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Microbial transformations in a cyclodextrin medium. Part 4. Enzyme vs microbial oxidation of cholesterol

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Abstract. A comparative study was conducted on two biocatalysts, resting *Rhodococcus erythropolis* cells and soluble cholesterol (CL) oxidase, both catalysing CL oxidation in a cyclodextrin (CD) medium. The enzyme-mediated sterol oxidation was clearly enhanced by the dimethylated β-cyclodextrin (Dimeb), as in the microbial oxidation. However, the microbial transformation was subject to a larger enhancement effect (enhancement factor of approx. 6) than the enzymic one (enhancement factor of approx. 2), with respect to the corresponding transformations with no CD. Rate vs substrate concentration curves of the microbial and enzymic systems were found to be Michaelis-Menten-like with corresponding Michaelis constant (K_m) values of approx. 0.25 and 0.5 g/l. The larger Dimeb-induced effect exerted on the microbial system was interpreted by a stronger affinity of Dimeb to the microbial cell. This CD-cell interaction was manifested through a slightly inhibited microbial growth and a limited leakage of cellular proteins and CL oxidase.

Introduction

Cyclodextrins (CDs) are increasingly being used for enhancing the bioavailability of organic substrates to biocatalysts: microbial cells or enzymes. Microbe-mediated transformations of water-insoluble steroids (Uvardy et al. 1985; Hesselink et al. 1989; Singer et al. 1991; Jadoun and Bar 1993; Schlosser et al. 1993) as well as of toxic aromatic compounds (Bar 1989) have been investigated in CD-supplemented media. Enzyme-mediated conversions were first introduced by Szejtli et al. (1985), who demonstrated an enhanced hydrolysis of olive oil or triolein by lipases in the presence of the methylated β-CD, Dimeb. More recently, Otero et al. (1991) investigated the enzymatic hydrolysis of insoluble *p*-nitrophenylbutyrate in solutions of β-CD. Another enzyme-mediated hydrolysis was reported by Pekic and Lepojevic (1991), who studied the action of β-glucosidase on deacetyl lanatoside in solutions of β- and γ-CDs. A different type of enzymatic reaction – a steroid hydroxylation – was successfully performed by Woerdenbag et al. (1990) on β-CD-complexed 17-β estradiol, using phenoloxidase.

All of these reported biotransformations have concentrated primarily on the effect of CDs on the substrate and not on the biocatalyst itself. Yet CDs, natural or chemically modified, can definitely interact with cells and enzymes by virtue of either their complexing ability and/or their surface activity (Szejtli 1988). Studies on CD-cell interactions are so far very few and involve primarily wall-less cells such as animal and *Mycoplasma* cells. Indeed, natural CDs as well as the methylated β-CD, Dimeb, were shown to complex and extract membrane components from erythrocytes, thus leading to their haemolysis (Irie et al. 1982). Similarly, Dimeb and the hydroxylpropylated β-CD, Hyprob, were recently shown to inhibit and promote the growth of *Mycoplasma capricolum,* respectively (Greenberg-Ofrath et al. 1993). With respect to enzymes, interactions between these and CDs are certainly possible through their amino acid side chains since proteins would not normally fit in a CD cavity. The thermodynamically favorable interaction between β-CD and single amino acids such as L-tryptophan and L-tyrosine has been investigated (Matsuyama et al. 1987). Complexation of human serum albumin with β-CD as well as solubilization and stabilization of several proteins by certain CDs have been demonstrated (Brewster et al. 1989, 1991).

When a cell or enzyme functions as a biocatalyst, interaction with CDs may alter their catalytic activities and consequently affect differently the kinetics of the biotransformations. Oxidation of cholesterol (CL) to cholest-4-en-3-one is a good example of a bioconversion that can be catalysed by the microbe *Rhodococcus erythropolis* or by the enzyme CL oxidase derived from

Dedicated to Prof. Jochanan Blum on the occasion of his 60th birthday

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it. The microbe-mediated oxidation of the sterol has already been the subject of a thorough and systematic investigation (Jadoun and Bar 1993), where it was shown that among all CDs examined, Dimeb exerted a remarkably enhancing effect on the biotransformation. The present study was aimed at comparing the performances of resting cells and soluble enzyme, both catalysing CL oxidation in Dimeb-supplemented media.

