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The importance of linoleic acid and linolenic acid as precursors of hexanal and *trans*-2-hexenal in black tea

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During tea fermentation, linoleic acid in the neutral fat fraction, and linolenic acid in both the neutral fat and phospholipid fractions from leaves decreased. The addition of linoleic or linolenic acid to leaf macerates during fermentation resulted in an increase in hexanal or *trans*-2-hexenal in the volatile fraction. Tracer experiments showed the direct conversion of linoleic-U-^{14}C and linolenic-U-^{14}C acids to labeled hexanal and *trans*-2-hexenal, respectively, which were identified as 2,4-DNPH derivatives.

Further conversion of hexanal and *trans*-2-hexenal into hexanoic and *trans*-2-hexenoic acids during tea fermentation was suggested by the increases in these compounds after the addition of hexanal and *trans*-2-hexenal to leaf macerates.

Volatile carbonyl compounds constitute one of the important groups in black tea aroma. Previous papers (*1*, *2*), showed that formaldehyde, acetaldehyde, isobutyraldehyde, isovaleraldehyde, 2-methylbutanal, methional and phenylacetaldehyde were formed from glycine, alanine, valine, leucine, isoleucine, methionine and phenylalanine, by interaction with oxidized polyphenols during tea fermentation (process of oxidation). However, amino acids are presumably not the precursors of some important carbonyl compounds, i.e. hexanal and *trans*-*2*-hexenal but validation of this hypothesis must be made. Formation of these aldehydes also increased considerably during tea fermentation, but decreased under an atmosphere of nitrogen (*3*).

Major et al. (*4*, *5*) reported that fresh leaves of *Ginkgo biloba* L. produced 2-hexenal when they were ground in the presence of air, and that linolenic acid was converted into 2-hexenal in the leaves. Drawert et al. (*6*, *7*) reported that hexanal and 2-hexenal were produced enzymatically with participation of atmospheric oxygen, from linoleic acid, and linolenic acid, respectively, in apples and grapes.

A number of volatile carbonyl compounds (C_1–C_{12} saturated and unsaturated) have been isolated from reverted or oxidized soybean oils, and the volatile substances isolated from autoxidized C_{18} unsaturated fatty acids have been shown to be mainly carbonyl compounds (*8*).

This paper investigates whether or not linoleic acid and linolenic acid might be precursors of hexanal and *trans*-2-hexenal, respectively, in tea fermentation.

Abbreviations: 2,4-DNPH, 2,4-dinitrophenylhydrazone; TLC, thin-layer chromatography.

Materials and methods

Fatty acid analysis (9–12)

Samples of tea leaves used for the extraction of fatty acids were mature *Yabukita* ones harvested in the spring. These leaves were fermented at 25°C for 0, 1 and 2 hr after maceration in a mortar, then they were dried. One hundred grams of dried tea leaves was ground to a particle size smaller than 60-mesh, then it extracted with 1 liter of chloroform-methanol mixture (2 : 1, v/v) for 3 hr with stirring. The solvent was removed under reduced pressure to give a crude lipid fraction.

The crude lipid was separated into acetone-soluble and acetone-insoluble fractions. The acetone-soluble fraction was concentrated under reduced pressure in an atmosphere of nitrogen and this concentrate was dissolved in diethyl ether-petroleum ether mixture (1 : 1). After free fatty acids in the ether soluble fraction were extracted with 1% sodium carbonate, the sodium salt solution was acidified and extracted with diethyl ether. The faraction not extracted with sodium carbonate was saponified by alcoholic potassium hydroxide. After unsaponifiable matter was extracted with diethyl ether, the solution of residual potassium salts was acidified and fatty acids derived from the neutral fat fraction were extracted with diethyl ether.

The phospholipid fraction was repeatedly extracted from the acetone-insoluble fraction with chloroform-methanol (1 : 1) until the residue lost its orange color. The combined extracts were concentrated in vacuo, and 160 ml of 20% HCl was added to the concentrate, then the mixture was treated in a boiling water bath for 28 hr under nitrogen. Fatty acids liberated from the phospholipid fraction were extracted with diethyl ether in the usual manner.

