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Generation of Superoxide Anion Radical from Atmospheric Organic Matter

Nobuyuki Takeda and Kiyoshi Teranishi

Public Health Institute of Hyogo Prefecture, Arata-cho 2-1, Hyogo-ku, Kobe 652, Japan

Atmospheric organic matter(AOM) derived from suspended particulates is carcinogenic to mouse skin(Hoffman and Wynder 1968). For assessment of polluted air to human health, various chemical and biological quantitative determinations have been carried out(Hughes et al. 1980, Chrisp and Fisher 1980), however, AOM contains several hundred organics that have not yet been tested individually for carcinogenic activity and only 30-40% of the components has been identified(Van Cauwenberghe and Van Vaeck 1983). Recently, much attention has been focused on a relation between oxygen radical and carcinogenesis(Ames 1983). Nakayama et al. (1983 and 1984) suggested a possible role of free radicals and subsequently formed active oxygens such as superoxide anion radical(O_2^-), hydrogen peoxide(H_2O_2) and hydroxyl radical(·OH) in aromatic amine and tabacco carcinogenesis, in both the initiation and promotion steps. Furthermore, these oxygen radicals have long been thought to be involved in cancer caused by radiation. It is, therefore, currently important to clarify whether AOM generates oxygen radicals or not. In this report, we demonstrate the generation of superoxide anion radical (O_2^-) from AOM and describe the contribution of the acidic fraction of AOM to O_2^- generation.

MATERIALS AND METHODS

Suspended particulate matter(SP) was collected at the roof of our institute(Kobe, Japan) by a high-volume air sampler for 24h in August, 1986. AOM was extracted with benzene/ethanol(4:1) by a Soxlet extractor. The solution was evaporated to dryness and the residue(AOM) was dissolved in 2.5ml of chloroform/methanol(2:1). The sample solution waf stored at -20℃. Mean value ± S.D. was 55±24μg/m³ for atmospheric SP level and 6.5±2.8μg/m³ for AOM. SP contained 12.8±5.5% of AOM. (Table 1). AOM was separated into neutral plus basic, ether-soluble and water-soluble acidic fractions by acid-base partitioning. AOM(52mg) was transferred into a separatory funnel with 200ml of both diethyl ether and 0.1N-NaOH, and shaken for 5min. The organic layer was washed, dried and evaporated(the neutral+basic fraction). Aqueous layer was acidified with 10ml of 1N-HCl and extracted twice with 30ml of diethyl ether. The ether layer was dried and evaporated(the ether-soluble acidic fraction). The aqueous layer was passed through SEP-PAK C_{18} cartridge(Waters, U.S.A.). The cartridge was washed with 5ml of water. The water-soluble acidic fraction was eluted with 5ml of methanol. O_2^- was detected by reduction of nitro blue tetrazolium(NBT) according to the method of Nakayama et al. (1984). An appropriate amount of AOM and

Table 1. Daily variation of atmospheric SP and AOM level

Date	Air (m^3)	SP (mg)	SP/Air ($\mu g/m^3$)	AOM (mg)	AOM/Air ($\mu g/m^3$)	AOM/SP (%)
8/01	2592	125	48.2	11.60	4.48	9.3
/04	2447	60	24.5	15.77	6.44	26.3
/05	2486	79	31.8	10.16	4.09	12.9
/06	2462	198	80.4	30.55	12.41	15.4
/07	2507	171	68.2	15.79	6.30	9.2
/18	2582	128	49.6	13.87	5.37	10.8
/19	2615	120	45.9	11.70	4.47	9.8
/20	2534	107	42.2	13.10	5.17	12.2
/21	2498	250	100.1	23.56	9.43	9.4

its individual fraction were redissolved in dimethyl sulfoxide. The solution(0.1ml) was added to 3ml of 100mM sodium phosphate buffer (pH7.4) containing 50μM NBT, 100μM EDTA and 0.06% Triton X-100 in a photometric cell, mixed immediately and incubated at 37℃. Blue-formazan which is the reduction product of NBT was measured by reading the absorbance at 560nm. To determine the acid component of AOM, the substance was dissolved in 2ml of diethyl ether/ethanol(2:1) and titrated with 0.1N-KOH ethanolic solution using phenolphthalein as an indicator. It was expressed as acidity(μl of 0.1N-KOH ethanolic solution required to neutralize 1mg of AOM).

