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USE OF WATER-INSOLUBLE ENZYME DERIVATIVES IN SYNTHESIS AND SEPARATION [1]

by

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SUMMARY

USE OF WATER-INSOLUBLE ENZYME DERIVATIVES IN SYNTHESIS AND SEPARATION [1]

by

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Water-insoluble enzyme derivatives have been prepared by **1. adsorption** on inert carriers or ion exchange resins, **2. entrapping in gel lattices**; 3. covalent binding to insoluble polymeric carriers and 4. **covalent crosslinking** using bifunctional reagents. The kinetic behavior of immobilized **enzyme systems** is dominated by several factors not encountered in the kinetics of free enzymes: a) effects of the chemical nature of the carrier, stemming from the modified environment within which the immobilized enzyme is located, **b) steric restrictions** imposed by the carrier and c) diffusional control on **the rate** of substrate penetration. Thus the anomalous pH-rate dependencies and Michaelis constants of several polyelectrolyte-enzyme derivatives could be related to the unequal distribution of ionic species between the charged

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"solid phase" polyelectrolyte-enzyme particle and the surrounding solution. Proteases bound to polyelectrolyte carriers have been shown to exhibit restricted specificity towards protein substrates. The flow rate at which substrate perfuses through an enzyme column has been shown to affect the degree of conversion of substrate to product, the apparent Michaelis constant and the apparent rate constant. Anomalies in the pH-dependence of activity of papain-collodion membranes could be explained by the generation of local pH-gradients within the membrane due to the liberation of hydrogen ions in the course of hydrolysis of an ester substrate. Kinetic analysis has shown that under stationary state conditions, a membrane-bound enzyme could not attain its maximal activity, except when acting on a poor substrate. Immobilized enzymes have been used to attain better regulation of enzymic processes and for the preparation of enzyme electrodes. Enzyme columns have been utilized in devising automated analytical procedures and in affinity chromatography - for single-step purification procedures of specific inhibitors; reversal of the latter procedure has been employed for the purification of enzymes. Large scale enzyme-columns operated continuously on an industrial basis have been shown to be superior to the batch processes utilizing native enzymes.

Abstract

Water-insoluble enzyme derivatives have been prepared by 1. adsorption on inert carriers or ion-exchange resins; 2. entrapping in gel lattices; 3. covalent binding to insoluble polymeric carriers and 4. covalent crosslinkirg using bifunctional reagents. The kinetic behavior of immobilized enzyme systems is dominated by several factors not encountered in the kinetics of free enzymes: a) effects of the chemical nature of the carrier, stemming from the modified environment within which the immobilized enzyme is located, b) steric restrictions imposed by the carrier and c) diffusional control on the rate of substrate penetration. Thus the anomalous pH-rate dependencies and Michaelis constants of several polyelectrolyte-enzyme derivatives could be related to the unequal distribution of ionic species between the charged "solid phase" polyelectrolyte-enzyme particle and the surrounding solution. Proteases bound to polyelectrolyte carriers have been shown to exhibit restricted specificity towards protein substrates. The flow rate at which substrate perfuses through an enzyme column has been shown to affect the degree of conversion of substrate to product, the apparent Michaelis constant and the apparent rate constant. Anomalies in the pH-dependence of activity of papain-collodion membranes could be explained by the generation of local pH-gradients within the membrane due to the liberation of hydrogen ions in the course of hydrolysis of an ester substrate. Kinetic analysis has shown that under stationary state conditions, a membrane-bound enzyme could not attain its maximal activity, except when acting on a poor substrate. Immobilized enzymes have been used to attain better regulation of enzymic processes. Enzyme columns have been utilized in devising automated analytical procedures and in affinity chromatography - for single-step purification of specific inhibitors; reversal of the latter procedure has been employed for the purification of specific inhibitors. Large scale enzyme-columns operated continuously on an industrial basis have been shown to be superior to the batch processes utilizing native enzymes.

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Enzyme derivatives, in which the biologically active protein is bound to a water-insoluble polymeric carrier, have been used to achieve better control of enzyme reactions, and packed in columns in continuous processes. Highly specific adsorbents could be prepared by binding a protein to a biologically inert carrier.[1-4] Such preparations have found wide application in the field of immunochemistry,[5] in the isolation of purified antibodies and more recently, in the isolation of specific enzyme inhibitors. Analogous procedures have been employed in the purification of enzymes.

Enzyme-carrier conjugates have served as simple model systems for the investigation of some of the principles underlying the kinetic behavior of enzymes immobilized in biological membranes and in subcellular particles.[1,3,4]

In the following a brief summary is presented of the most commonly employed preparative procedures. The various factors which can affect the behavior and the apparent kinetic parameters of an immobilized enzyme system are discussed. Current developments and trends in the application of water-insoluble enzyme derivatives are surveyed.

Preparation of Water Insoluble Enzyme Derivatives

Four principal methods can be used for the preparation of water-insoluble derivatives of biologically active proteins.[1-4]

i. Adsorption on inert carriers or synthetic ion exchange resins. This method, despite its inherent limitations, has been successfully applied in several cases.

ii. Inclusion into gel lattices, the pores of which are too small to allow the escape of the entrapped protein. This method has been mainly employed for the immobilization of enzymes for analytical purposes, using for example acrylamide or starch gels.

iii. Covalent binding of proteins to a suitable water-insoluble carrier, via functional groups non-essential for their biological activity.

iv. Covalent cross-linking of the protein by an appropriate bifunctional reagent.

The methods available for the immobilization of proteins have been recently summarized in several reviews.[1-4] Some of the more important methods involving the covalent binding of proteins to water-insoluble polymeric carriers are listed below.

The amino groups on proteins have been utilized to effect covalent linking to several carboxylic polymers via the corresponding azides.[5-9] (Fig. 1) or by activation of the polymer carboxyls by carbodiimides[10-12] (Fig. 2) or by Woodward's Reagent K (N-ethyl-5-phenylisoxazolium-3'sulfonate).[13] More recently, cellulose activated by s-trichlorotriazine (cyanuric chloride)[14-16] or by a dichloro-sym-triazinyl dyestuff (Procion brilliant orange MGS),[17] and Sephadex or Sepharose, activated by cyanogen bromide,[18-20] (Fig. 3) have been successfully applied to the immobilization of several enzymes. A polymeric acylating reagent, ethylene-maleic anhydride (1:1) copolymer (EMA), has been successfully used for the preparation of polyanionic water-insoluble derivatives of enzymes, antigens and protein enzyme inhibitors.[3-4, 21-23] (Fig. 4)

In the case of acidic proteins, containing large excess of carboxyl groups, immobilization could be achieved by coupling the protein to aminoethyl cellulose through soluble carbodiimide activation of the protein carboxyls.[24]

Proteins containing relatively large amounts of aromatic amino acids have been coupled to the polydiazonium salts derived from p-aminobenzyl cellulose,[5,25] poly-p-amino-styrene,[5,26] the m-amino-benzyloxymethyl ether of cellulose,[10,14,27,28] a p-amino-DL-phenylalanine copolymer[29,30] (Fig. 5) and, more recently, a synthetic resin, S-MDA[23,31], the S-MDA resins were prepared by the condensation of dialdehyde starch (a commercially available periodate oxidation product of starch) with p,p'-diamino diphenyl methane, and the subsequent reduction of

the Schiff's base type polymeric product (Fig. 6).

Insolubilization of enzymes and other proteins has also been attained by their covalent crosslinking making use of bifunctional reagents.[1,3,32] Two types of crosslinking reagents have been commonly used: a) bifunctional reagents possessing two identical functional groups such as glutaraldehyde,[32-35] bisdiazobenzidine,[1,30,36] 1,5-difluoro-2,4-dinitrobenzene,[37,38] diphenyl-4,4'-diisothiocyanate-2,2' disulfonic acid,[39] 4,4'-difluoro-3,3'-dinitrodiphenyl sulfone,[40] phenol-2,4-disulfonyl chloride,[41] etc., and b) bifunctional reagents possessing two different functional groups, or groups of differing reactivities such as toluene-2-isocyanate-4-isothiocyanate;[42] 3-methoxydiphenyl methane-4,4'-diisocyanate,[42] trichloro-s-triazine,[14,15] etc. (see Fig. 7)

A new method involving the adsorption of a protein as a monolayer onto colloidal silica particles, followed by intermolecular crosslinking with glutaraldehyde has been recently reported.[43]

Stability of Water Insoluble Enzyme Derivatives

The existing data on the stability of immobilized enzyme derivatives is still rather inadequate. It has been shown that insoluble preparations of trypsin, subtilopeptidase A and papain bound to a p-aminophenylalanine-leucine copolymer or to S-MDA resins (q.v.)[23,31] and EMA-derivatives of trypsin and chymotrypsin,[1,3,23] retained most of their enzymic activity after being stored in aqueous suspension for several months at 4°. Ethylene-maleic acid copolymer (EMA) derivatives of trypsin, chymotrypsin papain and subtilopeptidase A could be lyophilized without loss of activity[23] whereas p-aminophenylalanine-leucine copolymer and S-MDA derivatives of trypsin and subtilopeptidases A and B, lost most of their activity on lyophilization.[3,23,31] Lyophilized powders of ethylene-maleic acid copolymer derivatives of several proteolytic enzymes exhibited high shelf stabilities, even at room temperature.[23]

Columns of ethylene-maleic acid derivatives of trypsin, chymotrypsin and kallikrein could be used repeatedly (up to 60 operations) at 4-8°C without loss of specific activity.[44] Improved stabilities toward alkaline pH's up to 10.7 were exhibited by the ethylene-maleic acid derivatives of several enzymes,[1,3,21,23] conversely polycationic derivatives of a number of enzymes exhibited improved stabilities in the acid pH-range.[24] In a few instances retention of activity in the presence of high urea concentrations has been reported.[1,3,21]

In the case of immobilized derivatives of proteolytic enzymes, the increased stability in the alkaline pH-range could most probably be related to the prevention of autodigestion due to attaching the enzyme to the insoluble matrix. No simple rules, regarding all the factors determining the stability of immobilized enzymes emerge, however, from the known data.

