RAPID COMMUNICATION Jpn. J. Cancer Res. 83, 923–928, September 1992

High Correlation between Lipid Peroxide Radical and Tumor-promoter Effect: Suppression of Tumor Promotion in the Epstein-Barr Virus/B-Lymphocyte System and Scavenging of Alkyl Peroxide Radicals by Various Vegetable Extracts

Hiroshi Maeda,[1,4] Takato Katsuki,[2] Takaaki Akaike[1] and Ritsu Yasutake[3]

[1]*Department of Microbiology, Kumamoto University School of Medicine, Honjo 2-2-1, Kumamoto 860,* [2]*Department of Laboratory Medicine, College of Medical Science, Kumamoto University, Kuhonji 4-24-1, Kumamoto 860 and* [3]*Faculty of Education, Kumamoto University, Kurokami 2-40-1, Kumamoto 860*

We examined the ability of hot-water extracts of 66 vegetables and plants to suppress tumor promotion, as well as to scavenge lipid peroxide radicals *in vitro*. To assess the effect against tumor promotion (transformation) *in vitro*, we used the phorbol myristate acetate/Epstein-Barr virus/B-lymphocyte system. To assess the lipid radical-scavenging effect, the luminol-enhanced chemiluminescence method using the *tert*-butyl hydroperoxide/heme system was used, which generates more alkyl peroxide radical (ROO•) than alkyl (R•) and alkoxyl (RO•) radicals. The results showed a significant correlation between the anti-tumor-promoting effect and the lipid radical-scavenging effect ($r=0.82$). We found that boiled extracts of green leaves of carrot, crucifers, and beans (black bean, red bean, mung bean, and soybean) had the greatest anti-tumor-promoter and radical-scavenging activities. Cold-water extracts of vegetables generally exhibited only about 10% or less of the activity of the hot-water extracts.

Key words: Lipid peroxide radicals — Tumor promoter — EA antigen of EB virus — Hot-water extract of vegetable — Radical scavenger

Many reports on carcinogenesis indicate a significant role of oxygen radicals (e.g., superoxide, hydroxyl radical, and singlet oxygen) and alkyl peroxide-derived radicals (R•, RO•, ROO•).[1–4] Recently, the alkyl peroxide radical was shown to have a tumor-promoter effect[5,6]; this radical can be generated by the reaction between methemoglobin and alkyl hydroperoxide compounds (ROOH).[6,7] Alkyl hydroperoxide can be formed by reaction of oxyradicals (such as superoxide or hydroxyl radical) with lipids or by the oxidation of unsaturated lipids. Alkyl hydroperoxide radical was demonstrated recently to exhibit a potent cytotoxicity.[7] Oxygen radicals are known to damage DNA[8,9]; 8-hydroxydeoxyguanosine is a well known product of oxidative DNA damage.[10,11] Virus infection also causes activation of oxygen radical generation as the result of a host's over-reactive immune response, with deleterious consequences.[12–15]

Epidemiological studies have led to suggestions of a link between colorectal cancer and consumption of high-fat diets: fecal stasis that might occur in the sigmoid colon could be associated with lipid peroxidation reactions, and subsequent production of lipid radicals may result in epithelial mucous cell damage. A diet high in red meat (heme-containing) is also associated with a high incidence of colorectal cancer,[16–20] and alkyl hydroperoxides plus heme yield alkyl peroxide radicals, as we have recently demonstrated,[7] as follows:

$$Fe^{3+}=O\ (\text{porphyrin})+ROOH \rightarrow Fe^{4+}=O\ (\text{porphyrin})^{+\cdot}+ROH$$

$$Fe^{4+}=O\ (\text{porphyrin})^{+\cdot}+ROOH \rightarrow Fe^{4+}=O\ (\text{porphyrin})+ROO\cdot+H^{+}$$

