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STUDIES ON VIRUS REPLICATION

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COMMISSION OF THE EUROPEAN COMMUNITIES

STUDIES ON VIRUS REPLICATION

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SESSION I

SYNTHESIS OF VIRAL RNA

SYNTHESIS OF TOGAVIRUS RNA

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The present Virus Replication meeting has been arranged by the Commission of European Communities, more precisely by its swine fever virus group. The causative agent of one of the diseases, European swine fever or hog cholera has been identified as a member of the toga-family of viruses (26) and this is why I have been asked to summarize the present knowledge on the synthesis of togavirus RNAs.

Most of the data which will be discussed have been obtained with arthropod-borne togaviruses of the serological group A (alphaviruses 65), Sindbis (SIN) and Semliki forest virus (SF) being the most exhaustively studied representatives. Members of the serological group B (flavoviruses 65), such as dengue, Japanese encefalitis, St. Louis encefalitis viruses and others have been investigated, too, and of the nonarbo togaviruses (25) rubella virus has been analyzed. For the sake of clearness in presentation I should like to review in the first place results obtained with the alphagroup of togaviruses.

Togaviruses are spherical enveloped single-stranded RNA viruses. The RNA is in one piece and is infectious when extracted and assayed appropriately. In the virion it was shown to play a structural role since treatment of isolated nucleocapsids with pancreatic RNase resulted not only in a reduction in sedimentation coefficient and RNA content (33) but also in size (5). RNA extracted from the virion shows properties indicating significant secondary structure which is necessary for infectivity. When heating of an RNA preparation was followed by rapid cooling, the residual infectivity increased with salt concentration. Slow cooling resulted in a better survival than rapid cooling. These conditions did not favour survival of poliovirus RNA, included for comparison (54). With other alphaviruses hyperchromicity has been demonstrated for the RNA (WEE: Tm = 57.5° C (57); SIN: Tm = 57.5° C (43); SFV: Tm = 65° C (10), in contrast to ribosomal RNA serving as a marker. When SIN virus RNA was

chromatographed on columns of cellulose CF-II at different temperatures, the ratio of the amounts of RNA eluting in the single-stranded peaks to those eluting in the double-stranded peak increased at higher temperatures (I8). When prepared from the virion or its nucleocapsid alphavirus RNA sediments at 40 (57) to 49 S (50) on sucrose gradients and moves as a homogeneous fraction on polyacrylamide gels (57,I0, I4). To avoid confusion arising from the difference in S_{20}- values given by different authors this nucleic acid will be termed "virion RNA" in this paper. From infected cells an additional single-stranded RNA species possessing a sedimentation coefficient of 26 S could be extrated (2I, 7, 53, 57). Virion RNA could be transformed into the 26 S ("interjacent") species by treatments which are known to break hydrogen bonds (heating/rapid cooling, urea, DMSO 57, I3, I2). However, if precautions had been taken to warrant virion RNA preparations free of hidden breaks, no changes towards a lower sedimentation coefficient were observed after heating or treatment with denaturing agents (I). Based on the observation that both RNA species have the same base ratios (20, 53, 57), it has been suggested that they may differ in configuration (57) or might be polymerization/depolymerization products (9, I3, I4, I2). Recently, it was shown for SIN virus by hybridization-competition experiments using double-stranded RNA isolated from infected cells that 26 S RNA represents a unique fraction of the viral genome, containing I/3 of the base sequences in the parental strand (50).

Little is known on the primary structure of togavirus RNA. Since the terminal RNA oligonucleotide sequences are accessible for characterization by several techniques, viral RNAs have also been studied in this respect. Poly(A) sequences were observed in the genome of togavirus (alphagroup) representatives, namely EEE (?), SIN (I7, 30, 3I) and SF (I5, II). They were also found in poliovirus (2) and Columbia SK-virus, but lack in vesicular stomatitis virus which is negative-stranded (3I). As togaviruses do not possess a virion associated polymerase (3), it was suggested that parental RNA might serve as the initial messenger molecule during infection. From polysomes of SIN-infected cells poly (A) strands could be liberated (I6). The size of the polyadenylic fragment in virion RNA was estimated as being 50 - I00 nucleotides in length (2, 30, II). However, using membrane-binding

and sequential extraction at different pH, a still greater heterogeneity among the SIN RNA species was found with respect to their poly (A) content, which ranged from 60 - 250 nucleotides (I7). Virion and interjacent RNA differ in their polyadenylic acid content, the former containing I00 - II0 and the latter 63 - 67 residues (II). - The poly (A) sequence of SIN virus is not located at the 3'- terminus but about 6% in from it; the virion RNA possesses a 3'-terminal uridine residue (I5).

After infection of a cell, a double-stranded RNA sedimenting at I6 - 20 S after RNase treatment (2I, 53, I9, 38) can be extracted as the first species. This has been determined either by labelling early (one hour) in infection (I9) or by using short pulses (2I,9); also in vitro it was the earliest and main product of a virus specified polymerase (37, 36, 58, 35, 38). This double-stranded RNA, which is termed replicative form (RF) has been demonstrated after extraction by serological methods (60). By most workers it has been produced after RNase treatment of a RNA with a polydisperse sedimentation profile (52, 2I, 53, 4I, 8), the replicative intermediate (RI) which shows properties of both single- and double- stranded RNA (I9). The question of whether the double-stranded RNAs are actually present as such or are artifacts of deproteinization and isolation has been answered by their demonstration in infected cells using fluoresceine-labelled antibodies against double-stranded polyribonucleotides (6I). Replicative internediates of alphaviruses have been found to sediment between I4 - 30 S (42, I9, 32); they are considered as molecules consisting of a double-stranded core (the RF) with single-stranded tails of nascent RNA attached to it, which can be removed by controlled degradation with pancreatic RNase. Kinetic and pulse chase experiments in vitro and in vivo have shown that the double-stranded RNAs are the precursors of all single-stranded species (47, 38).

With the aim of obtaining information on the sequence of events during viral RNA synthesis, the RF has been studied in detail, since it can be isolated with comparative ease from RNase-treated extracts of infected cells. Further evidence for its double-stranded nature comes from its high melting temperature Tm = I03° C in 0.I5 M NaCI (53); II0°C in 0.2I M phosphate;

97°C in 0.02 M KCl (cf.20). The concept of a single, homogeneous RF had to be abandoned when more than on species of double-stranded RNA was identified (35, 51). The RFs with estimated molecular weights of 8.8×10^6 (23.5 S - RF I), 5.6×10^6 (20.1 S - RF II) and 2.9×10^6 (16 S - RF III) could be separated from each other by gradient centrifugation and gel electrophoresis and their molar ratios determined throughout the infection cycle. The two smaller forms RF II and RF III were always found to exist in a I:I ratio in the cell, although varying in their total amounts. The molar ratio of RF I to the other forms was constant at 0.4 : I between 2 and 6 hours post infectionem, which is in the middle of the replication cycle. All three RFs could be liberated by RNase treatment from the peak of RIs, which have molecular weights of 8.8×10^6. This indicated that the two smaller forms (RF II and RF III) were joined together in the RI before enzyme treatment, a suggestion which is supported by the constant I:I proportion of their occurrence. The rates of label incorporation into the three RFs were determined; they can be considered as a measure of single strand synthesis from the respective templates. It was demonstrated that RF II was labelled 20 to 50 times more slowly than the other forms, which were labelled equally rapidly. This would imply that the corresponding single-stranded RNA species would be synthesized in comparatively small amounts. - From hybridization competition experiments it became clear that the minus strand of the smallest RF (RF III) is complementary to the single-stranded 26 S RNA species and the minus strand of RF II is complementary to the remainder of the viral genome. A precursor-product relationship has been postulated between RF I and the virion RNA and between RF III and the 26 S RNA found in infected cells (51). Early in the replication cycle (before 3 hours post infectionem) the 26 S RNA is the first single-stranded species detected after short pulses (21) and showing the highest rate of synthesis (53). When examined in vitro 4 hours post infectionem the 26 S and the virion RNA species were synthesized at an equal linear rate for about 10 min after which time 26 S RNA synthesis decreased (38). Later in infection, a relative increase in virion RNA synthesis was stated in vivo (51) and in vitro (38). A third species of virus-specific RNA with a base sequence complementary to the minus trand of RF II had to be expected, which, on the basis of its low rate of label incorporation, would occur in comparatively small amounts. Meanwhile,

a further single-stranded 33 S RNA has indeed been detected (35, 34, 38, II) which may well be the candidate for the RF II product. Since this species was isolated from polysomes of infected cells (34) and was shown to contain a polyadenylic sequence (II) there is strong suggestion that this molecule serves a messenger function, too.

In summarizing the data on RNA species encountered in alphavirus-infected cells, the following list can be made up :

I. The predominant infectious (64), single-stranded (parental or prospective) virion RNA, sedimenting at 40 (57) to 49 S (50).

2. A minor single-stranded 38 S RNA (35, II).

3. A minor single-stranded 33 S RNA (35, 34, II)

4. A single-stranded "interjacent" 26 S species (53).

5. A single-stranded 20 to 22 S RNA (32, 35, 38).

6. A polydisperse, partly double-stranded replicative intermediate (RI) sedimenting at I4 - 30 S (I9, 32) which contains a RNase resistant I6 S-core (I9).

7. A double-stranded replicative form (RF), sedimenting at about 20 S (20, 53), which is analogous to the resistant core produced by RNase treatment of the RI. It is heterogeneous, consisting of at least three forms (35, 5I).

8. Further double-stranded RNAs sedimenting at I5 S (49,60); these species were specifically detected in cells infected at high multiplicities with virus stocks containing incomplete particles which had been derived by repeated passages at high m.o.i. (29, 46).

Not all of these forms have been invariably detected by different authors; it has been demonstrated that their relative amounts can vary with the virus (SF, SIN) or cell culture (human, chick) employed (35).

From the experimental results published in about 40 papers during the last ten years, the following - although still incomplete - picture of the replication of alphavirus RNA emerges: when the genome of the infecting virion had been decoated it will serve as a messenger, coding for an enzyme which allows its conversion into a double-stranded form; both virus-specific RNA and protein have been found associated with rapidly sedimenting, detergent sensitive structures in the infected cell (20, 4, 56, 32, 23, 55, 68, 38). A membrane-associated replication complex containing the RNA polymerase with its products and consisting of unique membraneous vesicles (type I cytopathic vacuoles) has been isolated (22, 24). Two types of RI with nearly identical sedimentation characteristics are then formed, one of them being a continuous double-stranded molecule with nascent polynucleotide chains. Its minus strand is the template for the virion RNA. The other RI has a gap in a specific region of the plus strand with a corresponding RNase susceptible single-stranded nucleotide sequence. This explains the appearance of RF II and RF III after enzymatic hydrolysis, whose molecular weights add up to approximately the weight of RF I. The product of the smallest RF, the 26 S RNA, most probably is the messenger for the virion structural proteins, which are translated as a large polypeptide chain and cleaved subsequently (for literature see 62, 40). Since the minor 33 S (and 38 S) RNA are not prominent in infection they may be required only in small amounts and may have late regulatory function (35).

In the flavovirus genus of the togavirus family similar observations as those reviewed for the alphaviruses have been made. The genome appears slightly smaller and is denatured to slower sedimenting RNA species by urea treatment (6). Besides the virion progeny RNA, 26 S and double-stranded 20 S species have been detected in extracts of cells infected with JBE (67), Dengue (59) and SLE (63, 44) viruses. The only nonarbo togavirus representative studied, rubella virus, has equally been shown to contain single-stranded infectious RNA sedimenting at 38 - 40 S; in infected cells an additional (partially) double-stranded RNA was detected and indications for an interjacent species were found (66, 27, 28, 48).

More data on the RNAs of animal viruses classified with the togafamily on the basis of their size, ether-lability and substructure are certainly needed. A beginning was made in our laboratory (39) by studing mucosal disease/diarrhea virus of cattle (MD-virus), which serologically is closely related to hog cholera virus. The reason for selecting this virus was that it can be assayed by plaque titration whereas hog cholera virus must be quantitated by immunofluorescent focus counts. Using the phenol-SDS method an infectious RNA could be extracted from MD virus-infected cells, which was assayed in calf testicle cell monolayers after pretreatment with hypertonic buffers. From gradient centrifugations in 0.I M NaCl a value of 37 to 40 S was determined for the infectious RNA (Figure I). A peak of TCA-precipitable radioactivity was found to coincide with RNA-infectivity in a preparation, where 32P-labelled MD virus had been treated with sodium deoxycholate (Figure 2). Until now, only togaviruses have been demonstrated to yield infectious RNA after mild detergent treatment (25). The hydrodynamic properties of MD virus RNA were indicative for an essentially single-stranded molecule with secondary structure; lowering of the salt molarity resulted in a reduction in sedimentation coefficient, whereas Ca^{++} and Mg^{++} ions increased the velocity of the molecules in sucrose gradients. SIN virus RNA behaved accordingly under the conditions described (Figures 3a, b; 4a, b). Preliminary data obtained by J. Zeegers from our group on the incorporation of ^{3}H uridine into PK I5 cells infected with hog cholera virus show its dependence on the multiplicity of infection (Figure 5). A further characteristic is that incorporation starts only six hours post infectionem which is very late as compared with e.g. SIN virus.

In conclusion one may say that much more work is required especially on the nonarbo togavirus RNAs in order to learn whether the replication mechanisms found for alphaviruses are valid for them, too. However, rapid progress has been hampered in the case of hog cholera virus by tedious assay procedures, growth to only moderate titers and poor labelling under actinomycin D.

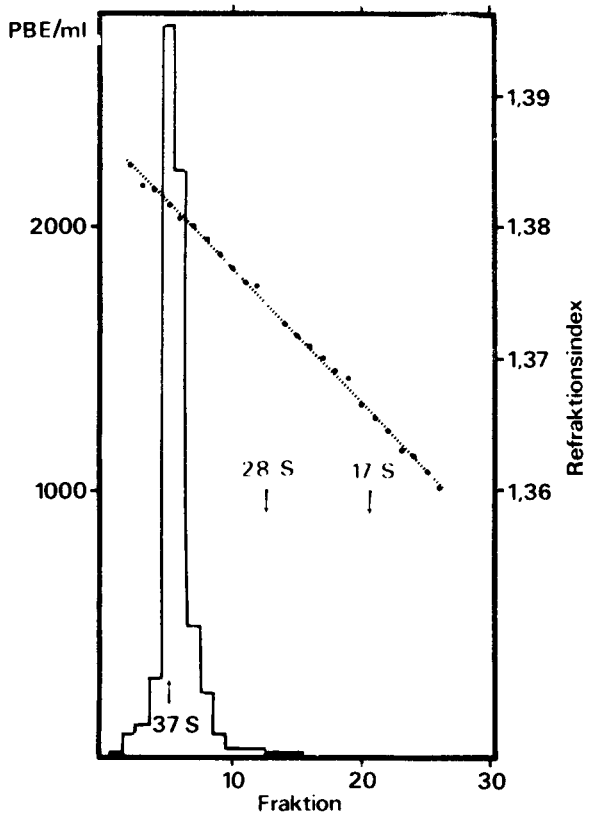


Fig. I. Distribution of infectious RNA (in plaque-forming units = PBE/ml) from an extract of cells (phenol-SDS-pronase) infected with MD virus. Rate zonal sedimentation was performed in a linear 15-30% sucrose gradient for 16 hours using a Spinco SW 25.1 rotor at 24.000 rpm (from Ref. 39).

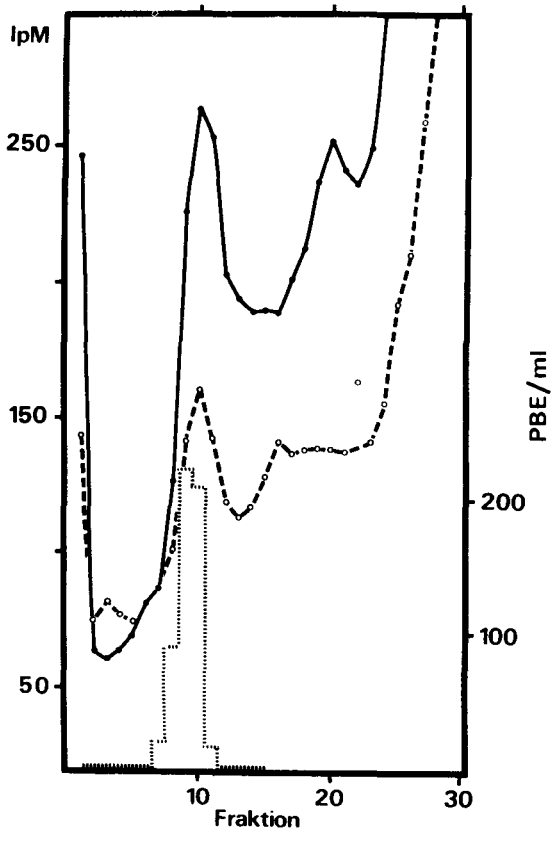
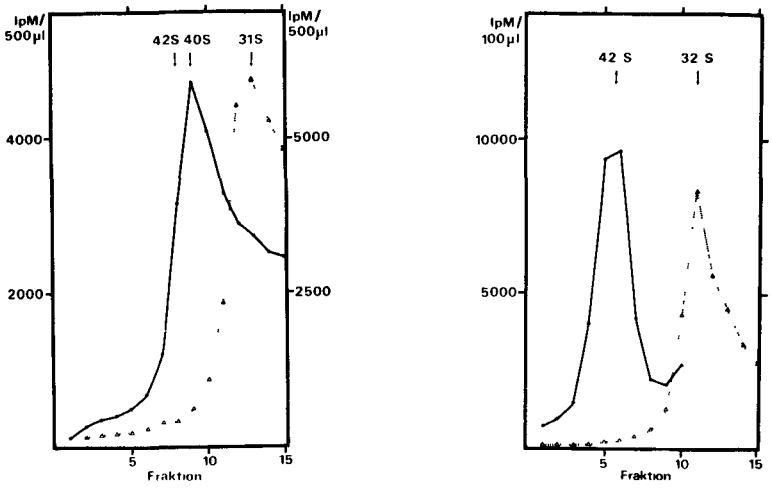


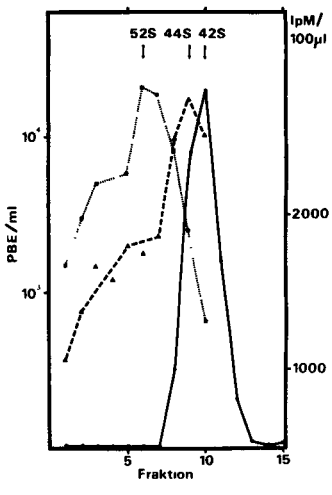
Fig. 2. Distribution of infectious (dotted column) and ^{32}P-labelled RNA extracted from purified MD virus using sodium deoxycholate. Technical data see Fig.I. Solid line: total radioactivity (Cerenkov); dashed line: TCA-precipitable radioactivity.



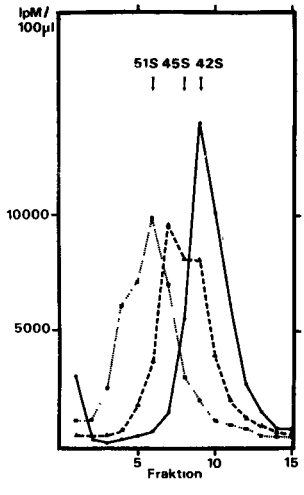
3 a

3 b

Fig. 3. Effect of ionic strength on the sedimentation behaviour of MD (a) and SIN virus RNA (b). Slower sedimentation occured in 0.001 M NaCl (dotted line) as compared with the standard gradient containing 0.1 M NaCl (solid line).



4 a



4 b

Fig. 4. Effect of Mg^{++} (dotted line) and Ca^{++}ions (dashed line) on the sedimentation behaviour of MD (a) and SIN virus RNA (b), with SIN virus RNA in O.I M NaCl (solid line) serving as a marker.

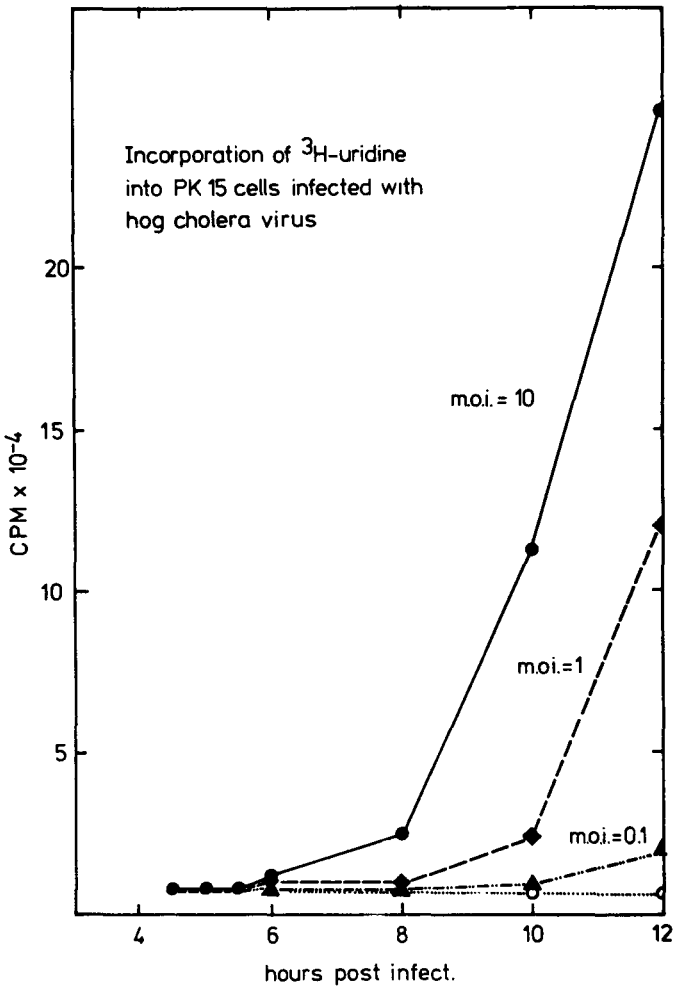


Fig. 5. Dependence of rate of label incorporation from the multiplicity of infection. Actinomycin D was added 10 min before infection of PK 15 cells in suspension with HC viruscontaining plasma from viremic pigs. At the times indicated samples were drawn and assayed for TCA precipitable radioactivity.

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DISCUSSION

Dr. S. Schlesinger: When you look at incorporation of the uridine why do not the uninfected cells incorporate ?
Do you have actinomycin D there ?

Dr. Horzinek: We work under actinomycin D.

Dr. S. Schlesinger: Is hog cholera virus not sensitive to actinomycin D ?

Dr. Horzinek: It is not. In our hands it was not, but perhaps Jean-Marie Aynaud can comment on this later. He has done the first actinomycin D experiments to my knowledge.

Dr. Scholtissek: Concerning the replicative intermediate structures of the RNA: have these RNA been analysed after treatment with phenol or with detergent ?

Dr. Horzinek: The usual extraction procedure included phenol and detergents.

Dr.Scholtissek: How can you exclude that these structures are artefacts caused by this extraction ? Because, for example Weisman, Oberg and Phillipson suggested an open structure in which the two strands of RNA template and growing strands are held together just by the polymerase ? And if you now use phenol you just get then the double-stranded parts; but these are artefacts.

Dr. Horzinek: Well, first of all I am commenting on results and interpretations presented by others. Then on the other hand it has been shown that using antibodies directed against double-stranded RNA you can immunologically identify double-stranded structures and I think under these conditions it is less likely that these double-stranded molecules would be artifacts.

Dr. Scholtissek: Concerning the analysis by these antibodies, I do not doubt the results and I do not doubt that you in your infected cells have double-stranded structures but the replicative intermediates I am sure are artifacts.

CHARACTERISTICS OF THE IN VITRO MULTIPLICATION OF HOG CHOLERA VIRUS

and

THE IN VITRO GENETIC MARKERS IN RELATION TO PATHOGENICITY FOR THE PIG

(a summary)

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I. - INTRODUCTION

With Rubella, Equine arteritis and Bovine diarrhea virus, Hog cholera virus (Swine fever virus) is classified as a non arbo-TOGAVIRUS (11). Hog cholera virus is a small envelopped RNA virus (40 - 50 nm). Viral particle appears roughly spherical, displaying an internal core and an envelopping membrane (10, 18).

II. - IN VITRO MULTIPLICATION OF HOG CHOLERA VIRUS

In vitro multiplication of Hog cholera virus has been observed in a variety of cells , mainly of porcine origin (bone marrow, leucocytes, spleen, testis, lung, kidney and trachea) but also of other mammalian origin (bovine, lamb, goat, guinea-pig, rabbit, dolphin, etc...) (15). Usually Hog cholera virus grows without cytopathic effect in tissue culture. In 1960, experiments reported growth of Hog cholera virus with cytopathic effect under special conditions (8) but cytopathic strains have been shown to be contaminated with porcine adenoviruses, parvoviruses or Bovine viral diarrhea virus (4, 9).

A technique for production of plaques by Hog cholera virus using secondary pig kidney cells under an agarose overlay was described (3). Hog cholera virus plaques only became visible after prolonged incubation of stained cultures with neutral red in the dark. In infected cultures, non cytopathological changes could be detected.

Hog cholera virus enhances the replication of Newcastle Disease virus (E.N.D. phenomenon) (5), Teschen virus (E.T.V. phenomenon) (5) and Rubella virus in pig testicle cells. This enhancement seems

to be a result of the suppression by Hog cholera virus of the production of interferon in swine testicle cells when they are infected with Newcastle Disease virus or Rubella virus. Inversely, in pig kidney cells, Hog cholera virus inhibits the normally cytopathogenic Newcastle Disease virus (H.E.I.C. phenomenon) (5).

Cytogenetic alterations in swine kidney cells persistently infected with Hog cholera virus have been described. The infected cells showed chromosomal doubling during interphase for four passages after infection (15). Acute and persistent infections of PK. 15 cells with Hog cholera virus extended the period of DNA synthesis 0,9 and 0,7 hours, respectively (17).

Replication of Hog cholera virus at 37°C in single growth cycle conditions in PK. 15 cells was studied by means of the highly reliable titration method of infectivity using the immunofluorescent microplaques techniques (1, 6, 14). Viral antigen was first detected 4 hours post infection and progeny virus 6 hours P.I. The latent period is followed by an exponential rise until the 15th hour. By means of electron microscopy using ultra thin sections of infected PK. 15 cells under single growth cycle conditions, new viral particles were observed at 10 hours post infection in extracellular spaces, in Golgi apparatus and also in cytoplasmic vesicles (18). The infectivity of cell-bound virus is 10 to 100 times that of the medium (6). Identification of antigen exclusively in the cytoplasm of infected cells suggested that Hog cholera virus replication was entirely extra-nuclear.

Hog cholera virus multiplies in the presence of DNA synthesis inhibitors : analog (BUDR, IUDR, FUDR) and mitomycine (1). Virus growth is not inhibited by actinomycin D. An enhancement of virus production (fig. 1 and fig. 2) is observed in presence of actinomycin and mitomycine (1). Probably, Hog cholera virus does not require the DNA-dependant RNA synthesis for viral replication. Hog cholera virus and B.V.D. virus have the same properties of inhibition by acriflavine, but equine arteritis virus and Rubella virus obviously do not (7). Studies of viral RNA replication is in progress (HORZINEK).

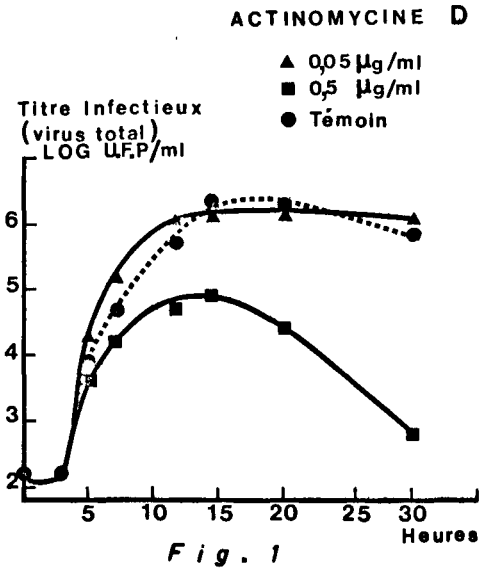

Fig. 1

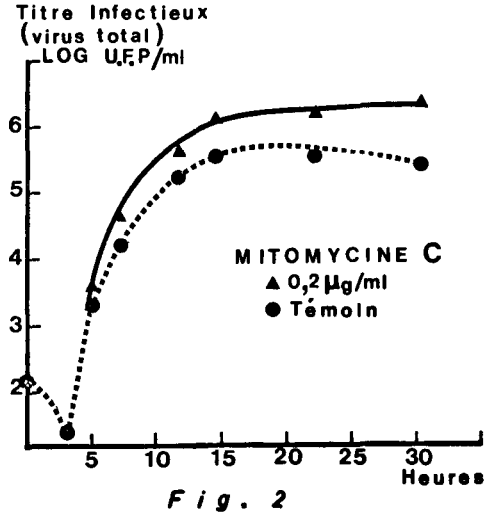

Fig. 2

In summary the main feature of Hog cholera virus replication is the absence of cytopathogenic activity. After virus infection, there is no inhibition of host cell synthesis and apparently cell growth is not affected. In these conditions often the labeling of viral material with radioactive precursors (nucleotides or aminoacids) is unefficient.

III. - THE IN VITRO GENETIC MARKERS OF THE VIRUS, CONNECTED TO PATHOGENICITY FOR PIG

Using in vitro genetic markers, we are now able to differentiate between virulent and attenuated strains of Hog cholera virus in the laboratory. Recently, two reliable and workable genetic markers were characterized (2) :

1) determination of the optimal temperature for maximum virus yield in tissue culture under conditions of single growth cycle (fig. 3).

2) heat infectivity resistance (30 minutes at 56°C) (fig. 4).

These two genetic markers are highly related to the virulence for pig.

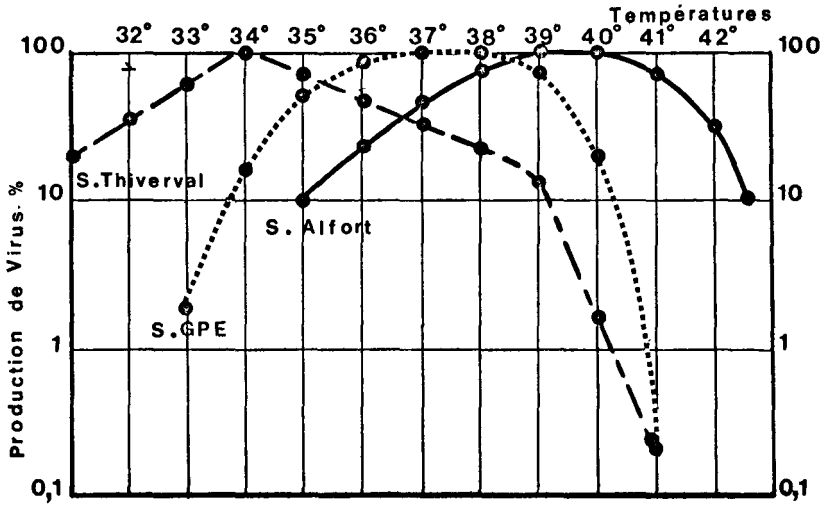
Températures
32° 33° 34° 35° 36° 37° 38° 39° 40° 41° 42°
100
10
1
0,1
Production de Virus %
S.Thiverval
S. Alfort
S.GPE

Fig. 3

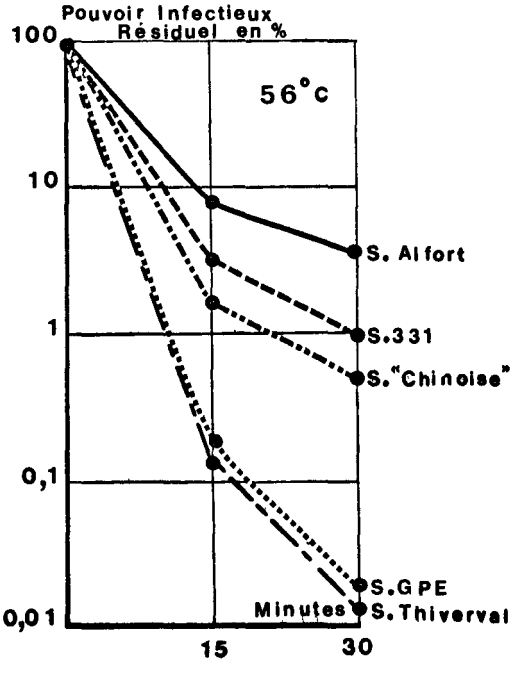
Pouvoir Infectieux
Résiduel en %
100
10
1
0,1
0,01
56°c
S. Alfort
S.331
S."Chinoise"
S.GPE
S.Thiverval
Minutes
15
30

Fig. 4

STRAINS	MARKER I optimal temperature for virus growth	MARKER II heat infectivity resistance (30 mn at 56°C)
FULLY VIRULENT STRAINS (ALFORT, ALD)	39 - 40°C = "HOT STRAINS"	1,5 log decrease in infectivity = "SOLID STRAINS"
LOW OR MODERATELY VIRULENT STRAINS	35 - 39°C	2 - 2,5 log decrease in infectivity
ATTENUATED STRAINS (THIVERVAL, GPE)	33 - 37°C = "COLD STRAINS"	3 - 4 log decrease in infectivity = "FRAGILE STRAINS"

Fully virulent strains are "hot" and "solid". Inversely completely attenuated strains are "cold" and "fragile". Low or moderately virulent strains and also some lapinized strains (chinese) are in an intermediate position. Being "hot", virulent strains can grow intensively in the pig, the normal temperature of which is about 39.0°C. Inversely, being "cold", the growth of attenuated strains is limited by the normal body temperature of the pig. Virus diffusion from cell to cell and from pig to pig is limited or suppressed by the fragility of attenuated strains. The "cold" (or "hot") marker probably is connected with some viral enzymatic functions occuring during viral replication, while the "fragile" (or "solid") marker is connected with general properties of structural proteins of the virion.

IV - APPLICATION OF THE IN VITRO GENETIC MARKERS TO DEVELOPMENT OF A NEW TISSUE CULTURE VACCINE : THE COLD STRAIN VACCINE

The in vitro genetic markers open the way for studies on cold strains of Hog cholera virus in regards to their practical use as vaccines.

Recently a cold strain was described (2, 12, 13) : isolated in tissue culture at 29 - 30°C, the U.610 clone (THIVERVAL strain) has the in vitro properties of being "cold" and "fragile". This strain

is attenuated for pig and has good immunogenic properties. Inoculated into sows on the 30th or 60th day of pregnancy in field conditions, this strain has no unfavorable effects on the development of pregnancy or on the quality of offspring. Innocuity for pig embryo suggests that cold strain "Thiverval" is devoid of residual virulence. Piglets born to these vaccinated sows showed normal growth rates and were observed to be fully susceptible to vaccination or to challenge carried out at the 3rd month of their life.

Concerning immunity, challenge resistance and anti Hog cholera virus neutralizing activity of high level (neutralization index ≥ 5) persisted and were constant during an observation period of 18 months. Diffusibility of vaccinal virus was not detectable in laboratory experimental conditions even after 18 months of cohabitation.

Concerning genetic stability of the cold strain : even after 10 serial passages in tissue culture at 37°C or in susceptible piglets, the in vitro and in vivo characters of the cold strain are not modified (13). Attenuation for pig and in vitro properties are apparently genetically stable.

DISCUSSION

Dr. Soholtissek : What is the physical particle relation to infectivity?

Dr. Scherrer : Excess of physical particles exists comparatively because when examining the cell preparations if they are infected the calculation seems to indicate that when a cell may get about 5 to 10 particles the ratio physical particles/infectivity appear then to be probably very near to one.

Dr. Rott: What is your interpretation about the actinomycine D effect ?

Dr. Aynaud : There are several interpretations : the first one is the reduction of interferon synthesis observed in others systems (arboviruses, measles virus). Actinomycin D reduces the general level of host cell protein synthesis and consequently the interferon synthesis.

The second one is, in presence of actinomycin D, the most part of host cell synthesis machinery (membranes and ribosomes) are available to viral synthesis.

Dr. Liess : What bothers me always is the question of dependency between optimal temperature for the multiplication of the hog cholera virus and what you called "cold" strain and "hot" strain. How did you check the virulence and dependence from different temperatures for multiplication ?

Dr. Aynaud : Now it is well established that "hot" strains are highly virulent for pig in contrast to "cold" strains which are not. These results are determined by means of the study of the influence of incubation temperature on the virus yield under one step growth cycle conditions in PK-15 cells.
I must notice that we have shown that virulence is also connected with two other genetic markers : heat resistance at 56°C of virus infectivity ("solid" strains are virulent, "fragile" strains are not), and the in vitro multiplication rate (fluorescent microplaques size). For example, the full virulent "ALFORT" which is able to kill pigs in one week is hot (maximum virus yield at 40°C), solid (1,5 log decrease in infectivity after 30 mn at 56°C), and large fluorescent plaque strain. Inversely, the completely attenuated "THIVERVAL" strain is a cold (maximum virus yield at 33-34°C), fragile (3-4 log decrease in infectivity after 30 mn at 56°C).

Dr. Liess : I am not quite satisfied because of the question whether virulence is judged from mortality or is it connected with clinical disease or is it connected with multiplication in the animal itself or how is virulence defined ?
For "lapinized" strains it is known that they multiply in the pig. We know for virulent strains that they can lead to death in some animals and in others they produce just a transcient clinical disease and then those animals recover. Now the question is the definition for virulence.
I think this is very necessary in order to find a correlation to an optimum of temperature.

Dr Aynaud : I call virulent strains, strains which are able to kill pigs in a week for example.
Low virulent strains cause irregular clinic symptoms, mortality, or lesions.

Frequently in fields, reproductive failures are associated with low virulent strains of swine fever.

Dr. Bachrach : Do you find evidence of defective interferring particles in hog cholera analogous to the defective interfering particles of poliovirus or of vesicular stomatitis virus having smaller pieces of RNA than in the infective particle ?

Dr. Aynaud : I did not look for such particles and I do not think that there is such a sort of particle because the ratio between infectious particles and physical particles is about one , in relation to Dr. Scherrer's observations in electron microscope.

Dr. Schlesinger : In your opinion is it possible to work with a plaque-purified strain of the virus ?

Dr. Aynaud : Yes, that's very easy.

Dr. Schlesinger : And do you get the same low infection with a plaque-purified strain ? and low multiplicity infection ?

Dr. Aynaud : We have isolated several clones by limited dilution techniques using fluorescence techniques. We did not observe differences between different clones of the same level of selection in regards to virulence for pig.

Dr. Horzinek : I should like to ask one question about the influence of actinomycine D. You found that you had a steeper slope of the decrease in titer in actinomycine D at higher concentrations as compared with those of lower concentrations. Could not this be a consequence of simply the decay of PK 15 cells which we know are very sensitive to actinomycine D with concomitant release of proteases? We know that all toga's are very sensitive to proteases.

Dr. Aynaud : With one microgram of actinomycien D by ml PK 15 cells are killed after 24 hours in our experimental conditions. PK 15 cells are very sensitive to actinomycine D.
I agree with you it is possible that in PK 15 cells affected by the drug toxicity, the concomitant release of proteases could have an indirect

inhibition effect on the virus production. Swine fever virus is highly sensitive to proteases and phospholipases.

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THE RNA POLYMERASES OF MYXOVIRUSES

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ABSTRACT

All negative strand RNA viruses, including the orthomyxoviruses and the paramyxoviruses, contain virion associated RNA transcriptases. The optimal requirements for the in vitro reaction catalysed by myxovirus transcriptases will be discussed. Details of the transcription process, such as the extent to which the genome is transcribed, and the nature of initiation have been determined only for orthomyxoviruses. The products of enzyme activity are small pieces of c.RNA. It is not known for certain what structural polypeptides constitute the transcriptase, as the enzyme is bound tightly to nucleocapsid structures, but high molecular weight proteins (P_1 and P_2 96,000, 83,000 daltons, influenza; P - 78,000 daltons, Sendai) are always associated with the transcription complex. While the biological activity of paramyxovirus transcriptases is concerned directly with the formation of complementary m RNA in infected cells, the role of orthomyxovirus transcriptases is less clear. Initiation of orthomyxovirus transcription in vivo is slow, is sensitive to actinomyxin D, and may require host cell factors.

RNA dependent RNA polymerases found in the cytoplasm of cell infected with both types of myxovirus resemble virion transcriptases, and may represent the accumulation of newly synthesised transcriptase in infected cells. The replication of orthomyxovirus RNA is also characterized by a stimulation of DNA-dependent RNA polymerase activity in host cell nuclei, and the development of RNA dependent RNA polymerase activity in nuclei.

INTRODUCTION

The term 'myxovirus' was adopted in the early days of virus taxonomy to describe those RNA containing animal viruses that adsorb

readily to muco-proteins (Andrews et al., 1955); other characteristics of these viruses that were originally considered to be important were their ability to agglutinate red cells (Hirst, 1942) and the presence of the enzyme neuraminidase as a structural component of the virus (Gottschalk, 1958). The original members of the group were the human and animal influenza viruses, Newcastle disease virus and mumps virus; some years later the newly recognized para-influenza viruses were added to the group (Andrews et al. 1959). On further investigation, it became apparent that significant morphological and biological differences existed between the influenza viruses and the remaining myxoviruses (Waterson, 1962). Although both types of virus contain a helical ribonucleoprotein surrounded by a lipid-containing envelope in which are embedded the radiating, spike-like surface glycoproteins, the influenza viruses possess a fragmented RNA genome (Davies & Barry, 1966), and there are differences both in the dimensions of the nucleocapsid and the average diameter of each type of virus particle. Unlike the para-influenza viruses, the multiplication of influenza viruses is characterized by a variety of co-operative phenomena (Barry, 1961 a,b) and is sensitive in its early stages to inhibitors of DNA function (Barry et al 1962; Barry, 1964). A further taxonomic problem arose when it was found that at least three other viruses - measles, rinderpest and distemper, were morphologically identical to the para-influenza viruses (Waterson et al., 1961; Cruickshank et al, 1962; Plowright et al., 1962), although they have no affinity for mucoprotein and lack neuraminidase.
Although all the viruses mentioned above are still known popularly as myxoviruses it is now the convention to classify viruses of the influenza type as 'ortho-myxoviruses', and viruses of the para-influenza type as 'paramyxoviruses' (Andrewes & Pereira, 1972).

Knowledge of the general principles of RNA virus replication has accumulated rapidly in recent years. The finding that small RNA containing viruses multiply normally in the absence of DNA synthesis (Simon, 1961; Cooper and Zinder, 1962) or DNA-directed RNA synthesis (Reich et al 1961) firmly established the concept that viral RNA replication is independent of the genetic material of the host cell. The detection of ribonuclease resistant, virus specific RNA in cells infected with EMC virus (Montagnier & Saunders, 1963) implied that viral RNA replicates by way of a double stranded base paired intermediate. The existence of such replicative forms has been established for many animal and bacterial RNA viruses (Bishop & Levintow, 1971) and it is now accepted that the replication of

viral RNA is a two stage process in which the first step is the synthesis of a complementary polynucleotide strand that acts as a template in the second step for the synthesis of progeny strands of viral RNA; in the case of the RNA tumour viruses, the complementary polynucleotide template is probably DNA (Temin, 1970). In those instances where the isolated viral RNA is infectious, the RNA serves as both genome and messenger RNA (plus strand), while the complementary RNA (virus strand) acts only as a template for plus strand formation. Cells infected with RNA viruses contain virus specific RNA dependent RNA polymerase(s) (Baltimore & Franklin, 1962; Baltimore et al 1963); these enzymes are not found in uninfected cells and they are responsible for the synthesis of both template and progeny RNA (replicase).

The RNA of the paramyxoviruses and probably also the orthomyxoviruses lacks infectivity, and the messenger RNA associated with polysomes of cells infected by these viruses is complementary to virion RNA (Pons, 1972, Vincent & Barry, 1974). The myxoviruses, as well as the rhabdoviruses (Huang et al., 1970) differ therefore from other RNA viruses in that v. RNA is not m. RNA, and they have been described collectively as 'Negative Strand Viruses'(Barry & Mahy, 1974). An essential requirement for this type of virus is the presence within the virion of an RNA polymerase capable of transcribing virion RNA into m. RNA following infection. Such virus associated transcriptases have been described for NDV (Huang et al, 1971), para-influenza I - Sendai (Robinson, 1971; Stone et al., 1971) and influenza viruses (Chow & Simpson, 1971, Penhoet et al., 1971). Among the rhabdoviruses, the RNA transcriptase of vesicular stomatitis virus (VSV) has been studied in detail (Baltimore et al., 1970).

Certain general features of the multiplication of myxoviruses can be stated. The paramyxoviruses contain a single stranded, non-infectious RNA of molecular weight 5×10^6 daltons (Kolakofsky, et al.1974). The first stage of RNA replication involves the transcription of v. RNA by the virion associated transcriptase with the formation of complementary polyribonucleotides that range in molecular weight from 3×10^5 to 2×10^6 daltons (Kingsbury, 1966; Bratt & Robinson, 1967); these products are probably monocystronic m RNA molecules. Replication of v. RNA proceeds from a replicative intermediate (Portner & Kingsbury, 1972), in which a virus induced RNA replicase is presumably involved. Both transcription

and replication are unaffected by actinomycin D.

The multiplication of orthomyxovirus RNA is less clear. These viruses contain an unknown number of single stranded RNA molecules. The total molecular weight of the RNA of each particle has been estimated to be 5×10^6 daltons, made up by 8 - 9 pieces (Skehel, 1972). Like the paramyxoviruses, influenza viruses have a transcriptase that catalyzes the formation of c. RNA in vitro (Bishop et al, 1971b), and the reaction is not affected by the presence of actinomycin D (Chow & Simpson, 1971), When cells are infected in the presence of actinomycin D however, no transcription occurs (Bean & Simpson, 1974). Influenza viruses cannot replicate in anucleate cell fragments (Cheyne & White, 1969), enucleated cells (Follett et al, 1974) or in cells in which there has been an inhibition of DNA function (Barry, 1964) or DNA transcription (Mahy et al, 1972). This evidence suggests that, unlike any other RNA virus, the replication of orthomyxoviruses may require the participation of the genetic material of their host cells.

Despite the existence of a number of RNA synthesising enzymes in preparations of virus and in infected cells, comparatively little is known of the details of myxovirus RNA replication. In the following sections of this paper, the properties of the virion associated transcriptases of myxoviruses will be reviewed first, followed by a consideration of the nature of polymerase enzymes found in infected cells.

VIRION TRANSCRIPTASES

Baltimore (1971) has summarized aptly the concept of how viruses carry out their initial transcription process. The discovery of virion associated DNA dependent RNA polymerase in vaccinia virus (Kates & McAuslan, 1967; Munyon et al 1967) led the way to the discovery of other virus associated RNA polymerases. In the case of the myxoviruses, RNA dependent transcriptases have been identified in both influenza A viruses (Chow & Simpson, 1971; Penhoet et al, 1971; Skehel, 1971) and influenza B viruses (Oxford, 1973), NDV (Huang et al, 1971) and Sendai virus (Robinson, 1971; Stone et al, 1971; Hutchinson and Mahy, 1972).

Properties of the Enzymes

General requirements for enzyme activity

Transcriptase activity is detected by the incorporation

of a radioactively labelled ribonucleoside triphosphate into acid insoluble product when the reaction mixture contains virus particles, detergents such as Triton N 101 or Noridet P40, divalent cations and all four ribonucleoside triphosphates. The complete ingredients of a typical reaction mixture used for the detection of transcriptase activity in the orthomyxovirus fowl plague virus (FPV) are listed in the footnote to Table 1. The following features of the system (Table 1) can be noted.

The reaction is dependent on the presence of all four ribonucleoside triphosphates, and only proceeds when the virus particles are disrupted by detergent, implying that the enzyme is located inside the virus particle. The template for the reaction is RNA, since incorporation is completely blocked by the presence of ribonuclease. Deoxyribonuclease has no effect on the reaction (Chow & Simpson, 1971). Inhibitors of DNA polymerases, such as actinomycin D, do not affect the reaction. Maximum activity for the FPV reaction depends on the presence of both Mn^{++} and Mg^{++} ions (see below). The levels of enzyme activity associated with different strains of influenza viruses vary over approximately a 20 fold range (Chow & Simpson, 1971).

Paramyxovirus transcriptases require approximately the same type of reaction conditions as those described for orthomyxoviruses (Robinson, 1971; Stone et al, 1971). The reaction is totally dependent on virions and Mg^{++} but does not require Mn^{++}; ribonuclease added to the reaction at zero-time completely inhibits the reaction, but it is unaffected by actinomycin D or deoxyribonuclease. A feature common to all paramyxovirus transcriptase assays reported so far is their relatively low specific activity. NDV polymerase has less than 3% of the activity of VSV (Huang et al, 1971); the level of activity quoted for NDV by Huang et al. (1971) is approximately 4% of that found with FPV (Table 1).

Effects of metal ions

In reactions primed by influenza WSN there is a 3 - 4 fold stimulation of activity when monovalent cations are present (Bishop et al, 1971a), and sodium, lithium, potassium, or ammonium ions are equally effective. Like WSN, the optimal concentration of monovalent cations for FPV is around 0.1 to 0.2 M (Fig. 1 C). Enzyme activity is inhibited at high (0.6M) concentrations of NaCl.

TABLE 1

REQUIREMENTS FOR IN VITRO RNA POLYMERASE ACTIVITY
OF INFLUENZA VIRUS (FPV) PARTICLES

Components	% Incorporation of 3H-UTP
Complete *	100
- ATP, GTP & CTP	2
- Mn^{++}	34
- Mg^{++}	42
- Mn^{++} & Mg^{++}	0
- NP40	0
- Dithiothreitol	60
+ RNase	0
+ Actinomycin D 200μg/ml	100
+ Rifampicin "	100
+ Streptlydigin "	100
+ Rifamycin AF/103 150μg/ml	100

* The complete assay mixture contained the following components:-

50mM Tris-HCL buffer (pH 8.2), 2.0mM each of ATP, CTP and GTP, 0.4mM 3M-UTP (42μCi/ mol), 8mM $MgCl_2$, 0.2mM $MnCl_2$, 5mM Dithiothreitol, 150mM KCL, 33mM NaCl and 0.5% Nonidet-P40. The total reaction volume was 150μl containing 50-150μg of viral protein.

Reaction mixtures were incubated at 31°C and duplicate samples of 50μl were removed and spotted on to Whatman GF-A glass fibre filters and dried at 60°C for 2 mins. The filters were then washed in 0.5NTCA containing 0.1M Sodium Pyrophosphate (x1), 0.5NTCA (x4) and finally in methanol and then dried at 60°C. When dry the filters were counted in a liquid scintillation counter in a toluene based scintillant. Background counts were removed and specific activities calculated. A typical 100% value was 5 nano-mol/mg Protein/Hour.

For the orthomyxovirus, there is some disagreement concerning divalent metal requirements. Chow & Simpson (1971) using influenza WSN report that there is an absolute requirement for Mn^{++} ions and Mg^{++} ions are not required. Both Penhoet et al. (1971) and Horisberger and Guskey (1974) using influenza NWS find that the enzyme is inactive when Mg^{++} is substituted for Mn^{++}. Bishop et al. (1971a) demonstrated that the WSN reaction requires both Mn^{++} and Mg^{++}; in the absence of Mn^{++}, 20% of optimal activity was obtained. In our experience with FPV (Figure 1), we find that when Mg^{++} is used as sole divalent cation (Figure 1A), a concentration between 6 - 8 m M Mg^{++} stimulates enzyme activity to about 65% of the level obtained at optimal Mn^{++} concentration, when the latter is used as sole source of divalent cation (Figure 1B). However, the levels of activity obtained at maximum concentrations of either divalent cation alone are less than 50% of the activity obtained when both ions are present at optimal concentration (0.2 m M Mn^{++}, 8 m M Mn^{++})(Figure 1D).

The metal requirements of paramyxoviruses have been studied in less detail. Sendai virus polymerase requires 10 m M Mg^{++} for optimum activity (Robinson, 1971), and almost no enzyme activity was found with $MnCl_2$ or Co Cl_2 over a concentration range from 0.1 to 10 m M.

The effects of temperature and pH

Both types of myxovirus transcriptase activity measured in vitro have a temperature optimum in the range 28-33°C (Bishop et al., 1971a, Stone et al., 1971). At 37°C, approximately 25% of maximum activity was obtained. When reactions initiated at 40, 45 or 55°C were shifted to 31°C after 5 hours incubation, no further increase in enzyme activity was noticed, suggesting that the enzyme or template has been inactivated.

The optimal pH for FPV transcriptase is 8.2 (Figure 2), similar to that for other myxovirus transcriptases (Bishop et al. 1971a, Stone et al 1971).

Kinetics of nucleoside triphosphate incorporation

The incorporation of ^{32}P-labelled nucleoside triphosphate catalyzed by influenza WSN transcriptase into acid insoluble material can proceed for at least 6 hours when incubated at 31-33°C

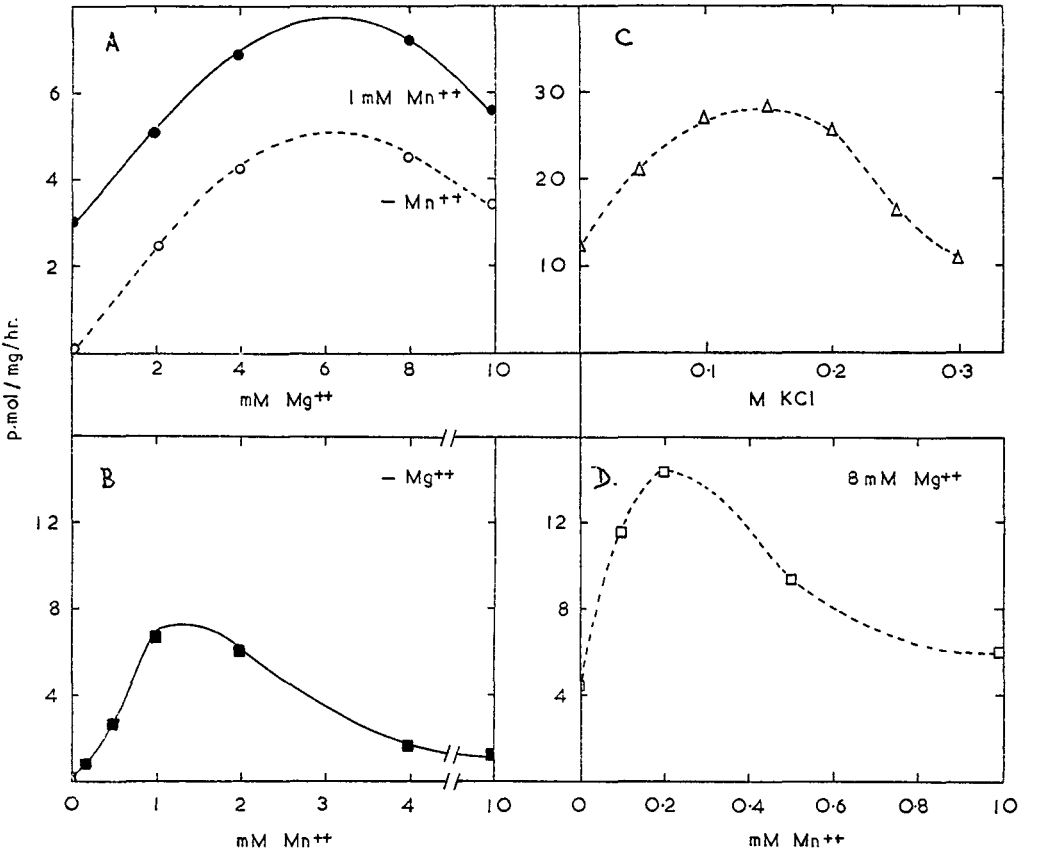

Figure 1 : Legend.

The effect of metal ion concentrations of the activity of fowl plague virus virion transcriptase. Using a standard reaction mixture (Table 1), the concentrations of the cations K^+, Mg^{++} and Mn^{++} were varied.

A. Effect of increasing Mg^{++} ion concentration alone (O– – –O) or in the presence of 1m M Mn^{++} (●– –●).

B. Effect of increasing Mn^{++} ion concentration.

C. Effect of increasing K^+ ion concentration.

D. Effect of increasing Mn^{++} concentration in the presence of 8 m M Mg^{++}.

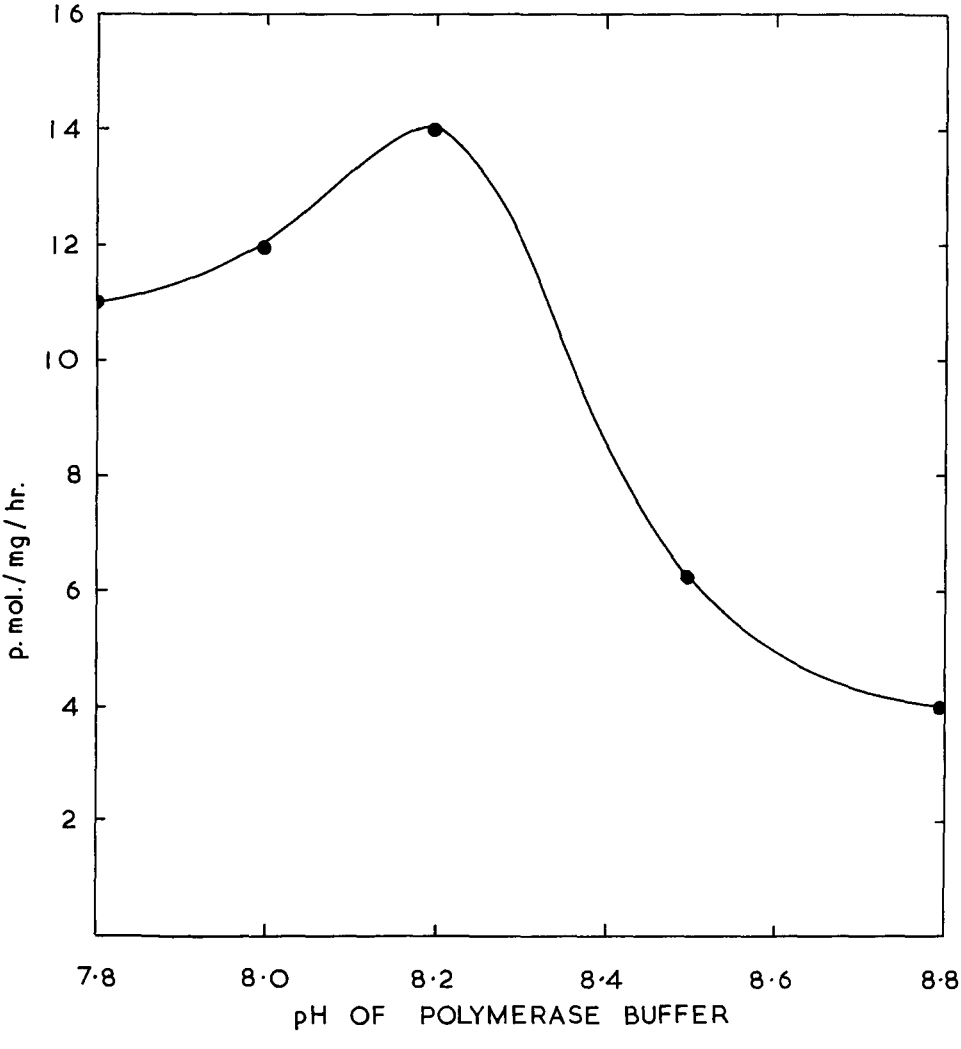

Figure 2. The effect of pH on the activity of fowl plague virion transcriptase.

(Bishop et al., 1971a). Using the reaction conditions shown in Table 1, the incorporation of ^{3}H-UTP by the FPV transcriptase is linear for at least one hour (Figure 3). The kinetics of Sendai transcriptase resemble those of the orthomyxoviruses (Stone et al., 1971; Hutchinson & Mahy, 1972). Termination of the reaction is probably not the result of exhaustion of substrate, but could possibly be attributed to inability of the enzyme to re-initiate, thermal instability of the enzyme, or loss of template. However, it has been found that during in vitro product synthesis, the virion RNA template is preserved (Bishop et al., 1971a).

Attempts to separate enzyme from template

The transcriptase activity of VSV is dissociated from the virus particles by treatment with Triton X-100 in high ionic strength buffer (Emerson and Wagner, 1972). Considerable enzyme activity could be restored by re-combining inactive sedimentable and non-sedimentable fractions. Attempts were made to solubilize the transcriptase of FPV by treatment of a purified virus suspension with Triton-high-salt solubilizer, composed of 3.74% Triton X-100 and 1.4 M NaCl. Samples were incubated at 28°C for 1 hour with occasional mixing. Both treated virus and controls were centrifuged at 40,000 rpm for 40 minutes in a Beckman 40.3 rotor. Resuspended pellets and supernatants were tested separately and after recombination for transcriptase activity (Figure 4). Despite repeated attempts using different solubilizer conditions, the transcriptase activity of FPV remains associated with virus cores.

Marx et al., (1974) report that the Sendai transcriptase complex consists of two polypeptides, the largest virion polypeptide and the nucleocapsid structural unit. The large polypeptide is evidently bound more tightly with nucleocapsid than the L polypeptide of VSV, because it could not be dislodged at very high ionic strength.

Nature of the transcription process

Detailed analysis of the amount of RNA involved in transcription, the extent of the transcription process and the nature of the initiation site have been reported only for myxoviruses. Bishop et al (1971b), using WSN found that only 7% of the virion RNA was involved in transcription and at least 14% of the genome was transcribed. Using the more active influenza A strain WS, at least 80% of the genome was trans-

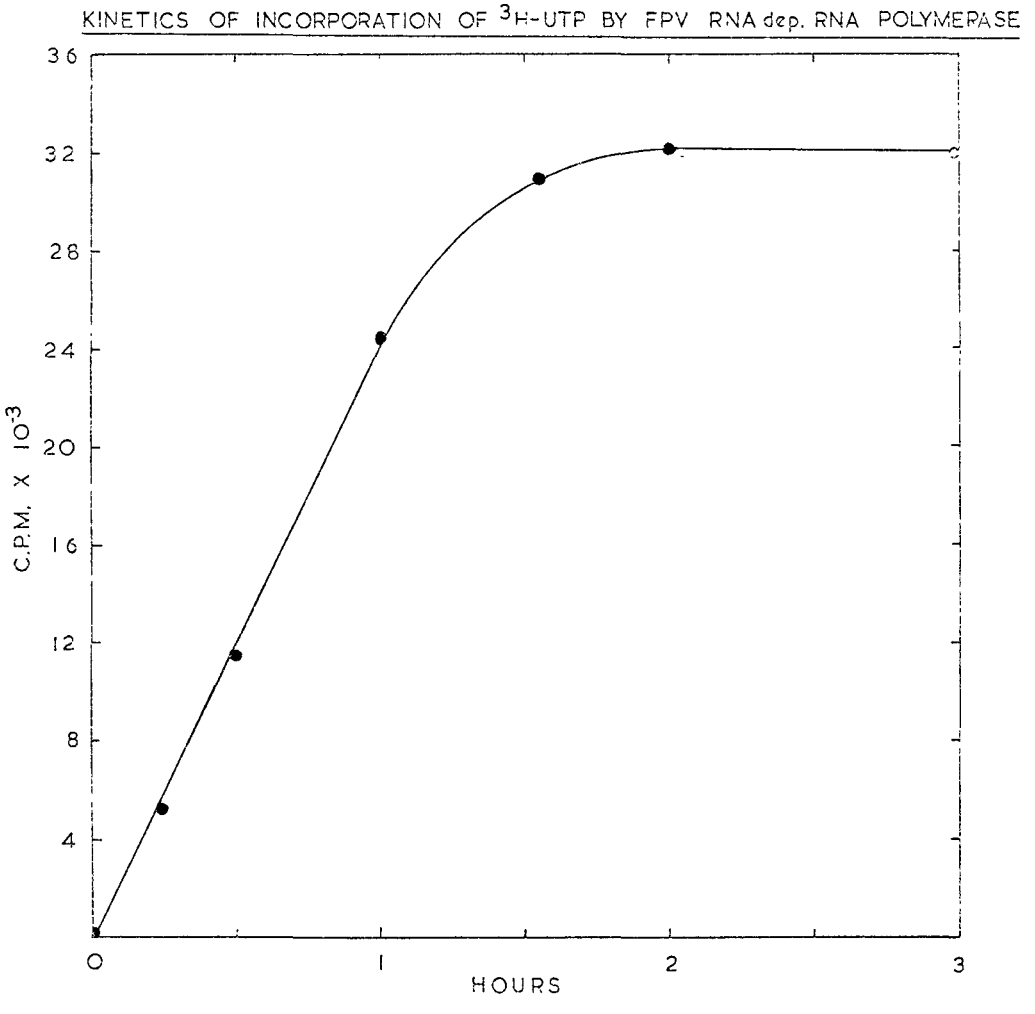

Figure 3. Kinetics of ^{3}H-UTP incorporation by fowl plague virion transcriptase. A ten fold reaction mixture (Table 1) was prepared and incubated at 31°C for the times indicated. Duplicate 50 µl samples were removed at each time point, and the TCA precipitable counts determined. The extent to which incorporation remains linear varies from 1-3 hours, depending on the nature of the preparation and the age of the virus.

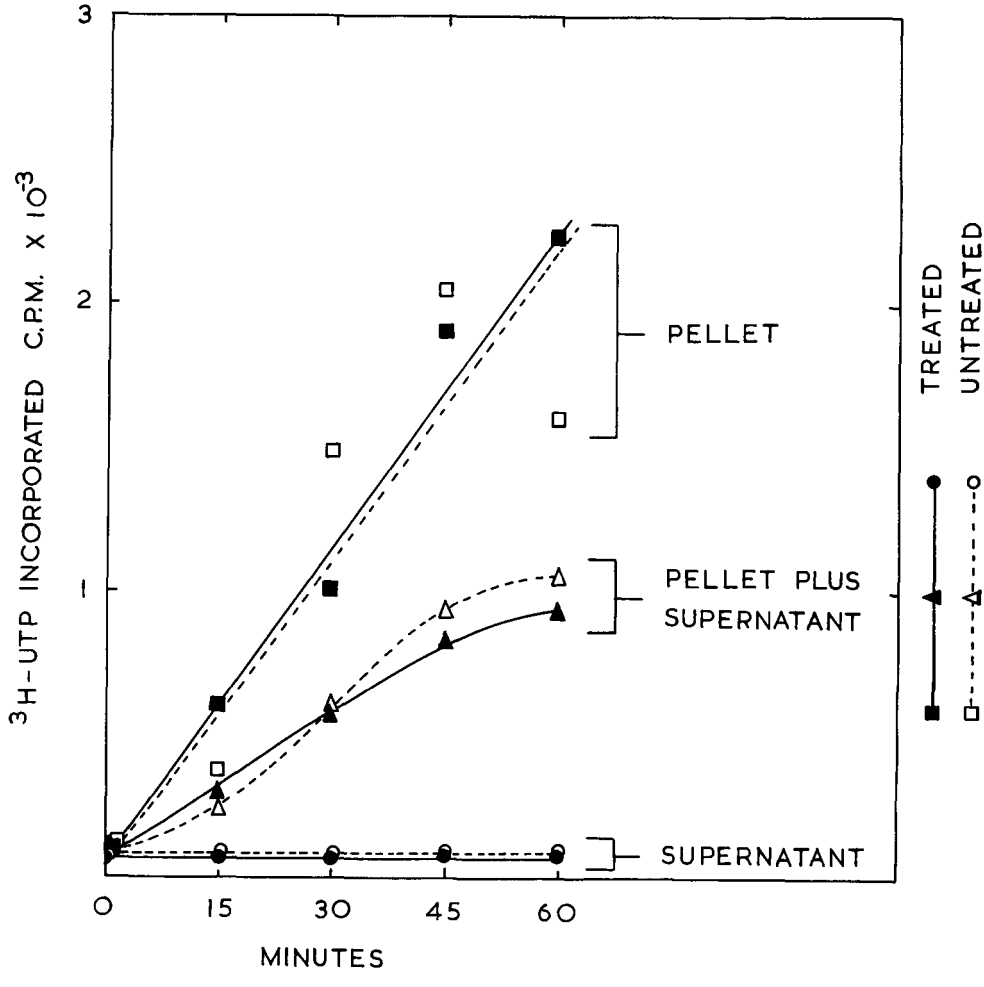

Figure 4. The effect of Triton X 100 and high salt on transcriptase activity of fowl plague virus. Suspensions of purified FPV in NT buffer (0.1 M NaCl, 0.01 M Tris HCl, pH 7.4) were mixed with an equal volume of 2 X Triton high salt solubilizer (3.74% Triton X-100; 1.2×10^{-3} M diothiothreitol, 18.7% glycerol, 1.44 M NaCl) or water and incubated at 28°C for 1 hour. Both suspensions were made up to 0.154 M NaCl, and centrifuged for 40 minutes at 40,000 rpm in Beckman 40.3 rotor. Supernatants were removed, and the pellets were resuspended in an equal volume of solvent containing 0.154 M NaCl. Using the standard reaction mixture, 0.1 ml samples of supernatant, pellet and mixtures of both were assayed for transcriptase activity at 13°C. Polymerase activity of supernatant fraction (circles) pellet (squares) and reconstituted supernatant plus pellet (triangles) for untreated (open symbols) N solubilizer treated FPV (closed symbols).

cribed, and the process appeared to be repetitive (Bishop et al., 1972). In vitro, the transcription process is slow, even with the relatively active WS transcriptase. In our experience, 40% of the template RNA of FPV is protected when the 2 hr. in vitro reaction product is self annealed. More than 95% of the product is complementary to template RNA (Carroll, personal communication). It is not known whether the varying extent to which transcription occurs for different strains of virus reflects differences in the enzymes concerned. It has been found (Bishop et al., 1974) that although there are multiple initiation sites for the influenza transcriptase, the initial sequence for each site is unique, beginning $pppG_pC_p$.

The nature of the transcription product

Paramyxovirus transcriptase synthesise RNA that is completely complementary to virion RNA (Huang et al., 1971, Robinson, 1971a). No detailed analysis of size or complexity of the transcriptase product has been reported.

Orthomyxovirus transcriptases also synthesise complementary RNA (Chow & Simpson, 1971; Penhoet et al. 1971; Skehel, 1971) and at least 95% of the product is complementary to viral RNA species (Bishop et al. 1971b). RNA was extracted from in vitro WSN transcriptase reactions at 10, 20 minutes and 2 hrs and subjected to polyacrylamide gel electrophoresis. It was found that the template was preserved throughout the reaction, and that the product RNA had essentially the same electrophoretic profile as the template. However, when the product was released from template by heat treatment it was found to occur as single stranded, ribonuclease sensitive polyribonucleotides of MW about 10^5 daltons (Bishop et al., 1971b).

For both types of transcriptase, the product thus appears to be completely complementary. This data suggests that product RNA species probably have some important function in infection, particularly as polysome associated RNA for both types of virus is complementary to the virion genome (Pons, 1972; Vincent & Barry, 1974).

The identity of the transcriptase

Sendai virus transcriptase was found to be associated with nucleocapsids (Robinson, 1971a). When nucleocapsids are separated

from other virion proteins by gradient centrifugation, the transcriptase activity had a 9 fold greater specific activity than when transcriptase was assayed as unfractionated, detergent disrupted virus. These enzyme active nucleocapsids contain only two polypeptides, a large polypeptide of MW 75,000 and the nucleocapsid polypeptide of MW 60,000 (Marx et al., 1974).

Biological function of transcriptases

The logical role of virion associated RNA polymerases in ortho - and paramyxoviruses is the synthesis of cRNA, which in turn would act as mRNA for the production of virus induced protein synthesis. However, the report by Siegert et al (1973) that influenza virus RNA is translated in a cell free system from E. coli casts doubt on the need for a virion transcriptase, at least in the case of orthomyxoviruses. However, it has also been claimed that cRNA and not vRNA is the true messenger for influenza (Kingsbury & Webster, 1973) as is the case with paramyxoviruses (Kingsbury, 1973).

It has been reported that primary transcription in vivo occurs in paramyxovirus infected cells, in the absence of host cell protein synthesis (Robinson, 1971b). The possible in vivo activity of influenza WSN transcriptase has been reported by Bean & Simpson (1973), who used ^{32}P-labelled virus to infect cells and monitored the conversion of labelled virus RNA into ribonuclease resistant RNA before and after annealing. They could not find any 'primary transcription' until 40-60 minutes after infection, and the process accelerated only after 90-120 minutes. An example of this type of experiment is shown if Figure 5. No transcription occurred in the presence of actinomycin D, and very little in the presence of cycloheximide.