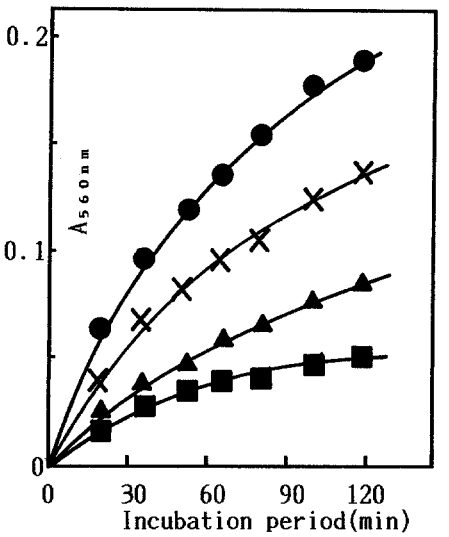
RESULTS AND DISCUSSION

To test whether or not AOM can generate O_2^-, the substance(0.3-1.2mg) was incubated with NBT at 37℃ up to 120min and the detection of O_2^- was attempted by measuring blue-formazan. As shown in Fig.1, the absorbance at 560nm increased proportionally with the amount of AOM added and the time incubated. The result indicates that AOM can reduce NBT to formazan, however, it was unknown whether the formation was due to the action of O_2^- generated or the organic component itself. The effect of superoxide dismutase(SOD, Sigma) was examined on the yield of the formazan in order to determine the reducing species. This is because the emzyme is known to scavenge the active oxygen as follows:

$$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

As shown in Fig.2, SOD decreased the yield of the product and completely inhibited the formation at the dose of 2μg. This result clearly demonstrates that the fromazan was produced by the action of O_2^- generated from the component of AOM via some chemical process. Thus, AOM was shown to be O_2^- generator under the physiological conditions.

AOM was separated into three chemical classes(the neutral+basic, the ether-soluble and the water-soluble acidic) and the individual fraction was studied for the contribution to O_2^- generation of the crude mixture. Results are shown in Table 2. Approximately a half amount of the organic material was recovered, on the contrary, only one fifth was did for the O_2^- generation. The difference between these recovery rates suggests the presence of some synergism of the individual component in the crude mixture and/or chemical denature of the component responsible for the O_2^- generation by that fractionation. The ether- and the water-soluble acidic fractions accounted for 80% of the sum of the organics recovered and did for almost all of the O_2^- generation



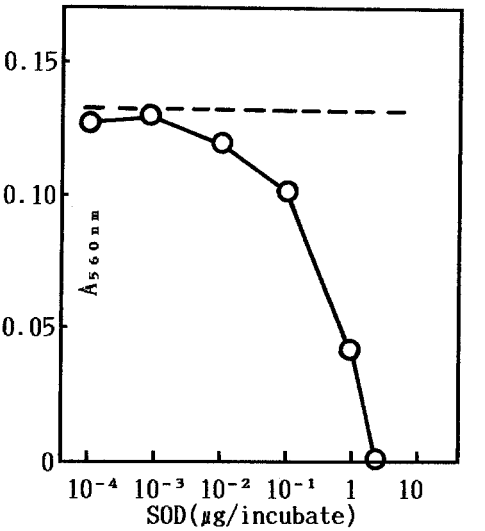


Figure 1. (left) Reduction of NBT by AOM. ● :1.2mg AOM/incubate, ✕ : 0.9mgAOM/incubate, ▲ :0.6mgAOM/incubate, ■ :0.3mgAOM/incubate. AOM used for the experiments shown in Figs. 2 and 3, and Table 2 was collected during August 11-15 and 25-29, 1986.

