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MACROMOLECULAR SYNTHESIS IN BLUETONGUE VIRUS INFECTED CELLS. I. VIRUS-SPECIFIC RIBONUCLEIC ACID SYNTHESIS

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ABSTRACT

HUISMANS, H. Macromolecular synthesis in bluetongue virus infected cells 1. Virus-specific ribonucleic acid synthesis. *Onderstepoort J. vet. Res.* 37 (4), 191-198 (1970).

Both virus-specific double-stranded and single-stranded ribonucleic acid (RNA) are synthesized during infection. The single-stranded RNA is formed in a large excess of double-stranded RNA and the rate of synthesis is maximal between 10 and 13 hours after infection. The single-stranded RNA is associated with the polyribosomes and consists of components with sedimentation constants varying between 12S and 22S. Hybridization of single-stranded RNA with double-stranded RNA indicated that the single-stranded RNA is probably messenger RNA. The secondary structure of the double-stranded RNA was verified by optical rotatory dispersion.

Introduction

The bluetongue virus (BTV) genome is comprised of segements of double-stranded ribonucleic acid (Verwoerd, 1969). This double-stranded ribonucleic acid (dsRNA) can be fractionated into five main components by means of sucrose gradient sedimentation analysis, and into ten components by electrophoresis on polyacrylamide gels (Verwoerd, Louw & Oellermann, 1970). When considering how replication of this dsRNA virus takes place, it is of prime importance to know whether or not the genome itself acts as messenger RNA (mRNA). Alternatively, the various components of the dsRNA can first be copied into single-stranded RNA (ssRNA) molecules which then act as templates for new viral protein synthesis.

The close similarity in structure that has been found to exist between the nucleic acids of BTV and reovirus could obviously reflect a similarity in mode of replication. In the case of reovirus it was shown that ssRNA components are synthesized which correspond in length to the fragments of denatured dsRNA (Watanabe & Graham, 1967). This ssRNA is associated with the polyribosomes in infected cells (Prevec & Graham, 1966) and each component hybridizes uniquely with a corresponding length of denatured dsRNA (Watanabe, Millward & Graham, 1968). This ssRNA is not a precursor of dsRNA (Watanabe, Kudo & Graham, 1967) and is therefore presumed to be mRNA.

The existence of such a mRNA molecule has not yet been proved in the case of BTV. In this paper results are presented which indicate that both ssRNA and dsRNA are synthesized during infection. The kinetics of the synthetic processes of the two types of RNA have also been investigated. A method for the isolation of ssRNA is described and results reported that were obtained during the characterization of the RNA by means of sedimentation analysis and electrophoresis on polyacrylamide gels.

Double-stranded RNA was also isolated from the infected cells, its optical rotatory dispersion characteristics determined and after denaturation it was hybridized with the isolated ssRNA. The hybridization product was further investigated by means of gel electrophoresis.

Materials and Methods

Virus

BTV Type 10 was used throughout this study. Methods for the production and assay of the virus have been described by Howell, Verwoerd & Oellermann (1967) & Verwoerd (1969). Partial purification of the virus was obtained by extracting virus-containing cells three times with an 0.002 M Tris-buffer, pH 8.8. The combined extracts were centrifuged for 1 hour at 100,000 × *g* and the virus pellets suspended in Eagle's medium.

Cells

The origin and cultivation of the L-cells that were used have been described by Verwoerd, Oellermann, Broekman & Weiss (1967).

Suspension cultures were prepared from L-cell monolayer cultures after 2 or 3 days growth. Cells were removed from the glass with an 0.25 per cent trypsin solution in phosphate buffered saline. Trypsin was removed by centrifugation and the cells then suspended in Eagle's medium at an approximate concentration of 1.50×10^6 cells/ml.

Extraction and analysis of cytoplasmic extracts

Cytoplasmic fractions were prepared according to the method of Bellamy, Shapiro, August & Joklik (1967). Cells were allowed to swell at 4°C for 15 min in RSB buffer (0.01 M NaCl, 0.01 M Tris HCl, 0.005 M $MgCl_2$, pH 7.3) and disrupted with 15 strokes of a tight-fitting Dounce homogenizer. Nuclei were removed by centrifugation at $1000 \times g$ for 2 min and the cytoplasmic fractions treated with sodium dodecyl sulphate (SDS), urea and sodium acetate to a final concentration of 1.0 per cent, 0.5 M and 0.1 M respectively. Samples were then layered in 0.2 ml volumes on 15 to 30 per cent sucrose gradients containing 0.1 M NaCl, 0.005 M Tris and 0.5 per cent SDS, pH 7.3. Centrifugation was carried out at $200,000 \times g$ and 25°C in a Spinco SW50 rotor. Gradients were fractionated in an Isco model D gradient fractionator. Ultraviolet (UV) absorbancy was monitored and in the case of radioactive labelled samples, the fractions were precipitated with 10 per cent trichloroacetic acid (TCA). Precipitates were collected on Millipore filters and counted in toluene scintillator solution in a Tricarb scintillation counter.

