

Vernonia anthelmintica (L.) Willd. Enzyme Studies.

Conversion of Epoxyoleic Acid to (+)-*threo*-12,13-Dihydroxyoleic Acid¹

1966

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Abstract

Vernonia anthelmintica (L.) Willd. seed was found to contain enzymes that were active only after the seed was ground. By deactivation of the enzymes, an oil rich in trivernolin (the triglyceride of epoxyoleic acid) was produced, and pure trivernolin was isolated. Acceleration of the enzyme activity altered the composition of the oil as evidenced by changes in free fatty acid content, iodine values, and oxirane oxygen (epoxy) content. Investigation showed that these changes were due, at least partially, to the conversion of epoxyoleic acid to (+)-*threo*-12,13-dihydroxyoleic acid, which was isolated in pure form. Pure (-)-*threo*-12,13-dihydroxyoleic acid was prepared by acetolysis of *V. anthelmintica* seed oil. Neither of these isomers had been obtained previously from mature *V. anthelmintica* seed.

Introduction

AT VARIOUS stages in the maturity of *Vernonia anthelmintica* (L.) Willd. seed there appear to be present four active principles of an enzymatic nature. Miwa et al. (1) reported the presence of two principles in maturing seed; one, an oxidative and, two, a dehydrating principle. A hydrolytic principle in the mature seed, active only after the seed is crushed, was discussed previously (2).

This paper elaborates on the behavior of the hydrolytic principle and on the activity of a fourth principle, a hydrating one, that is active when the mature seed is crushed and stored under a controlled experimental environment. The announcement of the presence of this hydrating principle responsible for the production of (+)-*threo*-12,13-dihydroxyoleic acid was made recently (3). The (+) and (-) isomers of *threo*-12,13-dihydroxyoleic acid were prepared previously by Chisholm and Hopkins (4) by acetolysis of the seed oils of *Malope trifida*, Cav. and *Vernonia colorata*, Drake, respectively.

Since this paper was submitted to the Editor, an article has appeared on the enzymatic production of (+)-*threo*-9,10-dihydroxyoctadecanoic acid in the spores of plant rusts, by A. P. Tulloch, Can. J. Biochem. Physiol., 41, 1115 (1963).

Experimental

Analytical Methods. The source of seed, analytical methods, Soxhlet, and rapid extraction techniques used in these investigations were described previously (2).

Thin-layer chromatographic procedures were essentially the same as published methods (5,6). The GLC analyses were done on a silicon rubber (SE-30) column using programmed temperatures as described by Herb et al. (7).

Hydrogenations were done in ethanol with 10% palladium on carbon powder as catalyst.

Deactivation of Enzymes. Both dry and steam heat were used in these experiments. The whole seed were heated in a forced-draft oven at 110C, 120C, 130C, and 150C for 8 and 20 hr. The seed were also heated by live steam at atmospheric pressure and by steam under pressure, as obtained in an autoclave, for 5 and 30 min.

Activation of Enzymes. Freshly ground seed samples were incubated under nitrogen at 28C. Samples in one experiment were sealed in individual flasks so that the available moisture was limited to the 7.8% naturally present in the seed. Seed in a second experiment were placed in open wide-mouthed bottles and covered loosely with aluminum foil; the bottles were placed over water in a vacuum desiccator, and the air was replaced by nitrogen. This provided the seed with a water-saturated nitrogen atmosphere. One sample from each experiment was extracted at weekly intervals for a 9 wk period.

Results and Discussion

The data in Table I show how the free fatty acid (FFA) content of *V. anthelmintica* seed oils rapidly increased when ground seed were air-equilibrated. There also appears to be a relation between the moisture of the seed and the rate of hydrolysis.

An immediate objective was the preparation of an oil with a low FFA content; therefore, studies were initiated to find an effective way to deactivate the enzymes.

The data in Table II show that dry heat required a temperature of 150C for 20 hr to completely deactivate the enzymes, while steam heat required only 30 min at 121C. In addition, dry heat at these conditions lowered the oxirane oxygen value of the oil from 3.9–3.7%. None of the steam treatments had any effect on the epoxy content.

