/minute/unit length of SFV DI, 42S and 26S RNAs. These values were computed as described in the text.

templates in the infected cell and that m (m<n), are actively synthesising 42S RNA at any particular point in time, then the total number of hits received by the active templates in one minute is m x 0.615 hits, but the average number of detectable hits received per template is m x 0.615/n. This expression is equivalent to the experimental result of 0.376 hits per minute/42S RNA template (Eqn. 4) and so the percentage of templates in use at any instant in time (given by m x 100/n) is calculated as follows:-

$$0.615 \times \frac{m}{n} = 0.376 \qquad \text{Eqn. 4}$$

or, on multiplying by 100 and re-arranging, Eqn. 4 becomes

$$\frac{m}{n} \times 100 = \frac{0.376 \times 100}{0.615} = 61.1\%$$

Therefore, it would appear that only 61.1% of all possible 42S RNA templates are involved in 42S RNA synthesis at any one time. A similar result was obtained for Sindbis wild type virus where repetition of the above calculations showed that the percentage of templates involved in 42S RNA synthesis was 65.5%.

The hypothesis predicts that increasing the relative amount of 42S RNA made will necessitate involvement of a larger fraction of the total pool of 42S RNA templates, the fraction unused in any instant decreases and hence $t_{37\%}$ should decrease. This is amply borne out by the results presented in Fig. 22 and Table 4, which shows that an increase in the molar 42S to 26S RNA ratio from 1 to 6, as observed in wild type infected cells to a value of 1 to 2 in N2 infected cells was sufficient to decrease $t_{37\%}$ for the 42S RNA template from 3.18 minutes to 1.87 minutes, the value expected for a template with a size of 4.35×10^6, the accepted value for 42S RNA being $4.2 \pm 0.2 \times 10^6$ (Simmons and Strauss, 1972a) and so each RNA is synthesised on a correspondingly sized template and no processing appears to be involved.

It is possible to extend these observations further and calculate the average number of polymerases per template involved in the transcription of 42S RNA. Knowing the proportion of inactive templates,

calculated above, and using the Poisson distribution, it is possible to estimate the average number of polymerases synthesising 42S RNA on each 42S negative strand RNA. In SFV infected cells it was shown that 61.1% of all the templates were involved in 42S RNA synthesis under the conditions used above or, alternatively, a fraction of 0.389 were not involved in 42S RNA synthesis. The average number of polymerases (z) can then be calculated using Eqn. 2 and substituting 0 for r and 0.389 for P_0, which gives an expression to determine the average number of polymerases bound per template, z, when there is a probability of 0.389 that a template will not have any polymerase bound.

$$0.389 = \frac{z^0}{0!} .e^{-z}$$

or, rearranging and solving for z

$$z = \ln (1/0.389) = 0.94$$

Therefore on average, there is approximately one polymerase on each 42S negative strand RNA which is involved in the synthesis of a complementary positive strand of 42S RNA. If this were the case, however, there would be a number of free 42S negative strand RNAs in the cytoplasm at a level which would represent 40% of the total number of 42S negative strand RNAs involved in 42S RNA synthesis. No such free 42S negative strand RNA has been detected in SFV infected cells (Martin and Burke). Similarly, for those 42S negative strand RNAs involved in 26S RNA synthesis, the 3' 2/3 of the 42S negative strand RNA would be single stranded the whole time, if it were assumed that 42S and 26S RNA synthesis took place on separate templates. This seems unlikely and there is good evidence that this is not in fact the case in either SFV (Martin and Burke) or Sindbis virus (Simmons and Strauss, 1972b) infected cells. In both of these studies the RIs were purified from infected cells and characterised by examining the double stranded RNAs remaining after RNAse digestion. In neither case was there any evidence for the existence of two discrete species of RI. However, varying the labelling period showed different labelling kinetics for those sequences corresponding to the nonstructural region of the 42S RNA, which were labelled more slowly than the region containing 26S RNA sequences. However, under equilibrium conditions

the molar ratio of the two segments was unity. Bearing in mind the results obtained above concerning the rates of synthesis of the two RNA species, it would seem reasonable to assume that both segments of the negative strand RNA are used i.e. 42S and 26S RNA are synthesised on the same template although the rate of 42S RNA synthesis is slower. If this is the case, then it is possible to calculate the average number of polymerase molecules actually sythesising RNA on each 42S negative strand RNA, knowing, that there is, on average, 0.95 polymerases synthesising 42S RNA on each 42S negative strand RNA and that the labelling ratio of 42S to 26S RNA is 1 42S RNA to each 2.84 26S RNAs. Assuming that the rate of transcription of 42S and 26S RNA is the same, then it is a simple step to calculate that if each template has 0.95 polymerases synthesising 42S RNA thenthere is, on average, 2.84 x 0.95 equal to 2.70 polymerases involved in 26S RNA synthesis per template and hence a total of 2.70 + 0.95 or 3.65 polymerases on each 42S -ve strand RNA. This is in contrast with a previous estimate of 8 - 9 obtained for SFV by an entirely different procedure (Martin and Burke). A similar value is obtained from the data for Sindbis virus infected cells, namely 3.43 polymerases per 42S negative strand RNA. Using this information it is possible to construct a model for the RIs found in alphavirus infected cells which is shown in Fig. 23. It is envisaged that initiation, and hence the RNA species synthesised, is under strict viral control (See Sections III and IV) but is not influenced by preceding events, in other words RIs are not designated as "26S or 42S RNA synthesising." This means that the ratio in which the two viral RNAs are sythesised is responsive to the prevailing conditions inside the infected cell. In this respect Fig. 23 could depict the same RI at different times with the central representation (ii) being that which predominates in normally infected cells, however should conditions change, such that less 42S RNA is required, then the left hand structure (i) might become dominant or conversely, if insufficient 42S RNA is present for viral needs, the right hand structure (iii) becomes common.

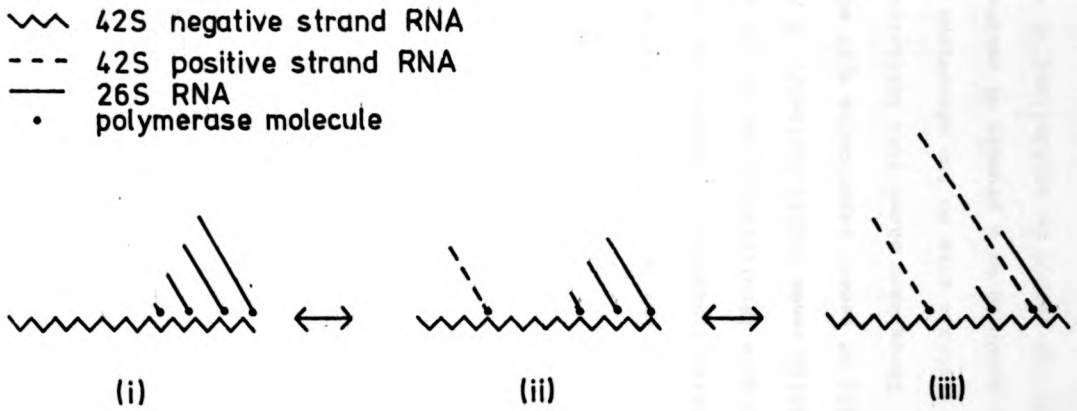

Fig. 23. Proposed structure for the configuration of RI's found in alphavirus infected cells under varying conditions of RNA synthesis.

Although the UV data is consistent with the model put forward above it does not discount the possibility of a ligase being involved. In this case the 42S negative RNA strand would be transcribed in two segments consisting of the 3' 2/3, which would terminate at the point of initiation of 26S RNA synthesis, and a second stage involving internal initiation and transcription of the 26S RNA sequences. Synthesis of 42S RNA would then be achieved by an RNA ligase, the activity of which would be virally controlled and would regulate the RNA ratio. It is unlikely that this ligase is cell specified as it would need to be under viral control but, additionally it seems unlikely that there are any ligases present in eucaryotic cells (Bedows et al). Although there is some evidence that poliovirus polymerase might possess some ligase-like activity (Yin), the presence of a viral specified ligase in Sindbis virus infected cells is unlikely for three reasons. Firstly, no mutant has been detected which sythesises 26S RNA and a second RNA, equivalent to the 5' 2/3 of the 42S RNA; such a mutant might have had a temperature sensitive ligase (See Section III). Secondly, no RNA equivalent to the 5' 2/3 of 42S RNA has been detected in wild type infected cells and thirdly, it has been suggested that a ligase would lead to a far higher recombination frequency in temperature sensitive mutants than has been observed to date (Pfefferkorn) and so the possibility of 42S RNA synthesis by ligation seems highly unlikely. A second possibility is suggested by the results of recent experiments with adenovirus (Westphal, personal communication). These have shown that initiation of all mRNA synthesis takes place at a single site on the adenovirus DNA and that functional mRNAs are then produced by a process of nucleolytic cleavage and ligation. Whereas 26S RNA cannot be synthesised by an analogous mechanism it is conceivable that initiation might take place at the 3' end of the 42S negative strand RNA followed by limited transcription. The RNA-polymerase complex might then leave the template RNA and re-associate at the region for initation of 26S RNA synthesis and transcription would then continue. Such a mechanism for the production of 26S RNA would also be consistent with the UV inactivation data.

Unlike the area of polypeptide synthesis, where picornaviruses and alphaviruses show a number of similarities, these two viruses differ in the way they replicate and transcribe their RNA because alphaviruses use a subgenomic RNA to code for the structural polypeptides whereas poliovirus does not. Alphaviruses, therefore, have a mechanism by which they can produce two different sizes of RNA from a single template. Although picornaviruses have no need for such a mechanism, VSV, a rhabdovirus containing one single stranded RNA of negative polarity, operates a mechanism which appears superficially similar and it might be useful to compare what is known concerning the transcription and replication of VSV and alphavirus RNAs. VSV contains a virion bound transcriptase (Baltimore et al.) which can transcribe five species of mRNA complementary to the single stranded RNA genome (Huang et al.) and in addition, is able to transcribe one complete copy of complementary RNA which can then act as a template for the further synthesis of virion RNA. The similarity between alphaviruses and VSV lies in the ability of the polymerase to transcribe the negative strand RNA in such a fashion as to generate both complete transcripts and subgenomic fragments. However, it does appear that VSV virion bound polymerase can only initiate at the 3' end of the virion RNA and must then transcribe all the genes sequentially (Ball; Ball and White; Breindl and Holland; Roy and Bishop) this is in obvious contrast to alphaviruses where internal initiation takes place. A possible explanation for this difference might lie in the observation that there appears to be little translational control in VSV infected cells and so the rate of polypeptide synthesis closely follows the level of the corresponding mRNA. The order of abundance of the polypeptides and their corresponding mRNAs is the same as the order in which the genes are transcribed (Villareal, Breindl and Holland), the first mRNA to be transcribed being the most abundant. This would suggest that after initiation of transcription the polymerase can terminate at the end of any gene, with increasing probability the further the gene is from the 3' end of the virion RNA; this would then generate the frequency distribution of VSV mRNAs seen in infected cells.

It is not known whether the polymerase terminates and reinitiates at the end of each gene or if the genes are transcribed as polycistronic messages and then processed before being translated, as happens in T7 infected cells (Dunn and Studier, 1973a; 1973b), but recently some evidence has accumulated in favour of a processing model. Firstly, in vitro coupled transcription - translation systems have been shown to make complementary RNAs larger than any single RNA and sometimes of a size equivalent to a complete transcript of the virion RNA (Breindl and Holland) and secondly, experiments designed to look at the structure of the cap on each of the five isolated mRNAs, suggested that the cap had been added to a 5' end terminating with a single phosphate group which would not be the case if the polymerase had initiated at the 5' end of each RNA when it would obviously have been a triphosphate (Rhodes and Banerjee). Nevertheless, although these experiments are suggestive of a processing mechanism the story is far from complete. Nevertheless, whereas in alphavirus infected cells processing of 42S RNA to generate 26S RNA would involve a great deal of wastage (approximately 2/3 of the genome), this is certainly not the case in VSV infected cells, where all segments are used, and it would be interesting to see how a RNA virus risks self-destruction by using a RNAse which is necessary for its replication... assuming that such a RNAse exists.

In this respect then, alphaviruses, along with possibly tobacco mosaic virus (Hunter et al.), which appears to resemble alphaviruses in some respects, seem to be unique in that they have two discrete initiation sites for RNA transcription on one template RNA and synthesise a sub-genomic RNA by internal initiation of transcription. With this knowledge it is now possible to consider the consequences of this unique property and how the virus might benefit from it.

SECTION III

CORRELATION OF LESIONS IN POLYPEPTIDE AND RNA SYNTHESIS IN RNA -VE TEMPERATURE SENSITIVE MUTANTS OF SINDBIS VIRUS

INTRODUCTION

Alphavirus Temperature-sensitive Mutants

(i) Advantages of Temperature-sensitive Mutants

Temperature sensitive mutants, or conditional lethal mutants are, as their name suggests, mutants which are able to grow at one temperature, the permissive temperature as if they were normal but on incubation at another temperature, the restrictive temperature, which is almost always higher than the permissive temperature, the lesion becomes apparent and the mutant fails to grow. Growth in this case is usually defined as production of infectious virus particles and so the virus may still be able to carry out a large number of functions at the restrictive temperature, such as RNA and protein synthesis, at levels comparable to those found in wild type virus infected cells. It is in instances like that that temperature sensitive mutants have their greatest use. If care is taken in their isolation the genome will contain a single point mutation and so, unless host dependant mutants are isolated, a virus with a mis-sense mutation is obtained i.e. a mutation in which one amino acid is replaced by another, hence a single gene and, therefore, a single polypeptide will be affected which should make it possible to allocate certain functions to specific genes. Since it is often easier to gain an insight into the events that take place in a normally infected cell by blocking some aspect of virus growth, the popularity of inhibitors of cellular or viral functions is not entirely unexpected. The temperature-sensitive mutants on the other hand are far more precise in their point of action than any inhibitor of protein or RNA synthesis. This will in turn facilitate the location of the lesion and hence the function of any specific polypeptide.

(ii) Isolation of Mutants

Any chemical which is capable of altering the structure of a nucleotide could be used as a mutagen. Those most commonly used for the preparation of alphavirus mutants were ethyl methane sulphonate, hydroxylamine, nitrous acid, N-nitro-N-methyl-N'-nitrosoguanidine and

5-fluorouracil. The virus was incubated with a low concentration of the mutagen for a short period of time, to reduce the possibility of generating double mutants, then diluted out and plaqued at the permissive temperature. At this point the isolation of the mutants could proceed in two different ways. Firstly, plaques appearing at the permissive temperature could be chosen at random, excised and used to grow up a virus stock. This could be checked for its ability to grow at the permissive and restrictive temperature and any stocks which grew equally well at both temperatures were discarded. Alternatively, a modification of this procedure used originally by Lake and Mackenzie (1973) and adapted by Atkins et al (1974) facilitated the isolation somewhat. The technique involved growing the mutagenized virus at the permissive temperature until plaques were just visible and then moving the virus infected cells to the restrictive temperature; under these conditions plaques derived from wild type virus continued to grow while those caused by temperature-sensitive mutants did not. If small plaques were now used to test for the presence of mutants the chance of finding them was increased ten fold over simply picking plaques at random (Atkins et al.). Once a virus had been shown to be temperature-sensitive its reversion frequency was calculated. This was done by simply growing the virus at the permissive temperature and titrating the virus yield at the permissive and restrictive temperatures. The reversion frequency is given by the ratio of the yield at the restrictive temperature to the yield at the permissive temperature and is a measure of how frequently the virus reacquires the ability to grow at the restrictive temperature. A reversion frequency of 5×10^{-3} to 10^{-4} is usually set as the upper limit to ensure that the results of a biochemical or genetic experiment are not confused by the presence of wild type virus derived from a mutant with a high reversion frequency.

Another source of temperature-sensitive mutants is A. albopictus cells although these have not been characterised to any great extent (Shenk et al.) These cells are passaged at 28°C and when persistently infected, they shed virus continuously at low levels; this virus proves to be temperature-

sensitive. Apparently, without the constant pressure on the virus to retain the ability to grow at 37°C, which is present when stocks are maintained in vertebrate cells, the virus adapts to the lower temperature to the point of losing its ability to grow at the higher temperature.

(iii) Genetic Characterisation

In order to learn more about the various mutations induced in the virus genome it is necessary to determine the number of different lesions involved. It would also be useful to construct a genetic map which would show the relative distances between the various mutations. Unfortunately, although such a map is undoubtedly useful it depends on the ability of the virus to demonstrate recombination i.e. for two mutant genomes inside an infected cell to approach each other sufficiently closely for the transcription enzymes to cross from one genome to the other at the appropriate time and so synthesise a wild type genome from two mutant ones. Obviously, this would happen more frequently when the mutations were widely separated and so it is possible to derive a map of the relative distances between the various lesions based on the frequency with which these events occurred. This in turn facilitates the assignment of lesions to specific polypeptides; unfortunately recombination has not yet been demonstrated in alphavirus infected cells (Burge and Pfefferkorn, 1966b; Tan; Atkins et al.).

Similar difficulties have been encountered in determining recombination frequencies for other RNA viruses. The only two systems that have shown high levels of recombination of their temperature-sensitive mutants have been those using reovirus (Fields and Joklik) and Influenza virus (Mackenzie). This result is not altogether unexpected since both these viruses have segmented genomes and hence the levels of intergenic recombination are high. This is probably due to reassortment rather than recombination. On the other hand experiments with other RNA viruses have shown low levels of recombination e.g. poliovirus 0.02% - 0.05% (Cooper, 1968) and VSV 0.31% - 3.4% (Pringle). This low level of recombination also appears to be true for the RNA phages (Valentine et al.).

Another method of characterisation involves identifying those mutants

with lesions in the same gene; this can be achieved by complementation studies. In theory, if a cell is infected with two mutants each with a single lesion in different proteins then it should be possible to assemble one functional set of proteins from the pool present inside the cell and progeny virus will be produced. Conversely, if both lesions are in the same gene, then it is not possible to assemble a complete set of functional proteins and the amount of virus produced is the same as that seen in single infections. Such complementation has been seen with mutants derived from the HR strain of Sindbis virus (Burge and Pfefferkorn, 1966b; Strauss et al., 1976). Burge and Pfefferkorn originally isolated 23 mutants and found five complementation groups but more recently Strauss et al., who isolated a further 89 mutants, increased this number to seven. On the other hand neither mutants of the AR 339 strain of Sindbis virus (Atkins et al.) nor SFV (Tan) could be shown to complement; the reason for this is not known.

(iv) Biochemical Characterisation

The first criterion investigated for the alphavirus temperature-sensitive mutants was their ability to synthesise RNA on continuous incubation at the restrictive temperature. The mutants were found to exhibit a continuous spectrum of values varying from 0% to 150% of the corresponding wild type control levels. For this reason the mutants have been classified into two or three different groups. Those mutants showing less than 10% of the wild type level of RNA synthesis at the restrictive temperature were defined as RNA -ve. Two different approaches have been used to classify the remainder; Atkins et al. termed them RNA +ve while Strauss et al. divided them into two parts, those with RNA synthesis between 10% and 60% of the wild type level were termed RNA $\pm$ve, but were assumed to have their lesion in the nonstructural region of the genome, while the remainder were defined as RNA +ve.

If the distribution of mutagenized sites were random then it would be expected that the number of RNA +ve and RNA -ve mutants would be a reflection of the ratio of the 42S RNA devoted to each of these functions. Assuming that all the nonstructural polypeptides are involved in RNA synthesis then the expected ratio of RNA +ve to RNA -ve mutants would be 1.8×10^6 to

$(4.2 - 1.8) \times 10^6$ or 0.43. In fact the ratio of RNA +ve to RNA -ve mutants found in each of the isolates described to date have been 0.39 (Atkins et al.), 0.68 (Strauss et al.), 0.44 (Burge and Pfefferkorn, 1966a) and 2.12 (Tan et al.). Whereas the first three values appear to be close to that expected on a random basis there appears to be some sort of bias present in the fourth ratio. Another example of non-random occurrences can be found if the ratio of RNA +ve to RNA -ve mutants derived from any particular mutagen is examined. Tan et al. found that hydroxylamine, for instance, gave rise to far more RNA +ve mutants than would be predicted on a random basis although there is no obvious reason why this should be so. Another extreme example of such a skewed distribution can be seen in the temperature-sensitive mutants of VSV isolated by Pringle. He found four complementation groups and the 169 mutants isolated were distributed such that group I contained 151 mutants, group II contained two, group III contained one and group IV contained 15 mutants. Nevertheless, the characterisation of the Sindbis virus RNA +ve mutants isolated by Atkins et al. has so far argued against such a skewed distribution being present in these mutants (see Section IV).

Very little has been published concerning the RNA -ve mutants. The experiments are complicated somewhat by the fact that these mutants must be incubated at the permissive temperature for the early part of the growth cycle for most experiments so that the virus can build up a sufficient pool of necessary enzymes. Once protein and RNA synthesis is under way the temperature-sensitive mutant infected cells can then be shifted up to the restrictive temperature, when it is hoped that its lesion will be expressed. Results suggest that the two RNA -ve mutants examined so far have a temperature sensitive lesion in that system responsible for 26S RNA synthesis. In addition one of these mutants, ts-11, synthesised large polypeptides with molecular weights of around 110,000 - 130,000 (Waite) while the second mutant, ts-24, synthesised a still larger polypeptide, p200 (Bracha et al.).

The RNA +ve mutants, on the other hand, have been extensively characterised and the three complementation groups (C, D and E) have now been assigned to lesions in specific polypeptides. Those mutants in group C

appear to have a lesion in the core protein which precludes the assembly of nucleocapsids (Burge and Pfefferkorn, 1968; Yin and Lockart). A lesion in the core protein can also cause the accumulation of p144 in some cases; this polypeptide contains the sequences of all three structural proteins (Schlesinger and Schlesinger, 1973; Scheele and Pfefferkorn, 1970).

Cells infected with group D mutants are unable to haemadsorb at the restrictive temperature suggesting the absence of active haemagglutinin on the cell surface. Similarly, group D mutant virus can haemagglutinate red blood cells at the permissive temperature but not at the restrictive temperature (Yin and Lockart). E1 has recently been recognised as the polypeptide with haemagglutinating activity (Dalrymple et al.) and hence group D mutants have a lesion in E1. The case for the group E mutants having their lesion in E2 is largely one of elimination. Although ts-20, the sole member of group E, produces a pE2 which cannot be cleaved at the restrictive temperature, indicative of a mutation in this polypeptide, ts-10 expresses a similar phenotype although it belongs to group D. However, ts-20 and ts-10 are in different complementation groups and so the lesion in ts-20 probably lies in E2.

This unusual behaviour of ts-10, namely the failure to cleave E2 even though its lesion is in E1, has led to the suggestion of an E1-pE2 complex which is non-functional because of the lesion in E1 (Bracha and Schlesinger, 1976b). However the authors make no suggestion as to how the lesion in E1 can affect the cleavage of pE2. Electron microscopic examination of cells infected with mutants representative of Groups D and E at the restrictive temperature provided the following observation. The nucleocapsids of the group E mutant were aligned along the plasma membrane as if in the process of budding through the membrane except that no extracellular virus could be detected. In the cells infected with the group D mutant, on the other hand, the nucleocapsids could be seen in the cytoplasm and were not attached to the membrane. In addition large areas of thickening of the plasma membrane were observed in cells infected with the group E mutant but were absent in Group D mutant infected cells. The size of this thickening was consistent

with its being the viral envelope glycoproteins. On the basis of these observations together with the fact that cells infected with group D mutants cannot haemadsorb at the restrictive temperature Brown and Smith (1975) concluded that the lesion in group D mutants resulted in failure to insert the viral haemagglutinin into the plasma membrane. Since cleavage of pE2 only takes place on maturation of the virus from the cell (Jones et al., 1974) it would seem reasonable to suppose that the failure of the E1-pE2 complex to appear at the surface of cells infected with group D mutants precludes the possibility of the virus maturing and cleaving pE2 and that this is the explanation for the observation that both group D and E mutants resemble one another in this respect.

