C, 70.57; H, 5.92. Found: C, 70.62; H, 6.01.

Birch Reduction of 4′-Methoxy-3′-methoxymethoxy-3-hydroxyflavone (7). One gram (3.05 mmol) of **7** was reduced with 250 mg (11 mmol) of Na in 50 mL of liquid NH_3 as described above. The combined products from three reactions were chromatographed on a 35 × 2.5 cm silica gel column using 15 and 20% EtOAc in hexane as eluant. The four products in order of elution were as follows.

(*a*) *1-(2-Hydroxyphenyl)-2-hydroxy-3-(4-methoxyphenyl)propanone* (**8**). 106 mg (4%) as an oil. NMR 3.1 (2 H, m, CH_2), 3.80 (3 H, s, OCH_3), 5.30 (1 H, m, C_2H), and 6.6–7.8 (8 H, m, ArH); IR (neat) cm^{-1} 3470, 2965, 1640, 1610, 1510, 1300, 1245, and 750; MS *m/e* (rel intensity) 272 (2), 254 (2), 151 (3), 134 (2), 123 (4), 122 (11) and 121 (100); *Oxime*, mp 138–9.5 °C; IR (KBr) cm^{-1} 3320, 3260, 1610, 1515, and 1250; MS *m/e* (rel intensity) 287 (2), 121 (100), 91 (34). Calcd for $C_{16}H_{17}NO_4$: C, 66.88; H, 5.96; N, 4.88. Found: C, 66.74; H, 6.27; N, 4.88.

(*b*) *2-Hydroxy-2-(4-methoxybenzyl)-2(3H)-benzofuranone* (**10**). 376 mg (1.39 mmol, 15%); mp 117–8.5 °C, lit. mp 120 °C (Chopin et al., 1964); IR (KBr) cm^{-1} 1720; MS *m/e* (rel intensity) 270 (8), 122 (13), 121 (100), 91 (7), 78 (12), and 77 (12).

(*c*) *1-(2-Hydroxyphenyl)-2-hydroxy-3-(4-methoxy-3-methoxymethoxyphenyl)propanone* (**9**). 336 mg (1.01 mmol, 11%) oil. Further purified by preparative TLC on silica gel. NMR 3.05 (2 H, m, CH_2), 3.45 (3 H, s, OCH_3), 3.85 (3 H, s, OCH_3), 5.15 (2 H, s, OCH_2O), 5.30 (1 H, m, C_2H), and 6.8–7.8 (7 H, m, ArH); IR (neat) cm^{-1} 3480, 2960, 1645, 1510, 1445, 1265, 1155, 1135, 1080, 1000, and 755; MS *m/e* (rel intensity) 332 (13), 182 (14), 181 (100), 151 (12), 137 (15), and 121 (33).

(*d*) *2-Hydroxy-2-(4-methoxy-3-methoxymethoxybenzyl)-2(3H)-benzofuranone* (**11**). 787 mg (2.38 mmol, 26%) oil. NMR 3.15 (2 H, b.s., CH_2), 3.45 (3 H, s, OCH_3), 3.75 (3 H, s, OCH_3), 5.15 (2 H, s, OCH_2O), and 6.7–7.7 (7 H, m, ArH); IR (neat) cm^{-1} 3380, 2960, 1715, 1610, 1460, 1255, 1140, and 1000; MS *m/e* (rel intensity) 330 (4), 314 (3), 182 (23), 181 (100), 151 (16), 137 (38), 121 (31), 88 (22), and 70 (44). *Dioxime*, mp 188–9.5 °C; IR (KBr) cm^{-1} 3260, 1515 and 1365; MS *m/e* (rel intensity) 360 (1), 342 (9), 207 (14), 181 (13), 177 (13), 163 (15), 151 (12), 148 (14), 137 (16), 121 (18), 120 (27), 119 (58), 92 (38), and 91 (100). Calcd for $C_{18}H_{20}N_2O_6$: C, 59.99; H, 5.59; N, 7.77. Found: C, 59.29; H, 5.51; N, 7.64.

1-(2-Hydroxyphenyl)-2-hydroxy-3-(4-methoxy-3-hydroxyphenyl)propanone (2). A solution of 250 mg (0.75 mmol) of **9** in 10 mL of MeOH containing 2 drops of 6 N HCl was heated at reflux for 15 min and then added to 100 mL of 0.5% $NaHCO_3$. Extraction with 3 × 25 mL of $CHCl_3$ and drying and evaporation of the $CHCl_3$ gave a yellow oil. Crystallization from EtOAc–hexane afforded 196 mg (0.68 mmol, 91%) of **2**: mp 114–5 °C; NMR 3.0 (2 H, m, CH_2), 3.80 (3 H, s, OCH_3), 5.30 (1 H, m, C_2H), and 6.7–7.8 (7 H, m, ArH); IR (KBr) cm^{-1} 3430, 3260, 1650, 1520, 1450, 1240, 1130, 1085, and 975. MS *m/e* (rel intensity) 288 (5), 256 (5), 270 (10), 138 (16), 137 (100), 122 (13), and 121 (33). Calcd for $C_{16}H_{16}O_5$: C, 66.66; H, 5.59. Found: C, 66.53, H, 5.56.

