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# Further Selective Redox Reactions on a Preparative Scale with Anaerobes and Facultative Anaerobes

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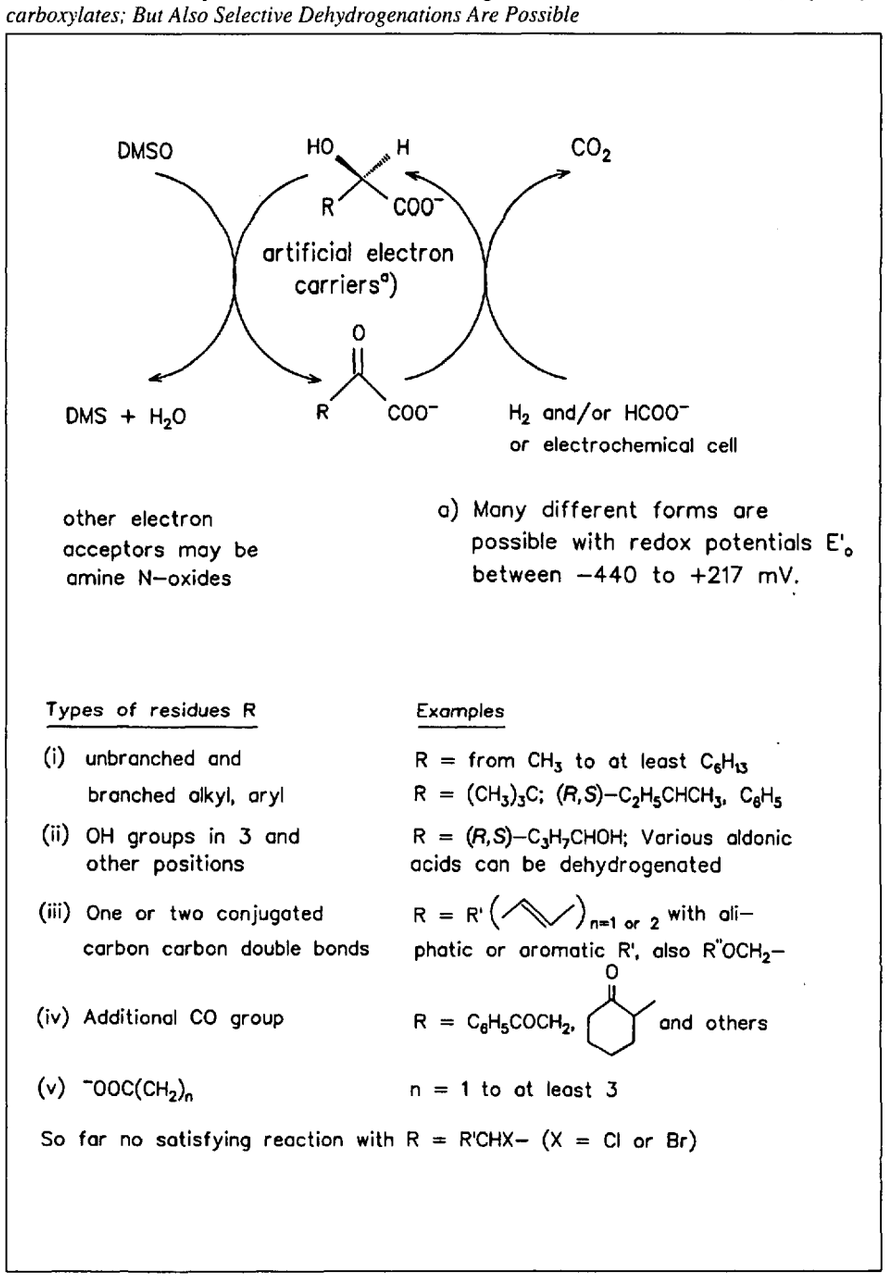
We showed that various anaerobes or anaerobically grown facultative bacteria are very effective catalysts for highly selective reductions at the expense of hydrogen gas, formate, or carbon monoxide. The productivity numbers are usually 1–3 orders of magnitude higher than those for yeasts with carbohydrates as electron donors, and new types of reactions can be catalysed [1–3]. But also selective dehydrogenations can be very effectively conducted with anaerobes. Often, but not always, non-pyridine-nucleotide-dependent enzymes are involved for which many different artificial electron carriers can be used. Many anaerobes also possess high activities of viologen-accepting pyridine-nucleotide-oxidoreductases (VAPORs) catalysing *Reaction 5* and/or *6* [4][5]. Mitochondria are especially active in the catalysis of *Reaction 5* [6]. Therefore, properly grown yeasts having high contents of mitochondria are *ca.* 50–60 times more effective in electrochemical cells than when used with carbohydrates as electron donors. The electrochemical cell delivers electrons which are transported into the cells by the viologens [7]. By this methodology, yeasts might even be suitable for selective dehydrogenations.

