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# FORMATION OF 2-HEXENAL FROM LINOLENIC ACID BY MACERATED *GINKGO* LEAVES

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**Abstract**—The content of linolenic acid and its fat-soluble derivatives in *Ginkgo* leaves has been determined. By utilization of uniformly $^{14}C$-labelled linolenic acid it has been shown that the linolenic acid in *Ginkgo* leaves is converted into 2-hexenal when the leaves are macerated in the presence of air. The conversion of linolenic acid to 2-hexenal under the conditions of temperature and pH existing in the *Ginkgo* leaf requires the presence in the leaves of an enzyme or other catalyst. This is not lipoxidase but is a hexane-insoluble, water-soluble substance. A preparation of this substance strongly catalyzes the absorption of oxygen by linolenic acid in water at 20°.

## INTRODUCTION

ALTHOUGH 2-hexenal is formed by the leaves of other trees under similar conditions,[1] the relatively high yield from *Ginkgo* leaves, as well as the relatively low pH of these leaves, made a study of the mode of formation of 2-hexenal from them of particular interest. Significant amounts of 2-hexenal are not present in intact *Ginkgo* leaves.[2]

Nye and Spoehr[3] have suggested that oleic acid, 3-hexenol or linolenic acid might be the precursor of 2-hexenal in leaves. Drawert *et al.*[4] have reported that in the presence of oxygen the content of linolenic acid in certain leaves decreased as 2-hexenal was formed. Studies with possible precursors which were isotope-labelled showed that in the air, small amounts of 2-hexenal were formed from linolenic acid during the maceration of fresh *Ginkgo* leaves.[5]

Since linolenic acid has been definitely identified as a constituent of *Ginkgo* leaves[6] it seemed advisable to explore more fully its role in the formation of 2-hexenal from *Ginkgo* leaves in the presence of oxygen. Since the oxidative enzyme, lipoxidase (lipoxygenase E.C. 1.13.1.13), is found in many plants[7] and is known to catalyze the oxidation of linolenic acid by oxygen[8] an investigation of its role, if any, in the production of 2-hexenal from *Ginkgo* leaves was undertaken.

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[2] R. T. MAJOR, O. D. COLLINS, P. MARCHINI and H. W. SCHNABEL, *Phytochem.* **11**, 607 (1972).
[3] W. NYE and H. A. SPOEHR, *Arch. Biochem.* **2**, 23 (1943).
[4] F. DRAWERT, W. HEIMANN, R. EMBERGER and R. TRESSL, *Ann. Chem.* **694**, 200 (1966).
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[7] A. L. TAPPEL, *Methods in Enzymology* (edited by S. P. COLOWICK and N. O. KAPLAN). Vol. 5, p. 539, Academic Press, New York (1962).
[8] A. L. TAPPEL, *The Enzymes* (edited by P. D. BOYER, H. LARDY and K. MYRBACK), 2nd Edition, Vol. 5, p. 275, Academic Press, New York (1963).

## RESULTS AND DISCUSSION

### *Linolenic Acid Content of* Ginkgo *Leaves*

Tsujimoto[6] determined the linolenic acid in *Ginkgo* leaves by extracting them with ether, saponification and conversion of the linolenic acid into its hexabromide. This method did not take into consideration the solubility of the hexabromide in ether and also in the large excess of bromine used in the analysis. Therefore, a somewhat different procedure, as described below, was adopted. A known weight of leaves was ground with ether in the presence of a known amount of uniformly $^{14}$C-labelled linolenic acid in an atmosphere of nitrogen. The extract was saponified and converted to the hexabromide with a large excess of bromine. Scintillation counting of the hexabromide provided the data necessary for calculation of the linolenic acid and the ether-soluble linolenate content of the leaves. By this procedure 79·9 mg of linolenic acid were found in 100 g of the *Ginkgo* leaves. At this point it must be noted that additional linolenic acid derivatives may be present in the leaves since only linolenic acid and those derivatives which were soluble in ether and could be saponified were determined by this method.

