Evidence for a Divided Genome in Nodamura Virus, an Arthropod-borne Picornavirus

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SUMMARY

This paper describes the physico-chemical properties of Nodamura virus. The virus infects mice and high yields (10⁶ LD₅₀/mg) were obtained from the muscle tissue. To allow for greater ease and accuracy in analysing the RNA, the virus was labelled by inoculating mice with [³²P]-phosphate during the incubation period. Purified virus was obtained from the muscle extracts by ammonium sulphate precipitation, differential ultracentrifuging and sedimentation of the SDS-disrupted virus pellet in a sucrose gradient. The virus particles were 29 nm in diam., sedimented at approximately 135 S in sucrose gradients and contained two species of RNA which sedimented at 22 S and 15 S. These values correspond to mol. wt. of 1.0 and 0.5 × 10⁶. Similar mol. wt. were obtained by polyacrylamide gel electrophoresis. The base ratios of the two RNA species were: 22 S: A = 22.4, C = 27.0, G = 27.6, U = 23.0; 15 S: A = 24.8, C = 28.2, G = 22.8, U = 24.2. Each RNA had low infectivity in mice but this was enhanced about 100-fold by mixing the two species. The RNAs may be present in two distinct particles since extraction of the 135 S peak with phenol gave the 22 S RNA alone, whereas phenol-SDS extraction liberated both the 22 S and 15 S species. Centrifuging in caesium chloride also fractionated the virus into components which contained either the 22 S or 15 S RNA. Preliminary evidence suggests that there is one major polypeptide, mol. wt. 35 × 10⁶, and two minor polypeptides in the virus.

INTRODUCTION

Nodamura virus is an arthropod-transmissible virus which is resistant to lipid solvents (Scherer & Hurlbut, 1967; Scherer, Verna & Richter, 1968) and resembles the vertebrate enteroviruses in its morphology, resistance to acid and buoyant density in caesium chloride (Murphy et al. 1970). The virus can multiply in mosquitoes, ticks and moth larvae without killing the host. Bees and wax moths are also susceptible to infection but these species die as a result of the infection (Bailey & Scott, 1973). Infected mosquitoes can transmit the virus to infant mice, which also die as a result of the infection. There is no certainty that there is a natural vertebrate host, but high levels of neutralizing antibody were found in pigs in Japan suggesting that this animal species may be ecologically associated with Nodamura virus (Scherer et al. 1968).

While the available information on the virus suggests that it belongs to the family Picornaviridae, decisive evidence is lacking, even concerning the nature of the nucleic acid. Its classification and relationship to other picornaviruses is thus obscure. The original
purpose of the work to be described was to provide information on the virus so as to estab-
ish its relatedness to other viruses. We have shown that the virus does, in fact, contain RNA
but of greater significance is our finding that it differs from other vertebrate and invertebrate
small RNA viruses in containing two species of RNA. Evidence has been obtained which
suggests that the two species may be present in different components of the virus.

METHODS

Growth of virus. Brain tissue from suckling mice was received as a frozen suspension from
Dr F. A. Murphy, Center for Disease Control, Atlanta, Georgia, U.S.A. This suspension
had an infectivity titre of $10^{6.9} \text{LD}_{50}/0.03 \text{ml}$ when inoculated intraperitoneally into 1- or
7-day old mice. It was passaged by inoculating the $10^{-5}$ dilution (i.e. $10^{5.5} \text{LD}_{50}$) intraperitoneally into 7-day-old mice and the muscle tissue removed from the mice as they became
paralysed. Virus labelled with $^{32}$P was obtained by inoculating each mouse intraperitoneally
with 10 to 40 $\mu$Ci $^{32}$P-phosphate, on one or more occasion, during the incubation period.

