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Hydroxylapatite-Catalyzed Degradation of Ribonucleic Acid†

H. G. Martinson*‡ and E. B. Wagenaar

ABSTRACT: Hydroxylapatite was found to catalyze the degradation of RNA at elevated temperatures. Single-stranded RNA was degraded considerably more rapidly than double-stranded RNA. DNA was not affected. Factors which decreased the adsorption strength of the RNA decreased the rate of degradation. Thus increasing the phosphate concentration of the surrounding buffer or substituting Cs^+ for Na^+ reduced the rate of hydroxylapatite degradation. The most likely mechanism appears to be a Ca^{2+} ion mediated hydrolysis which occurs primarily within a few angstroms of the crystal surface where the free Ca^{2+} ion concentration is several orders of magnitude higher than in the bulk solution. Caution is advised in the design and interpretation of experiments involving the chromatography of RNA on hydroxylapatite. However, the degradation of RNA by hydroxylapatite together with the ability of hydroxylapatite to fractionate nucleic acids according to size can be used for the controlled fragmentation of RNA preparations to preselected molecular weights in a simple one-step procedure.

Hydroxylapatite is becoming ever more widely used in the analysis of nucleic acids (Bernardi, 1971; Kohne and Britten, 1971; Muench, 1971). Much work now being done involves the use of elevated temperatures (Kohne and Britten, 1971) and many experiments are done which involve RNA. We have found previously that single-stranded RNA (ss-RNA)[1] but not DNA is degraded rapidly by hydroxylapatite in dilute buffer at high temperatures (Martinson, 1973c). We have now studied this phenomenon in greater detail in order first to try and elucidate the mechanism involved, second to assess its impact on the interpretation of conventional experiments, and third to explore the possibility of using the system as a convenient way to produce RNA fragments of defined size. It was found that adsorption on the hydroxylapatite surface is required for enhanced degradation to occur. Furthermore rigid ds-RNA is degraded much more slowly than ss-RNA. Consequently the extent of RNA cleavage is less for ds-RNA and probably also RNA–DNA hybrids than it is for ss-RNA during high-temperature chromatography on hydroxylapatite. Because the affinity of nucleic acids for hydroxylapatite decreases with molecular weight (Martinson, 1973c; Piperno *et al.*, 1972), degraded RNA fragments are released from the crystal surface when they become small enough. This limit size can be controlled by the eluting power of the buffer in which incubation is conducted and the system can therefore be adapted for the production of ss-RNA fragments of defined size.

Materials and Methods

The viral RNAs were gifts of Dr. L. Lewandowsky and Dr. C. A. Knight.

Hydroxylapatite chromatography was conducted as described previously (Martinson, 1973a,b) using either the Bio-Rad HT or HTP. Where indicated the hydroxylapatite was preincubated in 0.1 M phosphate in a boiling water bath for 30 min.

Linear sucrose gradients (0.25–0.65 M) which were 10 mM in Na_3EDTA and also either 10 mM in Tris-phosphate (pH 7.2) or 0.2 M in NaCl were run in the Beckman SW 41 rotor at 41,000 rpm. Fractions were collected by puncturing the bottom of the tube with a hypodermic needle and collecting a specified number of drops per fraction.

Polyacrylamide gel (5%) electrophoresis (Loening, 1967) was conducted at room temperature in 0.2% sodium dodecyl sulfate for 2 or 3 hr. Dr. G. Schultz kindly provided 4s and 5s size standards and Dr. R. B. Church provided the facilities.

Ribonuclease sensitivity was determined by adding 0.2 ml

† From the Department of Biological Sciences, University of Lethbridge, Lethbridge, Alberta, Canada T1K 3M4. *Received October 16, 1973.* This investigation was supported by Grant No. A-4926 from the National Research Council of Canada.

‡ Present address: Department of Biochemistry and Biophysics, School of Medicine, University of California, San Francisco, Calif. 94122.

[1] Abbreviations used are: ds-RNA, double-stranded RNA; ss-RNA, single-stranded RNA.

