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# The Stability of Polyuridylic Acid in Acid Solution*

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ABSTRACT: The acid stability of the phosphodiester bond in polyuridylic acid (poly-U) was investigated in solutions of low and high ionic strength using loss in biological messenger activity as a measure of bond hydrolysis. It was found that treatment of poly-U in acetic acid (0.01 M, pH 3.45) solution at 37° caused a rapid loss in messenger activity. By physical methods this loss was shown to be the result of fragmentation of poly-U through acid-catalyzed hydrolysis of the phosphodiester linkage.

Little or no loss in messenger activity was observed when the hydrolysis was attempted with acetic acid (0.01 M, pH 3.45) containing sodium chloride (0.2 M). The stability of various sizes of poly-U as a function of pH, temperature, and ionic strength was studied.

The development of an assay system from bacteria which will measure a specific biological property, namely, messenger activity, has provided a method of studying the relationship between chemical structure and biological function for ribonucleic acids (RNA) [see Nirenberg *et al.* (1963) and Speyer *et al.* (1963) for recent reviews]. The ability of polyuridylic acid (poly-U) to direct phenylalanine incorporation into peptide before and after a variety of chemical modifications to the polymer has been investigated (Michelson and Grunberg-Manago, 1964; Szer and Ochoa, 1964). However, the finding that there is an optimum size of poly-U in terms of its ability to function as an efficient messenger ribonucleic acid (m-RNA) in the amino acid incorporating system (Martin and Ames, 1962; Jones *et al.* 1964) and the observation that this messenger efficiency may vary with the nature of the terminating nucleoside end (Coutsogeorgopoulos and Khorana, 1964; Michelson and Grunberg-Manago, 1964; Nirenberg and Leder, 1964) indicate that, as a prerequisite to a study on the chemical modification of polyribonucleotides, it is necessary to know their general chemical stability. Thus, the effects of solvents, of pH, of temperature, and of ionic strength must be established.

In this communication[1] we wish to report on the acid stability of the phosphodiester bond in poly-U in solutions of low and high ionic strength.

## Materials

Adenosine triphosphate (ATP)[2] and guanosine triphosphate (GTP) were purchased from Pabst Laboratories, Milwaukee, Wis. Phosphoenolpyruvate and pyruvic kinase were obtained from California Corp. for Biochemical Research, Los Angeles, Calif. Phenylalanine (U-$^{14}$C) was the product of Nuclear Chicago Corp., Des Plaines, Ill. Poly-U was purchased from Miles Chemical Co., Elkhardt, Ind., and was also synthesized using polynucleotide phosphorylase purified as described by Singer and Guss (1962); the uridine diphosphate used in the synthesis was prepared chemically (Moffatt and Khorana, 1961). Sephadex G-200 was a product of Pharmacia Co., Sweden, and was sieved to remove particles smaller than 200–400 mesh size. All other chemicals were the commercially available reagent grade.

## Methods

The cell-free incorporating system used was similar to that described by Nirenberg (1963). Protein was determined using the method of Lowry *et al.* (1951).

*Standard Assay System.* The reaction mixture (0.25

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† Contribution No. 31 from The Syntex Institute for Molecular Biology, Stanford Industrial Park, Palo Alto, Calif. A portion of this work was carried out during D. H. R.'s stay in the Laboratory of Molecular Biology, National Institute of Neurological Diseases and Blindness, National Institutes of Health, Bethesda, Md.
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[1] Abbreviations used: ATP = adenosine triphosphate, GTP = guanosine triphosphate, TCA = trichloroacetic acid.
[2] A preliminary account of this work has been reported (Abell *et al.*, 1963).

