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Original Contribution

QUANTIFICATION OF LIPID PEROXIDATION IN TISSUE EXTRACTS BASED ON Fe(III)XYLENOL ORANGE COMPLEX FORMATION

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Abstract—Commonly used spectrophotometric methods for determining the extent of lipid peroxidation in animal tissue extracts, such as measurements of diene conjugation and thiobarbituric acid reactive substances (TBARS), have been criticized for their lack of specificity. This study shows that lipid hydroperoxides can be effectively quantified in animal tissue extracts using an assay based on the formation of a Fe(III)xylenol orange complex. Addition of H_2O_2, cumene hydroperoxides, or methanolic tissue extracts to an acidic reaction mixture containing 0.25 mM Fe(II) and 0.1 mM xylenol orange caused the formation of a broad Fe(III)xylenol orange complex absorbance peak at 560–580 nm with a corresponding decrease in the xylenol orange peak at 440 nm. Complex formation measured at 580 nm was saturable with both xylenol orange and Fe (II) concentration. Addition of ascorbic acid, GSH, and cysteine (0.3–5 mM) caused a saturable reduction of the Fe(III)xylenol orange complex. Formation of the Fe(III)xylenol orange complex was linear with the amount of tissue extract added. A significant correlation ($r = 0.88, p < 0.005$) existed between the xylenol orange method of estimating lipid peroxidation and the conventional TBARS assay in a series of animal tissues tested. The time course of increase in A580nm in tests using tissue extracts was typical of a free radical reaction; a lag phase was followed by a log phase. No increase in A580nm was observed up to 24 h when highly peroxidizable arachidonic acid was assayed. These results indicate that the formation of the Fe(III)xylenol orange complex reflects a chemical amplification of the original level of lipid hydroperoxides present in tissue extracts and that peroxidizable lipids do not influence the assay. The potential usefulness of the xylenol orange assay for comparative biochemical and toxicological studies of oxidative stress is discussed.

Keywords—Lipid hydroperoxide, Xylenol orange, Thiobarbituric acid reactive substance, Hydrogen peroxide, Oxidative stress, Free radicals

INTRODUCTION

Lipid peroxidation can be a major contributor to the loss of cell function under oxidative stress situations. For example, (i) peroxidation in microsomal membranes has been shown to lead to calcium release and uncontrolled activation of calcium-dependent proteases and lipases,[1,2] and (ii) peroxidation and permeabilization of mitochondrial membranes can induce disruption of cellular energetics.[3,4] In addition, accumulation of lipoperoxidation products in certain mammalian pathologic conditions is indicative of the involvement of oxygen radicals in the molecular mechanisms of these disorders.[5–10]

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The autoxidation of lipids (LH) leading to hydroperoxide formation (LOOH) can be summarized in the reactions below:

$$LH + R^{\cdot} \rightarrow L^{\cdot} \quad (1)$$

$$L^{\cdot} + O_2 \rightarrow LOO^{\cdot} \quad (2)$$

$$LOO^{\cdot} + LH \rightarrow L^{\cdot} + LOOH \quad (3)$$

where $R^{\cdot}$ is an initiator radical (such as $^{\cdot}OH$) and $LOO^{\cdot}$ is a lipid peroxide radical. Measurements of O_2 consumption, conjugated dienes, and decomposition products of LOOH (such as malondialdehyde, alkenes, and light emission by Russel reactions), have been extensively used as markers of lipid peroxidation.[5,11–14] Furthermore, methods for direct quantification of LOOH are available; the classical methods being the iodometric assay and peroxidase-catalysed reactions.[11,12,15,16]

Determination of lipid peroxidation in tissues sampled from animals exposed to stress conditions, such

as hyperoxia or xenobiotic intoxication, has been the goal of many researchers. Of the methods developed to date for the quantification of stable end products of peroxidation, all have limitations either of reproducibility, sensitivity, or accuracy. Currently the determination of conjugated dienes or of malondialdehyde (as TBARS[14,17]) are the spectrofluorometric methodologies of choice for many relevant studies of comparative biochemistry.[10,18-20] However, it has been shown that much of the diene-conjugated material in tissues does not contain the hydroperoxide functional group, and this suggests that the method is not measuring the products of lipid peroxidation exclusively.[5,21] In addition, the popular TBARS assay[14,22] has been criticized for its lack of specificity and accuracy,[23,24] even though many of the problems are caused by inappropriate modifications of the assay.[25]

This study describes a sensitive and cost-effective alternative for the determination of LOOH in animal tissue extracts. The methodology is based on the oxidation of Fe(II) by LOOH at acid pH in the presence of the Fe(III)-complexing dye, xylenol orange. The reaction has been used to assay peroxides in irradiated solutions[26-27] and to measure LOOH in low density lipoprotein (LDL), membrane preparations, or in plasma.[28-31] The incubation of previously peroxidized liposomes or LDLs with glutathione peroxidase (in the presence of phospholipase A_2 and reduced glutathione) resulted in a nearly 10-fold decrease in the formation of Fe(III)xylenol orange complex. This result demonstrated that the FOX assay (ferrous oxidation/xylenol orange method, as coined by Jiang et al.[28,29]) actually measured lipid peroxides. However, the assay has never previously been applied to assess LOOH in heterogeneous tissue extracts. This study presents the methodology for using a modification of the FOX assay with tissue extracts and shows a significant correlation between our method and the TBARS assay for quantifying lipid peroxides in extracts from several different tissue types. The study also further assessed the chemistry of the process, examining the reaction of peroxides with Fe(II) in the presence xylenol orange and the effect of reducing agents.

MATERIALS AND METHODS

Reagents

Ascorbic acid, thiobarbituric acid (TBA), 3,3′-bis[N,N-di(carboxymethyl)-amino-methyl]-o-resolsulfonephthalein (xylenol orange), butylated hydroxytoluene (BHT), cysteine and glutathione (GSH), and arachidonic acid methyl ester were obtained from Sigma Chemical Co., St. Louis, MO. Other reagents were of analytical grade. Ascorbate, cysteine, and GSH stocks of 0.1 M in 70 mM phosphate buffer pH 7.0 were prepared within minutes before use. Xylenol orange and ammonium ferrous sulfate stock solutions (1 mM each in deionized water) were also prepared immediately before use. Methanol was HPLC grade.

