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ALKALI-STABLE RNA FRAGMENTS FROM *CHLORELLA*

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Abstract—RNA was extracted from synchronous *Chlorella pyrenoidosa* cells at the stage of rapid cell expansion prior to nuclear division by a modified phenol method. RNA samples were hydrolyzed with KOH and applied to DEAE-cellulose columns. The columns were washed with 0·01 M Tris, pH 7·6 until the eluate contained no u.v.-absorbing material. The retained materials were eluted with a NaCl gradient. Three u.v.-absorbing fractions were distinguished by their order of elution; A, B, and C, respectively. They constituted 9, 2·5, and 1 per cent of the total hydrolysates as judged by absorption. Hydrochloric acid- and spleen phosphodiesterase-hydrolysates of the fractions were analyzed by column chromatography and paper electrophoresis. Two thirds of fraction A nucleosides and nine-tenths of fraction B nucleosides contained 2-*o*-methylribose. Fractions A and B were predominantly composed of purine nucleotides in 3′-5′-phosphodiester linkage. In fraction A the guanosine and cytidine were 2′-*o*-methylated, while the adenosine was not so substituted. Fractions A and B were dialyzable, but C was not, and exhibited hyperchromicity. Fraction C was also not dialyzable after RNase treatment and yielded only one nucleotide when hydrolyzed with spleen phosphodiesterase. About 95 per cent of this nucleotide was present as the 2′-*o*-methyl derivative. Acid hydrolysis of fraction C yielded only one u.v.-absorbing compound which appeared to be a free base. All three fractions gave orcinol reactions, but no diphenylamine reaction. 2,3-di-*o*-methylribose was synthesized and was also found to give an orcinol reaction.

INTRODUCTION

SMALL amounts of dinucleotides in alkaline hydrolysates of RNA from various animals and higher plants were first reported by Smith and Dunn.[1] They demonstrated that the dinucleotides were alkali-stable by virtue of a 2′-*o*-methyl group on one of the two nucleotides. The presence of this group blocks formation of the 2′,3′-cyclic phosphates which are necessary intermediate in either alkaline- or RNase-hydrolysis of RNA. 2′-*o*-Methylribonucleosides have been reported in RNA from *Anacystis nidulans*,[2] yeast,[3] and *Escherichia coli*.[4] These studies demonstrated that the 2′-*o*-methyl group is found on all of the major ribonucleotides from RNA. Also, it has been demonstrated that soluble RNA contains less 2-*o*-methylribose than ribosomal RNA in plant leaves.[5]

A series of dinucleotides and one trinucleotide have been separated from yeast RNA alkaline hydrolysates.[6] The same report also included data which indicated that 2′-*o*-methylribonucleotides give only slight reactions with orcinol. There was one report of the occurrence in nature of an RNA which was predominantly composed of 2′-*o*-methylribonucleotides[7]. This RNA was contained in ribonucleoprotein particles from *Saprospira grandis*.

This paper presents the results of a study of alkali-stable fragments from the RNA of

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Chlorella. The study was designed to search for oligonucleotide fragments and to attempt to determine the size, base composition, and distribution of *o*-methyl groups among the different nucleotides. Such information is pertinent to the interpretation of the role of 2′-*o*-methyl-nucleotides in such processes as protein synthesis.

RESULTS

A typical elution diagram obtained with a sodium chloride gradient on a DEAE-cellulose column of the material in a potassium hydroxide hydrolysate of *Chlorella*-RNA which was not removed from the column by prior exhaustive washing with 0·01 M Tris (pH 7·6) is shown in Fig. 1. Fractions A and B were each composed of several oligonucleotides which overlapped in the elution positions shown. Partially resolved peaks could be demonstrated in both

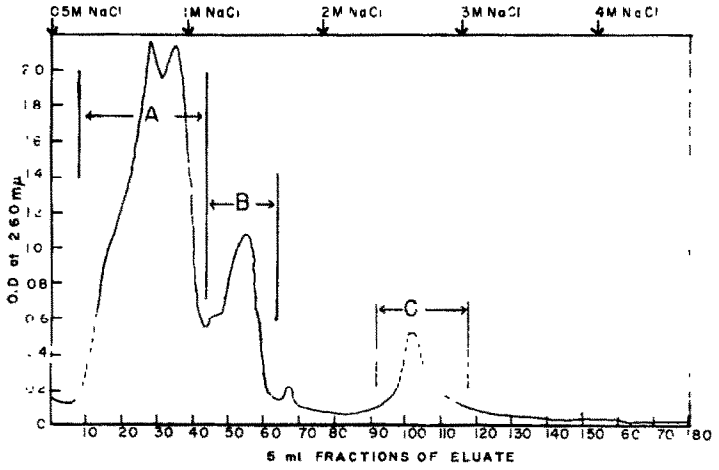

FIG. 1. DEAE-CELLULOSE COLUMN (1 × 25 cm) CHROMATOGRAM OF *Chlorella*-RNA AFTER HYDROLYSIS WITH 0·5 N KOH, 38°, 18 HR AND NEUTRALIZATION WITH PERCHLORIC ACID.

