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# Fermentation of 1,2-Propanediol and 1,2-Ethanediol by Some Genera of *Enterobacteriaceae*, Involving Coenzyme $B_{12}$-Dependent Diol Dehydratase

TETSUO TORAYA,* SUSUMU HONDA, AND SABURO FUKUI

*Laboratory of Industrial Biochemistry, Department of Industrial Chemistry, Faculty of Engineering, Kyoto University, Sakyo-Ku, Kyoto 606, Japan*

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*Klebsiella pneumoniae* (*Aerobacter aerogenes*) ATCC 8724 was able to grow anaerobically on 1,2-propanediol and 1,2-ethanediol as carbon and energy sources. Whole cells of the bacterium grown anaerobically on 1,2-propanediol or on glycerol catalyzed conversion of 1,2-diols and aldehydes to the corresponding acids and alcohols. Glucose-grown cells also converted aldehydes, but not 1,2-diols, to acids and alcohols. The presence of activities of coenzyme $B_{12}$-dependent diol dehydratase, alcohol dehydrogenase, coenzyme-A-dependent aldehyde dehydrogenase, phosphotransacetylase, and acetate kinase was demonstrated with crude extracts of 1,2-propanediol-grown cells. The dependence of the levels of these enzymes on growth substrates, together with cofactor requirements in in vitro conversion of these substrates, indicates that 1,2-diols are fermented to the corresponding acids and alcohols via aldehydes, acyl-coenzyme A, and acyl phosphates. This metabolic pathway for 1,2-diol fermentation was also suggested in some other genera of *Enterobacteriaceae* which were able to grow anaerobically on 1,2-propanediol. When the bacteria were cultivated in a 1,2-propanediol medium not supplemented with cobalt ion, the coenzyme $B_{12}$-dependent conversion of 1,2-diols to aldehydes was the rate-limiting step in this fermentation. This was because the intracellular concentration of coenzyme $B_{12}$ was very low in the cells grown in cobalt-deficient medium, since the apoprotein of diol dehydratase was markedly induced in the cells grown in the 1,2-propanediol medium. Better cell yields were obtained when the bacteria were grown anaerobically on 1,2-propanediol. Evidence is presented that aerobically grown cells have a different metabolic pathway for utilizing 1,2-propanediol.

Several papers have appeared concerning the metabolism of 1,2-propanediol in animals (14, 18), yeast (9), and bacteria (1, 6, 7, 10, 15, 19, 23, 28). The mechanism of its anaerobic metabolism is of great interest, since 1,2-propanediol is a highly reduced substrate compared with carbohydrates. Gaston and Stadtman have reported that whole cells of *Clostridium glycolicum*, which are capable of growing anaerobically on 1,2-ethanediol (or 1,2-propanediol) but not on glycerol, convert these 1,2-diols to the corresponding acids and alcohols by the following equation (6): 2 R—CH(OH)$CH_2$OH → R—$CH_2$COOH + R—$CH_2CH_2$OH + $H_2O$, where R = H or $CH_3$. Recently, Ichikawa et al. have reported that *Propionibacterium freudenreichii* is able to grow on 1,2-propanediol (or 1,2-ethanediol) under anaerobic conditions, forming propionic acid as an end product, with a yield of 0.5 mol/mol of 1,2-propanediol (10). Abeles et al. have described that cell suspensions of *Aerobacter aerogenes* (now known as *Klebsiella pneumoniae*) ATCC 8724 grown anaerobically on glycerol convert 1,2-propanediol to propionaldehyde, 1,2-ethanediol to acetaldehyde, and glycerol to β-hydroxypropionaldehyde (1). They isolated a coenzyme $B_{12}$ {adenosylcobalamin or Coα-[α-(5,6-dimethylbenzimidazolyl)]-Coβ-adenosylcobamide}-requiring enzyme responsible for the following dehydrations: R—CH(OH)$CH_2$OH → R—$CH_2$CHO + $H_2O$, where R = $CH_3$, H, or $HOCH_2$. They designated this enzyme dioldehydrase (diol dehydratase or DL-1,2-propanediol hydro-lyase [EC 4.2.1.28]) (2, 11).

To clarify the pathway of anaerobic fermentation of 1,2-diols, we have examined several genera of *Enterobacteriaceae* for their ability to grow anaerobically on 1,2-propanediol and 1,2-ethanediol. We found that several bacteria of this family are able to ferment these 1,2-diols under anaerobic conditions (T. Toraya, S. Kuno,

and S. Fukui, unpublished data). The presence of coenzyme $B_{12}$-dependent diol dehydratase in the extracts of these bacteria grown on 1,2-propanediol was also demonstrated (24). When *K. pneumoniae* ATCC 8724 was cultivated anaerobically in media supplemented with $CoSO_4$ (1 mg/liter of medium), the bacterium synthesized more vitamin $B_{12}$ in a glycerol–1,2-propanediol medium (90 μg/g of dry cells) than in a glycerol medium (35 μg/g of dry cells) or in a glucose medium (7 μg/g of dry cells) (T. Toraya, S. Honda, and S. Fukui, unpublished data). When grown aerobically, the bacterium produced much less vitamin $B_{12}$ (less than 5 μg/g of dry cells) in all three media. These results also suggest that coenzyme $B_{12}$-dependent diol dehydratase is involved in the anaerobic metabolism of 1,2-propanediol. This paper reports the pathway of anaerobic metabolism of 1,2-diols in these bacteria, with special emphasis on evidence for the involvement of diol dehydratase in an initial step of the fermentation. The presence of activities of other enzymes involved are also demonstrated here with cell-free extracts of *K. pneumoniae* (*A. aerogenes*) ATCC 8724.

