# THE ROLE OF METAL IONS IN THE γ-RADIOLYSIS OF DRY METAL-RIBONUCLEASE COMPLEXES

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Certain metal ions, when present in the solid enzyme, have been found to exert a protective effect on the inactivation of dry trypsin by ionizing radiation (Butler and Robins, 1963). These authors suggested that metal ions may affect the initial act of energy deposition and so diminish the amount of energy absorbed by the enzyme or by its active site. An alternative explanation appeared to be that metal ions protect because of their ability to act as scavengers of electrons and/or of hydrogen atoms (Riesz and White, 1965). To investigate this possibility, the reduction of cupric ions during the γ-radiolysis of $Cu^{2+}$-ribonuclease complexes was studied as a function of dose and of $Cu^{2+}$/ribonuclease ratio and compared with the survival of enzymatic activity. The results of experiments at room temperature and at $77^{o}$ K are consistent with the second of these alternatives.

## METHODS

Metal-ribonuclease complexes were prepared from aqueous solutions of bovine pancreatic ribonuclease (Sigma III A, deionized on a mixed bed column) at a concentration of 10 mg/ml. After adjusting the pH by addition of HCl solutions, the appropriate amounts of metal chloride were added in 1.0 ml of water to give the compositions and pH's shown in Table I. Mixtures were then freeze-dried.

Irradiations were performed in sealed pyrex vessels, evacuated for 2 hours at $10^{-3}$ torr. Experiments at $77^{o}$ K were also carried out in helium atmospheres at 50 torr to check control of sample temperature (Carter, Nelson and Augenstein, 1965). Samples were irradiated in a $Co^{60}$ source with dose rates of about 10 Mrads/day at room temperature and about 5 Mrads/day at $77^{o}$ K. Dose rates were determined by ferrous sulfate dosimetry. Enzymatic assays were carried out with cytidine 2':3'-phosphate as substrate (Crook, Mathias and Rabin, 1960). The dry irradiated samples were exposed to air for 1 hour before assay. ESR control experiments have shown that this exposure does not lead to any oxidation of $Cu^{+}$. Excess sodium ethylenediaminetetraacetate was added to the tris buffer and the activities of unirradiated metal-ribonuclease complexes and of native ribonuclease were found to be identical. Cuprous ion in irradiated copper-ribonuclease complexes was determined by dissolving them in air-free solutions of 2-2' biquinoline (50 mg/200 ml) in 50% acetic acid water (Felsenfeld, 1960). The molar optical density of the cuprous-biquinoline complex was 6800 at 540 mμ.

ESR measurements were carried out using a Varian V-4500 spectrometer with a 6 inch magnet which was regulated and scanned by "Fielddial".

## RESULTS AND DISCUSSION

Table I shows the effect of various metal ions, of pH prior to lyophilization, and of the $Cu^{2+}$/ribonuclease ratio on the mean lethal dose (the dose required to reduce enzymatic activity to 37% of its original value). The relative order of effectiveness is $Cu^{2+} > Ni^{2+} > Co^{2+} > Mn^{2+} \sim Zn^{2+} > Ca^{2+} \sim$ native ribonuclease. The maximum protective effect of cupric ions is reached when there are approximately two $Cu^{2+}$ ions per molecule of ribonuclease and falls off below this ratio. No significant change in protective effect occurs when the pH before lyophilization is varied from 1.6 to 5. Precomplexing $Cu^{2+}$ with ethylenediaminetetraacetate (1:1) removes most of the protective effect. A similar observation was reported by Butler and Robins for the case

of trypsin (Butler and Robins, 1963).

TABLE I

PROTECTIVE EFFECT OF METAL IONS AT 295°K

| Additive | $[M^{++}/RNase]$ | pH before Lyophilization | $D_{37}$ (Mrads) |
|---|---|---|---|
| None | | 5.30 | 36 ± 2 |
| $CaCl_2$ | 4.25 | 5.35 | 39 ± 2 |
| $ZnCl_2$ | 4.25 | 5.35 | 52 ± 5 |
| $MnCl_2$ | 4.20 | 5.33 | 52 ± 5 |
| $CoCl_2$ | 4.05 | 5.31 | 69 ± 4 |
| $NiCl_2$ | 3.94 | 5.23 | 76 ± 2 |
| $CuCl_2$ | 4.01 | 4.80 | 86 ± 4 |
| $CuCl_2$ | 4.05 | 1.60 | 76 ± 2 |
| $CuCl_2$ | 4.33 | 3.30 | 76 ± 2 |
| $CuCl_2$ | 1.96 | 3.30 | 75 ± 2 |
| $CuCl_2$ | 2.02 | 5.10 | 80 ± 4 |
| $CuCl_2$ | 0.99 | 3.00 | 55 ± 3 |
| $CuCl_2$ | 0.53 | 3.20 | 47 ± 3 |
| $CuCl_2$, EDTA (1:1) | 1.93 | 4.10 | 48 ± 2 |

Some qualitative ESR studies on $Cu^{2+}$-ribonuclease (4:1) irradiated in vacuo at 10 and 20 Mrads indicated a very marked decrease in the absorption due to cupric ions compared to the unirradiated sample.

