REPLICATION OF SEMLIKI FOREST VIRUS RNA IN INFECTED CHICK CELLS

by

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1. Classification, distribution and growth of Semliki Forest virus

Semliki Forest virus (SFV) is a group A arbovirus (or togavirus (Melnick, 1971)), first isolated in Uganda (Smithburn and Haddow, 1944). The arboviruses (arthropod-borne viruses) multiply in both vertebrates and blood-sucking arthropods. They are transmitted between arthropods and vertebrates during the feeding of the arthropod on a vertebrate host.

Classification of arboviruses is based on criteria different from the physical and chemical characteristics used in establishing most other groups of viruses. These criteria include: the circumstances of isolation, antigenic relationships with established members of the group, effect in laboratory animals and tissue cultures, and reduction in infectivity on exposure to sodium deoxycholate or ether (Casals, 1961).

Approximately 200 viruses have been designated arboviruses, of which 150 have been placed in 21 groups (Casals and Brown, 1954; Casals, 1957). Members of a group cross-react with each other in a serological test, and therefore probably have antigens in common, but each group is antigenically distinct. Groups A and B have been most extensively studied, and these contain many viruses of clinical importance to man.

Propagation of an arbovirus requires that there is at least one vertebrate species susceptible to infection by vector bite, and that a vertebrate host can circulate sufficient virus in the blood to infect other vectors. Maintenance of an arbovirus requires the intimate and frequent association of the vector and vertebrate host. It also requires that the vector prefers blood from an efficient host, and that sufficient numbers of the two are associated in an environment which will allow the virus to complete its extrinsic incubation (Casals and Clarke, 1965).

There are 19 arboviruses which have been assigned to group A, and within the group a number of sub-groups can be distinguished by haemagglutination-inhibition and complement-fixation tests (Casals, 1957, 1963), and by a neutralization test (Porterfield, 1961). By these criteria, SFV is placed in a sub-group of 10 viruses, also including Mayaro and Chikungunya. Another sub-group includes Venezuelan equine encephalitis, and a third sub-group, Western equine encephalitis (WEE) and Sindbis. Although these viruses have been placed in different antigenic groupings, very little difference has been demonstrated between their morphology, the structure of their components and their replication. Thus, data on all these viruses will be considered, where relevant.

The natural vector for all group A arboviruses is the mosquito, although there have been occasional reports of isolation from other arthropods. SFV has been biologically transmitted in the laboratory by the mosquito Aëdes aegypti (Woodall and Bertram, 1959).

Infection of man and domestic animals by arboviruses is usually tangential to the basic cycle between the vector and its host. Arboviruses may cause a variety of syndromes in man, but clinical disease is recognised in only a small proportion of infected persons. No known disease of man or lower animals has been associated in nature with SFV. However, neutralizing antibodies to SFV have been found in man (Smithburn and Haddow, 1944).

SFV has a wide host range under laboratory conditions. It grows well in the brains of newborn mice (Cheng, 196la), although its pathogenicity varies. Strains of the four original isolations of SFV were tested for virulence in laboratory animals. Some strains were lethal for mice, guinea-pigs and rabbits, and others only for mice (Bradish et al. 1971). SFV also grows in a variety of tissue culture cells. It multiplies with marked cytopathic effect in chick embryo fibroblasts (CEF), the human lines HeLa and Hep-2, baby hamster kidney (BHK21) cells, and mouse L cells (Zwartouw and Algar, 1968; Erlandson et al. 1967; Kääriäinen and Gomatos, 1969; S.I.T. Kennedy, 1972, personal communication). SFV has been grown in cultures of Aëdes aegypti mosquito cells, but yields are at least one hundred fold lower than those obtained from BHK or chick cells. The virus rarely produces any cytopathic effect in mosquito cells (Buckley, 1969; Peleg, 1969). Visible plaques are formed on monolayer cultures of CEF, BHK, rabbit kidney, HeLa and Vero (Green monkey) cells (Bergold and Mazzali, 1968; S.I.T. Kennedy, 1972, personal communication).

2. Chemical and physical characteristics of Semliki Forest virus

When examined with the electron microscope, SFV appears to be a spherical particle, approximately 70 nm. in diameter, bearing projections (Faulkner and McGee-Russell, 1968). RNA, protein and lipid are present in the virus particle in the proportions 6 : 63.4 : 30.6 (Ranki, 1972). However, carbohydrate comprises about 14% of the viral envelope protein (Spiro, 1969). The overall composition of the virion is thus RNA 5.6%, protein 59%, lipid 28.4% and carbohydrate 7%. The particle weight is approximately 6.5×10^7 (Ranki, 1972). The molecular weight of the RNA estimated from this data is 3.6×10^6.

The virus particle, which has a sedimentation coefficient of about 300S, consists of a lipoprotein envelope surrounding a ribonucleoprotein core, or nucleocapsid, which is released by treatment with detergents (Horzinek and Mussgay, 1969; Kääriäinen et al. 1969). The nucleocapsid, which has a sedimentation coefficient of about 150S, can also be extracted from infected cells (Dobos and Faulkner, 1969a; Acheson and Tamm, 1970a). When examined with the electron microscope, it appears to be a spherical particle about 40 nm. in diameter (Harrison et al. 1971). However, slightly acid conditions cause an irreversible shrinkage of the nucleocapsid to about 32nm. diameter (Söderlund et al. 1972). The symmetry of the nucleocapsid is unknown, but is thought to be cubic (Adelwahab et al. 1964; Klimenko et al. 1965; Acheson and Tamm, 1967).

Data from X-ray crystallography suggests that the nucleocapsid is surrounded by a protein-free lipid bilayer (Harrison et al. 1971). It is thought that envelope protein subunits protruding from this lipid layer are responsible for the "spikes" seen by electron microscopy. Evidence for this arrangement is the selective removal of the spikes by digestion with bromelain, a proteolytic enzyme, leaving the nucleocapsid intact (Oram et al. 1971; Compans, 1971). The nucleocapsid contains a single polypeptide (Strauss et al. 1968). It is rich in lysine (Simons and Käriäinen, 1970), and has a molecular weight of 32,000 - 37,000 (Acheson and Tamm 1970b; Simons and Käriäinen, 1970; Kennedy and Burke, 1972).

The envelope protein is hydrophobic (Helenius and Söderlund, 1972, personal communication) and has a molecular weight of 51,000 - 53,000 (Strauss et al. 1969; Simons and Käriäinen, 1970; Kennedy and Burke, 1972). It has been generally thought that the envelope protein consists of a single polypeptide, but there is a report that two have been separated (Schlesinger et al. 1972). However, only one of these was found in extracts of the infected cell, and it was suggested that one was derived from the other. Two envelope proteins have also been found in SFV grown in BHK 21 but not in chick cells (S.I.T. Kennedy, personal communication). The significance of these findings is not certain at present.

The viral envelope consists of glycoprotein (Burge and Strauss, 1970; Strauss et al. 1970), a feature apparently common to many cellular

and viral membrane proteins (Compans, 1971; Keller et al. 1970; Garon and Moss, 1971; Mountcastle et al. 1971; Ginsburg and Neufeld, 1969). The carbohydrate content is similar to that of a number of cellular glycoproteins (Spiro, 1969), consisting of glucosamine, mannose, fucose, galactose and sialic acid residues. The carbohydrate probably resides on the surface of the visible "spikes". This is indicated by the agglutination of the virus by concanavalin A, a phytohaemagglutinin which binds to certain sugar residues (Becht et al. 1972).

The composition of the lipid of the viral envelope is as follows: neutral lipid (mainly cholesterol) 31%, phospholipid (especially phosphatidyl choline and phosphatidyl ethanolamine) 61%, and glycolipid 8%. The molar ratio of cholesterol to phospholipid is 1 : 1 (Renkonen et al. 1971). The lipid content of the viral envelope is similar to, but not identical with, that of the plasma membrane of the infected or uninfected host cell. The viral envelope contains less free fatty acid and a little more cholesterol than the plasma membrane (Renkonen et al. 1971). In contrast to the protein of the virus envelope, which is virus-specified, the lipids are made before and during virus infection (Pfefferkorn and Hunter, 1963b; Kääriäinen and Söderlund, 1971). This is similar to the situation in other enveloped viruses, such as SV5, a paramyxovirus, which specifies its own glycoprotein but accepts host lipids (Klenk and Choppin, 1969).

Complement-fixing and haemagglutinating activities are associated with the infectious particle (Cheng, 1961b). Infectivity is reduced

by exposure to sodium deoxycholate, chloroform (Mayr and Bögel, 1961), or Tween 80 and ether (Mussgayand Rott, 1964; Theiler, 1957). Trypsin has no effect on the infectivity or haemagglutinating activity of the virus (Cheng, 1958). More than 60% of the viral phospholipid could be hydrolyzed by phospholipase C, with no reduction in viral infectivity (Friedman and Pastan, 1969). However, if incubation was continued after inactivation of the enzyme, the virus rapidly lost both infectivity and protein. These results all suggest that phospholipid is important for the structural and functional integrity of the virus.

The only species of RNA which can be extracted either from virus or nucleocapsid is a single-stranded molecule with a sedimentation coefficient of 42S (Friedman and Berezesky, 1967). The molecular weight of this RNA, estimated by polyacrylamide gel electrophoresis, is in the range $3.5 \times 10^6 - 4.5 \times 10^6$ (Cartwright and Burke, 1970; Dobos and Faulkner, 1970). A recent estimate is 4×10^6 (Levin and Friedman, 1971). Comparison with the RNA composition of SFV and the virus particle weight (above) suggests that there is only one 42S molecule in the virion. The proportions of bases in the virion RNA are uracil 19.5%, adenine 29.1%, guanine 25.5%, cytosine 25.9% (Kääriäinen and Gomatos, 1969).

3. Replication and morphogenesis of Semliki Forest virus

SFV replicates in the cytoplasm of infected cells, and matures by budding at the exterior plasma membrane. In vertebrate cells

which support the growth of SFV, the growth cycle is rapid. When chick cells were infected at a multiplicity of 20 plaque-forming units (p.f.u.) per cell, intracellular virus began to increase from about $3\frac{1}{2}$ hours after infection. The maximum intracellular titre of virus was reached about 8 hours after infection. Increasing the multiplicity of infection (m.o.i.) to 70 p.f.u. per cell reduced the time for the first appearance of newly-synthesized virus to $2\frac{1}{2}$ hours, and maximum yields were obtained $6\frac{1}{2}$ hours after infection (Mecs et al. 1967). In BHK 21 cells, the growth of SFV was somewhat faster, maximum yields of virus being obtained about 7 hours after infection with 20 p.f.u. per cell (Kääriäinen and Gomatos, 1969). The 2 to 4 hour latent period is followed by a period of exponentially increasing release of virus, as originally described for Western equine encephalitis (Dulbecco and Vogt, 1954). Yields of about 100 p.f.u. per cell are obtained from BHK cells, and up to 1000 p.f.u. per cell using CEF cells in suspension culture.

When a virus matures by budding at the cell surface, maturation and release of virus will be synchronous. Western equine encephalitis was found to be released within minutes of maturation in chick embryo cells (Rubin et al. 1955). Working with the same system, Morgan et al. (1961) described viral "precursors", about 30 nm. in diameter, surrounding cytoplasmic vacuoles, and in paracrystalline arrays. They suggested that the particle-lined vacuoles were sites of virus assembly. Acheson and Tamm (1967) saw similar vacuoles, paracrystalline

arrays and tubular structures surrounded by nucleocapsids, in chick embryo cells infected with SFV. However they were only seen late in infection, in cells showing marked cytopathic effect. It was concluded that they were not directly involved in virus formation. However, Grimley et al. (1968) described two types of vacuolar structure in cells infected with arboviruses, which they termed type I and type II cytopathic vacuoles (CPV-I and CPV-II). CPV-I were found as early as 3 hours after infection and were most prominent in the early stages of infection. CPV-II were seen 4 hours after infection, and their proportion to CPV-I rose during the later stages of infection. It was suggested that CPV-I were the sites of viral RNA replication, and that assembly of nucleocapsids took place on CPV-II.

More recently, membranes from infected cells have been fractionated by Friedman et al. (1972). When cells were pulse-labelled with ^{3}H-uridine before disruption, radioactive RNA was found in the same fraction as CPV-I and viral RNA polymerase activity. When cells were treated early in infection with cycloheximide or guanidine, which respectively inhibit viral protein and RNA synthesis, the formation of CPV-I was also depressed. By contrast, treatment with actinomycin D only delayed their formation slightly. Thus, these vacuolar structures appear to be sites of viral replication, as originally suggested by Morgan et al. (1961). Addition of cycloheximide rapidly inhibited the release of infectious virus, suggesting that protein synthesis is required for maturation (Scheele and Pfefferkorn, 1969a). Interestingly, budding of virus continued for at least two hours after

addition of the inhibitor, as visualized by electron microscopy (Friedman and Grimley, 1969).

It is possible to interrupt the budding of Sindbis virus from chick embryo cells by making the culture medium hypotonic (Waite and Pfefferkorn, 1970a). Restoring the ionic strength resulted in an instantaneous burst of virus release, suggesting that a very late step in maturation and release is being affected.

Cells infected with Western equine encephalitis were found to release a non-infectious subviral particle into the medium at the same time as infectious virus. Infectious RNA could be extracted from these particles with cold phenol, a treatment which does not release RNA from complete virions (Wecker and Schonne, 1964). These particles were not merely damaged virus, but apparently nucleocapsids released with incomplete envelopes later in infection, when cell damage became more marked (Grüschel and Wecker, 1965). Similarly, nucleocapsids extracted from Sindbis virus-infected cells are non-infectious, but contain infectious RNA (Dobos and Faulkner, 1969a).

Although defective particles of Sindbis virus have been found in BHK cells (Schlesinger et al. 1972), good preparations of SFV are apparently highly infectious. Cheng (1961a) reported a ratio of particles to plaque-forming units of 1 : 1 for SFV grown in baby mouse brain.

4. Effect of viral infection on host cell macromolecular synthesis

Infection with small RNA viruses such as poliovirus or Mengo virus results in a rapid and profound decrease in synthesis of cellular protein and RNA (Salzman et al. 1959; Penman et al. 1963; Fenwick, 1963). It is thought that a protein synthesized in the infected cell decreases the template activity of cellular DNA for DNA-dependent RNA polymerase (Martin and Kerr, 1968). The inhibition of protein synthesis results from accelerated breakdown of polysomes, possibly as a consequence of inactivation of host-cell messenger RNA.

Such rapid changes are not seen in cells infected with arboviruses. There is some reduction in host RNA synthesis during the exponential phase of growth of SFV in chick embryo cells (Taylor, 1965). In the presence of actinomycin D, the rate of host cell protein synthesis in chick cells infected with SFV is decreased to half of that in uninfected cells by 4 hours after infection, and to 10% by 12 hours after infection (Morser, 1972). The effect was faster at higher multiplicities of infection. Phospholipid synthesis was also decreased in BHK cells infected with Sindbis virus, but this was a secondary effect of the inhibition of host cell RNA and protein synthesis (Waite and Pfefferkorn, 1970b).

5. Semliki Forest virus protein synthesis

The molecular weight of the virion RNA of SFV is about 4×10^6. There is thus sufficient RNA to code for a protein with a molecular weight of approximately 4.5×10^5. There is at lease one virus-coded RNA polymerase, and possibly two (Martin and Sonnabend, 1967; Burge

However, the polymerase has not been identified

and hence the genetic information required to code for it is unknown. No genome products have been connected with other viral functions, such as the inhibition of host cell macromolecular synthesis.

Five polypeptides have been detected in animal cells infected with SFV and can be clearly distinguished by polyacrylamide gel electrophoresis. The estimated molecular weights of these are: I 98,000, II 77,000, III 62,000, IV 53,000 and V 36,500 (J. Morser et al., personal communication). Polypeptides IV and V correspond to the envelope and core proteins, respectively (Hay et al. 1968; Kääriäinen et al. 1969; Kennedy and Burke, 1972). When cells infected with SFV or Sindbis virus were treated with inhibitors of proteolytic enzymes, an accumulation of polypeptide I was observed, with a concomitant decrease in synthesis of core and envelope protein. This suggests that polypeptide I is a precursor to the core and envelope proteins (Pfefferkorn and Boyle, 1972; J. Morser, et al., personal communication). It has been shown by amino acid analysis and immunoprecipitation that polypeptide III and envelope protein are related. It has therefore been suggested from the molecular weights of the proteins that polypeptide I is a precursor to polypeptide III and core protein; and that polypeptide III is a precursor to envelope protein (Kennedy et al. 1972). No function has been proposed for polypeptide II.

Two virus-specific RNA molecules have been detected in polysomes extracted from SFV-infected cells, the major one having a molecular

weight of about 1.8×10^6 (see below). This could code for a protein of molecular weight about 200,000. It is suggested that this is a viral messenger RNA (Kennedy, 1972). The combined molecular weights of polypeptides I and II is about 175,000, which is close to the coding capacity of the viral messenger RNA. A polypeptide of molecular weight 165,000 was detected in SFV-infected chick cells treated with an inhibitor of chymotrypsin (Morser, 1972).

Poliovirus proteins are formed from a large precursor polypeptide by a specific cleavage mechanism (Jacobson et al. 1970). On the other hand, the genome of reovirus consists of pieces of double-stranded RNA, each of which codes for a separate protein (Bellamy and Joklik, 1967; Zweerink and Joklik, 1970). It appears that a third mechanism may exist in arboviruses, in which the principal messenger is not the genome RNA (though it must be translated initially, see below), but a molecule of about half this size, which is probably a polycistronic messenger. When cells were infected with Sindbis virus and pulse-labelled with radioactive amino acid, the first labelled virus to be released was preferentially labelled in the core protein. This suggests that assembly of the nucleocapsid is faster than modification of the cell membrane by addition of viral envelope protein. However, synthesis and release of virus is very rapid; labelled virus was detected within 20 minutes of addition of labelled amino acid (Scheele and Pfefferkorn, 1969b). Nucleocapsids were found to be assembled within three minutes of addition of labelled amino acids in SFV-infected cells (Friedman, 1968a). A large surplus of structural protein was made, over what is incorporated into complete virus.

6. Semliki Forest Virus RNA Synthesis

Several new RNA species are synthesized in cells infected with group A arboviruses. Three classes of RNA have been distinguished by centrifugation in sucrose gradients, with sedimentation coefficients of 42S, 26S and 20S (Friedman et al. 1966; Sonnabend et al. 1967; Cartwright and Burke, 1970). The 42S and 26S species are single-stranded, whereas the 20S RNA is largely, though not entirely, double-stranded. The first species to appear after infection was the 20S RNA, followed by 26S RNA and finally the 42S species.

The RNA of the arboviruses is replicated by an RNA-dependent RNA polymerase, characterized by its insensitivity to actinomycin D. This is in contrast to the DNA-directed RNA synthesis which takes place in uninfected cells (Reich et al. 1962). The virion RNA is infectious (Friedman et al. 1966), suggesting that at least part of it must be translated, to produce the viral RNA polymerase, and that transfer of information to another nucleic acid is not required for polymerase synthesis (Baltimore, 1971). As expected, no enzyme for transcription of RNA has been found in the virion of group A arboviruses.

A virus-specific RNA polymerase activity can be detected in infected cells (Martin and Sonnabend, 1967). These authors showed that 5'-triphosphates of guanosine, adenine, cytidine and uridine were converted into an acid-insoluble product, which was degraded by alkali. This RNA polymerase activity was not found in uninfected cells, and appeared at the time of

rapid viral RNA synthesis. The product was resistant to ribonuclease, suggesting that it was double-stranded, and sedimented at 20S. However, no single-stranded RNA product could be demonstrated.

The viral RNA polymerase in Sindbis virus-infected cells has been partially purified by centrifugation on discontinuous sucrose gradients by Sreevalsan and Yin (1969). These authors isolated a cytoplasmic structure containing membrane-associated RNA polymerase activity, which was not found in uninfected cells. The preparation incorporated nucleoside triphosphates in the absence of added template. The products of the polymerase reaction were analyzed by sucrose gradient centrifugation. A ribonuclease-sensitive species sedimenting at a similar rate to virion RNA was found, together with material which was partially ribonuclease-resistant, sedimenting as a disperse band between 18S and 28S. It was suggested that the enzyme preparation was catalyzing the synthesis of all the species of viral RNA made in the infected cell (Sreevalsan and Yin, 1969).

Labelling infected cells with ^{3}H-uridine for a brief period results in the appearance of heterogeneous RNA which is apparently membrane-bound and partially ribonuclease-resistant (Friedman, 1968a; Cartwright and Burke, 1970). This heterogeneous RNA has many properties in common with that found in bacterial cells infected with bacteriophages and which has been termed "replicative intermediate" (RI) (Fenwick et al. 1964; Kelly and Sinsheimer, 1964). An RNA of similar structure has been found in cells infected with picornaviruses, and its role in replication has been

demonstrated (Franklin, 1966; Baltimore, 1968). The RI of two picornaviruses has been shown to be infectious (Bishop and Koch, 1969). There is only one report of the RI of an arbovirus being infectious, that of Chikungunya virus, a close serological relative of SFV (Yoshinaka and Hotta, 1971).

Like virion 42S RNA, the 42S species extracted from infected cells is infectious. It has the same base composition as virion RNA (Sonnabend et al. 1967; Kääriäinen and Gomatos 1969) and the two are therefore probably identical. It is synthesized de novo in infected cells from the cellular nucleotide pool (Pfefferkorn and Hunter 1963b) and is found within two hours of infection (Wecker and Schonne, 1964; Kääriäinen and Gomatos, 1969).

The RNA of infecting virus appears to be converted rapidly to a double-stranded form. When infection was carried out with Sindbis virus containing ^{3}H-labelled RNA, label appeared in ribonuclease-resistant material within the first hour of infection. This material had a sedimentation coefficient of about 20S (Pfefferkorn et al. 1967). When protein synthesis was inhibited by cycloheximide, this conversion did not take place. Similarly, when mutants of Sindbis virus with a temperature-sensitive block in RNA synthesis were used, no conversion of parental RNA to a double-stranded form occurred at a nonpermissive temperature. These authors thought that breakdown and reuse of input RNA was not occurring, and that this conversion represented the initial step in RNA replication.

The 26S RNA found in infected cells is not generally thought to be infectious, although material sedimenting at 26S from WEE-infected cells

was reported to have a very low level of infectivity (Sreevalsan et al. 1968). Its molecular weight, as estimated from polyacrylamide gel electrophoresis, is $1.7 \times 10^6 - 1.8 \times 10^6$ (Cartwright and Burke, 1970; Dobos and Faulkner, 1970; Levin and Friedman, 1971).

Several theories have been proposed to account for 26S RNA. It has been suggested that 26S is a conformational variant of 42S RNA, and that viral RNA, released from the polymerase in the form of 26S, may be converted to the 42S form during incorporation into the virus particle (Sonnabend et al., 1967). However, this would require a radical conformational change, and the one report of this conversion being effected in vitro has not been substantiated (Sreevalsan et al. 1968). These authors suggested that 26S differed from 42S only by having a more open structure. However, if this were so, the 26S species should move more slowly than 42S RNA on polyacrylamide gel electrophoresis, whereas the reverse is the case (Cartwright and Burke, 1970).

It has also been suggested that 26S RNA is a precursor to 42S RNA. This is based mainly on three kinds of evidence: the size of 26S RNA, which is approximately half that of 42S RNA; the kinetics of synthesis of RNA species, 42S being formed later than 26S RNA, but at a similar rate (Cartwright and Burke, 1970); and on experiments in which 42S RNA has been claimed to be converted to a 26S form by denaturation with urea, dimethylsulphoxide or heat (Sreevalsan et al. 1968; Dobos and Faulkner, 1969b). More recently, it has been shown that if precautions are taken

in extraction of 42S RNA, no conversion to 26S occurs in denaturing conditions (Arif and Faulkner, 1972), and it has been suggested that the 26S RNA arose from hidden breaks in the viral RNA.

A special function for 26S RNA is suggested by experiments on Sindbis virus-infected chick embryo cells. When protein synthesis was inhibited by puromycin the synthesis of the 26S RNA was selectively inhibited (Scheele and Pfefferkorn, 1969b). However, once under way, RNA synthesis did not appear to require concomitant protein synthesis. A similar result was obtained using a mutant with a temperature-sensitive block in RNA synthesis, by shifting the temperature of infected cells from the permissive to the nonpermissive temperature when RNA synthesis was first detectable. This implies that the syntheses of 26S and 42S RNA are controlled by different gene products.

The 42S, 26S and 20S species of RNA are only incompletely separated by centrifugation, but may be completely resolved by polyacrylamide gel electrophoresis (Cartwright and Burke, 1970). Previously unrecognized species of RNA have been observed by this technique (Levin and Friedman, 1971). These have mobilities on polyacrylamide gel intermediate between those of 42S RNA and of 26S, and have been designated 38S and 33S, although sedimentation coefficients have not been determined by centrifugation. The molecular weights of these species, estimated from mobility on polyacrylamide gel electrophoresis, are 3.1×10^6 and 2.4×10^6 respectively. Their functions are unknown. However, a clue to the function of one of these species has been suggested. Polyribosomes have been isolated from

SFV-infected chick embryo cells, and have been found to contain RNA which co-migrates with 26S and 33S RNA on polyacrylamide gel electrophoresis (Kennedy, 1972).

As yet, there is no firm evidence on the role of the 38S RNA. If it is not an artefact of extraction, it may be the product of premature termination of transcription of the 42S RNA. Alternatively, it may have a role in control of processes in virus-infected cells (Levin and Friedman, 1971).

7. Mechanism of RNA Biosynthesis

It was stated above that the replication of RNA viruses can occur in the absence of DNA synthesis, suggesting that DNA intermediates are not involved in this process. A group which is an exception to this is the RNA tumour viruses, but only viral replication which occurs independent of DNA synthesis will be considered here.

In 1963, Montagnier and Sanders discovered double-stranded RNA in cells infected with encephalomyocarditis virus (EMC). This RNA was infectious, and therefore contained the EMC genome, presumably base-paired with its complement, and was thought to be a suitable candidate for the template for RNA replication. Double-stranded RNA has since been found in cells infected with many RNA viruses, and it is now thought to be a general feature in RNA virus replication.

Much of the early work on RNA replication was done with bacteriophage systems, and this will be discussed where relevant, as there seem to be many parallels between replication in bacteriophage and animal virus systems.

To account for the presence of virus-specific double-stranded RNA in infected cells, it was suggested that the input (positive) RNA strand was replicated to produce a complementary (negative) strand. Synthesis of progeny (positive) strands then took place, using the negative strand as template. This complex of RNA strands was termed the replicative intermediate (RI). This structure has properties of both double-stranded and single-stranded RNA, the former being due to the annealing of the positive strand with its complement. The single-stranded character of RI is thought to represent partially displaced "nascent" progeny strands (Erikson and Erikson, 1967; Robertson and Zinder 1969; Libonati and Floridi, 1969).

When cells infected with bacteriophage or picornaviruses were labelled for a short period with a radioactive RNA precursor, the first RNA form to be labelled was the RI (Kelly et al. 1965; Fenwick et al. 1964; Baltimore and Girard 1966). In the case of phage infected bacteria, a subsequent "chase" with unlabelled precursor results in the displacement of label from the RI and its appearance in completed genome RNA (Fenwick et al. 1964). Difficulties over the equilibration of nucleotide pools have prevented "pulse-chase" experiments with RNA in

animal cells. However, they have been performed using crude preparations of picornavirus RNA polymerases, and, again, RI appears to be the immediate precursor of progeny single strands in the *in vitro* replication of RNA.

Although the discovery of double-stranded RNA in virus-infected cells provided a starting point for studying the mechanism of replication of viral RNA, the role of the fully double-helical RNA, called "replicative form" (RF), is uncertain. The actively replicating structure appears to be the RI, and it has been suggested that the RF is the end product of replication, when initiation of synthesis of new progeny strands on a template ceases, and synthesis of the nascent strands is completed. Accumulation of RF *in vivo* follows that of RI and progeny single strands, suggesting that RF is a product rather than an intermediate of the replication process (Cartwright and Burke, 1970). Experiments with polymerase preparations from picornavirus-infected cells suggest that, *in vitro*, at least a portion of the RF population does not participate in the complete replication process (Plagemann and Swim, 1968; Girard 1969). RF appears to be metabolically stable, but pulse-chase experiments suggest that it is derived from RI.

There is evidence from bacteriophage systems to suggest that the formation of RF is also the first event in replication of input RNA, by synthesis of a strand complementary to the parental molecule. The first product of the *in vitro* reaction using Q_β phage RNA replicase is double-stranded RNA. The density labelling technique, originally developed for studying replication of DNA (Meselson and Stahl, 1958),

has been applied to studying the replication of an RNA phage (Kelly and Sinsheimer, 1967a). The input genome of MS2 phage is incorporated into a double-stranded form without breakdown or reincorporation. Both strands are then replicated, and synthesis of new duplexes continues at an exponential rate.

The kinetics of RNA synthesis in the infected cell suggest that a number of templates are synthesized soon after infection. It is estimated that there are about 500 templates present in the poliovirus-infected cell when RNA synthesis is at its maximum rate (Darnell et al. 1967). If RF is the initial template, then a number of molecules must be made early in infection.

8. Mechanism of synthesis of progeny single strands

Two modes of synthesis of viral RNA have been proposed, and these are best described by considering the transcription of double-stranded nucleic acid into single-stranded RNA by using DNA-dependent RNA polymerases. If the template is a synthetic DNA-RNA hybrid, the RNA is displaced by progeny strands, and only the DNA strand is conserved. This mode of replication is termed "semi-conservative" (Sinsheimer and Lawrence, 1964; Chamberlin and Berg, 1964). If double-stranded DNA is used as template, RNA is transcribed from the duplex, and both strands are retained. This is termed "conservative" replication (Hayashi, 1965).

Similarly, in conservative replication of RNA, the parental

positive strand remains in association with its complement throughout replication. In contrast, in semi-conservative replication of RNA, the parental positive strand is displaced by ongoing synthesis of progeny positive strands (Erikson and Franklin, 1966; Weissman et al. 1968). In systems where the mechanism of RNA biosynthesis has been studied, the great majority of it seems to be semi-conservative. The following evidence supports this conclusion.

1. The RI obtained after pulse-labelling in vivo (Kelly and Sinsheimer, 1967 b; Erikson, 1966) and in vitro (Girard, 1969) is highly resistant to pancreatic ribonuclease. This suggests that labelled strands are extensively hydrogen-bonded with template, which would be expected in semi-conservative replication. In conservative replication, one would only expect a small amount of the nascent progeny strand to be hydrogen-bonded to template (Baltimore, 1969; Hayashi, 1965).

2. Treatment of RI with ribonuclease gives rise to a base-paired, ribonuclease-resistant "core" structure from which nascent progeny strands have been removed (Baltimore and Girard, 1966; Bishop and Koch, 1969). The positive strand in this core turns over rapidly in vivo (Billeter et al. 1966) and in vitro (Weissman et al. 1964b). If replication were conservative, no turnover of the positive strand in the template would be expected, except during turnover of total template, which is an order of magnitude slower than the synthesis time of a molecule of poliovirus RNA in HeLa cells (Baltimore, 1969).

3. When the ribonuclease-resistant core of RI was denatured, 70% to 90% of the intact RNA molecules were negative strands (Bishop et al. 1969). If replication were conservative, one would expect intact positive and negative strands in the core.

4. In bacteriophage systems, parental RNA is rapidly converted to a ribonuclease-resistant form in vivo, which is progressively (though not completely)converted to a ribonuclease-sensitive form during replication (Weissman et al. 1964a; Erikson et al. 1964; Kelly et al. 1965). This implies that after the initial formation of a strand complementary to the parental molecule, positive strands are continuously displaced from the replicating complex.

One cannot exclude the possibility that both mechanismsare operating simultaneously. The observation that labelled parental RNA does not completely become ribonuclease sensitive after its initial eclipse, as mentioned above, suggests that some of the parental strands are conserved. Similarly, studies on the infectivity of native (Bishop et al. 1969) and denatured (Francke and Hofschneider, 1969) RI are consistent with about 10% of replication in the poliovirus and M12 phage systems being of the conservative type. Denaturation was carried out using formaldehyde, and residual infectivity thought to be due to positive strands which were completely base-paired to complementary strands. As was pointed out, however, the residual infectivity may have been due not to the occurrence of conservative replication, but to "negative RI", that is, RI in which the positive strand is the template, and is therefore conserved, and nascent strands are negative. It is difficult to resolve this issue.

The presence of negative RI was first postulated because free negative strands were found among the initial products of the Q_β phage replicase reaction (Spiegelman et al. 1968). No nascent negative

strands could be found in f2 phage-infected cells (Robertson and Zinder, 1969), but the proportion of positive to negative strand synthesis is about 10:1 in favour of positive strands, so the proportion of negative RI need only be very small.

A variation of the semi-conservative mode of replication is the circular model proposed for foot and mouth disease virus (Brown and Martin, 1965; Wild et al. 1968) and bovine enterovirus (Clements and Martin, 1971). This mechanism was proposed to account for the discovery of RNA strands apparently of greater length than the genome RNA. These observations have not been made with any other animal viruses, however. These mechanisms will be considered further in discussion.

MATERIALS & METHODS

MATERIALS

1. Chemicals

Acrylamide and sodium dodecyl sulphate were obtained from British Drug Houses, Ltd., Poole, Dorset, England.

N,N' - Methylene bisacrylamide and N,N,N',N'-tetramethylethylene diamine were obtained from Eastman Organic Chemicals, Rochester, N.Y., U.S.A.

Acrylamide and N,N'-methylene bisacrylamide were recrystallised before use from chloroform and acetone respectively (Loening, 1967).

Tris (hydroxymethyl) amino methane (tris), as Trizma base, and 2-mercaptoethanol were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A.

Diethylpyrocarbonate was purchased as Baycovin from Bayer AG, Leverkusen, Germany.

Dimethylsulphoxide and phenol were obtained from Hopkin and Williams, Ltd., Chadwell Heath, Essex, England.

2,5 - Diphenyloxazole (P.P.O.), 1,4 - di (2-(5-phenyloxazolyl))-benzene (P.O.P.O.P.) and naphthalene, all scintillation grade, were obtained from Nuclear Enterprises (G.B.) Ltd., Edinburgh, Scotland.

Aerosil standard silica was obtained from Bush Beach, Segner and Bayley, Ltd., London, England.

Dioxan and toluene were obtained from Fison's Ltd., Loughborough, Leicestershire, England.

Ether was obtained from May and Baker, Ltd., Dagenham, Essex, England.

Phenol and ether were both redistilled before use.

Agarose was obtained from L'Industrie Biologique Française S.A., Gennevilliers (Seine), France.

Cellulose, Whatman CFII, was obtained from Griffin and George, Ltd., London, England.

Diethylaminoethyl-dextran (DEAE-dextran) was obtained from Pharmacia, Uppsala, Sweden.

Uridine-5-T (24 Ci/m mole) and Uridine-2-^{14}C (60mCi/m mole) were obtained from the Radiochemical Centre, Amersham, Bucks., England.

2. Biological materials

Bovine pancreatic ribonuclease, as ribonuclease 1-A (100 Kunitz units/mg.), and bovine albumin powder, Cohn fraction V, were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A.

Deoxyribonuclease (ribonuclease-free, electrophoretically purified) was obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A.

Actinomycin D was obtained from Merck, Sharpe and Dohme Research Laboratories (Division of Merck and Co., Inc.), Rahway, N.J., U.S.A.

Medium 199 was obtained from Wellcome Reagents, Ltd., Beckenham, England.

Calf serum was obtained from Biocult Laboratories, Ltd., Paisley, Scotland.

Earle solution was obtained in the form of the solid salts from Oxoid, Ltd., London, England.

Penicillin and streptomycin were obtained in the form of Crystamycin, which contained 600 mg. (1 million units) of penicillin to 1 g. streptomycin. Crystamycin was purchased from Glaxo Laboratories, Ltd., Greenford, England.

Colomycin (colistin sulphomethate sodium) was obtained in batches of one million units from Pharmax, Ltd., Crayford, Kent.

Goose erythrocytes were the gift of Dr. C.J. Bradish.

