When fed to male and female albino rats for 90 days at levels of 0.002, 0.2, and 2.0% in the diet, TPGS had no untoward effects on the general behavior, appearance, feed consumption, or growth rate of these animals. Hemograms and clinical chemistry determinations were within normal ranges and organ weights recorded at necropsy were normal. Microscopic examination of tissues from all organ systems revealed no pathologic changes due to the ingestion of TPGS. Animals bred after 112 and 175 days of treatment had reproductive indexes similar to their controls and produced offspring that were normal. Microscopic examination of the tissue from the parents and offspring revealed no pathology due to ingestion of the compound. Fetuses collected from pregnant females treated with TPGS during the period of organogenesis had no congenital abnormalities which could be attributed to the compound.

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Extraction, Purification, and Partial Characterization of a Tea Metalloprotein and Its Role in the Formation of Black Tea Aroma Constituents

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Linolenic acid, the major fatty acid in tea leaves, is known to be partially oxidized during tea fermentation to yield 2(*E*)-hexenal, a constituent of black tea aroma. Evidence has now been obtained which suggests that this transformation is mediated by an endogenous, heat-stable, metal ion containing protein rather than a true lipoxygenase. This metalloprotein has been purified by isoelectric focusing and has been found capable of producing a number of volatiles other than 2(*E*)-hexenal. The activity of this nondialyzable metalloprotein was found to be stable for over 10 min at 100 °C. Inhibition was achieved with various metal ion chelators and respiratory enzyme inhibitors. The purified tea metalloprotein gave a radioactive volatiles pattern for the oxidation of [^{14}C]linolenic acid which was very similar to that of cytochrome *c* and chlorohemin. A total of ten radioactive volatiles were detected and identified during the extraction and purification of this tea metalloprotein. Eight of these compounds are aldehydes, which are predictable oxidation products of linolenic acid. Two alcohols were found which were presumed to be formed from two of the aldehydes by the action of an endogenous tea alcohol dehydrogenase.

2(*E*)-Hexenal, a constituent of black and green tea aroma (Sanderson, 1972, 1975), is known to be formed from linolenic acid during tea fermentation (Gonzalez et al., 1972; Saijo and Takeo, 1972). This aldehyde results from the isomerization of 3(*Z*)-hexenal (Hatanaka and Harada, 1973; Kazeniac and Hall, 1970; Kajiwara et al., 1975; Hatanaka et al., 1976) which is the first formed volatile product of linolenic acid breakdown, and is responsible for the characteristic flavor of various foodstuffs (Harper, 1975). In black tea, the 2(*E*)-hexenal content has been shown to relate to flavor quality (Gianturco et al., 1974), a fact which makes the mechanism by which this compound is formed in black tea of considerable interest.

Something is now known of several mechanisms in biological materials for the breakdown of linolenic (and linoleic) acid to 2(*E*)-hexenal and other products. First, there is the peroxidation catalyzed by the enzyme lipoxygenase (linoleic acid:oxygen oxidoreductase, EC 1.13.11.12), which is known (Axelrod, 1974; Gardner, 1975; Parsons, 1974) to initiate the formation of peroxidized linolenic acid which in turn breaks down via enzymatic and nonenzymatic pathways to form several volatile and nonvolatile oxidation products. Most of the adequately verified lipoxygenase preparations have come from plants in the *Leguminosae* family. However, there are reports of lipoxygenase activity having been detected in many different plant families (cf. Axelrod, 1974).

Next, it has been shown (Gardner, 1975; Tappel, 1961, 1962; Blain, 1970; Eriksson et al., 1969; Grosch et al., 1974) that hematin compounds, i.e. heme-containing proteins like the cytochromes and peroxidase, will catalyze the nonenzymatic peroxidation of unsaturated fatty acids.

Finally, Grosch et al. (Grosch and Schwartz, 1971; Grosch et al., 1974) have shown that singlet oxygen can react with unsaturated fatty acids producing a number of volatile compounds including 2(*E*)-hexenal from linolenic acid.

Of course, the above-mentioned mechanisms for the breakdown of unsaturated fatty acids in plant materials are not mutually exclusive and there may be other mechanisms not yet discovered. Our investigation was carried out to determine the mechanism by which unsaturated fatty acids, especially linolenic acid, are broken down during black tea manufacture with particular at-

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tention to the above-mentioned possibilities.