Purification of virus. The infected muscle tissue from paralysed mice was extracted with a
mixture of 0.04 M-phosphate, pH 7.6, and carbon tetrachloride by grinding in the presence
of sand. This gave the best yield of virus ($10^6$ to $10^7 \text{LD}_{50}/0.03 \text{ml}$). Brain tissue contained
only about 1% of this amount of infective virus. Insoluble debris was removed by centri-
fuging at 2000 g for 5 min and the supernatant fluid was then precipitated with 50% saturated ammonium sulphate. The precipitate was separated by centrifuging at 2000 g for
30 min and then dissolved in 0.04 M-phosphate, pH 7.6. After clarification at 2000 g, the
virus was pelleted by centrifuging at 60000 g for 1 h. The pellet was resuspended in 0.04
M-phosphate and the insoluble debris removed at 2000 g. The supernatant fluid was made
1%, with SDS and centrifuged for 2 h in a 15 to 45% sucrose gradient in 0.04 M-phosphate or
tris-HCl, pH 7.6, at 30000 rev/min using the 3 x 23 ml rotor of the MSE ultracentrifuge.
The position of the virus was ascertained by infectivity, extinction and radioactivity measure-
ments. Recovery of virus at the different stages of the purification procedure was measured
by titrating the fractions in mice.

Sedimentation coefficient of the virus. A mixture of purified $^{32}$P-labelled Nodamura virus
and $^{3H}$-uridine labelled foot-and-mouth disease virus was centrifuged in a 15 to 45% sucrose gradient for 2 h at 30000 rev/min and the distribution of the two isotopes then
determined.

Fractionation of the virus in caesium chloride. The $^{32}$P-labelled virus was centrifuged in
preformed gradients prepared in tris or phosphate buffer, pH 8, and ranging from 1.30 to
1.70 g/ml. Centrifuging was continued for 6 to 17 h and the infectivity, radioactivity and
refractive index of the individual fractions was determined.

Sedimentation analysis of the virus RNA. The RNA was extracted by mixing the virus with
phenol alone, phenol-SDS or 2 M-guanidine. The aqueous layer, in the case of the phenol
extraction procedures or the entire solution in the case of the guanidine disrupted virus, was
then centrifuged immediately at 20000 rev/min for 16 h in 5 to 25% sucrose gradients
prepared in 0.1% SDS-0.1 M-acetate, pH 5.0. Alternatively, the RNA was first precipitated
in the presence of added BHK cell RNA by the addition of 2 vol. ethanol at $-20 \text{°C}$. After
storing overnight at $-20 \text{°C}$, the precipitate was collected at 2000 g and dissolved in 0.1%
SDS-0.1 M-acetate, pH 5.0, for subsequent sedimentation analysis. In some experiments the
precipitated RNA was resuspended in 1.1 M-formaldehyde in 0.01 M-phosphate, pH 7,
and heated at 63 °C for 15 min. The solution was then cooled rapidly in ice and centrifuged immediately at 20000 rev/min for 16 h in 5 to 25% sucrose gradients prepared in
Table 1. *Purification of Nodamura virus*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Vol (ml)</th>
<th>Infectivity (I.D&lt;sub&gt;50&lt;/sub&gt;/0.03 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle extract</td>
<td>50</td>
<td>7.8</td>
</tr>
<tr>
<td>50 % saturated ammonium sulphate precipitate</td>
<td>50</td>
<td>8.2</td>
</tr>
<tr>
<td>30000 rev/min pellet</td>
<td>2</td>
<td>8.6</td>
</tr>
<tr>
<td>Pellet in 1 % SDS</td>
<td>2</td>
<td>8.8</td>
</tr>
<tr>
<td>Peak fraction from sucrose gradient</td>
<td>1</td>
<td>8.4</td>
</tr>
</tbody>
</table>

Polyacrylamide gel electrophoresis of the virus RNA. The RNA was analysed in 2.4 \% polyacrylamide gels containing 0.4 \% agarose. RNA suspended in 50 μl of electrophoresis buffer containing 18 \% sucrose and 0.2 \% SDS was electrophoresed in 9 cm gels at 7 mA/gel for 2.5 h. The gels were frozen, sliced into 2 mm fractions with a gel slicer and the slices counted on a Panax solid scintillation counter. For mol. wt. determinations, BHK cell RNA was included as marker.

Analysis of base composition. Precipitated RNA was hydrolysed in 0.3 N-KOH for 18 h at 37 °C. After neutralization with HClO₄, the nucleotides were separated by the paper electrophoresis method described by Davidson & Smellie (1952). The dried papers were viewed under a Hanovia u.v. lamp and the light-absorbing zones were then cut from the papers and counted in vials containing PPO-POPOP in toluene, using a Packard Liquid Scintillation Counter, Model No. 3310.

