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Characterisation of rinderpest virus RNA and the action of actinomycin D on its replication

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Abstract. RNA extracted from purified rinderpest virus was characterised by sucrose gradient sedimentation and polyacrylamide gel electrophoresis. The predominant virion RNA species had a sedimentation constant of 46S and its estimated molecular weight was $4{\cdot}8 \times 10^6$ daltons. Consistently high amounts of UMP and AMP were detected. The melting-temperature profile of the virion RNA suggested absence of secondary structure.

The effect of actionomycin D on the replication of rinderpest virus in Vero cells was studied by following the viral RNA synthesis using labelled uridine as well as by infectivity titration. The viral RNA synthesis was not affected until 12 h following infection and was inhibited thereafter between 18 and 48 h to an extent of 25% at 5 and 10 μg levels of the drug. A 100 to 1000-fold reduction in the infectivity titres was observed in the presence of the drug. These results suggest that actinomycin D inhibits rinderpest viral RNA replication. Sedimentation analysis of viral RNA extracted from drug-treated cultures showed inhibition of the genome RNA of rinderpest virus.

Keywords. Rinderpest virus RNA; viral RNA synthesis in infected cells; infectivity titration; sedimentation analysis.

Introduction

Rinderpest virus, a member of the 'negative-strand' paramyxovirus group, produces a severe, often fatal disease of epidemic proportions in cattle, sheep, goats and swine. Although a large amount of information is available on the characterisation and replication of RNAs of other members of paramyxovirus group, such as Newcastle disease virus (Duesberg and Robinson, 1965), SV5 (Compans and Choppin, 1968), sendai virus (Barry and Bukrinskaya, 1968), measles virus (Schluederberg, 1971; Schluederberg *et al*., 1972) and mumps virus (East and Kingsbury, 1971) relatively much less is known about rinderpest virus (Wild *et al*., 1974). The sedimentation constant and molecular weight of other paramyxovirus RNAs have been reported to be around 50S and $5–7 \times 10^6$ daltons, respectively.

It is reported that, while paramyxoviruses are insensitive to the action of actinomycin D, the orthomyxoviruses are sensitive (Blair and Duesberg, 1970). In addi-

tion to an early report on the effect of this drug on Newcastle disease virus (Granoff and Kingsbury, 1964), there have been recent reports on the inhibitory effect of actinomycin D on measles (Schluederberg *et al*., 1972) and parainfluenza viruses (Dubois, 1975). This paper describes the characterisation of rinderpest virus (RPV) RNA and the effect of actinomycin D on the replication of RPV in Vero cells.

Materials and methods

Chemicals

Sucrose (density gradient grade), actinomycin D, pancreatic deoxyribonuclease (EC 3.1.4.5 type, I) and sodium dodecyl sulphate were obtained from Sigma Chemicals, St. Louis, MO, USA. Eagle's minimum essential medium (Earle Base) containing non-essential amino acids was obtained from Centron Research Laboratories, Bombay. 5-[^{3}H]-Uridine (9·7 Ci/mmol) and carrier-free [^{32}P]-orthophosphate (activity 10 mCi/ml) were obtained from the Bhabha Atomic Research Centre, Bombay. All other chemicals used were of analytical grade.

Cells and virus

African green monkey kidney cells (Vero), originally obtained from American type culture collection, were employed for all virus assays and virus production. The Kabete 'O' vaccine strain of rinderpest virus, used in the study, was from the stocks of Serum Institute, Bangalore, India. Cells were grown in Eagle's minimum essential medium (MEM) supplemented with non-essential amino acids, 4% goat serum and antibiotics. The virus used as inoculum had a litre of 10^{5} tissue culture infective dose 50/ml (50% end point). Infection was carried out for 1 h at 37° C. Cells were grown in sterile screw-capped tubes, for experiments with actinomycin D, with $0{\cdot}4 \times 10^{6}$ cells/tube.

