

ORIGINAL REVIEW

Biosynthesis of Leaf Alcohol

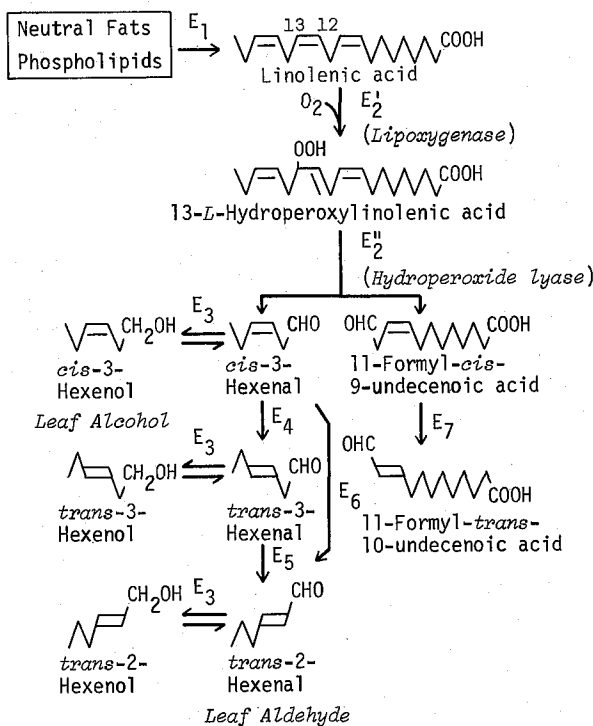
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INTRODUCTION

Leaf alcohol, *cis*-3-hexenol, and leaf aldehyde, *trans*-2-hexenal¹⁾, are widely distributed in fresh leaves, vegetables, and fruits, and they are responsible for the "Green Odor" characteristics of leaves. They are found in some insect excretions, as functioning attractants and repellents. Investigations on leaf alcohol have been carried out by Takei and Ohno *et al.* since 1938.¹⁻⁸⁾ From 1957, we have been studying leaf alcohol and leaf aldehyde from different approaches: synthetic chemistry, natural products chemistry, and plant biochemistry.⁹⁻¹⁰³⁾



Scheme 1 Biosynthetic pathway of leaf alcohol.

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We recently have focused our attention on the biosynthesis of leaf alcohol and aldehyde and have demonstrated the biosynthetic pathway with *Thea sinensis* leaves, as shown in Scheme 1. We know now that the leaf alcohol and aldehyde are produced from *cis*-3-hexenal.^{39, 42, 49)} This C₆-compound, *cis*-3-hexenal, and the C₁₂-compound, 11-formyl-*cis*-9-undecenoic acid,^{61, 62)} biosynthesized from linolenic acid by enzymatic oxygenative splitting through the addition of oxygen to the double bond between C-12 and C-13. In the oxygenative splitting reaction, two enzymes, *lipoxygenase* (E'_2) and *hydroperoxide lyase* (E''_2) are involved, and 13-L-hydroperoxylinolenic acid is an intermediate. From this *cis*-3-hexenal, *trans*-3-hexenal⁶⁰⁾ and *trans*-2-hexenal are formed by the isomerization and *cis*-3-hexenol is formed by reduction with *alcohol dehydrogenase* (E_3)*. The C₁₂ fragment isomerizes to give 11-formyl-*trans*-10-undecenoic acid,^{61, 62)} traumatic half aldehyde, which is known as the "wound hormone".

FORMATION OF "GREEN ODOR"

Changes in fatty acid contents.^{39, 72)} First, changes in fatty acid contents in lipids were examined. The Table I shows the result of leaves harvested in June and in November. The summer leaves showed a high activity for C₆-aldehyde formation and the winter leaves showed a low activity. Fatty acids were prepared from leaves blended for 3 minutes in a Waring blender or without blending. The homogenate was extracted with chloroform-methanol and separated into phospholipid, neutral fat, and free fatty acid fractions by acetone and ether fractionation. The phospholipids and neutral fats obtained were saponified with potassium hydroxide, then esterified with diazomethane. The obtained fatty acid methyl esters were analyzed and determined by GLC with a 20% PEG-adipate column, 3 mm by 1 m. In this table, the 0 and 3 in parentheses refer to minutes of blending time. With no blending, more than 50% of the fatty acids were found in the neutral fat fraction, and the rest in the

