THE PHYLOGENY OF THE RIBONUCLEASE–RIBONUCLEASE INHIBITOR SYSTEM: ITS DISTRIBUTION IN TISSUES AND ITS RESPONSE DURING LEUKAEMOGENESIS AND AGING*

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Summary

Acid RNAase, alkaline RNAase, and alkaline RNAase inhibitor appear to be widespread in higher animals. The livers of all mammalian species tested including that of a monotreme contained inhibitor active against bovine pancreatic RNAase. Chick liver contained inhibitor active against "activated" chick liver supernatant RNAase, but not against bovine pancreatic RNAase. The rat liver inhibitor showed partial activity against chick liver supernatant RNAase.

Within one species, all activities measured (alkaline RNAase, acid RNAase, and inhibitor) were high in tissues with high levels of cell proliferation or protein synthesis. During aging, the inhibitor levels in three different tissues dropped, and the ratio of inhibitor to alkaline RNAase decreased. In the thymus of mice becoming leukaemic, inhibitor levels rose, and the ratio of inhibitor to alkaline RNAase increased.

The widespread distribution of the inhibitor and its response to changes in the metabolic state of the tissue in this and other work suggest that the system of a labile sensitive protein inhibitor restricting the activity of the stable alkaline RNAase of the cell supernatant might represent a control point in cytoplasmic RNA catabolism.

I. INTRODUCTION

A specific and sensitive protein inhibitor of alkaline RNAase, first clearly demonstrated by Roth, has been found in several tissues of a variety of mammalian species (Roth 1956; Shortman 1962*a*, 1962*b*; Imrie and Hutchinson 1965; Girija and Sreenivasan 1966). The inhibitor is found in the supernatant fraction of tissue homogenates, and is usually present in excess over alkaline RNAase, which therefore exists primarily as an inactive complex, referred to as latent RNAase (Roth 1956, 1963; Shortman 1961; Roth and Hurley 1966). The inhibitor may represent a control point in the regulation of RNA catabolism in the cell. However, an argument against such a fundamental role has been the apparent restriction of this inhibitor to mammalian species. A different RNAase inhibitor has been reported in *Bacillus subtilis* (Nishimura 1960; Smeaton and Elliott 1967) and inhibitory materials have been found in plant tissues (Bernheimer and Steele 1955), but no inhibitor was detected in *Escherichia coli* (Shortman, unpublished data), or in chick liver (Roth 1962). This paper reports the existence of an RNAase inhibitor in a marsupial, in a monotreme (the echidna), and in chick liver and suggests a wider distribution than

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previously believed. It also reports enzyme and inhibitor levels in various mammalian organs in both young and old as well as in leukaemic animals, to test for possible correlations between RNAase and inhibitor levels and the metabolic state of the tissue.

II. Materials and Methods

(a) *General Basis of the Assays*

Mammalian tissue homogenates display two distinct peaks of RNAase assay, at pH 5·8 and pH 7·8. These undoubtedly reflect the activities of several enzymes (de Lamirande and Allard 1959; Maver *et al.* 1959; Roth 1959; Reid and Nodes 1959; Beard and Razzell 1964; Vaes and Jacques 1965). These peaks are the basis of the "alkaline RNAase" and "acid RNAase" activities investigated in this study. Both RNAase activities are partially restricted by binding within subcellular granules, and alkaline RNAase is also restricted by the inhibitor (Roth 1956; Reid and Nodes 1959; de Duve and Beaufay 1959). The assay techniques were therefore designed to distinguish between the total or potential activity of a homogenate and the proportion actually free or expressed.

Assay methods were derived from those previously described (Shortman 1962*a*, 1962*b*). RNAase activity was measured by the release of acid alcohol-soluble, ultraviolet-absorbing oligonucleotides and nucleotides from repurified yeast RNA. Each activity was assayed under conditions of optimal pH, as well as conditions of RNA concentration and ionic strength which gave an optimal and linear response for pancreatic RNAase (Kraft 1967). An additional alkaline RNAase activity described by Rahman (1966) was suppressed under these conditions of pH and ionic strength. Activity was expressed relative to a standard amount of crystalline bovine pancreatic RNAase included in every experiment, to eliminate the effects of any day to day variation in conditions, and to provide a basis for comparison with results of other laboratories. Inhibitor activity was normally measured by the extent of inhibition of a standard amount of bovine pancreatic RNAase.