In the present study we examined the lipid radical-scavenging potential of cold- or hot-water extracts of various vegetables by using an alkyl peroxide radical-generating system, which consisted of water-soluble *tert*-butyl hydroperoxide (*t*-BuOOH) as a representative alkyl hydroperoxide and methemoglobin.[7] These extracts of various fresh vegetables were also tested for their capacity to neutralize transformation of human B-lymphocytes by Epstein-Barr (EB) virus. In this system, the tumor promoter phorbol myristate acetate (PMA) and *n*-butyric acid were added to Raji cells, which harbor the EB virus. Raji cells are stimulated to produce greater amounts of early antigen (EA) in this system upon addition of PMA,[21,22] i.e., about 0.03% of the cells before addition vs. 30% after addition. EA may thus be regarded as a transformation-associated antigen corresponding to tumor antigen. Therefore, the ability of the extracts to suppress EA formation in the presence

[4] To whom correspondence should be addressed.

of PMA was interpreted as being due to the presence of anti-tumor-promoting activity in the vegetables.

The present study may provide important clues to two separate problems: one is whether the anti-tumor-promoter effect is correlated with lipid radical-scavenging capacity, i.e., whether the lipid radical (alkyl peroxide radical) functions as a tumor promoter. The second involves practical questions: which vegetables have high lipid radical-scavenging capacity and anti-tumor-promoter activity, and are there any effects of heat on the protective capacity of these vegetable extracts.

We confirmed by spin-trapping ESR spectroscopy that *tert*-butyl peroxide radical (an alkyl peroxide radical) is the major radical species generated in the model used in this study (*t*-BuOOH and hemoglobin/methemoglobin).[7] We chose *t*-BuOOH (Sigma Chemical Co., St. Louis) for the present study because other alkyl hydroperoxides such as α-linoleate were not sufficiently soluble in water. Anti-alkyl peroxide radical activity was measured by using a chemiluminescence multichannel analyzer (Berthold Model LB 9505 AT, Wildbad, Germany). Hemoglobin (methemoglobin) used in the experiment was purified from human red blood cells as described previously.[7] The assay mixture contained 250 μl of 0.01 *M* phosphate-buffered 0.15 *M* saline, 50 μl of 10 m*M* diethylenetriamine-pentaacetic acid, and 50 μl of 100 m*M* *t*-BuOOH; 50 μl of water extract sample and 50 μl of 100 μ*M* luminol were added and mixed well. Then, after addition of 50 μl of hemoglobin (1 mg/ml), the chemiluminescence assay was started. The rate, peak intensity, and peak area of chemiluminescence were recorded. The average chemiluminescence count was about 10^7 cpm in 5 min. The dilution factor of vegetable extracts which induced a 50% reduction in chemiluminescence intensity