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Formation of (*E*)-Hex-2-enal and (*Z*)-Hex-3-en-1-ol by Fresh Leaves of *Brassica oleracea*

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The production of (*E*)-hex-2-enal and (*Z*)-hex-3-en-1-ol by fresh leaves of cabbage has been shown to be due to a series of reactions, starting with the action of lipoxygenase on linolenic acid. The 13-hydroperoxide of linolenic acid thus formed decomposes due to the action of inactivated hemoprotein enzyme catalyst to give (*Z*)-hex-3-enal. This can then both isomerize to give (*E*)-hex-2-enal and it can also be reduced by alcohol dehydrogenase/NADH to (*Z*)-hex-3-en-1-ol. No obvious explanation could be found for these reactions occurring almost exclusively in the outer leaves of the plant.

(*E*)-Hex-2-enal (*trans*-hex-2-enal) and (*Z*)-hex-3-en-1-ol (*cis*-hex-3-en-1-ol) are extremely common aroma products of green leaves, so much so that they have been termed "leaf aldehyde" and "leaf alcohol". Many examples can be quoted of their production from fresh foliage (e.g., Walbaum, 1918; Bedoukian, 1963; Hatanaka and Ohno, 1971; Major et al., 1972; Major and Thomas, 1972), vegetables (e.g., Schormuller and Grosch, 1962; Forss et al., 1962; Eriksson 1967; Fleming et al., 1968; MacLeod and MacLeod, 1968, 1970a; Kazeniac and Hall, 1970; Buttery et al., 1971), and fruits (e.g., Winter and Sundt, 1962; Anderson and von Sydow, 1964; Drawert et al., 1966; Anjou

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and von Sydow, 1967). Indeed, the considerable majority of lists of aroma and flavor compounds of plants contain these two compounds, and generally they bestow a characteristic fresh green, leafy aroma. Interestingly, their occurrence crosses to the animal kingdom where they are produced by some insects as attractants or repellents; for example, the main active principle of the defensive secretions of black ants (Bevan et al., 1961) and of cockroaches (Wallbank and Waterhouse, 1970) is (*E*)-hex-2-enal.

With such a wide distribution, the biogenesis of these compounds by plants is of much interest—they themselves do not appear to be present as such in the intact plants. In addition, they are significant aroma and flavor compounds in plant foods with quite low odor threshold values [of the order of 0.02 ppm for the aldehyde and 0.07 ppm for the alcohol (Buttery et al., 1971)]. Consequently, a number of workers have studied the pathways of formation of these important compounds, in particular Kazeniac and Hall (1970) and Hatanaka and co-workers (e.g., Hatanaka and Harada, 1973; Kajiwara et al., 1975; Sekiya et al., 1976; Hatanaka et al., 1976). The former used tomatoes as the substrate for their investigations, whilst the latter have worked mainly on *Thea sinensis* (tea) leaves. Both these sets of workers have described overall schemes for the biogenesis of (*E*)-hex-2-enal and (*Z*)-hex-3-en-1-ol from linolenic acid via an enzymic route involving lipoxygenase, and recently Hatanaka et al. (1976) have confirmed the site for such reactions in *Thea sinensis* as the chloroplasts.

Similar overall results have been obtained by other workers (e.g., Major and Thomas, 1972; Tressl and Drawert, 1973; Grosch and Schwarz, 1971), although often with slight differences in detail most likely imposed by minor inherent differences in the various plant systems studied.

Here we report our findings with respect to the cabbage (*Brassica oleracea*) system, which has not before been investigated in this context. Our previous studies of the chemical flavor composition of cabbage which provoked interest in this particular problem provided the following relevant data: (1) that (*E*)-hex-2-enal and (*Z*)-hex-3-en-1-ol are relatively important to cabbage flavor, bearing in mind their flavor potency, in being present to the extents of approximately 1.5 and 4.5%, respectively, in flavor extracts (MacLeod and MacLeod, 1968); (2) that the compounds are almost certainly formed enzymically (MacLeod and MacLeod, 1970b); (3) that they are probably produced from a precursor that is a relatively small molecule (MacLeod and Nussbaum, 1977, MacLeod and Pikk, 1978); (4) that they are produced almost exclusively by the older, outer leaves of the cabbage plant (MacLeod and MacLeod, 1970c). The aims of this project were thus to determine the pathway of formation of the two compounds by cabbage, to ascertain how this compared with proposed schemes for other plants, and to answer questions raised by some of the observations noted above.

RESULTS AND DISCUSSION

The previous work which had suggested an enzymic mode of formation of (*E*)-hex-2-enal and (*Z*)-hex-3-en-1-ol involved a comparative survey of the volatile flavor components of fresh and preserved (dehydrated) cabbage (MacLeod and MacLeod, 1970b). Dehydrated cabbage, in which enzymes are inactivated by blanching prior to preservation, produced neither the aldehyde nor the alcohol. Similar results were obtained in comparing fresh and frozen Brussels sprouts (MacLeod and MacLeod, 1970a). These previous results were confirmed more formally here by experiments in which suitable extracts (i.e., simple distillates of chopped, macerated cabbage) were examined following heat-inactivation of the cabbage enzymes. No hexenol or hexenal was obtained, whilst control experiments in which enzymes were not inactivated produced good amounts of the two compounds.