3. Media

5% growth medium. Medium 199 was supplemented with calf serum to a final concentration of 5% (v/v), and adjusted to pH 7.4 with 5% (w/v) sodium bicarbonate solution. Penicillin and streptomycin were added to final concentrations of 60 µg. (100 units) per ml. and 100 µg. per ml. respectively.

Maintenance medium was identical to growth medium except that the concentration of calf serum was 2% (v/v) instead of 5%.

4. Buffers

TNE was 0.05M-tris, 0.1M-sodium chloride, 0.001M-ethylene diamine tetraacetate (disodium salt) (EDTA), adjusted to pH 7 at 20°C with hydrochloric acid.

TE, TN were as for TNE, but omitting NaCl and EDTA respectively.

Phosphate-buffered saline (PBS) was as described by Dulbecco and Vogt (1954).

Electrophoresis Buffer. This was prepared as described by Loening (1967).

A 10x concentrated stock solution was prepared as follows:

tris	43.5g.
$NaH_2PO_4.2H_2O$	46.8g.
EDTA $Na_2.2H_2O$	3.7g.

made up to 1000 ml. with glass distilled water.

For use as electrophoresis buffer, the stock solution was diluted 10x and SDS added to a final concentration of 0.2% (w/v).

Standard Saline Citrate (SSC) was 0.15M-sodium chloride, 0.015M-trisodium citrate, adjusted to pH7 with HCl.

Citrate buffer (for haemagglutination assay) was 0.045M-citric acid, 0.155M-trisodium citrate, 0.15M-NaCl.

TNM buffer was 0.01M-tris, buffered to pH 7.5 with HCl, 0.1M-NaCl and 0.002M-$MgCl_2$.

Borate-saline buffer was 0.05M-boric acid, 0.12 M-NaCl, pH 9.0.

5. Scintillation mixtures

Toluene scintillation mixture consisted of 6g. P.P.O. and 0.73g. P.O.P.O.P. dissolved in 1 l. toluene.

Toluene-triton scintillation mixture consisted of toluene scintillation mixture and Triton X-100, blended in the proportions 2:1.

Gel scintillation mixture consisted of a mixture of 50g. naphthalene, 7g. P.P.O., 150 mg. P.O.P.O.P., 30 ml. absolute alcohol and 200 ml. toluene, made up to 1 l. with dioxan. 35 g. silica gel was added and the mixture blended for 2 min.

METHODS

1. Preparation of primary chick embryo fibroblasts

Cells were prepared as described by Walters et al. (1967).

2. Determination of radioactivity incorporated into chick cell monolayers

Incorporation of radioactivity into monolayers was determined as described by Skehel et al. (1967), except that cells were washed with cold PBS instead of saline containing uridine.

3. Growth of Semliki Forest virus in suspension culture

Virus was grown in high density suspension cultures of chick embryo fibroblasts, as described by Kennedy and Burke (1972).

4. Preparation of radioactive Semliki Forest virus

Virus was grown in suspension culture in the presence of actinomycin D, 0.1 µg./ml., and ^{3}H-uridine, 10µCi/ml.

5. Virus purification

Cells and debris were removed from the suspension culture by centrifugation at 15,000 g for 30 minutes at 4°C. Calf serum was added to 5% (v/v) to the clarified supernatant. Virus was purified either (a) partially, by centrifugation through a linear sodium potassium tartrate gradient, or (b) more extensively, by a combination of ammonium-sulphate precipitation and centrifugation on continuous and discontinuous sucrose

gradients. The virus was kept as cool as possible without freezing, throughout the purification.

(a) The clarified supernatant was concentrated by pressure filtration using an Amicon ultrafilter, and the concentrated fluids were layered onto a preformed 5-50% (w/v) sodium potassium tartrate gradient, prepared in borate-saline buffer, pH 8.5. The gradient was centrifuged at 75,000 g for $2\frac{3}{4}$ hours. An opalescent band was observed about two-thirds of the way down the tube. Some aggregated material was also observed just below this band, but which had little HA activity, and was therefore probably non-viral. The gradient was fractionated by pumping from the bottom of the tube and aliquots of each fraction were assayed for HA activity, infectivity and radioactivity (Fig. 1). Peaks of all three parameters were coincident. Therefore, HA activity alone was sometimes used to detect the virus-containing fractions in subsequent experiments. The recovery of infectious virus was as shown in Table 1.

(b) Step 1. Ammonium-sulphate precipitation

Saturated ammonium sulphate was prepared in Earle's balanced salt solution and adjusted to pH7.5 with NaOH. The chilled solution was added dropwise to the virus suspension to a final concentration equivalent to 60% saturation. The opaque suspension was stirred for 15 min. at 4°C and the precipitate collected by centrifugation at 15,000 g for 20 minutes. The supernatant was discarded and the precipitate resuspended in one-twentieth the starting volume of TNE buffer. The suspension was dialyzed against 100 volumes of TNE buffer overnight at 4°C. The dialysate was

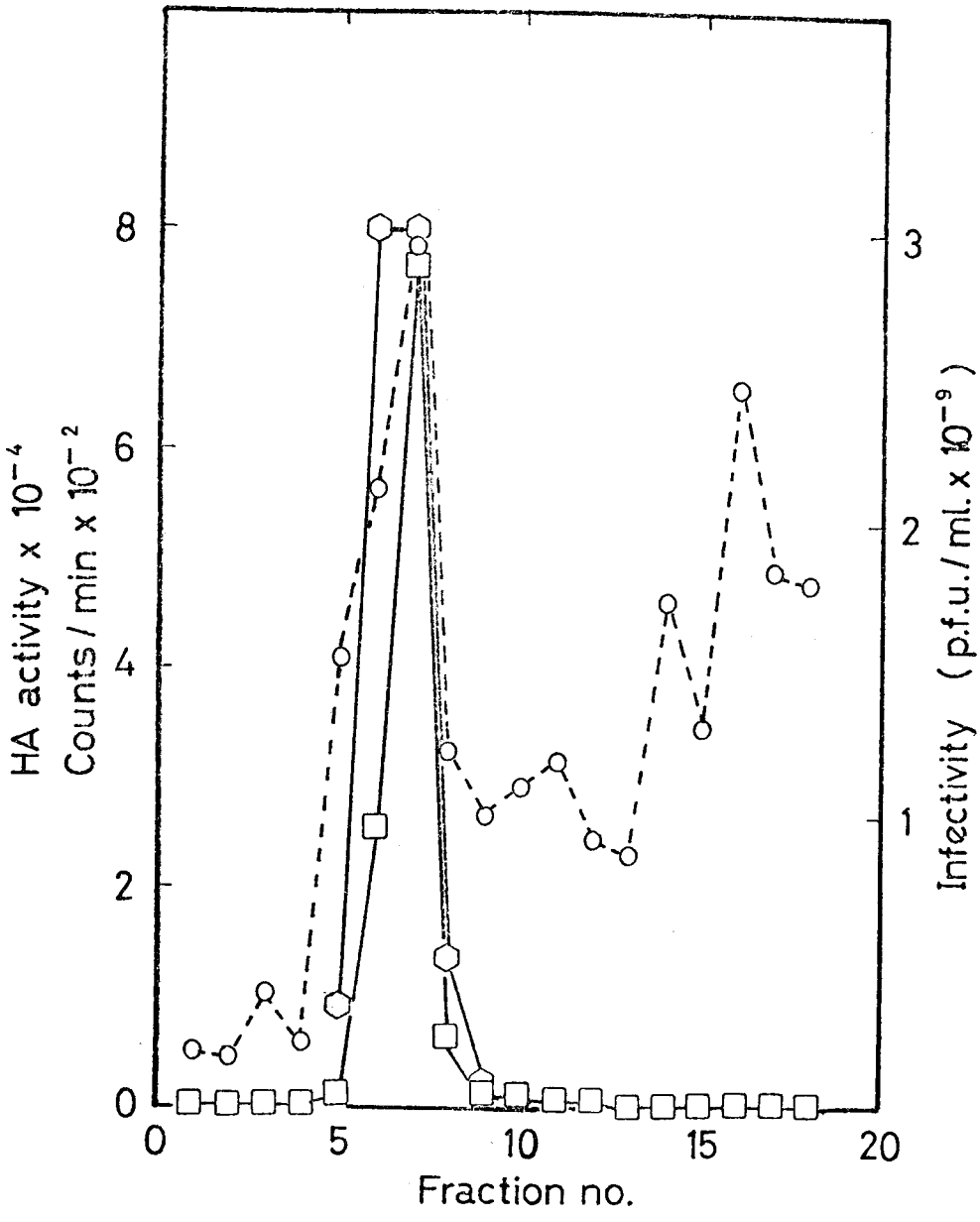


Fig. 1. Partial purification of SFV by tartrate gradient centrifugation. SFV was grown in suspension in the presence of ^{3}H-uridine and the clarified suspension (170 ml.) was concentrated (to 4 ml.) by ultrafiltration using an Amicon pressure filter. The concentrated suspension was layered onto a preformed, 20 ml., linear 5-50% sodium-potassium tartrate gradient, prepared in borate-saline buffer, pH 8.5. The gradient was centrifuged in an MSE 3 x 23 ml. swing-out rotor at 75,000 g for $2\frac{3}{4}$ hours, at 4°C. After centrifugation, gradients were unloaded downwards by pumping and fractions of about 1 ml. collected. Aliquots of fractions were taken for measurement of TCA-insoluble radioactivity (O-O), haemagglutinating activity (□-□), or infectivity (⬡-⬡). Centrifugation was from right to left.

Table 1. Recovery of Semliki Forest virus during purification

Scheme (a)

	Infectivity (p.f.u./ml.)	Total infections virus	Recovery (%)
Clarified suspension	4.2×10^7	6.7×10^9	(100)
Concentrated suspension	8.7×10^8	5.6×10^9	84
Gradient fractions 5–8	-	6.8×10^9	102

Scheme (b)

	HA titre	Total HA units	Recovery (%)
Clarified suspension	200	4.6×10^5	(100)
$(NH_4)_2 SO_4$ precipitate, redissolved	6,000	1.02×10^6	220
After discontinuous sucrose gradient	8,220	9.9×10^4	21
After continuous sucrose gradient	8,220	4.9×10^4	11

Suspension-grown SFV was purified according to Scheme (a) or Scheme (b), as described in Methods section 5.

centrifuged at 15,000 g for 30 min. and the supernatant collected.

Step 2. Discontinuous sucrose-gradient centrifugation

2 ml. 20% (w/v) sucrose was layered over 2 ml. 45% (w/v) sucrose in a 50 ml. centrifuge tube. Solutions were in TNE buffer containing 0.5% calf serum. 40 ml. virus suspension from step 1 was layered over the sucrose and centrifuged at 120,000 g. for 3 hours at 4°C. The supernatant was then removed, and the light-brown viral pellet resuspended in 1.0-1.5 ml. TNE buffer using a pipette.

Step 3. Continuous sucrose-gradient centrifugation

Resuspended virus was layered onto a preformed 20-55% (w/v) linear sucrose gradient, prepared in TNE buffer containing 0.5% calf serum, which was then centrifuged at 75,000 g for 15 to 18 hours at 4°C. The opalescent band containing virus was collected, diluted 4 - 5 fold in TNE buffer containing 0.5% calf serum and centrifuged at 150,000 g for 3 hours at 4°C. The supernatant was discarded and the light-brown virus pellet gently resuspended in TNE buffer. This purified preparation was not stored for any period exceeding a few hours at 4°C. Usually, it was used immediately. Typical recoveries during the purification scheme are shown in Table 1.

6. Extraction of RNA from purified virus

The suspension of purified virus was extracted with 1% 2-mercaptoethanol, 3.3% SDS and phenol. The phenol phase was re-extracted with TNE buffer containing 2% SDS and the aqueous wash pooled with the first aqueous extract. Phenol was removed by three washes

with ether, final traces of which were removed by evaporation in a stream of nitrogen.

7. Infectivity and haemagglutination assays for Semliki Forest virus

Infectivity was determined by plaque assay on monolayers of chick embryo fibroblasts as described by Walters et al. (1967). Haemagglutination (HA) activity was estimated using a modification of the method of Clarke and Casals (1958). Because of its greater buffering capacity at pH 5.8, citrate-saline buffer replaced phosphate-saline buffer.

8. Plaque titration of infectious RNA

Infectious RNA extracted from SFV was assayed by a modification of the method of Crick et al. (1966). Dilutions of RNA for inoculation were made in chilled 2M-$MgSO_4$ in 0.01M-tris-HCl buffer, pH 7.5, containing 1 mg./ml. DEAE-dextran (Pagano and Vaheri, 1965), and the assay was carried out on monolayers of chick embryo fibroblasts.

9. Preparation of radioactive viral RNA

The method used was a modification of that of Levin and Friedman (1971). Cells were infected with SFV at 10 p.f.u./cell, and ^{3}H-uridine was used as an RNA precursor rather than ^{3}H-uridine and ^{3}H-adenosine. After extraction of nucleic acids, two volumes of ethanol were added and the extract stored at -20 $^\circ$ C.

10. Preparation of cellular RNA

Monolayer cultures of chick embyro fibroblasts were incubated at 37°C in the presence or absence of ^{14}C-uridine (2μCi) for 18 to 24 hours. RNA was extracted as for virus-infected cells, but with phenol in the absence of SDS.

11. Treatment of cell extracts with deoxyribonuclease

When cells were treated with SDS, large amounts of DNA were extracted, which made extracts viscous. The viscosity, which makes manipulation of solutions difficult, was decreased by treatment of extracts with deoxyribonuclease (DNase). Ethanol precipitates of cell extracts were collected by centrifugation at 1500 g for 15 minutes at 4°C. The precipitate was washed with 70% ethanol in TNM buffer and redissolved in TNM buffer. DNase was added to a concentration of 10 μg/ml. and the extract incubated for 30 minutes at room temperature, with occasional gentle shaking. After incubation, two volumes of ethanol were added and the sample stored at -20°C.

12. Sucrose gradient centrifugation of RNA

Linear gradients of sucrose were prepared in TNE buffer or TNE buffer containing 0.5% SDS (TNES). Gradients were formed at room temperature and allowed to equilibrate at the running temperature. Gradients in TNE buffer were run at 4°C, and those in TNES at 20°C.

Up to 120 µg. RNA were layered onto gradients in the same buffer without sucrose. Gradients were unloaded by upward displacement with 50% - 65% (w/v) sucrose. Absorbance at 254 nm. was monitored using an LKB photocell and recorded by a Servoscribe pen recorder (Smiths Instruments, Ltd., Cricklewood, London).

13. Preparation of polyacrylamide gels

The method used was based on that described by Loening (1967). For preparation of gels containing agarose, the gel components, except for ammonium persulphate and TEMED, were mixed at double the final required concentration and brought to 45°C. An equal volume of melted 1% agarose was added, also at 45°C. Ammonium persulphate and TEMED were added, and the solution transferred immediately to 20 cm. Perspex tubes, sealed at the bottom ends. The surface was not overlaid and the gels were left at room temperature for one hour to solidify and polymerize. Gels were overlaid with water and could be stored at 4°C for several weeks.

14. Electrophoresis of RNA on polyacrylamide gels

Before electrophoresis, about 5 mm. was sliced from the top end of the gel, to remove a region of agarose and unpolymerized acrylamide. Gels were electrophoresed in an apparatus constructed so that most of the length of the gel tube was immersed in buffer. Gels were electrophoresed at 100 V. for about 30 minutes before the sample was

applied, to remove electrolytes. Buffer was recirculated at about 5 ml. per minute. Samples were dissolved in TNE or TE buffer containing 0.1 or 0.2% (w/v) SDS, 5-10% (w/v) sucrose and about 0.01% (w/v) bromophenol blue. Samples containing up to 50 µg. RNA in a volume of 100 µl. were applied without removing buffer from gels, using an Eppendorf plunger pipette. Gels were run at 100 V. (about 6 V. per cm.) at room temperature. Each gel drew about 7 mA.

15. Analysis of polyacrylamide gels after electrophoresis

Gels were removed from tubes and washed in distilled water for about 1 hour. When unlabelled chick cell RNA was used as a marker, the gels were analyzed by absorption of ultraviolet light. The gels were laid on thin-layer plates impregnated with a fluorescent dye (Polygram CEL 300 UV_{254}) and observed under ultraviolet light. RNA bands were dark purple against a bright green background. Quantities of RNA down to 10 µg. could be detected in this way. The gels were frozen and fractionated into 1 mm. slices using a Mickle slicer modified so that methanol at -15°C could be circulated through the supporting platform. Fractions were dissolved in 0.2 ml. hydrogen peroxide (100 volumes) at 70°C, and radioactivity determined in toluene-triton scintillator.

16. Chromatography of RNA on cellulose columns

Single- and double-stranded RNA were fractionated by chromatography on CF11 cellulose, as described by Bishop and Koch (1969). Columns

11 cm. x 1.5 cm. were packed under gravity with CF11 cellulose, suspended in TNE buffer to which was added ethanol to 35% (v/v) (35% ethanol buffer). Samples contained not more than 0.5 mg./ml. and were loaded in 35% ethanol buffer. The column was eluted by gravity under a head of buffer about 10 cm. high. This gave a mean elution rate of about 1 ml./min. Runs were at room temperature, and 1 ml. fractions were collected in tubes containing 2-mercaptoethanol (0.1 ml., 10% v/v).

17. Salt fractionation of RNA

As a first step in separation of RNA species, single- and double-stranded RNA were separated by treating cell extracts with IM-NaCl in TE buffer at -20°C for 18 hours by the method of Bishop and Koch (1967) or with 2M-LiCl at 4°C for 48 hours (Baltimore and Girard 1966). In both cases, precipitates were collected by sedimentation at 1,500 g for 20 min. and washed with the corresponding salt (IM-NaCl or 2M-LiCl in TNE buffer). The precipitate was dissolved in TNE buffer, and 2 volumes of ethanol added to this solution and the supernatant fraction. RNA was precipitated at -20°C.

18. Denaturation of RNA with dimethylsulphoxide

The method used was based on that of Iglewski and Franklin (1967). Samples were dissolved in TE buffer and 6 volumes DMSO added. The solution was incubated at 37°C for 20 min. and quickly cooled on ice. One-tenth of a volume of 10 x TNE buffer was added, and RNA precipitated with 2 volumes of ethanol at -20°C.

19. Measurement of radioactivity in TCA-insoluble material

The amount of radioactivity present in a sample of RNA was usually measured by determination of the radioactivity insoluble in cold, 5% TCA, in the presence of a suitable co-precipitant (or carrier). During the course of this work, it was noticed that the amount of radioactivity recovered in TCA-insoluble material depended both on the nature of the co-precipitant, and on the procedure used for washing the precipitate. Therefore, experiments were carried out to compare yeast RNA and BSA as co-precipitants for RNA, and to test different procedures for washing the precipitates.

The RNA used in these experiments was double-stranded RNA, prepared from SFV-infected chick cells, which had been labelled between 2 and 6 hours after infection. The double-stranded RNA was purified by treatment with IN-NaCl, as described in methods section 17, and precipitated with ethanol. The ethanol precipitate waz redissolved in 3 x TNE buffer, and 0.25 ml. 10% TCA added to 0.25 ml. samples. Either 100 µg. BSA or 250 µg. yeast RNA were added, from stock solutions containing 1 mg./ml. Ice-cold 5% TCA was added to a final volume of 1.5 ml. and the mixtures chilled in ice for 30 minutes. The precipitates were collected by centrifugation at 500 g for 10 minutes. The supernatants were carefully decanted and kept, and the precipitates were washed successively with 1 ml. 5% TCA, 1 ml. ethanol and 1 ml. ether, all ice- cold. After removing the ether, the precipitates were allowed to dry at room temperature and then dissolved in 0.5 ml.

0.2M-NaOH at 37°C for 1 hour. The test-tubes were washed with 0.5 ml. water, and the washings were added to the redissolved precipitates. The radioactivity in the redissolved precipitates, and in aliquots of the supernatants obtained by washing the precipitates, was measured using gel scintillator.

When BSA was used as carrier, no radioactivity was found in the supernatants obtained either from the initial precipitation with TCA, or in the TCA wash (Table 2). Nearly 60% of the radioactivity in the precipitate was reproducibly solubilized by washing with ethanol when BSA but not RNA was used as carrier. However, only about 60% of the radioactivity precipitated in the presence of BSA was precipitated when yeast RNA was used as carrier (Table 2). This may have been due to the quality of the commercial sample of yeast RNA used, since the supernatant obtained after precipitation in the presence of yeast RNA, but not BSA, was cloudy in appearance. Both the cloudiness and the radioactivity in the supernatant remained, even after repeated centrifugation.

In order to check that BSA was not causing the precipitation of TCA-soluble material, single-stranded RNA was extracted from uninfected chick cells, which had been grown in the presence of ^{14}C-uridine, and the RNA was treated with ribonuclease A (10 µg./ml., 37°C, 15 min., in 3 x TNE buffer). The solution was treated with cold 5% TCA, and yeast RNA (250 µg.) was added as carrier. The precipitate was allowed to form for 30 minutes in the cold, and collected by centrifugation, as

Table 2. Comparison of different washing procedures for TCA-precipitated RNA

Washing procedure	Coprecipitant	Radioactivity in precipitate after wash (%)			
		1st TCA	2nd TCA	ethanol	ether
2 x TCA, ethanol, ether	BSA	100	99	45	43
2 x TCA, ethanol, ether	RNA	64	62	60	60

(^{3}H)-labelled SFV double-stranded RNA was dissolved in 3 x TNE buffer and samples were treated with 5% TCA. Precipitates were collected in the presence of either 100 µg. BSA or 250 µg. yeast RNA as carrier. Precipitates were washed with 1 ml. ice-cold 5% TCA, or with 1 ml. 5% TCA, 1 ml. ethanol and 1 ml. ether. Radioactivity in the precipitates was expressed as a percentage of that in both the precipitate and the combined supernatants.

described above. All the ^{14}C radioactivity was found in the supernatant, as expected, since the single-stranded RNA had been treated with ribonuclease. BSA (100 µg.) or yeast RNA (250 µg.) was then added to samples of the supernatant, and after 30 minutes in the cold, the precipitates were collected by centrifugation. No radioactivity had been precipitated either by BSA or yeast RNA, showing that BSA did not cause the precipitation of TCA-soluble material.

Thus, the procedure adopted in subsequent experiments for the precipitation of TCA-insoluble material was to use BSA as co-precipitant, and to wash the TCA precipitates once with cold 5% TCA. Precipitates were allowed to dry at room temperature before they were digested with NaOH.

20. Digestion of RNA with ribonucleases

It was observed during the course of this study that different batches of ribonuclease A had different specific activities. Experiments were therefore performed in order to determine the activity of the batch used under different conditions, and to find conditions which gave the maximum difference in effect on single-stranded and double-stranded RNA.

(a) Effect of buffer and time of incubation on digestion of single-stranded RNA with ribonuclease A

RNA was extracted with phenol from uninfected chick cells which had been labelled with ^{14}C-uridine, and the RNA was precipitated in three batches with ethanol. The ethanol precipitates were redissolved

in 3 x TNE, 3 x TN or SSC buffer (described in the Materials section). Pancreatic ribonuclease was added to a final concentration of 10 µg./ml. and the reaction mixtures were incubated at 37°C for different times. The reactions were stopped by adding an equal volume of chilled 10% TCA and cooling the mixture in ice. BSA was added as carrier, and radioactivity in TCA-insoluble material was measured as described in the previous section. The enzyme showed no significant difference in activity in the three buffers tested (Fig. 2; Table 3). Thus, in subsequent experiments, these three buffers were used interchangeably.

(b) Effect of concentration of enzyme

Chick cell RNA, labelled with ^{14}C, was prepared as described above. Double-stranded RNA was prepared from SFV-infected cells which had been labelled with ^{3}H-uridine between 2 and 6 hours after infection. The two preparations were treated with different concentrations of ribonuclease A in 3 x TN buffer, or with ribonuclease A and ribonuclease T1 in SSC buffer. Reactions were stopped by addition of an equal volume of chilled 10% TCA, and the radioactivity in TCA-insoluble material was measured as described above (Methods, 19). The rate of digestion of the chick cell RNA was approximately proportional to the concentration of ribonuclease A present, whether or not ribonuclease T1 was present (Fig. 3; Table 4).

21. Conditions for annealing of RNA

Samples of RNA were dissolved in buffer containing 0.3 M-NaCl (3 x TNE or 2 x SSC buffer) and incubated at 65°C in thin-walled 2 ml.

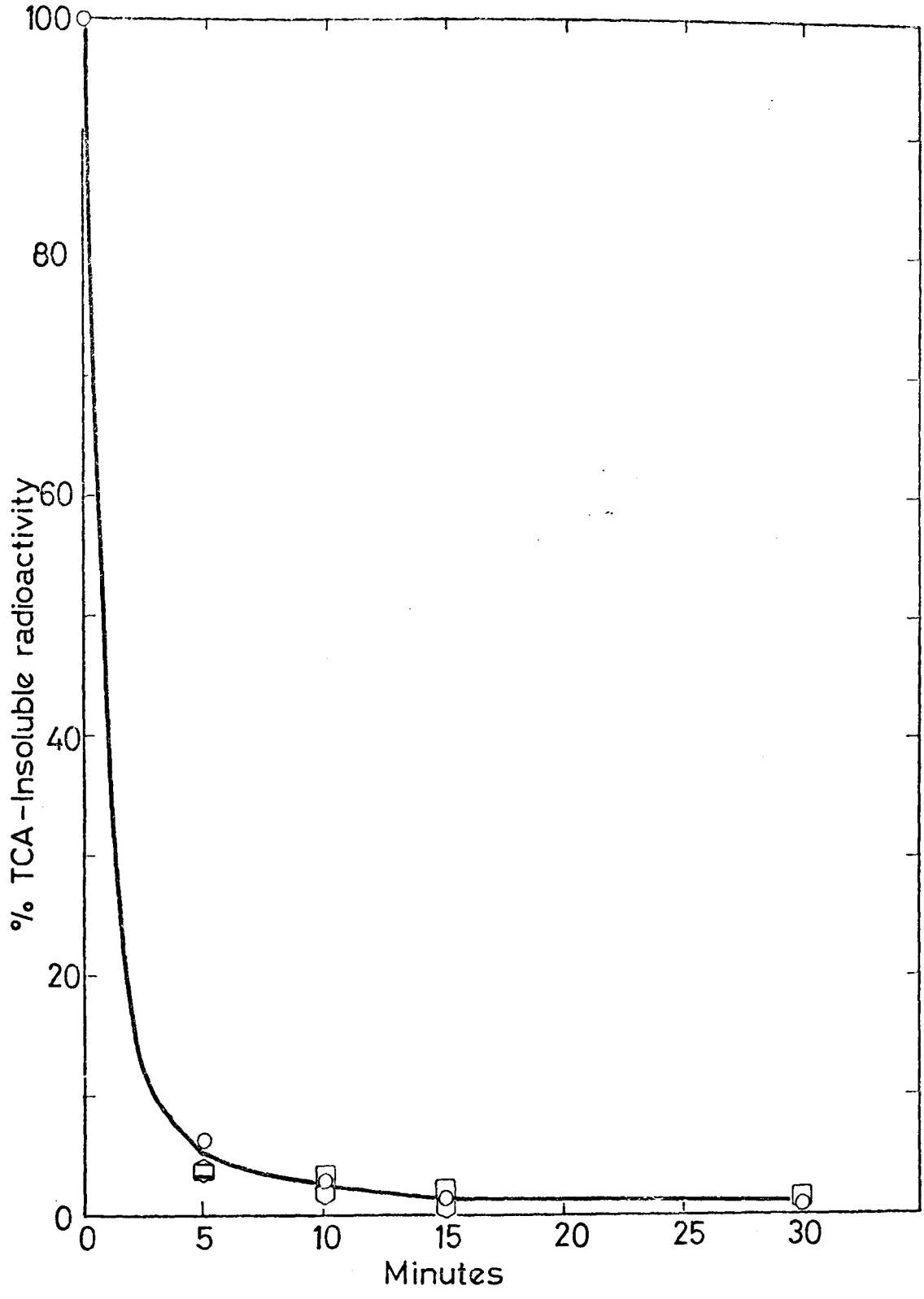


Fig. 2. Digestion of single-stranded RNA by ribonuclease in different buffers. ^{14}C-labelled RNA was extracted from uninfected chick cells and dissolved in 3 x TNE buffer, (o-o) or 3 x TN (without EDTA) (p-p) or SSC buffer. (○-○). RNase A was added to a final concentration of 10 µg./ml. Reactions were stopped by the addition of an equal volume of cold 10% TCA, and cooling in ice. Bovine serum albumin (100 µg.) was added as carrier. Radioactivity was measured in precipitates and in supernatants.

Table 3. Effect of buffer on digestion of single-stranded RNA by pancreatic ribonuclease

Incubation time (min.)	TCA-insoluble radioactivity (%)			
	5	10	15	30
3 x TNE	6.2	2.7	1.2	0.7
3 x TN	3.6	3.2	2.2	1.2
SSC	3.8	1.8	0.6	-

RNA samples were treated with pancreatic ribonuclease (10 µg./ml.) for stated times at 37°C. The value for TCA-insoluble radioactivity was derived from radioactivity in the final alkali-solubilised precipitate as a percentage of radioactivity in both this precipitate and in the TCA supernatant.

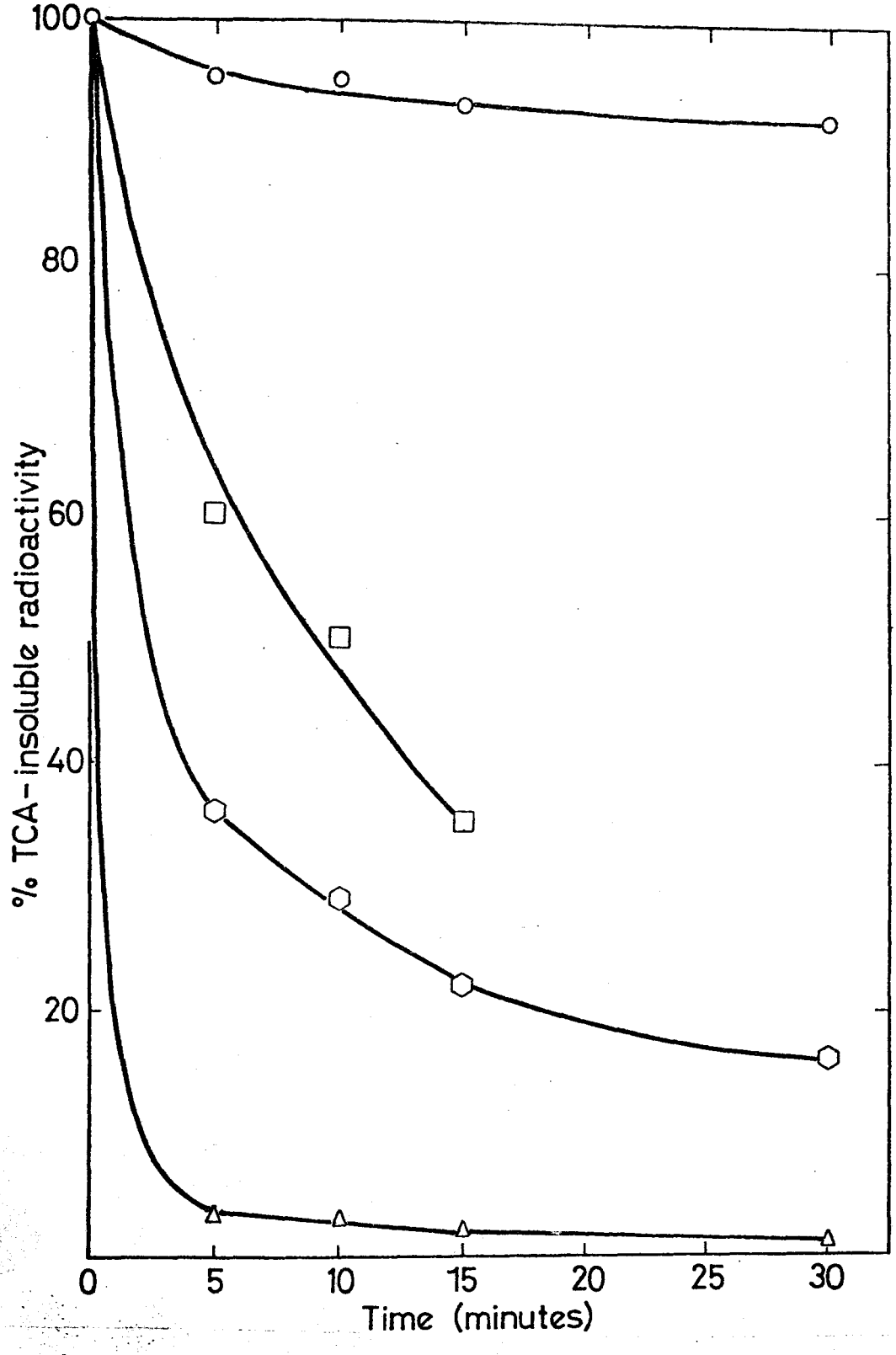


Fig. 3a. Effect of enzyme concentration on digestion of RNA with ribonuclease. Single-stranded RNA was extracted from uninfected chick cells which had been labelled with ^{14}C-uridine. The RNA was incubated in 3 x TN buffer at 37°C with RNase A at 1 (□-□), 2 (⬡-⬡), or 10 (Δ—Δ) µg./ml. Double-stranded RNA was prepared from SFV-infected chick cells, which had been labelled with ^{3}H-uridine. The RNA was purified by treatment with 2M-LiCl, and incubated with 10 µg./ml. RNase A in 3 x TN buffer at 37°C (○-○). The proportion of radioactivity insoluble in cold, 5% TCA was measured.

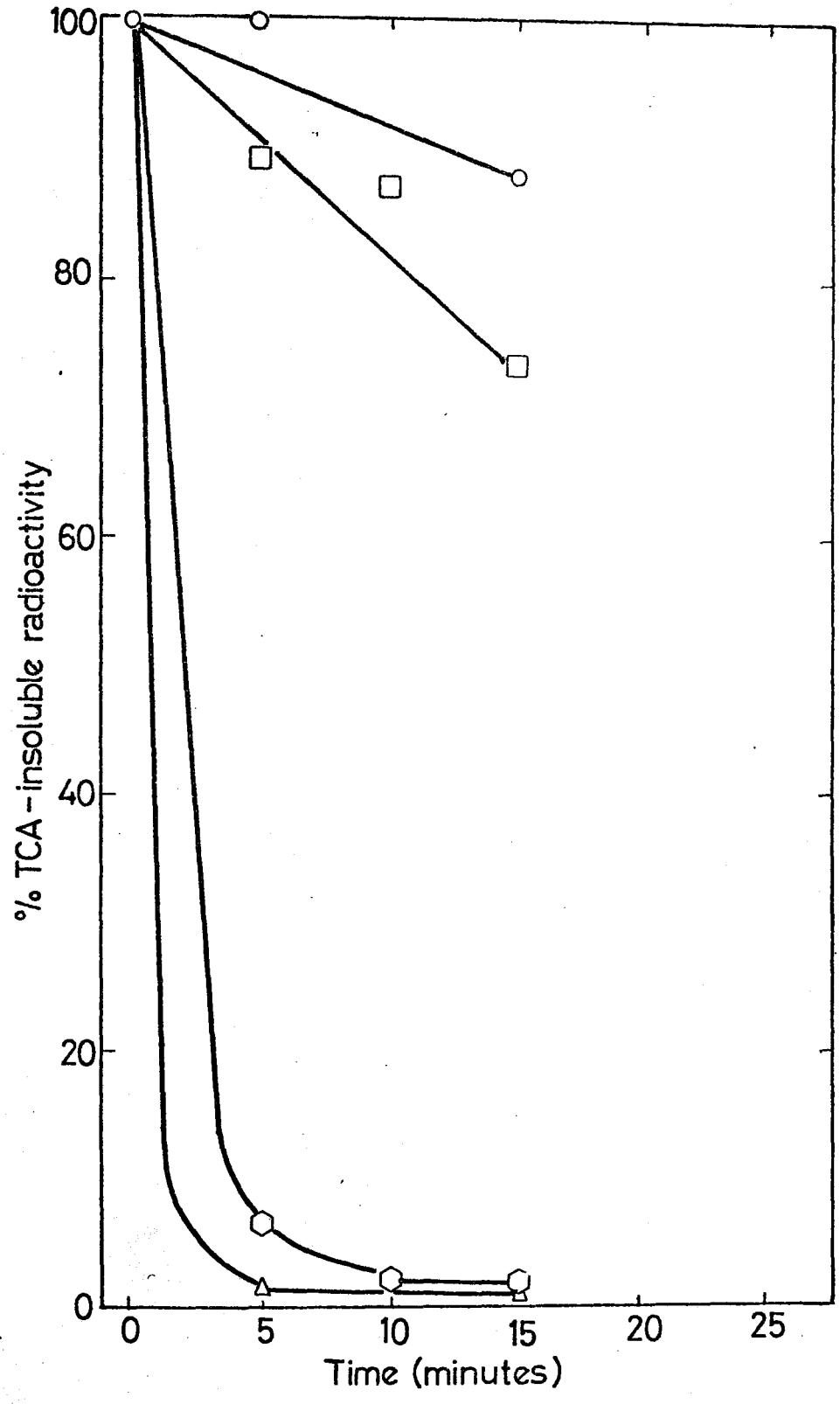


Fig. 3b. The combined effect of RNase A and RNase T1 on digestion of RNA. Single- and double-stranded RNA were prepared as in Fig. 3a, and incubated at 37°C in SSC buffer with RNase A (50 µg./ml.) and RNase T1 (170 units/ml.). ⬡—⬡, single-stranded RNA; ○—○, double-stranded RNA. RNA was also incubated with RNase A (100 µg./ml.) and RNase T1 (340units/ml.). △—△, single-stranded RNA; □—□, double-stranded RNA. The proportion of radioactivity insoluble in cold, 5% TCA was measured.