Kinetic Behavior of Immobilized Enzymes

The kinetic behavior of immobilized enzyme systems is dominated by several factors, not encountered in the kinetics of free enzymes a) effects of the chemical nature of the carrier, stemming from the modified environment within which the immobilized enzyme is located, b) steric restrictions imposed by the carrier and c) diffusion control on the rate of substrate penetration.

Effects of Charged Carriers on Kinetic Behavior

The pH-activity profiles of the polyanionic derivatives of several proteolytic enzymes acting on their specific low molecular weight substrates have been shown to be displaced towards more alkaline pH-values by 1 - 2.5 pH units, at low ionic strength ($\Gamma/_2 \approx 0.01$) as compared to the native enzymes.[23,45] Polycationic derivatives of the same enzymes exhibit the reverse effect, i.e. displacement of the pH-activity profile towards more acidic pH-values.[3,24] These anomalies are abolished at high ionic strength ($\Gamma/_2 \geq 1$). To illustrate this phenomenon the pH-

activity profiles of a polyanionic derivative of trypsin (EMA-trypsin) and of the polyanionic and polycationic derivatives of chymotrypsin (EMA-chymotrypsin and polyornithyl chymotrypsin) are shown in Figs. 8 and 9. Charged derivatives of papain,[23,24] ficin,[9] and subtilopeptidase A (subtilisin Carlsberg)[23,24] exhibit similar behavior. Furthermore, the apparent Michaelis constant (K_m) of a polyanionic derivative of trypsin (EMA-trypsin), with the positively charged substrate benzoyl-L-arginine amide (BAA) has been found to be markedly lower, at low ionic strength, than that of the native enzyme[45] (Figs. 10 and 11). Similar effects have been reported for the polyanionic derivatives of papain (EMA-papain,[24] ficin[9] (CM-cellulose-ficin) and bromelain[46] (CM-cellulose-bromelain) using benzoyl-L-arginine ethyl ester and substrate. Again, the perturbation of the apparent Michaelis constant is abolished at high ionic strength[45,46] (see Fig. 11). K_m values closely similar to those of the native enzyme have been demonstrated with uncharged substrates for the polyanionic and polycationic derivatives of chymotrypsin and for a polyanionic derivative of papain.[3,24]

These phenomena have been explained as resulting from the unequal distribution of ionic species between the charged "solid-phase", the polyelectrolyte-enzyme particle and the surrounding solution.[45]

Thus the local hydrogen-ion concentration in the domain of the charged enzyme derivative could be described by Eq. (1)

$$a^{i}_{H^+} = a^{o}_{H^+} e^{z\varepsilon\psi/kT} \qquad (1)$$

where $a^{i}_{H^+}$ and $a^{o}_{H^+}$ are the hydrogen ion activities in the polyelectrolyte-enzyme derivative phase and the external solution, ψ - the electrostatic potential in the domain of the charged immobilized enzyme particle, ε - the positive electron charge, z - a positive or negative integer, of value unity in the case of hydrogen ions, k - the Boltzmann constant and T - the absolute temperature.

It follows from Eq. (1) that the local pH in the domain of a polyanionic enzyme derivative will be lower than that measured in the external solution. The

reverse will be true for a polycationic enzyme derivative. Consequently the pH-activity profile of an enzyme immobilized onto a charged carrier will be displaced towards more alkaline or towards more acid pH-values, for a negatively or positively charged carrier, respectively, i.e.

$$\Delta pH = pH^{i} - pH^{o} = 0.43 \frac{z \varepsilon \psi}{k T} \qquad (2)$$

where ΔpH is the difference between the local pH within the polyelectrolyte-enzyme particle (pH^{i}) and the pH of the external solution (pH^{o}). pH^{i} is deduced from the pH-activity profile of the native enzyme (see Figs. 8 and 9).

The dependence of enzymic activity on pH is related to the dissociation of ionizing groups participating in the enzymic catalysis mechanism[47,48,49] (e.g. the acidic limb of the pH-activity curve of chymotrypsin associated with the ionization of histidine-57[47-50]). The displaced pH-activity profiles of a polyelectrolyte enzyme derivative can therefore be alternatively represented in terms of changes in the values of the apparent acidic dissociation constants (pK_a (app)) of the "active-site" ionizing group effected by the polyelectrolyte "microenvironment" of the enzyme derivative, i.e.

$$pK_a' = pK + 0.43 \frac{z \varepsilon \psi}{k T} \qquad (3)$$

or

$$\Delta pK_a = pK_a' - pK_a = 0.43 \frac{z \varepsilon \psi}{k T} \qquad (4)$$

pK_a and pK_a' are the apparent dissociation constants for the native enzyme and polyelectrolyte enzyme derivative, calculated from the appropriate pH-activity profile.

The model summarized in Eqs. (1) to (4) explains satisfactorily most of the known data on the pH-dependence of activity of chemically modified enzymes: The pH-activity profiles of both acetylated and succinylated, enzymically active derivatives of trypsin and chymotrypsin are displaced towards higher pH's.[3,45,51,52] A similar alkaline shift in the pH-optimum has been reported for insoluble preparations of chymotrypsin coupled to Sepharose[18,19] (by means of cyanogen bromide activation of the polysaccharide carrier). These effects are most probably due to the increase in the net negative charge on the protein, resulting from the acylation of amino groups, and thus to an increase in the values of pK_a (app) of the active-site histidines of these enzymes (see Eqs. (3) and (4) and References 47 - 50). Water-insoluble derivatives of enzymes bound to electrically neutral carriers such as p-amino-DL-phenylalanine-leucine copolymer,[29,30] S-MDA resin[23,31] (see Figs. 5 and 6) and p-amino-benzyl cellulose via the polydiazonium salts derived from these resins, often show pH-activity profiles - displaced towards more alkaline pH's.[31] This anomaly is somewhat unexpected in view of the assumed chemistry of binding, i.e., coupling of the polymeric diazonium salt to the tyrosyl residues on the protein, via azo bonds. Amino acid analysis of acid hydrolyzates of several such derivatives has, however, revealed among the missing amino acids not only tyrosine but also lysine and arginine.[31] It seems, therefore, that in this case as well, the increase in pK_a(app) is due to an increase in the net negative charge on the protein, effected by the disappearance of lysyl-ϵ-amino and guanido groups at some stage of the coupling reaction. In addition, the "acid-strengthening" effect of the azo-bond[53-56] on the hydroxyl ionization of "coupled" tyrosyl residues might also have contributed to the increase of net negative charge on the enzymes, and thus to the displacement of the pH-activity curves.

The changes in the values of the apparent Michaelis constants (K_m) of polyelectrolyte enzyme derivatives acting on charged low-molecular weight substrates (c.f. Figs. 10 and 11) can be related to the unequal distribution (c.f. Eq. (1)) of substrate between the charged enzyme particle and the external solution (Eq. (5))

$$[S]^{i} = [S]^{o} e^{z\varepsilon\Psi/kT} \qquad (5)$$

$[S]^{i}$ and $[S]^{o}$ are the substrate concentrations in the domain of the polyelectrolyte enzyme particle and the external solution. It follows from Eq. (5) that $[S]^{i} > [S]^{o}$ when polyelectrolyte enzyme derivative and substrate are of opposite charge; the enzyme derivative will attain the limiting rate, V_{max}, at lower bulk concentration of substrate as compared to the native enzyme, and the value of the Michaelis constant for the immobilized enzyme (K_m') will be correspondingly lower. For substrate and polyelectrolyte enzyme particle of the same charge, the opposite will be true, i.e. $[S]^{i} < [S]^{o}$ and K_m' of the enzyme derivative will be higher. This can be seen by introducing Eq. (5) into the Michaelis-Menten rate equation

$$v = \frac{V_{max}[S]}{K_m + [S]}$$

to obtain Eq. (6)

$$v' = \frac{V_{max}[S]^{o} e^{z\varepsilon\Psi/kT}}{K_m + [S]^{o} e^{z\varepsilon\Psi/kT}} \qquad (6)$$

Primed symbols indicate quantities pertaining to the polyelectrolyte enzyme derivative. From Eq. (6) $v' = \frac{1}{2} V_{max}$ when $[S]^{o} = K_m e^{-z\varepsilon\Psi/kT}$. Thus the value of external substrate concentration $[S]^{o}$, at which half-maximum velocity is attained, leads to an apparent Michaelis constant K_m', related to the Michaelis constant of the native enzyme by the expression

$$K_m' = K_m e^{-z\varepsilon\Psi/kT} \qquad (7)$$

Eq. (7) can be rewritten as

$$\Delta pK_m = pK_m' - pK_m = \log \frac{K_m}{K_m'} = 0.43 \frac{z\varepsilon\Psi}{kT} \qquad (8)$$

Action of Immobilized Enzyme Derivatives on High Molecular Weight Substrates

Most of the water insoluble enzyme conjugates investigated so far were derivatives of proteolytic enzymes. The activities of immobilized proteases toward protein substrates, as determined by the standard procedures (Kunitz's casein digestion method, appearance of non-protein nitrogen, potentiometric determination of the number of peptide bonds split, ninhydrin, etc.) are usually lower than those of the crystalline enzymes, when compared on a weight basis.[1,3,23] Water-insoluble derivatives of polytyrosyl trypsin[29] and papain[30] hydrolyzed casein at 15 and 30% of the rates which would have been expected from the amount of active bound enzyme (determined by a rate assay with low molecular weight substrate). Similar results have been obtained with carboxymethyl cellulose conjugates of ficin[9,57] bromelain[8] and trypsin[7] and with polyanionic polycationic and polyalcohol derivatives of papain[23,24] and subtilopeptidase A.[23,24] In the majority of cases the lowering in proteolytic activity can be attributed to steric hindrance induced by the carrier.[1,3,23,31]

