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A HYDROLYTIC PROCEDURE FOR RIBONUCLEOSIDES AND ITS POSSIBLE APPLICATION TO THE SEQUENTIAL DEGRADATION OF RNA

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SUMMARY

Ribonucleosides are converted quantitatively to the corresponding bases by reacting successively with periodate and cyclohexylamine. The hydrolysis of the glycosidic linkage of the nucleoside is presumably due to the formation of a double Schiff base between the periodate oxidized nucleoside and cyclohexylamine. This method has been also applied to the hydrolysis of the terminal nucleoside of s-RNA. The molar ratios of the free bases (uracil:adenine:cytosine, 1:2.8:6.1) thus obtained from the terminal nucleosides of RNA were found to be the same as those of the terminal nucleosides (uridine:adenosine:cytidine, 1:2.9:6.1) obtained by alkaline hydrolysis of RNA of the same preparation. The phosphoester linkage between the diesterphosphate and the 5′-hydroxyl group of the oxidized terminal nucleoside of RNA was also found to be hydrolyzed during the cyclohexylamine treatment, thus exposing a new terminal 3′-phosphate. It has been shown that, following prostatic phosphomonoesterase treatment, the periodate and cyclohexylamine reactions can be repeated and thus a possible method for the sequential degradation of RNA is provided. An application of this method to the sequential degradation of phenol extracted s-RNA and preliminary results thereof have been discussed.

Abbreviations: RNA, ribonucleic acid; DNA, deoxyribonucleic acid.

INTRODUCTION

Most procedures for the hydrolysis of ribonucleosides involve the use of either strong acid or strong alkali at rather high temperatures[1]. WHITFELD[2] has reported a milder procedure in which the ribonucleoside is first oxidized with periodate. Free base is obtained after incubation of the periodate oxidation products with glycine buffer at pH 10 for 18 h at 37°. This procedure has also been applied to the degradation of di- and tri-nucleotides. Uracil and 5-bromouracil have been obtained when periodate oxidized uridine (or 5-ribosyluracil) is treated with bromine[3].

The present communication reports a hydrolytic procedure for ribonucleosides in which the periodate oxidation products of ribonucleosides are treated with cyclohexylamine. Quantitative conversions of the nucleosides to the corresponding bases are obtained. This procedure has also been applied to the hydrolysis of the terminal nucleosides of RNA. Use of cyclohexylamine for this purpose has been suggested by HAKOMORI[4]. The phosphoester linkage between the diester phosphate and the oxidized terminal nucleoside is also hydrolyzed under these conditions, thus exposing a new terminal 3′-phosphate. Successive treatments with phosphomonoesterase and a repetition of the periodate and cyclohexylamine procedure may provide a method for the sequential degradation of RNA. Preliminary results in the application of this procedure to the sequential degradation of the phenol extracted RNA from yeast[5] will be discussed.

EXPERIMENTAL AND RESULTS

Material and methods

The nucleosides were purchased from the California Corporation for Biochemical Research, Los Angeles, California. s-RNA from yeast was prepared according to the method of MONIER *et al.*[5] Prostatic phosphomonoesterase was kindly supplied by Dr. G. SCHMIDT.

The paper electrophoretic experiments were carried out according to the procedure of CRESTFIELD AND ALLEN[6]. Whatman No. 1 paper which was washed with hot 2 *N* acetic acid and rinsed with distilled water was used throughout the experiments[7].

Periodate oxidation of ribonucleosides

To 0.5 ml of a 0.2 *M* solution of uridine (100 μmoles), 1 ml of a 0.2 *M** solution of potassium periodate (200 μmoles) was added. In contrast with reports from many laboratories[2,8], the periodate oxidation was found to be completed immediately after the reagents were added together. Similar experiments using adenosine and cytidine were carried out. Because of the low solubility of guanosine in aqueous solutions, a more dilute solution (0.01 *M*) was used.