Infectivity of the RNA. Samples (0.03 ml) of the RNA, prepared directly from virus by extraction with phenol-SDS or collected from sucrose gradients were diluted in sterile 0.04 M-phosphate pH 7.6 and inoculated intracerebrally into 7-day-old mice. The animals were observed daily for signs of paralysis.

Polyacrylamide gel electrophoresis of virus protein. The intact virus or separated virus protein was analysed in SDS-acrylamide gels as described in detail by Burroughs et al. (1971).

**RESULTS**

**Purification of [³²P]-labelled virus**

Each of the procedures used in the purification of the virus, namely 50 \% saturated ammonium sulphate precipitation of the mouse muscle extracts, differential ultra-centrifuging and sedimentation in a sucrose gradient following treatment of the pelleted virus with 1 \% SDS, gave good recovery of virus (Table 1). A sharp peak of [³²P] was obtained in the sucrose gradient which coincided with the peaks of infectivity and extinction (Fig. 1(a)). Although the recovery of infectivity was good, the titre of the purified preparations fell rapidly when stored at 4 °C. In view of this instability, subsequent experiments were made with freshly purified preparations whenever possible.

The sedimentation coefficient of the virus in sucrose gradients, relative to that for foot-and-mouth disease virus, was determined by centrifuging a mixture of [³²P]-Nodamura virus and [³H]-uridine labelled foot-and-mouth disease virus for 2 h at 30000 rev/min in a 15 to
Fig. 1. (a) Sedimentation of [3P]-labelled Nodamura virus in a 15 to 45% sucrose gradient showing the distribution of infectivity, radioactivity and absorbency. The virus was centrifuged at 30,000 rev/min for 2 h. Fractions 1 to 8 and 14 to 19 were combined in pairs, 1 + 2, 3 + 4, etc., for titration, the remaining fractions were titrated individually; (b) co-sedimentation of purified [3P]-labelled Nodamura virus and [3H]-uridine labelled foot-and-mouth disease virus under the same conditions as in (a), showing the distribution of radioactivity. ■ — ■, infectivity; ●—●, E_{260}; ○—○, [3P]; ▲—▲, [3H].

45% sucrose gradient. From the distribution of radioactivity (Fig. 1(b)) and accepting a value of 146S for the sedimentation coefficient of foot-and-mouth disease virus (Strohmaier, 1971), the relative sedimentation coefficient for Nodamura virus was estimated to be 135S. A portion of the peak fraction from the preparative sucrose gradient was examined in the
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Fig. 2. Electron micrograph of Nodamura virus stained with phosphotungstic acid.

electron microscope. Particles with a diam. of 29 nm were observed (Fig. 2). This diam. is the same as that reported by Murphy et al. (1970).

Nature of the virus nucleic acid

Murphy et al. (1970) concluded that Nodamura virus was a picornavirus on the basis of its size, morphology and buoyant density in caesium chloride. We have now obtained direct evidence that the virus contains RNA by examining the aqueous layer following extraction of the $^{32}$P-labelled virus with SDS-phenol. The aqueous extract was centrifuged in a 5 to 25 % sucrose gradient in 0.1 M-acetate-0.1 % SDS and the distribution of radioactivity examined. The profile in Fig. 3 (a) shows the presence of two species of RNA, both of which were hydrolysed to slowly sedimenting molecules by treatment with 0.1 µg ribonuclease per ml (Fig. 3(b)).

When phenol alone was used for the extraction, about 30 % of the $^{32}$P was released into the aqueous layer. Two subsequent extractions of the phenol layer with STE (0.15 M-NaCl, 0.01 M-tris, 10^{-3} M-EDTA, pH 7.6) released up to a further 10 % of the radioactivity. However, if this was followed by extraction of the phenol layer with STE containing 1.0 % SDS almost all the remaining $^{32}$P was released into the aqueous layer. A typical fractionation is given in Table 2.

