The Use of the Cetyltrimethylammonium Cation in Terminal Sequence Analyses of Ribonucleic Acids*

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ABSTRACT: Several complicated manipulations need to be carried out before a new cycle is started in the periodate–amine stepwise degradation of ribopolynucleotides. The cetyltrimethylammonium (CTA) cation is used here to simplify these manipulations. The CTA salt of transfer ribonucleic acid is soluble in 1 M chloride solutions and insoluble in 0.2 M chloride solutions. This property is used to precipitate quantitatively the polynucleotide from its released terminal base and from phosphomonoesterase in the periodate–amine stepwise degradation of tRNA. Desalting techniques were devised which permitted quantitative measurements of the released bases. Interfering ions were removed with ion exchangers. The bases recovered in acid solution were analyzed by cation-exchange chromatography using a hydrochloric acid elution system. Alternatively, the base analyses may be made directly on cation exchangers at pH 4.7 without prior desalting.

The terminal nucleoside of a polyribonucleotide releases its purine or pyrimidine base after reaction with periodate in the presence of an amine, giving rise to a 3′-terminal phosphate group. Enzymatic removal of this 3′-phosphomonoester end group yields a new terminal nucleoside. Hence, as originally proposed (Whitfeld, 1954; Brown *et al.*, 1955), repetition of the process gives a stepwise degradation procedure for determining nucleotide sequences in RNA. Further contributions from many different laboratories (Ogur and Small, 1960; Yu and Zamecnik, 1960; Khym and Cohn, 1961; Cohn and Khym, 1962) and particularly from the detailed study of Neu and Heppel (1964) permitted practical use of this procedure. The refined procedure has been applied not only to oligoribonucleotides but also, with some additional modifications, to the nucleotide sequence determination in larger polyribonucleotides (Yu and Zamecnik, 1960; Neu and Heppel, 1964; Hunt, 1965; Whitfeld, 1965; Steinschneider and Fraenkel-Conrat, 1966a,b). In these latter determinations, the separation of the polynucleotide from its released base involves several complicated manipulations before a new cycle can be started. We have used the CTA[1] cation to simplify these steps.

Dutta *et al.* (1953) observed that nucleic acids readily form complexes with CTA that are soluble in concentrated chloride solutions (*ca.* 1 M) but insoluble in more dilute solutions (<0.3 M). We have used this property to precipitate quantitatively the parent nucleic acid from its released terminal base and from phosphomonoesterase in the periodate–amine stepwise degradation of tRNA.

Experimental Section

Materials. The source of tRNA was a commercial preparation of "soluble" RNA from *Escherichia coli* B obtained from General Biochemicals, Inc. This material was purified on a DEAE-cellulose column (Holley *et al.*, 1961) to remove contaminants of low molecular weight; it was then precipitated with ethanol, dried, dissolved in water, and stored in a frozen state. Before use in sequence studies, aliquots were hydrolyzed in alkali and terminal nucleosides were determined by the method of M. Uziel and W. E. Cohn (in preparation). The material gave approximately 1 nmole of adenosine/optical density unit measured at 260 mμ.[2] The only other terminal nucleoside present in significant amounts was cytidine. Our first experiments were carried out on 1 μmole of tRNA so as to facilitate quantitative identification of the released base in each cycle. Later, we found that 0.1-μmole amounts of tRNA could be used if the released base was identified and quantitated by the sensitive system of M. Uziel and W. E. Cohn (in preparation).

Alkaline phosphomonoesterase (BAP-C) was purchased from the Worthington Corp. Sodium periodate, ethylene glycol, and lysine monohydrochloride[3] were obtained from Matheson Coleman and Bell. Cetyltrimethylammonium bromide (hexadecyltrimethylammonium bromide) was purchased from Eastman Organic Chemicals. Ion-exchange resins (200–400 mesh, 8% cross-linked) were obtained from the Bio-Rad Laboratories, and Acid Alumina Woelm was obtained from Alupharm Chemicals.

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[1] Abbreviations used: CTA, cetyltrimethylammonium; CCA is used to indicate the base sequence for the intact terminus of tRNA; AMP, adenosine 5′-monophosphate; GMP, guanosine 5′-monophosphate.

[2] All measurements of absorbancy were made at 260 mμ unless otherwise stated.

