Ion-Exchange Chromatography of a Dinucleotide Preparation from Controlled Alkaline Hydrolysis of Ribonucleic Acids

By KIMIHIKO SATOH AND YASUO INOUE

Department of Chemistry, Tohoku University, Sendai, Japan

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With the aim of preparing the 16 possible ribodinucleotides derived from the four principal ribonucleotides, we made a kinetic study to determine the optimum conditions for a partial alkaline hydrolysis of RNA. Satisfactory results were obtained when the hydrolysis of RNA (1 g.) in 0.2M-sodium hydroxide (100ml.) at 37° was ceased approx. 10 min. after the start of the reaction. The relative yields of the monomer, dimer and trimer fractions were approx. 41:30:15 in E_{260} units, indicating that the reaction conditions were reasonably close to those required from kinetic considerations. The partial alkaline hydrolysate was chromatographed on a column of DEAE-Sephadex A-25 in the presence of 7M-urea. The dinucleotide fraction thus obtained was subjected to a subsequent chromatography on Dowex 1 (X2) under acidic conditions to separate the mixture according to base composition and base sequence. The results were satisfactory, and most of the 32 dinucleotides [i.e. 16 $XpYp(3')$ plus 16 $XpYp(2')$] were fractionated by this single chromatographic procedure. The present method should be useful for further study of oligonucleotides as a time-saving method for the simultaneous preparation of a variety of dinucleotides. Further, examination of the present chromatographic pattern has provided several empirical criteria useful for the identification of oligonucleotides other than dimers appearing in the elution profile of Dowex 1 (X2) column chromatography under similar conditions.

Current investigations of the primary structure of nucleic acids and the physicochemical studies made in the last few years have evoked interest in the chemistry of oligonucleotides, including the synthetic studies of short-chain ribonucleotides of known sequence. Various approaches to oligoribonucleotide synthesis have been extensively pursued by organic chemists and biochemists, and chemical syntheses of dinucleoside monophosphates, dinucleotides bearing 3'- or 5'-phosphomonoester groups and trinucleotides have been established (Michelson, 1959*a,b*; Lohrmann, Söll, Hayatsu, Ohtsuka & Khorana, 1966; Holý & Smrt, 1966; Cramer, Scheit & Rhaese, 1966; Griffin & Reese, 1968; for review see Cramer, 1966). Synthetic reactions with the aid of ribonucleases of different specificities have also been used to prepare doublets or triplets (Heppel, Whitfield & Markham, 1955; Egami & Sato-Asano, 1958; Schiet & Cramer, 1964; Grünberger, Holý & Šorm, 1968; Sekiya, Furuichi, Yoshida & Ukita, 1968; Egami, Uchida, Arima & Koike, 1968).

With the aim of studying the structure and reactivity of oligoribonucleotides, we have undertaken a systematic investigation of the large-scale preparation of di- and tri-nucleotides obtainable by

enzymic digestion of RNA (Aoyagi & Inoue, 1968*a,b*). Although these preparative methods by degradation of RNA with pancreatic ribonuclease (EC 2.7.7.16) or Taka-diastase ribonuclease T₁ (EC 2.7.7.26) are useful for certain purposes, the number of di- and tri-nucleotides available is limited. For a better understanding of base-composition- and base-sequence-dependent properties of oligonucleotides, a comprehensive method for the preparation of a wider variety of such nucleotides might be useful to those wishing to study the structure and reactivity of oligonucleotides. We have therefore extended the anion-exchange chromatographic procedures originally reported by Volkin & Cohn (1953) and Tomlinson & Tener (1962) to the fractionation of controlled alkaline hydrolysates of RNA. A partial alkaline hydrolysis of RNA produces the 16 possible dinucleotides consisting of the four major mononucleotides, and for each dinucleotide there are two position isomers arising from the non-specific cleavage of an intermediate dinucleotide bearing a terminal 2',3'-(cyclic)-phosphate group to a mixture of 2'- and 3'-terminal phosphomonoester groups. Thus a total number of dinucleotides formed by non-enzymic hydrolysis of RNA is 32 [i.e. 16 $XpYp(2')$ plus 16

XpYp(3')*]. One might imagine that a complete resolution of the mixture of these 32 dinucleotides must be difficult or even unfeasible, since it contains six pairs of sequence isomers, and 2'-UMP and 3'-UMP are known to be eluted as a single peak on column chromatography on Dowex 1 (X2). However, we have found that a large number of dinucleotides can be fractionated even by a single column-chromatographic operation in satisfactorily high homogeneity. When the peaks obtained by this Dowex 1 (X2) anion-exchange chromatography were inhomogeneous, supplementary column chromatography on DEAE-cellulose at neutral pH was carried out. Homogeneity of the samples thus obtained was again checked by paper chromatography and by base analysis of the hydrolysate.

