OBSERVATIONS ON THE CONFIGURATION OF NUCLEOTIDES NEAR THE 3'-HYDROXY END OF ADAPTER RNA*

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It has been established that adapter (or transfer) RNA has a 3'-hydroxy end consisting of one adenylic followed by two cytidylic acids (...pCpCpA) (Hecht, Stephenson and Zamecnik, 1959). Adapter RNA from which this trinucleotide has been partially or completely removed can be rebuilt with the aid of a cytoplasmic incorporation factor, and the appropriate nucleoside triphosphates (Cannallakis and Herbert, 1960; Preiss, Dieckmann and Berg, 1961; Daniel and Littauer, 1963). These studies demonstrate that part or all of this terminal grouping is particularly susceptible to degradation. RNA nucleotides complexed in a hydrogen-bonded double helical structure are strongly protected against enzymatic degradation (Geiduschek, Moohr and Weiss, 1962; Cantoni et al., 1962). The susceptibility of the terminal nucleotides on adapter RNA to enzymatic degradation suggests that they may exist in the form of an exposed nonhydrogen-bonded single polynucleotide chain. Quite different considerations have also led to the speculation that the AC-terminal grouping is a single chain (McCully and Cantoni, 1962; Zubay, 1962b). It was our purpose to determine as nearly as possible which portion of the terminal grouping is exposed by measuring the susceptibility of adapter

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RNA from Escherichia coli to snake venom phosphodiesterase. It seemed likely that this exonuclease would remove 5'-nucleotides step by step until it reached a point where the secondary structure interfered with orderly degradation.

RESULTS

The hydrogen-bonded secondary structure of adapter RNA is stabilized by low temperature and high Mg^{++} (e.g. Marciello and Zubay, 1964; Nishimura and Novelli, 1963). All degradation studies were performed in the presence of 0.01 M $MgCl_2$. In Fig. 1 the rate of degradation is measured at several different temperatures and compared with the rate of degradation of polyuridylic acid. At 20° C the reaction appears to cease after about 3.7% of the optical density is released to the acid soluble supernatant. Further addition of enzyme does not increase this appreciably. At 5° C the reaction takes longer, but it appears to level off at nearly the same value as at 20° C. At 37° C the reaction proceeds further and does not show the same sharp leveling off effect. Polyuridylic acid is readily degraded at both 20° and 37° C under the same conditions.

The products from the 6 hour, 20° C enzymatic digestion of adapter RNA were analyzed in detail. Acid soluble material was analyzed chromatographically and the results of two determinations are summarized in Table 1. A $\frac{CMP}{AMP}$ ratio of 1.14 was found. The incorporation of CTP(α-P^{32}) and ATP(α-P^{32}) into the partially degraded adapter RNA was studied with the cytoplasmic incorporation system described by Preiss, Dieckmann and Berg (1961)(see Fig. 2). The limiting ratio approached was C/A of 1.24. It was found that the capacity of the resynthesized RNA to combine with leucine was precisely the same as that of native RNA.

The RNA in which CTP(α-P^{32}) had been incorporated was studied further (see nearest neighbor analysis in caption of Fig. 2). The RNA