[3] Attempts to remove all yellow impurities from lysine solutions were unsuccessful.

Preparation of the CTA Salt of tRNA. A. MICROMOLE SCALE. Analyses were always performed in duplicate at room temperature by using glass or Lusteroid (cellulose nitrate) tubes with a total volume of approximately 18 ml. A Teflon-coated $^3/_8 \times {}^1/_8$ in. stirring bar was added to each tube (the bars remain in the tubes throughout the analyses, even during centrifugation) followed by 4 ml of tRNA (~230 units/ml), 2 ml of H_2O, 2 ml of 1 M NaCl. After thorough mixing over a magnetic stirrer, 2 ml of 0.1 M CTA Br was introduced, and each tube was stirred for about 2 min. The CTA–tRNA precipitates were collected by centrifugation (8 min at 2000 rpm in an International portable centrifuge, Model PR-1, used at room temperature or at full speed in clinical model centrifuges), and the supernatants were discarded.

B. ONE-TENTH MICROMOLE SCALE. One-tenth the amounts of solutions used in A were placed in 2-ml conical tubes. The small $^3/_8$-in. stirring bars were used again, and the CTA–tRNA precipitates were collected by supporting the 2-ml tubes in larger Lusteroid tubes and spinning them in a clinical centrifuge.

The Stepwise Degradation Procedure. For analyses at the micromole level the following operations were performed.

(1) The CTA–tRNA salts were dissolved in 1.5 ml of 1 M lysine monohydrochloride (pH 8.1) containing 0.025 M $NaIO_4$ (the lysine–IO_4 solution was freshly prepared before each use). After thorough mixing, the contents of the tubes were centrifuged briefly to carry down liquid adhering to the sides, stoppered, and then incubated at 46° for 2 hr in a dry heating block.[4] (2) The tubes were cooled to 23–24°, and 0.1 ml of 1 M ethylene glycol was then mixed with the solution to convert excess IO_4^- to IO_3^-. After about 5 min the CTA–tRNA salt was precipitated by the rapid addition of 9 ml of H_2O. The contents of the tubes were immediately stirred for about 2 min and then centrifuged. The supernatant solution containing the released base (2a) was saved for further treatment. The precipitated CTA–tRNA salt was washed to remove any remaining base by stirring in 5 ml of 0.2 M NaCl–0.01 M CTA Br for about 0.5 min. After centrifugation, the supernatant (2b) was saved again and the base fractions were combined. In some cases the ethylene glycol was not added until the base fractions were combined. (3) NaCl (1 M)–0.2 M lysine monohydrochloride–0.005 M ethylene glycol was added to dissolve the CTA–tRNA salt. Alkaline phosphatase (0.05 ml, 12 units) was mixed into the samples, and after a brief centrifugation the stoppered tubes were incubated at 37° for 3 hr. (4) The enzyme was removed by the addition of 11–12 ml of H_2O to precipitate the CTA–tRNA salt again. After a stirring period of about 2 min, the tubes were centrifuged and the supernatants were discarded. The CTA–tRNA salts were dissolved in 2 ml of 1 M NaCl and reprecipitated by the addition of 9 ml of H_2O. After another 2-min stirring period the samples were again centrifuged and the supernatants were discarded. The CTA–tRNA salt was suspended in 5 ml of 0.2 M NaCl–0.01 M CTA, stirred briefly, and recentrifuged. After the supernatant had been discarded, the tRNA samples were ready for a new cycle, starting with step 1.

We found that the conditions given in steps 1–4 must be followed closely. In step 1 the CTA–tRNA readily dissolves in 1.5 ml of 1 M lysine monohydrochloride but will not do so if one-half this amount is used. In step 2, if the volume of water added is insufficient to dilute the chloride concentration to 0.2 M or less, the CTA–tRNA precipitate becomes tacky and difficult to redissolve. The same precautions apply to steps 3 and 4.

In the one-tenth scale experiments the amounts of reagents added and volumes used for washing were all decreased by the appropriate factor, and in step 2 the base analyses were made directly from fraction 2a (the second fraction, 2b, was discarded). The aliquots were placed directly on a cation-exchange resin without further treatment and analyzed.