Preparation of [^{32}P]-*rindenpest virus RNA*

Two-day old confluent cultures of Vero cells in Roux bottles were infected with rinderpest virus. After 36 h at 37° C, the medium was removed and replaced by phosphate-free medium containing 100 μCi/ml of carrier-free [^{32}P] orthophosphate. At 60 h post-infection, the medium was removed, centrifuged for 10 min at 1,000 g to remove cellular debris. It was then layered on to a discontinuous gradient containing 3 ml of 60% (w/v) sucrose and 3 ml of 15% (w/v) sucrose in 0·01 M Tris buffer containing 0·1M NaCl and 0·001M EDTA, pH 7·4 and centrifuged for 2 h at 70,000 g in a Beckman SW 25 1 rotor. The virus band at the interface was pooled, mixed with the buffer and layered on to a 25–60% (w/w) sucrose linear gradient and centrifuged for 12 h at 1,05,000 g in a Beckman L2–50 ultracentrifuge in a SW 27 rotor. The band showing a buoyant density of 1·21–1·22 g/cm^{3} on the gradient was collected and used for extraction of virion RNA by Sod. dod. SO_4-phenol in the presence of 200 μg of yeast RNA and 40 μl of 2-mercaptoethanol. RNA was precipitated from the aqueous phase by the addition of 1/10 volume of 2M sodium acetate and two volumes of ethanol and kept overnight at — 20° C. The precipitated RNA was washed with 70% ethanol in Tris-NaCl-EDTA buffer,

dried and dissolved in 0·5 ml of the same buffer. To check whether there was any contamination with DNA, the RNA was digested with pancreatic DNase. Only about 2–3% of the total radioactivity present in RNA was digested on incubation with pancreatic DNase showing that the rinderpest virus RNA sample was not associated with any DNA.

Sucrose gradient sedimentation analysis of rinderpest virus RNA

[^{32}p]-virion RNA was centrifuged on a 15–30% sucrose gradient for 4 h at 1,60,000 g and 4°C in a Beckman SW 50 rotor. [^{3}H]-labelled Vero cell ribosomal RNAs were used as markers for sedimentation constant and molecular weight determinations. From the sucrose gradient data, the S value of rinderpest virus RNA was calculated according to the proportionality of Martin and Ames (1961). The molecular weight of rinderpest virus RNA was estimated from the S value using the empirical relation of Spirin (1961).

Polyacrylamide gel electrophoresis

A modification of the method followed by Kolakofsky *et al*., (1975) was employed. Gels of 2% polyacrylamide containing 0·5% agarose were polymerised in 9 cm (5 mm internal diameter) tubes in a buffer containing 0·4M tris, pH 7·8, 0·2M sodium acetate and 20 mM EDTA. Electrophoresis of [^{32}P]-rinderpest virus RNA was carried out for 2 h at 5 mA/gel at 5° C in the above buffer containing 0·2% Sod. dod. SO_4. The gels were frozen and cut into 2 mm slices. The gel slices were digested in vials overnight with 1 ml of 30% H_2O_2 and counted in a Beckman LS–100 liquid scintillation spectrometer after addition of 5 ml of Bray's fluid Bray, 1960). Labelled ribosomal RNAs extracted from Vero cells were used as markers for molecular weight determination.

Base-composition

The [^{32}P-]-virion RNA was digested in a sealed tube with 0·2 ml of 0·3M KOH at 37° C for 18 h and subjected to paper electrophoresis at pH 3·5 (0·5% pyridine and 5% acetic acid (v/v)). The base-com position of the virion RNA was calculated by measuring the distribution of radioactivity among the four ribonucleotides. The procedure was essentially similar to the method followed for measles, canine distemper and vesicular stomatitis viruses(Yeh, 1973; Waters and Bussell, 1974; Bishop *et al*., 1975).

Melting temperature study of rinderpest virus RNA

The virion RNA was extracted from unlabelled, purified rinderpest virus using Sod. dod. SO_4-phenol without addition of carrier yeast RNA. The aqueous phase, after complete separation from the phenolic phase, was dialysed at 4° C against Tris-NaCl-EDTA buffer for 48 h. The melting temperature of the RNA was determined by monitoring the changes in absorbance at 260 nm.

