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Properties of Thiobarbituric Acid-reactive Materials obtained from Lipid Peroxide and Tissue Homogenate

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In order to classify the 2-thiobarbituric acid (TBA)-reactive materials in various tissues, control and CCl_4-intoxicated rat liver homogenates as well as methyl linoleate hydroperoxide (MLHPO) and malonaldehyde were subjected to the TBA reaction under both aerobic and anaerobic conditions.

The TBA reaction of malonaldehyde proceeded regardless of the absence of oxygen, while the TBA value obtained from MLHPO catalyzed by tissue under anaerobic conditions was as low as 15% of the aerobic value, and tissue homogenate, even prepared from CCl_4-intoxicated rat liver, gave practically no color development under anaerobic conditions.

However, after an aerobic preincubation, MLHPO and tissue homogenate produced TBA-reactive materials which could react with TBA even under anaerobic conditions. These TBA-reactive materials are not malonaldehyde itself but seem to be some further-oxidized and more polar lipid hydroperoxide.

The addition of Fe to the TBA reaction medium seems to negate the requirement for oxygen. The magnitude of the TBA value obtained anaerobically from tissue with Fe addition was similar to the TBA value obtained aerobically from tissue without Fe addition.

Keywords——TBA-reactive material; methyl linoleate hydroperoxide; effect of oxygen; peroxide value; TBA value; CCl_4-intoxicated rat liver

Introduction

The 2-thiobarbituric acid (TBA) method has been used to determine lipid peroxidation in stored fats or tissue homogenate, together with measurements of the peroxide value (POV) and conjugated diene content.[1-6]

The TBA value has long been believed to reflect only the peroxidation of fatty acids having more than three positions of unsaturation,[7,8] but it was recently found that methyl linoleate, which has only two double bonds and was thought to be insensitive to the TBA method, showed color development with TBA under suitable conditions.[9] Thus, the TBA reaction shows wide variance in "quantitative" results depending on the reaction conditions. Various authors have therefore questioned whether the TBA value can be utilized as a reliable index of lipid peroxidation.[10-12] However, the TBA method has been the most widely used for evaluating peroxidation of animal tissue because of its simplicity in operation and high sensitivity.[13-15]

Little is known about the TBA-reactive materials in tissue homogenate. Malonaldehyde is a well-known chromogen, but some other materials are believed to exist in tissue homogenate[16] and affect the TBA value directly or indirectly. In order to classify some of these TBA chromogens, we set out to investigate the effect of oxygen and iron on the color development in the TBA reaction of tissue homogenates prepared from normal or CCl_4-intoxicated rat liver. Preincubated homogenate was also employed as well as fresh and oxidized methyl linoleate hydroperoxides (MLHPO).

Materials and Methods

Methyl linoleate (more than 99% pure) and lipoxygenase were obtained from P-L Biochemicals, Inc.

Ethyl ether was peroxide-free grade and TBA, sodium dodecyl sulfate (SDS) and other reagents were of reagent grade from Wako Pure Chemicals Ind., Ltd. CCl_4 which was of reagent grade, was distilled before use. Malonaldehyde (MA) was prepared by the hydrolysis of 1,1,3,3-tetraethoxypropane (obtained from Tokyo Kasei Kogyo Co., Ltd.).[17]

Thin layer chromatography (TLC) and preparative layer chromatography (PLC) plates were prepared with Kieselgel F_{254} obtained from E.Merck.

For measuring color intensity, a Hitachi 556 dual-wavelength, double-beam spectrophotometer was used. Oxygen concentration in the reaction medium was measured with a Beckman 0260 model oxygen analyzer. Ultracentrifugation was carried out with a Hitachi model 40P.

Preparation of MLHPO and Air-exposed MLHPO——Pure methyl linoleate dissolved in ethanol was suspended in 0.2M borate buffer (pH 9.0) with the addition of lipoxygenase. The mixture was allowed to stand for a week at 5°C with occasional shaking until methyl linoleate was virtually undetectable. After neutralization, MLHPO was extracted with *n*-hexane. The extract was dried over Na_2SO_4 and concentrated under nitrogen. The residue was subjected to PLC, developed twice at 5°C with *n*-hexane–ethyl ether (7:3). The zones corresponding to MLHPO (Rf=0.7) were scraped off and eluted with peroxide-free ethyl ether. The resulting solution was evaporated to dryness under nitrogen. The residue was weighed and dissolved in ethyl ether to make a concentration of 1 mg/ml.