Treatment of Base Fractions. COMPLETE DESALTING PROCEDURE. The combined base fractions (total volume of approximately 15 ml) of each cycle were put through 5 cm × 1 cm^2, Dowex 50 (Na^+) form columns to remove CTA and lysine. The samples were washed through the columns with 25 ml of water and collected in 200-ml volumetric flasks. After an addition of 25 ml of 1.0 M NH_4OH, the samples were diluted to volume and percolated at about 4 ml/min through 6 ml of Dowex 1 (hydroxide) form resin contained in 1.33 cm × 4.5 cm^2 columns. The samples were washed on to the columns with 25 ml of 0.1 M NH_4OH followed by water to remove excess NH_4OH. The purine or pyrimidine base was removed from these columns by suspending the entire resin bed in 10 ml of 1 M acetic acid. Thorough stirring (~2–3 min) removes evolved CO_2. The resin bed was drained, and the process was repeated twice by first adding 10 ml and then 5 ml more of 1 M acetic acid. The columns were then washed with water until free of acid, and the combined fractions were run through a 3 cm × 1 cm^2 column of Dowex 1 (acetate) resin to remove residual IO_3^-. After a 20-ml water wash, the combined effluent containing the bases was vacuum distilled to dryness and then dissolved in 3 ml of 1.5 M HCl. This solution was used directly for analysis by cation-exchange chromatography (Cohn, 1949).

PARTIAL DESALTING PROCEDURE. The combined base fractions of each cycle were percolated through a 4.5 cm × 1 cm^2 column of anionotropic acid alumina oxide to remove IO_3^-. After a 25-ml water wash, 0.3 ml of 0.1 M NH_4OH was added to the effluents, which were then passed through a 5 cm × 1 cm^2 column of Dowex 50 (Na^+) form resin to remove CTA and lysine. After a water wash, the combined base fractions were vacuum distilled to dryness and then dissolved in 3 ml of 0.1 M HCl prior to analysis by cation-exchange chromatography. With this procedure about 3 mequiv of NaCl appeared in the base fraction; however, it was found that this amount of salt does not interfere with the subsequent analyses.

C. DIRECT ANALYSIS OF BASE FRACTIONS. Since less

[4] Neu and Heppel (1964) heated samples for 1.5 hr in a liquid bath.

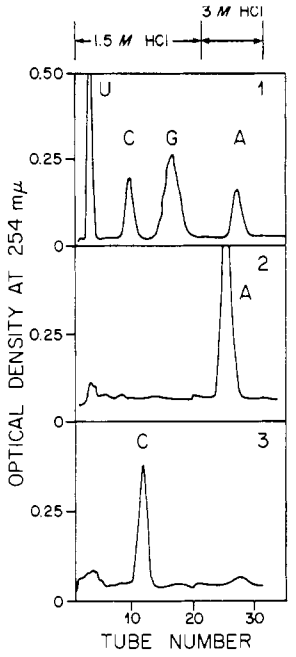

FIGURE 1: ISCO tracings showing separation of purine and pyrimidines by cation exchange. Column: 18 cm × 0.28 cm² Dowex 50 (H^+). Eluting solutions as shown at about 0.5 ml/min. Chart speed of ISCO 0.5 in./hr. Sorbed material of curve 1: 3.2 units of U, 2.5 units of C, 2.8 units of G, and 2.0 units of A as measured at 260 mμ recovered from 3 ml of 1.5 M HCl; curve 2: 0.87 μmole of A recovered from tRNA from the first cycle of the degradation procedure; and curve 3: 0.90 μmole of C recovered from tRNA from the third cycle of the degradation procedure.

than 10 nmoles of base is needed for analysis (Cohn and Uziel, 1965), a sample may be taken directly from the supernatant of 2a and applied to the analytical column. In this system, ultraviolet-absorbing reagents and reaction products do not interfere with measurement of the bases.

Cation-Exchange Chromatography. In experiments using 1 μmole of tRNA, purine and pyrimidine bases present in amounts as low as 2–3 absorbancy units were easily detected with ordinary laboratory apparatus using narrow diameter columns (18 cm × 0.28 cm²) of Dowex 50 (H^+) form resin. The effluent was monitored qualitatively at 254 mμ with an ISCO ultraviolet analyzer, Model UA, and measured quantitatively in a Model DB Beckman spectrophotometer at 260 mμ after 5-ml fractions had been collected.