Isolation of labelled ssRNA

The method used for the isolation of ssRNA is very similar to the one described by Prevec, Watanabe, Gauntt & Graham, (1968). A suspension culture of L-cells was inoculated with partially purified BTV at an input multiplicity of 50 plaque-forming units per cell. Actinomycin D (0.5 μg/ml) and cycloheximide (2 μg/ml) were added respectively 8 and $11\frac{1}{2}$ hours after

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infection. From 10 to 12 hours after infection the cells were labelled with 2 μCi/ml ^{3}H-uridine. Cells were harvested by centrifugation and stored at −20°C.

Single-stranded RNA was isolated from the polysomes extracted from these infected cells. Cells were suspended in STM buffer (0.1 M NaCl, 0.01 M Tris and 0.015 M $MgCl_2$, pH 7.4) at 4°C. After 15 min, BRIJ 58 (0.5 per cent) was added and the cells disrupted with a Dounce homogenizer. Nuclei were removed by low speed centrifugation and the cytoplasmic extract, containing the polyribosomes, layered in 3 ml volumes on 15 to 13 per cent sucrose gradients in the centrifugation tubes of the Spinco SW27 rotor. The gradients were prepared from ribonuclease-free sucrose in STM buffer. Centrifugation was carried out at 24,000 rpm for 2 hours at 4°C. The fractions of each gradient that contained the polyribosomes were pooled and the polyribosomes collected by centrifugation for 18 hours at 20,000 rpm in the SW 27 rotor. Single-stranded RNA was obtained by disruption of the polysome complexes with 0.1 per cent SDS and deproteinized by two phenol extractions at room temperature followed by two ether extractions to remove phenol residues. The RNA was precipitated overnight by 2 volumes ethanol at −20°C and the precipitates dissolved in STE buffer containing 0.01 M NaCl.

Isolation of dsRNA

Double-stranded RNA was isolated from infected cells as previously described (Verwoerd & Huismans, 1969).

Optical rotatory dispersion

Optical rotatory dispersion (ORD) measurements were made with a Jasco model ORD/UV 5 recording spectropolarimeter. A cell of 1 cm path length was used. Unfortunately an accessory for controlling the temperature in the cell was not available. In order to allow measurements at different temperatures, water was circulated from a temperature regulated waterbath through a copper holder in which the cell fitted tightly. Even though the holder was isolated, heat loss did occur. This necessitated determination of the temperature in the cell itself and made it impossible to investigate the ORD characteristics of dsRNA at a temperature higher than 90°C. To avoid artifacts the absorbance of the RNA solution was kept below 2 optical units per ml during ORD measurements. All rotation angle readings were expressed in terms of specific rotation and plotted against wave length.

Gel electrophoresis

Electrophoresis in polyacrylamide gels was carried out according to a slight modification of the method of Loening (1967) as described by Verwoerd *et al.* (1970). Electrophoresis of dsRNA was performed in 10 cm length columns at 10 V/cm for 4 hours. Electrophoresis of ssRNA was carried out for only 2 hours.

Hybridization

Hybridization was done according to a modification of the method previously described by Verwoerd & Huismans (1969). Labelled ssRNA and unlabelled dsRNA, both dissolved in STE buffer containing 0.01 M NaCl, were mixed in appropriate amounts and heated for 5 min in tightly stoppered glass tubes in a waterbath at 97°C. The salt concentration was then raised to 0.3 M NaCl and the mixture immersed in a waterbath at 72.5°C for 30 min. The hybridization product was separated from unhybridized ssRNA by precipitating the latter overnight at a salt concentration of 1.0 M NaCl. The different dsRNA components in the hybridization product were identified by means of gel electrophoresis.

Results

Virus-specific RNA synthesis in the presence of Actinomycin D

In order to detect synthesis of virus-specific RNA components by means of a labelling technique, it is necessary to suppress cell-specific RNA synthesis during the labelling period. This can be done by selective inhibition of cellular RNA synthesis by Actinomycin D. Unfortunately the replication of BTV is also sensitive to high concentrations of the drug (Verwoerd, 1969) and at concentrations where replication of the virus is unaffected, inhibition of cellular RNA synthesis is incomplete. It was therefore important to determine whether virus-specific RNA synthesis could be differentiated from cellular RNA synthesis at the Actinomycin D concentration used.

Actinomycin D at a final concentration of 0.1 μg/ml was added to two L-cell suspension cultures and one of these infected with BTV. Between 8 and 10 hours after infection both cultures were labelled with ^{14}C-uridine (0.05 μCi/ml). The cytoplasmic fractions of the two cultures were obtained and the RNA components identified by means of sedimentation analysis. The results are shown in Fig. 1. As can be seen both the

