

On the Free Radical-Induced Aggregation of Ribonuclease – A Pulse Radiolysis Study Using the Light Scattering Detection Method

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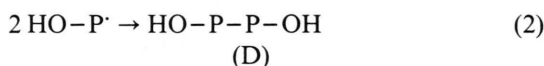
The aggregation of bovine ribonuclease (RNase) induced by either $\cdot\text{OH}$ or Br_2^- radicals has been studied. These radicals, which were generated by pulse radiolysis of aqueous solutions with 16 MeV electrons, readily attack the enzyme thus producing RNase-radicals which subsequently combine. At initial radical concentrations low enough (≤ 0.1 radical per enzyme molecule) to prevent multimerizations other than dimerization, rate constants for the latter process were determined from the increase of the light scattering intensity after the pulse: $2k_2 = (2.2 \pm 0.3) 10^6 \text{ l/mol s}$ (Br_2^- initiated dimerization) and $(5.4 \pm 0.4) 10^6 \text{ l/mol s}$ ($\cdot\text{OH}$ initiated dimerization). The G-values for dimerization are: 1.3 (Br_2^-) and 0.85 ($\cdot\text{OH}$). Transient optical absorption measurements revealed the existence of phenoxyl radicals of tyrosine ($\text{TyrO}\cdot$) that decayed with a rate constant of $2k_2 (\text{total}) = (5.5 \pm 0.7) 10^6 \text{ l/mol s}$ (Br_2^- -case). The difference between k_2 and $k_2 (\text{total})$ presumably indicates the occurrence of disproportionation.

Introduction

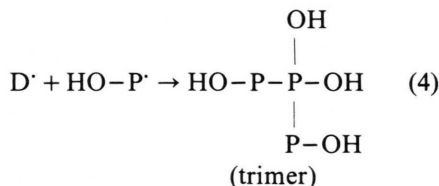
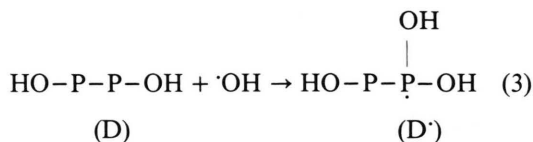
During recent years Rayleigh light scattering (LS) measurements have been used frequently in conjunction with flash photolysis and pulse radiolysis to study kinetic and dynamic phenomena related to molecular size changes in macromolecules [1, 2]. The LS detection method has proved especially useful for investigating aggregation processes in biopolymers such as DNA [3, 4] and plasma proteins [5]. Quite recently the radiation-induced dimerization of lysozyme was studied [6].

This paper reports on the free radical-induced aggregation of ribonuclease (RNase), an enzyme that, like many other proteins, is capable of forming aggregates out of a monomolecularly dispersed distribution in dilute solution under the influence of quite different external forces. For example, mechanical forces imposed on RNase solution by stirring or shaking induce aggregation. On the other hand, high energy radiation, such as X-rays or γ -rays, can be used quite effectively to generate aggregates in RNase solutions [7–13]. In the latter case, strong indications were obtained for the fact that in dilute

aqueous solution the aggregation of RNase occurs according to a free radical mechanism comprising the attack of amino acid residues by the free radical intermediates, stemming from the radiolysis of water, and the subsequent combination of macroradicals, thus produced, *e. g.*,



where P stands for RNase. This way, aggregation proceeds as a stepwise process involving the initial formation of dimers according to reaction (2) and the subsequent formation of trimers, tetramers etc., *e. g.*,

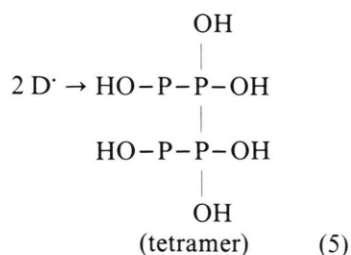


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In neat aqueous solutions OH radicals were found to be quite effective in inducing aggregation, whereas hydrated electrons and H-atoms proved to be rather ineffective [11]. To study the kinetics and the extent of the aggregation of RNase, we have, therefore, irradiated N₂O-saturated solutions with γ -rays or 16 MeV electron pulses. N₂O converts hydrated electrons to OH radicals: $e_{\text{aq}}^- + \text{N}_2\text{O} + \text{H}_2\text{O} \rightarrow \cdot\text{OH} + \text{OH}^- + \text{N}_2$. Moreover, N₂O-saturated solutions containing 0.1 M KBr have been irradiated. In this case $\cdot\text{OH}$ radicals readily react with Br⁻ forming bromine atoms which subsequently combine with Br⁻:



Br₂^{·-}, reportedly, reacts with amino acids with high selectivity [14]. According to Adams *et al.* [15], in the case of RNase, only a few residues (methionine, tyrosine, histidine, and phenylalanine) should show any appreciable reactivity.

