Effects of Linoleic Acid Hydroperoxides and their Secondary Products on the Growth of *Escherichia coli*

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Received June 5, 1970

Autoxidized lipids have been reported to be toxic. But which compound of autoxidized lipids, hydroperoxides or their secondary products, is really toxic seems to be not still clear. Autoxidized linoleic acid was fractionated by column chromatography and thin-layer chromatography (TLC). The purity of linoleic acid hydroperoxide (LAHPO) was confirmed by TLC and polarography.

The effect of purified hydroperoxide and the secondary products on the growth of *Escherichia coli* was tested at the beginning and after 2 hr of incubation. LAHPO at 10^{-3} M concentration showed complete inhibition, when added at the initial stage or at the early log phase. The secondary products at about 0.035% concentration when added at the beginning of incubation showed complete inhibition, but when added at the early log phase they showed no inhibitory effect.

The inhibitory effect of LAHPO was partially retarded by the addition of antioxidants, but the inhibitory effect of the secondary products could not be retarded by such compounds. These results show that the inhibition mechanism by LAHPO may be of radical type whereas the inhibitory effect of the secondary products may be of different mechanism.

Polyunsaturated fatty acids undergo autoxidation, and hydroperoxides (ROOH) are formed as primary oxidation products.¹⁾ The so-called secondary degradation products of lipid peroxidation (presumed to be a mixture of aldehydes, ketones, alcohols, etc.) are believed to be formed largely from hydroperoxide decomposition.²⁾ Autoxidized lipids have been reported to be toxic, but which compound of autoxidized lipids is really toxic seems to be not still clear.³⁾ Many experiments have been done on the effect of autoxidized products on living animals, cells, enzymes and biological active substances. Lipid peroxides are known

to be formed by many noxious agents such as carbon tetrachloride,⁴⁾ ultraviolet light,⁵⁾ and ionization radiation,⁶⁾ and the reaction is catalyzed by such agents as ascorbate,⁷⁾ NADPH,⁸⁾ ferrous,^{9,10)} cuprous, cobaltous, and manganous ions.¹¹⁾ Such peroxides formed may be important as a factor causing general

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membrane damage,⁴¹ and may be of importance in the overall metabolism of the endoplasmic reticulum or its destruction.¹²¹ Such cellular damage may be the result of oxidative destruction of SH groups,¹³¹ proteins¹⁴¹ and particularly enzymes.¹⁵¹ As a result, existence of peroxide lipids in tissues may cause deleterious changes in the cell metabolism. However, in most of these experiments, crude materials have been used. Therefore the necessity to find the effects of separated pure compounds of autooxidation products on the cell metabolism is inevitable.

This study has been done by using *Escherichia coli* (*E. coli*) B cells to see the effects of pure linoleic acid hydroperoxides (LAHPO) and the secondary products of peroxidation on their growth.

MATERIALS AND METHODS

Materials. Linoleic acid was purchased from Tokyo Kasei Co. and used without further purification.

E. coli B was kindly supplied by Dr. Kito of our institute.

Oxidation of linoleic acid. Linoleic acid (4g) was rotated as a thin film on the inner surface of a 100 ml round bottomed flask at 37°C for 60 hr. No catalyst was added during oxidation.

Separation of the products of autooxidation. The autooxidation mixture was subjected to column chromatographic separation as described by Frankel *et al.*¹⁶¹ by using 20% methanol in benzene as an immobile solvent and 2% methanol in benzene as a mobile solvent. Ether was used to elute the secondary products. The suspending medium of the hydroperoxide fractions was changed from 2% methanol in benzene to methanol by repeated addition and evaporation of me-

thanol in 50 ml portions. The peroxide value (POV)¹⁷¹ and absorbance of LAHPO by using an extinction coefficient value $E_{233}^{mM}=25.25$ ¹⁸¹ were used to measure the yield of LAHPO.

The secondary product fractions eluted with ether was similarly treated to get the sample in methanol. The concentration was determined by the weight after evaporating the solvent.

Thin-layer chromatography. Silica gel F_{254} , 0.25 mm plates (Merck) were used. The plates were preheated at 150°C for 1 hr before use. The solvent system consisted of petroleum ether-ether-acetic acid (8:7:0.5). Linoleic acid, LAHPO and the secondary products were exposed to iodine vapour. For further purification of LAHPO, 0.75 mm preparative plates (silica gel) and the same solvent system were used. The bands were peeled off and the components were recovered with methanol.

Polarography. A Yanako P8 Polarograph (Yanagimoto) was used to get current-voltage curves. The experiment was carried out at 20°C. The electrolyte solution consisted of 0.3 M lithium chloride in methanol-benzene (50:50, v/v).¹⁹¹ Deaeration was done with argon gas for 20 min before current voltage curves were obtained.

