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THE OXIDATION OF GLYCOLS BY ACETIC ACID BACTERIA

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SUMMARY

1. Resting cells of 14 different strains of acetic acid bacteria oxidized 1,2-ethanediol, DL-1,2-propanediol, DL-1,3-butanediol, meso-2,3-butanediol and 1,4-butanediol.

2. The oxidation of 22 different glycols was studied with resting cells of *Gluconobacter oxydans* (suboxydans).

3. The end products of the oxidation of the following glycols with resting cells of either *Gluconobacter oxydans* (suboxydans) or *Acetobacter aceti* (liquefaciens) have been isolated and chemically identified: 1,2-ethanediol to glycollic acid, 1,3-propanediol to β-hydroxypropionic acid, 1,4-butanediol to succinic acid, 1,5-pentanediol to glutaric acid, 1,6-hexanediol to adipic acid, 1,7-heptanediol to pimelic acid and DL-1,3-butanediol to DL-β-hydroxybutyric acid. The oxidation of 1,4-butanediol and 1,5-pentanediol occurred in two steps.

4. *Acetobacter aceti* (liquefaciens) was unable to grow in a medium with DL-1,3-butanediol as sole carbon source. This compound inhibited growth in culture media containing either ethanol or glycerol.

5. All glycols which were oxidized by resting cells were also oxidized by the particulate fraction. D(−)- and L(+)-1,2-propanediol, D(−)- and L(+)-2,3-butanediol were oxidized to acetol, D(−)- and L(+)-acetylmethylcarbinol, respectively.

6. A soluble NAD-linked primary alcohol dehydrogenase oxidized monohydric primary alcohols and ω-diols. DL-1,3-Butanediol was oxidized slowly at C-1.

7. A soluble NAD-linked secondary alcohol dehydrogenase oxidized monohydric secondary alcohols and the secondary alcohol function of the following glycols: meso-2,3-butanediol, DL-2,3-butanediol, DL-1,2-propanediol, L(+)-1,2-propanediol, meso-3,4-hexanediol and (−)-3,4-hexanediol. Meso-2,3-butanediol and meso-3,4-hexanediol were oxidized to L(+)-acetylmethylcarbinol and (+)ethylpropionylcarbinol.

8. Both soluble dehydrogenases were purified and separated by chromatography.

INTRODUCTION

Acetic acid bacteria, which appear to be uniquely endowed with the capacity for oxidizing a great variety of carbohydrates and derivatives, also oxidize several glycols.

BROWN[1] showed that *Acetobacter aceti* oxidized 1,2-ethanediol to glycollic acid. This was later confirmed with *A. pasteurianus* and *A. kützingianus* by SEIFERT[2] and with *A. xylinum*, *A. aceti*, *Gluconobacter suboxydans* and *G. melanogenus* by VISSER 'T HOOFT[3]. BANNING[4] reported that many strains were able to form oxalic acid from 1,2-ethanediol.

With unshaken cultures of a variety of strains KLING[5] showed that the D(—) form of DL-1,2-propanediol was oxidized to acetol. This 50% conversion was probably a coincidence since VISSER 'T HOOFT[3] gave evidence that both isomers were oxidized by *A. xylinum* and *G. suboxydans*. BUTLIN AND WINCE[6] convincingly showed that thoroughly aerated cultures of *G. suboxydans* oxidized both isomers nearly quantitatively to acetol. COPET, FIERENS-SNOECK AND VAN RISSEGHEM[7] and VAN RISSEGHEM[8], again using poorly aerated unshaken cultures, reported that *A. xylinum*, *A. aceti* and *G. suboxydans* oxidized D(—)-1,2-propanediol, another strain of *G. suboxydans* oxidized the L(+) form.

From KLING's results[9] with unshaken cultures of *A. xylinum* and *A. aceti* it can be deduced that the D(—) form of DL-2,3-butanediol was oxidized preferentially to D(—)-acetoin. His results were confirmed by GRIVSKY[10] using the same species. UNDERKOFLER, FULMER, BANTZ AND KOOI[11] confirmed that the D(—) form was oxidized quantitatively by *G. suboxydans*, but the L(+) form was not attacked. Meso-2,3-butanediol was oxidized by *G. suboxydans*[12] with the formation of L(+)-acetoin. From the results of VISSER 'T HOOFT[3] with *A. xylinum* and *G. suboxydans* the same conclusion can be drawn (although this author thought that he was using the DL-form). According to GRIVSKY[10] only D(—)-acetoin would be oxidized further to diacetyl by *A. aceti*.

VAN RISSEGHEM[13] found that *A. aceti* and *A. xylinum* oxidized meso-3,4-hexanediol to L(+)-ethylpropionylcarbinol. D(+)-3,4-Hexanediol was oxidized to D(—)-ethylpropionylcarbinol. The L(—) isomer was not attacked. Both isomers of ethylpropionylcarbinol were oxidized to dipropionyl.

Growing cultures of *A. aceti*, *A. xylinum* and *G. suboxydans* did not oxidize 1,2-butanediol[7,9], 1,2-pentanediol[7] and 1,2-hexanediol[7].

CUMMINS[14] reported on the oxidation of the following glycols by resting cells or cell-free extracts: DL-1,2-propanediol, 2-methyl-2-nitro-1,3-propanediol, 2-butyne-1,4-diol, 1,2,4-butanetriol, 2-butene-1,4-diol, 1,3-pentanediol, 1,5-pentanediol, hexyleneglycol, 1,2,6-hexanetriol, 2,5-hexanediol, cyclohexane-1,4-diol. The following compounds were not oxidized: 2,5-dimethyl-hexyne-3-diol-2,5, pentaerythritol, dipropyleneglycol, diethyleneglycol, thiodiethyleneglycol, styreneglycol and cyclohexane-1,4-diol.

GOLDSCHMIDT AND KRAMPITZ[15] have briefly reported on an NAD-linked 2,3-butanediol dehydrogenase from *G. suboxydans* which lacks specificity.

Many of the previous experiments have been carried out with growing cultures, often poorly aerated and extending over periods up to 3–6 months. Knowledge on the enzymology of these glycols is negligible. In the present paper we want to report on the oxidation of several straight-chain glycols by acetic acid bacteria and on the nature of the enzymes which effect the primary catabolic step. It is an extension of the previous work of this laboratory on the biochemistry and enzymology of these bacteria (for reviews, see DE LEY[16,17]).

METHODS AND MATERIALS

Bacteria used

We used the same strains of acetic acid bacteria which have previously been studied by DE LEY[17]. The same cultural conditions were adopted, as well as the

nomenclature of the strains. *Acetobacter aceti* (paradoxus) and *A. aceti* (peroxydans) were grown in the liquid medium of ATKINSON[18] under vigorous aeration[19]. All the other strains were grown on solid glucose media. *Pseudomonas fluorescens*, strain 488 of the Institute of Biology, Prague, was obtained through the courtesy of Dr. O. LYSENKO. It was grown for about two days at 30° under vigorous aeration in a liquid medium containing 0.5% peptone and 0.25% yeast extract (Difco).

Preparation of cells

Cells were harvested after 2–3 days of growth at 30° and washed three times by suspending in 0.01 M phosphate buffer (pH 6.2) and centrifuging at 12000 × *g* for 15 min. They were finally suspended in 0.02 M phosphate buffer (pH 6.2) in a concentration of 100 mg living cells (wet wt.) per 1.8 ml and used at once for manometric experiments. Possible contamination was checked for by the criteria prescribed by JOUBERT, BAYENS AND DE LEY[22].

