Extra-Weak Chemiluminescence of Organ Homogenate and Blood in Tocopherol-Deficient Rats

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Summary Extra-weak chemiluminescence was detected in the organ homogenate and blood of tocopherol-deficient rats by use of a newly devised single photon counting apparatus. The spectrum distribution showed that the chemiluminescence had emission peaks at wavelengths between 500 and 650 nm corresponding to the simultaneous transition of singlet molecular oxygen \(^{1}O_2\), \(2[1\Delta_g] \rightarrow 2[3\Sigma_g^-]\). This chemiluminescence was quenched by the presence of free radical scavengers, butyl hydroxytoluene and \(d-\alpha\)-tocopherol. It was stimulated by \(D_2O\) and \(^{1}O_2\)-emission enhancer, 1,4-diazabicyclo[2,2,2]octane. The chemiluminescent intensities in tissues of rats fed a tocopherol-free diet for seven months were higher than those of rats fed a normal diet which contained 15 mg of tocopherol per 100 g of diet. The considerable increase of light emission was observed especially in liver, kidney, heart, lung and brain homogenates. The intensity of tocopherol-deficient liver chemiluminescence corresponded to \(22 \times 10^3\) photons per sec·cm\(^2\). The results indicated that the chemiluminescence was directly related to the generation of \(^{1}O_2\) involving free radical reactions in the tocopherol-deficient rat tissues.

Key Words tocopherol-deficiency, chemiluminescence, lipid peroxidation, singlet oxygen, free radical, thiobarbituric acid assay, hemolysis

Recent studies have revealed the close relationship of tissue damage, membranous lipid peroxidation, aging, evolution of free radicals, and particularly the participation of active oxygen analogues such as singlet oxygen \((^{1}O_2)\), superoxide and hydroxy radical in the deteriorative process of tissue lipids (1–4). The thiobarbituric acid (TBA) assay is one of the most commonly used methods for the determination of lipid peroxides. In this process malondialdehyde (MDA), the artificial product from lipid peroxides, is measured. However, no evidence has been presented that MDA could be found in all oxidizing systems (5, 6). The difficulties of measuring the lipid peroxides and free radicals in living tissues lie in studying the

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mechanism of tissue lipid peroxidation.

It is known that a very weak chemiluminescence is accompanied by the oxidation process of some organic compounds. The same chemiluminescent phenomenon can also be expected to occur under physiological conditions. We have recently discovered that rat blood and organ homogenates emit a spontaneous chemiluminescence which is enhanced by the progression of in vivo lipid peroxidation (7-9). A more recent report by Boveris et al. (10) has indicated that in situ and perfused rat livers emitted a spontaneous chemiluminescence which was increased by infusion of exogenous hydroperoxides. In that report the photon counting system employed was similar to ours. Tocopherol deficiency has been reported to cause a progress of lipid peroxidation in animal tissues (11-13). Thus, the measurement of chemiluminescence of tocopherol-deficient rat tissues would be interesting with respect to the antioxidative nature of tocopherol.

This paper deals with the characteristics of extra-weak chemiluminescence which is spontaneously emitted from organ homogenates and blood of tocopherol-deficient rats.

MATERIALS AND METHODS

Animals and diets. Male Wistar rats\(^1\) weighing about 100 g were fed either commercial pellet rations\(^2\) containing 15 mg of tocopherol per 100 g of diet (normal diet) or tocopherol-free rations\(^3\) which basically consisted of similar components as the normal diet except for the absence of tocopherol. After feeding for seven months, rats were dissected under ether anesthesia. Blood was collected via heart puncture, and organs were excised. Ten percent (w/v) organ-physiological saline homogenates were prepared with a glass homogenizer under ice cold conditions, and then they were immediately submitted to chemiluminescent assay.