Air-exposed MLHPO was made as follows: One-tenth ml of ether solution of MLHPO was pipetted into a test tube, freed from the solvent and left to stand at room temperature under air. After a certain period of time, the oily layer was subjected to the determination of chemical indices. When necessary, the oily layer was washed with water to remove water-soluble materials.

Preparation of Liver Homogenate and Incubated Homogenate——Livers removed from normal and CCl_4-intoxicated rats were homogenized in a Potter-Elvehjem Teflon homogenizer with 9 volumes of 1.15% KCl solution.

The incubated homogenate was prepared as follows: The incubaton mixture consisted of 5% liver homogenate of normal rat, 1 mM ascorbic acid and 0.1M phosphate buffer (pH 6.0) as a final concentration. The mixture was incubated at 37°C for 60 min.[14] An aliquot of the mixture was assayed to determine the TBA value[13] and was thus confirmed to contain sufficient TBA-reactive materials. The mixture was then centrifuged at 105000**g** for 60 min to obtain a particle fraction, which was washed with water to remove MA. The MA-free precipitate was resuspended in 1.15% KCl solution to make a homogenate which gave a TBA value corresponding to 10 nmol/0.5 ml or more of MA. This homogenate was designated as "*in vitro* peroxidized homogenate."

Preparation of CCl_4-intoxicated Rats——Male rats of the Wistar strain were fed with a commercial diet *ad libitum*. CCl_4 was administered orally *via* a stomach tube to rats at a dose of 0.4 ml per 100 g body weight, and the rats were killed by decapitation 24 h after CCl_4 administration.[18]

Analytical Procedures——The TBA reaction of MLHPO and air-exposed MLHPO with the addition of tissue as a catalyst[19,20] was performed at pH 3.5[9] and that of tissue homogenate (fresh and *in vitro*-peroxidized), at pH 2.[16]

Peroxide determination was conducted according to Swoboda and Lea[21] with a slight modification.[18] Standardization was done by preparing a known concentration of *tert*-butyl hydroperoxide which had itself been standardized by titration.[22]

The solvent system for TLC was *n*-hexane and ethyl ether (7:3) on a volume basis, and the Rf of MLHPO was 0.35. Detection of peroxides on a TLC plate was done by spraying a mixture of 10% KI in ethanol–acetic acid–chloroform (5:3:2).[9] TBA-positive spots were visualized by heating for 15 min after spraying of the plate with 20% acetate buffer (pH 3.5) followed by 0.6% TBA. All spray reagents were freshly prepared before used.

The Procedure for the TBA Method under Anaerobic Conditions——The anaerobic TBA-reaction was performed in Thunberg tubes. Homogenate or MLHPO was put into the main tube with all reagents. Air in the tube was evacuated at 16 mmHg for 3 min by means of an aspirator and the tube was filled with nitrogen gas (high purity grade). By a further repetition of this gas exchange the oxygen concentration was reduced to 0.02 ppm.

Results

The Effect of Oxygen on the TBA Reaction of Fresh Tissue

MLHPO (more than 96% pure), tissue homogenate and MA were subjected to the TBA method both under aerobic (conventional, about 8 ppm oxygen) and anaerobic (about 0.02 ppm oxygen) conditions (Table I). The TBA reaction of MA proceeded regardless of the absence of oxygen, while the TBA value obtained for MLHPO catalyzed by tissue under anaerobic conditions was as low as 15% of the aerobic value. Moreover, fresh tissue homogenate gave practically no color development by the TBA method under anaerobic conditions.

TABLE I. Effect of Oxygen on the TBA Values of Tissue Homogenate, Methyl Linoleate Hydroperoxide (MLHPO) with Tissue, and Malonaldehyde (MA)

	Tissue homogenate		MLHPO +Tissue	MLHPO +$FeCl_3$	MA
	Normal	CCl_4			
Aerobic condition	4.2±0.3	34.0±3.5	17.1±0.9	8.0±0.3	4.2±0.1
Anaerobic condition	0.4±0.1	1.0±0.2	2.1±0.4	11.6±0.8	4.3±0.1

The reaction mixture consisted of 1 % H_3PO_4 (3ml), 0.6 % TBA (1ml) and 10 % tissue homogenate (0.5ml) or MA (4.3nmol). When MLHPO (150nmol) was used, it consisted of 20 % acetate buffer (pH 3.5, 1.9ml), 0.6 % TBA (1ml) and 10 % tissue homogenate (0.25ml) or $FeCl_3$(0.5μmol). All reaction mixtures were heated with or without previous gas-exchange as described in "Methods". The values are means±S. D. of 3 determinations expressed as nmol MA.