Animals

Adult Wistar rats and CD mice were used; rats were sacrificed using CO_2 gassing and mice by decapitation. All tissues were rapidly dissected out and immediately frozen in liquid N_2. Adult golden-mantled ground squirrels (*Spermophilus lateralis*) were captured at White Mountains, California, by Dr. C. Frank (Carleton University) and maintained on a semi-synthetic diet high in linoleic acid content for a period of 7 weeks as described previously.[32] Squirrels were sacrificed by cervical dislocation and tissues were frozen immediately in liquid N_2. Adult red-eared slider turtles (*Trachemys scripta elegans*) were obtained from Wards Natural Science (Mississauga, Ontario) and maintained in large tanks with dechlorinated water at 7°C for at least 3 weeks prior to experimentation. Turtles were decapitated, and liver and red skeletal muscle were quickly excised and frozen in liquid N_2.

Xylenol orange assay (or FOX-modified assay)

The standard assay for all tissue extracts is as follows. Frozen tissue samples were rapidly weighed and homogenized in 100% cold (5°C) HPLC grade methanol (1:5 w:v except for mouse heart, which was 1:10) using a Janke and Kunkel Ultraturrax homogenizer. Homogenates were centrifuged at 1000 × g in a benchtop centrifuge, and then supernatants were removed for assay. For the standard assay, the following reagents were added sequentially: 0.25 mM $FeSO_4$, 25 mM H_2SO_4, 0.1 mM xylenol orange, and water to a total of 0.9 ml. A sample of tissue extract (2–100 μL) was then added, and the final volume was adjusted to 1 ml with water. Blanks were prepared by replacing tissue extract with water. Samples were incubated at room temperature until the reaction was complete (30 min to 2 d), and absorbance at 580 nm was then read (against water except when stated in the text). With tissue samples, a further addition was then made of 5 μL of 1 mM cumene hydroperoxide or 10 μL of 0.8 mM H_2O_2, and absorbance at 580 nm was again read. Levels of lipid hydroperoxides are expressed as cumene hydroperoxide equivalents.

In initial experiments, we determined that the blank A580 nm (0.04–0.05 Abs) of acidic media containing xylenol, Fe(II), and no tissue extracts (or peroxides) was not different from the A580 nm recorded in acidic media containing only xylenol orange. Moreover, pre-

incubation of the acidic reaction medium over 60 min before addition of peroxides or mouse liver extract caused only minute changes (not statistically significant) in the final A580 nm (not shown). These data indicated that Fe(II) in the acidic reaction media did not oxidize before addition of tissue extracts/peroxides.

Control experiments replaced tissue extracts with 0.5 mM arachidonic acid, and the A580 nm was followed for 48 h. Stock solution of arachidonic acid was prepared just prior to use as 50 mM in anoxic (bubbled with N_2) 19:1 chloroform:methanol.

TBARS measurements

A modification of the methodology described by Uchiyama and Mihara[22] was used. Frozen tissue samples were homogenized (1:10, w:v) in cold 1.1% phosphoric acid. Then 0.4 ml of homogenate was mixed with 0.4 ml 1% TBA/50 mM NaOH/0.1 mM BHT solution and O.2 ml of 7% phosphoric acid (final pH was 1.6–1.7 in all cases). Subsequently, samples were heated for 15 min at 100°C, and then 1.5 ml of butanol was added. Finally, tubes were vigorously vortexed and centrifuged for 5 min in a benchtop centrifuge. The organic layer was removed and placed in cuvettes; absorbances at 532 and 600 nm were measured using a Lambda 4B Perkin Elmer spectrophotometer. For the blanks, 3 mM HCl replaced the TBA solution. Results were calculated in a way that minimized background interference: sample (A532–A600) − blank (A532–A600). Final TBARS values were expressed using the extinction coefficient of 156 mM − 1 cm.

RESULTS AND DISCUSSION

The mechanism involved in Fe(III)xylenol formation

Initial studies involved the use of hydroperoxides instead of tissue extracts to study the mechanism of the Fe(III)xylenol orange complex formation. The reaction of H_2O_2 with Fe(II) (at acid pH) in the presence of xylenol orange induces the formation of a Fe(III)xylenol orange complex.[26] This reaction was completed after approximately 30 min of incubation at 25°C as described by Jiang et al.[29] An acid solution of xylenol orange shows only one peak at 440 nm, and this absorbance increases linearly with the increase in xylenol orange concentration (Fig. 1A). A broad peak at 560–580 nm is formed after the addition of H_2O_2 to the reaction mixture containing 0.25 mM Fe(II) (Fig. 1B). An increase in xylenol orange concentration enhanced the formation of the Fe(III)xylenol orange complex (measured at 580 nm), which saturated at about 0.04 mM xylenol orange (Fig. 1A). A H_2O_2-dependent decrease in A440 nm values was also seen. Similar to previous observations,[28,29] the extinction coefficient for H_2O_2 at 580 nm was 4.0×10^4 M^{-1} cm (results not shown).

Figure 2 shows the dependence of Fe(III)xylenol orange complex formation (resulting from addition of 8 μM H_2O_2 in media containing 0.1 mM xylenol orange) on Fe(II) concentration. A saturable effect was observed and the data values could be fitted to a Hanes plot (Inset to Fig. 2). The equation below describes the formation of Fe(III)xylenol orange:

$$\text{Fe(III)xylenol} = [\text{Fe(III)xylenol}_{(\max)} \cdot \text{Fe(II)}]/[K' + \text{Fe(II)}]$$

where K′ is the concentration of Fe(II) able to induce half-maximal Fe(III)xylenol orange formation. The value of K′ was 0.02 mM. These results suggest that a binding process is involved in the reaction mechanism; the binding of xylenol to Fe(III) probably accounts for these results.

