ENZYMATIC HYDRATION OF cis-9,10-EPOXYOCTADECANOIC ACID BY CELL-FREE EXTRACTS OF GERMINATING FLAX RUST UREDOSPORES[1]

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Received January 23, 1963

Gas chromatographic analysis of the fatty acids from flax rust (Melampsora lini) uredospores (Frear, 1960; Tulloch and Ledingham, 1962) has shown that cis-9,10-epoxyoctadecanoic acid is the major fatty acid constituent. The flax rust uredospore lipids have also been shown to be rapidly metabolized upon the initiation of germination (Frear, 1960). The present report describes the results of experiments which indicate that the initial step in the metabolism of cis-9,10-epoxyoctadecanoic acid by germinating flax rust uredospores after hydrolysis of triglycerides is an enzymatic hydration with resulting inversion of configuration to form threo-9,10-dihydroxyoctadecanoic acid.

Materials and Methods

cis-9,10-Epoxyoctadecanoic and trans-9,10-epoxyoctadecanoic acids were prepared by the resin-peracetic acid treatment of oleic and elaidic acids, respectively (DuPont Bulletin P61-454).

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[1] Research supported in part by National Science Foundation Grant G-23635. Published with the approval of the Experiment Station director as Journal Article No. 25,

Uniformly C^{14}-labeled cis-9,10-epoxyoctadecanoic acid (2.4 x $10^{-3}\mu c/\mu$ mole) was prepared in a similar manner from uniformly labeled oleic acid (Nuclear Chicago Corp.). erythro-9,10-Dihydroxyoctadecanoic acid was prepared from oleic acid (Wiberg and Saegebarth, 1957), and threo-9,10-dihydroxyoctadecanoic acid was prepared from cis-9,10-epoxyoctadecanoic acid (Swern, 1948). After recrystallization, these acids were converted to methyl esters (Schlenk and Gellerman, 1960) for further purification by preparative gas or thin-layer chromatography. The epoxy and dihydroxy acids used were then obtained by saponification of the corresponding purified methyl esters (Hopkins and Chisholm, 1959). The melting points of the purified threo-9,10-dihydroxyoctadecanoic acid (m.p. 93°C), erythro-9,10-dihydroxyoctadecanoic acid (m.p. 131°C), cis-9,10-epoxyoctadecanoic acid (m.p. 55.5°C), and trans-9,10-epoxyoctadecanoic acid (m.p. 52°C) agree to within 1°C of reported values (Hopkins and Chisholm, 1959; Morris and Holman, 1961; Swern, 1952).

Flax rust uredospores were germinated at 16°C on demineralized water, lyophilized and stored in sealed glass vials at -17°C. Lyophilized spore mats were homogenized at 0-4°C with a glass tissue homogenizer and a homogenizing medium of 0.05 M phosphate buffer pH 7.0 (10 ml/g lyophilized spore mat). The resulting crude homogenate was centrifuged at 10,000 x g for 10 min. to remove whole spores and large cell fragments. The supernatant from this centrifugation was heated for 10 min. at 50°C, rapidly cooled and again centrifuged at 10,000 x g for 10 min. The supernatant from the heat treatment was 30% saturated with

ammonium sulfate and the precipitate removed by centrifuging at 20,000 x g for 15 min. The resulting supernatant was used for enzyme studies. The protein concentration of each fraction was determined (Lowry, Rosenbrough, Farr and Randall, 1951).

The enzyme reaction mixture consisted of 1 ml. of 0.002 M cis-9,10-epoxyoctadecanoic acid (2μ moles), 0.4 ml. of 0.1 M Tris buffer pH 7.0 (40 μ moles), 0.1 ml. of enzyme and demineralized water to a final volume of 1.5 ml. All enzyme reactions were run at 25°C. Enzymatic activity was stopped, and the fatty acids extracted with 6.0 ml. of 2:1 chloroform:methanol (Folch, Lees and Stanley, 1957). Boiled enzyme was used as a control.

Enzyme activity was followed qualitatively and quantitatively by the rate of substrate disappearance and product formation after the conversion of the reaction products to methyl esters (Schlenk and Gellerman, 1960), and their separation by thin-layer chromatography (Morris, Holman and Fontell, 1961).

In experiments where uniformly labeled cis-9,10-epoxyoctadecanoic acid was used, the radioactivity was located by autoradiographs of the thin-layer chromatograms and the activity of each spot determined quantitatively (Snyder and Stephens, 1962).

Results and Discussion

Thin-layer chromatograms of the methyl esters of the enzymatic reaction products demonstrated the presence of only one slow moving compound in addition to the faster moving unreacted substrate methyl ester. When radioactive substrate was used, only the same two spots showed any radioactivity. The migration of the slow moving compound corresponded to

that of known threo and erythro-9,10-dihydroxyoctadecanoic acid methyl esters.

The products of several 20 min. enzymatic reactions were pooled and recrystallized several times from carbon disulfide. The melting point of the recrystallized reaction product (92°C) corresponded to within 1°C of the value reported for threo-9,10-dihydroxyoctadecanoic acid (Hopkins and Chisholm, 1959). The melting point was unchanged by admixture with known threo-9,10-dihydroxyoctadecanoic acid.

