Stepwise Degradation of Polyribonucleotides†

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ABSTRACT: A method for the preparative stepwise degradation of polyribonucleotides has resulted from the development of a new reaction sequence employing the periodate oxidation–β elimination–dephosphorylati on procedure. The method consists of a series of degradation cycles each of which involves (i) the periodate oxidation of the 3′-terminal of the polynucleotide, (ii) the removal of the excess periodate by reaction with rhamnose, (iii) the cleavage of the oxidized terminal nucleoside from the chain and the enzymatic dephosphorylation of the polynucleotide product in one step, and (iv) the separation of the polynucleotide from the reactants and other products. The reaction sequence has a number of advantages over previously used procedures in which the periodate oxidation and the β-elimination steps were usually combined in the one reaction mixture and the dephosphorylation was effected in a separate reaction. The conditions for quantitative reaction in each step of the degradation cycle have been perfected by using mononucleotides, dinucleoside phosphates, and adenosine tetra-, penta-, hexa-, and heptanucleotides as substrates, and the degradation procedure has been tested by carrying out nine cycles on a dodecanucleotide, T-ψ-C-A-A-U-U-C-C-C-C-G, obtained from a ribonuclease T_1 digest of $tRNA^{Asp}$. Polynucleotide sequence information may also be obtained during the stepwise degradation by isolating and analyzing the nucleoside fragment produced in each cycle. Thus, from the analyses of nine degradative cycles, the 3′-terminal nonadecanucleotide from yeast initiator tRNA was shown to be m^1A-A-A-C-C-G-A*-G*-C-G-G-C-G-C-U-A-C-C-A, a result that eliminates the sequence ambiguity in the stem region of the published sequence of this tRNA. The reaction sequence used in the degradative procedure also allows for the removal of more than one nucleotide from a polynucleotide chain in a single reaction mixture, and this technique has been demonstrated by the conversion of pA-A_5-A to A-A_2-A in 80% yield.

Since the original suggestion (Whitfeld and Markham, 1953; Brown *et al.*, 1953) that periodate oxidation and subsequent β elimination might be used as a method for the successive degradation of polyribonucleotides, the degradation procedure has been studied in a number of laboratories as a potential method for the sequence analysis of RNA (Ogur and Small, 1960; Yu and Zamecnik, 1960; Neu and Heppel, 1964; Steinschneider and Fraenkel-Conrat, 1966; Weith and Gilham, 1967, 1969, 1970; Uziel and Khym, 1969). These studies have been directed mainly at the problems of effecting quantitative yields or recoveries in the four steps of the degradative system: the periodate oxidation of the 3′-terminal diol group, the cleavage of the terminal nucleoside moiety, the enzymatic removal of the terminal phosphate group on the polynucleotide, and the separation of the polynucleotide from the released nucleoside base. The usual reaction sequence employed has involved the treatment of the polynucleotide with periodate together with a primary amine to effect, in the one reaction mixture, the oxidation step, the β-elimination reaction, and the subsequent conversion of the nucleoside fragment to the corresponding base. A number of physical methods have been developed for the separation, at this stage, of the polynucleotide fragment from the released base, and for the isolation and identification of the base. The polynucleotide must then be dephosphorylated prior to the next degradative cycle and, thus, each reaction cycle is maintained separate from successive cycles by carrying out the dephosphorylation in a separate step. In earlier studies in this laboratory on the structure of the 3′-terminal sequence of bacteriophage ribonucleic acids this was also the general reaction sequence used. However, in more recent work directed toward the development of an automatic polynucleotide sequencing device, a new reaction technique has been indicated (Weith and Gilham, 1970; Rosenberg *et al.*, 1971). A cycle of degradation using this procedure consists of (i) the periodate oxidation of the polynucleotide at 0°, (ii) the removal of the excess periodate at 0° with rhamnose, (iii) the amine-catalyzed removal of the oxidized nucleoside moiety and the enzymatic dephosphorylation of the polynucleotide in the same reaction mixture at 45°, (iv) the separation of the dephosphorylated polynucleotide from the nucleoside fragment, and (v) the identification of the purine or pyrimidine base contained in the nucleoside fragment. This degradation procedure has several advantages over the earlier approach in that (i) the total reaction time is reduced, (ii) the polynucleotide is not exposed to periodate at the high temperature during the β-elimination step thus avoiding possible side reactions, (iii) the released nucleoside fragment contains all of the original carbon atoms of the ribose moiety thus permitting the introduction of radioactive label into the fragment to increase sensitivity in sequence analysis applications (Schwartz and Gilham, 1972), and (iv) the cycles of degradation are controlled by the presence or absence of periodate and by the two temperatures used, and this allows a number of degradation cycles to be specifically carried out in one reaction mixture. The present work contains the details of this degradation procedure and its application to adenosine oligonucleotides and tRNA fragments.