Table I. Changes in fatty acid contents in tea leaves

Fraction	Blending time (min)			
	0	3	0	3
	Linoleic acid		Linolenic acid	
Free fatty acid	trace (trace)	trace (trace)	trace (trace)	trace (trace)
Neutral fat	55.6 (18.5)	23.5 (15.8)	218.3 (164.0)	99.3 (145.8)
Phospholipid	16.8 (13.8)	13.3 (3.5)	11.5 (8.4)	10.5 (1.4)
Total lipid	72.4 (32.3)	36.8 (19.3)	229.8 (172.4)	109.8 (147.2)

mg/100 g fresh tea leaves.

On 6th of June, () on 26th of November.

* *trans*-2-hexenol.⁸¹⁾

phospholipid fraction. The ratio of linolenic acid to linoleic acid was about 3 in summer leaves and 5 in winter leaves. When the summer leaves were blended for 3 minutes, large quantities of linoleic acid and linolenic acid, about a half of the initial content, disappeared from these two lipid fractions, neutral fat and phospholipids. But no free fatty acids were found. In the winter leaves, blending caused a far lower decrease in fatty acid content in lipid fractions compared with the summer leaves. These results indicate that linolenic acid and linoleic acid hydrolyzed are converted to compounds other than free fatty acids.

Hexenal and hexenal formation during the blending.^{39,72)} In Fig. 1, the broken line indicates the total linolenic acid content in lipids. During the blending of summer tea leaves, linolenic acid content decreased. Therefore, we expected that C₆-compounds, such as *trans*-2-hexenal and *cis*-3-hexenol derived from linolenic acid would increase during the blending of leaves with decreasing linolenic acid content. In addition to linolenic acid determination, C₆-compound determinations were performed using essential

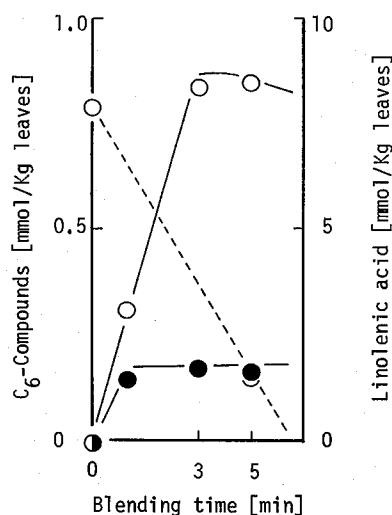


Fig. 1. Effect of blending time on the formation of *cis*-3-hexenol, *trans*-2-hexenal in tea leaves.

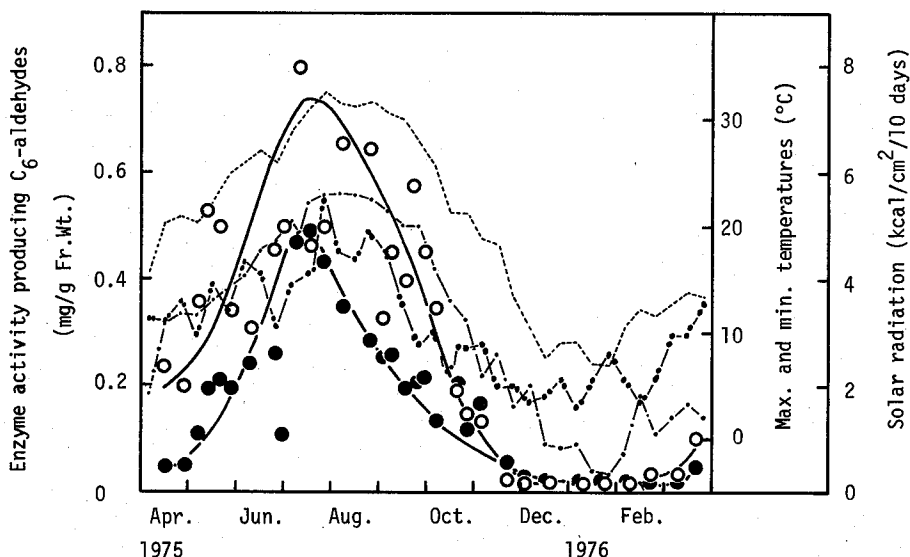


Fig. 2. Seasonal changes in the activity of enzyme system producing *cis*-3-hexenal in fresh leaf homogenate.