Effect of actinomycin D on rinderpest virus RNA synthesis

In the first series of experiments, the drug was added at a concentration ranging from 1–10 μg/ml to the injected cultures and allowed to grow from $1\frac{1}{2}$ to 48 h.

In the second series, the Vero cells were preincubated with the drug (5 μg/ml) for 2 h, after which the drug was removed by washing and the cells were infected. [^{3}H]-Uridine (10 μCi/ml) was added afer infection and incubated for 4 h at 37° C. The third set of experiments was aimed at locating a drug-sensitive phase. Concentrations of actinomycin D (1 to 10 μg/ml) as mentioned above were added at 6 h intervals upto 24 h and at 12 h intervals between 24 and 48 h post-infection and viral RNA synthesis was followed. Uninfected cell-cultures with the same concentrations of the drug and infected cultures without the drug served as controls. The samples were processed with 0·5 ml of 0·1M tris buffer, pH 7·4 containing, 0·001M magnesium chloride, 0·1% Sod. dod. SO_4. Equal volumes of cell-lysate (0·1 ml), spotted on Whatman 3MM paper (1·8 cm^2), were used to determine cold trichloroacetic acid insoluble radioactivity. Processing was done with 10% trichloroacetic acid first for 10 min and then twice with 5% trichloroacetic acid. The papers were then dried thoroughly and the radioactivity determined in a Beckman LS–100 liquid scintillation spectrometer.

In the last experiment, [^{3}H]-labelled RNA was extracted from rinderpest virus-infected cells grown in medium containing actinomycin D (5 μg|ml) from 1 $\frac{1}{2}$ to 48 h post-infection and compared with labelled RNA isolated from infected cells exposed to actinomycin D for 2 h before incroporation. The two RNAs were subjected to rate zonal centrifugation on 15–30% sucrose gradients as described earlier (Prakash *et al*., 1978).

Infectivity titration

The released virus was harvested from rinderpest virus-infected cells grown for 48 h in a medium containing actinomycin D (1 to 10 μg/ml) from 1 $\frac{1}{2}$ to 48 h post-infection. The virus samples pooled from drug treated and control cultures were titrated using Vero cells and the tissue culture infective dose 50 was calculated by the method described by Karber (1931).

Results

Characterisation of rinderpest virus RNA

The sucrose gradient profile of the [^{32}P]-labelled virion RNA shows the presence of a major and a minor RNA species (figure 1). Using the Vero cell ribosomal RNAs as markers, the sedimentation constant of the predominant rinderpest virus RNA species was calculated to be 46S. The minor peak was observed between the 18–28S region. The molecular weight of the virion RNA, calculated from the sucrose gradient data, was found to be $4{\cdot}8 \times 10^6$ daltons. The virion RNA migrated mainly as a single peak on polyacrylamide gel (figure 2). Using the reference values obtained for the 28S and 18S ribosomal RNAs (Weinberg and Penman, 1970) and for 4S RNA (Sober, 1970), the molecular weight of the virion RNA run on polyacrylamide gels was calculated to be $4{\cdot}75 \times 10^6$ daltons. The distances moved by the virion RNA and the marker RNAs have been plotted against the logarithm of molecular weight (inset of figure 2).).

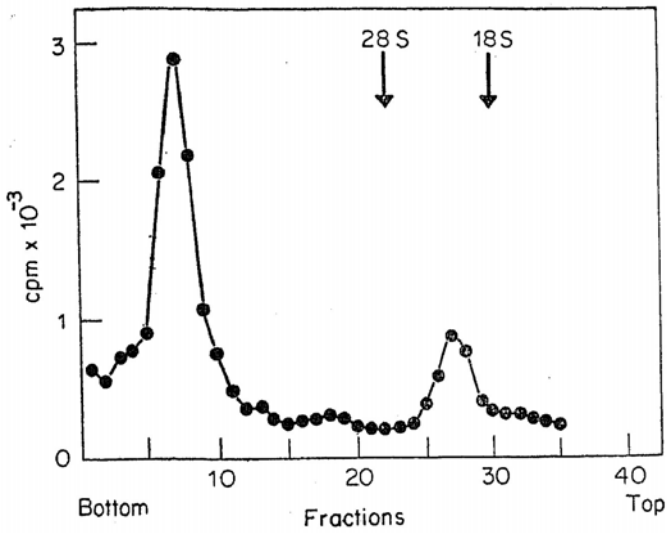

Figure 1. Profile of [^{32}P]-rinderpest virus RNA on 15–30% sucrose gradient.The arrows indicate the positions of [^{3}H]-labelled 28S and 18S rRNAs (markers) run in parallel.

