Some Factors Affecting Ribonucleic Acid Chain-Length Estimation with *Bacillus subtilis* Exonuclease*

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ABSTRACT: An exonuclease from *Bacillus subtilis* degrades different RNA species at characteristic rates. The rate differences may be interpreted as relating to differences in RNA chain length.

Some experiments reflecting on the validity of this interpretation are described. No indication of endonuclease contamination has been found. The method seems to be useful over a 30-fold range of RNA chain length.

An exonuclease isolated from *Bacillus subtilis* culture media has been employed to compare the chain lengths of different RNA species (Riley, 1969). A population of radioactive RNA particles, for example, 16S rRNA, is mixed with a nonradioactive population of RNA particles, for example, 28S rRNA. The mixture is degraded with exonuclease. Comparison of the specific activities of the acid-soluble degradation products to those of the original materials allows the mean chain lengths of the two populations to be compared. In many cases this direct comparison is the matter of most immediate interest. The method is based on the assumption that differential rates of degradation of the mixed populations of RNA species are dominated by differing end-group frequencies. This approach to chain-length estimation offers some advantages. It can be used to investigate materials, for example, rapidly labeled RNA, which cannot be conveniently prepared in large amounts. Second, because all RNA particles are quantitatively degraded (and without perceptible delay) the chemical nature of the point of attack, the 5′-chain terminus, is unlikely to affect the results. The enzyme may be expected to impose uniformity, with respect to phosphate substitution at the 5′-chain terminus, before the chains are degraded to an extent that is measurable in these experiments.

The interrelationships among the apparent mean chain lengths of rapidly labeled and rRNA of HeLa cells and *Escherichia coli* strongly suggest the emergence of a simple pattern of structures based on assembly from smaller subunit chains. Some additional experiments relating to possible difficulties of interpretation are described below.

Materials and Methods

Isolation of Exonuclease. The method was adapted from Nakai *et al.* (1965) and Okazaki *et al.* (1966).

Bacillus subtilis (Marburg strain) was grown in two 1-l. batches of broth (peptone water granules 15 g/l., yeast extract powder, 5 g/l., Oxoid Ltd., London) in swirling culture overnight at 37°. Cells were removed by centrifugation and the supernatant was brought to 20% saturation with ammonium sulfate. Any precipitate forming after 90 min at room temperature was discarded. The supernatant was adjusted to 65% saturation with ammonium sulfate and allowed to stand overnight at 4°. The precipitate was dialyzed against 0.05 M Tris-acetate (pH 6.8). NaCl was added to 0.05 M and the solution applied to a 9 × 1 cm column of DEAE-Sephadex A-50 (Pharmacia Ltd., London). The column was washed with 100 ml of 0.05 M Tris-acetate–0.05 M NaCl before eluting with 200 ml of buffer containing a linear gradient of 0.05–0.5 M NaCl.

The ammonium sulfate precipitate contained three distinct enzyme activities degrading RNA at pH 9 in the presence of calcium ions. One was not properly adsorbed under the conditions described. The activity eluting at about 0.15 M NaCl was discarded as this enzyme, possibly because of endonuclease contamination, exhibits no differences in rate of attack on different RNA species. The enzyme activity eluting at about 0.3 M NaCl was employed in the experiments described. The product was free from detectable phosphodiesterase activity against bis(*p*-nitrophenyl)phosphate. Repeated chromatography assists in reducing the pigment content of the enzyme but does not affect the results obtained by its use.

If required the enzyme may be further purified by electrophoresis through 3% w/v polyacrylamide gel at pH 9.0. The enzyme migrates to the anode. The enzyme employed in the experiments described was not subjected to electrophoresis.

Isolation of RNA. rRNA was isolated by procedures described previously (Riley, 1969). R17 RNA was isolated from purified bacteriophage (Fenwick, 1968). 4S and 5S RNA were prepared from a 2 M NaCl extract of total nucleic acids of lysozyme-treated *E. coli.* Excess salt was removed by ethanol precipitation followed by washing with ethanol–water (8:2, v/v). The 4S and 5S RNA were isolated by electrophoresis through a 3-cm column of 3% w/v acrylamide gel at pH 7.4. The cells had been cultured for 18 hr in the presence of 10 μCi of [8-^{14}C]adenine.

