Studies on the formation and function of SFV proteins

by

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BROKEN TEXT AND SOME POOR QUALITY IMAGES IN ORGINAL THESIS.

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INDEX

Results

INTRODUCTION

Semliki Forest virus (SFV) is a group A togavirus that was first isolated from mosquitoes in Uganda (Smithburn and Haddow, 1944). Since then, two further isolations, both from mosquitoes, have been reported (Casals and Clarke, 1965).

The togaviruses are enveloped RNA viruses that can multiply in both vertebrates and arthropods. Previously togaviruses (toga means envelope) were classed as arboviruses, (arbo stands for arthropod borne). The name was changed because the arbovirus group included viruses that had different structures and that replicated in many diverse ways. The togavirus group contains about 200 viruses and has been subdivided, on the basis of antigenic relationships, into 3 major subgroups designated A, B and C, and several smaller subgroups (Casals, 1962). Earlier studies further subdivided them by their susceptibility to inactivation by lipid solvents, in particular ether (Andrewes and Horstmann, 1949), and the detergent sodium deoxycholate (Theiler, 1957).

Different group A togaviruses, sometimes called alpha-viruses, have similar morphological and physico-chemical properties as well as a marked similarity in their mode of replication. As well as SFV, other group A togaviruses that have been extensively studied include Sindbis, Chikungunya, Mayaro viruses and Eastern, Western and Venezuelan equine encephalitis viruses. Data obtained from studies on these closely related viruses is therefore relevant to SFV.

SFV possesses a number of properties which make it suitable for biochemical study, in particular its wide host range. Also SFV has not been associated with any known illness in man, although antibodies which neutralise it have been found in the sera of some human beings. It can be grown easily in a wide variety of laboratory host systems, including chick embryo (Taylor 1952), mouse brain (Cheng, 1961), mosquitoes (Woodall

Subsequently the RNA$^-$ mutants of Sindbis Virus were reallocated between three complementation groups, instead of two (Marcus and Zuckerbraun, 1970).

and Bertram, 1959) and several types of vertebrates and arthropod cells in culture (Henderson and Taylor, 1960). In these systems, SFV has a relatively rapid growth cycle; for example about 8 hours in chick embryo cells (Taylor, 1965). The virus can be assayed by plaque formation (Miles and Austin, 1963) and by haemagglutination of red blood cells either from geese or from 24 hour old chicks.

SFV is relatively resistant to nucleases and proteases (Cheng, 1958). Thus inactivation from this source during growth and purification is not a serious problem. The subsequent discussion of the properties and replication of SFV will suggest that it is a relatively simple virus. Therefore it provides a simple model system for the formation and function of lipoprotein membranes (Strauss, Burge, Pfefferkorn and Darnell, 1968). The synthesis of the viral RNA and proteins provides a useful model for the control of the formation of these macromolecules in eucaryotic cells.

Temperature sensitive (conditional lethal) mutants of SFV (Tan, Sambrook and Bellet, 1969), Sindbis (Burge and Pfefferkorn, 1966a) and Eastern equine encephalitis virus (Zebovitz and Brown, 1970) have been isolated. The mutants of Sindbis virus have been assigned to five complementation groups (Burge and Pfefferkorn, 1966b). Burge and Pfefferkorn (1967) showed that two of these complementation groups contain viruses that do not synthesise RNA at the restrictive temperature (RNA^--mutants), while the viruses in the remaining three do synthesise RNA (RNA^+-mutants). The mutants of SFV have also been divided into RNA^+ - and RNA^- classes both by Lomniczi and Burke (1970) and Tan et al, (1969).

1) Virus Structure

Electronmicroscopic studies indicate that the shape of SFV is spherical, with a diameter of 60-80nm including a border of radial

projections 11nm long (Acheson and Tamm, 1967). The internal structure of the virus particles was shown to consist of a nucleoid 28nm in diameter, separated by a narrow space from a membrane 7.5nm thick. These values are similar to the values reported by Faulkner and Mcgee-Russell (1968) and by Simpson and Hauser (1968a). It has been suggested that the nucleoid possesses cubical symmetry (Osterreith, 1968; Horzinek and Mussgay, 1969); however, there is still some disagreement on this point (Simpson and Hauser, 1968b), because of the difficulty in interpreting electronmicrographs.

The radial electron density distribution in Sindbis virus particles was determined by Harrison, David, Jumblatt and Darnell (1971) from measurements of spherically averaged X-ray diffraction. These authors suggested that the lipids are organised in a bilayer, with the polar groups at 21nm and 25.8nm from the centre. From this proposal and the known lipid composition of Sindbis virus (David, 1971), it was deduced that there is enough lipid present to form a bilayer around the core. These authors then calculated that less than 10% of the area at radii between 21 and 25.8nm is occupied by components other than lipid. Therefore protein probably does not form a bridge across the bilayer. The relatively low electron density of the outer region shows that there is considerable solvent interpenetrating the outer protein or carbohydrate.

The sedimentation coefficient of Semliki Forest virus in the ultracentrifuge is about 350S (Cheng, 1961; Kennedy, 1973). The sedimentation coefficient of the core particle has been estimated by Kennedy (1973) to be 151S confirming earlier results obtained by centrifugation on a sucrose density gradient (Friedman and Berezesky, 1967).

2) Chemical composition of virus particles.

Similar results have been obtained on the chemical composition of two group A togaviruses, Venezuelan equine encephalitis virus (Johnson and Wechter, 1962) and Sindbis virus (Pfefferkorn and Hunter, 1963). If it is assumed the analysis accounted quantitatively for all components, the composition of Sindbis virus is:- protein 65.6%,RNA 5.8% and lipid 28.6%. The lipid fraction is composed of 76.2% phospholipid and 24.8% cholesterol. Friedman and Pastan (1969) reported the lipid composition of SFV was sphingomyelin 26.5%, lecithin 34.0%, phosphatidylethanolamine 22.8%, phosphatidylinositol 15.0% and phosphatidylserine 0.8%. By comparison with membrane fractions derived from the host cell, it has been shown that the lipid composition of the plasma membrane fraction is closest to that of the virus, (David, 1971; Renkonen, Kaariainen, Simons & Gahmberg, 1971). Since the phospholipid component of Sindbis virus is derived from pre-existing host cell material (Pfefferkorn and Hunter, 1963b) it has been suggested that the virus acquires its phospholipid component when it buds through the plasma membrane (Acheson and Tamm, 1967), possibly at specific sites (David, 1971).

3) RNA of the virus particle

RNA extracted from purified SFV has a sedimentation coefficient of 42S (Sonnabend, Martin and Mecs, 1967), and a molecular weight, determined by polyacrylamide gel electrophoresis, of 4×10^6 (Levin and Friedman 1971). This RNA was shown to be single-stranded because of its sensitivity to ribonuclease, the dependence of its sedimentation rate on ionic strength and its precipitation by a high concentration of salt. The 42S virion RNA was reported by Svensalsan, Lockart, Dodson and Hartman (1968) and by Dobos and Faulkner (1970) to be converted by agents that break hydrogen

bonds into 26S fragments. Cartwright and Burke (1970) showed that 26S RNA was formed by heating RNA from virus particles and proposed that it was an intermediate in the formation of the 42S RNA. In contrast Arif and Faulkner (1972) demonstrated that the apparent fragility of the 42S RNA depended on the technique used for extraction and that the genome of Sindbis virus was a single polynucleotide chain with a molecular weight of 4.0×10^6.

Pfefferkorn and Hunter (1963b) reported that the base compositions of Sindbis virus RNA was: adenine 29.6 ± 1.0%, cytosine 24.9 ± 0.9% guanine 25.8 ± 1% and uracil 19.7 ± 3%. A similar composition for the RNA extracted from SFV particles was found by Sonnabend et al (1967): adenine 27.4%, cytosine 24.4%, guanine 26.1%, and uracil 22.2%. Johnston and Bose (1972a, b) demonstrated the presence of an adenlyate-rich segment in the 42S RNA of Sindbis and suggested that it was correlated with its possible function as a messenger RNA.

Virus RNA extracted from SFV or Sinbis virus particles did not stimulate incorporation of precursors into protein in an in vitro protein synthesizing system, (Kerr, Sonnabend and Martin, 1971), although an "initiation complex" was formed when Sindbis RNA and ribosomes were mixed (Cogniaux-leClerc, 1971). When RNA from Sindbis virions was mixed with ribosomes from chick embryo fibroblasts a complex that had a sedimentation coefficient of 70-75S could be identified (Cogniaux-leClerc, 1971). Aurintricarboxylic acid but not sodium fluoride inhibited this reaction. On addition of GTP, transfer RNA and an energy source a complex with a sedimentation coefficient of 100S was formed. It was suggested that an "initiation complex" was being formed (Cogniaux-leClerc, 1971).

The RNA from SFV particles was shown to be infectious by Sonnabend et al (1967). This suggests that the 42S RNA or part of it, was

translated after infection. It also suggests that the virus particles do not contain an essential RNA polymerase activity.

4) Proteins of the virus particle

There has been some disagreement over the number of proteins found in purified SFV particles. Friedman (1968b) reported that the purified virus contained three proteins, two in the core, one in the envelope. In contrast to this Hay, Skehel and Burke (1968), showed that only two proteins were present in the virus, one in the envelope and the other in the core. Using highly purified cores only one protein could be found (Acheson and Tamm, 1970) and it was suggested that the second protein was an artefact arising from incomplete reduction of the proteins, and subsequent dimerization (Kennedy and Burke, 1972). The presence of one envelope protein, and one protein in the core particle is a common feature of all the group A togaviruses investigated, including Sindbis virus (Strauss et al, 1968), Mayaro virus (Dorsett and Acton, 1970) and Chikungunya virus (Igarishi, Fuhuoko, Nithiuthai, Hou and Fukai, 1970). In contrast the group B toga viruses contain three virion proteins, two in the envelope and one in the core (Trent and Quereshi, 1971). The molecular weights of the proteins have been determined by several groups. The envelope protein has a molecular weight of $50\text{-}53 \times 10^3$ while the core protein has a molecular weight of $30\text{-}34 \times 10^3$. Recently the envelope protein from Sinbis virus has been shown to consist of two components, of molecular weight 53,000 and 45,000 (Schlesinger, Schlesinger and Burge, 1972a). The second component, E2, of lower molecular weight, was only found in the purified virus and not in the infected cell. Therefore it was suggested that E2 might be derived from E1. Defective particles of Sindbis virus can be isolated by passaging the virus serially in baby hamster kidney (BHK) cells. (Schlesinger, Schlesinger and Burge, 1972b).

This defective virus appears to differ from the normal virus in haemagglutinating properties. It is possible that the presence of the second envelope protein is related to this defectiveness.

The amino acid composition of the envelope and core protein of SFV has been determined by Simons and Kaariainen (1970), and the N-terminal amino acid of the two proteins has been identified by Kennedy and Burke (1972). The core protein is rich in lysine and arginine; an expected result because of its association with the RNA in the nucleocapsid. Maps of the peptides produced by tryptic digestion of the envelope and core proteins have been published containing the expected number of spots. It has been estimated that the nucleocapsid contains about 270-300 molecules of the core protein, while there are about 570-580 molecules of envelope protein in one virus particle. The number of spikes bordering the virus particle has been estimated from electron micrographs. If the envelope protein is contained in the spikes then each spike contains 2 molecules of the protein.

The envelope protein was shown to be a glycoprotein containing glucosamine, mannose, galactose, fucose and sialic acid (Burge and Strauss, 1970; Strauss, Burge and Darnell, 1970). If there are two envelope proteins then these results are not invalidated, but represent the mean of their individual compositions (Schlesinger et al, 1972a). The carbohydrate was arranged in three types of chain, two of which were terminated with fucose or sialic acid. The total molecular weight of the carbohydrate moiety was 7,200 and of the three types of chain, 2,800, 2,490 and 1,740. By comparing the glycopeptides of the envelope protein from Sindbis virus with those derived from the membrane protein of vesicular stomatitis virus, Burge and Huang (1970) demonstrated when the viruses were grown in the same type of host cells, the glycopeptides were

the same. They suggested from this that the carbohydrate moiety was synthesised by host specified glycosyl-transferases. This result was confirmed by using an in vitro system for assaying fucose and sialic acid transferases (Grimes and Burge, 1970) derived from uninfected cells. This system had the capability of adding fucose or sialic acid to the envelope protein of Sindbis virus.

5) Biological activity of sub-viral components

Several different sub-viral particles can be generated by mild enzymatic or chemical treatments of the virus. The surface properties of SFV that have been investigated are the ability to agglutinate goose red blood cells, the specific antigenicity, and the ability to precipitate concanavalin A - a plant lectin that binds to certain sugar residues (Oram, Ellwood, Appleyard and Stanley, 1971). In Sindbis virus the neutralising antibodies are directed against antigens in the viral envelope (Bose and Sagik, 1970). All these properties are destroyed if the virus is treated with certain proteolytic enzymes. The virus is resistant to trypsin (Cheng, 1958), but bromelain, (Compans, 1971), caseinase C (Osterieth and Calberg-Bacq, 1966) and pronase (Kennedy, in press) all destroyed the surface properties mentioned above. The spikes were removed, and the remaining particle was no longer infectious, although the RNA contained inside the nucleocapsid was still intact and infectious. Analysis of the proteins that remained after the enzyme treatment, by polyacrylamide gel electrophoresis, showed that the envelope protein had been destroyed. This experiment demonstrates that the envelope protein is necessary for the virus particle to attach and penetrate the plasma membrane of the host cell. The particle remaining after these treatments still contains the majority of the lipid, and under the electron microscope it is shown to consist of the nucleocapsid surrounded by a smooth

membrane (Igarishi et al, 1970). The buoyant density of the particle is consistent with this.

The haemagglutinin can be released in an active form by treating the virus with either nonidet P40 or tween 80/ether (Mussgay and Roth, 1964). The two techniques disrupt the virus in different ways releasing components with contrasting properties. The treatment with tween 80/ ether releases a haemagglutinin with a buoyant density of 1.28g/cc, while the nonidet P40 treatment releases one with a buoyant density of 1.19g/cc (Igarishi et al, 1970). The haemagglutinin released with nonidet P40 can be considered as part of the envelope consisting of the envelope protein still embedded in the lipid membrane; while the remaining particle is identical with the nucleocapsid. In contrast the tween 80/ether technique liberates haemagglutinin fragments that had been freed from the lipid. The nucleocapsid remaining was still surrounded by the lipid bilayer.

The whole virus particle is sensitive to treatment with phospholipase C, about 45% of the phospholipid being released, but the infectivity is retained (Friedman and Pastan, 1969). This experiment shows that there must be spaces between the envelope protein sufficiently large to allow the enzyme access to the underlying phospholipid. It also raises the question of the biological function of the phospholipid. These authors suggested that the phospholipid stabilised the virus structure. In contrast to these results Kennedy (in press) reports that whole virus particle is resistant to phospholipase C, but if the envelope protein is removed by treatment with pronase, all of the phospholipid can be destroyed with phospholipase C, liberating the nuclèapsid. The difference in sensitivity to phospholipase C may be due to the amount of disrupted virus in the preparation.

6) Morphogenesis of SFV

Spherical particles, with a diameter of about 30nm, supposed to be viral "precursors" were found in the cytoplasm of cells infected with Western equine encephalitis virus (Morgan, Howe and Rose, 1961). They were partly scattered in the cytoplasm, but also regularly arranged around cytoplasmic vacuoles. Acheson and Tamm (1967) reported the existence of similar vacuoles and tubular structures surrounded by nucleocapsids during the course of infection by SFV, but only late during the growth cycle when the rate of production of virus had already decreased. These authors also studied the process by which the virus acquired its peripheral membrane and suggested that the virus either passed into the lumen of the vacuoles or was extruded through the plasma membrane. Erlandson, Babcock, Southam, Bailey and Shipkey (1967) published electron micrographs showing that the virus pushes the plasma membrane outwards into a bud before the membrane seals over and the virus particle is released. Higashi, Matsumoto, Tabata and Nagatomo (1967) studied the interaction of ferritin-labelled antibodies with VERO cells infected with Chikungunya virus, and concluded that host cell membrane, which had been modified during infection so as to contain viral antigen, was elaborated into the virus envelope.

By using the technique of freeze-etching of samples in preparation for electron microscopy, the movement of host protein out of the area of the plasma-membrane that is being elaborated into the viral envelope can be seen. (M.R.F. Waite, personal communication). The process of resealing the holes in the leaflets of the host cell membranes can be visualised; and it was demonstrated that the inner leaflet was sealed over before the outer leaflet.

The release of infectious virus from infected cells depends on the composition of the extracellular fluids. At least two different parameters

are involved. The release of Sindbis virus can be slowed down by lowering the osmotic pressure of the culture medium, and it can also be inhibited if the ionic strength of the culture medium is reduced (Waite and Pfefferkorn, 1968; Waite and Pfefferkorn, 1970a). When the virus release was inhibited by the use of culture medium of low ionic strength, protein, phospholipid and infectious RNA synthesis, assembly of nucleocapsids and viral modification of the plasma membrane were all normal. When the inhibition was eased, infectious virus was detected within 20 sec and was not dependent on osmotic pressure, de novo protein or RNA synthesis or a functional energy metabolism (Waite and Pfefferkorn, 1970a).

If infected cells are pulsed with radioactive amino acids followed by a chase, during the logarithmic phase of virus growth, radioactive virus is maximally released within one hour. Initially (within ten minutes) more newly synthesized protein is found in the nucleocapsid than in the envelope of the released virus, while after one hour the situation is reversed (Scheele and Pfefferkorn, 1969a). Despite this rapid release of the labelled structural proteins a large quantity of them accumulate in the infected cell. This could be explained if only some of the newly synthesized proteins were available for construction of virus particles.

After their synthesis both structural proteins appear to be immediately sequestered in virus precursors. Intracellular nucleocapsids are assembled within 3 min in cells infected with SFV (Friedman, 1968c) or within 10 to 15 min in cells infected with Sindbis virus (Ben-Ishai, Goldblum and Becker, 1968). Envelope protein can be detected incorporated into the plasma membrane in a similar length of time. (S.I.T. Kennedy, personal communication). In the case of SFV, no viral proteins could be detected in the soluble cell extracts.

The host cell membrane is not only altered by the presence of viral

antigen, but the cells become agglutinable with concanavalin A (Becht, Rott and Klerk 1972). Also gander erythrocytes can be haemadsorbed onto the membrane (Burge and Pfefferkorn, 1967). Haemadsorption was shown by Burge and Pfefferkorn (1968) to depend on the formation of a functional envelope protein, by showing that cells infected with one of the mutants from one of the RNA $^{+}$ complementation groups (group D) did not haemadsorb at the non-permissive temperature.

Nucleocapsids, apparently identical with the core present in the virus particle, can be found in the cytoplasm of infected cells and have a sedimentation coefficient of 140S. Late in infection they can be found in massive crystalline arrays (Acheson and Tamm, 1967). The nucleocapsids were shown by Hay et al (1968), to contain only one protein, the core protein, and the 42S RNA. Temperature sensitive mutants of both Sindbis virus and SFV have been found that do not form nucleocapsids at the restrictive temperature. All this class of RNA^{+} Sindbis mutants were shown to be members of the same complementation group (C) by Burge and Pfefferkorn (1968). The failure to form nucleocapsids, detected by the presence of particles that sediment at 140S at the restrictive temperature, by two mutants of SFV was correlated with the absence under the electron microscope of the crystalline arrays and single nucleocapsids from the cytoplasm of infected cells (Tan, 1970).

7) Association of viral replication with membranes

Most, if not all, virus directed synthesis of macromolecules appears to be intimately associated with host cell membranes. Unique cytoplasmic structures bounded with membrane and called cytopathic vesicles type I, are formed during the early logarithmic phase of SFV growth. (Grimley, Berezesky and Friedman, 1968). They are characterized by a regular series of membrane buds or spherules that are attached to the inside of the

vesicles. High resolution autoradiography suggests that viral RNA is synthesized on the outer membrane of these vesicles. Friedman and Grimley (1969) described a second type of cytopathic vesicle found late in infection that was connected with nucleocapsid assembly.

Caliguiri and Tamm (1970b) showed that in HeLa cells infected with poliovirus, RNA and protein synthesis were taking place on distinct structures. The membranes were separated by equilibrium centrifugation of cytoplasmic extracts on a discontinuous sucrose density gradient. Using similar techniques Grimley, Levin, Berezesky and Friedman (1972) showed that a similar separation existed in cells infected with SFV. The band involved with RNA synthesis consisted of a purified preparation of type I cytopathic vesicles. An alternative approach, employed by Bose and Brundige (1972), is to isolate membranes from specific parts of the cells, such as the plasma membrane and the endoplasmic reticulum. These authors demonstrated that the envelope protein and the core protein were associated with the plasma membrane, while no viral structural proteins were detectable in a smooth membrane fraction.

8) RNA synthesis

Several virus-specific RNA species can be identified in the cytoplasm of cells infected with SFV or Sindbis virus. They are named for convenience by their sedimentation value on sucrose gradients, although in some cases the sedimentation coefficient has not been determined. Originally 3 species of RNA could be identified in cells infected with group A togaviruses. These were two species of single-stranded RNA, 26S and 42S, and one double-stranded species, 20S RNA (Friedman, Levy and Carter, 1966; Pfefferkorn, Burge and Coady, 1967). This 42S RNA was shown to be identical to the 42S RNA of the mature virion. Indeed RNA extracted from Sindbis infected cells is infectious (Scheele and Pfefferkorn, 1969b).

The 26S RNA found in infected cells was not shown to be the same as the 26S RNA produced by heat treatment of the 42S RNA, although it was thought to be a precursor of it (Cartwright and Burke, 1970).

By using more sophisticated techniques of analysis, several more species of RNA have been identified. Levin and Friedman (1971) found four single-stranded species which have been termed 42S, 38S, 33S and 26S, several totally double-stranded species, and a multi-stranded species (replicative intermediate), by fractionating the viral RNA extracted from cells infected with either Sindbis virus or SFV. The molecular weights of these species have been estimated by B. Martin (unpublished observation) as 26S: 1.8×10^6, 33S: 2.3×10^6 and 38S: 3.1×10^6. The role of the 26S, 33S and 38S RNA species during the course of infection is not known, but none of the four single-stranded species would hybridise with 42S RNA from virus particles. This would suggest that all the single-stranded species free in the infected cells are of the same polarity as the 42S virus RNA (positive).

The 26S and 33S RNA, in the form of a ribonucleoprotein complex have been extracted from the membrane bound polysomes of infected cells (S.I.T. Kennedy, personal communication), who suggested that they were the messenger RNAs. The RNA, extracted from polysomes was shown to contain adenylate-rich sequences by Eaton, Donaghue and Faulkner (1972). Although the RNA species were not identified, the RNA was shown to be in a ribonucleo protein complex with a sedimentation coefficient of 65S. These could correspond to 65S particles, previously thought to be precursors of the nucleocapsid, which are known to contain 26S RNA (Friedman and Berezesky, 1967; Dobos and Faulkner, 1969.)

Three classes of totally double-stranded RNA were identified by Stollar, Shenk and Stollar (1972). These authors prepared anti-body

against double-stranded RNA and used it to precipitate the RNA found in infected cells. Before precipitation this RNA had been analysed by sucrose density gradient centrifugation. Peaks of double-stranded RNA were found with sedimentation coefficients of 12S, 16S and 20S. The amount of these three classes of double-stranded RNA varied depending on the type of cell from which the RNA was extracted. It was suggested that the differing inhibition of host cell protein synthesis might be due to the variation in quantity of double-stranded RNA. The multiple forms of double-stranded RNA raise the question of whether there is a different type involved in the synthesis of each of the species of single-stranded RNA.

The predominant type of RNA produced after a short pulse of radioactive uridine is a heterogenously sedimenting RNA, apparently membrane bound, which is partially resistant to ribonuclease and contains minus strands, (Friedman, 1968; Kaariainen and Gomatos, 1969). This material has the properties of replicative intermediate, similar to that isolated from cells infected with other viruses (Franklin, 1966; Baltimore, 1968).

The first newly synthesized RNA species that can be detected following infection in SFV infected cells is the double-stranded RNA (Cartwright and Burke, 1970). After preparing infectious Sindbis virus containing radioactive RNA of a very high specific activity, its fate after infection was followed (Sreevalsan, 1970). The RNA became attached to membranes during the latent period following infection, was converted to a ribonuclease resistant form, and was subsequently slowly detached from the membrane. The RNA from an RNA$^-$ temperature sensitive mutant still became associated with the cellular membranes, even at the non-permissive temperature at which no RNA synthesis occurs. But the detachment was restricted in the absence of RNA synthesis.

26S RNA probably has a special function in Sindbis virus multiplication

(Scheele and Pfefferkorn, 1969b). If cellular protein synthesis was inhibited 1½ hours after infection, the synthesis of infectious 42S RNA continued, but that of 26S RNA was selectively inhibited. A corresponding, preferential inhibition of the formation of 26S RNA was seen in cells infected with a temperature sensitive mutant defective in RNA synthesis. After starting RNA synthesis at the permissive temperature, despite shifting the infected cultures to the restrictive temperature, the synthesis of 42S RNA continued, whereas the formation of 26S RNA ceased. This implies that the synthesis of 26S and 42S RNA is controlled by two different genome products.

9) RNA polymerase

A new enzyme activity can be detected in cells infected with group A togaviruses. This enzyme is a RNA-directed RNA polymerase (RNA nucleotidyl transferase, E.C.2.7.7.6.), and can be found after infection even in cells treated with actinomycin D (Lust, 1966; Martin and Sonnabend, 1967). The enzyme was found in the large particle fraction, and can be fractionated by centrifugation on sucrose density gradients. (Sreevalsan and Yin, 1969). Martin and Sonnabend (1967) demonstrated that the enzyme can be assayed *in vitro*, but that the product was totally double-stranded. By isolating the RNA polymerase on a sucrose density gradient Sreevalsan and Yin (1969) demonstrated that it had the capability of synthesizing 42S and 26S single-stranded RNA as well as the 20S double-stranded RNA.

The RNA polymerase is membrane bound (Yin and Sreevalsan, 1969). All attempts so far to liberate the enzyme from the membrane have resulted in either a partial or complete loss of activity. The cytopathic vesicles (type I), when isolated by equilibrium centrifugation on a discontinuous sucrose density gradient, possess RNA polymerase activity when assayed

in vitro. (R.M. Friedman, personal communication).

The activity of the RNA polymerase prepared from cells infected with either Sindbis virus or SFV is not dependent on continuing protein synthesis. This contrasts with the RNA polymerase of picornaviruses, whose continued formation is inhibited if protein synthesis is halted (Nehrenfeld et al, 1970). These results are confirmed if protein synthesis is inhibited with canavanine, an analogue of arginine (Ranki and Kaariainen, 1970). Once infection has been established by the temperature sensitive RNA$^-$ mutants of SFV at the permissive temperature, the temperature of incubation can be stepped up to the restrictive temperature without inhibiting RNA synthesis (Lomniczi and Burke, 1970). The RNA$^-$ mutants of Sindbis virus have been placed in at least two complementation groups (Burge and Pfefferkorn, 1966b). This suggests that at least two gene products are involved in the synthesis of RNA.

Originally it was assumed that the replication of a single-stranded RNA must involve a double-stranded intermediate (Montagnier and Sanders, 1963). There was no agreement as to the number of enzymes required for the synthesis of viral RNA (Spiegelman, Haruna, Holland, Beaudreau, and Mills, 1965; Lodish and Zinder, 1966). It was suggested that two enzymes were essential, one to form a double-stranded replicative form from the input strand and one to produce new progeny strands. Alternatively, one enzyme may suffice for RNA synthesis, but be composed of two different polypeptides. Finally, one cistron may be required to neutralise a cellular activity prejudicial to viral RNA synthesis (e.g. ribonuclease) while the other provides polymerase.

More recently it has been shown that the replicase of the RNA bacteriophage Qβ consists of four protein subunits. Three of these are host specified, and only one is virus specified (Kondo, Gallerani and

Weissman, 1970). The replicase can transcribe strands of either polarity, but has a higher affinity for negative strands. It has been suggested that the active template is not double-stranded, but a loose complex of enzyme, template and progeny RNA (Weissman, Feix and Slor, 1968). According to this view the extensive hydrogen-bonded structure of isolated replicative form and replicative intermediate arise during the extraction procedure (Borst and Weissman, 1965).

Baltimore (1971) has suggested that animal viruses can be divided into six classes on the basis of the way in which messenger RNA is formed. Two of those six classes included viruses with single-stranded RNA as their genetic material. The typical member of one of these classes (IV) is poliovirus, while vesicular stomatitis virusis representative of the other class (V). Class IV consists of single-stranded RNA viruses whose messenger RNA is identical in base sequence to virion RNA. In contrast class V consists of single-stranded RNA viruses whose messenger RNA is complementary in base sequence to the virion RNA. Class V viruses have a virion polymerase, but not infectious RNA, while class IV viruses have infectious RNA but no virion polymerase. A feature of this classification is that it explains why some viruses have infectious RNA, while others do not. On the basis of the infectivity of the virion RNA, Baltimore (1971) placed the togaviruses in class IV.

10) Protein synthesis

The envelope and core proteins are readily detected in cells infected with Sindbis virus (Strauss, Burge and Darnell, 1969), SFV (Hay et al, 1968) Mayaro virus (Dorsett and Acton, 1970) and Chikungunya virus (Igarishi, 1970). It is interesting to note, that the reported second envelope glycoprotein, was not found in infected cells (Schlesinger et al,

1972a). Many other polypeptides have been found by analysing material from infected cells by polyacrylamide gel electrophoresis. The best defined species have molecular weights of about 65,000 and 95,000. Several other processes are known to be associated with infection by the virus, such as the RNA polymerase, the effective inhibition of host cell protein synthesis and the reorganisation of the host cell membranes. Gene products connected with these functions have not been identified.

The division between class IV and class V animal viruses (Baltimore, 1971) apparently extends to the translation of the messenger RNA and the formation of the proteins. Influenza virus (a class V virus) produces six or seven different species of messenger RNA (Skehel, 1972). It has been suggested that when each of these RNAs is translated, a primary gene product is synthesized. Friedman (1969) claimed that the structural proteins of SFV were primary gene products on the basis of two tests. The two tests employed were to investigate the protein labelled in infected cells during pulse-chase experiments and during treatment with amino acid analogues. In the test cells the same proteins could be detected in the same amounts as in the control cells.

In contrast Jacobson and Baltimore (1968) showed that poliovirus proteins are synthesized from large precursors by specific cleavages, and suggested that the messenger RNAs of animal viruses will only possess one initiation site leading to monocistronic translation.

By the use of the same tests, amino acid analogues and very short labelling periods, Jacobson, Asso and Baltimore (1970) demonstrated the presence of a single transcript precursor in HeLa cells infected with poliovirus. A single transcript precursor occurs when a polycistronic messenger RNA encodes a single polypeptide chain. The single transcript

precursor is then cleaved to yield the final gene products. A precursor-product relationship has been established by showing a movement of radioactivity between certain polypeptides in poliovirus infected cells.

Strauss et al (1969) reported a large number (12-16) of non-structural proteins found in cells infected with Sindbis virus, and suggested that the polypeptide of molecular weight 65,000 was a precursor. Both Burrell, Martin and Cooper (1970) and Igarishi (1970) proposed that the polypeptide of molecular weight 95,000 was a precursor of the structural proteins. A large protein was seen to accumulate in cells infected with a temperature sensitive mutant of Sindbis, defective in nucleocapsid synthesis (Scheele and Pfefferkorn, 1970).

If there is a cleavage mechanism operating then the specific cleavages needed may be caused by proteolytic enzymes. Therefore when the proteolytic enzymes are inhibited,there should be an accumulation of the precursor. Using an inhibitor of proteolytic enzymes, Pfefferkorn and Boyle (1972) demonstrated that there was an accumulation of a high molecular weight polypeptide. This result was confirmed by Ranki (1972) by growing the virus in the presence of canavanine. In neither case was a movement of label from the postulated precursor to the product demonstrated.

11) Purpose of the present study

The work reported in this thesis was carried out to determine the mode of synthesis of the structural and non-structural proteins of SFV. It was hoped to be able to discover whether any precursor-product relationship existed between any of the proteins specified by the virus. The parameters of growth of the virus were studied in order to establish the optimum experimental conditions. Then the proteins found in cells infected with SFV were separated by polyacrylamide gel electrophoresis and sub-

sequently attempts were made to identify their function. The RNA polymerase was partially purified, in order to identify the RNA and protein structures involved in the synthesis of the RNA. Also the proteins contained in other membrane fractions were analysed, so that the process of glycosylation of the viral proteins could be investigated. Finally it was hoped from these experiments to construct a model of the way in which RNA and protein synthesis occurred in SFV infected cells.

MATERIALS

(a) Biological Materials

Medium 199 as defined by Morgan, Morton and Parker (1950) and basal Eagle's medium as defined by Eagle (1955) and modified by Macpherson and Stoker (1962) were purchased as ten fold concentrates from Wellcome Reagents Ltd., Beckenham, Kent.

Calf Serum was purchased from BioCult, Paisley, Scotland. Dialysed calf serum was prepared by dialysis overnight against a hundred fold excess of Earle's salt solution.

Growth medium consisted of Media 199 supplemented with calf serum to a final concentration of 5% (^{v}/v), adjusted to pH7.4 by addition of 5% (w/v) sodium bicarbonate, and contained 100 unit/ml of cristamycin.

Maintenance medium was identical in composition to growth medium, except that the calf serum content was 2% (^{v}/v)instead of 7%.

MEM consisted of Eagle's basal medium supplemented with 10% (^{v}/v) calf serum, 1% (w/v) glucose and 2.95g/l tryptose phosphate both adjusted to pH7.4 by addition of 5% (^{w}/v) sodium bicarbonate, and contained 100 units/ml of crystamycin.

Earle's balanced salt solution, as defined by Earle (1943) was purchased from Oxoid Ltd., London. The salt mixture was dissolved in distilled water, sterilised, adjusted to pH7.4 with 5% (^{w}/v) sodium bicarbonate, using 0.001% phenol red as an indicator of pH. Earle's salt solution contained 100 units/ml of crystamycin.

Earle's salt solution containing dialysed calf serum and actinomycin D (EDA) was prepared by supplementing Earle's balanced salt solution with 2% (^{v}/v) dialysed calf serum and 1µg/ml actinomycin D. The calf serum was dialysed to remove low molecular weight constituents that would reduce incorporation of radioactive precursors.

Plastic Petri dishes (5cm diameter) were purchased from Sterilin Ltd., Richmond Surrey.

Antiserum Preparations against the envelope and core proteins of SFV and preimmune antisera were kindly prepared by Dr. S.I.T. Kennedy.

Goose erythrocytes were a gift from Dr C.J. Bradish, Microbiological Research Establishment, Porton, Wilts., and were washed three times by centrifugation in dextrose/gelatin/veronal medium (0.058% veronal, 0.38% sodium veronal, 0.06% gelatin, 0.02% $CaCl_2$, 0.012% $MgSO_4 7H_2O$, 0.85% NaCl and 1% (w/v) glucose). The erythrocytes were resuspended to a concentration of 5% in the same medium, and were stored at $4^{\circ}C$.

Actinomycin D was the gift of Merck, Sharpe and Dohme Research Laboratories, Rahway, New Jersey, U.S.A.

Colomycin was bought from Pharmax Ltd., Crayford, Kent.

Crystamycin was purchased from Glaxo, Greenford, Middx. and Cycloheximide was purchased from Sigma Ltd., London.
Puromycin was bought from Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A.

Trypsin 1: 250 for preparation of chick embryo fibroblast cultures and tryptose phosphate broth wereobtained from Difco Laboratories, East Molesley, Surrey.

Bovine serum albumin (BSA), fraction V, was obtained from Armour Pharmaceutical Co., Ltd. Eastbourne, Sussex.

Ovalbumin, trypsin, a-chymotrypsinogen, carbonic anhydrase, pepsin, transferrin, phosphorylase A and B-galactosidase for use as molecular weight standards and the enzymesribonuclease (pancreatic), and cytochrome c were purchased from Sigma Ltd., London.
All media were checked for sterility before use.

(b) Chemical materials

2-Mercaptoethanol, reduced glutathione, trizma base, DL-p-fluorophenyl-alanine (FPA), sodium lauroyl sarcinosate, DL-ethionine azotryptophan, L-leucine, D-glucosamine, N-acetyl glucosamine, adenosine triphosphate (ATP), cytidine triphosphate (CTP), guanosine triphosphate (GTP) and uridine triphosphate (UTP) were purchased from Sigma Ltd., London. The nucleoside triphosphates were checked for the presence of mono and diphosphates by thin layer chromatography.

Nicotinamide adenine dinucleotide (reduced; NADH), nicotin amide adenine dinucleotide phosphate (reduced; NADPH), Phosphoenol pyruvate (PEP) and pyruvate kinase were obtained from Boehinger and Soene GmbH, Mannheim, Germany. The PEP was checked for purity by a coupled enzymic assay.

N-fluoracetyl glucosamine (FNAG) was a kind gift from Dr G.G. Butchard, Dept. of Biochemistry, Oxford University, Oxford.

Cleland's reagent (dithiolthreitol) L-canavanine sulphate, L-azetidine-2-carboxylic acid and tosyl-L-phenyl alanyl chloromethane (TPCK), all grade A,, were purchased from Calbiochem Ltd., London.

N,N,N^1,N^1-tetramethylethylene diamine (TEMED) and NN^1-methylenebisacrylamide were obtained from Eastman Kodak Co., Rochester, New York, U.S.A. Acrylamide (purum) was obtained from Fluka AG, Buchs, Switzerland. The acrylamide was recrystallised from chloroform, and the NN^1-methylenebis-acrylamide from acetone before use (Loening, 1967).

Coomassie brilliant blue R was purchased from G.T. Gurr Ltd., London.

Sodium Lauroyl (or dodecyl) sulphate (SDS), chloramine T, specially pure dansyl chloride (1-dimethylaminonapthalene-5-sulphanyl chloride) and Urea (aristar) were bought from B.D.H. Chemicals Ltd., Poole, Dorset.

Dextran Sulphate 500 and Sephadex G25 were bought from Pharmacia (Gt.)

Britain) Ltd., London.

Phenol, diethyl ether, and ethanol were redistilled before use.

Agarose was bought from L'industrie Biologique Francaise S.A., Gennevillies, Seine, France.

(c) Radioactive materials

The following radio chemicals were all obtained from Radiochemical Centre, Amersham, Bucks.

[2-^{3}H] -AMP (aqueous solution containing 50% ethanol; 4 Ci/mMol)

[^{14}C] - formaldehyde (aqueous solution; 15Ci/mMol)

[^{3}H] L-fucose (aqueous solution; 2.8Ci/mMol)

[^{3}H] D-glucosamine hydrochloride (aqueous solution; 2.3Ci/mMol)

[5-^{3}H] GTP (aqueous solution containing 50% ethanol; 8Ci/mMol)

[^{125}I] iodide (carrier free; 110mCi/ml).

[4,5-^{3}H] L-leucine (aqueous solution containing 2% ethanol; 19Ci/mMol)

[^{14}C] L-leucine (aqueous solution containing 2% ethanol; 300mCi/mMol)

[4,5-^{3}H] L-lysine monohydrochloride (aqueous solution containing 2% ethanol; 300mCi/mMol)

[^{14}C] L-lysine monohydrochloride (aqueous solution containing 2% ethanol; 300mCi/mMol)

[^{3}H] L-methionine (aqueous solution; 150mCi/mMol)

[^{14}C] L-methionine (freeze dried; 50mCi/mMol)

[^{35}S] L-methionine (aqueous solution containing 0.04% 2-mercaptoethanol; 20Ci/mMol)

[^{14}C] L-phenylalanine (aqueous solution containing 2% ethanol; 405mCi/mMol)

[^{14}C] L-proline (aqueous solution containing 2% ethanol; 265 mCi/mMol)

[^{14}C] L-threonine (aqueous solution containing 2% ethanol; 208mCi/mMol)

[^{14}C] DL-tryptophan (freeze dried; 50mCi/mMol)

[^{14}C] L-tyrosine (aqueous solution containing 2% ethanol; 507mCi/mMol)

[2,3-^{3}H] L-valine (aqueous solution containing 2% ethanol; 1.5Ci/mMol)

[^{14}C] L-valine (aqueous solution containing 2% ethanol; 225mCi/mMol)

[5-^{3}H] -uridine (aqueous solution; 3Ci/mMol)

[^{14}C] -uridine, (aqueous solution; 405mCi/mMol)

[^{3}H] (1mCi/ml) and ^{14}C (1mCi/ml), reconstituted protein hydrolsates were obtained from Schwarz BioResearch, Orangeburg, New York, U.S.A.

(d) Scintillation Fluid Materials

2,5-Diphenyloxazole (P.P.O.), 1,4-di(2-(5-phenoloxazlyl)-benzene (P.O.P.O.P.) and napthalene (scintillation grade) were obtained from Nuclear Enterprises (G.B.) Ltd., Edinburgh. Aerosil standard silica was bought from Bush Beach Segner Bayley Ltd., London. Triton-X-100 was purchased from B.D.H. Chemicals Ltd., Poole, Dorset.

Gel scintillation cocktail was a mixture of napthalene (50g), P.P.O. (7g) P.O.P.O.P. (150mg), absolute alcohol (30ml), toluene (A.R.) (200 ml), made up to 1 litre with dioxan (A.R.).Silica gel (35g) was added and the mixture blended for 2 minutes.

Triton-toluene scintillation cocktail was a mixture of toluene (A.R.) (670ml), triton-X-100 (330ml), P.P.O. (4g), and P.O.P.O.P. (500 mg).

Plastic scintillation vials were bought from Packard Instruments Ltd., Caversham, Berks.

METHODS

1. Preparation of chick embryo cells

Primary chick embryo cells were prepared using a modification of the method described by Porterfield and Allison (1960). Eleven day old chick embryos were used. After removal of the heads and legs the torsos were washed with phosphate buffered saline (PBS) pH7.4 (Dulbecco and Vogt, 1954). The torsos were finely minced before being suspended in 0.1% trypsin in PBS (4ml per embryo) and stirred at 37°C for 20 min. The supernatant fluids were decanted and stored at 4°C while the remaining tissue was retrypsinized another three times. The supernatant fluids were pooled, made 2% (v/v) with respect to calf serum and filtered through a 40 mesh stainless steel filter. The cells were sedimented at 500g for 20 min at 4°C before being resuspended in growth medium (2ml per embryo). The cells were first filtered through a grade 0 sintered glass filter and then through a grade 1 sintered glass filter.

The number of cells was determined by diluting 0.5 ml of the cell suspension with 4.5 ml of PBS and 1 ml of this dilution was mixed with 3 ml of PBS and 1 ml of 0.1% trypan blue. This material was then counted using a Neubauer haemocytometer.

For monolayer cultures the cells were diluted to 5×10^6 cells/ml or 3×10^6 cells/ml, and 30 ml aliquots of the former concentration were dispensed on to 15cm glass Petri dishes while 3 ml aliquots of the latter concentration were dispensed on to 5cm plastic Petri dishes. Cultures were incubated at 37°C in a humid atmosphere of 5% carbon dioxide/95% air, before use 18-24 hours later. At confluence the 5cm plastic Petri dishes contained 2×10^7 cells and the 15cm glass Petri dishes 1.2×10^8 cells.

