peared within seconds and grew for a number of minutes. There were 17.3 ± 1.2 bubbles in the lower 3 mm of gel in each glass chamber. Bubbles within 1 mm of the surface were not counted because they were smaller, more numerous, and difficult to see through the meniscus. The system was allowed to stabilize overnight. The next morning the chamber was flushed with helium for several minutes without altering ambient pressure. Photographs of bubbles were taken serially before and after flushing.

Before the pressure chamber was flushed with helium, there was no change in bubble size. Soon after it was flushed all the bubbles grew, and subsequently stopped growing, as indicated for two bubbles in Fig. 1. The change in bubble volume appeared to depend on the initial bubble volume and the bubble's distance from the gel surface and from other bubbles. The growth of five bubbles in three separate experiments was measured with similar results. These data suggest that switching from breathing nitrogen-oxygen to breathing helium-oxygen during the treatment of decompression sickness would make the disease worse rather than better. This conclusion is also supported by studies of the change in size of subcutaneous pockets containing various gases in rats (*5*). Other related experiments in animals have involved venous air embolism (*6*), pneumothorax (*7*), and bowel obstruction (*7*).

The converse experiment was performed, in which the initial saturation was by helium and the chamber atmosphere was subsequently changed to nitrogen. Bubbles near the surface became smaller and those deep in the gel disappeared. The histories of two bubbles are shown in Fig. 2. A total of four bubbles were measured in three experiments with similar results. These data support work by Keller and Buhlmann (*8*), in which the decompression time in humans was shortened by switching from helium-oxygen to nitrogen-oxygen breathing during decompression.

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Pancreatic Ribonuclease: Enzymic and Physiological Properties of a Cross-Linked Dimer

Abstract. *Monomeric ribonuclease A has very low activity toward typically double-stranded RNA's; the dimeric form of ribonuclease A obtained by cross linking the enzyme by dimethyl suberimidate has more than 78 times the activity of the monomer toward polyadenylate · polyuridylate and 440 times the activity of the monomer toward the double-stranded RNA of a virus from* Penicillium chrysogenum. *The half-life of the dimer in the bloodstream of the rat is 12 times that of the monomer.*

Interest in the properties of dimeric ribonucleases has been stimulated by the finding of D'Alessio and Leone and their colleagues (*1*) that the ribonuclease of bovine seminal plasma is a dimer. The seminal enzyme is similar in some of its properties to the more readily dissociable dimer that is formed (*2*) when solutions of pancreatic ribonuclease A in 50 percent acetic acid are lyophilized. A special property (*3*) of such dimeric structures, with two active sites, is the ability to hydrolyze double-stranded RNA's at an appreciable rate under conditions of salt concentration where the action of the pancreatic monomer is minimal. Since there is increased interest in the roles of double-stranded RNA's in animal cells (*4*, *5*) and in bacteria (*6*), we have studied the enzymic properties of a cross-linked dimeric enzyme that is readily prepared from bovine pancreatic ribonuclease A.

The cross linking, through specific reaction with NH_2 groups of ribonuclease A, has been accomplished with the bifunctional reagent dimethyl suberimidate $[CH_3OC(=NH)(CH_2)_6C(=NH)OCH_3]$ under conditions similar to those described by Hartman and Wold (*7*) for cross linking of ribonuclease A by adipimidate. To a stirred solution of 100 mg of ribonuclease A (Worthington, RAF grade) in 10 ml of 0.1*M* sodium phosphate, *p*H 10 at 25°C, 10 mg of dimethyl suberimidate dihydrochloride (Pierce Chemical) was added in 2-mg portions at 5-minute intervals; the *p*H was maintained at 10 by additions of 0.1*N* NaOH. After 1 hour, the reaction was stopped by the addition of 0.5 ml of 0.2*M* ammonium acetate. The dimeric fraction was isolated by gel filtration on a column (1.5 by 80 cm) of Sephadex G-100 with 0.9 percent NaCl as eluent [figure 3 in (*7*)]. The yield of dimer (eluted between 75 and 100 ml) was about 50 mg. The solution was frozen for storage.

A molecular weight of about 27,000 for the dimeric fraction was obtained by sodium dodecyl sulfate gel electrophoresis (*8*). The number of free NH_2 groups remaining in the dimer was measured photometrically by the picryl sulfonic acid method of Satake (*9*). Ribonuclease A (*10*) has one alpha NH_2 group and ten epsilon NH_2 groups of lysine residues; the photometric method gave 11 groups for the monomer; the cross-linked dimer contained 19 free NH_2 groups, which indicated that the suberimidate had imidylated three NH_2 groups in the dimeric structure. This result is comparable to that obtained by Hartman and Wold (*7*) with dimethyl adipimidate; they found that two NH_2 groups reacted with the bifunctional reagent to form a bridge between the two monomers and that two additional NH_2 groups reacted monofunctionally with the reagent. Their dimer had 135 percent of the activity of the monomer toward 2′,3′-cyclic cytidylate.

