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NUCLEAR EVENTS DURING INFECTION OF AVIAN FIBROBLASTS WITH INFLUENZA VIRUS

by

John Robert Stephenson, B.Sc., (Aberdeen)

LIBRARY

This thesis is presented for the degree of Doctor of Philosophy, in the Department of Biological Sciences, University of Warwick, February 1975.

CONTENTS

DIAGRAMS

ACKNOWLEDGEMENTS

I would like to thank the following for their helpful cooperation during the past 3 years; without it much of the work presented in this thesis would have been impossible. Dr N.J. Dimmock - for the selection of an interesting and demanding research topic and his expert judicious guidance throughout its execution. Professor D.C. Burke - for his encouragement and support throughout my stay in his research group. Dr R.J. Avery, Dr S.I.T. Kennedy and the remainder of the Virus Group for providing such an amiable and stimulating environment in which to work. Dr S.I. Koliais for the gift of poliovirus Type I. Dr S.I.T. Kennedy for the gift of Semliki Forest virus (ts +). Dr D. McCahon for the gift of FP/BEL virus. Dr E.A.C. Follett for growing and enucleating BSC-1 cells. Mr M.R. Lee for help in the growth and purification of ^{32}P labelled virus at considerable risk to his own personal virulence. Miss C. Penny for growing HeLa cells. Mrs Valerie Strickler for her expert typing. The Cancer Research Campaign for granting me funds to keep my own metabolism in order.

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.....the timeless in you is aware of life's timelessness,
And knows that yesterday is but today's memory and tomorrow is today's dream.
And that that which sings and contemplates in you is still dwelling within the bounds of that/first moment which scattered the stars into space.

(Kahlil Gibran - 'The Prophet')

SUMMARY

The work presented in this thesis is an attempt to evaluate the molecular mechanisms of both host and viral nucleic acid metabolism in the nucleus of avian fibroblasts infected with influenza virus.

The first section describes the alteration of the processing of rRNA in the nucleus of virus infected cells.

The second section describes the kinetics of virus adsorption at 4° and room temperature and the occurance of micropinocytosis in CEF cells at 4°.

The third section describes the movement of viral nucleic acid into the nucleus of infected cells, its association with the cellular DNA and the subsequent exit of the viral RNA from the nucleus.

ABBREVIATIONS

All abbreviations in this thesis are in accordance with those described in 'Units, Symbols and Abbreviations'. A guide for Biological and Medical editors and authors, published by the Royal Society of Medicine; and all chemical formulae are written in the format described by the Chemical Society (Journal of the Chemical Society (1936) p. 1067) with the following additions:

AMD	-	actinomycin D
CEF	-	chick embryo fibroblasts
c.p.m.	-	counts per minute
DN'ase (or DNase)	-	deoxyribonuclease
d.p.m.	-	disintegrations per minute
EDTA	-	ethylenediaminetetra-acetic acid
EM	-	electron microscopy
FMDV	-	foot and mouth disease virus
FPV	-	fowl plague virus
FPV/BEL	-	a recombinant between FPV and A/BEL/42 virus, see materials section for details
HA	-	haemagglutinin
HAU	-	haemagglutinin units
HEPES	-	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
NDV	-	Newcastle disease virus
PAGE	-	polyacrylamide gel electrophoresis
PBS	-	Phosphate buffered saline
PBS	-	" plus 0.5mM $CaCl_2$ and 0.5 mM $MgCl_2$
p.f.u.	-	plaque-forming unit
POPOP	-	1,4-di(2-(5-phenyl-oxazolyl))-benzene
PPO	-	2,5-diphenyloxazole

cRNA	-	RNA with a sequence complementary to that of the virion genome
hnRNA	-	heterogenous nuclear RNA
mRNA	-	messenger RNA
rRNA	-	ribosomal RNA
tRNA	-	transfer RNA
vRNA	-	RNA from the genome of a virus
RN'ase (or RNase)	-	ribonuclease
RNP	-	ribonucleoprotein
SDS	-	sodium dodecyl sulphate
SFV	-	Semliki forest virus
SSC	-	Standard saline citrate
TCA	-	trichloroacetic acid
TEMED	-	N,N,N',N'-tetramethylethylenediamine
Tris	-	Tris (hydroxymethyl) aminomethane
VSV	-	Vesicular stomatitis virus

GENERAL INTRODUCTION

The economic and psychological importance of influenza virus

Human influenza has been known to occur in pandemic form from as early as 1510 A.D., with at least 30 pandemics recorded between 1510 and 1930. The morbidity of this disease is extremely high, usually involving from 30 to 80 per cent of the entire human population. Fortunately the mortality rate is usually low, although it should be realised that a mortality rate of 3% such as occurred in the pandemic of 1918-19 represents the death of 20 million people (Thomson and Thomson, 1933). With the advent of antibacterial chemotherapy, the mortality rate had been reduced to 0.1% by 1957, the year of the "Asian" influenza pandemic. However, because of the rapid, almost universal spread of the disease in an exponentially growing population, this still represents the death of several million people. Thus influenza is responsible for more human deaths than any one other virus disease. Similarly, because of its universal occurrence, rapid rate of dispersal in an industrial economy and high morbidity, influenza is responsible for more industrial inefficiency than any other single cause, including absenteeism, strikes, incompetent management and nationalization.

Influenza viruses also infect many other vertebrates of which Fowl plague virus (FPV) is one of the most virulent. It infects nearly all types of birds with a mortality of 30%. However, in domestic fowls and game birds, the mortality rate can be as high as 100% (Todd and Rice, 1930). After the first reported outbreak in 1878 in Italy and the subsequent rapid spread over the rest of the world, Fowl plague is now only thought to be endemic in Egypt. The last serious outbreak occurred in 1924 in America.

Structure of Fowl plague virus

The purpose of both this section and the one following, is not to

The economic and sociological importance of influenza virus

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Structure of Fowl plague virus

The purpose of both this section and the one following, is not to

give an exhaustive review of the relevant literature, but to outline the processes of replication and the virion structure in order to facilitate the discussion of data presented later. The structure of FPV has recently been reviewed in detail by other authors (Flamand and Bishop, 1975; Skehel, 1974b), as has the replication cycle (Hoyle, 1966; Flamand and Bishop, 1975; Burke and Russell, 1975).

FPV is an avian virus of the myxovirus group. Its genome consists of single stranded ribonucleic acid (RNA) in a ribonucleoprotein (RNP) complex, which is surrounded by a lipo-protein envelope, the external proteins of which are glycosylated.

The genome of FPV consists of single stranded RNA of total molecular weight of about 4.0×10^6 daltons. There has been much discussion in past years over whether or not the genome is segmented, but for all purposes here it will be assumed to be segmented. The size of the RNA pieces range from 2.0×10^5 to 7.0×10^5 daltons although their exact number and molecular weight has not been resolved. However, the RNA from a related influenza virus, X-31, has been more closely characterized by Skehel (1973). This RNA can be resolved into 3 distinct size classes, each containing at least 2 distinctive species. At present the function of any one segment is not known, nor is its sequence, although the terminal nucleotides have been determined, e.g. all segments contain a unique 5' terminal nucleotide 5'-p p p Ap (Young and Content, 1971), and uridine at the 3' terminus (Lewendowski et al., 1971). The RNA of the virion is not infectious and appears to be of complementary sequence to virus-specific RNA acting as messenger in infected cells. In the virus particle the nucleic acid is tightly complexed with the nucleoprotein (NP) of mol. wt. 35,000 to form a helical RNP complex. Also associated with this RNP, but in much smaller amounts, are two

other proteins, P1 and P2 (Mol. wts. 94,000 and 81,000 respectively). Surrounding the helical RNP is a shell of matrix (or membrane) protein of mol. wt. 25,000. The RNA dependent RNA polymerase activity is associated with this core but has not been assigned to any single protein or combination of proteins.

Surrounding the shell of matrix protein is a layer of glycoproteins embedded in a lipid bilayer. This lipid bilayer appears to have the same constitution as that of the host cell and is derived directly from it. These lipids differ from those in the host plasma membrane in one respect; they appear to be rather more sterically inhibited in both their lateral and vertical movement. Inserted in this membrane are 2 glycoproteins, the haemagglutinin (HA), responsible for the attachment of virus particles to cell surface receptors, and the neuraminidase (NA), an enzyme whose function is unknown, but is probably involved in the release of progeny virus from the cell. Recently the HA and NA of a closely related influenza virus (X-31) has been closely characterized by Skehel et al., (1975). The HA spike consists of 3 subunits of mol. wt. [illegible] arranged in a trimer, 160 Å long, having an equilateral triangular cross-section of height 50 Å. Each of the 3 subunits consists of 2 glycopeptides of estimated mol. wt. 46,000 (HA1) and 29,000 (HA2). One end of the trimer is thought to be hydrophobic and is probably inserted in the lipid bilayer. The other end contains the antigenic determinants. The neuraminidase consists of a tetramer of 4 coplanar cubes of side 40 Å with a tail of about 100 Å extending on one side from the point of contact of all 4 cubes. The base of the tail is hydrophobic and is presumably the point of attachment to the virus lipid bilayer, whereas the antigenic sites appear to be located on the vertices of the coplanar cubes. Each

subunit of the tetramer has an identical molecular weight of 45,000 daltons.

The replication cycle of FPV

The influenza virus particle attaches to sialic acid receptors on the plasma membrane of the host cell by the haemagglutinin protein of the virus envelope. The virus is then absorbed into the cell. This mechanism of absorbtion is not clearly understood and will be discussed more fully in Section II of the results. On penetration of the host plasma membrane, recognisable virus particles are no longer visible by electron microscopy (EM), presumably because the particle is digested, although it is not known whether the RNA is released or it remains as an RNP complex.

The virion specific messenger RNA is transcribed from the genome by a virion associated RNA-dependent RNA polymerase after a lag of 30 min. The activity of this polymerase is sensitive to inhibitors of DNA function such as actinomycin D (AMD) *in vivo* but not *in vitro*. This sensitivity to AMD of the *in vivo* polymerase activity also correlates with the complete inhibition of virus replication if cells are treated either before or during the first 2 h of infection with many such inhibitors or if the cells are enucleated. This sensitivity is unique amongst the RNA viruses except the tumour viruses, although tumour viruses, unlike the influenza viruses are sensitive to inhibitors of DNA synthesis as well. Some workers report that the addition of AMD late in infection also inhibits virus replication, but to a lesser extent. From about 1½ h after infection virion RNA is synthesised preferentially. The mechanism of this changeover is unknown, but it is sensitive to cycloheximide and therefore probably involves the synthesis of a new protein. Recently there has been some evidence that virus mRNA is

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synthesised in the cytoplasm and virion RNA (vRNA) in the nucleus. This will be discussed in greater detail in Section III of the results. Unlike infections with poliovirus, FPV causes no sudden decrease in host RNA synthesis although it does decline later in infection, i.e. from about 4 h post infection.

The first proteins to be synthesised are P2, NP and a virion-specific, non-structural protein of 23,000 mol. wt (NS1) (Skehel, 1973). Later in infection, all virion proteins are synthesised as well as 2 non-structural proteins, NS1 and NS2 (11,000 mol. wt). All proteins are synthesised in the cytoplasm, although P1, P2, NP, and NS1 are found in the nucleus, with NS1 concentrated in the nucleolus. The haemagglutinin is synthesised as a precursor of 70,000 daltons on the rough endoplasmic reticulum (ER) along with the neuraminidase. Both proteins are then transferred via the smooth ER to the plasma membrane where the HA precursor is cleaved to form the 2 polypeptides found in the mature virion. The matrix protein is synthesised close to the plasma membrane where it is inserted directly into regions already containing HA (Hay, 1974). Nothing else is known about the location and mechanism of synthesis of the other virus specific polypeptides. Again unlike poliovirus, no sudden cut-off in protein synthesis of the host cell is observed, although after 4 h infection, the main proteins synthesised are viral.

Mature virus is released from the cell after about 4 h post-infection, although the mechanism of virus assembly and release is virtually completely obscure. All that is known, is that virions appear to bud from the plasma membrane of the host cell carrying with them some host membrane components, and that this process can be inhibited by blocking neuraminidase activity.

The problems of curing influenza

Like all other virus-induced diseases of higher vertebrates, it has so far proved impossible to adequately treat cases of influenza by chemotherapy. With every anti-viral agent tried so far the concentration needed to produce an anti-viral effect is very close to the toxic level for the host cell. This is probably because the majority of virus-required synthetic functions are carried out by host cell mechanisms (Hoyle, 1968a).

Most virus infections can be readily prevented (in theory at least) by immunization of the potential host. Influenza virus is an excellent immunogen and theoretically, immunization should produce excellent results (Smith et al., 1933; Andrewes and Smith, 1939; Stuart-Harris et al., 1938). However even under ideal conditions, protection was achieved in only 80% of cases; protection lasted only a year and immunization at times when no pandemic was threatened produced undesirable side effects resulting in more "inconvenience" than if the population had not been immunized. The most important reason for this unsatisfactory record for anti-influenza vaccination is the remarkable frequency with which spontaneous antigenic variations occur. This plasticity (Smith, 1952) far exceeds that of most animal viruses although foot-and-mouth-disease virus and rhinovirus also have a very broad antigenic range. This antigen plasticity makes it impossible therefore to stock-pile vaccines. Recently the WHO have established a global network to isolate and identify new serotypes as they arise, but the system is far from satisfactory as new strains spread very fast in a highly industralised world.

This process of change is that of "antigenic drift" whereby the antigenic properties of the virus slowly change between pandemics, probably due to the selection, under immunological pressure, of spontaneous

virus mutants (Burnet, 1960). The second process, that of "antigenic shift", is the sudden large change of antigenicity, which causes the pandemics. Two of the theories which attempt to explain this phenomenon, involve an extension of the antigenic drift mechanism or the participation of influenza viruses from other species. The latter mechanism is given considerable support from two areas of research. Firstly, high genetic recombination rates are observed from mixed infections of influenza virus (Burnet and Lind, 1949; Hirst, 1962). This high recombination rate is thought to result from the fact that the genome is segmented into several (5-9) pieces of single-stranded RNA (Pons and Hirst, 1968; Deusberg, 1968; Bishop et al., 1971; Skehel, 1971). This enables the virus genes to re-assort during a mixed infection without going through the less efficient classical recombination machinery. Secondarily, Laver and Webster (1973) have shown that tryptic peptide maps of the surface glycoproteins of human influenza viruses isolated before and after a pandemic show absolutely no similarities. However the peptide map of the glycoproteins from virus isolated after the pandemic show considerable similarities to animal viruses in current circulation. This cross-infection is thought to occur in mainland China where humans, pigs and birds live in close proximity.

One of the most promising attempts at circumventing the problem of antigenic change is the production of subunit vaccine, i.e. vaccine against the virus core proteins which do not undergo antigenic change. This may make it possible to produce a non-toxic vaccine against all major types of influenza virus (Davenport et al., 1959; Hennessy and Davenport, 1966). However these studies have not been significantly extended, probably due to the difficulties of producing large amounts of vaccine.

Influenza virus infection as a model for cellular control mechanisms

The understanding of control mechanisms in eukaryotic cells is

probably the most stimulating, demanding and potentially rewarding task facing modern molecular biology. However the complexity of a typical mammalian cell with a coding capacity for approximately 10^7 proteins provides an almost insurmountable obstacle to such a study. The study of virus infection should simplify these problems as they contain very much less genetic information - only about seven genes in the case of influenza. Because of this simple structure the virus relies almost completely on the synthetic and metabolic processes of the host cell. Therefore the virus has to ensure that these host processes are efficiently used for the production of progeny virus. Thus virus infection offers an excellent system for the study of the control mechanisms of these processes. Furthermore Hoyle (1968c) has suggested that influenza virus is not a parasite in the classical sense, but an escaped cellular gene that has evolved a mechanism for rapidly transmitting information in an emergency to large sections of the population. If this were true, the evaluation of the nature of this information and its mode of transport would be of prime importance.

Influenza virus offers many advantages over other systems in the study of both virus multiplication and the control of cellular metabolism. Although influenza virus has a high morbidity rate, the mortality rate is very low (0.1%) and usually only the aged and infirm are fatalities. (Hopefully this does not therefore include many of the active scientific community!) If workers restrict themselves to working with recently derived strains, both they and the population at large will already be immune, due to 'wild' infections. Even if extremely virulent strains (such as the avian influenza virus FPV) are used, the chances of them escaping from the laboratory are very small as most are very heat sensitive (Horsfall, 1955) and labile to dehydration or exposure to sunlight (Edward, 1941). Large quantities of virus can be conveniently

grown in the allantoic sac of fertile hens eggs (Miller, 1944) and FPV can be conveniently grown in CEF monolayers (Stulberg and Schapira, 1953). Both FPV and WSN can be easily assayed for infectivity in CEF monolayers and all influenza viruses are easily and quickly assayed by haemagglutination of erythrocytes (Salk, 1944). Purification of influenza virus is readily achieved without loss of biological activity, by differential centrifugation, adsorption and elution from erythrocytes or less satisfactorily by many other methods (see Hoyle, 1968b for details).

Unlike most other RNA viruses, influenza does not cause cut-off of host cell RNA, DNA and protein synthesis completely. It is therefore more likely to reflect the true host cell control mechanisms, than viruses with a more abrupt effect such as poliovirus, Semliki Forest virus etc. Furthermore, the virus genome is in several distinct pieces which can be easily separated and therefore it should be possible to identify single virus induced proteins corresponding to each of these pieces of RNA. Recently several pieces of evidence have shown that viral protein synthesis is under some form of temporal control. Skehel (1973) has shown that only some viral proteins are synthesised early in infection whereas later on in infection all proteins are synthesised. The synthesis of these early proteins also appears to be less sensitive to AMD (P.D. Minor - personal communication). Also some types of virus-specific mRNA (i.e. RNA complementary to the virion) appear in infected cells, before other species (Avery, R.J. and Dimmock, N.J. - personal communication). Therefore the study of expression of genetic information and its control in influenza virus infection may give insight into control mechanisms in the host cell. Probably the most significant feature of influenza virus multiplication is its dependance on the host cell nucleus: this is discussed further in the following section.

Possible functions for the nuclear phase of influenza virus replication

The nuclear phase in influenza virus infection is unique in its mechanism. Both the nuclear requiring DNA and RNA tumour viruses appear to require the host DNA polymerases for the production of progeny virus or provirus and, unlike influenza virus, are sensitive to inhibitors of DNA synthesis as well as DNA function. The details of the previously published data in this field will be dealt with at greater length in succeeding chapters; but here only the possible roles for the nuclear phase and its relevance to the understanding of host cell control will be discussed.

After entry into the host cell, there are several problems that a virus must overcome in order to achieve a productive infection.

1) Influenza virus is thought to be a negative stranded virus (i.e. with a genome of opposite sense to mRNA) with a virion associated polymerase, which synthesises RNA complementary to vRNA (cRNA) (Bishop and Obijeski, 1971; Chow and Simpson, 1971). This cRNA is found on host cell polysomes (Nayak, 1970; Pons, 1970) and is assumed to be mRNA. However the situation is more complex than with other similar RNA viruses, like NDV and VSV. Unlike these viruses, influenza virus vRNA is found on infected cell polysomes (Nayak, 1970; Scholtissek and Rott, 1970) and also stimulates some in vitro translation systems (Siegert et al., 1973) but not others (Kingsbury and Webster, 1973). Further, as mentioned above some virus proteins are synthesised before others. Therefore, upon uncoating, either the correct vRNA segments must be recognised and transcribed by the polymerase or only certain vRNA segments are recognised as messengers and translated.

2) At some time during infection the virus multiplication machinery must stop producing cRNA to use as mRNA and start using it for an entirely

different function, ie, that of a template for progeny vRNA.

3) The final problem of virus multiplication is that of correctly controlling the assembly of vRNA and virus proteins into the cell membrane in such a way as to produce complete infectious particles when the virus buds from the plasma membrane.

It will be realized therefore, that the problems of transcriptional and translational control, correct utilization of RNA and assembly, faced by the virus are similar to the problems of transcriptional and translational control, mRNA transport and membrane assembly faced by the host cell. The evaluation of these processes could be the key to understanding the long term management of eukaryotic cellular functions as well as understanding the control of virus multiplication.

Control mechanisms in eukaryotic cells are at best poorly understood at present. Most information at present is available on translational control mechanisms, and as these are cytoplasmic and also appear to be of little importance in influenza virus multiplication, they will not be dealt with here. Transcriptional control is poorly understood at the moment. Histones have been implicated (Bradbury et al., 1974), but no definite evidence is yet available. Messenger RNA species are formed by the transcription of DNA to form a heterogeneous population of nuclear RNA (Williamson et al., 1973). The heterogeneous nuclear RNA (hnRNA) is synthesised very rapidly but has a short half life of only about 5-15 min (Darnell, 1968). Messenger RNA is formed by sequential cleavage of hnRNA (MacNaughton et al., 1974). As there appears to be no change in the pattern of heterogeneous nuclear RNA throughout the life cycle of the cell, it would appear that precursors to all mRNA species are synthesised at all times, and broken down if they are not immediately

needed. Therefore transcriptional control may not in fact exist, but the important controls may occur at the level of the destruction of the presumptive messenger species before they are exported to the cytoplasm. This control of mRNA export has been the postulated role for the post-transcriptional addition of poly A sequences to hnRNA observed in all eukaryotic cells so far studied (Edmonds et al., 1971). The notable exception to this rule is histone mRNA which does not contain any poly A tracts. Recently, Jelinek et al., (1974) have shown that virtually all poly A synthesis occurs in the nucleus (with the exception of certain viral messengers e.g. VSV and poliovirus). Influenza virus mRNA has now been shown to contain poly A (M. MacNaughton - personal communication; R. Krug - manuscript in preparation). If influenza virus RNA does not contain short tracts of poly U which would enable the cytoplasmic virion polymerase to form poly A tracts when transcribing the vRNA, as is the case with VSV (see discussion in Bannerjee et al., 1974) then the virus may have to enter the nucleus to have its mRNA charged with poly A to enable it to be correctly translated.

The packaging of new host mRNA into RNP complexes-informosomes, occurs in the nucleus (Georgiev et al., 1972). The virus could make use of this same process for the assembly of its own RNP cores. In fact it has been known for some time that the proteins of the RNP complex along with NS1 appear in the nucleus of influenza virus infected cells (Breitenfeld and Schaf er, 1957; Krug and Etkind, 1973; Taylor et al., 1970). This complex then moves into the cytoplasm to finally arrive at the plasma membrane where virus assembly occurs (Hay and Skehel, 1974).

Therefore it is easy to justify the existance of a nuclear phase in influenza virus replication and it is not difficult to see that study

of this nuclear phase, will not only give an insight into the replication of the virus but into the nature of the control of host cell macromolecular synthesis in general. It is with these facts and postulates in mind that the data in this thesis is presented. The only mystery that remains is how do so many RNA viruses manage to replicate at all without a nucleus as demonstrated by their multiplication in enucleate cells (Follett et al., 1974; Pollack and Goldman, 1973; Kelly et al., 1974).

MATERIALS

Radiochemicals and isotopes

These were obtained from the Radiochemical Centre, Amersham, U.K. and had the following specific activities.

^{198}Au] colloidal gold, 2-6 mCi/mg Au; L-[4,5-^{3}H] leucine, 40-60 Ci/mmol; L-[^{3}H] methionine, >5 Ci/mmol; L-[^{35}S] methionine, [^{32}P] orthophosphate, 171 Ci/mg P; [2-^{14}C] uridine, 60 mCi/mmol, [2-^{14}C] cytidine, >50 mCi/mmol, [^{3}H] cytidine, >30 Ci/mmol.

Drugs

Actinomycin D and Amino C (Macdoal) were obtained from George Kemp, Medical Supply Services, High Wycombe, Bucks.

Electrophoresis chemicals

Acrylamide, N-N'-methylene bisacrylamide and N,N,N',N',-tetramethylethylenediamine (TEMED) were obtained from Kodak Ltd., Liverpool.

Scintillants

2,5-Diphenyloxazole (PPO); 2,4-di(2(5-phenyl-oxazolyl))-benzene (POPOP) and naphthalene (all scintillation grade), were obtained from Nuclear Enterprises (G.B.) Ltd., Edinburgh.

Enzymes and substrates

Ribonuclease T_1 (Grade XII), pancreatic ribonuclease A and deoxyribonuclease, Type I were obtained from the Sigma Chemical Co., St Louis, Missouri, U.S.A. Fetuin was prepared as follows. Saturated $(NH_4)_2SO_4$ (1 litre) at 5° was added slowly to foetal calf serum (1 litre) at 5° with gentle stirring. The precipitate was redissolved in 200 ml of water at 5° and reprecipitated with saturated $(NH_4)_2SO_4$. The precipitation was repeated twice and the final precipitate was dissolved in no more than 200 ml of distilled water and dialysed at 4° for 16 h against flowing distilled water. Any precipitate formed was removed and the solution allowed to stand 5-7 days in the cold room, after which any

Radiolabelled compounds

These were obtained from the Radiochemical Centre, Amersham, U.K. and had the following specific activities.

[^{198}Au] colloidal gold, 2-6 mCi/mg Au; L-[4,5-^{3}H] leucine, 30 Ci/mmol; [methyl-^{3}H] L-methionine, >5 Ci/mmol; L-[^{35}S] methionine, >100 Ci/mmol; [^{32}P] orthophosphate, 121 Ci/mg P; [2-^{14}C] uridine, 60 mCi/mmol; [5'-^{3}H] uridine, 24 Ci/mmol, [2-^{14}C] thymidine, >50 mCi/mmol, [methyl-^{3}H] thymidine, 15-30 Ci/mmol.

Stains

Coriophosphine O and Azure C (Macneal) were obtained from George T.Gurr, Searle Scientific Services, High Wycombe, Bucks.

Electrophoresis components

Acrylamide, N-N'-methylene bisacrylamide and N,N,N',N',-tetramethylenediamine (TEMED) were obtained from Kodak Ltd., Liverpool.

Scintillants

2,5-Diphenyloxazole (PPO); 1,4-di(2(5-phenyl-oxazolyl))-benzene (POPOP) and naphthalene (all scintillation grade), were obtained from Nuclear Enterprises (G.B.) Ltd., Edinburgh.

Enzymes and substrates

Ribonuclease T_1 (Grade III), pancreatic ribonuclease A and deoxyribonuclease, Type I were obtained from the Sigma Chemical Co., St Louis, Missouri, U.S.A. Fetuin was prepared as follows. Saturated $(NH_4)_2SO_4$ (1 litre) at 5° was added slowly to foetal calf serum (1 litre) at 5° with gentle stirring. The precipitate was redissolved in 200 ml of water at 5° and reprecipitated with saturated $(NH_4)_2SO_4$. The precipitation was repeated twice and the final precipitate was dissolved in no more than 200 ml of distilled water and dialysed at 4° for 16 h against flowing distilled water. Any precipitate formed was removed and the solution allowed to stand 5-7 days in the cold room, after which any

additional precipitate was removed. Final solution (12 mg/ml) was sterilized by filtration.

Metabolic inhibitors

Cycloheximide was obtained from the Sigma Chemical Co., and Actinomycin D was a gift from Merck , Sharpe and Dohme (U.K.), Hoddesdon, Herts.

Tissue culture media

199 and Eagle's BHK media were obtained from Wellcome Research Laboratories, Beckenham, Kent. 199 also contained 75 units/ml of crystamycin (Glaxo, U.K) and 50 units/ml of Mycostatin (E.R. Squibb and Sons, London). The tryptose phosphate broth was omitted from Eagle's BHK medium. Earle's solution was obtained from Oxoid Ltd., London S.E.1. and HEPES buffer from the Sigma Chemical Co. Calf serum (membrane filtered) was obtained from Tissue Culture Services, Slough, Bucks, U.K.

Viruses

A/FPV/Rostock/34 ($H_{av}1$ $N_{av}1$) (referred to in future as FPV) was grown by inoculating 11 day embryonated chicken's eggs with 10^4 p.f.u. per egg and harvesting the allantoic fluid after 24 h growth at 37°. Allantoic fluid was clarified by centrifugation at 1,500 g for 20 min at 4°. Virus stocks were snap frozen at -70°.

The recombinant, having the HA of A/FPV/Dutch/27 ($H_{av}1$ $N_{eq}1$) virus and the neuraminidase of A/BEL/42 (HO N1) (designated hereafter as FPV/BEL) was a gift from Dr D. McCahon. The virus was plaque purified 3 times and stocks grown as for the above Rostock strain.

Semliki Forest virus (ts +) (SFV), grown in the brains of suckling mice was a gift from Dr S.I.T. Kennedy. Sendai virus was a gift from Dr R.J. Avery. Poliovirus type I (LSc 2ab) was a gift from Dr S.I. Koliais.

METHODS

Buffers

Lysing buffer: Na_2 EDTA (0.001 M), Na acetate (0.01 M), NaCl (0.05 M), pH adjusted to 4.6 with acetic acid, sterilised and then saturated with diethylpyrocarbonate (L Buffer).

Proteinase buffer 1: Tris (0.01 M), $MgCl_2$ (0.001 M) pH adjusted to 7.8, sterilised and saturated with diethylpyrocarbonate.

Proteinase buffer 2: as above but including Sucrose (0.25 M).

Phosphate buffered saline (PBS): NaCl (0.130 M), KCl (0.028 M), Na_2HPO_4 $(2H_2O)$ (0.0075 M), KH_2PO_4 (0.0147 M).

Wash buffer: Tris (0.01 M), NaCl (0.05 M), $MgCl_2$ (0.002 M), pH adjusted to 7.6, and sterilised.

Standard saline Citrate (SSC): NaCl (0.15 M), Na tri) citrate (0.015 M), pH adjusted to 7.0 with HCl and sterilised.

Acetate buffer (AB): Na acetate (0.01 M), pH adjusted to 4.6, sterilised and saturated with diethyl pyrocarbonate.

Membrane lysing buffer: Sucrose (0.25 M), Tris (0.05 M), Na acetate (0.001 M), pH adjusted to 7.4.

Membrane gradient buffer: Tris (0.001 M), Na acetate (0.0005 M) pH adjusted to 8.6.

RNA gradient buffer: NaCl (0.01 M), Tris (0.02 M), Na_2 EDTA (0.001 M) pH adjusted to 7.0, sterilised and saturated with diethyl pyrocarbonate.

Where indicated, buffers were sterilised by autoclaving at 15 p.s.i. for 30 mins.

Preparation of primary chick embryo fibroblast cells (CEF cells)

The method used was that of Moreau et al., (1973). Embryos from 11 day hen's eggs were removed, decapitated, and chopped by hand. A suspension of cells was prepared by repeated incubations

Buffers

Lysing buffer:- Na_2 EDTA (0.001 M), Na acetate (0.01 M), NaCl (0.05 M). pH adjusted to 4.6 with acetic acid, sterilised and then saturated with diethylpyrocarbonate (L Buffer).

Fractionation buffer 1: Tris (0.01 M), $MgCl_2$ (0.001 M) pH adjusted to 7.4, sterilised and saturated with diethylpyrocarbonate.

Fractionation buffer 2: as above but including sucrose (0.25 M).

Phosphate-buffered saline (PBS): NaCl (0.139 M), KCl (0.028 M), Na_2HPO_4 ($2H_2O$) (0.0075 M), K H_2PO_4 (0.00147 M).

DNase buffer: Tris (0.01 M), NaCl (0.05 M), $MgCl_2$ (0.002 M), pH adjusted to 7.5, and sterilised.

Standard Saline Citrate (SSC): NaCl (0.15 M), Na (tri) citrate (0.015 M). pH adjusted to 7.0 with HCl and sterilised.

Acetate buffer (AB): Na acetate (0.01 M), pH adjusted to 4.6, sterilised and saturated with diethyl pyrocarbonate.

Membrane lysing buffer: Sucrose (0.25 M), Tris (0.05 M), Mg acetate (0.001 M), pH adjusted to 7.4.

Membrane gradient buffer: Tris (0.001 M), Mg acetate (0.0005 M) pH adjusted to 8.6.

RNA gradient buffer: NaCl (0.01 M), Tris (0.02 M), Na_2 EDTA (0.001 M) pH adjusted to 7.0, sterilised and saturated with diethyl pyrocarbonate.

Where indicated, buffers were sterilised by autoclaving at 15 p.s.i. for 20 mins.

Preparation of primary chick embryo fibroblast cells (CEF cells)

The method used was that of Morser et al., (1973). Embryos from 11 day hen's eggs were removed, decapitated, and chopped by hand. A suspension of cells was prepared by repeated incubations

with 0.1% trypsin in PBS at 37° for 20 min. The cell suspensions were combined, filtered through 40 mesh stainless steel guaze, pelleted by centrifugation, resuspended in 199 medium (with 5% v/v calf serum, 100 units/ml penicillin and 100 units/ml streptomycin) and finally passed through a grade 0 and then a grade 1 sintered glass filter. Suspensions were diluted in 199 medium to the desired concentration, seeded on plastic petri dishes and incubated overnight at 37° in 95% air/5% CO_2 before use.

Preparation of HeLa cells

The method employed was that of Koliais and Dimmock (1973). Cells were grown to confluence at 37° in 1 litre glass bottles (5×10^7 cells per bottle) and passed every seventh day by trypsinization to form subcultures at a 1 in 6 dilution. Cultures were treated every 2-3 months with Kanamycin to prevent formation of colonies of mycoplasma.

Preparation of enucleate BSC-1 cells

These were prepared by Dr E.A.C. Follett as described previously (Follett et al., 1974).

Growth and purification of ^{32}P labelled FPV/BEL virus

Batches of 16 twelve day old de-embryonated eggs were prepared as described by Bernkopf (1949). Standard medium (Fazekas de St Groth and White, 1958) was added and each egg was inoculated with 0.1 ml of FPV/BEL virus suspension containing approximately 10^4 p.f.u. The open end of the egg was sealed with aluminium foil and wax. Eggs were labelled overnight by incubation with 0.5 mCi of ^{32}P at 37°.

Medium from the eggs was clarified by centrifugation at 1500 g for 10 min at 4° and virus was precipitated by stirring at 4° for 30 min in 60% saturated $(NH_4)_2SO_4$ in phosphate buffered Earle's saline. After centrifugation at 20,000 g for 15 min, the virus pellet was resuspended in 4 ml of phosphate buffered saline containing 0.5 mM $MgCl_2$ and

0.5 mM $CaCl_2$ (PBS/Ca/Mg). The suspension was loaded onto five 60 ml 15-40% linear sucrose gradients containing 0.1% BSA and centrifuged at 90,000 g for 1½ h at 20°. The gradient was fractionated into 2 ml aliquots and assayed for HA and total radioactivity. Where these activities formed a co-incident peak, the fractions were pooled. A typical gradient is shown in Fig. 1. The virus was precipitated with $(NH_4)_2SO_4$ and dialysed overnight at 4° against PBS/Ca/Mg. Table 1 shows the purification and recovery from a typical preparation. The virus RNA had a specific activity, on average of about 5.0×10^5 d.p.m./µg.

Growth and purification of ^{32}P-labelled Semliki Forest virus (SFV)

SFV was grown in a suspension of CEF cells, labelled with ^{32}P (20 µCi/ml) in phosphate-free Earle's saline, buffered with HEPES, and purified on sucrose gradients containing 0.1% calf serum as described by Kennedy (1974).

Growth and purification of ^{32}P labelled Sendai virus

0.1 ml of a 10^{-3} dilution of infectious allantoic fluid in PBS/Ca/Mg was injected into each of 24 eleven day embryonated eggs. After 24 h incubation at 37°, each egg was injected with 0.3 mCi ^{32}P ortho-phosphate and incubated for a further 24 h. The allantoic fluid was harvested, clarified by centrifugation at 1500 g for 10 min at 4° and the virus pelleted by centrifugation at 75,000 g for 45 min at 4°. The pelleted virus was purified as follows after the method of Moore and Burke (1974). The pellet was resuspended by vigorous shaking and sonication in 3 ml of PBS/Ca/Mg and loaded onto a linear gradient prepared as follows. Equal volumes of 60% sucrose (w/v) in 0.1 M tris (pH 7.3), 45% (w/w) sodium, potassium tartrate in 0.1 M tris (pH 7.3) and glycerol, were mixed to form the heavy component. 1 in 2 dilution of the heavy component in 0.1 M tris (pH 7.3) formed the light component. A linear gradient was formed from these

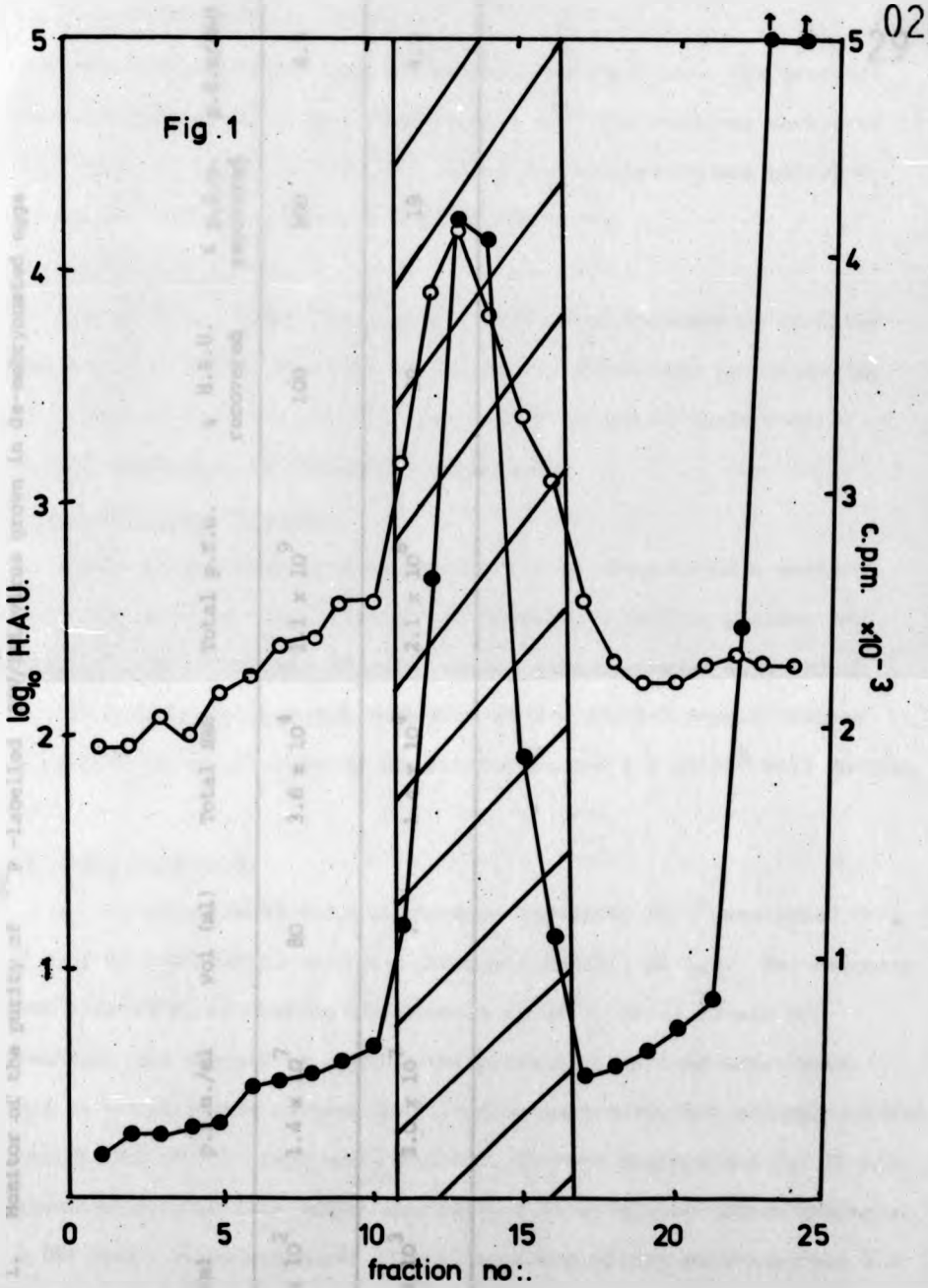


Fig. 1. Purification of FPV/BEL virus by centrifugation at 90,000 g for 1½ h at 20° on a 15-40% sucrose gradient containing 0.1% BSA. The gradient was then fractionated into 2 ml aliquots. Sedimentation is from right to left and the shaded area shows the fractions pooled. O - O H.A.U. per fraction, ● - ● radioactivity per 10 µl sample from each fraction.

