Hydrolysis of N-(2-Hydroxy-3-aryloxypropyl)-N-phenylformamide (IIIa,b)——N-(2-hydroxy-3-phenoxy or *p*-tolyloxypropyl)-N-phenylformamide was hydrolysed in 4 N HCl under reflux for 12 hr. The solution was extracted with ether twice and the aqueous layer was evaporated. The residue was treated with 6 N NaOH and extracted with ether. The extract was evaporated and the residue was recrystallized from ethanol to give N-(2-hydroxy-3-phenoxy or *p*-tolyloxypropyl)aniline(IVa,b). IVa; *Anal.* Calcd. for $C_{15}H_{17}NO_2$: C, 74.05: H, 7.04: N, 5.76. Found: C, 74.39: H, 7.12: N, 5.57. IVb; *Anal.* Calcd. for $C_{16}H_{19}NO_2$: C, 74.68: H, 7.44: N, 5.44. Found: C, 74.34: H, 7.55: N, 5.99.

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Hemin-Catalyzed Peroxidation Test for Rapid Evaluation of Antioxidant Activity

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A rapid method for evaluating antioxidant activity was developed. By this method as many as twenty samples can be evaluated simultaneously. Moreover, the procedure is facile and gives quick result. The test system consisted of linoleate emulsion dispersed in phosphate buffer (pH 7.0) containing hemin. The rapid oxygen uptake due to hemin-catalyzed oxidation of linoleic acid was manometrically measured with a Gilson differential respirometer. The rate of decrease in the oxygen uptake was used for evaluating antioxidant activity of samples. The activity of browning products formed by the reaction between amino acid and sugar was determined by this method, and the results were compared with those obtained by the active oxygen method.

Keywords——antioxidant evaluation; oxygen uptake; linoleic acid; hemin; browning product; tocopherol

A number of methods have been known for evaluating oxidative stability of fatty materials and antioxidant activity.[2] The Schaal oven test, the active oxygen method (AOM) and many procedures measuring oxygen uptake are typical methods widely used. However, these conventional methods are time consuming and laborious.

Hamilton and Tappel[3] developed a polarographic, hemoglobin catalyzed oxygen uptake method for determining antioxidant activity. Berner *et al.*[4] modified this method to determine activity in animal fats. Cort[5] also published a paper describing a similar procedure to screen compounds having antioxidant activity and to study synergism using safflower oil as substrate. In these methods, the rate of oxygen uptake was measured with an oxygen analyzer. Thus, only one sample can be determined with one instrument although time required for the test is very short. So, we attempted to develop a new system which can evaluate many samples simultaneously in a short time. As a result, very efficient method was developed. The procedure consists of a hemin peroxidation system and the amount of oxygen consumption in the system is measured manometrically with a Gilson differential respirometer

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which can treat 20 samples at the same time. To confirm the feasibility of this method, the antioxidant activities of tocopherol and browning products prepared from amino acids and dihydroxyacetone were evaluated by this method, and compared with those obtained by the active oxygen method.

Experimental

Materials——Butylated hydroxyanisol (BHA), linoleic acid and corn oil were obtained from Katayama Chemical Co.; *dl*-α-tocopherol and a natural tocopherol mixture (tocopherol content: *ca* 80%) from Eisai Co.; crystalline hemin (Type I) from Sigma Co. Safflower oil and lard which were not added with any antioxidants were obtained from a supplier. All of the amino acids (L-form) and dihydroxyacetone are the products of Tanabe Seiyaku Co. *tert*-Butylhydroquinone (TBHQ) was prepared from BHA as follows: Oxidation of BHA with a mixed acid (nitric acid: acetic acid, 1: 2) led to the formation of *tert*-butylbenzoquinone. Reduction of the quinone with flower tin and hydrochloric acid gave *tert*-butylhydroquinone (mp 126—127°) in a yield of 64% based on starting material.

Preparation of Browning Oil——Browning oils were prepared according to the method previously described by the authors.[6,7] Five milliliters of corn oil was added to a test tube containing 1 mmol of dihydroxyacetone and an equimolar amino acid. The oil was heated at 150° for 5 min. The insoluble materials formed were removed by filtration. The filtrate was used as antioxidant sample of browning oil.

