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# STUDY OF IMMOBILIZED α-STEROID DEHYDROGENASE IN WATER–METHANOL MEDIA
## Cofactor Regeneration in Situ

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The present paper deals with the use of immobilized α-steroid dehydrogenase in water–methanol media. The cofactor molecule is grafted at the site of the enzyme and regenerated with oxygen using phenazine methosulfate (PMS) as an electron carrier. From our results, the possibility of using immobilized steroid dehydrogenase in water–methanol solutions and the feasibility of the regeneration and retention of the pyridinic cofactors have been shown.

### INTRODUCTION

Immobilization and stabilization of enzyme systems by various methods (1) have increased the possibilities in enzyme applications. But little attention has been paid to steroid dehydrogenases. Cremonesi et al. (2) have described the preparation of 20 β-hydroxysteroids by using a dehydrogenase. The work of Larsson and Mosbach (3) has involved dehydrogenase in steroid-transforming microorganisms immobilized in polyacrylamide. Studebaker and Slocum (4) have described the enzymatic catalysis of a three-step steroid conversion. The specific dehydrogenation of steroid has an important industrial potentiality, and it is of interest in organic biochemistry.

However, a principal limiting factor of the use of dehydrogenases is the low solubility of steroids in water. Several authors have described the use of stabilized enzyme in nonaqueous media. For example, Weetall and Vann (5) have studied trypsin in organic solvents, and Buckland et al. have investigated the enzyme transformation of cholesterol in nonaqueous solvents (6). The work of Cremonesi et al. (2) gives a nice example of an enzymatic process in a two phase system.

A second limiting factor in the further development of the use of steroid dehydrogenase is the regeneration of cofactor. Wykes et al. (7), Davies and Mosbach (8), Coughlin et al. (9), May and Landgraff (10), Campbell and



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Chang (11), and Chambers et al. (12) have described systems allowing the retention and the regeneration of pyridinic cofactors in enzyme systems. Gestrelius et al. (13), Mansson et al. (14), and Legoy et al. (15) have shown the advantages of artificially fixing a normally dissociable coenzyme acting as a cosubstrate in a permanent position in the immediate vicinity of the active site of the enzyme.

The present paper deals with the use of immobilized $\alpha$-steroid dehydrogenase in water–methanol media. The cofactor molecule is grafted at the active site of the enzyme and regenerated with oxygen using phenazine methosulfate (PMS) as an electron carrier.

## MATERIALS AND METHODS

The enzyme was immobilized within an artificial proteinic membrane. The membrane was prepared according to a previously described cross-linking method (15). An aliquot of 1.5 ml of a solution containing 42 mg/ml albumin, 3.6 mg/ml glutaraldehyde in 0.02 M phosphate buffer, pH 6.8, was prepared. After 30 min of "precross-linking", 1 mg of $\alpha$-steroid dehydrogenase (*Pseudomonas testosteroni*, Sigma) and 5 mg of $NAD^+$ were added to the solution. The resulting solution was spread on a flat glass plate. After 3 h, complete insolubilization occurred, and a membrane was produced. The enzyme membrane was thoroughly rinsed with distilled water. The membrane area and thickness were 20 $cm^2$ and 70 $\mu$, respectively. The activity yield was equal to 45%. The membrane was stored in aqueous conditions at 4°C.

Enzyme activity, in the absence of cofactor regeneration, was spectrophotometrically measured at 340 nm in a 0.166 M pyrophosphate buffer, pH 9.0, solution containing 0.26 mg/ml of $NAD^+$. For both native and immobilized enzymes to start the reaction, the androsterone solution in methanol was added in the cuvette. In the presence of cofactor regeneration, enzyme activity was directly obtained by measuring the evolution of androsterone concentration with an enzyme method using $\alpha$-steroid dehydrogenase. When immobilized, 20 $cm^2$ of membrane bearing 1 mg of enzyme was tested in a batch of 26 ml. The solution was continuously circulating in a flow cuvette. When regeneration of the cofactor was carried out, 0.02 mg/ml of PMS was introduced in the reaction medium. When superoxide dismutase (bovine blood, Sigma) was used, 1 mg was introduced in a 20 $cm^2$ membrane.

## RESULTS AND DISCUSSION

The resulting enzyme activity after immobilization was studied as a function of the $NAD^+$ concentration used during the immobilization process

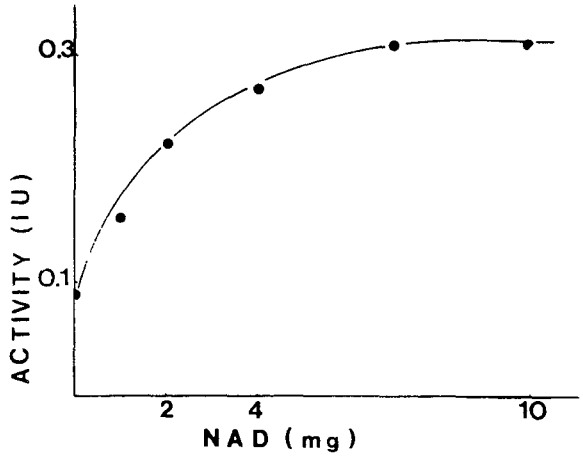


FIG. 1. Artificial α-steroid dehydrogenase membrane. Resulting enzyme activity after immobilization as a function of the amount of $NAD^+$ introduced during the immobilization process.