Experimental Section

Materials. *Escherichia coli* alkaline phosphatase (Grade BAPF, Worthington Biochemical Corp., Freehold, N. J.) was assayed by the method of Garen and Levinthal (1960), and the unit of activity is that quantity of enzyme that liberates 1 μmol of *p*-nitrophenol from *p*-nitrophenyl phosphate per min at 25°. The dinucleoside phosphates, A-A and G-A, were purchased from P-L Biochemicals Milwaukee, Wis. The adenosine oligo-

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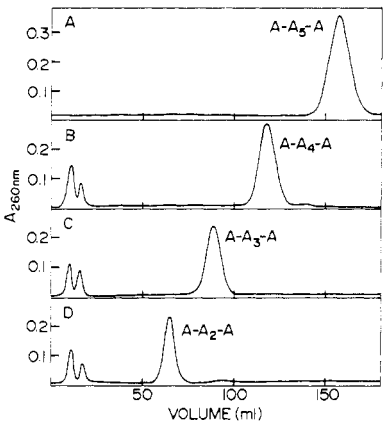

FIGURE 1: Elution patterns from the column chromatography of the products from the degradation of adenosine oligonucleotides: (A) A-A_5-A before degradation; (B), (C), and (D) products from a single cycle of degradation carried out on A-A5-A, A-A4-A, and A-A3-A, respectively. Each separation was made on a column of Dowex 1-X2 (−400 mesh) ion-exchange resin with dimensions of 100 × 0.4 cm and the elution was carried out at 12 ml/hr, under pressure, with 200 ml of 40% ethanol solution containing a linear gradient of 0.3–0.5 M ammonium chloride which had been brought to pH 8 with ammonium.

nucleotides were prepared from a pig liver nuclei ribonuclease digest of poly(A) as described previously (Bennett *et al.*, 1973). The dodecanucleotide from the ribonuclease T_1 digest of yeast $tRNA^{Asp}$ was isolated by the method of Keith *et al.* (1970) and Gangloff *et al.* (1971) while the nonadecanucleotide corresponding to the 3′ terminus of yeast initiator tRNA was kindly provided by Dr. M. Simsek and Dr. U. L. RajBhandary, Department of Biology, M. I. T. The chromatographic materials, Dowex 1-X2, Dowex 1-X4, and beaded polyacrylamide, were purchased as AG 1-X2 (-400 mesh), AG 1-X4 (-400 mesh), and Bio-Gel P-2 (200–400 mesh), respectively, from Bio-Rad Laboratories, Richmond, Calif. *N,N,N′,N′*-Tetramethylglycina mide (TMGA[1]) was obtained from Eastman Kodak Co., Rochester, N. Y., and converted into its hydrochloride. The base was mixed with water and brought to pH 5 with concentrated HCl. The mixture was rendered free of water by evaporation *in vacuo* and the hydrochloride was recrystallized from butanol. Cyclohexylamine hydrochloride was prepared in a similar way and recrystallized from water. The TMGA–cyclohexylamine reagents were prepared by adjusting a solution of the two hydrochlorides to pH 8 at 45° with concentrated NaOH.

Periodate Oxidation. The rates of periodate oxidation under various conditions were determined using nucleoside 5′-phosphates and dinucleoside phosphates, and the products were analyzed by paper chromatography after stabilization by reduction with sodium borohydride. The nucleotide or dinucleoside phosphate (2 μmol) was dissolved in 250 μl of water, dilute sodium chloride, or buffered amine solution and was treated with 250 μl of 0.04 M sodium periodate at various temperatures. At regular intervals of time 20-μl samples of the reaction mixture were withdrawn and each sample was treated with 20 μl of 0.1 M sodium borohydride. After 1 hr at 0° the sample was applied to Whatman No. 3MM chromatographic paper and eluted with ethanol–1 M ammonium acetate (7.5:3, v/v) which had been saturated with sodium borate and adjusted to pH 10 with ammonia (Plesner, 1955). The products were then cut out and extracted, and their amounts were determined spectrophotometrically. The R_F values of the various products are listed in Table I.

[1] Abbreviation used is: TMGA, *N,N,N′,N′*-tetramethylglycinamide.