Fatty acids from the various lipid fractions were converted to methyl esters by reacting them with diazomethane, after which they were analyzed by gas chromatography.

Fermentation of tea leaves after the addition of linoleic acid or linolenic acid

Six hundred grams of young *Hatsumomiji* leaves harvested in the summer, were withered on a net for fourteen hr at room temperature. One third of the withered-leaf sample was macerated in a mortar for 30 min after adding 0.5 g linoleic acid or linolenic acid which had been dispersed in 50 ml M/15 phosphate buffer (pH 7.0) containing 0.5% Tween 80, then it was fermented at 25°C for 2 hr.

One third of the leaf sample was macerated without the addition of fatty acids and was fermented as reported above. For comparison, an emulsion containing 0.5 g of either linoleic acid or linolenic acid, dispersed in the same buffer solution, was kept at 25°C for 2.5 hr.

From these samples, volatile fractions were collected using the procedure described in a previous paper (*3*) and the neutral fraction was analyzed by gas chromatography. These experiments were repeated twice.

Fermentation of tea leaves after the addition of hexanal or trans-*2-hexenal*

Fifty grams of *Hatsumomiji* leaves were withered and fermented after adding 50 mg of hexanal or *trans*-2-hexenal as described above. Hexanal and *trans*-2-hexenal were purified by gas chromatography. After volatile fractions had been collected by the usual method, acidic fractions were separated by extraction with 5% sodium hydroxide and were methylated by treatment with diazomethane in ether for gas chromatographic analysis.

Tracer experiments using radioactive linoleic acid or linolenic acid

Fifty grams of young *Benifuji* leaves were maceratee with 10 μCi linoleic acid-U-^{14}C or linolenic acid-U-^{14}C (both specific activities, 100 mCi/mmole) in 20 ml phosphate buffer solution. Other conditions were the same as those described for unlabeled fatty acids.

Methanol soluble materials were extracted from fermented leaves with 500 ml methanol, after which they were concentrated and subjected to steam distillation under reduced pressure. The distillate was extracted with diethyl ether. The ether layer was separated into acidic, basic and residual neutral fractions by extraction with 5% NaOH and 3% HCl, respectively. After 50 mg of unlabeled volatile fractions from the tea leaves had been added to the residual neutral fraction, 2,4-DNPHs were prepared by treating the mixture with 2,4-dinitrophenylhydrazine.

2,4-DNPHs were streaked on a 20×20 cm thin-layer silica gel plate and developed with benzene-ligroin-ethylacetate (6 : 6 : 1). After development, zones of silica gel, each 5 mm wide were scraped off the plate, after which they were eluted with acetone, then assayed for radioactivity using an Aloka 2π gas flow counter, model JDC-10413.

Gas chromatography

The gas chromatograph used was a Hitachi model K-53 equipped with a flame ionization detector. Conditions of gas chromatography are shown in the footnote to Table 1 and in Fig. 1 and 4.

Results

Changes in fatty acids during tea fermentation

Fatty acids in each lipid fraction were analyzed at various stages of fermentation and the results are shown in Table 1. In the process of fermentation, the amounts of lauric, myristic and linolenic acids in both the neutral fat and phospholipid fractions decreased. Oleic and linoleic acids in the neutral fat fraction also decreased during fermentation. It is not likely that the decreases observed for these fatty acids in the neutral fat and phospholipid fractions are accompanied by increases in free fatty acids. Therefore, these fatty acids in the neutral and phospholipid fractions must be converted to other compounds.

Volatile compounds of tea leaves fermented after the addition of linoleic acid or linolenic acid

The volatile fraction obtained from tea leaves, fermented after the addition of linoleic acid or linolenic acid, was analyzed (Fig. 1-C, or -D).

