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Application of Urethane Prepolymers to Immobilization of Biocatalysts: Δ^1-Dehydrogenation of Hydrocortisone by *Arthrobacter simplex* Cells Entrapped with Urethane Prepolymers

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Acetone-dried cells of *Arthrobacter simplex* having appreciable steroid Δ^1-dehydrogenase activity were immobilized by mixing the cell suspension with water-miscible urethane prepolymers synthesized from toluene diisocyanate and polyether diols. The entrapped cell activity in the transformation of hydrocortisone to prednisolone was affected by the properties of urethane prepolymers, such as the isocyanate group content in prepolymers, the molecular weight of polyether diols and the ethylene oxide content in diols. The addition of 10% of organic solvents, such as methanol and glycols, to the aqueous reaction mixture enhanced the solubility of the substrate greatly and the reaction rate of the immobilized cells. The activity of immobilized cells remained high even in the system containing 30% of methanol, which drastically inhibited the activity of free cells. The presence of an electron acceptor, phenazine methosulfate or 2,6-dichlorophenolindophenol, significantly stimulated the steroid conversion with entrapped cells, as well as free cells. The stability of the cells over repeated reactions was greatly improved by immobilization.

Bioconversions of steroids are one of the most representative subjects in the field of biotechnology. Biochemical reactions *in vivo* of highly lipophilic or water-insoluble substances, such as steroids, are generally mediated by membrane-associated enzymes in hydrophobic circumstances. Hence, *in vitro* reactions in hydrophobic circumstances—uses of immobilized biocatalysts and organic solvents or water-organic solvent mixtures—will be of great interest and importance for the conversion of such lipophilic compounds from both fundamental and practical viewpoints.

Buckland *et al.*[1] reported conversion of cholesterol to cholestenone by the cells of *Nocardia* sp. suspended in a water-immiscible solvent. Carrea *et al.*[2] used a water-organic solvent system for the transformation of steroids with immobilized hydroxysteroid dehydrogenase. Recently, we have successfully applied gel-entrapped cells of *Nocardia rhodocrous* and *Arthrobacter simples* to the conversion of 4-androstene-3,17-dione to 1,4-androstadiene-3,17-dione[3] and 3β-hydroxy-Δ^5-steroids to 3-keto-Δ^4-steroids[4] in a water-insoluble organic solvent system (benzene–*n*-heptane, 1 : 1 by volume) and the transformation of hydrocortisone to prednisolone[5] in a methanol-containing aqueous system, respectively. These facts strongly indicate the utility of the gel-entrapped biocatalysts for the conversion of highly lipophilic compounds.

Of various methods now available for inclusion of enzymes as well as microbial cells inside polymer matrices, two novel techniques developed recently in our Laboratory—the photo-crosslinkable resin prepolymer method[6] and the urethane prepolymer method[7]—will be very suitable for this purpose.

Some advantages of the prepolymer methods are that not only inclusion of a biocatalyst in gels can be achieved by a very simple and mild technique but also tailor-made gels of desired physical and chemical properties can be ob-

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tained by the use of appropriate prepolymers.

Photo-crosslinkable resin prepolymers of hydrophilic or hydrophobic nature have been successfully utilized for the entrapment of enzymes,[6,8~10] organelles[11,12] and microbial cells.[3~5,13~15] Urethane prepolymers are also useful for preparing gel-entrapped biocatalysts of different properties.[7,16]

This paper describes the application of urethane prepolymers for the entrapment of acetone-dried cells of *Arthrobacter simplex* and the conversion of hydrocortisone to prednisolone with so immobilized cells.

MATERIALS AND METHODS

Organism. Arthrobacter simplex ATCC 6946 was grown at 30°C, as described previously.[5]

Urethane prepolymers (PU). Urethane prepolymers used were synthesized from toluene diisocyanate and polyether diol.[7] The general formula of urethane prepolymers and their properties are shown in Fig. 1 and Table I, respectively. The contents of NCO and ethylene oxide in the prepolymers affect the degree of cross-linking in the formed gels and the hydrophobicity of the gels, respectively. These prepolymers were supplied from Toyo Rubber Industry Co., Osaka.