oil prepared from blending leaves by steam distillation followed by ether extraction. During the blending, *trans*-2-hexenal, indicated by the open circles and solid line, increased dramatically. *cis*-3-Hexenal, indicated by the dark circles, increased initially at one minute and then reached a plateau. On the other hand, in the first three minutes, 4 μ moles of linolenic acid were lost from lipids and 1 μ mole of *trans*-2-hexenal and *cis*-3-hexenal was newly formed, indicating that one-quarter to one-third of the lost linolenic acid was converted to C₆-compounds, such as *trans*-2-hexenal and *cis*-3-hexenal. In the procedures used here, *cis*-3-hexenal, which is a labile compound in the homogenate, was not detected because of isomerization to *trans*-2-hexenal.

Seasonal changes in the C₆-aldehyde forming activity.^{52,54)} Next we examined the seasonal changes in C₆-aldehydes forming activity from linoleic acid and linolenic acid using tea leaf homogenates. Linolenic acid or linoleic acid was incubated at 35°C for 10 minutes with the leaf homogenate in a sealed flask. At the end of incubation time, the headspace vapor gas was analyzed by GLC as having 20% PEG 20M column in 3 mm by 3 m. Hexanal formation is represented by the dark circles and hexenal formation, by open circles. The dotted lines indicate averages of maximum and minimum temperature for 10 days (Fig. 2). The solid line indicates solar radiation. C₆-Aldehyde forming activity began to increase in late March to April. In July to August, the activity reached a maximum, then gradually decreased and disappeared completely in December. The activity changes were parallel to the temperature changes.

Localization of C₆-aldehyde formation activity.⁴⁷⁾ Since the summer leaves exhibited high activity for C₆-aldehyde formation, we fractionated the tea leaves into subcellular fractions. Tea leaves harvested in August were homogenized with McIlvaine's buffer, pH 6.3, containing 0.4 M sucrose for 3 minutes, and the homogenate was filtered through three layers of gauze. Then the filtrate was subjected to successive centrifugation at 1,000 g, 4,000 g and 19,000 g. The activity for C₆-aldehyde formation in each fraction was determined by the headspace method. Most of the activity for C₆-aldehyde formation was localized in the 1,000 g pellet, which was the chloroplast-rich fraction (Table II). Washing the chloroplast-rich 1,000 g pellet with McIlvaine's buffer, pH 6.3, repeatedly, did not cause a significant decrease in activity. Therefore, the activity for C₆-aldehyde formation was concluded to be localized in the chloroplast lamellae membrane. In the following experiments, the

Table II. Localization of C₆-aldehyde formation activity

Fraction	C ₆ -Aldehydes [μ mol]	
	Hexenals	Hexanal
1000 g pellet*	117.5 (90)	22.3 (76)
4000 g pellet	10.7 (8)	3.6 (12)
19000 g pellet	2.9 (2)	1.1 (4)
Supernatant	0.4 (0)	2.4 (8)

* Chloroplasts

pellet obtained by centrifugation of the 1,000 *g* supernatant at 4,000 *g* for 10 minutes was used routinely as the chloroplast-rich fraction.