Very little work has been done with RNA -ve mutants to date. Since these mutants have lesions in the enzymes responsible for RNA synthesis it was considered that they might provide information on the mechanisms by which alphaviruses control the rate at which they synthesise the 26S and 42S RNAs. Specifically it was hoped that they might answer the question of whether one or two polymerases were required for the synthesis of 26S and 42S RNA. For this reason a number of mutants have been characterised with respect to the effect of temperature shift on the rate of synthesis of the two species of RNA. In addition, the polypeptides synthesised by these mutants after shift up to the restrictive temperature were examined in the hope of characterising the defect of the mutants biochemically. The results of these experiments and their consequences for the mechanism of transcription are discussed in the following pages.

RESULTS

(i) RNA Synthesis by an RNA -ve Mutant Under Permissive, Restrictive and Shift-Up Conditions

Before it became possible to do any meaningful experiments with the temperature sensitive mutants it was necessary to characterise their lesions in some way. For this reason, a number of mutants were examined in order to see how the rate at which they synthesised RNA varied with the different conditions used. These were - incubation entirely at the permissive temperature, a shift-up to the restrictive temperature after six hours at the permissive temperature and continuous incubation at the restrictive temperature. RNA synthesis was measured by labelling with $[^3H]$-uridine for thirty minute intervals over a five hour period starting three hours post infection and measuring the amount of label incorporated into TCA insoluble products. The results of such an experiment with one mutant, E268, is shown in Fig. 24 and Table 6. Firstly, it can be seen from Table 6 that, whereas wild type virus synthesised RNA equally well on continuous incubation at 30°C or 39°C, incorporation of $[^3H]$-uridine on continuous incubation of E268 infected cells at 39°C was at the same level as that in mock infected cells whereas at 30°C RNA synthesis approached the levels seen in wild type infected cells. Similar results were seen with all RNA -ve mutants tested (results not shown). Hence, as has been suggested previously (Burge and Pfefferkorn, 1966a), these mutants probably have a defect in one or more of the enzymes involved in RNA synthesis. Further evidence for this theory was found in the results shown in Fig. 24. In wild type infected cells (Fig. 24a), after a shift up to 39°C at six hours post infection the rate of RNA synthesis doubled and continued to increase over the next two hours, however, in cells infected with E268 there was an initial small increase in the rate of RNA synthesis, presumably a temperature effect, but after this the rate declined slowly such that, at eight hours post infection, incorporation of $[^3H]$-uridine was less at 39°C than at 30°C (Fig. 24b). This was interpreted to mean that any more polymerase synthesised after shift up was

Incubation Temperature	$[^3H]$ c.p.m. incorporated by: wild type	E268	Uninfected
30°C	211064	130742	6045
39°C	278111	5820	5859

Table 6. Incorporation of $[^3H]$-uridine by wild type virus or E268 infected cells at restrictive and permissive temperatures. Duplicate cultures of BHK cells infected with wild type virus or E268 or mock infected were incubated at 30°C or 39°C for 3h. The medium was replaced with HEDA containing $[^3H]$-uridine for 4h and then the amount of label incorporated into TCA insoluble products was determined.

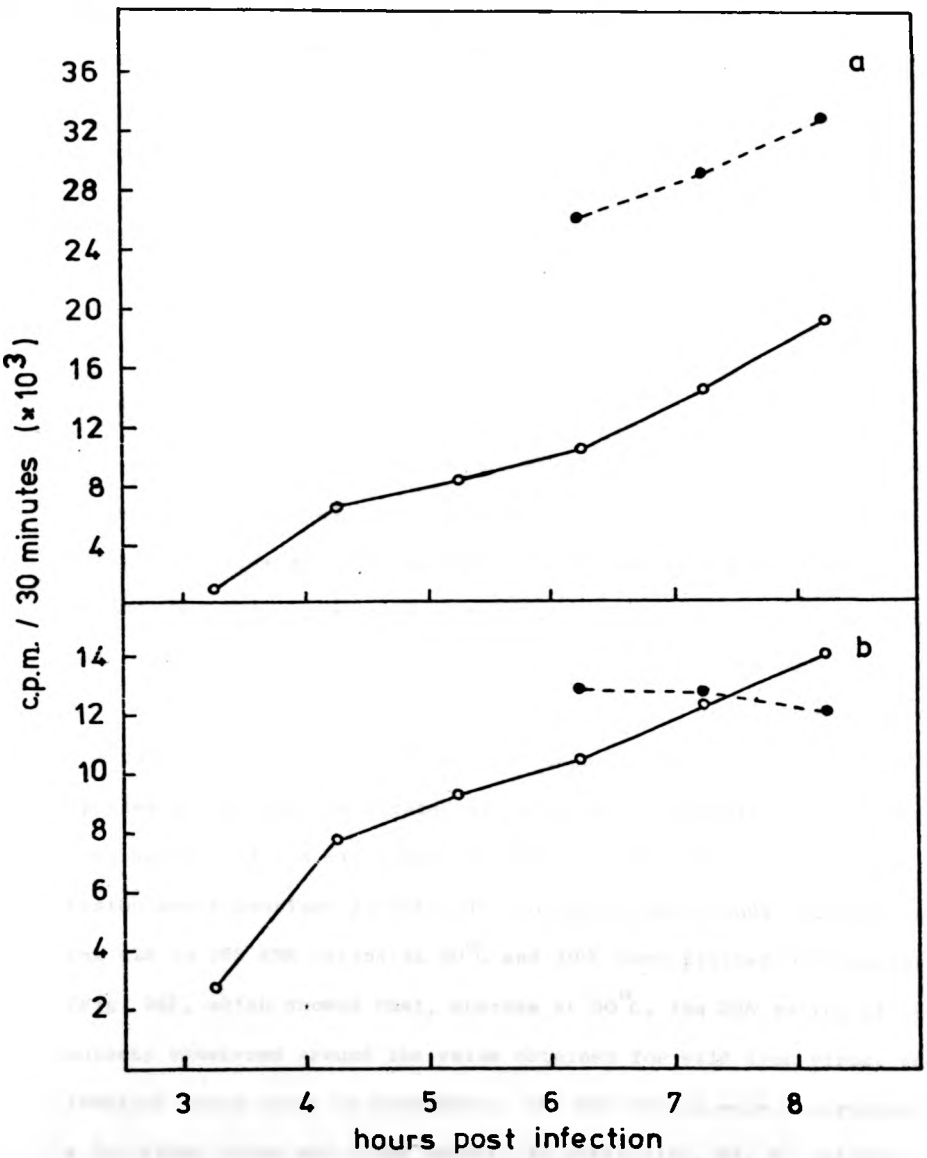

Fig. 24. Rate of incorporation of [^{3}H] -uridine into TCA insoluble products by wild type or E268 infected CEF cells. Replicate cultures of CEF cells were infected with wild type virus (a) or E268 (b) and incubated at 30°C. Triplicate cultures at 30°C (o —— o) were labelled for 30 mins. at 3,4,5,6, 7 and 8 hours post infection with HEDA containing [^{3}H] -uridine (2μCi/ml). Parallel sets of cultures were shifted up to 39°C (● --- ●) at 6h and similarly labelled at 6,7 and 8h post infection. At the end of the labelling period the incorporation of label into TCA insoluble products was determined.

non-functional and that the polymerase already present underwent a decrease in activity. It was not possible to distinguish between the following two alternative explanations for this loss in activity. Firstly, it could be due to complete inactivation of a portion of the pool of polymerases or, secondly, all the polymerases might have undergone a slight loss in activity.

(ii) The 42S to 26S RNA Ratio of RNA -ve Mutants at 30°C and Under Shift-Up Conditions

In order to determine whether the drop in $[^{3}H]$ -uridine incorporation represented a general loss of RNA synthetic activity or, alternatively, whether it represented a specific loss of 42S or 26S RNA, RNA -ve mutants were chosen at random and used to infect duplicate cultures of BHK cells at the permissive temperature. At six hours post infection half the cultures were shifted to 39°C then, at eight hours post infection, the RNA was labelled with $[^{32}P]$ -orthophosphate. The amount of label in each species of RNA was quantitated as described in Materials and Methods, (Section V, vii and viii) and the results obtained for the fifteen mutants tested are summarised in Table 7. To see if any trends could be detected, the 42S to 26S RNA ratios at 30°C and 39°C were plotted on a histogram (Fig. 25), which showed that, whereas at 30°C, the RNA ratios of the mutants clustered around the value obtained for wild type virus, when labelled under shift up conditions, the RNA ratios were distributed over a far wider range and three mutants in particular, N2, N7 and E268 showed very large changes in their RNA ratio. In the case of E268, for instance, the RNA ratio increased nine fold. Whereas these three mutants showed a dramatic increase in the RNA ratio, it should be noted that all mutants underwent a similar, although less pronounced, change. It was apparent, therefore, that all mutants showed a relative decrease in the rate of 26S RNA synthesis and in the case of three mutants in particular this was pronounced. It is important to note that of the fifteen mutants tested not one showed a relative decrease in the synthesis of 42S RNA.

To examine the changes in the pattern of RNA synthesis following a

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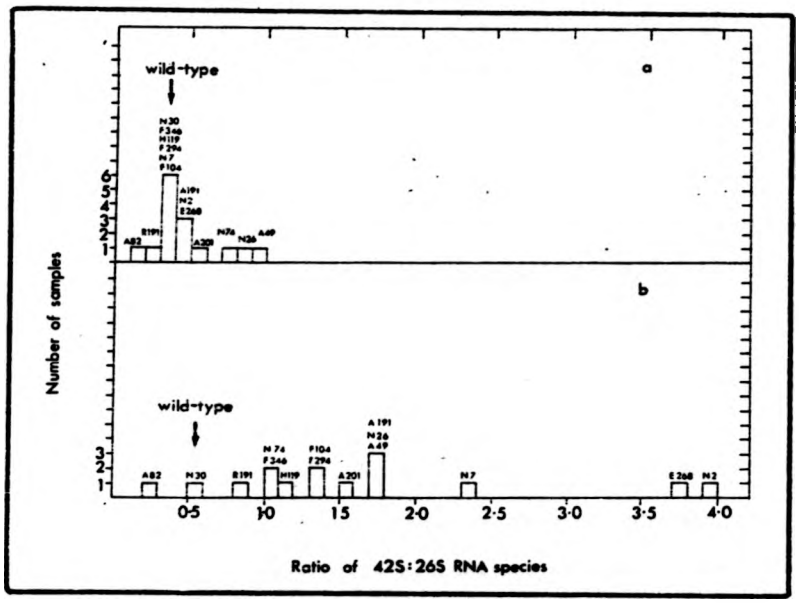
To examine the changes in the pattern of RNA synthesis following a

Mutant	42S to 26S RNA ratio: On continuous incubation at 30°C	42S to 26S RNA ratio: Under shift up conditions
A49	0.95	1.70
A82	0.11	0.26
A191	0.43	1.72
A201	0.53	1.54
E268	0.42	3.78
F104	0.31	1.34
F294	0.38	1.34
F346	0.31	1.10
H119	0.34	1.12
N2	0.48	4.00
N7	0.34	2.36
N26	0.89	1.73
N30	0.36	0.53
N74	0.73	1.08
R191	0.24	0.87
Wild type	0.39	0.56

Table 7. The 42S to 26S RNA ratio of mutants incubated under permissive or shift-up conditions. Duplicate cultures of BHK cells were incubated at 30°C for 6h after which time half were shifted up to 39°C for a further 2h. The cells were then labelled for 30 min with [^{32}P] -orthophosphate and the amount of radioactivity incorporated into each type of RNA was determined.



a, labelled at 30°C; b, labelled under shift up conditions

Fig. 25. Histogram showing distribution of mutants with respect to ratio of 42S to 26S RNA synthesis at 30°C and under shift-up conditions. Duplicate cultures of BHK cells infected with RNA -ve mutants were incubated at 30°C for 6h and then half were shifted up to 39°C. At 8h p.i. the cells were labelled for 30 min with [^{32}P] -orthophosphate and the amount of radioactivity incorporated into each type of RNA was determined.

shift in a little more detail, the experiment was repeated in a slightly modified form with N2 and wild type infected cells. In this case the RNA was labelled at six, seven and eight hours post infection, after the cultures had been shifted up to 39°C at six hours post infection, and the amount of label incorporated into 42S and 26S RNA determined at these various times (Fig. 26). It can be seen that the change in RNA ratio observed in N2 infected cells under shift up conditions was due to a combination of two effects namely, a progressive slow decrease in the rate of 26S RNA synthesis accompanied by a rapid increase in the rate of 42S RNA synthesis. No such effect was seen in wild type virus infected cells where the rates of synthesis of both types of RNA increased gradually after the shift up presumably due to the increase in temperature. However, this increase was not as large as that seen in the rate of synthesis of 42S RNA in N2 infected cells.

The change in RNA ratio seen in N2 infected cells is unlikely to be due to the cessation of functional polymerase synthesis per se since there is a large pool of previously synthesised enzyme already present and so it was assumed that the loss of 26S RNA synthesis was due to a temperature sensitive lesion in a polypeptide that was specific for the synthesis of 26S RNA. A similar observation has been made for other RNA -ve temperature sensitive mutants of Sindbis virus (Scheele and Pfefferkorn, 1969b; Bracha et al.).

(iii) Polypeptide Profiles of RNA -ve Temperature Sensitive Mutants at 30°C and Under Shift-Up Conditions

In order to assign certain roles in RNA synthesis to specific non-structural polypeptides it was necessary to correlate a lesion in RNA synthesis with a corresponding aberrant or missing nonstructural polypeptide. The mutants examined above for their RNA phenotype were also examined for their polypeptide phenotype under similar conditions namely, duplicate cultures of infected and mock infected cells were incubated at 30°C for six hours and then half the cultures were shifted to 39°C. At eight hours post infection the cells were pulse labelled with [^{35}S] -

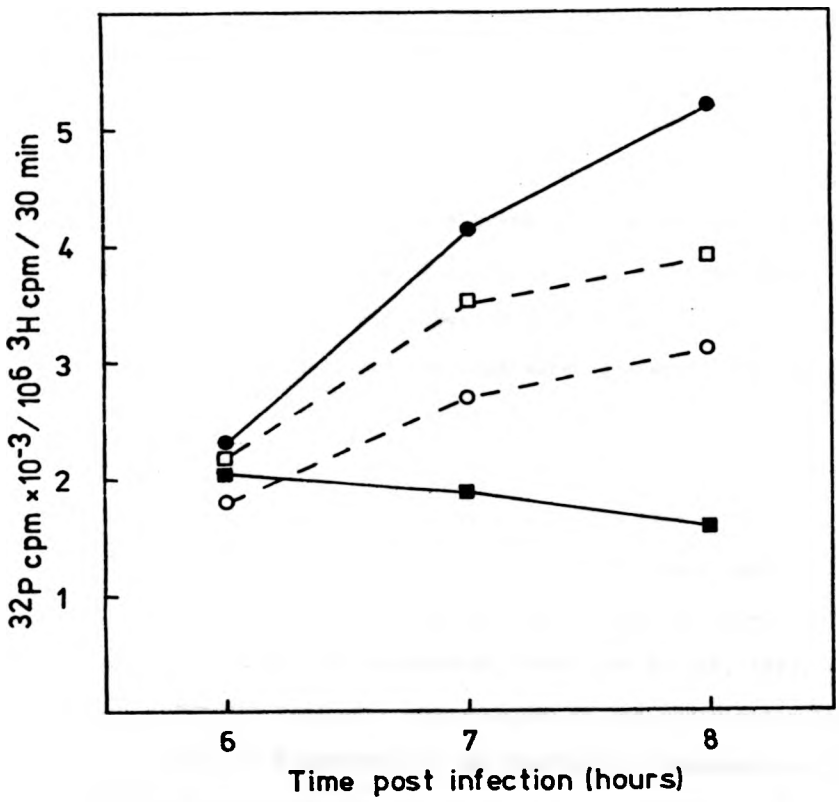


Fig. 26. Effect of shift up conditions on the rate of synthesis of 42S and 26S RNA in wild type and N2 infected CEF cells. Replicate cultures of CEF cells infected with N2 (● —— ●, ■ —— ■) or wild type virus (O --- O, □ --- □) were incubated at 30°C for 6h. The cultures were then shifted up to 39°C and labelled with [^{32}P] - orthophosphate for 30 min. intervals at 6,7 and 8h post infection. At the end of the labelling period the nucleic acids were extracted and the amount of radioactivity incorporated into 42S (O, ●) and 26S (□ , ■) RNA was determined.

methionine for thirty minutes and the labelled polypeptides were then separated on polyacrylamide gels and the profiles examined to see if any new polypeptides could be detected. Three mutants were found that synthesised precursors of the nonstructural polypeptides at the restrictive temperature but not at the permissive temperature; F294 synthesised p150 while N2 synthesised p215 (Fig. 27); N7 also synthesised p215 (C. Clegg, personal communication). A comparison of the tryptic peptide map of p215 from N2 infected cells incubated under shift up conditions and from wild type infected cells labelled in the presence of zinc ions is shown in Fig. 28. When the map of p215 from N2 infected cells was prepared it was not possible to isolate p215 from wild type infected cells in sufficient quantities to use it as a control. The two maps were, therefore, prepared under slightly different conditions and at different times but it is quite obvious, nevertheless, that the two polypeptides are related. There thus appeared to be a good correlation between a dramatic loss of 26S RNA synthesis, shown by N2, N7 and E268, and accumulation of p215 shown only by N2 and N7. A similar observation has been made with the mutants originally isolated by Burge and Pfefferkorn, where one mutant, ts24, showed a rapid loss of 26S RNA synthesis when shifted to the restrictive temperature and synthesised a polypeptide, at the restrictive temperature, termed p200, which was unrelated to the structural polypeptides, and was therefore assumed to be a precursor of some of the nonstructural polypeptides (Bracha et al.). On the other hand synthesis of p150 in F294 infected cells at 39^{o}C did not cause any such dramatic change in the 42S to 26S RNA ratio. It seemed, then, that a temperature-sensitive mutation in a polypeptide responsible for the synthesis of 26S RNA could also result in the accumulation of p215 when it was synthesised at the restrictive temperature.

The processing of these polypeptides was then examined in more detail for the three mutants F294, E268 and N2. The reversion frequency of N7 was found to be rather high (Atkins, personal communication) and so, because N2 appeared to be a similar mutant with a much lower reversion frequency, it was not considered necessary to repeat the experiments with N7. Four cultures infected with one of the three mutants were incubated at 30^{o}C for

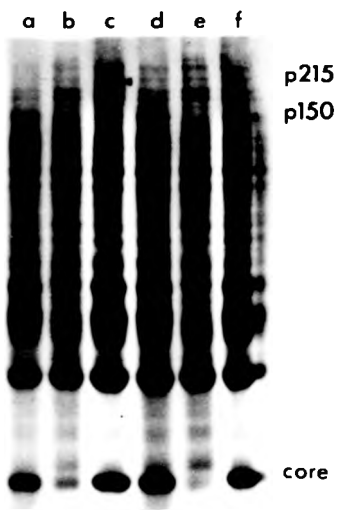


Fig. 27. Polypeptide profiles of N2 and F294 infected cells under shift up conditions. Triplicate cultures of CEF cells were infected with N2 (a, b and c) or F 294 (d,e and f). Two cultures of each (a,c,d and f) were incubated at 30°C while one of each (b and e) was incubated at 39°C. At 6h post infection one of each culture was shifted up to 39°C (c and f) and all six cultures were labelled with $[^{35}S]$ -methionine for 30 mins. at 8h post infection. The cells were then extracted and electrophoresed on polyacrylamide gels.

Fig. 28. Tryptic peptide maps of p215 and the large polypeptide synthesised in N2 infected CEF cells at the restrictive temperature. Ten vial cultures of N2 infected cells were incubated at 30°C for 6h and then shifted up to 39°C for a further hour. The medium was then replaced with labelling medium containing 250μCi/ml of $[^{35}S]$ -methionine for 2h. The polypeptides were extracted, electrophoresed and tryptic peptide mapped as described. The map of p215 is the same as that shown in Fig. 7.

six hours after which time two cultures were shifted up to 39°C for thirty minutes and then all four cultures were labelled with [^{35}S] -methionine for fifteen minutes. One of the cultures labelled at 30°C was shifted up and chased for thirty minutes at 39°C while the second was chased at 30°C; similarly one culture labelled at 39°C was shifted down to 30°C and chased for thirty minutes while the second was chased at 39°C. This gave all four possible combinations for labelling and chasing at 30°C and 39°C (Figs. 29 and 30). It was difficult to see nsp82 in this experiment for two reasons, firstly there was only a short chase period and so only a small amount of p76 had been converted to nsp82 and secondly, temperature sensitive mutants do not inhibit host cell protein synthesis to the same degree as wild type virus and so it was not always easy to see the rather diffuse band that corresponded to nsp82. However, at 30°C, N2 synthesised all three nonstructural polypeptides (Fig. 29 lane a). On the other hand, if the cultures were incubated at the restrictive temperature at any time during the experiment, either during the pulse or the chase period, this was sufficient to prevent the synthesis of the nonstructural polypeptides and cause the accumulation of p215 (lanes b, c and d). A similar effect was seen in F294 infected cells. On continuous incubation at 30°C (lane e), all three nonstructural polypeptides were synthesised. On the other hand, if the cultures were chased at 39°C, irrespective of the labelling temperature, only small amounts of nsp60 and nsp89 were produced although this was not accompanied by the appearance of p150, which was probably degraded during the chase period. By contrast, p76 was seen under all conditions, albeit faintly in some cases.

The results of a similar experiment performed with E268 infected cells (Fig. 30) showed that nsp60 and p76 were produced under all conditions of pulse and chase but, whereas at 30°C nsp89 was stable (lanes a and c), when chased at the restrictive temperature (lanes b and d), no nsp89 was detected. This suggested that in E268 infected cells incubated at the restrictive temperature, the dramatically altered 42S to 26S RNA ratio and the instability of nsp89 might be two different phenotypic

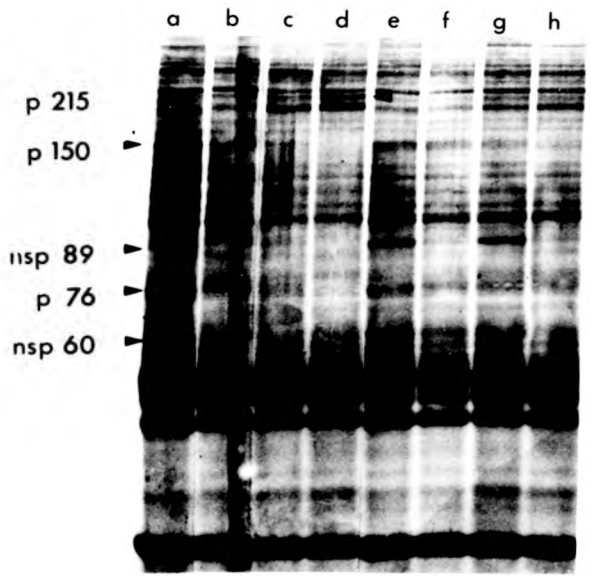


Fig. 29. Polypeptide profiles of F294 and N2 infected cells under shift-up and shift-down conditions. Replicate cultures infected with N2 (a-d) and F294 (e-h) were incubated at 30°C for 6h and then two of each (c,d,g and h) were shifted to 39°C. At 7h post infection the medium was replaced with labelling medium for 15 min. At the end of this period two cultures were shifted up to 39°C (b and f) and two shifted down to 30°C (c and g). The labelling medium was replaced with chase medium for 1h and then the cultures were prepared for polyacrylamide gel electrophoresis.