Table 4. Effect of enzyme concentration on digestion of single- and double-stranded RNA by RNase A and RNase T1.

(a) Single-stranded RNA

		TCA-insoluble radioactivity (%)			
Incubation time (min.)		5	10	15	30
RNase A (µg./ml.)	RNase T1 (units/ml.)				
1	-	*60	50	35	-
2	-	36	29	22	16
10	-	3.6	3.2	2.1	1.2
50	170	3.8	1.8	0.6	-
100	340	1.2	-	1.7	-

(b) Double-stranded RNA

		TCA-insoluble radioactivity (%)			
Incubation time (min.)		5	10	15	30
RNase A (µg./ml.)	RNase T1 (units/ml.)				
10	-	*95	95	93	92
50	170	99	87	88	-
100	340	89	-	73	-

Incubations were in 3 x TNE buffer (RNase A) or in SSC buffer (RNase A and RNase T1).

* TCA-insoluble radioactivity was expressed as a percentage of that in the precipitate added to that in the TCA supernatant.

glass tubes, fitted with screw-caps. After incubation, samples were cooled quickly in ice. In order to estimate the amount of annealing, aliquots of RNA samples were treated with ribonuclease (10 µg./ml. RNase A, 34 units/ml. RNase T1, 37°C, 15 min. in SSC or TNE buffer), before and after incubation. Ribonuclease-resistant material was estimated by precipitation with cold, 5% TCA, as described in Methods, section 19.

22. Estimation of radioactivity in mixtures of ^{3}H and ^{14}C

When samples contained both ^{3}H and ^{14}C, the radioactivity which was due to each isotope was estimated using the channels-ratio method of Hendler (1964).

RESULTS

RESULTS

In all the experiments described which involved infection of cells, the beginning of infection was measured from the time of inoculation with virus. Cells were infected at a multiplicity of about 10 p.f.u. per cell, unless otherwise stated.

I. Effect of actinomycin D on RNA synthesis and growth of Semliki Forest virus in chick cells

1. Effect of actinomycin D on cellular RNA synthesis

The purpose of these initial experiments was to find conditions which suppressed cellular RNA synthesis, while allowing synthesis of Semliki Forest virus RNA and the maturation of virus. Therefore, monolayers of chick cells were treated with different concentrations of actinomycin D, and pulse-labelled with ^{3}H-uridine for 30 minute periods at different times after addition of the inhibitor.

It was found that treatment with actinomycin D at concentrations of up to 0.01 µg./ml. for $3\frac{1}{2}$ hours had a negligible effect on the rate of RNA synthesis, compared with untreated cells, whereas synthesis of RNA was 95% inhibited within $2\frac{1}{2}$ hours in cells treated with 0.5 µg./ml. (Fig.4). The small, residual incorporation of ^{3}H-uridine into TCA-insoluble material in cells treated with actinomycin D has been observed before, and was found to be RNA of low molecular weight, as judged by its low sedimentation coefficient and its high electrophoretic mobility

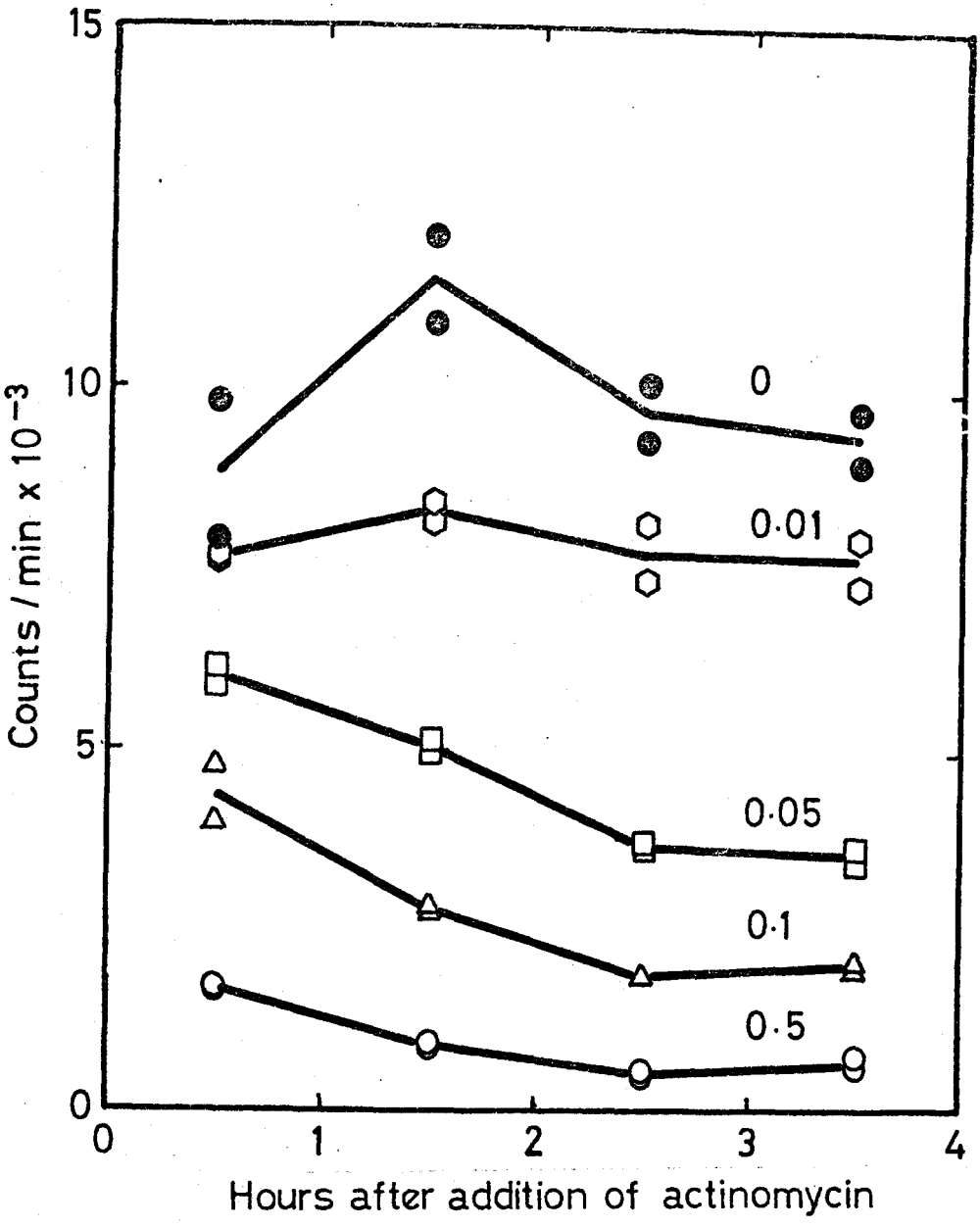


Fig. 4. Effect of actinomycin D on RNA synthesis in chick embryo fibroblasts. Monolayers of cells on 65 mm. glass dishes were incubated at 37°C with 0, 0.01, 0.05, 0.1 or 0.5 µg./ml. actinomycin D in maintenance medium. Starting at the times shown, duplicate cultures at each concentration of actinomycin were labelled with 1 µCi ^{3}H-uridine for 30 minutes. Cultures were washed with cold saline, cold TCA, ethanol and ether, and radioactivity insoluble in TCA was measured.

on polyacrylamide gel (Martin and Brown, 1969; Gandhi and Burke, 1970; Stern and Friedman, 1970). In order to demonstrate that actinomycin D-resistant RNA synthesis would not be a source of error in studying virus RNA synthesis, uninfected chick cell monolayers were treated with actinomycin D (1 µg./ml.) and labelled with ^{3}H-uridine. RNA was then extracted from the cells using phenol and SDS, and analyzed by chromatography on a CF11 cellulose column and by polyacrylamide gel electrophoresis (Fig. 5). Labelled material was eluted from the CF11 cellulose column in the way expected for RNA of low molecular weight (see Results, section II3). The labelled material also had a high electrophoretic mobility, and therefore would not be a source of error in the examination of virus-specified RNA (see Results, section II3).

2. Effect of actinomycin D on RNA synthesis in infected cells

Chick cell monolayers were infected with SFV at a multiplicity of 10 p.f.u. per cell, and actinomycin D (0.5 µg./ml.) was added to the infected monolayers one hour later. As a control, a duplicate set of monolayers was mock-infected by adding maintenance medium to them at the same time as virus was added to the infected cells. As shown in Fig. 6, the rate of RNA synthesis in the infected cells continued to increase for at least 7 hours after infection. By contrast, the rate of RNA synthesis in the control cells was depressed to less than 15% of that in the infected cells by 7 hours after infection. However, the rate of RNA synthesis in uninfected cells

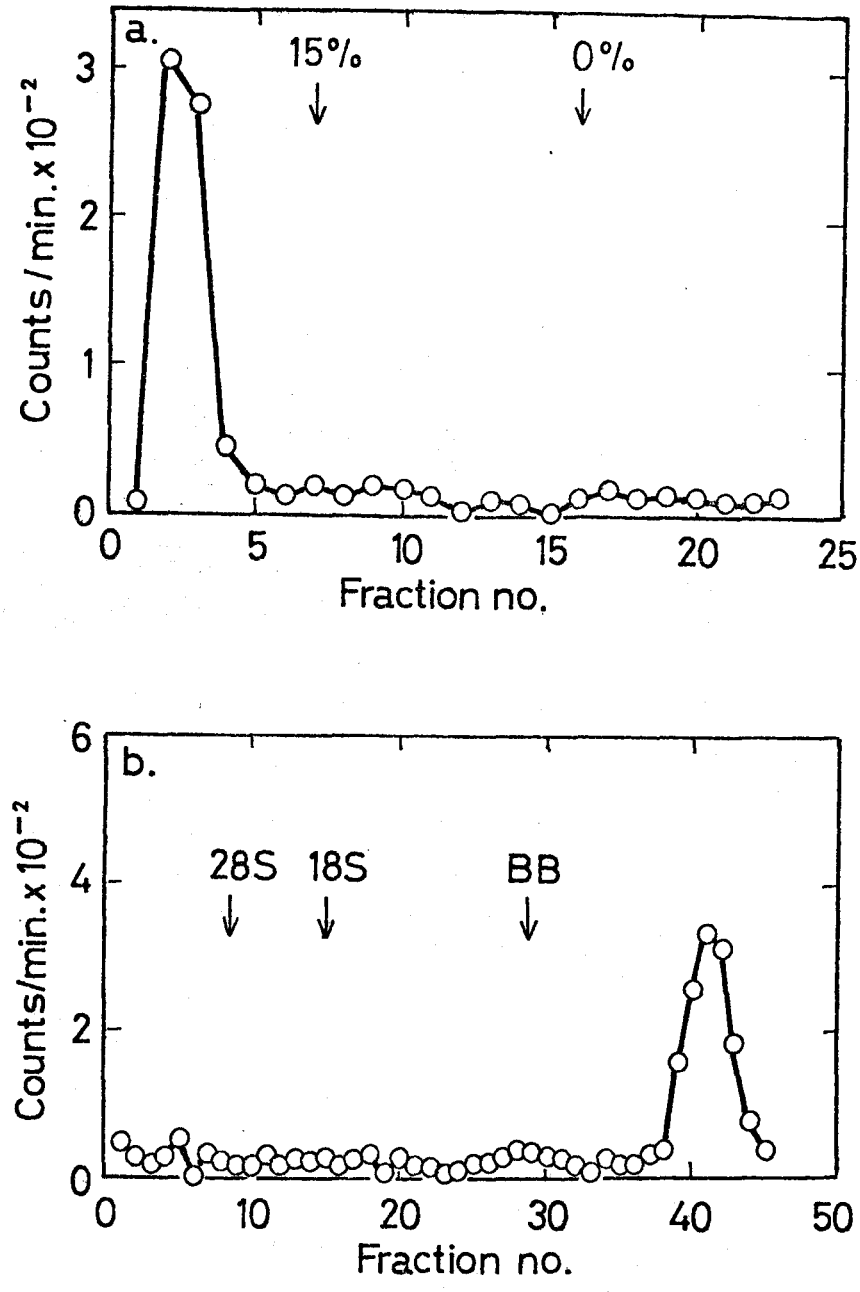


Fig. 5. RNA synthesized in uninfected chick cells in the presence of actinomycin D. A monolayer culture of cells was incubated at 37°C with maintenance medium containing actinomycin D (1 µg./ml.) for 2.5 hours, and then with the same medium containing ^{3}H-uridine (200 µCi/ml.) for 0.5 hours. The monolayer was washed 3 times with TNE buffer, and RNA was extracted from the cells using phenol and SDS.
(a) RNA was chromatographed on a CF11 cellulose column (10 x 1.5 cm.), which was eluted stepwise with 35%, 15% and 0% ethanol in TNE buffer. The arrows indicate the changes in elution buffer to 15% and 0% ethanol. Radioactivity was measured in aliquots of 2 ml. fractions.
(b) The extract was analyzed by electrophoresis on 2.0% polyacrylamide gel containing 0.5% agarose. Electrophoresis was for 1¾ hours at 100V. The arrows represent the positions of the 28S and 18S ribosomal RNA species, detected optically, and of bromophenol blue dye (BB).

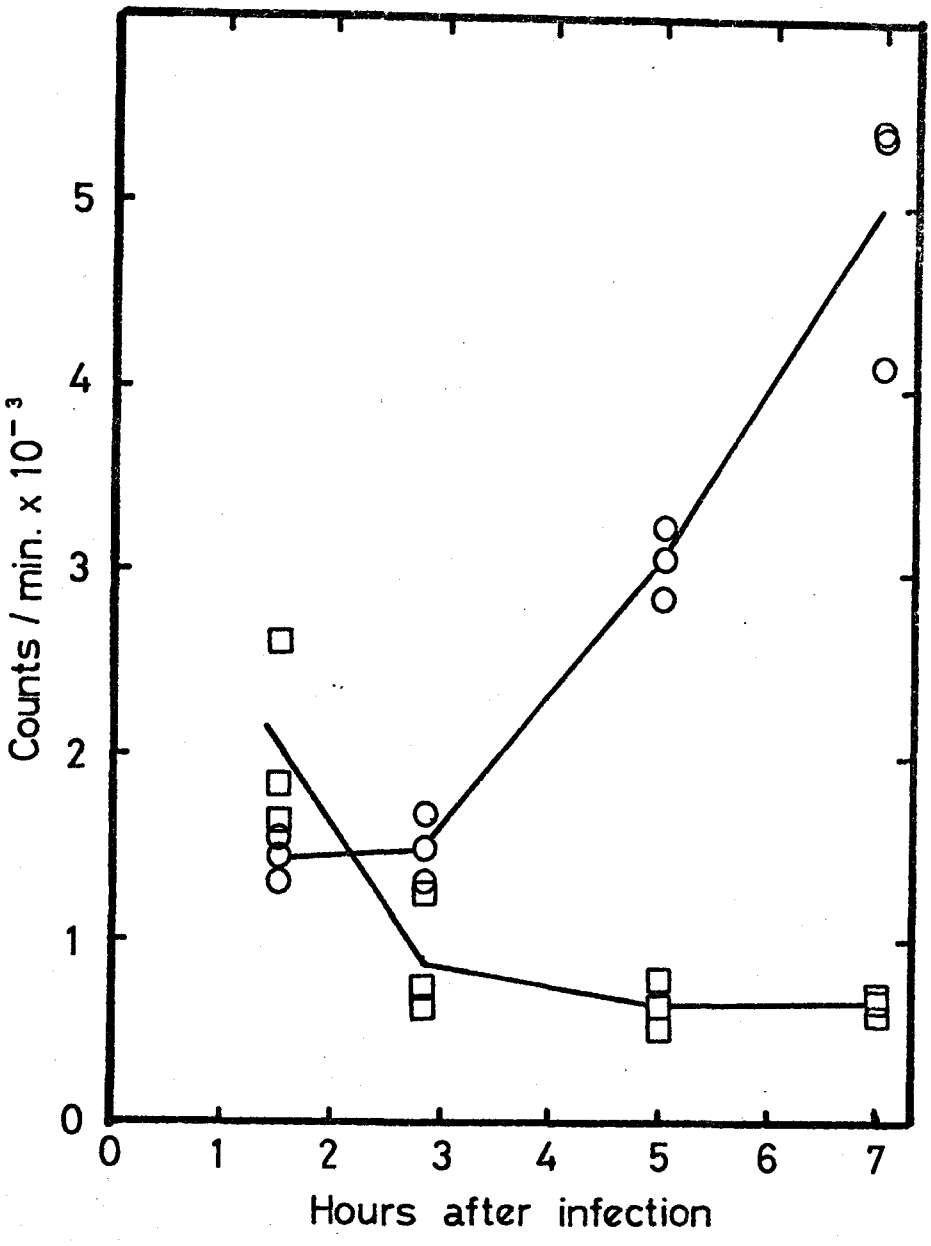


Fig. 6. Effect of actinomycin D on synthesis of RNA in infected and uninfected chick cells. Cultures were either infected with SFV (O-O) or mock-infected by incubating with maintenance medium (□-□). Cultures were then washed with maintenance medium, and incubated at 37°C with medium containing actinomycin D (0.5 µg./ml.). At the times shown (after infection or mock-infection), cultures were labelled with ^{3}H-uridine (1 µCi/ml.) and incubated for 1 hour. Cultures were washed with ice-cold TCA, and radioactivity in TCA-insoluble material was determined.

was consistently greater than that in infected cells for about the first 3 hours of infection. This was probably due to suppression of cellular RNA synthesis by the infecting virus.

3. The biological activity of virus grown in the presence of actinomycin D

Although RNA was synthesized in infected cells in the presence of actinomycin D, it was important to know whether or not the growth of infectious virus was impaired in the presence of the inhibitor. Therefore, suspension cultures of chick cells were infected with SFV and treated with different concentrations of actinomycin D. The infectivity and HA activity of virus released into the culture fluids was measured after 18 hours' incubation.

Treatment of cells with actinomycin D at concentrations up to 0.05µg./ml. had no significant effect either on the infectivity of the released virus or on the yield of virus as measured by HA titration (Table 5). The HA titre of the virus grown in the absence of actinomycin D was slightly higher than that obtained from cultures grown in the presence of the inhibitor, but since a 2-fold difference was the smallest difference that could be detected by this technique of HA titration, the difference observed was probably within the limits of experimental error. This is further supported by the observation that when virus was frozen and stored at -20°C for 24 hours, the HA titres of all the samples were the same, and were slightly lower than the initial titres.

Table 5. Effect of Actinomycin D on HA titre and infectivity of Semliki Forest Virus

Actinomycin D (µg./ml.)	Infectivity (p.f.u./ml., x 10^{-8})	HA titre	
		Before storage	After storage at -20°C
0	4.0	256	56
0.01	7.8	128	56
0.05	5.9	128	56

SFV was grown in 50 ml. suspension cultures of chick cells for 18 hours at 35°C. Actinomycin D at the appropriate concentration was added immediately after the period of virus absorption.

II. RNA specified by Semliki Forest virus in chick cells

1. Analysis of RNA by sucrose gradient centrifugation

In order to determine the composition of the RNA specified by SFV, chick cells were infected in the presence of actinomycin D (1 µg./ml.) and labelled with ^{3}H-uridine between 2 and 6 hours after infection. RNA was extracted from the cells using phenol and SDS, and the extract was analyzed by centrifugation on a linear 6 to 25% gradient of sucrose (Fig. 7). Two peaks of radioactivity were seen, whose positions were distinct from the peaks of ultraviolet absorption, which represented the species of ribosomal RNA. The sedimentation coefficients of the two radioactive peaks were about 23S and 40S. The pattern obtained was similar to that obtained by Sonnabend, Martin and Mécs (1967), who identified the faster-sedimenting peak as virion RNA, and showed that the slower-sedimenting peak contained a mixture of species.

2. Analysis of RNA by polyacrylamide gel electrophoresis

Cartwright and Burke (1970) showed that better resolution of SFV-specified RNA species could be obtained by polyacrylamide gel electrophoresis than by sucrose gradient centrifugation. In order to determine whether any improvement in resolution could be obtained, RNA was extracted from infected cells which had been labelled with ^{3}H-uridine between 2 and 6 hours after infection, and the extract was analyzed on polyacrylamide gels of different concentration. When electrophoresis was performed on 2.5% polyacrylamide gel, two peaks of radioactivity were observed (fig. 8). However, more peaks of

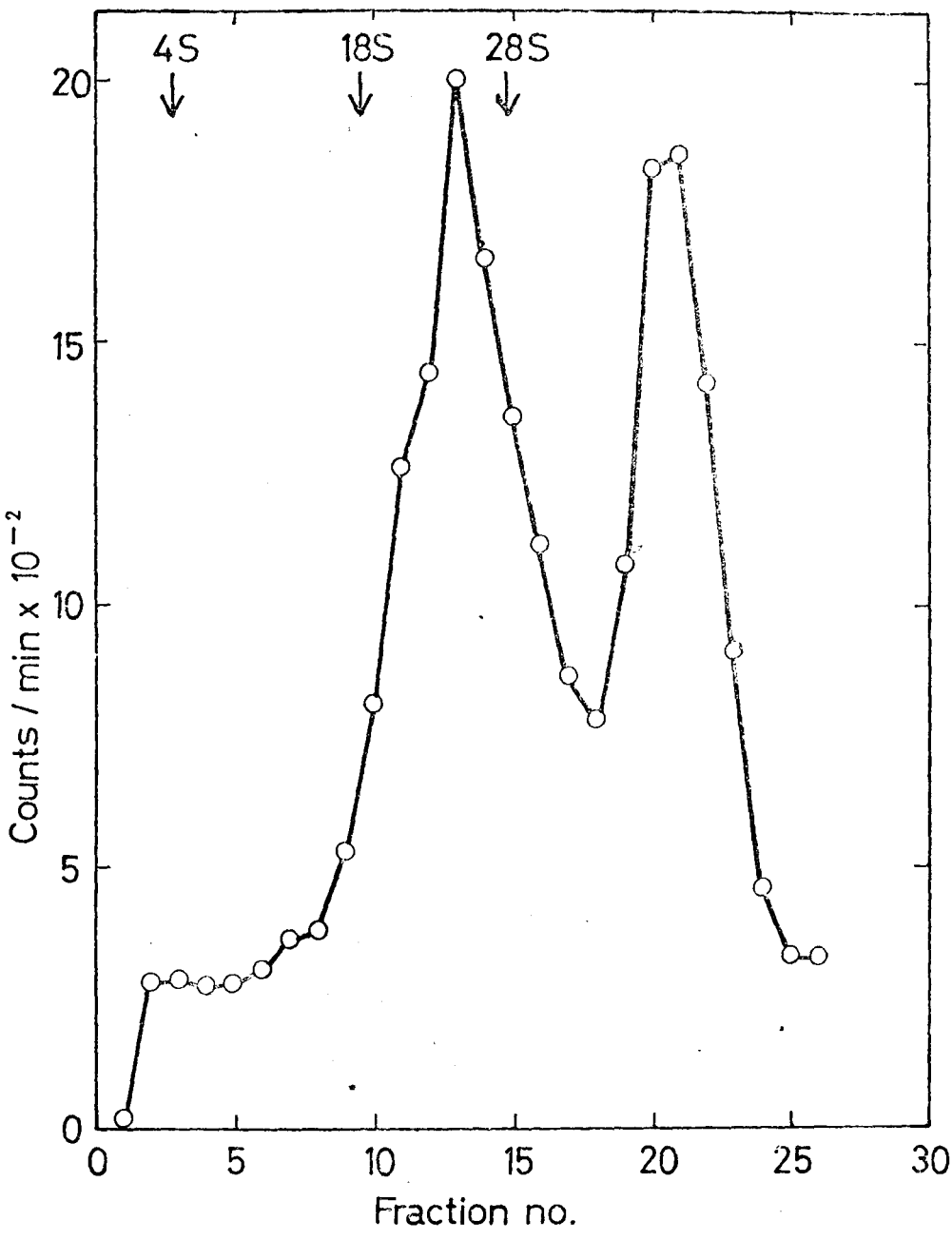


Fig. 7. Sucrose-gradient centrifugation of SFV-specified RNA. Monolayer cultures of chick embryo fibroblasts were infected with SFV in the presence of actinomycin D (1 µg./ml.) and labelled with ^{3}H-uridine between 2 and 6 hours after infection. RNA was extracted from the cells using phenol and SDS. The extract, containing 120 µg. RNA and 220,000 c.p.m., was layered over a preformed 12 ml. sucrose gradient (6–25% sucrose in TNE containing 0.5% SDS) and centrifuged at 200,000 g for 3 hours at 20°C, in an MSE 6 x 14 ml. swing-out rotor. The gradient was fractionated by upward displacement, and uv absorbance monitored continuously. The arrows represent peaks of uv absorbance, corresponding to ribosomal RNA species. Radioactivity was measured in aliquots of fractions.

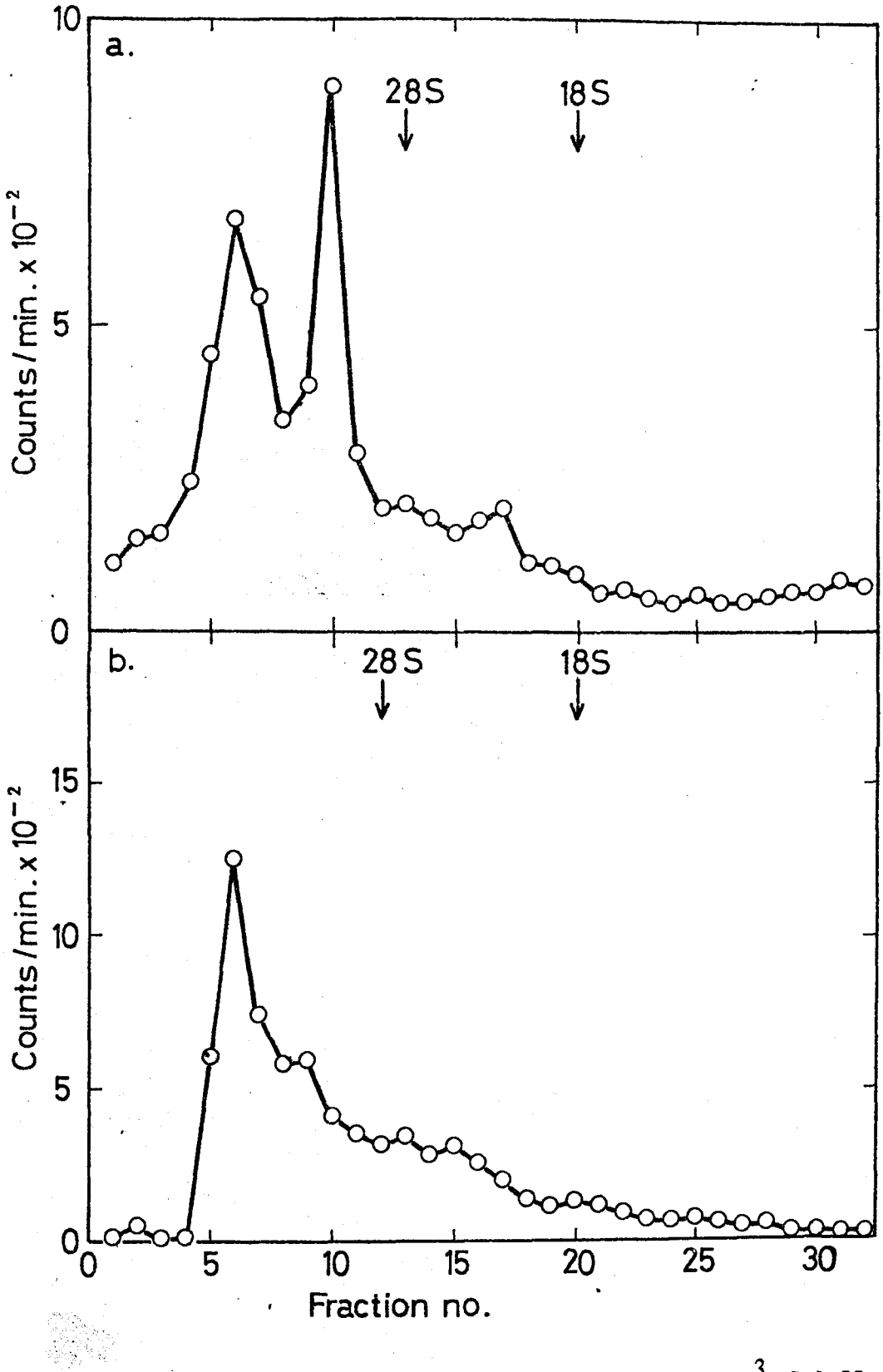


Fig. 8. Polyacrylamide gel electrophoresis of SFV RNA. ^{3}H-labelled RNA was (a) prepared as in Fig. 7 or (b) extracted from purified SFV and analyzed by electrophoresis on 2.5% polyacrylamide gel. The arrows mark the positions of species of ^{14}C-labelled chick ribosomal RNA, which was co-electrophoresed with the virus-specified RNA.

radioactivity were detected when electrophoresis was carried out on 2.4%, 2.2%, or 2.0% polyacrylamide gels (Fig. 9). These patterns were similar to those reported by Levin and Friedman (1971). The gels at all concentrations except 2.5% contained 0.5% agarose for structural support, and the agarose itself may have contributed to the improved resolution on these gels. The identities of these peaks are discussed in the next section.

3. Identification of SFV-specified RNA species resolved by polyacrylamide gel electrophoresis

In order to identify the different species of RNA separated by polyacrylamide gel electrophoresis, three approaches were used. First, RNA was extracted from purified virus. Second, RNA extracted from infected cells was fractionated into double- and single-stranded forms by chromatography and on the basis of differential solubility in high concentrations of salt. Third, RNA extracted from infected cells was fractionated by sucrose gradient centrifugation.

(a) Identification of virion RNA

Virion RNA was prepared from SFV grown in suspension-cultured chick cells, in the presence of ^{3}H-uridine and actinomycin D (0.1 µg./ml.). Virus was concentrated and partially purified by centrifugation through a linear gradient of sodium potassium tartrate. An opalescent band was

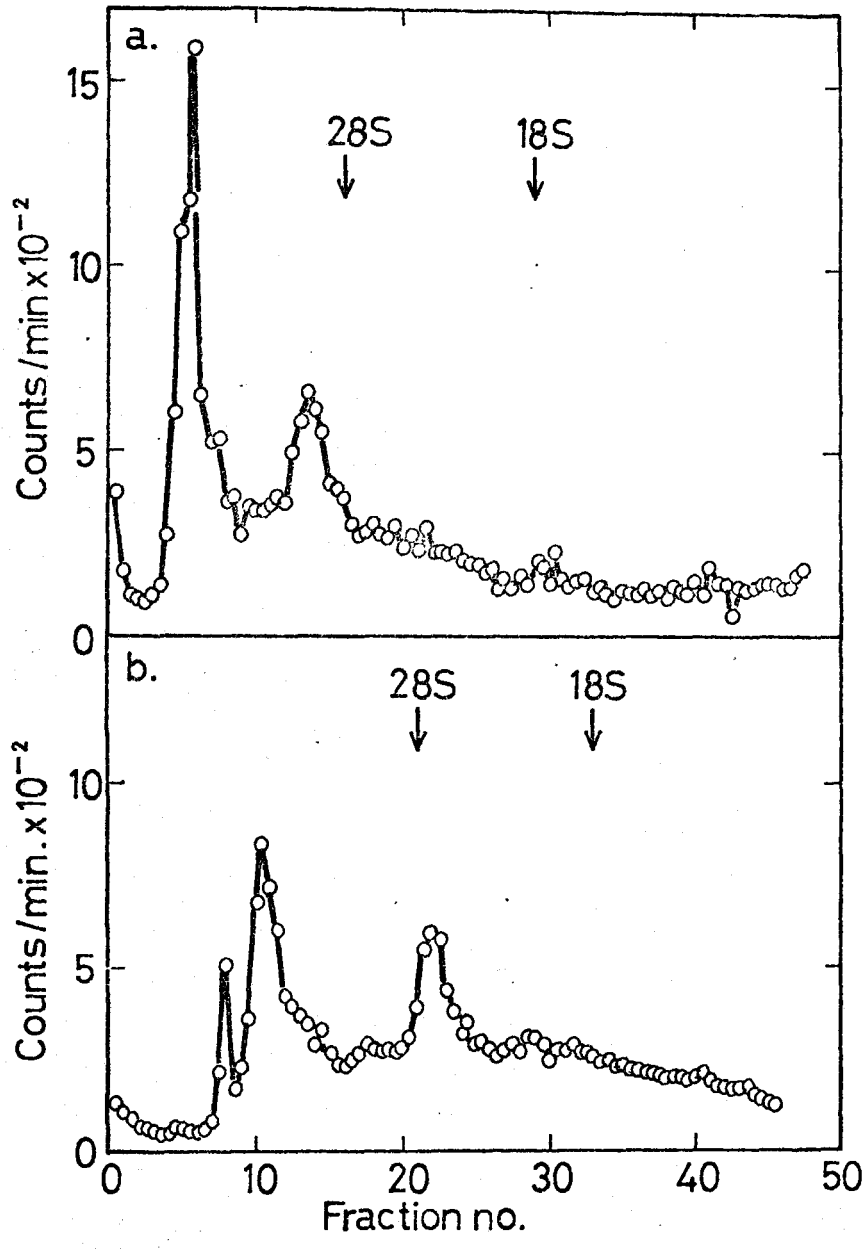
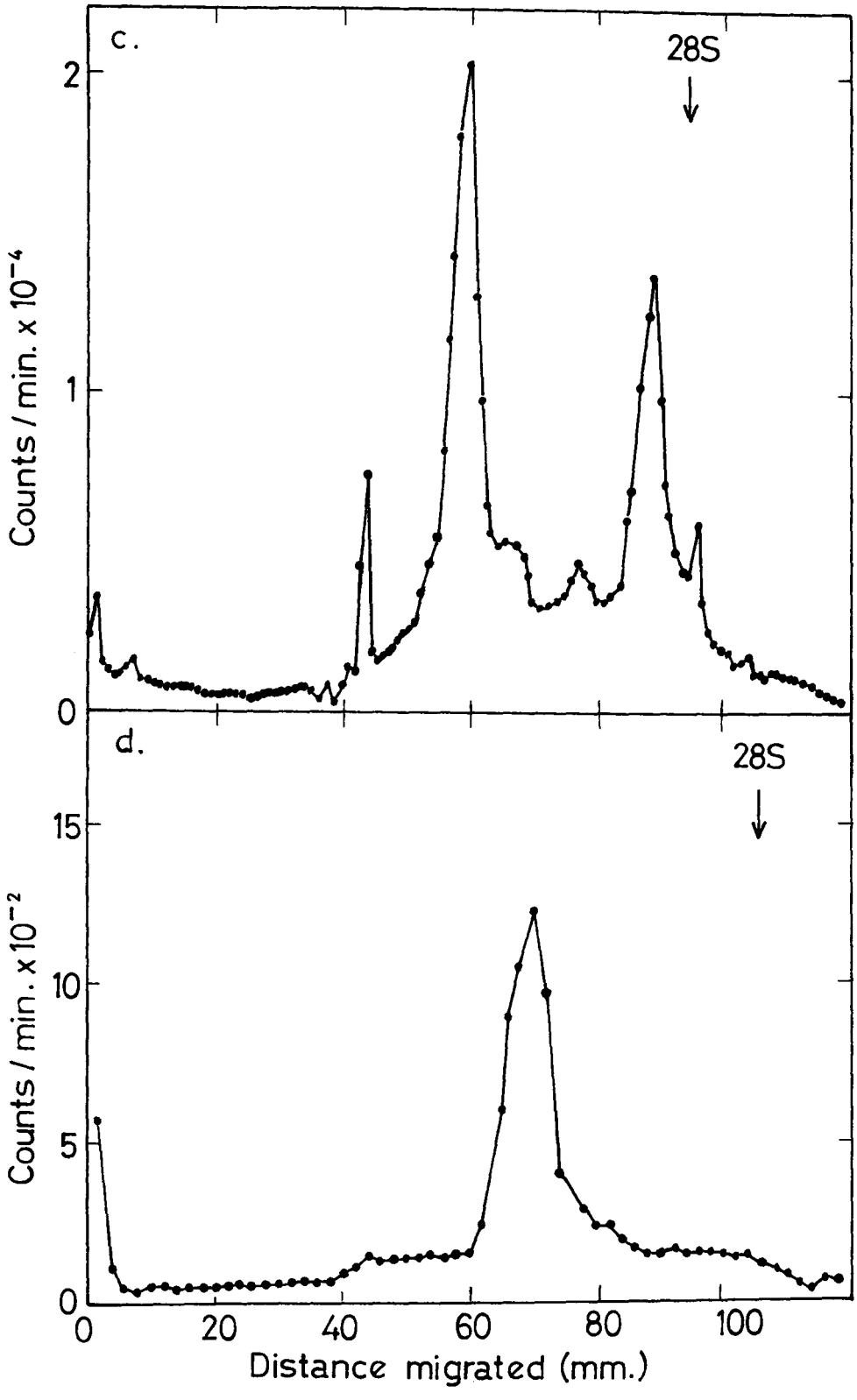


Fig. 9. Polyacrylamide gel electrophoresis. SFV-specified RNA was labelled and prepared as in Fig. 7, and analyzed by electrophoresis on (a) 2.4%, (b) 2.2% and (c) 2.0% polyacrylamide gel. RNA was also prepared from purified ^{3}H-labelled SFV, and analyzed on 2.0% gel (d). All gels contained 0.5% agarose. Electrophoresis was at 100V. for (a) 3 hours, (b) $3\frac{1}{2}$ hours, (c) $5\frac{2}{3}$ hours and (d) $6\frac{1}{2}$ hours. The arrows mark the positions of the 28S and 18S species of chick ribosomal RNA, which were detected optically.


c.
28S
2
1
0
Counts / min. x 10⁻⁴
d.
28S
15
10
5
0
Counts / min. x 10⁻²
0
20
40
60
80
100
Distance migrated (mm.)

seen about two-thirds of the way down the centrifuge tube. This band contained virus, as described in Methods (section 5a). The virus-containing band was collected and dialyzed against 0.01 M-Tris-HCl buffer, pH 7.4, at 4°C, overnight. RNA was extracted from this material using phenol and SDS. The extract was analyzed by electrophoresis on 2.5% polyacrylamide gel, and a single peak of radioactivity was obtained (Fig. 8b), whose mobility corresponded to the peak at Fraction 6 in Fig. 8a.