Figure 2. (right) Effect of superoxide dismutase(SOD) on the reduction of NBT by AOM. - - - - - - : without SOD, O——O : with SOD. SOD from bovine erythrocyte(Sigma) was used. An aliquot(20μl) of the diluted enzyme solution was added. The mixture containing 1mg of AOM was incubated for 90min both with and without SOD.

Table 2. Fractionation of AOM and O_2^- generation

Fraction	Weight mg	Weight %	Superoxide Radical A_{560nm}/mg/90min	Superoxide Radical %Contribution
Crude mixture(AOM)	52.0	100	0.159	100
Neutral+Basic	6.4	12.4	0.007	0.5
Ether-soluble acidics	17.8	34.2	0.029	5.8
Water-soluble acidics	5.0	9.6	0.237	14.3
Sum of individual	29.2	56.2		20.6

A 1mg-aliquot of each fraction was determined for the O_2^- generation. Values for the contribution of the individual were expressed as a percentage of the activity of the crude mixture(AOM).

recovered. The rate of O_2^- generation was approximately 8 fold greater for the water-soluble acidic fraction than for the ether-solubles. Although the sum of the individual contribution was very low, the acidics water-soluble were shown to play the most inportant role in the O_2^- generation of AOM.

O_2^- generation by AOM was further studied for the dependency on the acid content. Daily levels of these two properties were measured and the correlation coefficient was calculated. As shown in Table 3, both the levels varied day by day widely. Mean value was 0.062 for O_2^-

Table 3. Daily variation of O_2^- generation and acidity of AOM.

Date	O_2^-(A_{560nm} /mg AOM)	Acidity(0.1N-KOHμl/mg AOM)
8/01	0.040	15.6
/04	0.030	8.1
/05	0.082	18.2
/06	0.132	25.3
/07	0.046	26.4
/18	0.064	15.1
/19	0.033	10.7
/20	0.024	8.1
/21	0.105	18.6

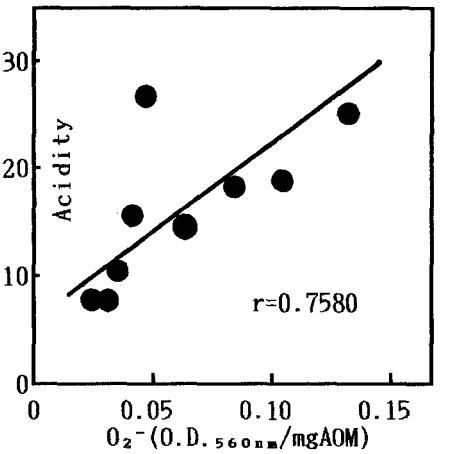


Figure 3.(right) Correlation between O_2^- generation and acidity of AOM. A 1mg-aliquot of AOM was used for both the determinations.

generation and 17.3 for the acid content. The coefficients of variation were 60.5% and 44.4%, respectively. The correlation coefficient was found to be 0.7580 and the value was statistically signifcant at $p<0.05$. Thus, O_2^- generation by AOM was demonstrated to be dependent on the amount of the acidic components of AOM (Fig. 3). The probable acidic components are polyphenols, because 1)in the separation step, a typical dark brown color of the alkarine solution of the acidic fraction disappeared on acidification, being suggested to be compounds of polyphenolic nature and 2)the authentic compounds are known to produce active oxygens(O_2^- and H_2O_2) by their autoxidation(Nakayama et al. 1984). These organic acidic materials should be further characterized and identified for the elucidaton of their effects for human health.

In the course of this study, carbonyl content of AOM was also examined for the relation to O_2^- generation, since these compounds seemed to generate the active oxygens(Levin et al. 1982). As a result, no significant correlation was observed($r=0.1339$, $p<0.05$). Some of them (alkanals and alkadienals) become potent O_2^- generators in the presence of xanthine oxidase(unpublished data). The extent of their contribution in vivo remains to be determined.