These results are strikingly different from those obtained with VSV. In the latter case, complete primary transcription occurs within 4 minutes of infection (Flamand & Bishop, 1973), and the process is unaffected by either actinomycin D or cycloheximide. Since the influenza virion transcriptase is insensitive to actinomycin D (Table 1), the implication of Bean & Simpson's experiment is that primary genome transcription in influenza infected cells requires host cell factors that must be synthesised after infection; transcription proceeds normally only after

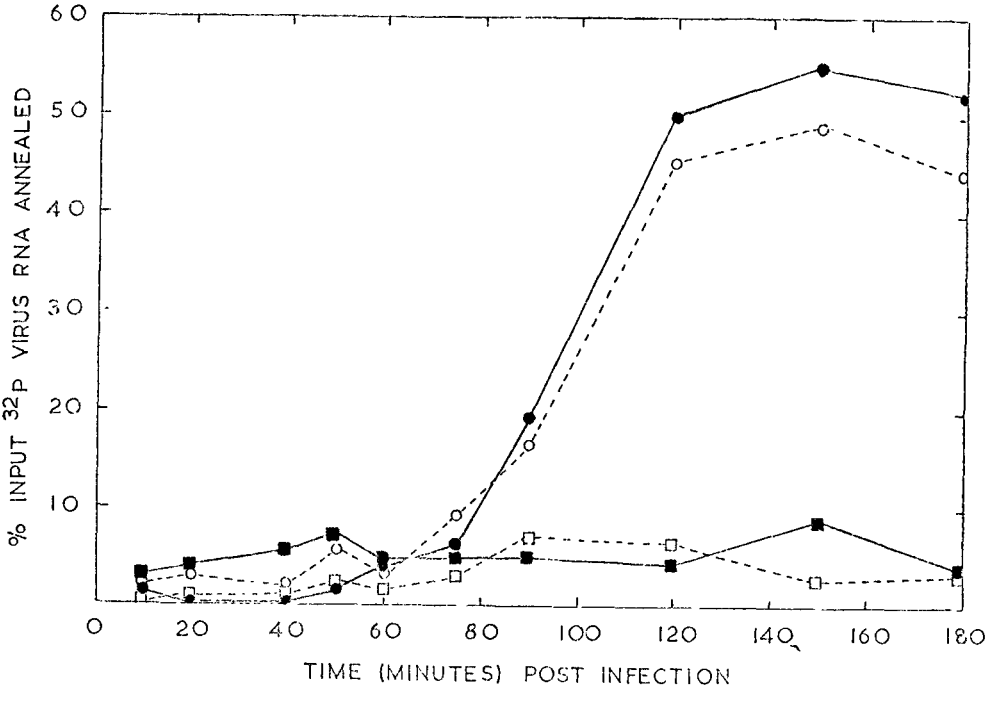

Figure 5. Primary transcription in vivo in chick embryo cells infected with FPV virus. ^{32}P-labelled FPV of high specific activity was prepared in the following way. Monolayer cultures of CEF, pre-incubated in phosphate free 199 medium, supplemented with 50 C/ml ^{32}P, were infected with FPV and the medium was harvested 24 hours later. The virus was purified by conventional methods and used to infect monolayers of CEF at high multiplicity. At intervals following infection, cells were harvested, separated into nuclear and cytoplasmic fractions (Hastie & Mahy, 1973) and extracted for RNA. At each time point, the amount of ribonuclease resistant ^{32}P-RNA was determined for both nuclear (open symbols) cytoplasmic fractions (closed symbols), before or after annealing (ref.Bean & Simpson,1973).

cellular RNA synthesis and protein synthesis has occurred.

Conclusions

All negative strand viruses have virion associated transcriptases. The properties of these transcriptases are summarized in Table 2. The rhabdoviruses have been included in this Table because they contain by far the most active enzyme (Huang et al. 1971), and they have been studied in greater detail than either of the two myxovirus transcriptases. Certain interesting differences are apparent between the rhabdo- and orthomyxovirus enzymes, notably that the enzyme of VSV can be separated from its template and characterized separately, that it has distinct and different initiation sites, and that it is undoubtedly responsible for primary transcription with the formation of mRNA. Paramyxovirus transcriptions have low activity; transcription occurs in cells in the absence of protein synthesis implying that, like the VSV enzyme, paramyxovirus transcriptases undertake primary transcription. The role of orthomyxovirus transcriptases in primary transcription is unknown.

RNA POLYMERASES IN INFECTED CELLS

RNA dependent RNA polymerase activity associated with myxoviruses was reported first for influenza viruses by Ho and Walters (1966), and later for NDV (Scholtissek & Rott, 1969b) and Sendai virus (Mahy et al., 1970). As will be outlined below, it now appears that the predominant polymerase activity detected in cells infected with either type of myxovirus closely resembles the virion transcriptase, and might represent the accumulation of newly synthesised transcriptase in infected cells.

The replication of orthomyxovirus RNA is complicated by the existence of at least three types of polymerase in infected cells. Early in the replicative cycle, there is a stimulation of DNA-dependent RNA polymerase activity in host cell nuclei (Borland & Mahy, 1968; Mahy et al., 1972), which may be related to the unusual sensitivity of influenza virus growth to inhibitors of DNA function (Barry, 1964). RNA dependent RNA polymerase activity has been detected in both the microsomal and nuclear fractions of infected cells (Ho & Walters, 1966; Ruck et al., 1969; Scholtissek & Rott, 1969a; Skehel & Burke, 1969; Mahy & Bromley, 1970). The microsomal enzyme synthesises RNA that is complementary to virion RNA (Scholtissek, 1969), and may represent newly synthesised transcriptase

Table 2

Properties of Negative Strand Virus Transcriptases

	Rhabdoviruses (VSV) *	Orthomyxoviruses (Influenza)	Paramyxoviruses (NDV, Sendai)
Template	s.s. RNA, single molecule 3.8×10^6 M.W.	s.s. RNA, 7–9 pieces $\sim 5 \times 10^6$ M.W.	s.s. RNA, single molecule 5×10^6 M.W.
Enzyme requirements			
Temp. in vitro	28 C	28–31 C	28–31 C
" in vivo	36–40 C	N.T.	N.T.
pH	8.0	8.2	8.0
divalent cations	Mg^{++}	Mn^{++}, Mg^{++}	Mg^{++}
Transcription			
Repetitive	+	+	N.T.
Complete	+	+	N.T.
Sequential	+	–	N.T.
Initiation	Multiple	Multiple	N.T.
Initiation sequances	Distinct & distinguishable ($A_pC_pG_p$; G_pC_p ; $G_pG_pC_p$) etc.	Same and unique (G_pC_p)	N.T.
Structure of enzyme			
Transcription complex	L, NS, NP	P_1, P_2, NP, X	P, NP
Separation from template	+	–	–
Possible transcriptase	L	P_1, P_2	P
Nature of product	Multiple, smaller than template C.RNA	As for VSV	As for VSV
In vivo activity	Immediate	Delayed	+

rather than replicase. The nuclear RNA dependent RNA polymerase is most active in cells at the time of maximal virus RNA synthesis, and less than 40% of its product is complementary to virion RNA (Hastie & Mahy, 1973).

The properties of myxovirus polymerases from infected cells will now be considered briefly.

Paramyxovirus polymerases

Scholtissek and Rott (1969b) were the first to report paramyxovirus polymerase activity in infected cells. They found that the cytoplasm of NDV infected chick cells contain an RNA dependent RNA nucleotide transferase, not present in uninfected cells. The enzyme activity was first detected at 3 hours after infection, and reached its highest activity at 5 hours after infection; it was not inhibited by actinomycin D. Mahy et al., (1970) detected a similar activity associated with the 'microsomes' of chick cells infected with Sendai virus. In this case, maximum detectable activity was 18 hours after infection, and the reaction was actinomycin D insensitive.

General properties of the enzymes

Both NDV and Sendai virus induced polymerases have general requirements for activity that resemble closely those described for virion transcriptases (see above). Enzyme activity *in vitro* is dependent on Mg^{++} ions and all four nucleoside triphosphates. The enzyme from cells has a higher specific activity than the enzyme from virions, but the kinetics of the reaction are similar (Stone et al., 1971).

Nature of the product

Both nearest neighbour analysis (Scholtissek & Rott, 1969) and annealing procedures (Mahy et al., 1970) indicate that the product of the paramyxovirus polymerases is complementary to virion RNA. Sedimentation analysis indicates that it is variable in size, and smaller than the 50S virion RNA.

Nature of the enzyme

Both transcriptive and replicative intermediates have been isolated from Sendai virus infected cells (Portner & Kingsbury, 1972;

Bukrinskaya 1973). The transcriptase complexes have a structure resembling viral nucleocapsids (Stone et al., 1972). These transcriptases have physical properties that would place them in the 'microsomal' fraction of cells, and they represent the source of microsomal polymerase. The complex contains two polypeptides, the viral nucleocapsid structural unit and the largest virion polypeptide (MW 75,000). From the foregoing, it appears that the polymerase activity isolated and characterized from cells infected with paramyxoviruses represents the synthesis and accumulation of nucleocapsids in infected cells. Thus the cellular and viral enzymes are identical; no information is available concerning a possible replicase for the production of v.RNA.

Orthomyxovirus polymerases

Compared to the virion transcriptases, little is known of the properties of RNA-dependent RNA polymerases found in cells infected with orthomyxoviruses, particularly concerning the optimal conditions for the in vitro reaction, the nature of the product synthesised by the enzymes or the biological function of the enzymes.

Ho and Walters (1966) detected RNA polymerase activity in the microsomal fraction of influenza virus-infected cells; the in vitro activity of this enzyme was inhibited by ribonuclease but not by deoxyribonuclease. The enzyme activity was not inhibited by the presence of actinomycin D, but treatment of infected cells at any time during the first hour following infection with this drug prevented its appearance. The polymerase required Mg^{++} ions and all four ribonucleoside triphosphates, but not the addition of an RNA template.

The findings of Ho and Walters were confirmed and extended by Scholtissek and Rott (1969a), Page et al., (1969), Skehel and Burke (1969) and Mahy and Bromley (1970). In each case, the properties reported for the enzyme are very similar. Each enzyme was detected in the cytoplasmic fraction of cells, they were inhibited by ribonuclease, required all four triphosphates, and could not be purified from associated template. More recently, it has been shown that the cytoplasmic enzyme is associated with ribonucleoprotein complexes, similar in morphology to the viral internal components (Compans & Caliguiri, 1973), and it is highly likely that

this cytoplasmic enzyme is a transcriptase. This conclusion is supported by the finding that the *in vitro* product of the enzyme is 85–100% complementary to virion RNA (Scholtissek, 1969).

Nuclear RNA dependent RNA polymerase

Several authors reported the presence of RNA polymerase activity in infected cell nuclei (Scholtissek & Rott, 1969a, Skehel & Burke, 1969), but the levels of activity were low and could represent contamination of nuclei with microsomes. Careful purification of nuclei, using cytochrome C reductase as an assay for cytoplasmic contamination, indicate that nuclei from chick embryo fibroblasts (CEF) infected with FPV contain an RNA dependent RNA polymerase (Hastie & Mahy, 1973). Although the *in vitro* requirements for the nuclear RNA dependent RNA polymerase resemble those required by the cytoplasmic enzyme, annealing reactions indicate that only 40% of the *in vitro* reaction product is complementary to virion RNA. This finding suggests that the nuclear enzyme may have replicase activity.

Time of appearance of RNA dependent RNA polymerases

Scholtissek & Rott (1969a) reported that in both microsomes and impure nuclei of CEF infected with FPV, polymerase activity continues to increase up to 9 hours after infection. Using purified nuclei, Hastie & Mahy (1973) found that the nuclear enzyme activity increased rapidly from one hour after infection reached a maximum at 3–4 hours and then declined; the microsomal enzyme increased from 2 hours after infection and reached a maximum at 5–6 hours after infection.

Armstrong & Barry (1974) have examined both the activity and intracellular localization of various RNA-dependent RNA polymerases (*in situ* polymerase activity) by incubating whole, fixed cells with the RNA dependent RNA polymerase reaction mixture and determining the distribution of polymerase activity by autoradiography. A peak of enzyme activity was detected at 3 hours after infection in the nucleoplasm; a second peak of activity was detected at 6 hours in the cell cytoplasm (Figure 6).

DNA dependent RNA polymerase

Evidence for the participation of nucleoplasmic DNA dependent RNA polymerase II in the multiplication of orthomyxoviruses has been reviewed recently by Mahy et al (1974). The evidence is based mainly

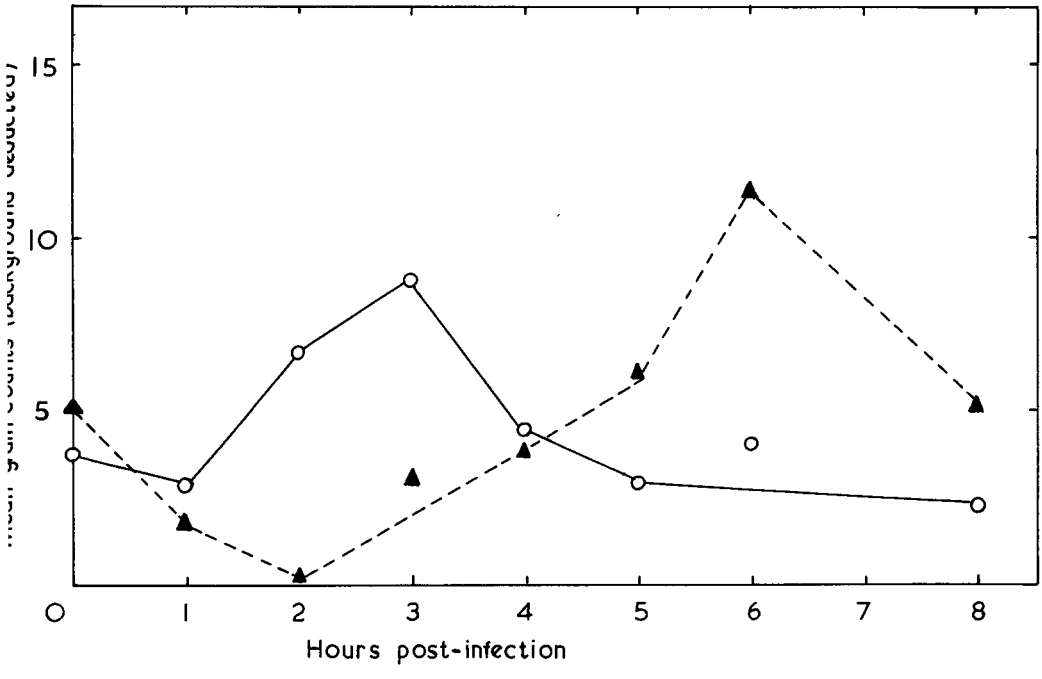

Figure 6. Incorporation of (^{3}H)-uridine triphosphate into the nuclei and cytoplasm of cells infected with FPV, fixed at various times after infection and incubated with an RNA dependent RNA polymerase reaction mixture containing actinomycin D. Infected and control CEF cells were fixed at hourly intervals in cold alcohol-acetone. Cell strips were incubated with the reaction mixture at 32° for 45 minutes, fixed and washed in TCA, and prepared for autoradiography. Cytoplasmic and nuclear grains were counted for 100 cells at each time point. The background levels (4.60 grains / cell for cytoplasm, 1.97 grain / cell for nucleus) were deducted from mean values. O———O nuclear grains ▲- - - -▲ cytoplasmic grains.

on the sensitivity of virus replication to treatment with α-amanitin, a bicyclic octapeptide from the toadstool, Amanita phalloides, that inhibits DNA transcription in vitro by binding specifically to the RNA polymerase, form II of eukaryotic cells without affecting polymerase I (Kedinger et al., 1970).

The replication of FPV in CEF is inhibited by α-amanitin, if the drug is added during the first two hours following infection at concentrations similar to those used to inhibit cellular DNA dependent RNA polymerase (Mahy et al., 1972). Of two periods of increased RNA synthesis in infected cells only the first, occurring from 0 to 2 hours after infection, is sensitive to α-amanitin. During this early period there is a stimulation of the activity of DNA-dependent RNA polymerase II.

Acknowledgements

I am indebted to all my colleagues both for providing data and helpful comments during the preparation of this manuscript. My particular thanks are due to Dr. McGeoch for useful discussions, and to Mr. A.R. Carroll who generously provided the data presented in Table 1, and Figures 1-3; Mr. R.H. Raper for Figure 4 and Miss S.E. Glass for Figure 5.

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DISCUSSION

Dr. Scholtissek : What evidence do you have that P-1 protein in Sendai virus is the polymerase ?

Dr. Barry : We have no direct evidence. The American group led by Kingsbury claim that the replicative complex from infected cells and the virion transcriptase itself consists on the nucleoprotein, and a large molecular weight protein MW 75.000.

Dr. Scholtissek : One comment concerning the corresponding protein of influenza virus. There is other evidence, or there are some suggestions at least, that the P proteins of influenza are not polymerases. Caliguiri for example has purified the enzyme and finds that it contains only nucleoprotein.

Dr. Barry : There may be problems concerning the interpretation of Compans and Caliguiri's experiment, firstly because they could not ressolve protein X, which we find reproducibly in cells and virus. Secondly, their conclusions are based on a pulse-labelled experiment and it could be that P proteins are in the enzyme complex, but not labelled under the conditions of the experiment.

Dr. Scholtissek : I see, but other evidence by Klenk indicates that

when cells are fractionated, practically all the P protein stayed in the supernatant, but this fraction is free of enzyme activity.

Dr. Barry : I do not have any strong views concerning the role of P proteins in enzyme function. I have mentioned them as possible candidates for this job, since they are the only structural polypeptides to which a function has not yet been assigned.

Dr. S. Schlesinger : Did I understand you correctly ? Does 60% of ^{32}P-labelled virion RNA become double-stranded ?

Dr. Barry : Because I was hurrying, I may have missed a crucial point in my explanation of this experiment. The infecting virus is labelled with ^{32}P, and at intervals after infection the total cell RNA is extracted and self-annealed.

Dr. S. Schlesinger : My question had to do with the fact that the ratio of infectivity of particles in influenza is usually quite low. It seems to me that there is an awful lot of RNA which is not really going to produce virions, but is still able to be transcribed into the complementary strand.

Dr. Barry : I do not think that this experiment indicates how much input virion RNA is actually transcribed. What is contributing to the double stranded RNA is newly synthezised complementary RNA. Its production may be initiated and continued by only a small amount of the total input RNA. Once produced however, newly formed c RNA may soak up any excess, non-functional virion RNA.

Dr. S. Schlesinger : Where does the figure of 60% come from ? Is it the total amount of input RNA converted into RNAse insensitive material after self-annealing[2].

Dr. Barry : Yes.

Dr. Becker : I am wondering about the fragmented genome, and whether or not it would be worthwhile to check this problem again. I happened to see a short while ago a reprint from Ruth Kravineff on reovirus double stranded RNA, and this I think agreed that it has a fragmented genome.

When she changed the conditions of extraction, she found that the genome is circular and complete. It is my interpretation that the virion might contain a restriction enzyme that always cleaves the RNA in the same position. What is the possibility of testing this hypothesis, at least in the case of influenza virus, to see whether or not you have the complete genome which is always fragmented in the same position, and this is why we have always six to seven fragments? I also have a second question. What is the mechanism of replication or synthesis of the plus strand ? Is it the same as we think about other single-stranded RNA viruses ?

Dr. Barry : To take the second question first - all the RNA dependent RNA polymerases we detect in cells infected by either class of myxovirus makes predominantly, if not totally, complementary RNA. Nobody has found reaction conditions where there is a significant increase of virion RNA. The replicase has not yet been identified. In answer to the first question, it is possible that the RNA in the virion is a single polyribonucleotide. Several years ago, Sato claimed that RNA released from influenza virus by very gentle extraction procedures and examined by electron microscopy was much longer than any of the RNA fragments identified by gel electrophoresis. The possibility remains that the RNA obtained from influenza viruses after detergent treatment and phenol extraction is the product of a restriction enzyme. There is no information on this point.

Dr. M. Schlesinger :Is it possible to look at the product of your in vitro reaction on gels and get some indication of its size ?

Dr. Barry : This problem has been examined by Bishop and colleagues. When the reaction mixture is placed directly on gels, the product has migratory properties similar to template RNA. However, in this case, it is not clear whether this is completed, new product, or whether it is partly formed products still associated with template. When the product is melted before electrophoresis, it is found to be much smaller than any template RNA. However, this analysis was carried out on a sub-optimal reaction product, and needs to be repeated, using the best available virus and reaction mixture.

Dr. M. Schlesinger :I would like to ask one further question. In the infected cell, do all these different fragments show up in equal molar quantities ?

Dr. Barry : No.

Dr. M. Schlesinger :You said they were monocystronic ?

Dr. Barry : Yes, because polysome associated complementary RNA for both types of virus is predominantly 15-20 S, even though the para-influenza virus template is 50 S.

Dr. M. Schlesinger :You do not know whether one species occurs in greater amounts than another .

Dr. Barry : Gel electrophoresis suggests that RNA of approximately 18 S is relatively more abundant than RNA that is 20 S or larger.

Dr. Van Oirschot : Do you have any information about the role of the nucleocapsid in the in vivo transcriptional process ?

Dr. Barry : The so-called "microsomal" enzyme found in infected cells, and described originally by Ho and Walters (1966), appears to contain the ribonucleoprotein nucleocapsid, as shown by Compans and Caliguiri. Furthermore, these workers could not detect P protein in this complex. Since it has so far proved impossible to separate enzyme activity from nucleocapsid, it would seem that either the ribonucleoprotein is the enzyme, or there is another protein present that is of a similar molecular weight.

REPLICATION OF MYXOVIRUS RNA

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Myxoviruses contain single-stranded RNA as genetic material. In contrast to parainfluenza viruses, which have a single RNA-molecule with a molecular weight of about 7 x 10^6 (1), influenza virus RNA was found to exist and to function in pieces (2,3,4). Regardless of this fundamental difference in the structure of RNA both types of RNA have to be replicated in such a way that the virion RNA functions first as a template for the viral RNA polymerase to form the complementary RNA, which again serves as template either for the same, or a modified, or a totally different viral enzyme to form the progeny virion RNA.

I shall mainly concentrate my talk on the in vivo synthesis of myxovirus RNA considering the following problems:

1. What is the time course of the appearance of viral RNA polymerase activity ?
2. What is the time course of the appearance of viral RNA ?
3. What is the stability of viral RNA in vivo ?
4. Are the virion RNA and complementary RNA synthesized by completely different enzymes ?
5. Does the viral enzyme synthesizing the virion RNA use the same nucleoside triphosphate pools as the cellular enzymes do ?
6. How far can the synthesis of viral RNA be influenced by inhibitors ?

I want to select one virus of each group of the myxoviruses as examples: for the influenza virus I want to talk about results obtained with fowl plague virus (FPV), which is an influenza A virus. For the parainfluenza viruses I will present data obtained with the Newcastle disease virus (NDV).

1. Time course of appearance of viral RNA polymerase

Fig. 1 (left) shows the appearance of the FPV RNA polymerase activity in chick embryo cells after infection (5). The enzyme activity slightly preceeds the appearance of the RNP-antigen in the infected cells. Up to 20% of the enzyme activity is tightly bound to the nuclear fraction. In our hands only complementary RNA is synthesized by the enzyme preparation in vitro (6). Even the enzyme of the nuclear fraction produces between 50 and 80% complementary RNA. When the nuclei of FPV-infected cells were incubated with (^{3}H)-GTP, there was no significant radioactive material hybridizable with non-labelled complementary RNA (unpublished).

Fig. 1 (right) shows the corresponding data for NDV. The NDV-RNA polymerase, too, produces in vitro mainly complementary RNA. All viral components, including polymerase, are synthesized at a slower rate compared with FPV-infected cells (7).

2. Time course of appearance of viral RNA in vivo

In the case of influenza viruses actinomycin D cannot be used to trace viral RNA synthesis, since this antibiotic does not only inhibit cellular RNA synthesis but it also completely suppresses the production of influenza viruses (8,9,10). Therefore we used the method of specific hybridization of newly synthesized labelled viral RNA with a surplus of either non-labelled virion RNA or complementary RNA. The experiment is done in the following way: at different times after infection a pulse with labelled uridine is given, the total RNA is extracted by phenol, and aliquots are hybridized with the various non-labelled viral RNA samples. The results are presented in Fig. 2. Two hours after infection there is a maximal production of complementary RNA, one hour later mainly virion RNA is being synthesized. Double-stranded RNA is found only in very low amounts (11).

With parainfluenza viruses the time course of virus-specific RNA can be determined with the aid of actinomycin D, since these viruses multiply in the presence of the antibiotic (8,9). Kingsbury (12) was the first one who used the technique to follow NDV-RNA synthesis. Fig. 3 demonstrates the time course of NDV-RNA synthesis in our system (13). The curve is comparable to that of Fig.1 (right). Kingsbury (14) found

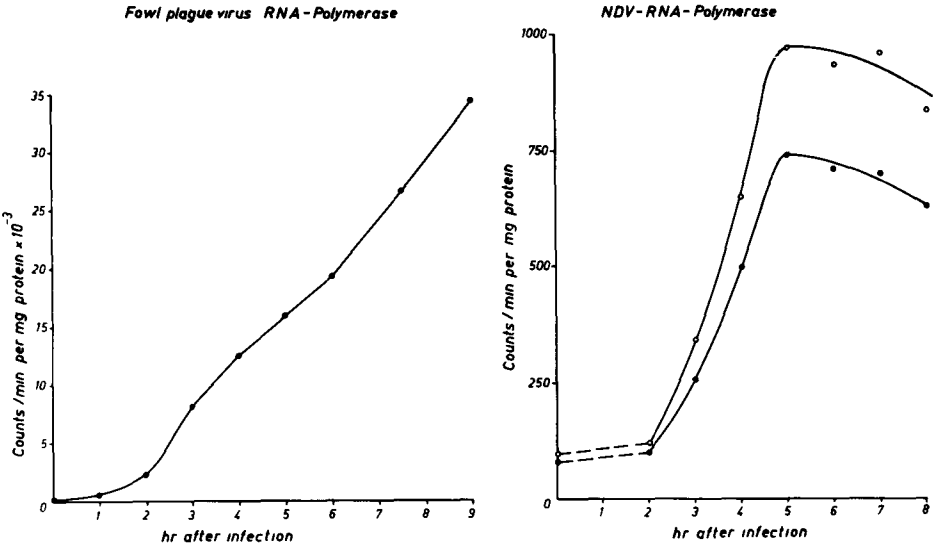

Fig. 1. Appearance of RNA polymerase activity in the cytoplasm of cells infected either with FPV (left) or NDV (right).

Cytoplasmic fractions were prepared at the times after infection as indicated on the abscissa. They were incubated with (^{3}H)-GTP and cofactors for 10 min in the case of FPV (left), or either for 3 min (•) or 5 min (o) in the case of NDV (right). The radioactivity was determined in RNA.

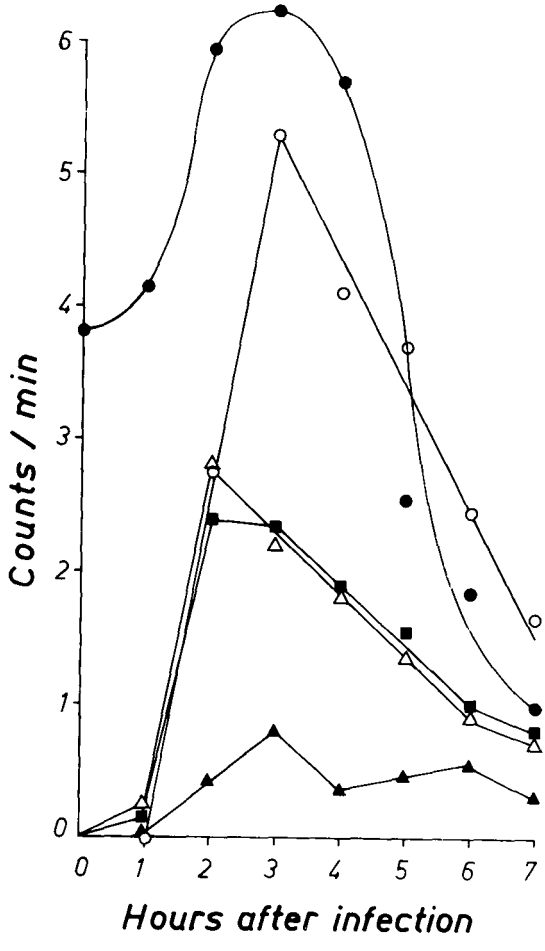

Fig. 2. Synthesis of virion and complementary RNA of FPV in vivo.

To 6 infected cultures 10 μCi (^{3}H)-uridine, each, was added at the times as indicated on the abscissa; 30 min later total RNA was isolated and dissolved in 1 ml Tris-HCl (5 mM) plus EDTA (1 mM). Aliquots of 0.1 ml were used for the determination of total RNA (●, x 10^{-4}), virion RNA (o, x 10^{-3}), complementary RNA (△, x 10^{-3}), double-stranded RNA (▲, x 10^{-3}), and self-annealed RNA (■, x 10^{-3}).

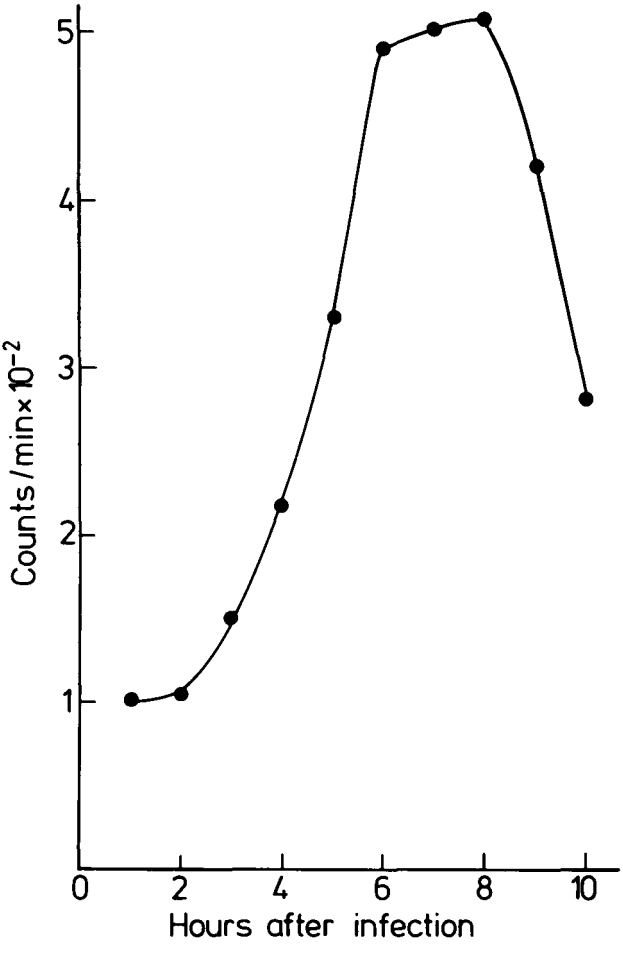

Fig. 3. Time course of NDV-RNA synthesis in vivo.

Viral RNA was measured by adding (^{14}C)-uridine (1 µCi/culture) to the infected cultures at the times indicated on the abscissa. Half an hour later the cultures were processed for the determination of the radioactivity in the RNA. Cellular RNA synthesis was suppressed by addition of 5 µg/ml of actinomycin D.

that most of the in vivo synthesized NDV-RNA is complementary to virion RNA. Most of this RNA has a sedimentation constant of 18 S (15). Fig. 4 shows a corresponding sucrose gradient profile (16).

3. Stability of myxovirus RNA

Since virus-specific RNA also functions as messenger for protein synthesis, we wanted to know how stable the different kinds of viral RNA are in vivo. Since there is no specific inhibitor for viral RNA synthesis available like actinomycin D for cellular RNA synthesis, pulse-chase experiments had to be performed. In animal cells, however, labelled nucleosides cannot be used for such pulse-chase experiments, since the nucleoside triphosphate pools are too large to be sufficiently diluted with the non-labelled nucleoside after the pulse in order to follow the fate of the newly synthesized macromolecule. For this reason we have incubated infected cells with glucosamine either prior to the pulse with (^{3}H) - uridine or immediately thereafter. Glucosamine specifically reduces the UTP-pool (17). In the case of FPV we incubated the infected cells with (^{3}H) -uridine at 4°. At this temperature uridine is phosphorylated to UTP but is not further metabolized (18). After washing off the surplus of (^{3}H) - uridine, the infected cells were further incubated with glucosamine at 37°. Now the viral RNA becomes labelled by the prelabelled (^{3}H) - UTP. After 30 or 40 min a surplus of non-labelled uridine is added, the uptake of which is facilitated by lowering the UTP-pool by glucosamine (19). This stops further incorporation of labelled UTP into RNA. Now the fate of labelled viral RNA can be studied (16).

Table 1 shows that under these conditions FPV-RNA, the virion as well as the complementary RNA, is completely stable for at least 90 min. Fig. 4. shows corresponding data for NDV. The sucrose gradient pattern of NDV-RNA does not change significantly during a chase of 140 min. Thus both types of viral RNA are not catabolized significantly during a reasonable time of the infectious cycle.

4. The enzyme(s) synthesizing virion and complementary RNA of FPV

If two completely different enzymes were involved in the synthesis of virion and complementary RNA, one should not be able to find a single gene mutant in which both steps are inhibited. We have been searching for temperature sensitive mutants of FPV, and Table 2 presents

data on such a mutant induced by 5-fluorouracil (20). At the permissive temperature (33°) this mutant synthesizes both kinds of RNA. If we shift the temperature at 4 hr after infection from 33° to the non-permissive temperature of 40°, virion as well as complementary RNA synthesis is completely abolished. As soon as we come down to 33° again, synthesis of both types of RNA is resumed. These results strongly suggest that either both enzymes are identical or that they contain at least one common virus-specific protein which is necessary for enzyme activity in vivo. There are no comparable data available for NDV.

TABLE 1

STABILITY OF FPV-RNA IN VIVO

Counts/min in	Cold chase (min.)		
	0	30	90
Total RNA	15,800	13,000	10,600
Virion RNA	2,200	2,100	2,150
Complementary RNA	1,550	1,380	1,450

After infection for 2.5 hr, each culture was incubated for 1 hr at 4° with 25 µCi (^{3}H) - uridine. After removal of surplus (^{3}H) - uridine the cultures were incubated with 10 mM glucosamine at 37° for 30 min (= pulse). Thereafter, 0.1 mg/ml of non-labelled uridine was added (= cold chase). Each value is the mean for the RNA component analysed on five separate cultures.

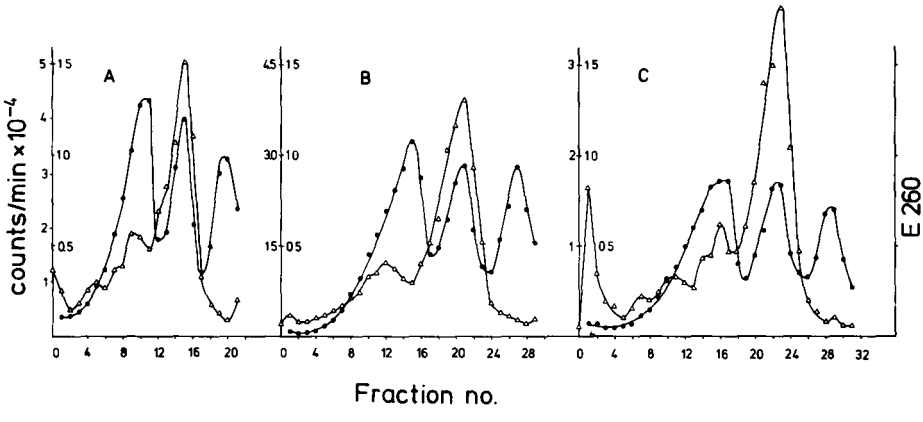

Fig. 4. Stability of NDV-RNA.

To NDV-infected cells 5 hr after infection 2 µg/ml of actinomycin D and 10 mM glucosamine were added. A pulse with 25 µCi/culture of (^{3}H) - uridine was started 90 min later. After 40 min part of the cultures were processed (17) (A) or received 0.1 mg/ml non-labelled uridine to stop further incorporation. Either 50 min (B) or 140 min (C) later the other cultures were processed for isolation of RNA and sucrose gradient analysis (16).

TABLE 2

SYNTHESIS OF VIRAL RNA BY A TEMPERATURE-SENSITIVE MUTANT OF FPV

	0 – 4 hr 33°	0 – 5.5 hr 33°	0 – 5.5 hr 40°	0 – 4 hr, 33° 4 – 5.5 hr, 40°	0 – 4 hr, 33° 4 – 5.5 hr, 40° 5.5 – 6.5 hr, 33°
Virion RNA	20.0	22.7	0.0	0.6	9.9
Complementary RNA	4.2	3.3	0.0	0.6	1.8

Chick embryo cells were infected with the ts-mutant and were incubated either at 33° or at 40° at the times after infection as indicated. Each value is the average of three cultures, which were labelled with 25 µCi (^{3}H) – uridine, each, starting 1 hr before the cultures were processed. The values are expressed as % radioactivity in each virus RNA strand compared with total newly synthesized RNA (about 100,000 counts/min/sample). The corresponding background values of non-infected control cells were subtracted before calculation (11).

5. Nucleoside triphosphate pools used for viral RNA synthesis

In order to get an idea whether different nucleoside triphosphate pools are used for viral and cellular RNA synthesis we have to manipulate these pools and look how far this manipulation affects virus and cellular RNA synthesis. We used for this purpose again glucosamine which is known to reduce specifically the UTP-pool (17). When Earle's medium containing glucose is used for incubation, there is no significant inhibirory effect on viral RNA synthesis (Table 3), although no infectious virus is produced (21), as will be shown later on by Dr. Rott. If, however, glucose is replaced by fructose as energy source of the medium, glucosamine is about 10 times more effective concerning the effect on the UTP-pool, because fructose does not interfere with the uptake of glucosamine as glucose does (22). Under these conditions the amino sugar specifically interferes with the production of viral RNA synthesis leaving cellular RNA synthesis intact (Table 3). The effect is most pronounced on the synthesis of virion RNA and less on complementary RNA (22). In the Semliki Forest virus system actinomycin D can be used to suppress cellular RNA synthesis; and (^{3}H) - adenosine was investigated instead of (^{3}H) - uridine to label the viral RNA. Therefore with this virus system it could be shown that the effect of glucosamine on viral RNA synthesis can be counteracted by adding uridine to the culture medium, but not by guanosine or cytidine (22). Thus viral RNA synthesis is inhibited because the UTP-pool becomes rate limiting specifically for viral RNA synthesis.

This observation can be explained in two ways: either the viral RNA polymerase has a lower affinity for UTP as compared to the cellular enzyme using the same UTP-pool, or different and more or less independent UTP-pools are used for the synthesis of viral and cellular RNA. To study this problem further we have performed the following experiment: We incubated cells infected with FPV from 4 to 5 hr after infection with (^{3}H) - uridine, isolated total RNA, hybridized the labelled virion RNA with a surplus of non-labelled complementary RNA and after digestion with RNase the acid soluble material (= cellular plus complementary RNA) and acid precipitable RNA (= virion RNA) were digested further by alkali to the 2',3' - nucleotides. Then we determined the ratio of the specific radioactivity of CMP to UMP. The radioactivity found in CMP is obtained from the de novo synthesis of CTP via the UTP-pool. If the cellular enzyme uses the same pyrimidine nucleoside triphosphate pools as the viral enzyme then

TABLE 3

EFFECT OF GLUCOSAMINE ON FPV-RNA SYNTHESIS IN VIVO

Exp. No.	Medium 10 mM	Glucosamine mM	Pulse from .... to .... hr after infection	Virion RNA % of total	Complementary RNA % of total
1	glucose	0	3 - 4	23.3	4.6
		5	3 - 4	21.8	8.8
	fructose	0	3 - 4	21.0	4.7
		5	3 - 4	6.7	4.1
2	fructose	0	3 - 4.3	37.0	10.6
		5	3 - 4.3	3.0	3.0

Glucosamine was added immediately after infection of chick embryo cells with FPV. The medium contained either 10 mM of glucose or fructose as energy source. For further details see Table 2.

the ratio of (^{3}H) - CMP to (^{3}H) - UMP should be the same for both types of RNA. If the ratio is different for both types of RNA then the viral enzyme uses other pools. As shown in Table 4 the ratios of the specific activities of (^{3}H) - CMP to (^{3}H) - UMP are completely different demonstrating that two different pyrimidine nucleoside triphosphate pools are used for viral and cellular RNA synthesis (23). This observation does not rule out the possibility that in addition there might be a difference in the affinity of the two enzymes for the same substrates.

TABLE 4

RATIO OF THE SPECIFIC RADIOACTIVITY OF CMP/UMP AFTER LABELLING OF FPV VIRION - AND CELLULAR RNA BY (^{3}H) - URIDINE

	Virion RNA	Cellular RNA
Dpm in CMP	9,795	10,665
Dpm in UMP	166,615	292,454
% of CMP in RNA	25.2	30.2
% of UMP in RNA	32.0	18.4
Spec. activity CMP/UMP	0.075	0.022

Chick embryo cultures were labelled with 50 µCi of (^{3}H) - uridine, each, from 4 to 5 hr after infection with FPV. Thereafter total RNA was extracted by phenol plus 0.5% sodium dodecyl sulfate at room temperature. After hybridization with a surplus of non-labelled complementary RNA the perchloric acid-precipitated (=virion RNA) as well as soluble material (= cellular plus about 10% complementary RNA) was digested by 0.5 M KOH at 65° for 45 min. Radioactivity was determined in CMP and UMP. For the calculation of specific radioactivity the base composition of fowl plague virus was taken from Rott et al. (26); that for total cellular RNA from Scholtissek (27).

The multiplication of NDV also can be inhibited by glucosamine when glucose is replaced by fructose in the medium (22).

Although we have not yet studied the NDV-RNA synthesis under these conditions, the effect of the amino sugar might also involve viral RNA synthesis.

6. Inhibition of viral RNA synthesis by antibiotics

I mentioned at the beginning that the multiplication of influenza viruses can be inhibited by actinomycin D, while that of parainfluenza viruses cannot (8,9,10). If the antibiotic is added later in the infectious cycle when the influenza RNA polymerase is already present, virus maturation is not much impaired.

In Table 5 data are presented which demonstrate that FPV-virion RNA synthesis proceeds for at least some time when actinomycin D is added to the infected cultures 2 or 3 hr after infection, while synthesis of complementary RNA is almost completely suppressed. Thus we know that this antibiotic interferes with the production of complementary RNA in vivo, although we do not know anything about its mechanism. In vitro actinomycin D is without the effect. We can isolate the enzyme from actinomycin treated cells and synthesize the complementary RNA in the test tube. Therefore, it must have been synthesized in the actinomycin D-treated cells, although it is not functional in vivo (11).

If we inhibit protein synthesis 2 or 3 hr after infection with FPV by adding cycloheximide we suppress specifically the production of virion RNA, but not that of complementary RNA (Table 5). We have interpreted this observation by assuming that we need a viral protein for the switch from synthesizing mainly complementary RNA to virion RNA. This viral protein has to be continuously synthesized and might remove the virion RNA as template for the synthesis of complementary RNA. If we stop the synthesis of this protein, then production of complementary RNA prevails (11). A candidate for such an effect would be the NP-protein.

Other antibiotics like mithramycin (24) and α-amanitin (25) have a similar effect as actinomycin D concerning viral RNA synthesis. All these antibiotics under comparable conditions do not interfere with the production of NDV-RNA in vivo.

Concluding remarks

The first virus-specific activity found in chick embryo

TABLE 5

INHIBITION OF FPV-RNA SYNTHESIS IN VIVO BY ACTINOMYCIN D AND CYCLOHEXIMIDE

Addition at 2 hr after infection	Radioactivity in RNA	Counts/min
None	total RNA	42,000
	virion RNA	3,400
	compl. RNA	1,600
Cycloheximide 10 µg/ml	total RNA	37,800
	virion RNA	320
	compl. RNA	1,700
Actinomycin D 2 µg/ml	total RNA	1,700
	virion RNA	1,000
	compl. RNA	80

Chick embryo cells were infected with FPV. Two hr later either cycloheximide or actinomycin D as indicated were added. The pulse with 25 µCi (^{3}H) - uridine per culture was started 3 hr after infection. The cultures were processed 1 hr later. For further details see Table 2.

cells infected either with an influenza (FPV) or parainfluenza virus (NDV) is the RNA-dependent RNA polymerase synthesizing viral complementary RNA. In the case of FPV the synthesis of complementary RNA preceeds that of virion RNA. There is at least one common viral protein involved in the synthesis of virion and complementary RNA. In NDV-infected cells mainly complementary RNA with a sedimentation value of about 18 S accumulates. All viral RNA once synthesized is stable during the infectious cycle. The multiplication of both viruses can be inhibited by glucosamine, when glucose of the medium is replaced by fructose. For FPV it is shown that the effect is on viral RNA synthesis. It is suggested that virion RNA synthesis gets its precursor supplies from different pyrimidine nucleoside triphosphate pools than the corresponding cellular enzymes. Concerning the effect of various antibiotics on viral RNA synthesis, there are fundamental differences between influenza and parainfluenza viruses. Actinomycin D, mithramycin and α-amanitin specifically interfere with the production of

influenza complementary RNA in vivo, while cycloheximide prevents production of virion RNA, when added 2 to 3 hr after infection. All these antibiotics have no effect on the production of NDV-RNA under comparable conditions. Especially these and other differences mentioned have to be taken into consideration when one deals with the molecular biology of myxoviruses as a whole group.

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DISCUSSION

Dr. M. Schlesinger : In the mutant that you mentioned where do you think the mutation is? Do you think that the mutation is in a structural protein ? Do you denature it by bringing the infected cells to high temperature and after lowering the temperature do you need de novo synthesis of this protein ?

Dr. Scholtissek : The polymerase is not denatured at the non permissive temperature, it is functional when checked in vitro. The polymerase of the mutant works in vitro but in vivo it is not functional. There is some similarity to the actinomycin D effect. We do not denature the polymerase at 40°C.

Dr. Van der Zeijst : I should like to ask what do you think of the controversary results about the polarity of RNAs in viral polysomes and the functioning of the viral RNAs as a messenger RNA in the cells?

Dr. Scholtissek : People call the influenza viruses negative stranded viruses because the RNA complementary to viral RNA should be the messenger RNA. But it could well be that for example one piece of the virion RNA functions as template for the production, for example of the nucleocapsid protein while another piece of RNA which is complementary to the virion RNA might function for the synthesis of another virus specific protein for example, for the matrix protein as found by Kingsbury. So it is difficult to define influenza virus as a negative strand virus at the moment. Furthermore contradictory results have been published concerning the kind of RNA bound to polysomes : Nayak found mainly virion RNA with polysomes, while Pons found complementary RNA. Even this point is not clear.

Dr. Barry : It seems that this question is directed both to Dr. Scholtissek and myself. I disagree slightly in that both by reason of circumstantial evidence and because of some data we have ourselves, that the RNA associated with polysomes of influenza virus infected cells, particularly with fowl plague, is more than 90% complementary and contains poly A. I think that the Hofschneider-experiment has to be considered in the light of the fact that many preparations of RNA from influenza virus show a certain degree of self complementarity. So one could argue that the translation you see in an in vitro system using influenza RNA is due to a low level of complementary RNA that has been incorporated into the virion. We find

looking in virus particles that the sort of RNA that is there is not only virion RNA but there is also a small amount of cellular RNA. This is not complementary. This is ribosomal RNA and it is in the virus particles. So it is my impression that the assembly process of influenza viruses is such that quite frequently it incorporates other RNA. Sometimes this may be complementary RNA, sometimes it's cellular ribosomal RNA. So my reading of the Hofschneider-result is that this is contributed by a small proportion of complementary RNA accidently incorporated into a certain proportion of virions.

Dr. Scholtissek : Yes, this is a possible explanation of this discrepancy tool. There are other explanations, I mean, we ourselves did not find any complementary RNA virions. It was not more than 0.5.% which is just background of the method. But this again might be strain-dependent. I know for example from people looking for complementarity of the Newcastle disease virus RNA that there were differences between 3 and 20% self complementary depending on the strain used. It could be that there are also influenza strains which incorporate the complementary RNA, because our own studies on in vitro experiments using nucleocapsid protein and virion RNA as well as complementary RNA had shown that both strands have the same affinity to the NP protein.

Dr. Becker : You mentioned polysomes and RNA in the polysomes and different sorts of RNA on the polysomes. I have the feeling that people are using the word "polysome" in a very loose manner these days. We all know especially from the experiments of Baltimore about ten years ago that RNA can bind to a protein and sediment non-specifically so to say in the polysome region. Now if you look at the RNA which sediments together with the polysomes you might make a mistake if you think that all the RNA is there because it is bound to ribosomes. If you really want to know what is associated with the ribosomes you have to fix the polysome region with glutaraldehyde and then run in a Caesium chloride gradient and see what proportion of the radioactivity will sediment at a density of 1.54 which is the density of the ribosomes or the polysomes. Now when we did experiments of that sort in another system we discovered that although we have a beautiful profile of polysomes and RNA sedimenting with the polysomes; only a small amount of radioactive RNA is really bound to ribosomes ; so if people are discussing polysomes these days I think that they have to

subject a more stringent test ; this is one thing ; the other thing is, I would like perhaps to ask Dr. Scholtissek and Dr. Horzinek how this technique of replacing glucose with fructose would be helpfull in labelling the classical swine fever virus which is, I understand, very hard to label these days.

Dr. Scholtissek : You might get radioactivity into this RNA if you apply this method. I have labelled different kinds of RNA at the moment only using glucose in the medium, but the labelling is about ten times better when glucose is replaced by fructose. Concerning the polysomes, I completely agree with you that one has to be very careful in talking about the virus-specific RNA found in the polysome region. I do not think that anybody has done this very stringent experiments. And another point is that it is also very difficult to remove the RNP antigen, this means nucleocapsid + RNA which might migrate into the polysome region.

Dr. Becker : Not if you fix it with glutaraldehyde. This complex should have an entirely different density I believe so. I did not do any experiments but the ribosome-RNA complex has a very distinct property to bound at particular densities. But may I ask you to give perhaps a little more detail about this fructose experiment. How much you use and how general is this phenomenon ?

Dr. Scholtissek : I first have to say that it is very important to know the enzymatic state of the cell. For example when we used chick embryo cells 22 hours after seeding we had completely different results compared with cells 48 hours after seeding. It is very important that the corresponding enzymes are very active in these cells. If you are working with liver cells you better use galactosamine, because liver cells have high levels of galactokinase and also converts galactosamine into the corresponding intermediates depleting the cell of UTP. So we have to check the cells very carefully in order to be sure that they work. For example we also tried KB cells and in the KB cells the effect is much less pronounced, but here again if you replace glucose by fructose you might be able to succeed. So, if at the first trial you do not get a corresponding result you then have to play a little bit around with the method and you might also use other energy sources like pyruvate for example. Pyruvate is also a very good compound which you can use as energy source and which does not

interfere with the uptake of glucosamine. And if glucosamine does not work you might use or try other amino sugars. With 2-dioxyglucose, I should mention you get a very similar effect. It is a dogma that 2-dioxy glucose is not further metabolized than to the level of the 6-phosphate. Cori and others have published this already several years ago and people are using 2-dioxy glucose as a probe to study transport. But we found that we can deplete the UTP and we get all the activated 2-dioxy glucose intermediates in our cells in large quantities.

STRUCTURE AND SYNTHESIS OF INFECTIOUS PANCREATIC NECROSIS (IPN) VIRUS RNA

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ABSTRACT

Data are presented concerning the structure of IPN virus genome, the kinetics of both single-stranded and double-stranded virus-directed RNA synthesis in infected FHM cells, and the effect of temperature on virus replication and virus-directed RNA formation.

The IPN virus RNA was found to be double-stranded according to several physico-chemical characteristics, namely : resistance to pancreatic RNase, low density in Cs_2SO_4(1.616), sharp melting curve with a Tm of 89°C in hypotonic buffer, and base composition. By gel electrophoresis, the virus RNA was resolved into three peaks corresponding to double-stranded molecules with mol. weights ranging from 2.85×10^6 to 2.55×10^6.

In FHM cells (temperature optima : 34°C) the optimal temperature for virus replication was found to be close to 20°C. IPN virus replication in FHM cells was unaffected by 0.5 ug Actinomycin per ml., a concentration which reduces the amount of host-cell RNA synthesis to negligible levels within 5 hours. Under these conditions of selective inhibition, the synthesis of virus-directed single-stranded and double-stranded RNA was first detected 5 hours post infection. Maximum amounts of total RNA were found at 16 hours after infection. On further incubation these amounts decreased, suggesting that a degradation process, affecting most probably the viral messenger RNA, prevails from this time over any further synthesis. By 16 hours post-infection, the amounts of double-stranded molecules had also reached a maximum, representing about 20% of total RNA. We noticed that there was a strict temporal relationship between virion synthesis and double-stranded RNA formation.

When infected cells were maintained at 20°C for three hours and then shifted to supraoptimal temperatures (24°C, 26°C, 28°C)

it was found that virus multiplication was inhibited by 15% after a shift to 24°C and by 80% after a shift to 26°C. A shift to 28°C effected a complete block in virus multiplication. As could be expected, the overall virus-directed RNA synthesis after shifting the cells to 24°C, was not significantly affected. At 26°C the apparent rate of synthesis was considerably reduced, and at 28°C no virus directed RNA synthesis could be detected.

When infected cells were shifted to 28°C at different times after infection, virus directed RNA synthesis was either apparently suppressed without delay (late shifts) or slightly enhanced during a space of time and then supressed (early shifts), suggesting that some chain termination may occur even at 28°C. At 20°C and at 28°C we noticed that the rate of degradation of single-stranded RNA was very much the same. Therefore, it seems reasonable to assume that supraoptimal temperatures affect directly the mechanisms involved in viral RNA transcription and replication.

INTRODUCTION

Infectious pancreatic necrosis virus is the etiologic agent of an acute disease which may cause high mortality specifically among young salmonid fishes (WOLF et QUIMBY 1967 ; WOLF et al. 1960). Although the classification of the virus has been a matter of controversy (MALSBERGER et CERINI 1963 ; ARGOT 1969 ; NICHOLSON 1971 ; KELLY et LOH 1972), data have accumulated which indicate that the virus may well be classified among reolike viruses, specially because of double-strandedness and segmentation of its RNA genome (COHEN et al. 1973), size, symetry and structure of the capsid (COHEN et SCHERRER 1972) and cytoplasmic site of replication (MOSS et GRAVELL 1969).

A few studies have already been published concerning the multiplication of IPN virus in fish cell cultures (MALSBERGER et CERINI 1965, MOSS et GRAVELL 1969). Therefore, the main characteristics of the viral growth-cycle are at present known. However, the biochemistry of virus replication is still unknown although some attempts have been made to analyse the synthesis of viral RNA (NICHOLSON 1971) or viral proteins (PIPER et al. 1973) in the infected cells.

In our laboratory we have focused our attention since a couple of years on the effects of temperature on virus replication since

the elucidation of a viral thermosensitive step can supply much information on the mode of IPN replication and especially on the nature of the virus-cell interactions. The IPN-FHM system exhibits the following particularity: the temperature at which virus synthesis is completely shut off, i.e. 28°C, is a perfectly normal temperature for cell metabolism, since we usually grow FHM cells at 28°C. Such a situation is rarely encountered in homeothermic systems where non permissive temperatures for the viruses are usually supra-optimal for the host-cells (ZEBOVITZ and BROWN, 1967). This study should provide us with some preliminary data for subsequent work on interferon induction by IPN virus in FHM cells. Indeed we already know that IPN virus is a good interferon inducer not only at permissive temperatures but also at non permissive temperatures i.e. at 28°C, 30°C or 34°C (SCHERRER et al. 1974). Knowing the mechanism by which temperature shuts off virus multiplication would enable us to determine which viral structure (or which viral function) is sufficient to trigger interferon synthesis in the infected cell.

In the present work we intend to recall first some data obtained in our laboratory concerning the structure of the viral genome ; concerning the effect of temperature on IPN virus multiplication, the study presented here is still in progress and the results thus far obtained permit us to elaborate some interesting hypothesis.

RESULTS

A - Properties of the virus RNA

Viral RNA labelled with ^{32}P or ^{14}C was extracted from purified virus by phenol-SDS (MONTAGNIER 1968 ; HAREL et MONTAGNIER 1971) and analysed using a variety of different techniques. All the results obtained in our laboratory (COHEN et al. 1973) indicate that the viral genome is double-stranded and that it may consist of a single size-group of RNA segments having a mol. Wt. ranging from 2.85×10^6 to 2.55×10^6.

a) Resistance to hydrolysis by RNase.

Resistance of the virus RNA to hydrolysis by pancreatic RNase A has been tested by measuring the amount of nucleic acid rendered soluble after treatment with the enzyme, in different buffers. It is shown in table I that, at a molarity greater than 0.1 SSC ^{14}C labelled virus-RNA is resistant to hydrolysis, but at lower molarities (0.1 SSC or 0.01 SSC) the RNA becomes highly susceptible to the enzyme. It is also shown in

table I that, at low ionic strength (10^{-2} M-tris Hcl, pH 7.4) the resistance of IPN RNA is markedly increased by Mg^{2+}.

b) Melting behaviour.

To obtain information on the melting behaviour, the virus RNA was heated in 0.1 SSC at a given temperature for 10 min. and then rapidly cooled in ice. Subsequently the percentage of radioactivity rendered acid soluble, after treatment with pancreatic RNase (10 µg/ml. ; 0.2 M NaCl ; 30 min. ; 37°C) was determined by precipitation with 5% TCA. As it is shown if fig. 1 the viral RNA exhibited a sharp melting profile with a Tm value of 89°C. Thus, the Tm value for IPN virus RNA is slightly higher than that reported for reovirus type 3 (SHATKIN 1965).

TABLE 1

RNase susceptibility of IPN virus RNA

RNA	Buffer	Acid-insoluble radioactivity (ct/min)	Hydrolysed (%)
RNA only	1 x SSC	8961	-
RNA + RNase (10 µg/ml)	2 x SSC	8003	11
RNA + RNase (10 µg/ml)	1 x SSC	6850	24
RNA + RNase (10 µg/ml)	0.1 x SSC	791	91
RNA + RNase (10 µg/ml)	0.01 x SSC	709	92
RNA only	0.01 M-tris HCl, pH 7.4	2720	-
RNA + RNase (10 µg/ml)	0.01 M-tris HCl, pH 7.4 10^{-3} M-Mg^{2+}	2420	12
RNA + RNase (10 µg/ml)	0.01 M-tris HCl, pH 7.4 5×10^{-4} M-Mg^{2+}	2006	26
RNA + RNase (10 µg/ml)	0.01 M-tris HCl, pH 7.4 10^{-4} M-Mg^{2+}	77	97

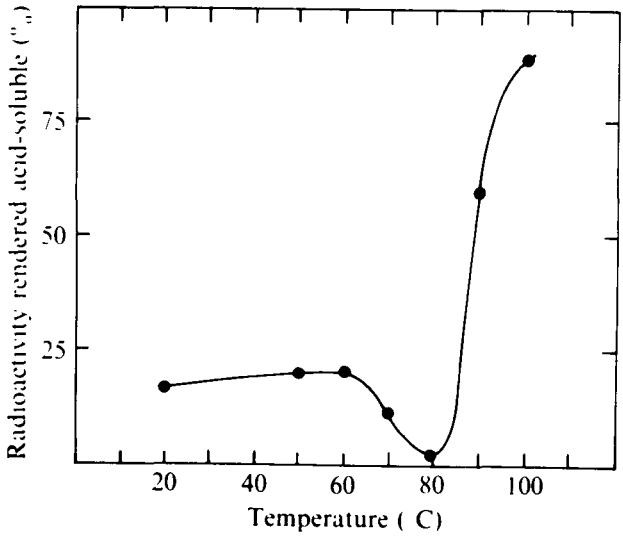

Figure 1 : Thermal denaturation of IPN RNA. The virus nucleic acid extracted by phenol-SDS-pronase, resuspended in 15 mM NaCl, 1.5 mM sodium citrate, was heated at indicated temperatures for 10 min. and then rapidly cooled in ice. The percentage of radioactivity rendered acid-soluble after treatment with pancreatic RNase (10 µg/ml, 0.2 NaCl, 30 min., 37°C) was determined by precipitation with 5% trichloracetic acid.

c) Density in caesium sulfate and sedimentation analysis in sucrose gradients

As determined by equilibrium density gradient centrifugation in CS_2SO_4, the buoyant density of IPN virus RNA was found to be 1.615 (fig. 2). In a 5% to 20% sucrose gradient, a single pic of RNA was resolved. The sedimentation coefficient was calculated to be close to 14 S, using 28 S and 18 S BHK's rRNA as markers (fig. 3). Additionally, the distribution of the label remained unchanged when the sample was reacted in 1 x SSC with pancreatic RNase A before being applied to the gradient. Under the same conditions, the BHK's markers were completely hydrolysed. When the digestion was performed in 0.1 or 0.01 SSC the radioactivity and E 260 were found exclusively at the top of the gradient, indicating that virus RNA and marker RNA had been hydrolysed.

d) Base composition

For analysis of the base composition, virus RNA was precipitated with 5% TCA, collected by centrifuging, washed twice with ethanol to remove TCA and hydrolysed in 0.3 N-KOH for 18 h. at 37°C. The hydrolysate was neutralized with perchloric acid and the ribonucleotides separated by two dimensional cellulose thin-layer chromatography (SCHERRER et al. 1966). Table II presents the results concerning the base composition of total virus RNA (whole virus) as well as RNA obtained after banding in sucrose gradients. The results indicate a perfect base pairing with A/U and G/C equal to 1.

e) Polyacrylamide gels electrophoresis of virus RNA

Electrophoresis of ^{32}P virus RNA in 5% acrylamide gels for 17 h.to 20 h. revealed two closely positionned bands located close to the position occupied by the L class of reovirus RNA (fig. 4). By electrophoresis in 2.5% acrylamide a third intermediate peak could be resolved.

Using reovirus type 3 RNA as mol. weight standard, the estimated mol. weight of IPN RNA segments was found to be in the range of 2.85×10^6 (segment I) to 2.55×10^6 (segment III). These values are obtained either with virus solubilized in urea-SDS or with virus RNA extracted by phenol.

Evidence was also obtianed that, RNA which had denatured by heating at 100°C in 0.1.SSC and quick cooling, migrated about twice

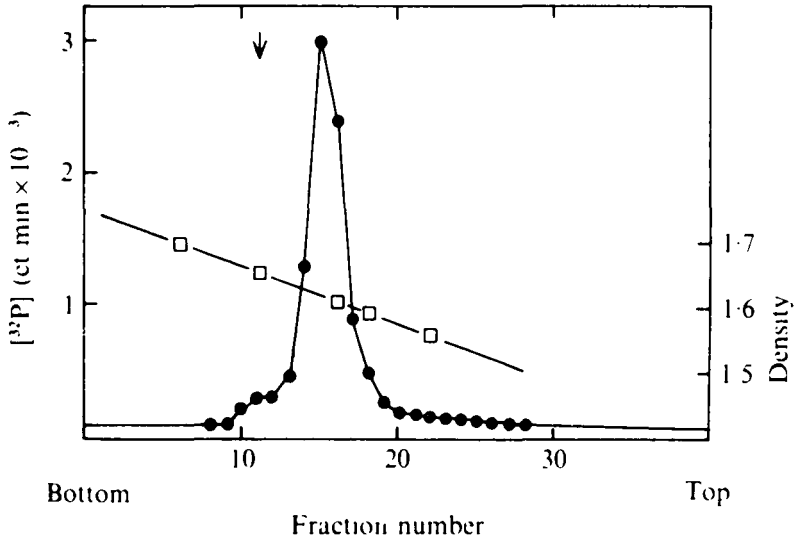

Figure 2 : Isopycnic sedimentation in caesium sulfate of RNA extracted from purified virus. Centrifugation was continued for 2 days in the Spinco SW 65 rotor at 40,000 rev/min. Density was determined by measuring the refractive index. The arrow indicates the position of ^{3}H ribosomal RNA used as a marker.

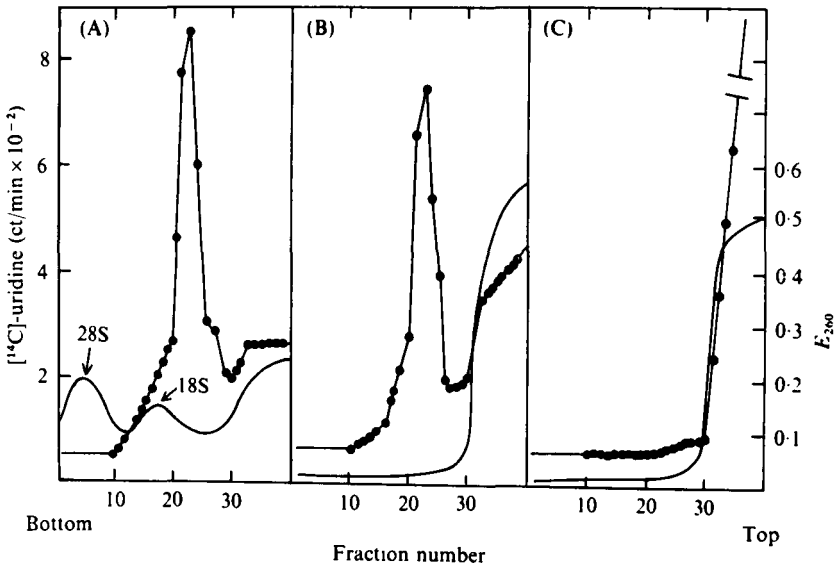

Figure 3 : Sedimentation pattern of IPN RNA. ^{14}C-uridine-labelled RNA extracted by phenol-SDS-pronase, resuspended in 150 mM NaCl, 15 mM sodium citrate (A and B) or in 15 mM NaCl, 1.5 mM Sodium citrate (C), was layered on to 5%-20% sucrose gradient and centrifuged for 17 hours at a 24,000 rev/min. in the Spinco SW 25 rotor. BHK ribosomal RNA was used as a marker.

A : before treatment with pancreatic RNase

B and C : after treatment with pancreatic RNase (10 µg/ml, 30 min. at 37°C) ●—● ^{14}C count ——— E_{260}

Table 2 : Nucleotide composition of IPN virus RNA

RNA source	^{32}P activity (%)				A/U	G/C
	A	U	G	C		
IPN Virus (whole virus) (1)	24.1 ± 0.8	22.4 ± 0.1	26.2 ± 0.9	27.2 ± 0.7	1.07	0.96
IPN Virus (sucrose gradient ^{14}S purified RNA) (2)	23.5 ± 0.6	23.5 ± 0.4	27.0 ± 0.4	26.0 ± 0.8	1.00	1.04
Reovirus 3 (whole virus) (3)	34.8 ± 0.3	23.0 ± 0.3	21.8 ± 0.6	20.4 ± 0.4	1.48	1.04
Reovirus 3 (sucrose gradient purified RNA) (4)	27.2 ± 0.4	26.5 ± 0.8	22.8 ± 0.7	23.4 ± 0.2	1.03	0.97

(1) Six determinations on a single sample of IPN virus
(2) Nine determinations on two separately produced samples of IPN virus
(3) Five determinations on a single sample of virus
(4) Four determinations on a single sample of virus

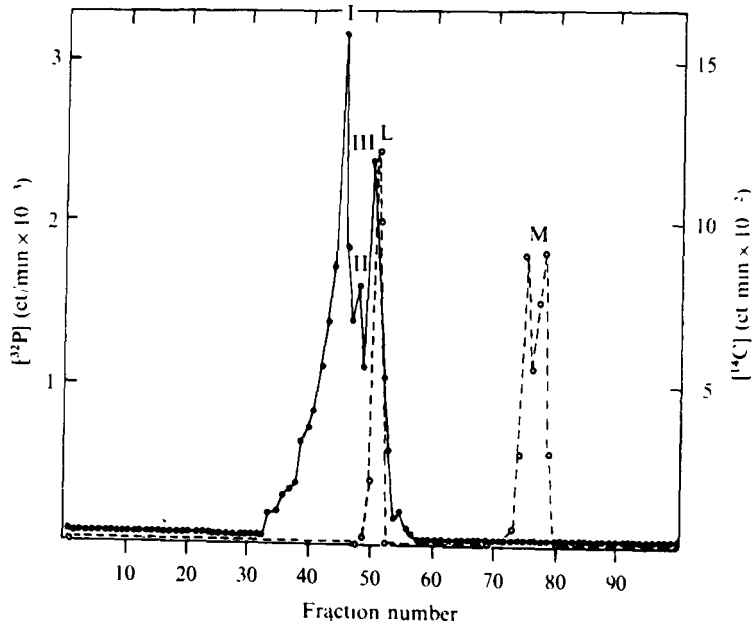

Figure 4 : Electrophoregram of ^{32}P - labelled IPN virus RNA (•——•), and ^{14}C - labelled reovirus RNA (o- - o); 2.5% gel, 20 h migration from left to right (anode). The RNA of the two viruses was extracted by the urea-SDS method. Polyacrylamide gel electrophoresis was performed by the method of LOENING (1967).

as quickly as the native RNA and showed the same peak pattern although the peaks were broader.

B - Effect of temperature on virus replication and virus-directed RNA synthesis

All the experiments reported here were performed in FHM cells, an established cell-line from a cyprinid fish : the Fathead Minnow (GRAVELL and MALSBERGER 1965). These cells grow optimally between 28°C and 34°C, but they can be perfectly maintained at temperatures as low as 15°C. At 20°C, their multiplication is very slow but effective.

IPN virus multiplies to high titers in FHM cells between 15°C and 26°C ; but at 28°C and above, one does not detect any multiplication.

I - IPN virus multiplication at 20°C

a) Infectivity

Fig. 5 represents a typical one-step growth curve of IPN virus at 20°C ; once the virus had been absorbed, infected cells were maintained in suspension throughout the experiment. It should be pointed out that this same curve could be obtained in cells that were infected and incubated in monolayers. In all instances, new virus formation was first detected at 4-5 hours after infection, and maximum virus yields were obtained 10-15 hours after infection.

b) Viral RNA synthesis

IPN virus infection does not inhibit host-cell RNA synthesis during the first 7-8 hours after infection (unpublished data). In a preliminary experiment we tested the effect of Actinomycin D on FHM cells to determine the lowest concentration that could markedly inhibit 14-C Uridine uptake by non infected cells. Fig. 6 shows that the cellular uptake was completely shut off 4 hours after the addition of 0.5 µg of Actinomycin D per ml. At that concentration, 24 hours virus yields were reduced by 40% to 60%. A concentration of 0.5 µg of Actinomycin D per ml. was used in all subsequent experiments aimed to analyse specifically virus-directed RNA synthesis. In such experiments, ^{14}C-Uridine was added to the cells 3 hours after Actinomycin D, in order to minimize the level of labelled cellular RNA.

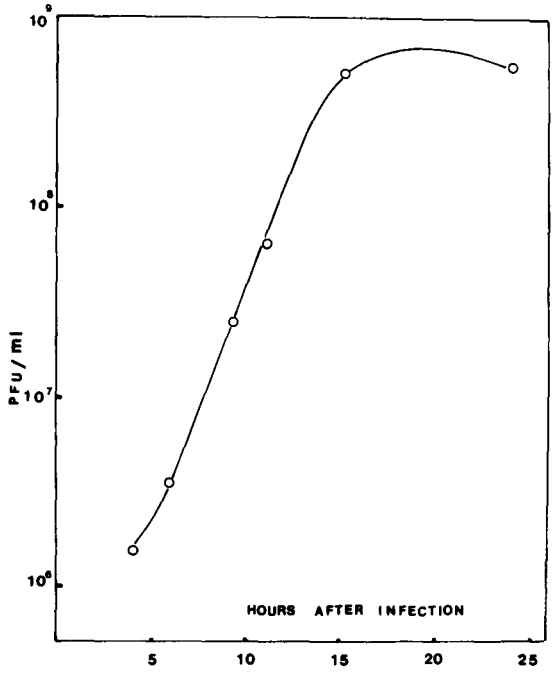

Figure 5 : IPN virus growth curve in FHM cells at 20°C. FHM cells are infected in monolayer with IPN virus at a multiplicity of 10 PFU/cell. After a 45 min adsorption, cells are washed twice with fresh Eagle's Minimum Essential Medium (MEM, Stocker's modification). Infected cells are then trypsinized and washed by two centrifugations in MEM and finally suspended in MEM supplemented with 0.3% tryptose phosphate and 4% foetal calf serum. Cells are adjusted at 2.10^6 cells/ml, and incubated at 20°C in precisely regulated water-bath. 0.2 ml aliquotes of the suspension are collected at intervals and titrated after two cycles of freezing and thawing.

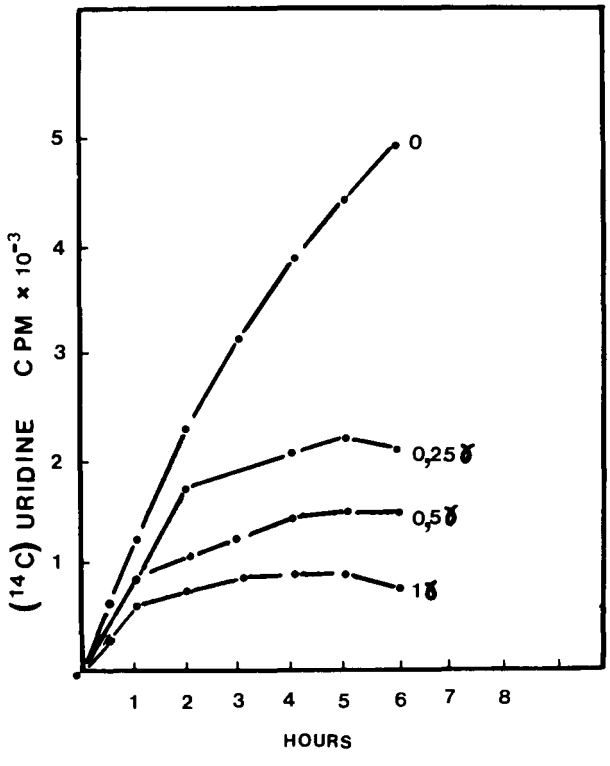

Figure 6 : Effect of different concentrations of Actinomycin D on ^{14}C-uridine uptake by FHM cells at 20°C. 24 hours-old FHM monolayers are trypsinized, washed and suspended as above in MEM containing the indicated concentrations of Actinomycin D. At zero time, 0.1 µCi/ml of ^{14}C-uridine was added. Every hour 0.2 ml of the suspension is precipitated by 5% cold Trichloracetic acid. The precipitate is washed, dried and counted on a Liquid scientillation counter (Intertechnique).