The RNA released into the aqueous layer by extracting the virus with phenol alone gave a single peak at 22S in sucrose gradients (Fig. 3(c)) compared with the two peaks obtained with phenol-SDS extracts. The 15S peak was not produced from the 22S species by the extraction procedure since shaking the 22S RNA with phenol-SDS did not alter its rate of sedimentation. Treatment of the virus with 2 M-guanidine, a method we have used for extracting the RNA from foot-and-mouth disease virus, also released the two species of RNA (Fig. 3(d)).

As it seemed possible that the 15S RNA could have arisen from the 22S by cleavage within virus particles formed early during the growth of the virus, preparations were made from mice which received the $^{32}$P-phosphate either on the first day after inoculating the virus or on the day prior to onset of paralysis and collection of the mouse tissue. The viruses were purified and the RNA extracted by phenol-SDS was analysed by sucrose gradient centrifuging. Both preparations gave essentially the same profile of radioactivity (Fig. 4(a), (b)).
Fig. 3. Sedimentation of $[^{32}\text{P}]$-labelled Nodamura virus RNA and BHK cell RNA in a 5 to 25% sucrose gradient showing the distribution of extinction and radioactivity. The RNA was centrifuged at 20000 rev/min for 16 h. (a) RNA extracted with SDS-phenol; (b) RNA treated with 0.01 µg ribonuclease per ml before sedimentation; (c) RNA extracted with phenol only; (d) RNA released with 2 M-guanidine. ○—○, $E_{260}$; □—□, $[^{32}\text{P}]$.

Table 2. Extraction of RNA from $[^{32}\text{P}]$-labelled Nodamura virus

<table>
<thead>
<tr>
<th>Sample</th>
<th>$[^{32}\text{P}]$, (ct/min)</th>
<th>% original radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus</td>
<td>90500</td>
<td>100</td>
</tr>
<tr>
<td>Aqueous layer after phenol extraction</td>
<td>28570</td>
<td>32</td>
</tr>
<tr>
<td>First STE wash of phenol layer</td>
<td>4430</td>
<td>5</td>
</tr>
<tr>
<td>Second STE wash of phenol layer</td>
<td>3430</td>
<td>4</td>
</tr>
<tr>
<td>First SDS-STE wash of phenol layer</td>
<td>38950</td>
<td>43</td>
</tr>
<tr>
<td>Second SDS-STE wash of phenol layer</td>
<td>7560</td>
<td>8</td>
</tr>
</tbody>
</table>

Similarly, virus was heated at 37 °C for 24 h (during which time more than 2 logs of infectivity were lost) prior to extraction of the RNA. This preparation gave a profile which was different from that obtained with RNA from freshly prepared virus in that there was proportionately less radioactivity in the 22 S peak. These ‘lost’ counts appeared as a shoulder on the light side of the 15 S peak but there was no conversion of 22 S to 15 S molecules.

The base composition data suggest that the 22 S RNA is not converted to the 15 S RNA since the base compositions of the two species would be the same if one 22 S RNA molecule was converted to two molecules of 15 S RNA. In fact, they differed quite markedly (Table 3). However, if breakdown of the 22 S RNA to 15 S RNA and small fragments were occurring, the two RNAs could have different base compositions. In the event of this type of breakdown, however, we would expect to detect the small breakdown products in sucrose gradients of the isolated RNA.
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Fig. 4. Sedimentation of $^{32}$P-labelled Nodamura virus RNA and BHK cell RNA in a 5 to 25% sucrose gradient showing the distribution of absorbency and radioactivity. The RNA was centrifuged at 20000 rev/min for 16 h. (a) RNA prepared from virus labelled during first 24 h of incubation period; (b) RNA prepared from virus labelled during last 24 h of incubation period. ●—●, $E_{260}$; ○—○, $^{32}$P.

Mol. wt. of the two RNA species

The relative sedimentation coefficients of the RNAs in sucrose gradients were determined by sedimenting $^{32}$P-labelled virus RNA with BHK cell RNA. The distribution of radioactivity and extinction showed that the virus RNAs sedimented at 22S and 15S, compared with 28S and 18S for the cell RNAs (Fig. 3(a)). These relative sedimentation coefficients for the virus RNAs correspond to mol. wt. of 1.0 and $0.5 \times 10^6$, respectively (Spirin, 1964). Similar values were obtained by polyacrylamide gel electrophoresis (Fig. 5).