Reactions of cyclohexylamine with periodate oxidized ribonucleosides

Various amounts of cyclohexylamine were added to individual samples of the periodate oxidized ribonucleosides. The final concentrations of the cyclohexylamine in the reaction mixture were 0, 0.06, 0.12, 0.24, 0.48, 0.96 and 1.92 *M*. (The molar ratios of the cyclohexylamine and the oxidized ribonucleosides used in these experiments were 0, 0.9, 1.8, 3.6, 7.2, 14.4 and 28.8.) Samples of the reaction mixture were spotted for paper chromatography immediately after the addition of cyclohexylamine.

* See NOTE ADDED IN PROOF.

The chromatograms were developed with either isobutyric acid–0.5 *M* ammonium hydroxide (10:6, v/v) (see ref.[9]) or isopropanol–acetic acid–water (60:30:10, v/v/v) (see ref.[10]) solvents. As shown in Fig. 1A, the quantity of the free base formed increases with the increasing concentration of cyclohexylamine and is substantially quantitative at 1.92 *M* cyclohexylamine (No. 7, Fig. 1A). The amounts of the free bases recovered range from 93 to 100 % of the four ribonucleosides used in the experiments.

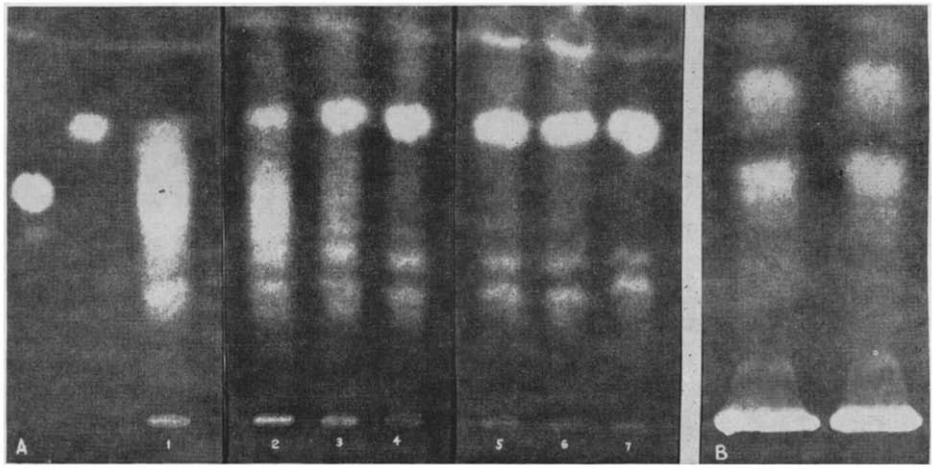

Fig. 1. A. Uridine treated with periodate and cyclohexylamine. First and second column, from left, reference compounds uridine and uracil respectively. Number 1 to 7, aliquots of periodate oxidized uridine to which cyclohexylamine was added to a final concentration of 0, 0.06, 0.12, 0.24, 0.48, 0.96 and 1.92 *M* respectively. Small quantities of U.V.-absorbing material behind uracil which have not been identified are presumably the oxidized ribose and its reaction product with cyclohexylamine. Paper chromatograms were developed with isobutyric acid–0.5 *N* ammonium hydroxide solvent ascending for 12 h. B. RNA treated with periodate and cyclohexylamine. The heavy white spot at the origin is the bulk of the RNA molecules. The three U.V.-absorbing spots are, from the top, uracil, adenine and cytosine respectively. The paper was developed in butanol–water solvent ascending for a total of six 12-h periods.

Hydrolysis of terminal nucleosides of s-RNA with periodate and cyclohexylamine

Samples of 20 mg of s-RNA were dissolved in 0.2 ml water and were oxidized with 0.2 ml periodate (0.2 *N*). It is known that only the terminal free nucleosides of RNA are oxidized by periodate[11]. Cyclohexylamine was added to a final concentration of 1 *M*. Some of the samples were spotted for ascending paper chromatography and others were dialyzed overnight at 4°. The papers were developed with either butanol–water (84:16, v/v) (see ref.[8]) or isopropanol–acetic acid–water solvents. When the butanol–water solvent was used, the paper was dried after a 12-h development and resubmitted to the same solvent. This process was repeated several times in order to separate the free bases from the bulk of the RNA molecules. As shown in Fig. 1B, the bulk of the RNA molecules remained at the site of application. The u.v.-absorbing material with the highest R_F value was identified as uracil, that with the second R_F value as adenine, and that with the lowest R_F value as cytosine. The identification of these compounds was made by comparison of their R_F values, electrophoretic mobilities at pH 3.5 and u.v.-absorbing spectra at pH 1 and pH 13 with those of known reference compounds. The molar ratios of the free bases obtained are 1:2.8:6.1