## MATERIALS AND METHODS

**Chemicals.** Propionyl phosphate was prepared by reaction of $K_2HPO_4$ with propionic anhydride by the procedure described by Stadtman for the preparation of acetyl phosphate (21). All other chemicals were obtained from commercial sources and were used without further purification. Acetyl phosphate (Sigma Chemical Co.) and propionyl phosphate were assayed colorimetrically by the method of Lipmann and Tuttle (12).

**Bacteria and growth.** Bacterial strains were obtained from the American Type Culture Collection (*K. pneumoniae* ATCC 8724 and 13882) and the Faculty of Agriculture, Kyoto University, Kyoto, Japan (*Citrobacter freundii* AKU 0009 and *Citrobacter intermedium* AKU 0010). Bacteria were grown statically (without aeration) at 30 or 37°C in a complex medium containing 5.4 g of $KH_2PO_4$, 1.2 g of $(NH_4)_2SO_4$, 0.4 g of $MgSO_4 \cdot 7H_2O$, 2.0 g of yeast extract, 2.0 g of tryptone, and an appropriate growth substrate (7.6 g of 1,2-propanediol, 9.2 g of glycerol, or 9.0 g of glucose) in 1 liter of tap water (25). The medium was adjusted to pH 7.1 with KOH. Cells harvested in the late exponential phase were used for the fermentation balance experiments and enzyme activity measurements.

**Fermentation balance studies.** The harvested cells were washed twice with 0.1 M potassium phosphate buffer (pH 7.1). For fermentation balance experiments with whole bacteria, the cells were suspended in 10 ml of the same buffer to a cell concentration of about 10 mg of dry cells per ml and incubated at 37°C for 1 h with 1.0 mmol of an appropriate substrate (final concentration, 0.1 M) under a He atmosphere. The cells were removed by centrifugation, and the products formed were analyzed with a supernatant solution, as described below.

**Test of the pathways of 1,2-propanediol and 1,2-ethanediol fermentations with cell-free extracts.** A complete reaction mixture was composed of 20 μmol of an appropriate 1,2-diol or aldehyde (final concentration, 10 mM), 20 μM coenzyme $B_{12}$, 0.5 mM NAD, 10 mM ADP, 10 mM $MgCl_2$, 0.05 mM coenzyme A (CoA), 0.04 M potassium phosphate buffer (pH 7.5), and crude extracts containing 5 mg of protein, in a total volume of 2 ml. The mixture was incubated at 30°C for 1 h under a He atmosphere. The reactions were terminated by adding 3 ml of 0.1 N HCl, and the products formed were analyzed as follows.

**Analyses of the fermentation products.** Propionaldehyde and acetaldehyde were determined colorimetrically by the 3-methyl-2-benzothiazolinone hydrazone method (26). To assay propionate and acetate, a small sample of the medium was evaporated to dryness in vacuo under alkaline conditions, acidified with concentrated HCl, and extracted successively with diethyl ether. The amount of propionic acid and acetic acid in the ether extracts was determined by gas chromatography with a Yanagimoto model GCG-550T gas chromatograph equipped with a hydrogen flame ionization detector and a stainless steel column (0.3 by 225 cm) of 15% diethylene glycol succinate polyester on Neopak AS (60 to 80 mesh; Nishio Kogyo Co., Ltd., Tokyo, Japan). Column temperature and the flow rate of a carrier gas ($N_2$) were kept at 100°C and 30 ml/min, respectively. Under the conditions, these two acids were well separated. 1,2-Propanediol and 1,2-ethanediol were estimated with diol dehydratase by the method of Toraya et al. (26). *n*-Propanol and ethanol were determined spectrophotometrically by measuring the increase in the absorbance at 340 nm (formation of NADH) after reaction with yeast alcohol dehydrogenase (EC 1.1.1.1) and NAD (3).

**Preparation of cell-free extracts.** Late-exponential-phase cells were washed twice with 0.05 M potassium phosphate buffer (pH 8.0) and disrupted in the same buffer by sonication at 20 kHz for 5 min with a ultrasonic disintegrator (Kaijo Denki Co., Ltd., Tokyo, Japan). The resulting homogenate was centrifuged for 30 min at 20,000 × *g*, and the supernatant solution was used as a crude extract after overnight dialysis against 500 volumes of 0.05 M potassium phosphate buffer (pH 8.0). Protein concentration was determined by the procedure of Lowry et al. (13), with crystalline bovine serum albumin as standard.

