Free Radical Reactions with Simple Biochemical Systems

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Free radical processes are an integral part of biochemical processes and as such play a role in the development of a variety of biological effects and pathological disorders. For example, free radical mediated lipid peroxidation has been implicated in cellular aging processes, liver damage induced by chlorinated hydrocarbons and alcohol, cellular damage by air pollutants and oxygen toxicity (*1* and references therein). In addition, free radical processes are known to be involved in radiation-induced cellular lethality (*2* and references therein) and most probably involved in the initiation of the carcinogenic and mutagenic action of ionizing radiation. The carcinogenic effect of certain polycyclic hydrocarbons is also thought to be mediated through free radical processes. For example, certain chemical carcinogens are converted to active forms by enzymatic processes involving free radical reactions (*3*). This same activation process can be achieved by exposing the biological system and "inactive" carcinogen to ionizing radiation (*4*).

The above paragraph outlines some biological effects involving free radical processes. Most of the effects mentioned are undesirable. By understanding the mechanism of free radical reactions with biochemical systems, means of controlling these reactions should be able to be devised, and thus be of benefit to mankind. An example of this is in the radiation therapy of cancer where radiation sensitizing and protective agents are being used in attempts to increase tumor cell kill while preserving acceptable levels of normal tissue injury (*5*). These sensitizing and protective agents act at the free radical level.

Ionizing Radiation—A Convenient Means of Generating Free Radicals

As we have seen in the preceding papers in this symposium ionizing radiation provides a convenient way of generating free radicals. Therefore radiation chemical methods can be used readily to study free radical reactions in whatever chemical system is of interest to you. It is important to realize here that ionizing radiation can be used as a "tool" to study free radical reactions.

Radiation Chemical Methods in the Study of Free Radical Reactions

In order to avoid repetition of what has been described in the preceding papers I will only briefly survey this topic in this section. Since most biochemical systems we deal with are aqueous solutions, the radiation chemistry of water becomes of prime importance when discussing the generation and reactions of free radicals in such systems. When water is ionized, the resulting ions undergo a variety of ion-molecule reactions to produce what are known as the primary water radicals, namely $OH^{\cdot}$, the hydroxyl radical; e^-_{aq}, the solvated electron, and $H^{\cdot}$, the hydrogen atom.

$$H_2O \rightsquigarrow H_2O^+ + e^- \xrightarrow{H_2O} {}^{\cdot}OH, e^-_{aq}, {}^{\cdot}H \qquad (1)$$

These species are themselves very reactive, and therefore short-lived, and in the absence of added solute will react with each other to form the permanent products of water radiolysis, hydrogen (H_2), and hydrogen peroxide (H_2O_2).

Immediately following an absorbed dose of radiation of 1000 rad there will be ~3 μM of $^{\cdot}OH$ and e^-_{aq} radicals produced and ~0.5 μM $^{\cdot}H$ atoms. The hydroxyl radical is a strong oxidizing agent while the hydrated electron is a strong reducing agent. The hydrogen atom can react either as a reducing agent or an oxidizing agent. Fortunately, there exist several solutes which will react rapidly with $^{\cdot}OH$ and slowly with e^-_{aq} and vice versa. Thus by adding an appropriate concentration of such a solute to the system under study it is possible to study the reactions of one radical independently of the other. Scavenger systems which allow us to do this are listed in Table 1.

Of course the reaction of a primary radical with a solute will produce a solute radical which may or may not be reactive with the system under study. For example, both tertiary butyl alcohol and bromide ions are excellent $^{\cdot}OH$ scavengers; however, the secondary radicals produced by OH reaction, namely

$\cdot CH_2(CH_3)_2OH$ and $\cdot Br_2^-$, differ widely in their respective reactivities. The tertiary butyl alcohol radical is relatively unreactive, whereas the $\cdot Br_2^-$ radical anion is a strong oxidizing agent.

Free Radical Reactions with Amino Acids, Peptides, and Enzymes

The object of this paper is to discuss free radical reactions in biochemical systems as studied using radiation chemical techniques. To attain this end I have chosen *not* to give an exhaustive survey of this subject but rather to illustrate it principally with specific examples from my own work (in collaboration with several colleagues) on free radical reactions with enzyme systems. Before doing this, however, I will *briefly* describe the nature and function of enzymes in biochemistry.