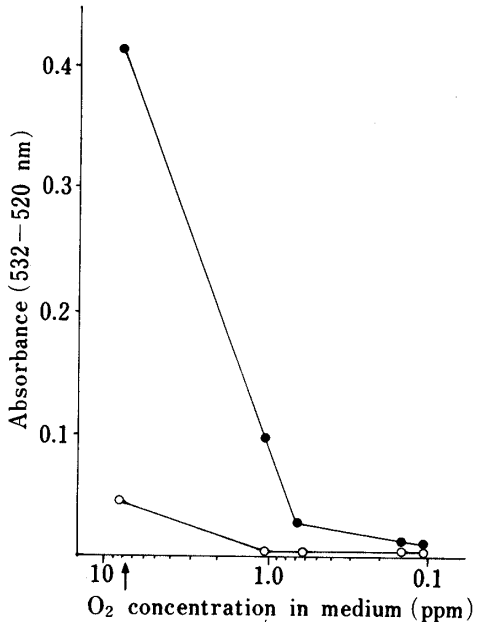

Fig. 1. Dependency of TBA Coloration of Fresh Control and CCl_4-intoxicated Rat Liver Homogenates on Oxygen Concentration in the Reaction Medium

—○—, control; —●—, CCl_4-intoxicated. ↑, indicates the dissolved oxygen concentration under air (about 8 ppm). Reaction conditions are described in "Methods".

Asakawa *et al.*[20] reported that MLHPO cannot yield a sufficient TBA value unless an appropriate catalyst is added with TBA. However, as we reported previously,[19] the characteristics of color development, such as oxygen requirement, depend on the nature of the catalyst employed. In fact, the effect of anaerobic conditions could be nullified by the use of $FeCl_3$ as a catalyst (Table I).

The correlation of TBA value of tissue homogenate with oxygen concentration is shown in Fig. 1. The TBA values of CCl_4-intoxicated liver homogenate appeared to depend on the oxygen concentration.

The Effect of Oxygen on the TBA Reaction of "*in Vitro*-peroxidized Homogenate"

It is known that MA content increases with the aerobic incubation of tissue homogenate, and the measurement of MA in a soluble supernatant is considered to be the principle of the TBA method. In such a method, alterations occurring in the insoluble fraction of homogenate cannot be taken into account.

Since MA can react with TBA without oxygen, as shown above, MA should be

TABLE II. Effect of Oxygen on TBA Value of Air-exposed Methyl Linoleate Hydroperoxide and "*in Vitro*-peroxidized Homogenate"[a]

	Air-exposed MLHPO	*In vitro*-peroxidized homogenate
Aerobic conditions	18.5±0.5	7.3±0.1
Anaerobic conditions	18.2±0.2	5.7±0.1

a) "Air-exposed MLHPO" (exposed at 17—20°C) and "*in vitro*-peroxidized homogenate" are described in "Methods". The composition of the reaction medium is described in Table I. Values represent means±S. D. of 3 determinations.

TABLE III. Effect of pH Change on TBA Values of Air-exposed Methyl Linoleate Hydroperoxide[a)]

	pH 3.5	pH 1.0
Whole sample	18.5±0.5	4.9±0.1
Water soluble	2.4±0.3	1.1±0.1
Residue	13.8±1.0	3.6±0.4

a) "Air-exposed MLHPO" was washed twice with water (0.5ml). The water layers were collected and added to 2ml of 20 % acetate buffer (pH 1.0 or 3.5) and 0.6 % TBA (1.0ml). The residue was added to 2.5ml of 20 % acetate buffer (pH 1.0 or 3.5), 0.6 % TBA (1ml) and 7 % SDS (0.5ml). Both reaction mixtures were subjected to the usual treatment. Values represent means±S. D. of 3 determinations.

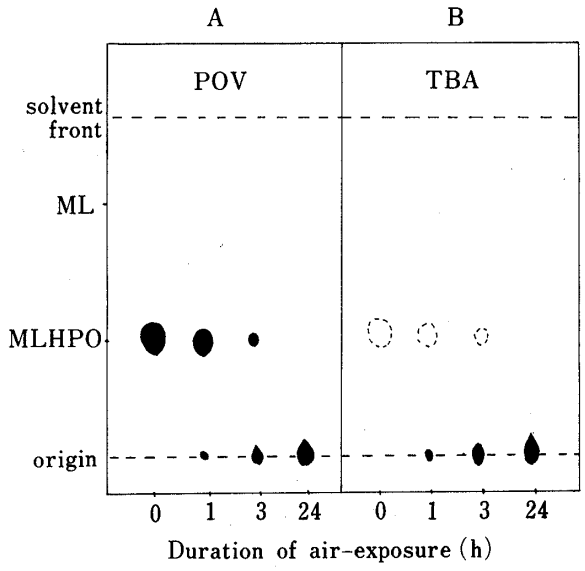

Fig. 2. Thin Layer Chromatograms of Methyl Linoleate Hydroperoxide exposed to Air for a Certain Period before Development