The peak of hexanal in Fig. 1-C increased in comparison to that in Fig. 1-B (the reference) and the peak of *trans*-2-hexenal in Fig. 1-D increased in comparison to that in Fig. 1-B. As shown in Fig. 1-A, no volatile compounds corresponding

Table 1

Changes in fatty acids in each fraction during tea fermentation

Fatty acid [a]	Free fatty acid fraction Fermentation time (hr)			Neutral fat fraction Fermentation time (hr)			Phospholipid fraction Fermentation time (hr)		
	0	1	2	0	1	2	0	1	2
Lauric	—	—	—	0.7	0.2	—	1.1	0.7	0.4
Myristic	0.2	0.1	0.1	2.0	1.5	0.7	5.3	1.8	2.4
Stearic	—	—	—	—	—	—	0.8	1.7	2.4
Oleic	—	—	—	4.9	3.8	1.1	1.6	1.8	2.4
Linoleic	0.2	0.3	0.8	4.2	1.4	0.6	10.2	14.6	11.7
Linolenic	0.2	0.2	0.2	22.6	10.2	4.2	27.2	27.0	18.3

Each value is the average peak area (cm^2) of two gas chromatograms obtained under the following conditions: Column packing, 20% poly-ethylene glycol adipate on Chromosorb W (60–80 mesh); Column temperature, 180°C; He flow rate, 30 ml/min; attenuation range of detector, 5×10^2. Under these conditions 1 cm^2 of peak area corresponded to 6.2 μg of lauric acid.

[a] Fatty acid was identified by comparing its log retention time with that of the authentic fatty acid ester.

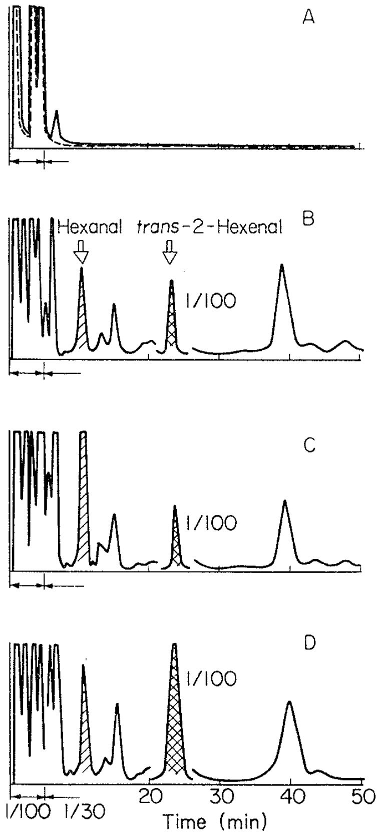

Fig. 1. *Changes in volatile components of tea leaves with the addition of linoleic acid or linolenic acid.* Conditions of gas chromatography were as follows: Column packing, 23% Carbowax 6000 on Chromosorb W (60–80 mesh); Column temperature, 90°C isothermal; He flow rate, 58 ml/min; attenuation range of detector, 1/100 or 1/30. A, the solid line shows volatile components of linoleic acid kept at 25°C for 2.5 hr, the dotted line shows those of linolenic acid kept at 25°C for 2.5 hr; B, volatile components from normally fermented leaves; C, volatile components from fermented leaves after adding linoleic acid; D, volatile components from fermented leaves after adding linolenic acid.

Table 2

Distribution of radioactivity in volatile fractions of tea leaves fermented after adding radioactive linoleic acid or linolenic acid

Fatty acid added	Volatile fractions				
	Acidic	Basic	Carbonyl	Neutral (carbonyl-free)	Total
Linoleic acid	5500[a](13)[b]	trace (0)	31400 (72)	6800 (16)	43700 (100)
Linolenic acid	5700 (16)	trace (0)	21400 (60)	8500 (24)	35650 (100)

[a] Radioactivity measured (cpm).

[b] Percentage of total radioactivity detected in volatile fractions.

to hexanal or *trans*-2-hexenal were detected in an emulsion containing either linoleic acid or linolenic acid.

These facts suggest that during tea fermentation, the linoleic acid or linolenic acid added to tea leaves was decomposed to hexanal and *trans*-2-hexenal, respectively, as reported in experiments with apples and some other plant (*6*, *7*).

