Evaluation of Antioxidants by a Rapid Polarographic Method[1]

J. W. HAMILTON[2] and A. L. TAPPEL, Department of Food Science and Technology, University of California, Davis, California

Abstract

A simple, rapid, precise, and reproducible method and an adaptable apparatus for polarographic measurement of lipid peroxidation and antioxidant activity is described. This method measures antioxidant activity within a few min time as compared to the hours or days needed for several widely used methods. Peroxidation rates were measured for different lipids as a function of amount of lipid, and amount of biocatalyst. The method is applicable to the detection and measurement of the antioxidant effects exhibited by weak antioxidants and has been satisfactorily used for the measurement of the antioxidant activity of different concentrations of powerful antioxidants.

Introduction

THE LARGE AMOUNT of effort expended in testing antioxidants has emphasized the need for rapid and simple methods. Some of the accepted methods like the Active Oxygen Method (1), oxygen absorption (2), and the modified ASTM Bomb Method (3) require many hours for antioxidant measurements. Other accepted methods like the Shaal oven method and the direct wt method (4) require days. A number of techniques for evaluation of antioxidants have incorporated the hematin compounds, particularly hemoglobin, as the lipid peroxidation catalysts (5,6).

This paper describes a polarographic method for measuring antioxidant efficiency which can be completed in min. Lipid peroxidation is recorded as the decrease in dissolved oxygen measured polarographically. The powerful biocatalyst, hemoglobin, is used for rapid initiation of the reaction.

Experimental

Apparatus similar to that described by Packer (7) was used. The complete apparatus with a 10 m amp recorder (Varian G-11A) is shown in Figure 1. Descriptions of the silver and platinum electrodes and the bridge circuit will be given since the original source (7) is not readily available.

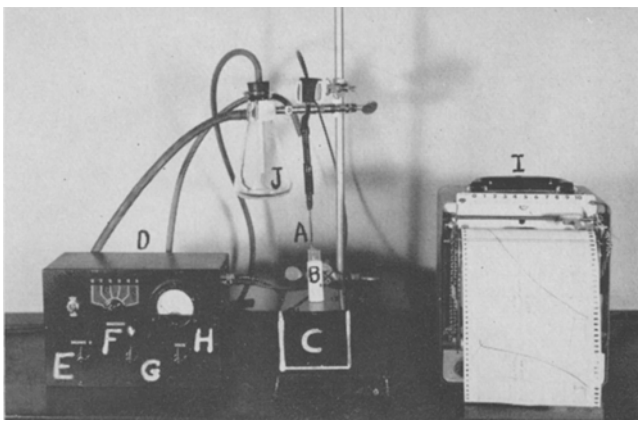

FIG. 1. Polarographic apparatus. A. Silver and platinum electrodes. B. Reaction tube. C. Synchronous motor. D. Bridge circuit. E. Zero control. F. Sensitivity coarse switch and fine adjustment. G. Polarizing voltage control. H. Voltmeter. I. 10 m amp recorder. J. Suction to empty reaction tube.

For the platinum electrode a piece of 1-mm bore soft glass tubing is heated and drawn. A 1 in. piece of #32 size platinum wire is heated until red hot and then dropped into the drawn-out glass tube and the tube tapped gently. The tube is heated at the tip until the glass melts and surrounds the platinum. The platinum tip and glass are trimmed so that the wire is sealed flush with the end. The exposed tip is polished with a fine abrasive until microscopic examination reveals a smooth polished wire tip firmly sealed in the glass with no air bubbles in the seal. The glass tube is filled with mercury, and a soft iron wire is inserted into the mercury to carry the current generated to the center of a shielded cable. A short length of sterling silver wire is used as the other electrode and is connected to the ground of the shielded cable and thus to the bridge circuit shown in Figure 2. For the reaction vessel a short tube, capacity approx 3 ml, is attached to a 60 revolution/min synchronous motor by a plastic bushing to provide a rotating cuvette. The suction device is used to empty and assist in cleaning the cuvette.

