Flavin-Linked Dehydrogenation of Ether Glycols by Cell-Free Extracts of a Soil Bacterium

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ABSTRACT

PAYNE, W. J. (University of Georgia, Athens), AND R. L. TODD. Flavin-linked dehydrogenation of ether glycols by cell-free extracts of a soil bacterium. *J. Bacteriol.* 91:1533–1536. 1966.—Cell-free extracts of bacterium TEG-5 grown on tetraethylene glycol dehydrogenated a variety of ether glycols and nonylphenoxy and secondary alcohol ethoxy derivatives. Nicotinamide nucleotides did not serve as electron acceptors, but ferricyanide was effective. Dialysis of crude extract depressed activity with tetraethylene glycol, which was restored then by flavine adenine dinucleotide (FAD) or boiled extract supernatant fluid (BES) but not by other flavins. Precipitation of extract protein at pH 4.0 at 80% ammonium sulfate saturation dissociated FAD and yielded an inactive fraction. Activity was restorable by FAD and BES but not by other flavins. Ethylene glycol was not dehydrogenated by the acid ammonium sulfate fraction with FAD. Atabrine inhibited tetraethylene glycol oxidation, and the inhibition was relieved by FAD but not by other flavins. Tergitols which have sulfated ethoxy side chains on secondary alcohols were not dehydrogenated, but those with free ethoxy side chains on identical alcohols were.

Fincher and Payne (3) isolated from soil enriched with triethylene glycol a bacterium which grows on a variety of ether glycols as the sole carbon source. This aerobic, nonsporeforming, gram-negative rod has not been classified despite considerable study, but it may be considered a member of the *Pseudomonas-Achromobacter* group, and has been designated TEG-5. Payne (6) later observed that sonic extracts of cells grown on tetraethylene glycol dehydrogenated a number of ether glycols by coupling the dehydrogenation to ferricyanide reduction.

This paper presents the results of experiments designed to demonstrate that (i) ether glycols attached to secondary alcohols or phenoxy compounds are substrates for dehydrogenation, (ii) dehydrogenation of ether glycols is accomplished by a flavoprotein, and (iii) the terminal hydroxyl group on the ether glycol must be uncovered for catabolism to be initiated.

MATERIALS AND METHODS

Cell-crops of bacterium TEG-5 were obtained by culturing on tetraethylene glycol minimal medium as previously described (3). Cell-free extracts were prepared by alumina grinding [frozen cells, 1.0 g; levigated alumina, 2.5 g; 0.01 M tris(hydroxymethyl)-aminomethane (Tris) buffer (pH 8.0), 2.0 ml] and

centrifugation in the cold. Dissociation of flavin was accomplished by precipitation of protein from crude extract at pH 4.0 with 80% ammonium sulfate saturation. The precipitate was suspended in 0.04 M phosphate at pH 7.0; but unlike the enzymes in the preparations used by Benziman and Galanter (1), which were stable through three successive precipitations, activity was greatly diminished by more than one precipitation of enzymes in the TEG-5 extracts. After acid ammonium sulfate precipitation, the extracts were most stable and active when stored at pH 7.0 in phosphate buffer.

Assays were made of the ability of riboflavine (RF), flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), and the supernatant liquid from boiled extract (BES) to restore the capacity for ferricyanide reduction to the acid ammonium sulfate fraction. BES was prepared as described by Benziman and Galanter (1). The manometric assay for inhibition by Atabrine and its reversal by FAD with phenazine methosulfate (PMS) as the intermediate electron carrier was also performed as described by those authors.