TABLE I: R_F Values of Oxidation Products.[a]

Nucleotide	Unreacted Nucleotide	Oxidized and Reduced Product[b]	Oxidized β-Eliminated and Reduced Product[c]
pA	1.00	1.70	2.90
pC	1.04	2.15	3.00
pG	0.67	1.90	2.80
pU	1.17	2.25	3.00
Ap	1.78		
Gp	0.55		
A-A	1.19	2.35	
G-A	0.38	1.25	

[a] The R_F values listed are relative to the R_F value of pA, and were measured on Whatman No. 3MM paper with the solvent, ethanol–1 M ammonium acetate (7.5:3, v/v) which had been saturated with sodium borate and adjusted to pH 10 with ammonia. [b] These products correspond to the dialcohol derivatives formed by treating the nucleotide with sodium periodate at 0° and then with sodium borohydride in the absence of a primary amine. [c] These products correspond to the methylene dialcohol derivatives (Schwartz and Gilham, 1972) formed by oxidation with periodate and the removal of the excess with rhamnose, followed by treatment of the reaction mixture at 45° in the absence of primary amines and the subsequent reduction of the product with sodium borohydride.

Destruction of Excess Periodate. A mixture of 250 μl of water or buffered amine solution and 125 μl of 0.04 M sodium periodate was added to 0.04 M rhamnose (125 μl) at 0°. At various times 25-μl samples of the reaction mixture were withdrawn and each sample was treated with 0.01 M adenosine 5′-phosphate (25 μl) for 1 hr at 0° and then with 0.1 M sodium borohydride (25 μl) for 1 hr at 0°. Each sample was then analyzed by paper chromatography as described above to determine the amount of nucleotide that had been oxidized and reduced.

β-Elimination and Dephosphorylation Reactions. The dinucleoside phosphate or oligonucleotide (about 100 nmol) in 150 μl of 0.05 M sodium chloride was treated at 0° with 20 μl of 0.2 M sodium periodate for 1 hr followed by 20 μl of 0.4 M rhamnose at 0° for 30 min. To this mixture was added 60 μl of 0.5 M TMGA·HCl (pH 8, 45°) containing various concentrations of the primary amine hydrochloride. In the case of the degradation of oligonucleotides 40 μl of phosphatase solution (50 units/ml) was also added. The reaction mixtures were kept at 45° for various times. In the case of the dinucleoside phosphates the extent of reaction was determined by paper chromatographic analysis of the products (Table I) while the reaction products of the various oligonucleotides were analyzed by ion-exchange chromatography (Figure 1).

Stepwise Degradation of A-A_4-A. For each cycle of degradation the oligonucleotide (about 100 nmol) was dissolved in 150 μl of water and treated with 20 μl of 0.2 M sodium periodate at 0° for 1 hr. Rhamnose (0.4 M, 20 μl) was then added and the solution was kept at 0° for a further 0.5 hr. The amine solution, 60 μl of 0.5 M TMGA–2 M cyclohexylamine hydro-

chloride (pH 8 at 45°) and 40 μl of phosphatase solution (50 units/ml) were added. The mixture was kept at 45° for 90 min, and the products were then separated by passage through a gel filtration column. The column of Bio-Gel P-2 (65 × 1 cm) was prewashed with 0.2 M sodium acetate in 5% aqueous butanol and then with 1 mM sodium chloride in 5% aqueous butanol before the fractionation of the products from each cycle of degradation. The reaction mixture was applied to the column with a few milliliters of the dilute sodium chloride solution and this was followed by 5 ml of the same salt solution. Elution was then effected with 0.2 M acetic acid at a flow rate of 25 ml/hr. The elution pattern from a typical separation is shown in Figure 2. The fractions containing the oligonucleotide were combined and evaporated to dryness *in vacuo* in a small tube. The product was then redissolved in 150 μl of water and subjected to the next degradation cycle as described above. After each cycle and separation step a portion of the oligonucleotide product was removed and analyzed by ion-exchange chromatography (Figure 3).