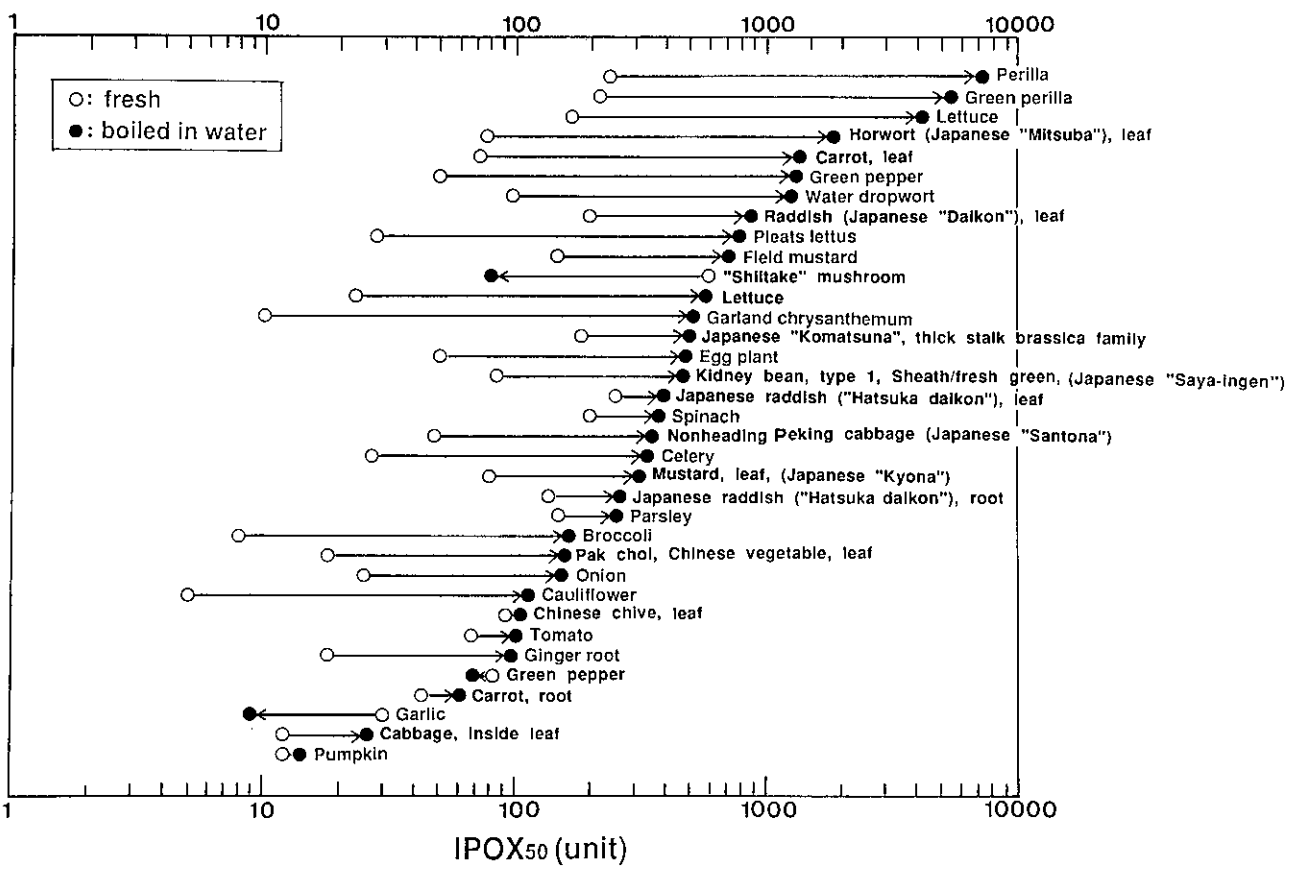

Fig. 1. Alkyl peroxide radical-scavenging activity of various vegetable extracts. A given amount of *t*-BuOOH (5.0 μmol) was reacted with the methemoglobulin / hemoglobin system, which generates predominately alkyl peroxide radicals.[7] Various cold-water vegetable extracts after homogenization or hot-water vegetable extracts after 5 min boiling were tested for anti-alkyl radical activity by the chemiluminescence method using luminol (see ref. 7). $IPOX_{50}$ is defined as the 50% inhibitory dilution (fold) of vegetable extracts toward alkylperoxide radical formation, as described in the text. Higher values indicate greater lipid radical-scavenging potency.

(peak height) was defined as one $IPOX_{50}$ (50% peroxide radical inhibitor activity) unit.

All vegetables were obtained reasonably fresh from local grocery stores. They were homogenized in a ceramic mortar and pestle at room temperature with 2 parts of vegetable to 3 parts of 0.01 *M* phosphate-buffered 0.15 *M* saline, pH 7.3 (by weight). Beans were milled to flour, which was soaked in water for a few hours before use; root vegetables were used after mincing. Samples were subjected to centrifugation with a clinical centrifuge for 10 min at 10,000*g* (Kubota KR-2000T, Kubota Inc., Tokyo); supernatants were filtered by using a membrane filter with a pore size of 0.45 μm before use as fresh materials. Hot-water extracts of these materials were prepared by boiling the vegetables in a 5-fold excess of water (w/w) for 5 min. Bean flours and minced root vegetables with a 5-fold excess of water (by weight) were boiled for 5 min followed by homogenization; supernatants after centrifugation (10,000*g*) were used as samples of hot-water extracts. All samples were added to the assay mixtures for both $IPOX_{50}$ and IEA_{50} (50% EA-inhibitory dilution) after appropriate dilution with physiological saline (thus all samples are buffered).