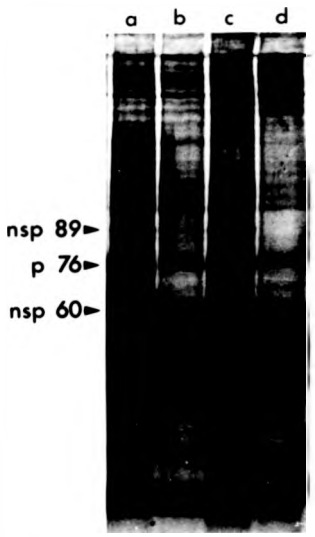


Fig. 30. Polypeptide profile of E268 infected CEF cells under shift-up and shift-down conditions. The experiment was carried out essentially as described in the legend to Fig. 29 namely four cultures of CEF cells infected with E268 were incubated at 30°C for 6h and then two (c and d) were shifted up to 39°C. At 7h post infection the medium in all four cultures was replaced with labelling medium for 15 min. and then one culture was shifted up to 39°C (b) and one shifted down to 30°C (c). All four cultures were then chased for a further hour and the cells extracted and prepared for polyacrylamide gel electrophoresis.

expressions of a single lesion in the genome of E268. A similar observation of polypeptide instability in cells infected with temperature-sensitive mutants of VSV has also been reported recently (Knipe et al.).

(iv) Instability of nsp89 in RNA -ve Mutant Infected Cells at 39°C

If the inability to synthesise 26S RNA at the restrictive temperatures was related to the inability to produce nsp89, in E268 infected cells, then it was of interest to see whether nsp89, which had been previously synthesised at 30°C in N2 infected cells would prove to be unstable when incubated at 39°C. To investigate this possibility, N2 or wild type infected cells were labelled with [^{35}S] -methionine at 30°C from five and a half to six hours post infection and then chased for a further thirty minutes to allow the stable end products to accumulate. These cultures were then shifted to 39°C and harvested after various times (Fig. 31). The results clearly show that, in wild type infected cells all nonstructural polypeptides are stable, except for p76 which is converted to nsp82, while in N2 infected cells, nsp89, which is present at the start of the experiment (lane d), has almost completely disappeared after one hour at 39°C and is completely absent after three hours at 39°C. Five other mutants were examined in a similar experiment and this showed that in all cases, after two hours at 39°C, the intensity of the band corresponding to nsp89 had decreased (Fig. 32), however, in only one mutant, F104 did this appear to be as quick and complete as it was in N2 infected cells. This again suggested that a rapid disappearance of nsp89, as in N2 and E268 infected cells, led to a dramatic change in the 42S to 26S RNA ratio whereas a slower rate of degradation, as in the other mutants with the possible exception of F104, was also paralleled by a smaller change in RNA ratio.

It was considered possible that nsp89 might have been undergoing some reversible modification which changed its electrophoretic mobility. To investigate this, a similar experiment to that described above was performed with N2 except that after a period of time at 39°C the cultures were returned to 30°C to see if nsp89 would reappear. The results shown in Fig. 33 suggest that, after ninety minutes at 39°C, nsp89 had

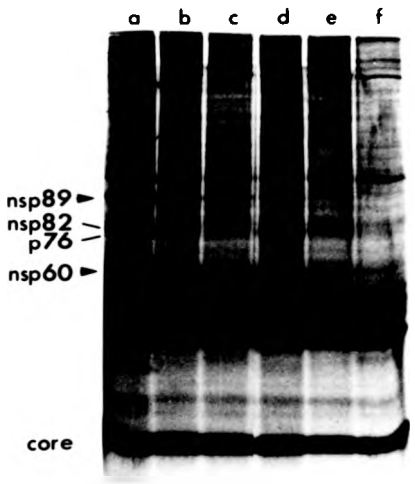


Fig. 31. Stability of nsp89 in N2 and wild type infected CEF cells. Triplicate cultures of CEF cells infected with wild type virus (a,b,c) or N2 (d,e,f) were incubated at 30°C for 5.5h. The medium was replaced with labelling medium for 30 min. and this, in turn was replaced with chase medium for a further 30 min. at 30°C. The cultures were then shifted up to 39°C and chased for a further Oh (a,d); 1h (b,e) and 3h (c,f). The cell extracts were then processed for polyacrylamide gel electrophoresis.

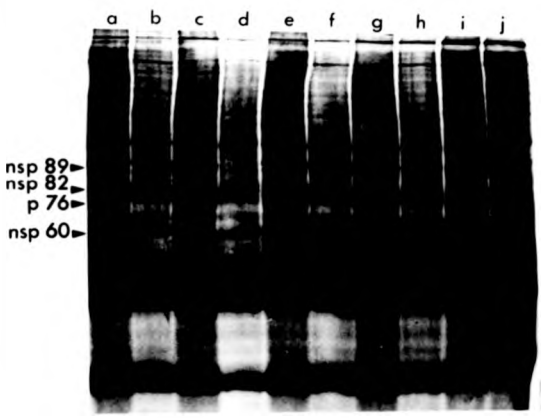


Fig.32. Stability of nsp89 in cells infected with RNA -ve mutants. Duplicate cultures of CEF cells infected with A201 (a,b), F104 (c,d), N26 (e,f), N74 (g,h) and R191 (i,j) were incubated at 30°C for 5h. The medium was replaced with labelling medium for 30 min. and this was in turn replaced with chase medium for a further 30min. at 30°C. Half the cultures were then shifted up to 39°C (b,d,f,h and j) while the remainder were chased at 30°C. All cultures were harvested at 8h post infection and prepared for polyacrylamide gel electrophoresis.

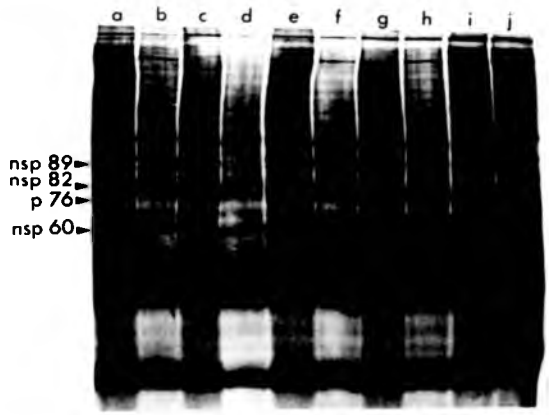


Fig.32. Stability of nsp89 in cells infected with RNA -ve mutants. Duplicate cultures of CEF cells infected with A201 (a,b), F104 (c,d), N26 (e,f), N74 (g,h) and R191 (i,j) were incubated at 30°C for 5h. The medium was replaced with labelling medium for 30 min. and this was in turn replaced with chase medium for a further 30min. at 30°C. Half the cultures were then shifted up to 39°C (b,d,f,h and j) while the remainder were chased at 30°C. All cultures were harvested at 8h post infection and prepared for polyacrylamide gel electrophoresis.

disappeared (lane c) but a further ninety minutes incubation at 30°C did not cause it to reappear (lane d), while nsp89 was still present in a culture maintained at 30°C for an equal length of time (lane b). It thus appears that nsp89 synthesised at 30°C in cells infected with N2, and also E268 (results not shown), becomes unstable and is degraded when cultures are shifted to 39°C.

It is therefore, tentatively suggested that nsp89 is in some way involved in the synthesis of 26S RNA on the basis of the following evidence:-

(a) On shift up to the restrictive temperature both E268 and N2 undergo a dramatic change in their 42S to 26S RNA ratio, which involves a decrease in the rate of synthesis of 26S RNA.

(b) N2 synthesises p215 at the restrictive temperature. Processing of p215 requires the first proteolytic cleavage to take place between nsp89 and nsp82 (Section I, vii) and it is presumably a temperature sensitive alteration in the conformation of one of these two polypeptides in p215 that prevents this cleavage.

(c) nsp89 when synthesised at 30°C in N2 infected cells become unstable and is degraded when shifted up to 39°C.

(d) E268 on incubation at the restrictive temperature synthesises nsp60 and p76 but does not make nsp89.

It should, however, be borne in mind that nsp89 is also unstable in other mutant infected cells, although not to the same degree, and so the assignation of the ability to regulate the synthesis of 26S RNA to nsp89 must remain tentative.

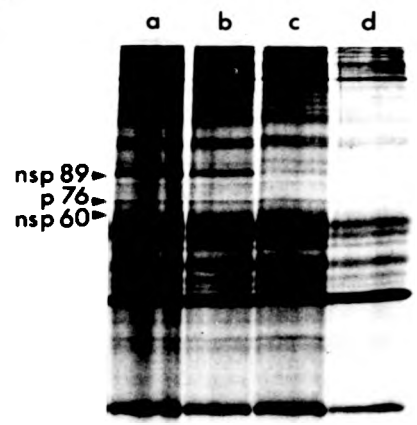


Fig.33. Nsp89 does not undergo reversible modification. Replicate cultures of N2 infected CEF cells were incubated at 30°C for 4h. The medium was then replaced with labelling medium for 30 min after which time this was replaced with chase medium for 30 min. and then two cultures (c and d) were shifted up to 39°C while the other two (a and b) were retained at 30°C. After a further 90 min. two cultures (a and c) were prepared for polyacrylamide gel electrophoresis and a third culture (d) was shifted down to 30°C. After a further 90 min. at 30°C both remaining cultures were harvested and the cell extracts prepared for polyacrylamide gel electrophoresis.

DISCUSSION

The results presented above suggest that N2, N7 and E268 have a similar lesion in their RNA synthetic machinery at 39°C which is necessary for the synthesis of 26S RNA. Two of these mutants synthesise p215, a precursor to all three nonstructural polypeptides, at 39°C while the third E268, synthesises nsp60 and p76 but does not appear to make any nsp89. Additionally, if N2 or E268 are allowed to synthesise their nonstructural polypeptides at 30°C, and are then shifted up to 39°C, it is found that nsp89 is specifically degraded, something which does not occur in wild type virus infected cells. How, then can all these phenotypic expressions be attributed to a single point mutation or must it be concluded that these mutants do, in fact, contain multiple lesions. The possibility of multiple lesions cannot be excluded but, on the other hand, the suggestion that a single lesion could be responsible for all the above observations would be more acceptable if it could be shown that one single amino acid change, derived from a single point mutation, could alter the thermal stability of a polypeptide. Such an observation has been reported for the thermal stability of the α-subunit of tryptophan synthetase derived from mutants of E. coli (Yutani et al.). These mutants synthesised α-subunits which differed from the wild type by a single amino acid change (Yanofsky and Horn). After a period of time at an elevated temperature these subunits had residual activities which varied from 20% to 200% of the corresponding wild type activity. Presumably these differences in activity after exposure to higher temperatures were due to a change in the three dimensional conformation of the polypeptide chain, which, in turn, led to a change in the enzymic activity. It is proposed, therefore, that E268, N2 and N7 have their temperature sensitive lesion in nsp89, a polypeptide responsible for the synthesis of 26S RNA. When this polypeptide is synthesised at 30°C its conformation is such that it has some enzymic activity but, when the temperature is raised, there is a change in the three dimensional configuration of the polypeptide and it is this conformational change that results in a reduction or loss of enzymic activity and increased sensitivity

to proteolytic digestion by exposure of protease sensitive sites. This proposed difference in the conformation at the two temperatures would then explain the instability of nsp89 when synthesised at 39°C but not at 30°C in cells infected with E268. In cells infected with N2 and N7 it is proposed that the lesion in nsp89 is near its junction with nsp82 and hence, when p215 is sythesised at the restrictive temperature, it adopts a conformation that does not allow access of the proteolytic enzymes to the cleavage site and so, because nsp82 must be removed before any further processing can take place, p215 accumulates. Similar observations have been made in work on the RNA +ve mutants of Sindbis virus (Strauss et al., 1969; Waite; Clegg, personal communication) and SFV (Keränen and Kääriäinen) where precursors to the structural polypeptides were shown to accumulate when temperature sensitive mutant infected cultures were incubated at the restrictive temperature. However, this result is not peculiar to alphavirus temperature sensitive mutants. Work with a temperature sensitive strain of poliovirus, LSc, demonstrated that an accumulation of high molecular weight polypeptides coincided with a corresponding reduction in the synthesis of smaller polypeptides, when the virus was grown at 39°C (Garfinkle and Tershak). Similarly, a temperature sensitive mutant of poliovirus with a lesion in one of the structural polypeptides has been shown to accumulate a high molecular weight precursor to the structural polypeptides when incubated at the restrictive temperature (Cooper et al., 1970b). It seems, then, that production of stable, uncleaved precursors at the restrictive temperature is a common occurrence in cells infected with temperature sensitive mutants of alphaviruses and picornaviruses. This is presumably due to the use of polycistronic messenger RNAs to code for their various polypeptides.

It is, therefore, possible that a single mutation in the area of the genome corresponding to nsp89 could give rise to the phenotypic responses described above but this would have been more convincing had it been possible to demonstrate that N2, N7 and E268 were members of a single

complementation group. Previous attempts to demonstrate complementation in these mutants have not met with any success (Atkins et al.). Similarly, Tan, who isolated a number of mutants of SFV, did not achieve any complementation, although mutants derived from the HR strain of Sindbis virus do complement (Burge and Pfefferkorn, 1966b; Strauss et al., 1976). A further series of experiments to define complementation groups was initiated after the recent report in which complementation was apparently facilitated by magnesium ions (Brawner et al.). Unfortunately, whereas magnesium ions could be shown to reduce the titre of Sindbis virus, reportedly due to aggregation of the virus particles, this did not increase yields of virus from cells mixedly infected with RNA +ve and RNA -ve mutants at the restrictive temperature (results not shown). The reason for this inability of the virus to complement is unknown.

It is convenient at this point to return to the question of the number of nonstructural polypeptides that are coded for by Sindbis virus before any consideration is given to their possible function. A recent report has suggested that there are four complementation groups for the RNA -ve mutants of Sindbis virus (Strauss et al., 1976). However, as the authors point out, assignment of complementation groups might be unreliable when production of uncleaved precursors is involved; this is the case with the complementation group they have called B. This group contains only one mutant, ts11, which accumulates large polypeptides with a molecular weight of 110,000 - 130,000 when incubated at the restrictive temperature after shift up (Waite). It is also difficult to predict how temperature sensitive mutants would behave in complementation experiments if the lesion were in a polypeptide that had two functions. For instance, the coat polypeptide of the MS2 type of RNA phage not only encapsidates the RNA to produce new virus particles but also serves as a feedback inhibitor for the synthesis of replicase (Lodish and Zinder; Gussin; Garwes et al.). However,the two functions would have to be essential rather than regulatory, as in MS2, if the two lesions were to behave as separate complementation groups. It is necessary, therefore, to view with

caution any extrapolation where the number of complementation groups is assumed to be synonymous with the number of polypeptides, especially in the case of viruses that produce their polypeptides by cleavage of polyproteins.

The actual number of nonstructural polypeptides is, perhaps, open to question but this is only of secondary importance compared to the number of functions performed by them in virus infected cells. The nonstructural polypeptides could express a number of enzymic activities and some of these possibilities are considered below:

(a) An enzyme activity responsible for the synthesis of 26S RNA. This is the only activity that has definitely been identified. It appears to be the primary lesion in N2, N7 and E268 but loss of 26S RNA synthesis also appears to be a secondary effect of other lesions seen in the remainder of the RNA -ve mutants. This possibility will be considered in more detail in the final discussion.

(b) An enzyme activity responsible for the synthesis of 42S RNA. No mutant has been found in which 42S RNA synthesis is inhibited specifically on shift up to 39°C. If 42S RNA synthesis is inhibited in any of the mutants examined it must also be the case that 26S RNA synthesis is inhibited more so for the 42S to 26S RNA ratio to increase in favour of 42S RNA after a shift as has been observed (Fig. 25). No such mutant has been found.

(c) A separate enzyme activity to synthesise the complementary RNA strand using virion RNA as a template. Such an activity has been suggested for a polymerase synthesised in picornavirus infected cells (Arlinghaus and Polatnick; Cooper et al., 1971). A similar activity also appears to be present in Qβ infected cells (August et al.; Eikholm and Spiegelman; Shapiro et al.) and could conceivably exist inside alphavirus infected cells as well. Under the conditions used in the experiments reported above such an activity would not have been detected and so this possibility must remain open.

(d) Two separate polypeptides are required for initiation and transcription. No mutants were detected that ceased to synthesise all RNA when

shifted up to the restrictive temperature, nevertheless all these mutants are RNA -ve and so, by definition, do not synthesise any RNA on continuous incubation at 39°C. Presumably once the polypeptides responsible for these functions have been synthesised at the permissive temperature they are moderately stable when the temperature is raised.

(e) One polypeptide might be required for inhibition of host cell DNA/RNA/protein synthesis. If a polypeptide were produced solely for this purpose then the mutant would not be RNA -ve as this mutation would not have affected the functioning of the polymerase. However, if this mutation caused the accumulation of a high molecular weight precursor this might then block polymerase synthesis indirectly. Alternatively it is possible that one of the polymerase polypeptides can inhibit host cell functions in addition to fulfilling its normal role.

(f) One polypeptide might be required for capping of the 5' terminus of the viral mRNAs with an inverted 5'-5' linkage which appears to be a universal phenomenon in eucaryotes (see Introduction to Section II). The negative stranded RNA and the double stranded RNA and DNA viruses which contain virion bound polymerases, are able to cap the RNA they synthesise e.g. vaccinia (Wei and Moss), reovirus (Furuichi et al., 1975b) and VSV (Abraham et al.). It is not known how positive strand RNA viruses are capped but to this extent poliovirus is an exception in that the 5' end of the RNA is blocked with a small polypeptide and not a cap (Lee et al.). Perhaps one of the Sindbis virus nonstructural polypeptides might have the ability to cap the various viral mRNAs, or alternatively, the capping enzymes could be host specified and would then be found in the cell cytoplasm. Such enzymes have been found in cell free extracts derived from L cells and wheat germ (Muthukrishnan et al.).

The paucity of knowledge concerning animal virus RNA polymerases is a direct reflection of the difficulty of purifying these enzymes and using them to construct cell free RNA synthesising systems. However, the above results have given an insight into how Sindbis virus might regulate the rate at which it synthesises 26S and 42S RNA. Although there are two

messenger sense RNAs synthesised in Sindbis virus infected cells only one thermolabile RNA synthesising system has been found, that responsible for the synthesis of 26S RNA (Fig. 25). It is possible to calculate the probability of failing to detect the 42S RNA polymerase, assuming it is present, as follows; fifteen mutants have been examined for their RNA ratio after a shift up to 39°C and these were chosen at random from the original 75 RNA -ve mutants isolated. If it is assumed that there are four complementation groups (the most unfavourable case) and that all groups are equally represented then there would be 75/4 or 19 mutants with the lesion in the polymerase responsible for the synthesis of 42S RNA. The probability, then, of not choosing any of these mutants after fifteen attempts is given by

$$\frac{56}{75} \times \frac{55}{74} \times \frac{54}{73} \times \quad \ldots \quad \times \frac{42}{61} = 0.0097$$

or less than 1 chance in one hundred and if there were only three complementation groups this probability would shrink to 1 in 1000. It seems unlikely, then, that such a mutant has remained undetected, especially if the following argument is taken into account. If one of the four complementation groups is assumed to be that responsible for 26S RNA synthesis then it would be expected that 25% of the mutants tested so far would be deficient in this way. In fact three such mutants, N2 N7 and E268, have been found and so these represent 3 x 100/15 or, 20% of the total number of mutants examined compared with an expected value of 25%. This makes the likelihood of having missed a mutant deficient in the synthesis of 42S RNA seem even more unlikely. It is conceivable, however, that for some unknown reason, the actual number of mutants with a lesion in the 42S RNA synthesising enzyme is less than could be expected by chance. However, a second explanation is suggested by Fig. 26 which shows that, along with a decrease in 26S RNA synthesis, there is a concomitant increase in 42S RNA synthesis. It is therefore proposed that there is a single enzyme responsible for both 42S and 26S RNA transcription and that the synthesis of 26S RNA is under positive control. This would explain the experimental observations that (a) there is no enzyme responible for 42S RNA synthesis,

because a lesion in the single polymerase would affect both types of RNA equally and (b) the apparent specific loss of 26S RNA synthesis, which would be due to a lesion in the control factor, which would then lead to an increase in 42S RNA synthesis at the expense of 26S RNA synthesis.

There are a number of ways in which this regulation could be achieved but all mechanisms must be based on the assumption that 26S RNA synthesis is under positive control i.e. the enzyme preferentially synthesises 42S RNA but under certain conditions will synthesise 26S RNA. This is, in turn, probably a manifestation of a higher affinity of the unregulated enzyme system for 42S RNA synthesis. The reverse is unlikely to be true for the following reason; if 26S RNA synthesis is preferred but a regulating polypeptide could cause the synthesis of 42S RNA then the observed phenotypes of temperature-sensitive mutants would be the reverse to that normally seen i.e. they would lose either 42S RNA specifically or both types of RNA equally but it is difficult to imagine how they could loose 26S RNA specifically. Possible mechanisms by which the rate of synthesis of the two species of RNA might be regulated are outlined below:

(a) a polypeptide subunit of the polymerase might facilitate internal initiation of transcription such that the affinities of the two sites become comparable in magnitude.

(b) a polypeptide subunit might enhance the rate of transcription of 26S RNA, but it is difficult to see how a subunit could affect the rate of transcription of 26S RNA specifically when the template for 26S RNA is also part of that for 42S RNA.

(c) a polypeptide subunit might enhance the rate of termination of 26S RNA over 42S RNA but again, as in (b), the terminal sequences of 26S and 42S RNA are the same and it is difficult to imagine where specifity could arise.

(d) a polypeptide might be present which can physically block the 3' end of the 42S negative strand RNA so preventing initiation of 42S RNA synthesis and leaving only the initiation site for 26S RNA synthesis open (a case of molecular Hobson's choice!) or alternatively a polypeptide

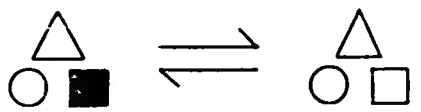
might open the initiation site for 26S RNA synthesis which would, in this case, be situated in a region of secondary structure of the 42S negative strand RNA.

The alternatives outlined in (d) are attractive for a number of reasons but differ from (a) in one very important detail which makes then untenable as a working hypothesis. If the regulator acts directly on the RNA by physically opening or closing an initiation site and is not part of the polymerase complex itself then those mutants which have a temperature sensitive lesion in this polypeptide but synthesise the remaining non-structural polypeptides (e.g. E268) should still be able to make 42S RNA, as the polymerase would, in theory, be unaffected; repeated attempts to show that E268 synthesises only 42S RNA on continuous incubation at the restrictive temperature have failed. The other polymerase activities, then appear to be dependent on the presence of a functional regulatory polypeptide and for this reason it is proposed that the regulatory polypeptide forms an essential part of the polymerase which exists as a dimeric or possibly trimeric enzyme. In addition, it is considered that all subunits must be functional before assembly to give an active polymerase can take place or conversely a single fault in any one of the functions of the polymerase is sufficient to render all polymerase functions inactive.