Virion RNA was also obtained from virus purified according to the scheme outlined in Methods, section 5b. This was prepared and kindly donated by Dr. S.I.T. Kennedy of this laboratory. The virion RNA obtained was analyzed by electrophoresis on 2.0% polyacrylamide gel, containing 0.5% agarose. A single peak of radioactivity was obtained (Fig. 9d), whose mobility corresponded to the peak at fraction 60 in Fig. 9c.

This latter preparation of virion RNA was also analyzed by sucrose gradient centrifugation. A single peak of radioactivity was obtained, with a sedimentation coefficient of about 43S (Fig. 10) corresponding to the faster-sedimenting peak observed when labelled RNA extracted from infected cells was centrifuged on a sucrose gradient, as described previously (Fig. 7). This result suggests that the virion RNA isolated by this procedure was intact. The virion RNA was also sensitive to ribonuclease A, as shown later (Section III, 1), confirming

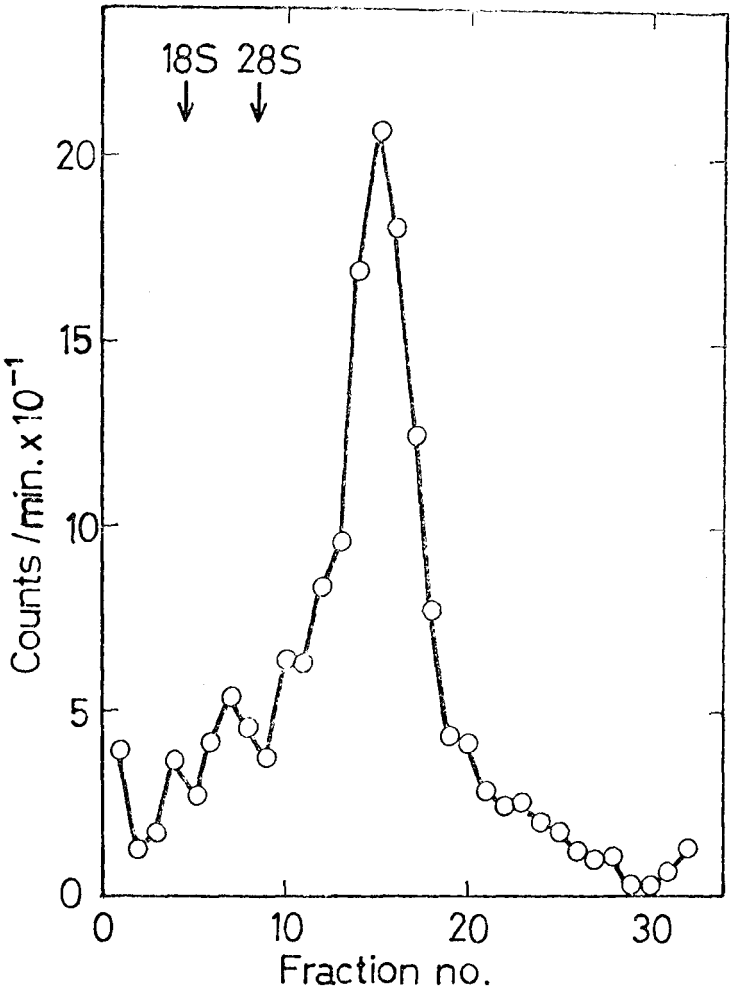


Fig. 10. Sucrose gradient centrifugation of SFV virion RNA. RNA was extracted from purified SFV and layered onto a preformed, 6 ml., 10-25% sucrose gradient, prepared in TNE buffer containing 1% SDS. The gradient was centrifuged at 120,000 g for 2.5 hours at 23°C in an MSE 3 x 6.5 ml. titanium swing-out rotor. The arrows mark the positions of the 18S and 28S chick ribosomal RNA species, which were detected optically.

that the RNA contained in the virion is single-stranded. Virion RNA will for convenience be called 42S RNA.

(b) Identification of other single-stranded species of RNA

Franklin (1966) showed that when nucleic acids were chromatographed on a column of CF11 cellulose, and eluted stepwise with decreasing concentrations of ethanol in a suitable buffer at pH7, DNA and low-molecular-weight RNA were eluted with 35% ethanol, and the majority of the single-stranded RNA was eluted with 15% ethanol. Double-stranded RNA (RF) and multi-stranded RNA (RI) were eluted with buffer containing no added ethanol.

This technique was employed to fractionate the RNA extracted from SFV-infected cells. Chick cell monolayers were infected with SFV in the presence of actinomycin D (1 μg./ml.), and labelled with ^{3}H-uridine between 2 and 6 hours after infection. The monolayers were then extracted with phenol and SDS, and RNA was precipitated from the extract by adding two volumes of ethanol and storing at -20°C overnight. The precipitated RNA was redissolved in TNE buffer (0.01M-tris-HCl, 0.1 M-NaCl, 0.001 M-EDTA, pH 7.0) and ethanol was added to 35%. The solution was chromatographed on a column of CF11 cellulose, which was eluted successively with TNE buffer containing 35%, 15% and 0% ethanol. 1 ml. fractions were collected, and the radioactivity in these fractions was measured (Fig. 11). A small amount of radioactive material was eluted with 35% ethanol. The majority of the radioactivity was eluted

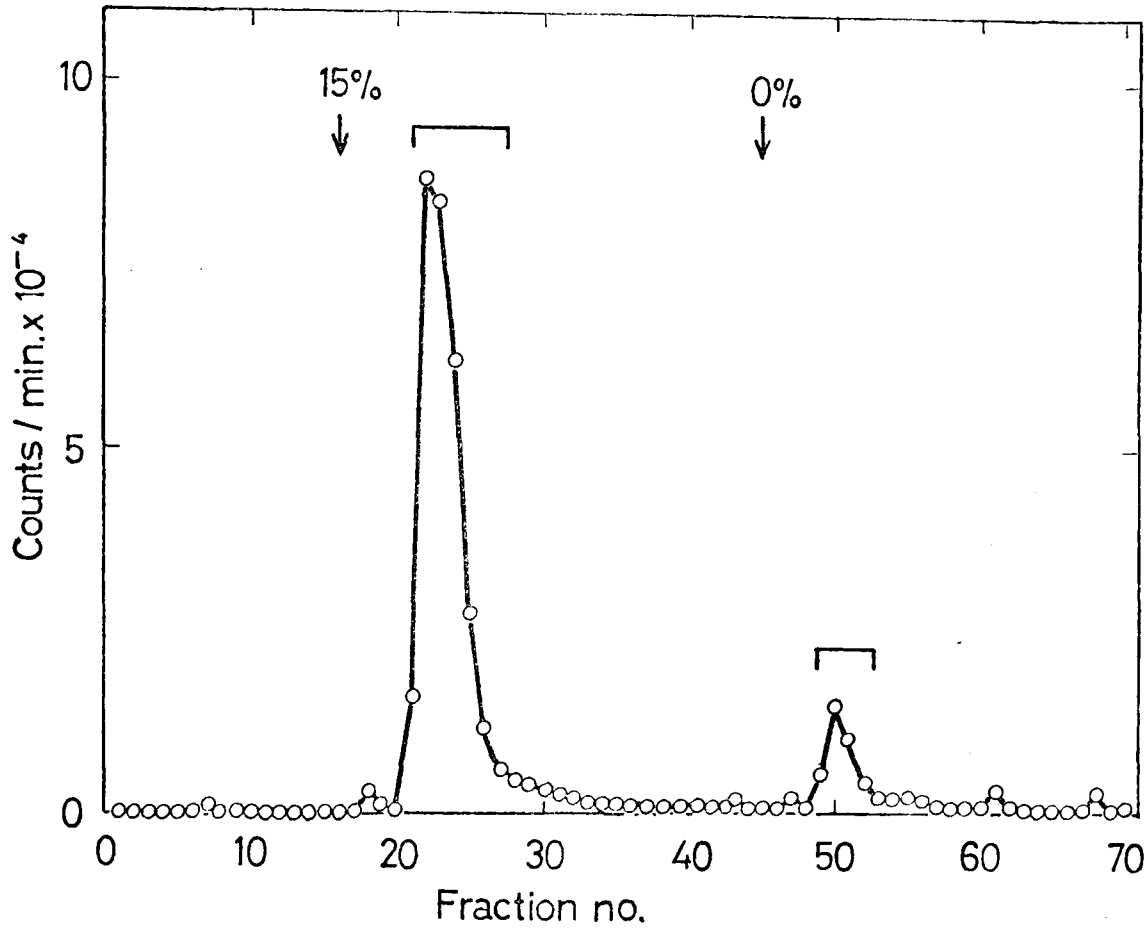


Fig. 11. Chromatography on CF11 cellulose. SFV-specified RNA was prepared from infected chick cells as described in Fig. 7. The RNA was precipitated with ethanol and redissolved in TNE buffer, to which ethanol was added to 35%. The RNA was chromatographed on a CF11 cellulose column, 11 x 1.5 cm., and eluted stepwise with 35%, 15% and 0% ethanol in TNE buffer. Other details as in Fig. 5a. Fractions were pooled as shown, and RNA recovered by precipitation with ethanol.

with 15% ethanol, while an intermediate amount of radioactivity was eluted with buffer containing no added ethanol.

The RNA which was eluted with 15% ethanol was sensitive to ribonuclease A, as shown later (Table 6), and this RNA was therefore single-stranded. When this material was analyzed on 2.0% polyacrylamide gel, only those species whose electrophoretic mobility was equal to or greater than that of 42S virion RNA were seen (Fig. 12). Two major peaks and two minor peaks of intermediate mobility were detected. The mobilities and proportions of these species were similar to those of the 42S, 38S, 33S and 26S species described by Levin and Friedman (1971).

The material eluted from CF11 cellulose with 15% ethanol was also analyzed by centrifugation on sucrose gradients, using chick cell RNA and 42S virion RNA as markers (Fig. 13). Two peaks of radioactivity were obtained, with sedimentation coefficients of 45S and 26S. The faster-sedimenting peak coincided with that of the 42S virion RNA. These peaks thus corresponded to virion RNA, and the interjacent RNA described by Martin (1967).

(c) Identification of double-stranded and multi-stranded RNA

Double-stranded and single-stranded RNA have different solubilities in high concentrations of certain salts, and this has been used as the basis of their separation. Montagnier and Sanders (1963)

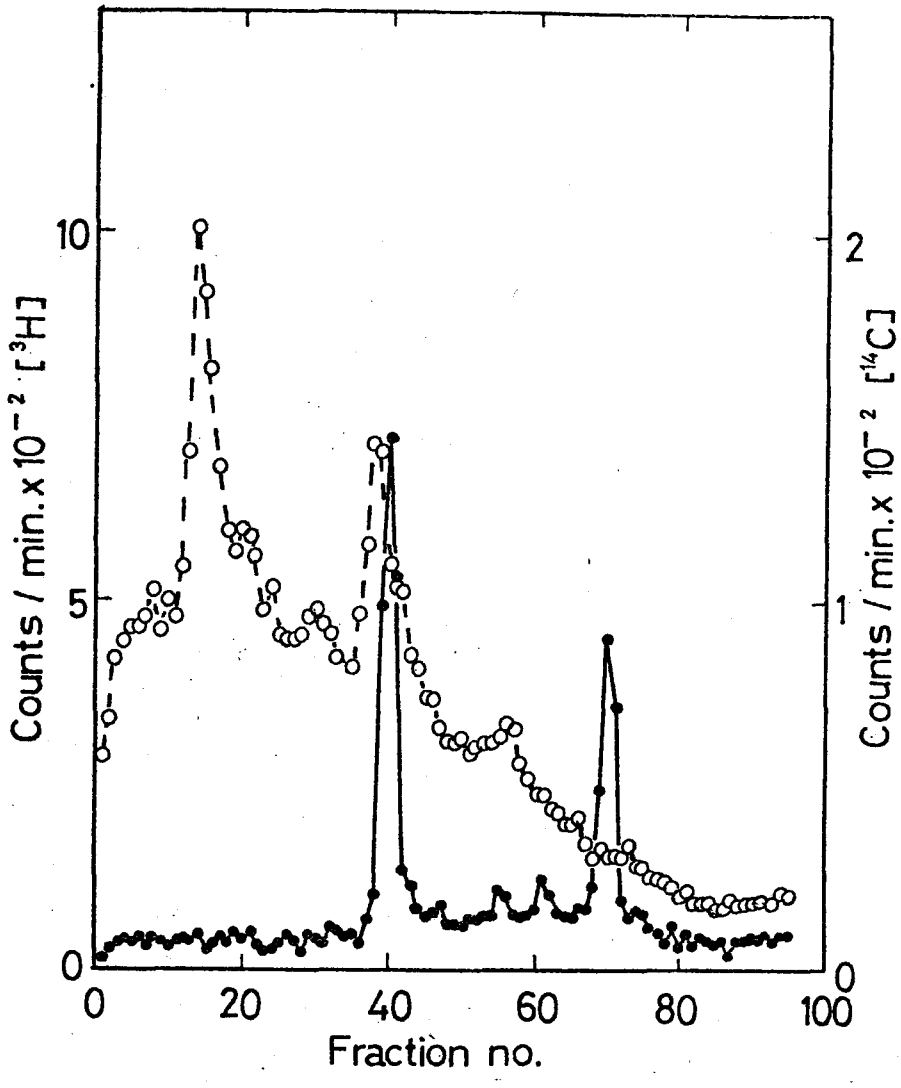


Fig. 12. Polyacrylamide gel electrophoresis of RNA after chromatography on CF11 cellulose. Fractions eluted with 15% ethanol were pooled as shown in Fig. 11, and RNA precipitated with ethanol. ^{14}C-labelled chick cell RNA was added to the ^{3}H-labelled RNA and electrophoresed on 2.0% polyacrylamide, 0.5% agarose gel, at 100V. for $6\frac{1}{2}$ hours. o–o, ^{3}H. ●–●, ^{14}C.

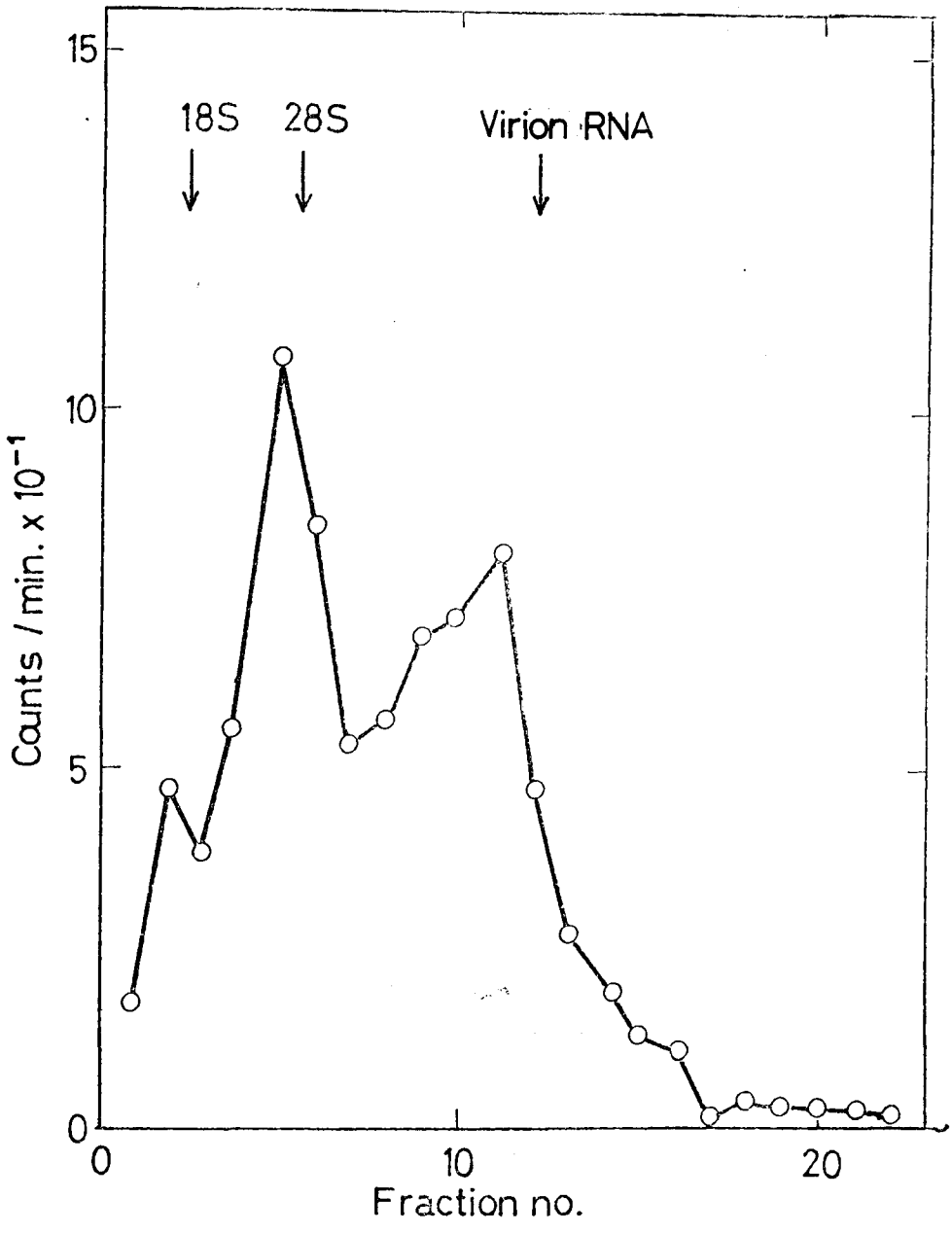


Fig. 13. Sucrose gradient centrifugation of RNA eluted from a CF11 cellulose column. RNA eluted with 15% ethanol (Fig. 12) was precipitated with ethanol redissolved in TNE buffer, and layered onto a preformed, 6 ml., 6-25% sucrose gradient, prepared in TNE buffer containing 1% SDS. ^{14}C-labelled RNA extracted from purified SFV was cosedimented as a marker. The gradient was centrifuged at 120,000 g for 2.5 hours at 23°C in an MSE 3 x 6.5 ml. swing-out rotor. Other details as in Fig. 10.

showed that double-stranded RNA was soluble in 1M-NaCl, while single-stranded RNA was insoluble. Bishop and Koch (1967) showed that poliovirus RI, which has properties of both single-stranded and double-stranded RNA, was co-precipitated with single-stranded RNA by 1M-NaCl. However, it was shown (Franklin, 1966; Bishop and Koch, 1967). that single-stranded RNA could be separated from other forms of RNA by chromatography on CF11 cellulose. Thus, a combination of salt precipitation and chromatography on CF11 cellulose provides a way, in principle, of separating double-stranded, multi-stranded and single-stranded RNA, and these techniques were applied to the separation of SFV-specified RNA extracted from chick cells.

Infected chick cell monolayers were labelled with either ^{3}H- or ^{14}C-uridine from 2 to 6 hours after infection in the presence of actinomycin D. RNA was extracted from the cells using phenol and SDS, and precipitated with ethanol. The precipitated RNA was redissolved in TNE buffer, and an equal volume of 2M-NaCl was added. The solutions were stored at -20°C for 18 hours, and thawed at 4°C. The flocculent precipitates were sedimented by centrifugation and the supernatants decanted and retained. RNA was recovered from the supernatants by precipitation with ethanol, and electrophoresed on 2.0% polyacrylamide gel. When the salt supernatant RNA labelled with ^{14}C was treated with ribonuclease A (10 µg./ml., 37°C, 15 min., in 3 x TNE buffer), and co-electrophoresed with untreated salt supernatant RNA labelled with ^{3}H, ~~identical~~ similar patterns of ^{3}H and ^{14}C radioactivity were obtained (Fig.14a).

The small difference in mobility of the major peak may have been due to the ribonuclease treatment of the ^{14}C-labelled material.

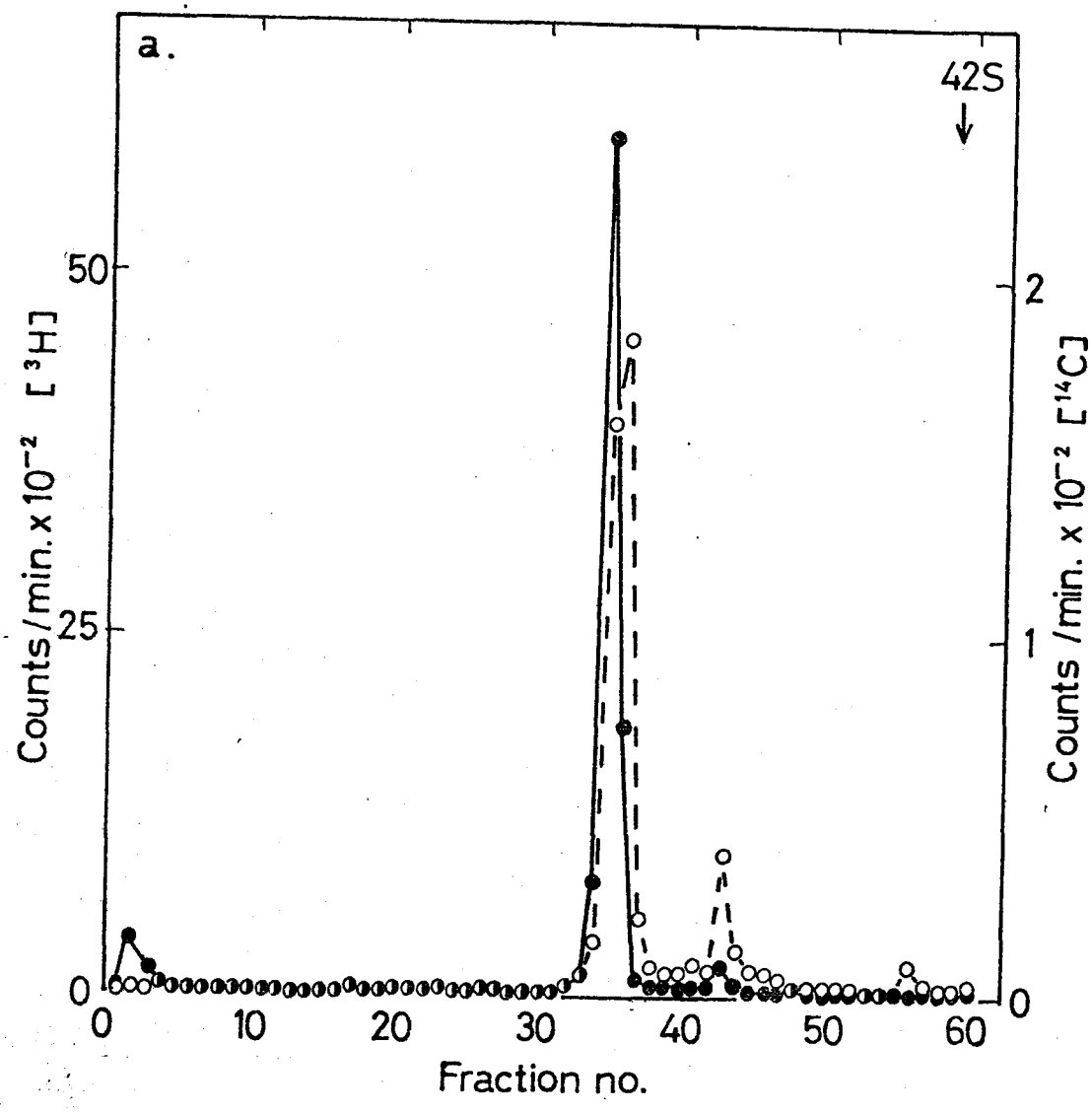
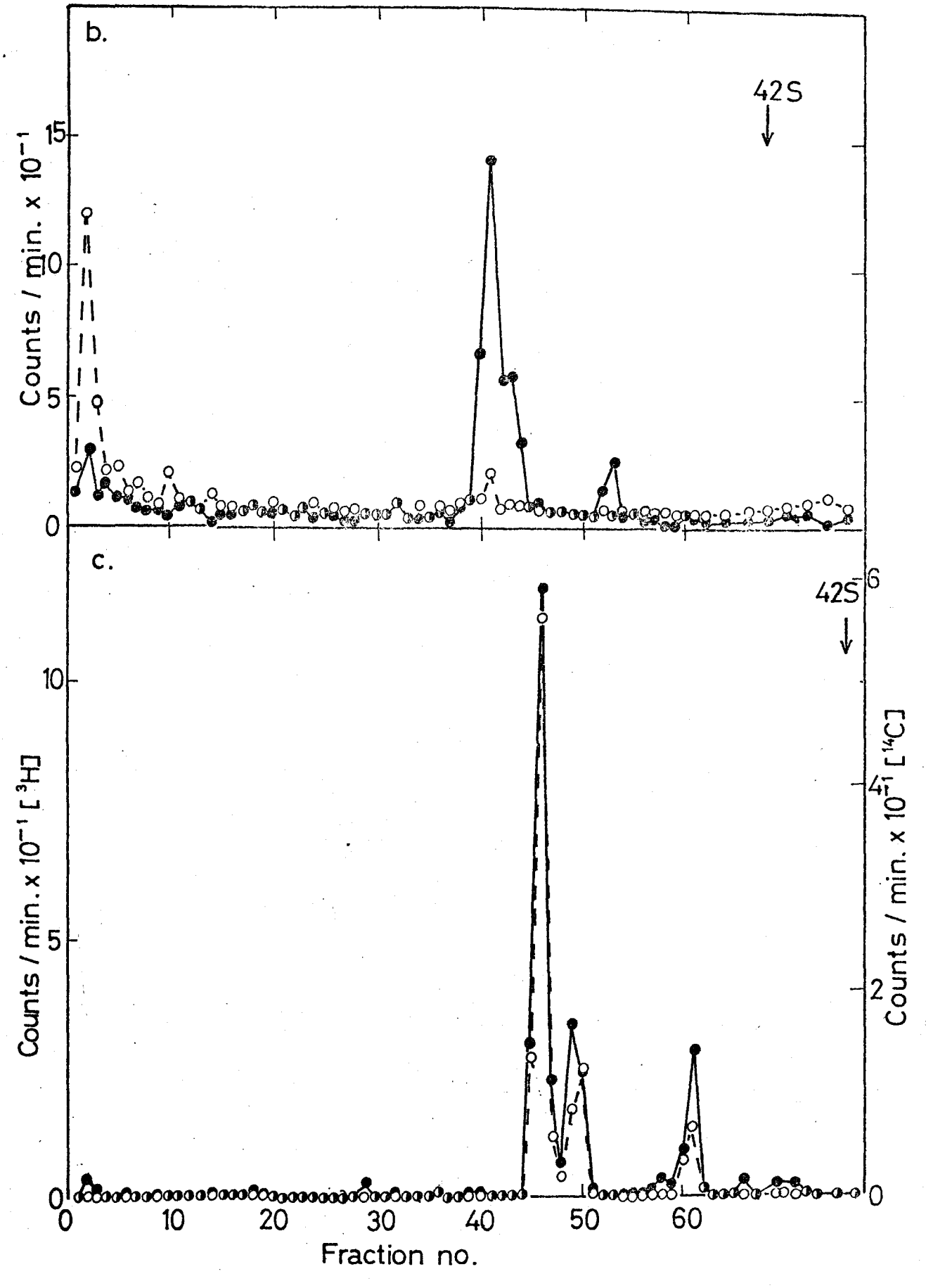


Fig. 14. Polyacrylamide gel electrophoresis of SFV-specified RNA, fractionated by treatment with 1M-NaCl, and chromatography on CF11 cellulose (Fig. 15). Samples were also treated with pancreatic RNase (10 µg./ml., 37°C, 10 min., 3 x TNE buffer) and analyzed by electrophoresis on 2.0% polyacrylamide, 0.5% agarose gel, 100V., $7\frac{1}{2}$ hours.
(a) ^{3}H-double-stranded RNA, and ^{14}C-double-stranded RNA treated with RNase;
(b) ^{3}H-multi-stranded RNA treated with RNase, and ^{14}C-multi-stranded RNA;
(c) ^{3}H-multi-stranded RNA, and ^{14}C-double-stranded RNA, both treated with RNase. ●—●, ^{3}H. o-o , ^{14}C.
The arrow indicates the position of 42S SFV RNA, estimated from gels run in parallel.


b.
42S
Counts / min. x 10⁻¹
15
10
5
0
c.
42S
6
4
2
0
10
5
0
Counts / min. x 10⁻¹ [³H]
Counts / min. x 10⁻¹ [¹⁴C]
0
10
20
30
40
50
60
Fraction no.

Three peaks of radioactivity were observed, all with lower electrophoretic mobility than 42S virion RNA, and the major species was the species of lowest mobility. It thus appeared that the RNA soluble in 1M-NaCl was double-stranded. This was confirmed by the resistance of this material to ribomuclease A (10 µg./ml., 37°C, 15 min., in 3 x TNE buffer)(Table 6). This RNA was completely resistant to these conditions of digestion, as measured by TCA-insoluble radioactivity.

The RNA in the ^{3}H- and ^{14}C-labelled salt precipitates was fractionated by chromatography before analysis. The salt precipitates were redissolved in TNE buffer, and ethanol was added to 35%. The solutions were chromatographed on a CF11 cellulose column, which was eluted successively with 35% , 15% and 0% ethanol in TNE buffer. The elution profiles are shown in Fig. 15. The majority of the radioactivity was eluted with 15% ethanol, and the remainder was eluted with buffer containing no added ethanol. When the material eluted with buffer containing no ethanol was electrophoresed on 2.0% polyacrylamide gel, the only radioactivity observed was at the origin of the gel, and only entered the first few fractions. When this RNA was treated with ribonuclease A (10 µg./ml., 37°C, 15 min. in 3 x TNE buffer), and co-electrophoresed on 2.0% polyacrylamide gel with RNA soluble in 1M-NaCl, three peaks with identical mobility and in the same proportions were observed (Fig. 14b, c).

Thus, a species of RNA was extractable from infected cells which

Table 6. Ribonuclease-resistance of SFV RNA fractionated by treatment with 1M-NaCl and chromatography on CF11 cellulose

Fraction	RNase resistance (%)
1. RNA soluble in 1M-NaCl	* 90
2. RNA insoluble in 1M-NaCl, and chromatographed on CF11 cellulose	
(a) eluted with 15% ethanol	1.4
(b) eluted with 0% ethanol	36

* Samples were treated with RNase A (10 µg./ml.) for 10 min. at 37°C in 3 x TNE buffer. Radioactivity was then measured in TCA-insoluble material.