(uracil:adenine:cytosine). When the isopropanol–acetic acid–water solvent was used, a single 12-h development was sufficient to achieve the separation of the free bases from the bulk of the RNA molecules. Since adenine and cytosine have similar R_F values in this solvent, an additional chromatographic procedure with butanol–water solvent system was required to separate these two compounds.

To each of the dialyzed samples of periodate oxidized, cyclohexylamine treated s-RNA, 10 units* of prostatic phosphomonoesterase was added, and the mixtures were incubated for 15 min at 37° in 0.1 *N* acetic acid buffer at pH 5.6. Thereafter, the samples were mixed thoroughly with equal volumes of 90 % phenol and centrifuged for 15 min at 3,000 rev./min at room temperature. The upper layer was recovered and to it were added 10 % by volume of 20 % potassium acetate at pH 5 and 2 volumes of 95 % cold ethanol. The samples were left in the deep freeze for at least 4 h. The RNA precipitates were recovered by centrifugation, dissolved in small amounts of water, and dialyzed overnight. After dialysis the RNA samples were treated with periodate and cyclohexylamine and were subjected to paper chromatography as described. The free bases obtained were presumably thus derived from the nucleotides next to the original terminal nucleosides of the s-RNA molecules. From these second nucleotides of the RNA molecules only adenine and cytosine were found, and the molar ratio of adenine to cytosine was 1:3.9. Control experiments were carried out in which either the periodate or the cyclohexylamine or the phosphomonoesterase treatments were omitted. No detectable base was found during the subsequent paper chromatography in any of the control experiments. These preliminary observations indicate that this procedure is promising in the sequence determination of RNA. Experiments are under way to carry the degradation further into the RNA molecules.

Alkaline hydrolysis of s-RNA

In order to verify the results on the terminal groups of RNA obtained by the periodate and cyclohexylamine treatments, a sample of RNA from the same preparation was hydrolyzed with alkali and the terminal nucleosides liberated were determined. 60 mg of s-RNA were hydrolyzed with 0.8 ml of 1 *N* KOH at room temperature for 24 h. Thereafter the solution was neutralized with concentrated perchloric acid and centrifuged at 3,000 rev./min for 10 min at 2°. The precipitate was washed with 0.5 ml of water and the suspension was again centrifuged in the cold. The combined supernatant solutions were spotted for paper chromatography in butanol–water solvent ascending for 12 h. The papers were then dried and resubmitted to the same solvent for a total of six 12-h periods. The nucleosides moved from the origin in this solvent system, whereas the nucleotides remained at the site of application. The area of the paper containing the nucleosides was cut horizontally into four approx. ½″ wide strips and eluted with water. The material eluted from the paper was resubmitted to paper chromatography with the isobutyric acid–0.5 *N* ammonium hydroxide solvent ascending for 12 h. The nucleosides thus completely separated from each other were identified as uridine, adenosine and cytidine by comparison of their R_F values and their u.v.-absorbing spectra at pH 1 and pH 13 with those of known reference compounds. A total of 1.94 μmoles of the nucleosides were obtained

* Each unit of prostatic phosphomonoesterase equals 10 μl of an enzyme preparation which is capable of hydrolyzing 1 μmole of adenosine-3′-phosphate in 15 min at 37° in 0.1 *N* acetic acid buffer at pH 5.6. No diesterase activity was detectable under these conditions.

and the molar ratios of uridine, adenosine and cytidine were found to be 1:2.9:6.1. These nucleosides were presumably derived from the terminal nucleosides of RNA molecules[12]. The molar ratios of the nucleosides were approximately the same as those of the bases derived from the terminal nucleosides of RNA by the periodate and cyclohexylamine method. Assuming that no branching of the s-RNA chain occurs, these data are consistent with a molecular weight of around 30,000.