Cultivation and the determination of the rate of growth of *E. coli*. The culture medium consisted of the components as described by Pizer.²⁰¹ For the determination of the rate of growth of *E. coli*, 2.5 ml of the mineral medium was taken to an incubating tube to which was added 0.03 ml of 20% glucose solution, 0.1 ml of the suspension of *E. coli* and 0.05~0.1 ml of the solution of the compounds under determination (Concentration of the compounds are shown in the figure. A small difference in the total volume among different experiments was neglected). These were incubated at 37°C. All the experiments were carried out in parallel form. At every hour a set of tubes were taken out and the contents were centrifuged at 5,000×g. The deposited *E. coli* cells were suspend-

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ed in 3.5 ml of water and the optical density at 600 m μ was measured.

The inhibitory effects of LAHPO and the secondary products on the growth of E. coli and the protection by some agents.

In all such determinations, each experimental unit consisted of 2.5 ml of mineral medium, 0.03 ml of 20% glucose solution, and 0.1 ml of the suspension of *E. coli* to which 0.05 ml of LAHPO solution (10^{-3} M final concentration) and the secondary product solution (1 mg/tube, about 0.035%) was added at the beginning of after 2 hr of incubation. In all determinations where the protection against the inhibitory activity were examined, 0.05 ml of the test compound (10^{-3} M final concentration), such as BHA (butyl hydroxy anizole), BHT (butyl hydroxy toluene), uric acid, tryptophan, tyrosine, alanine, ascorbic acid, etc. were added to the above mixture either at the beginning or after 2 hr of incubation, and the mixtures were further incubated. The effects were determined by the rate of growth.

RESULTS

Identification of hydroperoxides

The fractions obtained from column chromatography of the autoxidation mixture of

LAHPO were pooled and concentrated under reduced pressure. This material was further purified by preparative TLC and tested for purity by TLC.

The purified LAHPO were negative to the Folin phenol reagent. However when the LAHPO in methanol was diluted with water, incubated for 20 min at 37°C and subjected to the above reaction, it did produce positive blue color.²¹⁾ This confirms that the purified LAHPO did not contain the Folin phenol reagent-reactive substance, but may be decomposed to a reactive substance on incubation in aqueous medium. The purified LAHPO was also tested by polarography. Pure linoleic acid was not reduced polarographically under the conditions used, but LAHPO showed a clear wave with a half-wave potential at -0.8 V (Fig. 2), as reported by Willits *et al.*¹⁹⁾

By these identifications, LAHPO separated by column chromatography and further purified by preparative TLC was regarded to be practically pure for the foregoing experiments.

Effects of LAHPO and the secondary products on the growth of E. coli

FIG. 1. Schematic Presentation of the TLC of the Compounds Fractionated by Column Chromatography.

22~34, linoleic acid; 70~78, LAHPO; 114~116, the secondary products. Experimental conditions are shown in the text.

linoleic acid was identified by TLC as shown in Fig. 1. The fractions which contained

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FIG. 2. Polarogram of LAHPO.

Experimental conditions are shown in the text.

FIG. 3. Effects of Linoleic Acid, LAHPO and the Secondary Products on the Growth of *E. coli* When Added at the Initial Stage of Incubation.

Test samples were added at zero time of incubation. Experimental details are shown in the text.
A, control; B, 10^{-3} M linoleic acid; C, 10^{-4} M LAHPO; D, 0.05 ml methanol/tube; E, 6×10^{-4} M LAHPO; F, 10^{-3} M LAHPO; G, 1 mg sec. product/tube.

LAHPO and the secondary products in various concentrations were added to the incubating medium consisting of the components required for normal growth as described in the method. Samples were added at two points of the growth, at the beginning of incubation (Fig. 3) and after 2 hr of incubation (Fig. 4).

FIG. 4. Effects of LAHPO and the Secondary Products on the Growth of *E. coli* When Added at the Early Log Phase of the Growth Curve.

Test compounds were added after 2 hr of incubation. Experimental details are shown in the text.

A, control; B, 1 mg sec. product/tube; C, 10^{-4} M LAHPO; D, 6×10^{-4} M LAHPO; E, 10^{-3} M LAHPO.

The sample addition at 2 hr was considered to be most appropriate, as it is the early log phase of the growth. Linoleic acid had no effect on the growth in either case. LAHPO at 10^{-3} M showed complete inhibition when added at the initial stage or at the early log phase. The secondary products, when added at the initial stage at about 0.035% concent-

ration, showed complete inhibition, but when added at the early log phase showed no inhibitory effect (Fig. 4).