Table 1. Monitor of the purity of ^{32}P -labelled FPV/BEL virus grown in de-embryonated eggs

Sample	HAU/ml	p.f.u./ml	vol (ml)	Total HAU	Total p.f.u.	% H.A.U. recovered	% p.f.u. recovered	p.f.u/HAU ratio
Crude virus	4.5×10^2	1.4×10^7	80	3.6×10^4	1.1×10^9	100	100	4.5
Purified virus	2.0×10^3	3.0×10^7	7	1.4×10^4	2.1×10^8	39	19	4.2

two solutions and the virus loaded onto the gradient. The gradient was centrifuged at 90,000 g for 12 h at 4°. The band was harvested and dialysed against PBS/Ca/Mg. The virus suspension was pelleted as before and resuspended in 5 ml of PBS/Ca/Mg.

Haemagglutinin assay

Haemagglutinin (HA) was titrated in 0.25 ml aliquots by doubling dilutions in PBS/Ca/Mg. The end point was determined by estimating the interpolation between full agglutination and no agglutination of a 0.5% suspension of chicken erythrocytes.

Virus infectivity assays

Infectivity was assayed by plating 0.1 ml aliquots of a serial dilution of virus suspension on CEF monolayers on 5 cm plastic petri dishes. The monolayers (9×10^6 cells) were overlayed with 199 medium containing agar and incubated at 37° for 2-3 days. Plaques were counted after staining the monolayers for 1 h at 37° with neutral red.

Neuraminidase assay

0.1 ml of suitably diluted virus in PBS/Ca/Mg at 4° was added to 0.1 ml of calf fetuin in 0.2 M phosphate buffer, pH 5.9. The reaction was started by incubating the mixture at 35°. After 30 min the reaction was stopped by placing the mixture in an iced water bath. 0.1 ml of periodate reagent (4.3% sodium periodate, 62% orthophosphoric acid) was added, mixed well, and left at room temperature for 20 min. Arsenite reagent (10% sodium arsenite, 7.1% anhydrous sodium sulphate, 0.28% conc. sulphuric acid) (1 ml) was added, mixed well and then 2.5 ml of thiobarbituric acid reagent (0.6% sodium thiobarbiturate, 7% anhydrous sodium sulphate) was added. The mixture was boiled for 15 min and cooled rapidly to room temperature. The pink colour which developed was extracted in 4 ml of 5% (v/v) HCl in n-butanol and the phases clarified by centrifugation at 1,000 g for 10 min. The O.D.

of the organic phase was read at 459 nm immediately.

Preparation of antisera

New Zealand white rabbits were bled from the ear vein to produce pre-immune serum and then immediately inoculated intravenously with 5×10^4 HAU of A/FPV/Rostock/34 in PBS/Ca/Mg. Twenty one days later the animal was inoculated again and bled daily, commencing 5 days after the second inoculation. The HAS of the Rostock and Dutch strains of FPV are identical (Hoyle, 1968d), so this antiserum was used experimentally to neutralize the FPV/BEL recombinant.

Staining of subcellular fractions

Cells were stained with coriophosphine as follows. Cells or sub-cellular fractions were suspended in PBS, smeared onto a coverslip and allowed to dry. Then they were fixed for 10 min in Carnoy's fluid (60% absolute ethanol, 30% chloroform, 10% acetic acid), rinsed in 0.2% (v/v) phenol in PBS and stained for 5 min in 0.05% coriophosphine in PBS/phenol. Preparations were rinsed thoroughly in PBS/phenol and mounted in the same solution. When examined by U.V. illumination, DNA and double stranded RNA fluoresced green and single stranded RNA, brick-red.

For azure C staining, samples were prepared as before, fixed for 3 min in 50% acetic acid, rinsed for 3 min in absolute ethanol, then 3 times for 3 min in 3:1 ethanol/acetic acid and stained for 1 min in a solution containing 7 parts sodium citrate (10%) and 3 parts 0.1% azure C in 0.25 M sucrose. Samples were washed in sodium citrate (10%) and mounted. Nucleoli preferentially take up the stain; although nucleoplasm may have some colour, cytoplasm is unstained.

Nuclear fractionation

For fractionation into cytoplasmic and nuclear fractions, cell monolayers were washed once with cold PBS/Ca/Mg and scraped into the

same solution, then harvested by centrifugation at 1,000 g for 5 min. The cell pellet was resuspended in fractionation buffer (1 ml per 10^8 cells) and disrupted by 5 strokes of a stainless steel Dounce homogenizer having a clearance of 0.002 ins for 4 ins of the barrel length. The suspension was pelleted by centrifugation at 1,000 g for 5 min. The pellet was resuspended in fractionation buffer containing 0.25 M sucrose, rehomogenized by 5 strokes of the Dounce homogenizer and pelleted at 1,000 g for 5 min. This pellet was resuspended in fractionation buffer containing 0.25 M sucrose and 0.1% (v/v) Nonidet P40 (B.D.H., Poole, Dorset), given 3 strokes with the homogenizer and pelleted at 1,000 g for 5 min. This last step was repeated and the final pellet was designated the nuclear fraction. Nuclei harvested from cells 6 h or more after infection tended to be fragile, so only 3 strokes of the homogenizer were used in the first 2 resuspensions and during the detergent washes the nuclear pellet was resuspended with a mechanical mixer for 30 s.

For the cytoplasmic fraction, only the supernates from the first 2 stages were used as detergent treatment breaks lysosomes and releases nucleases and proteases. These supernates were pooled, centrifuged at 1,000 g for 5 min and the pellet discarded.

All procedures in this fractionation scheme were carried out at 4° or in an ice bath.

Criteria for purity of the nuclear fraction

When assayed by phase contrast microscopy, the proportion of unbroken cells or nuclei with visible cytoplasmic tags or other non-nuclear particles, to visible, clean, discrete nuclei was less than 1%.

Infected nuclei were conveniently assayed for cytoplasmic contamination by measuring HA in a sonically disrupted sample. This provided a

stringent test since the HA antigen is concentrated in the perinuclear cytoplasm (Breitenfeld and Schaf er, 1957). Table 2 shows a typical result, although levels of up to 1% HA in the nuclear fraction were tolerated.

The purity of the nuclear fraction was further checked by sedimentation through a sucrose cushion, as follows. Nuclei from 7.5 x 10^8 CEF cells were resuspended in 0.9 ml of fractionation buffer containing sucrose and detergent and mixed with 1.7 ml of buffer containing sucrose. This was loaded onto a cushion of 2.3 M sucrose in fractionation buffer and centrifuged at 125,000 g for 30 min at 4°. The nuclei formed a pellet and no material was left at the interface. However, a very faint band could be seen about 1/4 of the way into the sucrose cushion and this was removed and dialysed against PBS at 4°. The dialysate was extracted for nucleic acids as described below and run for 4 h on 2.4% P.A.G.E. A small peak of optical density at 260 nm was observed in the region where free degraded DNA normally migrates; no rRNA could be seen at all. The band in the sucrose cushion was judged to be from free DNA or chromatin released when some nuclei were broken during the fractionation procedure.

Extraction of nucleic acids

Nuclei were suspended in 100 volumes of L buffer; cytoplasmic fractions were adjusted to the same molarity of L buffer by the addition of concentrated buffer; and virus suspensions were mixed with approximately 200μg of total CEF nucleic acid after dialysis against L buffer at 4°. The solution was made 2% with respect to SDS (Specially Pure Grade - B.D.H., Poole, Dorset) and added to an equal volume of water-saturated redistilled phenol. The phenol contained 0.1% (w/v) 8-hydroxy-quinoline to act as an anti-oxidant. The mixture was then shaken at room temperature for 5 min and the aqueous phase separated by centrifugation at 3,400 g at 4° for 10 min.

Table 2. Distribution of haemagglutinin in subcellular fractions of 7.5×10^8 CEF cells harvested 8 h after infection with 5 p.f.u./cell of FPV.

Fraction	HAU/5 µl	Sample volume (ml)	Total HAU	% of homogenate
Whole cell homogenate	6.5×10^3	5	6.5×10^6	100
Cytoplasm	1.6×10^3	10	3.2×10^6	49
Pooled detergent washes	1.6×10^3	10	3.2×10^6	49
Nuclei	50	5	1.0×10^4	0.15

The aqueous phase was removed and kept on ice. The phenolic layer and interface were extracted with 20% of the original volume of L buffer, shaken and centrifuged as before. The aqueous layers were pooled, shaken with an equal volume of unsaturated phenol and 8-hydroxy-quinoline at room temperature for 5 min and centrifuged at 3,400 g for 5 min at 4°.

Sodium perchlorate was added to the aqueous phase to a final concentration of 1 M and the mixture shaken briefly with an equal volume of 4% (v/v) n-octonal in chloroform at room temperature. The mixture was centrifuged at 3,400 g for 2½ min at 4°. The aqueous layer was removed, extracted twice with a equal volume of cold 'Analar' diethyl ether and precipitated overnight at -20° by the addition of two volumes of redistilled ethanol. The nucleic acid was collected by centrifugation at 20,000 g for 1 h at 4° and dissolved in the required buffer.

All reagents except the phenol were precooled and all manipulations, except where indicated were carried out at 4°. This procedure resulted in the recovery of 75-80% of the TCA precipitable RNA from cells and 90-95% of the RNA from purified virions. The purity of the nucleic acid preparations were estimated by the ratio of absorbtion at 260 nm: 280 nm and at 260 nm:230 nm being 2.0 and 2.3 respectively.

Preparation of [^{14}C] -labelled RNA from poliovirus-infected HeLa cells

A monolayer of 5 x 10^7 HeLa cells was labelled by the addition of 25μ Ci of [^{14}C] -uridine in 10 ml of Eagle's BHK medium with 10% dialysed calf serum. After 2 h incubation at 37°, the medium was removed, the cells washed once with PBS and then infected with 10^{10} p.f.u. poliovirus type 1 (prepared as described by Koliais and Dimmock,

Fig. 2a. Electrophoresis of RNA of ^{14}C uridine labelled HeLa cells infected with poliovirus on 2.4% PAGE for 4 h. The numbered arrows indicate the positions of the 45S, 32S, 28S and 18S cellular RNA species, corresponding to molecular weights of 4.1×10^6, 2.1×10^6, 1.65×10^6 and 0.65×10^6 respectively. 'ds' indicates the position of a double-stranded viral RNA species of unknown molecular weight and 'ss' indicates the position of the single stranded viral RNA (molecular weight 2.6×10^6) (Weinberg and Penman, 1970).

Fig. 2b. Electrophoresis of RNA of ^{14}C uridine labelled HeLa cells treated for 1 h at 37° with 2 µg/ml AMD before infection with poliovirus. Electrophoresis and captions as for Fig. 2a.

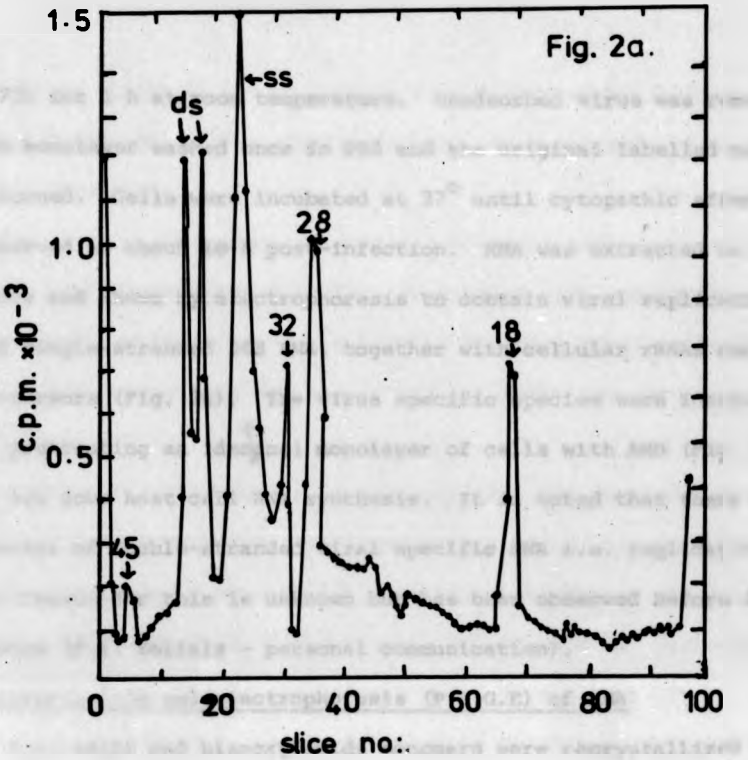
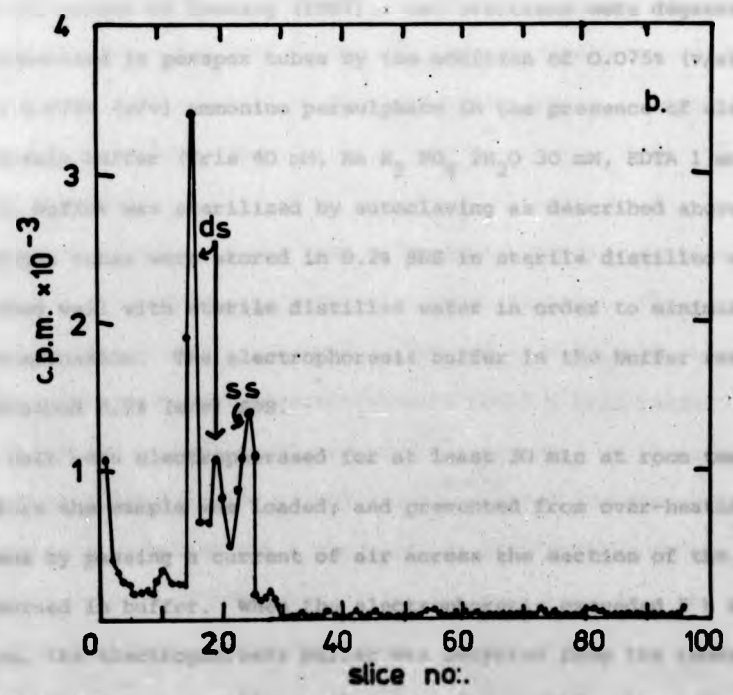


Fig. 2a.
1.5
1.0
0.5
c.p.m. ×10⁻³
ds
ss
28
32
18
45
0
20
40
60
80
100
slice no:.


b.
4
3
2
1
c.p.m. ×10⁻³
ds
ss
0
20
40
60
80
100
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1973) for 1 h at room temperature. Unadsorbed virus was removed, the monolayer washed once in PBS and the original labelled medium returned. Cells were incubated at 37° until cytopathic effects were observed at about 10 h post-infection. RNA was extracted as described above and shown by electrophoresis to contain viral replicative form and single-stranded 36S RNA, together with cellular rRNAs and their precursors (Fig. 2a). The virus specific species were identified by pretreating an identical monolayer of cells with AMD (Fig. 2b) to cut down host cell RNA synthesis. It is noted that there are two species of double-stranded viral specific RNA i.e. replicative form: the reason for this is unknown but has been observed before in this system (S.I. Koliais - personal communication).

Polyacrylamide gel electrophoresis (P.A.G.E) of RNA

Acrylamide and bisacrylamide monomers were recrystallized by the method of Loening (1967). Gel solutions were degassed and polymerized in perspex tubes by the addition of 0.075% (v/v) TEMED and 0.075% (w/v) ammonium persulphate in the presence of electrophoresis buffer (Tris 40 mM, Na H_2 PO_4 $2H_2O$ 30 mM, EDTA 1 mM, pH 7.8, buffer was sterilized by autoclaving as described above). Perspex tubes were stored in 0.2% SDS in sterile distilled water and washed well with sterile distilled water in order to minimize RN'ase contamination. The electrophoresis buffer in the buffer reservoirs contained 0.2% (w/v) SDS.

Gels were electrophoresed for at least 30 min at room temperature before the sample was loaded; and prevented from over-heating at all times by passing a current of air across the section of the gel not immersed in buffer. When the electrophoresis exceeded 5 h in duration, the electrophoresis buffer was recycled from the anode to the cathode through a cooling coil immersed in melting ice. Care was

taken at all times to ensure freedom from RN'ase contamination by wearing gloves and either sterilising all equipment and buffers or treating with SDS.

RNA samples were prepared as follows, 50μl samples, containing 10-50μg RNA dissolved in A buffer, were mixed with sucrose to a final concentration of 5% and bromophenal blue dye to a final concentration of 0.01% and loaded onto the top of the gel under the level of electrophoresis buffer.

After electrophoresis, gels were extruded and scanned at 260 nm in a Gilford spectrophotometer fitted with a linear transport mechanism. Subsequently the gels were frozen, cut into 1 mm slices and dissolved in 0.2 ml of 100 volume H_2O_2 by heating to 90^{o} for 2-4 h. Upon cooling the samples were mixed with a toluene based scintillant (PPO 0.6% (w/v), POPOP 0.073% (w/v), triton X-100 33.3% (v/v)) and radioactivity estimated with a Packard 'Tricarb' liquid scintillation spectrometer. When samples contained both ^{3}H and ^{14}C, radioactivity was determined by the channel-ratio method of Hendler (1964), using a computer programme for the calculations.

PAGE of proteins

The method employed was that of Ornstein (1964), and Davis (1964). Samples for electrophoresis were prepared as follows. Virus or subcellular fractions were suspended in PBS and made 5% with respect to SDS, 5% with respect to β-mercaptoethanol and 5 M with respect to urea. Nuclear samples contained 250μg protein (estimated by the method of Lowry et al., 1951) in 140μl and virus samples contained 10μg protein in 10μl. The samples were boiled for 5 min, made 5% with respect to sucrose and 0.01% with respect to bromophenol blue and then loaded on top of the gel, under the level of electrophoresis buffer.

Gels containing 10% acrylamide and 0.275% bisacrylamide were polymerized in perspex tubes by the addition of 0.075% ammonium persulphate in the presence of 3.3 M urea, 0.15% (w/v) SDS, 0.075% TEMED and 36.3% (w/v) tris buffered to pH 8.4 with HCl. Gels were prerun at 1 ma/gel overnight with a buffer containing 0.3% (w/v) tris, 1.45% (w/v) glycine, 0.1% (w/v) SDS, 20% (w/v) urea and 0.1% (v/v) Na mercaptoacetate in the buffer reservoirs. The sample was then boaded and run in the same buffer at 10 ma/gel for the required time. After electrophoresis the gels were sliced and assayed for radioactivity as described above for RNA.

Preparation of plasma membranes from CEF cells

The method employed was based on that described by Bingham and Burke (1972). All operations were performed at 4°. Cells were washed once in PBS/Ca/Mg and scraped into 25 ml aliquots of membrane lysing buffer, each containing 6 x 10^8 cells. The cell suspension was pelleted by centrifugation at 1500 g for 5 min, resuspended in 5 ml of fresh buffer and homogenized with a stainless steel dounce homogenizer (identical to that used in the nuclear preparations) ten times. Under these conditions 99% of the cells appeared disrupted, without visible damage to the nuclei when examined by phase microscopy. The homogenate was centrifuged at 4,000 g for 10 min and rehomogenized as before in a further 5 ml of membrane lysing buffer and sedimented as above. The supernatant fractions were pooled and recentrifuged at 4,000 g for 10 min. This procedure resulted in 92% of the mitochondria being pelleted but only 29% of the endoplasmic reticulum (ER) sedimented (Bingham and Burke, 1972).

The pooled supernatant fractions were pelleted by centrifugation at 104,000 g and 4° for 2 h and gently resuspended in 4 ml of membrane gradient buffer. The suspension was dialysed for 2 h against two 1 litre changes of membrane gradient buffer at 4° and loaded onto a linear 15 ml

5-25% (w/v) dextran T40 gradient in membrane gradient buffer with a 3 ml cushion of 40% dextran T40 at the bottom. The gradients were centrifuged at 82,000 g for 12 h.

The ER formed a sharp band between the 25% and 40% dextran and the plasma membrane formed a sharp band between the sample layer and the 5% dextran. By phase contrast microscopy, these bands were seen to consist of small, highly refractile vesicles about 1.5μm in diameter. Unfortunately when these experiments were performed, no electron microscopy facilities were available to study the fractions further.

TCA precipitation

After the final ethanol precipitation of the RNA extraction procedure, the samples were dried, dissolved in 0.02 x SSC and mixed with an equal volume of 10% (w/v) TCA in sterile distilled water. The samples were allowed to stand for 30 min on ice before being precipitated onto 25 mm (0.45μ pore size) plain white cellulose acetate filters by suction. The filters were washed by suction 3 times with 1 ml volumes of cold 5% (w/v) TCA and twice with 1 ml volumes of cold ether and finally dried. Radioactivity was estimated by the addition of 5 ml of a toluene based scintillant (0.6% (w/v) PPO, 0.073% (w/v) POPOP).

Ribonuclease digestion

Samples were prepared in 2 x SSC and incubated for 20 min at 37^{o} with 170 units/ml T, RN'ase and 50μg/ml pancreatic RN'ase. The reaction was stopped by the addition of an equal volume of ice-cold 10% TCA. TCA precipitable species were assayed as described above. The procedure digested at least 99% of virion RNA but caused only 2% degradation of double stranded RNA (Avery, 1974).

DN'ase digestion

Samples were shaken for 1 h at room temperature with 10μg/ml of DN'ase in DN'ase buffer and immediately loaded onto gels.

RNA-RNA annealing

Samples were prepared in 0.02 x SSC, boiled in sealed containers for 5 min and incubated at 65° for 2 h in 6 x SSC. This treatment caused maximum reassociation between virion RNA and RNA complementary to virion RNA from infected cells (Avery, 1974).

DNA-RNA annealing

DNA from CEF cells was prepared as follows. 5×10^9 cells were pelleted by centrifugation at 1500 g and resuspended in 20 ml of extraction buffer (NaCl 0.4 M, NaEDTA 0.1 M, pH 8.0) containing 4% (w/v) Na 4-aminosalycilate and 2% (w/v) SDS. An equal volume of water-saturated redistilled phenol containing 0.1% (w/v) 8-OH quinoline was added and shaken at room temperature for 5 min. The mixture was centrifuged at 3,400 g and 4° for 10 min. The phenolic layer was re-extracted with 4 ml of extraction buffer, centrifuged as before and the supernatant fractions pooled. The pooled supernates were re-extracted once more with an equal volume of phenol/8-OH quinoline. An equal volume of redistilled ethanol at -20° was added slowly to form an upper phase. The DNA precipitated at the interface and was spooled out on a glass rod. The DNA was redissolved by stirring gently overnight at room temperature in 10 ml of sterile distilled water. The solution was made up to 0.1 x SSC and incubated for 1 h at 37° with 50 μg/ml of pancreatic RN'ase (the RN'ase was preheated for ½ h at 90° in 0.2 M NaCl to remove any contaminating DN'ase). The solution was made 0.4 M with respect to NaCl and 4% with respect to sodium 4-aminosalicylate and extracted once with phenol/8-OH quinoline as before. Ethanol was added as before and the DNA spooled out and dissolved in 5 ml of sterile distilled water as described above. The DNA solution was fractured by passing it through a French press twice at 2×10^4 p.s.i. This procedure

gives pieces of DNA approximately 500 nucleotides long at a concentration of 3 mg/ml which complied with the criteria of purity stated for the extraction of RNA.

RNA was prepared from purified virions as described above, except that 200μg of highly polymerized yeast tRNA (from Sigma Chemical Co.) was used as carrier.

1 ml of the DNA solution was added to 0.1 ml of the virion RNA solution in 0.02 x SSC and boiled for 5 min. Then 1.1 ml of 12 x SSC was added and the mixture incubated at 65° for 21 h. The TCA precipitable RNA was assayed as previously described both before and after RN'ase digestion.

These conditions were more than adequate to guarantee 50% reannealing of the host DNA, assuming that chick DNA has a kinetic complexity no greater than that of calf thymus DNA (Britten and Kohne, 1966).

SECTION I

EFFECTS OF INFLUENZA VIRUS INFECTION ON THE SYNTHESIS OF HOST CELL rRNA

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EFFECTS OF INFLUENZA VIRUS INFECTION ON THE SYNTHESIS OF HOST CELL rRNA

INTRODUCTION

In this section, current data on the synthesis of nuclear RNA in the eukaryotic cell will be reviewed, along with the effect of influenza virus infection on these processes. The rationale for undertaking the investigation described in this section will be explained, and after a description of the experimental results, the data will be discussed in the light of present knowledge. Finally further possible investigations will be described.

Synthesis of mRNA

The initial precursor of the mRNA species in the uninfected cell is thought to be a species of heterogeneous RNA molecules found in the non-nucleolar fraction of the nucleus, the nucleoplasm (Penman, 1966). This heterogeneous nuclear RNA (hnRNA) has been studied for over a decade (Georgiev, 1964) and is characterized by its rapid synthesis and heterogeneity of size, having a distribution of mol. wt. from 10^5 to 2×10^7 daltons (Yoshikawa et al., 1964; Attardi et al., 1966; Scherrer et al., Warner et al., 1966). HnRNA is produced by transcription of the host DNA, by a DNA dependent RNA polymerase which is sensitive to AMD (Goldberg and Rabinowitz, 1962; Hurwitz et al., 1962) and α-amanitin (Weinberg, 1973). It has few methyl groups or rare bases and is very labile, with at most 10% of the population destined for export to the cytoplasm (Soeiro et al., 1963; Darnell, 1968).

It has been difficult to conclusively prove the precursor product relationship between hnRNA and mRNA as pulse chase experiments are impossible in eukaryotic systems. Treating cells with an AMD "chase" after a pulse label has been tried, but AMD interferes with the processing and transport of RNA molecules as well as stopping transcription (Warner et al., 1966). However hnRNA is probably the precursor to

mRNA since mRNA is synthesised in the nucleus, they are both heterogeneous in size, have the same base composition, contain poly A sequences and recent work has shown that haemoglobin mRNA sequences are found in hnRNA from anaemic duck erythrocytes (Melli and Pemberton, 1972).

The processing of hnRNA to mRNA is at present ill understood, but cleavage appears to occur at double-stranded regions (MacNaughton et al., 1974) and adenylic acid residues are added sequentially at the 3' end of the molecule to form a tail of 150-200 nucleotides in length. Although most mRNA molecules have a poly A tail (Adesnik et al., 1972) which is necessary for their efficient translation (Lee et al., 1971; Mendecki et al., 1972), there are exceptions. Histone messenger, for example, is synthesised in the nucleus, but has no poly A and appears to be transported and translated normally (Adesnik and Darnell, 1972). At present, little is known about the transport of mRNA to the cytoplasm, or even if processing of hnRNA has been completed before it is transported to the cytoplasm.

Synthesis of rRNA

Like mRNA synthesis, the synthesis of rRNA in eukaryotic cells originates by the transcription of a large, precursor molecule from DNA. This DNA is located exclusively in the nucleolus (Brachet, 1942; Bogoroch and Siegel, 1961; Perry et al., 1961) and its transcription is sensitive to AMD, although at a much lower concentration than that of hnRNA (Perry, 1964). However, unlike hnRNA, this initial transcript is recognisable as a discrete species and is cleaved via discrete intermediates to the mature rRNA molecules. The initial transcript is a molecule of 45S, which is rapidly methylated (Perry, 1962) and then cleaved to form a 41S molecule. This is split to form a

32S and a 20S molecule which form the mature 28S rRNA molecule, along with a 7S molecule, and the mature 18S rRNA molecule respectively (Weinberg and Penman, 1970). The discarded portions of the precursor are unmethylated (Weinberg et al., 1967) and the methylated bases are conserved throughout the processing procedure (Weinberg and Penman, 1970). The enzymes responsible for these cleavages are unknown in eukaryotic cells. Although recently it was shown that prokaryotic rRNA is synthesised from a single precursor molecule which is cleaved by RNase III which is specific for double-stranded RNA (Schlessinger et al., 1973).

The above scheme was determined from investigations of HeLa cells, but similar schemes for many protozoan and metazoan eukaryotes have been recently described, although not in so much detail (Grierson et al., 1970; Loening, 1968; Maden, 1971; Perry et al., 1970). In addition it has been shown that in L cells there are two precursors of the 45 S species, i.e. one of the sequence 47S - 46S - 45S. So in mammalian cells, the real precursor may be a very short lived 47S molecule, or one even larger (Tiollais, 1971).

Synthesis of other nuclear RNA species

The synthesis of 7S, 5S and transfer RNA will not be discussed as either their function is uncertain or the effect of virus infection on their synthesis is not known.

Role of rRNA in the synthesis and transport of mRNA

Studies with hybrid cells have shown that the expression of genetic information to form both surface antigens and soluble enzymes is dependent on an intact nucleolus (Harris et al., 1969; Harris and Cook, 1969; Ringertz and Bolund, 1969). Also it has been shown that that both heterokaryons and ordinary cells with irradiated nucleoli (i.e. inactive

nucleoli) are incapable of expressing new genetic information (Sidebottom and Harris, 1969; Harris, 1970). Therefore it appears that a functional nucleolus is essential for the expression of genetic information.

It would be attractive to suggest therefore, that nascent mRNA molecules must attach to nascent ribosomes before they are exported to the cytoplasm. Such charged ribosomes or polysomes could form, or be constituents of, the "informosomes" described by Georgiev (1964).

Effect of influenza virus infection on host RNA synthesis

Skehel and Burke (1969) found that the uridine pools in infected cells rose by 50% during the first 4 h of infection and then declined to 50% of the original level. The initial rise, but not the subsequent decline was found in mock-infected cells and was probably an artefact. The uridine incorporation into macromolecular species followed a similar pattern, but the final level was only 30% of the original. Borland and Mahy (1968) found a similar phenomenon, but their peak level was reached after only 2 h post-infection. However Mahy and his colleagues (1972) found that this rise in RNA synthesis could be divided into 2 phases, the first reaching a maximum at 1½ h post-infection and the second at 3 h post-infection. As the first phase was sensitive to α-amanitin, they concluded that the host DNA-dependent RNA polymerase was involved. As the second phase was insensitive to α-amanitin, they concluded that this was caused by the activity of a virus specific RNA-dependent RNA polymerase. Armstrong and Barry (1974) have recently shown by autoradiography that both these phases of RNA synthesis are confined to the nucleus.

Other RNA viruses have similar effects on host RNA synthesis. NDV causes a cut-off of RNA synthesis related to the virulence of the strain used, but this is evident only 5 h after infection and synthesis drops to only 50% of the original value at most (Wilson, 1968; Alexander

et al., 1973). Infection with SFV also has little effect with a 50% reduction in host RNA synthesis being reached 3 h after infection (Taylor, 1965). Similar results have been obtained with a variety of other viruses (Martin and Kerr, 1968).

However the picornaviruses cause profound alterations in the pattern of RNA synthesis in infected cells. Infection with poliovirus causes a rapid drop in the synthesis of the 45 S rRNA precursor (Darnell et al., 1967) and Weinberg et al., (1967) have shown that aberrant processing of the 45S species occurs, causing a build up of intermediates. The aberrant processing is not due to faulty methylation and occurs in the presence of guanidine indicating that it is mediated by the input virus. However virus infection has no effect on the synthesis of hnRNA and sometimes causes a slight stimulation (Willems and Penman, 1966). This may be a similar phenomenon to that reported by Mahy and his colleagues observed during influenza virus infection.

Effect of virus infection on host DNA-dependent RNA polymerase

In influenza virus infected cells, there is a rise in DNA-dependent RNA polymerase activity which corresponds to the rise in RNA synthesis reported above (Borland and Mahy, 1968; Mahy et al., 1972). The latter authors have shown that the increased activity of the nucleoplasmic polymerase is responsible for the initial rise in RNA synthesis, but this enzyme activity drops back to normal 8 h after infection. The fall in RNA synthesis observed is probably due to the drop in activity of the nucleolar RNA polymerase. The level of this enzyme remains constant for the first 3 h of infection and then drops rapidly to 20% of its initial activity by 9 h post-infection.

The effect of virus infection on host protein synthesis

Infection with FPV virus causes a depression in host cell protein synthesis, but relatively slowly, with the level of synthesis after 8 h

infection 30-40% of the original value (Scholtissek, 1965). This effect was inhibited by cycloheximide and puromycin, but not AMD or UV irradiation. Therefore it was concluded that this inhibition of host protein synthesis is dependent on virus protein synthesis but can make use of the RNA from the infecting virion. One curious aspect of this effect is that although it is sensitive to cycloheximide and puromycin, it is not sensitive to another protein synthesis inhibitor p-fluro-phenylalanine (Long and Burke, 1970).

Similar effects are found in many other virus infections (Martin and Kerr, 1968). NDV infection causes a slow cut-off of protein synthesis, related to the virulence of the strain studied, reaching about 50-60% of the original level of synthesis after 6-8 h of infection (Wilson, 1968; Reeve et al., 1971). SFV infection also causes a slow reduction in host protein synthesis, reaching a level of 10% 7 h after infection (Morser, 1972). Vaccinia virus may be an exception as the factor responsible for inhibiting host protein synthesis appears to be a component of the infecting virus particle (Moss, 1968). Infection with poliovirus appears to cause a much more dramatic effect. There is a rapid decline in protein synthesis, with only 10% of the original level remaining after 5 h infection. This effect appears to be caused by a disaggregation of ribosomes and a specific reloading with viral messengers (Darnell et al., 1967). Mengo virus infection causes very similar effects to that of poliovirus (Fenwick, 1963).

Virus proteins associated with the nucleus in influenza virus infections

The most obvious way for influenza virus to mediate the above effects on macromolecular synthesis is for a virion protein to enter the nucleus and interfere with either the action of the host DNA dependent RNA polymerase or with the transport of the RNA species out of the nucleus. Hoyle and Finter (1957) have shown by using ^{35}S-labelled virus,

that input virion protein does not enter the nucleus of the cell. Therefore these effects must be due to newly synthesised proteins and this would certainly correlate more closely with the time course of the inhibition of macromolecular synthesis.

Breitenfeld and Schaf er (1957) showed that the ribonucleoprotein antigen was found in the nucleus of the infected cell. Using fluorescent antibody, Dimmock (1969) discovered a virus specific antigen, not present in purified virions, concentrated in the nucleolus of the infected cells. Both the nucleocapsid protein (NP) and the non-structural protein (NS_1) have been found in the nuclear fraction of infected cells by other workers (Taylor et al., 1969; Lazarowitz et al., 1971). The NP is concentrated in the nucleoplasm (Krug, 1972) and NS_1 in the nucleolar fraction (Taylor et al., 1970). As NS_1 is also found in the polysome fraction from infected cells (Pons, 1972; Compans, 1973) it has been suggested that NS_1 is incorporated into newly synthesised ribosomes, but Krug (1972) showed that NS_1 is loosely bound to the ribosomes and is not a structural component. Krug and Etkind (1973) found a total of 4 virion specific peptides in the nucleus of infected cells. Along with NP and NS_1 they found another non-structural protein (NS_2) and the largest virion protein P, thought to be the polymerase.

Gross ultrastructural changes in the nucleolus of the infected cell

Concomitant with the appearance of virion proteins in the nucleus, gross ultrastructural changes can be seen by electron microscopy (Compans and Dimmock, 1969). These authors observed dense areas in the nucleolus 4 h after infection, followed by an expansion to give rise to numerous discrete masses by 12 h. It is interesting to compare these changes in structure with those observed after treating the cells with various inhibitors. Treatment with AMD produces segregation of the nucleolus into light and dark areas (Reynolds and Montgomery, 1963) with the forma-

tion of dense spots. Aflatoxin and other related inhibitors have similar effects (Sirtori and Bosisio-Bestetti, 1967). Herpes and vaccinia virus infections also cause nucleolar segregation. Thus, the presence of virus proteins in the nucleus is associated with changes in nucleolar structure, similar to those observed for metabolic inhibitors, therefore virus proteins could be acting in a manner analogous to metabolic inhibitors.