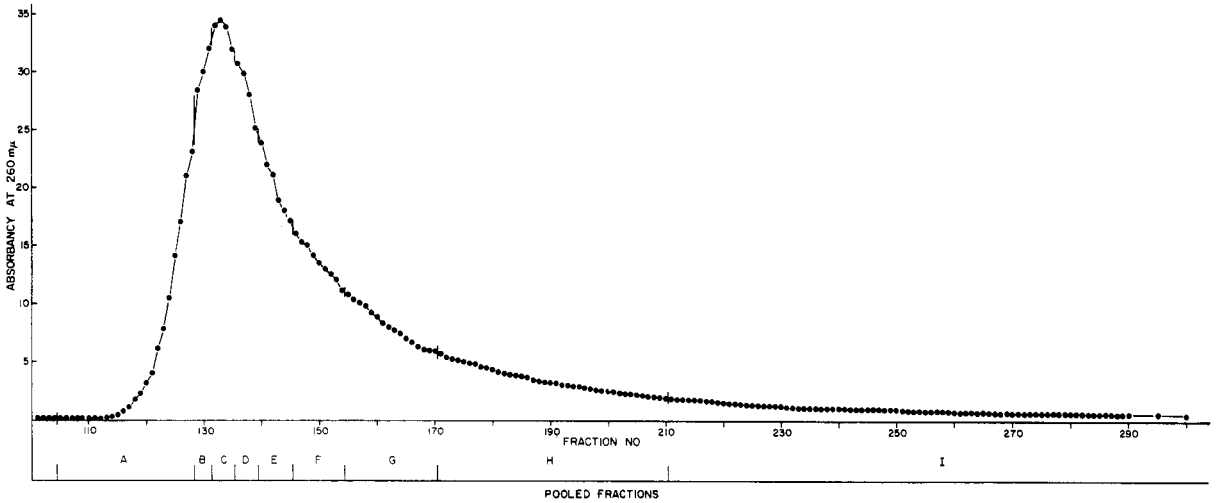


FIGURE 1: Sephadex G-200 was kept at 5° in Tris-HCl (0.01 M, pH 7.8) containing NaCl (1.0 M) for 2 weeks. After degassing, a column (109 × 2 cm) was prepared and allowed to equilibrate an additional 3 days. Poly-U (1212 OD, 4.8 ml) was placed on the column, and the column was developed with the same solvent. The flow rate was 10 ml/hr and 1-ml fractions were collected. The fractions were pooled as indicated on the lower portion of Figure 1 and dialyzed against Tris-HCl (0.001 M, pH 7.8) for 24 hr, followed by dialysis against water for 8 hr. The fractions were dialyzed each time against 5 changes of approximately 20 times their volume. All operations were carried out at 5°. The dialyzed fractions were lyophilized to dryness and redissolved into water to about 50 OD/ml for use.

ml) contained tris(hydroxymethyl)aminomethane-(Tris) HCl (25 μmoles, pH 7.8), magnesium acetate (3.5 μmoles), potassium chloride (12.5 μmoles), 2-mercaptoethanol (1.4 μmoles), ATP (0.25 μmole), GTP ($7.5 \times 10^{-3}$ μmole), phosphoenolpyruvate (1.87 μmoles), pyruvate kinase (4.0 μg), 19 L-amino acids ($5 \times 10^{-2}$ μmole), in addition to L-phenalalanine (U-$^{14}$C) ($5 \times 10^{-2}$ μmole; 2.5 or 5 mc/mmoles), poly-U (0.1–0.26 OD at 260 mμ)[3] or treated poly-U at the same concentration, and S-30 protein (0.5–0.6 mg.). The reaction mixture was incubated for 30 min, stopped by the addition of trichloroacetic acid (TCA) (3 ml, 10%), and heated for 20 min. The precipitated protein was then deposited and washed on Millipore filters. The radioactivity on the filter pads was determined on a Nuclear Chicago gas flow counter equipped with a Micromil window having approximately a 30% counting efficiency.

A typical acid hydrolysis of poly-U was carried out as follows. An appropriate fraction of poly-U was dissolved in water and a predetermined amount of acetic acid was added to yield a 0.01 M solution, pH 3.4. An aliquot of this solution was immediately removed and frozen, this being termed the zero-time (0) sample. The remaining solution was treated under various conditions, as indicated in the text. When samples were removed from the reaction solution, they were immediately frozen. Frozen samples from acetic acid treated poly-U were lyophilized to dryness and dissolved in Tris-HCl (pH 7.8, 0.01 M) at a concentration of 10 OD/ml for testing in the amino acid incorporating system. Where other buffers or salt solutions were used to maintain different pH values, the process was essentially the same, except that a known volume of acid, base, or buffer was added to neutralize the treated polymer just prior to freezing, and the lyophilization step was omitted. A standard sample of poly-U was used to test the efficiency of the amino acid incorporating system for each experiment reported. The loss in activity of treated poly-U, as reported, is measured as per cent change from the incorporation value found for the 0-time sample. In most of the experiments the incorporation of the 0-time sample was either the same or varied about 5% from the nontreated sample. The amounts of poly-U used in the incorporating system were below the saturation level.

Sucrose gradient techniques were performed essentially as described by Martin and Ames (1961). Velocity sedimentation measurements (Richards *et al.*, 1963) were performed using a Spinco Model E ultracentrifuge equipped with an ultraviolet optical system.[4]

## Results

*Fractionation of Poly-U on Sephadex G-200.* The elution pattern of a poly-U sample obtained from Miles Chemical Co. is shown in Figure 1.

*Messenger Activity of Sephadex-Sized Poly-U.* The effect of chain length on the messenger activity of poly-

[3] One OD unit is defined as that amount of material per milliliter of solution which produces an absorbancy of 1 in a 1-cm light path cell at 260 mμ.

