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MEMBRANE COARCTATION BY CALCIUM AS A REGULATOR FOR BOUND ENZYMES

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SUMMARY

The enzymic activity of spherical membranes formed by conjugates of trypsin and chymotrypsin with a polycarboxylic polymer decreases with increasing Ca^{2+} concentration in the surrounding solution. This phenomenon is reversible and attributed to the coarctation of the membrane structure rather than to changes in the intrinsic behavior of the bound enzymes. Coarctation decreases the swelling and increases the virtual cross-linking of the membrane so that the diffusion rate of the substrate to the catalytic sites is reduced. As a result the overal enzymic activity decreases and the observed reaction departs from the Michaelis–Menten kinetics. The activity of the trypsin conjugate decreases with increasing Ca^{2+} concentration unlike that of trypsin in free solution, because the effect of membrane coarctation masks the enhancement of tryptic activity by Ca^{2+}. The physical and chemical properties of these polycarboxylic membranes, which contain about 40% enzyme protein, resemble those of some cell membranes such as erythrocyte ghosts. The results suggest that a similar indirect regulation of the activity of bound enzymes *via* membrane coarctation by Ca^{2+} or other multivalent metal ions may occur in living systems also.

INTRODUCTION

The versatile role of calcium in cellular events has long been recognized. Some of the most fundamental physiological phenomena, such as muscle contraction, secretion by endocrine glands and intracellular communication are linked to membrane processes involving Ca^{2+}. *In vitro* experiments have demonstrated the propensity of various cellular membranes to form complexes with Ca^{2+} and the resulting changes in electrical resistance, permeability, *etc.* The wealth of experimental data notwithstanding our knowledge of the actual functions of Ca^{2+} is still exiguous. The goal of the present paper is to demonstrate by the results of simple model experiments that the activity of membrane bound enzymes can be regulated by a direct effect of Ca^{2+} on the membrane structure which can be of biological significance.

Abbreviations: TAME, *N*-tosyl-L-arginine methyl ester; TEE, L-tyrosine ethyl ester; BAEE, *N*-benzoyl-L-arginine ethyl ester.

Recently, Forstner and Manery[1,2] investigated the binding of Ca^{2+} by human erythrocyte membranes and found that most of the Ca^{2+} was bound to proteins rich in aspartic and glutamic acids rather than to lipids as had been postulated. They stated that "the physiological significance of the observed distribution of Ca^{2+} within membranes is as yet unknown" and "membrane protein–Ca^{2+} interaction has been largely unexplored to date". It has been known, however, that such membranes contain a large variety of enzymes and Green *et al.*[3] have long stipulated that all enzymes of glycolysis are embedded in the erythrocyte membranes.

We observed that when polycarboxylic trypsin and chymotrypsin conjugates were exposed to $CaCl_2$ solutions of different concentrations their physical properties underwent similar changes as those of erythrocyte ghosts[1] or calsequestrin, a Ca^{2+}-binding protein recently isolated by MacLennan and Wong[4] from the sarcoplasmic reticulum under similar conditions. The cross-linked enzyme conjugates and the ghosts showed increasing shrinkage (Palek *et al.*[5]) and hardening (Weed *et al.*[6]), tantamount to an increase of the degree of cross-linking, when the Ca^{2+} concentration was increased in the surrounding solution. This phenomenon is conveniently termed coarctation. This observation suggested that suitable membranes made of such enzyme conjugates might be employed to explore the effect of Ca^{2+} on the activity of enzymic membranes.

Most commonly enzymes are immobilized by covalent binding to solid matrices for use as specific biosorbents in affinity chromatography or as heterogeneous catalysts in biochemical reactors. Nonetheless, they can serve as simple models for studying enzyme action in restricted systems encountered in the cellular milieu, as was originally suggested by Katchalski[7]. A great deal of experimental data illustrates that the kinetic behavior of immobilized enzymes can be significantly different from that of the same enzymes in free solution due to micro-environmental and diffusional effects. Therefore, the study of the behavior of immobilized enzymes promises more reliable information about enzyme action in the natural cellular environment than the conventional methods with enzyme solutions.