Enzymes are large protein molecules essentially made up of a long chain of amino acids in a specific configuration. They control specific chemical reactions by forming a complex with a substrate, allowing chemical reactions to occur with the liberation of a product together with enzyme. In this sense, the enzyme is a catalyst.

$$E + S \rightleftarrows ES \rightleftharpoons E + P \qquad (2)$$

There is generally a specific site within an enzyme molecule to which a substrate can bind and at which the chemistry occurs. This site generally involves several amino acid residues lying in a specific configuration with respect to each other. These residues although physically located close to each other may be separated by many other residues in terms of their position in the polypeptide chain.

The experiments I will describe in the paragraphs below illustrate how ionizing radiation can be used as a tool to probe for the nature of the amino acid residues involved in the chemical and structural function of enzymes.

It is appropriate to start with an illustration that water derived free radicals are effective enzyme inactivators. This is readily demonstrated by comparing the radiation sensitivity of dry enzymes with those in dilute aqueous solution. Figure 1 compares the radiation inactivation of dry ribonuclease with the same enzyme in dilute aqueous solution. The increased sensitivity in solution relative to the dry state can be explained by the reaction of water free radicals, particularly $\cdot OH$ and $\cdot H$ radicals.

Reactions of Primary Water Radicals with Amino Acids, Peptides and Enzymes

During the past 10 to 15 years a great deal of information has been gathered regarding the kinetics and mechanism of reaction of the primary water radicals with amino acids and peptides. This subject has been reviewed by several authors (*6, 7*) and readers are referred to these papers for details.

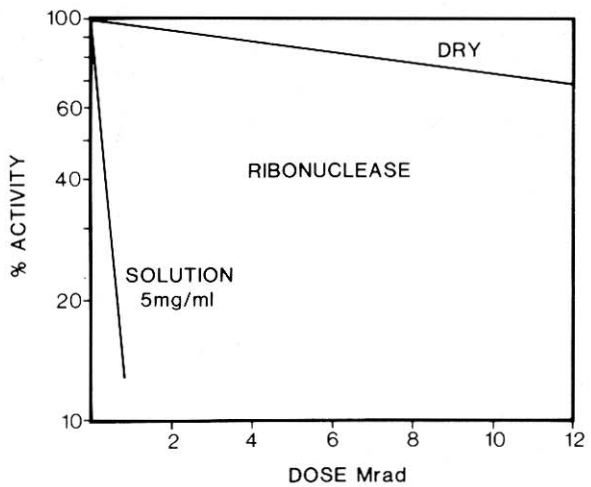

Figure 1. Inactivation of ribonuclease in the dry state and in aqueous solution by ^{60}Co γ-radiation. (Redrawn from Dertinger & Jung (*16*).)

There is a wide spectrum of reactivities encountered for each of the radicals with the 20 commonly occurring amino acids. The aromatic and sulfur-containing amino acids tend to be the most reactive, often reacting with diffusion controlled rates. The rate constants are pH dependent due to the various ionic equilibria in amino acid structures. Hydrated electrons will react with protonated amino groups and at carbonyl groups in peptides. Hydroxyl radicals and hydrogen atoms will often react by H-atom abstraction from carbon atoms alpha to the carboxyl group (in free amino acids) or to the peptide bond (in peptides). The simple aliphatic compounds glycine and alanine are the least reactive; however, when OH groups or branched chains are present the reactivity tends to be increased due to inductive effects.

Since an enzyme consists of many different amino acid residues, primary water radicals will react at many different sites on the enzyme because of the general lack of specificity of reaction of these radicals with amino acid moieties especially when in peptide form (*6, 7*). While by the use of appropriate scavenger systems (see Table 1) it is possible to assess the relative contributions of the radicals ($\cdot OH$, e^-_{aq}, $\cdot H$) to enzyme inactivation, it is *not* possible to state at which amino acid residues critical damage occurred (*8*).

Free radical inactivation of enzymes can occur by several possible mechanisms. Firstly, damage can occur to an amino acid residue or residues in the active site which is either involved in substrate binding or in the chemistry of the catalytic reaction. Secondly, damage can occur to residues which are essential to the structural integrity of the enzyme which if disrupted can result in loss of enzymic activity. By the use of radiolytically generated free radicals which are specific in their reactions with amino acids, it is possible to determine residues in an enzyme which are critical to enzyme function. The experimental approach in such an instance is to combine information from pulse radiolysis experiments on rates of reaction and sites of attack with stationary-state radiolysis enzyme inactivation data. In the section below I will describe the use of selective secondary radicals in probing enzyme structure and function.