Consideration of the data derived from E268, namely that it produces all three nonstructural polypeptides at 39°C but one of them, nsp89, is unstable (Fig. 30) along with the observation concerning its RNA phenotype under shift up conditions (Fig. 25) and the fact that on continued incubation at 39°C it does not make any RNA at all (Table 6) tentatively suggests that nsp89 is the polypeptide that is involved in facilitating the transcription of 26S RNA. Its mode of action is unknown but consideration of a number of alternatives suggests that control of the initiation of transcription would be a likely and convenient point at which to exert its effect. If the activity level of nsp89 dictates the amount of 26S and 42S RNA synthesised, then this polypeptide would obviously be a very useful means of regulating the amount of 26S RNA,

which codes for the structural polypeptides, and 42S RNA present inside the cell such that they are synthesised in the correct ratio of 230 structural polypeptides to 1 42S RNA for optimal assembly of the virus particle (Laine et al.). The mechanism by which the levels of 42S and 26S RNA could be adjusted, so that structural polypeptides and 42S RNA are synthesised in the correct ratio, is considered in the next section.

To summarise then, it is assumed that the three nonstructural polypeptides are synthesised in such a fashion as to form a single trimeric polymerase (Fig. 17 and Fig. 34) and that one of these polypeptides possibly nsp89, will facilitate the synthesis of 26S RNA under certain conditions but that the enzyme preferentially synthesises 42S RNA. The two polymerases could then be considered to exist in the cell in an equilibrium which could be adjusted by modulating the activity of the regulatory polypeptide (□, Fig. 34). This model is consistent with the experimental data but, as is true with all models, it serves only as a working hypothesis and is not necessarily unique. It will be extended further in the next section but it must be borne in mind that, whereas the above model is consistent with the experimental data, the actual molecular mechanisms by which the results are achieved might well be different.



42S FORM 26S FORM

Fig. 34. A schematic representation of the proposed relationship between the polymerases synthesising 42S and 26S RNA.

SECTION IV

EXPERIMENTS WITH RNA +VE TEMPERATURE SENSITIVE MUTANTS; EVIDENCE FOR THE REGULATION OF THE 42S TO 26S RNA RATIO BY A STRUCTURAL POLYPEPTIDE

INTRODUCTION

Synthesis of the Structural Proteins and Maturation of Sindbis Virus

(i) Strucutal Protein mRNA

Isolation of polysomes from Sindbis virus infected cells has shown that the majority of RNA present was 26S RNA (Mowshowitz; Simmons and Strauss, 1974a; Rosemond and Sreevalsan). Bearing in mind that structural protein synthesis predominates at the time when these experiments were performed, this was indicative of 26S RNA being the mRNA for the three structural proteins. This did in fact prove to be the case, as was demonstrated by cell free translation of the 26S RNA (Cancedda et al., 1974; Cancedda and Schlesinger; Simmons and Strauss, 1974b). The 26S RNA is a polycistronic mRNA which, in certain temperature-sensitive mutant infected cells incubated at the restrictive temperature, gives rise to a large polypeptide which contains the sequences of all three structural proteins (Schlesinger and Schlesinger, 1973). However, this polypeptide is not processed when the cells are chased at the permissive temperature (Scheele and Pfefferkorn, 1970) and so is presumed to be a dead end product. In wild type infected cells however, this large polypeptide is rarely seen, as the core polypeptide is cleaved nascently in both SFV (Clegg) and Sindbis virus (see Results Section I, viii; Fig. 10) infected cells; the envelope proteins, meanwhile, are translated on polysomes which are bound to the endoplasmic reticulum (Wirth et al.).

(ii) Fate of the Core Protein

The core protein is cleaved from the nascent polypeptide chain and then binds 42S RNA to form the nucleocapsid. This association is rapid and takes place such that nucleocapsid assembly is complete within ten minutes in SFV (Söderlund) and Sindbis virus infected cells (Ben-Ishai et al.). In addition, the observation that the core protein can be incorporated into complete nucleocapsids within three minutes of its synthesis (Friedman, 1968) and that free core protein cannot be detected in infected cells suggests that there is a pool of partially assembled nucleocapsids present in the cytoplasm (Söderlund). This would indicate that the core protein has a high affinity for the 42S RNA.

(iii) Fate of the Envelope Proteins

The envelope proteins are synthesised on the endoplasmic reticulum (Wirth et al.) but budding of the mature virus takes place from the plasma membrane (Brown et al.; Birdwell et al.). The method of translocation of the envelope proteins is not known but might well parallel that used for the transfer of host cell proteins destined for the plasma membrane.. In the process of translocation, the envelope proteins are glycosylated (Bose and Brundige). They contain approximately 8% by weight of carbohydrate the majority being mannose and glucosamine with smaller amounts of galactose, sialic acid and fucose (Sefton and Keegstra). Whereas the mannose, glucosamine, and galactose are uniformly distributed between E1 and E2, there appears to be four times as much fucose in E1 as in E2 (Sefton and Burge). The carbohydrate appears to be host cell specified since a comparison of the glycoproteins of VSV and Sindbis virus grown in CEF cells showed that the carbohydrate residues were very similar (Burge and Huang). In addition, sialyl and glycosyl transferases isolated from both infected and uninfected cells were equally active when Sindbis envelope proteins were used as substrates (Grimes and Burge). This glycosylation is essential for production of virus. Inhibition of glycosylation using tunicamycin (Leavi et al.) or glucosamine (Duda and Schlesinger) in cells infected with Sindbis virus or 2-deoxy-D-glucose in SFV infected cells (Kaluza et al.) leads to an inhibition of release of virus particles.

There is a certain amount of evidence to suggest that E1 and E2 are present as a complex inside the infected cell. This is based on temperature-sensitive mutant work described in the introduction to the previous section (Bracha and Schlesinger, 1976b), along with the kinetics of labelling of E1, E2 and pE2 (Sefton and Keegstra) and also the observation that anti-serum to E1 can prevent processing of pE2 to E2 (Jones et al., 1977). Presumably, then, the complex is derived from the envelope protein precursor which adopts a specific conformation and is then cleaved but the two proteins thus produced do not separate.

(iv) Maturation

Maturation takes place at the plasma membrane. The virus buds through the membrane in an area that has already been modified by the insertion of virus specified envelope proteins (Brown et al.; Acheson and Tamm; Birdwell et al.; Brown and Smith). Maturation requires the concomitant cleavage of pE2 to E2 (Jones et al., 1974). The fate of the fragment that is removed is not known while, on the other hand, the corresponding peptide from SFV infected cells is found in the virus particle (Garoff et al.). Electron microscopic examination of cells infected with SFV shows the presence of two types of vacuole. One of these vacuoles, CPV-I, appears to be involved in RNA synthesis (Grimley et al.) while the second type, CPV-II, has nucleocapsids clustered around it and appears to be involved in the maturation of the virus, perhaps by budding of the virus into the vacuole, followed by fusion of the vacuole with the plasma membrane. However, the amount of mature virus found inside the vacuoles is small and it was suggested that CPV-II, with its associated nucleocapsids, fused with the plasma membrane and only then would the nucleocapsids bud through the membrane (Grimley et al.). This attachment of the nucleocapsids to the vacuole while it is still in the cytoplasm may seem a little unusual but may well indicate the method by which the glycoproteins are transported to the plasma membrane. Grimley et al. suggested that the morphology of CPV-I and CPV-II resembles that of the vacuoles derived from the Golgi region which is now thought to be involved in the synthesis of secretory proteins and their transport to the plasma membrane (Palade). If this were the case it would seem reasonable to expect that the envelope proteins would have been inserted into the membranes of these vacuoles and then directed to the plasma membrane. During their passage through the cytoplasm they would then pick up the nucleocapsids, which would not be able to distinguish this vacuole from the plasma membrane. Such a mechanism would explain the origin of the CPV-IIs seen under the electron microscope and also why the virus need not code for any glycosyl transferases since the primary sequence would be sufficient to subvert the cellular machinery which would then process E1 and E2 as if they were simply secretory proteins.

They would then follow the normal paths of such proteins and appear, fully glycosylated, at the cell surface, courtesy of the host cell machinery and give rise to the large areas of cell membrane which appear to contain exclusively viral envelope proteins (Brown et al.; Acheson and Tamm).

This is consistent with the kinetics of appearance of the structural proteins in mature virus. After a short pulse with a radioactive amino-acid, and sequential sampling of the released virus during a chase period, it was found that there was preferential labelling of the core protein with respect to the envelope protein in virus released immediately after the end of the pulse but later in the chase period the ratio of label in core to that in envelope proteins approached that seen in equilibrium labelled samples (Scheele and Pfefferkorn, 1969a). It seems then that the time required to synthesise, glycosylate and transport the envelope proteins to the plasma membrane is longer than the time required to synthesise the core protein and assemble nucleocapsids, a result which is not entirely unexpected. Scheele and Pfefferkorn (1969a) also demonstrated that the rate of release of labelled virus, after infected cells had been treated with a radioactive amino acid for thirty minutes, was maximal in the hour following the pulse and declined rapidly therafter, indicating that the majority of the virus that was to be released had matured and was present in the extracellular medium within an hour of the synthesis of its constituent proteins, indicative of the close co-ordination of the various processes.

In order to determine what mechanisms, if any, operated inside Sindbis virus infected cells such as to regulate the rate of synthesis of the structural proteins and the 42S RNA they must encapsidate, the various temperature sensitive mutants have been analysed in this laboratory with respect to determining their temperature-sensitive lesions and the effect of these lesions on the RNA ratio. A proportion of this work was carried out by other members of this laboratory but, because of its relevance it is being included; all such work has been specifically acknowledged.

RESULTS

(i) Is the 42S to 26S RNA Ratio Under Viral Control?

In the previous section, the various results obtained were consistent with there being a regulatory polypeptide in Sindbis virus infected cells which was required for the synthesis of 26S RNA. It is obviously of interest to determine whether this polypeptide acts in a random fashion, such that the rate of 26S RNA synthesis is only dependent on the concentration of the polypeptide, or if its activity is in turn regulated by other factors so as to maintain 42S and 26S RNA at those levels required to produce virion components in the correct quantities. To investigate whether there was any control of the rate of synthesis of the two types of RNA, replicate cultures of Sindbis virus infected cells were pulse labelled with $[^{32}P]$ -orthophosphate for thirty minutes at hourly intervals throughout the growth cycle. The amount of radioactivity incorporated into each species of RNA was quantitated as described in Materials and Methods (Section V, vii and viii). The result of this experiment (Fig. 35) shows that the rates of synthesis of both 42S and 26S RNA parallel each other as if they were tightly coupled and that there might, then, be some sort of controlling effect present. A similar result has recently been reported for SFV infected cells (Lachmi and Kääriäinen, 1977). This effect might be fortuitous however, and so further evidence was required before viral control of rates of RNA synthesis could be considered as a possibility. In this respect again, temperature sensitive mutants have proved useful.

(ii) The RNA Ratio and Polypeptide Phenotype of Sindbis Virus RNA +ve Temperature Sensitive Mutants

The 42S to 26S RNA ratios determined at the restrictive temperature for the RNA +ve temperature sensitive mutants have already been published (Atkins et al.). The results were presented as a histogram illustrating the distribution of the mutants with respect to their RNA ratios. This histogram is repeated, with additional information, as Fig. 36. Since this was published various other pieces of information have been obtained.

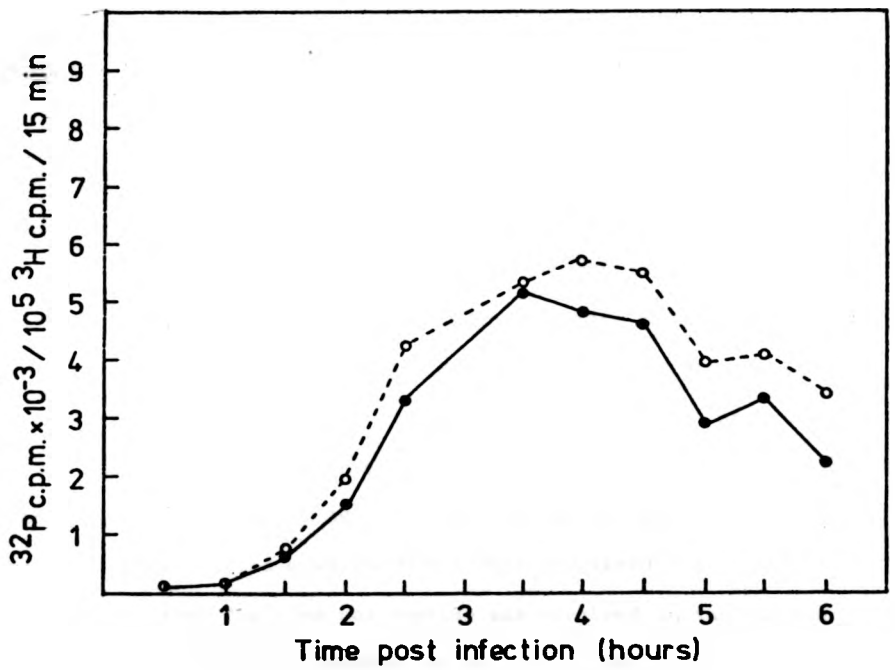
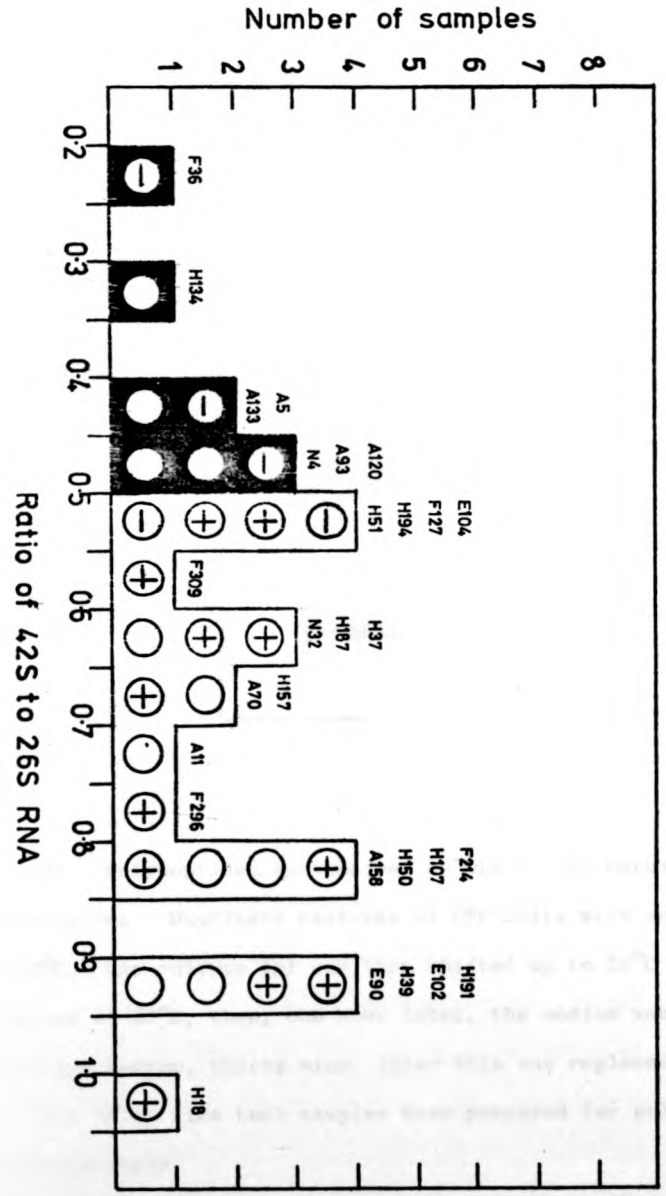


Fig. 35. Rate of 42S and 26S RNA synthesis during the growth cycle. Replicate cultures of CEF cells were pretreated with GMEM - P for 2 hours and then infected with wild type Sindbis virus. At ½ hourly intervals from ½ - 6 hours post infection one culture was labelled with [^{32}P] -orthophosphate for 15 min. The nucleic acids were purified and the amount of radioactivity incorporated into 42S (●——●) and 26S (○-----○) RNA was determined.

Firstly, seven of the mutants (shaded boxes in Fig. 36) at the extreme left hand end of the histogram synthesised p144 when incubated at 39°C (Brzeski et al., submitted for publication). This suggested that there was an attempt by the virus to correct for the lack of synthesis of structural polypeptides by increasing the level of the messenger RNA (26S RNA) coding for these structural polypeptides. In an attempt to determine which of the structural polypeptides was involved in regulating the RNA ratio experiments with zinc ions were used to determine the connection between polypeptide synthesis and RNA ratio. Under certain conditions zinc ions caused the accumulation of p120 which, in turn, resulted in a decrease in the amount of envelope proteins synthesised in wild type virus infected cells. However, the 42S to 26S RNA ratio was not affected by such treatment suggesting that neither p120 nor the envelope proteins were able to regulate the RNA ratio. In addition, shift down experiments performed with F36 infected cells, which had accumulated p144 at the restrictive temperature, showed that the presence of this polypeptide previously synthesised at 39°C, did not affect the RNA ratio after the cells had been shifted down to 30°C. This suggested, by a process of elimination, that the core polypeptide was involved in the regulation of the 42S to 26S RNA ratio (Brzeski et al., submitted for publication).

If the lack of core protein caused a change in the 42S to 26S RNA ratio in favour of 26S RNA, it was necessary to find out if the converse was true i.e. that the presence of excess core resulted in a relative increase in the rate of 42S RNA synthesis. It was considered that H18 would be a useful mutant to examine in this respect for two reasons. Firstly, H18 appears to have a lesion in one of the envelope polypeptides (Fig. 37 lane b) at the restrictive temperature but synthesis of the core protein appears to take place normally at both temperatures (lanes a and b), hence there will be a large amount of core protein accumulating in the cell. Secondly, if the rates of 42S and 26S RNA synthesis were to prove invariant and hence the change in RNA ratio were simply due to accumulation of 42S RNA then H18, which synthesises the greatest proportion

Fig. 36. Histogram showing the distribution of RNA +ve mutants with respect to their RNA ratio and also their ability to synthesise p144 and assemble nucleocapsids. This is adapted from an original figure published by Atkins et al. The shaded boxes represent those mutants which accumulate p144. Mutants which are able to assemble nucleocapsids are indicated by a +, those which do not, have a -. Empty circles indicate that that particular mutant was not examined.


Number of samples
1
2
3
4
5
6
7
8
Ratio of 42S to 26S RNA
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
1·0
F36
H134
A5
A133
N4
A93
A120
H51
H194
F127
E104
F309
H187
N32
H37
H157
A70
A11
F296
A158
H150
H107
F214
E90
H39
E102
H191
H18

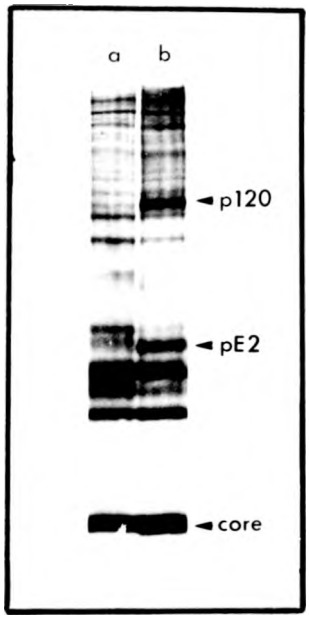


Fig. 37. Polypeptides synthesised by H18 at the permissive and restrictive temperatures. Duplicate cultures of CEF cells were infected with H18 for 5h at 30°C. One culture (b) was then shifted up to 39°C and the second (a) retained at 30°C, then, one hour later, the medium was replaced with labelling medium, thirty mins. later this was replaced with chase medium for 1h after which time both samples were prepared for polyacrylamide gel electrophoresis.

of 42S to 26S RNA at the restrictive temperature, should show this effect most strikingly.

To see if this was the case, the amount of $[^3H]$-uridine incorporated into mature virus released during the hour's pulse was determined and compared to the total incorporation over the same labelling period. Duplicate cultures of CEF cells infected with H18 were incubated at 30°C for four hours and then one of these was shifted up to 39°C. At this time both cultures were labelled with $[^3H]$-uridine for one hour. At the end of this period the amount of label released from the cell as mature virus was determined using the one step purification procedure (Fig. 38) of Scheele and Pfefferkorn (1969a) while the total incorporation of $[^3H]$-uridine into TCA insoluble products was determined on the same monolayers. The RNA ratio at the two temperatures was determined in parallel cultures by labelling with $[^{32}P]$-orthophosphate, extracting the RNA and separating the two species on polyacrylamide gels. The RNA ratio is shown in Table 8 and the amount of label released as mature virus is calculated as a percentage of the total label incorporated and is shown in Table 9. It is possible to calculate the amount of 42S RNA that would need to be released as mature virus at 30°C if the differences in RNA ratio of H18 at 30°C and 39°C were to be due simply to a block in the maturation of H18 at the restrictive temperature. By normalising the amount of 26S RNA to unity at the two temperatures the difference in the amount of 42S RNA made under the different conditions is 1.06 - 0.59 = 0.47 (Table 8). If it is assumed that the ratio in which 42S and 26S RNA were synthesised remained unchanged on shift up to the restrictive temperature, i.e. 0.59 molecules of 42S RNA to every 26S RNA, then the apparent increased amount of 42S RNA made, i.e. 0.47 times the total incorporation into 26S RNA, was due simply to accumulation of 42S RNA which would normally have been released as mature virus. It can be seen then, that the difference of 0.47 represents 0.47 x 100/(1 + 0.59) or 29.6% of the total RNA synthesised and remaining in the infected cell during the one hour pulse at 30°C. The amount of label actually released was only 0.5% of the total radio-

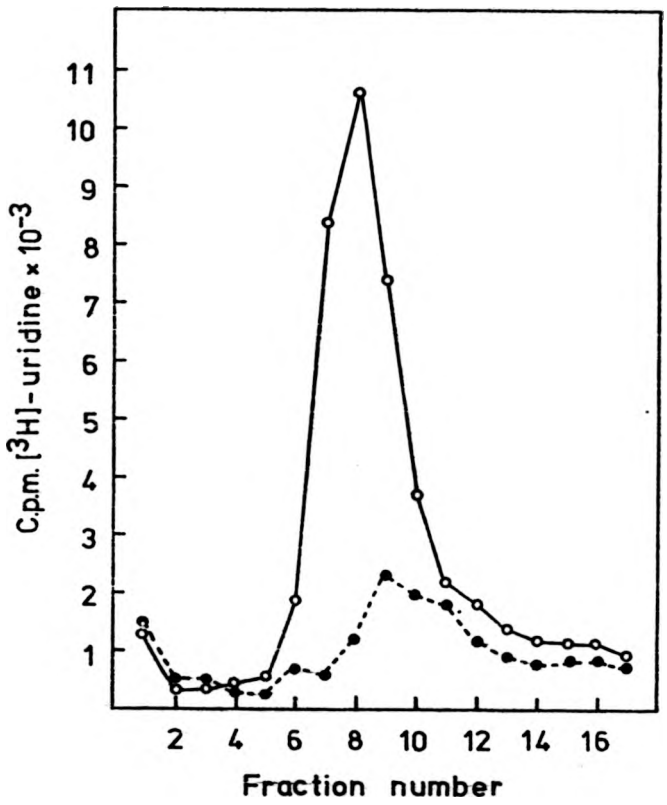


Fig. 38. Virus release by H18 infected CEF cells at the permissive and restrictive temperatures. Four cultures of CEF cells infected with H18 were incubated for 30°C for 4h, at which time two cultures were shifted up to 39°C (●-----●) while the remaining two were retained at 30°C (o———o). The medium was then replaced with HEDA containing $[^3H]$ -uridine for 1h. The monolayers were retained and the amount of $[^3H]$ -uridine incorporated into TCA insoluble products was determined (Table 9). The medium from each pair of cultures was pooled and then layered on a one step virus purification gradient (Scheele and Pfefferkorn, 1969a; see Section V,xii) and centrifuged for 3·5h at 60,000g. The gradient was fractionated directly into scintillation vials containing Triton/toluene scintillator and the amount of radioactivity present was determined by means of a liquid scintillator counter (Centrifugation was from right to left).