Subsequent to these simple experiments, it was found that instead of chopping the cabbage, shredding at high speed in a Waring blender not only produced greater amounts of the two compounds but also in general provided more reproducible results. Consequently, this method was used in all other experiments. These findings agree with those of Kazeniac and Hall (1970) who obtained about eight times as much (*E*)-hex-2-enal in this manner compared with hand-slicing of tomatoes. They suggested that more intimate mixing of air (necessary for reaction, see later) with the tomato particles on high-speed blending was probably the reason for this higher production.

The reason for suspecting formation of the hexenal and hexenol from relatively small molecules is based on the finding that in both cabbage and Brussels sprouts much more of the compounds were produced when the plants were grown under conditions of horticultural stress, when plants tend to biosynthesize their smaller molecules to greater extents (MacLeod and Nussbaum, 1977; MacLeod and Pikk, 1978). Detailed figures have been quoted (MacLeod, 1976).

Earlier work had also shown that (*E*)-hex-2-enal and (*Z*)-hex-3-en-1-ol were produced almost exclusively from the outer, older leaves of the cabbage with virtually none being formed from the inner leaves (MacLeod and MacLeod, 1970c). Again these results agree with the findings of Kazeniac and Hall (1970) who obtained increasing amounts (of aldehyde) with age of the tomato.

The fact that oxygen is necessary for the biogenesis of the two compounds by cabbage was shown by blending cabbage samples either in air or under a nitrogen atmosphere. Both compounds were produced to normal extents in the former case, but under nitrogen only negligible amounts were obtained. In addition, these experiments showed that both (*Z*)-hex-3-en-1-ol and (*E*)-hex-2-enal were formed in air in increasing amounts with time, although it did appear that a maximum was attained after about 2 h. Apart from this latter important point, indicating decomposition of the products after some time, these findings confirm those of previous workers (e.g., Kazeniac and Hall, 1970; Hatanaka and Harada, 1973).

Drawert et al. (1966) have suggested linolenic acid as precursor for these two compounds, and this was readily shown to be the case for cabbage as well as other plants, in that a cabbage sample to which linolenic acid was added gave more than twice the amounts of these two products compared with a blank to which no linolenic acid had been added. Other, similar fatty acids did not show the same effect. Linolenic acid was confirmed to be present in cabbage and in reasonable amounts ($\sim$30 mg %); no difference was observed between results obtained separately for inner and outer leaves.

Although (*Z*)-hex-3-enal is not an important volatile flavor component of cabbage, it was implicated in the formation of at least (*E*)-hex-2-enal by the following experiment. Blended cabbage was maintained at room temperature in a sealed container from which headspace samples were removed at 15-min intervals. The results are summarized in Table I and show that initially (*Z*)-hex-3-enal is formed in relatively large amounts which rapidly decrease accompanied by an increase in (*E*)-hex-2-enal. It is a reasonable deduction that the former rearranges into the latter, but, from the discrepancy in amounts, most of the (*Z*)-hex-3-enal is not being consumed in this manner

$CH_3—CH_2—CH\overset{Z}{=}CH—CH_2—CH\overset{Z}{=}CH—CH_2—CH\overset{Z}{=}CH—(CH_2)_7—COOH$

linolenic acid

lipoxygenase/O_2
(Reaction 1)

$CH_3—CH_2—CH\overset{Z}{=}CH—CH_2—CH(O—OH)—CH\overset{E}{=}CH—CH\overset{Z}{=}CH—(CH_2)_7—COOH$

13-hydroperoxide of linolenic acid

(Reaction 2)

$CH_3—CH_2—CH\overset{Z}{=}CH—CH_2—CHO$

(Z)-hex-3-enal

(Reaction 3) (Reaction 4)

$CH_3—CH_2—CH_2—CH\overset{E}{=}CH—CHO$ <--?--> $CH_3—CH_2—CH\overset{Z}{=}CH—CH_2—CH_2OH$

(E)-hex-2-enal (Z)-hex-3-en-1-ol

Figure 1. Proposed broad scheme for the formation by cabbage of (*E*)-hex-2-enal and (*Z*)-hex-3-en-1-ol from linolenic acid.

Table I. Effect of Time on the Production of Hexenals by Cabbage

time, min	GC peak areas in mm^2 (*Z*)-hex-3-enal	(*E*)-hex-2-enal	total hexenals
0	643	2	645
15	342	73	315
30	176	113	289
45	75	131	206

(the figures for total hexenals show this clearly). In the cabbage system at least it would appear that most of it is probably forming the major product of the two, (*Z*)-hex-3-en-1-ol. These results show some interesting parallel with the findings of Kazeniac and Hall (1970).