An additional effect might become prominent in the case of polyelectrolyte derivatives of enzymes, i.e., the electrostatic interaction between charged carrier and charged, high molecular weight substrate: in the digestion of casein by polyanionic derivatives of trypsin (EMA-trypsin, q.v.) the rate of hydrolysis has been found to depend on the carrier to enzyme ratio.[21] Preparations of high enzyme content (high enzyme:carrier ratio) showed caseinolytic activities close to those of native trypsin while preparations of low enzyme content had about 20% of the expected proteolytic activity. Raising the pH and thus increasing the charge density of the carrier led to a marked decrease in the caseinolytic activity of both EMA-trypsin preparations. The data conveyed that the number of peptide bonds split could be controlled by varying the charge density on the polyelectrolyte enzyme derivative. Furthermore, indications that the sites of attack as well as the rates of cleavage of a high molecular weight substrate might be affected by the chemical nature of the polymeric carrier could be found in the literature.[1,3,21,22,58-64] For example,

both water-insoluble polytyrosyl trypsin and EMA-trypsin attacked prothrombin. The polytyrosyl trypsin derivative like native trypsin, converted prothrombin to thrombin which was not digested any further. In contrast EMA-trypsin rapidly degraded the newly formed thrombin.[22] It was also found that while the clotting factor VII was activated by insoluble polytyrosyl trypsin it was not affected by EMA-trypsin.[1]

These observations have been substantiated in studies on the EMA-trypsin digestion of myosin and the meromyosins.[58-61] It was found that the first-order rate constants estimated for the EMA-trypsin digestion of myosin were about fifty-fold lower as compared to those of native trypsin, and about half as many peptide bonds were split by the polyanionic trypsin derivative. Moreover, different protein fragments were obtained on limited EMA-trypsin digestion of both heavy-meromyosin (HMM) and myosin. In these investigations, a new helical subfragment (HMM-subfragment-2) was isolated from EMA-trypsin digests of heavy meromyosin.[58, 59] The separation of the globular part of the myosin molecule, HMM-subfragment-1, as well as the isolation of its intact helical rod portion have been recently achieved by the controlled digestion of myosin with a water-insoluble papain derivative.[60-62] These studies were conclusive in establishing the currently accepted two-coiled-coils model of the myosin molecule.

It has been recently reported that whereas trypsin hydrolyzed the 15 lysyl peptide bonds in pepsinogen, the maximal number of bonds cleaved by EMA-trypsin never exceeded 10,[63] the same difference was observed when reduced carboxymethylated pepsinogen was used as substrate. These findings were confirmed by peptide mapping.

The data indicates that the chemical nature of the carrier, at least in the case of polyelectrolyte enzyme derivatives, may impose additional restrictions on the specificity of the bound enzyme. These restrictions probably result from charge interactions between carrier and different regions, or different sequences on the high molecular weight substrate molecule. Hence, in studies of protein

sequence, the restricted specificity displayed by the polyelectrolyte derivatives of proteases could be utilized to obtain overlapping peptides by a series of derivatives of one enzyme only.[1,3,4,23]

Enzyme Columns

Columns of immobilized enzymes have been employed for the continuous preparation of product, for regulation of the extent of conversion of substrate to product, and in automated analytical procedures.[1,3,4,29,57]

A modified form of the integrated Michaelis-Menten rate equation has been used by Lilly et al.[57] to describe the kinetics of substrate hydrolysis in an enzyme column.

Defining the residence time of substrate in the enzyme column - t, by

$$t = V_f / Q \qquad (9)$$

where V_f is the void volume of the column, Q the flow rate, and substituting Eq. (9) into the integrated Michaelis-Menten equation

$$S_0 - S_t = k_3 [E] t + K_m \ln (S_t / S_0)$$

Eq. (10) is obtained:

$$P S_0 - K_m' \ln (1 - P) - k_3 E \beta / Q = C / Q \qquad (10)$$

where $\beta = V_f / V_t$ is voidage of the column, $P = (S_0 - S_t) / S_0$ - fraction of substrate reacted in column, $C = k_3 E \beta$ - reaction capacity of column, V_t - total volume and S_0, S_t - k_3, E and K_m have their usual significance.

(Eq. (10) may be rearranged

$$P S_0 = K_m' \ln (1 - P) + C / Q \qquad (11)$$

If values of P are measured when various initial concentrations of substrate are perfused through the same column at an identical flow rate (i.e. Q = const.),

PS_0 plotted against (1 - P) will give a straight line if K_m and C are constant at this flow rate. The slope of the line will be equal to K_m and the intercept to C/Q. Thus K_m and C can be determined for any flow rate through the column. Kinetic data on the hydrolysis of benzoyl-L-arginine ethyl ester in packed columns of carboxymethyl cellulose-ficin when plotted according to Eq. (11) indicated that the apparent Michaelis constant (K_m') decreased with increasing flow rate and asymptoted towards a minimal value at high flow rates.[57] In general the value of the Michaelis constant was higher than that observed under comparable conditions in stirred suspensions of the carboxymethyl-cellulose ficin derivative.[9,57] The data also suggested that at very low values of Q there was a tendency for C to increase. These deviations from the behavior expected on the basis of Eq. (11) could be qualitatively explained as due to diffusion-limited transport of substrate into the enzyme particles.[65]

The efficiency of operation of biochemical reactors using immobilized enzymes in suspension in a continuous-feed stirred tank has been investigated[66,67] and compared to that in a packed bed. In a suspension of carboxymethyl cellulose-chymotrypsin in a 500 ml tank agitated by a turbine impeller, both the apparent Michaelis constant and V_{max} varied with the degree of agitation, indicating that the reaction rate was partly diffusion-controlled.[65,67] Similar phenomena have been observed in studies on the rate of utilization of dissolved oxygen by microorganisms[68] and of glucose by microbiological films,[69] which were found to be limited by the rate of diffusion of substrate. Under most conditions a packed bed has been shown to be more efficient than a continuous-feed stirred tank, but diffusion limitations of the reaction could be significant in either type of reaction.[65] In a packed bed this limitation could be overcome by increasing the linear velocity of the substrate solution through the bed.[66,67] One of the major problems in operating a packed bed, however, is the difficulty of getting adequate flow rates with the present enzyme-support materials. Increase of the flow rate through an immobilized-enzyme bed could be attained by the use of porous sheets, such as

filter paper or cloth to which the enzyme has been attached. Reactive sheets of chymotrypsin, lactate dehydrogenase, creatine kinase, pyruvate kinase and β-galactosidase have been described.[16,70,71] The kinetics of enzymes attached to porous sheets appear to be similar to those of immobilized enzyme in packed beds, of which they are a special case.

Enzyme Membranes

A systematic study of diffusion-controlled processes in immobilized enzyme systems was carried out by Goldman et al.[36,72,73] using papain-collodion membranes. The enzyme membranes were prepared by adsorbing papain on preformed collodion membranes and crosslinking the adsorbed protein with bisdiazobenzidine 2,2' disulfonic acid.[36,72] The thickness of the enzyme layers could be controlled by adjusting the amount of papain in the adsorption solution. Three-layer papain-membranes, consisting of two enzyme layers separated by a collodion layer; two-layer papain membranes consisting of an enzyme layer and a collodion layer, and one-layer papain membranes were prepared by this technique.[36,72]

The rate of hydrolysis of benzoyl-L-arginine ethyl ester (BAEE) by a papain membrane showed a monotonous increase with pH up to pH 9.6, in contradistinction to the bell-shaped pH-activity profile of the native enzyme acting on the same substrate (Fig. 12).[57] The pH-activity profile of papain membrane with benzoyl-L-arginine amide (BAA) as substrate on the other hand was similar in shape to that of the native enzyme.[72] The anomaly in the pH activity profile of the papain membrane acting on BAEE was diminished by grinding up the membrane; moreover, this anomaly could be abolished by increasing the buffer concentration and by forcing the substrate solution through the membrane under a pressure difference of 5 atmospheres (Fig. 13).[72]

These anomalies in the pH dependence of the esterase activity of membrane-

bound papain could be explained by the generation of local pH gradients in the membrane due to the liberation of hydrogen ions in the course of the ester hydrolysis (Eq. (12)):

$$R_1COOR_2 \rightarrow R_1COO^- + H^+ + R_2OH \qquad (12)$$

In the stationary state the local pH inside the membrane was shown, by the use of indicators, to be lower than the pH outside the membrane by several pH units.[72] In the hydrolysis of BAA, on the other hand, no hydrogen ions were liberated below pH 8 (Eq. (13)).

$$R_1CONH_2 \rightarrow R_1COO^- + NH_4^+ \qquad (13)$$

Comparative kinetic studies on membrane-bound and solubilized acetylcholinesterase derived from the electric tissue of Electrophorus Electricus revealed that the particulate enzyme exhibited anomolous pH dependence of the acetylcholinesterase activity similar to that observed in the membrane-papain/BAEE system and was probably due to the same type of local pH effects.[74]

The main feature of an enzyme-membrane as deduced from the experiments described above is therefore that in the domain of the membrane (totally devoid of mechanical stirring) the flow of substrate to the site of the enzyme-catalyzed reaction is solely diffusion-controlled. In other words the concentration gradients of substrate and product with the enzyme membrane determine its apparent activity.

