STUDIES ON THE REPLICATION OF RHINOVIRUS RNA.

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Thesis presented for the degree of

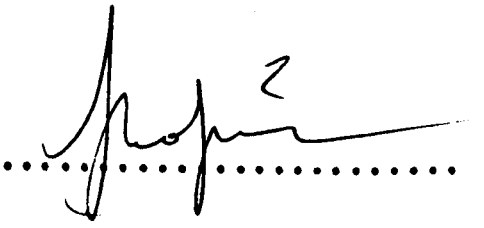
Doctor of Philosophy

University of Warwick

September, 1973

D E C L A R A T I O N

I hereby declare that this thesis has been composed by myself and has not been accepted in any previous application for a degree. The work of which it is a record has been done by myself and all sources of information have been specifically acknowledged by means of references.



ACKNOWLEDGEMENTS

I should like to express my gratitude to Professor D. C. Burke and N. J. Dimmock to whom I am indebted for their guidance, endless discussions and interest in this work.

I should also like to thank the following:

Drs. E. J. Stott and R. A. Killington for allowing me to draw on their experience of rhinoviruses;

Dr. R. A. Bucknall for providing unpublished data on 65-709;

Messrs S. Lolos and V. Vasiliadis for their pains-taking help in preparing the drawings for this thesis;

My colleagues in the department of Biological Sciences for many useful discussions;

Mrs. D. C. Kelly for her careful typing;

Miss H. Kucza for her support and encouragement during the last three years.

This thesis was undertaken through a studentship provided by Imperial Chemical Industries Limited to whom I extend my appreciation.

ABSTRACT

We have studied the multiplication of rhinovirus type 2 in human embryo lung cells.

The work which will be described in this thesis has been divided into three parts.

I RNA replication

II Effect of the antiviral compound I.C.I. 65-709 on viral RNA synthesis.

III Rhinovirus induced polymerase activity. The effect of I.C.I. 65-709 on polymerase activity.

GENERAL INTRODUCTION

Historical aspects - Classification

Intensive study of the common cold led to the finding that its main cause was a virus (Andrewes, 1965). In 1953, Andrewes et al. reported the in vitro culture of a virus obtained from a human being suffering from a cold. These experiments could not be repeated for about 8 years, then in 1960, Tyrrell and Parsons reported that viruses which could induce cytopathic effects in vitro could induce colds when inoculated into volunteers. They found that a temperature of 33°C in a medium with a low pH of 6.8 optimized the growth of the virus.

Among the numerous names suggested for the common cold viruses, rhinoviruses is the one generally accepted today (Virus Subcomm. Int. Nomencl. Comm., 1963). Rhinoviruses were defined as ether stable RNA-containing viruses which were smaller than 30nm in diameter and labile below pH 5 (Tyrrell & Chanock, 1963) and they were placed as a separate subgroup inside the picorna-virus group (International Study Group for Enteroviruses, 1963). However this definition included some other viruses such as foot and mouth disease virus (FMDV), vesicular exanthema virus (Wawrzkiewicz et al., 1968) and feline picornavirus (Burki, 1965). Studies on physicochemical properties of many picornaviruses revealed many differences among the members of the group and a reclassification has been suggested (Newman et al., 1973) in which physico-chemical properties are taken into account together with the biological properties.

Biological properties

The ratio of physical particles to p.f.u. has been estimated as 2,210 for rhinovirus type 2 and 6,500 for rhinovirus type 14 (Stott & Killington, 1972). These values are well below the particle : p.f.u. ratio of poliovirus (Korant et al., 1972).

Rhinoviruses can grow in the laboratory in human cells (H-strains) or in both human and monkey cells (M-strains) (Tyrrell & Bynoe, 1966). However it has been found that H and M strains may exist within the same serotype (Phillips et al., 1965) and it has also been reported that some H-strains can grow in monkey cells (Douglas et al., 1966).

The first plaque assay of rhinoviruses was described by Parsons and Tyrrell in 1961 who counted microplaques under fluid overlay. A year later Porterfield reported a plaque assay in human embryo lung cells under agar (Porterfield, 1962). However in 1966, Fiala and Kenny reported that increased Mg^{++} concentration enhanced plaquing and demonstrated the appearance of plaques in 2-3 days using HeLa cells. This assay has proved to be applicable to the 55 known serotypes tested (Connant et al., 1968; Fiala, 1968).

Rhinovirus infectivity can be neutralized by specific antisera and this reaction is the basis of classification within the rhinovirus group. The rhinovirus collaborative programme, which was initiated by the World Health Organisation, to compare all the reported rhinoviruses in cross neutralization tests has revealed 55 distinct serotypes during the first phase of its study (Kapikian et al., 1967). The number has been increased to 89 after the second phase (Kapikian et al., 1971) and 17 other candidates are currently being investigated (Stott & Killington, 1972). The existence of serological variation within a serological type and of some low level cross reactions between serotypes led to the further classification into antigenic groups (Fenters et al., 1966).

Immunodiffusion reactions between rhinoviruses and their antisera revealed one precipitin line (Acornley et al., 1968). However heating rhinovirus type 2 at 56°C for 30 min results in a failure of the virus to combine with neutralizing antibodies or induce antibodies (Doggett et al., 1963; Dimmock, 1967) suggesting a change or loss of antigenicity.

Rhinoviruses 1A and 2 have been used to demonstrate complement fixation (Mogabgab, 1962; Chapple et al., 1967) however the distribution of complement fixation antibody did not correlate with that of the neutralizing antibody and their specificity was uncertain.

Physicochemical properties

Rhinoviruses are similar to most picornaviruses as far as morphology, size and symmetry are concerned. They are of hexagonal shape with a diameter of 18 to 28 nm (Hamparian et al., 1961; Fiala, 1968; Blough et al., 1969; Kawana & Matsumoto, 1971). The hexagonal shape of the particles suggests icosahedral symmetry (Mayor, 1964; McGregor et al., 1966; Gerin et al., 1968).

Dimmock and Tyrrell (1964) first studied the sedimentation properties of rhinoviruses. They found a sedimentation coefficient in the range of 122S to 165S which did not significantly differ from the value obtained for poliovirus. Since then, velocity centrifugation has given conflicting results. Poliovirus was reported to have the same sedimentation rate with rhinovirus type 14 (McGregor & Mayor, 1971) and type 1A (Medappa et al., 1971). However in these reports the virions had first been purified by CsCl-gradient centrifugation and this may have increased the sedimentation rate of the rhinoviruses (Gerin et al., 1968). The best results were obtained by co-sedimentation studies. Two reports on such studies have recently appeared: Korant et al. (1972) estimated an S value of 150 for type 2, type 14 and equine rhinovirus by co-sedimentation, assuming the sedimentation coefficient of poliovirus to be 156S (Schaffer & Schwerdt, 1959; Schwerdt & Schaffer, 1955). Newman et al. (1973) used FMDV (146S) as a marker in sucrose gradients and found that rhinovirus type 2 and bovine enterovirus sedimented with a rate of 156S while rhinovirus type 4 and equine rhinovirus had a sedimentation coefficient of 150S.

The buoyant density of rhinoviruses has been

investigated by a number of groups (Rowlands et al., 1971; Newman et al., 1973; Chapple & Harris, 1966; Dans et al., 1966; McGregor et al., 1966; Gerin et al., 1968; Kojrant et al., 1970; Thomas et al., 1970). Rowlands et al. (1971) pointed out the errcrs which can arise when the viruses with the higher density are centrifuged for prolonged periods; for example, the buoyant density of FMDV increased from 1.43 g/ml to 1.46 g/ml when the sedimentation time was increased from 5 to 100 hours. The same people reported that the density of acid stable picornaviruses was not increased by prolonged sedimentation in CsCl. Nevertheless the buoyant density of rhinoviruses is placed by these investigators in the range of 1.38 - 1.41 g/ml. The wide range of density values could be explained by differences in experimental conditions used by the various groups as well as to the different strains of the virus used. It seems by co-sedimentation with FMDV that rhinoviruses have a buoyant density between that of enteroviruses (1.33 - 1.34 g/ml) (Mattern, 1962; Schaffer & Frommhagen, 1965; Mandel, 1962; Rowlands et al., 1971; Martin et al., 1970) and FMDV (1.42 - 1.51 g/ml) (Rowlands et al., 1971; Newman et al., 1973; Trautman & Breese, 1962; Brown & Wild, 1966; Liebermann & Gralheer, 1968). Equine rhinovirus has a higher density (1.45 g/ml) (Rowlands et al., 1971; Newman et al., 1973) and different base composition (see below) from human rhinoviruses and should therefore be classified in a separate sub-group (Newman et al., 1973).

Only one report has appeared on the molecular weight of the virus (McGregor & Mayor, 1971). Calculations based on sedimentation rate, diffusion coefficient and partial specific volume give a molecular weight of 7.1 x 10^6 daltons.

Chemical analysis of purified rhinovirus 14 indicates 70.2% of the virion is protein and 29.8% RNA (McGregor & Mayor, 1971).

Nucleic acid and proteins of the virion

The genetic material of rhinoviruses is single stranded RNA which has a molecular weight within the range of 2.4 - 2.8 x 10^6 daltons (McGregor & Mayor, 1971; Van Elsen et al., 1968; Nair et al., 1971; Koliais & Dimmock, 1973). Since the molecular weight of the virion is about 7 - 8 x 10^6 daltons and the RNA content is only 30% it is apparent that only one strand of RNA is contained per virion. Base ratio analysis of the RNA has shown rhinoviruses to have a uniquely high adenine content (Brown et al., 1970; McGregor et al., 1971; Newman et al., 1973) which distinguishes them from the other picornaviruses including equine rhinovirus. Since this thesis is directly concerned with the various RNA species induced by the virus, this topic will be discussed extensively in the chapters to follow.

Like the other picornaviruses, rhinoviruses possess four virion polypeptides (VP) (Korant et al., 1972; Medappa et al., 1971; Stott & Killington, 1973). VP1, VP2 and VP3 are the major polypeptides with molecular weights of 37,000, 26,000 and 24,000 daltons and VP4 a minor polypeptide with a molecular weight below 10,000 daltons. Korant et al. (1970) reported that rhinoviruses lacked the minor polypeptide but later agreed to its presence. This earlier failure to detect it was caused by a procedural artefact as they stated in a more recent report (Korant et al., 1972). A fifth polypeptide VP0 or E has been reported by Medappa et al. (1971), but it has not been detected by others, the significance of this finding remains to be solved in the future.

The proteins of equine rhinovirus have been studied by Korant et al. (1972) who found them all to be of higher molecular weight than human rhinovirus proteins.

An equivalent molar ratio of the four polypeptides has been calculated by Medappa et al. (1971) although Stott and Killington (1973) reported a 1:1:1:0.5 molar ratio which is similar to that found for mouse encephalo-

myelitis (ME) virus (Rueckert et al., 1969), bovine enterovirus (Johnston & Martin, 1971) and FMDV (Talbot et al., 1973).

Site of replication

All available evidence indicates that picornaviruses multiply in the cytoplasm. Cytoplasmic RNA replication was shown first by autoradiography (Franklin & Rosner, 1962; Hausen, 1962) and the RNA synthesizing enzymes are found together with viral RNA and viral proteins as large membrane bound aggregates in the cytoplasm of the cell (Holland & Bassett, 1964; Dalgarno & Martin, 1965; Eason & Smellie, 1965; Penman et al., 1964; Beker et al., 1963). Since the rhinovirus induced polymerase is found in the cytoplasm (Yin & Knight, 1973), it is likely that the cytoplasm is the site of replication of the rhinoviruses.

Multiplication cycle

The time course of the multiplication cycle of picornaviruses depends on a number of factors. Amongst the most critical is the type of virus, the multiplicity of infection (Baltimore et al., 1966), the type of cells in which the virus is growing and the nutritional or mitotic state of the cells. Studies on the early events of the replication of some rhinoviruses grown in HeLa cells have been done recently by Lonberg-Holm & Korant (1972). They find that although the rate of attachment to cells of rhinovirus type 2 is greater than that of type 14, the number of receptor sites is similar for each type. Competition experiments on the saturation of the receptor sites on the cell surface revealed that the sites for type 2 are different from those for type 14. However excess rhinovirus type 2 blocks attachment of type 1A and excess of type 14 blocks type 51, indicating that the different serotypes might fall within certain receptor families. Another interesting aspect revealed by Lonberg-Holm & Korant (1972) is the fact that attachment of some rhinoviruses to the cell, in

addition to the eclipse phase, is temperature dependent. Some efforts have been made to analyse the product of eclipse. It is believed to be a slower sedimentating particle (135S) which is not infectious. It lacks VP4 but still contains RNA. Upon CsCl centrifugation these particles lose polypeptide VP2. It is possible that the particles which lack polypeptide VP4 represent the first step of the virus uncoating procedure. However since the majority of picornavirus particles are non-infectious there is no way of determining if the 135S particles are degraded non-infectious particles or represent the early stages of productive infection.

The majority of the progeny virus remains cell associated when the plateau of virus production is reached. Over 90% of the virus is cell associated when the virus grows in KB or HEL cells and over 99% when it grows in HeLa cells (Thomas et al., 1970; Koliais & Dimmock, 1973; Fiala & Kenny, 1967; Stott & Heath, 1970). The amount of released virus is increased, without affecting the total virus yield, by a high concentration of magnesium ions (Stott & Heath, 1970).

MATERIALS AND METHODS

MATERIALS

1. Viruses

Rhinovirus type 2 was obtained from Dr. E. J. Stott, poliovirus type 1 (L Sc 2 and b) from Dr. M. S. Pereira and bovine enterovirus (VG-5-27) from Dr. S. J. Martin. Guanidine resistant rhinovirus mutants were prepared in this laboratory by Dr. N. J. Dimmock.

2. Cells

Human embryo lung (HEL) cells of the MRC5 line were obtained from Mr. J. P. Jacobs (National Institute for Medical Research, Hampstead Laboratories). Both L132 and HeLa cells were obtained from Dr. E. J. Stott. Baby hamster kidney cells (BHK 21, clone 13) were obtained from Dr. D. H. Watson. Chick embryo fibroblast (CEF) cells were routinely prepared in this laboratory from 11 days old fertilized eggs (Walters et al., 1967).

3. Biological materials

Bovine albumin (fraction V) was purchased from Sigma Chemical Co. St. Louis, Missouri, U.S.A.

Deoxyribonuclease (ribonuclease-free, electrophoretically purified) was obtained from Worthington Biochemical Corp., Freehold, N. J., U.S.A; pancreatic ribonuclease and pyruvate kinase from the Boehringer Corporation, London. Trypsin was obtained from Difco Laboratories, Detroit, U.S.A.

Eagle's minimal essential medium and 199 medium were purchased as ten-fold concentrates from Wellcome Reagents Ltd., Beckenham, England.

ATP, UTP, GTP and CTP (synthetic or from equine muscle) were purchased from Sigma Chemical Company Ltd., London.

Actinomycin D (AMD) was donated by Merck, Sharp and

Dohme Ltd., Rahway, New Jersey, U.S.A.

Penicillin and streptomycin were obtained in the form of crystomycin which contained 600 mg (10^6 units) of penicillin to 1 g streptomycin. Crystomycin was purchased from Glaxo Laboratories Ltd., Greenford, England. Kanamycin was obtained from Winthrop laboratories.

[^{3}H]-uridine labelled HeLa nucleolar RNA was a generous gift from Dr. A. Meager.

4. Chemical materials.

65-709 gluconate was kindly provided by I.C.I. Ltd., through Dr. R. A. Bucknall.

Sodium lauryl (or dodecyl) sulphate (SDS), ammonium persulphate, gentian violet, diethylpyrocarbonate and uridine were all purchased from B.D.H. Chemicals Ltd., Poole, Dorset.

Dimethylsulphoxide and phenol were obtained from Hopkin and Williams Ltd., Chadwell Heath, Essex, England. Phenol, ether and ethanol were redistilled before use.

Phospho(enol)pyruvic acid, guanidine hydrochloride and cycloheximide were obtained from Sigma Chemical Company Ltd., London.

NNN'N' - Tetramethylenediamine (TEMED) and NN' - - methylenebisacrylamide were both purchased from Eastman Kodak Company, Rochester, New York, U.S.A. Bisacrylamide was recrystalized before use from acetone as described by Loening (1967).

Acrylamide was a gift from Fluka, Switzerland.

Cleland's reagent (dithiothreitol) was obtained from Calbiochem., London.

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

2,5 - Diphenyloxazole (PPO), 1,4 - di(2 - (5 - phenyloxazolyl)) - benzene (POPOP) and naphthalene, all scintillation grade, were obtained from Nuclear

Enterprises (G.B.) Ltd., Edinburgh, Scotland. Aerosil standard silica was obtained from Bush, Beach, Segner and Bayley Ltd., London.

5. Radioactive materials

All radioactively labelled compounds were obtained from the Radiochemical Centre, Amersham.

6. Media

10% growth medium. Minimal essential medium was supplemented with 10% (v/v) calf serum and adjusted to pH 7.4 with 5% (w/v) sodium bicarbonate solution. Penicillin and streptomycin were added to final concentrations of 60 µg (100 units) and 100 µg per ml respectively.

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

Overlay medium was identical to maintenance medium except that 0.5% (w/v) of agar was included.

Storage medium. Minimal essential medium was supplemented with calf serum and dimethylsulphoxide to a final concentration of 10% (v/v).

7. Buffers and solutions

RNA extraction buffer was 0.05 M tris, 0.1 M sodium chloride, 0.0025 M ethylenediamine tetra acetate (disodium salt)(EDTA), adjusted to pH 7.5 at 20° with hydrochloric acid.

Electrophoresis buffer. This was prepared as described by Loening (1967).
A concentrated stock solution was prepared as follows:

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

made up to 1000 ml with glass distilled water. For use as

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

Acrylamide stock solution. This contained 15 g acrylamide and 0.75 g bisacrylamide in 100 ml of glass distilled water. It was stored in the dark at 5°C.

Standard saline citrate (SSC) was a solution of 0.15 M sodium chloride, 0.015 M trisodium citrate, adjusted to pH 8.45 with HCl.

Gentian violet stain was a solution of 1% (w/v) gentian violet and 20% (v/v) ethanol.

Phosphate buffered saline (PBS) was a solution of 0.15 M - NaCl, 3.4 mM - KCl, 10 mM - Na_2HPO_4 and 2 mM KH_2PO_4, pH 7.4 (Dulbecco and Vogt, 1954).

Formol saline consisted of 4% (v/v) formaldehyde in 85 mM NaCl, 0.1 M - Na_2SO_4.

8. Scintillation mixtures

Toluene scintillation mixture consisted of 6 g PPO and 0.73 g POPOP dissolved in 1 litre of toluene.

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

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

METHODS

1. Subculturing of cell lines.

HEL, HeLa, L132 and BHK 21 (clone 13) cells were all maintained in a similar way except that HeLa cells were treated with kanamycin to combat mycoplasma at 2 - 3 month intervals. Apart from the HEL cells, which were grown in flat bottles, all others were grown in both flat and roller bottles. The following method was used:

a. Trypsinisation. The medium from the confluent bottles was removed and the monolayer washed with approximately 10 ml trypsin solution (0.2% trypsin in PBS). 1 ml of the same trypsin solution was added and the bottles were incubated at 37°C until the cells came off the glass (usually 2-4 min). When all or nearly all the cells were detached, 10 ml of growth medium was added and the cells were suspended in this medium by pipetting, and the growth medium was then added. HEL cells were diluted 1 : 3 and the other cell lines 1 : 6. The freshly seeded bottles were either gassed with 5% CO_2 in air for 20 seconds or incubated with loose caps inside a gassed incubator.

b. Growth. The cells grow at 37°C. HEL were confluent after 3 - 4 days and the other cell lines after 7 days. HEL cells lasted up to 35 - 40 passages before cell division slowed down and the cells degenerated. The cells were maintained happily at 33°C with maintenance medium.

c. Preservation in liquid nitrogen. The cells were trypsinized as usual and suspended in 10 ml growth medium as described under (a). They were pelleted in the bench centrifuge for 2 min and the cells obtained from one confluent bottle were suspended in 1 ml of storage medium. They were then transfered to an ampoule which was sealed in a flame. The ampoule and contents were then gradually cooled at the rate of 1-2°C per min by placing in a polysterene container at -20°C for 1 h and at -70°C for

2 h. The ampoules were then placed in liquid nitrogen. To recover the cells the ampoules were thawed rapidly in a water bath at 37°C, and the cells transfered into a 150 ml bottle containing about 30 ml of growth medium. Cells were incubated at 37°C. The medium was replaced with fresh growth medium immediately after the cells stuck on the glass (usually between 6 - 11 h after they were thawed) since dimethylsulphoxide is toxic.

Since the HEL cells were being used continuously care was taken in order to have the required stocks. Many ampoules containing cells of early passage were therefore stored in the liquid nitrogen. An ampoule was recovered from the liquid nitrogen when the current cell line reached pass 30.

2. Preparation of virus stocks

a. Rhinovirus stocks were prepared using either L132 or HeLa cells in the following manner: 30 million cells were suspended in a roller bottle containing about 250 ml of growth medium with 0.1% bicarbonate. The bottles were rolled at 37°C and growth medium containing 0.2% bicarbonate was replaced after 2 days. When cells reached confluency (usually 3 days later) the growth medium was replaced with 50 ml of MEM and 0.2% bicarbonate containing no calf serum and the bottles were allowed to roll for 30 mins. The medium was then replaced with 10 ml of the virus suspension (10^7 pfu / ml) which was adsorbed at 33°C for 1 hour. 50 ml of 2% MEM were added and the bottles were rolled at 33°C for 24 hours. The bottles were then shaken vigorously and if all the cells were not detached from the glass 50 ml more of fresh medium was added and incubation continued for a further 24 hours at 33°C. The cells were broken by freezing and thawing and the cell debris was removed by low speed centrifugation. Stocks were kept frozen at -70°C without any significant loss of infectivity over a period of several months. The titre of the virus prepared by this method was around 10^8 pfu per ml as titrated with HeLa cells. The yield of virus

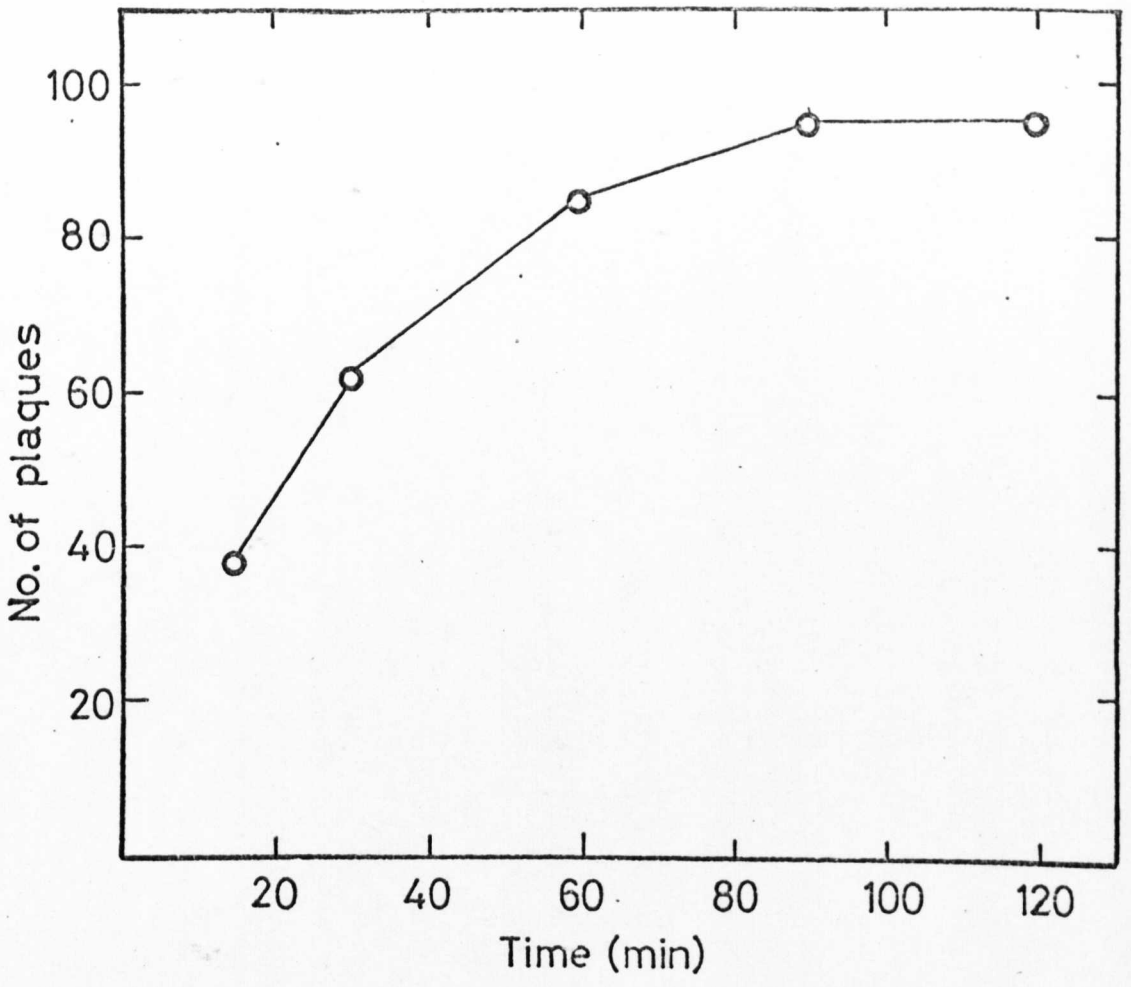


Fig. 1. Kinetics of virus adsorption by HeLa cells. Confluent monolayers were infected at the same multiplicity of infection and were overlayed at the indicated times after infection. The number of plaques counted 3 days later is plotted against the time the virus was allowed to adsorb.

from HEL cells was approximately 10 times less than L132 or HeLa cells.

b. Poliovirus. The best titres (up to 2 x 10^9 pfu / ml) were obtained when the virus was grown in HeLa cells using the method used for rhinovirus. The only difference was that the cells infected with poliovirus were rolled at 37°C.

c. Bovine enterovirus was grown in BHK 21 cells as described by Martin, Johnston and Clements (1970). Monolayers of BHK cells infected with virus were rolled at 37°C overnight with MEM without serum. The cell sheets were completely disrupted and the virus harvested and stored at -70°C.

d. Guanidine-resistant rhinovirus mutants. Rhinovirus was adapted to grow in the presence of guanidine hydrochloride by titrating the virus in HEL monolayers in rolled tubes containing 20, 60 or 100 µg / ml of inhibitor (Acornley et al., 1968). Virus growth was detected by cytopathic effects and the highest dilution of virus which grew at the highest concentration of guanidine was passaged again. Complete resistance was achieved by the fifth passage and stocks were prepared from virus of the seventh passage. Plaque titration in HeLa cells showed that the resistant mutant was not significantly inhibited by either 100 or 200 µg / ml guanidine.

3. Adsorption of virus by HeLa cells

In this experiment the kinetics of virus adsorption in HeLa cells were studied. The stock virus was diluted to 4 x 10^2 pfu / ml. 15 small plastic plates confluent with HeLa cells were infected with 0.2 ml of the diluted virus and incubated at 33°C for 15, 30, 60, 90 and 120 mins. After each of these periods the plates were washed and overlayed and incubated at 33°C. Plaques were counted as described in the next experiment.

The results obtained are shown in Fig. 1 which shows

Virus dilutions

10^{-7}

10^{-6}

10^{-5}

10^{-4}

Plate 1. Rhinovirus plaque assay in HeLa cells

that maximum virus adsorption occurred only after 90 mins adsorption at 33°C.

4. Assay of infectious virus

The infectivity of all virus samples was titrated by plaque assay:

a. Rhinovirus was titrated in monolayers of HeLa cells by a modification of the method described by Stott and Heath (1970). The cells obtained from one 1-litre flat bottle were seeded into 8 - 10 petri dishes (5 cm diameter) in 5 ml of growth medium and were incubated at 37°C for one day. The medium was removed and the plates were inoculated with 0.2 ml of virus diluted in maintenance medium. Virus was allowed to adsorb for 90 min at 33°C. The inoculum was then removed and the plates were incubated with 5 ml of overlay medium at 33°C for 3 days. The cells were then fixed with buffered formol saline, the agar removed and the cells stained with 1% gentian violet in 20% alcohol (Plate 1).

b. Poliovirus was titrated using the same plaque assay.

c. Bovine enterovirus was titrated in BHK cells as described by Martin et al., 1970. The cell sheets of the confluent monolayers were washed 3 times with calf serum-free medium and were inoculated with 0.2 ml of the virus dilutions. After 30 min incubation at room temperature the sheets were overlaid with 0.9% agar in Eagle's medium without calf serum. Plaques developed within 48 h at 37°C and were visualised by fixing with formol saline and staining with methylene blue.

d. Rhinovirus guanidine resistant mutants were titrated in the same way as the wild type rhinovirus. When 1 or 2 mM guanidine was included in the overlay no difference was obtained in the infectivity titres, although the same concentration of guanidine prevented the formation of plaques when wild type rhinovirus was assayed.

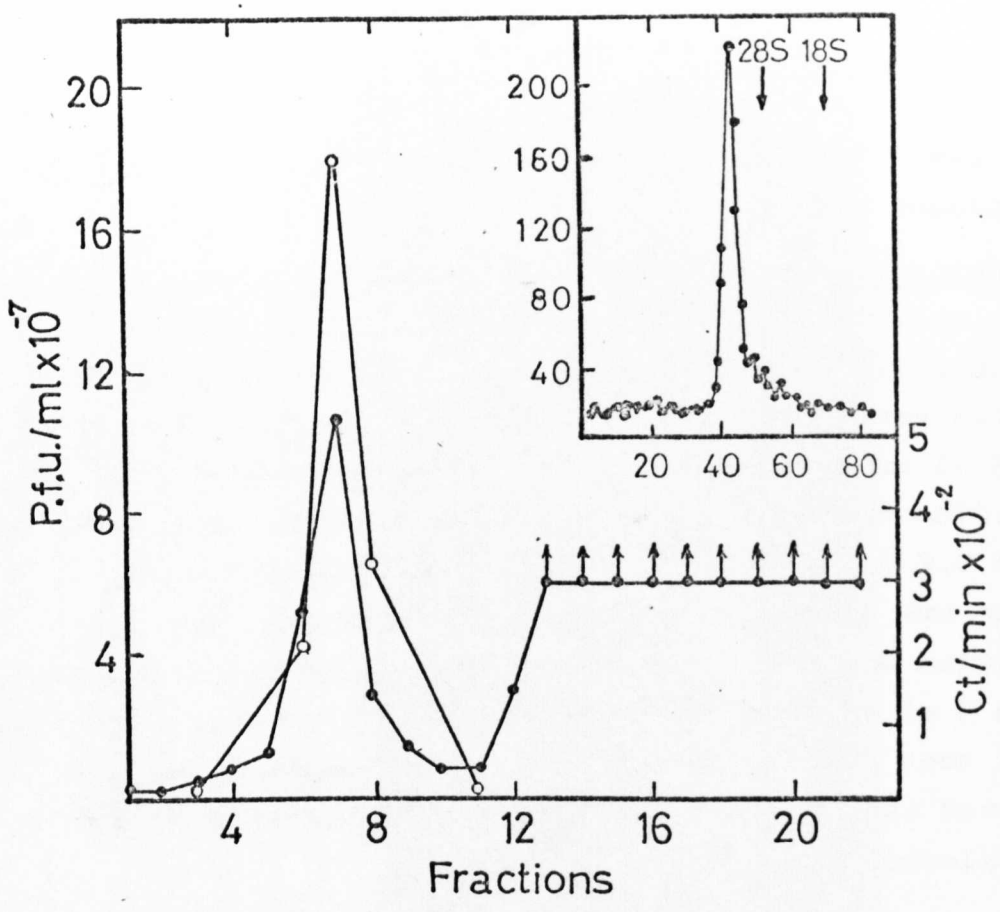


Fig. 2. Sedimentation of concentrated $[^3H]$-uridine labelled virus on a 15 to 45% sucrose gradient at 65,000 g for 3 to 5 h. Fractions of 1 ml were collected and the radioactivity of 100 µl samples was determined (●——●). Only the infectivity (o——o) of the peak fractions is shown. Inset: RNA was extracted from gradient fractions 6, 7 and 8 and electrophoresed on polyacrylamide gel. Ribosomal RNA markers from HeLa cells were co-electrophoresed and detected by optical density measurements.

5. Labelling and purification of rhinovirus

Confluent monolayers of L132 were infected as described above. 250 μC of [^{3}H]-uridine was added at the onset of the cytopathic effect. Virus was harvested after a labelling period of about 5 h. Purification of the labelled virus was carried out as described by Brown and Cartwright (1963) for FMDV. After the cell debris was removed by low speed centrifugation an equal volume of cold PBS saturated with ammonium sulphate was added, and the solution allowed to stand at 4°C for 1 h. The resulting precipitate was collected by centrifugation at 3,000 rpm for 30 mins and was resuspended in 50 ml of cold PBS. The suspension was then centrifuged for 10 min at 10,000 rpm using the 8 x 25 MSE angle rotor to remove insoluble debris and the virus was pelleted at 30,000 rpm for 1 h using the same rotor. The pellet was resuspended in 1 ml PBS containing 1% (w/v) sodium dodecyl sulphate (SDS) and it was layered on the top of a sucrose gradient (15-45% sucrose in PBS). The gradients were centrifuged for 3½ h at 25,000 rpm using the 3 x 23 ml MSE rotor. 1 ml fractions were collected by puncturing the bottom of the tubes containing the gradients. Radioactivity was determined by counting 0.1 ml of each sample in a liquid scintillation counter. The peak fractions were titrated for infectivity and the results shown in Fig. 2. A coincidence of infectivity and radioactivity is observed.

The peak fractions were pooled together, dialyzed overnight against PBS to remove sucrose and the volume was reduced by vacuum dialysis. Virion RNA was then extracted as described below and was analysed by polyacrylamide gel electrophoresis. A single peak of radioactivity was found (Fig. 2, inset) of MW around 2.8×10^6 daltons as estimated from the ribosomal RNA markers.

6. Effect of actinomycin D on cellular RNA synthesis

These experiments were carried out in order to

Duration of pulse after addition of AMD	HEL cells		L132 cells	
	CPM	%	CPM	%
Control (No AMD, labelled for 1 h)	12,000	100	63,000	100
1 - 2 h	5,400	45	18,000	28.5
3 - 4 h	230	1.9	700	1.1

Table 1. Effect of AMD on cellular RNA synthesis. Cells treated with 2μg/ml of AMD were labelled with [^{3}H]-uridine at the indicated times, and they were assayed for TCA-insoluble material.

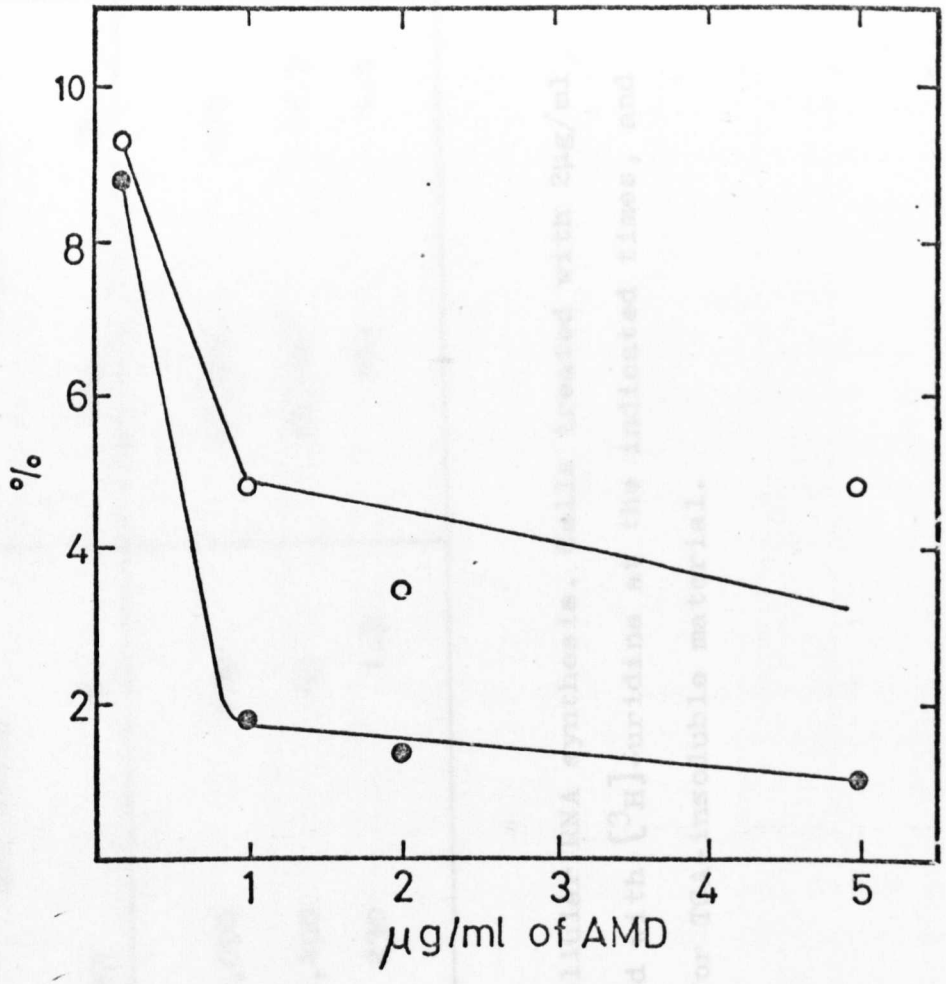


Fig. 3. Inhibition of ~~ribosomal~~ cellular RNA synthesis by AMD. Cells were treated for 3 h with the indicated doses of AMD; at the end of these periods they received a 1 h pulse of [^{3}H]-uridine. The percentage of the radioactivity of AMD treated cells to non treated is plotted against the drug doses (o—o HEL cells, ●—● L132 cells).

establish the conditions necessary for total inhibition of cellular RNA synthesis.

a. Time course of inhibition

Of six 50 mm Petri dishes confluent with HEL cells, four were treated with 2 µg / ml of AMD, and two served as untreated controls. Two of the AMD-treated cultures were labelled with 0.5 µc / dish of [^{3}H]-uridine between 1 and 2 h after the AMD was added and the other two between 3 and 4 h. The two control plates were similarly labelled for one hour. The same experiment was done in parallel using L132 cells. TCA-precipitable radioactive material was assayed using the method already described.