Cell-free enzyme preparations

Intact cells of either acetic acid bacteria or *Pseudomonas* (10 g of living cells per 50 ml 0.01 M phosphate buffer (pH 6.2)) were disrupted in the 10-kC, 250-W Raytheon Sonic Oscillator at 4° in H_2 atmosphere for 20 min. Intact cells and large cell debris were removed by centrifugation at 4° for 30 min at 10000 rev./min. The supernatant "crude extract" was centrifuged for 2 h at 105000 × *g* at 4° in a preparative Spinco ultracentrifuge. The gelatinous red or brown precipitate contained mainly small hull fragments (cell wall and cytoplasmic membrane) and the larger ribosomes. It was called "crude particles". The yellowish supernatant contained the "soluble enzymes".

Respirometry

The conventional Warburg respirometer was used at 30°. With resting cells each vessel contained 1.8 ml suspension as above, 10 μmoles substrate in 0.1 ml in the side-arm and 0.1 ml 20% KOH in the center well. Oxygen uptake was corrected for the endogenous respiration which was usually very small (about 0.4 μmole O_2/h). Oxygen uptake was never followed for more than 5 h. The results were expressed as mole O_2 uptake/mole substrate. The crude particle gel was suspended in 0.02 M phosphate buffer (pH 6.2) with a Potter–Elvehjem homogenizer to obtain a final turbidity of 400 units in the Klett colorimeter with filter 66, corresponding to about 2 mg protein/ml. 1.7 ml of the latter suspension and 30 μmoles $MgCl_2$ in 0.1 ml were added in the main Warburg vessel. The side-arm and the center well contained the ingredients as above. Final volume 2 ml.

When the end products of the oxidation had to be isolated and identified, the Warburg vessels contained in a final volume of 3 ml: 2.7 ml particle suspension (54 mg protein) in 0.02 M phosphate buffer (pH 6.2) and either 60 or 100 μmoles of substrate in the side-arm; KOH in the center well.

Dehydrogenase and reductase activity

The formation or disappearance of either $NADH_2$ or $NADPH_2$ was followed at 340 mμ in a Beckman spectrophotometer, equipped with an ERA and a Varian recorder. Dehydrogenase activity was measured in the system: 0.1–0.5 ml of enzyme

preparation, 15 μmoles $MgCl_2$ in 0.05 ml, 0.15 μmole NAD or NADP in 0.05 ml, 0.1 ml substrate (20 μmoles for secondary alcohol dehydrogenase or 200 μmoles for primary alcohol dehydrogenase). The final volume of 1.2 ml was made up with 0.05 M Tris–HCl buffer (pH 8.8). The reduction of keto and aldehyde groups was measured in the system: 0.1–0.5 ml of enzyme preparation, 15 μmoles $MgCl_2$ in 0.05 ml, 0.15 μmole $NADH_2$ freshly prepared in 0.05 ml, 20 μmoles substrate in 0.1 ml. The final volume of 1.2 ml was made up with 0.05 M phosphate buffer (pH 7.5). In both types of estimation the reaction was started by the addition of the substrate. The enzyme activity in the eluates of DEAE-cellulose columns was determined by recording the linear activity for 90 sec and calculating the increase in absorbancy in the period 15–75 sec. The formation of 1 μmole $NADH_2$ or $NADPH_2$ per min per l was defined as one International Unit per l (see ref. 21). Specific activity was defined as the number of units per mg protein, determined according to WARBURG AND CHRISTIAN[22].

Chemical and physical methods

Soluble Ca was determined titrimetrically according to WILLARD AND FURMAN[23]. Methyl-ketones were detected with the nitroprusside reagent[24]. Organic acids were detected by circular paper chromatography with the solvents: propanol – ammonia (80:20, v/v)[25], n-amylalcohol – 5 M formic acid (50:50, v/v)[26] and ethanol – water – ammonia (35:13:2, v/v)[27]. The chromatograms were sprayed with 2% ethanolic bromocresol green. Spraying with ammoniacal silver nitrate followed by heating at 105° for 5 min revealed the acids as white spots on a brown background[25]. Optical activity was determined with a Zeiss polarimeter (0°01), usually in a 2-dm tube at 5461 Å. When the content of the Warburg vessels (3 ml) was used, it was deproteinized with 1 ml 10% trichloroacetic acid and centrifuged. Optical rotation was measured against a blank consisting of the endogenous reaction mixture, treated in the same way. Melting points were determined in a Reichert "RCH" apparatus and are recorded uncorrected.

Chemicals

Acetylmethylcarbinol (82% D(−) and 18% L(+)) had previously been prepared by DE LEY[28] from DL-lactate with *A. aceti* (rancens) strain 23. It was repurified by distillation. DL-2,3-Butanediol, L(+)-1,2-propanediol, meso-3,4-hexanediol and (−)-3,4-hexanediol were from the collection of Mlle H. VAN RISSEGHEM and were obtained through the courtesy of Professor J. LÉONIS. Other alcohols, glycols, aldehydes and ketones were commercial products. They were purified by fractional distillation.

L-Erythrulose, dihydroxyacetone, acetol and (+)ethylpropionylcarbinol were prepared biochemically from meso-erythritol, glycerol, DL-1,2-propanediol and meso-3,4-hexanediol by oxidation with the crude particle fraction of *Gluconobacter oxydans* (suboxydans). The Warburg vessels contained 1.6 ml concentrated particle suspension, 30 μmoles $MgCl_2$ in 0.1 ml and 60 μmoles substrate in 0.3 ml. When O_2 uptake stopped, the suspension was centrifuged at 24000 × *g* for 1 h. 0.5 ml supernatant (15 μmoles of the keto-compound) was used to check for the $NADH_2$-linked reductase in the soluble fraction of *G. oxydans* (suboxydans).

Sephadex G-25 (Pharmacia, Sweden) was washed repeatedly with 0.01 M phosphate buffer (pH 6.5) to eliminate fine particles. DEAE-cellulose (Serva-Entwick-

lungslabor, Heidelberg, Germany) No. 27061 with a capacity of 0.68 mequiv/g was washed to eliminate fine particles and transformed into the free base with NaOH. After thorough washing with distilled water, it was charged with 0.1 M phosphate buffer (pH 6.5) and equilibrated with 0.01 M phosphate buffer (pH 6.5).

RESULTS

1. Oxidations with resting cells of various acetic acid bacteria

The pH optinum for the oxidation of DL-1,3-butanediol with resting cells of *A. aceti* (liquefaciens) strain 20* extended between 5.0 and 7.5. Therefore and also because acetic acid bacteria prefer a slightly acid pH for their oxidations, all Warburg experiments were carried out in 0.02 M phosphate buffer (pH 6.2). The oxidation of five diols by 14 different strains of acetic acid bacteria, representing a wide taxonomic variety, are summarized in Table I.

TABLE I

THE OXIDATION OF SOME GLYCOLS BY ACETIC ACID BACTERIA

Contents of the Warburg vessels: see text. The results are expressed as mole O_2 uptake/mole substrate after 180 min. When the reaction still continued after that time, it is shown by the sign >.