Measurement of extra-weak chemiluminescence. A synchronous single photon counting apparatus, Chemiluminescence Analyzer OX-7C experimentally manufactured by Tohoku Electronic Industries Co. (Sendai, Japan), was used. A Hamamatsu R878 photomultiplier with spectral response range of 300–650 nm was equipped and kept at \(-20^\circ\text{C}\) using a thermoelectronic cooler. A phototube output was connected to an amplifier-discriminator, adjusted for the synchronized single photon counting method (14) and connected to a frequency counter and a digital recorder. Then 2 ml of whole blood or 5 ml of organ homogenates were placed on a stainless-steel plate (5.3 cm diameter, 1.3 cm height, and 22.1 cm\(^2\) surface) and analyzed at 37\(^\circ\text{C}\) for 10 min. The emission intensity was expressed in terms of average counts per 10 sec of the 10-min measurement and corrected for background current (below 10 counts/10 sec).

\(^1\) From Nihon Rat Co., Urawa, Japan.
\(^2\) From Funabashi Farms Co., Chiba, Japan.
\(^3\) From Oriental Kobo Kogyo Co., Tokyo, Japan.
The spectral characteristics of observed emission were measured by interposing various Toshiba sharp-cut filters between the sample container and the photomultiplier. Tocopherol-deficient liver (500 mg) was homogenized in 4.5 ml of 0.9% NaCl-D2O solution, and the homogenate was subjected to filter spectrum analysis. The filters (Toshiba filter number, 50% transmittance wavelength in nm, and % transmittance) were as follows: (V-Y 50, 500, 81.4), (V-O 53, 530, 76.4), (V-O 55, 550, 62.5), (V-O 58, 580, 38.6), (V-R 61, 610, 16.8), (V-R 63, 630, 7.67) and (IRP-50, 400–650, 48.3). The typical wavelength variation of relative sensitivity of the phototube was (wavelength in nm and relative sensitivity): (515, 1), (540, 0.667), (565, 0.5), (590, 0.333), (620, 0.2) and (635, 0.117).

General assays. The degree of hemolysis induced by hydrogen peroxide was estimated by the method of Ikehata et al. (15) using a JASCO UVIDEC-320 spectrophotometer. The tocopherol content of plasma and the organs was determined by the procedure of Abe et al. (16, 17) using a Hitachi 635 high performance liquid chromatography equipped with a Hitachi 650-10S fluorescence spectrophotometer, column: LiChrospher SI-100 (Merck), mobile phase: isopropanol–n-hexane (0.5:99.5, v/v), flow rate 1.1 ml/min, internal standard: 2,2,5,7,8-pentamethyl-6-hydroxy chroman. The TBA-positive material in plasma and the organs was measured by the method of Yagi (18) and Ohkawa et al. (19), respectively, using a Hitachi 204 fluorescence spectrophotometer and a JASCO UVIDEC-320 spectrophotometer.

Chemicals. d-a-Tocopherol was purchased from Eisai Co. Butyl hydroxytoluene and 1,4-diazabicyclo[2,2,2]octane were obtained from Tokyo Kasei Kogyo Co. D2O was purchased from Merck. Other reagents were of analytical grade.

RESULTS

Spectral distribution of tocopherol-deficient liver chemiluminescence

The spectral distribution of the chemiluminescence of tocopherol-deficient liver homogenized in 0.9% NaCl-D2O was measured to characterize the emission species. When the filters, IRP 50 (400–650 nm, transmittance 48.3%) and V-Y 50 (500–650 nm, transmittance 81.4%), were used, the transmitted emission corresponded to about 100 and 96% of the total chemiluminescence, respectively, after correction with filter transmittance and phototube sensitivity. This indicated that the major part of the present chemiluminescence had a wavelength region lying between 500 and 650 nm. As shown in Fig. 1, there were three main emission bands at 500–550, 580–610, and 630–650 nm in the wavelength region of 500–650 nm. The red light chemiluminescence band around 630–650 nm gave the greatest contribution to total photoemission observed.