Degradation of RNA was performed at 37° in GC buffer (0.2 M glycine–3 × 10^{-3} M $CaCl_2$ adjusted to pH 9.0 with KOH) as previously described (Riley, 1969). Solutions of bovine plasma albumin (Armour Pharmaceutical Co., Eastbourne, England) were extracted with bentonite before use. The presence or absence of albumin in the reaction mixtures does not affect the results.

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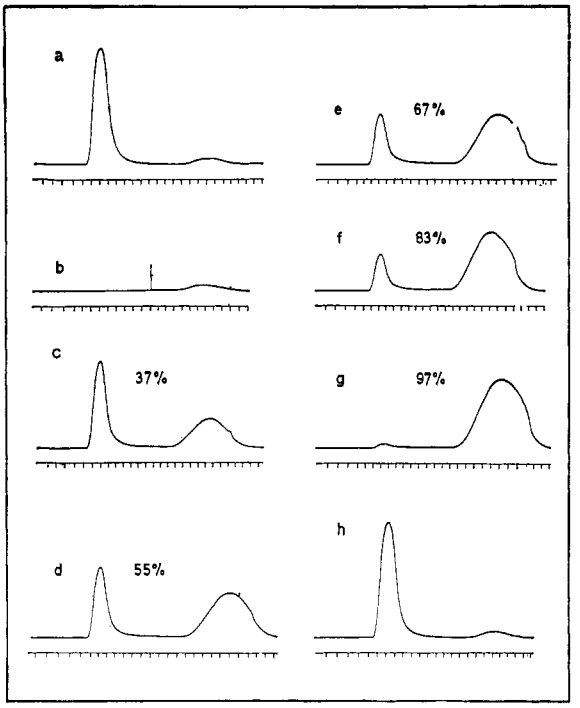

FIGURE 1: Size of exonuclease degradation products. *E. coli* 16S rRNA was incubated with exonuclease at 37° in GC buffer. After varying periods of time (up to 100 min) aliquots were cooled in ice before sampling to determine the degree of acid solubility. The degradation was stopped by the addition of two-tenths volume of 0.1 M EDTA. Aliquots (0.5 ml) were applied to a 10 × 1 cm column of Sephadex G-100 equilibrated with 10^{-3} M EDTA (pH 7). The ordinate shows the ultraviolet extinction of the column effluent. The lower scale indicates approximately 0.5-ml fractions. (a) Control RNA not incubated at 37°; (b) buffer and EDTA only; (c–g) products after the indicated degrees of degradation; (h) RNA incubated in GC buffer 100 min in absence of enzyme.

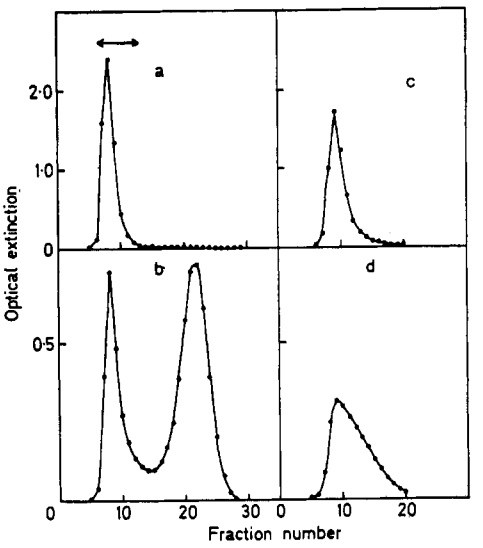

FIGURE 2: Progressive degradation. Polyuridylic acid was incubated in GC buffer in the absence (a) or presence (b) of exonuclease. The products were passed through a 10 × 1 cm column of Sephadex G-100 as in Figure 1. The indicated peak fractions from (a) and (b) were precipitated with ethanol and the products separated on 10 × 1 cm columns of Sephadex G-200; (c) undegraded poly(U); (d) degraded poly(U). The ordinate shows the optical extinction of the column effluents in a 2-mm light path.

Results

Absence of Endonuclease Contamination. It is essential that the end-group frequencies of RNA populations under comparison should remain substantially unaltered during the experiments. The strongest evidence for lack of endonuclease activity in the reaction mixtures is that different RNA populations are degraded at different and reproducible rates. In the presence of endonuclease any initial differences in end-group frequency would be rapidly swamped. The previously reported initial constancy in the rates of degradation of different RNA species is also relevant.