The above considerations have been emphasized in a theoretical analysis of the kinetic behavior of enzyme membranes, carried out by Goldman et al.[73] These authors showed that the substrate concentration-profile across the membrane, which determined the overall enzymic activity was dependent on both the flow of substrate into the membrane and the rate of its depletion resulting from the local enzyme reaction. It was shown that very few enzyme layers in

the membrane were utilized for hydrolysis of substrate, the effective concentration of which was nearly zero in the inner layers. A practical conclusion obtained from this model was that under stationary state conditions a membrane bound enzyme could not attain its maximal activity, V_{max}, except when acting on a relatively poor substrate, i.e. under conditions where breakdown of substrate was negligible in comparison to its rate of diffusion into the membrane.[73] These inferences from theory have been verified experimentally.[72,73]

Application

Of the variety of enzymes which have been immobilized onto water-insoluble carriers, the hydrolyzing enzymes and particularly the proteases have received by far the widest attention.[1,3,4,23]

In experiments aimed at obtaining well-defined fragments of complex proteins, better control of the enzymic degradations could be achieved by the use of insoluble derivatives of proteolytic enzymes. Water insoluble papain derivatives have been employed in studies concerning the structure of immunoglobulins.[75-77] Thus antibodies have been cleaved into F_{ab} and F_c fragments by water-insoluble papain in a two-stage process.[75] Limited digestion of the antibody with the preactivated insoluble enzyme derivative (after exhaustive washing to remove any excess of the cystein activator) yielded a product of unimpaired ability to precipitate antigen and having a sedimentation constant (6.25 S) identical with that of the intact antibody. On addition of reducing agent (cysteine or thioglycolate) subsequent to the removal of the insoluble enzyme, the 3.3 S fragments by Porter were obtained.[75,76] Moreover, immunoprecipitates obtained with insoluble papain treated antibody, dissolved on treatment with reducing agent and revealed in the ultracentrifuge two sedimenting peaks - a rapidly sedimenting peak shown to be a complex of one antigen molecule with several monovalent F_{ab} fragments and a slow peak lacking the antigen-binding capacity, i.e., the

F_c fragment. Native antibody-antigen precipitates did not dissolve on similar treatment .

Water-insoluble derivatives of various proteases have been recently applied to studies of protein structure. Cryofibrinogen (a highly clottable, cold-insoluble form of fibrinogen) was obtained by the limited digestion of purified fibrinogen, with a water-insoluble polyanionic derivative of thrombin.[78] The reversible reduction of disulfide bonds in trypsin and ribonuclease was studied by means of the carboxymethyl-cellulose derivatives of these enzymes.[79] Chymotrypsinogen was activated by means of water-insoluble trypsin derivatives, thus avoiding contamination of the system with the activating enzyme.[21]

Relatively few large scale applications of water-insoluble enzyme derivatives have been reported in the literature.

A water-insoluble trypsin derivative (with ethylene-maleic acid copolymer as carrier) was used for the selective absorption, at neutral pH, of trypsin inhibitors from crude animal-organ extracts.[44,80,81] The inhibitors were eluted at low pH. In addition the use of water insoluble derivatives of chymotrypsin and kallikrein (with the same carrier) led to the isolation of the Kunitz inhibitor from ox pancreas extracts; by subsequent use of insoluble trypsin, the specific trypsin inhibitors remaining in the extract were isolated.[44] Quantitative separation of the two inhibitors was achieved despite the similarity of their molecular weights (Kunitz inhibitor, M. Wt. 6513; specific trypsin inhibitor, M. Wt. 6000). Reversal of the above procedure, i.e., binding of kallikrein inhibitor to an ethylene-maleic acid copolymer, allowed the purification of trypsin and chymotrypsin.[80,81]

The inherent binding capacity of enzymes towards their specific substrates or inhibitors could thus form the basis for a new method of enzyme purification - affinity chromatography.[82] The crude enzyme preparation to be purified is passed through a column containing a resin to which a specific competitive inhibitor of the enzyme has been covalently attached. Proteins of no substantial affinity for

the bound inhibitor pass directly through the column, whereas the enzyme which recognizes the inhibitor is retarded in proportion to its affinity constant. Elution of the bound enzyme can be achieved by changing such parameters as salt concentration or pH, or by addition of a competitive inhibitor to the eluent.[82] Affinity chromatography procedures for the purification of staphylococcal nuclease, α-chymotrypsin, carboxypeptidase A[82,83] and papain[84] have been recently reported.

Immobilized derivatives of proteins have been used in an analogous fashion in immunochemistry for the isolation of purified antibodies.[5]

Enzyme columns prepared by adsorbing a mold aminoacylase (E.C. 3.5.1.14) on DEAE-cellulose or DEAE-Sephadex have been utilized for the resolution of acetyl-DL-methionine, into L-methionine and acetyl-D-methionine.[85-87] Under the appropriate conditions of flow rate and temperature, 100% resolution could be attained. Continuously operated aminoacylase columns are at present being utilized, for the large scale resolution of several racemic N-acyl amino acids.[88,89] In the batch processes previously employed, a purification step was necessary for separating the products in pure form. In the process utilizing an aminoacylase column, contamination of product by foreign protein and colored substances (originating from the crude enzyme extracts employed) did not occur. The isolation of pure product was thus simplified and the production costs reduced, concurrently with a considerable increase in the overall yields. The aminoacylase columns retained 60% of their activity after continuous operation at 50° for one month.[88,89] The loss of activity was most probably due to slow elution of the adsorbed enzyme. The columns could, however, be regenerated to their initial activity by recharging with enzyme.[89]

It should be pointed out in this context, that at high substrate concentrations, a considerable increase in the rate of elution of adsorbed enzymes is usually observed. This limitation can in principle be overcome by attaching the

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enzymic protein covalently to the insoluble support.

Immobilized enzymes and enzyme columns have also been employed to devise automated analytical procedures. The work in this area has been summarized elsewhere.[3]

Concluding Remarks

A survey of our current knowledge of immobilized enzymes demonstrates that only a limited number of systems have been thoroughly investigated. Most of the metabolically significant enzyme systems, or those of industrial and medical importance are still rather poorly represented. This paucity of data can be attributed mainly to the lack of general binding procedures by means of which reasonable yields of immobilized derivatives of widely differing protein species can be obtained. Recent advances in binding techniques have shown definite promise in this direction. Furthermore, the technology of immobilized enzyme systems is still in its infancy. Preliminary reports in the literature do show, however, an increasing interest in the study of both the theoretical and applied aspects of immobilized enzymes as well as the beginnings of a new technology.

The various hydrolases appear to be the most likely candidates for large scale application in immobilized form. Such systems, used in continuous processes for the degradation of naturally occurring macromolecules would have the obvious advantages of end products of higher purity and lower production costs. Hydrolases could, moreover, serve for the degradative removal of polymeric contaminants in fluids and extracts of biological origin, e.g. traces of antigenic protein contaminants in drugs.

In the field of biochemical analysis, immobilized enzymes could provide stable and reproducible analytical reagents to be used in an "off-the-shelf manner.

Application of insoluble enzyme derivatives in pharmacology, making use of such special properties as enhanced temperature stability, resistance to

proteolysis and stability to extremes of pH, could be advantageous for the prolongation of the effectiveness of the biologically active molecule under in vivo conditions.

Finally, the use of multi-enzyme biochemical reactors, carrying out continuously a sequence of biochemical processes can be foreseen.

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Legends to Figures

FIG. 1 Synthesis of carboxymethyl-cellulose azide (A) and carboxymethyl-cellulose-protein conjugates (B) (Ref. 6).

FIG. 2. Coupling of proteins to carboxylic polymers by the carbodiimide method. (Ref. 11).

FIG. 3. Coupling of proteins to Sephadex by means of cyanogen bromide. (Ref. 20).

FIG. 4. Coupling of proteins to a copolymer of ethylene and maleic anhydride (EMA) (Ref. 21).

FIG. 5. Preparation of the polydiazonium salt of an L-leucine-p-amino-DL-phenylalanine copolymer (A) and its coupling to a protein (B) (Ref. 29).

FIG. 6. Synthesis of S-MDA resin (Ref. 23).

FIG. 7. Some common crosslinking reagents.

FIG. 8. pH-Activity curves for trypsin and a polyanionic, ethylene-maleic acid copolymer derivative of trypsin (EMA-trypsin), at different ionic strength, using benzoyl-L-arginine ethyl ester as substrate (redrawn from the data of Ref. 45).

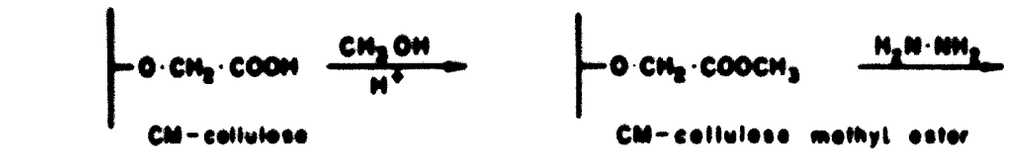
FIG. 9. pH-Activity curves of low ionic strength ($\Gamma/_1 = 0.008$) for chymotrypsin, a polyanionic derivative of chymotrypsin (EMA-chymotrypsin), and a polycationic, polyornithyl derivative of chymotrypsin, using acetyl-L-tyrosine ethyl ester as substrate (Ref. 3 and 23).

FIG. 10. Normalized Michaelis-Menten plots for trypsin and a polyanionic derivative of trypsin (EMA-trypsin) acting on benzoyl-L-arginine amide (Ref. 45).

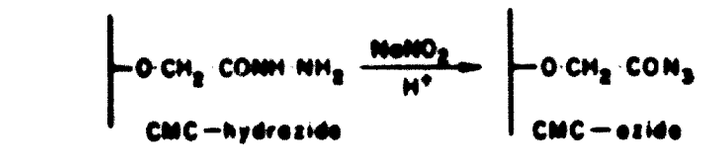
FIG. 11. Normalized Lineweaver-Burk plots for trypsin and a polyanionic derivative of trypsin (EMA-trypsin) acting on benzoyl-L-arginine amide (Ref. 45).

FIG. 12. pH-Activity curves for papain and two papain membranes using benzoyl-L-arginine ethyl ester as substrate. ▲ - ▲, a three-layer papain membrane; - o - o -, a one-layer papain membrane; - ● - ● -, native papain (Ref. 72).