### *Interaction of Linolenic Acid and Oxygen*

The interaction with oxygen of linolenic acid in water, and of buffered ammonium linolenate at pH 6·8, were studied at room temperature in a Warburg apparatus. Under such conditions there was no detectable absorption of oxygen.

It has been reported that 2-hexenal may be obtained in small quantities from linolenic acid and air at room temperature.[5] In our experiments no 2-hexenal could be detected by GLC or by 2,4-dinitrophenylhydrazine when linolenic and water were agitated in air at room temperature for several minutes. Moreover, uniformly $^{14}$C-labelled linolenic acid in water was homogenized in the presence of air and steam distilled. Any trace of radioactive 2-hexenal formed was trapped by the addition of synthetic, non-radioactive 2-hexenal and the whole converted into the methone derivative.[9] This was then scintillation counted. Only 0·0953% of the radioactivity was recovered as 2-hexenal methone in this experiment. The experiment was repeated in the absence of air or oxygen and in an atmosphere of nitrogen in order to see whether the above 0·0953% recovery of radioactivity was actually due to autoxidation of linolenic acid or because of some impurity in the linolenic acid. The experiment carried out in an atmosphere of nitrogen also produced almost the same recovery of radioactivity, namely 0·0848%. It seems quite clear that this arose as was the case when oxygen was present, from a radioactive impurity, presumably 2-hexenal, in the radioactive linolenic acid. As described later the radioactive linolenic acid used in our experiments was prepared by the addition of a minute quantity of a highly radioactive linolenic acid to a large quantity of non-radioactive linolenic acid. Since our experiments, described above, showed that the non-radioactive linolenic acid neither contained nor formed 2-hexenal under our experimental conditions the 2-hexenal obtained must have come from the minute quantity of the highly radioactive linolenic acid that was used. These findings suggest that the earlier observation of quick autoxidation of linolenic acid to 2-hexenal in the absence of a catalyst was probably due to the same impurity in the linolenic acid.[5]

Privett and Blank have reported that metals such as iron and cobalt catalyze the formation of aldehydes in the autoxidation of unsaturated fatty acids.[10] Since iron has been found

[9] E. C. HORNING and M. G. HORNING, *J. Org. Chem.* **11**, 95 (1946).
[10] O. S. PRIVETT and M. L. BLANK, *J. Am. Oil Chem. Soc.* **39**, 465 (1962).

in the leaves of many trees[11] its effect on the conversion of linolenic acid to 2-hexenal was examined. No 2-hexenal was detected by GLC when aqueous suspensions of linolenic acid at pH 4·4 were homogenized in the presence of ferrous sulfate; at a pH of 6·8 GLC indicated the possible presence of 2-hexenal among the products of the reaction. However, the experiment at pH 6·8 was repeated with uniformly $^{14}$C-labelled linolenic acid and the product isolated as 2-hexenal methone after the addition of non-radioactive 2-hexenal. There was only 0·095% recovery of radioactivity, showing that only a trace, if any, of 2-hexenal was formed under these conditions.

*Role of Lipoxidase in the Formation of 2-Hexenal*

Lipoxidase[7] is the best known enzyme which catalyzes the oxidation of linolenic acid in plants to hydroperoxides. Hamberg and Samuelsson have shown that the oxidation of linolenate at pH 9·0 in the presence of lipoxidase gives exclusively the 13-hydroperoxy derivative.[12] Lipoxidase is reported to be most active at pH 6·5–7·0.[8] The pH of decoctions of *Ginkgo* leaves is only 4·15;[2] the pH of aqueous suspensions of the leaves of most trees are considerably higher. Buffered aqueous mixtures at pH 4·4 and 6·8 which contained linolenic acid and lipoxidase were thoroughly blended. The effect of the addition of ferrous sulfate[13] to these systems was also explored. No 2-hexenal was detected in the preparations at pH 4·4 with or without iron. At pH 6·8 GLC indicated that no 2-hexenal was in the preparation without iron. When the experiment was repeated in the presence of ferrous sulfate GLC showed a few peaks, one of which might have been 2-hexenal. Moreover studies with a mixture of $^{14}$C-labelled linolenic acid, non-radioactive 2-hexenal, lipoxidase, water and ferrous sulfate at pH 6·8 showed on scintillation counting of the 2-hexenal methone derivative only a 0·137% recovery of the radioactivity.