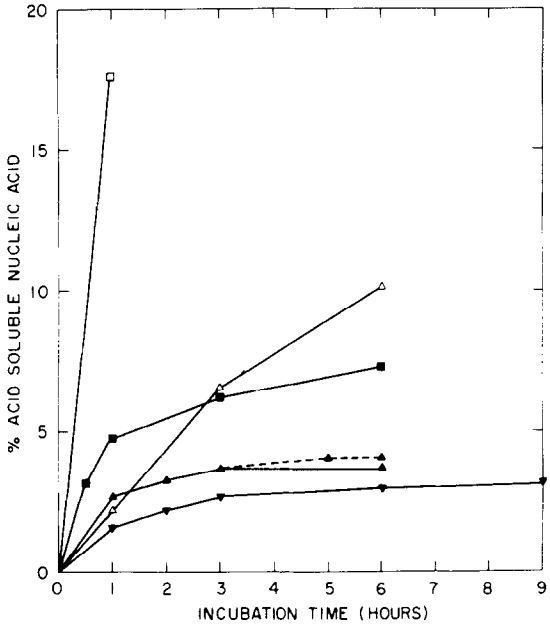

Fig. 1. Rate of digestion of nucleic acid by snake venom phosphodiesterase. Adapter RNA was prepared according to the method of Zubay (1962a), and was adsorbed to a DEAE cellulose column in 0.02 M NaAc-HAc buffer pH 5.4. The fraction eluting between 0.3 M to 0.8 M NaCl was dialyzed against water before use. High molecular weight polyuridylic acid was prepared according to the method of Singer and Guss (1962). Purified snake venom phosphodiesterase was obtained from the Worthington Biochemical Corp., Freehold, New Jersey. Digestion was carried out in 0.01 M $MgCl_2$ + 0.01 M tris·HCl, pH 7.6 containing 10 γ/ml of phosphodiesterase. The concentration of nucleic acid in all experiments was about 1 mg/ml. After incubation for a predetermined time and temperature, a 0.5 ml aliquot was chilled and treated with 2.5 ml 0.5 M perchloric acid. The resulting precipitate and supernatant are separated by centrifugation. The precipitate is hydrolyzed by suspending in 0.5 M perchloric acid and heating at 70° C for 50 minutes. The percentage of acid soluble nucleic acid is determined spectrophotometrically at 260 mμ. A zero time incubation blank is subtracted from all readings. —□— poly U at 37° C; —Δ— poly U at 20° C; —▼— RNA at 5° C; —▲— RNA at 20° C; ---▲--- RNA at 20° C with an equal quantity of enzyme added after 3 hours. —■— RNA at 37° C.

was degraded with alkali and the resulting 2′(3′) nucleotides analyzed chromatographically. The radioactivity of the various fractions was determined as follows: 83% in the C fraction, 11% in A, 4% in U, and 2% in G.

TABLE 1

Nucleotide composition analysis of venom phosphodiesterase digestant (adenylic acid = 1.00). Chromatographic separation of nucleotides was accomplished on a 15 cm x 0.3 cm column of 200-400 mesh Dowex 1 x 2 (Cl). Nucleosides, cytidylic and adenylic acids were eluted on a linear gradient from 0.001 M NH_4Cl to 0.005 N HCl; the remaining nucleotides were eluted on a linear gradient from 0.005 N HCl to 0.1 M NaCl + 0.001 M HCl.

	Exp. 1	Exp. 2
Cytidylic acid	1.13	1.16
Uridylic acid	0.175	0.225
Guanylic acid	--	--
Nucleosides	0.138*	0.225*

*Estimated as a mixture of adenosine and cytidine.

DISCUSSION

The degradation of adapter RNA by snake venom phosphodiesterase at 20° C or below in the presence of 0.01 M Mg^{++} appears to cease after the removal of approximately two residues, A and C, from the amino acid acceptor end of the RNA. This is indicated by the limited production of perchloric acid soluble material and the analysis of this product. The ratio C/A of 5'-nucleotides produced after termination of the reaction is 1.15. In incorporation studies, CMP and AMP are added back to the partially digested RNA; a limiting ratio of C/A of 1.25 is obtained. The resynthesized product has the same leucine acceptor activity as native RNA. The fact that there is about 20% more C than A found in these analyses suggests that in addition to complete removal of the terminal pCpA residues about 20% of the penultimate pC is also removed. As a check on these findings, radioactive CTP(α-P^{32}) was added back to the phosphodiesterase digested RNA followed by alkali digestion and analyses of the product. This technique which has been described by Josse, Kaiser and Kornberg (1961) results in the transfer of the original 5' radioactive phosphate to the 3'(2') position of the adjacent nucleotide. Analysis indicates the nearest neighbor of the