According to a mechanism proposed by Gupta,[26] Fe(III) is a product of the reaction between Fe(II) and H_2O_2 (Fenton reaction) (4) and between xylenol orange radical (xylenol$^\bullet$) (see reaction (5)) and Fe(II) (6). The reactions involving organic peroxides (LOOH) would be similar, and alkoxyl radicals (LO$^\bullet$) would substitute for $^\bullet$OH in the equations. However, the observed stoichiometry of 3 mol Fe(III)xylenol orange formed per mol of peroxide added[29] cannot be explained by Gupta's mechanism.

$$H_2O_2\ (\text{LOOH}) + \text{Fe(II)} + H^+ \rightarrow \text{Fe(III)} + H_2O + {}^\bullet\text{OH}\ (\text{LO}^\bullet) \quad (4)$$

$${}^\bullet\text{OH}\ (\text{LO}^\bullet) + \text{xylenol} + H^+ \rightarrow H_2O\ (\text{LOH}) + \text{xylenol}^\bullet \quad (5)$$

$$\text{xylenol}^\bullet + \text{Fe(II)} \rightarrow \text{xylenol} + \text{Fe(III)} \quad (6)$$

$${}^\bullet\text{OH}\ (\text{LO}^\bullet) + \text{Fe(II)} + H^+ \rightarrow \text{Fe(III)} + H_2O\ (\text{LOH}) \quad (7)$$

Xylenol orange addition to an acidic media containing H_2O_2 and Fe(II) and preincubated for 30 min does not cause an increase in the yield of Fe(III) compared with samples prepared with H_2O_2 as the last addition (there is actually a small decrease) (Table 1). Similar results were also obtained using cumene hydroperoxide (CHP) as the oxidizing agent. Furthermore, a 3 min H_2O_2 preincubation gave the same results as 30 min preincubation (not shown). This indicates that xylenol

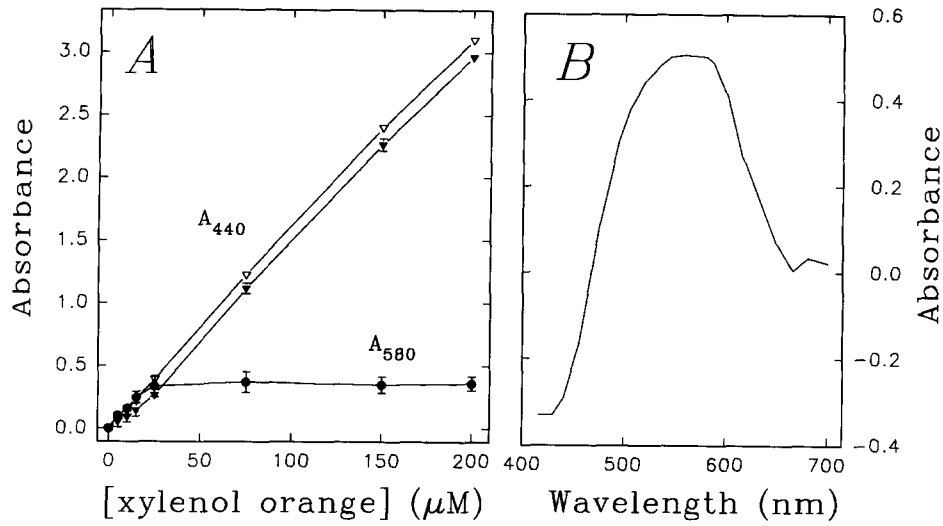

Fig. 1. (A) Effect of increasing concentration of xylenol orange on the absorbance peaks at 440 nm and 580 nm. Reaction media contained 0.25 mM $FeSO_4$, 25 mM H_2SO_4 and the concentrations of xylenol orange shown on the abscissa. Absorbance values at 440 nm were recorded (open triangles) prior to addition of 8 μM H_2O_2. Thirty min after H_2O_2 addition (incubation at 25°C), A440 nm and A580 nm values (filled triangles and circles, respectively) were recorded. Absorbances were read against water. All samples with absorbance above 1.6 were diluted with distilled water 2- to 3-fold. Then the recorded absorbance was multiplied for the dilution factor and used as the actual absorbance of the samples. Data are means ± SD, $n = 3$. (B) Wavelength spectrum of the Fe(III)xylenol orange complex induced by 10 μM H_2O_2 under the same conditions as in the principal panel, using 0.1 mM xylenol orange. The scan was performed against a blank containing no H_2O_2.

orange does not catalyze oxidation of Fe(II), and that Gupta's mechanistic proposal[26] was incorrect. Nevertheless, the xylenol orange radical is possibly formed in reaction media due to the extreme reactivity of 'OH. Alternatively, we postulate that 'OH (formed from the Fenton reaction) reacts with Fe(II) and produces Fe(III) in acid pH.[7] Ferrous iron may compete with xylenol for the reaction with 'OH (a similar reaction may occur involving radicals generated from CHP).

Moreover, a transient complex involving H_2O_2 and Fe(III) could still be formed in the reaction media. This could result in Fe(II), which is formally Fe (IV) or ferryl (found in Compound-1 from peroxidases).[33] This iron of high valence could oxidize Fe(II) and thus increase the overall yield of ferric iron [Fe(IV) + Fe(II) → 2 Fe(III)]. Although these reactions are

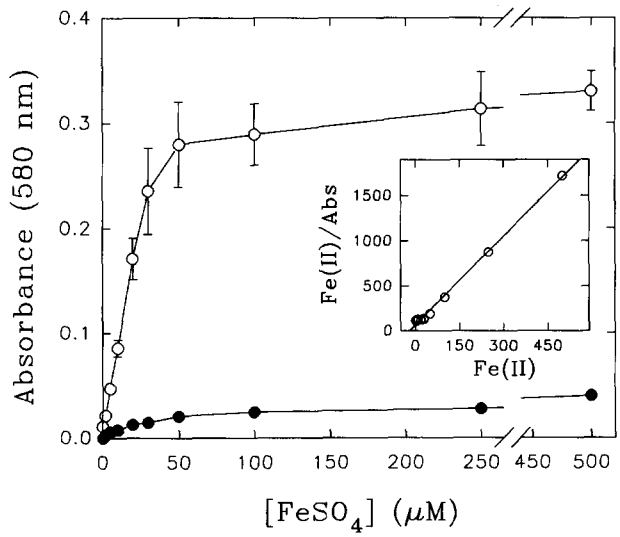

Fig. 2. Dependence of Fe(III)xylenol complex formation (measured as A580) on Fe(II) concentration. Reaction media contained 25 mM H_2SO_4, concentrations of Fe(II) shown on the abscissa, and 0.1 mM xylenol orange. Absorbance values at 580 nm were taken before (filled circles) and after addition of 8 μM H_2O_2 (open circles). Data are means ± SD, $n = 3$; where error bars are not shown, these are enclosed within the symbol used. The inset shows a Hanes plot of the open circle values after subtraction of the corresponding filled circle values.

Table 1. The Effect of 30-min Preincubation of Acid Medium Containing Peroxides (5 μM CHP or 8 μM H_2O_2) and Fe(II) on the Fe(III)Xylenol Orange Complex Formation

	Experimental Condition	
	With Preincubation[a]	Without Preincubation[b]
CHP	0.508 ± 0.019[c]	0.419 ± 0.018
H_2O_2	0.400 ± 0.022[c]	0.326 ± 0.021

Results are expressed as means ± SD ($n = 3$) of absorbance at 480 nm (reading against water).