The results (Table 1) indicate that the cellular RNA synthesis is inhibited by 98% when the cells are treated for 3 h with 2 µg / ml of AMD before labelling.

b. AMD concentration required for inhibition

Duplicate plates of HEL or L132 cells were treated for 3 h with increasing amounts of AMD. Two plates in each case did not receive any AMD and were used as controls. At the end of the three hours the cells were pulsed for 1 h with [^{3}H]-uridine (0.5 µc / plate), and the TCA-precipitable radioactive material was assayed.

The results (Fig. 3) show that total inhibition of cellular RNA synthesis occurs when they are treated with 1 µg / ml of AMD for 3 hours.

7. Infection and labelling of HEL cell monolayer cultures

Most of the experiments were carried out in HEL cell monolayers which were grown in 1 litre flat bottles on a surface area of 115 cm^2. This is occupied by between 40 and 50 million cells when the monolayer is confluent. A multiplicity of 5 - 10 p.f.u. / cell was used for most experiments; each bottle was infected with 5 ml of stock virus containing 10^8 p.f.u. / ml, and allowed to adsorb at 33°C for 1 hour. The inoculum was then removed, 30 ml of maintenance medium was added and incubation continued at the same temperature. AMD (1 µg / ml) was added 4 hours

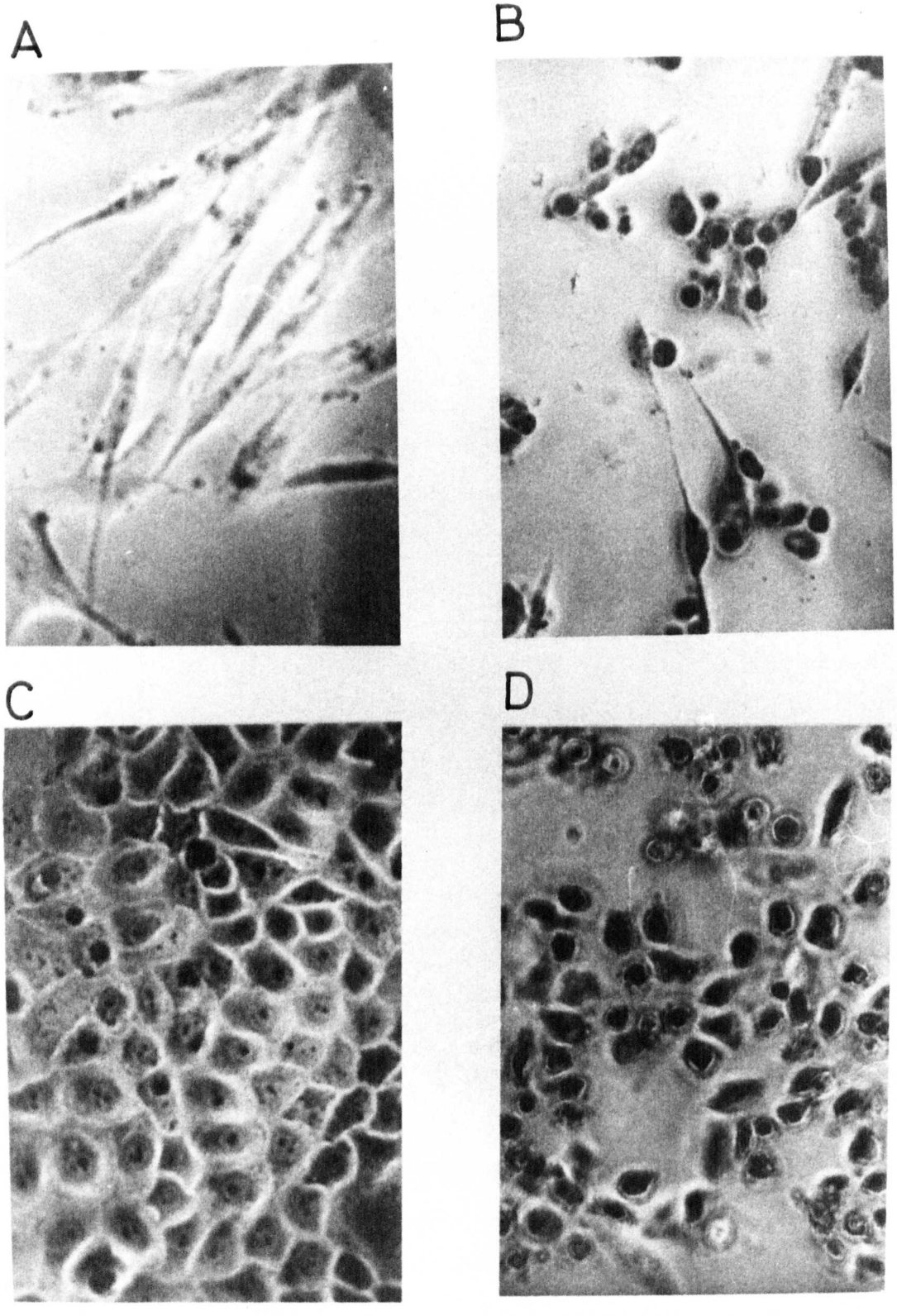


Plate 2. Human cells in tissue cultures. A. Non infected HEL cells. B. HEL cells infected with rhinovirus type 2 as seen in the microscope at 12 h p.i. C. Non infected L132. D. L132 cells infected with rhinovirus type 2 at 15 h p.i.

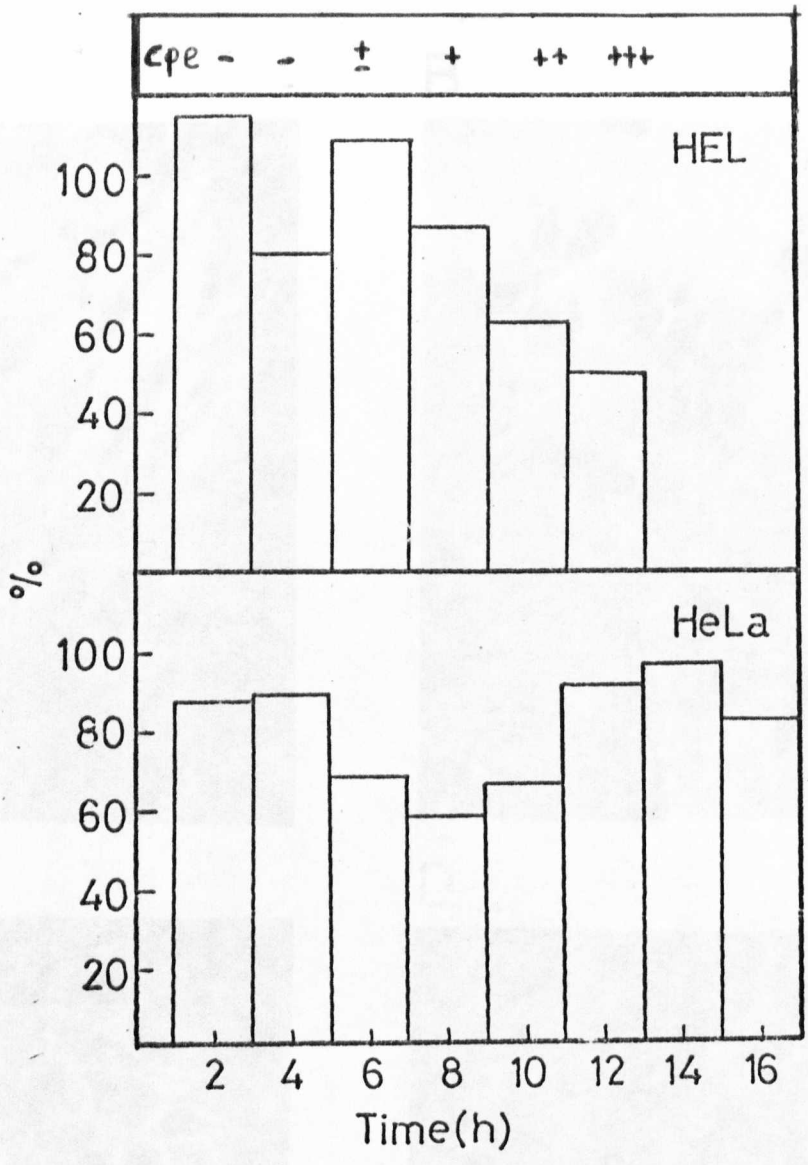


Fig. 4. Comparison of the acid-soluble uridine pools in cells infected with rhinovirus and non-infected cells. The vertical axes indicate the percentage of the acid soluble counts of the infected cells compared to that of the non-infected controls.

before the addition of the radioactive label. In experiments where it was necessary to add the label early in the replication cycle, the cells either were treated with AMD prior to infection or were given a higher dose of AMD. $[^3H]$-uridine or $[^{14}C]$-uridine was used to label the viral RNA. At the required time the AMD-containing maintenance medium was replaced with 5 ml of the same medium containing either 20 µc / ml of $[^3H]$-uridine or 5 µc / ml of $[^{14}C]$-uridine. The usual duration of the isotope pulse was of 2 hours, and when shorter pulses were required more radioactive label was added.

When the infected cells were observed under the light microscope a cytopathic effect was visible after 8 - 9 h post infection. Plate 2 shows infected and uninfected cells as they usually appear under the microscope at 12 h after infection.

8. Uridine uptake by infected cells

The incorporation of uridine into virus RNA depends upon the efficiency with which the cells are able to take up $[^3H]$-uridine into intracellular pools. Consequently to determine the variation in pool size, the acid-soluble $[^3H]$-uridine present in non-infected cells and in infected cells throughout the growth cycle was measured.

50 mm plastic Petri dishes confluent with HEL cells were infected and labelled with 0.2 µc / dish $[^3H]$-uridine at the times indicated in Fig. 4. Non-infected dishes were treated in parallel in exactly the same way. AMD (1 µg / ml) was present in all plates. At the end of each pulse infected and non-infected cells were assayed for TCA-soluble material as follows. After the monolayers were washed three times with cold PBS, 4 ml of cold 5% TCA were added to each dish for 30 min at 4°C. 0.5 ml of the TCA was assayed for radioactivity using triton-toluene scintillation mixture. The percentage of radioactivity of infected to non-infected samples was plotted in Fig. 4.

At the end of the incubation of each sample, the cells were observed under the light microscope and the c.p.e. was noted (upper part of Fig. 4).

It is evident that while the cells remained free of cytopathic effects, the pool of [^{3}H]-uridine varied by only 20%. At the onset of the cytopathic effect, the size of the radioactive uridine pool fell gradually to 50% of the control value. This probably reflected the numbers of cells which were detached from the monolayer, since only cells remaining attached were assayed.

The same experiment was repeated with HeLa cells. Similar results were obtained (Fig. 4).

9. Extraction of RNA

a. From infected cells

The infected monolayers were washed twice with cold extraction buffer solution containing 100 mM NaCl, 50 mM tris and 2.5 mM EDTA (pH 7.5) and the cells were lysed into 2 ml of the same buffer solution containing 1% (w/v) SDS. RNA was extracted by shaking at room temperature with two volumes of phenol saturated with extraction buffer solution. The aqueous phase was separated by centrifuging at 800 g for 7 mins. The phenol extraction was repeated one or two times until the resulting aqueous layer was clear. Residual phenol was removed from the final aqueous phase by three extractions with ether saturated with extraction buffer. A stream of nitrogen was passed over the sample to remove any remaining ether. The RNA was then precipitated with 3 volumes of redistilled ethanol at -20°C for over 10 hours.

b. From purified virions

The same extraction method was used. About 50 µg cold ribosomal RNA was added as carrier before the first phenol extraction.

10. Extraction of nucleolar RNA

Weinberg and Pennman (1970) showed that HeLa cell

ribosomal RNA, the 28S and 18S RNA's, were processed from a larger RNA molecule of 45S found in the nucleolus. The 45S molecule was cleaved specifically by nucleolytic enzymes in the nucleolus to give a series of RNA molecules which were precursors of ribosomal RNA. The RNA molecules found in nucleolar RNA had sedimentation coefficient values 45S, 41S, 36S, 32S, 28S, 24S, 20S and 18S. Usually only the 45S, 32S, 28S and 18S are found in nucleolar RNA, the other intermediate species being too short-lived to be detected. However under certain conditions (infection by poliovirus; Weinberg and Pennman, 1970, or in confluent monolayers of non-dividing HeLa cells) processing of the intermediates is inhibited so that detection of some short-lived intermediates is possible. Using this principle, nucleolar RNA labelled with $|^3H|$-uridine was prepared by Dr. A. Meager and was extracted according to the method described by Penman (1966) and Wagner et al., 1967.

11. RNA polyacrylamide gel electrophoresis

The system described by Loening (1967) was used. Polyacrylamide gels of 1.7 or 2.0% were supported by 0.5% agarose and polymerized in 10 cm tubes of 0.7 cm internal diameter.

a. Preparation of gels

2% polyacrylamide gels were prepared as follows: the following solutions were mixed in a 250 ml spherical flask:

6.1 ml of H_2O
7.5 ml of 5 x electrophoresis buffer
5.0 ml of acrylamide stock solution

The flask was placed into a 50°C bath and 18.6 ml of 1% hot (50°C) agarose were added before the whole mixture was degassed for 30 seconds. 0.025 ml of NNN'N' tetramethylethylenediamine and 0.25 ml of 10% ammonium persulphate were added before the mixture was transfered into the perspex electrophoresis tubes. Before the gels

polymerised 50 µl of H_2O were layered onto the top of each gel. After 30 mins the gels were extruded from the tubes and the slightly diluted end of the gel removed with a razor blade, leaving a clean flat surface of polyacrylamide/agarose mixture. The gels were drawn back into the tubes before use.

1.7% gels were prepared similarly, the proportion of each solution was different:

8.25 ml H_2O
8.80 ml 5 x buffer
5.00 ml acrylamide stock solution
22.05 ml 1% agarose

Details of the above solutions are given in the 'Materials' section above.

b. Preparation of the RNA sample for electrophoresis

RNA precipitated with ethanol was collected by centrifuging for 20 mins at 1,000 g. The ethanol was poured off and the RNA was dried with nitrogen. 0.1 to 0.2 ml of extraction buffer solution was added and 0.01 ml of the RNA solution was used for quantitative estimation of the RNA using absorbance at 260 nm. Up to 60 µg of RNA was electrophoresed on each gel. This RNA was made up to 5% sucrose before loading to prevent mixing with electrophoresis buffer.

c. Conditions of electrophoresis

Electrophoresis was carried out using a Shandon disc electrophoresis kit with the gels fitted vertically to connect the upper and lower buffer reservoirs which contained electrophoresis buffer plus 1% SDS. Extra care was taken to remove any air bubbles trapped at the bottom of the gels. The gels were electrophoresed for 1 h at 50 volts before the samples were loaded. The samples were electrophoresed at the same voltage for between 3 and 5 hours.

d. Detection of cellular RNA after electrophoresis

After electrophoresis the gels were extracted from the tubes directly into the cuvette of a Joyce-Loebl "Chromoscan" densitometer and were analysed for material

absorbing at 260 nm. Usually the DNA and the two ribosomal RNA species were detected.

e. Detection of viral RNA bands

Gels containing radioactive material were first scanned to detect the O.D. markers and were then frozen onto metal plates. Slices 1 mm thick were cut, placed in scintillation vials and dissolved in 0.2 ml of 100 volume hydrogen peroxide by incubating at 80°C for 1 to 3 h until the gels were completely dissolved. 10 ml of toluene/triton scintillation mixture were added to each sample which were then cooled at 4°C and counted in a Packard Tricarb series 3,000 liquid scintillation counter fitted with a teletype digital line printer. Simultaneous counting of $[^{3}H]$ and $[^{14}C]$ radioactivity was carried out using the channel-ratio method of Handler (1964) encoding the information in ASC-2 code onto punched tape. The results were calculated using an I.C.L. 1900 series computer.

12. Ribonuclease treatment of RNA

Salt concentration is the major factor which permits the selective degradation of single or double stranded RNA by ribonuclease (Baltimore, 1966). The following conditions were used for the experiment which involved ribonuclease treatment:

a. Degradation of both single and double stranded RNA

The RNA was dissolved in extraction buffer and it was incubated for 30 min at 37°C with 10 µg / ml of ribonuclease.

b. Selective degradation of single stranded RNA

The RNA was dissolved in double strength standard saline solution (SSC) pH 8.45 and was then incubated for 10 min at 37°C with 0.1 µg / ml of ribonuclease. Ribonuclease treatment was terminated by loading the RNA-RNAse mixture onto the top of the polyacrylamide gels and electrophoresing the samples immediately.

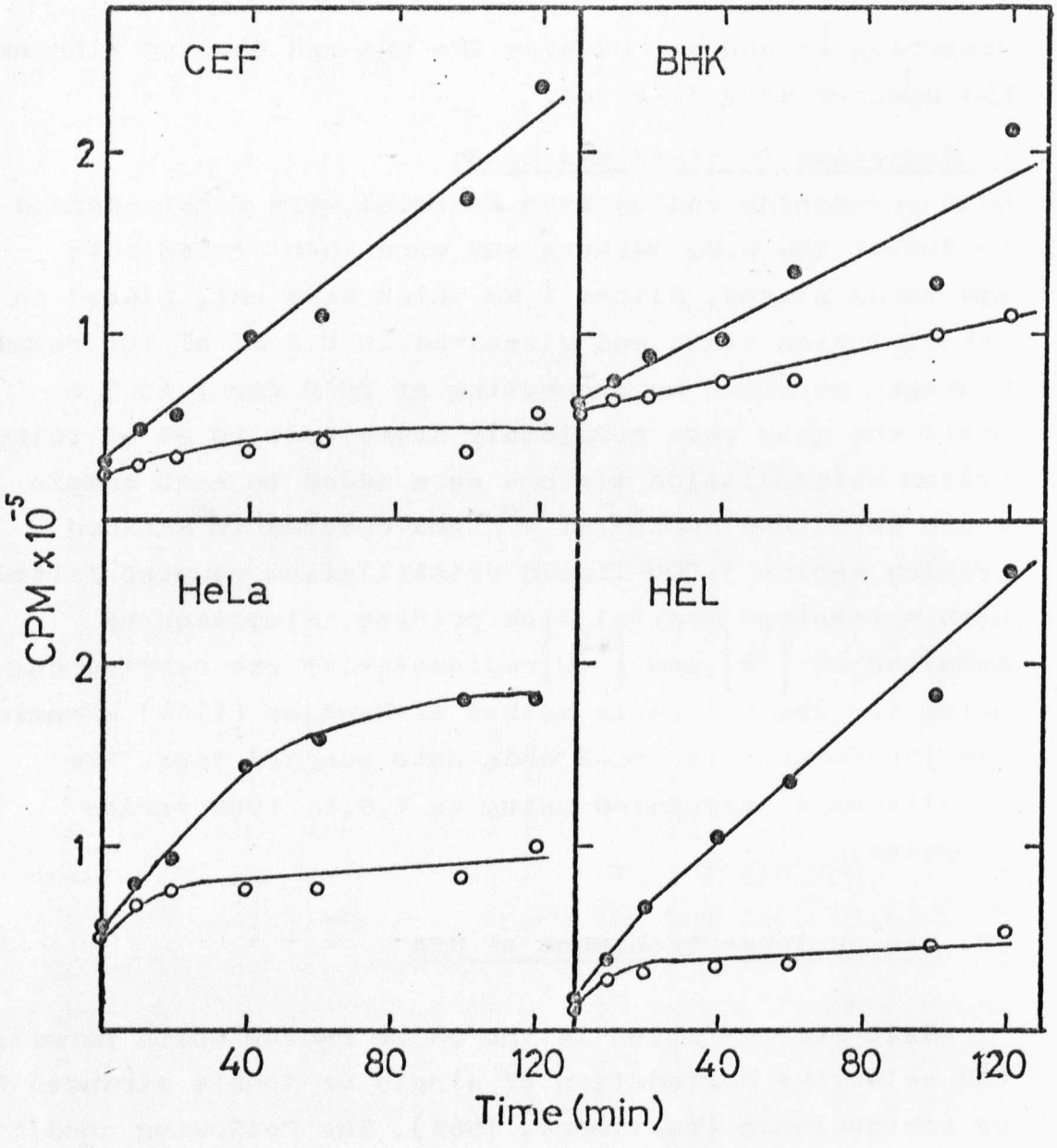


Fig. 5. Chase of $[^3H]$-uridine by cold uridine. The total acid-insoluble counts are plotted against the time after the cold uridine was added (●—● non-chased controls, ○—○ after the addition of 10^6 excess of cold uridine).

13. Ribonuclease treatment of infected broken cells

After the cell sheets were washed twice with extraction buffer, the cells were scraped from the glass into 2 ml of 2 x SSC solution. The cells were broken by freezing and thawing and were incubated at 37°C for 10 min in the presence of 0.1 or 10 µg / ml of RNase. The RNase treatment was terminated by addition of 1% SDS (w/v) and 2 volumes of phenol and the immediate extraction of RNA.

14. Deoxyribonuclease treatment of RNA extracted from infected cells.

Treatment of RNA with deoxyribonuclease (DNase) necessitated further purification by ethanol precipitation to remove traces of SDS which inhibit the enzyme. The reaction mixture contained 10 µg / ml DNase and 10 mM $MgCl_2$ in extraction buffer solution, and was incubated for 30 to 60 min at 37°C.

This procedure when applied to the RNA extracted from infected cells resulted in the complete degradation of the cellular DNA leaving the cellular or viral RNA intact.

15. Chasing of radioactive uridine

The possibility of chasing radioactive uridine with cold uridine was studied with HEL, HeLa, BHK and chick embryo fibroblast (CEF) cells.

50 mm plastic Petri dishes confluent with cells of each of the above cell types were incubated for 10 min with 1 ml of maintenance medium per dish containing 4 µC / ml of [^{3}H]-uridine. At the end of this period 2 dishes of each cell type were assayed for radioactivity incorporated into acid-insoluble material as described elsewhere. Into half of the remaining plates 0.1 ml of 10^{-3} M cold uridine was added and incubated at 37°C. At the time intervals indicated in Fig. 5 two chased and two control non-chased dishes were assayed for acid-insoluble material.

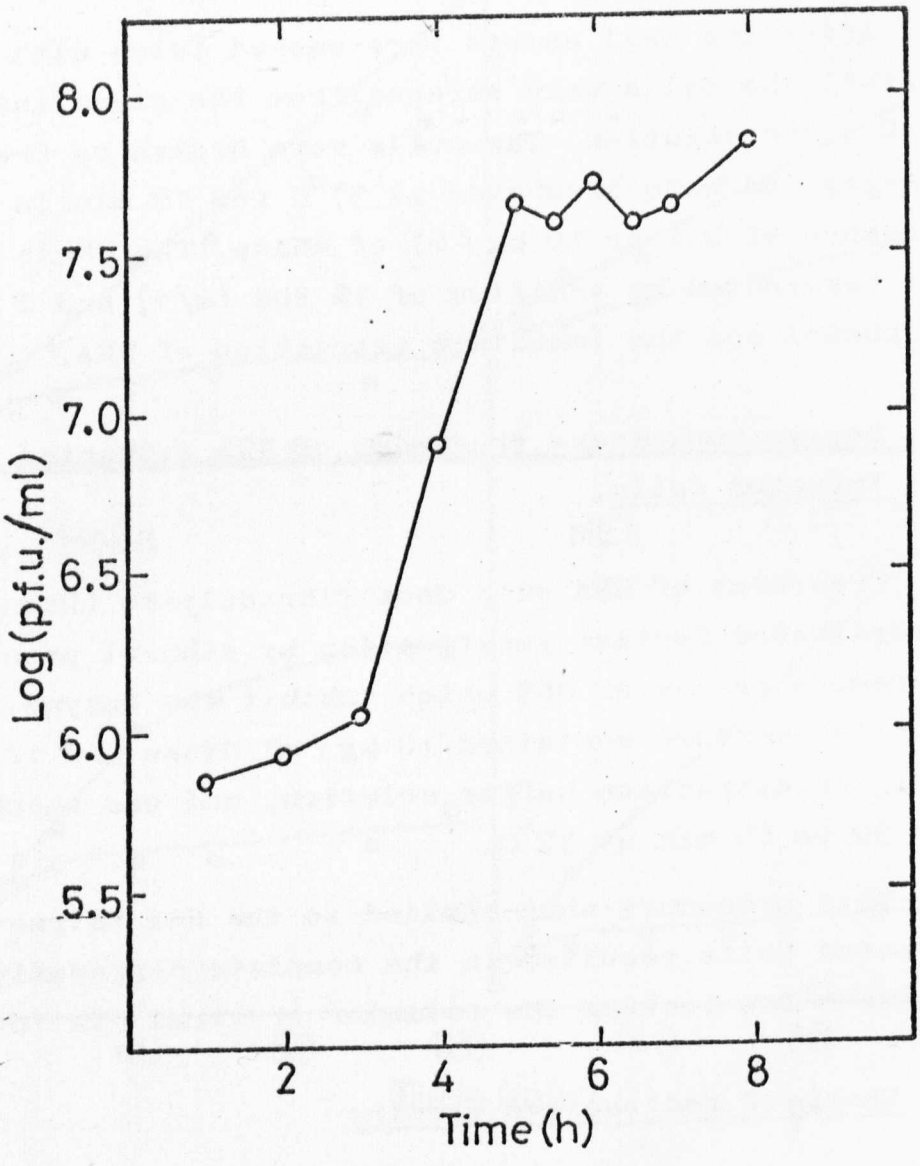


Fig. 6. Multiplication of bovine enterovirus in BHK cells. Infectivity represents the total yield of cell-associated and released virus.

Fig, 5 shows that the radioactive uridine label could be chased and that the efficiency with which the chase takes place depends on the cell type. The label was chased most efficiently in CEF and HEL cells.

When viral RNA was to be chased the infected cells were exposed to 10 µc / ml of [^{3}H]-uridine for 5 min at 9 h post infection otherwise the experimental conditions described above were used.

16. Multiplication of bovine enterovirus in BHK cells

The bovine enterovirus - BHK cells system has been used in one experiment in this thesis. The growth curve of the virus in these cells is described here.

Confluent BHK cells in 50 mm plastic Petri dishes were washed with buffered Earle's solution and were infected with 0.1 ml of virus at a multiplicity of 5 - 10 p.f.u. / cell. After 30 min incubation at room temperature the unadsorbed virus was removed and the cells were incubated at 37°C with Eagle's medium without calf-serum. At the intervals indicated in Fig. 6, dishes were removed from the incubator and were titrated for total virus infectivity as described above. Fig. 6 shows that virus infectivity was first detected at 3 h and then increased exponentially until 5 h when it reached a plateau.

A growth curve of bovine enterovirus in HEL cells is shown in "Results Part II".

17. Extraction of rhinovirus RNA-dependent RNA polymerase activity from infected cells

The cellular fraction used for the polymerase assays was extracted following the method described by Yin and Knight (1972): Monolayers of either HeLa or HEL cells were infected in the presence of AMD (1 µg / ml). At the end of 9 h the cell sheets of 5 bottles (about 225 x 10^6 cells) were washed twice with cold PBS and the cells of each bottle were scraped into 2 ml of PBS. The suspensions

were pooled together and collected by low speed centrifugation. The cell pellet was frozen at -70°C and cells were suspended in 10 ml of cold buffer containing 0.5 M tris (pH 7.2), 0.002 M $MgCl_2$ and 0.1 M NaCl at 4°C for 10 min. Disruption was completed with 14 strokes in a stainless-steel Dounce homogenizer with a clearance of 0.002 ins; the nuclei were removed by centrifugation at 800 g for 5 min. The supernatant fraction was spun at 30,000 g for 20 min and the pellet was suspended in 0.5 ml of buffer containing 0.05 M tris buffer pH 8.0 and 0.01 M NaCl. This fraction contained cytoplasmic membranes and it has been reported to contain the majority of the polymerase activity (Yin & Knight, 1972).

18. Assay of rhinovirus RNA-dependent RNA polymerase activity

The in vitro RNA polymerase assay described by Yin and Knight (1972) was used with few minor modifications. 0.1 ml of the enzyme sample was incubated at 33°C for 30 min with 0.1 ml of a mixture containing the following constituents: 0.25 μmoles of the 5' - triphosphates of adenine, cytidine and uridine; 0.002 μmoles of guanosine triphosphate; 10 μmoles of tris buffer pH 8.0; 1 μmole of $MgCl_2$; 0.25 μmoles of phosphoenolpyruvate; 5 μg of phosphoenolpyruvate kinase; 1 μg of AMD; 1.3 μmoles of dithiothreitol and 2.5 μc of [^{3}H]-guanosine-triphosphate. The reaction was terminated by adding 1 ml of ice cold solution containing 0.1 M sodium pyrophosphate and 6% TCA. The precipitate was washed and collected after 1 h by either of the two following methods:

Method 1 The precipitates were collected on Whatman GF/C glass fibre discs using a millipore 302S sampling manifold (Millipore (UK) Limited, Wembley, Middlesex). The discs were washed 3 times with 5 ml of 5% (w/v) TCA containing 0.1 M sodium pyrophosphate, 3 times with 5 ml of an ethanol-ether (3:1 volumes respectively) mixture and twice with 5 ml of ether. After drying in air for 15 min the discs were placed in scintillation vials and were counted

using triton-toluene scintillation mixture.

Method 2 The samples were spun in the 4L MSE centrifuge for 15 min at 2,000 rpm. The precipitates were washed 3 times with 5% TCA, 0.1 M pyrophosphate solution and collected each time by centrifugation. The final precipitate was dissolved in 0.5 ml 0.2 N NaOH and it was counted using 20 ml of the gel scintillation mixture.

Duplicate samples washed according to method 2 were more consistent than the duplicates obtained when method 1 was used.

19. Extraction and analysis of the RNA polymerase product

When the product in an RNA polymerase assay was to be analysed by polyacrylamide electrophoresis the reaction volume and the amount of reactants were doubled, the amount of [^{3}H]-GTP was increased 20-fold and cold GTP was omitted. After 30 min of incubation at 33°C, the reaction was terminated by addition of 2 ml of cold extraction buffer and 5 ml of phenol. The RNA was extracted as described above except that SDS was not used in the extraction buffer. RNA was then analysed by polyacrylamide gel electrophoresis as described in M 11.

20. Determination of protein concentrations

Protein concentrations were determined by a modification of the method of Lowry et al. (1951) described by Oyama and Eagle (1956) with bovine serum albumin (BSA) as standard.

21. RNA determination

RNA was determined by absorbance at 260 nm assuming that the relationship established for yeast RNA, where 20 OD_{260} was equivalent to 1 mg/ml RNA, was generally applicable.

22. Measurement of cellular macromolecular synthesis

After the 50 mm plastic Petri dishes with confluent cell sheets were treated as demanded by each particular experiment they received a pulse of 0.5 ml of either $[^{3}H]$-uridine or $[^{3}H]$-valine and $[^{14}C]$-thymidine respectively. At the end of the pulse the cell sheets were washed 3 times with 5 ml of ice cold PBS, 3 times with 5 ml of cold TCA (5% w/w) and twice with 2 ml of cold ethanol. The monolayers were then allowed to dry and the cells dissolved in 0.5 ml of 0.5 N NaOH. Radioactivity was counted using gel scintillation mixture.

23. Treatment of infected or non-infected cells with 65-709, guanidine or cycloheximide

I.C.I. 65-709 was most easily dissolved in dimethyl-sulphoxide at a concentration of 1 mg / ml. Before use it was diluted 10 times in sterile distilled water to a concentration of 100 µg / ml.

Guanidine and cycloheximide solutions were made in water at concentrations of 2 mg / ml and 15 mg / ml respectively and subsequently sterilised.

When either of the above compounds was to be used, they were added to the maintenance medium for the necessary period of time. When the effect of either of the above compounds was to be reversed, the maintenance medium which contained the compound was removed from the cells and the monolayers were then washed twice with maintenance medium and finally incubation resumed with prewarmed medium.

RESULTS, PART 1.

"RNA REPLICATION"

INTRODUCTION

Virus induced RNA species

The small RNA containing phages were discovered in the early 1960's (Loeb & Zinder, 1961; Straus & Sinsheimer, 1963; Parenchych & Graham, 1962). The techniques already developed with T-phages and the ease of working with bacterial systems are the major reasons why RNA has been better studied with RNA phages. Evidence which established that RNA replicates independently of the host cell's genetic material was provided by Doi and Spiegelman (1962) who showed by hybridisation experiments the absence of any homologous sequences between host DNA and phage RNA. This was confirmed by experiments which showed that viral RNA synthesis takes place in the absence of either host DNA synthesis (Cooper & Zinder, 1962; Simon, 1961) or DNA directed RNA synthesis (Reich et al., 1961). The existence of an RNA-dependent RNA polymerase was first detected in mammalian cells infected with mengovirus (Baltimore & Franklin, 1962) and poliovirus (Baltimore et al., 1963). However final proof of the nature and function of the enzyme require it to be purified. This has proved to be very difficult with animal virus systems because the enzyme is intimately associated with membranes (Horton et al., 1966; Friedman & Berezesky, 1967; Arlinghaus & Polatnick, 1967). However the purification of EMC virus induced polymerase in BHK 21 cells has been recently claimed (Rosenberg et al., 1972).

The fact that the replication of the single stranded DNA phage ϕ x 174 involved a double stranded DNA replicative form (Sinsheimer et al., 1962) led to the search for such replicative forms induced by RNA viruses. Montagnier & Sanders (1963) detected a 20S infective RNA in Krebs II ascites tumor cells infected with EMC virus which by criteria, such as ribonuclease resistance, solubility in NaCl, buoyant density in $CsSO_4$, thermal transition and sedimentation behaviour, established its

hydrogen bonded double helical structure. It was called replicative form (RF) in analogy with the ϕ x 174 replicative form. Baltimore et al. (1964) and Pons (1964) reported a double stranded RNA in HeLa cells infected with poliovirus. The finding that more than one species of RNA existed in infected cells was confirmed with other picornaviruses such as FMDV (Brown & Cartwright, 1964), ME virus (Hausen, 1965), mengovirus (Plagemann & Swim, 1966), bovine enterovirus (Clements & Martin, 1971), arboviruses such as Semliki forest virus (Sonnabend et al., 1964; Friedman & Sonnabend, 1965), plant viruses (Burdon et al., 1964; Mandel et al., 1964) and RNA phages (Ammann et al., 1964; Fenwick et al., 1964; Kaerner & Hoffmann-Berling, 1964; Kelly et al., 1965). It was realised soon after that while the RNase resistant material was sedimenting as a broad peak in sucrose gradients (extending between 14S and 30S) a sharp peak of RNA of around 16S was observed after treatment with RNase (Erikson et al., 1965). This behaviour led to the suggestion that another virus specific RNA might be present in the infected cells which after RNase treatment could yield material indistinguishable from RF (Erikson et al., 1965). It was also found that if cells infected with bacteriophage R-17 were exposed to a radioactive RNA precursor for a very short period of time, all the radioactive label is found in this heterogeneous, partially RNase resistant RNA. It was therefore called replicative intermediate (RI), (Erikson et al., 1964) and the proposal was made that these molecules consisted of a double stranded core to which was attached RNase-sensitive nascent single stranded RNA of variable size (Fenwick et al., 1964).

The existence of replicative intermediate was soon confirmed in cells infected with other bacterial viruses and discovered in cells infected with a number of animal viruses (Baltimore & Girard, 1966; Brown & Cartwright, 1964; Clements & Martin, 1971; Friedman, 1968). Both RF and RI have been purified using NaCl and LiCl precipitation (Montagnier & Sanders, 1963; Baltimore, 1966; Baltimore, 1968a),

various chromatographic methods (Ammann et al., 1964; Bishop, 1966; Bishop & Koch, 1967; Franklin, 1966; Kelly & Sinsheimer, 1967a; Pinck et al., 1968; Bishop & Koch, 1969; Robertson et al., 1968) and by exclusion filtration through agarose (Baltimore, 1968a; Erikson & Gordon, 1966). It should be pointed out here that RF and RI induced by picornaviruses are infectious (Baltimore, 1966; Baltimore, 1968a; Bishop, 1966; Bishop & Koch, 1967) in contrast to the RF from phages and arboviruses (Pons, 1964; Franklin, 1966). Phage RI has low specific infectivity (Kelly & Sinsheimer, 1967a). All three virus induced RNA species were better resolved when the polyacrylamide gel electrophoresis technique became available (Loening, 1967).

As far as rhinovirus induced RNA species are concerned the first reports appeared in the literature while this work was in progress. Sethi and Schwerdt (1972) analysed the RNA species synthesized in HeLa cells after infection with rhinovirus type 20 by sucrose gradient centrifugation. They detected the RF and single stranded RNA species and suspected the existence of RI as a minor shoulder at about 24-26S. However the limitations of the technique they used did not allow good resolution of the three species. Yin and Knight (1972) obtained the three RNA species from HeLa cells infected with rhinovirus type 2 using polyacrylamide gel electrophoresis; however the emphasis of this report was on the rhinovirus induced RNA polymerase with little attention to the RNA species induced in vivo.

Involvement of RF and RI in the replication cycle

The replication of RNA has been generally considered as a two step reaction (a) the synthesis of the template complementary to the infecting molecule catalyzed presumably by an RNA-dependent RNA polymerase (Enzyme I) and (b) the synthesis of the progeny strands from the template catalyzed presumably by a second enzyme (Enzyme II). Whether two different enzyme molecules or an enzyme with

two functions are involved in the completion of this reaction has not yet been established. The enzymatic activity associated with the first reaction seems to be virus coded since some animal virus (Burge & Pfefferkorn, 1967; Cooper et al., 1970) and phage (Lodish & Zinder, 1966) conditional lethal mutants lack this property. The evidence for a discrete second step in the above reactions comes from the fact that enzyme I mutants can carry out the enzyme II reaction for a substantial period of time under non-permissive conditions if enzyme I is first synthesized under permissive conditions (Lodish & Zinder, 1966). Cooper et al. (1970) have also shown that different temperature sensitive mutants of poliovirus are defective in distinct functions of RNA synthesis. This is good evidence for the presence of more than one enzymatic activity in the production of poliovirus RNA. A similar situation has been demonstrated for FMDV by the separation of the two enzymatic activities (Arlinghaus & Polatnick, 1969; Arlinghaus et al., 1972). However the criticism that the enzymatic reactions were studied with crude preparations is always valid.