The amount of cuprous ion formed on irradiation of $Cu^{2+}$-ribonuclease was more conveniently determined by the $Cu^{+}$-biquinoline method (Felsenfeld 1960). Figure 1 shows the percent survival of cupric ions for different $Cu^{2+}$-ribonuclease complexes as a function of dose at 22° C. The survival of enzymatic activity during irradiation of the same complexes is presented in Figure 2. Both enzymatic activity and remaining cupric ion follow an exponential survival curve except when the ratio of $Cu^{2+}$/ribonuclease is 1/2. In this case, marked deviations from exponential survival occur above 40 Mrads, indicating that cupric ions are less efficiently reduced when there is only 1 $Cu^{2+}$ for about 10 molecules of ribonuclease. The results of Figure 2 suggest that there is a correlation between this decreased efficiency of cupric

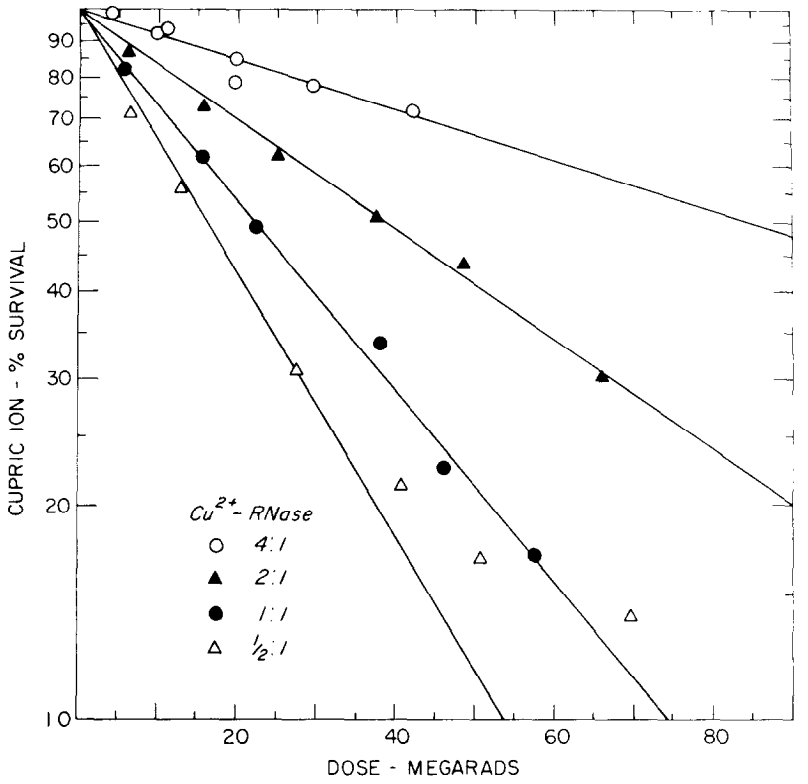


Figure 1. Survival of cupric ions at various $Cu^{2+}$/ribonuclease ratios. γ-Radiolysis at 295° K.

ion reduction and a decreased protective effect by cupric ions. Such a correlation would be fortuitous if the protective effect of metal ions were to be explained in terms of the ability of certain metal ions to quench the ultraviolet initiated emission of proteins at 77° K (Kuntz, Robins and Augenstein, 1965).

The $D_{37}$ dose, obtained from Figures 1 and 2, is the dose which would be required to inactivate all of the ribonuclease molecules or reduce all of the cupric ions, if these processes had continued at their initial rates. One can therefore calculate the initial G values (number of molecules involved in the specified process per 100 electron volts absorbed by the protein) for the loss of enzymatic activity and for cupric ion reduction. Table II summarizes the initial G values for experiments at 295 and 77° K.