The present paper also reports some new findings on the chromatographic behaviour of dinucleotides that can be conveniently employed for analysis of chromatographic patterns of oligonucleotides other than dinucleotides.

EXPERIMENTAL

Chemicals. Yeast RNA was purchased from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Ribonucleases T₁ and T₂ were respectively gifts from Dr H. Okazaki (Sankyo Co. Ltd.) and Dr T. Uchida (University of Tokyo). *Escherichia coli* alkaline phosphomonoesterase (EC 3.1.3.1) was kindly given to us by Dr S. Inoue (University of Tokyo). All other chemicals used were analytical grade.

Measurements. Extinction measurements were made on a Hitachi-Perkin-Elmer model 139 spectrophotometer with a 1 cm. quartz cell. For all pH measurements a Radiometer model TTT1b pH-meter was used. Optical-rotatory-dispersion measurements were made on a JASCO model ORD/UV-5 spectropolarimeter for identifying base sequences and for distinguishing between position isomers of terminal phosphate groups of dinucleotides.

RESULTS

Determination of apparent first-order rate constant of the alkaline hydrolysis of yeast RNA. Yeast RNA (0.5g.) was dissolved in 25ml. of water. To the RNA solution an equal volume of 0.4M-sodium hydroxide was added and the reaction was carried out at 37°. Constancy of temperature during the hydrolysis experiments was maintained by the use of a Taiyo Coolnit CL-15 unit. The hydrolysis reaction was followed, at appropriate time-intervals, by titrating phosphomonoesters produced on the alkaline hydrolysis of inter-ribonucleotide linkages with standard 5mM-sodium hydroxide after trans-

ference of a 2ml. sample into a flask containing 2ml. of 0.2M-hydrochloric acid. The reaction did not obey a strict pseudo-first-order rate law, since the reaction is composite in nature and the rate of cleavage of different internucleotide linkages is both base-composition-dependent (Kaltreider & Scott, 1962; Michelson, 1963; Bock, 1967; Lane & Butler, 1959) and base-sequence-dependent (Y. Inoue & K. Satoh, unpublished work). However, these differences were not great, under the conditions used, so that the hydrolysis of RNA, at least in the initial stage, can be approximated by the simple first-order rate law, and the apparent first-order rate constant was determined to be 0.11 ± 0.01 min.⁻¹. The reaction was found to be a general base catalysis, so that it is advisable to use a concentration of RNA not far from that of the present experiment for the practical production of dinucleotides by the method described in this paper.

Controlled alkaline hydrolysis of yeast RNA. Aqueous solution (200ml.) of RNA (4g.) was mixed with an equal volume of 0.4M-sodium hydroxide at 37° and the resulting solution was kept in a thermostatically controlled bath for approx. 10min. The reaction was stopped by adding 80ml. of 1.0M-hydrochloric acid to the reaction vessel at an appropriate time as recommended from kinetic considerations (see the Discussion section).

Fig. 1. Chromatographic separation of partial alkaline hydrolysates of RNA (4g.) according to chain length on DEAE-Sephadex A-25 (Cl⁻ form) in the presence of 7M-urea and 0.02M-tris-chloride, pH 7.6. Column dimensions were 3 cm. × 94 cm.; the eluent was a linear gradient of NaCl as shown (broken line); the fraction volume was 27 ml.; the average flow rate was approx. 80 ml./hr.

* Abbreviations: in oligonucleotides A, C, G and U represent adenosine, cytidine, guanosine and uridine respectively, and p to the left of a nucleoside symbol indicates a 5'-phosphate and to the right it indicates a 3'-phosphate (unless specified otherwise).

