A TITRIMETRIC METHOD FOR THE DETERMINATION OF MOLECULAR WEIGHT OF SMALL POLYNUCLEOTIDES

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The determination of the molecular weight of nucleic acids by physical methods has been criticized in view of the uncertain degree of aggregation which would result in high values (1). The effect of aggregation on the value obtained for the molecular weight of synthetic ribose polynucleotide by ultracentrifugation is clearly demonstrated in the studies of Ochoa on pure and mixed polymers and mixtures of these (2).

If the molecular weights obtained by physical methods were to represent "chemical" molecular weights, then the postulate of branching through triester phosphate (3) would seem to be inevitable in view of the high values found for end groups in various samples by titration and by enzymatic methods. Brown, Magrath, and Todd, working with model phospho-
triesters, found this grouping very unstable in aqueous solution and concluded that its presence in ribonucleic acid (RNA) was unlikely (4).

A "chemical" method for the determination of the molecular weight of RNA samples which is not affected by physical aggregation and which does not presuppose the absence of triester phosphate linkages is therefore desirable. In this paper we propose such a method through titration of total phosphate groups.

An examination of the various molecular structures proposed for RNA (see Fig. 1) reveals the fact that for each possible structure the number of titratable phosphoryl groups (P(OH)) is one more than the total number of phosphorus atoms (P). Hence the number of nucleotides per molecule is equal to

\[
P = \frac{P}{P(OH) - P}
\]

where P and P(OH) are expressed in terms of molar concentration. This formula applies whether the structure is an unbranched chain of nucleotides, or one which is branched through ribose (Fig. 1, C) or through triesterified phosphate (Fig. 1, B). In the use of this formula we have assumed 1 phosphorus atom to be present per nucleotide and the amount of cyclic phosphate at the end of the chains to be negligible.
**Preparation of Sample**—Several commercial specimens of yeast RNA were studied, as well as samples of pancreas RNA prepared in this laboratory (5). In each case the sample was converted to the salt of 1 cation and 1 anion in order to facilitate the quantitative determination of bound base. The specimens of yeast RNA were dissolved in \( m \) NaCl, with the careful addition of NaOH to a final pH of 6 to 7, and were then dialyzed in the cold room first against frequent changes of \( m \) NaCl and finally against distilled water until the dialysate became free of chlorides. This treatment served to exchange sodium for any other base bound to the phosphoryl groups and chloride for any anion which might be bound to amino groups. This exchange was shown to be quantitative in the case of one sample which was converted by this method from the sodium to the potassium salt and showed a negative flame test for sodium. The yield of RNA secured by this procedure varied from about 50 per cent for sample Y2 (the “high polymer” fraction of a commercial yeast RNA prepared by Chantrenne’s method (6)) to 0 per cent for a commercial specimen of yeast RNA (Boehringer).

All determinations were performed in duplicate or triplicate on aliquots of the same stock solution of the various RNA specimens. The titration and the enzymatic end group determination were made within a narrow time interval.

1 KCl was used throughout in Sample Y1.

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**Fig. 1.** Molecular structures proposed for RNA, showing the titratable phosphoryl groups (▼) for each structure to be one more than the number of phosphorus atoms.
Bound Base—Sodium and potassium were determined by flame photometry\(^2\) after ashing the nucleic acid solution with sulfuric and nitric acids.

Phosphate was determined by the Fiske and Subbarow method (7).

Chloride was determined in the RNA samples in order to permit a correction for any base present as chloride and for the titration of any amino groups combined as hydrochloride, since these would not be distinguished from phosphoryl groups by the titration.

The samples for the chloride determination were mixed with sodium bicarbonate and ashed at 600° in a muffle furnace. The ashed material was dissolved, neutralized with HNO\(_3\), and diluted to a known volume. Chloride was then precipitated by AgNO\(_3\) and determined by comparing the optical density of the AgCl suspension with that of known standards in a spectrophotometer.