The enzymic activities of the present dimer toward a variety of polynucleotides have been measured by the procedure of Anfinsen *et al.* (*11*). The *p*H-activity curve was first established for the dimer against yeast RNA and found to be the same as that of the

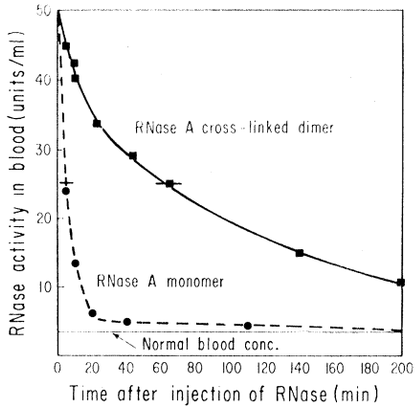

Fig. 1. Comparison of the half-life of ribonuclease A (RNase A) and the cross-linked dimer in the blood. At time zero, 750 units of ribonuclease activity in 1 ml of physiological saline were injected into the tail vein of the rat. At the indicated times, 0.1-ml samples of blood were drawn and added to 1 ml of H_2O; 0.1 ml was taken for assay against yeast RNA.

monomer. Substrate saturation curves showed that the two enzymes have the same Michaelis constant (K_m) for yeast RNA. The dimer is inhibited by the natural inhibitor of ribonuclease that is present in liver (*12*), but it takes about twice as much of the inhibitor per milligram of enzyme to affect the dimer to the same extent as the monomer.

For the primarily single-stranded RNA's listed in Table 1, assayed in 0.1*M* salt at 37°C, the activity per microgram of the dimer is in most instances about twice that of the monomer; from the data in the table, the specificity of the dimer for pyrimidine-containing nucleotides is similar to that of the monomer. In addition, with polyadenylate · polyuridylate [poly(A) · poly(U)] the products of the hydrolysis were examined by paper electrophoresis in 0.05*M* ammonium formate, *p*H 3.5 (*13*), 40 volt/cm; only uridylic acid was formed.

Michelson *et al.* (*14*) have emphasized the complexity of the secondary structures of synthetic polynucleotides; the higher activity of the dimer may reflect a greater ability to hydrolyze structured segments, since with typically doubled-stranded RNA's (the last three substrates in Table 1) the ratio of the activity of the dimer to that of the monomer rises to 36, > 78, and 440. The cross-linked dimer is far more effective in this respect than the dissociable dimer formed by simple aggregation (*2*) which hydrolyzes poly(A) · poly(U) only four times as fast as does the monomer (*3*).

The ready availability of a dimer with increased activity toward double-stranded RNA's has prompted us to study its half-life in the blood after intravenous injection in the rat. The effects of ribonuclease A monomer on cellular metabolism have been demonstrated in studies on the ability of the enzyme to penetrate intact cells (*15*), to inhibit protein synthesis in some instances (*16*), and to interfere with viral multiplication (*17*). However, the monomer, with a molecular weight of 13,683, has a half-life in the blood of only about 5 minutes, and a major proportion of the injected dose is excreted in the urine (*18*). With the thought that the dimer, with a molecular weight of about 27,000, should experience appreciably greater retention, the comparison shown in Fig. 1 was made. The dimer has a half-life of about 60 minutes; 1 day after injection, the ribonuclease activity in the blood is still twice the normal value. The enzyme is acting in vivo, as evidenced by a four-fold increase in the 260-nm absorbance of perchloric acid–precipitated blood 1 hour after the injection. The monomer is known to be extracted from the bloodstream by the liver (*18*). The dimer is similarly subject to endocytosis; after a single injection of 300 μg of the dimer the accumulation of maximal neutral ribonuclease activity in the liver [after inactivation of the ribonuclease inhibitor of the liver by *p*-hydroxymercuribenzoate (*12*)] was at 24 hours. The peak activity was 2.5 times the control value.

The cross-linked dimer of ribonuclease A thus has physiological properties that merit further examination.

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Table 1. Comparative tests on the acid-soluble nucleotides produced by the monomer and the cross-linked dimer of ribonuclease A with different substrates. The enzyme (0.01 μg) was incubated for 10 minutes at 37°C in 0.5 ml of 0.1*M* tris buffer at *p*H 7.5 in the presence of 1 mg of the different nucleic acids and 2 mg of bovine serum albumin. After addition of 5 ml of 10 percent perchloric acid, 0.125 percent in uranyl acetate, the soluble nucleotides were measured at 260 nm (*11*). For the purpose of comparison of the activities of the two enzymes, the approximate number of micromoles of nucleotides liberated per minute per microgram of enzyme were estimated using an extinction coefficient at 260 nm of 8500. The response with yeast RNA is linear for more than 30 minutes. Abbreviations: A, adenylate; G, guanylate; C, cytidylate; U, uridylate; I, inosinate; and RNase A, ribonuclease A.

Substrate	Activity		
	RNase A	Dimer	Dimer/monomer
Yeast RNA*	2.58	4.98	1.9
Poly(A)†	< 0.05	< 0.05	
Poly(G)	0.06	< 0.05	
Poly(I)	0.13	0.38	2.9
Poly(U)	1.16	2.97	2.6
Poly(C)	4.05	8.15	2.0
Copoly(A • C • U)	8.3	8.1	1.0
Copoly(A • C • G)	1.94	3.23	1.7
Copoly(C • U)	2.52	5.14	2.0
tRNA‡	1.68	4.50	2.7
Poly(I) • poly(C)	0.02	0.72	36
Poly(A) • poly(U)	< 0.01	0.78	> 78
RNA from a virus of *Penicillium chrysogenum*§	0.002	0.88	440

* Yeast RNA (Sigma type VI), was thoroughly dialyzed (*18*). † Synthetic polynucleotides were from Miles Laboratories. ‡ Calf liver tRNA (R7250) was from Sigma. § The double-stranded viral RNA was a gift from H. D. Robertson (*6*).

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