Degradation of RNA molecules in the presence of endonuclease would be expected to release materials of varied intermediate size. For this reason gel filtration of samples of reaction mixtures on Sephadex G-100 has been used as a means of distinguishing endo- and exonucleases (Birnboim, 1966) and as a qualitative test for contamination of exonuclease preparations by endonuclease (Sporn *et al.*, 1969).

Figure 1 shows the products at various stages of exonucleolytic degradation of *E. coli* 16S rRNA. The Sephadex G-100 column was equilibrated and eluted with 10^{-3} M EDTA (adjusted to pH 7.0 with Tris) to facilitate dissociation of any fragments. Endonuclease contamination is not indicated. This is in agreement with the inference drawn from earlier results. In Figure 1f the mean chain length of the undegraded material is about 260 nucleotides. In Figure 1g the RNA is almost completely degraded. This figure shows high particle weight impurities in the RNA and enzyme preparations.

Gel filtration in the absence of endonuclease can be used to distinguish progressive and processive exonucleases (Nossal and Singer, 1968). Progressive exonucleases reduce the chain length of all molecules present. The enzyme is released from the substrate after removing one, or a few, nucleotides and then attacks the next chain terminus at random.

Processive exonucleases remain attached to the substrate processing one molecule to the maximum extent before moving to the next. In principle the number of chains of intermediate size is equal to the number of active enzyme sites. As the degradation of short chains will be completed before degradation of long chains, the mean length of a heterogeneous population will increase continuously from the start of the experiment.

Figure 2 shows the degradation of polyuridylic acid. A high molecular weight fraction of poly(U) was isolated by preliminary fractionation on a Sephadex G-200 column. This fraction was degraded with *B. subtilis* exonuclease until half of the material was acid soluble. If the degradation was processive no change in heterogeneity with respect to chain length should be seen (Nossal and Singer, 1968). Progressive degradation, however, should remove approximately equal lengths from all RNA chains. The longer RNA chains suffer a relatively small, and the shorter chains a relatively large, change in chain length. Any initial variety in chain length will be amplified by progressive degradation. A proportion of the RNA chains may be expected to be so reduced in size as to penetrate the gel. Such an increase in heterogeneity is shown in Figure 2, indicating that the mode of attack is pro-

TABLE I: Competitive Inhibition by 2′,3′-Ribonucleotides.[a]

	% Act. Remaining at Stated Nucleotide Concentration										
	Sample Number										
	0	1	2	3	4	5	6	7	8	9	10
Adenylic acid	100	75	61	56	47	54	29	22	17	14	12
mM	0	0.02	0.04	0.06	0.08	0.21	0.42	0.63	0.84	1.05	2.09
Guanylic acid	100	37	35	28	26	31	18	17	15	14	10
mM	0	0.02	0.04	0.06	0.075	0.12	0.20	0.32	0.46	0.54	1.12
Cytidylic acid	100	97	96	94	91	84	74	71	66	62	42
mM	0	0.02	0.035	0.05	0.07	0.20	0.40	0.60	0.80	1.00	2.00
Uridylic acid	100	107	72.1	97	97	92	82	72	67	66	47
mM	0	0.02	0.05	0.07	0.09	0.25	0.50	0.75	1.00	1.25	2.50

[a] Competitive inhibition by 2′,3′-ribonucleotides. 2′,3′-Ribonucleotides were added to aliquots of radioactive *E. coli* 16S RNA, at a final concentration of 1.5 optical density units/ml in GC buffer. Equal amounts of enzyme were added to each tube. Samples 0–5 and 6–10 were incubated at 37° for 20 and 40 min, respectively. The degree to which the release of acid-soluble radioactivity was suppressed by the nucleotide was taken as a measure of enzyme inhibition. The RNA in the uninhibited controls was about 15% degraded.

gressive, like that of the other exonuclease present in *B. subtilis* culture media (Okazaki *et al.*, 1966).

Progressive degradation is advantageous. Selective removal of the smallest chains from a mixture is delayed, allowing observation of an initially constant rate of degradation. The parallel degradation of all chains may be useful in the location of any identifiable base sequence or chemical grouping. It may be used, for example, to determine the position of groups reacting with sodium periodate and isonicotinic acid hydrazide.

Reaction with Individual RNA Species. Under the conditions used to compare chain lengths, the enzyme is almost saturated with substrate. Reduction of substrate concentration reduces the absolute rate of reaction but increases the fraction of any RNA chain degraded in any interval of time. At low RNA concentrations the RNA chains are degraded at inconveniently high rates. In order to examine the degradation of single RNA species, it was necessary to inhibit the enzyme competitively.