**Determination of enzymatic activities in crude extracts.** Diol dehydratase (diol dehydrase or DL-1,2-propanediol hydro-lyase [EC 4.2.1.28]) was assayed by the 3-methyl-2-benzothiazolinone hydrazone method, as described previously (25, 26). Alcohol dehydrogenase activity (alcohol:NAD oxidoreductase [EC 1.1.1.1]) of the forward reaction was determined spectrophotometrically by measuring the change in absorbance at 340 nm. The reaction mixture was composed of 0.1 M alcohol substrate, 1 mM NAD (or NADP), 0.048 M potassium phosphate buffer (pH 8.0), and an appropriate amount of crude extracts in a total volume of 3.0 ml. The activity of the backward reaction was determined by the same procedure, except that 0.2 mM NADH (or NADPH) and 0.03 M aldehyde substrate were used instead of NAD and alcohol, respectively. The activities of CoA-dependent alde-

hyde dehydrogenase (aldehyde dehydrogenase [acylating] or aldehyde:NAD oxidoreductase [acylating CoA]; EC 1.2.1.10), phosphotransacetylase (phosphate acetyltransferase or acetyl-CoA:orthophosphate acetyltransferase [EC 2.3.1.8]), and acetate kinase (ATP: acetate phosphotransferase [EC 2.7.2.1]) were assayed by the procedures of Stadtman and Burton (5, 22), Stadtman (20), and Rose et al. (16, 17), respectively. All of the enzyme activities described above were determined with both two-carbon (C2) and three-carbon (C3) substrates, since these C2 and C3 compounds were assumed to be involved as intermediary metabolites in the pathways of 1,2-ethanediol and 1,2-propanediol fermentations, respectively.

## RESULTS

**Fermentation balance study with whole cells of *K. pneumoniae* ATCC 8724.** Table 1 summarizes the fermentation products formed from 1,2-diols and aldehydes by cell suspensions of *K. pneumoniae* ATCC 8724 which were cultivated in the three different growth substrates. The capability to ferment 1,2-diols was dependent on the prior growth history of the cells. 1,2-Propanediol-grown cells were able to convert 1,2-propanediol and 1,2-ethanediol to the corresponding acids and alcohols essentially quantitatively under a He atmosphere. A trace amount of the corresponding aldehydes was also detected. 1,2-Propanediol and 1,2-ethanediol are in the same oxidation states as propionaldehyde and acetaldehyde, respectively. Therefore, oxidation-reduction balance for this fermentation could be calculated to be approximately 1. This indicated that 1,2-diols or their metabolites undergo dismutation to give acids and alcohols as terminal products, with a yield of 0.5 mol of each product per mol of substrate consumed. Of course, it is impossible that 1,2-diols themselves are converted to acids and alcohols. Since propionaldehyde and acetaldehyde were also utilized by the cells to give the same fermentation products (Table 1), aldehydes would be the most likely candidates for this dismutation. Since this bacterium produces diol dehydratase (11, 25), a coenzyme $B_{12}$-requiring enzyme which catalyzes the conversion of 1,2-propanediol to propionaldehyde and the conversion of 1,2-ethanediol to acetaldehyde, it seems reasonable to assume that the diol dehydratase reaction is involved in the 1,2-propanediol and 1,2-ethanediol fermentations as the initial step and that both 1,2-diols are fermented by the common systems. The bacterial cells grown on glycerol were also capable of fermenting 1,2-propanediol, but those grown on glucose could not. These data indicated that the ability of cells to ferment 1,2-diols is dependent on the presence of diol dehydratase in the cells.

On the other hand, the capability to ferment aldehydes was independent of the prior growth history of the cells. This result suggested that the enzymes involved in the dismutation of these aldehydes are not inducible but rather constitutive.

**Pathway of 1,2-propanediol and 1,2-ethanediol fermentation.** The metabolism of 1,2-diols and aldehydes was tested under anaerobic conditions with cell-free extracts of *K. pneumoniae* ATCC 8724 grown on 1,2-propanediol. Cofactor requirements in the conversion of these substrates are shown in Table 2. The crude

TABLE 1. *Fermentation of 1,2-diols and aldehydes with whole cells of K. pneumoniae ATCC 8724*

| Cells grown with:[a] | Substrate | Amt of substrate consumed (mmol) | Amt of products formed (mmol) | | | Carbon recovery (%) | O/R balance[e] |
|---|---|---|---|---|---|---|---|
| | | | Acid[b] | Aldehyde[c] | Alcohol[d] | | |
| 1,2-Propanediol | 1,2-Propanediol | 0.99 | 0.51 (C3) | 0.00 (C3) | 0.50 (C3) | 102 | 1.02 |
| | 1,2-Ethanediol | 0.99 | 0.44 (C2) | 0.00 (C2) | 0.47 (C2) | 92 | 0.94 |
| | Propionaldehyde | 0.98 | 0.46 (C3) | | 0.45 (C3) | 93 | 1.02 |
| | Acetaldehyde | 1.00 | 0.49 (C2) | | 0.51 (C2) | 100 | 0.96 |
| Glycerol | 1,2-Propanediol | 0.61 | 0.27 (C3) | 0.01 (C3) | 0.22 (C3) | 82 | 1.23 |
| | Propionaldehyde | 0.92 | 0.42 (C3) | | 0.42 (C3) | 91 | 1.00 |
| Glucose | 1,2-Propanediol | 0.00 | 0.00 (C3) | 0.00 (C3) | 0.00 (C3) | | |
| | Propionaldehyde | 0.67 | 0.31 (C3) | | 0.32 (C3) | 94 | 0.97 |

[a] Glycerol-grown cells were washed twice with 0.9% KCl and inoculated into fresh media at an initial concentration of about 0.002 g of dry cells per liter. The maximal growth yields of the bacterium in complex media supplemented with 1,2-propanediol, glycerol, and glucose under anaerobic conditions were 0.18, 0.44, and 0.51 g of dry cells per liter, respectively. The growth in the medium without supplementation was 0.07 g of dry cells per liter.

