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THERMODYNAMICS AND KINETICS OF THE INTERACTION OF SYNTHETIC AND SEMI-SYNTHETIC POLYMERS WITH ENZYMES DURING THEIR IMMOBILIZATION AND MODIFICATION

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Abstract - The interaction of enzymes and polyelectrolytes leads to modification of the enzymes, changing not only their catalytical properties but also their stability. Carboxylic heterogenic exchangers "Biocarb" were synthesized from methacrylic acid and hexahydro-1,3,5-triacryloyltriazine. Proteins are sorbed on these exchangers with high selectivity and good kinetics (diffusion coefficient $\bar{D} \sim 10^{-9} - 10^{-10}$ $cm^2 \cdot sec^{-1}$). The sorption of α-amylase on Biocarb leads to its acid stabilization. Urease is sorbed on Biocarb with great capacity and on covalent immobilization completely retains its activity after repeated use in the conditions of a flow reactor.

A chromatographic method for determination of the equilibrium and kinetic constants of interaction between enzymes and soluble polyelectrolytes was developed. The kinetic constants of the formation ($k_1 = (8 \pm 4) 10^{-4}$ sec^{-1}) and dissociation ($k_{-1} = (4 \pm 2) 10^{-5}$ sec^{-1}) of the RNase-dextran-sulfate complex were determined. The thermodynamic analysis of the interaction showed that the inhibition of the enzyme in the complex is characterized by high energy intermolecular interaction and low specificity, whereas the activation of the enzyme is a more specific process accompanied by an increase in entropy.

The immobilization or modification of enzymes by synthetic polymers, in particular by polyelectrolytes, can be carried out either in the solid phase, or swollen gel or in the solution. Crosslinked polyelectrolytes are used in the former case, while linear or branched polyelectrolytes in the latter. The immobilized or modified enzymes acquire new properties, e.g. an increasing stability of the structure, resistance to inhibitors and some times higher activity. Although the modification of enzymes by crosslinked and soluble polyelectrolytes gives different new forms (insoluble or soluble), they still have many properties in common.

To immobilize enzymes, only those crosslinked polyelectrolytes should be used which do not cause structural deformation of the sorbed macromolecules and exhibit high sorption capacity with respect to enzymes. New carboxylic biosorbents "Biocarb" (1) satisfy these requirements. They are obtained by the copolymerization of methacrylic acid (MAA) with hexahydro-1,3,5-triacry-

loyltriazine (T) in low concentration acetic acid solutions containing large amount of the trivinyl component. These highly permeable ion exchangers exhibit a stable steric structure and their catalytic effect on enzymes is negligible; they immobilize native macromolecules in the voids filled with the solvent. The structure of the networks of macroreticular carboxylic KMDM cation exchangers (copolymers of MAA and N, N' - alkylene dimethacrylamides) is slightly less rigid. We mean by a macroreticular exchanger the exchangers, whose structure contains crosslinking agents with remotely spaced vinyl groups. Their synthesis is carried out in the presence of a good solvent. Biosorbents of the Biocarb type exhibit little change in the degree of swelling during titration (Fig.1). The most rigid network structure is displayed

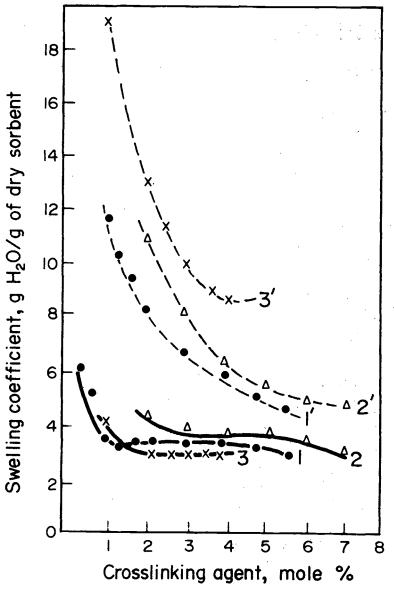

Fig.1. Swelling coefficients K_{SW} (g H_2O/g of dry sorbent) of carboxylic cation exchangers Biocarb (1 and 1'), KMDM-2 (2 and 2') and KMDM-6 (3 and 3'); H^+-form (1,2,3) and H^+-Na^+ form (1',2',3').

