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Antioxidative Activity against Active Oxygen Species[1)*1]

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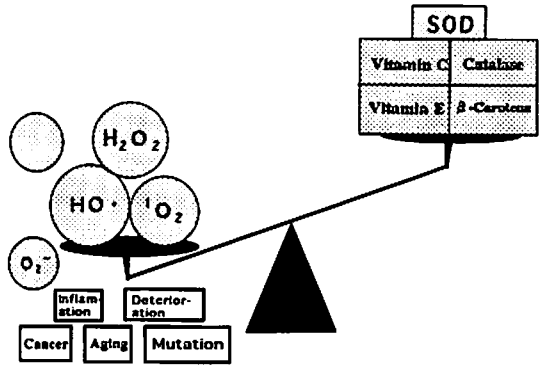
Detection and measurement of singlet oxygen and superoxide, two major active oxygen species from foods or biological tissues and cells in vivo, and analyses of light emitting species and their reaction processes have been investigated from the biochemical and food-chemical points of view. Based on knowledge obtained by such measurement and analyses, we are intending to search the methodology of measuring antioxidative activities of biochemically active compounds against the active oxygen species and its application.



Balance of Terror Scheme 1. Injuries resulted from Active Oxygens and Antioxidative Agents.

1 Introduction

We are pursuing detection and measurement of singlet oxygen and superoxide, two major active oxygen species (Fig. 1), from foods or biological tissues and cells in vivo primarily without external or artificial stimulation, and analyses of light emitting species and their reaction processes from the biochemical and food-chemical points of view.

Based on knowledge obtained by such measurement and analyses, we intend to search for methodology to measure antioxidative activities of biochemically active compounds against the active oxygen species and its application.

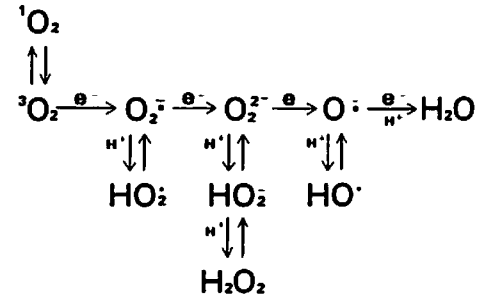


Fig. 1 Active Oxygen Species.

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2 Active Oxygen Species

Both singlet molecular oxygen ($^1O_2$) and superoxide (O_2^-) among the active oxygen species play important roles in various biological and chemical processes, and many biological effects, such as cancer, mutation, aging, and inflammation are considered to result from the active oxygen species (Scheme 1).

For detecting the former oxygen species ($^1O_2$), direct spectroscopic observation of near-infrared emission at 1.27 μm is one of the best ways, and this is the most reliable physical method (Fig. 2).[2-5] However, direct observation of $^1O_2$ in biological systems is still extremely difficult because of low emission quantum yields ($\leqq 10^{-6}$ einstein/mol) in spite of recent advances in detection techniques for the active oxygen species using highly sensitive detectors made with semiconductors. On the other hand, there is no direct spectroscopic method for detecting the latter oxygen species (O_2^-).

Cypridina luciferin analogues, 2-methyl-6-phenyl- and 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo [1,2-*a*] pyrazin-3-ones (CLA and MCLA) and 2-methyl-6-[*p*-[2-(sodium 3-carboxyl ato-4-(6-hydroxy-3-xanthenon-9-yl) phenylthioureyl-ene] ethyleneoxy] phenyl] -3,7-dihydroimidazo [1,2-*a*] -pyrazin-3-one (FCLA) were shown to be versatile tools for specific detection of $^1O_2$ and O_2^- (Fig. 3).[6]

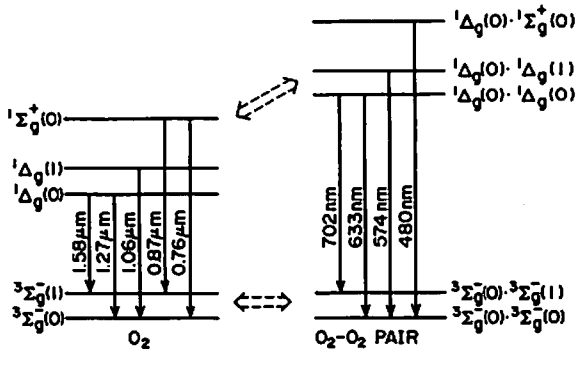


Fig. 2 Energy Diagram of Oxygen.

