A CYCLIC PATHWAY FOR THE BACTERIAL DISSIMILATION OF 2,3-BUTANEDIOL, ACETYL METHYL CARBINOL, AND DIACETYL

I. GENERAL ASPECTS OF THE 2,3-BUTANEDIOL CYCLE

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It is a well established fact that all organic compounds produced by living cells can be broken down by microorganisms. During the dissimilation of carbohydrates by organisms such as Aerobacter and certain members of the genus Bacillus 2,3-butanediol (2,3-butylen glycol) is formed as the chief product of the fermentation. There are many organisms in the soil that can use 2,3-butanediol or acetyl methyl carbinol (AMC, acetoin) as the sole source of carbon and energy for growth (LeMoigne et al., 1952; Sebek and Randles, 1952; Juni, 1952a). Studies in this laboratory have revealed a new pathway for the dissimilation of 2,3-butanediol by these organisms (Juni and Heym, 1953). Although the breakdown of 2,3-butanediol is an aerobic process, nearly all the reactions involving its oxidation to acetic acid can take place anaerobically as well as aerobically. The new pathway is a cyclic process involving two new intermediates, diacetyl methylcarbinol (DAMC) and acetylbutanediol (ABD). The cycle appears to be a method for generating acetic acid, a compound which these organisms oxidize readily. The present paper will be concerned with the general aspects of the reactions of this pathway.

MATERIALS AND METHODS

Cell Preparations

Bacteria capable of utilizing 2,3-butanediol or AMC as the sole source of carbon and energy were obtained by the enrichment technique. Mud from a local lake was inoculated into the following medium placed in shallow layers in Erlenmeyer flasks: 2,3-butanediol or AMC, 5 g; KH₂PO₄, 1.5 g; Na₂HPO₄, 13.5 g; MgSO₄, 0.1 g; NH₄Cl, 2.0 g; CaCl₂, 0.01 g; FeSO₄·7H₂O, 0.0005 g; thiamin hydrochloride, 0.01 g; water, 1,000 ml; pH 7.6. After several days of incubation at room temperature growth had taken place and the cultures were streaked on the same medium to which had been added 1.5 per cent agar. Two organisms were isolated in pure culture. One proved to be a Corynebacterium and the other appears to be identical with Micrococcus ureae. The latter organism was used in nearly all the studies described below although it has been shown that other bacteria capable of growing in the above medium carry out the same reactions. Cultures of Aerobacter aerogenes strain ATCC 8724 and Pseudomonas fluorescens strain A 3.12 were among the organisms that could grow in this medium. Cell suspensions were prepared by inoculating 400 ml of the medium, described above, contained in a 2.5-L Fernbach flask, with the scrapings from a nutrient agar slant of the organism suspended in 5 ml of nutrient broth. After shaking at room temperature for 18 to 24 hr the cells were centrifuged, washed with 0.8 per cent NaCl and then suspended in 10 ml of 0.8 per cent NaCl. Such cell suspensions retained their full activities when stored in the refrigerator for as long as a week. Cell-free bacterial extracts were prepared by suspending wet packed cells in twice their volume of water and subjecting the suspension to sonic vibration for 30 min with a Raytheon 10-kc oscillator. The resulting suspension was then centrifuged at 25,000 X G for 30 min and the precipitate discarded.

Manometric Studies

CO₂ evolution from bicarbonate buffer was followed in the conventional Warburg apparatus at 30 C, with 100 per cent CO₂ as the gas phase. When diacetyl was the substrate it was added to the side arms of both the experimental vessel and an identical control vessel. Flushing both flasks simultaneously resulted in the same loss of diacetyl from both vessels due to volatilization and made possible the estimation of the initial diacetyl concentration.
Methods for determining acetyl condensation products and related compounds

