The present findings suggest that *N*-ethylmaleimide acts as an "activator" by blocking the binding site for the formation of the dead-end enzyme–dihydroxyacetone phosphate complex[2].

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Enzymatically active membranes: Some properties of cellophane membranes supporting cross-linked enzymes

Some recent studies[1–3] have emphasized the biological interest of enzymes linked to insoluble macromolecules.

In a previous work, we have shown that bifunctional agents can be used to bind enzymes to insoluble sheets and membranes[4]. In this paper, we report the preparation of catalytically active cellophane–glucose oxidase (β-D-glucose:oxygen oxidoreductase, EC 1.1.3.4) membranes, using glutaraldehyde as the cross-linking agent[5,6], and we describe some properties of the bound enzyme as compared with the soluble one. Similar techniques have been employed successfully using other enzymes such as trypsin, chymotrypsin, urease, carbonic anhydrase, urate oxidase (uricase) and different supporting membranes.

Cellophane sheets (0.05 mm thick) are impregnated with an enzyme solution containing 6 mg/ml of protein in water. The water is evaporated by perventilation at 4°. The coupling between enzyme molecules is obtained by dropwise addition of a solution of 2.5% (w/v) glutaraldehyde in 0.1 M phosphate buffer (pH 6.8)[5] on both surfaces of the membrane. The preparation is then left overnight at 4°. The membrane is rinsed with a physiological saline solution until the excess of uncoupled protein and glutaraldehyde is completely eluted. This is controlled by the decay of the absorption at 280 mμ of the rinsing solution. Nitrogen determination shows that 0.1 mg of protein is bound to 1 cm^2 of cellophane.

In order to measure the activity of the bound enzyme, the membrane is dipped in a solution of glucose through which pure oxygen is bubbled. At intervals samples

of the solution are removed, and the activity is measured according to HYVARINEN AND NIKKILA[7] by following the drop of the reductive power of glucose due to its transformation to gluconic acid. The specific activity of the bound enzyme was found to be 12% of that of the native enzyme.

Qualitatively, the presence of enzyme on the membrane was detected using an histochemical stain specific for glucose oxidase[5]. The membrane was immersed in the absence of light in a solution of 0.1 M phosphate buffer (pH 6.9) containing M.T.T. tetrazolium salt (0.15 mg/ml), 0.1 mg of phenazine methosulfate and 5 mg of D-glucose. A purple color of the insoluble formazan develops throughout the entire depth of the membrane.

To test whether the enzyme remains firmly bound to the insoluble membrane and that the catalytic activity pertains to the cross-linked enzyme molecules, an active membrane was left for 24 h in a 0.03 M glucose solution. Then a sample of the solution was removed and the presence of glucose oxidase was detected in it using the M.T.T. test. No coloration was observed, indicating that the enzyme is firmly bound to the membrane even in the presence of a high concentration of substrate. Moreover, a powder of finely ground membrane was suspended in water. The suspension was thoroughly mixed and centrifuged. No coloration of the supernatant was noted when the M.T.T. test was carried out, demonstrating that no solubilization of active enzyme molecules takes place. Evidence that the cross-linking of proteins with glutaraldehyde proceeds through the formation of covalent bonds has been already reported[6].

Some comparisons between the enzyme activities in solution and in membrane were carried out by using substrate concentrations largely exceeding the K_m value of the enzyme for glucose, and the formation of gluconic acid was measured by an acidimetric method with a "pH-stat" using a 50 cm^2 membrane dipped into a solution of glucose (0.15 M) in phosphate buffer (0.005 M). The chosen pH was kept constant by the addition of NaOH (0.1 M) while pure oxygen was bubbled through the solution.

Fig. 1 shows the activity of glucose oxidase in solution and in membrane for

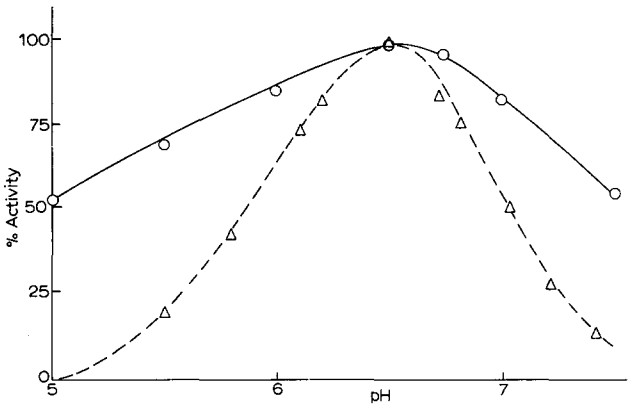

Fig. 1. Activities of glucose oxidase at different pH values between 5 and 8. Activity was measured in the pH-stat at 35° using 0.15 M glucose in 0.005 M phosphate buffer. Ordinate: percentage of activity at a given pH to activity at optimum pH. Abscissa: pH values. ○—○, enzyme in solution. △— — —△, enzyme cross-linked to cellophane.

various pH values at 35°. No significant shift of the apparent optimum pH is observed. This is contrary to the results that GOLDMAN *et al.*[8] have obtained working with papain and resembles the results of WHITTAM *et al.*[9] on apyrase bound on cellulose matrix. The catalytic activity of the bound glucose oxidase appears more susceptible to pH changes than in solution. Also the activity of the enzyme linked on the membrane seems to be, at an optimum pH, more temperature dependent. More work is needed in order to formulate a hypothesis explaining these results.

The binding to cellophane sheets increases the resistance of glucose oxidase to thermal denaturation. After 12 h of incubation at 37°, the catalytic activity of glucose oxidase in solution decreases, except when the enzyme is cross-linked to the membrane. Catalytic activity of stored dry membranes was tested after one month of storage at −25°, +4° and +20° and after 15 days at 37°. Under these conditions, no decrease in the activity of the bound enzyme was noted.

The same value of the Michaelis constant[10] was found for the membrane-bound enzyme as for the enzyme in solution (K_m = 0.013 M). Glucose oxidase in solution exhibits 1% activity on D-mannose, 0.15% on D-galactose and no activity on D-arabinose and D-fructose (where 100% denotes the activity of the enzyme on D-glucose). Identical values were found for the membrane-bound glucose oxidase.

In summarizing, these results tend to confirm that at optimum pH, the essential characteristics of the active molecules cross-linked to a membrane either remain unchanged or, at the most, are only slightly modified.

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