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Specific Growth of a Soil Microorganism on the Natural Isomer of α-Tocopherol*

C. T. Goodhue†

ABSTRACT: A microorganism with the ability to metabolize *d*-α-tocopheryl acetate has been isolated from soil. This organism grows selectively on *d*-α-tocopheryl acetate in the presence of various other optical isomers of *d*-α-tocopheryl acetate. A growth test based on this property was developed in which the following mean responses were observed: *d*-α-tocopheryl acetate, 100%; 2*dl*-α-tocopheryl acetate, 48%; racemic α-tocopheryl acetate, 28%; *l*-α-tocopheryl acetate, 3.0%.

A rapid and sensitive method for determination of the optical isomers of α-tocopherol is needed. Assays based on biological functions such as gestation-resorption, encephalomalacia, and nutritional muscular dystrophy in rats and chicks respond selectively to *d*-α-tocopherol, the naturally occurring isomer, but these assays are rather time consuming. In these assays, the unnatural isomer, *l*-α-tocopherol is only about 20% as active as *d*-α-tocopherol (Ames *et al.*, 1963; Dam and Sondergaard, 1964; Scott and Desai, 1964; Witting and Horwitt, 1964). Presently available evidence suggests that the epimeric configuration at the 2 position[1] is dominant in determining biological activity in higher organisms (Ames *et al.*, 1963).

Using enrichment culturing methods in a medium containing *d*-α-tocopheryl acetate, we have obtained soil bacteria that grow selectively on *d*-α-tocopheryl acetate in the presence of other optical isomers of α-tocopheryl acetate. A diagnostic test for the presence of *d*-α-tocopherol based on this property has been developed in which the calculated weight of bacteria at maximum growth is proportional to the amount of *d*-α-tocopheryl acetate initially in the culture medium. This relationship holds true even in the presence of optical isomers of α-tocopherol not found in nature.

Experimental Methods

Isolation of Bacteria. Conventional enrichment culturing methods were used (Hayaishi, 1955). Mud samples from a local pond were incubated without shaking at 30° in a medium containing, per liter, 0.35 g KH_2PO_4, 2.0 g $(NH_2)_2SO_4$, 1.0 g *d*-α-tocopheryl acetate, and 10.0 ml mineral salt solution. The *p*H value of the medium was adjusted to 7.0 with KOH. At first, the α-tocopheryl acetate was suspended in acacia. Later this was found to be unnecessary. The mineral salt solution contained, per 100 ml, 2.5 g $MgSO_4 \cdot 7\ H_2O$, 0.01 g $CaCl_2 \cdot 2\ H_2O$, 0.28 g $FeSO_4 \cdot 7\ H_2O$, 0.17 g $MnSO_4 \cdot H_2O$, and 0.006 g $ZnSO_4$. After 1 week, a portion of the culture was used to inoculate shake flasks containing the same medium. After three transfers at 1-week intervals, a pure culture was isolated on agar plates of the same medium.

The organism was an acid-fast, nonmotile rod (usually paired) averaging 0.3 × 1 μ after cultivation 4

* From the Research Laboratories of Distillation Products Industries, Division of Eastman Kodak Company, Rochester, N.Y. *Received April 30, 1965.* Communication No. 323.

† Present address: Research Laboratories, Eastman Kodak Company, Rochester, N.Y. 14650.

[1] We will use the following designations for configuration of the optically active centers of α-tocopherol:

Trivial	Configuration
d-α-tocopherol	2D,4′D,8′D-α-tocopherol
l-α-tocopherol	2L,4′D,8′D-α-tocopherol
2*dl*-α-tocopherol	2DL,4′D,8′D-α-tocopherol
racemic α-tocopherol	2DL,4′DL,8′DL-α-tocopherol

The configurations of the optically active centers of *d*-α-tocopherol were established by Mayer *et al.* (1963).