Materials and methods

Materials. The sources and specifications of all materials have been listed previously (Jadoun and Bar 1993). Heptakis-2.6-di-*O*-methyl-β-CD, abbreviated to Dimeb, was purchased from Chinoin as a pure substance with a water content of 1.5%. A *Nocardia erythropolis* CL oxidase (EC 1.1.3.6) was purchased from Sigma (St. Louis, USA) as an aqueous solution of 25 units (U).

Microorganism. Rhodococcus (formerly Nocardia) *erythropolis* ATCC 25544 was induced for oxidase activity and cultivated as previously described (Bar 1988; Goetschel and Bar 1992). Washed and lyophilized *Rhodococcus* cells were used as biocatalysts.

Microbial oxidation of cholesterol in the bioreactor. The procedure for the microbe-mediated oxidation of the sterol as well as the stirred 100-ml bioreactor are described in detail in Jadoun and Bar (1993). For each experiment, the baffled bioreactor, agitated by a paddle impeller at 500 rpm, was loaded with a suspension of *Rhodococcus* cells [2.5 g dry weight (DW/l)] and with a known amount of CL dissolved in an aqueous solution of Dimeb. Intermittently, aliquots (0.5 ml) were withdrawn and extracted with chloroform. These extracts were analysed for cholestenone and residual CL by gas chromatography as detailed elsewhere (Jadoun and Bar 1993).

Enzymatic oxidation of CL in the bioreactor. The previously mentioned bioreactor was loaded with 12.5 ml of a double-strength potassium phosphate buffer (0.1 M, pH 7.4) and with 12.5 ml of an aqueous Dimeb solution of CL. For the control experiments, the latter solution was substituted by a fine suspension of CL crystals, prepared by the dropwise addition of a known volume of a stock dimethyl formamide (DMF) solution of CL (100 mg/ml) to magnetically stirred 12.5 ml distilled water. The bioconversion was initiated by adding 200 μl of a stock solution of *N. erythropolis* CL oxidase (25 U/ml). The bioreactor, in a thermostatted water bath at 30° C, was agitated by a paddle impeller at 500 rpm. Intermittently, aliquots (0.5 ml) were withdrawn and analysed for substrate and product, as in the case of the microbial transformation.

Growth of R. erythopolis in Dimeb-supplemented nutrient broths. Indented 150-ml erlenmayer flasks were loaded with 50 ml nutrient broth (Difco) enriched with glucose (1%, w/v) and supplemented with Dimeb at 0, 5, 20 and 50 mM. The flasks were sterilized at 121° C for 15 min. It should be noted that Dimeb precipitates were observed following autoclaving [the solubility of Dimeb is known to decrease at an elevated temperature (Uekama and Irie 1987)] but they redissolved upon cooling to room temperature. The flasks were seeded with 5 ml of a 24-h-grown culture in glucose-enriched nutrient broth and incubated in a rotary shaker (150 rpm) at 30° C. Growth was monitored every 2 h by measuring absorbance at 600 nm.

Leakage of proteins from the microbial cells. Rhodococcus cells (2.5 g DW/l) were suspended in 50 ml phosphate buffer (0.1 M, pH 7.4) with and without Dimeb (20 mM). The suspension was placed in the bioreactor operating at 500 rpm and 30° C. Intermittently, aliquots (1.5 ml) were withdrawn, centrifuged and the protein content of the supernatant was estimated by measuring absorbance at 280 nm