2. Subculturing of continuous cell lines

Baby hamster kidney (BHK) cells, L-929 and human embryo lung (HEL)

cells were all passaged in a similar way. Cells were seeded in litre flow bottles ($1x10^6$ cells/bottle; 100ml of MEM) and were gassed for 15 seconds with a 5% carbon dioxide/95% air mixture. The bottles were incubated at 37°C until the monolayer had reached confluence. The fluids were poured off and 5ml (flow bottle) or 25ml (roller bottle) of 0.1% trypsin in 0.0004M ethylene diamine tetra acetic acid (EDTA) solution was added. The cell sheet was washed gently, the fluids poured off, and this procedure repeated. A small volume of fluid was left covering the monolayer. The bottle was allowed to stand until the cells started to detach from the glass, when 3ml (flow bottle) or 10ml(roller bottle) of MEM was added. The cells were resuspended in this medium by pipetting. Before being dispensed they were then counted by the same method used for chick embryo cells and diluted appropriately with MEM, either into bottles or onto Petri dishes. For monolayer cultures the cells were seeded at $0.5x10^6$ cells/plate in 3ml of MEM on 5cm plastic Petri dishes and $3x10^6$ cells/plate in 25ml of MEM on 15cm glass Petri dishes. The cultures were then incubated at 37°C in a humid atmosphere of 5% carbon dioxide/95% air, and were ready for use 18-24 hours later. At confluence the 5cm plastic Petri dishes contained $1x10^6$ cells and the 15 cm glass Petri dishes $5x10^7$cells.

3. Growth of Virus

Semliki Forest virus (SFV) the tst wild type strain, was kindly supplied by Professor F. Fenner, John Curtin School of Medical Research, Australian National University, Canberra, and Sindbis virus (AR 339) was the kind gift of Dr J. Porterfield, National Institute of Medical Research, Mill Hill. Virus was stored at 4°C if it was going to be used within 24 hours, otherwise it was supplemented to 10% (w/v) calf serum and kept at -70°C. Each fresh sample of virus was checked for bacterial contamination

by streaking on nutrient agar plates and incubating for 24 hours at 37°C.

a) Growth in brains of suckling mice

Three day old suckling mice were injected intracerebrally with 10^4 p.f.u. of SFV in 0.01ml of maintenance medium (Walters, Burke and Skehel, 1967). After 24 hours, when the mice were dying, they were killed with chloroform and the brains were removed. The brains were suspended in maintenance medium (1ml/brain) and homogenised using a micromixer (Measuring and Scientific Equipment, London). The virus suspension was clarified by centrifuging at 12,000g for 30 min at 4°C. The supernatant contained $1\text{-}2\times10^9$ p.f.u./ml.

b) Growth in suspension culture

SFV was grown in suspension cultures of chick embryo cells using a modification of the method of Zwartow and Algar (1968). A concentrated suspension of chick embryo cells ($5\times10^8\text{-}10^9$ cells/ml) was infected with SFV (1p.f.u./cell) at 4°C for 60 minutes. The infected cells were then diluted to a concentration of $2\text{-}3\times10^7$ cells/ml in Earle's solution containing 0.35% glucose, 0.4% sodium bicarbonate and 180 units/ml colomycin. The media had already been adjusted to pH7.5 with 10% carbon dioxide/90% air. The infected cell suspension was dispensed in 180 ml aliquots into 1L Erlenmeyer flasks, from which the air was then displaced with a mixture of 5% carbon dioxide/95% air, and the flasks tightly sealed. The cultures were incubated at 35°C for 18 hr rotated at 80rev/min by an orbital shaker (Gallenkamp Ltd., London). The cells and debris were removed by centrifuging at 15,000g for 30 min at 4°C. The supernatant fluid contained $2\times10^9\text{-}10^{10}$ p.f.u./ml.

In order to prepare radioactively labelled virus, [^{14}C]valine (10 μCi/ml) was added to the medium, after the 1 hour absorption period.

4. Virus Purification

Virus was purified by the method of Kennedy and Burke (1972).

5. Assay of infectious virus

The infectious titre of SFV was estimated by plaque assay on monolayer cultures of chick embryo cells. (Dulbecco, 1952). 0.5ml aliquots of 10 fold serial dilutions of the virus in maintenance medium were added to monolayer cultures of chick embryo cells (1.5×10^6 cells/culture), on plastic Petri dishes. After adsorption at 37°C for 60 min the virus inoculum was replaced by 4ml of overlay medium consisting of growth medium containing 0.9% Noble agar. The cultures were incubated at 37°C and the plaques were counted after 36 hours. To facilitate plaque counting the cells were stained by the addition of 2ml of 0.026% neutral red in Earle's solution (pH7.4) to the plates for 2 hours.

6. Assay of virus haemagglutinin

The haemagglutinating activity of virus samples was measured by a modification of the method of Clarke and Casals (1958). Virus was serially diluted in 0.25ml volumes of 0.4% BSA in a solution of 0.05M-borate+ NaOH(pH9.0) containing 0.12M-NaCl on lucite plates. The goose erythrocytes (0.3ml) were diluted in 10ml of 0.2M-citrate buffer (pH5.5) in 0.15M-NaCl, before adding 0.25ml to each well to give a final pH of 5.8.

7. Infection of monolayer cultures

Monolayers of cells were infected with SFV at a multiplicity of

infection (m.o.i.) of 10p.f.u./cell and incubated at 37°C for 1 hour. In some experiments, noted in the text, the m.o.i. was varied. The unadsorbed virus was removed by washing the cell sheets twice with maintenance medium. Further incubation was carried out in the presence of 2ml or 10ml of maintenance medium containing 1µg/ml of actinomycin D, for 5cm or 15 cm Petri dishes respectively.

When cultures are referred to in the text as "uninfected" they had been treated in the following way. Monolayers of cells were incubated at 37°C for 1hr in the presence of an equivalent quantity of maintenance medium to that of virus. Then the cell sheets were washed twice with maintenance medium before incubation further under the same conditions as the infected cells.

8. Measurement of macromolecular synthesis

In order to measure macromolecular synthesis, monolayers, in plastic Petri dishes were labelled for 1 hour with a radioactive precursor, and the radioactivity of the defatted, acid insoluble material was determined by a modification of the method described by Skehel, Hay, Burke and Cartwright (1967). After the pulse, the cells were washed three times with 3ml of cold PBS and then three times with 3ml of cold 10% (w/v) TCA. The acid insoluble precipitate was then washed three times with 3ml of cold ethanol and three times with 3ml of cold 20% acetone in 0.1M HCl, before being left to dry. The defatted residue was then dissolved in 1ml of 0.5M-NaOH and a 0.5ml portion taken for determination of radioactivity. The difference in radioactivity between identical samples was less than 3%.

a) RNA synthesis

[^{3}H]uridine (1µCi/culture) was added to triplicate cultures on plastic

Petri dishes at various times after infection, and the incorporation measured by the method described above.

b) protein synthesis

Three hours before the labelled precursor was added, the culture fluids were removed from triplicate plastic Petri dishes, and the cultures washed twice with 3ml of Earle's solution containing 2% (v/v) dialysed calf serum and 1µg/ml actinomycin D (EDA) before being incubated in 2ml of the same medium. At suitable times after infection 1µCi/culture of [^{3}H] valine was added and the incorporation measured by the technique described above.

c) glycoprotein and carbohydrate synthesis

Three hours before the labelled precursors were added, the culture fluids were removed from triplicate Petri dishes, the cultures washed twice with 3ml of EDA, before being incubated in 2ml of the same medium. After the three hours the fluids were again removed and were replaced by 2 ml of PBS containing 1µg/ml of actinomycin D Isotope, 5µCi/culture of [^{3}H] glucosamine and 1µCi/culture of [^{14}C]valine, were then added. After 1 hour the radioactivity of defatted, acid insoluble radioactivity was determined by the method described above.

9. Extraction of radioactive RNA

Monolayer cultures of chick embryo cells (1.25×10^{8} cells/culture) were infected with SFV, washed and incubated for 4 hours. The medium was removed and replaced with 2ml of maintenance medium containing 1µg/ml of actinomycin D and 5uCi/culture of [^{14}C] uridine. The cultures were then incubated 1 hour before being washed three times with cold PBS. The cells were scraped from the glass Petri dishes and centrifuged at 1,000g for 10 min at 4°C. They were then resuspended in 1ml of PBS

and 1ml of 1% ($^{W}/_{v}$) SDS before addition of 2ml of phenol saturated with 0.05 M-tris+HCl pH7.4, 0.1 M-NaCl and 0.001 M-EDTA (TNE buffer). The mixture was shaken vigorously for 10 min at room temperature using a Griffin flask shaker and the phases were then separated by centrifuging at 1000g for 10 min at room temperature. The resulting aqueous layer was removed and was re-extracted with 2ml of phenol saturated with TNE buffer. Residual phenol was removed from the final aqueous layer by three extractions with ether saturated with TNE buffer. A stream of nitrogen was passed over the sample to remove any remaining ether. The RNA was then precipitated by standing with two volumes of ethanol at -20°C for 18 hours, before being collected by centrifuging at 1000g for 25 min at 4°C. The RNA was then washed twice with ethanol, before being re-dissolved in an appropriate buffer. The RNA extracted by this method contained sufficient unlabelled cellular RNA to be analysed spectrophotometrically.

10. Assay of RNA polymerase

All reagents were dispensed with an Eppendorf automatic pipette. The assay tube contained in a final volume of 0.35ml:

tris + HCl pH 8.5	35.0μmol
$MgCl_2$	1.3μmol
KCl	4.0μmol
2-mercaptoethanol	7.0μmol
PEP	0.5μmol
pyruvate kinase	10.0μg
actinomycin D	1.0μg
dextran sulphate 500	3.5μg
ATP,CTP,UTP	50.0nmol
[^{3}H] GTP	0.5μCi; 1.0nmol

and an 0.1ml aliquot of the fraction being tested for polymerase activity. The assay mixture was incubated for 60min at 37°C before stopping the reaction by cooling to 4°C and adding 1ml of cold 0.05M-$Na_4P_2O_7$ in 10% ($^W/_v$) TCA and 0.1ml of 10mg/ml BSA. After standing for 30 min at 4°C, the precipitate was centrifuged at 1000g for 10 min at 4°C before being resuspended in 1ml of 0.05M-$Na_4P_2O_7$ in 10% ($^W/_v$) TCA. The centrifugation step was repeated twice. Finally the precipitate was collected on 25mm Whatman GF/C glass fibre discs, that had previously been soaked in 10% ($^W/_v$) TCA and dried, using a Millipore multifiltration apparatus. They were then washed three times with 3ml of cold 0.05M-$Na_4P_2O_7$ in 10% ($^W/_v$) TCA, three times with 3ml of cold ethanol, three times with 3 ml of cold ethanol/ether (3:1) mixture and finally three times with 3ml of cold ether. The discs were dried and the radioactivity determined. These conditions were varied in certain experiments as specified in the results.

All assays were peformed in triplicate, and the difference in radioactivity between members of a triplicate was less than 5%. The results were compared with an assay incubated in the absence of the three unlabelled nucleoside triphosphates. This value was the same as the value of a complete assay that was not incubated, and was subtracted from the assay value to give the activity dependent on the presence of all four nucleoside triphosphates.

11. Extraction of product from an RNA polymerase assay.

When the product synthesized in an RNA polymerase assay was to be analysed, the reaction volume and the amount of reactants were doubled, while the amount of [^{3}H] GTP was increased twenty-fold. The reaction mixture contained in a volume of 0.7ml:

tris + HCl pH8.5	70.0μmol
$MgCl_2$	2.6μmol
KCl	8.0μmol
2-mercaptoethanol	14.0μmol
PEP	1.0μmol
pyruvate kinase	20.0μg
actinomycin D	2.0μg
dextran sulphate 500	7.0μg
ATP, CTP, UTP	100.0nmol
$[^3H]$ GTP	10.0μCi; 5nmol

and a 0.2ml aliquot of the enzyme fraction. The reaction mixture was incubated at 37°C for 60 min before stopping by cooling to 4°C. The RNA was extracted by the phenol/SDS technique described above (Section 9).

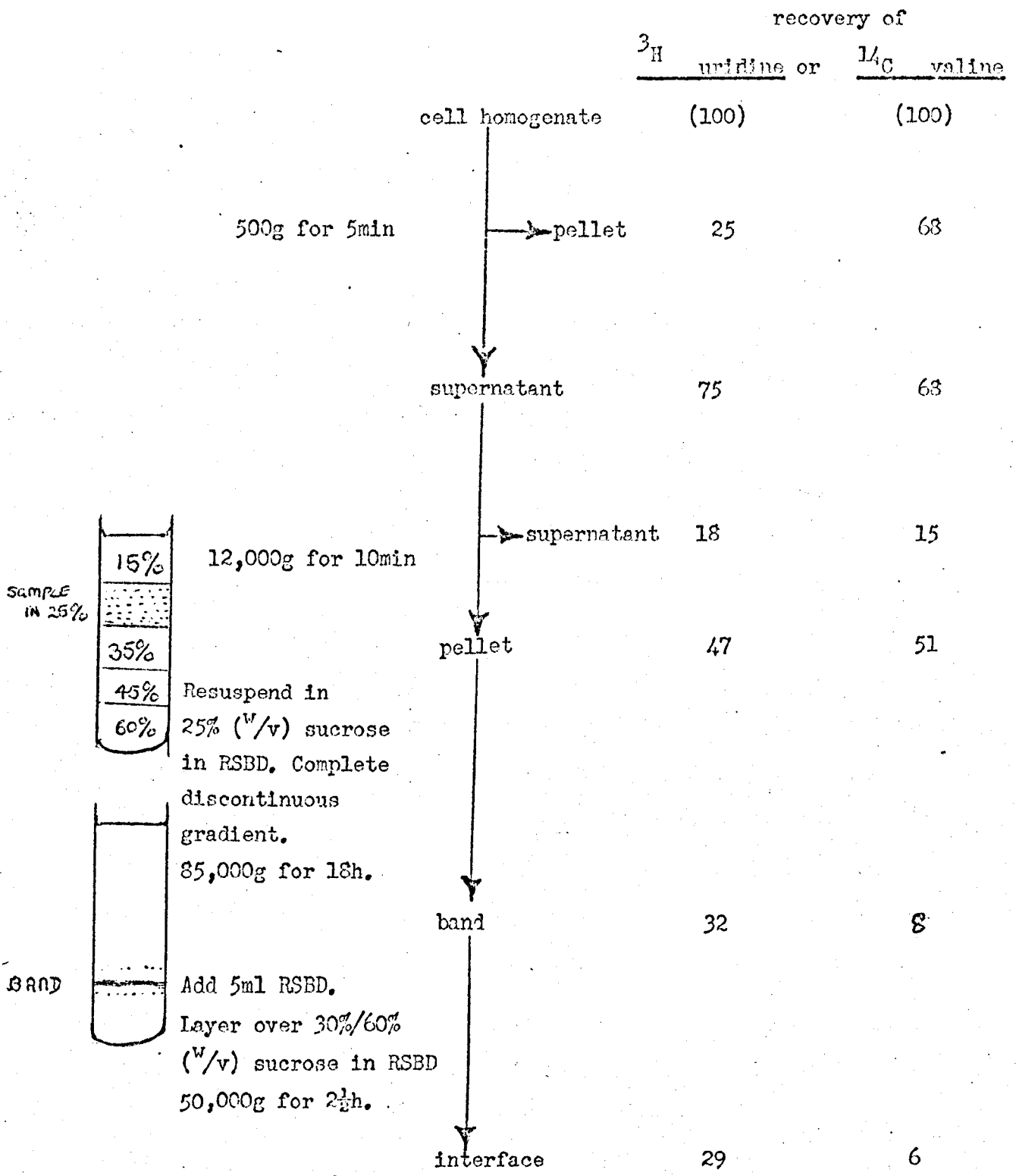
12. Extraction of RNA polymerase

The method involved is shown on a flow chart in table 1. At 5 hours after infection, chick embryo cell monolayers (10^9 cells) were washed twice with 10ml of cold PBS, and once with 10ml of cold reticulocyte standard buffer containing dextran sulphate (RSBD:0.01M-tris+ HCl pH7.4, 0.01M-KCl, and 0.0015M-$MgSO_4$ and 10μg/ml dextran sulphate 500). All subsequent operations were performed at 4°C using sterile solutions and apparatus. The sucrose solutions were autoclaved for 10min at 10lb/sq.in. while all other solutions were treated for 25min at 15lb/sq.in. Glass apparatus was autoclaved for 25min at 15lb/sq.in. except for the Dounce homogenisers which were washed twice with ethanol and once with ether and dried before use.

The cells were scraped from the glass Petri dishes and centrifuged at 1000g for 10min. Then the cells were resuspended in 2ml RSBD($4-5x10^8$ cells/ml) and disrupted in a tight-fitting, all glass Dounce homogenizer

Table 1.

The flow chart of the partial purification of the RNA polymerase from chick embryo cells infected with SFV, is shown. Also shown is the percentage recovery of acid precipitable radioactivity when the cells had been prelabelled with either [^{3}H] uridine (100% is equal to 4.3×10^{4} c.p.m.) or [^{14}C] valine for 10min (100% is equal to 8.7×10^{3} c.p.m.) before the polymerase was prepared.


recovery of
^{3}H uridine or ^{14}C valine
cell homogenate (100) (100)
500g for 5min
pellet 25 68
supernatant 75 63
supernatant 18 15
12,000g for 10min
15%
SAMPLE IN 25%
35%
45%
60%
pellet 47 51
Resuspend in 25% (W/v) sucrose in RSBD. Complete discontinuous gradient. 85,000g for 18h.
band 32 8
BAND
Add 5ml RSBD. Layer over 30%/60% (W/v) sucrose in RSBD 50,000g for 2½h.
interface 29 6

(4ml capacity) under conditions that gave maximum breakage of cells without damaging nuclei, as assessed by microscopic examination. Nuclei and cell debris were removed by centrifuging at 500g for 5 min. The supernatant fluids constituting the 500g supernatant fraction, were then centrifuged at 12,000g at 4°C for 10min. The 12,000g pellet was resuspended with 3 strokes of an all glass Dounce homogeniser, in 3ml of RSBD if it was to be assayed, or if further fractionation was to be carried out, in 3.5ml of 25% (W/v) sucrose in RSBD.

Further fractionation of the material was by a modification of the method described by Caliguiri and Tamm (1970). Either the 12,000g pellet, resuspended in 25% (W/v) sucrose, or the whole cell homogenate made up to 25% (W/v) sucrose was layered onto a preformed discontinuous gradient, consisting of 4.5ml of 60% (W/v) sucrose, 4.5ml of 45% (W/v) sucrose and 4.5ml of 35% (W/v) sucrose in RSBD. The sample was overlaid with 4.5ml of 15% (W/v) sucrose in RSBD and the tube was spun for 18 hours at 85,000g at 4°C in a 3x23ml swing out rotor (M.S.E.) Several bands were visible. The bottom of the tube was pierced and the gradient pumped out through the top by displacement with 65% (W/v) sucrose. The u.v. absorption was monitored on a Uvicord II ultraviolet absorptionmeter (LKB Instruments Ltd., Croydon, Surrey) with 0.3mm light path absorbing at 254nm linked to a servoscribe RE 511.20 potentiometric recorder (Smiths Industries Ltd., Wembley, Middlesex). Fractions of the gradient were collected in an LKB ultrorac fraction collector. When no record of the bands was required, samples were collected by aspiration with a Pasteur pipette.

Concentration and further purification of the RNA polymerase was obtained by diluting the fraction to 6ml with cold RSBD, and layering it over a preformed discontinuous sucrose gradient of 4ml 60% (W/v) sucrose under 4ml 30% (W/v) sucrose in RSBD. After centrifugation for 150 min at 50,000g at 4°C in the 6x15ml swing out rotor (M.S.E.), the tube

was pierced and fractions collected by the methods described above. In some experiments the diluted sample was layered onto a preformed linear 25-60% (W/v) sucrose gradient in RSBD, before being centrifuged under the same conditions.

13. Isolation of membranes by the M band technique

Cytoplasmic extracts, prepared as described in the previous section, were subjected to fractionation by the M band technique described by Tremblay, Daniels and Schaechter (1969). Sodium lauroyl sarcinosate in distilled water was layered onto a preformed discontinuous sucrose gradient consisting of 8ml of 15% (W/v) sucrose over 8ml of 40% (W/v) sucrose in 0.01M-tris+HCl (pH7.0), 0.1M-KCl and 0.01M-$MgCl_2$. Then 0.2M-$MgCl_2$ was added to the sample to a final concentration of 0.05M. The sample was layered immediately over the gradient and briefly mixed with the underlying layer of sodium lauroyl sarcinosate. The final concentration of sodium lauroyl sarcinosate in terms of the total layered volume of the sample was 0.2%. The tubes were immediately centrifuged in a 3x23 swing out rotor (M.S.E.) at 50,000g for 60min at 20°C. The magnesium lauroyl sarcinosate crystals, trapping some membrane material, formed a sharp white band (M band) at the interface between the 15% (W/v) and 40% (W/v) sucrose. The fractions were either collected from the gradient by the method described above, (Section 12), or a small Pasteur pipette was lowered gently through the gradient, and the M band was carefully sucked into it. RNA was extracted from the M band by the phenol/SDS technique described above (Section 9).

14. Isolation of endoplasmic reticulum and plasma membranes

The isolation of endoplasmic reticulum and plasma membranes was carried out by the method described by Bingham and Burke (1972).

15. Treatment of RNA with ribonuclease

The RNA samples to be treated with ribonuclease were dissolved in double strength standard saline citrate (0.3M-NaCl and 0.3M citric acid+ NaOH pH 7.0), and 20µg/ml bovine pancreatic ribonuclease, also dissolved in double strength standard saline citrate, was added to a final concentration of 1µg/ml. The reaction mixture was incubated at 37°C for 30 min. The reaction was stopped by cooling to 4°C and adding 1ml of cold 0.05M-$Na_4P_2O_7$ in 10% ($^W/_v$) TCA and 0.1ml of 10mg/ml BSA. The acid insoluble precipitates were then collected on glass fibre filter discs by the method described above (Section 10). If the RNA was required for further analysis the enzyme was removed by the phenol/SDS technique (Section 9).

16. Analysis of RNA using a sucrose density gradient

A linear gradient of sucrose (5-25% $^W/_v$) in 20ml of 0.1% SDS and TNE buffer was prepared. The sample of RNA to be analysed, dissolved in 1ml of 0.1% SDS in TNE buffer, was carefully layered on to the top of the preformed sucrose gradient and centrifuged at 50,000g for 16 hours at 4°C in a 3x23ml swing out rotor (M.S.E.). The u.v. absorption was monitored and 0.5ml fractions were collected by the technique described in Section 12. The radioactivity in the fractions was determined by the addition of 0.05M-$Na_4P_2O_7$ in 10% ($^W/_v$) TCA and 0.1ml of 10mg/ml BSA, and collecting the acid insoluble precipitates on glass fibre filter discs (Section 10). In all figures of sucrose gradients the numbering of fractions is from right to left, to maintain the convention that the bottom of the tube is to the left.

17. Extraction of proteins from cell monolayers

a) labelling of proteins

Cell monolayers infected with SFV at the required time after infection

were washed twice with either 3ml or 10ml of EDA (5cm plastic or 15cm glass Petri dishes) and were then incubated for 30min in the same medium. The fluids were replaced by either 0.5ml (plastic Petri dishes) or 2ml (glass Petri dishes) of EDA containing the radioactive amino acid. The quantity and type of amino acids are specified in the results section. The monolayers were then incubated for the length of the pulse at 37°. If the carbohydrate moiety of glycoproteins was to be labelled, after infection the cells were incubated in maintenance medium containing 1μg/ml actinomycin and lacking calf serum. During the pulse PBS replaced EDA as the medium in which the radioactive precursors were added. When protein synthesis up to three hours after infection was investigated, the cells were washed twice with PBS and were incubated in leucine free medium. This medium differed from maintenance medium in three ways. It was lacking leucine and was supplemented with dialysed calf serum to 2% ($^{v}/v$) and contained 1μg/ml of actinomycin D.

b) extraction method I

This method was used for monolayers in 15cm glass Petri dishes. At the end of the pulse the cultures were washed twice with 10ml of cold PBS and once with 10ml of cold hypotonic buffer (0.001M-tris + HCl pH 8.3, 0.001M-$MgCl_2$ and 0.001M-2-mercaptoethanol). All the cells were scraped off the glass and centrifuged at 1000g for 10min at 4°C. They were resuspended in 1ml of hypotonic buffer, before sonication for 1min at 1.2 amps with the $\frac{1}{8}$" probe of a Soniprobe (Dawe Instruments Ltd., London). The sample was kept in ice during sonication. The protein was then solubilised by addition of 1/10th volume 10% ($^{w}/v$) SDS, 1/10th volume 5M-urea and 1/10th volume 10% ($^{v}/v$) 2-mercaptoethanol, and boiling for 2min at 100°C. (Summers, Maizel and Darnell, 1965). The sample was then dialysed overnight against 1 litre of dialysis buffer (either 0.005M-tris+0.038M-

glycine pH8.4 containing 0.1% SDS and 0.01% Clelands reagent or 0.005M-sodium phosphate pH7.2 containing 0.1% SDS, and 0.01% Clelands reagent), and stored at -20°C. Samples dialysed against the latter,(phosphate buffer)gave sharper bands when they were analysed by polyacrylamide gel electrophoresis.

c) extraction method II

This method was used for cultures in 5cm plastic Petri dishes. At the end of the labelling period, the dishes were plunged into a bath containing a freezing mixture of solid carbon dioxide in methanol. The fluids froze instantaneously, and then 3ml of 10% ($^{W}/v$) TCA was added which also froze. The dish was placed at room temperature and allowed to thaw. The acid insoluble precipitate was washed three times with 3ml of 10% ($^{W}/v$) TCA, then three times with 3ml of ethanol, and finally three times with 3ml of 80% acetone containing 0.1M-HCl, before being allowed to dry. The sample was then dissolved in 0.4ml of 0.005M-sodium phosphate pH7.2, containing 1% ($^{W}/v$) SDS, 1% ($^{V}/v$)2-mercaptoethanol, and 0.5M-urea, and boiled for 2min at 100°C before storing at -20°C. Using extraction method II it was possible to obtain protein samples of higher specific activity than samples obtained using extraction method I. The protein bands on polyacrylamide gel electrophoresis were sharper using method II. The protein nature of the sample was confirmed in the way described by Hay et al (1968).

18. Preparation of molecular weight standards

a) Coomassie blue staining technique

The protein molecular weight standards were dissolved in PBS to a final concentration of 1mg/ml and then 1/10th volume of 10% ($^{W}/v$) SDS, 1/10th volume of 10% ($^{V}/v$)2-mercaptoethanol and 1/10th volume of 5M-urea

were added. The sample was treated at 100°C for 1min before dialysis overnight against 1 litre of dialysis buffer. The samples were then stored at -20°C.

b) Dansylation technique

The protein molecular weight standards (5mg) were dissolved in 1ml 0.01M-sodium phosphate buffer (pH7.2) and 0.5ml of 10% ($^{W}/v$) SDS in the same buffer was added. Dansyl chloride was dissolved in acetone at room temperature to a concentration of 100mg/ml. A 0.05ml aliquot was added to the sample which was then boiled for 2.5 min. Then 0.15 ml of 10% ($^{V}/v$)2-mercaptoethanol was added, and the sample was boiled for 1min more. After cooling to room temperature, the sample was passed through a Sephadex G-25 column equilibrated with 0.01M-sodium phosphate buffer pH7.2, containing 0.1% SDS. The Sephadex G-25 column was formed in a small pasteur pipette as described by Talbot and Yphantis (1971). The separation from the unreacted dansyl chloride could be followed by illuminating the column with a u.v. lamp. The protein band passed through the column in the void volume, and was recovered in 1.5ml before storage at room temperature. This method was used when it was wished to monitor the progress of the electrophoresis, as the marker proteins could be followed by illuminating the electrophoresis apparatus with a u.v. light.

c) ^{14}C radioactive labelling technique

The method was essentially that of Rice and Means (1971). Samples of the protein standards (0.2mg) were dissolved in 0.2ml of 0.2M-boric acid-NaOH pH9.0. To the solution, cooled in ice, 20µl of 0.05M-[^{14}C]-formaldehyde was added. This was followed in 30 sec by the sequential

additions of 5µl of freshly prepared 5mg/ml sodium borohydride. After a further minute an extra 30µl of 5mg/ml sodium borohydride was added. Then the sample was dialysed overnight against 1L of dialysis buffer before being stored at -20°C. This method was used to establish the molecular weights of unknowns labelled with [^{3}H] by co-electrophoresis.

d) [^{125}I] radioactive labelling technique

The method used was Stanley and Haslam's (1971) modification of the technique described by Marchalonis (1969). The sample was dissolved in 0.05M-sodium phosphate (pH7.2) at a concentration of 1mg/ml and a 100µl aliquot was mixed with 20µl of [^{125}I] (40µCi). A 10µl aliquot of a freshly prepared solution of chloramine T (14.1mg/ml) was added, and the reaction mixture was shaken vigorously for 30sec. The reaction was stopped by adding 20µl of potassium metabisulphite (13.9mg/ml) and 50µl of 0.1M-KI before dialysis overnight against 1L of dialysis buffer.

19. Polyacrylamide gel electrophoresis of proteins

The system used was a modification of that described by Laemmli (1970). Electrophoresis was carried out in 10% (^{W}/v) polyacrylamide gels cast to a height of 90mm, 120mm, or 190mm in 6mm internal diameter perspex tubes. In some experiments a 10mm long stacker gel of 3% (^{W}/v) polyacrylamide was formed on top of a 80mm long 10% (^{W}/v) polyacrylamide separation gel. The gels were polymerised at room temperature from the following reagents:

	3%	10%
acrylamide	3% (W/v)	10% (W/v)
NN^{1}-methylenebisacrylamide	0.09%	0.27%
SDS	0.1%	0.1%
urea	0.5M	0.5M
tris-HCl (pH 8.9)	0.375M	0.375M
TEMED	0.05%	0.05%
ammonium persulphate	0.06%	0.06%

(the concentrations are the final concentrations in the mixture.) While the gels were setting, 0.1ml of distilled water was layered carefully onto the top of the setting gels.

Electrophoresis was carried out using a Shandon disc electrophoresis kit (Shandon Scientific Co., Ltd. London) with the gels fitted vertically to connect the upper and lower buffer reservoirs. The lower buffer reservoir had been modified so that almost the complete length of the perspex tubes could be submerged in buffer, to provide greater cooling (Maizel, 1971). The electrophoresis buffer contained 0.05M-tris+0.38M-glycine pH8.4, 0.1% SDS and 0.1% 2-mercaptoethanol. A 0.1ml aliquot of 0.1M-reduced glutathione dissolved in dialysis buffer was layered onto the top of each gel. The gels were then pre-electrophoresed for 30min at 100V, with the positive electrode in the lower buffer reservoir, and the negative electrode in the upper reservoir. If the sample had been stored at -20°C, it was thawed and then boiled for 1min before being mixed with 1/5th volume of 50% (W/v) sucrose and 1/10th volume of 0.1% Bromophenol blue. The sample was then applied to the top of the gel (referred to as the origin in the Results Section). Electrophoresis was carried out for either 4$\frac{7}{8}$h (90mm gel), 8$\frac{1}{4}$h (120mm gel) or 11$\frac{1}{2}$h (190mm gel) at a constant potential difference of 100V. Immediately after electrophoresis

the gels were fixed in 12.5% ($^{W}/v$) TCA.

20. Polyacrylamide gel electrophoresis of RNA

The system used was a modification of those described by Dingman and Peacock (1968) and Summers (1969). Electrophoresis was carried out in 2% ($^{W}/v$) polyacrylamide, 0.5% agarose gels cast to a height of 190mm in 6mm internal diameter perspex tubes. To prevent solidification of the agarose, mixing and degassing was carried out at 50°C. The gels were polymerised at room temperature from the following reagents:

	2.0%
acrylamide	2.0%($^{W}/v$)
N,N^{1}-methylenebisacrylamide	0.1%
agarose	0.5%
tris	0.0278M
NaH_2PO_4	0.0332M
EDTA	0.001M
SDS	0.2%
TEMED	0.066%
ammonium persulphate	0.066%

(the concentrations are the final concentrations in the mixtures). While the gel was setting, 0.1ml of distilled water was layered carefully onto the top of the setting gels. The gels were stored overnight at 4°C before use the next day. The gels were extruded from the tubes and the agarose tip removed with a razor blade, leaving a clean flat surface of polyacrylamide/agarose mixture. The gels were drawn back into the tubes before use.

Electrophoresis was carried out in the same apparatus that was used

for protein polyacrylamide gel electrophoresis, except that the buffer was recirculated by pumping from the lower to the upper reservoirs. The electrophoresis buffer (E buffer; Bishop, Claybrook and Spiegelman, 1967) contained 0.0272M-tris+0.0332M-NaH_2PO_4 (pH7.8), 0.001M-EDTA and 0.2% SDS. The gels were then pre-electrophoresed for 1hr at 100V with the positive electrode in the lower buffer reservoir and the negative electrode in the upper reservoir. For actual electrophoresis the RNA sample was dissolved in 0.1ml of buffer containing 0.002M-tris+0.003M-NaH_2PO_4(pH7.8) and 0.2% SDS, mixed with 1/5th volume of 50% ($^w/_v$) sucrose, and 1/10th volume of 0.1% bromophenol blue, before applying to the top of the gel (referred to as the origin in the Results section). Electrophoresis was carried out for 7½hr at a constant potential difference of 100V. Immediately after electrophoresis the gels were washed for 3hrs in distilled water, before freezing at -20°C.

21. Detection of sample bands after electrophoresis

a) RNA optical density markers and dansylated proteins

Immediately after electrophoresis, the gels were held under a u.v. lamp in a dark room when the bands of the RNA optical density markers could be clearly seen. The distance from the origin could then be measured. If a more permanent record was required the gels could be scanned in a Chromoscan densitometer (Joyce, Loebl and Co., Ltd. Gateshead, Durham), to give a trace of the peaks.

Under a u.v. lamp the bands of dansylated protein could readily be detected by their fluorescence. The distance from the origin could then be measured.

b) staining

Immediately after electrophoresis, the gels were fixed in 12.5%

($^{W}/v$) TCA overnight. The protein bands were stained with Coomassie brilliant blue (a 1:20 dilution of 1% ($^{W}/v$) Coomassie brilliant blue in 12.5% ($^{W}/v$) TCA) for 6 hours. Excess stained was removed from the gels by washing with 7% ($^{V}/v$) acetic acid in 10% ($^{V}/v$) methanol for 3-4 days with several changes. (Maizel et al, 1970). The position of stained protein bands on the gels was recorded by scanning at 570nm in a Joyce Loebl Chromoscan.

c) autoradiography

Immediately after electrophoresis the gels were fixed overnight in 40% ($^{W}/v$) sulphosalicylic acid. They were then sliced longitudinally in an apparatus similar to the one described by Fairlands, Levinthal and Reeder (1965). The two internal slices were then vacuum dried onto a piece of Whatman 3MM filter paper, under an infra-red lamp for 18 hours. Finally the slices were exposed to Kodirex X-ray film (Kodak Ltd., London) for several days, before being developed according to the manufacturer's instructions.

d) determination of radioactivity in 1mm slices

After electrophoresis the gels were fixed in 12.5% ($^{W}/v$) TCA overnight and then immobilised for chopping by freezing at -70°C onto the brass platform of a laboratory gel slicer (Mickle Laboratory Engineering Co., Ltd., Comshall, Surrey) and covered with wetted filter paper. The gel slicer had been modified so that the platform was cooled to -10°C by circulating methanol from a cooling bath (Grant Instruments Ltd., Barrington, Cambridge). Slices (1mm thick) were cut, placed in scintillation vials, and dissolved in 0.2ml of 100 volume hydrogen peroxide by incubating at 60°C for 90 min. (Young and Fulhurst, 1965).

In order to check the slices for uniformity, a gel was prepared that

contained 1μCi [^{3}H] valine. The gel was frozen, sliced and the radioactivity in each slice was determined. The difference in radioactivity between slices was less than 2%. In all figures of polyacrylamide gels the numbering of fractions is from left to right; the left representing the origin, and the direction of migration being from left to right.

22. Determination of radioactivity

Two types of sample were counted for radioactivity:

(i) Samples in 0.5M-NaOH. These were counted in 10ml of gel scintillator.

(ii) samples precipitated onto filter discs and dissolved gel slices had 10ml of "Triton/toluene" scintillator added to them.

The samples were cooled to 4°C and counted in Packard Tricarb series 3000 liquid scintillation counter fitted with a Teletype digital line printer (Teletype Corporation, Skokie, Illinois, U.S.A.) The results were corrected for background radioactivity and expressed as counts per min (c.p.m.). Simultaneous counting of [^{3}H] and [^{14}C]radioactivity was carried out using the channel-ratio method of Hendler (1964) encoding the information in ASC-2 code onto punched tape. The results were calculated using an I.C.L. 1900 series computer, which printed out the results, and utilising the digital plotter facility, could also plot the results onto a graph. The programme was written in algol by myself.

The whole procedure, from the labelling of the proteins in vivo till the computer processing of the results was checked in the following ways. First uninfected cells were labelled simultaneous with [^{3}H] and [^{14}C] valine. On analysis of the gels, the electrophoretograms of the two isotopes /

could be superimposed upon each other. Then one culture of SFV infected cells, and one of uninfected cells were labelled with [^{3}H] valine. At the same time two equivalent cultures were labelled with [^{14}C] valine. Then the cells were harvested and the proteins extracted. The two samples from infected cultures were independently mixed with the sample from the uninfected cultures labelled with the other isotope. The resulting two mixtures were analysed. The same proteins could be detected in the same amounts in both samples. Therefore it was assumed in evaluating the results that no artefacts were introduced by the manipulations described above.

23. Enzymic determinations

a) lactate dehydrogenase

Lactate dehydrogenase (E.C.1.1.1.27) was assayed by the method of Meister (1950) by following the reduction of NADH on conversion of pyruvate to lactate. The reduction was followed spectrophotometrically at 340nm in a Unicam SP800 (Pye Unicam Ltd., Cambridge) linked to a servoscribe potentiometric recorder. A decrease in absorbance of 2.07 units/min was equivalent to the reduction of lumole of NADH.

b) succinate dehydrogenase

Succinate dehydrogenase (E.C.1.3.99.1) was measured by the method of Porteous and Clark (1965) by following the reduction of 2-(p-iodophenyl) -3-(p-nitrophenyl)-5-phenyl-tetrazolium hydrochloride in the absence of phenazine methosulphate at 490nm in the Unicam SP800 spectrophotometer. The enzyme was used as a marker for material from the mitochondrial fraction.

c) NADPH-cytochrome C reductase

NADPH-cytochrome C reductase (E.C. 1.6.2.3.) was assayed by the

method of Ragnotti, Lawford and Campbell (1969) by following the reduction of cytochrome C by the change in absorbance at 550nm, in the Unicam SP800 spectrophotometer. It was used as a marker for endoplasmic reticulum.

d) 5^1-nucleotidase

5^1-nucleotidase (E.C.3.1.3.5.) was estimated by the method of Avruch and Wallach (1971) by following the hydrolysis of [^{3}H] AMP. The remaining [^{3}H] AMP, after an incubation of 30min,was precipitated with 0.25M-$Ba(OH)_2$. The released [^{3}H] adenosine remained in the supernatant fluids and was mixed with 10ml of "gel" scintillator before determining its radioactivity. This enzyme was used as a marker for the plasma membrane.

24. Determination of protein

Protein concentrations were determined by the method of Lowry, Rosebrough, Farr and Randall (1951) using the continuous flow system. of Gibbs and Bright (1968) BSA (fraction V) was used as a standard. The protein determinations were corrected for the sucrose content of samples by the method of Gerhardt and Beevers (1968).

RESULTS

I. Conditions of virus growth

The results section is divided into three parts. The first part describes the conditions used for growth of SFV. The second covers the analysis of the proteins specified by SFV, while the final part discusses different sub-ceullular fractions and the viral RNA and protein associated with them.

The growth of SFV in different cell types and media and the kinetics of RNA and protein synthesis are described in the first section, as well as the effect of virus infection on host cell protein synthesis.

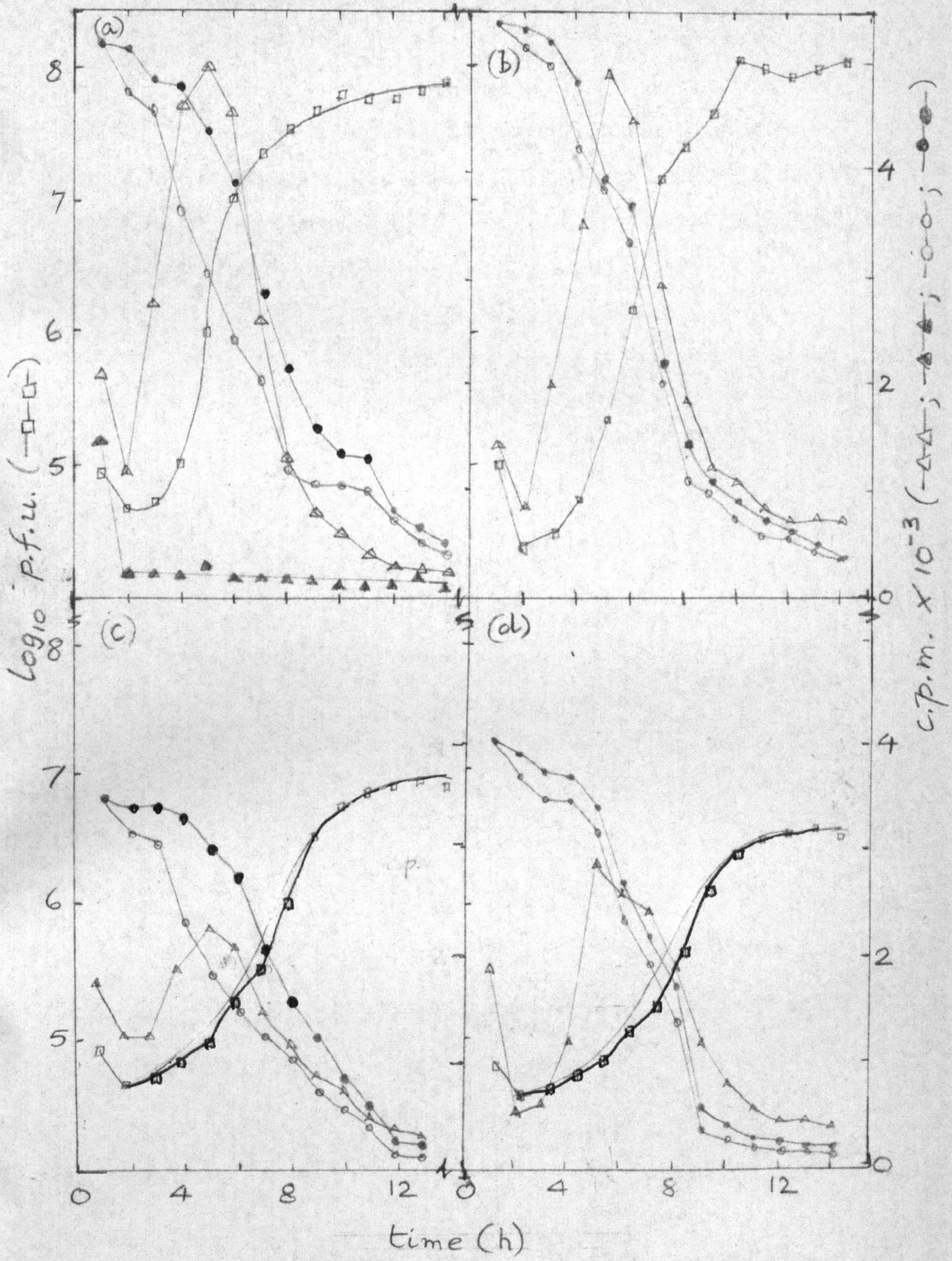
1. Growth of SFV in monolayer cultures

The growth of SFV in chick embryo cells, BHK cells, HEL cells, and L-929 cells, is shown in Fig.1. Time after infection refers to the time elapsed after the end of the absorption period. All the experiments were carried out using cells treated with actinomycin D. There was very little infectious virus released during the first three hours, after which the virus was released exponentially. The maximum yield of virus was obtained 10h after infection. The growth of the virus in all four types of cell showed the same kinetics, but the yield of released virus was different in the different cell lines (measured as infectious virus released/cell).