In the present work most data were obtained with supported spherical enzymic membranes containing trypsin or chymotrypsin covalently bound to a polycarboxylic matrix formed by the hydrolysis of a maleic anhydride vinyl methyl ether copolymer (Horvath, C., unpublished). The shells of protein conjugate were supported by glass spherules and this type of material has been termed pellicular immobilized enzyme (Horvath and Engasser[21]) because of their structural similarity to pellicular sorbents used in high performance liquid chromatography (Horvath[8]). After evaluating the Ca^{2+}-binding and swelling properties of the polycarboxylic trypsin conjugate, the esterolytic activity of both type of enzymic membranes was measured at different Ca^{2+} concentrations.

The results indicated that the Ca^{2+}-binding properties of the polycarboxylic trypsin conjugate were similar to those of erythrocyte ghosts so that this material could serve indeed as a suitable model. The activity of such enzymic membranes was found to decrease with increasing Ca^{2+} concentration. The effect was reversible and attributed to the coarctation of the membrane structure. The results of these model experiments suggest a regulatory mechanism by Ca^{2+} for membrane-bound enzymes which does not necessarily involve a direct action of Ca^{2+} upon the enzyme molecules and may have considerable biological significance.

EXPERIMENTAL

Materials

Trypsin, code TRL, chymotrypsin, code CDI, *N*-tosyl-L-arginine methyl ester (TAME), L-tyrosine ethylester (TEE) and *N*-benzoyl-L-arginine ethyl ester (BAEE) were purchased from Worthington Biochemical Co. $CaCl_2$, trihydroxymethylamine (Tris), dimethylformamide and acetone were analytical grade and supplied by Fisher Scientific Co. Double distilled water was used in all studies. Maleic anhydride–vinyl methyl ether copolymers were gifts of GAF Co. Glass beads were obtained from Potter Bros. Co. and screened to obtain the sieve fraction No. 230–270. $^{45}CaCl_2$ was obtained from New England Nuclear Co.

Methods

Preparation of pellicular immobilized enzymes. Glass beads were first coated with a very thin layer of strong anion-exchange resin (Horvath[8]). 10 g of such beads were coated with a mixture of 60 mg of maleic anhydride and vinyl methyl ether and 60 mg of a maleic acid–vinyl methyl ether copolymer dissolved in dimethylformamide containing acetone and the solvents were evaporated. In some experiments beads coated with the half amount of polymer mixture (thin coating) were also used. The enzyme resin was obtained by treating 5 g of polymer-coated beads with 5 ml of enzyme solution containing 100 mg protein in 0.1 M phosphate buffer, pH 7.0, in a shaker bath at 4 °C overnight. For the thin-coated beads the half amount of protein was used in the coupling reaction. The product was washed with phosphate buffer until the washing showed no esterolytic activity, dried *in vacuo* and stored at 5 °C. No decrease of activity was observed over a 2-month period.