Decomposition of radioactive linoleic acid and linolenic acid to volatile fractions during tea fermentation

The distribution of decomposition products from radioactive linoleic acid and linolenic acid, in volatile fractions from fermented leaves, is shown in Table 2. The highest radioactivity was present in the carbonyl fractions in both experiments. The radioactivity of each carbonyl fraction produced from radioactive linoleic acid or linolenic acid was approximately 0.008% or 0.005% of the total radioactivity administered.

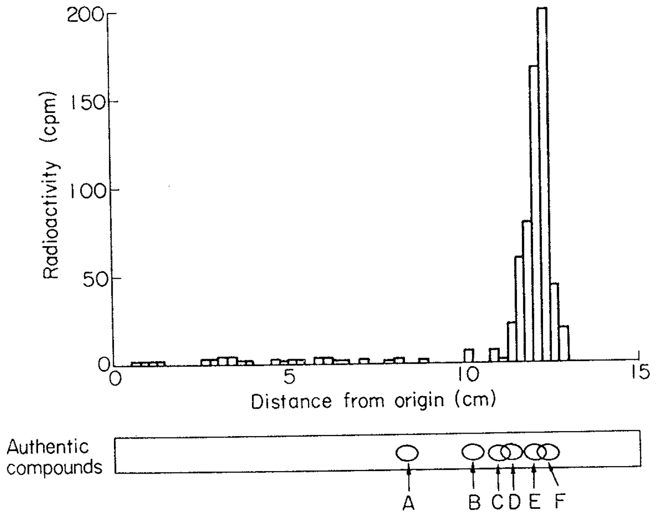

Fig. 2. *Thin-layer chromatography of 2,4-DNPH derivatives from the carbonyl fraction of tea leaves fermented with radioactive linoleic acid.* A, acetaldehyde 2,4-DNPH; B, propionaldehyde 2,4-DNPH; C, butyraldehyde 2,4-DNPH; D, valeraldehyde 2,4-DNPH; E, *trans*-2-hexenal 2.4-DNPH; F, hexanal 2,4-DNPH.

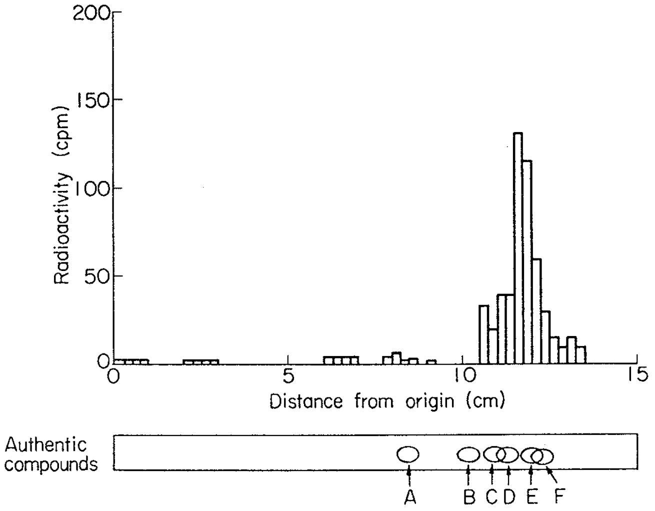

Fig. 3. *Thin-layer chromatography of 2,4-DNPHs from the carbonyl fraction of tea shoots fermented with radioactive linolenic acid.* A, B, C, D, E and F are the same as compounds mentioned in Fig. 2.

Decomposition products of linoleic acid and linolenic acid and their identification

An aliquot of 2,4-DNPH derivatives of the carbonyl fraction was subjected to TLC, after which radioactivities of components on the chromatograms were measured. At the same time the 2,4-DNPH derivative of each authentic carbonyl compound was developed. Results are shown in Fig. 2 and 3.