(b) *General Assay Procedure*

The following reagents were added to ice-cold, siliconed, glass reaction tubes and thoroughly mixed: buffer–salt solution as detailed below, 0·2 ml; additional reagents as detailed below, or water, 0·1 ml; a suitable dilution of tissue homogenate, pretreated as detailed below (or of standard bovine RNAase), 0·1 ml. The reaction was then initiated by addition of RNA [0·2 ml of a 1% solution of yeast RNA (Lights or Fluka) repurified as described previously (Shortman 1961) to remove proteins, low molecular weight material, and heavy metal ions]. The tubes were incubated at 37°C for 30 min. The reaction was stopped by transferring the tubes to an ice–water bath for 1 min, then precipitating undegraded RNA and protein with 0·6 ml of cold acidethanol (90% A.R. ethanol; 10% A.R. conc. HCl; 10% deionized water). The tubes were spun at 4000 *g* for 30 min at 4°C. A sample (0·5 ml) of the supernatant was diluted sixfold in water and the absorbance at 260 nm determined in a Hilger Uvispek spectrophotometer. Controls for RNAase assay consisted of zero time blanks for all samples, activity being measured as the increase in absorbance over the 30-min incubation, compared to the pancreatic RNAase standard. Controls for inhibitor assays included tubes with pancreatic RNAase alone, and tubes with homogenate added but pancreatic RNAase omitted to correct for any endogenous RNAase activity. The percentage inhibition of added pancreatic RNAase was then calculated, and inhibitor levels determined from a standard curve (Shortman 1961; Kraft 1967). All assays and controls were in duplicate.

(c) *Specific Assays*

(i) *Total Alkaline RNAase.*—This was measured after fully releasing activity from granules by freezing and thawing the homogenates six times. In addition, inhibitor was destroyed and the RNAase activated by including the sulphydryl reactant *p*-chloromercuribenzoate (*p*CMB, 0·1 ml, 0·01M, pH 8·0, Koch-Lights Laboratories) in the assay (Roth 1956; de Duve and Beaufay 1959; Shortman 1962*a*, 1962*b*). The buffer–salt solution for assay consisted of: Tris–HCl, pH 7·8, 0·05M; NaCl 0·24M; EDTA, pH 7·8, 0·05M.

(ii) *Free Alkaline RNAase Assay.*—This assay was an attempt to measure the actual RNAase activity in the cell cytoplasm. Care was taken not to disrupt granules and to protect inhibitor against inactivation. Fresh unfrozen homogenate was used. No additional reagents were added to the assay. The buffer–salt solution was as for total alkaline RNAase.

(iii) *Total Acid RNAase.*—The total acid RNAase was measured after releasing all enzyme from granules by freezing and thawing the homogenate six times. Inhibitor was not destroyed in order to keep alkaline RNAase (an enzyme with a broad pH optimum) suppressed. Acid RNAase is not affected by the inhibitor (Shortman 1962*a*, 1962*b*). No additional reagents were added to the assay. The buffer–salt solution consisted of: sodium acetate–acetic acid buffer, pH 5·8, 0·05M; NaCl 0·24M; EDTA, pH 5·8, 0·05M.

(iv) *Free Alkaline RNAase Inhibitor.*—This assay measured the excess inhibitor free to act on added pancreatic RNAase. In some assays chick liver supernatant RNAase was used in place of pancreatic RNAase. Fresh, unfrozen homogenate, diluted in 0·44M sucrose, 0·005M EDTA, was used. Bovine pancreatic RNAase (0·1 ml containing 0·005 μg), was included in the assay. The buffer–salt solution consisted of: Tris–HCl, pH 7·8, 0·05M; sucrose, 1·1M.

(v) *Units of Activity.*—One unit of RNAase activity was equivalent to the activity of 1 μg crystalline pancreatic RNAase, measured under the same conditions as free alkaline RNAase. One unit of inhibitor was the amount causing 50% inhibition of 2 μg crystalline pancreatic RNAase.

(vi) *Bovine Pancreatic RNAase.*—Pancreatic RNAase, grade 1A (Sigma), was dried over NaOH and H_2SO_4 in a vacuum dessicator, weighed, and made into a stock solution in 0·1% gelatine solution. Further dilutions were made in the same gelatine solution. The gelatine was prepared by dialysing a 0·5% solution against two changes of a solution containing 0·05M EDTA and 0·15M NaCl, and then against four changes of glass-distilled water.