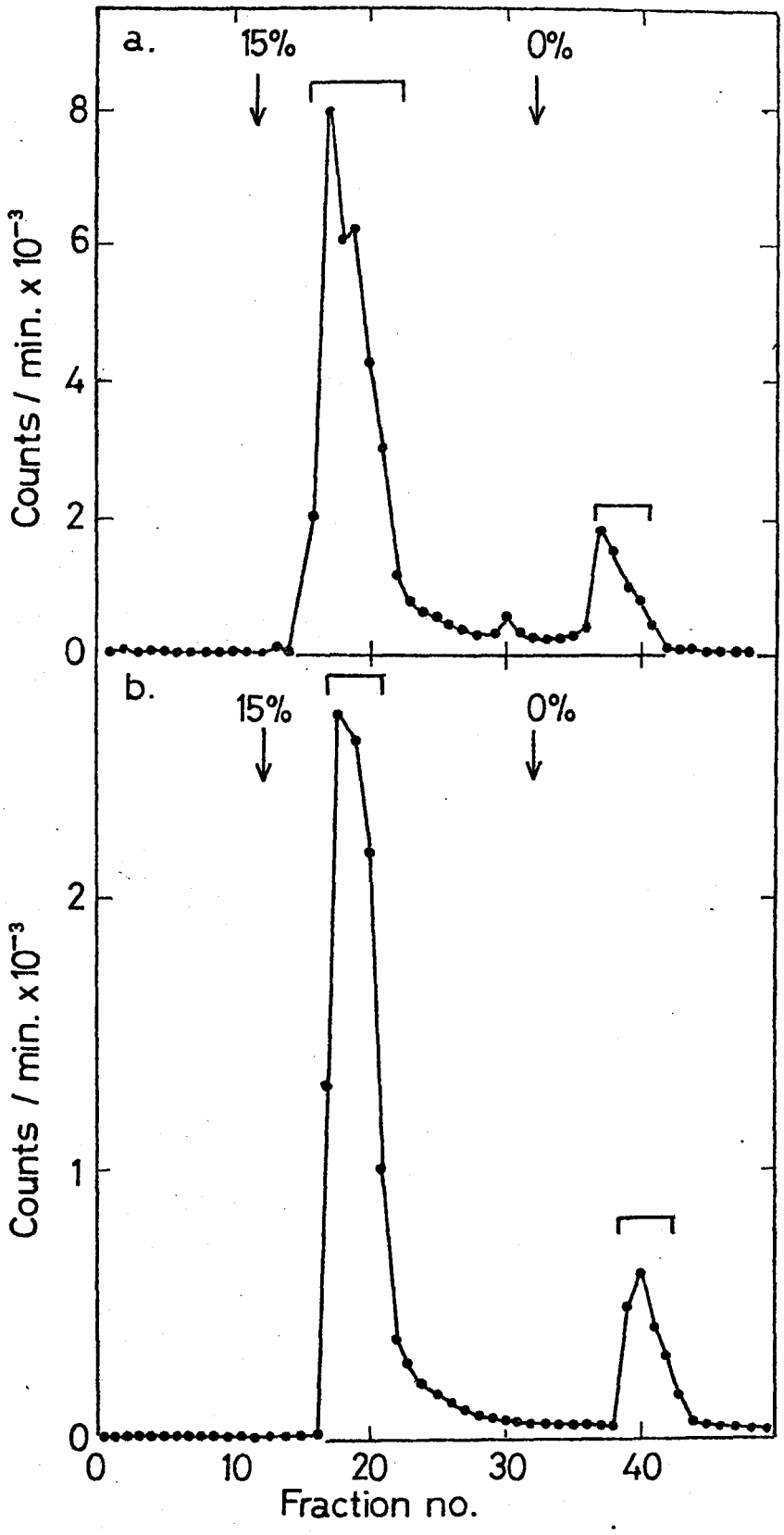


Fig. 15. Chromatography of salt-fractionated RNA on CF11 cellulose. RNA was extracted from SFV-infected chick cells, labelled 1 to 5 hours after infection with (a) ^{3}H-uridine or (b) ^{14}C-uridine. Extracts were treated with 1M-NaCl and the precipitates obtained were further fractionated by chromatography, as described in Fig. 5. Fractions were pooled as shown, and RNA recovered by precipitation with ethanol.

was precipitated by 1M-NaCl, which is a property of single-stranded RNA, but chromatographed on CF11 cellulose in the way expected of double-stranded RNA. This material was also partially resistant to ribonuclease (Table 6), and thus exhibited properties both of double-stranded and single-stranded RNA. Moreover, the ribonuclease-resistant "core" of this RNA appeared to be identical to double-stranded RNA extracted from infected cells.

Therefore, the radioactivity which was found at the origin of the polyacrylamide gel upon electrophoresis of an extract of infected cells was not simply an artefact caused by aggregation of RNA, but represented a complex, multi-stranded structure of RNA.

Because the multi-stranded RNA only entered the first few gel fractions, it was important to test whether or not the different species of virus-specified RNA were recovered in the same proportions by polyacrylamide gel electrophoresis as by other techniques. In order to do this, the relative amounts of radioactivity in multi-stranded, double-stranded and single-stranded RNA were compared with the relative recoveries of these species by CF11 cellulose chromatography and by salt precipitation (Table 7). It was found that the proportions of the different types of RNA were approximately the same, when estimated by polyacrylamide gel electrophoresis, as estimated from the recoveries using the preparative techniques. Therefore, polyacrylamide gel electrophoresis can reasonably be used to estimate the relative amounts of the different types of RNA found in SFV-infected cells.

Table 7. Comparison of the proportions of the different forms of RNA, estimated by polyacrylamide gel electrophoresis, CF11 cellulose chromatography and treatment with 1M-NaCl

% radioactivity in:	(a) PAGE	(b) CF11	(c) 1M-NaCl
DS RNA	8.1	22.0	9.8
MS RNA	13.5		90.2
SS RNA	78.4	78.0	

(a) Polyacrylamide gel electrophoresis. RNA from cells labelled 4 to 5 hrs. 10 min. after infection.

(b) CF11 cellulose chromatography. RNA from cells labelled 4 to 5 hrs. after infection.

(c) Treatment with 1M-NaCl. RNA from cells labelled 2 to 6 hrs. after infection.

4. Estimation of molecular weights of virus-specified RNA species by polyacrylamide gel electrophoresis

(a) Calibration of polyacrylamide gels

The behaviour of single-stranded RNA in the gel system employed was studied using chick embryo cell RNA. The sizes of the ribosomal RNAs have been characterized both by centrifugation and polyacrylamide gel electrophoresis. Their molecular weights have been estimated as 1.58×10^6 and 0.7×10^6 by polyacrylamide gel electrophoresis (Loening, 1968) and will be designated 28S and 18S RNA respectively. The mobility of these species and of bromophenol blue dye is shown in Fig. 16a. It may be seen that mobility was constant for each species during a 13 hour period of electrophoresis.

It has been shown that there is a linear relationship between electrophoretic mobility of species of RNA on polyacrylamide gels and the logarithm of the molecular weight, and that for gels of different concentration, the difference between the electrophoretic mobilities of the two RNA species is constant (Loening, 1969). Thus, when mobilities of RNA species are plotted against mobility at different gel concentrations, a family of straight lines is generated. The mobilities of the 28S and 18S species estimated in this experiment fall on one of these lines, suggesting that the linear relationship between mobility and log (molecular weight) holds true in the system used here.

(b) Molecular weights of the single-stranded RNA species

In order to estimate the molecular weights of the virus-specified single-stranded RNA species, ^{3}H-labelled virus-specified RNA extracted

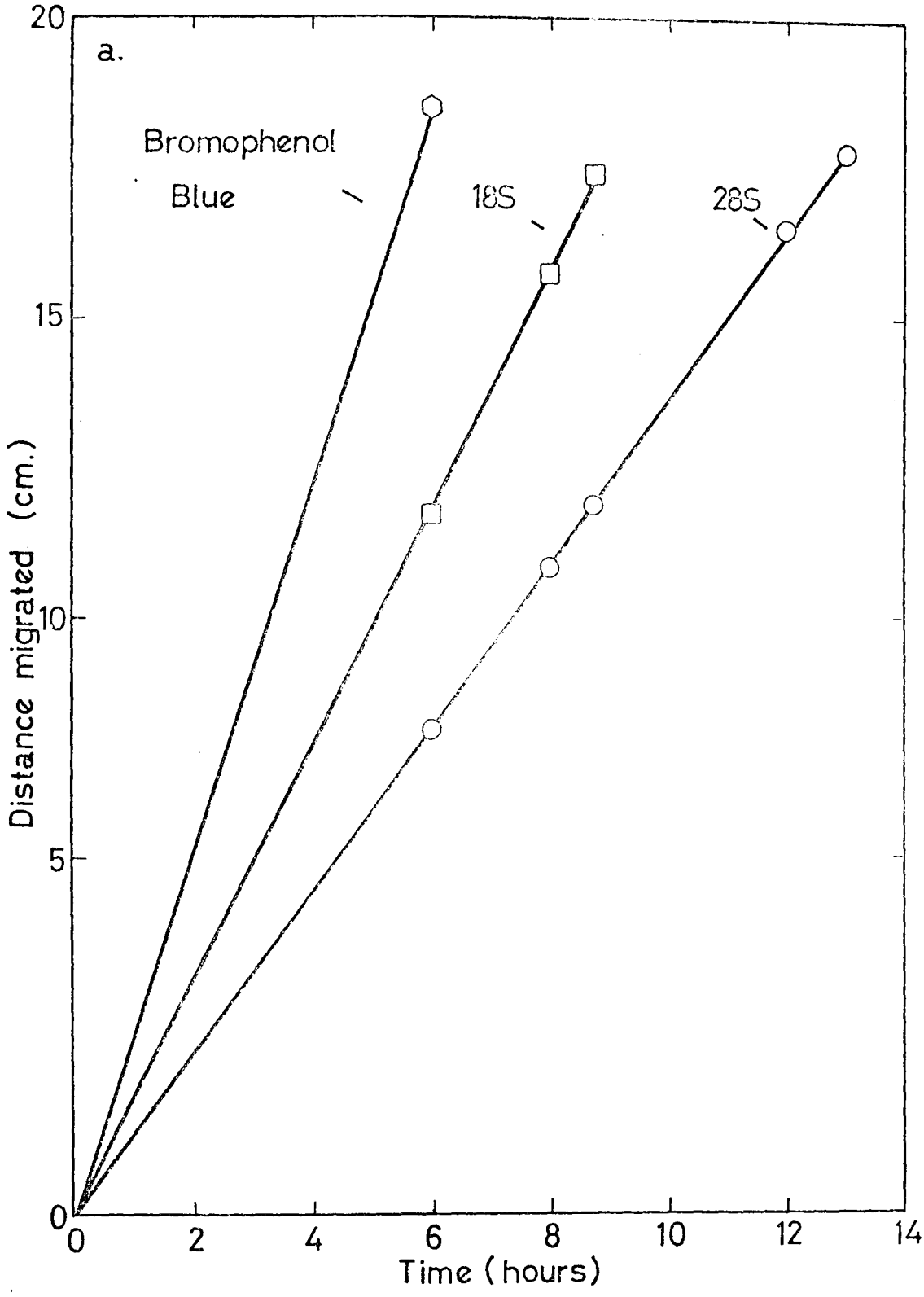
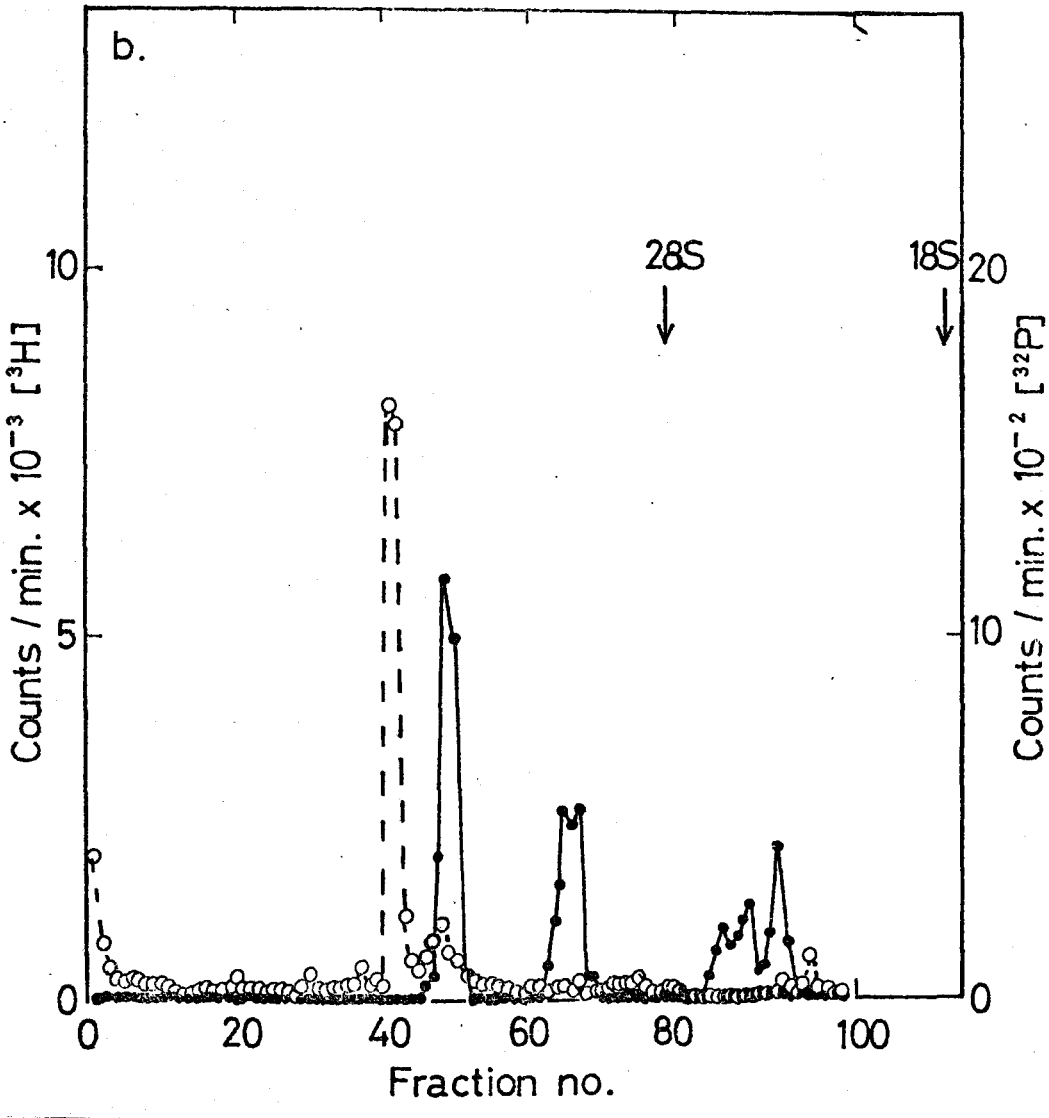


Fig. 16. a. Electrophoretic mobility of chick ribosomal RNA species and bromophenol blue on 2.0% polyacrylamide gel containing 0.5% agarose. At different times, the gel was extruded from the running tube, and the positions of the ribosomal RNA bands was determined by uv absorbance. The gel was replaced in the running tube between examinations, and electrophoresed at 100V. The overall length of the gel increased by 3% during the 13-hour run. The positions of the absorbing bands were corrected for expansion of the gel, assuming a uniform rate of expansion of the gel throughout the run.



b. Polyacrylamide gel electrophoresis of SFV and reovirus RNA. Double-stranded SFV RNA was prepared from total ^{3}H-labelled, SFV-specified RNA, by salt-precipitation and CF11 cellulose chromatography, and coelectrophoresed with RNA extracted from ^{32}P-labelled reovirus.

from infected cells was mixed with either unlabelled or ^{14}C-labelled RNA from uninfected chick cells, and the mixtures were electrophoresed on 2.0% polyacrylamide gel. The molecular weights were estimated by comparing the mobilities of the virus-specified RNA species with those of the chick 28S and 18S RNA species. A calibration line was obtained for each gel by plotting the logarithm of the molecular weights of the 28S and 18S chick RNA species against the distance migrated into the gel. The molecular weights of the virus-specified RNA species were estimated from their electrophoretic mobilities using this calibration line. The mean values obtained from a number of determinations were as follows:

Species	*Molecular weight ($\times 10^{-6}$)	No. of determinations
42S	3.97 ± 0.08	15
38S	3.05 ± 0.07	11
33S	2.31 ± 0.04	12
26S	1.76 ± 0.02	15

* ± standard error of the mean

(c) Molecular weights of the double-stranded RNA species

An attempt was made to estimate the molecular weights of the double-stranded species of RNA specified by SFV, by comparison with double-stranded reovirus RNA. Reovirus contains a double-stranded RNA genome consisting of several segments, which are in three size classes. The molecular weights of the reovirus RNA species have been

estimated by electrophoresis on 2.5% and 5% polyacrylamide gel (Shatkin et al. 1968).

The molecular weights of the SFV-specified double-stranded RNA species were estimated in a way similar to that used for the single-stranded species. Double-stranded RNA was prepared from ^{3}H-labelled RNA extracted from SFV-infected chick cells, by salt precipitation and CF11 cellulose column chromatography. The double-stranded SFV-specified RNA was mixed with ^{32}P-labelled reovirus RNA (^{32}P-labelled reovirus was kindly donated by Dr. W. Shepherd, of this department). The mixture was electrophoresed on 2.0% polyacrylamide gel and 8 peaks of radioactivity were detected, in 3 size classes (Fig.16b). The pattern was qualitatively similar to that obtained by Shatkin et al. (1968). A calibration curve was drawn from the mobilities of the ^{32}P-labelled peaks. However, in common with the result of Shatkin et al. (1968), it was found that the mobilities of the ^{32}P-labelled peaks did not fall on a straight line, when plotted against the logarithms of their known molecular weights. Because the mobilities of the SFV-specified double-stranded RNA species were all lower than those of the reovirus RNA species, it was not possible to estimate their molecular weights with accuracy. The best estimates that could be made, by extrapolation, were: 3.3×10^{6}, 2.6×10^{6} and 2.4×10^{6}. These values are all lower than would be expected if the SFV-specified double-stranded RNA species were twice the size of any of the single-stranded SFV-specified RNA species.

Thus, although electrophoresis on 2.0% polyacrylamide gel is useful for the separation of RNA species and for estimation of the molecular weights of single-stranded species of RNA, it is not suitable for determining molecular weights of double-stranded RNA species. The reason for this difference in behaviour of single-stranded and double-stranded RNA is not understood at present.

Therefore, a different approach was used to estimate the sizes of the SFV-specified double-stranded RNA species, which was to separate the strands of the double-stranded species and to examine the product by polyacrylamide gel electrophoresis. The results of this investigation are described later (Results, section III4).

5. Species of virus-specified RNA synthesized early in the infectious cycle

In order to determine which species of SFV-specified RNA were synthesized first after infection, chick cell monolayers were infected in the presence of actinomycin D and labelled with ^{3}H-uridine for 30-minute periods from the beginning of infection. RNA was extracted from the cells after each pulse, and analyzed by electrophoresis on 2.0% polyacrylamide gel (Fig. 17).

After an initial lag of about one hour, double-stranded and multi-stranded RNA were the first species of SFV-specified RNA to be

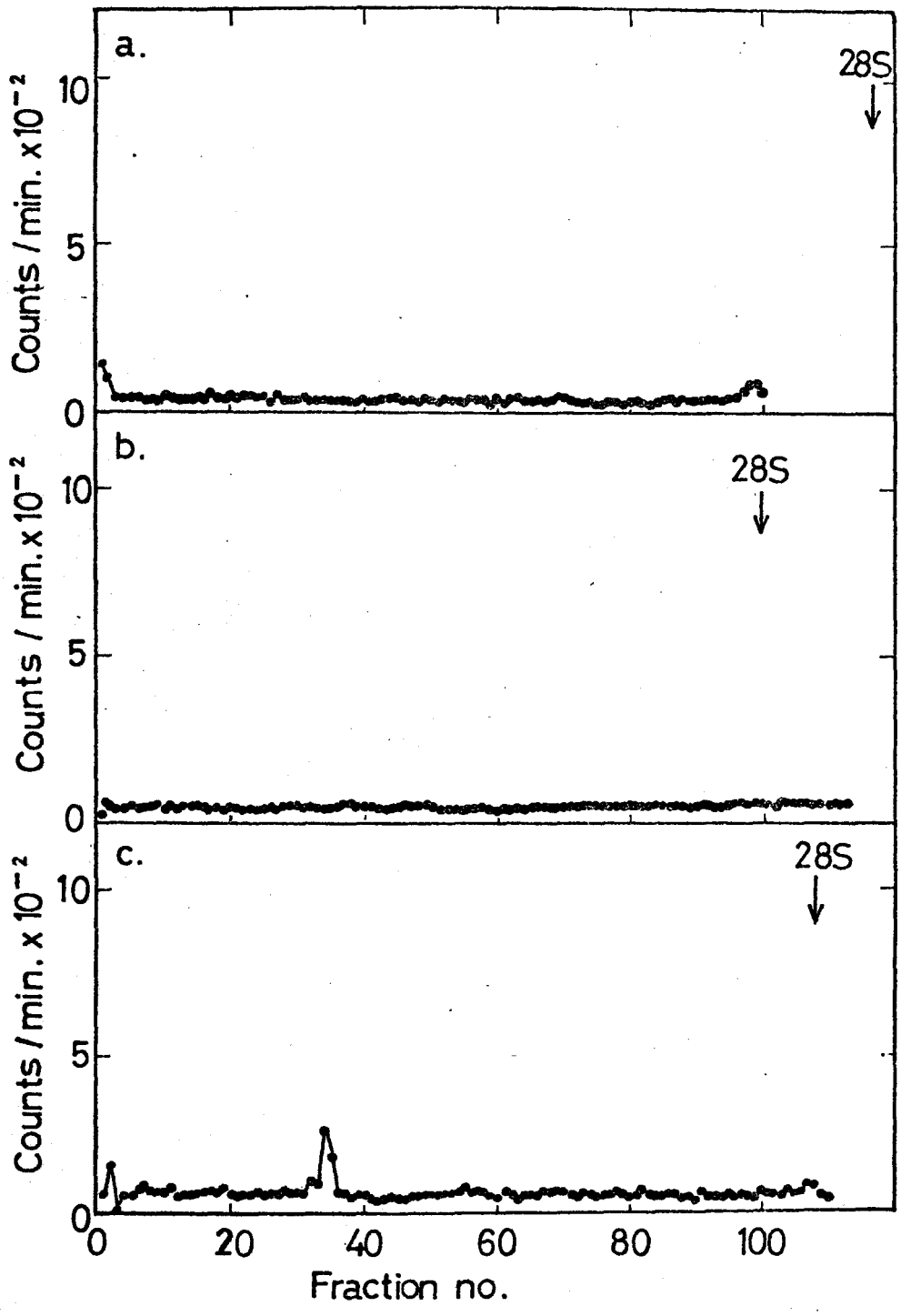
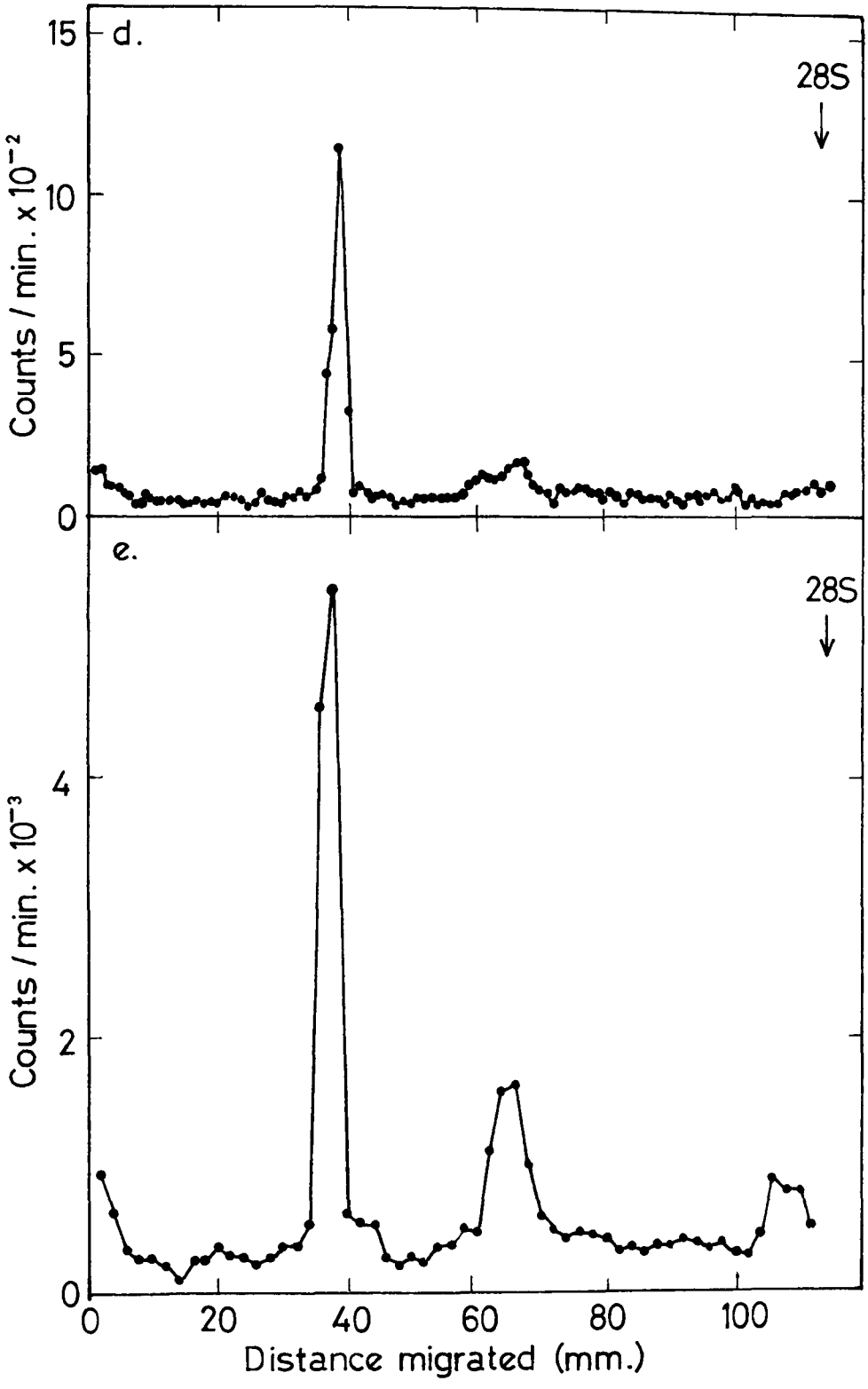
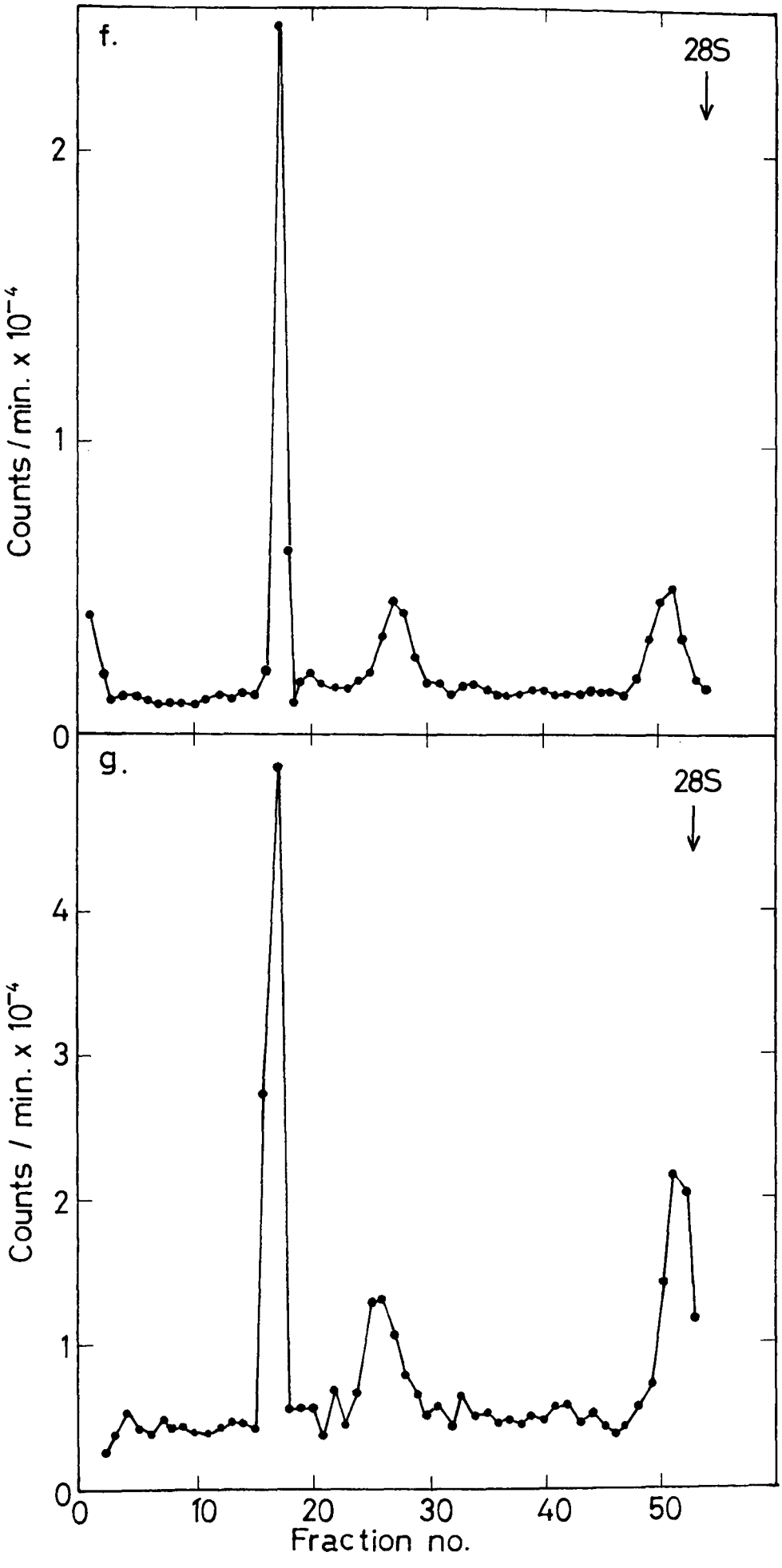


Fig. 17. Polyacrylamide gel electrophoresis of RNA labelled in 30-minute periods from the beginning of infection of chick cells with SFV. Cultures were incubated with maintenance medium containing actinomycin D (1 µg./ml.) at 37°C for 1 hour, and then infected with SFV. Separate cultures were labelled for 30 minute periods starting from the addition of virus until 11 hours after infection. RNA was extracted from cells with phenol and SDS, and electrophoresed on 2.0% polyacrylamide, 0.5% agarose gels, at 100V. for $6\frac{1}{2}$ hours. The arrow indicates the position of the chick ribosomal 28S RNA species, which was detected optically. The figures show RNA from cells labelled as follows: (a) 0–$\frac{1}{2}$ hour (b) $\frac{1}{2}$–1 hour (c) 1–$1\frac{1}{2}$ hours (d) $1\frac{1}{2}$–2 hours (e) 2–$2\frac{1}{2}$ hours (f) $2\frac{1}{2}$–3 hours (g) 3–$3\frac{1}{2}$ hours after infection.


d.
28S
15
10
5
0
Counts / min. x 10⁻²
e.
28S
4
2
0
Counts / min. x 10⁻³
0
20
40
60
80
100
Distance migrated (mm.)


f.
28S
Counts / min. x 10^{-4}
0
1
2
g.
28S
Counts / min. x 10^{-4}
0
1
2
3
4
0
10
20
30
40
50
Fraction no.

detected in infected cells, and their rates of synthesis increased at a logarithmic rate. No synthesis of single-stranded species was detected for another 30 minutes, after which time 42S RNA was observed (Fig. 17). The 26S, 33S and 38S species of RNA could be detected within the next 30 minutes. Since 42S RNA was the major species until $2\frac{1}{2}$ hours after infection, it is possible that synthesis of all the virus-specified single-stranded RNA species commenced at the same time, but that 42S RNA was detectable first, because it was labelled faster than the other species.

The synthesis of total virus-specified RNA in this experiment was estimated by measuring the specific activity of the TCA-insoluble radioactivity in each sample of extracted RNA (Fig. 18). Very little synthesis of RNA was observed until $1\frac{1}{2}$ hours after infection. A small peak in the rate of RNA synthesis was seen 3 hours after infection, which has been observed before, but whose significance is unknown (Taylor, 1965; Sonnabend et al. 1967).

Species of virus-specified RNA labelled by short pulses

The preceding section dealt with the time course and order of ppearance of viral RNA species, showing the pattern of events early the infectious cycle. In order to examine in more detail the events ich occur once RNA synthesis is established, infected cells were belled with ^{3}H-uridine for short periods at 5 hours after infection,

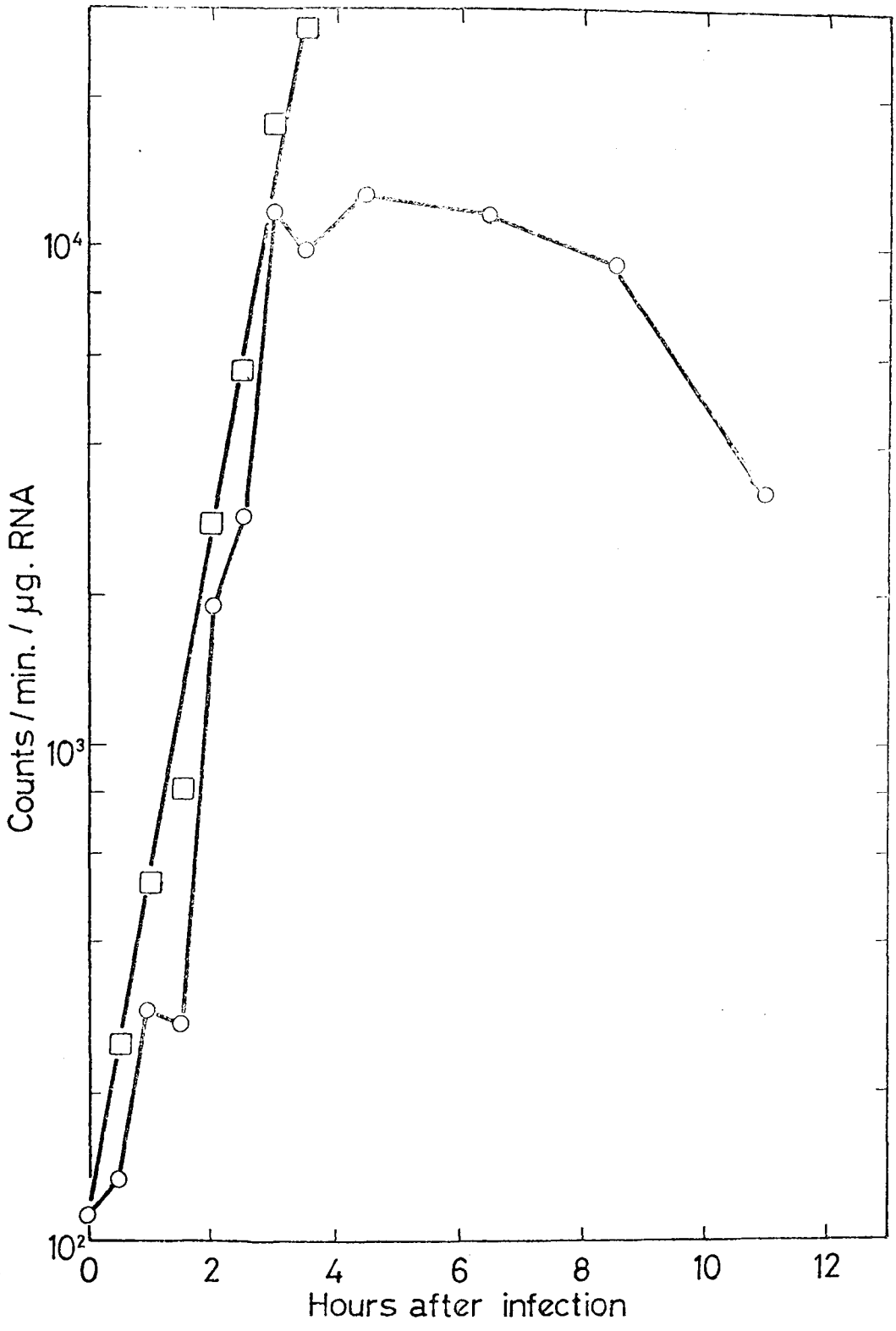


Fig. 18. Rate of labelling of RNA and accumulation of RNA during infection with SFV. Infected chick cells were labelled for 30-minute periods from the beginning of infection (Fig. 17). TCA-insoluble radioactivity and RNA were estimated in each sample. ○—○ , periodic incorporation. □—□, cumulative incorporation.

when virus-specified RNA synthesis was at its maximum rate. After each pulse, RNA was extracted from the cells and the specific activity of the extracted RNA was measured and plotted as a function of time (Fig. 19). After an initial lag of about 5 minutes, synthesis of RNA was linear for at least one hour, as was expected, since the rate of synthesis was constant at this time in the infectious cycle (Fig. 18). The initial lag in incorporation of radioactivity into RNA has been observed previously by Baltimore (1969) in poliovirus-infected HeLa cells, and by Skehel et al. (1967) in uninfected chick cells, and is thought to be due to the large size of the intracellular nucleotide pool, and the relatively slow rate of equilibration of extracellular uridine with this pool.