Reactions of Selective Secondary Radicals with Amino Acids, Peptides and Enzymes

The use of selective free radicals to identify amino acid residues in enzymes which are crucial to activity is analogous to classical biochemical methods of using reagents that attack specific functional groups. Such selective free radicals were first recognized about 10 years ago through an unexpected result in an experiment designed to demonstrate the protection of lysozyme, irradiated in an N_2O-saturated solution, against $\cdot OH$ radical inactivation by the OH scavenger CNS^- (*9*). The rate of enzyme inactivation (Fig. 2) was unaffected although all $\cdot OH$ radicals had been converted to the radical anion $\cdot(CNS)_2^-$ by reactions (3) and (4), viz.:

$$\cdot OH + CNS^- \rightarrow OH^- + \cdot CNS \qquad (3)$$

$$\cdot CNS + CNS^- \rightleftharpoons (CNS)_2^- \qquad (4)$$

The only explanation of this result was that the $\cdot(CNS)_2^-$

Table 1. Some Scavenger Systems and Resulting Reactive Radicals

Scavenger System	Reactive Radicals[a]
N_2	$\cdot OH$ (2.7), e^-_{aq} (2.7), $\cdot H$ (0.55)
N_2O	$\cdot OH$ (5.4), $\cdot H$ (0.55)
N_2 + t-butyl alcohol (0.1 *M*)	e^-_{aq} (2.7), $\cdot H$ (0.55)
N_2O + t-butyl alcohol (0.1 *M*)	$\cdot H$ (0.55)
$N_2O + CNS^-$ (0.1 *M*)	$\cdot(CNS)_2^-$ (5.4), $\cdot H$ (0.55)
$N_2O + Br^-$ (0.1 *M*)	$\cdot Br_2^-$ (5.4), $\cdot H$ (0.55)
$N_2O + HCOO^-$ (0.1 *M*)	$\cdot CO_2^-$ (5.4), $\cdot H$ (0.55)

[a] Numbers in parentheses are G-values (radicals/100 eV).

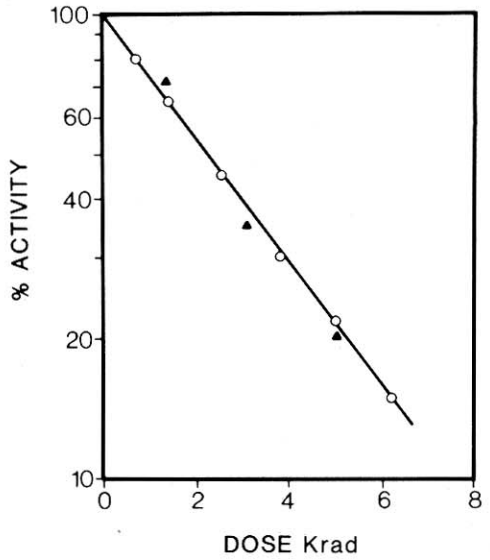

Figure 2. Decrease in the activity of lysozyme with irradiation in the presence of various radical scavengers. Enzyme concentration 7 *M* O, N_2O, pH 6.9; ▲, N_2O, pH 8.8, 10^{-2} *M* KCNS. (Redrawn from Adams et al. (*9*).)

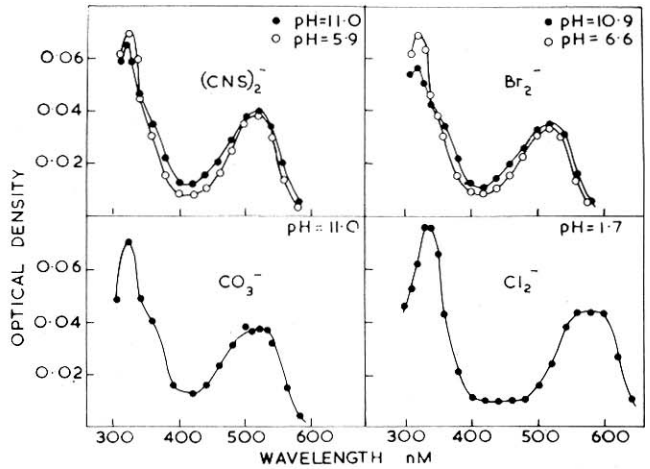

Figure 4. Transient spectra from pulse radiolysis of N_2O-saturated solutions of tryptophan (10^{-3} *M*) and the respective anions (10^{-1} *M*). Spectra measured 50 μsec after the pulse. Dose: 2400 rads. (Reproduced from (*10*) with permission of Radiation Research and Academic Press, Inc.)