*Evidence of an Oxidative Catalyst in* Ginkgo *Leaves*

The results that had been obtained up to this point have provided no evidence tha 2-hexenal is derived from linolenic acid in leaves. Proof that this conversion does take place when *Ginkgo* leaves are macerated in air was provided by the studies with radioactive linolenic acid which are described below. A known amount of uniformly $^{14}$C-labelled linolenic acid was homogenized in water with a known weight of *Ginkgo* leaves. The 2-hexenal that formed was converted into its methone derivative. The highly purified methone derivative was scintillation counted. Because of losses during purification by recrystallization this did not provide a quantitative indication of the amount of 2-hexenal formed in the experiment. Accordingly, a parallel experiment was carried out with *Ginkgo* leaves from the same tree and picked at the same time as those used in the tracer studies. These leaves were also homogenized in water in the presence of non-radioactive linolenic acid but the 2-hexenal was recovered as the 2,4-dinitrophenylhydrazone which gave essentially quantitative recovery of the 2-hexenal after the following correction was made. The yields obtained were corrected by the results of an independent study of the solubility of 2-hexenal 2,4-dinitrophenylhydrazone[1] in the solvent used in the crystallizations. Based on the reasonable but unproved assumption that the only source of carbon in the 2-hexenal was from carbons 13 to 18 of the linolenic acid, the percentage conversion of linolenic acid to 2-hexenal was

[11] C. S. Piper, *Soil and Plant Analysis*, p. 338, Interscience, New York (1947).
[12] M. Hamberg and B. Samuelsson, *J. Biol. Chem.* **242**, 539 (1967).
[13] A. I. Vogel, reports that ferrous sulfate converts peroxides into aldehydes, *A Textbook of Practical Organic Chemsitry*, 3rd Edition, p. 163, Longmans–Green, London (1957).

10·92%. The above experiment has established that 2-hexenal is formed by the oxidation of linolenic acid which takes place when the leaves are macerated in the presence of air. As the agitation of linolenic acid alone or in presence of lipoxidase does not produce any appreciable amount of 2-hexenal, it is evident that *Ginkgo* leaves contain an enzyme or enzymes or other catalyst, not lipoxidase, which is or are active at pH 4·15. It has been shown earlier that the yield of 2-hexenal is pH sensitive and also that not even a trace of 2-hexenal is formed when the leaves are macerated in presence of air after being steamed.[3] In view of these findings it seems quite probable that the leaves contain an enzyme or enzymes which is or are active at pH 4·15.

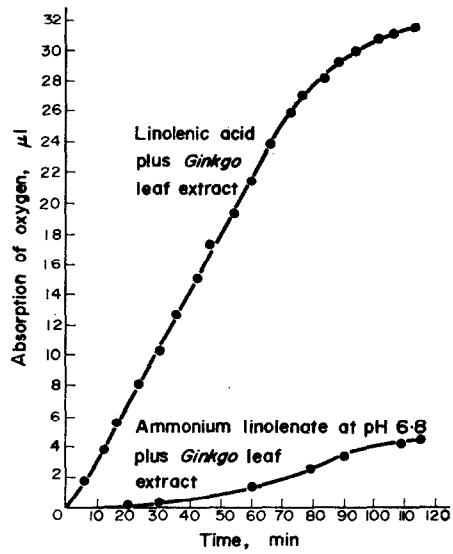


FIG. 1. ABSORPTION OF OXYGEN BY LINOLENATE IN PRESENCE OF DEFATTED *Ginkgo* LEAF EXTRACT.