Dimeb-induced leakage of CL oxidase from microbial cells. Suspensions (50 ml) of *Rhodococcus* cells (2.5 g DW/l) in potassium phosphate buffer (0.1 M, pH 7.4) solutions of Dimeb at varying concentrations of 5, 20 and 50 mM were agitated in the bioreactor operating under the same conditions of the microbial transformation. After 2 h, the suspensions were centrifuged and the clear supernatant liquid was separated from the cells. The supernatant liquid was placed again in the agitated bioreactor and 500 μl of a DMF solution of cholesterol (100 mg/ml) were added drowise in order to detect and quantify the oxidizing activity of any liberated CL oxidase. In parallel, the cells were resuspended in 50 ml of a buffer solution of Dimeb under identical initial concentrations and the oxidizing activity of these treated cells was tested following the drowise addition of 500 μl of a DMF solution of CL (100 mg/ml). Intermittently, aliquots (0.5 ml) from either reaction system were withdrawn and subjected to product analysis. The control experiments consisted of a regular 50 ml reaction system with cells (2.5 g DW/l) and CL (1 g/l) in buffer solutions of Dimeb at the same concentration employed for cell treatment.

Results

The phase-solubility diagram of CL in Dimeb solutions reported previously (Jadoun and Bar 1993) demonstrated clearly its strong solubilising power and, indeed, 10 and 50 mM Dimeb solutions were shown to dissolve completely up to 1 and 4 g CL/l, respectively. In order to conduct a comparative study on the performances of biocatalysts – free resting microbial cells and free soluble enzyme – the biotransformations were investigated primarily in media with a Dimeb concentration sufficiently high to completely dissolved all substrate and product.

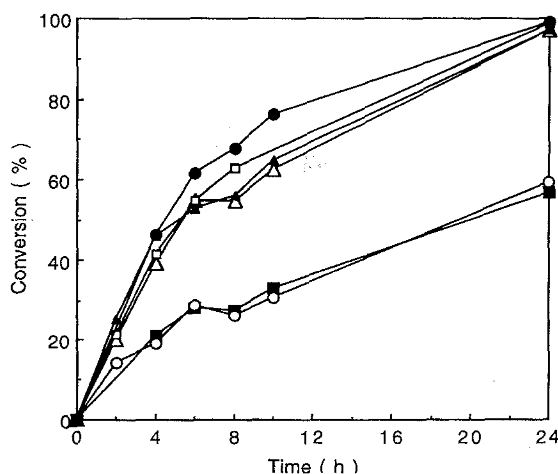


Fig. 1. Time courses of cholesterol (1 g/l) oxidation by soluble cholesterol oxidase [40 units (U)/l] in the stirred bioreactor (500 rmp) with media at various Dimeb concentrations (○, control; ■, 5 mM; △, 10 mM; □, 20 mM; ●, 30 mM; ▲, 50 mM) at 30° C

Effect of Dimeb concentrations on the enzymic oxidation

Figure 1 compares the time courses of CL (1 g/l) oxidation to cholestenone by freely soluble CL oxidase in buffer media in the presence and absence of 5, 10, 20, 30 and 50 mM Dimeb. It is seen that with the exception of the system with the low 5 mM Dimeb concentration, the profiles and the initial rates of the bioconversion in all other systems were quite similar. The total dissolution of the sterol by Dimeb at a concentration ≥10 mM (see above) enabled a significant enhancement of the enzymic oxidation as well as its completion after 24 h.

Comparative enhancement of microbial and enzymic oxidations

The enhancement of the microbe-mediated oxidation in the presence of various Dimeb concentrations has been presented previously (Jadoun and Bar 1993). This enhancement may be compared with that observed in the enzymic systems by introducing an enhancement factor E, which represents the ratio between the initial bioconversion rate in the presence of a given Dimeb concentration and that in the control experiment with no CD.