[4] The authors are indebted to Dr. E. Glen Richards of the University of California who kindly performed the ultracentrifugational analysis.

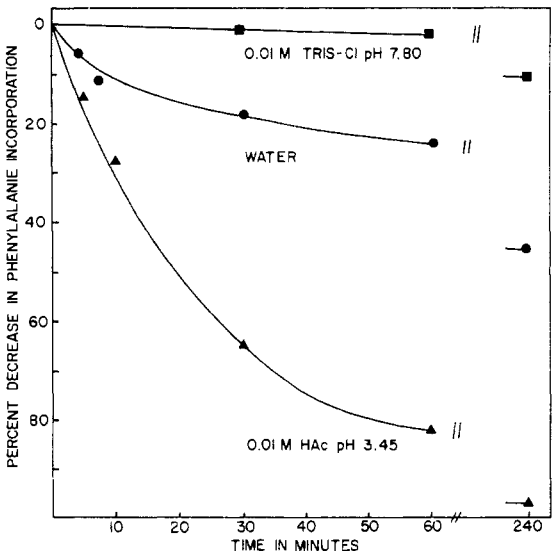


FIGURE 2: Samples of poly-U obtained from Sephadex chromatography. Fractions similar to B, C, and D, were desalted as described in Figure 1 and were dissolved (1 mg/ml) in Tris-HCl (0.01 M, pH 7.8), ■; glass-distilled water (pH 5.6), ●; and acetic acid (pH 3.45), ▲. These solutions were kept at 37° for 0, 10, 30, 60, and 240 min. After this time they were frozen and lyophilyzed to dryness, and the dry residue was dissolved in Tris-HCl (0.01 M, pH 7.8) for assay in the standard system.

TABLE I: Phenylalanine Messenger Activity of Sephadex-Sized Poly-U.[a]

| Poly-U Fractions from Sephadex Sizing | Phenylalanine Incorporation into Peptide (cpm/OD) Activity before Acid Treatment, 0 Min | Activity after Acid Treatment, 60 Min | % Loss in Activity |
|---|---|---|---|
| A | 47930 | 22460 | 48.8 |
| B | 51940 | 12350 | 76.2 |
| C | 49960 | 11800 | 76.4 |
| D | 46530 | 10620 | 77.2 |
| E | 38690 | 12530 | 67.6 |
| F | 33260 | 12870 | 61.3 |
| G | 27900 | 14220 | 49.0 |
| H | 18620 | 14050 | 16.8 |
| I | 6720 | 5900 | 12.2 |

[a] The poly-U fractions were obtained from Sephadex sizing shown in Figure 1. Acid treatment was carried out in acetic acid (0.01 M, pH 3.45) for 60 min at 37°. The samples were lyophilized to dryness and then redissolved, to equal absorbancy at 260 mμ, into Tris-HCl (0.01 M, pH 7.8) and assayed in the standard assay system. All analyses were carried out in duplicate and the results represent the average values.

ribonucleotides has been the subject of several investigations (Martin and Ames, 1962; Jones *et al.*, 1964). The various fractions of poly-U obtained from the Sephadex sizing described above were tested for their ability to stimulate the incorporation of phenylalanine into peptide using the standard assay system. In agreement with Jones *et al.* (1964), the results shown in Table I indicate that significant sizing of high molecular weight poly-U can be achieved simply with Sephadex G-200. It is assumed that fractionation by Sephadex provides a decreasing homologous series of poly-U molecules starting from fraction A. However, because of the large size of the molecules, accurate values for terminal phosphate, necessary for average molecular weight determinations, could not be achieved. From the studies carried out by Martin and Ames (1962), poly-U of approximately 450–700 nucleotide residues provides the most efficient messenger activity, this activity decreasing with size.

*The Effect of pH on Poly-U Messenger Activity.* The stability of the messenger activity of poly-U in distilled water and in acid and neutral solutions was investigated using the fractions obtained by Sephadex sizing. The effect of acetic acid (0.01 M, pH 3.45) on the messenger activity of the various fractions is shown in Table I. Acetic acid was chosen because in dilute solution it provides a moderate pH and can be readily removed by lyophilization.

Figure 2 demonstrates the rate of the loss in messenger activity for a fraction similar to the combined C and D fractions of Table I. Loss is most striking with dilute acid. The curves shown are from a single experiment; however, they are representative of many experiments using both Sephadex-sized and nonsized poly-U. When the per cent loss of activity in acetic acid (0.01 M, pH 3.5) is plotted on a log scale *vs* time, a straight line is obtained for approximately 60 min, the apparent first-order rate constant being 0.03 $min^{-1}$.

The effect of temperature on the rate of the loss of poly-U messenger activity for phenylalanine incorporation is indicated in Table II. These data demonstrate that, for poly-U, very little loss of activity occurs in dilute salt at pH 7.8, even at 50° for 30 min; however, when the pH is lowered, a consistent loss of activity is observed.