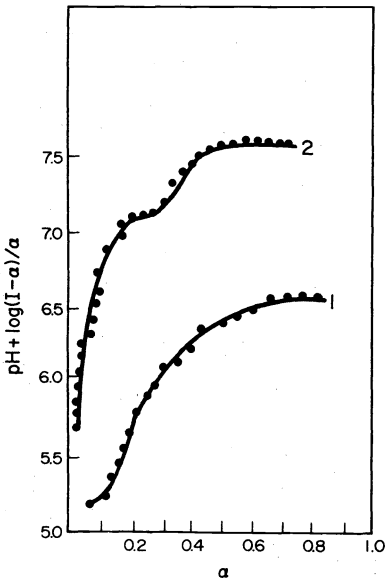

Fig.2. Potentiometric titration of Biocarb (1) and KMDM-2 (2) polyelectrolytes.

by the Biocarb cation exchangers containing a large percentage (6-7 mole%) of a crosslinking agent and by macroreticular cation exchangers containing methylene dimethacrylamide. The thermodynamic mobility of crosslinked structures can be followed by potentiometric titration of copolymer micrograins (1-5 μm) obtained by dispersing the crosslinked polyelectrolytes. In this case the kinetic difficulties of the titration are eliminated. The mobility of oligomers located between the knots can be observed from the conformational transition characteristic of polymethacrylic acid (PMAA) for which, in contrast to polyacrylic acid (PAA) the hydrophobic internal interaction decreases when the ionization rate increases (2). This is shown by the plateau region on the titration curve (Fig.2). The comparison of the mobility of oligomer chains shows

that the Biocarb sorbent is much more rigid than the macroreticular cation exchanger (3). It should be noted that commercial carboxylic cation exchangers obtained with divinylbenzene as a crosslinking agent are less effective than macroreticular exchangers or Biocarb sorbent. Commercial exchangers are permeable for macromolecules only at small amounts of the crosslinking agent; the mobility of their network is great, which leads to irreversible immobilization of the sorbed enzymes and to considerable inactivation. The investigation of the state of water in the Biocarb exchangers by the isopiestic method showed that the swelling of their H^+- and Na^+-forms are comparable as regards the amounts of sorbed water whereas the curves for water sorption versus partial pressure of water vapours are very different. This shows that the introduction of Na^+- ions in the exchanger leads to partial replacement of the "free" water by bound water.

Biocarb biosorbents are heterogeneous materials containing regions with high and low density. The investigation of the porosity of swollen polymers by small angle x-ray scattering showed that the radii of gyration for the Biocarb biosorbents range from 40 to 120 Å. Electron micrography is used to evaluate the size of the regions of high and low density of crosslinked structures. For this purpose the water in swollen sorbents was successively replaced by ethanol and ether. After this an epoxide composition, Araldite, was introduced and the sample was hardened for two days at 70°. Uranyl acetate was used as a contrasting agent. The microsections of 400-500 Å thick were examined. Fig.3 shows that for Biocarb the size of pores greatly exceeds that of insulin macromolecules and is 100-200 Å. Gel chromatography of inert macromolecules shows that Biocarb contains a certain amount of pores with a radius up to 1000 Å.