We have found that in both kinds of experiments the light scattering intensity (LSI) of RNase solutions increases upon irradiation indicating aggregation. The extent of LSI increase was more strongly pronounced when Br₂^{·-} induced aggregation. The experiments were performed both as stationary irradiations with ⁶⁰Co- γ -rays and as pulse irradiations with 16 MeV electrons produced by a linear accelerator. Gel electrophoresis was used for product analysis.

Experimental Part

(a) Materials

Chromatographically purified bovine pancreatic RNase, obtained from Sigma Chemical Co. (Type X11A) was used without further purification. RNase concentrations were determined spectrophotometrically at $\lambda = 278 \text{ nm}$ ($\epsilon = 9.7 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$). All other chemicals were obtained from E. Merck. Water was

purified by allowing it to pass through a set of Millipore cartridges.

(b) Preparation of Solutions

RNase was dissolved immediately before irradiation in 10⁻² M phosphate buffer solution (pH 7) containing either 0.1 M Na₂SO₄ or 0.1 M KBr. The solutions were filtered through a Millipore filter (pore size 0.22 μm). Saturation with N₂O was accomplished by blowing the gas over the surface of the liquid in the storage flask, which was occasionally gently shaken. Bubbling caused aggregation.

(c) Gel electrophoresis

In order to characterize irradiated RNase according to the molecular size, electrophoresis studies were carried out with polyacrylamide gel plates using a commercial setup obtained from LKB. Prior to electrophoresis RNase solutions were dialyzed against 0.01 M phosphate buffer (pH 7) overnight at 5 °C. After the addition of sodium dodecyl sulfate (2%) and 2-mercaptoethanol (5%) the solutions were heated to 100 °C for two minutes to denature and reduce the proteins. After electrophoresis the proteins were fixed with a solution of trichloro acetic acid (114 g/l) and sulfosalicylic acid (34 g/l) in a methanol/water mixture (volume ratio 3:7). For staining an amido black solution was used (concentration 5 g/l). The solvent was a mixture of water, ethanol and acetic acid (volume ratio 6:3:1). The neat solvent served as destaining agent.

(d) Irradiations

Continuous irradiations were carried out with ⁶⁰Co- γ -rays at an exposure dose rate of $1.0 \times 10^5 \text{ r/h}$. For pulse radiolysis experiments a L-band linear accelerator (Vickers) was used. The absorbed dose was determined with the aid of the Fricke dosimeter, using O₂ saturated 0.01 M Fe(II) solutions, or with the thiocyanate dosimeter. Details of the LS-detection method have been described elsewhere [1].

(e) Evaluation of light scattering data

A detailed treatment of light scattering data obtained from kinetic measurements has been presented elsewhere [1]. The special case of dimeriza-

tion has been referred to recently [6]. A similar treatment of data obtained at a scattering angle of 90° has been applied in this paper. It is assumed that in the Debye equation

$$\frac{K c I_p}{I - I_s} = \frac{1}{P_9 \bar{M}_w} + 2 A_2 c \quad (8)$$

K , c , I_p and P_9 remain constant upon irradiation, and that the second term can be neglected because of the low enzyme concentration.

$$K = (2 \pi^2 n^2 / \lambda^4 N_A) (dn/dc)^2$$

- I_p intensity of incident light;
 I LSI of the solution;
 I_s LSI of solvent;
 c enzyme concentration in g/cm³;
 P_9 particle scattering factor
 A_2 second virial coefficient
 N_A Avogadro's number.