(Fig. 1). The maximum observed by Legoy and Thomas (15) with alcohol dehydrogenase was not observed with α-steroid dehydrogenase. It is important to note that the best stabilization effect is obtained with NAD concentrations more than tenfold of the $K_m$ value. Hence, it seems that this stabilization is due not only to the cofactor (NAD) binding at the active site, but at other secondary sites as well. Due to the low solubility of the substrate, the use of nonaqueous solvent was needed. It was of interest to compare the relative stabilities of free and immobilized enzymes in the presence of pure methanol. A significant increase of the enzyme stability was observed after immobilization. Halflife times were 2 and 20 min for free and immobilized enzymes, respectively. The activity as a function of methanol concentration in the reaction medium was studied for native and immobilized enzymes (Fig. 2). An increase of the activity was observed until 5%; with higher concentrations a decrease of the activity was shown. The increase is due to the improvement of the substrate solubility linked to the presence of methanol. The decrease is due to the denaturation of the enzyme. The immobilized enzyme is less sensitive than the native one.

A limiting factor in the use of the described enzyme has been the consumption of the pyridinic cofactor. In our work, a chemical system of regeneration was used. Legoy et al. have shown with alcohol dehydrogenase by a fluorescence method that with the procedure described above the $NAD^+$ is immobilized at the active site level, when the NAD concentrations are large enough during the immobilization process (16). It was of interest to test if the same behavior is possible with α-steroid dehydrogenase.

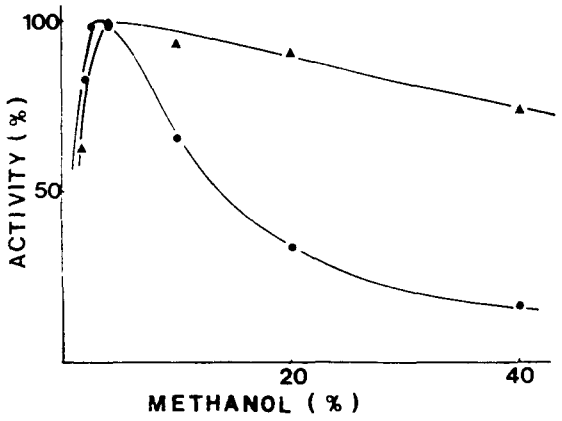


FIG. 2. Relative enzyme activity free (circles) and immobilized (triangles) as a function of methanol concentration in the reaction medium.

The enzyme membrane was tested in the absence of free NAD molecules, but in the presence of phenazine methosulfate. PMS and oxygen gave a regeneration of the cofactor molecules. The time variation of androsterone concentration was studied in these conditions (Fig. 3). The results showed evidence for a retention and regeneration of the cofactor at the active site. After 1 h of recycling, the conversion of substrate ceased and only about one-fourth of the total substrate had been converted. The occurrence of

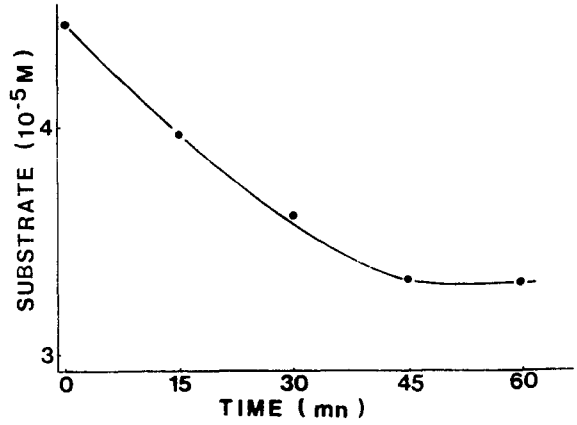


FIG. 3. Androsterone concentration in the reaction medium as a function of time when dealing with immobilized enzyme acting in presence of traces of PMS and of oxygen. The solution does not contain any exogenous $NAD^+$ molecules.

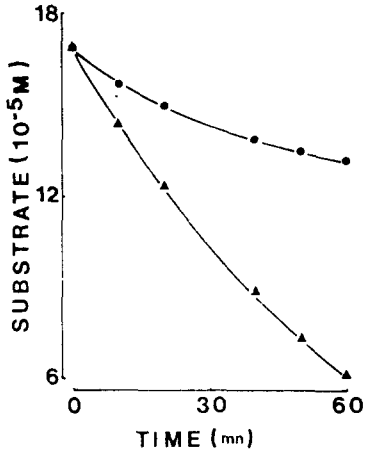


FIG. 4. Androsterone concentration in the reaction medium as a function of time under regeneration conditions when dealing with a membrane bearing both α-steroid dehydrogenase and superoxide dismutase (triangles) and with a membrane bearing only the first enzyme (circles).

superoxide anion in the reaction between reduced PMS and molecular oxygen has been discussed by Nishikimi et al. (17).

In order to overcome the possible denaturation effect of the superoxide anion in our steroid dehydrogenase system, superoxide dismutase was immobilized in the active membrane (1 mg per membrane of 20 $cm^2$). Experiments dealing with higher androsterone concentrations were performed with the new membrane (Fig. 4). Results were compared with data obtained from the same system without superoxide dismutase. A protective effect due to the presence of superoxide dismutase was observed. The possibility of using immobilized steroid dehydrogenase in water–methanol solutions and the feasibility of the regeneration and retention of the pyridinic cofactors have been shown.

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