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4-HYDROXYBENZYL ALCOHOL, A NATURALLY OCCURRING COFACTOR OF INDOLEACETIC ACID OXIDASE

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Abstract—4-Hydroxybenzyl alcohol, a previously unrecognized constituent of plants, has been isolated from muskmelon seedling extracts (*Cucurbita moschata*). The compound acts as a cofactor for indoleacetic acid oxidase both *in vitro* and in the oat coleoptile section test.

INTRODUCTION

INDOLEACETIC acid oxidase (IAAO) has been shown to require an organic cofactor of low molecular weight.[1,2] This requirement may be satisfied by several substituted phenols,[3] but with the exception of the work on the hydroxycinnamic acids by Gortner, Kent, and Sutherland[4] and that by Sharpensteen and Galston on an unidentified substance in peas,[5] naturally occurring IAAO cofactors have received little attention. An investigation of the phenolic substances in seedling extracts revealed a previously unrecognized constituent of plants,[6] 4-hydroxybenzyl alcohol. This report describes the isolation of this compound and studies on its activity as an IAAO cofactor.

RESULTS AND DISCUSSION

A new phenolic constituent of plants, 4-hydroxybenzyl alcohol, has been isolated from muskmelon seedlings (*Cucurbita moschata*) by extraction with aqueous methanol and subsequent fractionation with ether and sodium bicarbonate solution. The compound was obtained crystalline from the "neutral" fraction and was characterized by analysis, i.r. and u.v. spectra (Fig. 1) and comparison with a synthetic specimen. Although phenolic substances are often present in plant tissue as glycosides, it is believed that most of the 4-hydroxybenzyl alcohol isolated existed as such in the seedlings since the extraction method employed should not lead to glycoside hydrolysis.[7]

In a colorimetric IAAO assay, in which the Salkowski reagent as modified by Tang and Bonner[8] was used for determination of unreacted indoleacetic acid (IAA), 4-hydroxybenzyl alcohol showed increasing IAAO cofactor activity with increasing concentration

* Contribution No. 787.

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[2] G. A. MACLACHLAN and E. R. WAYGOOD, *Can. J. Biochem. Physiol.* **34**, 1233 (1956).
[3] P. L. GOLDACRE, A. W. GALSTON and R. L. WEINTRAUB, *Arch. Biochem. Biophys.* **43**, 358 (1953).
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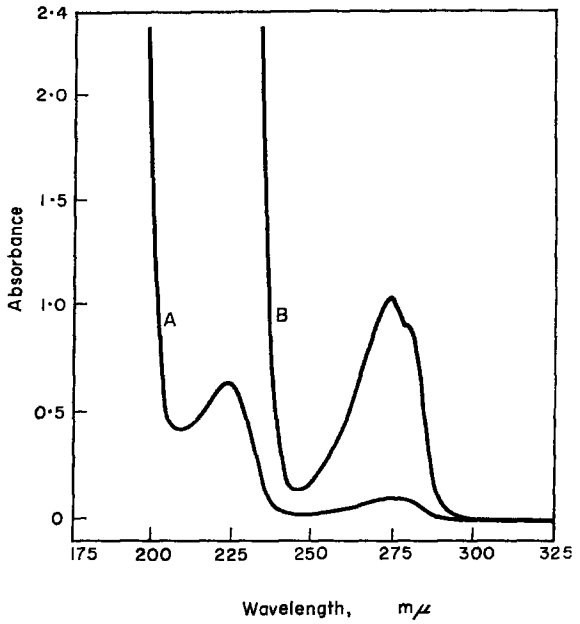

FIG. 1. UV ABSORPTION SPECTRUM OF 4-HYDROXYBENZYL ALCOHOL
A, 10 mg/l. in ethanol; B, 100 mg/l. in ethanol.

over the range of 10^{-6} to 10^{-4} M (Table 1). At concentrations greater than 10^{-4} M, however, 4-hydroxybenzyl alcohol interfered with color development. Accordingly, its optimal stimulatory level could not be determined by the colorimetric procedure. 2,4-Dichlorophenol, a compound often used as an IAAO cofactor, and 4-hydroxycinnamic acid, the

TABLE 1. ASSAY OF IAAO COFACTORS

Cofactor added to assay system* (M)		% IAA destroyed	
		20 min	60 min
None		6	11
4-hydroxybenzyl alcohol	7×10^{-4}	†	†
	1×10^{-4}	38	56
	1×10^{-5}	12	20
	1×10^{-6}	10	13
2, 4-dichlorophenol	7×10^{-4}	17	50
	1×10^{-4}	44	77
	1×10^{-5}	19	35
	1×10^{-6}	4	12
4-hydroxycinnamic acid	5×10^{-4}	9	11
	1×10^{-4}	11	31
	1×10^{-5}	28	58
	1×10^{-6}	13	27
2-hydroxybenzyl alcohol	1×10^{-4}	13	17
	1×10^{-5}	3	10
4-methoxybenzyl alcohol	1×10^{-4}	5	11