Since (*E*)-hex-2-enal and (*Z*)-hex-3-en-1-ol are produced enzymically from a long-chain fatty acid and oxygen is necessary for reaction, then it is a reasonable deduction that the lipid oxidation enzyme lipoxygenase (EC 1.13.1.12) is at least initially involved in the reaction pathway. Lipoxygenase activity in cabbage has been adequately described (Pinsky et al., 1971) and was confirmed in this project by the fact that when incubated with various fatty acids, cabbage extracts produced additional lipid oxidation products. No difference in activity was observed between extracts separately prepared from the inner and outer leaves of the plant.

It is possible at this stage on the evidence so far presented to construct a likely reaction scheme for the formation by cabbage of (*E*)-hex-2-enal and (*Z*)-hex-3-en-1-ol from linolenic acid although the individual steps require confirmation and clarification of detail (Figure 1).

Reaction 1. Lipoxygenase specifically catalyzes the oxidation by molecular oxygen of 1*Z*,4*Z*-pentadiene systems to *Z*,*E* conjugated hydroperoxides. In vegetables the substrate mainly resides in linoleic, linolenic, and certain other polyunsaturated fatty acids (Eriksson and Svenssen, 1969). Since linolenic acid alone has been proved to be

Table II. Amounts of Hexenals Produced (GC Peak Areas in mm^2) from Blended and Ether Extracted Cabbage Samples

	(*Z*)-hex-3-enal	(*E*)-hex-2-enal
freshly prepared	21	Tr
after standing 24 h	80	3

the substrate in this project, only this need be considered. Six hydroperoxide products have been predicted for linolenic acid (Badings, 1960) and four of these have been isolated and identified (Frankel, 1962), the main ones being the 9- and 13-hydroperoxides. Hamberg and Samuelsson (1965) have reported that at 0 °C the action of soybean lipoxygenase on linolenic acid provides about 20% of the 9-hydroxperoxide and about 80% of the 13-hydroperoxide isomer although it would be expected that proportions would differ depending on various circumstances such as pH, origin of enzyme, etc. With regard to this latter point, we also used soybean lipoxygenase and therefore would expect the 13-hydroperoxide to be the major product.

The presence of linolenic acid hydroperoxide(s) in cabbage extracts was indicated by ultraviolet spectrophotometry when characteristic strong absorption was shown at 236 nm. The same extracts immediately examined by gas chromatography showed the presence of small quantities of (*Z*)-hex-3-enal and traces of (*E*)-hex-2-enal. These amounts increased somewhat if the extract was allowed to stand for a long time (see Table II). These findings suggest the formation of the 13-hydroperoxide of linolenic acid (13-hydroperoxyoctadeca-9*Z*,11*E*,15*Z*-trienoic acid) since the conjugation in such hydroperoxides constitutes a chromophore with λ_{max} 234 nm (Hamberg and Samuelsson, 1965) and since only this particular hydroperoxide would be expected to decompose to form (*Z*)-hex-3-enal. These deductions were confirmed to some extent by incubating synthetic linolenic acid with commercial soybean lipoxygenase. In agreement with the

Table III. Amounts of Hexenals Produced (GC Peak Areas in mm^2) from Synthetic 13-Hydroperoxide of Linolenic Acid by Various Influences

	hexenals
hydroperoxide alone, blank	40
standing for 24 h	112
UV radiation	116
heat	117
copper ions	121
copper ions + heat	134
iron ions	142
iron ions + heat	160
peroxidase	61
catalase	97
heat-denatured peroxidase	593
heat-denatured catalase	620
acid-denatured peroxidase	738
hemin	812

result for the cabbage extract, an extremely rapid development (only a few minutes to reach maximum) of a strong UV absorption at 236 nm was observed, and furthermore small amounts of (*Z*)-hex-3-enal (but not (*E*)-hex-2-enal) were detected by gas chromatography. A more extensive examination of the UV spectrum showed an additional small peak at 282 nm, and a similar one was confirmed to be present also in the UV spectrum of the previously mentioned cabbage extracts. It was assumed that this was due to the (*Z*)-hex-3-enal since Tappel (1953) has shown that hydroperoxides decomposing thermally characteristically show decrease in UV absorbance at 232.5 nm and increase at 277.5 nm, this latter being typical of aldehyde absorption. In support, the UV spectrum of (*Z*)-hex-3-enal was found to show a peak at 284 nm.

Reaction 2. The above results indicate that the 13-hydroperoxide of linolenic acid is formed by cabbage and that this decomposes to (*Z*)-hex-3-enal. The deduction that (*Z*)-hex-3-enal is the first product from linolenic hydroperoxide is reasonable since it has already been shown to be an early product of reaction and itself then to react further to give (*E*)-hex-2-enal (Table I). Nevertheless this was confirmed by synthesizing specifically the 13-hydroperoxide of linolenic acid, treating it with cabbage extract and analyzing the mixture at once by gas chromatography, when large amounts of (*Z*)-hex-3-enal were obtained, far in excess of a control (cabbage alone).