The question of the role of RI and RF in the synthesis of RNA outlined above has been investigated by many workers. Montagnier and Sanders' assumption (1963) that RF itself could mediate the reaction is questionable. An outline of a scheme for RNA replication was put forward by Spiegelman et al. (1968). Initially, the input RNA (plus strand) is transcribed to give a partially double stranded replicative intermediate (RI) which is hydrogen bonded with the transcript. The plus strand of the RI then serves as template for the synthesis of minus strands and gives rise to multi-stranded structures containing nascent minus strands. These are the negative RI's. From the negative RI, negative RNA strands are released. These are converted to positive RI's which synthesize positive strands in the same way as minus strands are synthesized.

In summary:

$$SS^{+} \longrightarrow RI^{-} \longrightarrow SS^{-} \longrightarrow RI^{+} \longrightarrow SS^{+}$$

The theory can be extended in the light of more recent work to include RF which under normal conditions appears to be as much an end product obtained from RI as is single stranded RNA itself (Baltimore, 1968a; Girard, 1969). However there is disagreement in this area since Weissman et al. (1968), while agreeing with the general concept deny that any of the RNA molecules are joined by hydrogen bonding in the infected cell. They suggest that the replicative intermediate is a loose complex consisting of template, polymerase and nascent chains and that there is no hydrogen bonding between complementary RNA molecules. They believe that hydrogen bonds and hence RF's are created during the deproteinising extraction procedure. Spiegelman's model is supported by the observations that (a) it is possible to recover partially RNase resistant RNA (RI) from phage-infected cells without the use of phenol or detergents (Weissman et al., 1968) and (b) it is possible to isolate RF from extracts of poliovirus infected cells after treatment only with deoxycholate which is a very mild detergent (Baltimore et al., 1964; Bishop et al., 1965). However there is evidence to the contrary that (a) the early product of the standard in vitro replicase reaction possesses little if any double helical structure prior to the removal of protein with denaturants (Feix et al., 1967), (b) purified minus strands (Feix et al., 1968) and denatured RF (Weissman et al., 1967) are efficient templates in vitro while (c) neither intact RI nor RF can serve as templates in vitro (Weissman et al., 1967; Feix et al., 1968; Mills et al., 1966).

We have used a different approach to investigate the existence of hydrogen bonding between RNA molecules in the rhinovirus-HEL cell system. Treatment of disrupted infected cells with ribonuclease will degrade all single stranded RNA. Using this procedure, followed by RNA extraction, we find unchanged amounts of RF present

indicating that this structure is not an artefact of the deproteinising extraction procedure.

Another point to be considered is whether conservative or semi-conservative RNA replication occurs. The existing data suggest that the majority of RNA is replicated semi-conservatively (Kelly & Sinsheimer, 1967a; Erikson, 1966; Kelly & Sinsheimer 1967b; Girard, 1969; Billeter et al., 1966; Weissman et al., 1964a; Bishop et al., 1969; Weissman et al., 1964b; Kelly et al., 1965; Erikson et al., 1964; Lodish & Zinder, 1966). However the evidence does not exclude conservative replication and more recent data suggest that RNA is replicated by both mechanisms (Kelly & Sinsheimer, 1967a; Kelly & Sinsheimer, 1967b; Bishop et al., 1969; Francke & Hofschneider, 1969).

Finally I shall consider the circular model of replication proposed by Brown and Martin (1965). The fact that RNA extracted from cells infected with FMDV was found to be larger than the virion RNA could not be explained by any model in which the template is assumed to be linear. It was postulated, therefore, that the ingoing virus RNA codes for a complementary minus strand which takes up the configuration of a circle or is synthesized as a circular structure. The polymerase rotates around this structure synthesizing a long plus strand consisting of repeated sequences of virion RNA. This long plus strand is then chopped by an enzyme into pieces of approximately the size of virion RNA. Only the strands with the correct length are incorporated into virions. The possibility that the observed differences between the sedimentation of single stranded RNA extracted from infected cells and of virion RNA could be attributed to different secondary structures rather than difference in size was excluded by treatment of the RNA's with dimethylsulphoxide (Wild et al., 1968) or formaldehyde (Wild & Brown, 1970) prior to sedimentation. Further evidence in support of the circular replication model was provided by Clements and Martin (1971) when they reported that the same differences observed originally on the size of RNA induced by FMDV

were found in BHK cells infected with bovine enterovirus. In addition to this, they have shown that the size of the majority of RNA species varied with time and that early in infection longer RNA strands were synthesized.

We were unable to confirm Clements and Martin's findings and found that the majority of single stranded RNA in infected cells was the same size as virion RNA and that there was no difference in size between the RNA synthesized early and late in infection. However a small fraction of single stranded RNA may have been heterogeneous in size and further investigation to establish the role, if any, of this RNA is necessary.

An interesting and unexpected finding which arose during this study of RNA replication was the different kinetics of synthesis of viral RNA which depended on the cell in which the virus was grown. This finding will be reported and discussed further below.

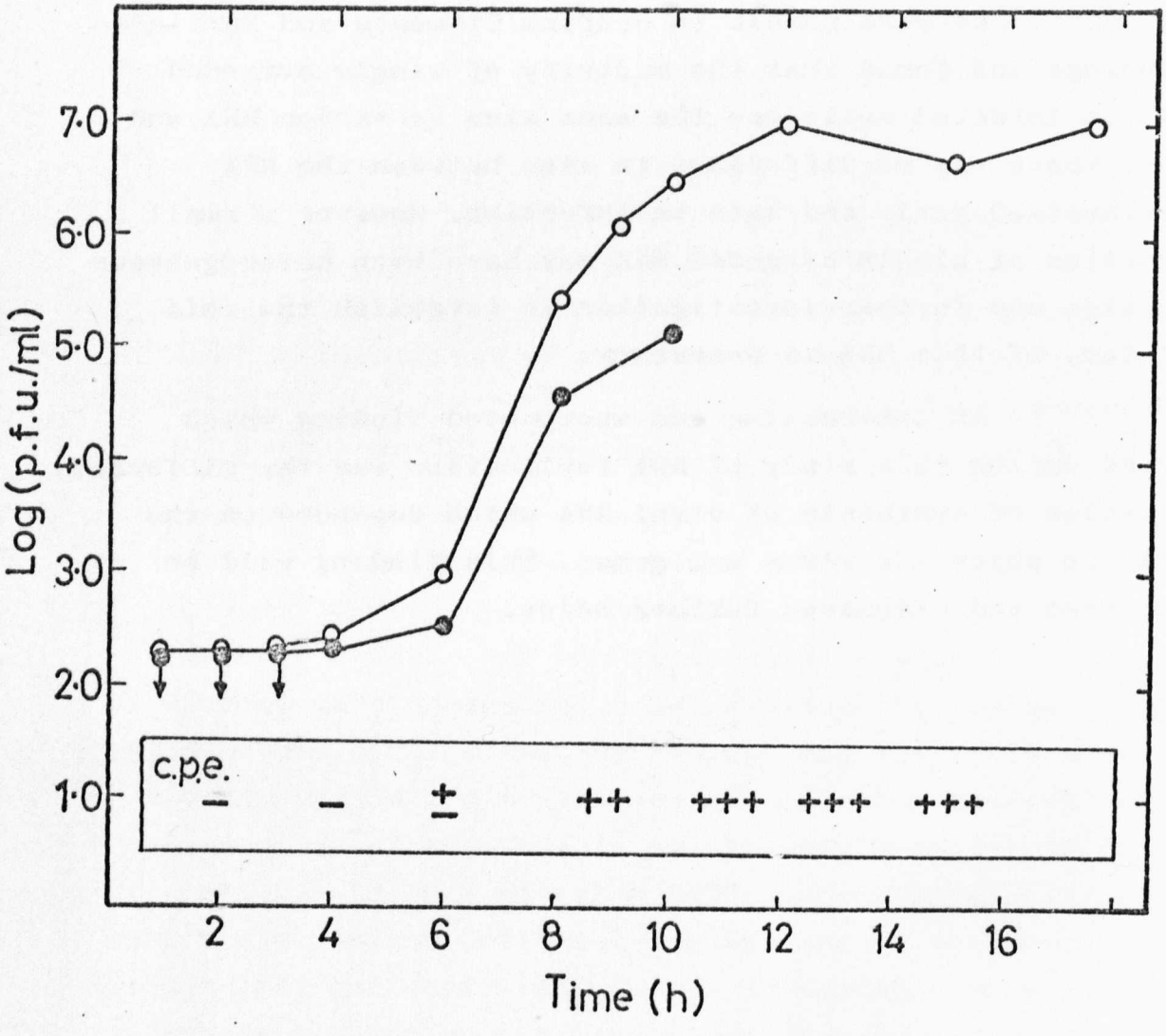


Fig. 7. Multiplication of rhinovirus in HEL cells inoculated with 5 to 10 p.f.u. / cell. The total yield of virus (o——o) and the yield of released virus is (o——●) presented. The virus caused a cytopathic effect which spread from about 10% of cells (±) to over 80% (+ ++).

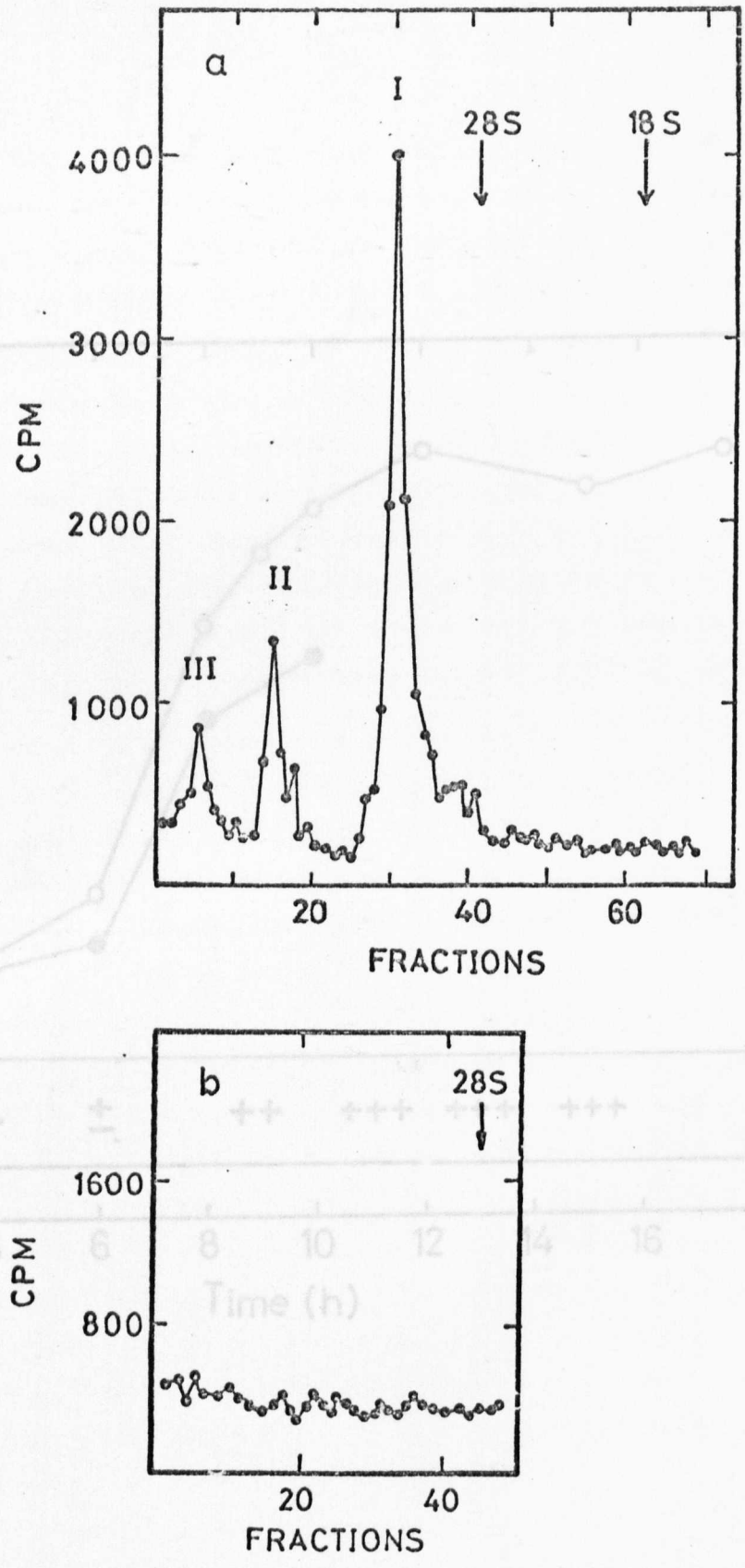


Fig. 8. Electrophoresis of RNA extracted (a) from infected HEL cells, (b) from non-infected cells. Cells were labelled with $[^3H]$-uridine from 5 to 7 h after infection in presence of AMD. The arrows are taken from the OD_{260} trace indicating the position of the ribosomal RNA markers.

RESULTS

A. General aspects of virus multiplication.

1. Virus growth in human embryo lung (HEL) cells

Confluent monolayers of HEL cells in small plastic petri dishes were inoculated with virus at multiplicity of 5 to 10 p.f.u./cell. The virus was allowed to adsorb for 90 min at 33°C and the residual virus was removed by washing the cell sheets three times with maintainance medium. The cells were then incubated at 33°C with 2 ml of the same medium. At the intervals indicated in Fig. 7 the cells were observed in the microscope for cytopathic effects (c.p.e.) and at the same times samples were collected for titration of total and cell associated virus as follows:

a. For total virus, the cells were scraped into the culture fluid and frozen and thawed to release cell associated virus. After clarification at 1000*g* the fluids were titrated for infectivity.

b. For released virus, the culture fluids were titrated for infectivity.

Fig. 7 shows the two growth curves. Infectious virus was first detected at 4 h p.i. after which it increased exponentially until a plateau was reached at 11 h. Over 90% of the virus remained cell associated. These conditions of virus growth were used in the following experiments on the synthesis of viral RNA.

2. Virus-induced RNA species

RNA was extracted from cells labelled with [^{3}H]-uridine from 5-7 h p.i. and analysed by polyacrylamide gel electrophoresis (Fig. 8a). Three major RNA species were identified which were absent from extracts of uninfected cells (Fig. 8b).

The fastest (I) had a mobility identical with that for virus RNA (see Fig. 9) and migrated to a position between the 28S ribosomal RNA and a "marker" of cellular DNA which was frequently observed in the optical density trace. The species of intermediate mobility (II) migrated

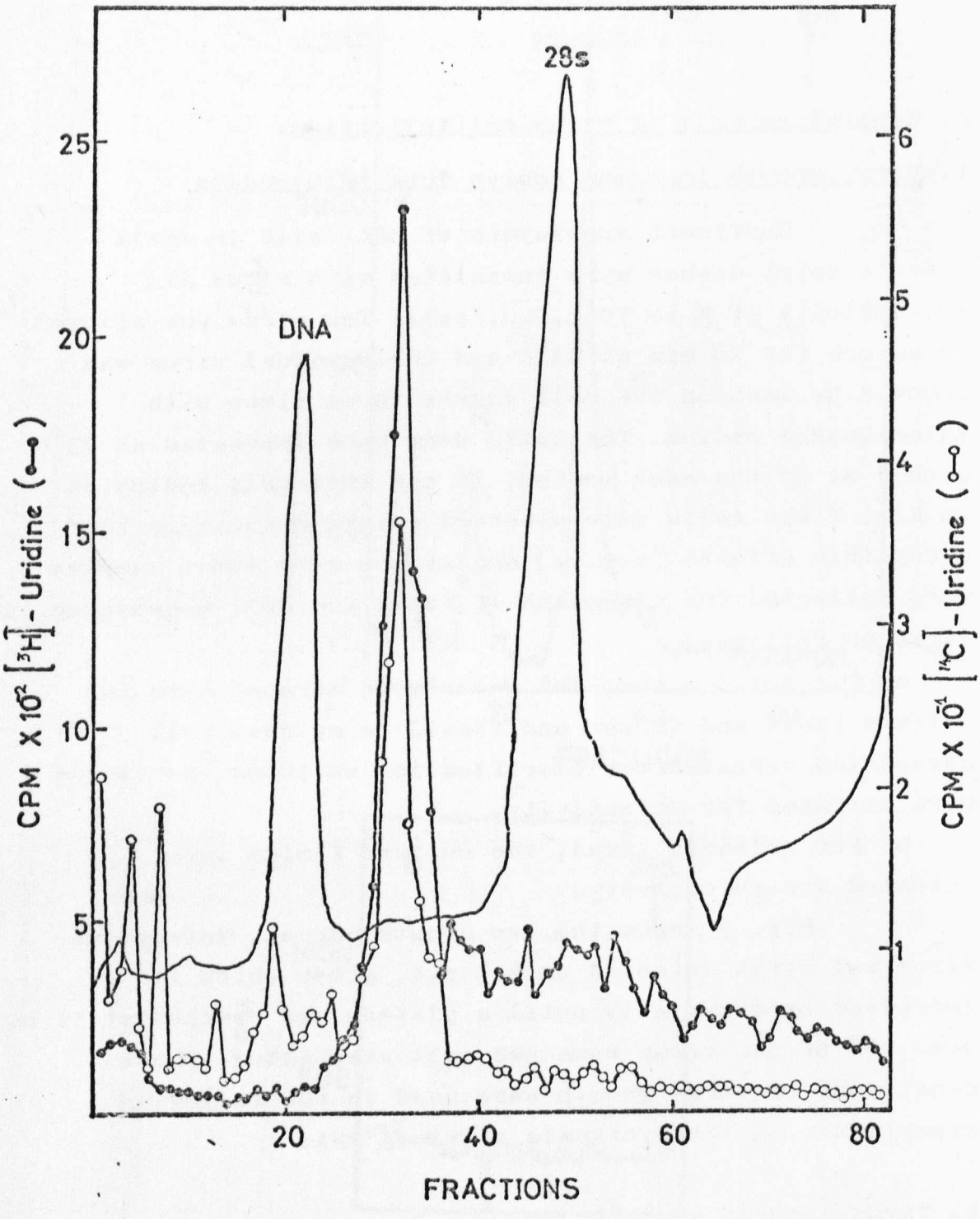


Fig. 9. Co-electrophoresis of RNA extracted from HEL cells labelled with [^{14}C]-uridine (O——O) from 9 to 11 h after infection and virus RNA labelled with [^{3}H]-uridine (●——●). The continuous line is the optical density trace at 260 nm, indicating the position of the 28S ribosomal RNA and the marker DNA.

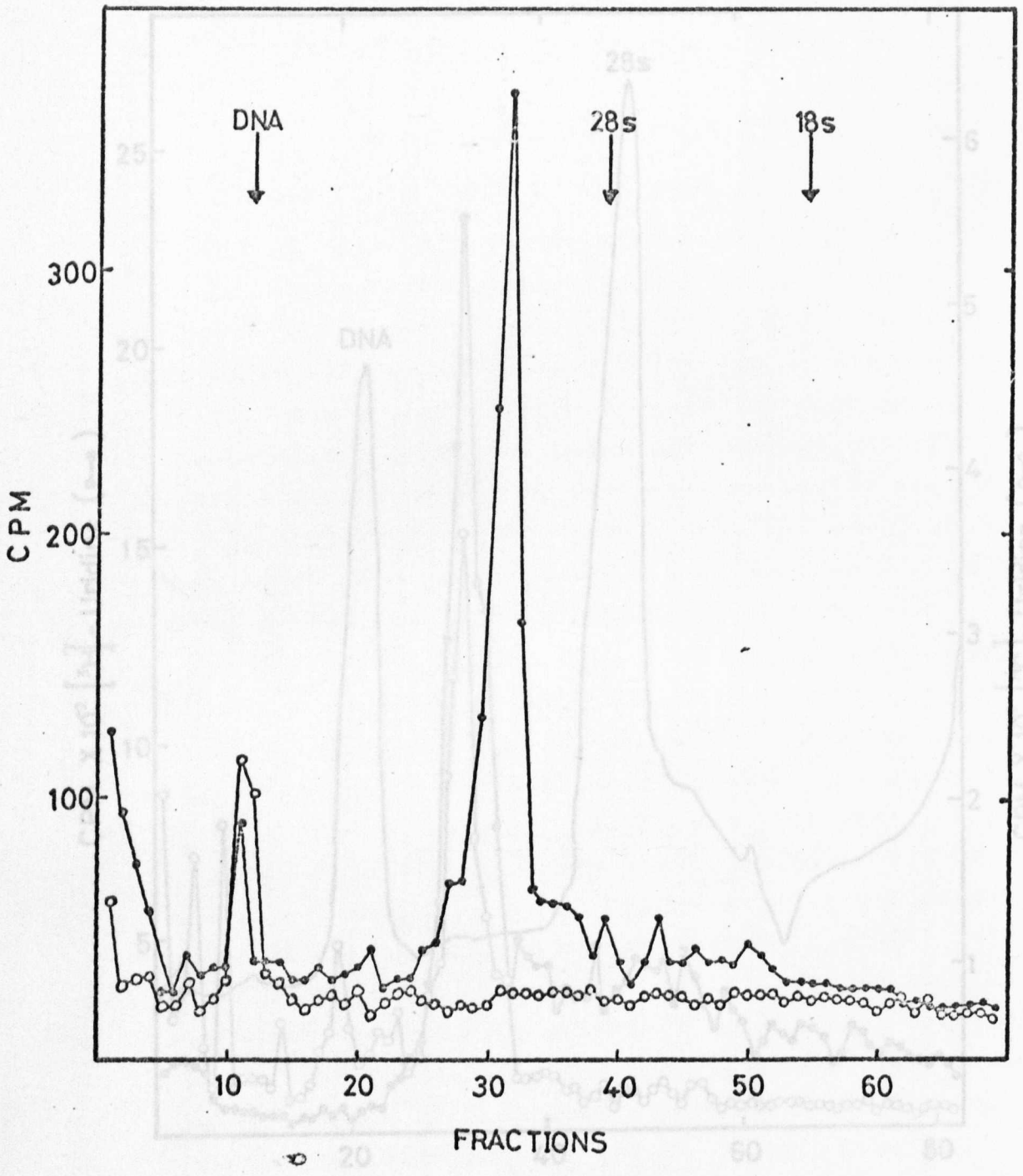


Fig. 10. Electrophoresis of RNA extracted from infected HEL cells labelled with [^{3}H]-uridine from 5 to 7 h after infection (●——●); untreated RNA; (○——○), RNA treated with RNase (0.1 µg / ml). ~~[illegible] The arrows~~ indicate the positions of the 28 S and 18S ribosomal RNAs and the marker DNA.

close to the DNA marker, and the slowest (III) entered the gel to a variable extent.

Treatment of the extract with DNase before electrophoresis did not alter the position of any of the virus RNA species and served only to remove the peak of cellular DNA referred to above.

3. Electrophoresis of virion RNA together with RNA extracted from infected cells.

RNA was extracted from purified virions labelled with [^{3}H] -uridine. The RNA extracted from infected HEL cells was labelled with [^{14}C]-uridine from 9 to 11 hours after infection. A mixture of the two was analysed by polyacrylamide gel electrophoresis (Fig. 9). The fastest migrating RNA from the cell extracts (Peak I) migrated exactly to the same position as the peak obtained from the purified virions indicating that peak I contributes RNA to infectious progeny virus particles.

4. Ribonuclease resistance of virus RNA.

Information on the structure of the virus RNA species was obtained by RNase treatment. RNA extracted from infected cells was dissolved in 2 x SSC and incubated with 0.1µg/ml RNase. Electrophoresis of the RNase-treated extract showed that the fastest and slowest peaks had been degraded, while the middle peak had increased in size (Fig. 10 and Table 2).

	[^{3}H] RNA cpm		
Treatment	Multi-stranded	Double stranded	Single stranded
None	254 (100%)	76 (100%)	938 (100%)
0.1µg RNase/ml	47 (19%)	164 (216%)	0 (0%)

Table 2. Treatment with RNase of rhinovirus RNA from infected cells.

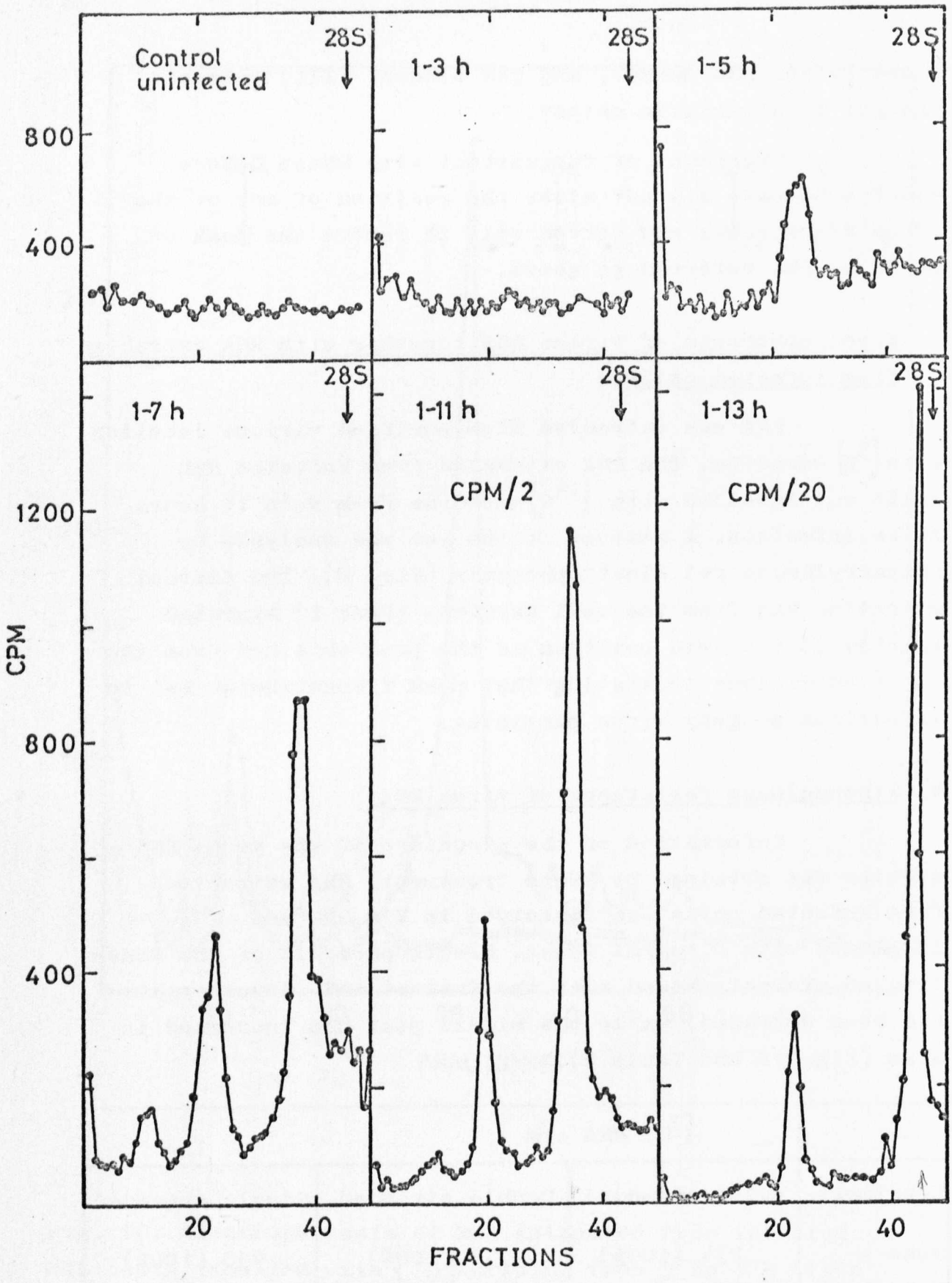


Fig. 11. Synthesis of virus RNA in HEL cells. Cells were labelled with [^{3}H]-uridine continuously for the times indicated and harvested immediately. The arrows indicate 28S ribosomal RNA.

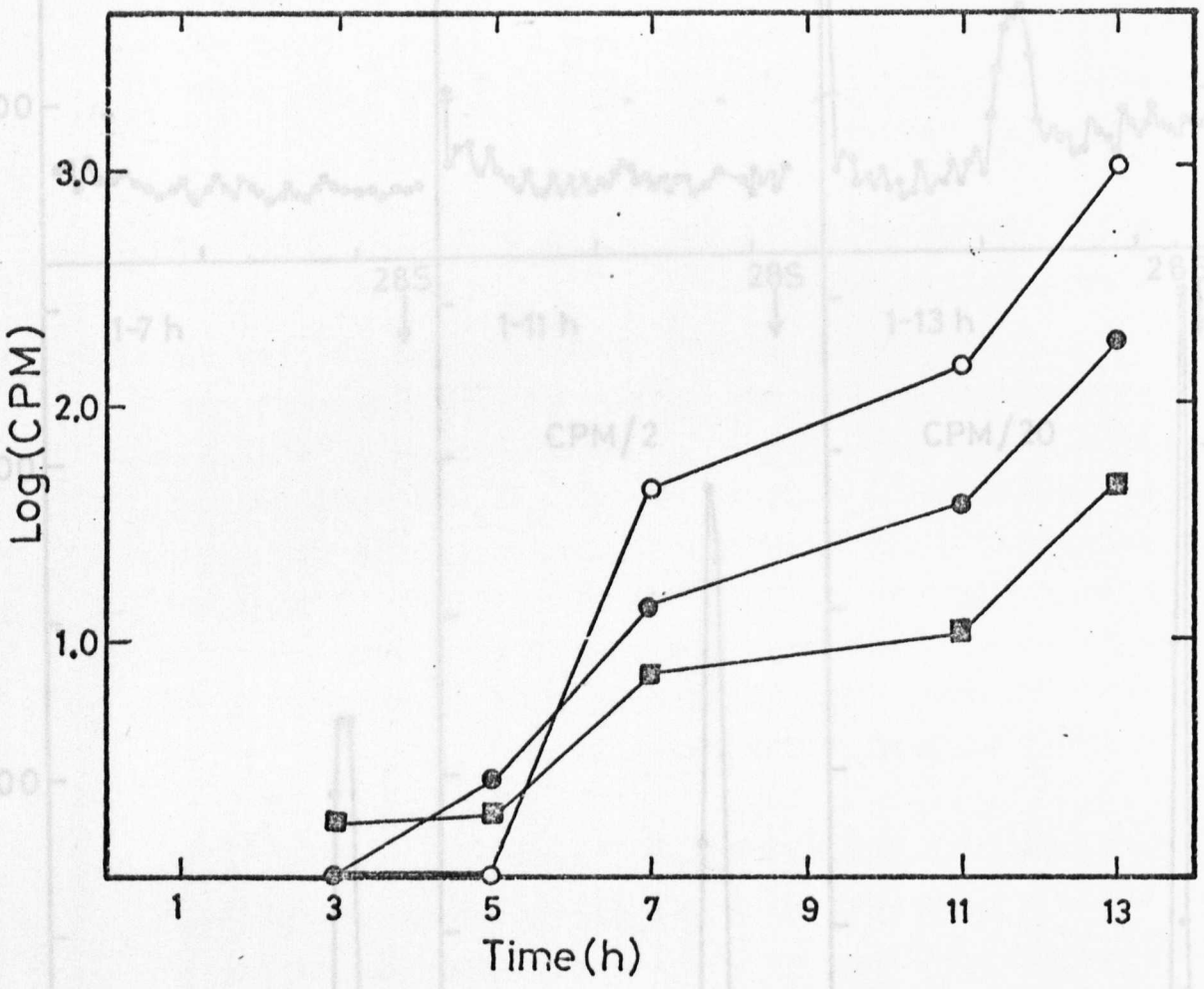


Fig. 12. Course of synthesis of virus RNA. Each point represents the total $[^3H]$-uridine incorporated into a species of RNA pulsed continuously up to the times indicated. Only radioisotope above the background level of the gel is included. Different preparations were normalised with reference to internal optical density markers of ribosomal RNA. (o——o SS, ●——● DS, ■——■ MS, RNA.)

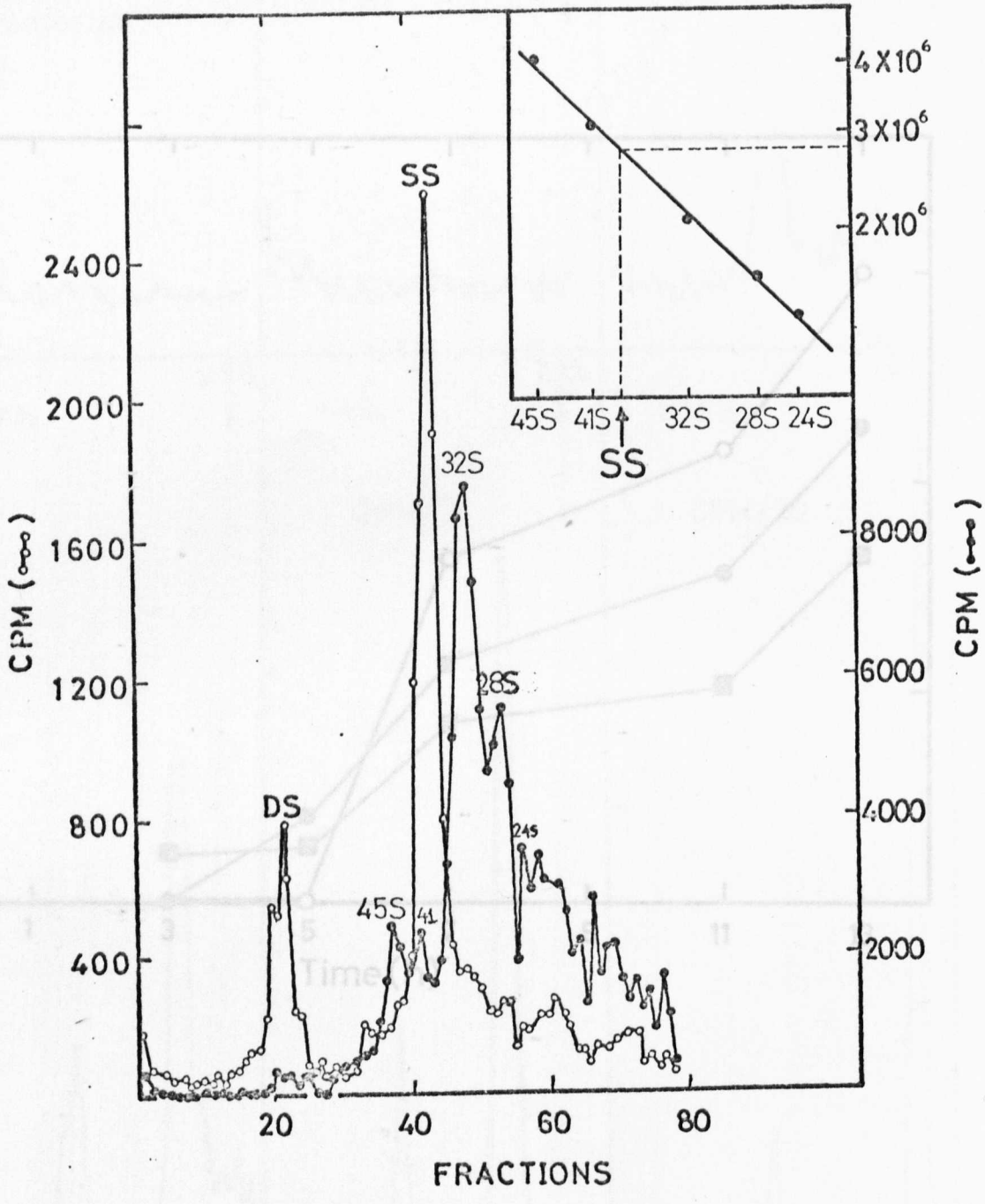


Fig. 13. Co-electrophoresis of nucleolar RNA extracted from HeLa cells labelled with $[^3H]$-uridine (●——●) and viral RNA extracted from infected HEL cells labelled with $[^{14}C]$-uridine from 9 to 11 h after infection (○——○). Inset: The distance migrated by each RNA species is plotted on the abscissa against the log. of its molecular weight.

The latter RNase resistant peak was therefore designated as double stranded (DS) RNA. The fast-moving peak was RNase sensitive and migrated to the same position as virion RNA and was designated single stranded (SS) RNA. Since the slowest moving peak was hetero-disperse and appeared to be degraded by RNase treatment into double stranded RNA it was designated as multi-stranded RNA (MS).

5. Time course of the synthesis of virus RNA's.

RNA was extracted from cells labelled continuously with [^{3}H]-uridine during the multiplication cycle, and analysed by electrophoresis on polyacrylamide gel (Fig. 11). The mean radioactivity above background in peaks of MS, DS and SS RNA was calculated and normalized by reference to the optical density of the ribosomal RNA present in each preparation (Fig. 12). Radioactivity at the origin suggested that MS could be detected at 3 h after infection, DS was not detected until 5 h and SS RNA until 7 h. MS and DS increased exponentially from 3 h and SS RNA from 5 h up to the end of the experiment at 13 h. This was surprising since by 11 h, the production of infective virus particles had reached a plateau and the majority of cells showed cytopathic effects.