From the results of TLC, the major radioactive zone in Fig. 2 was tentatively recognized in F as corresponding to authentic hexanal 2,4-DNPH, while that in Fig. 3 seemed to be E, corresponding to authentic *trans*-2-hexenal 2,4-NDPH. However, these could not be absolutely identified from the available data.

For further identification of the radioactive F and E fractions, zones corresponding to F or E were scraped off and extracted with acetone. After being mixed with 50 mg of authentic hexanal or *trans*-2-hexenal 2,4-DNPHs, respectively, they were recrystallized from dilute ethanol and their melting points were determined. As shown in Table 3, constant melting points were observed for both the hexanal and *trans*-2-hexenal 2,4-DNPH derivatives. Moreover, their specific radioactivities were not lowered by recrystallization. From these data it is evident

Table 3
Specific radioactivity of hexanal and trans-*2-hexenal 2,4-DNPH derivatives*

Recrytallization No.	Hexanal 2,4-DNPH		*trans*-2-Hexenal 2,4-DNPH	
	mp (°C)	sp. activity (cpm/mg)	mp (°C)	sp. activity (cpm/mg)
1	106.0	82	137.0	28
2	107.0	77	136.0	25
3	106.0	72	137.0	29
4	106.0	70	137.0	32

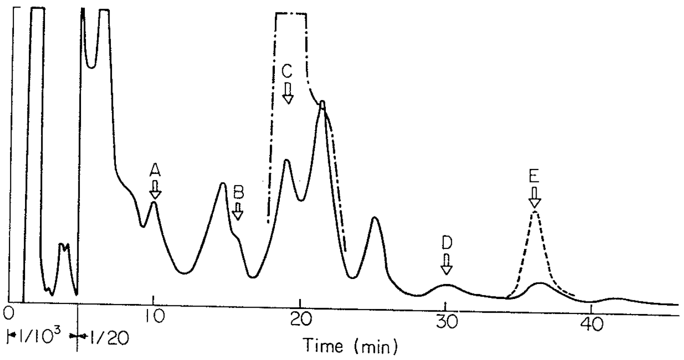

Fig. 4. *Gas chromatograms of the methyl esters of volatile acids from tea leaves fermented with added hexanal or* trans-*2-hexenal.* Conditions of gas chromatography were as follows: Column packing, 23% Carbowax 6000 on Chromosorb W (60–80 mesh); Column temperature, 90°C isothermal; He flow rate, 40 ml/min; attenuation range of detector, $1/10^3$ or 1/20. A, B, C, D and E indicate methyl esters of n-C_5, iso-C_5, n-C_6, *cis*-3-C_6 and *trans*-2-C_6 acids, respectively. ——, gas chromatogram of normally fermented leaves; – – –, the peak increased by the addition of hexanal; - - - -, the peak increased by the addition of *trans*-2-hexenal.

that a portion of the radioactive linoleic acid and linolenic acid changed to hexanal and *trans*-2-hexenal during tea fermentation.

Changes in the volatile acidic fraction of tea leaves fermented after the addition of hexanal or trans-*2-hexenal*

To determine the oxidative products of hexanal and *trans*-2-hexenal, tea leaves were fermented in the presence of these aldehydes. Characteristic gas chromatogram patterns were obtained with each aldehyde as shown in Fig. 4. Gas chromatograms indicated that peak C (the methyl ester of caproic acid) markedly increased on the addition of hexanal and that peak E (the methyl ester of *trans*-2-hexenoic acid) also increased on the addition of *trans*-2-hexenal.

Discussion

Linoleic acid and linolenic acid added to tea leaves were decomposed to hexanal and *trans*-2-hexenal, respectively, during tea fermentation, and these aldehydes were the main products in the volatile fractions. Since analyses of these fatty acids in tea leaves showed that their proportions decreased during tea fermentation, we believe that a portion of linoleic acid and linolenic acid present in tea leaves, respectively, changes to hexanal and *trans*-2-hexenal as major products. Volatile carbonyl compounds are known to be produced from unsaturated fatty acids by autoxidation.