In many respects the main problem with reaction 2 is not so much that it occurs, but how it occurs, and much previous work has ignored this aspect. In this investigation the 13-hydroperoxide of linolenic acid was freshly prepared and its degradation due to the effect of various influences was examined. Initially reagents tested for activity were simplistic ones, e.g., heat, different pH, metal ions, UV radiation. Those that functioned at all gave the same product, (*Z*)-hex-3-enal, sometimes together with a much smaller proportion of (*E*)-hex-2-enal, and results are quoted for these in Table III. Although extra amounts of hexenals over a blank experiment (synthetic hydroperoxide alone) were obtained in these cases, amounts were insufficient to account for the quantities known to be produced naturally by cabbage. The most effective of the reagents tested were metal ion catalysts, particularly iron, and it was observed that their activities were increased to minor extents by heat treatment in addition. Various enzymes (catalase and peroxidase) were also studied, but again these were only slightly effective. However, in these cases, application of heat as well caused considerable increase in the amount of hexenals obtained, as shown in Table III. Both heat and enzyme were necessary to form these large amounts, either without the other was much less active.

Table IV. Amounts of Products Formed (GC Peak Areas in mm^2) by Adding Synthetic (*Z*)-Hex-3-enal to Cabbage Extracts

	(*Z*)-hex-3-enal	(*E*)-hex-2-enal	(*Z*)-hex-3-en-1-ol
cabbage alone, blank	12	144	472
cabbage + (*Z*)-hex-3-enal	23	296	807

Table V. Amounts of Individual Hexenals Produced (GC Peak Areas in mm^2) from Synthetic 13-Hydroperoxide of Linolenic Acid with Time and Heat Treatment

hydroperoxide	(*Z*)-hex-3-enal	(*E*)-hex-2-enal
after standing 1 h	40	ND[a]
after standing 24 h	109	3
after refluxing 0.5 h	90	27

[a] ND, not detected.

Hemoprotein enzymes, such as peroxidase and catalase, can function as catalysts in two different ways. One is to act as an enzyme in the normal manner whilst the other is to catalyze nonenzymically the oxidation by molecular oxygen of unsaturated compounds. Eriksson has reasonably suggested that the increased catalytic activity of such enzymes on denaturation (by heat or acid) is due to conformation changes in the protein whereby the catalytically active heme group [presumably, as with hemoglobin and myoglobin, held in hydrophobic pockets within the globin molecule (Rosen, 1970)] becomes more favorably exposed to the substrate molecules for reaction (Eriksson et al., 1971). This would seem to be true since increased exposure of the heme group tends to lead to increased nonenzymic activity (Eriksson et al., 1971). Taken to extremes this would mean that a completely isolated heme group would have greatest activity. The compound hemin [chloroprotoporphyrin IX iron (III)] is a reasonable approximation to this ideal and we did find that of all reagents studied this was indeed the one which gave greatest decomposition of linolenic hydroperoxide (Table III). When added to a cabbage extract it also gave excess hexenal and hexenol products over amounts normally obtained.

It seems a reasonable suggestion, therefore, that an inactivated hemoprotein enzyme catalyst can be responsible for the production of (*Z*)-hex-3-enal from the 13-hydroperoxide of linolenic acid for cabbage at least and probably for some other systems also. Calculations indicate that about the correct amount of hexenals is produced by heat-inactivated hemoprotein enzymes.

Reaction 3. From previously mentioned results (see Table I), it appears that (*Z*)-hex-3-enal isomerizes to (*E*)-hex-2-enal in cabbage. This was proved to be the case by adding synthetic (*Z*)-hex-3-enal to a cabbage preparation and analyzing the products. The results are shown in Table IV and it can be seen that much more (*E*)-hex-2-enal was produced by cabbage to which (*Z*)-hex-3-enal had been added. The fact that both hexenals were sometimes obtained from synthetic linolenic hydroperoxide as described under reaction 2 tends to suggest that the isomerization might proceed to some extent spontaneously or due to the particular influence under examination (Table III). However, in these model system experiments (*Z*)-hex-3-enal was always the major product and even after standing for 24 h it was only very slowly transformed into (*E*)-hex-2-enal. Table V shows this, in which selected relevant data from Table III for the two individual hexenals are given. Even heat treatment for 30 min caused

Table VI. Amounts of Products Formed (GC Peak Areas in mm^2) by Adding Synthetic (*E*)-Hex-2-enal or (*Z*)-Hex-3-en-1-ol to Cabbage Extract

	(*Z*)-hex-3-enal	(*E*)-hex-2-enal	(*Z*)-hex-3-en-1-ol	(*E*)-hex-2-en-1-ol
cabbage alone, blank	12	144	472	ND[a]
cabbage + (*E*)-hex-2-enal	11	554	470	78
cabbage + (*Z*)-hex-3-en-1-ol	15	140	935	ND

[a] ND, not detected.

only modest conversion. This type of behavior was certainly not the case for cabbage (see Table I) and therefore there must be some factor in the plant which provokes this rapid isomerization. Unfortunately all attempts to locate and identify this failed. Whatever the cause, the isomerization is swift and little (*Z*)-hex-3-enal is normally present in the aroma volatiles of cabbage, although this is not always the case with other plant systems.

