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Factors Influencing the Activity of Succinate Dehydrogenase in Membrane Preparations from *Micrococcus lysodeikticus*

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1. Some properties of succinate dehydrogenase [succinate–(acceptor) oxidoreductase, EC 1.3.99.1] in membrane preparations from *Micrococcus lysodeikticus* (N.C.T.C. 2665) were investigated. 2. In the spectrophotometric assay system adopted the reaction velocity was shown to be proportional to the amount of membrane added. Dichlorophenol-indophenol, reduced photochemically in the presence of phenazine methosulphate, or enzymically by the membrane-bound enzyme, was shown to undergo reoxidation in the dark. 3. The membrane-bound enzyme was found to be inactivated at temperatures above 10°C. 4. The specific activity of membrane-bound succinate dehydrogenase was found to increase between two- and three-fold in diluted membrane preparations equilibrated at 0°C for 6h. Membranes treated with sodium deoxycholate showed no enzyme activation on dilution but displayed maximal activity, all activity being sedimentable at 103000*g*. The increase in specific activity observed on dilution could be partially inhibited by fixation with glutaraldehyde, or by the presence of bovine serum albumin. 5. The addition of Mg^{2+} or Ca^{2+} ions to membrane suspensions caused an overall depression of enzyme activity. 6. The results suggest the presence of an 'inhibitor' that affects the expression of membrane bound succinate dehydrogenase activity.

Several reports on the fractionation of bacterial membrane systems have been published (Ferrandes, Chaix & Ryter, 1966; Fitz-James, 1967; Reaveley, 1968; Ghosh & Murray, 1969; Reaveley & Rogers, 1969). The fractionation methods usually yield a minor small vesicle fraction [the so-called 'mesosome' fraction (Fitz-James, 1960)] which has been shown to differ from the major membrane fraction in several respects. A differential distribution of components of the respiratory chain between major and minor fractions has been shown in several organisms (Ferrandes *et al.* 1966; Reaveley, 1968; Ghosh & Murray, 1969; Reaveley & Rogers, 1969). Various membrane fractions from *Micrococcus lysodeikticus* showed different amounts of succinate dehydrogenase [succinate–(acceptor) oxidoreductase, EC 1.3.99.1] activity. The purpose of this investigation was to determine to what extent differences in activity could be accounted for by variations in membrane preparation procedure or conditions of the enzyme assay.

MATERIALS AND METHODS

Chemicals. Lysozyme (EC 3.2.1.17) was obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.) and deoxyribonuclease (EC 3.1.4.6) from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.), DCIP,* PMS and sodium dodecyl sulphate were from BDH Chemicals Ltd. (Poole, Dorset, U.K.), tris, disodium succinate (enzyme grade) and sodium deoxycholate were from Koch–Light Laboratories Ltd. (Colnbrook, Bucks., U.K.), glutaraldehyde was from Taab Laboratories (Reading, Berks., U.K.) and bovine serum albumin Fraction V was from Armour Pharmaceutical Co. Ltd. (Eastbourne, Sussex, U.K.). All other chemicals and reagents were of analytical grade.

Preparation of 'standard' membranes. Membrane preparation was based throughout on the method of Salton & Freer (1965). Cells of *Micrococcus lysodeikticus* (N.C.T.C. 2665) were grown from a 10% inoculum in 2 litre conical flasks containing 500ml of a medium containing 5% Bacto-Peptone, 0.1% Difco Yeast Extract, 0.5% NaCl, pH7.2, on an orbital shaker at 30°C (150 rev./min). After 18h of growth cells were harvested at 4°C (2500*g* for 25min) and washed once with distilled water and once with 50mM-tris–HCl buffer, pH7.5. (This buffer, referred to as 'tris', was used throughout this work, unless otherwise stated.)

Cells were resuspended in tris buffer to approx. 50mg dry wt./ml. Cell walls were removed with lysozyme at a concentration of 100μg/ml by incubation at 30°C for 45min. Deoxyribonuclease was added to the lysed protoplast suspension to decrease the viscosity. Membranes were sedimented from the total lysate by centrifugation at 38000*g* for 50min at 0°C and kept at 0°C during all subsequent procedures, unless otherwise stated. Membrane fractions were washed six times with tris buffer by

* Abbreviations: DCIP, 2,6-dichlorophenol-indophenol; PMS, phenazine methosulphate.

resuspension and recentrifugation and finally resuspended in tris buffer to 10–15mg dry wt. of membrane/ml. This suspension is referred to as 'standard' membranes, and was always prepared within 18h of harvesting the cells.