Labelling temperature	[^{32}P] c.p.m. incorporated in: 42S RNA	26S RNA	42S to 26S RNA ratio
30°C	562	954	1 : 0.59
39°C	961	902	1 : 1.06

Table 8. The 42S to 26S RNA ratio of H18 under permissive and shift-up conditions. Duplicate cultures of H18 infected CEF cells were incubated at 30°C for 4h. One culture was shifted up to 39°C and both cultures were labelled with [^{32}P] -orthophosphate for 1h after which time the amount of radioactivity incorporated into each species of RNA was determined.

Temperature	[^{3}H] c.p.m. incorporated: per monolayer	in released virus	Virus released, as % of total incorporation
30°C	1.13×10^6	5.46×10^3	0.48
39°C	1.08×10^6	1.10×10^3	0.11

Table 9. Percentage of [^{3}H]-uridine released as mature virus during a 1h pulse. Four cultures of H18 infected CEF cells were incubated at 30°C for 4h. Two cultures were shifted up to 39°C and then all four cultures were labelled with [^{3}H]-uridine for 1h. The amount of virus released into the medium was determined as described in the legend to Fig. 38 and Section V, xii. The incorporation of label into TCA insoluble products was determined as described in the legend to Fig. 19. The values given above are the average of duplicates and have been corrected for incorporation shown by mock infected controls.

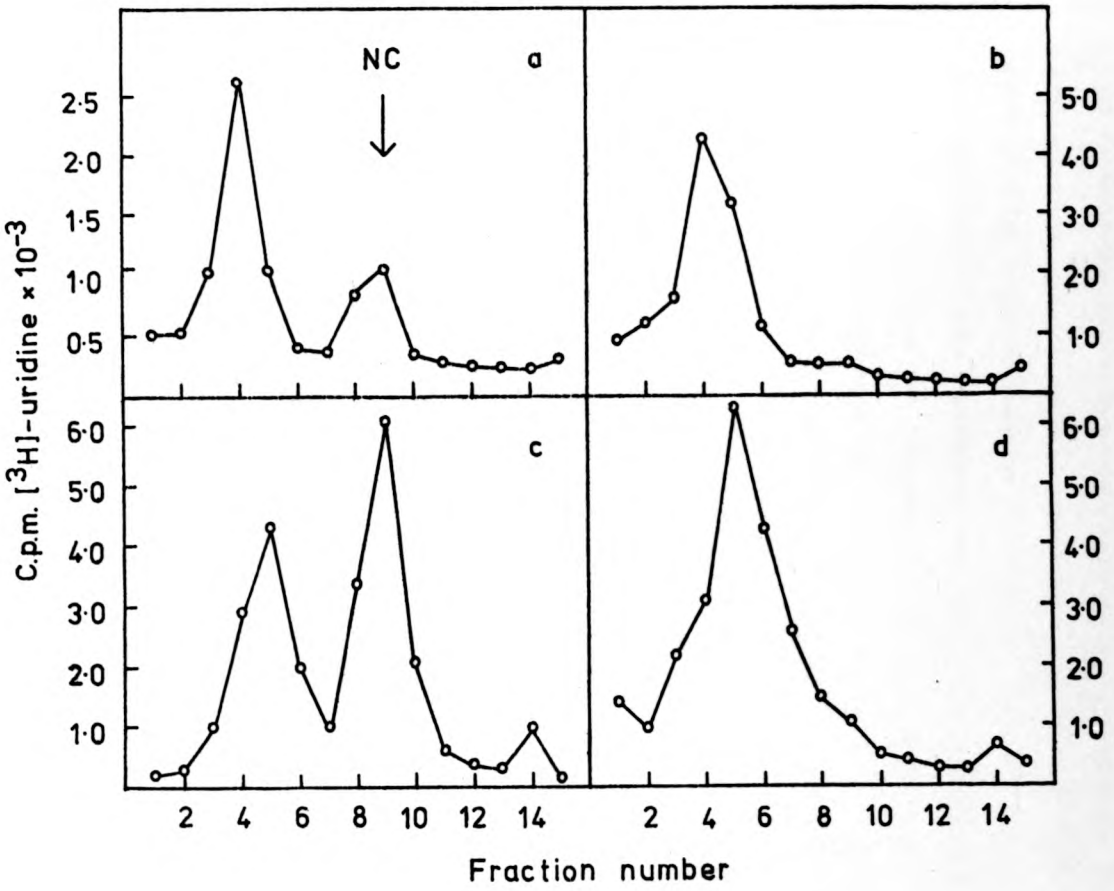
activity remaining inside the cell after a one hour pulse at 30°C. This in turn suggests that the increased RNA ratio reflects a relative increase in the rate of 42S RNA synthesis and is not due to accumulation of 42S RNA which can no longer leave the cell as mature virus. It is difficult to take into account the precise effect of temperature on the RNA ratio, however, but it is unlikely to make a great deal of difference to the calculation, certainly not of the magnitude required to render these conclusions invalid. Therefore, it seems reasonable to conclude that, in H18 infected cells, in the presence of large amounts of the core protein, the relative rate of 26S RNA synthesis is reduced at 39°C compared to the rate at 30°C. This alteration of the RNA ratio in the absence of the synthesis of functional envelope proteins is similar to that seen in the experiments using zinc ions and would again suggest that the core polypeptide is able to regulate the rate at which 26S RNA is synthesised. In this case it would be acting in an analogous manner to the feedback inhibitors of many of the metabolic pathways found in cells. (The rather low proportion of 42S RNA which was released as mature virus during the hour's pulse was probably due to poor growth of the mutant even at the permissive temperature. A parallel experiment performed with wild type virus at the same time as that with H18 showed that the wild type virus released approximately 5% of the total amount of radioactivity incorporated at 30°C. This does not affect the conclusions drawn above, however.) Although the above results are consistent with the idea of core being a regulator of 26S RNA synthesis, all the experiments so far have constituted negative evidence. It was necessary to show that a defect in the core polypeptide caused a relative increase in the amount of 26S RNA synthesised. This was achieved by examining the ability of the RNA +ve temperature sensitive mutants to assemble nucleocapsids (another criterion for the functionality of the core polypeptide) at the restrictive temperature. The results of these experiments are detailed below.

(iii) Ability of the RNA +ve Temperature Sensitive Mutants to Assemble Nucleocapsids at the Restrictive Temperature

CEF cells infected with RNA +ve temperature sensitive mutants at the restrictive temperature were labelled with [^{3}H] -uridine from 6 - 8 hours post infection. The post-nuclear supernatants were layered onto sucrose gradients which were centrifuged and then fractionated. The gradient profile obtained for wild type virus, was used as a positive control, and F36, a mutant that synthesised p144 at 39^{o}C and hence did not make core or nucleocapsids was used as a negative control (Fig. 39 a and b). The labelling pattern obtained was similar to that already described for Sindbis virus (Ben-Ishai et al.; Burge and Pfefferkorn, 1968; Yin and Lockart). The faster sedimenting peak, labelled NC in Fig. 39, was assumed to be nucleocapsid because it sedimented in a similar position to marker nucleocapsid, prepared by treating purified virus with Triton N101, run on a parallel gradient. The nature of the more slowly sedimenting peak is not known but has been observed by other workers (Burge and Pfefferkorn, 1968; Friedman). These experiments were repeated for a selection of the RNA +ve temperature sensitive mutants and the results are summarised in Fig. 36. The results show that those mutants which do not cleave p144 at the restrictive temperature are unable to assemble nucleocapsids; this is not a surprising result and is presumably due to the very low levels of core present in infected cells at the restrictive temperature. On the other hand nearly all the mutants tested which are able to cleave p144 also assemble nucleocapsids with the exception of two mutants, H51 and E104. These mutants are also situated at the left hand end of the histogram, immediately adjacent to those mutants which synthesise p144. The nucleocapsid gradient profile of H51 is shown in Fig. 39d along with that of F127 (Fig. 39c) a mutant with a RNA ratio similar to that of H51; it seems then that both H51 and E104 have a temperature sensitive lesion in the core polypeptide which renders them incapable of assembling nucleocapsids at the restrictive temperature. The RNA ratio of these mutants, at the restrictive temperature, is altered such that they synthesised relatively more 26S RNA than wild type infected cells under the same conditions. However, the only defective polypeptide in cells

C.p.m. [3H]-uridine × 10-3
NC
a
b
c
d
Fraction number

Fig. 39. Nucleocapsid gradient profiles from cells infected with F36, H51, F127 and wild type virus. CEF cells infected with wild type or mutant virus were incubated at 39°C for 6h after which time the medium was replaced with HEDA containing [^{3}H] -uridine 10μCi/ml and the cells harvested two hours later. The post nuclear supernatants were prepared as described and centrifuged on linear 15% -30% w/v sucrose gradients in TN and centrifuged at 40,000g for 14 hours. The gradient fractions were TCA precipitated, collected on glass fibre filters and counted in a liquid scintillation counter. (a) wild type virus, (b) F36, (c) F127 and (d) H51. (Centrifugation was from left to right).


NC
a
b
c
d
C.p.m. [3H]-uridine × 10-3
2·5
2·0
1·5
1·0
0·5
5·0
4·0
3·0
2·0
1·0
6·0
5·0
4·0
3·0
2·0
1·0
2 4 6 8 10 12 14
2 4 6 8 10 12 14
Fraction number

infected with H51 and E104 appears to be the core polypeptide and so this experiment, together with those presented above, suggests that the core polypeptide might in some way be involved in the regulation of the 42S to 26S RNA ratio.

DISCUSSION

A number of observations that were made when working with the RNA +ve temperature sensitive mutants suggest that at least one of the structural polypeptides might have the ability to regulate the rate of synthesis of its messenger RNA (26S RNA). This is most strikingly shown in the clustering, at the left hand end of the histogram, of those mutants which synthesise p144 and therefore do not make any structural polypeptides. The evidence from other experiments with zinc ions, with the mutant H18 and the results of the nucleocapsid survey are all consistent with the hypothesis that the core polypeptide is responsible for regulating the rate of 26S RNA synthesis. From a theoretical standpoint this is attractive for a number of reasons. Firstly, there appears to be little or no translational control of SFV 26S RNA (Lachmi and Kääriäinen, 1977) and so any regulation of structural polypeptide synthesis must take place at the level of transcription of the corresponding messenger RNA. Secondly, the core polypeptide is not membrane bound, unlike the envelope polypeptides, and hence is not physically restrained in the same way i.e. any free core polypeptide could permeate throughout the cell cytoplasm whereas the envelope polypeptides are always found attached to membranes. Thirdly, the core polypeptide, under normal conditions, has a very short half life as a free entity inside alphavirus infected cells as it is very rapidly incorporated into nucleocapsids (Söderlund; Ben-Ishai et al.). It has been suggested that there is a pool of free 42S RNA, or more probably, partially assembled nucleocapsids which quickly pick up the newly synthesised core polypeptides. With this in mind Fig. 34 has been modified to take account of the regulatory structural polypeptide and is presented as Fig. 40. The regulatory polypeptide is assumed to be core for the various experimental and theoretical reasons outlined above. It is assumed that the ability of core to regulate the RNA ratio is mediated through one of the nonstructural polypeptides rather than core having a separate activity of its own. Obviously this can only be confirmed by examination of the different

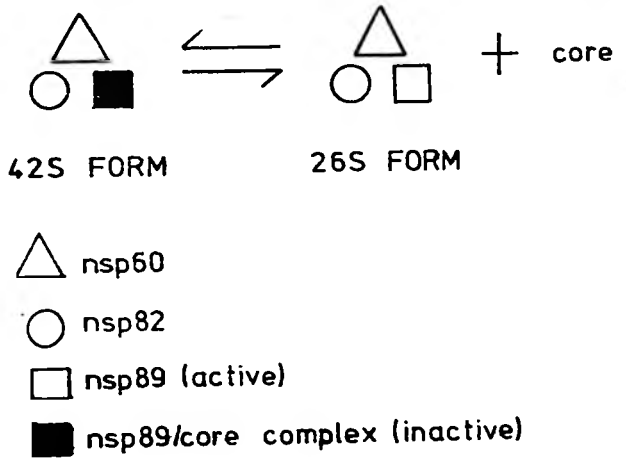


Fig. 40. Schematic representation of the proposed mode of regulation of 42S and 26S RNA synthesis by the core protein.

effects that these various polypeptides would have on a cell free RNA synthesising system.

The method by which the structural polypeptide might regulate the activity of the nonstructural polypeptides is unknown. Two possibilities have been examined by investigating whether the nonstructural polypeptides were in any way altered when synthesised in cells infected with RNA +ve mutants at the permissive or restrictive temperature. Firstly, the mutants were examined to see if the amounts of p76 and nsp82 reflected the proportions of 26S and 42S RNA synthesised under different conditions of incubation; it was found that the polypeptide ratio was constant. Secondly, the nonstructural polypeptides were examined to see if any of them could be specifically phosphorylated and whether this corresponded with an altered RNA ratio however, no such differences were observed (results not shown). As a working idea it will be assumed that core has the ability to bind to nsp89 and render its ability to facilitate the synthesis of 26S RNA temporarily inoperative, possibly by some mechanism akin to allosteric modification. This mechanism would allow the virus to synthesise both RNA and structural protein in the correct ratios for virus assembly by the simple expedient of linking the rate of synthesis of both 26S and 42S RNA to the level of free core polypeptide i.e. core which is not complexed with 42S RNA, present inside the infected cell at any instant in time. It is proposed that regulation is achieved in the following manner. The rate of 26S RNA is positively controlled, by nsp89, at the expense of 42S RNA. The polymerase exists as an equilibrium between two forms, the "26S" form, containing an active nsp89 which allows the polymerase to initiate internally and synthesise 26S RNA, or the "42S" form, in which nsp89 has been temporarily inactivated by core, which then initiates terminally and synthesises 42S RNA. The position of the equilibrium is affected by the structural polypeptides - most probably the level of free core present in the cytoplasm of the infected cell (Fig. 40). It is envisaged that the model would work in the following fashion. If, for some reason, the rate of core synthesis is higher than the corresponding rate of 42S RNA

synthesis then some will escape encapsidation and this free core is then able to bind to nsp89 rendering it inactive; the equilibrium is then shifted to the left, in favour of the "42S" form, which has a two fold effect. Firstly, the rate of synthesis of 26S RNA is reduced in an attempt to reduce the rate of synthesis of the structural polypeptides and secondly, the level of 42S RNA increases. This increase in 42S RNA synthesis serves to remove the free core as nucleocapsids. This, in turn, decreases the concentration of free core, the nsp89-core complex then dissociates and results in a shift in the equilibrium to the right in favour of the "26S" form and the restoration of the rate of 42S RNA synthesis to its previous level. Hence, the rate of 42S RNA synthesis was increased when the level of unencapsidated core rose but once the imbalance had been corrected its rate of synthesis was reduced again. This model therefore provides a mechanism by which the amount of 42S RNA is regulated such that there is just sufficient to use all the core polypeptide that is present. This, in turn ensures optimal co-ordination of the RNA and protein synthetic machinery.

As mentioned previously, there are a number of theoretical reasons why the rate of synthesis of the two RNAs might be regulated by one of the structural polypeptides. For this reason, it is interesting to note that Cooper (1973) has come to a similar conclusion regarding a hypothetical regulator of poliovirus RNA and protein synthesis, termed the equestron, which he proposed would be related to one of the structural polypeptides. There is, similarly, a small amount of evidence that structural polypeptides, or their precursors, can affect RNA synthesis in poliovirus infected cells. For instance, RNA synthesis is sensitive to guanidine in vivo but not in vitro and this inhibitory effect can be nullified, in vivo, by certain mutations in the structural polypeptides suggesting that a structural polypeptide is in some way linked with RNA synthesis (Cooper et al., 1970a). A second piece of evidence concerns the apparent instability of RNA synthesis after the addition of cycloheximide to cells infected with poliovirus (Ehrenfeld et al.). Recently, isolation and partial

purification of poliovirus RNA polymerase at various times after the addition of cycloheximide showed that loss of polymerase activity *in vitro* correlated well with the loss of a structural polypeptide precursor from the polymerase preparation (Roder and Koschel). On the other hand, this should be contrasted with the results reported by Lundquist *et al.* who found that in their hands a similar purification procedure gave rise to a polymerase from untreated cells which consisted essentially of a single polypeptide, NCVP4. This purified preparation contained very little in the way of structural polypeptides or their precursors but still retained the ability to synthesise RNA. The presence of regulatory mechanisms in poliovirus infected cells is, then a little uncertain. Nevertheless, because of the inability of poliovirus to regulate the rate at which it synthesises the nonstructural polypeptides it appears, instead, to regulate their half life (Ehrenfeld et al.). If this half life were dependant on the level of structural polypeptides, as suggested by Roder and Koschel, then this would appear to represent a solution to the problem of regulation for picornaviruses. The synthesis of a large number of structural polypeptides would result in an increased half life for the polymerase population leading to increased RNA synthesis while a decrease in the level of the structural polypeptides will have the reverse effect. A second area in which poliovirus might be able to regulate the rate of synthesis of its RNA and protein is by direct interaction of a small polypeptide with the RNA, an area which is not open to alphaviruses. Picornaviruses are one of the few plus-strand viruses which appear to lack a cap structure at the 5' end of their RNA, instead they would seem to have a protein covalently linked to this region of the RNA (Lee et al.). What significance this has for any regulatory mechanisms remains to be seen.

DISCUSSION

This final discussion will be concerned with collating the relevant results described above, together with previously reported observations of events in alphavirus infected cells, where relevant, in order to provide a model for the growth of Sindbis virus and SFV in vertebrate cells. This model will put into context the various observations reported above and show how they might act as regulators in alphavirus infected cells in order to co-ordinate the different processes taking place. This model includes the following assumptions:-

(a) All three nonstructural polypeptides are associated with a single trimeric polymerase. Both nsp89 and nsp60 have been identified as components of the Sindbis virus RNA polymerase (S.I.T. Kennedy, personal communication); the presence of nsp82 was not definitely established. However, in this case nsp82 was included but the model is not dependent on its presence and remains unchanged if it is omitted. No function has yet been determined for nsp82 but it could possibly be involved in complementary RNA strand synthesis either on its own or in combination with one or both of the remaining nonstructural polypeptides.

(b) There is only one polymerase which is able to synthesise both 42S and 26S RNA. This seems the most likely explanation for the RNA -ve mutant data shown in Fig. 25 (Section III, ii). No RNA -ve mutant has been found which is deficient in 42S RNA synthesis; in addition, a loss of 26S RNA synthesis is accompanied by an increase in 42S RNA synthesis (Fig. 26), for this reason it is proposed that ...

(c) The polymerase would, under normal conditions, synthesise only 42S RNA but for the presence of a regulator which facilitates the synthesis of 26S RNA at the expense of 42S RNA. This regulator is temperature sensitive in N2, ^{N7} and E26S infected cells and ceases to function efficiently under shift up conditions, hence the rate of 26S RNA synthesis slows while that of 42S RNA rises.

(d) The regulator of 26S RNA synthesis is nsp89. The observation that N2 synthesises p215 at the restrictive temperature, together with the

instability of nsp89 either when synthesised at 39°C in E268 infected cells, or when made at 30°C and incubated at 39°C in N2 infected cells, are all consistent with this proposal.

(e) The RNA ratio is regulated by one of the structural polypeptides probably core. The RNA +ve histogram suggests that a lesion which prevents the synthesis of any of the structural polypeptides leads to an increase in the synthesis of 26S RNA. Results of experiments with inhibitors and with the mutant H18 are all consistent with the regulatory polypeptide being core. Similarly two RNA +ve mutants, H51 and E104, which are unable to assemble nucleocapsids at the restrictive temperature, presumably due to a temperature sensitive defect in the core polypeptide, also have a 42S to 26S RNA ratio biased in favour of 26S RNA synthesis. Although it is possible that core might modulate the activity of nsp89, no modification of any of the nonstructural polypeptides has been detected and so it is assumed, for the purposes of the model, that core binds either to nsp89 or to one of the other component(s) of the polymerase and prevents nsp89 functioning as a regulator of 26S RNA synthesis. The actual means by which this modification takes place does not affect the basic working of the model as long as the modification is rapidly reversible.

(f) The control mechanism is dependant on the level of free core polypeptide. It is proposed that there are two functions for a newly synthesised core polypeptide, it can either associate with 42S RNA to produce nucleocapsids (Ben-Ishai et al.; Söderlund) when there is sufficient 42S RNA present or alternatively, if the unencapsidated 42S RNA level is low, it might associate with, or modify, nsp89. These two possible fates for the core polypeptide can be represented as an equilibrium (Fig. 41) although the rapid association of core with 42S RNA suggests that core has a high affinity for 42S RNA and hence the equilibrium for the reaction will be well over to the right.

The bare bones of the proposed control system is shown in Fig. 42; its postulated mechanism is as follows. At equilibrium core and 42S RNA

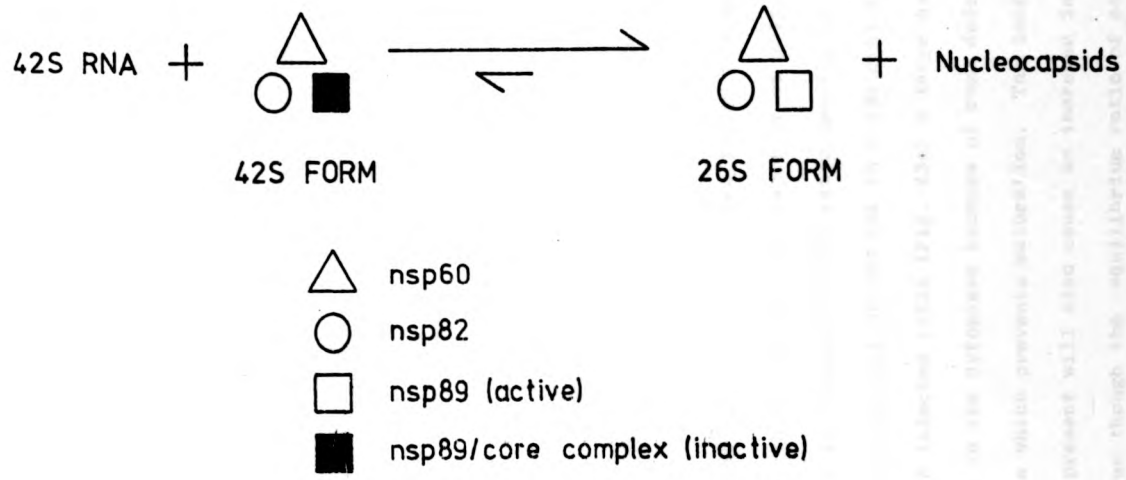


Fig. 41. Schematic representation of the proposed equilibrium between 42S RNA, core and nucleocapsids governing the rate of 42S and 26S RNA synthesis.

are being made in the correct ratio of approximately 230 to 1 respectively, If the rate of 42S RNA synthesis should now exceed that of core, the level of free core decreases, this in turn brings the equilibrium in Fig. 42 towards the right by activating nsp89, leading to more frequent internal initiation and increasing the rate of 26S RNA synthesis. The increase in the level of 26S RNA leads to a similar increase in the level of core which will eventually exceed that of 42S RNA, the level of free core will now rise and this will inactivate nsp89, the equilibrium will move back to the left and the original balance will have been restored.