No structural changes in the nucleoplasm of influenza virus infected cells have been recorded, although late in infection, nuclei do become more fragile (see methods section for details).

The data presented above has shown that the cut-off in host cell RNA and protein synthesis during influenza virus infection is associated with the presence of virus specific proteins in the nucleus, one of which (NS_1) is found in both the nucleolus and on polysomes. This association may be related to the appearance of gross ultrastructural changes in the nucleolus. Further, as virus multiplication is not only dependent on an intact nucleus, but is completely inhibited by concentrations of AMD too low to affect the nucleoplasmic DNA-dependent polymerase, it seemed likely that the nucleolus had an important role in virus replication. Therefore it was decided to investigate the pattern of rRNA synthesis in influenza virus infected cells.

RESULTS

Preparation of subcellular fractions

Cytoplasmic fractions were prepared as described in the "Methods" section. As has already been discussed above, it was possible that virus multiplication might have an effect on nucleolar RNA metabolism as a virus specified antigen is found there and virus multiplication is sensitive to low doses of AMD (Barry et al., 1962) which characteristically inhibit only nucleolar RNA synthesis. Therefore attempts to prepare purified nucleoli were made as follows.

Nuclei from infected or uninfected cells were obtained as described in 'Methods' and resuspended in a high salt buffer with a high Mg^{++} concentration (NaCl 0.5M, $MgCl_2$ 0.05 M, Tris 0.01 M pH 7.4) at 1 ml per 10^8 cells in order to prevent aggregation. (This method was never as successful for CEF cells as that reported with HeLa cells - Penman, 1969). The nuclear suspension was homogenized rapidly in a stainless steel Dounce homogeniser (clearance 0.0005") ten times and then incubated at 37° with 10 ug/ml of DN'ase for 1 h. The solution was cooled, made 0.85M with respect to sucrose and centrifuged at 1,500 g for 5 min at 4°. This yields a pellet designated "nuclear membranes". The supernatent fraction was then spun at 3,300 g for 20 min. The pellet was termed the "nucleolar pellet" and the supernate the "nucleoplasmic fraction". All the fractions were examined by phase-contrast microscopy, and light microscopy after staining with azure C and coriophosphine O.

Under phase-contrast conditions the nuclear membrane fraction appeared as large clumps of dense fibrous material packed close together with many unbroken nuclei present; the nucleolar pellet contained small highly refractile particles and long thin fibres of irregular shape and size; and the nucleoplasmic fraction contained the same refractile particles and fibres, but in fewer numbers than in the nucleolar pellet.

When stained by azure C the nuclear membrane fraction was not stained except in the nucleoli of the unbroken nuclei. In the nucleolar pellet and the nucleoplasmic fraction only the refractile particles were stained, but many of the fibres in both fractions had one or two dark staining areas similar in size to the refractile particles. When stained with coriophosphine O, the nuclear membrane fraction was unstained, except for the whole nuclei, the nucleoplasm of which was a bright green. The nucleoli were mostly unstained, although a few had a faint brick-red colouration. In the nucleolar pellet, the refractile particles did not stain, but the fibres stained bright green. The areas previously stained with azure C either did not stain or were a very faint brick-red. The staining pattern in the nucleoplasmic fraction was similar to that of the nucleolar pellet.

Therefore we can assume that the refractile particles are nucleoli as they stain characteristically of RNA and the fibres in the nucleolar pellet and the nucleoplasmic fraction are chromatin fibres with nucleoli still embedded, as they stained characteristically of DNA. The fibres in the nuclear membrane fraction were assumed to be sheets of membranous material as they did not stain with either nucleic acid dyes.

Attempts were made to improve the fractionation by breaking the chromatin strands with prolonged homogenization (30-50 strokes) and vigourous sonication. Either of the methods (or both used together) increased the number of broken nuclei, but made no significant change to either the integrity of the chromatin strands or their distribution in the sub-nuclear fractions. Similarly changes in the conditions of centrifugation had little effect on improving the separation of nucleoli from chromatin. Increasing the concentration of DNase and the incubation time gave no significant improvement in the degree of digestion of the

chromatin.

RNA in the three fractions was prepared and analysed by PAGE (Fig. 3). It is obvious from these profiles that the nucleolar pellet is still heavily contaminated with chromatin as the background on these gels is very high, indicative of large amounts of hnRNA present in this fraction. In fact the overall features of the profile from the nucleolar pellet are indistinguishable from those of the nuclear membrane fraction which includes unbroken nuclei and large pieces of chromatin. The nucleolar supernatent (nucleoplasm) would be expected to contain all the hnRNA as this should be released on DN'ase digestion of the chromatin. However there is less hnRNA in this fraction than in the nucleolar fraction, indicating that most of the chromatin has been undigested and has pelleted with either the nuclear membrane fraction or the nucleolar pellet. The rRNA species present in the nucleolar supernate are presumed to come from free ribosomes in the nucleus.

Therefore, as sub-nuclear fractionation was unsuccessful in yielding a good recovery of pure nucleoli, it was decided to continue the investigation using highly purified nuclear fractions.

Time course of single cycle infection of FPV in CEF cells

In order to determine the time course of infection under the conditions used, the HA titre from infected cells was measured at various times after infection (Fig. 4). There is an initial lag of 1-2 h before HA is detected. The HA titre then rises exponentially to reach a plateau by 6 h and remains at that level for up to 9 h, which is the period under investigation.

Effects on host cell cytoplasmic rRNA

The effect of influenza virus infection on rRNA synthesis was examined by pulsing the cells for 2 h at various times after infection

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Fig. 3d.

Fig. 3. Electrophoresis of RNA from sub-nuclear fractions from 3 x 10^9 CEF cells. Cells were seeded at 1.5 x 10^8 per 11 cm petri dish and labelled for 2 h with 400 μCi 5-^{3}H uridine in Eagle's BHK medium with 10% calf serum dialysed against PBS (3 ml per plate). Subnuclear fractions were prepared and RNA extracted. 50 μl of RNA was electrophoresed on 2.4% gels for 4 h at 6 ma/gel and 50 v. Arrows represent the positions where DNA and rRNA (26S and 18S) were detected by absorbance at 260 nm. Electrophoresis is from left to right. a) RNA from the nuclear membrane fraction, b) RNA from the nucleolar supernatant, c) RNA from the nucleolar pellet.

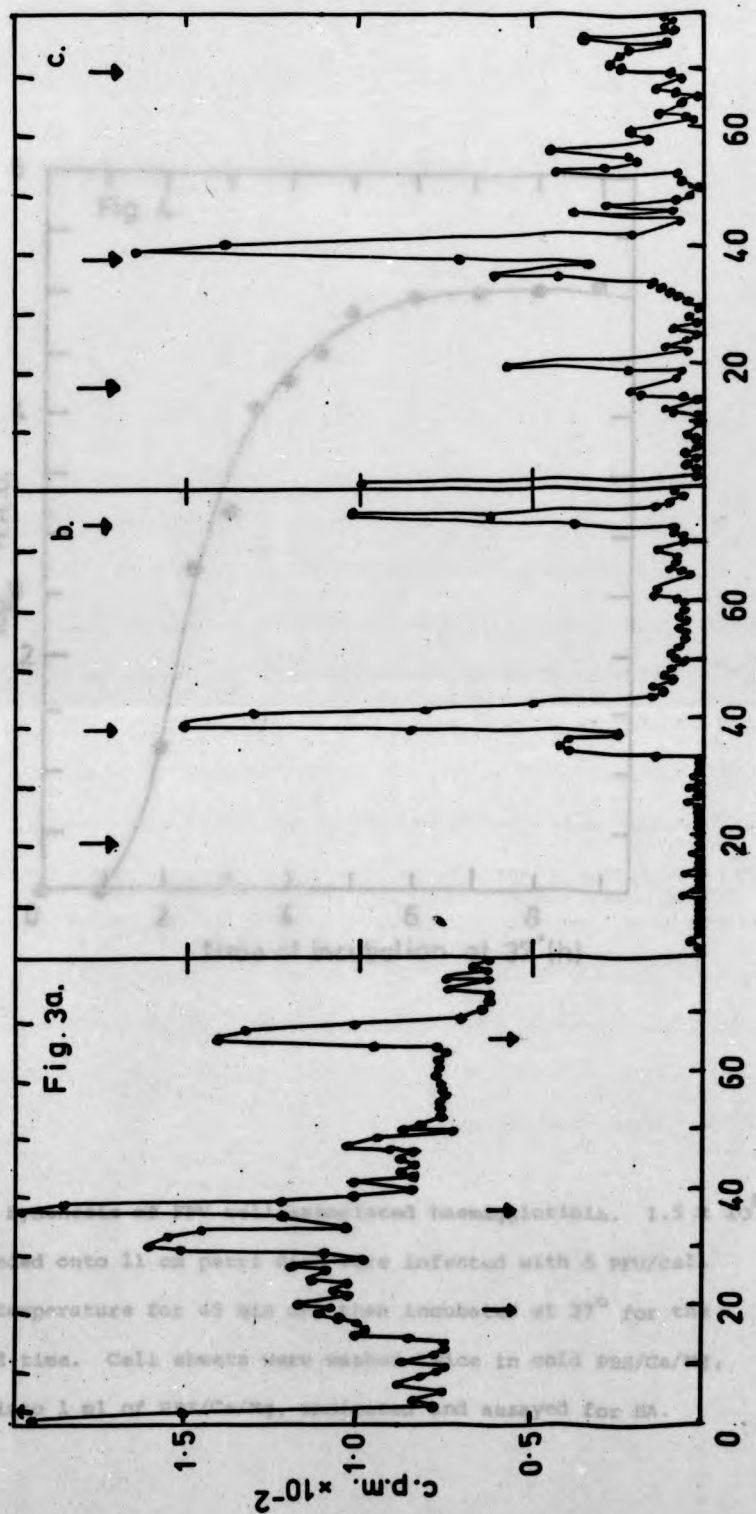


Fig. 3a.

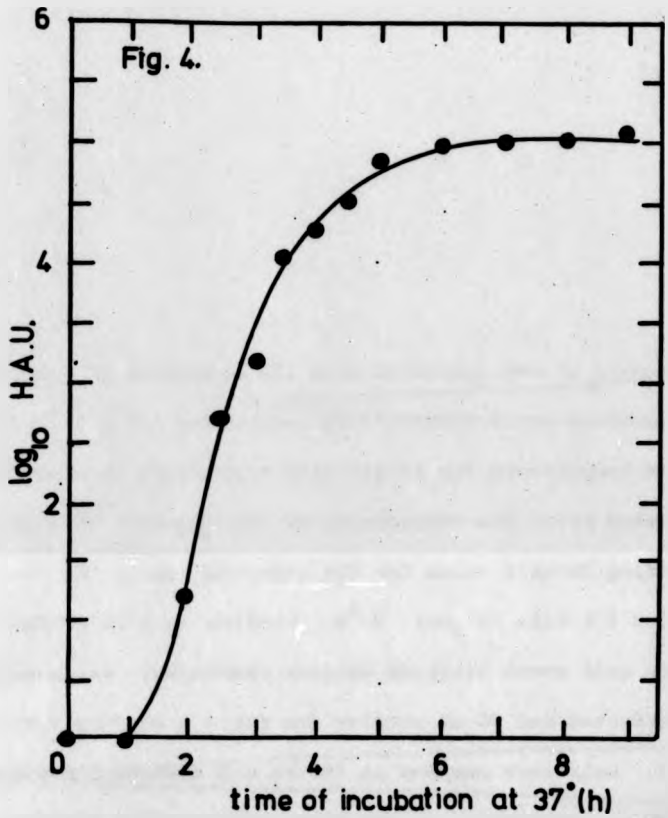
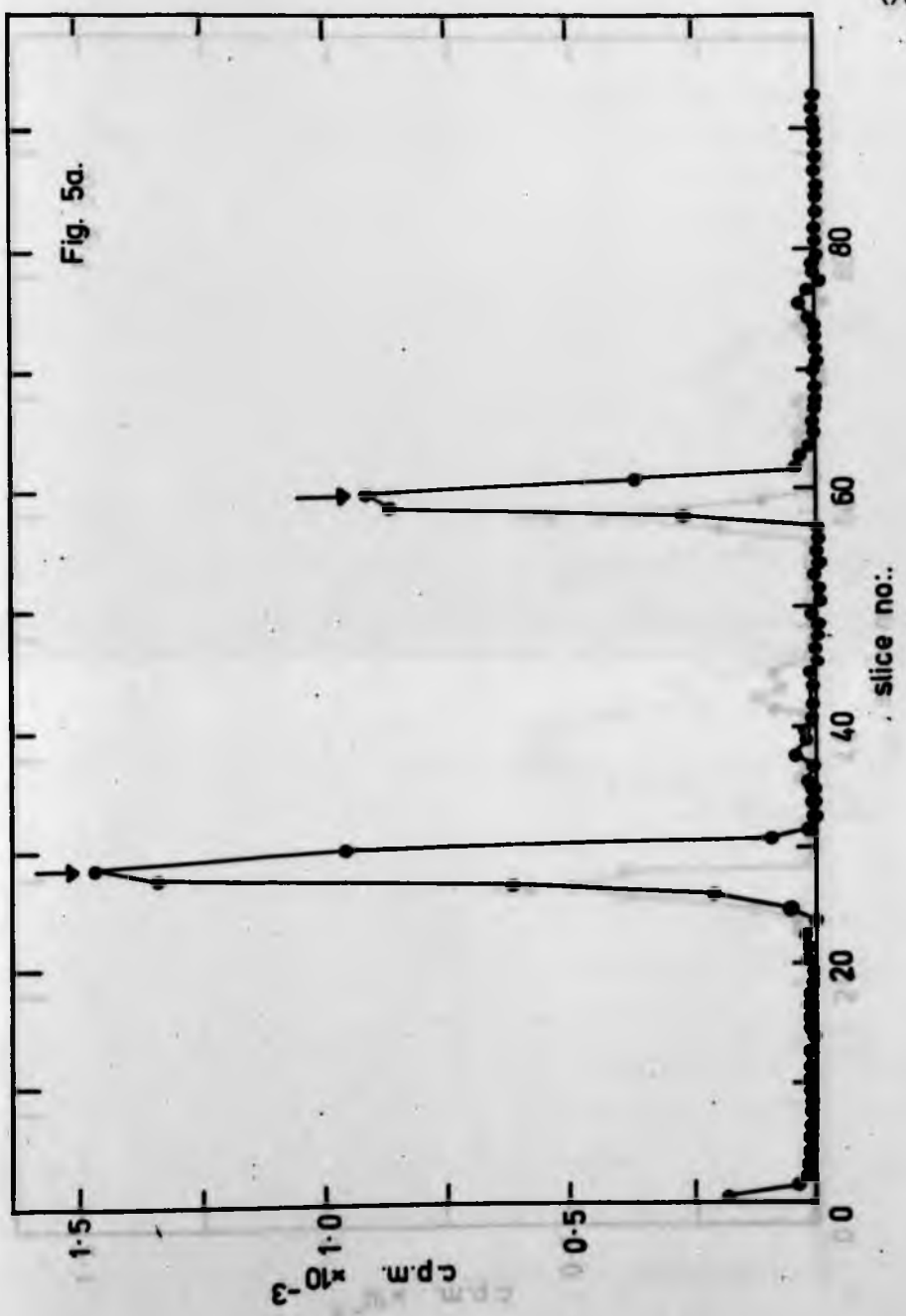


Fig. 4. Synthesis of FPV cell associated haemagglutinin. 1.5×10^8 cells seeded onto 11 cm petri dish were infected with 5 PFU/cell at room temperature for 45 min and then incubated at 37° for the specified time. Cell sheets were washed twice in cold PBS/Ca/Mg, scraped into 1 ml of PBS/Ca/Mg, sonicated and assayed for HA.

Fig. 5. Electrophoresis of RNA extracted from the cytoplasm of uninfected and infected cells. Five 11 cm petri dishes, each containing 1.5 x 10^8 cells were infected at room temperature for 45 min with 5 PFU/cell in 5 ml PBS/Ca/Mg. They were each incubated after the replacement of the inoculum with 20 ml of 199 medium containing 5% calf serum for the required time. The monolayers were then labelled for 2 h with 20 µCi 5-^{3}H uridine in 3 ml of Eagle's BHK medium containing 10% calf serum dialysed against PBS/Ca/Mg. Cells were fractionated, RNA extracted and 50 µl samples run for 4 h on 9 cm 2.4% PAGE at 6 ma/gel and 50 v. Gels were scanned at 260 nm and assayed for radioactivity as described in 'methods'. a) RNA from cultures mock infected with PBS/Ca/Mg. b) RNA from cultures infected with FPV and pulsed 4-6 h after infection. c) RNA from infected cultures pulsed 8-10 h after infection. Arrows show positions of absorbance at 260 nm.



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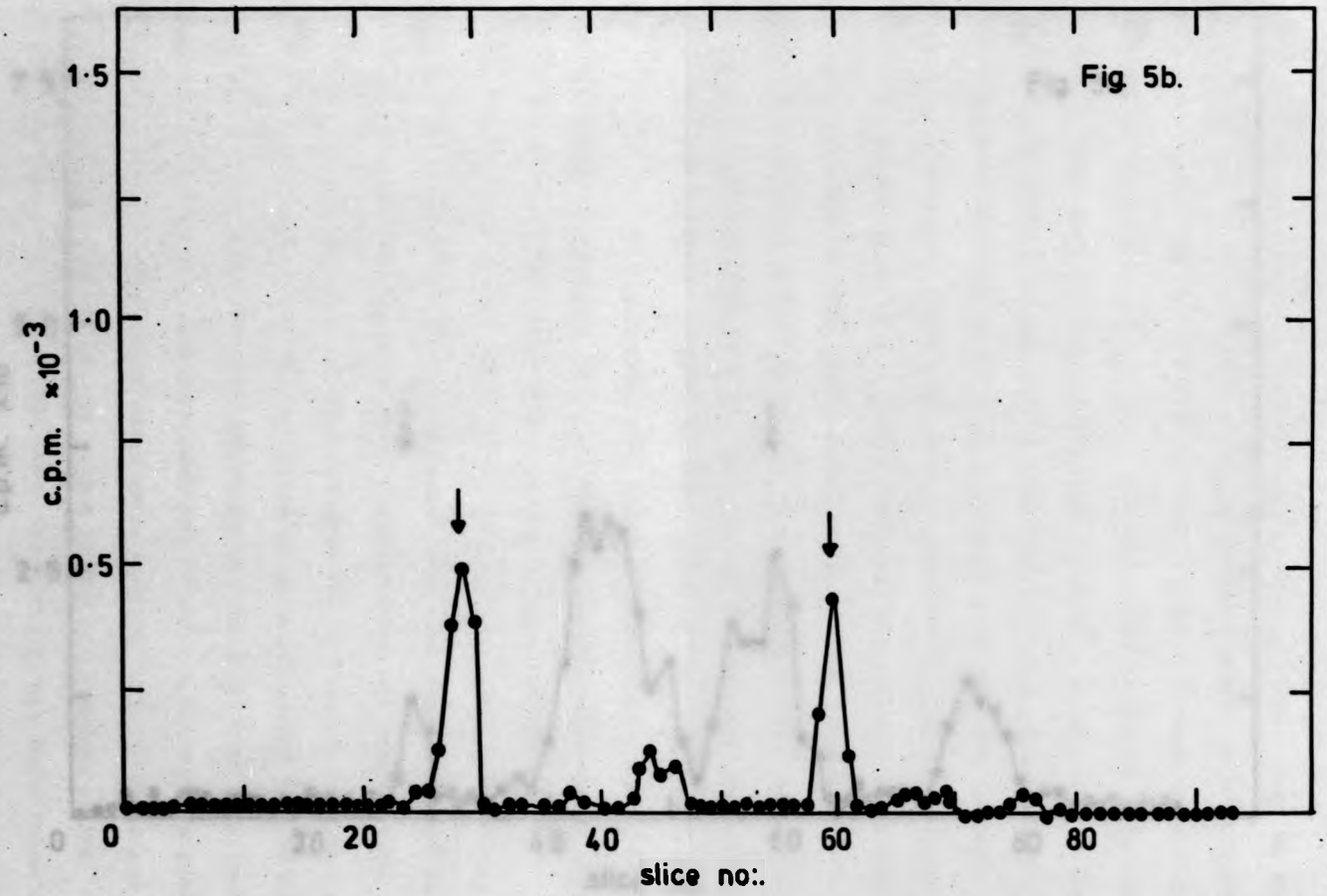
Fig. 5b.
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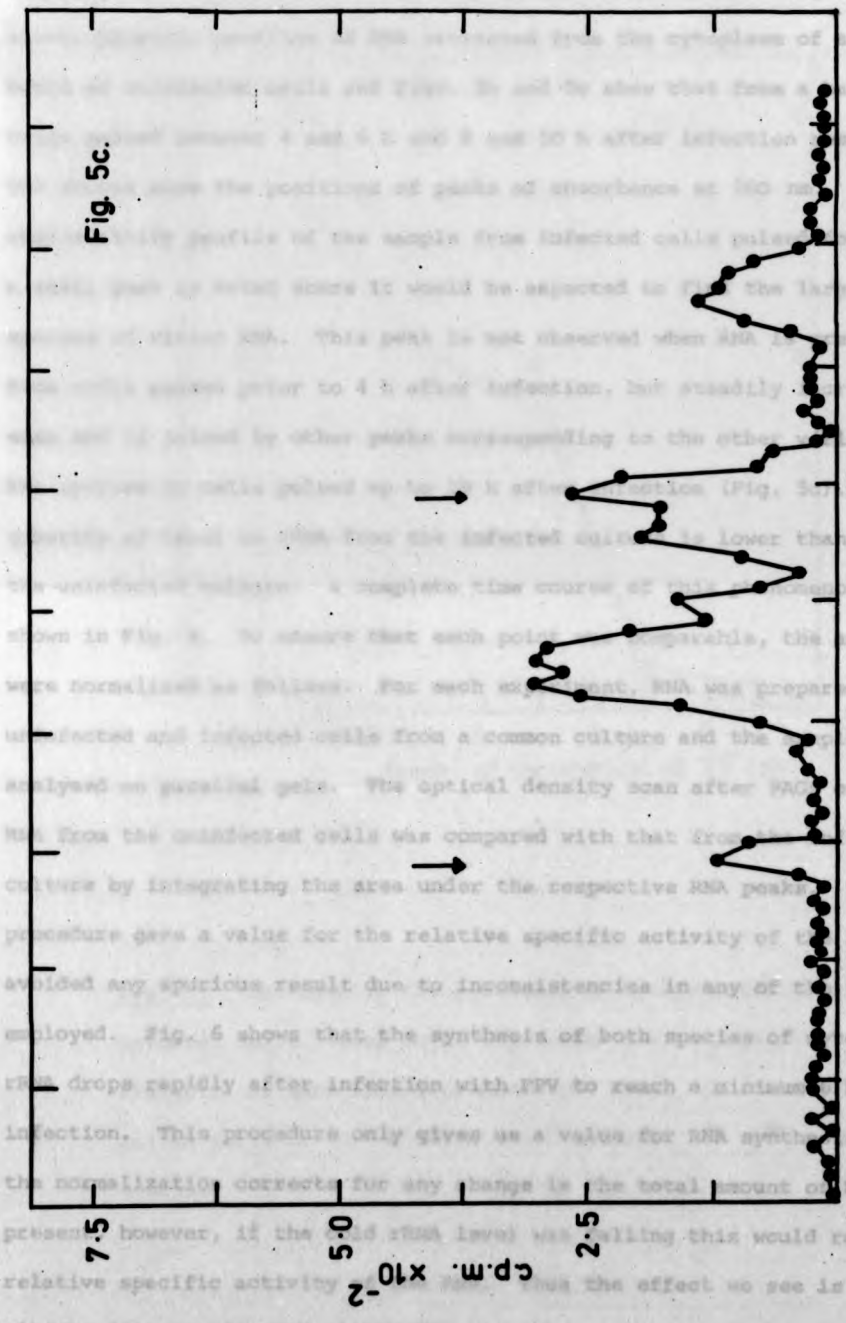
Fig. 5c.
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and comparing the synthesis with that of uninfected cells. Fig. 5a shows electrophoretic profiles of RNA extracted from the cytoplasm of a typical batch of uninfected cells and Figs. 5b and 5c show that from a batch of cells pulsed between 4 and 6 h and 8 and 10 h after infection respectively. The arrows show the positions of peaks of absorbance at 260 nm. On the radioactivity profile of the sample from infected cells pulsed for 4-6 h, a small peak is noted where it would be expected to find the largest species of virion RNA. This peak is not observed when RNA is prepared from cells pulsed prior to 4 h after infection, but steadily increases in size and is joined by other peaks corresponding to the other virion RNA species in cells pulsed up to 10 h after infection (Fig. 5c). The quantity of label in rRNA from the infected culture is lower than that of the uninfected culture; a complete time course of this phenomenon is shown in Fig. 6. To ensure that each point was comparable, the activities were normalized as follows. For each experiment, RNA was prepared from uninfected and infected cells from a common culture and the samples analysed on parallel gels. The optical density scan after PAGE of the RNA from the uninfected cells was compared with that from the infected culture by integrating the area under the respective RNA peaks. This procedure gave a value for the relative specific activity of the rRNA which avoided any spurious result due to inconsistencies in any of the techniques employed. Fig. 6 shows that the synthesis of both species of cytoplasmic rRNA drops rapidly after infection with FPV to reach a minimum 6 h after infection. This procedure only gives us a value for RNA synthesis and the normalization corrects for any change in the total amount of RNA present; however, if the cold rRNA level was falling this would raise the relative specific activity of the RNA. Thus the effect we see is the minimum decrease in rRNA synthesis.

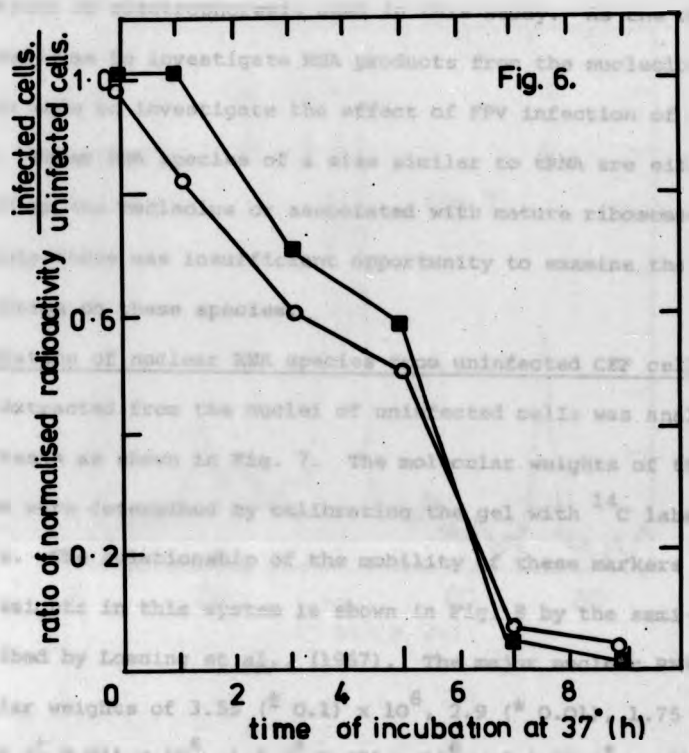

Fig. 6. **Decrease in the synthesis of cytoplasmic rRNA in FPV infected cells. Each point represents the midpoint of a 2 h pulse, the point of zero time represents the level from uninfected cells pulsed for 2 h. o - o 1.55 x 10^6 species, ■ - ■ 0.65 x 10^6 species.**

Other cytoplasmic species such as tRNA are not resolved under the conditions of electrophoresis used in this study. As the object of this study was to investigate RNA products from the nucleolus no attempt was made to investigate the effect of FPV infection of tRNA synthesis. Other RNA species of a size similar to tRNA are either synthesised in the nucleolus or associated with mature ribosomes, but unfortunately there was insufficient opportunity to examine the effect of virus infection on these species.

Characterization of nuclear RNA species from uninfected CEF cells

RNA extracted from the nuclei of uninfected cells was analysed by electrophoresis as shown in Fig. 7. The molecular weights of the major RNA species were determined by calibrating the gel with ^{14}C labelled RNA markers. The relationship of the mobility of these markers to their molecular weights in this system is shown in Fig. 8 by the semi-logarithmic plot described by Loening et al., (1967). The major nuclear RNA species had molecular weights of 3.55 ($\pm$ 0.1) x 10^6, 2.9 ($\pm$ 0.01), 1.75 ($\pm$ 0.05) x 10^6, 1.55 ($\pm$ 0.01) x 10^6, 1.1 ($\pm$ 0.02) x 10^6 and 0.65 ($\pm$ 0.05) x 10^6 daltons. Standard errors from at least 5 experiments are shown in parenthesis. The 1.55 x 10^6 and 0.65 x 10^6 species had the same molecular weights as the species found in the cytoplasm.

The RNA species described above were assumed to be rRNAs and their precursors for the following reasons. The values shown correspond reasonably well to those obtained from avian cells by Perry et al., (1970) and correspond to an overall scheme for rRNA transcription and processing similar to that described for HeLa cells by Weinberg and Penman (1970), except that only the 1.75 x 10^6, 1.55 x 10^6 and 0.65 x 10^6 species were detected by optical density measurements. Although it is impossible to prove this scheme by pulse chase experiments, because of

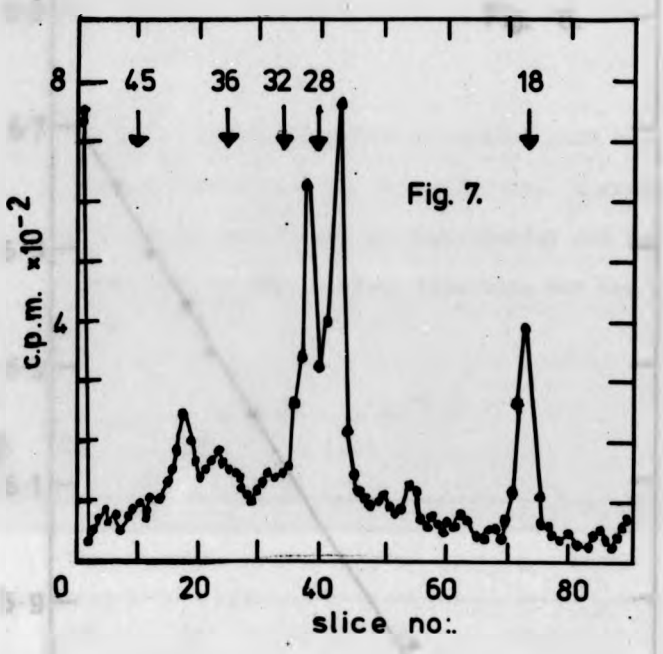

Fig. 7. Electrophoresis of RNA from nuclei of uninfected CEF cells prepared as described in Fig. 5. ^{14}C-labelled RNA from poliovirus-infected HeLa cells was co-run on the same gel. The mobility of this RNA is indicated by arrows surmounted by the respective sedimentation coefficient.

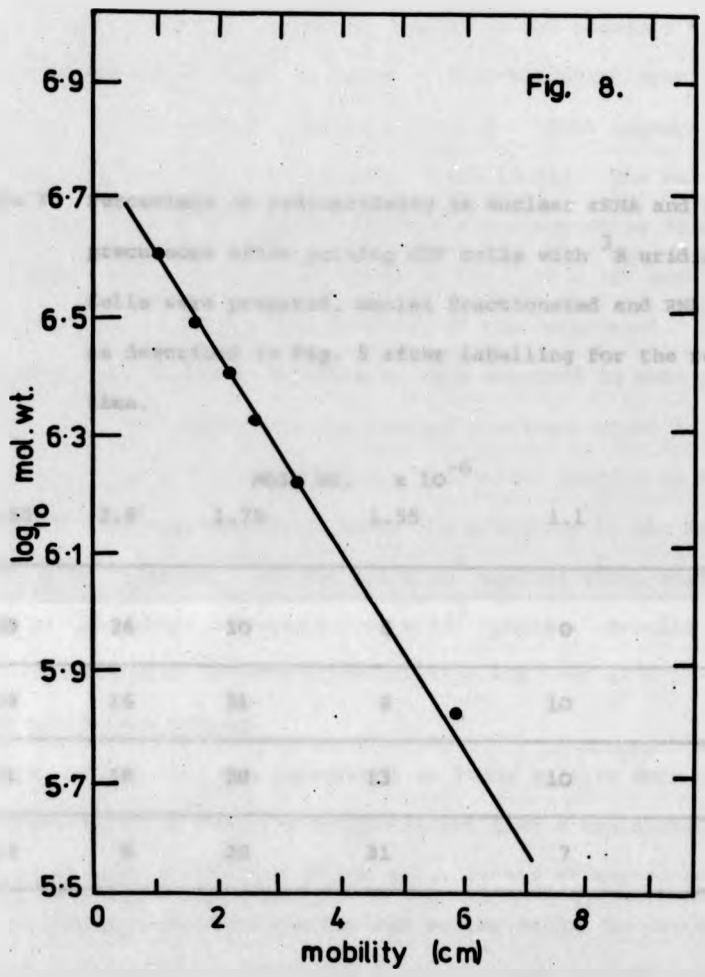

Fig. 8. Calibration of the electrophoresis of single-stranded RNA on 2.4 % PAGE by plotting the mobility of ^{14}C-uridine labelled RNA from poliovirus infected HeLa cells against $\log_{10}$ of their mol. wts. Electrophoresis was for 4 h at 6 ma/gel and 50 v.

Table 3. Percentage of radioactivity in nuclear rRNA and its precursors after pulsing CEF cells with ^{3}H uridine. Cells were prepared, nuclei fractionated and RNA extracted as described in Fig. 5 after labelling for the required time.

Pulse (Min)	Mol. Wt. x 10^{-6} 3.55	2.9	1.75	1.55	1.1	0.65
15	59	26	10	5	0	0
30	29	16	31	8	10	6
45	21	18	28	13	10	10
120	13	8	22	31	7	18

the large uridine pools in eukaryotic cells, it was possible to pulse the cells for different times in order to discover which species were labelled first. This data is shown in Table 3. Label appears preferentially in the 3.55 x 10^6 and 2.9 x 10^6 species after 15 min. The relative amount of label in the 1.75 x 10^6 species reaches a maximum after 30 mins and then declines. The proportion of label in the 1.55 x 10^6 and the 0.65 x 10^6 species rises throughout the duration of the experiment. This pattern is consistent with a system (similar to that observed in HeLa cells) in which the 3.55 x 10^6 species is the initial precursor which is then cleaved to form the 2.9 x 10^6 species. The latter species is divided into the 1.75 x 10^6 species which forms the precursor to the mature ribosomal 1.55 x 10^6 species; and the 1.1 x 10^6 species which forms the precursor to the mature ribosomal 0.65 x 10^6 species. Results from later experiments with ^{3}H methionine labelled RNA were also consistent with this maturation scheme.

It is noticed that the background on these gels of nuclear RNA is much higher than that of comparable RNA from the cytoplasm and that a considerable amount of label is located at the top of the gel. It was attempted to resolve this heterogenous background and the RNA at the origin by running on 20 cm gels at 6 ma/gel and 100 v for 8 h or by running on 9 cm 2.0% gels for 4 h at 6 ma/gel and 50 v.

On the 20 cm 2.4% gels no improved resolution was observed, although on the 2.0% gels all of the label moved into the gel but still formed a heterogenous profile. Therefore it was assumed that this RNA corresponded to the hnRNA which is thought to be the precursor of mRNA.

Effect of FPV infection on nuclear rRNA synthesis

In order to determine the origin of the effect of FPV infection on the decrease of newly synthesised rRNA in the cytoplasm, the maturation

of rRNA in the nucleus was examined during the first 10 h of infection. Electrophoretic profiles obtained by pulsing cells with ^{3}H uridine at various times after infection are shown in Fig. 9. There is a decline in the synthesis of rRNA and its precursors, but the decline of the synthesis of the initial transcript and its immediate product occurs later and to a lesser extent than that of the mature rRNA species and their immediate precursors. This effect is clarified in Fig. 10 where the synthesis is compared to that in parallel uninfected cultures. Unfortunately it was difficult to obtain reliable data on the 1.1×10^6 species due to the small amounts present, but where it was detected its activity followed that of the 1.75×10^6 species. This preferential decrease of the mature rRNAs and their immediate precursors can be explained by an alteration in the processing mechanism of the cell so that non-specific breakdown of the initial transcript occurs instead of a specific cleavage. This aberrant processing is thought to involve the degradation of the transcript to species of mol. wt. less than 0.5×10^6 or to cause a random cleavage as no discrete new species were observed at any one time during infection.