The methyl esters of erythro-9,10-dihydroxyoctadecanoic acid and threo-9,10-dihydroxyoctadecanoic acid have recently been separated by differences in their ability to form borate complexes with thin-layer chromatography (Morris, 1962). Such a thin-layer chromatogram showing that the reaction product is threo-9,10-dihydroxyoctadecanoic acid rather than erythro-9,10-dihydroxyoctadecanoic acid is shown in Figure 1.

Radioactivity measurements of the rate of substrate disappearance and product formation as shown in Figure 2 demonstrate that cis-9,10-epoxyoctadecanoic acid is converted to threo-9,10-dihydroxyoctadecanoic acid and that no significant non-enzymatic hydration occurs during the course of the reaction with a boiled enzyme control.

A 3.3 fold purification of the enzyme, as shown in Table 1, has been achieved. The enzyme appears to be soluble and fairly heat stable.

Thin-layer chromatography studies have qualitatively shown that trans-9,10-epoxyoctadecanoic acid as well as the methyl esters

of both cis and trans-9,10-epoxyoctadecanoic acid are not readily hydrated by the partially purified enzyme.

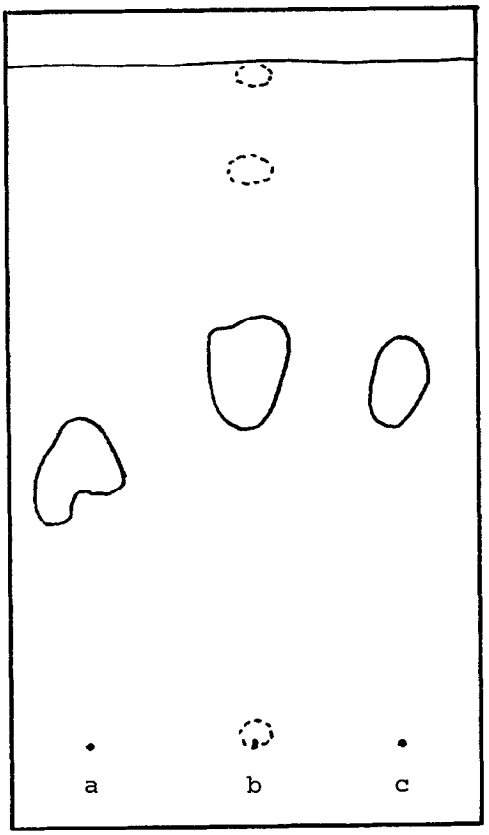

Figure 1. Identification of Reaction Product. a) methyl ester of known erythro-9,10-dihydroxyoctadecanoic acid; b) methyl esters of reaction products; c) methyl ester of known threo-9,10-dihydroxy-octadecanoic acid. The figure represents a tracing of an actual thin-layer chromatogram. Dotted lines indicate minor constituents.

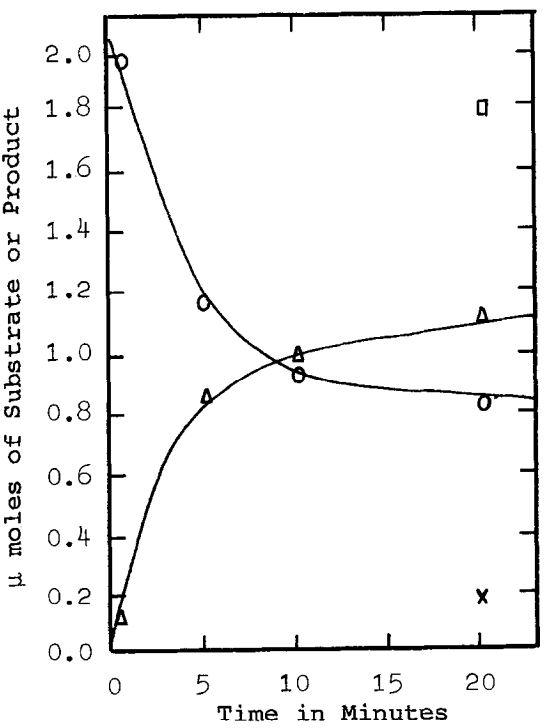

Figure 2. Rate of Enzymatic Hydration of cis-9,10-Epoxy-octadecanoic Acid and Formation of threo-9,10-Dihydroxy-octadecanoic Acid. O, cis-9,10-epoxyoctadecanoic acid; Δ, threo-9,10-dihydroxyoctadec-anoic acid; □, cis-9,10-epoxy-octadecanoic acid control; X, threo-9,10-dihydroxyoctadec-anoic acid control.

Table 1: Purification of cis-9,10-epoxyoctadecanoic acid hydrating enzyme from germinating flax rust uredospores.

Fractionation procedure	Activity (units[1]/ml)	Total units	Protein (mg/ml)	Specific Activity (units/mg protein)
Crude homogenate	22.8	111.7	9.8	2.3
Supernatant	6.6	51.5	2.4	2.7
Heat treated supernatant	6.8	36.0	1.8	3.9
30% $(NH_4)_2SO_4$ supernatant	6.0	27.0	0.8	7.8

[1] Unit of enzyme = amount that catalyzes the transformation of 1 μ mole of cis-9,10-epoxyoctadecanoic acid / min.

Autoradiographic analysis of thin-layer chromatograms from studies with uniformly labeled cis-9,10-epoxyoctadecanoic acid and crude cell-free homogenates indicate that the threo-9,10-dihydroxyoctadecanoic acid may be further metabolized to a monohydroxy acid.

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