In an effort to discern whether the rapid loss of messenger activity was the result of pH rather than a direct effect of acetic acid, the messenger activity of poly-U was tested after treatment with HCl. In order to maintian a constant ionic strength, poly-U (1 mg/ml) was dissolved in KCl (0.01) and a predetermined amount of dilute HCl was added to give a final pH of 2.5. After 60 min at 37°, a sample was neutralized and tested for its messenger activity. At this pH, 90% of the activity was lost. Poly-U (1 mg/ml) was kept at pH 5.8 and 3.45 in sodium citrate buffer (0.01 M) for 60 min at 37°, as

TABLE II: Loss in Phenylalanine Messenger Activity of Poly-U as a Function of Temperature.[a]

| Temp. (C°) of Solution | Tris-HCl (0.01 M, pH 7.8) Time in Solution (min) 0 | 30 | Water[b] Time in Solution (min) 0 | 30 | Acetic Acid (0.01 M, pH 3.45) Time in Solution (min) 0 | 30 |
|---|---|---|---|---|---|---|
| 2 | 12,640 | 12,735 | 11,395 | 9,260 | 9,852 | 8,749 |
| 22 | 13,829 | 12,479 | 10,292 | 9,551 | 10,628 | 7,026 |
| 37 | 11,748 | 11,570 | 9,930 | 9,480 | 9,310 | 3,560 |
| 50 | 12,752 | 11,832 | 10,282 | 8,959 | 9,963 | 1,233 |

[a] The poly-U was not sized on Sephadex. It was dialyzed against sodium EDTA (0.001 M, pH 7.0) and against water, both for 16 hr at 5°. The volume of EDTA and water used was about 200 times the volume of the poly-U solution. The activities are indicated as cpm/10 μg of poly U. [b] The pH at room temperature was between 5 and 6.

TABLE III: Possible Formation of a Nonnucleotidic Inhibitor for the Standard Assay System.[a]

| Poly-U Samples | Treatment of Poly-U pH | Time (min) | Poly-U Assayed in Standard System (μg) | Phenylalanine Messenger Activity (cpm) |
|---|---|---|---|---|
| 1 | 3.4 | 0 | 1 | 554 |
| 2 | 3.4 | 60 | 1 | 72 |
| 3 | 3.4 | 0 | 2 | 1324 |
| 4 | 3.4 | 60 | 2 | 192 |
| 5 | 7.8 | 0 | 1 | 581 |
| 6 | 7.8 | 0 | 2 | 1324 |
| 1 + 5 | | | 2 | 1376 |
| 2 + 5 | | | 2 | 733 |

[a] Poly-U (1 mg/ml) was kept in acetic acid and Tris-HCl, 0.01 M, pH 3.45 and 7.8, respectively, for the intervals indicated. Samples were removed and were tested in the standard assay system. Samples treated with acetic acid were lyophilized prior to assaying.

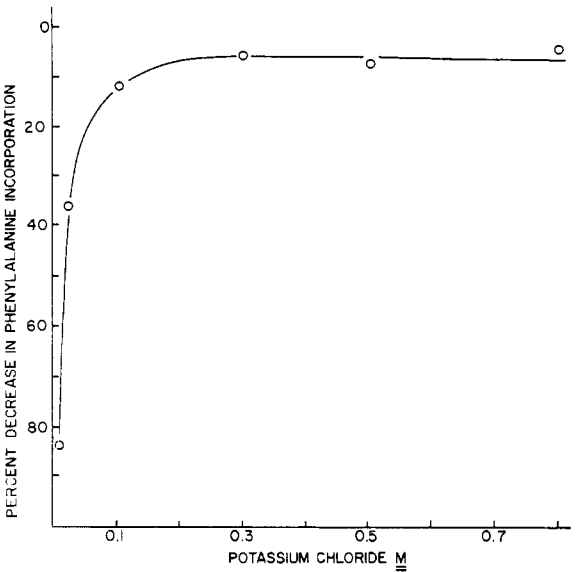


FIGURE 3: Poly-U (1 mg/ml) was dissolved in KCl at the concentrations indicated. These solutions were adjusted to 0.01 M, pH 3.45, with a predetermined amount of acetic acid, and the reaction was kept at 37° for 60 min. After this time, each sample was brought to neutrality with base and assayed in the standard system.

well as in Tris–maleic acid (0.01 M), pH 5.8 and 7.8, under the same conditions. The loss in messenger activity for these pH values was 5, 21, 7.7, and 7.6%, respectively. A possible reason for the inability to correlate the loss in activity of these buffers with acetic acid and water under the same conditions could be due to the differences in ionic strength.

