Ribonucleic Acid Content of Encephalomyocarditis Virus

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SUMMARY

The RNA content of encephalomyocarditis virus was found to be 31.7%, a figure obtained by combining results from nitrogen and phosphorus determinations which gave 32.0%, from extinction measurements at 260 nm. on perchloric acid hydrolysates which gave 31.3% and from orcinol determinations which gave 31.8%. Specific extinction coefficients obtained were $E_{260}^{1\%}$ for the virus in $0.1\text{ M-KCl+0.02 M-phosphate buffer, pH 8.0}$ and $E_{260}^{1\%}$ for its RNA in $0.1\text{ M-tris+HCl, pH 7.2}$.

INTRODUCTION

Encephalomyocarditis (EMC) virus is a picornavirus and also one of the Columbia SK group of viruses; these are assumed to contain 25 to 30% RNA (Fenner, 1968). The determinations reported here were made as part of a comprehensive study of the structure of EMC virus, particularly since previous values of about 30% for its RNA content (Faulkner et al. 1961) were made on virus the purity of which has been questioned (Kaighn, Moscarello & Fuerst, 1964) and with non-viral RNA and protein standards. Moreover, Scraba, Kay & Colter (1967) have reported that highly purified Mengo virus, also a member of the Columbia SK group, apparently contains only 21% RNA; this value appears to be incorrect. The present investigation, made using EMC virus known to be of rigorously established purity (Burness, 1969), shows conclusively that EMC virus, at least, contains 30% rather than 20% RNA.

METHODS

Purified EMC virus used was a cloned, large plaque variant of the k2 strain (Hoskins & Sanders, 1957) grown and purified as described previously (Burness, 1969).

Virus RNA and protein were prepared by stirring 5 mg. purified virus in 2 ml. $0.1\text{ M-NaCl+0.02 M-phosphate buffer, pH 8.0}$ for 2 min. at room temperature with an equal volume of water-saturated, redistilled phenol (Gierer & Schramm, 1956), and separating the aqueous and phenol layers by centrifugation at $3000\text{ g}$ for 5 min. The upper layer containing the RNA was extracted a second time with phenol before precipitating the RNA by addition of 2 volumes of ethanol at $4^\circ$. After standing for at least 30 min. the precipitate was collected by centrifugation, dissolved in 2% (w/v) sodium acetate and the RNA precipitated twice more in the same way, after which it was washed twice with 70% (v/v) ethanol, dissolved in water and stored at $-20^\circ$.

The interface and lower layer from the first phenol extraction were extracted a second time with water to remove the last traces of contaminating RNA, after which the protein

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was precipitated from the phenol layer by the addition of 10 volumes of ethanol, containing 0·1 M-ammonium acetate, precooled to −20°. After standing overnight at 4°, the protein formed a flocculent precipitate. This was collected by centrifugation at 3000 g for 10 min., washed twice with ethanol, dissolved in 67 % (v/v) acetic acid, dialysed for 2 hr periods against several litre volumes of distilled water at 4° and finally stored as a lyophilized powder over P₂O₅.

RNA concentration was determined using a modified orcinol method (Hurlbert et al. 1954). Samples containing 2 to 20 μg. RNA in 0·3 ml. were heated at 100° for 30 min. with 0·6 ml. orcinol reagent (0·4 % (w/v) orcinol + 0·1 % (w/v) FeCl₃·6H₂O in concentrated HCl) and after cooling the extinction was measured at 675 nm. in semimicro cells. EMC virus RNA was used as a (primary) standard until the amount of yeast RNA (secondary standard) to produce the same colour was known (see RESULTS) when the latter replaced the virus RNA as a standard.