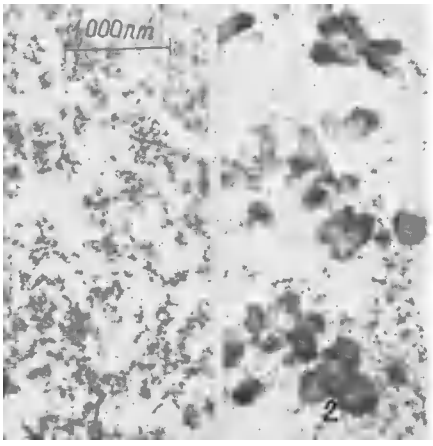

Fig.3. Micrographs of the Biocarb exchanger (I) and the same exchanger with sorbed insulin (2).

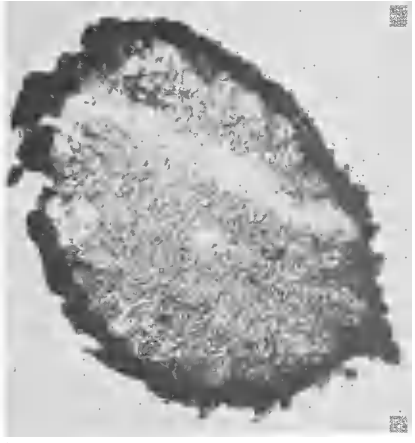

Fig.4. Distribution of sorbed protease terrilytin dyed with Amidoschwarz in Biocarb.

Since Biocarb biosorbents and macroreticular carboxylic KMDM cation exchangers exhibit heterogeneous porosity, proteins are distributed in them nonuniformly, as is shown in Fig.4 for terrilytin protease isolated from Asperg. terricola culture liquid. To observe protein distribution in the sorbent grains, they were placed in a 20% solution of gelatin melted at 37° and

cooled. Their microsections were dyed with Amidoschwarz. The degree of protein penetration into Biocarb grain depends on the molecular mass (MM) of the biopolymer, its morphology and electrochemical properties of its macromolecules as well as on the pH of the solution. Thus, for cytochrome C the penetration depth of macromolecules was half of the grain radius and for haemoglobin the protein distribution was uniform. The dispersion of the Biocarb biosorbents to the size of several microns makes it possible to carry out uniform sorption of very great amounts of protein throughout the grain Fig.5 shows that the amount of bound serum albumin is 5 g per gram of sorbent. Thus, a gel microcapsule is formed, which consists of a network filled with the protein and the solvent; the weight fraction of the copolymer is only 20%.

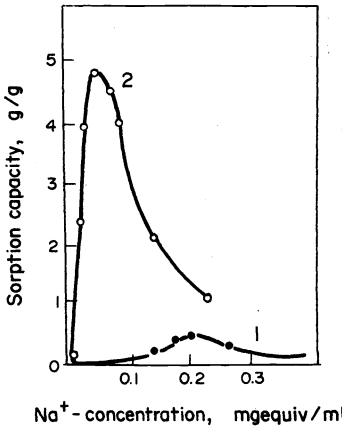

Fig.5. Sorption capacities, m (g/g), of KMDM-2 cation exchanger for serum albumin. Grain diameter: I , 100 μm; 2, 1-5 μm.

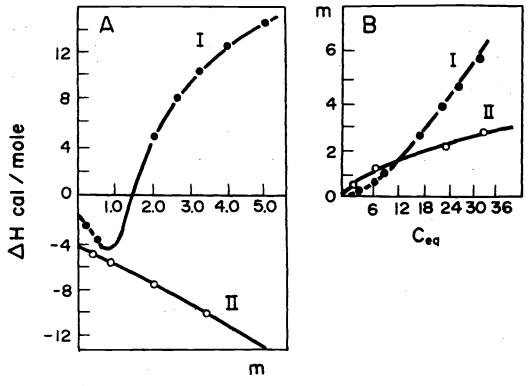

Fig.6. Differential enthalpies (A) and sorption isotherms (B) of haemoglobin for KMDM-2 (1) and KADM-2 (11) exchangers. m is sorption capacity, g/g. Ceq is equilibrium concentration of protein in solution.