Measurement of enzymic activity. The pH-stat method (Jacobsen *et al.*[9]) was used to measure the esterolytic activity of both soluble and immobilized enzymes. The instrument consisted of ABU-1 automatic buret, TTT-1 titrator, SBR-2 recorder and Model 26 pH-meter (Radiometer Co.). The reaction vessel was thermostated to maintain the temperature at 25 ± 0.1 °C, except when the temperature profile of enzyme activity was measured. All the measurements but those for obtaining the pH profile of the enzyme activity were carried out at pH 7.0 and 8.0 with chymotrypsin and trypsin, respectively. The total liquid volume was 10 ml and vigorous stirring was employed. The titrant was 0.1 M NaOH. Each measurement was made in duplicate using 30 and 40 mg of pellicular trypsin and chymotrypsin resin, respectively. The amount of soluble enzyme was chosen to obtain reaction rates comparable to those measured with the immobilized enzymes. The initial substrate concentration in the reaction mixtures was $5 \cdot 10^{-3}$ M, except when the effect of substrate concentration on the initial reaction rate was studied. When the effect of Ca^{2+} was investigated, buffer solutions containing $CaCl_2$ at different concentrations were employed. In all experiments with pellicular enzyme conjugates, the enzymic activity was measured after contacting the beads with the buffer or Ca^{2+}-containing buffer at room temperature under occasional stirring for 10 min. In some experiments the activity of the beads was determined at $3 \cdot 10^{-2}$ M $CaCl_2$ concentration in the Tris buffer, then the beads were washed portionwise first with 30 ml of $2 \cdot 10^{-2}$ M EDTA solution in the Tris buffer subsequently with 30 ml of the buffer proper.

Determination of water uptake and Ca^{2+} binding. Bulk polycarboxylic trypsin

conjugate has been prepared in a similar procedure as the pellicular conjugate. The amount of bound protein was determined from the nitrogen content obtained by the Kjeldahl method. Assuming a nitrogen content of 14.9% for trypsin, the protein content was calculated as 39% of the dry conjugate.

About 50-mg quantities of the finely ground material were placed into 5-ml nitrocellulose centrifuge tubes (Beckman), and dried *in vacuo* in order to establish the dry weight of the resin. Then 4 ml of $5 \cdot 10^{-2}$ M Tris buffer, pH 7.0, containing $^{45}Ca^{2+}$ of known radioactivity but different concentrations of $CaCl_2$ were added to each tube. The contents of the tubes were thoroughly mixed and stored at 25 °C for 3 h. Subsequently the tubes were centrifuged in a Beckman Model L-265 ultracentrifuge at 48000 rev./min at 15 °C for 30 min. After removing the supernatant the weight of both the swollen enzyme gel and the supernatant was determined. The radioactivity of both the starting solutions and the supernatants was measured in a Beckman Model CPM-200 scintillation counter using Aquasol (New England Nuclear) with an accuracy of better than 1%.

The water uptake by the gel was calculated directly from the weights of the dry gel and the swollen gel after centrifugation and expressed as mg of water taken up by mg of dry gel. The Ca^{2+} binding by the polyacidic protein conjugate itself was calculated with the assumption that both the density and the Ca^{2+} concentration in the interstitial volume of the swollen gel equal those of the supernatant. Thus, the μequiv of Ca^{2+} bound per mg of dry trypsin conjugate, U, were calculated by the following equation

$$U = 8(1-\alpha)C/W$$

where α is the ratio of the number of counts in the supernatant to that in the starting solution, C is the molar Ca^{2+} concentration and W is the weight in mg of the dry polymer which was contacted with 4 ml of starting solution.

RESULTS AND DISCUSSION

In many respects our trypsin and chymotrypsin conjugates with poly maleic acid–vinyl methyl ether copolymer showed chemical properties similar to those of polycarboxylic trypsin gels made with other types of large molecular weight polyanhydrides as described by Katchalski and collaborators[10–12], Fritz *et al.*[13] and by Conte and Lehman[14]. The effect of Ca^{2+} on those immobilized enzymes, however, has not been investigated yet.

Therefore, it was necessary to obtain quantitative data on the Ca^{2+}-binding and swelling properties of our trypsin conjugate. The measurements were performed with the bulk product that had the same composition as the membraneous material. It contained 39% (w/w) protein, covalently bound to the polyanionic matrix formed by the hydrolysis of the maleic anhydride–vinyl methyl ether copolymer. The finely divided protein conjugate was equilibrated with $CaCl_2$ solutions of different concentrations in 0.05 M Tris buffer, pH 7.0. Subsequently the gel phase was separated from the supernatant by ultracentrifugation at $200000 \times g$. As seen in Fig. 1*, about 97% (w/w) of the swollen gel consisted of water at Ca^{2+} concentrations lower than 10^{-3} M. Thus, the amount of water in the swollen gel was greater than 30 times the