Table 1. Base ratios and extinction coefficient of hydrolysed EMC virus RNA

<table>
<thead>
<tr>
<th>Base</th>
<th>(a)</th>
<th>(b)</th>
<th>(c)</th>
<th>(d)</th>
<th>(e)</th>
<th>(f)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Base ratio</td>
<td>Base ratio</td>
<td>Nucleotide mol.wt</td>
<td>a/100 x e</td>
<td>E₂₆₀</td>
<td>a/100 x e</td>
</tr>
<tr>
<td>Guanine</td>
<td>24·4±0·2</td>
<td>23·6</td>
<td>363·2</td>
<td>88·6</td>
<td>11·2</td>
<td>2·7</td>
</tr>
<tr>
<td>Adenine</td>
<td>25·8±0·2</td>
<td>27·4</td>
<td>347·2</td>
<td>89·6</td>
<td>15·0</td>
<td>3·9</td>
</tr>
<tr>
<td>Cytosine</td>
<td>25·1±0·3</td>
<td>23·5</td>
<td>323·2</td>
<td>81·1</td>
<td>7·6</td>
<td>1·9</td>
</tr>
<tr>
<td>Uracil</td>
<td>24·8±0·3</td>
<td>25·6</td>
<td>324·2</td>
<td>80·4</td>
<td>7·4</td>
<td>1·8</td>
</tr>
</tbody>
</table>

Total of column (d), average nucleotide mol.wt = 339·7
Total of column (f), average nucleotide E₂₆₀ = 10·3

In 0·1 M-NaOH, E₄₁₀ at 260 nm. = \( \frac{103}{339.7} \times 10,000 = 303 \)

Column (a) is in mole per cent and is the mean and standard error of the mean of 16 determinations.
Column (b) is base composition obtained by Faulkner et al. (1961).
Column (e), E₂₆₀ is molar extinction coefficient in m-mole/1. x 10⁶ in 0·01 M-NaOH; figures from Beaven, Holiday & Johnson (1955).

The RNA concentration was determined in perchloric acid hydrolysates from its extinction coefficient in this solvent, calculated in a similar manner to that described in Table 1, by summation of the extinction coefficients of the individual nucleotides.

Protein concentration was measured by a scaled down version of the procedure of Lowry et al. (1951). EMC virus protein was used as a (primary) standard until the amount of bovine serum albumin (secondary standard) to produce the same colour was known (see RESULTS) when the latter replaced the virus protein as a standard.

Base analysis of RNA was made on both virus and its RNA by hydrolysing samples containing 500 μg. RNA at 100° for 1 hr with 20 μl. 1 M-HCl (Smith & Markham, 1950), separating the hydrolysis products by chromatography on Whatman no. 1 paper using isopropanol-HCl (Wyatt, 1951), eluting the spots, located with an ultraviolet lamp, in 0·1 M-HCl and using ultraviolet spectrophotometry to estimate the amount of each base present.

Total phosphorus was determined by a modification of the method of Fiske & Subbarow (1925). Samples containing about 4 μg. phosphorus in 0·2 ml. were digested with 0·5 ml. microanalytical reagent grade 5 M-H₂SO₄ by heating for 2 hr longer than required to drive off the water and, after cooling, each was diluted with 3·8 ml. water, heated on a boiling water bath for 10 min. to convert any pyrophosphate formed to orthophosphate and then 0·5 ml. 2·5 % (w/v) ammonium molybdate and 0·5 ml. reducing agent (5 g. 1-amino-2-
naphthol-4-sulphonic acid + 16 g. sodium metabisulphite + 10 g. anhydrous sodium sulphite dissolved in 250 ml. warm water) was added and the whole heated a further 10 min. at 100° (Griswold, Humoller & McIntyre, 1951). After cooling, the samples were made up to 5 ml. and the extinction read at 820 nm. Adenosine monophosphate (AMP) was found to be a convenient standard since its exact concentration could be checked each time by its extinction at 260 nm.

Inorganic phosphate concentration in chromatography eluates was estimated by mixing thoroughly between additions 2 ml. sample, diluted to contain about 6 μg. phosphorus/ml., with 0·1 ml. 5 M-H₂SO₄, 0·2 ml. 2·5% (w/v) ammonium molybdate and 0·1 ml. aminonaphthol-sulphonic acid reducing agent described above, and after 10 min. reading the extinction at 820 nm. Blanks and standards containing water and 6 μg. phosphorus/ml., respectively, were run at the same time.