Effects of 1O2-emission stimulants and free radical scavengers on chemiluminescence

To further elucidate the participants of light emission, the effects of 1O2-emission stimulants, e.g. 1,4-diazabicyclo[2,2,2]octane (DABCO) and D2O, and free

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Fig. 1. Filter spectrum distribution of chemiluminescence in tocopherol-deficient liver. Tocopherol-deficient rat liver (500 mg) homogenized with 4.5 ml of 0.9% NaCl-D_2O was used for the measurement. The explanation of filter spectrum analysis is described in MATERIALS AND METHODS. The light transmitted by the filters is expressed as relative percentage of 630–650 nm light emission detected by the photomultiplier after correcting for peak transmission of each filter and phototube sensitivity at each wavelength.

Fig. 2. Effect of 1O_2-emission stimulants and free radical scavengers on chemiluminescence of tocopherol-deficient liver. (A) Tocopherol-deficient liver, 500 mg, homogenized with 4.5 ml of physiological saline, (B) tocopherol-deficient liver, 500 mg, homogenized with 4.5 ml of 0.9% NaCl-D_2O. Abbreviations: DABCO, 1,4-diazabicyclo[2,2,2]octane (100 μM); BHT, butyl hydroxytoluene (100 μM); Toc, d-α-tocopherol (200 μM).

radical scavengers, e.g. butyl hydroxytoluene (BHT) and d-α-tocopherol, were studied using the liver homogenate prepared from tocopherol-deficient rats. As shown in Fig. 2-A, the chemiluminescence was stimulated by the addition of DABCO, and effectively quenched by the presence of BHT. When the tocopherol-deficient liver was homogenized with D_2O-substituted physiological saline, the emission intensity was markedly enhanced (Fig. 2-B), and this chemiluminescence was diminished by the addition of tocopherol.

**Chemiluminescence of organ homogenates in tocopherol-deficient rats**

The chemiluminescent intensities of organ homogenates, α-tocopherol contents

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Table 1. Organ chemiluminescent intensity, α-tocopherol content and TBA reactants in rats bred on a normal diet and a tocopherol-free diet for seven months.a

| Organ     | Group   | α-Tocopherol content (µg/g wet wt.) | Chemiluminescent intensity (counts/10 sec) | TBA reactants (nm MDA/g wet wt.) |
|-----------|---------|-------------------------------------|-------------------------------------------|----------------------------------|
| Liver     | Normalb | 20.0 ± 3.4                          | 42 ± 16                                   | 844 ± 194                        |
|           | Toc-freec | 0.2 ± 0.1d                          | 173 ± 28a                                | 999 ± 184                        |
| Kidney    | Normal  | 11.2 ± 2.4                           | 120 ± 27                                 | 544 ± 79                         |
|           | Toc-free | 0.2 ± 0.1d                          | 334 ± 56e                                | 828 ± 372                        |
| Heart     | Normal  | 28.2 ± 3.5                           | 137 ± 44                                 | 486 ± 118                        |
|           | Toc-free | 0.4 ± 0.1d                          | 445 ± 147e                               | 651 ± 196                        |
| Lung      | Normal  | 28.2 ± 4.9                           | 294 ± 56                                 | 382 ± 54                         |
|           | Toc-free | 0.4 ± 0.1d                          | 576 ± 158e                               | 462 ± 76                         |
| Brain     | Normal  | 11.8 ± 1.1                           | 76 ± 46                                  | 851 ± 358                        |
|           | Toc-free | 2.4 ± 0.3d                          | 182 ± 44e                                | 1,160 ± 411                      |
| Testes    | Normal  | 14.0 ± 3.3                           | 94 ± 57                                  | 581 ± 230                        |
|           | Toc-free | 0.3 ± 0.1d                          | 123 ± 62                                 | 970 ± 302                        |
| Adipose tissue | Normal | 37.9 ± 7.6                          | 119 ± 29                                 | 1,347 ± 479                      |
|           | Toc-free | 0.8 ± 0.1d                          | 162 ± 46                                 | 1,437 ± 568                      |

a Means ± SD for five rats. b Normal diet group. c Tocopherol-free diet group. d p < 0.01. Between normal and tocopherol-free diet groups. e p < 0.05. Between normal and tocopherol-free diet groups.