6. Estimation of the molecular weight of the SS viral RNA by coelectrophoresis with nucleolar RNA as marker.

[^{3}H]-uridine labelled nucleolar RNA extracted from HeLa cells was coelectrophoresed together with RNA extracted from infected cells which were labelled with [^{14}C]-uridine between 9-11 h p.i.. The molecular weights of six of the nucleolar RNA species had been previously determined: these were 4.1×10^6, 3.1×10^6, 2.1×10^6 and 1.4×10^6 for the 45S, 41S. 32S and 24S respectively (Weinberg & Penman, 1970), and 1.65×10^6 and 0.65×10^6 for the 28S and 18S ribosomal RNA's (Petermann & Pavlovec, 1966). In agreement with Loening (1969) the migration of the marker RNA's was proportional to the $\log_{10}$ molecular weight (Fig. 13, inset). The viral SS RNA migrated between

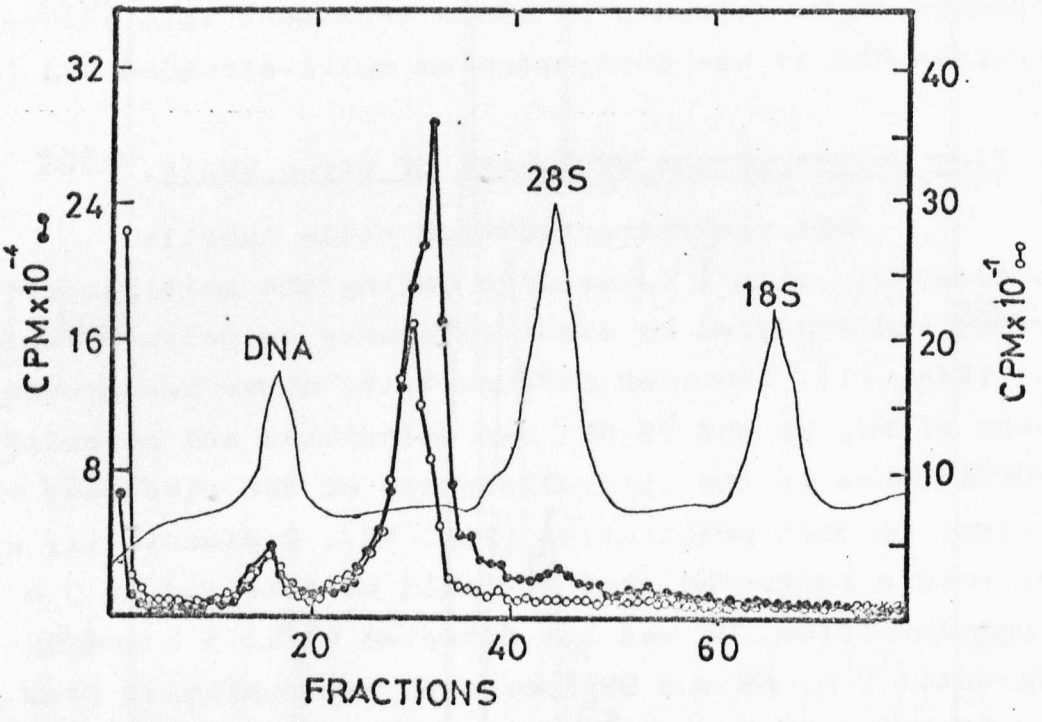


Fig. 14. Co-electrophoresis of RNA from cells infected with rhinovirus or with poliovirus. Both were labelled with uridine from 9 to 11 h after infection. The rhinovirus RNA was $[^{14}C]$ labelled (o——o) and poliovirus RNA $[^{3}H]$ labelled (o——●). The continuous line is the trace at $^{OD}260$.

the 41S and 32S marker RNA (Fig. 13) and was calculated to have molecular weight of 2.8×10^6 daltons.

7. Comparison of SS viral RNA from HEL cells infected with rhinovirus or with poliovirus.

By sedimentation, native or denatured poliovirus type 1 RNA is found to be larger than rhinovirus RNA (Nair & Lonberg-Holm, 1971; Sethi & Schwerdt, 1972) but these RNA's have not been compared by polyacrylamide gel electrophoresis.

Parallel cultures of HEL cells were infected with rhinovirus or poliovirus at a multiplicity of 10 p.f.u./cell. The rate of growth of both viruses was similar as judged by the appearance of cytopathic effects. Both cultures were labelled from 9 to 11 h p.i.; the culture infected with rhinovirus was labelled with [^{14}C]-uridine and that infected with poliovirus with [^{3}H]-uridine. RNA extracts were electrophoresed together (Fig. 14). Rhinovirus single stranded RNA was clearly larger than that of poliovirus, The MW of rhinovirus RNA was estimated as 2.8×10^6, by reference to ribosomal RNA's (MW's of 0.67×10^6 and 1.64×10^6: Petermann & Pavlovec, 1966) and to poliovirus RNA (MW 2.6×10^6, Tannock et al., 1970; Granboulan & Girard, 1969).

The same results were obtained when the experiment was repeated with the isotopes reversed (i.e. with rhinovirus [^{3}H]-uridine labelled RNA and poliovirus [^{14}C]-uridine labelled RNA).

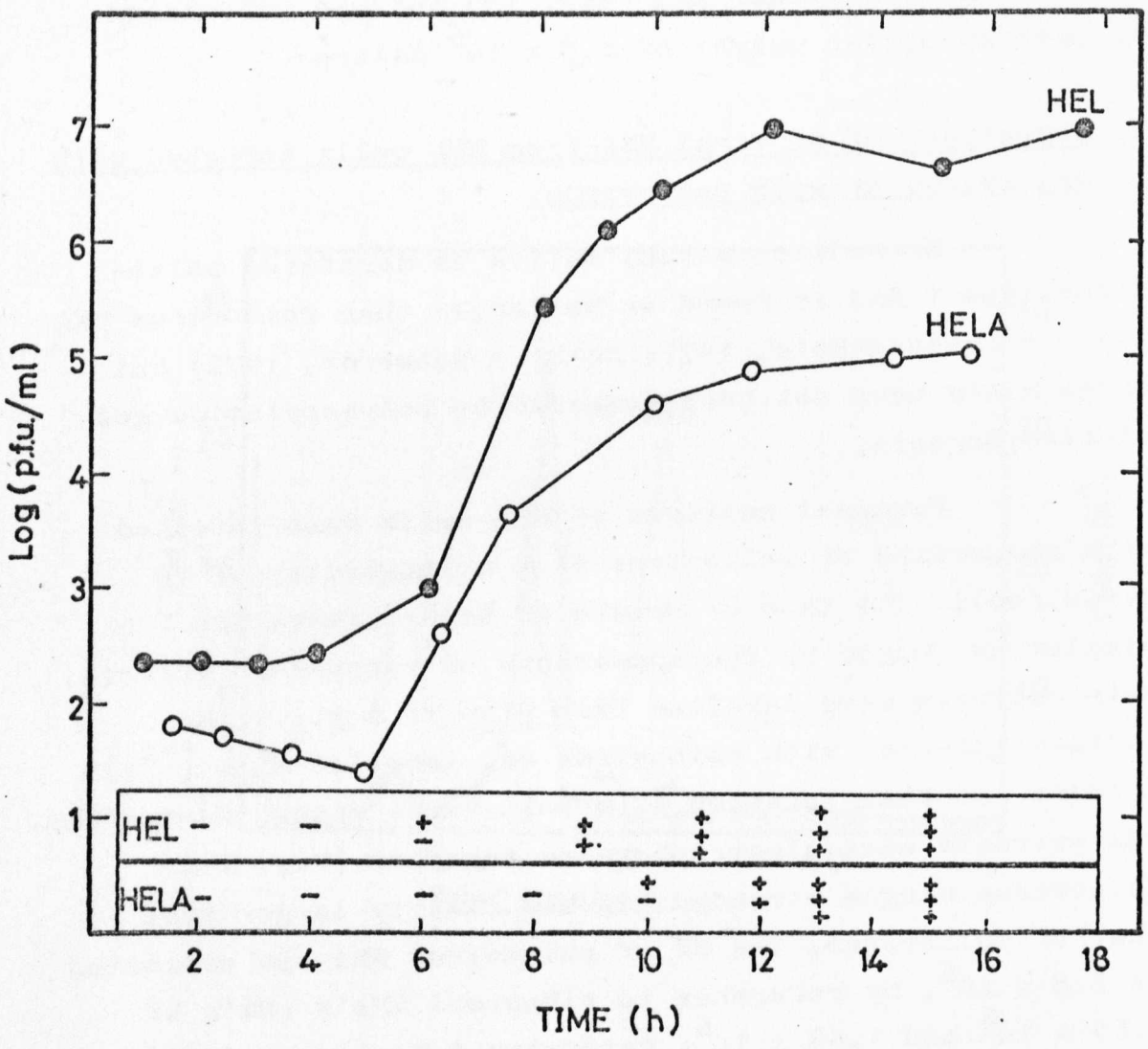


Fig. 15. Multiplication of virus in HeLa cells compared with that in HEL cells. The total yield of cell-associated and released infectivity is presented. The virus caused a cytopathic effect which spread from about 10% of cells (±) to over 80% (+++).

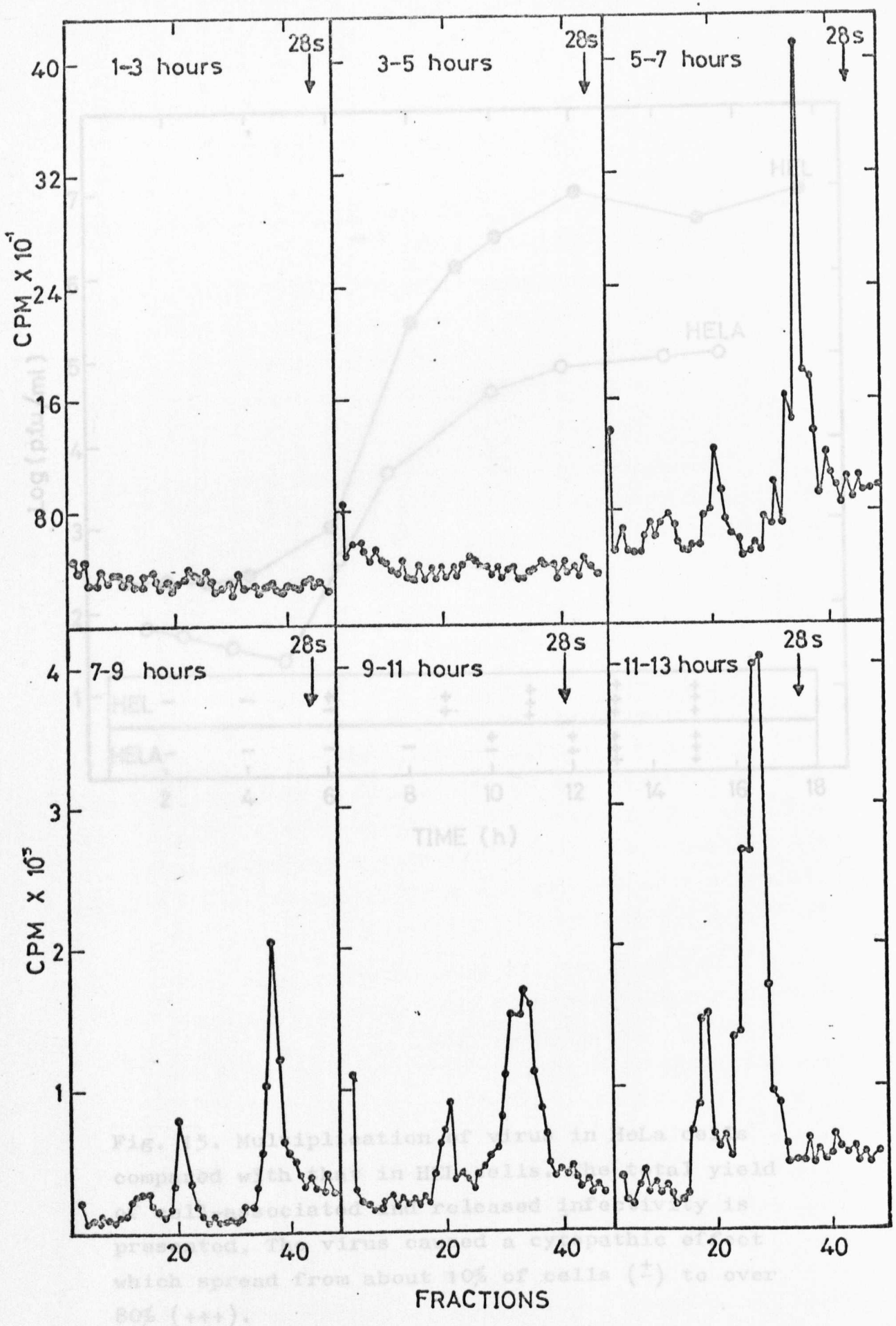


Fig. 16. Electrophoresis of virus RNA in HEL cells synthesized during consecutive 2 h pulses of $[^3H]$-

Pulse time (h)	MS	DS	SS
1 - 3	1.7	0.8	0
3 - 5	1.8	0.5	0
5 - 7	1.2	1.3	5.6
7 - 9	10.0	10.5	47.8
9 - 11	10.6	15.2	73.4
11 - 13	11.7	37.2	135.5

Table 3. Synthesis of rhinovirus RNA in HEL cells. The radioactivity under each peak was calculated and normalised with respect to the 28S ribosomal marker. Data from Fig. 16.

Pulse time (h)	MS	DS	SS
5 - 7	10.0	9.3	80.8
7 - 9	6.1	12.7	81.2
9 - 11	2.5	13.9	83.6

Table 4. Percentage of total rhinovirus RNA synthesized as multi-stranded (MS), double stranded (DS) or single stranded (SS) species during infection of HEL cells.

B. Virus multiplication and RNA replication in diploid and heteroploid cells.

The study of the time course of synthesis of viral RNA in HEL cells was reported in section A5. The observation that viral RNA synthesis increased exponentially even after the virion production reached a plateau, led us to the following experiments.

1. Virus multiplication in HEL and HeLa cells.

The growth of the virus was followed in both HEL and HeLa cells. The two growth curves were obtained under identical conditions described in section A1. The two growth curves were essentially similar (Fig. 15) and in both systems the virion production reached a plateau at about 11 h after infection.

2. Viral RNA replication in diploid and heteroploid cells.

In the experiment of section A5 the [^{3}H]-uridine was present continuously during the replication cycle because the aim of that experiment was to obtain information on the appearance and accumulation of the 3 viral RNA species. In the two following experiments cells were pulsed with [^{3}H]-uridine for consecutive intervals of 2 h to enable us to determine the peak of viral RNA synthesis in both HEL and HeLa cells

a. Replication in HEL cells.

The RNA from infected HEL cells pulsed with [^{3}H]-uridine for consecutive intervals of 2 hours was extracted and analysed by polyacrylamide gel electrophoresis (Fig. 16). The area of radioactivity in peaks of virus RNA was calculated and normalized as described earlier (Table 3). Synthesis of virus RNA was first detected during the 5 - 7 h pulse. DS and SS RNA continued to increase exponentially even during the last pulse from 11 to 13 h. The synthesis of MS RNA increased until the 7 - 9 h pulse and then remained constant.

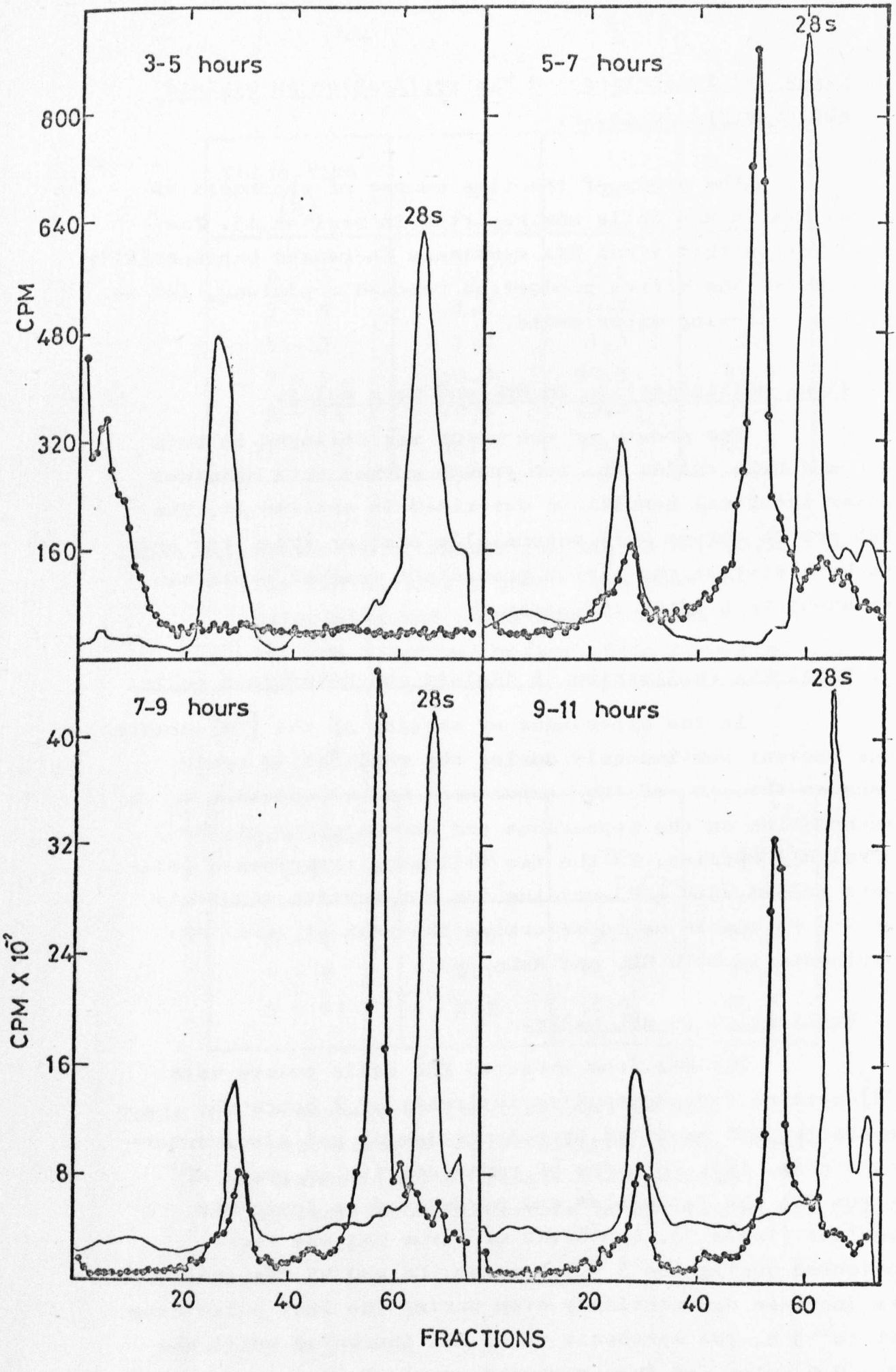


Fig. 11, continued over page

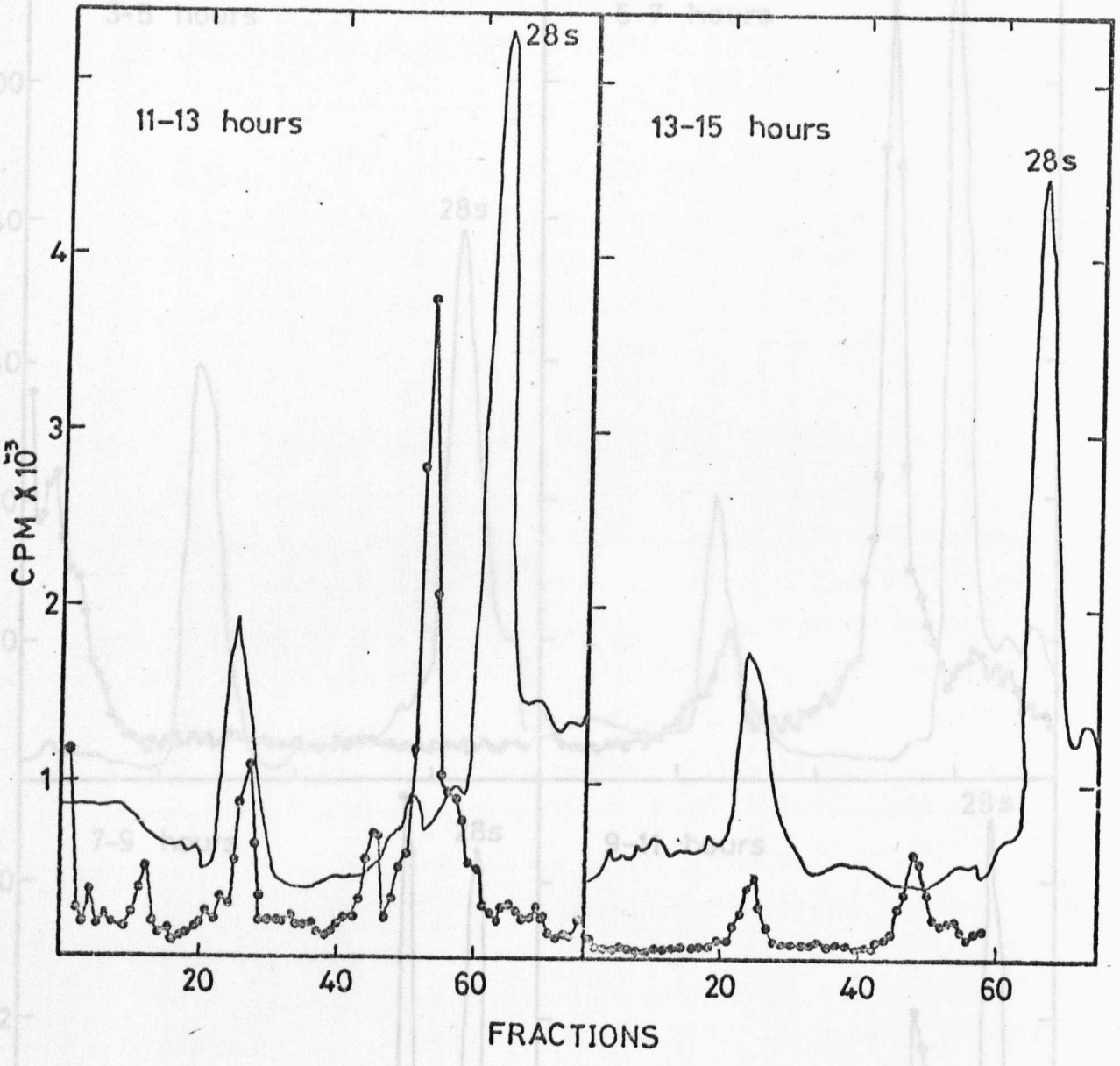


Fig. 17. Electrophoresis of virus RNA synthesised in HeLa cells. The continuous line is the OD_{260} trace, indicating the position of the 28S ribosomal RNA and the 'marker' DNA.

Pulse time (h)	MS	DS	SS
3 - 5	100.0	0	0
5 - 7	1.4	15.8	125.4
7 - 9	7.1	139.0	618.1
9 - 11	9.9	147.0	561.9
11 - 13	43.4	90.9	523.8
13 - 15	3.3	57.8	91.5

Table 5. Synthesis of rhinovirus RNA in HeLa cells. The radioactivity under each peak was calculated and normalised with respect to the 28S ribosomal RNA. Data from Fig. 18.

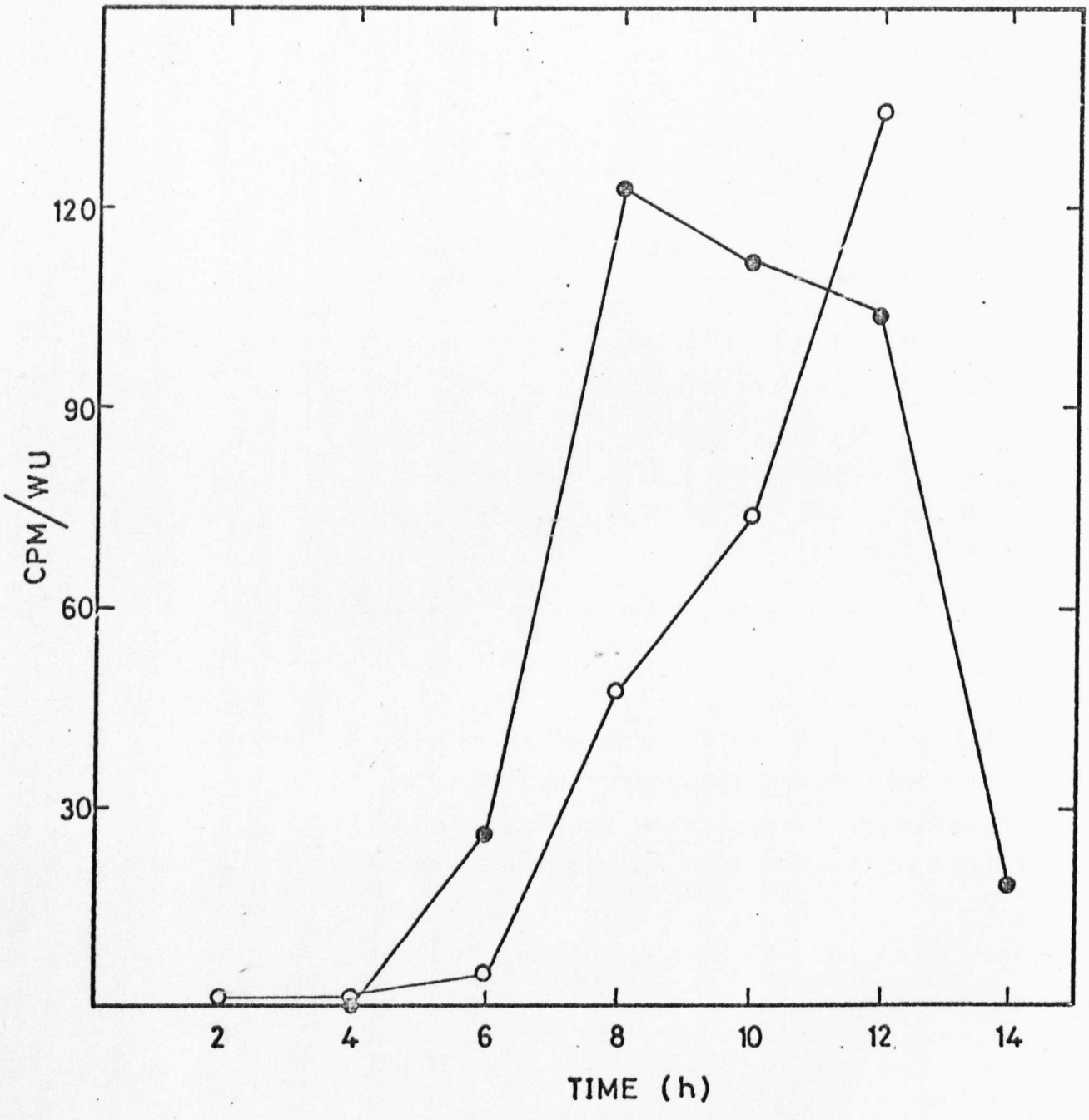


Fig. 18. Time course of synthesis of viral SS RNA in HEL (O——O) and HeLa (●——●) cells. Each point represents the middle of a 2 h pulse of $[^3H]$-uridine and is the normalised sum of radioactivity under each peak. Data is taken from Tables 3 and 5.

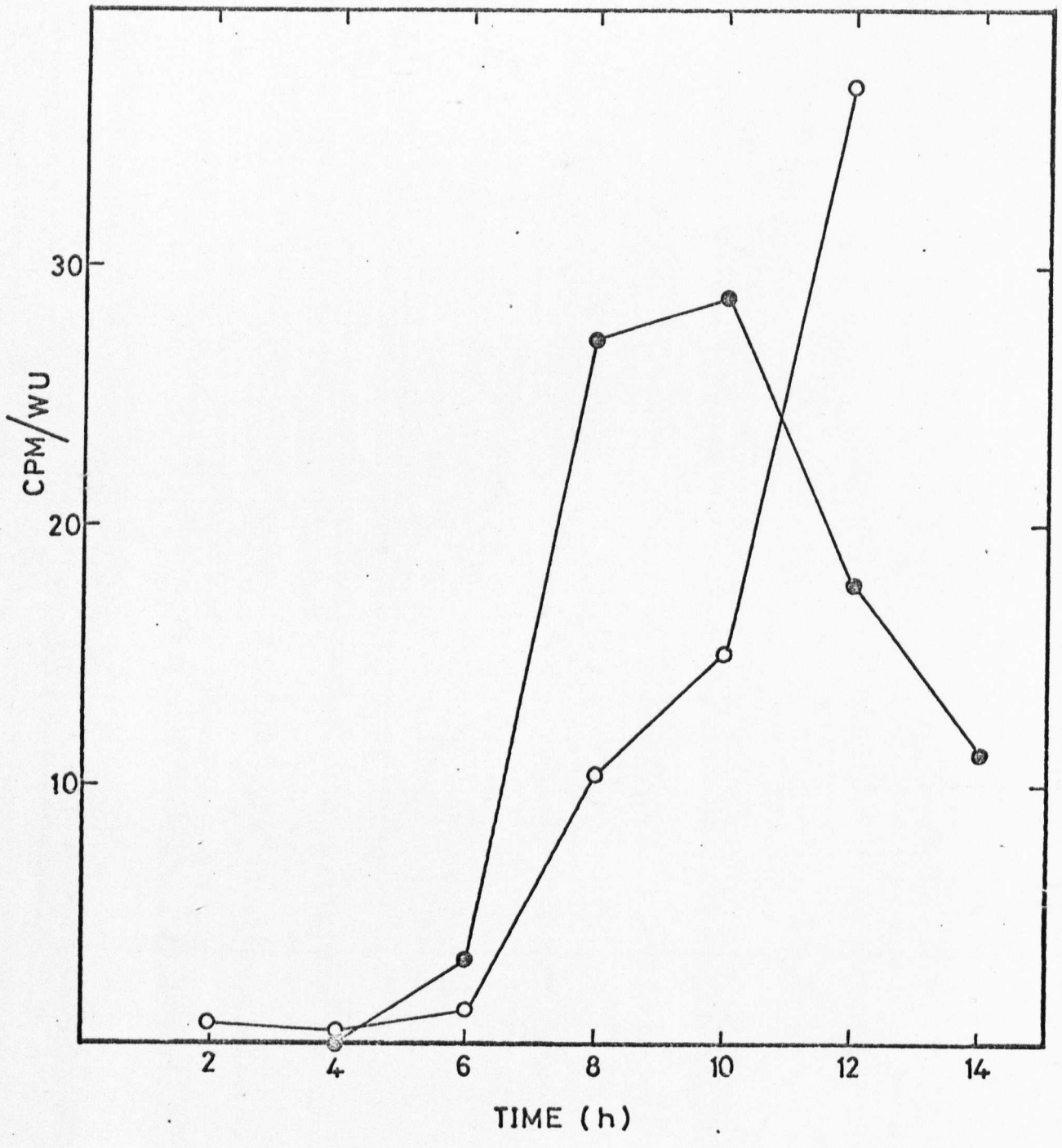


Fig. 19. Time course of synthesis of viral DS RNA in HEL (O—O) and HeLa (●——●) cells. Each point represents the middle of a 2 h pulse of [^{3}H]-uridine and is the normalised sum of radioactivity under each peak. Data is taken from Tables 3 and 5.

Pulse time (h)	TCA - precipitable cpm
Non-infected	1,600
3 - 5	4,600
5 - 7	55,000
7 - 9	13,000
9 - 11	10,000

Table 6. Synthesis of rhinovirus RNA in L132 cells. The total TCA-precipitable counts are presented.

Over a large number of experiments using different concentrations of [^{3}H]-uridine, different batches of virus and cells of different passage number, the proportion of each RNA species expressed as a percentage of the sum of the species was remarkably constant (Table 4). The predominant species was SS RNA. From its first appearance until late in infection, SS RNA comprised approximately 80% of the total. DS RNA remained constant at about 10% while the percentage of MS RNA declined from 10 to 2.5%.

b. Replication in HeLa cells.

This experiment was done in parallel with the experiment described above in which HEL cells were used. Fig. 17 shows the analysis of viral RNA's extracted from infected HeLa cells pulsed for consecutive periods of 2 h. The radioactivity in peaks of virus RNA was calculated and normalized as described earlier (Table 5).

The results from tables 3 and 5 were plotted separately for SS and DS RNA as they appear with the time after infection (Fig. 18, 19). The important point from these graphs is that, in contrast to the situation in HEL cells, the synthesis of the viral RNA in HeLa cells reached a plateau at 8 h after infection and later on declined.

c. Replication in L132 cells.

In this experiment viral RNA synthesis was measured in infected L132 cells, which is another heteroploid human cell line, as a function of the time after infection. The infected cells were pulsed with [^{3}H]-uridine for consecutive 2 h intervals, in the presence of AMD and were assayed for TCA-insoluble material (Table 6). Viral RNA which was first detected during the 3 - 5 h pulse increased until the 5 - 7 h pulse and then declined.

In conclusion the kinetics of rhinovirus RNA synthesis in heteroploid cells is different from those in

diploid cells.

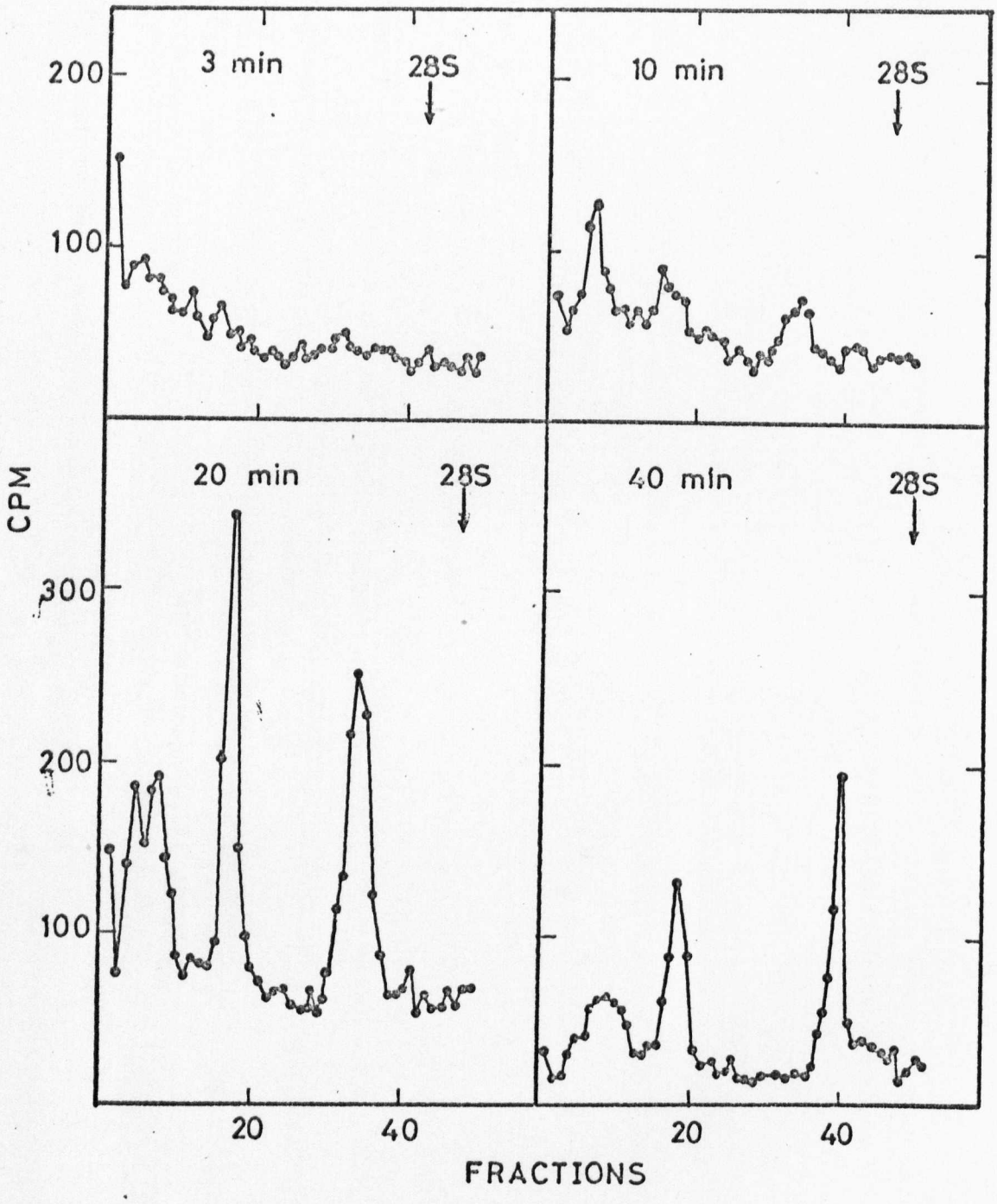


Fig. 20, continued over page

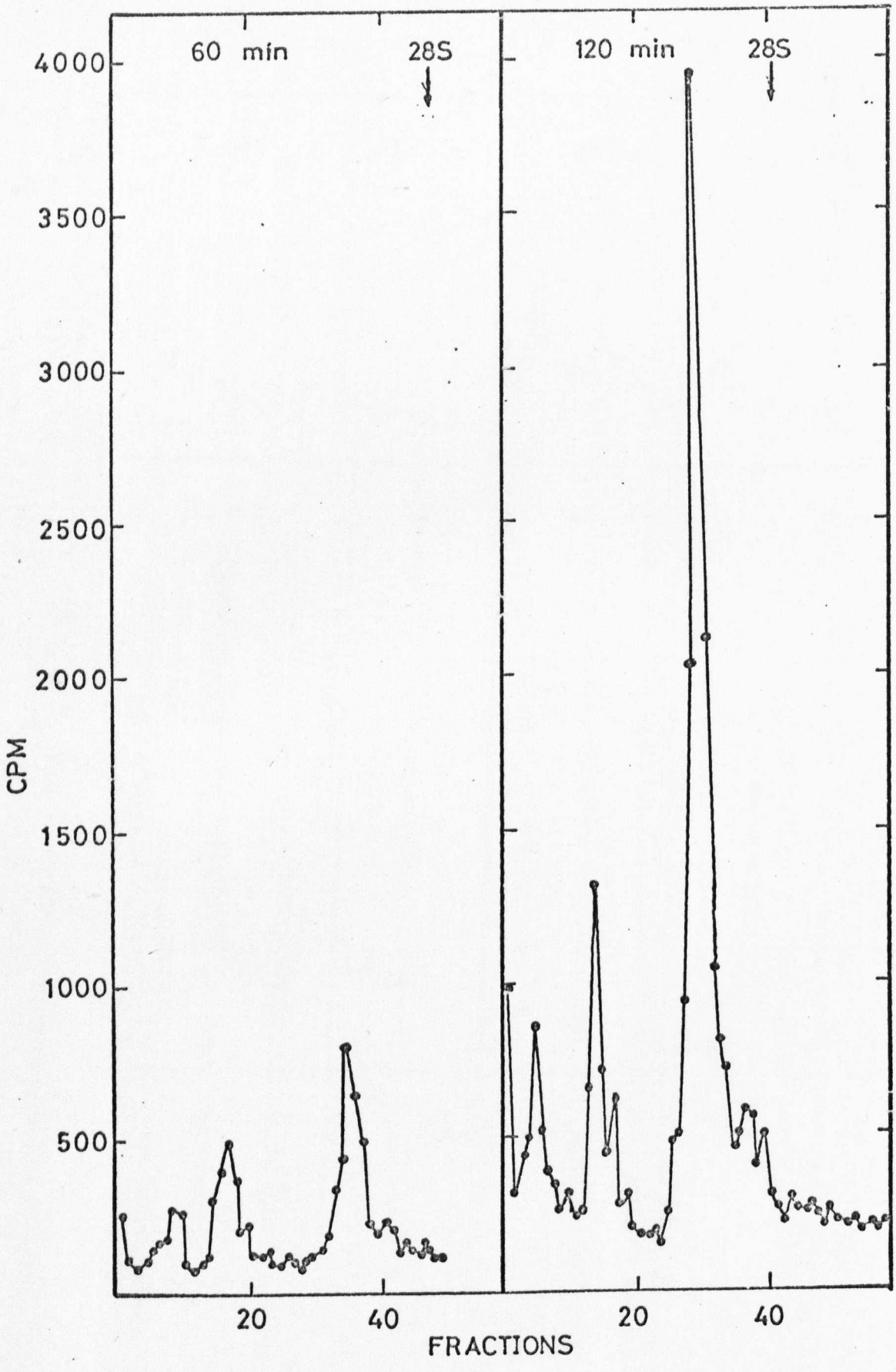


Fig. 20. Electrophoresis of RNA extracted from infected HEL cells which were pulsed with [^{3}H]-uridine for 3, 10, 20, 40, 60 and 120 min.