*Effect of Ionic Strength on the Acid Stability of Poly-U.* From Figure 3, it is clear that increasing the concentration of KCl considerably reduces the effect of acid on poly-U. This protection is rapidly lost below 0.15 M KCl. When poly-U was treated with acetic acid (0.01 M, pH 3.45, 37°, 60 min) in 4 M urea, no significant difference in the loss in messenger activity could be detected when compared with the control without urea. Thus, the cpm/mg of poly-U in 4 M urea and acetic acid (0.01 M, pH 3.45) dropped from 7000 to 1100 cpm (84%), while the control, which was not treated with urea, dropped from 5500 to 710 (87%).

*Formation of an Inhibitor to the Standard Assay System.* The possibility that small amounts of nonultraviolet-absorbing material present as a contaminant in the poly-U might yield, upon treatment with acid, an inhibitor to the standard assay system was tested. The results shown in Table III demonstrate that no inhibitor is formed by the treatment indicated. When samples A and E are combined on an equal optical density basis, the combined activity is almost the sum of the two separate activities, and a similar additive

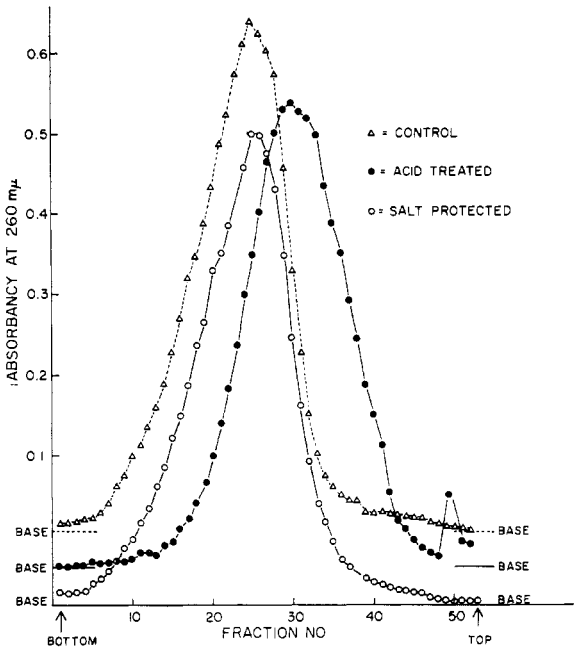


FIGURE 4: Samples of poly-U, obtained from combined fractions B, C, and D of Figure 1, were subjected to sucrose density gradient centrifugation after treatment with acetic acid (0.01 M, pH 3.45, 60 min), ●; a similar treatment with acetic acid which contained NaCl (0.2 M), ○; or no acid treatment, △. The acid-treated samples were neutralized with base and 26 OD (0.2 ml) of each was placed on the top of a sucrose density gradient, 4.6 ml (20 to 5% in sucrose containing Tris-HCl, 0.01 M, pH 7.8; NaCl, 0.2 M) prepared as described by Martin and Ames (1961). After 16 hr of centrifugation in a Spinco Model L ultracentrifuge at 35,000 rpm, 0–4°, a small hole was punctured in each tube and fractions of 10 drops (approximately 0.09 ml) were collected. A known amount (0.08 ml) was removed from each fraction and diluted, and its absorbancy was determined at 260 mμ.

effect is found for the acid-treated sample B and the nontreated sample E. The activity is thus approximately proportional to the active poly-U added. If an inhibitor were formed which directly affected the protein-synthesizing apparatus, one would expect the activity to be lowered, if not to the extreme of 80%, at least to some proportional value. This was not the case.

*Possible Transesterification of the 3′→5′ Phosphodiester Bond.* Acetic acid treated (0.01 M, pH 3.45, 60 min, 37°) poly-U (10 mg) was hydrolyzed with pancreatic ribonuclease (100 μg) in Tris-HCl (1 ml, pH 7.8, 0.1 M) for 8 hr. Chromatography on diethylaminoethyl-cellulose (DEAE-cellulose) in 7M urea using the method described by Tomlinson and Tener (1962) yielded an elution pattern similar to a ribonuclease hydrolyzed, nonacid-treated control. The principle nucleotidic component was uridine 3′-phosphate. The evidence indicates that under these mild acidic conditions extensive transesterification (Rammler and Khorana, 1962) does not occur.

*Acid-Catalyzed Hydrolysis of the Phosphodiester Bond in Poly-U.* The possible acid-catalyzed hydrolysis of the phosphodiester bond was investigated. Analysis for gross alteration in the molecular structure of acid-treated poly-U (60 min, 37°) was conducted in the following manner.

SUCROSE DENSITY GRADIENT CENTRIFUGATION. Samples of poly-U, treated in low and high ionic strength and nonacid treated, were subjected to centrifugation in a sucrose density gradient (Martin and Ames, 1961). The results are depicted in Figure 4.