Stepwise Degradation of T-ψ-C-A-A-U-U-C-C-C-C-Gp. The polynucleotide fragment (15 $ODU_{260\,nm}$) from $tRNA^{Asp}$ was processed through a cycle of degradation and separation as described above except that no periodate was added to the reaction mixture. This step served to remove the 3′-terminal phosphate group prior to the first periodate oxidation. The dephosphorylated dodecanucleotide was then subjected to nine degradative cycles as described above for A-A_4-A. The yields (in $ODU_{260\,nm}$) of the oligonucleotide recovered after each cycle were 1, 11.3; 2, 8.9; 3, 7.7; 4, 6.6; 5, 5.3; 6, 4.8; 7, 4.3; 8, 3.8; and 9, 3.2. The progress of the stepwise degradation was followed by determining the 3′-terminal nucleoside of the oligonucleotide obtained after five cycles (T-ψ-C-A-A-U-U), and after nine cycles (T-ψ-C). A sample of each of these two oligonucleotide fractions was treated with 0.25 M sodium hydroxide at 37° for 20 hr. The alkaline hydrolysate was then diluted to 1 ml with water and analyzed by a new ion-exchange chromatographic system (G. T. Asteriadis and P. T. Gilham, unpublished data). A column of Dowex 1-X4, -400 mesh (100 × 0.2 cm), was prewashed with 20% ethanol under pressure before the following additions were made by means of a sample injection loop: 0.3 ml of 1 M sodium hydroxide, the alkaline hydrolysate, and two 0.5-ml portions of 20% ethanol. Separation was effected by elution, under pressure at 10 ml/hr, with 200 ml of 20% ethanol containing a linear gradient of 0–0.5 M ammonium chloride that had been brought to pH 10 with ammonia. The retention volumes of the four nucleosides are cytidine, 24 ml; adenosine, 35 ml; uridine, 45 ml; guanosine, 55 ml. Under these conditions the oligonucleotide fraction obtained after five cycles gave 93% uridine and 7% cytidine as its terminal nucleoside while the product obtained after nine cycles was shown to contain 92% cytidine and 8% adenosine at its 3′ terminus.

Stepwise Degradation of m^1A-A-A-C-C-G-A-G*-C-G-G-C-G-C-U-A-C-C-A.* The nonadecanucleotide (15 $ODU_{260\,nm}$) from yeast initiator tRNA was carried through nine cycles of degradation as described above. The yields (in $ODU_{260\,nm}$) of oligonucleotide obtained after each cycle were 1, 13.2; 2, 11.8; 3, 10.0; 4, 8.1; 5, 6.9; 6, 6.3; 7, 4.9; 8, 4.6; 9, 4.0. After the gel filtration separation of the products from each degradative cycle the fractions corresponding to the released nucleoside fragment (Figure 2) were combined and evaporated to dryness. The product was then dissolved in 10 ml of water and heated at 100° for 1 hr to complete the conversion of the nucleoside fragment to the corresponding purine or pyrimidine base. Relatively good recoveries of adenine, uracil,

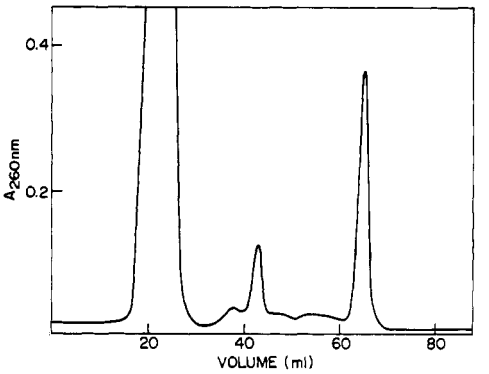

FIGURE 2: Elution pattern from the separation by gel filtration of the products from a cycle of degradation carried out on A-A_4-A. The column of Bio-Gel P-2 had dimensions 65 × 1 cm and, after the reaction mixture was applied in dilute sodium chloride, the elution was carried out at 25 ml/hr with 0.2 M acetic acid. The peaks with retention volumes of 22, 43, and 65 ml correspond to the oligonucleotide, iodate, and nucleoside fragment, respectively. For the recovery of the oligonucleotide, fractions corresponding to elution volumes 16–28 ml were combined and, for the isolation of the nucleoside fragment, fractions corresponding to elution volumes, 40–80 ml, were combined.

and cytosine can be achieved by this procedure while the yield of guanine is somewhat lower. The identity of each base was determined by ion-exchange chromatography and the amount obtained was estimated spectrophotometrically. The chromatographic system developed specifically for this purpose is indicated in Figure 4. The bases and the amounts (nmol) obtained from the degradation cycles were 1, A (45); 2, C (38); 3, C (32); 4, A (32); 5, U (30); 6, C (34); 7, G (12); 8, C (22); 9, G (10).