Pulse duration min	Total viral RNA		MS		DS		SS	
	cpm	%	cpm	%	cpm	%	cpm	%
3	13.3	100	11.7	88.0	1.3	9.7	0.3	2.3
10	47.8	100	18.2	38.1	16.4	34.3	13.2	27.6
20	59.1	100	18.9	32.0	17.6	29.8	22.6	38.2
40	156.5	100	39.9	25.5	52.3	33.4	64.3	41.1
60	136.7	100	18.7	13.6	42.8	31.3	75.2	55.1
120	649.3	100	49.5	7.6	102.9	15.8	496.9	76.6

Table 7. Viral RNA synthesis in HEL cells as detected by increasing the duration of the $[^3H]$-uridine pulse. The figures represent the normalised radioactivity under each peak in Fig. 20.

C. The role of the three viral RNA species in replication.

According to their electrophoretic mobility and RNase sensitivity the three species of virus RNA in infected cells were designated MS, DS and SS RNA. In this section experiments will be described which investigate the relationship between MS, DS and SS RNA during replication, H-bonding between strands in MS and DS RNA and the circular model of replication.

1. Short [^{3}H]-uridine pulses.

Fenwick et al. (1964) first used short uridine pulses in order to elucidate the active intermediate(s) of RNA replication. Theoretically if [^{3}H]-uridine is present in the infected cells for shorter time than the time required for the synthesis of a new strand of RNA, then all label is expected to be located in the active replicative intermediate of the replication process.

Infected HEL cells were pulsed with [^{3}H]-uridine from 3 to 60 mins as indicated in Fig. 20. The pulses were all terminated at 11 h and RNA was extracted and analysed by polyacrylamide gel electrophoresis (Fig. 20). During the shortest pulse (3 min) all label above background is found in the position to which MS RNA usually migrates. After the 10 min pulse, MS RNA is still the predominant species although DS and SS RNA are easily recognised. The percentage of radioactivity in the peaks of MS, DS and SS RNA was calculated from the sum of normalized radioactivity in each peak (Table 7). MS RNA, which is the main species after the shortest pulse (88% of the total RNA) decreased gradually as the labelling period was increased (only 7.6% for the 120 min pulse). DS RNA which was 9.7% of the total viral RNA after the 3 min pulse increased to a constant value of 30% in the longer pulses and dropped down again to 15% after the 120 min pulse. The SS RNA increased constantly with the duration of the pulse, from 2.3% after the 3 min to 76.6% after the 120 min pulse.

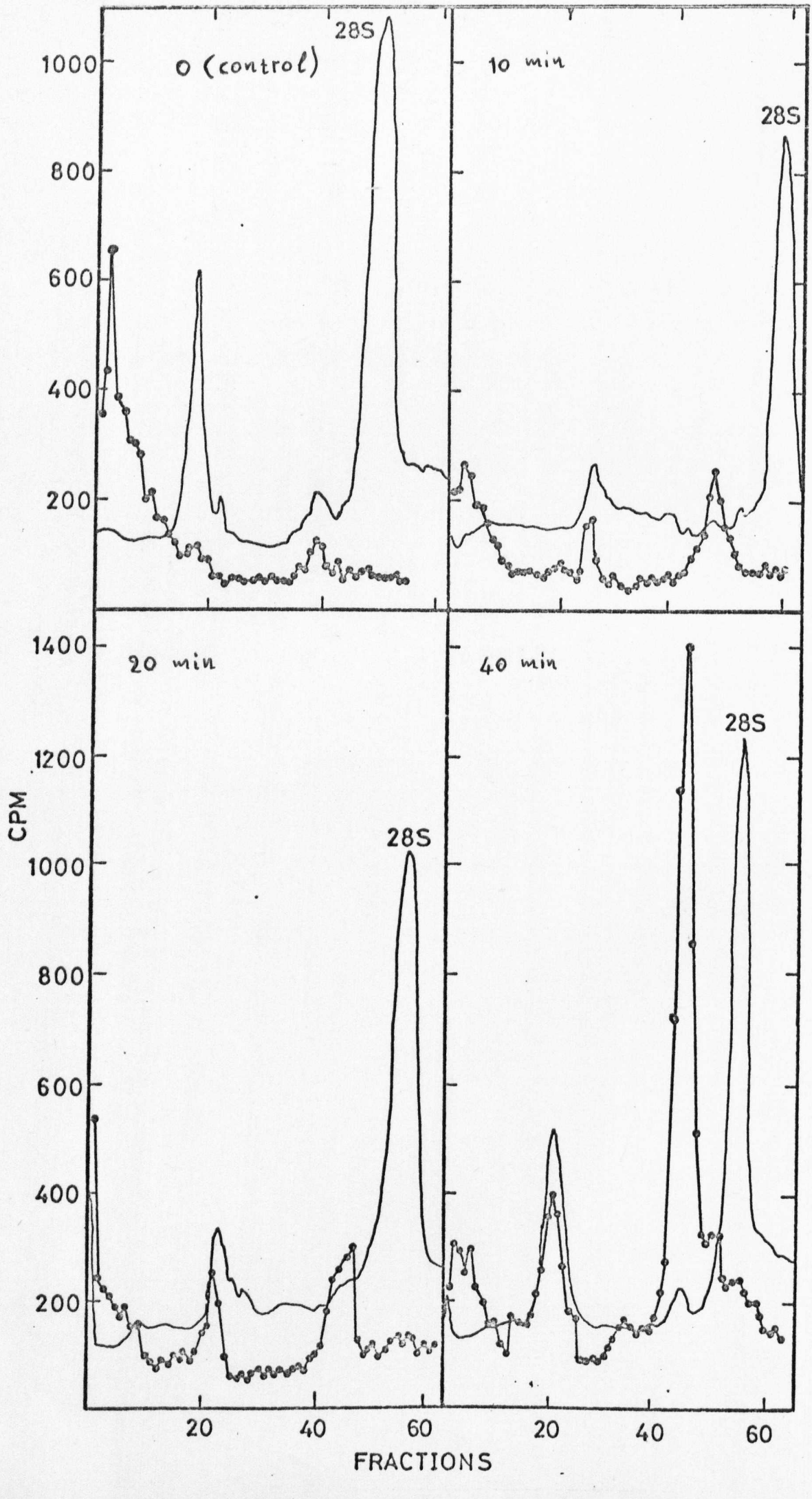
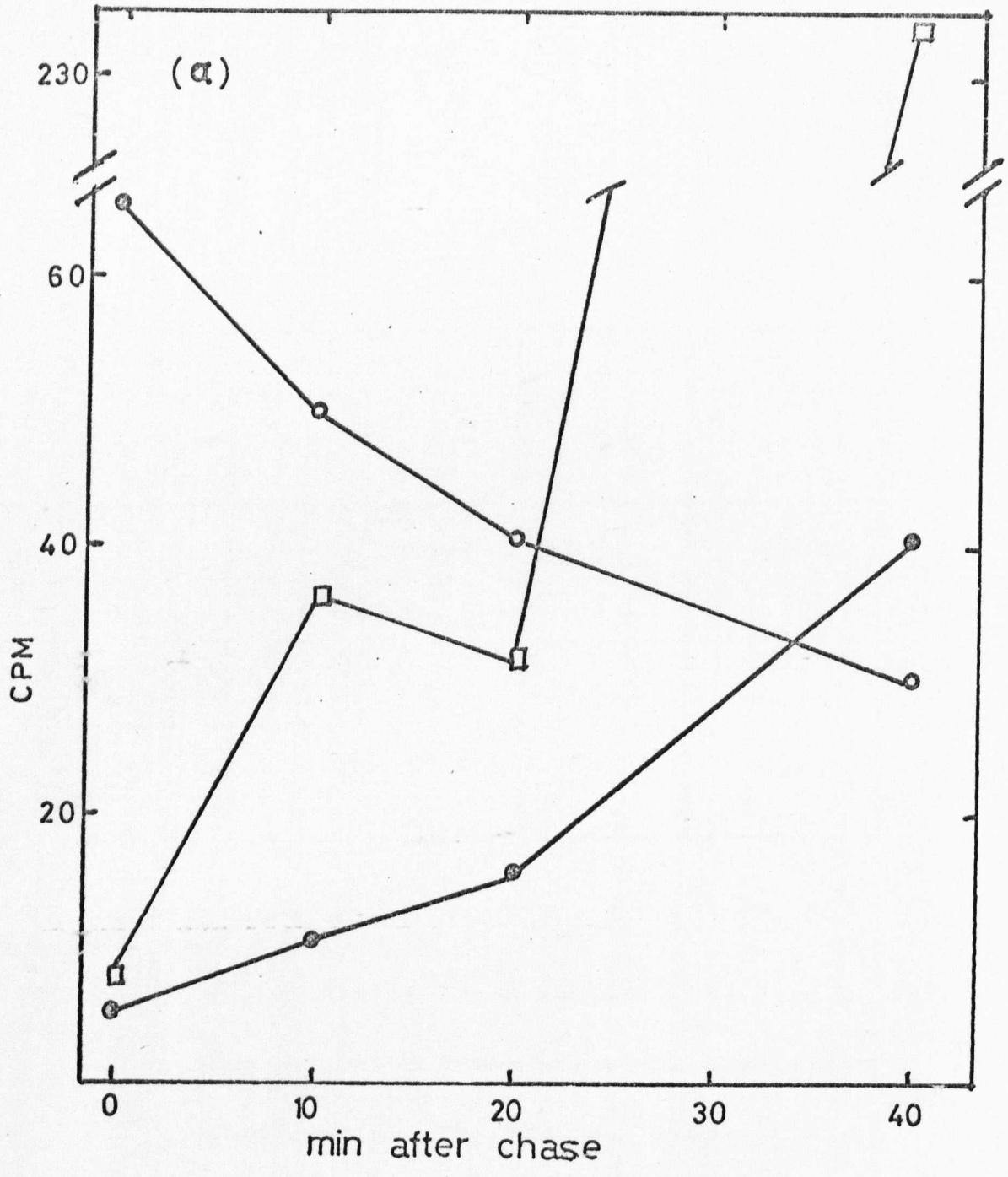

0 (control)
28S
10 min
28S
20 min
28S
40 min
28S
CPM
1000
800
600
400
200
1400
1200
1000
800
600
400
200
20
40
60
20
40
60
FRACTIONS

Fig. 21. Electrophoresis of RNA extracted from infected HEL cells. Cells were labelled for 5 min with 10 μc / ml of [^{3}H]-uridine; at the end of this period they received a 10^6-fold excess of cold uridine and the RNA was extracted under standard conditions at the indicated times. Continuous line represents the OD_{260} trace indicating the position of the 28S ribosomal RNA and the 'marker' DNA.

Time after chase (min)	MS	DS	SS
0	65.6	5.1	8.0
10	50.0	10.6	36.7
20	40.8	15.5	31.4
40	30.1	41.1	234.3

Table 8. Radioactivity found in viral RNA species at the indicated times after the addition of cold uridine. The figures represent the normalised radioactivity under each peak in Fig. 21.


(a)
230
60
40
20
CPM
0
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20
30
40
min after chase

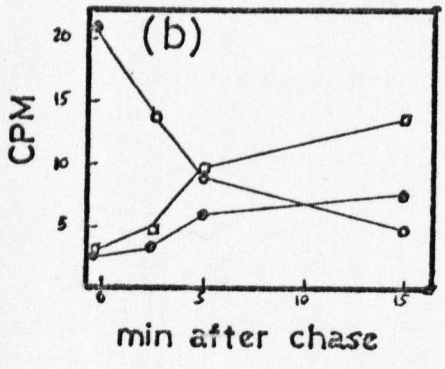

(b)
CPM
min after chase

Fig. 22. (a) Chase of viral RNA in vivo by addition of excess of cold uridine. Data obtained from Fig. 21. (o—o) RI, (●—●) RF, (□—□) SS RNA.
Fig. 22 (b) is reproduced from McDonnell and Levintow (1970) and illustrates the distribution of the label between the 3 poliovirus RNA species after chase.

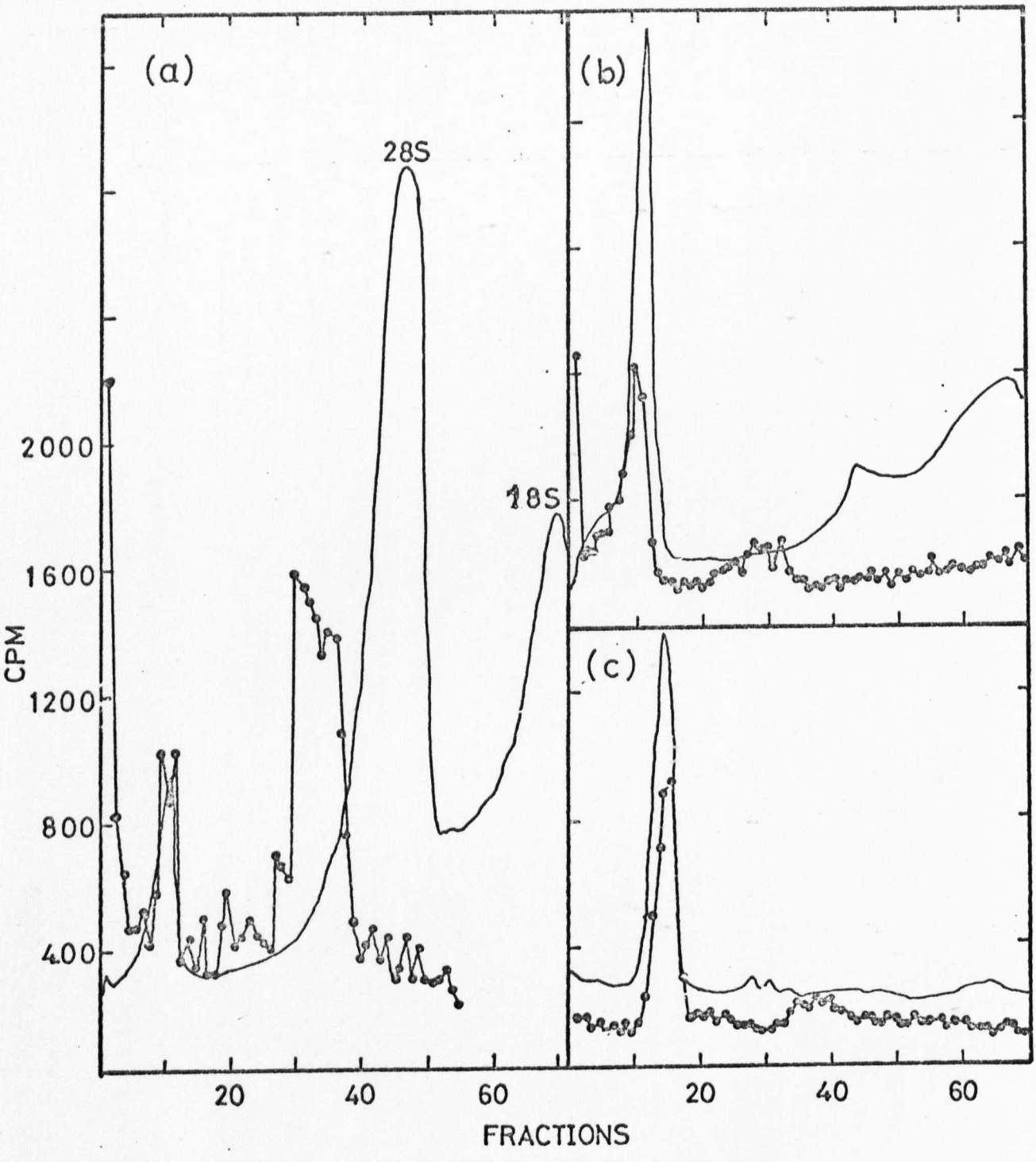


Fig. 23. Treatment of disrupted infected cells with ribonuclease before extraction of RNA. Infected HEL cells were labelled with [^{3}H]-uridine from 9 to 11 h after infection and the extracted RNA was analysed by electrophoresis. (a) control, (b) and (c) treated with 0.1 and 10 µg / ml of RNase respectively. The continuous line is the OD_{260} trace indicating the position of the 'marker' DNA and the ribosomal RNA in the control figure.

2. Chase of [^{3}H]-uridine labelled viral RNA by cold uridine.

Using HEL cells we have shown that it is possible to chase [^{3}H]-uridine with an excess of cold uridine (see "Methods"). Using the same conditions we have attempted to investigate the relationship between MS, DS and SS RNA during replication.

At 9 h after infection HEL cells were labelled for 5 mins with 10μc/ml of [^{3}H]-uridine. At the end of this period the cells received a 10^6-fold excess of cold uridine and the RNA was extracted at various times after chase (Fig. 21). The radioactivity in each RNA species was calculated and normalized (Table 8). Initially the major species was the MS RNA. During the 40 min chase the MS RNA decreased 2-fold while the DS and SS RNA increased 8-fold and 30-fold respectively (Fig. 22a). This is in close agreement with the data obtained from the in vitro chase of poliovirus RNA synthesis by McDonnell & Levintow (1970) from where the Fig. 22b was obtained for comparison.

3. Hydrogen bonded RNA in replication.

The aim of this experiment is to demonstrate directly whether H-bonding between RNA molecules is an integral part of the replication process (Spiegelman et al., 1968) or artefact resulting from the extraction procedure (Weissman et al., 1968).

Infected HEL cells were labelled with [^{3}H]-uridine between 9 - 11 h p.i.. The cells were then washed twice with cold extraction buffer and 2 ml of 2 x SSC were added to cover the cell sheet. The monolayer was scraped into the SSC solution and the cells disrupted by freezing and thawing. At this point the cell extracts were divided into three parts, two of which were treated with RNase (0.1 and 10μg/ml) for 10 min at 37°C. RNA was then extracted from the three samples using our routine phenol-SDS extraction method and was analysed by polyacrylamide gel electrophoresis (Fig. 23).

Complete degradation of the SS RNA species,

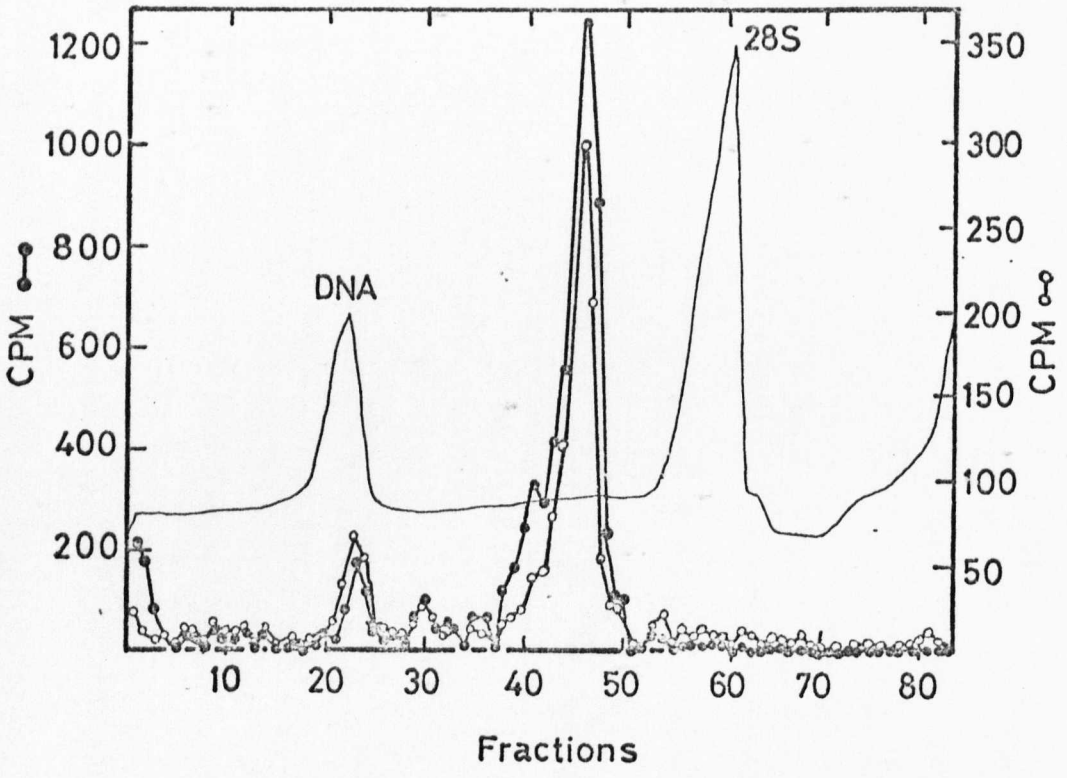


Fig. 24. Co-electrophoresis of RNA from rhinovirus-infected HEL cells labelled from 5 to 7 h with [^{3}H]-uridine (●—●) or from 9 to 11 h with [^{14}C]-uridine (○—○). The continuous line is the OD_{260} trace, indicating the position of 28S ribosomal RNA and the 'marker' DNA.

both viral and ribosomal occurred after ribonuclease treatment at either concentration of the enzyme. The MS RNA was partially degraded with 0.1µg/ml of RNase and was totally degraded when the concentration of RNase was increased to 10µg/ml. The DS RNA remained intact after such treatment indicating that hydrogen bonding was present in the preparation before the phenol extraction.

4. Size of SS RNA synthesized early and late in infection.

Evidence has been provided that early in infection some picornaviruses synthesize SS RNA which is larger than the RNA of the virion (Brown & Martin, 1965; Clements & Martin, 1971). With this evidence these authors proposed a 'rolling-circle' model for the synthesis of picornavirus RNA. Since this matter is fundamental to theories of RNA replication we examined the size of SS RNA synthesized by rhinovirus and by the bovine enterovirus used by Clements & Martin (1971).

(a) Rhinovirus in HEL cells

Rhinovirus RNA was labelled at 5 to 7 and at 9 to 11 h postinfection with [^{3}H]- or [^{14}C]-uridine, respectively and analysed by electrophoresis on a single polyacrylamide gel (Fig. 24). The SS RNA labelled at either time migrated to the same position in the gel and in peaks of similar width.

(b) Bovine enterovirus in HEL cells

In HEL cells the exponential production of virions ended at 7 h after infection. Accordingly, RNA was labelled from 0.5 to 4 h and from 4 to 7 or 9 to 11 h postinfection with [^{3}H]- or [^{14}C]-uridine, respectively. Co-electrophoresis showed that SS RNA extracted at either time migrated identically (Fig. 25a).

(c) Bovine enterovirus in BHK cells

The occurrence of SS virus RNA larger than virus particle RNA has only been reported in BHK cells. Since we failed to find such RNA in infected HEL cells

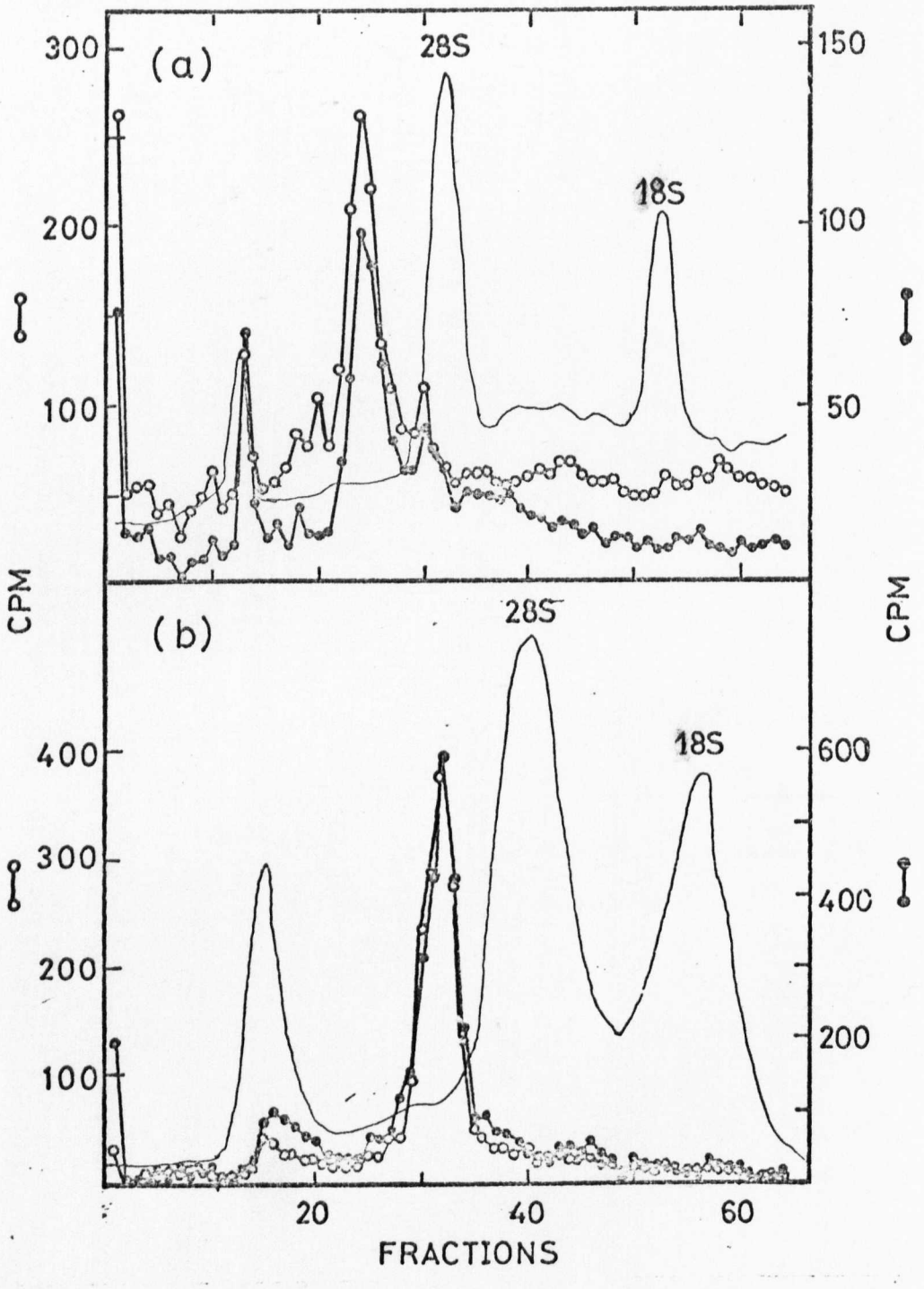


Fig. 25. Co-electrophoresis of RNA from bovine enterovirus-infected cells (a) HEL cells labelled from 0.5 to 4 h with $[^{14}C]$-uridine (●——●) or from 4 to 7 h with $[^{3}H]$-uridine (o——o). (b) BHK cells labelled from 0.5 to 4 h with $[^{3}H]$-uridine (●——●) and from 4 to 6 h with $[^{14}C]$-uridine (o——o). The continuous line is the optical density trace at 260 nm.

we examined the system described by Clements & Martin (1971), that of BHK cells infected with bovine enterovirus.

In BHK cells the exponential production of virus particles started at 3 h and reached a plateau at 5 h after infection. Accordingly, we labelled from 0.5 to 4 h and from 4 to 6 h after infection with [^{3}H]- or [^{14}C]-uridine, respectively (Fig. 25b). Co-electrophoresis showed that the peaks of virus SS RNA coincided and had similar distributions. SS RNA obtained when cells were labelled from 0.5 to 2.5 h was also of the same size.

DISCUSSION

Growth of rhinovirus in HEL cells

We have found that HEL cells provide a satisfactory system for studying rhinovirus multiplication, although the diploid cells are less convenient to use than continuous cell lines and more fastidious in their growth requirements.

The growth of the virus in these cells is very similar to that reported by Stott and Heath (1970) in suspensions of the continuous line of L132 cells infected with the same virus. No virus is detected before 4 hours after infection. Virus then increases exponentially until 11 h after infection when the plateau of virus multiplication is reached.

A major advantage of the HEL cells was the consistently higher yield of virus RNA relative to cellular RNA. Viral RNA could be detected by optical density at 260nm and was as much as 4% of the total ribosomal RNA present in infected HEL cells.

Our work on the synthesis of viral RNA in HEL cells indicated that the kinetics were different from those in HeLa cells. The importance of this observation in relation to transcriptional control, which might be relevant to the diploid and heteroploid state of the cells, is discussed below.

Virus RNA species

We did not detect any RNA synthesis in non-infected HEL cells treated for 3 h with 1μg/ml AMD.

The use of the polyacrylamide gel electrophoresis technique, especially when the concentration of the acrylamide was brought down to a minimum (1.7%) has been proved to offer good resolution of the various RNA species. The results presented in this study clearly establish that three RNA species are consistently found in HEL cells

infected with rhinovirus type 2. This is in agreement with the results obtained by Sethi and Schwerdt (1972) who detected only double and single stranded RNA species in HeLa cells infected with rhinovirus type 14. The limited resolution by sucrose gradient centrifugation is the obvious explanation for their failure to detect the third RNA species. We are also in agreement with the results reported by Yin and Knight (1972) who resolved by polyacrylamide gel electrophoresis the three RNA species obtained from HeLa cells infected with rhinovirus type 2.

Structure of the viral and virus induced RNA species

Pancreatic ribonuclease has been used to study the structure of these RNA species since it will attack single more readily than double stranded regions of RNA. We have worked out conditions in which RNase would selectively degrade only the SS RNA leaving the DS RNA intact. In our experience the concentration of salt is the critical factor for this selective degradation of SS RNA.

In the experiment illustrated in fig. 10, it is clearly shown that while the species with the fastest electrophoretic mobility is RNase sensitive, the one with intermediate mobility is RNase resistant. The third species which did not form a discrete peak and migrated only a short distance into the gel upon electrophoresis was found to be degraded by RNase and this resulted in an increase in the peak of the RNase resistant species (Table 2).

This behaviour of the virus induced RNA species on ribonuclease treatment is in agreement with the results published by Baltimore (1968) for poliovirus and therefore we conclude that the three RNA species (in order of electrophoretic mobility) are single stranded, double stranded and the third consisted of a double stranded core from which single strands are tailing off.

Size of virion RNA

The molecular weight of the RNA of various rhinovirus strains has been studied by a number of workers. Only lately has agreement been reached that the MW of the RNA of rhinoviruses is in the region of 2.2 - 2.8 x 10^6 daltons. In 1968, McGregor and Mayor on the basis of measurement of the length of a ribonucleoprotein strand by electron microscopy estimated the MW of the RNA of rhinovirus type 14 as 4 x 10^6 daltons. However the same workers in 1971 reported that "evidence suggests that the association of a single stranded nucleic acid with a protein can lead to errors in the estimation of molecular weight based on linear density" and reported a value of 2.1 x 10^6 daltons based on hydrodynamic methods and 2.2 x 10^6 daltons based on the sedimentation of the RNA in sucrose gradients.

Our estimate of the MW of rhinovirus type 2 RNA is 2.8 x 10^6 daltons. This is based on coelectrophoresis with the precursors of the ribosomal RNA from HeLa cells. The MW's of the ribosomal RNA's are well established (Weinberg & Penman, 1970) and have been used for the calibration of the polyacrylamide gels. The value of 2.8 x 10^6 daltons lies within the range of MW's reported by several investigators (Brown et al., 1970; Nair & Lonberg-Holm, 1971; Sethi & Schwerdt, 1972). It should be pointed out that the value of 2.8 x 10^6 daltons has been obtained after electrophoresis of native RNA. This value must therefore be regarded as a minimum estimate since the absolute MW can only be measured after the secondary structure has been destroyed. Fenwick (1968) and Boedtker (1968) have showed that a more precise relationship between sedimentation rate and MW for a number of different RNA's could be obtained if the configurational restraints are diminished by reacting the RNA with formaldehyde. Boedtker (1971) has shown that the MW's of Qβ and R17 phage RNA as measured by polyacrylamide gel electrophoresis are significantly larger after treatment with 1.1M formaldehyde. Nevertheless

Brown et al. (1970) and Nair and Lonberg-Holm (1971) estimated the MW of rhinovirus type 2 RNA being within the MW range of 2.4 - 2.8 x 10^6 daltons after treatment of the RNA with formaldehyde or dimethylsulphoxide.

Earlier reports have compared the size of SS RNA of rhinovirus with poliovirus: McGregor and Mayor (1971), Nair and Lonberg-Holm (1971) and Sethi and Schwerdt (1972) found that both native and denatured poliovirus type 1 RNA sedimented faster on sucrose gradients than did rhinovirus RNA from type 14, type 2 and type 20, respectively. However our results from coelectrophoresis of native RNA from cells infected with rhinovirus type 2 or poliovirus type 1 showed that the rhinovirus SS RNA was the larger. Clearly there is a discrepancy which indicates that movement of these RNA molecules in sucrose gradients or polyacrylamide gels cannot be determined solely by the molecular weight of the RNA. Such an observation has already been made with the "26S" RNA of Semliki Forest virus, which by polyacrylamide electrophoresis is larger than 28S ribosomal RNA (Kennedy, 1972).

Function of virus induced RNA species

The single stranded RNA extracted from infected cells has an electrophoretic mobility identical to the RNA found inside virions (Fig. 9). This, and by analogy with the fact that the base ratio of the single stranded RNA synthesized in poliovirus-infected cells almost matches that of virion RNA (Zimmerman et al., 1963), leads us to the conclusion that the single stranded RNA found in infected cells is the end product of the RNA replication process and will be incorporated within the virions during maturation. This conclusion is further supported by experiments on the kinetics of RNA synthesis which are discussed below.

When a labelled precursor of RNA is present continuously in infected cells the amount of radioactivity present in each of the RNA species observed at a given time represents the resultant synthesis, degradation and

interconversion. If we assume that the degradation of SS RNA occurs to the same extent as the degradation of the single tails of the MS RNA, then any observed difference between the two species will be due to the conversion of the species with the lower rate of increase to the other with the higher rate. We have found that while the SS RNA increased 1000-fold between 5 and 13 h after infection, the multi-stranded species increased only 250 times during the same period (Fig. 12). This is in agreement with the postulated precursor-product relationship between multi-stranded and single stranded species. No conclusion is possible from this experiment as far as DS RNA is concerned (which increased 500 times in the same period) since the degradation pattern will be totally different. The situation is further complicated since it is possible that DS RNA is derived from MS RNA species.

Evidence that the MS RNA is the basic precursor in replication is also shown by the [^{3}H]-uridine short pulse experiment (Fig. 20). After infected cells were exposed to the radioactive RNA precursor for as little as 3 min all the label was present in the region of the polyacrylamide gel in which the MS RNA runs. By increasing the period of exposure, the label is found in increasing amounts in the other species of RNA. These results are in agreement with those of Baltimore (1968) who found that after exposure of infected HeLa cells with poliovirus to [^{3}H]-uridine for 2.75 min 67% of the label was found in the MS RNA. From this experiment two observations regarding the DS RNA can be made: the initial synthesis of DS RNA followed similar kinetics to the SS RNA a fact which is against any intermediate role of the DS RNA in the replication process. However, later in infection DS RNA is accumulated at a much slower rate than the SS RNA. This is an indication that the DS RNA is not a consistent by-product of the replication.

The conclusive evidence that MS RNA is the intermediate of the replication comes from the chase experiment (Fig. 21).

Baltimore, in his review article (1969), has presented evidence that radioactive uridine cannot be chased in mammalian cells with an excess of cold uridine. He suggests that the intracellular uridine is phosphorylated and cannot escape from the cells, hence addition of cold uridine therefore will not lower the amount of radioactivity in the cellular pool of nucleotides. Secondly, because the cell itself has a larger internal pool of uridine nucleotides than the maximal amount of uridine it is able to take up from the medium, the nucleotide pool cannot be extensively diluted by adding a large excess of cold uridine. Nierlich (1967) has shown that exogenously supplied radioactive uracil enters the intracellular pools of RNA precursors in Escherichia coli only as nucleotides are removed from these pools by net synthesis of RNA. He went on to show that the chase of radioactive RNA precursor by an excess of cold precursor was also impossible in procaryotic cells.

We have shown that the ability to chase uridine in eukaryotic cells varies with the cell type and that it is possible to do useful pulse chase experiments (Fig. 5). Although it seems that this brings ourselves into a controversial position with Baltimore we believe that the difference lies in the experimental conditions of the chase. While Baltimore measured the radioactivity incorporated into the RNA only shortly after the addition of the cold uridine (his last sample was measured only 12 min after the chase was started) we extended this period to 120 min. In the case of HEL or HeLa cells we have shown that for the first 10 to 15 min the cold uridine retarded but did not stop the incorporation of radioactive uridine into the RNA. However, after the first 10 min, virtually no more radioactivity is incorporated into RNA. Of the cells tested only BHK cells followed the kinetics predicted by Baltimore. Surprisingly enough we found that chick embryo fibroblasts are the most suitable cell type for chase experiments (Fig. 5). It can be argued that chase with a delay of approximately 10 min could not

be of any use. However the experiment illustrated in Fig. 21 shows for the first time direct evidence on the role of MS RNA in an animal virus system in vivo. Initially label is present only in MS RNA. After the addition of cold uridine the label in MS RNA decreases and label in SS and DS RNA increases. The kinetics are in fact similar to those of the short-pulse experiment and again DS RNA accumulated in the same manner in which it accumulated in the pulse experiment. Therefore DS RNA has no intermediate role in RNA replication.