and TBA reactants in the rats bred on a normal diet and a tocopherol-free diet are shown in Table 1. By the feeding of a tocopherol-free diet for seven months, α-tocopherol contents were significantly decreased as compared with those of the normal diet group. γ-Tocopherol existed in normal diet rats, amounting to 0.81 µg/g in liver, but this was not detected after tocopherol deficiency. Even the organ homogenates of the normal diet group emitted an extra-weak spontaneous chemiluminescence. The emission of liver homogenate in the normal group had an intensity corresponding to about 5.5 × 10³ photons/sec·cm². In the tocopherol-free diet group, the chemiluminescent intensities of organ homogenates were significantly enhanced. A considerable increase in chemiluminescence was especially observed in liver, heart, kidney, lung and brain homogenates. This increase was found to be 2.4–4.1-fold of the normal group. The intensity of tocopherol-deficient liver chemiluminescence corresponded to 22.0 × 10³ photons/sec·cm². The TBA reactants tended to increase with the tocopherol deficiency.

Chemiluminescence of blood in tocopherol-deficiency rats

The chemiluminescent intensities, α-tocopherol contents, TBA reactants and hemolytic percentages of blood in the rats bred on a normal diet and a tocopherol-free diet are shown in Table 2. In the tocopherol-deficient rats, the α-tocopherol contents were significantly less, and the chemiluminescent intensities and TBA
reactants tended to increase. \( \alpha \)-Tocopherol which existed at the level of 30 \( \mu g/100 \text{ml} \) in plasma of the normal diet group ceased to exist with tocopherol deficiency. The degree of hemolysis was considerably greater in red blood cells of the tocopherol-free diet group.

**DISCUSSION**

We first noted that the progression of *in vivo* lipid peroxidation induced by the feeding of autoxidized oil to rats could be monitored by the measurement of chemiluminescence of blood and organ homogenates, in which a highly sensitive photon counting apparatus was used (7–9).

In the present investigation, we studied the chemiluminescent nature of tocopherol-deficient rat tissues. Generally, deteriorative lipid peroxidation is found in the tocopherol-deficient animal tissues, and the evidence has been shown as a function of TBA reactants (20, 21), fluorescent pigments (13), and hemolytic percentages (22).

The generation of \( ^1\text{O}_2 \) as the major light-emitting species was confirmed from the filter spectrum distribution of chemiluminescence (Fig. 1). The spectrum showed three emission bands in the visible wavelength region corresponding to the simultaneous transition of dimol \( ^1\text{O}_2, 2[1\Delta_g] \rightarrow 2[3\Sigma_g^-] \), with a vibrational quantum number of (2, 0) for 512–530 nm, (1, 0) for 570–595 nm, and (0, 0) for 620–640 nm, respectively (23). This information indicates the chemiluminescence directly reflects the \( ^1\text{O}_2 \) generation. The chemiluminescence was quenched effectively by BHT and tocopherol, while on the other hand, it was stimulated by DABCO and \( \text{D}_2\text{O} \) (Fig. 2). This demonstrates the involvement of \( ^1\text{O}_2 \) and free radicals in the chemiluminescence of tocopherol-deficient tissues. It is known that BHT scavenges free radicals (6), tocopherol quenches \( ^1\text{O}_2 \) and free radicals (24, 25), DABCO stimulates \( ^1\text{O}_2 \)-dimol emission (26), and \( \text{D}_2\text{O} \) has an elongative effect on the lifetime of \( ^1\text{O}_2 \) (27). \( ^1\text{O}_2 \) is a very powerful and specific oxidant whose chemical action is related to harmful biological effects as in the photodynamic reaction, lipid peroxidation,

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hemolysis of erythrocytes, destruction of the enzymes and nucleic acids, etc. (2).