Biosynthetic pathway of C₆-aldehydes from ¹⁴C-labeled fatty acids.⁴⁹⁾ When uniformly labeled ¹⁴C-linolenic acid was incubated with isolated tea chloroplasts, two radioactive peaks were found at positions of *cis*-3-hexenal and *trans*-2-hexenal in head-space vapor gas with radio gas chromatograph, as shown by the spiked line (Fig. 3). Other radioactive peakes, such as hexanal, alcohols, and short chain aldehydes, were not found. When incubation was prolonged, radioactivity in *cis*-3-hexenal decreased, and radioactivity in *trans*-2-hexenal increased. When ¹⁴C-labeled linoleic acid was used, hexanal was labeled by ¹⁴C, but no other volatile compounds were labeled. Thus, the six-carbon aldehydes were produced from linolenic acid or linoleic acid in the presence of isolated tea chloroplasts. When the reaction mixture of 1-¹⁴C-labeled linolenic acid and isolated tea chloroplasts was incubated, extracted with ether and the extract analyzed by gas liquid chromatograph, five major radioactive peaks were found. One peak was an unreacted substrate peak. Two others were 11-formyl-*cis*-9-undecenoic acid and 11-formyl-*trans*-10-undecenoic acid, as presented here. After cleaving linolenic acid and linoleic acid, these two compounds were expected to be moiety twelve-carbon. Other radioactive peaks were azelaic half aldehyde, 9-formyloctanoic acid, and unknown compound. Radioactivity in 11-formyl-*cis*-9-undecenoic acid decreased with prolonged incubation time, while 11-formyl-*trans*-10-undecenoic acid increased. These results indicate that *cis*-3-hexenal and 11-formyl-*cis*-9-undecenoic acid were first formed from linolenic acid and then *cis*-3-hexenal was isomerized to *trans*-2-hexenal and 11-formyl-*cis*-9-undecenoic acid to 11-formyl-*trans*-10-undecenoic acid.

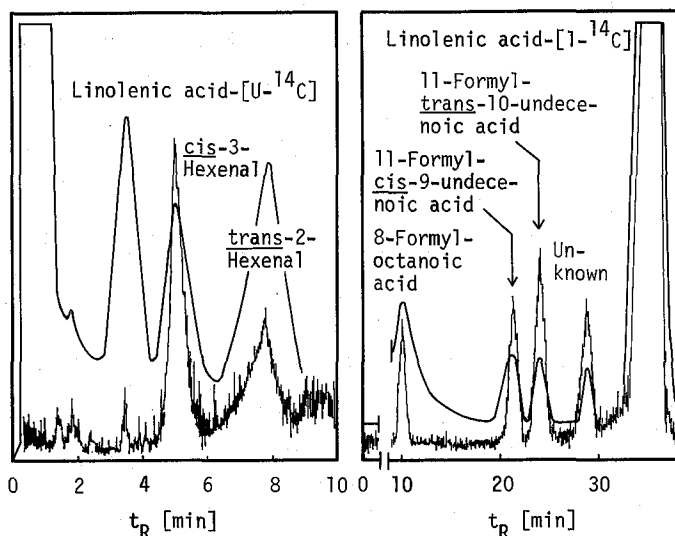


Fig. 3. Radio gas chromatograms of *cis*-3-hexenal and *trans*-2-hexenal from linolenic acid [^U-¹⁴C] (left) and of 11-formyl-*cis*-9- and -*trans*-10-undecenoic acid from linolenic acid [1-¹⁴C] by isolated tea chloroplast.

Distribution of an enzyme system producing *cis*-3-hexenal and *n*-hexanal from linolenic and linoleic acids in some plants.⁶⁷⁾ The activity of the enzyme system ($E'_2 + E''_2$) producing C_6 -aldehydes from C_{18} -unsaturated fatty acids was investigated using about 40 plants. Green leaves of dicotyledonous plants belonging to the *Sphenopsida*, *Pteropsida*, *Theaceae* and *Leguminosae* showed a high enzyme activity but edible leafy vegetables and fruits and monocotyledonous plants showed a low activity as shown Table III. Seasonal changes in the enzyme activities were observed. The concentrations of *cis*-3-hexenal and *trans*-2-hexenal and the enzyme activities showed a correlation; high concentrations were observed in the summer but they were low in the winter.

Solubilization and properties of the enzyme-cleaving 13-L-hydroperoxylinolenic acid in tea leaves.⁸²⁾ The membrane bound *hydroperoxide lyase* (E''_2) which catalyses the cleavage of 13-L-hydroperoxides of linolenic and linoleic acids to C_6 -volatile aldehydes (hexenals and *n*-hexanal) was found to be localized in the chloroplast lamellae of tea leaves. It was selectively solubilized from the lamellae with 0.5% (w/v) Tween 20. The enzymatic cleavage of the hydroperoxides occurred even under anaerobic conditions. The optimal pH of E''_2 was 7–8. The common structural features shown by substrates of E''_2 were the presence of a 13-L-hydroperoxy group at ω -6 with a conjugated *trans*, *cis*-diene at ω -7 and ω -9 in a C_{18} -fatty acid. E''_2 had an apparent K_m of 2.5 and 1.9 mM for 13-L-hydroperoxylinolenic and 13-L-hydroperoxylinoleic acids, respectively. No significant differences were found between chloroplast and solubilized (E''_2).