Immobilization method. Standard immobilization method was as follows: 2 g of urethane prepolymer was melted at 60°C (if necessary) and cooled to room temperature. To the melted prepolymer were added 200 mg of acetone-dried cells of *A. simplex* suspended in 6 ml of chilled 20 mM potassium phosphate buffer (pH 7.0) and mixed quickly. The mixture was immediately layered on a glass plate framed with plastic tape (to make the dimension of the resin, 10 × 7 cm), and allowed to stand for 1 hr at 4°C to complete gelation according to the following reaction:

$$-R-NCO + OCN-R- + H_2O \longrightarrow -R-NHCONH-R- + CO_2$$

The resin film formed was cut into small pieces (each approximately, 5 × 5 mm) and used for the transformation of hydrocortisone (Fig. 2).

TABLE I. PROPERTIES OF URETHANE PREPOLYMERS USED

Prepolymer	M_w of polyether diol	NCO content in prepolymer (%)	Ethylene oxide content in polyether diol (%)
PU-1	1486	4.0	100
PU-2	2529	3.1	57
PU-3	2529	4.2	57
PU-4	2529	5.6	57
PU-5	2627	2.7	91
PU-6	2627	4.0	91
PU-7	2627	5.6	91
PU-8	2616	2.7	100
PU-9	2616	4.0	100
PU-10	2616	5.6	100
PU-11	4285	4.0	73

Enzyme reaction. Unless stated otherwise, the enzyme reaction was carried out as follows: To 18 ml of 20 mM potassium phosphate buffer (pH 7.0), was added 2.0 ml of methanol dissolving 20 mg of hydrocortisone. An electron acceptor, if necessary, was added directly to the reaction mixture. The reaction was initiated by addition of the immobilized *A. simplex* cells (corresponding to 200 mg acetone-dried cells) or free cells (200 mg), and continued at 30°C with shaking (120 strokes/min). The concentration of hydrocortisone in the immobilized cell system was obtained by dividing the weight of hydrocortisone by the sum of the volumes of the solution and the cell-entrapping gels. The activity (μmol·hr^{-1}·mg dry cell^{-1}) was calculated from the initial linear phase in the time course of the steroid conversion.

Analysis. Analysis of the product was carried out by thin-layer chromatography as described previously.[5] A typical chromatographic pattern is illustrated in Fig. 3.

Partition coefficient. Partition coefficients of hydrocortisone between the gels and the reaction solution were calculated from the following equation.[3]

$$P = \left(\frac{C_0 - C_1}{C_1}\right)\left(\frac{V_0}{V_1 - V_0}\right),$$

where P is the partition coefficient; C_0, the initial concentration of hydrocortisone in the solution; C_1, the final

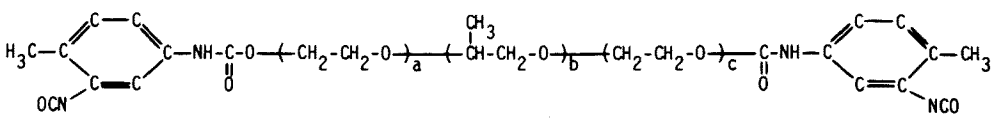

FIG. 1. Structure of Urethane Prepolymers Used in This Study.

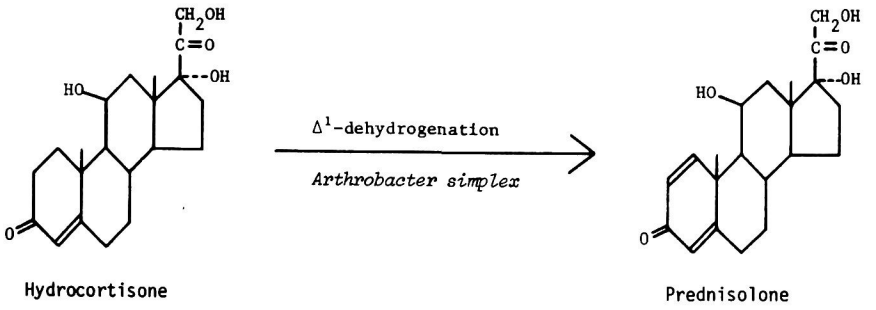

FIG. 2. Hydrocortisone Dehydrogenation Catalyzed by *Arthrobacter simplex*.