Table I shows that the enzyme is subject to partial product inhibition. No significant further inhibition is afforded by cytidine 3′-phosphate when the enzyme is fully inhibited by adenosine 3′-phosphate. In partial competitive inhibition the inhibitor is thought to act at a different site from the active center (Haldane, 1930). It is, however, possible that the enzyme preparation is a mixture of exonucleases sensitive and insensitive to product inhibition.

Although the four common nucleotides behave differently as inhibitors, the effect of substrate base composition on the rate of degradation by the exonuclease remains unknown. The base compositions of the rRNAs examined do not vary widely, particularly with regard to the purine to pyrimidine ratio. The simplicity of the data obtained on the comparative rates of reaction of different RNA species (Riley, 1969) argues against the view that variation in base composition is an important consideration.

Figure 3 shows the degradation of varying concentrations of radioactive *E. coli* 23S rRNA in the presence of maximally inhibiting concentrations of adenosine 3′-phosphate. Over this range of RNA concentration the rate of reaction varies with the concentration of initially identical end groups. The following experiments in the presence of inhibitor were performed at RNA concentrations of less than 0.2 optical density

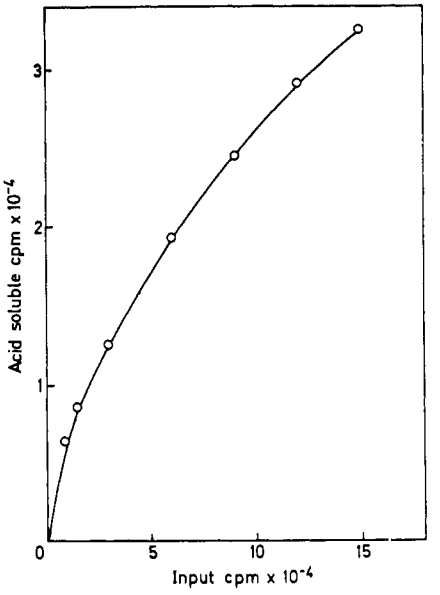

FIGURE 3: Effect of RNA concentration on the rate of RNA degradation in the presence of adenylic acid. Varying quantities of radioactive *E. coli* 23S rRNA (1.67×10^5 cpm/optical density unit) were mixed with equal quantities of exonuclease in GC buffer containing 1.5 mM adenylic acid and 1 mg/ml of bovine plasma albumin. The degradation was stopped after 20 min by cooling to 0° and addition of $HClO_4$. The ordinate shows the total radioactivity made acid soluble.

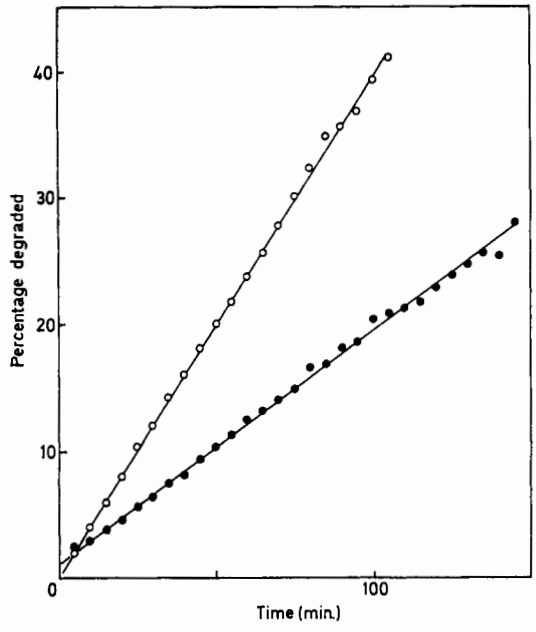

FIGURE 4: Constancy of early rate of RNA degradation. Radioactive HeLa 28S and 16S ribosomal RNA at a concentration of 0.16 and 0.08 optical density unit per ml, respectively, were incubated with equal concentrations of exonuclease in GC buffer containing 1.5 mM adenylic acid and 1 mg/ml of bovine plasma albumin. The ordinate shows the degree to which the 28S (●) and 16S RNA (○) were degraded to acid-soluble products at the times shown in the abscissa.