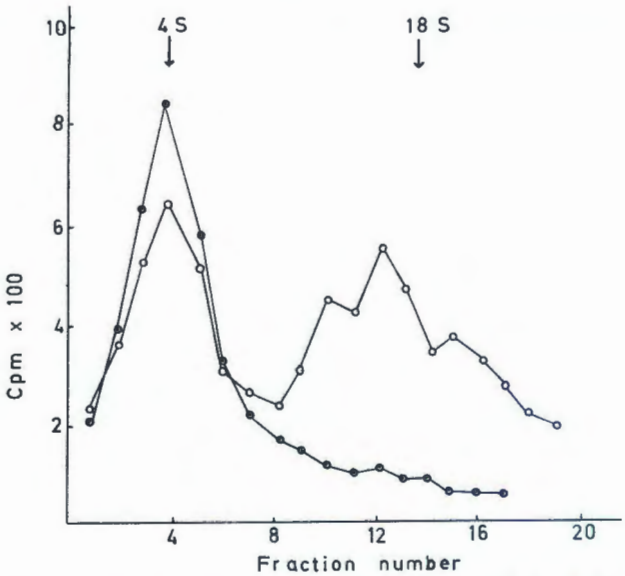

FIG. 1 Sucrose density gradient sedimentation analysis of the RNA synthesized in an infected and an uninfected L-cell suspension culture in the presence of 0.1 μg/ml Actinomycin D. Sedimentation is from left to right. The position of added 4S and 18S markers are indicated by arrows. The infected culture is indicated by (—○—) and the uninfected by (—●—)

infected and uninfected cultures contain a labelled RNA component with a sedimentation constant of 4S. This is probably cellular transfer RNA (tRNA). Cellular ribosomal RNA (18S and 28S) is however unlabelled. Virus-specific RNA sediments in a heterogeneous peak between 10S and 25S. No labelled cellular RNA components are present in this sedimentation range and a good differentiation between labelled virus-specific and cell-specific RNA can therefore be obtained in the presence of 0.1 μg/ml Actinomycin D.

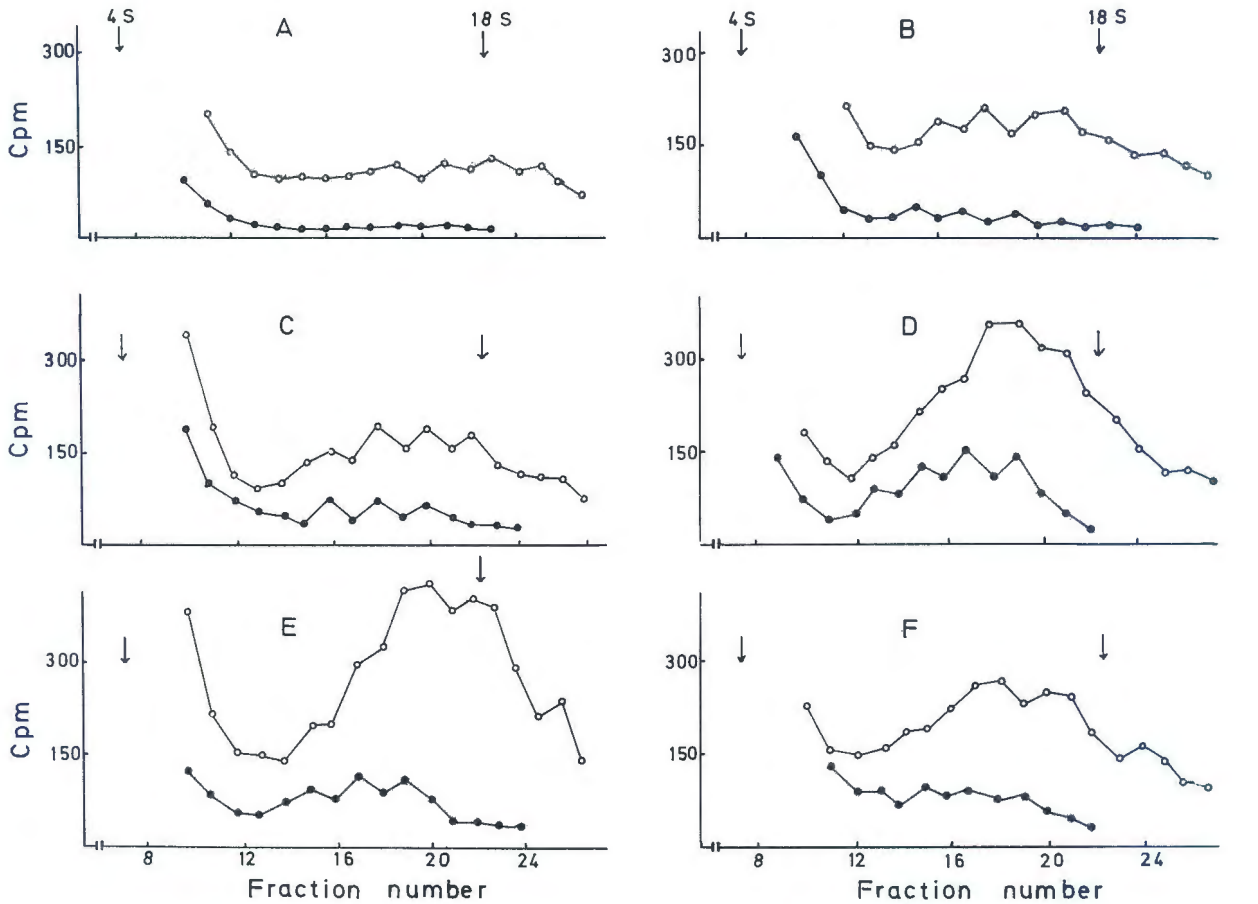

FIG. 2 Sucrose density sedimentation analysis of virus-specific RNA synthesized at different intervals after infection (p.i.). The different labelling periods: A, 1-3½ h p.i.; B, 3½-6 h p.i.; C, 6-8½ h p.i.; D, 8½-11 h p.i.; E, 11-13½ h p.i.; F, 13½-15 h p.i. Distribution of counts before (—○—) and after (—●—) RNase treatment