Resting cells of *Proteus vulgaris* and *P. mirabilis* reduce at the expense of formate and/or hydrogen gas an extremely broad range of 2-oxocarboxylates to enantiomerically pure (2*R*)-hydroxycarboxylates. The *Scheme* shows examples. Product concentrations up to 250 mM have been obtained. The yields are always greater than 95% and the ee values are higher than 98% [8–10]. Artificial electron mediators such as viologens or cobalt sepulchrate in catalytic concentrations of 1–5 mM accelerate the reductions and stabilise them at least up to 400–600 h. The biocatalyst can also be immobilised [11]. With a series of (2*R*)-hydroxy-3-enoates prepared for the first time, we demonstrated that by epoxidation, hydroxylation, or addition of bromine or iodine, products with three chiral centers can be obtained with reasonable-to-excellent diastereoselectivity [12]. Also 5-methoxypentono-1,4-lactones of the L-*arabino*-, D-*ribo*-, L-*lyxo*-, and D-*xylo*-series were prepared [13]. Further interesting starting materials for selective

Scheme. *Reductions of 2-Oxo-acids with* Proteus vulgaris *or* P. mirabilis *Lead to (2R)-Hydroxycarboxylates; But Also Selective Dehydrogenations Are Possible*

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synthesis should be (2*R*)-hydroxy-4-oxo-carboxylates [10], since the activated C(3) position and the 4-oxo group are points for further reactions under the influence of the chiral center at C(2).

In the following three groups of new examples for the use of *Proteus* and *Clostridia* species in selective redox reactions will be shown:

*i*) Use of two *Proteus* species for selective dehydrogenations of (2*R*)-hydroxy-carboxylates to 2-oxo-carboxylates [14] [15], *ii*) preparation of some chiral succinate derivatives, and *iii*) use of crude extracts from *Clostridium thermoaceticum* or partially purified systems from it for the effective regeneration of NADH, NAD, NADPH, and NADP demonstrated by various selective reactions leading to the production of (*R*)- and (*S*)-isocitrate, ribulose-5-phosphate and others.

The non-pyridine-nucleotide-dependent enzyme (2*R*)-hydroxycarboxylate-viologen-oxidoreductase (HVOR), present in *P. vulgaris* or *P. mirabilis* catalysing the aforementioned reductions, is also able to dehydrogenate quantitatively a great variety of (2*R*)-hydroxy-carboxylates to 2-oxo-carboxylates (*Scheme*). This was used for the preparation of pyruvate from (*R*)-lactate. On an industrial scale, racemic and stereochemically pure forms of lactate are much cheaper than pyruvate.

For various synthetic or biosynthetic methods, pyruvate is an interesting building block. Compared with other small nonchiral building blocks pyruvate is relatively expensive. In the last six years, more than 30 patents dealt with the preparation of pyruvate. Pyruvate formation by fermentation usually does not lead to high space time yields. But also yields as such and concentrations are often not very high. This can be seen from a recent paper in which pyruvate is produced from gluconate [16].

As recently shown, *P. vulgaris* and *P. mirabilis* can be grown with activities up to 30 U/mg protein in the crude extract for reductions of 2-oxo-carboxylates and *ca.* 5–6 U/mg protein for dehydrogenations of (2*R*)-hydroxy-carboxylates [14]. The purification of the enzyme with specific activities up to *ca.* 1000 U/mg protein has been described [9]. Further properties of this new type of a Mo-containing enzyme have to be published. The natural electron carrier of the HVOR is not known.