The slow decline in synthesis of the critical precursors could have been due to the decrease in the size of soluble uridine pools as described by Skehel and Burke (1969). This possibility was tested as follows. 1.5×10^8 cells were infected and labelled as described in Fig. 5. Monolayers were washed 3 times in PBS/Ca/Mg and then scraped into 5 ml of PBS/Ca/Mg and sonicated. 5 ml of 10% TCA was added, the cells left to stand on ice for 45 min and then centrifuged at 1,000 g for 5 min at 4°. The supernate was removed and 1 ml of 5% TCA added. The precipitate was removed by centrifugation as before. The supernates were pooled and 100 µl aliquots assayed for radioactivity in a triton-

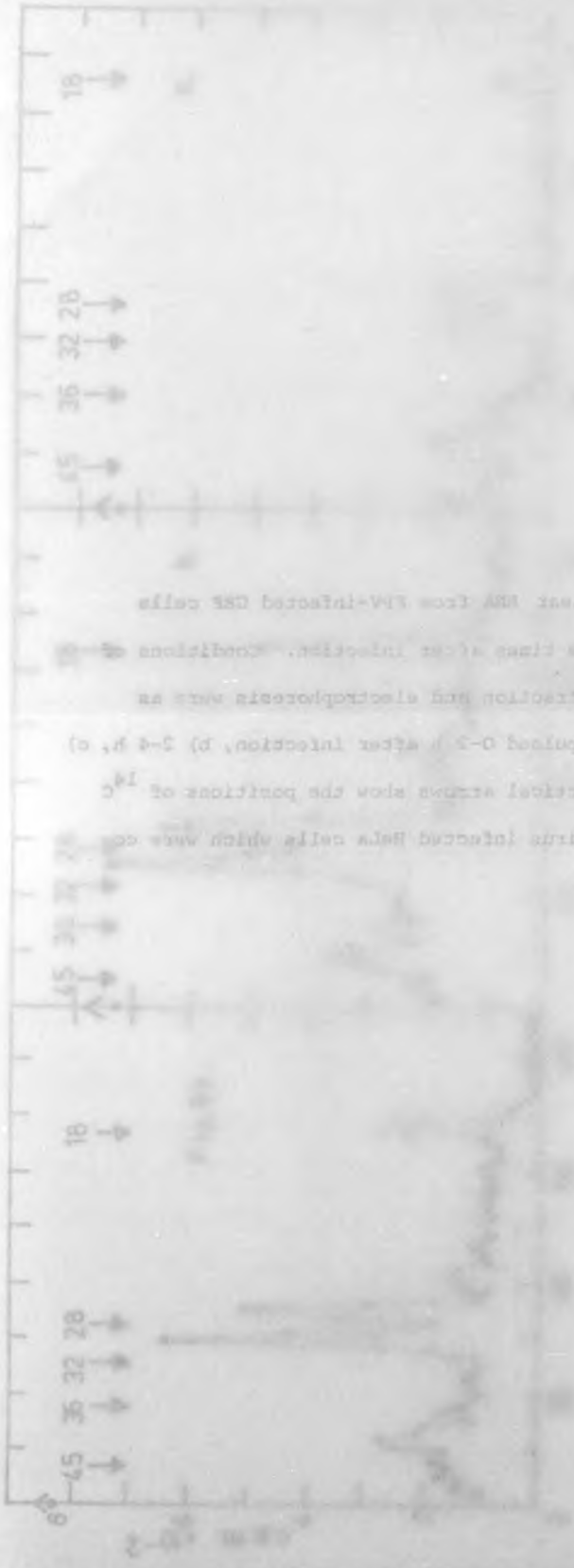
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Fig. 9. Electrophoresis of nuclear RNA from FPV-infected CEF cells pulsed with ^{3}H uridine at various times after infection. Conditions of infection, fractionation, RNA extraction and electrophoresis were as described in Fig. 5. a) cells pulsed 0-2 h after infection, b) 2-4 h, c) 4-6 h, d) 6-8 h, e) 8-10 h. Vertical arrows show the positions of ^{14}C uridine labelled RNA from poliovirus infected HeLa cells which were co-electrophoresed.

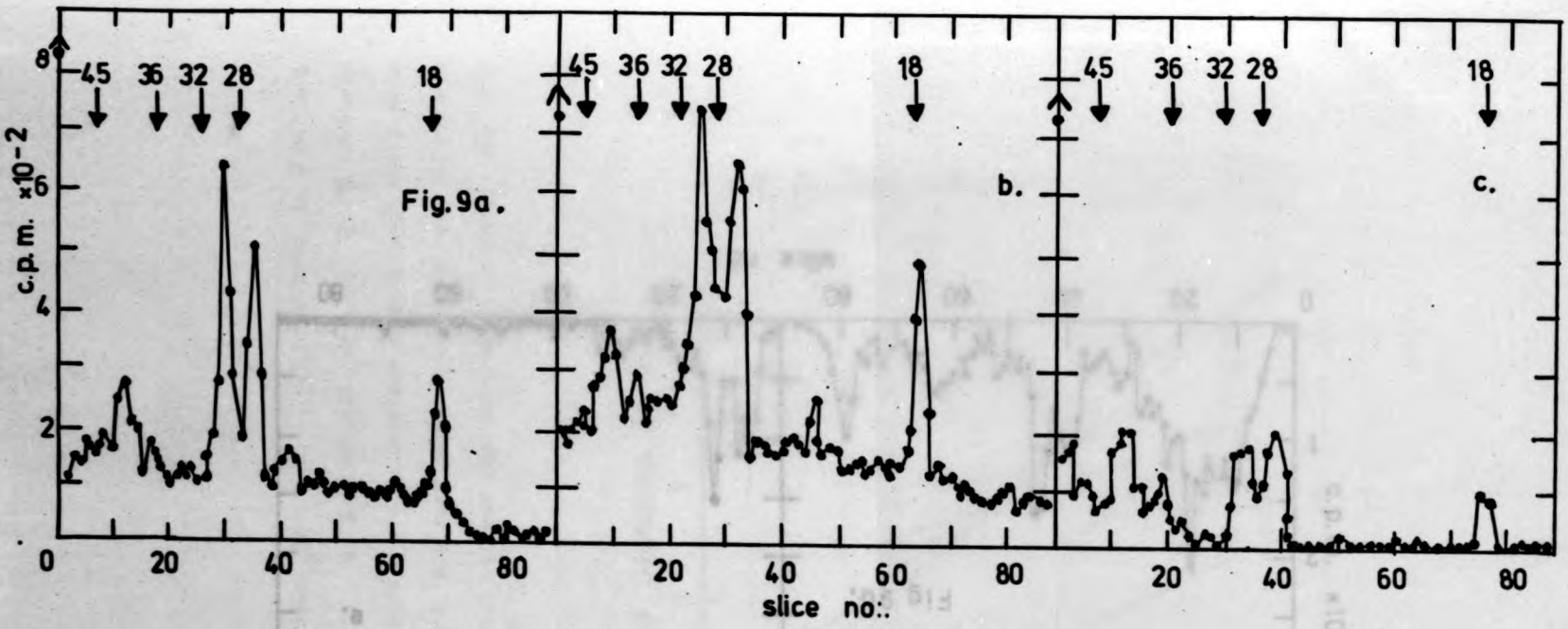
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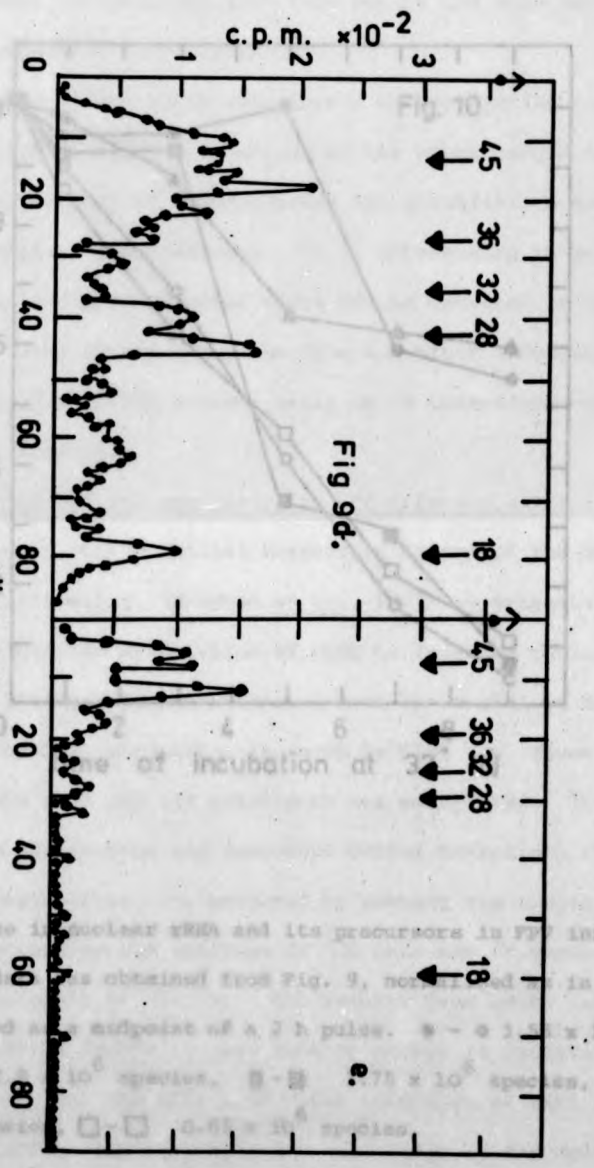
Fig. 9a.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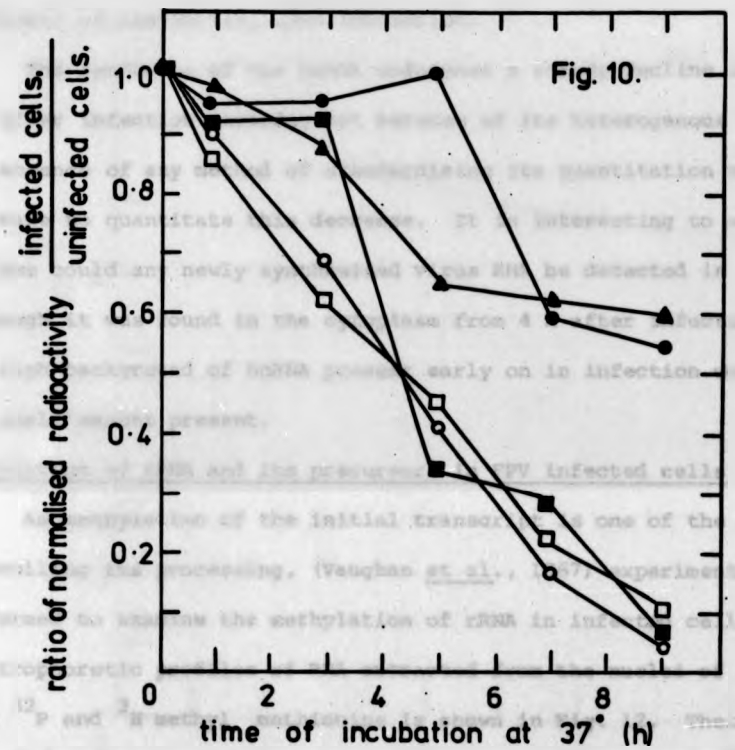

Fig. 10. Decrease in nuclear rRNA and its precursors in FPV infected CEF cells. The data was obtained from Fig. 9, normalised as in Fig. 6 and plotted as a midpoint of a 2 h pulse. ● - ● 3.55 x 10^6 species, ▲- ▲ 2.9 x 10^6 species, ■-■ 1.75 x 10^6 species, O-O1.55 x 10^6 species, □-□ 0.65 x 10^6 species.

toluene based scintillant. Fig. 11 shows that the soluble uridine pools in these cells fall at about the same time and to the same extent as the synthesis of the initial rRNA transcript.

The synthesis of the hnRNA undergoes a steady decline as well from 4 h after infection onwards, but because of its heterogenous nature and the absence of any method of standardizing its quantitation no attempt was made to quantitate this decrease. It is interesting to note that at no time could any newly synthesised virus RNA be detected in the nucleus although it was found in the cytoplasm from 4 h after infection. However the high background of hnRNA present early on in infection would mask any small amount present.

Methylation of rRNA and its precursors in FPV infected cells

As methylation of the initial transcript is one of the factors controlling its processing, (Vaughan et al., 1967) experiments were performed to examine the methylation of rRNA in infected cells. The electrophoretic profiles of RNA extracted from the nuclei of cells labelled with ^{32}P and ^{3}H methyl methionine is shown in Fig. 12. These profiles show that only the rRNA and its precursors are methylated. In order to determine if the methylation had decreased during infection, the specific activity of the methylation was measured by summing the counts in each of the peaks, correcting for spillage of ^{32}P into the ^{3}H channel (4.5%) and measuring the ratio of $^{3}H:^{32}P$. The results from cells labelled at 4-6 h post-infection (Table 4) show that no change in methylation occurred at a time when the effect of virus infection of rRNA processing was well established. Theoretically the multiples of the molecular weight and the $^{3}H/^{32}P$ ratio of the precursors should equal the sum of the multiples from the immediate products (Weinberg and Penman, 1970), indicating

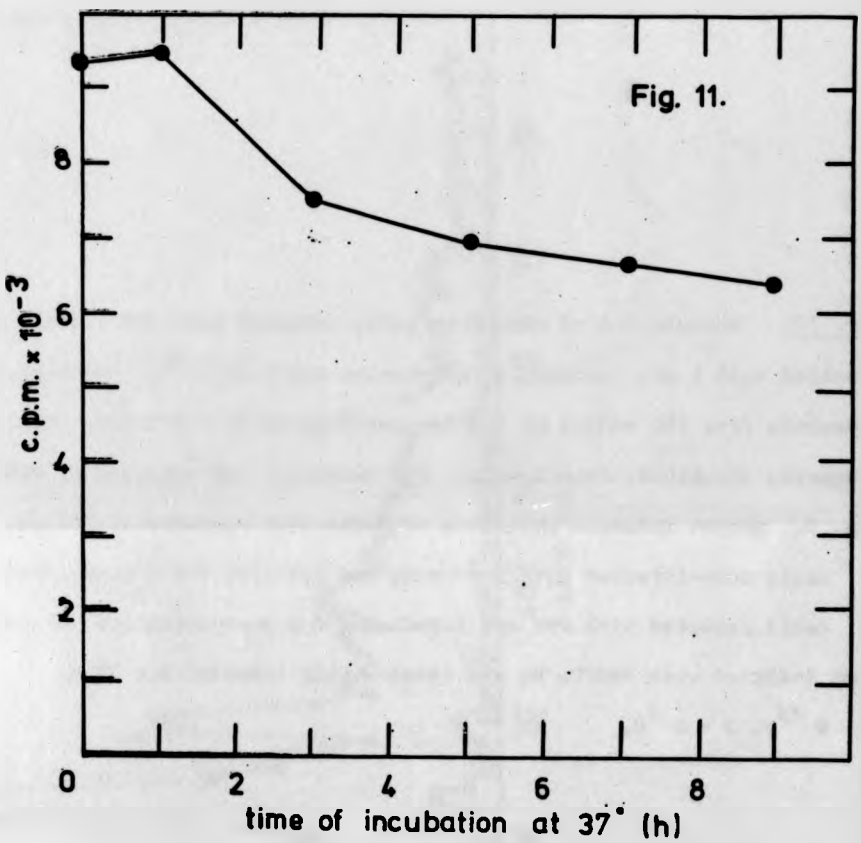

Fig. 11. Effect of FPV infection on the soluble uridine pools in CEF cells. Each point represents the midpoint of a 2 h pulse. The value at zero time after infection represents the value obtained from cells mock-infected with PBS/ca/Mg.

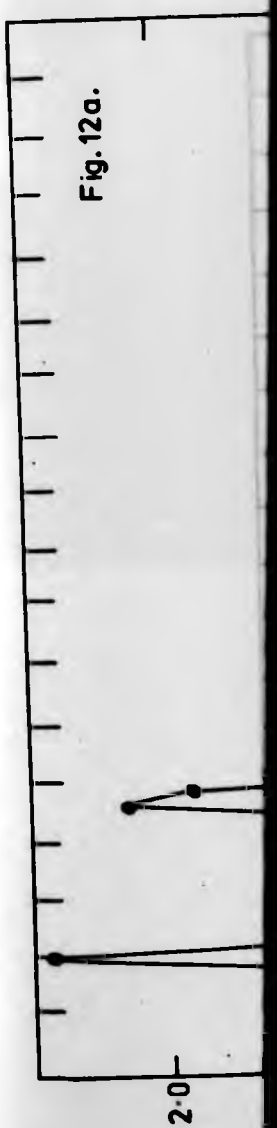

Fig. 12. Methylation of rRNA from cells infected with FPV. Cells were labelled with 1 mCi methyl-^{3}H methionine and 1 mCi ^{32}P orthophosphate in phosphate free 199 medium at the desired time after infection. Cells were prepared, incubated, fractionated, RNA extracted and analysed by PAGE as in Fig. 5. Arrows indicate positions of peaks of absorbance at 260 nm.
a) cells mock-infected with PBS/Ca/mg and labelled 4-6 h post-infection, b) cells infected with FPV and labelled 4-6 h post-infection, c) cells mock infected with PBS/Ca/Mg and continuously labelled for 24 h.
● - ● ^{32}P, o - o ^{3}H.

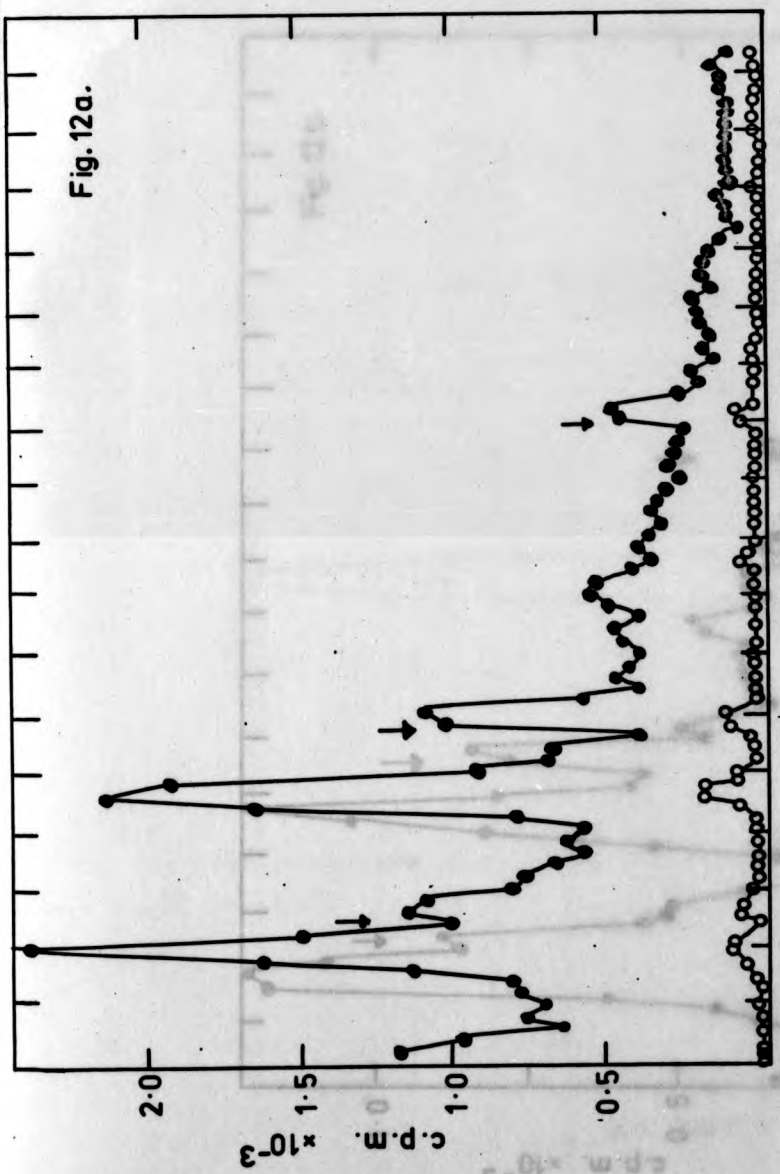

Fig. 12a.

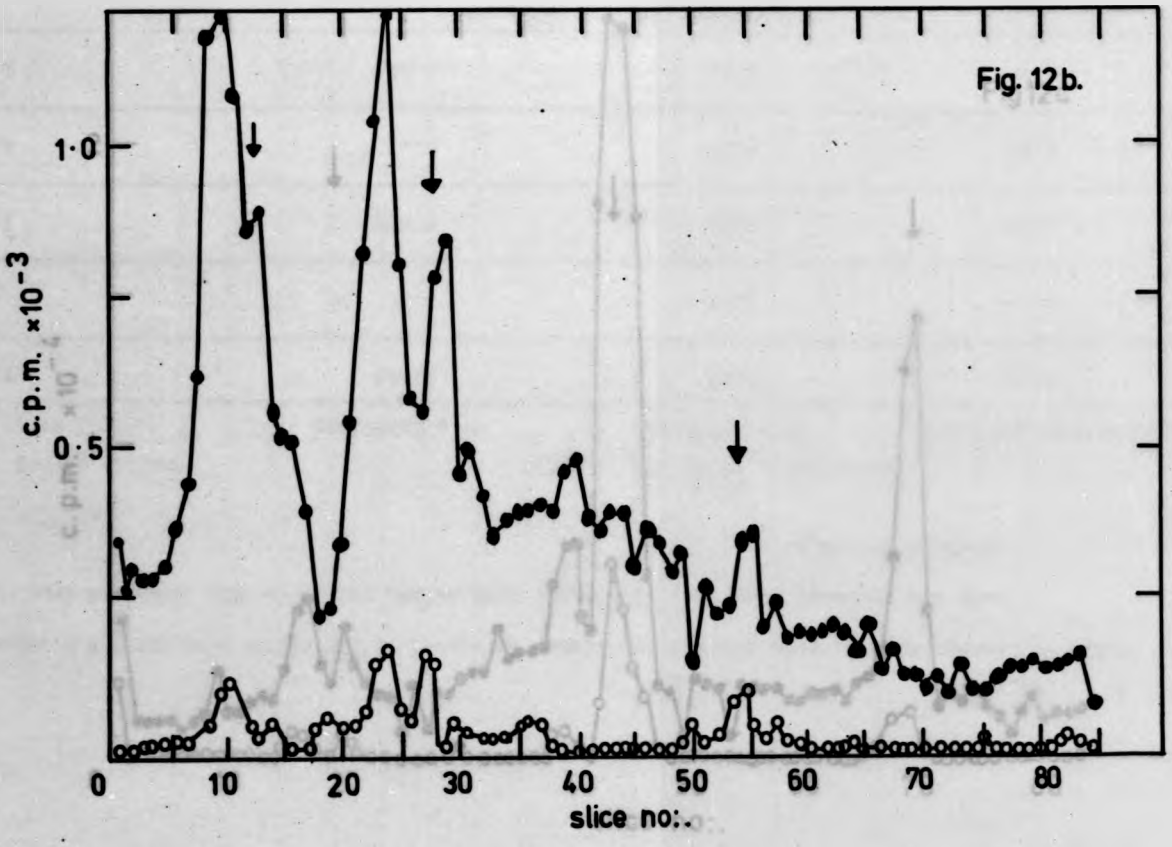

Fig. 12b.

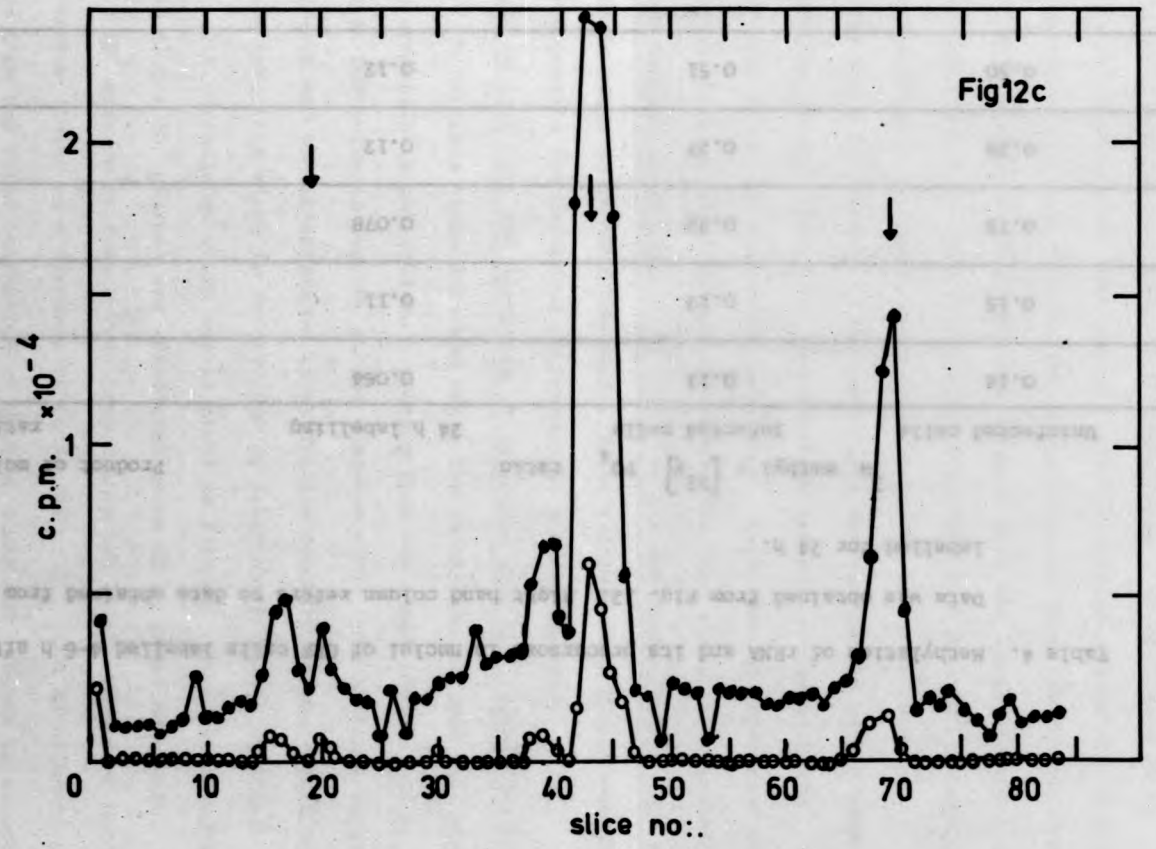
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Table 4. Methylation of rRNA and its precursors in nuclei of CEF cells labelled 4-6 h after infection. Data was obtained from Fig. 12. Right hand column refers to data obtained from cells continuously labelled for 24 h.

Mol. wt. ($\times 10^{-6}$)	^{3}H methyl : $[^{32}P]$ PO_4 ratio			Product of mol. wt. and ^{3}H:^{22}P ratio $\times 10^{-4}$
	Uninfected cells	Infected cells	24 h labelling	
3.55	0.14	0.13	0.064	5.0
2.9	0.15	0.12	0.11	-
1.75	0.19	0.22	0.078	3.8
1.55	0.29	0.27	0.12	4.1
0.65	0.50	0.51	0.12	1.4

a true precursor-product relationship. This was not the case. However, if the cells are labelled to equilibrium in this case 24 h, (Table 4 - last column) the product from the initial transcript (5.0×10^4) is approximately equal to the sum of the products from the mature rRNA species ($4.1 + 1.4 \times 10^4$. Also the product from the large rRNA (4.1×10^4) approximates to that from its immediate precursor (3.8×10^4). Unfortunately it was difficult to obtain reliable data on the 2.9×10^6 species and the 1.1×10^6 species. The above data are consistent with a rRNA maturation scheme similar to that outlined for HeLa cells in which methylated bases are conserved throughout. This type of experiment provides no information on the accuracy of methylation, but since the processing of the initial transcript (3.55×10^6) to its immediate product (2.9×10^6) proceeds normally in infected cells, it is unlikely that faulty methylation is responsible for defective processing to smaller species.

Effect on host cell RNA synthesis of infection with U.V. irradiated FPV

In vaccinia virus infected cells the inhibition of host cell protein synthesis is mediated by a protein from the input virion. If a similar mechanism is present in FPV infected cells, the decrease in rRNA synthesis and the interference in maturation described above should occur if the infecting virus is inactivated by irradiation with U.V. light.

U.V. irradiated virus was prepared as follows. 10 ml of allantoic fluid (3.0×10^9 pfu/ml) was irradiated in an 11 cm petri dish with 6.1×10^3 ergs/cm^2/s from a U.V. lamp calibrated by the method of Hatchard and Parker (1956) at a distance of 15 cm (Fig. 13). 0.1 ml aliquots were taken from the virus suspension at various times and allowed to adsorb for 45 min at room temperature onto 3 cm plastic petri dishes containing 9.0×10^6 cells. The monolayers were incubated overnight at 37° in 199 medium and the cell associated HA production assayed. Using the standard

determined by Gandhi and Burke (1970) the time of maximum interferon production was estimated and the virus stock was irradiated for that period (see Fig. 13). Interferon production was used as a standard as this is a function which is dependent on the integrity of FPV as indicated by virion polymerase activity, but not on infectivity; thus paralleling the situation with vaccinia virus as closely as possible.

Fig. 14 shows the electrophoretic profile of RNA extracted from the cytoplasm and nucleus of cells infected with U.V. irradiated FPV. Table 5 shows the ratio of synthesis of rRNA and its precursors in nuclei and cytoplasm from cells infected with U.V. irradiated FPV to that in parallel uninfected cultures. No effect was observed on the synthesis of the rRNA precursors or on their subsequent maturation to mature rRNA in the nucleus. Neither was any effect on the appearance of rRNA in the cytoplasm observed.

Ribonuclease resistant RNA species in infected and uninfected CEF cells

During the RNA extraction procedures, no incubation with DNase is performed, so it is possible that although the preparations are labelled with uridine (which is not present in DNA), some of the label could be incorporated into DNA via intermediate metabolic pathways. Treating the RNA preparations with a mixture of T_1 and pancreatic RN'ases would remove all single stranded species (except poly A tracts) and expose double-stranded RNA or DNA labelled with uridine. Fig. 15 shows the electrophoretic profiles of RNase treated RNA preparations from the cytoplasm and nuclei of uninfected cells and cells pulsed 8-10 h after infection with FPV. In both the uninfected and infected cytoplasmic fractions no species of RNase resistant RNA could be detected either by radioactivity or optical density measurements except at the bottom of the gel in the region of the marker dye. These species were thus of very small molecular weight and assumed to be the breakdown products of the enzyme digestion.

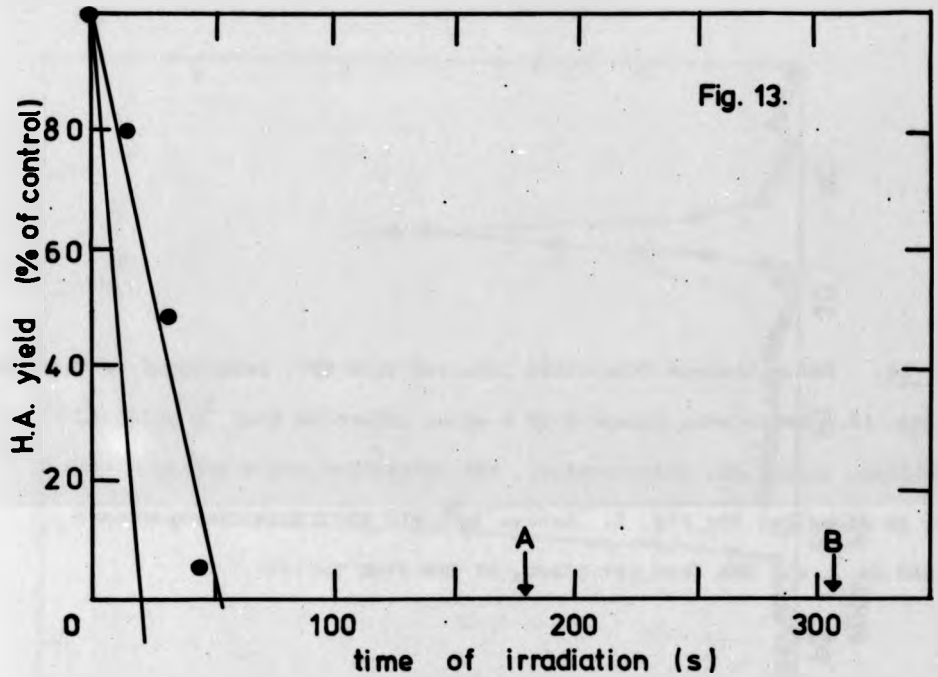

Fig. 13. U.V.-irradiation of FPV. (———) irradiation of FPV in the system described by Gandhi and Burke (1970), (● - ●) irradiation of FPV in the system described in the text. 'A' represents the time of irradiation to produce the maximum amount of interferon in the Gandhi and Burke system. 'B' represents the estimated time of irradiation to produce the maximum amount of interferon in the system described in the text.

Fig. 14. RNA extracted from cells infected with FPV, irradiated as described in Fig. 13. Cells were pulsed 8-10 h after infection with ^{3}H uridine. Labelling, infection, fractionation, RNA extraction and electrophoresis were as described for Fig. 5. Arrows indicate positions of absorbance at 260 nm . a) RNA from cytoplasm, b) RNA from nuclei.

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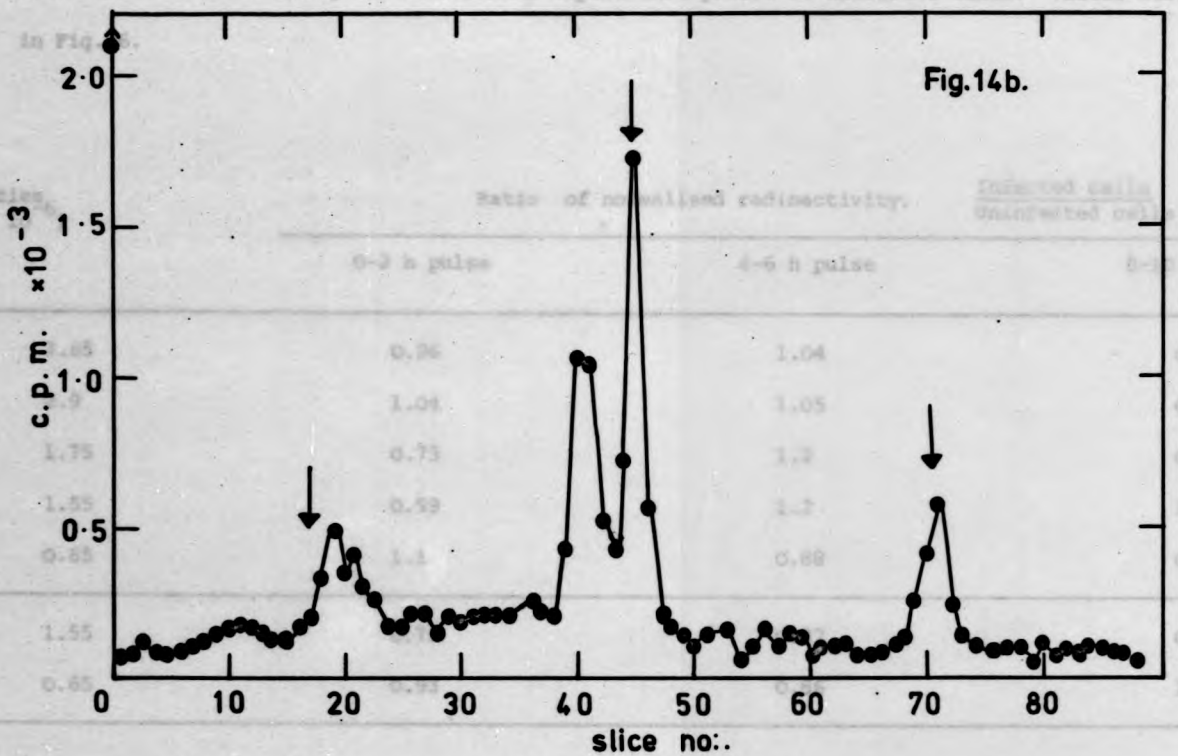
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Table 5. Effect on the synthesis of rRNA and its precursors by infection of CEF with U.V. irradiated FPV. Data was obtained from Fig. 14 and similar experiments pulsed at different times. Values were normalised as in Fig. 6.

RNA species (mol. wt. x 10^{-6})		Ratio of normalised radioactivity. $\frac{\text{Infected cells}}{\text{Uninfected cells}}$		
		0-2 h pulse	4-6 h pulse	8-10 h pulse
Nuclear	3.55	0.96	1.04	0.86
	2.9	1.04	1.05	0.92
	1.75	0.73	1.2	0.90
	1.55	0.99	1.2	1.06
	0.65	1.1	0.88	0.74
Cytoplasmic	1.55	0.73	0.82	0.76
	0.65	0.93	0.86	1.1

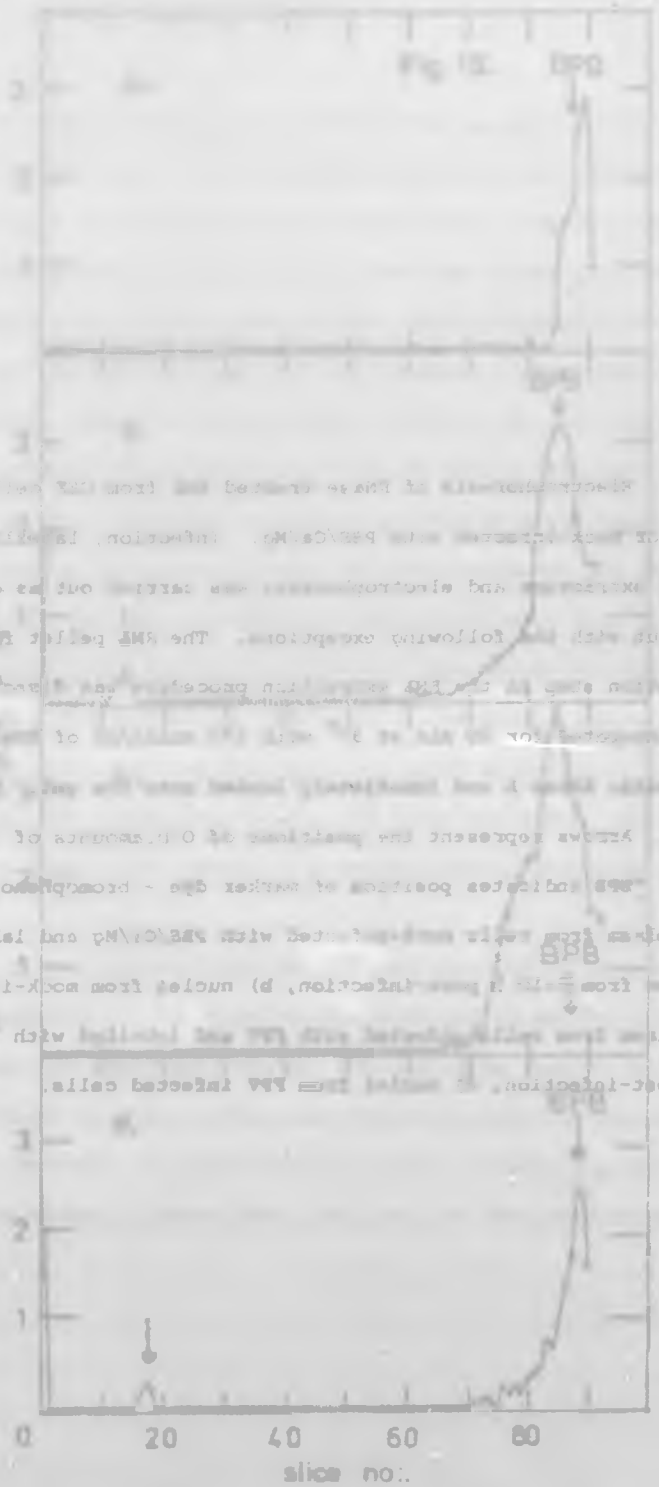
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Fig. 15. Electrophoresis of RNase treated RNA from CEF cells infected with FPV or mock-infected with PBS/Ca/Mg. Infection, labelling, fractionation, RNA extraction and electrophoresis was carried out as described in Fig. 5, but with the following exceptions. The RNA pellet from the ethanol precipitation step in the RNA extraction procedure was dissolved in 2 x SSC and incubated for 20 min at 37° with 170 units/ml of RNase T, and 50 µg/ml of pancreatic RNase A and immediately loaded onto the gel. The PAGE was for 3½ h. Arrows represent the positions of O.D. amounts of RNase resistant species. 'BPB indicates position of marker dye - bromophenol blue.
a) cytoplasm from cells mock-infected with PBS/Ca/Mg and labelled with ^{3}H uridine from 8-10 h post-infection, b) nuclei from mock-infected cells, c) cytoplasm from cells infected with FPV and labelled with ^{3}H uridine from 8-10 h post-infection, d) nuclei from FPV infected cells.