* pCa, the negative logarithm of the Ca^{2+} concentration is used in the graphs throughout.

dry weight of the conjugate even after ultracentrifugation. This compares with the 20- and 30-fold weight gain reported for the swelling of cross-linked dextrane, with an exclusion limit of mol. wt. 200000, and of a trypsin polyacrylic acid conjugate (Conte and Lehman[14]). The uptake of water by the dry resin, however, decreased with increasing Ca^{2+} concentration as shown in Fig. 1. The appearance of the gel changed from that of a soft mucilage to that of a harder gel as the concentration of Ca^{2+} increased. The reduced water content and rigidness of the wet gel were manifestations

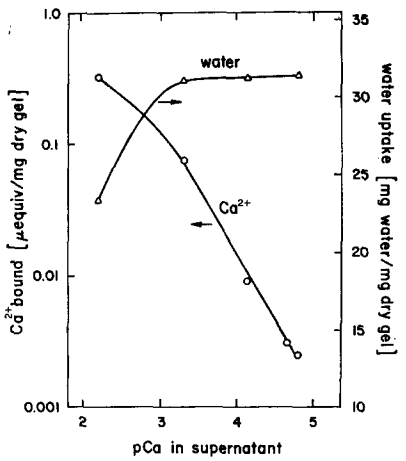


Fig. 1. Uptake of water and binding of Ca^{2+} by polycarboxylic trypsin conjugate as a function of the Ca^{2+} concentration in $5 \cdot 10^{-2}$ M Tris buffer, pH 7.0.

of the coarctation of the originally soft swollen gel by Ca^{2+}, which has also been observed with natural membranes, such as erythrocyte ghosts.

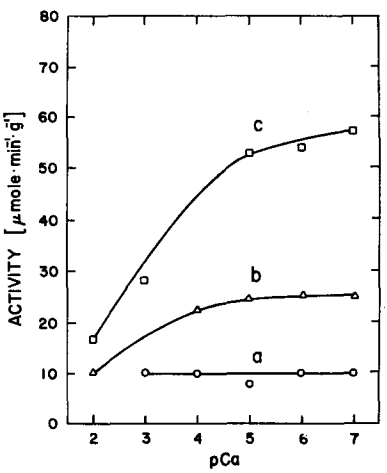
The Ca^{2+} binding by the polycarboxylic trypsin conjugate is also shown in Fig. 1. The corresponding dissociation constant has been evaluated from a reciprocal plot (Edsall and Wyman[15]) and found to be $2 \cdot 10^{-4}$ M. The Ca^{2+}-binding capacities of human erythrocyte ghost, calsequestrin and the polycarboxylic trypsin were of the same order of magnitude at the pCa value of the blood plasma which is about 2.9. The dissociation constants for membrane and calsequestrin-bound Ca^{2+} were reported to be in the range of 10^{-4} to 10^{-3} M (Kwant and Seeman[16]) and $4 \cdot 10^{-5}$ (MacLennan and Wong[1]), respectively. These values are commensurable with that obtained for the polycarboxylic trypsin conjugate. Nevertheless, the data reported in the literature were obtained with Tris buffers less concentrated than 10^{-2} M, while our measurements were made with $5 \cdot 10^{-2}$ M Tris buffer. Since it has been shown by Gent *et al.*[17] that the Ca^{2+} binding by ghosts decreased 74% when the ionic strength increased from 0.01 to 0.06, our results give a rather low estimate in comparison to the literature data obtained with natural membranes.