As revealed in Tables 1 and 2, the tissue chemiluminescent intensities were increased evidently in the tocopherol-deficient rats. The enhancement of chemiluminescence was accompanied by the accumulation of TBA reactants, the decrease of tocopherol contents, and the increase of hemolytic percentages. This fact implies that tissue deterioration induced by tocopherol deficiency is adequately detected by the present chemiluminescent technique. The chemiluminescent phenomenon has been observed in several in vitro systems, such as NADPH-induced microsomal lipid peroxidation (28, 29), ascorbic acid-induced microsomal lipid peroxidation (29), xanthine oxidase and aldehyde oxidase (30), linoleate lipoygenase (31), and hydroperoxide-supplemented cytochrome c (26).

In conclusion, the evidence presented here may provide support for the use of the chemiluminescent technique as a monitoring method for tissue lipid peroxidation accompanied the formation of $^{1}$O$_2$ and free radicals in animal tissues.

REFERENCES

1) Pryor, W. A. (1978): The formation of free radicals and the consequence of their reactions in vivo. Photochem. Photobiol., 28, 787–801.
2) Krinsky, N. I. (1979): Biological roles of singlet oxygen, in Singlet Oxygen, ed. by Wasserman, H. H., and Murray, R. W., Academic Press, New York, pp. 597–641.
3) Willson, R. L. (1979): Hydroxy radicals and biological damage in vitro: What relevance in vivo? in Oxygen Free Radicals and Tissue Damage, Chiba Foundation Symposium 65, Excerpta Media, New York, pp. 19–42.
4) Bors, W., Saran, M., Lengfelder, E., Michel, C., Fuchs, C., and Frenzel, C. (1978): Detection of oxygen radicals in biological reactions. Photochem. Photobiol., 28, 629–638.
5) Gray, J. I. (1978): Measurement of lipid peroxidation: A review. J. Am. Oil Chem. Soc., 55, 539–546.
6) Logani, M. K., and Davies, R. E. (1980): Lipid oxidation: biologic effects and antioxidants—a review. Lipids, 15, 485–495.
7) Miyazawa, T., Kaneda, T., Takyu, C., Yamagishi, A., and Inaba, H. (1979): Chemiluminescence of blood and tissue homogenates in the autoxidized oil-fed rats. Proc. Jpn. Conf. Biochem. Lipids, 21, 366–369.
8) Miyazawa, T., and Kaneda, T. (1980): Estimation of the proceeding of tissue lipid peroxidation by the measurement of chemiluminescence. J. Am. Oil Chem. Soc., 57, 128A.
9) Miyazawa, T., Kaneda, T., Yoda, B., Goto, Y., Takyu, C., Yamagishi, A., and Inaba, H. (1980): The changes in tissue chemiluminescence of rats administered with oxidized oil. Proc. Jpn. Conf. Biochem. Lipids, 22, 222–224.
10) Boveris, A., Cadenas, E., Reiter, R., Filipkowski, M., Nakase, Y., and Chance, B. (1980): Organ chemiluminescence: noninvasive assay for oxidative radical reactions. Proc. Natl. Acad. Sci. USA, 77, 347–351.
11) Rieley, C. A., Cohen, C., and Lieberman, M. (1974): Ethane evolution, a new index of lipid peroxidation. Science, 183, 208–210.
12) Hafeman, D. G., and Hoekstra, W. G. (1977): Lipid peroxidation in vivo during vitamin E and selenium deficiency in the rat. J. Nutr., 107, 666–672.
13) Desai, I. D., Fletcher, B. L., and Tappel, A. L. (1975): Fluorescent pigments from uterus of vitamin E-deficient rats. *Lipids*, **10**, 307–309.

14) Shimizu, Y., Inaba, H., Kumai, K., Mizuno, K., Hata, S., and Tomioka, S. (1973): Measuring methods for ultra-low light intensity and their application to extra-weak spontaneous bioluminescence from living tissues. *IEEE Trans. Instrum. Meas.*, **IM-22**, 153–157.

