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PREPARATION AND PROPERTIES OF PENICILLIN AMIDASE IMMOBILIZED IN POLYELECTROLYTE COMPLEXES

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(1) Immobilization of penicillin amidase (acylamide amidohydrolase, EC 3 5 1 4) from *Escherichia coli* was carried out on negatively charged particles of the water-soluble polyelectrolyte complex formed by poly(4-vinyl-*N*-ethylpyridinium bromide) and poly(methacrylic acid) in the ratio 1 3 The enzyme was covalently cross-linked to the polycation which was previously modified by cyanuric chloride (2) Kinetic parameters of benzylpenicillin hydrolysis, catalyzed by immobilized penicillin amidase, were determined and it was shown that with a given method of immobilization, the catalytic efficiency of enzyme action changes slightly The reaction proceeds in homogeneous aqueous solution without any diffusional difficulties (3) Deformation of the pH optimum of catalytic activity, an increase of k_{cat} in alkaline media and a sharp increase of the inhibition constant from 2 10^{-5} to 1 10^{-3} M caused by the product of the reaction (phenylacetic acid) show a remarkable effect of the negatively charged shell of the polyelectrolyte complex on kinetic parameters of the reaction (4) Immobilization of the enzyme in polyelectrolyte complex particles leads to the appearance of new properties of the biocatalyst, immobilized penicillin amidase can be reversibly converted to the insoluble state with a slight change in pH and ionic strength of the solution Transition of the enzyme into the insoluble state results in interruption of the reaction The conditions for the phase separation of immobilized enzyme in solutions of salts are determined by the composition of polyelectrolyte complex and the nature of the low molecular weight electrolytes Dissolution of the precipitate leads to quantitative recovery of the initial catalytic activity (5) The self-regulating enzymatic system was simulated Control of the activity in the system takes place according to the following scheme accumulation of product → change in ionic strength of the solution → alteration of microenvironment of the enzyme → decrease in catalytic activity

Introduction

It is well known that immobilization of enzymes can result in a considerable change in their properties, namely, kinetic parameters of the catalyzed reactions, the pH optimum of the catalytic activity, the stability of enzymes, etc [1–3] Each method of immobilization gives a specific set of new properties to a chosen enzyme This set of properties is determined finally by the interaction between the enzyme and a support

A proper choice of the enzyme and the method of its immobilization permits the realization of the purposeful synthesis of new catalysts with unique properties and, thus, the successful solution of important technological problems

Another important aspect of the use of immobilized enzymes is the attempt to simulate the complex processes in a living cell As is known in vivo, most enzymes are incorporated in a membrane, cytoplasm and the mitochondrial matrix where their microenvironment differs appreciably from that in which

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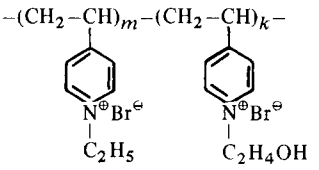
the properties of native enzymes are studied in vitro [4]

Incorporation (immobilization) of enzymes in a structure of water-soluble non-stoichiometric polyelectrolyte complexes seems to be a very effective way for studying such problems It is well known that stoichiometric compounds (1 1 polyelectrolyte complexes) are usually precipitated when polyions with opposite charges interact in equimolar ratios However, the situation is quite different with those non-stoichiometric polyelectrolyte complexes which are soluble in water [5,6] A particle of such a polyelectrolyte complex, being the product of completed intermolecular reactions, consists of a nucleus formed by sequences of salt bonds between the units of oppositely charged polyelectrolytes (1 1 polyelectrolyte complex) and the hydrophilic shell – ionized groups of the polyelectrolyte being incorporated in the polyelectrolyte complex to excess [7] The methods of preparation of such compounds are based on direct mixing of the corresponding solutions of polycationic (polyamines and polyammonium salts) and polyanionic (e g , polycarboxylic, polyphosphoric acids) components It is noted that the hydrophilic shell of polyelectrolyte complex particles can be formed by both the polyanions and polycations Specific structures of polyelectrolyte complex particles lead to the appearance of a number of extraordinary properties of such compounds in aqueous solutions Thus, by changing the pH or ionic strength of the solution, phase separation of polyelectrolyte complex solutions can be achieved [8] The existence of a homogeneous solution of polyelectrolyte complexes is determined, as a rule, by the composition of their particles which varies within a sufficiently wide range Therefore, by varying the composition of polyelectrolyte mixtures, it is possible to prepare polyelectrolyte complex solutions with given properties in a rather simple way [6]