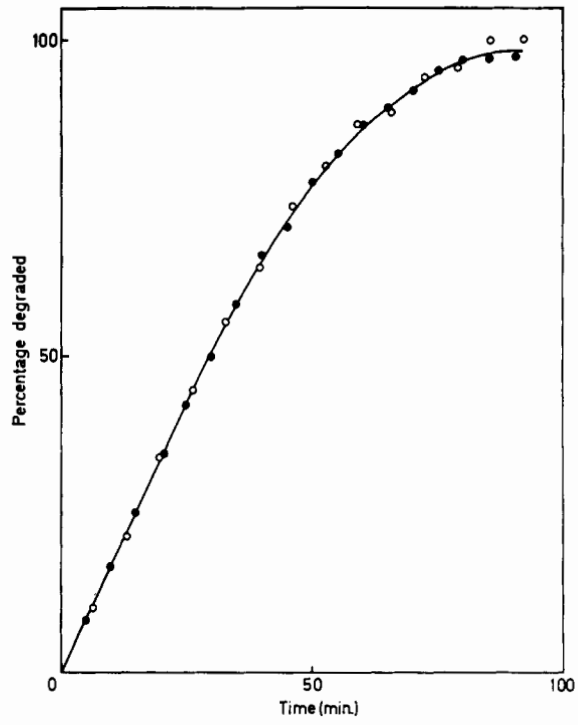

FIGURE 5: Comparison between the courses of degradation of HeLa 28S (●) and 16S ribosomal RNA (○). The radioactive RNAs were degraded with exonuclease under conditions described in the legend to Figure 3. The time scale of degradation of the 28S RNA was adjusted to align the curves at the point at which each RNA was 50% degraded.

unit[1]/ml where the rate of reaction is most sensitive to end-group concentration and where any contribution to product inhibition by the RNA is greatly reduced.

Figure 4 shows that the early rate of degradation of HeLa rRNA is constant. The relationship between end-group concentration and reaction rate under product-inhibited conditions is not linear. This was taken into account by using twice as much 28S RNA as 16S RNA (on an optical extinction basis). Assuming that native 28S RNA behaves as if its polynucleotide chains are twice as long as those of 16S RNA (Riley, 1969), this measure should equate the effective end-group concentrations in the two reaction mixtures. It will be seen that the comparative rates of reaction (on a percentage basis) are in agreement with this assumption.

The rate at which the inhibited enzyme attacks RNA is consistent with the relative rates of reaction with the uninhibited enzyme. This would be expected if a uniform enzyme activity were being observed. Similarly the early constancy of reaction rates is also reflected in the relative rates of reaction of different RNA species in mixtures. In mixtures the relative rates of degradation of two species remains constant until the smaller is 50% degraded. Factors which might have been expected to disturb this early constancy of reaction rate or to make a coincidental contribution to it, will be discussed below.

Possible Appearance of Further End Groups. From Figure 3 it can be expected that any increase in the number of end groups, at least under product inhibited conditions, would produce an increased rate of reaction.

Figure 5 compares the full course of degradation of HeLa 28S and 16S rRNA. In this experiment the time scale of degradation of 28S RNA was adjusted to make the two curves coincide at the point at which each was 50% degraded. No evidence was found either for endonuclease attack or for any hidden end groups in 28S RNA (Riley, 1969) which are exposed substantially before degradation of the most vulnerable chains approaches completion.

Heterogeneity of Chain Length. The ability of the exonuclease to degrade completely any RNA presented for measurement is important. Cessation of degradation at any undefined point (possibly at a variety of undefined points in rapidly labeled RNA) might lead to such inconstancy in the relative reaction rates as to make arbitrary the choice of any particular value. Where such incomplete reaction is avoided by choice of a suitable exonclease, variation in reaction rate attributable to heterogeneity in chain length might be seen. The fall of reaction rate after 50% degradation of HeLa rRNA (Figure 5) is unlikely to be due to heterogeneity in chain length. *E. coli* 16S RNA shows the same effect, and this RNA is considered to be a highly uniform population of single chains.

Short RNA chains are degraded at correspondingly high rates. A mixture of electrophoretically purified, radioactive *E. coli* 4S and 5S RNA was degraded 14 times faster than total *E. coli* K12 rRNA. Assuming a value of 1500–1600 nucleotides for the rRNA (McIlreavy and Midgley, 1967) the mean chain length of the 4S and 5S RNA may be estimated

[1] Optical density unit = that amount of RNA which when dissolved in 1 ml of water has, at 260 mμ, an optical extinction of 1.0 in a 1-cm light path.

at about 110 nucleotides. A correction factor is used to calculate the relative rates of degradation from the relationship between the specific activities of the initial materials and the degraded products (Riley, 1969). This factor becomes both more important and more difficult to determine with increasing disparity between the chain lengths to be compared. The value of 110 nucleotides is in good agreement with the expected value of about 80 nucleotides.