15) Ikehata, H., Koyanagi, K., and Wakaizumi, M. (1968): Studies on the hemolysis caused by vitamin E deficiency. II. Hemolysis test with hydrogen peroxide. *Vitamins* (in Japanese), **37**, 37–43.

16) Abe, K., and Katsui, G. (1975): Determination of tocopherols in serum by high speed liquid chromatography. *Vitamins* (in Japanese), **49**, 259–263.

17) Abe, K., Ohmoe, M., and Katsui, G. (1976): Rapid and micro-method for the determination of tocopherols in liver. *Vitamins* (in Japanese), **50**, 453–457.

18) Yagi, K. (1976): A simple fluorometric assay for lipoperoxide in blood plasma. *Biochem. Med.*, **15**, 212–216.

19) Ohkawa, H., Onishi, N., and Yagi, K. (1979): Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.*, **95**, 351–358.

20) Zalkin, H., and Tappel, A. L. (1960): Studies on the mechanism of vitamin E action. IV. Lipid peroxidation in the vitamin E-deficient rabbit. *Arch. Biochem. Biophys.*, **88**, 113–117.

21) Kornbrust, D. J., and Mavis, R. D. (1980): Relative susceptibility of microsomes from lung, heart, liver, kidney, brain and testes to lipid peroxidation: Correlation with vitamin E content. *Lipids*, **15**, 315–322.

22) Bunyan, J., Green, J., Edwin, E. E., and Diplock, A. T. (1960): Studies on vitamin E. 5. Lipid peroxidation in dialuric acid-induced haemolysis of vitamin E-deficient erythrocytes. *Biochem. J.*, **77**, 47–51.

23) Inaba, H., Shimizu, Y., Tsuji, Y., and Yamagishi, A. (1979): Photon counting spectral analyzing system of extra-weak chemi- and bioluminescence for biochemical applications. *Photochem. Photobiol.*, **30**, 169–175.

24) Fragata, M., and Bellemare, F. (1980): Model of singlet oxygen scavenging by \( \alpha \)-tocopherol in biomembranes. *Chem. Phys. Lipids*, **27**, 93–99.

25) McCoy, P. B., Fong, K.-L., Lai, E. K., and King, M. M. (1978): Possible role of vitamin E as a free radical scavenger and singlet oxygen quencher in biological systems which initiate radical-mediated reactions, in *Tocopherol, Oxygen, and Biomembranes*, ed. by de Duve, C., and Hayaishi, O., Elsevier/North-Holland Biomedical Press, Amsterdam, pp. 41–57.

26) Cadenas, E., Boveris, A., and Chance, B. (1980): Low-level chemiluminescence of hydroperoxide-supplemented cytochrome c. *Biochem. J.*, **187**, 131–140.

27) Kruk, I., Lichszteld, K., and Michalska, T. (1979): The extra-weak spontaneous chemiluminescence during decomposition of hydrogen peroxide. *Z. Phys. Chemie. Leipzig*, **260**, 371–375.

28) Nakano, M., Noguchi, T., Sugioka, K., Fukuyama, H., Sato, M., Shimizu, Y., Tsuji, Y., and Inaba, H. (1975): Spectroscopic evidence for the generation of singlet oxygen in the reduced nicotinamide adenine dinucleotide phosphate-dependent microsomal lipid peroxidation system. *J. Biol. Chem.*, **250**, 2404–2406.

29) Wright, J. R., Rumbaugh, R. D., Colby, H. D., and Miles, P. R. (1979): The relationship between chemiluminescence and lipid peroxidation in rat hepatic microsomes. *Arch. Biochem. Biophys.*, **192**, 344–351.

30) Arneson, R. M. (1970): Substrate-induced chemiluminescence of xanthine oxidase and *J. Nutr. Sci. Vitaminol.*
aldehyde oxidase. Arch. Biochem. Biophys., 136, 352–360.

31) Nakano, M., and Sugioka, K. (1977): Mechanism of chemiluminescence from the linoleate-lipoxygenase. Arch. Biochem. Biophys., 181, 371–383.