TABLE I
Evidence of Enzyme Activity

Seed accession		Ground seed air-equilibrated	FFA (as epoxyoleic)
No.	Moisture		
1	7.8	Days	%
		0 ^a	2.1
		7 ^b	25.2
2	9.2	0 ^a	2.1
		7 ^b	38.5
		14 ^b	50.2

^a Extracted immediately after grinding.

^b Exposed to room conditions (air-equilibrated) for stated period of time before it was extracted.

¹ Presented in part at the AOCs meeting in Chicago, Ill., 1961; and Toronto, Canada, 1962.

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TABLE II
Heat Deactivation of Enzymes

Time heated hr	Temp heated C	% FFA in oil from ground seed air equilibrated	
		0 Days	8 Days
8	120	0.7	4.3
20	120	0.6	2.3
8	130	0.7	2.0
20	130	0.6	1.1
8	150	0.6	0.8
20	150	0.5	0.5
10 ^a	100 ^b	1.0	1.9
5 ^a	104 ^c	1.1	1.2
10 ^a	121 ^c	0.9	1.2
30 ^a	121 ^c	1.1	1.0

^a Minutes. ^b Live steam. ^c Autoclave.

Vernonia oil that contains mainly trivernolin, and that is practically free of divernolin and vernolic acid, can now be prepared. From such an oil, trivernolin can be produced quickly and simply as described by Krewson et al. (2).

Since divernolin and vernolic acid were obtained from an oil that contained 38% FFA (2), experiments were started to determine if the enzymes could be activated to produce optimum yields of these two compounds and also to produce monovernolin.

Fifty-g samples of freshly ground seed were incubated as described previously in this paper. A nitrogen atmosphere was used to prevent mold growth. After one week of incubation, it was necessary, owing to changes in the oil composition, to extract the petroleum ether extracted seed with ethyl ether in order to obtain all of the oil. The effect of time on the properties of oils prepared from seed incubated with a limited amount of moisture and in a water-saturated atmosphere is illustrated in Figures 1, 2, and 3. The rapid increase in FFA content of the oils before reaching a plateau at about the 4th week is shown in Figure 1. The oxirane oxygen content of the oil from the sample that was incubated with a limited amount of available moisture decreased gradually during the 9 wk period; that of the sample incubated in a water-saturated atmosphere decreased rapidly and leveled off at about the 6th week (Figure 2). Figure 3 shows that the iodine values of the oils from both experiments increased steadily during the nine-week period. As can be seen in all three figures, the changes were more dramatic in the oils prepared from seed incubated in a water-saturated atmosphere; the moisture content of this seed had increased to 16.2% by the 9th wk.

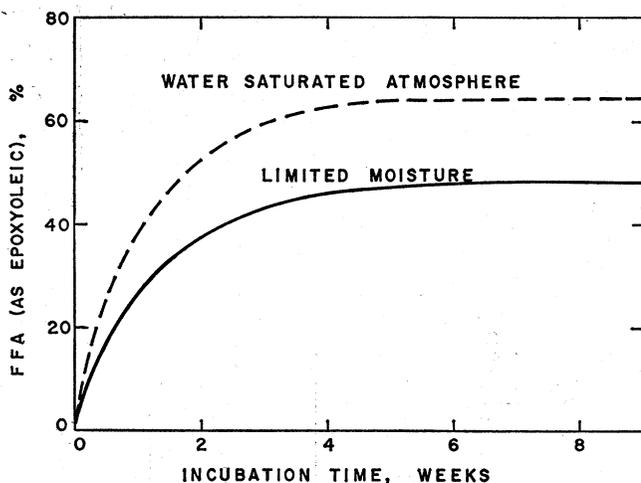


FIG. 1. The effect of time on the FFA content of oils prepared from seed incubated with limited moisture and in a water-saturated atmosphere.