[b] In this column C3 = propionic acid and C2 = acetic acid.

[c] In this column C3 = propionaldehyde and C2 = acetaldehyde.

[d] In this column C3 = *n*-propanol and C2 = ethanol.

[e] O/R balance, Oxidation-reduction balance.

TABLE 2. *Cofactor requirements of the metabolism of 1,2-diols and aldehydes in cell-free extracts of K. pneumoniae ATCC 8724 grown anaerobically on 1,2-propanediol*

| Substrate | Reaction mixture | Amt of substrate consumed (μmol) | Amt of products formed (μmol) | | | Carbon recovery (%) | O/R balance[d] |
|---|---|---|---|---|---|---|---|
| | | | Acid[a] | Aldehyde[b] | Alcohol[c] | | |
| 1,2-Propanediol | Complete | 20.0 | 7.6 (C3) | 2.3 (C3) | 9.5 (C3) | 97 | 0.80 |
| | −Coenzyme $B_{12}$ | 0.7 | 0.0 | 1.2 | 0.0 | | |
| | −NAD | 20.0 | 0.0 | 19.9 | 0.0 | 100 | |
| | −NAD, + NADP | 20.0 | 8.4 | 1.7 | 9.7 | 99 | 0.87 |
| | −ADP | 20.0 | 9.6 | 1.1 | 9.6 | 102 | 1.00 |
| | −ADP, + AMP, + $PP_i$ | 20.0 | 9.1 | 0.7 | 9.8 | 98 | 0.93 |
| | −CoA | 20.0 | 1.4 | 17.2 | 1.6 | 101 | 0.88 |
| | −$MgCl_2$ | 20.0 | 5.7 | 8.3 | 5.6 | 98 | 1.02 |
| | −$MgCl_2$, + $MnCl_2$ | 20.0 | 1.5 | 15.1 | 1.7 | 92 | 0.88 |
| | +$NaHSO_3$ | 20.0 | 0.0 | 19.5 | 0.0 | 98 | |
| 1,2-Ethanediol | Complete | 15.6 | 7.1 (C2) | 1.7 (C2) | 7.3 (C2) | 103 | 0.97 |
| | −Coenzyme $B_{12}$ | 1.4 | 0.0 | 0.7 | 0.0 | | |
| | −ADP | 14.6 | 5.1 | 1.0 | 6.0 | 83 | 0.85 |
| | −ADP, + AMP, + $PP_i$ | 9.3 | 3.2 | 0.8 | 3.5 | 81 | 0.91 |
| | −$MgCl_2$ | 7.0 | 2.1 | 1.2 | 2.1 | 74 | 1.00 |
| Propionaldehyde | Complete | 17.3 | 9.7 (C3) | | 8.7 (C3) | 106 | 0.90 |
| | −Coenzyme $B_{12}$ | 17.8 | 10.0 | | 8.4 | 103 | 0.84 |
| Acetaldehyde | Complete | 18.9 | 10.1 (C2) | | 10.1 (C2) | 107 | 1.00 |
| | −Coenzyme $B_{12}$ | 19.6 | 9.6 | | 10.2 | 101 | 0.94 |

[a] In this column C3 = propionic acid and C2 = acetic acid.
[b] In this column C3 = propionaldehyde and C2 = acetaldehyde.
[c] In this column C3 = *n*-propanol and C2 = ethanol.
[d] O/R balance, Oxidation-reduction balance.

extracts were able to convert 1,2-propanediol and 1,2-ethanediol only in the presence of added coenzyme $B_{12}$, whereas the conversions of aldehyde substrates to acids and alcohols were not dependent on coenzyme $B_{12}$. This indicated that the initial step in the fermentation of 1,2-diols is the conversion of 1,2-diols to the corresponding aldehydes by a coenzyme $B_{12}$-dependent diol dehydratase. It should be noted that 1,2-propanediol and 1,2-ethanediol metabolism in crude extracts showed the same cofactor requirements. This constitutes additional evidence that these two 1,2-diols are utilized by the bacterium by a common metabolic pathway.

When sodium bisulfite was added to the reaction mixture, aldehydes were trapped as bisulfite adducts and were not further converted to acids or alcohols. Accumulation of aldehydes was also brought about when either NAD or CoA was omitted from the reaction system (Table 2). These findings are consistent with the proposed position of aldehydes as intermediates and suggested that NAD and CoA are obligatory cofactors for the dismutation of aldehydes to acids and alcohols. NADP was able to replace NAD at least partially. Furthermore, omission of $MgCl_2$ or replacement of $MgCl_2$ by $MnCl_2$ also resulted in partial accumulation of aldehydes. These cofactor requirements led us to examine the pathway from aldehydes to acids via acyl-CoA and acyl phosphates involving CoA-dependent aldehyde dehydrogenase, phosphotransacetylase, and acetate kinase or related enzymes. Since the fermentation balance studies were performed under anaerobic conditions, oxidation and reduction should have been balanced. The results shown in Tables 1 and 2 indicated that this was the case; that is, 50% of the aldehydes were reduced to the corresponding alcohols through the action of alcohol dehydrogenase. Thus, aldehydes must accept the reducing equivalents which are produced by the oxidation of aldehydes to the carboxylic acid of the same carbon number.