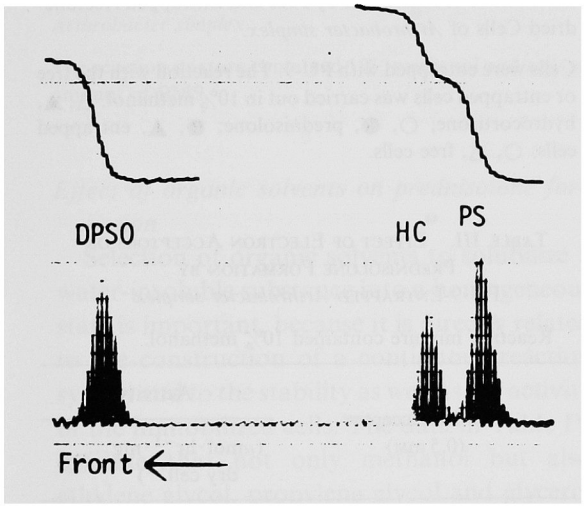

FIG. 3. Typical Thin-layer Chromatographic Pattern of the Reaction Mixture.

DPSO, diphenylsulfoxide (internal standard); HC, hydrocortisone; PS, prednisolone.

concentration of hydrocortisone in the solution after the gels are added; V_0, the volume of the solution; and V_1, the volume of the system (solution plus gels).

Chemicals. Flavin adenine dinucleotide was obtained from Wako Pure Chemicals Industries, Ltd. Other chemicals were obtained as described previously.[5]

RESULTS

Effect of properties of urethane prepolymers on activity of immobilized Arthrobacter simplex

Acetone-dried cells of *A. simplex* were used in this study because the cells stoichiometrically converted hydrocortisone to prednisolone.[5] To select a desirable urethane prepolymer for the entrapment of the acetone-dried cells, 11 kinds of urethane prepolymers with different molecular weight of diol, NCO content and/or ethylene oxide content (Table I) were examined. As shown in Table II, the cells entrapped with PU-3, PU-6 and PU-9 had a high activity of the steroid transformation. PU-1, PU-2 and PU-5 did not form sufficiently strong gels under the conditions employed. On the other hand, PU-10 crosslinked too rapid to make a thin film of the gel. Furthermore, appropriate mixtures of hydrophilic PU-9 and hydrophobic PU-3 could be used to prepare the gels with various hydrophobicity. As shown in Fig. 4, the activity of the cells entrapped with these prepolymer mixtures was similar, although the partition coefficient of hydrocortisone between gels and

TABLE II. PREDNISOLONE FORMATION BY *Arthrobacter simplex* ENTRAPPED WITH DIFFERENT TYPES OF URETHANE PREPOLYMERS

Transformation reaction was carried out with 10% methanol.

Prepolymer	Reaction rate (μmol·hr^{-1}·mg dry cell^{-1})
PU-1	—
PU-2	—
PU-3	0.257
PU-4	0.176
PU-5	—
PU-6	0.268
PU-7	0.095
PU-8	0.108
PU-9	0.270
PU-10	—
PU-11	0.162

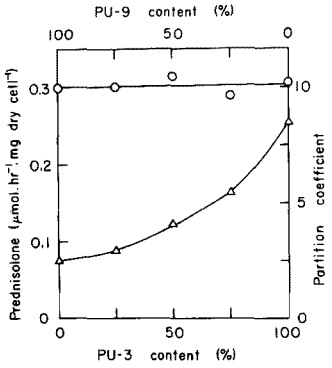

FIG. 4. Effect of Gel Hydrophobicity on Hydrocortisone Transformation by Entrapped Acetone-dried Cells of *Arthrobacter simplex* and on Partition Coefficient for Hydrocortisone.

Cells were entrapped with various proportions of PU-3 and PU-9. ○, transformation activity; △, partition coefficient.