For the increase of the light scattering intensity I as a consequence of the increase of the molecular weight from $\bar{M}_{w,0}$ to $\bar{M}_{w,t}$ at time t , and finally to $\bar{M}_{w,\infty}$, following equations are obtained:

$$\frac{\bar{M}_{w,\infty}}{\bar{M}_{w,0}} = \frac{I_\infty - I_s}{I_0 - I_s} = \frac{U_\infty - U_s}{U_0 - U_s} \quad (9)$$

$$\frac{\bar{M}_{w,\infty} - \bar{M}_{w,0}}{\bar{M}_{w,\infty} - \bar{M}_{w,t}} = \frac{I_\infty - I_0}{I_\infty - I_t} = \frac{U_\infty - U_0}{U_\infty - U_t} \quad (10)$$

Here, account is taken for the fact that the measured signal voltage U is proportional to the LSI. Equations describing the change of the LSI according to a pseudo 1st order process, e. g.,



(P^* and P denote enzyme radicals and intact enzyme molecules, respectively) or to a 2nd order process



are readily derived with the aid of Eqn. (10): pseudo 1st order case:

$$\ln \frac{\bar{M}_{w,\infty} - \bar{M}_{w,0}}{\bar{M}_{w,\infty} - \bar{M}_{w,t}} = \ln \frac{U_\infty - U_0}{U_\infty - U_t} = k_1 t \quad (13)$$

and 2nd order case:

$$\frac{U_\infty - U_0}{[P]_0 (U_\infty - U_t)} - \frac{1}{[P]_0} = 2 k_2 t. \quad (14)$$

If the increase in LSI is exclusively due to dimerization, the initial concentration of enzyme radicals can be estimated as follows: for the average molecular weight $\bar{M}_{w,\infty}$ measured after irradiation Eqn. (15) holds:

$$\bar{M}_{w,\infty} = \frac{([P]_0 - [P^*]_0) M_0^2 + ([P^*]_0/2) (2 M_0)^2}{([P]_0 - [P^*]_0) M_0 + ([P^*]_0/2) 2 M_0} \quad (15)$$

which gives

$$\frac{\bar{M}_{w,\infty}}{M_0} = 1 + \frac{[P^*]_0}{[P]_0}. \quad (16)$$

With the aid of Eqn. (9) one obtains:

$$[P^*]_0 = [P]_0 \frac{U_\infty - U_0}{U_0 - U_s}. \quad (17)$$

Thus, rate constants k_2 can be evaluated using Eqn. (14) and (17).

If, again, the aggregation is considered being limited to dimerization the dependence of the extent of LSI increase on the absorbed dose can be expressed by Eqn. (18):

$$\left(\frac{U_\infty - U_0}{U_0 - U_s} \right)_D = \frac{20 G(X) D}{N_A [P]_0} \quad (18)$$

$G(X)$ 100 eV yield of dimerization;

D absorbed dose in eV/cm³.

Eqn. (18) is based on $[\text{Dimer}]_\infty = [P^*]_0/2$ and $[\text{Dimer}]_\infty = 10 G(X) D / N_A$.

Experimental Results

(a) Gel electrophoresis measurements

Electrophoretic separations of radiolysis products were carried out with several RNase samples which had been irradiated in the presence of KBr or Na₂SO₄. Products corresponding to the molecular weights of dimers, trimers, tetramers and pentamers were detected at absorbed doses above 100 krad. Typical results obtained at irradiations with γ -rays are shown in Fig. 1, which presents electrophoresis patterns of unirradiated and irradiated RNase. At absorbed doses of 26 and 44 krad spots corresponding to dimers are recognized. At 62 krad a spot related to trimers becomes detectable. Fig. 2 shows a plot of the logarithm of the molecular weight of standard proteins vs. the relative mobility. The latter is defined as the ratio of migration distances

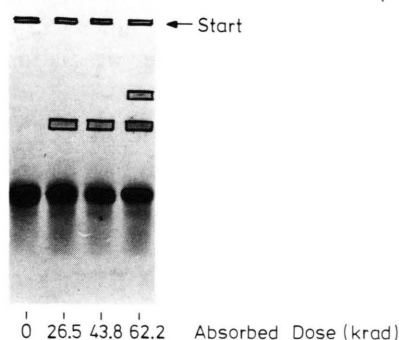


Fig. 1. Gel electrophoresis of RNase irradiated with ^{60}Co - γ -rays at a concentration of 4×10^{-4} mol/l in N_2O saturated aqueous solution containing 0.1 mol/l KBr at pH 7 at room temperature. Absorbed doses are indicated at the pattern.