Separation of the four common bases is shown in Figure 1 (curve 1). In procedure A, samples in 3 ml of 1.5 M HCl were washed into the column with an additional 3 ml of 1.5 M HCl. This same reagent was continued according to the schedule shown in Figure 1 to remove any uracil (U), cytosine (C), or guanine (G) that was present. Adenine (A) was removed from the exchanger with 3 M HCl. In procedure B, where 3 mequiv of NaCl is present, the column was equilibrated with 0.1 M HCl and the sample was sorbed on the column with 3 ml of 0.1 M HCl followed by an additional 3-ml wash of 0.1 M HCl before being switched to the 1.5 M HCl and 3 M HCl elution system of Figure 1. In this method, U is eluted with the 0.1 M HCl reagent; and C, G, and A follow closely the pattern and position shown in Figure 1.

The separation of some common purine and pyrimidines by use of nanomole quantities of each compound is shown in Figure 2. The effluent is continuously monitored with a modified nucleotide analyzer (Anderson *et al.*, 1963; M. Uziel and W. E. Cohn, in preparation). The width and peak-height method is used to calculate the moles of each component (Anderson *et al.*, 1963).

Results

Reproducibility of Data in the Determination of the CCA Terminus of tRNA. Six independent sequence determinations of the CCA terminus of tRNA were carried out. The amounts of recovered base found at each cycle in these experiments are shown in Table I. Typical chromatographic profiles from which the amounts were calculated are shown in Figure 1 (curves 2 and 3).

Determination of Terminal Group as Nucleoside in Each Cycle. Aliquots were taken from the original tRNA solution as well as at the end of step 3 when terminal nucleosides are present. These samples were hydrolyzed in alkali and then analyzed for terminal nucleoside content. The presence of nucleosides other than the expected adenosine and cytidines is tabulated in Table II.

One-tenth μmole Scale for Determining the CCA Terminus of tRNA. Approximately 92 absorbancy units of tRNA precipitated as its CTA–tRNA salt were subjected to five cycles of the degradative procedure. Analysis by cation-exchange chromatography showed quantitative elimination at each stage. The base released at

TABLE I: Precision of Sequence Method in Determining the CCA Terminus of tRNA.[a]

Cycle	Base[b]	nmoles[c]	Range
1	A	890	870–920
2	C	910	820–920
3	C	900	870–930

[a] About 930 absorbancy units of tRNA was used in each single determination. Alkaline hydrolysis of starting material yielded 930 nmoles of adenosine/930 absorbancy units taken for analysis. [b] Base analysis by procedure A is not sensitive enough to measure any but the major component of each cycle. The other components were present but could not be determined accurately by this procedure (see Table II). [c] Numbers represent average nanomoles found for six determinations.