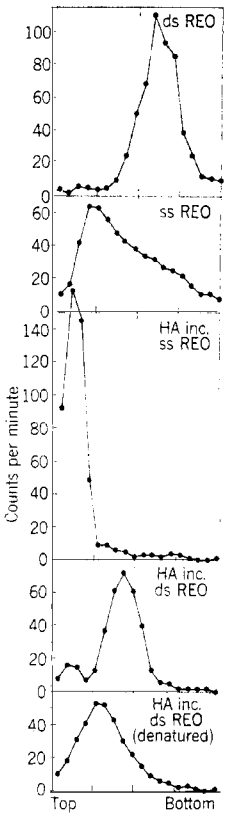

FIGURE 1: Degradation of ss- and ds-RNA by hydroxylapatite; 0.1 μg of reovirus ds-[^{3}H]RNA in 1.85 ml of 10^{-3} M Na_3EDTA were divided into aliquots and treated as follows. ds REO: 0.2 ml was mixed with an equal volume of 10^{-3} Na_3EDTA and then run on a sucrose gradient (0.2 M in NaCl–EDTA) for 10 hr. A similar aliquot was assayed by ribonuclease digestion and found to be 87% resistent. ss REO: 0.625 ml was heated for 1 min in a boiling water bath and 0.2 ml was run on a sucrose gradient as above. Another 0.2 ml was found to be only 4% resistent to ribonuclease. HA inc. ss REO and HA inc. ds REO: the remainders of the "boiled" (ss) and original (ds) stocks were each mixed with 0.8 ml of 0.1 M NaCl and loaded on 20 mg of preincubated HTP. The columns were rinsed successively with 0.5 ml each of water then 20, 40, and 60 mM potassium phosphate and finally three times with 1 ml of 20 mM potassium phosphate. After layering on an additional 0.1 ml of 20 mM buffer both ends of the columns were closed and the temperature was raised to 70° and maintained for 20 hr. The temperature was then reduced and the columns were eluted with three 0.1-ml aliquots of 0.1 M cesium phosphate. The eluate from the columns which had been loaded with ss-RNA was loaded directly on to the sucrose gradient and run for 10 hr (HA inc. ss REO). To the other eluate was added 0.425 ml of 10^{-3} M EDTA; 0.2 ml was then brought to 0.4 ml with 10^{-3} M EDTA and run on a sucrose gradient (HA inc. ds REO) and another 0.2 ml was assayed with ribonuclease (66% resistent). HA inc. ds REO (denatured): the remainder was heated in a boiling water bath for 1 min and then run on a gradient and assayed with ribonuclease (7% resistant) as above.

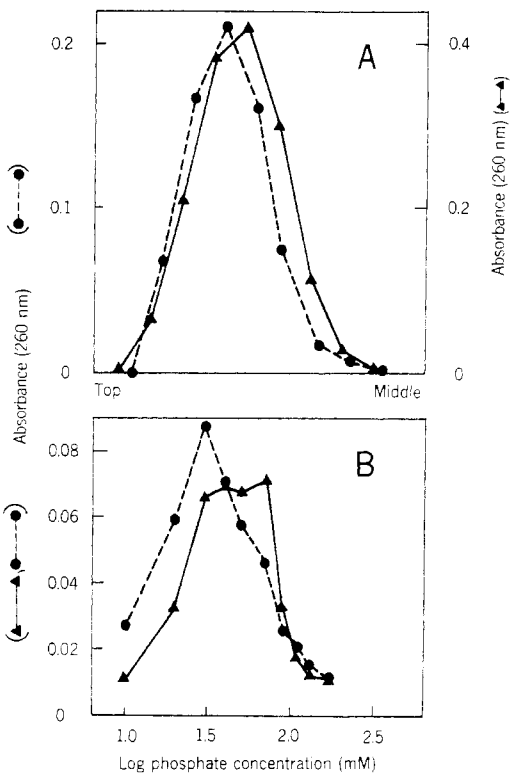

FIGURE 2: Fractionation of ss-RNA fragments according to size on hydroxylapatite. Two preparations of cucumber virus 4 RNA fragments were compared in size (A) by sucrose density gradient centrifugation (10 mM Tris-phosphate–EDTA, 23 hr) and (B) by hydroxylapatite chromatography (30 μg of RNA on 30 mg of HT, 0.5 ml of potassium phosphate/step). The two preparations were about 19,000 and 21,000 molecular weight, respectively. The fragmented RNA was prepared as described in the legend to Figure 3.

of the RNA solution (monovalent cation concentration less than 0.06 M, total RNA about 0.01 μg) to 0.4 ml of 0.2 M sodium phosphate (0.3 M Na^+) containing 0.08 μg of pancreatic ribonuclease. The mixture was incubated 1 hr at 37° after which 0.15 ml of DNA carrier (25 μg) and 0.2 ml of 50% trichloroacetic acid were added and mixed. Total RNA was determined by immediately withdrawing 0.4 ml for scintillation counting. Ribonuclease digested, acid soluble, and therefore presumably single-stranded RNA was determined by centrifuging the remainder at 2500 g for 1 hr at 0° and withdrawing 0.4 ml of the supernatant for counting.