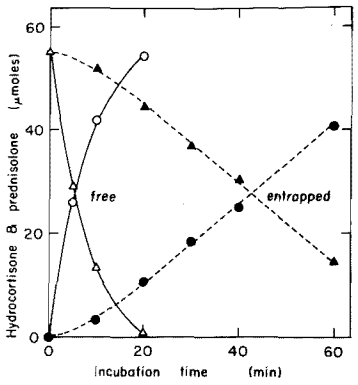

FIG. 5. Time Course of Transformation of Hydrocortisone to Prednisolone by Free and Entrapped Acetone-dried Cells of *Arthrobacter simplex*.

Cells were entrapped with PU-9. The reaction with the free or entrapped cells was carried out in 10% methanol. △, ▲, hydrocortisone; ○, ●, prednisolone; ●, ▲, entrapped cells; ○, △, free cells.

solution increased with increasing content of PU-3. PU-9 was chosen as prepolymer for subsequent experiments. Figure 5 illustrates a typical time course change in the amounts of hydrocortisone consumed and prednisolone formed. The reaction system was the homogeneous solution containing 10% methanol. Complete conversion of hydrocortisone to prednisolone was observed by both the free and the immobilized cells. The transformation rate by the entrapped cells was 0.230 μmol·hr^{-1}·mg dry cell^{-1}, while that of the free cells was 1.25 μmol·hr^{-1}·mg dry cell^{-1}.

Effect of electron acceptors on prednisolone formation

In the previous paper,[5] we described that the presence of an electron acceptor stimulated the dehydrogenation of the steroid with the entrapped cells as well as the free cells. As shown in Table III, the activity of the cells immobilized with PU-9 was also accelerated by the addition of phenazine methosulfate (PMS), menadione or 2,6-dichlorophenolindophenol. Especially, PMS was most effective for the reaction. Pyridine nucleotides, resazurin, methylene blue and potassium ferricyanate were not effective. Similar effects of the electron acceptors were also reported in steroid dehydrogenation reactions.[17~19] Figure 6 shows the relationship between the concentration of PMS and the activity of the entrapped cells. Under the experimental conditions employed, the Δ^1-dehydrogenation reaction with the immobilized cells was accelerated with the increasing concentration of PMS in the reaction mixture. Thus, we employed 0.5 mM PMS for subsequent experiments.

TABLE III. EFFECT OF ELECTRON ACCEPTORS ON PREDNISOLONE FORMATION BY PU-9-ENTRAPPED *Arthrobacter simplex*

Reaction mixture contained 10% methanol.

Electron acceptor (0.5 mM)	Activity (μmol·hr^{-1}·mg dry cell^{-1})	(%)
None	0.196	100
Phenazine methosulfate	0.367	187
Menadione	0.324	165
2,6-Dichlorophenolindophenol	0.309	158
FMN	0.259	132
FAD	0.232	118
Methylene blue	0.179	91
Resazurin	0.177	90
Potassium ferricyanate	0.166	85
NADP	0.139	71
NAD	0.133	68

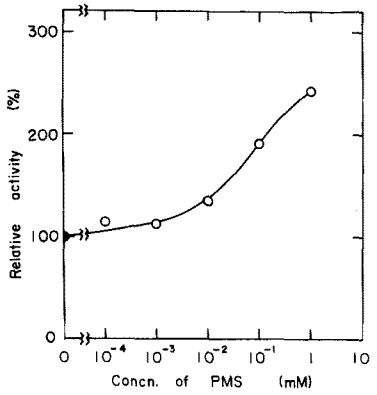

FIG. 6. Effect of PMS Concentration on Hydrocortisone Transformation by PU-9-entrapped Acetone-dried Cells of *Arthrobacter simplex*.

The reaction mixture contained 10% methanol and a given amount of PMS.

Effect of organic solvents on prednisolone formation

Selection of organic solvents to solubilize a water-insoluble substance into a homogeneous state is important, because it is directly related to the construction of a continuous reaction system and to the stability as well as the activity of the immobilized cells. The data in Table IV indicate that not only methanol but also ethylene glycol, propylene glycol and glycerol at 10% are effective on the prednisolone formation. On the contrary, the reaction did not proceed with 10% of *n*-butanol and *n*-amyl alcohol. Furthermore, the effect of each concentration of ethylene glycol, propylene glycol and methanol was examined on the steroid conversion by the cells (Fig. 7). The entrapped cells showed the maximum activity at 10% methanol or propylene glycol and 10~30% ethylene glycol. On the other hand, the activity of the free cells attained the maximum at 0~5% methanol, then being markedly reduced in the higher concentration range of methanol. These results indicate that the immobilization appreciably protects the cells from the denaturation caused by methanol and that a higher concentration of hydrocortisone can be dosed in the immobilized cell system than the case of the free cells. Methanol was chosen as solvent in subsequent experiments because it permitted a high solubility of hydrocortisone and prednisolone.