EXPERIMENTAL SECTION

Materials. (a) Fresh green tea leaf was obtained from experimental tea plantings (Co and Sanderson, 1970) and was stored at −40 °C until required. (b) [U-^{14}C]Linolenic acid (99+% cis) was obtained from Applied Science Laboratories, Inc., State College, Pa. (c) Soybean lipoxygenase was obtained from Miles-Servac (PYT) Ltd., Berks, England. (d) Protease enzymes (Rhozyme-62; activity factor 3.65) were obtained from Rohm and Haas, Philadelphia, Pa. (e) HOQNO (2-*n*-heptyl-4-hydroxyquinoline *N*-oxide) was obtained from Sigma Chemical Co., St. Louis, Mo. (f) Hemin (chlorohemin), recrystallized, was obtained from the Nutritional Biochemical Corp., Cleveland, Ohio.

Methods. (a) Peroxidase, catalase, and lipoxygenase activities were determined by the spectrophotometric assays described in the Worthington Enzyme Manual (1972). Lipoxygenase activity was also determined by a polarographic method described by Restrepo et al. (1973).

(b) Radioactive gas chromatographic headspace analyses were made according to the procedures described by Co and Sanderson (1970).

Sample solutions were placed inside a 100-ml double-necked flask, water jacketed at 95 °C. Nitrogen gas was passed through the system until 1 l. of headspace volatiles had passed through the trap. The trap was a 7-in. piece of stainless steel tubing, 0.25 in. o.d., packed with 5% Apiezon L on Gas-Chrom Q, 60–80 mesh, and cooled with dry ice. Chromatography was done on the Barber Colman Model 5000 GC unit utilizing a single 18 ft × 0.25 in. stainless steel column packed with 10% Carbowax 20M on Gas-Chrom Q, 60–80 mesh; helium carrier gas at a flow rate of 40 ml/min; temperature programmed from 70 to 210 °C at 2 °C/min. The electrometer attenuation was set at 64 × 10^{-11} A for all runs. When radioactive fatty acids were used in model systems, the GC column effluent was split such that the counter tube exit port had a flow rate of 32 ml/min and the quench gas (propane) flow rate adjusted to ~3.2 ml/min. The Barber Colman Model 5190 radioactivity monitor was precalibrated with [^{14}C]toluene (New England Nuclear) under the following conditions: combustion temperature, 680–700 °C; transfer temperature, 210 °C; operating plateau at 1700–1750 V with a count rate of 1 K/min with a 10% error. These conditions were maintained for each model system experiment employing ^{14}C-labeled fatty acid.

(c) Crude soluble tea enzymes were extracted and purified by the method described by Coggon et al. (1973). Fresh frozen green tea leaf (50 g) was mixed with 25 g of Polyclar AT and 10 ml of Tween 80 in prechilled (4 °C) phosphate buffer (250 ml, 0.05 M, pH 7.2) and the mixture was homogenized continuously in a cold base Waring Blendor (0 °C) for 5 min. The homogenate was then centrifuged for 10 min at 4 °C and 10 000*g*. The supernatant was passed through a jacketed (4 °C) Sephadex G-50 coarse column (6 × 3 cm) and the CST enzymes filtrate was then subjected to successive ammonium sulfate fractionation. The precipitated protein from each fraction was resuspended with 50 ml of cold (4 °C), 0.05 M, pH 5.5 McIlvaine's buffer and subsequently frozen until used in radioactive model system studies. Ten milliliters of the 40–60% fraction was dialyzed overnight against cold (4 °C) distilled water in dialysis membrane tubing. This clear, yellow solution was placed onto the LKB Model 8102, 440-ml capacity, isoelectric focusing column containing a pH gradient maintained with Ampholine (pH 3.5–10.0) in a sucrose gradient. Direct current voltage was applied at 400 V and maintained for ~48 h until a current drop of 3 mA was observed. The column contents were scanned by the LKB Uvicord-4700 at 254 nm and collected by a Gilson fraction collector at a rate of 150 drops/tube until 70 tubes were collected. Each fraction (tube) was then assayed for peroxidase, catalase, and lipoxygenase activities.