An increase in the rate of viral RNA synthesis was detected before the exponential release of virus. The maximal synthesis of RNA was at 4-5h after infection. Because these experiments were done in the presence of actinomycin D there was no appreciable synthesis of RNA which was host-directed after 1h (Fig.1a). However the run down of protein synthesis was much slower, and the proportion of the total protein synthesis that was virus-directed varied between the cell types. SFV is known to inhibit host cell protein synthesis (Strauss, Burge and Darnell, 1969) and Fig.1 shows that the amount of inhibition was found to depend on the cell type. The inhibition was greatest in BHK cells and least in L-929

Figure 1.

SFV was grown in chick embryo fibroblasts (Fig.1a), L-929 cells (Fig.1b), BHK-21 cells (Fig.1c) and HEL cells (Fig.1b) that had formed monolayers in 5cm Petri dishes. At the times indicated, released virus (–□——□–), RNA synthesis in uninfected (–▲——▲–) and infected cells (–△——△–), and protein synthesis in both uninfected (–●——●–) and infected cells (–○——○–) was assayed. The chick cell monolayers consisted of $2x10^6$ cells, while the other types of monolayer consisted of $1x10^6$ cells.


(a)
(b)
(c)
(d)
$\log_{10}$ p.f.u. (—□—□—)
c.p.m. $\times 10^{-3}$ (—△—△—; —●—●—; ---○---○---; —●—●—)
time (h)
0
4
8
12
5
6
7
8
2
4

cells.

The virus could form plaques within 36h in all four cell types, but the size of the plaques varied. The plaques were largest in BHk-21 cells and smallest in L-929 cells.

2. Incubation of infected cultures at different temperatures.

The growth of SFV in chick embryo cells at 30°C, 37°C and 39°C is shown in Fig.2. At 30°C RNA synthesis reached a maximum at 7h after infection while maximum yield of virus was not reached until 13h. At 39°C the virus grew much faster, RNA synthesis reaching a maximum by 4h, while the maximum titre of virus was released by 8h.

The rate of protein synthesis in infected chick embryo fibroblasts was compared with that of uninfected cells. Between 2 and 3h after infection the rate of protein synthesis in the infected cells fell rapidly, and then subsequently decreased slowly over the next 10h. The decrease in the rate of protein synthesis is less rapid in the uninfected cells, and is due to the effect of the actinomycin D.

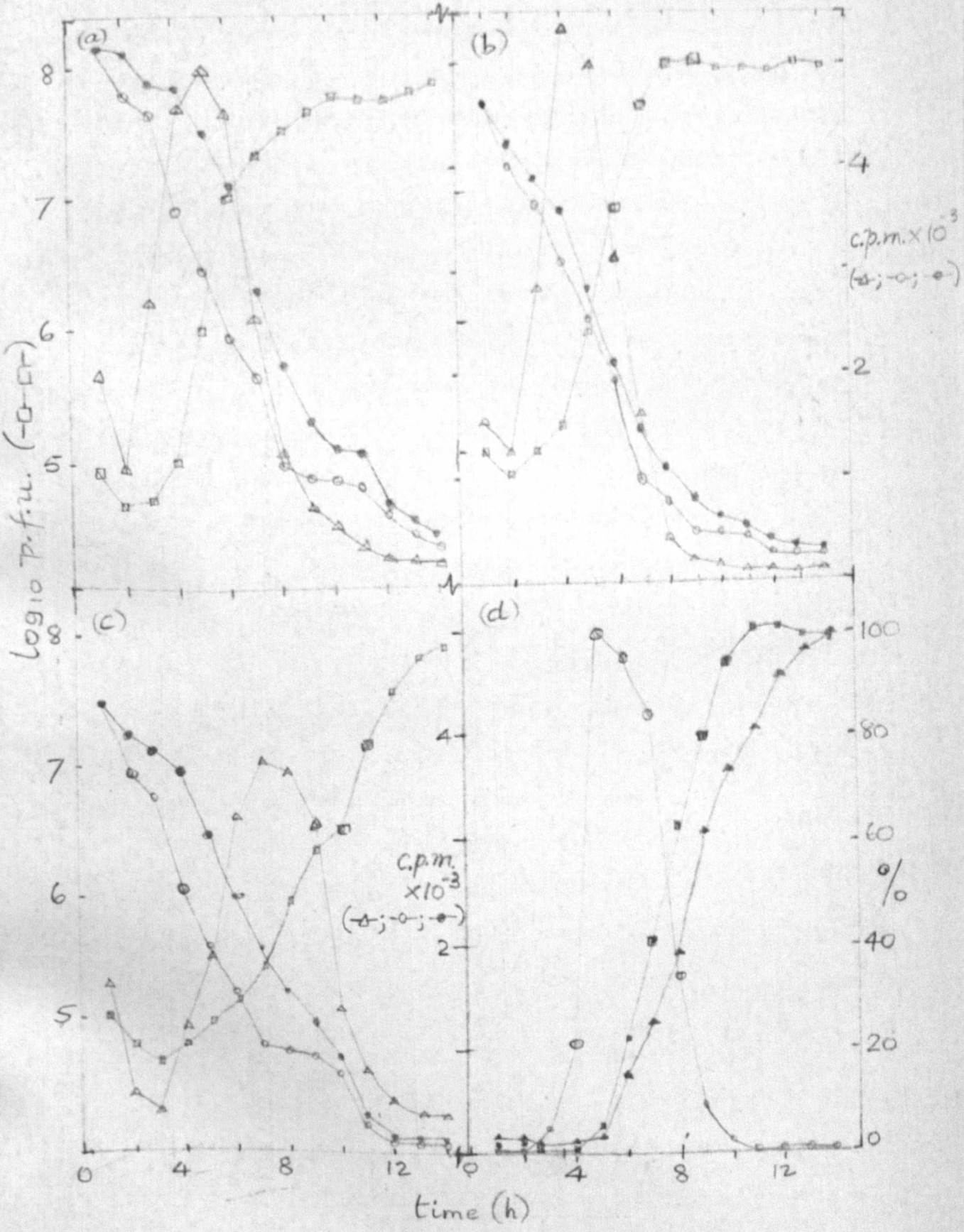
The release of virus haemagglutinating activity, and of lactose dehydrogenase activity can also be used to measure virus growth (Fig.2d). The increase in both these parameters paralleled the release of infectious virus. Both techniques are less sensitive than plaque assays, but are quicker. RNA polymerase activity, assayed *in vitro*, reached a maximum at 5h after infection (Fig.2d). The rise in activity followed the appearance of viral RNA, but enzyme activity was not detectable as early in the growth cycles, presumably because of the insensitivity of the assay (Martin and Sonnabend, 1967).

3. Multiplicity of infection

Monolayers of chick embryo cells were infected with SFV at multiplicity

Figure 2.

SFV was grown in monolayer cultures of chick embryo fibroblasts in 5cm Petri dishes incubated at 37°C (Fig.2a), 30°C (Fig.2c) and 39°C (Fig.2b). Similar assays to those carried out in Fig.1 were completed at the times indicated. Released virus (—□—□—); RNA synthesis in infected cells (—Δ—Δ—); protein synthesis in uninfected cells (—● —●—) and infected cells (—○—○—). Three other parameters were measured in the experiments shown in Fig.2d. SFV was grown in monolayer cultures of chick cells in 15cm glass Petri dishes. The cultures were incubated at 37°C and at the times indicated intracellular RNA polymerase activity (—○—○— ; 100% is equivalent to 4973 cpm/mg/60min) and extracellular haemagglutinin (—■—■— ; 100% is equivalent to log $_2$8 haemagglutinin units) and LDH activity(—▲—▲— ; 100% is equivalent to 3.1 I.U. of LDH activity) were measured.


(a)
(b)
(c)
(d)
log10 p.f.u. (-□-□-)
c.p.m. ×10⁻³ (-△-; -○-; -●-)
%
time (h)
0
4
8
12
5
6
7
8
2
4
20
40
60
80
100

of infection (m.o.i.) that varied from 0.1p.f.u./cell to 100p.f.u/cell. The monolayers were washed very carefully to remove any virus that had not been absorbed after the 1h period of infection. The kinetics of virus release were then measured by assaying the infectious particles in the supernatant fluids. The higher the m.o.i. the faster the growth of the virus (Fig.3). This result is similar to that reported by Baltimore, Girard and Darnell (1966) for polio virus.

4. Effect of different culture media

Medium 199, used for culturing chick embryo cells, contains between 20 and 150µg/ml of each amino acid. In order to decrease the dilution of the radioactive amino acids with non-radioactive amino acids present in the medium, both EDA, PBS and maintenance media lacking calf serum, were used as the culture medium prior to and during the labelling period. The effect of this change in culture medium on the growth of SFV, and the incorporation of [^{3}H]uridine, [^{3}H] glucosamine or [^{3}H] valine into acid insoluble material was studied. Provided the change was made at least 3½h after infection no reduction in the yield of infectious virus was observable (Table 2). But the rate of incorporation of [^{3}H] valine, [^{3}H]uridine and [^{3}H] glucosamine was significantly increased by incubating the cultures in a medium depleted of other metabolites.

The incorporation of [^{3}H] uridine, [^{3}H] glucosamine and [^{3}H] valine into an acid insoluble precipitate of uninfected cultures of chick embryo cells was followed over three hours. The rate of incorporation of the radioactive precursor was constant after an initial lag of 3min (Fig.4). The initial period was probably required for the radioactive precursor to equilibrate with the intracellular pools of precursors.

Figure 3.

Chick embryo fibroblasts, in monolayer culture in 5cm plastic Petri dishes were infected with varying m.o.i. of SFV, all contained in 0.5ml of maintenance medium. After 1h the virus was removed, and the cell sheets were very carefully washed five times with 5ml of maintenance medium. Then the cultures were incubated at 37°C in 1ml of maintenance medium. At the times indicated after infection, the culture fluids were assayed.

Symbols; m.o.i. of 0.1 p.f.u./cell (-●-●-); of 1 p.f.u./cell (-▲-▲-); of 5 p.f.u./cell (-■-■-); of 10 p.f.u./cell (-○-○-); of 50 p.f.u./cell (-△-△-); and of 100 p.f.u./cell (-□-□-).

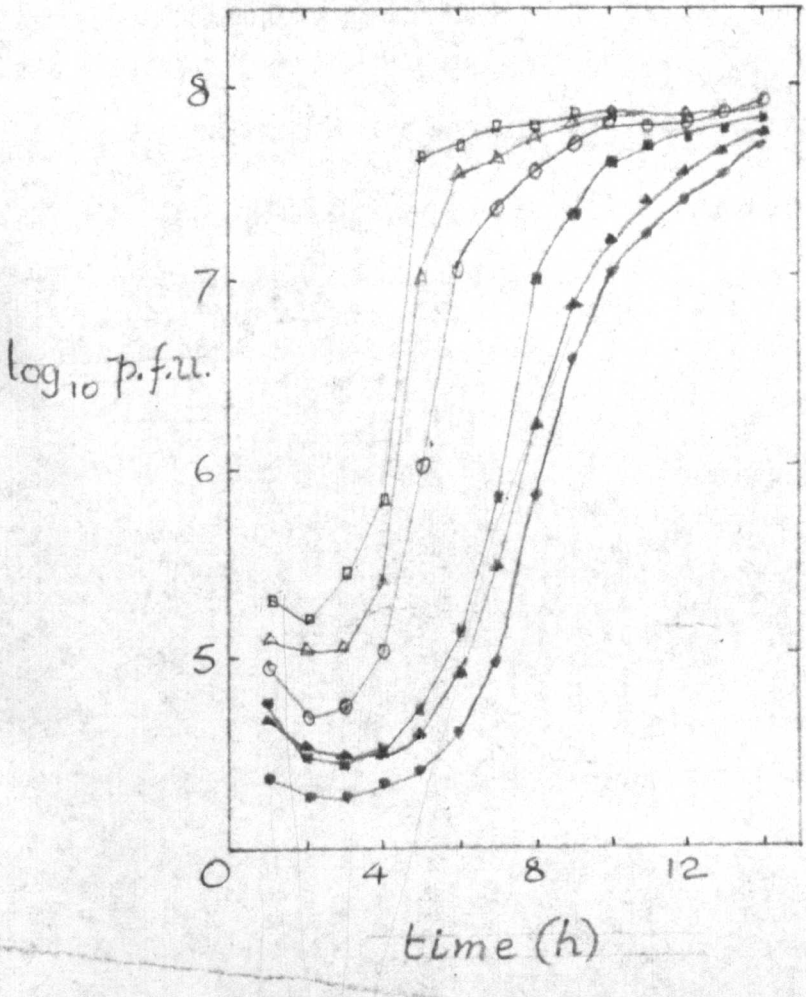

8
7
6
5
0
4
8
12
log10 p.f.u.
time (h)

Table 2.

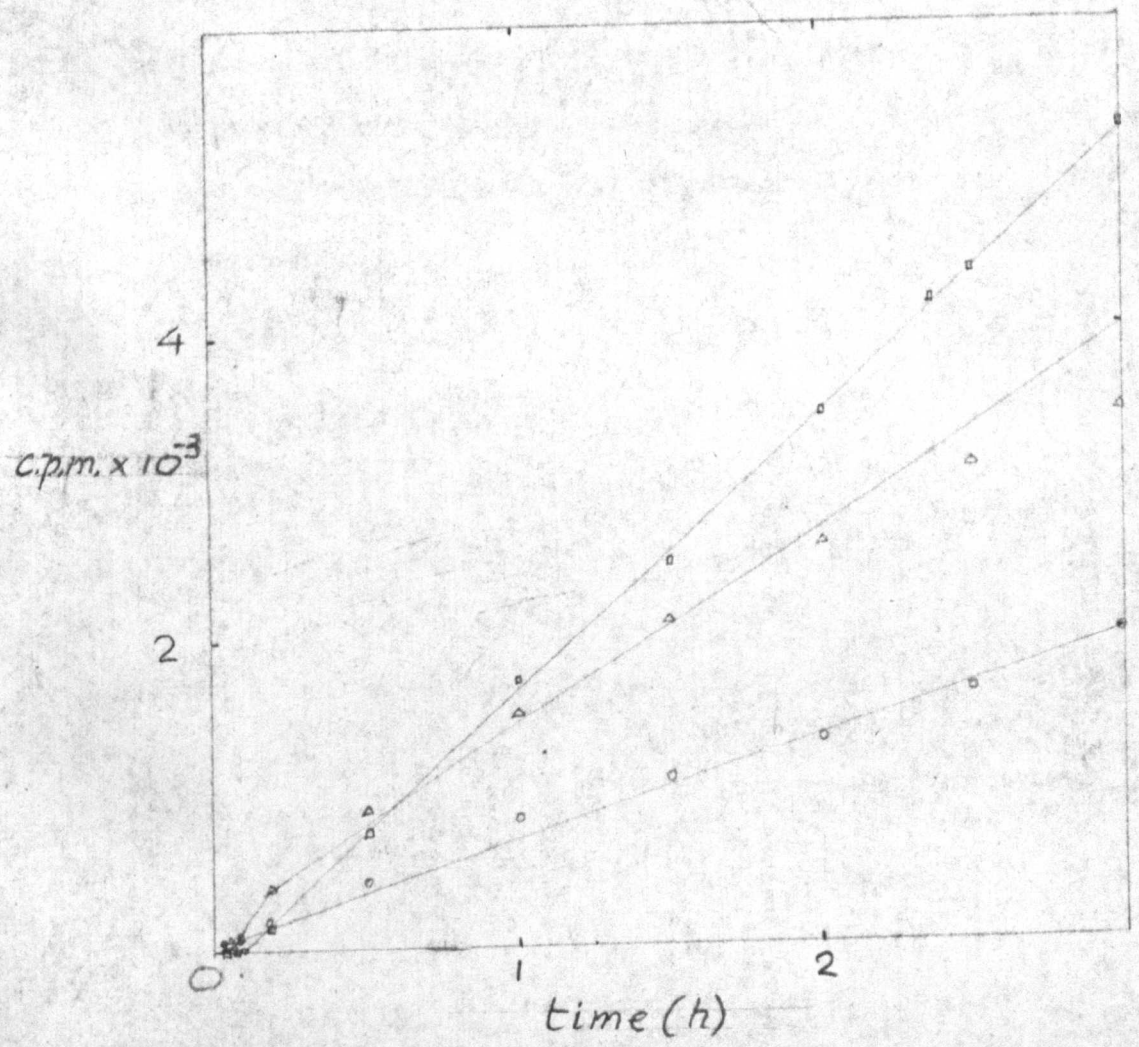
Chick embryo cells, 1h and 3½h after infection with SFV, had the medium of incubation charged from maintenance medium, containing 1µg/ml actinomycin D, to either EDA, or PBS containing 1µg/ml actinomycin D, or maintenance medium lacking calf serum but containing 1µg/ml actinomycin D. Then 4h after infection 1µCi/culture of [^{3}H] uridine, [^{3}H] valine or [^{3}H]glucosamine was added, and 30min later the incorporation into acid precipitable material was measured. Also the yield of infectious virus 9h after infection under the various conditions was measured.

	incorporation (c.p.m.) of			
	uridine	valine	glucosamine	p.f.u.
medium changed after 1h to:				
maintenance medium	3,178	2,976	1,528	3×10^8
maintenance medium - calf serum	2,819	1,750	1,643	9×10^7
EDA	1,786	899	772	3×10^6
PBS	1,123	562	681	8×10^5
medium changed after $3\frac{1}{2}$h to:				
maintenance medium	2,765	2,718	1,326	2×10^8
maintenance medium - calf serum	2,974	2,895	1,865	1×10^8
EDA	4,826	4,923	7,514	2×10^8
PBS	4,735	4,665	9,163	9×10^7

all media used in this experiment contained 1µg/ml actinomycin D.

Figure 4.

Uninfected cultures of chick embryo fibroblasts in 5cm plastic Petri dishes were incubated for 2h in the presence of maintenance medium that contained 1µg/ml of actinomycin D. The culture fluids were then replaced by either more maintenance medium containing actinomycin either with or without calf serum, or EDA or PBS containing 1µg/ml actinomycin D. After 30min radioactive uridine, valine or glucosamine were added to the respective cultures. Incorporation into an acid-insoluble precipitate of the uridine (-□-□-); valine (-△-△-) and glucosamine (-○-○-) was followed.


4
2
c.p.m. x 10⁻³
0
1
2
time (h)

II Virus-specified protein synthesis

The synthesis of virus specified proteins in chick embryo cells was studied using a double-labelling method. The term 'virus-specified protein synthesis' refers merely to the synthesis of proteins which are not present in uninfected cells and does not indicate the mechanism by which it occurs. First the virus-specified proteins were analysed at different times during the growth cycle, in different cell types, and at different temperatures of incubation. Then the carbohydrate content and amino acid composition of these proteins was investigated. Finally, by the use of protease inhibitors and amino acid analogues, the mode of formation of the virus-specified proteins was studied.

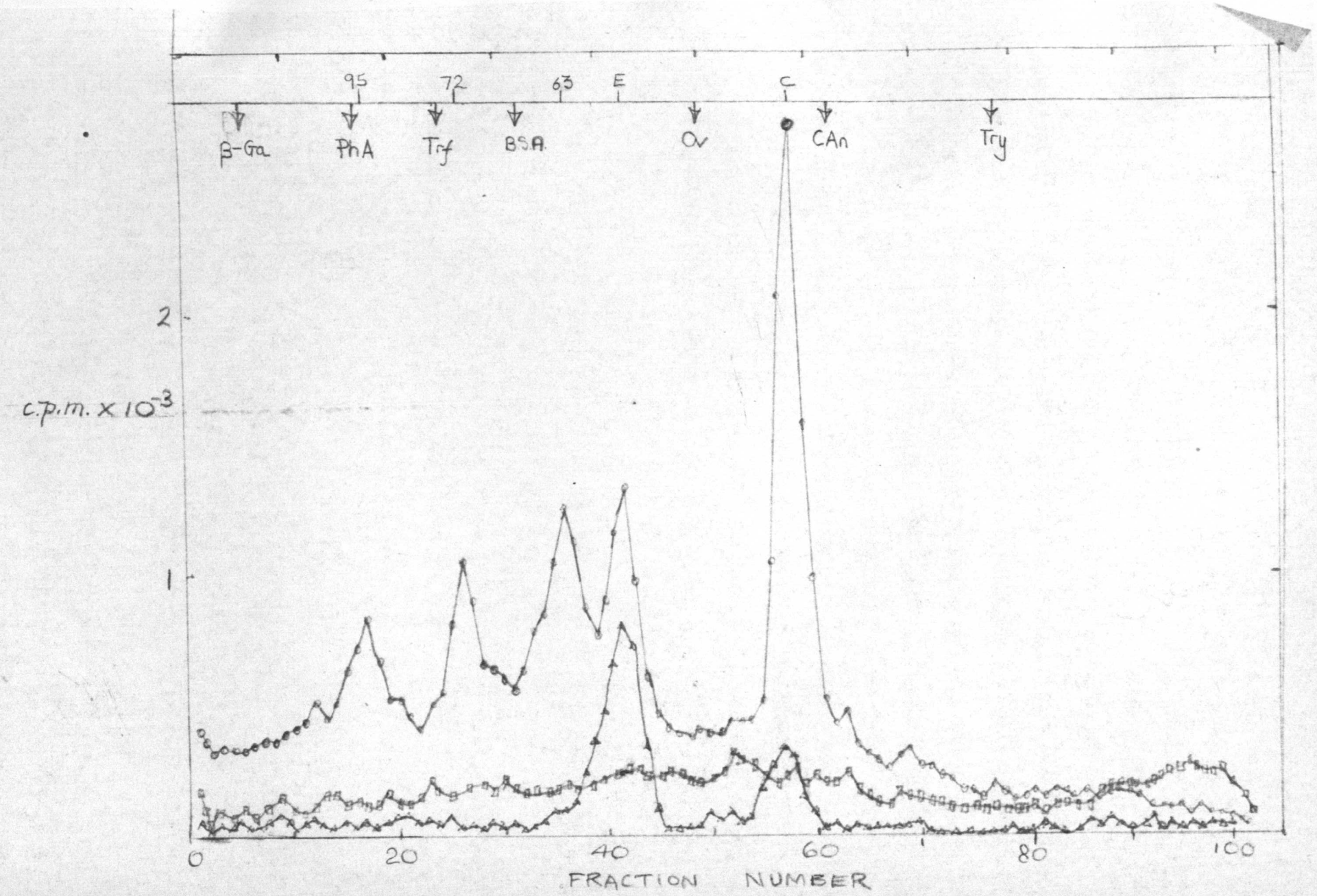
1) Virus-specified protein synthesis in chick cells

The proteins were labelled for a 1h period between 6 and 7 hours after infection, extracted and analysed by polyacrylamide gel electrophoresis. Five virus-induced proteins could be detected in cultures of chick embryo cells (Fig.5). It is apparent that no discrete proteins were being formed in the uninfected cells. This is because the cells had been treated with actinomycin D. In the infected cells, virus-specified protein synthesis accounted for over 85% of all protein synthesis.

Two of the peaks labelled with [^{3}H] amino acid hydrolysate comigrated with the two proteins of the purified virus labelled with [^{14}C] valine (Fig.5). These two polypeptides are labelled envelope (E) and core (C). The other three peaks are identified as NVP95, NVP72 and NVP63 (NVP stands for non-virion polypeptide). The molecular weights of these proteins were determined by the method of Shapiro, Vinuela and Maizel (1967) These authors reported that a linear relationship existed between the logarithm of the molecular weight of proteins and the relative electrophoretic

Figure 5.

Infected (-O-O-) and uninfected (-□-□-) chick embryo cells were labelled with 10μCi/culture of [^{3}H] and 2μCi/culture of [^{14}C] amino acid hydrolysate respectively, between 6 and 7h after infection. The samples were mixed before the proteins were extracted by method I and analysed on 9cm polyacrylamide gels. Marker proteins were run to the positions indicated on the bar (β-Ga:β-galactosidase, PhA; phosphorylase A, Trf; transferin, BSA; bovine serum albumin, Ov; ovalbumin, CAn; carbonic anhydrase, and Try; trypsin). Also included is an analysis on 9cm polyacrylamide gels of the proteins from purified SFV (-Δ-Δ-) labelled with [^{14}C] valine. Please note that in some figures of sucrose gradients or polyacrylamide gels there are two different ordinate scales.


95
72
63
E
C
β-Ga
PhA
Trf
BSA
Ov
CAn
Try
2
1
c.p.m. x 10⁻³
0
20
40
60
80
100
FRACTION NUMBER

mobilities of their SDS complexes. The molecular weights of the marker proteins were taken as follows: trypsin 23,680; α-chymotrypsinogen 25,000; pepsin 35,000; carbonic anhydrase 34,500; ovalbumin 44,800; bovine serum albumin 68,000; transferrin 77,000; phosphorylase A 95,000; and β-galactosidase 135,000 (Darnall and Klotz, 1972). With some of these proteins, multiple bands were observed; in which case the major band was taken as corresponding to the quoted molecular weight. The molecular weights of the virus-specified proteins were estimated to be:

Virus-specified protein	molecular weight
NVP 95	95,000
NVP 72	72,000
NVP 63	63,000
E	53,000
C	36,500

The molecular weight of the core protein is higher than the values previously reported (Hay et al, 1968; Strauss et al, 1968)

The system of polyacrylamide gel electrophoresis described in the methods section was compared with the phosphate system (Summers et al, 1965) and the high urea/phosphate system (Russell and Skehel, 1972). The resolution of protein bands was better in the tris/glycine system used than in the other two systems. In none of these systems, nor in the discontinuous tris/glycine system with a stacker gel (Laemmli, 1970) could a second envelope protein, of molecular weight 45,000 be detected in the virus particle (S.I.T. Kennedy, unpublished observations). The resolution of 10% ($^{W}/v$) tris/glycine polyacrylamide gels was compared with that of 7.5% ($^{W}/v$) and 5% ($^{W}/v$) gels and was found to be better. Using the 5% ($^{W}/v$) and 7.5% ($^{W}/v$) gels NVP 72 and NVP 95 could not be

separated completely. Thus, the 10% ($^{W}/v$) tris/glycine system was chosen for routine analysis of protein samples.

Inclusion of a sulphydryl reductant in the extraction medium was found to be essential in order to prevent dimerisation of the core protein. If reduced glutathathione was not pre-electrophoresed into the matrix of the gel, the envelope peak split into two peaks and the core was partially dimerised. These changes in pattern were believed to be due to the reduction of intra- and intermolecular disulphide bonds.

The protein sample from infected and uninfected cells shown in Fig.5 was analysed on long gels, run so that the Bromophenol blue marker dye was retained at the bottom of the gel (Fig.6) In contrast to the findings of Strauss et al (1969) no material migrating with the bromophenol blue tracker dye could be found. A small peak, which might correspond to protein 5 described by Hay et al (1968) could occasionally be detected. No other material with a molecular weight lower than that of the core was identified.

This sample was also analysed on a 10% ($^{W}/v$) polyacrylamide gel cast with a stacker gel of 3% ($^{W}/v$) polyacrylamide above it. No material was detected in the stacker gel or at the interface with the separation gel (Fig.7). Therefore, in chick embryo cells infected with SFV, only 5 virus-specified polypeptides could be detected reproducibly. Whichever extraction method was used, the whole cell was analysed, unlike all previous methods in which a nuclear fraction was removed before analysis. Thus the counts incorporated into each protein represent the relative rates of synthesis of these proteins. Since the amino acid hydrolysate labelled with tritium was used as the precursor in this experiment, the

Figure 6.

The same protein sample used in Fig.5 from infected (—○-○—) and uninfected (— □-□-) cells, was analysed on a 19cm polyacrylamide gel. The arrow labelled Brb marks the position of the bromophenol blue tracker dye at the end of the period of the electrophoresis.

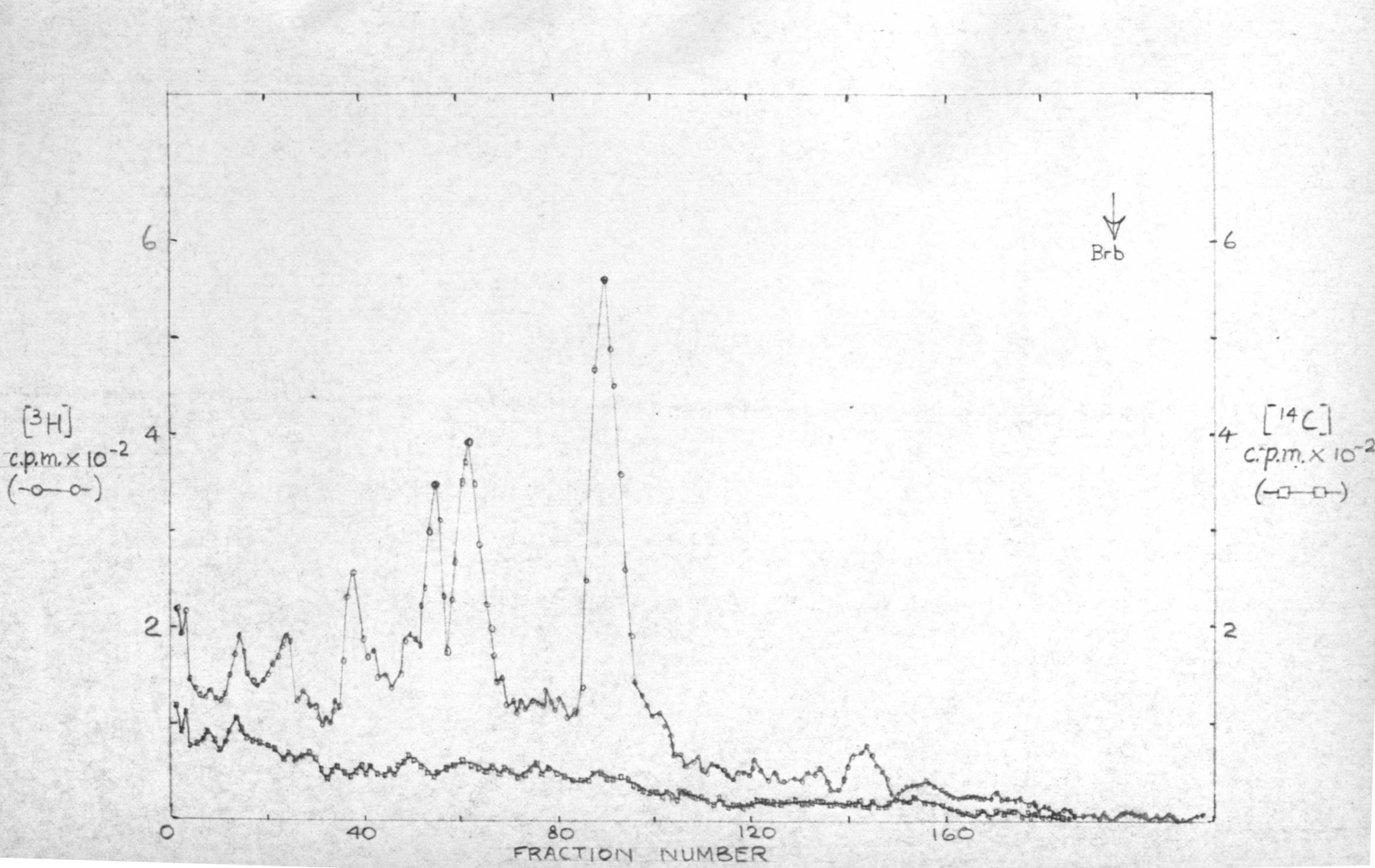
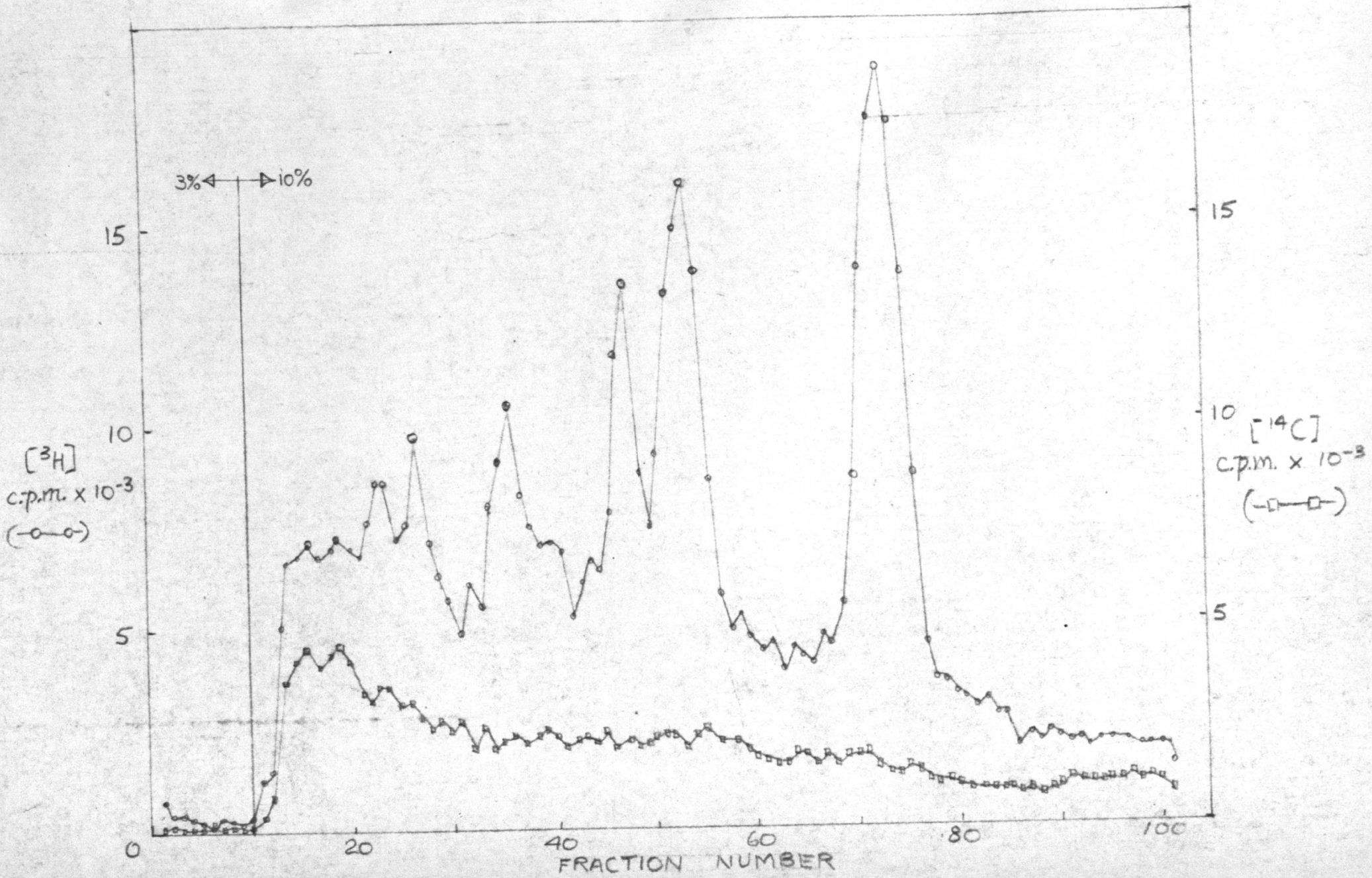

[3H]
c.p.m. x 10-2
[14C]
c.p.m. x 10-2
Brb
6
4
2
0
40
80
120
160
FRACTION NUMBER

Figure 7.

The same protein sample used in Fig.5, from infected (-O -O-) and uninfected (-□-□-) cells was analysed on a 8cm 10% ($^{W}/v$) polyacrylamide gel with a 1cm 3% ($^{W}/v$) stacker gel formed on top of the separation gel. The vertical bar represents the interface between the gels of different concentration.


3%
10%
15
10
5
0
20
40
60
80
100
FRACTION NUMBER
[3H]
c.p.m. x 10-3
[14C]
c.p.m. x 10-3

relative areas of the peaks approximate to the molar ratio in which these proteins are formed. It can be seen that there is no simple relationship between the quantities of these proteins. From a series of gel patterns such as Fig.5, the rate of synthesis of each of the SFV specific proteins at different times after infection were estimated by comparison with the pattern obtained from uninfected cells. The kinetics of appearance of the different virus-specified proteins appeared to be similar (Fig.8). The same virus-specified proteins were observed at all times investigated from 3h to 13h after infection. This was true for cultures incubated at 30°C, 37°C or 39°C. During the course of infection the amount of structural proteins labelled during a 1h period increased relative to the amount of NVP 95, NVP 72 and NVP 63 labelled. The counts under all five peaks of the virus-specified proteins were totalled and expressed as a percentage of the radioactivity in the whole length of the gel. This percentage is a measure of the proportion of protein synthesis that is virus-induced, and rises during the course of infection to over 95% of all protein synthesis (Fig.9a). Thus the inhibition of host cell protein synthesis is greater than indicated by Fig.2 because that figure indicates total protein synthesis rather than just viral. The inhibition of host cell protein synthesis was dependent on the m.o.i. (Fig.9b). The higher the m.o.i. the greater the inhibition.

The following events take place after infection of chick embryo cells with SFV. Between two and three hours after infection host protein synthesis is severely inhibited, while SFV protein synthesis rises rapidly and infectious virus begins to be released exponentially into the culture fluid. SFV protein synthesis reaches a maximum at seven hours after infection, and after eleven hours no more virus was produced, although SFV-specified protein synthesis continues. At times later than three hours after infection virus-specified protein synthesis was readily detected.

Figure 8.

A series of samples similar to that shown in Fig.5 were labelled for different 1h periods after infection. They were analysed by polyacrylamide gel electrophoresis, and the percentage of label in each of the five virus-specified protein peaks was calculated with respect to the total label detectable on the gel. Core protein (-▲-▲-), envelope protein (-●-●-), NVP 63 (-□-□-), NVP 72(-△-△-) and NVP 95 (-○-○-).

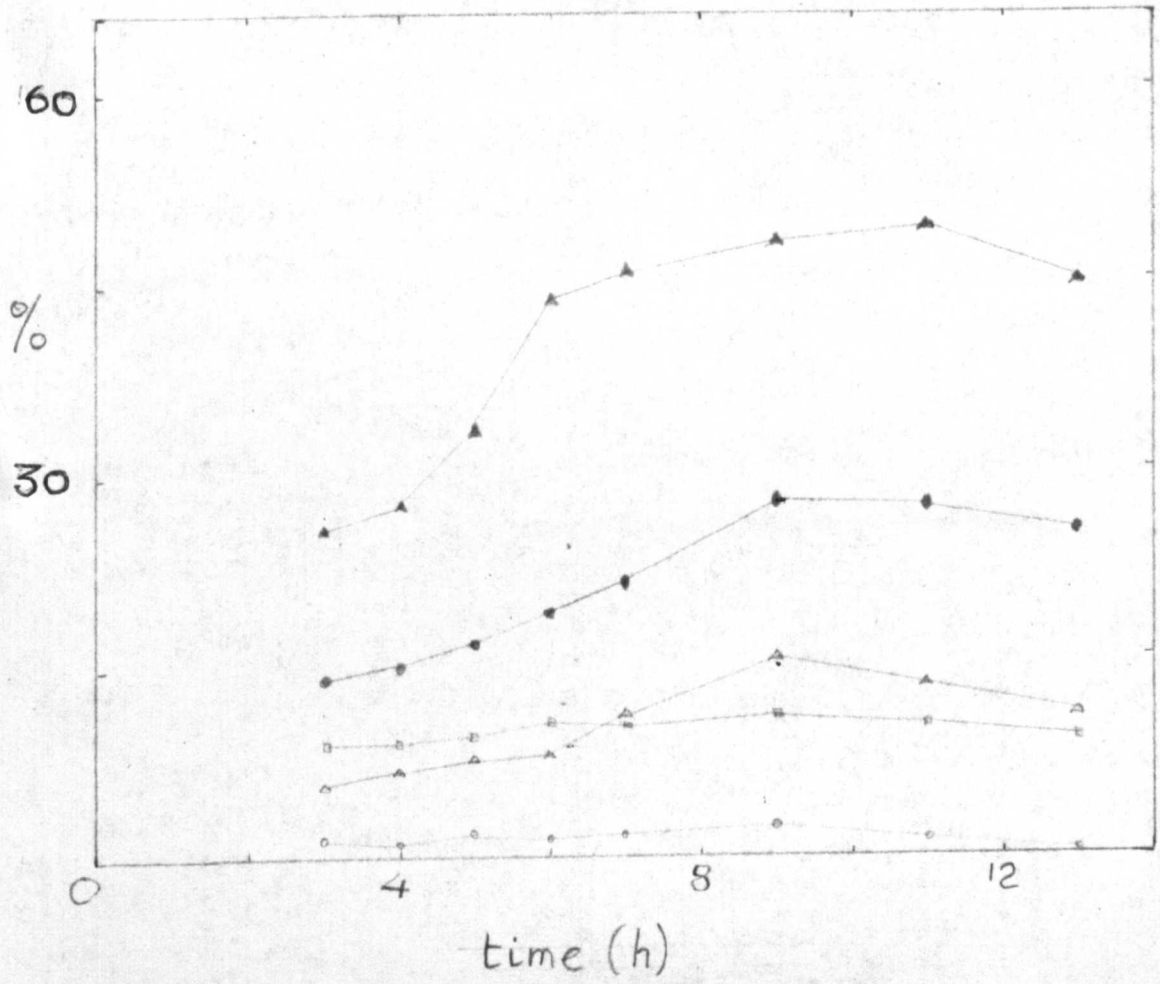
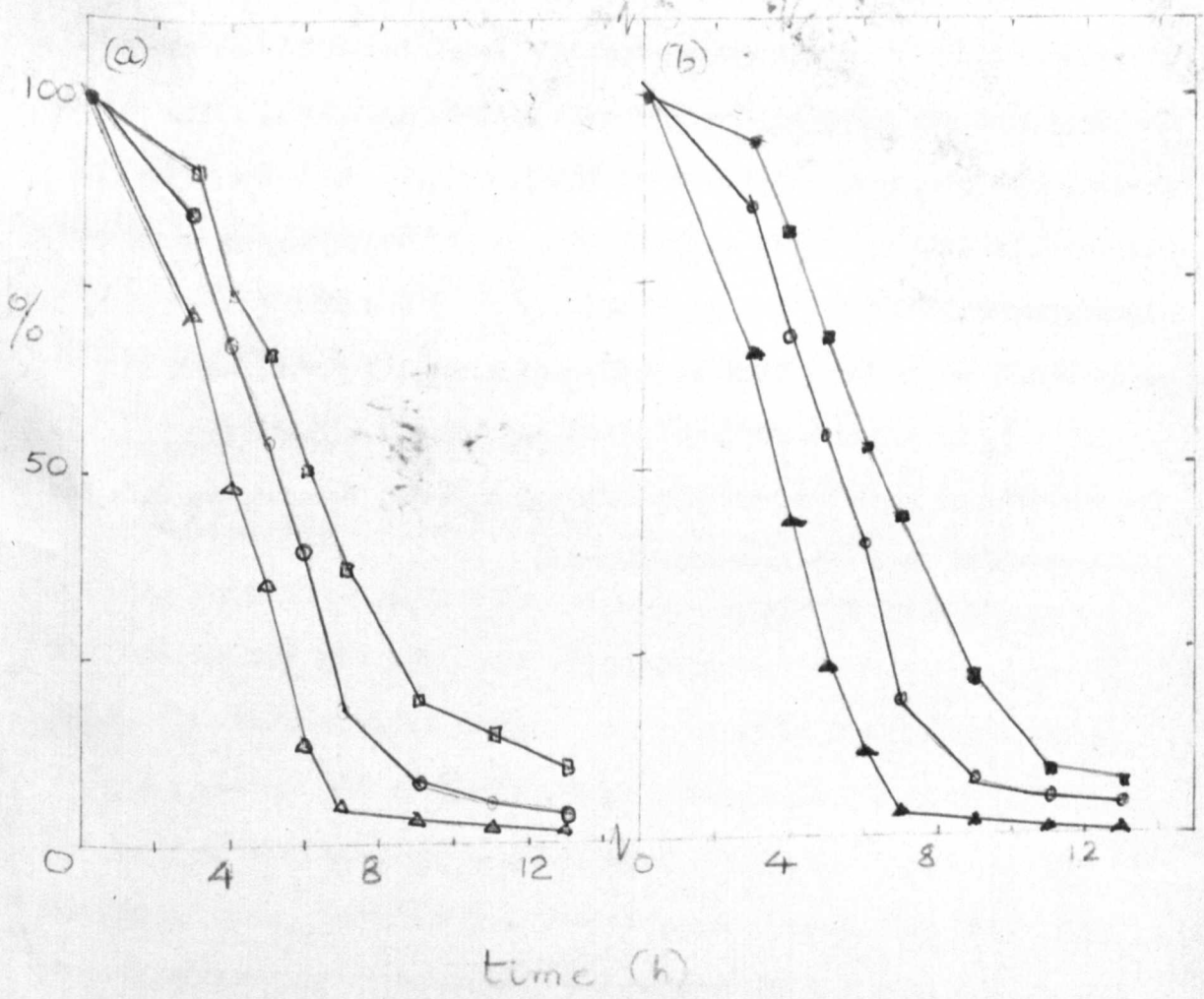

60
%
30
0
4
8
12
time (h)

Figure 9.