Nitrogen was determined by two methods both relying on digestion of the sample with microanalytical reagent grade 5 M-H₂SO₄ to convert bound nitrogen to ammonia, the amount of which was determined by heating with ninhydrin in a test tube (Jacobs, 1960) for concentrations of 4 to 40 μg. nitrogen, or by using the Technicon autoanalyser (see RESULTS) for quantities of 1 to 20 μg. AMP was used as a standard for the reason given earlier under total phosphorus determination.

Chromatography on Sephadex G 100, prepared and packed in 0·05 M-NaCl to form a 1·5 x 6 in. column, was used to remove inorganic nitrogen and phosphorus. The virus sample (1 ml.), containing 20% (w/v) sucrose to help to load the sample on the column and frequently a few drops of bromocresol purple to locate the elution volume of low molecular weight material, was washed from the column with 0·05 M-NaCl in 3 ml. fractions, which were monitored at 260 nm. and at 595 nm. to locate the virus and bromocresol purple respectively. If indicator was not added, the samples were analysed for inorganic phosphate content by the method described above.

Spectrophotometric measurements were made either manually with a Unicam 500 or else with a Perkin Elmer model 137 recording spectrophotometer.

Materials. All chemicals used were supplied by B.D.H. Ltd, Poole, Dorset, except the AMP, yeast RNA and bovine serum albumin which came from Calbiochem Corporation, Los Angeles, California.

RESULTS

Base composition of EMC virus RNA

Determinations of base composition were made on HCl hydrolysates from several different preparations of both virus and isolated RNA. The values, corrected for 5% destruction of pyrimidine bases (Markham & Smith, 1951), differed slightly from those obtained for preparations of uncloned EMC virus (Faulkner et al. 1961) of less certain purity than those used in the present report (Table 1, columns (a) and (b)).

Specific extinction coefficients of EMC virus RNA

The ultraviolet absorption spectrum of EMC virus RNA (Fig. 1) has a maximum at 258 nm., a minimum at 231 nm., E₂₅₈: E₂₃₁ = 2.14 and E₂₅₈: E₂₆₀ = 2.02. These figures were corrected for scattering by using as a base the extrapolated line drawn connecting the values for extinction between 360 and 320 nm. through the virus peak region (Beaven & Holiday, 1952).

Specific extinction coefficients, E₁ % , at 260 nm. = 267 and 303 in 0·5 M-HClO₄ and 0·1 M-NaOH, respectively, were calculated for hydrolysed DNA of the base composition given in
Table 1, column (a). The base composition obtained by Faulkner et al. (1961) was calculated to give an $E_{1\%}$ at 260 nm. = 305 in 0.1 M-NaOH demonstrating that the differences in the two base ratios reported (Table 1) have little effect on the estimation of RNA by extinction measurements.

Determinations of the specific extinction coefficient at a particular wavelength for unhydrolysed RNA required knowledge of the extinction at that wavelength for a solution of known RNA concentration. The extinction, corrected for scattering, was measured by diluting 0.2 ml. stock RNA in water with 0.1 ml. 1.0 M-tris + HCl, pH 7.2 and 0.7 ml. water and scanning in a recording spectrophotometer. The RNA concentration was determined by incubating 0.2 ml. of the same stock RNA with 0.1 ml. 1 M-NaOH at 37° for 20 hr, diluting to 1 ml. with water and measuring the extinction at 260 nm., corrected for scattering, from which, knowing the calculated extinction coefficient of hydrolysed RNA in 0.1 M-NaOH (Table 1), the RNA concentration was calculated. An $E_{1\%}^{10}$ at 260 nm. = 221 in 0.1 M-tris + HCl, pH 7.2 and a hyperchromicity (the increase in extinction caused by alkali hydrolysis) = 1.38 were obtained.

