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FORMATION OF 2-HEXENAL BY LEAVES

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Abstract—By means of GLC and by precipitation as the 2,4-dinitrophenylhydrazone the amount of 2-hexenal formed from leaves of several plants when ground in two ways was determined. The effect on 2-hexenal formation of changing the pH of the solutions in which the grinding took place was investigated. The results obtained suggest that different enzymes or other catalysts are involved in the formation of 2-hexenal in leaves of different species. Results obtained with *Ginkgo* leaves indicate that 2-hexenal formation is not effected by a dehydrogenase since dehydrogenase inhibitors did not interfere with the formation of 2-hexenal in extracts of these leaves.

INTRODUCTION

IT HAS been shown that 2-hexenal is formed when the leaves of the tree, *Ginkgo biloba* L. are ground in the presence of oxygen at room temperature. This aldehyde is an inhibitor of the growth of fungi with an ED_{50} of $30 \times 10^{-3}\%$.[1] There was some variation in the amount of 2-hexenal formed from *G. biloba* and also considerable variability in the amount of this aldehyde which was obtained from the leaves of other trees.[2] Nye and Spoehr[3] have found that the yield of 2-hexenal from leaves varied with the fineness to which the leaves were ground. These studies have now been extended.

Since enzymatic reactions are generally affected by pH, the effect of pH on the formation of 2-hexenal has been investigated. Major, Marchini and Boulton[2] have found that when *Ginkgo* leaves were macerated in ligroine in the air, 2-hexenal was produced, while similar treatment of leaves of *Ailanthus glandulosa* gave no 2-hexenal. The question arises as to whether this is due to the greater acidity of *Ginkgo* leaves or due to different oxidative enzymes in the two kinds of leaves, one of which is poisoned by ligroine.

The possibility that 2-hexenal was formed by the action of a hydrogenase on precursors was studied. We have also investigated the effects of various enzyme inhibitors on the formation of 2-hexenal from macerated leaves.

RESULTS

Fresh leaves of the *Ginkgo* tree as well as those of other trees were ground in a ball-mill as described by Major, Marchini and Boulton.[2] For comparison, similar leaves were ground in a blender. If any 2-hexenal were found on grinding, generally somewhat more was found

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[1] R. T. MAJOR, P. MARCHINI and T. SPROSTON, *J. Biol. Chem.* **235**, 3298 (1960).
[2] R. T. MAJOR, P. MARCHINI and A. J. BOULTON, *J. Biol. Chem.* **238**, 1813 (1963).
[3] W. NYE and H. A. SPOEHR, *Arch. Biochem.* **2**, 23 (1943).

when the leaves were ground in the blender than in the ball-mill. The 2-hexenal was determined by gas chromatography, and from the weight of the 2,4-dinitrophenylhydrazone formed. As previously reported by Nye and Spoehr,[3] we found considerable variability in our yields. However, highest yields were obtained from leaves of *Albizzia julibrissins*, *Ailanthus glandulosa* and *G. biloba*.

The pH of the leaf purees varied from 4·15 (*G. biloba*) to 5·7 (*A. glandulosa*) in the variety of trees investigated. The use of buffered solutions for puree preparations showed that hexenal yields remained high over the range of pH 3·7–5·0 in the case of *G. biloba* and 6·0–7·4 in the case of *A. glandulosa* and dropped sharply at higher values. Yields of 2-hexenal from leaves of *A. glandulosa* dropped markedly at lower pH values (Table 1); the effect of lower pH values on *Ginkgo* leaves was not studied. However, these results suggest that unless there were purely buffer-ion effects different enzymes may have catalyzed the formation of 2-hexenal in the leaves of the different species. This possible difference in the enzymes which catalyze the formation of hexenal by different leaves is suggested also by the earlier finding that ligroine interferes with the formation of 2-hexenal from leaves of *A. glandulosa* but not from those of *G. biloba*.[2]

Since 2-hexenal does not seem to be present in significant amounts in intact, live leaves, it was of interest to determine how it was formed. Since hexenal is formed when leaves are