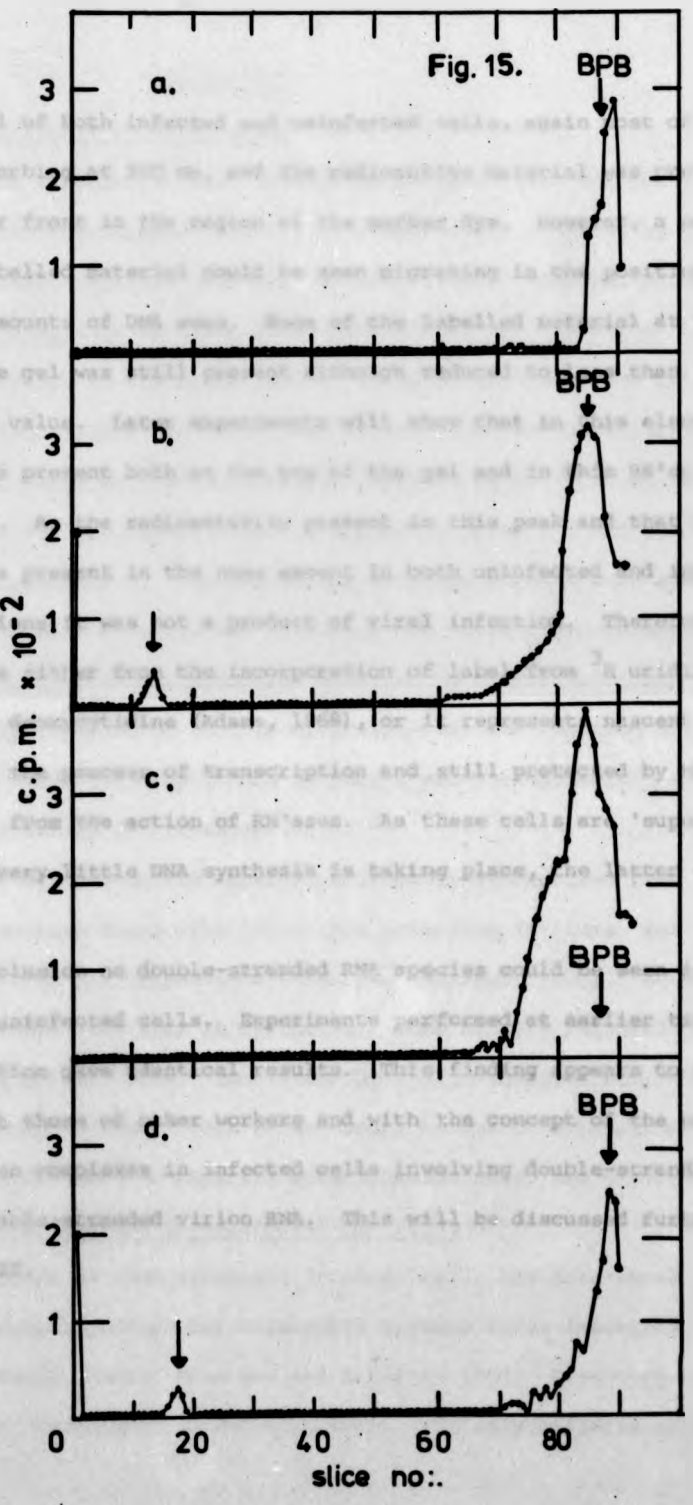

Fig. 15.

In the nuclei of both infected and uninfected cells, again most of the material absorbing at 260 nm, and the radioactive material was present at the buffer front in the region of the marker dye. However, a small amount of labelled material could be seen migrating in the position where O.D. amounts of DNA were. Some of the labelled material at the origin of the gel was still present although reduced to less than 10% of its original value. Later experiments will show that in this electrophoresis system DNA is present both at the top of the gel and in this RN'as resistant peak. As the radioactivity present in this peak and that on top of the gel is present in the same amount in both uninfected and infected RNA preparations it was not a product of viral infection. Therefore its origin can be either from the incorporation of label from ^{3}H uridine into DNA via deoxycytidine (Adams, 1968), or it represents nascent RNA molecules in the process of transcription and still protected by their DNA template from the action of RN'ases. As these cells are 'superconfluent' and very little DNA synthesis is taking place, the latter explanation is favoured.

In conclusion no double-stranded RNA species could be seen in either infected or uninfected cells. Experiments performed at earlier times during infection gave identical results. This finding appears to be at variance with those of other workers and with the concept of the existence of replication complexes in infected cells involving double-stranded or partially double-stranded virion RNA. This will be discussed further in Section III.

DISCUSSION

HA production in FPV infected cells

Both the kinetics of cell associated HA production and the total amount obtained corresponded closely with the values obtained by other workers in similar systems (Borland and Mahy, 1968; Long and Burke, 1970).

Reduction in synthesis of cytoplasmic rRNA during FPV infection

The decrease in the appearance of newly synthesised rRNA in the cytoplasm is evident within the first hour of infection and parallels the production of virus HA; both reaching a maximum by 6-7 h after infection. Viral RNA species are observed in the cytoplasm after 4 h and by 8 h post-infection they dominate. The sum of the decrease in host rRNA synthesis and of the increase in virus RNA synthesis can account approximately for the overall decline in RNA synthesis observed by Skehel and Burke (1969). As the primary interest of this investigation was the function of the nucleolus in virus infection, tRNA synthesis and its subsequent appearance in the cytoplasm was not investigated.

Similar effects on the appearance of newly synthesised rRNA in the cytoplasm have been found with poliovirus infection (Willems and Penman, 1966) and for herpesvirus infection (Roizman, 1970). Both these authors found that the fall in cytoplasmic rRNA closely followed the appearance of intact virus. The latter work also showed a similar effect on tRNA in the cytoplasm. This effect could be due to alterations in the synthesis or transport of rRNA and these possibilities are discussed below.

Synthesis of nuclear RNA in uninfected CEF cells

The pattern of rRNA synthesis in avian cells has been shown to follow the overall pattern for eukaryotic systems first described in HeLa cells (Perry, 1962; Scherrer and Darnell, 1962; Greenberg and Penman, 1966; Zimmerman and Holler, 1967). The only differences lie in

the apparent mol. wts. of some of the species.

These results compare closely with those obtained from chicken liver by Perry et al.,(1970) except that the initial transcript in their system was slightly larger (3.92×10^6 daltons). It is interesting to insert the values for the molecular weights of rRNAs and their precursors into an evolutionary table, as done by Loening (1968). The size of the smaller rRNA is very highly conserved throughout eukaryotic evolution, implying that its structure is of fundamental importance. By comparing the sizes of both the larger rRNA species and the initial transcript, Loening compiled a table of evolutionary hierarchies closely comparable to that achieved by taxonomic considerations. The values obtained here from embryo avian fibroblasts put birds underneath lower mammals such as the rabbit and above the amphibia. Similarly one could argue that embryonic avian fibroblasts are more 'primitive' that adult liver cells as the sizes of the larger rRNA and the primary transcript reported here are smaller than the mol. wts. of RNA from adult chicken liver (Perry et al., 1970). However no firm conclusions can be drawn until absolute molecular weights are determined.

Because of the polydisperse nature of hnRNA and the lack of convenient probes in the chicken system, such as there are in the immunoglobulin and haemoglobin systems, no further characterization of hnRNA was attempted.

Effect of FPV infection on nuclear RNA synthesis

The synthesis of the initial rRNA transcript and that of its immediate product remained constant for the first 3 to 4 h of infection, whereas that of the mature rRNA species and their immediate precursors decreased from the outset of infection. The eventual decrease in the synthesis of the two primary precursors was closely paralleled by a

decrease in the soluble intracellular uridine pools. This decrease in precursor pools probably caused the fall-off in synthesis of the initial transcript.

However the fall-off in synthesis of the mature rRNAs and their immediate precursors is too rapid for the same argument to apply. This decrease must be caused by an interference at the step of splitting the 2.9×10^6 molecule into the immediate precursors for the mature rRNAs. In the HeLa cell system methylation is known to be a controlling factor in processing of rRNA precursors (Vaughan et al., 1967), but no alteration in total methylation of rRNA precursors occurred in FPV infected cells. However the data presented gave no information on the possibility of aberrant methylation occurring. In E.coli the cleavage of the rRNA precursor is effected by an endonuclease specific for double-stranded regions of the RNA (Schlessinger et al., 1973). It is possible that the virus infection is affecting a similar enzyme function in CEF cells. No additional discrete RNA molecules were observed in infected nuclei, so it could be postulated that the enzyme is totally degrading the 2.9×10^6 molecule or cleaving it randomly instead of at a specific point.

It is possible that the decline in host protein synthesis could cause the above effect of rRNA processing as the initial transcript is bound to proteins as it is being synthesised and all rRNA precursors are found as ribonucleoproteins in vivo (Higashi et al., 1968). Thus the orientation of the RNA in the complex could make available specific sites for cleavage. However if this was the case, there must be a specific effect on certain ribosomal proteins as the synthesis of the first two precursors is unaffected.

No effect on rRNA transport was observed as the rate of decline of synthesis of both the 1.55×10^6 species and the 0.65×10^6 species in the nucleus exactly paralleled that of their appearance in the cytoplasm.

The observed effect on the processing of host rRNA correlates well with the appearance of a non-virion, virus-specified protein in the nucleolus and with the onset of morphological changes similar to those observed in the presence of RNA synthesis inhibitors. Therefore it can be postulated that the presence of the virion specified protein causes a breakdown in the normal rRNA maturation scheme by either altering the tertiary structure of the precursor or by changing the specificity of the enzyme or enzymes responsible for cleavage.

Thus infection with FPV causes similar changes in rRNA synthesis as that found with herpesvirus. However they are quite different from those changes caused by poliovirus. Whereas poliovirus does cause an alteration in processing, it does so by causing a buildup of precursors; this is not found in FPV infection. Also poliovirus causes a decrease in the synthesis of the initial transcript and interferes with the transport of ribosomes from the nucleus; none of these effects are observed in FPV infection.

Effect of FPV infection on hnRNA synthesis

No appreciable effect on hnRNA was observed until 4 h after infection. A similar effect was observed with poliovirus (Penman et al., 1966; Soeiro et al., 1966) although these observers found a slight increase in hnRNA synthesis within the first hour of infection. From 4 h post-infection onwards, FPV caused an overall decrease in the synthesis of hnRNA. It is difficult to reconcile the results above with those of Borland and Mahy (1968) who found an increase in nucleoplasmic DNA dependent RNA polymerase activity during the first 2 h of infection which then reverted to normal by 8 h post-infection. However, if it is assumed that the polymerase is acting as normal and the virus is inducing an increase in nuclease activity so that there is a net decrese in the synthesis of complete hnRNA chains, these results can be reconciled.

Synthesis of viral RNA in the nucleus of infected cells

Although the high levels of hnRNA synthesis early in infection prevents the detection of viral RNA synthesis, they should be low enough after 6 h for synthesis of viral RNA to be observed. This was not the case, although by 4 h post-infection viral RNA could be detected in the cytoplasm. These results appear to conflict with those of Avery (1974) and Armstrong and Barry (1974) who found newly synthesised virion RNA in the nucleus. These data also conflict with data present in Section III which shows virion RNA transcription in the nucleus. It is concluded that the majority of virus RNA is in the cytoplasm and that the absence of observable amounts of viral RNA in the nucleus reflects the relative insensitivity of the techniques employed.

Effect of infection with U.V.-irradiated FPV on RNA synthesis in CEF cells

If a protein from the infecting virus particle causes the observed interferance of the processing of rRNA, virus made non-infectious by U.V.-inactivation should have the same effect as live particles. The data presented above demonstrate that host rRNA synthesis, like host protein synthesis (Long and Burke, 1970) is not affected by infection with U.V. inactivated virus although such virus will induce interferon (Gandhi and Burke, 1970).

Double-stranded RNA species in FPV infected cells

Double-stranded RNA is well known to have toxic effects on cells as well as inhibiting translation (Cordell-Stewart and Taylor, 1973; Lee et al., 1971). Therefore an investigation was pursued to discover if an accumulation of double-stranded RNA could cause the observed effect on rRNA synthesis. The data showed that no such species of RNA were found at any one time in either the nuclei or cytoplasm of infected cells.

This was somewhat surprising as many workers have found double-stranded virus-specific RNA in FPV infected cells. However, this apparent

inconsistency may be a peculiarity of the different analytical systems and will be discussed more fully in Section III.

Suggestions for further investigation

In order to complete the story, the effect of FPV infection on tRNA synthesis could be elucidated as has been done with herpesvirus (Roizman, 1970). However, it is unlikely that this would throw any light on either the mechanism of virus replication or on the control processes in eukaryotic cells, unless the methods developed by Sueoka and Tetsuo (1962) for separating the different tRNA species were used.

At present the evidence that the virus specified protein NS_1 causes the inhibition of rRNA processing is only circumstantial. Unfortunately the experiments done with the poliovirus system to distinguish whether the effect on rRNA maturation is dependent on virus RNA synthesis, cannot be performed as FPV is insensitive to guanidine. No other RNA synthesis inhibitors are available which specifically inhibit FPV RNA synthesis although α-amanitin is a possibility under investigation.

It should be possible to prepare purified NS_1 and either see if it causes the same effect *in vivo*, or prepare a crude nucleolar preparation and assay for *in vitro* RNA synthesis both with and without NS_1.

It is difficult to envisage a positive rationale behind this effect of virus infection on rRNA synthesis. It should be in the interest of the virus to have as many ribosomes available as possible. However, if new messengers are only transported from the nucleus on new ribosomes, as suggested by Harris, such a mechanism would prevent the expression of any host defence mechanisms such as interferon. This is even more plausible when it is considered that interferon is only produced by irradiated FPV (Gandhi and Burke, 1970) and such virus does not cause any inhibition of host rRNA synthesis.

SECTION II

ATTACHMENT AND PENETRATION OF VIRUS

INTRODUCTION

Structural characteristics of the eukaryotic plasma membrane relating to virus attachment

The initial event in a viral infection is the collision of the virus particle with the host cell plasma membrane. The structure of this membrane must be of crucial importance to the success of the infective process. No attempt will be made here to exhaustively review the structure of eukaryotic membranes as this has been done recently elsewhere (Green, 1971; Racker, 1970) but only to refer to those functions directly related to virus attachment.

Singer and Nicholson (1972) have visualized the eukaryotic membrane as a fluid mosaic of protein, within a "sea" of lipid. These proteins can either be inserted into one side of the lipid bilayer only, or traverse the membrane completely. Recent research has shown that the membrane lipids are capable of very rapid lateral movement (Poste and Allison, 1973) at about 4 μm/s; although virtually no vertical movement occurs. However most experiments have been done with microvesicles in which the hydrophilic portions of the molecule would be sterically inhibited. If the experiments are repeated with large membrane sheets, the vertical 'flip-flop' of the lipid molecules is increased by up to five orders of magnitude. By using sophisticated spectroscopic methods, Linden et al., (1973) found that membrane lipids were not homogeneously arranged but formed a mosaic of well ordered and fluid regions.

The proteins in the membrane appear to move laterally about 100 times slower than the lipids. Nothing is known of their vertical movement through the membrane although it is difficult to imagine how the polar regions of a large molecule could traverse the non-polar region in the centre of the lipid bilayer as the lipid molecules appear to do.

Proteins which lie exclusively on one side of the membrane, and others which traverse the membrane, have been described for human erythrocytes (Tillack et al., 1972). Glycophorin is of particular interest as it spans the membrane. The exterior hydrophilic portion is covalently linked to about 30 oligosaccharide residues in a highly branched structure which forms several antigenic sites. The centre is hydrophobic and is bound tightly to membrane lipid while the interior hydrophilic portion appears to be in contact with cytoplasmic proteins.

The membrane structure of the enveloped viruses closely resembled that of the cell membrane in many ways. The lipids are derived from those in the host plasma membrane (Frommhagen et al., 1959; Kates et al., 1962) although their position in the virus envelope imposes severe restrictions on their lateral movement (Lenard and Compans, 1974). Similarly all of their envelope proteins are glycosylated as are those of other lipid containing animal viruses such as SFV.

Therefore the overall picture of the eukaryotic membrane is one of an extremely mobile structure, covered on the outside with proteins on which are various receptors or recognition sites, some of which are in the form of oligosaccharides. The impermeable lipid bilayer is penetrated at several points by protein, some of which also have a receptor function. The membrane of an infecting enveloped virus has a very similar structure, also containing a lipid bilayer of like consistency to the cell membrane and studded with glycoproteins which interact with some of the cell receptor sites.

Attachment of virus to cell receptors

Picornaviruses and myxoviruses have both been studied intensively as regards their mechanism of attachment. These two virus classes represent two very different mechanisms of host-virus interaction.

In vivo poliovirus has a very narrow host range, multiplying only in the nervous and intestinal tissues of primates (Holland, 1961). Cells which support virus replication contain a specific virus receptor protein on their plasma membrane (Holland, 1962) which combines with a specific protein (VP4) on the virus envelope. The specific viral receptor is found only in cells which support infection (Holland, 1961) and accounts for the narrow host range of poliovirus.

The myxoviruses on the other hand bind to a very wide range of host cells. Many attempts have been made to isolate a protein from susceptible cells with the characteristics of a specific receptor. Kathan *et al.*, (1961) isolated a glycoprotein, but unlike the receptor of poliovirus only the sialic acid residues and not the whole protein acted as the receptor (Gottschalk and Graham, 1958). If the true receptor for myxoviruses is in fact sialic acid, this would explain the broad host range for this class of virus. The virus attaches to these receptors by the haemagglutinin protein which constitutes one type of spike on the virus coat (Laver and Valentine, 1969; Webster and Darlington, 1969).

Broadly speaking, the adsorption behaviour of other virus types follows that of poliovirus for the non-enveloped viruses, and that of the myxoviruses for the enveloped viruses and the large poxviruses.

In general, the first stage of virus attachment to the host cell results from Brownian motion, although it appears that a virus particle has to collide several times before it is firmly bound (Allison and Valentine, 1960). The initial binding appears to be electrostatic in nature (Tolmach, 1957; Philipson, 1963), although it is difficult to understand how this can be as the net charge on both the virus particle and the cell surface is negative (J.J. Skehel - personal communication) and large amounts of free energy must be pumped into the system if the massive hydration shells, which surround charged particles in aqueous media, are to be removed. However, frequency of attachment rates for

virus-cell interactions as high as 3.5×10^{-9} ml/cell have been estimated (Ogston, 1963).

The initial attachment of most viruses appears to be unaffected by temperature changes from 4°-37°, with the notable exception of rhinoviruses whose attachment rate is markedly temperature sensitive (Lonberg-Holm and Korant, 1972). During this first stage of attachment the virus is sensitive to neutralization by antibody. Some viruses which are acid sensitive, such as the myxoviruses and herpesvirus, can also be neutralized by low pH during this stage of attachment. Thus the virus appears to be still on the outside of the cell. With influenza virus, this first stage of attachment is reversible and can be totally abolished by treating the cells with a bacterial neuraminidase called receptor destroying antibody. With influenza virus (Ishida and Ackermann, 1956) and perhaps with vaccinia virus (Smith and Sharp, 1961) there is a second stage in the attachment process. This stage is irreversible, temperature independent and insensitive to receptor destroying enzyme, but the virus still remains sensitive to antibody.

After attachment of virus particles to the cell, a considerable number elute off at temperatures around 37°. With poliovirus infection this occurs by a proteolytic mechanism resulting in the loss of VP4 from the virus particle rendering it non-infective. In the case of influenza virus elution, this is achieved by the neuraminidase on the outer surface of the virus (Webster and Darlington, 1969) which cleaves off sialic acid residues from the cell surface, but leaves the virus particle infective.

Penetration of the cell by infecting virus

The mechanism whereby animal viruses penetrate their host cell after adsorption has been a subject of controversy ever since investigations were started over 15 years ago. The reasons for this are many. Although we now have a reasonable conception of the gross ultrastructure of membrane systems, our knowledge of the transport processes across

these membranes by small molecules or macromolecular complexes, such as a virus, is minimal. A further problem is the speed at which penetration takes place. Thorne (1962) has shown that penetration of foot and Mouth disease virus (FMDV) is 90% complete in 3 min at 37°; Marcus (1959) showed that the entry of NDV is complete in less than 5 min at 37° and Simpson et al., (1969) showed that 60% of virus-like particles from VSV preparations were taken up by cells within 5 min at 37°. Another difficulty arises in the use of the electron microscope, which in theory should be a powerful tool in such studies. Whereas it is relatively easy to visualize whole virus particles by E.M., once inside the cell and eclipsed (i.e. presumably modified in some way) it is impossible to recognise discrete virus specific particles. The possible exception to this is vaccinia virus which uncoats to produce a discrete core before DNA is released.

There are two major theories of virus penetration which may in fact describe two parts of the same process, although their exponents seem to be not quite so compromising. One theory, first described by Fazekas de St Groth (1948) as 'Viropexis' involves the engulfment of the virus particle in a phagocytotic vesicle and the subsequent breakdown of both vesicle and virus by cell hydrolases. The other major theory, first enunciated by Hoyle and Finter (1957), involves the fusion of the outer coat of lipid-containing viruses with that of the membrane of the cell, thus injecting the nucleocapsid into the cytoplasm in a manner analogous to the T-even phages of bacteria. The second theory, by definition does not attempt to describe the method of penetration by viruses which do not have a lipid containing outer coat. A third method of entry described for poliovirus by Dunnebacke et al., (1969) involves the virus particle passing right through the plasma membrane as a complete entity. As very little additional evidence is supplied for this last theory, it will not be discussed further.

By far the greatest amount of evidence favours the viropexis theory and involves almost exclusively studies with the electron microscope, although Joklik (1964) carried out some biochemical studies on the fate of poxviruses which, he concluded, fitted only the viropexis concept. Practically every class of virus has been shown to be present in pinocytotic vacuoles by E.M. (see review by Dales, 1965). Simpson et al., (1969) have shown by E.M. that VSV is taken up very rapidly by pinocytosis at a rate which correlates closely with the rate of appearance of transcription of the viral genome (Flamand and Bishop, 1973). E.M. studies of RNA tumour viruses by Dales and Hanafusa (1972) have shown that virus was adsorbed at 4° and penetrated by viropexis at 37°. The evidence by these authors is more convincing than most as they accounted for all the virus particles in their system. Recently Dourmashkin and Tyrrell (1974) have shown E.M. pictures demonstrating that electron micrographs apparently showing fusion of influenza virus with the cell membrane are artefacts due to tangential sectioning. These authors conclude that all virus particles enter the cell by viropexis.

The theory of virus entry by fusion is attractive, not only in that it has a precedent in the penetration of the T-even phages, but in that it is the exact reversal of virus assembly and release, at least for influenza viruses (Compans and Dimmock, 1969). Initially Hoyle and Finter (1957) concluded that influenza virus became disrupted so as to leave viral envelope and HA on the cell surface, allowing penetration of the internal nucleocapsid. Hoyle (1962) prepared cytoplasmic particles (which are released in vivo by chorioallantoic membrane cells - CAM) and showed E.M. pictures of virus fusing with the cytoplasmic particles causing disruption of the particle inside the cell. E.M. studies by Morgan and Rose (1959) yield more convincing pictures of influenza virus particles merging with cell membranes.

Some viruses definitely do fuse with the plasma membrane of the host cell and discharge their genomes into the cell (Apostolov and Almeida, 1972), but these are notably the haemolysin-containing paramyxoviruses and it is unclear whether this process is responsible for a productive infection.

However exponents of both the above theories do not attempt to answer one important problem of animal virus infection, namely that we cannot tell which particle of the many on the virus surface actually causes the infection. The electron microscope cannot distinguish between particles which are infectious and those which are not, neither can infecting virus components be recognized once they have passed the cell membrane. The evidence presented for either theory which relies on electron microscopy may be invalid or even misleading. This question is particularly important for animal viruses as their particle to infectivity ratio is very much greater than unity. There is a further important objection to the viropexis theory. If the virus is taken up in a phagocytotic vesicle it is still outside the cell, i.e. on the wrong side of the plasma membrane. These vesicles can then fuse with lysosomal vacuoles (see review by Allison and Davies, 1974) and are thus subject to attack by cellular hydrolases. There is no evidence to suggest that virus components are in any way resistant to these enzymes. The cell itself is not broken down by its own hydrolases as it contains them in the membrane bound lysosomes. Therefore the only way a virus could prevent itself from being attacked by these hydrolases would be to insert itself actually into the cytoplasm where it would be as immune as the host cell. Therefore cellular hydrolases would not be implemented in the specific, limited degradation of the virus particle which occurs during the eclipse phase of infection.

Project outline

The aim of this experimental programme was to study the initial events in influenza virus synthesis. In order to ensure that a comprehensive picture of this part of the replication cycle was built up, it was necessary to carefully characterize the adsorption phenomena in our system. It is very difficult to assay most of the processes which have been postulated to occur in virus attachment and penetration. However micropinocytosis, which is thought to be important (see reviews by Allison and Davies, 1974; Dimmock, 1974), is easily studied by monitoring the uptake of colloidal gold and this process is studied in the system used for virus adsorption.

RESULTS

Kinetics of attachment

In order to study the initial events in virus replication, it is important to synchronize the infectious process. Normally this is done by adsorbing the virus for a given period of time at a temperature lower than that necessary for multiplication. In the previous section this adsorption was done at room temperature (about 20°) and the kinetics of adsorption at this temperature were investigated. Fig. 16 shows that adsorption was complete by 45 min and had a half time of reaction of 25 min. The recombinant virus FPV/BEL was used as this was more suitable than FPV for later experiments described in Section III. Other experiments (see Section III) indicated that at room temperature, not only was virus firmly attached to the cells, but that virion RNA had penetrated the nucleus and was probably on its way out again after 1 h post-infection. Kinetics of absorption were therefore investigated at 4° in an attempt to slow down these processes to facilitate their further analysis. At 4°, adsorption was complete by 60 min and had a half time of reaction of 10 min (Fig. 17). Therefore attachment at 4° is as fast, if not faster than at room temperature.

Sensitivity of virus attachment to neutralizing antibody

The above results suggested that virus was attached to the cells firmly enough not to be dislodged by successive washing; but they do not indicate whether the virus has penetrated the cell or is still attached to the outside. In order to investigate this phenomenum infected cells were treated with neutralizing antibody: only if virus had penetrated the cell would plaques appear. The antiserum was titrated as described in Table 6. Complete neutralization (greater than 97%) of the virus was achieved at a 10^{-3} dilution and 10^{-2} dilution was used routinely throughout the investigation.

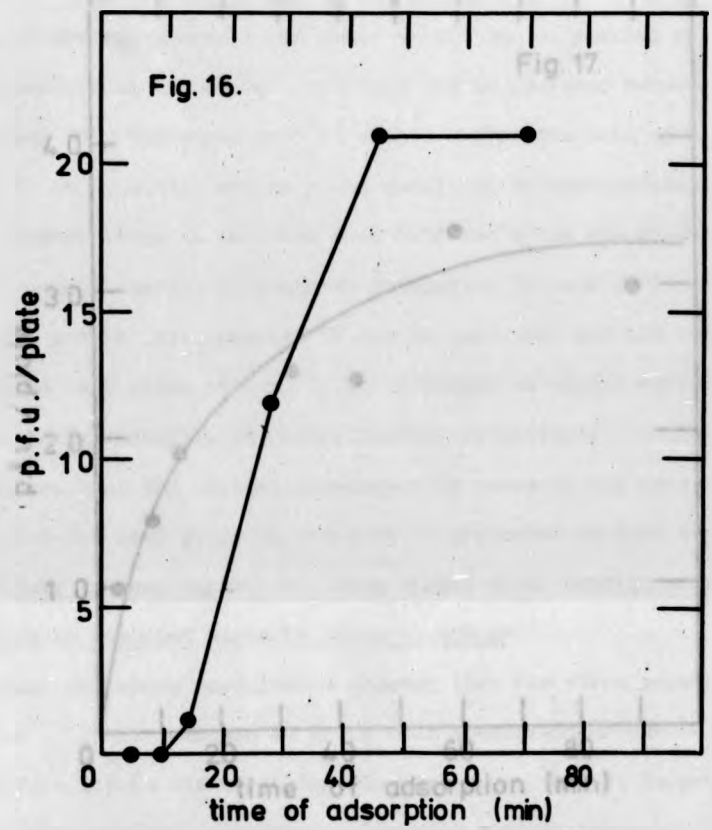

Fig. 16. Kinetics of attachment of FPV/BEL to CEF cells at room temperature. 25 PFU of purified FPV/BEL in 0.1 ml PBS/Ca/Mg were allowed to adsorb for the required time to 9 x 10^6 cells on 5 cm plastic petri dishes. The cells were washed 4 times in PBS/Ca/Mg and incubated at 37° in 199 medium under agar until plaques appeared (about 2 days). Each point is an average of duplicate samples.

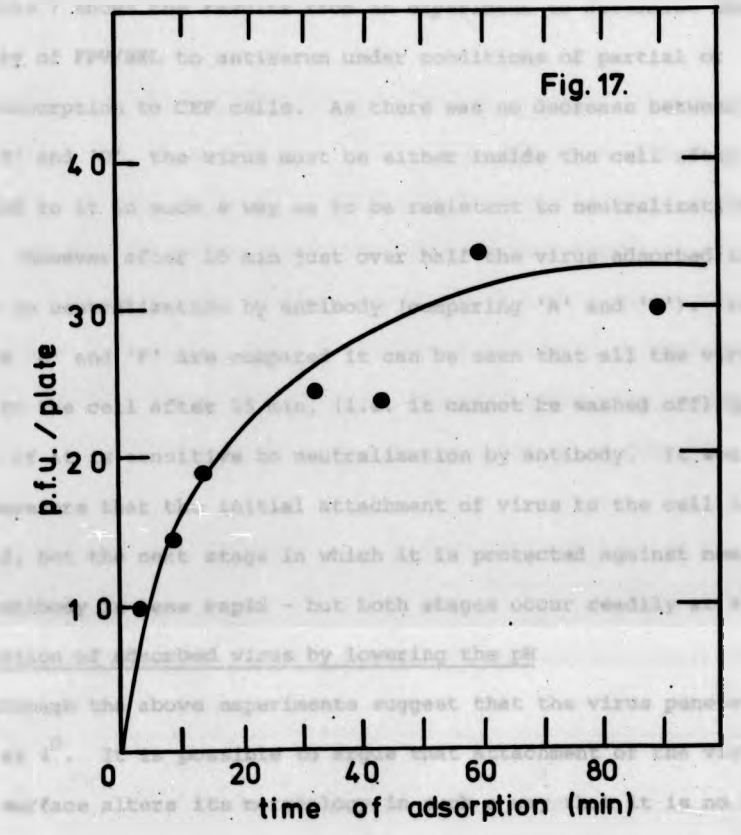

Fig. 17. **Kinetics of attachment of FPV/BEL to CEF cells at 4°**
9 x 10^6 cells on 5 cm plastic petri dishes were precooled in a 4°
cold room for 30 min and then adsorbed for the required time with
40 PFU of purified FPV/BEL in 0.1 ml PBS/Ca/Mg. The cells were
washed 4 times in cold PBS/Ca/Mg and incubated as in Fig. 16. Each
point is an average of duplicates.

Table 7 shows the results from an experiment to determine the sensitivity of FPV/BEL to antiserum under conditions of partial or complete absorption to CEF cells. As there was no decrease between samples 'B' and 'C', the virus must be either inside the cell after 1 h at 4° or bound to it in such a way as to be resistant to neutralization by antibody. However after 15 min just over half the virus adsorbed is sensitive to neutralization by antibody (comparing 'A' and 'D'). Further, if samples 'E' and 'F' are compared it can be seen that all the virus is attached to the cell after 15 min, (i.e. it cannot be washed off) but about 50% of it is sensitive to neutralization by antibody. It would appear therefore that the initial attachment of virus to the cell is very rapid, but the next stage in which it is protected against neutralization by antibody is less rapid - but both stages occur readily at 4°.

Neutralization of adsorbed virus by lowering the pH

Although the above experiments suggest that the virus penetrates the cell at 4°. It is possible to argue that attachment of the virus to the cell surface alters its morphology in such a way that it is no longer neutralizable by antibody, although it is still outside the cell. Huang and Wagner (1964) have shown that the infectivity of herpes simplex virus can be rapidly and efficiently inactivated by lowering the pH of the medium. A similar phenomenon has been demonstrated for influenza virus by Tovarmitsky and Shishkina (1943) and used for an extensive study of the adsorption kinetics of FMDV by Thorne (1962). N.J. Dimmock and D. Price (personal communication) have shown that a drop of at least two orders of magnitude in the infectivity of FPV/BEL can be achieved by treating suspensions of the virus for 1 min at 4° with PBS buffered to pH 3 with HCl.

Table 8 shows the results of treating CEF cells to which FPV/BEL had been adsorbed, with acidified PBS. Again we see a very similar

Table 6. Titration of antiserum against FPV/BEL. 0.9 ml of virus in PBS/Ca/Mg containing about 350 p.f.u. was mixed with 0.1 ml of antiserum, diluted in PBS/Ca/Mg to give the final concentrations shown below. The mixtures were incubated by gentle shaking at 4° for 1 h and then 0.1 ml aliquots were allowed to adsorb for 1 h at 4° on precooled 5 cm petri dishes containing 9×10^6 CEF cells. The monolayers were washed 4 times with cold PBS/Ca/Mg and incubated as in Fig. 16.

Final antiserum concentration	p.f.u. per dish (av. of 4)
10^{-2}	0
10^{-3}	0
10^{-4}	2
10^{-5}	37
10^{-6}	25
10^{-7}	29

Table 7. Sensitivity to antibody of FPV/BEL adsorbed to CEF cells at 4°. Virus, cells, antiserum and PBS/Ca/Mg were precooled to 4°. 50 p.f.u. virus was added to cells in 0.1 ml of PBS/Ca/Mg. Antibody was added in 1 ml volumes of a 1/100 diln. in PBS/Ca/Mg. Washes were each of 2 ml. A- virus adsorbed for 15 min and 1 ml PBS/Ca/Mg added for 1 h; B- virus adsorbed for 1 h and 1 ml of PBS/Ca/Mg added for 1 h; C- Virus added for 1 h and antiserum added for 1 h; D- Virus added for 15 min and antiserum added for 1 h; E- virus added for 15 min, washed twice in cold PBS/Ca/Mg and 1 ml PBS/Ca/Mg for 1 h. F- virus added for 15 min, plates washed twice with cold PBS/Ca/Mg and antiserum added for 1 h. All plates were washed twice in cold PBS/Ca/Mg and incubated as in Fig. 16.

Sample	p.f.u. per plate (av. of 4)
A	47
B	47
C	44
D	21
E	44
F	24

Table 8. Sensitivity to acidified PBS/Ca/Mg of FPV/BEL adsorbed at 4° to CEF cells. 9 x 10^6 cells on 5 cm petri dishes, virus suspensions, acid PBS/Ca/Mg and neutral PBS/Ca/Mg were all precooled to 4°. The virus was adsorbed to the monolayers for the stated time, treated for 1 min at room temperature with 1 ml of PBS/Ca/Mg at pH 3, neutralized by the addition of an equivalent amount of NaOH, washed twice in cold PBS/Ca/Mg and incubated as in Fig. 10. The control contained 0.9 ml of acidified PBS/Ca/Mg and 0.1 ml of virus (2 x 10^3 p.f.u.) which was left at 4° for 1 min and then neutralized with NaOH. 0.1 ml aliquots were adsorbed onto cells for 1 h at 4° and incubated as above.

	time of adsorption (mins)	p.f.u. per dish (av. of 2)
Washed only	0	71
	10	165
	60	178
Treated with acid PBS/Ca/Mg before wash	0	36
	10	57
	60	131
Control		2

result to that obtained with antibody, i.e. the virus attaches very rapidly and then becomes resistant to acid inactivation at a slower rate. It was concluded therefore that as the virus is resistant to both antibody and low pH when adsorbed to CEF cells at 4° it has penetrated the cell.