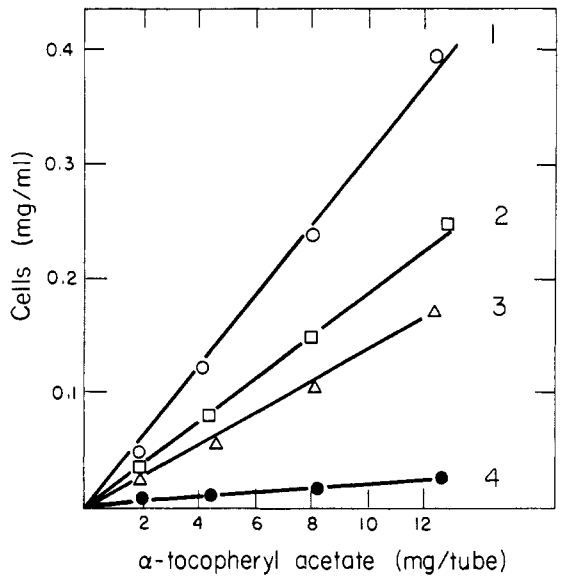

FIGURE 1: Growth response of isolate A-7 to optical isomers of α-tocopheryl acetate. Growth conditions are described in the text. Curve 1, *d*-α-tocopheryl acetate. Curve 2, 2*dl*-α-tocopheryl acetate. Curve 3, racemic α-tocopheryl acetate. Curve 4, *l*-α-tocopheryl acetate.

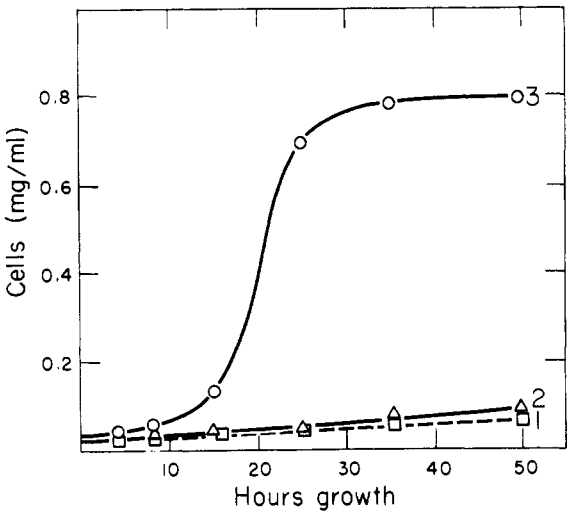

FIGURE 2: Nonadaptability of isolate A-7 to *l*-α-tocopheryl acetate. Growth conditions are described in the text. Curve 1, growth on *d*-α-tocopheryl acetate. Curve 2, growth on *l*-tocopheryl acetate. Curve 3, growth of organisms that previously have been grown 6 days on *l*-α-tocopheryl acetate and then used to inoculate a fresh medium of *l*-α-tocopheryl acetate.

days on the above-mentioned medium. The organism showed no anaerobic growth in a nutrient agar stab. It did not hydrolyze gelatin; it did not reduce sulfur or produce indole. On the basis of these tests, we have tentatively assigned the organism to the genus *Mycobacterium* (Breed *et al.*, 1957). The organism will be referred to subsequently as "isolate A-7."

Growth Measurements. To obtain an inoculum essentially free of α-tocopherol or α-tocopheryl acetate, isolate A-7 was grown 4 days in the usual medium containing 12 mg *d*-α-tocopheryl acetate per ml. The culture was shaken at 200 rpm on a New Brunswick Model G-10 Gyratory Shaker at 30°.

Measurements were made in 125-ml Erlenmeyer flasks with test tube side arms. The culture flasks contained 10 ml of 3% agar gel (as a plate at the bottom of the flask) and 30 ml of the mineral salts medium. The agar promotes uniform growth on the oily substrate. The sterile salts medium was added to sterile flasks containing the agar gel. At this point, turbidimetric readings at 650 mμ were obtained for all flasks. Optical isomers of α-tocopheryl acetate were added in purified pentane (distilled from zinc and KOH) solution (2 mg ml). The amount of α-tocopheryl acetate was varied from 0 to 12 mg per flask. Pentane was removed by evaporation. Each flask then was inoculated with 0.2 ml of the inoculum culture and incubated exactly 48 hours at 30° with shaking at 200 rpm. At the end of the incubation period, turbidimetric measurements at 650 mμ were obtained. The following controls were run for each optical isomer or mixture of isomers tested: (1) no α-tocopheryl acetate, inoculated; (2) α-tocopheryl acetate (12 mg/ml), no inoculation. All values were obtained in triplicate and averaged. Control values were subtracted and the adjusted optical density values at 650 mμ were converted to cellular dry weight per ml by means of a standard curve. Relative activities of the various mixtures of optical isomers were determined as ratios of the slopes of growth response curves.