Assays of anti-tumor-promoter effect in the PMA/EB virus/B-lymphocyte system were described by Koshimizu *et al.*,[24,25] with EA as a marker.[22-24] Briefly, EB virus genome-carrying human B-lymphoblastoid Raji cells (5×10^5 cells/250 μl) were added to 50 μl of water extract samples. After addition of 250 μl of RPMI 1640 medium containing 10% fetal calf serum (Filtron Pty, Brooklyn, Australia), 40 μg of PMA, and 88 μg of *n*-butyrate, the cells were incubated for 36–40 h. EA values were compared with those in the presence of *n*-butyrate plus PMA, but without water extracts, as a positive control, in which about 30% of cells usually exhibited EA. The negative control (without both *n*-

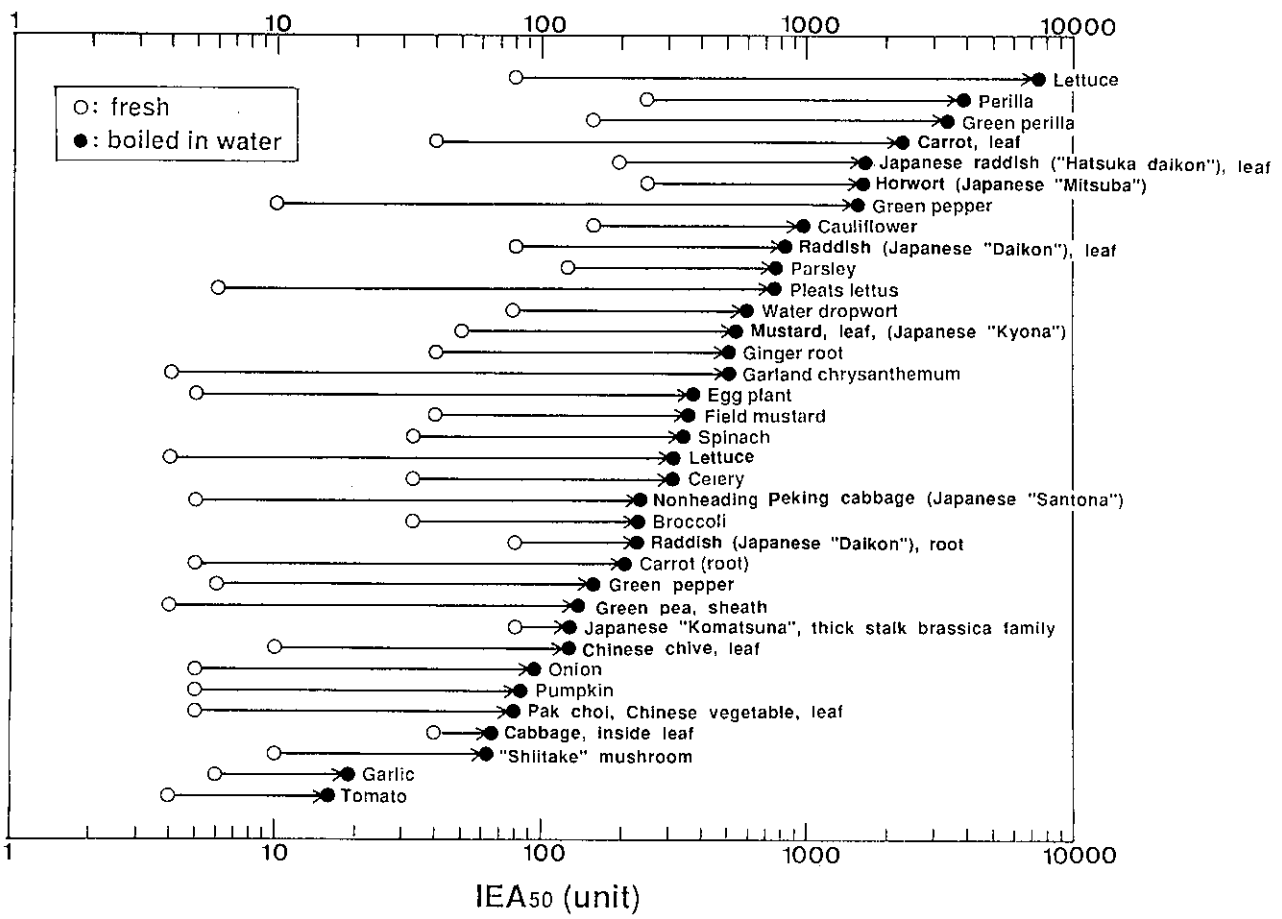

Fig. 2. Inhibitory activity of various vegetable extracts on EA formation (see Fig. 1 legend and assay system description in the text). IEA_{50} is defined as the 50% inhibitory dilution (fold) of vegetable extract toward EA-formation, as described in the text. Note the great increase in the values for heated extracts.