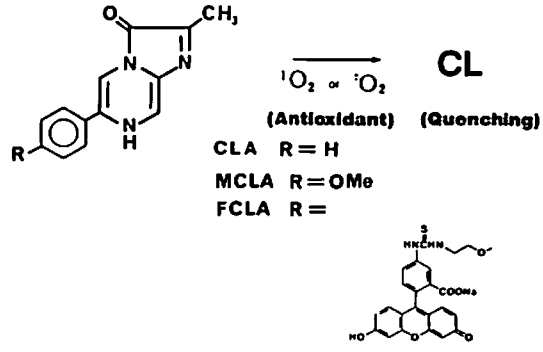


Fig.3 *Cypridina* Luciferin Analogues.

3 Singlet Oxygen[2-5]

Many biochemical sources of $^1O_2$ were known, such as the reaction of KBr with H_2O_2 in the presence of a peroxidase and peroxidation of lipids.[7]

Among the active oxygen species, $^1O_2$ alone gives light emission directly in the near-infrared (IR; around 1.27 μm) and red regions. Both O_2^- and H_2O_2 do not give light emission directly but some oxidation reactions by them result in biophoton emission indirectly.

The red luminescence from $^1O_2$ sometimes overlaps with that from the excited carbonyls. Hence, spectra in red region are apt to give ambiguous conclusion. For identifying $^1O_2$ generation, near-IR luminescence spectroscopy is much superior to visible spectroscopy, since almost no emission gives rise around 1.27 μm except from $^1O_2$. We developed two kinds of ultra-high sensitive instruments (trans-impedance amplifier (TIA) and charge integrating amplifier (CIA) systems) for detecting near-IR emission from $^1O_2$ and made an application for measuring reaction rate constants of some chemiluminescent compounds and superoxide dismutase (SOD) with $^1O_2$ using the near-IR instruments and a flow reaction cell (Table 1). The rate constants were measured by quenching the 1.27 μm emission with the quenchers (Q) and

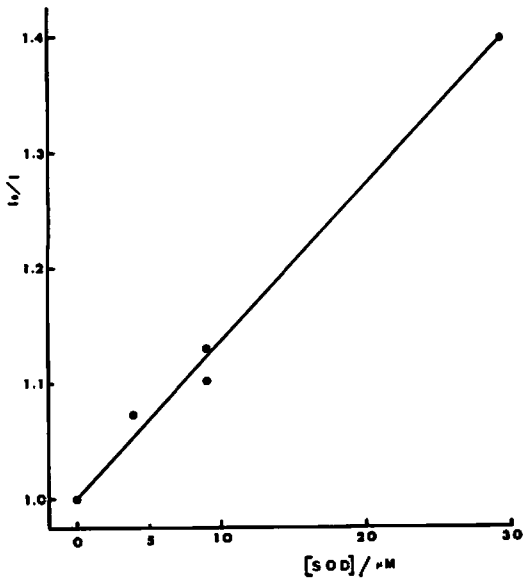


Fig. 4 Stern-Volmer Plot ($^1O_2$-SOD System).

calculated from the Stern-Volmer (equation 1),

$$I_0/I=1+k_q\tau\,[Q] \qquad (1)$$

where [Q], τ, and I_0/I are the concentration of the quencher, the lifetime of $^1O_2$, and the ratio of the emission intensity of $^1O_2$ in the absence and presence of the quencher, respectively (Fig. 4).