Ether glycol substrates used in these studies included di-, tri-, and tetraethylene glycol, polyethylene glycol (PEG) 200, PEG 300, 400, 600, 1,000, and 4,000 (these figures indicate the average molecular weights), nonylphenoxy ethylene glycols (with 10 to 20 repeating ethoxy units), and dipropylene polyethylene glycols (with 10 to 20 repeating ethoxy units), all provided by Jefferson Chemical Co., Austin, Tex. Tergitol 15-S-3S and 15-S-9 were provided by the

Union Carbide Co., South Charleston, W.Va. These Tergitols are mixtures of linear, C_{11} to C_{15} secondary alcohols with either three- or nine-unit ethoxy side chains. Those with the three-unit ethoxy side chain (15-S-3S) are esterified with sodium sulfate at the terminal hydroxyl group. Ethylene glycol was provided by Jefferson Chemical Co. The chemical structures of the significant substrates used in these studies are:

where $\text{R}-\text{CH}-\text{R}' = C_{11}$ to C_{15} .

RF was obtained from Eastman Organic Chemicals, Rochester, N.Y. FMN, FAD, and PMS were purchased from Sigma Chemical Co., St. Louis, Mo., and Atabrine from the Winthrop Chemical Co., New York, N.Y.

RESULTS

Di-, tri-, and tetraethylene glycols and the straight-chained PEG preparations up to that with an average molecular weight of 600 were rapidly dehydrogenated by crude, cell-free extracts as previously noted (6). The nonylphenoxy and dipropylene derivatives were dehydrogenated as well, but less rapidly. Di-, tri-, and tetraethylene glycols were catabolized at essentially identical rates, six- to eightfold faster

than any of the derivatives. Nicotinamide nucleotide coenzymes were not reduced, but ferricyanide served well as an electron acceptor, with the use of crude extract dialysed for 3 hr against 0.01 M Tris (pH 8.0). If dialysis of the extracts was continued until approximately one-third the activity of the crude extract was lost, FAD or BES, but not RF or FMN, restored the activity (Fig. 1). Further dialysis yielded systems not responsive to added FAD or BES.

Acid ammonium sulfate precipitation, however, yielded a fraction with very little activity with tetraethylene glycol as the electron donor and ferricyanide the acceptor. FAD restored activity in proportion to the quantity provided (Fig. 2), but RF and FMN were inactive. BES restored

FIG. 1. Restoration by FAD and BES of ferricyanide-linked dehydrogenation of tetraethylene glycol by a dialyzed, cell-free extract. Complete reaction mixtures contained: Tris buffer (pH 8.0), 300 μ moles; tetraethylene glycol, 10 mg; $\text{Fe}(\text{CN})_6^{3-}$, 6 μ moles; KCN, 10 μ moles; (X) crude extract, 0.2 ml; (●) dialyzed extract, 0.2 ml; (Δ) dialyzed extract plus BES, 0.2 ml; (○) dialyzed extract plus FAD, 0.06 μ mole; and water to bring the final volume to 3.0 ml. KCN stimulates ferricyanide reduction in this system, perhaps by complexing an inhibitory free metal or diverting electron flow from cytochrome oxidase to the artificial acceptor as suggested by Webb (7).

FIG. 2. Proportional restoration by FAD of ferricyanide-linked dehydrogenation of tetraethylene glycol by an acid ammonium sulfate fraction precipitated from cell-free extract. Complete reaction mixtures contained: (Δ) crude extract system as in Fig. 1; ($\circ$) phosphate buffer (pH 7.0), 300 μ moles; extract protein, 8 mg; tetraethylene glycol, 10 mg; $\text{Fe}(\text{CN})_6^{3-}$, 6 μ moles; KCN, 10 μ moles; FAD, 0.06 μ mole; and water to 3.0 ml; ($\bullet$) same with only 0.03 μ mole of FAD; ($\times$) same with no FAD or with either FMN or RF, 0.06 μ mole.

activity as well and in proportion to the quantity provided. The presence of FAD (and FMN) in the BES was readily demonstrated chromatographically.

Ethylene glycol was dehydrogenated slowly by crude extracts with ferricyanide as the electron acceptor. The acid ammonium sulfate fraction did not dehydrogenate ethylene glycol, however, with or without FAD.