butyrate and PMA) usually showed about 0.03% positive cells. The dilution of samples required to reduce the efficiency of EA induction to half of the positive control, i.e. 15% in this case, is defined as one IEA_{50} unit.

The ability of 37 vegetable extracts to neutralize alkyl peroxide radical generation ($IPOX_{50}$) was tested by chemiluminescence measurement; the results are shown in Fig. 1. Note that 31 (about 90%) of the extracts

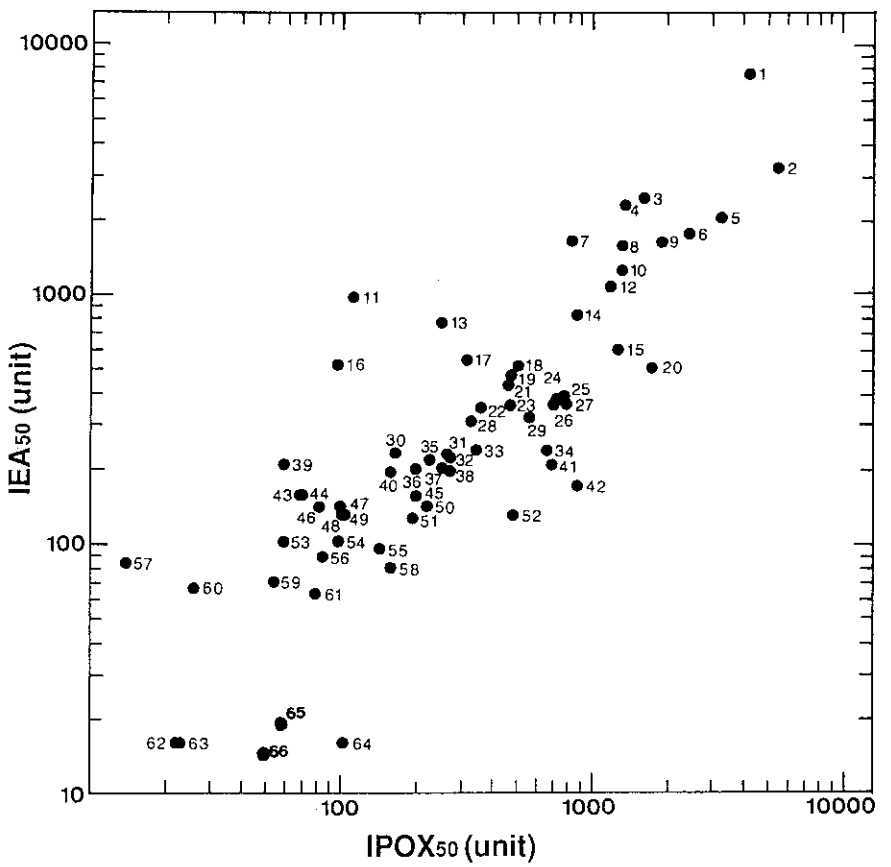

Fig. 3. Correlation between anti-tumor-promoter effect (IEA_{50}) and alkyl peroxide radical-scavenging effect ($IPOX_{50}$) of various hot-water extracts of vegetables, beans, mushrooms, and root vegetables. The correlation factor was 0.82. (1) Japanese lettuce ("chisha"), (2) green perilla, (3) black bean, (4) carrot leaf, (5) red bean (Japanese "azuki"), (6) mung bean (green bean), (7) radish (Japanese "daikon") leaf, (8) Japanese hot pepper ("shishito") leaf, (9) horwort (Japanese "mitsuba") leaf, (10) fern sprout with stalk ("warabi"), (11) cauliflower, (12) edible burdock ("kaori-gobo") root, (13) parsley, (14) Japanese radish ("hatsuka daikon") leaf, (15) water dropwort, (16) ginger root, (17) mustard leaf (Japanese "kyona"), (18) garland chrysanthemum, (19) soybean, (20) stalk of butlerbur, (21) kidney bean, type 1, sheath/fresh green (Japanese "saya-ingen"), (22) spinach, (23) eggplant, (24) cabbage, outside green leaf, (25) lotus root, (26) mustard leaf, (27) lettuce, (28) celery, (29) leaf lettuce (*Lactuca sativa*), (30) broccoli, (31) Japanese radish ("hatsuka daikon") root, (32) rape / brassica leaf (Japanese "mibuna"), (33) nonheading Peking cabbage (Japanese "santona"), (34) plum (Japanese "ume"), (35) taro root (Japanese "satoimo"), (36) kidney bean, type 2, fresh green/sheath (Japanese "ingen"), (37) potato, (38) Japanese hot pepper (Kyoto var. 1), (39) carrot root, (40) Japanese hot pepper (Kyoto var. 2), (41) brassica family vegetable ("hiroshimana") leaf, (42) sweet potato, (43) green pepper, (44) red pepper, (45) asparagus, (46) green pea, (47) green pea sheath, (48) kidney bean, type 3, fresh green sheath (var. "master-ingen"), (49) Chinese chive leaf, (50) red onion, (51) kidney bean, type 3, var. Japanese "toramame," (52) Japanese "komatsuna," thick stalk brassica family, (53) Japanese champignon ("shimeji" mushroom), (54) "enoki" mushroom, (55) onion, (56) bamboo shoots, (57) pumpkin, (58) pak choi, Chinese vegetable leaf, (59) onion, miniature type, (60) cabbage, inside leaf, (61) "shiitake" mushroom, (62) Japanese radish ("daikon") root, (64) tomato var. 1, (65) tomato var. 2, (66) tomato var. 3. See Figs. 1 and 2 and text for details.