In the present paper, some properties of the enzyme, incorporated in particles of water-soluble polyelectrolyte complexes are reported Penicillin amidase (acylamide amidohydrolase, EC 3 5 1 4) from *Escherichia coli* has been used as a model enzyme The kinetics and mechanism of action of the native enzyme have been studied in detail previously [9,10] The immobilization of penicillin amidase has been carried out by covalent attachment to the modified



polycation of a structure such as follows
where the proportions are k = 0 05–0 10 and m = 0 95–0 90

Further, the water-soluble polyelectrolyte complex has been obtained by mixing of immobilized penicillin amidase and an excess of poly(methacrylic acid), the polyelectrolyte particles being formed by the polycation, poly(4-vinyl-*N*-ethylpyridinium bromide) and poly(methacrylic acid) (the latter being excess)

Materials and Methods

Penicillin amidase from *E coli* was isolated and purified using a technique described previously [9] A commercial preparation of benzylpenicillin and cyanuric chloride from Koch-Light Laboratories Ltd (U K) were used, ethylene chlorohydrin and mineral salts from Reakhim (U S S R) were of the highest purity available phenylmethylsulfonyl fluoride (PMSF) was supplied by Sigma (U S A)

Poly(methacrylic acid) was prepared by radical polymerization The polymer was fractionated by adding ethyl acetate to a solution of poly(methacrylic acid) in methanol The fraction with $\bar{M}_{w_r}$ = 260 000 was used (molecular weights, $\bar{M}_{w_r}$, of polyelectrolytes were measured by light scattering [6]

The preparation of poly(4-vinylpyridine) was described in Ref 11 A fractionated sample with $\bar{M}_{w_r}$ = 40 000 was used Poly(4-vinylpyridine) was dissolved in methanol (1 mol/l) and modified by treatment with ethylene chlorohydrin under an N_2 atmosphere at 60°C for 10 h The degree of alkylation, β, calculated from the infrared spectra by the relative absorption at λ = 1600 and 1640 cm^{-1}, was equal to 5% Subsequently, the polymer was dissolved in methanol and completely alkylated under an N_2 atmosphere with ethyl bromide (60°C, 10 h) The modified polycation obtained was reprecipitated in absolute ether The degree of sample alkylation was equal to 100%

Penicillin amidase, covalently attached to the polyacrylamide gel after a preliminary modification of the native enzyme by acrolein, was prepared

according to Ref 12 A preparation of penicillin amidase incorporated in fibres of cellulose triacetate was kindly given to us by Dr Marconi

Penicillin amidase was immobilized in particles of water-soluble polyelectrolyte complex in the following way A solution of cyanuric chloride in dioxane was added while stirring to an aqueous solution of modified polycation (0 1 g/ml) The final concentration of cyanuric chloride was 50 mM in 50% dioxane Then an excess of penicillin amidase was added to the solution obtained, and the mixture stirred and incubated at room temperature for 10–12 h (pH 8 4) To separate the excess of the native enzyme an equimolar amount of poly(methacrylic acid) was added to the modified polycation solution Insoluble polyelectrolyte complex (1 1) precipitate was isolated by filtration or centrifugation and washed with 0 1 M NaCl until the washing solution completely lost enzymatic activity The excess of poly(methacrylic acid) was added to the precipitate of (1 1) polyelectrolyte complex to form a (3 1) polyelectrolyte complex and the ionic strength of the final solution reached the value of 0 5 M Solubilization of the precipitate was observed During all operations, the pH was maintained at a value of 6 0–8 5 by adding 0 1 M NaOH The prepared solution was diluted with distilled water to reach a concentration of 0 1 M NaCl and then concentrated by ultrafiltration to a volume of 10–15 ml The obtained preparation of immobilized penicillin amidase was a homogeneous transparent solution The amount of incorporated enzyme was varied from 0 5 to 50 μmol/g of modified polycation