Participation and properties of lipoxygenase and hydroperoxide lyase in volatile C_6 -aldehyde formation from C_{18} -unsaturated fatty acids in isolated tea chloroplasts.⁸⁴⁾ Isolated tea chloroplasts utilized linoleic acid, linolenic acid and their 13-hydroperoxides as substrates for volatile C_6 -aldehyde formation. Optimal pH values for oxygen uptake (E'_2), *hydroperoxide lyase* (E''_2) and the overall reaction $E'_2 + E''_2$ from C_{18} -fatty acids to C_6 -aldehydes were 6.3, 7.0 and 6.3, respectively. Methyl linoleate, linoleyl alcohol and γ -linolenic acid were poor substrates for the overall reaction, but linoleic and linolenic acids were good substrates (Fig. 4, 5). The 13-hydroperoxides of the above fatty acids and alcohol also showed substrate specificity similar to that of fatty acids. Oxygen uptakes (relative V_{max}) with methyl linoleate, linoleyl alcohol, linolenic acid, γ -linolenic acid and arachidonic acid were comparable to or higher than that with linoleic acid. In winter leaves, the activity for C_6 -aldehyde formation from C_{18} -fatty acids was reduced to almost zero. This was due to the reduction in oxygenation. The findings presented here provide evidence for the involvement of *lipoxygenase* and *hydroperoxide lyase* in C_6 -aldehyde formation in isolated chloroplasts.

Oxygen-isotope effect in enzymatic cleavage reaction of 13-L-hydroperoxylinoleic acid to hexanal and 11-formyl-*cis*-9-undecenoic acid⁸⁶⁾ *Hydroperoxide lyase* E''_2 solubilized with Tween 20 from tea chloroplasts⁸²⁾ was shown to catalyze cleavage reaction of 13-L-hydroperoxy-*cis*-9, *trans*-11-octadecadienoic acid (13-L-hydroperoxylinoleic acid) to hexanal, and 11-formyl-*cis*-9-undecenoic acid by identification of cleavage products using authentic specimens synthesized through an unequivocal route. An oxygen-isotope effect was first observed in the cleavage