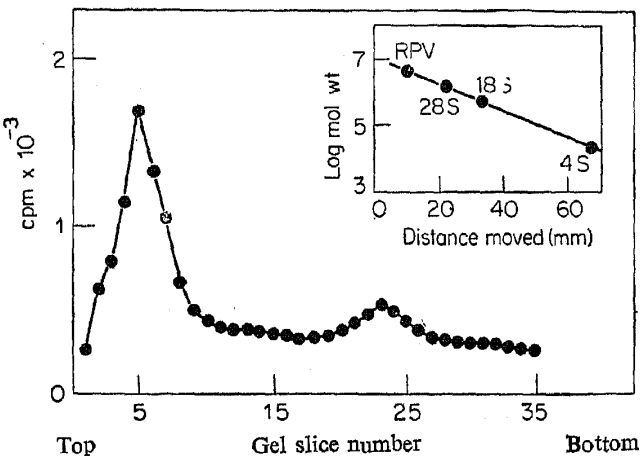

Figure 2. Characterisation of [^{32}P]-virion RNA on 2% polyacrylamide gels containing 0·5 % agarose. The numbers on the x-axis represent gel slice numbers. The inset shows the distances moved by the labelled virion RNA and marker RNAs plotted against the logarithm of molecular weight.

Base-composition

The distribution of radioactivity amongst the ribonucleoside monophosphates of the [^{32}P]-labelled virion RNA is shown in table 1. UMP was found to be predominant, though a high percentage of AMP was also found. The base-ratio clearly indicates the single-stranded nature of the virion RNA.

Table 1. Base-composition of rinderpest virus RNA.

Ribonucleoside monophosphate	% of total radioactivity
C_p	20 ± 0·5
A_p	27 ± 0·5
G_p	24·5 ± 0·5
U_p	28·5 ± 0·5

Melting temperature study

The melting temperature profile suggested that the virion RNA does not possess any secondary structure. Although a slight increase in absorbancy was observed when the temperature was raised from 30–40° C, it remained stationary thereafter indicating that virion RNA does not possess any secondary structure.

Effect of actinomycin D on the replication of rinderpest virus

When actinomycin D was present from 1½ to 48 hpost-infection, viral RNA synthesis was not affected upto 12 h but was subsequently inhibited (figure 3).

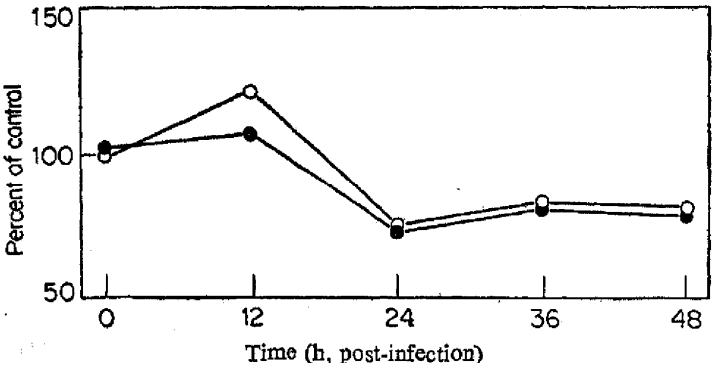

Figure 3. Effect of higher concentrations of actinomycin D on viral RNAsynthesis when present from 1½ to 48 h post-infection, O—O 5 μg/ml of drug used, ●—● 10 μg/ml of the drug used.