Fractionation according to chain length. The partial alkaline hydrolysate was diluted to 2.5–3 vol. with water, and urea was added to give 7M-urea solution. The pH of the solution was adjusted to 7.6 with the minimum amount of tris-chloride. The solution was then applied on a column (3cm. $\times$ 94cm.) of pre-equilibrated DEAE-Sephadex A-25. The column was then eluted with 10l. of 0.1–0.27M-sodium chloride linear gradient in 7M-urea at pH 7.6 (adjusted with 0.02M-tris-chloride) at a flow rate of approx. 80ml./hr. The fraction volume was 27ml. This method is essentially the same as that reported by Rushizky, Bartos & Sober (1964) (see also Tener, 1967). The results are shown in Fig. 1.

Fractionation of dimer fraction according to base sequence. On the basis of the pioneering work by

Volkin & Cohn (1953), the dinucleotide mixture, successfully fractionated according to size, can be subfractionated by chromatography on Dowex 1 (X2; Cl⁻ form) at pH values where the bases are partially or fully protonated. The dinucleotide fraction was diluted to approx. 5 vol. and adsorbed on a column of Dowex 1 (X2). The details of the experiment are described in Fig. 2 legend. The fractions in each peak in the chromatogram (Fig. 2) were combined and neutralized to pH 7 immediately after elution. This solution was then diluted to approx. 10 vol. with water and adsorbed on a small column (approx. 1.7cm. $\times$ 12cm.) of DEAE-Sephadex A-25 (HCO₃⁻ form). Each column was washed well with freshly prepared 0.02M-ammonium hydrogen carbonate (about 2l.), and the dinucleotides were eluted with 1.0M-ammonium hydrogen

Fig. 2. Chromatography of a dinucleotide mixture (salt-free) (17500 E_{260} units) on Dowex 1 (X2; Cl⁻ form). The column dimensions were 3.2cm. $\times$ 137cm.; the eluent was a linear gradient of NaCl in 5mM-HCl, pH 2.4, from 0.02M-NaCl (5l.) to 0.5M-NaCl (5l.) and then 0.5M-NaCl (4l.) to 0.9M-NaCl (4l.); the fraction volume was 7ml.; the flow rate was 80ml./hr.

carbonate. Water and ammonium hydrogen carbonate were removed by freeze-drying, and white fluffy residues of the dinucleotides were obtained in the form of diammonium salts.

Several attempts were also made to separate the 16 dinucleoside monophosphates (XpY) obtained by dephosphorylation of the mixture of 32 dinucleotides with *E. coli* alkaline phosphomonoesterase. Chromatography under neutral conditions on DEAE-cellulose anion-exchanger showed poorly resolved elution patterns. Dowex 1 (X2) [or AG-1 (X2)] column chromatography at acidic pH values (pH 2.9–3.5) is again recommended for the fractionation of dinucleoside monophosphates, although a complete separation cannot be attained.

Identification of dinucleotide preparations. The base sequences of the dinucleotides were determined from their relative positions in a Dowex column chromatogram (Fig. 2) at an acidic pH (the primary elution sequences of nucleotides at pH 2.5–3.5 are in the order CMP, AMP, UMP and GMP), by base composition and known specificities of pancreatic ribonuclease and ribonuclease T_1 , and by, when necessary, *E. coli* alkaline phosphomonoesterase treatment followed by the hydrolysis with ribonuclease T_2 . Column chromatography of the

ribonuclease digests of the dinucleotide mixture was carried out with the aim of simplifying the elution pattern and ascertaining the assignment of the peaks in Fig. 2. The results are reproduced in Fig. 3. In some cases optical-rotatory-dispersion titrations at the pH range of the pK_a of the secondary phosphate were made to determine the position of the terminal phosphate groups (Inoue & Satoh, 1969).

The homogeneity of the peaks was verified by paper chromatography in a manner similar to that reported by Aoyagi & Inoue (1968a). Spectrophotometric constants were also used for the dinucleotides obtainable from the ribonuclease T_1 digest (Aoyagi & Inoue, 1968a) and pancreatic ribonuclease digest (Stanley & Bock, 1965). Where there was inhomogeneity due to overlapping of more than two components, supplementary column chromatography on DEAE-cellulose was carried out at neutral pH (for experimental procedure, see Aoyagi & Inoue, 1968b).