Preparations of 'equilibrated' membranes. 'Standard' membranes were diluted in tris buffer to give 200–500μg dry wt. of membrane/ml and kept at 0°C for at least 6h. This suspension was termed 'equilibrated' membranes.

Glutaraldehyde fixation. 'Standard' membrane suspensions (10–15mg dry wt. of membrane/ml) were diluted with glutaraldehyde in tris buffer to give a final concentration of approx. 5mg dry wt. of membrane/ml of buffered glutaraldehyde (0.5% glutaraldehyde in tris buffer). The 'standard' membrane suspension was rapidly diluted and mixed in the fixative solution to prevent aggregation. The membrane suspension was then immediately centrifuged for 50min at 38000*g* and the membrane pellets were resuspended and washed twice in tris buffer to remove glutaraldehyde before resuspension in tris buffer to give 10–15mg dry wt./ml. All operations and reagents were at 0°C.

Deoxycholate extraction. Deoxycholate extraction was performed essentially as reported by Salton, Freer & Ellar (1968). 'Standard' membrane suspensions were extracted six times with 1% (w/v) sodium deoxycholate in tris buffer at 0°C and washed three times in tris buffer to remove residual deoxycholate. Membrane residues were sedimented at 38000*g* for 1h at 0°C during extraction and washing and then resuspended in a volume of tris buffer equivalent to that of the 'standard' membrane suspension before extraction.

Succinate dehydrogenase assay. The procedure for assay of succinate dehydrogenase was based on the spectrophotometric method of Ells (1959) with succinate as substrate, KCN as an inhibitor of the terminal oxidase, PMS as intermediate electron acceptor, and DCIP as terminal electron acceptor. The test cuvette (1cm light-path) contained 0.06ml of 2.5mM-DCIP, 0.30ml of 10 mM-KCN, 0.15ml of PMS (3mg/ml, freshly prepared), 0.60ml of 20mM-disodium succinate and membrane suspension. Tris buffer was added to give a final volume of 3.00ml. All reagents except for PMS were present in the 'blank' cuvette. Membrane suspensions were brought rapidly from 0°C to assay temperature, and the reaction was initiated with succinate. Decrease in E_{600} was recorded in a Pye Unicam SP.800 spectrophotometer fitted with temperature-controlled cuvette holders and a recorder. The reaction velocity, expressed as ΔE_{600}/min was measured over the linear part of the plot, which occupied the first 50% of the reaction. Assay was performed at 15°C unless otherwise stated. To minimize the photoreduction of DCIP found to occur in the presence of PMS the test cuvette was covered with aluminium foil until the reaction had been initiated.

RESULTS

Factors affecting the assay of succinate dehydrogenase in membrane preparations. In the assay system adopted the reaction velocity was found to be proportional to the amount of membrane added, no reduction of DCIP occurring in the absence of succinate. It was found that the reduction of DCIP by membrane preparations in the absence of PMS amounted to approx. 10% of that observed in the complete assay system (considerably higher values were found in assays of total lysate activity).

To measure only the PMS mediated reduction of

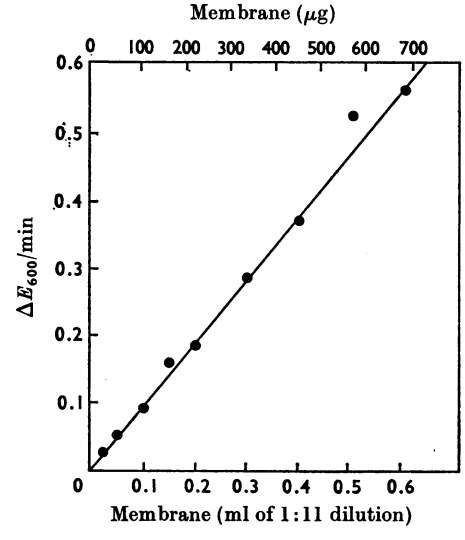

Fig. 1. Relationship of succinate dehydrogenase activity to the amount of membrane present in the assay. 'Standard' membrane suspensions were diluted (1:11) and equilibrated in tris buffer at 0°C for 12h before assay at 30°C.

