

### Studies on the structural basis of ribonuclease activity

Since the fundamental studies of SUMNER and of NORTHROP AND KUNITZ on the protein nature of enzymes, most investigators in the field have tacitly assumed the relative inviolability of enzyme structure, both as regards the covalent linkages between amino acids in the peptide chains and also with relation to geometrical configurations imposed by non-covalent binding forces. Teleological reasoning has led to theories in which the protein structure as a whole was assumed to play a dominant role in catalytic action by transferring energy to the catalytic center through a resonance chain made up of regular hydrogen bonded structures<sup>1,2,3</sup>.

It has, however, been known for some time that certain groupings (*e.g.* epsilon-amino groups on the lysine residues of pepsin<sup>4</sup>) could be shown to be non-essential in catalytic activity and in more recent years it has been possible to modify, more drastically, certain biologically active proteins without apparent loss in activity. Thus, insulin<sup>5,6</sup>, ACTH<sup>7</sup>, TMV<sup>8</sup>,  $\alpha$ -chymotrypsin<sup>9</sup>, and ribonuclease<sup>10</sup> have been found to exhibit their normal behavior even after removal with carboxypeptidase of one or more amino acid residues from the carboxyl-terminal ends of the peptide chains. In the case of the first three listed, such experiments are partly equivocal since activity tests (which might involve resynthesis) must necessarily be performed upon actively metabolizing tissues or upon intact organisms. The studies on  $\alpha$ -chymotrypsin and ribonuclease, however, were performed using *in vitro* methods and are therefore of particular interest since they clearly suggest that the painstaking evolution of a unique structure for these enzymes (and by inference of other enzymes as well) may have been directed towards the fulfillment of more subtle biological requirements than catalytic activity alone.

Previous studies have shown that a number of enzymes (*e.g.* trypsin<sup>11</sup>, subtilisin<sup>12</sup>, pepsin<sup>13</sup>) maintain activity even when tested in strong urea solutions where, according to considerations by KAUZMANN<sup>14</sup> and SCHELLMAN<sup>15</sup>, secondary hydrogen-bonded structures are likely to be seriously distorted. However, the absence of adequate physical and chemical data on these proteins has precluded a proper appraisal of this rather surprising phenomenon.

Recent investigations on ribonuclease structure<sup>16</sup> have suggested that this molecule exists in solution as a monodispersed<sup>17</sup> unit consisting of a single peptide chain of 128 amino acids<sup>18</sup>, cross-linked through four disulfide bonds. It has been found that restricted subtilisin digestion leads to a modified, fully active derivative<sup>19</sup> in which a new N-terminal end group can be demonstrated, the peptide chain which it terminates being attached to the main body of the molecule by disulfide bonding<sup>20</sup>. Restricted pepsin digestion, on the other hand, appears to completely inactivate the enzyme, even after the cleavage of one or two peptide bonds<sup>21</sup>. Complete loss of activity is also produced upon rupture of the disulfide linkages with performic acid<sup>16</sup>.

A series of experiments have now been conducted in this laboratory with the purpose of characterizing this enzyme under different conditions. They include (1) measurements of optical rotation and its dispersion (unpublished material, SCHELLMAN, compare<sup>22</sup>), (2) measurement of viscosity (unpublished data of HARRINGTON), (3) determinations of the rate of exchange of peptide bond hydrogen atoms (<sup>23,24</sup>, and unpublished data by AA. HVIDT), and (4) measurements of enzymic activity against RNA and uridine-2',3'-phosphate.

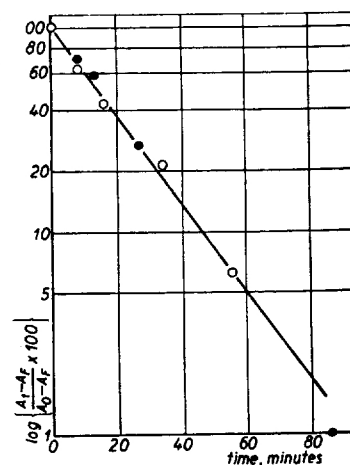
The results are summarized in Table I where a comparison is made between native RNase, RNase in 8 M urea or 2.5 M guanidine chloride, and oxidized RNase.

TABLE I

	Native	8 M urea	Oxidized
Exchangeable peptide-bonded hydrogens (theoretical-127)	70 rapidly exchangeable	All rapid*	All rapid
Intrinsic viscosity in (g/100 ml) <sup>-1</sup>	0.033	0.089	0.116
$[\alpha]_D^{20}$	74.0	108	91.1
$\lambda_C$	2330	2170	2220

\* Measurements made in 2.5 M guanidine chloride.

Fig. 1. First order plot of change in viscosity of RNA with time during RNase action in presence and absence of 8 M urea. RNA (2% in 8 M urea, pH 5.0, 20° C) + RNase (2.6  $\gamma$ /ml 8 M urea, pH 5.0): O. Same without urea ●.  $A_0$  = initial viscosity;  $A_f$  = final viscosity.



All the data indicate that the enzyme molecule denatured by urea or guanidine chloride approaches in shape and disorientation the molecule produced by performic acid oxidation.

The activity of RNase toward RNA (Schwarz, dialyzed against 0.2 M acetate buffer, pH 5.02) was determined by measuring the change in viscosity with time. The results shown in Fig. 1 indicate that ribonuclease is fully active in 8 M urea solution (the enzyme having been incubated in 8 M urea for two days at 5° C prior to testing against RNA, also dissolved in 8 M urea). Determinations of non-precipitable<sup>16</sup> and dialyzable  $e_{260}$  absorbing material during the reaction and after maximum viscosity change also indicated that the hydrolysis of the substrate had proceeded unimpaired.

The enzyme was further tested against the synthetic substrate, uridine-2',3'-phosphate<sup>25</sup> in the presence and absence of urea as above. The course of the reaction in this case was followed by a microspectrophotometric method developed by Dr. FRED M. RICHARDS. The hydrolysis of this substrate appears to be somewhat slower in urea than in its absence although an essentially linear production of uridylic acid with time was demonstrable by means of paper chromatographic analysis<sup>26</sup> of the reaction mixture.

The above data, considered together, suggest that only a relatively small part of the ribonuclease molecule is directly involved in catalytic activity and, that in the conversion from the native to the extended form, this part, the active center, may be protected from deleterious unfolding by restricting cross linkages. It seems impossible, at any rate, that an ordered secondary structure is responsible for its properties as a catalyst. The data further support the possibility that a considerable part of the enzyme structure may be superfluous from the catalytic standpoint, a possibility that is also suggested by the autodigestion experiments on pepsin previously published by PERLMANN<sup>27</sup>.

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