When the same sequence of operations was carried out under identical conditions in the absence of cyanuric chloride, the (3 1) polyelectrolyte complex obtained had no enzymatic activity This fact indicates that immobilization of penicillin amidase in the polyelectrolyte complex (3 1) can proceed only by covalent cross-linking of the enzyme to the polyelectrolyte complex particles

Assay of native and immobilized penicillin amidase

Penicillin amidase was assayed with benzylpenicillin as substrate, titrating the liberated phenylacetic acid with 0 01 M KOH in a pH-stat (TTT-1c, Radiometer, Denmark) All experiments were carried out at 25 ± 0 2°C in 0 1 M NaCl in non-buffer medium The kinetics of enzymatic hydrolysis were studied under conditions of substrate excess in comparison with the enzyme ($[S]_0 >> [E]_0$) V and K_m values for hydrolysis of benzylpenicillin catalyzed by different samples of penicillin amidase were determined by initial rate analysis for the enzyme immobilized in polyacrylamide gel and fibres of cellulose triacetate and from progress curves (using an initial substrate concentration of 0 4 10^{-4} M for native enzyme and enzyme incorporated in polyelectrolyte complex [13] In both cases, the treatment of experimental data was performed using the least-squares method by the computer PDP 8/E Determination of the 6-aminopenicillanic acid concentration was performed using the indicator reaction with *p*-dimethylaminobenzaldehyde [14] The samples were centrifuged before assay where necessary

Results and Discussion

Determination of the kinetic parameters of hydrolysis catalyzed by immobilized penicillin amidase

The concentration of the active sites of the enzyme was determined by titration of penicillin amidase with PMSF (Fig 1) according to a previously developed method [15] On the basis of the data

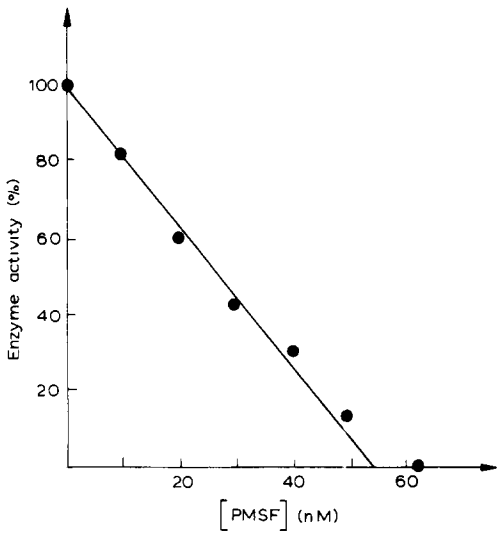


Fig 1 Determination of the concentration of active centres of immobilized penicillin amidase by titration with PMSF Conditions incubation of the enzyme was carried out at 25°C in 0 01 M phosphate buffer (pH 6 0, 0 1 M NaCl) for 10 min Penicillin amidase was assayed at 25°C, pH 7 5, 0 1 M NaCl, $[\text{benzylpenicillin}]_0$ = 5 mM