showed a great increase (about 10–50 fold) in this ability upon boiling the vegetables for 5 min, whereas 2 showed a significant decrease. The remaining four showed little change.

Similarly, 35 vegetable extracts were tested for inhibitory activity against EB virus-induced EA formation. The results again showed a great increase in anti-EA formation activity (IEA_{50}), usually 10 fold or more, upon heating (Fig. 2).

Most green-leaf vegetables had both $IPOX_{50}$ and IEA_{50} values of more than 100 units, and the activity was found mostly in hot-water extracts, not in the solid materials that precipitated upon centrifugation after heating (data not shown). Also note that carrot and pumpkin had extremely low $IPOX_{50}$ and IEA_{50} values. These vegetables are known to contain large amounts of β-carotene, which scavenges singlet oxygen but not alkyl peroxide radicals, as we have demonstrated by using an ESR spin-trapping method.[7] In this study we did not examine the effect of cooking time, although it is expected to be important from the above results.

$IPOX_{50}$ and IEA_{50} values are shown in Fig. 3 for hot-water extracts of representative vegetables, beans, and mushrooms. Values for three beans (black bean, azuki/or red bean, and mung bean) were quite high, followed by soybean. These findings suggest that the role of vitamin C (L-ascorbate), which is inactivated to a great extent by heating, is quite small. Other components may be more significant in this respect.

When 66 vegetable and other plant foods were tested and the two parameters, IEA_{50} and $IPOX_{50}$, were plotted against each other, we found an extremely high correlation ($r=0.82$). This result indicates that alkyl peroxide radical has a tumor-promoter effect. Many hot-water extracts of vegetables showed a potent inhibitory effect in terms of both parameters. It is not clear why the two activities became higher upon heating; it might be due to the thermal destruction of vegetable cell walls and subcellular compartments, liberating more components, or/and thermal chemical reaction producing more potent radical-scavenging antioxidants.

