Note

Separation and Quantitation of 2,3-Butanediol Isomers ((-), (+), and *meso*) by a Combined Use of Enzyme and Gas ChromatographySadaharu Ui, Hiroshi MASUDA
and Hiroyuki MURAKIDepartment of Fermentation Technology,
Yamanashi University, Kofu, Yamanashi 400, Japan

Received May 29, 1984

There exist three isomeric forms of 2,3-butanediol ((-), (+), and *meso*), which are separated and identified as the optically active- and the *meso*-isomer by gas chromatography.¹⁾ However, quantification of each of the enantiomeric pairs ((-) and (+)) has not yet been done.

Brevibacterium saccharolyticum C-1012 is known to possess a strictly stereospecific (+)-2,3-butanediol dehydrogenase from the preceding reports.^{2,3)} This enzyme catalyses the following reaction:

In the presence of adequate amounts of NAD and hydrazine and in an alkaline condition, the equilibrium of the reaction is much in favour of acetoin formation. We attempted to apply this reaction to the analysis of butanediol isomers.

The organism was cultivated by the same procedures as those previously mentioned under aerobic conditions.⁴⁾ Preparation of cell-free extract was done in the same way as previously mentioned.⁴⁾ Phosphate buffer (0.1 M, pH 8.0) containing 2% protamine sulfate was added dropwise with continuous stirring to the cell-free extract to achieve a final ratio of 0.05 mg of protamine sulfate per mg of protein. After stirring for 20 min, the precipitate was removed by centrifugation. The protamine-treated extract was used as the enzyme preparation. The activity was measured at 25°C from the linear increase in absorbance at 340 nm produced on the addition of enzyme preparation to a reaction mixture containing 1 M hydrazine-0.2 M glycine buffer, pH 9.8, 2.5 μ mol NAD, 50 μ mol (+)-butanediol, and 0.02 ml of suitably diluted enzyme solution in a total of 3.0 ml. Protein was estimated by the method of Lowry *et al.*⁵⁾ The specific activity of the enzyme used was 2.80 U/mg (expressed as μ mol of NADH formed per min per mg protein). Though the enzyme frozen at -17°C (pH 8.0) is stable, repeated freezing and thawing during storage must be avoided because of the injurious effect on the enzyme activity.

As shown in Fig. 1, only the (+)-butanediol was almost completely removed from the reaction mixture by the enzyme reaction. Therefore, the amount of (+)-butanediol could be determined by a combined use of this enzyme reaction and the gas chromatographic method reported by Manius *et al.*,¹⁾ that is, by subtracting residual optically active-butanol ((-)-butanediol) in the reaction mixture from the total optically active-butanol in the blank mixture (containing water in place of the enzyme). By this procedure the other isomers ((-) and *meso*) are also determined, because the enzyme does not act on them at all. An example of optimum conditions examined is summarized as follows: Reaction mixtures containing 1 M hydrazine-0.2 M glycine buffer (pH 9.8), 0.25 ml of (+)-butanediol solution (3.3~22.2 mM), 0.05 ml of the enzyme solution (0.08 mg of protein) and excess NAD (about 1.5 times the molar quantity of each butanediol concentration) in a total volume of 1.5 ml was kept standing at 30°C for 20 min in sealed test tubes. In the described conditions, the amount of

FIG. 1. Gas Chromatogram for Enzymic Removal of only (+)-2,3-Butanediol.

(+)-Butanediol peak a falls off to b during the enzyme reaction, while peaks c and d for (-) and *meso*-butanediol, respectively, are not changed by the enzyme reaction. The reaction mixtures contained 1 M hydrazine-0.2 M glycine buffer (pH 9.8), 10 μ mol NAD, 5.0 μ mol (+)-butanediol with A or 5.1 μ mol (-) and 5.3 μ mol *meso*-isomer with B and 0.05 ml of the enzyme solution in a total volume of 1.5 ml. The reaction times were 20 min at 30°C. The working conditions of gas chromatography (Hitachi Type K-53; 2 m stainless steel column packed with PEG-600) were as previously described.⁴⁾ All three isomers of 2,3-butanediol used were obtained according to the procedures previously described.⁴⁾

TABLE I. EFFECT OF THE PRESENCE OF EXCESS ACETOIN ON THE DEHYDROGENATION OF (+)-BUTANEDIOL

(+)-Butanediol ^a (mm)	(±)-Acetoin (mm)	Residual butanediol ^{b,c} (%)
3.33	0	<2
3.33	16.7	2
3.33	33.3	<2
3.33	66.7	2

^a Butanediol (0.45 mg) was added.

^b Determinations are as described in the text.

^c $\frac{\text{(Residual butanediol)}}{\text{((+)-Butanediol added)}} \times 100 (\%)$.

removed (+)-butanediol reached 98% value for each concentration of the initial amount. Accordingly, the quantification of three individual isomers of butanediol could be attained easily. The sensitivity limit and the precision of this analysis is dependent on the efficiency of the gas chromatograph used.

In the case of the removal of (+)-butanediol from a sample such as a fermentation liquid or wine, in most

cases, containing acetoin, the influence of acetoin must be considered. However, the results (Table I) show that the acetoin, even in considerably high concentrations, does not have any appreciable influence on the dehydrogenation of (+)-butanediol.

Contrary to the procedure mentioned above, the removal of only (–)-butanediol from the mixture of the isomers can also be attained by using a butanediol dehydrogenase. The butanediol dehydrogenases from *Bacillus coagulans* ATCC 8038, *Bacillus cereus* IAM 1072 etc. appear to be suitable for this purpose, because according to the preceding report²⁾ they are unable to oxidize (+)-isomer.

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