Hemin-Catalyzed Oxygen Uptake Test——Oxidation was followed by manometric measurement of oxygen consumption. Linoleic acid (0.5 ml), 0.1 M phosphate buffer pH 7.0 (3.0 ml), 20% Tween 20 (0.5 ml), ethanol (0.1 ml) and water (0.1 ml) were placed in a respirometer flask. Antioxidant samples were added by dissolving either in ethanol or in water. A control without antioxidant was run in each test. In the sidearm of the flask was placed 0.2 ml of hemin solution (2.5 mg hemin dissolved in 100 ml water containing 0.1 ml of 10% KOH). The flasks containing the systems were attached to the manometer of a Gilson differential respirometer, Gilson Medical Electronics. The systems were equilibrated and emulsified with shaking at 37° for 5 min. The valves were closed and the content in sidearm was transferred to the main part of flask. Oxygen uptake per 30 min or 60 min was taken as a measure of oil stability.

Active Oxygen Method (AOM)——Twenty milliliters of safflower oil or lard was placed in a test tube of an AOM apparatus, Kuramochi Kagaku Co. The test tubes were set in the apparatus maintained at 97.8° ± 0.1°. Air was bubbled into the oil at a constant rate of 2.33 ml/sec. Peroxide value of the oil was determined by conventional KI–$Na_2S_2O_3$ titration procedure at appropriate intervals. For evaluating effectiveness of tocopherol, the time required for the lard to attain a peroxide value of 100 meq/kg was taken as AOM-time. In the case of determing antioxidant activity of browning oil, safflower oil containing 5% browning oil was oxidized under the AOM-condition for 6.5 hr. Then, peroxide value of the oil was determined and the data thus obtained was used for evaluating the activity.

Result and Discussion

In the hemin-catalyzed oxygen uptake method, the effect of hemin concentration on the oxygen uptake was first investigated. The amount of hemin added was varied from 1 μg to 20 μg while the amount of linoleate was kept constant at 0.5 ml. As shown in Figure 1 A, oxygen uptake was proportionally accelerated with increasing the hemin concentration and with extending the incubation period. Figure 1B shows plots of the oxygen uptake *versus* amount of hemin at the incubation periods of 30 min and 60 min. Oxygen uptake increased almost linearly with increase of hemin concentration. When the amount of hemin added was above 10 μg, the rate became too rapid to operate the volmometer of respirometer for reading oxygen uptake of 20 samples. So, the hemin concentration was fixed at 5 μg per 0.5 ml of linoleic acid.

Figure 2 shows the typical curves of the oxygen uptake of linoleic acid with and without tocopherol. Blank determination, using either hemin or lipid emulsion alone, resulted in little or no oxygen uptake throughout the incubation period. In the presence of antioxidants, oxygen absorption was decreased without any induction period characterized by little or no oxygen uptake. The rate of oxidation of linoleic acid was generally satisfactorily constant

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within a single experiment. From the results described above, the hemin-catalyzed oxygen uptake method using Gilson respirometer was found to be useful for evaluation of antioxidant. In this paper, oxygen uptake during 60 min-incubation period was used as an index of antioxidant activity.