* By colorimetric IAAO assay as described in Experimental.
† Poor color development.

cofactor from pineapple,[4] showed their maximal activity in this assay at 10^{-4} M and 10^{-5} M, respectively. At comparable levels 2-hydroxybenzyl alcohol (saligenin), which has been isolated previously from several plant species,[6] was a much less effective IAAO cofactor than 4-hydroxybenzyl alcohol (Table 1). Another known plant constituent structurally related to 4-hydroxybenzyl alcohol, 4-methoxybenzyl alcohol (anisyl alcohol), had no cofactor activity at concentrations between 10^{-6} and 10^{-4} M.

In a manometric IAAO assay, 4-hydroxybenzyl alcohol gave its greatest stimulation of oxygen absorption at 5×10^{-3} M, while 2,4-dichlorophenol had its optimum effect at 8×10^{-4} M (Fig. 2). At these optimum levels both compounds stimulated oxygen uptake at about the same rate during the first 20 min of incubation; however, subsequent oxidation

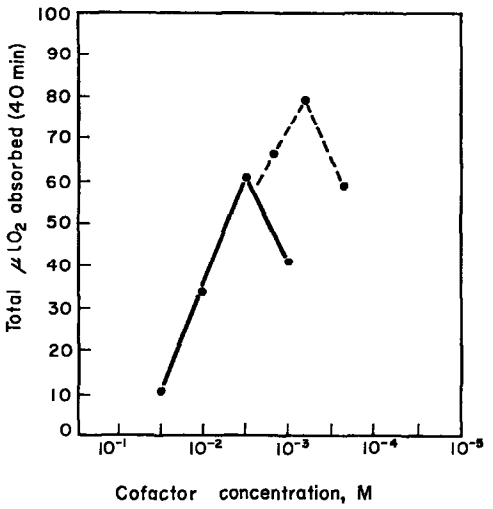

FIG. 2. OXIDATION OF IAA IN THE PRESENCE OF VARYING CONCENTRATIONS OF COFACTOR 4-hydroxybenzyl alcohol (————), 2,4-dichlorophenol (- - - - -). (For details see Experimental.)

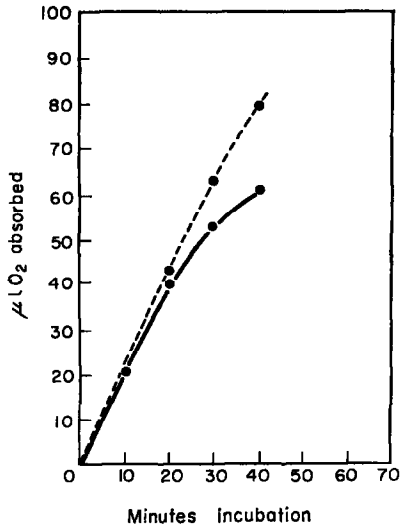

FIG. 3. OXIDATION OF IAA AT OPTIMUM COFACTOR CONCENTRATION 4-hydroxybenzyl alcohol (————), 2,4-dichlorophenol (- - - - -). (For details see Experimental.)

in the presence of 4-hydroxybenzyl alcohol was considerably slower than in the presence of 2,4-dichlorophenol (Fig. 3). This probably is due to the instability of 4-hydroxybenzyl alcohol and its tendency to form condensation products under the reaction conditions.

At concentrations from 0·1 to 100 mg/l., 4-hydroxybenzyl alcohol did not affect the endogenous growth of oat coleoptile sections in the standard straight growth assay (Table 2). At 10 and 100 mg/l., however, the compound strongly inhibited IAA-induced section growth. This level of 4-hydroxybenzyl alcohol seems relatively high, but it is apparently not unphysiological since up to 9 mg/kg fresh weight of plant tissue was isolated from muskmelon seedlings. Thus, the results of the section assay provide evidence that 4-hydroxybenzyl alcohol can function as an IAAO cofactor *in vivo*. They also suggest that the endogenous auxin in the oat coleoptile may not be IAA, since endogenous section growth is not inhibited by levels of cofactor which strongly inhibit IAA induced growth.