Direct Degradation of pA-A_5-A to A-A_2-A. The adenosine heptanucleotide (50 nmol) was dissolved in water (150 μl) and treated with 0.025 M sodium periodate (20 μl) for 1 hr at 0° and then with 0.025 M rhamnose (20 μl) for 30 min at 0°. To

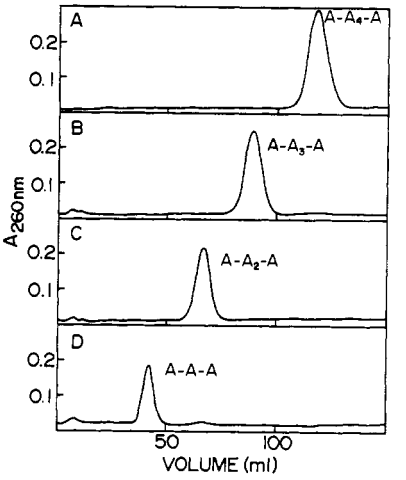

FIGURE 3: Elution patterns from the chromatographic analysis of the stepwise degradation of A-A_4-A. Each separation was carried out on a column (100 × 0.4 cm) of Dowex 1-X2 (-400 mesh) ion-exchange resin by elution at 12 ml/hr, under pressure, with 200 ml of 40% ethanol solution containing a linear gradient of 0.3–0.5 M ammonium chloride which had been brought to pH 8 with ammonia. (A) Sample of the starting oligonucleotide, (B), (C), and (D) samples of the oligonucleotide products withdrawn after the first, second, and third degradative cycle, respectively.

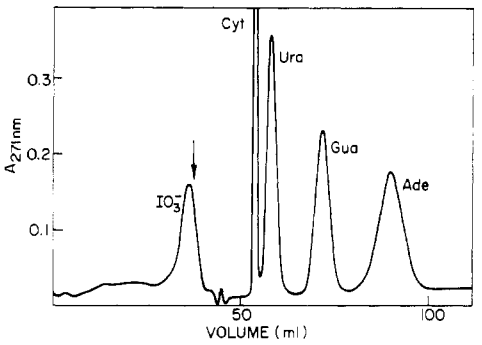

FIGURE 4: Elution pattern from the chromatographic separation of a mixture of sodium iodate, cytosine, uracil, guanine, and adenine. The column of Dowex 1-X2 (~400 mesh) had dimensions 40 × 0.4 cm and the following additions were made *via* a sample injection loop: 2 ml of 1 M sodium hydroxide, a mixture of 2 ml of 1 M sodium hydroxide and 10 ml of water containing the nucleotide bases and sodium iodate, and 5 ml of 0.05 M sodium chloride–0.1 M sodium hydroxide. The column was then eluted at 32 ml/hr, under pressure, with 0.05 M sodium chloride–0.1 M sodium hydroxide. At the point indicated by the arrow the solvent was changed to 20% ethanol containing 0.1 M ammonium chloride–0.5 M ammonium hydroxide.

this solution was added 60 μl of 0.5 M TMGA–2 M cyclohexylamine hydrochloride (pH 8 at 45°) and 40 μl of alkaline phosphatase (50 units/ml) and the mixture was kept at 45° for 90 min. The solution was then cooled to 0° and treated with 20 μl of 0.05 M sodium periodate at 0° for 2 hr and then 20 μl of 0.05 M rhamnose at 0° for 1 hr. A further 20 μl of the phosphatase solution was added and the mixture was then kept at 45° for 90 min. The solution was then treated at 0° with 20 μl of 0.15 M sodium periodate for 2 hr and then 20 μl of 0.15 M rhamnose for 1 hr. The temperature was then raised to 45° and the reaction mixture was kept at this temperature for 90 min. Samples of the reaction mixture were removed after each cycle of degradation and the products were analyzed by ion-exchange chromatography as described above. The yields of products after the first cycle were: A-A_4-A, 99%; after the second cycle, A-A_3-A, 90%; and A-A_4-A, 10%; and, after the third cycle, A-A_2-A, 80% and A-A_3-A, 20%.

Results and Discussion

Periodate Oxidation. The conditions necessary for the complete oxidation of the cis glycol group were established by using 5′-nucleotides and dinucleoside phosphates as substrates, and they were determined by observing the disappearance of the starting material and the concomitant formation of the corresponding dialdehyde in each case. The oxidation products were stabilized by reduction with sodium borohydride prior to the paper chromatographic analysis of the reaction mixtures (Table I). The oxidation studies were performed in those solvents that were to be used in the subsequent study of the degradation of oligonucleotides: water, 0.05 M sodium chloride, 0.1 M TMGA·HCl (pH 8 at 45°), or 0.1 M TMGA–0.4 M cyclohexylamine hydrochloride (pH 8 at 45°). In the case of water or dilute salt solutions containing 0.02 M periodate the oxidation was complete within 15 min at 0° whereas, in the buffered amine solutions, quantitative oxidation required about 1 hr.