(d) **Preparation of Samples for Radioactive Gas Chromatographic Headspace Analysis.** Labeled linolenic acid (10 μCi; 720 mCi/mmol specific activity) was diluted to 2.0 ml with ethanol or benzene and 100 μl was pipetted into the headspace flask and the solvent evaporated under a slow stream of nitrogen gas. This level of labeled linolenic acid was used in all subsequent model system experiments unless otherwise stated.

(1) *Tea Leaf Homogenates.* Whole green tea leaf (50 g) was homogenized with 250 ml of glass-distilled water, and 50 ml of the resulting homogenate was incubated with labeled fatty acid for 30 min under maximal stirring rates.

(2) *Crude Enzyme Preparation.* Samples were taken during the enzyme purification and they were monitored for radioactive volatile formation by the procedures previously described. The equivalent of one-fifth of the total volume of crude enzyme extraction was used in model system experiments with labeled linolenic acid.

(3) *Isoelectrically Focused Tea Enzymes.* Samples (1.0 ml) of the various fractions were incubated with labeled fatty acid in a total of 30 ml of 0.05 M (pH 5.5) McIlvaine's buffer using the conditions described above. Variations in this study included the passage of a focused enzyme fraction over a 130 mm × 7.5 mm column packed with AG 50W-XI cation exchange resin to remove possible inorganic ion contaminants; overnight dialysis using Spectropore dialysis tubing (mol wt cutoff 12 000–14 000) against glass-distilled water; digestion by protease enzymes (Rhozyme-62 concentrate); and utilization of various inhibitors.

(e) **Identification of the Linolenic Acid Oxidation Products.** (1) *Identification by Gas Chromatography.* Reference compounds were obtained from commercial suppliers and other laboratories. The retention times of various labeled aldehydes were consistent with those obtained from the corresponding authentic compound using a Carbowax 20M column.

(2) *Identification by GC/IR.* GC effluents were analyzed directly using a Norcon Model 201 GC/IR Identifier, Norcon, Norwalk, Conn. Reference IR spectra were obtained from authentic compounds.

(3) *Identification by 2,4-DNPH (2,4-Dinitrophenylhydrazine) Derivatization.* Preparation and TLC identification of labeled aldehyde–DNPH derivatives were made according to the procedures described by Urbach (1963), Schormuller et al. (1969), and Major and Thomas (1972).

RESULTS AND DISCUSSION

Production of Volatile Compounds from Linolenic Acid in Fresh Green Tea Flush. To date, ten compounds (carbonyls and alcohols) have been identified as products of the oxidative degradation of linolenic acid in tea leaves. The compounds identified are listed in Table I, and they are all consistent with the volatiles expected to be formed from linolenic acid in metal-catalyzed reactions (Parsons, 1974). Currently, only two of the ten volatiles, 2(*Z*)-hexenal and 3(*Z*),6(*Z*)-nonadienal, remain to be detected in either green tea aroma (Yamanishi, 1975) or black tea aroma (Sanderson, 1975).

In the first set of experiments, whole green tea flush was macerated and allowed to ferment. The results showed

Table I. Identification of Volatile Radioactive Components Produced from [^{14}C]Linolenic Acid in Tea Systems

Peak designation	Compd name	Evidence[a]
1	Ethanal	GC-IR, t_R, 2,4-DNPH
2	Propanal	GC-IR, t_R, 2,4-DNPH
3	2(*E*)-Pentenal	GC-IR, t_R
4	3(*Z*)-Hexenal	GC-IR, t_R, 2,4-DNPH
5	2(*E*)-Hexenal	GC-IR, t_R, 2,4-DNPH
6	2(*Z*)-Hexenal[b]	GC-IR
7	3(*Z*)-Hexenol	GC-IR, t_R
8	2(*E*)-Hexenol	GC-IR, t_R
9	3(*Z*),6(*Z*)-Nonadienal[b]	GC-IR
10	2(*E*),6(*Z*)-Nonadienal	GC-IR, t_R

[a] Abbreviations: GC-IR, gas chromatography followed by vapor phase infrared analysis; t_R, retention time; 2,4-DNPH, 2,4-dinitrophenylhydrazine derivative. [b] Tentative identification.