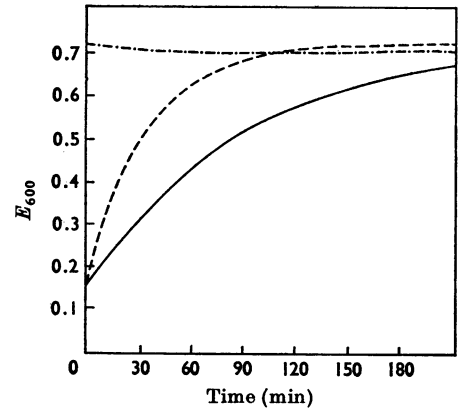

Fig. 2. Reoxidation of DCIP after photoreduction in the presence of PMS. The assay system without enzyme was exposed briefly to sunlight and reoxidation was recorded continuously at 25°C (----); reoxidation after photoreduction in the presence of 0.67% sodium dodecyl sulphate was also recorded (——). ·–·–·–·, Assay system without enzyme and protected from sunlight.

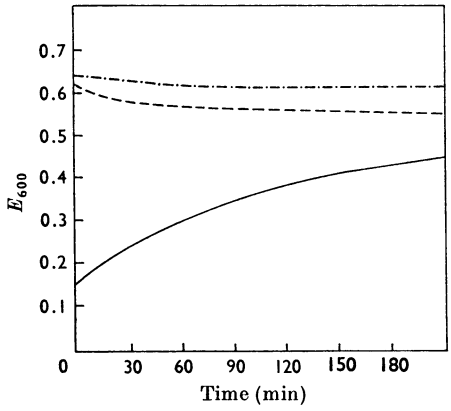

Fig. 3. Reoxidation of DCIP at 25°C after enzymic reduction, in the absence of photoreduction. The enzyme was inactivated by 0.67% sodium dodecyl sulphate after enzymic reduction of DCIP (——) or before addition of enzyme to the assay system (– – – –). ·–·–·–·, Assay system without enzyme and protected from sunlight.

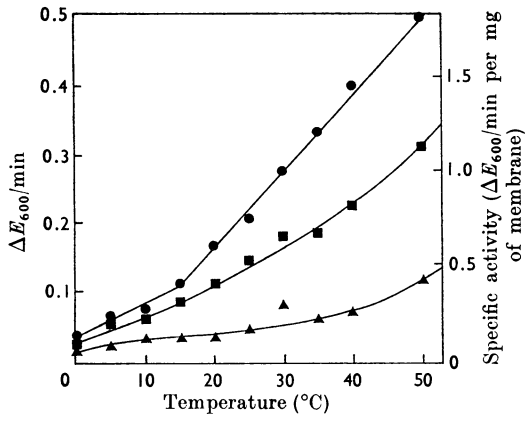

Fig. 4. Effect of assay temperature on the activity of succinate dehydrogenase in membrane suspensions equilibrated for 12h at 0°C in various diluents. ●, Tris buffer; ■, tris buffer containing 10mg of bovine serum albumin/ml; ▲, tris buffer containing 12mM-disodium succinate.

DCIP, it is essential to omit PMS from the 'blank' cuvette. The inclusion of PMS necessitates the elimination of either enzyme, substrate or terminal acceptor from the 'blank'. Under these latter conditions the overall reaction rate is a measure of both PMS-mediated and non-PMS-mediated dye reduction.

The enzyme reaction rate was found to be dependent on the concentration of PMS, and within the range of PMS tested (0.05–0.30mg/ml of assay mixture), the rate of dye reduction was proportional to the amount of membrane added. This result, for PMS at a concentration of 0.15mg/ml (as in the adopted assay system), is illustrated in Fig. 1.

It was found that spontaneous reduction of DCIP in the presence of PMS occurred on exposure to sunlight, a reaction that did not appear to be grossly affected by the other constituents of the assay system. This observation necessitated the protection of the test cuvette from direct light during assay. After photoreduction, the dye is reoxidized in the dark, the rate being proportional to the concentration of PMS. This process can be monitored at 600nm, being unaffected by irradiation at this wave-length. From Fig. 2 it can be deduced that the rate of reoxidation is also proportional to the concentration of reduced DCIP. After enzymic reduction of DCIP under assay conditions, reoxidation was again evident, at a rate similar to that observed for the photoreduced dye (see Fig. 3). In the experiment illustrated in Fig. 3, enzyme was inhibited after dye reduction by addition of sodium dodecyl sulphate to a final concentration of 0.67% (w/v).