Further evidence for the presence of a factor in *Ginkgo* leaves that catalyzes the oxidation of linolenic acid was obtained by the following experiment. *Ginkgo* leaves were defatted by repeated grinding with *n*-hexane in an atmosphere of nitrogen. The residue was then blended with water, filtered and concentrated to a small volume *in vacuo* at room temperature. This concentrate was used in a study of its catalytic effect on the absorption of oxygen by linolenic acid in water and also by buffered ammonium linolenate at pH 6·8. Only negligible absorption of oxygen occurred with the buffered solution and the concentrate but very marked absorption took place with linolenic acid in water plus the defatted *Ginkgo* leaf concentrate as illustrated in Fig. 1. Again this points to the presence in *Ginkgo* leaves of an oxidizing enzyme or other catalyst which is active at pH 4·15. Since the completion of this work it has been noted that Grossman *et al.* have recently reported that alfalfa leaves not only contain an enzyme or enyzmes which catalyze the oxidation of linolenates and carotene at pH 6·5–6·8 but also a fraction which catalyzes the oxidation of carotene at pH 4·0–4·5.[14]

[14] S. GROSSMAN, A. BEN AZIZ, P. BUDOWSKI, I. ASCARELLI, A. BERTTER, Y. BIRK and A. BONDI, *Phytochem.* **8**, 2287 (1969).

## EXPERIMENTAL

*Materials.* The leaves of *Ginkgo biloba* were freshly picked. The non-radioactive linolenic acid used was a high-purity grade (Hormel Institute, Austin, Minnesota). The uniformly $^{14}$C-labelled linolenic acid was prepared by dissolving about 0·3 $\mu$g of uniformly $^{14}$C-labelled linolenic acid (Amersham/Searle Corp., Arlington Heights, Illinois) in 3 g of the non-radioactive linolenic acid. The lipoxidase, assaying 8200 units/mg, was from Sigma Chemical Company, St. Louis, Missouri. Synthetic 2-hexenal[1] was purified by preparative GLC on a Carbowax column. For scintillation counting polyethylene vials were used, each containing 12 ml of a solution made from 2,5-diphenyloxazole (4 g) and *p*-bis[2-(5-phenyloxazolyl)]-benzene (50 mg), (Nuclear Enterprises, Inc.) in 1 l. of scintillation grade toluene.

*Linolenic acid content of* Ginkgo *leaves. Ginkgo* leaves (100 g), 120 mg of uniformly $^{14}$C-labelled linolenic acid ($888 \times 10^3$ dis./min) and 350 ml of ether were homogenized under $N_2$ in a blender for 3 min; the ether extract was removed with a siphon. The residue was further extracted with $3 \times 125$ ml portions of ether; the solvent was removed from the combined extract over a hot water bath in a stream of nitrogen. The residue was saponified by refluxing with a solution of 1 g KOH, 10 ml EtOH and 2 ml $H_2O$ for 1 hr. The reaction mixture was cooled and diluted with 100 ml $H_2O$. All steps up to this point were carried out in an atmosphere of nitrogen. The solution was subjected to continuous extraction with ether to remove the unsaponifiable matter. The aq. solution was cooled in ice, acidified with HCl and extracted with ether. The ether extract was washed twice with water, concentrated to 20 ml, cooled in ice, and treated with 3·0 ml of $Br_2$. Since the hexabromide did not crystallize out in 3 days in the refrigerator, the solvent and excess bromine were blown off with $N_2$ at room temp.; the residue was treated with 10 ml of ether and left overnight. The precipitated hexabromide was separated by centrifugation, washed twice with ether and recrystallized four times from EtOH and once from a mixture of ethyl acetate and benzene; the melting point rose to 179–180° (uncor.) (reported[6] 177–178°). This hexabromide (1 mg) showed 1602 counts/min over a background of 133 counts/min leaving 1469 counts/min as net count per mg. The efficiency of the counting was 90%; thus the radioactivity of the hexabromide was 1632 dis/min/mg. The weight of linolenic acid including the ether soluble saponifiable derivatives of linolenic acid expressed as linolenic acid in 100 g of *Ginkgo* leaves was therefore calculated to be 79·9 mg.