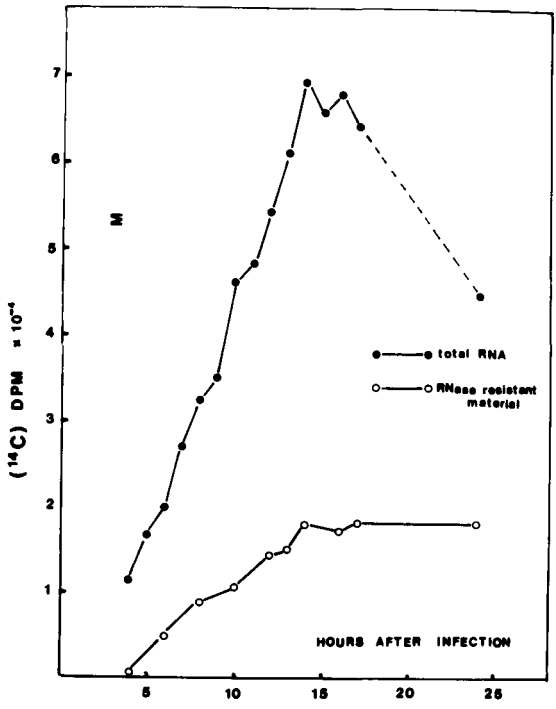

Figure 7 : Kinetics of total and RNase resistant IPN RNA synthesis in FHM cells (20°). Cells are infected as above. The medium contains 0.5 µg of Actinomycin D per ml. 2 hours after the addition of Actinomycin D, ^{14}C-uridine is added at a concentration of 2 µCi/ml. Total RNA synthesis is evaluated by counting the total radioactivity of 0.2 ml aliquotes after precipitation by 5% TCA. Duplicate samples are made for determination of RNase-resistant RNA : RNA is extracted with phenol-SDS-pronase, and precipitated with 5% TCA after or before treatment with pancreatic RNase (10 µg/ml, 30' at 37°C) in 300 mM NaCl 30 mM sodium citrate pH 7, 4 buffer.

Fig. 7 shows the time-course of ^{14}C-Uridine uptake by infected cells into TCA-precipitable material : total RNA on one hand, RNase-resistant RNA on the other hand. The results clearly show that only a fraction of total RNA is RNase-resistant (25% at the 15th hour) ; therefore the curve for total RNA represents mainly the synthesis of single-stranded, and probably messenger RNA.

Maximum amounts of total RNA were found at 16 hours after infection. On further incubation these amounts diminished, suggesting that a degradation process, affecting most probably the viral messenger RNA, prevails from this time over any further synthesis.

Now, if one considers double-stranded RNA synthesis, we observe that the curve is in good correlation with the time course of virion synthesis (fig. 5) ; this might indicate that newly formed double-stranded RNA is rapidly coated into virions.

II - Effect of supra-optimal temperatures

a) Infectivity

We first studied IPN growth-curves in a range of temperatures comprised between 20°C and 28°C (fig. 8). We would like to emphasize that, in all instances, the cells were infected and maintained at 20°C during 3 hours before being shifted to different supra-optimal temperatures. Under these conditions reproducible results were obtained. Two main important facts may be retained :

- there is a sharp decrease in the virus yield around 27°C.
- there is no detectable virus production at 28°C.

If we let the yield at 20°C be 100%, the following comparative figures are obtained : 73% (24°C), 47% (25°C), 22% (26°C), 1% (27°C) and 0% (28°C).

In figure 9 we plotted these percentages against corresponding temperatures : the abrupt drop in virus yield between 26°C and 28°C is very clear ; this sharp slope suggests that only one limiting factor might be responsible for IPN virus thermosensitivity.

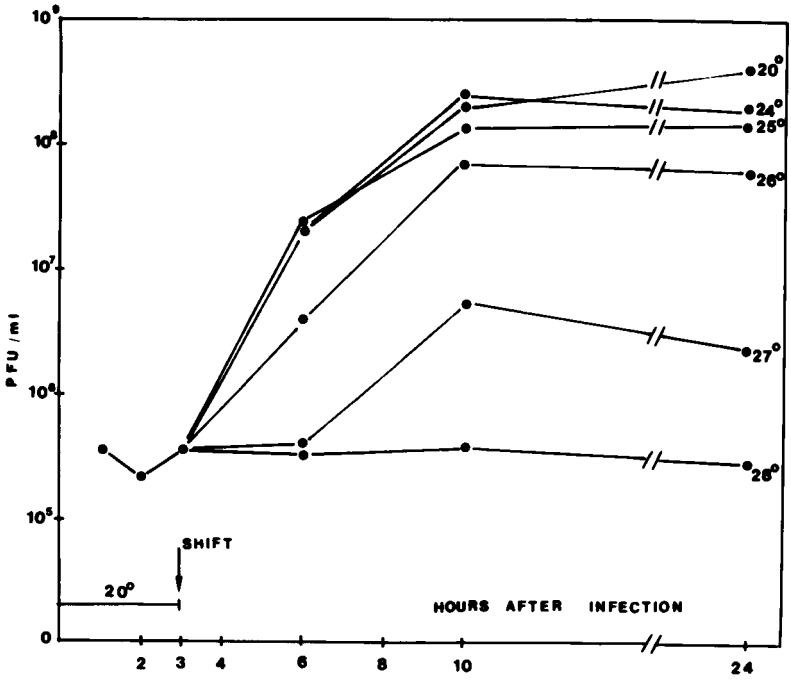

Figure 8 : Influence of incubation temperature on IPN virus multiplication in FHM cells. The experimental procedure is the same as above (see legend to fig. 5) ; all manipulations during the first three hours are carried out at a strictly controlled temperature of 20°C. At 3 hours post-infection suspended infected cells are divided into 6 different flasks for further incubation at the indicated temperatures.

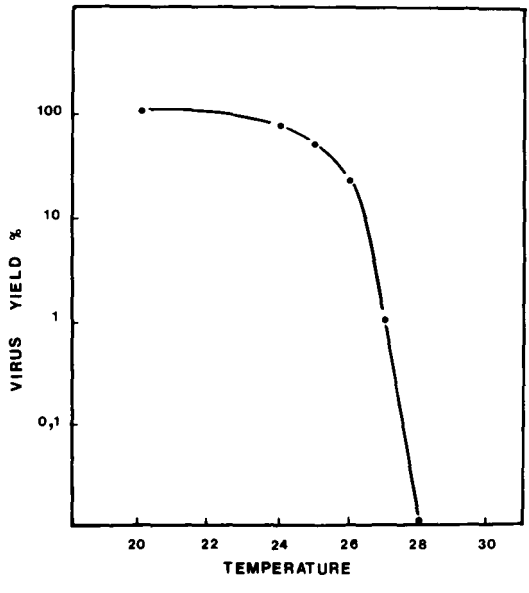

Figure 9 : IPN virus yield at supraoptimal temperatures as compared to 20°C. The figures obtained in the experiment shown in fig. 8 have been expressed in per cent of the yield at 20°C, and plotted against the corresponding temperatures.

Before going further, let us come back to the experimental procedure shown in fig. 8 : starting the growth cycle at 20°C for 3 hours turned out to be a necessary condition for the reproducibility of the results.All procedures including virus adsorbtion on monolayers, washings, trypsinization, resuspension of the cells and beginning of the incubation were carried out at 20°C. In any other condition where the initiation of infection did not proceed at this controlled temperature, we observed that comparative yields as a function of temperature were variable, particularly in the range of 25°C - 27°C ; at 26°C, for instance, virus yields ranged between 20% and less than 1% as compared to 20°C. At 28°C, however, virus multiplication was always completely shut off.

This might indicate that the thermosensitive function is expressed very early in the replicative cycle.

b) Viral RNA synthesis

It seemed of interest to determine whether virus RNA synthesis was, or not, directly affected by supra-optimal temperatures. Therefore, we measured the uptake of ^{14}C Uridine in infected cells at 20°C, 24°C, 26°C and 28°C (fig. 10 A and B) ; the conditions used for infection and incubation were the same as those used in the above infectivity experiments (fig. 8) in that infected cells were kept at 20°C during the first 3 hours post-infection. Taking into account the amount of label in non-infected cells (fig. 10 B), we calculated the percentages of total RNA synthesized at each temperature as compared to 20°C ; here we got : 77% (24°C), 17% (26°C) , 0% (28°C).

These results indicate that virus-directed RNA synthesis is inhibited by supra-optimal temperatures to the same extent as virus particles synthesis. Hence, it becomes clear that viral RNA transcription is very likely involved in the thermosensitivity of IPN virus.

III - Temperature-shift experiments

We can reasonnably assume that the increasing virus-yield inhibition that we observe when temperatures increases reflects the progressive alteration of a same function. Since this effect was complete at 28°C , we chose 28°C as the restrictive temperature to carry out

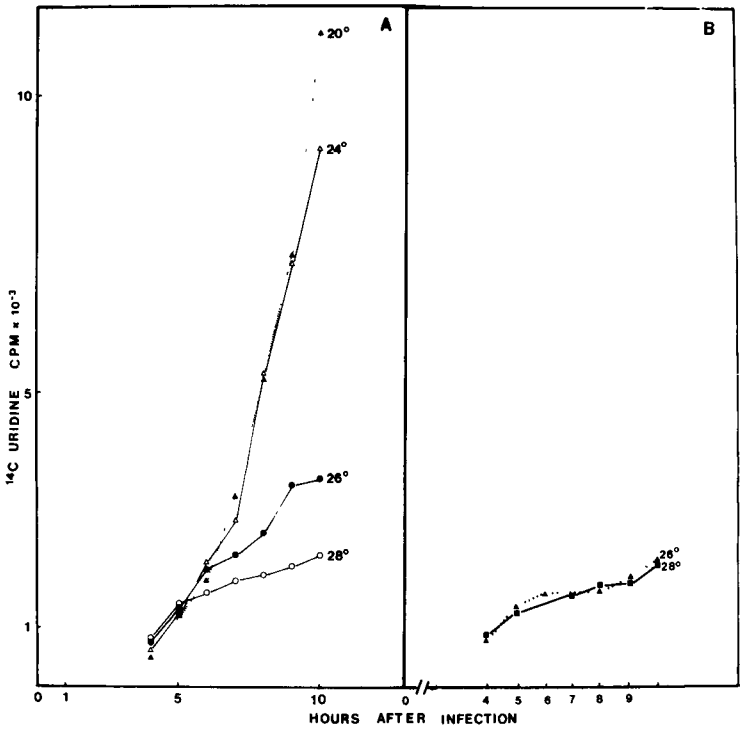

Figure 10 : IPN virus-directed RNA synthesis as affected by supraoptimal temperatures. Infection and incubation of infected cells are processed as previously (see legend to fig. 8) ; at 3 hours post-infection, the suspended cells are shifted at the indicated temperatures. At intervals the amount of total RNA is measured by counting the radioactivity incorporated into TCA-precipitable material. Panel A : ^{14}C label in infected cells ; panel B : ^{14}C label in mock-infected cells.

temperature-shift experiments, where either infectivity, or RNA synthesis was followed.

a) Infectivity

- Shift-down experiments ($28^{\circ}C$ to $20^{\circ}C$)

Table 3 shows the result of a typical experiment in which cells which had been infected and incubated at $28^{\circ}C$ were shifted to $20^{\circ}C$ at different times after infection. Virus yields were titrated by 24 hours after infection. The results indicate that, at least, during the early steps of the growth cycle, the temperature-dependent inhibition is perfectly reversible ; it is remarkable that slightly higher 24 hours-yields are found for those samples which were shifted at 1 and 2 hours after infection ; this small but quite reproductible difference may probably result from a better adsorbtion and/or penetration of the virus at $28^{\circ}C$ as compared to $20^{\circ}C$.

Fig 11 shows some more details concerning the reversibility of the $28^{\circ}C$ inhibition : here we shifted the infected cells from $28^{\circ}C$ to $20^{\circ}C$ at 6 hours after infection and we measured by infectivity assay the time at which newly synthesized virus began to appear (Actinomycin D was added to avoid any inhibiting or delaying effect due to interferon). It is seen that after the shift a latent period of about 4 hours ensues before virus growth starts, as for the control curve at $20^{\circ}C$. We also notice that the slope of the curve after the shift is nearly the same as that of the control. Therefore it seems that at $28^{\circ}C$ virus development is blocked at some very early step. We know that this step is neither adsorption, nor penetration since IPN virus can induce interferon very efficiently at $28^{\circ}C$.

- Shift-up experiments ($20^{\circ}C$ to $28^{\circ}C$)

Fig. 12 shows the results of experiments where infected cells had been shifted from $20^{\circ}C$ to $28^{\circ}C$ at different times between 1 and 6 hours after infection. It is clearly seen that a shift to $28^{\circ}C$ effects a sudden arrest in virions synthesis since the infectious titer at 20 hours post-infection is the same as that found at the time of transfer. It is important to notice that IPN virus is quite stable when exposed to $28^{\circ}C$ during 24 hours or even more ; so, the thermosensitive function, if it is unic, is required as well for the very early steps of infection, as for

TABLE 3

Reversibility of the 28°C-inhibition in the early steps of infection. The numero of each sample is the time at which infected cells have been shifted from 28°C to 20°C.

Sample	inf. titer at the time of transfer PFU/ml	inf. titer at 20 hrs PFU/ml
1	4×10^4	4.2×10^7
2	3.3×10^4	3.2×10^7
3	4.5×10^4	2×10^7
4	3.5×10^4	1.4×10^7
Control (20°C)	-	2.4×10^7

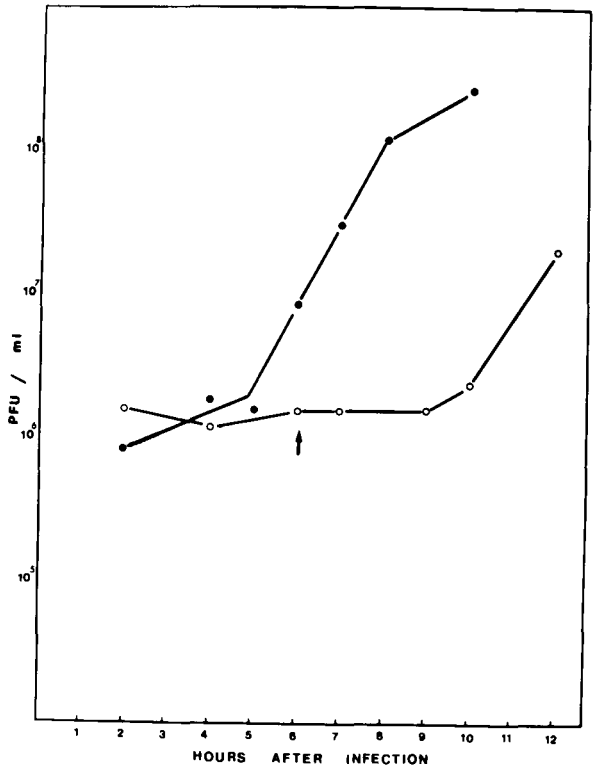

Figure 11 : Shift-down experiment from 28°C to 20°C : virus yield.
The arrow indicates the moment at which shift was made.
●——● Control virus yield in infected cells at 20°C.
o——o Virus yield in infected cells shifted 6 hours after infection.

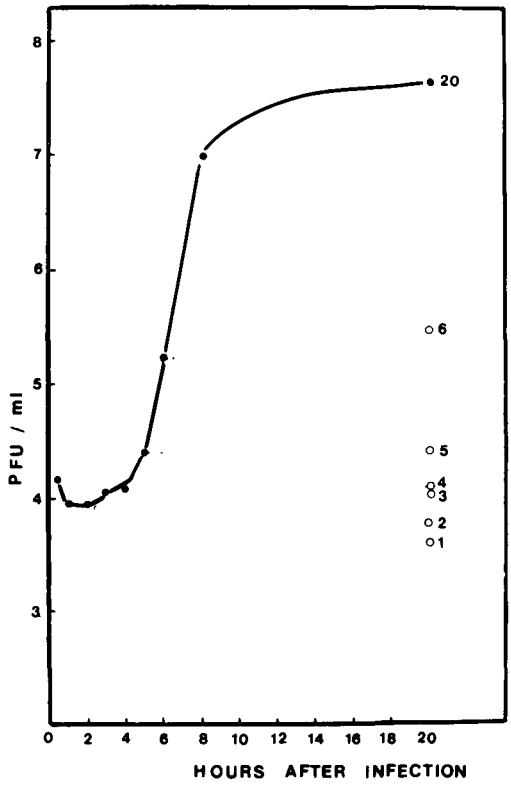

Figure 12 : Shift-up experiment from 20°C to 28°C : Virus yield. During an usual one step growth cycle at 20°C (•——•) samples of infected cells are transfered to 28°C every hour up to the 6th hour after infection. At the 20th hour, virus yields are titrated (o).

the continuation of the exponential growth.

Finally, we examined the fate of virus-directed RNA synthesis following temperature-shifts from 20°C to 28°C. Fig. 13 shows that the control curve at 20°C is identical to that of Fig. 3 ; at the times indicated by arrows, 3 ml aliquots of the suspended infected cells were transferred to 28°C and the amount of TCA-precipitable radioactive material was determined : when the cells are shifted before the 10th hour, it is clear that RNA synthesis goes on during a space of time ; moreover we notice a slight increase in the rate of RNA synthesis for about 30 minutes ; then RNA synthesis ceases abruptly and a very quick degradation process takes place. After 9 or 10 hours after infection, shifts are immediately followed by a severe reduction in the amount of TCA-precipitable material.

DISCUSSION

A - Structure of the genome

All the results obtained in our laboratory lead to the conclusion that the bulk of IPN virus RNA is double-stranded. Such a conclusion would agree with results obtained previously by ARGOT (1969) ; MOSS et GRAVELL (1969) ; SCHERRER et COHEN (1971), but is in contrast to NICHOLSON (1971) and KELLY et LOH (1972). We are inclined to think that the different results could be accounted for either by a difference in the method used to extract the viral RNA (hot-phenol method by KELLY et LOH without reextraction of the interphase) or by contamination with cellular RNA. In fact it is known that for some multicomponent nucleic-acid systems, selective trapping of nucleic acid may occur in the protein layer and thus the isolated nucleic acid may not be representative of the composition that existed in the virion (DIENER et SCHNEIDER, 1968). During our studies we noticed that an important part of the viral RNA is indeed trapped in the interphase and that a successful recovery of this RNA could be achieved when the protein was treated with pronase. In our hands, virus RNA extracted by the cold phenol-SDS-pronase method was relatively resistant to pancreatic RNase since the percentage of hydrolysis in 1 x SSC buffer never exceeded 40%. Additionally we found that the total yield of viral RNA was better at low temperature than high temperature.

Sucrose gradient analysis of the virus RNA indicate that the bulk of the viral genome is double-stranded since it is resistant

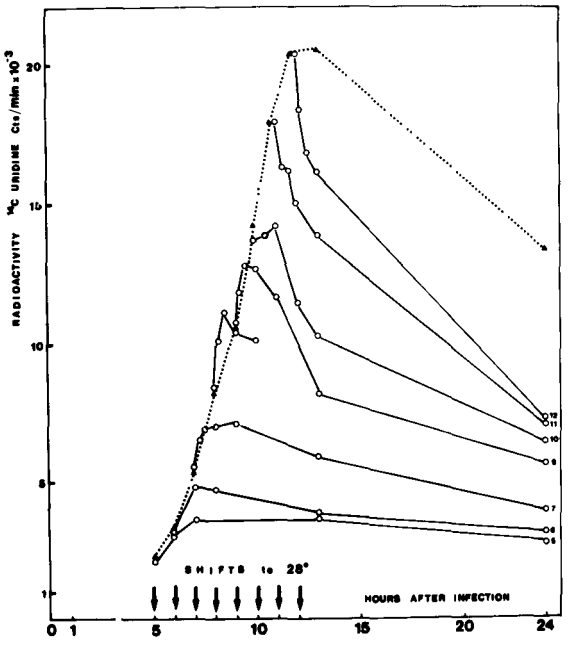

Figure 13 : Shift-up experiment from 20°C to 28°C : Virus directed RNA synthesis. For the experimental procedure, see legend to figure 7. At the moments indicated by arrows, 3 ml aliquotes of infected cells are shifted to 28°C, and the amount of TCA precipitable ^{14}C-labelled material is determined. Control curve at 20°C (▲·····▲) ; curves after shifts to 28°C (o——o).

to hydrolysis by pancreatic RNase in 1 x SSC buffer. However we always noticed a slight increase in the proportion of the radioactivity found at the top of the gradient after treatment with RNase ; this may indicate that a single-stranded component is also present within the virion.

Polyacrylamide gel electrophoresis indicate that the virus RNA consists of a single size group of RNA segments having a mol. Wt. between 2.9 x 10^6 and 2.5 x 10^6. Although the peaks are rather broad in comparison to the reovirus RNA's (SHATKIN, SIPE et LOH, 1968), the size distribution of the segments does not seem to be a random distribution suggesting a fragmentation of a single molecule.

B - Effect of temperature on virus replication and virus-directed RNA synthesis

We have shown that in FHM cells, the virus yield drops abruptly between 26°C and 28°C (fig. 8 and fig. 9), and we also demonstrated that this virus yield inhibition could be correlated with a decrease in viral RNA synthesis (fig. 10 A and B). Single-stranded RNA seems to be primarily involved in this process.

We demonstrated that the thermosensitive event must involve a very early step in virus growth, since as long as infected cells were maintained at 28°C, neither virus formation, nor viral RNA synthesis could be detected (fig. 11). Moreover, we showed that the temperature-inhibition process was perfectly reversible, at least up to the 6th hour of the growth cycle (table 3 and fig. 11).

Temperature shift-up experiments showed that during a virus growth cycle at 20°C, any shift to 28°C immediately abolishes the synthesis of new virions (fig. 12). On the other hand, the RNA-synthesis pattern appeared to be more complex (fig. 13) : up to the 10th hour after infection shifting the cells from 20°C to 28°C resulted in a RNA stimulation during about 30', followed by a rapid breakdown of labelled material.

We first thought that such a stimulation could be due to chain termination by an enzyme which is unable to reinitiate transcription at 28°C. This hypothesis must reasonnably be ruled out because the time necessary to transcribe an IPN-RNA molecule should be far less.

We know (SHEHEL and JOKLIK, 1969) that in Reovirus, the largest piece of RNA which corresponds roughly in size to the IPN RNA pieces is transcribed by Reovirus-associated transcriptase in 8 minutes.

In our opinion, the stimulation would rather result from a two-step process in IPN RNA transcription : only the first step would be thermosensitive, the second step being allowed to go on as long as products from the first step are present. As far as comparison with Reovirus is possible, the first step might be the transcription of "plus" strands from parental double-stranded RNA, and the second the replication of "plus" strands into progeny double-stranded RNA (SCHONBERG et al., 1971 ; SAKUMA and WATANABE, 1971).

When we started this work we had two important questions in mind : first, is the thermosensitivity an instrinsic property of IPN virus, or is it dependent on FHM cells ? Secondly, is there more than one thermosensitive step involved ? The question of intrinsic thermosensitivity was primary in our search due to our inability to isolate a "hot" mutant. In neither non-treated IPN virus, nor mutagenized stocks (utilizing nitrous acid, hydroxy-lamin, or Proflavin) could we isolate a virus able to overcome the 28°C inhibition (unpublished results) ?

Our results to the first question came from experiments carried out with other cell lines in which IPN virus grows perfectly well: EPC cells (Epithelioma Papulosum Carpio) and BB cells (Brown Bull-head), (MALSBERGER 1966). We observed that the pattern of IPN virus inhibition by temperature was nearly the same in these two different cell lines as in FHM cells ; thus at 28°C there was no viral multiplication at all, while either cell, like FHM cells grows optimally at 28°C. These observations favor the assumption that supraoptimal temperatures directly affect viral functions.

Now can we answer the question : is there only one viral function involved ? If so, what is this function ?

Until now, all data can be explained assuming that only one thermosensitive function is involved : this function, very likely to be one of transcription, plays a role at all steps during virus infection ; indeed the results of fig. 8 and fig. 9 indicate that this function is necessary throughout the exponential growth period. On the other hand

shift-down experiments (fig. 7) show that it is also required at a very early time after infection.

The existence of a IPN virus-associated transcriptase has not yet been shown. If such an enzyme is found it will be of interest to verify whether or not its activity is restricted at 28°C and above.

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DISCUSSION

Dr. Scholtissek : Did you find any replicase activity with your virus particles ?

Dr. Scherrer : This was not found until now. In fact we tried to detect a polymerase associated with the virions but our attempts were unsuccessful until now. Perhaps we do not possess the best technique to activate the enzyme. Occasionnally we must use the methods which have been successful in the case of reovirus, namely by heating the virions under various conditions or by treating the virus particles with chymotrypsin or trypsin.

Dr. Rott : Is polymerase active at 28°C ?

Dr. Scherrer : I think so because RNA synthesis continues during a space of time after a shift to 28°C.

Dr. Rott : Only the RNA synthesis but not the polymerase activity. The question is : do you have a RNA polymerase activity at this high temperature ?

Dr. Scherrer : We must say that RNA appears after a shift to 28°C and so we can conclude that probably the RNA polymerase activity must be found at this temperature.

REPLICATION AND TRANSLATION OF FOOT-AND-MOUTH DISEASE VIRUS RIBONUCLEIC ACID

by

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ABSTRACT

The replication and translation of foot-and-mouth disease virus (FMDV) ribonucleic acid (RNA) have been studied in cultures of baby hamster kidney (BHK) cells and in cell-free systems. A virus-specific RNA polymerase is formed, which catalyzes the replication of both double- and single-stranded FMDV RNA. After its isolation from cells, the polymerase is still active in synthesizing FMDV RNAs in a suitable cell-free system, and this activity is inhibited by antibody from convalescent animals. The crude polymerase is resolved in sucrose gradients into fractions with differing activities : a 20-70 S fraction that synthesizes 20 S double-stranded RNA and a 100-300 S RNA that synthesizes 37 S viral RNA.

The translation of FMDV RNA has also been studied *in vivo* and in a cell-free system. As has been firmly established for several picorna viruses, the translation of FMDV RNA in cells produces large molecular weight precursor proteins that are cleaved into smaller capsid and noncapsid proteins. Most of the cleavage products of FMDV protein are analogous in molecular weight and molar ratio to those of other picornaviruses, indicating a similarity in genetic maps. One of the major noncapsid proteins $NCVP_5$ (57,000 daltons) appears to be a component of FMDV RNA polymerase. The three major capsid proteins VP_1, VP_2, and VP_3 have different amino acid compositions as well as different sequences of amino acids at their carboxyl terminal ends. Finally, a protein synthesis-initiating factor (I factor) isolated from infected cells promotes the synthesis of precursor-like protein in a cell-free system programmed with FMDV RNA.

INTRODUCTION

Investigations on the molecular biology of foot-and-mouth disease (FMD) are providing knowledge [1] that may lead to new measures for controlling the disease. Information has already accrued which

indicates quite conclusively that the molecular events in FMDV multiplication are analogous to those for several other mammalian picornaviruses, e.g., poliovirus, encephalomycarditis (EMC) virus, human rhinovirus (HRV), and mengovirus[2]. This conclusion is not unexpected because these viruses are classified together on the basis of similarities in size, structure, content of protein and ribonucleic acid (RNA), and other characteristics [3]. None has a demonstrable constituitive enzyme, but each contains enough RNA (2.6×10^6 daltons) to code for 220,000 to 260,000 daltons of protein, most of which has been accounted for in their stable translation products. There is enough information for about five functions: the known viral proteins, two RNA polymerase factors, a postulated but as yet unidentified host-synthesis repressor [2,4], and a possible maturation factor [4]. The picornavirus capsid and noncapsid proteins and virus-specified RNA polymerase(s) have been the objects of intensive investigation and characterization. The purpose of the present paper is to review the progress which has been made, principally that by workers in the author's group, toward elucidating the molecular events in the replication and translation of FMDV RNA. Comparisons are made with results reported by others on related picornavirus diseases.

REPRESSION OF HOST CELL SYNTHESIS

Before replication and translation of FMDV RNA can begin, the virus must interfere with certain host processes that need to be supressed or redirected into viral multiplication pathways. The impairment of host cell protein and RNA synthesis by virus infection is well known for several small RNA viruses [2]. With foot-and-mouth disease virus (Type A_{12}) infection of baby hamster kidney (BHK) cells (passage 21, clone 13), protein synthesis inhibition exceeds RNA synthesis inhibition throughout the infectious cycle [5]. Early in infection (90 minutes postinfection [PI]) and at peak virus production (300 minutes PI), protein synthesis is inhibited approximately 50 and 80%, respectively. By contrast, host cell RNA synthesis inhibition at 90 minutes PI is only 20%; this inhibition increases to 50% at 300 minutes PI concomitant with the loss of 45 S ribosomal precursor RNA and appearance of 37 S viral RNA.

Although protein and RNA inhibition are substantial, the most pronounced early inhibition of host cell metabolism detected after FMDV infection is the interruption of RNA methylation [6,7,8]. Thus, methylation of 45 S precursor ribosomal RNA is inhibited by 50% at 60 minutes PI

and leads to the disappearance of ribosomal precursor particles in the nucleus. Apparently this methylation inhibition, which interrupts the processing of 45 S RNA into ribosomal RNA, may, in part, be responsible for the observed decrease in extractable polysomes at 150 minutes PI, thus enhancing host protein synthesis inhibition. However, by 180 minutes PI, a >400 S class of viral-specific polysomes (actinomycin D-insensitive) has formed; these contain nearly 80% of the radioactive amino acids incorporated during a 2-minute *in vivo* pulse.

Methylation of transfer RNA (tRNA) is also inhibited. Methylation of tRNA which is depressed by 30% at 60 minutes PI is restimulated so that it approaches the normal level at 120-150 minutes, followed by a rapid decline thereafter to a 60% inhibition at 240 minutes PI. The period of restimulated tRNA methylation just precedes the appearance of maximal amounts of viral-specific RNA polymerase(s) (210 minutes PI) and may be associated with their synthesis.

REPLICATION OF FMDV RNA

In vivo FMDV RNA Synthesis:

The replication of most small RNA animal viruses occurs in the cytoplasm of cells. With FMDV infection, this conclusion is based on the distribution within BHK subcellular fractions of ^{3}H-uridine pulse-labeled virus-specific RNA as well as of FMDV-induced RNA polymerase activity [9]. Foot-and-mouth disease virus-specific RNA is defined as RNA synthesized in the infected cell in the presence of 5 μg/ml of actinomycin D, which inhibits DNA-dependent host-cell RNA synthesis by 95-98%. Three FMDV-specific RNAs are resolved on sucrose gradients: 37 S single-stranded viral RNA, 20 S ribonuclease-resistant double-stranded RNA, and heterogeneous RNA in the 20-70 S region of the gradient (Fig. 1A). The ratio of 20 S double-stranded RNA to 37 S single-stranded RNA increases during the replication cycle, and both are infectious (Fig. 1B). The rate of virus-specific RNA synthesis is highest at 300 minutes PI, coincident with the time of maximum virus production.

The *in vivo* synthesis of FMDV RNA is inhibited by guanidine added at 200 μg/ml [10]. The mechanism of this inhibition remains obscure; in polio infections, the guanidine marker maps in the coat protein region [4], indicating that viral RNA synthesis is in some manner dependent

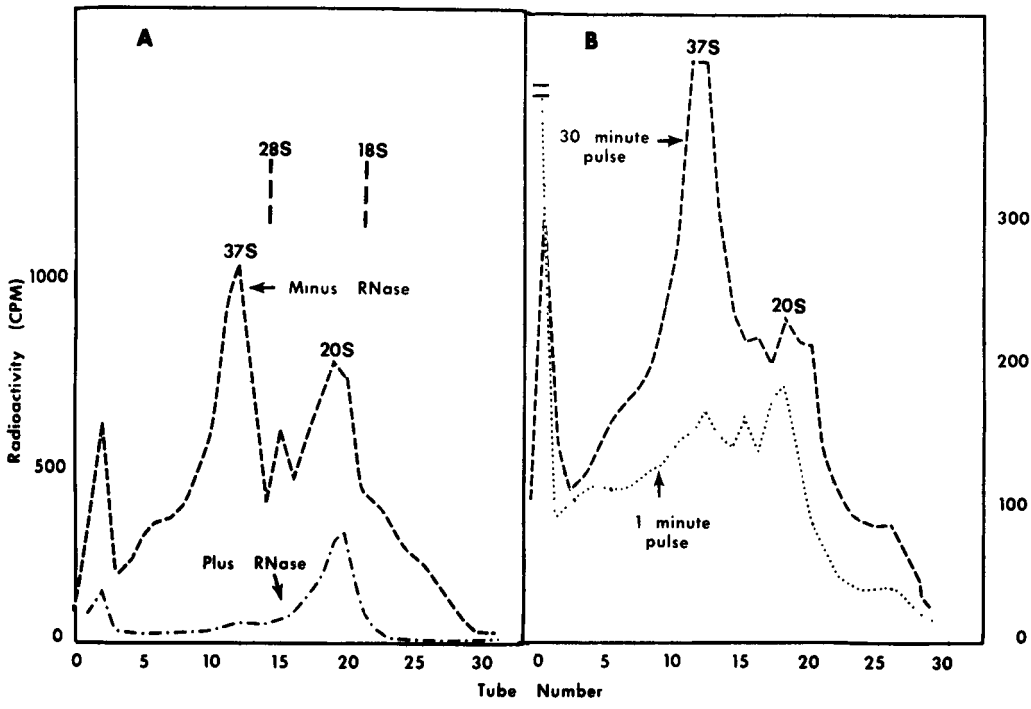

Figure 1 : Sucrose gradient analysis of FMDV-specific RNA 5 hours after infection of BHK cells with type A_{12} virus in the presence of actinomycin D. The RNA was extracted with 1% sodium dodecylsulfate (SDS) at pH 5.1. A) Infected cells were pulsed with ^{3}H-uridine for 15 minutes before harvest. Aliquots of each gradient fraction were incubated with and without RNase, and the trichloracetic acid insoluble radioactivity was measured; B) Infected cells were pulsed with ^{3}H-uridine for 1 or 30 minutes before cell harvest, RNA extraction, and gradient centrifugation. Adapted from [12].

upon the cleavage or conformation of the virion proteins.

Cell-Free FMDV Synthesis:

An RNA-dependent RNA polymerase that is active in the cell-free synthesis of virus-specific RNA can be obtained from FMDV-infected BHK cells but not from normal cells [10,11,12]. This polymerase is most active when isolated 210 minutes PI. The RNA polymerase is dissociated from cytoplasmic membranes, but not from its viral-specific RNA templates, by means of deoxycholate (DOC) with dextran sulfate present as an inhibitor of ribonuclease (RNase). The enzyme is not dependent upon additional FMDV RNA template, but it does require the four ribonucleoside triphosphates, phosphoenolpyruvate, pepkinase, and Mg^{++}. During a 60-minute cell-free reaction, the enzyme produces (Fig. 2A) the same three classes of virus-specific RNA that are formed *in vivo*: a 37 S viral RNA, 20 S double-stranded RNA, and heterogeneous RNA. Polymerase isolated late in infection (5 hours PI) produces considerably less 37 S viral RNA and more 20 S double-stranded RNA (Fig. 2B)[9].

The cell-free synthesis of FMDV-specific RNA can be inhibited in several ways. It is inhibited by Mn^{++} but is unaffected by actinomycin D, indicating the absence of a host cell DNA-dependent RNA polymerase [10]. It is also inhibited moderately by polyamines (putrescine> spermine> cadaverine) and more strongly by basic polyamino acids or basic protein (polylysine, polyornithine, polyarginine, or salmine)[13]. This inhibition and the binding between purified FMDV RNA and polyornithine are relieved by polyanions (e.g., polyglutamic acid and heparin sulfate), indicating a direct interaction of the basic compounds with the RNA template of the polymerase complex.

It is more significant (see TRANSLATION OF FMDV RNA) that the *in vitro* synthesis of FMDV-specific RNA can be inhibited by antibody to a virus-infection-associated (VIA) antigen that arises in infected or convalescent animals but not in animals vaccinated with inactivated virus vaccines [14] (Table 1). The inhibition is cross-type specific; type O and A antibodies are equally inhibitory to polymerase derived from type A infection. No 37 S viral RNA is detected at the end of an antibody-inhibited reaction; there is only a small peak corresponding to 20 S RNA. Controls have shown that the absence of 37 S RNA is not caused by RNase as

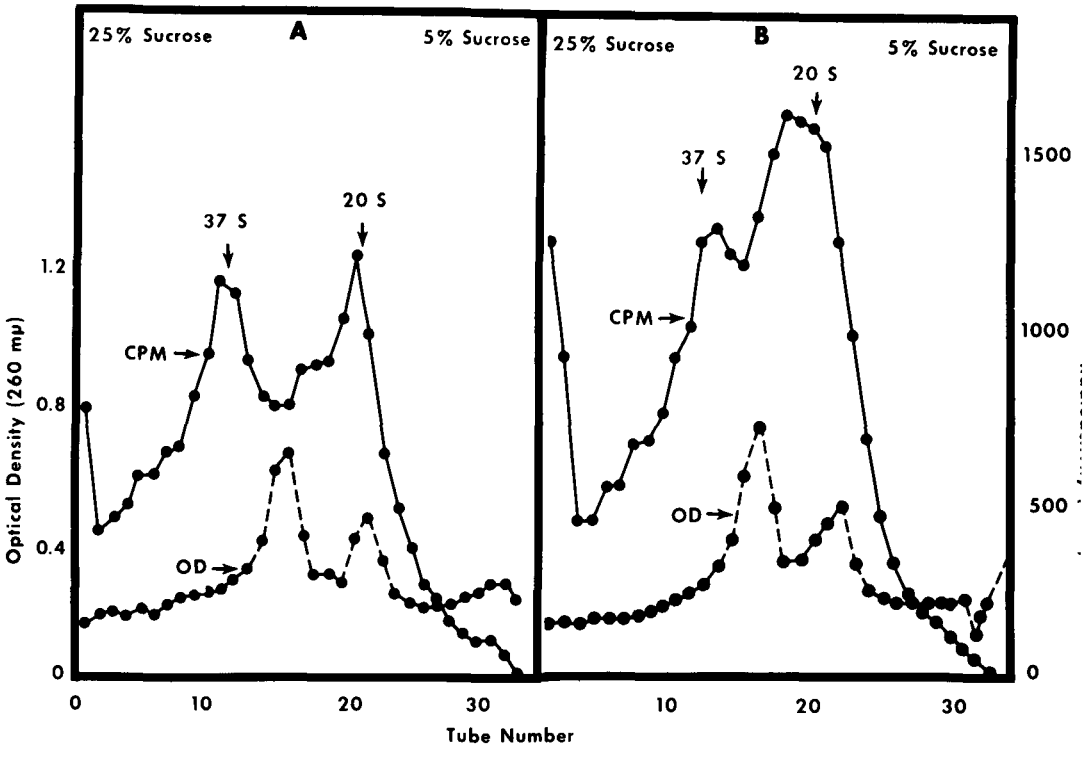

Figure 2 : Sucrose gradient analysis of FMDV-specific RNA from 60-minute, cell-free reaction mixtures utilizing FMDV-RNA polymerase that had been harvested from BHK cells A) 2.5 hours postinfection and B) 5 hours postinfection. The RNA was extracted with SDS at pH 5.1. Adapted from[9] .

TABLE 1

EFFECT OF ANTIBODY ON THE ACTIVITY OF FMDV RNA POLYMERASE[a]

ADDITIVE	CPM ^{3}H-UTP INCORP./MG PROTEIN[b]
	(% OF CONTROL)
NONE (CONTROL)[b]	100
NORMAL SERUM	96
ANTISERUM (INFECTED A)	15
" (INFECTED O)	19
" (INACTIVATED A)	106
" (INFECTED + VIA Ag)[c]	100
NORMAL γ- GLOBULIN	104
IgG (INFECTED A)	10

a) Adapted from [14]: using antibody from guinea pigs and type A polymerase.

b) Ranged from 25,000 to 100,000 CPM for different controls.

c) Treated antiserum had no residual free antibody to virus-infection-associated (VIA) antigen.

a contaminant. These results indicated that FMDV RNA polymerase and VIA antigen have a common antigenicity.

Early in the cell-free reaction (e.g., after 5 minutes), its contents, before deproteinization, are distributed in two broad s-rate classes, a 20-70 S class and a 100-300 S class, both partly RNase resistant; after deproteinization, all the RNA has an s-rate of under 60 S and is partly RNase resistant [15]. After a 30-45 minute cell-free reaction, the 100-300 S heterogeneous RNA-containing component disappears concomitant with a marked slowing or cessation of RNA synthesis. The reaction restarts, however, when more of the polymerase-RNA complex is added. These results

provide evidence that only those RNA chains that have been initiated *in vivo* are being completed in the cell-free system. It is postulated that the 100-300 S component represents complexes of polymerase(s) with various size classes of replicative intermediate structures analogous to those reported for bacteriophage R17 RNA synthesis [16]. Electron micrographs of the R17 replicative intermediates were described as containing single to triple branched RNA structures, the branches representing growing RNA strands.

Evidence for two polymerase activities in FMD has been obtained by resolving the DOC-treated polymerase-RNA complex on a sucrose gradient into three fractions that have differing activities: a light 20-70 S fraction that synthesizes predominantly the 20 S RNase-resistant RNA, a 100-300 S fraction that synthesizes predominantly 37 S viral RNA, and a heavy (>300 S) pellet that synthesizes both the 20 S RNase-resistant RNA and 37 S viral RNA [17]. The pellet is postulated to be comprised of the native polymerase complex still bound to cellular particulates. A similar fractionation of RNA polymerase activities has been reported in mengovirus infection [18], and a requirement for two RNA polymerase factors in poliovirus RNA replication is predicted on the basis of two sets of temperature-sensitive (*ts*) mutants with differing polymerase defectiveness [4]. One set is defective in the synthesis of both double- and single-stranded RNA, and the other is defective only in the synthesis of single-stranded RNA. These mutant sets occupy loci at the 3' end of the poliovirus genome.

TRANSLATION OF FMDV RNA

In Vivo Synthesis of FMDV Protein:

As has been established for poliovirus, EMC virus, HRV, and other picornaviruses, the translation of FMDV RNA in cells produces large molecular weight precursor proteins that are subsequently cleaved into smaller capsid virus proteins (VP) and noncapsid proteins (NCVP) [2,19]. Mapping of these proteins on the virus genome has been reported for poliovirus by using *ts* mutants that are defective in different viral functions [4] in conjunction with the use of inhibitors to interrupt virus-directed protein synthesis [4,19]. Mapping by the latter procedure, which also provides information on post-translational cleavage of the primary products, has also been described for EMC virus and HRV-1A [19]. Although mapping of the FMDV genome by using inhibitors has not been completed, analysis of the ^{35}S-methionine pulse-labeled translation products chased with unlabeled

methionine for successively increasing intervals has been reported [20]. The results have provided information on the number of proteins translated, their molecular weights, time of appearance and disappearance, and molar ratios of FMD VP_{0-4} and $NCVP_{1,1a,2-11}$. The proteins were analyzed by SDS polyacrylamide gel electrophoresis (PAGE) in the presence of ^{14}C-amino acid labeled, poliovirus-infected cell cytoplasm or of ^{3}H-leucine labeled, Maus Elberfeld virus protein markers. Seven of the FMDV-specific proteins, $VP_{0,1,3}$ and $NCVP_{5,9-11}$, have molar ratios close to unity [20] and are considered to represent stable cleavage products. The number of stable proteins and their total molecular weight (224,000 daltons) are both within the limits of information that can be specified by the FMDV genome (2.6×10^6 daltons).

The order of translation and of cleavage as well as the molecular weights of the virus-specific proteins of poliovirus, EMC virus, HRV-1A, and FMDV, are depicted in Fig. 3. The FMDV proteins were positioned by analogy with the genetic maps reported for the other three picornaviruses [4,19]. The similarities are striking and indicate that these four antigenically-unrelated viruses have codons specifying protease-sensitive peptide bonds at nearly identical positions in their genomes. The slight differences that exist may be important in the differential stability of their virions.

Of particular importance in FMD is $NCVP_5$ which has a molecular weight of 57,000 daltons. From the weight of evidence now available, it is almost inescapable that $NCVP_5$ is a virus-specified constituent of FMDV RNA polymerase: 1) FMDV RNA polymerase and VIA antigen have a common antigenicity; 2) $NCVP_5$ co-electrophoreses with purified VIA antigen [21] (Fig. 4); 3) the size of FMD $NCVP_5$ and its apparent 3' end locus are characteristics that have been predicted for poliovirus RNA polymerase factors from mappings with *ts* mutants [4] (FMD $NCVP_5$ is analogous to polio $NCVP_4$, Fig. 3); and finally, 4) the emergence of $NCVP_5$ is delayed in the presence of guanidine [20], which is also known to inhibit the *in vivo* activity of FMDV RNA polymerase.

However, the large size of viral RNA polymerases and the borrowing, for example, by Qβ RNA polymerase of 3 host components for its completion [22] suggest a similar requirement for native FMDV RNA polymerase. This hypothesis is supported by the fact that purified VIA antigen,

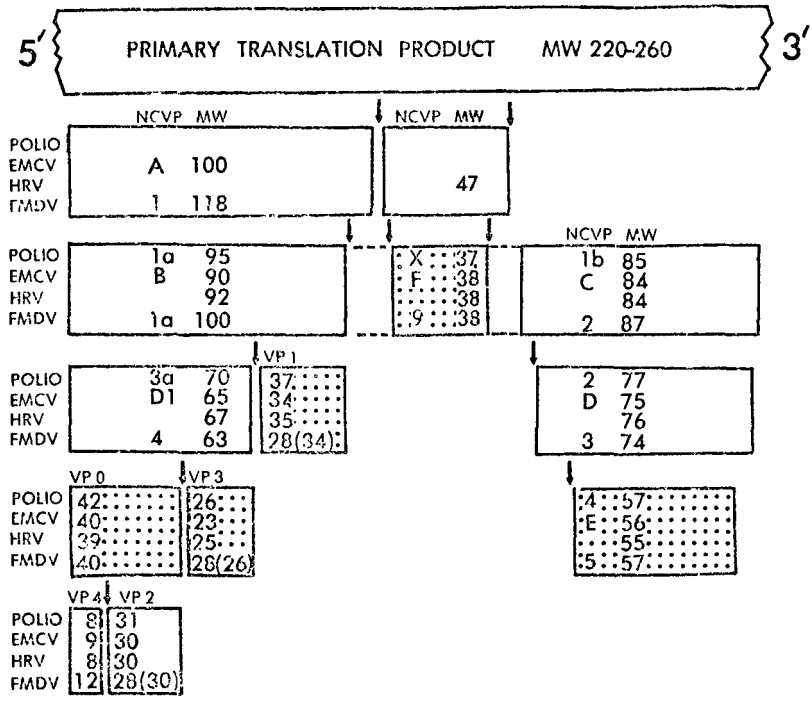

Figure 3 : Legend.

Comparison of the order of translation (lateral) and of post-translational protein cleavages (top to bottom) for poliovirus, EMC virus, HRV-1A, and FMDV. Translation begins at the 5' end of the RNA. The poliovirus genetic map is adapted from [4] and [19], and that for EMC virus and HRV-1A from [19]. That for FMDV is a tentative construction based on these maps and on date in [20].

NOTES :

a) A and B for EMCV as well as 47 and 38 for HRV may be alternate early cleavage noncapsid proteins [19]. The capsid proteins of EMCV and for HRV are generally designated as ε, α, β, γ, and δ rather than VP_{0-4}.

b) Molecular weights (MW) are in thousands. Alternate values [27] for FMD VP_{1-3} are in parenthesis, VP_4 may be a group specific antigen of FMDV [28].

c) Stippling designates stable cleavage products before virus maturation. Several low-MW noncapsid proteins mapping in the middle two gap regions [19] are not shown.

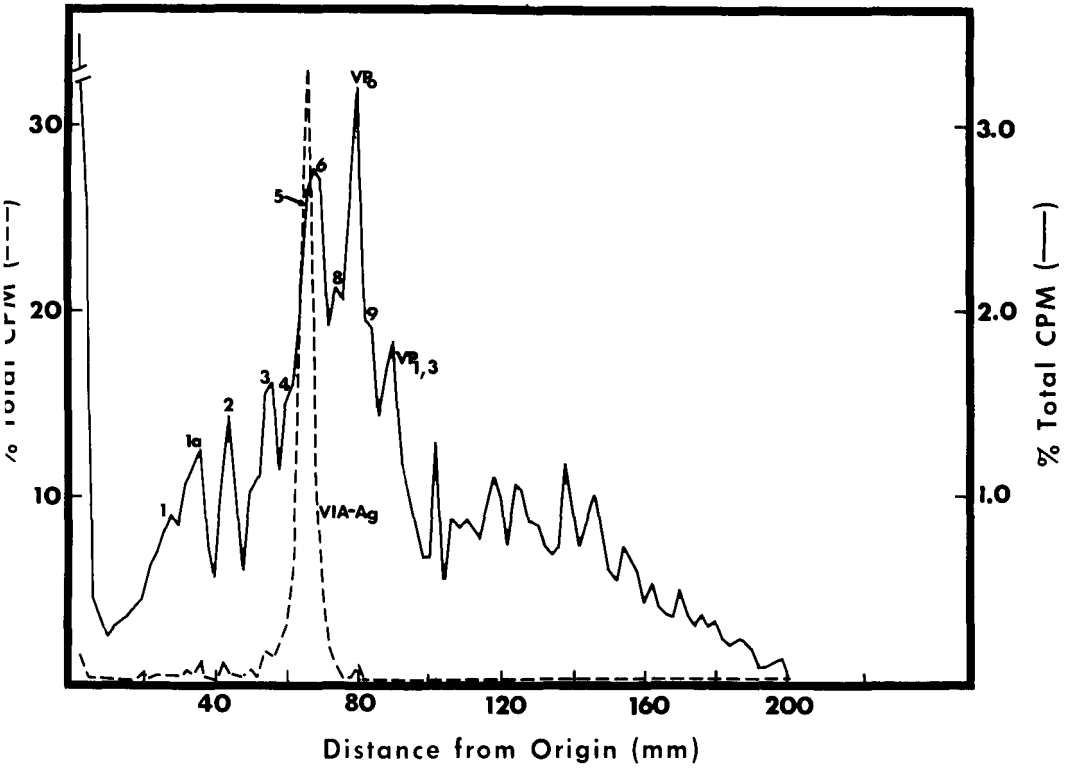

Figure 4 : SDS-7.5% polyacrylamide gel electrophoregram of purified ^{35}S-methionine-labeled virus-infection associated antigen (VIA-Ag) versus ^{3}H-methionine *in vivo*-labeled FMDV-infected cell cytoplasm harvested 360 minutes postinfection. (Courtesy of G.F. Vande Woude).

which is presumably identical to FMD $NCVP_5$, has no polymerase activity. In addition, actinomycin D markedly depresses the production of active FMDV RNA polymerase, suggestive of the inhibited synthesis of messenger RNA for host protein co-factor(s) of the enzyme.

Although FMD VP_{1-3} appear to have nearly identical molecular weights by SDS-PAGE(Fig. 3), they were resolvable from virions into separate VP_1, VP_2, and VP_3 fractions under discontinous PAGE conditions and were found to have differing amino acid compositions and C-terminal sequences [23]. As expected, the weighted average amino acid composition of these three major capsid proteins was quite similar to the amino acid compositions of the total protein of the virion. From carboxy peptidase-A analysis, the C-terminal sequences were: (- - - serine-glutamine) for VP_1; (- - - glutamic acid) for VP_2; and (- - - glutamine-alanine-leucine) for VP_3.

Cell-free Synthesis of FMDV Protein:

A protein synthesis-initiating factor (I factor), which promotes the synthesis of proteins in a cell-free system programmed with 37 S FMDV RNA, can be isolated from infected BHK cells (Fig. 5) [24,25]. The I factor is obtained by 1 M KCℓ treatment of ribosomes from infected BHK cells. The system also requires ribosomal subunits from normal BHK cells. Without programming by viral RNA, incorporation of amino acids is only about one-third of that achieved with the added template. The proteins synthesized *in vitro* during a viral RNA-programmed, 60-minute reaction are of the high molecular weight NCVP type and are not converted in appreciable amounts into lower molecular weight proteins, indicating that this *in vitro* system is deficient in the required cleavage proteases [25]. An I factor from normal guinea pig liver had practically no activity with FMDV RNA as the template although the factor was very active in the presence of polyuridylic acid in synthesizing polyphenylalanine, in accord with the multiplicity of UUU codons in this template.

The *in vitro* protein product specified by FMDV RNA was not otherwise compared with FMDV-specific proteins made *in vivo*. It is known, however, that many of the tryptic peptides from a ^{14}C-labeled product of poliovirus RNA-directed *E.coli* cell-free synthesis co-migrate with those derived from ^{3}H-labeled poliovirus capsid and noncapsid proteins during the cation exchange chromatography or electrophoreses [26].

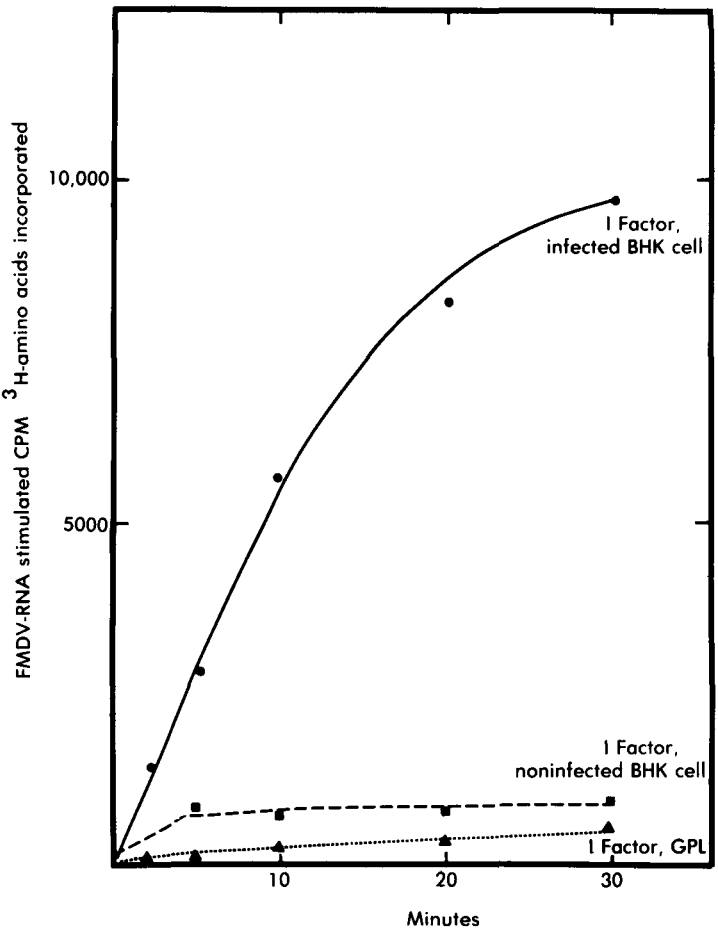

Figure 5 : Incorporation of a mixture of 18 ^{3}H-labeled amino acids into TCA-insoluble polypeptides in the presence of I factors and FMDV RNA (300 µg/ml) after subtracting any incorporation in the absence of exogenous FMDV RNA message. The I factors are from FMDV-infected BHK cells, noninfected BHK cells, or normal guinea pig liver (GPL). Adapted from [25] .

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I wish to acknowledge Drs. R.B. Alinghaus, R. Ascione, K.M. Cowan, J. Polatnick, and G.F. Vande Woude, who carried out most of the research on the molecular biology of foot-and-mouth disease that is reported in this review. I am especially indebted to Dr. Vande Woude for providing Figure 4 of the text.

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DISCUSSION

Dr. Scholtissek : I have a comment on your fractionation studies concerning the RNA polymerase. You got two fractions, one synthesizing single-stranded RNA and the other fraction synthesizing double-stranded RNA. With our influenza enzyme we had the following experience. When we used the crude enzyme, we got synthesis of single-stranded RNA, but when the enzyme was purified about two-hundred fold, we synthesized exclusively double-stranded RNA. We have interpreted these results in the following way: the polymerase not only has to synthesize RNA along the template but it also has to displace it from the template and then this newly synthesized RNA has to be stabilized by some proteins or something else. You would not have double-stranded RNA apart because double-stranded RNA is the end of the reaction. If you purify the enzyme, this type of protein or factor is lost, and then you form the double-stranded structure. Could it not be that the synthesis of the single-stranded RNA has killed this stabilizing factor and the other fractions might not have it. This could explain the results shown in the slides.

Dr. Bachrach : In addition, it was reported by Dietzschold that FMDV RNA polymerase synthesizes mainly plus strands in vitro. I think that there is a temporal relationship: at an early time there has to be negative strands and at a later time virus strands. Lazarus has reported that double-stranded FMDV RNA is formed exclusively in a cell-free system during the

first two minutes, after which the proportion of double-stranded form decreases to about 20% of the total RNA which is synthesized.

Dr. Liess : I am not quite sure whether I am allowed to ask this question, but Dr. Bachrach mentioned quite a number of virus diseases which are exotic to the USA, and there is still one virus disease in the USA which is present as far as I know. This is hog cholera. Now could you tell us perhaps if you are informed on what is being done about hog cholera in the USA, particularly concerning the diagnostic point of view as well as the control of the disease.

Dr. Bachrach : I will try to answer your question in some detail. You may know that for many years Canada has been free from hog cholera but not the United States. About 25 years ago the United States used the so-called simultaneous method of vaccination of pigs, inoculating live virus in one hind leg and antiserum into the other. This procedure, as you might expect, did not eliminate hog cholera, but appeared rather to contribute to its spread. Some twenty years ago the so-called "inactivated" vaccines of the crystal-violet type were tried. About fifteen years ago live-virus vaccines for hog cholera, modified by passages in tissue cultures or in rabbits, were developed. The programme with these modified live-virus vaccines was also unsuccessful. Consequently, approximately ten years ago our State and Federal governments started a programme of eradication, without the use of vaccines, which has virtually eliminated hog cholera in the United States at present. Elimination of the disease has been approached on a State-by-State basis. The last outbreak occurred some three months ago in one of our southern states, at a time when 49 of 50 states were considered hog cholera free. This interrupted a period in excess of seven months since hog cholera has been last confirmed in the United States.

Dr. Liess : It is very difficult to get information from the USA, and I think this will have some implication for the European countries too. This can stimulate our thinking about what to do.

Dr. Bachrach : Hog cholera would appear to be a simple disease to eliminate. There is only one immunological type of virus and only one natural host. Yet, it could not be eliminated by the vaccination procedures that I mentioned. Hog cholera has been much more amenable to slaughter, quarantine

and sanitary measures, and it appears that such practices will succeed before too long in the complete elimination of hog cholera from the United States.

Dr. Leiss : There is still one question which we discussed this morning in our working group. The question is the connection to BVD-MD in cattle, and I just wonder whether you in the USA ever checked for antibodies in the pig population for BVD-MD antibodies in the cattle. Is there any idea what is going on in the pig population in this sense ?

Dr. Bachrach : I cannot answer that.

Dr. Liess : Because this is very important to us at least.

Dr. Bachrach : Mucosal disease ?

Dr. Liess : Yes.

Dr. Bachrach : I do not know what the serological surveys have shown for that, but I would suspect that they have been carried out. But we hope that it will soon be an academic question in the United States insofar as hog cholera is concerned.

Dr. Aynaud : Are there methods to differentiate between attenuated strains of swine fever and, attenuated and non cytopathic strains of mucosal disease virus ? This point is important for the diagnosis of latent infections of pigs in the field. In other words, how can you differentiate between an attenuated or low virulent strain of swine fever and a non cytopathic strain of mucosal disease isolated in pigs ?

Dr. Bachrach : I cannot answer that. This problem is studied by our organization in Ames, Iowa, which is working on mucosal disease and hog cholera. They instituted fluorescent antibody studies for this, and I presume that they can differentiate by this method.

Dr. Horzinek : May I ask one question on picorna's again ? Have you ever tried to immunize cattle with non-viral proteins such as the polymerase ? Would this protein protect against a challenge infection ?

Dr. Bachrach : We have not done precisely that. However, when you degrade foot-and-mouth disease virus by acidification or by heating into the 12 S component and virus protein number 4, the degraded virus is very poorly immunogenic. It is difficult to prepare much purified virus protein 4, because the latter constitutes only 6 percent of the total virus protein.

Dr. Horzinek : And if you treat cattle with the virus-induced or infection-associated-antigen, are they protected against foot-and-mouth disease ? You apparently have no type-specificity in this polymerase, so it would be a candidate for protection.

Dr. Bachrach : There is no report that this antigen is immunogenic for foot-and-mouth disease.

Dr. Van Bekkum : Mr. Chairman, if I may in reply to two of the points that have been raised previously. First of all, the question Dr. Liess asked.

I should perhaps point out that bovine virus diarrhea has been isolated in the United States from swine in the fields. So the diarrhea problem is probably the same as in Europe.

Secondly, with regards to the point raised by Dr.Aynaud, I should perhaps point out that in our experience all non-cytopathogenic strains of bovine virus diarrhea produce plaques using the same technique but with bovine testis cells that we used for swine fever virus. That is to say incubation under an overlay, staining with neutral red and keeping the cells in the dark for a couple of days. Thank you.

SUMMARY OF THE SESSION

P. J. Enzmann

The nucleic acids of Togaviruses and picornaviruses serve as both, genome and mRNA. The data which indicate that the viral RNA functions indeed as a messenger depend primarily upon the fact that RNA extracted from virus particles is infectious. The start of RNA-replication requires a step involving the synthesis of protein coded by the viral genome. During this process the virus-specific RNA-replicase is synthesized. The replicase seems to be identical with the transcriptase in the case of Togaviruses and picornaviruses. In contrast to this system, myxoviruses need as a first product in the course of virus replication a new species of RNA which functions as a mRNA on the ribosomes. These viruses require a new enzyme, the transcriptase, to synthesize RNA which is complementary to virion-RNA. The transcriptase is associated with the virion. In the course of infection these viruses produce another enzyme, the replicase which is necessary to synthesize virion-RNA.

Messenger RNAs in the Togavirus-group and picornavirus-group are at least in part polycistronic, whereas in the myxovirus-group there are monocistronic mRNAs because the genome is fragmented. During the replication of Togavirus-RNA several double-stranded structures appear in the infected cells. One of these, the replicative intermediate form (RI) of RNA consists of the minus strand which is associated with several nascent (+)-strands of varying length but is largely present in single-stranded form inside the cell. After ribonuclease treatment of this RNA form, a double-stranded RNA with single-strand breaks in the (+)-strand arises which is called replicative form (RF). It is not yet decided finally whether there is another replicative form of RNA which occurs as complete duplex without single-strand breaks. I think there is no real explanation until now in which way the (-)-strand RNA arises.

In systems which work with polycistronic mRNAs the product of translation is a precursor protein. Specific proteolytic enzymes have to cleave the precursor to get functional polypeptides. This cleavage has been shown definitly by the precursor-product relationship.

Now I would propose that we discuss the papers of this afternoon instead of giving a whole summary, because these papers are too heterogeneous.

GENERAL DISCUSSION

Dr. Bachrach to Dr. Scholtissek :

You indicated that the mutant shuts off both complementary and viral RNA synthesis. The work reported by Cooper using polio mutants gave evidence for two RNA polymerase factors. How do you reconcile this sort of difference ?

Dr. Scholtissek : My data can be interpreted in such a way that the two enzymes synthesizing a minus strand and a plus strand are different in the sense that some additional proteins are complexed to one common virus specific protein and so far I cannot say that one and the same enzyme is synthesizing both strands. The only thing I can say is that there is one common protein involved in the synthesis of both strands.

Dr. M. Schlesinger :I'd like to ask Dr. Scholtissek and possibly Dr. Rott also to comment on the use of glucosamine because we have found that there is a certain hazard in using glucosamine. With envelope-viruses, where there are carbohydrates attached to the envelope-proteins and these attachments are essential for maturation and secretion of viruses, the addition of glucosamine inhibits those attachments and therefore essentially inhibits viral maturation and replication.

Dr. Scholtissek : That is true. In glucosamine-treated cells infected by Semliki-Forest virus, and using a glucose containing medium, the effect is mainly on the production of glycoproteins. In glucose containing medium, we do not have an effect on viral RNA, and under these conditions, we get a large primary translation product which is wrongly glycosylated and we get the normal splitting into the NP protein. The splitting into the carbohydrate containing envelope polypeptides, however, does not occur at the right sites. So we get a whole bunch of different molecules with different molecular weights which are - and these molecules are rather unstable - broken down at least partially during further incubation of these cells. Therefore we don't get any particles because we don't get the right envelope proteins. I might mention here some observations we have made with vesicular stomatitis virus. We can't inhibit the multiplication or the production of infectious vesicular stomatitis virus under identical conditions, although we have the same effect on the synthesis of vesicular stomatitis virus glycoproteins. Now, if we isolate the virus particles from the

supernatant medium, we get the normal yield of infectious virus and these virus particles now contain wrongly glycosylated glycoproteins. So these viruses can tolerate the incorporation of the wrong glycoproteins while the Semliki-Forest virus does not, and also influenza virus does not tolerate the incorporation of these wrong glycoproteins. It depends very much on the virus strain you are looking for!

Dr. M. Schlesinger : To illustrate that point further, in my laboratory, we looked at the appearance of the envelope proteins in Sindbis virus-infected BHK-cells in the presence of glucosamine using glucose in the medium and there we found the two envelope proteins accumulated as individual polypeptides. They do not chase into the carbohydrate-containing forms but they are stable and furthermore by subfractionating such infected cells into various membrane fractions, we do find those non-glycosylated envelope polypeptides moving into the plasma membranes.

Dr. Bachrach : I could follow up something that Dr. Scholtissek was speaking about. That is cleavage of proteins. Some interesting observations have been made on the treatment of foot-and-mouth disease virus with trypsin. One of its peptides will be cleaved, and depending upon the strain, there will be a large drop in infectivity or no drop in infectivity.

Dr. Scholtissek : I have a question to this last point concerning these two different strains. In one you got cleavage with loss of infectivity, and with the other strain, cleavage without loss of infectivity. Do you know whether the cleavage was at the same site ?

Dr. Bachrach : We presume that it is close to the same site because the products are similar in molecular weight.

Dr. Becker : We are dealing here with essentially two different phenomena. On the one hand we have viruses which can shut off cellular function very efficiently and replicate in the whole cell, on the other hand there are groups of viruses which replicate very well but they do not shut off cellular functions. It is of great importance to understand what is controlling the cell mechanism since one of these factors could be the initiation factor which may assist the ribosomes to discriminate between viral and cellular messenger RNA.

Dr. Bachrach actually started to talk about the initiation factor, which can be extracted from the ribosomes. Work is now being done by Dr. Kaempfer in the United states on what he calls the "Initiation factor 3" in mammalian cells. This is a very complicated multipeptide component which binds to the 40S ribosomal sub-unit and determines whether the sub-unit will attach to a messenger RNA molecule and become an active ribosome. One of the

suggestions that he makes is that the virus can perhaps modify one or more peptides in this initiation factor 3 (IF3), which will make the ribosome discriminate between cellular and viral messenger RNA. This pertains to viruses which can shut off the mechanism of whole cell but it is not yet very clear, so maybe Dr. Bachrach can tell us a little bit more about this initiation factor.

Dr. Bachrach : I can't contribute much more about it. I factor has not been characterized further in our laboratory other than being in the supernatant from the one molar KCl-shock of infected ribosomes. Without this initiation factor, the cell-free system is inactive.

Dr. Scholtissek : I want to comment a little bit more on what Dr. Bachrach said concerning the initiation factor. Dr. Wengler in our institute has done experiments with Semliki-Forest virus-infected cells. After the hypertonic shock of the infected cells, the Semliki-Forest viral specific RNA still stayed on the polysomes synthesizing specifically Semliki-Forest virus proteins; but in the non-infected cells the cell-specific mRNA was lost from the polysomes and after this shock, they did not synthesize any more cell-specific protein. This indicates that somehow the virus specific RNA has a high affinity to the polysomes. We don't know anything about the initiation factor because these experiments were done in vivo, but it might be explained in a very similar line.

Dr. Becker : I am not so sure that this has any connection with the initiation factor 3 (IF3) because, according to Dr. KAEMPFER, and this is true of other systems, the factor is released from the 40 S ribosomal sub-unit the moment the active ribosome is formed on the message. Afterwards the initiation factor can re-cycle so that the number of IF 3 molecules in a cell actually determines the number of ribosomes which can bind messenger RNA and initiate polysome formation. This is a factor which is very well controlled in infected cells. This initiation factor is obviously important since it also has to do with the phenomena of interferon and sensitivity of cells to viruses. These two phenomena seem to be tied to the question of the specificity of IF 3.

I have another question which has to do with guanidine sensitivity. Many years ago it was claimed that a membrane is synthesized in the cytoplasm of polio virus infected cells. At a certain stage these were called virus-synthesizing bodies, which had a sort of membrane.

Dr. PENMAN published a paper on increased choline uptake by poliovirus infected cells. The question is, if a membrane is synthesized under the control of the virus, we probably also need glycoproteins. Did anyone look for a glycoprotein in the case of picornaviruses and if so, could it be that guanidine sensitivity has to do with the formation of this membrane ? According to papers published in the last few years, in the presence of guanidine, the membrane structure which contains the RNA polymerase is not synthesized. After removing the guanidine, the whole membrane comes together once again and becomes functional.

Dr. Scholtissek : I can report on preliminary data Dr. Kalúza has obtained in our institute. He has studied poliovirus multiplication under the influence of 2-Deoxy-glucose. Under certain conditions he can inhibit the multiplication of poliovirus. Our idea, of course, is that it has to do with this membrane formation. But I will not follow this up too much.

Dr. Bachrach : I presume that Dr. Becker was talking about the so called "guanidon"?

Dr. Becker : I was very curious about the specific inhibitory effect of guanidine on poliovirus replication.

Dr. Bachrach : One can hypothesize that guanidine might interfere with post-translational cleavages by inhibiting proteases. I think guanidine is known to have some inhibiting effect upon proteases. Non-capsid virus protein 5 in FMD appears late in the presence of guanidine, and therefore the active polymerase may not be present when it should be in the infection-cycle.

Dr. Scholtissek : Concerning the regulation how virus can or might switch off cell functions, I would like to call your attention to a recent result by Schweiger et al. on T7 phage of E. coli. He found that after virus infection, a phosphokinase activity occurred and this enzyme phosphorylated several cell specific proteins. So they switch their function now in such a way that these cells are specifically synthesizing T7 specific proteins and they do not any more synthesize the cell specific proteins. I guess this is one thing that we really should look for whether we have induction, for example, of such general enzymes like phosphokinase which might then somehow change cellular proteins that they function in a different way.

Dr. M. Schlesinger to Dr Bachrach :

There was a report by a group from Dupont that they found a cleavage enzyme - an enzyme that would cleave the polio non-virion protein, the precursor, and they could only isolate this from infected cells. Do you know if that result has been repeated, or do you have any information ?

Dr. Bachrach : Not much additional information, other than that the work was done by Bruce Korant at Dupont on a polyprotein produced during poliovirus infection. He was able to reproduce in vitro - as I recall - the cleavages that occur in vivo.

I might switch now to something that I didn't talk about previously. That is a control mechanism in foot-and-mouth disease which contributes to the shut off of protein synthesis after virus infection. Dr. Vande Woude showed that the earliest and strongest inhibition in the infected cell was the sub-methylation of 45 S ribosomal precursor RNA and also sub-methylation at a certain time of transfer RNA. The sub-methylation of the ribosomal precursor RNA would stop the processing of the ribosomal precursor RNA into ribosomal RNA, and hence contribute to the shut off of normal protein synthesis. The temporal sequence of methylation of the transfer RNA was such that it appeared to be related to the synthesis of the RNA polymerase. There was a decrease early in the methylation of transfer RNA, followed by an increase in methylation just prior to the formation of the RNA polymerase and then a decrease after that.

SESSION II

Synthesis of Viral DNA

MOLECULAR EVENTS IN THE REPLICATION OF AN ICOSAHEDRAL CYTOPLASMIC DEOXYVIRUS
I. VIRAL RNA SYNTHESIS

by

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ABSTRACT

Frog virus 3 may be a convenient model for study of the replication of members of icosahedral cytoplasmic deoxyviruses (Iridovirus) including African swine fever virus.

Viral DNA synthesis commences about three hours post-infection and continues throughout the replication cycle. Host DNA and RNA synthesis is rapidly depressed. The electrophoretic pattern of virus specific RNA transcripts in infected cells has been determined. When analyzed by gel electrophoresis, the transcripts separated in a size range of 16-6s with the bulk migrating around 16s. These messenger RNA's contain polyadenylic acid tracts of about 150-200 nucleotides. In the absence of viral DNA replication, the rate of transcription is reduced but the molecular size species correspond to those produced in normal infection. There is no evidence of distinct early and late RNA transcripts. These aspects will be contrasted with other viral systems.

INTRODUCTION

The icosahedral cytoplasmic deoxyviruses (Iridoviruses) can, perhaps, be considered as a viral supergroup in which a number of icosahedral viruses from insects, amphibians, fish and swine probably belong to various sub-groups within it. Characteristics of these viruses include:

a) icosahedral symmetry of the virion
b) a minimum size of about 130 nm
c) replication apparently confined to the cytoplasm

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d) a double-stranded DNA genophore of molecular weight 100-130 million daltons

e) a single structural unit membrane associated with the virus nucleoid

Of these viruses the most important economically is African swine fever virus (ASFV).

The biochemistry of icosahedral cytoplasmic deoxyviruses (ICDV) has been the subject of a recent review (20) in which we pointed out that knowledge of the biochemistry of their replication is fragmentary. Frog virus 3 (FV_3) has been the most intensively studied and it will be of great interest to see if it can be taken as a representative model system for an understanding of the general aspects of replication of the whole group and in particular for ASFV.

FV_3 replicates in the cell cytoplasm (7, 20). Its genome is a double-stranded DNA of molecular weight 100 to 130 x 10^6 daltons (15). The programming of DNA synthesis by FV_3 resembles that of a number of DNA virus that replicate in the nucleus in that it continues throughout the onset of the virus maturation phase. In contrast, poxvirus DNA synthesis reaches a peak and declines prior to the appearance of progeny virus (18, 19). The main features of poxvirus transcription are biphasic transcription coupled to DNA synthesis (18, 19) and the presence of a virion-associated transcriptase that will synthesize early in RNA in vivo and in vitro (13, 14). In this report we will describe some general aspects of FV_3 transcription and compare and contrast it with the transcriptional events of other deoxyviruses.