Figure 2 shows the E factors for the microbial and enzymic oxidation of 1 g CL/l for various Dimeb concentrations. The former values were determined from the preceding paper and the latter were calculated from Fig. 1. Figure 2 delimits a monophasic region (Dimeb ≥ 10 mM) with totally dissolved steroids from a biphasic region of liquid-solid systems with a Dimeb concentration (< 10 mM) too low to dissolve all the sterol. It is clearly seen that the enhancement factor observed in the microbial sytems is considerably larger (≅6.2) than that in the enzymic systems (≅2) and both are virtually independent of Dimeb concentration in the homogenous systems. Interestingly, Dimeb at 5 mM exerted only a slight effect (E = 1.1) in the enzymic system in contrast to the significant enhancement (E = 5.8) experienced by the microbial system.

Rate vs substrate curves for microbial and enzymic oxidations

The initial enzymic (40 U/l) oxidation rates of homogeneous Dimeb (50 mM) solutions of 0.25, 0.5, 1.0 and 1.5 g CL/l were determined in the bioreactor after 15 or 30 min and they are plotted in Fig. 3. The rates increased up to a CL concentration of 1 g/l, above which they levelled off. Figure 3 shows equally initial microbial (2.5 d DW/l) rates of various CL concentrations totally dissolved in the same medium. The curve is clearly steeper and levels off at a substrate concentration of approx. 0.5 g/l. Obviously, comparison of the absolute magnitudes of the enzymic and microbial rates cannot be made, given the different nature and amounts of biocatalysts.

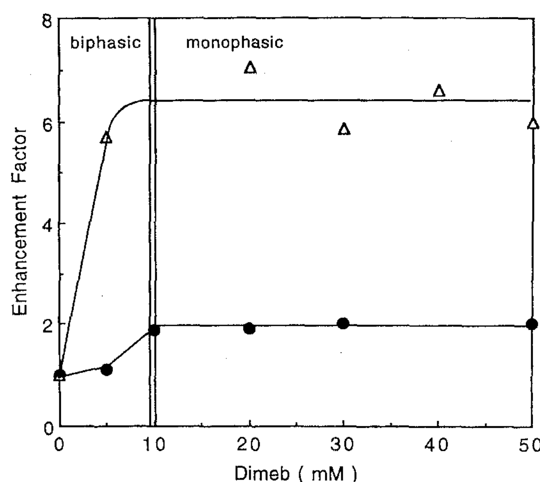


Fig. 2. Enhancement factors of cholesterol (1 g/l) oxidation by *Rhodococcus* cells [△, 2.5 g dry weight (DW)/l] or soluble cholesterol oxidase (●, 40 U/l) in the stirred bioreactor (500 rpm) with media at various Dimeb concentrations at 30° C

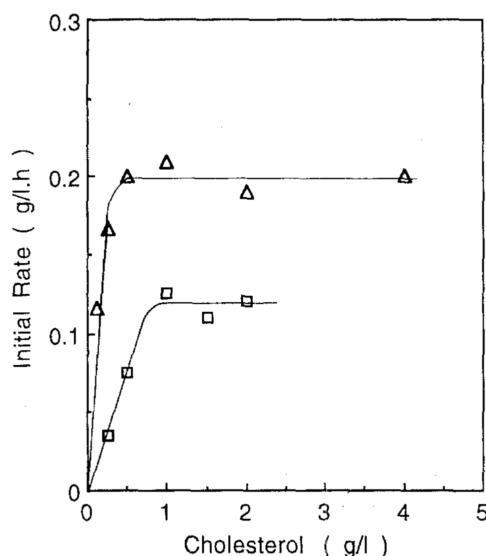


Fig. 3. Initial oxidation rates of various cholesterol concentrations by *Rhodococcus* cells (△, 2.5 g DW/l) or soluble cholesterol oxidase (□, 40 U/l) in the stirred bioreactor (500 rpm) at 30° C

Even though the data in Fig. 3 could not be satisfactorily fitted to Lineweaver-Burk lines, both curves in Fig. 3 appear similar to Michaelis-Menten curves in the sense that they consist of a monotonous increase and levelling-off of the rates. With this reservation in mind, apparent Michaelis constant (K_m) values, can be derived straightforwardly from the substrate concentrations at mid-maximal rates, and they are 0.25 and 0.5 g/l for the microbial and enzymic catalysts, respectively.