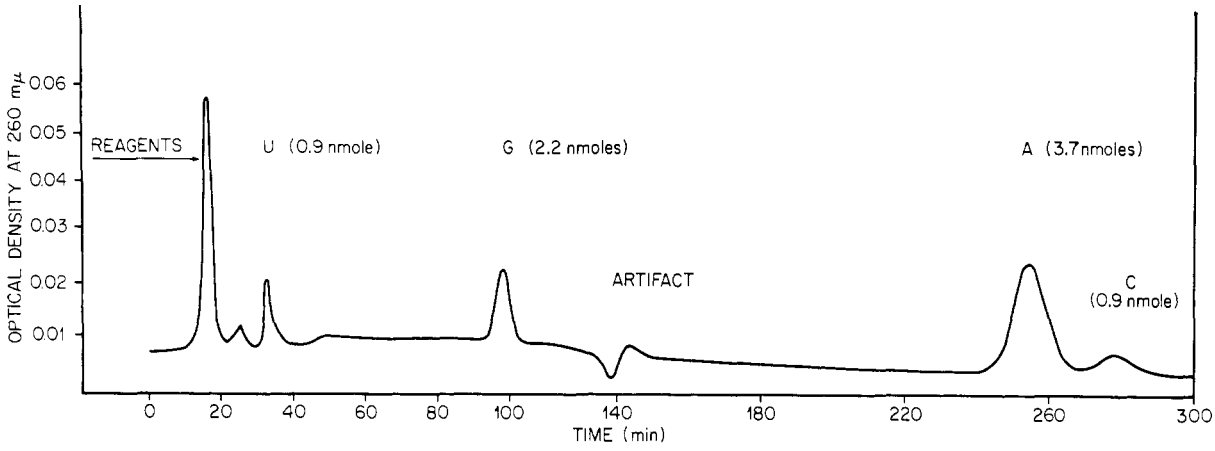

FIGURE 2: A typical base analysis without prior removal of excess reagents. From a total volume of 1.2 ml, a 100-μl sample (step 2a cycle 4) was dried under a stream of nitrogen gas at 45°. The residue was dissolved in 50 μl of 0.4 M ammonium formate (pH 4.7) and analyzed by cation-exchange chromatography at pH 4.7. The elution sequence is uracil (33 min, 0.9 nmole), guanine (98 min, 2.2 nmoles), adenine (256 min, 3.7 nmoles), and cytosine (278 min, 0.9 nmole). This represents an over-all recovery of 91%. The artifact is due to a concentration disturbance when salts are eluted from the exchanger and is routinely experienced.

each cycle was analyzed by procedure C. Cycles 4 and 5 gave 91 and 100% over-all recovery, respectively. Typical elution patterns from which the amounts of base were calculated are shown in Figure 2. The data for the first three cycles are similar to that of Table II. Figure 2 is an example of cycle 4, which is expected to contain a mixture of bases since the tRNA used was not homogeneous.

Removal of Phosphomonoesterase. To ascertain the distribution of phosphomonoesterase after it had been used to remove the terminal phosphate, phosphatase activity (by AMP hydrolysis[5]) was measured before and after step 3 and in the precipitate and supernatant from step 4. There was no loss of activity during the incubation with tRNA, and no phosphatase activity (<0.3% formation of Ado) was detected in the final tRNA precipitate, although the washings were very active.

Discussion

The conditions used for the release of purine or pyrimidine base in these experiments were essentially those of Neu and Heppel (1964). Lysine monohydrochloride was chosen as the amine source, since it readily dissolves precipitated CTA–tRNA and can serve as the buffer material in the enzymic reaction of step 3. The pH of lysine buffers drops considerably in the presence of $NaIO_4$, and in the reaction with an oxidized nucleoside a further drop will be encountered (Khym and Cohn, 1961; Cohn and Khym, 1962). A large excess of lysine was used to stabilize the final pH at 7.3–7.5 after release of purine or pyrimidine in step 1. The pH is also of importance in the isolation of the base in procedures A and B. If the pH is much below 7 in the passage of these fractions over Dowex–sodium resin, large losses of C, A, and G can occur. If the initial pH is much higher during this step, the resin columns will not adsorb lysine quantitatively. As lysine is sorbed on these columns, the pH increases to give a final pH of about 9.5. In procedure A, losses (particularly of cytosine) are encountered in the sorption of base fractions on Dowex 1 (hydroxide) resin if the total chloride concentration is much greater than 0.015 M and if the pH is less than 9.

A source of concern in the initial experiments was the solubility of purines and pyrimidines in the presence of precipitated CTA–tRNA. This was easily checked by performing steps 1 and 2 in the presence of known amounts of authentic compounds and analyzing the

[5] The activity of enzyme was estimated by the measurement of adenosine formed from the adenosine 5′-monophosphate substrate. The colorimetric reagent usually used to standardize the phosphatase solution could not be used because of its insolubility in the presence of small amounts of CTA.

TABLE II: Nanomoles of Terminal Group Determined as Nucleoside in Each Cycle.[a]

	Nucleosides Found				
Cycle	Ado	Cyd	Guo	Urd	Total
1	930	70			1000
2	30	840	10	100	980
3		780	70	120	970

[a] Nanomoles of nucleoside per 928 absorbancy units of tRNA taken for analysis. Figures of cycle 1 represent end-group analyses of the starting material.

supernatant fractions for recovery. Even guanine, which has an extremely low solubility, was recovered quantitatively if 1.5 μmoles were added to the micromole scale experiment. Because of solubility problems, GMP was used as a source of guanine which is readily released from GMP in step 1. Spectrophotometric ratios as well as chromatographic position were used to identify purine and pyrimidine in these systems.