Stability of immobilized Arthrobacter simplex

The immobilized cells were stable at −20°C over 120 days without an appreciable loss of the activity. The free cells also showed almost the similar stability.

When the free and immobilized cells were

TABLE IV. EFFECT OF ORGANIC SOLVENTS ON PREDNISOLONE FORMATION BY PU-9-ENTRAPPED *Arthrobacter simplex*

Reaction mixture contained 0.5 mM PMS.

Organic solvent (10%)	Activity (μmol·hr^{-1}·mg dry cell^{-1})	(%)
None	0.34	100
Methanol	0.45	132
Ethanol	0.29	85
n-Propanol	0.12	35
n-Butanol	0	0
n-Amyl alcohol	0	0
Ethylene glycol	0.48	140
Propylene glycol	0.56	165
Trimethylene glycol	0.42	124
Glycerol	0.65	191

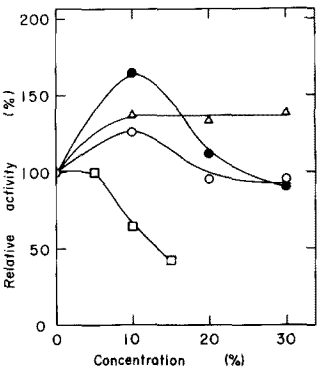

FIG. 7. Effect of Solvent Concentration on Hydrocortisone Transformation by Free and PU-9-entrapped Acetone-dried Cells of *Arthrobacter simplex*.

The reaction mixture contained 0.5 mM PMS. ○, methanol (entrapped cells); □, methanol (free cells); △, ethylene glycol (entrapped cells); ●, propylene glycol (entrapped cells).

used in the repeated reactions without any exogenous electron acceptor, the immobilized cells were found to be fairly stable than the free cells. The half-life of the free cells was about 160 min (8 batches of reaction), while that of the entrapped cells was 11 hr (11 batches of reaction) (Fig. 8). The total amount of prednisolone formed in the absence of PMS was 2.3 μmol/mg dry cell after 15 batches of reaction with the entrapped cells (total operational period, 15 hr), while that with the free cells was 1.8 μmol/mg dry cell after 10 batches of reaction (total operational period, 200 min). As described above, the addition of PMS markedly stimulated the steroid dehydrogenation. Figure 8 shows that the entrapped cells was stable over 30 batches of reaction (total operational period, 15 hr) without any loss of the activity in the reaction mixture containing 0.5 mM PMS and 10% methanol. On the other hand, the free cells were unstable even in the presence of PMS. The half-life of the free cells was 40 min (8 batches of reaction). The total amount of prednisolone produced in the presence of PMS by the entrapped cells was 8.0 μmol/mg dry cell after 30 batches of reaction (total operational period, 15 hr), and that of the free cells was 1.9 μmol/mg dry cell after 10 batches of reaction (total operational period, 50 min). Addition of PMS enhanced significantly the stability of the entrapped cells, but not that of the free cells. When the entrapped cells were used in the system containing 0.5 mM PMS and 10% propylene glycol, the cells retained the original activity even after 30 batches of reaction (operational period, 15 hr).

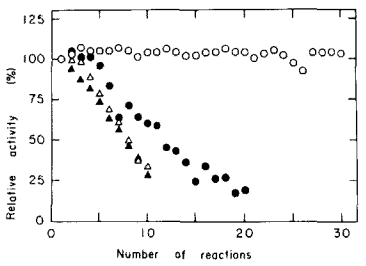

FIG. 8. Repeated Use of Free and PU-9-entrapped Acetone-dried Cells of *Arthrobacter simplex* on Hydrocortisone Transformation with or without PMS.