Effect of Dimeb on microbial growth

The growth curves of *R. erythropolis* in nutrient broths supplemented with 5, 20 and 50 mM Dimeb are plotted

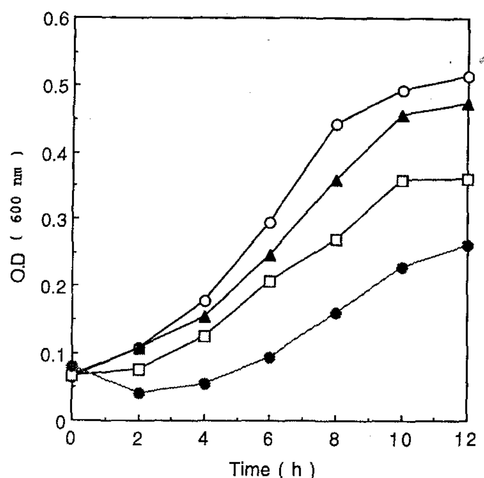


Fig. 4. Growth curves of *R. erythropolis* in shake flasks with glucose (1%, w/v)-enriched nutrient broth, supplemented with various Dimeb concentrations (○, control; ▲ 5 mM; □, 20 mM; ●, 50 mM) at 30° C: OD, optical density

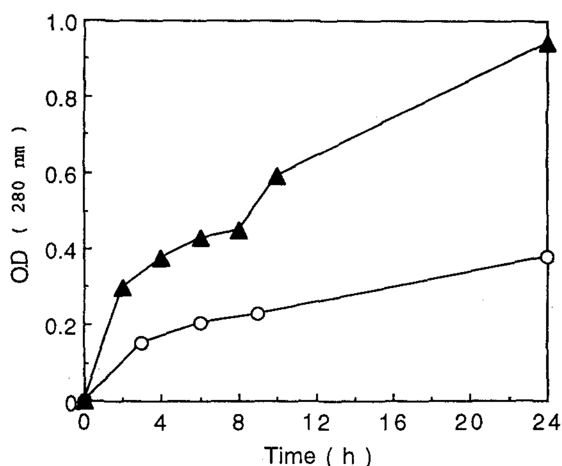


Fig. 5. Leakage of proteins from *Rhodococcus* cells (2.5 g DW/l) given in the stirred bioreactor (500 rpm) in the absence (○, control) and presence of 20 mM Dimeb (▲) at 30° C

in Fig. 4. *Rhodococcus* cells did grow in the presence of Dimeb but a progressively inhibitory effect was clearly exerted by increasingly greater Dimeb concentrations.

Effect of Dimeb on leakage of microbial proteins

Rhodococcus cells incubated in a phosphate buffer with and without 20 mM Dimeb showed a gradual leakage of proteinaceous material into the medium, as estimated by absorbance measurements at 280 nm (Fig. 5). The cells were grown in the bioreactor operating under the same conditions employed for microbial transformations. Figure 5 shows that the presence of Dimeb enhanced protein leakage to levels higher than those ascribable to normal cell lysis in the control experiment.

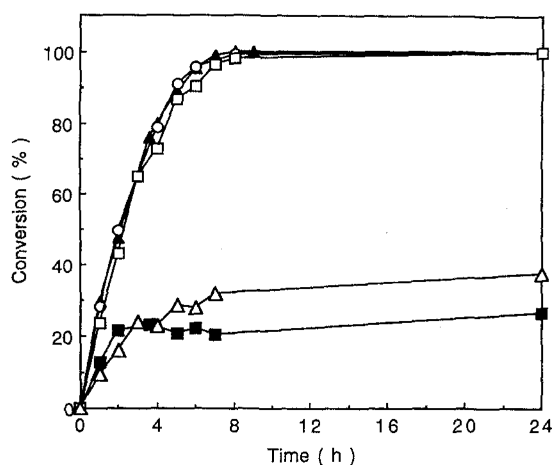


Fig. 6. Time course of cholesterol (1 g/l) oxidation following pretreatment of *Rhodococcus* cells (2.5 g DW/l) with Dimeb at 20 and 50 mM. For each system, the treated cells and the supernatant liquid were separately employed as biocatalysts for new biotransformations (see Materials and Methods). The control experiment was a biotransformation system with a Dimeb (20 mM) medium with untreated cells: ○, untreated cells; ▲, treated cells (20 mM); ■, supernatant (20 mM); □, treated cells (50 mM); △, supernatant (50 mM)