Variation of enzyme reaction velocity with temperature is illustrated in Fig. 4. In experiments conducted with membrane preparations equilibrated in tris buffer alone a point of inflexion was consistently observed ata pprox. 15°C. The temperature of assay that was finally adopted was 15°C, and the reasons for this choice are evident from the results presented below. The effect of temperature on enzyme activity is illustrated in Table 1. 'Equilibrated' membrane suspensions were rapidly brought to assay temperature and the change in enzyme activity at the particular temperature was recorded at intervals over the next 1h. At temperatures below 10°C the loss of activity over the first 1h was slight (less than 13%), most of this occurring within the first 20min. However, at temperatures exceeding 15°C, an appreciable and progressive loss of activity was noted over the first 1h (greater than 55% at 25°C).

Preincubation of membrane suspensions with succinate for 1h appeared to increase the initial rate of enzyme inactivation, and at temperatures up to 25°C resulted in an overall depression of activity (see Table 1). Equilibration of membrane suspensions in the presence of succinate also resulted in a depression of specific activity when compared with suspensions equilibrated in either buffered bovine serum albumin solutions or buffer alone. The depression was evident over the range of assay temperatures 0–50°C (Fig. 4). This result contrasts with the reported activation of mitochondrial succinate dehydrogenase by preincubation with substrate (Kearney, 1957).

Enzyme activity and membrane concentration. The specific activity of succinate dehydrogenase in

Table 1. *Time-course of thermal inactivation of 'equilibrated' membrane suspensions at various temperatures*

Membrane suspensions (228 μg dry wt. of membrane/ml) were equilibrated for at least 12 h at 0°C in tris buffer (Expt. *a*) and in tris buffer containing 12 mM-disodium succinate (Expt. *b*). The suspensions were then brought to the desired temperature and succinate dehydrogenase activity was assayed at that temperature over the next 1 h.

Temperature (°C)	Expt. type	100 × Succinate dehydrogenase activity (ΔE_{600}/min) Time ... 0	15 min	30 min	45 min	60 min
5	*a*	6.1	5.4	5.0	5.0	4.9
	b	4.2	4.0	3.7	3.1	2.2
15	*a*	10.1	9.2	8.7	8.4	8.1
	b	7.6	5.3	4.9	4.8	4.8
25	*a*	16.7	14.4	12.0	9.7	7.4
	b	12.3	8.3	7.8	7.2	6.3

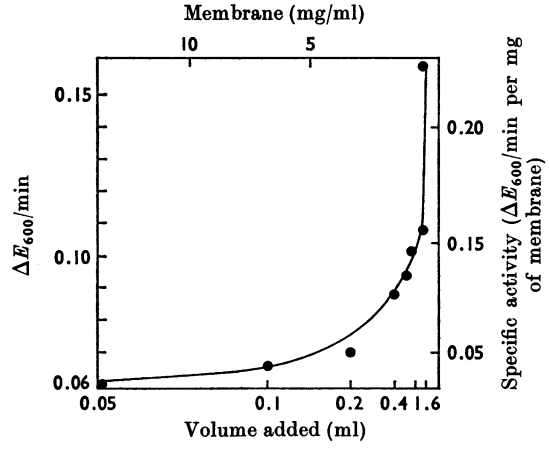

Fig. 5. Effect of membrane concentration on the activity of succinate dehydrogenase. 'Standard' membrane suspensions were diluted in tris buffer to various concentrations and equilibrated for 12 h at 0°C before assay at 0°C. The upper abscissa represents the membrane concentration in the suspensions and the lower abscissa the volume of that suspension present in the assay system. All determinations of enzyme activity were performed on the same dry wt. of membrane.