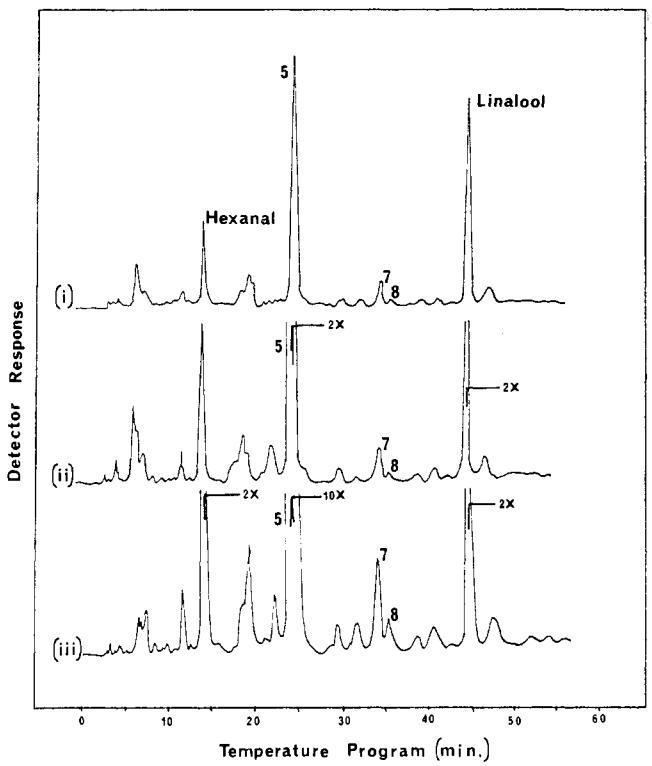

Figure 1. GC-headspace chromatograms showing the effect of maceration on the production of tea volatiles in green tea homogenates. For peak identification, see Table I: (i) unmacerated tea leaf; (ii) macerated tea leaf homogenate, 2-min fermentation; (iii) macerated tea leaf homogenate, 2-h fermentation.

(Figure 1) that there was a twofold increase in 2(*E*)-hexenal resulting from the maceration of the tea leaf (i.e., 2-min fermentation) and a tenfold increase resulting from a 2-h fermentation period. Various other peaks, including those of hexenal, 3(*Z*)-hexenol, 2(*E*)-hexenol, and linalool, were observed to increase over this period. The 3(*Z*)-hexenol is thought to arise from 3(*Z*)-hexenal by the action of an endogenous tea alcohol dehydrogenase (Hatanaka and Harada, 1973). These observed increases in aldehydes and corresponding alcohols are in agreement with results obtained by other workers (Sanderson, 1972; Saijo and Takeo, 1972; Kajiwara et al., 1975).

Carbon-14 labeled linolenic acid was used to confirm the origin of the volatiles produced during the fermentation or oxidation period. It appeared (Figure 2A) that only one radioactive peak, known to be 2(*E*)-hexenal, arises during