*Hexenal formation by* Ginkgo *leaves.* A mixture of 125 g of freshly picked *Ginkgo* leaves and 400 ml $H_2O$ was homogenized in a blender for 3 min;[15] the slurry was steam-distilled. The steam distillate (300 ml) was saturated with NaCl and exhaustively extracted with ether. The total extract (50 ml) was washed with 5 ml $H_2O$ to remove any NaCl and then treated with 10 ml of a solution of 1 g of 2,4-dinitrophenylhydrazine in 97·5 ml of abs. MeOH and 2·5 ml of 6 N HCl. After the mixture had stood overnight it was refluxed for 5 min and the solvents were evaporated over a water-bath under $N_2$. The residue was dissolved in 10 ml of refluxing MeOH; when cooled, it formed crystals overnight. The crystals were collected by centrifugation, washed with two or three drops of MeOH and then with $H_2O$ and dried. They weighed 54·5 mg; m.p. was 144–147°. The solid was recrystallized from 6 ml of MeOH. The recrystallized sample melted at 147–148° (m.p. of 2-hexenal 2,4-dinitrophenylhydrazione[1] is reported in the literature to be 147°);[16] the weight was 36·3 mg. Prior to this, the solubility of 2-hexenal 2,4-dinitrophenylhydrazone in 97·5% MeOH at room temp. had been found to be 2·6 mg/ml. With this correction the calculated yield of 2-hexenal dinitrophenylhydrazone obtained was 77·9 mg. This is equivalent to 27·5 mg 2-hexenal which was formed from the 125 g of *Ginkgo* leaves.

*Hexenal from* Ginkgo *leaves plus added linolenic acid. Ginkgo* leaves (125 g) 480 mg of linolenic acid and 400 ml $H_2O$ were treated as described above for *Ginkgo* leaves without added linolenic acid. A corrected yield of 91·1 mg of 2-hexenal 2,4-dinitrophenylhydrazone, m.p. 147–148°, was obtained; this indicated a total yield of 32·1 mg of 2-hexenal from the *Ginkgo* leaves and the added linolenic acid.

*Linolenic acid as precursor of 2-hexenal.* (1) *In the presence of* Ginkgo *leaves.* Fresh *Ginkgo* leaves (125 g) 480 mg of uniformly $^{14}$C-labelled linolenic acid with a total activity of $3552 \times 10^3$ dis/min, and 400 ml $H_2O$ were homogenized in a blender for 3 min and then steam distilled. The distillate (350 ml) was saturated with NaCl and extracted twice with 25 ml of ether. The methone of 2-hexenal was made from this extract by a modification of the method of Horning and Horning.[9] The ether extract was treated with 175 mg of methone dissolved in 5 ml of 50% aq. EtOH and 1 drop of piperidine. After this mixture had stood at room temp. for 30 min it was refluxed for 10 min and then the solvents were evaporated under $N_2$. The last traces of the solvents, as well as unreacted methone, were removed by means of a vacuum pump at 1 mm pressure at 100° for 15 min. The residue solidified; it was recrystallized by dissolving it in 10 ml of hot EtOH, concentrating to 0·5 ml on a steam bath in a stream of $N_2$, adding hot $H_2O$ to the point of permanent turbidity and removing the turbidity with a drop of EtOH. After this solution had stood overnight the crystals which formed were separated by centrifugation; they were washed with 1 ml of 50% EtOH. The solid was recrys-

[15] Larger yields of 2-hexenal were generally obtained from *G. biloba* leaves ground in a blender for 2 min than in a ball-mill for 4 hr.

[16] I. Heilbron, A. H. Cook, H. M. Bunbury and D. H. Hey, *Dictionary of Organic Compounds*, 4th Edition, Vol. 3, p. 1613, Oxford University Press, New York (1965).

talized four more times as described; 28·5 mg of 2-hexenal methone, m.p. 196–197° was obtained. TLC on silica gel showed that the crystals were pure; solvent systems of acetone–hexane, carbon tetrachloride–chloroform and chloroform–ethanol were used.