In order to determine the temporal order of synthesis of the virus-specified RNA species, the RNA extracted from pulse-labelled, infected cells was analyzed by polyacrylamide gel electrophoresis (Fig. 20). All the species characterized in long periods of labelling could be detected in 1 minute, the shortest period of labelling used. However, the distribution of radioactivity in the different species of RNA altered with the length of pulse. When cells were labelled for 1 or 2 minutes, most radioactivity was found in RI and RF (Fig. 21). When the period of labelling was 5 minutes or longer, however, most radioactivity was found in single-stranded species of RNA. The radioactivity in 26S RNA was approximately 1.5 times that in 42S RNA in all the labelling periods. Assuming that these species are labelled to the same specific activity in the cell, this means that

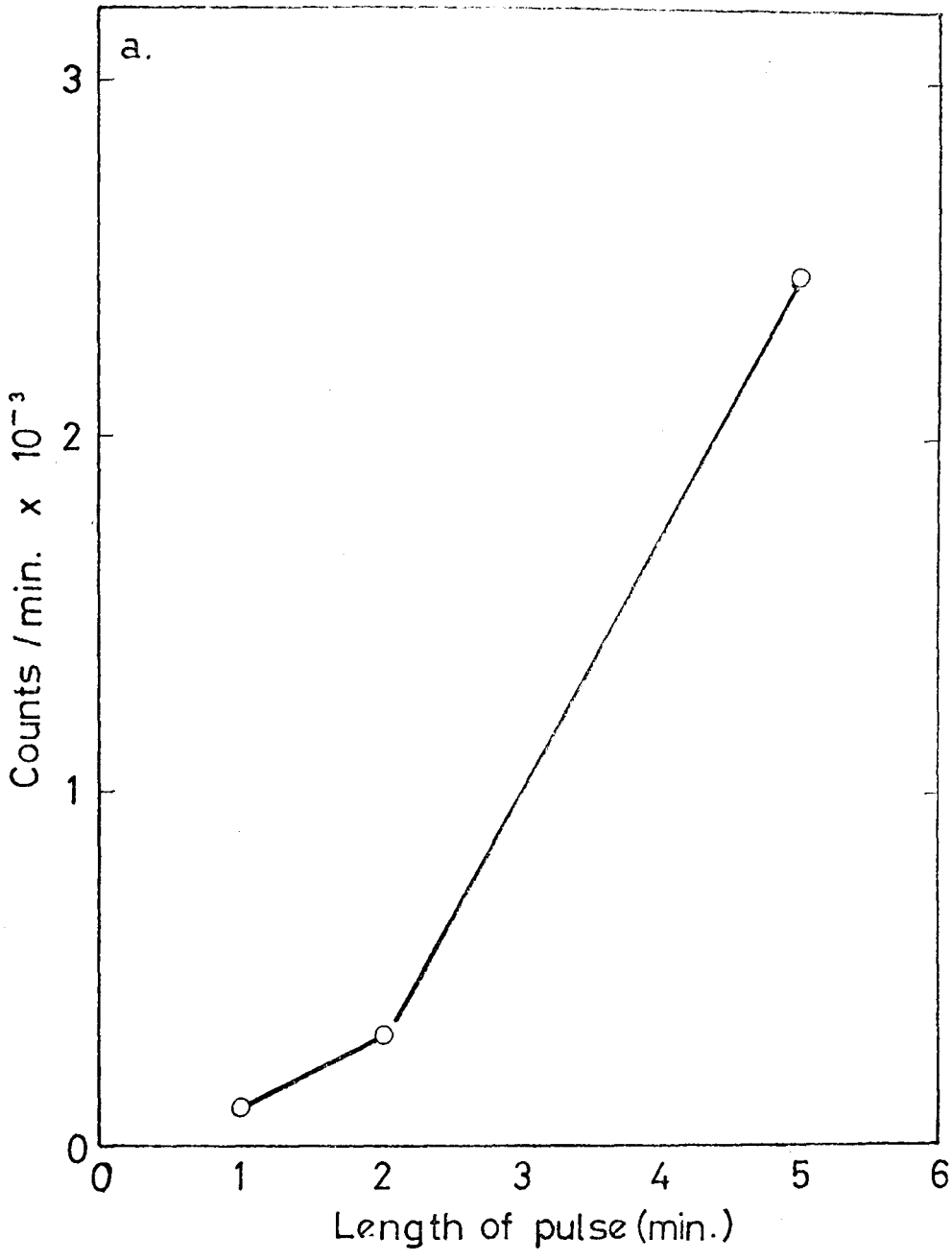


Fig. 19. Pulse-labelling of SFV-specified RNA. Chick cells were infected with SFV, and labelled with ^{3}H-uridine for 1, 2, 5, 10, 15, 30 or 70 minutes, 5 hours after infection. Radioactivity is expressed as counts/min./µg.RNA/mCi ^{3}H-uridine. Incorporation during labelling periods of up to (a) 5 minutes; (b) 70 minutes.

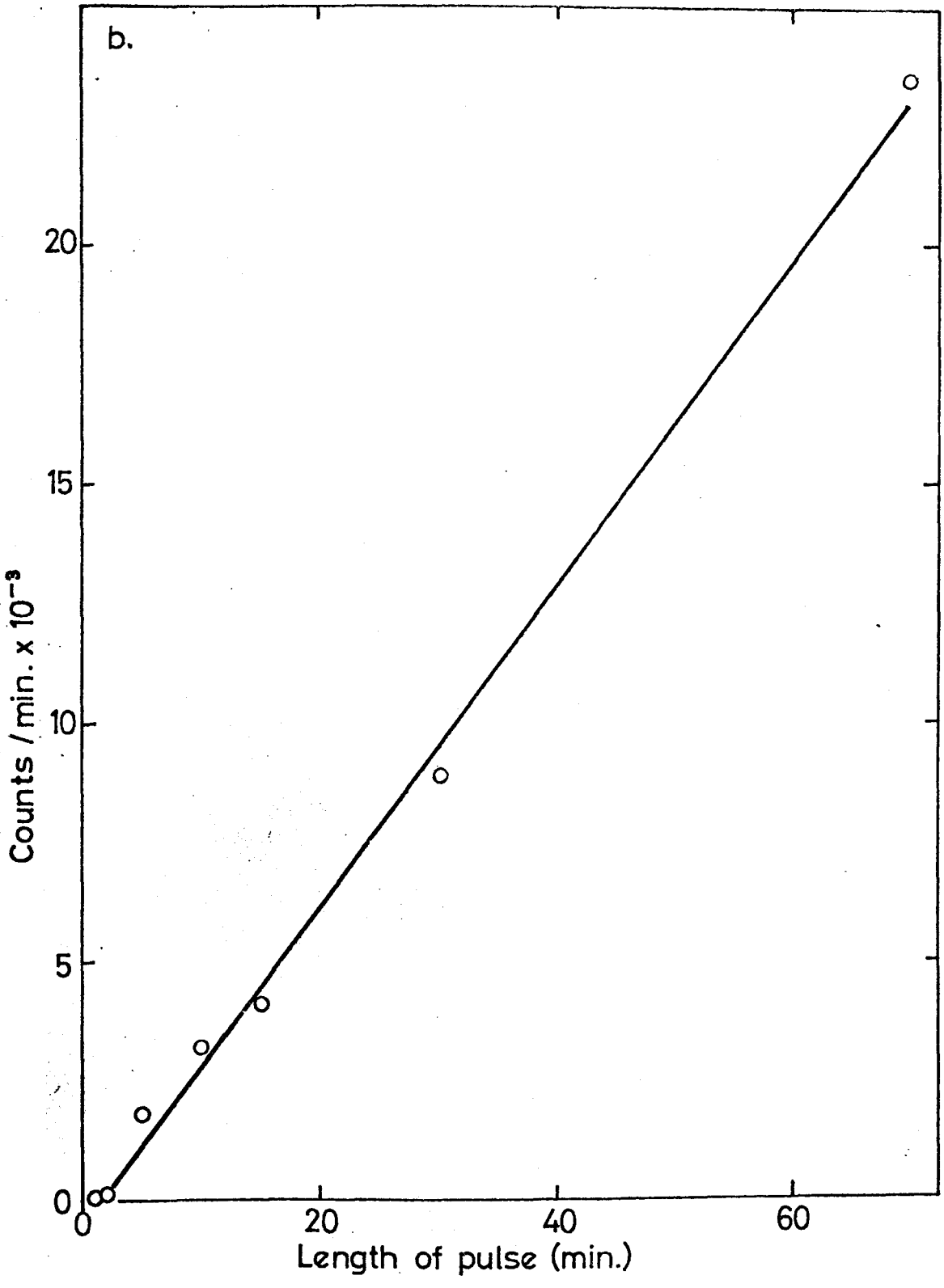
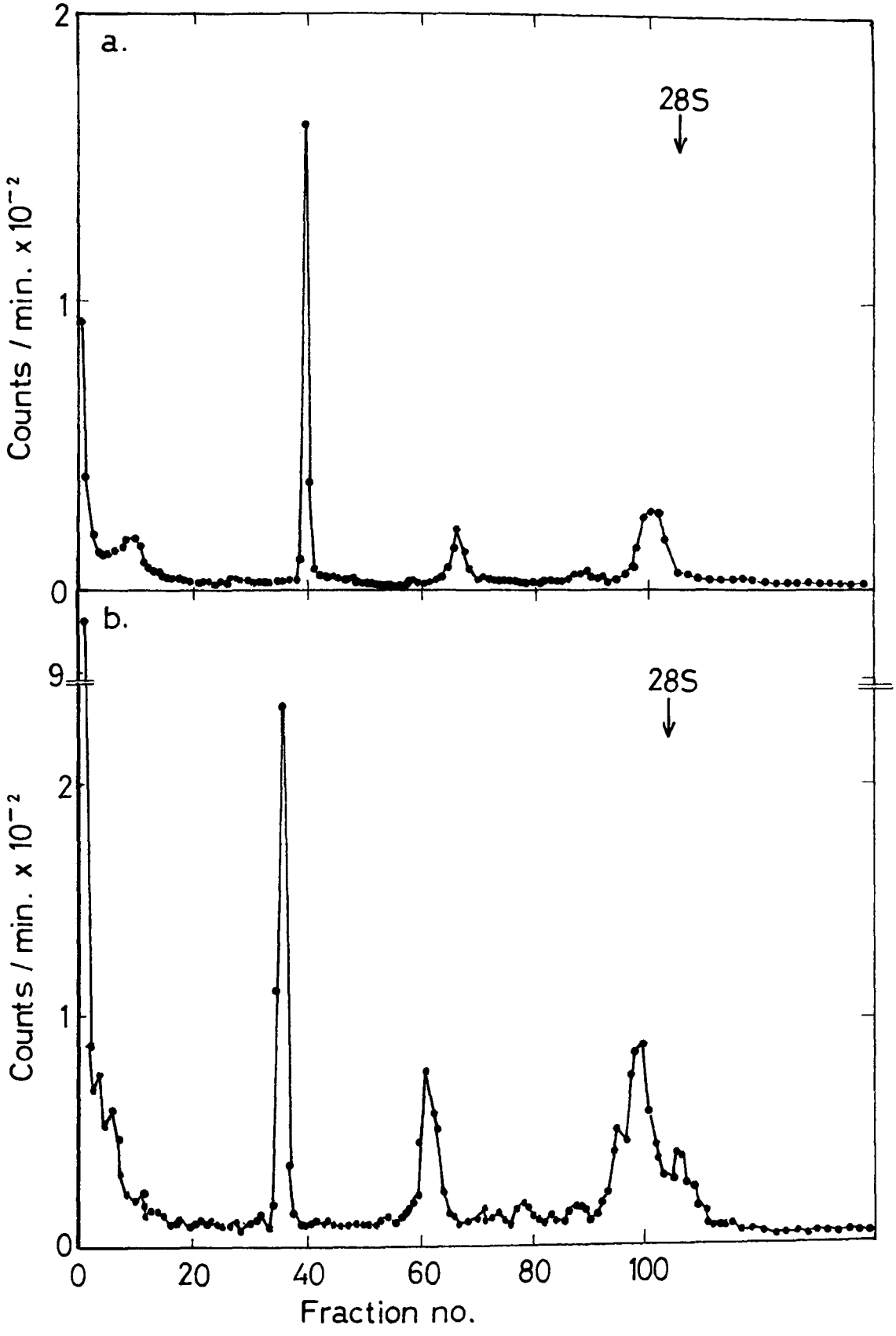
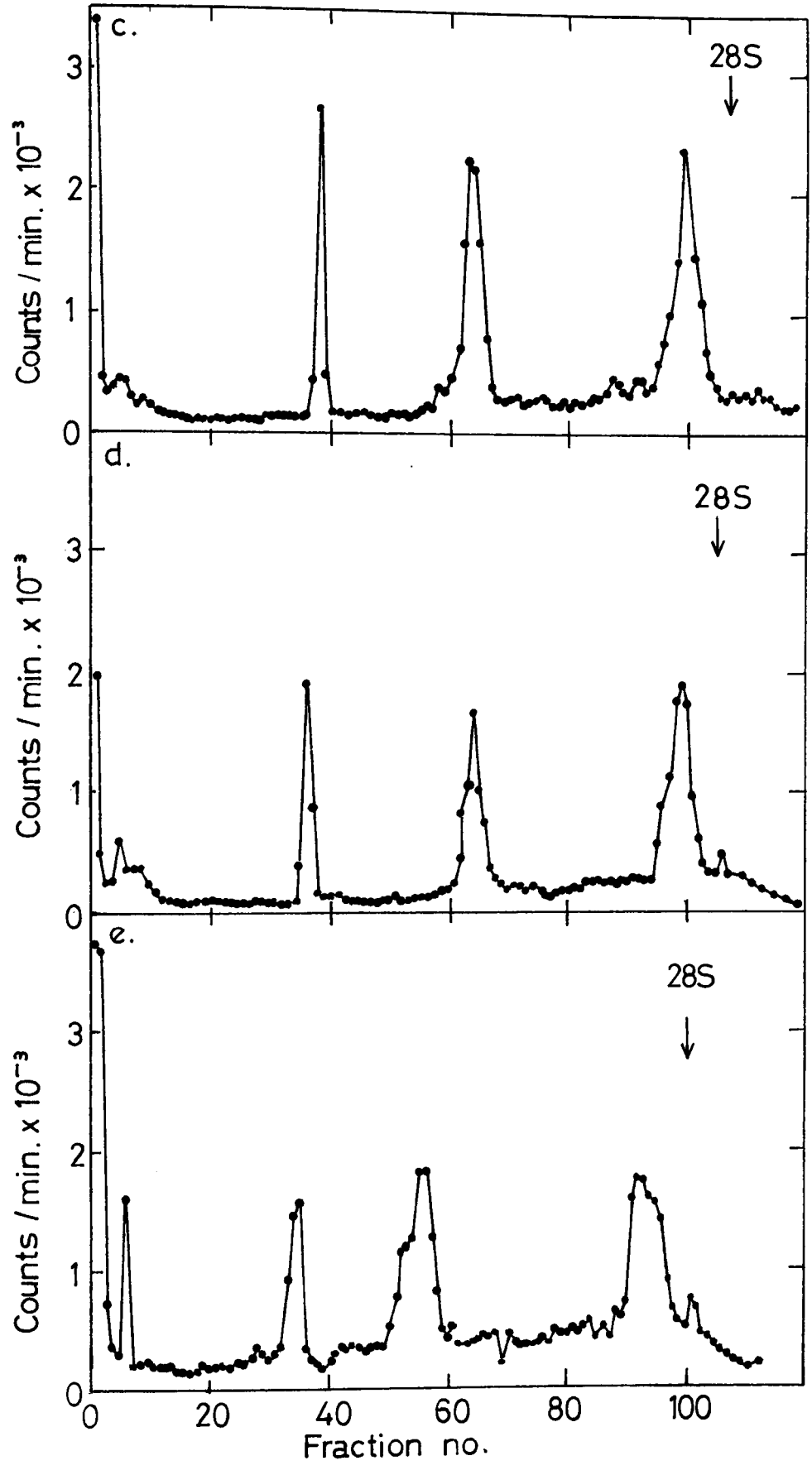
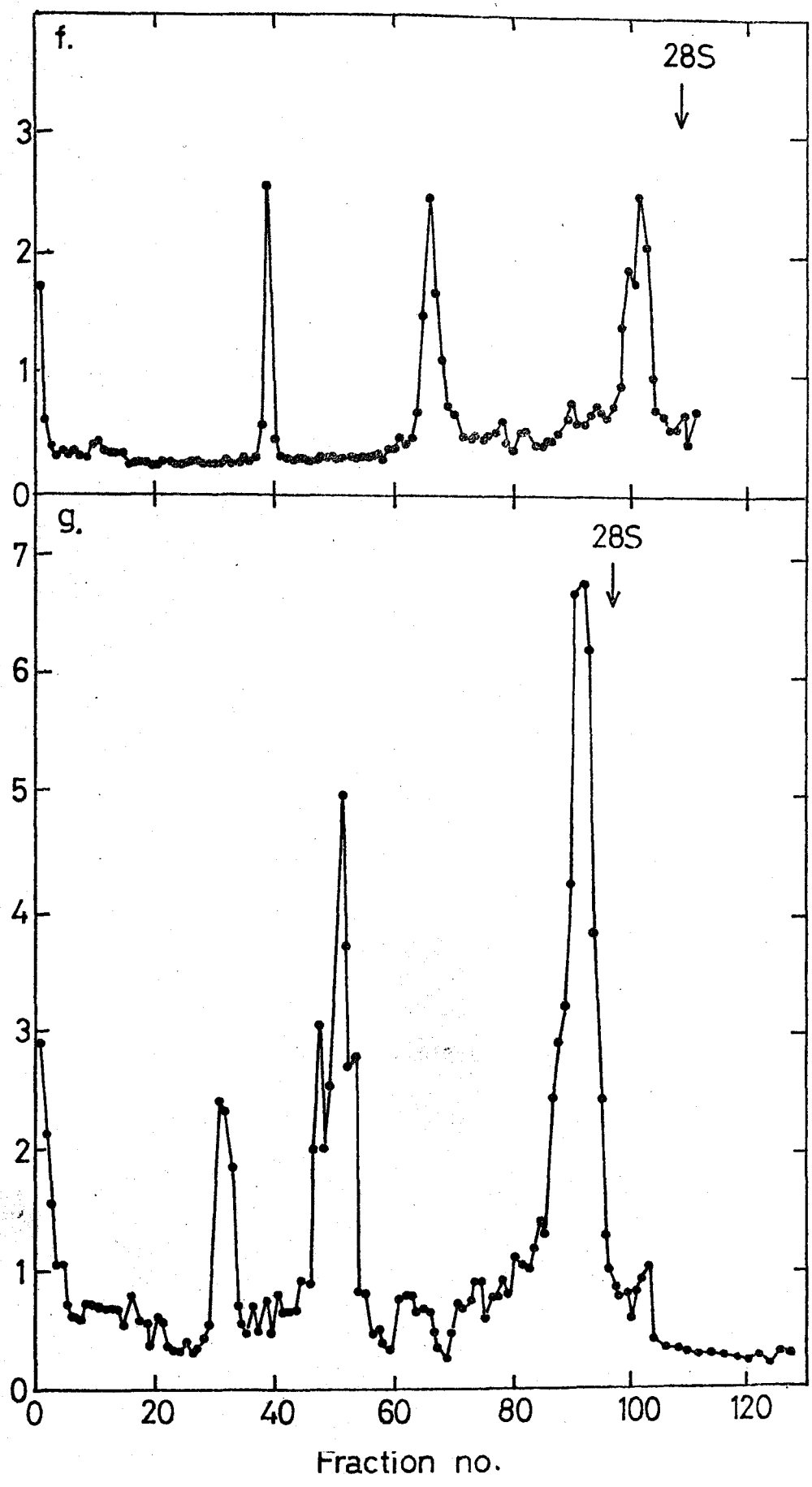

b.
20
15
10
5
0
0
20
40
60
Counts / min. x 10^{-3}
Length of pulse (min.)

Fig. 20. Polyacrylamide gel electrophoresis of pulse-labelled SFV RNA. Chick cells were infected with SFV and labelled with ^{3}H-uridine for 1, 2, 5, 10, 15, 30 or 70 minutes, at 5 hours after infection (Fig. 19). RNA was extracted and analyzed by electrophoresis on 2.0% polyacrylamide, 0.5% agarose gels, at 100V. for $6\frac{1}{2}$ hours. The cells were pulse-labelled for (a) 1 minute (b) 2 minutes (c) 5 minutes (d) 10 minutes (e) 15 minutes (f) 30 minutes (g) 70 minutes. ^{14}C-labelled chick cell RNA was coelectrophoresed with ^{3}H-labelled SFV RNA. The arrow marks the position of the chick ribosomal 28S RNA species.


a.
28S
Counts / min. x 10⁻²
2
1
0
b.
9
28S
2
1
0
0
20
40
60
80
100
Fraction no.


c.
28S
Counts / min. x 10⁻³
d.
28S
Counts / min. x 10⁻³
e.
28S
Counts / min. x 10⁻³
0
1
2
3
0
20
40
60
80
100
Fraction no.


f.
28S
3
2
1
0
g.
28S
7
6
5
4
3
2
1
0
0
20
40
60
80
100
120
Fraction no.

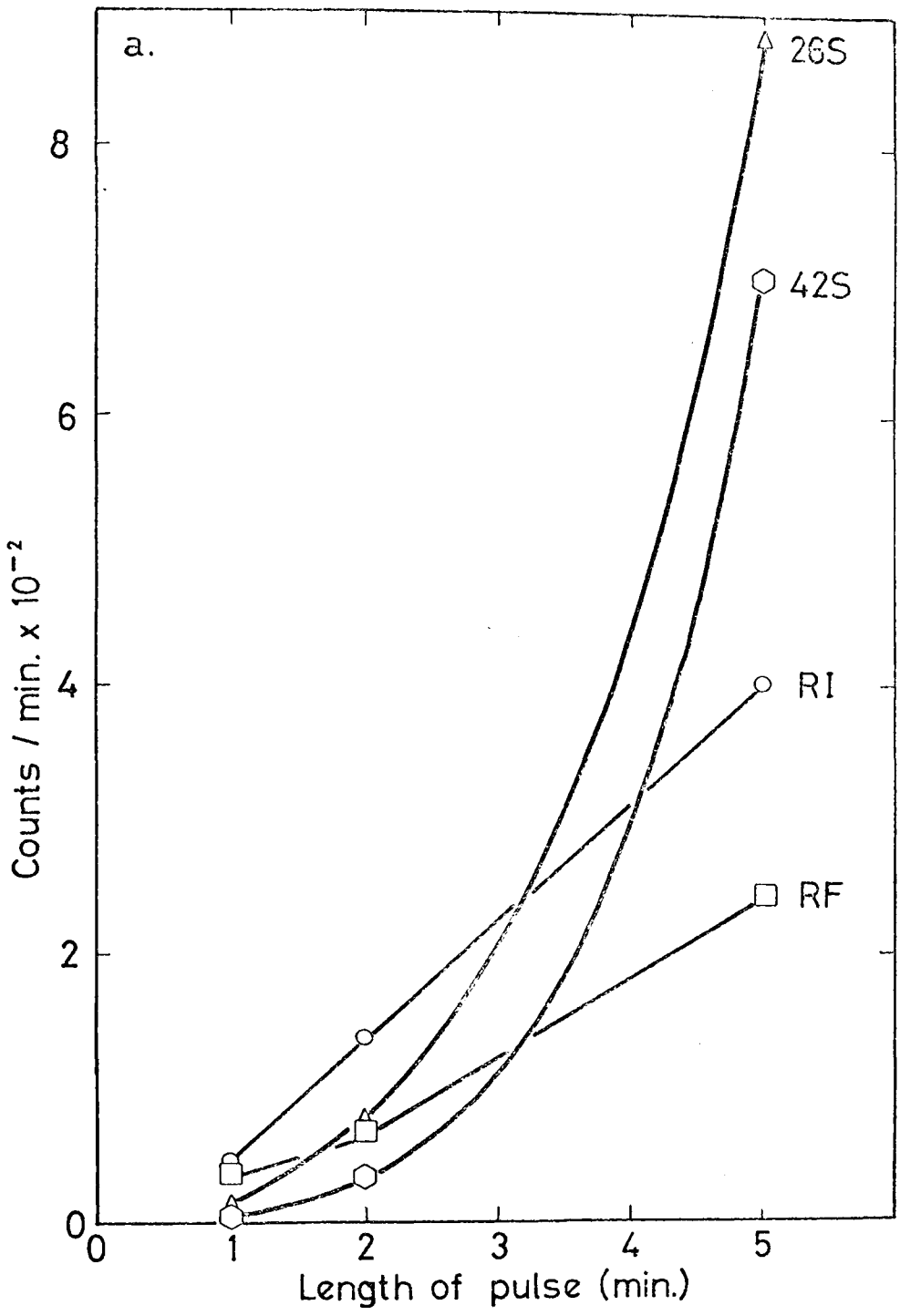
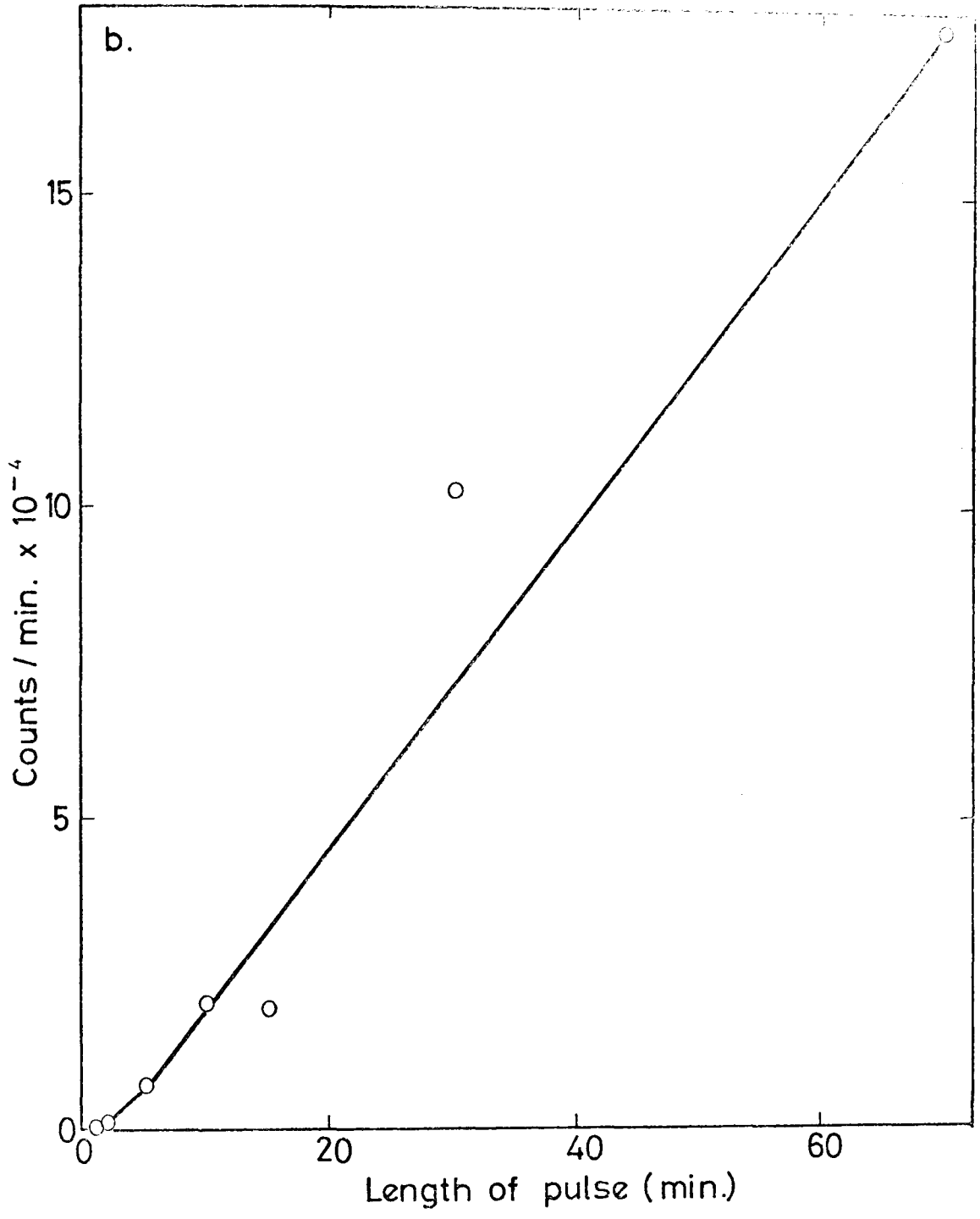
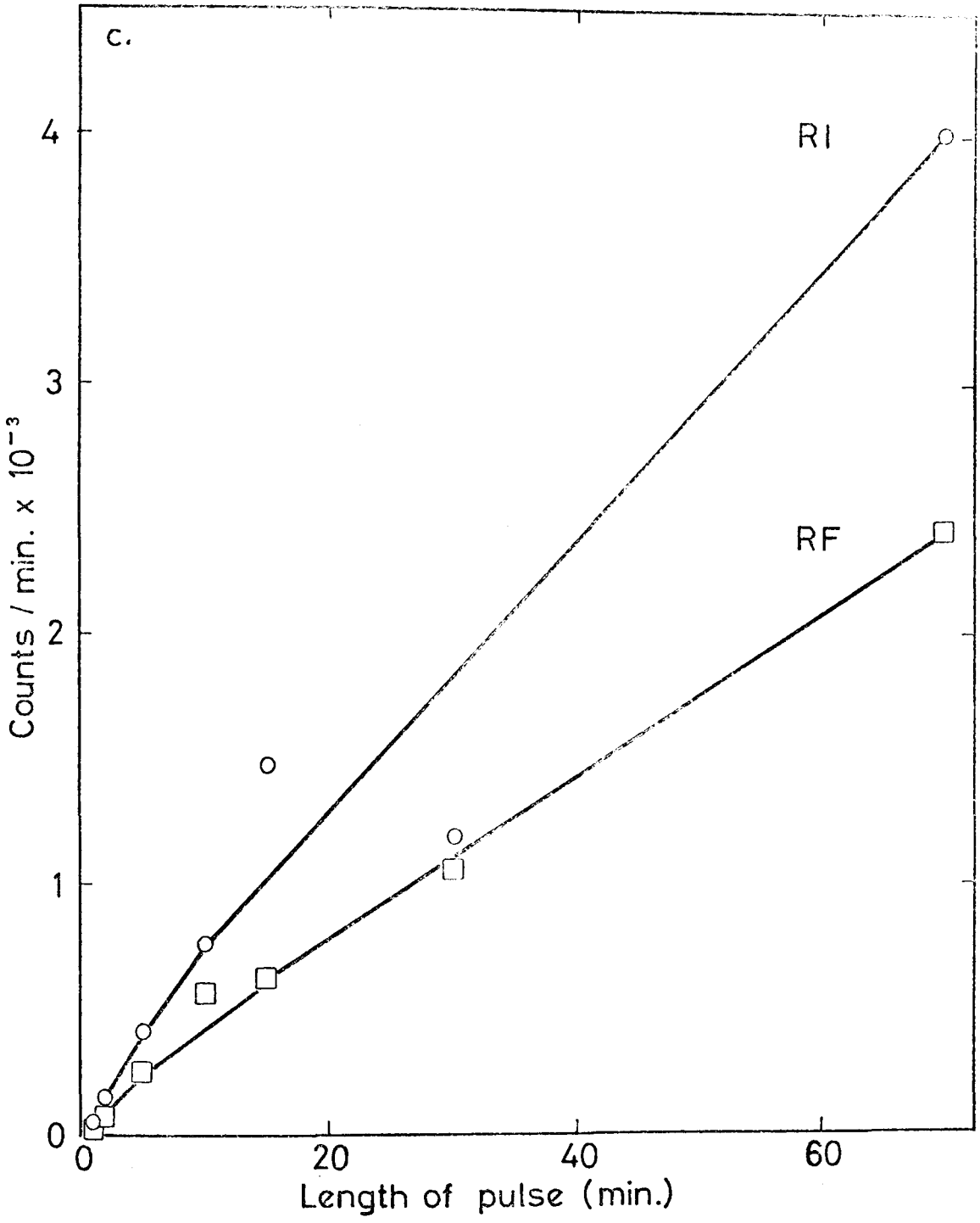


Fig. 21. Incorporation of RNA into different species of RNA during short pulses with ^{3}H-uridine. RNA in single-stranded (ss) RNA, double-stranded (RF) RNA and multi-stranded (RI) RNA were estimated from the electrophoretic profiles in Fig. 20. (a) Radioactivity in ss, RF and RI RNA, 1 to 5 min. pulses. (b) Radioactivity in ss RNA, 1 to 70 min. pulses. (c) Radioactivity in RI and RF RNA, 1 to 70 min. pulses.


b.
15
10
5
0
Counts / min. x 10^{-4}
0
20
40
60
Length of pulse (min.)


c.
RI
RF
Counts / min. x 10^{-3}
Length of pulse (min.)
0
1
2
3
4
0
20
40
60

26S RNA is being synthesized at about three times the rate of the 42S species, since 42S RNA is approximately twice the size of 26S RNA.

Thus, the pattern of synthesis of virus-specified RNA species when RNA synthesis was at the maximum rate was different from that early in the infectious cycle, in the following respects: (i) the accumulation of RNA was linear for at least one hour after the maximum rate of RNA synthesis was reached; (ii) at this time, the rate of synthesis of the single-stranded RNA species was greater than that of the double-stranded and multi-stranded species; (iii) 26S RNA was synthesized at a greater rate than 42S RNA; (iv) multi-stranded RNA was synthesized at a greater rate than double-stranded RNA.

7. Extraction of RNA in the presence of diethylpyrocarbonate

Öberg and Philipson (1971) found that when RNA was extracted from poliovirus-infected cells in the presence of 0·4 M-diethylpyrocarbonate (DEPC), about 80% less multi-stranded and 30% less double-stranded RNA were observed by polyacrylamide gel electrophoresis than when RNA was extracted in the absence of DEPC. They suggested that DEPC was preventing the formation of annealed structures which were generated during extraction with phenol and SDS.

When a similar experiment was performed with SFV-infected chick cells, similar patterns were obtained whether or not RNA was extracted

in the presence or in the absence of DEPC (Fig. 22). If the interpretation that Öberg and Philipson placed on their result is correct, this would suggest that the SFV-specified RNA species are present in the cell in the same proportions as after extraction, and that multi-stranded and double-stranded forms were not artificially "collapsed" or annealed during the extraction procedures.

