J. Lipid Research, April 1963
Volume 4, Number 2

A highly sensitive method for measurement of lipid hydroperoxides by iodimetry and amperometric endpoint*

KURT OETTE, M. L. PETERSON,† and R. L. MCAULEY‡

The Rockefeller Institute,
New York 21, New York

[Manuscript received November 19, 1962; accepted December 5, 1962.]

SUMMARY

A simple, accurate, and highly sensitive method is described for measurement of lipid hydroperoxides, utilizing standard iodimetric reactions and an amperometric endpoint. Measurement of 10^{-8} equivalents of hydroperoxides is feasible in samples of milligram size. Several parameters of the analysis have been defined.

This report describes a procedure that permits accurate and specific measurement of lipid peroxides by iodimetry in the small samples available in many biological studies. Martin's review (1) of methods for determination of organic peroxides indicates that most methods are based on reactions with various reducing substances. The most serious drawback of methods other than iodimetry can be ascribed to interfering substances in the reagents, in the solvents, and in the tissue extracts themselves. For specificity, therefore, we have chosen iodimetry, and for increased sensitivity, we have used an amperometrically determined endpoint that permits analysis of as little as 10^{-8} equivalents of lipid peroxides. A study of possibly troublesome side reactions has been made. These include oxidation of iodide by atmospheric oxygen and by contaminants in tissue extracts and in reagents, addition of iodine to plasmalogens and to olefins, loss of iodine by evaporation, and oxidation of thiosulfate by substances other than iodine.

This micro-iodimetric method undoubtedly has applications in the lipid field other than in analysis of peroxides; e.g., in measurement of plasmalogens, in determination of iodine numbers, in phenol titrations, and in measurements of periodate reduction. The many uses of iodimetry have been discussed by Kolthoff and Sandell (2).

* Supported in part by PHS Research Grant H-6222 from the National Heart Institute, U.S. Public Health Service.

† Present address: Washington University School of Medicine, Department of Medicine, St. Louis, Missouri.

‡ Present address: New England Deaconess Hospital, Laboratory of Pathology, Boston, Massachusetts.

METHODS

Principle. Hydroperoxides are reacted stoichiometrically with iodide as shown in equation I.

$$ROOH + 2I^- + 2\,H^+ \rightarrow ROH + H_2O + I_2. \quad (I)$$

Then thiosulfate is added in excess to the reaction mixture to reduce the iodine.

$$I_2 + 2S_2O_3^= \rightarrow 2I^- + S_4O_6^=. \quad (II)$$

Finally, the unreacted thiosulfate is determined by back-titration. Potassium iodate is added to the reaction mixture and reacts with iodide to form iodine,

$$IO_3^- + 5I^- + 6H^+ \rightarrow 3I_2 + 3H_2O, \quad (III)$$

which, in turn, oxidizes the excess thiosulfate (as in Reaction II). After all the thiosulfate has been oxidized, iodine is no longer reduced. Persistence of free iodine permits current to flow through the reaction mixture, by accepting electrons from the cathode, so that the completion of back-titration of thiosulfate can be detected amperometrically.

Reagents. Potassium iodate (analytical grade), dissolved in distilled water to make a stock solution of 0.1 N. Working solutions of 0.0001 N were made by successive dilutions of stock solution.

Sodium thiosulfate (analytical grade), dissolved in oxygen-free distilled water to make a stock solution of

0.1 N. Working solutions of 0.0001 N were made by successive dilution of stock solution. Since sodium thiosulfate solutions are not stable over long periods, fresh reagents were made monthly and stored at 4° in the dark.

Potassium iodide (analytical grade), powdered.

Glacial acetic acid (analytical grade), glass-distilled.

Chloroform and methanol, glass-distilled and tested to assure absence of materials reacting with iodine.

Nitrogen, pre-purified, bubbled through pyrogallol to remove traces of oxygen.

Reference Standards. Working standards of 0.0001 N were prepared by successive dilutions from 0.1 N solutions of iodine, benzoyl peroxide, *t*-butylpermyristate, and dilauroylperoxide. The latter two substances were generously provided by Dr. Leonard Silbert, Eastern Utilization Research and Development Division, U.S. Department of Agriculture, Philadelphia, Pennsylvania.

Equipment. The titration assembly is illustrated in Fig. 1. The platinum wire electrodes (30 mm of coiled 32-gauge wire, distance between electrodes 1-2 mm) were held in the reaction mixture just beside the orifice of the nitrogen bubbler. The stream of nitrogen excluded atmospheric oxygen and mixed the reactants. A microburette (2 ml with 0.01 ml graduations) was fitted with narrow Teflon tubing (0.015 in. i.d.) to restrict the rate of outflow of reagent into the titration mixture.