The experiments discussed in this section supply the evidence on the intermediate role of MS RNA which can therefore be accurately designated as replicative intermediate (RI) (Erikson et al., 1964). These experiments also supply the evidence that the SS RNA found in the infected cells is the end product of the replication process. The question which remains unsolved is the one concerning the DS RNA which has all the characteristics of replicative form (RF) as described for the other virus-cell systems (Bishop & Levintow, 1971). It has been suggested that RF might be an artefact formed during extraction procedures (Weissman et al., 1968). In fact, as has been mentioned in the introduction, Weissman et al. believe that H-bonds between the RNA molecules are only formed during extraction of the RNA. We can exclude this possibility since we found that RF was still present after all RNA single strands were degraded with ribonuclease before the RNA was extracted with phenol (Fig. 22). Although this experiment has shown that H-bonding is not an artefact of the extraction, we do not know if the RF remaining after RNase degradation is real RF or degraded RI. This question could be answered by selectively labelling RF without RI by chasing uridine from RI to RF (and SS RNA). If RF remains after treating freeze-thawed cells with RNase followed by phenol extraction, then we can conclude that RF is a real entity in infected cells. A preparation containing only RI can be obtained by labelling with a brief pulse.

By treating this preparation with RNase in the same way we shall be able to determine whether or not multi-stranded RI has H-bonding between RNA molecules.

From the experiment in Fig. 22 we can determine the proportion of SS RNA which has been incorporated into virions. Since the RNA in released virions is resistant to RNase we can assume that the RNA in cell-associated virions is similarly resistant. Therefore the proportion of SS RNA remaining after RNase treatment of freeze-thawed cells represents encapsidated SS RNA and the remainder is not in mature virions. Thus 4% RNA is encapsidated and 96% is not.

Virus multiplication and RNA replication in diploid and heteroploid cells

The dynamics of synthesis of the three species of rhinovirus RNA in HEL cells are of interest since some aspects of these results are different from those obtained by other investigators working with similar systems. Sethi and Schwerdt (1972) found that the exponential increase of viral RNA synthesis in HeLa cells infected with rhinovirus type 2 is not maintained late in infection. Noble and Levintow (1970) have also reported a similar decline in RNA synthesis in HeLa cells infected with poliovirus. The experiments described in section B of this chapter demonstrate that although the multiplication of the virus is very similar in the two cell types (Fig. 15), different levels of viral RNA are observed late after infection. In HeLa and L132 cells the levels of viral RNA reached a maximum and then declined (Fig. 18, 19 and Tables 4, 5, 6) with kinetics very similar to those observed for the replication of poliovirus in HeLa cells (Noble & Levintow, 1970). However in HEL cells the levels of viral RNA continued to increase for at least 4 hours longer than in HeLa or L132 cells (Fig. 18, 19 and Tables 4, 5, 6). Since the pool of acid soluble uridine in HeLa cells has been shown to be constant (Fig. 4b) even when the

levels of viral RNA decline, the explanation of the different levels of viral RNA in diploid and heteroploid cells could possibly be the result of different cellular control of the replication of virus RNA. Control may be exerted by the action of ribonucleases, the low levels of viral RNA observed late after infection in HeLa cells being due to increased RNase activity. However we consider this to be an unlikely explanation since the change in viral RNA levels is shown in both single and double stranded RNA and it is unlikely that the nucleases degrade both types of nucleic acid with equal efficiency. If the difference obtained with diploid and heteroploid cells is not due to degradation of RNA by nucleases, then it may be due to different rates of viral RNA synthesis. It has been suggested recently that the enzyme responsible for the viral RNA synthesis (RNA-dependent RNA polymerase) in virus-infected cells contains virus modified cellular polypeptides (Rosenberg et al., 1972). It is also known that picornaviruses inhibit cellular protein synthesis and that the viral polymerase is unstable. Therefore if the continued synthesis of cellular protein synthesis is required it is possible that the rate at which the virus inhibits cellular protein synthesis could control the rate at which viral RNA polymerase synthesis and hence RNA synthesis can continue.

Circular model of replication

We have shown that cells infected with rhinovirus type 2 synthesize a species of SS RNA which has an electrophoretic mobility identical to that of virion RNA (Fig. 9). The details of synthesis are not known but evidently it is a closely regulated process since SS RNA molecules synthesized in infected cells during exponential increase of virus and during the plateau of virus production showed no detectable variation in size (Fig. 24). This feature is of particular interest since other picornaviruses, foot-and-mouth disease virus (Brown & Martin, 1965; Wild & Brown, 1970) and bovine

enterovirus (Clements & Martin, 1971), are reported to synthesize RNA of different molecular weights at different times during the multiplication cycle. We have repeated these experiments with bovine enterovirus and found that SS RNA synthesized in HEL or BHK cells at different stages of the multiplication cycle also showed no detectable variation in size (Fig. 25). It is still possible to argue that molecules of SS RNA larger than virion RNA were present in small amounts in the rhinovirus, bovine enterovirus or poliovirus infected cells represented in the electrophoretograms above. Such small amounts would be difficult to distinguish from the background scatter of radioactivity. The limited number of such molecules would only be found on our experimental conditions if the labelling of SS RNA of the same size as virion RNA was favoured. We conclude that our data provide no evidence to support the "rolling - circle" hypothesis for picornavirus RNA replication and do not conclusively disprove it. However the hypothesis of a circular model of replication is supported by the findings of other investigators. Agol et al. (1972), using electron microscopy of purified preparations of the RF of EMC virus found three categories of molecules which were of linear, of circular and of unknown structure. On the basis of electron microscopy they have calculated the MW of these circular structures to be $5.25 \pm 0.28 \times 10^6$ daltons. This value is expected to be twice as much as the MW of the SS RNA of the same virus. Indeed the MW of SS RNA of EMC has been calculated by an independent method to be 2.7×10^6 daltons (Burness & Clothier, 1970).

Yogo and Wimmer (1973) have found that while the 3'-end of the plus strand of the RF of poliovirus RNA contains a polyA region, which is longer than the corresponding region of the virion RNA, the complementary (minus) strand contains the polyU region at the 3'-terminus. An explanation of this finding might be that the poliovirus RF forms circles since its single stranded 3'-termini are complementary to each other.

RESULTS PART II

"Effect of the antiviral compound ICI 65,709 on viral RNA synthesis."

INTRODUCTION

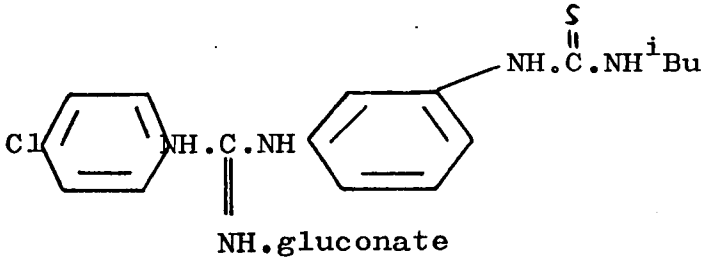
Several natural or synthetic selective inhibitors of picornavirus multiplication have been described during the last 15 years. The term selective inhibitor is generally employed to characterize inhibitors which display antiviral activity in concentrations much lower than those producing harmful effects to host cells.

The best studied inhibitors are of both biological and synthetic origin. The first class includes the inhibitors contained in bovine sera (Takemori et al., 1957; Takemori et al., 1958), normal horse sera (Takemoto & Habel, 1959) and dextran sulphate (Rightsel et al., 1961); they are of large molecular size and act on an early stage of cell-virus interaction. Among the synthetic inhibitors guanidine (Rightsel et al., 1961; Loddo, 1961; Ueda et al., 1961), 2-(α-hydroxybenzyl)-benzimidazole (HBB) (Hollinshead & Smith, 1958; Tamm et al., 1961; Eggers & Tamm, 1961a; Eggers & Tamm, 1961b) and gliotoxin (Rightsel et al., 1964) are best characterized; they are of small molecular weight and act on a later stage of viral synthesis.

The compound which we have studied was provided by ICI Ltd. and it has been designated as 65-709. It was shown by ICI investigators (Bucknall, unpublished results) that 65-709 possesses a selective antiviral activity with a therapeutic ratio for rhinovirus type 2 when grown in HEL cells of 500. The therapeutic ratio is defined as the ratio of the 50% cytopathic concentration of the compound to the 50% virus inhibitory concentration. R. A. Bucknall carried out preliminary studies on 65-709. It had greatest activity against picornaviruses and vaccinia virus and showed slight activity against arboviruses and herpes simplex virus. It was not active against respiratory syncytial virus or equine rhinovirus. The antiviral activity was greatest in HEL cells and was reduced in either diploid primary monkey kidney cells or heteroploid human cells in culture. 65-709

appeared to inhibit a replicative or maturation process since it neither inactivated virions nor prevented their penetration into the host cell. The compound did not appear to bind to cellular components or cause irreversible changes to the cell since antiviral activity was absent after removing the drug from the culture medium.

The compound, 1 - p - chlorophenyl - 3(3-isobutylthioureido) phenyl guanidine gluconate, (MW 571.5) is designated ICI 65,709 or in this thesis 65-709 and has the chemical formula:



The mode of action of guanidine is still an unsolved problem despite the impressive amount of data which have appeared in the literature during the last ten years. Nevertheless the various suggestions made to explain its activity will be presented here. It is generally agreed that guanidine neither inactivates extracellular virions nor interferes with early events of viral infection (Crowther & Melnick, 1961; Ueda et al., 1961; Carp, 1964; Pringle, 1964; Eggers et al., 1965). The inhibition of cellular protein and RNA synthesis in infected cells is not inhibited by guanidine (Baltimore et al., 1963; Bablanian et al., 1965), although it has been demonstrated that guanidine prevents the viral induced inhibition of DNA synthesis in infected HeLa cells (Powers et al., 1969). It was suggested in 1961 by Crowther and Melnick that guanidine might inhibit a hypothetical virus-coded non-virion enzyme necessary for viral replication and two years later the viral RNA polymerase was discovered by Baltimore and Franklin (1963). It was shown shortly after that guanidine affects the development of enzyme

activity in picornavirus infected cells (Eggers et al., 1963; Baltimore et al., 1963). This was a discovery of some importance because it was thought at that time that guanidine led to the formation of defective viral RNA, however since guanidine inhibits development of the RNA polymerase it means that it prevents synthesis of any viral RNA. It should be pointed out here that guanidine does not inhibit polymerase activity when assayed in a cell free system (Baltimore et al., 1963). Consequently it was suggested that guanidine prevented the synthesis of the enzyme. This however has been challenged by more recent experiments as will be discussed below.

Lwoff and co-workers (1962) believed that guanidine acted allosterically upon the monomers of the replicase in such way that it prevented them from aggregating to form the active polymers (Lwoff et al., 1963; Lwoff & Lwoff, 1964; Lwoff, 1965). Very little evidence exists in support of Lwoff's suggestion; the observation that presence of guanidine for a very short time during exponential replication is enough to show its inhibitory effect is the evidence that guanidine acts on the morphogenesis of the enzyme rather than its synthesis. However Baltimore et al. (1963) and Caliguiri et al. (1965) demonstrated that maximum inhibition is obtained only when guanidine is present throughout the growth cycle. This has also been confirmed in an experiment with rhinovirus type 2 in HEL cells.

Caliguiri and Tamm (1968a) found that treatment of infected cells with guanidine resulted in a decrease of the ribonuclease resistant part of the RI. This has been explained by assuming that the target of guanidine is the initiation of synthesis of new RNA strands. Strands already initiated are able to be completed even in the presence of guanidine. At the same time Baltimore (1968) has presented evidence that guanidine blocks the processing of newly synthesized viral RNA from the replication complex. This hypothesis is strengthened by the observation that the RNA which is made in the presence of guanidine is

found in a structure which sediments more rapidly than either viral polyribosomes or replication complex which Baltimore called the guanidon. This hypothesis has however been challenged by further experiments of Caliguiri and Tamm (1968b) who showed that viral RNA molecules, even when guanidine is present, were released from the replication complex and were subsequently incorporated into viral polyribosomes and then into mature virions.

Finally Koschel and Wecker (1971) presented experimental data which support the possibility that guanidine prevents the generation of functional viral polymerase from precursor material. Their assumption was based on Jacobson and Baltimore's model of translation (1968) according to which the viral genome is transcribed in one piece, which is translated so as to form a single polypeptide. Cleavage of this polypeptide occurs while it is still growing to give large precursors proteins which are further cleaved into mature functional proteins. This specific cleavage is believed to be inhibited by guanidine (Koschel & Wecker, 1971).

As discussed above all the available data implicate viral RNA polymerase as the principal target for the antiviral activity of guanidine. However genetic analysis of the poliovirus genome carried out by Cooper's group (Cooper, 1968; McCahon & Cooper, 1970) has shown that the site of guanidine resistance is located in the coat protein region of the genome. This was surprising since the RNA deficient mutants mapped at a different location and one would have expected these and the guanidine marker to map in the same place. To explain these findings one can suggest that polypeptides from the cistrons known to give deficient RNA replication and guanidine markers both contribute to polymerase activity.

Some experiments are described in this section involving the two structurally related compounds, 65-709 and guanidine. Guanidine was used for comparative purposes and evidence is presented to show that the modes of action of the two compounds are not identical.

To establish whether 65-709 and guanidine share the same site of action, rhinovirus guanidine resistant mutants were used. Melnick et al., in 1961, were the first to observe development of resistance to guanidine when they cultivated poliovirus for several passages in the presence of increasing concentrations of guanidine. This was soon after confirmed in several laboratories (Tamm & Eggers, 1962; Loddo et al., 1962; Ledinko, 1963; Veda, 1963; Carp, 1963; Horodniceanu et al., 1963; Pringle, 1964). Rhinovirus resistant mutants were first isolated by Acornley et al., (1968). Guanidine-dependent mutants of poliovirus have also been obtained after repeated passage of resistant virus in the presence of guanidine (Loddo et al., 1963; Ledinko, 1963; Nakano et al., 1963). The fact that resistance and dependence to guanidine is controlled by the virus genome has been established by the following observations:

(a) Resistance to guanidine is retained even after several passages of the virus in the absence of guanidine (Melnick et al., 1961; Ueda et al., 1963; Ledinko, 1963; Horodniceanu et al., 1963, 1964).

(b) The viral progeny from cells infected with the RNA extracted from resistance or dependent mutants retained the appropriate character (Horodniceanu et al., 1964; Loddo et al., 1962; Nakano et al., 1963; Eggers & Tamm, 1963).

(c) After treatment of type 1 and 2 poliovirus with the mutagen hydroxylamine, a statistically significant increase of the proportion of resistant mutants was recorded (Klein & Teodoresku, 1968).

A further aspect of the guanidine mutants that should be considered is the action of the so called antiguanidines which are compounds antagonistic to the action of guanidine (Philipson et al., 1966; Lwoff, 1965; Loddo et al., 1966). Most of these compounds contain a methyl group, an amino group or both. Such compounds as choline, methionine and trimethylamine are antiguanidines. The mechanism by which these compounds antagonize guanidine inhibition is not

known. While the resistance character of guanidine mutants is affected by antiguanidines, dependence is not.

Efforts to isolate mutants to 65-709 by Bucknall were unsuccessful and he showed that antiguanidines do not antagonize the inhibitory effect of 65-709.

PAGE

NUMBERING

AS ORIGINAL

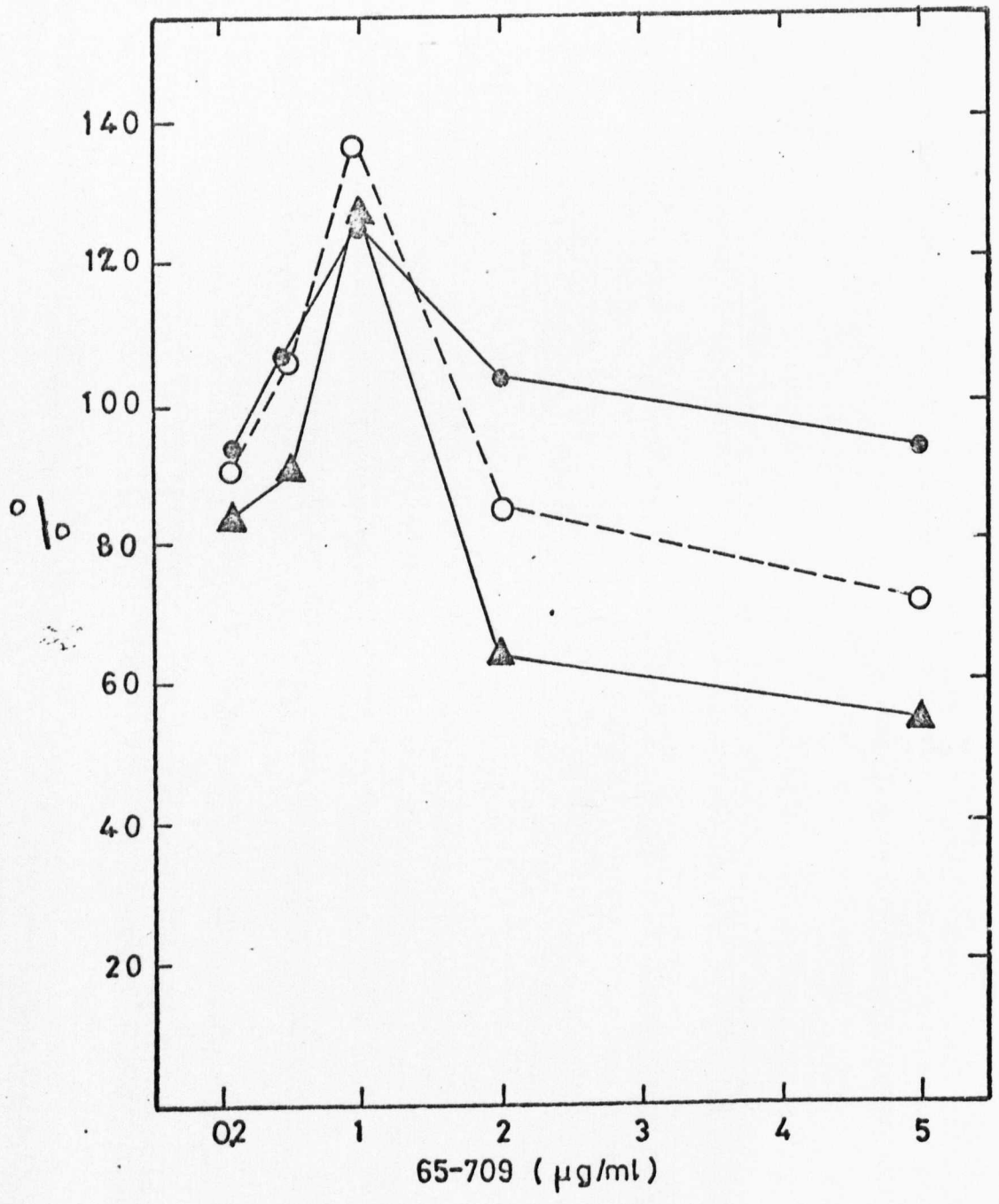
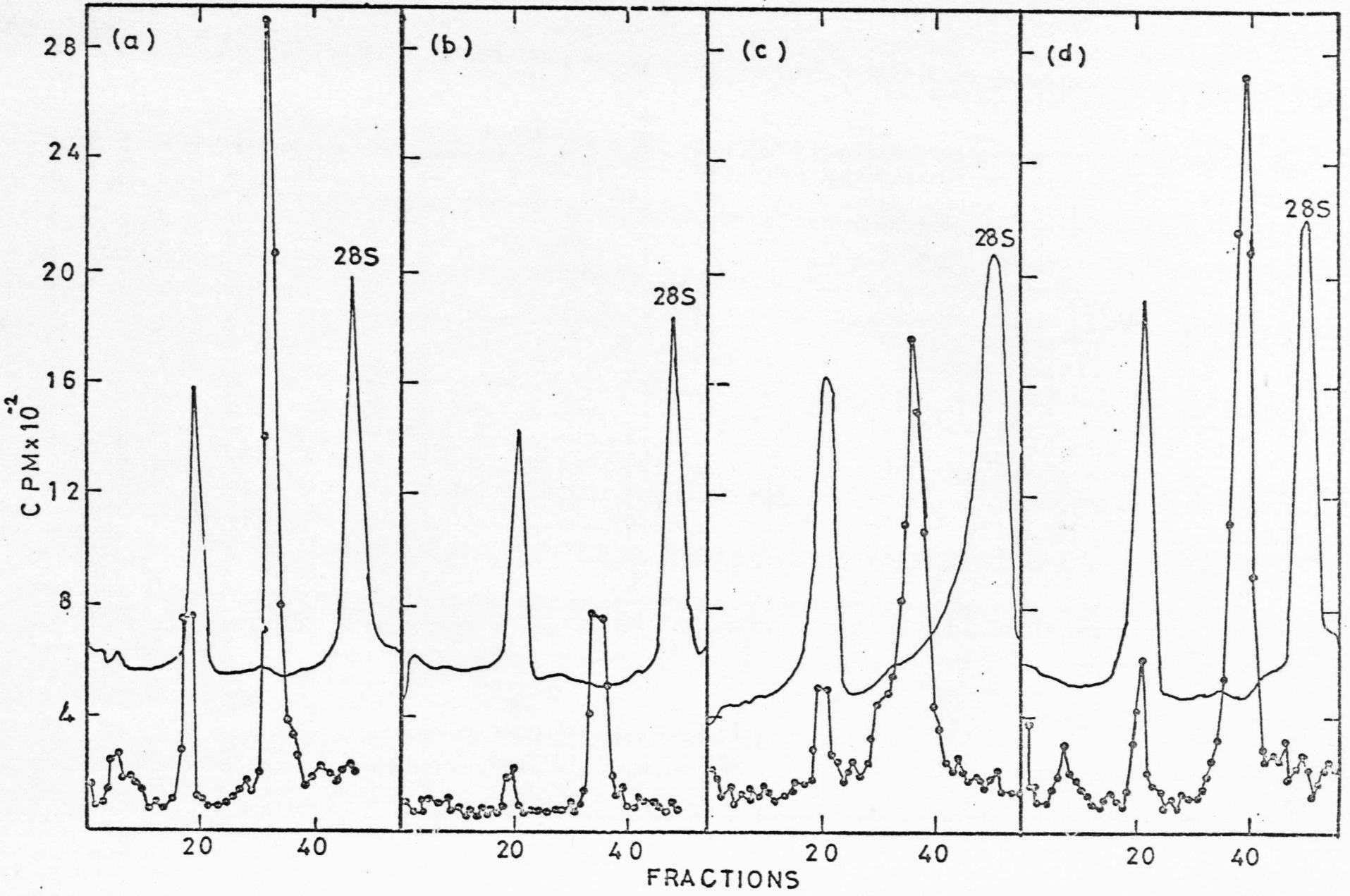


Fig. 26. Effect of 65-709 on RNA, DNA and protein synthesis in uninfected HEL cells. Cells were incubated with 65-709 for 9 h and pulsed for 2 h with radioactive precursors.
(●——● [^{3}H] -uridine; ○——○ [^{14}C] -thymidine; ▲——▲ [^{3}H]-valine).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FRACTIONS

Fig. 27. Inhibition of rhinovirus RNA synthesis by 2 µg / ml 65-709 added for different times during virus multiplication. All cultures were pulsed with [^{3}H]-uridine from 9 to 11 h after infection. The continuous line is the optical density trace at 260 nm indicating the position of 28S ribosomal RNA and DNA extracted together with viral RNA. (a) control, (b) 65-709 added between 1 to 11 h p.i., (c) 65-709 added between 7 to 11 h p.i. and (d) 65-709 added between 7 to 8 h p.i.

Time of exposure to 65-709 (h)	RI	RF	SS	Total
0 to 11	70	80	67	69
7 to 11	85	65	54	59
7 to 8	26	47	24	27

Table 9. Percentage inhibition of the synthesis of rhinovirus RNA by 65-709. Infected cells were treated with 2μg/ml 65-709 as indicated and pulsed with [^{3}H]-uridine from 9 to 11 h. The extracted RNA was electrophoresed and the radioactivity under each peak normalised with respect to optical density markers.

RESULTS

1. Effect of 65-709 on macromolecular synthesis in non-infected HEL cells. The effect of 65-709 on the synthesis of cellular DNA, RNA and protein was determined by incubating HEL cells in the presence of 0 to 5 μg / ml of 65-709 for 9 h at 37°C and then by pulsing with [^{3}H]-uridine or a mixture of [^{14}C]-thymidine and [^{3}H]-valine. Fig. 26 shows the effect of 65-709 on the incorporation of acid-soluble radioactivity during a 2 h pulse. Cellular RNA synthesis was not inhibited at the concentrations used but along with DNA and protein synthesis showed an increased activity at 1.0 μg / ml. DNA synthesis fell by 15% and protein synthesis by 36% at a concentration of 2 μg / ml.

2. Inhibition of viral RNA synthesis by 65-709. HEL cells were infected at 33°C and virus allowed to adsorb for 1 h. Time after infection was measured from the beginning of adsorption. Virus was removed and replaced with warm medium containing 2 μg / ml 65-709. Cells were pulsed with [^{3}H]-uridine for 2 h from 9 to 11 h post infection and the RNA was then extracted and analysed by polyacrylamide electrophoresis (Fig. 27a,b). Radioactivity under each peak of RNA was summed and normalised by reference to the internal markers of ribosomal RNA measured by adsorption at 260 nm. The overall inhibition of synthesis of viral RNA was 69%. Individually, RI fell by 70%, RF by 80% and SS RNA by 67% (Table 9).

RNA synthesis was inhibited by the addition of 65-709 well after viral RNA could be detected in the cell. When 65-709 was added at 7 h post infection and the cells pulsed from 9 to 11 h the overall inhibition was 59% and the RI, RF and SS RNA fell by 85%. 65% and 54% respectively (Table 9).

65-709 added at 1 h also inhibited the viral cytopathic effect which extended to over 80% of the cells by 11 h after infection.

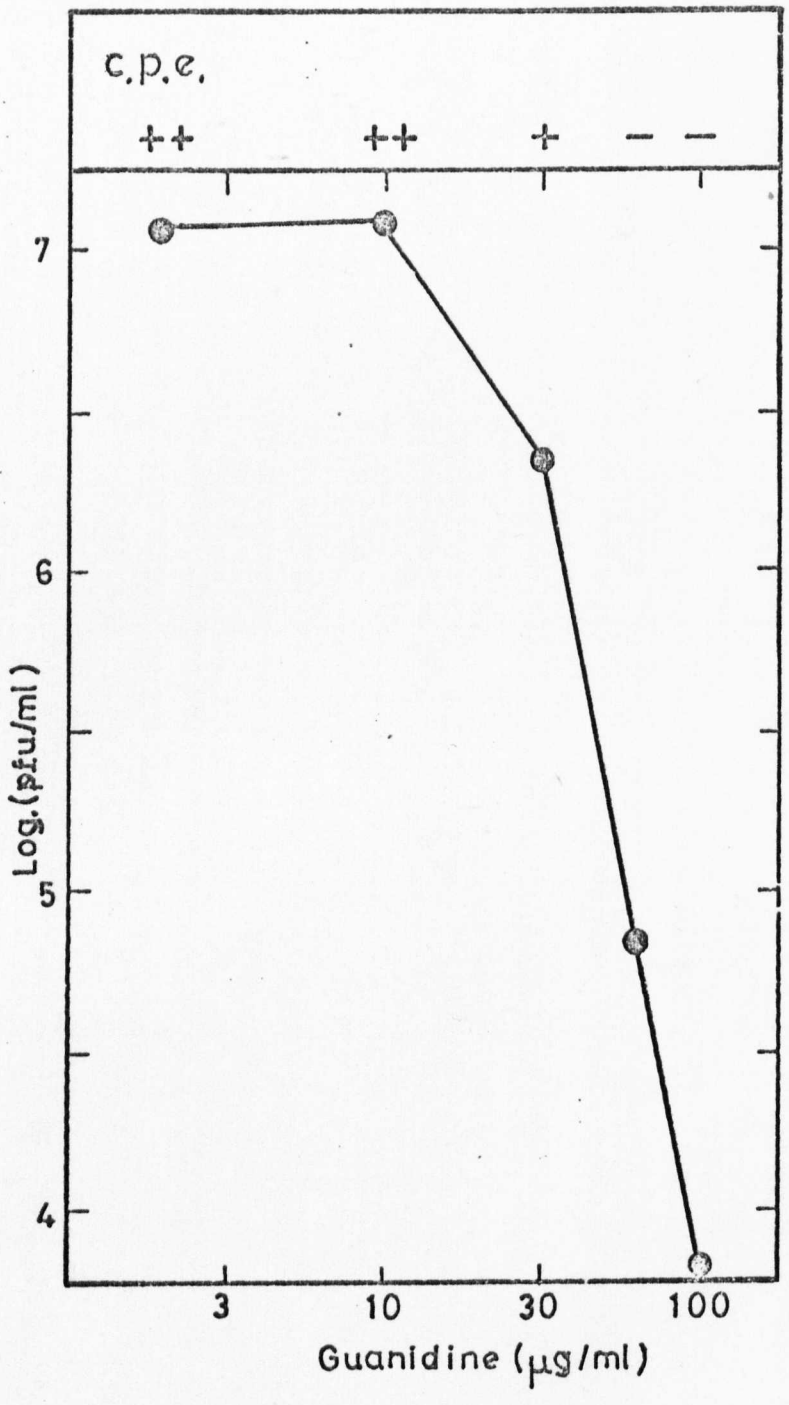


Fig. 28. Inhibition of rhinovirus multiplication by guanidine. The yield of combined intra- and extra-cellular virus is presented. Cytopathic effects at the time of harvest (21 h) is indicated by + (50% of cells affected) or ++ (over 80% of cells affected).

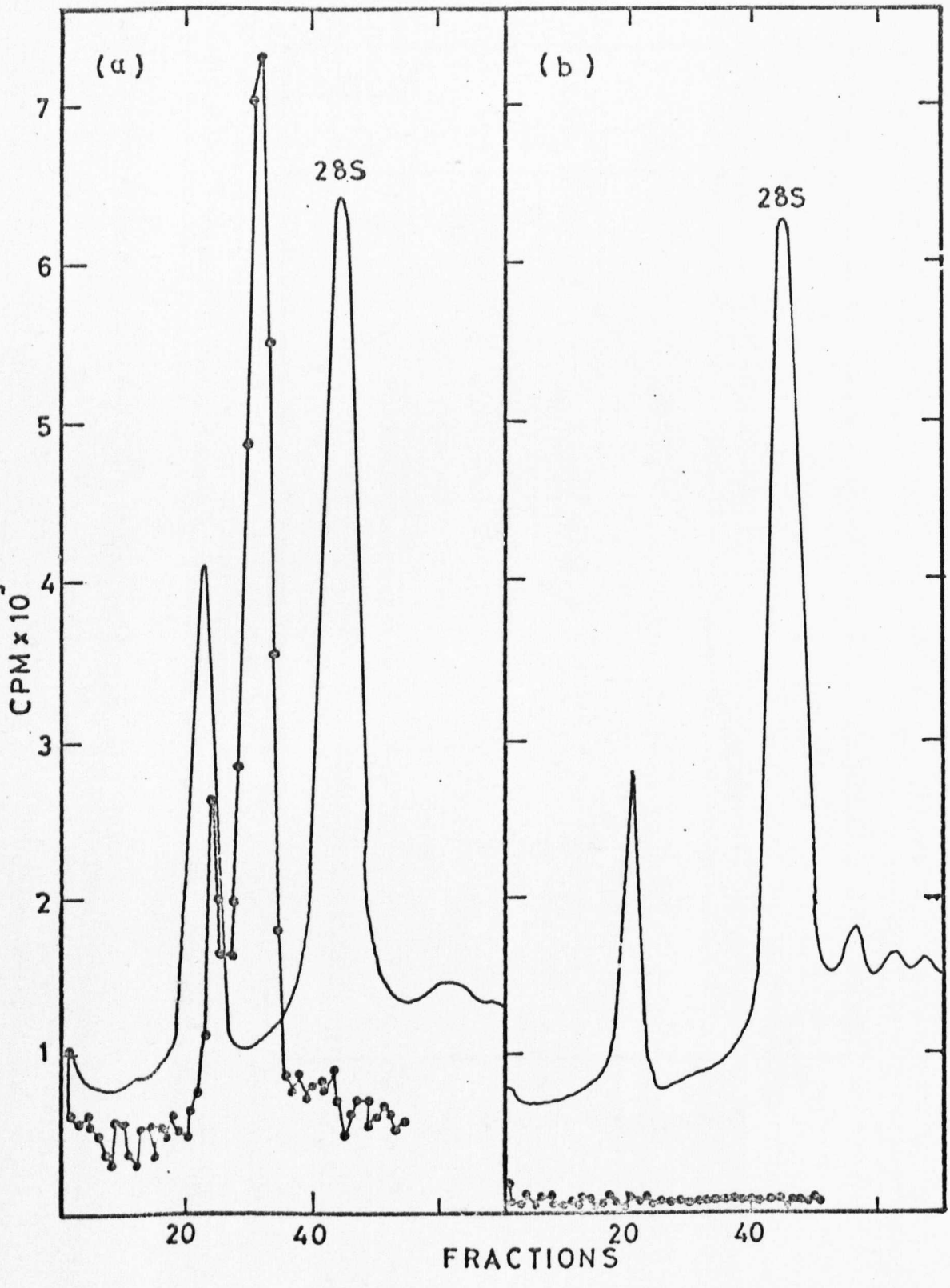


Fig. 29, continued over page

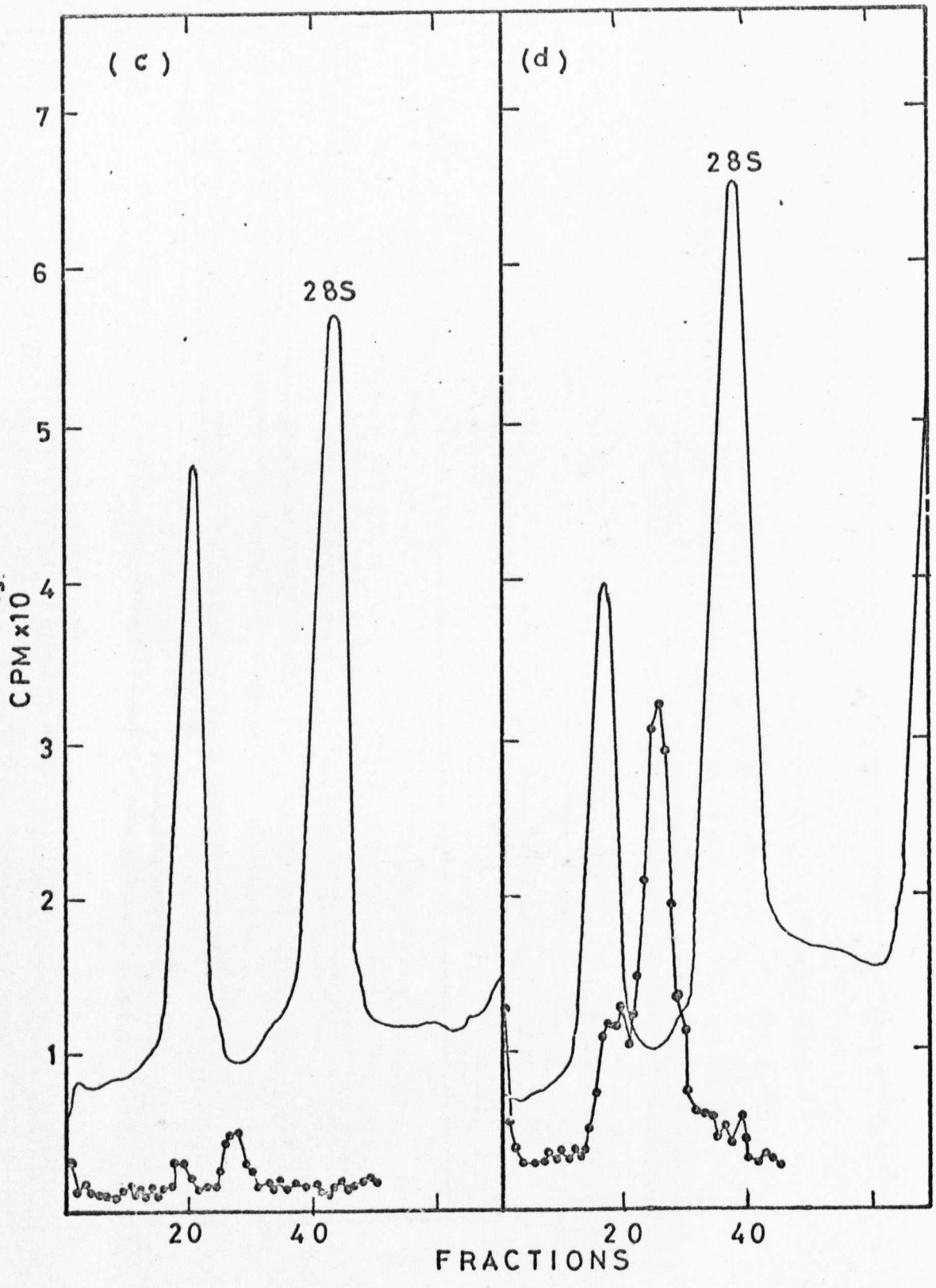


Fig. 29. Inhibition of rhinovirus RNA synthesis by guanidine (100 µg / ml). All cultures were infected and pulsed from 9 to 11 h with [^{3}H]-uridine. The continuous line is the optical density trace at 260 nm. (a) control, (b) guanidine included between 1 to 11 h p.i., (c) guanidine added between 7 to 11 h p.i. and (d) guanidine added between 7 to 8 h p.i.