The reaction system used for the determinations consisted of 10 or 30% (v:v) lipid emulsified in a phosphate buffer with a minimal amount of solid emulsifier (Myrj 53), and hemoglobin catalyst. The phosphate buffer contained a concentration of 0.01M KCl and sufficient K_2HPO_4 and KH_2PO_4 to yield a concentration of 0.1M and a pH of 7.2. The following lipids were used at various times: linoleic acid (Nutritional Biochemicals Co.), stripped lard (Distillation Products Co.), and methyl linoleate (Pacific Vegetable Oil Corp.). Stripped lard oxidized at a satisfactory rate, however, some difficulty was encountered in preparing and maintaining satisfactory emulsions. Linoleic acid and methyl linoleate were equally satisfactory and were used for nearly all determinations. The emulsion was stable for 30 min or longer; since slow oxidation occurs without addition of catalyst fresh emulsions should be prepared at 30 min intervals. The emulsions were prepared by use of either a hand piston homogenizer or a glass

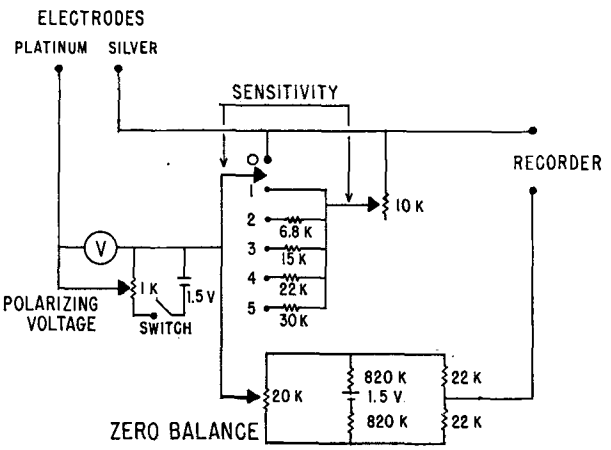

FIG. 2. Bridge circuit.

[1] Supported in part by Grant RG-5759 from the National Institutes of Health.
[2] On sabbatical leave from University of Wyoming, Laramie, Wyo.

tissue homogenizer. The materials to be tested were either added in weighed quantities to the emulsion or added in a buffer solution, if soluble, replacing a like amount of buffer. If they were soluble in organic solvents the desired amount was added to the emulsifying vessel and the solvent removed by evaporation prior to addition of the emulsion or emulsion components.

The electrode tip should be dipped into dilute hydrochloric acid and rinsed prior to use and should remain in a fixed location in the cuvette during the determinations. The emulsion at 26–30C is shaken in air to saturate with air and 2 ml is pipetted into the cuvette. The concentration of oxygen of air saturated water at 26C is 240 μM. The solubility of oxygen in the lipid phase is much greater. Water comprises 90% of the emulsion system and the lipid 10%. We maintained a relatively constant temp, used only comparative measurements, and expressed the results in relative amounts of oxygen consumed during lipid peroxidation.

The polarizing voltage was adjusted to 0.7V. When the electrode is in position and the emulsion added the pen is adjusted to 100 on the recorder with the sensitivity coarse and fine resistors. A 2×10^{-5}M hemoglobin solution was measured by micropipette, and amounts of 5–20 μl were transferred to a small loop glass rod and added to the emulsion with thorough stirring. Blank determinations, using same amount of hemoglobin were run at the beginning and near the end of the experimental period. Duplicate determinations were made and the average time values of two runs were used.

The results are conveniently expressed as protective indices (PI) calculated for the time needed to utilize 90% of the dissolved oxygen. The protective indices are the ratios of the time required for reaction of 90% of the dissolved oxygen in the antioxidant system divided by the time for the control. The time required for the reaction of oxygen varied somewhat from one day to another, however, if the control and test reactions were run during the same experimental period the protective indices for the same reaction were nearly identical from one experimental period to another. Several antioxidants or different concentrations of the same antioxidant can be evaluated within a short period of time with precision.