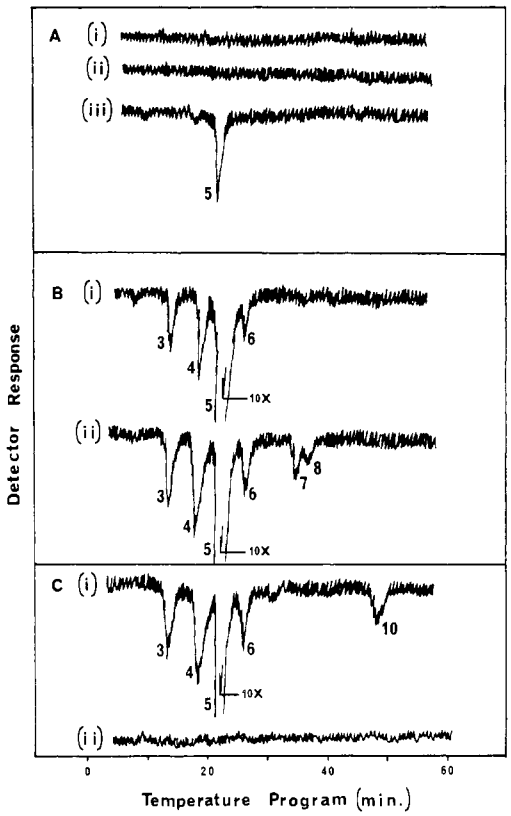

Figure 2. GC-headspace radiograms showing the formation of 2(*E*)-[^{14}C]hexenal and other volatiles from [^{14}C]linolenic acid (30-min aeration). For peak identification, see Table I. (A) Green tea homogenate system: (i) 10 μl of [^{14}C]linolenic acid in buffer; (ii) same as A (i) with the homogenate heated prior to addition; (iii) same as A (i) with the homogenate added. (B) Increased level of [^{14}C]linolenic acid: (i) 100 μl of [^{14}C]linolenic acid with the green tea homogenate; (ii) same as B (i) but with a 2-h aeration. (C) Crude enzyme purification step: (i) 100 μl of [^{14}C]linolenic acid with the Sephadex filtrate fraction added; (ii) same as C (i) but heated prior to aeration.

fermentation and it was shown by control experiments that this is a direct result of the fermentation. This observation is in complete agreement with results reported by Gonzalez et al. (1972). However, when the level of labeled linolenic acid was increased tenfold, not only was there a tenfold increase in the production of 2(*E*)-hexenal, but three additional compounds were detected as seen in Figure 2B (i). Two of these, 3(*Z*)-hexenal and 2(*E*)-pentenal, are aldehydes which are known oxidation products of linolenic acid (Tappel, 1962). The third new peak, 2(*Z*)-hexenal, is a newly identified component of this system. When the aeration period was increased from 30 min to 2 h, two additional peaks were detected as seen in Figure 2B (ii). These have been identified as the alcohols 3(*Z*)-hexenol and 2(*E*)-hexenol whose presence is presumed to be due to the action of tea alcohol dehydrogenase (Hatanaka and Harada, 1973; Kajiwara et al., 1975; Hatanaka et al., 1976) on the corresponding aldehydes formed directly from linolenic acid.

Production of Volatile Compounds from Linolenic Acid by Fractions from a Tea Enzyme Purification Scheme. A crude enzyme extract was prepared which, after passage through a short column of Sephadex G-50 (to remove residual polyphenolic material), gave a radioactive volatiles pattern (Figure 2C) with labeled linolenic acid which was nearly identical with the patterns obtained with green tea homogenates (Figure 2B). When this enzyme preparation was heated prior to incubation, no radioactive volatiles were detected (Figure 2C). Next, a

Table II. Incubation of Isoelectrically Focused Tea Catalyst (Tubes 49–51) with Labeled Linolenic Acid before and after Various Treatments[a]

Treatment	Conditions	Total radioact. by summing peak heights, arbitrary units, before/after
A. Heat	Immersion in boiling water for 10 min prior to incubation	65/68
B. Cation exchange	130 × 7.5 mm column of Bio-Rad AG 50W-XI	44/48
C. Dialysis	Spectropore mol wt 12 000–14 000 cutoff tubing against distilled water	44/45
D. Protease	1-h treatment with Rhozyme 62 concentrated prior to incubation	65/4
E. Protease (heat inactivated)	1-h hold period with heat-inactivated protease	65/3

[a] See Figure 5, part ii, for representative trace.

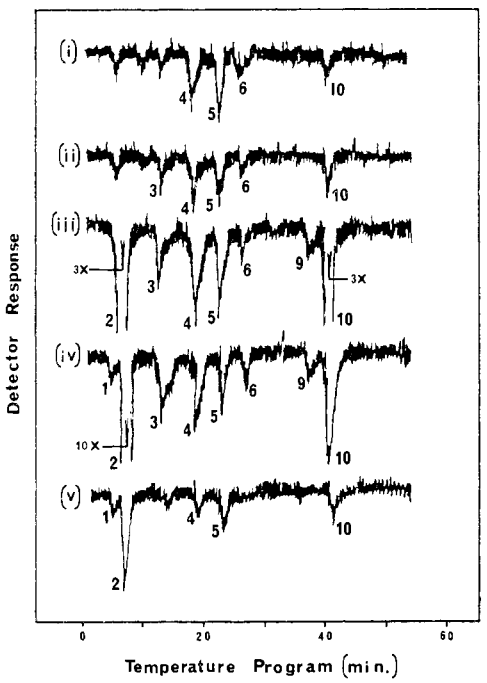

Figure 3. GC–headspace radiograms showing the effect on radioactive volatiles formation from [^{14}C]linolenic acid of various ammonium sulfate fractions. For peak identification, see Table I. The five radiograms were run on fractions having the following ammonium sulfate saturation level/peroxidase activity: (i) 0–20%/0 units; (ii) 20–40%/3 units; (iii) 40–60%/53 units; (iv) 60–80%/138 units; (v) >80%/40 units.