Inclusion of 1.0 μ mole/ml of Atabrine in reaction mixtures containing PMS inhibited by 32% the ability of a crude, dialyzed extract to oxidize tetraethylene glycol. FAD overcame this effect and enhanced the activity by 16% when 0.067 μ mole/ml was provided and 33% when 0.2 μ mole/ml was supplied (Table 1). RF and FMN did not relieve inhibition.

Apparently, the terminal hydroxyl group on an ethoxy chain must be free for metabolism by the enzymes in TEG-5 extracts to take place (Fig. 3). A mixture of compounds consisting of secondary alcohols linked through their hydroxyl groups to nine-unit ethoxy chains were substrates for dehydrogenation, but such a mixture of similar compounds with sulfated three-unit ethoxy chains attached were not. The only significant difference in these preparations as far as the enzy-

TABLE 1. Reversal by FAD of Atabrine inhibition of PMS-linked oxygen uptake

Addition	Effect
None	0
Atabrine, 1.0 μ mole/ml	32% inhibition
Atabrine + FAD, 0.067 μ mole/ml	16% stimulation
FAD, 0.067 μ mole/ml	30% stimulation
Atabrine + FAD, 0.2 μ mole/ml	33% stimulation
FAD, 0.2 μ mole/ml	45% stimulation

FIG. 3. Suppression of ferricyanide-linked dehydrogenation of ethoxy compounds by the presence of a sulfate esterified to the terminal hydroxyl group. Complete reaction mixtures contained: Tris buffer (pH 8.0), 300 μ moles; crude extract, 0.2 ml; appropriate Tergitol, 20 mg; $\text{Fe}(\text{CN})_6^{3-}$, 6 μ moles; KCN, 10 μ moles; and water to bring the final volume to 3.0 ml.

matic activity is concerned is the presence of the sulfate on the terminal hydroxyl group.

DISCUSSION

Results presented here satisfy several criteria for designating as a flavoprotein the dehydrogenase produced by growing bacterium TEG-5 on tetraethylene glycol. Dissociation of activity by acid ammonium sulfate precipitation and its restoration by FAD or FAD-containing BES, but not by RF or FMN, supports that conclusion. Moreover, the specific alleviation of the inhibitory effects of Atabrine by FAD are consistent with the hypothesis that our system is a flavoprotein.

The partial dissociation of FAD by dialysis that we observed is analogous to the partial removal by phosphate washing of FAD from the

malic dehydrogenase of particles from *Mycobacterium avium* experienced by Kimura and Tobari (4).

That the presence of a sulfate ester on the terminal hydroxyl group of the ethoxy chain attached to linear secondary alcohols of the Tergitol 15-S-3S prevented catabolism suggests that this carbinol is a site for enzyme attachment. Past experience by many workers with FAD-linked dehydrogenation of various substrates would not favor the possibility than an "alcohol dehydrogenase" type reaction occurs at the hydroxyl on the terminal ethoxy group, since this type of reaction is more likely to be nicotinamide adenine dinucleotide-linked. The product of this dehydrogenation is, of course, not yet known; nor is there any body of information available now concerning the metabolic products of dissimilation of ether compounds. MacRae and Alexander (5) reported the cleavage by a *Flavobacterium* of the ether bond in 2,4-dichlorophenoxyalkyl carboxylic acids, and suggested either reductive or hydrolytic cleavage. We have not yet designed experiments to determine whether there is cleavage of the ether bonds before dehydrogenation. Ethylene glycol, which might be postulated as an intermediate, is not metabolized by the flavoprotein dehydrogenase in extracts of TEG-5. De Ley and Kersters (2) reported that extracts of acetic acid bacteria oxidized ethylene, diethylene, and triethylene glycols, as well as a great many other glycols. The alcohol dehydrogenases involved appeared to be linked to cytochrome 553 without participa-

tion of nicotinamide, flavin, ubiquinone, or free metal cofactors.

Projected studies with a variety of trapping and analytical techniques may provide us the needed identity of the initial product of dehydrogenation.

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