The above results show that the polycarboxylic trypsin conjugate has a significant Ca^{2+}-binding capacity and its physical properties change with increasing Ca^{2+} concentration as if the cross-linking of the polymer network would have been increased. This coarctative effect of Ca^{2+} is similar to that described for human erythrocyte ghost and other membrane proteins which contain a large amount of glutamic

and aspartic acids and have comparable Ca^{2+}-binding properties. The numerical values for Ca^{2+} binding indicate that the polycarboxylic trypsin conjugate may serve as a suitable model for the interaction of Ca^{2+} with membrane proteins located in erythrocytes or in the sarcoplasmic reticulum.

The enzymic properties of both the polycarboxylic trypsin and chymotrypsin conjugates were investigated with supported spherical membranes made of these materials. The spherical membranes were formed *in situ* on the surface of 50–μm diameter glass spherules having a thin primary coating of a strong anion-exchange resin in order to facilitate the adhesion of the polycarboxylic membrane to the glass surface. About 40% of the actual conjugate was protein so that the amount of protein was about 3.5 mg/g of coated glass beads (thick membranes). In some experiments the membrane thickness and the amount of protein conjugate per g of product was about the half of the above value (thin membranes). Since the glass surface was sealed by the polystyrene type ion-exchange resin, no Ca^{2+} was assumed to leach out of the glass during the experiments. Although this pellicular configuration of immobilized enzymes has originally been developed for use in packed beds, it is evidently a very convenient and suitable system to serve as an experimental model for cellular membranes.

In order to assess the effect of coarctation on the enzymic behavior of the membranes, the esterolytic activity of polycarboxylic chymotrypsin conjugates was measured toward L-tyrosine ethyl ester at different Ca^{2+} concentrations in the external solution. Fig. 2 shows the activity of membraneous chymotrypsin conjugates of different thickness and that of chymotrypsin in free solution as a function of the Ca^{2+} concentration. As seen, the activity of the dissolved chymotrypsin is unaffected by Ca^{2+} in contradistinction to the activity of both the thick and thin polycarboxylic chymotrypsin membranes which decreases with increasing Ca^{2+} concentration. The



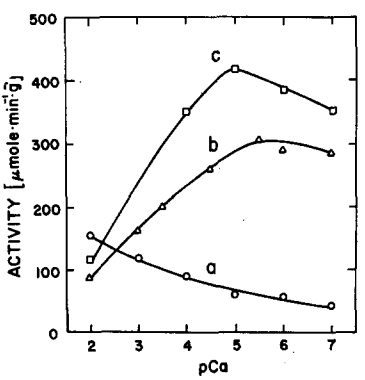
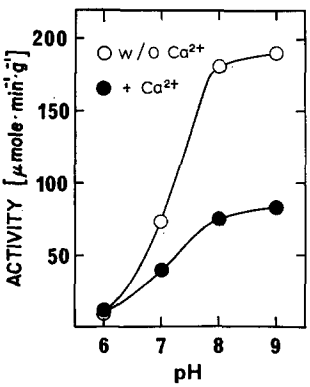


Fig. 2. Graph illustrating the effect of Ca^{2+} concentration on the activity of chymotrypsin in free solution (a) and in thin and thick polycarboxylic membranes (b and c), respectively. Substrate: $5 \cdot 10^{-3}$ M TEE in $1 \cdot 10^{-2}$ M Tris buffer, pH 7.0.

Fig. 3. Graph illustrating the effect of Ca^{2+} concentration on the activity of trypsin in free solution (a) and in thin and thick polycarboxylic membranes (b and c), respectively. Substrate: $5 \cdot 10^{-3}$ M TAME in 10^{-2} M Tris buffer, pH 8.0.

same set of experiments was performed also with polycarboxylic trypsin conjugates and BAEE as substrate. The results are shown in Fig. 3. It is known that the activity of trypsin is enhanced in the presence of Ca^{2+} (Sipos and Merkel[18]) and is shown also by the plot of the activity of trypsin in free solution against the Ca^{2+} concentration in Fig. 3. Therefore, the dramatic decrease in the tryptic activity in both the thin and thick membranes with increasing Ca^{2+} concentration as shown in Fig. 3 was quite unexpected.

