Determination of the Molecular Weight of Bovine Enterovirus RNA by Nuclease Digestion

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SUMMARY

The mol. wt. of a [\(^{32}\)P]-labelled bovine enterovirus RNA has been determined by digesting with pancreatic RNase and separating the resulting oligonucleotides using a two-stage fractionation method on DEAE-Sephadex in 7 M-urea at pH 7.6 and pH 3.0. We have estimated the number of nucleotides as 8612 ± 55. This corresponds to a mol. wt. of 2.93 ± 0.02 x 10^6 which is in agreement with estimates obtained by sedimentation and gel electrophoresis techniques.

INTRODUCTION

The mol. wt. of RNA is often determined by sedimentation on sucrose density gradients (Spirin, 1963) or electrophoresis on acrylamide gels (Peacock & Dingman, 1968; Loening, 1969). Both procedures depend on a comparison with other RNA molecules of known mol. wt. such as ribosomal RNA. However, unexpected effects on sedimentation or electrophoretic behaviour can result from configurational and ionic influences (Gesteland & Boedtker, 1964; Bishop, Claybrook & Spiegelman, 1967). Attempts to overcome conformational differences by use of denaturants such as dimethyl sulphoxide (Kolakofsky & Bruschi, 1973) and formaldehyde (Boedtker, 1968, 1971; Fenwick, 1968; Kaper & Waterworth, 1973) have not always been successful. In view of these inherent difficulties the reliability of such comparative methods has been questioned.

Direct methods for mol. wt. determination such as end group analysis or complete sequencing (Gilham, 1970) have had limited application in the study of virus RNA molecules. The most accurate method, total base sequencing, is suitable only for short RNA strands. However, sequencing techniques have been adapted to estimate the number of nucleotides in the RNA strand. Sinha, Fujimura & Kaesberg (1965) made such an estimate for R17 RNA when they separated the oligonucleotides in a pancreatic RNase digest of [\(^{32}\)P]-labelled RNA. More recently, Repik & Bishop (1973) have shown that a similar estimate can be made with the larger vesicular stomatitis virus RNA by using controlled nuclease digestions of RNA labelled separately with each of the tritiated nucleotide precursors.

In this paper we report an accurate estimation of the number of nucleotides present in the RNA of a bovine enterovirus. Our approach is essentially the same as that described by Sinha et al. (1965), although this paper was unknown to us until the work reported here was near completion. In our experiments [\(^{32}\)P]-labelled RNA obtained from purified bovine enterovirus was exhaustively digested to yield a mixture of oligonucleotides. A two-stage fractionation on DEAE-Sephadex columns equilibrated with 7 M-urea in buffers at pH 7.6 or pH 3.0 separated individual oligonucleotide isopliths. Quantitative fractionation allowed
the [\(^{32}\)P] activity proportional to a single nucleotide to be determined. This activity and that representing the total activity of the digest allows the number of nucleotides in the RNA strand to be calculated. This method is based on the following assumptions: (1) the [\(^{32}\)P] activity is randomly distributed throughout the population of RNA molecules; (2) the RNA isolated from a preparation of virus particles is homogeneous in both size and sequence. The results indicate that, in the case of bovine enterovirus, these assumptions are valid.

**METHODS**

*Cell cultures.* Monolayers of BHK 21 cells were grown in 2·5 l roller bottles as described by Martin, Johnston & Clements (1970).

*Virus.* The virus used in this investigation was a bovine enterovirus (serotype VG-5-27; McFerran, 1962) which had undergone four passages in BHK 21 cells.

*Isotopic labelling.* Confluent monolayers of BHK 21 cells were pre-incubated with phosphate deficient medium for 2 h and infected at a multiplicity of 1 p.f.u./cell. After 0·5 h adsorption, 50 ml of phosphate deficient medium containing [\(^{32}\)P]-orthophosphate (20 \(\mu\)Ci/ml) was added. Virus, harvested after 16 h, was purified using the method described by Hoey & Martin (1974).