Detection of peroxides on plate A was done by spraying a KI solution, and TBA detection on plate B was done by heating after spraying TBA reagents as described in "Methods".

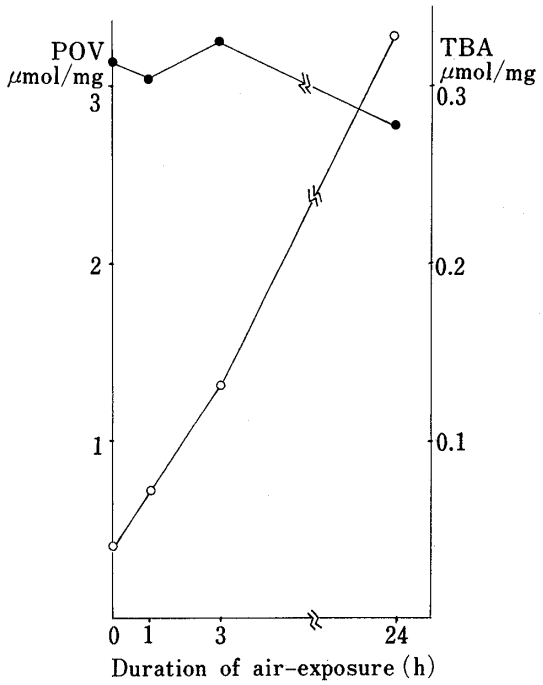

Fig. 3. Effect of Air Exposure of Methyl Linoleate Hydroperoxide on Its Peroxide and TBA Values

—●—, peroxide value; —○—, TBA value. Methyl linoleate hydroperoxide (0.1 mg) was exposed to air at 25—28°C (ambient temperature). The TBA reaction was carried out under anaerobic conditions.

removed from the incubated homogenate, so as to investigate the reactivity of the insoluble fraction with TBA. For that purpose, "*in vitro*-peroxidized homogenate" was subjected to the TBA reaction under aerobic and anaerobic conditions. "Air-exposed MLHPO" was also tested.

From Table II, it can be seen that the oxygen requirement for color development of both *in vitro*-peroxidized homogenate and air-exposed MLHPO was much less than that of the original materials. In other words, on aerobic incubation, both MLHPO and tissue homogenate produced TBA-reactive materials which may be similar to MA and can react with TBA even under anaerobic conditions.

The Properties of Air-exposed MLHPO

To investigate the requirement for oxygen in the TBA reaction, the properties of "air-exposed MLHPO" were examined in detail in comparison with those of "*in vitro*-peroxidized homogenate".

The lack of oxygen requirement in the TBA method for air-exposed MLHPO is apparently similar to that of MA shown in Table I. Thus, to determine whether MLHPO was decomposed to MA by air-exposure or not, air-exposed MLHPO was washed with water. The resulting aqueous phase and the residue were tested by the TBA method under various pH conditions. Table III shows that most of the air-exposed MLHPO is insoluble in water and contains almost no MA, because MA is expected to react with TBA equally at pH 3.5 and 1.0,[9,16] and even the water-soluble fraction still shows pH dependency. There may be no MA in oxidized MLHPO, though UV-irradiated linolenate liberates MA spontaneously.[23,24]

Figure 2 illustrates the thin layer chromatograms of MLHPO after exposure to air for 1 to 24 h. The A plate was treated with KI to visualize the peroxides and the B plate with TBA. Two peroxide regions were observed.

The region of lower *Rf* became more intensely colored by KI than the upper region with increase of the exposure time. The upper region corresponds to MLHPO itself and TBA reaction occurred only in the lower region. These findings indicate that some kinds of TBA-reactive materials other than MLHPO and MA exist in air-oxidized MLHPO,[25-28] and MLHPO itself cannot react directly with TBA.

The changes of anaerobic TBA value and peroxide value (POV) of MLHPO after exposure to air are shown in Fig. 3. The POV is almost independent of the duration of exposure, but the TBA value obtainable without oxygen increases significantly with the progress of exposure. From these results it is clear that POV and TBA values represent different stages of lipid peroxidation.