Reactivity of SOD to $^1O_2$ was proven experimentally. Hence, the reaction rate constant for SOD is similar to that with O_2^-.

4 Superoxide[7-16)]

Superoxide is considered to be a main cause of biological damages[6)] by reactions such as peroxidation of RX to give $ROO_2\cdot$ radicals, addition reaction to $R_2C{=}X$ to give $^-X\text{-}CR_2\text{-}OO\cdot$, electron substitution of Y to give $Y^{+\cdot}$ and O_2^{2-}, and electron donation to X to give $X^{-\cdot}$ and O_2.

Most living things are considered to possess well-arranged antioxidant systems in order to protect themselves against the toxicity of oxygen, although the full details are under being clarified. Antioxidant activities and mechanisms were well established for major antioxidants known to be highly active even in trace amounts, such as ascorbic acid, tocopherols, and superoxide dismutase (SOD). In living things, however, there are many antioxidative compounds whose activity per mole is not high, but the total activity is comparable to those for major antioxidants because of their abundance.

They would play some important roles in the organisms, such as anticancer, anti-inflammation, and oxidative stress relief at ischemia-reperfusion (Scheme 1). We developed a highly sensitive method for measuring rate

Table 1. Quenching Constants of Some Quenchers with $^1O_2$ and O_2^- at 25 ℃

	$^1O_2$				O_2^-	
Quencher	$k_q/10^7(M^{-1}s^{-1})$	solv.[a]	pH/method[b]		$k_2/10^7\,(M^{-1}s^{-1})$[c]	solv./pH
CLA	63.0	A	7.1	E	10.8	A 7.1
MCLA	294	B	——	E	25.4	A 7.1
FCLA	80.0	A	7.1	E	8.5	A 7.1
Luminol	140	A	7.1	E	0.156	A 7.1
	93.0	C	10.1	E	0.151	C 10.1
	~3	D	11.8[d]	F		
SOD	273	A	7.1	E		
	260	D	——	F		

a) A: 25 mM Phosphate buffer. B: Distd. water. C: 25 mM Glycine buffer. D: D_2O.

b) E: Near-IR emission spectra of $^1O_2$. F: $^1O_2$-Oxidation of bilirubin (ref. 17).

c) Based on the rate consts. k_1 for $2\,O_2^- \rightarrow O_2 + O_2^{2-}$ ($k_1 = 10^2\,M^{-1}s^{-1}$; ref. 18) and k_3 for the reaction of SOD and O_2^- ($k_3 = 2 \times 10^9\,M^{-1}s^{-1}$; ref. 19).

d) pD value (see ref. 17).

constants of such antioxidants with superoxide (O_2^-) (*Cypridina* chemiluminsecence method)[8-11] and applied the method to some biologically active compounds, such as amino acids, peptides, and proteins; anticancer catechins from tea-leaves; and oxygen-stress relievers at ischemia-reperfusion (chlorophyll derivatives).[12-16]

[*Cypridina* chemiluminsecence method][8-10] A method for measuring reaction rate constants of antioxidants with O_2^- was developed using a quenching experiment of chemiluminescence (CL) of *Cypridina* luciferin analogue (CLA) and O_2^- by SOD. The values of the reaction rate constant k_2 were determined for some chemiluminescent compounds including CLA (Table 1).