FIG. 13. pH-Activity curves for a one-layer papain membrane acting on benzoyl-L-arginine ethyl ester under different conditions. 1 - papain membrane in absence of buffer; 2 - papain membrane in presence of buffer; 3 - papain membrane through which substrate in buffer was forced under a pressure difference of five atmospheres; 4 - native papain (Ref. 72).


O·CH₂·COOH
CH₃OH
H⁺
CM-cellulose
O·CH₂·COOCH₃
H₂N·NH₂
CM-cellulose methyl ester

A)


O·CH₂·CONH·NH₂
NaNO₂
H⁺
CMC-hydrazide
O·CH₂·CON₃
CMC-azide

B)

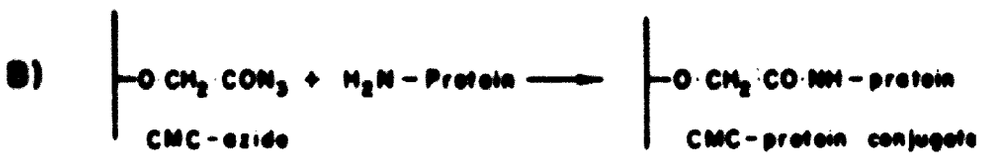
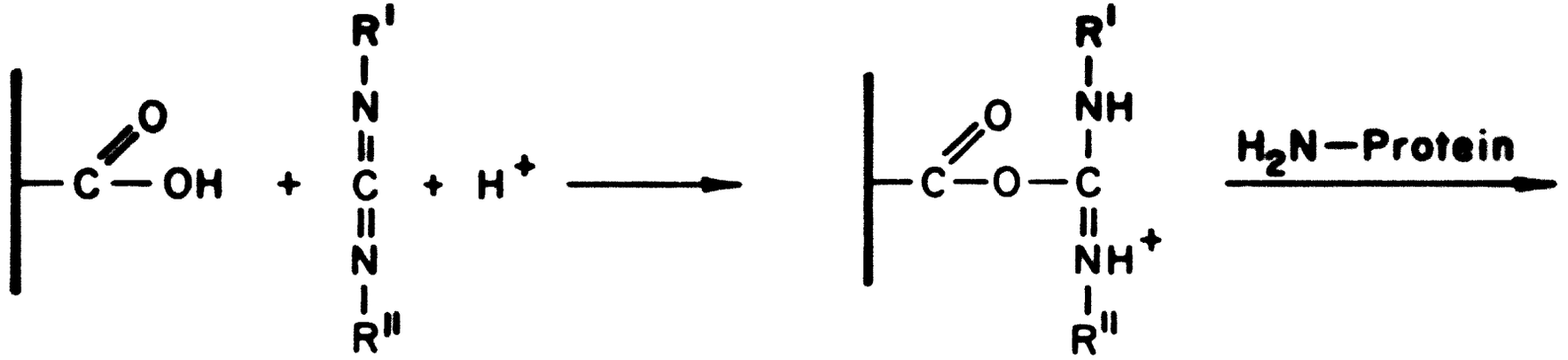

O·CH₂·CON₃ + H₂N-Protein
CMC-azide
O·CH₂·CO·NH-protein
CMC-protein conjugate

Fig. 1



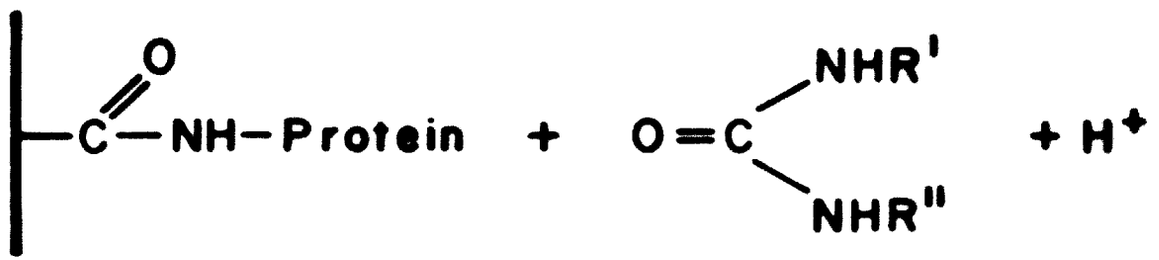


Fig. 2

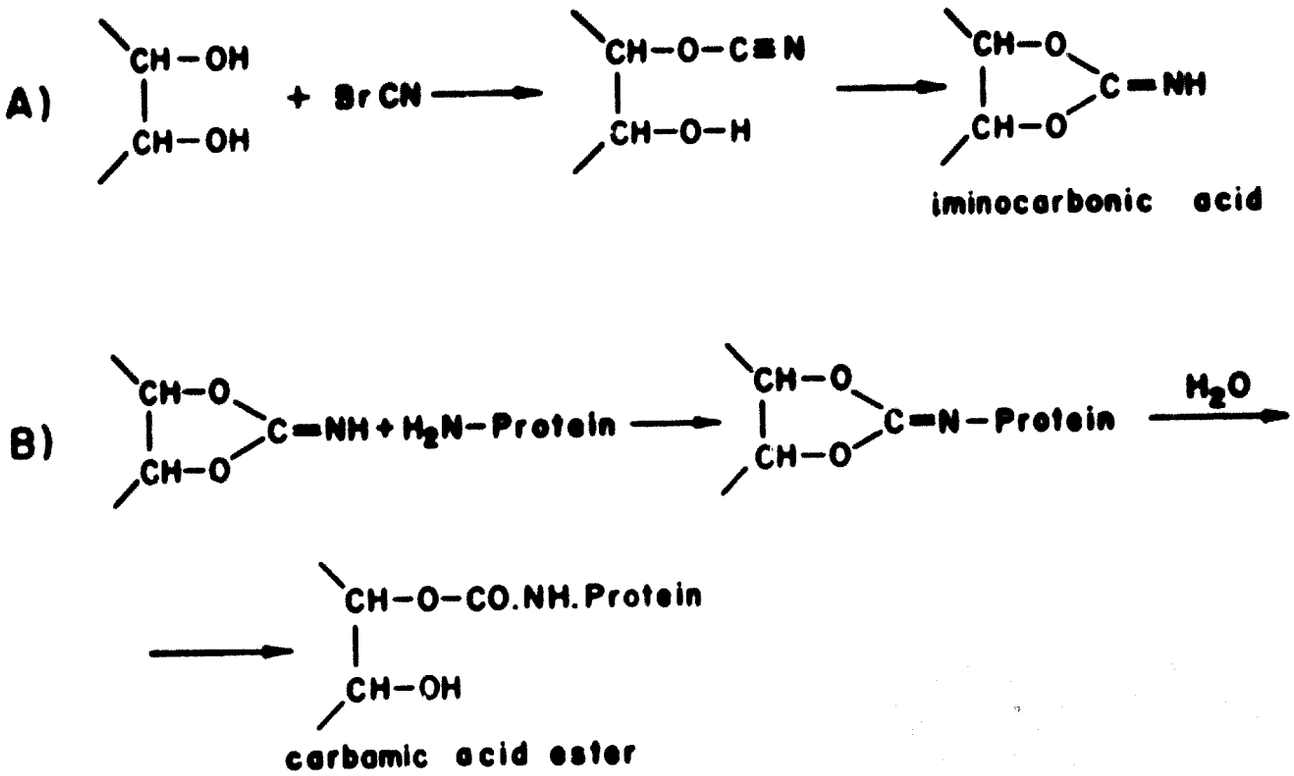

A)
CH–OH
CH–OH
+ BrCN
CH–O–C≡N
CH–O–H
CH–O
C=NH
CH–O
iminocarbonic acid
B)
CH–O
C=NH + H2N–Protein
CH–O
CH–O
C=N–Protein
CH–O
H2O
CH–O–CO.NH.Protein
CH–OH
carbamic acid ester