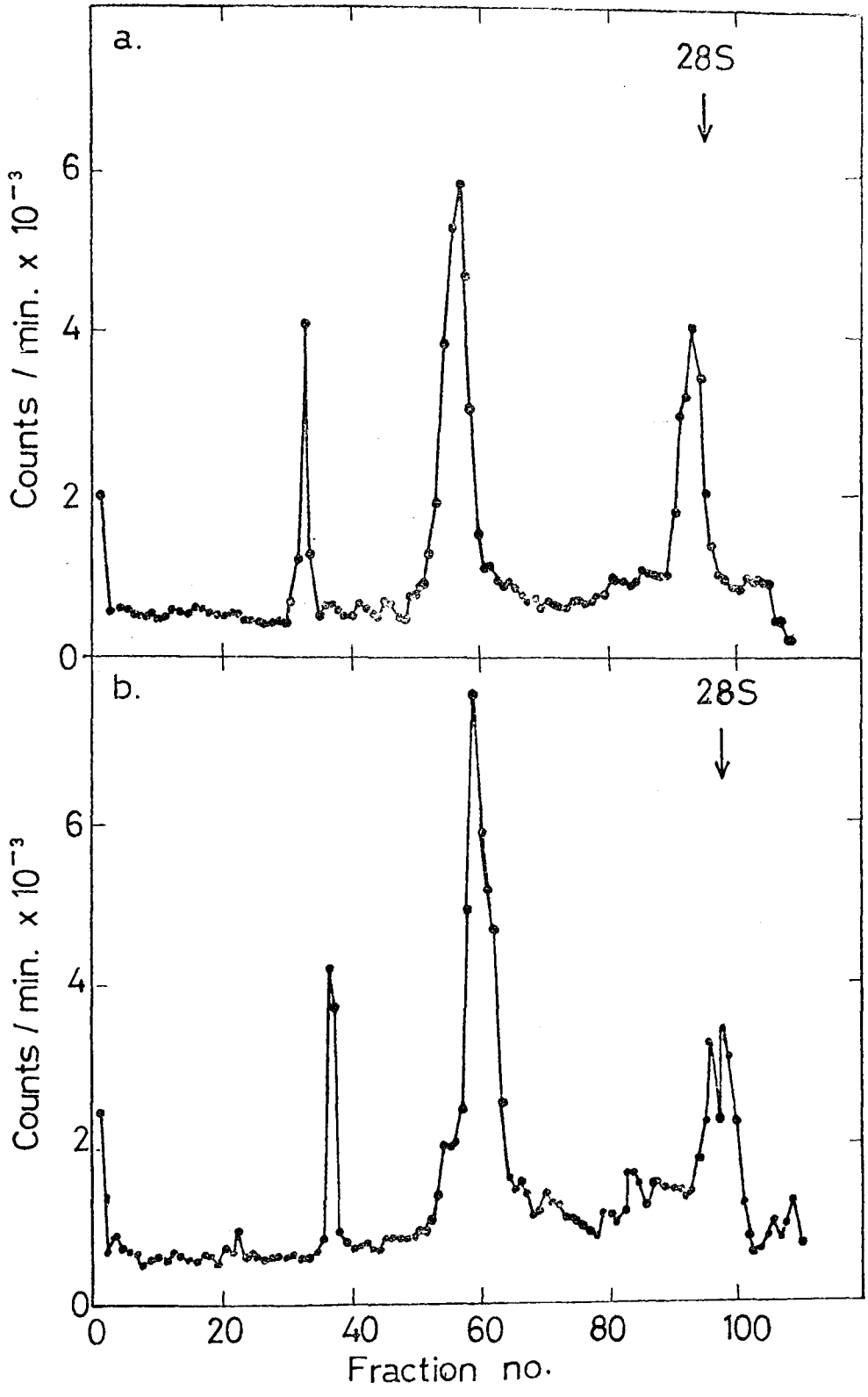


Fig. 22. Polyacrylamide gel electrophoresis of SFV-specified RNA extracted in the presence or absence of diethylpyrocarbonate (DEPC) (0.4%). Chick embryo fibroblasts infected with SFV were labelled with ^{3}H-uridine between 2 and 5 hours after infection. RNA was extracted with phenol and SDS, in (a) the presence or (b) the absence of DEPC. RNA was analyzed on 2.0% polyacrylamide, 0.5% agarose gels, at 100V. for $6\frac{1}{2}$ hours. The arrows indicate the positions of chick ribosomal RNA species, which were detected optically.

III. Some properties of the RNA species specified by Semliki Forest virus

1. Annealing experiments

Experiments were performed to detect the presence of labelled RNA whose nucleotide sequence was complementary to that of 42S virion RNA. As a positive control, ^{14}C-labelled polycytidylic acid (poly C) was annealed to unlabelled polyguanylic acid (poly G). Before annealing, ^{14}C-poly C was found to be about 0.1% resistant to ribonuclease (10 µg./ml. RNase A and 34 units /ml. RNase T1, 37°C, 15 min. in 1 x SSC buffer). After annealing at 65°C to a ten-fold excess of poly G, the ^{14}C-radioactivity was 96% resistant to the same treatment with ribonuclease (Table 8). This figure was taken to represent the extent of annealing. By contrast, less than 1% self-annealing could be detected with 42S virion RNA (Table 8). In order to prepare virus-specified single-stranded RNA for annealing, infected cells were labelled with ^{3}H-uridine between 2 and 6 hours after infection, or between $2\frac{1}{2}$ and 3 hours after infection, or for 1 minute at 5 hours after infection. RNA was extracted from the cells, and single-stranded RNA was purified by salt precipitation and chromatography on a CF11-cellulose column. The labelled, single-stranded RNA obtained by this procedure was incubated under annealing conditions with known amounts of unlabelled 42S virion RNA. No significant annealing could be detected between any of these labelled preparations and 42S virion RNA (Table 8). Thus, very little (less than 1%) RNA complementary to SFV virion RNA occurs either in the

Table 8. Annealing of single-stranded RNA

Sample	Concentration* (µg./ml.)	Time at 65 C (hours)	Annealing (%)+
^{14}c-poly C + poly G	300	1	96.2
^{3}H-42S RNA	400	8	0
Unlabelled 42S RNA + ^{3}H-SS RNA, labelled:			
(a) 2-6 hours	29	3	0
(b) $2\frac{1}{2}$-3 hours	200	5	1.2
(c) for 1 minute	150	5	0

* refers to concentration of unlabelled RNA, except in the case of ^{3}H-42S RNA.

+ % annealing was taken as % RNase-resistant radioactivity after incubation, minus that before incubation.

virus particle, or free in the infected cell from the earliest time in the infection cycle that sufficient single-stranded RNA could be prepared for the annealing assay. The implications of these observations to the mechanism of virus RNA biosynthesis will be considered in the Discussion section.

2. Ribonuclease resistance of multi-stranded RNA

In order to determine the ribonuclease-resistance of multi-stranded RNA, purified preparations were required. RNA was prepared from cells which had been labelled between 2 and 6 hours after infection (continuously-labelled RNA), or for 1 minute at 5 hours after infection (pulse-labelled RNA). The RNA was extracted from these cells using phenol and SDS, and the multi-stranded RNA in each preparation was purified by salt precipitation and three cycles of chromatography on CF11 cellulose. The elution profiles obtained by chromatography of the continuously-labelled RNA are shown in Fig. 23. Similar profiles were obtained for the pulse-labelled RNA. The purified preparations of multi-stranded RNA were treated with ribonuclease (10 µg./ml. RNase A and 34 units/ml. RNase T1, 37°C, 15 min. in SSC buffer), and the proportions of radioactivity which remained insoluble in TCA are shown in Table 9. The significance of the differences in ribonuclease-resistance of the two preparations of multi-stranded RNA is considered in the Discussion section.

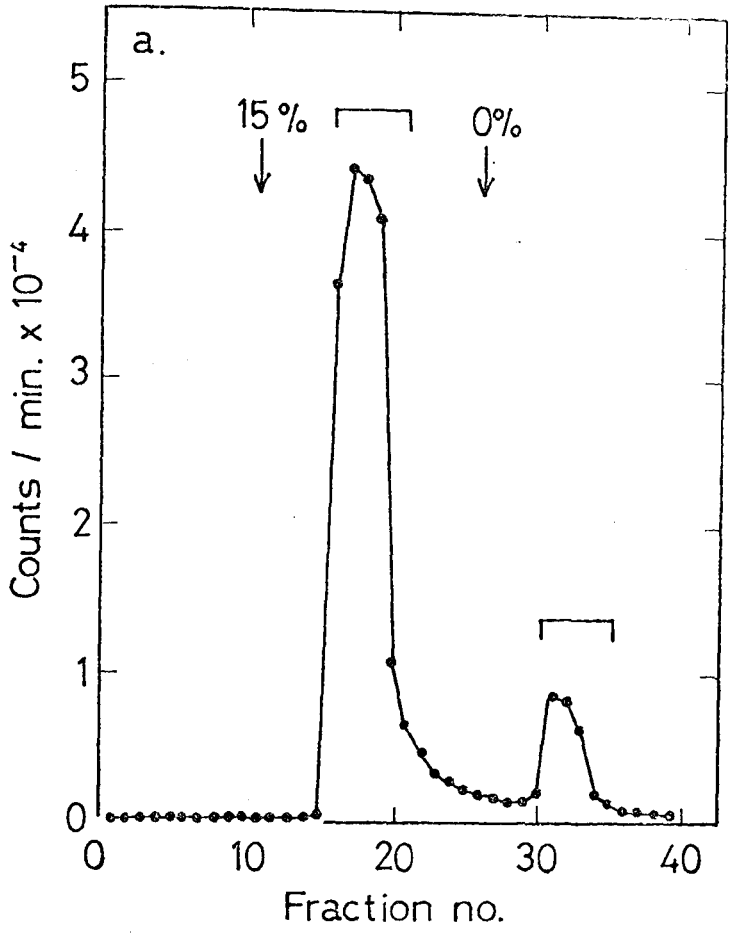


Fig. 23. Purification of multi-stranded RNA by chromatography on CF11 cellulose. Chick cells infected with SFV were labelled 2 to 6 hours after infection, and RNA was extracted using phenol and SDS. Single- and multi-stranded RNA were precipitated with 1M-NaCl, and the precipitated RNA was redissolved in TNE buffer. Ethanol was added to 35%, and the RNA was fractionated by chromatography on an 11 x 1.5 cm. column of CF11 cellulose. The column was eluted stepwise with 35%, 15% and 0% ethanol in TNE buffer. (a) Fractions were pooled as shown, and the RNA eluted with 0% ethanol (fractions 31-34) was recovered by precipitation with ethanol. The precipitated RNA was redissolved, and chromatographed again as above. (b) The RNA eluted with 0% ethanol was recovered and chromatographed a third time. (c) The RNA eluted with 0% ethanol was recovered by precipitation with ethanol.

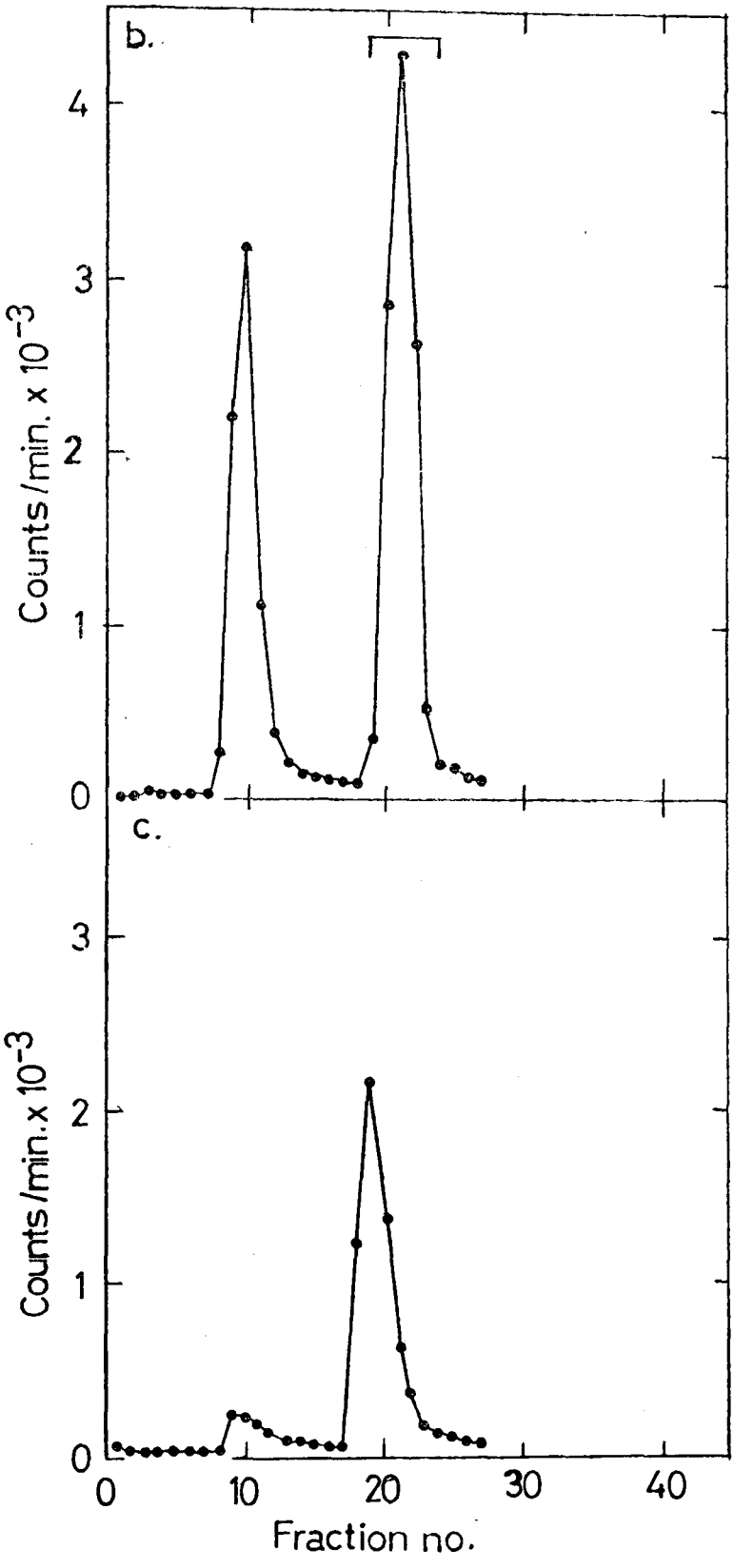

b.
c.
Counts/min. x 10⁻³
Counts/min.x 10⁻³
0
1
2
3
4
0
10
20
30
40
Fraction no.

Table 9. Ribonuclease-resistance of multi-stranded RNA

Labelling time after infection with SFV	RNase-resistance (%)
2-6 hours	30
$2\frac{1}{2}$-3 hours	31
for 1 minute, at 5 hours	70

Multi-stranded RNA was purified by precipitation with 1M-NaCl, and chromatography on CF11 cellulose columns.

3. Proportion of single-stranded RNA in multi-stranded RNA

Multi-stranded RNA has properties both of single-stranded and double-stranded RNA, as shown by its chromatographic behaviour on CF11 cellulose, and by its behaviour in solutions containing 1M-NaCl (Results sections II3(b) and (c)). The single-stranded RNA content of labelled multi-stranded RNA may be estimated from its ribonuclease-resistance, R, using the following equation:

$$R = \frac{\text{proportion of radioactivity in hydrogen-bonded form}}{\text{total radioactivity}}$$

If the multi-stranded RNA is uniformly labelled, there will be the equivalent of 2 single-stranded RNA molecules in the ribonuclease-resistant "core". If double-stranded RNA is 100% resistant to ribonuclease, and if single-stranded RNA is 0% resistant to ribonuclease, then the number of single-strand equivalents, N, is given by the equation:

$$R = \frac{100 \times 2}{2 + N} \qquad (1)$$

However, double-stranded RNA was found to be about 93% resistant to the conditions of ribonuclease digestion used, and about 1% single-stranded RNA remained TCA-insoluble under the same conditions (Table 4). Using these values, a second equation may be derived:

$$R = \frac{93 \times 2 + N}{2 + N} \qquad (2)$$

Rearranging equation (2),

$$N = \frac{186 - 2R}{R - 1} \qquad (3)$$

Substituting the value of R (30%) for continuously-labelled multi-stranded RNA:

$$N = \frac{126}{29}$$
$$= 4.3$$

The significance of this figure for the mechanism of virus RNA replication is considered in the Discussion section.

4. Separation of strands of virus-specified double-stranded RNA

As shown above (Results section II4b), the system of polyacrylamide gel electrophoresis used in this study was not suitable for the estimation of molecular weights of SFV-specified double-stranded RNA molecules. Therefore a different approach was tried, which was to separate the strands of double-stranded RNA, and to examine the single-stranded product by polyacrylamide gel electrophoresis. Double-stranded RNA was prepared from SFV-infected cells which had been labelled between $2\frac{1}{2}$ and 3 hours after infection in the presence of actinomycin D. The RNA was extracted from the cells using phenol and SDS, and single-stranded and multi-stranded RNA was precipitated with IM-NaCl. The supernatant RNA was chromatographed on a CF11 cellulose column three times, taking the material eluted with buffer containing no ethanol in each case. The RNA was recovered after the final chromatography by precipitation with

ethanol, and redissolved in TE buffer. Six volumes of DMSO were added (to a final concentration of 85.7%) and the solution was incubated at 37°C for 15 minutes. The solution was cooled quickly in ice, and RNA was recovered by precipitation with ethanol. Before treatment with DMSO, SFV double-stranded RNA was 93% resistant to ribonuclease (10 µg./ml. RNase A and 34 units/ml. RNase T1, 37°C, 15 min. in 1 x SSC buffer), but only 0.7% resistant to ribonuclease after treatment with DMSO.

The RNA which had been treated with DMSO was examined by electrophoresis on 2.0% polyacrylamide gel, containing 0.5% agarose. Unlabelled chick cell RNA was included as a marker, and ^{3}H-labelled SFV 42S virion RNA was electrophoresed on a gel run in parallel (Fig. 24). Three major peaks were observed, two of which corresponded to viral 42S and 26S RNA. The mobility of the third species was intermediate between that of the 33S and 38S viral RNA species, and its molecular weight, estimated from its electrophoretic mobility, was 2.8×10^{6}. These data suggest that SFV double-stranded RNA consists of three species, with molecular weights 8.0×10^{6}, 5.6×10^{6} and 3.6×10^{6}.

However, in addition to the three major species in the DMSO-treated double-stranded RNA, two minor peaks were observed, whose electrophoretic mobilities corresponded to SFV 33S and 38S RNA. Also, the proportion of the 42S peak to the two other major peaks was less than the proportion of the largest SFV double-stranded RNA species to the other two double-stranded species (c.f. Fig. 14), which suggests that some degradation has occurred.

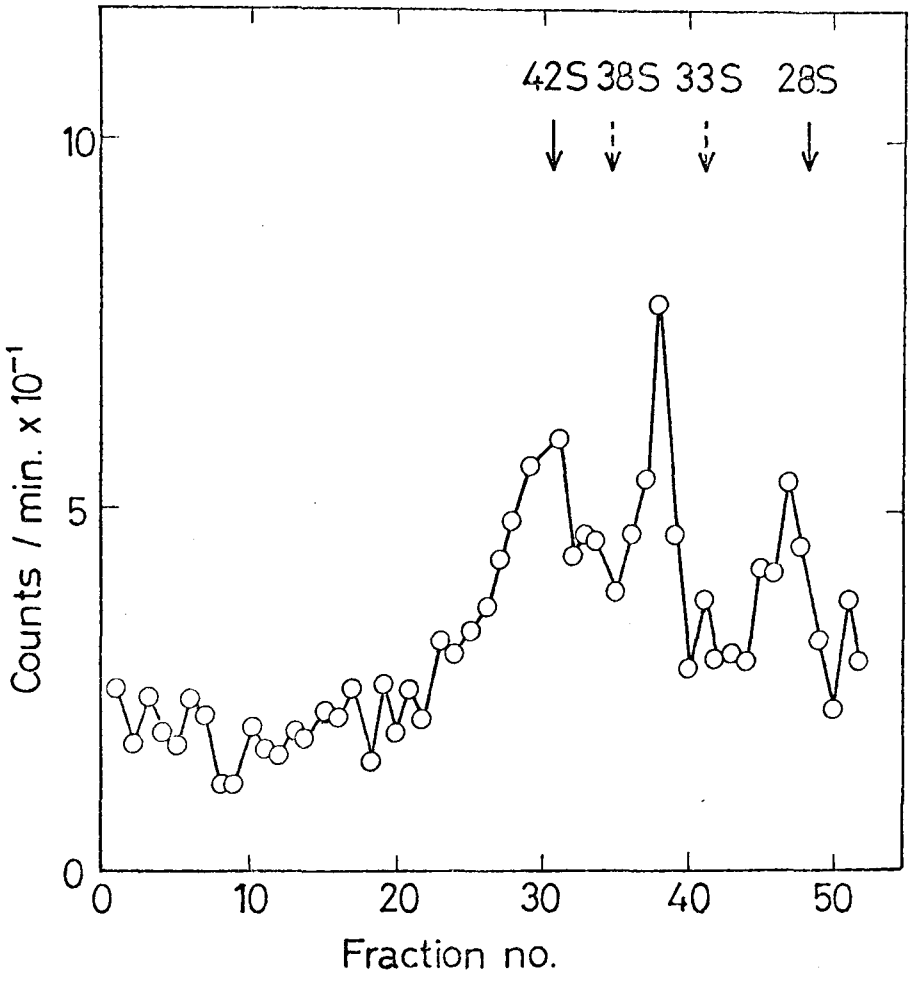


Fig. 24. Polyacrylamide gel electrophoresis of the single-stranded product obtained by separating the strands of SFV double-stranded RNA. SFV-infected chick cells were labelled with ^{3}H-uridine $2\frac{1}{2}$ to 3 hours after infection, and RNA was extracted using phenol and SDS. Single-stranded and multi-stranded RNA were precipitated with 1M-NaCl, and double-stranded RNA was recovered from the supernatant by precipitation with ethanol. The ethanol precipitate was redissolved in TE buffer, and treated with DMSO (6 volumes DMSO, 37°C, 15 min.). The solution was cooled quickly in ice, and RNA was recovered by precipitation with ethanol, after adding 0.1 volumes of 10 x TNE buffer. The RNA was electrophoresed on 2.0% polyacrylamide, 0.5% agarose gel, at 100V. for $6\frac{1}{2}$ hours. Unlabelled chick cell RNA was coelectrophoresed as a marker, and the position of 28S ribosomal RNA was detected optically. SFV 42S RNA was electrophoresed on a gel run in parallel. The positions of the 38S and 33S RNA species were those expected from the mobilities of the 42S and 28S species.

DISCUSSION

DISCUSSION

I. Introduction

The subject matter of this thesis is the replication of viral RNA in cells infected with Semliki Forest virus. In order to be able to study this problem easily, the experimental system must fulfil certain requirements. First, the virus must grow rapidly and reproducibly. This requirement is satisfied by the SFV-chick cell system. Second, it is necessary that cellular and virus-directed RNA synthesis can be readily distinguished. This can be done by using actinomycin D, which inhibits cellular RNA synthesis, and it follows that virus-directed RNA synthesis must be unaffected by this inhibitor. Many observations have shown that actinomycin D inhibits cellular RNA synthesis by more than 95%, but several groups have reported a continued, low rate of RNA synthesis in the presence of the inhibitor (Martin and Brown, 1967; Gandhi and Burke, 1970; Stern and Friedman, 1970). The product of this synthesis is a partially ribonuclease-resistant molecule, with a mean sedimentation coefficient between 7S and 12S, depending on the type of cell from which it is obtained, and it has a high electrophoretic mobility on polyacrylamide gel. However, in the present study, the inclusion of suitable controls made it possible to eliminate this actinomycin-resistant RNA synthesis as a source of error (Results section I 1).

It is also important that actinomycin D does not affect virus-directed RNA synthesis. However, since the virus yield was actually

increased in the presence of actinomycin D (Taylor, 1965), an effect on viral RNA synthesis is unlikely.

Having established a suitable system in which to study virus-directed RNA synthesis, it was necessary to develop separation procedures for the species of RNA which are found in the infected cell. Three fractionation techniques were employed: polyacrylamide gel electrophoresis, chromatography on CF11 cellulose columns, and precipitation of single-stranded RNA with high concentrations of salt.

Electrophoresis on low-concentration polyacrylamide gel containing agarose was found to be a useful analytical technique, by which four single-stranded, three double-stranded, and multi-stranded RNA species could be separated. These constituted all the RNA species which have been identified to date in cells infected with group A arboviruses (Sonnabend et al., 1967; Cartwright and Burke, 1970; Levin and Friedman, 1971; Stollar et al., 1972; Simmons and Strauss, 1972b). The molecular weights of the single-stranded species could be determined by their electrophoretic mobilities in this gel system.

Chromatography on CF11 cellulose columns was used in the present work to separate single-stranded RNA from double-stranded and multi-stranded RNA. Low-molecular-weight RNA, including all the cellular RNA synthesized in the presence of actinomycin D, was eluted with 35% ethanol, while single-stranded RNA larger than 18S ribosomal RNA was eluted with 15% ethanol (Results section I1 and II3). Double-stranded and multi-

stranded RNA were eluted together in buffer containing no ethanol.

In order to separate virus-specified double-stranded RNA from multi-stranded RNA, fractionation with high concentrations of salt was used, since double-stranded RNA was soluble in 1M-NaCl, whereas multi-stranded RNA was insoluble. Thus, by combining salt fractionation with CF11 cellulose chromatography, it was possible to separate single-stranded, double-stranded and multi-stranded species of RNA with very little manipulation.

II. Virus-specified RNA species in infected cells

1. Single-stranded RNA

Four species of single-stranded RNA specified by SFV were found in infected chick cells, which corresponded with the 42S, 38S, 33S and 26S species identified by Levin and Friedman (1971) in Sindbis virus-infected cells. The molecular weights of these species, determined by polyacrylamide gel electrophoresis, were 4.0×10^6, 3.1×10^6, 2.3×10^6 and 1.8×10^6. These values are close to those estimated by Levin and Friedman (1971), who also used polyacrylamide gel electrophoresis. However, the molecular weights of the 42S and 26S RNA species of Sindbis virus have also been estimated by centrifugation on sucrose gradients containing DMSO or formaldehyde, and by competition hybridization. The values obtained by these techniques were $4.3 \pm 0.3 \times 10^6$ for 42S RNA, and 1.6×10^6 for 26S RNA (Simmons and Strauss, 1972a). Thus, molecular weight values estimated for the 42S and 26S RNA species were

close to those determined by other physical techniques. The 42S RNA found in infected cells was of the same size as RNA extracted from virions. In addition, no single-stranded RNA was found in infected cells which was complementary to virion RNA, and so the 42S RNA species found in infected cells was probably virion RNA.

2. Multi-stranded RNA

The material which entered the first few gel fractions was characterized as multi-stranded RNA, and it could be purified by a combination of salt precipitation and chromatography on CF11 cellulose columns (Results section II3). The multi-stranded RNA had properties which would be expected of an intermediate in RNA synthesis. It was the RNA species most highly-labelled during exposure of infected cells to a radioactive RNA precursor for short times (Fig. 20); it was labelled early in infection, before labelled single-stranded RNA could be detected (Fig. 17); it was partially resistant to ribonuclease (Results section III 2), and the ribonuclease-resistant core had the same electrophoretic pattern as double-stranded RNA extracted from infected cells (Fig. 15). These properties are similar to those described for the replicative intermediates of RNA bacteriophages such as Qβ (Pace et al. 1967) and the picornavirus, poliovirus (Erikson et al. 1964; Baltimore and Girard, 1966).

Additional evidence for the SFV-specified multi-stranded RNA being an intermediate in RNA replication is as follows. During the

synthesis of single-stranded RNA in the infected cell, partially completed molecules must be present. Exposure of cells to a radioactive RNA precursor for a short period of time (less than that required for the synthesis of an RNA molecule) would thus result in most of the label being present in partially-completed or "nascent" molecules. The time to synthesize a molecule of Sindbis virus RNA has been estimated as about 1 minute (Simmons and Strauss, 1972). It was found in the present study that when SFV-infected cells were exposed to ^{3}H-uridine for 1 minute when viral RNA synthesis was at the maximum rate, most radioactivity was incorporated into multi-stranded RNA (Fig. 20). The evidence therefore suggests that nascent RNA molecules occur in a multi-stranded or double-stranded form. If this were not so, one would expect to find heterogeneous single-stranded RNA, corresponding to partially-completed viral RNA molecules, and no trace of these is found. Since multi-stranded RNA is partially single-stranded, this RNA probably contains the nascent molecules. Unfortunately, it has not proved possible to demonstrate the transfer of radioactivity from multi-stranded RNA to single-stranded RNA by a pulse-chase experiment in animal virus-infected cells, although this has been done in phage-infected cells (Fenwick et al. 1964). Pulse-chase experiments have been attempted both in animal virus-infected cells (Cartwright and Burke 1970) and with uninfected animal cells in culture (Warner et al. 1966), but without success. This difficulty is thought to be due to the relatively slow rate of equilibration of extracellular nucleotides with the immediate precursors of RNA (Skehel et al. 1967; Baltimore, 1969). However, pulse-chase experiments have been performed with partially-purified RNA polymerases

obtained from cells infected with mengovirus (Plagemann and Swim, 1968) and poliovirus (Girard, 1969), and it was demonstrated in both cases that a multi-stranded species is the precursor to single-stranded RNA in vitro. The evidence summarized above is sufficient to indicate that multi-stranded RNA is an intermediate in SFV-directed RNA synthesis, and it will hereafter be referred to as replicative intermediate, or RI.

In spite of the evidence for multi-stranded RNA being RI in virus-infected cells, there has been some controversy over whether or not the extensive hydrogen-bonded secondary structure of isolated multi-stranded and double-stranded RNA is an artefact of the extraction procedure (Borst and Weissman, 1965; Bishop et al. 1965; Weissman et al. 1968). It was suggested that extraction of RNA from cells with phenol or detergents facilitated the formation of hydrogen bonds between regions of RNA which had complementary nucleotide sequences. In favour of the proposal that hydrogen bonding occurred during extraction, Öberg and Philipson (1971) showed that when RNA was extracted from poliovirus-infected HeLa cells in the presence of DEPC, which prevents the formation of new hydrogen bonds, less multi-stranded and double-stranded RNA were found in the extract than when cells were extracted using phenol and SDS without DEPC. However, in the present study, extraction of SFV-infected chick cells in the presence or absence of DEPC made no difference to the composition of the extracted RNA (Fig. 22). This suggests, first, that RNA strands which are hydrogen-bonded after extraction are at least in close association in the cell; and second, that the relative proportions of RI, double-stranded RNA and single-stranded RNA in extracts of SFV-infected chick

cells are the same as their cellular counterparts, whatever the precise structure of the different forms of RNA in the cell.

3. Double-stranded RNA

The double-stranded RNA found in SFV-infected cells was characterized by its resistance to ribonuclease (Table 4) and by its being converted to a ribonuclease-sensitive form by denaturation with DMSO (Results section III 4). The double-stranded RNA could be purified by chromatography on CF11 cellulose columns and on the basis of its solubility in IM-NaCl (Results section II 3c). Three species of double-stranded RNA were found in SFV-infected chick cells by polyacrylamide gel electrophoresis, and for convenience these will be called DSI, DSII and DSIII, in order of decreasing size. Treatment of the double-stranded RNA with ribonuclease under conditions which completely digested single-stranded RNA to TCA-soluble fragments had no effect either on the electrophoretic mobilities or on the relative proportions of the three double-stranded RNA species (Results, section II 3c). The sizes of the species of double-stranded RNA, as estimated by separation of the strands using DMSO, and examination of the single-stranded product by polyacrylamide gel electrophoresis, were 8.0×10^6, 5.6×10^6 and 3.6×10^6 (Fig. 24). Using sucrose gradient centrifugation, Simmons and Strauss (1972b) have recently estimated that the molecular weights of the double-stranded species of RNA from Sindbis virus-infected BHK cells are 8.8×10^6, 5.6×10^6 and 2.9×10^6. They concluded that the largest and smallest of these are duplexes of the Sindbis virion RNA and Sindbis virus 26S RNA, respectively. These findings are consistent with the present work.

The existence of multiple species of double-stranded RNA in cells infected with group A arboviruses was first discovered by Levin and Friedman (1971), but these species were only partially resolved by their technique of polyacrylamide gel electrophoresis. However, Stollar et al. (1972) succeeded in demonstrating the existence of three species of double-stranded RNA in extracts of chick cells infected with Sindbis virus, using the technique of sucrose gradient centrifugation and an immunochemical binding assay for double-stranded RNA. The pattern of double-stranded RNA species found by these workers was very similar to the electrophoretic pattern of SFV-specified double-stranded RNA from chick cells, reported in the present work. Stollar et al. (1972b) also found that the proportions of the three double-stranded RNA species varied as a function of time, such that the ratio of DSI:DSII:DSIII was about 1 : 0.4 : 0.4 in the first half of the infection cycle (1 to 6 hours after infection), and about 1 : 0.6 : 0.6 in the second half (6 to 11 hours after infection) (Stollar et al. 1972). It was also found that the proportions of the double-stranded RNA species were different in chick, BHK and mosquito cells, infected with Sindbis virus. Only two species of double-stranded RNA were found in BHK and mosquito cells, and, in the latter, the larger species of RNA was predominant. In BHK cells, the ratio of the larger to the smaller species of double-stranded RNA varied from 1 : 0.5 in the first half of the infection cycle to 1 : 1.1 in the second half (Stollar et al. 1972). However, in these same experiments, the replication kinetics of Sindbis virus in BHK and chick cells were found to be almost identical (R.W. Schlesinger, 1972, personal communication).

Stollar et al. (1972) proposed three alternative explanations for the presence of different proportions of the double-stranded RNA species in different infected cells. First, that the different proportions of double-stranded RNA may be related to the different cytopathic effects of infection in different cells. This suggestion was prompted by the finding of Ehrenfeld and Hunt (1971) that poliovirus-specified double-stranded RNA was a potent inhibitor of protein synthesis in vitro. Second, that the different species of double-stranded RNA may represent stages in degradation of RI, which is known to turn over in poliovirus-infected cells (Baltimore, 1969). Third, that the multiple forms of double-stranded RNA might be a reflection of the presence of a different RI for each single-stranded species of RNA. These alternatives will be considered further in section III.

In the very recent paper by Simmons and Strauss (1972), it was reported that three forms of double-stranded RNA were found in Sindbis virus-infected chick and BHK cells, both by sucrose-gradient centrifugation and polyacrylamide gel electrophoresis. These workers also found that the proportions of the different species of double-stranded RNA varied during the infection cycle such that the ratio of labelling of DSI : DSII : DSIII varied from 1 : 0.45 : 0.22, early in the infection of chick cells with Sindbis virus, to 1 : 1.7 : 0.9 by 6 hours after infection. However, in the present study, the ratio of DSI : DSII : DSIII labelled in SFV-infected chick cells was about 1 : 0.4 : 0.2 by 6 hours after infection. Simmons and Strauss (1972b) also found that the double-stranded RNA's were labelled in different proportions after short

exposures of Sindbis virus infected BHK cells to ^{3}H-uridine. After a 1-minute pulse, the ratio of DSI : DSII : DSIII labelled was 1 : 0 : 2.2. However, in SFV-infected chick cells, a 1-minute pulse was found, in the present study, to label the three double-stranded RNA's in the proportions 1 : 0.27 : 0.13. DSI was never found to contain less than about twice the radioactivity in DSII or DSIII. These differences between the observations of Simmons and Strauss (1972b) and those made in the present study may be due either to the different viruses used in the two studies, or to the different extraction procedures used. Since Simmons and Strauss prepared double-stranded RNA by treatment of unfractionated virus RNA with ribonuclease, their preparations would contain a mixture of ribonuclease-resistant "cores" of RI, as well as the double-stranded RNA present in infected-cell extracts. In the present study, the ribonuclease-resistant cores of the RI extracted from infected cells at different times after infection were not examined. However, when chick cells were labelled with ^{3}H-uridine between 2 and 6 hours after infection with SFV, the RI extracted at this time yielded a ribonuclease-resistant core which contained three RNA species in identical proportions to those found in the double-stranded RNA extracted from infected cells. This suggests that the different results were not due to the different extraction procedures. Thus, the difference between the present data and that of Simmons and Strauss (1972b) appears to reside in the different viruses used.

The role of double-stranded RNA in virus replication has been studied in cells infected with poliovirus, and it has been found that the synthesis of double-stranded RNA follows that of single-stranded RNA and

RI in a way which suggests that it is a consequence rather than an intermediate of RNA replication (Baltimore and Girard, 1966; Noble and Levintow, 1970). However, in cells infected with group A arboviruses, the infecting genome appears to be converted to a double-stranded form as the first event in replication (Friedman et al. 1966; Pfefferkorn et al. 1967). In the present study, it was found that double-stranded RNA was labelled more rapidly than multi-stranded RNA both early in infection and during short exposure of infected cells to ^{3}H-uridine when viral RNA synthesis was at its maximum rate (Results sections II 5,6). Thus, it appears that double-stranded RNA is an intermediate in RNA synthesis in cells infected with group A arboviruses. In addition, the continued labelling of double-stranded RNA when viral RNA synthesis is at its maximum rate (Fig. 19) suggests that there is turnover of RI, as in poliovirus-infected cells (Baltimore, 1968), and that double-stranded RNA is probably also an end-product of RNA synthesis.

Further evidence for the involvement of double-stranded RNA in viral RNA synthesis is given by the finding in the present work that the product obtained by treatment of RI with ribonuclease was identical to the double-stranded RNA extracted from SFV-infected cells, when examined by polyacrylamide gel electrophoresis. Thus, RI and double-stranded RNA are structurally, although not necessarily functionally, related. Double-stranded RNA will for convenience be referred to as replicative form or RF, in common with the double-stranded RNA found in cells infected with other single-stranded-RNA viruses.