When the RNAs were first treated with formaldehyde at low ionic strength before centrifuging, as described by Boedtker (1968), values of 17S and 12S were obtained. These values
Table 3. Base composition of Nodamura virus RNAs

<table>
<thead>
<tr>
<th>RNA species</th>
<th>Adenylic acid</th>
<th>Cytidylic acid</th>
<th>Guanylic acid</th>
<th>Uridylic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>22S</td>
<td>22.4 ± 0.3</td>
<td>27.0 ± 0.5</td>
<td>27.6 ± 0.9</td>
<td>23.0 ± 1.0</td>
</tr>
<tr>
<td>15S</td>
<td>24.9 ± 0.3</td>
<td>28.2 ± 0.8</td>
<td>22.8 ± 1.4</td>
<td>24.1 ± 0.9</td>
</tr>
</tbody>
</table>

Fig. 5. Polyacrylamide gel electrophoresis of [$^{32}$P]-Nodamura virus RNA showing the distribution of radioactivity. The position of marker BHK cell RNA is shown by the arrows. The RNA was electrophoresed in 9 cm gels for 2.5 h at 7 mA/gel. ○-○, [$^{32}$P].

correspond to mol. wt. of about 2 and 1 x 10^6. While we cannot be certain of the reason for the discrepancy in the values obtained with the different centrifuging methods, work with a number of RNAs has shown that the extent of unfolding of the RNA molecules with formaldehyde is influenced by the GC content. With RNAs possessing high GC content (e.g. 28S RNA, 64%), the rate of sedimentation following treatment with formaldehyde is higher than expected, whereas those RNAs with a lower GC content behave normally (Newman, Rowlands & Brown, 1973). Nodamura virus RNA has a GC content which is somewhat higher than those of, for example, poliovirus and the rhinoviruses, which give the same mol. wt. by both methods.

Infectivity of the RNAs

The infectivity of the virus RNAs can be measured by intracerebral inoculation of 7 day old mice. As with most of the picornaviruses we have examined, 10^8 LD$_{50}$ of virus gave about 10^{2.5} LD$_{50}$ of RNA. All of this infectivity was precipitated by 2 vol. ethanol at -20°C and could be recovered quantitatively.

The RNA extracted from [$^{32}$P]-labelled virus was precipitated with 2 vol. ethanol in the presence of cell RNA. The re-dissolved precipitate was then centrifuged in a 5 to 25% sucrose gradient in 0.1 M-acetate-0.1% SDS. Samples of each fraction were assayed for radioactivity and infectivity (Fig. 6). The distribution of these activities showed that
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whereas two clearly defined peaks of \(^{32}P\) were obtained, the infectivity sedimented as a broad band with the maximum infectivity midway between the two radioactive peaks. This suggested that, as with the split genome plant viruses, each RNA had low infectivity which was enhanced by mixing with the second species of RNA. To test this idea, each peak of RNA (fraction 10 of the 22 S RNA and fraction 16 of the 15 S RNA; Fig. 6) was precipitated with 2 vol. ethanol and recycled on a separate gradient. The infectivity of each RNA peak, alone or mixed with the second species, was then measured.

The results of four experiments of this type showed that each RNA had low infectivity which was enhanced 10- to 100-fold by mixing with the second species of RNA (Table 4).

### Table 4. Infectivity of Nodamura virus 22 S and 15 S RNA singly and together

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Infectivity of RNA (log LD(_{50})/0.03 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>22 S</td>
</tr>
<tr>
<td>1</td>
<td>1.3</td>
</tr>
<tr>
<td>2</td>
<td>2.3</td>
</tr>
<tr>
<td>3</td>
<td>2.1</td>
</tr>
<tr>
<td>4</td>
<td>0.3</td>
</tr>
<tr>
<td>Non-infective*</td>
<td>0.1</td>
</tr>
<tr>
<td>Non-infective†</td>
<td>3.2</td>
</tr>
</tbody>
</table>

* 10-fold dilution of RNA. † 100-fold dilution of RNA.

In one experiment, dilutions of the 22 S and 15 S RNAs which were non-infective killed mice when mixed before inoculation. In another experiment in which the 22 S RNA obtained by
extracting the virus with phenol alone was mixed with 15S RNA prepared from SDS-phenol extracted RNA, the infectivity of the individual RNAs was enhanced more than 100-fold (Table 4).