Kinetics of virus-specific RNA synthesis

It was important to determine whether both single-stranded and double-stranded RNA are synthesized during infection and if so, in what amounts they are produced at various intervals after infection. A suspension culture of L-cells was infected with BTV in the presence of 0.1 μg/ml Actinomycin D. At various intervals after infection samples containing about 10^8 cells were removed from the infected culture and labelled with ^{14}C-uridine (0.05 μCi/ml) for a 2½ hour period. Sucrose gradient sedimentation analysis was used to identify the labelled RNA components in the cytoplasmic extracts. Centrifugation was at 200,000 × *g* and 25°C for 5¾ hours. Half of each of the different cytoplasmic extracts was also treated for 30 min at 37°C with 2 μg/ml pancreatic ribonuclease (RNase) and analysed in the same way. The results are summarized in Fig. 2.

The labelled tRNA peak is not indicated in the figures and only the virus-specific RNA components are shown. As can be seen the virus-specific RNA consists of both RNase-sensitive and RNase-resistant components. Only dsRNA is resistant to RNase treatment, therefore probably both ssRNA and dsRNA are synthesized during infection. Both types of RNA are clearly heterogeneous and comprised of several components with varying sedimentation rates. It can furthermore be seen from Fig. 2 that the rates at which the two types of RNA are synthesized depend on the time after infection.

This result is more clearly demonstrated in Fig. 3.

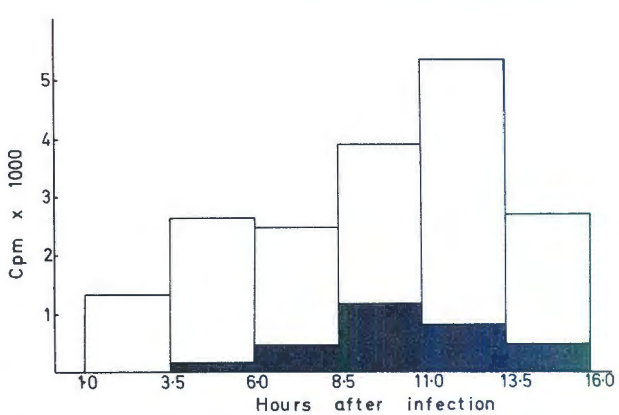

FIG. 3 Variation in rate of incorporation of label into virus-specific RNA at different 2½ hour intervals after infection. The height of the different rectangles indicates the amount of label incorporated into total virus-specific RNA in the relevant pulse period. The fraction of this RNA which is RNase resistant is indicated by the shaded area of these rectangles. The unshaded areas therefore indicate the amount of label incorporated into RNase sensitive ssRNA.

The areas of the different rectangles in the figure are directly proportional to the amounts of label incorporated into virus-specific RNA in the relevant 2½ hour pulse periods. The shaded area of each rectangle indicates the fraction of the virus-specific RNA which is RNase resistant. The incorporation values were calculated from the same experimental results that are

summarized in Fig. 2. The incorporation of label into virus-specific RNA was derived from the total amount of label associated with the RNA in the Fractions 12 to 27 of the centrifugation patterns in Fig. 2. The amount of dsRNA was similarly derived from the label associated with the RNase-resistant RNA in these fractions. All incorporation values were corrected by subtraction of the small amount of label which was present in Fractions 12 to 27 of identically treated non-infected control cultures. These corrections were necessary due to some overlap of the labelled tRNA peak. The difference between the amount of label incorporated into virus-specific RNA and into dsRNA (the unshaded areas in Fig. 3) indicates the amount of ssRNA synthesized in a certain pulse period. It can be seen from Fig. 3 that ssRNA synthesis commences very shortly after infection. The rate of synthesis increases until a small reduction is observed between 6 and 8½ hours after infection. This reduction is followed by a second increase in the rate of ssRNA synthesis until the rate is at its maximum between 11 and 13½ hours after infection. The kinetics of dsRNA synthesis is different. The first detectable amount of dsRNA is formed more than 3½ hours after infection. The rate of synthesis is very much slower than that of ssRNA and reaches a maximum between 8½ and 11 hours after infection.

Isolation of ssRNA

The virus-specific RNA components in Fig. 2 consist of a mixture of double-stranded and single-stranded molecules. In order to attempt a further characterization of the ssRNA it is necessary either to separate the two types of RNA or selectively inhibit the synthesis of dsRNA during the labelling of ssRNA. Selective inhibition of dsRNA synthesis is possible in the case of reovirus by treatment of infected cells with cycloheximide at a late stage in the infection cycle (Watanaba *et al.*, 1967). It was found that the same method could be used for the production of BTV ssRNA but it had the disadvantage that the ssRNA was always contaminated with labelled tRNA which was undesirable in certain experiments. Separation of ssRNA from dsRNA and tRNA, however, yielded satisfactory results. It was observed that a large amount of ssRNA was associated with the polyribosomes in the infected cells and it was therefore possible to obtain the virus ssRNA by extraction from isolated polyribosomes.