[a] Reactions were started by the addition of 0.1 mM xylenol orange after the preincubation period.

[b] Reactions were started by the addition of CHP or H_2O_2.

[c] Significantly different (Student t-test, $p < 0.01$) from data obtained without preincubation.

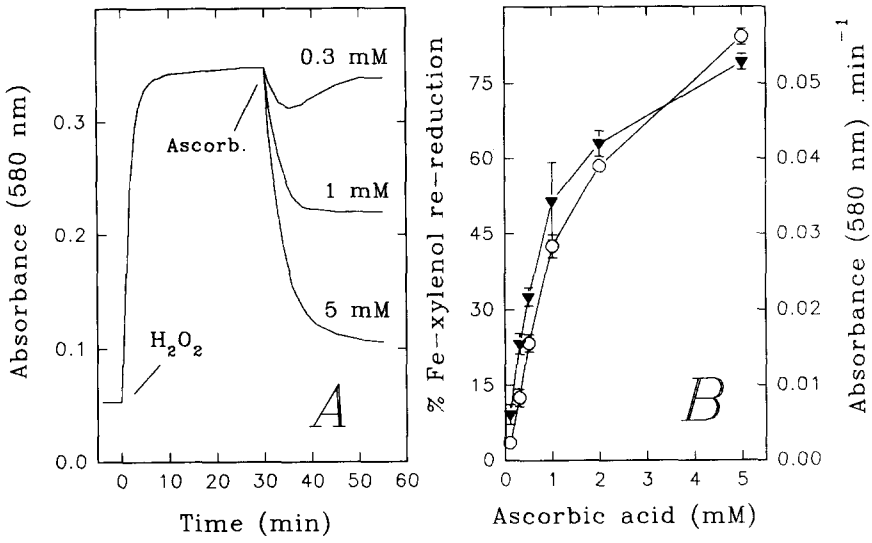

Fig. 3. The effects of ascorbate on the rereduction of Fe(III)xylenol orange complex. (A) The effect of the addition of 8 μM H_2O_2 to the reaction medium followed by the addition of different concentrations of ascorbate. Other reaction conditions were as in Figure 1. (B) The percentage of the maximal level of rereduction (triangles; left axis) and of the rate of Fe(III)xylenol orange rereduction (circles; right axis) on ascorbic acid concentration. Data are means ± SD, n = 3.

of a speculative nature, they could help to explain the stoichiometry of the process of peroxide-induced Fe(III)xylenol orange complex formation.

Addition of ascorbic acid (0.3–5 mM) to the reaction mixture after the H_2O_2-induced Fe(III)xylenol orange complex formation caused a decrease in absorbance at 580 nm due to the reduction of Fe(III) (Fig. 3A). Interestingly, at low ascorbic acid concentration the Fe(III)xylenol orange reduction effect was only transient. This is probably an incidental phenomena since the consumption of ascorbate would result in gradual reoxidation of Fe(II). The dependence of the rates and amount (% of maximum) of Fe(III) reduction on ascorbic acid concentration exhibited saturating profiles. Half maximal rates of Fe(III)xylenol orange complex reduction were obtained with about 1 mM ascorbic acid (Fig. 3B). Reduction of the Fe(III)xylenol orange complex could also be performed with GSH and cysteine (Table 2). Moreover, ascorbic acid, GSH, and cysteine were also able to reduce Fe(III)xylenol orange produced from the oxidation of Fe(II) by CHP.

A new method for quantifying in vivo lipid peroxidation

The next experiments were designed to test whether the xylenol orange assay could be used to quantify lipid hydroperoxides (or more properly, FOX reactive substances) in tissue extracts. Addition of 2 to 70 μL of a methanol extract (1:5 w/v) of mouse liver to the reaction medium (see Materials and Methods) caused the formation of the Fe(III)xylenol orange complex. Linearity was maintained up to 30 μL of liver extract added, and saturation was attained at higher volumes (Fig. 4). This shows the importance of choosing an appropriate sample volume of tissue extract to use and of determining an optimal sample volume before beginning extensive measurements (e.g. 5 μL was chosen for use with mouse liver extracts for further assays). A linear relationship was also observed with increasing amounts of extracts from turtle liver (TL), mouse kidney (MK) (Inset to Fig. 4), rat brain (RB), squirrel liver (SL), squirrel white adipose tissue (SWAT), turtle red muscle (TM), rat lung (RLn), or mouse heart (MH)

Table 2. The Effect of Reducing Agents on the Re-reduction of the Fe(III)Xylenol Orange Complex Formed by the Action of 5 μM CHP or 8 μM H_2O_2

	Oxidant of Fe(II)	
Reductant	CHP	H_2O_2
Cysteine		
0.2 mM	3.8 ± 0.2 (3)	4.4 ± 0.6 (3)
1 mM	18.1 ± 2.9 (4)	0.8 ± 1.4 (4)
GSH		
0.2 mM	8.3 ± 0.8 (3)	8.5 ± 1.8 (3)
1 mM	33.8 ± 1.6 (3)	30.8 ± 2.5 (3)

Results (means ± SD; n values shown in brackets) are expressed as percent of maximal loss of absorbance at 580 nm after addition of the reducing agent. A580 before reducing agent addition was 0.35. Reaction conditions are the same as described in Figure 3.

(not shown). Complete stabilization of the A580 nm was observed after 30 min to 2 h in most cases; SL and RB required 3–4 h to reach maximum A580 nm, and SWAT required 24–36 h. The time course of increase in A580 nm for a given volume of tissue extract was typical of a free radical reaction: a lag phase followed by a log phase increase in absorbance during a propagation-type phase (Fig. 5).