**Percentage RNA from phosphorus and nitrogen content of virus**

Purified virus samples for analysis were eluted in 0.05 M-NaCl from a G-100 Sephadex column ahead of the inorganic phosphate used in the virus purification (Fig. 2). In some experiments samples from the chromatographic peak were separately digested in concentrated H$_2$SO$_4$ and half were used for phosphorus and half for nitrogen determination, respectively; for the purposes of calculation they were corrected to the same extinction at 260 nm. Alternatively, peak samples were combined, centrifuged at 144,000 g for 1 hr and
the virus resuspended in 0.1 M-NaCl before digestion in concentrated \( \text{H}_2\text{SO}_4 \) for phosphorus and nitrogen determinations. Since this added ultracentrifugation had no effect on the per cent RNA obtained, it appeared that Sephadex chromatography alone effectively removed inorganic nitrogen and phosphate from the samples.

The phosphorus and nitrogen content of EMC virus RNA was calculated to be 9.11 and 15.58 %, respectively, from its base composition (Table 1). The amino acid composition of the virus protein (A. T. H. Burness & I. U. Pardoe, in preparation) gave a nitrogen content of 13.29 %. The weight of RNA and thus of RNA nitrogen was calculated from these figures knowing the number of \( \mu \text{g} \) phosphorus present in a preparation. The difference between total and RNA nitrogen was assumed to be protein nitrogen from which the weight of protein present was calculated. The combined weight of RNA and protein was assumed to be equal to the weight of virus present, leading to a value of 32.6 ± 0.6 % (mean and standard error of mean (S.E.M.), 7 determinations) for the RNA content of EMC virus.

**Nitrogen determinations with Technicon autoanalyser**

The Technicon amino acid analyser quantitatively records the increase in extinction at 570 nm. produced when ammonia, in addition to amino acids, reacts with ninhydrin. Only the autoanalyser part is required for samples containing a single component, the chromatographic column being by-passed. This arrangement was investigated as a possible sensitive method for estimating ammonia produced during the digestion of nitrogen-containing samples with concentrated \( \text{H}_2\text{SO}_4 \).

Samples for analysis were digested with 25 \( \mu \text{l} \) 5 M-\( \text{H}_2\text{SO}_4 \) for 2 hr longer than the time required to drive off the water, cooled, diluted with 10 ml. water and then allowed to be drawn into the autoanalyser for 4 min. with a 5 min. wash with water between samples, at a pumping rate of 0.5 ml./min.

Using similarly treated standards, containing known amounts of nitrogen, it was established that the colour produced at 570 nm. was directly proportional to the ammonia, or to
the nitrogen content before digestion (Fig. 3). The upper limit of useful concentration was 3 µg. nitrogen/ml. above which the extinction exceeded 1.0 and was difficult to measure. The CuSO₄ and HgO present in the catalyst (Jacobs, 1960) added to aid digestion caused inhibition of colour formation. In its absence, 100% leucine nitrogen was nevertheless recovered suggesting the lack of catalyst did not result in incomplete digestion. The main advantages of the autoanalyser technique for nitrogen determination compared with other methods were that no titration or neutralization of excess acid was required and that it was more sensitive.

Four virus samples were analysed for nitrogen content in this way and when combined with phosphorus determinations gave a mean value of 30.9% for the RNA content for EMC virus.

![Fig. 3. Relationship between nitrogen content and extinction at 570 nm. Nitrogen, in AMP which was used as a standard, or in virus, was converted by digestion with concentrated H₂SO₄ to ammonia which was estimated by the colour produced in its reaction with ninhydrin in a Technicon autoanalyser.](image)

**Percentage RNA from RNA and protein determinations**

*Primary and secondary standards.* The same extinction at 675 nm. in the orcinol procedure was given both by 851 ± 10 µg. (S.E.M., 10 determinations) of EMC virus RNA and by 1000 µg. yeast RNA which, knowing this correction factor, replaced virus RNA as a standard in subsequent orcinol method determinations of the RNA content of EMC virus. The yeast RNA was always adjusted to the same extinction at 260 nm. before use.

The same extinction at 750 nm. using the Lowry et al. (1951) procedure was given both by 983 ± 5 µg. (S.E.M., 4 determinations) of EMC virus protein, E₅₀₆₅ at 280 nm. = 1.48 in 0.1 M-NaOH (A. T. H. Burness & I. U. Pardoe, in preparation), and by 1000 µg. of a preparation of bovine serum albumin which was subsequently used as a secondary standard, after adjusting each time to the same extinction at 280 nm. before use.