MATERIALS AND METHODS

Virus

Crude FV_3 stocks were grown in FHM monolayers (9). Confluent monolayers were infected (moi = 0.1 or less) and incubated at 28°C for 72 hours. Cells were harvested, washed in phosphate-buffered saline (PBS), disrupted by sonic vibration, and cellular debris removed by centrifugation (10 min, 2.500 xg). The crude supernatant was layered over 30% W/V sucrose in 1x standard reticulocyte swelling buffer (RSB) and centrifuged (30 mins, 25.000 RPM in an SW27 rotor). The pellet was diluted approximately 100 fold into PBS and was stored at -70° in ampules. Stocks

used in these experiments contained 2 x 10^9 to 1 x 10^{10} plaque forming units (PFU) per ml (17).

Electrophoresis

Unless otherwise specified, all electropherograms refer to 1.7% acrylamide -0.5% agarose gels prepared according to Peacock and Dingman (21). Running buffer was 0.1 Tris-borate pH 8.3, 2.5×10^{-3} M EDTA. Gels were 13 cm in length and were run approximately 3 ½ hours at 5V/cm. A tracking dye, bromphenol blue was used and gels were aligned relative to the dye position. RNA samples were applied in 12% glycerol 0.4x running buffer with or without dimethyl sulfoxide (DMSO, Fisher Scientific Co.) as stated. Gels were cut into approximately 2 mm slices and were incubated 90 mins at 60°C with 0.5 mls of a mixture of 9:1:10 protosol (New England Nuclear), water, and toluene. The digested material was then chilled for 1 hr at -20° prior to counting in toluene based scintillation fluid (Spectrafluor, Amersham). RNA could be extracted from gel slices with greater than 90% efficiency by shaking the fractions in 1 ml of TSM buffer (0.01 M tris pH 8.0, 0.1 M NaCl and 0.001 M $MgCl_2$) at 37° for 4 to 5 hrs.

Hybridization

Hybridization was performed at 66° in 4x saline-citrate (SSC, 0.6 M NaCl, 0.06 M sodium citrate) 0.1% sodium dodecyl suflate (SDS) for 24 hrs using DNA trapped on filters (27 mm type B-6, Bac-T-Flex, Schleicher & Schuell, Inc.) as described by Green *et al.* (10); filters contained 20 µg of the indicated DNA.

DNA

FV_3 DNA was extracted from virus purified according to Tan and McAuslan (27). Purified virus in 1x RSB were exposed to DNase I (100 µg/ml) at 28° for 30 min. The virus was then rebanded in a 20-55% sucrose gradient (27) and the viral band was concentrated by centrifugation through 30% sucrose (see above). The viral pellet was resuspended in 1x RSB containing 0.02 M EDTA and 0.1% SDS. Self-digested pronase was added (1 mg/ml) and digestion was allowed to proceed for 30 min at 37° prior to a second addition of an equal amount of pronase. After another 60 mins, the DNA was extracted with an equal volume of cold phenol-chloroform (1:1). The aqueous phase was re-extracted with the phenol-chloroform mixture and the final supernatant was extracted twice with ethyl ether. DNA was precipitated in 70% ethanol and was exhaustively dialyzed against 0.1 x SSC

prior to use in hybridizations.

FHM DNA

Nuclei were prepared from FHM (minnow) monolayer cells (9) by the NP_{40} method detailed below. DNA was extracted from the isolated nuclei by the same method used to isolate DNA from FV_3 except that the nuclei were not exposed to DNase digestion.

Hybridization in formamide

Hybridization in 50% formamide was performed exactly as described by Bachenheimer and Roizman (2).

Infection of cells

Unless stated to the contrary, all infections were carried out at an input multiplicity of 20 pfu/cell on monolayers. Monolayers were grown in plastic flasks at 28° in Eagle's Minimal Essential Medium (F-11 Grand Island Biological Co., N.Y.) with added glutamine and 10% inactivated fetal calf serum. The cells were drained and washed in fresh medium containing 0.02 M $MgCl_2$ and 2% inactivated fetal calf serum. Virus was applied in a small volume of this adsorption medium and allowed to absorb for 1 hr. After adsorption, the inoculum was removed and fresh medium was added.

Labeling of infected cells

At the indicated times after adsorption, samples were incubated with tritiated uridine or adenosine (10 uCi/ml, New England Nuclear) for the indicated labeling periods. Incorporation was stopped by the addition of excess cold precursor and the cells were placed on ice. Monolayers were removed with a rubber policeman. Cells were collected and centrifuged at 600 g for 4 mins and the pellet was washed in cold buffer A (0.01 M tris pH 8.5, 0.003 M $CaCl_2$, 0.01 M Mg acetate, 0.15 M KCL). At this point the cells were either fractionated into cytoplasm and nuclei or whole cell samples were disrupted by addition of sodium dodecylsulphate (SDS) to 1% concentration. Whole cell lysates were then precipitated in 5% TCA and filtered. Extraction of RNA from whole cell samples by the host phenol technique is described below. Cells to be fractionated were collected, resuspended in cold NP_{40} buffer (Buffer A + 0.5% NP_{40}), and held at 0° for 5 mins. Under these conditions, cells lysed spontaneously with less than 1% of thymidine labeled DNA appearing in the cytoplasmic fraction.

The nuclei were removed by centrifugation (600 g, 3 min) and the cytoplasmic fraction was brought to 1% SDS (see below for extraction of RNA). The nuclei were also lysed in 1% SDS and both fractions were precipitated in 5% TCA and filtered on GF/C glass filters. The air-dried samples were digested and counted as described for acrylamide gel slices.

Preparation of RNA from whole cells

Infected cells were labeled, collected, and washed as described above. Immediately after washing in cold buffer A, the cell pellet was resuspended in pH 5.0 acetate buffer (0.05M). The cells were lysed in SDS (1%) and were extracted in hot phenol as described by Scherrer (24). After ethanol precipitation, the pellet of purified RNA was digested with RNase-free bovine pancreatic DNase I (Worthington, 25 ug/ml) at 37° for 30 mins. The reaction mixture contained 0.01 M tris pH 8.0, 0.04 M NaCl, and 0.01 M $MgCl_2$. RNA was re-extracted at 24° with an equal volume of phenol and chloroform and was re-precipitated in ethanol. Between 65 and 80% of the incorporated uridine label was recovered by this method.

Preparation of RNA from cytoplasmic extracts

The supernatant from cytoplasmic fractions containing 1% SDS was extracted with an equal volume of phenol and of chloroform. Phases were separated after extraction by centrifugation at 5° for 15 mins at 4,400 xg. Interphase material was re-extracted twice with fresh buffer A and the aqueous extracts were combined. RNA was precipitated by addition of 3-4 volumes of ethanol and allowed to stand at -4° for at least 24 hours. Unless otherwise stated, cytoplasmic viral DNA was removed by $LiCl_2$ precipitation. The RNA in ethanol was collected by centrifugation at 4,500 xg for 30 mins. The precipitate was resuspended in 0.01 M tris pH 8.0 and brought to 2M $LiCl_2$. The RNA was allowed to precipitate for 12 hours at 4°. The precipitate was spun down at 4,500 xg for 30 mins and was resuspended in 0.01 M tris pH 8.0 buffer and was again precipitated in ethanol and stored at -20° prior to use.

Binding to polydeoxythymidylate-cellulose

Poly dT-cellulose was kindly supplied by Dr. S. Kerwar. Purified RNA was applied to a 1x 4 cm column of poly dT-cellulose in 0.01 M tris pH 7.5 containing 0.5 M KCL. The column was washed with 5 column volumes of this application buffer. The majority of the input RNA eluted in the application wash and contains ribosomal and transfer RNA as well as

mRNA and mRNA fragments which lack poly adenylic acid sequences (1). The column was then washed (5 column volumes) with the same buffer containing 0.01 M KCL. Less than 3% of the input RNA was eluted in this wash. The final wash was with buffer without salt and the RNA was eluted which bound to the poly dT-cellulose due to poly adenylic acid sequences. In the first wash, 1-2% of the total adenine labeled RNA was resistant to RNase digestion at high salt. The second wash contained 0.3 to 0.8% and the final wash contained 11-12% RNase resistant RNA. RNase digestion was carried out at 37° for 30 mins in 0.32 M NaCl, 0.01 M EDTA with 14 units/ml of RNase T1, (Miles Laboratories) and 3.2 μg/ml of RNase A (Seravac Laboratory).

RESULTS

Effect of FV_3 infection on host RNA synthesis

A number of papers have drawn attention to the observation that FV_3 infection of cultured cells causes a rapid inhibition of host RNA synthesis (11). As FV_3 appears to replicate in discrete cytoplasmic factories (16, 20), incorporation of tritiated uridine into the nuclear fraction of infected cells during a brief exposure period gives an approximate measure of host RNA synthesis, and incorporation into the cytoplasmic fraction is indicative of the rate of viral RNA synthesis. The rate of nuclear RNA synthesis in infected FHM monolayers declines approximately 80% within 4 hrs after adsorption of FV_3 whereas incorporation into the cytoplasmic fraction increases (Fig. 1). However, although inhibition of synthesis is significant, nuclear RNA production is not completely eliminated in the infected cells. Furthermore, the rate of the residual nuclear RNA synthesis is 10 to 20% of the rate of the total RNA synthesis. These preliminary experiments indicate that although host RNA synthesis is inhibited to a great extent, the residual synthesis of host species occurs at a significant rate relative to viral RNA synthesis. Furthermore, the processing of host RNA to 28S and 18S species was not affected by FV_3 infection (data not shown).

Viral RNA in FV_3-infected cells

Since labelled host RNA will contribute significantly to the overall species of RNA isolatable from infected cell, it was important to measure contamination by host RNA and to positively identify viral species by hybridization. Contamination by host species was successfully reduced by labelling for only a brief period and removing nuclei intact from the disrupted infected cells. RNA from the cytoplasmic extracts of

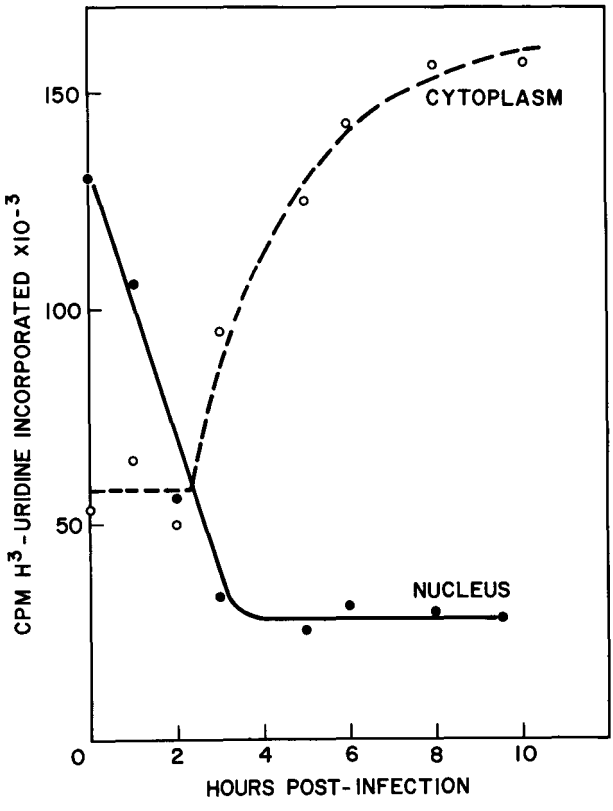

Figure 1 : Effect of FV_3 infection on uridine incorporation into nuclear and cytoplasmic fractions. Infected FHM monolayers were labeled at the indicated times after infection for 15 mins with tritiated uridine. Mock-infected controls which were labelled and sampled in parallel with the infected cells exhibited a constant ratio of cytoplasmic to nuclear incorporation although this ratio (c/N) varied from 0.4 to 0.8 in different experiments. o – – o cytoplasm, ●——● nucleus.

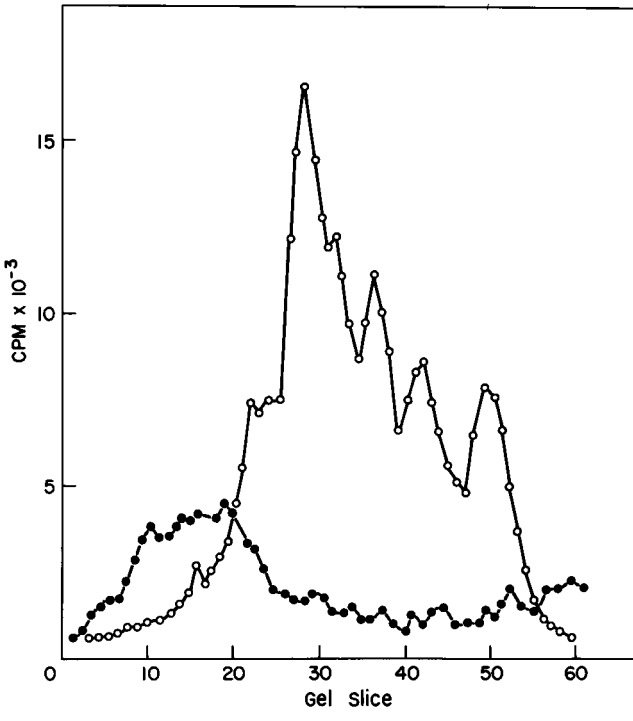

Figure 2 : Electrophoresis of RNA from the cytoplasmic fraction of uninfected and FV_3 infected FHM cells. FHM monolayers were infected with FV_3. At 5 hours post-infection, cells were labelled with tritiated uridine (50 µCi/ml) for 15 minutes. Monolayers were rinsed with cold PBS containing 0.01 M uridine and cytoplasmic fractions prepared according to Methods. The cytoplasmic RNA was extracted and analyzed by acrylamide-agarose electrophoresis. An equal number of mock-infected cells were processed in the same way. o——o, RNA from infected cells; •——•, RNA from mock-infected cells. The arrows indicate the position of C^{14}-labelled HeLa cell 28S, 18S, and 4S markers run simultaneously.

briefly labelled infected cells form a series of peaks with the predominant species migrating at a position expected for a species with a sedimentation coefficient of 16S (Fig. 2). In contrast much less RNA of the same size species was obtained from uninfected cells. Very similar patterns were obtained from infected FHM and BHK cells. Evidence for the viral origin of RNA in the 16-8S peaks was provided by hybridization of the RNA extracted from the gels (Fig. 3). Such RNA contained relatively little RNA hybridizable to host DNA (Fig. 4). This combination of a short-labelling period to reduce the time available for host RNA to be transported from the nucleus and separation of intact nuclei from the cytoplasm permits the isolation of labelled viral RNA with reduced levels of labelled host RNA.

"Early" viral RNA

In a number of deoxyvirus systems distinct "early" RNA species whose synthesis is independent of viral DNA synthesis are transcribed prior to initiation of DNA synthesis (see 18). To clearly separate any such species from "late" RNA, DNA synthesis was inhibited in order to permit transcription of only early species. In this system, DNA synthesis, as measured by a rise in thymidine incorporation into the cytoplasmic fraction starts at 3 hours and continues for a further 12 hours. When hydroxyurea or cytosine arabinoside was added to cultures prior to or at the time of infection, FV_3 DNA synthesis was inhibited (20); synthesis of viral specific RNA was greatly reduced compared to normally infected cells but virus specific RNA was synthesized over a long period of time (Fig. 5). When DNA synthesis was prevented indirectly by inhibition of protein synthesis (6) incorporation of uridine into RNA was markedly depressed (Fig. 6). This process was irreversible.

To compare the RNA species made, RNA from normally infected cells was compared with RNA from cells pretreated with hydroxyurea or cytosine arabinoside or cycloheximide (Fig. 7).

It can be seen that if viral DNA synthesis is prevented by hydroxyurea or cytosine arabinoside, the pattern of RNA synthesized in the cytoplasm is similar to that of normal viral RNA. There is no evidence of a distinct early RNA species, at least not in the size range 28-4S. The case of RNA made by cycloheximide treated infected cells is not as clear due to the consistently low levels of incorporation. However, there appears

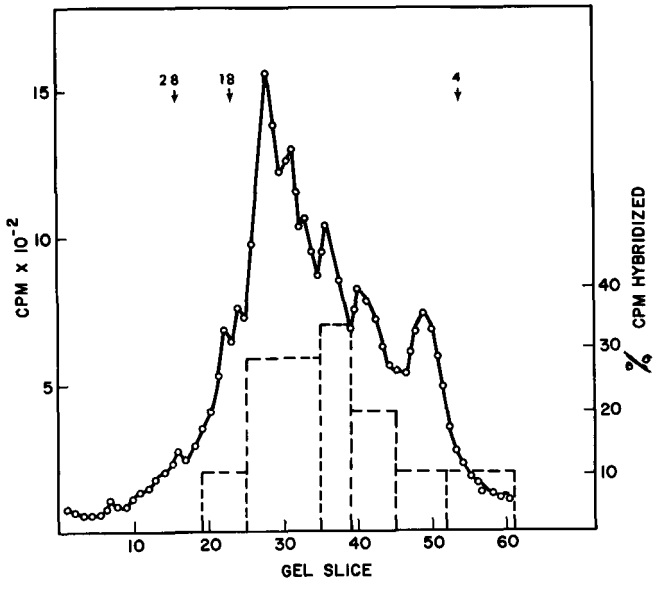

Figure 3 : Hybridization to FV_3 DNA of RNA extracted from acrylamide-agarose gels. Cytoplasmic RNA was prepared from FHM monolayers infected with FV_3. The cells were labelled with tritiated adenosine for 30 mins. between $5\frac{1}{2}$ and 6 hours post-infection. Purified RNA was resuspended from an ethanol pellet into 80% DMSO in 0.4 x running buffer prior to electrophoresis. The RNA from gel slices was extracted (see Methods).

RNase free DNase (2 µg/ml) was added to each fraction and allowed to incubate 30 mins. at 22° to remove any trace amounts of DNA. Samples of 0.5 ml were removed from each fraction and precipitated in TCA (5%) and filtered (o——o).

Fractions were pooled as indicated, re-extracted in phenol-chloroform (24°), and precipitated in ethanol. The precipitated RNA was hybridized against FV_3 DNA.

Background binding (120-231 cpm) to blank filters is subtracted and data is presented as the % of input CPM hybridized (- - -) for the pooled fractions. Maximum hybridization of the input counts corresponds to 2,887 cpm (35.2%). No significant hybridization was found in fractions 1-10 and 11-19 (less than 0.5%).

Arrows indicate position of C^{14} HeLa RNA marker species run simultaneously.

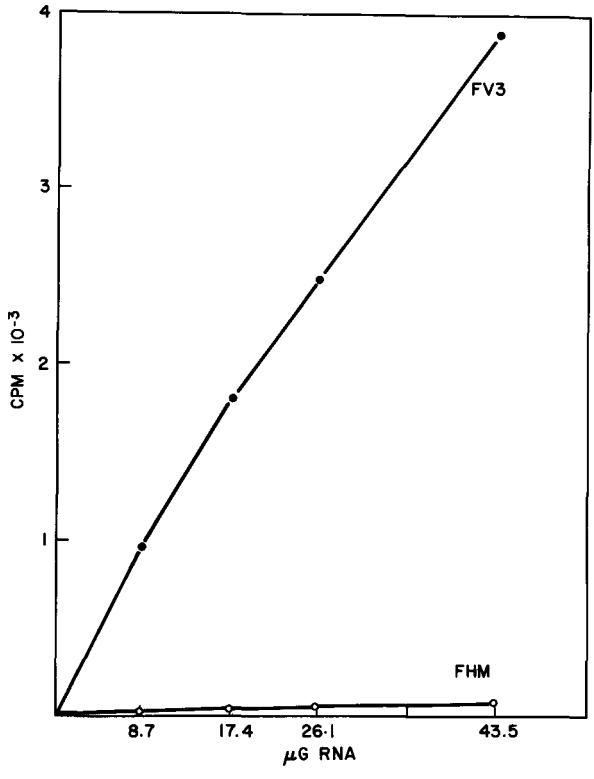

Figure 4 : Host RNA and viral RNA in briefly labelled cytoplasmic RNA of infected FHM cells. See legend to Fig. 3 for preparation of RNA. Increasing amounts of purified labelled RNA 994 cpm/µg were hybridized against duplicate filters of FHM DNA, FV_3 DNA and HeLa DNA (as a control) with 20 µg of DNA per filter. Backgrounds of 65 to 131 cpm were subtracted (●—●, FV_3 DNA; O—O, FHM DNA).

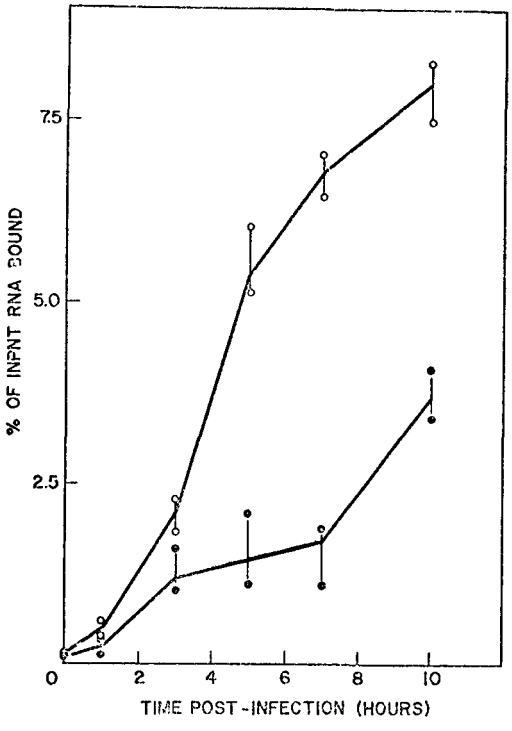

Figure 5 : Legend.

Effect of inhibition of DNA synthesis on viral RNA synthesis. FHM monolayers were infected with FV_3 at a moi of 10. Immediately after the 1 hour adsorption period, half of the monolayers were exposed to medium containing 1 μCi tritiated uridine per ml and 10^{-4}M cold uridine. The other half received the same medium with 3.3×10^{-4}M hydrowyurea. At the times indicated, 1.5×10^8 cells were harvested from each group. The cells were not fractionated. The total cellular RNA was extracted by the hot phenol SDS method. The purified RNA was digested with DNase prior to hybridization against filters containing either FV_3 DNA (20 μg) or no DNA. The hybridization was run in duplicate and at two different concentrations of RNA of each time sample. Each point is the average of duplicates at one RNA combination and is presented at the percentage of input RNA hybridized. The specific activity of the RNA samples was from 98.5 to 421.7 cpm/μg and the background 46 cpm average. The "zero" time point sample is RNA from uninfected cells, 0.11% of which bound to FV_3 DNA filters. (o——o infected cells without hydroxyurea; •——• infected cells plus 10^{-4}M hydroxyurea).

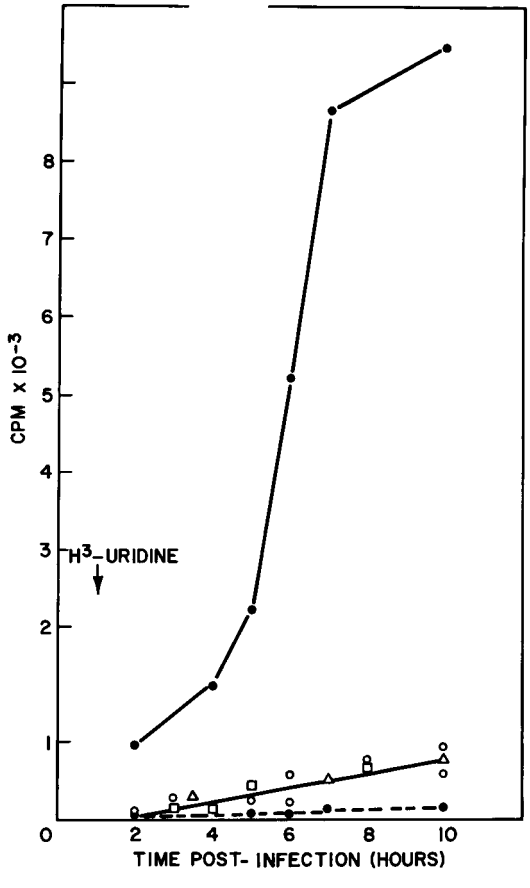

Figure 6 : Effect of cycloheximide on RNA synthesis in infected FHM monolayers. Four sets of FHM monolayers were exposed to cycloheximide for two hours and one group was not. All cells were infected with FV_3 at a moi of 5 except for one control set of monolayers which was exposed to cycloheximide but not infected. H^3-uridine (1 μCi/ml) was added to all cells at 1 hour post-infections. At the times indicated, duplicate monolayers were sampled, the cells harvested and acid precipitable counts were determined (see Methods) from whole cell extracts.

A, (●——●) infected cells, no cycloheximide
B, (□- - □) infected cells, 10 μg/ml cycloheximide
C, (o——o) infected cells, 100 μg/ml cycloheximide
D, (Δ- -Δ) uninfected cells, 100 μg/ml cycloheximide
E, (● – –●) infected cells, 100 μg/ml cycloheximide washed out at 2 hrs. P.I.

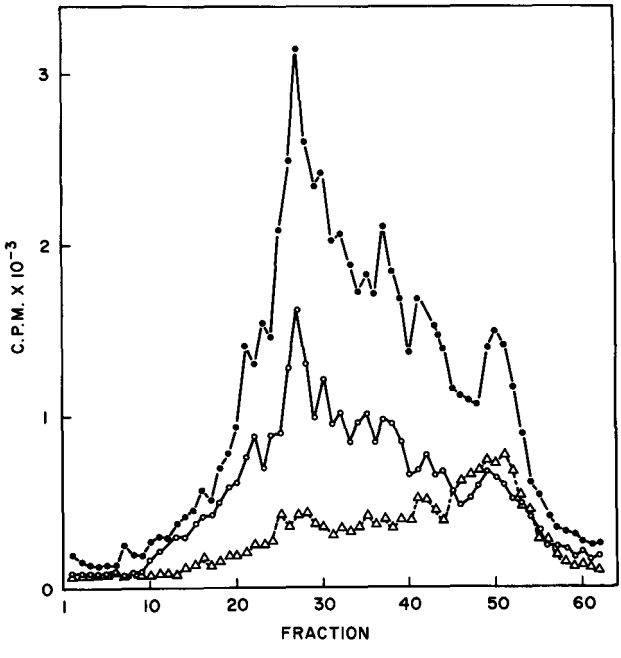

Figure 7 : FV_3 RNA from cells normally infected and exposed to hydroxyurea or cycloheximide. Hydroxyurea (10^{-4}M) was added to one set of FHM monolayers and cycloheximide (100 µg/ml) to another 1 hour before infection with FV_3 (moi = 30). All three groups were infected and at 8½ hrs post-infection all were labelled with tritiated uridine (10 µCi/ml) for 15 minutes. Inhibitors were present continuously from the time of their addition. RNA was purified from cytoplasmic extracts and electrophoresized. A - normal RNA specific activity 1,343.8 cpm/µg, 9.2% of input hybridized to FV_3 DNA filters (• - - •). B - cells exposed to hydroxyurea, specific activity 618.1 cpm/µg. 4.6% of input RNA hybridized to FV_3 filters (o - - o). C - cells exposed to cycloheximide, specific activity 169.0 cpm/µg RNA (Δ- -Δ) 3.8% of input RNA hybridized.

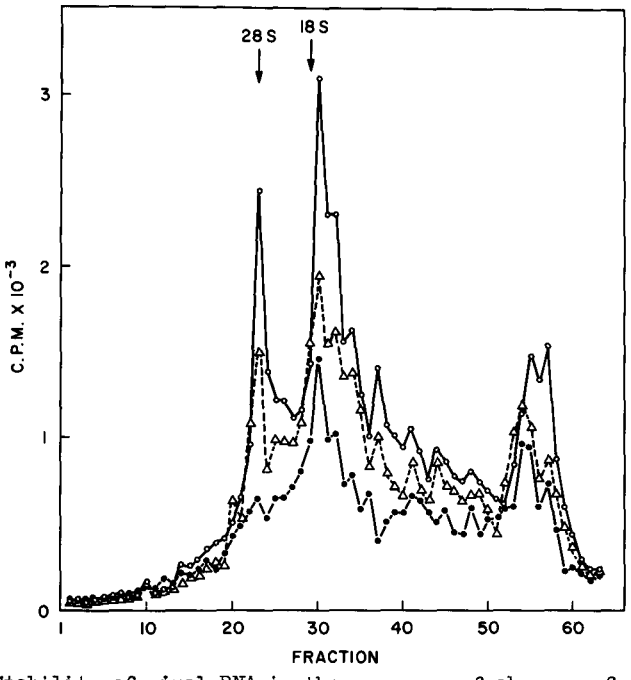

Figure 8 : Stability of viral RNA in the presence of absence of cytosine arabinoside. Infected FHM monolayers were incubated (15 minutes) with H^3-uridine, six hours after infection (see Methods). RNA was purified from cytoplasmic extracts from one third of the cells immediately after labelling. The remaining cells were washed with medium containing cold uridine (10^{-3}M), divided into two groups (with and without 10^{-4}M cytosine arabinoside), and were allowed to incubate for 6 hours in medium containing cold uridine (10^{-3}M). During the 6 hours incubation period after labelling, there was no decrease in incorporated label for either group as measured by TCA precipitable counts in whole cells from duplicate monolayers sampled at 1, 4, and 6 hours after labelling (Table 1). Cytoplasmic RNA purified from the two groups of cells 6 hours after labelling was compared with RNA isolated immediately after labelling by gel electrophoresis. HeLa cell RNA (C^{14}) was used as a size marker. The amount of label applied to gels for the 3 samples was: RNA taken immediately after labelling, 29,000 cpm applied (●——●); RNA from cells after incubated 6 hours after labelling, no cytosine arabinoside, 50,000 cpm applied (○——○); RNA from cells incubated 6 hours after labelling, plus 10^{-4}M ARA-C, 40,000 cpm applied (△- -△).

to be a larger proportion of the RNA in the 10-4S region in the samples of cycloheximide treated cells. Similar results were obtained at high (100 ug/ml) or low (10 µg/ml) concentrations of cycloheximide.

As there is no biological assay to demonstrate the intactness of these RNA species, it is difficult to prove that they are not produced by degradation. The following experiment was designed to show that the cytoplasmic RNA (viral) species observed are stable within the infected cell and do not undergo extensive degradation in the presence of cytosine arabinoside.

Infected cells were briefly incubated with H^3-uridine late in infection. One sample was taken immediately after labelling and the remainder were washed with medium containing excess cold uridine. Half of these remaining cells were allowed to continue the course of infection in the presence of cold uridine while cytosine arabinoside was added to the other half. Addition of cytosine arabinoside to the infected cells rapidly inhibited incorporation of uridine from the internal pool, whereas the normally treated cells continued incorporation at a declining rate for at least 6 hours (Table I).

The pattern of viral RNA observed immediately after labelling (Fig. 8) was still present 6 hrs after the cold uridine chase. The only major difference is the appearance of 28S RNA in the cytoplasm as would be expected from the fact that the synthesis and processing of host RNA continues after infection. Analogous "pulse-chase" experiments using actinomycin D (10^{-4}M) to block RNA synthesis in place of cytosine arabinoside give similar results (data not shown).

Polyriboadenylic acid in viral RNA

It has been suggested that polyadenylic acid sequences at the 3' end of messenger RNA's might be involved in transport of RNA from the cell nucleus to the cytoplasm (5). However, poxvirus messenger RNA contains polyadenylic acid sequences which are unlikely to be involved in such a transport process. It was of interest to know if FV_3 RNA also contains polyadenylic acid sequences particularly in view of the possibility that they might be synthesized by a virion encapsulated enzyme as appears to be the case for poxvirus (8, 3).

TABLE I

Effects of Cytosine Arabinoside on Incorporation into Cytoplasmic RNA after Briefly Labelling Infected Cells

Hours after Addition of Cold Uridine	+ ARA-C*	- ARA-C
0	40,101	38,509
1	190,172	202,885
4	209,689	301,896
6	194,089	343,550

See legend to Figure 8. Cytoplasmic extracts (see Methods) were made from 2 monolayers for each time point. Net incorporation into the cytosine arabinoside-treated cells stopped within one hour of addition of drug plus excess cold uridine. In the absence of cytosine arabinoside cells continued to incorporate label from the internal pool of uridine at a declining rate.

* H^3-uridine incorporated into TCA precipitable cpm.

The majority of FV_3 specific RNA contains polyadenylic acid sequences of sufficient length to bind to poly dT-cellulose. FHM monolayers were infected with FV_3 and labelled with either tritiated uridine or adenosine for 15 minutes at 6 hours post-infection. Purified RNA ($1x10^6$ cpm) from the cytoplasmic fraction was passed over a poly dT-cellulose column. The bound fraction was eluted (see Methods) and brought to 4 x SSC + 0.1% SDS. Approximately 10% of this RNA was hybridizable to FV_3 DNA. Most (70%) of the hybridizable RNA was found in the fraction binding to dT-cellulose. Table II summarizes experiments which support the conclusion that at least some of the viral RNA contains polyadenylic acid. Furthermore, the RNase resistant sequences (polyadenylic acid) occurs in all size classes of viral RNA, although certain species contain more resistant material than others (Fig. 9). The RNase resistant material from FV_3 RNA has a size distribution which is about the same as that of a wide variety of other viral and cellular mRNA's (apparent size 150-200 nucleotides, c.f. Fig. 10 and ref. 2).

TABLE II

Poly-A in FV_3 RNA (%)

RNA	Total CPM	TCA Precipitable CPM after RNase	CPM Bound to dT Cellulose after RNase
H^3-A RNA	1.7×10^6	2.6×10^5 (15.1%)	2.2×10^5 (12.7%)
H^3-u RNA	4.4×10^5	1.4×10^4 (3.2%)	9.2×10^2 (0.2%)
H^3-A RNA eluted from gel	2.0×10^4	2.4×10^3 (11.8%)	- - - - - - - -
H^3-A RNA eluted from hybrid	2.9×10^4	- - - - - - - -	3.1×10^3 (10.8%)

Presence of polyadenylic acid sequences in RNA from FV_3 infected cells.

RNA was prepared at 6 hours post-infection from the cytoplasm of FV_3 infected FHM monolayers after 15 minutes labelling with either tritiated uridine or adenosine. Part was subjected to RNase digestion (37° for 30 minutes with 1.6 µg/ml of RNase A and 7 units/ml of RNase T, in 0.32 M NaCl, 0.02 M EDTA) and the RNase digest then passed over a poly-dT cellulose column (Methods). Aliquots of bound material were hydrolyzed greater than 99% after 18 hours in 0.6 M KOH at 37°. Another portion of the labelled RNA was subjected to electrophoresis then eluted from gels and pooled. (See legend to Fig. 9 for details of elution of RNA from gels). Such RNA labelled with tritiated adenosine was hybridized to FV_3 DNA (20 ug/filter) in 50% formamide - 5x SSC at 43° for 24 hours (2). The hybridized viral RNA was eluted from washed filters in 0.01 M tris pH 7.5 0.01 M EDTA by shaking for 1 hour at 37°. Of the input RNA (5×10^5 cpm) 5.7% was recovered from hybrids. The eluant RNA also bound to polyU filters (36.3% of input) (23) prior to RNase digestion. The RNA was digested and was passed over poly-dT-cellulose using the methods described above. CPM refers to acid precipitable counts.

DISCUSSION

One of the characteristics of FV_3 which has been singled out for attention is its ability to severely inhibit host cell function (4). Although the virus does cause a rather rapid decrease in host cell RNA synthesis, residual RNA synthesis continues at a significant level. The infected cell continues to produce ribosomal and messenger RNA and ribosomal

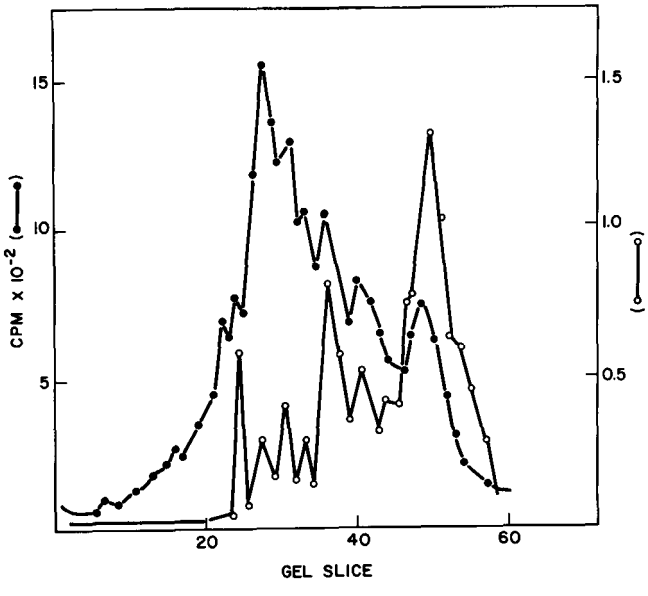

Figure 9 : Distribution of RNase resistant RNA in viral RNA. RNA was purified from the cytoplasm of infected FHM monolayers after 15 mins. labelling with tritiated adenosine. The sample was applied for electrophoresis in 80% DMSO – 0.04 x running buffer. RNA from gel slices was eluted (see Methods). The eluant was digested with DNase (2 µg/ml) for 30 minutes at 25° and 0.5 mls was precipitated in TCA (●—●). The remainder was brought to 0.32 M NaCl, 0.02 M EDTA and digested with 3.2 µg/ml RNase A, 14 units/ml RNase T, at 37° for 30 minutes followed by TCA precipitation (o—o). Total recovery (%) of RNase resistant cpm from the eluant material is given in Table 1 (third line).

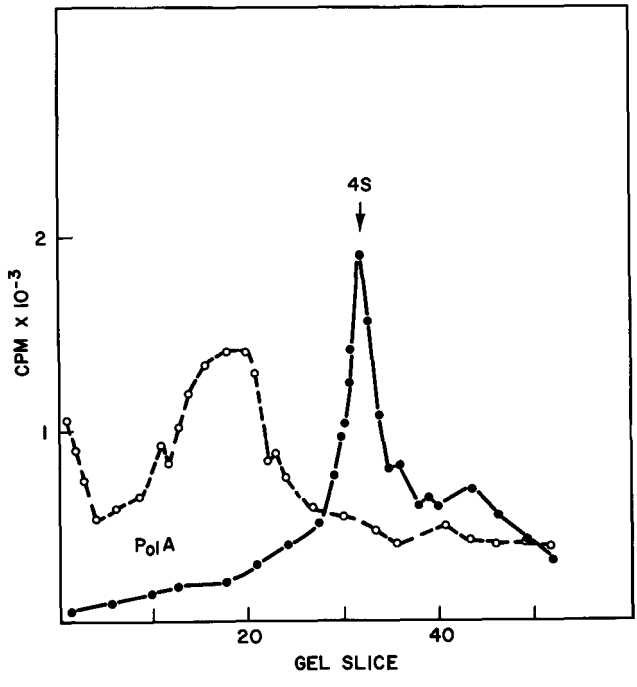

Figure 10 : Size of RNase resistant viral RNA. RNA was purified from the cytoplasm of infected FHM monolayers after labelling 15 minutes with tritiated adenosine. The RNA was digested first with DNase, then with RNase (see legend to Fig. 9), and was precipitated in ethanol with cold polyadenylic acid carrier. The RNA was denatured in 80% DMSO prior to application to acrylamide gels. Electrophoresis was carried out on 11 cm gels of 10% acrylamide at 150 volts. Tritiated transfer RNA was run simultaneously as a size marker. The size of the RNase resistant material was calculated from its migration relative to the 4S marker to be an average of 60,000 daltons, or about 175 nucleotides.

RNA appears to be processed normally from precursor molecules (unpublished observations). FV_3 RNA is transcribed at a comparatively low rate in fact about one tenth of that of poxvirus in the same cell (data not cited). This makes it difficult to readily study the pattern of FV_3 transcription.

Because of the problems implicit in the cytoplasmic replication of nucleic acids, it is of interest to compare the modes of transcription of the two virus groups represented by poxvirus and frog virus 3. The transcriptional pattern of poxvirus can be understood in terms of the sequential events of uncoating (14, 18) and the presence of a virion associated transcriptase (13). Frog virus 3 appears to be uncoated in a single step independent of protein synthesis (25) and to date no virion RNA polymerase has been detected.

The "early" messenger RNA of poxvirus is transcribed in a distinct wave of synthesis preceding DNA replication; this RNA is readily distinguished from "late" RNA species by size (14, 28). FV_3 RNA produced in cells infected in the presence of inhibition of viral DNA synthesis appears to be identical to RNA from normally infected cells. In agreement with this observation is a report that most, if not all, viral proteins are produced in the absence of viral DNA synthesis (6). Because of the high input multiplicity of infection and the efficiency of inhibition of viral DNA synthesis by cytosine arabinoside, it is unlikely that we were measuring RNA synthesized from "breakthrough" of some viral DNA synthesis. A very similar situation occurs in Herpes virus infected cells where parental DNA is transcribed for the production of most viral structural proteins (23).

At least some FV_3 transcripts contain stretches of polyadenylic acid and in this regard FV_3 resembles poxvirus and a great many other viruses. However, we were unable to detect any virion-associated enzyme which might give rise to these polyadenylic acid tracts when various assay methods described for poxvirus (12, 3) were applied to purified or crude virus.

FHM cells infected with FV_3 under conditions that permit extensive FV_3 replication (20) do not show a distinct burst of FV_3 RNA synthesis preceding DNA synthesis nor can any such burst be magnified by infection in the presence of cycloheximide as is the case for poxviruses (18).

The very low rate of RNA synthesis in FHM cells pretreated with cycloheximide and infected with FV_3, precludes extensive studies and determination whether the 6S RNA species (Fig. 7) represents pre-early messenger. We can only point out that (i) it does appear to be the predominant species unlike the case for RNA produced in the presence of cytosine arabinoside, that (ii) the available evidence on FV_3 uncoating which is not extensive (25) gives no indication of why RNA made in the presence of cycloheximide should differ from RNA made in the presence of cytosine arabinoside; currently there is no early enzymatic marker of FV_3 that could be used to readily test the function of this RNA.

ACKNOWLEDGEMENT

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DISCUSSION

Dr. S. Schlesinger :When you got some RNA in the presence of cycloheximide did you see if that RNA would hybridize with the viral DNA or was it just host ?

Dr. McAuslan : It was difficult to get enough RNA under these conditions for hybridization so we have not done that. But I want to point out that other people, Gravell for one, have found a considerable amount of RNA that is synthesized in the presence of cycloheximide and this suggests that either the virus does carry a transcriptase or uses a pre-existing RNA polymerase in the cell. I think it would be very interesting to sort this one out. Not only for hog virus but also for African swine fever virus.

Dr. Scholtissek : Does the viral DNA contain poly DT structures ? I mean if this is the case you do not need a special enzyme to put a poly A at the end.

Dr. McAuslan : I don't know if that is the case. We have not looked to see if there are DT regions in FV_3 DNA. In the poxviruses there has been some change in the results. Initially Kates reported that they found evidence for poly DT regions in Pox DNA. More recently this has been corrected. A poly A polymerase distinct from the transcriptase has been demonstrated. We have looked for such an enzyme in FV_3. As far as I know no one has conclusively demonstrated a poly A polymerase or a transcriptase, in FV_3.

Dr. Bachrach : Is there a double-stranded ribonuclease associated with this virus ?

Dr. McAuslan : Yes, I'll talk about that later to-day.

We found ribonuclease acitivity associated with FV_3. This activity can degrade single or double strand RNA. Perhaps we can discuss later its possible function. Certainly it will add to the problem of transcription. I might point out that we did some experiments at Plum Island where we looked for such an activity in African Swine fever virus. We found no trace at all which is a pity since it would have been a characteristic marker of the virus.

IN-VITRO SYNTHESIS OF HERPES SIMPLEX VIRAL DNA IN ISOLATED NUCLEI

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SUMMARY

Nuclei from Herpes simplex virus (HSV) infected cells were isolated and incubated in-vitro in the presence of the four deoxynucleoside triphosphates, (including ^{3}H-TTP), 6 mM Mg^{++} and 8% (w/v) sucrose. Under these conditions, the nuclei continue to synthesize DNA. $CsCl_2$ density gradient analysis revealed that most (90%) of the in-vitro synthesized DNA banded at the density of HSV-DNA (1.718 g/ml). The rest banded at the density of cell DNA (1.700 g/ml). The in-vitro synthesized viral DNA molecules were heterogenous in size and their sedimentation coefficient ranged from 65S to 4S.

Nuclei isolated from HSV infected cells treated in-vivo with metabolic inhibitors were used to study the nature of the inhibitory effects exerted by several antiviral drugs.

INTRODUCTION

Herpes simplex virus (HSV) DNA, a linear molecule with a molecular weight of about 10^8 daltons (Becker et al, 1968), is replicated semiconservatively (Kaplan and Ben Porat, 1964) in the nuclei of infected cells (Munk and Sauer, 1964) by a virus coded DNA polymerase (Kier et al, 1966; Miller et al,1973; Weissbach, 1973). However, the replicative form of HSV-DNA has not yet been isolated. To obtain information on HSV-DNA replication, we have studied DNA synthesis in nuclei isolated from HSV infected cells and incubated under in-vitro conditions, an approach which was also used by Radsak (1973) and Kolber (1973). We have also investigated the nature of the DNA molecules synthesized in-vitro in infected nuclei which were treated in-vivo with inhibitors of DNA replication. These studies demonstrated that under in-vitro conditions, the infected nuclei synthesize mainly short double stranded DNA molecules which have the density of HSV-DNA.

RESULTS

In-vitro DNA synthesis in isolated nuclei

Preliminary studies with nuclei from actively growing uninfected BSC_1 cells were carried out to determine the conditions for in-vitro DNA synthesis which would eliminate the synthesis of cellular DNA. The conditions selected for in-vitro DNA synthesis by E. coli DNA polymerase (Goodman and Spiegelman, 1971) were found suitable for the study of DNA synthesis in the nuclei from HSV-infected cells. No DNA synthesis was found in uninfected nuclei (not shown), while ^{3}H-TMP was incorporated into DNA molecules by nuclei from infected cells (Fig. 1A). The synthesis of DNA in-vitro was dependent on the presence in the reaction mixture of the four deoxyribonucleoside triphosphates, since ommission of either one or three of them prevented ^{3}H-TTP incorporation into DNA (Fig. 1A). The synthesis of DNA in the nuclei isolated from infected cells was also dependent on the presence of Mg^{++} in the reaction mixture, 6 mM being the optimal concentration (Fig. 1B). The in-vitro synthesized DNA molecules were extracted with phenol and characterized by banding in $CsCl_2$ gradients. It was found (Fig. 2) that most of the DNA molecules synthesized in-vitro, had a density of 1.718 g/ml, which is the density of double-stranded HSV-DNA. The remaining DNA banded at the density of cellular DNA. These results indicate that nuclei isolated from HSV infected cells continue to synthesize both viral and cellular DNA in-vitro, similarly to the synthesis of DNA in infected cells in-vivo.

The nature of the in-vitro synthesized DNA

Two methods were used to characterize the in-vitro DNA product: a) analysis of the DNA molecules by centrifugation in sucrose gradients, to determine the molecular size and, b) characterization of the DNA by elution from hydroxylapatite columns. The nuclei incubated in the reaction mixture in-vitro were separated from the supernatant fluid by centrifugation and the supernatant fractions were analyzed by centrifugation in sucrose gradients. The results of a typical experiment are presented in Figure 3. It was found that even after 5 min of in-vitro DNA synthesis, most of the radioactivity banded at the top of the sucrose gradient with a coefficient of about 4S. However, only part of the in-vitro synthesized DNA molecules were released into the supernatant fluid of the reaction mixture. Some radioactive DNA also banded in the position between intact HSV-DNA (65S) and the low molecular weight DNA (Fig. 3A). Analysis of DNA synthesized during a 2- min period revealed a pattern essentially resembling that

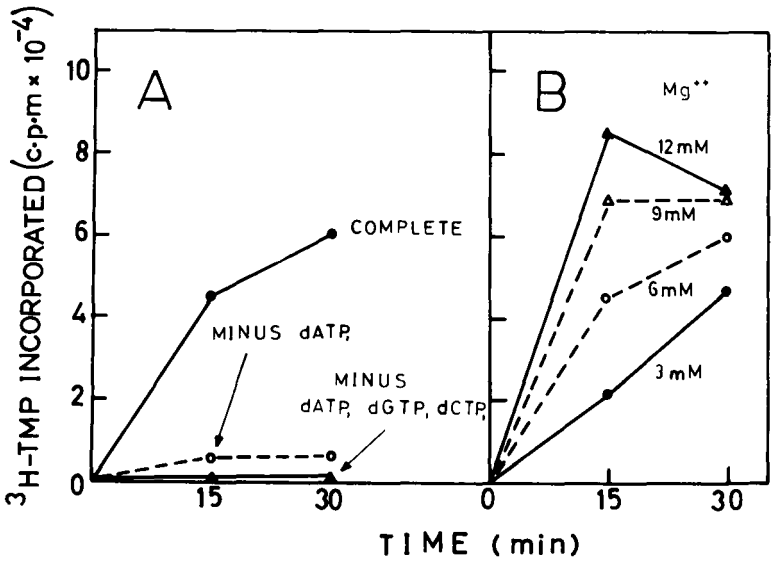

Figure 1 : In-vitro DNA synthesis in nuclei isolated from HSV infected cells.

Four day old monolayers of BSC_1 cells were infected with the HF strain of herpes simplex virus at 10 pfu/cell. The cells were incubated in modified Eagle's medium without serum. Under such conditions, the virus growth cycle is completed within 18 hrs after infection. The infected cells were scraped into reticulocyte standard buffer (RSB: 0.01M Tris.HCl, pH 7.7, 0.01M KCl, 0.015M $MgCl_2$) and homogenized in a glass Dounce homogenizer. After centrifugation for 2 min at 800 rpm in a PR-2 refrigerated centrifuge, the nuclear pellet was washed in phosphate buffer (0.075M phosphates, pH 7.4 containing 8% w/v sucrose [RNase free]). The nuclei were resuspended at a concentration of 10^6 nuclei per 100 µl phosphate buffer to which was added 3 mM mercaptoethanol, 6 mM $MgCl_2$, 40 µM each of dATP, dCTP, dGTP (Sigma Co., St. Louis, Missouri USA) and 50 µCi ^{3}H-TTP (specific activity, 15 Ci/mmole; The Radiochemical Centre, Amersham, England), in a final volume of 200 µl phosphate buffer. The reaction mixture was incubated at 37°C and duplicate samples of 25 µl each were removed at different time intervals to tubes containing 10% (w/v) trichloroacetic acid (TCA).

A. Requirement of deoxyribonucleoside triphosphates for in-vitro DNA synthesis.

B. Effect of Mg^{++} concentration on in-vitro DNA synthesis.

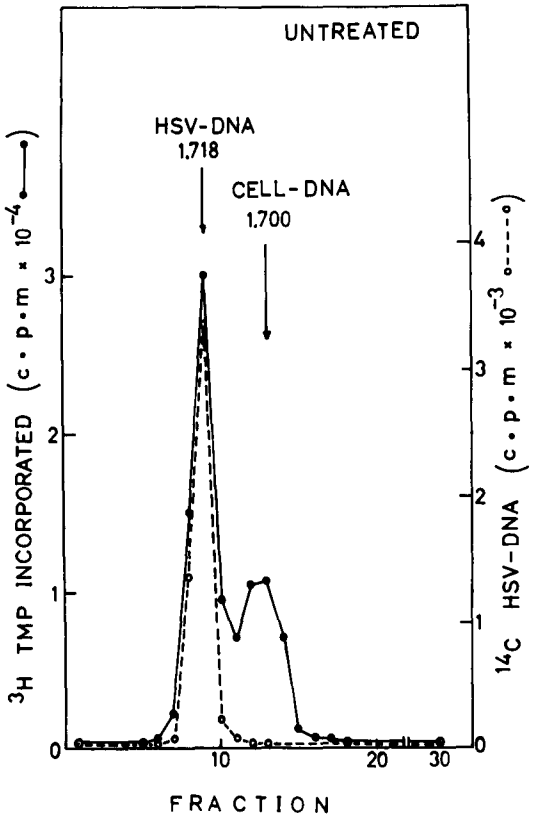

Figure 2 : Characterization of in-vitro synthesized DNA.

To suspensions of infected nuclei incubated in-vitro for 10 min, sodium dodecyl sulfate (SDS) (1% w/v final concentration) and pronase (1 mg/ml) were added and the suspensions were further incubated overnight at 37°C. The DNA was extracted with phenol and afterwards extracted several times with chloroform: isoamyl alcohol (1:25) solution until the interphase was clear. The DNA was precipitated in ethanol and kept at 4°C. When needed, the DNA centrifuged for 30 minutes at 2000 rpm in a PR-2 centrifuge and dissolved in TEN buffer (0.2M Tris.HCl, pH 8.0, 0.001M EDTA and 0.8M NaCl). ^{14}C-HSV-DNA (0 - - - 0) was added to serve as a marker and $CsCl_2$ crystals were added to bring the density to 1.70 g/ml. The gradients were centrifuged in the R50 Ti rotor at 40,000 rpm for 48 hrs at 20°C in the Beckman preparative ultracentrifuge. The gradient was collected dropwise, the density and radioactivity in the fractions were determined.

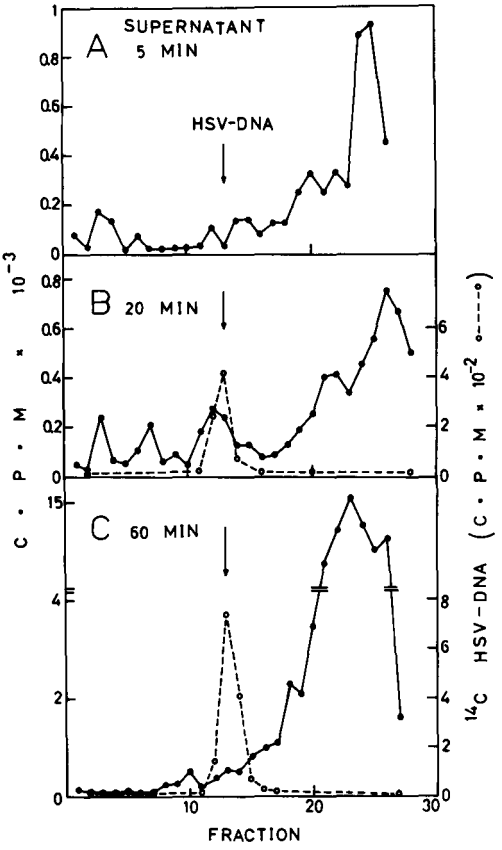

Figure 3 : Sucrose gradient analysis of in-vitro synthesized DNA released from the nuclei.

Infected nuclei were incubated for 5 (A) 20 (B) and 60 (C) min under in-vitro conditions. The nuclei were centrifuged at the end of the incubation period (for 2 min at 800xg) and the supernatants were layered on top of sucrose gradients, (5 - 25% (w/v) sucrose (crystalline density gradient, ribonuclease free; Schwarz and Mann, Division of Becton and Dickenson, Orangeburg, New York), made in TEN buffer (0.01M Tris.HCl, pH 7.4, 0.001M EDTA, 0.1M NaCl)). The gradients were centrifuged at 39,000 rpm for 2 hrs in the SW41 rotor of the Beckman model L-2 ultracentrifuge. ^{14}C-HSV-DNA (o - - - - o) was added as a marker. The gradients were collected dropwise and the TCA precipitable radioactivity was determined.

of intact HSV-DNA (Fig. 3B). It should be noted that in Fig. 5A and B, only a fraction of the labeled DNA sedimented in the sucrose gradients as large DNA molecules (larger than 100 x 10^6 daltons). Analysis of the DNA molecules synthesized for a 60 min period, showed that most of the in-vitro synthesized DNA molecules banded at about 20S, while some radioactive DNA also banded at 65S (Fig. 3C). At this time, most of the newly synthesized DNA had been released from the nuclei, since no more DNA appeared on further incubation.

The in-vitro synthesized DNA molecules present in both the supernatant and the pellet fractions of the nuclei, which were incubated for 5, 15, and 30 min, were characterized by chromatography on hydroxylapatite columns. It was found (Fig. 4), that the in-vitro synthesized DNA molecules were divided into two species: molecules which were eluted by 0.2M phosphate and, DNA molecules which were eluted by 0.4M phosphate. After in-vitro DNA synthesis for 5 min, the two DNA species were found in equal amounts in the supernatant fraction. On further in-vitro incubation, the amount of radioactive DNA eluted by 0.2M phosphate remain almost constant, while the DNA molecules eluted by 0.4M phosphate gradually increased (Fig. 4A). Analysis of the nuclear pellet (Fig. 4B) revealed a low level of radioactive DNA which was eluted by 0.2M phosphate, and remained constant throughout the in-vitro incubation. Most of the radioactive DNA in the nuclei was eluted by 0.4M phosphate and it increased linearly during a 30 min incubation period in-vitro (Fig. 4B). This result indicated that most of the in-vitro synthesized DNA consists of double-stranded molecules which are eluted from a hydroxylapatite column by 0.4M phosphate (Gordin et al, 1973). It is not yet known whether the DNA molecules which were eluted by 0.2M phosphate are the precursors for the double-stranded DNA molecules.

The in-vitro synthesized DNA molecules which were eluted by 0.2M and 0.4M phosphate were further analyzed by centrifugation in $CsCl_2$ density gradients. As shown in Figure 5, the labeled DNA molecules extracted by 0.4M phosphate from the hydroxylapatite column banded as double-stranded HSV-DNA molecules at a density of 1.72 g/ml (Fig. 5B and D). These preparations also revealed minor peaks of cellular DNA. The radioactive DNA molecules eluted from the columns by 0.2M phosphate also banded as double-stranded DNA molecules (Fig. 5A and C). It should be noted that a smaller peak of single-stranded DNA molecules banded at a density of

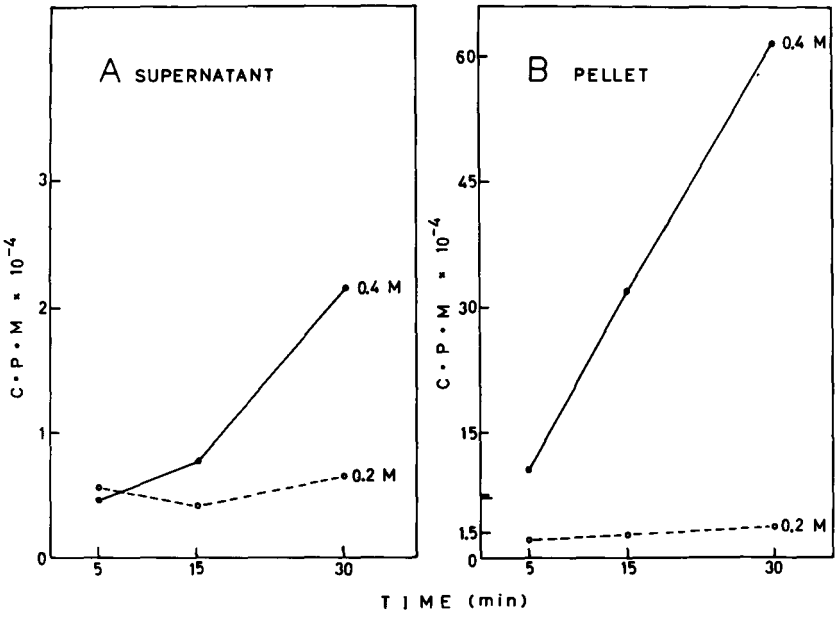

Figure 4 : Chromatography of in-vitro synthesized DNA on hydroxylapatite columns.

The nuclei from HSV infected cells were incubated in-vitro for 5, 15, and 30 minutes. The nuclear preparations were centrifuged, and the supernatants (A) and nuclear pellet (B) were treated with sarcosyl (3% w/v) and pronase (500 µg/ml preincubated for 2 hrs at 37°C). The two preparations were layered onto hydroxylapatite columns (Gordin et al, 1973) and eluted stepwise with 15 ml each of 0.01M, 0.2M and 0.4M sodium phosphate solutions. The TCA precipitated radioactivity in each preparation was determined.

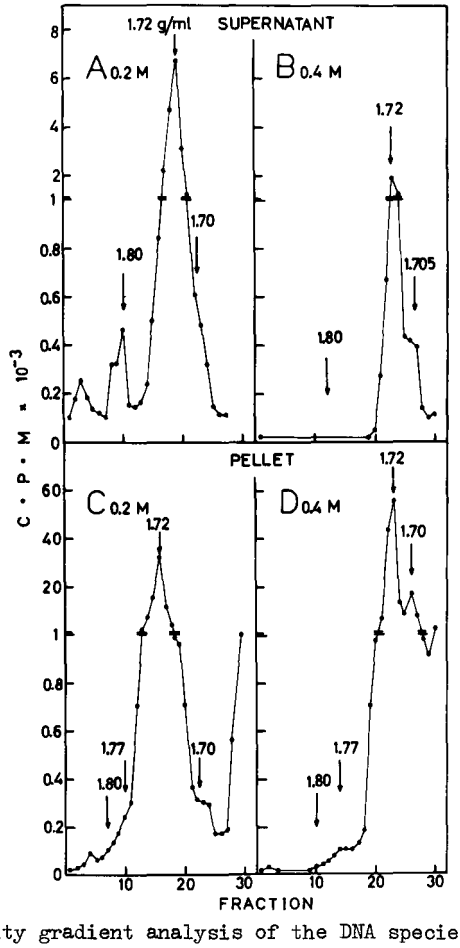

Figure 5 : Density gradient analysis of the DNA species eluted from hydroxylapatite columns.

The DNA preparations from a 30 min reaction in-vitro were eluted from hydroxylapatite columns by 0.2M and 0.4M phosphate. Samples of each were transferred to $CsCl_2$ (initial density 1.72 g/ml) and centrifuged for 48 hrs at 40,000 rpm in the Beckman model L-2 preparative ultracentrifuge. Heat denatured and native HSV-DNA were used as density markers (1.80 and 1.718 g/ml respectively). The gradients were collected and the radioactivity in each fraction was determined. The density of different fractions in each gradient was determined by weighing 100 µl samples.

A. Labeled DNA from the supernatant fraction after elution from hydroxylapatite column by 0.2M phosphate.

B. As above, but eluted with 0.4M phosphate.

C. Labeled DNA from the nuclear pellet after elution from hydroxylapatite column by 0.2M phosphate.

D. As in C, but eluted with 0.4M phosphate.

1.80 g/ml (the density of single-stranded HSV-DNA) with an additional peak close to the bottom of the gradient (Fig. 5A). It is not yet known whether the DNA molecules, which were eluted by 0.2M phosphate were originally eluted as single-stranded DNA molecules and afterwards reassociated to form double-stranded DNA molecules. Further studies are in progress using DNA nucleases to determine the amount of single-stranded DNA molecules in these preparations.

Effect of metabolic inhibitors on in-vitro DNA synthesis

To study the effect of metabolic inhibitors on the expression of viral functions, HSV infected cells were treated in-vivo with cytosine arabinoside (ara-C), cordycepin, and distamycin A. All three inhibitors were shown to inhibit HSV replication in-vivo (Levitt and Becker, 1967; Becker et al, 1972; Becker and Olshevsky, 1973a). At 18 hrs after infection, the nuclei were isolated and their ability to synthesize DNA in-vitro was studied (Fig. 6). It was found that treatment of the infected cells with ara-C allowed DNA synthesis to continue for 10 min in-vitro before ^{3}H-TMP incorporation was stopped. Similarly, after treatment with cordycepin, the nuclei had a low level of DNA synthesis, while distamycin A had a marked inhibitory effect. To characterize the in-vitro synthesized DNA, the nuclei were extracted with phenol and the DNA molecules were analyzed by banding in $CsCl_2$ gradients. It may be seen that in ara-C treated cells, only cellular DNA molecules were synthesized under in-vitro conditions (Fig. 7B), in contrast to the synthesis of viral DNA in untreated HSV infected nuclei (Fig. 7A). Cordycepin, on the other hand, prevented the virus mediated inhibition of cellular DNA synthesis (Becker and Olshevsky, 1973a), and although the level of DNA synthesis was reduced (Fig. 6) both cellular and viral DNA molecules were synthesized in-vitro in nuclei from cordycepin treated cells (Fig. 7C). In this respect, the in-vitro system is similar to DNA synthesis in-vivo (Fig. 7D). These results are taken to indicate that the synthesis of nucleic acids in the infected nuclei in-vitro reflects the presence of the virus coded DNA polymerase.

DISCUSSION

The results of the present study demonstrate that nuclei isolated from HSV infected cells are capable of synthesizing HSV-DNA in-vitro. The infected nuclei continue to incorporate ^{3}H-TMP into HSV-DNA as determined by $CsCl_2$ density gradient analysis of the labeled DNA molecules

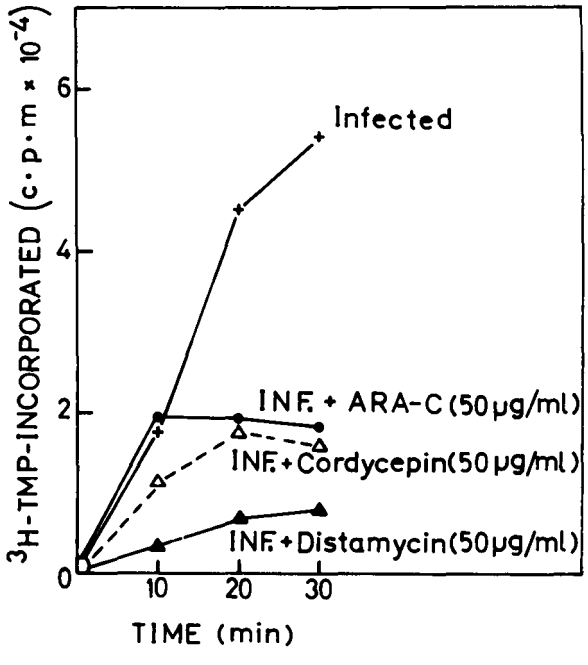

Figure 6 : In-vitro DNA synthesis in nuclei isolated from HSV-infected cells treated in-vivo with inhibitors.

HSV infected cells were treated with 50 µg/ml of ara-C, cordycepin, and distamycin A, added at 3 hrs post-infection, and incubated at 37°C. At 18 hrs p.i., the cells were harvested, infected cells serving as a control. The nuclei were isolated and incubated under in-vitro conditions. The amount of ^{3}H-TMP incorporation into DNA was determined.

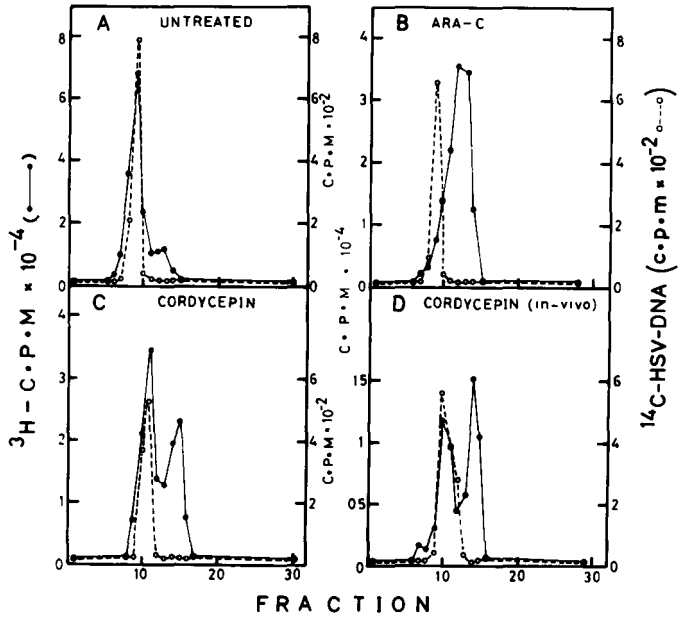

Figure 7 : Analysis of DNA synthesized in-vitro by nuclei from HSV infected cells treated with metabolic inhibitors in-vivo.

The DNA synthesized in-vitro for 10 min by infected untreated nuclei (A) and nuclei from ara-C (B) and cordycepin (C) treated HSV infected cells was extracted with phenol, as described in the legend to Figure 2. Cordycepin treated HSV-infected cells labeled with ^{3}H-thymidine (specific activity, 2 Ci/mM, Nuclear Research Centre, Negev) for 15 hrs in-vivo were also extracted with phenol (D). The DNA preparations (• —— •) were mixed with ^{14}C-HSV-DNA (o - - - o) and centrifuged in $CsCl_2$ density gradients. The distribution of the radioactive DNA in the density gradients was determined after centrifugation for 48 hrs at 40,000 rpm in a preparative Beckman L-2 ultracentrifuge.

synthesized in-vitro. The major in-vitro DNA product (more than 90%) banded at the density of HSV-DNA (1.718 g/ml), while the rest of the labeled DNA banded at the density of cellular DNA (1.700 g/ml). In this respect, the in-vitro DNA synthesis resembled the synthesis of DNA in the nuclei under in-vivo conditions (Becker and Olshevsky, 1973a,b). It is of interest that although we selected in-vitro conditions, which would eliminate the synthesis of cellular DNA in uninfected nuclei, cellular DNA was synthesized in the HSV-infected nuclei. This result suggests that the synthesis of cellular DNA in the infected nuclei might be carried out by the virus coded DNA polymerase, which functions under the in-vitro conditions. This concept is further supported by the use of ara-C (Fig. 7B). In the presence of ara-C, viral DNA synthesis is completely inhibited (Levitt and Becker, 1967) while the translation of proteins from the viral mRNA transcribed from the viral parental DNA, continues (Becker and Olshevsky, 1973b). In nuclei from ara-C treated HSV infected cells only cellular DNA is synthesized. This suggests that the viral DNA polymerase is synthesized in the ara-C treated cells together with the viral structural peptides. In the absence of viral DNA, the virus coded DNA polymerase is capable of synthesizing only cellular DNA. Since the affinity of the viral DNA polymerase for the cellular DNA template is restricted, DNA synthesis under in-vitro conditions takes place for only 10 min.

Nuclei from cells infected with polyoma virus (Winnacker et al, 1971; 1972, Magnusson et al, 1972, 1973) and adenovirus (Sussenbach and van der Vliet, 1972; van der Vliet and Sussenbach, 1972) synthesize viral DNA under in-vitro conditions. In these systems, intact viral DNA molecules were synthesized in-vitro, and were separated from the host cell DNA. Unfortunately, herpesvirus DNA is too large to be effectively separated from cellular DNA by the Hirt procedure (Hirt, 1967) which leads to the precipitation of both the viral and cellular DNAs. To avoid some of the difficulties, we therefore analyzed the labeled DNA molecules, which were released from the nuclei during incubation in-vitro. These analyses revealed that most of the in-vitro synthesized DNA molecules are double-stranded, and have a low sedimentation coefficient in sucrose gradients. Some radioactive TMP was also found to be incorporated into the DNA molecules which banded at 65S, the position of intact HSV-DNA, while some radioactive DNA molecules sedimented further into the sucrose gradient in the position of cellular DNA. The nature of these labeled DNA molecules is under study.

Analysis of the labeled DNA by chromatography on hydroxylapatite showed that the amount of double-stranded DNA molecules synthesized in the nuclei gradually increased after the start of in-vitro DNA synthesis.

It is of interest that part of the in-vitro synthesized DNA molecules were eluted by 0.2M phosphate, the conditions for elution of single-stranded HSV-DNA molecules (Gordin et al, 1973). Nevertheless, in the $CsCl_2$ density gradient, these viral DNA molecules banded at the density of double-stranded DNA molecules, except for a small peak of single-stranded DNA molecules. It is not known whether the DNA molecules, which are eluted by 0.2M phosphate are indeed single-stranded molecules which self-anneal to form double-stranded molecules. Further studies are needed to characterize the in-vitro synthesized DNA molecules and to determine the mechanism of viral DNA replication.

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DISCUSSION

Dr. Labonnardiere : You told us about the alkali-sensitive regions in Herpes virus DNA and if I heard right you assumed that these regions of DNA might correspond to the initiation points of replication such in OKAZADI's model. Is that right ?

Dr. Becker : Yes.

Dr. Labonnardiere : So why do you think this would be special to this virus whereas I think for bacterial DNA for instance where OKAZAKI's model might imply as well here are no such alkali-sensitive regions.

Dr. Becker : The working hypothesis is that this supposed "RNA" sequence or ribonucleotide containing sequences in the viral DNA might be part of the mechanism of replication of the viral DNA. The ribonucleotide sequences are in one strand since there is only one strand which contains these alkaline sensitive regions. This sequence is a signal for the DNA polymerase to initiate on the opposite strand. The deoxyribonucleotides are then polymerized and the turning point for the enzyme to continue to synthetize DNA on the opposite strand is controlled by a second ribonucleotide sequence. In other words when there is the opening of the duplex, the polymerase will go to the first to the alkali-sensitive region and then the polymerase will turn back with the synthesis of the OKAZAKI piece.

So opposite to the alkali-sensitive region on the new strand of DNA should be a gap opposite to the ribonucleotide sequence. At the end we will find ourselves with two progeny DNA molecules: in one molecule the old strand will contain the alkali-sensitive sequences and the new strand will be alkali-resistant. In the second molecule the old strand will be alkali-resistant and the new strand alkali-sensitive.

This is just a working hypothesis but we are trying to find out if indeed we have the pieces of DNA synthetized this way both in vivo and in vitro.

Dr. Scholtissek : It is known that OKAZAKI pieces have about 3000 nucleotides. Does this correspond to the size of DNA alkaline treatment which you get ? Are these pieces of DNA also about 3000 nucleotides big ?

Dr. Becker : Well they seemed to be different sized. So the spacing

of this region is different. But this does not rule out the possibility that they are not the only signals for DNA synthesis. We think there are four or five alkali-sensitive regions but this is not yet settled.

Dr. Scholtissek : Can you get your in vitro system free of ribonucleotide-triphosphates because if you can and you do not supplement your system with the ribonucleotide-triphosphate you should be able to get either only the pieces or no DNA synthesis in-vitro ?