The observations regarding the effect of Ca^{2+} on the behavior of the tryptic and chymotryptic membranes could be explained by Ca^{2+}-induced changes in the active structure of the immobilized enzymes *per se*. The investigation of the enzyme structure inside the polymer matrix, where a large number of phenomena may affect the enzyme catalyzed reaction, is beset by great difficulties. Yet, from some simple measurements, such as that of the pH profile and temperature profile, possible changes can be inferred. The pH profiles of both polycarboxylic enzyme conjugates were measured with and without Ca^{2+} in the external solution. The results shown in Figs 4 and 5 suggest that neither trypsin nor chymotrypsin inside the membrane was affected by Ca^{2+} since no shift in the pH of maximum activity was observed. Nevertheless, the pH profile of the polycarboxylic trypsin conjugate was different from that of trypsin in free solution as discussed by Levin *et al.*[19] earlier. As expected in view of the previous results the activity of both enzymes was reduced in the presence of Ca^{2+} almost in the whole pH range investigated.



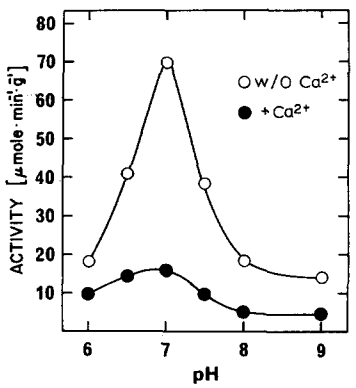


Fig. 4. pH profile of polycarboxylic trypsin membrane as measured with $5 \cdot 10^{-3}$ M BAEE without Ca^{2+} and with $3 \cdot 10^{-2}$ M $CaCl_2$ in 10^{-2} M Tris buffer.

Fig. 5. pH profile of polycarboxylic chymotrypsin membrane as measured with $5 \cdot 10^{-3}$ M TEE without Ca^{2+} and with $3 \cdot 10^{-2}$ $CaCl_2$ in 10^{-2} M Tris buffer.

Sipos and Merkel[18] observed that dissolved trypsin underwent conformational changes in the presence of Ca^{2+} because its activity showed a sharp maximum at 60 °C, although without Ca^{2+} in the incubation mixture the maximum tryptic activity was at a temperature slightly above 40 °C. A similar phenomenon was observed with the polycarboxylic trypsin conjugate as shown in Fig. 6 by the plots of activity of the polycarboxylic trypsin membrane against temperature as measured with and without Ca^{2+} in the external solution. It is seen that the corresponding activity maxima are at the same temperatures as reported for the dissolved trypsin. The distinct activity maximum at 60 °C strongly suggests that trypsin molecules

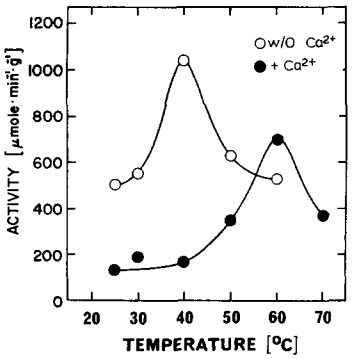


Fig. 6. Plots of the activity of polycarboxylic trypsin membrane against the temperature in the absence of Ca^{2+} and in contact with $3 \cdot 10^{-3}$ M $CaCl_2$ solution. Substrate: $5 \cdot 10^{-3}$ M TAME in $5 \cdot 10^{-2}$ M Tris buffer.

undergo the same conformational changes in the presence of Ca^{2+} not only in free solution but also when they are bound covalently to the polymer matrix.