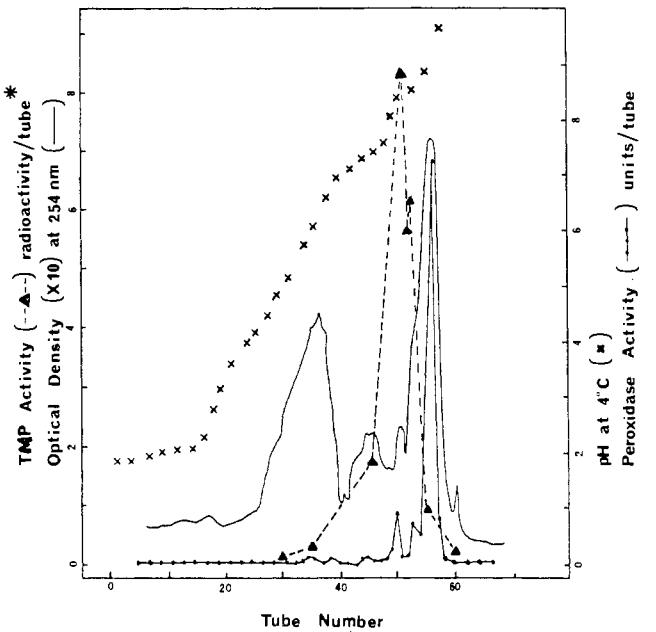

Figure 4. Isoelectric focusing of a portion of the 40–60% ammonium sulfate fraction. Note: TMP (tea metalloprotein) activity is defined as the total peak height of all radioactive volatiles detected on the radiogram (in arbitrary units) per tube of isoelectrically focused protein fraction.

series of ammonium sulfate fractions, taken at 20% saturation increments, was monitored for their ability to produce radioactive volatiles from labeled linolenic acid. Dramatic changes in the volatiles pattern (Figure 3) occurred as a result of this fractionation. Enzyme assays of the various fractions revealed (Figure 3) that maximal peroxidase activity coincided with maximal volatiles formation in the 40–60 and 60–80% ammonium sulfate fractions. No lipoxygenase-like activity could be detected using a polarographic assay in either the crude enzyme preparation or the ammonium sulfate fractions. The formation of radioactive volatiles is considered to be a more sensitive assay than the polarographic one and is used throughout the remaining purification work as the main assay method.

Further purification was then attempted by isoelectric focusing of the 40–60% ammonium sulfate fraction. Enzyme assays revealed (Figure 4) that maximal peroxidase was recovered at an isoelectric point of pH 9.6 and lesser amounts at pH 8.7 and 8.4. Neither catalase activity nor lipoxygenase activity (polarographic assay) was detected in any of the fractions collected. Small amounts of lipoxygenase activity, however, were detected by a spectrophotometric assay in fractions containing the highest levels of peroxidase activity. When some of the fractions from tubes 30 through 60 were incubated with labeled linolenic acid, maximal radioactive volatiles formation (Figure 5, ii) occurred in a fraction, tubes 49–51, which was not associated with maximal peroxidase activity (Figure 4).

The purified linolenic acid oxidation factor from the isoelectric focusing run was characterized in the following way. Incubation of the active fraction in boiling water for 10 min had no effect on the ability of this fraction to produce volatiles (Table II, part A). Passing the active fraction through a short cation exchange column caused no change in either the pattern or amount of radioactive volatiles formed on incubation with labeled linolenic acid (Table II, part B). Dialysis of the active fraction using Spectropore membrane tubing showed (Table II, part C) that the linolenic acid oxidation factor had an apparent molecular weight greater than 14 000. These results are