Biotype	*Strain*	*1,2-Ethanediol*	*1,2-Propanediol*	*Meso-2,3-butanediol*	*DL-2,3-Butanediol*	*1,4-Butanediol*
Acetobacter aceti	*Peroxydans group*					
	peroxydans (8618)	> 0.45	> 0.70	0.50	> 0.70	> 3.4
	paradoxus (P)	> 0.38		> 0.1	> 0.1	
Acetobacter aceti	*Oxydans group*					
	mobilis (6428)	> 0.21	> 1.1	> 4.5	1.0	> 3.3
	estunensis (E)	> 0.4	> 0.1	> 3.2	> 0.2	> 3.9
	lovaniensis (13)	> 0.5	> 0.8	> 4.7	> 0.8	> 3.7
	vini acetati (4939)	> 0.1	> 0.2	2.5	> 0.4	> 2.5
	rancens (23 kl +)	> 0.2	> 0.1	> 0.7	> 0.08	> 0.4
	rancens (D)		> 0.8	> 1.5	> 1.0	
Acetobacter aceti	*Mesoxydans group*					
	mesoxydans (8622)	> 0.1	> 0.5	> 3.0	1.0	> 1.7
	xylinum (8747)	> 0.5	> 0.7	> 2.0	1.0	> 1.8
	aceti (Ch31)		0.5	> 4.0		> 2.3
	liquefaciens (20)	0.7	> 1.2	0.5	1.0	> 3.7
Gluconobacter oxydans	*Suboxydans group*					
	melanogenus (8086)	0.7	> 1.0	0.5	1.0	> 1.3
	suboxydans (SU)	0.6	0.5	0.5	0.9	1.9

DL-*1,3-Butanediol*: All the strains of *Gluconobacter* and of the mesoxydans group of *Acetobacter* oxidized this substrate quickly to DL-β-hydroxybutyric acid (see below). The oxidation rate with strains of the oxydans and peroxydans groups of *Acetobacter* depended on the strain used: from 0.04 mole O_2/mole substrate/h for *A. aceti* (paradoxus) to 1.5 moles O_2/mole substrate/h for *A. aceti* (mobilis). The end point of the fast strains was again 1 mole O_2/mole substrate, indicating the formation of DL-β-hydroxybutyric acid.

* This strain was previously tentatively classified as a *Gluconobacter* due to the uncertainty of its flagellation. Its infrared spectrum (kindly examined by Dr. A. W. SCOPES) showed it to be an *Acetobacter*.

1,4-Butanediol: The most clear-cut case was represented by the suboxydans strain, which oxidized the substrate to the succinate stage only. The melanogenus strain oxidized more slowly, but also leveled off to the same final stage. Succinate was not oxidized further since these bacteria do not possess a Krebs cycle[29]. All the other strains (*A. aceti* (rancens 23 kl+) excepted, which was very slow with all substrates) took up O_2 far beyond the succinate stage, in some cases to near-completion.

Meso-2,3-butanediol: was oxidized to acetoin by the *Gluconobacter* strains and *A. aceti* (liquefaciens, 20). The end product was detected by the Voges–Proskauer reaction. All the strains of the mesoxydans and oxydans groups of *Acetobacter* oxidized the substrate far beyond the acetoin stage. The strains of the peroxydans group were very slow.

DL-*1,2-Propanediol* was oxidized to acetol (see below) by *G. oxydans* (suboxydans, SU) and by *A. aceti* (xylinum, 8747). The oxidation rate with the other strains varied widely, from hardly any oxidation at all as with *A. aceti* (rancens, 23 kl+) and *A. aceti* (estunensis, E) to very fast as with *A. aceti* (liquefaciens, 20) and *G. oxydans* (melanogenus 8086).

1,2-Ethanediol was oxidized very slowly by all strains. Only the *Gluconobacter* strains and *A. aceti* (liquefaciens, 20) reached an upper level of about 0.6 mole O_2/mole substrate after 3 h. Oxalic acid could not be detected by paper chromatography, only glycollic acid.

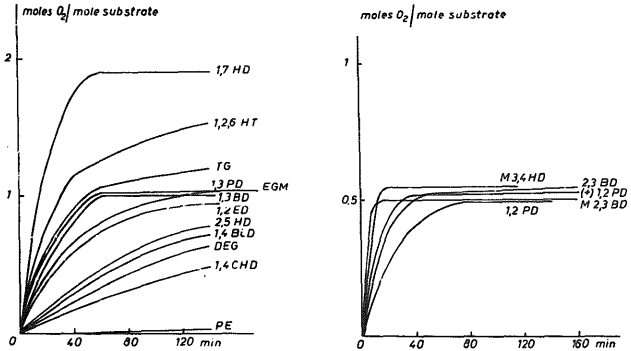
2. *Oxidations with resting cells of G. oxydans (suboxydans) strain SU*

In addition to the above substrates, the oxidation of the following glycols was studied with resting cells of the suboxydans strain: 1,3-propanediol, 1,5-propanediol, 1,6-hexanediol, 1,7-heptanediol, diethyleneglycol, triethyleneglycol, 2-ethyl-2-nitro-1,3-propanediol, 2-ethyl-2-amino-1,3-propanediol, pentaerythritol, 1,4-butinediol, 1,2,6-hexanetriol, 2,5-hexanediol, 1,4-cyclohexanediol, ethyleneglycol-monomethylether, DL-2,3-butanediol, L(+)-1,2-propanediol and meso-3,4-hexanediol. The results are represented in Figs. 1 and 2. The contents of the vessels, in which acids had been formed, were centrifuged, the supernatant decationized with Amberlite IR-120(H^+) and analyzed by paper chromatography. The results are summarized in Table II. From the O_2 uptake and the paper chromatographic evidence, we can put forward the following reactions:

1,2-ethanediol	→ glycollic acid
1,3-propanediol	→ β-hydroxypropionic acid (hydracrylic acid)
1,4-butanediol	→ succinic acid
DL-1,3-butanediol	→ DL-β-hydroxybutyric acid
1,5-pentanediol	→ glutaric acid
1,6-hexanediol	→ adipic acid
1,7-heptanediol	→ pimelic acid
ethyleneglycol-monomethylether	→ α-methoxy acetic acid

Since the C_4–C_7 ω-diols were apparently oxidized to the corresponding dicarboxylic acids, the intermediate formation of an ω-hydroxy carboxylic acid seemed possible. This was found to be the case with 1,4-butanediol and 1,5-pentanediol. The oxidation of two concentrations of both substrates by intact cells of the suboxydans strain is shown in Fig. 3. After 1 mole O_2/mole substrate had been taken up, the oxidation

rate decreased, even more so when the initial substrate concentration was lower. Circular paper chromatography with the solvent propanol–ammonia (80:20, v/v)



Figs. 1 and 2. Oxidation of several glycols by resting cells of *Gluconobacter oxydans* (suboxydans) strain SU. Content of the Warburg vessels, see text. Abbreviations used in this and the following figures: 1,2 ED: 1,2-ethanediol; 1,2 PD: DL-1,2-propanediol; (+)1,2 PD: L(+)-1,2-propanediol; 1,3 PD: 1,3-propanediol; 1,4 BD: 1,4-butanediol; 1,3 BD: DL-1,3-butanediol; 2,3 BD: DL-2,3-butanediol; M 2,3 BD: meso-2,3-butanediol; 1,4 BiD: 1,4-butinediol; (—)AMC: D(—)acetoin; 1,5 PD: 1,5-pentanediol; PE: pentaerythritol; 1,6 HD: 1,6-hexanediol; M 3,4 HD: meso-3,4-hexanediol; 2,5 HD: 2,5-hexanediol; 1,2,6 HT: 1,2,6-hexanetriol; 1,4 CHD: 1,4-cyclohexanediol; 1,7 HD: 1,7-heptanediol; EGM: ethyleneglycol monomethylether; DEG: diethyleneglycol; TG: triethyleneglycol.

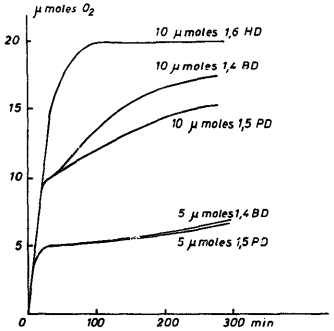


Fig. 3. The step-wise oxidation of some ω-diols by resting cells of *G. oxydans* (suboxydans) strain SU. Content of the Warburg vessels: see text. Substrate concentrations used are indicated near the curves. For abbreviations, see Fig. 1.

of the vessel content showed that from 1,5-pentanediol an acid with $R_F = 0.55$ had been formed which we believe to be δ-hydroxyvaleric acid. After 300 min the O_2 uptake had reached 1.8 moles O_2/mole substrate. Another Warburg vessel was then

TABLE II

PAPER CHROMATOGRAPHIC ANALYSIS OF THE CONTENT OF WARBURG VESSELS AFTER THE OXIDATION OF SEVERAL GLYCOLS BY RESTING CELLS OF *G. oxydans* (SUBOXYDANS) STRAIN SC

Content of Warburg vessels and procedure: see text.