In initial work, RNA and protein determinations were made at the same time on identical samples of virus. However, it was found more convenient to carry out the two kinds of determinations at different times on samples of known extinction at 260 nm. and to correct for any differences in such readings for the purpose of calculation.
RNA content of EMC virus

RNA content using the orcinol and Lowry procedures

Assuming that the only constituents of EMC virus were protein and RNA, after correction for the differences in extinction between the primary and secondary standards, the RNA content of EMC virus was found to be \(31.8 \pm 0.4\%\) (S.E.M., 15 determinations) using orcinol and Lowry et al. (1951) procedures.

RNA content from extinction of hydrolysates

Preparations of EMC virus of known extinction at 260 nm. were hydrolysed using 0.5 M-HClO₄, the precipitated protein removed by centrifugation and the extinction at 260 nm. of the supernatant fluid measured. The RNA content, estimated from the \(E^{1%}_{1cm}\) for RNA from EMC virus hydrolysed with HClO₄, the procedure for calculation of which is similar to that given in Table 1 for NaOH hydrolysates, combined with further protein determinations by the method of Lowry et al. (1951) gave a value of \(31.3 \pm 0.3\%\) (S.E.M., 15 determinations) RNA in EMC virus.

Specific extinction coefficient of EMC virus

Most virus preparations used for RNA and protein determinations were of known extinction in 0.1 M-KCl + 0.02 M phosphate buffer, pH 8.0. Assuming only RNA and protein were present in the virus, an \(E^{1%}_{1cm}\) at 260 nm. = 77.4 \(\pm\) 0.57 (S.E.M., 31 determinations) was calculated, after correction for scattering.

DISCUSSION

Several different methods gave the same percentage RNA composition for EMC virus within experimental error; the combined figure from all techniques was \(31.7 \pm 0.23\%\) (S.E.M., 39 determinations). These results are very similar to the value for EMC virus of \(31.8 \pm 1.1\%\) (5 determinations) which can be calculated from the work of Faulkner et al. (1961) and to that for the closely related ME virus of \(31.4 \pm 0.9\%\) (5 determinations) found by Rueckert & Schäfer (1965).

Although similar values were found for the per cent RNA in EMC virus both by nitrogen and phosphorus, and by RNA and protein determinations, it cannot be concluded that all the nitrogen present in the virus was derived only from nucleotide or amino acid sources. The specific extinction coefficient of EMC virus protein used in this study involved weighing, so that if there were a component attached, or similarly soluble in phenol and ethanol, to the virus protein, it would be weighed as part of the latter. The possible presence of such a component, however, while affecting the figure for per cent protein would not affect that for RNA or for virus specific extinction coefficient.

However, a non-nitrogenous component unattached to or behaving in its solubility unlike both RNA and protein would affect the value obtained for RNA content of the virus and would remain undetected by the methods used in this study. A partial specific volume of 0.678 ml./g. has been measured for EMC virus (Burness & Clothier, 1970) from which an RNA content of about 32% can be calculated (Markham, 1962). This suggests that a non-nitrogenous component, if present, represents only a small fraction of the virus.

Further work (Burness & Clothier, 1970) with EMC virus has shown that the virus has a particle weight of \(8.51 \times 10^6\). A single piece of RNA comprising 31.7% of this would have a molecular weight of 2.7 million which is significantly higher than 2.0 million generally assumed for picornaviruses (Fenner, 1968), and which gives the RNA genome coding...
capacity for a further three polypeptide chains, each containing in excess of 200 amino acids. Confirmation of this size for EMC virus RNA is presently being sought.

I thank Dr F. Kingsley Sanders for his continued advice and encouragement, Dr P. Faulkner for many helpful discussions, Miss Joy H. Moss for preparing the crude virus, and Mr D. S. Walter and Miss Ingrid U. Pardoe for most competent technical assistance.

REFERENCES


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