Dr. Becker : When we add ribonucleotide-triphosphates to the in vitro DNA synthesizing reaction mixture containing nucleotides from infected cells we do not stimulate it. However, we did not try to eliminate completely the ribonucleotides from the nuclei or dialyse them out. I do not think the ribonucleotide-triphosphates are going to influence the reaction.

Dr. Scholtissek : But I mean you need some RNA synthesis in any case, in order to start the DNA synthesis according to your model.

Dr. Becker : We do not know the DNA polymerase under in vitro conditions completes what was initiated in vivo.

Dr. Scholtissek : But then I want a different question. At the beginning you showed a slide in which you showed that about 10 to 15 percent of the DNA synthetized in vitro banded at a density of the cellular DNA. Did you also hybridize this material with viral DNA because it could be if you synthetize your DNA in pieces that just by chance that the G + C content might be distributed at random during the total length of DNA in such way distributed that you might end up with viral DNA with a lower density ?

Dr. Becker : We spent quite a long time on studying the density of fragmented DNA molecules; it was found that all the HSV-1 banded sharply at the density of 1.718. Nothing in any experiments banded at a lower density. So whatever happens that there are no sketches of a dA-dT sequence more than twenty or twenty-five. I think of twenty nucleotides because Herpes DNA is very sensitive to Distamycin A which preferentially binds to poly-dA sequences and requires DNA with about twenty nucleotides in size. But more than that, by no means you can fractionate the DNA in fragments of Herpes virus DNA to find a fraction which will band at a lower density.

The DNA which appears at density of 1.70 apparently is cellular.

Dr. McAuslan : How positive are you that this was in fact Herpes DNA that you were looking at ? Did you actually hybridize it against Herpes DNA ? Or are you only going on faith in the density ?

Dr. Becker : Although we are quite confident that the DNA which appears is synthesized in the Herpes virus infected cells and bands at the density of 1.718 is only Herpes DNA, we are doing hybridizing this DNA to viral DNA.

Dr. McAuslan : A second question concerns this question of nicks in the Herpes DNA. I seem to remember that Dr. BICKEN from England mentioned that if they prepared DNA from freshly grown Herpes virus they could see no evidence of any nicks. Whereas if they stored the virus for some time nicks appeared. And I am wondering if these experiments with a former made have really been done at the same time and at the same batch of Herpes virus as the alkaline denaturation.

Dr. Becker : In parallel and on the same preparations and in so many times that it seems to be real we have alkali-sensitive sequences in fresh and old virus. As a rule we do this experiment on fresh virus. It is our observation that on storage of the purified virions the viral DNA is fragmented due to double strand breaks. The reason for the degradation of the DNA is not yet known.

Dr. Bachrach : I wonder if in purified DNA from Herpes virus you find ribo-C there and if you analyze also, is there an enzyme which will hydrolize that ribo-C runs ?

Dr. Becker : We can demonstrate the presence of ribo-C only if we label the cells with radioactive uridine. The uridine is incorporated into deoxy-C but there is also a small amount of radioactivity in a base which runs in chromatography to a postition very close to the ribo-C marker. This is done by digestion with a variety of DNAses, to isolate the ribo-C. This is as far as we have got. But as the label did not run exactly as ribo-C, I still have some reservations about it. Incidentally, Dr. Herbert Rozenkranz at Columbia University, New York, had found a similar situation

in T5 DNA and also in Herpes virus DNA.

Dr. M. Schlesinger :I think this is something Dr. Bachrach was asking about. There is an enzyme in E. coli that will split a deoxynucleotide, ribonucleotide double strand hybrids, namely ribonucleic H. Have you tried this or can you get this or does it work ?

Dr. Becker : Not yet. It could be that this is a very short sequence; Dr. Rosenkranz estimates that it contains not more than 20 ribonucleotides in one stretch.

Dr. McAuslan : Can you tell us a little bit more about the mechanism of action of synthesizing an enzyme after inhibiting DNA synthesis and can you refresh my memory about some studies I think you reported about the synthesis of Herpes DNA in the presence of inhibitors ?

Dr. Becker : Under in vitro conditions nuclei from infected cells treated with inhibitors which affect the DNA (e.g. Distamycin) do not synthesise DNA at all. Nuclei from cells treated with inhibitors which do not affect the DNA but inhibit a biosynthetic step (e.g. ara-C and hydroxyurea) a DNA polymerase is synthesised which synthesises DNA for a period of 10 minutes only and stops. The DNA product has a density of cellular DNA.

Dr. S. Schlesinger :Two question; the first: how is it that you prevent the uninfected cells from incorporating thymidine ? and the second is: as I understand the inhibition experiments were all done by killing the cells by inhibitors, what happens if you treat the nuclei with the inhibitor ?

Dr. Becker : The conditions for the in vitro DNA synthesis of those used for E. coli DNA polymerase assays. By testing a variety of conditions for DNA polymerase activity in vitro synthesising we found conditions which shut off the uninfected cells but not the infected ones. We showed that 90% of the in vitro synthesised DNA had viral specificity and 10% was cellular DNA. When we used inhibitors to inhibit in vitro DNA synthesis, we had found that only cellular DNA was synthesised in vitro. It is possible that the same enzyme might be responsible for the cellular DNA synthesis and this will explain why in vivo we get the synthesis of viral and cellular DNA since the latter was completely shut off due to the viral infection.

This is the answer to the first question.
The answer to the second question: If you add inhibitors to the isolated nuclei in vitro the inhibition depends on the mode of action of the inhibitor e.g. Distamycin which binds to DNA and will inhibit nuclei in vitro while ara-C or hydroxyurea, which function only in vivo, will have no effect in vitro.

Dr. Scholtissek : I have one additional question to the first one; may I ask you - did you try to label your viral DNA in vitro by using non-labelled deoxy nucleoside-triphosphate and one or two labelled ribonucleoside-triphosphates ?

Dr. Becker : Not under these conditions.

Dr. Scholtissek : But this would be a good experiment to show that RNA synthesis is necessary.

Dr. Becker : Addition of ribonucleoside-triphosphates to the in vitro reaction mixture had no effect on the extent of TMP-H^3 incorporation into DNA.

MODIFICATIONS OF SOME MAIN PROPERTIES OF THE AFRICAN SWINE FEVER VIRUS

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The knowledge of some properties of the African Swine Fever virus (ASFV) brings some interesting tests for the practical diagnosis and control of the disease in the field.

Among them, one can find :

a) the ability for hemadsorption (Malmquist and Hay, 1960)

b) the fixation of fluorescent antibodies (Heuschele and all, 1966 - Boulanger and all, 1967 - Carnero and all - Sanchez Botiza and all. 1970 - 1971).

c) the cytopathic effect in cell cultures

d) the ability of the virus adaptating surviving in ticks (argasidae) (Sanchez-Botiza, 1963)

The three first properties are considered as essential and stable for the identification of the virus and they constitute the basis of the present techniques used for the diagnosis of African Swine Fever.

It is the ability of the virus to survive in ticks, as observed in Spain (Sanchez Botiza, 1963) which could explain the transmission of the disease the mechanism of which being ignored at this time, and lead to the new sanitary rules for the control of the disease.

Nevertheless, the ability for hemadsorption, the fixation of fluorescent antibodies, anti_african swine fever virus and the cytopathic effect changed to some extent during the epizooty and induced new difficulties for the rapid identification of the virus, and subsequently for the control of the disease.

Modifications of the ability of the ASFV for hemadsorption

The modifications which were observed could be related to some changes in the antigenecity of the virus due to the persistence of the epizooty.

The antigenic composition of the ASFV is still not enough known. The existence of some specific soluble antigens which could be revealed by different

immunological reactions (complement fixation, immuno precipitations in agar gel, inhibitions of hemadsorption, etc.) was demonstrated. But one does not know the native antigens of a complete virion and what are the antigens produced by the infested cell during the viral replication and which are not incorporated in the virion.

The mechanism of hemadsorption as observed by Malmquist and Hay (1960) in ASFV infected leucocytes cultures is not well known. One could think of a specific antigen produced by the infected cell and associated in the mechanism of viral replication.

This hemadsorbing antigen (infectious antigen) does not seem to be essential for the growth and the maturation of the complete virion.

This hypothesis was confirmed by the presence in the natural field of non-hemadsorbing ASFV strains, as it appears in vitro after a certain number of passages of the virus on cell cultures.

It was shown that the virus ability for hemadsorption changed during the epizooty. It is interesting to remind that during the two first years (1960 - 1962) of the epizooty in Spain, the wild ASFV always produced regularly the hemadsorption in leucocyte cultures.

Since 1963 and 1964 (Sanchez Botiza, 1965) one observed for the first time non-hemadsorbing wild ASFV strains (0,09 to 0,21 % which produced a cytopathic effect without the expected hemadsorption). During the following years (from 1965 up to 1974) 0.32 to 5.19 up to a total of 216 of non hemadsorbing ASFV strains were isolated each year.

Table 1 shows the figures established for the non hemadsorbing virus strains from pigs which died from African Swine Fever, and the number of field samples which required one, two, three or four passages on leucocyte cultures to produce the hemadsorption.

In parallel, it was observed that during the epizooty the time required to produce the hemadsorption reactions was increased (table 2).

These observations prove the existence in the natural conditions of non hemadsorbing viral strains. The identification of the virus can be confirmed by inoculation to pigs and by immuno fluorescence. On another side, Coggins (1968) succeeded in isolating the non-hemadsorbing fraction from two ASFV strains and more recently Pini and Wagenaar (1974) isolated a non-hemadsorbing strain.

TABLE I

NECESSARY PASSAGES FOR HEMADSORPTION AND NON HEMADSORBING VIRUS STRAINS

Year	Total Number of positive samples from the Field	Number of samples and necessary passages for hemadsorption								Samples with non-hemadsorbing virus	
		First Passage		Second Passage		Third Passage		Fourth Passage			
		Samples	%	Samples	%	Samples	%	Samples	%	Number	%
1965	909	841	92,51	50	5,50	6	0,66	2	0,22	10	1,10
1966	2.184	2.044	93,58	103	4,71	7	0,32	-	-	30	1,37
1967	2.413	2.295	95,10	67	2,67	2	0,08	-	-	49	2,03
1968	1.332	1.199	90,01	102	7,65	8	0,60	-	-	23	1,72
1969	1.144	1.076	94,05	43	3,75	-	-	-	-	25	2,18
1970	1.345	1.260	93,68	60	4,46	10	0,74	-	-	15	1,11
1971	1.428	1.308	91,50	86	6,02	14	0,98	1	0,07	19	1,33
1972	911	842	92,42	53	5,81	6	0,65	1	0,10	9	0,98
1973	622	594	95,49	21	3,37	5	0,80	-	-	2	0,32
1974*	462	413	89,39	25	5,41	-	-	-	-	24	5,19
TOTAL	12.750	11.872	93,11	610	4,78	58	0,45	4	0,03	206	1,61

* First six months

TABLE 2

PERCENTAGE OF POSITIVE HEMADSORPTION REACTIONS FOR AFRICAN SWINE FEVER DURING THE PERIOD OF OBSERVATION OF THE LEUCOCYTE CULTURES IN SAMPLES FROM THE FIELD

Year	Total number of samples	Days								
		1	2	3	4	5	6	7	8	more than 8
1970	1.354	60,0	17,3	6,3	3,2	3,0	1,4	1,0	0,4	7,30
1971	1.443	53,5	16,2	9,0	5,8	2,3	1,3	1,2	0,3	10,4
1972	929	49,0	22,9	8,6	4,7	2,5	1,4	0,7	0,3	9,9
1973	1.413	37,9	19,1	13,3	8,8	7,0	4,1	2,2	1,7	4,8

In this table one can observe the progressive increase during the disease of the necessary time for the ASFV to perform the hemadsorption.

T A B L E 3

NUMBER OF NECESSARY PASSAGES ON LEUCOCYTE CULTURES FOR OBTAINING THE DISAPPEARANCE OF THE HEMADSORPTION ABILITY OF THE VIRUS.

Strain	Number of passages
EH	40
Cba x R	50
B_{86}	50
CD	60
AL	70
LG	80
Cba	90
L	100
PV	150
VP	230

In vitro most of the ASFV strains can loose their hemadsorbing property after a variable number of passages on cell cultures.
Table 3 shows the results on 11 strains, of the study of the relationships between the serial passages on leucocyte cultures and the hemadsorbing ability. From these results the loss of the hemadsorbing ability is obtained after 40 to 80 passages on leucocyte cultures for eight strains. For the three other strains, the loss of the hemadsorption property is observed respectively after 100, 150 and 230 passages on leucocyte cultures.

Modification of the ability of the wild virus for the fixation of fluorescent antibodies

Among the main modifications of the properties of the wild virus, one finds the loss of ability for fixing the fluorescent antibodies.
During the 1968 and 1969 years, it was possible to identify the virus in animals died from African Swine Fever by the direct immunofluorescence. In 1973, it was only possible to identify by this technique, the viral antigen in 55% of the identified breaks of African Swine Fever.
The virus present in tissues of died animals from African Swine Fever, can now escape the diagnosis through the presence of anti-ASFV antibodies which block the fixation of fluorescing anti-ASFV antibodies.
The blockade of the virus by antibodies and the frequent formation of antibody-antigen complexes which cannot be revealed by fluorescing antibodies is due to numerous building of antibodies which co-exist with the virus and react in fixing to the peripheral surface of the virus.

Modifications in the cytopathic effect

The cytopathic effect of the wild ASFV on the leucocyte cultures was estimated to be the most constant character. All the field strains (from 1961 up to 1971) produced a cytopathic effect during the first passage on leucocyte cultures. In 1972 and 1973, wild strains appeared which did not produce cytopathic effect on a primary culture but only after several identical passages.
So, it was possible to isolate in our laboratory ASFV strains which did produce a cytopathic effect only ten passages on leucocyte cultures. The identification of the virus was performed by immunofluorescence; one of the two viral strains does not induce hemadsorption and behalves as a non-hemadsorbing non-cytopathic strain

The other viral strain produces only hemadsorption after 7 or 8 passages.

The titer of the virus after the tenth passage on leucocytes cultures sets up $10^{-5.6}$ to $10^{-6.5}$.

The loss of cytopathic effect is an important change if one takes in account that although the percentage of strains presenting such a loss is presently weak, the presence of non-hemadsorbing and non-cytopathic ASFV strains could raise some difficulties in diagnosis and localising the disease.

Impact of the modifications of the properties of the wild ASF VIRUS on the diagnosis of the disease

In addition to the changes in the properties of the virus, the clinical framework of the African Swine Fever changes in some extent during the epizooty. The delay between the natural inoculation of the virus into the animal and its death is presently shorter than some years ago. Now, most of the breaks present a clinical phase of 14 days. Previously death occured between 6 to 8 days after infection.

Presently the subacute form of the disease is very frequent and the number of subclinical and chronical forms is increasing. The general mortality decreased and the symptons and lesions loosed their first typical characteristics.

Due to these changes in the clinical aspects, difficulties arived not only on the field but also at the level of the speed of the assessment of the laboratory diagnosis.

On the field, the new clinical forms can be ignored or considered as other pig diseases clinical forms. At the laboratory level, the usual improved techniques for the diagnosis of ASFV can loose presently their efficiency in assessing a quick diagnosis of the subclinical and subacute chronical forms of the disease.

The technique which was usually applied before the presence of the modifications of the virus for the quick diagnosis of the African Swine Fever was based on the presence of direct immunofluorence on homogenates from tissues and organs (Bool, Ordas and Sanchez Botiza 1969). This method requires only 2 to 3 hours. The negative samples are submitted to the inoculation in leucocytes cultures in view of the hemadsorption test.

Presently the virus provokes, during the new clinical forms of the

disease, the formation of high levels of antibodies and consequently a high percentage of pigs died from African Swine Fever contains the virus and the antibodies.

The presence of antibodies at the tissue level or the small titers of virus which can be demonstrated in the subacute or chronical forms lead to the loss of sensitivity of the application of the direct immunofluorescence technique on homogenated for the quick diagnosis of the disease.

In 1970, 76% of the samples were positive to this test. In 1973, only 55% were positive.

Nowadays, the frequency of the suspect samples for ASFV where the virus is blocked by the antibodies needs a new technique for the quick diagnosis of the antibodies extracted from organs usually sent to the diagnostic laboratory.

The new method set up and performed in our laboratory is based on the identification of IgG anti-ASFV extracted from tissues of animals which died from a disease, this by indirect immunofluorescence. The sensitivity of our method raises 80% of the African Swine Fever cases. Presently the search of antibodies is more sensitive for the diagnosis than the identification of the viral antigen in tissues by means of immunofluorescence.

The concomittant use of both tests lead to a great progress : direct immunofluorescence for the viral antigen and indirect immunofluorescence for the identification of tissular antibodies. Recent studies permit the diagnosis of 93.4% of the cases of African Swine Fever in 3 to 4 hours.

Adaptation and survivance of the ASFV in the tissues of ticks

The finding of the virus in ticks (ornithodorus erraticus) during the spanish epizooty (Sanchez Botija, 1963) shows the transmission of African Swine Fever to pigs by this vector. This finding was confirmed in Tanzany by Plowright (1969) with Ornithodorus Moubata present on wild pigs (Phacochoerus, Potamochoerus). The study of this contaminating mechanisms, unknown in Spain by those times, contributed to improve the knowledge on the disease in Africa.

The studies of the survival of the virus in Ornithodorus showed its adaptation in the tissues of this arthropod where he could survive several years as a virus "tanker".

The adaptation and the survival of the virus in the tick during the Spanish epizooty was certainly facilitated because the virus which came in the Iberic peninsul had its natural "tanker" in African ticks.

On the basis of such observations new sanitary control measures against the disease were taken including the measures against the development of this arthropod.

SUMMARY OF THE SESSION

B.R. McAuslan

If we look at the programming of DNA for various viruses we see some differences. Pox viruses show a distinct short phase of DNA synthesis followed by the maturation of virions. While it is said to replicate entirely in the cytoplasm, no-one yet, I believe, has been able to get viral production from enucleated cells. One does get viral DNA and proteins made in such artificially enucleated cells, but not mature virus, so one is not sure of the significance of the role of the nucleus in the replication of cytoplasmic viruses. In fact, I believe that there is some evidence that pox virus DNA may in fact enter the nucleus and perhaps integrate with its DNA. If we look at the nuclear viruses such as Adeno virus and Herpes viruses, their DNA is synthesised over a much longer period of time and virus maturation then follows a few hours behind DNA synthesis. Nevertheless, viral DNA synthesis and viral maturation are proceeding simultaneously through the maturation cycle. If we now shift attention to the icosahedrial cytoplasmic deoxy viruses such as frog virus and presumably the other viruses, these apparently replicate in the cytoplasm like pox virus but the programming of their macromolecular synthesis resembles more the herpes viruses. In fact, these ICDV's are a sort of missing link between pox viruses and herpes viruses, although that's not to employ the phrase in evolutionary sense - perhaps a missing piece of the mosaic rather than a missing link. If one wants to study DNA synthesis per se, then the animal viruses are probably not the best systems for us to work with, although as Dr. Becker has so neatly shown it is feasible to study viral DNA synthesis using the nuclei from infected cells. However, I think what is easiest to approach and what is most interesting among the animal DNA viruses is the number of structural aspects one uncovers when one takes a close look at viral DNA per se. For example, in the Parvo viruses, there is now evidence that there are complementary sequences at the end of the DNA so that it can form a sort of pan handle structure. In the pox viruses from sedimentation data of Berns, it appears that there could be some interesting cross-linking so that it forms a hairpin structure.

If we consider Herpes simplex virus DNA there is the evidence that at least one strand appears to be extensively nicked, but

we don't know the significance of that at this time. Herpes simplex virus DNA is infectious and incidentally, Dr. Bachrach and Adeldinger and co-workers showed some time ago that African swine fever virus DNA is also infectious. It would be interesting to see if other members of the ICDV group have infectious DNA, and this could be done very readily with the frog virus. Frog virus DNA has been reported by Kelly, whose work is in press, to be extensively nicked also, so that one only gets about 10% of the total DNA molecules that come out as intact strands.

Again, we know almost nothing about the significance of all these unusual structural aspects of the various DNA viruses, but I suggest that we certainly should continue a study of the physical chemistry of the DNA of all these virus groups and especially African swine fever virus, where to date, all of the work has been almost exclusively concerned with the epidemiology for obvious practical reasons. Finally, I would like to point out that some of the more complex DNA viruses such as pox and frog virus, adeno virus, perhaps herpes virus, have associated enzymes that appear to be essential components of the virion. At least some of these enzymes are nucleases. Pox virus, for example, has one or two nucleases associated with the particle. Adeno virus has been reported to have an endonuclease associated with the pentons. Frog virus has two endonuclease activities within the particle. Pox virus also has a DNA dependent ATPase within it and frog virus has an ATPase in it. Now, for want of a better idea, one usually says that such enzymes must have some sort of role in repair or re-combination or packaging of DNA or modifying the DNA in some way that it can initiate replication. In fact, we really don't know anything about the function of these DNA associated enzymes and I think that this is one area which will need some continual investigation.

SESSION III

SYNTHESIS OF VIRAL PROTEINS

IN VITRO SYNTHESIS OF VIRAL PROTEINS USING VIRAL mRNA

by
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UTRECHT

INTRODUCTION

Until now we have occupied ourselves mainly with the synthesis of viral nucleic acids. From now on we will focus on the synthesis of viral proteins. Yet, this means in practice first the synthesis of a nucleic acid, the mRNA, which is translated into a protein by the normal machinery of the cell. The strategies which have been used to obtain the translation of the viral genome into proteins are varied. DNA-viruses have a pattern which is rather like that of the normal cell; animal RNA-viruses, however, behave in a more complex way (see Table I).

A first criterion for the classification of RNA-viruses according to their replication mechanism, is the polarity of the viral genome which can be plus (+), minus (-) or double-stranded ($\pm$). We define plus as the polarity of mRNA. Connected with this criterion is the occurrence of a RNA polymerase in the virion, which is obligate in -stranded and double-stranded RNA-viruses. A second criterion is the occurrence of a multipartite genome or in the case of -stranded viruses the synthesis of several mRNA's on a larger genome. A third criterion is the occurrence of post-translational cleavage of viral precursor proteins.

From Table I we see that using these criteria there are 6 different combinations in 7 different structural groups of animal RNA-viruses.

What is the sense of this variety ? If we may be teleological for a moment we perceive several ways which Nature has found to circumvent the use of polycistronic mRNA, the commonly used mechanism in bacteria and their viruses, but unknown in eucaryotic cells. However, research in virology is much more complicated than philosophy. Stringent proofs for the schemes given in Table I, require the use of advanced techniques to assay the information content of mRNA's.

TABLE I

Classification of RNA-viruses according their replication mechanism. The protein cleavage found in reovirus has been ignored, as it is not essential for virus replication.

Virus group	Polarity of viral RNA	RNA polymerase in virion	Multipartite viral genome	Number of mRNA's	Post translational cleavage
picornavirus	+	no	no	one	yes
reovirus	±	yes	yes	more	no
togavirus	+	no	no	more (?)	yes
orthomyxovirus	+ & - (?)	yes	yes	more	no
paramyxovirus	-	yes	no	more	no
rhabdovirus	-	yes	no	more	no
oncornavirus	+	no	no	more (?)	yes

In this paper I will summarize some results of the use of in vitro protein synthesizing systems and demonstrate that this tool is very useful for this purpose. In addition these systems can contribute to the elucidation of regulatory processes connected with virus replication, like the influence of virus infection on the RNA and protein synthesis of the host cell on one side and the working mechanism of interferon on the other side.

The cell-free systems

At this moment there are some standard cell-free systems in use for the assay of putative mRNA's. In general these systems are crude lysates of animal cells. By preincubation under conditions which favour protein synthesis, ribosomes are freed from their endogenous mRNA, thus making them available for the translation of added mRNA's. Then low molecular weight compounds are removed (by dialysis or gel filtration) in order to be able to measure incorporation into protein of added radioactively labelled amino acids. All necessary compounds are added, together with fractions which are supposed to contain mRNA. Experiments of this type are designed to answer the questions of 1) whether there is a stimulation of protein synthesis and - if this is the case - 2) whether the newly formed polypeptides can be identified or correlated with known viral proteins.

Systems derived from rabbit reticulocytes (33, 34, 37, 38), Krebs II ascites cells (3-5, 12, 18, 21, 31, 32, 37, 38, 68, 77), Ehrlich ascites cells (15, 45), L-cells (12, 15, 16, 18, 21, 22, 32, 36, 37), HeLa-cells (15, 37), Chinese hamster ovary cells (37, 38) and Sarcoma 180 cells (27) have been used.

More recently a wheat germ system has been introduced. Wheat germs do not contain polyribosomes and it appeared that a lysate can faithfully translate a number of mRNA's from animals and their viruses giving a very low background incorporation and thus a prominent stimulation of protein-synthesis (14, 38, 51, 53). This facilitates in particular the analysis of the products which are formed.

Before looking at the results which have been obtained I want to discuss two problems in more detail, namely:

1. How can one get mRNA and how much is needed ?

2. How can the polypeptide products formed in vitro be identified ?

Isolation of mRNA

The most direct method is to isolate polyribosomes and dissociate them in order to liberate mRNA. Standard methods are now available to isolate polyribosomes from cells in culture (19). If the approximate molecular weight of a mRNA is known one can make an estimate of the sedimentation coefficient of the polysomes in which it will be present. The number of ribosomes which are attached to a mRNA is dependent on the equilibrium between initiation and elongation (and release) during protein synthesis. About one ribosome is present per 90 nucleotides (i.e. 30,000 daltons) mRNA (23, 71). Moreover for eucaryotic polyribosomes the following relationship exists between the number of ribosomes (n) and the $s_{20,w}$ of the polysomes (S_n) (44):

$$\log S_n = \log 80 + 0.60 \log n$$

When combining these two data we obtain

$$\log S_n = \log 80 + 0.60 \log \frac{MW_{RNA}}{3x10^4}$$

were MW_{RNA} is the molecular weight of the mRNA.

If, for example, the MW_{RNA} of $2.6x10^6$ for poliovirus is inserted into this formula, a maximum $s_{20,w}$ of 1160 can be calculated for the virus specific polysomes. In fact a sedimentation coefficient of 400 S is found early in infection (47). This discripancy could be due to a different ratio between initiation and elongation for viral mRNA as has been found during the late infection of HeLa cells be adenovirus-2 (48).

It is evident from these calculations that some predictions can be made about the position of virus specific polysomes in a density gradient. Recently, several groups of investigators employed ts-mutants, which cannot form nucleocapsids under restrictive conditions for the study of togavirus-specific polysomes (30, 41, 56). We found that there is no need for such an elaborate approach. If one assumes that the 26 S RNA (1.5×10^6 daltons) serves a messenger function in alphavirus infection, a sedimentation coefficient of 840 S can be calculated. Confusion with viral

nucleocapsids sedimenting at about 150 S is therefore not likely to occur. In fact in our hands Semliki forest virus polyribosomes have sediment coefficients of 400 S and more, provided that so called "runn of" of ribosomes from polysomes which would reduce their size is prevented. This can be done by the addition of cycloheximide to cells before lysis which "freezes" the polysomes.

Another way of obtaining mRNA consists of extraction of the whole RNA content of the infected cell with subsequent isolation of the appropriate fractions.

For most RNA-viruses one can easily discriminate between host cell RNA-synthesis and viral RNA-synthesis using actinomycin D. The detection of oncornavirus specific polysomes, however, is not so simple. In this case rather intricate methods like hybridization of DNA transcripts from the virion RNA with polysomal RNA are required (17, 73). From DNA-viruses which do not depress cellular RNA synthesis mRNA has also to be isolated by hybridization with viral DNA. This has been done for simian virus 40 (55).

In some cases it is possible to synthesize the mRNA's *in vitro*, a technique which has been exploited *e.g.* for reoviruses (2, 21, 37, 66) and vacciniavirus (3, 18, 28, 29). From these *in vitro* systems one can obtain large amounts of mRNA. This is convenient as generally at least about 1 µg of viral mRNA is needed to obtain enough incorporation. In most cases this amount is not saturating.

Identification of products

There are several methods which have been used to correlate proteins formed *in vitro* to viral proteins. First coelectrophoresis can be used as a indication for the identity of two proteins. A more stringent proof however should be given by analysis of tryptic digests. Also immunoprecipitation can be used (34, 63, 75).

It has appeared that in cell-free systems no post-translational proteolytic cleavage occurs (5, 21, 31, 36, 37). On this place I should mention another method which can be used to assay mRNA: the injection of mRNA into *Xenopus laevis* oöcytes. In the case of encephalomyocarditis

virus mRNA all the normal cleavage products were formed (35).

Survey of results obtained in cell-free systems

I should like to give a short review on the kind of information one can obtain using these in vitro systems.

DNA-viruses

1. Poxvirus

RNA can be obtained by in vitro incubation of vaccinia virus. It is complementary to a part of the viral DNA and it has properties similar to those of early mRNA (28, 29). Its messenger function has been directly proven by the stimulation of protein synthesis which it exerts in vitro (3, 18). The products, however, have not yet been compared with authentic viral coded proteins.

2. Papovaviruses

Recently the translation in a wheat germ system of simian virus 40 mRNA, purified by oligo (dT)-cellulose chromatography from infected cells, has been described. A polypeptide which was in size and tryptic peptide composition identical to the major capsid protein of the virion was synthesized (51). The mRNA could be further purified by hybridization to the viral DNA. Several other peptides which were also formed have to be analyzed into more detail.

RNA-viruses

1. Picornaviruses

There is now ample evidence that the viral RNA (molecular weight 2.6×10^6) has one initiation site for protein synthesis. The translation product, a "polyprotein", is processed by proteolytic cleavage to smaller proteins. The stable fragments are produced in equimolar amounts (8, 13, 26, 52). Detailed studies of the products of cell-free protein synthesis have clearly shown indeed that there is only one initiation site for protein synthesis (45, 68). Moreover, larger proteins than the capsid proteins, containing the same tryptic peptides as the capsid proteins, were produced (4, 12, 15). However, no translation product equivalent to more than 60% of the viral genome could be found. Several discrete proteins are formed which all initiate at the beginning of the genome, but which are

prematurely terminated. No proteolytic cleavage was observed (5, 31). As has been mentioned before all normal cleavage products has been found in Xenopus oöcytes (35). An interesting observation was that an initiation factor from Krebs II ascites cells is necessary for protein synthesis directed by encephalomyocarditis RNA but not by globin RNA (77). This might be a factor which determines the ability of cells to support virus replication.

2. Reoviruses

The study of the functioning of reovirus mRNA's has been greatly facilitated by the possibility to prepare in vitro the messengers for all ten pieces of the double-stranded genome (2, 66). Direct evidence that the RNA transcribed in vitro is indeed functional mRNA has come from tests in cell-free systems (21, 37). Proteins were formed which co-electrophoresed with the reovirus specific proteins. When the mRNA's were fractionated, medium sized and short proteins were formed after addition of medium sized and short messengers respectively (21). No proteolytic cleavage of the μ_1 protein to μ_2 occured (21, 36, 37).

3. Togaviruses

This group will be discussed in the last section of this paper.

4. Orthomyxoviruses

The virion contains an RNA-polymerase (10, 46, 67). Strands of the same and of reversed polarity have been detected in viral polysomes (43, 50). In an Escherichia coli cell-free system the RNA of influenza virus was translated into a polypeptide antigenetically identical with the viral ribonucleoprotein (61, 62). On the other hand no messenger function could be found in an eucaryotic system, whereas in this system the matrix protein was synthesized after addition of RNA from infected cells (34). If indeed some viral RNA strands are + and others - it should be possible to determine in this way the polarity of all strands.

5. Paramyxoviruses

These viruses have -stranded genomes. The virions contain a RNA-polymerase (24, 54, 55, 72). From infected cells polysomes have been isolated which contain mRNA which can hybridize with the viral RNA (6). Newcastle disease virus codes for at least seven mRNA's having a mole-

cular weight from 0.55 to 1.53×10^6 (11). Until now these species have not been tested separately. Unfractionated preparations were able to direct the synthesis of a protein immunologically identical to one of the virion polypeptides (33).

6. Rhabdoviruses

Rhabdoviruses contain a single molecule of RNA that is complementary to the mRNA's (25). A RNA-polymerase is present in the virion (1). By sucrose density gradient centrifugation the mRNA's could be separated into a homogeneous 28 S species and a heterogeneous group, containing at least 3 species, of 13-15 S (25, 57). Recently, it could be demonstrated that the 28 S species *in vitro* directs the synthesis of one of the viral proteins, the L-protein. In agreement with the heterogeneity of the 13-15 S RNA, these code for 3 or 4 other viral proteins (38).

7. Oncornaviruses

Oncornaviruses contain a 60-70 S genome which is not multipartite in the same sense as the genomes of reoviruses and orthomyxoviruses. It can be split, however, after denaturation into smaller RNA's (see Ref. 9 for a discussion). Evidence for a +stranded genome is the ability of mRNA from infected cells to hybridize with DNA complementary to the viral RNA (17, 73). The 35 S messenger seems to code for a large precursor protein which is split into the viral proteins (75). Until now a poor translation has been obtained using eucaryotic cell-free systems (42, 74). Surprisingly, more success was obtained in an *Escherichia coli* system in which it was possible to translate the RNA of several oncornaviruses before and after denaturation of the RNA (20, 63).

Conclusion and prospects

From this survey it is clear that a considerable amount of information has been obtained with the aid of cell-free systems. There are some difficulties in translating very large genomes until the end. However, conclusive results are in this case obtained by comparison of tryptic peptides. When better methods have been developed to separate RNA's of about the same size, it will be possible to correlate all proteins of many virus groups to mRNA. Moreover, more light will be shed on the controversial results which have been obtained with orthomyxoviruses. Interesting questions like the specificity of cellular enzymes for virus replication (77)

and the working mechanism of interferon await an answer. Evidence for an effect of interferon on the level of translation in vitro has been obtained by several groups (16, 22, 32). An exact mechanism, however, has not yet been established.

Finally, we can ask ourselves the question: what is the potential usefulness of these techniques for work with the viruses of hog cholera or African swine fever ? Dr. McAuslan later at this symposium will give us the answer for African swine fever virus (or rather for iridoviruses in general). In the first paper at this meeting given by Prof. Horzinek, we have heard that togaviruses (to which hog cholera virus belongs) seem to use mRNA molecules that are smaller than the whole genome which has a molecular weight of about 4.4×10^6 (64).

From virus specific polysomes of togavirus infected cells several different types of mRNA have been isolated (30, 41, 56). The major species is a 26 S RNA, having a molecular weight of 1.5×10^6. This RNA is large enough to code for all structural proteins, which have been demonstrated to origin by proteolytic cleavage from a large precursor protein (7, 39, 40, 49, 58-60, 65, 69, 70, 76). A possible model has been given recently by Morser & Burke (40). They have summarized a number of findings in a scheme which is reproduced in Fig. 1.
The scheme is partly based on pulse chase experiments of proteins in virus infected cells. A strict proof, however, requires the comparison of tryptic digests as has been done by the author of the following paper, Dr.Schlesinger, who in fact has done the work to prove all but the two upper lines of the scheme (58-60).

Whether the 26 S species is really the mRNA for the precursor of the structural proteins and what the role of the other RNA's is, is one of the questions which can be resolved by an in vitro system. In our laboratory we try to answer this question by examining the products of cell-free synthesis of Semliki forest virus RNA's.

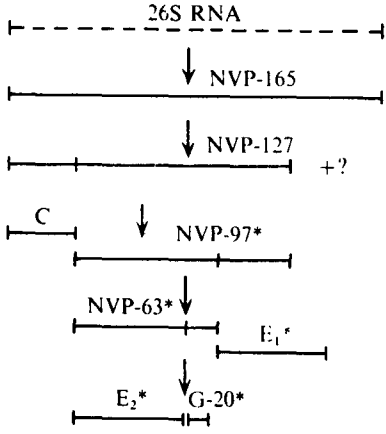

Fig. 1 : A possible cleavage mechanism for the production of the virus-specified polypeptides of togaviruses. C is the capsid protein. E_1 and E_2 are the two envelope proteins. NVP = non-virion polypeptide. C-20 and the other starred polypeptides are glycosylated. The direction of translation is from left to right (Taken from Ref. 40).

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DISCUSSION

Dr. McAuslan : Could you just briefly tell us a few of the features of the best system available now for translation of proteins ? I think we mentioned before that this wheat germ system is particularly a good one. What are currently the best systems to use ?

Dr. Van der Zeijst :There are two aspects. The first is that we want to get a stimulation which is as large as possible and therefore the wheat germ system seems to be fairly appropriate. On the other hand, we want to be sure to have got a system in which RNA can be translated.
There have been some results from Lodish group about reovirus messenger RNA's which were translated in several systems. The system in which most of the RNAs were translated is the reticulocyte system which has, on the other hand, a rather high background which is reduced by the addition of exogenous messenger RNA. The results which have recently been obtained with simian virus 40 indicate that in this case the wheat germ is the system of choice. However, Dr. Schlesinger has got a very clear result using the reticulocyte system. It is necessary to have all systems in the lab. - at least, most of them.

Dr. M. Schlesinger :Let me just make a comment about the wheat germ system which we tried to use without success. I can report that the people at MIT in the laboratory of Harvey Lodish informed me that they had also heard from a number of laboratories throughout the country, in fact, throughout the world, that had been unsuccessful in using the wheat germ system and apparently the trick is that in trying to obtain a wheat germ system that has good capacity for synthesizing proteins in vitro, one has to disrupt the wheat germ in a very gentle way and most people grind the wheat germ very vigorously with sand. Routinely, that leads to inactive extracts - it certainly did in our laboratory. When I talked to the Lodish group they said that the problem people had was that in fact, they weren't making the extracts properly and that it wasn't so critical - that it's true that some sources of wheat germ are better, but that the main problem was how you made the extracts. I was told that there was one laboratory at Cold Spring Harbour that had sent all over the world for 30 different kinds of wheat germ and when they finally learned how to make the extract, they all were essentially the same.

Dr. Scholtissek : Dr. Schlesinger, you just told us how not to treat the wheat germ but what is the trick ?

Dr. M. Schlesinger :We've not had success in our laboratory but I was told that one has to very gently break open the cells - the most gentle grinding possible.

Dr. Barry : I understand that in some systems, the presence of any double-stranded RNA is inhibitory. You may have mentioned this early in your talk - I didn't hear it. Could you comment perhaps on the importance and relative sensitivity of systems to the presence of double-stranded RNA ?

Dr. Van der Zeijst : In fact, I didn't mention experiments because of lack of time but there have been some experiments about inhibition of single-stranded systems by double-stranded RNA. However, no comparisons have been made to my knowledge between the translation of endogenous RNA and translations of viral RNA. These are the experiments which have yet to be published.

Note added : There is, however, one report (H.D. Robertson and M.B. Mathew (1973) Proc. Nat. Acad. Sci. U.S.A. 70, 225-229) in which such a comparison has been made. No difference in sensitivity for double-stranded RNA of the translation of mouse globin RNA and encephalomyocarditis virus RNA could be found.

FORMATION OF RNA AND PROTEIN IN CELLS INFECTED WITH STANDARD AND DEFECTIVE SINDBIS VIRUS

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SUMMARY

Sindbis virus is a relatively simple enveloped virus. The RNA of the virion is single stranded with a molecular weight of 4 X 10^6 daltons; it is infectious and can be translated in vitro. The major species of viral mRNA found in infected cells has a molecular weight of 1.6 X 10^6 daltons (26S) and contains only one-third the nucleotide sequences of the virion RNA. The 26S RNA is believed to code for the three viral structural proteins. Two of these proteins (E1 and E2) are glycoproteins and are located on the surface of the virion : the third protein is found as part of the nucleocapsid. The capsid protein and E1 accumulate in chicken embryo fibroblasts or BHK cells infected with Sindbis virus. E2, itself, does not accumulate in these cells; instead a larger molecular weight precursor (PE2) accumulates and is slowly converted to E2. The conversion of PE2 to E2 includes changes in carbohydrate content.

When Sindbis virus is passaged at high multiplicity on BHK cells, defective-interfering (DI) particles accumulate after 8 or 9 passages. Furthermore, coinfection of cells with early and late passage virus causes a marked reduction in viral yield. It has not been possible to separate DI particles from standard virions, but RNA isolated from late passages is heterogeneous compared to early passage virion RNA. BHK cells infected with late passage virus have a number of properties distinct from cells infected with early passage virus.

One of the most striking is the appearance of a new species of RNA of mol. wt. 9 X 10^5 daltons (20S) accompanied by a decrease in 26 S RNA. Nucleic acid hybridization experiments have demonstrated that the 20 S RNA is related to 26S RNA. It differs from 26S RNA, however, in its ability to function as a messenger. 20 S RNA is not found on polyribosomes *in vivo* and is not translated *in vitro*. Cells infected with late passage Sindbis virus show a marked inhibition in the formation of intracellular nucleocapsids. This inhibition is correlated with a decrease in the synthesis of capsid protein.

INTRODUCTION

Togaviruses are a relatively new group in the taxonomy of animal viruses. Members of this group are classified together based on similarity in size and structure; they are quite diverse in their disease potential for man and animals. A number of these viruses are extremely pathogenic and for this reason have not been amenable to detailed examination. Sindbis virus and Semliki Forest virus are two members of the group A togaviruses that show minimal hazard to man. Studies with these viruses have provided valuable information on the properties of togaviruses. We have been investigating the structure and replication of Sindbis virus. I will discuss two aspects of these studies : the processing of the viral proteins, and the effects of defective-interfering particles on viral replication in BHK cells.

The RNA of Sindbis virus is single stranded with a molecular weight of about 4 X 10^6 daltons (49S) (1). The virion RNA is infectious - a property that distinguishes the togavirus group from other RNA enveloped viruses. The major species of viral RNA synthesized in infected cells is not virion RNA, but an RNA 26S of molecular weight 1.6 X 10^6 daltons. This RNA has the same polarity as virion RNA and contains only one-third the nucleotide sequences of virion RNA (1). 26S RNA in found on polyribosomes in infected cells (2, 3) and can be translated *in vitro* (4). It is believed to be the messenger RNA for the structural proteins of the virion.

Sindbis virions contain three distinct proteins (5). These proteins were identified after disruption of the virus and separation of the polypeptides by polyacrymalide gel electrophoresis (Fig. 1). Two of the proteins (E1 and E2) are glycoproteins with apparent molecular weights of about 50,000 daltons and are located on the surface of the virion (5, 6). The relationship of E1 to E2 in the virion is not known, however E2 may be located external to E1. In experiments reported by Sefton, et al. (7) the intact virion was labeled with iodine using lactoperoxidase; a procedure that should label only those proteins exposed to the external environment (8, 9). Only E1 and E2 were iodinated, and E2 was labeled more extensively than E1. When the virion was disrupted before treatment with lactoperoxidase the two glycoproteins were labeled equally and the internal capsid protein (c) was also iodinated. The latter protein is associated with the RNA in the nucleocapsid. In the virion a lipid bilayer separates the nucleocapsid from the envelope proteins (10). The lipid and envelope of the virion are acquired as the nucleocapsid buds from the plasma membrane.

FORMATION OF SINDBIS VIRUS PROTEINS

There are a number of experiments to suggest that the discrete virion polypeptides are formed by post-translational cleavage of large molecular weight precursors in a manner analogous to that established for picornaviral proteins (11 - 13). Although E1 and the capsid protein accumulate in infected cells, E2 is not detected in these cells after a short pulse of radioactive amino acids. Instead a significant proportion of the radioactivity is found in a protein with a molecular weight of approximately 60,000 daltons (Fig. 1). This observation led us to suggest that E2 was formed by cleavage of the larger molecular weight protein and that the conversion was an essential and rate-limiting step in the budding process (5). We have obtained evidence to support the first part of this hypothesis from kinetic studies and by a comparison of tryptic peptides of the proteins. Figures 2 and 3 show the results of an experiment in which chicken embryo fibroblasts were labeled for 20 min with a mixture of ^{14}C-labeled amino acids five hours after they had been infected with Sindbis virus.

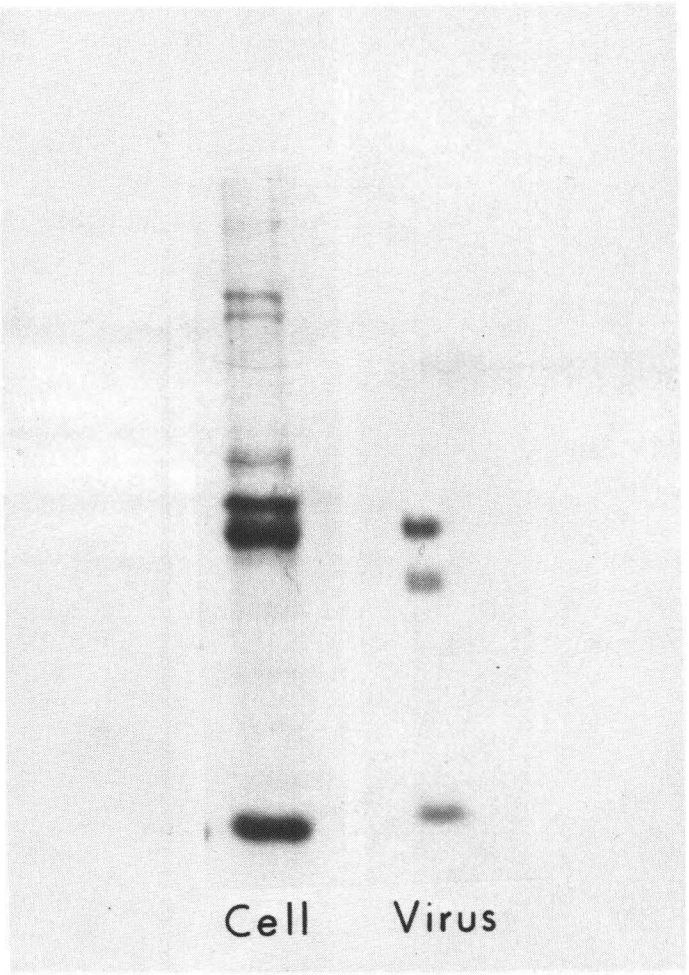

Figure 1 : Autoradiogram of an extract from ^{14}C-labeled cells infected with Sindbis virus and of ^{35}S-labeled purified virus electrophoresed in a SDS-acrylamide slab gel. Chick embryo cells, infected with virus for 6 hours, were labeled with a mixture of ^{14}C-amino acids for 10 Minutes. Details of the procedures have been described (5).

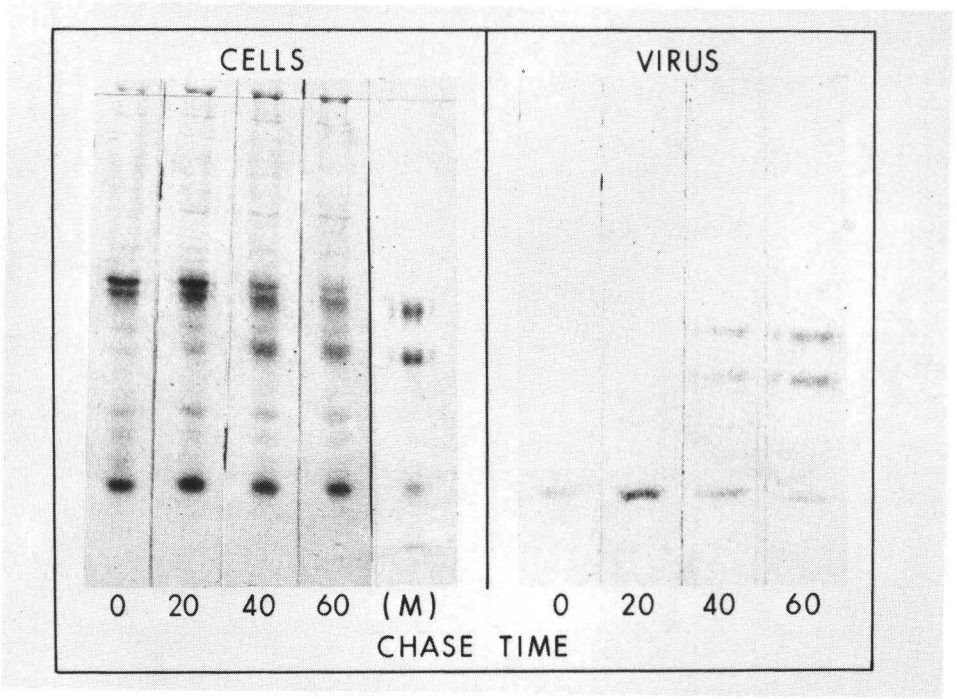

Figure 2 : Autoradiograms of ^{14}C-labeled viral proteins at several periods after a 20-min pulse of radioactive amino acids. The details of the pulse-chase experiment, slab gel electrophoresis, and autoradiography, have been described (14).

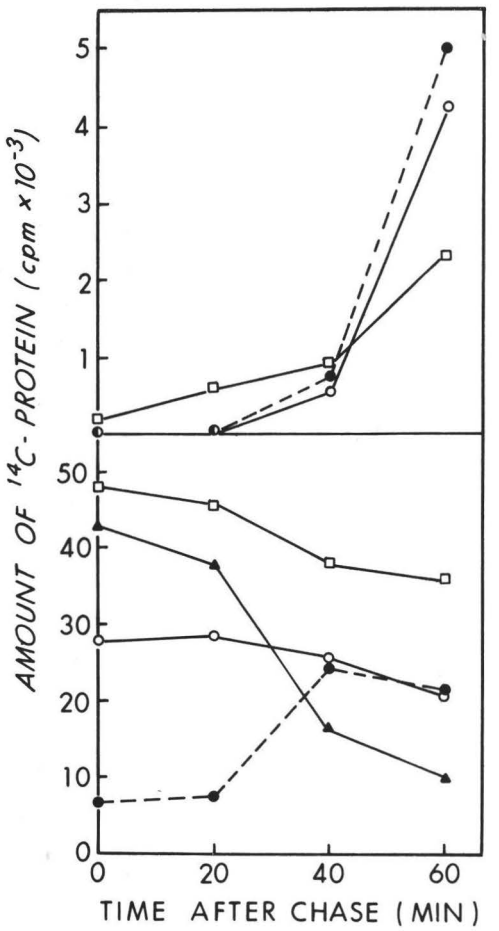

Figure 3 :

The fate of labeled virus-specific proteins after a 20-min pulse of ^{14}C-amino acids. The amount of radioactivity in each protein at several chase times was quantitated from the autoradiograms shown in Fig. 2. Top, Data obtained for the proteins in the virion; bottom, data obtained for the proteins in the infected cells. After the 20-min pulse, the total acid-insoluble counts per minute in the cells were 1.37 X 10^5. They decreased to 1.2 X 10^5, 1.05 X 10^5 and 0.9 X 10^5 at 20 min, 40 min, and 60 min, respectively, during the chase. The total acid-insoluble counts per minute recovered in the virus after purification by sucrose gradient centrifugation were 234 after the 20-min pulse. They increased to 600, 2.330 and 8.950, respectively, at the 20-, 40-, and 60-min chase time. Capsid protein, □——□; E1, O——O; E2, ●——●; the putative precursor to E2 ▲——▲ .

The radioactivity was then chased by the addition of an excess of unlabeled amino acids. After the 20 min pulse most of the radioactive protein was represented by the capsid proteins, E1 and the putative precursor to E2, PE2. During the chase period the decrease in isotope in PE2 was accompanied by an increase in E2. The amount of radioactivity detected in the purified virions increased during the chase period. In this time interval, however, only a small fraction of the total radioactivity appeared in virions.

Infected cells were also labeled with ^{14}C-arginine and the labeled proteins purified by electrophoresis in polyacrylamide gels (14). The fingerprints of the tryptic peptides obtained from the different proteins proved that PE2 and E2 were closely related and that E1 was distinct (Fig. 4).

Subsequent studies by Sefton et al. have provided evidence to support the hypothesis that the conversion of PE2 to E2 is an important step in virion formation. They analyzed the carbohydrate content of PE2 and E2 and found that the conversion involves the addition of galactose and fucose as well as the loss of mannose (15). Furthermore, iodination of infected cells using lactoperoxidase labeled both E1 and E2 but not PE2 (7). Thus, when PE2 is converted to E2, it becomes more exposed to the outside surface of the cell.

A larger molecular weight viral protein has been detected in BHK cells ((16) and Fig. 5) but not in chicken embryo fibroblasts. We established the relationship of this protein (B1) to the envelope proteins by analyzing the ^{14}C-arginine-labeled tryptic peptides (17). Although B1 is metabolically unstable its role as an essential intermediate in the formation of virion proteins has not yet been determined.

It has not been possible to accumulate larger molecular weight viral proteins under normal conditions of Sindbis virus infection even by using very short pulses of radioactive amino acids or by the addition of amino acid analogues (18).

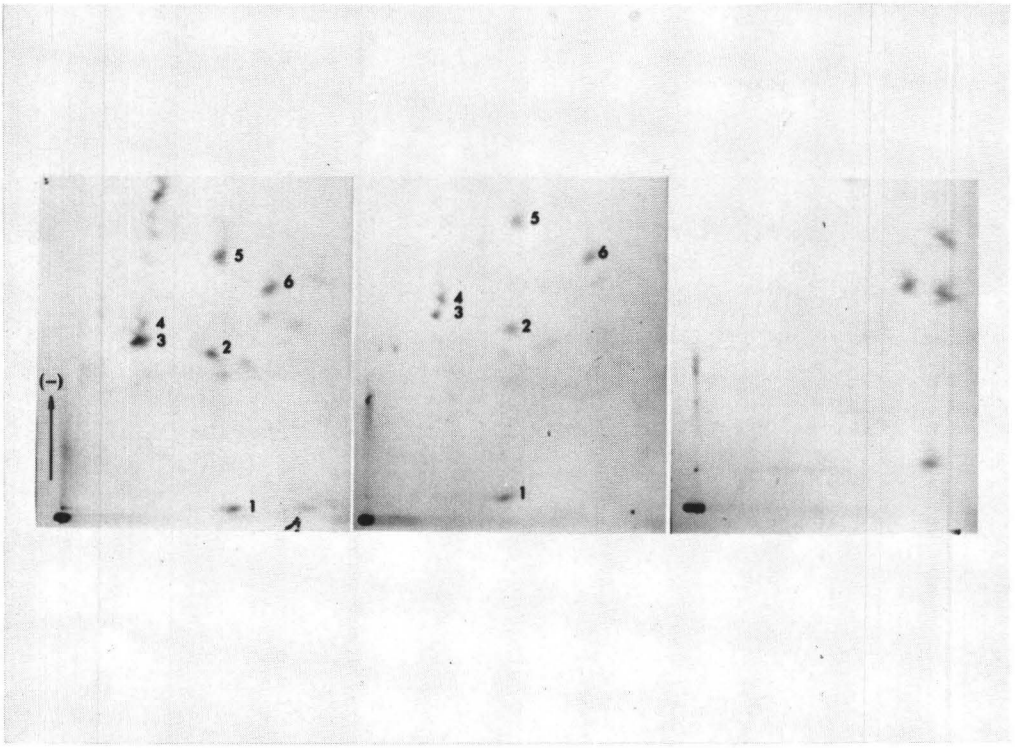

Figure 4 : Autoradiograms of tryptic-peptide fingerprints. ^{14}C-arginine-labeled proteins were purified on SDS-polyacrylamide gels prior to digestion with trypsin. The fingerprint of protein E1 is on the right, E2 in the center, and the proposed precursor on the left.

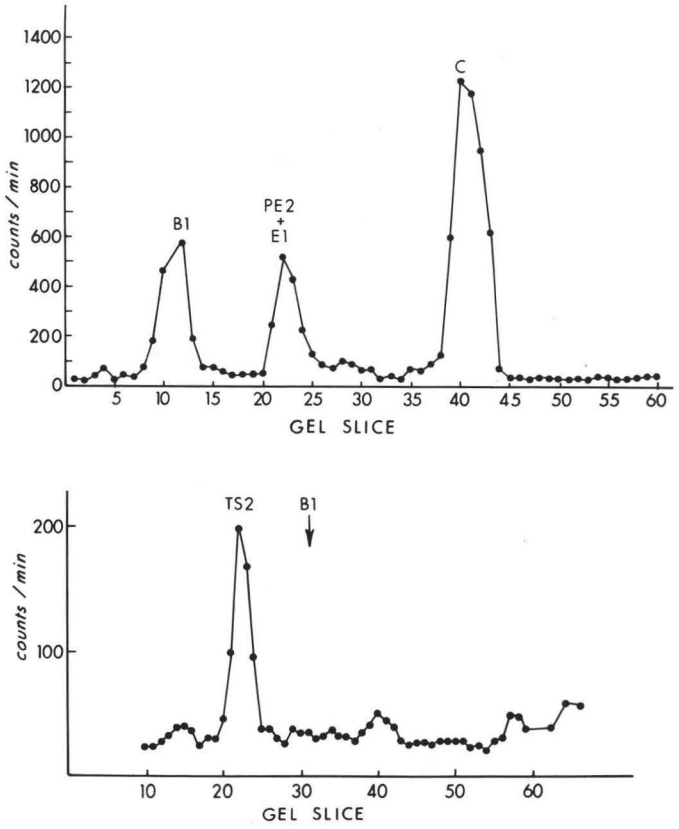

Figure 5 : SDS-acrylamide gel electrophoretic patterns of viral proteins from infected cells. The bottom panel shows the accumulation of a large molecular weight protein when chicken embryo fibroblasts were infected with the ts-2 mutant of Sindbis virus at non-permissive temperature (17). The gel was 7.5% acrylamide and the arrow indicates the mobility of B1 run in a parallel gel. The top panel is the pattern of viral proteins on a 10% polyacrylamide gel from BHK cells infected with Sindbis virus (17).

Snyder and Sreevalsan have reported that high molecular weight proteins accumulate in aged chicken embryo fibroblasts infected with Sindbis virus (19). The strongest evidence that there may be a single site for the initiation of protein synthesis comes from results obtained with a particular class of temperature-sensitive (ts) mutants. At non-permissive temperature these mutants accumulate a protein (ts-2 protein) of molecular weight greater than 130,000 at the expense of the virion proteins ((16, 18) and Fig 5). Our analyses of the ^{14}C-arginine labeled tryptic peptides from the ts-2 protein established that it contained peptides of the capsid protein as well as those of the envelope proteins (17). It has been difficult to reconcile the results with the ts mutant with the fact that the amount of capsid protein found in infected cells always exceeds the amount of envelope proteins by at least three-fold. The possibility must be considered that the single initiation site is a peculiarity associated with the temperature-sensitive mutation. The existence of the ts-2 protein, however, proves that the capsid protein can be covalently linked to the envelope proteins; therefore, these proteins must be translated from the same mRNA. Studies on the in vitro translation of 26S RNA have established that this RNA codes for the capsid protein (4); consequently, it must also code for the envelope proteins.

DEFECTIVE INTERFERING PASSAGES OF SINDBIS VIRUS

The appearance of defective-interfering (DI) particles is now a wellrecognized phenomenon associated with high multiplicity passaging of many different viruses in tissue culture (20). In those examples where DI particles have been well-characterized, they have been shown to contain less nucleic acid than the standard virion. One of the most interesting features of these defective particles is their ability to interfere with the replication of homologous standard virus; they show little or no effect on heterologous virus.

We reported several years that when Sindbis virus was passaged at high multiplicity in BHK cells, D1 particles accumulated after 8 or 9 passages (21). The presence of these particles was readily detected by their ability to inhibit the replication of early passage virus (table 1). We have not been able to separate D1 particles from standard virions (22) and this has made their characterization difficult. Preparations of purified Sindbis virus that contain D1 particles have a decreased PFU to CPM ratio compared with standard virus (table 2). This finding indicated that D1 particles must contribute significantly to the mass (radioactivity) of purified virions. RNA isolated from standard virions contained a single peak of radioactivity corresponding to the molecular weight of 4×10^6 daltons reported for virion RNA, but the RNA obtained from D1 passages was always heterogeneous (22). The degree of heterogeneity was variable but there did appear to be a correlation between the amount of RNA found outside the peak of virion RNA and the ability of the preparation to interfere with the yield of standard virus (22). We do not yet know the reason why the viral RNA is heterogeneous. It is possible that the defective particle population is heterogeneous or that some of the particles are unstable and the small pieces of RNA represent degraded virion RNA.

Infection of BHK cells with late passage virus alone or coinfection with late and early passages led to a marked drop in virus production (tables 1 and 2). We observed, however, that cells from such infections incorporated amounts of ^{3}H-uridine into RNA that were completely unrelated to the degree of inhibition of virus production. In an experiment in which the virus yield was reduced by about 90 per cent there was no effect on the rate of RNA synthesis (fig. 6). Despite the large accumulation of RNA very little of the RNA was encapsidated in cells infected with D1 particles. Figure 7 presents a sucrose gradient profile of the distribution of viral-specific RNA in cell extracts treated with 0.002 M EDTA to dissociate polyribosomes and permit more clear visualization of the nucleocapsid.

Table 1

Inhibition of Virus Yield by Defective Particles

Passage added (MOI)[a]	Yield PFU/ml x 10^8
6 (2.3)	8.7
(9.2)	10
(23)	13
(92)	12
6 (9.2) + 8 (2.5)	2.1
(6.2)	1.3
(25)	0.52
6 (9.2) + 9 (.34)	1.5
(.85)	0.45
(3.4)	0.25
8 (2.5)	0.87
(6.2)	0.65
(25)	0.4
9 (0.34)	0.37
(0.85)	0.27
(3.4)	0.17

a) Samples of virus from the passages indicated were added in a final volume of 0.4 ml to monolayers of 10^7 BHK cells. After 1 hr adsorption at 37°, 7.5 ml of Eagle's medium plus 6% fetal calf serum were added to each dish. The media were harvested 11 hr later.

Table 2

Virus yields, radioactivity and PFU/CPM ratios for early and late passage infections. *

Passage	PFU/ml x 10^9	CPM/ml	PFU/CPM x 10^5
2	30	180,000	1.7
8	0.6	24,300	0.25

* Cells were labeled with ^{3}H uridine (24.3 Ci/mM, 50 μc/ml) between 5 and 10 h postinfection. Labeled virus produced during this interval was purified through a sucrose gradient.

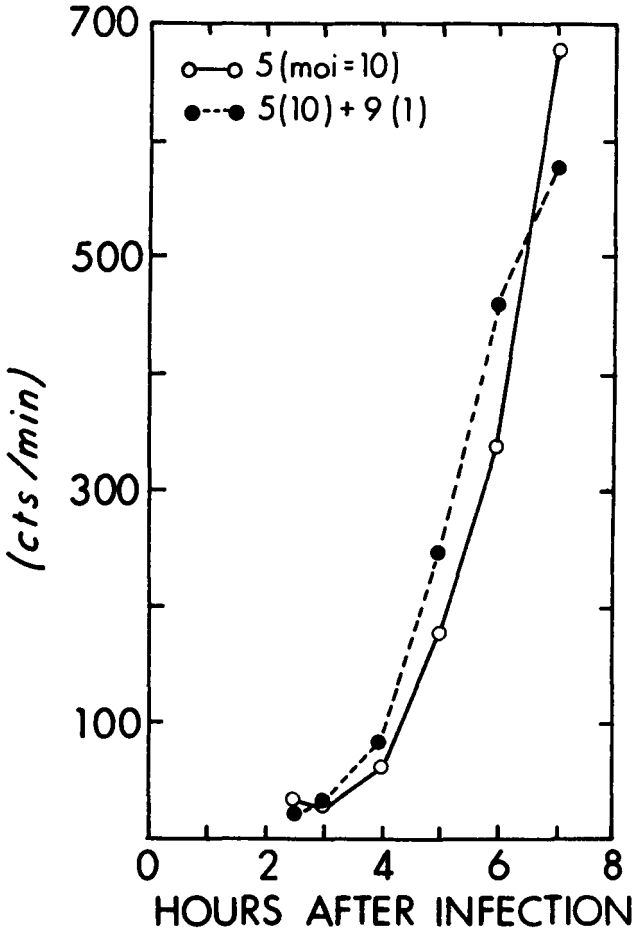

Figure 6 : Comparison of the kinetics of ^{3}H-uridine incorporation following infection with passage 5 (MOI = 10) or coinfection with passages 5 (MOI = 10) and 9 (MOI = 1). ^{3}H-uridine (1 μCi/ml, 2 x 10^{-5}M) was added 2 hours after infection. Actinomycin D (1 μg/ml) was present from the time of infection.

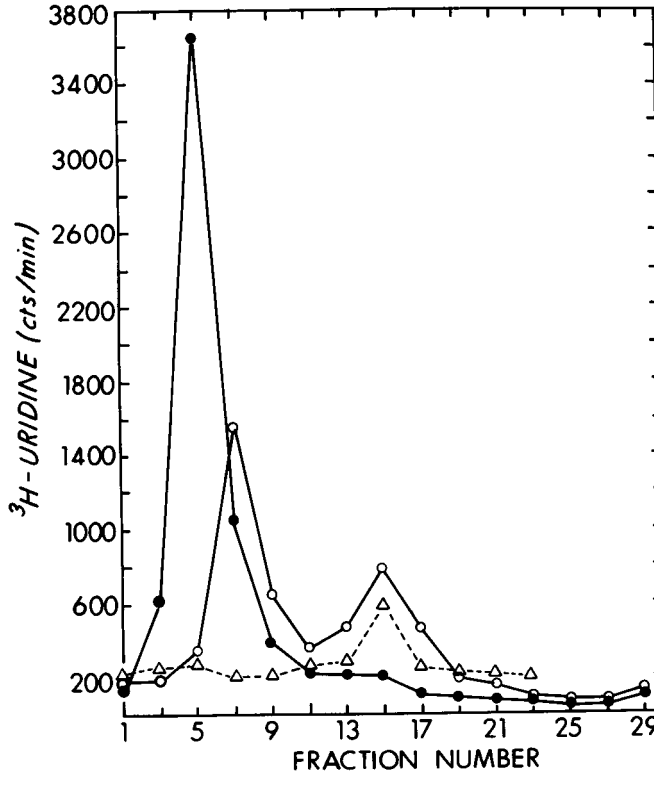

Figure 7 : Comparison of the distribution of viral-specific RNA in extracts prepared from cells infected with passage 5 or coinfected with passages 5 and 9 under interference conditions. Cells were labeled with ^{3}H uridine between 1 and 12 h after infection in the presence of 0.25μg/ml Act D. Extracts were prepared and a portion was layered onto a 15-30% sucrose gradient and centrifuged for 2 h at 34,000 rpm. Passage 5 (—O—); passage 5 + 9 (—●—); ^{14}C amino acid labeled viral nucleocapsid (Δ).

^{14}C-Amino acid-labeled viral nucleocapsid obtained by Nonidet P40 disruption of purified standard virus was run concurrently to obtain more certain identification of the nucleocapsid. In the cells infected with standard virus about 40% of the RNA was present in structures sedimenting with viral nucleocapsid. When cells were coinfected with passages 5 and 9 a maximum of 8% of the RNA was in the nucleocapsid region.

In many experiments we did observe a decrease in viral RNA synthesis, but it was never as extensive as the decrease in virus yield. Differences in the amount of viral RNA synthesized were not as striking as the difference in the species of RNA (Fig. 8). When cells were infected with standard virus, the main fraction of RNA had a molecular weight of about 1.6×10^6 daltons (26S). The larger molecular weight species of RNA corresponded to virion RNA (49S). When cells were infected with late passage virus, the peak of 26S RNA was greatly diminished, and was replaced by a new peak of RNA with a molecular weight of 0.86×10^6 daltons. This RNA is probably identical to the 20S RNA species reported by Eaton and Faulkner (23).

We have determined that this new species of RNA (20S) is related to 26S RNA by sequence homology. 20S RNA was able to compete with ^{32}p-labeled 26S RNA in hybridization experiments, but only to the extent of 50 per cent (Fig. 9). This is the expected result if the defective RNA is homogeneous at it is only half the molecular weight of 26S RNA.

Cells infected with late passage virus also showed differences in viral-specific proteins. The amount of capsid protein synthesized by these cells relative to PE2 and E1 was less than that produced by cells infected with standard virus (Fig. 10). The finding of a decreased amount of capsid protein in cells infected with both standard and defective virus provides an explanation for the reduced levels of assembled nucleocapsids in these cells.

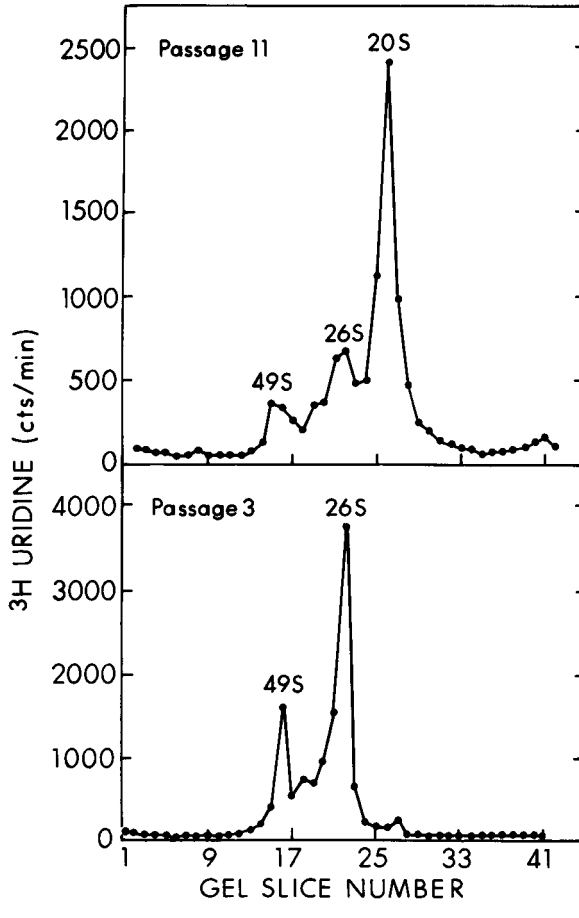

Figure 8 : Comparison of the viral specific, intracellular RNA species synthesized in cells infected with early or late passage virus by polyacrylamide gel electrophoresis.

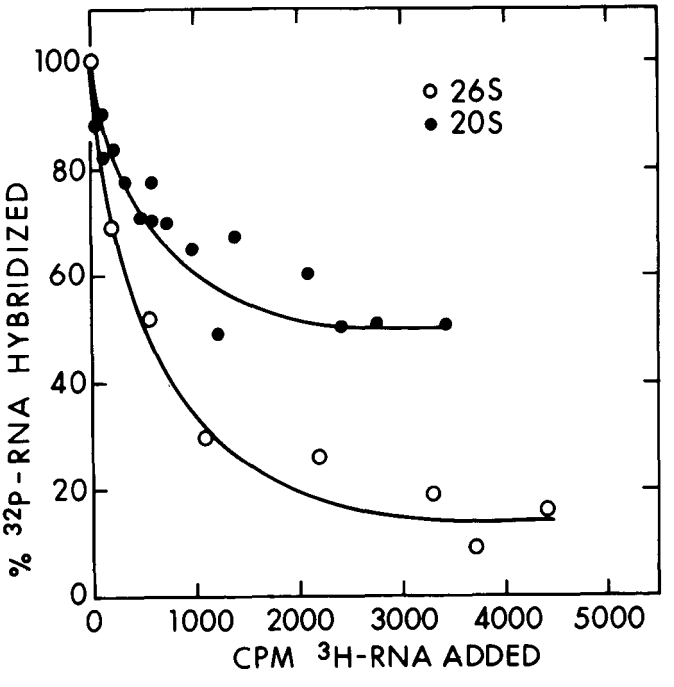

Figure 9 :

Determination of the degree of sequence homology between 26S and 20S RNA by competition hybridization. Hybridization was performed in sealed capillary tubes in a volume of 50μ liters of 4 x SSC (SSC = 0.15 M NaCl, 0.015 M sodium citrate). Each sample contained unlabeled viral-specific dsRNA (2.5μg), 500 counts/min of ^{32}P labeled 26S RNA, and increasing amounts of ^{3}H-uridine labeled 26S (—o—) or 20S (—•—) RNA. Approximately 0.8μ liters of diethylpyrocarbonate was present in each annealing mixture. Samples were heated for 3 minutes in an ethylene glycol bath at 110-120°C. Annealing was carried out at 68°C for 17 hours. Following annealing, samples were either immediately TCA precipitated or incubated with 40μg/ml ribonuclease A in 2 x SSC for 30 minutes at 37°C and then TCA precipitated.

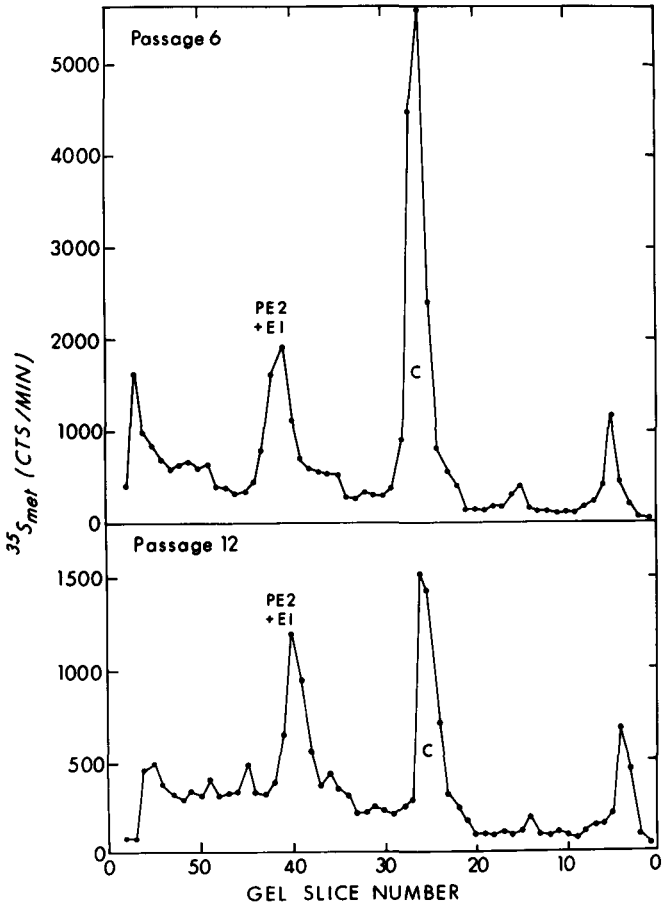

Figure 10 : Comparison of the viral specific proteins synthesized in cells infected with passage 5 and passage 11 virus by polyacrylamide gel electrophoresis. (For this experiment, passage 5 virus was first passaged at low multiplicity to dilute out any DI particles that might be present this early in passaging). Cells were pulse labeled with ^{35}S met ($10^{-5}M$) for 40 minutes at 5 hours after infection and extracts were prepared with Nonidet P40.