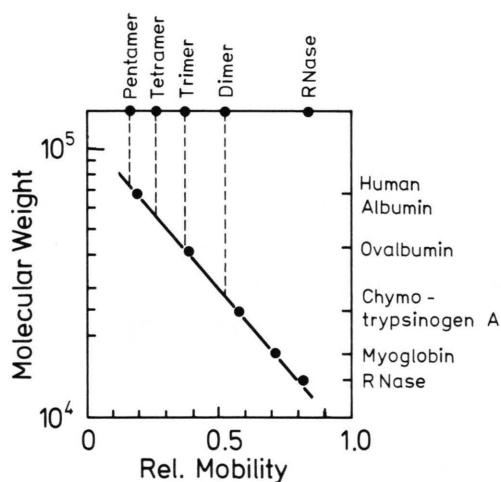


Fig. 2. Gel electrophoresis of RNase irradiated with ^{60}Co - γ -rays at a concentration of 4×10^{-4} mol/l in N_2O saturated aqueous solution containing 0.1 mol/l KBr at pH 7 at room temperature. Absorbed dose: 108 krad. Plot of the molecular weight of standard proteins (indicated at the right coordinate) vs. the relative mobility. The positions of spots observed at the electrophoresis are indicated at the upper abscissa.

of the sample to that of bromophenol blue. The relative mobility of the products is shown at the upper abscissa. It can be seen that the experimental points correspond fairly well to molecular weights expected for the various multimers. The experiments revealed, moreover, that the yield of dimers, trimers etc., increases linearly with dose. In accordance with earlier findings it can be concluded, therefore, that the aggregation of RNase is a step-

wise process which is initiated by the radicals produced by the radiolysis of water. A comparison of the result, obtained with RNase solutions containing either Na_2SO_4 or KBr showed that Br_2^- radicals are more efficient in initiating aggregation than $\cdot\text{OH}$ radicals.

From our gel electrophoresis studies it can be concluded that at relatively low absorbed doses ($< \text{ca. } 20$ krad) multimerization processes other than dimerizations do not occur, or occur only to a negligible extent. Moreover, it can be concluded that covalent bonds are formed between RNase molecules.

(b) Light scattering measurements

Irradiations of N_2O saturated RNase solutions containing either 0.1 M KBr or 0.1 M Na_2SO_4 with a 50 ns pulse of 16 MeV electrons led to an increase of the LSI after the pulse. Typical oscilloscope traces are shown in Fig. 3 (a₁) and (b₁). The LSI increase

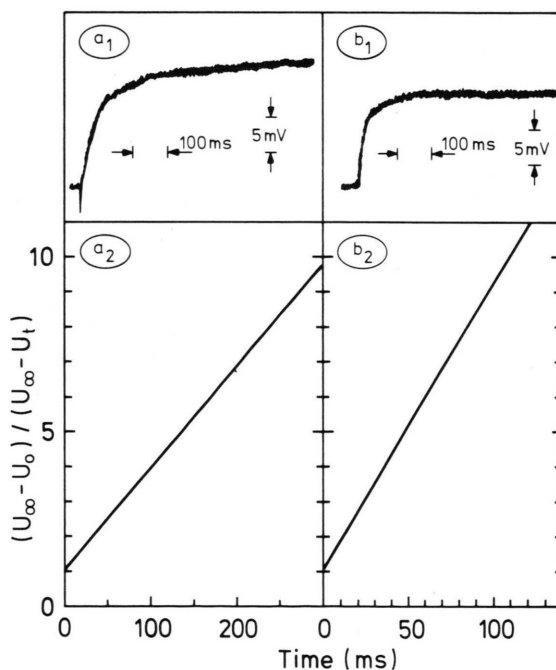


Fig. 3. Aggregation of RNase (4×10^{-4} M) in N_2O saturated solution initiated by Br_2^- - (a) or $\cdot\text{OH}$ -radicals (b). Increase of the LSI after irradiation with a 50 ns pulse of 16 MeV electrons (absorbed dose: ca. 5 krad). Oscilloscope traces depicting the LSI change after the pulse (a₁) and (b₁). 2nd order plots according to Eqn. (14). Additional experimental conditions: pH 7, 10^{-2} M phosphate buffer. [KBr]: 0.1 M (a) and $[\text{Na}_2\text{SO}_4] = 0.1$ M (b). U_0 : 597 mV (a₁) and 700 mV (b₁).

follows 2nd order kinetics as can be seen from Fig. 3 (a₂) and (b₂), where plots according to Eqn. (14) are presented.