Studies on micropinocytosis

For some time there has been controversy whether influenza virus (or any other virus) enters the host cell by fusion of its membrane with that of the host cell, or enters by an operation called viropexis, which could be similar in operation to that of micropinocytosis. In the previous paragraph it has been shown that influenza virus can enter cells at 4°. It is difficult to envisage any energy requiring processes taking place at this temperature, so such mechanisms as phagocytosis and macropinocytosis are probably not important in influenza virus infection. However micropinocytosis and membrane fusion are energy independent (Allison and Davies, 1974) and could operate at this temperature. It is difficult to assay the extent of a complex process such as membrane fusion, but micropinocytosis is easily assayed by the uptake of colloidal gold.

Fig. 18 shows the uptake of radiolabelled colloidal gold over a period of 24 h by CEF cells. Under these conditions the cells appear to take up gold faster at 37° than at 4°, although the final level reached differed by only two-fold. However these conditions were not those used in the virus adsorption experiments described above. Results from experiments done at 4° in 0.1 ml volumes are shown in Fig. 19. Under these conditions, uptake is very rapid and reaches a maximum after 10 min. The fall off towards the end of the incubation is probably due to the cells dehydrating and dying as the volume of overlay is very small. Fig. 19 also shows that the addition of influenza virus did not stimulate pinocytosis at 4°.

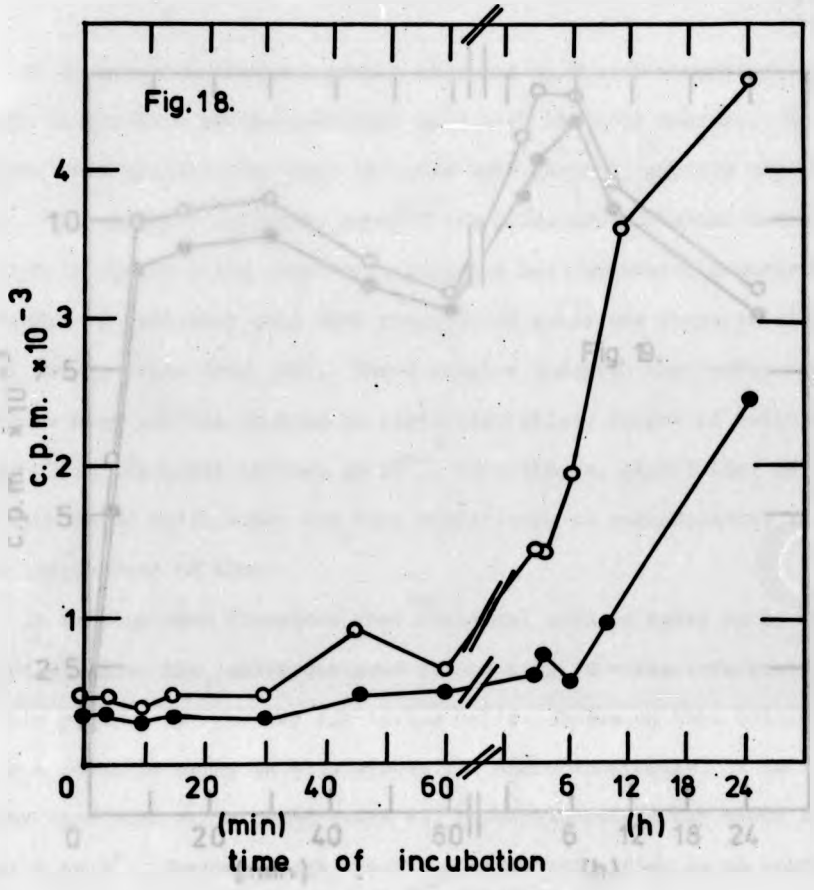

Fig. 18. Uptake of colloidal gold ^{198}Au by CEF cells. 9×10^6 cells were incubated on 5 cm petri dishes with 3 ml of 199 medium containing 1 µCi of colloidal ^{198}Au for the required time. The sheets were then washed four times with cold PBS/Ca/Mg, dissolved in 1 ml of 0.2 M NaOH for 15 min at room temperature and the whole sample counted by the Cerenkov method. Samples counted at 80% gain without scintillant gave an efficiency of 25% as compared to those assayed in triton-toluene based scintillant. O-O samples incubated at 37°, ● - ● samples incubated at 4°. All points are the average of duplicates.

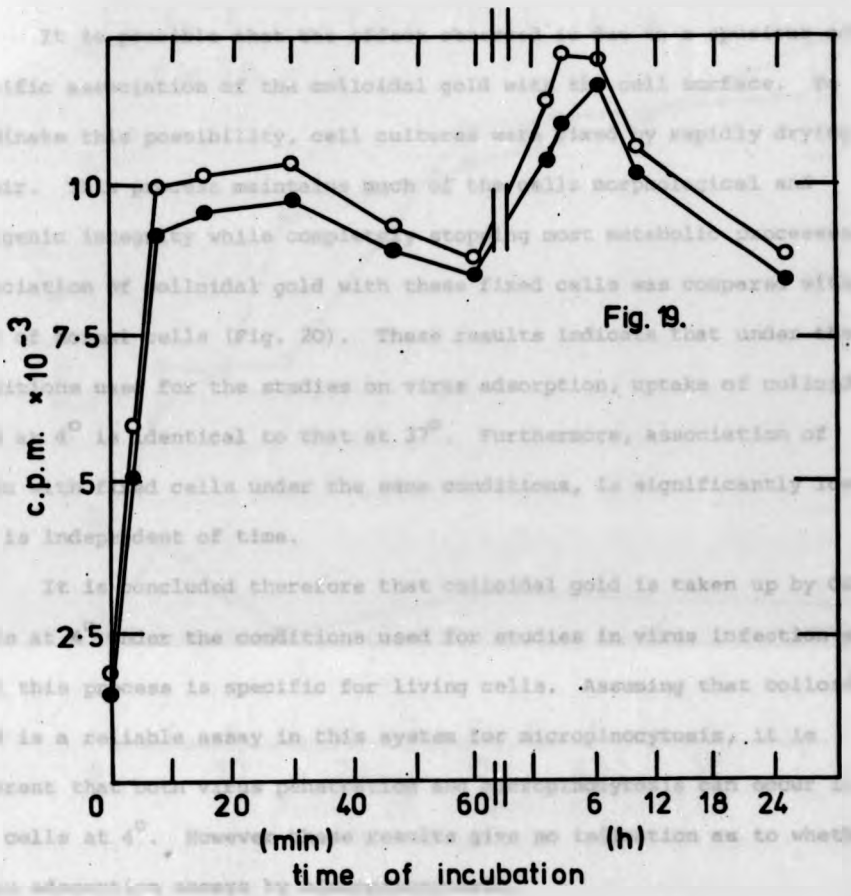

Fig. 19. Uptake of colloidal gold ^{198}Au by CEF cells at 4° in 0.1 ml volumes. O - O cells incubated for the required time at 4° with 0.1 ml PBS/Ca/Mg containing 1 µCi ^{198}Au. ● - ● cells incubated at 4° with 0.1 ml PBS/Ca/Mg containing 10^7 PFU of FPV/BEL. Cultures were prepared, harvested and assayed for radioactivity as in Fig. 18.

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tion occurs by micropinocytosis.

It is possible that the effect observed is due to a spurious non-specific association of the colloidal gold with the cell surface. To eliminate this possibility, cell cultures were fixed by rapidly drying in air. This process maintains much of the cells morphological and antigenic integrity while completely stopping most metabolic processes. Association of colloidal gold with these fixed cells was compared with that of normal cells (Fig. 20). These results indicate that under the conditions used for the studies on virus adsorption, uptake of colloidal gold at 4° is identical to that at 37°. Furthermore, association of ^{198}Au with fixed cells under the same conditions, is significantly lower, and is independent of time.

It is concluded therefore that colloidal gold is taken up by CEF cells at 4° under the conditions used for studies in virus infection and that this process is specific for living cells. Assuming that colloidal gold is a reliable assay in this system for micropinocytosis, it is apparent that both virus penetration and micropinocytosis can occur in CEF cells at 4°. However these results give no indication as to whether virus adsorption occurs by micropinocytosis.

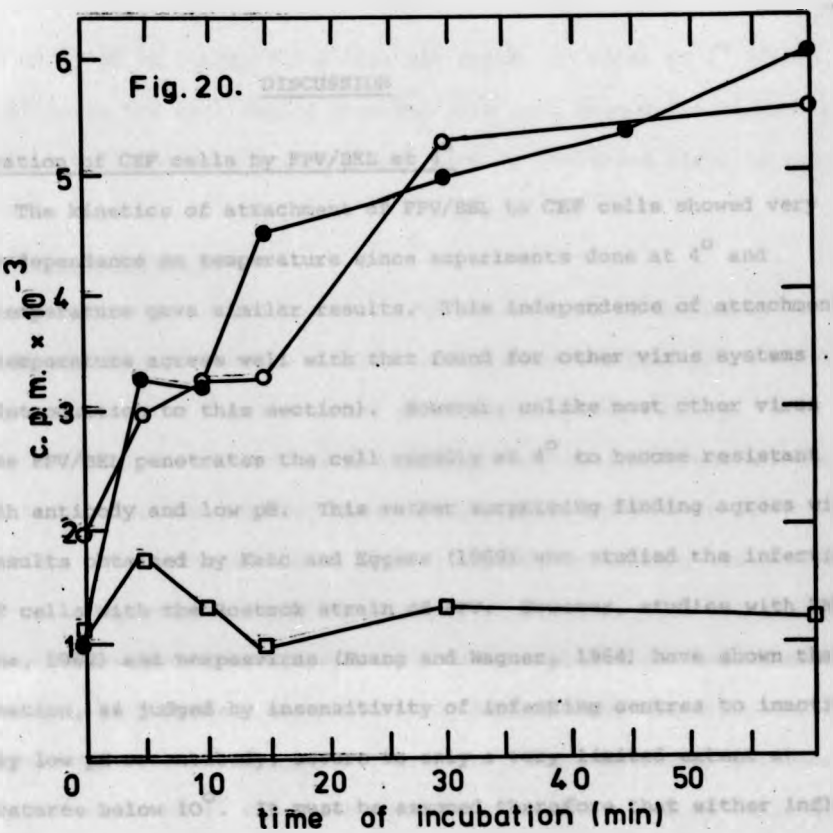

Fig. 20. Uptake of colloidal ^{198}Au gold at 4° by fixed and viable CEF cells. Cultures were prepared, harvested and assayed for radioactivity as in Fig. 18. O - O cultures incubated at 4° with 0.1 ml PBS/Ca/Mg containing 1 uCi ^{198}Au, ● - ● cultures incubated at 37° with 0.1 ml PBS/Ca/Mg containing 1 uCi ^{198}Au, □-□ cultures fixed by drying in a current of air at room temperature for 1 h, precooled, and then incubated at 4° with 0.1 ml PBS/Ca/Mg containing 1 µCi ^{198}Au.

DISCUSSION

Penetration of CEF cells by FPV/BEL at 4°

The kinetics of attachment of FPV/BEL to CEF cells showed very little dependence on temperature since experiments done at 4° and room temperature gave similar results. This independence of attachment upon temperature agrees well with that found for other virus systems (see Introduction to this section). However, unlike most other virus systems FPV/BEL penetrates the cell rapidly at 4° to become resistant to both antibody and low pH. This rather surprising finding agrees with the results obtained by Kato and Eggers (1969) who studied the infection of CEF cells with the Rostock strain of FPV. However, studies with FMDV (Thorne, 1962) and herpesvirus (Huang and Wagner, 1964) have shown that penetration, as judged by insensitivity of infecting centres to inactivation by low pH or antibody, occurs to only a very limited extent at temperatures below 10°. It must be assumed therefore that either influenza virus penetrates the host cell by a different method from FMDV and herpesvirus or it is very much more efficient in its penetration process. The penetration of many other viruses has been studied (see review by Dales, 1965), but these involve almost exclusively E.M. studies and suffer from the limitations mentioned above.

If influenza viruses penetrate the cell by fusion, the viscosity of the cell membrane will be an important factor in this penetration. Viscosity measurements on some membranes indicate that they have a viscosity similar to olive oil, i.e. about 100 times that of water. Reduction of the temperature of olive oil from 37° to 10° increases its viscosity by only four-fold and the extreme change of molecular structure from the fluid to the crystalline state does not take place until -6° is reached. Therefore at 4° there is enough fluidity in membrane structure for fusion to take place at only slightly reduced rates. However, cell

fusion mediated by Sendai virus does not appear to occur at 4° (Okada, 1962) although the cell fusion observed with such haemolytic viruses may occur by a different process than that used by influenza virus to enter the host cell.

Studies of pinocytosis

If viropexis is the mechanism for penetration of influenza at 4° it is important to determine whether this process can take place at this temperature as virus attachment and penetration occurs readily. Both phagocytosis and macropinocytosis require an energy source and are thus temperature sensitive, so they cannot play a major role in virus adsorption. Micropinocytosis, a process capable of forming vesicles of 70-100 nm - big enough to contain a virus, does not require energy and is thus the most probable endocytotic process employed in virus penetration at 4°. This process is conveniently monitored by observing the uptake of radiolabelled colloidal gold. A decrease in the uptake of colloidal gold by CEF cells at 4° was observed when compared to that of cells at 37° - as found by Davies et al., (1973). However when the gold was adsorbed under the conditions used for virus adsorption experiments, an equal sharp rise in uptake was observed both at 37° and at 4°. This uptake was not observed in cell sheets which had been fixed by drying in air, and was unaffected by the presence of virus. Therefore micropinocytosis does occur in this system at 4° and could be the mechanism for virus penetration. However it has not been shown that membrane fusion cannot occur at 4°, so the question of the mechanism of virus penetration remains unanswered.

Suggestions for further experimentation

These studies would be usefully complemented by electron microscopy, to compare this experimental system with those described for most viruses, in particular the recent study of influenza in CAM cells by Dourmashkin

and Tyrrell (1974): although such studies would of course be open to the objections to this technique mentioned above. It would be interesting to repeat these experiments using a range of influenza viruses and other host cells to establish if penetration of the host cell at 4° by the influenza viruses is in fact widespread throughout the class. Tagging the virus components with fluorescent antibody would also give an indication as to the final location of various viral antigenic components and also remove many of the objections to artefactual situations which could arise in the experiments described above. Experiments along these lines are presented in the next section.

INTRODUCTION

The experiments described in this section were designed to investigate the mechanisms of transcription of the viral RNA in influenza virus infected cells. The transcription of RNA of other viruses will be briefly discussed first in order that their transcription mechanisms can be compared with those postulated for influenza virus. It will become evident that it is impossible to discuss the transcription of viral RNA without also involving a discussion of the mechanisms of RNA replication; therefore the strategies of both events will be discussed. Only the mechanisms elucidated for the f2 RNA phages, poliovirus, rhabdoviruses and reovirus will be discussed as they represent the major types of transcriptional mechanisms which may be used during influenza virus multiplication.

SECTION III

INTRACELLULAR LOCATION OF RNA FROM THE INFECTING VIRION AND ITS TRANSCRIPTION

Throughout this section the term virion RNA (vRNA) refers to RNA which has the same base sequence as the infecting virion. Complementary RNA (cRNA) refers to RNA which is complementary in sense to vRNA, i.e. has a base sequence complementary to vRNA.

Multiplication of RNA bacteriophages

The mechanisms of transcription and replication of this class of virus will be only briefly discussed as they appear to have little relevance to processes occurring during influenza virus multiplication: however a great deal is known about their multiplication and it will be useful to bear in mind some basic concepts which could apply to influenza virus. These mechanisms have been reviewed elsewhere (Sugiyama et al., 1972; Kozak and Nathans, 1971).

Most work in this area deals with the f2 group of phages. f2 Phage has a genome of mol. wt. 1.1×10^6 which codes for 3 proteins, the coat protein (mol. wt. 1.5×10^4), the maturation protein (mol. wt. 3.8×10^4) and the replicase (mol. wt. 5×10^4). The maturation protein is encoded

INTRODUCTION

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Throughout this section the term virion RNA (vRNA) refers to RNA which has the same sense as RNA found in mature virions. Complementary RNA (cRNA) refers to RNA which has the opposite sense to vRNA, i.e. has a base sequence complementary to vRNA.

Multiplication of RNA bacteriophages

The mechanisms of transcription and replication of this class of virus will be only briefly discussed as they appear to have little relevance to processes occurring during influenza virus multiplication: however a great deal is known about their multiplication and it will be useful to bear in mind some basic concepts which could apply to influenza virus. These mechanisms have been reviewed elsewhere (Sugijama et al., 1972; Kozak and Nathans, 1971).

Most work in this area deals with the f2 group of phages. f2 Phage has a genome of mol. wt. 1.1×10^6 which codes for 3 proteins, the coat protein (mol. wt. 1.5×10^4), the maturation protein (mol. wt. 3.8×10^4) and the replicase (mol. wt. 5×10^4). The maturation protein is encoded

at the 5' end, the coat protein, centrally and the replicase at the 3' end. The genome also acts as a mRNA and is transcribed disproportionately. The replicase is synthesised early in infection and its synthesis is then shut off. Coat protein is produced throughout infection in gradually increasing amounts until it becomes the major product, late in infection. The maturation protein is synthesised at a constant level at all times but at a much lower rate than the other two virus proteins.

The virus uses several transcriptional and translational control processes. The shut-off of replicase synthesis is caused by the coat protein binding at the initiation site of the replicase gene and preventing ribosomes binding. This only occurs when the concentration of coat protein molecules is such that they form a certain configuration at the initiation site. Thus replicase synthesis is only switched off at the correct moment. This is an example of translational control by a messenger product.

The reduced synthesis of maturation protein is because the RNA at the initiation site is in a configuration which makes ribosome binding at this site less efficient than at the other sites. The configuration of the RNA also decreases the synthesis of the replicase 'in vitro'. However 'in vivo' the ribosome unwinds the initiation site of the replicase gene as it transcribes the initial segment of the coat protein gene. This ensures that the synthesis of replicase does not outstrip that of coat protein early in infection and vice-versa.

As well as acting as message, the phase genome is the template for the synthesis of cRNA from which new virion RNA is synthesised. Therefore both replication and translation cannot occur at the same time as they proceed in opposite directions. This problem is overcome in Qβ phage infection by the replicase protein binding to the ribosome binding site and preventing further ribosomes from binding. Then all

the ribosomes are allowed to run off and the genome is then replicated. Therefore control of phage protein and RNA synthesis is achieved merely by an elegant configuration of the RNA and the specificity of the interactions between virus proteins and virus RNA.

One aspect of virus RNA synthesis which is completely obscure is that of how the replicase changes its specificity from making cRNA from vRNA to making vRNA from cRNA.

Multiplication of poliovirus

The study of picornavirus multiplication has yielded most information about the mechanisms of animal virus infection. However, this class of virus is very different from influenza virus and therefore its multiplication processes will only be discussed briefly in order to highlight some concepts which may be useful in evaluating the mechanism of influenza virus infection.

Poliovirus contains a single-stranded RNA genome of mol. wt. about 2.6×10^6. The RNA is infectious and like the genome of RNA phages function as a message and as a template for cRNA synthesis. A unique feature of picornavirus replication is that the genome is transcribed to form one large polypeptide which is subsequently cleaved by a series of specific proteases to form the functional proteins. Thus all virus specific proteins are produced in equal numbers at all times. One of the products of translation and cleavage is the replicase. This enzyme may synthesise both cRNA and vRNA. Hence this enzyme may exist in two forms or there may be two separate replicases. The replicase has not been purified.

Picornaviruses have the same problem as the RNA phages, i.e. before the replicase can transcribe the input RNA, it must ensure that all ribosomes have been cleared before transcription can start. No control mechanism, similar to that of the RNA phages, has been described yet, and if none exists it may explain the low infectivity to particle

ratio of these viruses.

Again like the RNA phages cRNA is produced in much smaller amounts than vRNA. How this is achieved is unknown, nor is it understood how the virus knows when to switch from making cRNA to making vRNA. In general there appears to be no control over the amount of virus specific protein or RNA produced at any one time, although the assembly of virus particles is made quite efficient by combining all processes involved in multiplication into large "replication complexes".

Further details of picornavirus replication can be found in reviews by Brown and Hull (1973) and Baltimore (1969).

Multiplication of Rhabdoviruses

The multiplication of vesicular stomatitis virus (VSV) will be used as a model system as it has been extensively studied. The details of the replication cycle has been recently reviewed elsewhere by Flamand and Bishop (1974) and only the conceptual principles will be discussed here.

VSV is a negative stranded enveloped RNA virus containing a virion associated RNA-dependent RNA polymerase which specifically transcribes the virion genome. Three proteins associate with an RNA of mol. wt. 3.8×10^6 to make up a ribonucleoprotein core. Approximately 2×10^3 molecules of N protein are tightly bound to the RNA and probably protect it from nuclease digestion. The L protein is probably the virion transcriptase and the exact amount present in the core is unknown, although it is much less than the N protein. The third core protein is the NS protein which is phosphorylated and has no known function. The outer envelope contains the G glycoprotein in a lipid bilayer beneath which is the M protein.

Transcription is a property of the inner core and only occurs if L protein is present although complete transciption does not occur

in the absence of NS protein. The function of the N protein is unknown, but it is essential for transcription. The role as protector against nuclease attack may be important as the RNA is very tightly stretched and has no double stranded regions. Somehow the N protein must be displaced during transcription and immediately replaced to maintain the integrity of the transcription complex. The mechanism for this process is unknown, neither is it known if the N protein has an additional role to play, perhaps as a cofactor for the replicase.

'In vitro' transcription is complete, repetitive and sequential, although viral proteins are made in disproportionate amounts. 'In vivo' only 20% of the infecting particles are transcribed. Transcription is at a linear rate of one complete genome every 90 s. Initial transcription is unaffected by pretreating with AMD, cycloheximide or other inhibitors of protein synthesis. The mRNA species are transcribed individually in pieces approximately equivalent in coding capacity to that required by the virus polypeptides. The virus mRNA species contain poly A at the 3' end (which is not derived from poly U sequences in the virion RNA) and are methylated on synthesis in a manner analogous to reovirus messengers (see paragraph below). The VSV messengers are produced at different rates and hence some transcriptional control mechanism must operate.

Viral proteins are produced in differing quantities which reflect the frequency of messenger production. The mechanism of control is unknown but it could reflect a difference in the rate of initiation or elongation of the mRNAs - possibly induced by the secondary structure of the virion RNA; or the presence of "transcription stop" sequences in the nascent mRNA which inhibit the further action of the replicase on the genome at a specific site. Alternatively a form of product inhibition

control could operate, similar to that described above for f2 translation. All or any one of these roles could be performed by the NS protein or the poly A sequences or the methylated bases.

45 min from the start of infection, secondary transcription starts, i.e. the production of cRNA from newly synthesised vRNA. This process is insensitive to AMD, but is sensitive to cycloheximide. It is assumed the nascent vRNA is rapidly assimilated to form mature virions, as these are released from the cell at the same time. Perlman and Huang (1973) performed the following elegant experiment to investigate the mechanism of replication. They showed that the addition of cycloheximide at any time during infection to cultures at the permissive temperature of both wild type and a temperature sensitive, RNA minus mutant did not affect mRNA synthesis but immediately inhibited the synthesis of vRNA. This suggests that the continued synthesis of a protein is necessary for the change from transcription to replication. If the ts mutant was shifted to the non-permissive temperature, replication was unaffected but transcription was stopped. Therefore the lesion is in the transcriptase and either the replicase is a different enzyme or the transcriptase has a separate active site for replication which is activated by the protein factor. If cycloheximide was added at the time of the temperature shift, further synthesis of vRNA was stopped but mRNA synthesis now reverted to the level in the wild type. This data can be explained if we postulate that two new proteins are needed for replication; one of which is a replicase which in the presence of the second protein forms vRNA. In the absence of the second protein the replicase acts as a transcriptase. An alternative, although conceptually more difficult, hypothesis is that the transcriptase can be modified by the activation of a second active site by a new protein to make vRNA. In the absence of this new protein, the second active site will make mRNA. As replication is sensitive to

cycloheximide the synthesis of this new protein must be continuous. It is possible that the activation of the new replicase, or the modification of the transcriptase, could be mediated by the nascent N protein forming a replication complex with cRNA in such a manner that it not only activates the replication enzyme but causes it to make complete vRNA by aligning all the cRNA species in an RNP complex. Alternatively the NS protein in its phosphorylated or part-phosphorylated form could similarly activate the replication enzyme and line up the various cRNA species so that on replication a complete vRNA molecule was formed.

Replication of reovirus

Reovirus, like influenza virus has a segmented RNA genome, although unlike influenza virus, reovirus RNA is double stranded. The replication of reovirus will be discussed here to compare aspects of its replication with that of influenza virus. Further details of the events occurring during reovirus infection can be obtained from reviews by Shatkin (1971) and Flamand and Bishop (1974).

The genetic material of reovirus consists of 10 double stranded pieces of RNA in equimolar amounts. These are grouped into 3 size classes, Large (L)-3 pieces, medium (M)-3 pieces and small (S)-4 pieces. The L class of RNA codes for virion proteins λ_1, λ_2 and λ_3, the M class for μ_1, μ_2 and μ_3 and the S class for σ_1, σ_2, σ_3 and σ_4. σ_3 and σ_2 form the nucleocapsid core and the remainder form the virus shell.

Transcription only commences when the outer proteins are either removed or modified by host proteases, although synthesis of host protein is not necessary. Transcription 'in vivo' occurs only within the subviral cores and only the negative strand of double stranded RNA is transcribed. It is thought that the transcriptase accomplishes this by recognising a modified base on the strand opposite the one to be transcribed

The synthesis of mRNA is the dominant event early in infection, but declines later on. On transcription, the mRNA is methylated and this appears to cause an increase in transcription rate, but not translation rate. There appears to be some form of transcriptional control 'in vivo' as only 4 messengers are produced early on , but all 10 are produced later on. However 'in vitro' all 10 messengers are produced at all times. This difference in transcription patterns may reflect the fact that 'in vitro' transcription occurs within the sub-viral cores and viral proteins may effect a control within the complex but not on the isolated enzyme: certainly if ϑ3 is added 'in vitro' it causes a depression in transcription.

Some form of translational control also takes place as μ proteins are made in greater amounts than ϑ proteins although the reverse is true for the synthesis of the corresponding M and S messengers.

The switch from messenger production to viral RNA production occurs late in infection. This process is sensitive to inhibitors of protein synthesis and therefore must involve the synthesis of a new replicase or a protein to modify the transcriptase. Replication, like transcription, operates only in sub-viral cores, but the replicase acts at first only on the single stranded positive strand (mRNA) to produce negative strands, after which more positive strands are produced. However no details of the mechanism of this process are known. The assembly process of reovirus is complex as it must involve the accurate collection of 10 pieces of RNA and 4 proteins to form a core. The recombination frequencies of reovirus indicate that at some stage in infection a pool of all RNA species exists, but how these are gathered together in an orderly manner is not known.

Multiplication of influenza virus

a) Character of the genome: The influenza virus genome is a single stranded RNA molecule of total mol. wt. 4 x 10^6 which is segmented into several unique fragments. The sense of the influenza virion genome has recently been a subject of controversy since it was reported that RNA from purified virions stimulates an E.coli 'in vitro' protein synthesising system (Seigert et al., 1973). However the opposite result has been reported by Kingsbury and Webster (1973) using a mammalian 'in vitro' protein synthesising system. These latter workers did not find messenger activity in vRNA but RNA from infected cells did have messenger activity. However it was not determined whether this RNA was virion sense or cRNA or both.

b) The nuclear phase: Unlike all other RNA viruses, except the tumour viruses, influenza virus multiplication is dependent on the integrity of the host cell nucleus. Breitenfeld and Schaf er (1957) first demonstrated that an antigen corresponding to the virion ribonucleoprotein complex (RNP) was found in the nuclei of infected cells. At about the same time Hoyle and Finter (1957) showed that the input virion RNA entered the nucleus and they suggested it associated with the host DNA, but the techniques then available could only give equivocal results. They also showed tentatively that virion protein did not enter the nucleus immediately upon infection. Recently as many as 4 virion proteins have been found in association with the nucleus, NP, P, NS_1, and NS_2 (Taylor et al., 1970; Lazarowitz et al., 1971; Dimmock, 1969; Krug and Etkind, 1973).

Studies with inhibitors of DNA function such as AMD, mitomycin C or U.V. irradiation (Barry et al., 1962; Barry, 1964; Rott et al., 1965; Nayak and Rosmussen, 1966) showed that virus fails to replicate in the presence of these compounds. However, cells treated with inhibitors which stopped only DNA synthesis, supported virus infection (Nayak and

Rasmussen, 1966), unlike the RNA tumour viruses which are sensitive to this inhibitor. Recent results obtained with camptothecin (Kelly et al., 1974b) and with AMD blocked cells fused with dormant erythrocytes (Kelly and Dimmock, 1974) also demonstrated this sensitivity to DNA inhibitors. Influenza virus multiplication is also sensitive to α amanitin indicating that the nucleoplasmic polymerase could be necessary for virus replication (Mahy et al., 1972). Cordycepin, an analogue of adenosine does not however inhibit virus replication (Mahy et al., 1973) indicating that the nuclear poly A synthetase is possibly not necessary for virus multiplication. However this latter effect seems to be dependent on the cell system studied (Rochovansky and Pons, 1974). The exact effect of these inhibitors of virus function is uncertain although Ghendon et al., (1970) have shown that the virion RNA in the nucleus of the infected cells is broken down by nucleases induced by U.V. irradiation. Additionally Borland and Mahy (1968) demonstrated that the nucleoplasmic DNA dependent RNA polymerase is stimulated in infected cells and this is an obvious target for DNA inhibitors. Although virus infection is most sensitive to AMD early in infection, Gregoriades (1970), Scholtissek and Rott (1970) and Skehel (1973) have shown that the drug causes a decrease in virus production and RNA and polypeptide synthesis throughout infection. Bean and Simpson (1973) have shown that AMD inhibits the transcription of input virus genomes.

The failure of influenza virus to grow in non-nucleated cell fragments (Cheyne and White, 1969) or enucleate cells (Follett et al., 1974; Kelly et al., 1974a) has conclusively demonstrated the necessity of a host nucleus for successful virus multiplication.

Despite the plethora of data presented above, the role of the nucleus in virus infection is still unknown. The sensitivity to DNA inhibitors cannot be explained solely by the induction of host nucleases as the virus

RNA appears to be in an RNP complex. Primary transcription of the virus RNA is sensitive to AMD but not cycloheximide (Bean and Simpson, 1973), therefore newly synthesised host RNA is not needed in a messenger capacity. A possible explanation is that the host RNA synthesis is necessary to provide primers for the transcription of viral RNA to cRNA.

c) Primary transcription: Transcription of the viral genome is thought to be achieved by the virion associated transcriptase. This enzyme has an optimum activity 'in vitro' at 31° in the presence of Mg^{2+} and Mn^{2+} and has an absolute requirement for all nucleotide triphosphates especially ATP (Bishop et al., 1971). 95% of the product formed 'in vitro' is complementary to vRNA and 80% of the genome is transcribed (Bishop et al., 1972). A peculiarity of the 'in vitro' reaction is that a high proportion of the product is double stranded and the species gained on melting the RNA have a lower overall size than vRNA; in fact resembling RNA obtained from virions produced late in infection which have a lower infectivity than normal virus particles (Barry and Davies, 1968). Therefore it appears that the 'in vitro' reaction lacks some unspecified factor. The virion transcriptase activity has been shown to be a property of a complex containing all species of viral RNA along with the NP, P1 and P2 proteins only (Schwartz and Scholtissek, 1973; Hefti et al., 1974).

'In vivo' primary transcription has several important differences from that observed 'in vitro'. Very few (about 3%) of the infecting particles are transcribed (Bean and Simpson, 1973). Also there appears to be a lag phase of about 45 min between infection and the start of transcription which is not due to the time taken for the absorption process (see previous section). Probably the most puzzling difference is that 'in vitro' transcription is insensitive to AMD (Chow and Simpson,

but is sensitive 'in vivo' (Bean and Simpson, 1973). This fact suggests that primary transcription requires a nuclear function and recently no virion proteins or cRNA had been detected in enucleate cells (Kelly et al., 1974a; Follett et al., 1974). It is not known whether primary transcription takes place in the nucleus or cytoplasm, or whether the 'in vivo' process copies the whole genome.

d) Secondary transcription: 'In vivo' there is a sudden rise in the production of cRNA (Bean and Simpson, 1973) from about 1½ h after infection. This increase in transcription rate, unlike that observed earlier in infection is sensitive to inhibitors of protein synthesis. The second phase of transcription could be either due to the transcription of sequences not previously transcribed, or to an increase in the number of vRNA molecules caused by replication, with the consequent rise in transcription rate. In the first case the new protein would act as a cofactor to change the specificty of the transcriptase and in the second case the new protein required would either be a replicase or a factor to convert the transcriptase to a replicase.

Again it is unknown whether secondary transcription takes place in the nucleus or the cytoplasm, although an RNA dependent RNA polymerase, which is insensitive to AMD 'in vitro', has been isolated from both the nuclei and the cytoplasm of infected cells (Hastie and Mahy, 1973). However both these cellular polymerase activities reach a peak when virus is being released from the cell, it is possible that either or both activities could represent viral templates and enzymes ready to be packaged into progeny virus. Recently there have been reports of vRNA synthesis in the nucleus. Armstrong and Barry (1974) reported that there was a stimulation of RNA synthesis in the nucleus of infected cells. But these authors did not distinguish between viral RNA synthesis and host RNA synthesis, neither was it clear if the comparatively low levels of viral RNA synthesis occurring in influenza virus infection could

have been detected in their system. Avery (1974) used hybridization studies to show that both vRNA and cRNA were found in nucleus and cytoplasm; cRNA being predominant in the cytoplasm and vRNA in the nucleus late in infection. However, since the RNA was labelled with pulses of 1 h it is difficult to determine whether the RNA is synthesised in a particular cell fraction or whether it is transported there and accumulates.

e) Synthesis of viral protein: It can probably be assumed that vRNA does not direct virus protein synthesis (see above). However, cRNA is found early on during infection (Nayak, 1969; Bean and Simpson, 1973). This cRNA can be isolated from infected cell polysomes (Nayak, 1969), has the same size distribution as vRNA (Pons, 1972) and contains tracts of poly A about 50-200 nucleotides long (Etkind and Krug, 1974); although Nayak (1969) found both vRNA and cRNA on infected cell polysomes. The production of both messengers and proteins is dependent on host nuclear function (see paragraph b).

Skehel (1973) found that during the first 2 h of infection only some proteins were produced, namely P2, NP and NS. Later on in infection, all proteins were produced and their relative molar quantities controlled. This author also presented evidence that the synthesis of the messengers for the late proteins, but not the early proteins was dependent on the synthesis of a protein factor. P.D. Minor (personal communication) has also shown that only the early proteins are synthesised in the presence of a critical low dose of AMD.

Therefore it appears that protein synthesis is controlled both temporaly and quantitatively in infected cells and that this control is probably at the level of transcription.

Experimental programme

The experiments described below were designed to follow the fate of the input RNA and proteins in infected cells. The mechanism and subcellular localization of primary and secondary transcription was also studied and its sensitivity to various drugs investigated. This programme attempted to investigate the nuclear events in influenza virus multiplication and their possible role in the control of virus directed events.

RESULTS

Fate of the proteins of infecting virus

Having established in Section II that attachment and penetration can occur at 4°, the next objective was to determine where the various virion components go, on infection. As outlined in the introduction, the host nucleus plays an essential part in virus multiplication. It was judged important therefore to examine the presence of infecting virus components in nuclear and cytoplasmic fractions.

The location of virus proteins in cells incubated at 4° was studied as other experiments have shown (see later results in this section) that at higher temperatures infection occurs so fast that it is impossible to analyse the initial phenomena of infection. This was achieved by purifying virus labelled with ^{35}S methionine and examining electrophoretically the subcellular location of virion polypeptides after infection at 4° (Fig. 21). Although not all the proteins are labelled with ^{35}S methionine it does appear that the NP and M proteins go preferentially to the nucleus on infection. Unfortunately there was insufficient labelled material to draw any conclusions about the P1 and P2 proteins which are the putative virion polymerases.

Fate of the neuraminidase

As electrophoretic analysis of proteins from input virus in infected cell fractions was not clear cut, the biological activity of the haemagglutinin and neuraminidase was measured in cell fractions. There was too little haemagglutinin from the input virus to detect in infected cells, but the level of neuraminidase activity was sufficient to be assayed (Table 9). It was surprising to find a high level of neuraminidase associated with the nucleus at zero time of adsorption. This is assumed to be due to a non-specific association of virus with the nuclear fraction

Fig. 21. Electrophoresis of proteins from subcellular fractions of CEF cells infected with ^{35}S-labelled FPV/BEL. Virus was prepared and purified as described for ^{32}P-labelled FPV/BEL except that ^{32}P was replaced by 200 µCi ^{35}S methionine or 100 µCi ^{3}H leucine as required. Cells were prepared and seeded at 6 x 10^{7} per 11 cm petri dish (5 dishes per experiment). Virus was adsorbed at 4° (1 ml per plate containing 1 PFU/cell) for 1 h. Cells were fractionated and proteins prepared as described in methods. Electrophoresis was performed in the urea system for 5 h at 3 ma/gel and 80 v. Migration was from left to right. a) Proteins from the cytoplasm of cells infected with FPV/BEL, b) proteins from the nuclei of infected cells. Arrows represent the positions of co-run proteins from ^{3}H leucine labelled purified virions. NP - nucleocapsid protein, HA_2 - small haemagglutinin subunit, M - membrane protein. The neuraminidase and large haemagglutinin subunit do not label with ^{35}S-methionine in this virus. The P proteins were not present in large enough amounts to be resolved under these conditions.