Results and Discussion

In this method any of a number of lipids might be used. We found linoleic acid, methyl linoleate, and lard quite satisfactory. These lipids peroxidize rapidly enough to make possible rapid determinations but not so rapidly that the antioxidant effects of relatively weak antioxidants cannot be detected. Typical oxidation curves are shown in Figures 3, 4 to illustrate the variables of: 1) kind and amount of lipid present in the emulsion, and 2) the amount of hemoglobin added. Since hemoglobin is a powerful lipid peroxidation catalyst the amount added greatly affects the oxidation rate. We use 10% v/v of lipid in the emulsion since this emulsion appears to be more sensitive to antioxidant effects, oxidizes at a slightly faster rate, and the emulsion is more stable than is a 30% lipid emulsion. If we are testing strong antioxidants we use 1 or 2×10^{-7}M hemoglobin, and if a very weak antioxidant we use 0.5×10^{-7}M hemoglobin. Except in unusual situations 1×10^{-7}M hemoglobin is used as this gives a fast control reaction, usually less than 2 min for 90% oxygen uptake, and a good degree of sensitivity. As can be seen from Figure 4 the length of time required for 90% oxygen uptake varies from 96 sec for 2×10^{-7}M hemoglobin to 211 sec for 0.5×10^{-7}M hemoglobin when the emulsion contains 10% methyl linoleate.

TABLE I

Reproducibility of Lipid Peroxidation Measurements

Date	Lipid	Hemoglobin μM	Time for reaction of 90% of dissolved oxygen, sec
9/28/61	Methyl linoleate	.050	130, 131, 132
11/8/61	Methyl linoleate	.10	102, 104, 105, 105, 105,
9/28/61	Methyl linoleate	.20	75, 76, 76, 78, 80
10/3/61	Linoleic acid	.10	131, 132
9/6/61	Stripped lard	.10	176, 176, 176, 177, 178, 178, 182
9/6/61	Stripped lard	.20	138, 142

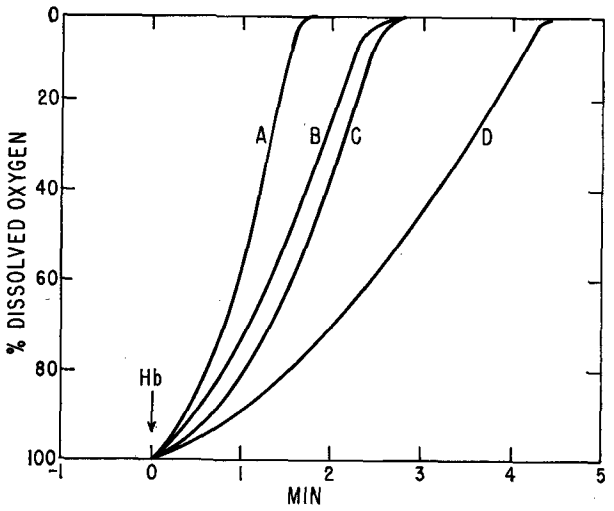

FIG. 3. Rates of oxygen reacting in peroxidation of linoleate emulsions with hemoglobin catalyst. A. 10% linoleic acid, 2×10^{-7}M hemoglobin. B. 10% linoleic acid, 1×10^{-7}M hemoglobin. C. 30% linoleic acid, 2×10^{-7}M hemoglobin. D. 30% linoleic acid, 1×10^{-7}M hemoglobin.

Data to indicate the reproducibility of results for blank determination run at different intervals during a single day are shown in Table I. These results indicate the excellent reproducibility of results by this method when the same emulsion is used. In actual