As mentioned earlier, labelled uridine was added at 12 h intervals and incubated for 4 h at 37° C. No significant inhibition was observed with a concentration of 1 μg/ml while the extent of inhibition increased with increasing concentrations of the drug, reaching a value of 20 and 25% with 5 and 10 μg/ml of actinomycin D, as compared to control. Pretreatment of Vero cells with the drug for 2 h before infection did not affect viral RNA synthesis at all.

No inhibition was observed with drug concentrations, of 1, 2 and 3 μg|ml added after infection at various intervals while a concentration of 4 μg|ml caused an inhibition of only 3 to 5%. Addition of higher concentrations (5 and 10 μg|ml) resulted in an inhibition of 10–15% (figure 4). Inhibition of viral RNA synthesis was observed between 18 and 48 h post-infection indicating the occurrence of a drug-sensitive phase in this period after infection.

The effect of the presence of actinomycin D (5 μg/ml) between $1\frac{1}{2}$ and 48 h post-infection on the different species of virus-specific RNA in infected cells was studied next (figure 5a) and compared with the virus-specific RNA synthesised in infected cells treated with the drug for 2 h before addition of labelled uridine (figure 5b). An inhibition of 30% of the virion RNA species in the 45S region

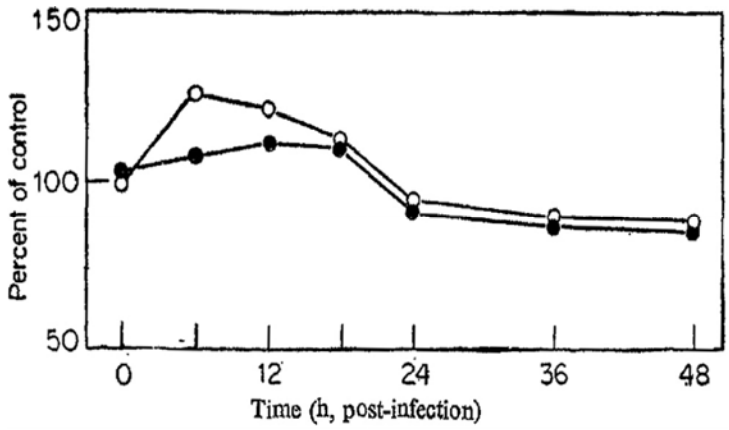

Figure 4. Profiles of viral RNA synthesis showing drug-sensitive phase. O—O 5 μg/ml of actinomycin D and ●—● 10 μg/ml of actinomycin D, added at various times after infection.

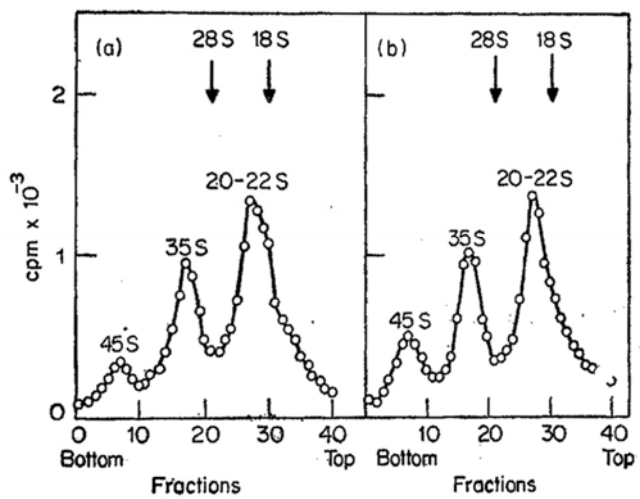

Figure 5. Sucrose gradient analysis of viral RNA species produced in infected cells. (*a*) Drug present from $1\frac{1}{2}$ –48 h post-infection. (*b*) Drug treatment for 2 h before addition of labelled uridine.

was observed while the 35S and 20–22S RNA species were not affected. This observation was highly reproducible with the 45S, 35S and 20S RNA species occurring in a similar ratio with each preparation. Incorporation with [^{3}H]-uridine (10 μCi/ml) was carried out for 8 h at 37° C (in both the experiements) between 40 and 48 h post-infection. Actinomycin D at 5 μg/ml concentration shuts off 95% of the host RNA synthesis. All the results obtained have been based on trichloroacetic acid-insoluble radioactivity. The experiments were not carried out beyond 48 h post-infection as maximal viral RNA synthesis has been shown to be between 24 and 48 h post-infection (Prakash *et al*., 1978).