The column was washed with 0·01 M Tris buffer, pH 7·6, until eluate fractions had little absorbance at 260 mμ. Sodium chloride solutions were added, by way of a reservoir, to a 500 ml closed mixing flask filled with 0·01 M Tris, pH 7·6, at the designated points in the elution.

fraction A and B by making changes in the sodium chloride gradient. The early eluting fraction (A) always contained about three times as much u.v.-absorbing material as fraction (B). Fraction C was always a symmetrical peak. Fractions A, B and C contained 9, 2·5, and 1 per cent, respectively, of the total alkaline hydrolysates as judged by u.v. absorption at 260 mμ.

The only free nucleoside mono-, di-, or triphosphates which were bound to the resin under the conditions used were ADP and ATP, which elute in the same position as fraction A. The possibility that fraction A was composed in part of ADP and ATP was eliminated in two ways: (1) The RNA was chromatographed on DEAE-cellulose before hydrolysis. Low- and high-molecular-weight RNA column fractions were then separately hydrolyzed in potassium hydroxide and the hydrolysates were found to contain about the same percentage of fractions A and B as hydrolysates of crude RNA. However, fraction C was only found in hydrolysates from high-molecular-weight RNA. (2) Samples of fractions A, B, and C were hydrolyzed to nucleosides with spleen phosphodiesterase and alkaline phosphatase. These enzymes were

unable to hydrolyze ADP or ATP to nucleosides in control experiments. Thus, any nucleosides produced could not have come from such contaminating nucleoside polyphosphates as ADP or ATP.

2′-Substituted nucleosides released by treatment with spleen phosphodiesterase and alkaline phosphatase were separated from ordinary ribonucleosides by electrophoresis in a borate buffer. Fractions A, B, and C contained 65–70, 90, and 95 per cent, respectively, of 2′-substituted nucleosides (average of two experiments). Fractions A, B, and C were tested for deoxyribose by the diphenylamine reaction and none was detected. However, all three fractions gave orcinol tests for ribose. These were 0·8, 0·8, and 1·0 mole of orcinol ribose per mole of total phosphorous in fractions A, B, and C, respectively. Eighty-five per cent of fraction A's, and 55 per cent of fraction B's orcinol reactivity and OD at 260 mμ were lost, when the fractions were dialyzed against distilled water for 24 hr. When dialyzed in the same way fraction C lost only 17 per cent of its orcinol reactivity, and a hyperchromic increase in extinction at 260 mμ of 3 per cent was observed. Treatment of fraction C with massive levels of RNase failed to change its properties.

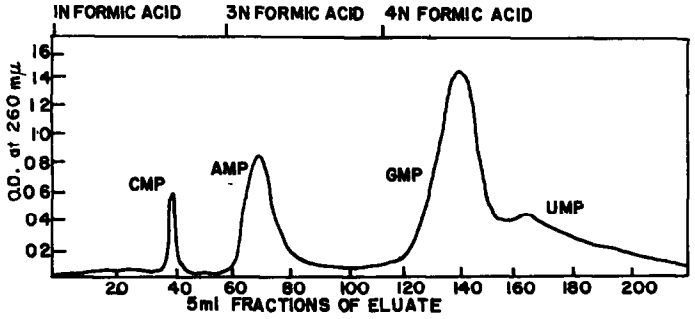

FIG. 2. DOWEX-1-FORMATE COLUMN (1 × 25 cm) CHROMATOGRAM OF FRACTION A AFTER HYDROLYSIS WITH SPLEEN PHOSPHODIESTERASE, 15 UNITS, pH 7·4, 38°, 24 HR.

Formic acid solutions were added, by way of a reservoir, to a 500 ml closed mixing flask filled with distilled water at the designated points in the elution.