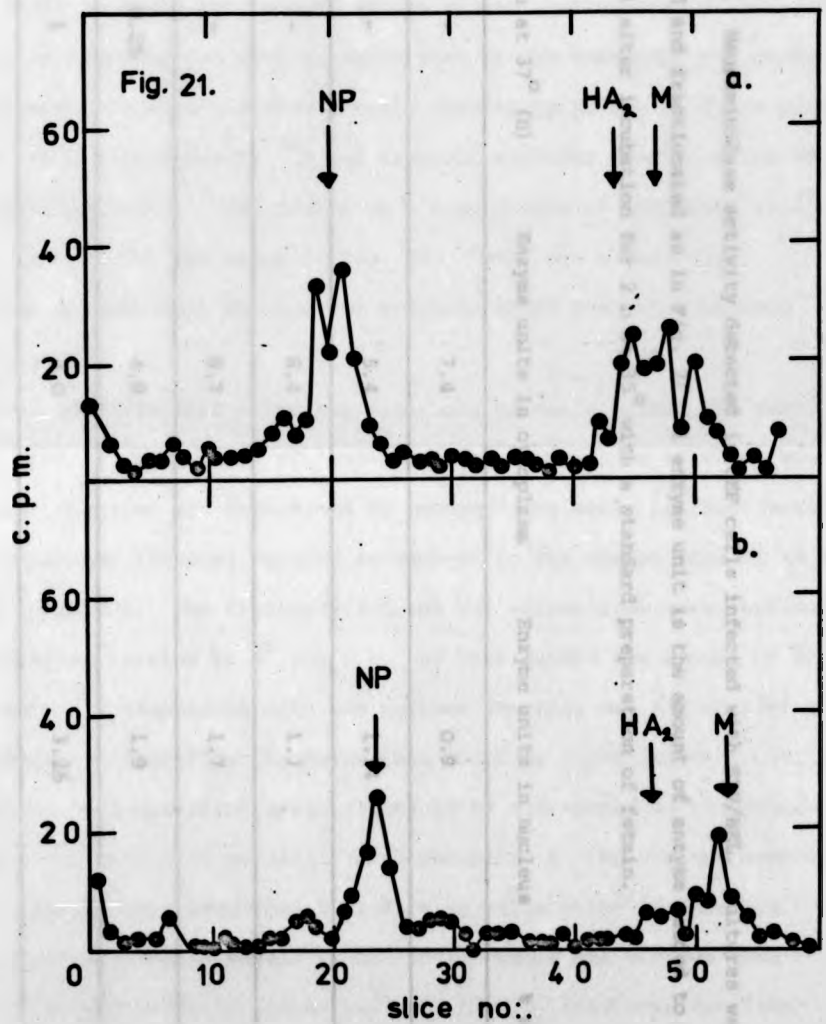
Fig. 21.
a.
b.
NP
HA2
M
c.p.m.
60
40
20
0
10
20
30
40
50
slice no:.

Table 9. Neuraminidase activity detected in CEF cells infected with FPV/BEL. Cultures were prepared, infected and fractionated as in Fig. 21. 1 enzyme unit is the amount of enzyme needed to produce an O.D of 1 after incubation for 2 h at 35° with a standard preparation of fetuin.

Time at 4° (h)	Time at 37° (h)	Enzyme units in cytoplasm	Enzyme units in nucleus	% enzyme activity in nucleus
0	-	7.4	0.9	17
0.25	-	5.4	1.14	17
1	-	8.4	1.3	13
3	-	8.7	1.7	16
1	0.25	4.8	1.9	28
1	1	3.0	3.25	52
Infected cells	-	0	0	-

and is examined further in the section below on RNA location. During the period at 4° the level of neuraminidase associated with the nucleus remained constant, but on warming to 37° it moved to the nucleus.

Fate of the RNA from infecting virus

In order to study the initial stages in the replication of influenza virus, it is essential to know in which part of the host cell the genome first appears. This can be conveniently studied by infecting cells with purified virus labelled with ^{32}P and assaying cellular fractions for TCA soluble radioactivity. The results of a time course of infection at 4° analysed in this way are shown in Fig. 22. There was a very rapid association of vRNA with the nuclear fraction which reached a maximum after 1 h at 4°.

It was possible that virus was attaching to nuclei after the cells had been broken. This level of 'non-specific' association of virus with the nuclear fraction was determined by homogenizing cells in fractionation buffer containing labelled virions equivalent to the amount present in a standard infection. The fractionation and RNA extractions were continued as before after leaving at 4° for 1 h. By this method the amount of TCA precipitable ^{32}P associated with the nuclear fraction was 13% of that in the homogenate, significantly below that found by infecting whole cells. The level of 'non-specific' association can be attributed to the binding of whole virus particles to sialic acid receptors on the nuclear membrane. Further controls were performed by infecting cells with ^{32}P labelled Sendai virus and Semliki forest virus (SFV). These RNA viruses were judged to be good controls since they both have a lipid envelope like influenza virus and both have been shown to grow in enucleate cells and thus do not require a nuclear function as does influenza virus (S.I.T. Kennedy, personal communication). When Sendai virus was adsorbed to CEF at 4° for 3 h under standard conditions only 20% of the virion ^{32}P RNA was in the nuclear fraction and when a similar experiment

and is examined further in the section below on RNA location. During the period at 4° the level of neuraminidase associated with the nucleus remained constant, but on warming to 37° it moved to the nucleus.

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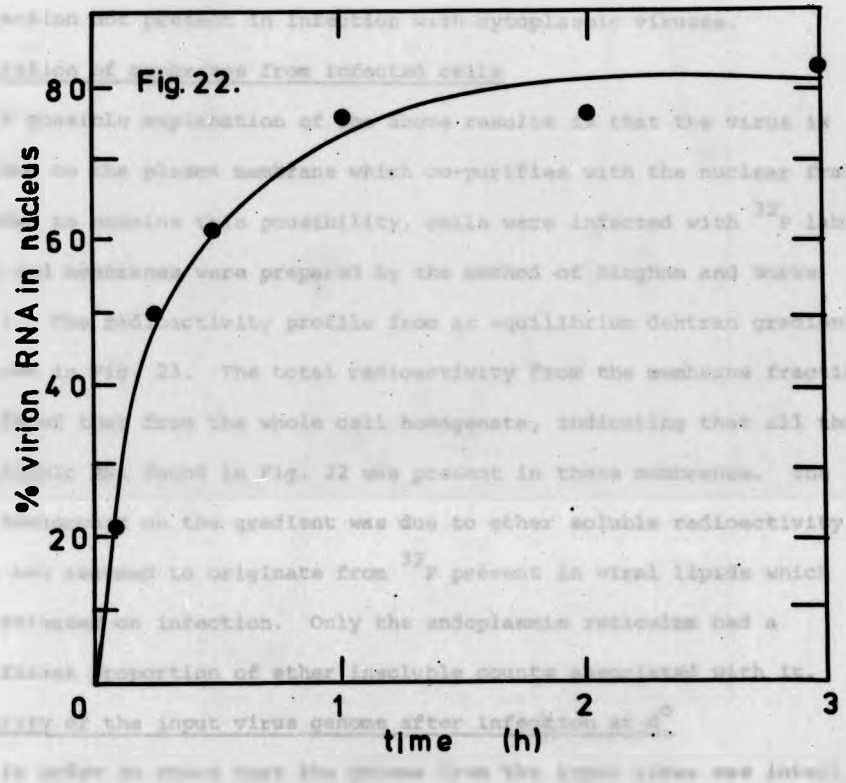

Fig. 22. Time course of the association at 4°C of ^{32}P-labelled RNA from purified FPV/BEL virus with the nuclear fraction of CEF cells. Monolayers were prepared, infected and fractionated as in Fig. 21. RNA was extracted and assayed for TCA insoluble species as described in methods.

was done with SFV, only 12% of the labelled virion RNA was in the nucleus. Thus it appears that the high level of association of influenza virus RNA with the nuclei of cells infected at 4° is the result of a specific interaction not present in infection with cytoplasmic viruses.

Preparation of membranes from infected cells

A possible explanation of the above results is that the virus is attached to the plasma membrane which co-purifies with the nuclear fractions. In order to examine this possibility, cells were infected with ^{32}P labelled virus and membranes were prepared by the method of Bingham and Burke (1972). The radioactivity profile from an equilibrium dextran gradient is shown in Fig. 23. The total radioactivity from the membrane fraction was 15% of that from the whole cell homogenate, indicating that all the cytoplasmic RNA found in Fig. 22 was present in these membranes. The high background on the gradient was due to ether soluble radioactivity which was assumed to originate from ^{32}P present in viral lipids which were released on infection. Only the endoplasmic reticulum had a significant proportion of ether insoluble counts associated with it.

Integrity of the input virus genome after infection at 4°

In order to check that the genome from the input virus was intact, the RNA recovered from infected cells was analysed by PAGE (Fig. 24). The virion RNA present in both the nuclear and the cytoplasmic fractions has the same electrophoretic distribution as the RNA extracted from purified virions grown in either de-embryonated or embryonated eggs (Fig. 25).

Characterization of virus RNA at the top of the gel

An interesting feature of the input virion RNA extracted from the nuclei of infected cells is that a significant proportion is found in the first few fractions of the gel (see Fig. 24b). The presence of macromolecular species at the origin of PAGE is always suspect; there-

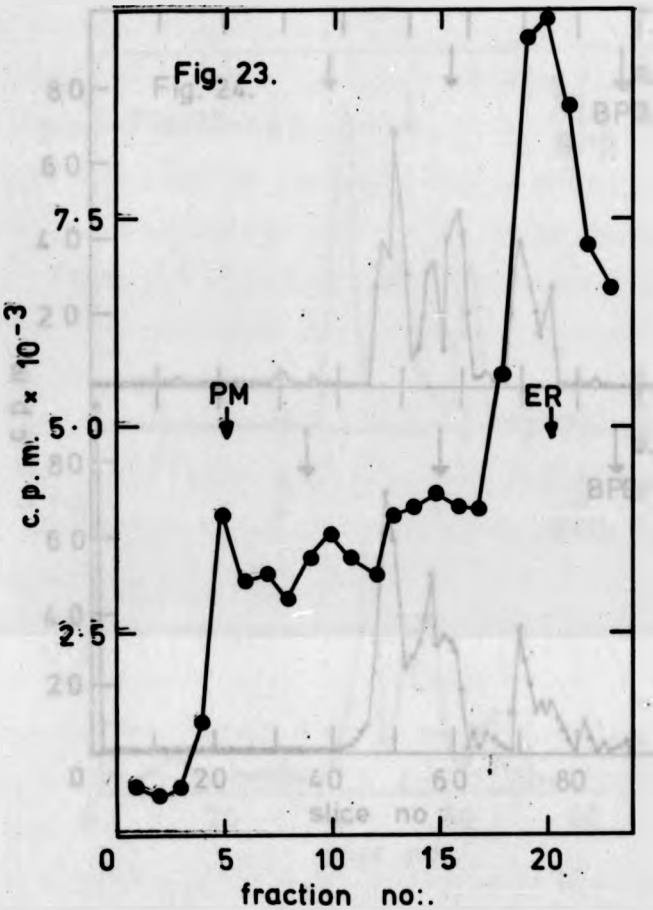

Fig. 23. Centrifugation of cellular membranes on dextran T40. Membranes were prepared from CEF cells which had been infected for 3 h at 4° with ^{32}P-labelled FPV/BEL as described in Fig. 21. The gradient was fractionated into 1 ml aliquots, all of which were assayed for radioactivity by the Cherenkov method. Sedimentation was from left to right. Positions of visible bands of plasma membranes (PM) and endoplasmic reticulum (ER) are arrowed.

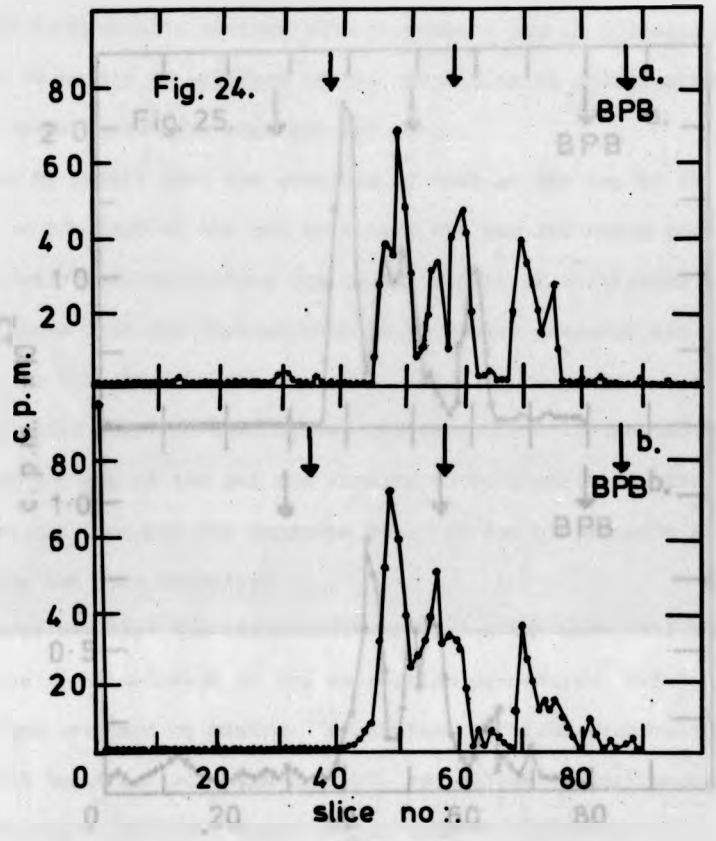

Fig. 24. Electrophoresis of labelled input FPV/BEL RNA from sub-cellular fractions of CEF cells infected for 3 h at 4°. Cultures were prepared, infected, fractionated and RNA extracted as described in Fig. 21. RNA was electrophoresed for 3 h at room temperature on a 2.4% gel at 6 ma/gel and 50 v. Arrows represent the positions of O.D. amounts of rRNA from the host cell. BPB indicates the position of the buffer front by the presence of bromophenol blue dye. Electrophoresis is from left to right. a) RNA from cytoplasm of infected cells, b) RNA from the nucleus of infected cells.

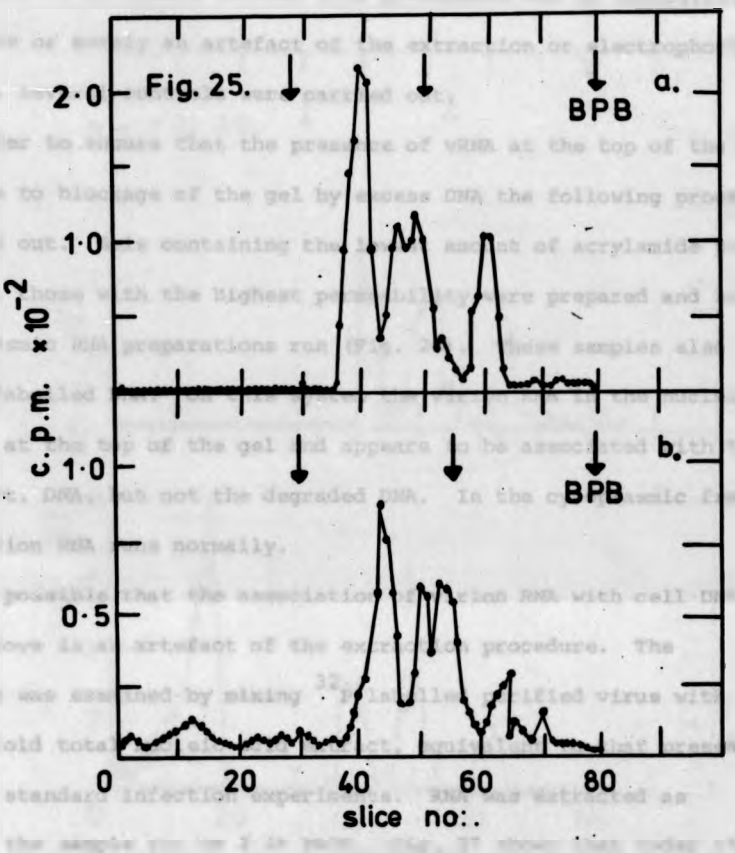

Fig. 25. Electrophoresis of RNA extracted from purified FPV/BEL. Conditions of electrophoresis were as described in Fig. 24. a) RNA from virus grown in de-embryonated eggs. b) RNA from virus grown in embryonated eggs.

fore in order to determine whether this phenomenon was of biological significance or merely an artefact of the extraction or electrophoretic procedures, several controls were carried out.

In order to ensure that the presence of vRNA at the top of the gel was not due to blockage of the gel by excess DNA the following procedure was carried out. Gels containing the lowest amount of acrylamide possible (2.2%) i.e. those with the highest permeability were prepared and nuclear and cytoplasmic RNA preparations run (Fig. 26). These samples also contained labelled DNA. On this system the virion RNA in the nucleus still runs at the top of the gel and appears to be associated with the high mol. wt. DNA, but not the degraded DNA. In the cytoplasmic fraction all the virion RNA runs normally.

It is possible that the association of virion RNA with cell DNA reported above is an artefact of the extraction procedure. The possibility was examined by mixing ^{32}P labelled purified virus with an amount of cold total nucleic acid extract, equivalent to that present during the standard infection experiments. RNA was extracted as before and the sample run on 2.4% PAGE. Fig. 27 shows that under these conditions no virion RNA is located at the top of the gel where cell DNA has been shown to run. A more rigorous check on the extraction procedure was made by precooling the cells to 4° for 6 h to ensure the shutdown of their metabolic processes and then extracting RNA immediately after the addition of labelled virus. Fig. 28 shows that under these conditions the virion RNA does not associate with cellular DNA.

Therefore the association of viral RNA with cell DNA after infection at 4° appears to be a significant phenomenon and is not an artefact of the extraction or electrophoretic systems.

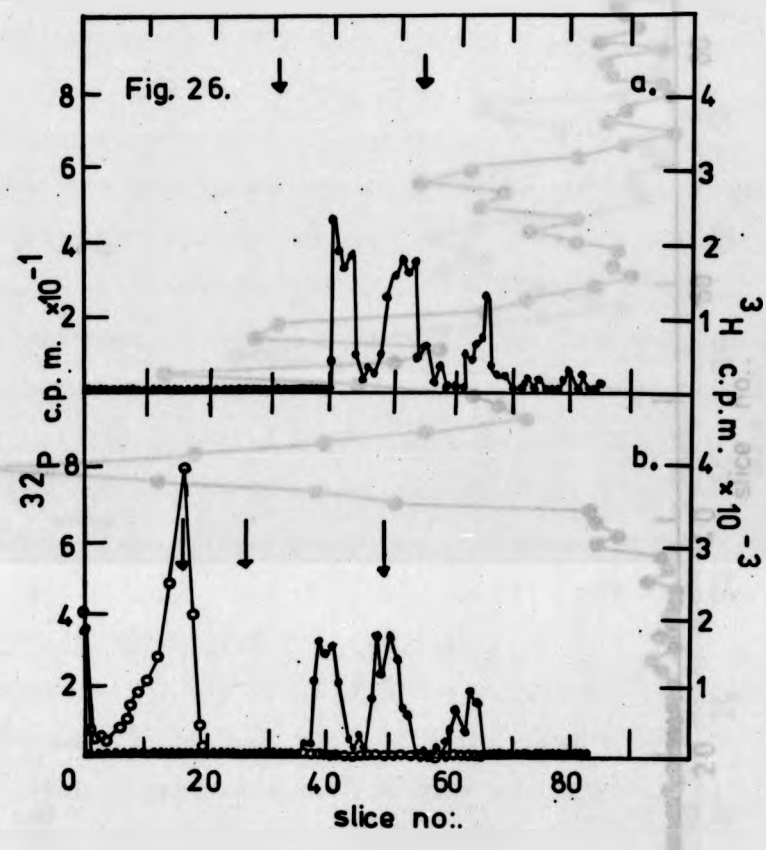

Fig. 26. Electrophoresis of virion RNA from infected cells prepared as in Fig. 24, but run for 3½ h on a 2.2% gel. Before infection with virus, the cells were incubated for 1 h at 37° with 50 µCi ^{3}H thymidine. a) cytoplasmic RNA, b) nuclear RNA. ● - ● ^{32}P, o - o ^{3}H. Arrows represent O.D. amounts of nucleic acid.

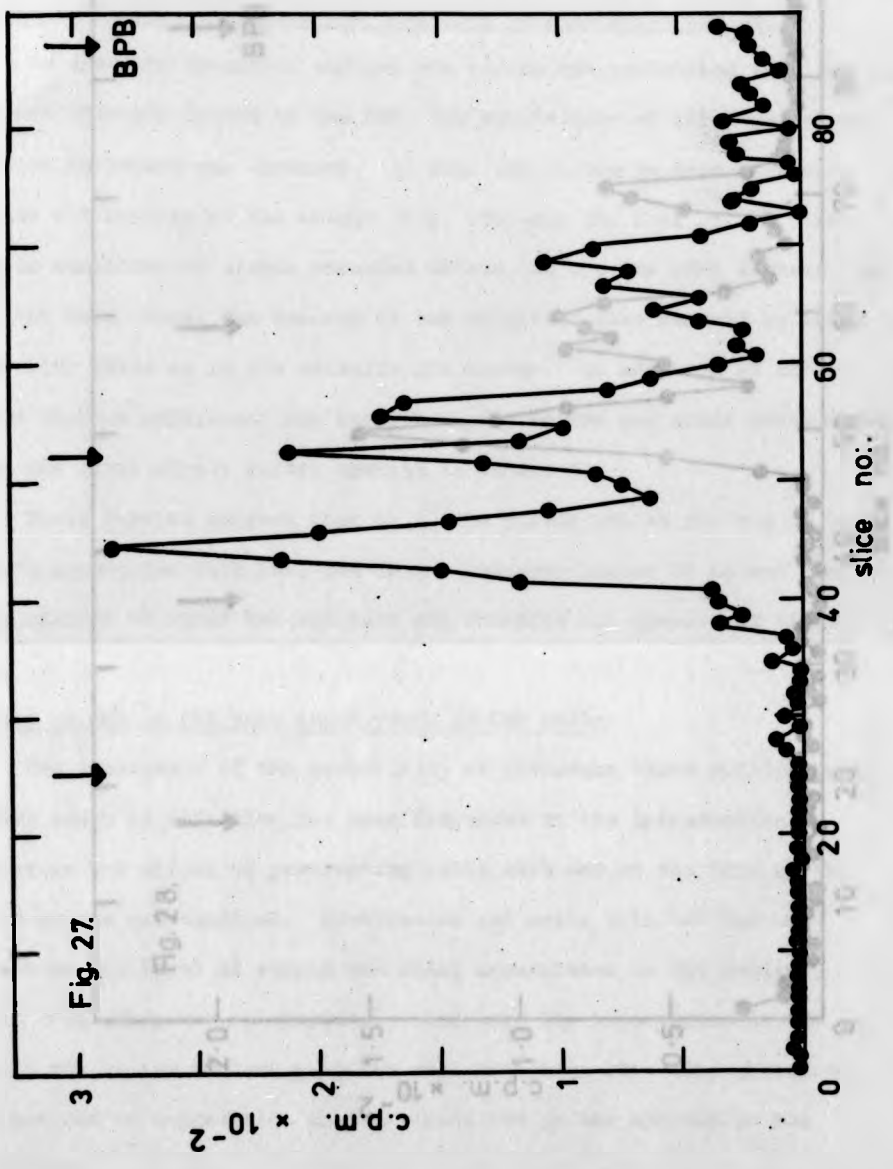

Fig. 27. Electrophoresis of virion RNA extracted in the presence of cold total cell nucleic acids from 3 x 10^8 CEF cells. Conditions of electrophoresis were as for Fig. 24.

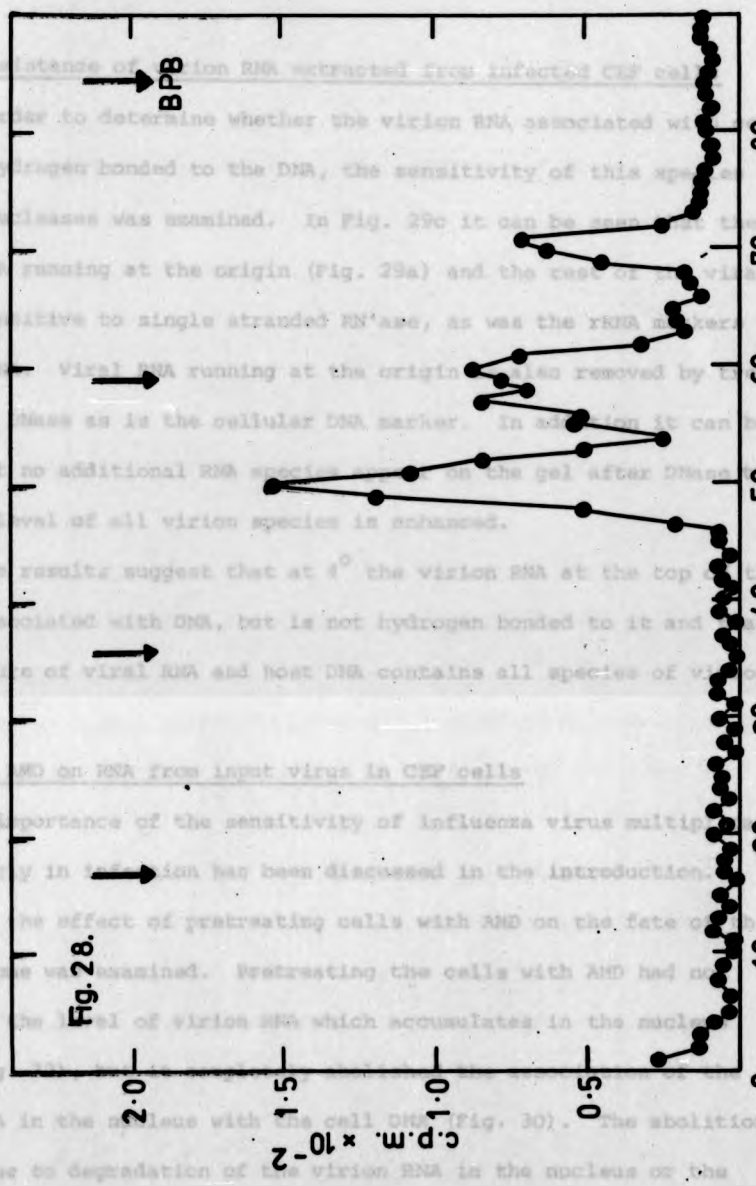

Fig.28.

Fig. 28. **Electrophoresis of virion RNA extracted from CEF cells pre-cooled at 4° for 6 h immediately after the addition of ^{32}P virus. Conditions of infection, RNA extraction as for Fig. 21, and electrophoresis as in Fig. 24.**

Enzyme resistance of virion RNA extracted from infected CEF cells

In order to determine whether the virion RNA associated with cellular DNA was hydrogen bonded to the DNA, the sensitivity of this species to various nucleases was examined. In Fig. 29c it can be seen that the virion RNA running at the origin (Fig. 29a) and the rest of the viral RNA is sensitive to single stranded RN'ase, as was the rRNA markers but not the DNA. Viral RNA running at the origin is also removed by treatment with DNase as is the cellular DNA marker. In addition it can be noted that no additional RNA species appear on the gel after DNase treatment and that the level of all virion species is enhanced.

These results suggest that at 4° the virion RNA at the top of the gel is associated with DNA, but is not hydrogen bonded to it and that this mixture of viral RNA and host DNA contains all species of virion RNA.

Effect of AMD on RNA from input virus in CEF cells

The importance of the sensitivity of influenza virus multiplication to AMD early in infection has been discussed in the introduction. Therefore the effect of pretreating cells with AMD on the fate of the virus genome was examined. Pretreating the cells with AMD had no effect on the level of virion RNA which accumulates in the nucleus at 4° (Fig. 32), but it completely abolished the association of the virion RNA in the nucleus with the cell DNA (Fig. 30). The abolition was not due to degradation of the virion RNA in the nucleus or the cytoplasm.

Effect of cycloheximide on RNA from input virus in CEF cells

Pretreating CEF cells with cycloheximide had no effect on the appearance of total virion RNA in the nucleus (Fig. 32). However the presence of this drug immediately before virus infection did abolish the association of virion RNA in the nucleus with cell DNA (Fig. 31). No breakdown of virion RNA species was observed in either the nucleus

Fig. 29.

Fig. 29. Electrophoresis of virion RNA prepared from the nuclei of CEF cells infected with ^{32}P-labelled FPV/BEL as in Fig. 24. a) control, b) sample treated with DNase (10 μg/ml), c) sample treated with RNase (T_1 -170 units/ml, Pancreatic - 50 μg/ml).

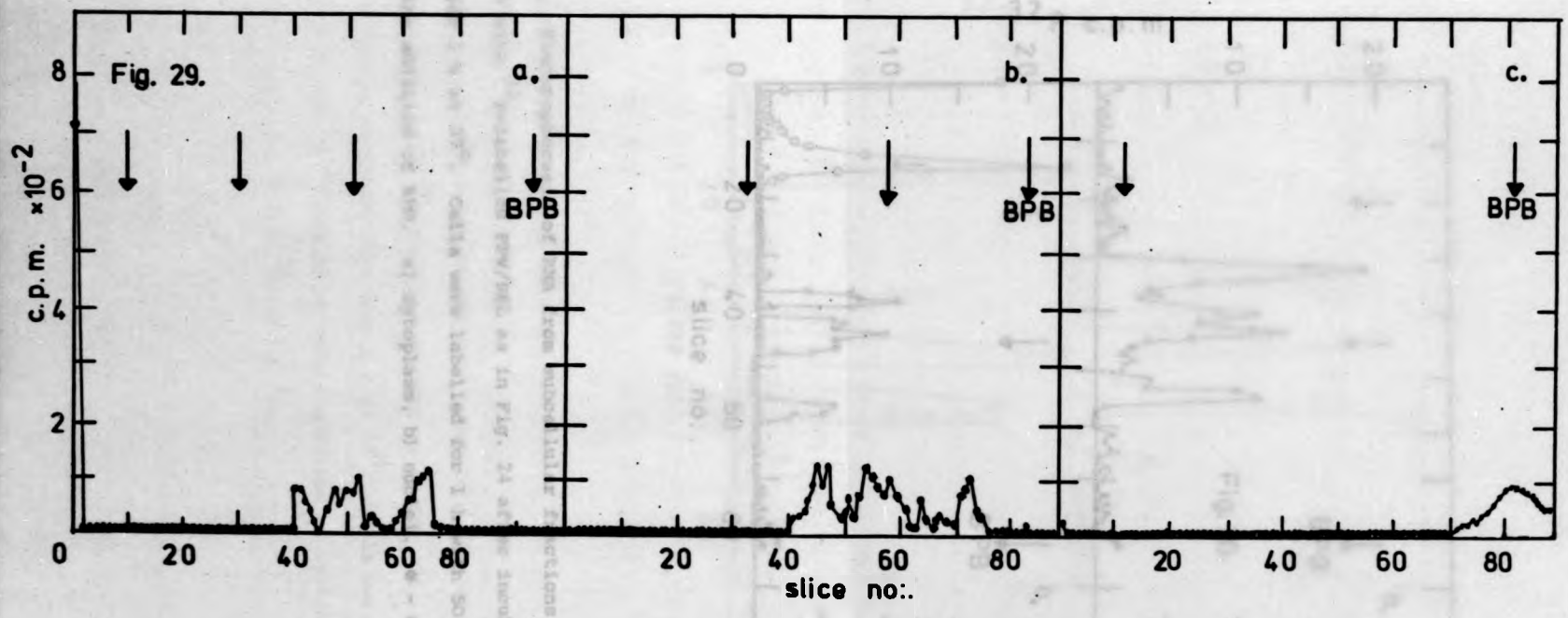

Fig. 29.

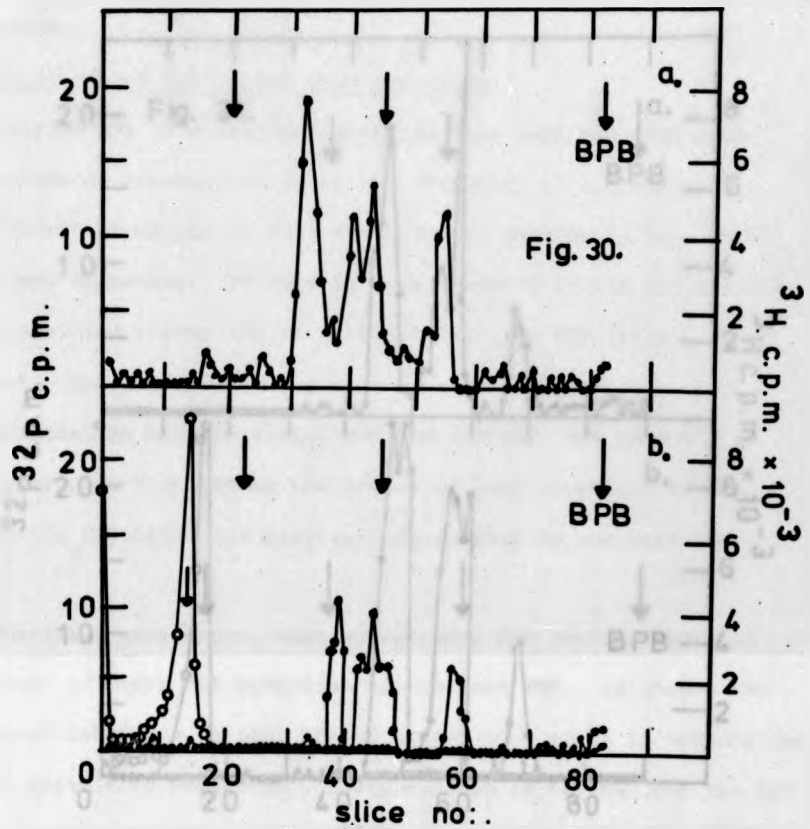

Fig. 30. Electrophoresis of RNA from subcellular fractions of CEF cells infected with ^{32}P-labelled FPV/BEL as in Fig. 24 after incubation with 1 µ of AMD for 1 h at 37°. Cells were labelled for 1 h with 50 µCi ^{3}H thymidi before the addition of AMD. a) cytoplasm, b) nuclei. ● - ● ^{32}P, o - o ^{3}H

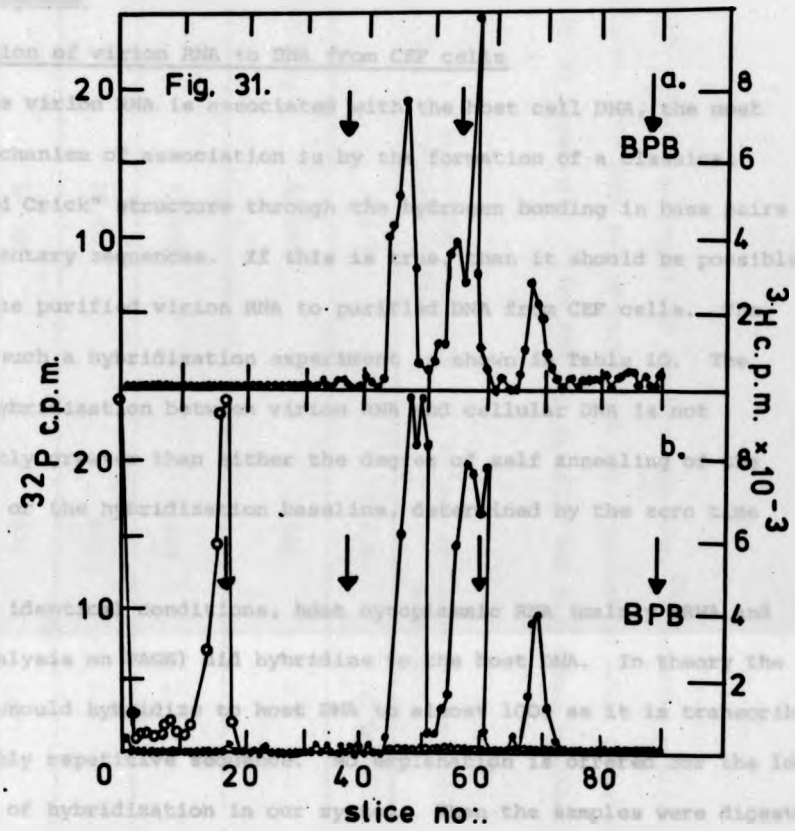

Fig. 31. Electrophoresis of RNA from subcellular fractions of CEF cells infected with ^{32}P-labelled FPV/BEL as in Fig. 24, after incubation with 200 µg/ml cycloheximide from 1 h at 37°. Cells were labelled for 1 h with 50 µCi of ^{3}H thymidine before addition of cycloheximide. a) cytoplasm, b) nucleus. ● - ● ^{32}P, o - o ^{3}H.

or the cytoplasm.

Hybridization of virion RNA to DNA from CEF cells

If the virion RNA is associated with the host cell DNA, the most obvious mechanism of association is by the formation of a classical "Watson and Crick" structure through the hydrogen bonding in base pairs of complementary sequences. If this is true, then it should be possible to hybridize purified virion RNA to purified DNA from CEF cells. The result of such a hybridization experiment is shown in Table 10. The level of hybridization between virion RNA and cellular DNA is not significantly greater than either the degree of self annealing of the virion RNA or the hybridization baseline, determined by the zero time control.

Under identical conditions, host cytoplasmic RNA (mainly rRNA and tRNA by analysis on PAGE) did hybridize to the host DNA. In theory the host rRNA should hybridize to host DNA to almost 100% as it is transcribed from a highly repetitive sequence. No explanation is offered for the low efficiency of hybridization in our system. When the samples were digested for a longer period of time in order to reduce the background level, a similar set of results were obtained.

Therefore it can be concluded that the association of input influenza virus RNA with host DNA is not by classical 'Watson and Crick' hydrogen bonded base pairs.