Fig. 3

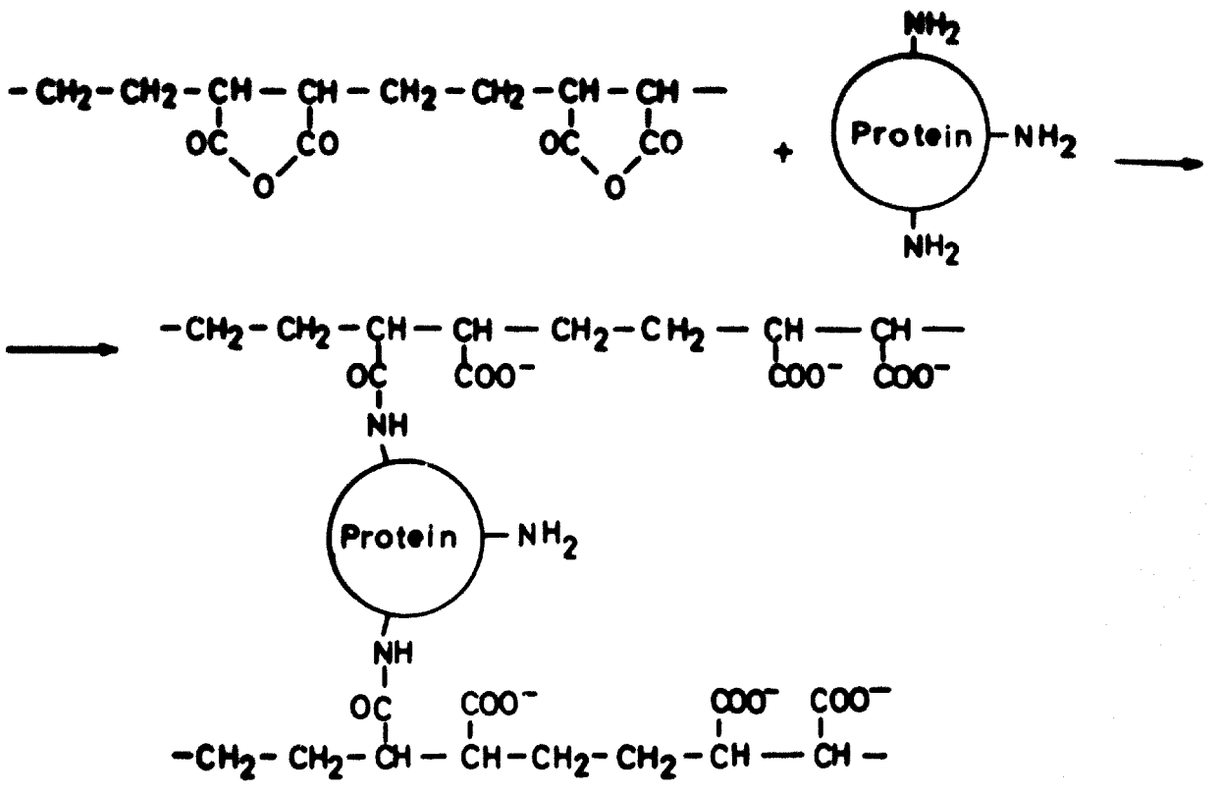


Fig. 4

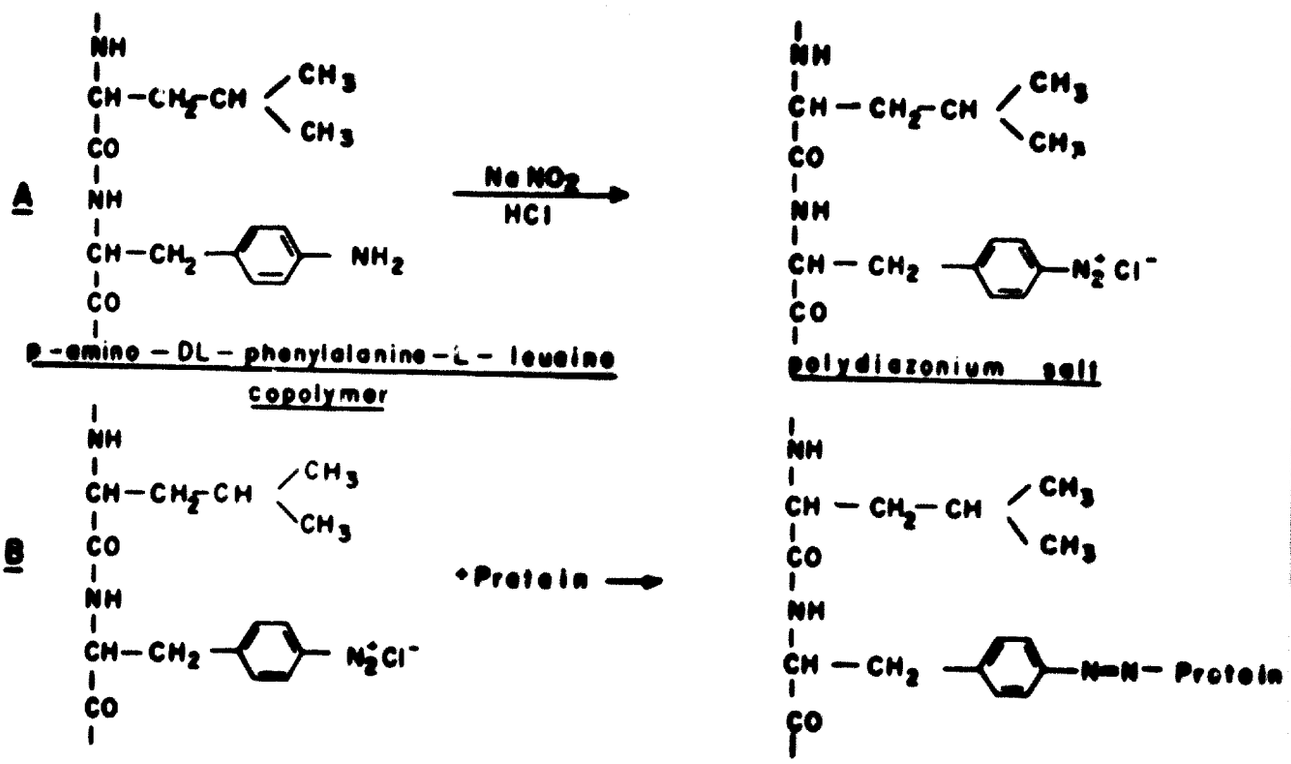

A
NaNO2
HCl
p-amino-DL-phenylalanine-L-leucine copolymer
polydiazonium salt
B
•Protein
Protein

Fig. 6

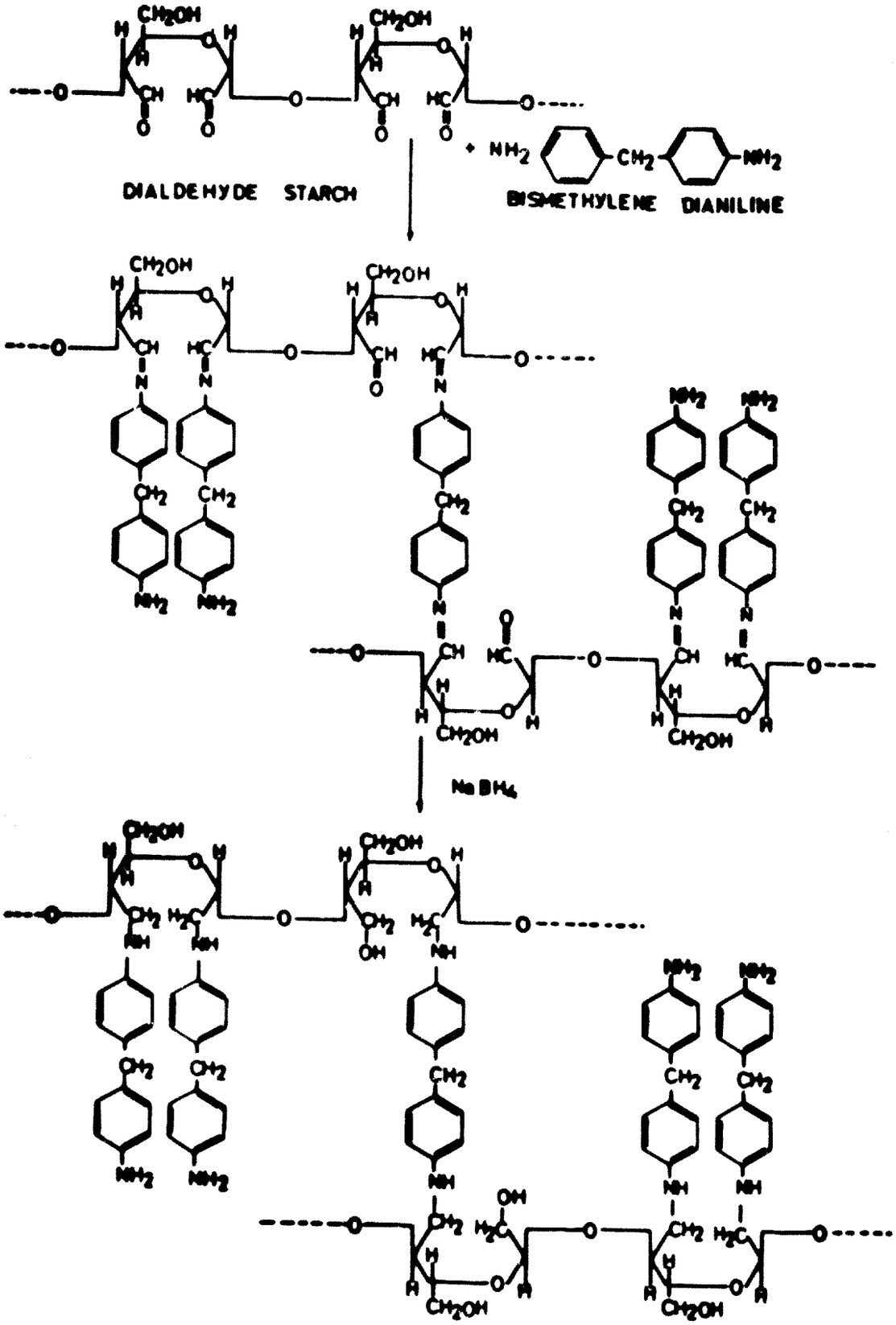
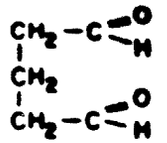
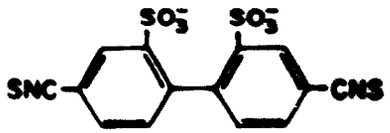

DIALDEHYDE STARCH
BISMETHYLENE DIANILINE
NaBH4
S-MDA RESIN

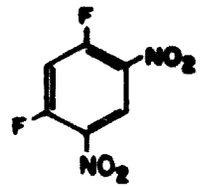
Fig. 6



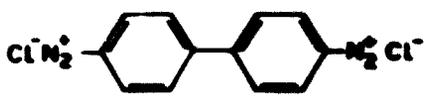
glutaraldehyde



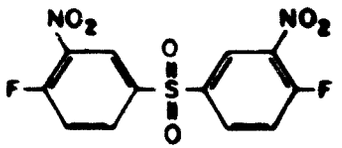
diphenyl-4,4'-diisothiocyonate-2,2'-disulfonic acid