Reaction 4. In addition to being the precursor for the (*E*)-hex-2-enal, (*Z*)-hex-3-enal is also the precursor for (*Z*)-hex-3-en-1-ol, as proved by the results in Table IV. This is a straightforward reduction, and it was shown that the enzyme system (alcohol:NAD oxidoreductase, EC 1.1.1.1) was active for synthetic (*Z*)-hex-3-enal in converting it efficiently into (*Z*)-hex-3-en-1-ol. The rate of reaction was found to be slower than that for the reduction of (*E*)-hex-2-enal, which agrees with a previous report that double bonds further from the functional group lower the rate (Eriksson, 1968). The appropriate enzyme activity in cabbage was indicated by accomplishing by means of a cabbage preparation a range of similar aldehyde/alcohol conversions, as well as the one under investigation here (Table IV). Thus, (*E*)-but-2-enal and hexanal were converted into excess of the corresponding alcohols compared with a control cabbage blank with no additions. Both these alcohol/aldehyde pairs are normally produced by cabbage (MacLeod and MacLeod, 1968).

Many factors affect this seemingly simple enzyme/substrate reaction, but as a generalization the equilibrium constant for the reaction is in favor of the alcohol (Eriksson, 1968). Our results (see the blank, Table IV) support this, in that very much more (*Z*)-hex-3-en-1-ol is obtained from cabbage than (*Z*)-hex-3-enal. It can also be deduced from these same figures that this enzymic conversion is more efficient or quicker than the isomerization of (*Z*)-hex-3-enal to (*E*)-hex-2-enal by the unknown factor(s).

Further Reaction Possibilities. It might be expected that the alcohol:NAD oxidoreductase system would also convert the produced (*E*)-hex-2-enal in cabbage to (*E*)-hex-2-en-1-ol, but although the reaction is possible and it was achieved in model systems, it does not seem to occur normally to any significant extent in cabbage since no (*E*)-hex-2-en-1-ol could be detected in extracts (see e.g., Table VI). The reason for this is unknown. However, this does raise the point as to whether (*E*)-hex-2-enal and (*Z*)-hex-3-en-1-ol are the final products of this series of reactions. In addition, there is the possibility that one is intermediate in the formation of the other. These are problems which in general have not been considered before, but it was readily shown that both compounds are the main end products of this reaction scheme for cabbage and that they do not interconvert. When excess synthetic (*Z*)-hex-3-en-1-ol was added to a cabbage extract and compared with a control of cabbage extract alone, no extra products were detected and nor were there any increases in amounts of any components. Results for compounds significant to this investigation are given in Table VI. Similar results were also obtained when (*E*)-hex-2-enal was added to a cabbage extract, and these are also given in Table VI. However, in this case there was one difference in that a small proportion of (*E*)-hex-2-en-1-ol was obtained, due presumably to the cabbage alcohol dehydrogenase system acting to a slight extent on the excess (*E*)-hex-2-enal. Since (*E*)-hex-2-en-1-ol does not occur to any significant extent, if at all, in cabbage aroma extracts and since only small proportions were formed from an excess of (*E*)-hex-2-enal by the action of cabbage extract, then this reaction has little, if any, importance or relevance. However, why it does not occur or why it occurs to such a small extent compared with model systems [enzyme + (*E*)-hex-2-enal] is obscure, particularly bearing in mind the facility of the reaction for the 3-*Z* system in cabbage.

In conclusion, the proposed scheme for the production by cabbage of (*E*)-hex-2-enal and (*Z*)-hex-3-en-1-ol from linolenic acid as outlined in Figure 1 is upheld by the results reported here. A number of reasonable reagents for accomplishing these transformations in cabbage have been determined and evaluated, although this does not, of course, preclude the possibility of some alternative, untested circumstances also functioning.

EXPERIMENTAL SECTION

Materials and Reagents. Cabbages were purchased from a local retailer as necessary; in general, outer leaves only were used. Chemicals were of analytical or high purity grade. Enzymes and coenzymes were as follows: lipoxygenase ex soybean (M_r 102 400; activity 50 000 units/mg), peroxidase ex horseradish (40 000; 200), catalase ex beef liver (251 000; 1200 Keil units/g), NAD and NADH ex yeast (purities, 98%), alcohol dehydrogenase ex yeast (activity 200 units/mg). All but the last, which was obtained from Boehringer Mannheim GmbH, Germany, were from Koch-Light Ltd., England.

Apparatus. (*a*) *Gas Chromatography.* A Pye-Unicam Series 104 instrument with FID was used, equipped with a 1.5 m × 4 mm id glass column packed with 12% PEG (M_r 15 000) coated on 100–120 BSS mesh acid-washed Diatomite C. A column temperature of 110 °C and a carrier gas (nitrogen) flow rate of 30 mL/min were used. Generally 2 μL of samples was injected at an attenuation setting of 50 (i.e., 5×10^{-11} A f.s.d.). A quantitative standard of hexan-1-ol (0.4% w/v aqueous solution) was added to all samples before injection into the GC. (*b*) *Ultraviolet Spectrophotometer.* A Pye-Unicam SP 800 double-beam recording instrument was used, employing at various times constant temperature cell holder, automatic cell change, and program controller.

Cabbage Analysis. Cabbage was analyzed for its volatile aroma components as described previously (MacLeod and MacLeod, 1968), and identifications of compounds relevant to this study were confirmed by comparison of relative t_R with standards. It was shown in particular that (*E*)-hex-2-en-1-ol, which elutes just after (*Z*)-hex-3-en-1-ol, was not detected.