3. Reversibility of 65-709 inhibition of viral RNA synthesis. Cells were treated with 2 μg / ml 65-709 from 7 to 8 h post infection and then washed twice, re-incubated in fresh medium and pulsed with [^{3}H]-uridine from 9 to 11 h. Inhibition of total viral RNA synthesis was 27%, which was half the value obtained by treatment with 65-709 from 7 to 11 h (Fig. 27c,d and Table 9). A similar recovery of RNA synthesis was also demonstrated when 65-709 was present from 0 to 3.5 h and cells pulsed from 5 to 7 h. Other experiments where an increase in RNA synthesis was evident at 1 h after removal of 65-709 indicated that recovery from inhibition was a rapid process.

4. Inhibition of rhinovirus multiplication by guanidine. Since 65-709 is a substituted guanidine it was important to establish whether or not 65-709 and guanidine had identical modes of action. The first experiments examined some parameters of guanidine inhibition of the multiplication of rhinovirus type 2 in HEL cells.

Rhinovirus was grown in the presence of increasing concentrations of guanidine for 21 h. The combined yield of extra- and intra-cellular virus was titrated by plaque assay. Significant inhibition was observed at a concentration of 33 μg / ml and inhibition increased to over 99.9% at 100 μg / ml (Fig. 28). The latter concentration was used in the experiments on RNA synthesis described below to ensure maximum inhibition.

5. Inhibition of rhinovirus RNA synthesis by guanidine. The extent of inhibition by guanidine added at different times and for varying periods was investigated by pulsing with [^{3}H]-uridine from 9 to 11 h after infection. When guanidine was present from 1 to 11 h RNA synthesis was inhibited completely and when present from 7 to 11 h it was inhibited by 95% (Fig. 29a,b,c).

Inhibition was reversible since it was found that

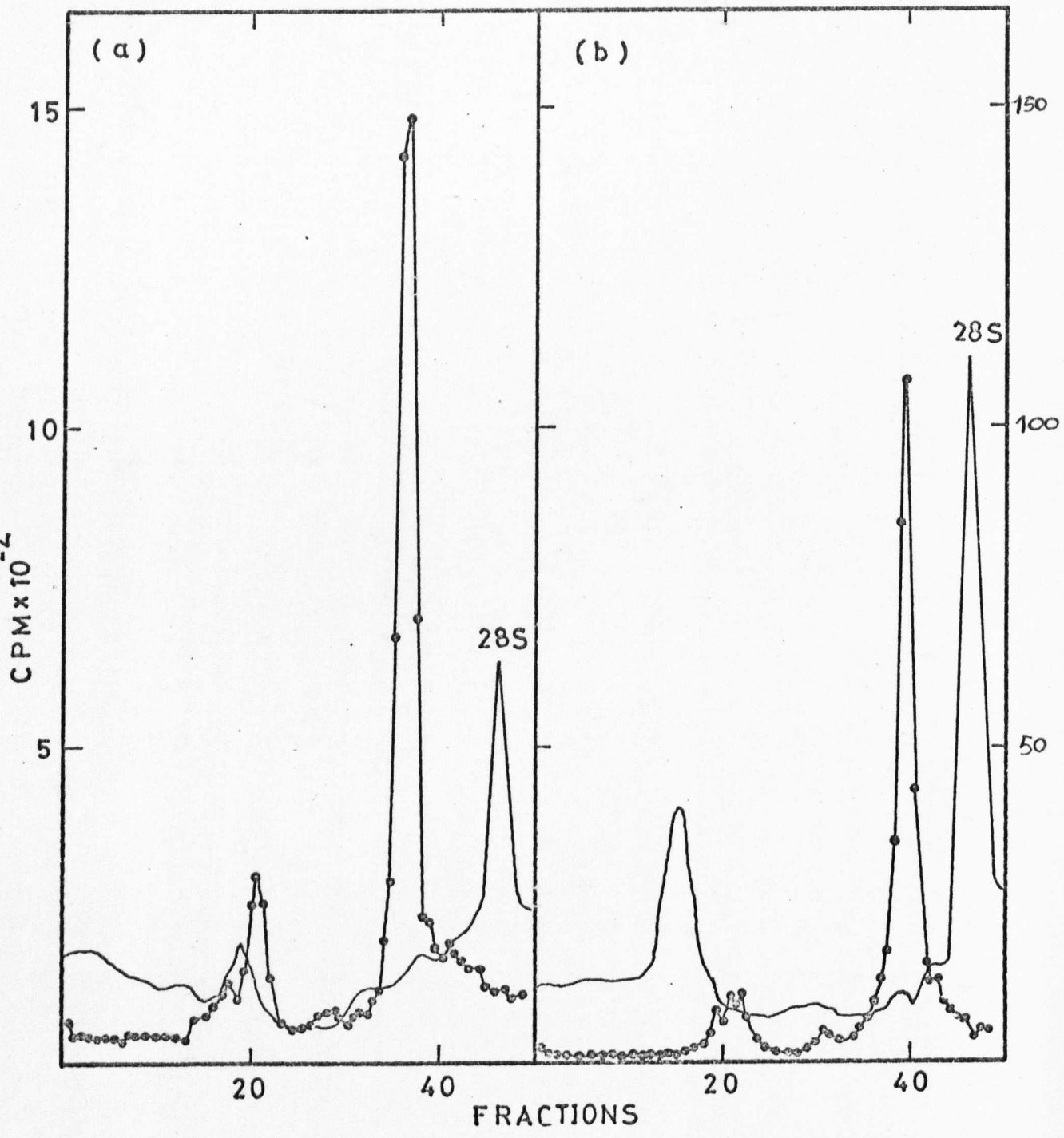


Fig. 30. Reversal of guanidine inhibition of rhinovirus RNA synthesis. Cells were pulsed from 9 to 11 h with [^{3}H]-uridine. The continuous line is the optical density trace at 260 nm. (a) control, (b) guanidine included in the medium between 0 to 3.25 h p.i.

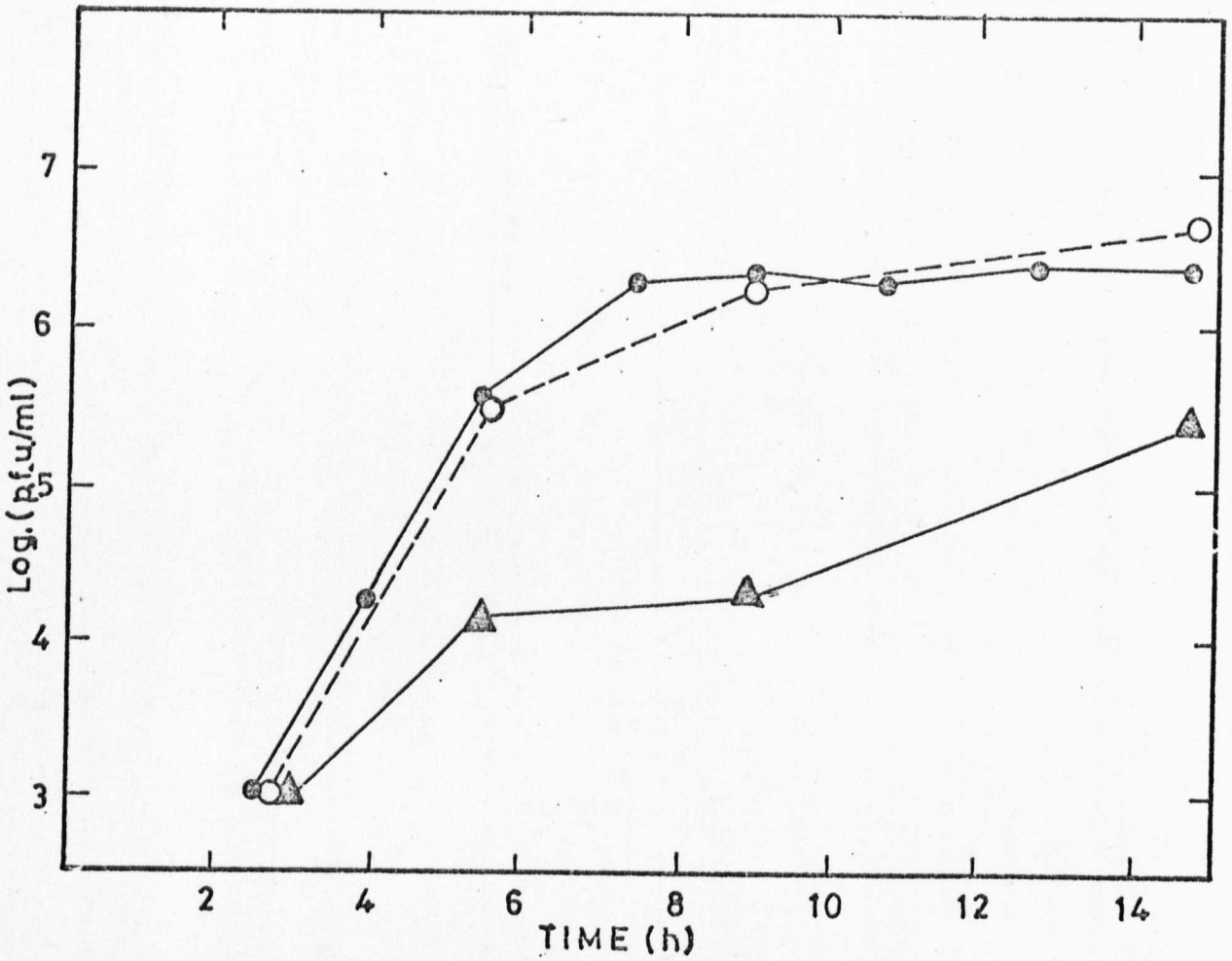


Fig. 31. Effect of 65-709 and guanidine on the multiplication of bovine enterovirus in HEL cells. Both 65-709 and guanidine were present continuously from 0 h. (●——●) control, (○——○) in the presence of 65-709, (▲——▲) in the presence of guanidine.

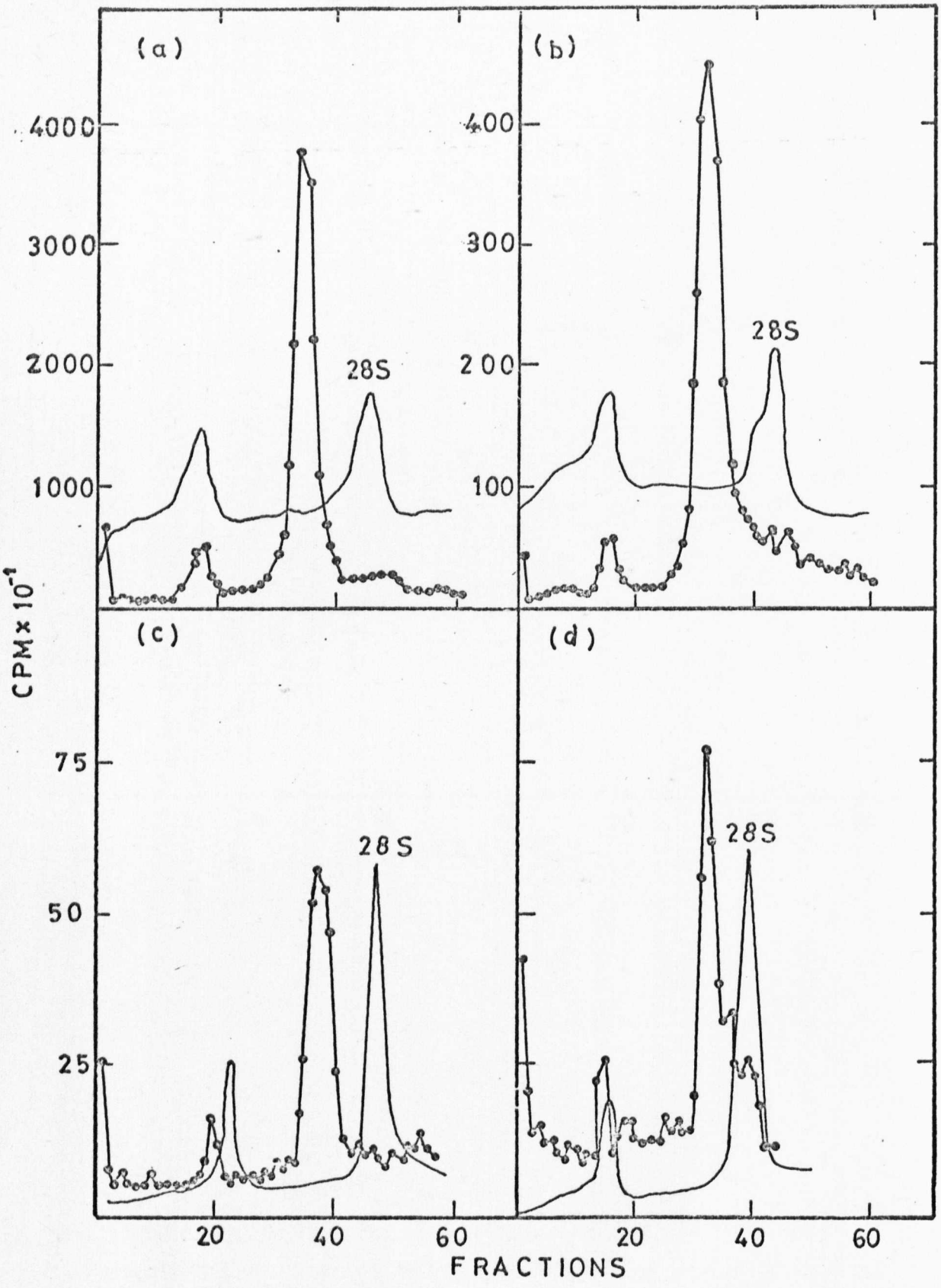


Fig. 32. Effect of 65-709 on poliovirus and bovine enterovirus RNA synthesis. 1 μg / ml 65-709 was present continuously in the treated cultures from 0 h and all cultures were pulsed from 9 to 11 h with $[^3H]$-uridine. The continuous line is the optical density trace at 260 nm. (a) control poliovirus RNA, (b) Poliovirus RNA in the presence of 65-709, (c) control bovine enterovirus, (d) Bovine enterovirus RNA in the presence of 65-709.

after a pulse of guanidine from 7 to 8 h, RNA synthesis was 49% of the control (Fig. 29d). Provided that sufficient time was allowed following reversal of inhibition, complete recovery of RNA synthesis was observed even when guanidine was added at the time of infection. Fig. 30 shows that after a pulse of guanidine from 0 to 3.25 h, normalised RNA synthesis measured at 9 to 11 h was 1.6-fold greater than the control.

6. Inhibition of bovine enterovirus and poliovirus multiplication by 65-709 or by guanidine. Bucknall et al., (personal communication) have shown that 65-709 inhibited the multiplication of a wide range of viruses in HEL cells, although the greatest activity was against rhinoviruses. We have extended these results by investigating the extent of inhibition of two other picornaviruses, bovine enterovirus and poliovirus by 65-709 or guanidine.

(a) Cytopathic effects

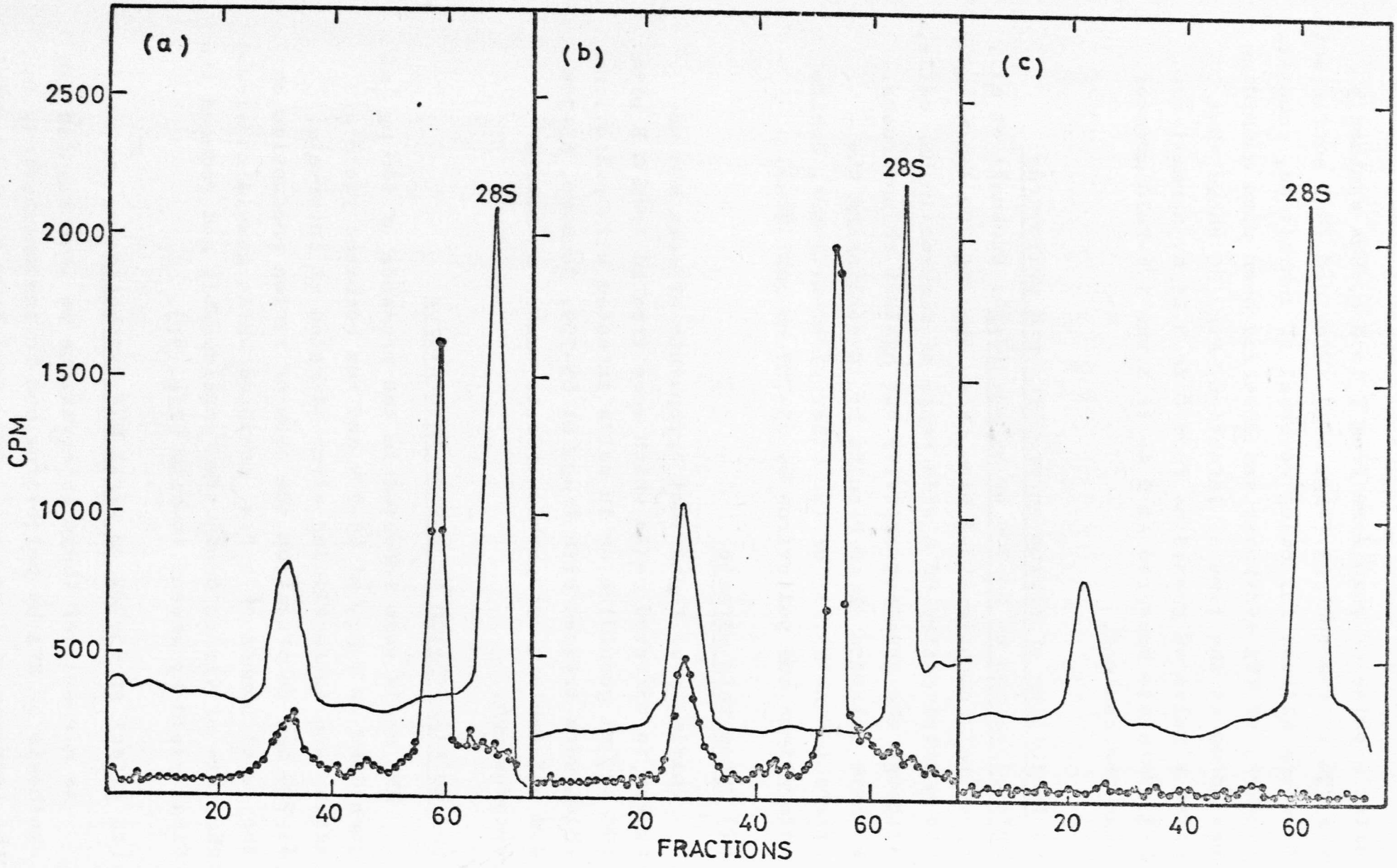
Initially we found that cytopathic effects did not appear in infected cells which were treated from 0 h with 100 μg / ml guanidine or in cells infected with poliovirus which were treated with 1 μg / ml 65-709. However, 65-709 did not inhibit cytopathic effects caused by bovine enterovirus.

(b) Multiplication of bovine enterovirus

HEL cells were infected in the presence of 100 μg / ml guanidine or 1 μg / ml 65-709 and the combined yield of extra- and intracellular virus titrated at intervals. 65-709 had no effect on the rate of virion production or the total number of p.f.u. produced while guanidine slowed the rate of virion production considerably and reduced the final yield by around 10-fold (Fig. 31).

(c) Effect of 65-709 on viral RNA synthesis

As a result of these observations we investigated the synthesis of RNA by poliovirus and bovine enterovirus in the presence of 1 μg / ml 65-709 added from 0 h (Fig. 32).


(a)
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FRACTIONS

Fig. 33. Effect of guanidine and 65-709 on the RNA synthesis by a guanidine resistant rhinovirus mutant. Guanidine (100 µg / ml) and 65-709 (2 µg / ml) were present from 0 h and cells were pulsed with [^{3}H]-uridine from 9 to 11 h. The continuous line is the optical density trace at 260 nm. (a) control, (b) viral RNA in the presence of guanidine (c) viral RNA in the presence of 65-709.

We found that poliovirus RNA synthesis was reduced by 86% and that bovine enterovirus was unaffected by the presence of 65-709.

7. Effect of 65-709 on RNA synthesis by a guanidine-resistant rhinovirus mutant

The resistance of bovine enterovirus to 65-709 but not to guanidine indicated that the modes of action of the two compounds were not identical. However, since rhinovirus replication was inhibited by both compounds we selected a mutant which was resistant to guanidine to see if resistarce to 65-709 was acquired simultaneously.

RNA synthesis by the guanidine-resistant rhinovirus was measured in the presence of 100 μg / ml guanidine and 2 μg / ml 65-709 (Fig. 33). The result was unequivocal: guanidine had no effect while 65-709 inhibited RNA synthesis. We concluded that the modes of action of guanidine and 65-709 in inhibiting RNA synthesis of rhinovirus were not identical.

DISCUSSION

The substituted guanidine compound 65-709 specifically inhibited the replication of rhinovirus type 2 RNA, the production of virions and cytopathic effects in HEL cells. RNA replication could be inhibited at any time during virus multiplication and the effect of the drug was reversed by removing it from the culture medium. In these respects the anti-viral activity of 65-709 and guanidine itself are similar.

The maximum inhibition of virus RNA synthesis by 65-709 varied between experiments from 72% (Fig. 27) to over 90% (Fig. 33) and had a mean value over a large number of experiments of 85%. The extent of inhibition of virion formation under single-step growth conditions was comparable with inhibition of RNA synthesis. Under the same conditions guanidine was the more effective inhibitor, no RNA synthesis being detected and virion formation being reduced by over 99.9%. However, since 30 μg / ml guanidine were required to inhibit virion formation to the same extent as 2 μg / ml 65-709, the latter was considerably more effective on a weight basis. On a molar basis, 65-709 (mol. wt. 571.5) was 90-fold more effective than guanidine (mol. wt. 95.5). Despite its relatively small inhibitory effect, 65-709 was able to prevent the appearance of gross cytopathic changes in cells when added at the time of infection, even though over 80% of cells in non-treated cultures were degenerating.

A comparison of the normalised radioactivity incorporated into single-stranded RNA, replicative form and replicative intermediate, indicated that 65-709 inhibited all RNA species equally. The same result was obtained when the drug was added at different times during replication and with different lengths of treatment. Guanidine inhibited RNA synthesis completely with the relatively long treatment of our experimental conditions. However, it would have been of interest to repeat with

65-709, Baltimore's (1969) finding that inhibition by guanidine was initially selective and that RF synthesis was spared. While we do not have this information, the continued synthesis in the presence of 65-709 of appreciable amounts of all three RNA species indicated that 65-709 did not selectivcly inhibit RNA synthesis.

Since 65-709 and guanidine both inhibited virus RNA synthesis, it was important to determine whether or not their modes of action were identical. This was achieved by selecting a rhinovirus mutant which was resistant to guanidine and finding that the mutant had acquired no resistance to 65-709. This result was corroborated by the failure of 65-709 to support the growth of a poliovirus guanidine-dependent mutant (Dimmock, N. J., unpublished results), by the failure to obtain rhinovirus mutants resistant to or dependent on 65-709 (Bucknall, R. A., unpublished results) and by the absence of inhibition of bovine enterovirus RNA synthesis and virion production in HEL cells under conditions where guanidine did cause inhibition.

RESULTS PART III

"Rhinovirus induced polymerase activity.
The effect of ICI 65,709 on polymerase activity."

INTRODUCTION

The first RNA-dependent RNA polymerase to be detected was induced by mengovirus in mammalian cells (Baltimore & Franklin, 1962). The evidence which shows that this is the enzyme responsible for viral RNA replication comes from the facts that it is found only in infected cells, its activity is not inhibited by AMD and the RNA which is synthesized in vitro has physicochemical properties identical to those of the virus specified RNA's obtained in vivo.

An RNA-dependent polymerase has been purified by several investigators working with bacteria infected with RNA phages (Haruna & Spiegelman, 1965; August & Eoyang, 1967; Feix et al., 1968; Kondo et al., 1970). The purified polymerase has been used to analyse RNA replication in vitro. The template requirement is one of the most striking properties of the purified enzyme. It will only transcribe single stranded RNA from the parental virus (Haruna & Spiegelman, 1965). Not even the RNA of a closely related but serologically distinct virus can be transcribed. It can use both plus or minus strands but has a greater affinity for minus strands. Infectious RNA is synthesized when non-infectious minus RNA strands are used as template (Feix et al., 1968).

The subunit structure of the Qβ replicase has been elucidated by Kondo et al. (1970) and Kamen (1970). Working independently they both found that purified Qβ replicase consisted of four polypeptides α, β, γ and δ of molecular weight 70,000, 65,000, 45,000 and 35,000 daltons respectively. The Qβ RNA has been estimated to code for 150,000 daltons of protein. The MW of the virion structural proteins is 90,000 daltons, leaving a spare coding capacity for a protein of 60,000 daltons. Since the total MW of the four replicase subunits is much higher than 60,000, the investigators predicted and soon afterwards confirmed that some of the replicase poly-

peptides are of host origin. In fact both Kondo et al. (1970) and Kamen (1970) showed that only polypeptide β is virus specified, while polypeptides α, γ and δ are present in non-infected cells and are host cell specific proteins. The host cell components appear essential for polymerase activity since Kamen (1970) found that neither complexes of α and β or γ and δ were active. However activity was restored by mixing the two inactive components. In addition the host components are not found associated together in uninfected cells.

It has been proved very difficult to purify RNA polymerases from eukaryotic cells infected with animal viruses. This is probably due to the relatively low amount of the enzyme activity in these systems and particularly to the fact that the replicase is firmly bound to smooth cytoplasmic membranes. Lack of an enzyme preparation capable of using a synthetic polyribonucleotide template has also hindered work on purification of the enzyme. Recently Rosenberg et al. (1972) reported the isolation of α poly C - dependent RNA polymerase from BHK 21 cells infected with EMC virus. Treatment with 20 mM dithiothreitol and 0.05% triton X-100 separated the membrane material from the replication complex. At this stage the template RNA became sensitive to the action of micrococcal ribonuclease which freed the replicase from its template while permitting the synthesis of poly G upon addition of poly C template. SDS polyacrylamide gel electrophoresis of the enzyme revealed five polypeptides of MW 72,000, 65,000, 57,000, 45,000 and 35,000. Four of the polypeptides seem to have identical molecular weights to the Qβ replicase polypeptides. At this stage it is not known whether all five polypeptides are true subunits of the enzyme.

Yin and Knight (1972) have detected polymerase activity from HeLa cells infected with rhinovirus type 2. The fractionation procedure described by these investigators has been followed to obtain the enzyme fraction used in our studies. The enzyme assay described in the same report

has been used for polymerase activity assays.

As it has been mentioned in the introduction of "Results Part II", the various hypotheses to explain the mode of action of guanidine involve inhibition of the RNA-dependent RNA polymerase activity. This, together with the fact that viral RNA synthesis is effected by 65-709 (Results Part II) led us to the experiments described in this section. A sensitive assay to study the products of the replication process in vitro was first established and the effect of 65-709 on RNA-dependent RNA polymerase in vivo and in vitro was then investigated.

Reaction mixture	Source of enzyme fraction	CPM/mg protein/h at 33°C
complete	-	8,120
complete	Non infected cells	42,360
-ATP,-CTP,-UTP	Infected cells	92,960
$-Mg^{++}$	"	85,400
completed but incubated at 0°C	"	78,160
complete	"	195,680

Table 10. Rhinovirus induced polymerase assay.

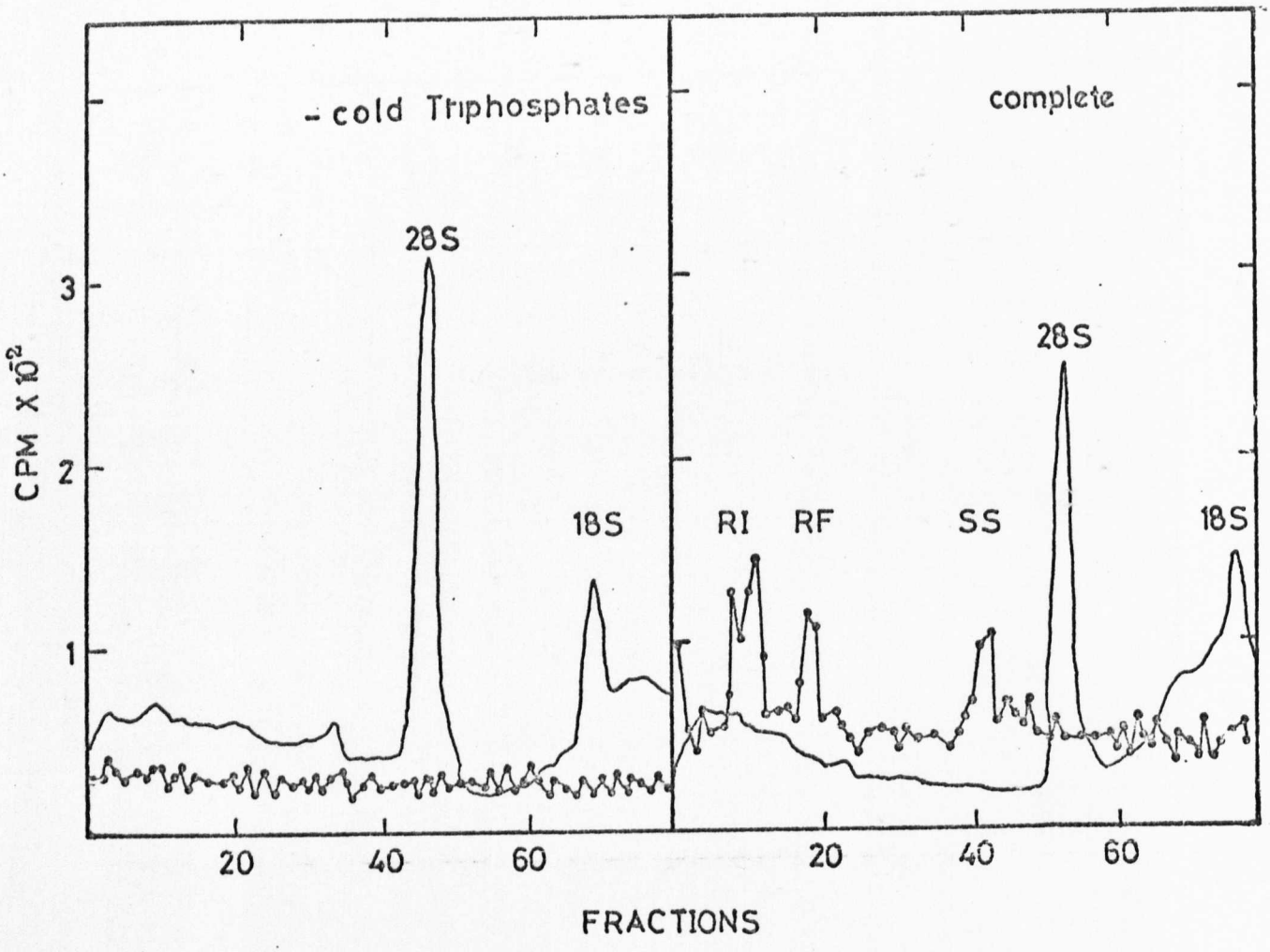

Fig. 34. Electrophoresis of the polymerase products. The polymerase fraction extracted from infected HEL cells was assayed with the complete polymerase assay mixture and an assay mixture from which the 3 cold triphosphates were omitted. The RNA was then extracted and analysed by polyacrylamide gel electrophoresis. The continuous line is the OD_{260} trace indicating the position of 28S and 18S ribosomal RNA.

RESULTS

A. Studies on rhinovirus induced RNA polymerase.

Some experiments are described in this section which establish a sensitive assay for the effect of 65-709 on the rhinovirus induced RNA polymerase.

1. Polymerase activity as measured by TCA-precipitable radioactivity.

Yin and Knight (1972) have reported that rhinovirus-induced RNA-polymerase activity obtained from infected HeLa cells is dependent on the presence of both the four nucleoside triphosphates and magnesium ions. The Yin and Knight fractionation procedure was applied to an extract of HEL cells infected with rhinovirus type 2 and the enzyme fraction was then assayed using the assay mixture described by the same investigators (Table 10). There was considerable incorporation of [^{3}H]-GTP with the complete assay mixture but controls reached nearly 50% of this value. Various methods were applied in order to wash the radioactivity from the controls without much success. Infected HeLa cells gave similar results.

2. Analysis of polymerase products

The question was asked whether any of the radio-activity which was precipitated by TCA was specifically incorporated into rhinovirus RNA.

An enzyme preparation was split into two portions, one which was assayed with a complete polymerase assay mixture, the other with an assay mixture from which the three cold triphosphates were missing. The RNA from these preparations was extracted with phenol and was analysed by polyacrylamide gel electrophoresis. The results in Fig. 34 show that three species of RNA could be distinguished from the complete assay mixture. However, no radioactivity above background was present when the three cold triphosphates were omitted. We concluded that the

control TCA-precipitable radioactivity was not incorporated into virus-induced RNA and is presumably adsorbed non-specifically to cellular material or incorporated in low molecular weight RNA.

3. Comparison of RNA's synthesized in vivo and in vitro.

The RNA species synthesized in vitro were identified by co-electrophoresis with RNA induced in vivo. The RNA preparations were labelled in vivo with $[^{14}C]$-uridine and in vitro with $[^{3}H]$-GTP. These were mixed and electrophoresed on polyacrylamide gel. Fig. 35 shows an absolute coincidence of the RI, RF and SS RNA prepared in vivo with the 3 RNA peaks obtained in vitro. The amount of SS RNA obtained in vitro is very small in relation to that obtained in vivo; this will be considered during the discussion of this section.

4. Time course of polymerase activity.

The RNA-dependent RNA polymerase activity induced in HEL cells after rhinovirus infection was assayed at various times after infection and the products of the in vitro assay were analysed by polyacrylamide gel electrophoresis (Fig. 36). The number of counts under the 3 peaks of RNA were calculated and normalised with respect to the 28S ribosomal RNA present in each preparation (Table 11). The total radioactivity in the peaks was normalised and plotted against time after infection (Fig. 37). Polymerase activity was first detected at 6 h after infection. This was also the earliest time that RNA synthesized in vivo was detected (see Chapter I). Total polymerase activity increased until 9 h p.i, and then appeared to decrease.

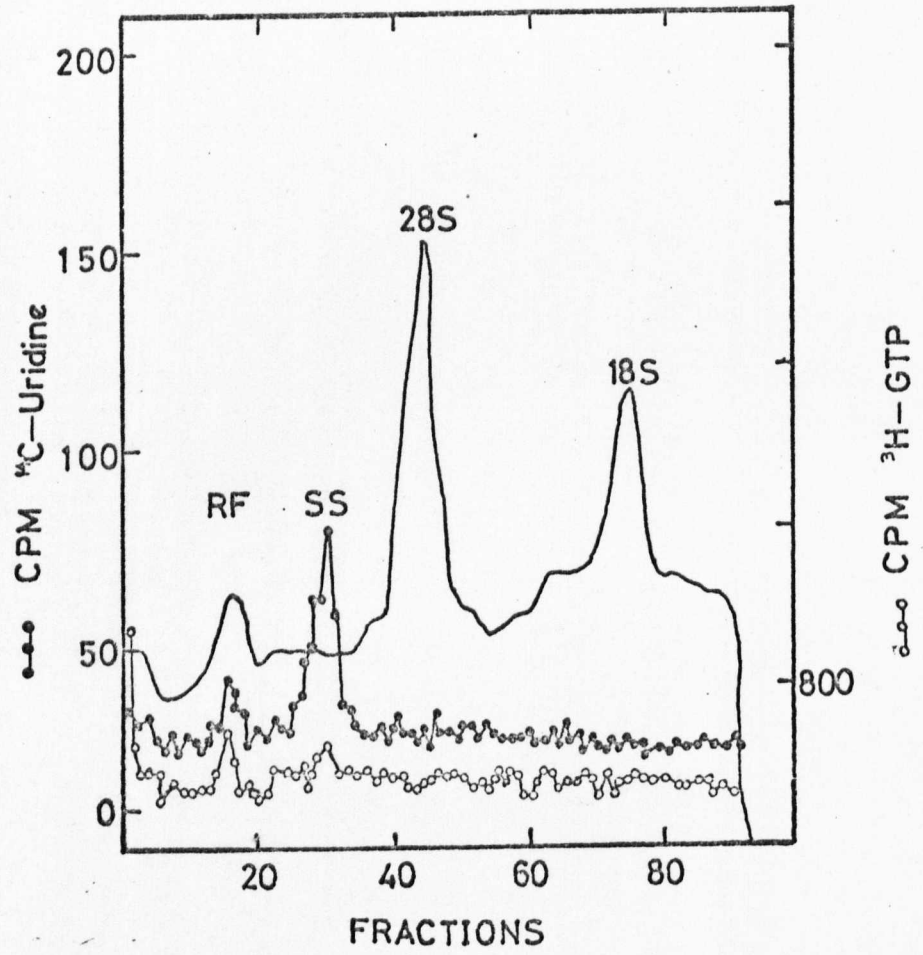

Fig. 35. Co-electrophoresis of RNA prepared in vivo (●——●), and in vitro (○——○). The continuous line is the OD_{260} trace indicating the position of the ribosomal RNA.