The Effect of Fe on the TBA Reaction of Tissue Homogenate

The "*in Vitro*-peroxidized homogenate" and "air-exposed MLHPO" could react with TBA in the absence of oxygen. When the TBA reaction of MLHPO was carried out in the presence of $FeCl_3$ or $FeCl_2$, it proceeded regardless of the absence of oxygen.[22,29] Thus, the TBA reaction of tissue homogenate was carried out with $FeCl_3$ under aerobic and anaerobic conditions (Table IV). The TBA value of control tissue homogenate obtained under aerobic conditions increased with Fe addition but such an increase was not brought about by CCl_4-intoxicated homogenate. The TBA value obtained from CCl_4-intoxicated homogenate seems to be sufficiently elevated by some stimulator induced by CCl_4. The value obtained with Fe addition to the tissue under anaerobic conditions seemed to reflect the peroxide level, and the magnitude of the TBA value was similar to the TBA value obtained from tissue without Fe addition under aerobic conditions.

Discussion

Questions have been raised regarding the validity of the TBA method as an index of oxidative deterioration of tissue,[5,30,31] because MA had been regarded as the only TBA-reactive

TABLE IV. Effect of Fe Addition on TBA Value obtained from Control and CCl_4-intoxicated Homogenate under Aerobic and Anaerobic Conditions[a]

	Aerobic condition		Anaerobic condition	
	None	Fe addition	None	Fe addition
Control	3.6±0.2	24.6±2.4	0.3±0.1	4.3±1.3
CCl_4	51.8±1.2	40.5±6.6	1.3±0.1	24.4±5.4

a) The composition of the reaction was as described in Table I except for Fe addition. CCl_4-intoxicated rat was orally given 0.4ml CCl_4/100g body weight 24 h prior to sacrifice.

material in tissue for a long time, and MA is only generated by aerobic preincubation of tissue which can show poor reproducibility.

Recently, TBA reaction directly applicable to tissue homogenate was reported by several authors.[16,32,33] However, the nature of TBA-reactive materials other than MA in tissue homogenate was still obscure and the reaction occurring in the TBA reaction of tissue homogenate seemed to be somewhat different from that of lipid hydroperoxide.

In the preceding paper, we reported that some of the above differences are caused by differences of Fe catalysts.[19] In fact, the data presented here indicate that lipid peroxide in tissue has similar properties to lipid hydroperoxide in view of the fact that both cannot sufficiently react with TBA anaerobically to develop color unless they are aerobically incubated previously, and addition of Fe is likely to negate the requirement for oxygen in both cases.

The necessity for oxygen in the TBA reaction in which MA is the ultimate chromogen is not unreasonable, because the lipid peroxidation process consists of radical production by proton abstraction, hydroperoxide formation by oxygen absorption, further oxidative alteration, and decomposition to MA.[7,8] The further oxidized products are considered to be bicyclic peroxides which can liberate MA under heating or acidic conditions,[31] and such bicyclic peroxides can be formed from lipids containing three or more double bonds. However, a cyclic peroxide which has a β-dioxygen functionality on non-tertiary carbons could react with TBA, therefore it is also reasonable that the further oxidized MLHPO could give a positive TBA test. In fact, linoleic acid peroxidized by lipoxygenase produced these cyclic peroxides.[34]

The reason why the addition of Fe can overcome the requirement for oxygen is obscure, but the addition of Fe enhances the 532 nm absorbance in the TBA assay of cyclic peroxides[35] and added Fe also increases the TBA value of air-exposed MLHPO (data not shown). Therefore, Fe seems to promote the conversion of some MA-precursor (further-oxidized MLHPO) to MA.

The observed POV on TLC of air-exposed MLHPO (Fig. 2) is likely to correspond to Mihelich's 1,2-dioxolanes[34] involved in lipohydroperoxide. The lack of alteration of POV during air exposure (Fig. 3) seems inconsistent with the assumption that MLHPO may decompose to MA-precursor, but if cyclic peroxide production correlates with oxygen absorption, such a constant POV during air exposure would be reasonable.

The present data are mostly refinements of features of the TBA reaction which are generally known and recognized, but it is noteworthy that they also provide evidence for the existence of certain types of precursors of substances which will give a positive TBA reaction. The properties of TBA-reactive materials and the participation of $FeCl_3$ in the TBA reaction should be investigated more precisely to define the validity of the TBA method as the measure of lipid peroxidation in tissues.

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