<table>
<thead>
<tr>
<th>Method</th>
<th>Acetyl-methylcarbinol</th>
<th>Diacetylmethylcarbinol</th>
<th>Diacetyl</th>
<th>Acetylbutadiol</th>
<th>2,3-Butanediol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Westerfeld (1945)</td>
<td>1.0*</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>0</td>
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<tr>
<td>White et al. (1946)</td>
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<td>1.0†</td>
<td>1.0†</td>
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<td>0</td>
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<tr>
<td>Prill and Hammer (1938)</td>
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<td>0</td>
<td>1.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Barker and Summersen (1941)</td>
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<td>0.0†</td>
<td>0.0†</td>
<td>1.0†</td>
<td>0</td>
</tr>
<tr>
<td>Molybdate color†</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CH₃COOH after HIO₄ oxidation‖</td>
<td>1.0</td>
<td>3.0</td>
<td>2.0</td>
<td>2.0</td>
<td>0</td>
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<tr>
<td>CH₃CHO after HIO₄ oxidation‖</td>
<td>1.0</td>
<td>0</td>
<td>1.0</td>
<td>2.0</td>
<td>0</td>
</tr>
</tbody>
</table>

* The figures in the table represent equivalents in the given method for the various compounds when compared with the reaction obtained with an equivalent of the compound for which the test was originally devised.
† No reaction occurs without NH₄OH for diacetylmethylcarbinol. However, NH₄OH is not required for the determination of diacetyl.
‡ High concentrations of diacetyl do interfere in this reaction; a green color is formed, however. To eliminate interference from high diacetyl concentrations, distill about 30 per cent of the solution. This will remove all diacetyl and leave better than 95 per cent of the acetylbutadiol remaining undistilled.
§ Diacetylmethylcarbinol reduces acid molybdate to give a typical molybdenum blue color. This method will be published shortly.
‖ Volatile acid determined after periodate oxidation according to Juni (1932b). Acetaldehyde determined after periodate oxidation according to Deamelle and Naudet (1945) without previous distillation, or according to Stots (1943) after preliminary distillation.

Chemical Methods

The complete series of chemical methods used in these studies is outlined in Table 1. Because some of these reactions and compounds are new, several of the methods will be discussed briefly.

Diacetylmethylcarbinol (DAMC). This compound reacts in a manner similar to diacetyl in the quantitative Voges-Proskauer test of Westerfeld (1945) since it is oxidized to diacetyl under alkaline conditions. It also reacts as diacetyl in the method of White et al. (1946), and it is, therefore, not possible to distinguish between these compounds using these two methods. DAMC can be determined in solutions containing both DAMC and diacetyl by the consecutive use of the methods of White et al. (1946) and that of Prill and Hammer (1938) since the latter method is specific for diacetyl. A new quantitative colorimetric test based upon the specific reduction of ammonium molybdate to molybdenum blue by solutions of DAMC has been devised in this laboratory and will appear, together with other information about the properties of DAMC, in another paper in this series.

Acetylbutadiol (ABD). ABD yields acetaldehyde quantitatively in strong sulfuric acid and can, therefore, be determined as lactic acid according to the method of Barker and Summersen (1941). Interestingly enough, ABD is still another compound that reacts as AMC in the Voges-Proskauer test.

Acetic acid. Acetic acid was determined either manometrically as described above or by titration with 0.1 N NaOH after steam distillation. The only volatile acid formed was shown to be acetic acid by chromatography on celite using the method of Bueding and Yale (1951).

Diacetyl (Eastman) was purified by fractional distillation. AMC (Eastman), a racemic mixture, was converted to the dimer by storing at -18°C for 2 weeks and the crystalline product washed repeatedly with ether to remove traces of diacetyl. 2,3-Butanediol was obtained from the Celanese Corporation of America and appeared to be entirely the meso-isomer. The synthesis of DAMC has already been described (Juni and Heym, 1950).

RESULTS

Growth on 2,3-butenediol and related compounds. All the bacteria capable of growing in the 2,3-butanediol-mineral-thiamin medium described could also grow with AMC in place of 2,3-butanediol. The fact that diacetyl could not replace 2,3-butanediol is not surprising since it is well known that diacetyl is inhibitory for the growth of many species (Myrvik and Volk, 1954). One of the organisms isolated (the Corynebacterium) could not grow in the absence of thiamin. While the others did not have an absolute requirement
for thiamin, their growth in this medium was stimulated by the addition of this vitamin.