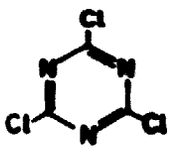
1,5-difluoro-2,4-dinitrobenzene



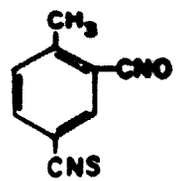
bis diazobenzidine



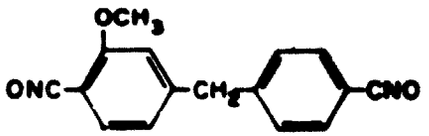
4,4'-difluoro-3,3'-dinitro diphenyl sulfone



trichloro-s-triazine (cyanuric chloride)



toluene-2-isocyonate 4-isothiocyonate



3-methoxy diphenyl methane-4,4'-diisocyonate

Fig. 7

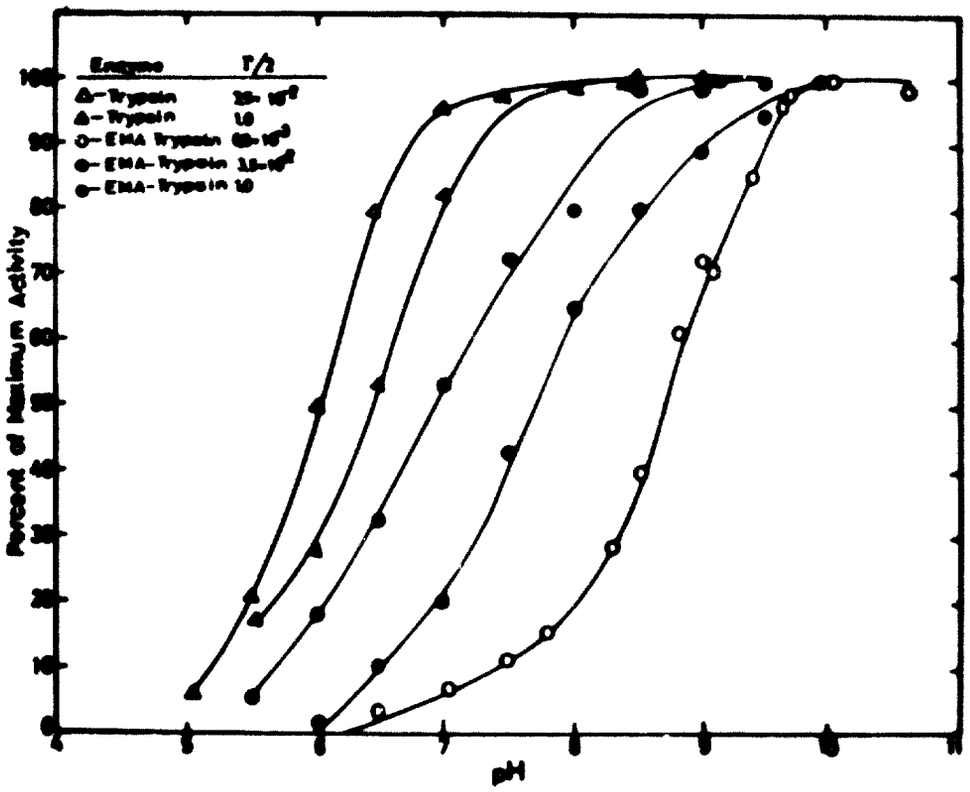


Fig. 8

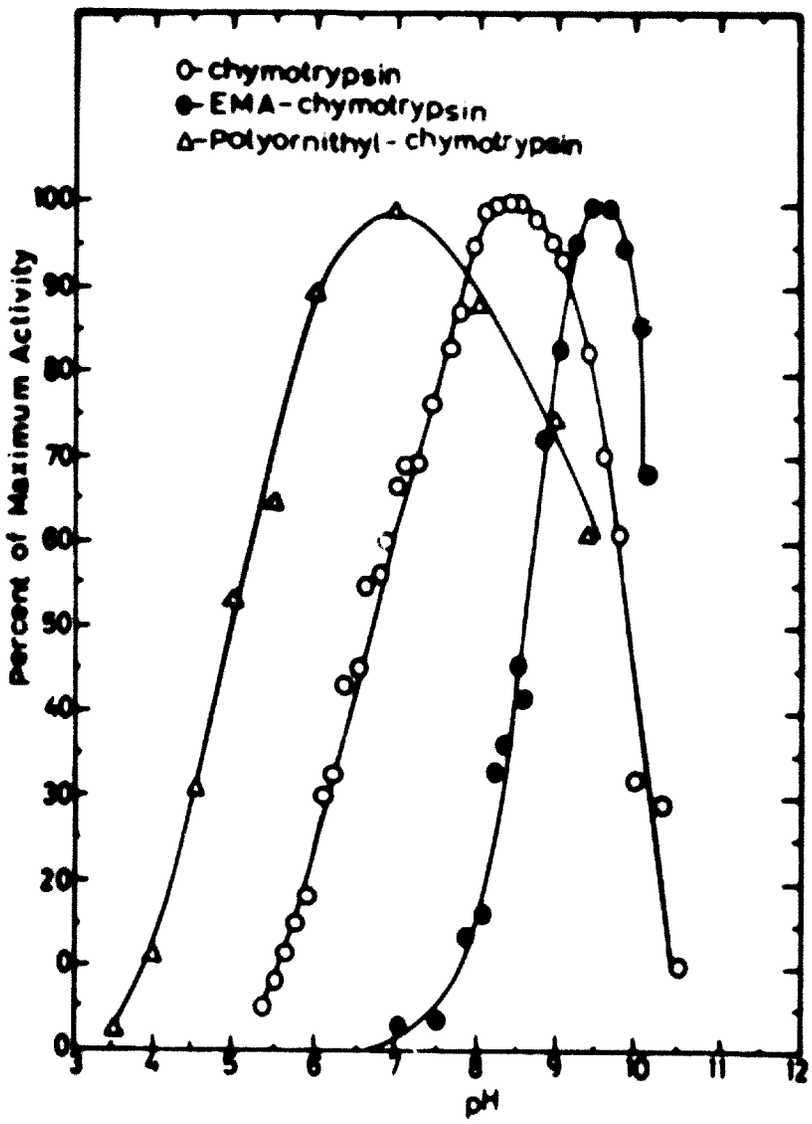

O- chymotrypsin
●- EMA - chymotrypsin
Δ- Polyornithyl - chymotrypsin
Percent of Maximum Activity
100
90
80
70
60
50
40
30
20
0
0
3
4
5
6
7
8
9
10
11
12
pH