Comp. und	R_F *in n-propanol – ammonia (80:20)*	R_F *in n-amylalcohol – formic acid (50:50)*	R_F *in ethanol – water – acetic acid (35:13:2)*
Oxidation product from 1,2-ethanediol	0.31	0.47	
Glycollic acid	0.31	0.47	
Oxalic acid	0.05		
Oxidation product from 1,3-propanediol	0.39		
β-hydroxypropionic acid	0.40		
Oxidation product from 1,3-butanediol	0.48	0.71	
β-hydroxybutyric acid	0.48	0.71	
Oxidation product from 1,4-butanediol			0.71
Succinic acid			0.70
Oxidation product from 1,5-pentanediol after { 1 mole O_2/mole substrate	0.55		
{ 1.8 mole O_2/mole substrate	0.15		
Glutaric acid	0.15		
Oxidation product from 1,6-hexanediol			0.78
Adipic acid			0.78
Oxidation product from 1,7-heptanediol			0.82
Pimelic acid			0.82

analyzed, which showed that the latter acid had disappeared and was replaced by an acid with the same R_F as glutaric acid. The oxidation thus occurred in two stages as represented by:

$$CH_2OH\cdot(CH_2)_3\cdot CH_2OH \longrightarrow COOH\cdot(CH_2)_3\cdot CH_2OH \longrightarrow COOH\cdot(CH_2)_3\cdot COOH$$

The results with 1,4-butanediol were similar and indicated that γ-hydroxybutyric acid was an intermediate in the formation of succinic acid, according to the reactions:

$$CH_2OH\cdot(CH_2)_2\cdot CH_2OH \longrightarrow COOH\cdot(CH_2)_2\cdot CH_2OH \longrightarrow COOH\cdot(CH_2)_2\cdot COOH$$

The oxidation of 1,6-hexanediol and 1,7-heptanediol showed no inflexion point, apparently both ends of the molecule were oxidized equally rapidly.

The end products of the oxidation of diethyleneglycol, triethyleneglycol, 1,4-butinediol, 1,2,6-hexanetriol, *cis*-1,4-cyclohexanediol and 2,5-hexanediol have not been identified, due partially to the slow oxidation and partially to the lack of reference compounds.

From the oxygen uptake with the following glycols, the reactions below could be put forward:

DL-1,2-propanediol	⟶ acetol
L(+)-1,2-propanediol	⟶ acetol
meso-2,3-butanediol	⟶ D(−) or L(+)-acetoin
DL-2,3-butanediol	⟶ DL-acetoin
meso-3,4-hexanediol	⟶ (+) or (−)ethylpropionyl-carbinol

The following compounds were not oxidized by resting cells of the suboxydans strain: 2-ethyl-2-nitro-1,3-propanediol, 2-ethyl-2-amino-1,3-propanediol and pentaerythritol. CUMMINS[14] reported that 2-methyl-2-nitro-1,3-propanediol and pentaerythritol were not oxidized by another strain of suboxydans.

3. Chemical identification of the oxidation products of some glycols by intact cells of G. oxydans (suboxydans) and A. aceti (liquefaciens)

3a. The oxidation product from DL-*1,3-butanediol*

A. aceti (liquefaciens) was unable to grow on a liquid medium with DL-1,3-butanediol as sole carbon source. FULMER, UNDERKOFLER AND BANTZ[12] and BUTLIN AND WINCE[6] had obtained similar results with *G. oxydans* (suboxydans) and meso-2,3-butanediol and DL-1,2-propanediol respectively. We tried to grow our strain on a medium with either ethanol or glycerol and in the presence of the diol. In these cultures, containing either 0.5, 1 or 2.5% of DL-1,3-butanediol, only trace amounts of acid were formed (estimated as soluble Ca and corrected for acetate formation). In concentrations of 5% or more of the diol there was even no growth. In cultures with glycerol, amounts as small as 0.5% of the diol inhibited growth. It was thus attempted to prepare the oxidation product with resting cells. The yield depended on the initial substrate concentration, 2.5% being optimal. When a new portion of 2.5% of the substrate was added after the first had been oxidized to completion, there was no further reaction. A similar phenomenon had been observed by BUTLIN AND WINCE[6] in the oxidation of 1,2-propanediol to acetol. Concentrations of 10% or more of the diol nearly abolished the reaction.

The following procedure was adopted for the isolation of DL-β-hydroxybutyric acid. 4 g of glucose-grown *A. aceti* (liquefaciens) were suspended in 100 ml of a 3% sterile solution of DL-1,3-butanediol in distilled water. The suspension was shaken at 30°. Titration with 0.1 N NaOH showed that 70% of the theoretically expected acid had been formed after 18 h. The bacteria were centrifuged off, the supernatant was saturated with ammonium sulphate, acidified with 5 ml of 25% sulfuric acid and continuously extracted for 6 h with ether. The ether phase was distilled, the remaining pale yellow acid oil dissolved in 7 ml aq. dest. and neutralized with 1 N NaOH. After distillation *in vacuo*, the crystalline Na salt was dissolved in the minimal amount of absolute ethanol and poured slowly under stirring in 50 ml of acetone. The crystals were filtered off and washed several times with ether. Melting point after two recrystallisations from hot ethanol: 163–164° (reported[30]: 163–164°).

The following additional criteria were used for identification. (a) The *p*-phenylphenacylester had a melting point of 105–106° after three crystallisations from acetone. Authentic *p*-phenylphenacylester of DL-β-hydroxybutyric acid had a melting point of 105–105.5°. The mixed melting point remained unchanged. (b) The equivalent

weight of the dry sodium salt was determined by potentiometric titration in glacial acetic acid with acetous perchloric acid[31] and was found to be 126.0. Theoretically expected: 125.9.

3b. The oxidation product from 1,2-ethanediol

The same procedure as above was followed, starting with a mixture of 1.5 % ethanediol, 4 % $CaCO_3$ and resting cells of *G. oxydans* (suboxydans). After ether extraction the Ca glycollate was purified twice by precipitation from acetone. The free acid was prepared with a column of Amberlite IR-120 (H^+) and concentrated *in vacuo* until crystals started to form. Crystallisation was continued at 4°. M.p. of the free acid: 73–75° (reported: 70–80° (see ref. 32) and 79° (see ref. 31)). M.p. of the *p*-bromophenacylester: 137–138° (reported[31]: 138°).

3c. The oxidation product from 1,3-propanediol

This oxidation product was prepared and the Na salt isolated as described *sub 3a*. M.p. of the Na salt: 141–142° (reported[30] for Na β-hydroxypropionate: 142°). The equivalent weight of the Na salt, determined according to VOGEL[31], 112.2 (calculated for Na β-hydroxypropionate: 112.0).

3d. The oxidation product from 1,4-butanediol

A mixture containing 2 g of resting cells of the suboxydans strain and 0.5 g of the diol in 50 ml distilled water was shaken for 2 days at 30°. The bacteria were centrifuged and the supernatant extracted with ether. The ether phase was distilled and the remaining acid was recrystallized from hot water. M.p.: 185–187° (reported[31] for succinic acid: 185°). M.p. of the *p*-bromophenacylester: 211–212° (reported[31]: 211°).