Sebek and Randles (1952) have shown, and we have confirmed their observations, that the enzymes concerned with the metabolism of 2,3-butanediol and its oxidation products are adaptive in nature. Cells grown in the presence of 2,3-butanediol or AMC rapidly oxidize 2,3-butanediol, AMC, and diacetyl, while those grown in nutrient broth cannot oxidize the above compounds. It was reported (Juni, 1952a) that cells of Corynebacterium grown on AMC could not oxidize diacetyl. Dried cells of the organism could readily oxidize diacetyl, however, and permeability factors seem to be involved here. Freshly harvested resting cell suspensions of other species oxidize diacetyl as well as 2,3-butanediol and AMC.

The particular medium employed in these experiments was used because of its very great buffering capacity. When smaller concentrations of phosphate were used (0.1 to 0.5 per cent), it was noticed that optimal growth (6 to 8 g wet weight of cells per L) was not obtained. In such cases it could be shown that the pH had dropped to as low as 4.0. Analysis of the medium, after removal of the cells, showed that considerable quantities of acetic acid had accumulated in spite of the fact that aerobic conditions, as required for growth, were maintained. Furthermore, it was shown that cells harvested from a medium in which acetic acid had accumulated could readily oxidize acetate. Analysis of the growth medium for acids other than acetic acid revealed a substance which reacted like lactic acid in the Barker and Summerson (1941) test. This material was subsequently shown not to be lactic acid but rather acetylbutanediol (ABD), an intermediate in the dissimilation of 2,3-butanediol.

Stanier and Fratkin (1944) reported that a strain of A. aerogenes with which they were working is capable of oxidizing 2,3-butanediol, AMC, and diacetyl. This particular strain (ATCC 8724) grows well on the AMC-mineral medium described above. Other strains of A. aerogenes cannot grow on this medium, however. Happold and Spencer (1952) were unable to observe AMC oxidation with their strain of A. aerogenes.

The pathway for the dissimilation of 2,3-butanediol and related compounds. Figure 1 is an outline of the cyclic pathway for the dissimilation of 2,3-butanediol. All the steps have been studied rather intensively and more detailed information concerning the individual reactions and the new intermediates will be presented in future papers of this series.

Step A of figure 1, the reversible oxidation of 2,3-butanediol to AMC, is catalyzed by a diphosphopyridine nucleotide (DPN)-linked enzyme that has already been studied in some detail in several species (Aubert and Gavard, 1953; Randles, 1954; DeMoss et al., 1951). Sonicate extracts of cells grown on 2,3-butanediol or AMC contain large concentrations of this dehydrogenase. Step B, the reversible oxidation of AMC has not yet been completely demonstrated. Randles and Harary (1954) have studied a DPN-linked enzyme in A. aerogenes and Micrococcus pyogenes var. aureus that will catalyze the reduction of diacetyl to AMC but they have been unable to demonstrate the reversibility of this reaction. It does appear certain, however, that diacetyl is formed from AMC since acetylbutanediol (ABD) accumulates in the growth medium (3-5 μmoles per ml) when cells are grown or incubated in AMC (see figure 2) and diacetyl is the precursor of ABD, as shown below. Furthermore, Sebek and Randles, (1952) have been able to detect diacetyl in the medium when a Pseudomonad was grown with 2,3-butanediol as the

![Figure 1. Reactions of the 2,3-butanediol cycle.](http://jb.asm.org/Downloaded from http://jb.asm.org on September 23, 2017 by guest)
carbon source. This step is now being investigated in our laboratory.