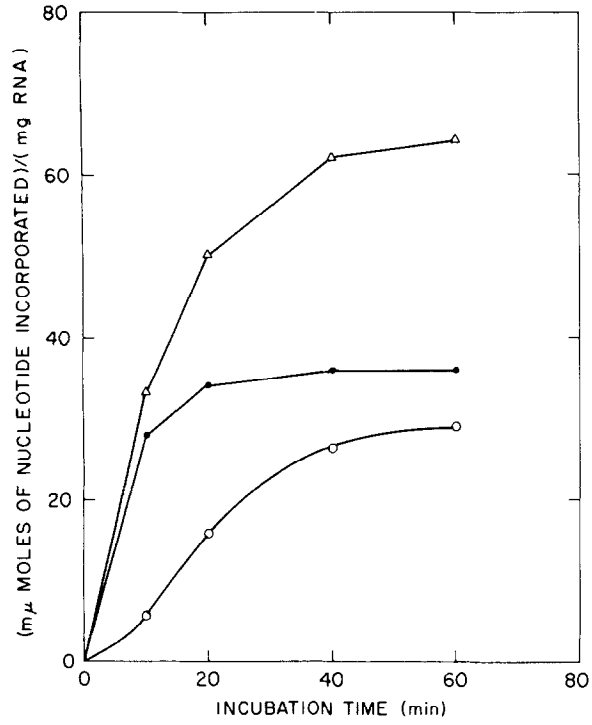

Fig. 2. Incorporation of ATP and CTP into venom diesterase digested adapter RNA. O ATP* incorporation in the presence of CTP; ● CTP* incorporation; Δ CTP* and ATP* incorporation. Approximately 100 mgs of RNA were digested for 6 hours at 20° C according to the procedure described under Fig. 1. The incubation mixture was chilled and twice shaken and extracted with an equal volume of 88% phenol. The RNA was precipitated from the aqueous layer with acetate buffer and ethanol, resuspended in water and reprecipitated in the same manner. The precipitation step was repeated once more and the final precipitate was dissolved in water and dialyzed overnight at 5° C. CTP(α-P^{32}) and ATP(α-P^{32}) were prepared according to a procedure described elsewhere (Takanami and Okamoto, 1963). Incorporation of CTP(α-P^{32}) and/or ATP(α-P^{32}) was carried out and measured according to the procedure of Preiss, Dieckmann and Berg (1961) except that tris buffer was used in place of phosphate buffer in the incubation.

Nearest neighbor base composition analysis.--Incubation mixture contains 5 mg of venom phosphodiesterase digested RNA. One μmole CTP(α-P^{32}), 1 μmole ATP, 20 μmoles phosphoenol pyruvate, 0.5 mg pyruvate kinase, 50 μmoles $MgCl_2$, 600 μmoles KCl, 1000 μmoles tris buffer, pH 7.8 in a total volume of 5 ml. Mixture was incubated for 60 minutes at 37° C and then chilled and shaken with an equal volume of 80% phenol. RNA was precipitated from the topmost aqueous layer by adding 2 volumes of ethanol. The precipitate was dissolved in water, and reprecipitated by adjusting to 0.1 N.HCl. The precipitate was washed twice by ethanol, then degraded in 0.5 N KOH at 37° C for 20 hours. The resulting 2′(3′) nucleotides were separated by chromatography as described above (see Table 1) and the radioactivity of each fraction determined by plating and counting.

added C to be 83% C, 11% A, 4% U, and 2% G. Lagerkvist and Berg (1962) have shown that the fourth nucleotide from the amino acid end of total E. coli adapter RNA is about 70% A and never C. These results comply with the interpretation that in most cases only the first two residues have been removed. In about 20% of the cases the second C has been removed, accounting for the large quantity of A found in the nearest neighbor analysis.

It seems likely that the secondary structure of the adapter RNA inhibits the removal of the second C by venom diesterase for several reasons: 1) Additional enzyme added after 3 hours incubation increases acid soluble nucleotides only slightly; 2) polyuridylic acid which has no hydrogen-bonded secondary structure undergoes extensive degradation under identical conditions; 3) at higher temperatures we should expect the secondary structure to be weakened, particularly near the ends of the polynucleotide chain. Accordingly at 37° C degradation proceeds much further without leveling off after 3 hours. Under exhaustive conditions venum phosphodiesterase can completely degrade adapter RNA to mononucleotides (Nihei and Cantoni, 1963).

Preiss, Dieckmann and Berg (1961) have studied the venom phosphodiesterase degradation at 37° C. They have shown that degraded material can incorporate C and A and that the limiting ratio of C/A incorporated approaches 1 to 2 depending on the amount of phosphodiesterase used in the degradation step. As a higher value for the ratio is approached, the total amount of A that can be incorporated drops to about 2/3rds of its value for less extensive reaction. This is consistent with our results and suggests that at 37° C degradation has proceeded beyond the third residue for a fraction of the material.

It is of interest that the 3'-hydroxy end of adapter RNA is readily susceptible to attack by the venom diesterase studied here and other degradative enzymes such as B. subtilis RNase and bovine pancrease RNase (Nishimura and Novelli, 1963). In contrast, poly-

nucleotide phosphorylase, which also attacks polynucleotides at the 3'-hydroxy end and is also blocked by excessive secondary structure in the substrate (Grunberg-Manago, 1959), fails to attack most adapter RNA chains even to the extent of removing a single terminal adenylate residue (Singer et al., 1960).

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