The reaction induced by tissue extracts was also strongly inhibited by the addition of 4 mM BHT (not shown). In the case of the H_2O_2-driven reaction (in the absence of tissue extracts) BHT addition had no effect on the time course of the A580 nm increment. These results suggested that unsaturated lipids present in the tissue extracts might undergo BHT-sensitive peroxidation during the FOX-modified assay and cause the observed A580 nm increase. To test whether the assay measures lipid hydroperoxides or peroxidizable lipids, tissue extracts were replaced with 0.5 mM arachidonic acid, a highly peroxidizable lipid (20:4), and changes in A580 nm were followed over time. The concentration of 0.5 mM arachidonic acid was chosen to match/surpass the concentration of unsaturated lipids that may occur in tissues after 1:100–1:1000 dilutions.[34] Although a substantial increase in A580 nm was observed, this phenomena occurred only after 24 h of incubation in the acidic media of the FOX-modified assay (Inset to Fig. 5). When excess arachidonic acid concentration was used (5 mM), the increase in A580 nm started only after 11–12 h incubation.† These results indicate that FOX-modified assay, when applied to tissues extracts, would not be affected by peroxidizable lipids if shorter term incubation periods (up to 24 h) are used. However, extracts from tissues incubated for longer periods (such as SWAT) may suffer some interferences from lipid peroxidation. The effect of BHT described above could be alteratively explained as due to a quenching of the reaction (7) (when substrates are LO$^{\bullet}$ species, but not $^{\bullet}$OH); BHT could react with LO$^{\bullet}$, resulting in LOH species and BHT radical, and diminishing Fe(III) formation.

The effect of increasing the volume of turtle liver extract (1:5 w/v) on the wavelength spectrum is shown in Figure 6. The A440 nm peak, typical of reduced xylenol orange (at acidic pH), decreased with increasing sample volume, whereas the A560–580 nm peak increased. The ratio of absorbances between 440 nm

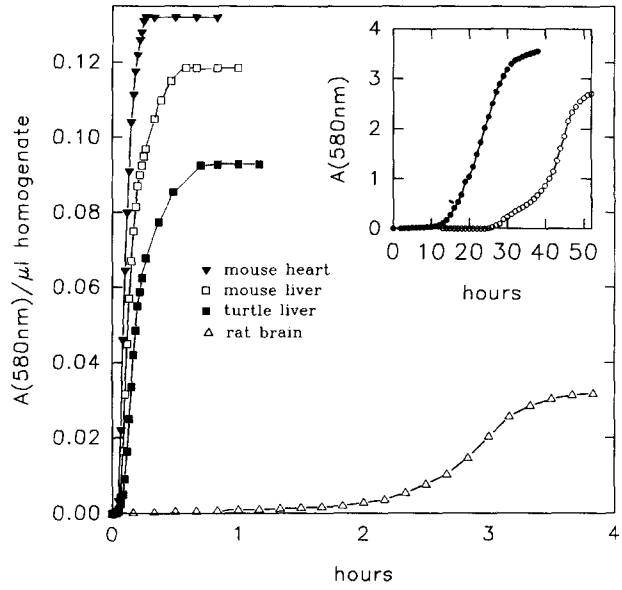

Fig. 5. Typical time course for increases in A_{580} for mouse heart (5 μl), mouse liver (10 μl), turtle liver (10 μl), and rat brain (100 μl). Experimental conditions were the same as Figure 4. Inset shows a typical time course for 0.5 mM (open circles) and 5 mM (filled circles) arachidonic acid under the same experimental conditions of the principal panel. Recorded absorbance values over 1.6 were corrected as in Fig. 1.

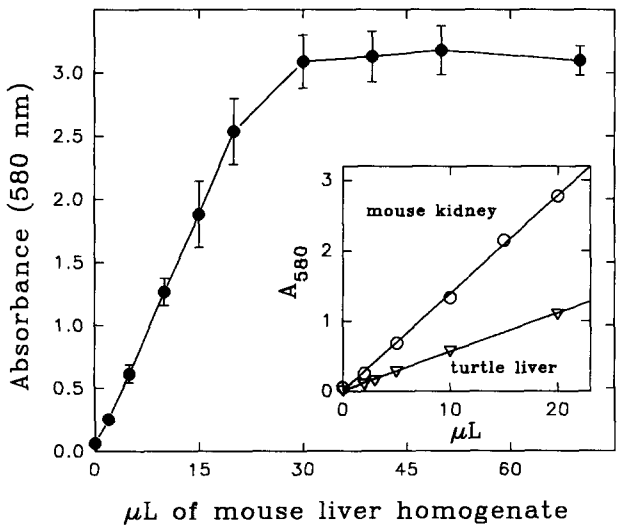

Fig. 4. Effect of increasing volumes of mouse liver extract on the on Fe(III)xylenol complex formation. Reaction media (1 ml) containing 25 mM H_2SO_4, 0.25 mM $FeSO_4$, 0.1 mM xylenol orange and extracts were incubated at 25°C for 1–3 h. For samples with absorbance values above 1.6; see procedure on legend to Fig. 1. Values are the means ± SEM, $n = 3$ independent measurements. The inset shows the dependence of the reaction on homogenate volume for turtle liver and mouse kidney extracts under the same conditions as used in the principal panel.

† Because even the purest commercially available lipids are not totally LOOH-free,[5,38] we cannot rule out that contaminating products of arachidonic acid autoxidation could have partially contributed to the A580 nm increase. When an older stock solution of arachidonic acid was used after storage for 5 to 6 months at 4°C (a condition that favors lipid autoxidation and LOOH formation) before use in the FOX-modified assay (at 5 mM archidonic acid), a fast increase in A580 nm occurred that reached maximal levels within few minutes (not shown).

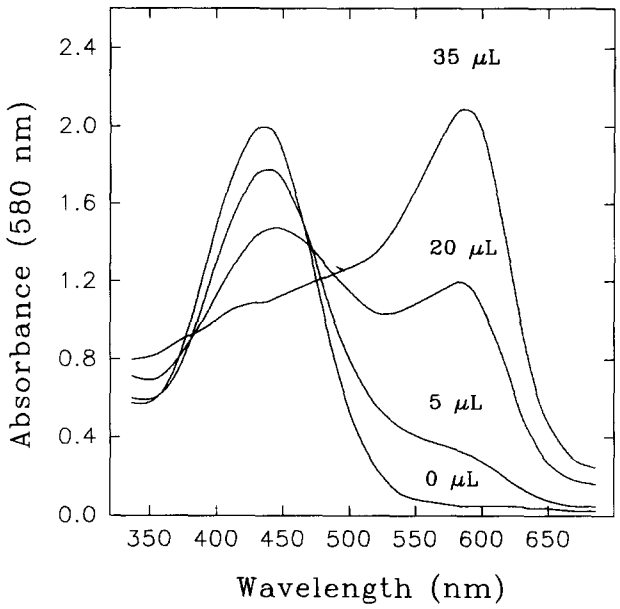

Fig. 6. The effect of different volumes of turtle liver extracts on the wavelength spectra of xylenol orange. Experimental conditions were the same as Figure 4.

and 580 nm are responsible for a series of color changes (from dark yellow to purple-blue) seen when increasing the volume of tissue extract or the concentration of H_2O_2.