The relationship of RF to RI is also indicated by the pulse-labelling experiments, in which SFV-infected cells were exposed to ^{3}H-uridine for short periods (Results section II6). When infected cells were labelled for 1 minute, more radioactivity was found in RF than in single-stranded RNA. If all the RF were the product of turnover of RI, then the maximum amount of radioactivity that would be expected in RF is

$$\frac{2}{2 + N} \times S ,$$

where S is the radioactivity in single-stranded RNA, and N is the number of nascent molecules per RI. Assuming that the average length of a nascent molecule is one half the length of a completed molecule, then N is (2 x 4.2 =) 8.4, since the number of single-stranded RNA molecules per RI is about 4.2 (see Results section III3). Thus, even if the turnover time of RI were as low as the average time to synthesize a molecule of RNA (about 75 seconds; see Discussion section III 1), then the most radioactivity one would expect to find in RF is about 20% of that in single-stranded RNA. The turnover time of poliovirus RI is about 10 minutes (Baltimore, 1969), and if the turnover time of SFV RNA were similar, then only a few per cent of the radioactivity in single-stranded RNA would be expected in RF. However, after infected cells were pulse-labelled with ^{3}H-uridine for 1 minute, the radioactivity in RF was 130% of that in single-stranded RNA (Figs. 19, 20a). Therefore, the great majority of the radioactivity in RF after a 1-minute pulse is not derived by turnover of RI. Consistent with this was the observation that the radioactivity

in RF was greater than that in RI, early in infection (Fig. 17). Bearing in mind the structural relationship between RI and RF mentioned in the previous paragraph, it is probable that RF has an intermediate role in SFV RNA synthesis, as a precursor of RI.

III. Mechanism of virus RNA replication

1. Time taken to synthesize a molecule of virus RNA

It is possible, in principle, to estimate the time required to synthesize a virus RNA molecule (the "synthetic time") by studying the distribution of label between species of RNA after pulse-labelling infected cells with a radioactive RNA precursor. When RNA is being synthesized at a constant rate, all the nascent RNA molecules which are present in the cell are completed, and replaced by new nascent molecules, during one synthetic time. If the average length of a nascent molecule is half that of a completed molecule, then the number of nucleotides added to the nascent molecules completed in this time is the same as the number of nucleotides entering new nascent molecules. Therefore, assuming that extracellular uridine equilibrates instantaneously with intracellular uridine nucleotides, when infected cells are exposed to ^{3}H-uridine for one synthetic time, equal amounts of label will be present in nascent RNA molecules and in completed molecules. When SFV-infected cells were pulse-labelled with ^{3}H-uridine, equal amounts of label were found in single-stranded RNA and in RI after a little more than 2 minutes (Fig. 20a).

However, the assumption that extracellular uridine equilibrates with intracellular uridine nucleotides is not justified, for reasons discussed earlier (Discussion section II 2). In fact, the rate of labelling of RNA was found to increase 2-fold in a 2-minute pulse, compared with that in a 1-minute pulse, and another 2-fold by 5 minutes after exposure of infected cells to ^{3}H-uridine (Fig. 19). Since label appears in nascent molecules before completed molecules, the increased rate of labelling of RNA will be reflected in an increase in the relative amounts of radioactivity in RI and single-stranded RNA. For a given increase in the rate of labelling of RNA, the ratio of the radioactivity in nascent molecules : completed molecules will depend on the synthetic time; a longer synthetic time will result in a larger proportion of radioactivity in nascent molecules than a shorter synthetic time. The ratio of the radioactivity in nascent molecules : completed molecules can be estimated from Fig. 20a, if it is assumed that the RI contains all the nascent molecules. Since the overall rate of labelling of RNA is also known (Fig. 19a), the synthetic time for an RNA molecule can be estimated. The calculation is outlined in the Appendix. In this way, the synthetic time for an RNA molecule was found to be about 75 seconds. However, this value represents the average synthesis time for all single-stranded species of RNA. In order to estimate the synthetic times for the 26S and 42S RNA species to a first approximation, the contribution of the minor species will be discounted. Since the 42S RNA is about 2.3 times the size of the 26S RNA, and since the rate of labelling of 26S RNA is about 1.5 times that of 42S RNA (Fig. 20c), (2.3 x 1.5 =) 3.37 molecules of 26S RNA are synthesized per 42S molecule. The synthesis times of the 42S RNA (T_{42}) and the 26S RNA (T_{26}) may then be estimated

from the following equation:

$$\frac{3.37}{437} T_{26} + \frac{1}{4.37} T_{42} = T$$

If the rates of transcription are the same for both 26S RNA and 42S SNA, then

$$T_{26} = 0.45\ T_{42}$$

If the value of T is taken to be 75 seconds, then

$$\frac{3.37 + 2.25}{4.37} T_{26} = 75 \text{ seconds}$$

and $T_{26} = 58$ seconds

Thus, $T_{42} = 130$ seconds

Using a different method, Simmons and Strauss (1972b)have recently estimated that the synthesis time for Sindbis virus 26S RNA in BHK 21 cells was about 50 seconds. Thus, the rates of RNA synthesis of group A arboviruses in these different cell systems are comparable.

2. Mechanism of virus RNA replication

Three possible mechanisms of replication of virus RNA were considered in the Introduction section (pp. 22-23). These were, first, conservative replication, in which both strands of the template are conserved; semi-conservative replication, in which only the strand

complementary to the progeny strands is conserved; and non-conservative, in which first one strand of the template and then the other is alternately displaced by progeny strands.

No firm evidence for one of these mechanisms was found in the present study, but the one which is most in accord with the present data is the semi-conservative mechanism. The reasons for this conclusion are two-fold. First, the RI obtained from infected cells which had been pulse-labelled with ^{3}H-uridine for 1 minute was 70% ribonuclease-resistant (Results Section III 2). In semi-conservative or non-conservative replication, the newly-synthesized portions of the nascent strands would be expected to remain in close association with the complementary strand until they are displaced by elongation of the following strand. If replication were conservative, very little radioactivity would be expected to be ribonuclease-resistant after labelling for a short period (less than the time required to synthesize a molecule of RNA). This evidence is not conclusive, because it is possible that the newly-synthesized portions of the nascent strands are hydrogen-bonded to the template, and are therefore ribonuclease-resistant. However, Hayashi (1965) found that, during the synthesis of an RNA molecule on a double-stranded DNA template, which is a conservative process, the length of a nascent RNA molecule which was hydrogen-bonded to the template was about 80 nucleotides long. If the replication of SFV RNA were conservative, the number of nucleotides, x, hydrogen-bonded to the template, can be estimated from the ribonuclease-resistance, R, of pulse-labelled RI.

If there are 12,000 nucleotides in a molecule of 42S RNA, and the template for its synthesis is the same length, and assuming that the average length of a nascent strand is $\frac{1}{2}$ that of a completed RNA molecule, then

$$R = \frac{\text{proportion of radioactivity in hydrogen-bonded form}}{\text{total radioactivity}}$$

Since the synthetic time for a molecule of viral RNA is about 1 minute (Discussion Section III 1), approximately all the nascent molecules will be labelled in 1 minute, but not the template strand. Thus, the proportion of single-stranded RNA labelled will be approximately $\frac{1}{2}$ n, where n is the number of nascent strands on the RI. Making an approximate correction for the increased rate of labelling in this time (about 1.4 times; see Appendix), the proportion of radioactivity in the nascent strands will be $\frac{1}{2} \times \frac{1 \text{ n}}{1.4}$

Thus,

$$R = \frac{100 \times \frac{nx}{12000}}{\frac{nx}{12000} + \frac{1}{2}\frac{n}{1.4}}$$

The value of R for R1 pulse-labelled for 1 minute is 70%, from which x is found to be 10,000. If no correction is made for the increased rate of labelling, x becomes 14,000. Thus, nearly all of the complementary

strand would be hydrogen-bonded to the nascent strand. But if the length of nascent strand hydrogen-bonded to template in conservative replication of RNA were similar to that found by Hayashi for the synthesis of RNA on a DNA template, or about 80 nucleotides, this would correspond to about 125 nascent strands per template.

The number of nascent strands estimated in the present study was 8.4 (Discussion, p. 79), which argues against the conservative mechanism of replication for SFV RNA.

The second piece of evidence discounts the non-conservative mode of replication in the synthesis of SFV RNA. Since each strand of the template is displaced alternately in this mechanism of synthesis, the product would consist of equal proportions of RNA of complementary nucleotide sequence. However, no RNA complementary to SFV virion RNA was found in single-stranded RNA extracted from infected cells (Results Section III 1). Thus, by elimination, most of the viral RNA synthesis in SFV-infected cells is semi-conservative. However, the possibility that small amounts (about 1%) of either conservative or non-conservative replication occur cannot be eliminated at present.

3. Model for the replication of Semliki Forest virus RNA

Any model for the replication of SFV RNA must take account of the presence of multiple forms of single-stranded RNA and RF in SFV-infected chick cells. The simplest way to do this is to postulate that all the single-stranded forms of SFV RNA can be synthesized using one template. The model proposed in this study is shown in Fig. 25. After a description of the model, the reasons for believing that it may operate in SFV RNA replication will be described.

The features of this model are as follows. Synthesis of all four single-stranded species of RNA takes place on one RI, which will be called 42S RI. The synthesis of 42S RNA is initiated at site A, under the direction of a protein which normally recognizes site D as a termination signal, but occasionally recognizes site C for termination. The synthesis of 26S RNA is initiated at site B, and the protein which controls this, normally recognizes site C as a termination signal, but occasionally "reads through" to site D. According to this model, the relative amounts of the single-stranded RNA species are controlled by the relative efficiencies of initiation at sites A and B, and of termination at sites C and D. The origin of the 33S and 38S minor species of single-stranded RNA may be explained as mistakes in the synthesis of 42S and 26S RNA, such that a molecule whose synthesis is initiated at A is prematurely terminated at C; and one whose synthesis is initiated at B is not terminated at C, but synthesis continues to D. In Fig. 25, 33S is shown as region AC, and 38S as region DB, but this choice is arbitrary.

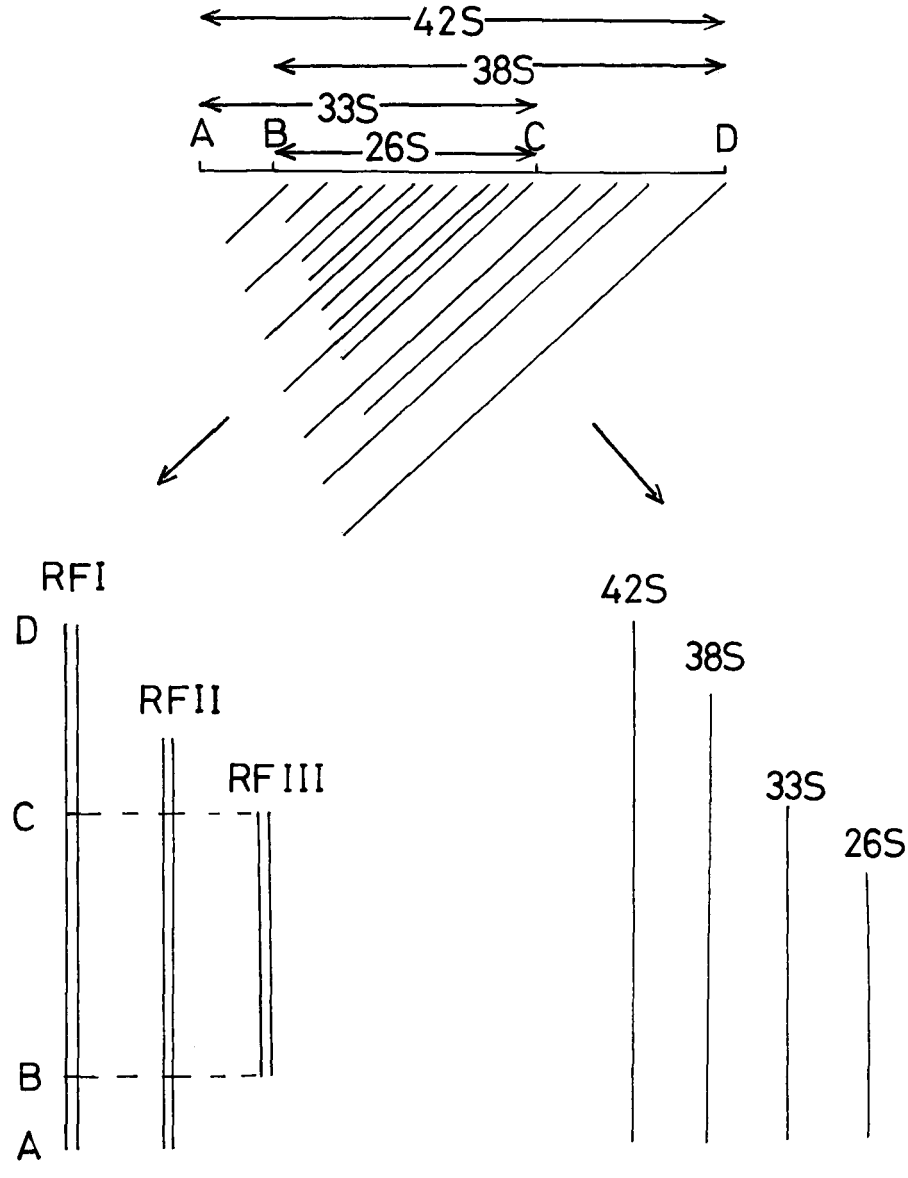


Fig. 25. Proposed model for the replication of SFV RNA. A and B represent initiation sites for RNA synthesis on the template strand, and C and D represent termination sites. Diagonal lines represent nascent molecules of RNA.

The origins of the double-stranded species of viral RNA will now be considered. DSI, DSII and DSIII will be called RFI, RFII and RFIII, according to the nomenclature of Simmons and Strauss, 1972b. RFI is easily accounted for, both as the precursor and the product of the 42S RI. RFIII, the smallest RF corresponds in length to 26S RNA, and it may be formed either as the precursor and the product of a 26S RI, or by degradation of the 42S RI. The presence of a 26S RI is considered unlikely, in view of the inability of Simmons and Strauss (1972b) to resolve different species of RI by sucrose gradient centrifugation. Thus, RFIII probably arises by degradation of the 42S RI. RFII cannot arise from a single-stranded species of corresponding length, since no single-stranded RNA species of molecular weight 2.8×10^6, which would probably be derived from RFII, was found free in the infected cell. Thus, RFII probably arises by degradation of the 42S RI.

The predictions of this model will now be compared with the data obtained in this study. If the majority of the RFI labelled between 2 and 6 hours after infection results from the turnover of the 42S RI, then a proportion of these RF's will be expected to have breaks in the plus-strand at points B and C (Fig. 25), depending on the RNA species whose synthesis was the last to be initiated. If the last molecule to be synthesized were 26S RNA, then breaks would be expected at both B and C. If the last molecule were 33S or 38S RNA, one break at either B or C would be expected. However, if 42S RNA were the last molecule to be

synthesized, no breaks would be expected in the plus-strand. The proportion of RFI containing these single-strand breaks can be estimated from the proportions of the single-stranded RNA species in the infected cell. Since the ratios of 26S : 33S : 38S : 42S RNA, labelled between 2 and 6 hours after infection, were approximately 1.5 : 0.25 : 0.25 : 1.0 (Fig. 22), then the relative rates of initiation of synthesis of the RNA species were in the same proportions. Thus, single-stranded breaks would be expected in (1.5 + 3 =) 50% of RFI's, at both B and C, in the plus-strand. Since 33S and 38S RNA comprise about 10% each of the labelled single-stranded RNA, approximately 10% of the RFI's will have one break at B, and about 10% will have one break at C. If the strands of an RFI are separated, the proportions of label expected in single-stranded RNA's of different sizes can be estimated, and these are shown in Table 10. Similar calculations may be made for RFII and RFIII. If RFII is formed by degradation of 42S RI, it will contain the same proportion of breaks at B and C as RFI. Since RFIII probably contains no internal initiation or termination signals according to this model of RNA synthesis, no single-strand breaks are expected in RFIII, and only 26S RNA would be expected upon strand-separation of RFIII (Table 10). The ratio of labelling of RFI : RFII : RFIII was 1 : 0.4 : 0.2 (Discussion, p. 74). From the molecular weights of the RF's (Results section III 4), the molar ratio of RFI : RFII : RFIII were estimated to be about 1 : 0.6 : 0.6. Thus, the proportions of different-sized products which would be expected upon strand-separation of total RF could be estimated, and compared with the proportions obtained experimentally (Fig. 24). The comparison is shown in Table 10 and it can be seen that the observed and expected ratios

Table 10. Ratios of radioactivity expected in RNA species of different size after strand separation of double-stranded RNA

RNA species (Mol.wt. x 10^{-6})	4.0	3.1	2.8	2.3	1.8
From RF I (minus strand)*	1	0	0	0	0
(plus strand)	0.3 x 1	0.1 x $\frac{3.1}{4}$	0	0.1 x $\frac{2.3}{4}$	0.5 x $\frac{1.8}{4}$
	1.30	0.08	0	0.06	0.23 (I)
From RF II (minus strand)	0	0	1	0	0
(plus strand)	0	0	0.4 x $\frac{2.8}{4}$	0.1 x $\frac{2.3}{4}$	0.5 x $\frac{1.8}{4}$
	0	0	1.28	0.06	0.23
+ x 0.6	0	0	0.77	0.04	0.14 (II)
From RF III (minus strand)	0	0	0	0	1 x $\frac{1.8}{4}$
(plus strand)	0	0	0	0	1 x $\frac{1.8}{4}$
	0	0	0	0	0.90
+ x 0.6	0	0	0	0	0.54 (III)
Total (I) + (II) + (III)	1.3	0.08	0.77	0.10	0.91
Ratios of RNA species :	1	: 0.06	: 0.59	: 0.08	: 0.70
Observed ratio :	1	: 0.08	: 0.67	: 0.08	: 0.64

Radioactivity in different species was estimated by making the following assumptions:

(i) that no breaks occurred in minus strands;

(ii) that 50% of RFI had 2 single-stranded breaks, at sites B & C on the plus-strand (Fig. 25);

(iii) that 10% of RFI had 1 single-strand break at site B and 10% had 1 break at site C;

Table 10 (continued)

(iv) that RFII had the same proportion of breaks as RFI;

(v) that RFIII had no breaks

* proportions of radioactivity in different species were estimated with respect to 42S RNA

\+ the proportions of radioactivity in products From RFII and RFIII were multiplied by 0.6, because molar ratio of RFI : RFII : RFIII = 1 : 0.6 : 0.6.

are quite close.

Simmons and Strauss (1972b) have very recently proposed a model for the replication of Sindbis virus RNA in BHK21 cells, and this model is similar in some respects to the model proposed in the present study. The major differences between the models are, first, that Simmons and Strauss proposed that the synthesis of 42S and 26S RNA take place on different RI's, one of which is the template only for 42S RNA synthesis, and the other of which produces 26S RNA and an RNA species with a molecular weight of 2.8×10^6; and second, in the model of Simmons and Strauss, RFII and RFIII are produced from the RI which synthesizes 26S, by the action of ribonuclease at a susceptible site. The model of Simmons and Strauss is compared with the one proposed in the present study, in the following discussion.

First, the three RF's found in extracts of SFV-infected chick cells occur in different proportions. Therefore, if 42S and 26S RNA are only made on different templates, as in the model of Simmons and Strauss, control is required for the synthesis of different amounts of the templates, and/or for the relative amounts of the different species of RNA which are made on these templates.

The first molecule of 26S RNA to be synthesized in the infected cell must be synthesized on a template of the length of 42S RNA, corresponding to RFI, unless 42S RNA splits into smaller pieces in vivo. However, the

work of Arif and Faulkner (1972) and of Simmons and Strauss (1972a) suggests that the 42S RNA of Sindbis virus is a continuous polynucleotide. Therefore, splitting of the 42S RNA in the cell is unlikely, because this would require specific and controlled enzymic digestion of the 42S molecule. Since at least one molecule of 26S RNA is probably synthesized on an RFI template, there is no reason why all the 26S RNA cannot be synthesized in the same way.

In the second place, according to the model of Simmons and Strauss, RFII and RFIII are obtained from the RI which is the template for 26S RNA synthesis, by intracellular degradation. But the molar ratio of RFI to either RFII or RFIII is 1 : 0.6, so that if 42S RNA is synthesized on a template corresponding to RFI, and 26S RNA is synthesized on a different template, corresponding to RFIII, as proposed by Simmons and Strauss, then the relative rates of initiation of 26S and 42S RNA synthesis would be (1.5 + 0.6 =) 2.5 : 1. However, the relative rates of initiation according to the model proposed here are 1.5 : 1.

A problem which is not readily solved by the model of Simmons and Strauss is the origin of the 33S and 38S single-stranded RNA species, which were identified by Levin and Friedman (1971), and observed in the present work. However, the model of RNA replication proposed in this study accounts for these minor RNA species very easily, as mistakes in initiation and termination of RNA synthesis.

Another problem that is difficult to solve using the Simmons and Strauss model is that of the composition of the single-stranded product

obtained by denaturation of total RF. According to the model of Simmons and Strauss, none of the RF's should contain single-strand breaks, since the different RF's are produced by cleavage at all the points where these breaks occurred, on the RI which synthesizes 26S RNA. Thus, one can estimate the proportions of the single-stranded RNA species that should be obtained upon strand-separation. RFI should yield only 42S RNA, RFII should yield only RNA of molecular weight 2.8×10^6 (2.8×10^6 RNA) and RFIII should yield only 26S RNA. Then, the ratio of radioactivity in 42S RNA : 2.8×10^6 RNA : 26S RNA should be:

$$1 : 0.6 \times \frac{2.8}{4.0} : 0.6 \times \frac{1.8}{4.0}$$

$$= 1 : 0.42 : 0.27$$

But the ratio of radioactivity in 42S RNA : 2.8×10^6 RNA : 26S RNA found experimentally was 1 : 0.68 : 0.65, which is not compatible with the predicted ratio.

There are several other ways in which the two models for RNA synthesis can be distinguished. First, according to the model of Simmons and Strauss, synthesis of 42S RNA and 26S RNA is initiated at the same point, at one end of the template. However, in the model proposed in this study, the synthesis of 26S RNA is initiated internally. Work is currently in progress to locate the 26S RNA sequence on 42S RNA (S.I.T. Kennedy, personal communication).

Second, the products expected on separation of the strands of the individual RF's are different for the two models. Therefore, the two models could be distinguished by examining the single-stranded components of the separated RF's.

Third, according to the model proposed in this study, most of the label in the 42S RNA obtained from RF should be in minus-strand RNA, because of the breaks in the plus-strand of RFI. The actual proportion expected is 1/1.32, or 75% (see Table 10). But, according to the Simmons and Strauss model, both the strands of RFI are intact, and therefore the proportion of minus-strand 42S RNA expected is 50%.

Fourth, according to the model proposed in this study, 26S plus-strand RNA should be complementary to the minus-strands of RFI, RFII and RFIII. However, according to the Simmons and Strauss model, 26S plus-strand RNA should be complementary to minus-strand RNA from RFI and RFIII, but not from RFII. Thus, it is possible to perform experiments which will distinguish between the two models of RNA synthesis.

In conclusion, the evidence presented here supports the inclusion of the group A arboviruses in animal virus class IV (Baltimore, 1971), which also includes the picornaviruses. Viruses in class IV have a genome of infectious, single-stranded RNA, and produce messenger RNA of the same polarity as the genome (i.e. plus-strand RNA). However, the replication of SFV appears to be more complex than that of other representatives of class IV, insofar as replication involves the production of multiple

messenger RNA's. In addition to the virion RNA itself, there appear to be two messenger RNA species in SFV-infected chick cells (Kennedy, 1972). Therefore, control is required for the synthesis of products of different size from one template. Arboviruses share this characteristic with members of animal virus class V, which includes the paramyxoviruses, the rhabdoviruses and the myxoviruses. For example, vesicular stomatitis virus (VSV), a rhabdovirus, produces multiple messenger RNA's in the infected cell (Huang et al. 1970), which are complementary to parts of the virion RNA. Yet an RNA complementary to the whole of the virion RNA must also be synthesized, as the template for replication.

A similar control problem thus exists in cells infected with VSV and SFV, namely the production of messenger RNA's smaller than the complete genome as well as replication of the complete genome. The model of RNA synthesis described in this study provides, in principle at least, a solution to this problem. It thus appears that SFV, and probably other group A arboviruses, represent a group which is transitional between class IV and class V viruses.

APPENDIX

APPENDIX

Estimation of the time required to synthesize a molecule of viral RNA

As explained earlier (Discussion, Section III 1), if RNA is being synthesized and labelled at a constant rate, the label in nascent RNA molecules (RI) after one synthetic time equals that in completed molecules (single-stranded RNA). However, the rate of labelling of RNA increased during the first few minutes after addition of ^{3}H-uridine to infected cells, even though RNA synthesis was at a constant rate by this time after infection (Results section II 6). This increase in the rate of labelling will result in a greater proportion of label in nascent than in progeny RNA strands, and the ratio of label in RI:single-stranded RNA at different times after addition of the isotope will depend on the synthetic time (Discussion Section III 1). The ratios of label in RI : single-stranded RNA at different times after addition of ^{3}H-uridine, and the increases in rate over corresponding time intervals, are shown in Table 11.

The correction for the increase in rate of labelling of RNA was made by estimating the ratio of label in RI : single-stranded RNA for different theoretical values of the synthetic time. As an illustration, the calculation is shown for a synthetic time of 2 minutes. If the synthetic time is 2 minutes, then in 0.5 minutes ($\frac{1}{4}$ of a synthetic time) the nascent strands will have extended by $\frac{1}{4}$ the length of a completed molecule, and $\frac{1}{4}$ of the nascent molecules originally present will have

Table 11a. Ratios of radioactivity in RI and single-stranded RNA, and increased rate of labelling after short exposure of SFV-infected cells to ^{3}H-uridine

Time after addition of ^{3}H-uridine (min)	RI:SS	Rate of labelling of RNA (cpm/min.)	Increase in rate of labelling of RNA	
			Incremental	Cumulative
0	-	330	-	-
0.5	2.0 : 1	450	1.36	1.36
1.0	1.6 : 1	600	1.33	1.81
1.5	1.3 : 1	875	1.46	2.64
2.0	1.1 : 1	1100	1.26	3.3

SFV-infected cells were pulse-labelled 5 hours after infection (Results Section II 6). Rates of labelling were estimated from Fig. 21a.

Table 11b. Proportions of radioactivity expected in RI and single-stranded RNA after short periods of labelling, estimated for different synthetic times

Synthetic time (min.)	Radioactivity in RI/SS expected at different times after addition of isotope			
	½ min.	1 min.	1½ min.	2 min.
1	3.4	1.2	0.8	-
1.25	4.5	1.8	1.0	-
1.3	4.8	2.0	1.1	-
1.5	5.5	2.3	1.4	1.0
2.0	7.7	3.5	2.2	1.4

Proportions of radioactivity in RI and SS RNA were estimated from Fig. 26.

been displaced (Fig. 26). The label in the nascent molecules can be estimated to a first approximation by considering the increase in the rate of labelling over increments of time. Thus, over the first 0.25 min., the average rate of labelling is taken to be 1, and over the second 0.25 min., the average rate of labelling is 1.4. A total of $\frac{1}{2}$ the nascent molecules will be labelled in $\frac{1}{4}$ of a synthetic time, and $\frac{1}{8}$ of this label will be displaced into completed molecules (Fig. 26). If the amount of label in RI, when nascent molecules are completely labelled at the initial rate, is called unity, then in 0.5 min. the label in the nascent strands is

$$\frac{15}{64} \times 1.4 + \frac{13}{64} \times 1 \text{ units}$$

and the label in completed molecules is

$$\frac{1}{64} \times 1.4 + \frac{3}{64} \times 1$$

Thus, the ratio of the label in Rl : SS is

$$\frac{34}{64} : \frac{4.4}{64} = 7.7 : 1$$

Similarly, during the next 0.5 min., the rate of RNA synthesis increases to 1.8 times the initial rate (Table lla), and the total labelling time is now $\frac{1}{2}$ of a synthetic time (Fig. 26). The ratio of RI : single-stranded RNA after this period is

$$69.6 : 20 = 3.5 : 1$$

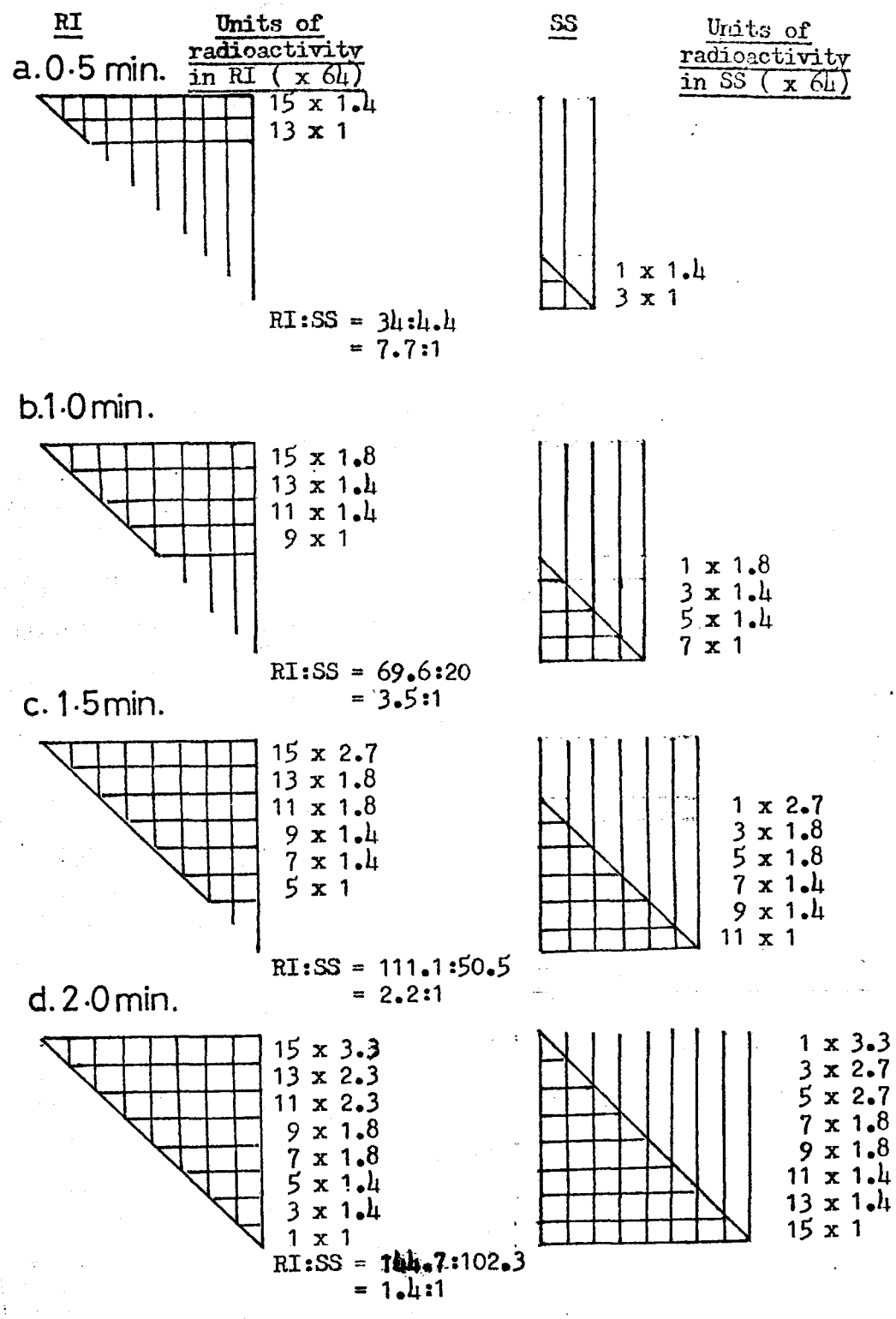


Fig. 26. Ratios of radioactivity expected in RI:SS after short periods of labelling, estimated for a synthetic time of 2 min. Calculations were made using the data in Table IIa. (a) $\frac{1}{2}$ min., (b) 1 min., (c) $1\frac{1}{2}$ min., (d) 2 min.

The ratios after 1.5 min. and 2 min. will be

$$111.1 : 50.5 = 2.2 : 1$$

and

$$144.2 : 102.3 = 1.4 : 1$$

Similar calculations were performed for theoretical synthetic times of 1, 1.25, 1.3 and 1.5 min. The ratios of RI : single-stranded RNA expected at different times after addition of the isotope are shown in Table 11b. If new templates are synthesized from the pool of newly-labelled single-stranded RNA, then, after the time required to synthesize a new RI, the label in RI will be greater than was estimated from the overall rate of labelling. Thus, the ratio of RI : single-stranded RNA does not decrease as rapidly as expected, with time. Taking this into account, the synthetic time is certainly less than 1.5 minutes, and is probably about 1.25 minutes, or 75 seconds.

SUMMARY

SUMMARY

1. RNA synthesis was studied in chick cells infected with SFV, to determine the sizes and the structures of the RNA species made, and the mechanism of replication of SFV RNA.

2. Four single-stranded (SS), three double-stranded (DS) and multi-stranded (MS) species of RNA were detected in infected cells. Methods were described for their separation.

3. The molecular weights of the SS species of RNA were 4.0×10^6, 3.1×10^6, 2.3×10^6 and 1.8×10^6, the largest of which was virion RNA. The molecular weights of the three DS RNA species, determined by separation of the strands of the DS RNA and analysis of the product, were 8.0×10^6, 5.6×10^6, and 3.6×10^6.

4. Viral RNA synthesis was detected within $\frac{1}{2}$ hour after infection, and was logarithmic for about 3 hours. The rate of RNA synthesis was linear for the next 5 hours, and then decreased rapidly.

5. DS and MS RNA were the first viral species detectable, at 1 hour after infection, No SS RNA was detected up to $1\frac{1}{2}$ hours after infection, when 42S RNA was detected.

6. During the linear phase of RNA synthesis, all species of RNA could be detected after labelling infected cells for 1 minute. The ratios of labelling of the SS species of RNA were the same for pulses varying between 1 minute and 70 minutes. More label was detected in DS and MS RNA in pulses up to 2 minutes, but in pulses of 5 minutes or more, the SS RNA species were more highly labelled.

7. No alteration of the pattern of viral RNA species was seen when RNA was extracted in the presence of DEPC, suggesting that no major alterations in the structure of RNA were taking place during extraction. It was concluded that DS and MS RNA represent viral RF and RI, respectively.

8. The synthetic times for 26S and 42S RNA molecules were estimated to be 58 seconds and 130 seconds, respectively.

9. The evidence suggests that the mechanism of SFV RNA replication is largely semi-conservative. A model for the replication of SFV RNA is described, which involves the synthesis of all the species of SS RNA on one template, corresponding in length to 42S RNA.

10. No SS RNA complementary to virion RNA was detected in infected cells, suggesting that viral messenger RNA was of the same polarity as virion RNA.

11. Thus, SFV appears to belong to animal virus class IV, although it shares some features with class V viruses.

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ABBREVIATIONS

ABBREVIATIONS

BHK	baby hamster kidney
BSA	bovine serum albumin
CEF	chick embryo fibroblast
CPV	cytopathic vacuoles
DEAE	diethylaminoethyl
DEPC	diethylpyrocarbonate
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DS	double-stranded RNA
EDTA	ethylene diamine tetraacetate, disodium salt
EMC	encephalomyocarditis virus
HA	haemagglutinating activity
m.o.i.	multiplicity of infection
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
p.f.u.	plaque-forming units
poly C	polycytidylic acid
poly G	polyguanylic acid
P.O.P.O.P.	1,4 - di(2-(5-phenyloxazolyl))-benzene
P.P.O.	2,5 - diphenyloxazole
RF	replicative form
RI	replicative intermediate
RNA	ribonucleic acid

RNase	ribonuclease
SDS	sodium dodecyl sulphate
SFV	Semliki Forest virus
SS	single-stranded RNA
SSC	standard saline citrate
TCA	trichloroacetic acid
TE	tris-EDTA buffer
TN	tris-NaCl buffer
TNE	tris-NaCl-EDTA buffer
TNES	tris-NaCl-EDTA-SDS buffer
TNM	tris-NaCl-$MgCl_2$ buffer
tris	tris (hydroxymethyl) amino methane
VSV	vesicular stomatitis virus
WEE	Western equine encephalitis virus