A linear relationship between the extent of LSI increase and the absorbed dose was observed for the dose range investigated (up to about 10 krad). This can be seen from Fig. 4. Radiation-chemical yields for dimerization were evaluated from the straight lines according to Eqn. (18) as $G(X) = 1.3$ for the Br_2^- -initiated reaction and as $G(X) = 0.85$ for the $\cdot\text{OH}$ -initiated reaction.

Rate constants for dimerization were estimated using Eqns. (14) and (17): $2k_2 = (2.2 \pm 0.3) 10^6$ l/mol s and $(5.4 \pm 0.4) 10^6$ l/mol s for Br_2^- and $\cdot\text{OH}$ -initiated processes, respectively. The radiation-chemical yields and the rate constants presented above were estimated on the basis that at low absorbed doses multimerizations other than dimerizations can be neglected. This is possible if the average number of radical sites per enzyme molecules is sufficiently low. Actually at a dose of 10 krad, e.g., this number is about 0.1, if it is assumed that all $\cdot\text{OH}$ or Br_2^- radicals attack RNase.

It is noteworthy that molecular oxygen completely suppresses aggregation. Upon irradiating air-saturated RNase solution with doses up to 30 krad no change in the LSI was observed.

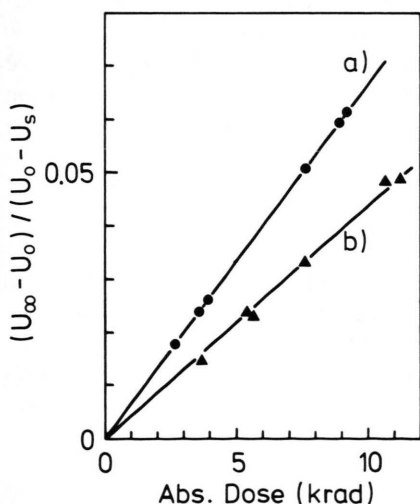


Fig. 4. Increase of the light scattering intensity as a consequence of the reaction of Br_2^- (a) or $\cdot\text{OH}$ (b) with RNase. Plot of the extent of LSI increase vs. the absorbed dose according to Eqn. (18). Experimental conditions: N_2O -saturated RNase solution (4×10^{-4} M) phosphate buffer: 10^{-2} M pH 7. (a): $[\text{KBr}] = 0.1$ M; (b) $[\text{Na}_2\text{SO}_4] = 0.1$ M.

(c) Optical absorption measurements

Pulse radiolysis experiments with N_2O -saturated, KBr containing RNase solution yielded the following results:

The optical absorption spectrum of the Br_2^- radical was formed during the pulse. It decayed rapidly, and simultaneously a long-lived transient possessing the absorption spectrum shown in Fig. 5 was formed. This spectrum strongly resembles that of the phenoxyl radical of tyrosine ($\text{TyrO}^\cdot$) reported by other

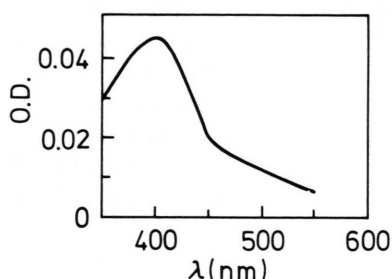


Fig. 5. Transient optical absorption spectrum recorded with a N_2O -saturated aqueous solution of RNase (1.9×10^{-4} M) containing 0.1 M KBr at pH 7 (10^{-2} M phosphate buffer) 100 μs after irradiation with a 50 ns pulse of 16 MeV electrons (absorbed dose: 3.1 krad).