*Isolation of virus RNA.* Purified virus was dissolved in 0·5 ml 0·1 M-NaCl, 0·01 M-EDTA, 0·01 M-tris-HCl (pH 7·4). Addition of 0·1 ml of a solution containing 8 M-urea and 1 % (w/v) SDS followed by incubation for 15 min at 37 °C released the RNA from the virus (Clements & Martin, 1970).

*Fractionation of virus RNA.* Samples of RNA isolated from the virus were analysed by sedimentation on sucrose density gradients. Five to 25 % (w/v) gradients of sucrose in 0·1 M-sodium acetate/acetic acid buffer (pH 5·0) were centrifuged at 55000 g for 16 h at 4 °C. Electrophoresis on polyacrylamide-agarose gels (2·2 %) was carried out as described by Clements & Martin (1971).

*Digestion of virus RNA with pancreatic RNase.* [\(^{32}\)P]-labelled virus RNA, precipitated in the presence of BHK ribosomal RNA with ethanol, was dissolved in 2·5 ml 0·01 M-EDTA, 0·01 M-tris-HCl (pH 7·5). 20 \(\mu\)g of pancreatic RNase was added to give an enzyme:substrate ratio of 1:12. The mixture was incubated for 24 h at 37 °C.

**Chromatography on DEAE-Sephadex in 7 M-urea**

*First Fractionation.* DEAE-Sephadex (A-25) was packed in a 2·6 × 30 cm column and equilibrated with a 7 M-urea, 0·02 M-tris buffer adjusted to pH 7·6 with HCl, which contained 0·1 M-NaCl. The pancreatic RNase digest was quantitatively added to the column in 100 \(\mu\)l portions. The column was eluted by an approx. linear gradient formed by continuously mixing 500 ml 0·5 M-NaCl with 500 ml 0·1 M-NaCl in the pH 7·6 buffer. The eluant was monitored spectrophotometrically using a Unicam SP500 equipped with a 1 mm path length flow cell. Fractions (5 ml) were collected directly into plastic vials for which the BTL fraction collector was adapted.

*Second fractionation.* A second DEAE-Sephadex column of similar dimensions was equilibrated with a 7 M-urea solution adjusted to pH 3·0 with HCl. The sample to be added was prepared by pooling the fractions from the first fractionation which contained the octa- or heptanucleotide activity. The salt concentration was diluted threefold with a neutral 7 M-urea solution before the sample (200 ml) was added. The column was re-equilibrated before starting the NaCl gradient formed by mixing 250 ml 0·5 M-NaCl with 250 ml 0·1 M-NaCl in 7 M-urea buffer (pH 3·0). Five ml fractions were collected.
Fig. 1. Sedimentation through a 5 to 25% (w/v) sucrose density gradient of RNA isolated from purified bovine enterovirus.

Radioactive determination. The fractions, collected in plastic vials, were counted for Cerenkov radiation in an Intertechnique liquid scintillation counter for 10 min. A computer program was devised so that a decay and background correction was applied to each fraction.

Base composition analysis. Mononucleotides resulting from the hydrolysis of $[^32P]$-labelled RNA from purified virus by 0·3 n-KOH treatment at 37 °C for 18 h were separated using paper electrophoresis (Davidson & Smellie, 1952). The bands of paper which contained the mononucleotide material were counted in water for Cerenkov radiation.

Materials. Minimal Eagle's medium without phosphate was obtained from Bio-cult Laboratories, Glasgow. Carrier free $[^32P]O_4$ was purchased from the Radiochemical Centre, Amersham, Buckinghamshire. Pancreatic RNase was obtained from Worthington Biochemical Corporation, New Jersey, U.S.A. Plastic vials for fraction collection and direct radioactive counting were purchased from Sterilin Ltd, Richmond, Surrey.