Table III. Distribution and activity of enzyme system

Plant no. and common name	Class	Family	Genus and species	Organ*
1. Hair moss	Musci	Polytrichaceae	<i>Pogonatum inflexum</i>	L
2. Horsetail	Sphenopsida	Equisetaceae	<i>Equisetum arvense</i>	L
3. Osmund	Pteropsida	Osmundaceae	<i>Osmunda japonica</i>	L
4. Bracken		Polypodiaceae	<i>Pteridium aquilinum</i>	L
5. Ginkgo	Ginkgopsida	Ginkgoaceae	<i>Ginkgo biloba</i>	L
6. Chinese black pine	Coniferopsida	Podocarpaceae	<i>Podocarpus macrophylla</i>	L
7. Melon	Dicotyledoneae	Cucurbitaceae	<i>Cucumis melo</i>	L
8. Cucumber			<i>Cucumis sativus</i>	F
9. Cabbage		Cruciferae	<i>Brassica oleracea</i> var. <i>capitata</i>	L (green)
10. Cauliflower			<i>Brassica oleracea</i> var. <i>botrytis</i>	L (pale green) floret
11. Radish			<i>Raphanus sativus</i>	L
12. Sakaki		Theaceae	<i>Cleyera japonica</i>	L
13. Tea			<i>Thea sinensis</i>	L
14. Camellia			<i>Camellia japonica</i>	L
15. Sasanqua			<i>Camellia sasanqua</i>	L
16. Japanese persimmon		Ebenaceae	<i>Diospyros kaki</i>	L
17. Strawberry		Rosaceae	<i>Fragaria grandiflora</i>	F (red)
18. Japanese apricot				L
19. Peach		Amygdalaceae	<i>Prunus mume</i>	L
20. Japanese wistaria			<i>Prunus persica</i>	L
21. False acacia		Leguminosae	<i>Wisteria floribunda</i>	L
22. Alfalfa			<i>Robinia pseudoacacia</i>	L
23. White clover			<i>Medicago sativa</i>	L
24. Soybean			<i>Trifolium repens</i>	L
25. Kidney bean			<i>Glycine max</i>	L
26. Mulberry		Moraceae	<i>Phaseolus vulgaris</i>	L
27. Japanese maple		Aceraceae	<i>Morus bombycis</i>	L
28. Spinach			<i>Acer palmatum</i>	L (green)
29. Potato		Chenopodiaceae	<i>Spinacia oleracea</i>	L (red)
30. Egg plant		Solanaceae	<i>Solanum tuberosum</i>	L
31. Tomato			<i>Solanum melongena</i>	tuber
32. Lettuce			<i>Lycopersicon</i> <i>esculentum</i>	L
33. Banana	Monocotyledoneae	Compositae	<i>Lactuca sativa</i>	F (Pink)
34. Onion		Musaceae	<i>Musa paradisica</i>	L
35. Duckweed		Liliaceae	<i>Allium cepa</i>	L
36. Rice		Lemnaceae	<i>Lemna polyrrhiza</i>	L
37. Wheat		Gramineae	<i>Oryza sativa</i>	L
			<i>Triticum aestivum</i>	L

* L: leaf. F: fruit.

† Plant materials were harvested in late May-July 1976, except for alfalfa in September.

‡ Total hexenals: *cis*-3- and *trans*-2-hexenal.

Biosynthesis of Leaf Alcohol

producing C₆-aldehydes in summer

Activity of enzyme system producing C ₆ -aldehydes†									Isomerization rate [‡]	
Fresh tissue homogenate (μg/g fr. wt)				4000 g precipitate fraction (μg/g ppt.)				Degree of activity	Fresh tissue homogenate	4000 g pellet
<i>cis</i> -3-hexenal	<i>trans</i> -2-hexenal	total hexenals‡	<i>n</i> -hexenal	<i>cis</i> -3-hexenal	<i>trans</i> -2-hexenal	total hexenals‡	<i>n</i> -hexenal			
0	0	0	19	90	16	106	230	L	0	2
1004	57	1061	1507	1776	481	2557	1877	H	5	21
214	107	321	966	3816	54	3870	2298	H	33	1
85	321	406	702	290	458	748	1580	H	79	61
0	24	24	6	132	63	195	48	L	100	32
577	165	742	109	1710	284	1994	388	H	22	14
2921	316	3237	430	—	—	—	—	H	10	—
26	14	40	42	162	27	189	62	L	35	14
0	0	0	88	24	8	32	23	L	0	25
532	123	655	52	150	71	221	117	M	19	32
555	33	588	36	600	347	947	184	M	6	37
49	7	56	12	48	19	67	48	L	13	28
1012	93	1105	357	216	101	317	781	H	8	32
854	14	868	292	2544	189	2733	1403	H	2	7
332	279	661	318	3000	134	3134	1785	H	42	4
339	5	392	150	498	0	498	604	M	2	0
105	7	112	61	792	102	894	209	L	6	11
621	79	700	141	0	1712	1712	3427	H	11	100
11	21	32	55	108	115	223	101	L	66	52
692	61	753	256	1512	95	1607	1137	H	8	6
623	85	708	342	3000	521	3521	1115	H	12	15
386	14	400	52	—	—	—	—	M	4	—
68	9	77	154	288	27	315	56	L	1	9
1037	263	1300	439	5376	499	5875	1124	H	20	8
1029	127	1156	509	—	—	—	—	H	11	—
566	104	670	110	36	324	360	250	M	16	90
299	70	369	1375	552	402	954	995	H	19	42
1025	410	1435	61	360	324	684	209	M	29	47
820	58	878	124	576	133	709	144	M	7	19
769	9	778	368	2820	101	2921	1510	H	2	3
555	2	557	287	3372	303	3675	3014	H	0	8
179	86	265	73	96	0	96	94	L	33	0
0	0	0	27	96	16	112	41	L	0	14
224	38	262	30	—	—	—	—	M	15	—
26	0	26	6	240	38	278	71	L	0	14
171	203	374	81	348	0	348	174	L	54	0
0	0	0	6	60	8	68	17	L	0	12
0	175	175	84	360	177	537	115	L	100	33
38	0	38	30	12	8	20	38	L	0	40
54	0	54	36	510	55	565	200	L	0	10
26	0	26	1	300	8	308	17	L	0	3
288	57	345	15	0	55	55	27	L	17	100