TABLE I

KINETIC PARAMETERS OF HYDROLYSIS OF BENZYLPENICILLIN BY DIFFERENT PREPARATION OF PENICILLIN AMIDASE

Conditions pH 7 5, 25°C PA, penicillin amidase, PEC, polyelectrolyte complex

	Native PA	PA covalently bound with polycation	PA incorporated in PEC	PA incorporated in fibres of cellulose triacetate	PA immobilized in polyacrylamide gel
k_{cat}	50	33	20	–	–
$K_m(\mu M)$	9	9	8	430	530
$K_p(\mu M)$	20	–	1 000	–	–

obtained values of the catalytic constants (k_{cat}) of benzylpenicillin hydrolysis catalyzed by preparations of the enzyme at different stages of immobilization were calculated (Table I) When enzyme is linked to polycation, k_{cat} decreases from 50 to 33 s^{-1} and further to 20 s^{-1} when the polyelectrolyte complex (3 1) is formed The interaction of enzymes with polyelectrolytes frequently leads to alteration of the k_{cat} value This can be explained by the effect of charged groups of macromolecules on the microenvironment of the enzyme active centre [16]

As seen in Table I, the value of K_m for benzylpenicillin is not increased, showing the absence of diffusion problems during the action of the immobilized enzyme This property of immobilized penicillin amidase is advantageous in comparison with those of other samples of this enzyme, e g , those of the enzyme immobilized in polyacrylamide gel or in fibres of cellulose triacetate (Table I)

Thus, when penicillin amidase is incorporated in polyelectrolytes, the catalytic efficiency of the enzyme action changes slightly and the reaction can be performed in a homogeneous aqueous solution It should be noted that charged chains of the polyanion forming the complex shell do not prevent substrate penetration to the enzyme active centre

pH dependence of the catalytic activity of immobilized penicillin amidase

When penicillin amidase is incorporated in polyelectrolyte complexes an appreciable deformation of the pH optimum of catalytic activity occurs and an increase of k_{cat} in alkaline media takes place (Fig 2) Such pH optimum broadening is wellknown in the literature and can be explained as a result of the redistribution of H^+ and OH^- [13,18,19]

It is interesting to note that although the enzyme is bound to the polycation and is within the nucleus of the polyelectrolyte complex, a shift of the pH dependence of k_{cat} to the alkaline region shows that catalytic properties of the enzyme are strongly affected by negatively charged groups of poly(methacrylic acid) (poly(methacrylic acid is in excess in the (3 1) polyelectrolyte complex) The fact that the shift of pH dependence is also observed at sufficiently high ionic strength of the solution (0 1–0 15 M NaCl) supports the suggestion of a high density of negative charge on the polyelectrolyte complex shell

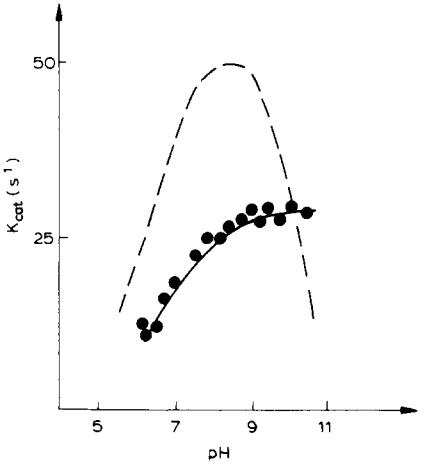


Fig 2 pH dependence of k_{cat} for benzylpenicillin hydrolysis catalyzed by native (– – –) and immobilized (•) penicillin amidase Conditions 25°C 0 1 M NaCl The pH dependence of k_{cat} for the native enzyme is represented according to Ref 17

Such a change in the microenvironment of the enzyme incorporated in polyelectrolyte complexes causes another interesting consequence As follows from the data of Table I, the K_m value for benzylpenicillin hydrolysis does not alter after immobilization by the method described, but the constant (K_p) of competitive inhibition by the reaction product (phenylacetic acid) increases by 2 orders of magnitude Under the conditions of the experiment (pH 7 5), both substrate and product (see Scheme I) are charged negatively However, the charge of phenyl acetate, situated in the immediate vicinity of the hydrocarbon part of the molecule affects binding with the enzyme active site much more strongly than does the peripheral charge of a molecule of benzylpenicillin