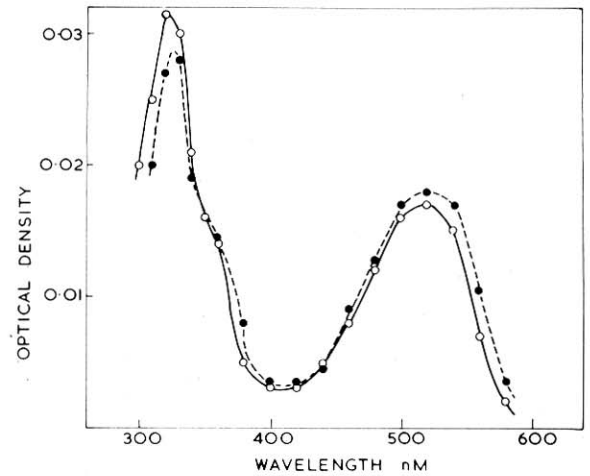

Figure 3. Comparison of product spectrum from reaction of $(CNS)^-_2$ with tryptophan with that from reaction of $(CNS)_2^-$ with lysozyme. Solid line: 10^{-3} *M* tryptophan, 10^{-1} *M* KCNS, pH 5.9, N_2O-saturated. Dashed line: lysozyme 2 mg/ml, 10^{-1} *M* KCNS, pH 5.4, N_2O-saturated. Spectra measured 50 μsec after pulse. Dose in each case, 1050 rads. (Reproduced from (*10*) with permission of Radiation Research and Academic Press, Inc.)

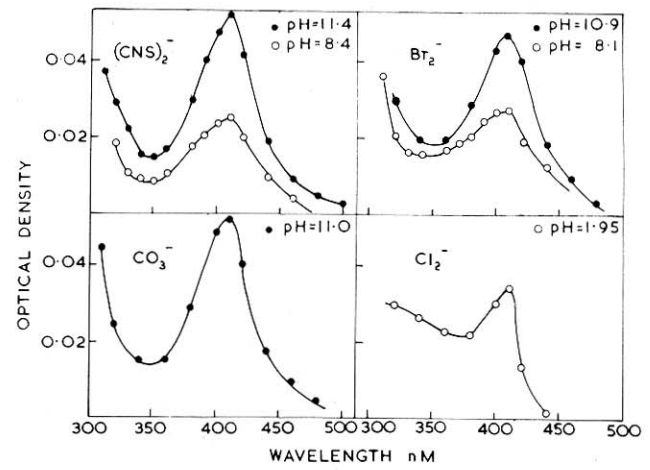

Figure 5. Transient spectra from pulse radiolysis of N_2O-saturated solutions of tyrosine (10^{-3} *M*) and the respective anions (10^{-1} *M*). Spectra measured 50 μsec after the pulse. Dose: 2400 rads. (Reproduced from (*10*) with permission of Radiation Research and Academic Press, Inc.)

radical anion must be inactivating the enzyme. Upon a survey of the reactivity of $\cdot(CNS)_2^-$ with amino acids using the pulse radiolysis technique it became apparent that at neutral pH $\cdot(CNS)_2^-$ reacted with tryptophan with a rate 50 to 100 times faster than with any other amino acids. Thus $\cdot(CNS)_2^-$ is a *selective* secondary radical. The kinetic data with individual amino acids would imply that $\cdot(CNS)_2^-$ would oxidize tryptophan residues in lysozyme. The fact that this indeed occurs was demonstrated by measuring and comparing the transient spectra of the products following reaction of $\cdot(CNS)_2^-$ with tryptophan and lysozyme (see Fig. 3). Not only do the shapes of the spectra coincide but also the yield in the case of lysozyme indicates that *all* $\cdot(CNS)_2^-$ radical anions had reacted with tryptophan residues. There are eight tryptophan residues in lysozyme and subsequent experiments (*9*) demonstrated that Trp-108 was the residue crucial to activity, in agreement with the conclusions of classical enzyme biochemistry.

Following the discovery of the selectivity of $\cdot(CNS)_2^-$ reactivity with amino acids, a search for similar selective radicals was made. It was found that several radical anions derived from halide ions and the carbonate ion possessed similar selectivities. The reactivities of these selective radicals with the most reactive of the amino acids at pH 7 are shown in Table 2. It should be stressed that these reactivities are pH-dependent. For details see Adams et al. (*10*).