(vii) *Tissue Homogenates.*—Tissues were removed into ice-cold saline. After drying and rapid weighing, 10 or 20% homogenates in 0·44M sucrose were prepared at 4°C, seven strokes of a motor-driven Teflon homogenizer being required. This was the minimum treatment to give >98% cell disruption as assessed by phase-contrast microscopy. Tissue supernatant fraction was prepared by centrifugation at 50,000 *g* for 1 hr.

(viii) *Protein Estimation.*—Protein determinations were performed according to the method of Lowry *et al.* (1951). Bovine serum albumin (Armour Pharmaceutical Co.), dissolved in 0·147M NaCl, was used as a primary standard.

(ix) *DNA Estimation.*—DNA was estimated according to the method of Burton (1956), slightly modified in that the optimum time of hydrolysis for the samples studied was found to be 8 instead of 17 min.

III. Results

In a small phylogenetic survey, the levels of free and total alkaline RNAase, total acid RNAase, and free or "excess" inhibitor were assayed in the liver homogenates of several species. The range of mammals tested included a marsupial and the monotreme echidna which may be considered a primitive mammal. Bovine pancreatic RNAase was the test enzyme of inhibitor activity. The results are given in Table 1.

RNAase activity was observed in all cases at both acid and alkaline pH. The livers of all mammals tested, including the possum and the echidna, had free inhibitor activity and most alkaline RNAase was in the inactive, latent state. However, both the toad and the chick displayed only marginal inhibitor activity, in agreement with the results of Roth (1962). In addition, in the liver homogenates of both these species, the bulk of the alkaline RNAase appeared to be in a free or uninhibited state.

The immediate conclusion that chick and toad liver lack inhibitor was questioned because it is known that mammalian and *Bacillus subtilis* inhibitors have restricted ranges of activities; the latter inhibitor is inactive against bovine pancreatic RNAase (Smeaton and Elliott 1967). The normal assay using bovine pancreatic enzyme may have failed to detect an inhibitor of different specificity. Roth (1962) tested chick liver

supernatant against chick liver mitochondrial RNAase and found no inhibitory activity. The mammalian inhibitor is found mainly in the supernatant fraction, however, and a supernatant RNAase would appear to be a more appropriate test enzyme. In fact, when chick liver supernatant was assayed, as opposed to the total homogenate, latent alkaline RNAase was detected, activated either by *p*CMB or by heating. This would suggest the presence of some substance with properties similar to the mammalian supernatant inhibitor. Accordingly, chick liver supernatant fraction was tested, not against bovine pancreatic RNAase, but against an "activated" RNAase derived from chick liver supernatant.

TABLE 1

LEVELS OF RIBONUCLEASE AND RIBONUCLEASE INHIBITOR IN LIVER HOMOGENATES OF DIFFERENT SPECIES

Species	No. of Samples	$10^3 \times$ Free Inhibitor Level* (units/mg homogenate protein)	No. of Samples	$10^3 \times$ RNAase Level (units/mg homogenate protein)		
				Free Alkaline RNAase	Total Alkaline RNAase	Total Acid RNAase
Rat	8	40	6	0·025	3·3	1·7
Mouse	10	63	4	0·002	4·2	4·0
Possum (*Trichosurus vulpecula*)†	1	11	1	n.d.‡	1·3	1·3
Echidna (*Tachyglossus aculeatus*)†	1	38	1	<0·002	2·0	4·4
Toad (*Bufo marinus*)	2	0·3	2	1·0	1·5	5·9
Chicken	2	0·9	2	2·6	2·2	3·6

* Activity measured against bovine pancreatic RNAase.

† Samples from these animals were frozen once prior to free inhibitor and free alkaline RNAase determinations.

‡ n.d., not determined.

An enriched but still crude chick supernatant RNAase preparation was made by: (1) centrifugation of chick liver homogenate in 0·44M sucrose for 1 hr at 80,000 *g*; (2) maintaining the supernatant at 80°C for 5 min at pH 4·5 to inactivate any substance similar to mammalian RNAase inhibitor; (3) bringing the preparation to pH 7·8. This preparation contained denatured protein but was usually assayed in this form. Removal of the denatured material by centrifugation removed a variable proportion of the RNAase activity. In several experiments the RNAase activity in the clarified soluble fraction showed the same characteristics as the sediment.