Each batch was carried out for 30 min with the entrapped cells in the presence of 0.5 mM PMS (○), for 60 min with the entrapped cells in the absence of PMS (●), for 5 min with the free cells in the presence of 0.5 mM PMS (△), or for 20 min with the free cells in the absence of PMS (▲).

DISCUSSION

In the cases of bioconversions of lipophilic compounds, it is desirable to perform enzyme reactions in solutions containing an organic cosolvent or in an appropriate organic solvent system. The use of such a reaction medium will improve the poor solubility in water of the substrate or other reaction components of hydrophobic nature and shift an unfavorable thermodynamic equilibrium to a desired direction.

However, attempt to make free enzymes function in an organic solvent system or a water-organic solvent mixture have been unsuccessful. The catalytic activity of enzymes dramatically decreased and their substrate specificity disappeared. One of the most probable mechanisms of the enzyme inactivation is considered to be conformational change of enzyme molecules upon introduction of an organic solvent into solution. In general, immobilization is one of effective and universal approaches for enzyme stabilization. Obviously the structure of protein molecule linked to a rigid support will be much more rigid and therefore unfolding of the high dimensional structure leading to inactivation will be much more difficult to be accomplished than in the case of the free counterpart. Entrapment of enzymes in suitable gels also renders them more stable than the free counterparts. The mechanism of stabilization of enzymes mechanically entrapped in polymeric supports would be explained by possible hindered transitional movement of the enzyme molecules due to multi-point interactions with gels. It is well known that many enzymes function *in vivo* in a membrane-bound state and that the stability of such membrane-bound enzymes is generally much greater than that of the enzymes released from membrane. Inclusion of enzymes within

suitable gels seems to give microenvironment analogous to that of membrane-bound enzymes. Furthermore, when microbial cells or organelles are entrapped in gels, it will obviously stabilize membrane-bound enzymes by preventing their solubilization after lysis of the cells or organelles. In the case of acetone-dried cells of *Arthrobacter simplex* used in this study, the marked operational stability of the gel-entrapped cells would result from this effect.

It should be noted that influences of the property of gels on the stability and activity of biocatalysts entrapped in the gels are very complicated. Klibanov[20] claims that immobilization of an enzyme to a support whose surface partially excludes an organic cosolvent will stabilize the immobilized enzyme. In this case, the concentration of organic cosolvent in the microenvironment of the enzyme will be less than in a bulk solution and, as a consequence, stabilization of the immobilized enzyme will be observed. This stabilization could be obtained by using gels of suitable hydrophilic nature, but low affinity for hydrophobic substrates of such hydrophilic gels will decrease the apparent activity of the gel-entrapped biocatalysts. In the case, where the incorporation of substrates and/or diffusion rate of thus uptaken substrates through gels will be rate-limiting, the use of appropriately hydrophobic gel material will have good effects. In a previous paper from our Laboratory,[4] the effectiveness of such hydrophobic gel-entrapped *Nocardia rhodocrous* cells was ascertained for the conversion of 3β-hydroxy-Δ^5-steroids to the corresponding 3-keto-Δ^4-steroids in a suitable organic solvent system (a benzene–*n*-heptane mixture). In these cases, the activity of the gel-entrapped cells related closely to the partition coefficients of substrates between the gels and the external solvent.

However, the results mentioned in this paper did not show such effect of the gel hydrophobicity, although the prepolymers with different enthylene oxide content (for example, PU-3, PU-6 and PU-9) would give the gels of different hydrophobicity. When the gels with various hydrophobicity were prepared by mixing hydrophilic PU-9 and hydrophobic PU-3, no difference in the conversion rates for hydrocortisone was obtained in spite of the marked difference in the partition coefficients for hydrocortisone. These results may be due to the facts that the concentration of hydrocortisone uptaken into any cell-entrapping gels is much higher than *Km* value for the free cells (0.64 mM). Even in the most hydrophilic PU-9 gel, the concentration of hydrocortisone was estimated to be 2.43 mM from the partition coefficient for the steroid. Swelling of the gels with high contents of PU-9 under the reaction conditions also seems to enhance the permeation of the steroid into gels.

The advantages of the entrapment of microbial cells with urethane prepolymers are not only its simplicity and convenience but also the protective effect of gels against denaturation by an organic solvent and improvement of the operational stability of the activity.

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