We have also observed the presence of a protein migrating between B1 and PE2 in extracts of cells infected with late passage virus, but not with standard virus (Fig. 11). This protein was stable in a pulse-chase experiment although the metabolic instability of B1 was apparent. It is tempting to suggest that this new protein might account for the decrease in capsid protein; on a quantitative basis, however it does not compensate for the loss of counts in the capsid protein. We have not fingerprinted this protein to determine its relation to the capsid protein.

In our initial studies we considered that D1 particles of Sindbis virus would be analogous to those described for poliovirus (24, 25). We suspected that the 20S RNA was a deleted form of 26S RNA, that it was deleted in those sequences that coded for capsid protein, and that it might code for the new polypeptide associated with late passage infections. Although 20S RNA does have 50 per cent of the nucleotide sequences of 26S RNA and contains PolyA (26) it apparently does function as an effective viral mRNA. Very little, if any, 20S RNA has been found on polysomes of infected cells. A comparison of the polysome profiles from cells infected with early and late passage virus is shown in Fig. 12. When cells were infected with passage 3 virus almost all of the viral RNA was found in the heavy region of the gradient (peak II). In contrast, when cells were infected with passage 9 or passage 11 virus the labeled RNA shifted toward the top of the gradient (peak I).

RNA was extracted from the two regions of the gradients and was analyzed by polyacrylamide gel electrophoresis (Figs. 13 and 14). Peak II from the gradient of passage 3-infected cells contained the two expected peaks of RNA — 26S viral mRNA and 49S virion RNA. The latter was most probably present in nucleocapsids which sediment at 140S. 49S and 26S RNA were also detected in the heavy polysome region of both passage 9- and passage 11- infected cells. In passage 9- infected cells 20S RNA represented 41 per cent of the total ^{3}H-uridine-labeled RNA but none of it was found in this region of the gradient.

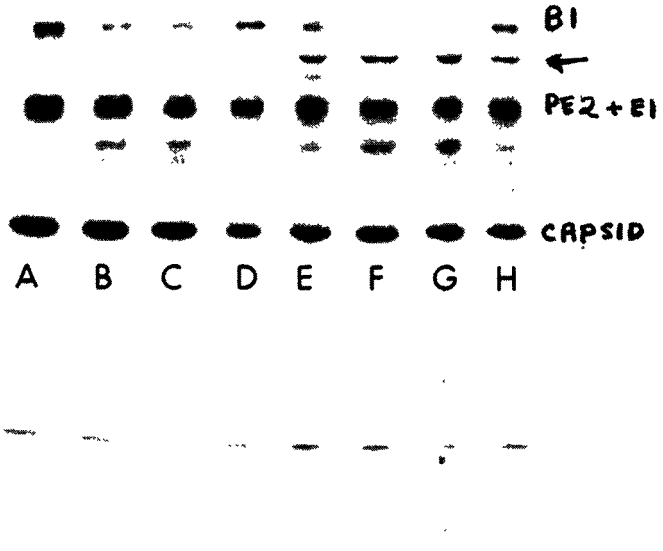

Figure 11 : Autoragiograms of ^{35}S met-labeled intracellular viral proteins of early and late passage virus. Two experiments are included in this gel. The samples labeled D and H refer to a 40 minute pulse labeling of cells infected with passages 5 and 11, and are identical to those analyzed in Fig. 10. Samples labeled A-C and E-G refer to a 30 minutes pulse, followed by a 30 and 60 minute chase with 10^{-4}M methionine, in cells infected with passages 5 and 11, respectively.

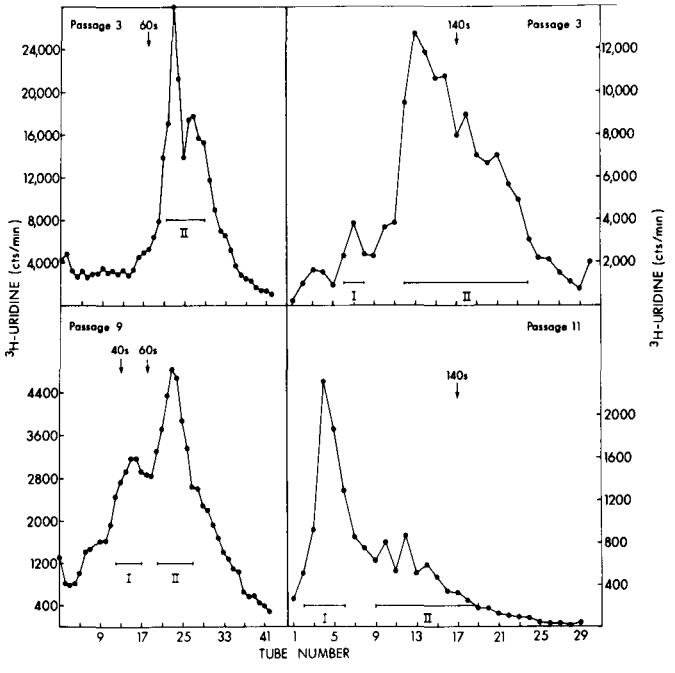

Figure 12 : A comparison of polysome profiles obtained for cells infected with early and late passage virus. Cells were labeled with ^{3}H-uridine (20 μC/ml) in the presence of actinomycin D (1μg/ml) from 1 to 12 hours postinfection. Cell lysates were centrifuged through 10-40% sucrose gradients for 200 minutes at 40,000 rpm. Fractions were pooled as indicated for RNA analysis.

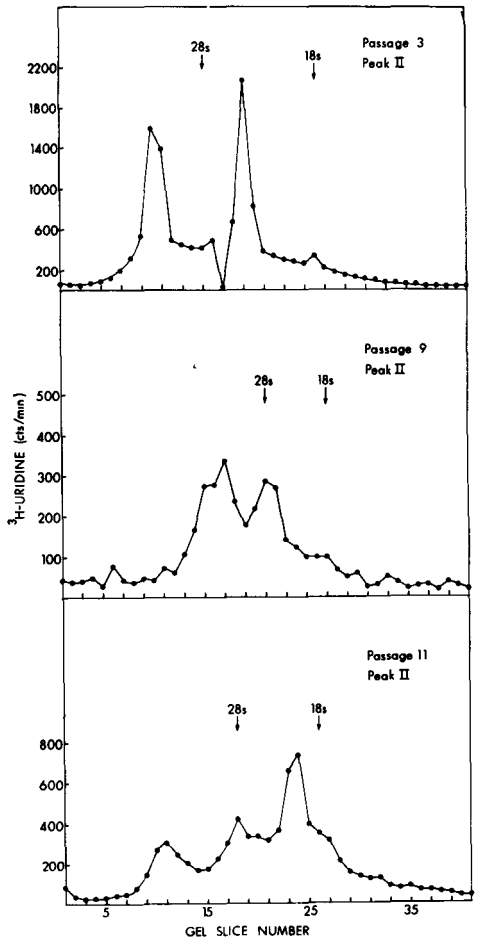

Figure 13 : Polyacrylamide gel electrophoresis patterns of RNA isolated from region II (polysome region) of the sucrose gradients displayed in Fig. 12. ^{3}H-uridine labeled RNA was electrophoresed through 1.8% polyacrylamide-agarose gels for 2-1/2 hours at 60 volts and 5 ma/gel. ^{14}C-uridine labeled BHK ribosomal RNA markers were coelectrophoresed with each viral RNA sample analyzed.

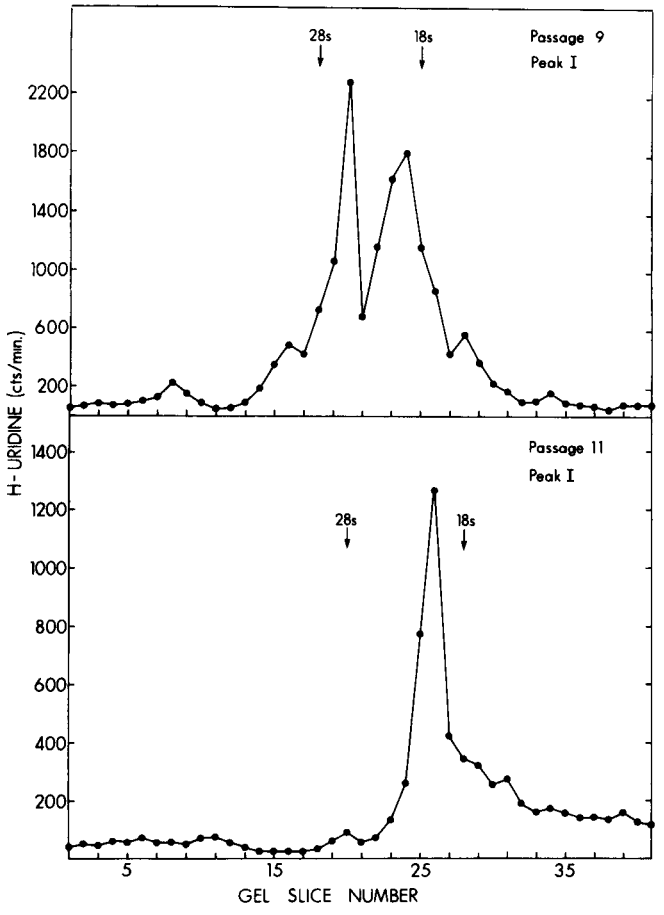

Figure 14 : Polyacrylamide gel electrophoresis patterns of RNA isolated from region 1 of the gradients illustrated in fig. 12. The conditions of electrophoresis were identical to those described in fig. 13.

Essentially all of it was present in peak I (Fig. 14). In cells infected with passage 11 virus 20S RNA represented 71 per cent of the total ^{3}H-uridine-labeled RNA. At least 70 per cent of this RNA was found in peak I of the gradient. Although the remaining 30 per cent was actually in the polysome region its presence there was most probably due to contamination from the large peak in region I (Fig. 12).

Further support for the inability of 20S RNA to serve as an mRNA was obtained using cell-free systems from rabbit reticulocytes or Krebs ascites cells (4). Both Sindbis virion RNA and viral RNA (26S) prepared from infected cells stimulated the incorporation of ^{35}S-methionine into protein. When similar experiments were performed with viral RNA prepared from cells infected with preparations of Sindbis virus containing D1 particles or with purified 20S RNA no stimulation of protein synthesis was observed (Table 3).

The major product obtained from the in vitro translation of Sindbis viral RNA was identified as capsid protein by its mobility in polyacrylamide gels (Fig. 15) and by an analysis of the fingerprints of ^{35}S-methionine-labeled tryptic peptides. Although no stimulation of protein synthesis was observed with RNA from cells infected with D1 particles, the reaction mixtures were also subjected to electrophoresis in polyacrylamide slab gels. No protein bands distinct from those present in the endogenous reaction were detected.

These results and our observations that cells infected with preparations of Sindbis virus containing D1 particles show decreased cytopathic effects (26) suggest that D1 particles of Sindbis virus may not be capable of producing viral products by themselves. In this respect, they resemble D1 particles of vesicular stomatitis virus rather than those of poliovirus (20).

TABLE 3

Stimulation of protein synthesis in cell-free extracts by Sindbis viral RNA

Cell-free System	Source of RNA	μg RNA added	cpm Incorporated into Protein
1. reticulocyte	—	—	12,910
	from cells infected with passage 2	7.0	57,612
	from cells infected with passage 9	8.0	8,974
2. ascites	—	—	2,579
	from std. virions	2.5	8,281
	from cells infected with passage 9	8.0	2,309
	20S RNA from passage 9 infection	3.7	2,299

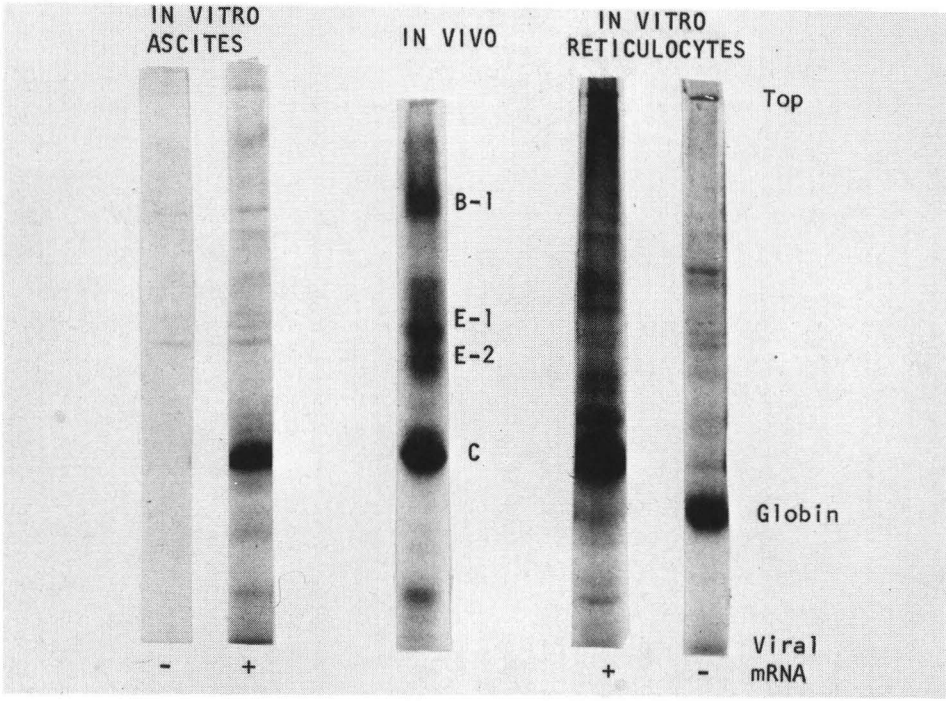

Figure 15 : Autoradiogram of ^{35}S met-labeled proteins synthesized by cell-free extracts from reticulocytes and ascites cells. Details of the methods have been described (4).

CONCLUSION

The studies presented here raise a number of questions about the processing of Sindbis viral RNA and protein. Infection of BHK cells with Sindbis virus containing DI particles affected capsid protein and envelope proteins in a disproportionate manner. This result provides further evidence that is inconsistent with there being a single initiation site for the synthesis of virion proteins. Instead, it suggests that the regulation of capsid protein synthesis may be different from that of the envelope proteins.

One of the essential definitions of DI particles is that they possess a deleted form of the viral genome (20). Although we have not identified this form in Sindbis virions, we have established that 20S viral RNA is a deleted version of 26S viral RNA.

In cells infected with standard Sindbis virus, virion RNA is transcribed and replicated so that the predominant species of viral RNA in the cell is 26S RNA. More detailed knowledge of this process is required if we hope to understand how in cells infected with DI particles the formation of 20S RNA replaces that of 26S RNA.

20S RNA not only does not contain all of the genetic information of 26S RNA but it also does not function as an efficient messenger. Undoubtedly one of the important factors in the interference of Sindbis virus formation by DI particles is the diminished amount of 26S RNA. A significant decrease in 26S RNA would greatly curtail the synthesis of the viral structural proteins.

DI particles have been isolated from a number of different viruses. They are providing a useful probe in the analysis of viral replication but they have also been attracting considerable interest as a possible factor in persistent viral infections or in slowly progressing

neurological diseases (27). The establishment of a persistent infection of Sindbis virus in mouse embryo cells was shown to depend on maintaining a balance among infectious virus, DI particles, and interferon (28). More recently Doyle and Holland (29) have been able to transform the rapidly fatal disease caused by injecting mice with VSV into a slowly progressing one by also injecting large quantities of DI particles of VSV. These two examples suggest that DI particles may have an important role in determining the outcome of viral infections.

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DISCUSSION

Dr. ROTT

RNA of Semliki forest virus in Germany behalves like 26S RNA of Sindbis virus in the United States.
Dr. Bangler in our group isolated the 26S RNA from a polyribosomal fraction and tested it in an in vitro system but in this case using the Krebs II ascitis tumor cells. This 26S RNA stimulated protein synthesis and in the slide of the polyacrylamide gel you can see that capsid protein is formed. Fingerprints of the tryptic peptides of virion nucleocapsid protein and the product synthesized in vitro are identical. Thank you.

Dr. HORZINEK

To my knowledge 20 S RNA that was single-stranded, was isolated by several groups from infected cells. What would be your interpretation, would there be defective interfering particles that they did not know about or is 20 S RNA also produced normally during infection ?

Dr. S. SCHLESINGER

I see no evidence for any 20S RNA with early passage virus. In some preparations of RNA we did see more heterogeneity in the RNA than I showed in the slide and there could be some degradation of RNA to 20S. We have characterized the 20 S RNA from defective passage infections by its molecular weight and by the fact that it has half the sequences of 26S RNA so I believe that it is homogeneous.
I might point out that in a paper by Eton and Faulkner on defective interfering particles, they claimed that they could see the 20S RNA after five passages, even though the accumulation of DI particles themselves did not appear until later.

Dr. SCHOLTISSEK

In Semliki-Forest viruses Dr. Simons from Helsinki found a third envelop glycoprotein, the E 3.
Did you find this also in your system ?

Dr. S. SCHLESINGER

We never had any evidence from more than three proteins. Either by isotopes or by staining our gels with protein stain.

Dr. SCHOLTISSEK

But I saw very little bump on the left side.
Could not this be the E 3, because this should have a very small molecular weight and it could occur in equal molar amounts compared with the other envelope components.

Dr. S. SCHLESINGER

I really have not looked for it.

Dr. SCHOLTISSEK

Because if it is there it should be there very low amounts.

Dr. SCHLESINGER

It might have run off the gels.

Dr. ROTT

There are defective particles found in influenza virus and rhabdoviruses and now also in togaviruses after undiluted passage of the viruses. And the question now arises if there is a general mechanism underlying the formation of these incomplete or defective particles or not. What is your feeling about the mechanism of the formation of these defective particles in togaviruses ?

Dr. S. SCHLESINGER

One really has to ask several questions. With regard to the formation of the particles, why do deletions occur during the synthesis of viral nucleic acids ? Secondly, how do defective particles interfere with normal virus replication ? And finally is the mechanism of interference going to be similar for all the different viruses ?
There is a big difference between the polio-defective virus, on one extreme, and the enveloped viruses using VSV as the other extreme. Defective interfering particles of polio and I would guess it to be true for all of the picornaviruses are able to carry out many functions of the normal virus but are not able to assemble into intact virions, whereas with VSV, and we think it is true also for Sindbis, the defective interfering particles by themselves are not able to carry out any viral functions.

Dr. ROTT

Is it possible to isolate this defective particles in pure form ?

Dr. S. SCHLESINGER

We have not ever been able to separate them from the normal virus.

Dr. ROTT

But it should be possible to isolate the RNA, the 20 S RNA from the 49 S RNA ?

Dr. S. SCHLESINGER

I can isolate the 20 S RNA but this is found in the cell. The RNA in the virion is very heterogeneous and as yet I do not know the reason for the heterogeneity.

Dr. DALSGAARD

You mentioned into the beginning of your report that the P.E.2. protein was a precursor of the E 2 protein. I should like to know whether you know if these proteins are antigenetically related using immunological techniques.

Dr. S. SCHLESINGER

We haven't tried to do this type of experiment.

Dr. SCHOLTISSEK

You mentioned that the smaller molecular weight RNA's are breakdown products but I guess you can exclude this possibility because you get a 50 % competition only; if this would be breakdown products you should get 100 % competition.

Dr. S. SCHLESINGER

The smaller RNA's were from the virions. The 50 % competition was between the 20 S and 26 S RNA in infected cells.

Dr. HORZINEK

Has anybody looked at the morphology of interfering particles. Do they differ structurally, do they agglutinate ?

Dr. S. SCHLESINGER

They seem to agglutinate very well; we have some evidence that they are more labile; when we try to look in the electron microscope for defective particles we find much degradation compared to the normal virus where we got very nice particles.

BIOGENESIS OF THE INFLUENZA VIRUS ENVELOPE

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Institut für Virologie, Justus Liebig-Universität,
Giessen, Germany.

There are close relationships between the structure and replication of a virus on one hand and the structural and physiological properties of the host cell on the other hand. Such relationships are particularly obvious in the case of enveloped viruses. The envelopes of many viruses are derived from areas of the plasma membrane which are composed of cell-specific as well as virus-specific constituents. Such envelopes, therefore, can be considered as pieces of a plasma membrane modified specifically by the virus.

Analysis of the biogenesis of an enveloped virus should throw some light on the synthesis of the virus-specific envelope components and on their interaction with cellular membrane constituents. Such studies might also provide information on the possible role of the structural and functional membrane modifications for the pathogenicity of a virus.

Influenza virus is a suitable object for such studies, because its structure has been well characterized and because its envelope proteins can be easily detected in the infected host cell.

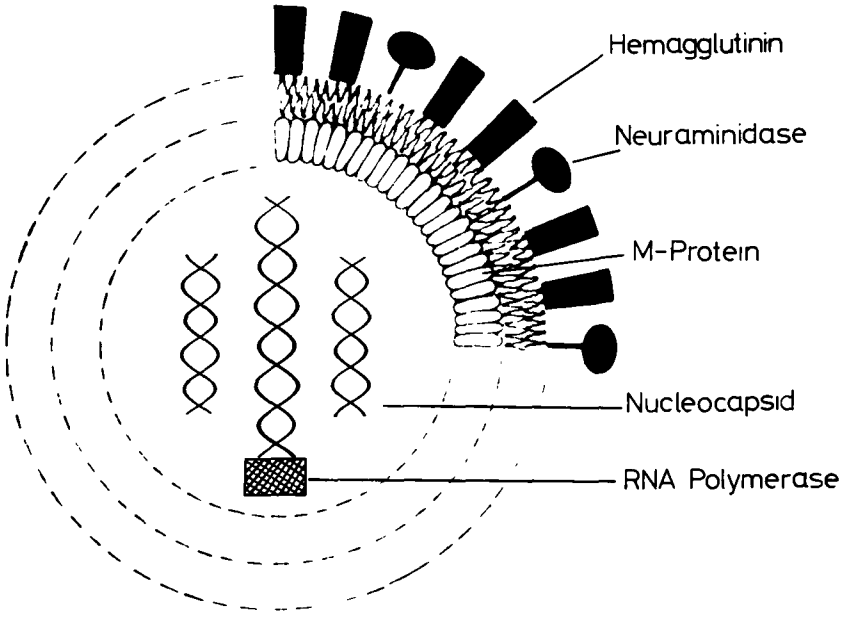

Figure 1. Schematic structure of influenza virion.

Composition of influenza virus envelope

The envelope of influenza virus meets all the criteria for cellular membranes. Morphologically it possesses a trilaminar or unit membrane structure. Chemically it is composed of lipids, proteins, and carbohydrates, the carbohydrates being covalently linked to proteins and to lipids (for ref. see Klenk, 1973).

It is generally accepted that the lipids are derived from the host cell. So far not a single enzyme involved in lipid metabolism has been found to be coded for by the viral genome. It was not surprising therefore that the composition of the virus lipids resembles that of its host cell plasma membrane.

A substantial portion of the total polar lipids is glycosylated (Klenk and Huang, 1973). Some of these glycolipids are classical heterophilic antigens, e.g. the Forssman-antigen, which are present in the corresponding host cell (Springer and Tritel, 1962 ; Rott et al., 1966). If such host cell antigens are found in the enveloped viruses, it is supposed that carbohydrate moieties represent the antigenic determinant. There is no doubt that these glycolipids are host-specific.

In contrast to the lipids the envelope proteins in influenza virus are specified by the viral genome. This holds true for weights of 50,000 and 30,000, respectively, which are linked by disulfide bonds. HA_1 is rich in carbohydrate and represents the peripheral part of the spike. It is responsible for virus adsorption. HA_2 appears to be located on the inner side of the spike and it is supposed to interact with the lipid layer by a hydrophobic segment.

The neuraminidase spike is built up by four glycoprotein subunits with a molecular weight of about 60,000. Like the hemagglutinin it possesses a hydrophobic part which enables the spike to interact with the lipids of the envelope.

Lipids are arranged in a bilayer structure as could be shown by spin-labelling experiments (Landsberger et al., 1971, 1973).

Beneath the central lipid bilayer an unglycosylated protein, the membrane or M-protein is located (Compans et al., 1970 ; Schulze, 1970). As will be shown later it has a definite membrane affinity. This supports the concept that the M-protein is an integral part of the viral envelope.

Biosynthesis of virus-specific envelope components

According to our present knowledge the virus-specific envelope components of influenza virus are synthesized independently.

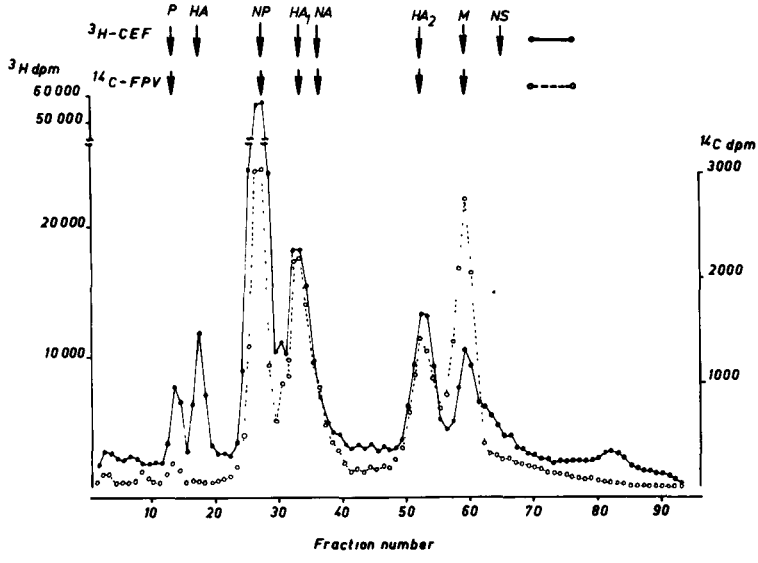

Figure 2. Polyacrylamide gel electrophoresis of polypeptides of fowl plague virus (^{14}C-amino acids o—o) and fowl plague virus infected cells (^{3}H-amino acids (•—•).
Cells were labelled 4 hr after infection.

In cells infected with fowl plague virus two other virus-specific proteins can be detected in addition to the structural proteins of the virion (Fig. 2). The function of protein NS is not known. Protein HA is a glycoprotein of an apparent molecular weight of 76,000. It has been suggested to be a precursor molecule from which glycoproteins HA_1 and HA_2 are derived by proteolytic cleavage (Lazarowitz et al., 1971). Such a precursor-product relationship can be demonstrated by the pulse-chase experiments shown in Fig. 3 where glycoproteins HA_1 and HA_2 are formed during the chase period on the expenses of glycoprotein HA. This can also be shown, if infected cells are kept at 25° C (Klenk and Rott, 1973). If infected cells are labelled at this temperature 24 hr p.i. for 1 hr with radioactive amino acids one finds glycoprotein HA in addition to proteins P and NP, but in relatively much higher amounts than under similar labelling conditions at 37° C (Fig. 4). The small glycoproteins HA_1 and HA_2 are missing. However, this accumulation of HA is reversible. If the pulse is followed by a chase period at 37° C, it can be seen that there is a drastic decrease of HA, and HA_1 and HA_2 are being formed. If the chase at 37° C is performed in the presence of a protease inhibitor, HA does not decrease and HA_1 and HA_2 are not being formed. These experiments show that glycoprotein HA_1 and HA_2 arise by proteolytic cleavage of glycoprotein HA.

The biosynthesis of these glycoproteins has been also inhibited at another stage. High doses of D-glucosamine and 2-deoxy-D-glucose inhibit the formation of influenza virus glycoproteins, whereas carbohydrate-free polypeptides are still being synthesized (Kaluza et al., 1972 ; Klenk et al., 1972). If glycosylation is inhibited by one of these sugars, a protein (HA_o), not present under normal conditions, can be detected (Fig. 5). We assume that this protein (molecular weight 64,000) is the unglycosylated or incompletely glycosylated polypeptide of glycoprotein HA. We have obtained fairly good evidence that this concept might be correct (Klenk et al., 1972) : (1) There is a similarity in the amino acid composition of HA and HA_o. (2) If the inhibitor is removed, the block of glycosylation can be released. Under these conditions HA_o is shifted to a position on polyacrylamide gels similar to that of HA. Since radioactive carbohydrates fail to label HA_o whereas they are readily incorporated into HA, it is suggested that HA_o might be carbohydrate-free.

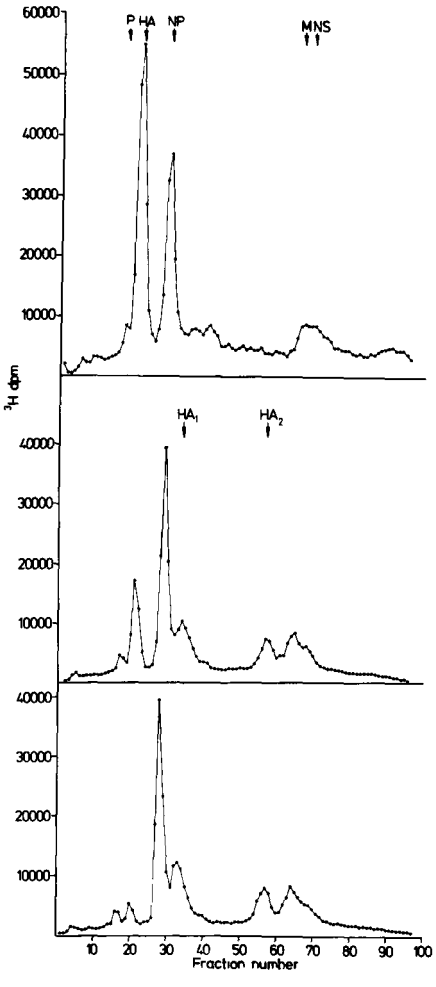

Figure 3. Polyacrylamide gel electrophoresis of FPV polypeptides synthesized in chicken embryo cells. Cells were labelled 4 hr after infection by a 10 min pulse with ^{3}H-leucine. Top : cells were scraped off immediately after the pulse ; middle : the pulse was followed by a chase period with cold leucine for 15 min ; bottom : the chase period was 60 min.

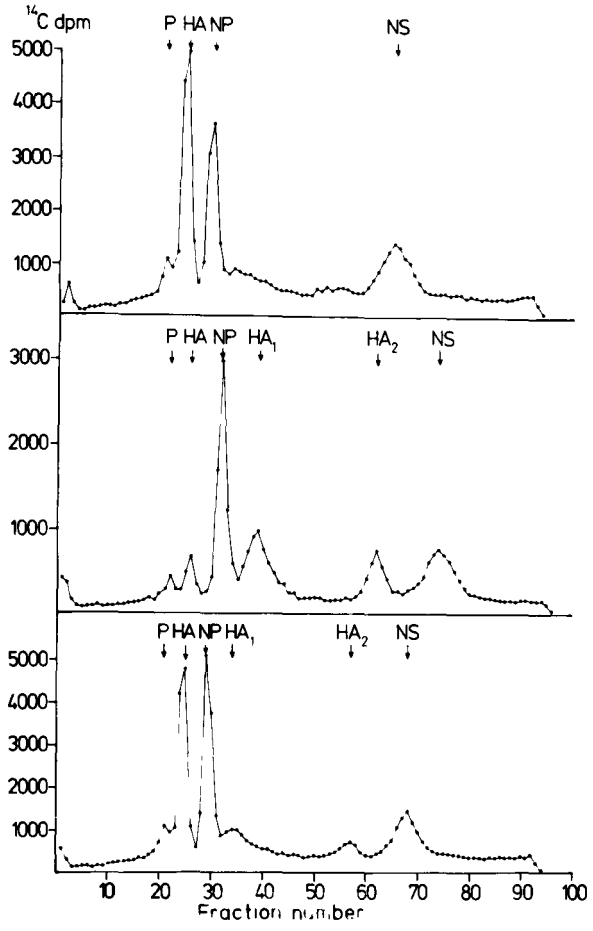

Figure 4 : Polyacrylamide gel electrophoresis of FPV proteins synthesized at 25°C.

Cells were labelled 24 hr post infection by a 1 hr pulse with ^{3}H-valine. Top : cells were scraped off immediately after the pulse ; middle : the pulse was followed by a 1 hr chase at 37° C ; bottom : the pulse was followed by a 1 hr chase at 37° C, and the media added to the cells during the chase period contained diisopropylfluorophosphate (20 µmol/ml).

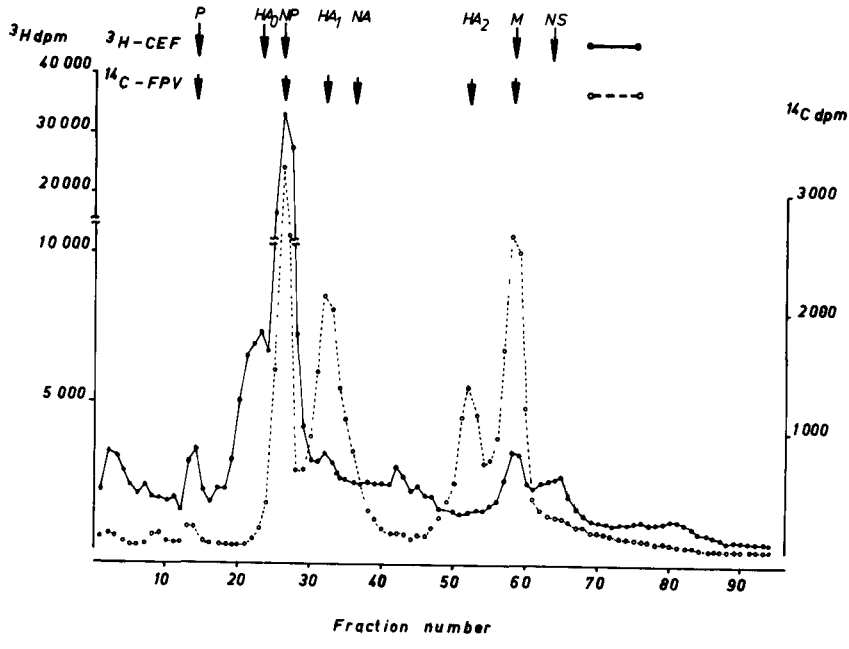

Figure 5. Polyacrylamide gel electrophoresis of FPV proteins synthesized in chick embryo cells in the presence of glucosamine (20 µmol/ml). Cells were labelled at 4 hr after infection with ^{3}H-amino acids. Marker virus labelled with ^{14}C-amino acids was coelectrophoresed on the same gel.

Further information on the relationship of HA_o to HA could be obtained from an analysis of the intracellular location of these proteins. Cells were homogenized and cytoplasmic extracts were separated into cell fractions by equilibrium centrifugation on sucrose gradients (Compans, 1973 ; Klenk et al., 1974).

The proteins of the rough membranes labelled at 4 hr p.i. for 10 min with (^{3}H)-amino acids are shown in Fig. 6. After the short pulse in the absence of glucosamine there is a large peak of the uncleaved glycoprotein HA. After the chase HA can no longer be detected either in the uncleaved or in the cleaved form. This indicates that HA has completely disappeared from this fraction. Corresponding experiments which were performed in the presence of high doses of glucosamine indicate that HA_o like HA disappears from the rough membrane.

The smooth membranes (Fig. 7) contain a significant amount of the uncleaved glycoprotein HA after a short pulse. After the chase the hemagglutinin-protein is still present in this fraction but now almost exclusively in the cleaved form as HA_1 and HA_2. In the presence of glucosamine, after the short pulse only HA_o is visible in the smooth membranes. After the chase the corresponding cleaved products called HA_{o1} and HA_{o2} can be detected in addition to HA_o. The data indicate that on smooth membranes HA and HA_o stay longer than on rough membranes and that they are cleaved there.

In conclusion, cell fractionation studies have revealed that the hemagglutinin glycoprotein migrates from the rough to the smooth endoplasmic reticulum. There it accumulates with the other constituents of the virus envelope. It can be assumed that these glycoproteins then migrate further to the plasma membrane, where they are incorporated to form the viral envelope. In the course of this migration the glycoprotein HA is cleaved by host cell-specific proteases into the smaller glycoproteins HA_1 and HA_2.

The behaviour of HA_o further underlines a close relationship between HA and HA_o. The membrane affinity of HA_o together with its lack of carbohydrates suggest that the carbohydrates are not

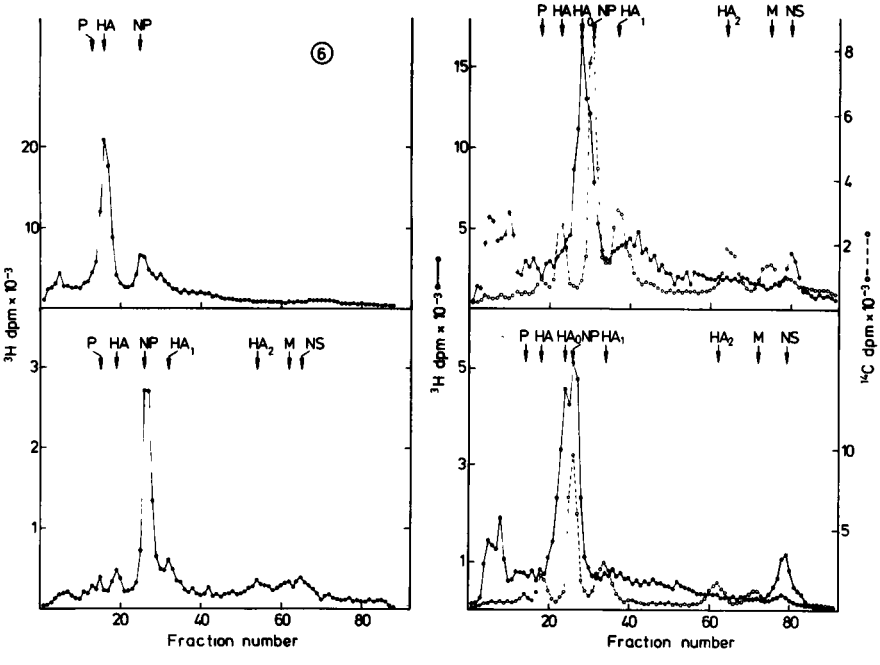

Figure 6. Polypeptides of fowl plague virus associated with rough membrane fraction after a 10 min pulse with ^{3}H-leucine, ^{3}H-valine, and ^{3}H-tyrosine at 4 hr post infection and after a pulse followed by a chase period with cold amino acids. The virus-specific polypeptides of ^{14}C-amino acid-labelled whole cells were included as internal markers.

Upper left panel : cells were labelled by a pulse ; lower left panel : cells were labelled by a pulse followed by a chase ; upper right panel : cells incubated in the presence of glucosamine (20 µmol/ml) were labelled by a pulse ; lower right panel : cells incubated in the presence of glucosamine were labelled by a pulse followed by a chase.

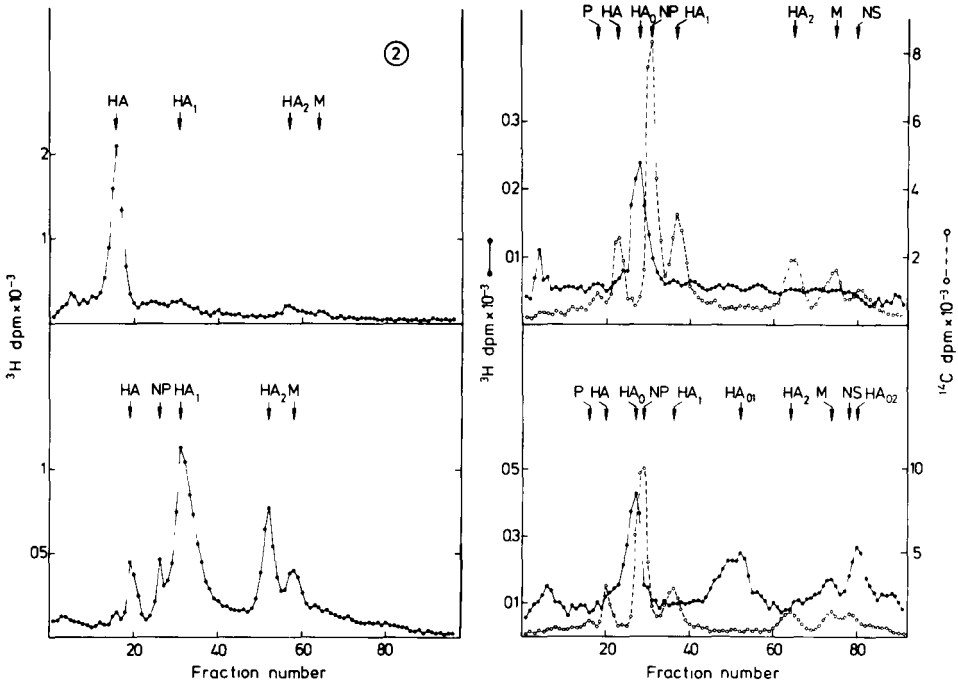

Figure 7. Polypeptides of FPV associated with smooth membrane fraction. Details as in Fig. 5.

essential for binding of glycoproteins to lipid membranes and for their transport. For hemagglutinating activity, however, the carbohydrate moiety appears to be necessary, because HA_0 does not exert hemagglutination.

Incorporation studies with radioactive sugars and amino acids suggest differences in the carbohydrate content of the uncleaved and cleaved hemagglutinin glycoprotein (Klenk et al., 1974). Such experiments showed that the incorporation of glucosamine in HA is 1.5 times as high as in HA_1 and HA_2, whereas that of fucose is only 0.5 times as high. These data are compatible with the idea that in the course of glycoprotein synthesis carbohydrates are attached in a stepwise manner and that fucose is added later than glucosamine. This means that glucosamine presumably forms the carbohydrate core of the oligosaccharide side chains. It must be added to the hemagglutinin quite rapidly at a time, when the polypeptide is still located on the rough endoplasmic reticulum, because so far it has not been possible to detect the unglycosylated polypeptide under normal condition.

As has been pointed out already the available evidence suggests that the structure of the carbohydrate side chains of the viral glycoproteins is determined to a good deal by the glycosyl transferases of the host cell. It is possible, however, that in addition to these enzymes the viral neuraminidase plays an important role in designing the carbohydrate moiety of the viral glycoprotein. There are some indications (Schulze, 1974) that the absence of neuraminic acid is essential for a functional hemagglutinin which enables it to interact with the neuraminic acid containing virus receptor of the host cell.

In addition to the hemagglutinin glycoproteins the smooth membranes also contain the neuraminidase and the M-protein, whereas the other virus-specific proteins are lacking in this cell fraction. The smooth membranes, therefore, contain all envelope proteins, a fact which shows again the membrane affinity of these viral proteins.

The envelope components and the nucleocapsid apparently move to the cell membrane through different routes. Whereas the envelope proteins are transported along cellular membranes, the nucleocapsid is always found in soluble form.

Assembly of influenza virus envelope

The envelope of influenza virus is assembled in a budding process at the plasma membrane (for ref. see Klenk, 1974). During this process the unit membrane of the viral envelope is continuous with and morphologically similar to the plasma membrane of the host cell. On the other hand, the areas of virus budding show several virus-specific modifications. On their outer surface they are studded with spikes, which react with anti-hemagglutinin antibodies. They differ from the normal cell membrane also by the absence of neuraminic acid. On their inner side these regions carry an additional layer which is not found in the normal cell membrane and which is most likely formed by protein M.

There are some indications that protein M might be rate limiting for virus assembly. At 25° C mature virions are not formed, and protein M is the only virus constituent which cannot be detected in infected cells under these conditions (Klenk and Rott, 1973). Moreover cell fractionation studies revealed that the smooth membranes of infected cells contain all envelope proteins. Protein M, however, is found in lower amounts than in the virus envelope (Stanley et al., 1973; Klenk et al., 1974). This suggests that only a small fraction of the membrane areas with hemagglutinin glycoproteins contain also the M protein and that only these areas are converted into viral envelopes.

No significant amounts of host cell proteins can be found in the membrane of the virus particle. The viral envelope must, therefore, be derived from segments of the plasma membrane from which the host cell proteins are excluded. It is not yet understood, how the virus-specific glycoproteins reach the surface of the plasma membrane.

Influenza virus-induced membrane modification

The close interactions between viral envelope and cellular membrane cause alterations of the structure and function of the cell surface. Such a modification of the membrane structure is, for instance, a rearrangement of receptor sites for Concanavalin A which render the cells agglutinable by this phytagglutinin (Becht et al., 1971, 1972 ; Rott et al.,

1972, 1974), a phenomenon which is well known for transformed cells. There is good evidence that the agglutinability of infected cells by Con A is mediated through viral spikes incorporated into the cell membrane. This concept is supported by the finding that isolated virus particles can be readily flocculated by the lectin and that removal of the viral glycoproteins by proteases leaves a spikeless particle which is no longer agglutinable by this phytagglutinin (Klenk et al., 1972).

The essential role of this kind of membrane changes brought about by the viral envelope component is underscored in experiments where 2-deoxy-D-glucose interfered with the synthesis of glycoproteins. Administration of this antimetabolite immediately after infection of chick fibroblasts with fowl plague virus did not impair synthesis of viral polymerase and RNP-antigen, but depressed the formation of viral hemagglutinin and neuraminidase and prevented the host cells to be agglutinated by Con A (Kaluza et al., 1972 ; Rott et al., 1974).

All data available suggest that binding of the phytagglutinin to the outer layer of the cell membrane has no significant effect on the synthesis of the membrane-associated hemagglutinin and neuraminidase, but interferes with virus release and probably with viral maturation. Treatment of infected cells with Concanavalin A appears to shift virus budding from the cell surface to intracellular vesicles, and one has the impression that budding takes place through the membranes of these vacuoles (Rott et al., 1974) (Fig. 8). Therefore it might be conceivable that intracellular membranes represent the site of second choice - and therefore a less efficient one - for the formation of envelope material after the location of optimal maturation had been disturbed.

The structural alterations of the cell membrane are accompanied by functional changes of the cell membrane as can be demonstrated by an altered uptake of low molecular weight precursors into the cell. We could show that the uptake of uridine into the cell is stimulated after infection with influenza virus, although there is no change in the uptake of adenosine, which is a good internal control. As far as uridine is concerned only the velocity of the uptake is changed but not the apparent K_m value (Rott et al., 1974). Similarly the uptake and incorpora-

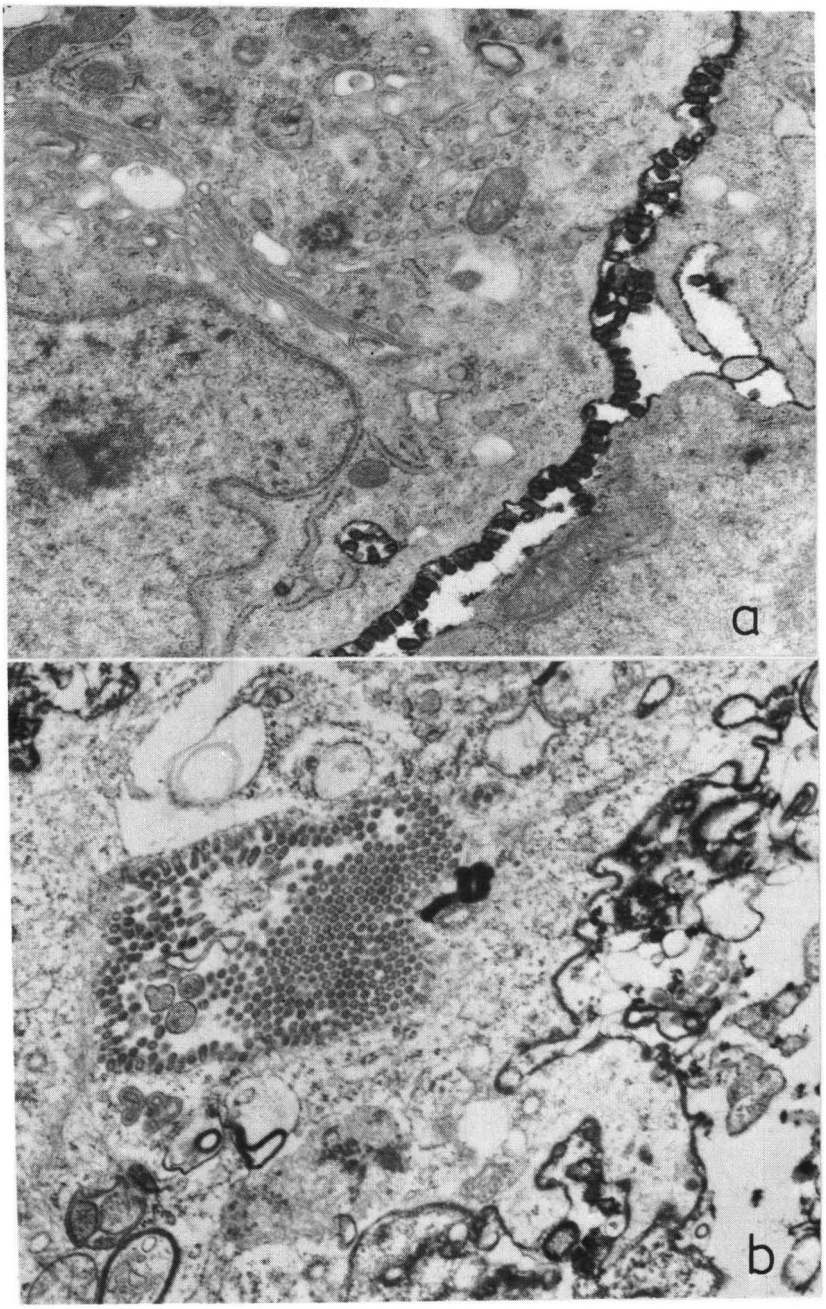

Figure 8 : Electron micrographs of fowl plague virus-infected chick embryo cells.

(a) cells were fixed for thin sectioning and stained with ruthenium red 8 hr after infection.

(b) immediately after infection the cells were treated with 50 µg/ml Concanavalin A. 8 hr after infection cells were fixed and stained (Electron microscopy : M. Reinacher).

tion of sugar derivatives like glucosamine and 2-deoxy-D-glucose into acid-insoluble material is enhanced after infection with influenza virus (Scholtissek, 1973).

Besides these different characteristics of a membrane altered by the insertion of virus-specific components one has to take into consideration that the appearance of these viral structures at the cell surface means that the cell as a whole carries a different antigenic mosaic. Since such a cell can no longer be recognized by the organism as "self", the described maturation process of the virus envelope should cause immunopathological reactions, which may have repercussions on the course of the disease.

Acknowledgement

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MOLECULAR EVENTS IN THE REPLICATION OF AN ICOSAHEDRAL CYTOPLASMIC DEOXYVIRUS

II. Regulation of FV_3 Protein Synthesis

by

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SUMMARY

Since FV_3 messenger transcription does not appear to involve distinct early and late phases, it raises the question of whether there is any regulation of FV_3 protein synthesis. This question will be approached from consideration of synthesis of virus structural proteins and from virus induced enzymes. Thymidine kinase and endoribonuclease are both induced by FV_3. Thymidine kinase appears to be an early function which is also synthesized at late times. Ribonuclease appears to be a typical late function. Most virus structural proteins seem to be synthesized at both early and late times but all can be considered as early functions. In this respect the system resembles Herpesvirus. Evidence for these points will be drawn from the data published by others and from some recent original experiments.

INTRODUCTION

There is no clear distinction between "early" and "late" mRNA transcripts of frog virus 3 (FV_3) as determined by their electrophoretic mobilities on polyacrylamide gels (see Paper I). With the reservation that the specific functions of various FV_3 messenger RNA transcripts, identified only by their apparent molecular sizes, have not been determined it appears that most FV_3 proteins could be synthesized under the direction of input DNA templates. This raises the question of what controls there are, if any, on the biosynthesis of FV_3 proteins in particular and of icosahedral cytoplasmic deoxyvirus (ICDV) proteins in general. During frog virus 3 replication, 17 virion structural polypeptides are synthesized and at least 2 new enzyme activities are elicited (13). We will outline some regulatory aspects of their synthesis using data from experiments already published and from recent work.

MATERIALS AND METHODS

Virus. Procedures for the production and purification of frog virus 3 were those published (13).

Cells. The cell line used to study induction of thymidine kinase was a thymidine kinase deficient mouse cell (LTK^-) (12).

Thymidine Kinase. The methods used were exactly as described by Aubertin and Longchampt (2).

DNA Synthesis. Virus-infected LTK^- cells were continuously labelled with H^3-thymidine (0.1 μC/ml). Methods to determine incorporation of label into nuclear and cytoplasmic fractions have been described in detail (11, 2). Using the system described, there was negligible incorporation into uninfected cells. Upon fractionation of infected cells, most of the viral DNA associated with nuclei (see 11).

Structural Polypeptides. Isotopic labelling and analysis of structural polypeptides was conducted as described by Tan and McAuslan (13) or by others (7). In some experiments infected cells were labelled for 2 hours with S^{35}-methionine (30 μC/ml; Sp act. 150 Ci/mM) and the polypeptides in the soluble fraction (100,000 xg supernatant of sonically disrupted cells) resolved by polyacrylamide slab electrophoresis (9) and detected by autoradiography.

RESULTS

Induction of Thymidine Kinase. In LTK$^-$ cells infected with FV_3 (m.o.i. 100 or 10 pfu/cell), viral DNA was synthesized and thymidine kinase activity elicited. The increase in thymidine kinase activity was parallel to the increase in viral DNA synthesis (Fig. 1). These date are essentially in accord with those of Aubertin and Longchampt (9) except that we detected a much earlier increase in activity; this increase terminated at 12-14 hours post-infection. When hydroxyurea was added at the time of infection, neither the rate of increase in kinase activity nor its subsequent termination was affected significantly (Fig. 1).

If the messenger for kinase is stable, we would possibly have an indirect approach to determining if mRNA transcribed from input virions in the presence of cycloheximide was functional. To determine the stability of messenger for induced kinase, actinomycin D (5 μg/ml final concentration) was added to cells at times after FV_3 infection and the activity of thymidine kinase (TK) was determined. Actinomycin D added before or after the onset of kinase synthesis rapidly arrested any further increase in activity (Fig. 2).

Assuming actinomycin D was inhibiting at the transcriptional level and not the translational level (cf Ref. 6), the results suggest that the messenger for kinase has a very short life. This is in contrast to the messenger for TK induced by herpes simplex or poxvirus (5, 10) and in contrast to other RNA transcripts of FV_3 (1).

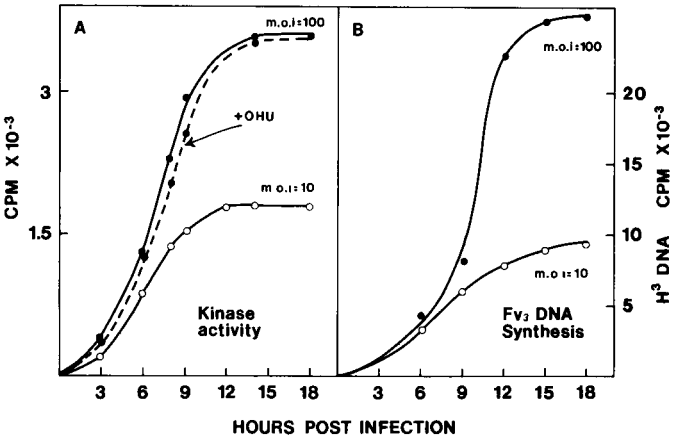


Figure 1 : a) FV_3 induction of thymidine kinase. Increase in kinase activity at an m.o.i. of 100 (•); increase in kinase activity at an m.o.i. of 100 when 20 μg hydroxyurea (OHV)/ml added at the start of infection (•—•) increase in kinase activity at an m.o.i. of 10.

b) incorporation of H^3-thymidine into FV_3 infected LTK^- cells.

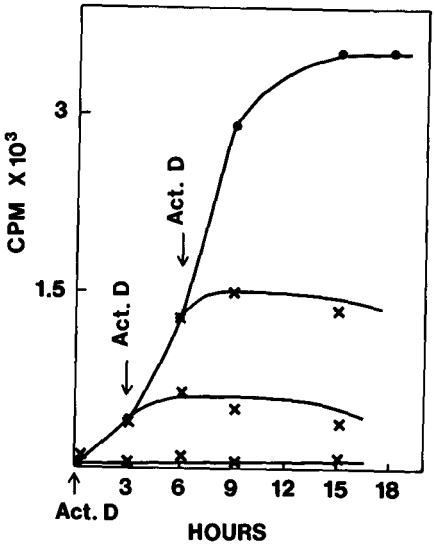


Figure 2 : Effect of actinomycin D on FV_3 induction of thymidine kinase in LTK^- cells. Increase in thymidine kinase with no actinomycin added (•) or with actinomycin D (5 μg/ml) added at 0,3 or 6 hours post-infection as indicated.

Induction of Ribonuclease. The properties of a novel FV_3- induced endoribonuclease that degrades double-strand RNA has been described (8). The appearance of this activity is dependent upon FV_3 DNA synthesis and it is classed, therefore, as a late function. Because the assay measures only endonucleolytic cleavage it is difficult to measure precisely the kinetics of its synthesis during the virus replication cycle.

Structural Polypeptides. The major virion structural polypeptides (VSP) are shown in Figure 3. VSP 1, 2, 7, 9 are probably internal polypeptides associated with the virus nucleoid and VSP 3, 5, 10, 14 are probably outer coat polypeptides as they are readily removed by mild detergent treatment of virions (3).

The structural polypeptides are synthesized throughout the infection cycle. If infection is initiated in hydroxyurea to prevent viral DNA synthesis, all major structural polypeptides are still synthesized (Fig. 4). Therefore they are early gene products. It is noteworthy that we can also detect by the slab gel method many other viral polypeptides that are probably non-structural. Our preliminary studies on the effect of actinomycin D on their synthesis suggests that the messengers for many viral proteins are not very stable (or that actinomycin D has a direct effect on translation); this is at variance with our observations on stability of the messenger profile (1).

DISCUSSION

From determination of the RNA transcripts by gel electropheresis, the overall pattern of messengers does not appear to change qualitatively during the replication cycle, suggesting that most FV_3 proteins could be synthesized at early times. This is supported by studies on synthesis of virion structural polypeptides under conditions where viral DNA synthesis is inhibited. All virion structural polypeptides so far detected are early viral gene products; a somewhat similar situation is true for herpesvirus protein synthesis (4). There is some evidence that the rates of synthesis of the different FV_3 polypeptides during the replication cycle change and that these changes in rate may be mediated by other viral functions (7).

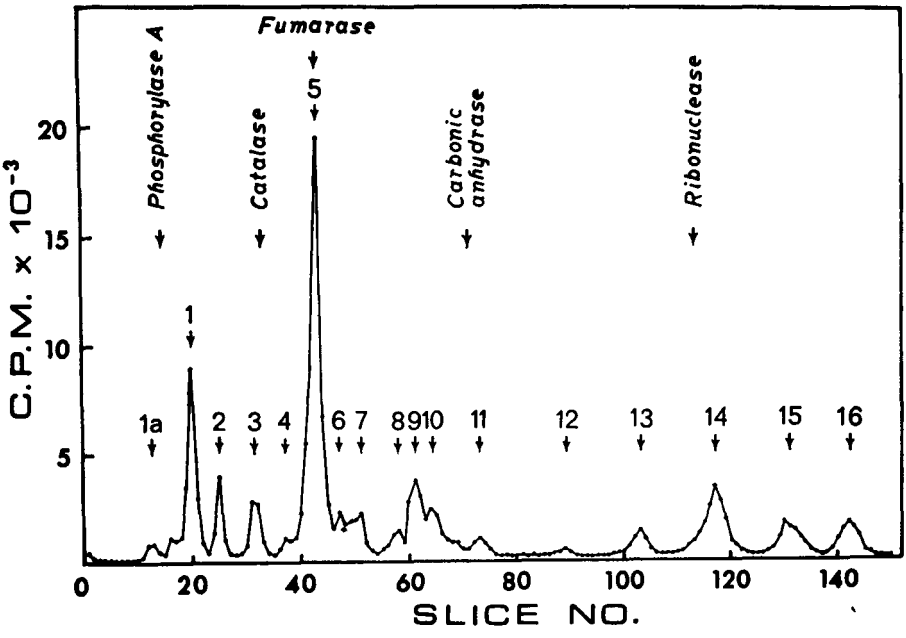
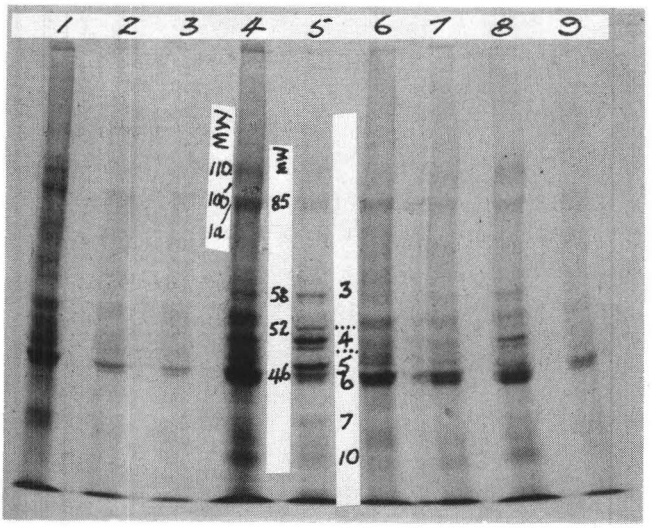


Figure 3 : The structural polypeptides of FV_3. FV_3 was grown in BHK cells incubated with H^3-labelled amino acids. Viral polypeptides were analyzed on polyacrylamide gels (13).

A



B

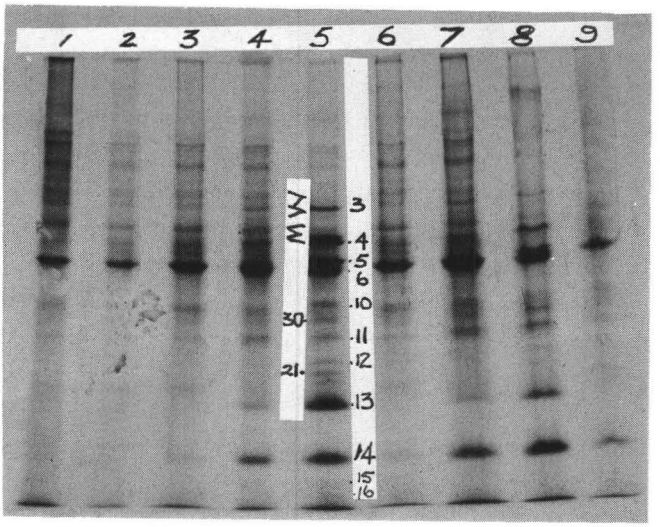


Figure 4 :

FV_3 polypeptides infected LTK^- cells. FV_3 infected LTK^- cells either untreated or inhibited with hydroxyurea or actinomycin D incubated with S^{35}-methionine for2 hours at different times after infection. The soluble supernatant fraction from disrupted cells was analyzed on 10% polyacrylamide slab gels (9) and visualized by autoradiography.

Gel 1, uninfected LTK^- cells; gel 2, LTK^- plus heated FV_3.

Gels 3, 4, 5 represent LTK^- cell plus heated FV_3 to reduce background, plus life FV_3 (50 pfu/cell). Cells were incubated with S^{35} methionine 1-3 hours p.i. (gel 3), 5-7 hours (gel 4) or 9-11 hours (gel 5).

Gel 6, 7, 8 represent LTK^- cells plus heated FV_3 plus life FV_3 plus hydroxyurea. Cells were incubated with S^{35} methionine 1-3 hours (gel 6); 5-7 hours (gel 7) or 9-11 hours (gel 8).

Gel 9 represents LTK^- cells plus heated FV_3 plus life FV_3. Actinomycin D was added at 5 hours and the cells incubated with S^{35} methionine from 7-9 hours. Fig. 4a, 10% gels. Fig. 4b, 15% gels.

However, if one considers non-virion proteins, for example, induced thymidine kinase and ribonuclease, there are clearly other modes of regulation.

Thymidine kinase is an early function whose messenger is short lived but which is synthesized throughout the replication cycle. This is in contrast to both herpesviruses and poxviruses. Ribonuclease seems to be a late function. The role of this activity, which is incorporated into virions is a mystery. It is conceivable that it cleaves certain viral transcripts to produce functional messengers. From the studies we have begun with FV_3, it is clear that no single approach is likely to give the complete picture of the control of ICDV protein synthesis.

ACKNOWLEDGEMENT

Part of this work was conducted at the Roche Institute of Molecular Biology.

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DISCUSSION

Dr. S. SCHLESINGER

I was a little bit confused about what you said with the unstable messenger RNAs. I do not remember in your talk this morning that you had evidence that the messengers were unstable.

Dr. MCAUSLAN

As we look at the transcripts on the gells they seem to be very stable. At least the six hours or more after actinomycine D, if we now look at the specific function for one of the enzymes this seems to be synthesized from an unstable messenger.

Dr. S. SCHLESINGER

But you define an unstable as a fact that the polypeptide is not continuing the synthesis. But there could be other explanations.

Dr. MCAUSLAN

Such as.

Dr. S. SCHLESINGER

Translations control.

Dr. MCAUSLAN

Yes but the evidence for actinomycine affecting translation is not exactly strong at the moment; but on the other hand if we look at the structural polypeptides some of them seem to be synthesized in the presence of actinomycine D and some certainly not.
What I am saying is, that just looking at transcripts on our gels is too rough a method, we have to get down to each specific function and ask the question about each one. Overall the regulation in this virus and perhaps in all viruses of this group seems to be most unusual and just about all polypeptides can be considered as early functions.

We do not see in this system a late shift with the one exception of the ribonuclease situation. And for that we can not even imagine what this function would be, except that it might convert some messenger to a state suitable for translation as described recently for T7.

Dr. S. SCHOLTISSEK

I have a rather theological question.
To what does the virus need timing in Kinase for ?
Because normal cell has a thymidine Kinase and further more thymidine and the TMP synthesis denovo in the cell; and this is used for DNA synthesis.

Dr. MCAUSLAN

In no case of any DNA virus, poxvirus, herpesvirus, has there been a good argument for the function of thymidine Kinase, and the only thing that I can suggest to get myself out of tricky situations Dr. SCHOLTISSEK is that one really has consider infection of the whole organism; maybe when viruses infect an animal they may start their replication in macrophages for example which do not normally undergo division. There may have a good supply of thymidine and thus a virus which can induce thymidine kinase has a selective advantage. It's the best I can do for a suggestion at short notice.

SYNTHESIS OF PROTEINS IN HERPES SIMPLEX VIRUS INFECTED BSC-1 CELLS

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SUMMARY

Analysis of ^{35}S-methionine labeled herpes simplex virions by electrophoresis in polyacrylamide gels revealed 12 distinct peptides by autoradiography. These bands corresponded to the 12 major bands detectable by staining with Coomassie blue. Electrophoresis of homogenates of infected cells labeled with ^{35}S-methionine for long periods revealed 12 major peptide bands which corresponded to the structural viral peptides and several nonstructural peptides. However, labeling for short periods with the radioactive amino acids revealed 39 peptide bands. These include the stable structural and nonstructural peptides as well as short-lived nonstructural peptides. Different rates of synthesis were found for the different peptides synthesized in the infected cells. Arginine deprivation affected the amount of peptides synthesized and only some of the structural peptides were synthesized. This might explain the lack of virion formation in the absence of arginine.

INTRODUCTION

Recent studies by Honess and Roizman (1973) using high resolution polyacrylamide gel electrophoresis described 49 peptides to be synthesized in herpes simplex virus (HSV) infected cells. These were 23 structural, 16 nonstructural, and 10 unclassified peptides, which comprise 75% of the genetic information inscribed in HSV-DNA (molecular weight 100 x 10^6 daltons (Becker, et al 1968)) assuming asymetric transcription.

In the present study, we used ^{35}S-methionine to label the peptides synthesized in HSV infected cells which were studied by radioautography after electrophoresis in acrylamide gels. From the analyses, we concluded that 39 peptide bands can be detected in the electropherograms. However, only 12 major peptides were detected in the analyses of purified virions by radioautography. The additional 10 bands were detected by Coomassie blue staining (Becker and Olshevsky, 1972) since they were hardly labeled by ^{35}S-methionine. Thus, attention was focused on the major viral structural and nonstructural peptides synthesized in HSV infected cells. In addition to the 12 major virion structural peptides, 9 nonstructural peptides were detected. The rest, 18 peptides were found in minimal amounts which did not allow accurate determinations or were detected only after short pulses with ^{35}S-methionine, indicating short lived peptides.

RESULTS

Electropherograms of infected cells and purified virions

a. Purified virions

Analysis of ^{35}S-methionine labeled purified herpes simplex virions by radioautography, revealed the presence of 12 major structural peptides (Roman numerals in Fig. 1). These labeled peptides are identical with the 12 major peptides which were detected by staining electropherograms of purified HSV with Coomassie blue (Becker and Olshevsky, 1972). In the stained electropherograms, 10 additional peptide bands were detected (which stained very faintly) bringing the total number of virion-associated peptides to 22. This is in accord with the 24 peptides described by Gibson and Roizman (1974). However, the absence of these 10 minor peptide bands from the radioautograms of purified herpes virions may indicate that they are present in small amounts, undetectable by the present technique, or they are contaminants which the virions obtain from the infected cells.

The molecular weights of the peptides detected by radioautography were determined (Table 1) by their position in the gels in respect to markers myosin M.W. 210.000, bovine albumin M.W. 67.000, egg albumin M.W. 45.000, and pepsin M.W. 35.000. Peptide 15, the major capsid protein of the virions (previously designated II and also VP5 by Honess and Roizman(1973) has a molecular weight of 190.000 daltons in our analyses and 155.000 daltons as determined by Honess and Roizman (1973). The discrepancies could be accounted for by differences in the positioning of the viral peptides in respect to the markers used for the construction of the reference gel. Nevertheless, the present estimations of the molecular weights of the viral structural peptides are higher than those published previously by us (Olshevsky and Becker, 1970 a; Becker and Olshevsky, 1972). If we sum up the total molecular weights of the 12 major viral structural peptides which were detected in the analysis of purified virions, it comes to 1.55×10^6 daltons. This requires 28% of the genetic information encoded in one strand of HSV-DNA (5.6×10^6 daltons unique amino acids) assuming assymetrical transcription.

b. Infected cells

In Figure 1, we compared the structural virus peptides with the peptides detectable in the infected cells and the cytoplasmic and nuclear fractions. A total number of 39 peptides were detected in electropherograms of cells which were labeled for long and short periods. Certain peptides were detected only after very short pulses with ^{35}S-methionine and comprise the group of unstable,presumably nonstructural, peptides. The peptides detectable after long labeling periods, are the stable, presumably the structural peptides. The total number of 39 labeled peptides which appear in the infected cells can be compared to 49 peptides (described by Honess and Roizman (1973). As can be seen from Figure 1, 21 peptide bands were detectable in the electropherogram of infected cells which were labeled with ^{35}S-methionine for 15 hrs (throughout the virus growth cycle). Peptides numbered 6 (M.W. 310.000), 9 (M.W. 260,000), 13 (M.W. 210.000), 14 (M.W. 200.000), 17 (M.W. 180.000), and 33 (M.W. 45.000), are nonstructural stable peptides which can be found in the infected cells and their function if not yet known.

Table 1. Herpes Simplex Virus Peptides

N°.	Peptide M.W.* x 10^{-3}	Honess & Roizman**	Becker & Olshevsky***	Rate of Synthesis during Infection ****		
			Structural Peptides	I	D	C
1						
2	350					
3						
4	320	VP 1-2	I			+ NS
5	320					
6	310					
7	285	3				
8						
9	260					
10	240					
11	230					
12	220					
13	210					
14	200	VP 4	IIa			+
15	190	VP 5	II capsid		+	
16						
17	180				+	NS
18	165	VP 7	III a		+	
19	150	VP 8	III glycoprotein	+		
20	140					+ NS
21	130				+	NS
22	120			+		NS
23	110	VP 12	IV a		+	
24	100	VP 14	IV glycoprotein	+		
25	94					+ NS
26	85					
27	80					+ NS
28	74	VP 15	V glycoprotein			+
29	70					
30	60	VP 16	VI capsid	+		
31	55					
32	51					
33	45		VII internal	+		
34	41				+	
35	36				+	NS
36	33	VP 22	VIII capsid	+		
37	31	VP 23			+	NS
38	27				+	NS
39	21		IX			+

* Total molecular weight 5.504.000 (total (calculated) 5,6 x 10^6 daltons of unique amino acid sequences) Total major viral peptides 1.550.000 Daltons = 28% of the information in the viral genome.