TABLE 1. 2-HEXENAL OBTAINED FROM LEAVES AT VARIOUS pH VALUES

Buffer	pH	% × 10^3 2-hexenal
Ailanthus glandulosa		
None	5·7	74·8
d	1·5	none
a	4·0	112·0
a	4·2	31·2
a	4·3	76·0
a	5·3	68·0
a	5·3	71·2
a	5·35	96·4
a	6·2	87·2
a	6·2	81·6
b	6·5	36·8
a	6·6	47·7
b	7·1	28·0
b	7·4	32·8
c	8·6	none
Ginkgo biloba		
none	4·15	14·6
none	4·15	46·0
d	3·7	13·0
a	4·4	37·6
a	5·0	25·0
a	6·2	2·0

[a] McIlvaine's citrate–phosphate (0·1 M citrate–0·2 M disodium phosphate).
[b] 0·1 M KH_2PO_4, pH adjusted with 1 M NaOH.
[c] 1 M KCl–1 M H_3BO_3, pH adjusted with 1 M NaOH.
[d] 1 M KCl–1 M HCl, ratios were adjusted to the desired pH.

damaged in an atmosphere containing oxygen, it seemed possible that it was formed by the dehydrogenation of some compounds in leaves such as 2-hexen-1-ol,[4] in the presence of a dehydrogenase. However, when the coenzyme, nicotinamide adenine dinucleotide (NAD), was ground with *Ginkgo* leaves in the absence of oxygen, only traces of 2-hexenal was detected in the steam distillate.[5] The same result was obtained if methylene blue was substituted for NAD.

TABLE 2. YIELDS OF 2-HEXENAL FROM *Ginkgo* LEAVES IN THE PRESENCE OF INHIBITORS

Inhibitors	Molar concentration of the inhibitor in aqueous solution	% × 10^3 of hexenal formed
Iodoacetamide	$2{\cdot}33 \times 10^{-3}$	35·0
Arsenosobenzoate (Na^+)	$2{\cdot}18 \times 10^{-3}$	37·2
p-Chloromercuribenzoate (Na^+)	$3{\cdot}28 \times 10^{-3}$	16·8
p-Chloromercuribenzoate (Na^+)	$6{\cdot}25 \times 10^{-5}$	25·0
Copper sulfate hydrate	10^{-2}	36·0
Hydrogen sulfide	*a*	12·0
Potassium ferricyanide[b]	10^{-2}	15·2
Carbon monoxide	*a*	16·5

[a] Saturated the cold water with the gas used.
[b] Mixture had strong odor of HCN.

Further evidence that points to the unlikelihood that 2-hexenal is formed by the dehydrogenation of a precursor in the leaves was obtained by studying the effect of dehydrogenase and other enzyme inhibitors on the formation of hexenal (Table 2). Enzyme inhibitors which operate by combination with sulfhydryl groups[6] have been studied. Little, if any, evidence of inhibition was noted. In addition, the effects of the following compounds on the formation of hexenal were studied: copper sulfate, hydrogen sulfide, carbon monoxide and potassium ferricyanide. No real evidence of change in hexenal formation was found with these compounds. All of these would be expected to inhibit metallo-porphyrin containing enzymes.[7]

Further investigations of the formation of 2-hexenal are reported in a subsequent paper[8].

EXPERIMENTAL

Methods of analysis of 2-hexenal in leaves. (I) *Grinding the leaves.* (A) The reaction vessel described previously[2] was filled with a mixture of 250 g of fresh leaves and 1000 ml H_2O. This mixture was ball-milled for 4 hr and then steam distilled until 250 ml of distillate had been collected.

(B) Fresh leaves (100 g)were ground in an *Oesterizer* blender with 350 ml H_2O until the leaves were well homogenized (generally 2 min). The mixture was steam distilled immediately, collecting 100 ml.

(II) *Analytical procedures.* (A) *GLC.* The column was a copper tube 3 m long packed with 10% Carbowax 20 M on Haloport F and was operated at 105°.