Results

The secondary structure of RNA is apparently an important factor in governing its susceptibility to hydroxylapatite degradation. Figure 1 illustrates the results of an experiment in which reovirus ds- or ss-RNA was incubated at high temperature on hydroxylapatite and then sedimented in sucrose for size comparison with unincubated RNA. The first panel of Figure 1 (ds REO) shows the distance migrated by reovirus ds-RNA after sedimentation. This RNA was 87% resistent to digestion by ribonuclease. The next panel (ss REO) shows the sedimentation pattern of heat-denatured reovirus RNA (4% ribonuclease resistent). The broad profile and early peak indicate that the double-stranded material had a large number of single-strand nicks. This was not unexpected because the RNA was labeled to a high specific activity with tritium and had been stored for some time (see Martinson, 1973d). The bottom three panels of Figure 1 show the sedimentation behavior of native and denatured reovirus RNA which had been incubated on hydroxylapatite at 70° for 20 hr in 20 mM potassium phosphate. Degradation is relatively slow at this temperature and phosphate concentration. It can be seen that the ss-RNA (HA inc. ss REO) was degraded to a very small size and remained near the top of the gradient while the ds-RNA (HA inc. ds-RNA) was much less degraded. Furthermore the ds-RNA was still 66% ribonuclease resistant after the 70° incubation. However, the possibility remained that the number of single-chain scissions introduced in the two cases was actually the same but that the duplex structure merely masked this situation for the ds-RNA. In order to check for this a portion

TABLE I: Inhibition of RNA Degradation by Various Salts.[a]

Type of Degradation	Experimental Conditions[b]	Relative Ability of Various Salts to Inhibit Degradation
Ca^{2+} ion degradation in solution	25 μg/ml of TMV, 1 mM Ca^{2+}, 97°, 30 min. Supplemented with KP or KCl at concentrations indicated at right	KP, 10 mM < 100 mM KCl, 15 mM < 150 mM
	25 μg/ml of TMV, 0.1 mM Ca^{2+}, 97°, 60 min. Supplemented as indicated at right	10 mM CsP < 10 mM KP < 10 mM NaP < 10 mM Tris-P < 150 mM KCl
Hydroxylapatite degradation	7 μg of TMV, 16 mg of HT, 102°, 4 or 30 min. Columns prewashed at room temperature with NaP concentration shown at right	NaP, 10 mM < 20 mM < 30 mM < 40 mM
	7 μg of TMV, 16 mg of HT, 102 or 80°, 5 min. Columns prewashed with indicated KP concn	KP, 10 mM < 100 mM
	10 μg of TMV, 16 mg of HT, 90° for 10 min or 102° for 5 min. Columns prewashed as indicated at right	150 mM KCl < 10 mM Tris-P < 10 mM KP < 10 mM NaP < 10 mM CsP

[a] Abbreviations used are: TMV, tobacco mosaic virus ss-RNA; NaP, sodium phosphate; KP, potassium phosphate; CsP, cesium phosphate; Tris-P, Tris-phosphate. [b] Ca^{2+} ion degradation was conducted by incubating the RNA at an elevated temperature in the presence of a low concentration of $CaCl_2$. Hydroxylapatite degradation was conducted essentially as described in the legend to Figure 1. In all cases the amount of degradation was determined qualitatively by chromatography on fresh hydroxylapatite.

of the hydroxylapatite-incubated ds-RNA was heat denatured (7% ribonuclease resistent) and sedimented on sucrose along side the others [HA inc. ds REO (denatured)]. The bottom panel of Figure 1 shows that in fact relatively few single-chain scissions were introduced into the duplex molecules. Comparison with the sedimentation profiles of unincubated (ss REO) and incubated (HA inc. ss REO) ss-RNA shows that only the longest chains were fragmented significantly and the fragments were still considerably larger than those produced from the ss-RNA incubated on hydroxylapatite. Thus it is clear that the double-stranded configuration confers a measure of chemical stability on the phosphodiestor linkage in RNA. Additional experiments have shown, however, that eventually ds-RNA does become fully degraded. Similar experiments with ss-DNA showed no detectible degradation whatever.