The synthetic polynucleotides poly(A), poly(C), and poly(U) were completely degraded by the enzyme, suggesting that in GC buffer none of these molecules were permanently folded so as to hide the 5′-end group. When attempts were made to compare the mean lengths of these synthetic polyribonucleotides to that of radioactive *E. coli* rRNA, the relative rate of degradation of synthetic polynucleotides fell continuously throughout the course of the reaction. This is consistent with the heterogeneity in chain length expected in the synthetic polyribonucleotides. Rapidly labeled RNA from HeLa cells, however, shows by this test no markedly greater heterogeneity in chain length than HeLa 16S rRNA (Riley, 1969; Figure 2).

Structural Influences. In a trivial sense it is obvious that the structure of RNA chains must affect the rate of reaction. Comparison of reaction rates with DNA is not practicable as the enzyme preparation apparently contains endonuclease activity for DNA. It degrades single-stranded circular DNA from the bacteriophages ϕX 174 (Okazaki *et al.*, 1966) and ZJ2 (W. T. Riley, unpublished data). The question remains as to whether structural differences between RNA chains affect the rate of reaction. Apart from certain, apparently stoichiometric, effects of hidden end groups, none of the observed variations in reaction rate are readily assignable to such undisclosed structural differences.

RNA from the bacteriophage R17 has by physical measurement (Mitra *et al.*, 1963; Gesteland and Boedtker, 1964) and chemical estimation (Sinha *et al.*, 1965) a particle weight of about 1.1×10^6. This is very close to the value assigned to *E. coli* 23S rRNA (Stanley and Bock, 1965). The bacteriophage RNA is, however, more condensed, sedimenting at about 27 S. Moreover, unlike ribosomal and rapidly labeled RNA, it shares with DNA the property of dissolving in 2 M NaCl, which may suggest that its surface lacks sites of hydrogen bonding to neighboring particles. R17 RNA is degraded more slowly than *E. coli* 23S RNA. If the observed rate is regarded as coincidental, this could suggest a structural effect. Two different preparations of radioactive R17 RNA gave chain lengths, as determined by exonuclease degradation, of $2.06 \times$ *E. coli* K12 23S RNA and $1.84 \times$ *E. coli* K12 total rRNA. These results are consistent with the view that the 23S RNA has two 5′-end groups exposed to exonuclease attack (Riley, 1969) and that the particle weight of R17 RNA is close to that of *E. coli* 23S RNA. These results do not, of course, exclude the possibility of further end groups in R17 RNA which are not available for immediate attack by the enzyme.

A possible explanation of this, perhaps surprising, lack of structural effect may lie in all these RNA species being highly condensed in the presence of excess calcium ions.

Discussion

No preparative method consistently yields undegraded RNA. Batches are selected on the basis of their behaviour on preparative sucrose gradient sedimentation. Those in which the ratio of the larger to the smaller rRNA is substantially different from 2:1, or with badly shaped peaks, are discarded. Fresh RNA is prepared for each experiment and used immediately. The selected RNA samples usually resist sedimentation changes after incubation at pH 9.0, extraction with EDTA, incubation in 80% dimethyl sulfoxide and sedimentation in the presence of urea. Where changes occur a characteristic pattern is followed (W. T. Riley, in preparation). There is no reason to suspect that the materials examined differ from those prepared by other workers.

The experiments, and remarks, about endonuclease activity refer to endonuclease of low specificity. Highly specific endonuclease attack has been invoked to account for certain changes in the apparent particle weight of labeled RNA fractions in nucleoli (Liau *et al.*, 1968), but this interpretation seems to be partly based on the view that the particles concerned were initially single RNA chains. Low degrees of exonuclease degradation alter the sedimentation characteristics of rRNA (W. T. Riley, in preparation). The results are interpretable partly as conformational changes and partly as separation of substructures. Different linkages vary in their susceptibility to exonuclease so that differences in stability do not necessarily suggest differences in secondary structure affecting reaction with an endonuclease.