Titration—Aliquot portions of the RNA solutions containing 65 to 130 \(\mu\)moles of RNA P were titrated with 0.5 N NaOH\(^3\) from a Scholander microburette (8), the tip of which was immersed in the sample contained in a small beaker. A stream of CO\(_2\)-free nitrogen provided stirring as well as an atmosphere free of CO\(_2\). The pH was determined after each small addition of alkali with a Beckman pH meter (glass electrode). The end point of the titration was chosen to be the inflection point (determined graphically) of the titration curve near pH 8.

End Group Determination—The proportion of terminal nucleotide to total phosphate was determined by two methods, one of these being a calculation from the titration curve by the equation developed by Seraidarian (9) in the laboratory of Dr. Gerhardt Schmidt.\(^4\) The second method is the estimation of glycol groups which appear when phosphomonoesterase sets free the phosphate bound to carbon 3 of ribose in the terminal nucleotide. The procedure used was that described by Schmidt et al. (10). The preparation of the enzyme is described in an accompanying paper (11).

Calculation—The dimensions of the dissociation constants of the various acidic and basic groups found in RNA (12) are such that titration from the acidic range to pH 8 would include the primary phosphoryl groups, acid-bound by the amino groups (HCl in our experiments) and the secondary phosphoryl groups. Hence “total titratable phosphoryl groups” (i.e. P(OH)) may be determined by adding milliequivalents of base found in the preparation to milliequivalents of NaOH used in titration to the inflection point and subtracting milliequivalents of chloride. The last step serves also to correct for any sodium left in solution as NaCl.

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\(^2\) The Perkin-Elmer internal standard flame photometer was used. The standards contained appropriate amounts of phosphoric and sulfuric acids as well as lithium.

\(^3\) The NaOH was prepared with precautions to keep it free from CO\(_2\) and silica.

\(^4\) This calculation assumes that all the secondary phosphoryl groups have the same pK. The equation used is quoted by Schmidt (18).
It must be pointed out here that a zwitter ion formed between the amino and phosphate groups would give a titration of amino groups not accounted for by the subtraction of Cl⁻. However, the phosphate group involved in such a zwitter ion would bind no base, and the deficiency in base would exactly account for the uncorrected amino titration.

Results

Table I presents the experimental findings obtained with several RNA preparations, the calculated values for titratable P(OH), and also the number of nucleotides per molecule, calculated by means of the equation P/(P(OH) - P). Column 3 of Table II shows the per cent of terminal nucleotide calculated on the assumption that the molecule is not branched through triesterified phosphate, while Column 4 gives the per cent of end groups calculated for a molecule branched once via triester phosphate. Column 5 shows the proportion of end groups calculated by Seraidarian's method from the titration curve (9), while Column 6 gives the corresponding values determined by measurement of the glycol groups liberated by phosphomonoesterase (10).

It is evident, from inspection of Table II, that the values obtained for end groups by calculation from the titration curves are in fair agreement with those obtained by glycol determinations after treatment with phosphomonoesterase. Furthermore, both of these values approach those ex-
expected for a straight chain rather than one branched through triester phosphate. Our results provide no information regarding possible branching from carbon 2 of ribose.

**DISCUSSION**

The results show that the RNA preparations we have examined are nucleotide polymers averaging seven to sixteen nucleotides (molecular weight 2200 to 5500). Because of the great variety of conditions used in the preparation and fractionation of samples, it is difficult to compare our values with those obtained by others with physical methods. When dif-

**Table II**

<table>
<thead>
<tr>
<th>Sample</th>
<th>No. of nucleotides per molecule</th>
<th>End groups (per cent of total P)</th>
<th>Found</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Calculated from Column 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Straight chain or branching via</td>
<td>1 branch via triester P assumed*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Y1</td>
<td>8.0</td>
<td>12.5</td>
</tr>
<tr>
<td></td>
<td>Y2</td>
<td>15.7</td>
<td>6.4</td>
</tr>
<tr>
<td></td>
<td>Y3</td>
<td>7.3</td>
<td>13.7</td>
</tr>
<tr>
<td></td>
<td>P1</td>
<td>9.8</td>
<td>10.2</td>
</tr>
<tr>
<td></td>
<td>P2</td>
<td>7.8</td>
<td>12.8</td>
</tr>
</tbody>
</table>