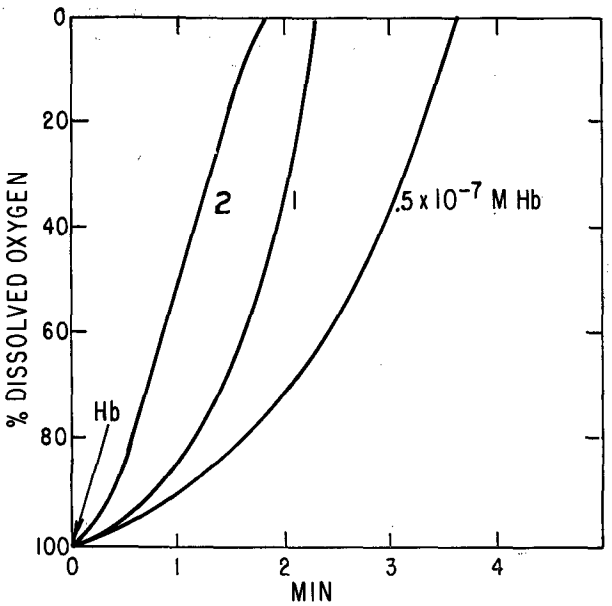

FIG. 4. Effect of increased hemoglobin on the rate of oxygen reacting. The emulsion is 10% methyl linoleate and hemoglobin concentrations are 2, 1, and 0.5×10^{-7}M.

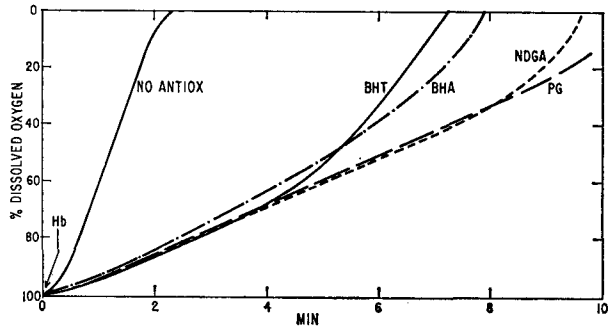

FIG. 5. Polarographic evaluation of antioxidants. The reaction system is 10% methyl linoleate, the catalyst is 1×10^{-7}M hemoglobin. Antioxidants at 1×10^{-4}M are: butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), nordihydroguaiaretic acid (NDGA), and propyl gallate (PG).

practice over a 3 month period some variability in the times required to consume 90% of the dissolved oxygen was noted. These variations for different dates using the same sample and amounts of lipid, similar buffer, and hemoglobin catalyst varied from 90–141 sec with a mean value of 112 and a standard deviation of ±21. This variation was largely the result of the technique used. We measured the buffer and lipid with graduated cylinders and some loss of material occurred using the emulsification process. This variation is not serious when it is realized that the protective index values were calculated using experimental and blank values obtained each period with the same emulsion. The values obtained for the same protective index determination performed on different occasions were nearly identical.

It should be emphasized that this method measures only the antioxidant activity exerted during a brief portion of the total period of lipid peroxidation, however, it is very useful in determining the existence or absence of antioxidant activity or peroxidizability by certain variations in system components. We have found it useful as a means of indicating and measuring the antioxidant effects of low concentrations of known powerful antioxidants present in the reaction system. Data on some of the powerful antioxidants that are widely used in industry are shown in Figure 5. The protective indices calculated from the polarographic measurements of some antioxidants are shown in Table II. The antioxidants greatly extend the time required to utilize 90% of the dissolved oxygen and the reaction curves are slightly variable in shape. It is interesting to note that despite the addition of a powerful antioxidant the reaction proceeds, in most instances, until 90% of the oxygen is utilized. In the

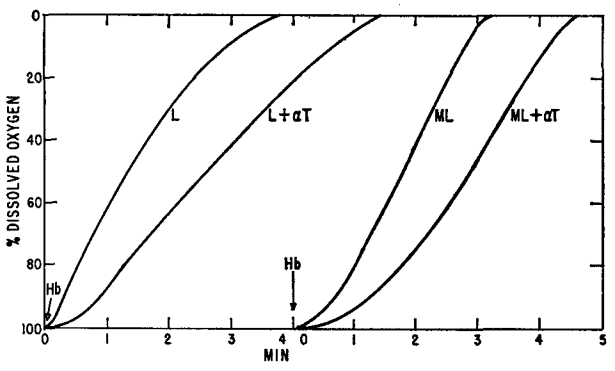

FIG. 6. Polarographic measurement of the antioxidant activity of α-tocopherol. Emulsions were 10% lard (L) and 10% methyl linoleate (ML). Alpha-tocopherol (α-T) was 1×10^{-3}M.