** J. Virol. p. 1352, Figure 3, 1973

*** Oncogenesis and Herpesviruses. Eds., P.M. Biggs, G. de The, & L.N. Payne, p. 421, 1972. I - increase, D - decrease, C - constant, VP - viral peptide, NS - non-structural peptides

**** Calculated from Figure 3 (Arginine +)

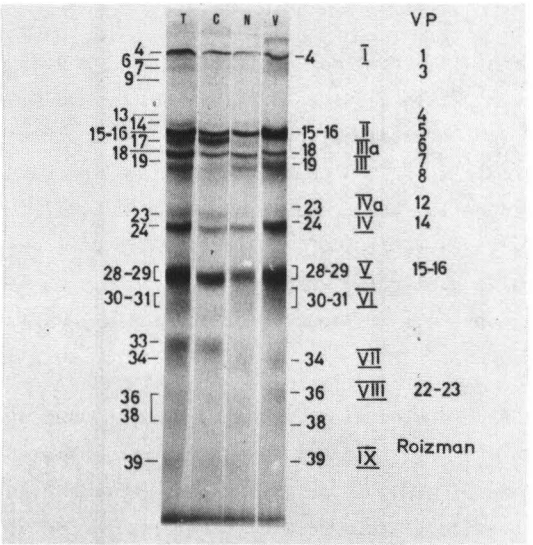

Figure 1 : Electropherograms of HSV infected, ^{35}S-methionine labeled, cells and virions. BSC_1 cell monolayers were infected with HSV (10 pfu/cell) and labeled for 15 hrs (starting at 3 hrs p.i.) with ^{35}S-methionine (10 μCi/ml) in Eagle's medium containing $^1/_{40}$ of the regular methionine concentration ^{35}S-methionine (specific activity of 100 Ci/mmole) purchased from the Radiochemical Centre, Amersham, England. The infected cells were harvested by scraping into reticulocytes standard buffer (RSB) and sonicated. Part of the cells were homogenized in a Dounce homogenizer and the nuclei were separated by centrifugation (800xg for 5 min) and resuspended in RSB. HSV was purified from nuclei on infected cells by centrifugation in sucrose gradients (12 - 52 % w/w) as previously reported (Becker and Olshevsky, 1972). Samples from homogenized cells (T) cytoplasmic (C) and nuclear (N) fractions as well as from the purified virions (V) were electrophoresed in 7,5% polyacrylamide gels as previously described (Olshevsky and Becker, 1970a). The gels were sliced longitudinally, dried on a filter paper, and put in contact with X-ray film. The exposed film was developed and the bands were detected. The arabic numbers (4 to 38) designate the position of bands detectable in a number of analyses. Missing numbers indicate peptides synthesized and detectable only during short pulses with ^{35}S-methionine. The roman numerals designate the major viral structural peptides previously detected in analyses of purified virions (Becker and Olshevsky, 1972). VP designate the virus structural peptides determined by Honess and Roizman (1973).

It is of interest that peptides 6, 9, 13, 17, 23 and 33 are found mainly in the cytoplasm, while peptide 19 (M.W. 150.000) is detectable mainly in the nuclei of the infected cells. The other peptides can be found in the nuclei and cytoplasm. Of the peptides which are confined to the cytoplasm of the infected cells, the function of peptides 6, 17, and 33 are not yet known. Peptide 23 (M.W. 110.000) is peptide IVa of the virion structural peptides. Peptide number 19, which is found mainly in the nuclear fraction, is a glycoprotein previously designated III, which is present in the virion envelope.

The nature of the labeled peptides which band in the upper part of the polyacrylamide gel (peptides 1 to 9) and have high molecular weights (from 350.000 to 260.000) is not yet known. Peptide 4 (M.W. 320.000) which is the major peptide in this region, can be found in both the cytoplasmic and nuclear fractions of infected cells as well as in purified virions. The function of these peptides in herpes virions is still to be studied.

c. Quantitative analyses

The long labeling of HSV infected cells with ^{35}S-methionine allows the analysis of the long-lived viral peptides (Fig. 2). It was found that the viral structural peptides were detected, as well as several nonstructural proteins. The profile shown in Figure 2 can be used for the computation of the quantity of the different peptides by determining the area underneath each peak. This analysis is not very accurate because some peptides band in close proximity to each other. However, it provides a useful analytical tool.

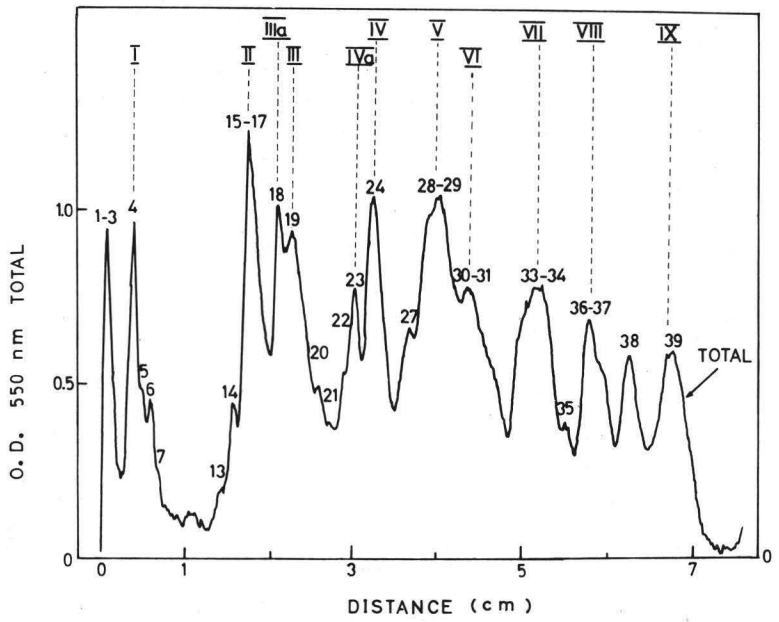

Figure 2 : Radioautography of ^{35}S-methionine labeled herpes simplex virus proteins synthesized in infected cells. BSC_1 cell monolayers were infected with HSV (10 pfu/cell) and labeled (3 to 18 hrs post infection) with ^{35}S-methionine (10 µCi/ml; specific activity 100 Ci/mmole purchased from the Radiochemical Centre, Amersham, England) in Eagle's medium containing $^1/_{40}$ of the regular methionine concentration. At the end of the labeling period, the cultures were washed with reticulocyte standard buffer (RSB) (10^{-2}M Tris., 10^{-2}M KCl, 15 x 10^{-4} MgCl, pH 7.7), scraped into 1 ml RSB and sonicated for 1 min. The sample was treated with sodium dodecylsulfate, urea, and 2 mercaptoethanol. The samples were electrophoresed in 7.5% polyacrylamide as previously described (Olshevsky and Becker, 1970). At the end of the electrophoresis, the gels were fixed in acetic acid (7.5% w/v), sliced longitudinally, dried and exposed to X-ray film for several days. The film was developed and scanned in a Gilford Spectrophotometer and the optical density of the bands was determined at a wavelength of 550 µm.

Analysis of proteins synthesized at different stages of the virus growth cycle

To obtain information on some of the short-lived virus coded peptides, the PSV infected cells were labeled with ^{35}S-methionine for 3 hr periods during the virus growth cycle. At the end of the labeling period, the cells were harvested, homogenized by sonication, and analysed by electrophoresis in acrylamide gels. A similar labeling procedure was done with HSV infected cells which were maintained in an arginine deficient medium, to study the effect of arginine deprivation on the synthesis of the viral structural and nonstructural peptides (Fig. 3). Every electropherogram was scanned in a Gilford spectrophotometer and analyzed by computing the area underneath each band of the labeled peptides.

The results presented in Figure 3 (Arginine +) demonstrate that a large number of peptides (29) were synthesized in the infected cells. The additional 10 peptides were detected only when short pulses with ^{35}S-methionine were given to the infected cells (not shown). Most of the peptides were synthesized throughout the virus growth cycle, but differed in the rate of synthesis (Fig. 3). This was determined by a quantitative analysis of the areas underneath each band. The different rates of synthesis of the various peptides are presented as arbitrary units of area, which indicate the relative amounts of peptides synthesized. The peptides were divided into three major groups (Fig. 4). Group A constitutes those peptides (Fig. 4 A) having a rate of synthesis which increases during the growth cycle. In this group, we find peptide 19 (glycoprotein III), 23 (peptide IVa), 24 (peptide IV glycoprotein), 30 (peptide VI, a capsid peptide), 33-34 (peptide VII, internal arginine rich protein), and 36 (peptide VIII, a capsid protein VIII). The latter peptide differs from the others in that it decreases in amount towards the end of the virus growth cycle.

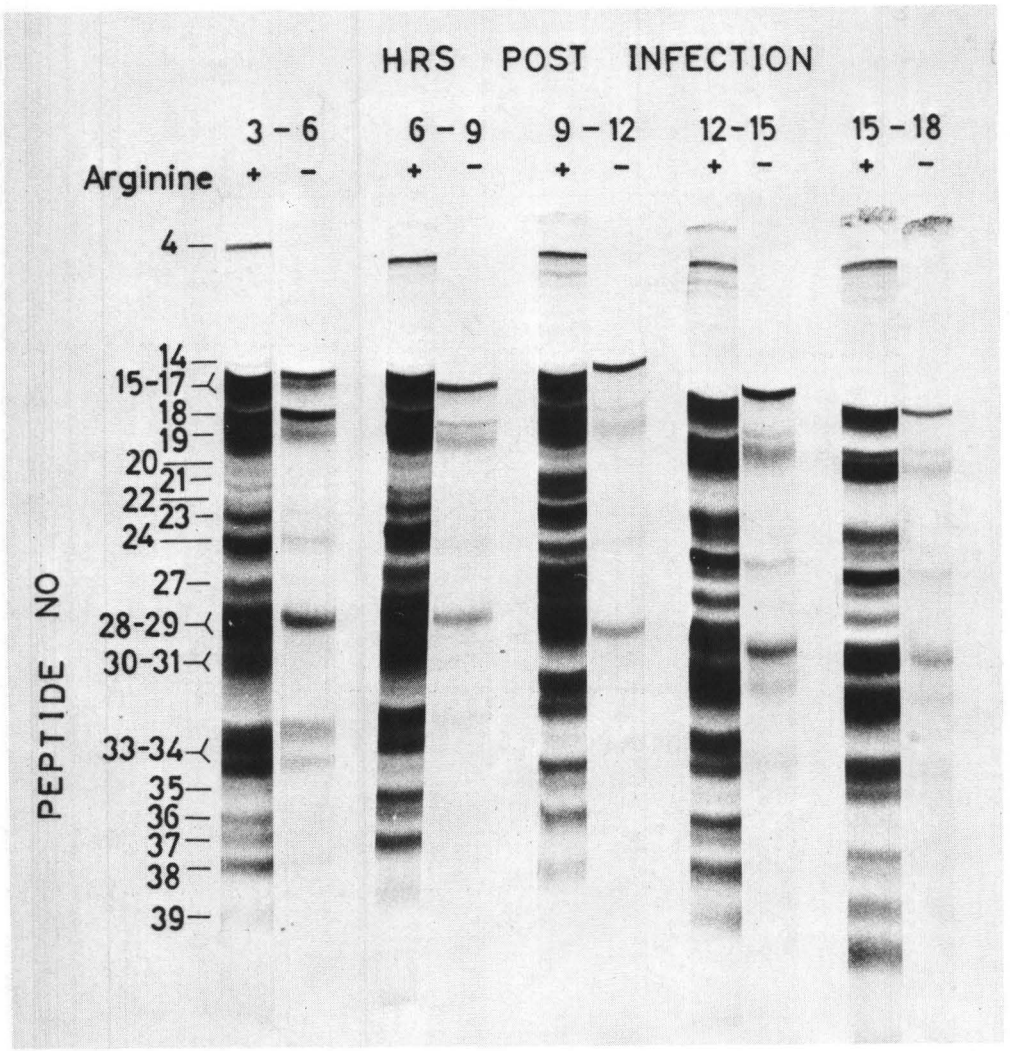

Figure 3 : Synthesis of proteins during different stages of the virus growth cycle in the presence and absence of arginine. BSC_1 cultures were infected with HSV (10 pfu/ml) and incubated for 3 hrs at 37°C. The medium was then removed and replaced in 5 cultures with Eagle's medium containing arginine and $^1/_{40}$ methionine. In another group of 5 cultures the medium was replaced by Eagle's medium containing $^1/_{40}$ methionine but without arginine. To each culture, ^{35}S-methionine was added for a 3 hr period starting at 3, 6, 9, 12, and 15 hrs after infection. At the end of the labeling period, the cells were washed, harvested, sonicated, and the samples were electrophoresed in polyacrylamide gels. Radioautograms were prepared as described in the legend to Figure 1.

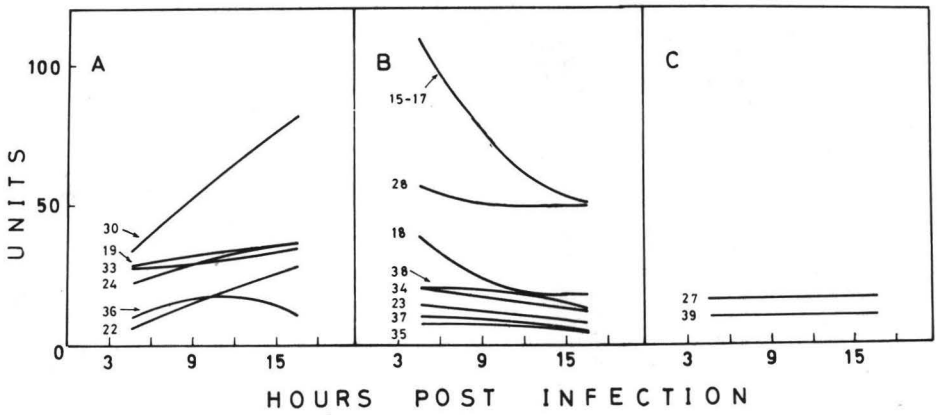

Figure 4 :

Quantitative analysis of electropherograms presented in Figure 3. To obtain quantitative data on the peptides synthesized in HSV infected cells, the radioautograms were scanned in a Gilford Spectrophotometer at a wavelength of 550 µm and the tracings were analyzed by computing the areas underneath each band with the aid of graf/pen sonic digitizer and a PDP-15/20 computer.

Group B peptides (Fig. 4 B) are characterized by a high rate of synthesis early in infection and a gradual decrease thereafter. These peptides are : 15-16 (capsid protein II), 17 (nonstructural (NS) peptide), 18 (virion associated peptide, III a), 21 (NS peptide), 23 (peptide IV a, virion associated), 34 (NS peptide), 35 (NS peptide), 37(NS peptide), and 38 (NS peptide). It is of interest that the major structural peptides II, III a, IV a, which are part of the viral capsid were synthesized at high rates early in infection and the rate decreased toward the end of the virus growth cycle (Fig. 1). The other peptides are nonstructural peptides.

Group C peptides (Fig. 4 C) are synthesized at a constant, relatively low rate, and consist of peptide 4 (NS peptide), 14 (peptide IIa, virion associated), 20, 25, 27, 39, (NS peptides), and 28-29 (peptide V, virion glycoprotein) (Table I, Fig. 4 C).

Effect of arginine deprivation on the synthesis of viral proteins

The results presented in Figure 3 (argine -) reveal that HSV infected cells synthesize less viral structural peptides than cells which were incubated in arginine containing medium. Peptides 15-17, which are the capsid protein II, were synthesized in detectable amounts throughout the virus growth cycle. The same is true for peptide 18 (peptide III a), 19 (glycoprotein III), 24 (glycoprotein IV), 28-29 (glycoprotein V), and 33-34 (peptide VII). Other peptides, especially the high molecular weight peptides (1 - 14) were not detectable by the present technique in the absence of arginine in the medium.

Virion associated peptides :

With the aid of ^{35}S-methionine and radioautography of polyacrylamide gel electropherograms it was possible to determine the number of the peptides present in the purified virions. This analysis was compared to previous analyses in which the virion associated proteins were determined by Coomassie blue staining. The latter technique revealed 12 major and 10 minor peptides. In the radioautograms of ^{35}S-methionine labeled peptides, the 12 major peptides were detectable while the 10 minor ones were not. It is not clear whether this is because the 10 minor peptides were not synthesized or whether the exposure time of the polyacrylamide gels was not sufficient, or the methionine content of these peptides is low. Whatever the explanation may be, this result suggests that the 12 major peptides are the structural peptides of the virion. Some of the 10 minor peptides correspond to certain nonstructural peptides found in the infected cells, which suggests that they may have been taken up nonspecifically by the virions.

The 12 major viral peptides are similar to the 12 peptides (VP) described by Gibson and Roizman (1974) and Honess and Roizman (1973). The visible peptide bands which can be seen in their autoradiograms, may also be regarded as the major viral peptides (Table 1). It seems that the same viral peptides were detected by Roizman's and by our groups.

Synthesis of viral peptides in HSV infected cells :

Analyses of electropherograms of HSV infected cells labeled for short and long periods with ^{35}S-methionine revealed a total of 39 peptides. Of these peptides, 14 were with molecular weights higher than 200.000 daltons. This is in contrast to Honess and Roizman (1973) who found only three peptides with molecular weights higher than 200.000 daltons. This difference could be accounted for either by the difference in the cells used for the study (BSC_1 in this study and HEp-2 by Honess and Roizman (1973)) or due to the difference in the virus strains.

In addition, in the analyses of Honess and Roizman (1973), the different peptides were better separated from each other than in the present study.

Labeling of cells for long periods (e.g. 15 hrs, Fig. 1 and 2) revealed the stable virus structural peptides and only a few stable nonstructural peptides like peptide 17 (M.W. 180.000) which was synthesized in large amounts shortly after infection and its rate of synthesis then gradually decreased. The nature of these peptides is not yet known. Labeling for shorter periods of time revealed more peptides in the infected cells. This indicates that certain viral structural peptides are synthesized throughout the virus growth cycle at a diminishing rate.

The total molecular weight of the genetic information encoded by HSV-DNA corresponds to 5.6×10^6 daltons of unique amino acids (Olshevsky and Becker, 1970 a; Honess and Roizman, 1973). The sum of the molecular weights of the 39 peptides described here, was calculated to be 5.5×10^6 daltons. If all the peptides synthesized in the HSV infected cells are indeed unique viral peptides, then almost all the viral genome is transcribed and translated in the infected cells. Further studies are still needed to identify the function of each nonstructural peptide and its relation to the virus replicative cycle.

Effect of arginine deprivation on the synthesis of viral proteins :

Previous studies on the effect of arginine deprivation on the replication of herpes simplex virus (Becker et al,1967; Alshevsky and Becker, 1970 b) showed that HSV-DNA is replicated in infected cells incubated in an arginine deficient medium although virions are not formed. Analysis of ^{35}S-methionine labeled HSV infected cells by electrophoresis in acrylamide gels revealed that most of the viral structural and nonstructural peptides were synthesized in the absence of arginine (as was also shown by Mark and Kaplan, 1972). The proteins were transported from the cytoplasmic to the nuclear fractions of the infected cells (not shown). However, the synthesis of certain peptides was markedly affected while others were affected to a lesser extent. Under these conditions, virions are not formed.

ACKNOWLEDGEMENTS

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We are indebted to Dr. G. Zeichek for his help in the use of the computer program.

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DISCUSSION

Dr. DALSGAARD

I just wondered, Dr. Becker, for the sake of resolution in polyacrylamide gel electrophoresis is a very complex mixture to separate. Have you considered the use of two-dimensional polyacrylamide gel electrophoresis, possibly also including a gradient in the gel ?

Dr. BECKER

You are absolutely right that the resolution could be improved by using better techniques, but we wanted to get an idea first of all from a technique that we know and we have used for quite a while. We plan to extract from the gels the heavy bands and resolve it by a different technique to find how many different peptides are present in each band. One of the methods is the hydroxy apatite resolution of SDS treated proteins.

Dr. VAN OIRSCHOOT

I have just read in your introduction that 75% of the viral genome is transcribed, and this is in contradiction with the book of Tooze where they say only 50% of the viral genome is transcribed during the whole efficient cycle, in human epithelium cells.

Dr. BECKER

What we could do obviously is to calculate the total amount of information that is present in a 50 million daltons molecular weight single-stranded DNA, assuming that the single-stranted DNA is transcribed. The total amount of unique amino acids coded by HSV is 5,6 million daltons. Now if we sum up all the peptides that we have seen here, we obtain a figure which is about 100% of that - 5,5 million daltons molecular weight. However, we have the feeling that some of the peptides, especially those with molecular weights higher than 200.000 daltons (Peptide No. 2 is 200.000 molecular weight) might be cellular proteins, and we are not absolutely sure if this is a group of cellular membrane glycoprotein. We do not know if the herpes virus really completely blocks the synthesis of cellular proteins.

Dr. SCHOLTISSEK

Do you have any indications whether some of the non-structural proteins are precursors of structural proteins - this means that they might be split to structural proteins ?

Dr. BECKER

We did not find precursors to the viral peptides.

Dr. MCAUSLAN

Dr. Horzinek raised the possibility of using frog viruses as tools to switch off the synthesis that we have done and then to look at the synthesis of other viral proteins and all I can do is to recommend to try in your system that most of the frog viruses have very funny effects on different systems, for example, it would inhibit right messenger transcription in the cells readily infected with pox virus that would not inhibit messenger which is transcribed from coated cores; we don't know what it does to herpes virus systems, but my guess is it would probably stop it because it does inhibit first ARN polymerase activity fairly extensively so it is said the question is reliable to influenza virus activity; I have two conflicting reports, one group says that free infection with frog virus will imply polymerase activity and also other enzyme implications, and another group said it doesn't, so it would be interesting for some expert to do this, but anyway it might be an interesting tool and even for something like African swine fever it might be worthwhile to try with just all the background of the first proteins and then infection with African swine fever and then with the polypeptides simply synthesized.

The technique of quantitative immunoelectrophoresis (crossed (immunoelectrophoresis) and its application in the study of viral antigens

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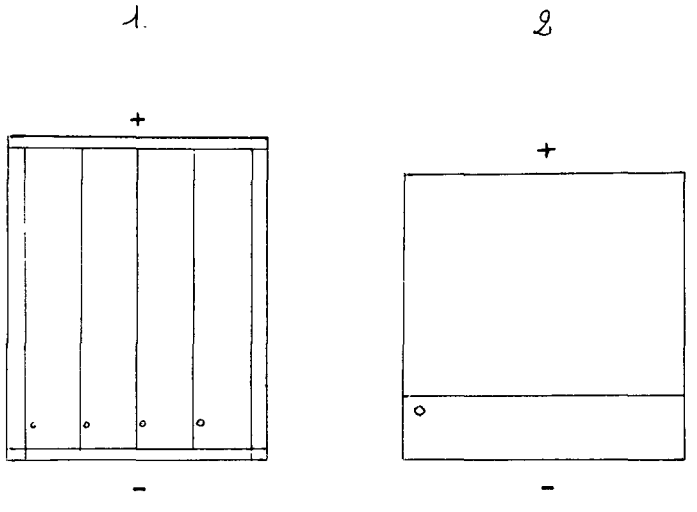
Crossed immunoelectrophoresis was introduced in 1965 as an analytical tool for the study of human serum proteins (1). The technique was modified the following year (2), and recently a team from the Protein Laboratory at the University of Copenhagen have published a book on the method (3), and a lot of sophisticated modifications.

In principle the method is a combination of electrophoretic separation of proteins in agarose gel and subsequent electrophoresis in an antibody-containing gel. The macro-technique adapted by us is performed as follows : 15ml of a heated 1% agarose solution (55°) in barbital buffer is poured onto a glass plate (90 x 110 x 1,5 mm) placed on a horizontal table. When the gel is congelated four wells are punched. After placing the plate in an electrophoresis aparatus with a cooling plate, the samples are applied and the plate is connected to the buffer vessels by means of paper wicks. First dimension electrophoresis is carried out using 8 V/cm. The temperature of the cooling water is 15°C, and the electrophoresis is run for 1½ hour. By means of a 15 cm long razor blade a 5 mm broad edge all round the gel is cut away. The rest of the gel is divided into four slabs (20 x 100 mm), and each of the slabs is transferred to an agarose coated glass plate (100 x 100 x 1,5 mm). The glass plate is placed on the horizontal table and 12 ml of an antibody-containing gel (55°C) is poured onto the rest of the plate, adjacent to the first dimension slab. After congelation the second dimension electrophoresis is carried out using 1,5 V/cm, at 15°C overnight. After the electrophoresis the gel is pressed (filter paper), washed, pressed, dried and stained by Coomassie brilliant blue, R. 0,5% solution in 10% solution of glacial acetic acid in 50% ethanol.

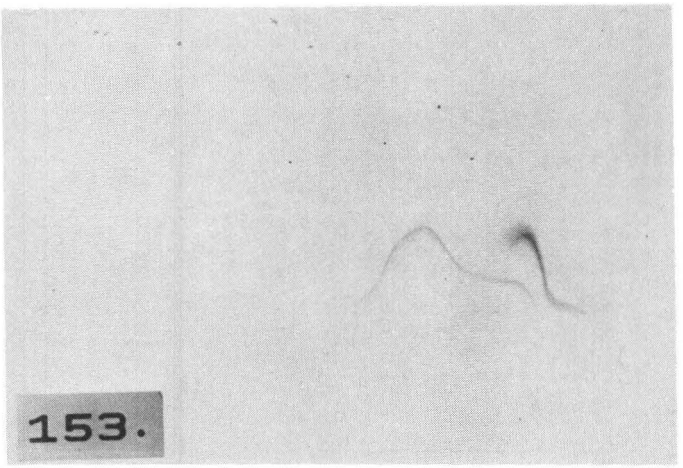
Destaining is performed in the solvent. By means of pressing and swelling the gel, the whole procedure can be carried out in the course of 2 hours, which means that if you start the first dimension electrophoresis in the afternoon of day 1, you will obtain the final result at noon of day 2.



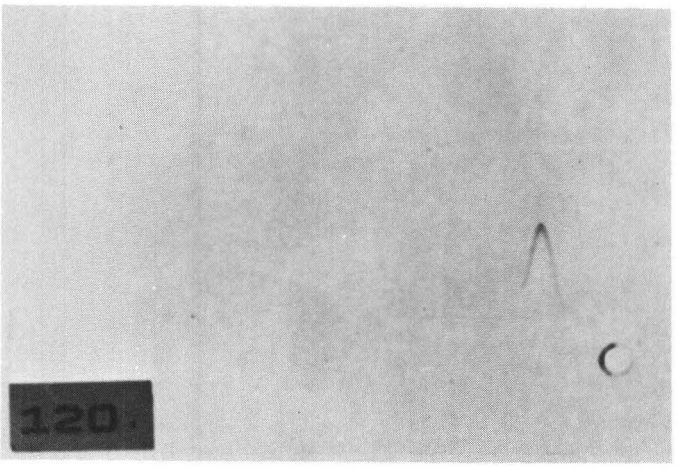
Crossed immunoelectrophoresis is superior to the classical immunoelectrophoresis according to Grabar and Williams because it provides a more powerful resolution. Furthermore, the area enclosed by an individual precipitate is proportional to the antigen/antibody ratio of the system. With a standard polyvalent antiserum it is thus possible to separate and quantitate more than 30 serum proteins by a single run.

Crossed immunoelectrophoresis has recently been used in the characterization of herpesvirus hominis type 1 and 2 antigens (4). In our laboratory we have adapted this technique for the study of the antigens of HCV and BVDV.

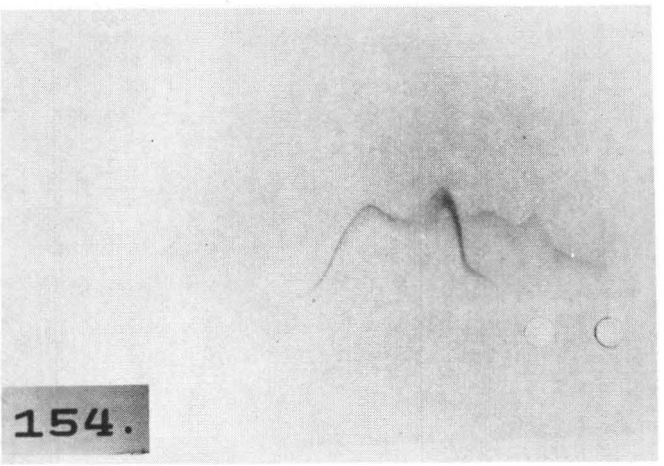
The antigens are obtained in the following way : Monolayers of susceptible cells are infected with the virus. 48 hours after infection (maximum fluorescent titre) the cells are scraped off the glass, packed and treated with a 10% solution of triton X-100 in neutral tris buffer. The mixture is sonicated and centrifuged at 100.000 g for one hour. The supernatant contains the solubilized antigens, and it is used in crossed electrophoresis without further purification. Control plates are always included. The control antigen is the same amount of non-infected cells prepared as described above. Using a hyperimmune serum against HSV (prepared in Brescia by Nardelli) it is possible to demonstrate 3 antigens specific of HCV. The same antiserum reacts with one antigen specific BVDV, and by means of a modification of the crossed electrophoresis called tandem electrophoresis (3) it has been demonstrated that this antigen of BVDV is identical to one of the antigens of HCV.



Antigen : 15 µl HSV (see text for preparation)
Antibody : 500 µl swine anti HCV (Nardelli)



Antigen : 15 µl BVDV (see text for preparation)
Antibody : same as above



Antigen : left hole 15 µl HCV; right hole 15 µl BVDV
Antibody : same as above

Note the fusing to a double peak indicating identity.

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DISCUSSION

Dr. KNORN MELGAR

You can also do this technique in slides and you can produce the slides so stained that you don't need to make a photograph on your electropherograms. I also used this technique for characterising corona viruses, TG virus, immunoglobulins specific and I only used the slides in LKB diffusion chamber and that goes very well. But I was a little worried about your first picture because you said that the immunoglobulin was at a very high level, and I don't think so. Could I see once your first diapositive ?

Dr. DALSGAARD

This is true. You can make it directly and put it into the projector but for the 10 x 10 plate you need a large projector.

Dr. KNORN MELGAR

But I wondered about what you said. How much time do you electrophorese it ? Eight hours, or longer ?

Dr. DALSGAARD

In the second dimension ? I electrophorese overnight. And then once the precipitates have formed then they are precipitated.

Dr. KNORN MELGAR

Do you characterize also the immunoglobulins ?

Dr. DALSGAARD

You can characterize the immunoglobulins, but then you have to alter the electric net large of the immunoglobulins because the reason why you can use this technique is that the immunoglobulins, namely the IgG fraction, in the second dimension at this pH of 8.6 they will not move, whereas the antigens they will move under these conditions and of course this counts for the IgG - here, it will not move the correct way so you cannot determine the correct amount of IgG in this system.

This you can do in another way. If you carbamylate your IgG fraction or you can carbamylate your antiserum here, then you will alter the electric net charge and you can alter the pH of the medium to have a pH of 6 or 5 where the immunoglobulines will move up.

Dr. KNORN MELGAR

But that doesn't comply with your first slides.

Dr. DALSGAARD

IgG is a heterogeneous population. There is anodic IgG and cathodic IgG and what you see in my picture is the anodic IgG.

Dr. KNORN MELGAR

But you must also see the anodic IgG.

Dr. FROST

I have a question concerning your second graph with the Hog Cholera antigen. I think you have only two antigens demonstrated and one with a dimeric form showing identity.

Dr. DALSGAARD

I agree, this is one possibility that a dimeric form may have another electrophoretic mobility in the system and then you will see them fuse in the system. This is a great possibility, but as I told you, these data are very preliminary and we have a lot of work to do. This is a good explanation, I believe it too.

Dr. HORZINEK

Could you comment on the sensitivity of the method with respect to microgram nitrogen detected antigen ?

Dr. DALSGAARD

This I could not tell you because I am not familiar with the microgram antigen detectible but I can say it's at least five to six times more sensitive than normal immuno diffusion because this is a forced transmission of antigens. In normal immuno diffusion, you will have a circular diffusion to all sides but in this system you just have one unidirectional movement of the antigens and this means alone six times more sensitivity.

Dr. CORTHIER

You say that it is more sensitive. I don't understand why. I agree if you say that it is more precise and quantitative but sensitive I don't agree because you delute this more than in a simple electrophoresis.

Dr. DALSGAARD

No, you include quite a lot of antibody into your gel in the second dimension - about half a milliliter in the second dimension - for a normal two microliters serum sample in the first dimension. In normal electrophoresis in the long well you only have about 50 microliters for two microliters in the first dimension, so this corresponds in sensitivity very well to the normal technique, but it is five times more sensitive because it's a unidirectional movement of the antigen, if you have a normal hole for immunodiffusion, your substances will diffuse radially out and you have your antibody here. Only this antigen will be available for precipitation. The rest of it is lost, whereas in this system, the whole antigen is forced into the antibody.

Dr. VAN OIRSCHOT

These hog cholera and BVD-MD antigens, are they membrane associated antigens ?

Dr. DALSGAARD

This we do not know. We know that they are associated to the infection, because we see them in our infected cell isolates and we do not see them in control cells, but whether they are incorporated into the envelope or not, anyway, Dr. Matthaeus is probably going to argue that tomorrow.

Dr. VAN OIRSCHOT

How do you get the hog cholera antigen ?

Dr. DALSGAARD

We propagate the hog cholera in primary pig kidney cells. We could use PK 15 cells but they were not so good. After 48 hours of infection, we discard the supernatant and then we scrape the cells together and pack them so that we end up with 1 milliliter of infected cells from about 8 or 10 roller bottles which represents quite a large concentration. And then we treat with sonication and triton X-100.

Dr. MCAUSLAN

Dr. HORZINEK and I have just been talking about the possibility of using a radio labelling technique as well. Would it be possible to have the antigens labelled up with iodine or something ?

Dr. DALSGAARD

This has been done already for the studies of IgE antibodies in connection with allergic reaction. There is a group in Copenhagen working on that and since the IgE level is normally quite low, they have tried to label it with iodine, and you can make autoradiography of these plates, especially if you cool them which is a more sensitive technique.

SUMMARY OF THE SESSION

S. Schlesinger

I just would like to say a few words in summary of this afternoon's meeting. I was struck by the fact that we talked about a spectrum of different viruses this afternoon, but what came through at least to me in a very clear way were the number of different approaches that were used. These different approaches are a reflection of both the complexity of the viruses that are being studied and the extent of our knowledge. In the case of frog virus and herpes virus, it is clear that one is still in the stage of identifying the viral proteins both the number of viral specific proteins in the infected cells, the number of proteins is the virion itself, the time at which these proteins are being synthesized during replication, and if both the amounts of proteins and the time at which they are synthesized is regulated. In the case of influenza virus (and also in the case of Sindbis virus) it is now possible to localize these proteins in the cell, particularly in the case of the glycoproteins; it's possible to follow their movement between the smooth and the rough membranes. It has been clear both this afternoon and in the previous lectures that the use of inhibitors has been a very important approach to the study of viral replication. Inhibitors will be particularly valuable for the study of the viruses that are less well-known. The studies with in vitro protein synthesis are now just beginning to be explored. Techniques are being worked out so that this approach can be used to answer questions about viral replication, about viral RNA, and about the proteins of the virus. I think defective interfering particles will also turn out to be very valuable in analysing the different processes in viral replication. It will be important to use these diverse approaches in trying to obtain more information about hog cholera virus and African swine fever virus.

SESSION IV

VIRAL ANTIGENS

HOG CHOLERA VIRAL ANTIGENS (BRIEF REVIEW) AND SOME RECENT RESULTS ON THE RELATIONSHIP TO ENVELOPE ANTIGENS OF BOVINE VIRAL DIARRHEA-MUCOSAL DISEASE VIRIONS

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B. Roeder and J. Frost

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Although Hog Cholera (HC) antigens have been demonstrated by a variety of methods, little is known on the character of these antigens, their origin and their relation to viral structures. For a long time most of the studies pointed at the demonstration of soluble antigens by immunodiffusion techniques using pancreatic or spleen tissue from HC infected pigs as source of antigens. Since Molnar (1954) first published results which showed the presence of precipitating antibodies in pig sera, many attempts were undertaken to make the agargel diffusion techniques of use for the diagnosis of HC by either demonstrating antigens in organ tissues or antibodies in blood sera. Apparently there are still some laboratories in which the agargel diffusion test is believed to be of value for HC diagnosis when it is worked with pieces of pancreas or crude pancreas suspensions.

It was not earlier than 1966 when van Aert first described a method for extraction and partial purification of a precipitating HC antigen preparation and later opened the way for studies on the physico-chemical and biological characterization of organ derived soluble antigens (van Aert, 1970).

Although the agargel diffusion test had meanwhile lost its former significance for the practical diagnosis of HC after the introduction of the immunofluorescence technique applied to organ sections of HC suspected animals, Matthaeus et al. (1970, 1971) tried further to elucidate the nature and HC specificity of antigens which are demonstrable in pancreas tissue of HC infected pigs and which react with antisera prepared not only against HC virus but for comparison against the virus of Bovine Virusdiarrhea-Mucosal Disease (BVD-MD), too. An important result obtained by Matthaeus (1972) was the isolation of an antigenic component which showed identical immunoelectrophoretic behaviour when tested in parallel with BVD-MD antigen in the same run of experiment. Besides these HC and BVD-MD specific antigen-antibody reactions Matthaeus (1972) described unspecific precipitations of non-antigen-antibody character which can hardly be distinguished by agargel diffusion, and therefore he recommended the additional use of auxiliary techniques like immunoelectrophoresis. We probably can expect to hear more informations from Dr. Matthaeus which he is going to present in the following paper, on the nature of the soluble HC antigen and its relation to viral structures. But it may be said here already that future work on soluble HC antigens needs more efforts for the large scale production of HC virus in tissue culture in order to avoid reactions which are due to host components like enzymes or other metabolic substances present e.g. in pancreas tissue.
From the practical as well as from the theoretical point of view surface antigens of HC virions are at least as interesting as soluble antigens. Envelope antigens of HC virus offer the possibility to study antigenic strain differences even in relation to BVD-MD virus by neutralization techniques using tissue cultures as indicator system. The latter is important since it is known that BVD-MD virus infects pigs which might harbour the virus for a rather long period without development of any sings of illnes but with induction of antibodies (13, 14).

Within a WHO/FAO programme on comparative virology a working group on small enveloped RNA viruses deals with the differentiation of HC and BVD-MD virus strains by means of seroneutralization. One of the most interesting results obtained by Dr. Aynaud and his coworker, Dr. Corthier, was the finding of one strain of HC virus (No. 331) obtained from Dr. Mengeling (U.S.A.),

which reacted to a higher degree of neutralization with antiserum prepared against the BVD-MD reference virus strain NADL than other strains of HC virus. Though testing for antigenic variation employing Grade I - antisera prepared against purified strains of HC and BVD-MD virus in gnotobiotic pigs or calves, respectively, is important and certainly should be carried on, the question arises whether strain differences can be detected by the application of virus neutralization tests to sera from pigs exposed to virus by natural infections.

With this question in mind it should be allowed to report on recent investigations which we had the opportunity to carry out within in programme of serological surveys for the detection of latently pig breeding herds during the year 1972. At that time the epidemiological situation of HC in the Federal Republic of Germany was characterized by a slightly higher incidence of clinical HC than in the foregoing years, but did not lead to an extensive use of HC vaccines as it did in feeder lots during the second half of 1973. Therefore when the blood samples were taken the epidemiological situation could be described as not abnormal compared with the development experienced in 1973 when the number of clinical HC outbreaks rose tremend ously. Using a neutralization technique which might be simply called NIF test (neutralization and immunofluorescence test : Liess and Frey, 1971) and which employs constant virus doses, blood sera sampled in about 400 pig breeding herds were screened at an initial dilution of 1 to 5. Such herds in which one or more sera with neutralizing activity could be detected were called positive. For comparison an equal number of herds was randomly selected where no neutralizing sera could be found (NIF negative herds). Neutralizing titres of NIF positive sera were determined for HC virus strain Alfort as well as for BVD-MD virus strain 1138/69 which is a highly cytopathogenic strain and can be used in the microliter system as described previously (Frey and Liess, 1971). The qualitative results of neutralization tests with both viruses can be seen in Figure 1. If one considers only those sera from NIF positive herds which showed neutralizing activity against HC virus and the group of sera from NIF negative herds and compares the results of BVD-MD neutralization in both groups it can be seen that less half the percentage of sera in the NIF negative group shows neutralizing activity against BVD-MD virus compared with the NIF psoitive group of sera.

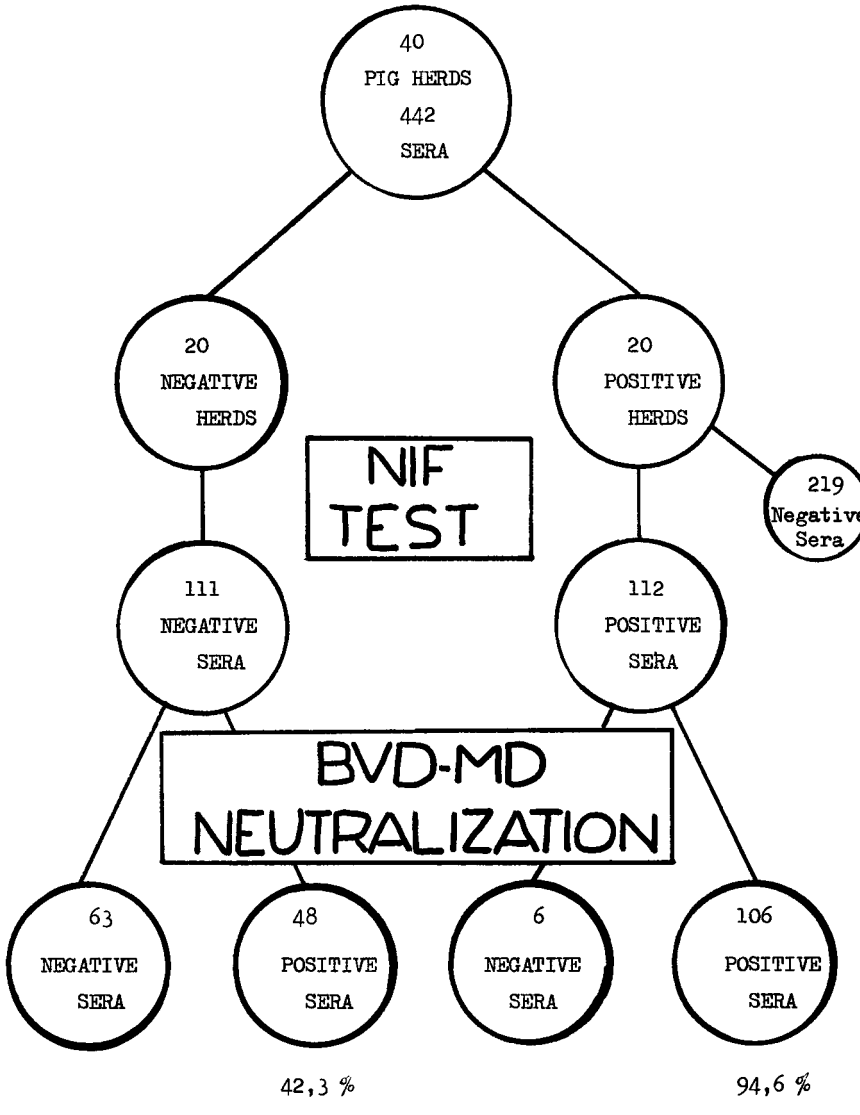

Figure 1 : Percentage of BVD-MD neutralizing antibodies in pig sera from the field with or without neutralizing activity against HC virus (strain Alfort)

These results justify the question whether the NIF test picked those sera which derived from pigs infected with HC virus previously and which cross reacted with BVD-MD virus or the other way around. An approach to answer this question might be offered by the comparison of the neutralizing titres of individual sera against both viruses. Figure 2 shows first the range of titres obtained with both viruses. The lines drawn in Figure 3 connect the titres measured for neutralizing antibodies against HC and BVD-MD virus in a total of 50 individual pig sera showing a rather scurile graph with all kinds of combination ranging from very low titres against one virus and high titres against the other one. Some sera showed titres which differed for less than one $\log_2$. Sera with complete absence of neutralizing activity against the other one, some of them piling up to 1/640 and more in the case of antibodies against BVD-MD virus, are not included in this figure.

It might be allowed to cast a view on experimental results described in the litterature with respect to measurable antibody formation after experimental infections of pigs with HC and / or BVD-MD virus. While some investigators were not able to demonstrate neutralizing antibodies against BVD-MD virus after primary HC virus inoculation before challenge (Figure 4), others were able to do so if they used virus strains which had undergone only a few passages in tissue culture after primary isolation (Figure 5). If we compare these experimental results with the results of serological testing of sera from pigs which were exposed to natural infections in the field, all these combinations are reflected (Figure 6).

One of the question which arises from the review of some published reports mentioned and which puzzles with regard to high titres of antibodies against BVD-MD virus and low titres against HC virus is whether the results published by Sheffy, Coggins and Baker (1961) reflect natural conditions in so far as HC infections in the field induce specific antibodies to a lower titre if BVD-MD antibodies were formed already and which rises to an even higher titre secondarily. If this is so future meetings like this one today can hardly devote to Hog Cholera only and treat BVD-MD virus infections in cattle as of secondary importance.

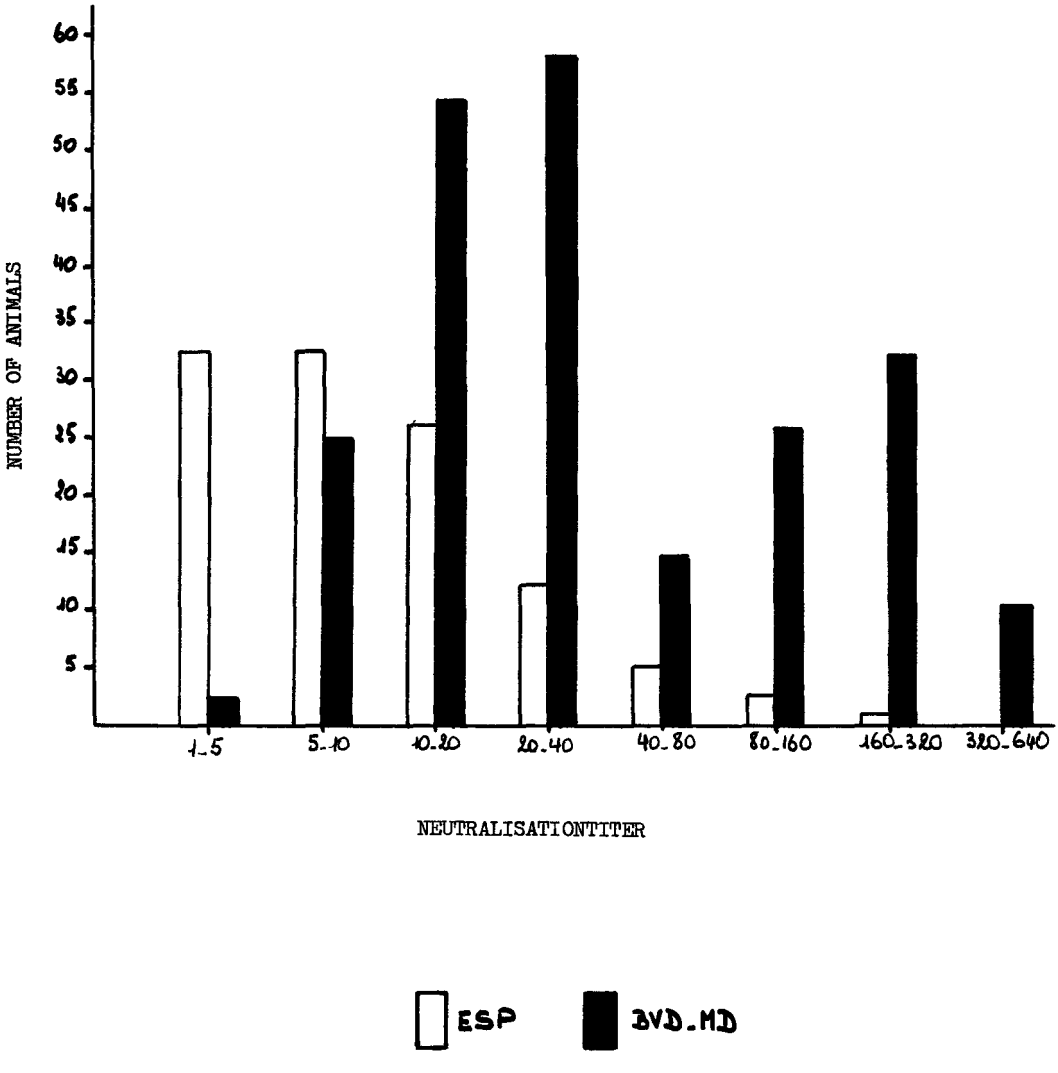

Figure 2 : Range of neutralizing titres against BVD-MD and HC virus, respectively, measured in pig sera from the field.

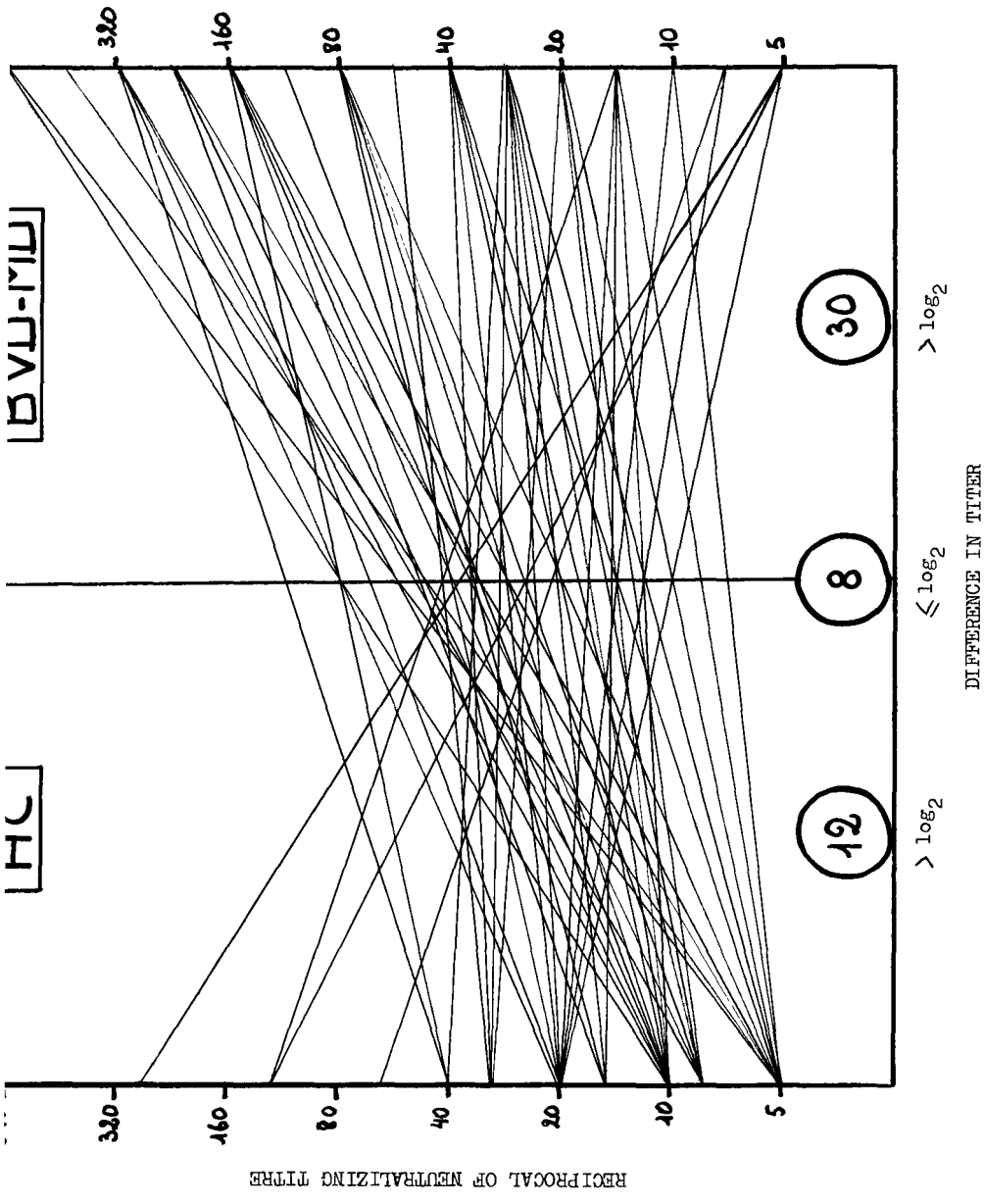

Figure 3 : Differences of neutralizing titres against BVD-MD and HC virus in fifty individual pig sera from the field.

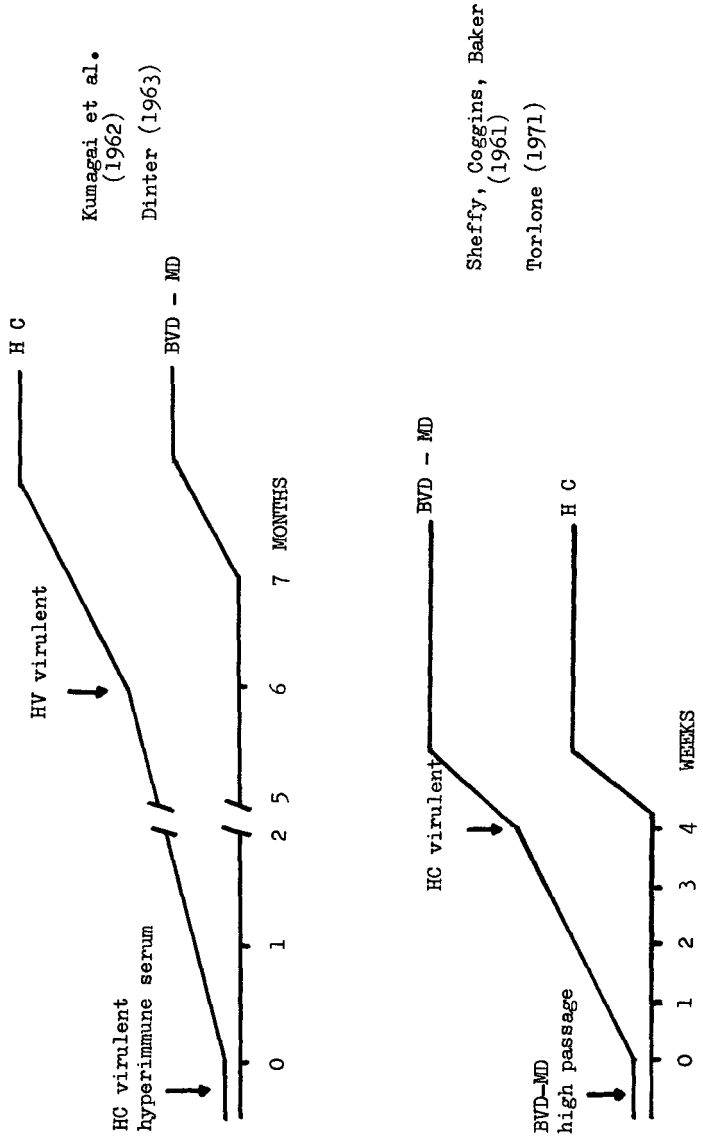

Figure 4 : Patterns of neutralizing antibody formation against HC and BVD-MD virus after experimental infection and booster by virulent HC virus of pigs.

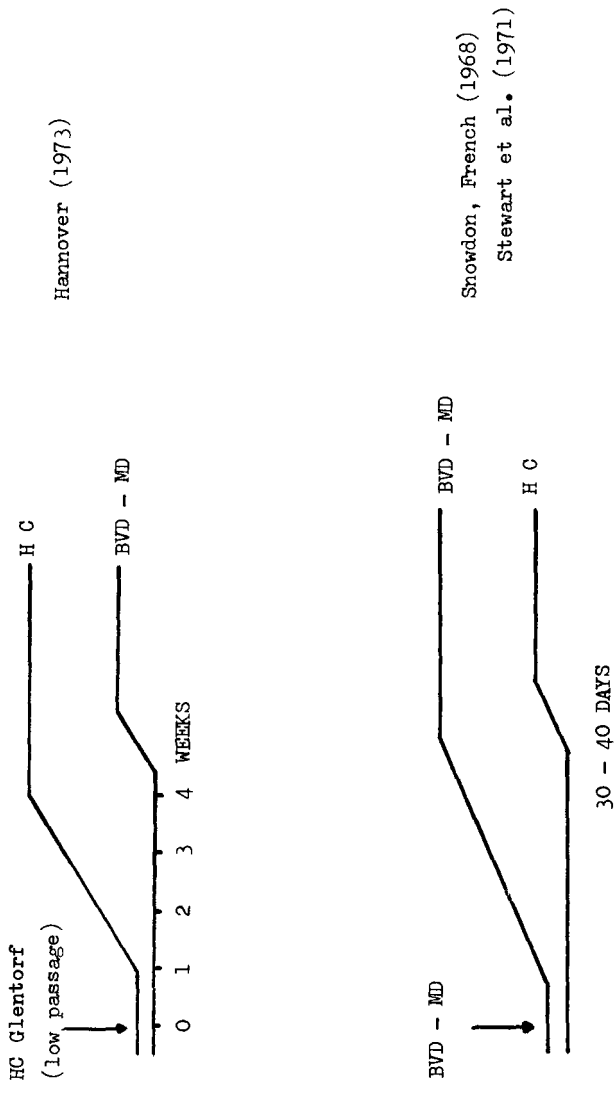

Figure 5 : Patterns of neutralizing antibody formation against HC and BVD-MD virus after primary inoculation of pigs with low tissue culture passaged HC or BVD-MD virus.

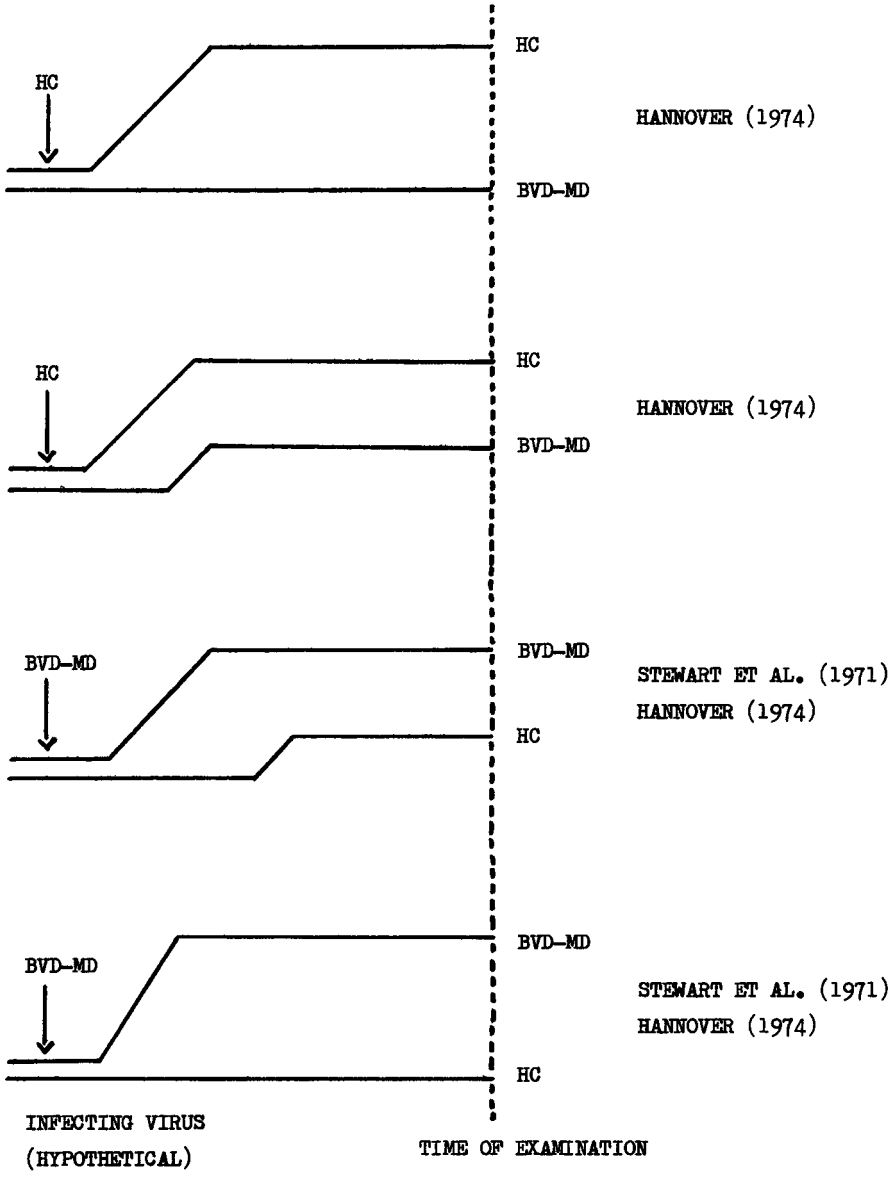

Figure 6 : Patterns of serological responses of pigs to hypothetical natural infections with HC or BVD-MD virus as revealed by differences of neutralizing antibody titres.

Finally for future work on antigenic variation of HC and BVD-MD virus the suggestion drawn from the results reported here might be given to use not only antisera from experimentaly infected pigs or calves but selected sera from the field, too, particularly those ones which show monospecific activity against one strain of virus.

Knowledge of the antigenic variation of HC virus and its relation to BVD-MD virus in pigs has some practical implications, especially with regard to the question of latent HC infections in pigs. This question employed us in the last few months during experiments designed to find out the percentage of pigs which are to be expected to react clinically to the Glentorf strain of HC virus after contact to inoculated pigs. The virus strain had been found to produce no clinical signs of illness in sows but to be able to induce malformations in foetuses. Of nine weaning pigs six developed febrile reactions of a few days duration and neutralizing antibodies first to HC virus and secondly with a delay of about 2 to 6 weeld to BVD-MD virus, too. The remaining four pigs died several weeks later sporadically after long periods of undulating fever and with no signs of any formation of neutralizing antibodies (Figure 7). We were not able yet, to evaluate all the leucocyte fractions collected in intervals in order to look for viremia. This is on the programme for the near future.

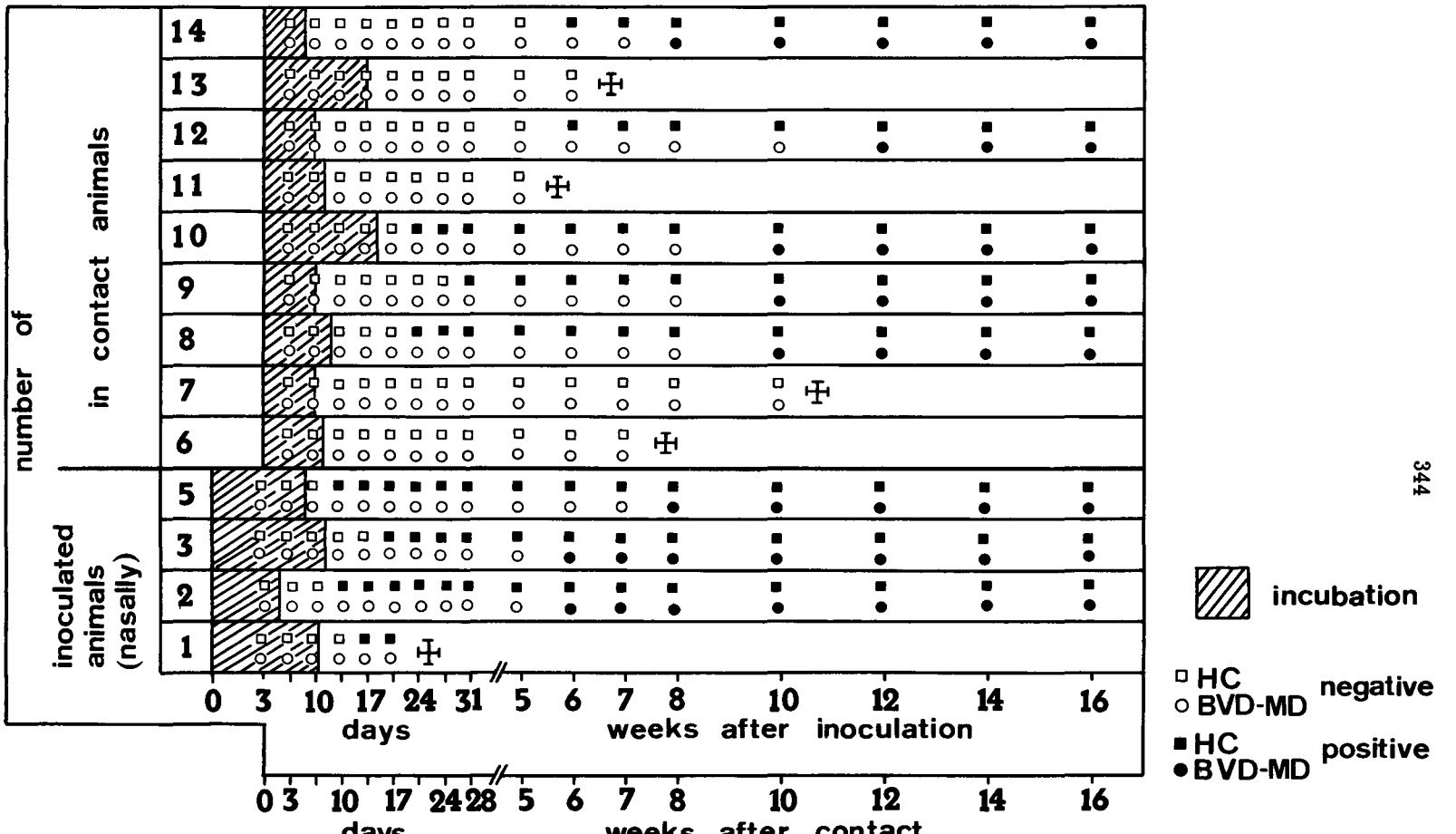

Figure 7 : Experimental HC infection, strain Glentorf, in pigs after nasal inoculation or contact and neutralizing antibody formation against HC and BVD-MD virus.

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DISCUSSION

Dr. NEWBY

Do you know anything about the antigenic response in these pigs which are not responding to the virus ? Do they respond to other antigens ? Have you tried to work on this ?

Dr. LIESS

No; we do not know this yet, but we have some indications from other experiments that they are able to respond, though I have not a clear proof, but we know that they are not agammaglobulinemic.

Dr. VAN BEKKUM

Does this glentorf strain produce normal immunofluorescence in the PK 15 cells ?

Dr. LIESS

Yes, there is no difference whatsoever. Perhaps I might say that it is a strain which does not multiply very well at 37° C and we have to try whether it is capable of multiplying at lower temperatures.

Dr. VAN BEKKUM

You showed us some antibody patterns in swine that might develop under different conditions. Would you expect the patterns to change if one used a different virus strain ? Viral diarrhea or hog cholera virus ?

Dr. LIESS

We are well advised to use other strains, particularly one which is characterized like strain No. 331, one which appears to be more related to BVD-MD virus.

Dr. KNORN MELGAR

Did you look also for the antibody class responding to BVD-MD and also for the antibody class responding to HC ?

Dr. LIESS

No, not yet. But there is certainly something which can be done and the question is what kind of antibody class are we expecting ?

Dr. KNORN MELGAR

There is a publication that the IgM response will be very specific against the pathogenic strain. You are using, in fact, your antibodies, but the IgG class is not so specifically directed against the antigens.

Dr. LIESS

This is probably a question of time. IgG is produced later during the run of the infection and we see that the quality of BVD apparently appears with a delay of at least 14 days compared with the appearance of antibodies against HC virus.

Dr. VAN BEKKUM

What is your reason for assuming that the glentorf strain is hog cholera virus ?

Dr. LIESS

Because it produces malformations and it was isolated from HC field outbreaks.

Dr. KNORN MELGAR

Did you also look for a specific neutralizing antibody against SMEDI virus ?

Dr. LIESS

No, we did not.

Dr. TERPSTRA

Refering to the case which you mentioned in which you got piglets with a persistent viremia without producing antibodies; at what stage of pregnancy was the sow infected ? Was this perhaps a case of immunotolerance due to infection of the sow at a very early period in gestation ?

Dr. LIESS

Are you refering to those pigs which were infected by contact ? Than the sow was not inoculated. The pigs were in contact with inoculated pigs and at an age of eight weeks they did not have, as far as we know, any experience with HC virus before, so I don't think that there was any contact intrauterin.

Dr. CORTHIER

Do you think it may be that you infected the sow with a strain of hog cholera virus that looks like the 331 ? We observe that this type of strain in certain cases produces a very small amount of antibodies against Alfort strain and like this, you cannot detect it by cour technique.

Dr. LIESS

This virus strain produced in the other animals which reacted serologically titres of more than 1/600 measured against the Alfort strain of HC virus.

ON THE SPECIFITY OF ANTIGENS OF HOG CHOLERA VIRUS DETECTED BY THE AGARGEL DIFFUSION TEST AND IMMUNOFLUORESCENCE

by

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Tübingen, German Federal Republic

SUMMARY

Extracts from various organs and cells of infected and noninfected animals and tissue-cultures, respectively, were fractionated by precipitation, ion-exchange, gelfiltration and centrifugation. In the agargel diffusion test, these fractions were compared with the precipitating antigens, derived from the pancreas of hog cholera virus (HCV)-diseased animals, as well as tested with regard to their capacity to adsorb fluorescein-labelled antibodies of anti-HCV-hyperimmune sera. Analogous experiments were performed using purified intact or splitted HCV. Immunoelectrophoretic studies of the HCV revealed a precipitating behaviour differing from that of the precipitating HCV-pancreatic antigens. It is discussed that the antigens detected in the agargel diffusion test by reaction of porcine anti-HCV-hyperimmune sera against pancreas of infected pigs and in the immunofluorescence against infected cultured cells do not represent virus-specific structural components, but rather disease-specific cell antigens.

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abbreviations used :
HCV = hog cholera virus; MDV = mucosal disease virus; ADT = agargel diffusion test; IE = immunoelectrophoresis; IF = immunofluorescence; HC-PAS = porcine anti-HCV- hyperimmune sera; HC-RAS = rabbit anti-HCV immune sera; MD-BAS = bovine anti-MDV hyperimmune sera; prec. HCV-O-antigens = precipitating HCV-antigens from pancreas of diseased pigs.

INTRODUCTION

Togaviruses have a genome of single-stranded RNA with a molecular weight of 3 to 4 x 10^6 daltons. These viruses consist of an isometric nucleocapsid surrounded by a lipidprotein envelope. Most of the investigations, so far, showed that the nucleocapsid contains a structural protein while the envelope contains host cell lipids and virus-specified polypeptides including one or more glycopeptides.

Within the togavirus family different genera, namely alphaviruses, flaviviruses and rubiviruses have to be distinguished. It is possible that the complex of hog cholera virus (HCV)/mucosal disease virus (MDV) represents a particular genus within this virus family. The equine arteritis virus and the lactic dehydrogenase virus of mice are further ungrouped viruses that may probably be togaviruses.

Studies about the structure of the alpha- and flaviviruses suggest that the HCV may possibly consist of two to three structural proteins, which may be distinguished serologically as distinct viral subunits. The usual way to demonstrate such antigenic subunits of a virus is to isolate the subunits after having splitted the purified and concentrated virus and to produce monospecific antisera with them. However, at present this is not a practicable way with regard to the HCV because the large-scale cultivation of highly titred HCV encounters difficulties. Suitable experiments are in progress to eliminate these difficulties.

With the aid of the agargel diffusion test (ADT), immunoelectrophoresis (IE), and immunofluorescence (IF), therefore we tried in the meantime to analyse antigens of the HCV in the following systems using porcine anti-HCV hyperimmune sera (HC-PAS) and bovine anti-MDV hyperimmune sera (MD-BAS), partly :

- extracts of pancreas and spleen from HCV-infected and noninfected animals;
- extracts from HCV-infected and non-infected homogenized PK 15 cells;
- homogenized lymphocytesand leucocytes from normal and HCV-diseased pigs;
- split products of purified and concentrated HCV.

The diagnosis of HCV was performed in the past with the aid of the ADT on the basis of antigens detectable in organs (pancreas, spleen, lymphatic glands) of HCV-infected pigs. Immune precipitation lines were formed by the reaction of preferably pancreatic tissue as antigen against HC-PAS or isolated γ-globulins thereof. This diagnostic technique was problematical but it was frequently used before the fluorescence method was introduced. A lot of papers with contradictory results dealth with it. This is the reason that we started studies on the technical conditions and specify of the ADT.

The aim of our experiments was to isolate the precipitating HCV-antigens from the lymphatic organs and the pancreas of infected animals (prec. HCV-O-antigens), in order to characterize them and to determine whether there is a structural relationship between these prec. HCV-O-antigens and the HCV itself. It is of interest to know whether these antigens are viral structural proteins or cellular metabolic products (cellular antigens) produced in the course of the infection.

RESULTS

Specifity and Properties of the Prec. HCV-O-antigens :

Our experiments revealed the following results : we were able to demonstrate two specific antigens in the pancreas and spleen in different amounts (14).

Both antigens are noninfectious; they are proteins without any prosthetic group. After heating for 30 min at +56° C or treatment with proteases (trypsin, pronase P) and detergents (helarin, zephirol, tween 80 deoxycholate) they lose their property to react with HC-PAS and MD-BAS in the ADT (16) and in the IE. In the purified state these antigens are very labile, but may be stabilized by ammonium sulfate to a limited degree (1).

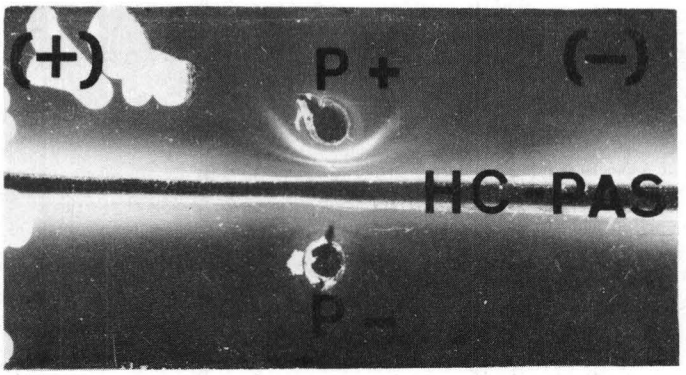

Figure 1 : Immunoelectrophoretic precipitation lines of pancreas from HCV-infected pigs in the β_o-globulin region.
Both antigens do not show serological relationship with each other, and one of them gives a cross-reaction with MD-BAS (15).