Steps C and D involve the synthesis of diacetyl-methylocarbinol (DAMC) from diacetyl. As diagrammed in C, diacetyl is split with the addition of diphosphothiamin (DPT) and water to form acetic acid and a reactive acetaldehyde-DPT complex. The reactive complex then undergoes an acyloin-type condensation with diacetyl (reaction D) to form DAMC (equation 1). This reaction is completely analogous to the synthesis of α-acetolactate from pyruvate (equation 2) (Juni, 1952c; Dolin and Gunsalus, 1951).

\[
\begin{align*}
\text{O} & \quad \text{O} \\
& \quad \text{C} - \text{C} - \text{CH}_3 \\
& \quad \text{H} \\
& \quad \text{O} \quad \text{O} \\
& \quad \text{CH}_3 - \text{C} - \text{CH}_4 + \text{CH}_4\text{COOH} \\
& \quad \text{C} = \text{O} \\
& \quad \text{CH}_4 \\
\end{align*}
\]

(1) \[2\text{CH}_4 - \text{C} - \text{C} - \text{CH}_3 + \text{H}_2\text{O} \rightarrow \]

\[
\begin{align*}
& \quad \text{H} \\
& \quad \text{O} \quad \text{O} \\
& \quad \text{CH}_3 - \text{C} - \text{C} - \text{OH} + \text{CO}_2 \\
& \quad \text{C} = \text{O} \\
& \quad \text{CH}_4 \\
\end{align*}
\]

(2) \[2\text{CH}_4 - \text{C} - \text{C} - \text{OH} \rightarrow \]

The enzyme system catalyzing the synthesis of DAMC has been partially purified and shown to carry out reactions C and D quantitatively. The condensation appears to be irreversible, and DPT and a divalent cation such as magnesium stimulate the partially resolved preparation. This enzyme system shows no activity on pyruvate nor does the α-acetolactate-forming enzyme from Aerobacter show any activity on diacetyl. DAMC has been synthesized chemically in this laboratory (Juni and Heym, 1956) and shown to be identical with the enzymatically-formed material.

Dihydriodiphosphopyridine nucleotide (DPNH) can reduce DAMC to ABD, as shown in step E of figure 1. This reaction appears to be irreversible since incubation of ABD and DPNH does not result in any detectable DPNH. DAMC reductase has been shown to be different from 2,3-butanediol dehydrogenase. Once formed, ABD is hydrolyzed to 2,3-butanediol and acetic acid, as shown in step F. ABD has also been synthesized for the first time in this laboratory and further details concerning its properties will appear shortly.

It can thus be seen that the cyclic process described above serves as a source of acetic acid. All organisms found to be capable of growing on 2,3-butanediol or AMC as the carbon source can also grow with acetate in place of the four carbon compounds. It seems likely that some Aerobacter strains cannot grow on AMC because of their inability to grow on acetate.

**Anaerobic operation of the cycle.** Cells grown aerobically on 2,3-butanediol can degrade AMC, diacetyl, DAMC and ABD anaerobically. In all cases the end products are acetate and 2,3-butanediol. Table 2 shows the balances obtained as a result of the anaerobic activity of freshly harvested resting cell suspensions on the various intermediates of the cycle. When AMC is the substrate it is first dismutated to diacetyl and 2,3-butanediol as shown in equation 3.

\[
\begin{align*}
& \quad 2\text{CH}_4\text{CHOHCOCH}_3 \rightarrow \text{CH}_3\text{COCOCH}_3 \\
& \quad \text{C} \quad \text{C} - \text{OH} + \text{CO}_2 \\
& \quad \text{C} = \text{O} \\
& \quad \text{CH}_4 \\
\end{align*}
\]

(3) \[2\text{CH}_4\text{CHOHCOCH}_3 \rightarrow \text{CH}_3\text{COCOCH}_3 + \text{CH}_3\text{CHOHCHOHCH}_3 \]

With diacetyl or DAMC as the substrate a reduction must take place in order to form ABD (step

**TABLE 2**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>End Products Formed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acetic acid</td>
</tr>
<tr>
<td></td>
<td>(Theoretical)</td>
</tr>
<tr>
<td>Acetylmethylcarbinol, 33.3 μM</td>
<td>22.2</td>
</tr>
<tr>
<td>Diacetyl, 24.6 μM</td>
<td>32.8</td>
</tr>
<tr>
<td>Diacetyl-methylocarbinol, 15.4 μM</td>
<td>25.6</td>
</tr>
<tr>
<td>Acetylbutanediol, 21.6 μM</td>
<td>21.6</td>
</tr>
</tbody>
</table>