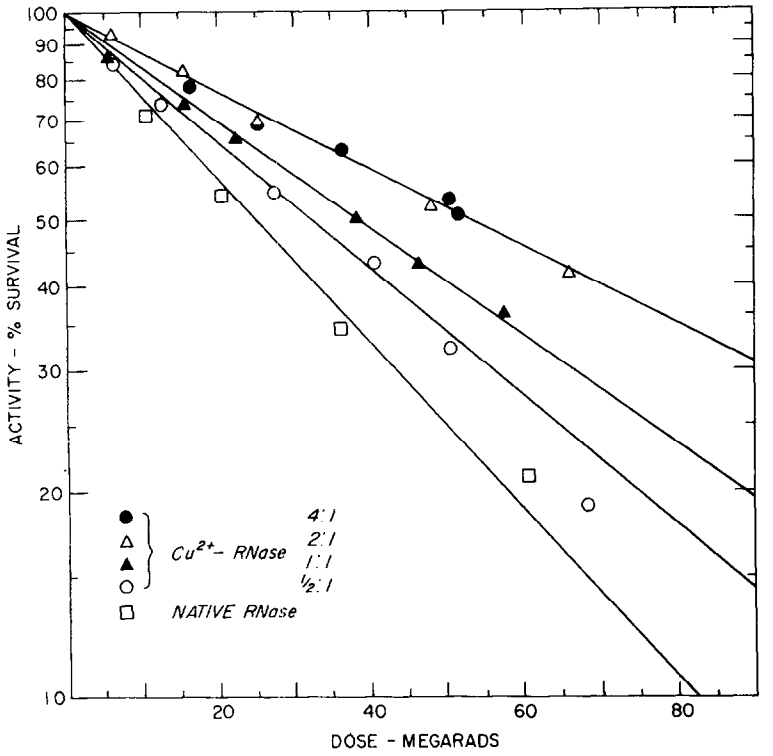


Figure 2. Survival of enzymatic activity at various $Cu^{2+}$/ribonuclease ratios. $\gamma$-Radiolysis at 295° K.

TABLE II

| | T°K | Loss of Activity G(-RNase) | G(-$Cu^{2+}$) | $Cu^{2+}$ Ions Reduced / RNase molecules protected |
|---|---|---|---|---|
| Native | 295 | 1.95 ± .10 | — | — |
| $Cu^{2+}$ - RNase, 4:1 | " | 1.02 ± .05 | 2.32 ± .12 | 2.3 ± .2 |
| " 2:1 | " | 0.94 ± .05 | 2.54 ± .13 | 2.5 ± .2 |
| " 1:1 | " | 1.28 ± .06 | 2.17 ± .11 | 3.2 ± .6 |
| " ½:1 | " | 1.50 ± .07 | 1.53 ± .08 | 3.4 ± 1.0 |
| Native RNase | 77 | .64 ± .06 | — | — |
| $Cu^{2+}$ - RNase, 4:1 | " | .43 ± .04 | 1.0 ± .10 | 4.8 ± 2.0 |

Several significant features of Table II may be noted:

(A) The ratio of ($Cu^{2+}$ ions reduced) / (ribonuclease molecules protected) at room temperature is approximately constant over an 8-fold range of variation of the $Cu^{2+}$/ribonuclease ratio.

(B) Reduction of cupric ions and protective effect occur at liquid nitrogen temperature. Among species responsible for cupric ion reduction at room temperature one may consider small organic radicals, radicals migrating in the protein molecule by successive H atom transfer reactions, hydrogen atoms and subexcitation electrons. At 77°K hydrogen atoms and subexcitation electrons can diffuse freely in organic solids, while larger organic radicals remain trapped in their original lattice sites (Bensasson, et al., 1963). The occurrence of reduction and protection at 77°K excludes small radicals or migrating polymer radicals as the responsible species at that temperature.

(C) The G values for cupric ion reduction of the 4:1, 2:1 and 1:1 complexes at room temperature are almost as large as the G value of 2.5 for total radical production in irradiated ribonuclease as measured by ESR (Hunt and Williams, 1964). Measurements of the number of free radicals on carbon in irradiated $Cu^{2+}$-ribonuclease (4:1) by the method of exposure to tritiated hydrogen sulfide (Riesz, White and Kon, 1966) indicate that the presence of cupric ions decreases the carbon-radical yield by a factor of about 10. These results taken together indicate that essentially for every cupric ion reduced, the formation of one trapped free radical is prevented. The approximately 10-fold reduction in the number of free radicals on carbon is accompanied by a decrease in the G-value for loss of enzymatic activity from $1.95 \pm .10$ to $1.02 \pm 0.05$ (Table II). This suggests that the free radicals observed at room temperature may be responsible for a part of the loss of enzymatic activity. It remains for further study to assess the relative contributions to the inactivation of the enzyme made by two possible processes; loss of conformation of the enzyme in the solid state caused by processes prior to the formation of stable free radicals, or reactions of the trapped free radicals on dissolving the enzyme in water.

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