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# Acceleration of Lipid-Peroxidation by 12-Keto Oleic Acid[1]

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The formation of lipohydroperoxide from polyunsaturated fatty acid was accelerated by the addition of 12-keto oleic acid (12-KOA) in the state of oil–in–water system. Saturated or hydroxy analogues failed to show such prooxidant action.

Increasing concentration of added 12-KOA was found to promote the rate of peroxidation.

The acceleration of peroxidation is not affected by either the esterification of 12-KOA or the preincubation of keto acid with albumin.

Taking the fact that 12-KOA is easily absorbable through intestine contrary to less-absorbability of lipohydroperoxide into consideration, it is suggested that keto acid produced in edible oil will act as potent prooxidant *in vivo*.

It is well known that edible oil containing polyunsaturated fatty acids (PUFA) has a tendency to be deteriorated by heat, oxygen or ultraviolet light to yield lipohydroperoxide, cyclic monomer or oligomer and many other secondary products. The toxicity of lipohydroperoxide and cyclic substances has been reported by several investigators.[3]

Among the secondary products having an oxygen function in acyl molecule, hydroxy and epoxy acids are not likely hazardous so far reported. But the long chain keto acid having a carbonyl group in the middle part of acyl chain has become of interest, since Kokatnur, *et al.*[4] showed that the addition of a small amount 12-keto oleic acid (12-KOA) to the feed during administration of a vitamin E–deficient diet to chickens shortened the period required for the production of encephalomalacia due to vitamin E–deficiency. On the basis of the fact that an intravenous injection of lipohydroperoxide to chickens produced promptly the same cerebellar disorder as caused by the feeding 12-KOA, Nishida, *et al.*[5] postulated that nutritional encephalomalacia may be initiated by accumulation of sufficient, though still immeasurable, levels of lipohydroperoxide *in vivo* and the aggravating effect of keto acid was assumed to be due to its activities as prooxidant on the dietary lipid and destructive agent to biological antioxidants. 12-KOA-induced stimulation of peroxidation of corn oil was recognized in the oily phase by Bhalerao, *et al.*[6]

To consider the physiological action of keto acid produced in oxidized edible oil, it became necessary to investigate the prooxidative character of keto acid in the phase of oil–in–water system and the effect of esterified or protein bound keto acid.

## Experimental

**Preparation of Fatty Acids**——PUFA was prepared from linseed oil by the conventional method. It contains 27.2% of oleic, 17.2% of linoleic and 49.5% of linolenic acids. The contamination of saturated

1) This report has been presented at Meeting of Tohoku Branch, Pharmaceutical Society of Japan, Sendai, July 1968.
2) Location: *Aobayama, Aramaki, Sendai.*
3) N. Matsuo, "Lipids and Their Oxidation," ed. by H.W. Schultz, The Avi Publishing Company, Inc., Westport, Connecticut, 1962, p. 321.
4) M.G. Kokatnur, S. Okui, F.A. Kummerow, and H.M. Scott, *Federation Proc.*, **19**, 421 (1960).
5) T. Nishida, H. Tsuchiyama, M. Inoue, and F.A. Kummerow, *Proc. Soc. Exptl. Biol. Med.*, **105**, 308 (1960).
6) V.R. Bhalerao, M.G. Kokatnur, F.A. Kummerow, and L.G. Zirkler, Jr., *J. Am. Oil Chemists' Soc.*, **39**, 28 (1962).

(mainly palmitic) acid was 6.1%. Ricinoleic acid obtained from caster oil was purified by means of column chromatography of silicic acid[7] and the purity was 99.1% by gaschromatographic analysis.

Chromic acid oxidation of hydroxy acid and caster oil to obtain keto acid and its glyceride was preformed according to Nichols and Shipper.[8] *Anal.* Calcd. for $C_{18}H_{32}O_3$ (12-keto oleic acid): C, 72.96; H, 10.88. Found : C, 73.29; H, 10.97. mp 39—39.5°. The unsaturated keto acid was hydrogenated under a stream of hydrogen gas with 5% Pd on C as catalyst. *Anal.* Calcd. for $C_{18}H_{34}O_3$ (12-keto stearic acid): C, 72.44; H, 11.48. Found: C, 72.47; H, 11.40. mp 82.0.