Application of 4-hydroxybenzyl alcohol to 12-day-old muskmelon seedlings either by foliar spray or in lanolin paste at concentrations up to 1 mg per plant failed to affect seedling growth significantly. This result was anticipated since the endogenous level,

(9 mg/kg) is probably near optimal for IAAO stimulation, and so addition of extra 4-hydroxybenzyl alcohol to the seedling would not be expected to cause any appreciable further affect. The fact that 4-hydroxybenzyl alcohol at 10 mg/l. caused almost as much inhibition of IAA induced oat coleoptile section growth as did the 10 fold higher concentration (Table 2) lends support to this conclusion.

TABLE 2. INHIBITION OF IAA INDUCED GROWTH OF OAT COLEOPTILES BY 4-HYDROXYBENZYL ALCOHOL

Concentration of IAA and 4-hydroxybenzyl alcohol used		Length of coleoptile sections 20 hr after treatment (mm)	Amount of IAA induced growth (mm)	% Inhibition of IAA induced growth
IAA μg/l	4-hydroxybenzyl alcohol mg/l			
	0	7·2		
	0·1	7·3		
0	1·0	7·4		
	10·0	7·2		
	100·0	7·8		
	0	10·4	3·2	
	0·1	10·2	3·0	4·5
50	1·0	9·8	2·6	18
	10·0	8·3	1·1	67
	100·0	8·1	0·9	74

In the IAAO modifying system in pineapples, 4-hydroxycinnamic acid was shown to be a cofactor while the closely related compounds, 3,4-dihydroxycinnamic acid (caffeic acid) and 4-hydroxy-3-methoxycinnamic acid (ferulic acid) act as inhibitors.[4] By analogy, 3,4-dihydroxybenzyl alcohol and 4-hydroxy-3-methoxybenzyl alcohol (vanillyl alcohol) which could be produced from 4-hydroxybenzyl alcohol by oxidation, should also act as inhibitors, and this was found to be so (Table 3). It has recently been reported that IAAO activity in peas may be modified by certain flavonoids.[9,10] Interestingly here also, the inhibitors, kaempferol glycosides, may be produced from the cofactors, apigenin derivatives, by an oxidative reaction.

TABLE 3. ASSAY OF IAAO INHIBITORS

Additions to assay system*			% IAA destroyed	
D C P		Inhibitor (M)	20 min	60 min
0		0	6	19
		0	23	46
	1×10^{-5}	4-hydroxy-3-methoxy-benzyl alcohol	7	7
	1×10^{-6}		13	42
	1×10^{-5}	4-hydroxy-3-methoxy-cinnamic acid	6	9
5×10^{-5} M	2×10^{-6}		17	47
	1×10^{-5}	3,4-dihydroxybenzyl alcohol	10	10
	1×10^{-6}		7	16
	1×10^{-5}	3,4-dihydroxycinnamic acid	0	0
	1×10^{-6}		0	6

* Colorimetric IAAO assay as described in Experimental.

[9] M. FURUYA, A. W. GALSTON and B. B. STOWE, *Nature*, **193**, 456 (1962).
[10] F. E. MUMFORD, D. H. SMITH and J. E. CASTLE, *Plant Physiol.* **36**, 752 (1961).

EXPERIMENTAL

Isolation of 4-hydroxybenzyl alcohol

4-Hydroxybenzyl alcohol was isolated from muskmelon seedlings by the following procedure. Muskmelon seeds (*Cucurbita moschata var.* Hale's Best Jumbo) (W. Atlee Burpee Company, Philadelphia, Pennsylvania), 4·54 kg, were planted in vermiculite in flats in a greenhouse. After 12–14 days, when the cotyledons had fully developed, the seedlings (45 kg fresh wt.) were uprooted, washed free from vermiculite, frozen with solid CO_2, and ground in an 8-in. Micro Pulverizer (Pulverizing Machinery Company, Summit, New Jersey). The resulting powder was added to about 30 l. 50% methanol : water (v/v) solution, and the slurry allowed to stand at 5° for two days. The slurry was then centrifuged and filtered, and the clear solution was concentrated to a volume of 2–3 l. *in vacuo* at 40°. After acidification with 40 ml HCl, the solution was filtered, and the clear brown filtrate extracted four times with one-liter portions of diethyl ether. The ether extracts were combined, extracted three times with 100-ml portions of 5% $NaHCO_3$ solution, dried over anhydrous Na_2SO_4, and the ether removed *in vacuo* on a "Rinco" evaporator. The yield of "neutral" residue was 1·7–2·5 g.

The bicarbonate-soluble material was recovered by acidification of the bicarbonate extracts with 15 ml concentrated HCl followed by extraction with ether. Evaporation of the ether from these extracts gave 1·4–1·6 g of brown oily residue which partially crystallized. Trituration of the oil with ether gave 0·15–0·3 g of white solid which was identified as fumaric acid by U.V and I.R spectra. The bicarbonate soluble fraction was not investigated further.