Four similar experiments to that shown in Fig.8 were carried out, one in which the cultures were incubated at 30°C, and one at 39°C, one in which the m.o.i. was 1 p.f.u./cell and finally one where the m.o.i. was 100 p.f.u./cell. The label on the five virus-specified protein peaks was summated, and then subtracted from the total detectable on the gel. The remainder was taken as the host cell protein synthesis. This was expressed as a percentage of the total label. Fig.9a shows the effect on host cell protein synthesis of incubation at different temperatures during virus growth; 30°C (-□-□-), 37°C (-○-○-) and 39°C (-△-△-), while Fig.9b shows the effect of different m.o.i.; 1 p.f.u./cell (-■-■-), 10 p.f.u./cell (-○-○-) and 100 p.f.u./cell (-▲-▲-). The comparisons reported here are only approximate, because the data has been assembled from separate experiments.


(a)
(b)
100
50
0
%
4
8
12
time (h)

This represents a rapid and major conversion of the protein synthesising system of the host cell, from its normal uninfected state to a virus directed state. Indeed late after infection only two proteins, the core and the envelope account for over 50% of protein synthesis.

The virus-specified proteins detectable in infected BHK, L-929 and HEL cells were analysed by the same techniques. The same five proteins could be identified, but they were present in different amounts. When infected BHK cells were compared with chick embryo cells NVP 95 was more prominent and NVP 72 less prominent (Fig.10). The relative amounts of NVP 95 and NVP 72 found in L-929 and HEL cells were similar to those found in BHK cells. This could be because viral replication proceeds by different mechanisms in the different cells, or because the proteins are broken down at different rates in the different cells.

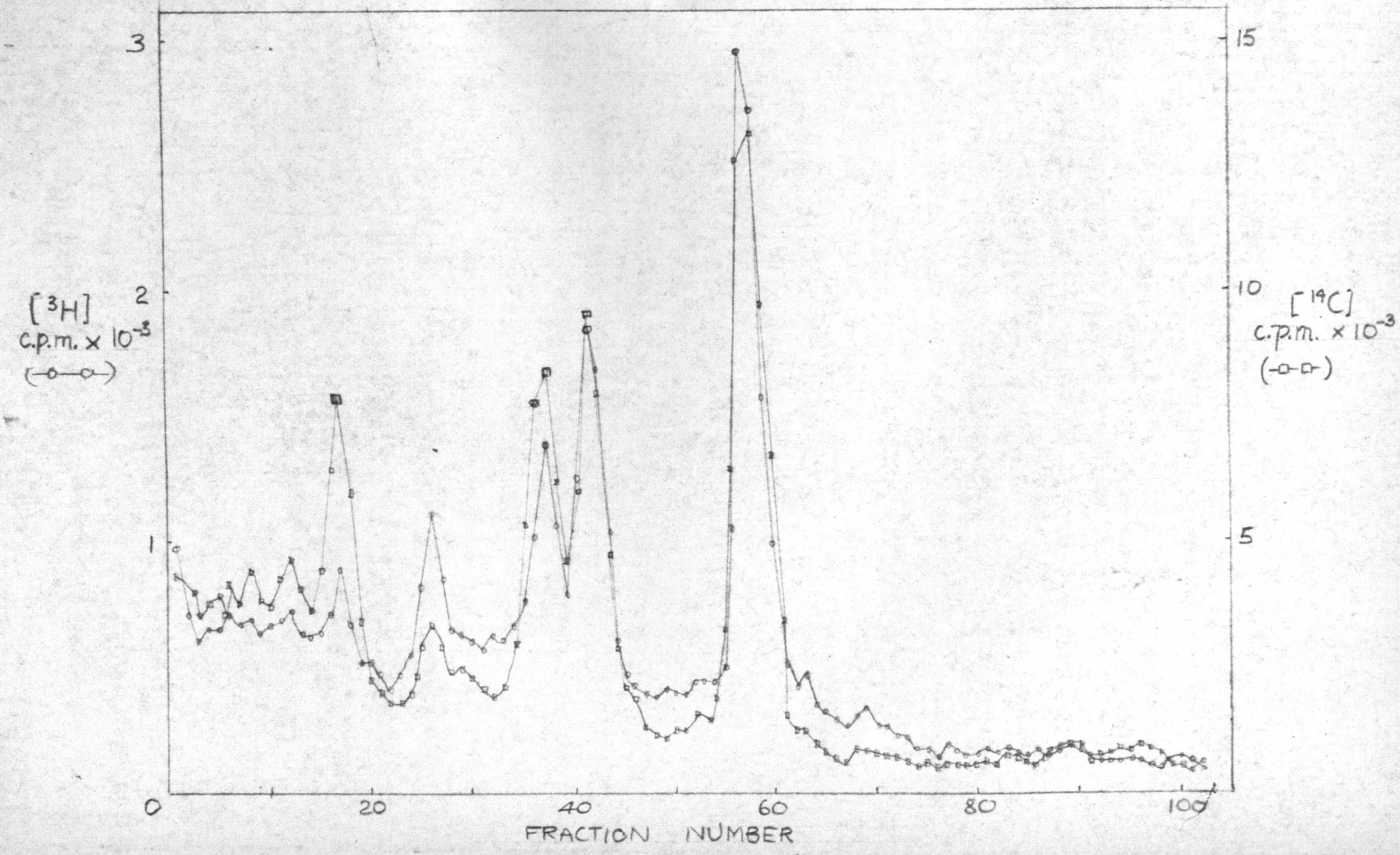
2. Comparison of Sindbis virus and SFV replication

The kinetics of replication of Sindbis virus in chick embryo cells was investigated by a series of experiments similar to those shown in Fig.2. The maximum yield of virus was reached by 12h after infection, while the rate of RNA synthesis was at a maximum at 5h after infection. The yield of Sindbis virus/cell was about double the figure for SFV. In the plaque assay, Sindbis virus formed larger plaques than SFV. The plaques were easily detectable by 24h, whereas SFV plaques took longer to be formed. The inhibition of host cell protein synthesis caused by infection with both Sindbis virus and SFV was similar.

Virus-specified proteins detectable 6 hours after infection of chick embryo cells with either Sindbis virus or SFV were compared using the technique of polyacrylamide gel electrophoresis. The electrophoretograms of the proteins from cells infected with the viruses were nearly identical

Figure 10.

Infected chick embryo cells (-o -o -) and BHK-21 cells (- □ - □ -) were labelled with 10μCi/culture of [^{3}H] valine and 4μCi/culture of [^{14}C]valine respectively, between 6 and 7h after infection. The samples were mixed, and extracted by method I before analysis on 9cm polyacrylamide gels.


3
2
1
0
[3H]
c.p.m. x 10-3
15
10
5
[14C]
c.p.m. x 10-3
20
40
60
80
100
FRACTION NUMBER

(Fig.11). It had previously been reported by Friedman (1970) that SFV and Sindbis virus-specified RNA species had the same sedimentation coefficients. Taking these results together they add support to the hypothesis that Sindbis virus and SFV replicate by the same mechanism.

3. Proteins formed early in the viral replicative cycle

At early times after infection virus-specified proteins are difficult to identify because they are synthesized in small amounts compared with the host proteins. Two methods were employed to try to overcome this. The first method utilised the reversible inhibition of SFV replication by guanidine reported by Friedman (1968b), following on earlier observations by Summers, Maizel and Darnell (1965) who had reported that the replication of polio virus was inhibited by guanidine. Guanidine resistant mutants of polio virus have been isolated (Ledinko, 1963) and these can be assigned to a specific locus on the genetic map (Cooper, 1968). It was known that when polio virus replication was prevented by the presence of guanidine, the inhibition of host cell protein synthesis still developed. Chick embryo fibroblasts were infected with SFV in varying concentrations of guanidine and after eight hours the yield of virus was measured (Table 3). Guanidine severely inhibited SFV growth. After four hours duplicate cultures were washed four times with maintenance medium and incubated for another four hours before assaying virus yield. In contrast to the results reported by Friedman (1968) the inhibition was not reversible. Thus it was not possible to use this approach.

The second approach to the problem of virus-specified proteins formed early in infection was to pre-treat the chick embryo cells with actinomycin D for six hours before infection. The cultures were labelled from the end of the period of infection for one, two or three hours. The proteins were analysed by polyacrylamide gel electrophoresis and the results were expressed

Figure 11.

Chick embryo fibroblasts infected with SFV (-O -o -) and with Sindbis virus (- □ - □-) were labelled from 6 - 7h after infection with 10μCi/culture of [^{3}H] valine and 4μCi/culture of [^{14}C] valine respectively. The samples were mixed and extracted by method I before analysis on 9cm polyacrylamide gels.

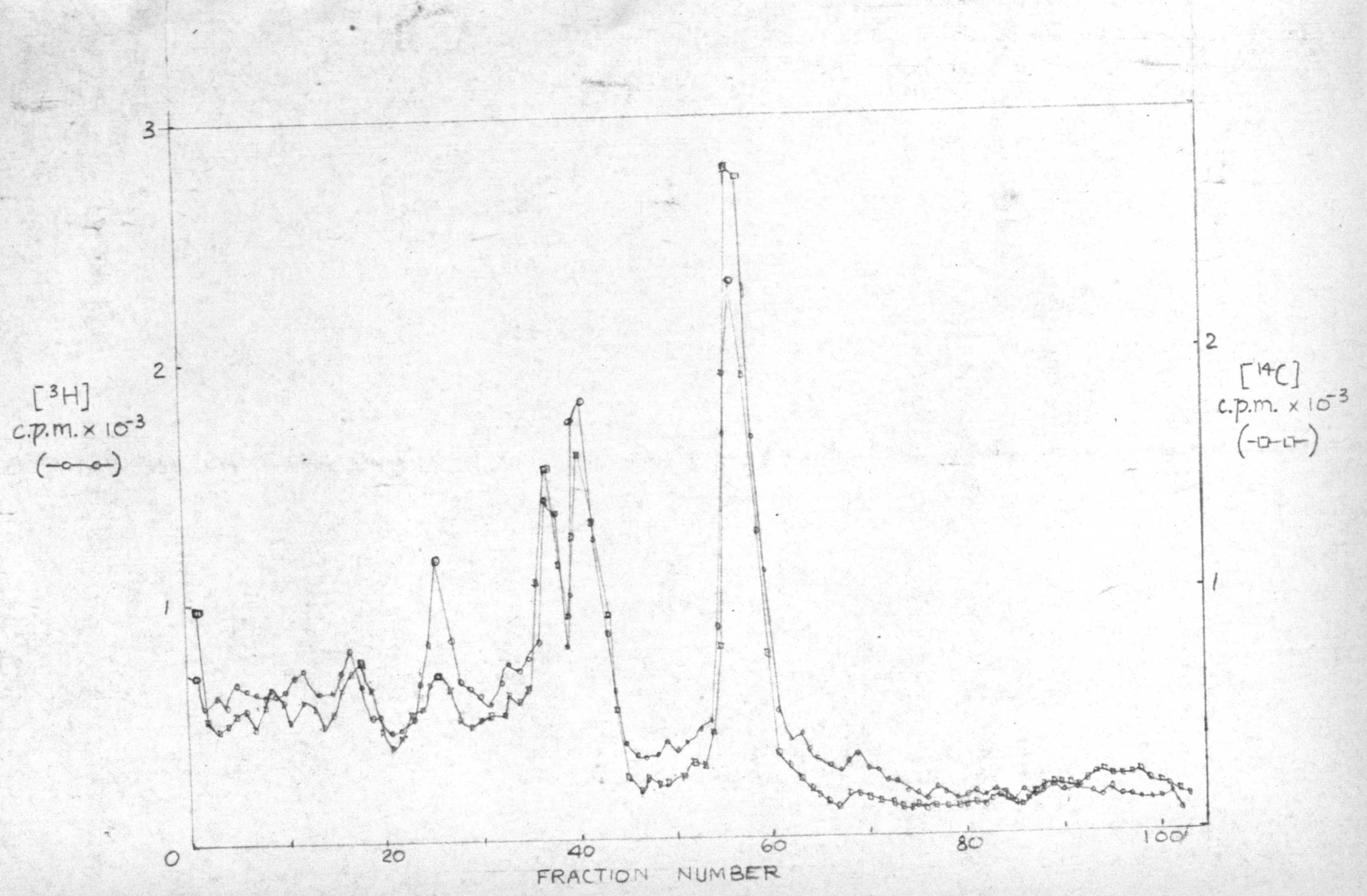

[3H]
c.p.m. x 10-3
(—o—o—)
[14C]
c.p.m. x 10-3
(-□-□-)
3
2
1
0
2
1
20
40
60
80
100
FRACTION NUMBER

Table 3.

The yield of infectious virus was measured 8h after infection, from chick embryo cells when varying concentrations of guanidine had been present continuously. Some of the cultures were washed four times with maintenance medium, 4h after infection, before continuing the incubation in maintenance medium containing 1µg/ml actinomycin D. Again 8h after infection the virus yield was assayed.

treatment:	Yield p.f.u. continuous	Yield p.f.u. washed after 4h
untreated	5×10^{8}	3×10^{8}
+ 0.005M-guanidine	2×10^{5}	3×10^{5}
+ 0.01M-guanidine	9×10^{4}	1×10^{5}
+ 0.05M-guanidine	5×10^{4}	5×10^{4}
+ 0.1M-guanidine	3×10^{4}	5×10^{4}

as a double-label ratio (Fig.12). No virus-specified proteins could be detected until the third hour after infection, when the five proteins normally detected could be identified. In an attempt to increase the sensitivity of the technique the cultures were incubated at 30°C from 30min before infection until the proteins were extracted. Still no distinct proteins formed early during viral infection could be detected.

4. Amino acid composition of NVP 72 and 63

Cultures of chick embryo cells infected with SFV were labelled with [^{3}H] lysine and [^{14}C]valine simultaneously. The proteins were extracted and analysed by polyacrylamide gel electrophoresis. The five virus-specified protein peaks contained differing ratios of [^{3}H] lysine to [^{14}C] valine counts (Fig.13). In contrast the ratio of [^{3}H] valine to [^{14}C] counts obtained when the proteins were extracted from uninfected cells was constant along the length of the gel (Fig.13b). The difference in ratio of the five virus-specified proteins was taken to represent the difference in the relative amounts of the two amino acids that the five proteins contained.

This experiment was repeated with seven other [^{14}C] amino acids. The results were expressed as the ratio of [^{3}H] lysine to the [^{14}C] amino acid counts for each of the four protein peaks that contained sufficient counts. Unfortunately NVP 95 did not contain enough counts for the results to be accurate for this polypeptide. The ratios obtained from this experiment were compared with those predicted from the amino acid analysis of the envelope and core reported by Kennedy and Burke (1972). A correction factor was needed to bring the prediction into line with the result. From the two correction factors an average correction factor was

Figure 12.

Cultures of chick embryo cells were pretreated for 6h with 1μg/ml actinomycin D in maintenance medium. Then they were infected with 20 p.f.u./cell for 1h, or mock infected, both still in the presence of 1μg/ml actinomycin D. Following the end of the infectious period the cells were washed twice, 50μCi [^{3}H] leucine was added to the infected cultures and 20μCi [^{14}C]leucine to the uninfected cultures in leucine free medium. A pair of infected and uninfected cultures were mixed and the proteins extracted by method I at 1h(-□-□-), 2h (-Δ-Δ-) and 3h (-○-○-) after infection. The samples were analysed by polyacrylamide gel electrophoresis on 9cm gels.

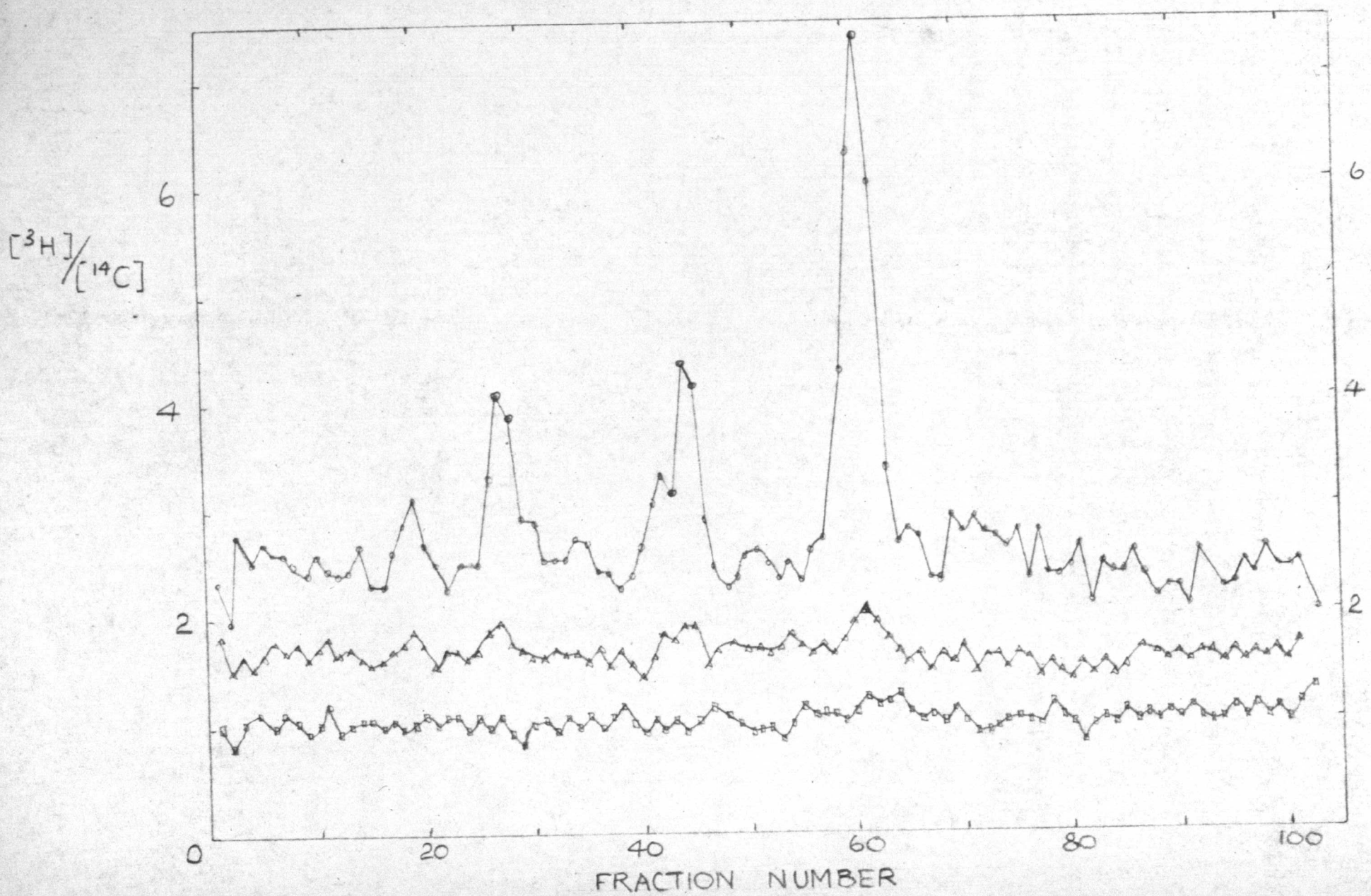
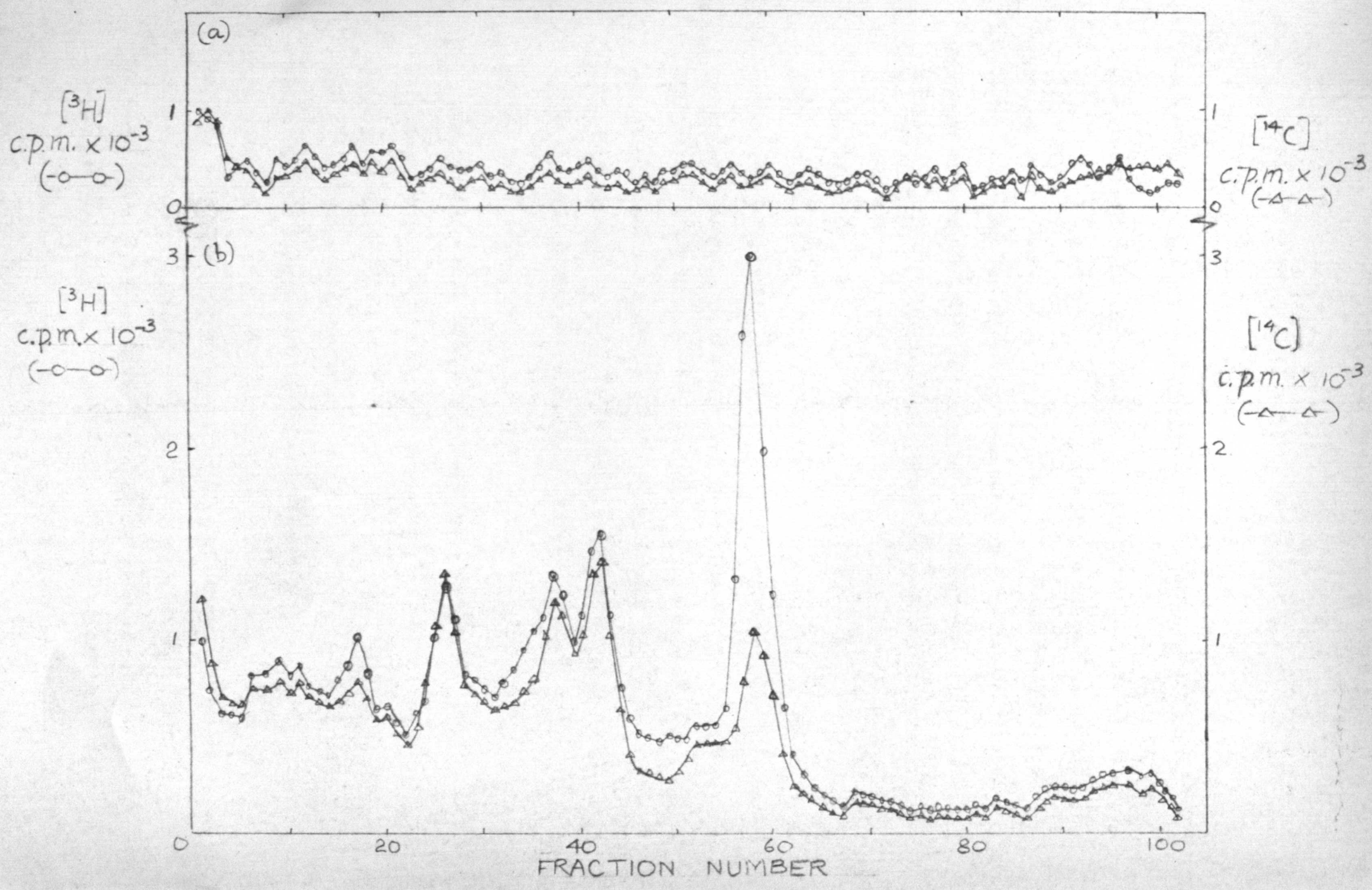

[3H]/[14C]
6
4
2
0
20
40
60
80
100
FRACTION NUMBER
6
4
2

Figure 13.

Infected and uninfected cultures of chick embryo fibroblasts were separately labelled with 10μCi/culture of [^{3}H] lysine (-o-o-) and 4μCi/culture of [^{14}C] valine (-Δ-Δ-). Fig.13a shows the sample extracted by method I from the uninfected cells and analysed by polyacrylamide gel electrophoresis on 9cm gels, while Fig.13b shows the comparable experiment with the same from uninfected cells.


(a)
(b)
[3H]
c.p.m. x 10-3
(-o-o-)
[14C]
c.p.m. x 10-3
(-Δ-Δ-)
0
1
2
3
20
40
60
80
100
FRACTION NUMBER

deduced for each amino acid. The ratios for NVP 72 and NVP 63 were multiplied by the average correction factor to give an absolute ratio with respect to lysine. All the values were then expressed as a ratio with respect to tryptophan (Table 4), because tryptophan is the amino acid present in the smallest quantities.

Because the values in Table 4 were deduced by comparison with those obtained by Kennedy and Burke (1972) from an independent amino acid analysis, they have been corrected for factors such as differential transport into cells, different specific activity of the labelled amino acids, and different pool sizes. The partial amino acid compositions of the four proteins are unremarkable, except for the high content of lysine contained in the core protein. The values for NVP 63 and the envelope protein are very similar, while those for the core and NVP 72 are substantially different. NVP 72 does not contain enough lysine to account for the amount present in the core. It is probable that the envelope protein is related to NVP 63, while neither of this pair is related to either the core protein or NVP 72.

5. Carbohydrate content of the virus-specified proteins

The carbohydrate content of the virus-specified proteins was investigated by labelling BHK-21 cells infected with SFV with [^{3}H] glucosamine and [^{14}C] valine simultaneously. The growth of the cells was halted by incubating them in medium containing no serum for 12h before infection, and the proteins were extracted and analysed by polyacrylamide gel electrophoresis. The results were compared with a similar experiment with proteins extracted from uninfected BHK cells (Fig.14). No significant peaks of protein containing glucosamine were found in the uninfected cells, but three of the five virus-specified proteins contained substantial amounts and the other two contained a small amount. The purified core protein had

Table 4.

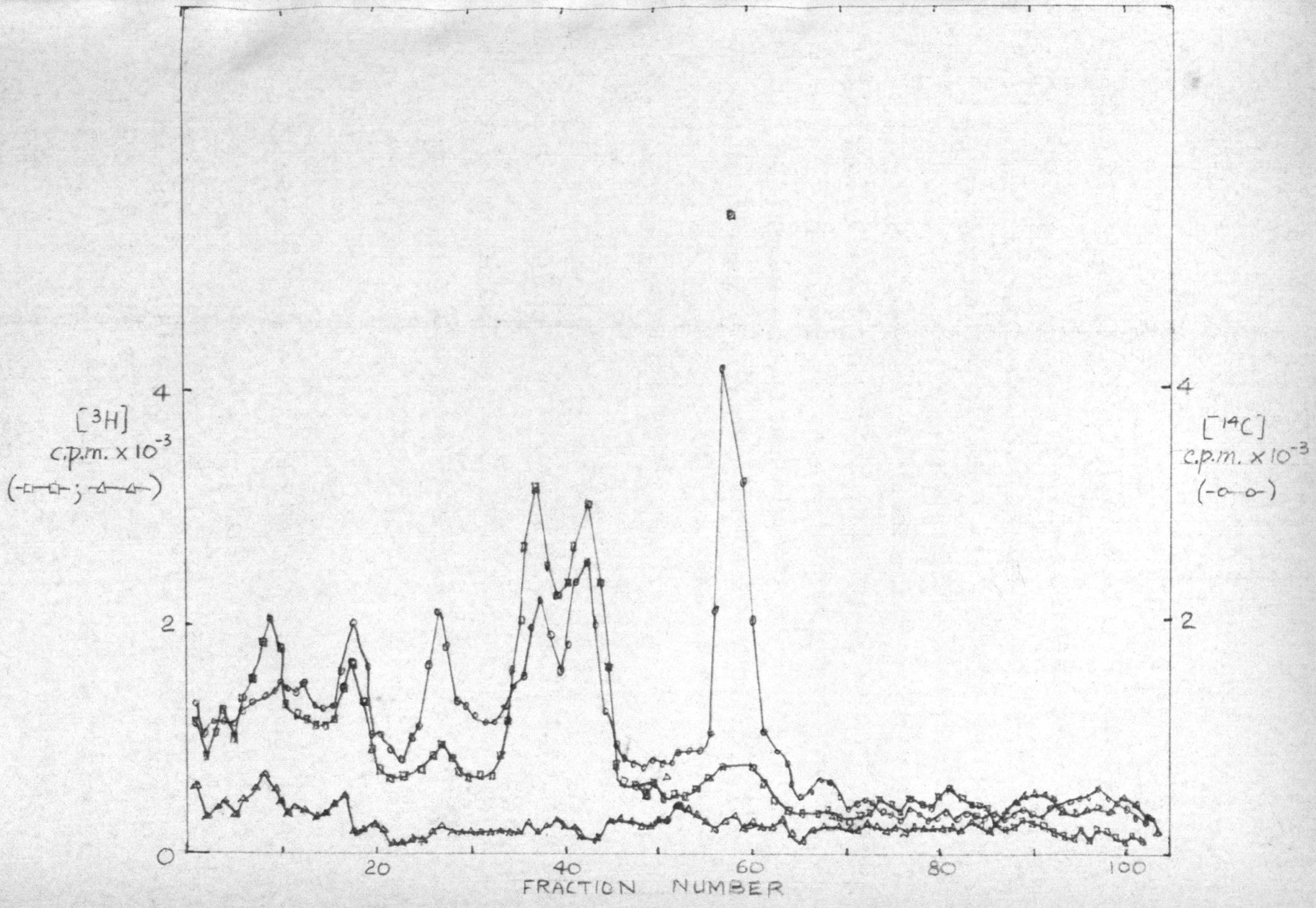
The partial amino acid composition of NVP72, NVP63, and the structural proteins were calculated by the method described in the text, from a series of experiments such as the one shown in Fig.13.

X : tryptophan

X	NVP72	NVP63	Envelope	Core
lysine	3.12	5.34	5.38	14.3
threonine	4.84	8.76	8.61	7.04
tyrosine	3.44	3.85	3.86	2.58
leucine	4.24	5.29	5.26	4.25
methionine	2.08	1.71	1.72	2.87
proline	4.35	6.65	6.67	9.25
phenylalanine	1.44	3.02	2.93	2.69
valine	4.61	7.08	7.14	7.44

Figure 14.

BHK-21 cells were incubated in the presence of maintenance medium lacking calf serum but containing 1μg/ml actinomycin, until 5½h after infection, when the medium was changed toPBS containing 1μg/ml actinomycin D. Then uninfected and infected BHK-21 cells were separately labelled with both 50μCi/culture of [^{3}H] glucosamine and 5μCi/culture of [^{14}C]valine between 6 and 7h after infection. The proteins were extracted by method I and analysed on 9cm polyacrylamide gels. Symbols used for [^{3}H] glucosamine label from infected cells (-□-□-), for [^{14}C]valine label from infected cells (-○-○-), and [^{3}H]glucosamine from uninfected cells (-△-△-).


[3H]
c.p.m. x 10-3
(-□-□-; -△-△-)
4
2
0
[14C]
c.p.m. x 10-3
(-o-o-)
4
2
20
40
60
80
100
FRACTION NUMBER

previously been shown not to contain any carbohydrate at all (Kennedy and Burke, 1972). Therefore the ratio of [^{3}H] to [^{14}C] counts in the core was taken as the level due to metabolism, some of which reappeared in the core protein. The ratio of counts for NVP72 was the same as for the core, suggesting that NVP72 also contained no glucosamine. However the ratio for the envelope protein and the NVP63 suggested that both contained glucosamine, the envelope slightly less than NVP63. The high molecular weight protein (NVP95) might also contain a small amount of glucosamine.

These experiments were repeated using fucose in place of glucosamine (Fig.15). Again, no distinct proteins containing fucose could be identified in the extracts from uninfected cells. In infected cells only the envelope protein and NVP 63 contained fucose, which was present in about the same amounts. There was no fucose present in NVP 95. The presence of similar amounts of glucosamine and fucose in NVP 63 and the envelope protein again suggests the two proteins are related in some way.

6. The effect of FNAG on viral protein synthesis

N-Fluoro-acetyl glucosamine (FNAG) had previously been reported to be a non-toxic inhibitor of glycoprotein synthesis (Kent, 1972) N-acetyl glucosamine (NAG) had been observed to inhibit viral growth slightly, but its effect appeared to be a non-specific inhibition of all macro-molecular synthesis (Scholtissek, 1972). The yield of SFV was estimated after being grown for 9h in the presence of varying concentrations of FNAG and NAG (Fig.16). FNAG was a much more effective inhibitor of viral growth than NAG, depressing virus yield by a factor of 100 times more at the same concentration. The effect of either 0.0005M- FNAG or 0.0005M-NAG on the incorporation of radioactive precursors into RNA, protein and carbohydrate was investigated in chick embryo fibroblasts infected with SFV. FNAG did

Figure 15.

Uninfected and infected BHK-21 cells were separately labelled with 50μCi/culture [^{3}H] fucose and 5μCi/culture of [^{14}C] valine between 6 and 7h after infection. The proteins were extracted by method I and analysed on 9cm polyacrylamide gels. Symbols employed for [^{3}H] fucose label from infected cells (-□-□-), for [^{14}C] valine label from infected cells (-○-○-), and for [^{3}H]glucosamine from uninfected cells (-Δ-Δ-).

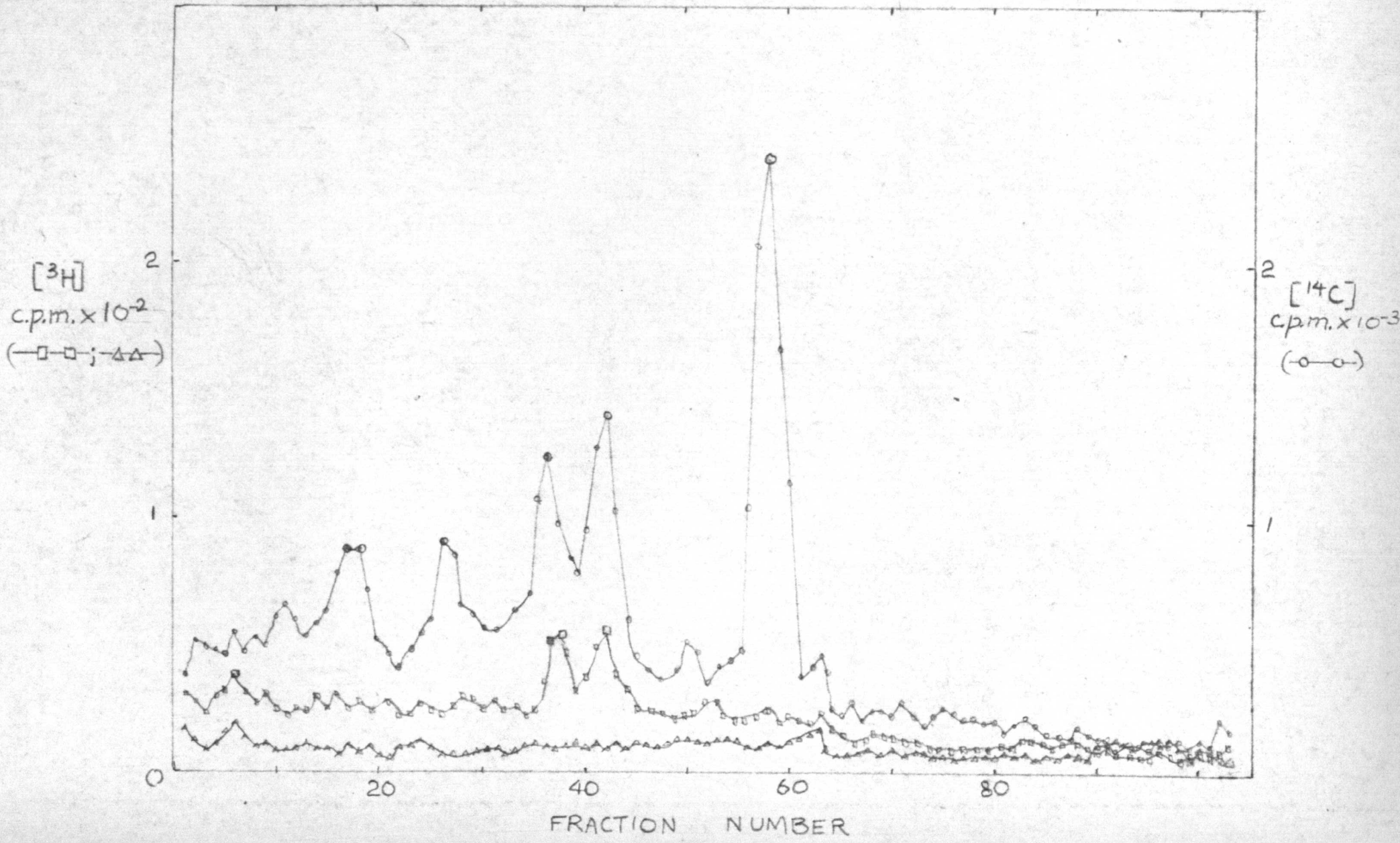
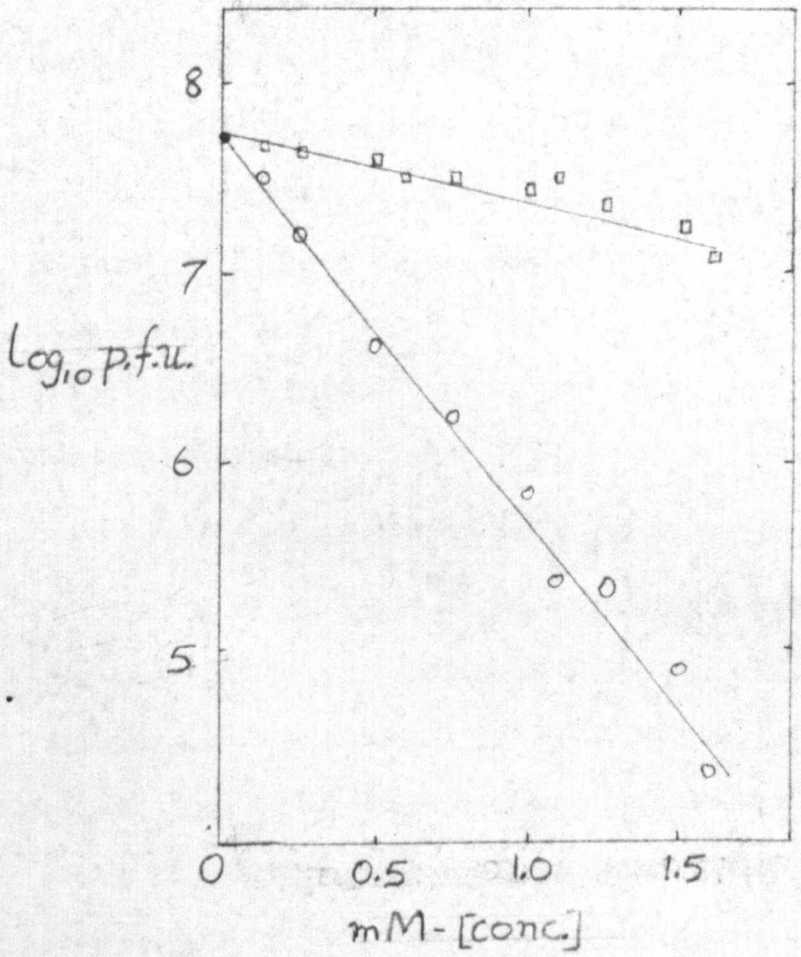

[3H]
c.p.m. x 10-2
(—□—□—; -△-△-)
2
1
0
[14C]
c.p.m. x 10-3
(-o—o-)
2
1
20
40
60
80
FRACTION NUMBER

Figure 16

The yield of infectious virus was measured after growth for 9h in the chick embryo vells in the presence of varying concentrations of FNAG (-o-o-) and NAG (-□-□-).

FIG. 16



not inhibit RNA or protein synthesis but altered the rate of glucosamine incorporation (Table 5). This contrasted with the slight depression of all these activities produced by the same concentration of NAG. Therefore FNAG was assumed to be a specific inhibitor of carbohydrate and glycoprotein synthesis.

Chick embryo cells, infected with SFV, were labelled with [^{3}H] valine after prior treatment with 0.5mM FNAG. The proteins were extracted and mixed with a similar preparation from infected cells labelled with [^{14}C] valine but which had not been treated with FNAG. The mixed extract was analysed by polyacrylamide gel electrophoresis (Fig.17). In the material from treated cells there was less NVP 63 and envelope protein and more NVP95 than in the material from control cells. This could be because NVP 95 is converted into NVP 63 and envelope protein.

7. Virus-specified proteins formed during long labelling periods

In all of the previous experiments the time that the radioactive precursor had been in contact with the cells was 1h. The electrophoretograms obtained represent the total protein synthesised during that period, less the proteins that are lost due to export from the cell, or due to degradation. The proteins that were labelled during a 9h period lasting from 3h until 12h after infection, were compared with the proteins labelled during a 1h period from 6h to 7h after infection. The envelope and core proteins made up a much higher proportion of the total protein during the long labelling period in comparison with the short period (Fig.18). Since it is probable that the only proteins released from the cell are the viral structure proteins, the other proteins must be 'degraded' in some ways. The 'degradation' could be of two sorts: either total breakdown of fragments of polypeptides no longer required, or specific cleavage of a large intermediate to form an active final product. In order to

Table 5.

The incorporation of [^{3}H] uridine, [^{14}C] valine and [^{3}H] glucosamine into acid precipitable material was measured in cultures of chick embryo cells 6h after infection with SFV. The cultures had been incubated in the presence either of 0.0005M-FNAG or of 0.0005M-NAG. The released virus was also assayed 9h after infection.

	incorporation of			Yield
	uridine	valine	glucosamine	p.f.u.
uninfected	377	5,689	6,836	-
infected	4,826	4,923	7.514	7×10^7
infected + 0.0005M-NAG	4,791	4,723	7,263	6×10^7
infected + 0.0005M- FNAG	4,754	4,691	2,435	1×10^6

values in c.p.m.

Figure 17

SFV was grown in chick embryo fibroblasts in the presence (–Δ–Δ–) and absence (–○–○–) of 0.0005M-FNAG. The cells were labelled from 6–7h after infection with 50μCI/culture of [^{3}H] leucine. The proteins were extracted by method II and analysed on 12cm polyacrylamide gels.