Since the location of the pH profiles of both enzyme conjugates were unaffected and the temperature profiles of polycarboxylic trypsin and trypsin in free solution were equally affected by Ca^{2+}, we inferred that the reduction in the enzymic activities of the membranes was not caused by changes in the intrinsic behavior of the bound enzymes. Therefore we attributed the observed behavior to the coarctative effect of Ca^{2+} which manifests itself in an increasing cross-linking of the membrane structure due to formation of Ca^{2+} bridges between carboxyl groups and a decreasing void fraction inside the membrane as shown in Fig. 1. Indeed, the similarity between the plots for the water uptake in Fig. 1 and for the activity of the chymotrypsin conjugate in Fig. 2 strongly suggests a relation between the decreasing void volume and activity. The relationship may be much more pronounced as indicated by the comparison because the enzyme activity was measured in 10^{-2} M buffer and, in contrast, the water uptake was measured in $5 \cdot 10^{-2}$ M buffer. Since the Ca^{2+} binding, *ie.* the membrane coarctation, is greater at lower ionic strength (Gent *et al.*[17]) this can explain why the Ca^{2+} concentration where the curve bends downward is lower for the activity than for the void fraction.

It is known that the diffusivity inside of a porous medium decreases with the void fraction (Satterfield[20]). Therefore, the most plausible explanation for the reduced enzymic activities of the membranes in the presence of Ca^{2+} is the decrease in substrate diffusivity inside the membrane. Several observations support this exposition. As seen in Fig. 2 the activity of both the thin and thick polycarboxylic chrymotrypsin membranes decreases with increasing Ca^{2+} concentration. Nonetheless, the relative decrease in the reaction rate is significantly greater with the thick membrane in which diffusional effects are expected to play a greater role. The activity maxima of the polycarboxylic trypsin membranes in Fig. 3 can be explained by the antagonistic effects of the enhancement of the activity of bound trypsin at low Ca^{2+} concentrations, where coarctation is negligible and, of the reduction of substrate diffusivity at high Ca^{2+} concentrations where coarctation is pronounced. It is noted that no maxima have been observed on the corresponding plots for chymotrypsin, which is not activated by Ca^{2+}. As seen in Fig. 4, the tryptic activity is the same at pH 6.0 with

and without Ca^{2+} in the external solution. Apparently, at such relatively low external pH the rate of reaction inside the membrane is so low that a decrease in substrate diffusion due to coarctation does not affect the overall rate of reaction. On the other hand the chymotryptic activity is lower in the presence than in the absence of Ca^{2+} at pH 6.0 as shown in Fig. 5, because the rate of the intrinsic reaction is sufficiently high to be affected by reduction of the internal substrate diffusivity due to the membrane coarctation.

These observations are in agreement with the theory that the relative decrease in the overall rate due diffusion limitations is greater when the intrinsic rate of reaction inside the membrane is higher. Thus, the flattening of the activity *vs* pH curves in the presence of Ca^{2+} as shown in Figs 2 and 3 is also explainable by diffusion limitations inside the membrane as a result of coarctation by Ca^{2+}. It should be pointed out that the interaction between internal diffusion and Michaelis–Menten type reaction is in determining the overall reaction behavior of such membranes is very complex particularly when electrical effects in the charged membrane may also play a role. Therefore, no direct correlation could be expected between the water uptake and activity.

The measurement of initial rates at various substrate concentrations in the absence and presence of Ca^{2+} further indicated that the reduction of the diffusivity of the substrate inside the membrane is the major factor in decreasing the enzyme activity. Fig. 7 shows Lineweaver–Burk plots obtained with thin polycarboxylic chymotrypsin membranes. Without Ca^{2+} in the external solution a straight line was obtained indicating that the reaction followed the Michaelis–Menten kinetics. The effect of membrane coarctation on the overall rate of reaction *via* the reduction of diffusivity of the substrate in the enzymic medium, however, is seen in the plots obtained at different Ca^{2+} concentrations in the external solution ranging from 10^{-3} to 10^{-2} M. The departure from the straight line is in agreement with the results obtained with the theoretical model for the effect of internal diffusion controlled reaction (Engasser, J.-M. and Horvath, C., unpublished). These findings further corroborate our previous statement that the transport rates through membranes formed by the polycarboxylic enzyme conjugates were reduced by the effect of Ca^{2+} on the membrane structure. As a result the enzymic activity of the membrane decreased and the kinetics of the reaction departed from the Michaelis–Menten scheme.