Infectivity titration

The results are given in table 2. In all the experiments, the drug was present from 1^1 to 48 h post-infection. The infectivity of virus was not significantly affected by a concentration of 1 μg/ml of the drug while a concentration of 2 μg/ml caused a 10-fold reduction in the titre of virus as compared to virus pooled from cultures not treated with the drug. A 100–1000-fold reduction in the titre was observed with higher concentrations of the drug.

Table 2. Effect of actinomycin D on the infectivity of rinderpest virus.

Actinomycin D concentration (μg/ml)	Titre (TCID 50)*
..	$10^{-5.1}$
1	$10^{-4.8}$
2	$10^{-4.2}$
3	$10^{-3.3}$
4	$10^{-2.6}$
5	$10^{-1.8}$
10	$10^{-1.8}$

* $TCID_{50}$ tissue culture infective dose 50% end point.

Discussion

The sedimentation constant of 46S and the estimated molecular weight of 4·8 × 10^6 daltons of rinderpest virus RNA are in general agreement with values obtained for virion RNAs of sendai (Kolakofsky *et al*., 1975), measles (Schluederberg, 1971), and mumps viruses (East and Kingsbury, 1971). We did not attempt denaturation of the virion RNA with dimethyl sulphoxide or formaldehyde as they are known

to denature RNAs to varying degrees (Arif and Faulkner, 1972; Kolakofsky and Bruchi, 1973). Moreover such studies have been made with nucleus-associated RNA in cells infected with rinderpest virus (Wild *et al*., 1974). The minor RNA species sedimenting slower than 46S in rinderpest virions might arise due to aggregation of degraded virion RNA or could possibly be RNA associated with the virion RNA.

The base-composition analyses have shown that UMP and AMP are present in high amounts with the former being predominant in rinderpest virion RNA. Similar observations with UMP being the most predominant have been reported for the closely related measles, canine distemper virus RNAs (Waters and Bussell; 1974; Yeh, 1973) and other well-characterised paramyxoviruses (Compans and Choppin, 1968; Duesberg and Robinson, 1965). The random distribution of radioactivity amongst the four ribonucleoside monophosphates indicates the single stranded nature of linderpest virus RNA and this observation is in agreement with the findings of the melting temperature studies.

Actinomycin D could be inhibiting the replication of rinderpest virus by shutting off the transcription of the host cell genome which may be required for rinderpest virus replication. Alternatively, it is possible that the drug may bind to a double-stranded replicative form of RNA during the replication of rinderpest virus RNA and thus bring about inhibition of rinderpest virus. Recently, data have been obtained (Prakash, 1979, unpublished results) to show the occurrence of double-stranded forms in rinderpest virus-infected cells as shown by RNase resistance. Similar inhibitory effect of actinomycin D has been reported in the case of influenza virus (Barry, 1964). Actinomycin D has also been shown to bind to RNA *in vitro* (Semmel and Huppert, 1965). Further, the inhibition of the genome RNA species (45S) and not the 35S and 20–22S RNA species suggests clearly that actinomycin D may be acting at the level of replication and not transcription. As a result of this, it is quite understandable that the infectivity of RPV is also affected to a significant degree. One objection that could be raised is that viral RNA synthesis is surprisingly detectable even in cultures where the infectivity is very markedly affected. This could be due to the fact that the drug inhibits the synthesis of the 45S (genome) strands only and not the 35S and 20–22S RNA species. Since it is well known that about 90% of the virus specific RNA is in the + strands as far as paramyxoviruses are concerned (Blair and Duesberg, 1970), the drug apparently does not affect the + strands (35S and 20–22S) synthesis of RPV. As Vero cells are quite resistant tothe toxic effects of high concentrations of actinomycin D (Schluederberg *et al*., 1971), the results cannot be ruled out by stating that the observed inhibition could be due to the presence of actinomycin D for a long period. Similar work, where the drug is present from 1–24 h post-infection, has been done for closely related measles virus (Schluederberg *et al*., 1972). While inhibition of measles, Newcastle disease and parainfluenza viruses was observed with concentrations of 1 μg/ml and lower (Barry, 1964; Schluederberg *et al*., 1972; Dubois, 1975) our results show that inhibition of RPV occurs only with slightly higher concentrations.