3e. The oxidation product from 1,5-pentanediol

A mixture, containing 2 g of resting cells of the liquefaciens strain and 50 mg of 1,5-pentanediol in 40 ml distilled water was shaken as above. When greater concentrations of the substrate were used, the oxidation did not go to completion. The free acid was isolated after ether extraction as above. It was recrystallized from benzene and had a m.p. of 96–97.5° (reported[31] for glutaric acid: 97–98°). M.p. of the di-*p*-phenylphenacylester, prepared according to VOGEL[31]: 151–152° (reported[31]: 152°).

3f. The oxidation product from 1,6-hexanediol

The same procedure as *sub 3d* was followed. M.p. of the free acid: 151–152° (reported[31] for adipic acid: 152°). M.p. of the *p*-bromophenacyl ester: 153.5–154.5° (reported[31]: 155°).

3g. The oxidation product from 1,7-heptanediol

This oxidation product was again obtained in the same manner. The free acid had a m.p. of 105° (reported[31] for pimelic acid 105°). The *p*-phenylphenacylester had a m.p. of 145–145.5° (reported[31]: 146°).

4. Oxidations by the crude particle fraction of G. oxydans (suboxydans)

Most of the glycols investigated were oxidized by this fraction. Some typical experiments are illustrated in Fig. 4 and the results are summarized in Table III.

TABLE III

OXIDATION OF SEVERAL GLYCOLS BY THE PARTICULATE FRACTION OF *G. oxydans* (SUBOXYDANS) STRAIN SU

Content of the Warburg vessels: see text. The oxygen uptake is recorded after 200 min and expressed as mole O_2 uptake/mole substrate. The nature of the presumed end products is concluded from oxygen uptake and paper chromatography, optical rotation and chemical tests.

Substrate	*O_2 uptake*	*Presumed end product*	*Nitroprusside test*	*$[\alpha]_{546}^{25}$ of Warburg vessel content*	*c in water*	*Reported $[\alpha]_{546}^{20}$*
Diols with primary OH groups						
1,2-Ethanediol	1.0	Glycollic acid				
1,3-Propanediol	0.95	β-Hydroxypropionic acid				
1,4-Butanediol	2.0	Succinic acid				
1,5-Pentanediol	2.0	Glutaric acid				
1,6-Hexanediol	1.98	Adipic acid				
1,7-Heptanediol	1.99	Pimelic acid				
Penta-erythritol	0.1					
2-Ethyl-2-nitro-1,3-propanediol	0					
2-Ethyl-2-amino-1,3-propanediol	0					
2-Amino-2-(hydroxymethyl)-1,3-propanediol	0					
1,4-Butinediol	>0.7					
Ethyleneglycol monomethylether	0.95					
Diethyleneglycol	>0.8					
Triethyleneglycol	>1.1					
Glycols with either secondary or primary and secondary OH groups						
DL-1,2-Propanediol	0.50	Acetol		0°	0.11	0°
L(+)-1,2-Propanediol	0.55	Acetol		0°	0.11	0°
DL-1,3-Butanediol	0.98	β-Hydroxybutyric acid				
Meso-2,3-butanediol	0.54	L(+)-Acetoin	+	+72°	0.22	+69°
DL-2,3-Butanediol	0.53	DL-Acetoin	+	0°	0.13	0°
(+,−,−) Acetoin	>0.06	Diacetyl				
Meso-3,4-hexanediol	0.51	(+)-Ethylpropionylcarbinol	+	+88°	0.17	+88°
2,5-Hexanediol	>0.6					
1,2,6-Hexanetriol	>1.4					
cis-1,4-Cyclohexanediol	>0.3					

ω-Diols with 2 and 3 carbon atoms were oxidized to the corresponding hydroxycarboxylic acids; ω-diols with 4–7 carbon atoms were oxidized to the corresponding dicarboxylic acids. Just as with intact cells the C_4 and C_5 ω-diols showed an inflexion point when 1 mole O_2/mole substrate had been taken up; C_6 and C_7 ω-diols were oxidized at once to the stage of 2 moles O_2/mole substrate. Fig. 5 illustrates an experiment. The presumed γ-hydroxybutyric acid was oxidized faster when the concentration of both enzyme and substrate was higher. Paper chromatography showed that the end products of the oxidation with crude particles and with intact cells were the same. The oxidation product from DL-1,3-butanediol was expected to be DL-β-hydroxybutyric acid as judged from the O_2 uptake. It was isolated by ether extraction and identified by the m.p. of its Na salt: 164–165°.

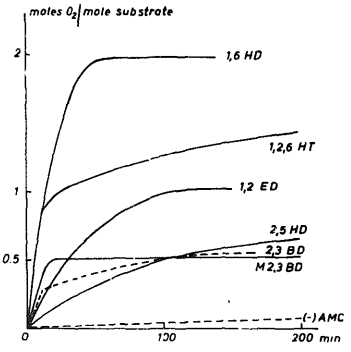


Fig. 4. Oxidation of some glycols by the particulate fraction of *G. oxydans* (suboxydans) strain SU. Content of the Warburg vessels: see text. Abbreviations as in Fig. 1.

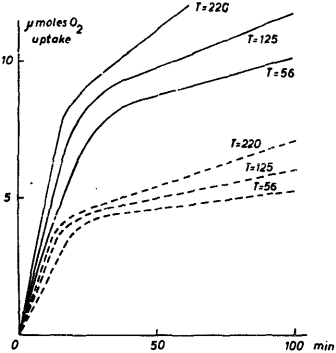


Fig. 5. Oxidation of two concentration levels (5 μmoles per Warburg vessel: lower broken lines and 10 μmoles per Warburg vessel: upper full lines) of 1,4-butanediol by several concentrations of the particulate fraction of *G. oxydans* (suboxydans) strain SU. T indicates the concentration of the particulate fraction expressed as Klett units with filter 66. Content of the Warburg vessels: see text.

Both D- and L-1,2-propanediol were oxidized to acetol, as evidenced by O_2 uptake, lack of optical rotation after oxidation ($c = 0.11$ in water, $l = 2$) and the reduction of the Fehling reagent. Lactaldehyde formation is ruled out since L(+)-1,2-propanediol ought to yield an optically active lactaldehyde. The end product showed again no optical activity.

Meso-2,3-butanediol was oxidized at the D-OH-group, since L(+)-acetylmethylcarbinol was formed. Meso-3,4-hexanediol was oxidized to (+)-ethylpropionylcarbinol.

The following experiments show that both D- and L-2,3-butanediol were oxidized to D- and L-acetoin. The D isomer was oxidized about 6 times as fast as the L form. The oxidation curve of DL-2,3-butanediol showed an inflexion point at 0.25 mole O_2/mole substrate (see Fig. 4). Supposing that only the D isomer had been oxidized, 30 μmoles of D(—)-acetoin and 30 μmoles of L(+)-2,3-butanediol had to be expected. The content of the Warburg vessels (60 μmoles substrate) after deproteinisation was

levorotatory, $\alpha = -0.07°$. The specific rotation of the end product, corrected for the rotation of L(+)-2,3-butanediol, was calculated to be $[\alpha]_{546}^{25} = -61°$ ($c = 0.066$ in water) which was in good agreement with the known value for D(−)-acetoin[10]. After 200 min the oxidation stopped when 0.5 mole O_2/mole substrate had been taken up. Two explanations are possible: either L(+)-2,3-butanediol was oxidized to L(+)-acetoin or D(−)-acetoin had been oxidized to diacetyl. The former explanation appeared to be the true one since (a) the reaction mixture was optically inactive, (b) only traces of diacetyl were found, and (c) D(−)-acetoin in separate experiments was oxidized only very slowly.

Since 2,5-hexanediol, *cis*-1,4-cyclohexanediol and 1,2,6-hexanetriol continued to take up oxygen even after 5 h, no effort was made to determine the nature of the end products. Finally it may be mentioned that the oxidation rates for all the above substrates were stimulated by Mg^{2+}.