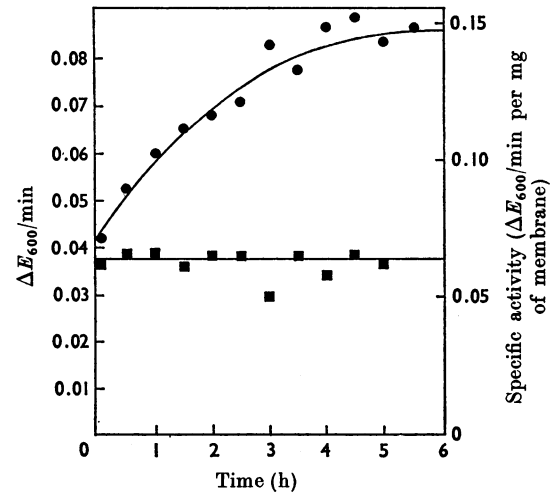

Fig. 6. Time-course of 'activation by dilution'. ●, Change in enzymic activity of a dilute membrane suspension (367 μg dry wt. of membrane/ml) prepared from a 'standard' membrane suspension (11.7 mg dry wt. of membrane/ml) by dilution in tris buffer at 0°C; ■, corresponding plot for the 'standard' membrane suspension All assays were performed at 15°C on the same dry wt. of membrane.

membrane suspensions was found to be dependent on membrane concentration. Over a range of concentration from 15 mg to 200 μg dry wt. of membrane/ml the specific activity was found to increase up to sevenfold although usually a two- to three-fold increase was observed on dilution (see Fig. 5). This 'activation by dilution' appeared to reach an equilibrium approx. 6 h after initiation (see Fig. 6). No change in activity was observed during the 6 h of equilibration in the 'standard' membrane suspensions.

Effect of deoxycholate extraction. Activation of succinate dehydrogenase by dilution (Fig. 5) was not observed in membranes that had previously been extracted with deoxycholate. Nevertheless, the combined activity of both the deoxycholate-extracted residue (38000*g* sediment) and that of the deoxycholate washes (sedimentable at 103000*g*) was equivalent to that observed for 'equilibrated' membrane suspensions (Fig. 7). This effect was observed in deoxycholate residues obtained from both 'standard' membranes and those of membrane suspensions obtained directly from total lysates.

Effect of glutaraldehyde and bovine serum albumin.

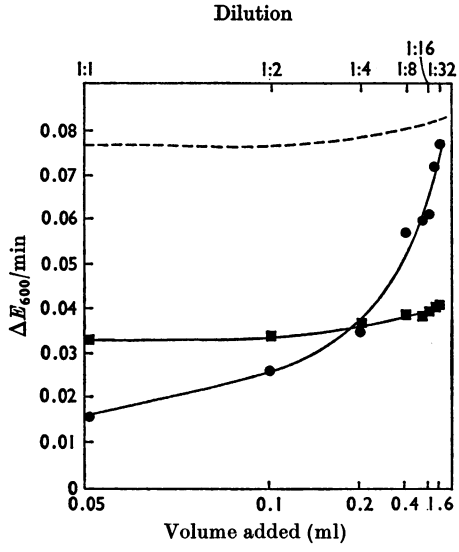

Fig. 7. Effect of dilution on the activity of succinate dehydrogenase in 'standard' and deoxycholate-extracted membrane suspensions. The upper abscissa represents the dilution factor of the 'standard' and deoxycholate-extracted membrane suspensions used, and the lower abscissa the corresponding volume of that suspension present in the assay system. The 'standard' (●) and corresponding deoxycholate-extracted membrane suspensions (■) were equilibrated at various dilutions in tris buffer for 12h at 0°C before assay at 15°C. ----, Total enzymic activity present in the deoxycholate-extracted membrane residues (38000*g* sediment) plus activity in the deoxycholate washes (sedimentable at 103000*g*).

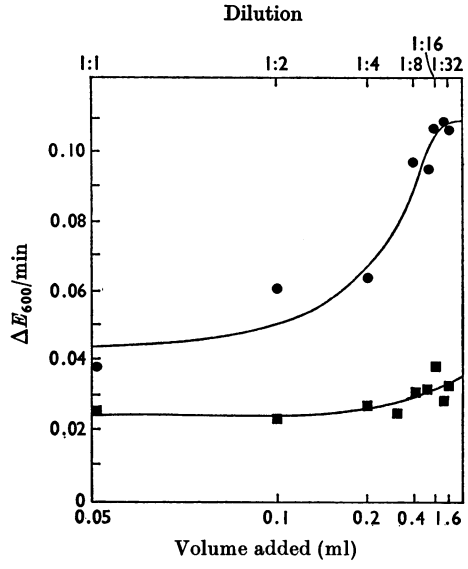

Fig. 8. Effect of dilution on the succinate dehydrogenase activity of 'standard' and glutaraldehyde-fixed membrane suspensions. The upper abscissa represents the dilution factor of the 'standard' and glutaraldehyde-fixed membrane suspensions, and the lower abscissa the corresponding volume of that suspension present in the assay system. The 'standard' (●) and glutaraldehyde-fixed (■) membrane suspensions were equilibrated at various dilutions in tris buffer for 12h at 0°C before assay at 15°C.