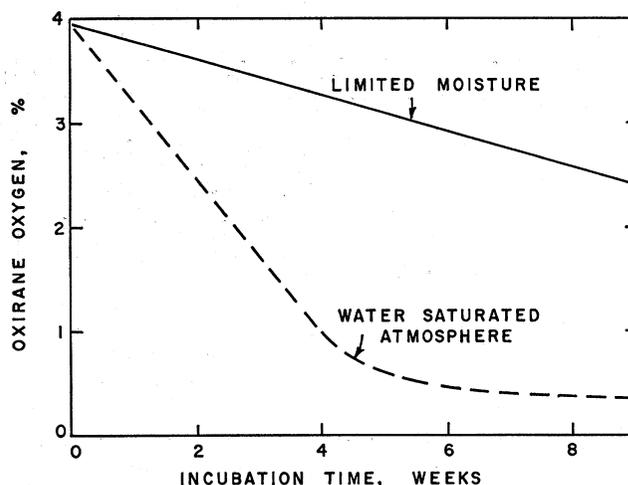


FIG. 2. The effect of time on the oxirane oxygen content of oils prepared from seed incubated with limited moisture and in a water-saturated atmosphere.

In order to determine the composition of the oil obtained from incubated seed, another sample of ground seed was incubated for 2 weeks in a water-saturated atmosphere and extracted as described. A portion of the extract was treated with diazomethane, and 90 mg of the resulting mixture of methyl esters and unreacted glycerides was fractionated by thin-layer chromatography (TLC), and the fractions located and recovered by known techniques (5). The composition, as shown in Table III, was determined by comparative TLC, GLC, and IR analyses. The composition, as indicated by the percentage in the table, represents the major portion of each fraction. No monovernolin was detected. GLC area analysis confirmed the fact that fraction 1 was approximately 30.4% dihydroxyoleate. Crystallization of the remaining oil from petroleum ether-ethyl ether at 0C, followed by recrystallization of the precipitate from ethyl acetate at 0C, acetone at -20C and 22C (twice) yielded a crystalline product (9.3% based on the weight of the oil) that proved to be (+)-*threo*-12,13-dihydroxyoleic acid. It had the following properties: mp 63-63.3C; $[\alpha]_D^{27} +19.0^\circ$ (in ethanol); oxirane oxygen, nil. Anal.: calc'd: C, 68.8%; H, 10.9%; found: C, 69.1%; H, 10.8%. The yield of this (+) isomer was increased to 32.2% by incubating ground seed with twice the weight of water.

In contrast, none of this isomer was found in oil obtained from ground seed autoclaved to deactivate

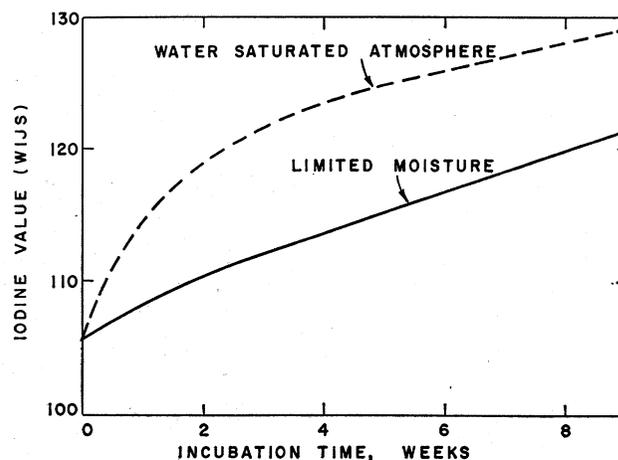


FIG. 3. The effect of time on the iodine values of oils prepared from seed incubated with limited moisture and in a water-saturated atmosphere.