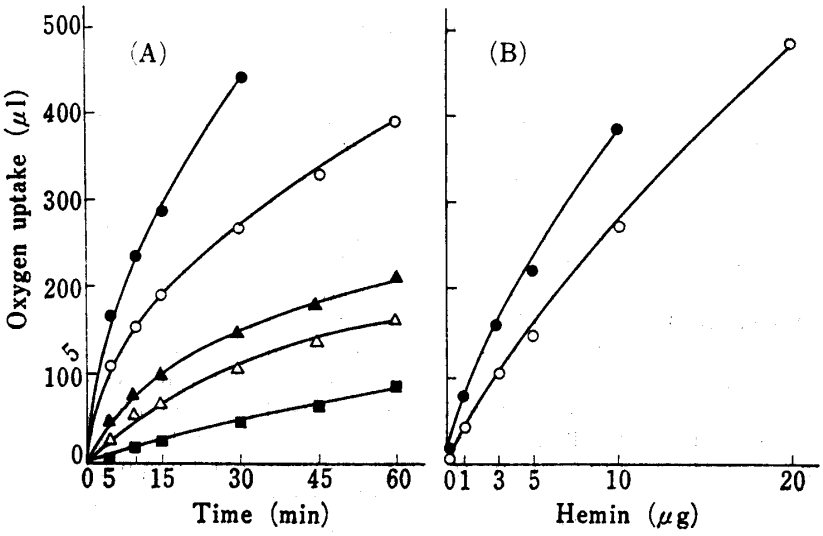

Fig. 1. (A) Time Course of Oxygen Uptake of Linoleic Acid in Various Hemin Concentration (●: 20 μg; ○: 10 μg; ▲: 5 μg; △:3 μg; ■: 1 μg), and (B) Effect of Hemin Concentration on Oxygen Uptake at Incubation Periods 30 min (○) and 60 min (●)

The points on the curves are averages of duplicate determinations. Oxidation was followed by manometric measurement of oxygen consumption. Linoleic acid (0.5 ml), ethanol (0.1 ml), and water (0.1 ml) were placed in a respirometer flask. In the sidearm of the flask was placed 0.2 ml of weak alkaline solution of hemin. The systems were equilibrated and emulsified with shaking at 37° for 5 min. The rapid oxygen uptake due to hemin-catalyzed oxidation of linoleic acid was manometrically measured with a Gilson differential respirometer at an interval of 5 min.

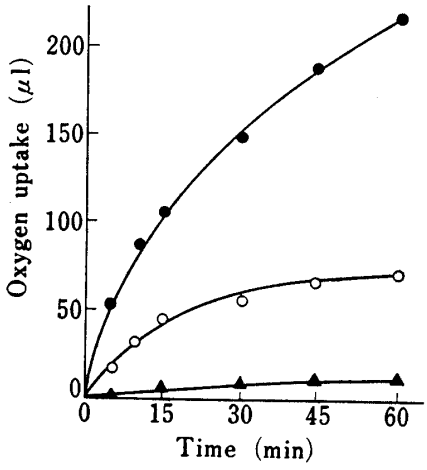

Fig. 2. Typical Curves of Oxygen Uptake of Linoleic Acid

(●: control without tocopherol, hemin 5 μg/linoleic acid 0.5 ml; ○: added with tocopherol, hemin 5 μg/linoleic acid 0.5 ml; ▲: blank, lipid emulsion alone).

The points on the curves are averages of duplicate determinations. Linoleic acid was subjected to hemin-catalyzed peroxidation as described in the Legend of Figure 1. Tocopherol was added as ethanolic solution at a concentration of 0.02% based on linoleic acid.

Table I. Effect of Known Antioxidants on Hemin-Catalyzed Oxygen Uptake of Linoleic Acid

Sample	Oxygen uptake (μl/60 min)
Control	243.5
α-Tocopherol	143.1
Natural tocopherol mixture	65.3
BHA	48.1
BHT	86.7
TBHQ	35.4

Data are averages of duplicate determinations. Linoleic acid was subjected to hemin-catalyzed peroxidation as described in the legend of Figure 1. Antioxidant samples were added as alcoholic solution at a concentration of 0.02% based on linoleic acid.

Activity of some antioxidants was measured by the hemin-catalyzed oxygen uptake method. As shown in Table I, all of the antioxidants tested lowered the oxygen uptake. The antioxidant activity was strongest with TBHQ, followed in decreasing order by BHA, natural tocopherol mixture, BHT, and α-tocopherol.

Antioxidant activity evaluated by the oxygen uptake method was compared with that by the active oxygen method. Table II shows effects of tocopherol level on the antioxidant

Table II. Antioxidant Activity of Tocopherol

Tocopherol Level (%)[a]	O_2 uptake[b] (μl/60 min)	AOM-time[c] (hour)
—	248.2	9.8
0.002	211.8	12.3
0.005	135.6	15.1
0.01	92.3	28.1
0.02	69.7	36.3
0.05	31.4	44.7
0.10	14.6	52.3

a) Concentrations based on oil substrates.
b) Linoleic acid was subjected to hemin catalyzed peroxidation as described in the Legend of Figure 1. Natural tocopherol mixture was added as ethanolic solution.
c) Time to reach peroxide value of 100 meq/kg in active oxygen method described in the text.

activity. In the oxygen uptake method, the uptake apparently decreased with increasing tocopherol level. It was observed that the addition of tocopherol at a concentration of 0.1% gave rise to the formation of an induction period (about 10 min). On the other hand, AOM-time determined by the active oxygen method extended with increasing tocopherol level. Thus there observed close agreement between results of the both methods.