* See Fig. 1.
† Calculated according to Seraidarian (9) from the titration curve.4
‡ Glycol groups determined according to Schmidt et al. (10).

fusion methods were used for the determination of molecular weights, values of 10,300 to 23,250 were obtained for unfractionated samples of yeast RNA (13, 14). Owing to the fractionation procedure (6) used in the preparation of Samples Y1 and Y2, and to the dialysis of all samples against strong salt solution, our samples might be expected to have higher molecular weights than the unfractionated starting material. Kunitz (15) reported a molecular weight of about 87,000 (240 nucleotides) for a sample of commercial RNA, presumably twice precipitated with glacial acetic acid. Our samples show molecular weights of much smaller dimensions.

The range of molecular weight suggested by our results for RNA specimens is even lower than that reported by others for the ribonuclease-resistant or "core" fraction of RNA. This result is noteworthy in view
of the fact that our preparations were subjected to dialysis against strong salt solution, which should result in a greater loss of the smaller fragments than the usual procedure of dialysis against water (1). Bacher and Allen (16) found molecular weights of 2000 to 6500 (seven to twenty nucleotides) by sedimentation studies on "core" specimens. Kunitz (15), using a diffusion procedure, reported a value of 22,000 (65 nucleotides) for yeast RNA "core" fractions.

The agreement between the values for terminal nucleotides as calculated for an unbranched structure and those determined experimentally by the enzymatic and titration methods clearly indicates that none of the samples studied in this series is branched via triester phosphate to any measurable extent.

It is recognized that none of the preparations studied represents undegraded "native" RNA. Commercial yeast RNA is subjected during its preparation to drastic chemical treatment. The pancreas RNA, although prepared in the cold room within a pH range of 4 to 7 (5), was undoubtedly degraded by coprecipitated ribonuclease. However, it must be pointed out that branching through triester phosphate was suggested by others (3) on the basis of studies made on samples similar to those we used.

In order to secure RNA as the free acid for the sake of determining the titratable phosphoryl groups, some workers (3, 9) precipitated the nucleic acid with strong mineral acid. In our hands this procedure yielded preparations containing considerable quantities of sodium and large amounts of the acid anion. We believe our procedure to be more reliable since, in the determination of total phosphoryl groups, base already combined with phosphate is included. Moreover, correction is made for base present as chloride and for acid bound to amino groups.

The accuracy of this calculation of molecular size is related chiefly to the accuracy of the determinations of total phosphate and of total base before titration. The latter figure represents about 90 per cent of the titratable phosphoryl groups (see Table I). A study of the effect of an error of 2 per cent (which we believe to be the maximal) in the determination of individual items in our formula would influence the final figure for the number of nucleotides by a probable 30 per cent for an RNA with nine nucleotides. The error will increase with increasing molecular weight, and therefore this method as such is not suitable for the determination of molecular weights of larger polynucleotides.

We have taken no account of the possible presence of end groups with cyclic phosphate (17). The presence of such groups would have the effect of increasing the apparent molecular weight. However, the conclusion concerning branching through triester phosphate is not affected.
SUMMARY

1. A method is described for the determination of the total titratable phosphoryl groups of ribonucleic acid (RNA) samples.

2. An equation is developed for the calculation of the number of nucleotides per molecule of RNA, based on the molar concentration of total titratable phosphoryl groups and total phosphate.

3. The determination of the molecular weights of different partially degraded RNA samples is reported, the results varying from seven to sixteen nucleotides per molecule.

4. Branching through triester phosphate is excluded in the samples studied.

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