**Measurement of Peroxidation**——Oxygen Uptake: Oxygen uptake was measured with Warburg's manometer at 37°.

PUFA was suspended in 0.1M phosphate buffer (pH 7.4) using Tween 20 and 0.3 ml of this suspension containing 100 mg PUFA was put in the side arm of the flask. Additives such as keto, hydroxy acids or DL-α-tocopherol were also prepared into similar suspension and 2.0 ml of each was placed in the main chamber.

Peroxide Value: One g of PUFA and 50 mg of 12-KOA were suspended in 0.1M phosphate buffer (pH 7.4) with Tween 20. The final volume was adjusted to 10.0 ml with buffer. Incubation was carried out at 37° under air with constant shaking. To 1.0 ml of aliquot from reaction mixture was added 25 ml of the mixture of glacial acetic acid and $CHCl_3$ (3:2) and then 1 ml of saturated KI solution. The mixture was kept stand for 10 min in the dark. Thereafter 30 ml of $H_2O$ and 1 ml of starch solution were added and titrated with 0.01N $Na_2S_2O_3$.

TBA Value: To 0.2 ml of aliquot from the reaction mixture same as peroxide value was added 1.0 ml of 10% trichloroacetic acid. The mixture was heated at 100° for 10 min together with 6.0 ml of 0.35% TBA solution dissolved in 50% v/v acetic acid. The absorbance at 532 mμ was measured.

## Result

### Effect of 12-Keto Oleic Acid on Peroxidation of Unsaturated Fatty Acids

PUFA undergo denaturation resulting the elevation of peroxide value when left in air, heated or irradiated by ultraviolet ray. Effect of 12-KOA on the peroxidation of PUFA was confirmed by incubation of PUFA suspended in the phosphate buffer with 12-KOA. As shown in Fig. 1, peroxidation was markedly accelerated by the addition of small amount of 12-KOA. Since the increment of peroxide value does not occur at all with 12-KOA alone, the marked progress of peroxidation in the system added with 12-KOA indicates the prooxidant action of 12-KOA.

The action of 12-KOA on PUFA as prooxidant was examined by means of the other parameters of peroxidation, namely oxygen consumption and thiobarbituric acid (TBA) value. The result was shown in Table I and it was well compatible with the result obtained in Fig. 1. Since 12-KOA employed here was prepared from ricinoleic acid by chromic acid oxidation, the contamintion of trace amount of $Cr^{3+}$ is likely. Generally, peroxidation of PUFA is catalyzed by many divalent metals,[9] but $Cr^{3+}$ did not affect the rate of peroxidation even in the concentration of $3\times10^{-3}$M.

### Some Other Oxygenated Fatty Acids and Esters as Prooxidant

In order to examine whether the prooxidant effect of 12-KOA is characteristic to unesterified, unsaturated keto compounds or not, some other fatty acids with oxygen function and esterified keto acid were used. Ricinoleic acid and 12-keto stearic acid failed to show any prooxidant effect. Stearic and oleic acids did not of course catalyze the peroxidation (Fig. 2).

Methyl ester of 12-KOA and the glyceride which contains 12-KOA as acyl component was able to accelerate the peroxidation of PUFA (Table II). And the difference of substrate namely PUFA and their glyceride did not show any significant difference for the catalytic action of keto compound. Therefore, it is obvious that such prooxidant effect requires the presence of carbonyl group and double bond and that carboxyl function is not participating in a stimulation of peroxidation.

7) M. Mizugaki, "doctoral thesis," Tohoku University, 1966, p. 46.
8) J. Nichols and E. Shipper, *J. Am. Chem. Soc.*, **80**, 5705 (1958).
9) E.D. Wills, *Biochim. Biophys. Acta*, **98**, 235 (1965).

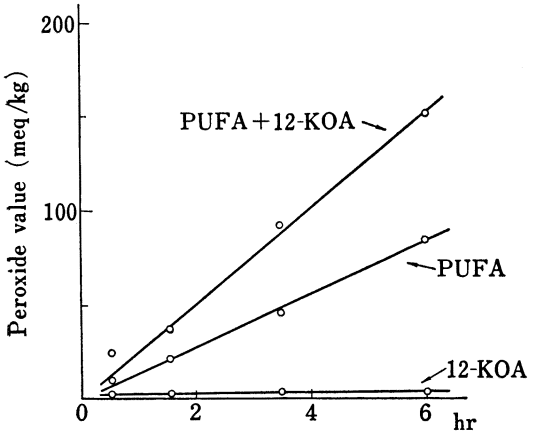


Fig. 1. Effect of 12-Keto Oleic Acid on Peroxidation of Polyunsaturated Fatty Acids

Polyunsaturated fatty acids (PUFA) (1 g) and 12 keto oleic acid (12-KOA) (50 mg) were suspended with Tween 20, respectively. The final volume was adjusted to 10.0 ml with 0.1 M phosphate buffer (pH 7.4). Incubation at 37° with constant shaking.