The conditions necessary for the complete removal of periodate were determined by measuring the amount of periodate remaining in the reaction mixture after exposure to rhamnose at 0° for various lengths of time. The remaining periodate was estimated by its capacity to oxidize adenosine 5′-phosphate. After exposure to the nucleotide for 1 hr the reaction mixtures were treated with sodium borohydride to stabilize by reduction any oxidized nucleotide prior to the separation of the products by paper chromatography (Table I). The destruction of periodate at 0° with a mole equivalent of rhamnose was complete within 1 hr in water or dilute salt solution and within 2 hr in the buffered amine solution.

In the application of these methods to the stepwise degradation of oligonucleotides the control of each degradative cycle depends on the absence of periodate during the β-elimination and dephosphorylation steps and, thus, it was necessary to establish that no β elimination takes place during the oxidation step or during the subsequent removal of periodate with rhamnose. To test this possibility the 5′-nucleotide or dinucleoside phosphate was oxidized with periodate at 0° in water, salt solution, or buffered amine solution and then treated with rhamnose for 2 hr at 0° and finally with sodium borohydride. Analysis by paper chromatography (Table I) showed that none of the possible nucleoside derivatives that could result from the β-elimination reaction were produced and, in the case of A-A and G-A oxidized in the presence of 0.1 M TMGA–0.4 M cyclohexylamine hydrochloride, no adenosine 3′-phosphate or guanosine 3′-phosphate was formed.

Stepwise Degradation. The studies on the conditions for the β-elimination reaction with the dinucleoside phosphates, A-A and G-A, showed that the treatment of the oxidized products with 0.1 M TMGA–0.4 M cyclohexylamine hydrochloride at 45° for 90 min resulted in complete degradation to Ap and Gp, respectively. In the case of the studies with the adenosine oligonucleotides the same conditions were selected except that phosphatase was also present in the β-elimination reaction mixture. Thus, it was possible to quantitatively convert A-A_5-A to A-A_4-A; A-A_4-A to A-A_3-A; and A-A_3-A to A-A_2-A (Figure 1).

In order to carry out a series of stepwise degradations it was necessary to develop a simple and rapid system for the isolation of the oligonucleotide fragment after each degradative cycle. Gel filtration on a column of beaded polyacrylamide fulfills the requirements of such a system in that it allows the rapid separation of the oligonucleotide together with the phosphatase from the other components of the degradation reaction: amine buffer, iodate, rhamnose, and its oxidation products, and the nucleoside fragment (Figure 2). The nucleoside fragment, at this stage, is presumed to be a morpholine derivative (Schwartz and Gilham, 1972) and, as such, tends to bind ionically to the column by virtue of the partial acidic character of the polyacrylamide gel. Thus, it is necessary to use elution with dilute acetic acid to recover the fragment, and the column then requires neutralization with sodium acetate solution prior to the next separation.

The degradative and separation systems were initially tested with the conversion of A-A_4-A to A-A-A in three separate cycles. The chromatographic analysis of a portion of the oligonucleotide product after each gel filtration step indicated that quantitative conversion was achieved in each reaction cycle (Figure 3). The recovery of the oligonucleotide from each cycle of degradation and separation was about 94%. In order to test the system with a polynucleotide containing all four nucleotide bases a similar series of degradations was performed on a dodecanucleotide isolated from a ribonuclease T_1 digest of yeast $tRNA^{Asp}$ (Keith *et al.*, 1970; Gangloff *et al.*, 1971). The fragment, T-ψ-C-A-A-U-U-C-C-C-C-Gp, after removal of the terminal phosphate group, was subjected to nine degradative cycles and the course of the degradation was followed by the analysis of portions of the remaining oligonucleotide isolated after the completion of five and nine cycles. The analytical method used to determine the 3′-terminal nucleoside of the oli-

gonucleotide in each case consisted of alkaline hydrolysis of the polymer followed by ion-exchange chromatography of the products. Thus, the 3′ terminus of the oligonucleotide obtained after five cycles was shown to consist of uridine (93%) and cytidine (7%) while the terminus of the material obtained after nine cycles was shown to contain cytidine (92%) and adenosine (8%).