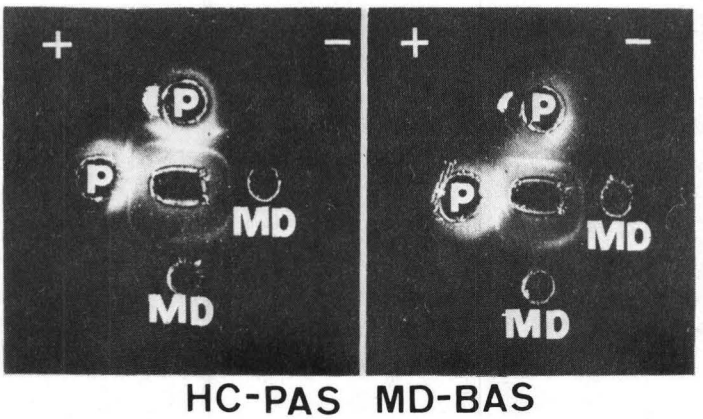

Figure 2 : Demonstration of two prec. HCV-O-antigens in the β_o-globulin region and their serological relationship to the soluble MDV-antigen using a combination of immunoelectrophoresis and agargel diffusion test.
It is possible, that there are some additional antigens.

The molecular weights of both antigens as determined by gelfiltration are 27 - 29 x 10^3 daltons (16). At least one of them appear in a dimeric form with a molecular weight of 56 - 59 x 10^3 (17). The monomeric and the dimeric forms exhibit uniform electrophoretic mobilities expressed by two immunoelectrophoretic precipitation lines one upon the other. Besides, one of these antigens seems to be able to transform and to change into forms differing in their net charges, i.e. electrophoretic mobilities.

In vivo immunisation experiments using purified and concentrated prec. HCV-O-antigens failed to protect pigs against HCV infection.

Reactions in order to characterise the chemical nature of the antigens in the precipitates formed in the ADT and in the IE, surprisingly resulted in hints of the presence of activities similar to those of trypsin and chymotrypsin, respectively. Attempts to demonstrate cholinesterase, carboxypeptidases, trypsin, and α-amylase gave negative results.

Experiments to Isolate Substances from Infected and Non-infected Organs and Cells Serologically Related to the Prec. HCV-O-Antigens

The hint for the protease character of the antigens prompted us to perform experiments with pancreas from noninfected pigs in order to detect antigens that may react with HC-PAS and MD-BAS in the ADT.

Fractions isolated by precipitation, ion-exchange chromatography and gelfiltration (1, 5, 16, 17) from extracts of powdered normal pancreas (3), noninfected as well as infected homogenised PK 15 cells (22) displayed a physical-chemical behaviour identical to that of the prec. HCV-O-antigens. These fractions partially cross-reacted in the ADT with MD-BAS and showed serological identity to β- and γ-chymotrypsins/-chymotrypsinogens, respectively; less to α-chymotrypsin/-chymotrypsinogen.

Moreover, an antigen could be demonstrated not only in leucocytes and lymphocytes, isolated from HCV-infected animals (4./7./9./10./14.dpi.), but also in those from clinical healthy ones. In gelfiltration experiments this antigen was detected in the 4 S region. It produced an identical precipitation reaction in the ADT with HCV-antigen-containing pancreatic tissue from infected pigs and cross-reacted with MD-BAS. The precipitation generally showed little intensity and mostly occurred only with the use of

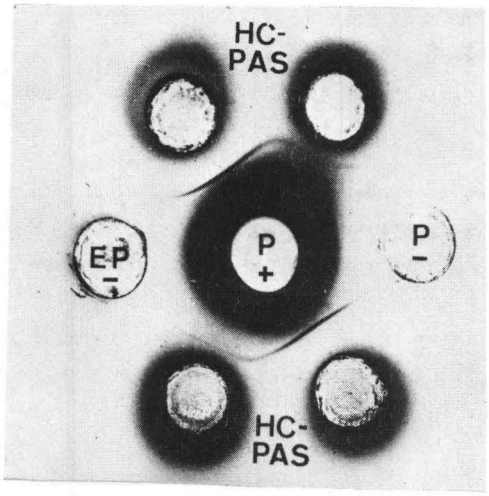

Figure 3 : Precipitation lines of concentrated extract (EP–) and residue (P–) from normal pancreas coalescing with prec. HCV-O-antigens (P+).

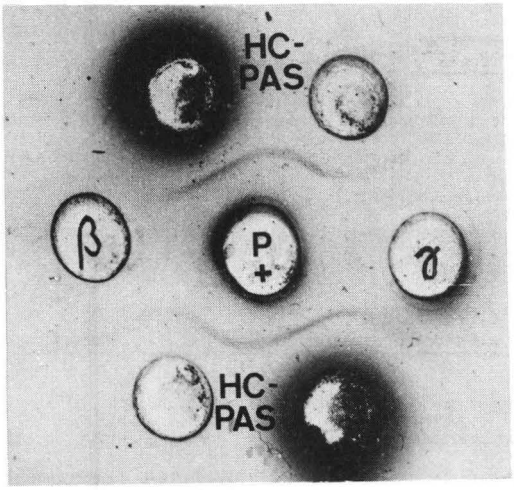

Figure 4 : Precipitation lines of β- and γ-chymotrypsin/-chymotrypsinogen (β, γ) isolated from normal swine pancreas confluent with prec. HCV-O-antigen (P+).

concentrated HC-PAS or preparations of γ-globulins thereof. Cells from infected animals reacted as antigens somewhat more intensively. In general, homogenates from lymphocytes displayed reactions more clearly than did those from leucocytes.

In sera and plasma from acutely (6. - 14. dpi.) and chronically (19. - 54. dpi.) infected pigs precipitating antigens could be demonstrated in small amounts. Reactions partially occurred not until after concentration and purification steps involving precipitation, ultracentrifugation, gelfiltration, rate zonal centrifugation and density gradient centrifugation in saccharose. The antigen only appeared in fractions that contained no virus.

Let me summarise:

We succeeded in demonstrating an antigen in pancreas and white blood cells from infected and noninfected pigs as well as in serum and plasma from infected animals, and, moreover, in infected and noninfected PK 15 cells that reacted with HC-PAS and MD-BAS. In this connection the question arises, whether this antigen represents a structural component of the HCV, at all.

Splitting of Purified HCV

HCV from infectious sera and infected tissue cultures was purified and concentrated by the above-mentioned procedures. Neither this antigen did react in the ADT and IE with HC-PAS or MD-BAS, nor after treatment with triton X-100, tween-ether, nonidet P 40, saponin, deoxycholate or other detergents and chemicals.

Immunoelectrophoretic Behaviour of Highly Virulent HCV

Against our experiments it could be argued that the HCV-preparations used may have contained not enough antigen for a positive reaction. However, material of all purification and concentration steps as well as the split products were controlled by virus-specific precipitation lines in the IE occurring with rabbit-anti-sera against HCV (HC-RAS). These sera were produced by hyperimmunising rabbits with HCV isolated from viremic swine sera and infected tissue cultures.

All these HC-RAS, in contrary to HC-PAS, were able to precipitate purified HCV as well as HCV in infectious sera or infected cell cultures in the α_2-globulin region (18). This result corresponded to the electrophoretic mobility of the virus reported by several authors (4, 6, 13).

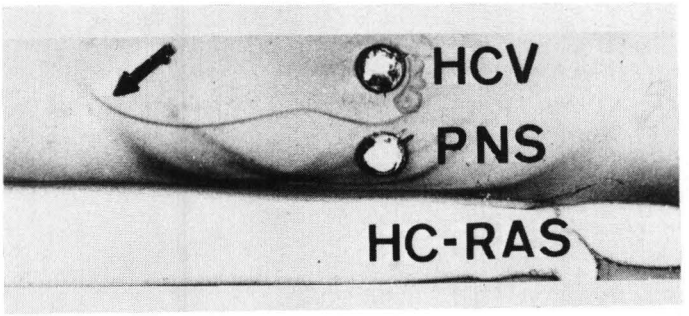

Figure 5: Immunoelectrophoretic precipitation line of HCV after adsorption of normal components in the agargel.

The adsorption in the agargel is produced by applying two holes for antigens. The hole nearest to the antibody trough is filled with pig normal serum (PNS) and the other one with HCV-preparations. The electrophoretically separated normal components adsorb the corresponding antibodies from the HC-RAS in the course of the diffusion process, while those antibodies directed against the virus are not prevented from diffusing and precipitating with the HCV.

This precipitation line was demonstrated to be specific for the virus by the following tests:

/reactions against HC-RAS were only demonstrable with infectious material as antigen;

/rabbit anti-sera against pig normal serum did not produce this specific line;

/antibodies in the HC-RAS against normal components were adsorbed by porcine normal serum or homogenised uninfected cell material in vitro or in the agargel (18);

/the antigen-antibody complex of the precipitate, extracted from the agargel, caused HC-infection in pigs.

The technique according to OSSERMAN (21) failed to demonstrate normal components in the virus-specific precipitation line.

There are some indications for some more virus-specific precipitates, which may be virus structural elements.

These can be demonstrated under distinct conditions in infectious sera or infected tissue cultures before and after treatment with triton X-100 and nonidet P 40, respectively using HC-RAS. Lipoproteids could be shown to be present. Further experiments are in progress to isolate and to characterise the antigens of these virus specific precipitates.

In all these immunoelectrophoretic analyses, HC-PAS did not react. The specific viral precipitate was only produced by HC-RAS. Virus-precipitating antibodies could not be detected in HC-PAS used for the precipitation of HCV-O-antigens in the ADT and in the IE. Mixing HCV-antigen with HC-PAS (one to three parts) the immunoelectrophoretic virus precipitation line was not affected. Obviously HC-PAS possess no virus-precipitating antibodies at all.

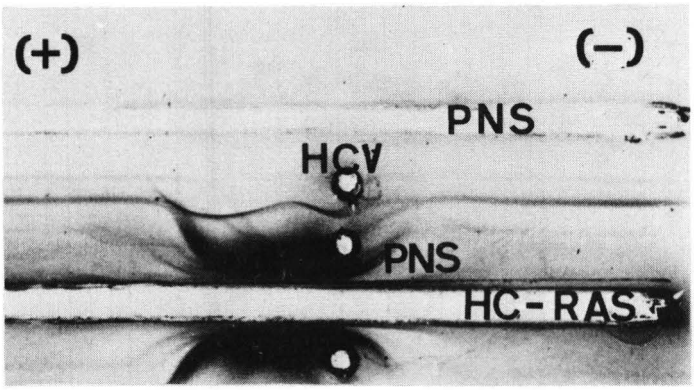

Figure 6: Immunoelectrophoretic precipitate of purified HCV using adsorption of normal components in the agargel and the technique of OSSERMAN.

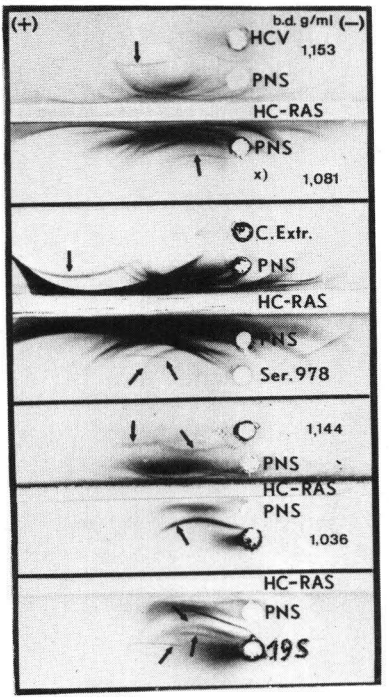

Figure 7: Immunoelectrophoretic virus-specific precipitation lines of various HCV-preparations using adsorption of normal components in the agargel ($^{x)}$ = after treatment with triton X-100).

Fluorescence-serological Reactions in Infected PK 15 Cells

The fluorescence method, without any doubt, is known to be specific for HC. The question arises whether it is specific for the virus or another antigen, which is connected with the disease like the prec. HCV-O-antigens.

The antibodies of the HC-PAS neutralise the HCV, and precipitate the prec. HCV-O-antigens, but lack obviously such precipitating activities for the virus itself. FITC-conjugated antibodies of HC-PAS produce fluorescence in HCV-infected PK 15 cells.

Antibodies of HC-RAS neutralise the virus too, but in contrast to HC-PAS, precipitate HCV. After conjugation fluorescence could not be demonstrated in infected cells. This is amazing.

To get some more information about the character of the antigen detected by immunofluorescence, the conjugates were treated by several distinct antigens (powdered organs; extracts from pancreas, and cells, infected and not; HCV-preparations) to adsorb corresponding antibodies. The reaction time was 30 min. at +18°C, the dilutions of the conjugates were 1:100 and 1:200.

The fluorescence was diminished by:

/extracts and powders from pancreas, isolated from normal and infected animals;

/extracts of uninfected and infected PK 15 cells;

/lymphocytes from normal and infected pigs;

/chymotrypsinogens and chymotrypsins (α-, β-, γ-), respectively.

No or only small effects on the fluorescence could be observed after adsorption of the conjugates with:

/HCV-preparations, containing intact or detergent-treated virus (virus-titer 10^7/ml);

/pig kidney powder;

/extracts of uninfected and infected PK 15 cells after treatment with triton X-100;

/extracts of powdered pancreas from infected and not infected pigs after treatment with triton.

Table 1: Adsorption of the fluorescing antibodies of conjugated HC-PAS 561 (30 min, +18° C)

PK 15 cells infected with HCV-ALD 10^4 fluoresc. dos. 24^h pi.

Virus controle	+++
conj. 561 (1:100; 1:200) adsorbed with	
HCV-ALD (tissue-culture; titer 10^{-6}/0.1 ml)	+++
HCV-ALD, concentrated	+++
HCV-ALD, concentrated + triton	+++
HCV (serum; titer 10^{-6}/0,1 ml)	++
HCV, concentrated	++
HCV, concentrated + triton	+++
ALD-infected PK 15 cells (conc. extract)	+
ALD-infected PK 15 cells (conc. extract) + triton	+++
powder from HCV-pancreas (or conc. extr.)	+
powder from normal pancreas (or conc. extr.)	+
powder from pig kidneys (trypsinised)	+++
normal lymphocytes	+
immune lymphocytes	+

The aberration of the fluorescence was seen in the background fluorescence (not infected cells) and the infected cells. On the one hand, the shining was diminished (qualitative process) thereby the weakly shining plaques disappeared (quantitative process).

DISCUSSION

The results of these experiments give rise to reflect on the specifity of the reactions in the ADT using material from organs of infected pigs and infected cells. It is felt, that it should be discussed, whether these reactions reveal structural components of the HCV or cellular substances, the production of which is possibly increased during the infection.

From the fact that HC-PAS does not show any precipitating activities with the HCV itself or its split products in contrast to the prec. HCV-O-antigens and antigens, isolated from organs of noninfected pigs as well as from infected and noninfected cells, makes us believe that in this system the reaction is not specific for viral structural antigen but indicates the presence of the disease. This assumption is supported by identical reactions in the ADT and in the IE, caused by proteases and their zymogens, such as the chymotrypsin group.

Immunofluorescence in HC-disease was studied by several authors (2, 7, 10, 12, 19, 20, 23-25). Only RESSANG and BOOL (23) made an effort to test the specifity of this method by virus adsorption experiments. They adsorbed the conjugate with viremic porcine serum and stated a reduction of the fluorescence without showing the absence of prec. HCV-O-antigens in the serum used for adsorption. Other authors examined the specifity of the test using other viruses and by covering the infected cells with HC-PAS that in any case contains antibodies against the pancreatic antigens, too. They then stated aberrations of the fluorescence and concluded the test to be specific for the virus. Up to this day, there is no proof that the immunofluorescence reaction is specific for the virus.

The presence of precipitating substances serologically related with the prec. HCV-O-antigens is demonstrated in concentrated material of noninfected cells and in normal pancreas. Therefore we think that this antigen is cell-related and that there is only a gradual difference between the reaction of material derived from noninfected and infected cells. In the immunofluorescence the same problem arises and this may be the reason that authors report on 'unspecific' fluorescence of leucocytes in normal animals. This 'unspecific' fluorescence in normal leucocytes could be explained to be specific for cellular antigen(s) in noninfected cells or tissues.

Considering this LINDENMANN (11) ought to be cited. In his article on interferon he says: ".... there is some reason to assume that during the process of a viral infection, there is not only produced virus, incomplete virus, soluble antigen, hemagglutinin, toxic products, lysozyme, cell detritus and interferon, but a lot of products which are worth to be investigated ...". It is the character of the prec. HCV-O-antigens and the properties of the precipitating antibodies of HC-PAS that suggest a connection to cellular substances produced in an increasing rate during the course of a viral infection. And this antigen seems to behave like chymotrypsin/chymotrypsinogen.

Studying the serological relationship between a mucosal disease of cattle and hog cholera, DARBYSHIRE (26) does not exclude the possibility that the antigens are cellular products which are liberated arising out of virus multiplication within infected cells. This product may be identical in the case of each virus and thus may act as an auto-antigen in the stimulation of specific antibodies. The antisera would possess this common antibody and it would be this which is detected in the gel diffusion reactions.

Moreover, it is interesting to state that the disturbance of the antibody production during the HCV-infection coincides with the viremic period. At this time (4. - 8. dpi.) high virus titers in blood are measured, but no or little antibody production occurs (8). The production of antibodies starts with decreasing virus titers, and this could be the explanation for the lack of virus-specific precipitating antibodies.

On the other hand, the most intense rate of lysis of white blood cells and cells in the lymphatics occurs between 8. - 12. dpi. (9). This is accurately the time that the prec. HCV-O-antigen is demonstrable. At the end of the third week of the infection, antibodies against this antigen being related to an antigen from lymphocytes and PK 15 cells can be detected. In the sera of animals vaccinated with dead vaccines, i.e. crystal violet vaccines, even applying 15 or 20 doses, one can detect neither precipitating nor fluorescent antibodies. Both antibody species appear after challenge infection, when the animals suffered some days from a mild form of the disease, including a virus replication phase.

Considering the above-mentioned findings, the fluorescent antigen seems to be more closely and directly related to the prec. HCV-O-antigens than to structural components of HCV.

Acknowledgements

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DISCUSSION

Dr. DALSGAARD

You mentioned in your summary that these antigens in the infected cultured cells do not represent virus specific structural components, but rather disease specific cell antigens. So I feel that - could it be possible that these antigens that cross-react with cell antigens could be incorporated into the viral capside in some way or another, as you see in influenza virus. The influenza virus surface carries antigens that are similar in some respects and cross-react serologically to most of the host antigens. Even if they are host antigens, they could be incorporated into the virus surface.

Dr. MATTHAEUS

We know that in polio virus synthesis part of the messenger RNA is transcribed into a gigantic polypeptide at the ribosomes and thereafter, this polypeptide is split into functional peptides by a protease. This enzyme may possibly act in hog cholera virus synthesis, and there is other evidence that a similar mechanism acts in toga viruses. I do not know which function this antigen has in the infection.

Dr. DALSGAARD

When you isolated the virion by density gradient centrifugation, you had a preparation of pure hog cholera virus, which react with a rabbit antiserum and my question is, did you try to degrade the isolated virus by Triton X 100 and then see how it reacted with the rabbit antiserum ?

Dr. MATTHAEUS

Yes, we did. In virus preparations treated with Triton X-100 or Nonidet P-40, we found the same precipitation line like against the virus non-treated. After sucrose density gradient centrifugation these precipitation lines are given by a split product at a buoyant density of about 1.10 - 1.125 g/ml.

I think that may be the envelope of the virus as the hog cholera virus particle itself shows a buoyant density of 1.15 - 1.16 in a sucrose density gradient.

Dr. TERPSTRA

We have examined spleens from quite a number of normal pigs in comparison with the hog cholera infected ones, and we have not been able to demonstrate a precipitating antigen in the spleens of the normal animals; I was interested in your remarks that you detected also precipitating antigen in non-infected pigs PK 15 cells. How consistent in this observation and under what circumstances and how long after seeding the cells did you examine those because we have examined also non-infected PK15 cells and we have not been able to demonstrate precipitating activity ?

Dr. MATTHAEUS

Concerning this question I wish to mention that we harvested non-infected cells as well as hog cholera-infected cells from fifty Roux-flasks. After washing each sort of these cells and disrupting them by sonification, insoluble particles were removed by centrifugation and extracted twice with buffer. The gathered supernatants were concentrated by vacuum-dialysis or salt precipitation (60% final concentration), and separated by ion-exchange chromatography. In this manner, we were able to demonstrate antigens, but we did not succeed in demonstrating them in harvested cells from five Roux-flasks, however.

Dr. TERPSTRA

How long after seeding was this ?

Dr. MATTHAEUS

It was eight days after. We trypsinized the cells and after three to four days, we got mono-layers completely (100%) covering the flasks. Then we added to the non-infected mono-layers the same maintenance medium as to the infected cells and harvested after four days' incubation.

Dr. TERPSTRA

In those cases where you have a mixture from a pooled sample from 15 bottles, did you check for infectivity in those samples - virus infectivity ?

Dr. MATTHAEUS

No, I did not, as I knew from experiments formerly made that the virus-titer in PK15 cells ranged between $10^{5.5}$ - $10^{6.0}$/0.1 ml as fluorescing unities.

ANTIGENIC COMPARISON OF SWINE FEVER VIRUS (SFV) AND MUCOSAL DISEASE VIRUS (MDV)

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INTRODUCTION

Mucosal disease virus (MDV) and Swine fever virus (SFV) are different in regards to their natural host, although their structural and antigenic properties are quite the same (1). Immunodiffusion (2), immunofluorescence (3) and complement fixation (4) tests provide evidence for an antigenic relationship between the two viruses.

In seroneutralization tests the relationship between the 2 viruses seems very weak. Only hyperimmune sera cross react (5), (6), (7), (8), (9).

This paper summarises a previous work (10). Using seroneutralization techniques we described antigenic difference between various strains of SFV (11) and we analyzed the antigenic relationship between each SFV serological variants and MDV.

I. SEROLOGICAL VARIANTS OF SFV

Tables 1 and figure 1 show results obtained by 3 different seroneutralization methods. An antigenic overlaps between studied strains is observed. However, two serological variants can be distinguished according to their antigenic properties :

Seroneutralizing methods	Studied strain	Studied sera : Thiverval strain antisera	331 strain antisera	Rabbit chinese strain antisera
Variable virus -constant sera Neutralization index Values for :	Alfort	3,4 (1)	1,8 (2)	3,5 (3)
	331	1 (1)	3,2 (2)	0,0 (3)
	Thiverval	3,5 (1)	-	-
Variable sera - constant virus : 50 % sera dilution for :	Alfort	28.000	1.000	-
	331	2.000	16.000	-
	Thiverval	20.000	1.000	-
Neutralization kinetics K values for :	Alfort	1,31 (4)	4,6	19,6
	331	0,37 (4)	17,0	8,2

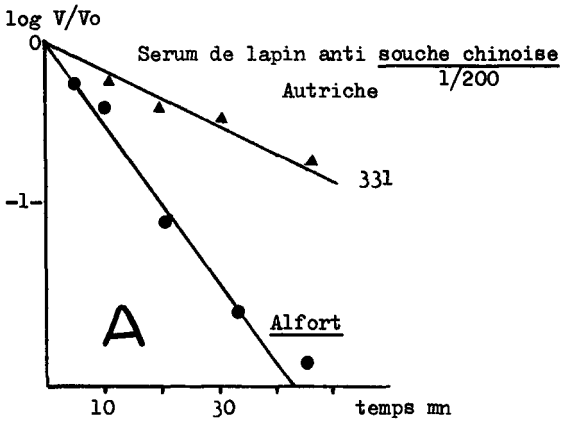
Table 1 : Characterization by different technics of serological differences between SFV strains

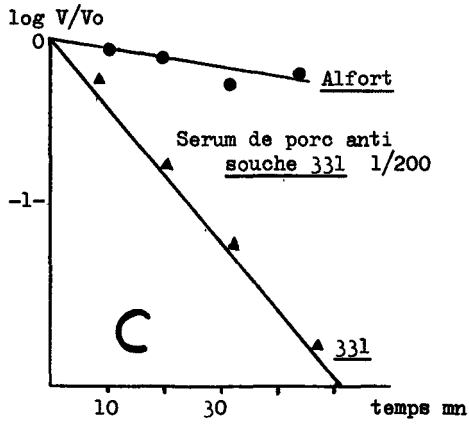
(1) final dilution 1/1600

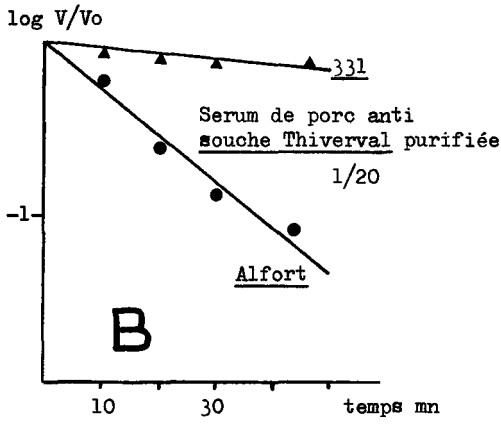
(2) final dilution 1/800

(3) final dilution 1/200

(4) sera prepared with purified Thiverval strain







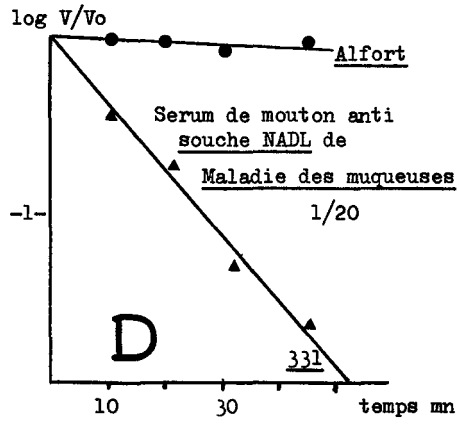


Figure 1 : Neutralization Kinetics

- the largest includes both virulent strains (Alfort) and attenuated strains (Thiverval, Chinese, GPE^-);
- the other includes 331 subacute SFV strains and field strains isolated from chronic disease (11).

The relationship between these 3 serological variants is symetric.

II. RELATIONSHIPS BETWEEN SFV AND MDV

a) Neutralizing activity of specific SFV antisera.

Twenty one serologically distinct MDV strains had been studied with SFV antisera specific for Alfort strain (table 2). We observed no specific neutralization. Using another seroneutralization method the NADL strain of MDV was slightly neutralized by SFV antisera with high homologous titer (table 3).

b) SFV neutralizing activity of specific MDV antisera.

Sera prepared with serologically distinct MDV strains had a neutralizing activity against the 2 serological variants of SFV (table 4). The serorelationship of the first variant (331) and MDV is more pronounced than with MDV and the second SFV variant (Alfort and Thiverval strains). These results are confirmed by neutralization kinetics.

CONCLUSION

The relationship between the seroneutralization antigens of SFV and MDV is summarized in figure 2.

Antibodies prepared against the two distinct serological SFV variants are not able to neutralize MDV, except some sera with high homologous titer. Antibodies prepared against serologically different MDV strains neutralize to a large extent the SFV variants. The relationship is more pronounced with the 331 subgroup in which there are various chronic SFV strains.

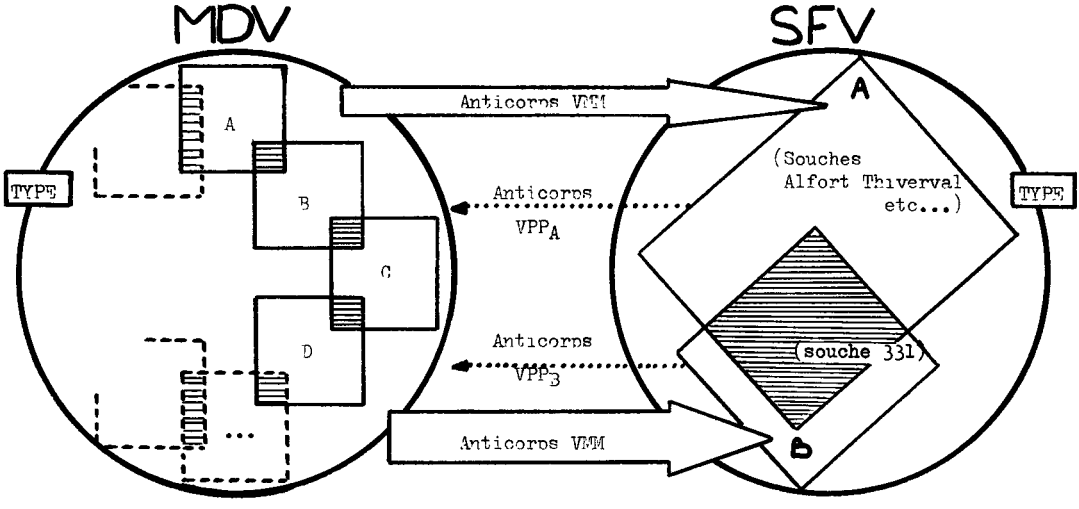


Figure 2 : Relationship between SFV and MDV

Virus	Souche	I.N.	Virus	Souche	I.N.
VPP	Alfort	6	VMM (2)	AR4	< 0,1
VMM (1)	T1	< 0,1		MM4	< 0,1
	T2	< 0,1		MM5	< 0,1
	T4	< 0,1	VMM (3)	I100	< 0,1
	T5	< 0,1		I153	< 0,1
	T6	< 0,1		I155	< 0,1
	T7	< 0,1		DK167	< 0,1
	T8	< 0,1		MQN	< 0,1
	TDVF	< 0,1	VMM (4)	NADL	< 0,1
	TA	< 0,1		Diam	< 0,1
	TP	< 0,1		C24V	< 0,1

Table 2 : Neutralizing activity of an anti Thiverval SFV strain (diluted 1/20) against MDV strains

(1) strains given by Dr GILBERT
(2) strains given by Dr FAYE
(3) strains given by Dr WELLEMANS
(4) U.S. strains

Sérums			Souches du virus de la : maladie des muqueuses	Peste Porcine	
Espèce animale	Spécifique		NADL	331	Alfort
Porc	Souche 331	1	< 1/4	1/6.000	
		2	1/150	1/50.000	1/1.000
Porc	Souche Thiverval		1/300	1/2.000	1/28.000
Lapin	Souche Chinoise Italienne	1	< 1/8	1/2.500	1/17.000
		2	1/200	1/8.000	1/12.000
Porc	Souche Chinoise "SUVAC"	1	N.D.	1/2.500	1/40.000
Porc	"LOUD"	1	< 1/4	1/120	1/16

Table 3 : Neutralizing activity of SFV antisera against MDV strains and SFV strains (the number indicate the 50% sera dilution)

Antisérums			Virus de la Maladie des muqueuses		Virus de la Peste porcine		
Provenance	espèce animale	Spécifique de la souche	titre vis à vis de la souche homologue	NADL	Alfort	Thiverval	331
Dr. Corthier	Mouton	NADL	2.000	2.000	160	160	1.200
et	Porc	NADL	28.000	28.000	1.800	3.000	6.000
Dr. Aynaud	Porc	NADL	3.000	3.000	50	-	2.700
Dr. Gilbert	Veau	C24V	-	8.000	500	2.000	5.400
Dr. Fernelius	Veau	CG 1220	32.000 (1)	5.000	1.600	3.500	4.000
	Veau	SAN	16.000 (1)	7.000	300	350	2.000
	Veau	HUB	-	4.000	800	1.000	2.200
	Veau	V W	16.000 (1)	3.000	900	1.600	4.000
Dr. Castrucci	Lapin	SAN	-	1.300	< 20	< 40	400
	Lapin	66/1237St	600 (2)	650	< 20	40	200
	Lapin	TVM2	300 (2)	250	< 20	20	150

Table 4 : Neutralizing activities of MDV specific antisera against MDV (NADL strain) and SFV

(1) Fernelius and al 1971

(2) Castrucci and al 1970

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DISCUSSION

Dr. LIESS

We have to look for a conclusion, and as far as I can see, you are thinking of intermediate types or strains of BVD/MD and hog cholera virus. Is that right ?

Dr. CORTHIER

In my study, I have not seen strains of swine fever virus which are between the Mucosal disease and swine fever strain.

Dr. LIESS

I think it's very important to look for this question again and to do more investigation on this question as this is very important for future.

ANTIGENIC STIMULATION IN THE PIG

Bourne, F.J., Newby, T.J. & Brown, P.J.
Department of Animal Husbandry
University of Bristol

The immune system of the pig has many features in common with those found in other mammalian species, but it also possesses features that appear to be pecular to it.

Structural features of the pig IgA molecule have been reported and the possible consequences of this to the pig have been discussed (Bourne, 1973 & 1974).

In addition to this, recent work has shown the distribution of immunocytes in the pig differs from that described in other species (Brown & Bourne, 1974, in preparation) and the response of the pig to antigenic stimulation also shows a species peculiarity (Bourne, Newby & Chidlow, 1974).

Distribution of immunocytes in the pig

Using peroxidase conjugated antisera we have made a comprehensive study of the distribution of immunocytes producing A, G and M immunoglobulins in the pig body tissues (Brown & Bourne, 1974, in preparation).

The densest population of cells occur in the duodenum. IgA forming cells predominate at all levels of the gut but to a much lesser extent that found in other species and there is a large population of IgM and IgG containing cells.

In the adult gut the ratio of A to M cells varies from 1 : 1 to 2 : 1 although in the young animal in the first two weeks of life M cells predominate, the ratio being 2 : 3. Between 4-8 weeks the A cells increase in number relative to M and assume predominance.

There are less G forming cells than A or M but it would appear that the majority of immunoglobulin forming cells for all three classes occur in the gut with relatively few in other tissues such as the spleen or lymph nodes, which are regarded as major sites of immunoglobulin formation in other species.

Preliminary experiments performed in this laboratory on the site of formation of immunoglobulins would support the above view that the gut is a major source of immunoglobulins in the pig and contributes significantly to systemic as well as local immune systems.

A further extension of this work has been a study of the secretory pathways of immunoglobulin across the gut epithelium (Brown, Bradley & Bourne, 1974, in preparation).

Immunoglobulin IgA and IgM staining can be shown on the mucosal epithelium of crypts, whereas IgG staining is absent. Also epithelial cells have been demonstrated which probably represent a transcellular secretory pathway for immunoglobulins IgA and IgM. Such a pathway for IgG could not be shown. This would in part account for the high levels of IgA and low levels of IgG found in intestinal secretions (Bourne, Pickup & Honour, 1971).

Stimulation of pig mammary gland

Virtually all milk IgA and a large proportion of IgM and IgG has been shown to be locally formed in pig mammary tissue (Bourne & Curtis, 1973). It has further been demonstrated in other species that intramammary vaccination of antigen is necessary to produce IgA antibodies in colostrum (Genco & Tauban, 1969, McDowell, 1973).

We have investigated the influence of the route of vaccination on the development of local and systemic immunity in the pig and determined the class of immunoglobulin involved (Bourne, Newby & Chidlow, 1974).

Non-replicating antigens were used, either horse spleen ferritin, bovine gamma globulin, DNP-albumen or E.coli K88 antigen. Antibody activity was determined by using immunoabsorbents of the above antigens (Newby, Bourne, Chidlow & Steel, 1974). The intramuscular route was compared with the intramammary route of vaccination and in one experiment antigen was given orally. The following observations were made :

1. Intramammary vaccination is far superior to intramuscular vaccination in producing not only a local but a systemic response. Thus, if following intramuscular vaccination the total serum response is x mg/ml of antibody, the colostrym response in this animal would be 2x and the milk response only $^{1}/10$ x. Following intramammary vaccination, however, the serum response is increased two-fold to 2x and the colostrum response three-fold to 6x but the milk response shows a ten-fold increase.

2. Following stimulation by either the intramuscular or the intramammary route antibody activity is found in all three in all three classes of immunoglobulin, the major amount of antibody activity in a body fluid correlating with the major immunoglobulin class in that fluid.

3. Vaccination of one mammary gland gives rise to significant antibody activity in the other glands. The reasons for this are not clear, but our findings suggest that it is not due to passive transfer from serum but to local production. It may be due to a general dissemination of antigen from the site of injection, and a further possibility is that immunoglobulin producing precursor cells from the vaccinated areas migrate to other immunoglobulin producing areas. A combination of events may also occur since Griscelli, Vassali & McCluskey (1969) have shown that immunoglobulin producing precursor cells accumulate in areas that contain their specific antigens.

4. Oral vaccination of gilts with a living non-pathogenic strain of E.coli, possessing K88 antigen, led to local production of K88 antibody in pig mammary tissue. The dissemination of antigen from the gut to the mammary gland in this experiment is unlikely and provides further evidence of an interrelationship between the various immune systems.

5. The cellular mechanisms of antibody production basic to parenteral immunisation would appear to operate during local stimulation also.

6. In view of the importance of mammary secretions in providing both systemic and local intestinal tract immune protection to the young suckling pig, the above findings could be very important in developing vaccination schemes to enhance this protection. The possibility of a cellular interrelationship of the various immune systems also demands further intensive study.

7. The above experimental findings question the concept of distinct local and systemic humoral immune systems.

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SUMMARY OF THE SESSION

J.M. AYNAUD

Results of immunoprecipitation of the immuno electrophoresis test suggest that swine fever viral material contains at least 2, and perhaps 3 antigens, some of which seem to be directly related to mucosal disease virus. But among these antigens, we are unable to identify the structural components of the virus particle. Seroneutralization reactions, being directly related with the external surface of the virion, we can assume that on the viral envelope there exists at least one structural component having a common antigenic activity between swine fever and mucosal disease.

Results of immunological and immunochemical methods, and also classical biochemical methods, could be compared. In these conditions it will be perhaps possible to determine the localization and the function of the different structural proteins of the virus particle for swine fever and also mucosal disease. On this aspect, it will be suitable to determine first of all the number and the nature and the localization of different antigens involved in precipitation, neutralization or immunofluorescent tests. It will also be suitable to determine the nature and the function of the soluble antigen and its relation to porcine immunity.

These results have large practical applications in diagnostic and epidemiologic study of swine fever in pigs. For example, it would be very useful to differentiate easily between low virus strains of swine fever from mucosal disease strains in latent infection of pigs. With respect to swine fever eradication, this point is very important.

WORKING GROUPS

COMPOSITION OF THE WORKING GROUPS

CLASSICAL SWINE FEVER

Working group 1 : Virology

Chairman :	HORZINEK, M.C.	Utrecht
Participants :	BOURNE, F.J.	Langford-Bristol
	CESSI, D.	Brescia
	DALSGAARD, K.	Lindholm-Kalvehave
	ENZMANN, P.J.	Tübingen
	FOGLINI, A.	Teramo
	FROST, C.	Hannover
	KNORN-MELGAR, O.	Utrecht
	ROTT, R.	Giessen
	SCHLESINGER, S.	Saint-Louis (USA)
	TERPSTRA, C.	Lelystad

Working group 2 : Epidemiology

Chairman :	LIESS, B.	Hannover
Participants :	CORTHIER, G.	Thiverval-Grignon
	MURPHY, T.	Dublin
	NEWBY, T.J.	Langford-Bristol
	OVERBY, E.	Lindholm-Kalvehave
	ROSSI, G.	Teramo
	SCHERRER, R.	Thiverval-Grignon
	VAN AERT , A.	Uccle-Bruxelles
	VAN DER ZEIJST, B.A.M.	Utrecht
	VAN OIRSCHOT, J. Th.	Lelystad
	ZEEGHERS, J.J.W.	Utrecht

Working group 3 : Immunology

Chairman :	AYNAUD, J.M.	Thiverval Grignon
Participants :	BARRY, R.D.	Cambridge
	LA BONNARDIERE, C.	Thiverval-Grignon
	MATTHAEUS, W.	Tübingen
	RUTILI, D.	Perugia
	SCHOLTISSEK, C.	Giessen
	VAN BEKKUM, J.G.	Lelystad

AFRICAN SWINE FEVER

Working group 4 :

Chairman :	BOTIJA, C.	Madrid
Participants :	BACHRACH, H.L.	Plum Island
	BECKER, Y.	Jerusalem
	LARENAUDIE, B.	Alfort
	McAUSLAN, B.R.	Epping (Australia)
	OLLIAS, M.	Madrid
	ORDAS, A.	Madrid
	SOLANA, A.	Madrid

SUMMARY OF THE WORKING GROUPS ON CLASSICAL SWINE FEVER

WORKING GROUP 1 : VIROLOGY

C. TERPSTRA

Centraal Diergeneeskundig Instituut, LELYSTAD

During the meeting several research workers pointed out that the low yield of virus from infected cells was felt as a major draw back for studying the characteristics of the multiplication of hog cholera in vitro. There appears to be no agreement on the ratio of cell free virus to cell associated virus produced in the same cell system.

1. It was therefore felt that it would be worthwhile to study the factors influencing the yield of hog cholera virus and in doing so attention should be paid to the circumstances which might account for the discrepancies obtained so far by research workers.

2. In the case of Bovine viral diarrhea (BVD), antigenic differences have been observed between various field isolates. The same may apply to different strains of hog cholera. In view of the high incidence of neutralising antibodies against BVD in pigs, as was demonstrated in Germany, and considering the antigenic relationship between hog cholera and BVD virus, it is recommended by the group that further studies should be undertaken to identify the antigenic characteristics of various BVD and HC strains, in order to select those strains which are most antigenically distinct for both viruses.

3. In view of the antigenic relationship between BVD and HC-virus, it is recommended that studies should be undertaken to identify the structural protein(s) of these viruses which induce neutralizing antibodies.

4. In order to eradicate hog cholera in the countries of the EEC, it is recommended that the governments of the member-States should uniform their regulations on the control of hog cholera.
An appeal to this effect to the authorities concerned should come either from this meeting or from the research coordinating committee.

WORKING GROUP 2 : EPIDEMIOLOGY

B. LIESS

Institut für Virologie
der Tierärtzlichen
Hochschule - HANNOVER

Working group 2 discussed the epidemiological situation as far as Hog Cholera is concerned.

It was agreed that one of the main interests of the European countries and probably even countries outside the CEE is to control this permanent threat to the pig population because of clear reasons.

Furthermore it was agreed that a common policy of controlling HC does not exist and that there are only two ways in order to get a proper control over HC,(1) general vaccination or (2) eradication.

Though the group realised that certain implications might hinder immediate steps, eradication of HC should be the aim.
In order to come to a decision in one way or another, economists should be asked for advice on the least costly way to get information which can be used for recommendations. If general vaccination procedures prove to be less costly than eradication procedures recommendations should be put straight to O.I.E. In this case the recommendations should include that a certain standard vaccine taking the new Japanese tissue culture vaccine under consideration, too, might be recommended or work initiated on the development of a better vaccine preferably one which guarantees no spread of virus and that the best route of vaccination should be investigated.
If, however, a new meeting organised by CEE comes to the conclusion that eradication of HC in Europe should be followed immediate steps should be taken in order to improve and formulate the basis for an eradication programme which necessarily includes the introduction of standard techniques.

In this case the working group recommends to set up and support reference laboratories for culture i.e. PK (15) cell, selected virus strains and antisera which meets the requirements of the WHO/FAO programme on comparative virology. Moreover, the research programme must devote in this case not only to Hog Cholera, but to BVD-MD virus in pigs, too. Pathogenetic studies will be necessary in connection with immunology especially on the question, which factors are involved for the occurence of latently infected pigs.

It was felt, that work even on the Border disease in sheep, which might be caused by a BVD-MD related virus, should be supported.

WORKING GROUP 3 : IMMUNOLOGY

R.D. BARRY

Department of Pathology

University of CAMBRIDGE

Members of Working Group 3 agreed that their discussions were of benefit and interest to those present, and recommend that the policy of conducting small, informal discussion groups should be adopted at future meetings of this type. It was concluded, however, that better grouping of participants in relation to the topics discussed could have been arranged at this meeting, should be adopted for future meetings.

Working Group 3 discussions began with a brief introduction by each participant of his research interests. Those directly concerned with hog cholera virus then outlined in detail the principal problems encountered in their work. Extensive discussions followed from which emerged a number of practiced suggestions and proposals for future experimental work with HC virus.

The following general proposals for future meetings were agreed :

1) "Mixed" meetings, with participants drawn approximately equally from the field under consideration, and from related field should continue.

2) Meetings should begin with a review of the main topic(s). This would be of particular help to external specialists. In addition, participants should be provided in advance with a detailed statement of objectives, and where appropriate, with reference material.

3) Careful planning of working groups.

4) Possible topics for future meetings might include epidemiological aspects of disease, standardization of vaccination procedures, and the rationalization of diagnostic procedures.

SUMMARY OF WORKING GROUP 4 ON AFRICAN SWINE FEVER VIRUS

B. R. McAUSLAN

Division of Animal Genetics,

C.S.I.R.O. - EPPING

African swine fever virus (ASFV) which in the past has caused considerable economic loss in Spain and Portugal, now seems to be under control but not completely eliminated. Attempts at eradication should be continued and potentiated because vaccination against this virus is not possible.

The control of the disease is due in part to improvements in husbandry practices and to the development of techniques for the rapid diagnosis of the disease. The diagnostic methods employed depend to some extent on the clinical condition of the infected animals, but in general indirect immunofluorescence is the technique of choice.

For this technique, antibody is extracted from the spleen, lung, lymph nodes or kidneys of animals suspected to be infected and tested in vitro against cells infected with ASFV.
The value of this procedure lies in its specificity so that ASFV can be distinguished from classical swine fever. All diagnostic services in Spain have been centralised. Recently the applicability of the techniques of immunodiffusion and immunoelectro-osmophoresis to diagnosis are being investigated.

Despite the valuable improvements in husbandry practice the control of transmission of ASFV by the Argasid Tick still merits some study.
It is suggested that consideration be given to employing biological population control methods and chemical agents now in use by parasitologists to eradicate insects and other pests. Perhaps a detailed study of the Ornithodorus tick species and control of its population should be undertaken since it has been shown that ASFV can reside in such tick populations for at least 8 years.

Very little has been done on the molecular biology of this virus. However, detailed knowledge of its replication and macromolecular synthesis might be a valuable adjunct to studies of viral antigens and to the possible design of antiviral chemical agents. It is reported that some of the rifamycin antibiotics may be effective against ASFV and it would be most helpful to have more information about the efficacy and mechanism of action of these compounds.

Some specific recommendations are :

(i) Continuation of investigation into improved immunological techniques for the rapid detection of the disease, particularly where its manifestations are sub-clinical;

(ii) Investigation of the antigens synthesised by the virus in cultured cells and of the structural proteins of the virus. Such studies might complement the diagnostic tests;

(iii) To undertake a thorough study of the biology of the Argasidae - in particular into means of introducing biological population control or chemical eradication;

(iv) Study of the pathogenesis of the disease and of the immunological responses in infected animals should continue.

SUMMARY OF THE MEETING

Y. BECKER

It is a little difficult to summarise such a meeting which has dealt not only with major problems in the molecular biology of viruses, but also with the under currents which every one is aware of and which came out very clearly in the recommendations of the discussions groups. The question is how to apply the knowledge that was developed in Virology to two viruses, hog cholera and African swine fever viruses, which still remain a major problem. The object of this meeting was not only to try and bring into one room people with knowledge in different fields, but also to try and point out in the clearest way possible, where the problems are and what can and should be done to advance the field of swine fever viruses one step further. We all know that it is a tremendous task to eradicate a virus. We have many models of efforts to overcome virus infections in animals. A typical example is that of the foot and mouth disease virus, which has been studied for so many years. We know a lot about its molecular biology and antigenic variants and in spite of all the attempts to get rid of it, the virus still persists. Certain ways have been clearly indicated to us by Dr. BACHRACH, but these require a generalised effort in many countries. The most striking fact is that the two viruses we are concerned with - hog cholera (HC) and African swine fever (ASF) viruses - have been studied for quite some time and yet there are many aspects which we know very little about. This is one of the things that we would like to understand in order to see where the difficulties lie and what should be done to overcome them.

CHAPTER I - THE BIOLOGIVAL ASPECTS OF HOG CHOLERA AND AFRICAN SWINE FEVER VIRUSES

A. The persistence of the virus in nature and the economic problem

These two viruses persist all over the world and even these countries which have succeeded in eradicating the virus are under constant treat of its reentry. The persistence of the virus in nature, which means that it has one or two hosts poses a very important economic problem.
Obviously, there is a tremendous need for active veterinary services concerned with how to get rid of the virus infection and how to stop it from reentering the country.

The education of personnel is ovbiously a task which each country must undertake, and if this going to be on an international level, it must be centrally organised. This is something that various organisations must think about and invest in. There is also demand for diagnostic laboratories which always have to be ready for the appearance of new variants or new mutant strains, and we need active diagnostic services. Tremendous investment is needed for the control of diseased animals in the fields, and of course this concerns all possible hosts of virus infection that are in the country. This requires the development of new vaccines as well as standardization of the present vaccines and vaccination procedures which needs manpower and facilities.

This means that every country has to put aside part of its national budget so as to combat a disease and make life a little bit better for the people. Basic researoh is also obviously required to give us a better understanding of immunogens. This is actually why we decided to devote this meeting to the basic aspects of virology, because if you start with the practical aspects, you obviously get to the practical ones, but in addition, you try to develop a concept to overcome the disease and some of the practical problems which we all know exist.

B. The biology of the viruses

Fig. 1 summarizes some aspects of the biology of both HC and ASF viruses. Viruses are built in such a way that they can persist in nature. The virus is defined as being nucleic acid coated by protein, so that its genetic information is protected from the ill-effects of the conditions in which it exists. We start with the virus which is excreted and if we have susceptible animals they become infected. This means entry of the virus into susceptible cells in the host where virus replication takes place. This is actually what we have dealt with during entire meeting - all the rest of the discussion had to do with the question of how antigens stimulate the immunological system to yield antibodies. We heard about the unique system of the pig from Dr. BOURNE. More knowledge of this system is needed since we cannot speak about viruses and animals in general.

Once again we have to focus our attention on one animal, because we are dealing with two viruses which affect the same host - the pig. We understand the immunological machinery of this host which is different from others and so of course we can progress a little further.

CHAPTER II - VIRUS REPLICATIVE CYCLE

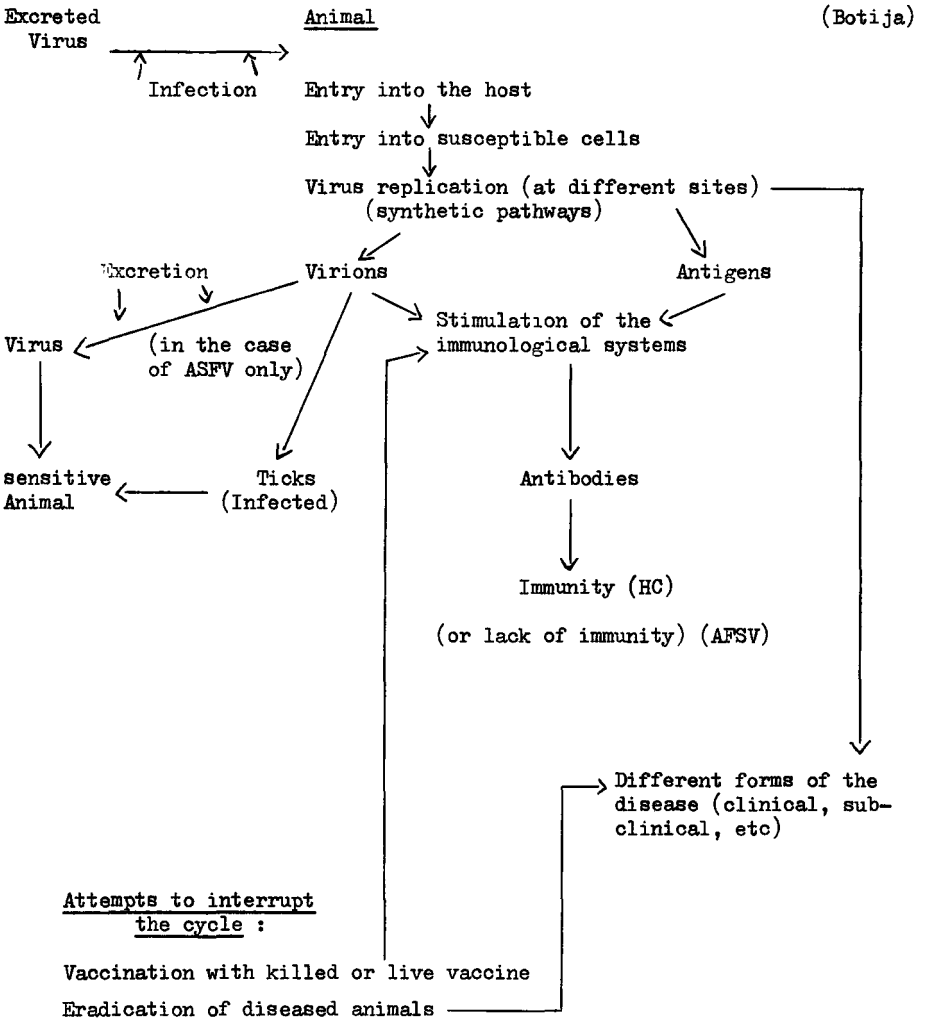
The virus replicates in the host and causes either a clinical or a sub-clinical infection. Once the virus replicates in the host, it re-cycles. It is released from the infected host and will again infects sensitive animals. This summarizes the major biological cycle for both viruses. In the case of the ASF virus, there is also a smaller cycle which Dr. BOTIJA explained to us in detail. The virus can persist in the tick and be transmitted to a sensitive animal.

One can attempt to interrupt the biological cycle by vaccination with a killed or live vaccine. On the other hand, we could try to eradicate the virus which means that we have to get rid of the host in order to get rid of the virus. Both the immunological and the eradication approach pose great problems since the virus may continue to persist. ASF virus can persist in ticks and if we do not eliminate this part of the cycle, then of course we do not eliminate the virus and nature will probably find other ways to help the virus to persist in different hosts. The epidemiology of the virus must be understood and characterised and as was said, we need a general international effort to overcome this problem. But as the subject of the meeting is virus replication, let us focus our attention only on the biological cycle of the virus and try to see what information obtained at this meeting can be utilised for an understanding of the particular problems.

I would like to divide the discussion into two parts :

a) hog cholera virus and
b) African swine fever and finally to try to draw certain conslusions.

Figure 1 : Some aspects of the biology of Hog Cholera and African swine fever viruses.



A. HOG CHOLERA VIRUS

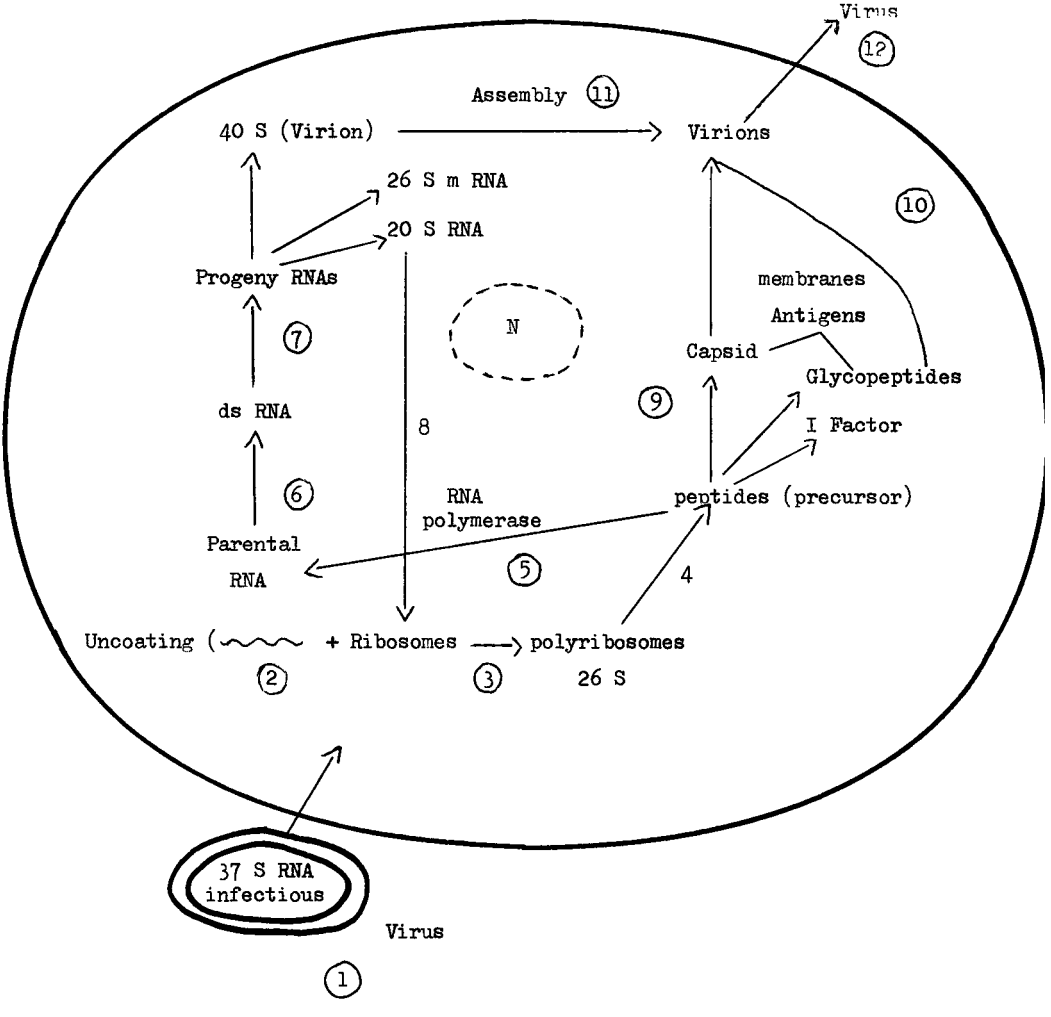
1. The replicative cycle

Fig. 2 summarizes the replicative cycle of an RNA virus. Understanding the replication of an RNA virus in the cytoplasm of the cell may assist in understanding some of the major problems that people have with HC virus, which is a Togavirus. A lot of work has been done on this group of viruses which was very clearly summarized by Dr. HORZINEK.

- Firstly in order to analyse and characterize the virus, it has to be purified. As Dr. HORZINEK told us, the virion contains 37 S single-stranded RNA. The morphology of the virus is known but we know very little about its antigenicity. In nature, there are viruses which are related antigenetically, but which differ in other properties. This is a very important point, because as in the case of Marek's disease which causes tumours in chickens, the problem of immunisation was solved by finding a turkey virus which shared antigens with Marek's disease virus. Immunisation of chickens with the turkey virus overcame a disease which was difficult to control before hand. By searching for viruses which share common antigens we may hit on a very useful approach in the case of other diseases, namely to find a virus which will replicate but will not cause disease and moreover which will block the entry of the virulent strains that we are so worried about. Thus if we know the nature of viruses from the Togavirus group, we may be able to utilise this approach.

- The second step in the infection of the cell is the uncoating and release of the parental RNA. Translation of parental RNA was summarized by Dr. HORZINEK. The third step is the interaction of parental message with the ribosomes to form polyribosomes which synthetise the peptides. Although we know a little about HC virus proteins and their synthesis, Dr. SCHLESINGER was able to bring us important data about the proteins of another virus of the myxovirus group. He described the translation of the genetic formation into a large polypeptide which was later cleaved to yield different proteins. One of these proteins is obviously the RNA polymerase.

Figure 2 : A. Togaviruses - Hog Cholera replicative cycle.



(See following page)

Figure 2 : Legend.

Molecular step	Description		Author
(1)	Virus structure and composition		Horzinek - Scherrer
(2)	Uncoating and translation		Horzinek
(3)(4)	Synthesis of viral proteins *in-vivo*		S. Schlesinger
	HC antigens (use of immunofluorescence)		Aynaud
(5)	RNA polymerase	Picorna virus	Bachrach
		Myxoviruses	Barry - Scholtissek
(6)(7)	RNA replication	Togaviruses	Horzinek
		IPN virus	Scherrer
	nucleotides poolsize - effect of glucosamine		Scholtissek
(8)	Polysomes formation		S. Schlesinger
(9)	Proteins : *in vivo* processing		S. Schlesinger
	in vitro synthesis		van der Zeijst
	antigens		Aynaud, Corthier, Matthaeus
	detection of antigens		Dalsgaard, Liess
(10)	Membrane formation in influenza virus		Rott
(11)	Assembly of virions		
(12)	Virus release.		

When we talk about proteins, we actually talk about antigens and Dr. AYNAUD was able to demonstrate to us by immunofluorescence the antigens of HC virus in the infected cell.

- The synthesis of the RNA polymerase is obviously one of the most important events in the life cycle of a RNA virus. RNA polymerase in the picornaviruses were described by Dr. BACHRACH. We heard about RNA polymerase in myxoviruses from Dr. BARRY and Dr. SCHOLTISSEK. It is very obvious that in the case of HC virus, the RNA polymerase is responsible for the replication of the RNA, but we don't know much about this enzyme. The enzyme will probably be isolated and characterised in the near future. After all, this enzyme is also antigenic and may be of interest to the immunologists.

The interaction of the RNA polymerase with the parental RNA (step 5) leads to the synthetic of double-stranded RNA (step 6) which we know is an essential part of the replicative cycle. We heard about RNA replication in a variety of systems. The replication of RNA in Togaviruses was very clearly summarised by Dr. HORZINEK. Dr. SCHERRER summarised the replication of RNA in a virus which replicates at low temperatures and Dr. SCHOLTISSEK brought us some of the problems of RNA pools in infected cells. One of the problems of course is how to label HC virus efficiently with radio-isotopes since there are always problems with the RNA pools. Dr. SCHOLTISSEK described how one can control the uridine pool and if this is possible, then one can label the virus more efficiently. I believe that Dr. HORZINEK has the right ideas.

The subsequent step (n° 7) is the synthesis of the various RNA molecules. From Dr. SCHLESINGER and from Dr. HORZINEK we heard about the translation of the genetic information and the replicative intermediates which are required for the synthesis of RNA and we have seen a variety of RNA species. I don't want to go into deteil, but we have a 40 S viral RNA, a 26 S and a 20 S RNA species. Each RNA species has its own function and the timing of the synthesis of these RNA molecules is easily controlled. Step n° 8 has to do with the association of the messenger RNA with the cellular ribosomes.

The virus utilizes the cellular machinery of the host in order to produce its own macro-molecules. A variety of proteins, peptides and glycoproteins are synthezised. We have heard about an initiation factor in the case of picornaviruses that Dr. BACHRACH brought up. The different glycoproteins and capsid proteins have specific functions in the cells and it is obvious that people will be interested in testing the genetic ability of the RNA species under in vitro condition. We heard from Dr. SCHLESINGER and from Dr. VAN DER ZEIJST about experiments in which one can study the ability of the RNA to be translated by ribosomes and yield the viral specific proteins. We now have better tools to understand this and may be in the future we will be able to develop systems in which the RNA could be translated under in vitro conditions. The peptides could then be used as antigens for immunisation, if they are immunogenic, and one would not have to grow viruses in cells any more.

Thus theoretically speaking one would be able to isolate the RNA polymerase and replicate the RNA under in vitro conditions. The RNA synthesized in vitro would be transferred into an in vitro protein synthesizing system and the proteins synthesized in vitro used to immunise an animal. This may be an efficient method but we have to wait for the future to tell us.

One we have the different proteins we certainly have to speak about antigens and I mentioned that Dr. AYNAUD, Dr. MATTHAEUS and Dr. CORTHIER described the different antigens and attempted to relate - which is what we actually need - the different peptides to the different antigens in order to separate them for further investigation. In the summaries of Dr. DALSGAARD and also Dr. LIESS we have seen that one can isolate antigens from infected cells, and study different kinds of antigen - antibody reactions under in vitro conditions.

One of the subsequent steps in the case of Togaviruses is the formation of the membrane. This includes integration of the glycoproteins which are synthesised into membranous formations because after all, the ribonucleoprotein complex of the virus must bud through a membrane. We heard from Dr. ROTT about his studies of influenza virus which is an entirely different virus, but the concepts are the same.

The concept is that the glycoprotein which is synthesised in the cell will find its way into a membrane structure and will be organised in this membrane either as a floating or as a fixed protein molecule. We certainly know very little about the formation of membranes in the Togaviruses, specifically in HC virus infected cells.

This brings us to step 11, which is the assembly stage. This means that the ribonucleoprotein complex must move in the direction of the membrane, which contains the viral glycoproteins and by passing through this membrane it will assemble into an enveloped and nature virion. This could take place in the cytoplasmic vesicles or at the surface of the cell, but finally the virus is released and the replication of an RNA virus is completed.

I hope you can all see that the research done on various other viruses has contributed much to our information in the field of this specific virus.

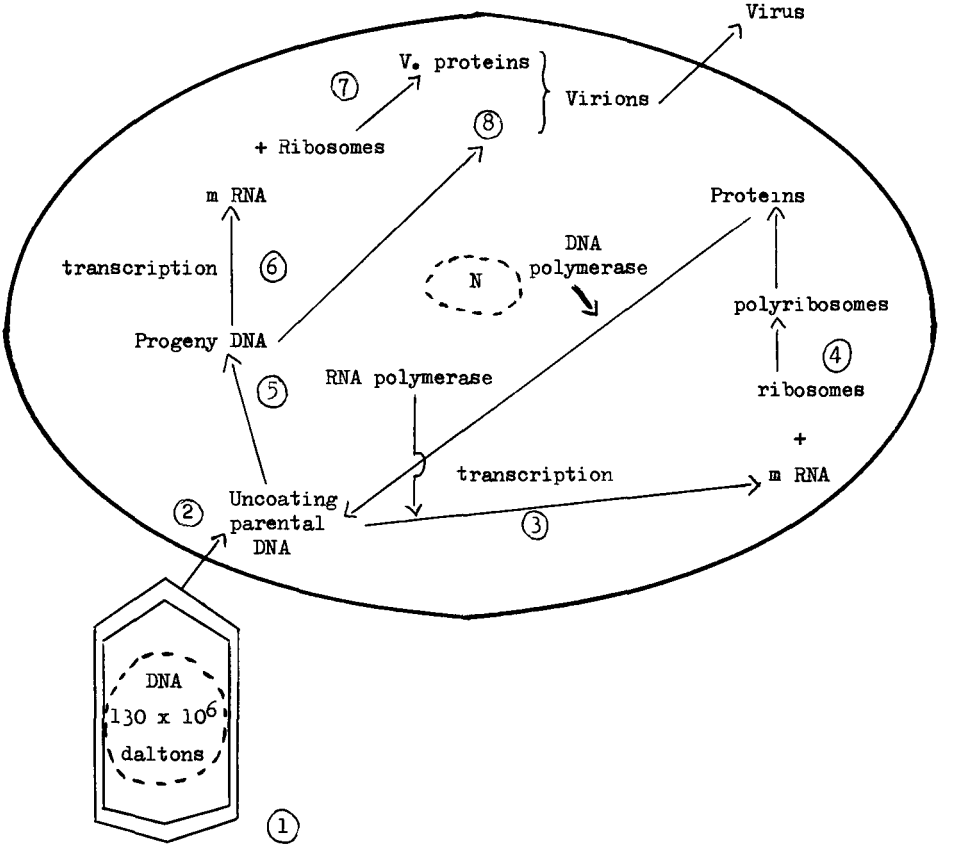
B. AFRICAN SWINE FEVER VIRUS

Here we have an entirely different virus, a DNA virus which poses a threat in many countries and yet little is known about it. One of the most amazing things about this virus is that when it infects an animal, the pig, no neutralizing antibodies are formed. The virus can circulate without the animals being able to develop a means to counteract the infection. We know very little about this group of viruses, the Iridoviruses, and we are very fortunate that we have Dr. McAUSLAN here to contribute to our knowledge in this field. I feel that we have to think about ways and means to use the knowledge on the iridovirus group to supplement the field of the African swine Fever virus.

1.The replicative cycle

In fig. 3 the replicative cycle of a cytoplasmic DNA virus has been presented. I am sure that this is known to everyone, but it can give us an idea of what information we already have, not as regard of ASF virus, but about the Iridovirus group and what problems we have to face. The virus contains a DNA genome of about 130 million daltons, which can synthesize a larger number of proteins.

Figure 3 : B. Iridovirus - African Swine Fever Virus.



(see following page)

Figure 3 : Legend.

Molecular step	Description	Author
(1)	Analysis of the purified virions	McAuslan
(2)	Uncoating	
(3)	Transcription	McAuslan
(4)	Polyribosomes formation - synthesis of viral proteins	
(5)	Replication of viral DNA	Becker
(6)	Transcription of progeny DNA	McAuslan
(7)	Viral structural proteins	Becker - McAuslan
(8)	Assembly	
(9)	Release of virus from infected cells	

It has a capsid and an envelope and this obviously makes it a virus with a very complex structure. People working in the field of ASF virus have great difficulty in purifying the virus as well as in growing it. If we cannot isolate and purify the virus, it is very difficult to label the viral components and obviously the information that can be obtained is very limited. We have to ask the questions : "What is the reason that a virus grows in such a way ? What are the methods that we have to develop in order to stimulate the virus to grow in larger quantities for its further analysis ?" Dr. McAUSLAN presented us with some analyses done on purified frog virus and we also have some idea about the structural proteins in some of the Iridoviruses. We know absolutely nothing about the ASF virus protein composition.

The second step in the cycle is of course the absorption and entry of the virus into the cell and the uncoating of the virus. Once again, we know very little about the uncoating of the ASF virion in the infected cell. The third step, as Dr. McAUSLAN indicated to us, is transcription. We do not know whether the enzyme responsible for transcription is an enzyme carried in the virus particle, or whether it is a cellular enzyme in the cytoplasm. But as a result, we know that we have messenger RNA. If we can block the initial transcription of the DNA by any means and Dr. BACHRACH indicated to us in the discussion that rifamycin derivatives can prevent the replication of the virus, then we may be able to do something about the transcription stage by the introduction of anti-viral substance.

The following step is the interaction of messenger RNA with ribosomes to form polyribosomes and I think I have indicated that one has to be very careful in defining polyribosomes - not what seem to be polyribosomes but what are indeed ribosomes. There are various interactions between messengers and proteins in the cytoplasm of cells and one has to be very careful in sorting these out.

One of the major proteins synthesised in the cycle is of course the DNA polymerase which will interact with the parental DNA and with the replicative DNA. I have been able to describe some experiments with another virus, a herpes virus which replicates in the nucleus and which can be studied under semi-in-vitro conditions.

Obviously these experiments could also be done with ASF virus infected cells and certainly with other Iridoviruses. Step n°3 is the synthesis of the progeny DNA and one lots of DNA is synthesised in the cell, the subsequent step is again transcription as was discussed by Dr. McAUSLAN. The genetic information is released by the synthesis of the messenger RNA from the parental DNA. We do not have to wait for the synthesis of structural proteins before the replication of the DNA. This is true for cytoplasmic DNA viruses, and also for the Iridoviruses and some of the nuclear viruses, including the herpesviruses.

Once again if we have transcription of the progeny which means that we have more and more proteins synthesised, more RNA synthesised, and more messenger associated to ribosomes, we have a cycle in which the virus takes over the cellular machinery. Dr. McAUSLAN mentioned that the Iridoviruses even control nuclear function and this is true also for the herpesviruses. The cell is shut off and the machinery is turned on for the virus by an initiation factor, a specific protein which is controlled by the virus and which makes the ribosomes recognize the viral messenger RNA. If there are still cellular messengers which are long lived in the cytoplasms, they eventually become degraded.We have a large variety of proteins as can be seen in the experiments of Dr. McAUSLAN and the paper by Dr. OLSCHEVSKY on herpes simplex virus. This means of course that we have more antigens than we except and in addition, by refinding our system, we may discover new proteins. There is the possibility that many of the proteins are not antigenic or are only slightly antigenic and they have nothing to do with the immune mechanism or the immunisation of the animal against the disease. But we must remember that we do not have neutralising antibodies and the question is why ? We have a large DNA genome which can synthetize more than 100 coat proteins, and yet in the animal these proteins do not express themselves. The main difficulty is that we cannot develop a vaccine and therefore we cannot act against the disease. Perhaps as Dr. BACHRACH indicated to us, the only way is just to eradicate the animal. This does not mean of course that we eradicate the virus, since all pigs would then have to be eradicated. Who knows whether this will be successful. There are more questions to be asked than answers brought here during this meeting.

CHAPTER III - GOALS

In concluding this summary, I hope that those who are not familiar with the DNA viruses, but are familiar with RNA viruses, and those who are familiar with other aspects of molecular virology, after being acquainted with the difficulties will be in a position to try and help those who are engaged in research on HC and ASF viruses. If possible, and I believe that there will be support, they could not only develop new concepts but also formulate experiments together.

This is one of the messages that we would like to come out with from this meeting. People can collaborate with each other if they wish and do experiments in other laboratories in order to help answer specific questions as well as to learn new techniques. What we would like to have, is the opportunity to obtain basic knowledge on the molecular biology of these two viruses. It is about time that we knew all the ins and outs of this group of viruses, which should be documented so that we can solve this problem at a molecular level and then use the information to solve some of the major problems which I have already discussed. We now have the tools of molecular biology in our hands and they are easy to learn. There is tremensous interest in the different groups to know all the techniques, and then obviously, when we have more people thinking and working in this field, it will be possible to study the molecular biology of these viruses. One of the things which we did not discuss here and which Dr. AYNAUD brought up are the different mutants of the viruses. Dr. AYNAUD has different viral strains in his laboratory which are both cols and hot strains. There are strains brought in from Japan which are being used for immunisation, but very little has been done on the genetics of the viruses. Here I think it is appropriate to repeat what I said before - we not only have to learn the genetics of these viruses, we also have to search for viruses which do not cause the disease in the pig, but replicate to a certain extent thus preventing sensitive cells from being infected by the wild strain of HC virus. In addition to this, it became clear at the meeting that we have to study the antigenic properties not only of HC virus, but also of the bovine mucosal and related viruses.

I have no doubt in my mind that there is a lot of work to be done and new techniques to be applied to the understanding of the antigenic properties as well. If we combine all the knowledge that we have gained here with the knowledge that we have in other fields, I am quite confident that we will be able to obtain basic information on some of the problems.

There are many more details that one could bring into the summary of the meeting but I don't think that this is necessary. All the details are in the papers and I want to congratulate the speakers on presenting very complicated information in a very clear way.

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The invited participants from the Laboratoire National de Recherches Vétérinaires,Estrada Benfica, 701, LISBONNE 4, were unable to participate in the meeting because of the practical situation in Portugal.

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