In Table III, the antioxidant effect of browning products was evaluated by both the oxygen uptake and the active oxygen methods. The activity was also compared with that

Table III. Antioxidant Activity of Browning Oil and Other Antioxidants

Sample	O_2 uptake[a] (μl/60 min)	POV[b] (meq/kg)
Control	243.6	26.4
Val-DHA[c]	113.5	6.1
Ile-DHA[c]	104.2	5.8
Leu-DHA[c]	98.7	5.5
Ala-DHA[c]	184.3	19.7
BHA[d]	46.3	18·3
Natural tocopherol mixture[d]	71.0	20.6

Data are averages of duplicate determinations.
a) Linoleic acid was subjected to the hemin-catalyzed peroxidation described in the legend of Figure 1.
b) Safflower oil was oxidized under the AOM-condition. At the termination of 6.5 hr-oxidation, the oil was titrated according to the KI-$Na_2S_2O_3$ titration procedure.
c) The browning oils were prepared by the reaction between each amino acid and dihydroxyacetone. These browning oils were directly added to the oil substrates at a concentration of 5%.
d) BHA or tocopherol was added as ethanolic solution at a concentration of 0.02% based on the oil substrates.

of BHA and natural tocopherol mixture. The order of effectiveness determined by the both methods seemed almost pararell in a series of browning oils. However, when the oxygen uptake method was employed, the activity of browning oils was considerably less than that

of BHA or tocopherol while the formers were more effective than the latter in the case of the active oxygen method.

In conclusion, the hemin-catalyzed oxygen uptake method using Gilson respirometer is facile and gives quick results. The experimental procedure is simple, and as many as 20 samples can be evaluated simultaneously without difficulty. In the experiment to examine the antioxidant activity of tocopherol, the result obtained by this method indicated almost the same tendency as that by the active oxygen method. On the other hand, when the activity values of different type of antioxidants were compared, their effectiveness was significantly different, depending upon whether the AOM or the oxygen uptake method was used. However, it may be reasonable that the relative effectiveness of antioxidants sometimes varies with the substrate. Anyway, in order to determine the antioxidant activity of a new substance, testing by several different evaluation methods seems necessary. The present method has a possibility to detect antioxidant activity of some substances which are inactive by other evaluation methods. The method is also useful as a screening test not only for the antioxidant activity of water-soluble substances but also for that of oil-soluble substances.

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Syntheses of O-α- and O-β-D-Galactopyranosyl-(1→6)-O-[α-D-glucopyranosyl-(1→4)]-D-glucopyranoses

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The title branched trisaccharides were synthesized from 1,2,2′,3,3′,4′,6′-hepta-O-acetyl-β-maltose (**1**). A modified Koenigs-Knorr condensation of **1** with 2,3,4,6-tetra-O-benzyl-α-D-galactopyranosyl chloride or 2,3,4,6-tetra-O-acetyl-α-D-galactopyranosyl bromide afforded a protected trisaccharide having α-D- or β-D-galactosidic linkage at the C-6 hydroxyl group in maltose, respectively. The yield was 37 or 52% from **1**. The configuration of new galactosidic linkages in the trisaccharides was confirmed by comparison of the molecular rotation of their corresponding undecaacetates with the value of calculation. Deacetylation of the undecaacetates afforded the title trisaccharides.

Keywords——trisaccharide synthesis; branched trisaccharide; maltose; D-galactose; modified Koenigs-Knorr reaction; molecular rotation

We reported previously about syntheses of new reducing trisaccharides having α-D- and β-D-(1→6)-galactosidic linkages on the C-6′ hydroxyl group of maltose.[2] As further extension of studies on syntheses of oligosaccharides having D-galactose,[3] we synthesized now the title new branched reducing trisaccharides from maltose derivative. A few trisaccharide syntheses binding monosaccharide to the C-6 hydroxyl group of maltose have been reported: Klemer[4] synthesized 6-O-β-D-glucopyranosylmaltose, which was later synthesized from different starting material.[5]

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