The dehydrogenation of (*R*)-lactate to pyruvate or of other (2*R*)-hydroxy acids needs from a microorganism only one enzyme when the reoxidation of the reduced electron acceptor can be achieved by chemical or electrochemical means, or if the electron acceptor is stoichiometrically applied.

$$RCHOHCOO^- + Acc_{ox} \rightarrow RCOCOO^- + Acc_{red} + 2H^+ \quad (1)$$

$$Acc_{red} - 2e \rightarrow Acc_{ox} \quad (2)$$

Two enzymes are necessary, if the electron carrier $Acc_{ox}$ is regenerated enzymatically.

We quantitatively prepared by various methods pyruvate from 500 to 650 mM solutions of (*R*)-lactate with *P. vulgaris* or *P. mirabilis*. Besides the already mentioned artificial electron carriers viologens or Co-sepulchrate, many others are rather active especially for dehydrogenation. We measured the relative activities of 14 different mediators with redox potentials between $E_0' = -440$ to +217 mV [15]. Again it turned out that neither NAD nor NADP are cosubstrates. Under various aspects anthraquinone-2,6-disulfonate (AQDS) was especially useful. It works not only very well with HVOR but also with dimethyl sulfoxide (DMSO) reductase. AQDS is stable, can be reisolated, and is also useful in the electrochemical cell. Furthermore, it has reasonable $K_m$ values and the $K_i$ values are rather high. That means, in the here described cases, the Acc mentioned in *Reaction 1* was AQDS.

Anaerobically grown *Proteus* species like *Escherichia coli* contain rather high activities of pyruvate formate-lyase. Therefore, it was necessary to block this enzyme. Otherwise the pyruvate formed from lactate would have been metabolised further. Based on the mechanism of this enzyme [17], we complexed the $Fe^{++}$ necessary for its activation with 5–10 mM concentrations of ethylenediaminetetraacetic acid (EDTA). This stabilising effect of EDTA for pyruvate was the prerequisite for the preparation of pyruvate from (*R*)-lactate according to *Reaction 1*.

The reoxidation of the reduced mediator can be achieved by various methods. Besides others, the following reactions catalysed by enzymes present in both of the applied *Proteus* species have been studied [15]:

$$DMSO + AQDS_{red} \rightarrow DMS + AQDS_{ox} + H_2O \quad (3)$$

$$R_3NO + AQDS_{red} \rightarrow R_3N + AQDS_{ox} + H_2O \quad (4)$$

The experiments have been carried out with *P. mirabilis* or *P. vulgaris* in a range from 1.8 g $l^{-1}$ to 14.4 g $l^{-1}$. In the latter case, the actual space time yield was *ca.* 128 mol $l^{-1}d^{-1}$. We assume that up to 40 g of cells can be applied per l in concentrations of 0.65M (*R*)-lactate, which should be converted in *ca.* 0.5 h to pyruvate. The experiments were usually conducted in deionised water without any buffer added. The pH value was kept constant by using a pH stat. Dimethyl sulfide formed from DMSO (*Reaction 3*) leaves the reaction vessel actually completely, if the bioconversion is carried out at 38°, *i.e.*, crude pyruvate solutions without other components can be obtained as soon as the cell debris, the proteins, and other cell components have been eliminated by filtration.

If *Reaction 4* is used for the regeneration of the oxidised AQDS, the amine is present in the same concentration as the formed pyruvate.

The biocatalyst which was not applied in an immobilised form is rather stable. Cells were 5 times reisolated and used again.

As can be judged from the abstracts of the 30 patents published in the last six years, most biocatalytic pyruvate preparations show space time yields of less than 0.5 mol $l^{-1}d^{-1}$. Only two of 18 procedures show values near 1. Usually the productivity numbers can not be checked, because the amount of cells used is not indicated. The concentration of formed pyruvate ranges from 12 to 590 mM.

By the same technique used for pyruvate formation (*R,S*)-glycerate can be converted very effectively in a mixture of (*S*)-glycerate and 3-hydroxypyruvate.

Other dehydrogenations of racemates led in addition to 2-oxo-carboxylates to the formation of new types of (2*S*)-hydroxy-carboxylates.