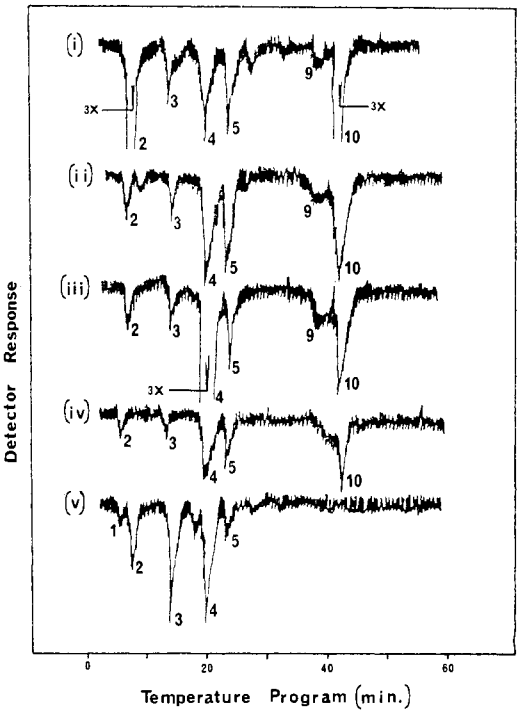

Figure 5. GC–headspace radiograms showing the similarity of the tea linolenic acid oxidation factor to heme and heme–protein (30-min aeration). For peak identification, see Table I: (i) 40–60% ammonium sulfate saturation; (ii) dialyzed isoelectrically focused tube 49; (iii) cytochrome *c* (40 μg); (iv) chlorohemin (8 mg); (v) lipoxygenase (10 mg), pH 5.5.

remarkably similar to those obtained by Major et al. (1974), who described the isolation of a heat-stable catalyst from Ginko leaves. They described the catalyst as stable to heat and extremes of pH but found the activity was lost after incubation with a bacterial protease. This last effect led Major et al. (1974) to conclude they had isolated an enzyme. Protease treatment of our active fraction from the isoelectric focusing run led to almost total loss of activity (Table II, part D). However, heat-inactivated protease was also capable of inactivating this same active fraction (Table II, part E).

Further work showed (Figure 5) that the radioactive volatiles pattern produced from linolenic acid by the active fraction from tea leaves was very similar to those produced by cytochrome *c* and chlorohemin. This similarity held even after heat treatment of all three of these materials (Figure 5). On the other hand, when soya lipoxygenase was tested under the same pH conditions, the volatiles pattern was different (Figure 5), and heat treatment destroyed the ability of the soya lipoxygenase to catalyze the production of volatiles from linolenic acid. These results suggested to us that the active factor in tea leaves responsible for the production of volatiles from linolenic acid was a metalloprotein (quite possibly a hematin), i.e. a protein (because of the way it behaves during isolation) with a metal containing catalytic moiety (because it is heat stable). The supposition that the tea catalyst is a metalloprotein was further supported by the results obtained (Table III) when various metal ion chelators and respiratory enzyme inhibitors were used (Appleby, 1969). It was also found (Table IV) that cytochrome *c* loses its ability to oxidize linolenic acid in the presence of both active and denatured forms of protease, as well as egg albumin and heated egg albumin. These results appear to put in question the contention of Major et al. (Major and Thomas, 1972; Major et al., 1974) that the catalyst in Ginko leaves which causes the oxidation of linolenic acid is an

Table III. Effect of Inhibitors on Linolenic Acid Oxidation Using Isoelectrically Focused Tube 49 Contents

Inhibitor used	Reported effective concn, M[a]	Concn used, M	Deg of inhibition, %
Sodium cyanide	10^{-3} to 10^{-5}	1×10^{-3}	60
α,α′-Dipyridyl	Fe^{2+} enzyme at 10^{-8}	1×10^{-6}	75
Sodium azide	Fe enzyme at 10^{-3}	5×10^{-4}	87
1,10-Phenanthroline	10^{-8}	2×10^{-8}	91
HOQNO[b]	10^{-5} to 10^{-8}	1×10^{-5}	94
		4×10^{-7}	91

[a] Appleby, 1969. [b] Abbreviation: HOQNO, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide.

Table IV. Effect of Protein on the Oxygen Uptake Associated with Catalyzed Oxidation of Linolenic Acid

Catalyst	Reagent added	Reaction time, min	Oxygen uptake
A. Lipoxygenase	(i) None	0	Fast (control)
	(ii) Protease	30	Slow
	(iii) Protease	60	Very slow
B. Cytochrome *c*	(i) None	0	Very fast (control)
	(ii) Protease	5	Very slow
	(iii) Heat-inactivated protease	5	Very slow
	(iv) Egg albumin	5	Slow
	(v) Heated egg albumin	5	Very slow

enzyme. We wonder if inactive protein might also "inactivate" the Ginkgo catalyst as was found to be the case with the tea leaf catalyst.