This fact indicates once more a considerable influence of the microenvironment of the enzyme on its catalytic properties

Effect of ionic strength and pH on the properties of immobilized penicillin amidase

The structural data, physico-chemical characteristics of polyelectrolyte complexes and results of the present work allow description of the influence of pH and ionic strength of the solution on properties of the immobilized enzyme in the following way (Fig 3) The high molecular weight of particles of the (3 1) polyelectrolyte complex ($M_r \approx 1\ 2\ 10^6$) and the high charge density on chains of the polyelectrolytes forming the complex result in a cooperative phase transition in these systems under slight variations of external conditions

Thus, an increase of the concentration of H^+ in the system leads to its binding with carboxylate anions, the total density of the negative charge of the polyelectrolyte complex particles decreases and folding of the polyelectrolyte chains followed by precipitation of the complex occur Phase separation in the

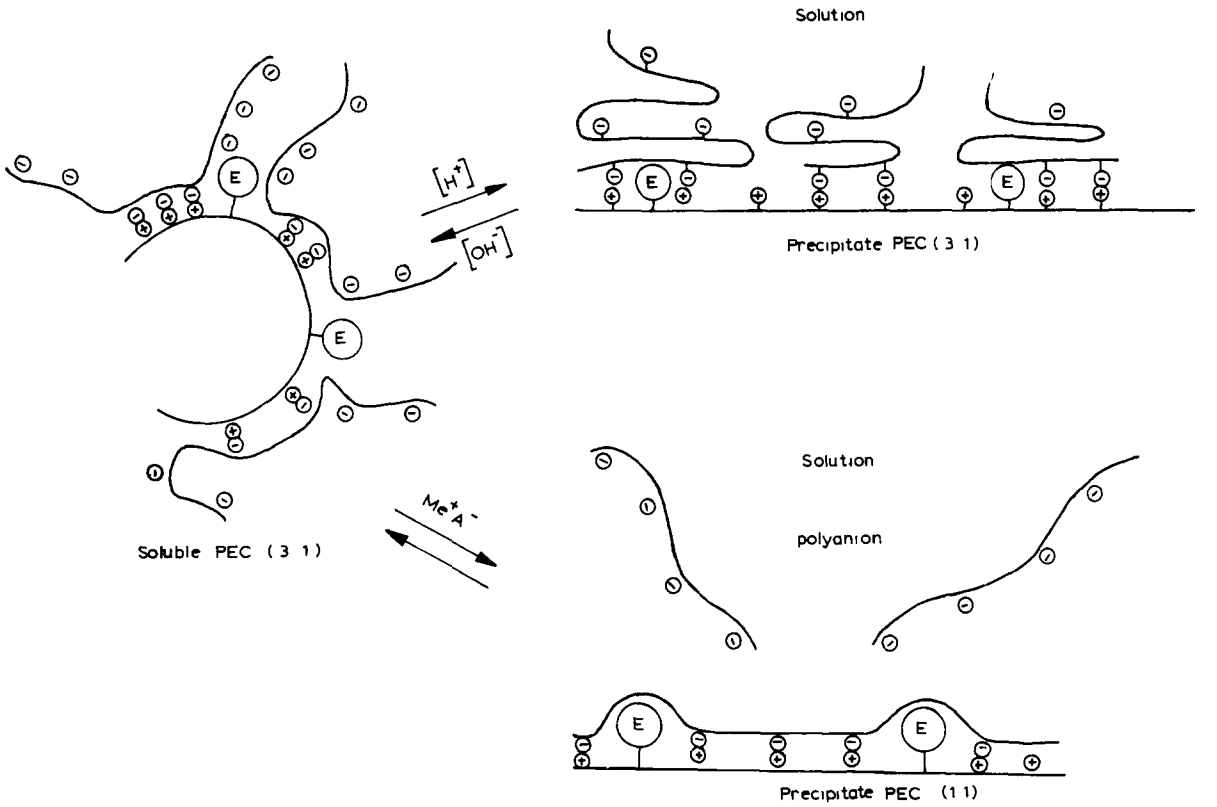


Fig 3 Influence of pH and ionic strength on the reversible transition between the soluble and insoluble forms of penicillin amidase immobilized in polyelectrolytes PEC, polyelectrolyte complex

system is observed within a narrow range of pH change, viz , approx 0 2 pH units An increase of pH causes redissolution of the precipitate and complete recovery of the initial enzymatic activity of immobilized penicillin amidase This process can be repeated continually