DISCUSSION

Kinetic consideration for the preparation of dinucleotides by partial alkaline hydrolysis of RNA.

Fig. 3. Chromatography of the ribonuclease T_1 digest of a dinucleotide mixture (7600 E_{270} units), obtained by partial alkaline hydrolysis of RNA in 0.2M-NaOH for 10 min. at 37°, on Dowex 1 (X2; Cl⁻ form). The column dimensions were 2.3 cm. $\times$ 142 cm.; the eluent was a linear gradient system of 5l. of 0.0M-NaCl in 5mM-HCl, pH 2.4, and 5l. of 0.5M-NaCl in 5mM-HCl; the fraction volume was 6 ml.; the flow rate was 60 ml./hr. —, E_{260} ;, E_{295} .

The average structure of RNA used may be represented by:

In 0.2M-sodium hydroxide at 37° (RNA concentration 1%) the observed apparent rate constant of

Assuming that every phosphodiester bond is hydrolysed at the same rate, then we can formulate the following rate equation:

$$\frac{dN_{n-i}}{dt} = 2k(N_n + N_{n-1} + N_{n-2} + \cdots + N_{n-i+1}) - (n-i+1)kN_{n-i} \quad (1)$$

where N_{n-i} is the concentration of a nucleotide with $n-i$ degree of polymerization and k is the first-order rate constant. Integration of eqn. (1) gives:

$$N_{n-i} = (i-1)N_n \exp[-(n-i+1)kt] - 2iN_n \exp[-(n-i)kt] + (i+1)N_n \exp[-(n-i-1)kt] \quad (2)$$

Therefore the concentration of monomer, dimer and trimer formed can be given by eqns. (3), (4) and (5) respectively:

$$[\text{monomer}] = N_1 = (n-2)N_n \exp(-2kt) - 2(n-1)N_n \exp(-kt) + nN_n \quad (3)$$

$$[\text{dimer}] = N_2 = (n-3)N_n \exp(-3kt) - 2(n-2)N_n \exp(-2kt) + (n-1)N_n \exp(-kt) \quad (4)$$

$$[\text{trimer}] = N_3 = (n-4)N_n \exp(-4kt) - 2(n-3)N_n \exp(-3kt) + (n-2)N_n \exp(-2kt) \quad (5)$$

If the average chain length of RNA, n , is large enough ($n \geq 20$), the formation and disappearance of the monomer, dimer and trimer fractions may be illustrated as a function of time by Fig. 4. As is clear from Fig. 4, during alkaline hydrolysis of RNA the concentration of the dimer fraction rises with time to a maximum followed by an exponential decay. Thus the time $t_{\max}$ can be defined and used to attain the maximum yield of the dinucleotide preparations. Differentiation of eqn. (4) gives:

$$\frac{d[\text{dimer}]}{dt} = -3k(n-3)N_n \exp(-3kt) + 4k(n-2)N_n \exp(-2kt) - k(n-1)N_n \exp(-kt) \quad (6)$$

at equilibrium $d[\text{dimer}]/dt$ equals zero, and if n is larger than, say, 20, we obtain the following relation:

$$t_{\max} = 2.303 \times \log 3 \times k^{-1} \quad (7)$$

0.11 min.⁻¹ gives $t_{\max} = 9.3$ min. Namely, if the reaction is stopped 9.3 min. after mixture of a solution of RNA and an equal volume of 0.4M-sodium hydroxide at 37°, the highest yield of dinucleotide mixture should be obtained. The relative magnitudes of the concentrations of mono-, di- and tri-nucleotides formed at $t_{\max}$, are in principle measurable quantities and can be used to test the validity of some assumptions made in the present treatment. The calculated proportions of mono-, di- and tri-nucleotide base concentrations are:

$$[\text{monomer}]:2[\text{dimer}]:3[\text{trimer}] = 44:29:15$$

Experimentally, beginning with a solution containing 4g. of RNA, the proportions of the total E_{260} values of monomer, dimer and trimer fractions obtained from a DEAE-Sephadex A-25 chromatography in 7M-urea were found to be:

$$\text{monomer:dimer:trimer} = 34\,000:25\,000:12\,600$$

Although these values are not directly related to the true concentrations of various species present in solutions, the results obtained seem to be satisfactory for a practical method of oligonucleotide preparations.