Effect of Dimeb pre-treatment on leakage of microbial CL oxidase

Rhodococcus cells oxidizing CL in a plain buffer medium were shown not to leak CL oxidase into the medium to any measurable extent. A similar behaviour was shown with cells previously agitated in a 5 mM Dimeb buffer solution for 2 h. When these "treated" cells were exposed to a fresh Dimeb medium with CL, the catalytic activities of the untreated and treated cells were found to be identical, whereas that of the liquid medium was vanishingly small. Preincubation of cells in buffer solutions of 20 and 50 mM Dimeb led, however, to some leakage of enzyme, as testified by the mild oxidizing activities of the supernatant liquids when exposed to the substrate (Fig. 6). Interestingly, the catalytic acivities of the treated cells, measured in fresh Dimeb media, remained high and comparable to that of untreated cells (Fig. 6).

Discussion

The well-known ability of CDs to bring about enhanced biotransformation as a result of enhanced dissolution of organic substrates has certainly been demonstrated again in this study. Indeed, Fig. 1 shows complete conversions and faster rates in enzymic systems with totally dissolved sterol whereas Fig. 2 shows that the bioconversion rates were larger in enzymic or microbial media supplemented with Dimeb than those without CD. When comparing the microbial and enzymic systems, the most prominent difference is the clearly larger enhancement factors (≅6) obtained in

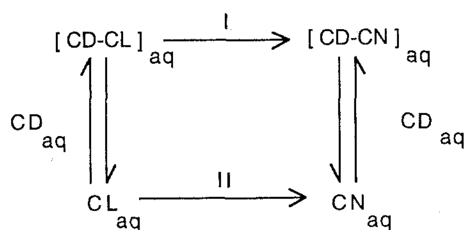
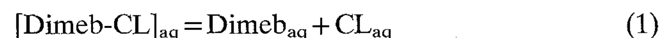


Fig. 7. Scheme of the various interacting species of cyclodextrin (CD), free substrate cholesterol (CL) and product (CN) and CD-complexed substrate ([CD-CL]) and product ([CD-CN]) in homogeneous biotranformation systems with Dimeb as CD. See text for explanation

the microbial systems. This difference cannot obviously be attributed to mere dissolution of the steroid reagents, as these were all dissolved in solutions with Dimeb ≥ 10 mM in accordance with their phase solubility diagram (Jadoun and Bar 1993). It appears then that some kind of interaction between Dimeb and the biocatalyst is at the origin of this differences. An indication of such an interaction was previously presented by Takahashi et al. (1991), who investigated the transphosphorylation of ADP by *Escherichia coli* cells in the presence of CDs. These authors found the reaction to be more accelerated by Dimeb than β-CD and suggested that Dimeb approached the cell membrane more efficiently.

In both biocatalytic systems, Dimeb forms with CL a soluble complex that undergoes a dissociation-complexation equilibrium (Eq. 1)



$$[\text{Dimeb-CL}]_{aq} = \text{Dimeb}_{aq} + \text{CL}_{aq} \qquad (1)$$

Both the free (CL_{aq}) and CD-complexed ($[\text{Dimeb-CL}]_{aq}$) sterol can in principle be substrates of the biotransformation (Fig. 7). The first is certainly the only one in reaction systems with no CD (path II in Fig. 7). In CD-supplemented media, $[\text{Dimeb-CL}]_{aq}$ could potentially serve as a reservoir of the free CL_{aq}, which is then bioconverted. In the latter case, increased CD concentrations would shift the equillibrium of Eq. 1 to the left, decrease CL_{aq} and consequently lower the bioconversion rates. However, Fig. 2 clearly shows that in homogenous solutions of a constant CL concentration, the initial rates were found to be independent of Dimeb loading, thus indicating that the complexed CL is presumably the primary substrate (path I in Fig. 7). Nevertheless, it appears reasonable to assume that some of the free CL is also reactive, as the relative biotransformation rates of the sterol in CD-free media are certainly not negligible.