NaHCO₃, 70 μM; cell suspension, 100 mg wet weight; substrate, as above; gas phase, 100 per cent CO₂; final volume, 1.6 ml; pH 6.5; incubation at 30 C, 10 hr.
Evidence for functioning of the cycle during aerobic growth. While it is easy to demonstrate the occurrence of the 2,3-butanediol cycle under anaerobic conditions the question arises as to the utility of this pathway during normal aerobic growth. The evidence to be presented will show that the cycle does indeed function under these conditions.

The accumulation of acetate and ABD in the growth medium is consistent with this concept. Evidence that AMC is metabolized faster by the anaerobic reactions than by direct oxidation is presented in figure 2, where a resting cell suspension was permitted to degrade AMC under aerobic conditions. The AMC oxidized was calculated from the following equation:

$$\text{CH}_3\text{CHOHCOCH}_3 + 5\text{O}_2 \rightarrow 4\text{CO}_2 + 4\text{H}_2\text{O}$$

It can be seen that the rate of disappearance of AMC is about six times faster than the rate of AMC oxidation. AMC is used as fast anaerobi-
cally as aerobically. Furthermore, considerable quantities of 2,3-butanediol accumulate during the oxidation as well as some ABD, one of the intermediates of the anaerobic cycle. The data in table 3 show that the rate of oxygen uptake in the presence of both AMC and acetate is almost identical to the rate of oxygen uptake in the presence of AMC alone. This indicates that the electron transport system in these cells is probably operating at its maximum rate which is much slower than the rate of the anaerobic reactions.

When 2,3-butanediol is the substrate, under aerobic conditions, it is oxidized more rapidly than acetate, as shown in table 4. It may also be seen that the presence of acetate exerts a slight "sparing" action on the oxidation of 2,3-butanediol. Since 2,3-butanediol is first oxidized to AMC it follows from the data presented above that the

### TABLE 3

The rates of oxidation of acetylmethylcarbinol and acetate by resting cell suspensions

<table>
<thead>
<tr>
<th>Additions</th>
<th>µM O2 Uptake/Hr</th>
<th>µM 2,3-Butanediol Used/Hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4.7</td>
<td>0</td>
</tr>
<tr>
<td>Acetylmethylcarbinol</td>
<td>18.4</td>
<td>0</td>
</tr>
<tr>
<td>Acetate</td>
<td>14.4</td>
<td>0</td>
</tr>
<tr>
<td>Acetylmethylcarbinol + acetate</td>
<td>18.2</td>
<td>0</td>
</tr>
</tbody>
</table>

Potassium phosphate buffer, 80 µM; cell suspension, 50 mg wet weight; acetylmethylcarbinol 57 µM; sodium acetate, 20 µM; gas phase, air; center well contained 0.1 ml of 2.5 N NaOH; final volume 1.6 ml; pH 5.5; incubation at 30 C, 1 hr.

### TABLE 4

The rates of oxidation of 2,3-butanediol and acetate by resting cell suspensions

<table>
<thead>
<tr>
<th>Additions</th>
<th>µM O2 Uptake/Hr</th>
<th>µM 2,3-Butanediol Used/Hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3.3</td>
<td>—</td>
</tr>
<tr>
<td>2,3-Butanediol</td>
<td>52.5</td>
<td>14.7</td>
</tr>
<tr>
<td>Acetate</td>
<td>37.7</td>
<td>0</td>
</tr>
<tr>
<td>2,3-Butanediol + acetate</td>
<td>51.0</td>
<td>10.5</td>
</tr>
</tbody>
</table>

Potassium phosphate buffer, 100 µM; cell suspension, 100 mg wet weight; 2,3-butanediol, 33 µM; sodium acetate, 20 µM; gas phase, air; center well contained 0.1 ml of 2.5 N NaOH; final volume, 1.6 ml; pH 6.4; incubation at 30 C, 1 hr.