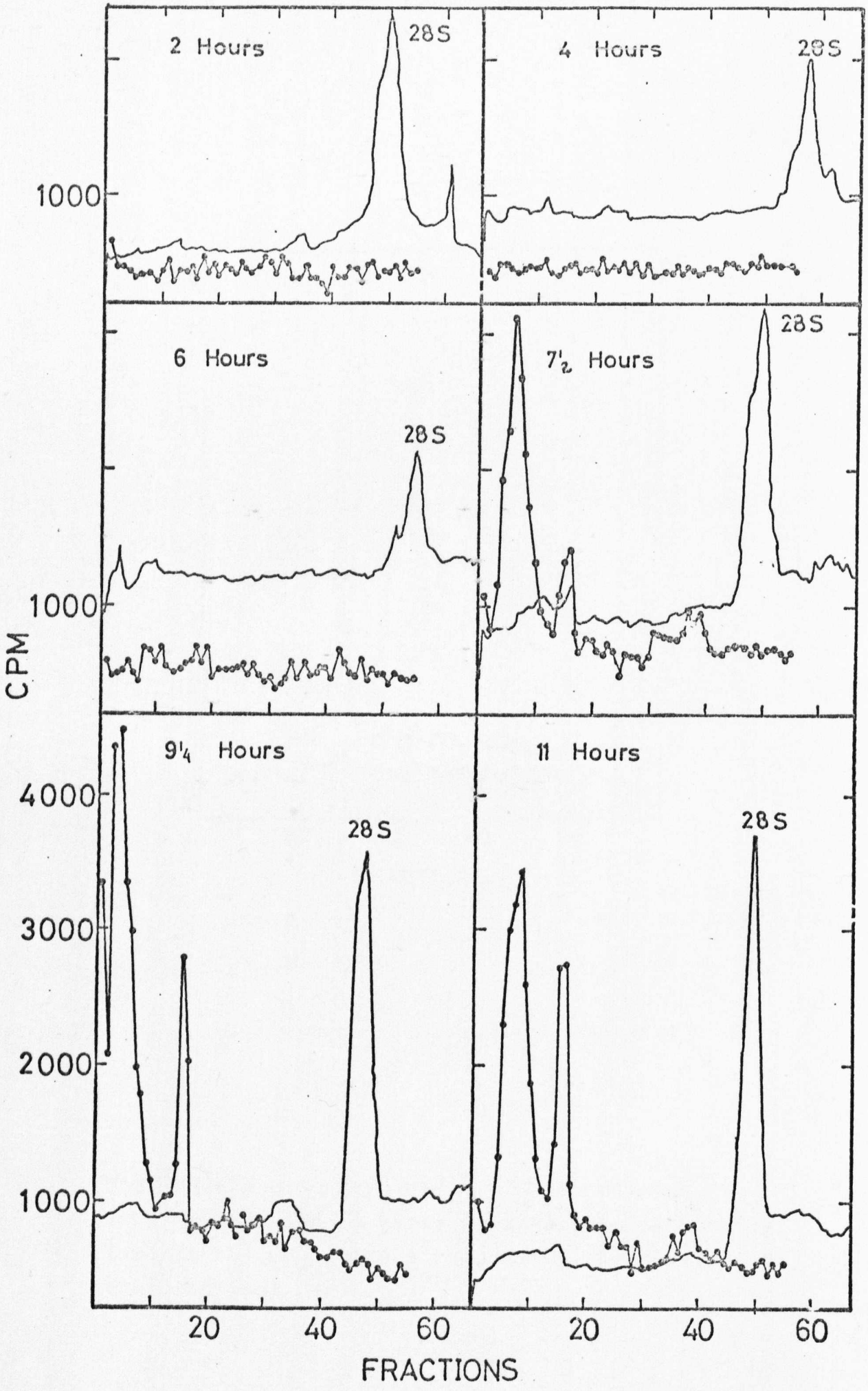
2 Hours
28S
4 Hours
28S
6 Hours
28S
7½ Hours
28S
9¼ Hours
28S
11 Hours
28S
1000
1000
4000
3000
2000
1000
CPM
20
40
60
20
40
60
FRACTIONS

Fig. 36. Electrophoresis of the polymerase products. Polymerase fraction was extracted at the indicated times after infection and was assayed with the complete polymerase assay mixture. The products of the polymerase are shown as analysed by polyacrylamide gel electrophoresis. The continuous line is the OD_{260} trace indicating the position of the 28S ribosomal RNA.

Time after infection (h)	CPM	%
2	88	5.4
4	211	13.0
6	820	50.4
7.5	1,009	62.1
9.25	1,624	100.0
11	1,429	88.0

Table 11. Rhinovirus induced polymerase activity at different times after infection as estimated from the products of the enzymic reaction. Data obtained from Fig. 36.

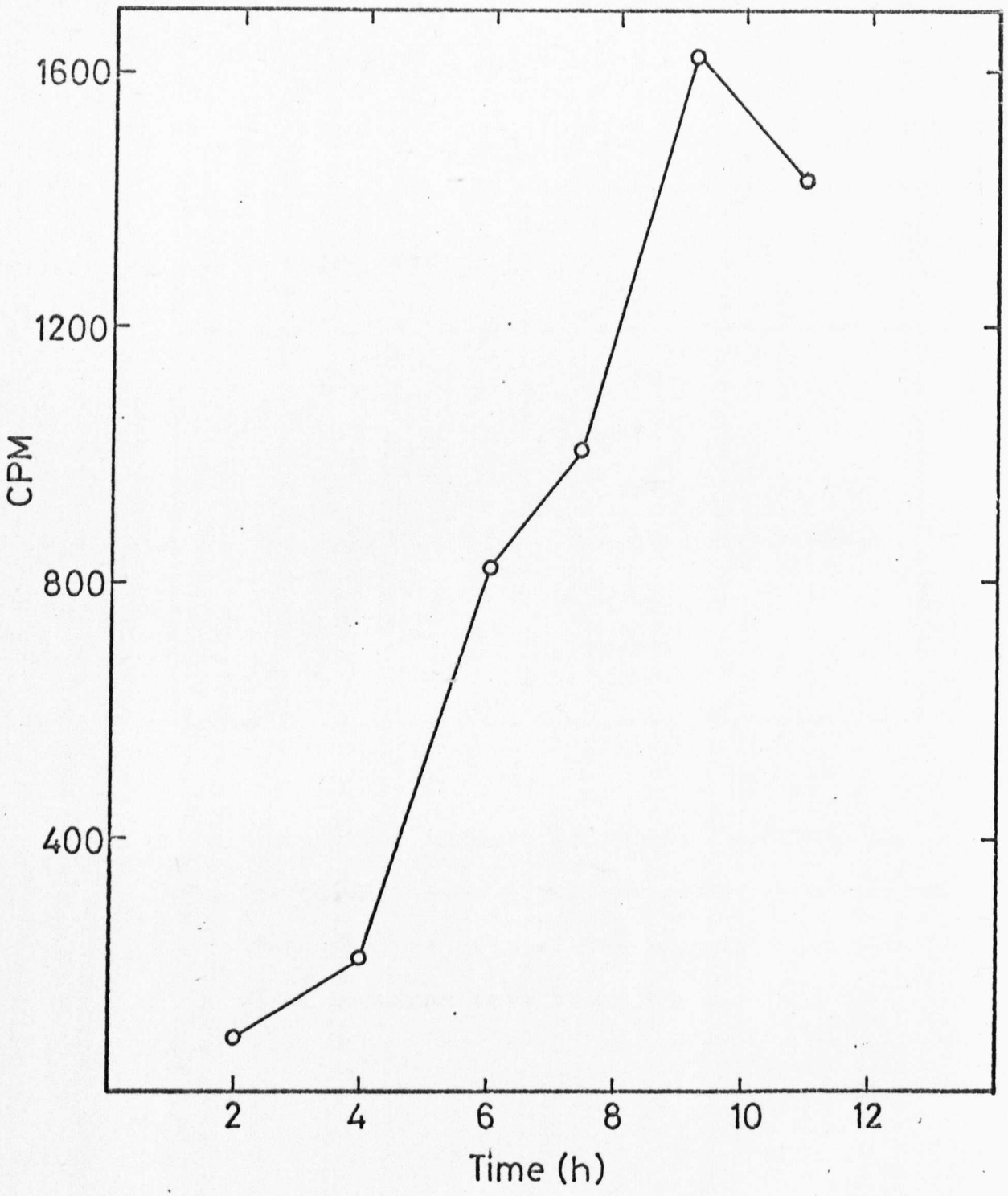

Fig. 37. Rhinovirus induced polymerase activity as a function of the time after infection. Data from Fig. 36.

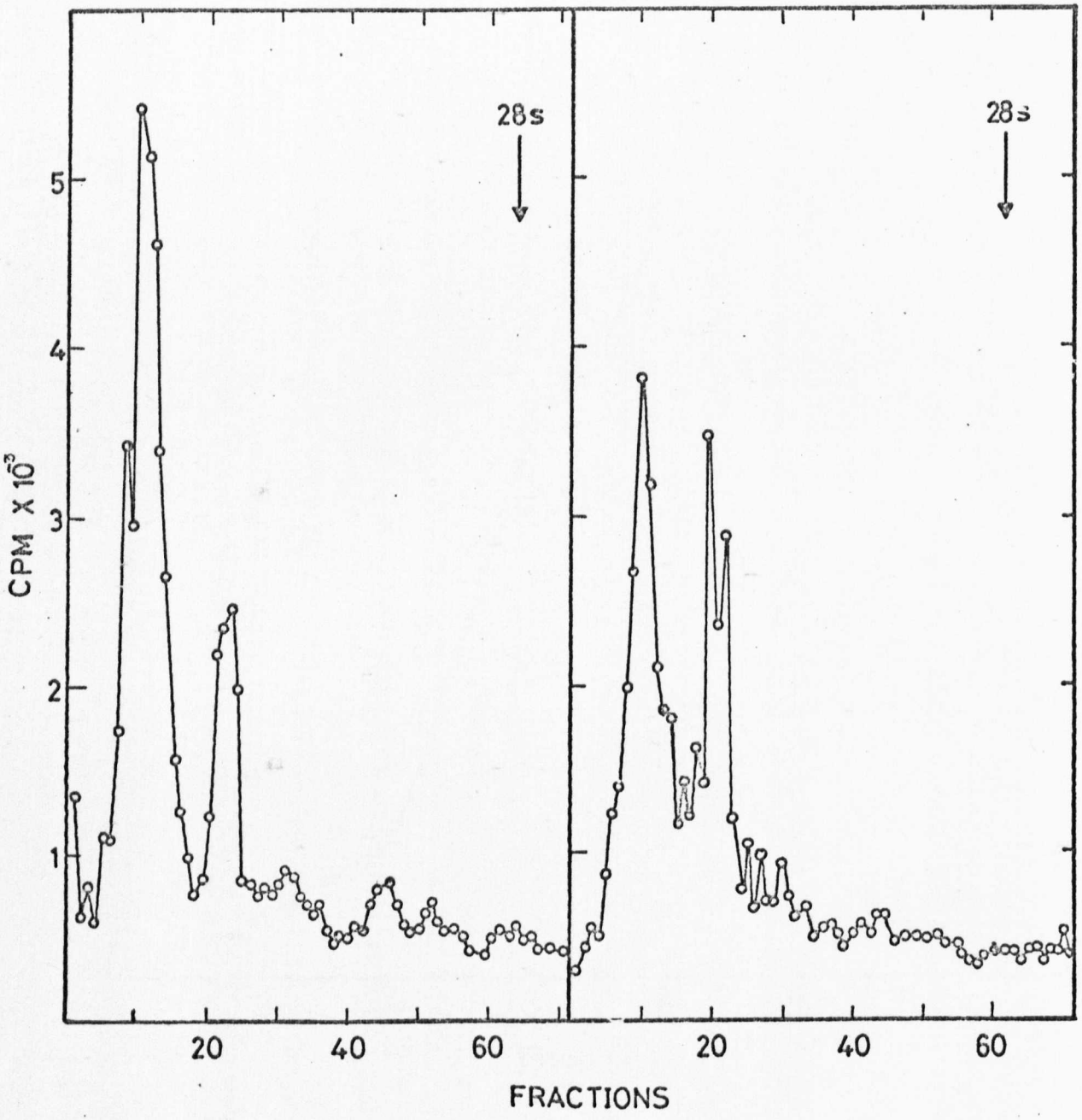

Fig. 38. Effect of 65-709 on polymerase activity in vitro. 10 µg / ml of 65-709 were included in the polymerase assay mixture. The products of the enzymic reaction are shown as analysed by polyacrylamide gel electrophoresis, (a) control (b) assayed in the presence of 65-709.

B. Effect of 65-709 on rhinovirus induced polymerase.

We have shown in 'Results Part II' that the antiviral compound 65-709 inhibited the replication of viral RNA in vivo. We also established an in vitro system for the study of RNA polymerase activity. In a continuing study to determine the mode of action of 65-709 we investigated the effect of 65-709 on polymerase activity in the following experiments.

1. Effect of 65-709 and guanidine on the polymerase activity in vitro.

Baltimore et al. (1963) reported that the activity of the polymerase induced by poliovirus in HeLa cells was not inhibited when it was assayed in the presence of guanidine.

The effect of 65-709 and guanidine on the polymerase induced by rhinovirus in HEL cells was first tested by measuring the TCA-precipitable counts after incubation of the enzyme with the usual assay mixture in the presence of either 65-709 or guanidine. Neither of the two compounds, even when used at concentrations 10 times greater than the inhibitory concentrations required in vivo, had the slightest effect on the activity of the enzyme. In order to confirm this result the products of the in vitro assay in the presence or absence of 65-709 were analysed (Fig. 38). No differences were detected.

2. Effect of 65-709 and guanidine on polymerase activity in vivo.

It has been shown in the previous experiment that neither 65-709 or guanidine affected the polymerase activity when the compounds were included in the assay mixture. Since both compounds inhibited the viral RNA synthesis in vivo (see Results Part II) their affect on the RNA-dependent RNA polymerase was tested in vivo.

Both compounds were added to infected HEL cells at

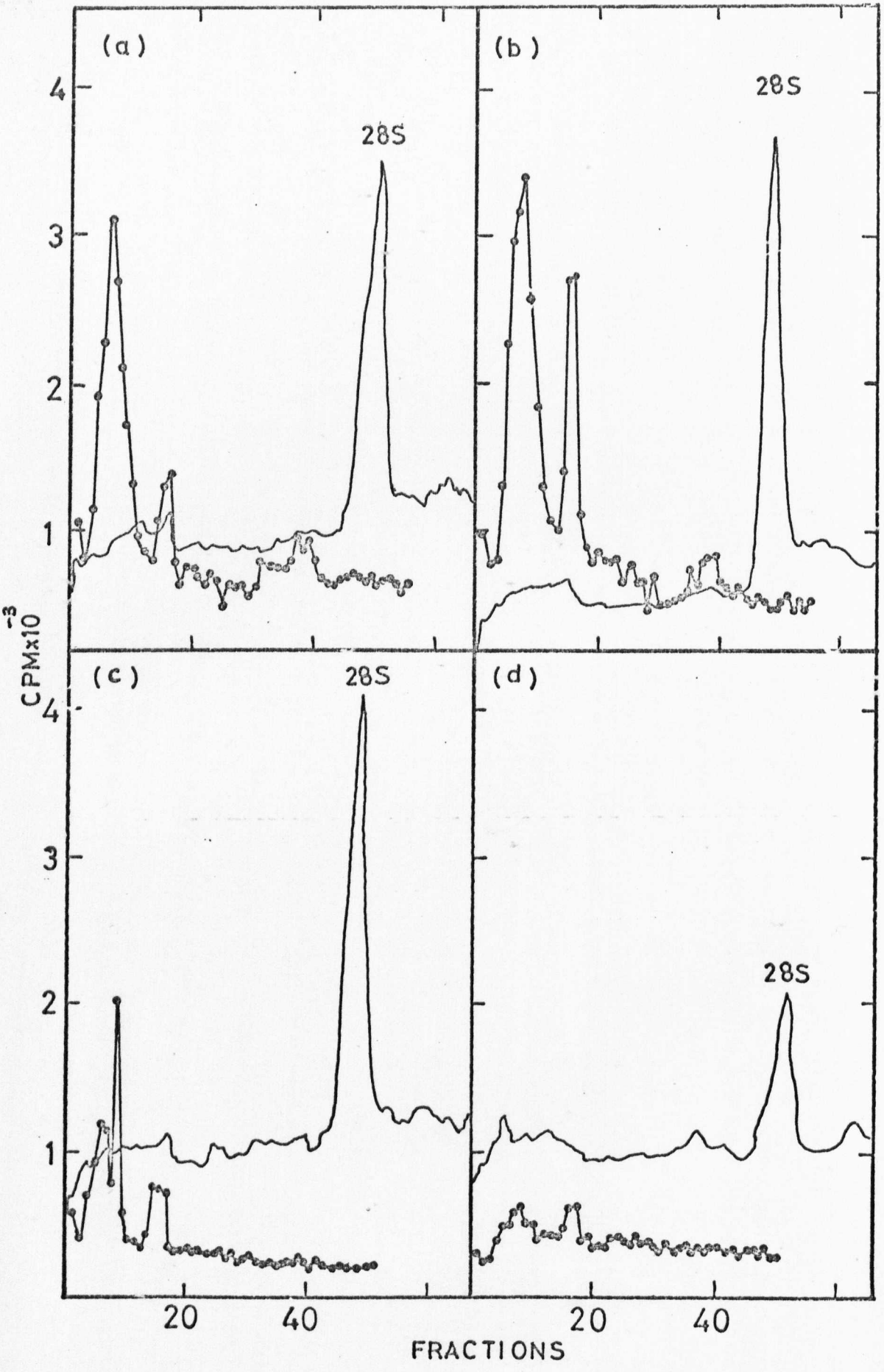
(a)
(b)
(c)
(d)
28S
28S
28S
28S
4
3
2
1
4
3
2
1
CPMx10^-3
20
40
20
40
FRACTIONS

Fig. 39. Effect of 65-709 and guanidine on polymerase activity in vivo. Both compounds were included in the medium of the infected HEL cells between 7.5 - 11 h after infection. The polymerase fraction was then extracted, assayed with the complete polymerase assay mixture and analysed by electrophoresis. (a) control at 7.5 h p.i., (b) control at 11 h p.i. (c) 65-709 treated cells and (d) guanidine treated cells. The continuous line is the OD_{260} trace indicating the position of the 28S ribosomal RNA.

Sample	CPM	%
Control at 11 h p.i.	1,429	100.0
Control at 7.5 h p.i.	1,009	70.6
65-709 treated between 7.5 - 11 h p.i.	330	23.1
Guanidine treated between 7.5 - 11 h p.i.	462	32.3

Table 12. Effect of 65-709 and guanidine on virus induced polymerase in vivo. The radioactivity above background (data from Fig. 39) was calculated and normalised as previously described.

Sample	TCA-precipitable CPM	%
Control cells	11,097	100
Cells treated with 65-709 for 3 h	8,344	75.2
Cells treated with cycloheximide for 45 min	380	3.4
Cells treated with 65-709 for 3 h and subsequently with cycloheximide for 45 min	372	3.3

Table 13. Effect of cycloheximide on cellular protein synthesis. 65-709 and cycloheximide were used at concentrations of 1μg/ml and 300μg/ml respectively. It is shown that the cycloheximide's inhibition of protein synthesis is not affected by pretreatment of the cells with 65-709.

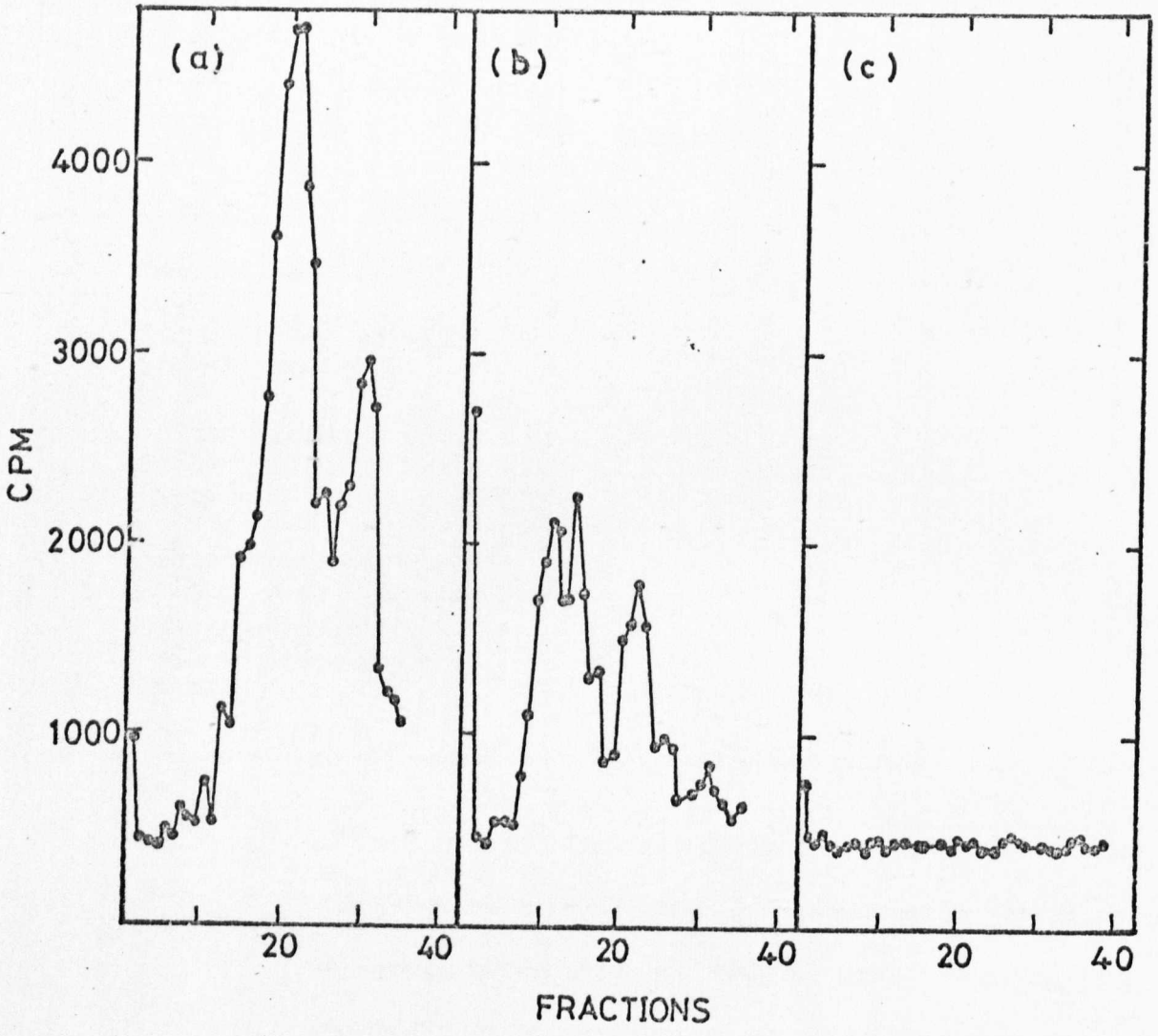

Fig. 40. Effect of cycloheximide on the polymerase activity in vivo. Cycloheximide was included in the medium of the infected cells at a concentration of 300 µg / ml. The products of the polymerase are shown as analysed by electrophoresis. (a) control, (b) and (c) infected cells treated with cycloheximide for 45 min and 3.5 h respectively.

7½ h after infection at the same concentrations used on previous occasions (1 µg / ml for 65-709 and 100 µg / ml for guanidine). The activity of the enzyme was tested at 11 h after infection and was compared with that of non-treated infected cells by extraction of the RNA from the assay mixture and analysis by polyacrylamide gel electrophoresis (Fig. 39). The radioactivity above background was calculated and normalized as previously described. (Table 12). Incubation of the infected cells with 65-709 or guanidine for 3½ h resulted in a 77% and a 68% inhibition of the polymerase activity respectively.

3. Effect of cycloheximide on protein synthesis in cells pretreated with 65-709.

In the last experiment of this section (Exp. B 5) cycloheximide is used to inhibit protein synthesis during a further investigation of the effects of 65-709 on the virus induced polymerase. This experiment was designed to show that protein synthesis is still inhibited by cycloheximide when the cells have been pretreated with 65-709 since it was possible that 65-709 might affect the uptake or action of cycloheximide.

Confluent monolayers of HEL cells were treated with 1 µg / ml 65-709 or left untreated for 3 hours. At the end of this period 300 µg / ml of cycloheximide were added to half of the plates and incubation continued for a further 45 min. During the last 15 min of the incubation period all plates received a pulse of [^{3}H]-protein hydrolysate (6 µc / plate) and the TCA-precipitable radioactivity was determined. Table 13 shows that the same inhibition of protein synthesis by cycloheximide was obtained whether or not cells were pretreated with 65-709.

4. Stability of polymerase activity.

Cycloheximide was used to inhibit protein synthesis so that the stability of existing polymerase activity could be determined.

When cycloheximide (300 µg / ml) was present in the infected HEL cells for $3\frac{1}{2}$ h (between $7\frac{1}{2}$ - 11 h p.i.) no polymerase activity was detected, as revealed by analysis of the polymerase products (Fig. 40). When cycloheximide was present for as little as 45 min the activity of the polymerase decreased by 54%. Similar results with poliovirus induced polymerase have been interpreted as indicating that the enzyme has a short half life in vivo (Baltimore, 1968b).

5. Does 65-709 prevent the synthesis or the maturation of the polymerase?

If 65-709 inhibits the synthesis of the polymerase, then removal of 65-709 and addition of cycloheximide to infected cells pretreated with 65-709 should result in the normal rapid decay of the small amount of the enzyme synthesized in the presence of 65-709. If the enzyme is synthesized in the presence of 65-709 but for some reason is not active, then removal of 65-709 might allow the inactive enzyme to attain an active configuration. Experimentally this could be shown by adding cycloheximide at the time of removal of 65-709 and would be reflected by an apparently lower rate of inactivation of polymerase activity than in the drug treated control.

Infected HEL cells were divided into four groups:
(a) was the control and did not receive any drug treatment.
(b) was treated with 300 µg / ml cycloheximide between 11 - 11.75 h p.i.
(c) was treated with 1 µg / ml of 65-709 between 7.5 - 11 h p.i. and with cycloheximide between 11 - 11.75 h p.i., and
(d) was treated with 65-709 between 7.5 - 11.75 h p.i.
At 11.75 h after infection the polymerase fraction was extracted and assayed with the complete assay mixture. The products of the enzymic reaction were extracted and analysed by polyacrylamide gel electrophoresis (Fig. 41). Cycloheximide alone reduced the polymerase activity to 46% (Fig. 41 a and b). When cycloheximide was added to 65-709 pretreated cells the polymerase activity was found to be

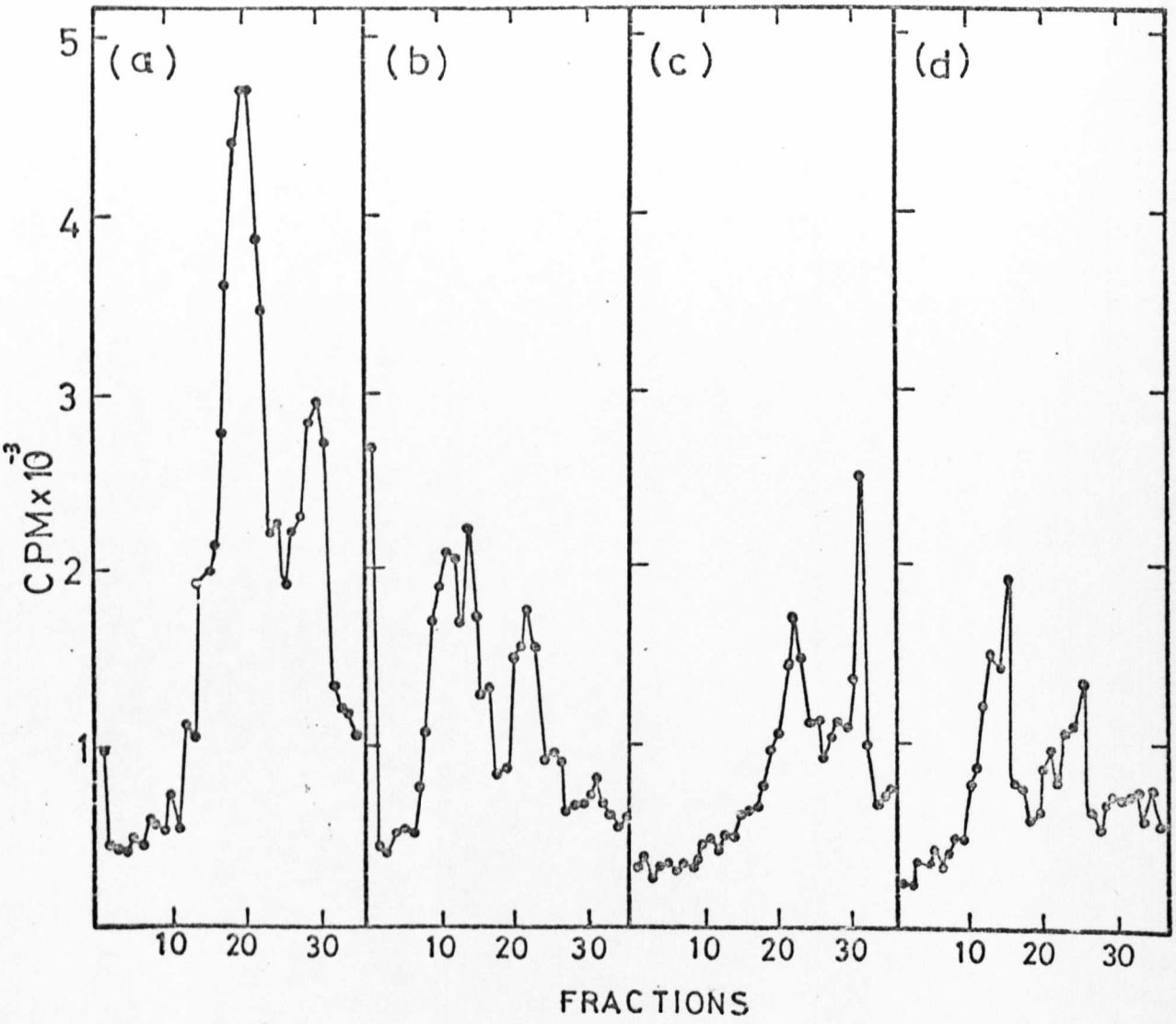

Fig. 41. The amount of inactivation of polymerase activity from infected cells pretreated with 65-709 compared with that of non-treated cells. (a) control, (b) infected cells treated with cycloheximide between 11 - 11.75 h p.i. (c) infected cells treated with 65-709 between 7.5 - 11 h p.i. and cycloheximide between 11 - 11.75 h p.i. and (d) cells treated with 65-709 between 7.5 - 11.75 h p.i.

Sample	CPM	%
Control at 11.75 h	44,660	100
Cells treated with cycloheximide between 11 - 11.75 h	20,880	46
Cells treated with 65-709 between 7.5 - 11 h and cyclo-heximide between 11 - 11.75 h	16,290	36
Cells treated with 65-709 between 7.5 - 11.75 h	11,950	27

Table 14. The amount of inactivation of polymerase from 65-709 pretreated cells by cycloheximide as compared to the rate of inactivation of the enzyme in non treated cells. Data from Fig. 41.

at the same level as the control and even higher (Fig. 41d and c). A summary of the results obtained is presented in Table 14. We consider that this evidence is consistent with the second hypothesis outlined above and indicates strongly that 65-709 allowed the synthesis of polymerase but that polymerase remained in an inactive form. Removal of 65-709 allowed the inactive polymerase to attain its functional configuration.

DISCUSSION

We have found that the requirements for the in vitro assay of the RNA-dependent RNA polymerase induced by rhinovirus type 2 in HEL cells were very similar to those described by Yin and Knight (1972). Incorporation of radioactive GTP was measured by TCA precipitation and the precipitates collected by filtration or centrifugation. The results obtained with the two procedures were very similar. Table 10 shows that the radioactivity obtained with the various controls was nearly 50% of that with the complete assay mixture. However, when RNA was extracted from the controls and electrophoresed on polyacrylamide gels no RNA species were resolved whereas with the complete reaction mixture viral RNA species were evident (Fig. 34). On the nature of the radioactivity obtained from the control polymerase assays our experimental data suggest:

a. Since no RNA was observed after electrophoresis its size must be less than 10S.
b. The appearance of this material is not cell specific since the same phenomenon was observed when viral polymerase obtained from HeLa cells was assayed.
c. The very low value of radioactivity obtained when the enzyme fraction was omitted suggests that a non-specific binding of the radioactive GTP to some cellular material which is TCA insoluble might occur.

No one has attempted so far to analyse the products of the rhinovirus-induced polymerase. We have used polyacrylamide gel electrophoresis to compare the viral RNA species prepared in vivo and in vitro. Fig. 35 shows that the viral RNA species prepared in vitro had identical electrophoretic mobilities to those synthesized in vivo but very little SS RNA was detected in the in vitro preparation. Among the explanations which could account for this are: a. The polymerase fraction contains ribonuclease which degrades the newly synthesized SS RNA. However since the single stranded regions of the RI are

also susceptible to ribonuclease (see Results Part I) we should expect to see a reduction in RI and to find that the predominant species was double stranded RNA. b. The newly synthesized RNA single strands are not released from the replicative intermediate. This process might require a cellular factor which was lost during the fractionation of the enzyme. In this case the ratio RI : RF should be much higher than that found in vivo. In fact the ratio RI : RF synthesized in vivo was about 1 while the corresponding ratio obtained in vitro was about 3.5. These values were consistent over a large number of experiments.

The kinetics of the synthesis of active enzyme appeared to be as expected: polymerase activity appeared at 6 h coincident with RNA synthesis which was also detected at 6 h after infection (Figs. 18 and 37). Only late in infection did the relationship between increasing polymerase activity in vitro and RNA synthesis in vivo diverge: while in vivo RNA synthesis continued to increase with time the polymerase activity reached a maximum at 9 h after infection and then declined. Whether this is a real difference cannot be decided at this stage since the two experiments were not carried out simultaneously. The point requires further experiments before it can be clarified.

It is known that poliovirus induced RNA polymerase has a half life of 15 mins (Baltimore, 1968b). Cycloheximide which is a potent inhibitor of protein synthesis has been used to study the stability of the rhinovirus induced enzyme. Although our experiments were not sufficiently extensive to determine with accuracy the half life of the enzyme it is apparent that the rhinovirus-induced polymerase was about as unstable as the poliovirus induced polymerase (Fig. 40).

When the compound 65-709 was included in the in vitro polymerase assay it did not affect polymerase activity. The same result was obtained even when the concentration of the compound was increased 10-fold over that used in

the in vivo experiments. Guanidine was also used at very high concentrations (1,000 μg / ml) and did not affect the activity of the enzyme. This finding is in agreement with Baltimore et al. (1963), who finds that guanidine does not affect the activity of the polymerase extracted from HeLa cells infected with poliovirus. However the amount of enzyme activity extracted from infected cells which had been treated with either 65-709 or guanidine was 23% and 32% respectively of the control value. The latter experiment clearly indicates that both compounds are only inhibiting the polymerase activity in vivo. This could mean that 65-709 and guanidine inhibited synthesis of the enzyme polypeptide(s) or that synthesis of the enzyme polypeptide(s) continues normally but mature active enzyme is not formed. If the second possibility was correct it was possible that preformed but inactive polymerase polypeptides could mature once the 65-709 block was removed. This would become apparent if total protein synthesis was inhibited. Through this experiment we found that the small amount of polymerase activity present in 65-709 treated cells was more stable than in control cells. Thus since there is no reason to suppose that 65-709 should enhance polymerase stability per se (although this must be checked) it appeared that inactive polymerase was synthesized in the presence of 65-709.

It would also be interesting to repeat these experiments to see if guanidine has a similar mode of action.

CONCLUSIONS

1. A system for the study of the replication of rhinovirus type 2 RNA has been established using diploid human embryo lung cells.

2. Three species of RNA in extracts of infected cells have been separated; in order of decreasing electrophoretic mobility they are single-, double- and multi-stranded RNA's.

3. Viral SS RNA was 4% of the ribosomal RNA present in infected HEL cells.

4. Viral SS RNA from extracts of infected cells has been shown to have identical electrophoretic mobility with virion RNA.

5. The possibility of chasing radioactive uridine with cold uridine in eukariotic cells has been studied and has been used to establish the intermediate role in virus replication of the MS RNA. Short pulse label technique led us to the same conclusion.

6. It has been established that hydrogen bonding between different viral RNA molecules is not an artefact of phenol extraction.

7. Only 4% of the viral SS RNA found in infected cell extracts late after infection is encapsidated into mature virions.

8. Treatment of RI by ribonuclease under defined conditions converts its electrophoretic mobility to that of RF.

9. The electrophoresis of mixtures of SS RNA synthesized early and late in infection showed no differences in size. This observation was confirmed with a bovine enterovirus.

10. The molecular weight of native viral RNA has been estimated by polyacrylamide electrophoresis to be 2.8×10^6 daltons. This has been done with some accuracy since HeLa nucleolar RNA's were used as molecular weight standards.

11. The native form of poliovirus type 1 SS RNA was smaller than rhinovirus RNA.

12. Some differences in the kinetics of synthesis of viral RNA's in HEL and HeLa cells were observed and the possibility that these differences reflecting an aspect of cellular control of virus transcription was discussed.

13. The antiviral compound ICI 65-709 inhibited the synthesis of all viral RNA species.

14. The antiviral activity of ICI 65-709 was compared with that of guanidine; some differences were found.

15. Guanidine-resistant mutants of rhinovirus were prepared. Since they were not resistant to ICI 65-709 it was concluded that the two compounds did not have identical modes of action.

16. An in vitro assay of the rhinovirus-induced RNA-dependent RNA polymerase has been established.

17. The rhinovirus polymerase was unstable in vivo and its half life was approximately as short as that of poliovirus-induced RNA polymerase.

18. Evidence was presented which suggested that the absence of SS RNA synthesis in vitro was due to the failure of RI to release newly synthesized strands of RNA rather than the degradation of RNA by ribonuclease.

19. Neither guanidine nor ICI 65-709 inhibited the synthesis of viral RNA in vitro.

20. Rhinovirus-induced RNA polymerase activity was inhibited by ICI 65-709 in vivo. Evidence was presented which suggested that ICI 65-709 inhibited the maturation of polymerase activity rather than the synthesis of polymerase polypeptide(s).

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ABBREVIATIONS

AMD	actinomycin D
ATP	adenosine triphosphate
BHK	baby hamster kidney
CEF	chick embryo fibroblasts
cpe	cytopathic effect
cpm	counts per minute
CTP	cytidine triphosphate
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
DS	double stranded
EDTA	ethylenediaminetetraacetic acid (di-sodium salt)
EMC	encephalomyocarditis
FMDV	foot and mouth disease virus
GTP	guanosine triphosphate
HBB	2-(a-Hydroxybenzyl)-Benzimidazole
HEL	human embryo lung
µc	microcurie
ME	mouse encephalomyelitis
MS	multi stranded
PBS	phosphate buffered saline
p.i.	post infection
pfu	plaque forming units
RF	replicative form
RI	replicative intermediate
RNA	ribonucleic acid
RNAse	ribonuclease
SDS	sodium dodecyl sulphate
SS	single stranded
SSC	standard saline citrate
TCA	trichloroacetic acid
tris	tris-hydroxy methyl amino methane
UTP	uridine triphosphate

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