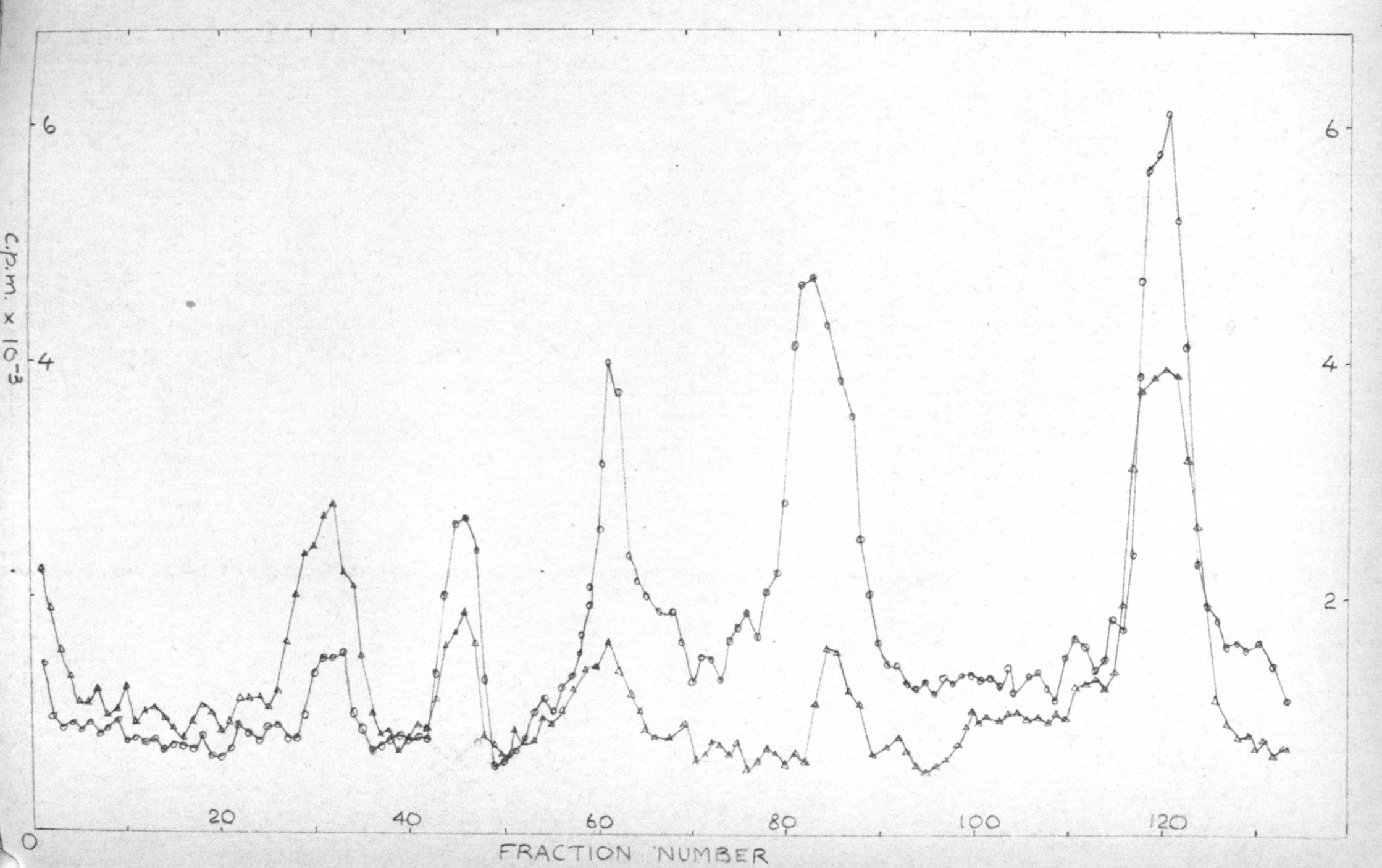
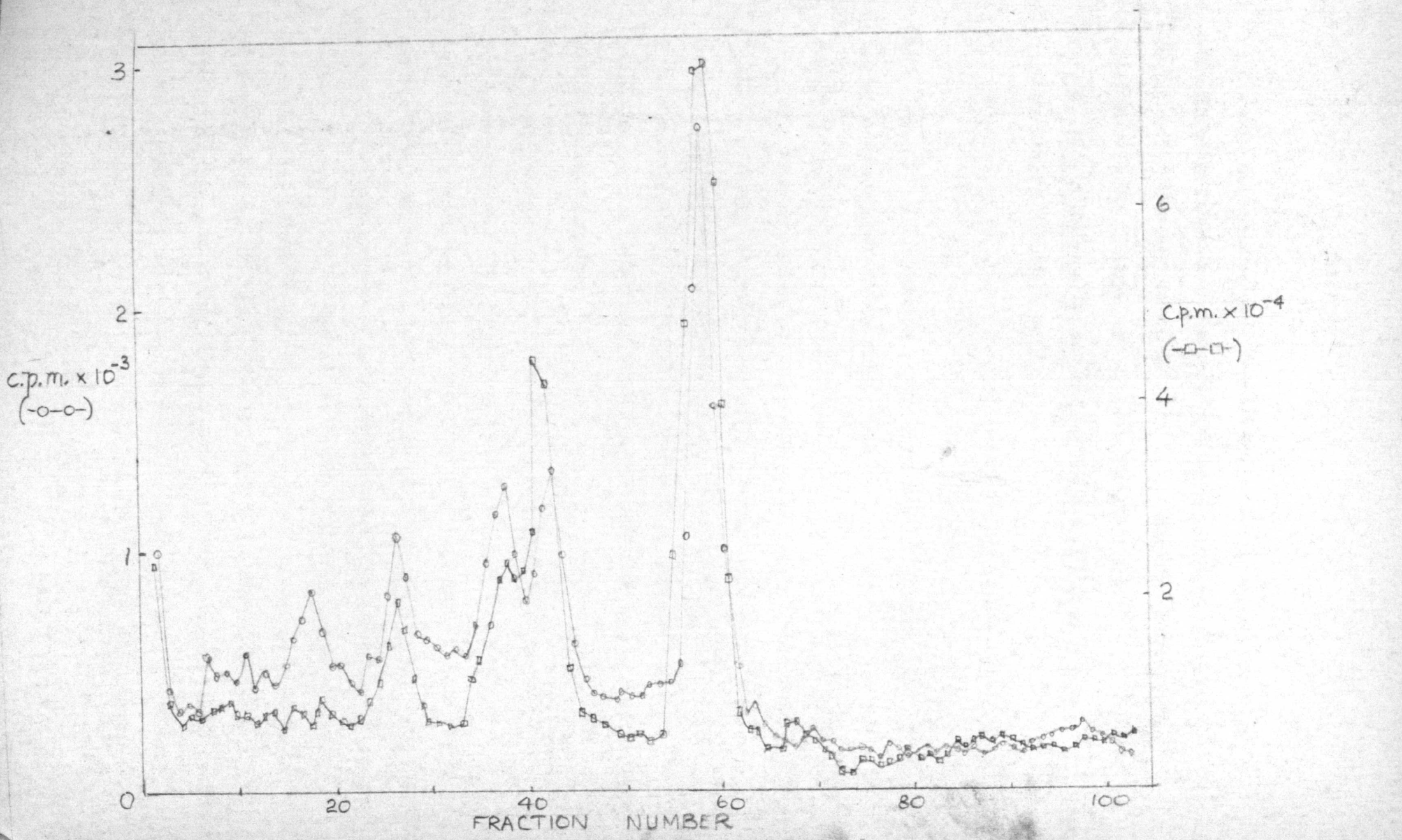

6
4
2
0
20
40
60
80
100
120
c.p.m. x 10-3
FRACTION NUMBER

Figure 18.

SFV grown in chick embryo fibroblasts, was labelled either for 1h from 6-7h after infection (—O—O-) or for 9h from 3-12h after infection (—□—□-) with 10μCi/culture of [^{3}H] valine. The proteins were extracted by method I, and analysed on 9cm polyacrylamide gels.


3
2
1
0
c.p.m. x 10^-3
(-o-o-)
6
4
2
c.p.m. x 10^-4
(-□-□-)
20
40
60
80
100
FRACTION NUMBER

demonstrate the second type of degradation, a movement of radioactive label from the precursor to the product has to be demonstrated. It is probable from this experiment that only NVP 95 and NVP 63 are precursors of other proteins.

8. Virus-specified proteins formed during short pulses and pulse-chase experiments

BHK-21cells, 6h after infection with SFV, were incubated in the presence of [^{3}H] leucine for 7min (pulse experiment). The proteins were extracted and analysed by polyacrylamide gel electrophoresis. The results were compared with proteins extracted from cells treated similarly, but which had been washed, and incubated for a further 53min in the presence of a high concentration of non-radioactive leucine after the7min labelling pulse (pulse-chase experiment). Finally both experiments were compared with proteins extracted from cells that had been labelled for 1h continuously with [^{3}H] leucine. The results, shown in Fig.19, demonstrate that a movement of label from NVP 95 to NVP 63 and the envelope protein is taking place.

These pulse-chase experiments were repeated using chick embryo fibroblasts infected with SFV, instead of BHK-21 cells. In chick embryo cells under normal conditions NVP95 is much less prominent than it is in BHK-21 cells. Therefore the increase in size of the NVP 95 peak during the short pulse is much more pronounced as is the reduction in size during the subsequent chase (Fig.20). The increase in NVP 72 is more obvious because the experiment was carried out in chick embryo cells. In chick embryo fibroblasts but not in BHK-21 cells there was a significant change in the size of the core peak during the chase period. A new, high molecular weight, protein peak was detectable in chick embryo fibroblasts during the short pulse period. This protein is labelled NVP 127 and has an

Figure 19.

Three monolayer cultures of BHK-21 cells on 5cm plastic Petri dishes were infected with SFV. 50μCi/culture of [^{3}H] leucine was added 6h after infection. Then 7min later the proteins of one culture were extracted by method II (short pulse: -O-O-), and a second culture was washed twice with EDA containing leucine, before being incubated for a further 53min in the presence of the same medium (chase: - □-□-). The third culture was a control, incubated in the presence of the radioactive leucine continuously for 1h (control: -Δ-Δ-). The proteins were extracted from each culture by method II, and analysed on 12cm polyacrylamide gels.

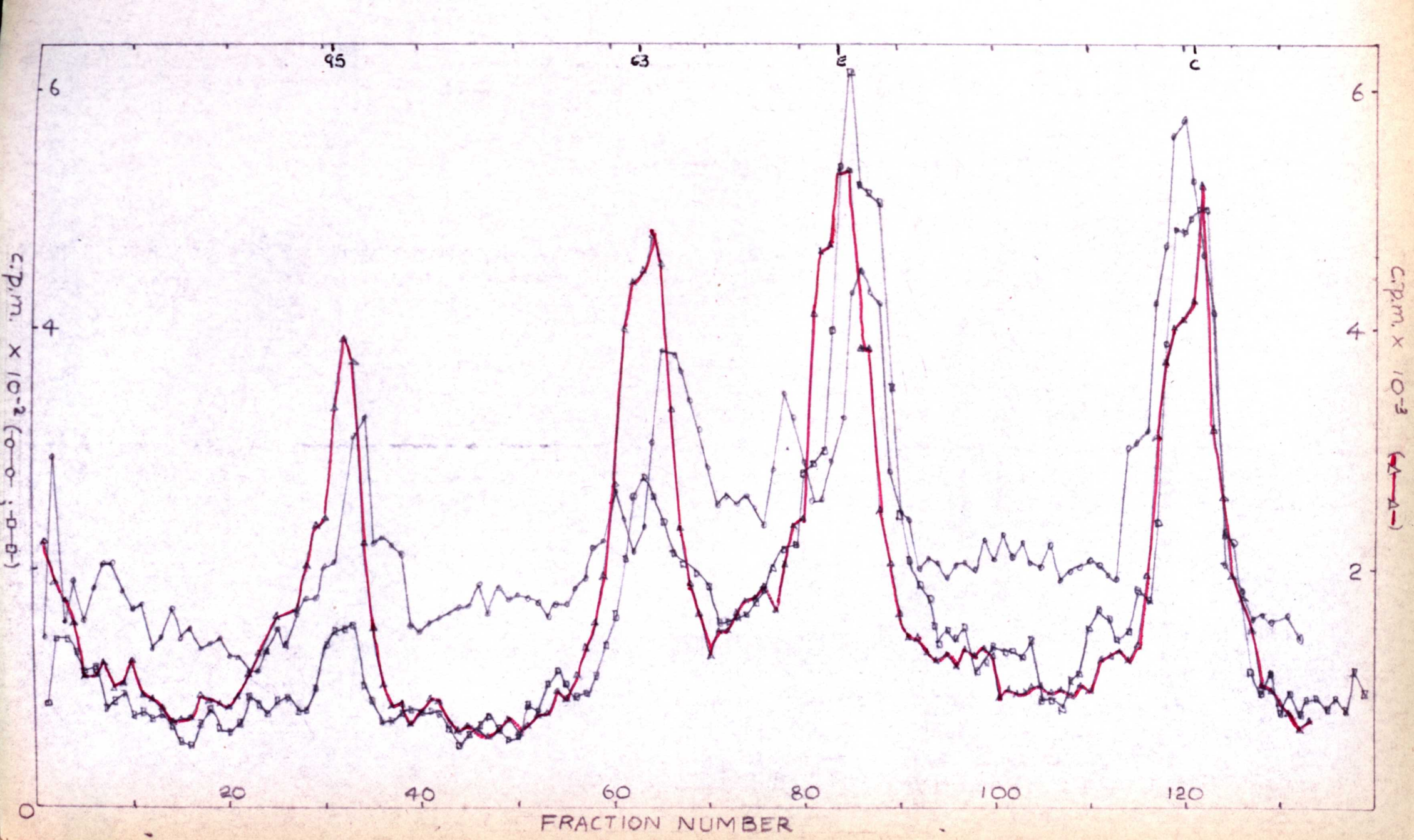
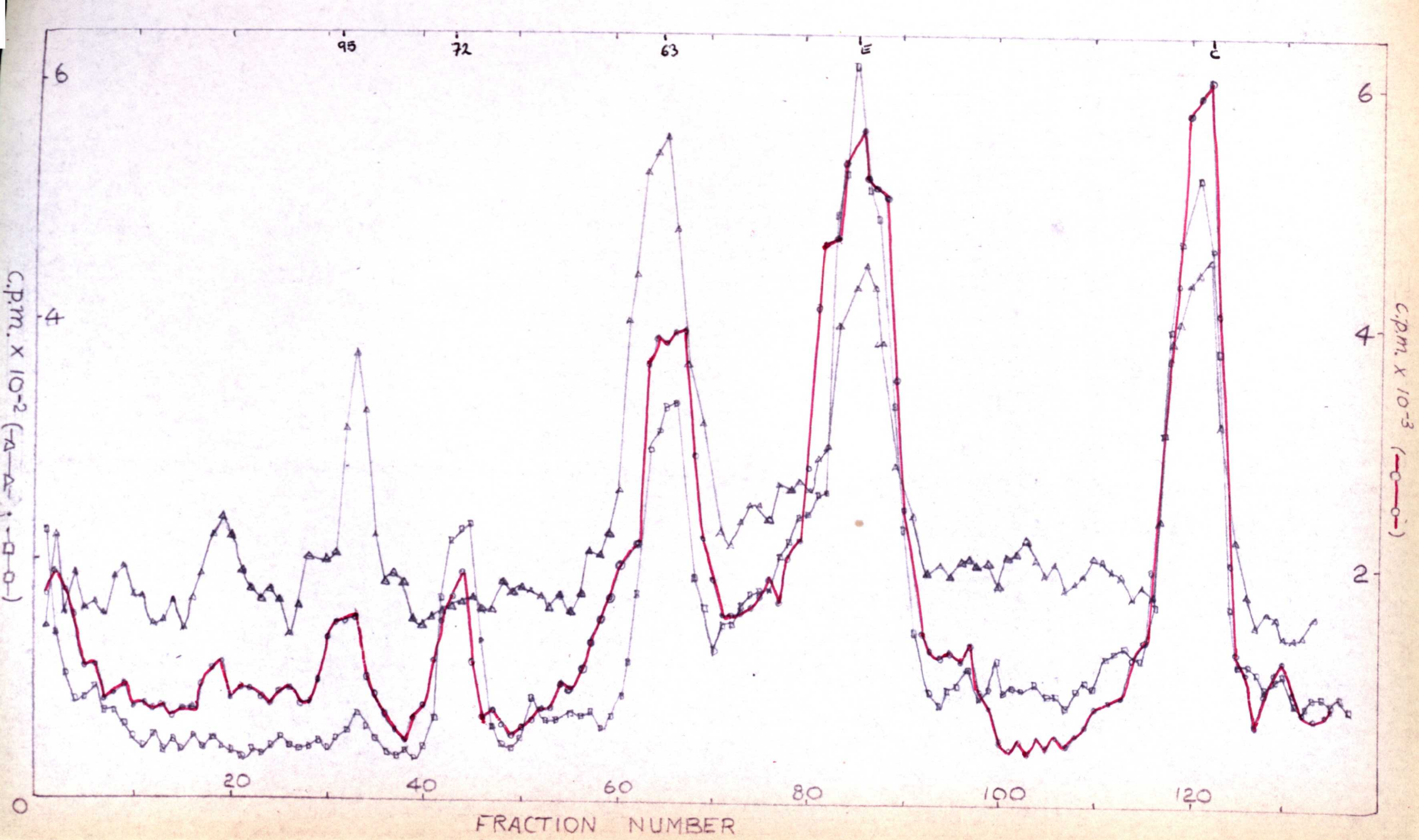

95
63
E
C
6
4
2
0
20
40
60
80
100
120
FRACTION NUMBER
c.p.m. x 10-2 (o—o—o—o—o)
c.p.m. x 10-3 (Δ—Δ—Δ)

Figure 20.

The same experimental procedure as in Fig.19 was used with SFV infected cultures of chick embryo fibroblasts on 5cm plastic Petri dishes. 50μCi of [^{3}H] leucine was added 6h after infection for a short pulse (-Δ-Δ-), chase (-□-□-) and control (-o—o—). The proteins were extracted by method II and analysed on 12cm polyacrylamide gels.


95
72
63
E
C
6
4
2
0
20
40
60
80
100
120
FRACTION NUMBER
c.p.m. x 10-2 (-Δ—Δ-; -□—□-)
c.p.m. x 10-3 (—○—○—)

estimated molecular weight of 127,000. NVP 95 and NVP 63 are precursors, in some way, to NVP 72 and the envelope and core proteins.

9. The effect of TPCK and DFP on virus-specified protein synthesis

Both tosyl phenylalanyl and chloromethyl ketone (TPCK) and diisopropyl fluoro phosphate (DFP) are inhibitors of proteolytic enzymes (Walsh and Wilcox, 1970). TPCK is a specific inhibitor of chymotrypsin (Pfefferkorn and Boyle, 1972) while DFP is a general inhibitor of serine proteases (Jacobson, Asso and Baltimore, 1970). The effect of varying concentrations of both TPCK and DFP on the rate of incorporation of ^{3}H valine into an acid insoluble precipitate in BHK-21 cells was measured. TPCK, even at the highest concentration used, had less of an inhibitory effect on protein synthesis, than DFP at the lowest (Fig.21), while both prevented the release of infectious virus. Therefore TPCK was used in the following experiments.

Pfefferkorn and Boyle (1972) had reported that TPCK prevented the breakdown of NVP 95 in chick embryo fibroblasts while tosyl lysylchloromethyl ketone (TLCK) did not. TLCK is an analogous inhibitor of trypsin. Therefore the cleavage was caused by an enzyme with a similar specificity to chymoatrypsin. In these experiments the cells were treated for 30min before the ^{3}H leucine was added with TPCK. Then a short pulse., and pulse-chase experiment were compared with an experiment in which the labelling period was 1h. These experiments were carried out in both chick embryo fibroblasts and BHK-21 cells 6h after infection with SFV. The proteins were extracted and analysed by polyacrylamide gel electrophoresis and the results are shown in Fig.22 (BHK-21 cells) and Fig.23 (chick embryo fibroblasts). Amongst the virus-specified proteins labelled during a short pulse in BHK cells, NVP127 could now be detected. Comparing the results from the short pulse with those of the pulse-chase experiment,

Figure 21.

SFV was grown for 6h in chick embryo fibroblasts in monolayer culture on 5cm plastic Petri dishes. The effect of varying concentrations of TPCK (−△−△−) and DFP (−○−○−) on incorporation of [^{3}H] valine into an acid insoluble precipitate was then measured. The yield of infectious virus 9h after infection was measured when TPCK (−▲−▲−) or DFP (−●−● −) had been present since 5½h after infection.

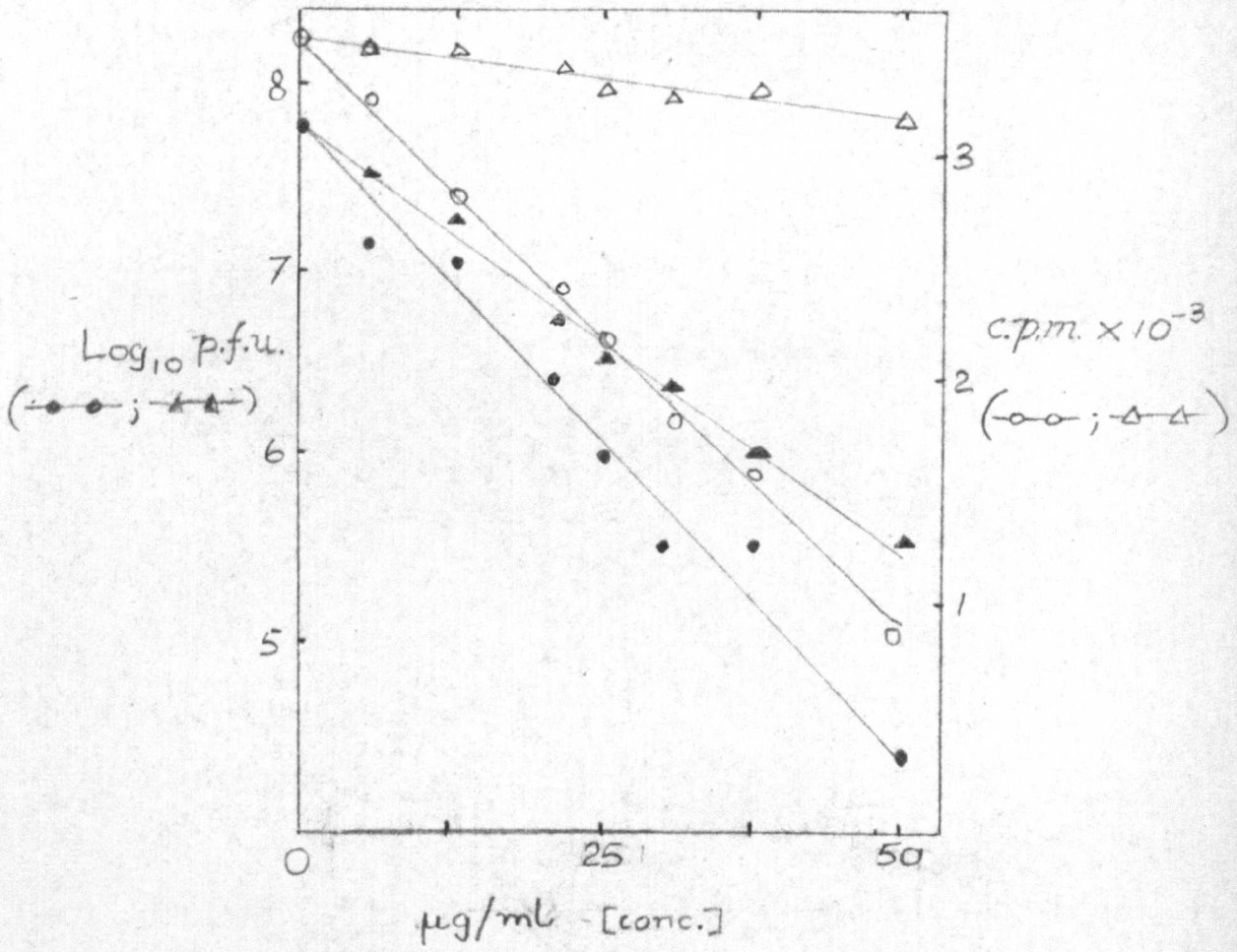

8
7
6
5
3
2
1
0
25
50
Log10 p.f.u.
(●—●; ▲—▲)
c.p.m. × 10⁻³
(○—○; △—△)
μg/ml [conc.]

Figure 22.

Three monolayer cultures of BHK-21 cells on 5cm plastic Petri dishes were infected with SFV. TPCK (20µg/ml) in EDA was added 5½h after infection. At 6h after infection, pulse-chase experiments (as in Fig.19) were carried out. The samples from the short pulse (—o—o—), chase (—□—□—) and control (—Δ—Δ—) were analysed on 12cm polyacrylamide gels.

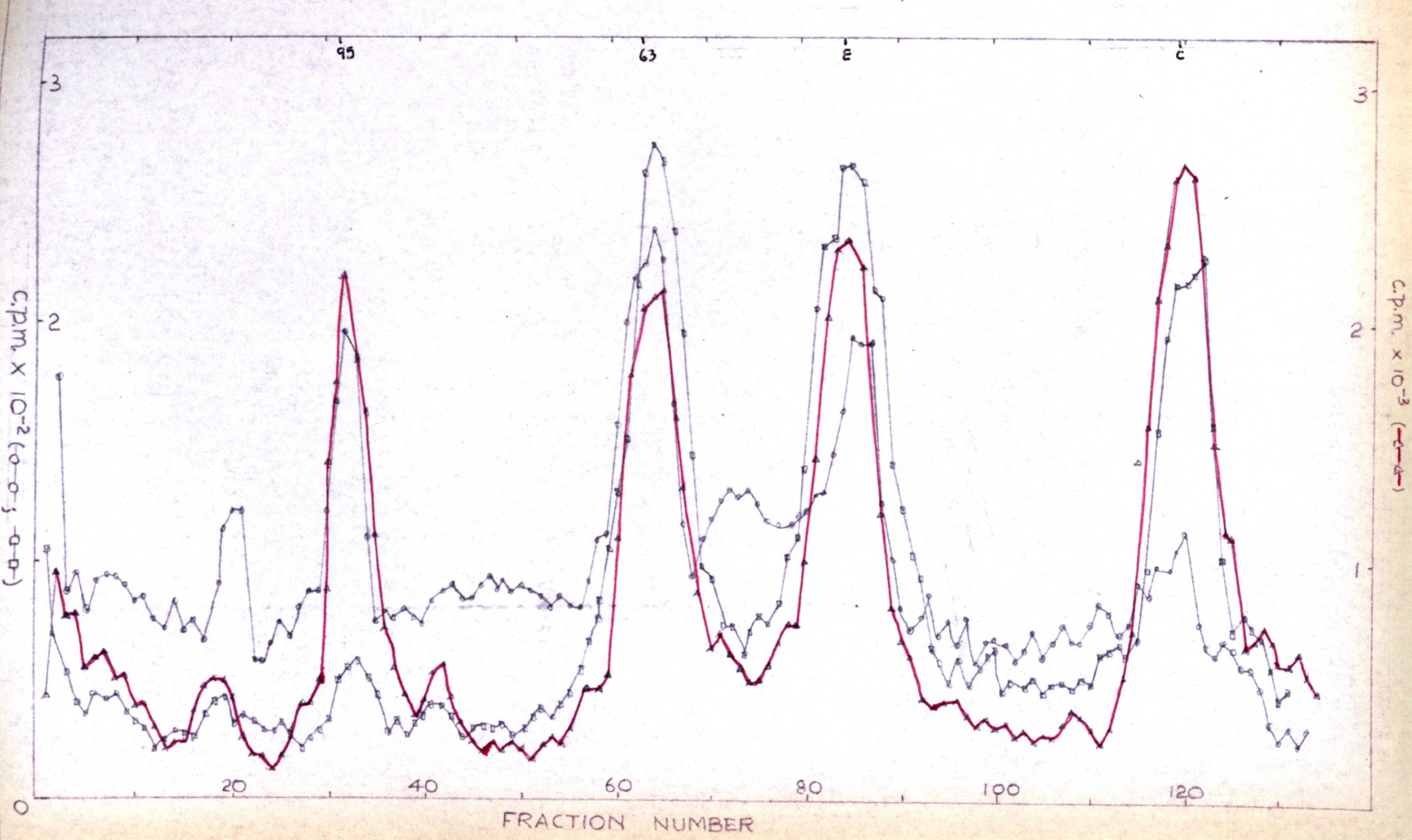
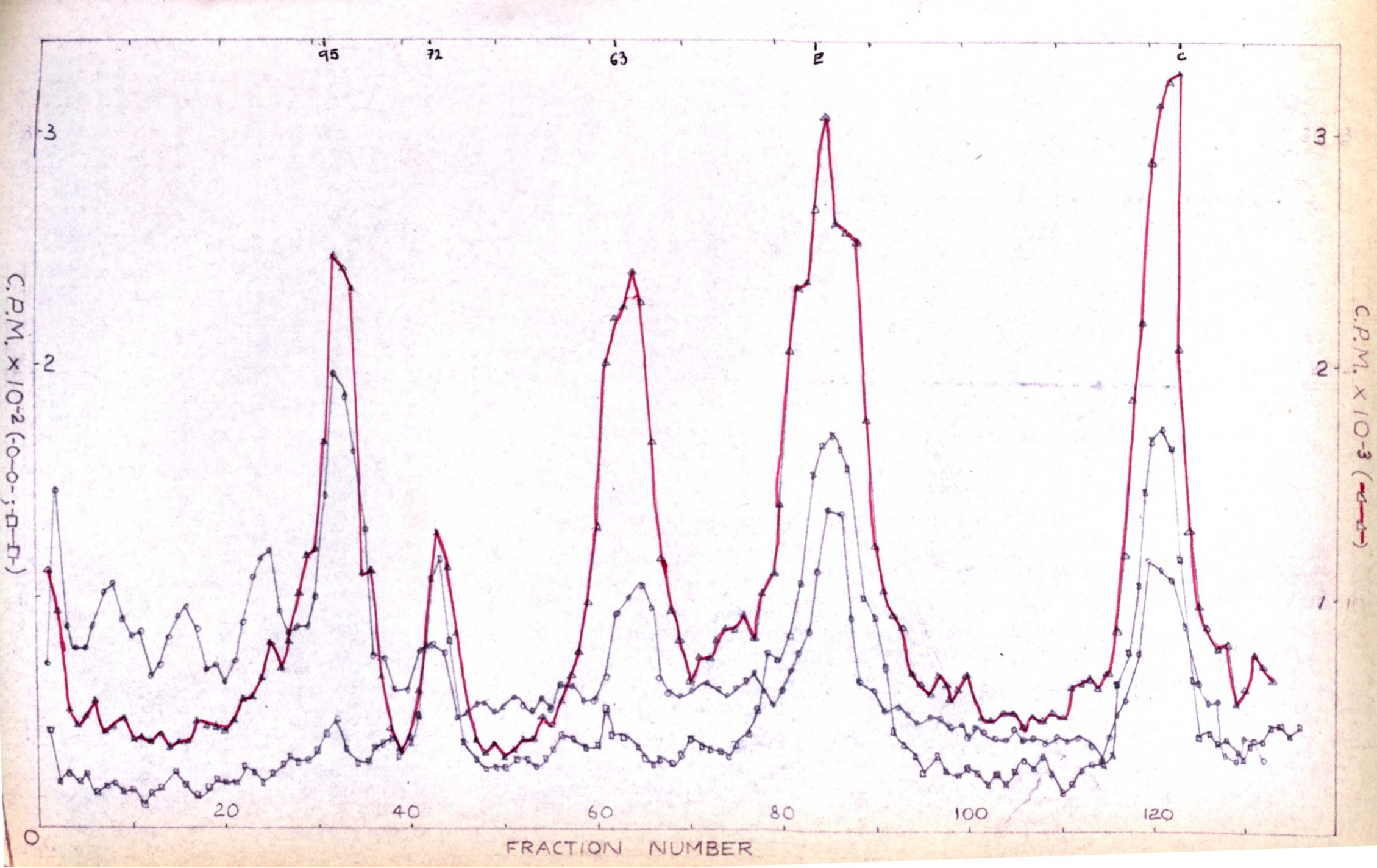

95
63
E
C
3
2
0
c.p.m. x 10-2 (o—o—o—o)
20
40
60
80
100
120
FRACTION NUMBER
3
2
1
c.p.m. x 10-3 (—Δ—Δ—)

Figure 23.

The experiment described in Fig.22 was repeated exactly, except that chick embryo fibroblasts were used instead of BHK-21 cells. The symbols used are (—○—○—) for the short pulse, (—□—□—) for the chase and (—△—△—) for the control.


95
72
63
B
C
3
2
1
0
20
40
60
80
100
120
FRACTION NUMBER
C.P.M. × 10-2 (-o-o-; -□-□-)
C.P.M. × 10-3 (-△-△-)

there was a real increase in the amount of core protein labelled during the chase. The core protein is probably derived from a large molecular weight precursor, maybe NVP 127.

When the proteins synthesised in chick embryo fibroblasts infected with SFV were analysed after a short pulse of [^{3}H] leucine in the presence of TPCK another high molecular weight peak could be detected. This peak had a molecular weight of 165,000 and was called NVP 165. This estimate of the molecular weight may be substantially inaccurate, as the value was based on extrapolation from a graph with β-galactosidase as the highest point (see Fig.5). An attempt was made to use human thyroglobulin (m.w. 165,000) as a marker, but the protein was broken into low molecular weight fragments. In the samples from both BHK and chick cells the peak of NVP 95 was unusually broad. This latter point is discussed later. Very little NVP 72, NVP 63, envelope or core protein was labelled during the short pulse while after the chase they predominated. Conversely after the chase NVP 165 and NVP 127 could not longer be detected while the amount of NVP 95 present was substantially reduced. These results confirm the hypothesis that NVP 127 and NVP 95 are precursors to NVP 72, NVP 63 and the structural proteins.

10. Virus-specified proteins synthesised in the presence of amino acid analogues

If the specific cleavage of NVP 165, NVP 127 and NVP 95 is due to a protease which recognises a particular sequence or three-dimensional configuration of the polypeptide, the cleavage might be prevented by replacement of appropriate amino acids with analogues. An experiment was performed with five analogues, all of which are known to be incorporated into proteins of eucaryotic cells. The analogues were p-fluorophenylalanine which is an analogue of phenylalanine, (Levintow, Thoren, Darnell and

Hooper, 1962), canavanine which is an analogue of arginine (Kreuse, White, Carter and McCoy, 1959), azetidine-2-carboxylic acid which is a proline analogue (Fowden and Richmond, 1963), ethionine which is an analogue of methionine (Rabinowitz, Olson and Greenberg, 1957) and azotryptophan which is a tryptophan analogue (Friedman, 1969). Chick embryo fibroblasts were incubated in the presence of the analogues for 20min before [^{3}H] leucine was added. The radioactive leucine was added 6h after the cells had been infected with SFV. The pulse was either halted after 7min or was chased for a further 53min in the absence of the analogues. The proteins were extracted and compared by analysis on polyacrylamide gels (Fig.24). The experiment was carried out in the presence of TPCK.

When TPCK was present, NVP 165, NVP 127 and NVP 95 could be labelled during the short pulse. During the chase the amount of NVP 72, NVP 63 and the core protein increased and the envelope protein became detectable. The amount of NVP 95 decreased and NVP 165 and NVP 127 was no longer present. Again the peak of NVP 95 was very broad in samples labelled during a short pulse, whether TPCK was present or not. If uninfected cells were treated in the same way before extraction and analysis of the proteins, no clearly defined peaks could be identified. The experiments add more weight to the hypothesis that NVP 165, NVP 127, NVP 95 and NVP 63 are precursors to NVP 72, and the virus structural proteins

11. <u>The effect of a temperature jump on the virus-specified proteins</u>

Baltimore (personal communication) showed that raising the temperature at which Hela cells, infected with poliovirus, were incubated inhibited virus-specified protein synthesis. When the proteins were analysed after the release of the inhibition there was a build up of the precursors. Cultures of BHK-21 cells, 6h after infection with SFV, had [^{3}H] leucine added. Immediately the Petri dishes were placed in a water bath at either

Figure 24.

The experiment described in Fig.23 was repeated exactly, except that amino acid analogues were present for 20min before the radioactive leucine was added. The analogues were used at the following concentrations: 0.0034M-azetidine -2-carboxylic acid, 0.002M-azotryptophan, 0.0033M-canavanine, 0.0018 M-ethionine and 0.0025M- FPA, and were present throughout the period of labelling. The symbols used are (-○ -○ -) for the short pulse, (-□-□-) for the chase, and (—△—△—) for the control.

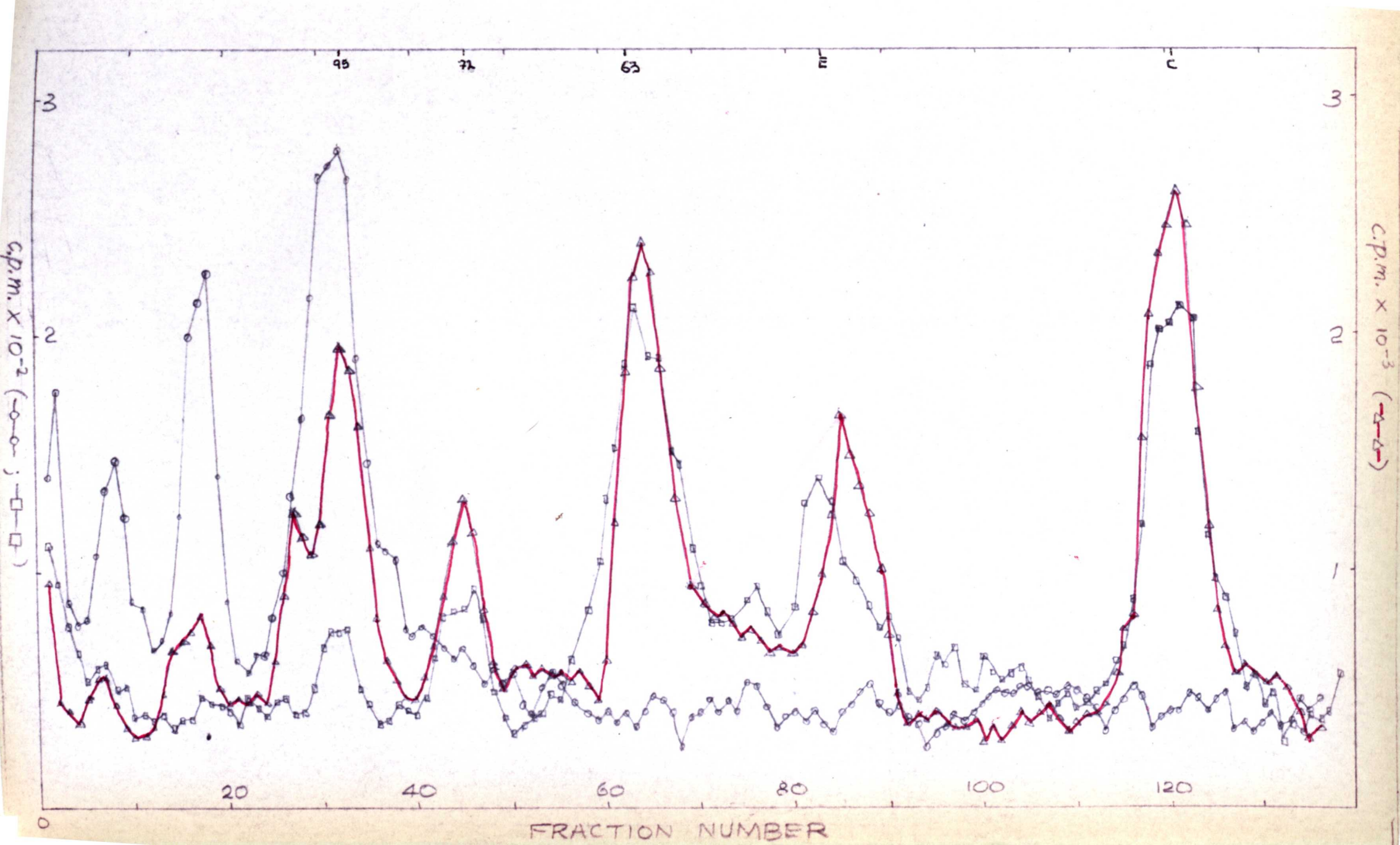
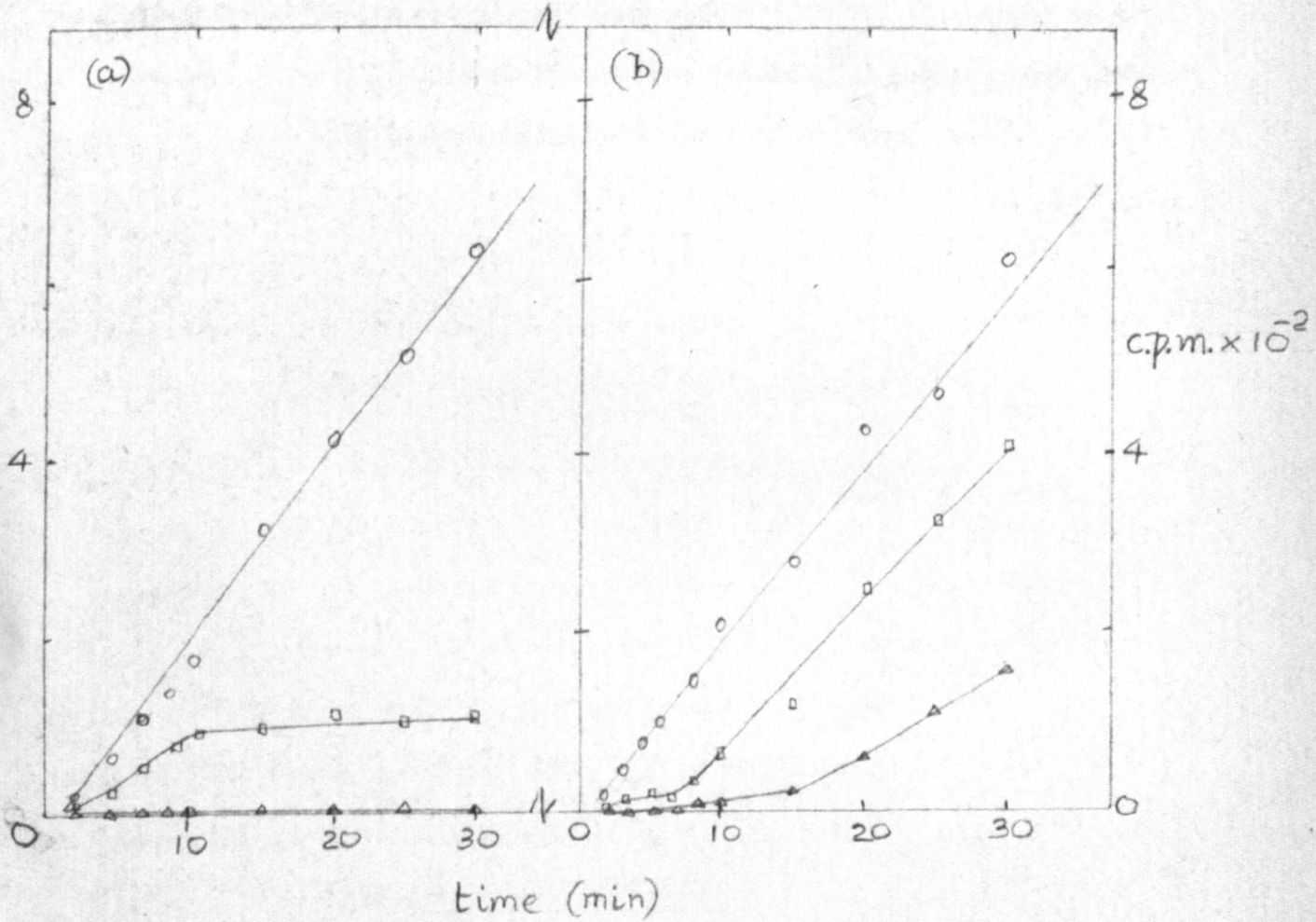

95
72
63
E
C
3
2
1
0
20
40
60
80
100
120
FRACTION NUMBER
c.p.m. × 10⁻² (o—o—o ; □—□—□)
c.p.m. × 10⁻³ (△—△—△)

Figure 25.