The patterns indicate a considerable change in the average molecular weight of poly-U treated in acid at low ionic strength compared with the nontreated control and a sample treated with acid in dilute salt.

ULTRACENTRIFUGATION ANALYSIS. Sedimentation analysis of acid-treated poly-U in a Spinco Model E

TABLE IV: Increase in Phosphomonoesterase-Susceptible Phosphate of Poly-U as a Function of Time of Acetic Acid Treatment.[a]

| Time of Acid Treatment (min) | Messenger Activity of Acid-Treated Poly-U (cpm/OD) | Phosphate Released (mμmole/OD) |
|---|---|---|
| 0 | 7205 | 0.38 |
| 10 | 6230 | 0.40 |
| 20 | 5260 | 0.41 |
| 30 | 4715 | 0.42 |
| 40 | 4355 | 0.43 |
| 50 | 3880 | 0.44 |
| 60 | 3165 | 0.44 |
| 90 | 2345 | 0.55 |

[a] Poly-U[b] (20 OD/ml) was treated with acetic acid (0.01 M, pH 3.45) and, after the intervals indicated below, aliquots were removed and lyophilized. The lyophilized samples were dissolved in Tris-HCl (0.3 ml, 0.01 M, pH 7.8) and a portion was tested for biological activity in the standard assay system. To the remainder was added *E. coli* alkaline phosphomonoesterase (3.5 μg of chromatographically purified enzyme, free of phosphodiesterase activity, obtained from Worthington Biochemical Corp., Freehold, N. J.). These samples were incubated for 6 hr at 37° after which time 0.25 ml of uranyl acetate (0.17%) in perchloric acid (1.7%) was added. After removal of the precipitate, the supernatant was assayed for inorganic phosphate (Ames and Dubin, 1960). The phosphomonoesterase-susceptible phosphate and messenger activity are given per OD unit. [b] The messenger activity of different lots of commercial poly-U varied considerably. This sample had less messenger per OD unit than others tested.

ultracentrifuge equipped with ultraviolet optics again revealed considerable hydrolysis of the polymer.

The photographic patterns obtained were converted into plots of absorbance *vs.* cell height with a Spinco Analatrol recording microdensitometer. Sedimentation coefficients were calculated from the slope of the line relating the logarithm of the distance between the axis of rotation and the position of the sedimenting boundary corresponding to half the total polymer concentration for each exposure time. The concentration of the polymer used for ultracentrifugational analysis was 0.94 OD/ml. The polymer used in the analysis was from the combined fractions B, C, and D from Figure 1. The centrifugation was carried out in Tris-HCl (0.01 M, pH 7.8) containing NaCl (0.2 M). The $s_{20,w}$ for the acid treated material was 5.59 (mol wt $1.9 \times 10^5$; see Richards *et al.*, 1963, for details on molecular weight calculation), while that for the nontreated material was $s_{20,w}$ 7.81 (mol wt, $4.1 \times 10^5$).

*Increase in Phosphomonoesterase-Susceptible Phosphate Groups.* Hydrolysis of the phosphodiester bonds in acid-treated poly-U would presumably yield additional phosphomonoesterase-susceptible phosphate groups when compared with the nontreated control. Because of the size of the polymers used, significant figures relating to the absolute number of phosphate groups released were not obtained.

The data in Table IV indicate that there is an increase in phosphomonoesterase-susceptible phosphate after treatment with acid. Several experiments were performed to test the biological activity of the enzymatically dephosphorylated poly-U (Harkness and Hilmoe, 1962). After removal of the phosphomonoesterase by phenol treatment (Kirby, 1956), by chloroform denaturation (Sevag, 1938), or by chromatography on Dowex-50, the dephosphorylated polymer was assayed in the standard system. In no instance was an increase in specific activity observed and, in many instances, the specific activity was reduced.

*Effect of Size on the Acid Stability of Poly-U.* The results shown in Table V demonstrate that a correlation exists between the degree of acid lability and the size of the molecule.

TABLE V: Effect of Acid on Poly-U of Different Sizes.[a]

| Poly-U Sample | Phenylalanine Messenger Activity (cpm) 0 min | 60 min | % Decrease in Activity |
|---|---|---|---|
| 1 | 51,940 | 12,350 | 76.2 |
| 2 | 18,620 | 14,050 | 16.8 |
| 3[b] | 67 | 68 | 0 |

[a] Poly-U samples 1 and 2 were fractions B and H obtained from Sephadex sizing (see Figure 1). Sample 3[b] has an average molecular size of 75 nucleotide residues as determined by terminal phosphate analysis. All samples were treated with acetic acid (0.01 M, pH 3.45, 37°, for 60 min) and assayed in the standard assay system. Activity is represented as cpm/OD. [b] Fraction 3 was a kind gift from Dr. Leon A. Heppel, National Institutes of Health, Bethesda, Md.