TABLE II

Protective Indices Determined by Polarographic Method [a]

Antioxidant	1×10^{-3}M	1×10^{-4}M	1×10^{-5}M
α-Tocopherol	1.5	.....	.....
Butylated hydroxytoluene	.....	3.7	1.2
Butylated hydroxyanisole	.....	4.2	.....
Propyl gallate	.....	5.7	3.8
Nordihydroguaiaretic acid	.....	5.2	2.8
N N'-Diphenyl p-phenylene diamine	11.8	2.5	1.8
1,2-Dihydro 6-ethoxy 2,2,4-trimethyl quinoline	.....	7.2	4.2
Hydroquinone	5.7	2.2	.....
Pyrogallol	3.4	2.0	.....
Quercetin	.....	.....	1.9

[a] Methyl linoleate (10%) + antioxidant + 1×10^{-7}M hemoglobin.

interest of time conservation we often found it necessary to decrease the concentration of these antioxidants in the emulsion. If for example we use 1×10^{-3} M NDGA the time required for 90% oxygen consumption would be an hour or more. Results obtained by use of the polarographic method can be supplemented by use of manometric techniques if it seems advisable to follow the antioxidant effects during several hours or days of the lipid peroxidation.

Stripped lard is a satisfactory lipid for use in the emulsion since it is readlly peroxidized. The shape of the reaction curves shown in Figure 6 indicates, certain differences between the rates and kinetics of the blank reactions using lard and methyl linoleate and 1×10^{-7}M hemoglobin. The differences in the shapes of the curves with lard and methyl linoleate are evident even when α-tocopherol is added to the system. α-Tocopherol has been shown by many investigators to be a weak antioxidant when tested by different methods. Manometric studies indicate that the protective indices of 1×10^{-3}M α-tocopherol (8), added to hemoglobin-catalyzed linoleic acid emulsion were 1.3 at the end of 0.5 hr and 1.6 after 1 hr. These values compare favorably with protective indices of 1.5 for 1×10^{-3}M α-tocopherol added to the methyl linoleate and lard emulsions as shown in Figure 5. These results indicate the close agreement between the protective indices for the same concentration of an antioxidant when included in different lipid systems and measured by the polarographic method. Further, the protective indices obtained by the polarographic method compared closely with the values obtained by the manometric method (6,8) regardless of whether a weak or a strong antioxidant is being tested.

We found the polarographic method readily adaptable to a study of the antioxidant properties of certain animal and plant fractions that were screened for antioxidant activity and synergism of certain amino acids, selenoamino acids, selenium compounds, and others. Many of these compounds are weak antioxidants and this method is readily applicable to determining weak antioxidant activity. The simplicity, ease, and speed of carrying out this determination, and the accuracy and reproducibility of this method are factors that indicate its usefulness for screening substances for antioxidant activity and measuring this activity.

REFERENCES

1. Official and Tentative Methods of the AOCS, Active Oxygen Method Cd 12-57.
2. Pohle, W. D., R. L. Gregory, and J. R. Taylor, JAOCS *39*, 226 (1962).
3. Gearhart, W. M., B. N. Stuckey, and J. J. Austin, JAOCS *34*, 427 (1957).
4. Olcott, H. S., and E. Einset, JAOCS *35*, 161 (1958).
5. Watts, B. M., Adv. Food Res. *5*, 1 (1954).
6. Lew, Y. T., and A. L. Tappel, Food Tech. *10*, 285 (1956).
7. Packer, L., Walter Reed Army Institute of Research, Report 143–57, 13 pp. (1957).
8. Tappel, A. L., W. D. Brown, H. Zalkin, and V. P. Maier, JAOCS *38*, 5 (1961).

[Received May 28, 1962—Accepted October 23, 1962]