At stationary state, the Stern-Volmer equation can be shown as Equation 2 for the reactions 3−5 (Fig. 5).

$$I_0/I = 1 + \{k_3/(k_1[O_2^-] + k_2[CLA])\}\ [Q] \qquad (2)$$

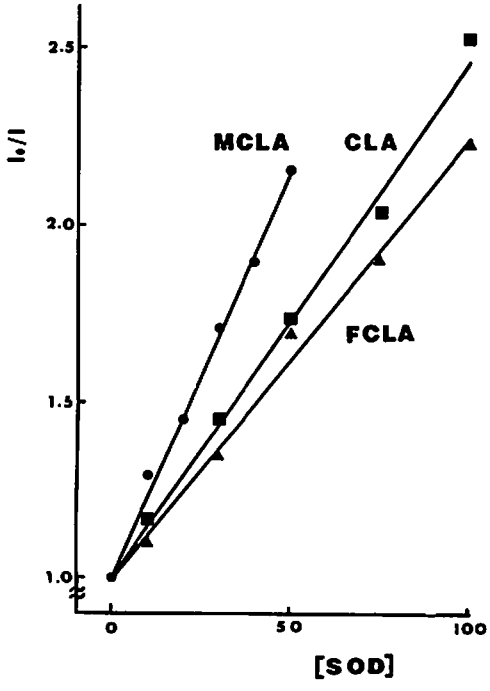


Fig.5 Stern-Volmer Plots for I_0/I-SOD/CLA, MCLA, or FCLA/O_2^-.

$$2\,O_2^- \xrightarrow{k_1} O_2 + O_2^- \qquad (3)$$

$$CLA + O_2^- \xrightarrow{k_2} P(\text{Oxyluciferin}) + h\nu \qquad (4)$$

$$Q + O_2^- \xrightarrow{k_3} O_2 + O_2^{2-} \text{ (or Product(s))} \qquad (5)$$

(Q: quencher or SOD)

where I_0/I is ratio of the emission intensity of CLA with O_2^- in the absence and presence of the quencher, and k_1, k_2, and k_3 are rate constants of the disappearance (3) and reactions with CLA (4) and with quencher (Q)(5) of O_2^-, respectively. By using k_2 value of CLA, k_3 values for quenching O_2^- with Q have been determined from these values. The results were shown in Table 2.

Equation 2 indicates that a plot of I_0/I vs [Q] gives a straight line with a slope equal to $k_3/(k_1[O_2^-] + k_2[CLA])$. From these values, k_3 or k_2 for quenching O_2^- with Q can be determined if the values of $k_1[O_2^-]$ and k_2 [CLA] are known. The values for k_1, k_2, and [CLA] are known, but those for $[O_2^-]$ and $k_1[O_2^-]$ are unknown. In order to eliminate the unknown terms, the I_0/I was plotted for two different concentration of CLA. From the slopes (A and B) of two straight lines plotted, each value of k_3 or k_2 can be calculated.

$$k_3/(k_1[O_2^-] + k_2[CLA]_1) = A \qquad (6)$$

$$k_3/(k_1[O_2^-] + k_2[CLA]_2) = B \qquad (7)$$

Therefore,

$$k_3 = k_2([CLA]_1 - [CLA]_2)/(1/A - 1/B) \qquad (8)$$

Many compounds are proven to have comparative antioxidative activity to ascorbic acid.[12-16]

Table 2. Rate Constants of Some Quenchers with O_2^{-} [a]