**Activities of enzymes involved in the anaerobic metabolism of 1,2-propanediol and 1,2-ethanediol.** Activities of enzymes implicated in the anaerobic metabolism of 1,2-diols were determined with cell-free extracts of *K. pneumoniae* ATCC 8724 grown anaerobically on 1,2-propanediol. Table 3 shows the alcohol dehydrogenase activity with C2 and C3 substrates and pyrimidine nucleotide coenzymes. The backward reaction (aldehyde → alcohol) was favored by the system. NADP(H) was able to replace NAD(H) at least partially. It should also be pointed out that C3 substrates were preferred to C2 substrates.

CoA-dependent aldehyde dehydrogenase activity was detected in the crude extracts. NAD and CoA were absolutely required for the oxidation of aldehydes. NADP was a far less effective coenzyme (13% as effective as NAD). Propionaldehyde was about 1.6 times better as a substrate than acetaldehyde. CoA was absolutely required for the arsenolysis of acetyl phosphate in the crude extracts of *K. pneumoniae* ATCC 8724. This was indicative of the presence of phosphotransacetylase in the extracts. Propionyl-CoA was attacked at a rate 0.70 as rapid as was acetyl-CoA. The presence of acetate kinase activity was also detected in the crude extracts. $MgCl_2$ was necessary for formation of acetyl phosphate from acetate and ATP. AMP and ADP were not able to replace ATP. Propionate was about 50% as active as acetate.

**Effects of growth substrates on the activities of enzymes.** Table 4 summarizes the activities of enzymes in crude extracts of *K. pneumoniae* ATCC 8724 cells grown on 1,2-propanediol, glycerol, or glucose. Coenzyme $B_{12}$-dependent diol dehydratase was an inducible enzyme, which preferred 1,2-propanediol to 1,2-ethanediol by a factor of about 1.7 (2). Alcohol dehydrogenase in this bacterium seemed constitutive, and the same order of substrate activity (C3 > C2) was obtained with all three cell-free extracts of cells grown on different growth substrates. The level of CoA-dependent aldehyde dehydrogenase activity was very high when the bacterium was grown on 1,2-propanediol. The difference in substrate activity between propionaldehyde and acetaldehyde was small. Even when grown on glycerol or on glucose, the bacterium still produced the enzyme, although its level was low. Phosphotransacetylase also seems to be a constitutive enzyme. The acetyl derivative was a little better substrate than the propionyl derivative. Essentially the same level of acetate kinase activity was obtained in the three extracts with acetate as a substrate. When propionate was used as a substrate, however, the activity was very low in the extracts of glucose-grown cells. The low relative activity of propionate was also observed in the extracts of glycerol-grown cells. These results on enzyme activities in crude extracts are consistent with the finding that aldehyde substrates were converted to acids and alcohols, but 1,2-diol substrates were not, with the extracts of glucose-grown cells.

TABLE 3. *Alcohol dehydrogenase activity in crude extracts of K. pneumoniae ATCC 8724 grown anaerobically on 1,2-propanediol*

| Direction | Substrate | Coenzyme | Alcohol dehydrogenase activity[a] |
|---|---|---|---|
| Forward | *n*-Propanol | NAD | 0.02 |
| | *n*-Propanol | NADP | 0.01 |
| | Ethanol | NAD | 0.01 |
| | Ethanol | NADP | 0.00 |
| Backward | Propionaldehyde | NADH | 0.11 |
| | Propionaldehyde | NADPH | 0.05 |
| | Acetaldehyde | NADH | 0.06 |
| | Acetaldehyde | NADPH | 0.02 |

[a] Specific activity, expressed as micromoles of NAD(P)H formed (forward) or consumed (backward) per minute per milligram of protein.

**Fermentation of 1,2-diols in other bacteria.** Besides *K. pneumoniae* ATCC 8724, some genera of *Enterobacteriaceae* were capable of growing anaerobically on 1,2-propanediol. To determine whether 1,2-propanediol was metabolized by the same pathway in these bacteria, the fermentation balance was studied with whole cells grown anaerobically on 1,2-propanediol (Table 5). 1,2-Propanediol and propionaldehyde

TABLE 4. *Effects of growth substrates on the activities of enzymes involved in the anaerobic metabolism of 1,2-propanediol and 1,2-ethanediol in crude extracts of K. pneumoniae ATCC 8724*

| Cells grown with:[a] | Activity of:[b] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Diol dehydratase[c] | Alcohol dehydrogenase | | Aldehyde dehydrogenase | | Phosphotransacetylase | | Acetate kinase | |
| | (C3) | C2 | C3 | C2 | C3 | C2 | C3 | C2 | C3 |
| 1,2-Propanediol | 1.9 | 0.06 | 0.11 | 0.29 | 0.45 | 0.23 | 0.16 | 1.4 | 0.7 |
| Glycerol | 0.4 | 0.10 | 0.22 | 0.11 | 0.09 | 0.26 | 0.17 | 1.4 | 0.4 |
| Glucose | 0.0 | 0.06 | 0.08 | 0.04 | 0.04 | 0.43 | 0.27 | 2.1 | 0.1 |

[a] The bacterial cells were obtained as described in the text and Table 1, footnote *a*.