Although the present methods have been developed as a preparative procedure for the specific reduction of the length of a polyribonucleotide chain they also permit nucleotide sequence information to be extracted from the polymer during its degradation. The nucleoside fragment that is produced in each degradation cycle is retarded during the polyacrylamide column separation and may be recovered by combining the appropriate fractions as indicated in Figure 2. The product is then heated to complete the conversion of the derivative to the corresponding purine or pyrimidine base and is then subjected to chromatographic separation for identification. The solvents used in the ion-exchange chromatographic system specifically developed for this purpose were chosen on the basis of their ability to effect the separation of the iodate anion as well as the four nucleotide bases (Figure 4). The system was applied to the analysis of the 3′-terminal nonadecanucleotide derived from a partial ribonuclease T_1 digest of yeast initiator tRNA. The sequence of the polynucleotide was known except for the order of the two nucleotides located at the eighth and ninth positions in from the 3′ terminus. In the published work on the determination of the sequence of the initiator tRNA the fragments that were obtained and characterized did not permit an unambiguous assignment of the nucleotide order in this area of the acceptor stem region (Simsek and RajBhandary, 1972). The analytical results obtained from nine degradation cycles in the present study indicate that the sequence of the 3′-terminal polynucleotide is m^{1}A-A-A-C-C-G-A*-G*-C-G-G-C-G-C-U-A-C-C-A.[2]

Multiple Degradation. The conditions developed for the stepwise degradation of polynucleotides are such that they can be applied to the removal of more than one nucleotide in a single reaction mixture without the isolation of the successive intermediates. This application arises out of the fact that the successive degradation cycles are controlled by the presence of periodate at 0° at which temperature no β elimination occurs, and the absence of periodate at 45°, the temperature at which the β elimination is effected. Thus, by achieving a careful balance between the periodate added and the amount of rhamnose used to destroy it, it is possible to carry out a number of degradative cycles in a single reaction mixture without the isolation of the intermediate polynucleotide products. This procedure has been demonstrated by the conversion of the heptanucleotide, pA-A_5-A, by three successive cycles in the one reaction mixture, to A-A_2-A in 80% yield.

Conclusion

In developing a procedure for the preparative stepwise degradation of polyribonucleotides it was considered important to define conditions under which each of the degradative steps could be effected quantitatively. The choice of rhamnose to remove the excess periodate in each cycle was made on the basis of its rapid rate of reaction with periodate and the fact that its oxidation products do not appear to affect either the polynucleotide or the subsequent steps in the degradation. The TMGA–cyclohexylamine–phospha tase mixture has been shown to effect both quantitative β elimination and dephosphorylation. The primary amine serves to catalyze the β-elimination reaction while the TMGA acts as the buffer to control the pH of the reaction mixture for the enzymatic step. The TMGA–cyclohexylamine mixture was originally used in the development, in this laboratory, of an automatic RNA sequencing device in which the successive reactions on the polynucleotide are carried out in the presence of a polystyrene anion-exchange resin. In this application the resin serves to separate the products and reactants from the polynucleotide after each step in the degradative cycle. Although other primary amines (for example, lysine) have been shown to effect quantitative β elimination (Neu and Heppel, 1964) the use of the TMGA–cyclohexylamine mixture was also preferred for the present work because, in contrast to lysine, this mixture does not react rapidly with periodate, a characteristic that permits its use in carrying out several degradative cycles in the one reaction mixture.

For preparative stepwise degradation the use of the polyacrylamide gel column constitutes a simple and efficient method for the separation of the polynucleotide from the reactants and other products from each reaction cycle, and it permits a complete cycle to be carried out in about 5 hr. In addition, the gel column separation yields the nucleoside fragment from the reaction cycle and this allows the determination of nucleotide sequence during the degradation procedure. In the application of the procedure to the determination of nucleotide sequences in unlabeled polynucleotides it should be possible to increase the sensitivity of the technique by the introduction of radioactive label into the products from each cycle. One approach would involve the removal, after each cycle, of a portion of the oligonucleotide for the determination of its 3′-terminal nucleoside using the periodate–sodium [^{3}H]borohydride method developed by RajBhandary (1968). An alternative procedure would exploit methods for the introduction of radioactive label into the nucleoside fragment that is produced in each reaction cycle (Schwartz and Gilham, 1972).