Fraction A nucleotides from a spleen phosphodiesterase digestion were separated on a Dowex-1-formate column (Fig. 2). The nucleotides were identified by their u.v. spectra and mobilities on paper chromatograms. The approximate base composition of fraction A per 100 mole of nucleotide bases was calculated from the areas under the curve to be adenine, 26; guanine, 54; cytosine, 16; uracil, 4. Thus adenine plus guanine was 80 mole per cent with guanine about double adenine. Each of the separated nucleotides from fraction A was treated with alkaline phosphatase and the nucleosides were then tested for the 2′-*o*-methyl group by subjecting them to electrophoresis in a borate buffer. The results in per cent 2′-*o*-methyl-nucleosides were adenosine, 15; guanosine, 95; cytidine, 90; uridine, no reliable values.

The hydrochloric acid hydrolysate of fraction B was chromatographed on a Dowex-1-formate column (Fig. 3). The purine bases and pyrimidine nucleotides were identified by their u.v. spectra and mobilities on paper chromatograms. The approximate base composition of fraction B, per 100 mole of nucleotide base, was calculated to be adenine, 45; guanine, 35; cytosine, 17; uracil, 3. Again, adenine plus guanine was about 80 mole per cent, but in this case adenine was present in higher amount than guanine.

Both hydrochloric acid- and spleen phosphodiesterase-hydrolysates of fraction C were

chromatographed on Dowex-1-formate columns (Fig. 4). After acid hydrolysis only one peak could be found. It was eluted by 0·04 N formic acid. After phosphodiesterase treatment one main peak, which gave an orcinol reaction, was eluted at a higher acid concentration and it was preceded slightly by a peak only 5 per cent as large. The nucleosides from these two peaks were tested for the presence of the 2'-*o*-methyl group by electrophoresis in borate. The major peak was 95 per cent 2'-*o*-methylnucleoside. The minor peak contained too little

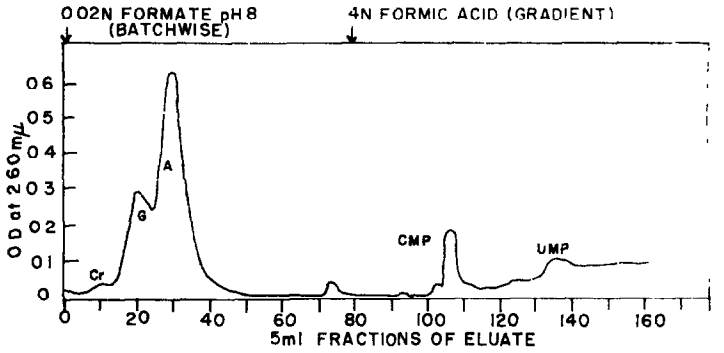

FIG. 3. DOWEX-1-FORMATE COLUMN (1 × 25 cm) CHROMATOGRAM OF FRACTION B AFTER HYDROLYSIS WITH 1 N HCl, 100°, 1 HR and ADJUSTMENT TO pH 9 WITH KOH.

Elution by (1) 0.02 M potassium formate buffer, pH 8·0; then (2) addition of 5 N formic acid, by way of a reservoir, to a 500 ml closed mixing flask filled with distilled water.

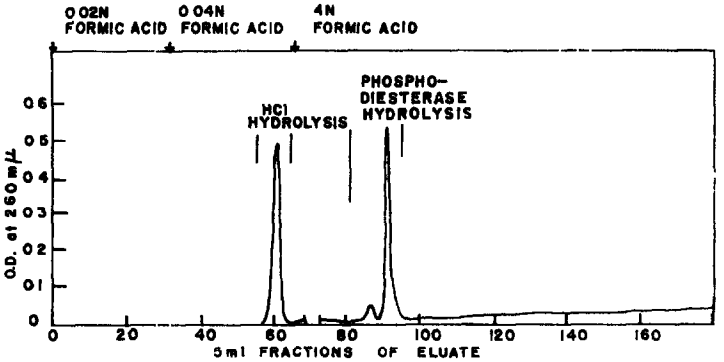

FIG. 4. DOWEX-1-FORMATE COLUMN (1 × 25 cm) CHROMATOGRAMS OF FRACTION C AFTER TREATMENT WITH (1) 1 N HCl, 100°, 1 HR AND ADJUSTMENT TO pH 9 WITH KOH; OR (2) SPLEEN PHOSPHODIESTERASE, 15 UNITS, pH 7·4, 38°, 24 HR.