In the autoxidation of soybean oil (linolenate containing oil), Selke et al. (*13*) reported that volatile aldehydes appeared in two-week-old oil aged at laboratory temperature (ca. 22°C) without protection from fluorescent light. As no volatile compounds corresponding to hexanal and *trans*-2-hexenal were detected in the linoleic acid and linolenic acid solutions kept at 25°C for 2.5 hr, it is probable that reactions involving the formation of hexanal and *trans*-2-hexenal from linoleic acid and linolenic acid, respectively, were not caused by autoxidation. Moreover, green tea manufactured from steamed tea leaves does not contain a large amount of hexanal and *trans*-2-hexenal (*14*), so they may be products of enzymatic action.

In a previous paper (*3*) we observed that *trans*-2-hexenal increased markedly to a maximum, followed by a gradual decrease during 4 hr of fermentation. Therefore, the aldehyde was not considered to be the end product. Results of the experiments using hexanal and *trans*-2-hexenal (Fig. 4) suggest that these carbonyl compounds are further oxidized to the corresponding acid.

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References

(*1*) Saijyo, R. and T. Takeo: The production of phenylacetaldehyde from L-phenylalanine in tea fermentation. *Agr. Biol. Chem.* 34: 222–226 (1970).

(*2*) Saijyo, R. and T. Takeo: The formation of aldehydes from amino acids by tea leaves extracts. *ibid.* 34: 227–233 (1970).

(*3*) Saijyo, R. and Y. Kuwabara: Volatile flavor of black tea. Part I. Formation of volatile components during black tea manufacture. *ibid.* 31: 389–396 (1967).

(*4*) Major, R. T., P. Marchini and A. J. Boulton: Observations on the production of α-hexenal by leaves of certain plants. *J. Biol. Chem.* 238: 1813–1816 (1963).

(*5*) Major, R. T.: The Ginkgo, the most ancient living tree. *Science* 157: 1270–1273 (1967).

(*6*) Drawert, F., W. Heimann, R. Emberger und R. Tressl: Enzymatische Bildung von Hexen-2-al-1 und Hexanal-1 bei der Aufarbeitung von Äpfeln. *Z. Naturforschg.* 20 b: 497–498 (1965).

(*7*) Drawert, F., W. Heimann, R. Emberger und R. Tressl: Über die Biogenese von Aromastoffen bei Pflanzen und Früchten II. Enzymatische Bildung von Hexen-(2)-al-(1), Hexanal und deren Vorstufen. *Ann. Chem., Justus Liebig* 694: 200–208 (1966).

(*8*) Hoffmann, G.: Vegetable oils, In *Symposium on Foods: Lipids and Their Oxidation.* Edited by H. W. Schultz. p. 215–229. The Avi Publishing Company, Inc., Westport, Connecticut (1962).

(*9*) Lee, F. A. and L. R. Mattick: Fatty acids of the lipids of vegetables. I. Peas (*Pisum sativum*). *Food Research* 26: 273–275 (1961).

(*10*) Mattick, L. R. and F. A. Lee: The fatty acids of vegetables. II. Spinach. *ibid.* 26: 356–358 (1961).

(*11*) Vorbeck, M. L., M. N. Albury, L. R. Mattick, F. A. Lee and C. S. Pederson: Lipid alterations during the fermentation of vegetables by the lactic acid bacteria. *ibid.* 28: 495–502 (1963).

(*12*) Lima, I. H., T. Richardson and M. A. Stahmann.: Fatty acids in some leaf protein concentrates. *J. Agr. Food Chem.* 13: 143–145 (1965).

(*13*) Selke, E., H. A. Moser and W. K. Rohwedder: Tandem gas chromatography-mass spectrometry analysis of volatiles from soybean oil. *J. Am. Oil Chemists Soc.* 47: 393–397 (1970).

(*14*) Yamanishi, T., A. Kobayashi, A. Uchida and Y. Kawashima: Studies on the flavor of green tea. Part VII. Flavor components of manufactured green tea. *Agr. Biol. Chem.* 30: 1102–1105 (1966).