The "neutral" fraction was taken up in 5–10 ml chloroform, and the solution placed in a refrigerator at 5° overnight. The white crystalline solid which separated was collected, washed with cold chloroform and air dried. The yield of crystals, m.p. 110–120°, varied between 0·25 and 0·4 g in three separate experiments. Recrystallization of this product from benzene gave white needles, m.p. 113–115°. (Found: C, 67·9; H, 6·3; O, 24·62. Calc. for $C_7H_8O_2$: C, 67·8; H, 6·45; O, 25·75%). M.W. (ebullioscopic method in acetone) 128 ± 4.

Identification of 4-hydroxybenzyl alcohol

The infrared spectrum (KBr pellet) showed strong absorption at 6·2, 6·25 and 6·6 μ (OH), absorption in the 3–4 μ (C = C, aromatic), bands at 8–8·3 μ (ether or phenolic OH) and strong absorption in the 12–12·5 μ (*para*-disubstituted benzene). The u.v. spectrum (Fig. 1) had λ max at 273 mμ (ε = 1280) and at 223 mμ (ε = 7920) and a shoulder at 279 mμ (ε = 1130).

An aqueous solution of the compound gave a blue color on the addition of 1% ferric chloride solution and a red color characteristic of phenols with Millon's reagent.[11] A spot of the solution on paper gave a yellow color with diazotized sulfanilic acid, a violet color with diazotized *p*-nitroaniline, and a brown color with 0·07% potassium permanganate spray reagent.[12] The R_f in benzene : acetic acid : water (2 : 2 : 1 v/v) was 0·19; in N-butanol: conc. ammonium hydroxide (4 : 1 v/v) 0·65.

These analyses suggested that the isolated compound might be 4-hydroxybenzyl

[11] F. FEIGL, *Spot Tests in Organic Analysis*, p. 182, Elsevier Publishing Co., New York (1956).
[12] R. J. BLOCK, E. L. DURRUM and G. ZWEIG, *A Manual of Paper Chromatography and Paper Electrophoresis*, p. 228, Academic Press, Inc., New York (1955).

alcohol.[13] 4-Hydroxybenzyl alcohol synthesized by reduction of 4-hydroxybenaldehyde[14] proved to be identical in all respects to the compound isolated from the muskmelon seedling extracts.

Colorimetric IAAO Assay

The effect of cofactors and inhibitors on IAAO activity was determined by use of the assay system described previously.[10] The assay cups, which were incubated in a Dubnoff metabolic shaker at 26°, contained 1 ml bean root IAAO (E), 2 ml solution (S) containing IAA at 10^{-3} M and $MnCl_2$ at 5×10^{-4} M in 0·01 M KH_2PO_4 buffer (pH 6·1), 0–5 ml inhibitor and/or cofactor solution and sufficient 0·01 M KH_2PO_4 buffer (pH 6·1) to bring the total volume to 10 ml. Residual IAA was determined colorimetrically.[8] Results are shown in Tables 1 and 3.

Manometric IAAO Assay

To the Warburg flasks were added 6·25 μM IAA, 3 μM $MnCL_2$ and 3–150 μM cofactor in a total of 2·5 ml 0·02 M phosphate buffer, (pH–6·1). 20% KOH, 0·2 ml, was placed in the center well and 0·5 ml bean root IAAO[10] in the sidearm. After equilibration at 30°, IAA oxidation was started by tipping in the IAAO from the sidearm. Results are shown in Figs. 2 and 3.

Oat coleoptile section assay

The effect of 4-hydroxybenzyl alcohol on the IAA induced growth of oat coleoptile sections was carried out using the method of Nitsch and Nitsch.[15] Groups of ten 5 mm sections were used per treatment. These were placed in 2 ml plastic cups to which had been added 1 ml phosphate–citrate buffer (pH 4·8) containing sucrose (1·79 g K_2HPO_4, 1·019 g citric acid monohydrate and 20 g sucrose in 1 liter of water) and IAA and/or 4-hydroxybenzyl alcohol at the desired levels. The cups were covered, oscillated gently on a "Gyrotory" shaker (New Brunswick Scientific Co., New Brunswick, New Jersey) at 25° for 20 hr, and the length of the sections then measured to the nearest 0·1 mm by use of a binocular dissecting microscope containing an ocular micrometer. Results are shown in Table 2.

[13] B. Dunning, Jr., F. DUNNING and E. E. REID, *J. Am. Chem. Soc.* **58**, 1565 (1936).
[14] W. H. CAROTHERS and R. ADAMS, *J. Am. Chem. Soc.* **46**, 1675 (1926).
[15] J. P. NITSCH and C. NITSCH, *Plant Physiol.* **31**, 94 (1956).