The incorporation of radioactive valine into an acid insoluble precipitate in infected BHK-21 cells, was measured after raising the temperature of incubation to 42.5°C (-□-□-) or 44°C (-△-△-), compared with continued incubation at 37°C (Fig.25a). A parallel series of cultures had the temperature altered for 30min before restoring the normal temperature of incubation (37°C). Radioactive leucine was added, and protein synthesis was measured in cultures held for 30min at 44°C (-▲-▲-), at 42.5°C (-■-■-) and control cultures maintained at 37°C (-○-○-; Fig.25b)

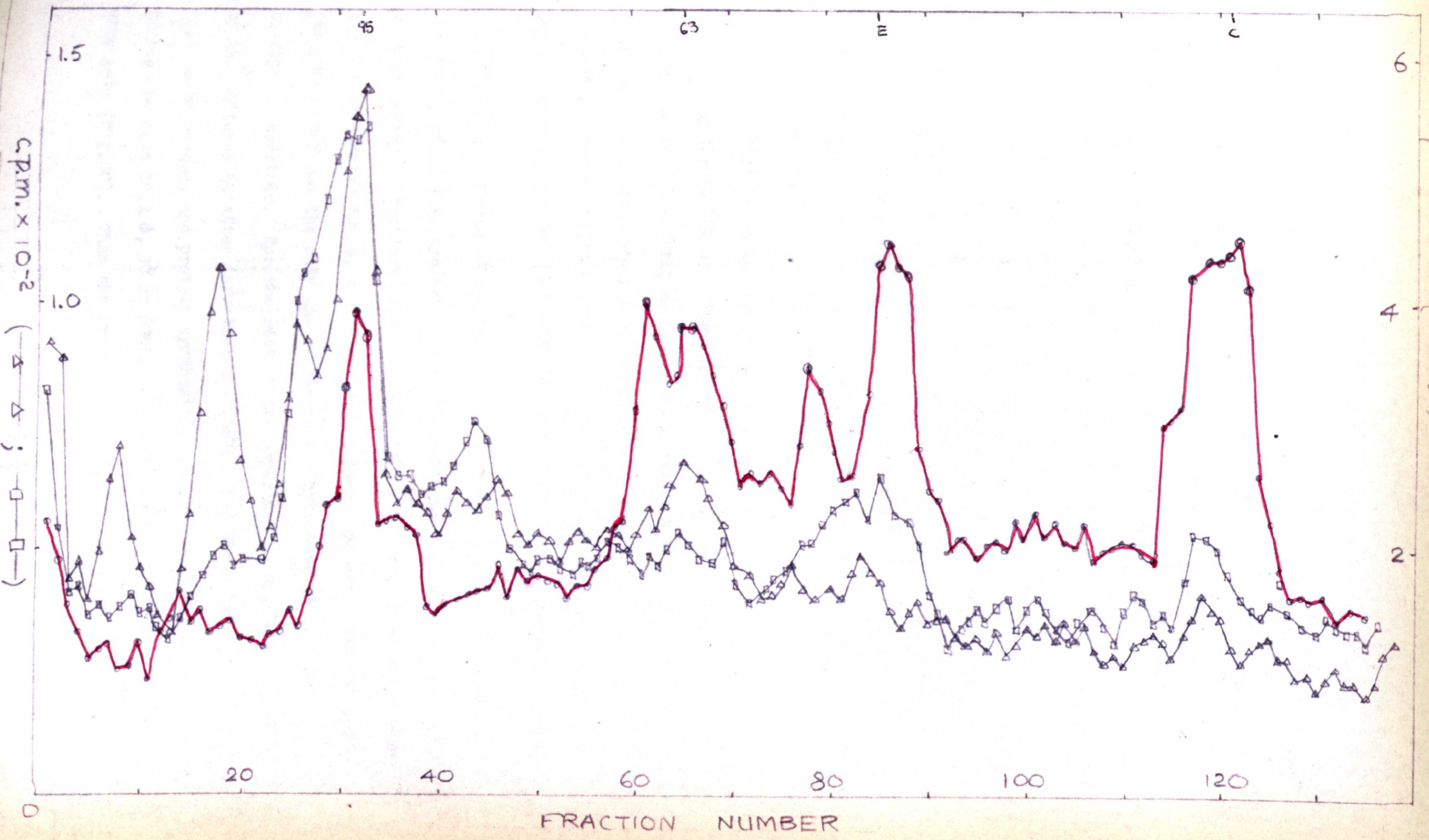

(a)
(b)
8
4
0
10
20
30
c.p.m. × 10⁻²
time (min)

42.5°C or 44°C. These temperatures were measured by a mercury thermometer immersed in the water bath, and are therefore not precise. At 2min intervals the incorporation of the radioactive leucine into an acid-insoluble precipitate was measured. At both temperatures the virus-specified protein synthesis was halted. By this time after infection, host cell protein synthesis had been almost completedly inhibited (see Figs. 2 & 8). At 44°C the inhibition of viral protein synthesis was almost immediate, but at 42.5°C it took 10min for inhibition of viral protein synthesis to become complete (Fig.25a). When the temperature was dropped back to 37°C protein synthesis restarted. The resumption of protein synthesis was followed by assaying the incorporation of radioactive leucine into an acid-insoluble precipitate (Fig.25b). After 30min at 44°C protein synthesis did not restart within 30min, while after treatment at 42.5°C for the same length of time protein synthesis immediately resumed.

The proteins formed during the 10min after the temperature was dropped from 42.5°C to 37°C were investigated, in the presence or absence of TPCK. The proteins were extracted and analysed by polyacrylamide gel electrophoresis. These results were contrasted with those from a 10min pulse at 37°C. At 42.5°C, in the absence of TPCK, the major protein detectable was NVP 95 (Fig.26). A small quantity of NVP 63 and the envelope and core proteins could also be identified. In the presence of TPCK at 42.5°C, it was possible to detect NVP 165 and NVP 127 as well as NVP 95 and NVP 63. NVP 95 appears to be split into two peaks, one coincident with that present under normal conditions, the other with a higher apparent mobility corresponding to an estimated molecular weight of 105,000. That is when the proteins were analysed there was a build up of precursors and there was a very much reduced quantity of radioactivity in the virus structural protein. The conclusion that can be derived from these results..

Figure 26.

The experiment described in Fig.22 was repeated exactly, except that the temperature of incubation was raised from 37°C to 42.5°C, when the TPCK was added 5½h after infection. After 30min the temperature was dropped to 37°C and radioactive leucine was added. The cultures were incubated at 37°C for 10min before the proteins were extracted. The symbols used are (-Δ-Δ-) for the short pulse in the presence of TPCK (-□-□-) for the pulse in absence of TPCK and (-○-○-) for the control, incubated continuously at 37°C.


95
63
E
C
1.5
1.0
0
6
4
2
c.p.m. x 10-2 (—△—△—□—□—)
c.p.m. x 10-2 (—○—○—)
20
40
60
80
100
120
FRACTION NUMBER

is that NVP 127 and NVP 95 are the precursors of the structural proteins.

12. The effect of inhibitors of protein synthesis on virus-specified protein synthesis.

The effect of five inhinitors of protein synthesis on the course of virus-specified protein synthesis was investigated. The five inhibitors used were sodium fluoride (NaF), N-butanol, aurinotricaroxylic acid (ATA), cycloheximide and puromycin. The NaF has been reported by Lin, Mosteller and Hardesty (1965a) to be an inhibitor of initiation in eucaryetic systemps, while ATA was suggested to act in a similar way by Cogniaux-leClerc (1971). Finally n-butanol was also suggested to be an inhibitor of initiation by Freedman, Hori and Rabinowitz (1967).

The inhibition caused by NaF has been reported to be readily reversed by removing the inhibitor. When protein synthesis is restored, the first proteins to be formed will be the primary transcripts of the RNA. As synthesis continues these precursors will be broken down into the final products. Wunner and Pringle (1972) used this method to investigate the way in which the proteins of Vesicular Stomatitis virus were synthesised.

First the effects of the inhibitors were investigated by measuring the decary of protein synthesis after NaF, n-butanol or ATA had been added to chick embryo fibroblasts infected with SFV (Fig.27a). Then after washing the monolayers twice, the recovery of protein synthesis was measured (Fig.27b) NaF was the only one of the three inhibitors that proved to be fully reversible. For the rest of the experiments, 0.01M-NaF was added to the cultures 6h after infection with SFV. Then 30min later, the cultures were washed, and protein synthesis resumed. The proteins synthesised during the next 10, 20, 30 or 60min were compared by analysis on polyacrylamide gels (Fig.28). Then the areas under the peaks were integrated and

Figure 27.

0.005M-ATA, 0.005M-n-butanol and 0·01M-NaF, were added to BHK-21 cells 5½h after infection with SFV. Incorporation of radioactive valine into an acid insoluble precipitate was measured (Fig.27a). After 30min parallel cultures were washed twice, and incubated in the presence of radioactive valine. Again the protein synthesis was assayed (Fig.27b). The symbols used are (-o-o-) for the control (untreated) cultures, (-□-□-) for the cultures treated with ATA, (-Δ-Δ-) for the cultures treated with n-butanol, and (-●-● -) for the cultures treated with NaF.

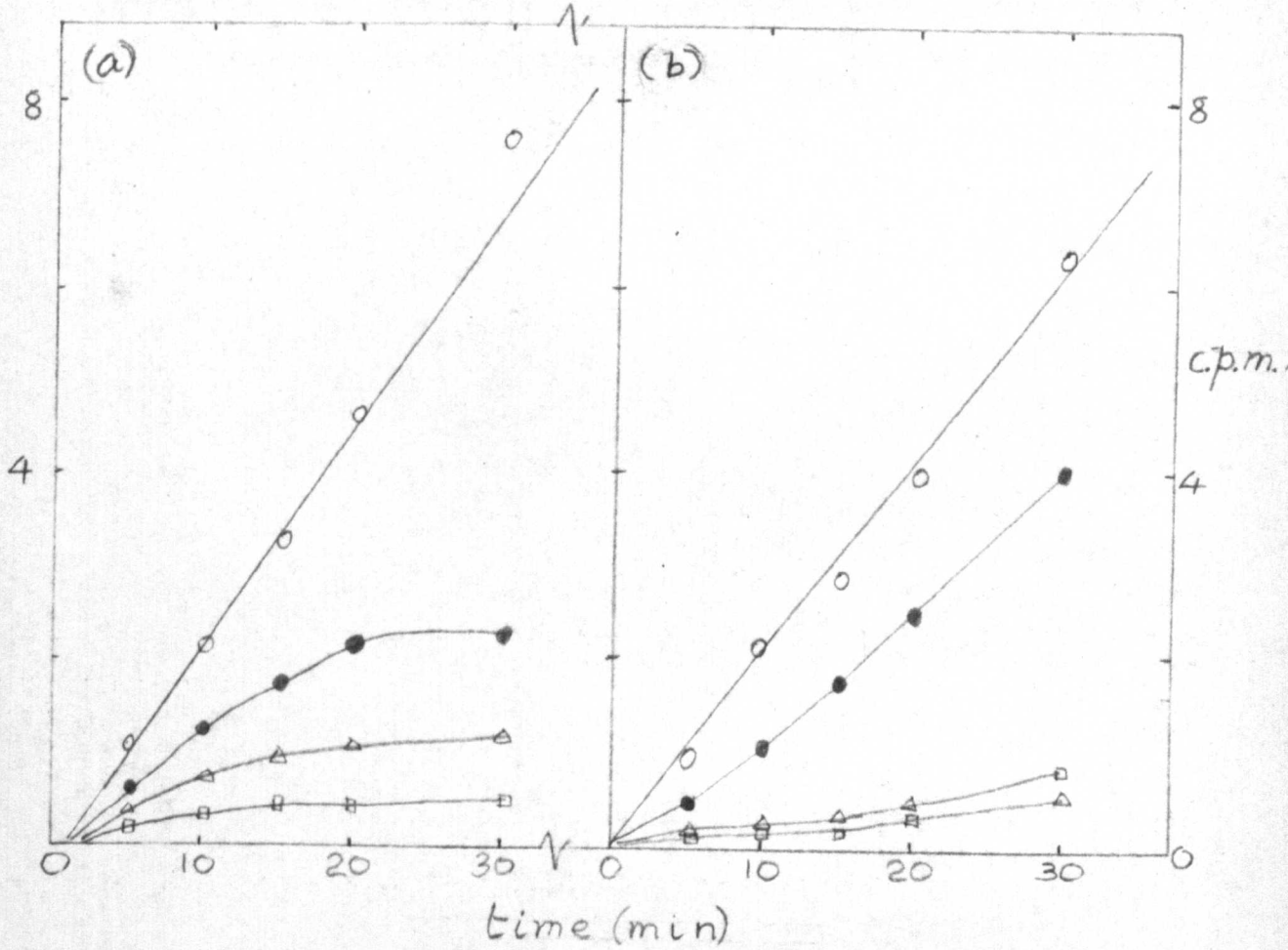
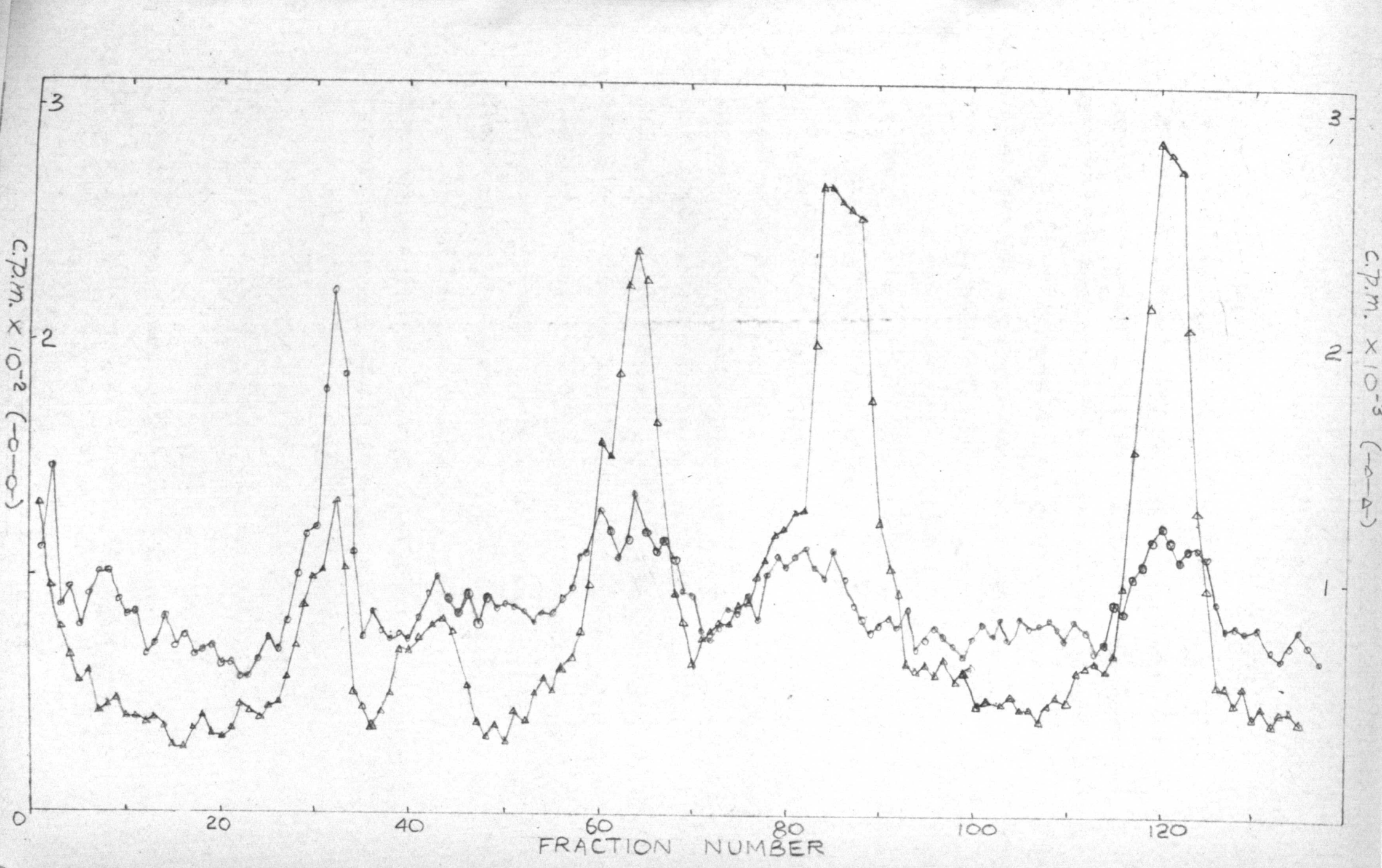

(a)
(b)
8
4
0
10
20
30
c.p.m.
time (min)

Figure 28.

Monolayer cultures of BHK-21 cells, 5½h after infection with SFV, were treated with 0.01M-NaF in EDA for 30min. Then the cultures were washed twice with EDA, and incubated with 50μCi/culture of [^{3}H] leucine. The proteins were extracted by method II after 10min (-○-○-) and 60min (-▲-△-), and analysed by electrophoresis on 12cm polyacrylamide gels.


C.P.M. X 10⁻² (—o—o—)
C.P.M. X 10⁻³ (—△—△—)
FRACTION NUMBER
0
1
2
3
20
40
60
80
100
120

plotted with respect to time (Fig.29). Host cell protein synthesis recovered very slowly, remaining at a very low level. Immediately after the resumption of protein synthesis NVP 95 was the major protein. In contrast to the other proteins there was no increase in the quantity of it during the rest of the labelling period. The quantities of NVP 72, and the envelope and core proteins increased continuously during the 60min labelling period, while NVP 63 reached its maximum after 30min. The simplest explanation of the results is that NVP 72,and the structural proteins are the final products while NVP 95 and NVP 63 are intermediates in the processing of NVP 165 and NVP 127.

Cycloheximide and puromycin are both antibiotics that prevent chain elongation during protein synthesis (Darken, 1964; Nathans, 1964; Sisler and Siegel, 1967). On addition of puromycin 3.5h after infecting chick embryo fibroblasts with Sindbis virus, further RNA synthesis was not prevented (Scheele and Pfefferkorn, 1969b). Similarly Friedman and Grimley (1969) showed that cycloheximide, added 4h after infection of chick embryo fibroblasts with SFV, did not inhibit further RNA synthesis. If the plausible assumption is made that the genetic information for the virus RNA polymerase is carried by the viral RNA, then the preceding results can be explained in two alternative ways. Either the synthesis of the polymerase proteins is resistant to the antibiotics or sufficient polymerase has been synthesised by 3.5h after infection to produce all the viral RNA. The proteins still being synthesised after addition of one of the antibiotics were analysed. If the amount of all of the five proteins is reduced equally, then it follows that the synthesis of all of them is sensitive to the inhibitors.

The resistance of viral RNA synthesis to puromycin and cycloheximide added 3.5h after infection with SFV was confirmed (Fig.30a) under

Figure 29.

The percentage of the various virus specified proteins was calculated from a series of experiments, such as the ones shown in Fig.28. The results are plotted as percentage of NVP 95 (-○-○-), NVP 72 (-△-△-), NVP 63 (-□-□-), envelope protein (-●-●-) and core protein (-▲-▲-) versus time after removal of NaF.

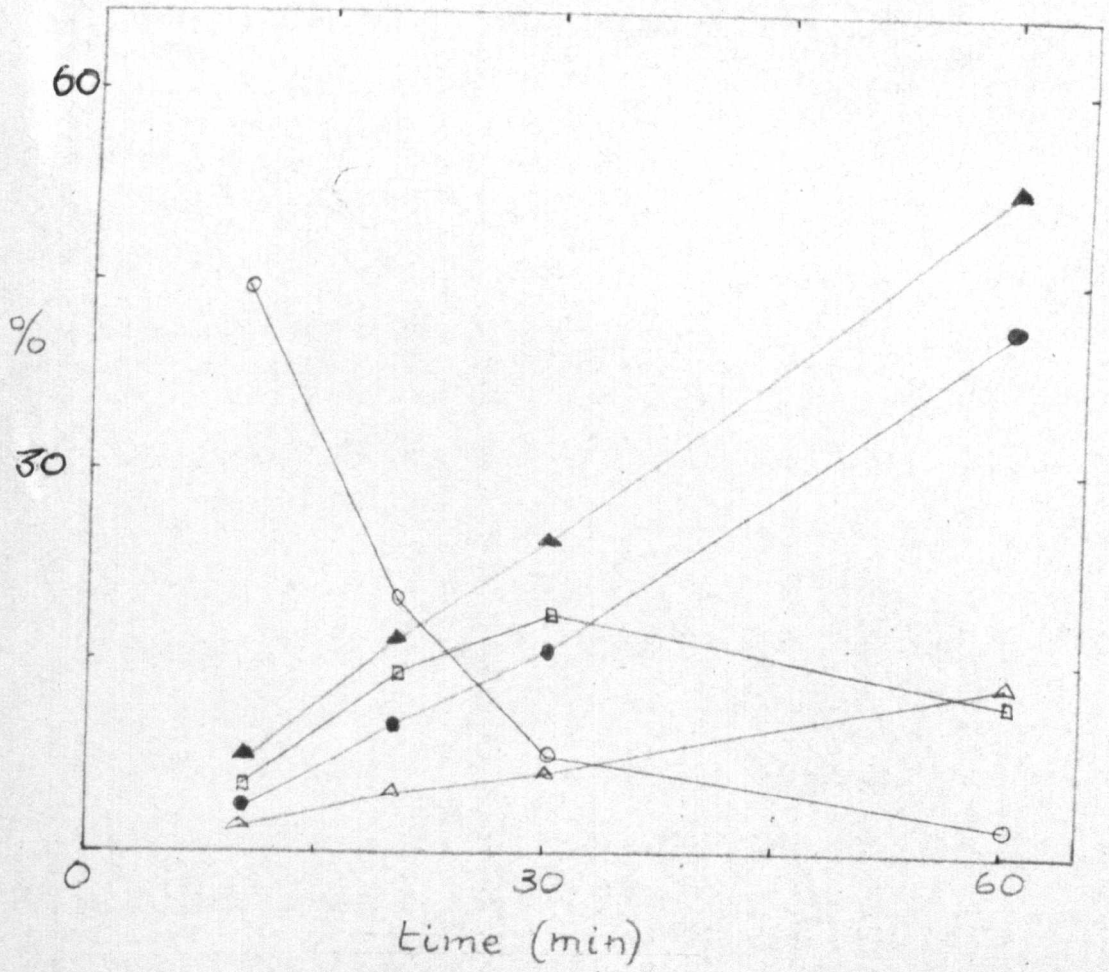
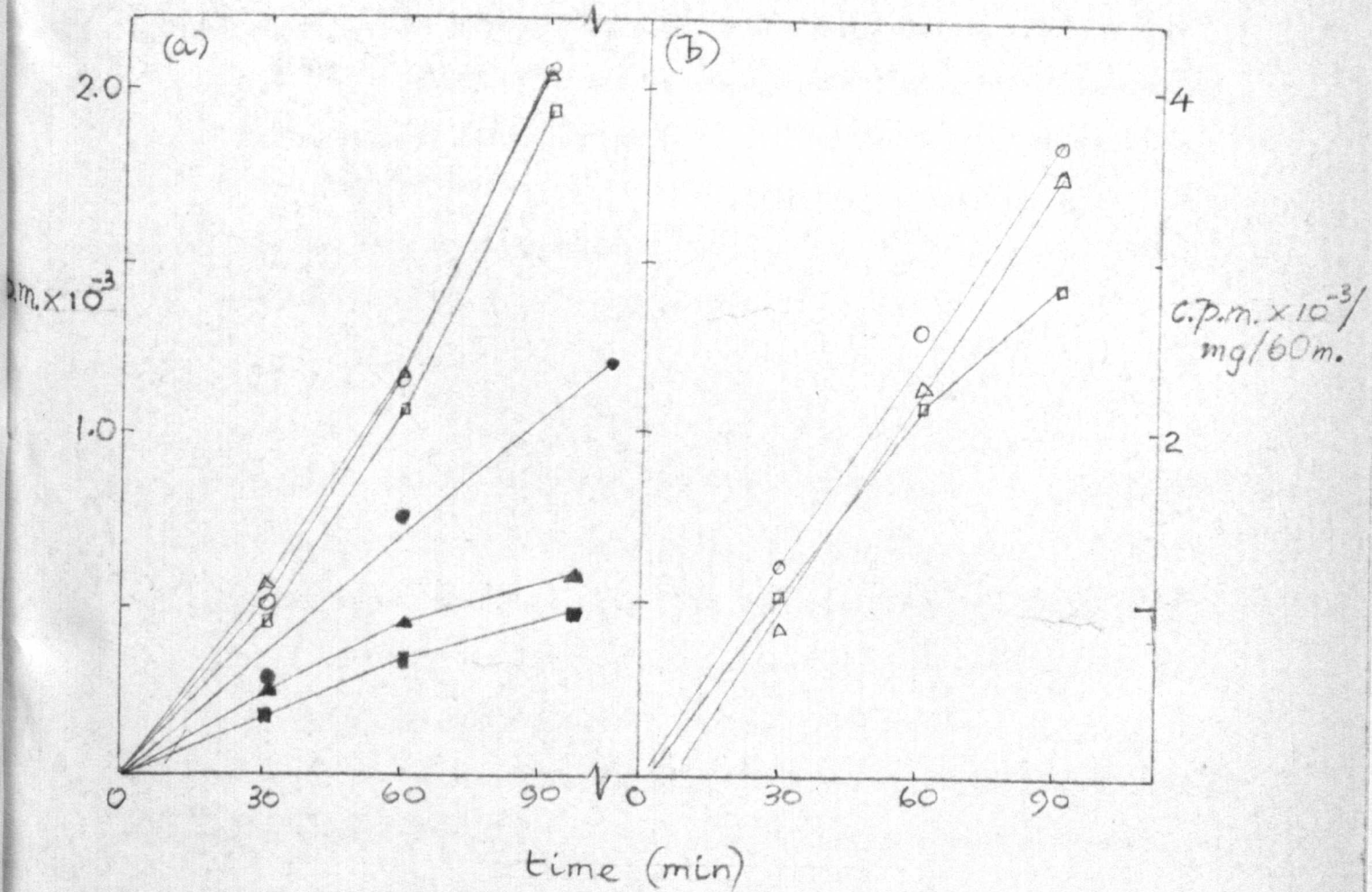

60
%
30
0
30
60
time (min)

Figure 30.

Cycloheximide (20µg/ml) and puromycin (50µg/ml), were added to monolayer cultures of chick embryo fibroblasts 3½h after infection with SFV. Incorporation of uridine into RNA and valine was measured (Fig.30a). The symbols used are (-O-O-) for RNA and (-●-●-) for protein synthesis in untreated cultures, (-Δ-Δ-) for RNA and (-▲-▲-) for protein synthesis in cycloheximide treated cells, and (-□-□-) for RNA and (-■-■-) for protein synthesis in puromycin treated cells. Also RNA polymerase activity in the homogenate of control cells (-O-O-), cycloheximide treated cells (-Δ-Δ-) and puromycin treated cells (-□-□-) was assayed (Fig.30b).


(a)
(b)
2.0
1.0
4
2
0
30
60
90
0
30
60
90
time (min)
c.p.m. × 10⁻³/
mg/60m.

conditions in which protein synthesis was inhibited over a period of 1h. After addition of puromycin or cycloheximide to the monolayer the amount of viral RNA polymerase activity in an in vitro system was assayed and was compared with that in untreated cells (Fig.30b). When the antibiotics were added 3.5h after infection, the RNA polymerase activity increased to reach a maximum 6h after infection. When the proteins were labelled, the experiment was carried out in the same way, except that radioactive lysine was present from 4-5h after infection. The proteins were then extracted and analysed by polyacrylamide gel electrophoresis. Compared with the normal electrophoretograms, electrophoretograms from the drug tested cells showed that all the proteins were proportionately reduced in quantity (Fig.31). This suggests that the synthesis of the polymerase proteins is completed before 3.5h after infection.

III. Virus-specified macromolecules associated with sub-cellular fractions

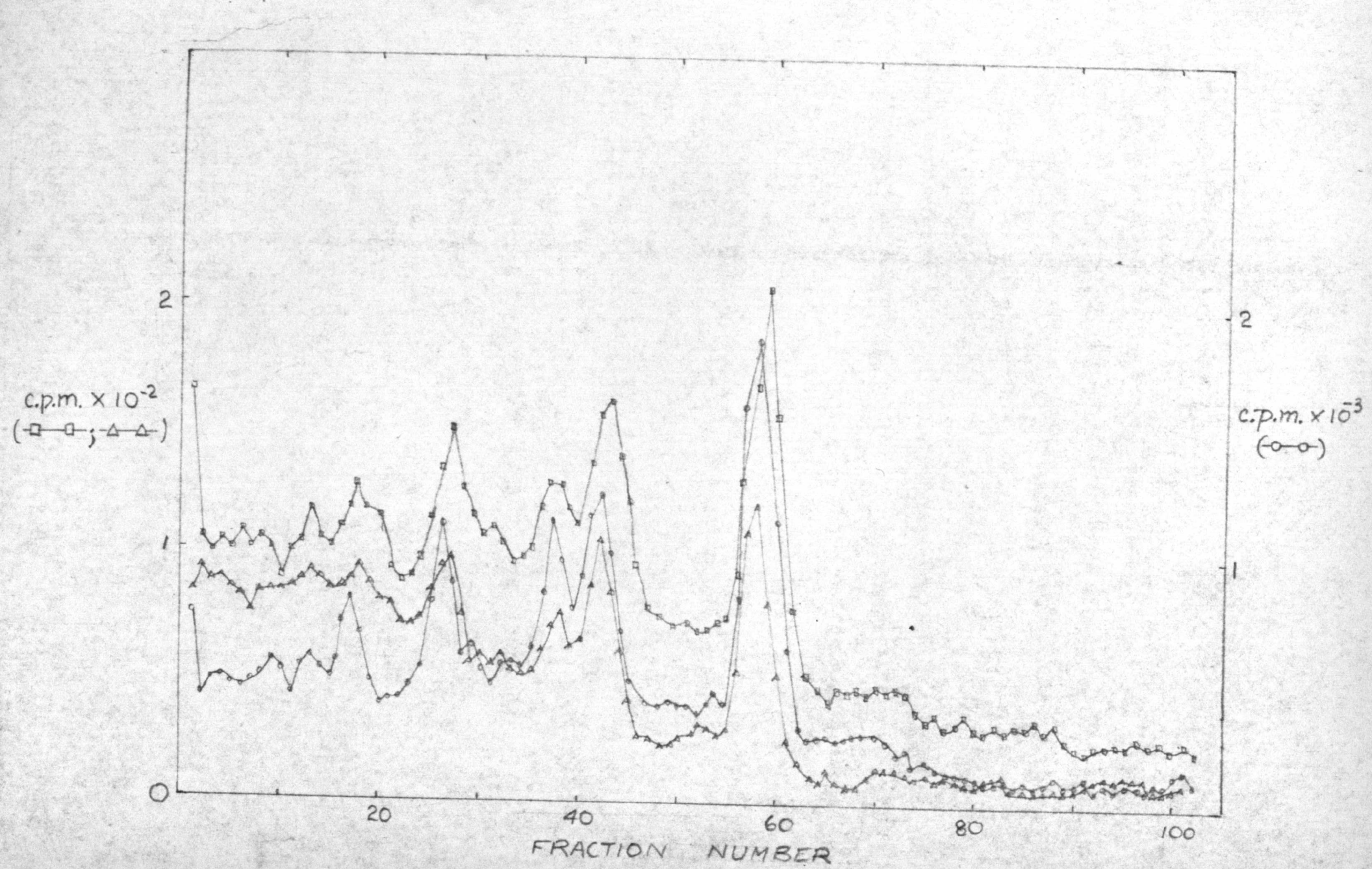
Three different techniques of sub-cellular fractionation were used to achieve different aims. The first technique was developed in an attempt to purify the RNA polymerase. In the second, homogeneous preparation of plasma and endoplasmic reticulum membranes were prepared and analysed. In the third the M band technique was used to isolate specific nucleoprotein complexes. In each case, the virus specified protein and RNA was analysed, in an attempt to assign functions to the different species. Finally glucosamine inhibition was used to try to investigate the relationships between the different species of RNA.

1. The partial purification of the RNA polymerase

The purification of the viral RNA polymerase was undertaken to try

Figure 31.

Cyclohexamide (20µg/ml) and puromycin (50µg/ml) were added to monolayer cultures of chick embryo fibroblasts 3½h after infection with SFV. 30min later 20µCi/culture of [^{3}H] valine was added to the cultures. Then 5h after infection, the proteins were extracted by method I and analysed on 9cm polyacrylamide gels. The symbols used are (-O-O-) for the proteins from untreated cells, (-Δ-Δ-) for proteins from cycloheximide treated cells and (-□-□-) for the proteins from puromycin treated cells.


c.p.m. × 10⁻²
(□—□; △—△)
c.p.m. × 10⁻³
(○—○)
2
1
0
20
40
60
80
100
FRACTION NUMBER

to identify the proteins that are involved in the replication of the viral RNA. The RNA polymerase is membrane bound, and this presents a problem in devising a procedure for purification. Another problem arises because the only way of identifying the RNA polymerase is by its activity in an *in vitro* system. Therefore no treatment can be used in the purification procedure that destroys the *in vitro* activity. The RNA polymerase activity was measured by the incorporation of [^{3}H] GTP into an acid-insoluble product. The properties of the particular assay system used in these experiments have been reported previously (Morser, 1971). Some important parameters of the RNA polymerase are shown in Table 6, demonstrating that activity is dependent on the presence of all 4 nucleosides triphosphates and magnesium ions. Addition of Dextran sulphate 500 doubled the incorporation of radioactive GTP, while no RNA polymerase activity was found in uninfected cells. The material formed was sensitive to ribonuclease. The flow chart for the partial purification is shown on Table 1. Also shown in Table 1 is the incorporation of [^{3}H] uridine and [^{14}C] valine into the acid precipitable material from various fractions. The isotope was present for 10min before the RNA polymerase was extracted. Using this short length of time it appears that there is a partial separation of the site of incorporation of the protein precursors from that of the RNA precursor. In Table 7 the total activity and the specific activity at each stage of the purification are shown. A very substantial purification has been achieved by the end of the procedure. The RNA polymerase sediments in the fraction that is most efficient at incorporating uridine into an acid insoluble product.

The activity of three marker enzymes was assayed in the fractions obtained at various steps of the partial purification of the RNA polymerase.

Table 6.

A preparation of the RNA polymerase from chick embryo cells infected with SFV was partially purified by the method shown in Table 1. The enzyme was then assayed in a complete *in vitro* system, and the activity compared with that in systems lacking various reagents. The complete assay contained the following reagents in a volume of 0.35ml:

tris+ HCl pH8.5	35.0μmol
$MgCl_2$	1.6μmol
KCl	4.0μmol
2-mercaptoethanol	7.0μmol
PEP	0.5μmol
pyruvate kinase	10.0μg
actinomycin D	1.0μg
dextran sulphate 500	3.5μg
ATP, CTP, UTP	50.0nmol
[^{3}H] GTP	0.5μCi; 1.0nmol

and 0.1ml of the enzyme . The effect of incubation in the presence of ribonuclease and at 0°C was investigated. Finally the equivalent fraction was prepared from uninfected cells, and was tested for RNA polymerase activity.

	specific activity c.p.m./mg/60min
complete from infected cells	93,246
-ATP, CTP and UTP	1,355
$-Mg^{++}$	1,627
-dextran sulphate 500	44,987
+ribonuclease	37,851
incubated at 0°C	1,263
not incubated	1,125
complete from uninfected cells	530

Table 7.

The total and specific activity of the RNA polymerase at the various stages of the partial purification was measured. The percentage recovery of the three marker enzymes (succinate dehydrogenase, NADPH: cytochrome C reductase, and 5' nucleotidase) was determined.

	succinate dehydrogenase	NADPH:cytochrome C reductase	5' nucleotidase	polymerase specific activity c.p.m./mg/60min	polymerase total activity c.p.m./60min.
cell homogenate	(100)	(100)	(100)	4,383	127×10^3
500g pellet	36	N.M.	31	3,214	31×10^3
12,000g pellet	51	0.38	34	15,700	600×10^3
12,000g supernatant	18.5	0	22	865	40×10^3
band from 1st gradient	41	21	5	98,274	200×10^3
interface from 2nd gradient	37	16	2	56,521	150×10^3

N.M. stands for not measurable, because the fraction was too cloudy.

Succinate dehydrogenase was used as a marker for mitochondrial material; NADPH-cytochrome C reductase as a marker for material from the endoplasmic reticulum; and 5' -nucleotidase for material from the plasma membrane. The results suggest that the fraction containing the RNA polymerase activity was mainly composed of mitochondrial material contaminated with a little material from the plasma membrane (Table 7).

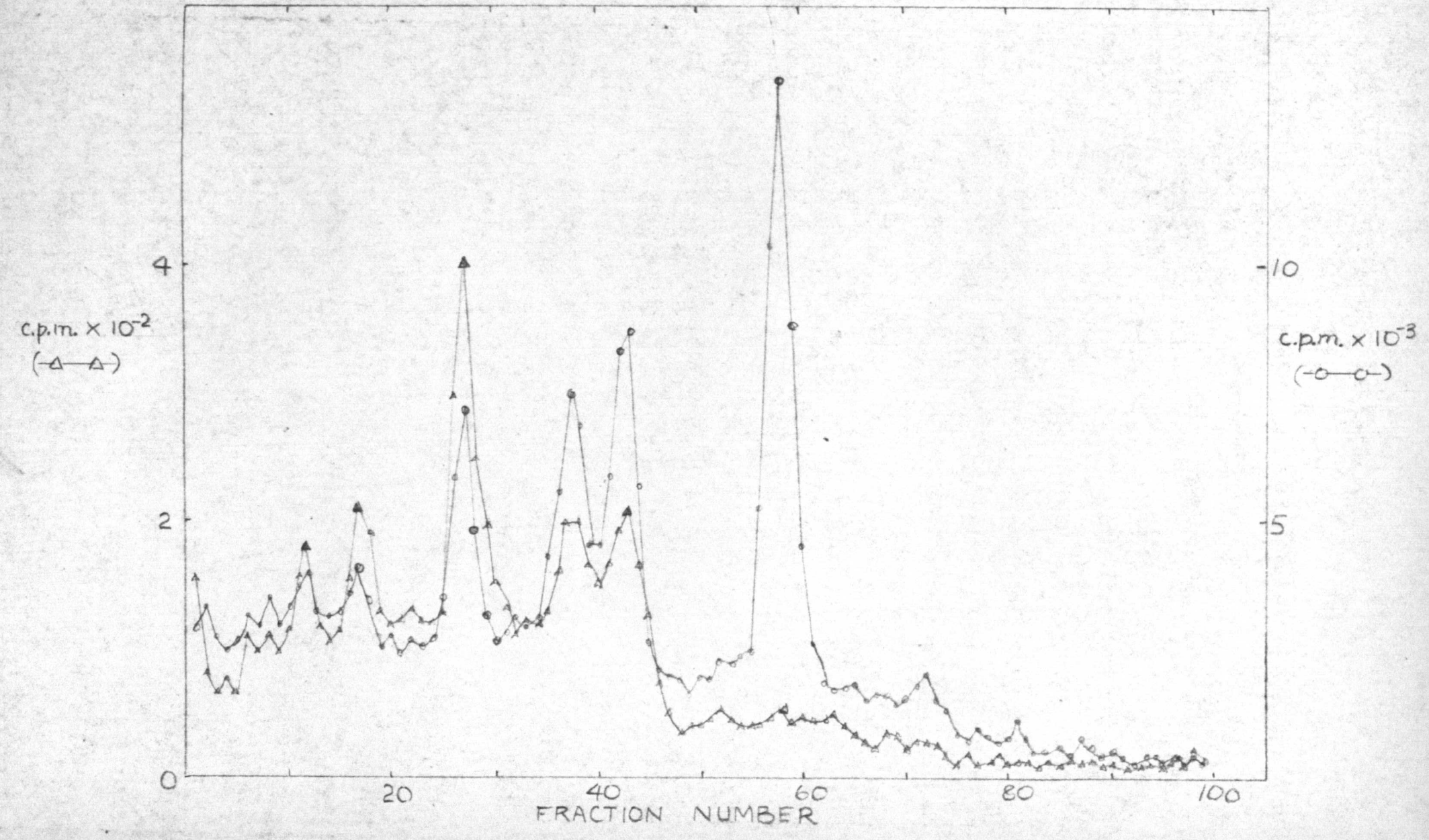
Chick embryo fibroblasts, infected with SFV, were labelled with [^{35}S] methionine from 1h after infection. The cells were harvested 5h after infection and the RNA polymerase was worked up by the purification method. After each step an aliquot was removed. The proteins were extracted from the samples and analysed by polyacrylamide gel electrophoresis. In Fig.32 the electrophoretogram of the sample from the homogenate is shown alongside the electrophoretogram of the final polymerase fraction. The polymerase fraction contained less NVP 63 and envelope protein than the homogenate and practially no core protein at all. This would suggest that these proteins are not connected with the RNA polymerase.

Antisera which specifically inactivates either the envelope or core proteins of SFV had been prepared. When the homogentate from the chick embryo cells infected with SFV was reacted with the core antiserum, only the core protein was precipitated. In contrast when the envelope antiserum was added to a homogenate prepared in a similar way from SFV infected cells, NVP 63 as well as the envelope protein was precipitated. (S.I.T. Kennedy, personal communication). The effect of these two preparations of antisera on the activity of the RNA polymerase was investigated.

The RNA polymerase was partially purified from chick embryo cells 5h after infection with SFV. The activity in a normal in vitro assay was compared with that in an assay that contained either the core or

Figure 32.

Chick embryo cells, infected with SFV, were labelled with 50μCi/ culture of [^{35}S] methionine from 1 - 5h after infection. The RNA polymerase fraction was prepared by the procedure detailed in Table 1. The proteins were extracted by method I, and were compared with the proteins in the total homogenate by analysis on 9cm polyacrylamide gels. The symbols used are (-o-o-) for the proteins from the homogenate and (-Δ-Δ-) for the proteins from the RNA polymerase fraction.


c.p.m. × 10⁻²
(-Δ—Δ-)
c.p.m. × 10⁻³
(-o—o-)
0
2
4
5
10
20
40
60
80
100
FRACTION NUMBER

envelope antisera. To show that any change observed was specific, pre-immune antiserum was added to the same concentration of protein as the specific antiserum. The antibody titre of the antisera prepared against the core was lower than that of the antiserum prepared against the envelope. Therefore more protein was added in the case of the former. The envelope antiserum reduced incorporation in the assay by 50%, but the control assay containing the pre-immune antiserum was reduced by a similar amount (Table 8). The decrease in activity was probably a non-specific neutralisation of some of the chick proteins (the antisera had been prepared in rabbits). In contrast the core antiserum stimulated the RNA polymerase activity by a small amount, but the equivalent quantity of pre-immune anti-serum increased incorporation by the same amount. The increase in incorporation was probably due to the non-specific binding of [^{3}H] GTP to the huge quantities of protein present in the assay. Neither of these preparations of specific antiserum had any specific effect on the activity of the RNA polymerase. This suggests that the proteins connected with the polymerase activity are not neutralised by these particular antisera. That is, the polymerase does not contain either NVP 63 or the structural proteins.