Some authors (Spahr and Gesteland, 1968; Spahr *et al.*, 1969; Szer, 1969) have reported nonrandom degradation of r- and bacteriophage RNA by an enzyme similar to or identical with an *E. coli* enzyme, ribonuclease IV. The enzyme involved is thought to be an endonuclease. However the purity of the enzyme preparation, the role of conformation in the observed changes, and the number of sites subject to attack remain uncertain.

Differences in the overall structure of RNA particles cannot readily account for the observations obtained by the use of *B. subtilis* exonuclease. Not only are the rates of degradation maintained up to 50% degradation of the particles, but there is no correlation between reaction rates and the kind of structural changes reported (Riley, 1969). Sedimentation properties may change with or without substantial changes in reaction rate, contrast the conversion of HeLa 28S and 16S rRNA into forms sedimenting near 8 S. The change in 28S RNA is accompanied by a twofold reduction in apparent chain length, that of 16S RNA is not. With *E. coli* B and *E. coli* K 12 23S RNA apparent chain lengths may differ without difference in sedimentation. Where there is reason to suspect differences in detailed chain structure, as between R17 RNA and rRNA, the results are open to a consistent and straightforward interpretation.

The results of chain-length estimation by exonuclease degradation can be accommodated in either of two ways. Either the method is simple and direct in its application, so that the results, though surprising, are meaningful. Or the method is complex and the apparently simple results are due to bizarre interactions between interfering factors, so that the apparently simple results are themselves bizarre. Such interactions would have to be maintained through a variety of different enzyme and nucleic acid preparations. There is no indication that either of the most obvious interfering agencies, endonuclease attack, or differential structural effects, are of any significance in the chain-length estimation

itself. So that this method unlike chemical methods, can be used to detect changes in end-group frequencies due to enzyme attack or other structural change.

Acknowledgments

I thank Professor H. Harris for his helpful comments. Mr. D. Morley helped with the experiments.

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Structure of Nucleic Acid–Poly Base Complexes*

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ABSTRACT: Complexes of DNA, RNA, and the synthetic polynucleotides, poly (A + U) and poly (I + C), with poly-L-lysine, prepared at high salt concentrations, have been studied.

The complexes containing DNA and the synthetic polynucleotides show anomalous circular dichroism, with greatly enhanced rotational strength, and in DNA and poly (A + U) an inversion of sign. Only relatively minor changes are found in the RNA complex. X-Ray diffraction studies of the complexes indicate that DNA remains in the B form, and appear to exclude any large degree of distortion of the helices, although poly (A + U) in the complex is evidently in the three-stranded form. No indication of periodicities in the range 30–150 Å were observed by low-angle X-ray diffraction. Reasons are given for believing that the anomalous optical activity may result from a liquid-crystal type of structure in the complexes. Electron microscopy shows that the salmon sperm DNA–polylysine complex is predominantly in the form of hollow doughnut-shaped particles, with a diameter of the order of 0.3 μ, and evidence of internal periodicity. With poly (A + U), cigar-shaped particles are found and, with poly (I + C) complexes, characteristic filaments. RNA in its complexes forms bundles of thin filaments, as well as long thick fibers, with a remarkably uniform diameter. They show no birefringence. The similarity of the DNA–polylysine particles to bodies found in chromatin of some kinds is noted.

The complexes formed between nucleic acids and the poly base, poly-L-lysine, have been widely studied as prototypic systems for nucleic acid–protein interactions. It has been established by Felsenfeld and his colleagues (Leng and Felsenfeld, 1966; Shapiro *et al.*, 1969) that if DNA and poly-L-lysine are allowed to react at high ionic strengths, where the interaction energy is diminished and the most stable conformations can form by "annealing," a high degree of specificity is manifested. The complexes formed under these conditions differ from those produced at low ionic strength in possessing a much higher thermal stability (Leng and Felsenfeld, 1966; Inoue and Ando, 1966), radically changed optical rotatory dispersion (Cohen and Kidson, 1968; Shapiro *et al.*, 1969) and circular dichroism (Shapiro *et al.*, 1969), and in manifesting a strong selectivity for DNA composition, with a strong preference for (A + T)-rich species. The high salt complexes are moreover particulate in nature, with apparently a rather narrow size distribution (Shapiro *et al.*, 1969). It is clear that it is these specific complexes which should be considered if such a simple system is to give information of general relevance to the specificity of the nucleic acid–protein recognition process.

Our object has been to examine the structural basis of the specific interaction between poly-L-lysine and helical poly-

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