Mechanism of Degradation. In most of the experiments to be discussed below the relative sizes of the various preparations of fragmented RNA were determined by hydroxylapatite chromatography. Figure 2 shows that in this size range (about 3s) hydroxylapatite chromatography (B) is as effective as sucrose density centrifugation (A) in comparing size differences. Similar experiments utilizing Sephadex chromatography and polyacrylamide gel electrophoresis have confirmed that hydroxylapatite fractionates according to size (see also Martinson, 1973c; Piperno *et al.*, 1972). When only a qualitative result was required the hydroxylapatite method was used because of its convenience and rapidity.

It is known that ss-RNA can be degraded by divalent cations (Butzow and Eichhorn, 1971) including Ca^{2+} (Huff *et al.*, 1964) and that such degradation is conformation dependent (Wintermeyer and Zachau, 1973). It was thus of interest to determine whether the hydroxylapatite-catalyzed degradation of RNA was merely mediated by Ca^{2+} ions leeched from the hydroxylapatite into solution. As a first test of this possibility it was necessary to determine whether the solubility of hydroxylapatite is sufficient to provide enough Ca^{2+} ions for the rapid hydrolysis of RNA in solution. To this end an extract of hydroxylapatite was prepared by boiling a sample of hydroxylapatite in water for 0.5 hr and then removing the solids by centrifugation. It was found that tobacco mosaic virus ss-RNA at 25 μg/ml was indeed degraded at high temperature (97°, 20 min) by the hydroxylapatite extract. The amount of degradation was intermediate to that given by 10^{-1} and 10^{-2} mM $CaCl_2$. No significant degradation occurred in the absence of Ca^{2+} ions. This result is consistent with those of Pak and Skinner (1968) who found that a solution in equilibrium with hydroxylapatite at 37° is about 10^{-1} mM in Ca^{2+}. However, the rate of degradation of RNA by the hydroxylapatite extract was much slower than that by hydroxylapatite itself which is considerably faster than even 1 mM $CaCl_2$. Furthermore if 1 mM solutions of $CaCl_2$ are also made 150 mM in KCl the degradation is effectively stopped while, in contrast, KCl was found to have little effect on the hydroxylapatite mediated degradation of RNA (see Table I). Thus the dissolving of Ca^{2+} ions from the hydroxylapatite into the bulk solution cannot account for the major portion of hydroxylapatite-catalyzed degradation of RNA.

The above results suggested that actual adsorption of the RNA on the hydroxylapatite was an important factor in hydroxylapatite degradation of RNA. This proposition was tested by comparing the protecting effectiveness of various salts which differ in their eluting power for nucleic acids on hydroxylapatite. The experimental procedure was basically the same as described above. Only qualitative results were sought and these are summarized in Table I. The most striking observation is the nearly complete reversal in protecting ability of the various salts depending on whether free Ca^{2+} ion or hydroxylapatite degradation were being tested. In the case of Ca^{2+} ion degradation, protection evidently depends on the ability of the monovalent cations to displace the Ca^{2+} from the RNA backbone. Thus the sequence, $Cs^+ < K^+ < Na^+$, reflects the increasing affinity of these cations for the RNA (Gordon, 1965). $Tris^+$ was even more effective, perhaps because, with its hydroxyls, it can act as a weak chelator for the Ca^{2+}. Finally, of course, the 150 mM KCl was the most effective simply because a tenfold higher K^+ concentration was used. For hydroxylapatite degradation, on the other hand, the 150 mM KCl was the poorest protector, in line with the fact

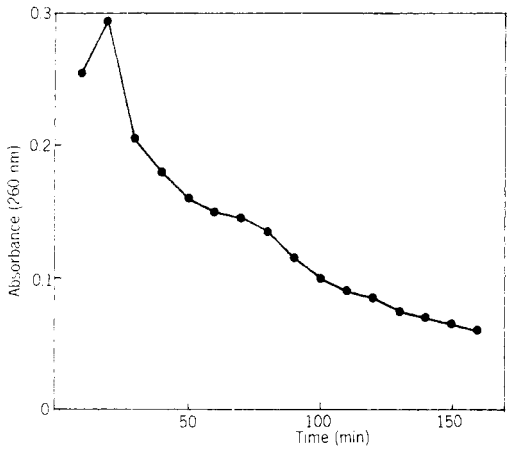

FIGURE 3: Hydroxylapatite degradation–elution of RNA. Cucumber virus 4 ss-RNA (190 μg, about 100,000 molecular weight) was loaded on a 7-mm diameter column of Bio-Rad HT (330 mg) and 10 mM preboiled potassium phosphate was pumped through at 0.2–0.3 ml/min for 30 min. The temperature was then raised to 70° and fractions were collected directly. The absorbance was determined for each fraction and then the beginning, middle, and end fractions were pooled and put through separate collection columns. Were the eluate passed directly through a collection column as is usually done the elution profile would be similar but reduced by about 70%. Acrylamide gel electrophoresis showed that the middle pool contained fragments of about 20,000 molecular weight. The other two portions of fragments were compared as shown in Figure 2.