## Discussion

The chemical instability of poly-U and RNA has been noted by several investigators (Richards *et al.*, 1963; Beard and Razzell, 1964). In general, this instability has been ascribed to the presence in poly-U of latent phosphodiesterase activity. In the present study, it was initially felt that the mild acidic conditions used were insufficient to cause extensive diester bond hydrolysis or hydrolysis of the glycosyl–pyrimidine linkage. This view was supported by acid hydrolysis studies on synthetic diribonucleoside phosphates (Rammler and Khorana, 1962). For these reasons, the rather precipitous loss in biological messenger activity of acid-treated poly-U was thought to have resulted from either partial modification of the polymer by contaminating phosphodiesterases and/or possible transesterification of the phosphodiester bond from the natural 3′–5′ linkage to the 2′–5′ linkage. The first possibility was excluded because (1) the rate at which the loss in activity occurred was independent of the method of deproteinization or the number of times a single type of deproteinization step was carried out, providing the salt concentration of the acid-treated material remained the same. These experiments were carried out despite the fact that no significant protein could be detected in any sample after Sephadex chromatography. (2) The loss in activity was dependent upon the apparent molecular weight of the poly-U. (3) No significant loss in activity occurred when the samples were stored in solutions of moderate ionic strength under conditions in which pancreatic ribonuclease is fully active. Although the second possibility was not completely eliminated, it was felt that the essentially total hydrolysis of the acid-treated poly-U by pancreatic ribonuclease to uridine 3′-phosphate indicated that no significant transesterification of the phosphodiester bond had occurred.

The absorbancy (260 mμ) per unit weight of poly-U varied somewhat from preparation to preparation indicating a possible contamination by nonnucleotidic material. It is conceivable that under acidic conditions used, these substances could form inhibitors of the amino acid incorporating system. This notion was ruled out because the activity of a mixture of acid-treated and nontreated poly-U was essentially the sum of the activities of the polymers assayed independently. In addition, the rate of loss of activity of a variety of poly-U samples was similar for samples isolated, treated, and assayed under standard conditions.

Initially, the possibility of major acid-catalyzed changes in the primary structure of poly-U was discounted because little or no difference in the Sephadex G-200 exclusion chromatographic pattern of the acid-treated and nontreated samples in solutions of low salt

concentration (Tris-HCl 0.01 M, pH 7.8) could be detected. In this regard, it is interesting to note that in one sucrose density analysis carried out in dilute Tris-HCl (0.01 M, pH 7.8) using the method described by Martin and Ames (1961), the patterns of the acid-treated poly-U and the nontreated sample were almost superimposable, the positions of the peaks being determined not only by volume but also by their refractive indices. However, Simha (1945) has shown that, in solutions of very low ionic strength, the sedimentation coefficient of the rigidly extended polymer is relatively independent of molecular weight, varying with the log of the molecular weight. When the sucrose density centrifugation was repeated at high ionic strength (0.2 M), the sedimentation patterns of the treated and untreated samples were easily distinguishable, indicating that a structural change had occurred upon acid treatment. Moreover, centrifugation of acid-treated poly-U in the analytical ultracentrifuge confirmed the presence of fragments smaller in size than the untreated poly-U.

Because of the acid stability of the glycosyl–pyrimidine bond, depyrimidination was not considered to be the primary route in disrupting the poly-U samples. The present data suggest that the degradation of poly-U in acid is due to the rupture of phosphodiester bonds. The ease with which this bond breakage occurred was unexpected. In low ionic strength, polyanions such as poly-U most probably exist in an extended form (Richards *et al.*, 1963), this extension resulting from repulsion of the negatively charged phosphate groups. One can speculate that in the extended form considerable internal strain on the phosphodiester bonds exists and that a driving force for the facile phosphodiester bond hydrolysis is the relief of this strain. Because the messenger activity of poly-U is dependent upon its size (Martin and Ames, 1962; Jones *et al.*, 1964), among other things, the lowering in size through hydrolysis of phosphodiester bonds should be reflected in the lowering of messenger activity. Conversely, in solutions of relatively high ionic strength, because of the shielding of the negative phosphate groups by counterions, poly-U could assume a more compact folded conformation. This should relieve strain within the molecule, which should be reflected in greater acid stability of the phosphodiester bond (see Figure 3). The data in Table I demonstrate that the messenger activity of poly-U on an equal optical density basis is a function of the size of the molecule. The apparent lower activity of nonacid-treated fraction A compared with B may be the result of fewer molecules of poly-U in the assay system, this following from its comparative large size and the fact that nonfractionated poly-U was limiting in the standard assay system. Assuming that the acid-catalyzed hydrolysis of poly-U is random, the lower per cent loss in activity of fraction A compared with B may be ascribed again to its size; that is, its hydrolysis in a given time would yield products which are larger than those of B treated under similar conditions. Fractions B, C, and D constitute approximately 30% of the total poly-U and on an equal optical density basis are the most efficient messengers. Ultracentrifugational analysis indicates an average molecular weight of 410,000 ($s_{20,w}$ 7.81). Martin and Ames (1962) have indicated that the true optimal chain length for poly-U is in the range of 450–700 nucleotide residues. In this analysis, discounting severe polydispersity of the fractionated samples B, C, and D, we have obtained an optimal size of 1300 nucleotide residues.