[4] Found in many plant leaves, T. YAMANISHI, J. TAKAGAKI and M. TSUMIMURA, *Bull. Agr. Chem. Soc. Japan* **20,** 127 (1956); *Chem. Abs.* **51,** 3934 (1957), T. Watanabe, *Nature, Lond.* **182,** 325 (1958).
[5] Since the completion of this study Hatanaka, *et al.* (A. HATANAKA, O. ADASKI, M. AMEYAMA, *Agric. Biol. Chem.* **34,** 1574 (1970)) have reported that allyl alcohol dehydrogenase from *E. coli* oxidized cis-2-hexenol in the presence of NADP but not NAD. However, -SH reagents strongly inhibited the oxidation.
[6] H. SUND and H. THEORELL, *The Enzymes,* 2nd Edition, Vol. 7, p. 46, Academic Press, New York (1963).
[7] M. DIXON and E. C. WEBB, *Enzymes,* 2nd Edition, p. 337, Academic Press, New York (1963).
[8] R. J. MAJOR and M. THOMAS, *Phytochem.* **11,** 611 (1972).

(B) 2,4-*Dinitrophenylhydrazones.* The steam distillates from the leaves were extracted with ether; these solutions were then dried. For each 100 g of leaves was then added 7 ml of a solution of 1·0 g of 2,4-dinitrophenylhydrazine, 2·5 ml of 6 N HCl and 100 ml MeOH. After 2 hr the solvents were evaporated to a small volume (2·7 ml/100 g of leaves). The 2,4-dinitrophenylhydrazone crystallized and was identified and checked for purity by the m.p. and by TLC on silica gel utilizing 6 : 1 hexane–ether as the developer.

Analytical method A generally gave somewhat higher results than method B, probably due to losses in method B in the crystallization procedure. When ball-milled leaves and method A was used, two runs with leaves of *Albizzia julibrissins* showed 38–50 $\times$ 10^{-3}% 2-hexenal, four runs with *Ailanthus glandulosa*, 18–25 $\times$ 10^{-3}% 2-hexanal and three runs with *Ginkgo biloba*, 11–27 $\times$ 10^{-3}% 2-hexenal; while by method B 24 $\times 10^{-3}$%, 10–18 $\times$ 10^{-3}% and 7–17 $\times$ 10^{-3}%, respectively, using one, three and seven runs each, respectively. For leaves ground by the blender only method A was used; one run with leaves of *A. julibrissins* gave 60 $\times$ 10^{-3}% hexenal, two runs with leaves of *A. glandulosa* gave 87–92 $\times$ 10^{-3}% hexenal and three runs with leaves of *G. biloba* gave 11–32 $\times$ 10^{-3}% hexenal. Leaves of other trees showed lower or no yields of hexenal when treated in the same way.

Effect of pH on 2-hexenal yields. Buffer solutions were used in place of water while grinding the leaves and the 2-hexenal content of the steam-distillates determined by GLC is shown in Table 1.

Effect of NAD and methylene blue on hexenal formation. Fresh *G. biloba* leaves (100 g) were placed in an air-tight stainless steel blender with 600 ml H_2O. The blender was evacuated and oxygen-free nitrogen was admitted. This was repeated three times, then the leaves were ground 8 min at 8000 rev/min. Then either 112 mg of NAD in 8 ml of pyrophosphate buffer (pH 8·8) or 1 g of methylene blue were added by injection; the mixture then stood at 20° for 15 min. The 2-hexenal content of the distillate was determined by GLC.

Effect of enzyme inhibitors on formation of hexenal. The inhibitors were added to *Ginkgo* leaves (100 g) with 300 ml H_2O and macerated for 5 min in the blender. Distillate (100 ml) was then collected from each by steam distillation and yields of hexenal determined by GLC are shown in Table 2.

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Key Word Index—Ginko biloba; Ginkgoaceae; *Ailanthus glandulosa*; Simarubaceae; biosynthesis; 2-hexenal.