that KCl has virtually no eluting ability, while the best protector, cesium phosphate, is known to be the most powerful elutor of the phosphate buffers (Martinson, 1973b). Thus it can be concluded that the hydroxylapatite-catalyzed degradation of RNA occurs only at or very near the crystal surface.

Degradation–Elution of RNA. The dual observations that hydroxylapatite degradation of RNA only occurs near the crystal surface and that hydroxylapatite fractionates nucleic acids according to size suggested the possibility that hydroxylapatite chromatography could be used to prepare RNA fragments of defined size. Thus if RNA adsorbed on hydroxylapatite is heated while a steady stream of dilute phosphate buffer is passed through the column, degradation of the RNA would be expected to continue until the RNA becomes so small that it is no longer tightly adsorbed. At that point further degradation would be nearly halted as the RNA fragments left the crystal surface and the continuous buffer flow washed them from the column. Figure 3 shows the time course of elution for fragments in one such experiment conducted at 70° with 10 mM potassium phosphate. Although the rate of fragmentation of RNA on hydroxylapatite is presumably size independent (see Butzow and Eichhorn, 1971) the method employed selectively collects fragments as soon as they reach a predetermined size. Thus the degradation can be continued indefinitely until the entire sample has been fragmented and the final length distribution will be independent of any size heterogeneity in the original sample. This allows high yields to be obtained. However, inevitably a certain proportion of cleavages occur near the ends of the adsorbed molecules yielding oligonucleotides smaller than the average fragment size produced. This factor is the main drawback and limits yields to about 70%. The bulk of the oligonucleotides can be removed from the preparation by passing the eluate from the high-temperature (fragmentation) column directly through another (collection) column maintained at a lower temperature. The desired fragments bind to the collection column because of their increased secondary and tertiary structure at the low temperature (Martinson, 1973c) but the oligonucleotides pass through because of their extremely small size. The fragmented RNA can then conveniently be eluted from the collection column with an appropriate buffer. Ideally the average fragment size eluted from the fragmentation column should remain constant from beginning to end. In practice, however, there is a slight tendency for larger fragments to be eluted as time goes on. This is illustrated by the two preparations of Figure 2 which differ in average size by about 10%. The smaller and larger fragments were collected from the 20 and 130 min eluted material, respectively, of Figure 3.

Discussion

In the previous section we have shown that RNA but not DNA can be degraded by hydroxylapatite at high temperatures. Furthermore ss-RNA is much more rapidly degraded than ds-RNA. This phenomenon can complicate the design and interpretation of some types of experiment but it does provide a new simple procedure for the controlled fragmentation of RNA for other purposes.

Mechanism. The basic characteristics of the degradation reaction (*e.g.*, resistance of DNA and ds-RNA) are similar to those of the well characterized divalent cation degradation of RNA which has been studied in several other systems (Butzow and Eichhorn, 1971; Huff *et al.*, 1964; Wintermeyer and Zachau, 1973). However, a major difference is the almost completely reverse order in which different salts which affect the hydroxylapatite reaction can be ranked compared to the Ca^{2+} ion degradation (Table I). The observation that in general the various salts inhibit hydroxylapatite degradation more effectively as their eluting power increases strongly suggests that adsorption is required for this kind of degradation to occur. The hydroxylapatite surface is known to have important catalytic properties of this type which are related directly to its surface geometry (Taves and Reedy, 1969). However, the particular stereochemistry involved in RNA degradation cannot be accounted for by using the Taves and Reedy (1969) model. Therefore either a new surface phenomenon is occurring or perhaps the crystal surface is in fact not directly involved in the degradation. We take the latter view. When hydroxylapatite is suspended in some buffer there exists, besides the small concentration of Ca^{2+} ions in the bulk solution (owing to the solubility of the hydroxylapatite), a very high concentration of Ca^{2+} ions within a few angstroms of the crystal surface held there by the requirements of electrostatic charge balance (Pak and Skinner, 1968). A rough calculation using the data of Pak and Skinner (1968) together with a surface area determination (Weikel *et al.*, 1954) gives a concentration of Ca^{2+} near the crystal surface (say within 100 Å) as high as 0.7 M or about four orders of magnitude higher than in the bulk solution. Clearly RNA which is adsorbed on the crystal surface is immersed in a medium of very high Ca^{2+} ion concentration which is capable of rapid RNA degradation. However, because these Ca^{2+} ions do not significantly leave the vicinity of the surface (Pak and Skinner, 1968) any RNA which is eluted is in effect protected since degradation can then only proceed at the rate characteristic of the cation composition of the bulk solution. Nevertheless if the eluted RNA is left in the presence of the hydroxylapatite for a long time it will eventually be degraded as it migrates randomly back and forth from the crystal surface. Presumably then the characteristics of competitive inhibition of degradation summarized for free Ca^{2+} in Table I also apply to hydroxylapatite degradation