The inhibitory activity of the original chick liver supernatant was then tested against this preparation and against a similar activity of bovine pancreatic RNAase [Fig. 1(*a*)]. Several other experiments gave the same results. The activity of rat liver supernatant on the two enzyme preparations is shown in Figure 1(*b*) for comparison. Clearly, chick liver supernatant contained a factor inhibitory to the activated chick liver RNAase, but had only little effect on the activity of the mammalian enzyme. The rat supernatant inhibitor was very effective against an alkaline RNAase from another mammalian species, but inhibited the chick enzyme to a much lesser extent.

Inhibition of chick liver supernatant RNAase by chick liver supernatant inhibitor gave an apparent plateau around 40% inhibition in several experiments [e.g. Fig. 1(*a*)]. This might indicate the presence of several RNAases, some unaffected by the inhibitor, although in the original liver supernatant all the RNAase activity was latent, or suppressed. Alternatively, the heat–acid treatment may have destroyed the potential of some RNAase molecules to react with the inhibitor without destroying enzyme activity. Finally, the curve may simply reflect the different kinetics of interaction of enzyme and inhibitor, since mammalian inhibitor acting on mammalian RNAase also gives what might be considered a "plateau" in the 90–99% inhibition range (Shortman 1961).

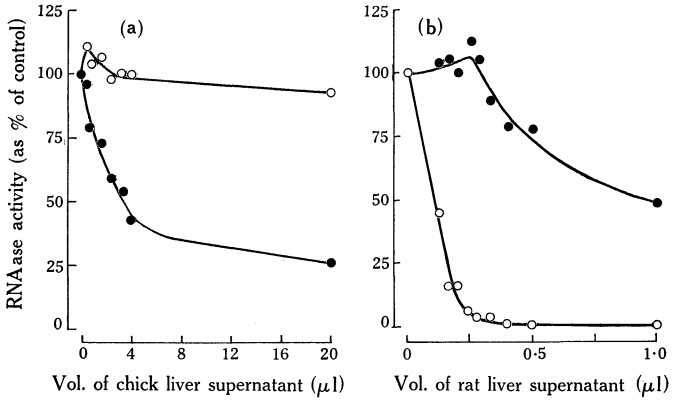

Fig. 1.—Inhibitory effect of chick liver supernatant (*a*) and rat liver supernatant (*b*) on crystalline bovine pancreatic RNAase (○) and crude "activated" chick liver supernatant RNAase (●). The activities of the two enzymes per assay tube were 0·0005 and 0·0008 units respectively in (*a*) and 0·002 and 0·0008 units respectively in (*b*), one unit being the same activity as that of 1 μg of crystalline bovine pancreatic RNAase, under the conditions of assay.

Comparable experiments were attempted on toad liver but it was not found possible to get a supernatant preparation containing alkaline RNAase which did not also contain large amounts of acid RNAase. Hence any effects were largely swamped by the presence of the latter enzyme.

The conclusion from the foregoing was that an alkaline RNAase inhibitor is probably widespread in the animal kingdom, although its specificities may vary. To provide further guidance to its role, different organs of a particular species were assayed, in the hope that some correlation could be obtained between the function or metabolic activities of tissues and the various enzyme activities. There have been other reports on the levels of RNAases in organs of the rat, but most of this earlier work was subject to two limitations: (1) the existence of the inhibitor and its extreme lability, especially to contaminants in commercial RNA (Shortman 1961), was not recognized; (2) the effect of both inhibitor binding and binding within subcellular granules was not considered, so no distinction could be made between "free" and "latent" RNAase activity.

The results of enzyme and inhibitor assays on various rat and mouse organs are given in Table 2. All organs had significant total acid and alkaline RNAase activities. In all organs a comparatively high level of free or "excess" inhibitor was measured. Most of the tissue alkaline RNAase was latent or inactive although a small but definite amount of free alkaline RNAase was always detected. In part this could have been due to the presence of different alkaline RNAases not affected by the inhibitor. The presence of a small amount of free alkaline RNAase activity is, however, consistent with the known kinetics of the interaction of the inhibitor with bovine