The 28·5 mg of 2-hexenal methone showed a total radioactivity of 31,260 dis/min or 1096·8 dis/min/mg. In the control experiment above in which non-radioactive linolenic acid was used with *Ginkgo* leaves 32·1 mg 2-hexenal, $C_6H_{10}O$ was obtained corresponding to 117·9 mg of 2-hexenal methone, $C_{22}H_{32}O_4$. The radioactivity of this amount of the methone would then show: 117·9 × 1096·8 dis/min/mg = 129,313 dis/min. The recovery of radioactivity as 2-hexenal methone based on the whole linolenic acid molecule was 129,313 ÷ 3,552,000 = 3·64%. Since it may be assumed, although not proved, that the 2-hexenal was formed from only one-third of the radioactive carbons of the linolenic acid, namely $C_{13}$ to $C_{18}$, the per cent conversion was 3 × 3·64 = 10·92%.

(2) *In the absence of* Ginkgo *leaves.* A mixture of 360 mg of uniformly $^{14}C$-labelled linolenic acid (total activity 2664 × $10^3$ dis/min) and 300 ml $H_2O$ in the absence of *Ginkgo* leaves was homogenized and then worked up as in the preceding experiment with *Ginkgo* leaves present. However, since the steam-distillate might have contained too little 2-hexenal to be determined readily, 24·5 mg of synthetic 2-hexenal, equivalent to 90·0 mg of 2-hexenal methone, was added to the steam-distillate. This mixture was extracted with ether and converted into the methone derivative with 130 mg of methone in 4 ml of 50% aq. EtOH plus a drop of piperidine. The 2-hexenal methone was purified as described in the previous experiment. A total yield of 24·2 mg of purified 2-hexenal methone was obtained which showed total radioactivity of 682·8 dis/min or 28·2 dis/min/mg. As reported above, the non-radioactive linolenic acid was not converted into 2-hexenal under these conditions but some 2-hexenal may be present in the radioactive linolenic acid, although extremely little by weight. The recovery of the radioactivity was 0·0953%.

(3) *In the absence of* Ginkgo *leaves and of oxygen.* The previous experiment was repeated with the same quantities of materials in the absence of $O_2$ but in an atmosphere of $N_2$. The pure 2-hexenal methone (20·3 mg) which was obtained showed a radioactivity of 509·3 dis/min. The recovery of radioactivity was 0·0848%.

*Effect of lipoxidase on hexenal formation from linolenate.* (a) *In the absence of iron.* The phosphate buffers used in these experiments were 5% solutions of potassium dihydrogen phosphate for pH 4·4 and a mixture of 0·1 M sodium hydroxide and 0·1 M potassium hydrogen phosphate for pH 6·8. A mixture of 150 ml of the suitable buffer, 500 mg of linolenic acid and 150 mg of lipoxidase was agitated in a blender for 3 min, steam distilled and the distillate (75 ml) was saturated with sodium chloride. The ether extract (5 ml) was dried over $MgSO_4$. No 2-hexenal was detected on GLC when either buffers at pH 4·4 or pH 6·8 was used. When the mixture was agitated for 25 min instead of 3 min, the same negative results were obtained.

(b) *In the presence of iron.* Two buffered solutions of 150 ml each at pH 4·4 and two buffered solutions at pH 6·8 were prepared. To each solution was added 500 mg of linolenic acid and 500 mg of ferrous sulfate. To one of the mixtures at each pH was added 150 mg lipoxidase; one mixture at each pH remained free of lipoxidase. Each mixture was homogenized and worked up as in the previous experiment with lipoxidase without iron. At pH 4·4 no steam volatile material was detected by GLC. However, at pH 6·8, both with and without lipoxidase, there was steam volatile material which showed peaks on GLC, one of which has the same retention time as 2-hexenal.

(c) *In the presence of iron with radioactive linolenic acid.* Two mixtures, one containing 100 mg lipoxidase and the other none, but each containing 360 mg uniformly $^{14}C$-labelled linolenic acid (2664 × $10^3$ dis/min), 360 mg of ferrous sulfate and 150 ml of the buffer solution at pH 6·8 were separately homogenized and then steam-distilled. To the steam-distillate of each 24·5 mg of 2-hexenal was added. The ether extract of each was converted into the methone derivative as described in earlier experiments in which no iron was used. With lipoxidase 18·2 mg of 2-hexenal methone, showing a radioactivity of 737·8 dis/min, was obtained. The recovery of the radioactivity in this case was 0·137%.