Formic acid solutions were added, by way of a reservoir, to a 500 ml closed mixing flask filled with distilled water at the designated points in the elution.

material for reliable values to be obtained. The u.v. spectra of the major and minor peaks were nearly identical. The best u.v. spectra obtained of the products of acid and enzymic hydrolysis are given in Fig. 5. These spectra have not been identified. The product of acid hydrolysis gave no orcinol reaction and it is assumed to be a free base. It had an R_f of 0·79 when chromatographed on paper in isobutyric acid:water:ammonium hydroxide (66:33:1) as compared to 0·63 for guanine and 0·89 for adenine.

2,3-Di-*o*-methyl-D-ribose was synthesized from D-ribose in order to test its reactivity in the

orcinol test. Ribose was protected in the 5 position by tritylation, then methylated. The products were hydrolyzed with acetic acid to remove the trityl and glycosidic methyl groups. *o*-Methylribose and di-*o*-methylribose were then separated by paper chromatography. Both methylated products were found to have a free reducing function and to be unable to complex borate. Yet both gave complete or nearly complete orcinol reactions. The dimethyl derivative, 2,3,-di-*o*-methylribose, gave 0·8 mole orcinol reaction per mole of reducing reaction.

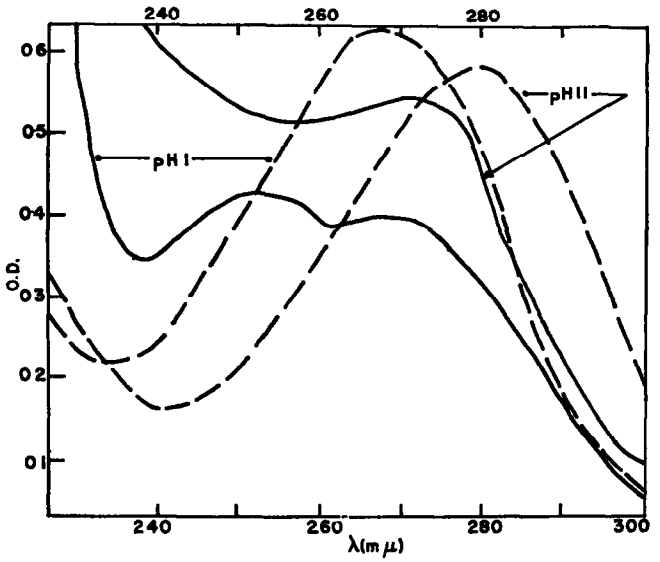

FIG. 5. ULTRAVIOLET SPECTRA OF PEAKS IN FIG. 4.
(1) Free base from fraction C acid hydrolysates (solid lines); (2) nucleotides from fraction C phosphodiesterase hydrolysates (broken lines).

DISCUSSION

The presence of large amounts of 2-*o*-methylribose in *Chlorella*-RNA raises the question of the function of the 2-*o*-methyl group. Various conclusions as to its distribution between the nucleotides and along the RNA molecule can be drawn from the data presented. In *Chlorella*-RNA the *o*-methyl group does not occur randomly within the molecule. Rather, it has a "clumped" distribution. This is evident from the fact that the alkali-stable fragments are not a series of oligonucleotides of increasing length and decreasing abundance. Instead, those isolated occur as three groups of oligonucleotides with different chain lengths (Fig. 1). The three groups are separated by chain length regions in which little if any fragments are found. The base composition of fractions A and B indicate that they are primarily purine oligonucleotides. Fraction A would seem to be predominantly trinucleotides since about two-thirds of the ribose is 2-*o*-methylated. Also guanosine, which is *o*-methylated, is about twice as abundant as adenosine, which is not *o*-methylated. Fraction B must be composed of molecules which contain about ten nucleotides, as evidenced by the fact that about 90 per cent of its nucleosides are methylated. Also fractions A and B were eluted from DEAE-cellulose (Fig. 1) at positions in which trinucleotides and decanucleotides, respectively, would be eluted. From Fig. 1 it is evident that a considerable gap exists between fraction B's elution position and that of fraction C. Fraction C's slow dialysis and high percentage of methylation indicate

it has a chain length of at least 20 nucleotides. Also, its position of elution from DEAE-cellulose (Fig. 1) was close to that of soluble RNA. The hyperchromicity which fraction C exhibits, upon dialysis against distilled water, indicates the presence of a hydrogen bond system.[7a] The presence of only one base in fraction C is especially interesting. The ease of release of this base by acid suggests it might be a purine derivative. The minor peak in fraction C phosphodiesterase-digests is probably the nucleoside.