[b] Activities of diol dehydratase, alcohol dehydrogenase (backward), aldehyde dehydrogenase, phosphotransacetylase, and acetate kinase were assayed with two-carbon and three-carbon substrates as described in the text and expressed in terms of specific activities (micromoles of substrate consumed or product formed per minute per milligram of protein).

[c] Data from Toraya et al. (25). This enzyme prefers 1,2-propanediol to 1,2-ethanediol by a factor of about 1.7 (2).

were converted to propionic acid and *n*-propanol in an almost quantitative yield by cell suspensions of all three bacteria tested. These bacteria were also able to produce diol dehydratase (24; Toraya, Kuno, and Fukui, unpublished data). Hence, it was evident that 1,2-propanediol is fermented in *K. pneumoniae* ATCC 13882, *C. freundii* AKU 0009, and *C. intermedium* AKU 0010 in the same manner as in *K. pneumoniae* ATCC 8724.

**Difference in the metabolism of 1,2-propanediol in aerobically and anaerobically grown cells.** *K. pneumoniae* ATCC 8724 was able to grow aerobically as well as anaerobically on 1,2-propanediol. The maximal growth of the bacterium under aerobic conditions was about 4.5 times higher than that obtained under anaerobic conditions. This suggested that the pathway of the aerobic metabolism of 1,2-diols was different from that of the anaerobic metabolism. As Table 6 shows, 1,2-propanediol was not metabolized at all by *K. pneumoniae* ATCC 8724 cells grown aerobically on 1,2-propanediol under the same conditions as those used in the experiments shown. This result indicated that diol dehydratase was not functional in aerobically grown cells. In the complete reaction system with cell-free extracts of aerobically grown cells (Table 6), 1,2-propanediol was converted to propionic acid and *n*-propanol, although the rate of conversion was much slower than that obtained with cell-free extracts of anaerobically grown cells (Table 2). This suggested that the cells grown aerobically on 1,2-propanediol contained some of the enzymes involved in the 1,2-diol fermentation. When coenzyme $B_{12}$ was omitted from the complete reaction mixture, 1,2-propanediol was not consumed. Thus, it was concluded that the above-mentioned systems for the 1,2-diol fermentation were not functional in the aerobically grown cells because of the low level of the intracellular coenzyme $B_{12}$, although the apoenzyme of diol dehydratase was present in the cells. Under aerobic conditions, 1,2-propanediol should be utilized by the bacterium through a metabolic pathway different from that observed in anaerobically grown cells.

## DISCUSSION

The metabolic pathway shown in Fig. 1 is

TABLE 5. *Fermentation of 1,2-propanediol and propionaldehyde by cell suspensions of some genera of Enterobacteriaceae grown anaerobically on 1,2-propanediol*

| Organism[a] | Substrate | Amt of substrate consumed (mmol) | Amt of products formed (mmol) | | | Carbon recovery (%) | O/R balance[b] |
|---|---|---|---|---|---|---|---|
| | | | Propionic acid | Propionaldehyde | *n*-Propanol | | |
| *K. pneumoniae* | 1,2-Propanediol | 0.98 | 0.49 | 0.00 | 0.46 | 97 | 1.07 |
| ATCC 13882 | Propionaldehyde | 0.99 | 0.44 | | 0.50 | 95 | 0.98 |
| *C. freundii* AKU | 1,2-Propanediol | 0.97 | 0.48 | 0.02 | 0.47 | 100 | 1.02 |
| 0009 | Propionaldehyde | 0.99 | 0.45 | | 0.41 | 87 | 1.10 |
| *C. intermedium* | 1,2-Propanediol | 0.99 | 0.50 | 0.00 | 0.45 | 96 | 1.11 |
| AKU 0010 | Propionaldehyde | 0.99 | 0.40 | | 0.43 | 84 | 0.93 |

[a] Glucose-grown cells were washed twice with 0.9 % KCl and inoculated into a fresh 1,2-propanediol medium at an initial concentration of about 0.002 g of dry cells per liter. The maximal growth yields of *K. pneumoniae* ATCC 13882, *C. freundii* AKU 0009, and *C. intermedium* AKU 0010 in a complex medium supplemented with 1,2-propanediol under anaerobic conditions were 0.17, 0.22, and 0.21 g of dry cells per liter, respectively.

[b] O/R balance, Oxidation-reduction balance.