The amperometric circuit of Potter and White (3) supplies about 175 mv to the electrodes. This voltage is obtained by using a 1.5 volt battery in a circuit containing three different resistors arranged in a T-pattern. The voltage is connected across the two top resistors (2×10^5 ohms, 1×10^6 ohms) and the electrodes are bridged across the smaller of these resistors. The circuit of the electrodes contains the third resistor (100×10^6 ohms) across which the electrometer is placed. Alternatively, a pH-meter may be used for the direct reading of emf, as described by Potter and White.

Technique. Hydroperoxides were reliably measured by the present procedure over the range of 0.01–0.40 μEq. Thus, the concentration of hydroperoxides in a lipid sample determined the amount of lipid taken for analysis (10 mg of a fat with peroxide number of up to 40, and less with more completely peroxidized samples).

The lipid sample was weighed in a 5-ml volumetric flask and taken to volume with chloroform. One milliliter of this solution was placed in a 20-ml glass-stoppered tube and about 150 mg of KI powder was added. Ten milliliters of 2% acetic acid in chloroform–methanol 1:3 was added, washing down the KI crystals from the sides of the tube. After flushing the solution with nitrogen, the tube was closed and shaken, then placed in the dark at room temperature for 1 hr. Iodine liberated (Reaction I) was completely reduced with an excess of 0.0001 N $Na_2S_2O_3$ (Reaction II) added from a standard micropipette. It is advisable to add the thiosulfate in 1-ml quantities in order to limit the amount of iodate required for back-titration. Thus,

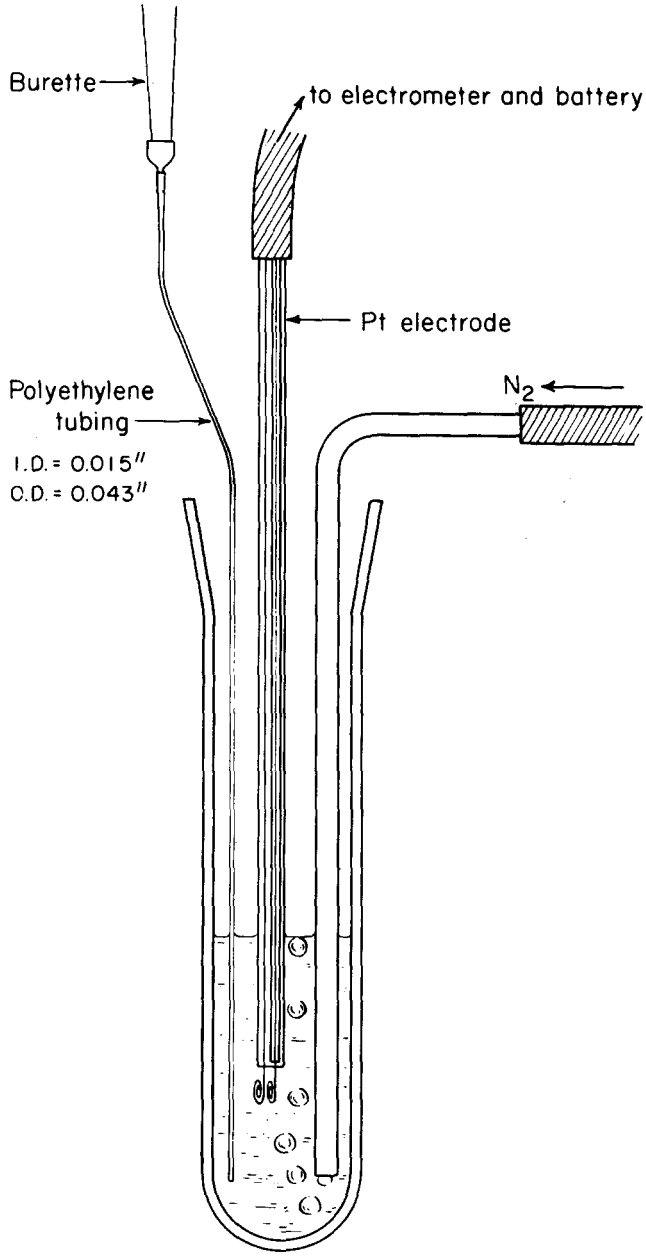

FIG. 1. Titration assembly. The titration is performed in a 20-ml tube. Bubbling nitrogen excludes oxygen and stirs the reaction mixture. Fine Teflon tubing restricts the rate of iodate addition. The endpoint of the reaction is measured by platinum wire electrodes connected with an amperometric circuit.

thiosulfate was added in 1-ml aliquots until visible iodine disappeared. Then, to check for complete reduction of iodine, the electrodes were immersed in the reaction mixture. If sufficient thiosulfate has been added, there will be no appreciable current flow.