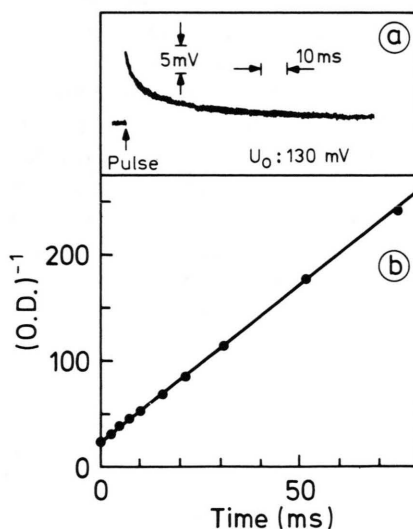
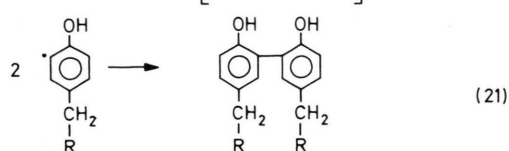
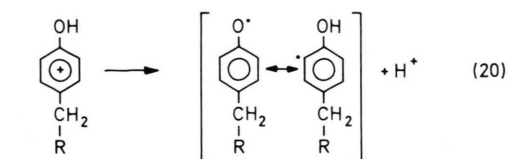
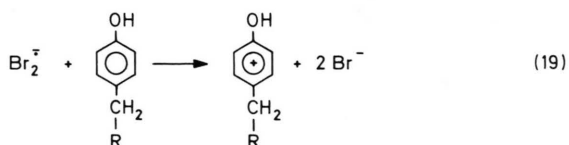


Fig. 6. Changes of the transient optical absorption observed at $\lambda = 400$ nm with a N_2O -saturated aqueous solution of RNase (4.0×10^{-4} M) containing 0.1 M KBr at pH 7. Absorbed dose: 3.6 krad. Pulse length: 50 ns. (a) Oscilloscope trace depicting the decay of the absorption which had been formed previously in the μs time range. (b) 2nd order plot: reciprocal optical density vs. time.

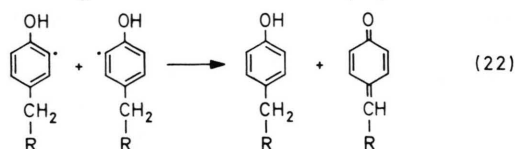
authors [14, 17, 18]. This spectrum decayed according to 2nd order kinetics. A typical oscilloscope trace illustrating the decay of the absorption at 400 nm is shown in Fig. 6 (a). The respective 2nd order plot is presented in Fig. 6 (b). For the evaluation of the rate constant the initial concentration of radicals must be known. We assumed that for the reaction of Br_2^- with RNase the same steric factors apply as for the reaction of OH radicals, which have been reported by Masuda *et al.* [16]. Then, about 70% of Br_2^- radicals should react with tyrosine, 17% with histidine and 12% with methionine. Considering that at $\lambda = 400$ nm only $\text{TyrO}^\cdot$ absorbs and that $[\text{TyrO}^\cdot] = 0.7[\cdot\text{OH}]_0$, where $[\cdot\text{OH}]_0$ is the concentration of OH radicals generated in the pulse, the concentration of enzyme radicals ($\text{TyrO}^\cdot$ -radicals) can be estimated from the absorbed dose and $G(\text{OH}) = 5.4$. Thus, $2k_2 = (5.5 \pm 0.7) 10^6 \text{ l/mol s}$ is obtained, a value about 2.5 times higher than the corresponding value obtained from LSI measurements.

Discussion

A rather straightforward interpretation is possible with the results obtained with KBr-containing solutions, because Br_2^- radicals are more selective in their reactions with RNase than $\cdot\text{OH}$ radicals. As has been pointed out, evidence could be obtained for the fact that phenoxyl radicals of tyrosine play a major role in the dimerization process. In this connection it is noteworthy that the radiation-induced formation of dityrosine in aqueous solutions of tyrosine has been reported recently [19]. A similar mechanism as proposed by these authors could also be operative in the present case.



It turned out that the rate constant for the decay of $\text{TyrO}^\cdot$ radicals is about 2.5 times greater than k_2 derived from LSI measurements. This finding can be explained in terms of a disproportionation reaction occurring in addition to reaction (21):



Upon the attack of RNase by OH radicals a broader spectrum of radicals is formed which, in part, can also combine to dimers. Therefore, multimerization processes will proceed *via* various different mechanisms in this case.

In conclusion it is interesting to note that the multimerizations of RNase and lysozyme induced by the attack of reactive radicals proceed in an analogous manner. In both cases stepwise aggregation occurs. The rate constants of dimerization lie in the same order of magnitude. The important role of tyrosine phenoxyl radicals on the multimerization process induced by Br_2^- radicals was established in both cases.

Acknowledgements

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