Growth curves were determined in media containing 20 mg per ml α-tocopheryl acetate. Inocula were obtained as described before.

Chromatography. The purity of the α-tocopheryl acetate samples was determined by thin-layer chromatography on silica gel G plates, 0.25 mm thick. The plates were developed in cyclohexane–ethyl ether (70:20) and sprayed with dilute H_2SO_4. Spots became visible after heating several minutes at 130°. The R_F values of α-tocopheryl acetate varied from 0.65 to 0.70. Samples that contained impurities were chromatographed on columns of Florisil (magnesium aluminum silicate) by elution with hexane–ethyl ether (95:5).

Results and Discussion

The results of a typical assay are shown in Figure 1. Between 0 and 12 mg α-tocopheryl acetate per ml, the weight of cells produced was directly proportional to the weight of substrate present. The responses of isolate A-7 to various optical isomers and mixtures of optical isomers are summarized in Table I. It is seen that, if the response to *d*-α-tocopheryl acetate is set at 100%, the response to *l*-α-tocopheryl acetate was not significantly different from zero, while the response to 2*dl*-α-tocopheryl acetate was approximately 50%. Therefore, the

TABLE I: Relative Response of Isolate A-7 to Optical Isomers of α-Tocopheryl Acetate.

Optical Isomer(s)	Response Mean[a]	Std. Error of Mean
d-α-Tocopheryl acetate	100	
2*dl*-α-Tocopheryl acetate	48	4.2
Racemic *d*-α-tocopheryl acetate	28	3.3
l-α-Tocopheryl acetate	3.0	2.5

[a] Five experiments.

presence of the 2*l* epimer does not appreciably affect growth on the 2*d* epimer. Furthermore, there was no significant growth on *l*-α-tocopheryl acetate even at 144 hours. This indicates that isolate A-7 is very sensitive to changes in configuration at the 2 position.

That isolate A-7 is also sensitive to changes in configuration at the 4′ and 8′ positions is indicated by the differences in response to the 2*dl*-α-tocopheryl acetate (2DL,4′D,8′D configuration) and to racemic α-tocopheryl acetate (2DL,4′DL,8′DL configuration). The response to racemic α-tocopheryl acetate (28%) was considerably less than 50%. If the optical specificity of isolate A-7 were restricted only to position 2, the response to the above-mentioned two isomeric mixtures would be identical since both mixtures contain an equal proportion of components with the 2D configuration.

It should be emphasized that the specificity of this test is an expression of the stereoselective growth of isolate A-7 on *d*-α-tocopheryl acetate. While the organism grows on substrates such as acetate and glucose, it does not adapt to growth on *l*-α-tocopheryl acetate (Figure 2). After growth for 6 days in a medium containing *l*-α-tocopheryl acetate (20 mg/ml), the cell yield reaches approximately 0.2 mg/ml. This is in contrast to a cell yield of 0.8 mg/ml obtained by growth for only 3 days on the same concentration of *d*-α-tocopheryl acetate. Because the organism can grow on substrates other than *d*-α-tocopheryl acetate, this test is practical only when samples of pure α-tocopheryl acetate are available.

The selective growth of isolate A-7 on *d*-α-tocopheryl acetate (versus other optical isomers) is probably due to the stereoselectivity of the enzymes that catalyze the degradation of α-tocopheryl acetate. By the use of these purified enzymes, it should be possible to develop an assay that is specific for *d*-α-tocopherol even in the presence of interfering substances such as might be found in natural products.

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