The feasibility of using these procedures for sequential analysis depends on the following: first, the use of CTA to separate the polynucleotide from the released base; second, the phosphomonoesterase must be active in the presence of this large detergent cation; and, third, the monoesterase must be completely removed or inactivated before a new cycle can begin. As we have demonstrated, the phosphatase is active and very soluble in the presence of CTA and remains so even when the tRNA is precipitated.

Overnight interruption of the degradative procedure was usually made after step 4. Samples were refrigerated before proceeding to a new cycle starting at step 1. tRNA can be recovered at any stage, and the CTA removed simply by passage through the sodium form of Dowex 50 or by precipitating with two volumes of alcohol in the presence of a salt such as sodium chloride (Ralph and Bellamy, 1964).

As noted in both Figure 1 and Table II, base components other than the major component expected appear in each cycle. These may arise from non-tRNA nucleic acid components (Staehelin, 1965) or may be cleavage products that result from phosphodiester-bond breakage. Steinschneider and Fraenkel-Conrat (1966b) found such bond breakage when they applied the periodate–amine degradation procedure to tobacco mosaic virus ribonucleic acid using amines other than aniline. The origin of these anomalous compounds can be more easily studied when this degradation procedure is applied to a homogeneous tRNA molecule or to other types of homogeneous RNAs. Also, the effect of repeated periodate treatment on all the rarer nucleosides needs to be investigated, as was done for pseudouridine by Tomasz and Chambers (1965) and Tomasz *et al.* (1965). With the improved sensitivity of our procedures, it is now possible to investigate further, quantitatively, the reactions that occur within the RNA molecule during periodate oxidation.

Acknowledgment

The authors wish to thank Mr. Chongkun Koh for the many nucleoside analyses that he carried out.

References

Anderson, N. G., Green, J. G., Barber, M. L., Ladd, F. C., and Clare, F., Sr. (1963), *Anal. Biochem. 6*, 153.

Brown, D. M., Fried, M., and Todd, A. R. (1955), *J. Chem. Soc.*, 2206.

Cohn, W. E. (1949), *Science 109*, 377.

Cohn, W. E., and Khym, J. X. (1962), *Colloq. Intern. Centre Natl. Rech. Sci. (Paris) 106*, 217.

Cohn, W. E., and Uziel, M. (1965), 150th National Meeting of the American Chemical Society, Atlantic City, N. J., Sept.

Dutta, S. K., Jones, A. S., and Stacey, M. (1953), *Biochim. Biophys. Acta 10*, 613.

Holley, R. W., Apgar, J., Doctor, B. P., Farrow, J., Marini, M. A., and Merrill, S. H. (1961), *J. Biol. Chem. 236*, 200.

Hunt, J. A. (1965), *Biochem. J. 95*, 541.

Khym, J. X., and Cohn, W. E. (1961), *J. Biol. Chem. 236*, PC9.

Neu, H. C., and Heppel, L. A. (1964), *J. Biol. Chem. 239*, 2927.

Ogur, M., and Small, J. D. (1960), *J. Biol. Chem. 235*, PC60.

Ralph, R. K., and Bellamy, A. R. (1964), *Biochim. Biophys. Acta 87*, 9.

Staehelin, M. (1965), *in* Proceedings of the 2nd Meeting of the Federation of European Biochemical Societies, Vol. 4, Tuppy, H., Ed., Oxford, Pergamon, p 1.

Steinschneider, A., and Fraenkel-Conrat, H. (1966a), *Biochemistry 5*, 2729.

Steinschneider, A., and Fraenkel-Conrat, H. (1966b), *Biochemistry 5*, 2735.

Tomasz, M., and Chambers, R. W. (1965), *Biochim. Biophys. Acta 108*, 510.

Tomasz, M., Sanno, Y., and Chambers, R. W. (1965), *Biochemistry 4*, 1710.

Whitfeld, P. R. (1954), *Biochem. J. 58*, 390.

Whitfeld, P. R. (1965), *Biochim. Biophys. Acta 108*, 202.

Yu, C.-T., and Zamecnik, P. C. (1960), *Biochim. Biophys. Acta 45*, 148.