TABLE 6. *Metabolism of 1,2-propanediol in whole cells and in crude extracts of K. pneumoniae ATCC 8724 grown aerobically on 1,2-propanediol*[a]

| System | Reaction conditions | Amt of 1,2-propanediol consumed[b] | Amt of products formed[b] | | |
|---|---|---|---|---|---|
| | | | Propionic acid | Propionaldehyde | *n*-Propanol |
| Whole cells[c] | Anaerobic | 0.00 | 0.00 | 0.00 | 0.02 |
| Cell-free extracts[d] | Anaerobic (complete system) | 7.9 | 2.9 | 0.8 | 4.4 |
| | Anaerobic (−coenzyme $B_{12}$) | 0.0 | 0.0 | 0.5 | 0.0 |

[a] *K. pneumoniae* ATCC 8724 cells cultivated aerobically on a rotary shaker (200 rpm) were used. The maximal growth yield was 0.81 g of dry cells per liter.

[b] The amounts of substrate consumed and products formed are expressed in millimoles for whole cells and micromoles for cell-free extracts.

[c] Fermentation balance experiments with whole cells were carried out as described in the text under anaerobic conditions.

[d] Experiments on the metabolism of 1,2-propanediol with cell-free extracts were carried out as described in the text under anaerobic conditions.

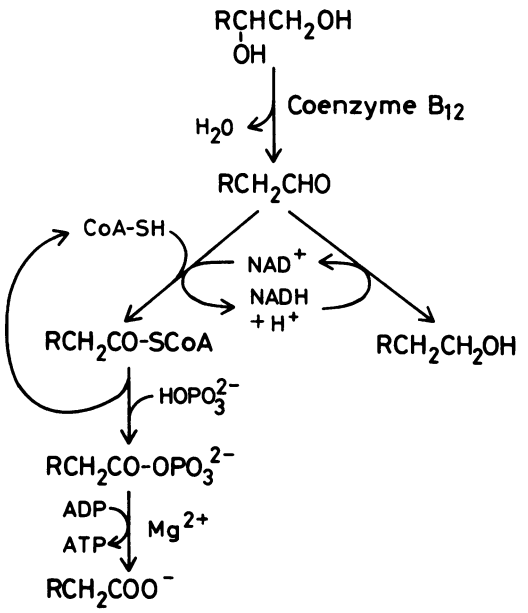


FIG. 1. *Schematic representation of the fermentations of 1,2-propanediol and 1,2-ethanediol. R = $CH_3$ or H.*

proposed for the fermentation of 1,2-propanediol and 1,2-ethanediol. The initial reaction is the coenzyme $B_{12}$-dependent dehydration of 1,2-diols to the corresponding deoxyaldehydes, which is catalyzed by diol dehydratase. Aldehydes are then oxidized to acyl-CoA by CoA-dependent aldehyde dehydrogenase. The next step is the transacylation from acyl-CoA to inorganic phosphate, which is mediated by phosphotransacetylase. The resulting acyl phosphates are finally hydrolyzed to carboxylic acids of the same carbon number by acetate kinase, with concomitant phosphorylation of ADP to ATP. This substrate-level phosphorylation is the only energy-generating step in the 1,2-diol fermentation. In these oxidation steps from aldehydes to acids, two equivalents of reducing power are produced. In this case, another mole of aldehyde acts as a hydrogen acceptor and consumes these two reducing equivalents to yield primary alcohols. The finding that the backward reaction (aldehyde → alcohol) was favored by the alcohol dehydrogenase system in cell-free extracts offers additional evidence that the system plays an essential role in reoxidation of reduced pyridine nucleotides with aldehydes. Thus, the following equation can be written to describe this fermentation: 2R—CH(OH)-$CH_2OH$ + ADP + $P_i$ → R—$CH_2COOH$ + R—$CH_2CH_2OH$ + ATP + $2H_2O$, where R = $CH_3$ or H. CoA and NAD are able to turn over in the system. Almost quantitative carbon recovery in acids and alcohols (Tables 1, 2, and 5) also supports this simple equation. For a net formation of 1 mol of ATP, 2 mol of 1,2-diols is consumed. In contrast, 2 mol of ATP is formed per mol of glucose in the homolactic fermentation. This lower energy yield in the 1,2-diol fermentation, based on the number of carbons fermented, would explain the poorer growth of bacteria in 1,2-diol media than in a glucose medium. Similar types of clostridial fermentations of ethanolamine and choline have been reported (4). In these fermentations, acetaldehyde is formed by elimination of ammonia from ethanolamine and of trimethylamine from choline. The phosphorylation is associated with the dismutation of the resulting acetaldehyde.

The conversion of 1,2-diols to aldehydes is catalyzed not only by diol dehydratase but also by glycerol dehydratase. These two coenzyme $B_{12}$-requiring dehydratases are similar in some catalytic and protein-chemical properties, but distinguishable in antigenic properties, in monovalent cation selectivity pattern, and in substrate specificity (24). We have reported that several bacteria of the *Enterobacteriaceae* produce diol dehydratase in a 1,2-propanediol medium and glycerol dehydratase in a glycerol medium (24). However, *K. pneumoniae* ATCC 8724 produces a single diol dehydratase, whether grown on 1,2-diols or on glycerol, although glycerol is a very poor inducer for this enzyme (25). Therefore, it can be concluded that the dehydratase involved in the 1,2-diol fermentation is a diol dehydratase.