§ H, L show high, moderate and low enzyme activity, respectively.

‡ Isomerization rate (%) = $\frac{\text{trans-2-hexenal}}{\text{total hexenals}} \times 100$.

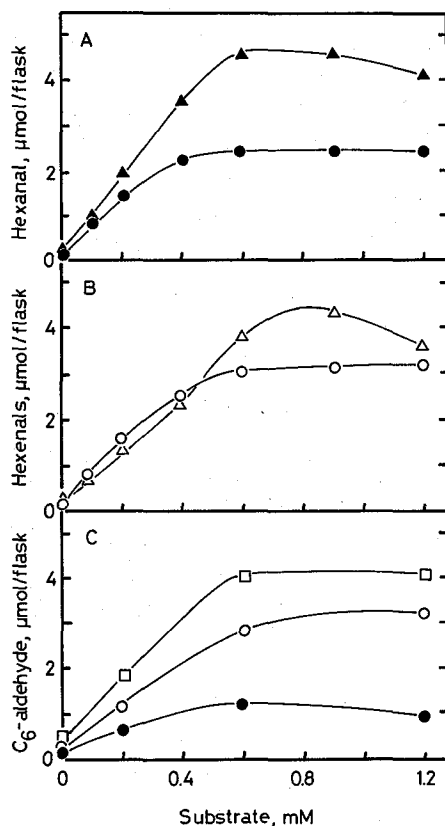


Fig. 4.

Fig. 4. C_6 -Aldehyde formation from LA, LNA and their 13-hydroperoxides by isolated tea chloroplasts. Ten milliliters of the chloroplast suspension was incubated at 35°C for 10 min with a single substrate (A or B) or mixed substrates (C) at the concentrations indicated in a sealed 50-ml flask. Numbers given as the substrate concentrations in (C) are the sums of LA and LNA (1:1). At the end of incubation, 6 ml of headspace vapor was analyzed by GLC as described in the text. A, hexanal formation from LA (●) or LAHPO (▲). B, hexenal formation from LNA (○) or LNAHPO (△). C, hexanal (●) and hexenal (○) formation from the mixture of LA and LNA as substrate. (□) shows the total C_6 -aldehydes (hexanal and hexenals) produced from the mixed substrates.

LNA: Linolenic acid, LA: Linoleic acid, LNAHPO: 13-*L*-Hydroperoxy-(*cis*, *trans*, *cis*)-9, 11, 15-octadecatrienoic acid, LAHPO: 13-*L*-Hydroperoxy-(*cis*, *trans*)-9, 11-octadecadienoic acid.

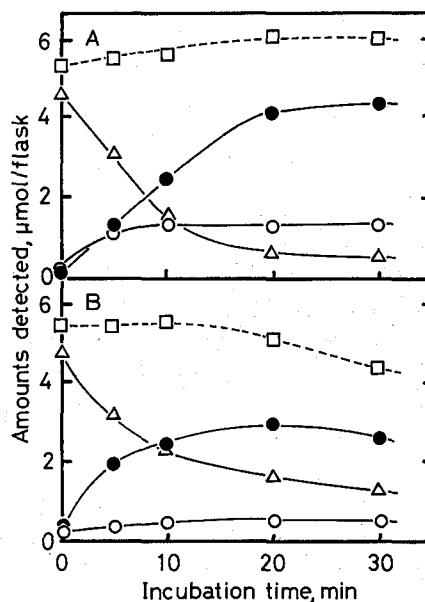
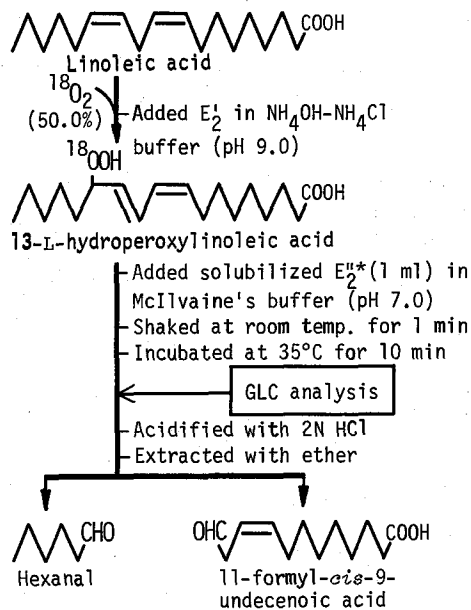