The experimental details of this isolation procedure are described under "Materials and Methods". It is known that low concentrations of cycloheximide stabilize polysome complexes by preventing the movement of the ribosomes relative to the messenger RNA (Stanners, 1966). Investigations were carried out to determine whether cycloheximide could also be used in the case of BTV to increase the polysome yield from infected cells. Cycloheximide (final concentration 2 μg/ml) was added 10½ hours after infection and the cells harvested 30 min later. The polyribosomes were extracted and separated from other cell components on a sucrose density gradient. Polyribosomes were identified on the gradient by UV absorbance at 260 nm, and compared to the amount of polyribosomes that could be isolated from BTV-infected cells that were not treated with cycloheximide. The results are shown in Fig. 4. Polyribosomes sediment in the broad heterogeneous peak which follows after the peak of free ribosomes at the 78S position. In Fig. 4 this part of the gradient is between Fractions 13 and 30. The absorbancy readings give a good indication of the amount of

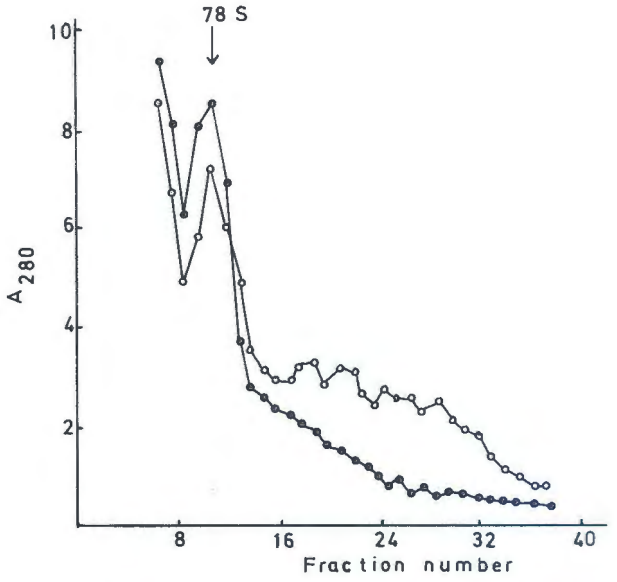

FIG. 4 Influence of cycloheximide on the polyribosome yield from virus-infected cells. The yields are compared by sucrose density analysis of polyribosomes isolated from infected cells that were treated with cycloheximide (—○—) and cells that were not treated with the drug (—●—)

polyribosomes present and as can be seen a considerable increase in the amount of polyribosomes isolated could be obtained by treatment with cycloheximide. This is in agreement with the results obtained in the case of reovirus (Prevec *et al.*, 1968).

For the production of labelled ssRNA the cycloheximide was always added 1½ hours after the start of the 2 hour labelling period between 10 and 12 hours after infection. Single-stranded RNA was extracted from the isolated polyribosomes as described under "Materials and Methods". Treatment of the isolated RNA with 5 μg/ml RNase at 37°C for 30 min indicated that no dsRNA was present. The RNA was still, however, contaminated with a relatively large amount of unlabelled ribosomal RNA.

Characterization of ssRNA

The ssRNA was first characterized by sedimentation analysis on sucrose gradients. Centrifugation was performed at 200,000 × *g* and 25°C for 5 hours in a Spinco SW 50 rotor. The fractionation pattern obtained is shown in Fig. 5. The results indicate that ssRNA can

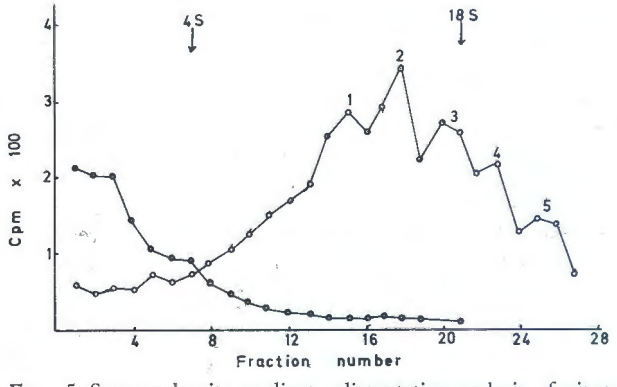

FIG. 5 Sucrose density gradient sedimentation analysis of virus-specific RNA associated with polyribosomes. The distribution of the counts before (—○—) and after (—●—) treatment with pancreatic RNase (2 μg/ml) for 30 min at 37°C is shown

be resolved into at least five components. A comparison of the sedimentation rates of these components with those of cellular tRNA and 18S ribosomal RNA gave approximate sedimentation constants of 12S, 14.5S, 17.5S, 20S and 23S for the Peaks 1 to 5 respectively. However, the different peaks in Fig. 5 are not homogeneous, therefore the sedimentation analysis yields little new information apart from indicating that the ssRNA is also comprised of RNA fragments of different molecular weights.