but are masked by the more important adsorption effects. This may explain why the sequence of salts for inhibition of hydroxylapatite degradation in Table I does not follow precisely the order of high temperature eluting ability of those salts (*i.e.*, KCl < NaP < Tris-P < KP < CsP).

Experimental Ramifications. The rapid degradation of ss-RNA on hydroxylapatite precludes the use of very high temperatures for phosphate gradient chromatography because mere oligonucleotides are eluted (Martinson, 1973c). However, if the low phosphate portion of a gradient is minimized and a few RNA chain scissions can be tolerated then a reasonably meaningful elution profile can be obtained at moderate temperatures (*e.g.*, 60°). Similarly thermal elution chromatography should not be attempted for ss-RNAs unless it is known that elution will be completed relatively rapidly and at moderate temperatures. During phosphate gradient chromatography degradation decreases during the course of the gradient owing to the increasing phosphate concentration. However, with thermal elution chromatography the rate of degradation rapidly increases as the temperature is raised. Because degradation is much slower for ds-RNA than for ss-RNA it is possible to run successful thermal chromatograms on the former (and presumably also DNA–RNA hybrid) provided some fragmentation of the single-chain product is permissible. If it is necessary to recover intact RNA no form of hydroxylapatite chromatography at elevated temperatures should be used although chromatography of RNA at room temperature may be "safe" (Bernardi, 1971; Muench, 1971).

Degradation–Elution of RNA. Frequently in hybridization studies, the use of fragmented RNA is preferable or even essential in order to gain certain types of information (*e.g.*, Dina *et al.*, 1973; Krueger and Kosky, 1973; Gillespie and Spiegelman, 1966; see also McConaughy and McCarthy, 1970). However, preparing large quantities of nucleic acid fragments of a defined size range can be time consuming (*e.g.*, Hell *et al.*, 1972). The controlled fragmentation of RNA on hydroxylapatite is rapid and inexpensive and gives a high yield of fragments whose size range is unaffected by any heterogeneity in the starting material.

The rate of hydroxylapatite degradation of RNA is easily controlled by varying the temperature. However, the predominant fragment size produced depends on the affinity of the RNA for the hydroxylapatite and this also is temperature dependent (Martinson, 1973c) besides being influenced by the nature of the elution buffer and the species of hydroxylapatite (Martinson, 1973b). Thus the desired rate should be selected first and then the fragment size. The latter is most easily controlled by varying the phosphate concentration. For example, 20 mM potassium phosphate yields predominantly 30,000 molecular weight fragments compared to about 20,000 for 10 mM potassium phosphate (at 70° on Bio-Rad HT).

The effects of numerous other parameters such as the RNA–hydroxylapatite load ratio or the flow rate of the elution buffer which may affect the degradation–elution reaction, or the degradation column–collection column temperature differential which would affect the size range of the retained fragments, have not been thoroughly investigated.

The fragments which are produced by degradation–elution are probably representative of the sequence distribution in the original RNA. Although the rate of phosphodiester cleavage is sequence dependent for degradation of RNA by Zn^{2+}, this effect appears to be primarily the result of the differing affinity of Zn^{2+} for the various bases coupled with differences in secondary structure of the RNA (Butzow and Eichhorn, 1971). Ca^{2+}, however, is not attracted to the bases (Singer, 1964) and exhibits no binding preference along the backbone (Shapiro *et al.*, 1969), while the secondary and tertiary structure in ss-RNA would be near minimal under the conditions of degradation–elution.

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