Fig. 9

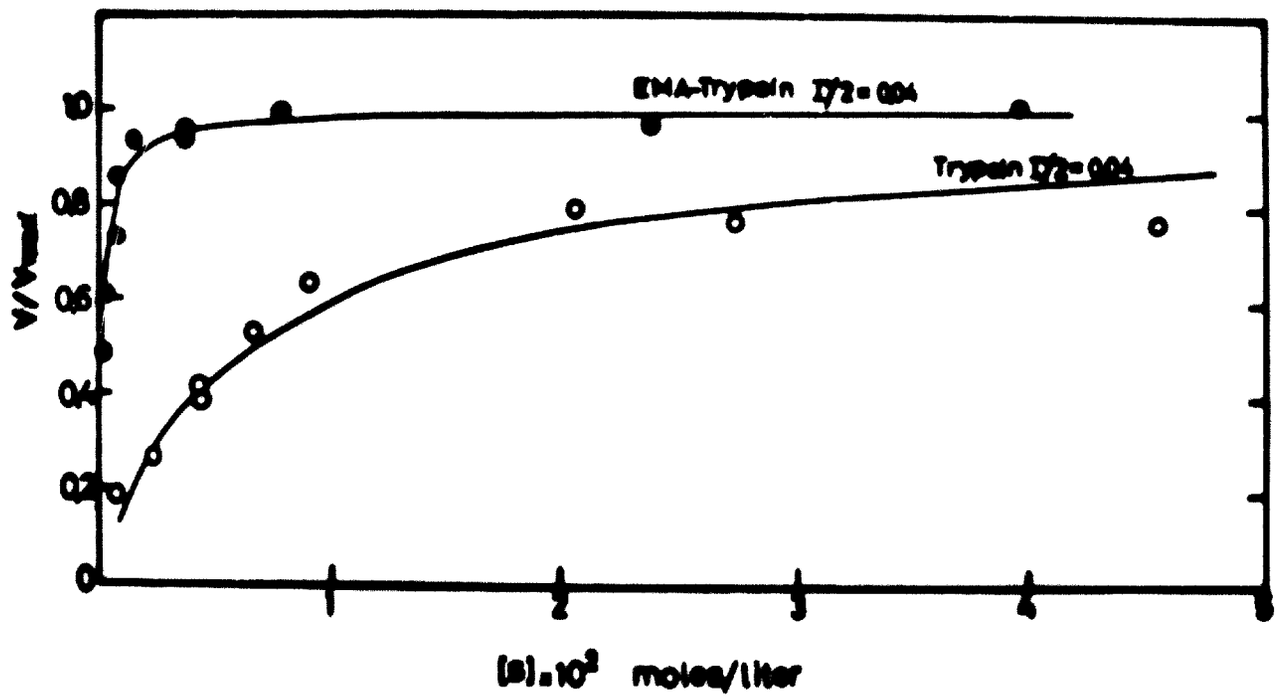

EMA-Trypsin
Trypsin
V/Vmax
0
0.2
0.4
0.6
0.8
1.0
1
2
3
4
5
[S].10³ moles/liter

Fig. 12

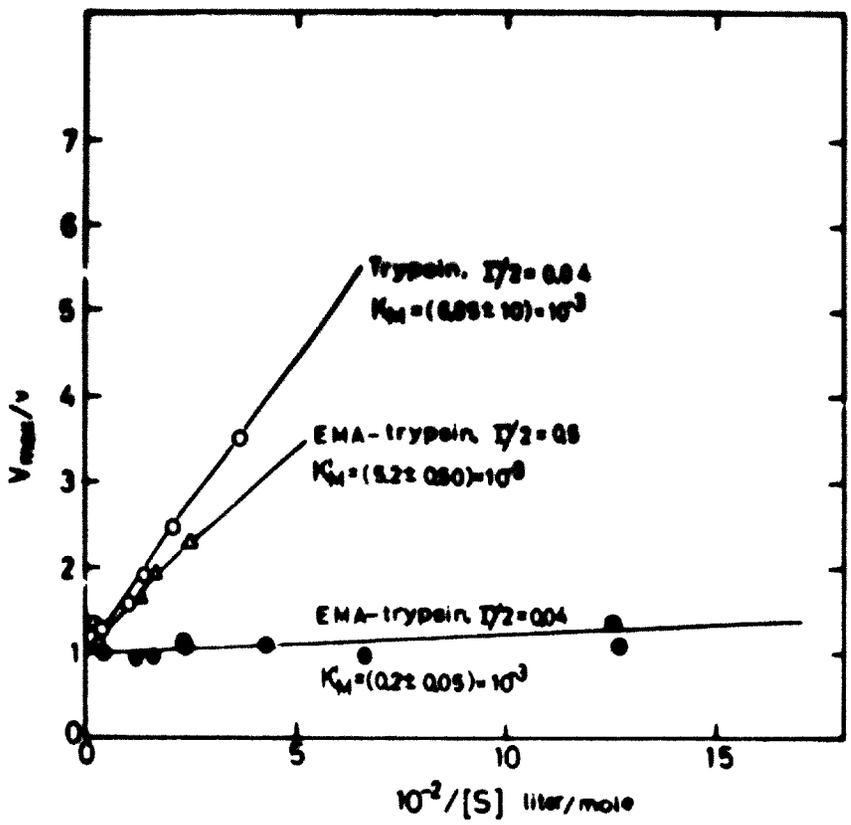

Trypsin, Γ/2 = 0.84
K_M = (6.8 ± 1.0) · 10^-3
EMA-trypsin, Γ/2 = 0.8
K_M = (5.2 ± 0.8.0) · 10^-3
EMA-trypsin, Γ/2 = 0.04
K_M = (0.2 ± 0.05) · 10^-3
V_max/v
10^-2/[S] liter/mole
0
1
2
3
4
5
6
7
0
5
10
15

Fig. 11

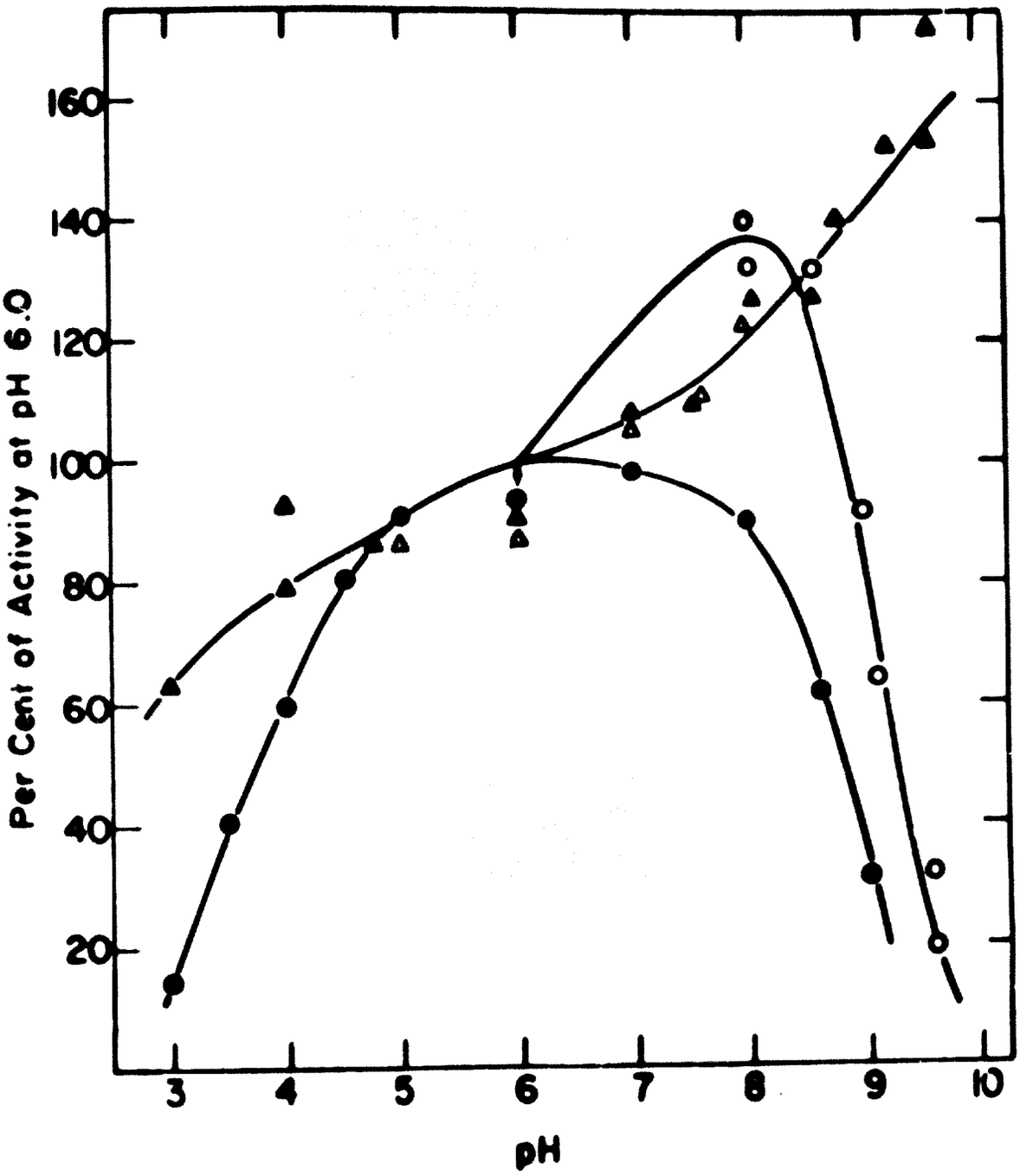

160
140
120
100
80
60
40
20
Per Cent of Activity at pH 6.0
3
4
5
6
7
8
9
10
pH

Fig. 12

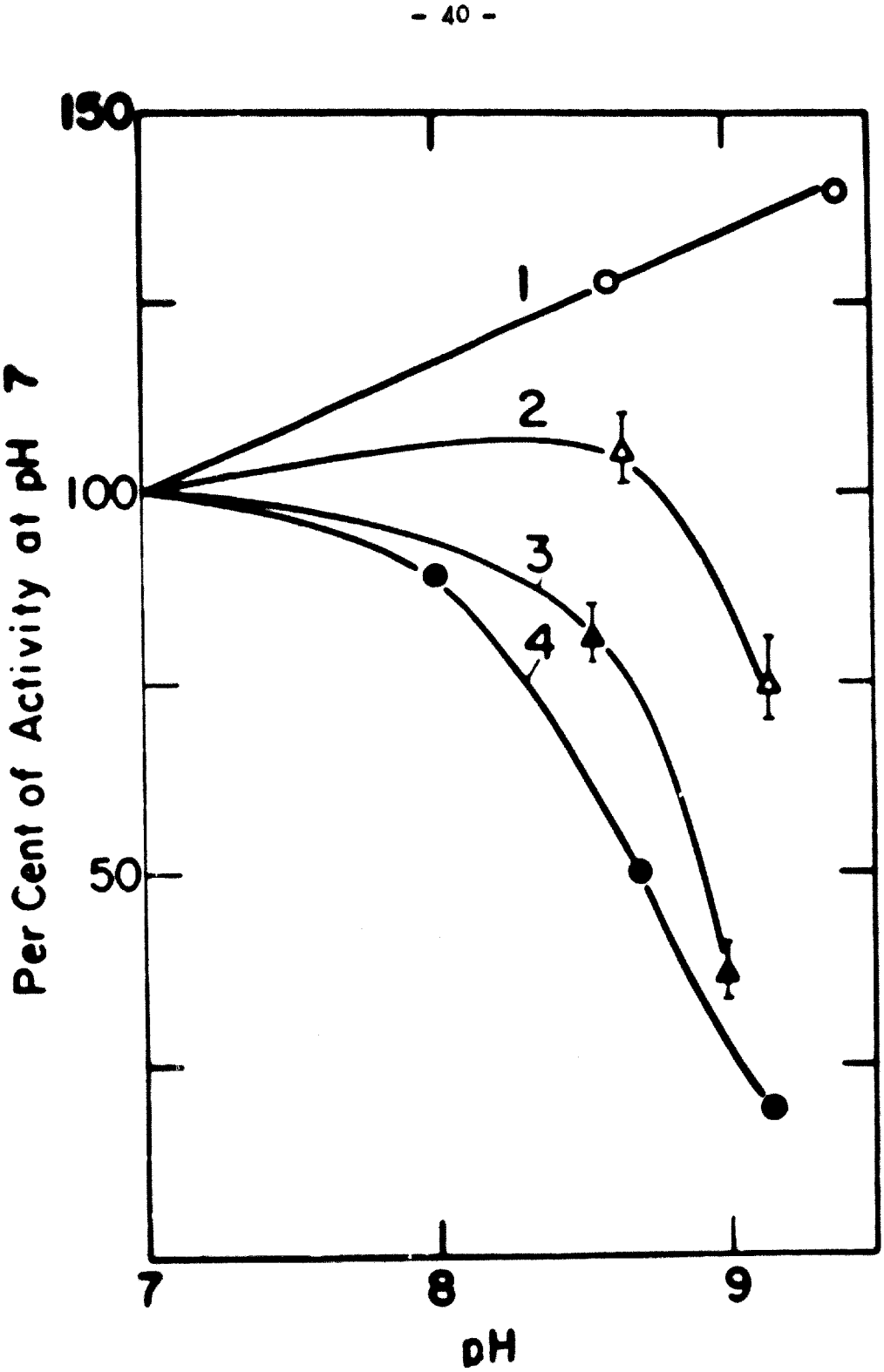


Fig. 13


4.4.72