5. *Soluble dehydrogenases*

Preliminary experiments showed that most glycols were oxidized by NAD with the "soluble-enzyme" fraction of the suboxydans strain. There was no reaction with NADP. The following purification procedure showed that two types of enzymes were involved: a primary and a secondary alcohol dehydrogenase. 2.5 ml 1 M $MnCl_2$ were added at 4° under stirring to 50 ml of the "soluble-enzyme" fraction, containing about 150 mg protein. After 2 h the precipitate was centrifuged off and the excess of $MnCl_2$ in the supernatant was eliminated by gel filtration on a Sephadex G-25 column. The eluate was saturated for 55% with ammonium sulphate. The precipitate was collected by centrifugation, dissolved in 5 ml 0.01 M phosphate buffer (pH 6.5) and the ammonium sulphate was eliminated by gel filtration on a Sephadex G-25 column, previously equilibrated with the same buffer. The resulting solution was adsorbed on a 2.5 × 20 cm column of DEAE-cellulose, previously equilibrated with 0.01 M phosphate buffer (pH 6.5). Proteins were eluted by an increasing NaCl-gradient in the same buffer. The mixing chamber contained 450 ml 0.01 M phosphate buffer (pH 6.5), the upper vessel contained 0.15 M NaCl in the same buffer. Fractions of 4 ml were collected. All manipulations were carried out at 4°. Fig. 6 represents the results of an experiment: a primary alcohol dehydrogenase was clearly separated from a secondary alcohol dehydrogenase. In both cases the specific activity had increased about 12 times. Fractions 62–66, representing the peak of the primary alcohol dehydrogenase, and fractions 83, 86 and 88, representing the peak of the secondary alcohol dehydrogenase, were collected and used to determine the enzyme specificity. The results are summarized in Table IV.

5a. *The primary alcohol dehydrogenase*

The following arguments showed that only one enzyme was concerned, and not a collection of closely related primary alcohol dehydrogenases, each with limited specificity.

(a) The reaction rates with *n*-propanol and 1,5-pentanediol were compared for each fraction of the curve. The ratio was always about the same, being (specific activity *n*-propanol/specific activity 1,5-pentanediol = 2.5).

(b) The reaction rates were measured with either *n*-propanol in the presence of 1,5-pentanediol, *n*-propanol in the presence of 1,7-heptanediol or each of the sub-

strates separately (Table V). The reaction rates were not additive. When two different enzymes are involved, the reaction rates would be additive, as illustrated in Table VI: the reaction rate with both *n*-propanol and meso-2,3-butanediol (0.150) was the sum of the rate with *n*-propanol (0.025) and meso-2,3-butanediol (0.125) separately. The former substrate was indeed oxidized by the primary, the latter by the secondary alcohol dehydrogenase. The oxidation of $NADH_2$ in the presence of acetaldehyde, propionaldehyde and β-hydroxybutyrylaldehyde (Table V) made it likely that the reactions were reversible and that the aldehydes were the end products from the primary alcohol dehydrogenase activity.

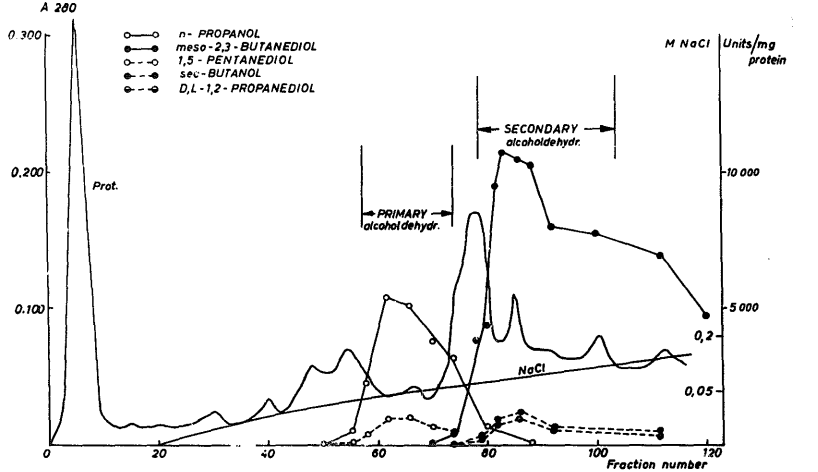


Fig. 6. Chromatographic separation on DEAE-cellulose–phosphate of the 0–55% ammonium sulphate saturated fraction of the "soluble enzymes" from *G. oxydans* (suboxydans) strain SU. The curve NaCl shows the NaCl gradient (left side of the right ordinate). In the curve Prot. the protein content is expressed as absorbancy at 280 mμ (left ordinate). The other curves represent the enzyme activity with the substrates indicated on the graph, determined as described in the text and expressed as units/mg protein (right side of the right ordinate).

5b. The secondary alcohol dehydrogenase

Arguments of the same type as above made it likely that only one enzyme was involved in the oxidation of the different substrates.

(a) The activity with meso-2,3-butanediol, *sec.*-butanol and DL-1,2-propanediol of the different fractions of the curve was proportionally the same.

(b) The reaction rates with mixtures of two substrates pointed to one single enzyme (Table VI).

The reaction product from monohydric secondary alcohols was obviously the corresponding ketone. With glycols this required further investigation. Preliminary experiments on the reversibility of the reaction with some ketones and $NADH_2$ (Table IV) suggested that indeed the secondary alcohol function was oxidized.

TABLE IV

SUBSTRATE SPECIFICITY OF THE PURIFIED SOLUBLE NAD-LINKED ALCOHOL DEHYDROGENASES

Purification: see text and Fig. 6. Estimation of enzyme activity: see text. The relative oxidation rates ware expressed against *n*-propanol or meso-2,3-butanediol, arbitrarily put at 100. The reduction of ketones and aldehydes was determined qualitatively with $NADH_2$ in the Beckman spectrophotometer, model DU at 340 mμ.

Primary alcohol dehydrogenase		*Secondary alcohol dehydrogenase*	
n-Propanol	100	Meso-2,3-butanediol	100
Ethanol	67	DL-2,3-Butanediol	195
n-Butanol	17	DL-1,2-Propanediol	9
iso-Butanol	3	L(+)-1,2-Propanediol	17
n-Hexanol	16	Meso-3,4-hexanediol	10
Allyalcohol	8	(−)-3,4-Hexanediol	64
DL-1,3-Butanediol	2	*Sec.*-butanol	12
Ethyleneglycol monomethylether	17	*Sec.*-propanol	1
1,2-Ethanediol	28	Cyclopentanol	2
1,3-Propanediol	22	Cyclohexanol	4
1,4-Butanediol	25	Cycloheptanol	30
1,5-Pentanediol	30	Cyclooctanol	29
1,6-Hexanediol	42	Glycerol	0
1,7-Heptanediol	67	Meso-erythritol	0
Glycerol	0	Na DL-lactate	0
DL-1,2-Propanediol	0	Na DL-β-hydroxybutyrate	0
L(+)-1,2-Propanediol	0	Na phosphoglycerate	0
Diethyleneglycol	0	Methanol	0
Triethyleneglycol	0	*Tert.*-butanol	0
2-Ethyl-2-nitro-1,3-propanediol	0		
2-Ethyl-2-amino-1,3-propanediol	0		
Methanol	0		
Tert.-butanol	0		
Reduction of aldehydes		*Reduction of keto groups*	
Acetaldehyde	++	Diacetyl	+++
Propionaldehyde	+	D(−)-Acetoin	++
β-Hydroxybutyraldehyde	++	Acetol	+
Glyceraldehyde	—	(+)-Ethylpropionylcarbinol	+
		Cyclopentanone	+
		Cycloheptanone	+
		Ethylmethylketone	+
		L-Erythrulose	—
		Dihydroxyacetone	—
		Na pyruvate	—