It is noteworthy that while the purified tea metalloprotein shows an unusually good heat stability, both the green tea homogenate and the crude enzyme preparation lose the catalytic activity upon heating (Figures 2A (ii) and 2C (ii), respectively). While our experimentation to date has not revealed the exact reason for this apparent discrepancy, it is presumed that some other component of these crude systems is responsible for the higher level of heat sensitivity and that the impurity is removed by the isoelectric focusing step.

CONCLUSION

The results of our investigation suggest that linolenic acid, and presumably other unsaturated fatty acids, are broken down in macerated tea leaf by oxidative reactions catalyzed nonenzymatically by endogenous metalloprotein compounds. The heat stability of this catalyst (Table II, part A) and the striking similarity of the volatiles formed by the purified tea catalyst and other hematin compounds (Figure 5) support this contention. The tea metalloprotein appears to have an apparent molecular weight greater than 14 000 and is purified by the usual protein purification techniques. However, the exceptional heat stability of the tea catalyst would appear to rule out an essential role for the protein portion of the tea catalyst.

The inhibitory effect of nonhematin proteins (enzymatically active or inactive) is not well understood but may result from a simple "masking" of the catalytically active face of the metal ion group. For example, introduction of nonenzyme protein, i.e. egg albumin, into reaction mixtures causes a marked reduction in catalytic activity. It is noteworthy that the catalytic activity of cytochrome *c* and chlorohemin toward the oxidative degradation of linolenic

acid is affected by treatment with other proteins in exactly the same way as the tea metalloprotein (Table IV).

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Tocopherols in the Unsaponifiable Matter of Coffee Bean Oil

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The unsaponifiable matter of coffee oil was fractionated by column chromatography. Thin-layer chromatography of the fractions indicated for the first time the presence of α- and (β + γ)-tocopherol. They were identified by UV, IR, NMR, and mass spectrometry. In oil of green coffee beans of different origin α- and (β + γ)-tocopherol were found to be present in concentrations of 89–191 and 252–465 μg/g of oil, respectively.

The chemical composition of the lipids in green coffee beans has received considerable attention. Several GLC studies on the fatty acid composition have been reported (Carisano and Gariboldi, 1964; Van de Voort and Townsley, 1974; Folstar et al., 1975). In the unsaponifiable matter diterpene alcohols of the kaurene type have been found (Djerassi and Bendas, 1955; Haworth and Johnston, 1956; Djerassi et al., 1959; Wahlberg et al., 1975) and the occurrence of *n*-nonacosane (Neu, 1948) and squalene (Kaufmann and Sen Gupta, 1964) has been established. Moreover, the composition of 4,4-dimethylsterols, 4-methylsterols, and 4-demethylsterols has been studied in detail (Nagasampagi et al., 1971). This paper reports the fractionation of the unsaponifiable matter of coffee oil, the identification of tocopherols, and the determination of the amount of these compounds in different samples of beans.

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EXPERIMENTAL SECTION

Materials. Florisil (60–100 mesh) was obtained from the British Drug Houses Ltd., Poole, England. Commercially prepared silica gel plates and Al_2O_3 for column chromatography as well as 2,2′-bipyridine, squalane, and α-tocopherol were purchased from E. Merck AG, Darmstadt, Germany. *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide was from Pierce, Rockford, Ill.

Isolation of the Unsaponifiable Matter. Green coffee beans (180 g) were coarsely ground in an Olland disk crusher. After cooling overnight in the refrigerator the material was ground more finely in a Retsch ZM I centrifugal mill, equipped with a sieve of 0.50-mm screen opening, and extracted for 24 h with petroleum ether (40–60 °C) in a Soxhlet. The solution was kept overnight in the refrigerator whereupon crystallized caffeine was filtered off by suction (0.2 μm membrane filter 11407, Sartorius GmbH, Göttingen, Germany). After evaporation a clear oil was obtained (20 g). According to the AOAC method (1965) the oil was saponified yielding 1.6 g of unsaponifiable matter.

Column Chromatographic Fractionation of the Unsaponifiable Matter. A slurry of 50 g of Florisil