Origin of the two RNA species

The preferential extraction of the 22S RNA with phenol pointed to the presence of two distinct components in the virus, one containing the 22S species and the second containing the 15S RNA. However, it was not possible to separate the virus into two components by sucrose gradient centrifuging. A single sharp peak was obtained (Fig. 1(a)) and the extracts prepared from individual fractions of the peak contained the same proportions of the two RNA species. However, caesium chloride gradient centrifuging gave separations which provided further evidence for the presence of more than one component.

Murphy et al. (1970) found a single infective peak at 1.34 g/ml when an extract of infected mouse muscle was centrifuged overnight in a caesium chloride gradient. We found that freshly purified [32P]-virus gave a single radioactive peak at the same density in a caesium chloride gradient buffered at pH 8 with either tris or phosphate. The major proportion of the infectivity was also present at 1.34 g/ml but a small amount of infectivity was found at about 1.55 g/ml (Fig. 7(a)). The overall recovery of infectivity was about 1%, irrespective of whether freshly purified virus or crude unfractionated muscle extract was used.

Purified [32P]-virus which had been stored for several days at 4 °C gave more than one radioactive peak. The results were somewhat variable but in most cases only a small amount of radioactivity was present at 1.34 g/ml and the major proportion was found in two peaks at about 1.6 and 1.5 g/ml. The peak at about 1.6 g/ml was quite clearly heterogeneous (Fig. 7(b)). So far we have been unable to determine the role of the separate peaks in infection because we did not recover any infectivity from caesium chloride gradients of stored preparations of purified virus. The high density values obtained for the two peaks indicated that protein was being detached from the virus during the centrifuging, since particles which had been fixed with 0.05% formaldehyde had a density similar to that of the freshly prepared virus.

The peaks at 1.6 and 1.5 g/ml in Fig. 7(b) were diluted with tris buffer and extracted with SDS-phenol. After mixing with BHK cell RNA and precipitating with 2 vol. ethanol at −20 °C, the preparations were centrifuged in 5 to 25% sucrose gradients. The 1.6 g/ml peak contained 22S RNA, whereas the 1.5 g/ml peak contained 15S RNA, indicating that the two peaks in the caesium chloride gradient were derived from different virus components (Fig. 8).

Polyacrylamide gel electrophoresis of virus protein

One major band (mol. wt. 35 x 10³) and two minor bands were obtained by polyacrylamide gel electrophoresis on SDS-gels. The virus thus resembles vesicular exanthema virus and the feline picornaviruses (J. N. Burroughs, personal communication) in its protein composition rather than the viruses of the enterovirus, cardio, rhino and foot-and-mouth disease virus subgroups (Brown & Hull, 1973).

DISCUSSION

Although Nodamura virus possesses several biological characteristics typical of the arboviruses, Scherer & Hurlbut (1967) showed the exceptional nature of this virus by demonstrating its resistance to ether and to chloroform. Murphy et al. (1970) characterized the virus as a picornavirus on the basis of its size, morphology and density in caesium chloride. However, they did not determine the type of nucleic acid in the virus and the initial purpose
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Fig. 7. Sedimentation of (a) freshly purified [32P]-labelled virus in a caesium chloride gradient buffered at pH 8 with tris-HCl showing the distribution of radioactivity and infectivity. Fractions 1 to 12 were combined in pairs 1 + 2, 3 + 4, etc., for titration; the remaining fractions were titrated individually. (b) [32P]-virus which had been stored for several days at 4 °C before centrifuging in caesium chloride buffered with tris at pH 8. ■—■, infectivity; ○—○, [32P]; □—□, density.
Fig. 8. Sedimentation of RNA prepared from the $[^{32}P]$-components equilibrating at densities of 1.58 to 1.63 and 1.51 g/ml as in Fig. 7(b). (a) 1.58 to 1.63 g/ml component RNA; (b) 1.51 g/ml component RNA; $\bigcirc$ --- $\bigcirc$, $[^{32}P]$. 

1.6 g/ml component

22S

1.5 g/ml component

15S
of this work was to examine the properties of the virus so that its relatedness to other picornaviruses could be established.