The loss of 'activation by dilution' after deoxycholate extraction could also be observed in 'standard' membranes subjected to mild fixation with glutaraldehyde (Fig. 8). However, to eliminate virtually all the 'activation by dilution', the degree of fixation required was such that an overall loss of about 30% of enzyme activity occurred, when compared with the activity of undiluted (i.e. 'standard') membranes.

The addition of bovine serum albumin to the diluent buffer also resulted in a depression of the enzyme activation normally observed on dilution of 'standard' membranes. The extent of depression depended on the concentration of bovine serum albumin and appeared to reach a maximum value of approx. 40% inhibition at a concentration of 10mg of bovine serum albumin/ml of suspension. The presence of bovine serum albumin (10mg/ml) in 'equilibrated' membrane suspensions caused an overall depression of enzyme activity when assayed at various temperatures between 0 and 50°C (Fig. 4). The point of inflexion observed to occur at 15°C with 'equilibrated' membranes was no longer evident.

Effect of bivalent cations. The influence of bivalent cations on the specific activity of membrane suspensions was investigated and the results are illustrated in Fig. 9. After dilution of 'standard' membranes in tris buffer containing either Mg^{2+} or Ca^{2+} ions, enzyme activity was observed to increase with time. However, the specific activity in suspensions containing bivalent cations was consistently lower than that of control suspensions. The extent of the cation-mediated depression of activity was dependent on the concentration of added ions and increased as the cation concentration was raised. The results of a similar experiment with deoxycholate-extracted membranes are shown in Fig. 10. Again, the presence of Mg^{2+} and Ca^{2+} ions caused an overall depression of specific activity when compared with control suspensions.

From the results presented in Figs. 9 and 10 it is evident that the presence of Mg^{2+} and Ca^{2+} ions does not influence 'activation by dilution', but does cause an immediate depression of enzyme activity.

DISCUSSION

Our results for *M. lysodeikticus* membrane preparations show that within the range of PMS concentrations examined the reaction velocity is

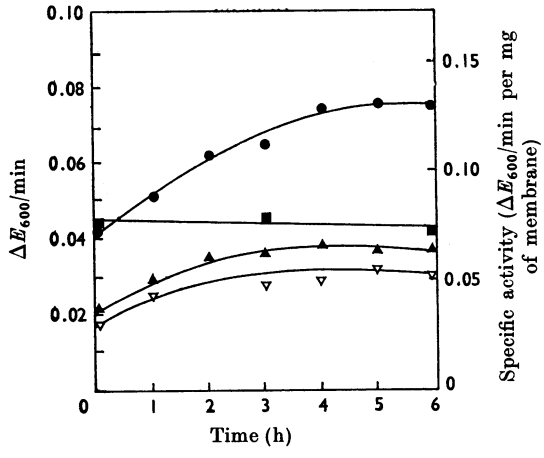

Fig. 9. Effect of Mg^{2+} and Ca^{2+} ions on the activity of succinate dehydrogenase in membrane suspensions. 'Standard' membrane suspensions (11.7mg dry wt. of membrane/ml) were diluted in tris buffer (●), tris buffer containing 40mM-$MgCl_2$ (▲) and tris buffer containing 40mM-$CaCl_2$ (▽) to 366μg dry wt. of membrane/ml. Enzyme activity was then recorded at intervals over the following 6h. ■, Activity in undiluted, i.e. 'standard', membrane suspensions. All assays were performed on the same dry wt. of membrane.