An attempt was made to obtain a better resolution by separating the various ssRNA components by means of electrophoresis on polyacrylamide gels. The result is shown in Fig. 6. At least six components can

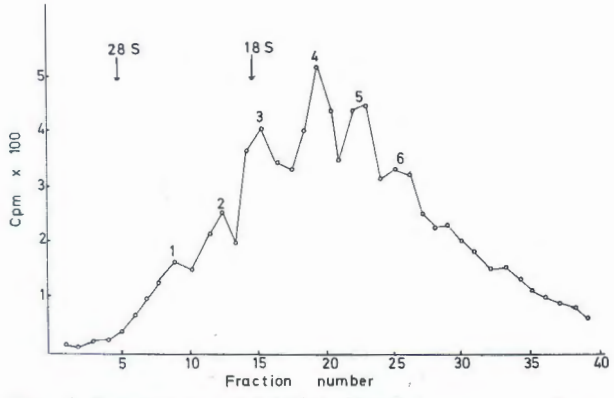

FIG. 6 Fractionation of labelled ssRNA by means of electrophoresis, on polyacrylamide gels. Migration is from left to right. The migration of labelled 18S and 28S ribosomal RNA markers is indicated by the arrows

be identified. If the distance of electrophoretic migration of these components is compared to those of the ribosomal RNA markers (18S and 28S), the sedimentation constants of the components, numbered 1 to 6 in Fig. 5, correspond approximately to respective mean values of 22.5S, 20S, 17.5S, 15S, 13S, and 12S. This compares very well with the values that were obtained by sedimentation analysis, except that the 14.5S and 12S components on sucrose density gradients (Fig. 5) are resolved into the three components 15S, 13S and 12S during gel electrophoresis. The resolution is, however, still unsatisfactory. The different peaks overlap, making an accurate estimation of the total number of components, and the relative amounts in which they are synthesized, impossible.

Unsatisfactory resolution in both cases is probably due to the contamination of the ssRNA with unlabelled ribosomal RNA. The interaction between these RNA molecules could influence both the sedimentation rate on sucrose density gradients and the electrophoretic mobility during electrophoresis.

Characterization of dsRNA by means of optical rotatory dispersion

For the investigation of the relationship between single-stranded and double-stranded RNA by means of hybridization experiments the isolation of dsRNA from infected cells was a prerequisite. The method employed has been described previously (Verwoerd & Huismans, 1969). Although the double-strandedness of the RNA isolated in this way has been proved convincingly by means of thermal denaturation curves, the resistance to RNase degradation and the base composition of the RNA (Verwoerd, 1969) it was decided to verify these results once more by investigation of the ORD characteristics of the molecule.

There is a characteristic difference between the ORD patterns of deoxyribonucleic acid (DNA), ssRNA and dsRNA. The multiple Cotton effects of nucleic acids have three peaks and two troughs in the spectrum range 190 to 300 nm. DNA differs from RNA in that DNA has a strong positive second peak near 228 nm whereas RNA has a rotation peak near zero at about 225 nm (Samejima & Yang, 1965). Single-stranded RNA differs from dsRNA mainly in the intensity of the peak at 280 nm. The magnitude of this peak for dsRNA is higher than that recorded for any ssRNA (Samejima, Hashizume, Imahori, Fujii & Miura, 1968). This characteristic difference makes an immediate identification of dsRNA possible.

The ORD of BTV RNA was investigated at different temperatures. Fig. 7 shows the ORD curves of dsRNA at 40°C and 90°C and at 70°C after slow

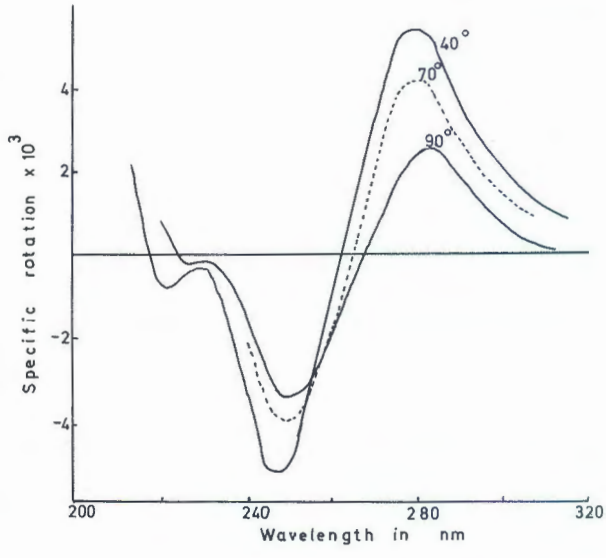

FIG. 7 Optical rotatory dispersion of BTV dsRNA at different temperatures. The ORD patterns shown are those at 40°C and 90°C as well as the pattern that was obtained after the dsRNA solution was allowed to cool slowly from 90°C to 70°C

cooling from 90°C. The pattern at 40°C, characterized by a high magnitude peak at 280 nm and a peak near zero at 225 nm, is typical for dsRNA in the region 210 to 300 nm. This pattern remained unchanged until a temperature of 78°C was reached. Further increase in the temperature resulted in the denaturation of the dsRNA into two ssRNA molecules, a change which was represented in the ORD pattern of the dsRNA by a decrease in the magnitude of the peak of 280 nm. The magnitude of this peak at 90°C in Fig. 7 is typical for ssRNA. After slow cooling from 90°C to 70°C partial renaturation of the complementary ssRNA molecules took place, resulting in a corresponding increase in the magnitude of the peak at 280 nm.