Fig. 5.

Fig. 5. Changes in the amounts of substrate and the products formed during C_6 -aldehyde formation. A chloroplast suspension (10 ml) first was incubated for 1 min at 35°C, then transferred to a sealed 50-ml flask which contained LA or LNA (final concentration, 0.6 mM). This mixture was incubated at 35°C. At the times indicated, 6 ml of headspace vapor from the sealed flask was analyzed by GLC. Immediately after headspace analysis, 1 ml of 2 N HCl was added to the reaction mixture, then the contents of the flask were treated with 5 ml of hexane. The hexane extract was concentrated to 2 ml, and 25 μ l was used in HPLC analysis to determine the C_{18} -fatty acid hydroperoxides. The rest of the concentrate was esterified with diazomethane, then analyzed by GLC to determine the unreacted C_{18} -fatty acids. A, hexanal formed from LA (●), LAHPO from LA (○), unreacted LA (△) and the total amount detected as LA, LAHPO and hexanal (□). B, hexenals formed from LNA (●), LNAHPO from LNA (○), unreacted LNA (△) and the total amount detected as LNA, LNAHPO and hexenals (□).

reaction of ^{18}O -labeled 13-L-hydroperoxylinoleic acid by solubilized E_2'' (Scheme 2, Fig. 6, Table IV). The ^{18}O -atom of hydroperoxide was not detected in carbonyl group of hexanal formed from ^{18}O -labeled 13-L-hydroperoxylinoleic acid.



Scheme 2 Procedure for the preparation of ^{18}O -labeled hydroperoxide and analysis of hexanal during E_2'' reaction.

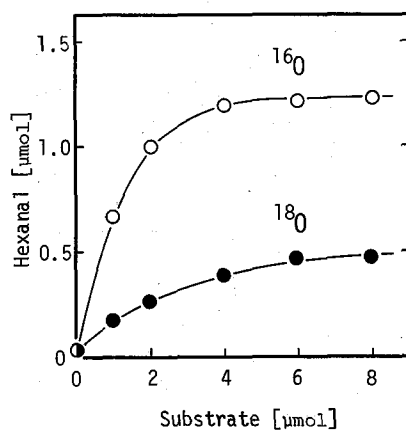


Fig. 6. Enzymatic formation of hexanal from ^{18}O - and ^{16}O -hydroperoxides by E_2'' .

Table IV Comparison of oxygen-isotope effect in E_2^s reaction by plant tissues.

Enzyme	Hexanal [μmol]	
	[^{16}O]* ⁵	[^{18}O]* ⁶
Tea leaves* ¹	2.42 (100)* ⁷	1.27 (52)
Tea chloroplasts* ²	3.68 (100)	1.62 (44)
Solubilized E_2^s * ³	2.70 (100)	1.23 (46)
Watermelon seedlings* ⁴	0.83 (100)	0.45 (54)

*1; 0.5 g fresh weight.

*2; 0.1 g [corresponded to 0.5 g leaves (fresh weight)].

*3; 1 ml (corresponded to 0.1 g chloroplasts).

*4; 10 ml (corresponded to 3 g fresh seedling).

*5; hexanal formation from [^{16}O]-13-L-hydroperoxide.*6; hexanal formation from [^{18}O]-13-L-hydroperoxide.

*7; numbers in parentheses represent relative values (%).

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