The fact that fractions A, B, and C all gave nearly quantitative orcinol reactions despite the high proportion of 2′-*o*-methylribose can be explained by the high percentage of purine nucleotides in all three fractions and the fact that synthetic 2,3-di-*o*-methylribose was also found to give an orcinol reaction almost as great as that which ribose gives. This reactivity of 2-*o*-methylribose is not entirely unexpected. Ethers alpha to the anomeric carbon of carbohydrates are unusually subject to hydrolysis due to the Lobry de Bruyn - Alberda van Ekenstein transformation, which is acid or base catalyzed. Thus, for example, 2-*o*-methyl groups do not interfere with osazone formation.[8,9] It is suggested that the precise conditions under which the orcinol reaction is performed may be critical. The 2-*o*-methyl group is quite stable to boiling for 1 hr in 1 N HCl,[7] but seems to be almost totally removed by boiling 40 min in 6 N HCl in the presence of orcinol and ferric ion.

It is not known whether fraction C is a part of a larger RNA molecule before hydrolysis. It is possible that fraction C RNA exists as such in the *Chlorella* cell, either in the cytoplasm or in some type of particle.

METHODS

Chlorella pyrenoidosa (Van Niel Z 2.2.1) was grown synchronously as described previously.[10] Cells were harvested at the 7 hr light stage, during rapid cell expansion, prior to nuclear division. RNA was isolated by a modified phenol procedure[10] from 100 g wet weight of cells for each experiment. In some cases the RNA was column chromatogrammed on diethylaminoethyl-cellulose (DEAE-cellulose) to separate low- and high-molecular-weight RNA.[10]

RNA samples were hydrolyzed with either (1) 0·5 N KOH, 38°, 18 hr; or (2) spleen phosphodiesterase, 15 units, Worthington Biochemical Co., Freehold, N.J., pH 7·4, 38°, 24 hr; or (3) pancreatic ribonuclease (RNase), 20 mg, California Biochemical Corp., Los Angeles, Calif., pH 8, 45°, 48 hr; or (4) 1 N HCl, 100°, 1 hr. Nucleotides were hydrolyzed to nucleosides with alkaline phosphatase and the nucleosides were tested for the 2′-*o*-methyl group by electrophoresis at pH 9·2 in borate buffer as described previously.[7] Ultraviolet-absorbing bands and blanks were eluted with 5 ml 1 N HCl each and the extinction at 260 mμ of each eluate was then measured on a spectrophotometer, Beckman, model DU.

Dowex-1-formate (10X), 200–400 mesh was used for the chromatography of nucleotides and purine bases on 1 × 25 cm columns. A closed 500 ml mixing flask, filled with water at the start of each gradient, was used. Paper chromatography of bases and nucleotides was run by the descending technique on Whatman No. 1 paper in isobutyric acid:NH_4OH:water (66:1:33).[11] Ultraviolet spectra were obtained with a Cary, model 14, recording spectrophotometer. A 1 cm light path was used for all optical density determinations.

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Orcinol reactions were run by adding 3 ml concentrated HCl and 0·3 ml 10% orcinol in ethanol (w/v) to 3 ml of aqueous sample. The samples were placed in a boiling water bath for 40 min, then cooled rapidly.[12] Diphenylamine reactions were done by the method of Dische.[13] Reducing sugar was determined by the method of Somogyi.[14]

Synthesis of 2,3-di-*o*-methyl-D-ribose. Five g of D-ribose was allowed to react with 10 g triphenylchloromethane (trityl-chloride) in anhydrous pyridine at 25° for 24 hr. Water was then added to the mixture and the tritylated-ribose was extracted with ether, then dried. The trityl-ribose was sealed with 30 ml methanol, 5 ml methyl iodide, and 5 g silver oxide and shaken at 25° for 24 hr. The suspension was centrifuged and the supernatant was dried. This product was heated with 80% acetic acid at 100° for 30 min., then lyophilized to remove the acetic acid. The residue was chromatographed by the descending technique on Whatman 3 MM paper with the upper phase obtained after shaking pyridine, ethyl acetate, and water (2:7:5).[15] Two bands of reducing sugar were detected by treatment with silver nitrate and ammonia. One had an R_{ribose} of 1·5 and the second an R_{ribose} of 1·7. Both gave reducing sugar tests.[14] The first had 1·0 mole orcinol reaction per mole reducing reaction. Both were unable to form a borate complex at pH 9·2, when tested by electrophoresis.[7] The chromatographic mobilities, coupled with the method of synthesis and chemical tests, indicate that the material with an R_{ribose} of 1·5 was a mixture of 2- and 3-*o*-methylribose, while the material with an R_{ribose} of 1·7 was 2,3-di-*o*-methylribose.

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