Preparation Methods. (*a*) *Cabbage Extracts.* Fresh cabbage leaves (100 g) were blended with 150 mL of distilled water or phosphate buffer (pH 6.8) in a Waring blender at high speed for 1 min at room temperature. The mixture was distilled until a 50-mL aliquot had been

collected. Fresh extracts were prepared for all experiments. This blending method was superior to chopping finely with a knife.

(*b*) *Headspace Sample.* Blended cabbage was maintained at room temperature in a flask sealed air-tight with a stopper containing a silicone rubber septum insert. Samples of headspace (5 mL) were collected at 15-min intervals with an air-tight syringe for injection into the GC.

(*c*) *13-Hydroperoxyoctadeca-9Z,11E,15Z-trienoic Acid (13-Hydroperoxide of Linolenic Acid).* This was prepared from linolenic acid and lipoxygenase based on methods previously described (Hamberg and Samuelsson, 1965; Gardner, 1970; Gardner and Weisleder, 1972). In addition, the reactants were incubated together at room temperature and the rate of formation of hydroperoxide was assessed by UV spectrophotometry. Further products were examined by gas chromatography as well.

Examination of Cabbage Extracts. (*a*) *Effect of Oxygen.* Blending was carried out in a N_2 atmosphere or as normal in the presence of air. Analysis was by GC. (*b*) *Effect of Time.* Samples as in (*a*) were allowed to stand for various lengths of time after blending before distillation and analysis by GC. (*c*) *Effect of Heat Treatment.* Cabbage was chopped manually and immediately plunged into boiling water for 5 min before distillation and analysis by GC. A control experiment without heat treatment was also performed. (*d*) *Addition of Linolenic Acid.* Various amounts of linolenic acid (25, 50, 75, 100, 150, 200, and 300 mg/100 g of cabbage) were added before blending, and samples were allowed to stand for 1 h before distillation and analysis by GC. A control experiment without any addition was also performed. Linoleic and oleic acids were similarly examined, and experiments were repeated using either the inner leaves alone or the outer leaves alone of cabbage. (*e*) *Addition of (Z)-Hex-3-enal, (E)-Hex-2-enal, (Z)-Hex-3-en-1-ol, (E)-But-2-enal, or Hexanal.* Blended cabbage was allowed to stand at room temperature for 1 h following the addition of 50 mg of one of the five named compounds. Samples were distilled and analyzed by GC as usual, and a control experiment without any addition was also carried out at the same time. (*f*) *Analysis for Hydroperoxides and Products.* A blended cabbage sample was immediately extracted with diethyl ether and the extract was concentrated and fractionated by column (alumina) chromatography. Fractions were examined by UV spectrophotometry and those showing hydroperoxide absorptions (λ_{max} 236 nm were also submitted to GC. The fractions were also allowed to stand at room temperature for 24 h and were then reexamined by GC.

Examination of Synthesized 13-Hydroperoxide of Linolenic Acid. (*a*) *Addition to Cabbage Extract.* Synthetic 13-hydroperoxide (50 mg) was added immediately to a freshly blended cabbage preparation. Following distillation the sample was analyzed by GC and compared with a control experiment without any addition of hydroperoxide. (*b*) *Decomposition by Various Agents.* Solutions of freshly prepared synthetic hydroperoxide (10 mg) in distilled water or pH 6.8 buffer (25 mL) were subjected to various influences and examined for any decomposition, usually after 1 h, by UV spectrophotometry (decrease in hydroperoxide absorbance at λ_{max} 236 nm) and by GC analysis following distillation. The control was an untreated hydroperoxide solution after standing at room temperature for 1 h. Solutions were examined after standing at room temperature for 24 h, after refluxing for 0.5 h, and after exposure to UV radiation for 1 h (in a UV spectrophotometer repetitively scanned from 190 to 450 nm). Chemical reagents tested included metal ions (e.g., iron, copper, calcium), 1 mg of a suitable soluble salt being added, and the mixture was allowed to stand 1 h; heat in addition was assessed by refluxing for the final 15 min of the hour. Hemin (chloroprotoporphyrin IX iron(III), 1 mg) was examined in the same manner. Enzymes tested were catalase and peroxidase, 1 mg being added, and the solution was allowed to stand for 1 h. Peroxidase was also used in combination with 1 mg of H_2O_2. For heat denaturation, enzyme solutions in pH 6.8 buffer were treated as recommended for maximum nonenzymic activity (Eriksson et al., 1971), catalase at 90 °C for 2 min, and peroxidase at 125 °C for 15 min. Acid-denatured peroxidase was obtained by treating a solution with hydrochloric acid to pH 2.1, again as recommended (Eriksson et al., 1971). Aliquots of denatured enzyme solutions (1 mL, containing 1 mg of enzyme) were added to the hydroperoxide solution, and the mixture was allowed to stand for 1 h before analysis.