Another interesting property of penicillin amidase incorporated in polyelectrolyte complexes is its capacity to undergo reversible transition from a soluble to an insoluble state with a slight change in ionic strength of the solution (Fig 3) As seen in Fig 4, which shows the curve of turbidimetric titration of the solution of the (3 1) polyelectrolyte complex with NaCl solution, complete transition to the insoluble state occurs when the concentration of low molecular weight electrolyte changes by 0 01–0 02 M The absolute value of the ionic strength (I^*) of the solution at which the phase separation occurs depends, as a rule, on the ratio polyanion polycation in polyelectrolyte complexes and can vary from 0 06 to 0 35 M NaCl During the phase separation, rearrangement of polyelectrolyte complex particles and transition of the excess of polyanion into the soluble state are observed The precipitate is found to be the insoluble stoichiometric (1 1) polyelectrolyte complex [6] The enzyme attached to chains of the polycation is precipitated, causing complete interruption of the catalytic reaction

The process represented schematically in Fig 3 is reversible and can be carried out repeatedly with quantitative preservation of the catalytic activity

When $I > 0\,4$ M (NaCl), redissolution of the precipitate is observed (Fig 4) caused by 1 1 polyelectrolyte complex dissociation [6]

The value of I^* also depends strongly on the nature of the salt added The effect of monovalent cations is analogous to that described above for Na^+ The value of I^* for the 3 1 polyelectrolyte complex changes slightly in the order $K^+ > Na^+ > Li^+$, in the range 0 22–0 29 M The bivalent cations Mg^{2+}, Ca^{2+} and Mn^{2+} lead to a sharp narrowing of the range of polyelectrolyte complex (3 1) stability, precipitation is observed already at $I^* \approx 0\,01$ M that is due to the more effective binding of these cations to carboxylate anions

Thus, incorporation of the enzyme in particles of water-soluble polyelectrolyte complexes results in the appearance of a new type of control of the catalytic

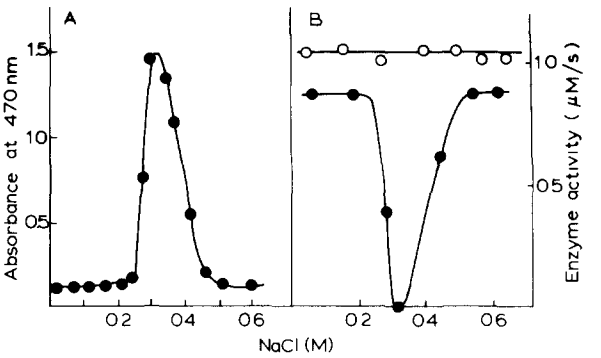
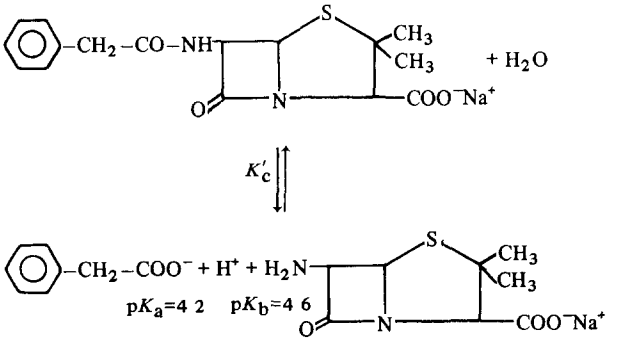