Although hazardous effect has been described in this report only for the organic portion of suspended particulates, the non-organics(more than 3/4 of SP, see Table 1) should also be considered as toxicant and/or synergists. One of them, iron ions are important as they may produce much stronger oxygen radicals(H_2O_2 and $OH\cdot$) from O_2^- via the Fentone type Harber-Weiss reaction as follows:

$$O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+}$$

$$H_2O_2 + Fe^{2+} \rightarrow \cdot OH + OH^- + Fe^{3+}$$

These radicals can cause lipid peroxidation(Tien and Aust 1982) and yield numerous toxic and mutagenic products such as lipid hydroperoxides, aldehydes and epoxides(Yamaguchi 1980, Yoshioka and Kaneda 1974). The deleterious reactions may indeed occur in lung, a target organ of SP, because it is rich in polyunsaturated lipids and the metal ions are abundant in SP. These oxidative damages also decreases cellular antioxidants such as glutathione, ascorbate and vitamin E.

The oxygen radicals directly or indirectly cause damage to DNA. Nakayama et al. (1985) reported that DNA single-strand breaks in human cells induced by cigarette smoke may be ascribed to active oxygens generated from cigarette smoke. The radical species can activate carcinogens such as benzo(a)pyrene and naphthylamine with or without peroxidized lipid radicals(Kodama 1985).

Mutagenic activity has long been used as an important index for the risk assessment of air pollution and numerous reports have been published for the mutagenicity of airborne organic materials. Recent findings show that both the initiation and promotion stages in carcinogenicity may involve oxidative damage to the organism caused by the active oxygens. Airborne particulate matter should be investigated for the quantitative estimation of the oxidative stress on human beings.

REFFERENCES

Ames BN (1983) Dietary carcinogens and anticarcinogens: oxygen radicals and degenerative diseases. Science 221:1256-1264

Chrisp CE, Fisher GL (1980) Mutagenicity of airborne particles. Mutation Res 76:143-164

Hughes TJ, Pellizzari E, Little L, Sparacino C, Kolber A (1980) Ambient air pollutants: collection, chemical characterization and mutagenicity testing. Mutation Res 76:51-83

Hoffman D, Wynder EL (1968) Chemical analysis and carcinogenic bioassay of organic particulate pollutats. In Stern AC(ed) Air Pollution, Vol.2, Academic Press, New York, p.230

Kodama M (1985) Active oxygens in carcinogenesis. Toxicology Forum 8:50-62

Levin DE, Hollstein M, Christman MF, Schwirs EA, Ames BN (1982) A new Salmonella tester strain(TA102) with A·T base pairs at the site of mutation detects oxidative mutagens. Proc Natl Acad Sci USA 79:7445-7449

Nakayama T, Kimura T, Kodama M, Nagata C(1983) Generation of hydrogen peroxide and superoxide anion radical from active metabolites of naphthylamines and aminoazo dyes: its possible role in carcinogenesis. Carcinogenesis 4:765-769

Nakayama T, Kodama M, Nagata C (1984) Generation of hydrogen peroxide and superoxide anion radical from cigarette smoke. Gann 75:95-98

Nakayama T, Kaneko M, Kodama M, Nagata C (1985) Cigarette smoke induceses DNA single-strand breaks in human cells. Nature 314:462-464

Tien M, Aust SD (1982) Comparative aspects of several model lipid peroxidation systems. In Yagi K.(ed) Lipid Peroxides in Biology and Medicene, Academic Press, New York, p.23

Van Cauwenberghe K, Van Vaeck L (1983) Toxicological implications of the organic fraction of aerosols: a chemist'view. Mutation Res 116: 1-20

Yamaguchi T (1980) Activation with catalase of mutagenicity of hydroperoxides of some fatty acids and hyrocarbons. Agric Biol Chem 44: 1989-1991

Yoshioka M, Kaneda T (1974) Studies of the toxicity of the autoxidized oils. Yukagaku 23:321-326

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