*P. mirabilis* catalyses also the selective dehydrogenation of aldonic acids being (*R*)-configurated in $\alpha$-position to the carboxylate group. We showed this by the preparation of the 2-oxo derivatives of L-arabinoic, D-ribonic, D-gluconic, L-mannonic, D-galactonic, and D-gulonic acid in 200 mM solutions. Also lactobionate was

successfully oxidised to 4-*O*-(β-D-galactosido)-D-*arabino*-2-hexulosonate. According to our expectation L-gluconate could not be dehydrogenated. The oxidation products have been isolated and characterised. It seems that most of them have never been obtained by synthesis. We assume that suitably configurated uronic acids and aldaric acids can be dehydrogenated in the same way. For the preparative conversions, DMSO was used as the electron acceptor.

So far, most preparative reversible redox reactions with whole cells have been conducted with yeasts. However, yeasts were hardly used for selective dehydrogenations with regeneration of NAD or NADP from the reduced forms. The production of pyruvate and the other 2-oxocarboxylates with *Proteus* species shows that redox enzymes, which are not pyridine-nucleotide-dependent, can be used very effectively in various ways for selective dehydrogenations. Usually, the pyridine-nucleotide-independent redox enzymes work with many different artificial mediators, which are depending on their redox potential suitable for quantitative reductions as well as quantitative dehydrogenations. Especially in the latter case, various regeneration methods are available which can not be used for pyridine-nucleotide-dependent enzymes. What is shown here for HVOR is also true for other non-pyridine-nucleotide-dependent redox enzymes, which are able to accept or deliver single electrons. The purification of the redox enzyme or enzymes is not necessary. By the use of suitable artificial electron carriers, the equilibrium constants of reactions can be changed by 10 and more orders of magnitude.

With *Clostridium formicoaceticum*, the preparation of a series of the following succinate derivatives was conducted on 10–20 mmol scale: (2*S*,3*S*)-(2,3-$^2$H)succinate, (2*S*,3*S*)-2-methyl(2,3-$^2H_2$)succinate, the corresponding (2*S*)-ethyl derivative and (2*S*)-chlorosuccinate as well as dimethyl (2*S*,3*S*)-2-methyl(3-$^2H_1$)succinate. The corresponding fumarate derivatives were reduced at the expense of formate.

*C. thermoaceticum* or crude cell extracts of it are very effective for the regeneration of all four forms of pyridine nucleotides.

$$2V^{+\cdot} + H^+ + NAD^+ \rightleftharpoons 2V^{++} + NADH \quad (5)$$

$$2V^{+\cdot} + H^+ + NADP^+ \rightleftharpoons 2V^{++} + NADPH \quad (6)$$

The formation of NADH in the crude extract is catalysed with 3.9 U/mg protein, that of NADPH, $NAD^+$, and $NADP^+$ with 9.5, 1.6, and 2.0 U/mg protein, respectively.

Also the reaction

$$HCOO^- + NADP^+ \rightarrow NADPH + CO_2 \quad (7)$$

with *ca.* 2.5 U/mg protein in the crude extract of *C. thermoaceti*cum is very effective. *C. thermoaceticum* can be grown with 10–15 g wet packed cells per l [8], *i.e.*, at least 2500–10000 U of the here mentioned enzymes can be prepared per l.

The above mentioned regenerations of NADPH, NAD, and NADP seem to be rather interesting compared to the methods suggested in the literature [19].

These VAPOR activities have been used for the preparation of various chiral compounds in concentrations given in mM in parenthesis: (3*S*)-hydroxy-acylates (120) or esters (200) from the corresponding 3-oxo-esters or 3-oxo-acids, ribulose-5-phosphate (300, 45% product formation) from glucose-6-phosphate, (2*R*)-isocitrate (100, 54% product formation) from 2-oxoglutarate by reductive carboxylation, as well as (2*S*)-isocitrate (50) after oxidative dehydrogenation of (2*R*)-isocitrate from a racemate of D,L-isocitrate. In all cases in which chiral centers were formed, the enantiomeric excess was ≥99%. Especially the isolation of the products formed by NADPH consumption is rather simple compared to procedures from the literature in which *e.g.* 6-phosphogluconate is a stoichiometrically formed by-product in the NADPH regeneration [19].

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