In order to find when the toxic effect of the secondary products becomes negative, the secondary products in the same concentration were added at every $\frac{1}{2}$ hr to the medium up to $2\frac{1}{2}$ hr. Complete inhibition was noted by additions prior to $1\frac{1}{2}$ hr. The products showed only a partial inhibitory effect when added at 2 hr and showed no significant effect when added at $2\frac{1}{2}$ hr (Fig. 5). Even when five times the amount of the secondary products was added at this time, no inhibition was observed.

FIG. 5. Decrease of the Inhibitory Effect of the Secondary Products on the Growth of *E. coli* with the Time of Addition.

Secondary products were added at the concentration of 1 mg/tube (otherwise stated) at every $\frac{1}{2}$ hr.

↓, time of addition; $\triangle-\triangle$, 5 mg/tube of the secondary products.

Protection of the growth of *E. coli* against LAHPO

Some substances were tested as protectants against the inhibitory activity of LAHPO (Figs. 6 and 7). All the compounds were tested under the same conditions as those of the proceeding experiment to get a comparison of the degree of protection. Although a complete protection does not seem to be attainable, BHT showed a remarkable protecting effect as shown in Figs. 6 and 7. BHA and uric acid showed the similar effect to BHT. Of the amino acids tested, tryptophan showed some effect but less effective than BHT as shown in Figs. 6 and 7. Tyrosine showed the similar effect to tryptophan. In all the above cases a similar

FIG. 6. Protective Effects of Some Components on the Inhibitory Effect of LAHPO When the Compounds and LAHPO were Added at the Initial Stage of Incubation of *E. coli*.

Test compounds and LAHPO were added at zero hr of incubation. Experimental details are shown in the text.

A, control; B, LAHPO+BHT; C, LAHPO+tryptophan; D, LAHPO+alanine; E, LAHPO.

FIG. 7. Protective Effects of Some Compounds on the Inhibitory Effect of LAHPO When the Compounds and LAHPO were Added at the Early Log Phase of the Growth of *E. coli*.

Test compounds and LAHPO were added after 2 hr of incubation. Experimental details are shown in the text.

A, control; B, LAHPO+BHT; C, LAHPO+tryptophan; D, LAHPO+alanine; E, LAHPO.

pattern of protection was observed whether the protecting agents were added at the initial stage or at the early log phase. Glutathione and ascorbic acid showed no protection against the inhibitory effect of LAHPO.

In the case of the secondary products, no such effect was observed in any compound tested.

DISCUSSION

Hydroperoxide concentrates have been obtained by various methods²² such as molecular

distillation, urea fractionation, reverse phase partition chromatography, adsorption chromatography, *etc.* Characterization and determination of hydroperoxides have been carried out by various methods such as POV,²³⁾ spectrophotometry,²⁴⁾ infrared analysis,²⁵⁾ polarography,¹⁹⁾ *etc.* The hydroperoxides used here were fractionated by column chromatography and confirmed to be pure by TLC and polarography. The secondary products used were a mixture of many substances but free from linoleic acid and LAHPO.

Under the experimental conditions used, a measurable effect of LAHPO and the secondary products was observed in the cell growth after 3 hr of incubation. Any damage to the normal cell metabolism may either cause a total stop or a decrease in the rate of cell division. Linoleic acid did not show any significant inhibitory effect. Methyl alcohol in which all water-insoluble compounds were dissolved had no perceptible effect upon the growth at the concentration used here as the solvent.

The protective effect of the antioxidants and some other compounds^{26,27)} having antioxidative properties suggests that the growth inhibitory activity of LAHPO may be of radical type and the protective effect may be the result of the interaction of LAHPO radicals with the protectant radicals to bring about the termination reaction. The observation that none of the aliphatic amino acids and electron donors like glutathione and ascorbic acid showed such a protective effect supports the above supposition. Glutathione and ascorbic acid generally do not show antioxidant activity against lipid oxidation when water is present in the system.²⁸⁾

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The similar protective effect of antioxidants could not be expected in the case of the secondary products. Because, their activity may not be of radical type.

Recently the toxic effect of oxidized lipid on the cell metabolism has been emphatically extended to the secondary products^{29,30} of peroxidation besides LAHPO. In the present experiments, the secondary products when added prior to 1½ hr, showed strong inhibition. However, the additions after 2½ hr

showed no inhibition. These results could be explained by the assumption that the toxic effect of the secondary products may be neutralized by some protective substances that may be accumulated at this stage.

It requires more direct experiments to clear the physiological role of the oxidized lipids in cell damage, and the difference in the physiological role between LAHPO and the secondary products in cell damage. Further study of this type of model system is in progress.

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