Under optimal conditions, diol dehydratase is induced to a level of 1 to 2% of the soluble protein of the *K. pneumoniae* ATCC 8724 cells (25). When assayed with added coenzyme $B_{12}$, the specific activity of diol dehydratase was the highest of all the activities of the enzymes involved in the 1,2-diol fermentation (Table 4). In fact, when the bacterium was cultivated anaerobically in a 1,2-propanediol–glycerol medium supplemented with $CoSO_4$ (0.1 to 1.0 mg/liter of medium), the maximal bacterial growth (0.24 g of dry cells per liter of medium) was only one-half of that in the same medium without $CoSO_4$ (0.47 g of dry cells per liter of medium), and eventually the cells were killed by the overproduced propionaldehyde (Toraya, Honda, and Fukui, unpublished data). The concentration of propionaldehyde accumulated in the medium was about 15 to 20 mM. In a glycerol medium, neither growth inhibition nor unusual accumulation of carbonyl compounds was seen. The bacterium synthesized coenzyme $B_{12}$ up to a level of about 90 μg/g of dry cells (determined as vitamin $B_{12}$) when grown anaerobically in a 1,2-propanediol–glycerol medium supplemented

with $CoSO_4$ (1 mg/liter of medium) (Toraya, Honda, and Fukui, unpublished data). However, since $Co^{2+}$ was not usually added exogenously to the normal medium for the bacterial growth, the coenzyme concentration in the cells grown in this medium was very low (approximately 3 μg/g of dry cells as vitamin $B_{12}$). The synthesis of a trace amount of coenzyme $B_{12}$ was still possible, because the medium was contaminated with a trace of $Co^{2+}$ from tap water and chemical reagents used for preparing the medium. The holoenzyme activity (assayed without coenzyme $B_{12}$) in the crude extracts prepared freshly in the presence of 1,2-propanediol as a stabilizer was only 1 to 2% or less of the total enzyme activity (assayed with added coenzyme $B_{12}$). Thus, in the cells grown in the normal medium (without $CoSO_4$), holoenzyme activity of diol dehydratase should be rate limiting in the overall fermentation.

The intact cells were able to ferment 1,2-diols in the absence of added coenzyme $B_{12}$. Coenzyme $B_{12}$ binds to this enzyme very tightly (11). Despite these facts, the requirement for the coenzyme in the in vitro conversion of 1,2-diols to acids and alcohols was nearly absolute. This suggests that the original holoenzyme activity was almost completely lost during preparation of crude extracts. In the absence of substrate, the holoenzyme of diol dehydratase is readily inactivated by oxygen, accompanying the irreversible cleavage of the carbon–cobalt bond of the coenzyme (27). Furthermore, coenzyme $B_{12}$ is extremely sensitive to light. Since cell-free extracts were prepared in normal light in the presence of air but in the absence of substrate (1,2-propanediol), these properties of the holoenzyme and the coenzyme suggest that the above-mentioned explanation for the absolute requirement for coenzyme $B_{12}$ in the in vitro conversion of substrates to products is very likely.

The metabolic pathway of the 1,2-diol fermentation described above was not functional in the cells grown aerobically on 1,2-propanediol. From the data in Table 6, it is evident that this is mainly due to the absence of functional holoenzyme of diol dehydratase. Since a low but significant level of apoenzyme is present even in the aerobically grown cells (25), it is likely that the intracellular concentration of coenzyme $B_{12}$ is not sufficient. This is consistent with the resuslts of Abeles and Lee (2), who reported that *A. aerogenes* (*K. pneumoniae*) ATCC 8724 cells grown aerobically on glycerol contain far less coenzyme $B_{12}$ than do anaerobically grown cells. Even when a small amount of active holoenzyme is formed from the apoenzyme and coenzyme $B_{12}$, it would be rapidly inactivated under aerobic conditions. Furthermore, the ability of the bacterium to synthesize $B_{12}$ under aerobic conditions (less than 5 μg/g of dry cells as vitamin $B_{12}$) is much lower than under anaerobic conditions in media supplemented with $CoSO_4$ (1 mg/liter of medium), almost irrespective of the growth substrates (Toraya, Honda, and Fukui, unpublished data).

The relative substrate activity of propionate to acetate in the acetate kinase reaction was dependent on the prior growth history of the cells (Table 4). These results may be explained by assuming that the bacterium produces more than two activating enzymes which differ in substrate specificity. However, it must be kept in mind that the data on substrate specificities and cofactor requirements for the enzymes involved in the 1,2-diol fermentation were obtained with crude extracts. Hence, quantitative comparison of them with the published data obtained with purified enzymes may be invalid.

Authentic samples of acetyl phosphate and propionyl phosphate were decomposed almost completely in the acidification step with concentrated HCl before extraction of acids with ether. Thus, the acyl phosphates accumulated in the absence of ADP must also be determined as the corresponding acids. This seems to be a possible reason why ADP was not apparently required for the overall conversion of 1,2-diols and aldehydes to acids and alcohols (Table 2) despite involvement of acetate kinase in the terminal and energy-generating step of the 1,2-diol fermentation.

## ADDENDUM

During the preparation of this manuscript, a paper by Hosoi et al. appeared which reports the same type of 1,2-propanediol fermentation in *Propionibacterium freudenreichii* (8).

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