An estimate of the sedimentation value for the RNA polymerase was calculated by the method of Martin and Ames (1961). Chick ribosomes (70S) were used as the reference marker. The RNA polymerase from chick embryo cells was purified by the method previously described until the second discontinuous sucrose gradient. Instead the preparation was layered over a preformed linear sucrose gradient, and centrifuged. Fractions from the gradient were analysed for RNA polymerase activity in vitro (Fig.33). The sedimentation coefficient of the fraction that contained maximum activity in the polymerase activity was calculated to be 270S. It must be

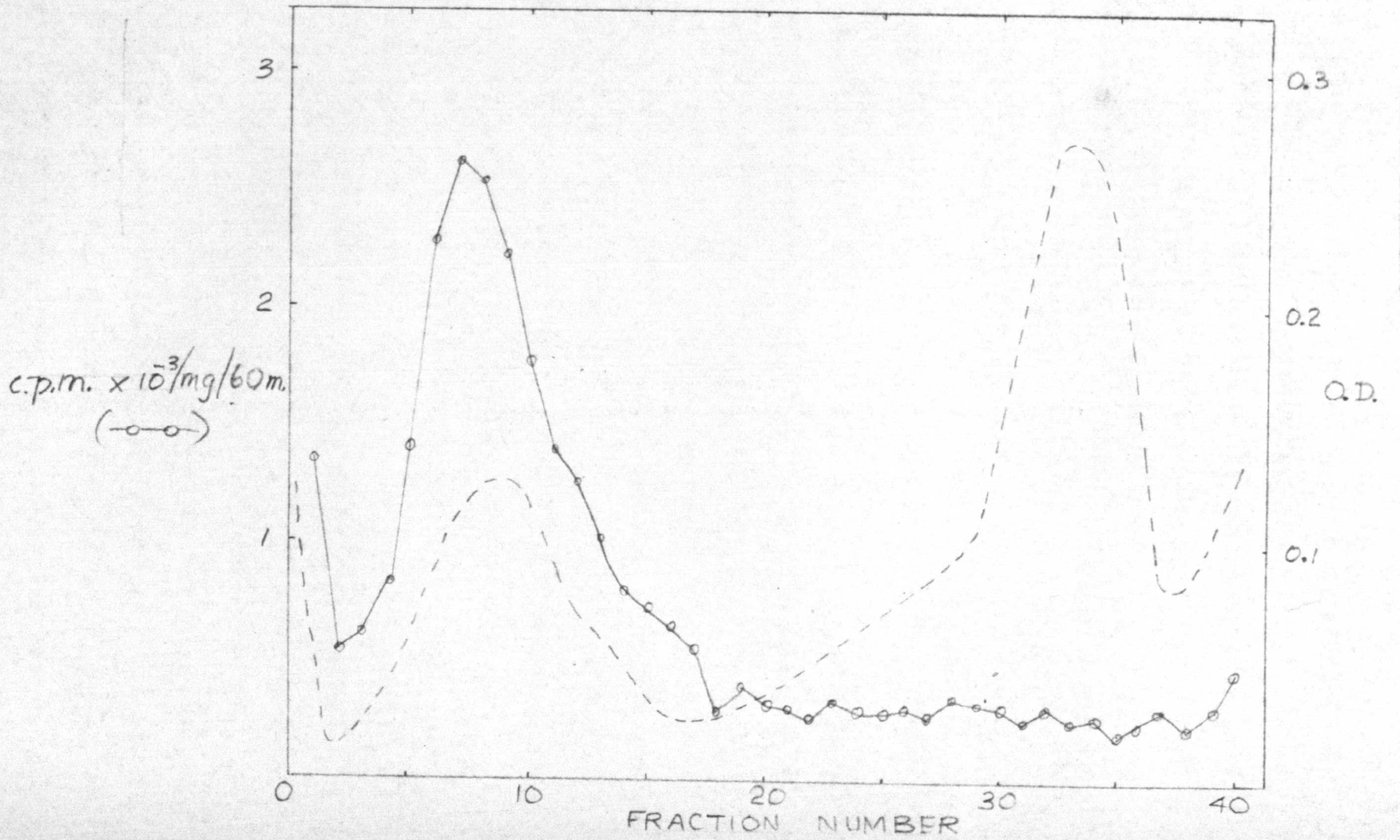
Table 8.

The activity of the partially purified RNA polymerase was assayed in the presence of antisera prepared against either the envelope protein or the core protein, or pre-immune antisera. In the case of the antiserum against envelope protein the assay contained an additional 40µg of protein while in the case of the antiserum against the core the protein there was an extra 250µg of protein present. Equivalent quantities of the appropriate pre-immune antisera were added to the control assays.

	specific activity c.p.m./mg/60min.
control	60,245
+ envelope antiserum	37,381
+ " pre-immune antiserum	39,253
+ core antiserum	65,420
+ " pre-immune antiserum	64,309

Figure 33.

The RNA polymerase, partially purified by the procedure described in Table 1, had its sedimentation coefficient estimated using 70S ribosomes as a marker. The polymerase activity (- O — O -) was assayed in the 0.5ml fractions collected from the sucrose gradient. The optical density (- - - - -) was determined by automatic monitoring of the unloading at 254nm.


c.p.m. x 10⁻³/mg/60m.
(—o—o—)
3
2
1
0
10
20
30
40
FRACTION NUMBER
0.3
0.2
0.1
O.D.

emphasised that this is only a very rough estimate of the real size of the entity that contains the RNA polymerase activity.

The RNA present in the partially purified RNA polymerase was analysed by polyacrylamide gel electrophoresis. Prior to the extraction of the polymerase, 5h after infection with SFV, radioactive uridine had been present for 1h. The polymerase was purified before the RNA was extracted. The RNA present in the homogenate was also extracted and analysed. There was far less single-stranded RNA present in the polymerase fraction than in the homogenate, while the proportion of replicative intermediate was increased (Fig.34). This suggests that the replicative intermediate is the structure connected with the RNA polymerase.

The RNA formed during a 1h incubation of the <u>in vitro</u> system was analysed by several different methods. The RNA was extracted and checked for sensitivity to ribonuclease (Table 6). Then both the ribonuclease treated and the untreated RNA were analysed by sucrose density gradient centrifugation and polyacrylamide gel electrophoresis. RNA labelled with [^{14}C] uridine was extracted from chick embryo cells infected with SFV and included to provide reference markers (Fig.35). The product of the polymerase reaction consisted of material that sediments with the major peak of RNA found in infected cells, sedimenting at 20-26S. When the product of the polymerase was treated with ribonuclease, the peak was much sharper and sedimented at 20S. When the RNA from infected cells was analysed by polyacrylamide gel electrophoresis the replicative intermediate and three totally double-stranded replicative forms could be separated as well as the single stranded. All of the multi- and double-stranded species were present in the product of the RNA polymerase reaction, but none of the single-stranded species. The major species was the replicative intermediate. When the product was treated with

Figure 34.

Chick embryo cells, infected with SFV, were labelled with 50μCi/culture of [^{3}H] uridine from 4 - 5h after infection. The RNA polymerase fraction was prepared by the method described in Table 1. The RNA was extracted by the phenol/SDS technique (—Δ—Δ—) and compared with the RNA present in the total homogenate (—o—o—) by electrophoresis on polyacrylamide gels.

The RNA species were identified by comparing their electrophoretic mobilities with those of the 28S and 18S RNA. Ribonuclease resistance was used to identify the replicative intermediate (R.I.) and the replicative forms (RF I, RF II and RF III).

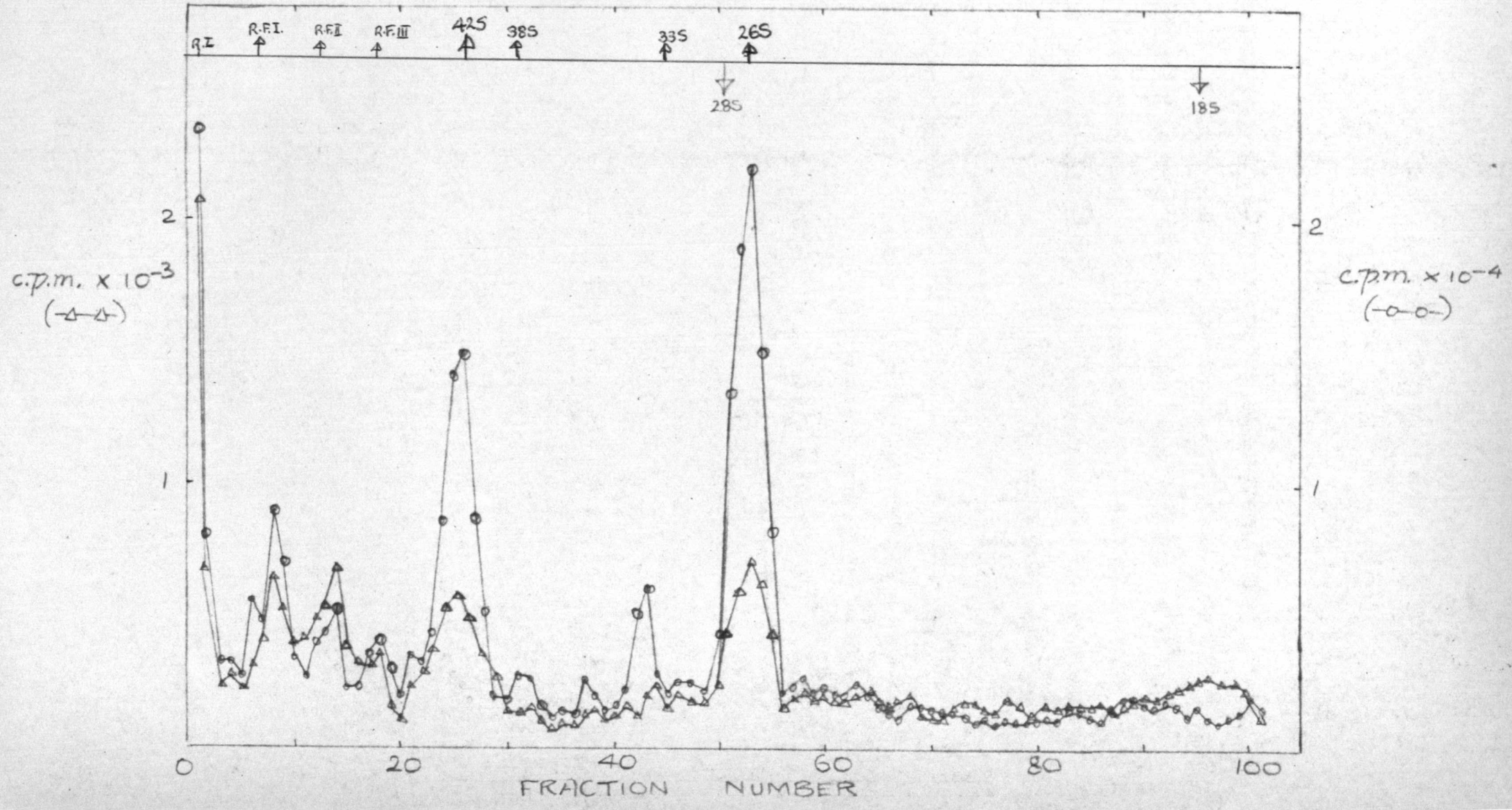
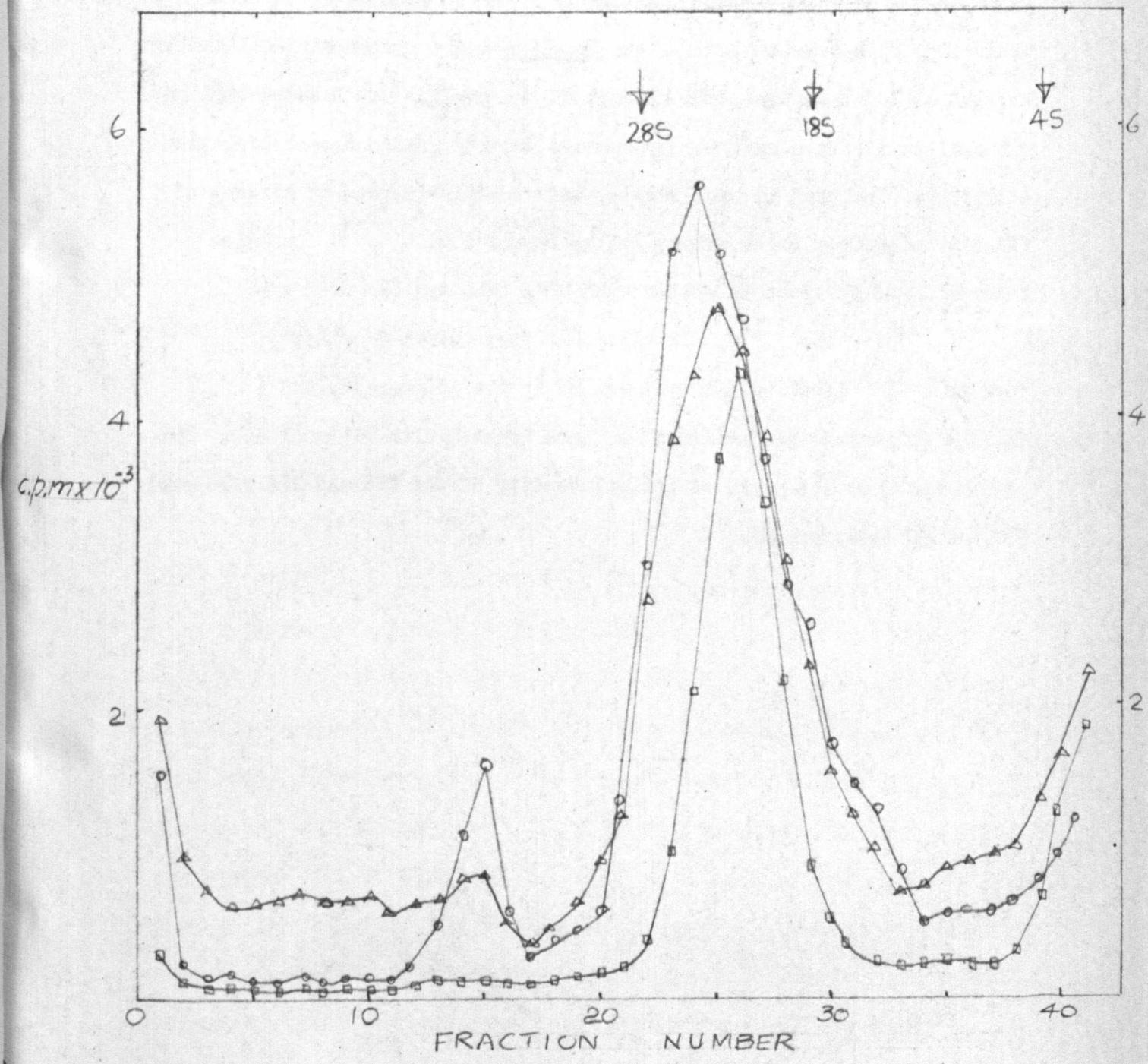

R.I
R.F.I.
R.F.II
R.F.III
42S
38S
33S
26S
28S
18S
2
1
c.p.m. x 10⁻³
(-Δ-Δ-)
2
1
c.p.m. x 10⁻⁴
(-o-o-)
0
20
40
60
80
100
FRACTION NUMBER

Figure 35.

The RNA polymerase fraction was prepared by the technique described in Table 1 from chick embryo fibroblasts infected with SFV. The RNA product synthesised in 60min in the in vitro assay system was isolated by the phenol/SDS technique. An aliquot of the product was treated with ribonuclease, before analysis by sucrose density gradient centrifugation (Fig.35.). Included in each sample, before analysis, was an aliquot of RNA extracted from chick embryo cells, labelled with [^{14}C] uridine between 4 and 5h after infection with SFV. The symbols used are (-o-o-) for the [^{14}C] labelled RNA from infected cells, (-△-△-) for the [^{3}H] labelled RNA synthesised by the polymerase, and (-□-□-) for the polymerase product that had been treated with ribonuclease. The arrows refer to the peaks of optical density of the 28S and 18S ribosomal RNA and 4S transfer RNA.


28S
18S
4S
6
4
2
0
10
20
30
40
c.p.m x 10⁻³
FRACTION NUMBER
[³H]
[¹⁴C]

ribonuclease before analysis on polyacrylamide gels, no replicative intermediate was found, but there was an equivalent increase in the three replicative forms (Fig.36). These results suggest that the replicative intermediate is the primary site for the incorporation of nucleotides in the in vitro system.

2. Virus-specified proteins present in the plasma membrane and endoplasmic reticulum

A good method for the preparation of an endoplasmic reticulum fraction and a plasma membrane fraction from chick embryo fibroblasts was described by Bingham and Burke (1972). An advantage of this method is that both fractions can be extracted from the same batch of cells, making a direct comparison of their properties possible. Chick embryo fibroblasts were labelled with [^{35}S] methionine from 5-7h after infection with SFV, before extraction of the membrane fractions.

Both the endoplasmic reticulum and the plasma membrane fractions, as well as intermediate steps, in the purification, were assayed for RNA polymerase activity. Neither of the final fractions possessed a significant amount of polymerase activity because most of the activity had been discuarded with the 4000g pellet (Table 9). The remainder of the activity was present in the 40,000g pellet, which was resuspended before centrifugation on the dextran gradient. Because of the low magnesium ion concentration in the solution for this gradient, it was not certain that it would have been possible to recover any RNA polymerase activity. However, the results do not contradict the theory that the polymerase is associated with the mitochondrial fraction. Also shown in Table 9 is the percentage recovery of the enzyme markers for the mitochondrial, endoplasmic reticulum and plasma membrane fractions at the various stages of the procedure. Finally

Figure 36.

An aliquot of the samples used in Fig.35 were analysed by polyacrylamide gel electrophoresis. The symbols used are (-o-o-) for the [^{14}C] SFV RNA from infected cells; (-Δ-Δ-) for the product of the polymerase, and (-□-□-), for the polymerase product after ribonuclease treatment.

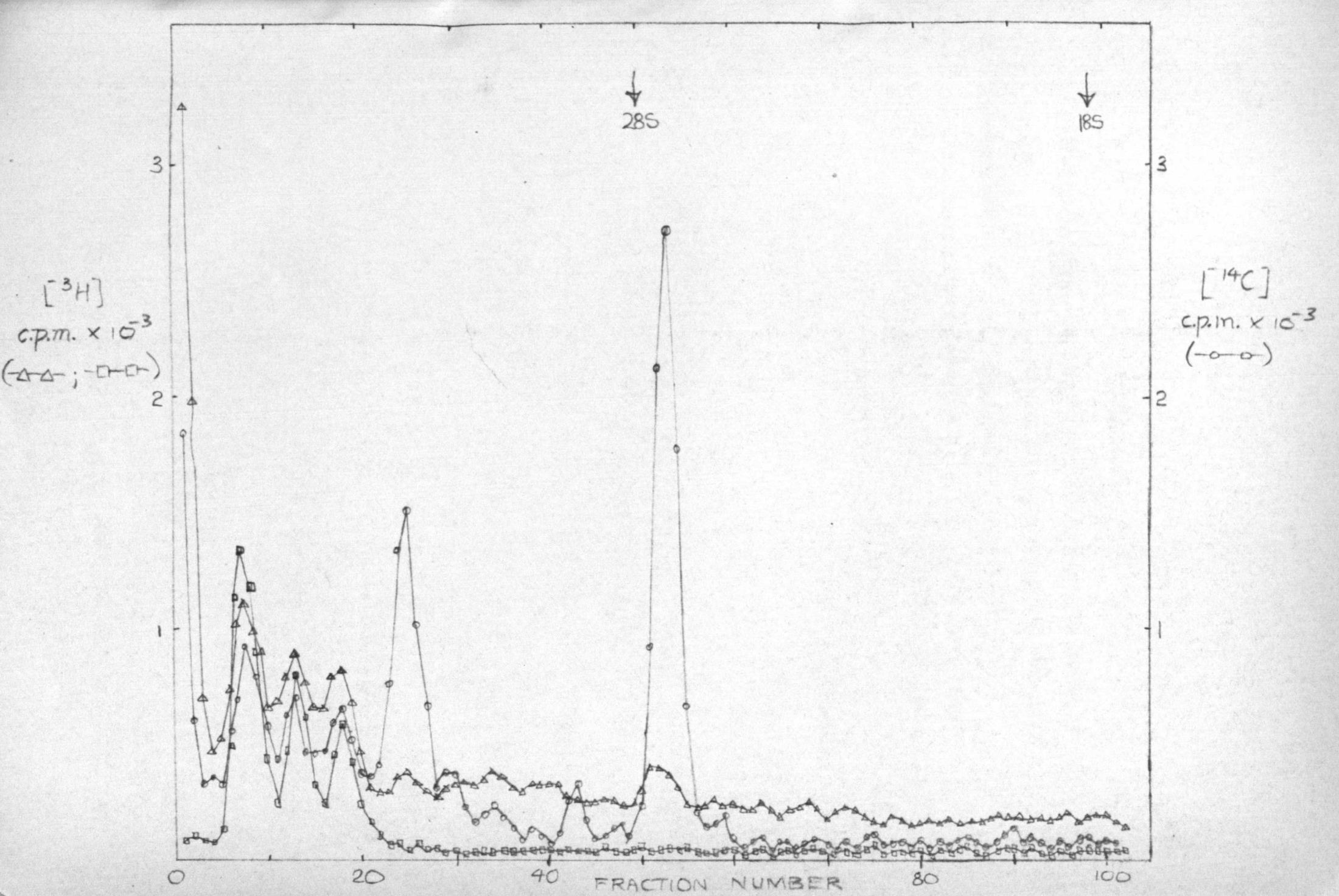

28S
18S
[3H]
c.p.m. x 10-3
(-Δ-Δ-; -□-□-)
[14C]
c.p.m. x 10-3
(-o-o-)
3
2
1
0
20
40
80
100
FRACTION NUMBER

Table 9.

Plasma membrane and endoplasmic reticulum fractions were from chick embryo fibroblasts 5h after infection with SFV.

The activity of the RNA polymerase was measured at various stages in the preparation of plasma membrane and endoplasmic reticulum fractions. Also the percentage recovery of the three marker enzymes (succinate dehydrogenase, NADPH: cytochrome C reductase. and 5' nucleotidase) was determined. The percentage recovery of labelled protein was also calculated when the cells had been labelled for 2h with 20μCi of [^{3}H] valine (100% is equal to 39,160c.p.m.)

	5' nucleotidase	NADPH:cytochrome C reductase	succinate dehydrogenase	polymerase specific activity c.p.m./mg/60min	recovery of valine
cell homogenate	(100)	(100)	(100)	6,173	(100)
4000g pellet	50	29	92	9,539	50
40,000g supernatant	5	3	0	475	8
E.R. band	2	27	0	0	5
P.M. band	31	0	0	0	0.3

the recovery is shown of acid precipitable radioactivity in the various fractions when the cells were labelled with [^{3}H] valine for 2h prior to extraction. The yield of plasma membrane was better than that of endoplasmic reticulum, but the two fractions contain the same amount of radioactivity.

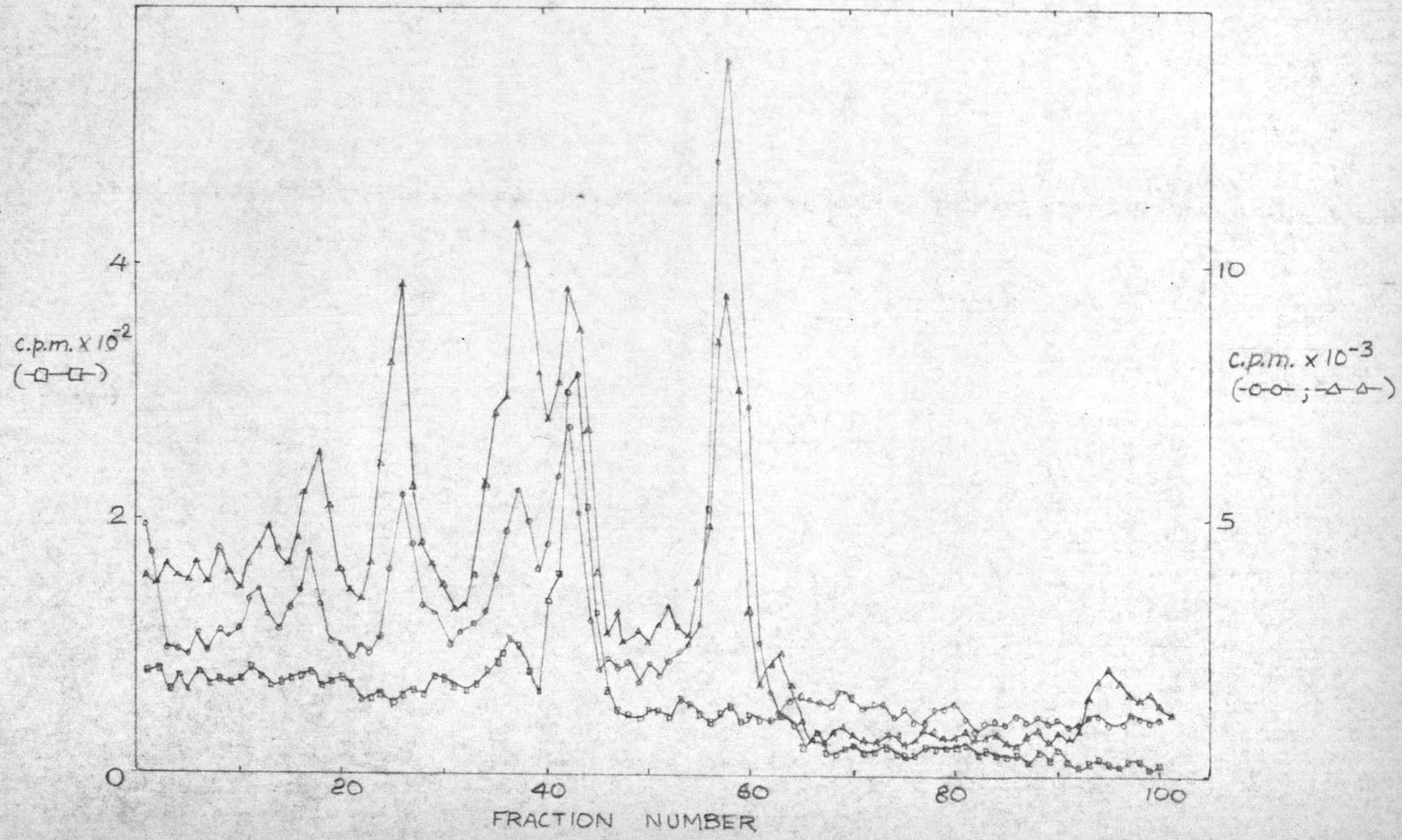
After the fractions had been collected from the final dextran gradient the material was concentrated by differential centrifugation. The pellet was resuspended and the proteins were extracted and analysed by polyacrylamide gel electrophoresis. The pattern of the proteins from the endoplasmic reticulum was very similar to that of the proteins from the homogenate (Fig.37). In contrast the plasma membrane contained only a very small trace of the envelope protein. Since the endoplasmic reticulum is the site of protein synthesis, it would be expected that all the virus specified proteins would be present in the same proportion as normal. The interesting result is the absence of all of the other proteins except the envelope protein from the plasma membrane. Despite the good yield of plasma membrane in this method and good labelling with [^{3}H] valine recovery of radioactivity in this fraction is very poor, making it difficult to detect any proteins present.

3. Virus-specified macromolecules present in an M band

The M band technique is a method of extracting complexes composed of nucleic acids and phospholipid membranes. It was first used by Trembley et al (1969) to extract the membrane bound replication complex of B. subtilis. In this method the sample is suspended in a buffer containing a high concentration of magnesium ions. A detergent, sodium lauroyl sarcinosate is then added to the sample. Fine white crystals of the magnesium salt of the detergent form. These are readily removed from the remaining material by centrifugation on a discontinuous sucrose gradient. The crystals form a fine white band at the interface between the two con-

Figure 37.

Chick embryo cells, infected with SFV, were labelled with [^{3}H] valine from 5 - 7h after infection. The endoplasmic reticulum and plasma membrane fractions were prepared. The proteins were extracted from them and compared with the proteins found in the total homogenate by electrophoresis on 9cm polyacrylamide gels. The symbols used are (-o-o-) for the total homogenate, (-□-□-) for the plasma membrane fraction and (-△-△-) for the endoplasmic reticulum.


c.p.m. x 10⁻²
(-□-□-)
4
2
0
20
40
60
80
100
FRACTION NUMBER
10
5
c.p.m. x 10⁻³
(-o-o- ; -Δ-Δ-)

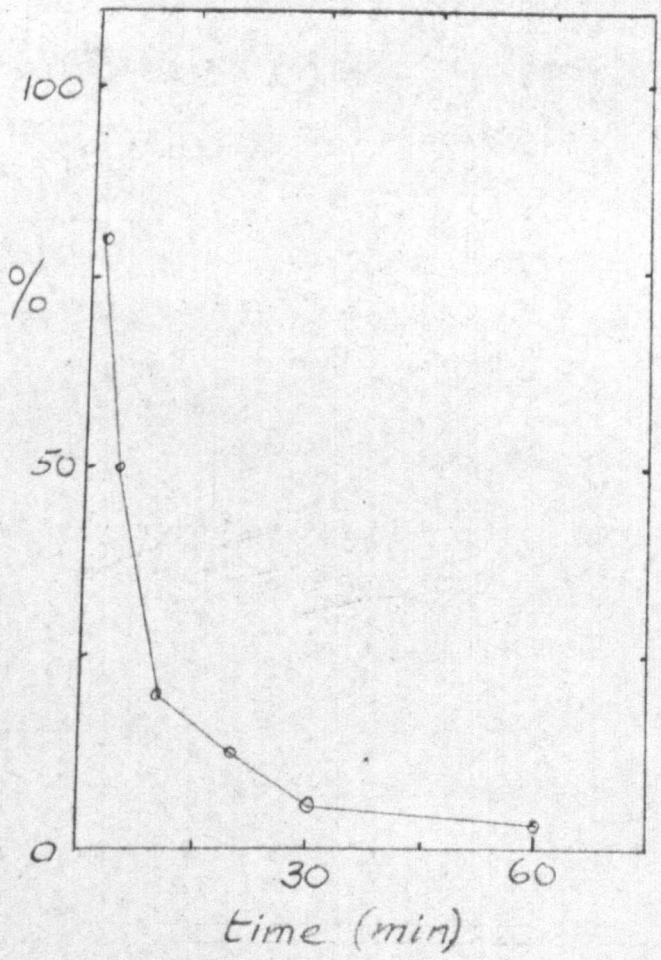
centrations of sucrose. This is the M band. When the crystals form, nucleoprotein complexes are trapped within them, and sediment with the M band. The account of the basis of the M band given here is based on that of Tremblay et al (1969). In a cell infected with SFV the only virus associated structure that has the correct properties to be extracted by the M band procedure is the RNA polymerase. The RNA polymerase had previously been shown to be membrane bound and to contain a nucleic acid complexed with it (Morser, 1971). The virus specific RNA and protein found after an M band extraction of SFV infected cells was investigated, in an attempt to identify the active components of the polymerase.

First the M band was shown to contain the primary site at which the incorporation of radioactive uridine into acid precipitable material took place. Chick embryo fibroblasts infected with SFV were labelled with [^{3}H]uridine for 1, 5, 10, 30 and 60min. In each case incorporation of uridine into the cells was halted 5h after infection. Then the percentage of acid precipitable radioactivity present in the M band was compared with the total incorporation. In a 1min labelling period 80% of the radioactivity that had been incorporated into acid precipitable material was present in the M band, while over a 60min labelling period this value had fallen to 4% (Fig.38). This result suggests very strongly that the primary site at which uridine is incorporated is extracted by the M band procedure.

Chick embryo fibroblasts, infected with SFV, were labelled with [^{35}S]methionine from 1-5h after infection. The proteins were extracted from the M band, after the cells had been fractionated by the M band technique and were analysed by polyacrylamide gel electrophoresis. In comparison

Figure 38.

Chick embryo fibroblasts, infected with SFV, were labelled with radioactive uridine for the times indicated, leading up to 5h after infection. M bands were prepared, and the acid precipitable radioactivity in them was determined. This was compared with the total amount of radioactive uridine incorporated into an acid insoluble precipitate in an equivalent period.


100
%
50
0
30
60
time (min)

with a protein sample from the total cell homogenate, far less envelope and core protein is present (Fig.39). There is an increase in the amount of NVP 95 and NVP 72 contained in the material extracted from the M band. Another sample from this batch was prepared by the method for the partial purification of the RNA polymerase. After the final step in that procedure the material was then extracted by the M band technique (see Table 1 for a flow chart). The proteins in the M band were compared with the other two samples by polyacrylamide gel electrophoresis (Fig. 39). The only major peak present was NVP 72, while a small amount of both NVP 95 and NVP 63 could be detected, but it was not possible to detect either of the virion proteins. These results confirm the earlier hypothesis that neither of the structural proteins nor NVP 63 are connected with the RNA polymerase. They also suggest that NVP 95 may not be associated with the polymerase.

A series of experiments using cells infected with SFV and labelled with radioactive uridine equivalent to those just described using a protein precursor were performed. The chick embryo cells were labelled with [^{3}H]uridine from 4-5h after infection with SFV, and three samples were prepared. These were a sample of the total homogenate, and the material extracted by the M band technique, either from the homogentate, or from the partially purified RNA polymerase fraction. The RNA was extracted from these samples by the phenol/SDS technique and analysed by polyacrylamide gel electrophoresis (Fig.40). The RNA contained in both of the fractions prepared by the M band technique was completely multi-stranded. That is it consisted of the replicative intermediate and the replicative form. There was a vast increase in the proportion of replicative intermediate in the RNA from the polymerase fraction. The replicative intermediate therefore seems to be located within the structure involved in RNA replication.

Figure 39.

Chick embryo fibroblasts, infected with SFV were labelled with [^{35}S] methionine, from 1 - 5h after infection. The RNA polymerase fraction was prepared, before being extracted by the M band technique. The proteins present in the M band (-□-□—) were analysed on 9cm polyacrylamide gels. A sample of the homogenate (-o-o-) was included for comparison, and was also extracted by the M band technique (—Δ-Δ-).

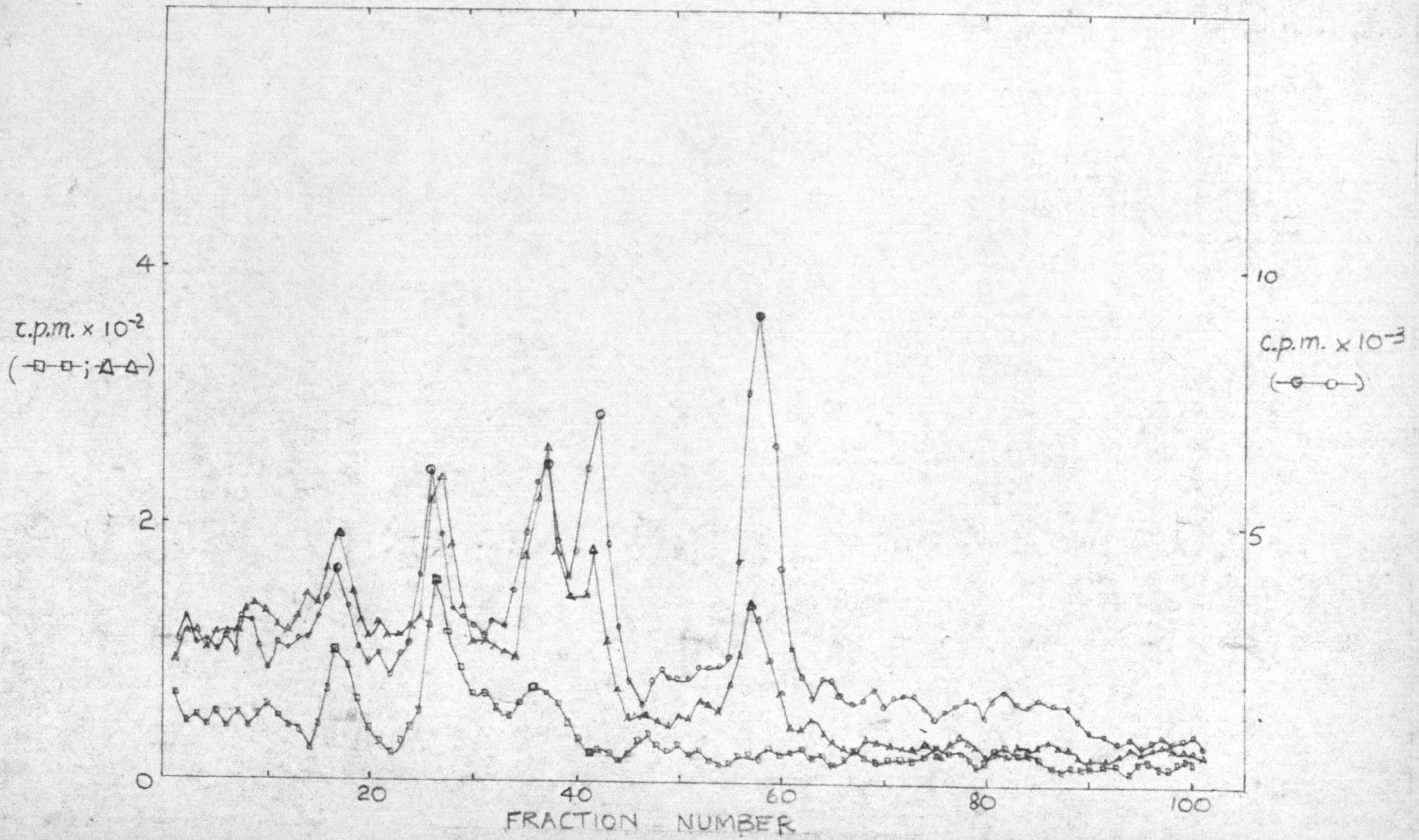
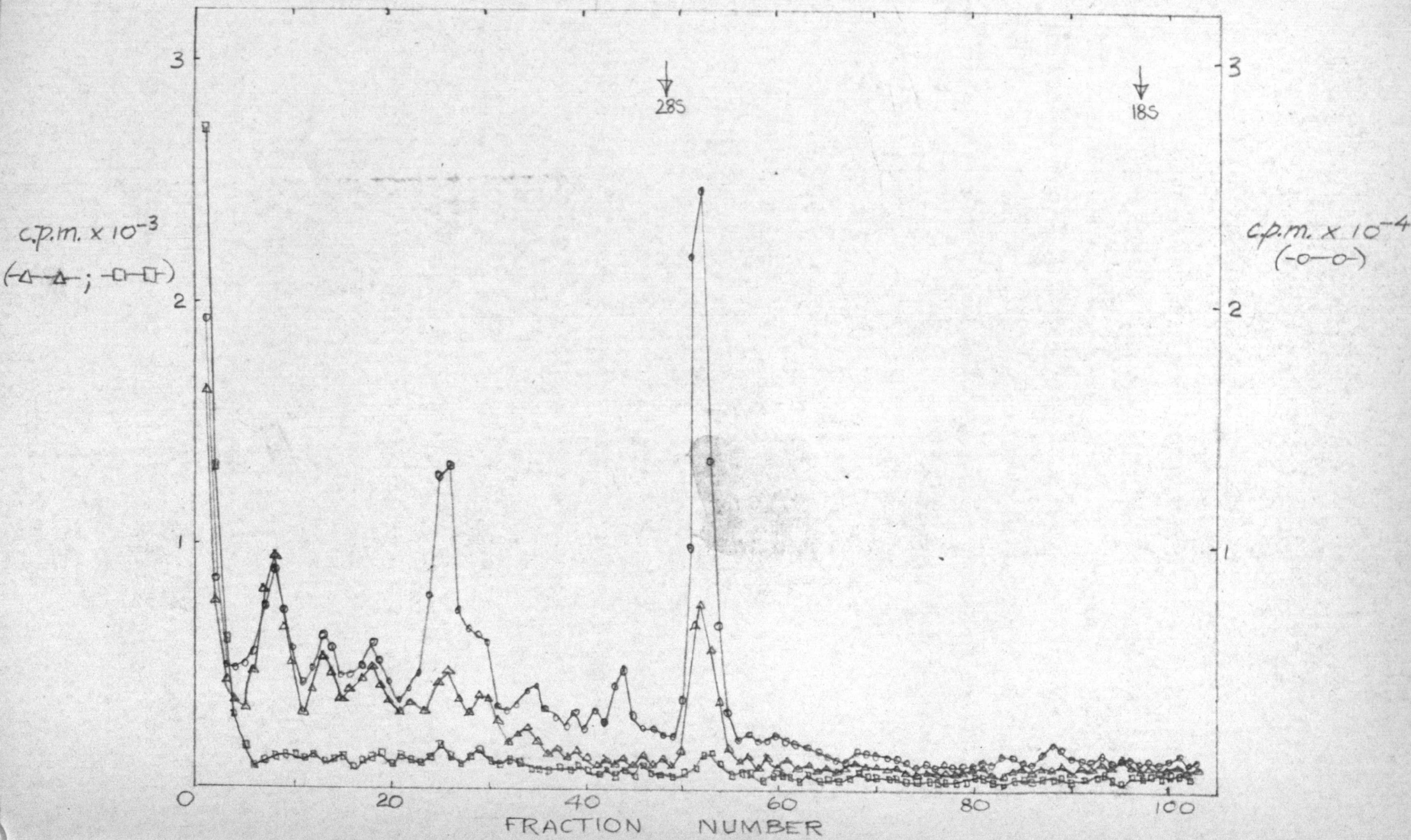

c.p.m. × 10⁻²
(□-□; △-△)
c.p.m. × 10⁻³
(○-○)
0
2
4
5
10
20
40
60
80
100
FRACTION NUMBER

Figure 40.

Chick embryo fibroblasts, infected with SFV were labelled with [^{3}H] uridine from 4-5h after infection. A sample of the RNA from the total homogenate (-o-o-) was compared with the RNA from the M band (-Δ-Δ-). Also an M band was prepared from a partially purified RNA polymerase fraction and the RNA analysed (-□-□-). In each case the RNA was extracted by the phenol/SDS technique and analysed by polyacrylamide gel electrophoresis.


28S
18S
c.p.m. x 10⁻³
(-△-△-; -□-□-)
c.p.m. x 10⁻⁴
(-o-o-)
3
2
1
0
20
40
60
80
100
FRACTION NUMBER

4. Effect of glucosamine on SFV replication

Kaluza, Scholtissek and Rott (1972) had reported that glucosamine inhibited the replication of certain enveloped viruses, amongst which was SFV. The cause of the inhibition was not connected with RNA replication, and it was suggested that the synthesis of haemagglutinin might be prevented. Previously Bosmann (1971) had shown that glucosamine was a general inhibitor of the synthesis of macromolecules in 3T3 cells. Protein, glycoprotein, RNA and DNA synthesis were all inhibited. The inhibition could be overcome by the addition of UTP. Bosmann suggested that the inhibition was caused by the removal of all UTP from the metabolic pools and conversion of it into UDP-glucosamine.

The inhibitor effect of glucosamine on SFV growth was confirmed by measuring the yield of infectious virus 9h after infection and growth in the presence of varying concentrations of glucosamine (Fig.41). Also shown in Fig.41 is the effect on virus yield of treating the cells before infection with glucosamine. Pre-treatment with glucosamine enhanced the inhibitory effect. RNA, protein and glycoprotein synthesis in chick embryo cells infected with SFV in the presence of 0.01M glucosamine was measured (Table 10). Provided the cells were pretreated with glucosamine all RNA, protein and glycoprotein synthesis was halted. Using this property of glucosamine, Scholtissek (1972) described a method of pulse labelling RNA. This method was used in these experiments. Chick embryo fibroblasts 4h after infection with SFV were placed at 4°C. No synthesis of macromolecules takes place at this temperature. Then 50min later, still at 4°C, some radioactive uridine was added and was allowed to equilibrate for 10min with the uridine mono-, di- and triphosphates. Finally the temperature of incubation was raised to 37°C and 0.01M glucosamine was added. Samples were taken at 5, 10, 30 and 60min after the temperature was raised.

Figure 41

Chick embryo cells were infected with SFV, and incubated in the presence of glucosamine. After 9h the yield of infectious virus was assayed (- o -o -). Also chick embryo fibroblasts were pretreated with glucosamine for 3h, before infection. Again the yield of infectious virus 9h after infection was assayed (-Δ -Δ -).