DISCUSSION

The reactions which lead to the formation of free base from the periodate oxidation product of ribonucleoside probably involve the formation of an intermediate which is a double Schiff base between the oxidized ribonucleoside and cyclohexylamine (the compound in parentheses, Fig. 2). BARRY AND MITCHELL[13] reported that crystalline

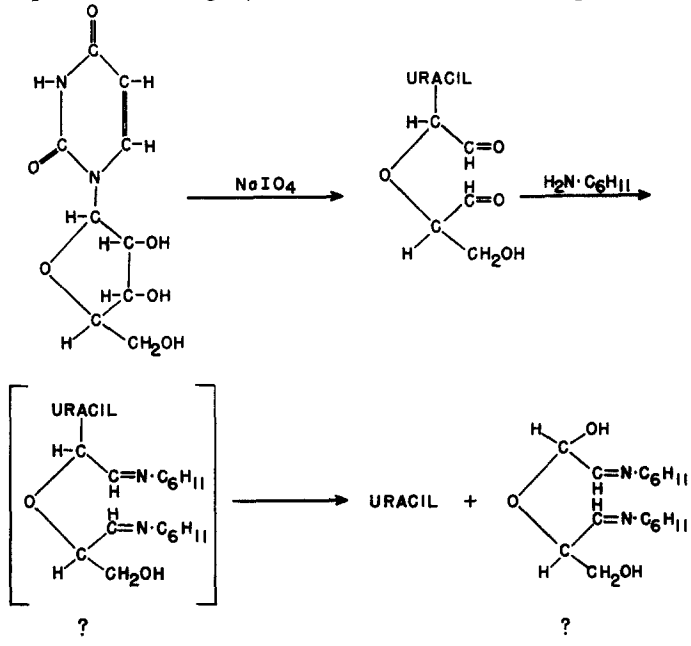

Fig. 2. Proposed reactions leading to the formation of free bases from the corresponding ribonucleosides when treated successively with periodate and cyclohexylamine.

Schiff base was formed when the periodate oxidized starch was treated with cyclohexylamine. The Schiff base formed was found to consist of two moles of cyclohexylamine/mole of the oxidized glucose unit of starch. GUTHRIE *et al.*[14] reported the formation of a similar compound when periodate oxidized methyl 4,6-O-benzylidene-2-D-glucoside was treated with cyclohexylamine. The hydrolysis of the glycosidic linkage then can be attributed to the nucleophilic property of the nitrogen atom of cyclohexylamine, which may result in a redistribution of electrons along the chain of the oxidized ribose molecule. The probable reactions leading to the formation of free base from ribonucleoside are described in Fig. 2.

Similar reactions with periodate and cyclohexylamine are conceivable for the hydrolysis of the glycosidic linkage of the terminal nucleoside groups of RNA (see Fig. 3). The hydrolysis of the phosphoester linkage between the diester phosphate

and the oxidized terminal nucleoside can also be attributed to the redistribution of electrons resulting from the formation of the Schiff base. The hydrolysis of this phosphoester linkage would result in the formation of a new terminal 3′-phosphate. In