RESULTS

Isolation of virus RNA

RNA was released from the virus by urea-SDS treatment and centrifuged on 5 to 25% (w/v) sucrose density gradients. Fractions which contained the virus RNA were pooled and diluted with two volumes of 0·1 M-sodium acetate/acetic acid buffer (pH 5·0). Virus RNA was precipitated with three volumes of ethanol at −20 °C. The purity of the RNA was established by sedimentation on sucrose density gradients and electrophoresis on polyacrylamide–agarose gels. Virus RNA sedimented as a sharp peak corresponding to 35S (Fig. 1). Electrophoresis on 2·2% gels (Fig. 2) also showed a single peak of activity equivalent to a mol. wt. of 2·8 × 10^6, as previously reported by Clements & Martin (1971).
Fig. 2. Electrophoresis on an acrylamide–agarose gel (2:2 %) of RNA isolated from purified bovine enterovirus.

**Base composition analysis**

Fifteen separate determinations were carried out on digest samples from three different virus preparations. The mean base composition was found to be A 26.8, C 26.4, G 23.8 and U 23.0 moles per cent.

**Fractionation on DEAE-Sephadex at pH 7.6**

The pancreatic RNase digest of virus RNA was applied to the column after its total activity had been estimated by counting 25 μl samples. At pH 7.6 the oligonucleotides are separated according to chain length (Work & Work, 1972). As the salt concentration increases oligonucleotides of increasing chain length are eluted. Fig. 3 shows a fractionation profile at pH 7.6 where it is seen that oligonucleotide peaks of up to eight bases in length were resolved. Minor peaks which occurred before and after the mononucleotide peak probably represented cyclic intermediates (Repik & Bishop, 1973). Further elution with 2 M-NaCl removed the remaining [32P] activity from the column. The recovery for this fractionation was always greater than 97 %.

The resolution of the larger oligonucleotides was improved by decreasing the fraction size to 0.5 ml. The fractions which comprised the octanucleotide peak were pooled and subjected to a second fractionation. In some experiments the heptamer peak was also further fractionated at pH 3.0, but we describe here only the octanucleotide fractionation.

**Fractionation on DEAE-Sephadex at pH 3.0**

Fractions which contained octanucleotide activity were pooled and the salt concentration decreased by dilution with three volumes of a neutral 7 M-urea solution. The 200 ml sample was added to the second column in a dropwise manner under gravity.

At pH 3.0 the octanucleotides are separated according to base composition (Work &
Mol. wt. of bovine enterovirus RNA

Fig. 3. Chromatography of pancreatic RNase digest of virus RNA on DEAE-Sephadex. First fractionation. A constant gradient, 0.1 M- to 0.5 M- NaCl (500 ml of each solution in 7 M-urea, 0.02 M-tris-HCl (pH 7.6) buffer was used. 5 ml fractions were collected. (●—●), [32P], ct/min/fraction; (- -), NaCl gradient.
Fig. 4. Chromatography of octanucleotide material on DEAE-Sephadex. Second fractionation. A constant gradient, 0·0 m- to 0·5 m-NaCl (250 ml of each solution in 7 m-urea-HCl (pH 3·0) buffer was used. 5 ml fractions were collected. (O–O), [32P], ct/min/fraction; (—), NaCl gradient.

Work, 1972). Fig. 4 shows a fractionation at pH 3·0 where five peaks of activity were eluted. Although the first four peaks eluted were equivalent in [32P] activity the fifth peak bore no integral, numerical relationship to the others. We consider it to be the result of contaminating monomer material, incompletely separated in the first fractionation. On the assumption that the first four peaks were individual isopliths we calculated the [32P] activity proportional to a single nucleotide. The results in Table 1 support this assumption since the estimated mol. wt. would be excessively large if the four peaks represented multiples of isopliths. Further, in one preparation the heptamers were separated into six peaks which had a precise, integral relationship to each other, indicating the presence of multiple isopliths in certain peaks. Moreover, estimations of [32P] activity proportional to a single nucleotide from either heptamer or octamer fractionations were similar (Table 1). The recovery for the fractionation at pH 3·0 was approx. 90%.
**Mol. wt. of bovine enterovirus RNA**

**Theory and calculations**

The theory underlying this method is represented schematically as follows:

- **Virus RNA $^{32}$P activity**
  - Pancreatic RNase
  - Digest containing a mixture of oligonucleotides of different length and base composition
    - Total A at time 0
    - Fractionation at pH 7.6
    - Total B (corrected)
  - Separation of oligonucleotides according to the chain length
    - Octanucleotide fractions pooled
  - Octanucleotide material
    - Total C (corrected)
    - Fractionation at pH 3.0
    - Total D (corrected)
  - Separation of octanucleotides according to base composition
  - Calculation:
    - $^{32}$P activity of octanucleotide = $X$
    - $^{32}$P activity proportional to single nucleotide = $X/8$
    - Correction for decay: $^{32}$P activity proportional to single nucleotide = $X'/8$
    - Correction for losses during fractionation: $^{32}$P activity proportional to single nucleotide = $X''/8$
    - Number of nucleotides in virus RNA strand
      - $\frac{\text{Total A}}{X''} \times \frac{\text{Total C}}{\text{Total D}}$

The number of nucleotides in the virus RNA strand is calculated knowing the $^{32}$P activity proportional to a single nucleotide, corrected for isotopic decay and losses occurring during both fractionations, and the total activity of the virus RNA in digest form.

The results of the four experiments are shown in Table 1. The number of nucleotides in the virus RNA strand was calculated as $8612 \pm 55$. When base composition analysis are considered a mol. wt. of $293 \pm 0.02 \times 10^6$ is estimated.

**DISCUSSION**

In this paper we report a direct method for determining the mol. wt. of the RNA from an animal picornavirus. As mentioned earlier this procedure is based on assumptions regarding the random distribution of $^{32}$P among the population of molecules present and regarding the purity of the virus RNA.

One of the major criticisms of the use of $^{32}$P as a means of determining the base composition or mol. wt. of RNA is that precursor pools of trinucleotides are not equally labelled.
Table I. Estimation of number of nucleotides in virus RNA

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Total $[^{32}P]$ activity (ct/min x $10^{-6}$)</th>
<th>% recovery of fractionations</th>
<th>Corrected $[^{32}P]$ activity ct/min/nucleotide</th>
<th>Number of nucleotides</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>First</td>
<td>Second</td>
<td></td>
<td></td>
</tr>
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<td>1</td>
<td>3.2444</td>
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<td>375.7</td>
<td>8636</td>
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<td>243.6</td>
<td>8684</td>
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<td>3</td>
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<td>98.42</td>
<td>148.0</td>
<td>8526</td>
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<tr>
<td></td>
<td>(octanucleotide)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2.8250</td>
<td>97.36</td>
<td>147.2</td>
<td>8572</td>
</tr>
<tr>
<td></td>
<td>(heptanucleotide)</td>
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</table>

Number of nucleotides = 8612 ± 55

Mol. wt. = 2.93 ± 0.02 x $10^6$

* Total activity of this preparation was estimated by summing activities of eluted fractions at a modified time zero.

(Repik & Bishop, 1973). However, our experiments suggest that the incorporation of $[^{32}P]$-precursors into a large population of molecules does result in random distribution of labelled nucleotides. This is supported by the equivalence of activity in the oligonucleotide isopliths. With regard to the purity of virus RNA, it is possible that complementary strands are encapsidated (Kingsbury, 1974) which would introduce inaccuracies into this method. Little information is available concerning the absolute homogeneity of the RNA. It should be noted that any heterogeneity in the RNA population will result in an overestimate of the mol. wt. by this method.

We have determined the number of nucleotides present in a bovine enterovirus RNA to be 8612 ± 55, equivalent to a mol. wt. of 2.93 ± 0.02 x $10^6$ when base composition analysis was considered. Estimates of picornavirus RNA by physical methods are in the range 2.6 to 2.8 x $10^6$ (Granboulan & Girard, 1969; Clements & Martin, 1971) with individual reports seldom being sufficiently well documented to permit a calculation of an accurate standard error. Where this has been done the range in terms of nucleotides is ± 300.

The reproducibility obtained in our work suggests that the method described here may be equally applicable to other virus RNA molecules where it is possible to obtain highly labelled $[^{32}P]$-preparations. The experimental procedure is relatively inexpensive and calculations straightforward.

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REFERENCES


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