IG. 38
2nd Copy

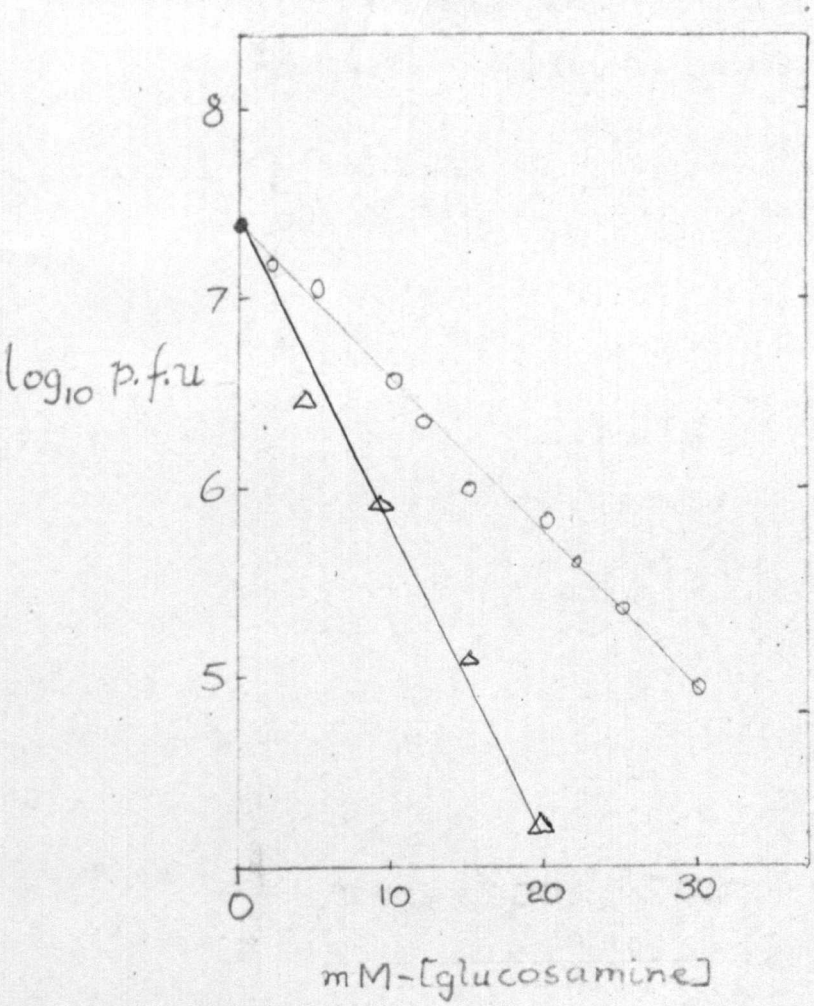


Table 10.

The effect of 0.0005M-glucosamine on incorporation of [^{3}H] uridine, [^{14}C] valine and [^{3}H] glucosamine into acid precipitable material in chick embryo fibroblasts 6h after infection with SFV, was investigated. The yield of infectious virus 9h after was measured. The glucosamine was either added at the end of the adsorption period, or else the cultures had been pretreated with glucosamine for 3h before infection.

	incorporation (c.p.m.) of			yield of
	uridine	valine	glucosamine	p.f.u.
untreated	3,270	4,391	8,756	8×10^7
+ 0.01M-glucosamine	3,196	3,728	4,129	3×10^6
pretreated 0.01M-glucosamine	307	452	276	8×10^5

RNA, which had been extracted from these samples, was analysed by polyacrylamide gel electrophoresis. In all of these samples only the replicative intermediate was present (Fig.42). As a control, cultures infected with SFV, that had been manipulated in the same way, had radioactive uridine added. The temperature was raised to 37°C but no glucosamine was added. Samples were taken at 2, 5, 10, 30 and 60min. and the RNA was analysed. Only the 5min sample was similar to those from the glucosamine treated cells (Fig.43). The replicative forms could be detected in the 10min sample, while all species of virus specified RNA were identified in the 30 and 60min samples from the control cells.

These results confirm the role of the replicative intermediate in virus specified RNA synthesis. They also suggest that the formation of the other RNA species probably requires active RNA synthesis, as they are not synthesised when glucosamine is present. Thus the single-stranded species and the double-stranded replicative forms do not appear to arise from passive breakdown of the replicative intermediate.

Figure 42.

Chick embryo fibroblasts were placed at 4°C 4h after infection with SFV. 50μCi/culture of [^{3}H] uridine was added 50min later, and allowed to equilibrate with the intracellular pools for 10min. Then 0.01M glucosamine was added, and the temperature raised to 37°C. The RNA was extracted after 5min (-o-o-) or 60min (-Δ-Δ-) by the phenol/SDS technique, and analysed by polyacrulamide gel electrophoresis.

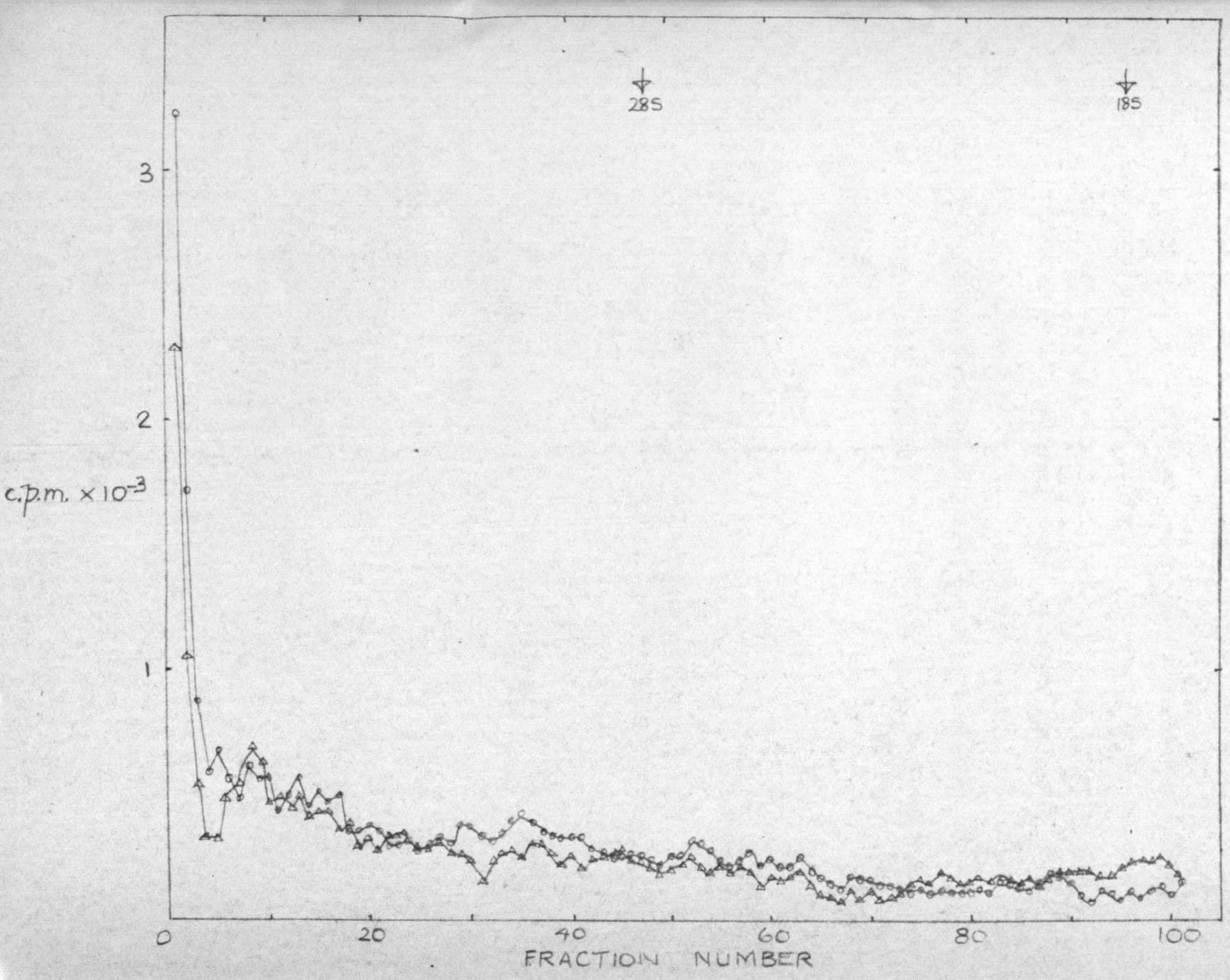
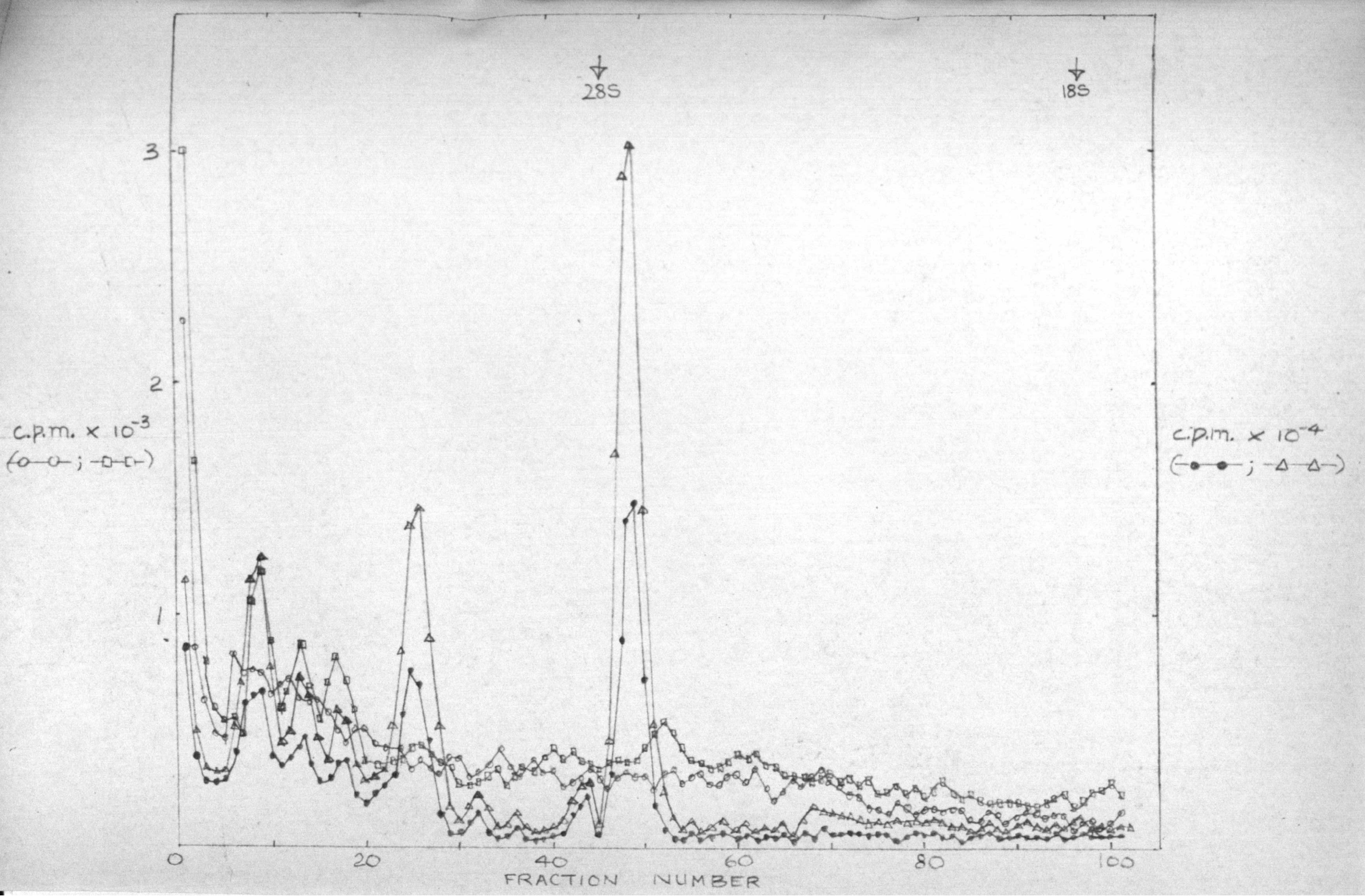

28S
18S
3
2
1
c.p.m. x 10⁻³
0
20
40
60
80
100
FRACTION NUMBER

Figure 43.

The experiments reported in Fig.42 were repeated exactly, except that no glucosamine was added. The RNA was extracted after 5min (-o-o-), 10min (-□-□-), 30min (-●-●-) and 60min (-Δ—Δ-).


28S
18S
3
2
1
c.p.m. x 10⁻³
(o—o ; □—□)
c.p.m. x 10⁻⁴
(●—● ; △—△)
0
20
40
60
80
100
FRACTION NUMBER

DISCUSSION

The growth cycle of group A togaviruses has been well studied. The results presented here for SFV confirm earlier work reported by Strauss et al (1969) for Sindbis virus and by Friedman (1968c) for SFV. At the start of the growth cycle there was a period in which no new infectious virus was produced. This was followed by a period in which virus was released exponentially and then ceased about 12h after infection, probably when cell death occurred. The timing of the growth cycle was shown to be dependent on both the temperature of incubation and the multiplicity of infection. An increase in either of these parameters shortened the cycle. The kinetics of the cycle was approximately the same in the 4 cell types investigated, although the yield of infectious virus varied considerably. The release of haemagglutinin and lactate dehydrogenase followed the same kinetics as the release of infectivity. Therefore either of these parameters can be used as a convenient marker for released virus. Neither is as sensitive as a plaque assay, but both are much quicker.

Infection of chick embryo fibroblasts with SFV caused a dramatic inhibition of host cell protein synthesis. The total rate of protein synthesis declined during viral infection to 50% of the normal level by 4h after infection, and to 10% by 12h after infection. The rate of viral protein synthesis was maximal between 6 and 8h after infection. Part of this inhibition was due to the presence of actinomycin D but parallel experiments with uninfected cells indicate that some of the inhibition was a direct effect of the virus infection. The extent of the inhibition of host cell protein synthesis varied between the different cell types. The size of the inhibitory effect caused by SFV infection was similar to that reported by Strauss et al (1969) for Sindbis virus and by Friedman (1968c) for SFV. The magnitude of the observed changes was dependent on multiplicity of infection, suggesting that it was dependent on the concentration of a product of the virus genome. Previously Waite and

Pfefferkorn (1970b) had shown that the inhibition of phospholipid synthesis was a secondary effect of Sindbis virus infection of BHK-21 cells, resulting from inhibition of host cell RNA and protein synthesis. Experiments with temperature sensitive mutants had shown that some viral RNA synthesis was essential for inhibition of host cell synthesis of macromolecules, but that functional virion proteins were not required. Double-stranded RNA is known to inhibit protein synthesis (Ehrenfeld and Hunt, 1971). Stollar, et al, (1972) have suggested that the varying quantities of double-stranded RNA in different cell types might be the cause of the differing extents of inhibition of protein synthesis. This hypothesis has been tested (W. Schlesinger, personal communication) by measuring the inhibitory effect of double-stranded RNA from chick and BHK-21 cells in an in vitro protein synthesizing system. These results were not consistent with the extent of the inhibitory effect measured in vivo, suggesting that the phenomenon was more complex.

SFV RNA synthesis started within 1hr of infection, reaching a maximum rate between 4 and 5h after infection. It preceded virus-specififed protein synthesis and the release of infectious virus. RNA polymerase activity measured in an in vitro system, was not detectable until 3h after infection, but its maximum activity was reached at 5h after infection. The in vitro system is very much less sensitive than the techniques used for detecting RNA synthesis in vivo.

Since all the experiments reported here were carried out in the presence of actinomycin D, host cell RNA synthesis was inhibited almost completely. The antibiotic was added concommitantly with the virus so that all RNA synthesis detected from 0.5hr after the end of the absorption period was virus directed. It also follows that any new proteins synthesized then are either translated from virus RNA or from long lived

host cell RNA, or finally from host cell RNA whose synthesis is resistant to actinomycin D. The last possibility can be set aside because it has been shown that the small amount of RNA still synthesized in the presence of actinomycin D is the same size as, or smaller than, transfer RNA (Gandhi and Burke, 1970).

Five virus-specified proteins could be readily detected from 3½hr after infection onwards. Two of these had the same mobilities in polyacrylamide gels as the two proteins in the virus particle. Unlike Schlesinger, et al (1972a) no second envelope protein could be detected. According to these authors, in their preparations of Sindbis virus two envelope proteins were present in equimolar quantities. The molecular weight of the two was 53,000 and 44,000 but the smaller, newly detected one, was not present in infected cells. They suggested that the better resolution of the Laemmli (1970) gel system enabled them to separate the two proteins. Using this gel system, or any of the several others tested, no second envelope protein could be identified in purified preparations of either SFV or Sindbis virus. Neither could it be detected in extracts from infected cells. The second envelope protein was not found by analysis of the virus-specified proteins extracted from cells infected with either the Sindbis or SFV series of temperature sensitive mutants under restrictive conditions (Scheele and Pfefferkorn, 1970; Lomniczi and Burke 1970). No other group A togavirus has been reported to have a second envelope protein. See, amongst others, Dorsett and Acton (1970; Mayaro virus) and Igarishi (1970; Chikungunya virus). The failure to find the second envelope protein could be because the polyacrylamide gel systems used did not have enough resolution or because it was not present. The first explanation

is unlikely since all the systems tested were able to cleanly separate the envelope protein (m.w. 53,000) from ovalbumin (m.w.44,800). A possible explanation for the detection of the second envelope protein in the virus prepared by Schlesinger et al, could be that there are defective particles present which contain the second envelope protein. Such defective virus particles have been produced by serially passaging the virus in BHK-21 cells (Schlesinger et al 1972b) and these authors suggested that the cause of the defect lay in the haemagglutinin. Despite attempts to reproduce the results of Schlesinger et al (1972a), two parameters were not checked. These were the possibility that the second envelope is formed in only some strains of virus, or that it was only produced when the virus was grown in certain types of cells.

Three of the five proteins found in chick embryo cells infected with SFV were not present in the virion. These were NVP95, NVP72, NVP63. NVP95 and NVP63 had been detected previously by several groups including Strauss et al, (1969) and Hay et al, (1968). These particular polypeptides had also been detected in cells infected with Chikungunya virus (Igarishi, 1970). Strauss et al (1969) had also claimed detection of about another ten proteins in cells infected with Sindbis virus. Most of these extra proteins were identified on the basis of minor fluctuations in the base line. Of these ten proteins only two have been found in the present work, NVP127 and NVP72. The group of polypeptides detected by Strauss et al (1969) that migrated with the electrophoretic front would presumably be lost by dialysis in the present experiments. The other proteins reported by these authors have not been detected.

Hay et al (1968) described four non-virion polypeptides extracted from cells infected with SFV. These had estimated molecular weights of 106,000, 63,000, 23,000 and 11,000. The protein with a molecular weight

of 106,000 probably corresponds to NVP95, while the protein of 63,000 is probably NVP63. In this work, occasionally a very small peak with the same as the protein of molecular weight 23,000 could be detected. Hay et al (1968) reported that the two smallest proteins were very minor components.

Using a double-labelling technique a partial amino acid composition was deduced for NVP72 and NVP63. The method gave absolute values, since the relative composition of the proteins, derived experimentally, could be compared with those obtained from the amino acid analysis of the envelope and core proteins (Kennedy and Burke, 1972). This method could be used generally to obtain a comparison of the amino acid composition of any proteins that are synthesized concurrently. They can then be labelled together, removing problems of different pool size, or permeability. In these experiments NVP72 was shown to be different in composition from NVP63 and the virion proteins. It was also possible to demonstrate that the core protein contained too much lysine to be derived from either NVP72 or NVP63. The envelope protein and NVP63 were very similar in amino acid composition. The similarities of the envelope protein and NVP63 extends to their carbohydrate composition; both were shown to contain glucosamine and fucose. The quantities of carbohydrate present in NVP63 and the envelope protein were almost the same, but the envelope protein contained slightly less glucosamine. When the homogenate prepared from cells infected with SFV was treated with antiserum that neutralises the envelope protein, both NVP63 and the envelope were precipitated. This antiserum does not precipitate the envelope protein from Sindbis virus grown in the same cells as SFV whose envelope protein can be neutralised. (S.I.T. Kennedy, personal communication). The antiserum is therefore probably active against the polypeptide portion of the envelope protein,

suggesting that the polypeptide components of NVP63 and the envelope protein are related.

If NVP63 and the envelope protein are structurally similar, then either one is a precursor to the other, or NVP63 is an end product that is not incorporated into the virus particle. When a long (9hr) labelling period was compared with a short (1hr) period, the proportion of NVP63 was decreased while that of the envelope protein was increased. This suggests that the envelope protein is an end product, and that NVP63 is being broken down, but it does not prove that NVP63 is being converted into envelope protein.

If this hypothesis is accepted, then what is the difference between NVP63 and the envelope protein? If the carbohydrate moiety is removed by enzyme treatment from the envelope protein, the mobility on polyacrylamide gels is not altered (Kennedy, 1973). However Ranki,Kaariainen and Renkonen (1972) have reported the existence of a carbohydrate rich fragment with an apparent molecular weight of 20,000. These authors suggest that in the conversion of NVP63 into envelope protein, this glycopeptide is specifically removed. If the fragment is carbohydrate rich, the very similar amino acid composition of NVP63 and the envelope protein is explained, because the loss of a few residues would be insufficient to change the overall composition greatly. The fragment may be identical to the minor protein with a molecular weight of 23,000 detected by Hay et al (1968). The non-additive values for the molecular weights (63,000-53,000≠20,000) could be because the apparent molecular weight of glycoprotein can vary on different concentration polyacrylamide gels, especially if the carbohydrate is a substantial portion of the molecule (Bretscher, 1971).

Concanavalin A is a plant lectin that specifically binds sugar

residues with theα-D-mannopyranoside, α-D-glucopyranoside andα-D-glucosaminyl residues at branched or terminal non-reducing ends of polysacharides (Goldstein, Hollerman and Smith, 1965). When the homogenate from cells infected with SFV was treated with concanavalin A, not only were NVP63 and the envelope precipitated, but NVP95 as well. This confirms the evidence obtained by labelling the virus infected cells with glucosamine, that NVP95 contains some carbohydrate. The relationship between NVP95 and the envelope protein and NVP63 was investigated further by the use of FNAG. Since NVP63 and the envelope protein contain carbohydrate, then an inhibitor of the glycosyl transferases should cause an accumulation of the non-glycosylated precursor. When the inhibitor of carbohydrate synthesis, FNAG, was present a protein the same size as NVP95 accumulated and there was less envelope protein. Again this suggests the hypothesis that NVP95 is converted into NVP63 and envelope protein and that this process is inhibited by the presence of FNAG.

Both Burrell et al (1970) and Strauss et al (1969) had previously proposed that NVP95 was a precursor to the structural proteins. This suggestion had been strengthened by the evidence that TPCK caused an accumulation of NVP95 (Pfefferkorn and Boyle, 1972). The pulse chase experiments in chick embryo fibroblasts and BHK-21 cells reported here demonstrate a movement of radioactive label from NVP95 to NVP63 and the envelope protein. In the presence of TPCK this movement of label was confirmed, as well as revealing two more non-virion polypeptides; NVP165 and NVP127. In the absence of TPCK it was not possible to show an increase in the amount of core protein during the chase, while it was possible to show this in its presence. By investigating the effects of amino acid analogues and of inhibiting virus directed protein synthesis by a temperature jump, it was possible to show that NVP95 and NVP63 were intermediates

in the conversion of NVP165 and NVP127 into the structural proteins and NVP72.

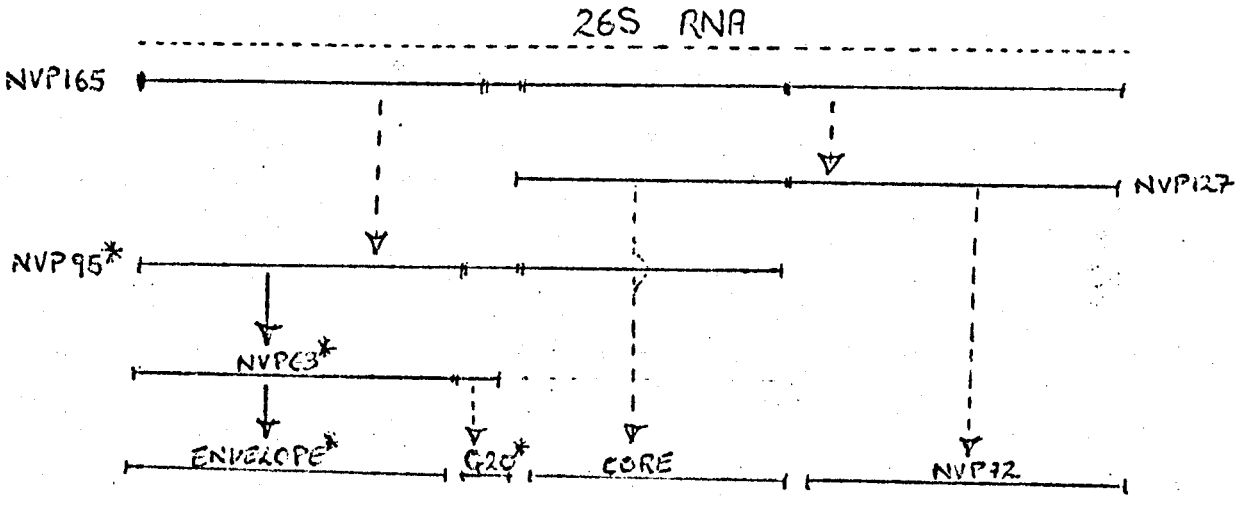
These results disagree with those reported by Friedman (1969) who suggested that the SFV proteins were primary gene products. The success reported here in unequivocally demonstrating a precursor-product relationship lies in the method used for extracting proteins. Method II (see Methods section) prevents any metabolic activity continuing extremely quickly, by the immediate freezing of the sample. The sample is then denatured completely by the treatment with TCA and acetone. Normal extraction methods take much longer before metabolic activity is halted. In the case of experiments carried out in the presence of chemicals such as TPCK and the amino acid analogues it is possible that the results could be due to a specific inhibition of incorporation into the lower molecular weight species. This possibility is unlikely because all the results, from the different techniques, are in agreement that the high molecular weight species are precursors to the remainder.

Two schemes are shown in Table 11 for the cleavage of the proteins. Although there is no evidence that NVP165 is a precursor of NVP127 or that the primary sequence of one of them is duplicated in the other, model A,proposes that NVP127 is the first cleavage product of NVP165. If it is assumed that NVP72 and the two virion proteins are the three unique end products, then the single transcript precursor would have a predicted molecular weight of 175,000. The estimated molecular weight of NVP165 could be somewhat inaccurate as it was obtained by extrapolation from the known markers. NVP165 is therefore proposed as the single transcript precursor translated from the 26S RNA. The 26S RNA has an estimated molecular weight of 1.8×10^6 which is just sufficient coding capacity for a protein of this size.

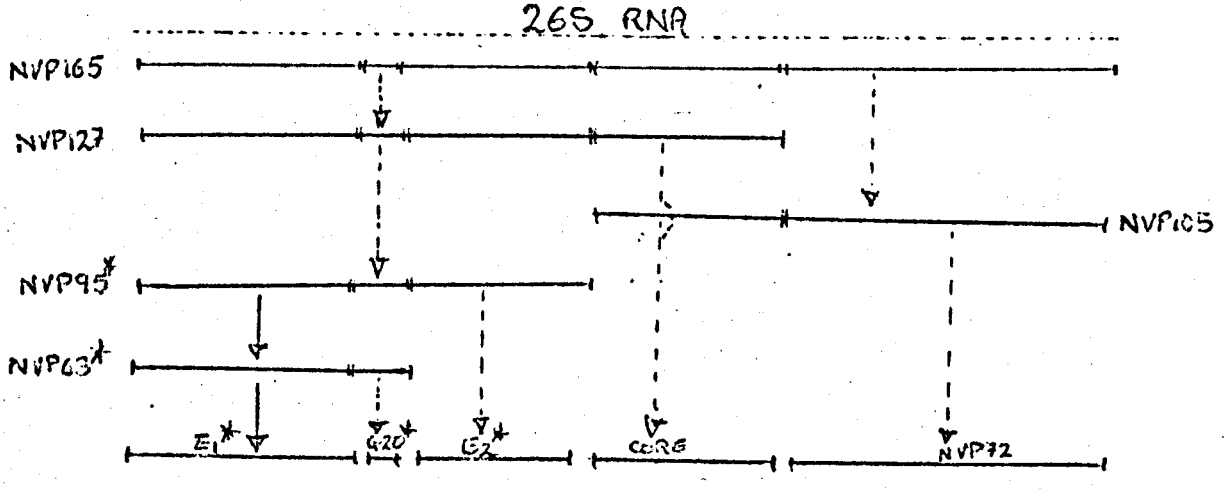
Table 11.

Two models of the formation of SFV proteins are shown. Polypeptides containing carbohydrate are marked (*).

Scheme A.



Scheme B.



It has to be recognised that the identification of NVP165 and NVP127 is doubtful for three reasons. These are the low counts in the peaks, the abnormal conditions under which they can be detected, and the difficulty in obtaining a reproducible pattern at the high molecular weight end of a gel.

A feature of the suggested model is that all of the possible cleavages can take place under different conditions. From an intensive study of the cleavages that produce the structural proteins in two different strains of poliovirus, Cooper, Summers and Maizel (1970) suggested that there could be ambiguity in the post-translational cleavages. It was proposed that the ambiguity arose because there are many possible sites of cleavage, and that these are used in varying combinations in different strains. A similar phenomenon may be taking place in the cleavage of the SFV proteins under the different conditions used in these experiments.

There are several doubtful parts of the proposed model A. First, although NVP63 is related to the envelope protein no movement of label could unequivocably be demonstrated between NVP63 and the envelope protein by pulse chase experiments. Even when processing of NVP63 was apparently inhibited completely, some label was present in the envelope protein. This agrees with the results reported by Ranki (1972) that labelling of the envelope protein could not be completely inhibited by canavanine treatment. Both of these observations could be explained, if there was a second envelope protein (see discussion below). Second, the molecular weights of the proteins do not add up correctly to fit the precursors. This scheme postulates that a precursor such as NVP127 is cleaved to produce NVP172 and the core protein (M.W.36,000). Addition of the molecular weights does not give 127,000. The molecular weights of these

three polypeptides are unlikely to be this inaccurate since none of them contain carbohydrate. Third, this shceme does not explain the very broad NVP95 peak under certain conditions, which in some experiments actually forms a double peak (see Fig.26).

These criticisms are removed in Scheme B, but 3 new assumptions are made. These assumptions are firstly that there are two envelope proteins, both of similar size, secondly the coding capacity of the 26S RNA has been under estimated, and finally that the broadening of NVP95 is due to a new protein, NVP105. If these assumptions are accepted then scheme B proposes that the single transcript precursor NVP165 is cleaved into NVP 127 and NVP105. It is suggested that NVP105 is broken down to yield NVP 72 and the core protein. If it exists NVP105 would be very similar in size to NVP95 and very difficult to separate from it. The accumulation of NVP127 and NVP105 (shown as a broadening of the peak of NVP95) occurs only when there is a real decrease in the rate of formation of core protein. This might suggest that the core protein is derived from both of them. Previously Igarishi (1970) had demonstrated the existence of a transient polypeptide of molecular weight 105,000 in cells infected with Chikungunya virus which might be same as NVP105. NVP127 is cleaved to release the core protein and NVP95, which is the precursor to both envelope proteins (E_1 and E_2). If there are two envelope proteins it is proposed that they have almost the same mobility on polyacrylamide gels. NVP95 is then broken into NVP63 and the second envelope protein (E_2). NVP63 is the direct precursor to the first envelope protein (E_1).

In both schemes NVP95 is the moiety that is glycosylated. If NVP 95 was a precursor to the core protein as in model A, this would pose problems either about the site of the glycosyl transferases or about the

core protein having to cross the membranes of the endoplasmic reticulum twice. This is because the glycosyl transferases are known to be sited on the external side of the membrane (Spiro, 1970). On the other hand if NVP95 contains both envelope proteins, then the carbohydrate moiety can be added on to the polypeptide on the exterior surface of the membrane. Then the completed envelope proteins only need to be inserted into the outer leaflet of the plasma membrane, and to migrate to the sites of virus release.

The only evidence on the order in which the genes are arranged along the RNA, is provided by the existence of both NVP127 and NVP95 or NVP105. It is suggested that the core protein sequence is common to both precursors, and will represent an overlap in their sequence. Experiments using pactamycin and emetine (antibiotics that inhibit protein synthesis) would enable the correct order of the genes to be deduced (Rekosh, 1972).

In order to differentiate between the schemes, the existence or absence of a second envelope protein needs to be unequivocably demonstrated. Also the conformation-independent molecular weight of the 26S RNA could be estimated. Finally any hypothesis rests upon the accuracy with which the molecular weights of the polypeptides can be estimated. In the case of any glycopeptide this problem is increased by the uncertainty of the behaviour of the carbohydrate in forming an SDS complex and its mobility upon polyacrylamide gel electrophoresis. Different glycoproteins behave differently; ovalbumin with a carbohydrate content of 2% migrates correctly while the glycoprotein derived from erythrocyte membranes by Bretscher (1971) has an apparent molecular weight three times its real molecular weight of 33,000. This glycoprotein has a carbohydrate content of 66%. In this discussion the estimated molecular weights of the glycoproteins have been used, taking no account of any possible effect

of the carbohydrate moiety on their mobility. In particular this would affect the estimated sizes of NVP95, NVP63 and the envelope protein decreasing the size of the polypeptide component. Previously Strauss et al (1970) had estimated that the carbohydrate content of the envelope protein of Sindbis virus was 14%. How this would affect its behaviour on electrophoresis is not known.

In both schemes the end products of the process are NVP72 and the virus structural proteins. What function does NVP72 have? From the various methods of sub-cellular fractionation it appears to be associated with the RNA polymerase. The ease with which the core protein was removed from the preparation with no loss of activity, suggests the the template for the polymerase is not the nucleocapsid particle. However the kinetics of its appearance and the inhibition of its synthesis on treatment with cyclohexamide or puromycin at 3½hr after infection, would suggest that NVP72 was not an essential part of the enzyme complex. It must be emphasised that this evidence is very circumstantial.

Burge and Pfefferkorn (1968) described the division of the temperature sensitive mutants of Sindbis virus into five complementation groups. Two of these complementation groups are RNA^- while the other three are RNA^+. One of the RNA^+ groups was shown to be defective in the core protein, and another in the envelope protein (Burge and Pfefferkorn, 1967). Incidentally, the biochemical tests used to differentiate the complementation groups would not have distinguished between two envelope proteins if indeed there are two envelope proteins. Indeed if NVP95 is the precursor to the two envelope proteins it may be possible to create only one complementation group for both envelope proteins. The third complementation group contains Ts20, the only mutant that is not more heat labile than the strain from which the mutants derived. Both Burge and Pfefferkorn (1968)

and Yin and Lockart (1968) had argued from this and the properties of the mutant, that the lesion could not be located in the structural proteins. It is possible that the lesion in this complementation group is concerned with NVP72.

The role of the 26S RNA as the major messenger RNA, and its translation into the structural proteins and NVP72 takes place only from 3hr after infection onwards. Before that the structural proteins cannot be detected. At early times after infection Scheel and Pfefferkorn (1969b) demonstrated that puromycin inhibited the formation of 26S RNA preferentially. A similar result was obtained by shifting cells infected with a RNA$^-$ temperature sensitive mutant from the permissive to the restrictive temperature. Under both conditions, the viral RNA produced was shown to be fully active biologically. From these experiments it appears that the RNA polymerase is completed early. Therefore it is quite probable that the RNA species that is the major messenger RNA at late times after infection (26S) is neither the first messenger RNA nor does it code for the RNA polymerase. The 26S RNA only represents a fraction of the coding capacity of the 42S RNA, which is the species present in the virion. Also present in the infected cell are 33S and 38S RNA species, both of which are single-stranded. Therefore any of these species could act as a supplementary messenger RNA at early times in the course of infection. Indeed 33S has been recovered from polysomes extracted from infected cells. But the 33S and 38S RNA have the same kinetics of appearance as the 26S RNA, arguing against them being the early messenger. The question of the identity of the messenger RNA and the genes that it carries will be resolved when in vitro protein synthesising systems have been successfully developed.

The single-stranded RNA species were shown to be derived from the

double- and multi-stranded species. The first species labelled was the replicative intermediate. The replicative intermediate was also the major RNA species extracted by the M band technique and was found in the polymerase fraction. From the replicative intermediate all the other RNA species are derived. There is not sufficient evidence to decide whether replication is conservative, semi- or non-conservative. Depending on this, the replicative forms are either an end product (as in polio virus replication) or an essential intermediate in the formation of the single-stranded species. Neither the composition of the three replicative forms is known, nor their individual roles in replication.

The partial purification of the RNA polymerase shows that it is a large membrane bound structure with a sedimentation coefficient of about 270S. It appears to be associated with material from the mitochondrial fraction. Inside infected cells the membranes are substantially reorganised. Vesicles appear, which Friedman (R.M. Friedman, personal communication) has shown to be the site of the RNA polymerase. These are probably the structures that are being purified. The enzyme purified by these procedures does not appear to be fully competent, in that no large amounts of the single-stranded species are synthesised in vitro. Unlike previous reports (Levin and Friedman, 1971) the enzyme does synthesise a product that is sensitive to ribonuclease. But this is the replicative intermediate. The reasons for the incomplete reaction in the in vitro system could either be because one of the substrates may be present in a limiting amount, or an essential element is lost in the purification procedure. The first is unlikely, because Morser (1971) investigating the kinetics of a similar polymerase preparation, demonstrated that the only material present in a limiting quantity was the enzyme itself. Therefore the RNA polymerase was probably damaged during the extraction.

The removal of NVP63 and the structural proteins from the RNA polymerase suggest that they are not the polymerase proteins. This was confirmed by the insensitivity of the *in vitro* enzyme assay to antiserum preparations that neutralise either the core or envelope proteins. Since all the remaining proteins that it has been possible to identify were precursors to the structural proteins, it follows that they are unlikely to be the polymerase proteins. This leaves, by exclusion NVP72 as a possible candidate for the polymerase protein. Other, as yet undetected virus specified polypeptides may be involved.

As well as the alterations of the internal membranes to form the RNA polymerase, the plasma membrane is also altered by the incorporation of the envelope protein into it. Bose and Brundige (1972) showed that as well as envelope protein, the core protein was present. In the results presented above, only the envelope protein was detected. This difference was probably due to the ionic conditions under which the extraction took place. In neither report was NVP63 detected in the plasma membrane, suggesting that the conversion of NVP63 into envelope protein took place in some other location. Both reports found several non-virion proteins present in the endoplasmic reticulum fraction, but Bose and Brundige (1972) failed to find any structural proteins. The nucleocapsid is assembled independently in the cytoplasm and migrates to the sites of virus release (Friedman and Berezesky, 1967). Since the arrival of the core particle and envelope protein at the site of budding is independent, how does one locate the other? Presumably by some modification of the internal surface of the plasma membrane, since it has been shown that there is a continuous lipid bilayer separating the envelope protein from the nucleocapsid (Harrison, Davis, Jumblatt and Darnell, 1971)

The evidence presented here suggests that SFV (and other group A togaviruses) should be placed in animal virus class IV (Baltimore, 1971). These viruses possess a genome of single stranded RNA and the messenger RNA is the same polarity as the virion RNA. But the replicative cycle appears more complex than that of poliovirus, a typical member of Class IV. In particular it appears that the replicative cycle can be divided into early and late phases, and there appears to be more than one species of messenger RNA. It is interesting to note that the proteins which might be specified by an early messenger, are the polymerase proteins. In class V viruses it is precisely these proteins that are in the virions. Thus the group A togaviruses in some respects seem to represent an intermediate between the Class IV and Class V viruses.

SUMMARY

1. The growth cycle of SFV in chick embryo fibroblasts, BHK-21, HEL and L-929 cells was described, as were the resultant effects on RNA and protein synthesis. The release of haemagglutinin and LDH from infected cells was assayed, and shown to parallel the release of infectious virus. The effect of altering the temperature of incubation on the growth of the virus, and the concommitant change in macromolecular synthesis , were investigated. There was a substantial inhibition of host cell protein synthesis from 3h after infection onward, under all the conditions investigated.

2. Five proteins could be readily detected in any of the four cell types when they had been infected with SFV for at least 3h. These five proteins were present throughout the growth cycle, whether the temperature of incubation was 30°C, 37°C or 39°C. Five proteins of the same size were also present in chick cells infected with Sindbis virus. Two of the five were the structural proteins, the other three were NVP 95, NVP 72 and NVP 63.

3. On the basis of its amino acid composition, its carbohydrate composition, and its kinetics of labelling, NVP 63 appears to be a precursor of the envelope protein. NVP 95 also contains some carbohydrate.

4. Pulse-chase experiments were carried out in infected chick and BHK-21 cells. The information from these experiments was supplemented with results from pulse-chase experiments in which the cells had been treated with TPCK, or NaF, or with amino acid analogues, or subjected to the reversal of a temperature jump. From these results, two further high molecular weight proteins became detectable, NVP 165 and NVP 127. It was possible to demonstrate that NVP 165, NVP 127 and NVP 95 and NVP 63 were precursors to NVP 72 and the structural proteins. Two models were proposed, based on the presence or absence of a second envelope protein.

5. The proteins present in the plasma membrane and endoplasmic reticulum were investigated. The only protein contained in the plasma membrane was the envelope protein, while all the proteins normally found in infected cells were present in the endoplasmic reticulum fraction.

6. On the basis of the proteins synthesised on inhibition with cycloheximide and puromycin, and because of the timing of their formation, it was suggested that none of the proteins so far identified was the RNA polymerase.

7. A method for obtaining a partially purified preparation of the RNA polymerase was described. The RNA and protein species associated with the enzyme were analysed. The M band technique was also utilised to obtain a further fractionation. By a combination of these methods, it was shown that the replicative intermediate is the first RNA species formed, while only NVP72 was associated with the RNA polymerase.

8. An in vitro assay for the RNA polymerase was described. The product was replicative intermediate and replicative form. It was shown that the assay was not sensitive to antiserum preparations that neutralised the core or envelope proteins.

9. A method for pulse labelling the RNA was described, using the inhibition of macromolecular synthesis caused by glucosamine. It was demonstrated that the replicative intermediate was the first RNA species formed.

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ABBREVIATIONS

1) ATA aurinotricarboxylic acid

2) ATP adenosine triphosphate

3) BHK baby hamster kidney

4) BSA bovine serum albumin

5) c.p.m. counts/min

6) C.T.P. cytidine triphosphate

7) DFP diosopropyl phosphate

8) EDA Earle's medium containing dialysed calf serum and actinomycin D

9) FNAG N-fluroacetyl glucosamine

10) FPA fluorophenyl alanine

11) GTP guanosine triphosphate

12) HEL human embryo lung

13) MEM minimal Earle's medium

14) m.o.i. multiplicity of infection

15) NAD nicotinamide dinucleotide

16) NAG N-acetyl glucosamine

17) NADP Nicotinamide dianucleotide phosphate

18) PEP phosphoenol pyruvate

19) p.f.u. plaque forming unit

20) P.O.P.O.P. 1,4 -di(2-(5-phenoloxazyl)-lenzene

21) P.O.P. 2,5, -diphenyloxazole

22) RNA ribo uclei acid

23) RSBD Reticulocyte standard buffer containing dextran sulphate 50

24) SDS sodium dodecyl sulphate

25) SFV Semliki Forest virus

26) TCA trichloracetic acid

27) TEMED NNN'N'-tetramethylenthylene diamine

28) TNE Tris NaCl, EDTA buffer

29) TPCK tosyl-phenylalanyl chloromethione

30) tris 2-amino -2-hydroxymethyl propane-2,3-diol

31) UTP uridine triphosphate