Excess thiosulfate was then back-titrated (Reaction III) with 0.0001 N KIO_3 from a microburette. As titration proceeded, the voltage across the circuit fell slightly as the concentration of thiosulfate dropped (Fig. 2). Near the end-point, the emf fell almost to zero, and beyond it there was a rapid increase in current flow as the concentration of iodine rose.

A "reagent blank" was treated in a similar manner. An aliquot of the lipid solvent was titrated as above in order to measure traces of oxidizing substances that cause iodine formation. The "reagent blank" remained remarkably constant for any batch of reagents, usually about 0.03 ml of 0.0001 N KIO_3.

Falsely high hydroperoxide values are characteristic of some tissue extracts because they contain substances, as yet unidentified, that consume thiosulfate. This necessitated measurement of a "thiosulfate blank." The usual procedure for measuring hydroperoxides was followed, except that oxidation of iodide (Reaction I) was prevented by adding thiosulfate to the reaction mixture at 4° directly after the addition of acetic acid in chloroform–methanol, then immediately back-titrating with iodate. When thiosulfate–consuming substances are present, the "thiosulfate blank" may amount to as much as 0.3 ml of 0.0001 N KIO_3.

Calculation. The amount of hydroperoxide in the sample was calculated as follows: mEq of hydroperoxide per kg of lipid[1] = μEq/g =

$$[V_1 - V_2 - (B_1 + B_2)] \times 10^{-1} \div W,$$

where V_1 and V_2 are the volumes (milliliters) of 0.0001 N $Na_2S_2O_3$ and 0.0001 N KIO_3, respectively; B_1 and B_2 are the volumes (milliliters) of iodate required for the "reagent blank" and "thiosulfate blank," respectively; and W is the weight (grams) of the lipid sample.

RESULTS

The accuracy of the method was determined with benzoyl peroxide, *t*-butylpermyristate, and dilauroylperoxide as reference compounds. The hydroperoxide values of these three compounds were found to be 98, 98, and 88% of theoretical, respectively. Corn oil was partially oxidized in a stream of air under UV-light; peroxide values determined by our microtechnique

[1] This value is commonly referred to as the "peroxide number" of a fat.

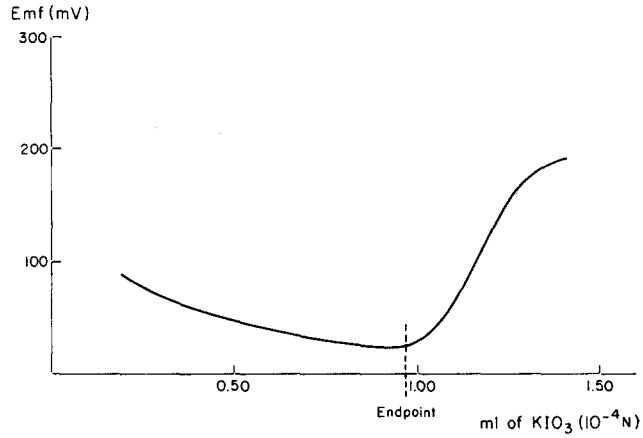

FIG. 2. Titration curve. With the titration of excess thiosulfate by iodate, the voltage across the circuit falls slightly. Beyond the endpoint, iodate reacts with iodide to produce iodine, which causes a rapid increase in current flow.

were 101% of the results obtained by the macro-iodimetric method of Wheeler (4).

Replicate determinations of hydroperoxide content in the same oxidized corn oil sample averaged 95.7 μEq of peroxide/g of fat over a series of sample sizes between 0.2 and 3 mg (0.02–0.3 μEq/sample). The standard deviation was +1.2 μEq/g, the range 94.2 – 96.8 μEq/g.

DISCUSSION

Reaction conditions were variously modified in order to define optimum times, temperatures, and concentrations. For samples containing less than 0.4 μEq of hydroperoxide, the reaction was found to be complete after 60 min at room temperature (Fig. 3). Although the reaction time could be reduced to 5 min by refluxing at 65°, results were always slightly higher than theoretical, suggesting that side reactions were occurring. Analyses of dilauroylperoxide standards gave low yields. That this was due to the presence of impurities in the material available rather than to an incomplete reaction is indicated by the flatness of the plateau from 60–120 min reaction time.