TABLE 2

INHIBITOR AND RIBONUCLEASE LEVELS IN TISSUES

Tissue	DNA/Protein Ratio	$10^3 \times$ Activity (units/mg homogenate protein) $\pm$ S.D.			
		Free Inhibitor	Free Alkaline RNAase	Total Alkaline RNAase	Total Acid RNAase
Rat*					
Liver	0·0098	40	0·025±0·009	3·3±1·0	1·7±0·4
Thymus	0·184	66	0·029±0·013	3·3±1·1	2·7±0·5
Spleen	0·092	29	0·13±0·09	6·6±1·8	5·0±0·3
Lymph nodes	0·192	48	n.d.‡	5·2±1·2	5·7±1·6
Heart	0·008	16	<0·0025	0·6±0·1	0·18±0·06
Brain	0·086	17	<0·0025	1·2±1·7	2·0±0·1
Mouse†					
Liver	0·018	63	0·02±0·03	4·2±2·6	4·0±0·2
Thymus	0·263	22	0·92±0·66	5·7±1·8	10·3±3·9

* Male Wistar rats 9 weeks old were used. The values for rat tissues are means of four or six determinations, except for thymus free inhibitor which represents 26 assays. No differences were detected in the levels between male and female rats.

† Mice used were 6-weeks-old females of the C3H strain. The numbers of determinations for each tissue were as follows: mouse thymus, free inhibitor assays 50, all other assays 20; mouse liver, free inhibitor assays 20, all other assays 4.

‡ n.d., not determined.

pancreatic RNAase, where an extremely large excess of inhibitor is needed for complete suppression of activity (Shortman 1961). The organs most active in cell division or protein production (thymus, spleen, and liver) had high RNAase and inhibitor activities as compared to heart and brain tissues. Values for mouse thymus and liver are given as an example of interspecies variation; mouse thymus had a very different inhibitor : alkaline RNAase ratio from rat thymus tissue although the liver ratios were similar. Table 3 indicates that most of the inhibitor and some proportion of all RNAase activities was present in the supernatant fraction in nearly all cases.

In order to see if levels in a given tissue would respond to changes in metabolic state, the effects of aging were investigated as shown in Table 4. Inhibitor levels decreased with age in rat liver, thymus, and lymph nodes as well as in C3H mouse thymus (Table 5). In contrast RNAase levels were higher in the lymphoid organs of older animals. The result in all cases was a change in the balance between alkaline RNAase and the inhibitor.

A second and more dramatic change in the state of a tissue is the development of spontaneous leukaemia in AKR mice. The disease is not apparent in young mice, but older animals show excessive and eventually fatal growth of lymphoid tissue. The rapidly growing leukaemic thymus has a much higher proportion of actively dividing

TABLE 3

SUPERNATANT INHIBITOR AND RIBONUCLEASE ACTIVITIES IN RAT TISSUE SUPERNATANTS

Tissue	Inhibitor Activity		Total Alkaline RNAase Activity		Total Acid RNAase Activity	
	(units/mg supernatant protein)	(% of total homogenate activity)	(units/mg supernatant protein)	(% of total homogenate activity)	(units/mg supernatant protein)	(% of total homogenate activity)
Liver	150	115	4·3	62	0·3	12
Heart	42	87	2·3	100	0·3	56
Thymus	240	91	5·0	30	2·0	18
Spleen	92	115	9·8	51	4·9	29

TABLE 4

EFFECTS OF AGE ON INHIBITOR AND RIBONUCLEASE ACTIVITIES IN RAT TISSUES

Age differences in the following activities were found to be significant at the 1% level according to Student's *t*-test: liver and thymus free inhibitor, thymus total acid RNAase, and lymph node total alkaline RNAase. The age differences in thymus total alkaline RNAase and lymph node total alkaline RNAase were significant at the 5% level

Tissue	Age (weeks)	No. of Samples	$10^3 \times$ Activity (units/mg homogenate protein)			DNA/Protein Ratio
			Free Inhibitor	Total Alkaline RNAase	Total Acid RNAase	
Liver	9	8	40	3·3	1·7	0·0095
	52	6	17	2·4	1·5	0·0098
Thymus	9	4	66	3·3	2·7	0·264
	52	5	23	5·8	6·5	0·173
Lymph nodes	9	6	48	10	9·3	0·192
	36	7	32	15	14	0·239

large and medium lymphocytes than the non-leukaemic thymus (Metcalf and Wiadrowski 1966). The changes observed in inhibitor and RNAase levels with age and associated leukaemia development are compared with the non-leukaemic C3H mice in Table 5.