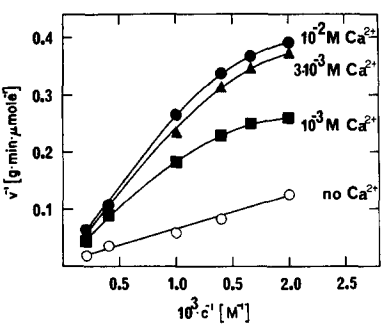


Fig. 7. Lineweaver–Burk plots for thin polycarboxylic chymotrypsin membrane obtained with TEE in the absence of Ca^{2+} and in contact with $CaCl_2$ solutions of different concentrations in 10^{-2} M Tris buffer, pH 7.0.

The coarctative effect has been found to be reversible by the following set of experiments. First, the activities of the pellicular trypsin and chymotrypsin conjugates were determined under standard conditions. Then they were immersed in $3 \cdot 10^{-2}$ M $CaCl_2$ solution for 30 min. Following, the beads were washed several times with $2 \cdot 10^{-2}$ M EDTA solution, then with $5 \cdot 10^{-2}$ M Tris buffer until all Ca^{2+} and EDTA were removed. The activities of both pellicular enzyme conjugates was the same within 5% before and after this treatment.

It was also investigated whether Ca^{2+} had a stabilizing effect on the polycarboxylic trypsin or chymotrypsin conjugates. Ca^{2+} *per se* stabilizes the active structure of both enzymes (Lazdunski and Delaage[22]; Chervenka[23]) and it was expected that the coarctation of the membrane decreased the degree of freedom of movement of both the enzyme molecules and polymer chains inside the membrane, consequently augmented the stability of the conjugates. Indeed, the pellicular trypsin conjugate showed only 0.5% decrease in activity after storage in $5 \cdot 10^{-2}$ M Tris buffer, pH 7.0, which was $3 \cdot 10^{-2}$ M for Ca^{2+}, at 15 °C for 144 h. On the other hand the control without Ca^{2+} showed a 15% loss of activity under these conditions.

It follows from these results that Ca^{2+} present in the substrate solution may impair the performance of biological reactors containing polycarboxylic enzyme conjugates (Horvath and Solomon[24], Horvath *et al.*[25,26]), therefore, the use of a Ca^{2+}-complexing agent in the substrate stream may be advisable. On the other hand the stability of wet polycarboxylic enzyme conjugates during storage could be improved by the addition of Ca^{2+} that can be removed prior to use.

The model experiments described in this study seem to corroborate Schoffeniels'[27] statement that "the molecular architectures responsible for the permeability characteristics of a living membrane are directly influenced by the concentration of Ca^{2+} in the surrounding medium". Since most cellular enzymes are bound to organelles (McLaren and Packer[28]) the above statement might be in view of the present results extended to chemical reactions in the cell whose enzymes may be regulated by Ca^{2+} either by a direct effect on the enzyme molecules, or by the coarctation of the medium or by both. Should the results of our model experiments be indeed of biological significance, a most intriguing task would be to find that or those agents which remove Ca^{2+} from the cellular membrane in living systems and play accordingly the complementary role to form a regulatory system.

The Ca^{2+}-binding properties of polycarboxylic substances may be utilized also in other form in physiological research. Soluble large molecular weight polycarboxylic derivates of biological substances can be easily prepared with polyanhydrides (Horvath, C., unpublished). Such conjugates, which combine a particular biological function with Ca^{2+}-binding properties, may be employed as probes to study membrane phenomena involving regulation by Ca^{2+}, for instance, drug–membrane interaction, cell adhesion, hormone action and muscle contraction.

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