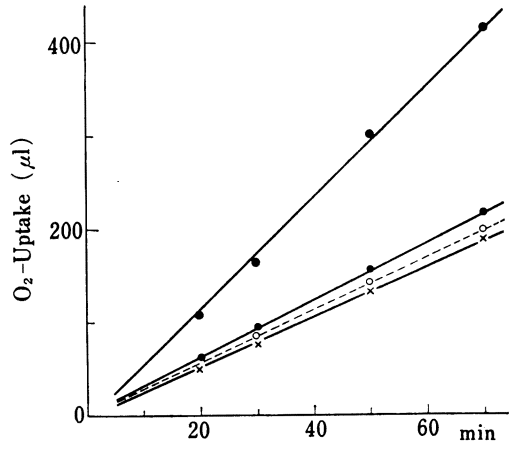


Fig. 2. Effect of Various Fatty Acids on Oxygen Uptake of Polyunsaturated Fatty Acids

$3 \times 10^{-3}$ M of each fatty acid was added.
—⊙—: PUFA+12-keto oleic acid
—●—: PUFA+12-keto stearic acid
—○—: PUFA+ricinoleic acid
—×—: PUFA

TABLE I. Oxygen Uptake and TBA Value at Final Stage

| Exptl. No. | $O_2$-Uptake (μl) 30 min | 60 min | final stage | TBA value (OD at 532 mμ) |
|---|---|---|---|---|
| 1. PUFA | 73 | 172 | 300 (95 min) | 0.448 |
| PUFA+12-KOA | 146 | 325 | 555 (95 min) | 1.380 |
| 2. PUFA | 69 | 182 | 280 (85 min) | 0.373 |
| PUFA+12-KOA | 116 | 303 | 454 (85 min) | 0.823 |

100 mg of PUFA and 2.3 μmole of 12-KOA were used. The final volume was adjusted to 2.2 ml with 0.1 M phosphate buffer (pH 7.4).

TABLE II. Acceleration of Lipid Peroxidation by 12-Keto Oleic Acid, Methyl 12-Keto Oleate and Its Glyceride

| PUFA | Additive | Carbonyl (μmole) | TBA value (mμmole) | | |
|---|---|---|---|---|---|
| + | — | — | 1.4 | — | mμmole/μmole of carbonyl |
| + | 12-keto oleic acid | 16.9 | 14.5 | 0.858 | |
| + | 12-keto oleic acid | 25.4 | 21.9 | 0.862 | |
| — | 12-keto oleic acid | 33.8 | 1.0 | 0.029 | |
| + | methyl 12-keto oleate | 17.2 | 15.4 | 0.894 | |
| + | methyl 12-keto oleate | 25.8 | 20.5 | 0.794 | |
| — | methyl 12-keto oleate | 34.4 | 1.2 | 0.035 | |
| + | 12-keto oleate glyceride | 14.4 | 11.6 | 0.805 | |
| + | 12-keto oleate glyceride | 21.5 | 16.3 | 0.758 | |
| — | 12-keto oleate glyceride | 28.8 | 1.0 | 0.035 | |

0.2 g of polyunsaturated fatty acids (PUFA) obtained from linseed oil and each amount of 12-keto oleic acid (12-KOA) and its derivatives were suspended in 0.1 M phosphate buffer (pH 7.4) with Tween 80. The final volume was adjusted to 3.0 ml with buffer. Incubation was carried out at 37° for 4 hours with constant shaking. 5 ml of $CHCl_3$ and 3 ml of 0.75 % and 2% TCA solution were added and shaken strongly. Then 4 ml of aqueous layer was heated at 100° for 10 min. The absorbance at 532 mμ was measured.