Furthermore it was tried to obtain larger amounts of some of the end products by coupling $NADH_2$ formation with a suitable acceptor. *N*-methylphenazinium-methosulphate, 2,6-dichlorophenolindophenol, methylene blue and ferricyanide reacted too slowly. A better method consisted in coupling with the particulate $NADH_2$-oxidase system from *Ps. fluorescens*. The "crude-particle" fraction of these bacteria was prepared in the same way as described for acetic acid bacteria. This fraction was devoid of dehydrogenases for secondary alcohols and glycols. The pH optimum for the coupled oxidation was about 7.6. In a typical experiment the Warburg vessels contained: 1.1 ml of the "soluble-enzyme" fraction of the suboxydans strain in 0.01 M phosphate buffer (pH 5.2), 0.5 ml of the "crude particle" suspension of *Ps. fluorescens* in 0.02 M phosphate buffer (pH 7.6) (turbidity 400 Klett units with filter 66), 0.3 ml Tris–HCl buffer (pH 7.6), 30 μmoles $MgCl_2$ and 2 μmoles NAD. The side arm contained 60 μmoles substrate. Final volume 3 ml and final pH 7.6.

TABLE V

NON-ADDITIVE REDUCTION RATE OF NAD BY MIXTURES OF SUBSTRATES FOR THE PRIMARY ALCOHOL DEHYDROGENASE OF *G. oxydans* (SUBOXYDANS) STRAIN SU

The reaction mixture contained: dialyzed "soluble-enzyme" fraction of the suboxydans strain (0.2 mg protein), 0.15 μmole NAD, 15 μmoles $MgCl_2$ and the amounts of substrate shown below in a final volume of 1 ml 0.05 M Tris–HCl buffer (pH 8.7). The results are expressed as the increase in absorbancy per min at 340 mμ in the Beckman spectrophotometer model DU.

Substrate	*Final molar concentration*	*Reaction rate*
n-Propanol	$1 \cdot 10^{-1}$	0.092
	$2 \cdot 10^{-2}$	0.090
	$1 \cdot 10^{-2}$	0.082
	$5 \cdot 10^{-3}$	0.076
1,5-Pentanediol	$1 \cdot 10^{-1}$	0.040
	$5 \cdot 10^{-2}$	0.034
	$2 \cdot 10^{-2}$	0.020
1,7-Heptanediol	$1 \cdot 10^{-1}$	0.060
n-Propanol and 1,5-pentanediol	$5 \cdot 10^{-2}$ + $1 \cdot 10^{-1}$	0.080
n-Propanol and 1,7-heptanediol	$5 \cdot 10^{-2}$ + $1 \cdot 10^{-1}$	0.080

TABLE VI

NON-ADDITIVE REDUCTION RATE OF NAD BY MIXTURES OF SUBSTRATES FOR THE SECONDARY ALCOHOL DEHYDROGENASE OF *G. oxydans* (SUBOXYDANS) STRAIN SU

Additive reduction rate for mixtures of two enzymes: the primary and secondary alcohol dehydrogenases. The reaction mixture contained: dialyzed "soluble-enzyme" fraction of the suboxydans strain (0.04 mg protein), 0.15 μmole NAD, 15 μmoles $MgCl_2$ and the amounts of substrate shown below in a final volume of 1 ml 0.05 M Tris–HCl buffer (pH 8.7). The results are expressed as in Table V.

Substrate	*Final molar concentration*	*Reaction rate*
Meso-2,3-butanediol	$4 \cdot 10^{-2}$	0.125
	$1 \cdot 10^{-2}$	0.115
	$4 \cdot 10^{-3}$	0.090
	$1 \cdot 10^{-3}$	0.030
n-Propanol	$2 \cdot 10^{-2}$	0.025
L(+)-1,2-Propanediol	$2 \cdot 10^{-2}$	0.030
Meso-2,3-butanediol and *n*-propanol	$4 \cdot 10^{-2}$ + $2 \cdot 10^{-2}$	0.150
Meso-2,3-butanediol and L(+)-1,2-propanediol	$4 \cdot 10^{-2}$ + $2 \cdot 10^{-2}$	0.100

Control vessel contained either no substrate, no NAD or no substrate and no NAD. A typical oxygen-uptake curve is represented in Fig. 7. After 10 h of oxidation the proteins were precipitated with trichloroacetic acid, final concentration 2 %. The contents of several Warburg vessels were pooled and dried overnight *in vacuo* over $CaCl_2$. The amount of the presumed end product was calculated from the O_2 uptake. Optical rotation was determined as before against the controls, treated in the same way. Table VII shows that meso-2,3-butanediol was oxidized to L(+)-acetoin, as in

the case of the particulate enzymes. Meso-3,4-hexanediol was likewise oxidized to (+)ethylpropionylcarbinol.

TABLE VII

END PRODUCTS OF THE OXIDATION OF TWO MESO-DIOLS BY THE NAD-LINKED SOLUBLE SECUNDARY ALCOHOL DEHYDROGENASE OF *G. oxydans* (SUBOXYDANS) STRAIN SU

Content of the Warburg vessels and procedure for the estimation of the optical rotation: see text.

Substrate	$[\alpha]_{546}^{25}$ *of the oxidation product*	*c in water*	*Nitroprusside test*	*Presumed end product*
Meso-2,3-butanediol	+62°	0.105	+	L(+)-acetoin
Meso-3,4-hexanediol	+84	0.066	+	(+)-ethylpropionylcarbinol

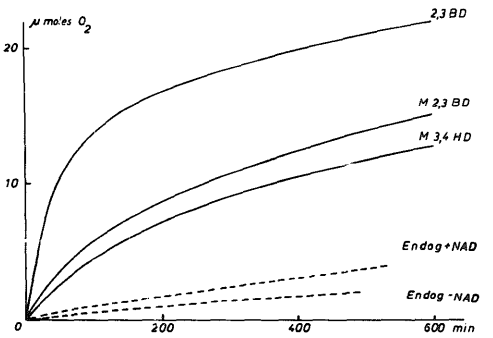


Fig. 7. Oxidation of some glycols in the presence of NAD and the "soluble enzymes" of *G. oxydans* (suboxydans) strain SU, coupled with the $NADH_2$-oxidative system of *Ps. fluorescens*. Abbreviations as in Fig. 1. 60 μmoles substrate. Content of the Warburg vessels: see text.

DISCUSSION

At least three enzymes are involved in the oxidation of aliphatic glycols by acetic acid bacteria: a soluble NAD-linked primary alcohol dehydrogenase, a soluble NAD-linked secondary alcohol dehydrogenase and one or more particulate oxidative systems. From previous experience in this laboratory[33,34] on the localisation of enzymes in the cell of acetic acid bacteria, it can be said that the soluble enzymes are located in the cytoplasm and the particulate one(s) are attached to the cytoplasmic membrane.