The present study is still preliminary; more plants, fruits, legumes, and grains, for example, and the effects of cooking conditions should be studied. However, the epidemiological evidence linking a high intake of green vegetables and a lower incidence of colon or breast cancer is consistent with these findings.[1, 18, 19] Epidemiological studies indicate an association between high fat and high iron storage in the body and a high incidence of cancer.[1, 16–20] As shown in this study, these two components would most likely yield alkyl peroxide radicals, which have a tumor-promotor effect, particularly in the case of fecal stasis.

The present study was designed to reflect actual human food habits, so that we used water extracts of vegetables instead of organic solvent extracts, which were used without cooking in other studies.[24, 25] The present study showed that various water-soluble components may be quite important in prevention of cancer, as is a high-fiber diet.[18–20] Furthermore, alkyl peroxide radical activity may be one of the major mechanisms for tumor promotion.

Many experimental studies have shown that dietary antioxidants suppress mutagenesis and carcinogenesis (for example, see ref. 26), and catechins, flavonoids, α-tocopherol, L-ascorbate, β-carotenes, lignins, and even chlorophyl have been implicated as responsible protective substances.[26] In a separate study we found that intensity of oxygen radical generation from various heterocyclic amines has a high correlation to mutagenicity in Ames' test ($r=0.88$).[27]

The values of the two parameters we measured ($IPOX_{50}$ and IEA_{50}) for these samples varied according to plant origin and growth (season and timing of harvest, specific part of plant) and so on even in the same species. For instance, the outer green part and colorless inside part of cabbage leaf had very different values (see. Fig. 3, No. 24 vs. No. 60). Although not indicated in the present data, various teas had generally high values of both parameters and warrant more investigations.

A part of this work was supported by a Grant-in-Aid from the Ministry of Education, Science and Culture (Monbusho), Japan. We wish to acknowledge Ms. Michiyo Nagano, Ms. Akemi Oka, Ms. Judith Gandy, Ms. Rie Yoshimoto, Ms. S. Ijiri, and Dr. Keizo Sato for their help in technical assistance, manuscript preparation, and discussions.

(Received May 15, 1992/Accepted July 3, 1992).

REFERENCES

1) Weinstein, I. B. Cancer prevention: recent progress and future opportunities. *Cancer Res.*, (Suppl) **51**, 5080s–5085s (1991).
2) Witz, G. Active oxygen species as factors in multistage carcinogenesis. *Proc. Soc. Exp. Biol. Med.*, **198**, 675–682 (1991).
3) Guengerich, F. P. Reactions and significance of cytochrome P-450 enzymes. *J. Biol. Chem.*, **266**, 10019–10022 (1991).
4) Guengerich, F. P. Role of cytochrome P-450 enzymes in chemical carcinogenesis and cancer chemotherapy. *Cancer Res.*, **48**, 2946–2954 (1988).