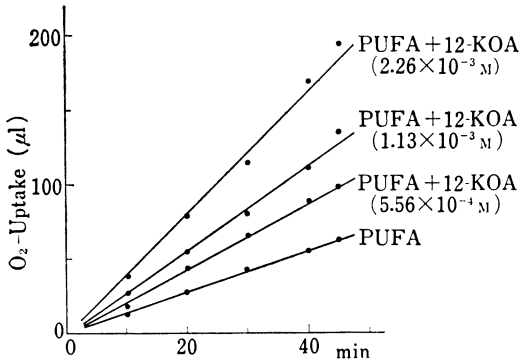


Fig. 3. Concentration of 12-Keto Oleic Acid (12-KOA) and the Promotion of $O_2$-Uptake of PUFA

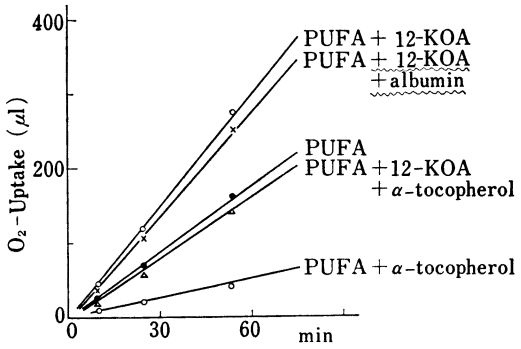


Fig. 4. Influence of $\alpha$-Tocopherol and Albumin on the Keto Acid–Induced Increase of $O_2$-Uptake

$1\times10^{-3}$M of $\alpha$-tocopherol, 15 mg of eggalbumin, $1\times10^{-3}$M of 12-KOA and 150 mg of PUFA were used. Preincubation at 37° for 60 min with constant shaking. ~~~ indicates preincubation.

Increasing concentration of 12-KOA added to constant amount of PUFA effected to increase the amount of oxygen uptake (Fig. 3). This is compatible with the observation of Bhalerao, *et al.*[6]

When ester or glyceride of 12-KOA was employed, the magnitude of increment is almost proportional to the equivalent of carbonyl value (Table II).

**Effect of α-Tocopherol and Albumin**

The addition of α-tocopherol, effective as an antioxidant even in nonenzymic peroxidation, into the reaction mixture markedly lowered the peroxidation induced by 12-KOA. Presence of albumin had little preventive effect on the peroxidation of PUFA stimulated by 12-KOA even in the state that 12-KOA was preincubated with 10 times (w/w) of albumin (Fig. 4).

## Discussion

Recently Wantland, *et al.* reported the production of various keto acids in thermally oxidized glyceride.[10] Since one of those keto acids, 12-keto oleic acid, was recognized to have a potent prooxidant action in oily and oil–in–water phase, it is probable that the peroxidation of co-existing PUFA in food is being accelerated during preservation and digestion.

The apperance of carbonyl compounds in the lymph of rats administered by oxidized oil has been recognized[11] while lipohydroperoxide itself is thought to be hardly absorbed through intestinal tract. Moreover, it was found that keto acid is efficiently absorbed through intestinal tract and esterified during absorption and that the retention of keto acid in blood is far longer than palmitic acid.[12] These facts indicate that the prooxidant action of keto acid against PUFA is also effective on PUFA existing in various tissues and biological component *in vivo*.

The stimulation of PUFA-peroxidation was not supressed by binding with albumin or esterification of 12-KOA, which also reveals the possibility of the promotion of peroxidation *in vivo*.

10) L.R. Wantland and E.G. Perkins, *Lipids*, **5**, 191 (1970).
11) A. Ueno, M. Inoue, M. Sugai, V.R. Bhalerao, and F.A. Kummerow, *Federation Proc.*, **19**, 19 (1960).
12) M. Sato, M. Mizugaki, R. Watanabe, and M. Uchiyama, The 89th Annual Meeting of Pharmacical Society of Japan, Nagoya, April 1969.

On the other hand, it was preliminarily recognized that 12-KOA reacts with SH-compounds such as cystein and glutathione to decrease free SH,[13] which will be reflected to the dimunution of antioxidative potency of animals and exaggerate the prooxidative effect of 12-KOA.

**Acknowledgment** A part of the expenses for the present series of experiments was defrayed by the Grant-in Aid for Scientific Research from the Ministry of Education. Authors thank the technical assistance of Messrs. Kenji Fukuzawa and Yoshikuni Suzuki.

13) M. Sato and M. Uchiyama, The 19th Meeting of Food Hygienic Society of Japan, Sendai, Octber 1969.