Fig. 43 shows the model in action in wild type infected cells (Fig. 43b) and in mutant infected cells at the restrictive temperature (Fig. 43 a and c). In wild type infected cells (Fig. 43b) there are low levels of free core and only rarely is one found bound to nsp89 generating a polymerase in the "42S" form. This gives rise to the normal wild type RNA ratio of 1 molecule of 42S RNA to approximately 6 26S RNAs. On the other hand F36 infected cells synthesise p144 at the restrictive temperature and so there are only low levels of core present in the infected cell (Fig. 43a), the amount of unencapsidated 42S RNA present is ample to remove the few core molecules that are made so very few indeed are able to bind to and inactivate nsp89, therefore the vast majority of polymerases present are in the "26S" form so biasing the RNA ratio heavily in favour of 26S RNA (approximately 10 molecules of 26S RNA to 1 42S RNA; Atkins et al.). Conversly, in H18 infected cells (Fig. 43c) a large number of nucleocapsids have accumulated in the cytoplasm because of some defect in envelope polypeptide synthesis which prevents maturation. The increase in the number of nucleocapsids present will also cause an increase in the concentration of free core, even though the equilibrium ratio of core to nucleocapsid remains unchanged, this will then bind to and inactivate nsp89 leading to an increase in 42S RNA synthesis in an attempt to remove the free core present (Fig. 36). A similar occurrence might take place in the RNA -ve mutant infected cells on shift up to the restrictive temperature. These

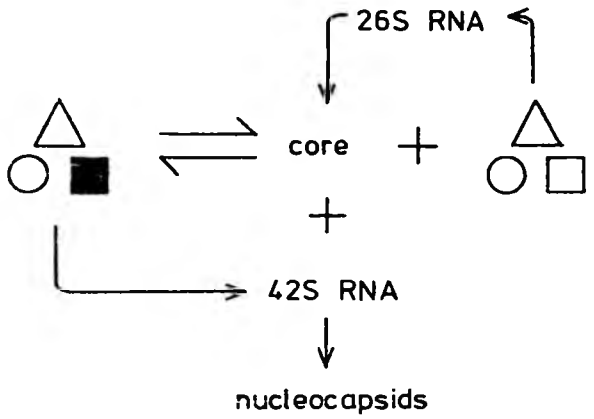


Fig. 42. Diagram representing the inter-relationships of the various viral products postulated to be involved in the regulation of the 42S to 26S RNA ratio. The symbols have the meanings defined in the legend to Fig. 40.

RNA
(polymerase)
42S FORM
26S FORM
42S negative
strand RNA
nucleocapsid
Cell nucleus

Fig. 43. Schematic representation of the structure of replicative intermediates inside cells infected with F36 (a), wild type virus (b) and H18 (c).

a
b
c
N
N
N
RNA polymerases
42S FORM
26S FORM
42S negative strand RNA
nucleocapsid
N Cell nucleus

mutants would not produce any more polymerase and the rate of RNA synthesis will level off (Section III, i; Fig. 24). However, 26S RNA is still translated in this situation and so the level of core continues to increase. This excess core will then shift the equilibrium in favour of the "42S" form by inactivating nsp89 and preventing internal initiation of transcription. Hence, all RNA -ve mutants would be expected to show some adjustment in their RNA ratio in favour of 42S RNA synthesis and this was found to be so (Fig. 25). This might explain the observation that nsp89 is unstable to some degree in all RNA -ve mutant infected cells. When the level of 42S RNA synthesis increases and a larger proportion of the total pool of nsp89 has been inactivated, they might become more sensitive to protease attack. This would then parallel the degradation of inactive nsp89 synthesised in E268 infected cells at the restrictive temperature (Section III, iii; Fig. 30).

One important question that has, of necessity, been almost completely ignored until this point is the identity of the polymerase which can use the 42S RNA as template to generate a complementary 42S negative strand RNA. This might involve nsp82 on its own or in combination with nsp89 and/or nsp60; additionally it might also involve some host cell specified polypeptides but this area is still virgin territory as far as alphavirus polymerases are concerned. It is necessary to point out that if the polymerase that synthesises 42S RNA (i.e. the basic trimer + core) were also responsible for the synthesis of the complementary 42S negative strand RNA, this would necessitate the presence of free core very early in the infectious cycle - even before 26S RNA had been synthesised. This would argue for the existence of a separate polymerase for the synthesis of the complementary RNA.

Another dilemma encountered by all viruses which have single stranded messenger sense RNA as their genetic material is the problem of synthesising complementary RNA on the input virion RNA which must first be cleared of ribosomes which are translating the necessary polymerase. The manner in which Qβ comes to terms with this dilemma appears to have been resolved

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(Kolakofsky and Weismann, 1971 a, b.). The polymerase is apparently able to bind at the initiation point for translation of the coat protein gene and prevent further initiation of protein synthesis. When all the ribosomes which were in the process of translating the RNA have terminated and the RNA is free of ribosomes, then the polymerase can bind at the 3' end of the messenger strand RNA and synthesise the negative strand. Obviously, it is only possible to speculate whether an analogous mechanism operates in animal virus infected cells but there is one piece of evidence which might suggest that such a possibility is worth investigating. One important similarity between alphaviruses, picornaviruses and the RNA phages is that all these RNAs have a single, normal initiation site for protein synthesis on their genomic RNA. The RNA phages contain three initiation sites for protein synthesis but only the coat protein initiation site is used under normal conditions (Hindley and Staples); similarly, alphaviruses have a second ribosome initiation site but this also appears to be cryptic. Hence, all that would be required to free the whole genome for replication in all three viruses would be one single binding event by one viral specified (polymerase ?) polypeptide.

As mentioned earlier, one of the unique properties of alphaviruses as animal viruses, is their ability to initiate transcription of RNA internally. Although this may prove to be true for flaviviruses also, these two groups of viruses are quite closely related and so this is not surprising. The interesting observations concern those plant viruses which also appear to transcribe subgenomic RNAs. Attempts to demonstrate the presence of coat protein in cell free systems translating TMV virion RNA had always met with very little success even though the coat protein always represented a major product in infected cells and is the only viral structural polypeptide. Recently, however, a subgenomic RNA has been isolated from TMV infected cells which appears to code solely for TMV coat protein in a cell free system (Hunter et al.; Siegel et al.). In the case of TMV at least, the virion RNA is infectious and the subgenomic

RNA is not required for infection. So the strategy here appears to be directly analogous to that employed during the alphavirus infectious cycle; however, the mechanism by which the smaller RNA is generated is not yet known. Nevertheless, this ability to generate a subgenomic RNA for the coat protein together with the observation that the coat protein gene is at the 3' end of the virion RNA (Hunter et al.; Beachy et al.) would suggest that both TMV and alphaviruses use very similar strategies in their growth cycles. Bearing in mind that alphaviruses have the ability to infect both vertebrate and invertebrate cells, while TMV infects plant cells and also remembering that both viruses are transmitted by insect vectors I would like to leave the reader with a question. Is the similarity between alphaviruses and TMV apparent so far, a case of convergent or divergent evolution?

SECTION V

MATERIALS AND METHODS

MATERIALS

All media, calf serum and HEPES were purchased from Flow Laboratories; [^{35}S] -methionine (350 - 500 Ci/mmol), [^{32}P] -orthophosphate (92 - 130 Ci/mg phosphorus), [^{3}H] -uridine (24 Ci/mmol), D- [6-^{3}H] -glucosamine hydrochloride (19 Ci/mmol) and L- [methyl - ^{3}H] -methionine (5.5 Ci/mmol were obtained from the Radiochemical Centre, Amersham, Bucks.; SDS (specially pure), acrylamide, molecular weight markers (53,000 - 265,000), NDS and yeast RNA from British Drug Houses Ltd. β-mercaptoethanol, iodoacetamide, DNAse I, RNAse A, ovalbumin, bovine serum albumin, human transferrin, phosphorylase a, Triton N101, agarose and cycloheximide were purchased from Sigma Chemical Co.; N,N' -methylenebisacrylamide from Eastman Organic Chemicals; Kodirex and RP Royal Xomat from Kodak; trypsin (treated with L- 1 - tosylamido - 2 - phenylethylchloromethyl ketone) and RNAse free DNAse from Worthington Biochemical Corp.;phenol from Koch-Light and the pre-coated silica gel plates for thin-layer chromatography from Schleicher and Schüll, Dassel, W. Germany. Actinomycin D was a generous gift from Merck, Sharpe and Dohme. All other chemicals were of the best grade available commercially.

Phenol/TNE was prepared by redistilling the commercial phenol into excess TNE. Ethanol and ether were redistilled prior to use in RNA extraction procedures and the acrylamide used for the separation of RNA was recrystallised from chloroform before use.

METHODS

(i) Growth of Virus Stocks

A constant problem with growth of virus stocks is the presence of defective interfering particles which could lead to confusing results if present in stocks of temperature sensitive mutants. In order to avoid this possibility all necessary passages of virus stock were carried out at low multiplicity and no stock was passaged more than twice in cell cultures, new stocks were always grown from plaques derived from the original isolate.

The original stocks of wild type or temperature sensitive mutants, originally isolated by Atkins et al. were serially diluted in medium 199 containing 2% calf serum and used to infect CEF cells for 1 hour after which time the virus was replaced with an agar overlay and plaques were allowed to develop at 30°C for the mutants or 37°C for the wild type virus. When plaques were visible the agar immediately above each plaque was removed using a Pasteur pipette and placed in 2mls of medium 199 containing 2% calf serum, disrupted using a vortex mixer and if not used immediately, the suspension was frozen at -70°C. This suspension was then used to inoculate CEF monolayers in 90mm plastic petri dishes, at an input multiplicity of 10^{-2} to 10^{-3} PFU/cell. The infected cells were then incubated at the appropriate temperature until the monolayers showed signs of a cytopathic effect (c.p.e.) usually 20 - 25 hours post infection when the fluids were harvested and plaque asssayed at 37°C for the wild type or both 30°C and 39°C for the mutants. The titre of the mutants was determined at each temperature and from this the reversion frequency was calculated as follows:-

$$\frac{\text{Titre of stock grown at } 39^{\circ}C}{\text{Titre of stock grown at } 30^{\circ}C}$$

and any stock with a reversion frequency greater then 5×10^{-3} was rejected (Atkins et al.). This stock was then used to infect monolayers of BHK cells, grown in roller bottles, at a multiplicity of 10^{-2} to 10^{-3} PFU/cell. After infection for one hour at the appropriate

temperature, the virus suspension was replaced by 50mls of medium 199 containing 2% calf serum and the infected cells incubated at the appropriate temperature until c.p.e. became apparent 18-24 hours later. This stock was again titrated and its reversion frequency calculated, if this was satisfactory these stocks were used without further passage. If the reversion frequency proved to be too high the stocks were rejected; no attempt was made to passage the stock further.

(ii) Preparation of Radioactively Labelled Polypeptides for Polyacrylamide Gel Electrophoresis.

In order to maintain efficient temperature control of the medium during the labelling period, all incubations were performed in scintillation vials which could then be mounted in special racks held in a water bath. The scintillation vials were sterilised by heating at 160°C for at least 4 hours and then, when cold 2mls of a suspension of CEF cells were added. The cells were present at 1 x 10^6 cells/ml in Glasgow modified Eagles medium (GMEM) which had only 1/10 the normal concentration of methionine and 5% calf serum (GMEM + met) in order to deplete the intracellular pool of methionine. The vials were incubated overnight at 37°C in a 95% air/5% CO_2 incubator by which time the cells had formed a monolayer which was approaching confluency. It was considered that such monolayers supported virus growth more efficiently than confluent monolayers.

The monolayers were washed once with pre-warmed GMEM which was buffered with 20mM HEPES to pH 7.4 and contained 2% calf serum and 1µg/ml actinomycin D but was lacking methionine (GMEM - met) and then infected with 0.25 ml virus, diluted in GMEM - met to give an infection multiplicity of 100 PFU/cell. The cells were then incubated at 30°C, 37°C or 39°C, depending on the experiment, for 1 hour after which time the virus was removed, the monolayers washed once with pre-warmed GMEM - met and then incubated with 1ml of GMEM - met. All times post infection are specified relative to the end of the absorption period. Labelling was carried out by washing the cells once with Earles salts solution, buffered with 20mM HEPES to pH 7.4 and containing 1µg/ml actinomycin D and 2% dialysed calf

serum (HEDA) and then incubating the cells with 0.5ml of HEDA containing 50μCi/ml [^{35}S] -methionine (labelling medium). If the samples were to be chased the radioactive medium was removed the monolayers were washed once with pre-warmed HEDA containing 2mM unlabelled methionine (chase medium) and then incubated with this chase medium for the required time. On completion of the experiment, the labelling or chase medium was removed and the scintillation vial was placed in a methanol/dry ice bath to terminate the reaction and 5% TCA (2mls) was added to the vials and allowed to freeze. The TCA was then thawed and removed, the monolayers were washed three times with 5% TCA containing 1% casamino acids, followed by two washes with ethanol/ether (3/1, v/v) and one wash with ether and then the fixed monolayers were air dried. They could be conveniently stored at -20°C at this point.

The monolayers were dissolved in 0.1ml of 50mM tris, 2% SDS, 1% mercaptoethanol pH 9.0. The solution was boiled for 3 minutes, made 0.2M in iodoacetamide by the addition of 25μl of 1M iodoacetamide and incubated at 37°C for 1 hour. The solution was then dialysed against 40mM tris, 40mM boric acid, 0.1% SDS pH 8.6 for 6 hours, aliquots of each sample were counted so that approximately equal counts could be loaded onto the gel and then sucrose was added to 3% and bromophenol blue was also added to act as marker. The samples were now ready for polyacrylamide gel electrophoresis.

Labelling of virus infected cells with [^{32}P] -orthophosphate was carried out essentially as described above. After ether drying, the cells were scraped off into 500 μl of nuclease buffer (10mM tris, 5mM $MgCl_2$, pH 7.4) the cells were sonicated (Dawes Soniprobe) position No. 2 for one minute at 4°C, DNAse I and RNAse A were added to 50μg/ml each and the reaction mixture incubated on ice for 20 minutes and then lyophilised. The samples were taken up in 50mM tris, 2% SDS, 1% 2-mercaptoethanol pH 9.0 and processed as described above.

(iii) Polyacrylamide Slab Gel Electrophoresis of [^{35}S]-Methionine Labelled Polypeptides

The samples were run on 20 x 18.5 x 0.15cm slab gels in an apparatus similar to that described by Studier using the discontinuous gel system of Laemmli. The concentration of the resolving gel was 7.5% acrylamide +0.2% N,N'methylene bis acrylamide (pH 8.6) with a stacking gel of 3% acrylamide + 0.8% N,N'-methylenebisacrylamide (pH 6.8). Results were found to be more reproducible if the acrylamide solutions were made up freshly each week. It was found that a teflon slot former gave superior results to one made from perspex. After assembly of the slab gel apparatus and addition of the electrophoresis buffer, the samples were added using a Hamilton microsyringe.. Sample volumes were adjusted such that approximately equal counts per minute were added to each lane where possible, in addition, if circumstances permitted the volume of sample added was the minimum possible commensurate with a reasonable exposure time for the autoradiograph, as it was felt that a sample height of more than 2mm, as measured in the sample slot, caused a decrease in resolution of the polypeptides, even though the gel system appeared to "stack" the proteins and the marker dye. The gel was then electrophoresed at 45V for 16 hours (drawing 20mA initially and 8mA finally), dried under vacuum at 80^{o}C onto Whatmans 3MM paper and autoradiographed using Kodirex film. To ensure close contact the dried gel and film were sandwiched between two glass plates and then the whole positioned under heavy weights for the duration of the exposure.

(iv) Molecular Weight Determinations

The discontinuous gel system of Laemmli proved to be unsuitable for molecular weight determinations and so the continuous gel system of Fairbanks et al. was used instead. Determinations were carried out using two different gel concentrations depending on the molecular weights of the polypeptides. Those with molecular weights in excess of 100,000 were separated on a 3.5% acrylamide + 0.093% N,N'-methylenebisacrylamide slab

gel. The markers used in this case were purchased from BDH and comprised of oligomers of a protein with a monomer molecular weight of 53,000 and provided a calibration line which was linear up to the molecular weight of the hexamer (318,000). The smaller polypeptides, with a molecular weight of less than 100,000 were separated on a 7.5% acrylamide + 0.2% N,N' methylenebisacrylamide slab gel. In this case the markers used were commercial preparations of proteins namely, phosphorylase a (92,000), human transferrin (77,000), bovine serum albumin (68,000) and ovalbumin (45,000). Labelled, virus specified polypeptides and molecular weight markers were electrophoresed on adjacent lanes of a polyacrylamide slab gel. The markers were visualized by staining with 0.05% Coomassie blue, 25% ethanol, 10% acetic acid and destaining with 10% ethanol, 10% acetic acid. The destained gel was dried onto a sheet of Whatman 3MM paper, which was then marked with radioactive ink and the radioactive virus specified polypeptides were detected by autoradiography.

(v) Fluorography of Polyacrylamide Slab Gels Containing $[^3H]$ Labelled Polypeptides

After electrophoresis the gel was washed with dimethyl sulphoxide (DMSO) for two successive 30 minute periods to remove the water. The gel was then transferred to a bath containing 20% w/v PPO in DMSO and soaked for 4 hours after which time the gel was washed in running tap water for at least one hour to remove the DMSO and to precipitate the PPO inside the gel. The gel was dried as described above and then flourographed by exposure to preflashed (to an OD of 0.15) Kodak RP Royal Xomat film at -70°C. This is essentially as described by Bonner and Laskey and by Laskey and Mills.

(vi) Tryptic Peptide Mapping

The various polypeptides that required tryptic peptide mapping were labelled as described in the respective figure legends using a higher concentration of $[^{35}S]$ -methionine (200 - 400 μCi/ml) and 10 vials of cells for each polypeptide. After TCA precipitation and drying, the monolayers

were dissolved and pooled, reduced, alkylated and dialysed as described above (V,ii). Discontinuous polyacrylamide gels were then constructed as above except that there were no slots in the stacking gel. The sample was layered on the top of the slab gel such that the whole width of the gel was used. After electrophoresis, the dried gel was marked with radioactive ink and autoradiographed for four hours. The required polypeptides were identified from the autoradiograph, located by means of the marker ink and excised using a scapel. The dried gel fragments were then rehydrated and the SDS removed by shaking overnight in 500mls of 20% ethanol, 10% acetic acid which was renewed for one hour the following day. The rehydrated gel fragments were then given two 30 minute washes with 10mM ammonium bicarbonate to neutralise the acid. The fragments were transferred to 15ml screw cap test tubes and the polypeptides were digested in situ with 8mls of 10mM ammonium hydroxide containing 50µg/ml of tryspin treated with L - 1 - tosylamido - 2- phenyl ethyl chloromethyl ketone for 6 hours at 37°C. The tubes were inverted occassionally and checked that the pH remained at about pH 8.0. The supernatant was removed, taking care to exclude as much filter paper fibre as possible, lyophilised and then dissolved in 100 µls of formic acid/methanol (4/1, v/v). Performic acid (25µl), prepared by the prior incubation of 19 parts formic acid and one part of 100 vol. hydrogen peroxide together for 2 hours at room temperature, was then added and the peptides oxidised at -8°C for 2 hours. The method of performic acid oxidation is essentially that described by Bray and Brownlee (1973). A known small fraction of the solution was then counted to ascertain the number of $[^{35}S]$ c.p.m. present after which 2mls of water was added and then the peptide preparation was lyophilised. The peptides were redissolved in 10mM NH_4OH such that the number of c.p.m. present in 5µl of the solution was the same as the molecular weight of the polypeptide, for instance the peptides derived from p150 were dissolved in a volume of 10mM NH_4OH such that 5µl contained 150,000 c.p.m.; a small amount of marker dye, xylene cyanol FF, was also added.

The peptides were then separated using 2 dimensional chromatography. 5µl aliquots of each peptide solution (i.e. 1 count per minute per dalton of the original polypeptide) were placed at the corner of 20cm x 20cm pre-coated silica gel plates and the peptides separated using methyl acetate/iso-propanol/25%NH_4OH (3:2:2 by volume) in the first dimension. The plates were then dried at 80°C for 30 minutes after which the second dimension was run using butan-1-ol/acetic acid/water (3:1:1 by volume) as a solvent. The plates were marked with specific co-ordinates and the dye was run to the same position on all plates. After drying, the plates were autoradiographed for 7-10 days using Kodirex film and developed in the usual manner.

In one particular case it was necessary to use a second method of separating the peptides due to insufficient resolution of a small number of spots. The final method settled upon was the following. The first dimension separation was carried out by electrophoresing the peptides at pH 2.1 using an acetic acid/formic acid/water buffer (80:20:900 by volume) for 3 hours at a constant current of 20mA (180 - 200v). The plates were dried at 80°C for 30 minutes and the second dimension separation was achieved by chromatography using a solvent consisting of butan -1-ol/ pyridine/water (3:1:1 by volume). The plates were autoradiographed as described above.

(vii) Preparation of RNA Samples for Polyacrylamide Slab Gel Electrophoresis.

To increase the incorporation of [^{32}P]-orthophosphate into RNA, the intracellular pool of phosphate was depleted by incubating monolayers of CEF cells or BHK cells in GMEM + 1/10 the normal concentration of phosphate and 10% dialysed calf serum (GMEM + P) overnight.

In the past essentially two different methods have been used. Originally, when conducting the screen of the RNA -ve mutants, BHK cells were set up in 1 litre flat bottomed Flow bottles (100 mls/bottle 2.5 x 10^5 cells/ml) for 24 hours, then the medium was replaced with GMEM + P

overnight. Alternatively, a concentrated suspension of CEF cells were diluted to 3 x 10^6 cells/ml in GMEM + P and 5cm plastic petri dishes were seeded with 3mls of suspension. If the experiment required quantitation of the level of RNA synthesis. $[^3H]$ -uridine was added to 5μCi/ml in order to label host cell rRNA. The following day the cells were washed once with pre-warmed GMEM containing 2% dialysed calf serum and 1μg/ml actinomycin D but lacking phosphate (GMEM -P) and then infected with 25 PFU/cell of virus at 30°C or 37°C for one hour after which time the virus suspension was replaced by GMEM -P (25mls/bottle; 4mls/dish) and the cells returned to the incubator. Labelling was carried out in phosphate free Earles salts solution containing 2% dialysed calf serum, 1μg/ml actinomycin D and buffered to pH 7.4 with 20mM HEPES (PFEDA). The monolayers were washed once with pre-warmed PFEDA and then labelled with PFEDA (10mls/bottle, 1ml/dish) containing 100μCi/ml of $[^{32}P]$ -orthophosphate. At the end of the labelling period the radioactive medium was removed, the monolayers washed three times with phosphate buffered saline (PBS) and twice with 50mM tris, 100mM NaCl, 1mM EDTA, pH 7.5 (TNE). The cells were scraped off the bottle, pelleted (350g, 5 minutes) and resuspended in 4mls TNE. The cells were lysed by sequential addition of 0.5mls of 10% napthalene -1, 5- disulphonic acid (NDS) and 0.5 mls of 10% sodium dodecyl sulphate (SDS), the viscous solution so obtained was extracted sequentially with equal volumes of phenol saturated with TNE (twice), chloroform/octanol (24/1, v/v once) and redistilled ether (twice). The remaining ether was removed using a stream of nitrogen gas and the nucleic acids precipitated by addition of 2.5 volumes of redistilled ethanol overnight at 4°C. The precipitated nucleic acids were pelleted (1,000g 10 minutes), washed twice with 50ml NaCl in 70% ethanol, drained and then dried using a stream of nitrogen gas. The dried nucleic acids were redissolved in 1ml DNAsing buffer (10mM tris, 100mM NaCl, 10mM $MgCl_2$ and 1mM EDTA, pH 7.2), RNAse free DNAse was added to 50μg/ml and the solution incubated at 37°C for 30 minutes after which 10% solutions of NDS and SDS were added in the same proportions as above and the nucleic acids were phenol extracted again as

described above. After removal of the ether, 2.5 volumes of redistilled ethanol were added and the RNA precipitated at -20°C overnight.