Substrates	$k_3/10^4$ $M^{-1}s^{-1}$	
	Our Results	Lit.[b]
(Sulfur compounds)		
$Na_2S \cdot 9H_2O$	23.9	150
$HSCH_2CH(NH_2)COOH$ (L-Cysteine: CySH)	28.4	270
$Me_2C(SH)CH(NH_2)COOH$ (DL-Pennycillamine)	12.4	
$HS(CH_2)_2CH(NH_2)COOH$ (DL-Homocysteine)	59.3	46
Glutathione (GSH)	66.5	67
$MeS(CH_2)_2CH(NH_2)COOH$ (L-Methionine)	6.42	0
$Me_2S^+(CH_2)_2COOH\ Br^-$ (Propiocethine)	0.184	
CyS-SCy (L-Cystine)	2.87	0
GS-SG (Glutathione oxidized)	3.02	0
$H_2N(CH_2)_2SO_3H$ (Taurine)	0.00	
(*Tea-leaf catechins*)		Rel.Act. AOA[c]
Epicatechin (EC)	1.25	1.00 1.00
Catechin (+C)	1.50	1.20
Gallocatechin (GC)	38.4	30.7
Epicatechin Gallate (ECg)	18.0	14.4 1.36
Epigallocatechin (EGC)	61.7	49.4 3.64
Epigallocatechin Gallate (EGCg)	74.9	59.9 3.73
Theaflavine (TF-1)	88.5	70.8
Theaflavine gallate (TF-2A)	36.5	29.2
Theaflavine gallate (TF-2B)	212.0	169.6
Theaflavine digallate (TF-3)	86.3	69.0
(Chlorophyll derivs.)		$IC_{50}/\mu g \cdot m\ell^{-1}$ [d]
Fe-chlorophyllin Na_3	——	6.4
Co-chlorophyllin Na_3	239	9.5
Ni-chlorophyllin Na_3	——	93
Cu-chlorophyllin Na_3	219	500
Mg-chlorophyllin Na_3	375	1400
Fe-chlorin e_6 Na_3	——	2.1
Zn-chlorin e_6 Na_3	347	850
Chlorin e_6 Na_3	536	>2500
Bovine SOD	220000[e]	0.28
Ascorbic acid (vitamin C)	299	2.7×10^5 [f]

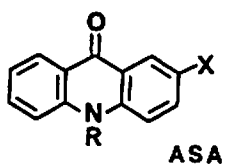
a) Based on the rate const., k_2 of CLA (see Table 1).
b) See ref. 20.
c) Antioxidative activity; see ref. 21.
d) SOD-mimic activity for oxygen stress relief (see ref. 22).
e) See ref. 19.
f) Rate constant ($M^{-1}s^{-1}$) at pH 7.4 (see ref. 23).

5 Oxidant Specificity of CLAs[27,28] and a Specific CL Agent, ASA[29,30]

Although *Cypridina* luciferin analogues (CLAs) have been said to react specifically with $^1O_2$ and O_2^-, no experimental evidence had been shown. When we examined the specificity of CLAs against representative oxidants, we found that CLAs certainly show specificity against $^1O_2$ and O_2^-; however, they give strong CL against some other oxidants. We have to say that some precaution is necessary for their experimental use.[27,28]

As shown in Table 2, SOD reacts with $^1O_2$ as well as O_2^-. Hence, the SOD addition effect could not be conclusive and we need the other additive experiments, such as NaN_3 or we need another chemiluminescence reagent specifically reactive to $^1O_2$ or O_2^-.

For this purpose, we developed a CL reagent, 9-acridone-2-sulfonic acid (ASA: Fig. 6), which gives CL specifically by O_2^-, not by $^1O_2$ and the others.[29,30] Unfortunately, this compound gives only weak CL in aqueous solution and some improvement is necessary in due course.



	R	X
ASA:	H	SO_3H
NHA:	H	H
NMA:	Me	H

Fig. 6 Superoxide-Sensitive Chemiluminescent Compounds (ASA and others).

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活性酸素種に対する抗酸化性（総説）

鈴木喜隆・金森悳雄・益子信郎・野本健雄・依田敏行

褐変、炎症、発ガン、突然変異、老化、死などの直接間接の原因となり、生体内や食品に大きな影響を与える活性酸素種の主要なメンバーであり、実際に与える害においても最も重要であると見做されている一重項酸素およびスーパーオキシドを物理・化学的に超微量で検出・定量する装置ならびに化学発光試薬を開発し、これら活性酸素種を抗酸化剤で消光する速度を測定する方法を考案した。さらにこの測定法を食品やその他の生理活性を有する物質に適用し、生理活性と「活性酸素に対する抗酸化性」との相関性を検討する方法論の開発を概説した。