There was a clear increase in inhibitor levels as AKR mice aged and became leukaemic, in contrast to the decrease with age seen with normal C3H mice. Total alkaline RNAase tended to decrease in the leukaemic animal but increase with age in

the normal mouse. The DNA levels indicate that, on a per cell basis, alkaline RNAase showed little change but the inhibitor levels showed a sixfold increase. On both the DNA and the protein basis there was a marked change in the balance of inhibitor to alkaline RNAase with the development of leukaemia, in the direction opposite to that found with aging.

TABLE 5

LEVELS OF INHIBITOR AND RIBONUCLEASE ACTIVITIES IN NORMAL AND LEUKAEMIC MOUSE THYMUS TISSUE

Number of animals of each type used given in parenthesis

Mouse Strain, Condition, and Number	Age (months)	$10^3 \times$ Activity (units/mg homogenate protein)			DNA/Protein Ratio
		Free Inhibitor	Total Alkaline RNAase	Total Acid RNAase	
AKR					
Normal (4)	3	20	7·0	13·8	0·24
Leukaemic (3)	7–9	46	4·6	13·2	0·090
C3H					
Normal (4)	3	24	8·7	13·2	0·26
Leukaemic (8)	7–9	18	10·9	14·9	0·32

IV. DISCUSSION

The finding of an RNAase inhibitor in chick liver, in contrast to previous indications (Roth 1962), suggests that a failure to detect inhibitor in a particular species using bovine pancreatic RNAase or some other arbitrary test enzyme may simply reflect inhibitor specificity for the appropriate supernatant alkaline RNAase. The lability of the inhibitor is another factor making detection difficult. The wide range of higher animals in which inhibitor was demonstrated in this study indicates that the system of a labile protein which is sensitive to sulphydryl reagent and which limits the activity of a stable alkaline RNAase in the cell sap is of widespread occurrence. It is of interest that in all tissues and species tested, inhibitor was in considerable excess, although, in accordance with the kinetics of interaction of inhibitor and enzyme (Shortman 1961), a small and possibly regulated enzyme activity was still detected. The normal state of tissue supernatant alkaline RNAase is thus as a largely inactive complex with the inhibitor, a complex which can in fact be isolated from tissues (Roth 1963). This complex of a stable component containing the active site and a labile, regulating component which is sensitive to sulphydryl reactant has many analogies with the allosteric enzyme systems with control functions in cellular metabolism (Stadtman 1966), and part of this paper explores the possibility for a controlling role for this system.

The results on the levels in different organs on the one animal lead only to the conclusion that in tissues where RNA levels and RNA metabolism are high, acid RNAase, alkaline RNAase, and inhibitor activity are all likely to be high. Since the active tissues contain both actively growing cells as well as phagocytic elements active in macromolecular degradation, studies on individual cell types are needed to extend this approach. These will be the subject of a subsequent paper.

The changes in organ levels with aging and in leukaemic thymus have created the impression that a low ratio of inhibitor to alkaline RNAase is associated with a change towards catabolic activity and a high ratio is associated with increased anabolism. There is strong support for this generalization in a number of other situations. Increased inhibitor levels have been recorded in regenerating rat liver (Shortman 1962), in mouse thymus regenerating after steroid-induced involution (Kraft 1967), and in rat adrenals stimulated with ACTH (Imrie and Hutchinson 1965). A decrease in inhibitor levels associated with increased catabolism or a drop in the rate of cellular proliferation has been found in the liver of starving rats (Girija and Sreenivasan 1966), in involuting mouse thymus after X-irradiation (Kraft, Shortman, and Jamieson 1969), or administration of [9-D]fluoroprednisolone (Wiernick and McLeod 1965). Data extending this generalization to the cellular level will be the subject of a later paper.

A response of the inhibitor to the metabolic state of the tissue seems to be a general phenomenon. This suggests that the inhibitor acts as a control point, the inhibitor–RNAase balance acting as one of several factors which determine cytoplasmic RNA levels. This control would be at the level of RNA breakdown, in contrast to most controls at the level of RNA synthesis. The evidence is, of course, purely circumstantial. There is no proof, for example, that cellular RNA catabolism is, in fact, due to RNAase action, rather than that of polynucleotide phosphorylase or some less specific phosphodiesterase. In addition, questions of enzyme specificity for different RNA species have been ignored. However, the data does justify a much closer study of the inhibitor and its interaction with alkaline RNAase.

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