Fig 4 Dependence of turbidity (A) and activity (B) of native (○) and immobilized (●) penicillin amidase (immobilized in polyelectrolyte complex) Conditions penicillin amidase was assayed at 25°C, pH 7 5, in 0 1 M NaCl $[S]_0$ = 5 mM In the case of the immobilized enzyme, the assay was performed on the supernatant after centrifugation of the sample

activity A change in ionic strength of the solution throughout the range 0 01–0 4 M does not result in considerable change in the native enzyme activity, while immobilized penicillin amidase under these conditions undergoes a reversible loss of activity within a very narrow range of I changes (Fig 4)

Self-regulating enzyme system

A self-regulating enzyme system can be created on the basis of high 'sensitivity' of the immobilized enzyme to a change in ionic strength of the solution The formation of charged particles takes place as a result of many chemical reactions catalyzed by enzymes The increase in I during the reaction up to I^* will lead to transition of the immobilized enzyme into the insoluble state, followed by interruption of the catalytic process Such a possibility was demon-



Scheme I

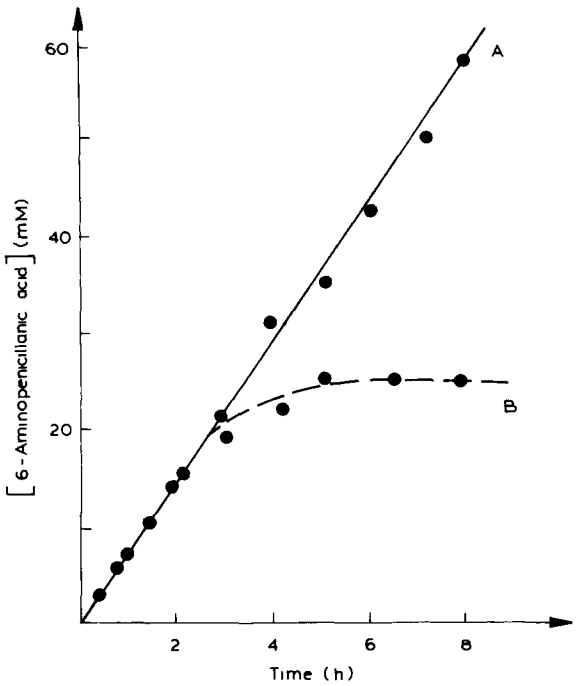


Fig 5 Progress curves of enzymatic hydrolysis of benzylpenicillin catalyzed by immobilized penicillin amidase Conditions 25°C, 0 1 M phosphate buffer, pH 7 5 $[S]_0$ = 100 mM, $[E]_0$ = 0 2 μM, I_0 = 0 20 M (A) and 0 25 M (B)

strated experimentally using the example of enzymatic hydrolysis of benzylpenicillin (Scheme I) When this reaction proceeds at a constant pH the ionic strength increases By varying the initial ionic strength of the solution, I_0, the reaction can be stopped at any degree of conversion The data presented in Fig 5 show that when I_0 = 0 25 M, the concentration of products formed cannot be higher than 26 mM (degree of conversion is 25% under the conditions specified) At this degree of hydrolysis the concentration stated was reached and the reaction stopped

A change in composition of the system (dilution of the solution or consumption of components of the reaction) leading to a decrease in ionic strength will start the hydrolysis again At lower values of I_0, the reaction proceeds in homogeneous solution until greater or complete substrate conversion is achieved

In comparison with the well known effects of enzyme inhibition by the reactions products when a direct interaction of product with enzyme is observed [20], in the present case control of the activity takes place indirectly by the following scheme accumulation of product → change in ionic strength of the solution → alteration of microenvironment of the enzyme → decrease in catalytic activity Since the enzyme in the cell is frequently in the environment of polyelectrolytes, the process of self-regulation of enzyme action, based on the same principles, can occur in vivo

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