The spectra of transient products from reaction of these radicals with tryptophan and tyrosine are shown in Figures 4 and 5. These are useful "fingerprints" which allow us to determine initial sites of attack of these radicals on enzyme structures and to allow us to observe changes in site of attack with changing conditions such as pH. Figure 6 illustrates this latter point for the enzyme papain where at neutral pH the principal sites of attack are tryptophan and cysteine residues, whereas at pH 10.5 tyrosine residues are the major site of attack.

Details of the application of these selective free radicals in determining amino acid residues crucial to enzymatic activity for several enzymes have been described in the literature, and some of the systems investigated and results obtained are summarized in Table 3.

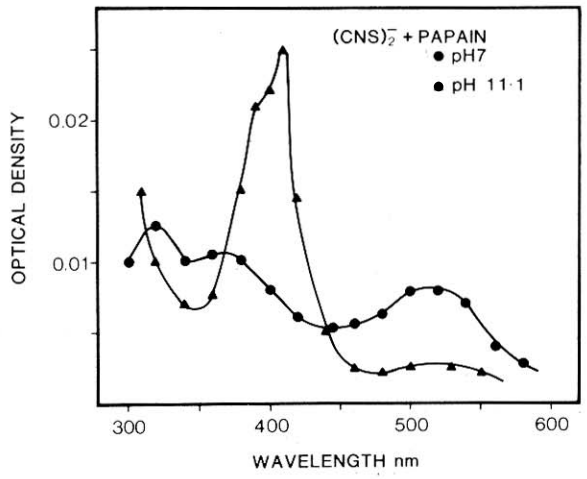

Figure 6. Transient spectra from pulse radiolysis of N_2O-saturated solutions of papain (0.75–1.0 mg/ml) containing KCNS (5×10^{-2} *M*). Spectra measured 50 μs after a 1000 rads pulse.

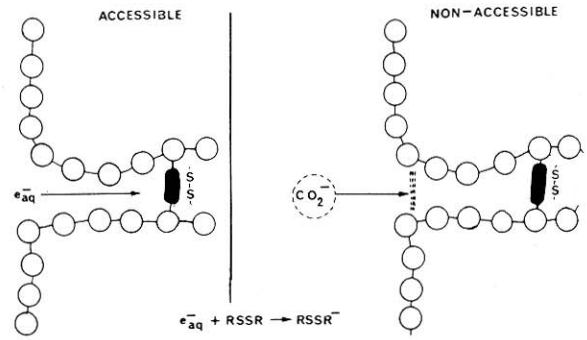

Figure 7. Structural inhibition of electron transfer from the formate radical ion to the S-S bridges in enzymes (see text).

Reaction of Selective Reducing Radicals with Amino Acids and Enzymes

The hydrated electron is a reducing radical which reacts rapidly with many amino acids, in particular histidine and oxidized cysteine, i.e., cystine. The formate radical ion, $\cdot CO_2^-$, formed by reaction of hydroxyl radicals with formate, viz.,

$$\cdot OH + HCOO^- \rightarrow \cdot CO_2^- + H_2O \qquad (5)$$

is a more selective reducing species than the hydrated electron. At neutral pH this radical reacts with disulfide bridges with a rate constant of $\sim 10^9\ M^{-1}\ sec^{-1}$ and with all other amino acids at $<10^7\ M^{-1}\ sec^{-1}$. In acid solution pH <4.5 a measureable reactivity with histidine ($\sim 10^7\ M^{-1}\ sec^{-1}$) can be seen using pulse radiolysis.

An interesting application of the selectivity of reaction of $\cdot CO_2^-$ with disulfide bridges is to probe enzymes for the relative accessibility of these structures. Unlike the hydrated electron, $\cdot CO_2^-$ can only react with accessible disulfide bridges since its size does not permit diffusion into the inner regions of the enzyme structure (Fig. 7). Pulse radiolysis studies have shown that the disulfide bridges in lysozyme are more accessible than those in ribonuclease or α-chymotrypsin (*11*). Pulse radiolysis of N_2O-saturated solution of ribonuclease containing formate shows no reaction of the $\cdot CO_2^-$ radical with the disulfide bridges in RNAse. However, by repeatedly pulsing the solution the enzyme structure can be broken up to expose the -S-S- bonds and allow for full reaction of the $\cdot CO_2^-$ radical at these sites (Fig. 8). An alternative way of doing the same thing is to heat the enzyme up to 55°C whereupon it loses its structural integrity.