Hybridization of ssRNA and dsRNA

Its association with polyribosomes already indicated that the ssRNA functions as mRNA during viral protein synthesis. Additional proof is, however, still required. If the ssRNA is indeed mRNA, each of the ssRNA components should be complementary to a corresponding length of one of the strands of the

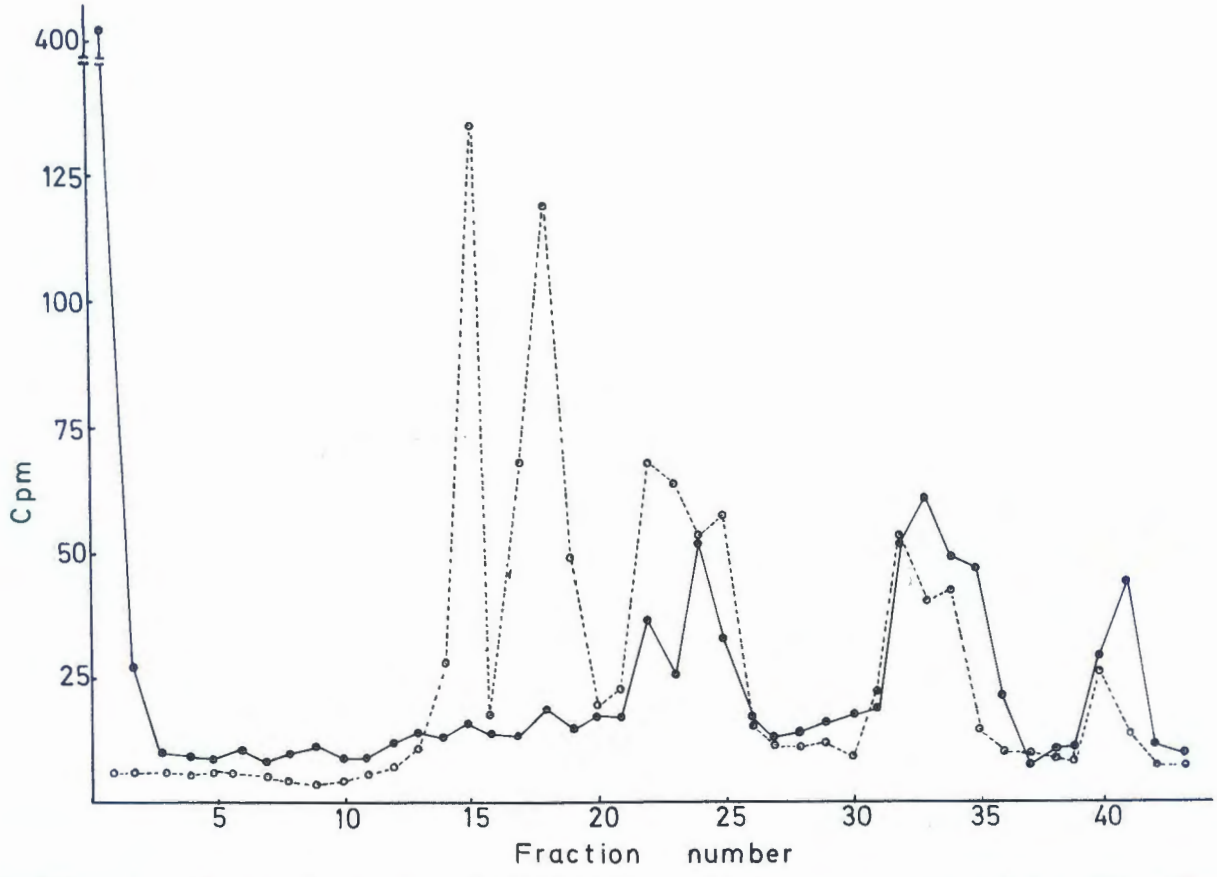

FIG. 8 Comparison of the electrophoretic patterns of labelled BTV dsRNA and the hybridization product of labelled ssRNA and denatured unlabelled dsRNA. Migration is from left to right. dsRNA is indicated by (—○—) and the hybridization product by (—●—)

various dsRNA components. Earlier hybridization experiments (Verwoerd & Huismans, 1969) suggested complementarity, but the hybridization product was not investigated or proved to be identical to dsRNA.

Labelled ssRNA and unlabelled dsRNA were hybridized and the hybridization product isolated as described under "Materials and Methods". The hybrid was characterized by electrophoresis on polyacrylamide gels. Fig. 8 shows a comparison of purified BTV dsRNA and the hybridization product. The ten dsRNA fragments of BTV RNA are under these conditions resolved in only five main peaks. The three lowest molecular weight components of these correspond to the components present in the hybridization product. The first two high molecular weight components are apparently absent. The hybrid also contained a component which moved only a very short distance into the gel and was therefore present in the top fraction. This component was repeatedly present in different preparations even after RNase treatment of the hybrid, and therefore probably consists of aggregates of dsRNA.