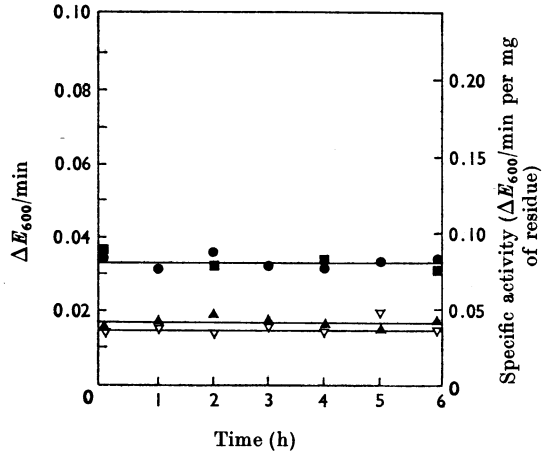

Fig. 10. Effect of Mg^{2+} and Ca^{2+} ions on the activity of succinate dehydrogenase in residues of deoxycholate-extracted membranes. Deoxycholate-extracted membranes (8.18mg dry wt. of residue/ml) were diluted in tris buffer (●), tris buffer containing 40mM-$MgCl_2$ (▲) and tris buffer containing 40mM-$CaCl_2$ (▽) to 256μg dry wt. of residue/ml. Enzyme activity was recorded at intervals over the following 6h. ■, Activity in undiluted deoxycholate-extracted membrane suspensions. All assays were performed on the same dry wt. of residue.

directly proportional to the amount of membrane added. Therefore, for estimates of comparative enzyme activity in such preparations, the assay system described by Ells (1959) and the modification used in this study, both using fixed concentrations of PMS, are acceptable.

Sensitivity of succinate dehydrogenase from *M. lysodeikticus* to temperatures greater than approx. 10°C has considerable bearing on the preparation methods adopted for membrane fractions. Since relatively rapid loss of enzyme activity occurs at room temperature (25°C), procedures involved in the production of 'standard' membranes must be carried out at 0°C.

The role of tris buffer in the observed thermal inactivation is not known. A diminished rate of inactivation does occur with a 100-fold decrease in the molarity of the buffer, but the membrane itself is known to undergo changes, resulting in the release of proteins, as a response to such a decrease in buffer molarity (Munoz, Nachbar, Schor & Salton, 1968; Munoz, Salton, Ng & Schor, 1969). Tris has been shown to retard substrate activation of succinate dehydrogenase in preparations obtained from higher plants (Hiatt, 1961). Similar inhibitory effects of tris may also be operative in the bacterial system described.

The 'activation by dilution' of succinate dehydrogenase observed in the present study shows some superficial resemblance to the 'allotopic' enzymes found in both bacterial (Munoz *et al.* 1969) and mitochondrial (Racker, 1967; Bruni & Racker, 1968; Bulos & Racker, 1968) membrane systems. An important difference between the 'activation by dilution' of bacterial succinate dehydrogenase and that of bacterial adenosine triphosphatase (Munoz *et al.* 1969) is that the increase in activity of the latter enzyme was shown to be the result of a transition from a 'bound' to a 'soluble' form. No such 'soluble' form could be demonstrated in the present study. The activation observed on dilution could be suppressed under conditions of mild fixation or increased soluble protein concentration. Extraction of membranes with deoxycholate resulted in the abolition of any 'activation by dilution', yet promoted maximal enzyme activity. These findings lend support to the possible presence of an 'inhibitor' associated with membrane-bound succinate dehydrogenase (cf. Warringa, Smith, Giuditta & Singer, 1958). Dilution of 'standard' membrane suspensions may result in the dissociation of 'inhibitor' leading to a corresponding activation of the membrane-bound enzyme. Deoxycholate extraction may also promote enzyme activity by removal of 'inhibitor' together with other membrane components (*see* Salton *et al.* 1968). Conversely, the presence of bovine serum albumin or fixation with glutaraldehyde (Bensch & King,

1961; Sabatini, Bensch & Barrnett, 1963; Bowes & Carter, 1966; Quiocho & Richards, 1966) may prevent the dissociation of inhibitor.

No evidence was found to indicate a role for bivalent cations in the observed activation of succinate dehydrogenase on dilution. Both Mg^{2+} and Ca^{2+} had no specific effect on the dilution phenomenon itself, although both of these cations caused an overall depression of enzyme activity. Since 'activation by dilution' of membranes was still evident in the presence of both Mg^{2+} and Ca^{2+}, it seems improbable that these ions play any role in the proposed association or dissociation of 'inhibitor'. Mg^{2+} or Ca^{2+} ions cause aggregation of *M. lysodeikticus* membranes dispersed by either ultrasound (Salton, 1967) or by detergents (Butler, Smith & Grula, 1967) and are involved in the binding of loosely associated membrane components (Munoz *et al.* 1968). Such mechanisms may account for the observed depression of enzyme activity in the presence of these cations. From the results presented above it is evident that careful consideration must be given to the history of membrane preparations used in comparative studies of membrane bound enzymes.

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