5) Taffe, B. G., Takahashi, N., Kensler, T. W. and Mason, R. P. Generation of free radicals from organic hydroperoxide tumor promoters in isolated mouse keratinocytes: formation of alkyl and alkoxyl radicals from *tert*-butyl hydroperoxide and cumene hydroperoxide. *J. Biol. Chem.*, **262**, 12143–12149 (1987).
6) Maples, K. R., Kennedy, C. H., Jordan, S. J. and Mason, R. P. *In vivo* thiyl free radical formation from hemoglobin following administration of hydroperoxides. *Arch. Biochem. Biophys.*, **277**, 402–409 (1990).
7) Akaike, T., Sato, K., Ijiri, S., Miyamoto, Y. and Maeda, H. Bactericidal activity of alkylperoxyl radicals generated by heme-iron-catalyzed decomposition of organic peroxides. *Arch. Biochem. Biophys.*, **294**, 55–63 (1992).
8) Kawanishi, S., Inoue, S. and Sano, S. Mechanism of DNA cleavage induced by sodium chromate (VI) in the presence of hydrogen peroxide. *J. Biol. Chem.*, **261**, 5952–5958 (1986).
9) Yamamoto, K. and Kawanishi, S. Site-specific DNA damage induced by hydrazine in the presence of manganese and copper ions. *J. Biol. Chem.*, **266**, 1509–1515 (1991).
10) Kasai, H. and Nishimura, S. Hydroxylation of deoxyguanosine at C-8 position by ascorbic acid and other reducing agents. *Nucleic Acids Res.*, **12**, 2137–2145 (1984).
11) Kasai, H., Okada, Y., Nishimura, S., Rao, M. S. and Reddy, J. K. Formation of 8-hydroxydeoxyguanosine in liver DNA of rats following long term exposure to a peroxisome proliferator. *Cancer Res.*, **49**, 2603–2605 (1989).
12) Oda, T., Akaike, T., Hamamoto, T., Suzuki, F., Hirano, T. and Maeda, H. Oxygen radicals in influenza-induced pathogenesis and treatment with pyran-polymer conjugated SOD. *Science*, **244**, 974–976 (1989).
13) Akaike, T., Ando, M., Oda, T., Doi, T., Ijiri, S., Araki, S. and Maeda, H. Dependence on O_2^- generation by xanthine oxidase of pathogenesis of influenza virus infection in mice. *J. Clin. Invest.* **85**, 739–745 (1990).
14) Maeda, H. and Akaike, T. Oxygen free radicals as pathogenic molecules in viral diseases. *Proc. Soc. Exp. Biol. Med.*, **198**, 721–727 (1991).
15) Haugen, A. and Harris, C. C. Interactive effects between viruses and chemical carcinogens. *Handb. Exp. Pharmacol.*, 249–268 (1990).
16) Yuspa, S. H. and Poirier, M. C. Chemical carcinogenesis: from animal models to molecular models in one decade. *Adv. Cancer Res.*, **50**, 25–70 (1988).
17) Willett, W. C., Stampfer, M. J., Colditz, G. A., Rosen, R. A. and Speizer, F. E. Relation of meat, fat, and fiber intake to the risk of colon cancer in a prospective study among woman. *N. Engl. J. Med.*, **323**, 1664–1672 (1990).
18) Stevens, R. G., Jones, D. Y., Micozzi, M. S. and Taylor, P. R. Body iron stores and the risk of cancer. *N. Engl. J. Med.*, **319**, 1047–1052 (1988).
19) Trock, B., Lanza, E. and Greenwold, P. Dietary fiber, vegetables, and colon cancer: critical review and meta-analyses of the epidemiologic evidence. *J. Natl. Cancer Inst.*, **82**, 650–661 (1990).
20) Cohen, L. A. Diet and cancer. *Sci. Am.*, **257**, 42–48 (1987).
21) Doll, R. and Peto, R. The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. *J. Natl. Cancer Inst.*, **66**, 1191–1308 (1981).
22) zur Hausen, H., O'Neill, F. J., Freese, U. K. and Hecker, E. Persisting oncogenic herpesvirus induced by the tumor promoter TPA. *Nature*, **272**, 373–375 (1978).
23) Yamamoto, N. and zur Hausen, H. Tumor promoter TPA enhances transformation of human leukocytes by Epstein-Barr virus. *Nature*, **280**, 244–245 (1979).
24) Koshimizu, K., Ohigashi, H., Tokuda, H., Kondo, A. and Yamaguchi, K. Screening of edible plants against possible anti-tumor promoting activity. *Cancer Lett.*, **30**, 143 (1986).
25) Ohigashi, H., Takamura, H., Koshimizu, K., Tokuda, H. and Ito, Y. Search for possible antitumor promoters by inhibition of 12-o-tetradecanoylphorbol-13-acetate-induced Epstein-Barr virus activation: ursolic acid and oleanolic acid from an anti-inflammatory Chinese medical plant, *Glechoma hederacreae L. Cancer Lett.*, **39**, 247 (1988).
26) Osawa, T., Namiki, M. and Kawakishi, S. Role of dietary antioxidants in protection against oxidative damage. *In* "Antimutagenesis and Anticarcinogenesis Mechanisms II," ed. Y. Kuroda, D. M. Shankel and M. D. Waters, pp. 139–153 (1990). Plenum Publishing Corp., New York.
27) Sato, K., Akaike, T., Kojima, Y., Ando, M., Nagano, M. and Maeda, H. Evidence of direct generation of oxygen free radicals from heterocyclic amines by NADPH/cytochrome P-450 reductase *in vitro*. *Jpn. J. Cancer Res.*, (1992), in press.