The results indicate that, with the possible exception of the two high molecular weight components, one of the strands of every one of the BTV dsRNA fragments is copied into a corresponding length of ssRNA.

DISCUSSION

An investigation was made of virus-specific RNA synthesis in BTV-infected cells. Actinomycin D was used to suppress most of the host cell RNA synthesis. The residual tRNA synthesized in the presence of Actinomycin D could be differentiated from virus-specific RNA by means of sedimentation analysis on sucrose density gradients.

It was shown that both ssRNA and dsRNA are synthesized after infection. The ssRNA was found to be associated with the polyribosomes in the infected cells. This suggested that the ssRNA functions as mRNA and is therefore synthesized by transcription of the viral dsRNA. This result would explain why ssRNA synthesis commences shortly after infection but attains its maximum rate of synthesis after that of dsRNA and also after most of the new infective virus particles have been synthesized (Huismans, 1970). The ssRNA synthesis that occurs shortly after infection is probably due to transcription of the parental virus genome. Between 6 and 11 hours after infection, a large amount of dsRNA is synthesized and this RNA also appears to be active as template for mRNA synthesis. This gives rise to the large increase in the rate of mRNA synthesis between $8\frac{1}{2}$ and $13\frac{1}{2}$ hours after infection.

The association of ssRNA with the polyribosomes in infected cells was utilized to isolate labelled ssRNA uncontaminated by tRNA or dsRNA. Cells were labelled between 10 and 12 hours after infection and the ssRNA isolated from the polyribosomes after treatment of the cells with cycloheximide 30 min before the end of the labelling period. The cycloheximide treatment significantly increased the amount of polyribosomes that could be isolated.

The ssRNA isolated in this way was shown to be heterogeneous and could be fractionated by sucrose gradient sedimentation analysis into at least five components with sedimentation constants in the sedimentation range 12S to 23S. However, it was not possible

to obtain a very satisfactory separation of all the different ssRNA components either by sucrose gradient analysis or by polyacrylamide gel electrophoresis. This made it impossible to establish with certainty the exact number of ssRNA components and the relative amounts in which they are synthesized. It is possible that relatively less of the two highest molecular weight components 20S and 22S are formed. This would mean that the rate of transcription of the different dsRNA components is controlled independently and that regulation of viral protein synthesis may well be exerted on a transcription level.

Further proof of the double-strandedness of BTV dsRNA was obtained by ORD measurements. The ORD pattern of BTV dsRNA showed a multiple Cotton effect with two peaks at 280 and 225 nm and a trough at 250 nm in the spectrum range 200 to 300 nm. The magnitudes of the peaks are in close agreement with those reported for the ORD of the dsRNA isolated from rice dwarf virus (Samejima *et al.*, 1968). The pattern appears to be characteristic for dsRNA. After heat denaturation of the dsRNA the ORD curve resembled that of ssRNA as shown by the decrease in magnitude of the peak at 280 nm at temperatures higher than 78°C.

The relationship between ssRNA and dsRNA was investigated by means of hybridization experiments. The results provided proof that the ssRNA consisted of copies of one of the strands of at least three of the five main dsRNA components of the virus. The absence of the two highest molecular weight components in the hybrid could possibly indicate that transcription of these fragments did not take place. However, this has not been proved satisfactorily and several other explanations could be advanced.

It is possible that the RNase-resistant component, which remained in the first fraction of the gel after a 4 hour electrophoresis period, consisted of aggregates of the absent dsRNA fragments in the hybridization product. It is difficult to explain, however, why only the high molecular weight components would form these aggregates. It could perhaps be due to incomplete hybridization of the dsRNA with the ssRNA, leading to the formation of "sticky ends", i.e. ssRNA sequences of a dsRNA fragment which could cross-link with other ssRNA pieces. In this way larger molecular weight molecules or aggregates could be formed. It is also quite reasonable to assume that incomplete hybridization and its secondary effects are more likely to occur with the larger molecular weight fragments.

Another explanation is that the different dsRNA fragments are not transcribed in equal amounts and that relatively more of the lower molecular weight molecules are synthesized. This would result in a corresponding larger proportion of the lower molecular weight dsRNA fragments in the hybridization product. As mentioned before, unequal transcription of the dsRNA genome is quite possible and it does appear in fact as if the higher molecular weight components of the dsRNA are copied less frequently than others. Due to the incomplete separation of the various ssRNA components it is, at this stage impossible to make a conclusive deduction in this respect.

Summary

A study of the virus-specific ribonucleic acid (RNA) components in bluetongue virus infected cells revealed that both double-stranded and single-stranded RNA are synthesized during infection. The single-stranded RNA is formed in a large excess of double-stranded RNA and the rate of synthesis is maximal between 10 and 13 hours after infection, about 2 hours later than for double-stranded RNA. The single-stranded RNA was found to be associated with the polyribosomes in infected cells and this characteristic was utilized for the isolation of the RNA. Single-stranded RNA consists of various components with sedimentation constants varying between 12S and 22S. These components are probably messenger RNA, as was shown by hybridization of single-stranded RNA with denaturated virus double-stranded RNA. Optical rotatory dispersion analysis of the viral double-stranded RNA components in infected cells verified the secondary structure of the bluetongue virus genome RNA.

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