It was found that later batches of DNAse contained RNAse activity which resulted in extensive degradation of the RNA and so the method was modified to eliminate this part of the procedure. After the cells had been washed, scraped off and pelleted the cells were suspended in 2mls of TNE and then 0.5mls of TNE containing 10% Triton N101 was added and the cells were maintained at room temperature for 2 minutes. Triton N101 lysed the plasma membrane, so releasing cytoplasmic RNA, without affecting the nuclear membrane. The nuclei were then pelleted (1,000g, 2 minutes), the supernatant was removed and made 2% in SDS. The sample was then phenol extracted as above; final precipitation of RNA took place at -20°C. This had the advantage of removing one step of the extraction, hence reducing losses, and removing the DNAsing step which had previously caused degradation of the RNA. The RNA profiles obtained by each method were identical. All aqueous solutions for RNA extraction were autoclaved before use to destroy RNAse.

(viii) Electrophoretic Separation of Viral Specified RNA on Polyacrylamide Slab Gels

The extracted RNA was pelleted (1,000g, 10 minutes) washed once with 50mM NaCl in 70% ethanol, re-pelleted, drained and then dried using a stream of nitrogen gas. The pellet was redissolved in a small volume, usually 100µl, of 0.2 x TNE + 0.2% SDS and a fraction counted for $[^{32}P]$ and also $[^{3}H]$ activity if present. Depending on the purpose of the experiment two approaches were adopted; either equal $[^{3}H]$ c.p.m. were loaded onto the gel, after correction for spill over of $[^{32}P]$ c.p.m. or alternatively, equal $[^{32}P]$ c.p.m. were loaded and the final results corrected by means of the $[^{3}H]$ c.p.m. After addition of sucrose, to 3% and bromophenol blue the samples were loaded onto a 20 x 18 x 0.15 cm polyacrylamide slab gel that had been pre-electrophoresed for 1 hour. The gel consisted of 1.7% acrylamide, 0.085% N,N' -methylenebisacrylamide and 0.5% agarose essentially as described by Levin and Friedman. The gel

was electrophoresed for 16 - 18 hours at 45V, and then dried onto a sheet of Whatman 3MM paper under vacumm at 80°C. The dried gel was marked with radioactive ink and then autoradiographed using Kodirex film. After development of the autoradiograph, the RNAs were located on the gel, by means of the radioactive marker ink. The RNA bands were cut out and counted using Triton/toluene scintillation fluid. When appropriate the number of counts of [^{32}P] incorporated into RNA could be expressed relative to a certain number of [^{3}H] c.p.m. hence taking into account any nonspecific losses occuring during extraction.

(ix) Determination of [^{3}H] -Uridine Incorporated into TCA Insoluble Products

Scintillation vials were seeded with 2 x 10^6 BHK cells/vial and incubated overnight in a 95% air/5% CO_2 incubator. The next day the medium was removed, the cells washed once with cold (4°C) medium 199 containing 2% calf serum, 1μg/ml actinomycin D and buffered with 20mM HEPES, pH 7.4 (199 ADH). The cells were infected with 20 PFU/cell of a temperature sensitive mutant or wild type virus for 1 hour at 4°C after which time the virus was removed, the monolayers were washed once with pre-warmed 199 ADH and then incubated in 1ml of 199 ADH at the appropriate temperature. The cells were labelled by removing the medium, washing once with HEDA (V,ii) and then labelling the cells for 30 minutes with 1ml HEDA + 2μCi [^{3}H] -uridine. At the end of the labelling period the medium was removed and the scintillation vial placed in a dry ice/methanol bath, 2mls of 5% TCA was added and allowed to freeze. At the end of the experiment the vials were removed from the freezing bath and the TCA was allowed to thaw. The cells were then washed three times with 5% TCA + 0.1M sodium pyrophosphate, twice with ethanol/ether (3/1, v/v) and finally once with ether and allowed to air dry. When dry, the monolayers were dissolved in 0.2ml of soluene (room temperature, 10 minutes) and then 4mls of acid scintillator was added and the amount of [^{3}H] incorporated was determined using a scintillation counter. All determinations were carried out in triplicate.

(x) UV Inactivation of the RNA Synthesised in Infected CEF Cells

CEF cells were diluted to 3 x 10^6 cells /ml in GMEM + P containing 5uCi/ml [^{3}H] -uridine, seeded at 9 x 10^6 cells/5cm plastic petri dish (3mls/dish) and incubated overnight. The following day the cells were infected (V,vii) and then incubated at the appropriate temperature. Prior to irradiation the cells were washed three times with phosphate free Earles salts solution containing 1µg/ml actinomycin D but lacking serum and irradiated for periods up to 14 minutes. The emission maximum of the lamp (Anderman and Co.) was at 256nm and the incident dose was 30 ergs/second/mm^2. The cells were then labelled with PFEDA containing 500 µCi/ml [^{32}P] -orthophosphate for one hour. The PFEDA contained 10µg/ml of cycloheximide to prevent further production of the polymerase (See Results Section II, i). The RNA was then extracted using the Triton N101 method described in V,vii above and the various species separated and quantitated as described in V, viii above.

(xi) Labelling and Identification of Nucleocapsids in Mutant Infected Cells

This was performed essentially as described by Burge and Pfefferkorn (1968). Nearly confluent monolayers of BHK cells in 1 litre flat bottomed flow bottles were infected at a multiplicity of 20 - 25PFU/cell for 1 hour at 39°C in a circulating water bath after which time the virus suspension was replaced with medium 199 containing 2% calf serum and 1µg/ml actinomycin D and the bottles returned to the water bath. At 6 hours post infection the medium was replaced with 10 mls of Earles salts solution containing 2% dialysed calf serum, 1µg/ml actinomycin D and 10 µci/ml [^{3}H] -uridine for 2 hours. At the end of the labelling period the cells were washed three times with cold (4°C) TNE, scraped from the bottle and pelleted at 4°C (350g, 5 minutes). The cells were resuspended in 1ml of 0.1 x TNE and allowed to swell on ice for 20 minutes after which time the cells were disrupted by the use of a tight fitting glass dounce (25 strokes). The nuclei and undisrupted cells were removed by centrifugation at 4°C (300g, 10 minutes) and the post nuclear supernatant made 2mM in EDTA, by addition

of 0.25 mls of 10mM EDTA, in order to disrupt the polysomes. A portion of the sample (300 - 500µl) was layered on top of a linear 15% - 30% w/v sucrose gradient in 50mM tris buffer containing 100mM NaCl, pH 7.4 (TN) and centrifuged for 14 hours at 40,000 g in a 6 x 14 titanium swing out rotor. The gradients were then fractionated by upward displacement, the fractions were precipitated by the addition of five volumes of 10% TCA and 200µg of RNA as co-precipitant and the precipitates collected on glass fibre filters, washed three times with 5% TCA + 0.1M sodium pyrophosphate twice with ethanol/ether (3/1, v/v) and once with ether. The discs were dried and then counted in a scintillation counter after addition of Triton/toluene scintillator. The nucleocapsid peak was identified by treating purified labelled virus with Triton N101 and then centrifuging it in a parallel gradient.

(xii) One Step Purification of Labelled Sindbis Virus

The gradient used here is an adaptation of that described by Scheele and Pfefferkorn (1969a). It combines features of both velocity and isopycnic centrifugation and consists of three separate sections. The bottom portion was a 0.5 ml cushion of 50% sucrose in 0.2M CsCl, 2mM tris, pH 7.8 followed by a 3ml density gradient (1.127 to 1.245 g/cm^3) formed by appropriate mixing of 1.5 ml of 25% sucrose (w/v) in PBS and 1.5 ml of 50% sucrose in 0.2M CsCl, 2mM Tris, pH 7.8. A second gradient was then formed on top of this consisting of a 6 ml linear 5% - 20% sucrose gradient in PBS. Medium (1 ml) containing released virus was layered on top of this gradient and the whole was centrifuged for 3.5 hours at 60,000g in a 6 x 14 titanium spin out rotor. The gradients were fractionated directly into scintillation vials, Triton/toluene scintillator was added and the radioactivity, corresponding to released virus, was determined by liquid scintillation counting.

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SECTION VI

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Up from Earth's Centre through the Seventh Gate

I rose, and on the Throne of Saturn sate,

And many Knots unravel'd by the Road;

But not the Knot of Human Death and Fate.

Rubaiyat of Omar Khayyam

Up from Earth's Centre through the Seventh Gate

I rose, and on the Throne of Saturn sate,

And many Knots unravel'd by the Road;

But not the Knot of Human Death and Fate.

Rubaiyat of Omar Khayyam

APPENDIX

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Synthesis of Sindbis Virus Nonstructural Polypeptides in Chicken Embryo Fibroblasts

HENRY BRZESKI AND S. IAN T. KENNEDY*

Department of Biological Sciences, University of Warwick, Coventry, CV4 7AL, England

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The identification of eight previously undescribed polypeptides in chicken embryo cells infected with Sindbis virus is reported. Seven of these polypeptides were distinguishable from the virus structural polypeptides and their precursors by their molecular weights and tryptic peptide maps. The eighth was closely related to pE2 (Schlesinger and Schlesinger, 1973), a precursor to one of the virus particle glycoproteins. Pulse-chase experiments and the use of an inhibitor of proteolytic cleavage allowed a division of the seven nonstructural (NS) polypeptides into three stable end products (NS p89, NS p82, and NS p60) and four precursors (p230, p215, p150, and p76). The labeling kinetics after synchronous initiation of translation indicated that synthesis of the NS polypeptides started at a single site and showed that the order of the genes coding for the NS polypeptides was (5′ → 3′) NS p60, NS p89, and NS p82. Short-pulse experiments under conditions of both synchronized and nonsynchronized translation suggested that cleavage of the primary translation product of the NS genes occurred only after its synthesis was completed and that the first cleavage removed the C-terminal polypeptide. From these and other experiments, we propose a detailed scheme for the synthesis and processing of Sindbis virus NS polypeptides.

The genome of alphaviruses such as Sindbis virus and Semliki Forest virus consists of single-stranded RNA that has a molecular weight of $4.2 \pm 0.2 \times 10^6$ and a sedimentation coefficient of about 42S (29, 23). In Sindbis virus particles, this RNA is encapsidated by core (C) protein in a nucleocapsid, which is surrounded by a lipoprotein envelope containing two glycoproteins (E1 and E2 [27]). In Semliki Forest virus particles, a third glycoprotein (E3) is present (15). Experiments in several laboratories have established that, in infected cells, a subgenomic RNA species (26S RNA; molecular weight, $1.8 \pm 0.2 \times 10^6$) acts as mRNA for the structural proteins (4, 6, 7, 30, 34). Studies in infected cells and with cell-free synthesizing systems have shown that the gene order (5′ → 3′) in 26S RNA is C, E3, E2, and E1 and that these proteins are synthesized from a single initiation site by a combination of nascent and post-translational cleavages (8, 9, 25, 28, 31).

Since the 42S RNA is infectious (13, 26), it must encode all the virus-specified polypeptides required for virus multiplication. These polypeptides comprise not only the structural proteins but also nonstructural (NS) polypeptides, some or all of which are components of the virus-specified RNA-dependent RNA polymerase (24, 32). Recent work has established that the nucleotide sequence of the 26S RNA is located inward from the 3′ end of the 42S RNA, and therefore the genes coding for the NS polypeptides must be situated in the 5′ terminal two-thirds of the genome (19, 34). In Semliki Forest virus-infected cells, several NS polypeptides have been identified (10, 17, 18, 20), and we have recently reported the presence of two virus-specified NS polypeptides in preparations of virus RNA polymerase purified from infected-cell lysates (11). Several lines of evidence, including cell-free translation studies with 42S RNA, as well as the kinetics of appearance of NS polypeptides in infected cells after synchronous initiation of translation (5, 10, 16, 21), suggest that synthesis of the NS polypeptides is initiated at a single site near the 5′ end of the genome and terminates internally before the start of the structural protein genes and that the primary translation product is then processed (5, 20). Many aspects of this overall scheme are, however, quite unclear. In particular, little is known about the processing of the primary translation product and, since almost all the available information has come from studies with Semliki Forest virus, we considered it pertinent to examine in some detail the expression of the NS genes of the 42S RNA of Sindbis virus.

In the present paper, we report the presence of three previously undetected NS polypeptides

in Sindbis virus-infected cells and show by tryptic peptide mapping and pulse-chase experiments that these polypeptides are synthesized via a series of precursors, the largest of which probably represents the entire translation product of the NS genes.

MATERIALS AND METHODS

Materials. Acrylamide, sodium dodecyl sulfate (SDS; especially pure grade), and molecular-weight markers (range, 53,000 to 265,000) were obtained from British Drug Houses Ltd; *N,N'*-methylenebisacrylamide from Eastman Organic Chemicals; trypsin (treated with L-1-tosylamido-2-phenylethylchloromethyl ketone) from Worthington Biochemical Corp.; *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) from Flow Laboratories Ltd; ovalbumin, bovine serum albumin, human transferrin, and phosphorylase *a* from Sigma Chemical Co; L-[^{35}S]methionine (350 to 500 Ci/mmol) from the Radiochemical Centre; and precoated silica gel plates for thin-layer chromatography from Schleicher and Schüll, Dassel, W. Germany. Actinomycin D was a generous gift from Merck, Sharpe and Dohme.

Virus. The wild-type AR339 strain of Sindbis virus was used throughout this work (1). Working stocks were prepared by infecting BHK, clone 13, cells in 2.5-liter roller bottles with (per cell) 0.1 PFU of virus that had been plaque-purified three times. After 1 h at 37°C the inoculum was replaced with 50 ml of medium 199 containing 2% calf serum, and incubation was continued for 24 h at 37°C. The extracellular fluid, which contained 0.5 to 1 × 10^9 PFU/ml, was stored at −70°C and used without further passage.

Cells and media. All experiments were performed in chicken embryo cells set up in glass scintillation vials as described previously (25). To increase the incorporation of radioactive methionine, the cells were seeded and incubated overnight in Glasgow modified minimal essential medium (GMEM) containing 5% calf serum and one-tenth the normal amount of methionine. After infection, cells were incubated in GMEM lacking methionine but containing 2% dialyzed calf serum and 1 μg of actinomycin D per ml, buffered to pH 7.4 with 20 mM HEPES (GMEM-met).

Radioactive labeling of cells. Cells were washed once with GMEM-met and then infected with 100 PFU of virus per cell for 1 h at 37°C. The inoculum was then replaced with GMEM-met. At the times specified in individual experiments, the medium was replaced with Earle salt solution containing 2% dialyzed calf serum, 1 μg of actinomycin D per ml, and 50 μCi of [^{35}S]methionine per ml, buffered to pH 7.4 with 20 mM HEPES (labeling medium). In experiments involving a chase period, the labeling medium was replaced with identical medium but containing 2 mM unlabeled methionine in place of the isotope (chase medium). The pulse or chase was terminated by removing the medium and placing the vial in a freezing mixture of methanol/dry ice. The cells were then processed for gel electrophoresis as previously described (10).

SDS-polyacrylamide gel electrophoresis. Samples were run on slab gels (20 by 18.5 by 0.15 cm) with an apparatus similar to that described by Studier (33). Samples were analyzed on 7.5% acrylamide + 0.2% *N,N'*-methylenebisacrylamide with the buffer systems of Laemmli (22). After electrophoresis (for 16 h at 45 V), the gels were dried under vacuum at 90°C and autoradiographed on Kodirex X-ray film.

Molecular-weight determinations. The continuous-gel system of Fairbanks et al. (12) was used. For molecular weights greater than 100,000, samples were run on gels containing 3.5% acrylamide + 0.093% *N,N'*-methylenebisacrylamide. Molecular-weight markers in this instance consisted of a commercial product (from British Drug Houses) comprised of oligomers of a protein of molecular weight 53,000. Molecular weights of less than 100,000 were determined with a gel containing 7.5% acrylamide + 0.2% *N,N'*-methylenebisacrylamide. Markers were phosphorylase *a* (92,000), human transferrin (77,000), bovine serum albumin (68,000), and ovalbumin (45,000). Labeled virus-specified polypeptides and markers were subjected to coelectrophoresis on the same gel, which was stained with Coomassie blue to visualize the markers and then dried and autoradiographed to reveal the position of the labeled polypeptides.

Tryptic peptide mapping. p230 and p215 were prepared by labeling infected cells for 30 min at 4 h postinfection in the presence of 0.1 mM $ZnCl_2$. p150, p76, and pE2 were prepared by labeling cells for 10 min at 4 h postinfection. NS p89, NS p82, NS p60, NS p59, E1 + E2, and C were prepared by labeling cells for 1 h at 3.5 h postinfection and then incubating the cells in chase medium for a further 1 h. In all cases, the polypeptides were purified by gel electrophoresis and digested in situ with trypsin, and the peptides were eluted, oxidized with performic acid, and fingerprinted by two-dimensional thin-layer chromatography as described before (10).

RESULTS

Identification of novel virus-specified polypeptides. Replicate cultures of chicken cells, infected or mock-infected, were labeled with [^{35}S]methionine for 30 min at either 4 or 8 h postinfection, and the polypeptides were extracted and analyzed on SDS-polyacrylamide gels. The results of this experiment are shown in Fig. 1. Early in infection (lane b), a large number of host polypeptides were still being synthesized, but there were also certain new polypeptides with mobilities different both from host polypeptides (lane a) and the structural polypeptides and their precursors (p120, pE2, E1 + E2, and C). These new polypeptides are designated p150, NS p89, NS p82, and NS p60. Later in infection (lane c), host protein synthesis was largely shut off, with structural protein synthesis accounting for a large percentage of total protein synthesis. At this time, the new polypeptides were made in smaller amounts

in Sindbis virus-infected cells and show by tryptic peptide mapping and pulse-chase experiments that these polypeptides are synthesized via a series of precursors, the largest of which probably represents the entire translation product of the NS genes.

MATERIALS AND METHODS

Materials. Acrylamide, sodium dodecyl sulfate (SDS; especially pure grade), and molecular-weight markers (range, 53,000 to 265,000) were obtained from British Drug Houses Ltd; *N,N'*-methylenebisacrylamide from Eastman Organic Chemicals; trypsin (treated with L-1-tosylamido-2-phenylethylchloromethyl ketone) from Worthington Biochemical Corp.; *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) from Flow Laboratories Ltd; ovalbumin, bovine serum albumin, human transferrin, and phosphorylase *a* from Sigma Chemical Co; L-[^{35}S]methionine (350 to 500 Ci/mmol) from the Radiochemical Centre; and precoated silica gel plates for thin-layer chromatography from Schleicher and Schüll, Dassel, W. Germany. Actinomycin D was a generous gift from Merck, Sharpe and Dohme.

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SDS-polyacrylamide gel electrophoresis. Samples were run on slab gels (20 by 18.5 by 0.15 cm) with an apparatus similar to that described by Studier (33). Samples were analyzed on 7.5% acrylamide + 0.2% *N,N'*-methylenebisacrylamide with the buffer systems of Laemmli (22). After electrophoresis (for 16 h at 45 V), the gels were dried under vacuum at 90°C and autoradiographed on Kodirex X-ray film.

Molecular-weight determinations. The continuous-gel system of Fairbanks et al. (12) was used. For molecular weights greater than 100,000, samples were run on gels containing 3.5% acrylamide + 0.093% *N,N'*-methylenebisacrylamide. Molecular-weight markers in this instance consisted of a commercial product (from British Drug Houses) comprised of oligomers of a protein of molecular weight 53,000. Molecular weights of less than 100,000 were determined with a gel containing 7.5% acrylamide + 0.2% *N,N'*-methylenebisacrylamide. Markers were phosphorylase *a* (92,000), human transferrin (77,000), bovine serum albumin (68,000), and ovalbumin (45,000). Labeled virus-specified polypeptides and markers were subjected to coelectrophoresis on the same gel, which was stained with Coomassie blue to visualize the markers and then dried and autoradiographed to reveal the position of the labeled polypeptides.

Tryptic peptide mapping. p230 and p215 were prepared by labeling infected cells for 30 min at 4 h postinfection in the presence of 0.1 mM $ZnCl_2$. p150, p76, and pE2 were prepared by labeling cells for 10 min at 4 h postinfection. NS p89, NS p82, NS p60, NS p59, E1 + E2, and C were prepared by labeling cells for 1 h at 3.5 h postinfection and then incubating the cells in chase medium for a further 1 h. In all cases, the polypeptides were purified by gel electrophoresis and digested in situ with trypsin, and the peptides were eluted, oxidized with performic acid, and fingerprinted by two-dimensional thin-layer chromatography as described before (10).

RESULTS

Identification of novel virus-specified polypeptides. Replicate cultures of chicken cells, infected or mock-infected, were labeled with [^{35}S]methionine for 30 min at either 4 or 8 h postinfection, and the polypeptides were extracted and analyzed on SDS-polyacrylamide gels. The results of this experiment are shown in Fig. 1. Early in infection (lane b), a large number of host polypeptides were still being synthesized, but there were also certain new polypeptides with mobilities different both from host polypeptides (lane a) and the structural polypeptides and their precursors (p120, pE2, E1 + E2, and C). These new polypeptides are designated p150, NS p89, NS p82, and NS p60. Later in infection (lane c), host protein synthesis was largely shut off, with structural protein synthesis accounting for a large percentage of total protein synthesis. At this time, the new polypeptides were made in smaller amounts

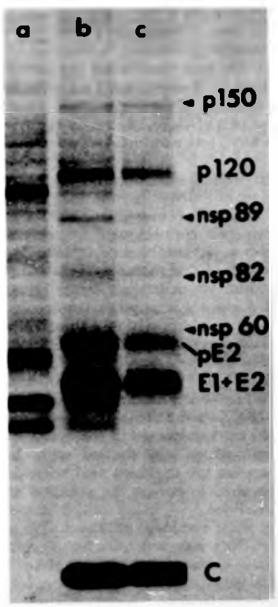

FIG. 1. *Synthesis of virus-specified polypeptides in chicken cells during a 30-min pulse. Infected and mock-infected replicate cultures of chicken cells were labeled with [^{35}S]methionine for 30 min and then processed for SDS-polyacrylamide gel electrophoresis. (a and b) Profiles from mock-infected and infected cells, respectively, labeled at 4 h postinfection. (c) Profile from infected cells labeled at 8 h postinfection. Migration in this and all subsequent electrophoretograms is from top to bottom. The identities of E1 + E2 and C were established from the positions of the structural proteins of purified virus particles run on a parallel gel (not shown).*

than at earlier times. Over the duration of the experiment, there were no apparent changes in the pattern of polypeptides from mock-infected cells.