Location of input virion RNA after incubation at 37°

The location of input vRNA in infected cells was investigated by infecting at 4° for 1 h (i.e. to give a maximum level of vRNA in the nucleus), and then incubating at 37° for the required time. RNA was assayed by measuring TCA insoluble radioactivity as in Fig. 22. Fig. 32 shows that vRNA moves rapidly out of the nucleus to reach a level of 20-

Table 10. Hybridization of CEF DNA with RNA from ^{32}P-labelled purified FPV/BEL virions. All results are the average of duplicates. CEF rRNA was prepared from the cytoplasm of cells labelled with ^{3}H uridine as in Fig. 5. Zero time control was obtained by incubating virion RNA and cellular DNA with RNase immediately after boiling.

Sample	Total TCA insoluble radioactivity	RN'ase resistant TCA insoluble material		TCA insoluble material after 1 h RNAse digestion	
	c.p.m.	c.p.m.	% of Total	c.p.m.	% of Total
Cellular DNA+virion RNA	51,250	1,380	2.7	444	0.86
Cellular DNA+virion RNA	49,400	1,060	2.4	435	0.88
Virion RNA	39,200	600	1.5	336	0.86
Virion RNA	40,000	870	2.1	326	0.81
Cellular DNA+ cellular RNA	37,900	5,230	13.8	1,430	3.8
Cellular DNA+ Cellular RNA	41,750	5,230	12.5	1,540	3.7
Zero time control	83,000	2,130	2.6	730	0.87

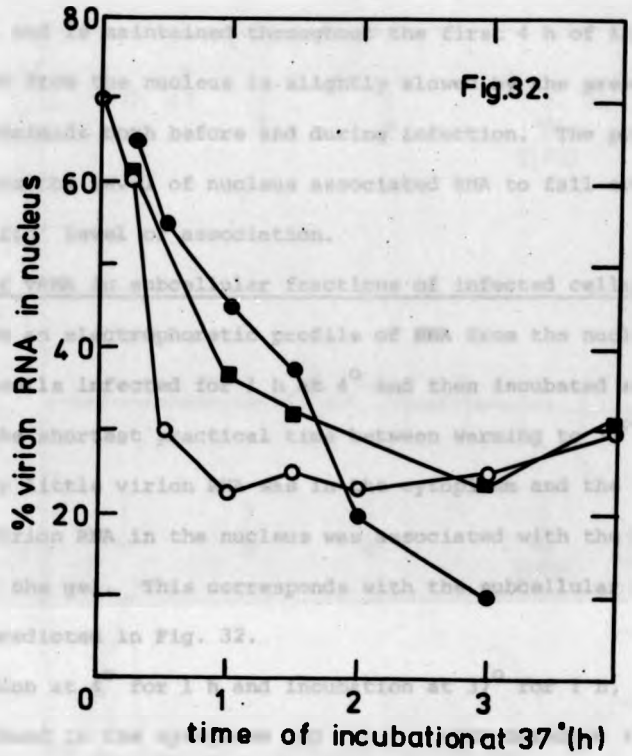

Fig. 32. Time course of the migration of ^{32}P labelled RNA of infecting FPV/BEL virus from the nucleus of CEF cells. Cells were infected at 4° for 1 h as in Fig. 21, then rapidly raised to 37° by the addition of 20 m of medium at 42°. Fractionation, RNA extraction and TCA precipitation were as described in methods. O - O cells without inhibitors, ● - ● cell incubated with 1 µg/ml AMD for 1 h at 37° before infection and upon warmi to 37°, ■ - ■ cells incubated with 200 µg/ml cycloheximide for 1 h at 37° before infection and upon warming to 37°.

30% after ½ h. This level is significantly above the 'non-specific' association level and is maintained throughout the first 4 h of infection.

The migration from the nucleus is slightly slowed by the presence of AMD and cycloheximide both before and during infection. The presence of AMD causes the level of nucleus associated RNA to fall eventually to the 'non-specific' level of association.

Electrophoresis of vRNA in subcellular fractions of infected cells at 37°

Fig. 33 shows an electrophoretic profile of RNA from the nucleus and cytoplasm of cells infected for 1 h at 4° and then incubated at 37° for 3 min (i.e. the shortest practical time between warming to 37° and harvesting). Very little virion RNA was in the cytoplasm and the majority of the virion RNA in the nucleus was associated with the cellular DNA at the top of the gel. This corresponds with the subcellular distribution of vRNA as predicted in Fig. 32.

After infection at 4° for 1 h and incubation at 37° for 1 h, most of the vRNA was found in the cytoplasm and all the vRNA detected in the nucleus was associated with cellular DNA (Fig. 34).

Location of vRNA in cells treated with AMD and warmed to 37°

Cells were incubated with AMD at 37° for 1 h, infected at 4° for 1 h with FPV/BEL and incubated again at 37° for 1 h in the medium containing AMD. Fig. 35 shows that no vRNA was present in the nucleus in any form. No degradation products of the vRNA could be seen in the nucleus as no radioactivity was present at the buffer front represented by the migration of the marker dye. All the vRNA present in these cells was in the cytoplasm: this RNA was characteristic of vRNA in size and no degradation products were seen. The subcellular distribution of vRNA in Fig. 32 corresponds with the results reported here.

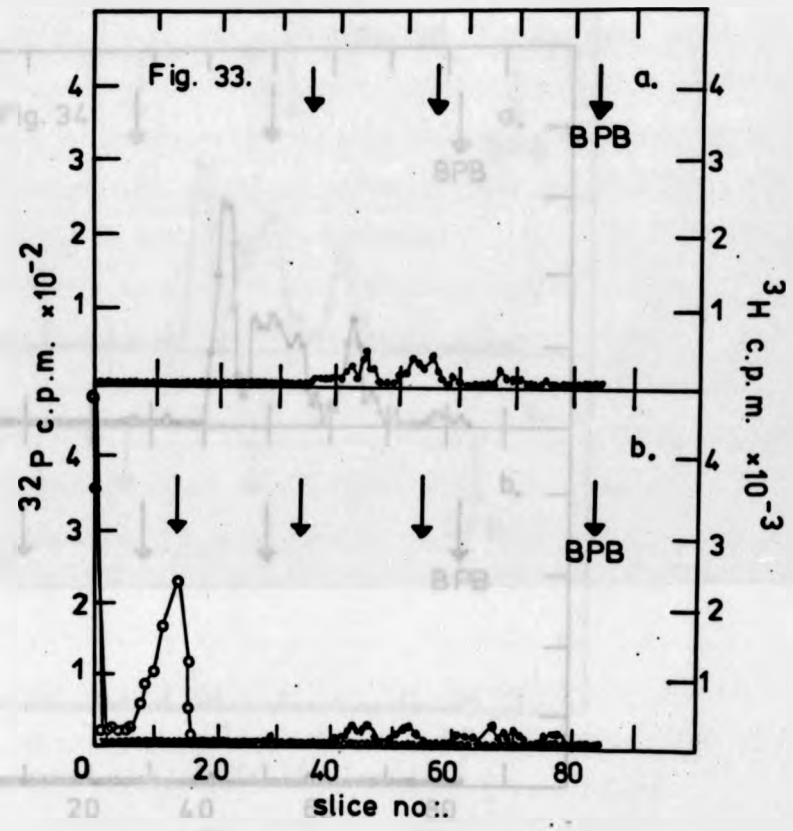

Fig. 33. Electrophoresis of RNA from subcellular fractions of CEF cells infected with ^{32}P labelled FPV/BEL for 1 h at 4° and then incubated at 37° for 3 min. Preparation of cells, adsorption, incubation, and fractionation as in Fig. 21, and RNA extraction as in methods. Electrophoresis as in Fig. 24. Cells were labelled before infection for 1 h with 50 µCi of ^{3}H thymidine. a) cytoplasm, b) nuclei. ● - ● ^{32}P, o - o ^{3}H.

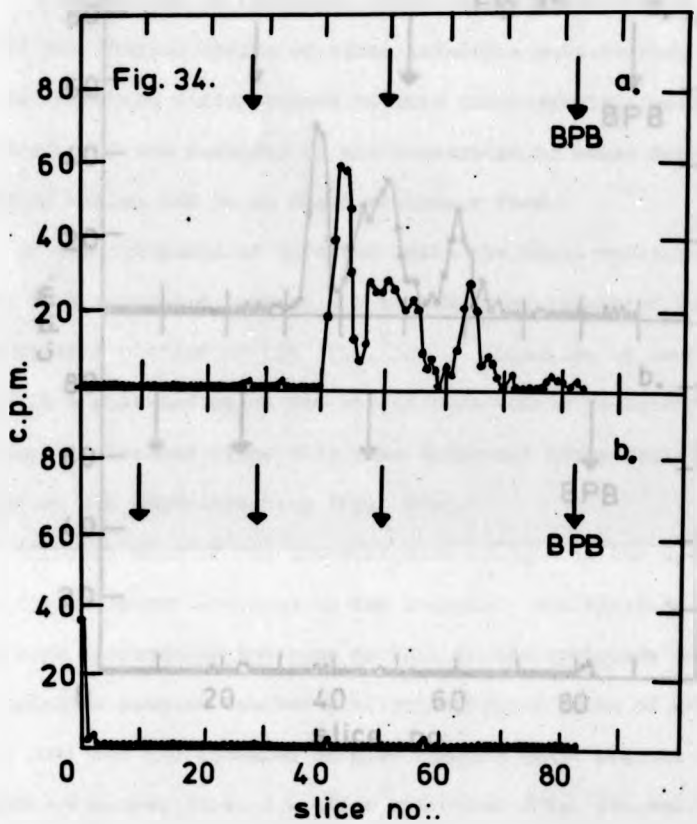

Fig. 34. Electrophoresis of RNA from subcellular fractions of CEF cells infected with ^{32}P labelled FPV/BEL at 4° for 1 h and incubated at 37° for 1 h. Infection, incubation and fractionation as in Fig. 21, RNA extraction as in 'methods' and electrophoresis were as in Fig. 24. a) cytoplasm. b) nucleus.

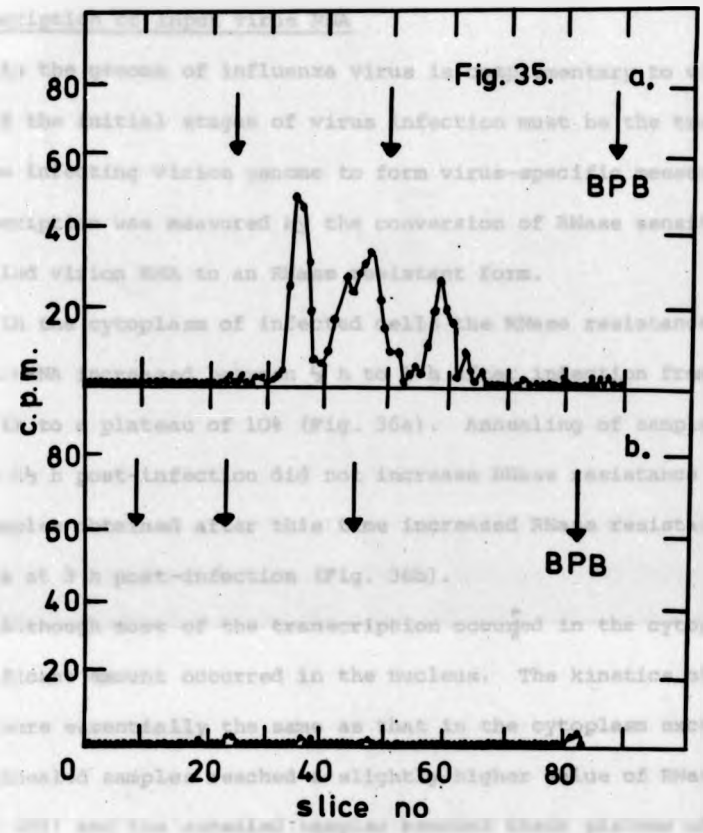

Fig. 35. Electrophoresis of RNA from subcellular fractions of CEF cells, pretreated for 1 h at 37° with 1 µg/ml AMD, infected with ^{32}P labelled FPV/BEL at 4° for 1 h and incubated at 37° for 1 h. Infection and fractionation as in Fig. 21, RNA extraction as in 'methods' and electrophoresis as in Fig. 24. a) cytoplasm. b) nucleus.

Transcription of input virus RNA

As the genome of influenza virus is complementary to viral mRNA, one of the initial stages of virus infection must be the transcription of the infecting virion genome to form virus-specific messengers. This transcription was measured by the conversion of RNase sensitive ^{32}P labelled virion RNA to an RNase resistant form.

In the cytoplasm of infected cells the RNase resistance of the input vRNA increased between ½ h to 1 h after infection from less than 1% to a plateau of 10% (Fig. 36a). Annealing of samples obtained up to 1½ h post-infection did not increase RNase resistance but annealing of samples obtained after this time increased RNase resistance by up to 36% at 3 h post-infection (Fig. 36b).

Although most of the transcription occurred in the cytoplasm, a significant amount occurred in the nucleus. The kinetics of transcription were essentially the same as that in the cytoplasm except that the non-annealed samples reached a slightly higher value of RNase resistance (i.e. 20%) and the annealed samples reached their plateau of RNase resistance sooner (i.e. 2 h after infection (Fig. 36c and d).

Effect of AMD and cycloheximide on transcription

Pretreatment of cells with AMD for 1 h before infection prevented all transcription in both the nucleus and the cytoplasm, as detected by conversion to RNase resistant forms, before and after annealing (Fig. 36).

Following pretreatment with cycloheximide, transcription could be detected in non-annealed nuclear preparations, but not in non-annealed cytoplasmic preparations. The transcription rate was lower than that in untreated cells and was still constant at 3½ h post-infection. Annealing of cytoplasmic and nuclear RNA samples from cycloheximide treated cells gave a higher transcription rate. Up to 1½ h post-infection, the transcription rate in the cytoplasm of treated cells approached that in un-

Fig. 36. Primary transcription of input ^{32}P labelled FPV/BEL in CEF cells. Infection, incubation, and fractionation as in Fig. 21, and RNA extraction were as in 'methods'. For each time point at least 2×10^4 c.p.m. were obtained from infected cultures. a) non-annealed RNase resistant RNA from cytoplasm, b) annealed RNase resistant RNA from cytoplasm, c) non-annealed RNase resistant RNA from nuclei, d) annealed RNase resistant RNA from nuclei. ● - ● cells without inhibitors, O - O cells treated with 1 µg/ml AMD at 37° for 1 h before infection and on subsequent incubation at 37°. □ - □ cells treated with cycloheximide for 1 h at 37° before infection and on subsequent incubation at 37°.

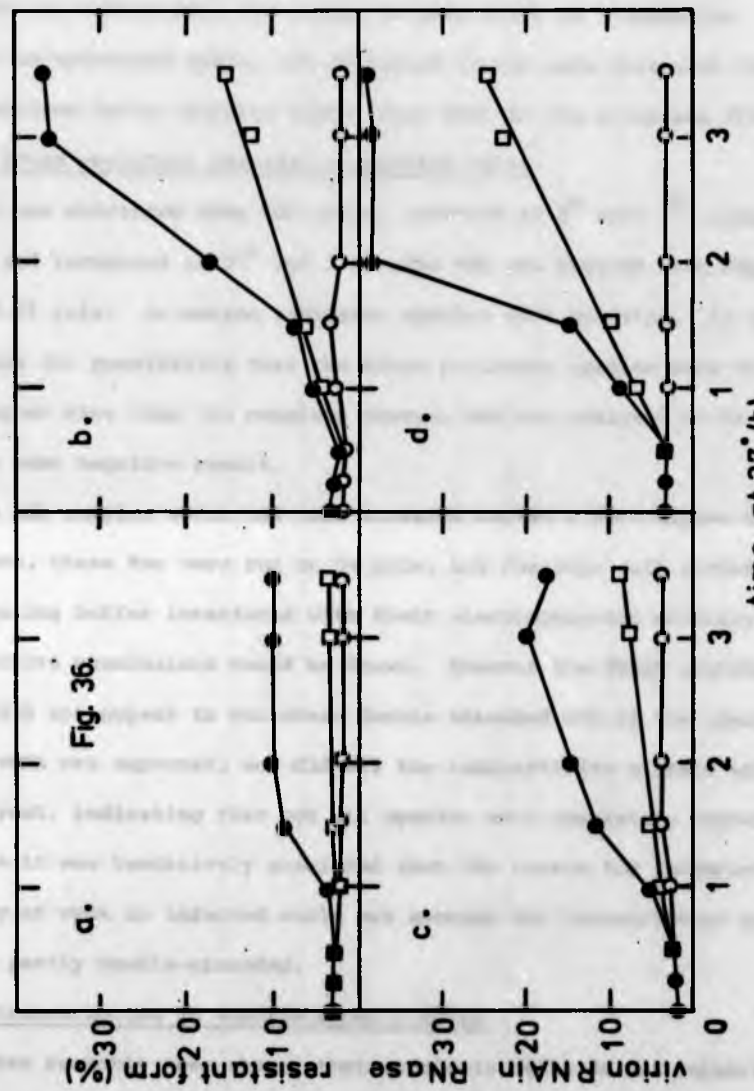

Fig. 36.

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treated cells, although the rate in the nucleus was slightly less than that in the control fractions. Transcription in cycloheximide treated cells did not demonstrate the change in rate after 1½ h infection observed in untreated cells, but continued at the same rate with the rate in the nucleus being slightly higher than that in the cytoplasm (Fig. 36).

PAGE of RNase resistant species in infected cells

RNA was extracted from CEF cells, infected at 4° with ^{32}P labelled FPV/BEL and incubated at 37° for 3 h. The RNA was treated with RNase and run on 2.4% gels: no enzyme resistant species were detected. In order to examine the possibility that the RNase resistant species were of a much smaller size than the complete genome, RNA was analysed on 5% gels with the same negative result.

Since RNA samples which had been annealed showed a much higher RNase resistance, these too were run on 5% gels, but the high salt content of the annealing buffer interfered with their electrophoretic mobility and no definitive conclusions could be drawn. However the RNase resistant species did not appear to run where double stranded RNA of the equivalent size to vRNA was expected, nor did all the radioactivity migrate with the buffer front, indicating that not all species were completely degraded. Therefore it was tentatively concluded that the reason for incomplete annealing of vRNA in infected cells was because the transcription complexes are only partly double-stranded.

Primary transcription in enucleate BSC-1 cells

It has recently been shown that enucleate cells do not support influenza virus replication (Follett et al., 1974; Kelly et al., 1974). In order to examine whether the presence of the nucleus is essential for primary transcription, enucleate BSC-1 cells were kindly prepared by Dr E.A.C. Follett, infected with ^{32}P labelled FPV/BEL and transcription assayed as above (Table 11).

Table 11. Primary transcription of ^{32}P labelled FPV/BEL in BSC-1 cells infected and assayed as in Fig. 36 except that m.o.i. = 10. (A) RNA from 2 x 10^6 enucleate cells, (B) RNA from 2 x 10^6 untreated cells. (C) RNA from 2 x 10^6 cells treated with cytochalasin B but not enucleated. Each figure is from average of duplicate samples.

	Non-annealed			Annealed		
Sample	Total radioactivity (c.p.m.)	RNase resistant radioactivity (c.p.m.)	RNase resistance (%)	Total radioactivity (c.p.m.)	RNase resistant radioactivity (c.p.m.)	RNase resistance (%)
A	8,730	40	0.5	8,850	144	1.4
B	6,900	90	1.3	7,730	194	2.0
C	4,400	80	1.8	5,050	184	2.9

It was difficult to draw any conclusions from these results as the level of transcription in these cells is very low. However the level of transcription in the enucleate cells was lower than that in either of the controls. Under these conditions RNA from purified virion has a resistance of 0.5% (non-annealed) and 1.5% (annealed). Therefore it was tentatively concluded that transcription does not occur in enucleate cells.

DISCUSSION

Subcellular location of RNA from infecting virions in CEF cells at 4°

Data presented in Section II has shown that at 4° FPV/BEL enters the host cell; as judged by the insensitivity of the infecting virus to neutralizing antiserum and acid pH. The data presented in this section shows that not only does virus enter the cell at 4° but the genome entered the nucleus as well. A similar phenomenon in influenza virus infected cells was suggested in experiments by Hoyle and Finter (1957). This effect was not due to contamination of the nuclear fraction by cytoplasmic membranes for the following reasons: 1) membranes isolated from infected cells contain little virion RNA and that which is present is in the E.R. and not the plasma membrane, 2) the level of 'non-specific' association of virion RNA with the nuclear fraction was only 12%, much lower than that observed when cells were infected normally, 3) other membrane bound viruses which do not require a nucleus for their multiplication, i.e. SFV and Sendai virus gave a level of nuclear association only marginally above the non-specific level.

Input virion RNA, isolated from both the cytoplasm and the nuclei of infected cells had an electrophoretic profile identical to that of RNA from purified virions. There was no apparent breakdown of vRNA inside the cell, unlike that observed with poliovirus infection (Dales, 1965), although the infection is relatively inefficient as even under optimum conditions, only 30% of the virus particles attach to the cells and of these particles about 50% are subsequently eluted off on warming to 37°. The protection of virion RNA against degradation is probably because it is present in the cell as an RNP complex. Certainly at least two virion proteins reach the nucleus and either or both could perform this protective function.

Probably the most intriguing finding in these studies is the association of the virion RNA with the DNA of the host cell. Evidence has been presented that this is not an artefact of either the electrophoretic system or of the extraction system. The latter is more difficult to prove however and it would have been useful to study the behaviour of a paramyxovirus under identical conditions. This experiment was tried but no conclusive results were obtained in the time available due to several technical difficulties.

The association of virion RNA with cellular DNA appears to be a function of the secondary structure of the DNA rather than its base sequence, for the following reasons: 1) no sequence complementarity could be detected between the virion RNA and cellular DNA by 'in vitro' hybridization, 2) 'in vivo' the complex was sensitive to both RNase and DNase, 3) the virion RNA associated with the macromolecular DNA at the top of the gel and not with the partially degraded DNA which entered the gel, 4) the complex is abolished completely by pretreatment with AMD, whose primary effect is to distort the DNA helix and thus alter its secondary structure. This association also appears to be dependent on a rapidly turned over host protein as it is sensitive to pretreatment of the cells with cycloheximide. However this result has not been repeated and it may be a spurious piece of data. If this data were true, it would be very difficult to explain the fact that only the second phase of virus transcription is sensitive to this drug.

Location of virion RNA after warming infected cultures to 37°

Immediately on warming to 37° all the virion RNA in the nucleus becomes associated with the high molecular weight DNA. Subsequent treatment with DNase reveals that all the major size classes of the virion RNA are associated with the DNA.

The virion RNA then moves rapidly out of the nucleus. A significant remainder (20-30%) stays in the nucleus throughout infection, all of which is associated with high molecular weight DNA. The exit from the nucleus is only slightly retarded by AMD or cycloheximide treatment and the final level reached in the presence of AMD is equal to the level of non-specific association and none of this RNA is associated with cellular DNA. The presence of vRNA from infecting virions in the nucleus of infected cells and its association with DNA reported here is unique for an RNA virus. The only similar phenomenon is the integration of tumour virus RNA into the DNA of the host cell, but this mechanism has been shown to differ from the mechanism in influenza virus infection by its drug sensitivity, its dependence on the cell replication machinery and the presence of sequences in the vRNA complementary to those in the host DNA.

It is interesting to note, that at the time of the exit of the RNA from the nucleus, the association of the input neuraminidase with the nuclear fraction was increasing. It could be one of the functions of the neuraminidase of influenza virus to assist the transport of virion RNA (presumably as an RNP) from the nucleus.

Throughout the time period studied, both at 37° and 4°, the input vRNA had a similar electrophoretic profile, both in the nucleus and the cytoplasm, to that of RNA from purified virions. No degradation was ever observed even in the presence of AMD.

Transcription of input virion RNA

Transcription of input virion RNA was shown to occur both in the nucleus and the cytoplasm of CEF cells. This transcription was completely abolished in both fractions by pretreatment of the cells with AMD. This finding was somewhat surprising since the 'in vitro' activity of the virion polymerase is insensitive to the drug (Ho and Walters, 1966). The initial

rate of transcription in both fractions was insensitive to treatment with cycloheximide but the enhanced rate, observed after 1½ h infection, was sensitive to this drug. Therefore the synthesis of a polypeptide, either coded for by the virus or by the host cell is not necessary for the initial transcription but is necessary for the enhanced rate to be observed.

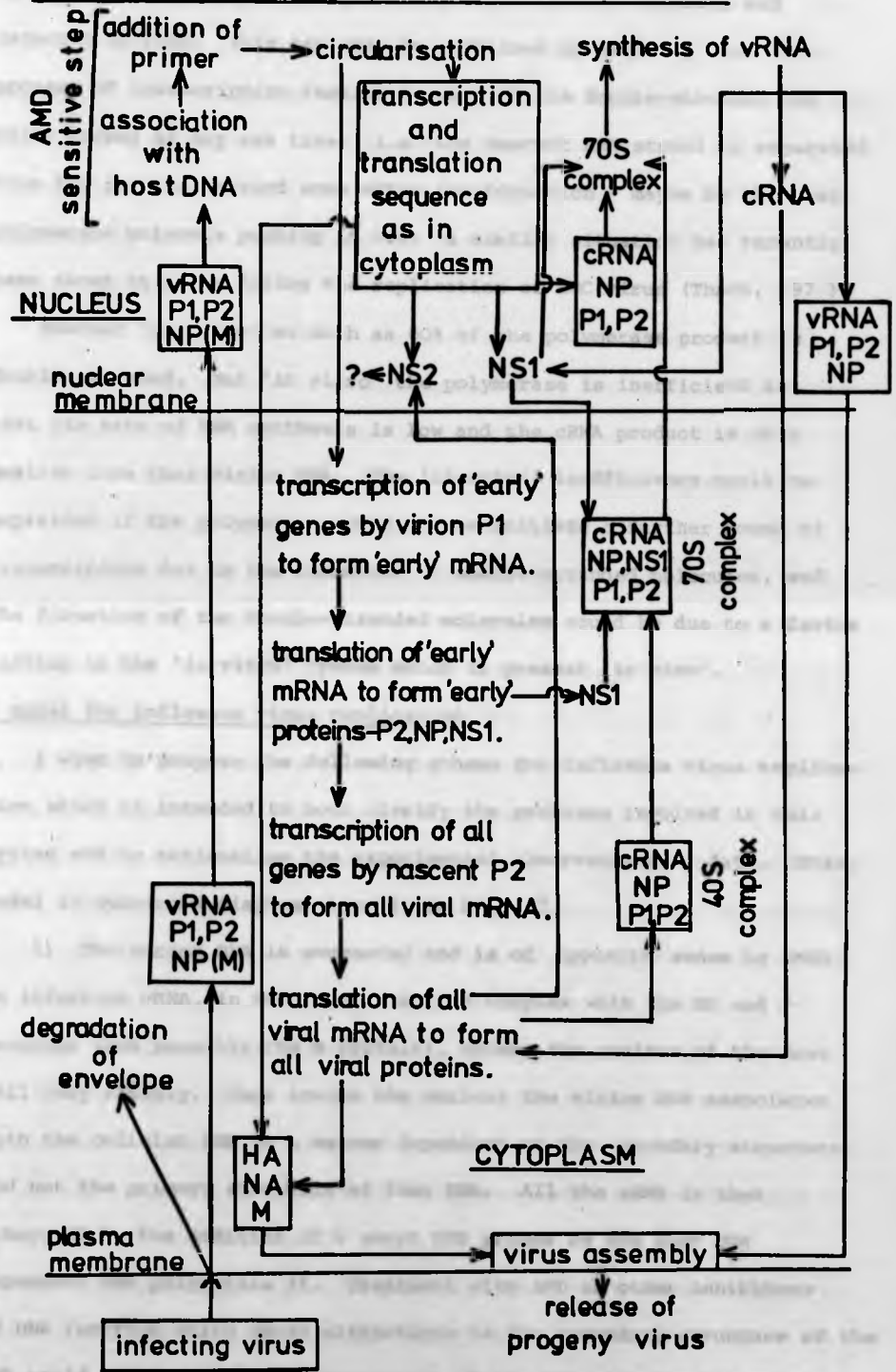
One of the striking aspects of the transcription of the influenza virus genome is a lag phase of at least 30 min before transcription commences. This correlates closely with the exit of the virus RNA from the nucleus, thus implying that the nuclear step is necessary before transcription can occur. This assumption is in agreement with the apparent absence of transcription in enucleate cells. It is interesting to compare VSV transcription with that of influenza virus. In VSV infected cells, although two rates of transcription are observed which show the same sensitivity to cycloheximide as influenza virus, transcription is insensitive to AMD and does not have a lag phase (Huang and Manders, 1972). Furthermore VSV does not need a nucleus to replicate as does influenza virus (Follett et al., 1974).

The data presented here corresponds with that from unfractionated cells presented by Bean and Simpson (1973), except that the biphasic rate of transcription was only observed when the RNA had been self-annealed and the final level of self-annealing was only 35-40% instead of 80-85% achieved by the above authors. The only explanation offered for this discrepency is that these authors worked with a different cell system to the one used in this investigation.

Structure of the transcription complex

A curious aspect of the transcription process is that very little (at the most 10%) of the input virion RNA is found in a double stranded form unless it is self-annealed. Similarly if RNA was extracted from infected cells incubated for 3 h (i.e. when transcription was maximal)

Fig. 37. A model for influenza virus multiplication.



and treated with RNase, very little RNase resistant material was detected by PAGE. This can only be explained by assuming that the process of transcription results in very little double-stranded RNA being formed at any one time; i.e. the nascent RNA strand is separated from the parental strand soon after its formation - maybe by the next polymerase molecule pushing it off. A similar situation has recently been shown to occur during the replication of EMC virus (Thach, 197).

However 'in vitro' as much as 60% of the polymerase product is double stranded. But 'in vitro' the polymerase is inefficient in that its rate of RNA synthesis is low and the cRNA product is of a smaller size than virion RNA. The 'in vitro' inefficiency could be explained if the polymerase could not re-initiate a further round of transcription due to the formation of double-stranded molecules, and the formation of the double-stranded molecules could be due to a factor lacking in the 'in vitro' system which is present 'in vivo'.

A model for influenza virus replication

I wish to propose the following scheme for influenza virus replication which is intended to both clarify the problems involved in this system and to rationalize the experimental observations to date. This model is summarized diagrammatically in Fig. 37.

1) The virion RNA is segmented and is of opposite sense to mRNA. On infection vRNA, in the form of an RNP complex with the NP and P proteins (and possibly the M protein), enters the nucleus of the host cell very rapidly. Once inside the nucleus the virion RNA associates with the cellular DNA in a manner dependent on the secondary structure and not the primary structure of that DNA. All the vRNA is then 'charged' by the addition of a short RNA primer by the host DNA dependent RNA polymerase II. Treatment with AMD or other inhibitors of DNA function which cause alterations in the secondary structure of the DNA would thus prevent the association of the RNA with the DNA or the

addition of the correct RNA primer, if it is host coded, or both. The nature of the RNA primer is unknown, but I would like to propose that the addition of the primer enables the RNA to circularize. This process would enable the virion polymerase to continually produce mRNA in a highly efficient manner. This circularization does not occur'in vitro' and could be one of the reasons for the inefficiency of the 'in vitro' polymerase reaction.

2) The charged vRNA molecules move out of the nucleus although a small percentage remain. The messengers coding for the early proteins (P2, NP and NS1) are synthesised in both nucleus and cytoplasm and are then all translated in the cytoplasm to form the early proteins. Thus the initial nuclear step would explain the lag observed in initial transcription, which does not occur with related viruses such as VSV.

3) The newly synthesised P2 protein could then act as a polymerase and synthesise mRNA for both early and late virion proteins. Thus the rise in transcription rate is observed after the first 90 min of infection and is sensitive to cycloheximide. A similar form of temporal control is observed in reovirus replication and that of the RNA phages. A possible mechanism for this control could be the variations in the secondary structure of the initiation sites of the vRNA species such that the efficiency of binding of the virion polymerase for the species coding for late viral proteins is low but the efficiency of binding for newly synthesised P2 is high. A similar situation occurs in the transcription of the RNA phages: however, unlike the RNA phages there appears to be no mechanisms for switching off the production of one mRNA species in preference to another, but all transcription is terminated at the same time.

4) P2 then associates with newly synthesised P1 and NP to form a 40S complex; then binds to NS1 to form a 70S complex (A.J. Hay -

personal communication). This binding of NS1 could then convert the transcriptases (P1 and P2) to replicases.

5) The binding of NS1 to the 40S complex causes it to have a high affinity for the nucleus. Therefore this 70 S RNP complex moves to the nucleus where vRNA synthesis takes place. On completion of vRNA synthesis, the cRNA template and NS1 dissociate from the complex. The RNP complex now has a low affinity for the nucleus and so it now migrates to the cytoplasm where it is incorporated into progeny virus particles. The transport from the nucleus could be a function of the virus neuraminidase.

Inadequacies of the model

1) This scheme does not explain the sensitivity of virus replication to AMD throughout infection. AMD added late in infection appears to stop vRNA synthesis although it only slows down protein synthesis (Gregoriades, 1970; P.D. Minor - personal communication). This effect could be explained if the input vRNA needed to be constantly 'recharged' in order to produce mRNA. If mRNA synthesis was thus blocked in AMD treated cells there would be insufficient to saturate the protein synthesising machinery and therefore there would be none to act as template for vRNA synthesis.

2) No explanation is offered for the function of NS2.

3) The appearance of NS1 in the nucleolus is not explained, although this protein could have a dual role as discussed in Section I.

4) P2 is present in virus particles and therefore, if it cannot take place in transcription early in infection as suggested by the above model, it must be inactivated in some way. There is a suggestion in the data in Section III that M protein from the input virus enters the nucleus and it thus could act as an inhibitor of P2 from input virus.

Predictions arising from the model

1) The 'charging' of the input vRNA should be sensitive to pretreatment with drugs which act on DNA dependent RNA polymerase II, such as α-amanitin.

2) Although the transcription of both early and late messengers can occur in the nucleus and the cytoplasm, vRNA synthesis can only occur in the nucleus.

3) Early in infection some messengers will be seen before others. There is already some indication that this may be so (Skehel, 1973).

4) If added to freshly homogenized cells, the virion RNP and the 70 S complex of cRNA, P1, P2 and NS1 will have an affinity for the nuclear fraction, whereas the charged virion RNA in its RNP complex, P2 and the 40 S complex of P1, P2 and NP will be relatively non-specific in their subcellular affinity.

5) The products of the 'in vitro' virion polymerase reaction could be charged by selectively removing various virus proteins such as M or adding others such as NS1.

6) The mutant described by Mackenzie and Dimmock (1973) in which the RNP antigen does not move out of the nucleus, should also be found to have a defective neuraminidase.

7) A similar experiment to that described by Perlman and Huang (1973) (see introduction to this section) with VSV could be performed with influenza viz. if a suitable mutant could be found, the inter-relationship between replication and transcription could be studied and perhaps it could be determined which proteins were involved and in what capacity.

CONCLUSIONS

1) The maturation pathway for CEF rRNAs has been described, and is similar in principle to that previously reported for other eukaryotic systems. The apparent mol. wts. of the mature rRNAs and their precursors correspond to the position of avian embryonic fibroblasts in a taxonomic evolutionary table.

2) Infection with influenza virus causes a random degradation of the 2.9×10^6 precursor RNA described in the above maturation scheme, instead of the controlled cleavage seen in uninfected cells. The alteration of the processing of the rRNA precursors is not due to lack of methylation or to alterations in the size of nucleotide pools. It is not caused by a protein from the infecting virus particle as U.V. irradiation of the virus blocks its effect on rRNA synthesis.

3) Attachment of influenza virus to CEF cells occurs very rapidly at 4°. This attachment occurs in two steps. In the first step the virus cannot be washed off, but is sensitive to neutralizing antibody and acid pH. In the second step the virus in insensitive to both these agents.

4) Micropinocytosis (as assayed by the uptake of radiolabelled colloidal gold) occurs in CEF cells at 4° under the conditions used for virus attachment and is thus a possible mechanism for virus infection.

5) At 4° input vRNA enters the nucleus of the host cell and becomes attached to the cellular DNA. This association with DNA is dependent upon the secondary structure and not the primary structure of the cellular DNA. The association is sensitive to AMD and cycloheximide and does not occur during infection with other related enveloped viruses. When in the nucleus the input vRNA appears to be accompanied by the NP and M proteins from the virus particle.

6) On warming to 37° the association of the vRNA with the cellular DNA is complete within 3 min, then most of the vRNA moves out of the nucleus, leaving a remainder, most of which is attached to DNA. The exit from the nucleus is only slightly retarded by treatment with AMD and cycloheximide, but the final level of RNA in the nucleus in the presence of AMD is down to that of 'non-specific association'.

7) Transcription of the input vRNA occurs mostly in the cytoplasm, is sensitive at all times to AMD and did not occur in enucleate cells. There is a lag of 30 min before transcription occurs which is not seen in related viruses such as VSV. The time-course of the lag correlates well with the exit of vRNA from the nucleus. From 30 min to 90 min post-infection the transcription rate is constant and is insensitive to cycloheximide. But from 90 min to 2½ h post-infection the transcription rate is increased and this increase is sensitive to cycloheximide. During the process of transcription, very little double stranded RNA was present. It was concluded therefore that in influenza virus infection, the association of the input vRNA with cellular DNA is an essential pre-requisite to transcription of the viral genome.

Roads go ever on,
Over rock and under tree,
By caves where sun has never shone,
By streams that never find the sea,
Over snow by winter sown,
And through the merry flowers of June,
Over grass and over stone,
And under mountains in the moon.

Roads go ever on,
Under cloud and under star,
Yet feet that wandering have gone
Turn back at last to home afar
Eyes that fire and sword have seen
And horror in the halls of stone
Look at last on meadows green
And trees and hills they long have known.

(J.R.R. Tolkien - The Hobbit)

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