When the concentration of acetic acid in the mixture was raised over 2%, values for the "reagent blank" were considerably increased. However, 2% acetic acid is more than adequate to provide the protons required in Reaction I. The presence of crystals of KI in the reaction mixture assures sufficient iodide for Reactions I and III. Use of a saturated aqueous solution of KI proved impracticable because of rapid

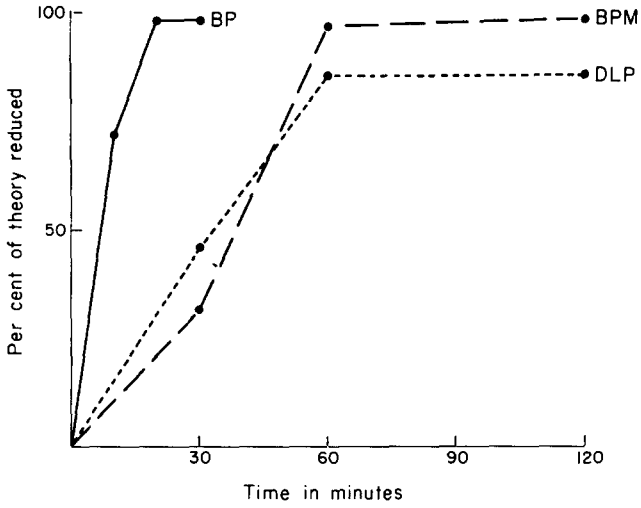

FIG. 3. Reaction time of standard peroxides. Under the conditions described, reduction of three standard peroxides was complete in 60 min. BP = benzoylperoxide, BPM = *t*-butylpermyristate, DLP = dilauroylperoxide.

formation of iodine in the reagent within minutes of preparing the solution.

Chloroform is the most satisfactory solvent for peroxidized lipids; neither methanol nor petroleum ether has sufficient solvent power, while acetone is unsuitable because it cannot be washed with water in order to remove nonlipid contaminants. These water-soluble contaminants lead to peroxide values that are too low, probably because of a reaction with iodate. During the titration in chloroform–methanol–acetic acid, the addition of aqueous reagents sometimes caused formation of a two-phase system. This did not affect the measurements, provided mixing was vigorous and the titration speed was reduced.

Autoxidative changes were minimized by continual flushing of extraction mixtures with nitrogen. When lipid extracts were dried over sodium sulfate, measurements of hydroperoxides were falsely high because of unidentified materials in the drying salt. For this reason, no drying agent was used after the chloroform solution had been washed.

The possibility of addition of iodine to unsaturated lipids has concerned previous workers using iodimetric methods in analysis of lipid peroxides. After addition of free iodine, however, we noted no iodination of double bonds in peroxide-free polyunsaturated fats under the analytical conditions described. This does not apply, however, to fats containing conjugated double bonds; conjugated eicosatetraenoic acid showed a rapid uptake of iodine. This addition could be greatly reduced, but not eliminated, by saturating the chloroform–methanol reaction mixture with KI. Accordingly, tissue extracts containing carotenoids and other olefins yield falsely low results for hydroperoxides. For liver and muscle, the magnitude of error introduced by these substances is in the range of 2.5 μEq/g, at the most. Although it is known that iodination of plasmalogens occurs readily (5), the acidity of our titration medium assures that only small amounts of plasmalogens are destroyed. Extracts of tissues rich in plasmalogens should be exposed to 20% acetic acid at room temperature for an hour to decompose the plasmalogens prior to analysis of peroxides in the usual manner.

Application of this highly sensitive assay of peroxides in biological studies has shown that lipid hydroperoxides can be detected in very low concentrations in samples of milligram size. Studies of hydroperoxide formation in vitamin-E-deficient rats will be reported in detail elsewhere.[2]

The authors gratefully acknowledge the advice and help of Dr. E. H. Ahrens, Jr., in preparing this manuscript.

REFERENCES

1. Martin, A. J. In *Organic Analysis*, edited by John Mitchell, Jr., I. M. Kolthoff, E. S. Proskaver, and A. Weissberger, New York, Inter-Sciences Publishers, Inc., 1960, vol. 4, p. 1.
2. Kolthoff, I. M., and E. B. Sandell. *Textbook of Quantitative Inorganic Analysis*. New York, Macmillan Co., 1956, pp. 585–605.
3. Potter, E. C., and J. F. White. *J. Appl. Chem.* **7**: 309, 1957.
4. Wheeler, D. H. *Oil and Soap (Egypt)* **9**: 89, 1932.
5. Rapport, M. M., and B. Lerner. *Biochim. Biophys. Acta*. **33**: 319, 1959.

[2] Oette, K. Manuscript in preparation.