Although the exact mechanism for the hydrolysis of poly-U is not known, it is assumed that it proceeds *via* attack of the ribose C-2′ hydroxyl on the adjacent protonated (neutral) phosphodiester to yield a transient phosphotriester (Michelson, 1963) which subsequently ruptures to yield the fragmented poly-U.

The stability of poly-U in alkaline solution has been briefly studied, and again considerable labilization (Brown and Todd, 1952) of this polymer was found in solutions of low ionic strength. Thus, poly-U (1 mg/ml) Tris-HCl (0.01 M, pH 9.0, 37°) loses about 80% of its messenger activity in 60 min.

The findings reported here point to the necessity of carefully defining the average size of poly-U in studies concerned with the enhancement or loss of its biological activity after specific biochemical or chemical treatment. They also indicate that, for meaningful interpretation of information derived from such experiments, the conditions used in handling the polymer should be presented in detail.

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# The Alkali-Stable Dinucleotide Sequences and the Chain Termini in Soluble Ribonucleates from Wheat Germ*

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ABSTRACT: There are sixteen possible alkali-stable dinucleotides having the general structure NxpNp in which N is any of the four major ribonucleosides and Nx is the 2′-*O*-methyl derivative of any of the four major ribonucleosides. Quantitative and qualitative data show that at least thirteen of these dinucleotides are detectable in alkali hydrolysates of wheat germ soluble ribonucleates (s-RNA). Two additional dinucleotides which contain the 2′-*O*-methyl derivative of the minor ribonucleoside, pseudouridine, have been identified. The proportions of the different alkali-stable dinucleotide sequences in wheat germ s-RNA contrast sharply with the corresponding proportions found for the 18S + 28S RNA from the same source. The alkali-stable dinucleotide sequences cumulatively account for about 2.6 mole % of the constituent nucleotides of wheat germ s-RNA, and, since the chain termini of s-RNA which appear as nucleosides and nucleoside diphosphates in alkali hydrolysates also cumulatively account for 2.6 mole % of the constituent nucleotides, there is an average of one alkali-stable dinucleotide sequence per s-RNA chain.

Smith and Dunn (1959a) isolated alkali-stable dinucleotides from the alkali hydrolysates of several ribonucleate specimens of animal, plant, and microbial origin. It was shown that there was 1 mole of *O*-methylribose per mole of dinucleotide and all available evidence supports their initial proposition that the sugar is 2′-*O*-methylribose (Smith and Dunn, 1959a; Hall, 1964; Honjo *et al.*, 1964; Singh and Lane, 1964a; Broom and Robins, 1965). The 2′-*O*-methylation confers alkali stability on the internucleoside phosphodiester bond which is linked to the adjacent 3′ position of the *O*-methyl nucleoside constituents of ribonucleate chains.

An examination of 18S + 28S RNA from wheat germ has shown that the *O*-methyl nucleoside constituents occur internally in ribonucleate chains (Singh and Lane, 1964b; Lane, 1965). About 90% of the *O*-methyl nucleosides in wheat germ 18S + 28S RNA are flanked by normal ribonucleosides in the ribonucleate chains and can be recovered as part of alkali-stable dinucleotides, NxpNp,[1] while the remaining 10% occur in clusters of two and can be recovered as part of alkali-stable trinucleotides, NxpNxpNp, after hydrolysis of the ribonucleates by alkali. The *O*-methyl nucleosides participate in all of the sixteen possible alkali-stable dinucleotide sequences and in at least six of the sixty-four possible alkali-stable trinucleotide sequences which can result from permuting the four principal ribonucleosides and their *O*-methyl derivatives (Lane, 1965).

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[1] The symbol N is used to designate a ribonucleoside residue and the symbol Nx is used to designate a 2′-*O*-methyl ribonucleoside residue. Oligonucleotides are abbreviated in accordance with the recommendations of the Journal of Biological Chemistry. The abbreviations 18S + 28S RNA and s-RNA refer to the 18S + 28S ribonucleates and amino acid acceptor ribonucleates, respectively.