The results described in this paper clearly establish Nodamura virus as a small RNA-containing virus. However, our results also show that it differs from all the small RNA viruses of vertebrates and invertebrates so far described in containing two species of RNA. The two species of RNA, sedimenting at 22S and 15S in sucrose gradients, were isolated from purified virus particles by extracting with phenol in the presence of SDS. It seems unlikely that the 15S species is a breakdown product of the 22S RNA since the proportion of the two RNAs was not significantly altered by incubating the virus at 37 °C until more than 99 % of the infectivity had been lost. Similarly, the base compositions of the two species are different; if scission of the 22S RNA (mol. wt. 1.0 x 10^6) into two molecules of 15S RNA (mol. wt. 0.5 x 10^6) were occurring, both RNA species would have the same base composition.

Although preparations of the individual RNAs, separated by centrifuging in sucrose gradients, had low infectivity in mice, mixtures of the two preparations were 10- to 100-fold more infective than the individual preparations. The 100-fold dilutions of each of the preparations were non-infective but they infected mice when mixed with similar dilutions of the second species. Each species of RNA thus appears to be necessary for infection.

We have examined the question as to whether the two RNAs are present in the same or different components. Individual fractions of the virus peak, isolated by sucrose gradient centrifuging, contained the same proportion of the two RNAs, suggesting that if the two species are present in different components, these must have identical sedimentation coefficients. However, extraction of the virus with phenol, without added SDS, liberated the 22S species alone and the 15S species was liberated only when SDS was added to the extraction mixture. These observations indicated that the virus contained two different components and further evidence for this view was obtained by centrifuging the virus in caesium chloride gradients. Murphy et al. (1970) found that extracts of infected mouse muscle gave a single infective peak at 1.34 g/ml. Although we confirmed this value with freshly purified virus, more than one radioactive peak was obtained with purified virus which had been stored at 4 °C for several days. The profiles were somewhat variable in different experiments but in most cases the major proportion of the radioactivity was found at about 1.60 and 1.50 g/ml. These high values indicated that protein was being detached from the virus particles during the centrifuging in caesium chloride. The particles with the higher density (1.60 g/ml) had presumably lost more protein than the less dense (1.50 g/ml) particles. However, differences in density would also be observed if the particles were penetrated to different extents by the caesium ions.

The peaks in the caesium chloride gradient appear to be derived from different components of the virus because the particles equilibrating at 1.60 g/ml contained the 22S RNA species, whereas those equilibrating at 1.50 g/ml contained the 15S RNA. Since the two components in freshly purified virus have the same sedimentation coefficient (135S) and the same density in caesium chloride (1.34 g/ml), they presumably contain the same proportion of RNA. If this is correct, one component will contain one molecule of 22S RNA (mol. wt. 1.0 x 10^6) and the second component will contain two molecules of 15S RNA (mol. wt. 0.5 x 10^6). The relative amounts of [32P] in the two RNAs would further suggest that twice as many particles contain 22S RNA as contain 15S RNA. Nevertheless, the possibility remains that the two components with densities of 1.60 and 1.50 g/ml were derived from a single component. To provide clear-cut evidence that the virus contains two components it will be necessary to separate them in an undegraded form.

We also considered the possibility that the virus isolated from the mice consisted of
Nodamura virus and a latent mouse virus. Since Nodamura virus grown in wax moth larvae also contains the same two RNA species (J. F. E. Newman & L. Bailey, unpublished observations) it seems unlikely that one of the RNA species in our purified preparations is derived from a mouse virus.

There are numerous examples of plant viruses in which the genetic information is distributed between two or more components (see references in review by Brown & Hull, 1973). The kind of information contained in each component has been elucidated with some of the viruses but the way in which the different components complement each other in the infection process remains to be investigated. Answers to this problem may be obtained by the use of leaf cell protoplasts in which there is the possibility of synchronous infection of a large number of cells (Takebe & Otsuki, 1969). Provided that a tissue culture system can be found which supports the replication of Nodamura virus, the observations described here suggest that this virus will provide an additional tool for investigating the problem of virus replication when genetic information is contained in more than one RNA species.

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REFERENCES


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