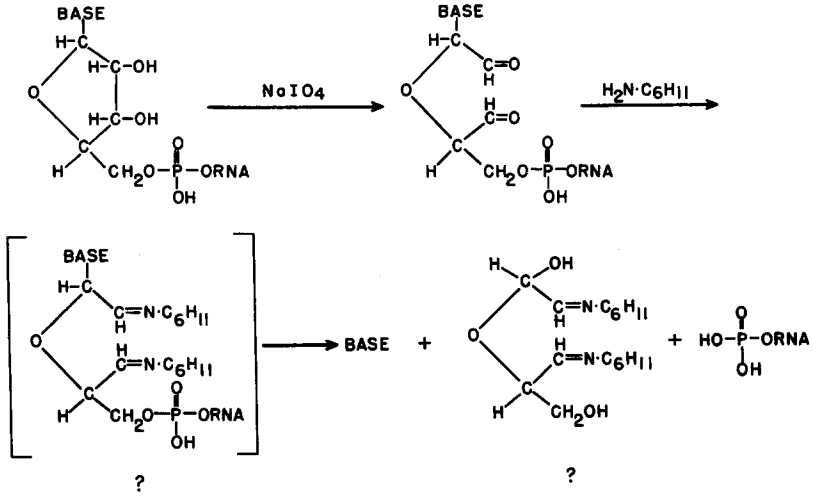

Fig. 3. Proposed reactions leading to the formation of free bases from the terminal nucleosides of RNA when treated successively with periodate and cyclohexylamine.

agreement with this postulation, BURTON AND PETERSON[15] report that after removing the purines from DNA, the phosphoester linkages of the original purine nucleotides in DNA become labile in the presence of diphenylamine. The formation of a compound between the aldehyde group of apurinic acid and diphenylamine was suggested as a possible mechanism for the hydrolysis of the phosphoester linkage.

After removal of the terminal phosphate with phosphomonoesterase, a new terminal nucleoside is exposed and the above procedure of periodate and cyclohexylamine reactions can be repeated, and thus a potential procedure is provided for the sequential degradation of RNA. In WHITFELD'S[2] procedure for the degradation of di- and tri-nucleotide, the periodate oxidized polynucleotide is incubated with buffer at pH 10 at 37° for 18 h. In the present studies the reactions are carried out at room temperature and are almost instantaneous, thus minimizing the danger of other chemical or enzymic degradation of RNA during the experiments. The terminal nucleosides of RNA determined with this procedure have been compared with those obtained from alkaline hydrolysis of RNA of the same preparation, and have been found to consist of similar proportions of uridine, adenosine and cytidine.

The phenol extracted RNA from yeast used in the present studies was found to be comparable with the soluble RNA (s-RNA) in its physical, chemical and biochemical properties[5]. s-RNA participates in protein synthesis by forming an amino acyl-RNA compound in which the amino acid is attached to the 3′ (or 2′) hydroxyl group of the terminal nucleoside adenosine[16–19]. s-RNA in its amino acid acceptor form presumably has a terminal sequence of -pCpCpA (see ref.[19]). SINGER AND CANTONI[20] have reported that the terminal nucleoside of s-RNA as extracted from rat liver consists of adenosine, cytidine and small amounts of other nucleosides, and studies the terminal nucleosides of s-RNA extracted from yeast were found to consist of uridine (10 %), adenosine (29 %) and cytidine (61 %). The discrepancies between

that adenosine represents at least 50 % of the total terminal nucleosides. In the present these results and those which indicate the terminal -pCpCpA structure for s-RNA may be explained by assuming that some of the terminal groups of s-RNA necessary for its amino acid transfer function were not present or were degraded during the preparation of s-RNA (see ref.[5]). It has been reported that the -pCpCpA end group can be reconstituted by incubation of s-RNA with ATP and CTP under suitable enzymic conditions[18]. s-RNA used in the present studies has not been so reconstituted. Further studies on sequence determination using s-RNA which has been partially purified[21] are now in progress.

NOTE ADDED IN PROOF

The reaction between the periodate oxidized RNA and cyclohexylamine was also carried out in 0.7 *N* sodium acetate buffer, pH 5. After the addition of cyclohexylamine to a final concentration of 1 *N*, the pH of the reaction mixture was found to be 6.2. The free bases thus obtained from the terminal groups of RNA were found to be the same as those obtained in experiments in unbuffered solutions (pH 11.9). To avoid possible chemical degradation of RNA at alkaline pH, it is desirable to include the acetate buffer in carrying out this reaction. The authors are indebted to Dr. W. E. COHN and Dr. J. X. KHYM for pointing out (personal communication) that certain amine induced cleavages of periodate oxidized nucleotide residues are favored at acid pH's.

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