Chromatography of the dimer fraction on a Dowex 1 (X2) column. By a method substantially the same as reported by Aoyagi & Inoue (1968a,b) for the preparation of pure di- and tri-nucleotides obtainable from ribonuclease digestions, a Dowex 1 (X2) column chromatography under acidic conditions was used for the separation of the dinucleotide

Fig. 4. Changes in concentrations of monomer, dimer and trimer fractions during the alkaline hydrolysis of polyribonucleotides.

fraction. CpCp is eluted in the first peak in the elution profile (Fig. 2) since this nucleotide is present in the least negatively charged form under the acidic elution conditions (pK_a of CMP is 4.2), whereas GpGp appears in the last peak, which is attributable to its greater affinity than UpUp for the resin surface. The present chromatographic results provide several relations that may be useful as criteria for identification of oligonucleotides other than dimers when chromatography is carried out at conditions similar to those used here.

(a) The 2'-position isomers are always eluted in earlier peaks than 3'-isomers, and the separation of the two position isomers is increased in the order:

(b) The separation of a pair of sequence isomers seems to be greatly influenced by the nature of pyrimidine base present in them, and thus the separation increases with the order:

By this analogy, the elution profiles of trinucleotides obtained from ribonuclease T_1 and pancreatic ribonuclease digestion may be understood (Aoyagi & Inoue, 1968a,b):

(c) The elution order of a pair of sequence isomers seems to be governed by the nature of the base closest to the phosphomonoester group, those having a purine at the 3'-termini being eluted faster than the corresponding sequence isomers, thus:

This relation may be applied to the prediction of the elution order for a given pair of trinucleotide sequence isomers. Those having purine base(s) closer to their 3'-termini are eluted faster than the corresponding sequence isomer(s):

(d) To supplement the relation (c), for a pair of isomers made up of only purine nucleotides, ApGp and GpAp, the former is eluted more slowly than the latter. Similarly, it follows that GpApUp is eluted faster than ApGpUp (cf. GpApCp $\approx$ ApGpCp).

The present chromatography readily provides us with most of dinucleotides bearing a 3'-terminal phosphate group: ApAp(3'), ApCp(3'), ApGp(3'), CpAp(3'), CpCp(3'), CpUp(3'), CpGp(3'), GpAp(3'),

GpGp(3'), GpUp(3'), UpAp(3'), UpGp(3') and UpUp(3'). The 2'-isomers of most of these dinucleotides are also obtainable from this chromatography. GpCp(2') and GpCp(3') are eluted in a single peak, and some peaks of ApUp, ApGp and GpAp overlap. However, rechromatography of these fractions on a column of DEAE-cellulose can separate some of these nucleotides. Therefore, when we combine the previous results obtained with dinucleotide preparations from ribonuclease T_1 digestion [ApGp(3'), CpGp(3') and UpGp(3')] and those from pancreatic ribonuclease digestion [ApCp(3'), ApUp(3'), GpCp(3') and GpUp(3')] with the present chromatographic results, we can now prepare all possible dinucleotides having 3'-terminal phosphate, and consequently 16 possible dinucleoside monophosphates by their enzymic dephosphorylation.

As a minor possible utilization of the present results, it should be noted that 2'-UMP can be easily prepared by either enzymic or non-enzymic hydrolysis of ApUp(2') or GpUp(2') followed by paper- or column-chromatographic separation of the hydrolysates. At the monomer level, 2'-UMP is hardly separated from the mixture of 2'- and 3'-isomers (see Fig. 3).

Finally, further modification of the present chromatographic analysis of partial alkaline hydrolysates may open up an approach to the automated computerized analysis of the nucleotide sequences of larger oligonucleotide fragments. The variation of the relative yields of the dinucleotides should be taken as a strong indication of the appreciable variation in rate constants of the specific base-catalysed hydrolysis of phosphodiester bonds linking the four principal nucleotides in the 16 possible combinations, although the observed relative yields are of course partly ascribed to nearest-neighbour frequencies in RNA used. Supporting evidence for the former possibility has been obtained by a preliminary kinetic study of the alkaline hydrolysis of several dinucleotides at low concentrations (0.01–0.1 mM).

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