The different enhancement effects exerted in the microbial and enzymic systems can now be interpreted in the light of the interaction of the predominantly complexed CL with the two biocatalysts. The Michaelis-Menten kinetic parameter K_m could well reflect these interactions. The Michaelis-Menten-like curves in Fig. 3 indeed show that the affinity of the substrate to be microbial cells ($K_m \cong 0.25$ g/l) is larger than that of the soluble enzyme ($K_m \cong 0.5$ g/l). As previously started, the curves in Fig. 3 do not exhibit a satisfactory fit to typical Michaelis-Menten kinetics of a single substrate and this can be reasonably attributed to a simultaneous participation of the free CL in both the enzymic and microbial systems.

The difference in substrate affinities to the biocatalysts forcibly directs our attention to the nature of the biocatalysts. In contrast to the system with free soluble enzyme, CL oxidase in the microbe is bound to the membrane (Smith and Brooks 1976), thus "immobilized" in a sense. The first protein is totally exposed to CD interactions, but the latter is only partly so, if at all. A possibly negative CD-free enzyme interaction, causing a reduced affinity or activity, may not take place in the "membrane-protected" enzyme. One should bear in mind that the fact that Dimeb enhanced the enzyme or microbial biotransformation does not necessarily demonstrate absence of negative CD-biocatalyst interaction. By comparing the kinetics in the presence and absence of CD, one in fact compares two different rate-determining processes: in a CD-free system, the rate is determined by the dissolution process of the insoluble sterol but in a CD-containing system, it is determined by the intrinsic bioconversion kinetics (Jadoun and Bar 1993). Such as comparison would be possible only with kinetics measured in a hypothetical homogeneous solution of 1 g CL/l with no CD. The aqueous solubility of CL is only 1.8 mg/l (Haberland and Reynolds 1973). Thus, the specific enzymatic activity of the free enzyme in a CD medium could have been perhaps higher in the absence of any CD interaction, but is perhaps lower than that of the "membrane-protected" enzyme in the CD-containing microbial system.

Another distinctive feature of the microbial catalyst is the cell wall. The wall of *Rhodococcus* cells contains a substantial amount of lipophilic mycolic acids (Sneath 1984), which render the microbial cells quite hydrophobic. Furthermore, Dimeb itself is certainly hydrophobic (Dimeb, unlike β-CD, is soluble in organic solvents) as well as surface active (Uekama and Irie 1987). Both these properties would certainly facilitate an interaction between the microbial cells and Dimeb. Indeed, Dimeb enhanced the leakage of cellular proteins (Fig. 5) and affected the growth rate of the microbe (Fig. 4). Leakage of the membrane-bound CL oxidase upon pre-treatment with 20 and 50 mM Dimeb (Fig. 6) was also observed, even though this leakage was quite mild and would not seriously affect the measured microbial initial rates.

We have not investigated whether Dimeb or [Dimeb-CL] enter the cell but we assume that these chemical species are too large to actually penetrate the cells. It is therefore believed that all Dimeb-induced effects exerted upon the microbial cells are primarily a result of interactions with the cell wall and membrane. Thus, a favourable interaction between microbial cells and the hydrophobic Dimeb could reasonably explain the stronger affinity of Dimeb-complexed substrate to the cells and, consequently, support a mechanism whereby the complexed substrate interacts directly with the mi-

crobial cells (path I in Fig. 7). The [Dimeb-CL] complex, once "adsorbed" onto the cell wall, releases (Eq. 1) free CL, which is soon bioconverted by the membrane-bound oxidase.

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