In the case of the experiment without lipoxidase, but with ferrous sulfate, 23·9 mg of 2-hexenal methone with 680 counts/min was obtained under the same conditions. This was equivalent to a radioactivity of 672·2 dis/min. The recovery of radioactivity was 0·095%.

*Preparation of an aqueous solution of an oxidation catalyst from* Ginkgo *leaves.* Fresh *Ginkgo* leaves (100 g) were placed in a specially constructed blender with an air-tight lid and an inlet and an outlet tube. and $O_2$-free $N_2$ was passed through the system for 1 hr. Freshly distilled hexane (AR hexanes-Mallinckrodt) (125 ml) was added while maintaining the $N_2$ atmosphere and the mixture was homogenized for 5 min. The hexane solution was siphoned out with a stream of $N_2$. The residue in the blender was completely defatted by repeated agitation with 125 ml portions of hexane until the solutions became colorless; all operations were carried out in a stream of $N_2$. The defatted tissue was agitated with 100 ml of distilled $H_2O$ for 20 min and the slurry was filtered. The filtrate (10 ml) was concentrated to 2 ml in a flash evaporator at room temp. This concentrated liquid was used for the studies of its catalytic effect on the absorption of oxygen by linolenic acid and of buffered ammonium linolenate.

*Effect of* Ginkgo *leaf extract on absorption of oxygen by linolenic acid.* A stock solution of ammonium linolenate (0·1098 N) was prepared by dissolving 1 g of linolenic acid in 32·8 ml of 0·1098 N ammonia solution. This was kept cold in an atmosphere of $N_2$. For studies of the oxygen absorption the ammonium linolenate was prepared by diluting 1·5 ml of the stock solution to 100 ml with the phosphate buffer (pH 6·8).

This solution (2 ml) was pipetted into the main compartment of each of three Warburg flasks. The linolenic acid solution was prepared *in situ* by diluting 3 ml of the stock ammonium linolenate solution to 100 ml with water, pipetting 1 ml of this diluted solution into the main compartment of each of three Warburg flasks, and neutralizing each with 1 ml of 0·003294 N HCl. In a side arm of each of the Warburg flasks was placed 0·2 ml of the defatted extract of the *Ginkgo* leaves.

The study of the absorption of oxygen by each preparation was carried out by the method described by Umbreit,[17] in air in a Warburg apparatus maintained at 20·0°. Filter paper with 0·2 ml of a 10% solution of KOH for the absorption of carbon dioxide were placed in the center cup of each Warburg flask; the upper portion of these cups were coated with Vaseline to avoid mixing the KOH solution with the solutions in the other compartments. The flasks were charged with the solutions in the various compartments and equilibrated with air at 20·0° for 20 min. Then the stop-cocks were closed, the solutions in the main compartment and in the sidearm of each flask were mixed, and readings were taken at 6 min intervals. The absorption of oxygen at intervals is shown in Fig. 1. After 800 min of shaking the linolenic acid in water had absorbed 54·5 $\mu$l while the linolenate at pH 6·8 had absorbed only 13·5 $\mu$l.

Three types of control (three flasks in each case) were used, namely (I) phosphate buffer at pH 6·8, (II) linolenic acid solution prepared in the same way as that prepared for the studies with the defatted *Ginkgo* leaf extract, and (III) ammonium linolenate solution in phosphate buffer at pH 6·8, in the same concentration as that used in the experiments with the defatted *Ginkgo* leaf extract. It was found that there was no absorption of $O_2$ in any of these control flasks even after shaking for 800 min. Separate experiments proved that the leaf extract did not absorb $O_2$ in the absence of linolenic acid.

*Acknowledgements*—The authors are much indebted to Mr. John L. Pratt for a grant to the University of Virginia in support of this research and for helpful discussions during the course of our study of the *Ginkgo* leaves.

[17] W. W. Umbreit, R.-H. Burrisand and J. F. Stauffer, *Manometric Techniques*, 3rd Edition, p. 1, Burgess, Minneapolis (1957).

*Key Word Index—Ginkgo biloba;* Ginkgoaceae; ljnolenic acid; 2-hexenal.