It would be predicted that addition of either H_2O_2 or cumeme hydroperoxide (CHP) to assays containing tissue extracts would cause similar increases in A580 nm to those seen in samples without tissue extracts. In the case of CHP addition to assays containing mouse liver extracts (Fig. 7) the A580 nm increase in samples with tissue extracts was 20% greater than the value in assays without liver extracts (a change in A580 nm of 0.512 as opposed to a change of A580 nm of 0.420). However, the addition of 8 μM H_2O_2 after Fe(III)xylenol orange complex had already been formed (via hydroperoxides present in mouse liver extracts) caused an increase in A580 nm that was approximately twice as great that seen in assays without tissue extract (a change in A580 nm of 0.717 as opposed to a change of A580 nm of 0.315). Very similar results were observed using turtle liver extracts. Thus, Fe(III)xylenol orange complex formation induced by H_2O_2 (mediated by $^{\bullet}OH$) manifests more interference caused by reducing/oxidizing agents present in the extracts than Fe(III)xylenol orange complex formation induced by cumene hydroperoxide (mediated by cumenoxyl radical).

In order to express the absorbance values from tissue samples in a quantitative manner we decided to use CHP equivalents as a working unit. By dividing the initial sample absorbance values by the absorbance contributed by CHP, the lipid hydroperoxides (as FOX reactive substances) may be expressed in CHP equivalents per gram wet weight of tissue. These CHP equivalents are relative values for tissue levels of FOX reactive substances. They would include A580 nm contribution by interfering oxidizing and reducing agents present in extracts in a way that would standardize the amount of interference between different tissue samples.

Finally, Figure 8 shows a comparison between this assay and the TBARS method for the determination of lipid peroxidation in several animal tissues. A significant correlation was found between the two methods ($r = 0.88$; $p < 0.005$). The data show, for example, that the three tissues containing the lowest concentration of TBARS (TM, RLn, and SWAT) were also the ones with the lowest levels of Fe(III)-xylenol orange complex formed. Furthermore, the absorbance changes observed in the xylenol orange assay in all samples caused by the CHP addition were approximately the same as that observed in Fig. 7. The FOX-modified assay and the TBARS method quantify different stages of the lipid peroxidation process; the FOX-modified assay quantifies the relative LOOH concentration whereas the TBARS assay measures several aldehyde products of LOOH decomposition.[14] Nonetheless, both estimate the relative "peroxidation profile" of the tissue.

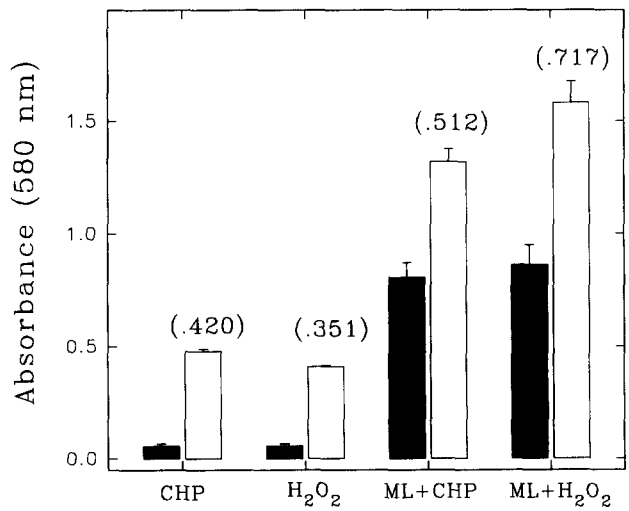

Fig. 7. Effect of addition of H_2O_2 or cumeme hydroperoxide (CHP) on the formation of the Fe(III)xylenol orange complex in 1 ml acid media, in the absence or in the presence of 5 μl of 1:5 mouse liver (ML) extract. Solid and open bars represent A_{580} before and after the addition of hydroperoxides, respectively. Concentrations of H_2O_2 and CHP were 8 and 5 μM, respectively. The reaction conditions were the same as in Figure 1. The addition of both peroxides was made 30 min after the addition of the extracts. Numbers in brackets represent the mean change in absorbance before and after addition of H_2O_2 or CHP. Data are means ± SEM, $n = 3$ or 4.

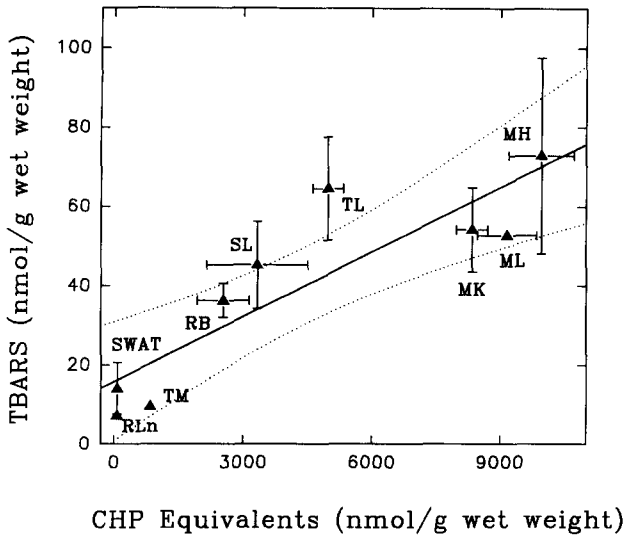

Fig. 8. Correlation between the xylenol orange method (measured as CHP equivalents) and the TBARS assay for quantifying the levels of lipid peroxidation in different animal tissue. The solid and dashed lines are computer generated linear correlation ($r = 0.88$; $p < 0.005$) and 95% confidence intervals, respectively, calculated from the means of values ($n = 9$); where error bars are not seen, these are enclosed within the symbol used. Tissues are: RLn, rat lung; SWAT, squirrel white adipose tissue; TM, turtle red muscle; RB, rat brain; SL, squirrel liver; TL, turtle liver; MK, mouse kidney; ML, mouse liver; and MH, mouse heart.