TABLE III

Composition of Oil from Incubated *Vernonia anthelmintica* Seed ^a

TLC fraction	Composition of total extracted oil	
		%
1	Dihydroxy + unknown material	31.0
2	1,3-Divernolin	19.8
3	Trivernolin	8.6
4	Non-epoxidized Triglyceride plus Trivernolin	2.4
5	Methyl Vernolate	16.7
6	Non-epoxidized Methyl Esters	11.1
7	Hydrocarbon	10.5

^a The oil was treated with diazomethane prior to the TLC fractionation.

the enzymes before incubation with twice the weight of water. A TLC comparison of this *Vernonia* oil with pure (+)-*threo*-12,13-dihydroxyoleic acid, pure vernolic acid, pure 1,3-divernolin, pure trivernolin, pure tristearin, and low FFA *Vernonia* oil showed that the major portion of the oil was trivernolin. In fact, the chromatogram was exactly like that of low FFA *Vernonia* oil.

In addition to isolating the (+) isomer, (-)-*threo*-12,13-dihydroxyoleic acid was prepared from *V. anthelmintica* seed oil by acetolysis (8), and purified according to the method of Chisholm and Hopkins (4). The (-) isomer had the following properties: mp 62.5–63°C; $[\alpha]_D^{27} -18.6^\circ$ (in ethanol); oxirane oxygen, nil. Anal.: C, 68.1%; H, 10.7%. A 1:1 mixture of the (+) and (-) isomers crystallized from petroleum ether-ethyl ether melted at 52.5–53°C.

TLC of the methyl esters of the (+) and (-) isomers of *threo*-12,13-dihydroxyoleic acid produced single spots that had the same migratory characteristics. Their infrared spectra were identical; each showed the typical pattern for an unsaturated dihydroxy ester with the *cis* configuration at the double bond (Fig. 4).

Hydrogenation of the (+) and (-) isomers gave the corresponding (+) and (-)-*threo*-12,13-dihydroxystearic acids; mp 97.8–98 and 98.5°C; $[\alpha]_D^{27} +23.8$ and -23.4° (in ethanol). Anal.: calc'd.: C, 68.3%; H, 11.5%; found: C, 68.4%; H, 11.4% and C, 68.4%; H, 11.5%. A 1:1 mixture of the (+) and (-) dihydroxystearic acids, crystallized from ethyl acetate, melted at 98.5–99°C.

The analyses for (+) and (-)-*threo*-12,13-dihydroxyoleic acid and for the corresponding stearic acids agreed with those reported by Chisholm and Hopkins

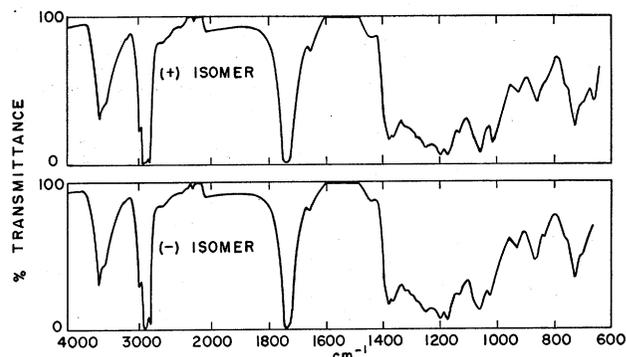


FIG. 4. Infrared spectra of methyl (+) and (-)-*threo*-12,13-dihydroxyoleate (CS₂, 1 mm cell).

(4) who showed that the hydroxyl groups were at the 12,13 positions and that the double bond in the unsaturated compound was at the 9,10 position (4,9).

The isolation of (+)-*threo*-12,13-dihydroxyoleic acid (I.V., 104.8; theory, 80.7) explains some of the results of the enzyme activation studies, especially the increase in iodine value, since it has been reported (10) that abnormally high values were obtained with some unsaturated hydroxy compounds.

These studies indicate that the conversion of epoxy to dihydroxy is due to an active hydrating principle, possibly enzymatic.

ACKNOWLEDGMENTS

GLC data by S. F. Herb and P. Magidman. TLC data by R. A. Barford. Drawings prepared by A. J. Menna. Photographic work by M. C. Audsley. Technical assistance by F. J. Oelshlegel, Jr., and D. B. Learn.

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[Received March 27, 1963—Accepted May 17, 1963]