Effect of Alcohol Dehydrogenase/NADH on Aldehydes. Aldehydes examined included (*Z*)-hex-3-enal, (*E*)-hex-2-enal, (*E*)-but-2-enal, and hexanal. The following procedure for the first is typical. (*Z*)-Hex-3-enal (20 mg) in pH 6.8 buffer (20 mL) was treated with alcohol dehydrogenase (3.7 mg) and cofactor NADH (1.3 mg). UV spectra were recorded at 5-min intervals over a period of 1 h against a blank solution lacking NADH, and reaction was assessed by decrease in NADH absorbance at 340 nm. The solution was then distilled and analyzed by GC.

Estimation of Linolenic Acid in Cabbage. A representative sample (500 g) of a whole cabbage was blended with 1 L of a chloroform/methanol mixture (2:1) for 2 min at high speed in a Waring blender. The mixture was filtered at the pump and the residue washed with 3 × 100 mL extracting solvent. Washings and filtrate were combined, transferred to a 1-L separatory funnel and allowed to stand for 15 min. The aqueous layer was discarded and the organic layer washed with 2 × 100 mL of distilled water. The extract was dried with anhydrous Na_2SO_4 and filtered through Whatman phase separating filter paper. The extract was concentrated by vacuum distillation to about 50 mL and made up accurately to 100 mL with extracting solvent. A 50-mL aliquot was evaporated to dryness under vacuum and methylated with methanol/BF_3. A known weight of linolenic acid was also methylated to determine the recovery of the procedure and to provide both qualitative and quantitative standard for subsequent gas chromatography. The fatty acid methyl esters were separated and analyzed by GC using a 2 m × 4 mm i.d. glass column packed with 10% PEGA on Diatomite C and operated at 200 °C with a 100 mL/min nitrogen flow rate. Samples of inner leaves alone and of outer leaves alone of cabbage were analyzed by the same procedure.

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$^{13}C-^{12}C$ Analysis of Vegetable Oils, Starches, Proteins, and Soy–Meat Mixtures

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The $^{13}C-^{12}C$ ratios for a number of vegetable oils, starches, and proteins have been determined. As expected, the values for animal proteins reflect the animals' diet. The possible application of using $^{13}C-^{12}C$ analysis in differentiating corn-fed animal protein (C_4 plant) from soy protein (C_3 plant) in soy–meat mixtures is discussed.

Measurements of the relative abundance of the naturally occurring stable carbon isotopes ^{12}C and ^{13}C have proven to be an important tool for evaluating the importance of specific chemical and physical processes in biochemical and geochemical cycles. Processes which discriminate against the heavier ^{13}C and favor use of the lighter ^{12}C cause isotopic fractionation to occur. With the development of sensitive mass spectrometric techniques, variations in the $^{13}C-^{12}C$ ratios of one part in ten thousand can be detected (Craig, 1953). Determination of $^{13}C-^{12}C$ ratios in organic matter may be carried out by combustion of relatively small amounts of material (5–10 mg) to CO_2. The ease with which CO_2 can be collected and purified has allowed stable carbon isotope measurement surveys to be undertaken in the biosphere and geosphere (Friedman and Irsa, 1967; Lerman and Troughton, 1976).

Surveys on the natural variations of $^{13}C-^{12}C$ ratios in the plant kingdom (Craig, 1953; Lerman and Troughton, 1976; Lerman, 1972; Minson et al., 1975; Bender, 1968; Smith and Brown, 1973; Tregunna et al., 1970; Lowdon, 1969; Garnier-Dardart et al., 1976; Smith and Epstein, 1971) have shown that the bimodal distribution of stable carbon isotope ratios ($^{13}C-^{12}C$) found to occur naturally in higher plants correlates closely with the two photosynthetic routes used by these plants in the fixation of atmospheric carbon dioxide. These biochemical routes, known as the C_3 (Calvin-Benson) and C_4 (Hatch-Slack) photosynthetic pathways, involve the initial fixation of CO_2 into respectively a three-carbon and four-carbon dicarboxylic acid product during the enzymatic carboxylation reaction. With average differences of approximately 14 parts per thousand (ppt) observed in the corresponding $\delta^{13}C$ values, the stable carbon isotope ratio is a direct indication of the carbon pathway used by the higher plant. Thus plants and plant products which are C_3 (such as wheat, oats, barley, potatoes, and soybeans) are readily distinguished from C_4 plants and plant products (such as sugar cane, corn, and sorghum) by their $^{13}C-^{12}C$ ratios.

The occurrence of natural ^{13}C labeling in plants and plant products has found application in many research investigations ranging from glucose metabolism studies in man (Lacroix et al., 1973) to substantiating incorporation of maize cultivation into an early North American culture (Vogel and Van Der Merwe, 1977).

Use of stable carbon isotope techniques may have direct applications in the food and drug industry (Lerman and Troughton, 1976; Haines, 1976). For example, the adulteration of carbohydrate products such as natural syrups and honeys by the undeclared addition of sugar cane or corn-derived products may be monitored using $^{13}C-^{12}C$ measurement (Doner and White, 1977).

Food chain investigations have shown that natural $^{13}C-^{12}C$ ratios of animal tissues (Minson et al., 1975; Parker, 1964; De Niro and Epstein, 1978) [as well as those of human tissues (Gaffney et al., 1978)] tend to follow

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