The results obtained with the RNA of rinderpest virus are quite similar to those reported for other paramyxoviruses thus bringing out their close relationship to

each other. It would also be not surprising if more and more paramyxoviruses are found to be sensitive to the action of actinomycin D as the replication of three or four members of this group have already been found to be affected by the drug in conflict with the general consensus.

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References

Arif, B. M. and Faulkner, P. (1972) *J. Virol*., **9,** 102.

Barry, R. D. (1964) in *CIBA Foundation Symposium—Cellular biology of myxovirus infections* eds. G. E. W. Wolstenholme and J. Knight (London: J. A. Churchill), p.51.

Barry, R. D. and Bukrinskaya, A. G. (1968) *J. Gen. Virol*., **2,** 71.

Bishop, D. H. L., Aaslestad, H. G., Clark, H. F., Flamand, A., Obijeski, J. F., Repik, P. and Roy, P. (1975) in *Negative-strand viruses*, eds. B. W. J. Mahy and R. D. Barry (London: Academic Press), **1,** 259.

Blair, C. D. and Duesberg, P. H. (1970) *Annu. Rev. Microbiol*., **24,** 539.

Bray, G. (1960) *Anal. Biochem*., **1,** 279.

Compans, R. W. and Choppin, P. W. (1968) *Virology*, **35,** 289.

Dubois, M. F. (1975) in *Negative-str and viruses* eds. B. W. J. Mahy, and R. D. Barry (London: Academic Press), **1,** 523.

Duesberg, P. H. and Robinson, W. S. (1965) *Proc. Natl. Acad. Sci. USA*, **54,** 794.

East, J. L. and Kingsbury, D. W. (1971) *J. Virol*., **8,** 161.

Granoff, A. and Kingsbury, D. W. (1964) in *CIBA Foundation Symposium-Cellular biology of myxovirus infections*, eds. G. E. W. Wolstenholme and J. Knight (London: J. A. Churchill), p. 96.

Karber, G. (1931) *Arch. Exp. Pathol. Pharmacol*., **162,** 480.

Kolakofsky, D. and Bruchi, A. (1973) *J. Virol*., **11,** 665.

Kolakofsky, D., Boy de la tour, E. and Delius, H. (1975) in *Negative-strand viruses*, eds. B. W. J. Mahy and R. D. Barry (London: Academic Press), **1,** 243.

Martin, R. G. and Ames, B. N. (1961) *J. Biol. Chem*., **236,** 1372.

Prakash, K., Antony, A. and Ramakrishnan, T. (1978) *Curr. Sci*., **47,** 42.

Schluederberg, A. (1971) *Biochem. Biophys. Res. Commun*., **42,** 1012.

Schluederborg, A., Hendel, R. C. and Chavanich, S. (1971) *Science*, **172,** 577.

Schluederberg, A., Williams, C. A. and Black, F. L. (1972) *Biochem. Biophys. Res.Commun*., **48**, 657.

Semmel, M. and Huppert, J. (1965) *Biochem. Biophys. Acta*, **103,** 702.

Sober, H. A. (1970) in *Handbook of biochemistry* (Cleveland:Chemical Rubber Co.). H128.

Spirin, A. S. (1961) *Prog. Nuc. Acid. Res*., **1,** 301.

Waters, D. J. and Bussell, R. H. (1974) *Virology*, **61,** 64.

Weinberg, R. A. and Penman, S. (1970) *J. Mol. Biol*., **47,** 169.

Wild, F., Underwood, B. and Brown, F. (1974) *Med. Microbiol. Immunol*., **160,**133.

Yeh, J. (1973) *J. Virol*., **12,** 962.