Product-precursor relationships among the novel polypeptides. Recently, evidence has been presented which suggests that a second cleavage scheme, distinct from the one that produces the structural proteins, operates for the NS proteins of Semliki Forest virus (10, 20). To see whether a similar scheme operates in Sindbis virus-infected cells, two experiments were performed. First, two infected and two mock-infected cultures were labeled for 10 min at 4 h postinfection, and then one of each type of culture was chased for a further 1 h. Extracts were prepared from these cultures and analyzed on an SDS-gel. Figure 2 shows the result of this pulse-chase experiment. The infected sample prepared immediately after the pulse (lane a) contained p150, NS p89, and two additional polypeptides (p215 and p76) not present in the respective mock-infected extract (lane c). After a chase in infected cells (lane b), radioactivity was found in NS p89, NS p82, and NS p60, whereas p215 had disappeared, and the amounts of p150 and p76 had decreased. In addition, an additional polypeptide, NS p59, with a mobility close to that of pE2, was detected after a chase. Together, these results suggest that NS p89, NS p82, NS p60, and NS p59 are stable end products and that p215, p150, and p76 may be precursors to them. In the second experiment, zinc ions, which have been reported to cause accumulation of uncleaved high-molecular-weight precursors (3), were added to infected and mock-infected cultures,

FIG. 2. *Synthesis of virus-specified polypeptides in chicken cells during short-pulse and pulse-chase conditions. (a and b) Infected and (c and d) mock-infected chicken cells were labeled for 10 min with [^{35}S]methionine at 4 h postinfection. One sample of each was processed immediately (a and c), while the others had labeled medium replaced with chase medium for a further 1 h (b and d).*

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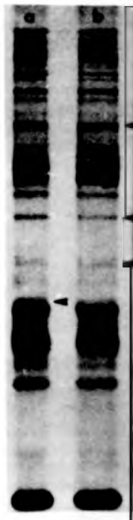

FIG. 3. *Effects of virus-specified poly chicken cells were in tion, the medium w taining various conce h postinfection, the m ing medium contain tion of zinc. After a processed for electrop 0.01 mM Zn^{2+}; (c) 0 Zn^{2+}.*

FIG. 4. *Calibratio calibration curve for determined with ca*

which were then pulsed with [^{35}S]methionine, and their proteins were analyzed (Fig. 3). Over the range of zinc chloride concentrations used in this experiment, there was no detectable change in the polypeptide profiles from mock-infected cells. With low concentrations of zinc (less than 0.01 mM), cleavage in infected cells appeared to occur normally (lanes a and b), but as the concentration was increased, the amounts of p150, NS p89, NS p82, and NS p60 decreased, while at the same time the amount of p215 increased and a further high-molecular-weight polypeptide, p 230, became clearly visible (lanes c and d). This result is entirely consistent with the tentative conclusions drawn from the pulse-chase experiments and suggests that p230 may be the precursor of all the stable end products.

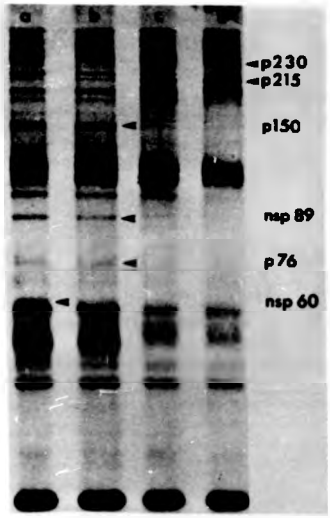

FIG. 3. *Effects of zinc ions on the synthesis of virus-specified polypeptides. Replicate cultures of chicken cells were infected and, at 3.5 h postinfection, the medium was replaced with medium containing various concentrations of zinc chloride. At 4 h postinfection, the medium was replaced with labeling medium containing the appropriate concentration of zinc. After a further 30 min, the cells were processed for electrophoresis. (a) 0.001 mM Zn^{2+}; (b) 0.01 mM Zn^{2+}; (c) 0.05 mM Zn^{2+}; and (d) 0.1 mM Zn^{2+}.*

Molecular weights of the novel polypeptides. The molecular weights of all the novel polypeptides, both stable end products and their putative precursors, were determined by using the continuous-gel system of Fairbanks et al. (12). The determinations were carried out in two parts as described in Materials and Methods. The two calibration curves are shown in Fig. 4. On the basis of these determinations, the novel polypeptides were designated p230 (molecular weight 230,000), p215 (molecular weight 215,000), p150 (molecular weight 150,000), NS p89 (molecular weight 89,000), NS p82 (molecular weight 82,000), p76 (molecular weight 76,000), NS p60 (molecular weight 60,000), and NS p59 (molecular weight 59,000).

Tryptic peptide mapping of the novel polypeptides. To further investigate possible precursor-product relationships, virus-specified polypeptides were isolated from infected cells and compared by the technique of tryptic peptide mapping. The conditions for the labeling of the polypeptides were essentially those described above for analytical gels, except that 10 vial cultures were used for each experiment. The peptide maps are shown in Fig. 5.

Several points emerge from this experiment.

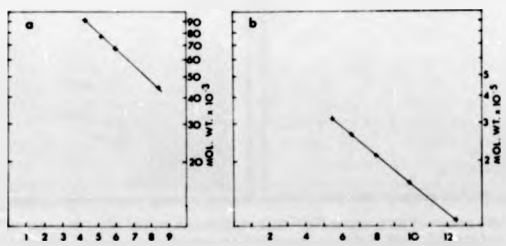

FIG. 4. *Calibration curves for the determination of the molecular weights of the novel polypeptides. The calibration curve for proteins with molecular weights of less than 100,000 (a) or more than 100,000 (b) were determined with commercial markers on 7.5 and 3.5% acrylamide gels, respectively.*

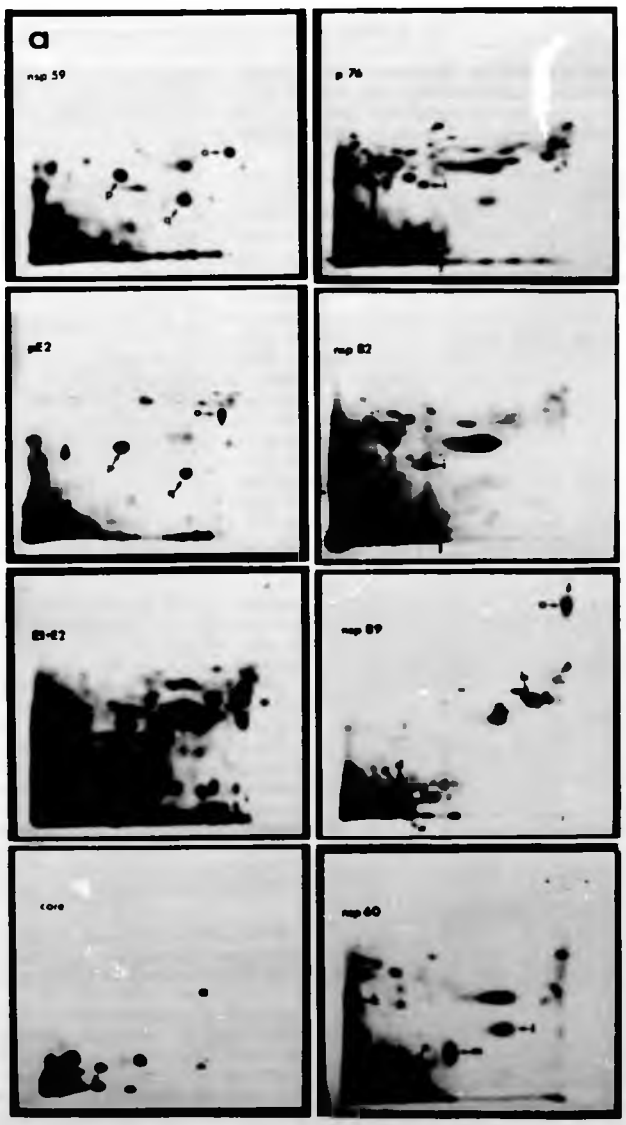

FIG. 5. *Tryptic peptide maps of virus-specified polypeptides. Each of the virus-specified polypeptides was labeled as described in Materials and Methods, purified by polyacrylamide gel electrophoresis, and digested with trypsin, and the performic acid-oxidized peptides were analyzed by two-dimensional chromatography. The first dimension is from left to right; the second is from bottom to top. In general, 1 cpm/dalton was applied. (a) Fingerprints of the structural proteins and their precursors, together with the putative NS polypeptides and p76. (b) Fingerprints of the NS polypeptides and their putative precursors. To aid comparisons, some of the most prominent spots in each map have been lettered (a–o). The significance of spot x is described in the text.*

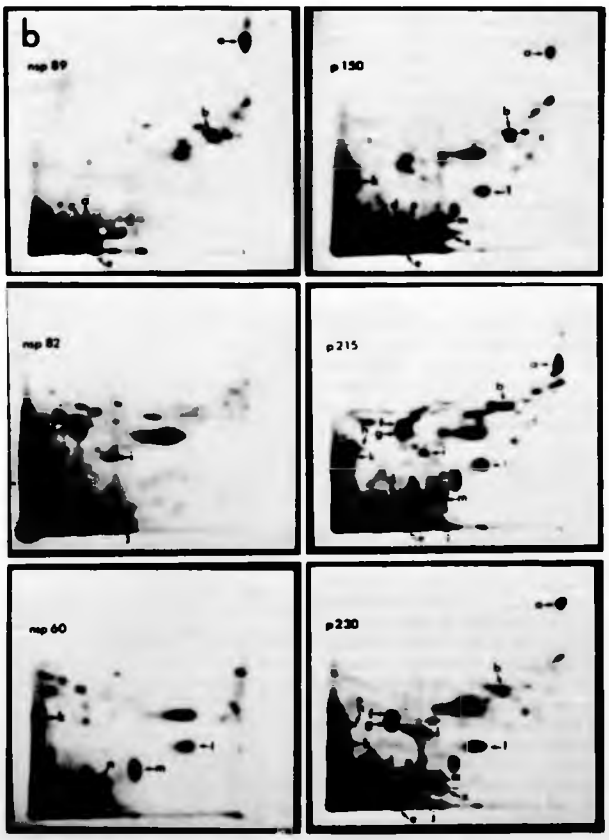

FIG. 5b.

First, comparison of the maps of NS p89, NS p82 and NS p60 show them to be unrelated to the virus structural proteins or their precursors (pE2, E1, E2, and C; Fig. 5a); therefore, these novel polypeptides can indeed be regarded as NS. Second, the map of NS p59 (Fig. 5a) shows a large number of spots (o, p, q, r, and s) that are also seen in pE2, so it is likely that NS p59 is some portion of the pool of pE2 that remains stable during chase (Fig. 2). A similar phenomenon has been observed in Semliki Forest virus-infected cells, in which there exists a protein that is apparently related to pE2 but which does not undergo cleavage to E2 + E3 (17). Third, comparison of the maps of NS p82 and p76 (Fig. 5a) shows a high degree of similarity (spots f, g, h, i, and j), suggesting that these proteins have a similar amino acid sequence. The apparent increase in molecular weight could be explained by a post-translational modification of p76, and experiments are in progress to determine exactly what this might be. There are, therefore, three stable NS proteins present in Sindbis virus-infected chicken cells. Fourth, the map of p150 (Fig. 5b) shows those spots characteristic of NS p89 and NS p60 (a, b, c, d,

e, k, l, m, and n) but not those of NS p82 (f, g, h, i, and j), whereas the map of p215 contains the spots characteristic of NS p89, NS p82, and NS p60 and so could be considered as a precursor to all three NS proteins. The map of p230 (Fig. 5b) shows a very high degree of similarity to that of p215, with the exception of one prominent spot (x). This spot is also present in the map of p150 and is absent from all the other maps. Taken together, these results strongly suggest that NS p89, NS p82, and NS p60 arise by cleavage of p230, p215, and p150. Indeed, with a molecular weight of 230,000, p230 may represent the entire translation product of the NS genes of the 42*S* RNA.

Ordering of the genes coding for NS p89, NS p82 and NS p60. Incubating cells in medium containing high salt prevents further initiation of protein synthesis but does allow continued polypeptide elongation (9). Removal of this block by replacement with isotonic medium, after all protein synthesis has terminated, results in synchronous initiation. To order NS p89, NS p82, and NS p60, salt synchrony was employed as follows. Four hours postinfection, initiation of protein synthesis was blocked by adding medium made hypertonic by increasing the concentration of NaCl by 225 mM. Ribosome runoff was then allowed to take place over a period of 40 min, by which time translation, as measured by incorporation of [^{35}S]methionine into trichloroacetic acid-precipitable material, had stopped. The block was then released by replacing the hypertonic medium with isotonic medium containing [^{35}S]methionine. After increasing periods of time, the label was removed and replaced with chase medium for a further 1 h to allow stable end products to accumulate. The results, shown in Fig. 6, indicate that the first NS protein to be synthesized was NS p60, which could be seen after a 4-min pulse (lane b); next to appear was NS p89, which was visible by 6 min (lane c); and last to appear was NS p82, which was not visible until 10 min (lane d) after release of the block. The gene order (5′ → 3′) is therefore NS p60, NS p89, and NS p82. These results are supported by the peptide maps, which demonstrate that NS p89 and NS p60 are adjacent to one another (in p150) and that NS p82 must therefore be terminal in p215 (and p230).

Orders of cleavage of the NS polypeptides from their precursors. As we have already observed, in a short pulse (Fig. 2, lane a) p76 and p150 are clearly visible, whereas only very small amounts of NS p89 can be seen (NS p60 is obscured by pE2). This suggests that translation of the entire NS genes occurs before cleavage of p230 commences and that the first cleavage is C-terminal, producing p76 and p150. To explore this possibility further, a modification of the salt synchronization technique was employed. After release from the salt block, cells were labeled for 2, 4, 6, 8, 10, and 12 min and then immediately processed for gel electrophoresis. The result of this experiment is shown in Fig. 7. Up to 8 min after synchronous initiation, no discernible NS polypeptides or their precursors were seen. However, after 10 min of incubation, p150 became visible and after 12 min, p215 and p150 were seen. By contrast, the C protein that is known to be nascently cleaved from the translation product of the 26S RNA (9) was visible by 4 min of incubation. This result clearly confirms the suggestion that cleavage only commences after translation of the NS genes has been completed.

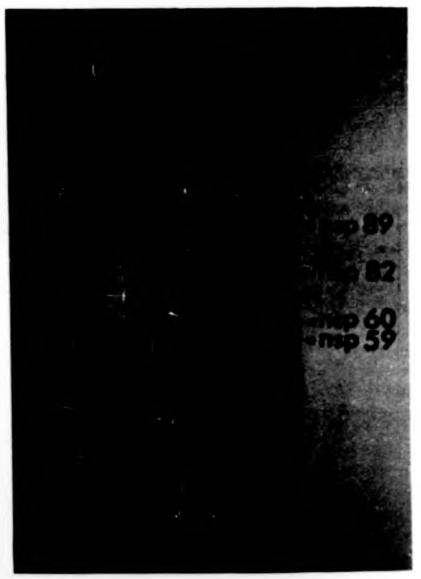

FIG. 6. *Synthesis of virus-specified polypeptides under pulse-chase conditions after synchronous initiation of protein synthesis. Replicate cultures of infected cells were incubated until 4 h postinfection, at which time the medium was replaced with medium containing an extra 225 mM NaCl for 40 min. The hypertonic medium was then replaced with labeling medium for (a) 2 min, (b) 4 min, (c) 6 min, (d) 10 min, and (e) 15 min. At the end of the labeling period, the medium was replaced with chase medium for a further 1 h, and the cells were extracted for gel electrophoresis. Approximately equal quantities (30 μg) of each extract were analyzed.*

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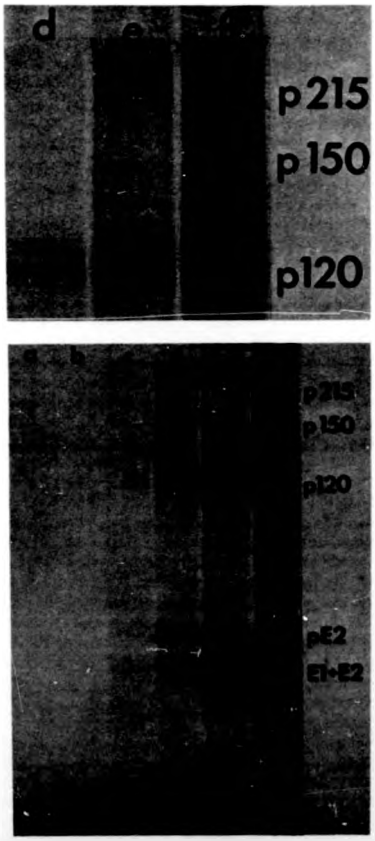

FIG. 7. *Synthesis of virus-specified polypeptides under short-pulse conditions after synchronous initiation of protein synthesis. Replicate cultures of infected cells were incubated until 4 h postinfection, at which time the medium was replaced with medium containing an extra 225 mM NaCl for 40 min. The hypertonic medium was then replaced with labeling medium for (a) 2 min, (b) 4 min, (c) 6 min, (d) 8 min, (e) 10 min, and (f) 12 min. After the pulse, incorporation was immediately stopped, as described in Materials and Methods, and extracts were prepared and analyzed by gel electrophoresis. Approximately equal quantities (50 μg) of each extract were analyzed. The upper panel is a magnified view of the top right-hand corner of the lower panel.*

DISCUSSION

The present work identifies eight novel polypeptides synthesized in chicken cells after infection with Sindbis virus. Most of these polypeptides are synthesized in proportionally greater amounts early in the virus multiplication cycle. Peptide mapping demonstrates that, of these eight polypeptides, only one, NS p59, is related to the structural proteins of the virus particle. NS p59 is closely related to pE2, possibly a subglycosylated variant of it. The remaining seven polypeptides are therefore NS and, as such, represent translation products of the 5′-terminal two-thirds of 42S RNA, i.e., that part of the 42S not represented in 26S RNA. Additional evidence that these seven polypeptides are indeed virus specified is provided by the observations that (i) they are readily detectable in infected but not uninfected hamster cells and (ii) in chicken fibroblasts infected with several RNA-negative mutants, a high-molecular-weight polypeptide, identical by tryptic peptide mapping with p215, accumulates at the nonpermissive, but not at the permissive, temperature (data not presented). Pulse and pulse-chase experiments (Fig. 1 and 2), together with the use of an inhibitor of post-translational cleavage (Fig. 3), permit a division of these seven polypeptides into three stable end products (NS p89, NS p82, and NS p60) and four unstable putative precursors (p230, p215, p150, and p76). Tryptic peptide mapping (Fig. 5) clearly confirms this precursor-product hypothesis and suggests the cleavage scheme shown in Fig. 8 for the expression of the NS genes of Sindbis virus. In this scheme, translation of the NS genes, which is initiated at or near the 5′ end of the genome (5, 16), produces p230. Although we cannot be certain that p230 represents the entire translation product of the NS genes, its molecular weight (230,000) is close to the theoretical coding capacity of the NS region of the 42S RNA (approximately 250,000, assuming that the total coding capacities are 420,000 for the 42S RNA and 175,000 for the 26S RNA). We propose that there are three cleavage sites (A, B, and C) in p230. Cleavage at C generates p150 and p76. Alternatively, p230 may be cleaved at A, generating p215 and fragment X. A careful comparison of the tryptic peptide maps of p230 and p215 in Fig. 5b shows a single peptide (x), which is present in p230 but absent from p215, and this peptide may therefore be characteristic of fragment X. This also indicates that fragment X is terminal in p230. Since the peptide characteristic of fragment X is present in p150, then this fragment must be at the extreme N-terminal end of p230. The fate of fragment X is, however, unclear. Analysis on gradient gels designed to resolve polypeptides in the molecular-weight range expected of X (7,500 to 20,000) have so far failed to provide evidence that fragment X is a stable end product and it may,

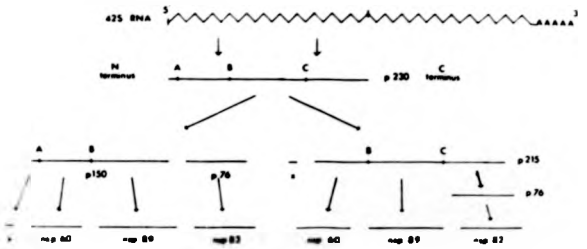

FIG. 8. *Proposed scheme for the synthesis and processing of Sindbis virus NS polypeptides.*

therefore, be degraded. We shall consider a possible role for fragment X later in the discussion. Cleavage of p150 at A and B generates fragment X, NS p60, and NS p89, and cleavage of p215 at B and C generates NS p60, NS p89, and p76. From short-pulse experiments either in unsynchronized (Fig. 2, lane a) or salt-synchronized cells (Fig. 7), it seems clear that the several cleavages outlined above only occur after translation of the NS genes has been completed. This situation contrasts with the one that operates during the synthesis of the structural proteins, in which there is evidence from both in vitro and in vivo studies that the C protein (the N-terminal protein) is nascently cleaved from the translation product of the 26S RNA (4, 6, 7, 9). The final step in the processing scheme converts p76 to NS p82. The nature of this conversion is unclear. The tryptic peptide maps of p76 and NS p82 are very similar (Fig. 5), and our evidence that p76 is indeed the precursor to NS p82 rests on pulse-chase data (Fig. 2). A possible modification of p76 that could result in an apparent increase in molecular weight is glycosylation, and experiments are in progress to investigate this and other possibilities. Salt synchronization experiments (Fig. 6) show that the gene order (5′ → 3′) of the NS genes in 42S RNA is NS p60, NS p89, and NS p82.

Since 42S RNA is infectious (13, 26) some, if not all, of the NS polypeptides described in the present work are extremely likely to be components of the virus-specified RNA-dependent RNA polymerase. The observation that this enzyme is associated with smooth membranes (14) raises the question of how the NS polypeptides could become associated with membrane. One explanation could be that fragment X plays a role analogous to the "signal sequence" present at the N-terminus of several proteins that are either components of, or are destined to cross, cellular membranes (2). Thus, the role of fragment X might be to anchor the NS polypeptide precursors in smooth membrane.

Recently, the synthesis and processing of NS polypeptides of Semliki Forest virus have been described (10, 17, 18, 20, 21). A comparison of the data in these reports with our present observations suggests that at least two of the Sindbis virus NS polypeptides, namely, NS p89 and NS p60, have counterparts in Semliki Forest virus-infected cells. Moreover, in one case, the NS polypeptides (designated NS p90 and NS p63) have been shown to be components of purified RNA polymerase (10). This observation, therefore, supports the idea mentioned earlier that NS p89 and NS p60 may be components of Sindbis virus polymerase. However, in addition to this correspondence, there is the observation of a third NS polypeptide (NS p82) in Sindbis virus-infected chicken cells, which Clegg et al. (10) did not detect in Semliki Forest virus-infected BHK cells. Four NS polypeptides have been identified or inferred in chicken cells infected with a temperature-sensitive mutant of Semliki Forest virus (21). Attempts to detect additional NS polypeptides in Sindbis-infected chicken cells by, for example, extending the labeling time of infected cells to 25 min after release from hypertonic block (more than twice the time required to label NS p82, the C-terminal polypeptide in p230) have not been successful. Because of the possibility that NS polypeptides might be masked by host or other virus-specified polypeptides, notably the structural polypeptides or their precursors, the final solution of not only the question of the number of NS polypeptides, but also the question of their function(s), may have to await the reconstruction of a membrane-associated polymerase capable of synthesizing 42S and 26S RNA of positive polarity and 42S RNA of negative polarity.

ACKNOWLEDGMENTS

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