Comparative measurements of Cu^+-induced peroxidation in liposomes have indicated that the molar levels of Fe(III)xylenol orange complex formation (converted as actual LOOH levels) are 6–9-fold higher than those of TBARS formation.[28,29] The molar ratio of Fe(III)xylenol orange (as CHP equivalents of FOX reactive substances)/TBARS was 70 to 174 (110 ± 16; mean ± SEM) in the several types of tissue extracts shown in Figure 8 (not including RLn and SWAT ratios). Also, considering that 3 mols of Fe(II) are oxidized per mol of LOOH in the presence of xylenol orange,[29] we estimate that the formation of Fe(III)xylenol orange complex during the FOX-modified assay is a 10–20-fold amplification of the original levels of LOOH in the tissue extracts. The mechanism for these free radical reactions in acid media have yet to be elucidated. This amplification property makes it possible to perform sensitive comparative studies of the extent of lipid peroxidation in vivo. Since BHT strongly quenches the amplification reactions, resulting in weaker A580 nm signal, we opted to not include it in the assay recipe, maintaining the high sensitivity of the FOX-modified assay.

It is noteworthy that the squirrel white adipose tissue (SWAT), which has high levels of lipids but a low metabolic rate,[32] had very low levels of FOX reactive substances (these levels of FOX reactive substances in SWAT are still an overestimation due to possible peroxidation during the long incubation period, 2 d, required in this tissue extract) and TBARS (Fig. 8). Considering that under normal conditions the levels of $O_2^{\bullet -}$ production and lipid peroxidation are highly influenced by organ metabolic rates,[35] this result was as expected.

An example of the applicability of the xylenol orange method in comparative biochemistry was obtained recently in a study of the anoxia tolerance of red-eared slider turtles. Studies of the natural tolerance of anoxia by reptiles[36] are excellent models for ischemia/reperfusion.[37] It was observed that hepatic levels of FOX reactive substances in turtles exposed to 20 h anoxia were significantly decreased as assessed by both the conjugated dienes and xylenol orange techniques (W. Willmore and K. Storey, unpublished results). This assay could be useful for comparative measures of lipid peroxidation in mammalian systems, such as perfused organs subjected to ischemia/reperfusion[18,10] or studies of iron overload.[3]

Thus, this study shows the usefulness of the formation of the Fe(III)xylenol orange complex (from the reaction of Fe(II) with peroxides in the presence of xylenol orange) for the measurement of lipid hydroperoxides in tissue extracts. This reaction has been previously used to measure levels of LOOH in biological systems only with membrane preparations[28–30] and with plasma.[32] We demonstrate here that LOOH levels in tissues extracts can also be quantified as CHP equivalents of FOX reactive substances using the assay. Due to a chemical amplification property of the FOX-modified assay, very low levels of LOOH can be detected. This assay cannot be used in fluids containing high levels of reducing agents because those agents could rereduce the Fe(III)xylenol orange complex (see Fig. 3 and Table 2). The positive correlation with the TBARS assay (Fig. 7) indicates that the xylenol orange assay is highly useful for comparative studies of oxidative stress.

BRIEF METHODOLOGY

Add sequentially the following reagents to a series of new glass tubes: 250 μL of 1 mM $FeSO_4$, 100 μL of 0.25 M H_2SO_4, 100 μL of 1 mM xylenol orange, and 450 μL of water. The iron stock solution must be prepared immediately before use. Then add 2 to 100 μL (2, 5, 7, 10, 15, 20, 30, 50, 75, and 100 μL) of methanolic tissue extract (prepared as described in Materials and Methods) to the tubes and bring the final volume to 1 ml with distilled water. Prepare blanks by replacing the tissue extract with water. Incubate samples at room temperature, for times ranging from 30

min to 24 h (12 h is recommended to "fatty" tissues, such as cerebral and adipose tissues), until the increase in A580 nm is maximum (cover samples to prevent evaporation). Read A580 nm values of samples and blanks and subtract blank values. Make sure that the plot of tissue sample volume vs. final A580 nm gives a linear response before reaching a plateau, as depicted in Figure 4.

For the second step of the assay, choose a volume of tissue extract that gives an A580 nm of 0.7–0.8 (e.g., 5 μL of the 1:5 ML extract). Note, however, that it is not recommended that more than 100 μl tissue extract be used even if this results in smaller A580 nm. After reading the A580 nm caused by the tissue extract, add 5 μL of 1 mM cumene hydroperoxide (5 nmol), wait 40–60 min for the reaction to reach a stable end point, and then reread absorbance at 580 nm. Determine the change in absorbance due to the addition of the 5 nmol CHP. Then divide the absorbance values for the tissue extracts by the absorbance contributed by 5 nmol CHP, to express the lipid hydroperoxides (as FOX reactive substances) in CHP equivalents per gram wet weight of tissue (CHPE/g wet weight) using the following formula: CHPE/g wet weight = (A580 nm sample/A580 nm 5 nmol CHP) $\times$ 5 nmol CHP $\times$ 1000/V_1 $\times$ 6, where V_1 = volume of sample extract used and the factor "6" presumes a 1:5 w/v methanolic extract.