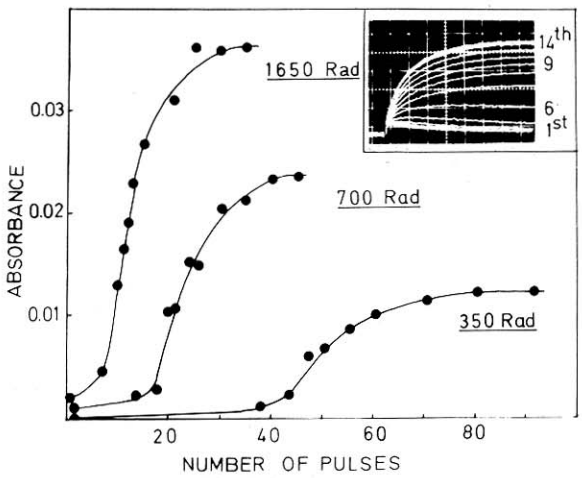

Figure 8. Electron transfer from the formate radical CO_2^-, to sulphur bridges in ribonuclease. *Main figure:* Effect of number of pulses on the absorption of RSSR$^-$ at 410 nm. Ribonuclease concentration 2.0 mg/ml, formate concentration = 4×10^{-2} *M*, pH 8.0. *Inset:* Oscillogram showing rate of formation of RSSR$^-$ at 410 nm. Each curve recorded after indicated number of prepulses. Dose per pulse = 2 Krad. Sweep speed 20 μseconds/cm. (Reproduced from (*8*) with permisssion from the Israel J. Chemistry and the Weizmann Science Press of Israel).

Table 2. Reactivity of Some Amino Acids with Radical-Anions

Radical Anion	Trypto-phan	Tyrosine	Histidine	ϕ-Ala-nine	Cysteine	Methio-nine
Br_2^-	77	2.0 pH 7.5	1.5 pH 7.6	0.1	18 pH 6.6	1.1 pH 7.3
$(CNS)_2^-$	27	0.5	0.1	0.1	5 pH 6.6	0.2
I_2^-	0.1	0.1	0.1	0.1	11 pH 6.8	0.1
CO_3^- pH = 1.8 ± 0.2	44	29	0.7	0.1	27	12
Cl_2^- pH = 1.8 ± 0.2	260	27	1.4	0.6	85	0.7

pH 7.0 ± 0.2 except where stated
Temperature 22°C
Rate constants, units of $1.0 \pm 0.1 \times 10^7\ M^{-1}\ sec^{-1}$
Inorganic salt concentration, 10^{-1} *M*, excepting iodide (5×10^{-1} *M*)
(Reproduced from (*10*) with permission of Radiation Research and Academic Press, Inc.)

Table 3. Some of the Enzymes Investigated by the Selective Free Radical Technique

Enzyme	Crucial Residues Identified	Reference
Lysozyme	TRP	(*9*)
Ribonuclease	HIS	(*17*)
Chymotrypsin	HIS	(*18*)
Trypsin	HIS	(*19*)
Papain	TRP, CYS	(*20*)
Carboxypeptidase	TYR (TRP?)	(*21*)
Subtilisions Carlsberg & Novo	HIS	(*22*)
Bovine Carbonic Anhydrase	TRP, TYR, HIS	(*23*)
Superoxide Dismutase	HIS	(*24*)
Yeast Alcohol Dehydrogenase	HIS, CYS	(*25*)

TRP = Tryptophan; TYR = Tyrosine; HIS = Histidine; CYS = Cysteine

Radiation Chemical Assay of Superoxide Dismutase Activity

Superoxide dismutase is an enzyme of widespread distribution in biological systems. This enzyme catalyses the disproportionation of superoxide radicals (*12*), and its physiological function is thought to be the dismutation of these radicals and hence enabling microorganisms to survive in the

presence of oxygen (*13*). The superoxide radical $\cdot O_2^-$ can be generated readily by irradiation of an O_2 saturated solution of water whereupon the hydrated electron is scavenged by O_2. This radical anion has a known absorption spectrum and decay rate. It is possible to observe $\cdot O_2^-$ directly using pulse radiolysis. By monitoring the rate of disappearance of O_2^- in the presence of added superoxide dismutase, one has a measure of enzyme activity (*14, 15*). If you have the necessary pulse radiolysis facility, this represents a quick and simple means of assaying superoxide dismutase activity.

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