Figure 3. Anaerobic dissimilation of diacetyl by resting cell suspensions as a function of time. Each vessel contained the following: NaHCO3, 80 µM; diacetyl, 37.7 µM; cell suspension, 100 mg wet weight; gas phase, 100 per cent CO2; final volume, 1.6 ml; pH 6.57; incubation at 30 C.

AMC formed aerobically from 2,3-butanediol is rapidly dissimilated via the anaerobic cycle even during aerobic growth.

Experiments with cell-free bacterial extracts capable of synthesizing DAMC from diacetyl indicate that diacetyl is oxidized neither in air nor anaerobically in the presence of electron acceptors such as ferricyanide. This evidence makes it seem likely that diacetyl must undergo anaerobic reactions before oxidation takes place.

Direct evidence for the accumulation of DAMC and ABD, intermediates in the 2,3-butanediol cycle, is shown in figure 3 which is a time curve for the anaerobic dissimilation of diacetyl by a resting cell suspension. As diacetyl disappears DAMC is rapidly formed and then subsequently reduced to ABD which starts to accumulate. It has been shown that diacetyl, in reasonable concentrations, is inhibitory for the enzyme that hydrolyzes ABD and hence this compound can accumulate in extremely large concentrations. Experiments with cell-free extracts have revealed that endogenous materials can provide hydrogens for the reduction of DAMC and it seems likely that this takes place when DAMC accumulates in high concentrations. When AMC is the substrate, however, no DAMC accumulates in the medium since the concentration of diacetyl never becomes very high.
DISCUSSION

It has been reported (Juni and Heym, 1954; Juni and Heym, 1956) that the synthesis of DAMC from diacetyl can be catalyzed by pyruvic oxidase preparations. In these cases diacetyl acts as an analogue of pyruvate. A reactive acetaldehyde, DPT complex is formed with pyruvate as well as with diacetyl (equations 9 and 10).

\[
\begin{align*}
\text{(9) } & \text{CH}_3\text{C} - \text{C} - \text{OH} \xrightarrow{\text{DPT}} \text{CH}_3\text{CHO-DPT}^+ + \text{CO}_2 \\
\text{(10) } & \text{CH}_3\text{C} - \text{C} - \text{CH}_3 \xrightarrow{\text{DPT}} \text{CH}_3\text{CHO-DPT}^+ + \text{CH}_3\text{COOH}
\end{align*}
\]

With pyruvic oxidase preparations the fate of the reactive acetaldehyde depends upon the experimental conditions. Under aerobic conditions, or anaerobically in the presence of a suitable electron acceptor such as ferricyanide, reactive acetaldehyde is oxidized to acetate (Schweet et al., 1951; Dolin, 1955; Gunsalus, 1954). Under anaerobic conditions, in the absence of an electron acceptor, reactive acetaldehyde condenses with either pyruvate or diacetyl to form \(\alpha\)-acetolactate or DAMC respectively (Juni and Heym, 1954; Juni and Heym, 1956).

In general, pyruvic oxidase preparations can catalyze several different kinds of acylon condensations. The DAMC-forming enzyme system that is part of the 2,3-butanediol cycle is specific for diacetyl, however, and is not identical with pyruvic oxidase. There is no evidence for diacetyl oxidation via the diacetyl mutase reaction described by Green et al. (1947) in pigeon breast muscle preparations.

SUMMARY

Bacteria able to grow with 2,3-butanediol or acetyl methylcarbinol as the sole source of carbon develop a cyclic mechanism for the conversion of these compounds to acetate. Diacetyl is an intermediate in the process and is converted to diacetyl methylcarbinol and acetate by a diphosphothiamin-catalyzed acylon condensation reaction. Diacetyl methylcarbinol is reduced to acetylbutanediol which, in turn, is hydrolyzed to 2,3-butanediol and acetate. All the reactions in the cycle can take place anaerobically. Evidence has been presented to show that this cyclic process operates during normal aerobic growth on the four carbon compounds.

Methods for the determination of diacetyl methylcarbinol and acetylbutanediol are described.

REFERENCES