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REFERENCES

1. Orrenius, S. D.; McConkey, D. J.; Bellomo, G.; Nicotera, P. Role of Ca^{2+} in toxic cell killing. *Trends Pharmacol. Sci.* **10:**281–285; 1990.
2. Geeraerts, M. D.; Ronveaux-Dupal, M.-F.; Lemasters, J. J.; Herman, B. Cytosolic free Ca^{2+} and proteolysis in lethal oxidative injury in endothelial cells. *Am. J. Physiol.* **261:**C889–C896; 1991.
3. Bindoli, A. Lipid peroxidation in mitochondria. *Free Radic. Biol. Med.* **5:**247–261; 1988.
4. Hermes-Lima, M.; Castilho, R. F.; Meinicke, A. R.; Vercesi, A. E. Fe(II)ATP complex-induced damage to liver mitochondria: Observation of lipoperoxidation-dependent and -independent phases of membrane permeabilization. *Mol. Cell. Biochem.* (in press).
5. Halliwell, B.; Gutteridge, J. M. C. Lipid peroxidation, a radical chain reaction. In: Halliwell, B.; Gutteridge, J. M. C., eds. *Free radicals in biology and medicine.* UK: Claredon Press; 1985: 189–276.
6. Kunio, Y.; Komura, S.; Ihara, N.; Abe, H.; Konishi, H.; Arichi, S. Serum lipid peroxide levels in rats with inherited cataracts. *J. Appl. Biochem.* **7:**202–206; 1985.
7. Wade, C. R.; Jackson, P. G.; Highton, J.; Van Rij, A. M. Lipid peroxidation and malondialdehyde in the synovial fluid and plasma of patients with rheumatoid arthritis. *Clin. Chim. Acta* **164:**245–250; 1987.
8. de Groot, H.; Littauer, A. Hypoxia, reactive oxygen, and cell injury. *Free Radic. Biol. Med.* **6:**541–551; 1989.
9. Catomeris, P.; Monforte, J. R.; Thibert, R. J.; Hood, I. Lipid peroxidation in postmortem blood of fire victims. *Microchem J.* **41:**271–277; 1990.
10. Ayene, I. S.; Al-Mehdi, A. B.; Fisher, A. B. Inhibition of lung tissue oxidation during ischemia/reperfusion by 2-mercapto-propionylglycine. *Arch. Biochem. Biophys.* **303:**307–312; 1993.
11. Pryor, W. A.; Castle, L. Chemical methods for the detection of lipid hydroperoxides. *Meth. Enzymol.* **105:**293–299; 1985.
12. Slater T. F. Overview of methods used for detecting lipid peroxidation *Meth. Enzymol.* **105:**283–293; 1984.
13. Jeejeebhoy, K. N. In vivo breath alkane as an index of lipid peroxidation. *Free Radic. Biol. Med.* **10:**191–193; 1991.
14. Kosugi, H.; Kikugawa, K. Potential thiobarbituric acid-reactive substances in peroxidazed lipids. *Free Radic. Biol. Med.* **7:**205–207; 1989.
15. Hicks, M.; Gebicki, J. M. A spectrophotometric method for the determination of lipid hydroperoxides. *Anal. Biochem.* **99:**249–253; 1979.
16. Cramer, G. L.; Miller, J. F., Jr.; Pendleton, R. B.; Lands, W. E. M. Iodometric measurement of lipid hydroperoxides in human plasma. *Anal. Biochem.* **193:**204–211; 1991.
17. Bird R. P.; Draper H. H. Comparative studies on different methods of malonaldehyde determination. *Methods Enzymol.* **105:**299–305; 1984.
18. Eckenhoff, R. G.; Dodia, C.; Tan, Z.; Fisher, A. B. Oxygen-dependent reperfusion injury in the isolated rat lung. *J. Appl. Physiol.* **72:**1454–1460; 1992.
19. Whiteley, G. S.; Fuller, B. J.; Hobbs, E. F. Deterioration of cold-stored tissue specimens due to lipid peroxidation: Modulation by antioxidants at high subzero temperatures. *Cryobiology* **29:**668–673; 1992.
20. Barja de Quiroga, G.; Lopez-Torres, M; Perez-Campo, R.; Abelenda, M.; Paz Nava, M.; Puerta, M. L. Effect of cold acclimatation on GSH, antioxidant enzymes and lipid peroxidation in brown adipose tissue. *Biochem. J.* **277:**289–292; 1991.
21. Smith, C. V.; Anderson, R. E. Methods for determination of lipid peroxidation in biological samples. *Free Radic. Biol. Med.* **3:**341–344; 1987.
22. Uchiyama, M.; Mihara, M. Determination of malonaldehyde in tissues by thiobarbituric acid test. *Anal. Biochem.* **86:**271–278; 1978.
23. Janero, D. R. Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. *Free Radic. Biol. Med.* **9:**515–540; 1990.
24. Bonnes-Taourel, D.; Guerin, M.-C.; Torreilles, J. Is malonaldehyde a valuable indicator of lipid peroxidation ? *Biochem. Pharmacol.* **44:**985–988; 1992.
25. Lapanna, D.; Cuccurullo, F. TBA test and "free" MDA assay in evaluation of lipid peroxidation and oxidative stress in tissue systems. *Am. J. Physiol.* **265:**H1030–H1031; 1993.
26. Gupta, B. L. Microdetermination techniques for H_2O_2 in irradiated solutions. *Microchem. J.* **18:**363–374; 1973.
27. Michaels, H. B.; Hunt, J. W. Determination of peroxides and hydroperoxides in irradiated solutions of nucleic acid constituents and DNA. *Anal. Biochem.* **87:**135–140; 1978.
28. Jiang, Z.-Y.; Woollard, A. C. S.; Wolf, S. P. Lipid hydroperoxide measurement by oxidation of Fe^{2+} in the presence of xylenol orange: Comparison with the TBA assay and an iodometric method. *Lipids* **26:**853–856; 1991.
29. Jiang, Z.-Y.; Hunt, J. V.; Wolf, S. P. Ferrous ion oxidation in the presence of xylenol orange for detection of lipid hydroperoxide in low density lipoprotein. *Anal. Biochem.* **202:**384–389; 1992.
30. Hunt, J. V.; Bottoms, M. A.; Mitchinson, M. J. Oxidative alterations in the experimental glycation model of diabetes mellitus are due to protein-glucose adduct oxidation: Some fundamental

differences in proposed mechanisms of glucose oxidation and oxidant production. *Biochem. J.* **291:**529–535; 1993.
31. Nourooz-Zadeh, J.; Tajaddini-Sarmadi, J.; Wolff, S. P. Measurement of plasma hydroperoxide concentrations by the ferrous oxidation-xylenol orange assay in conjunction with triphenylphosphine. *Anal. Biochem.* **220:**403–409; 1994.
32. Frank, C. L. The influence of dietary fatty acids on hibernation by golden-mantled ground squirrels (*Spermophilus lateralis*). *Physiol. Zool.* **65:**906–920; 1992.
33. Sutton, H. C.; Winterbourn, C. C. On the participation of higher oxidation states of iron and copper in Fenton reactions. *Free Radic. Biol. Med.* **6:**53–60; 1989.
34. Hilditch, T. P.; Williams, P. N. *The chemical constitution of natural fats* (4th ed.). London: Chapman and Hall; 1964.
35. Martin, V.; McCutcheon, L. J.; Poon, L.; Shen H.; Cory, C. R.; O'Brien, P. J. Comparative mammal model of chronic rate overload relationship of myocardial Ca-cycling to heart, metabolic and lipoperoxidation rates. *Comp. Biochem. Physiol. B* **106:**453–461; 1993.
36. Storey, K. B.; Storey, J. M. Metabolic rate depression and biochemical adaptation in anaerobiosis, hibernation and estivation. *Quart. Rev. Biol.* **65:**145–174; 1990.
37. Hermes-Lima, M.; Storey, K. B. Antioxidant defenses in the tolerance of freezing and anoxia by garter snakes. *Am. J. Physiol.* **265:**R646–R652; 1993.
38. Gutteridge, J. M. C.; Kerry, P. J. Detection by fluorescence of peroxides and carbonyls in samples of arachidonic acid. *Br. J. Pharmacol.* **76:**459–461; 1982.