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Magnesium Precipitation of Ribonucleoprotein Complexes. Expedient Techniques for the Isolation of Undegraded Polysomes and Messenger Ribonucleic Acid†

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ABSTRACT: A procedure is described for the precipitation of ribosomes from crude homogenates of animal tissues. The procedure was developed using chicken oviduct but has been used successfully with tissues from organisms as diverse as slime mold, insect, and mammal. Tissue is homogenized in the presence of detergent (Triton X-100) and a ribonuclease inhibitor, heparin; then after a 5-min centrifugation at 27,000*g*, the ribosomes in the supernatant are precipitated by addition of $MgCl_2$ to 100 mM, and collected 60 min later by a 10-min centrifugation at 27,000*g*. Ribosomes prepared in this way can be easily resuspended; polysomes are undegraded and biologically active, and they are suitable for the immunoprecipitation of a specific polysome class. In addition, EDTA-derived messenger ribonucleoprotein complexes can be precipitated by this method; however, tRNA is not precipitable. The recovery of translatable ovalbumin mRNA from hen oviduct is approximately 80% and the recovery of ribosomes is virtually 100%. Conditions are described in detail for the extraction of translatable ovalbumin mRNA with sodium dodecyl sulfate–phenol–chloroform, and for the removal of contaminating inhibitors of mRNA translation using high salt washes. The magnesium precipitation method is expedient, economical, and well suited for either large-scale isolation of polysomes and mRNA, or for the fractionation of many tissue samples.

The precipitation of ribosomes by Mg^{2+} was first reported by Takanami (1960). This technique has been used subsequently in several laboratories in conjunction with traditional ultracentrifugation methods during ribosome purification (Attardi and Smith, 1962; Gazzinelli and Dickman, 1962; Petermann and Pavlovec, 1963; Iwabuchi *et al.*, 1970). More recently, it has been used instead of ultracentrifugation for the isolation of ribosomes from crude tissue homogenates (Levy and Carter, 1968; Leytin and Lerman, 1969; Lee and Brawerman, 1971; Bitte and Kabat, 1972; Koka and Nakamoto, 1972; Dessev and Grancharov, 1973; Clemens and Pain, 1974). In this paper, the Mg^{2+} precipitation method is further adapted for the quantitative isolation of undegraded polysomes and translatable mRNA from homogenates of chicken oviduct tissue.

Methods

Buffers. Hepes,[1] a 100 mM stock solution, was prepared, adjusted to pH 7.5 with KOH, and treated with diethyl pyrocarbonate (see below). PB, 25 mM Tris–25 mM NaCl–5 mM $MgCl_2$, adjusted to pH 7.5 at room temperature with HCl: a 10X stock solution was prepared, treated with diethyl pyrocarbonate, and then diluted with distilled water. PBM, 25 mM Tris–25 mM NaCl–100 mM $MgCl_2$ (pH 7.5). 0.2PBM, 0.2PB, 1.0PB: buffers PB or PBM with sucrose added to give the molarity indicated; reagent grade sucrose was used but solutions were always treated with diethyl pyrocarbonate. A, buffer PB containing heparin at 1 mg/ml and 2% Triton X-100; prepared from a 10X stock of buffer PB, dry heparin, and a 10% solution of Triton X-100 in distilled water. B, buffer A (4 volumes) diluted with 1 M $MgCl_2$ (1 volume) to yield 0.2 M $MgCl_2$.

Diethyl Pyrocarbonate Treatment. Two drops (about 50 μl) of diethyl pyrocarbonate (Eastman) were added per 100 ml of solution. The mixture was shaken at room temperature and then placed in a boiling water bath for 15–30 min to decompose the remaining diethyl pyrocarbonate. While it was still hot, the solution was shaken vigorously to allow CO_2 and ethanol to escape; this step was repeated several times. Diethyl pyrocarbonate inactivates RNase and it has been used directly during tissue homogenization. Although intact RNA (Fedorcsák *et al.*, 1969) and polysomes can be prepared (Weeks and Marcus, 1969; Anderson and Key, 1971), this procedure destroys ovalbumin mRNA activity, probably due to the alkylation of RNA (Rhoads *et al.*, 1973; R. D. Palmiter, unpublished).

Magnesium Precipitation of Ribosomes. All procedures were at 0–2°. A 10% homogenate of tissue was prepared essentially as before (Palmiter, 1971). For each gram wet weight of finely minced tissue (either fresh or previously frozen at −20°), 9 ml of buffer A was added and the tissue was homogenized in a Dounce homogenizer (Kontes Glass Co.) with several strokes of a loose pestle followed by 10–20 with a tight pestle. The homogenate was centrifuged for 5 min at 27,000g_{max};

† From The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138. *Received April 8, 1974.* This research was supported by grants from the National Institutes of Health (HD 04701), National Science Foundation (GB 35608), and the Population Council (M74.62C).

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[1] Abbreviations used are: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; mRNP, messenger ribonucleoprotein.