1. The soluble NAD-linked primary alcohol dehydrogenase

The enzyme seems to require the $>CH \cdot CH_2OH$ group. That two carbons are required can be seen from the fact that methanol is not oxidized. A second H atom at C-2 is not absolutely required, since allyl alcohol is oxidized, but its presence improves the oxidation (compare allyl alcohol and *n*-propanol). The presence of an OH group or a second CH_2OH group at C-2 decreases the activity of the enzyme

(compare ethanol with 1,2-ethanediol and 1,3-propanediol, also *n*-propanol and 1,3-propanediol) and the presence of both groups, as in glycerol, completely prevents the activity of the enzyme. The presence of a secondary OH group alone in C-2 seems to be enough to prevent enzyme activity (compare *n*-propanol with 1,2-propanediol). The harmful effect of the second CH_2OH group is nicely illustrated in the series of the ω-diols from 1,3-propanediol to 1,7-heptanediol, where the enzyme activity increases with the chain length: the farther the second CH_2OH group is removed from the one to be attacked, the better the enzyme works. Also, when C-2 is completely surrounded by polar groups as in 2-ethyl-2-nitro-1,3-propanediol, 2-ethyl-2-amino-1,3-propanediol, pentaerythritol or Tris, the substrate is not attacked. Etherification of the second primary alcohol group has a further deleterious effect, as illustrated by the decrease of activity in the series 1,2-ethanediol, ethylene glycol monomethyl-ether and di- and triethylene glycol. Summarizing it can be said that the addition of a polar group on or in the vicinity of the C-2 of the $>CH\cdot CH_2OH$ structure decreases or can completely inhibit enzyme activity.

The end products of the oxidation are most likely the corresponding aldehydes as pointed out before. KING AND CHELDELIN[35] demonstrated the presence of relatively unspecific NAD- and NADP-linked aldehyde dehydrogenases in *G. oxydans* (suboxydans). These enzymes were detected in several other acetic acid bacteria by DE LEY AND SCHELL[19,36]. The oxidation products of the combined activities of both primary alcohol dehydrogenase and aldehyde dehydrogenases are probably the same as with the particulate enzymes.

2. The soluble NAD-linked secondary alcohol dehydrogenase

Glycols with a secondary OH group are oxidized by an enzyme with very wide specificity. The presence of an adjacent OH group improves the enzyme activity, *e.g.* 1,2-propanediol is oxidized faster than iso-propanol, both DL- and meso-2,3-butanediol are oxidized faster than *sec.*-butanol. Nevertheless this enzyme can not be called "glycol dehydrogenase" because monohydric compounds are quite readily oxidized. The presence of the carboxylic ion, as in lactate, phosphoglycerate and β-hydroxybutyrate, prevents enzyme activity. The presence of a third OH group as in glycerol, is likewise deleterious. The configurational specificity of this enzyme could not be established, due to lack of pure isomers. In both meso-2,3-butanediol and meso-3,4-hexanediol, it is the D-OH group which is oxidized. Apparently the C=O function prevents the oxidation of the L configuration in the resulting carbinols. However, also the L-OH can be oxidized as testified by the reaction with L(+)-1,2-propanediol and (−)3,4-hexanediol. According to VAN RISSEGHEM[13] the latter compound has the L configuration. Other stereoisomers were not available. It can tentatively be concluded that the secondary alcohol dehydrogenase can oxidize both L- and D-OH groups.

The end products of the oxidation appear to be the corresponding ketones. This was shown by direct proof for meso-2,3-butanediol and meso-3,4-hexanediol and also by the reversibility of the reaction with available ketones (see Table IV).

This enzyme appears to be different from the NAD-linked 2,3-butanediol dehydrogenases, which are specific for either D(−) or L(+) configuration, as found by TAYLOR AND JUNI[37] in several bacteria. LAMBORG AND KAPLAN[38] found an NAD-linked glycol dehydrogenase which oxidized glycerol and 1,2-propanediol in *Aerobacter aero-*

genes, Escherichia coli and *G. suboxydans*. Our purified enzyme did not oxidize glycerol.

3. The particulate oxidative systems

The oxidation of the glycols proceeds with the uptake of oxygen. Our previous experience with the particulate fraction makes it likely that electron transport occurs by way of the cytochrome system.

The oxidation of the primary alcohol group presents the following characteristics. (a) Oxidation of ω-diols depends on the distance between the terminal CH_2OH groups. When they are close together as in 1,2-ethanediol and 1,3-propanediol, only one of them is being oxidized. The resulting COO^- group apparently prevents further enzyme action. With greater distance, from 1,4-butanediol on, first one group is oxidized with the probable formation of an ω-hydroxyacid (γ-hydroxybutyric and δ-hydroxyvaleric acids), followed by the oxidation of the second CH_2OH group. The enzyme thus prefers to oxidize a molecule with a CH_2OH at the distal end over one with a COO^- group, illustrating again the harmful effect of the latter function. With longer molecules such as 1,6-hexanediol and 1,7-heptanediol, the negative influence of the carboxyl group becomes negligible and the oxidation proceeds with both types of molecules at once to the dicarboxylic acid. (b) The optical configuration has no importance as testified by the oxidation of both D- and L-1,3-butanediol.

It seems likely that the primary oxidation product of the particulate enzyme(s) is the corresponding aldehyde, which is then oxidized further by the same fraction to the acid. The aldehydes corresponding to all the compounds studied were not available to investigate this point, but it has been established that the particulate fraction readily oxidizes acetaldehyde (DE LEY AND SCHELL[36]), formaldehyde, DL-glyceraldehyde, propionaldehyde, iso- and *n*-butyraldehyde (unpublished results).

When stereoisomeric secondary alcohols were investigated, both forms were oxidized (D- and L-1,2-propanediol and D- and L-2,3-butanediol). HUFF[39] expressed doubt whether acetic acid bacteria produce acetol from 1,2-propanediol, because several tests used to identify acetol do not distinguish between this compound and lactaldehyde. There are, however, two arguments in favour of acetol. First, COPET *et al.*[7] crystallized a semicarbazide with the same melting point as HUFF's acetol-semicarbazide, while, according to HUFF, no crystals would be formed with lactaldehyde. Second, we found that the oxidation product from L(+)-1,2-propanediol was optically inactive as expected for acetol; lactaldehyde would have been optically active.

The elucidation of the nature of the particulate dehydrogenase(s) will have to await further study with solubilized preparations.

In previous papers from this laboratory it was pointed out that acetic acid bacteria decompose several substrates (*e.g.* hexoses, gluconate, 2-ketogluconate, lactate, some polyols, etc.) by two enzyme systems: one particulate, the other soluble. The same situation was again encountered for the oxidation of nearly all the glycols investigated.

4. Oxidations by resting cells of G. oxydans (suboxydans)

By comparing the results of the oxidations by intact cells with these of the particulate fraction, it is seen that the oxidation products are exactly the same in all the cases studied. Even details of the mode of oxidation were identical, such as the

two-step oxidation of 1,4-butanediol and of 1,5-pentanediol. Both intact cells and the particulate fraction have a pH optimum around 6. The soluble dehydrogenases have a pH optimum in the alkaline region. In neutral or slightly acid medium the reduction of the ketone or aldehyde group is favoured. From these reasons it follows that the oxidations of glycols effected by intact cells, are mainly, if not solely, the result of the enzymic activity on the cytoplasmic membrane. We believe that the formation of the oxidation products from glycols, as described by previous authors with other strains of acetic acid bacteria, can be understood in the same way.

Where our experiments with resting cells and particulate fraction overlapped those of other authors with thorougly aerated growing cultures and resting cells, the results qualitatively confirmed each other with the exception of L(+)-2,3-butanediol. UNDERKOFLER *et al.*[11] found that it was not oxidized, according to GRIVSKY[10] it was oxidized slowly and with the particulate fraction of our strain it was oxidized six times more slowly than the D(−) isomer. This difference probably is a matter of strain individuality.

5. Oxidations by other strains of Gluconobacter and Acetobacter

Representative strains of both biotypes have been selected, such as to cover the entire taxonomic range of the acetic acid bacteria. Table I shows that there is a wide range amongst the strains both in the rate of oxygen uptake and in the final stage of oxidation. Simplifying, it can be said that the basis types are represented by the acetate non-oxidizing *Gluconobacter* strains, which oxidize the glycols merely to the end products described in the present paper and by previous authors. Whether strains of *Acetobacter* will oxidize these compounds further or not and at what rate is a matter of strain individuality.

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