The linear dependence of the extent of the protein sorption on the square root of time permits the calculation of the diffusion coefficient $\bar{D}$ (Table I) from the law of diffusion into the sphere taking into account the relative radius of unsorbing grain.

Table 1

Protein diffusion into sorbents.

Protein	MM	Sorbent	Average radius of the sorbent grain, μm	$\bar{D}$ $cm^2 \cdot sec^{-1}$
Terrilytin (protease)	26,000	Biocarb	360	7.10^{-10}
Lysozyme	17,500	Biocarb	600	6.10^{-9}
Serum albumine	68,000	Biocarb	450	2.10^{-10}
α-amylase (Bac. subt.)	40,000	KMDM	360	8.10^{-10}

Investigation of the temperature dependence of the initial linear parts of sorption isotherms permits the calculation of the sorption enthalpy from the equation of isobars, wich in combination with the thermodynamic potential gives the entropy of interaction. Data in Table 2 show that the entropy is the main reason for the selective sorption of proteins by Biocarb. Apart from the hydrophobic interaction, other mechanisms also determine the increase in the entropy during sorption. The impossibility of interpreting all the sorption phenomena from the hydrophobic properties was shown by an increase in the positive entropy on passing to aqueous-alcohol solutions. We advance an alternative conception of polyfunctional interaction (4), wich is based on the sorption model of organic ions capable of forming both electrovalent and weak bonds with the sorbent. This gives rise to a set of microstates of the sorbent-organic counterion system or to increasing entropy in sorption. In some cases the increased entropy can be accounted for by the degradation of associates when the protein passes from solution into the sorbent.

Analysis of differential functions of sorption can be made by microcalorimetry. Differential enthalpies of sorption and isotherms of haemoglobin sorption on macroreticular sorbents KMDM-2 and KADM-2 (in the latter MAA is rereplaced by AA) shown in Fig.6 indicate that positive ΔH and ΔS correspond to the cooperative effect of sorption on the first sorbent whereas the statistical sorption corresponding to the Langmuir isotherm is revealed by the usual decrease in enthalpy.

Table 2

Thermodynamic functions of protein sorption by Biocarb (25^{o})

Protein (enzyme)	ΔG kcal/mole	ΔH kcal/mole	ΔS kcal/mole.degree
Ribonuclease	-3.5	-2.0	+ 4.4
Terrilytin	-5.5	0	+ 17
Thermolysine	-5.4	-12.9	- 24
Pepsin	- 4.5	+ 4.9	+ 32
Serum albumin (bovine)	- 4.0	+ 5.4	+ 32
Insulin	- 5.3	- 4.4	+ 5.0

Persumably, the cooperative effect favours the polyfunctionality and increases the number of microstates of the protein-sorbent system. The competing selectivity of protein sorption by biosorbents is extremely high and depends on the volume concentration of carboxylic groups in the sorbent. Fig.7 shows that the selectivity coefficient of proteins in crosslinked copolymers including MAA, T and hydroxypropyl methacrylamide (HPMA) increases on passing to Biocarb from the copolymers that contain a lower amount of carboxylic groups but exhibit high hydrophilic properties and high permeability for macromolecules. Fig.8 shows the pH dependence of the selectivity of insulin sorption by Biocarb and a crosslinked copolymer of MAA and HEMA (MK-30). Biocarb sorbs a much greater quantity of insulin than does MK-30.

The immobilization of enzymes by Biocarb was carried out for two purposes: to stabilize enzymes, in particular with respect to acid denaturation, and to

prepare an insoluble form of enzyme suitable for prolonged performance. The first approach is of considerable interest, especially, for the solution of a medical problem: the preparation of a modified form of hydrolytic enzyme capable of passing through the stomach without inactivation. It is also necessary to prepare a modified enzyme form that would be capable of catalyzing the hydrolysis of macromolecular substrates in the intestine. Both the stabilization and the maintenance of the catalytic activity of enzymes to macromo-

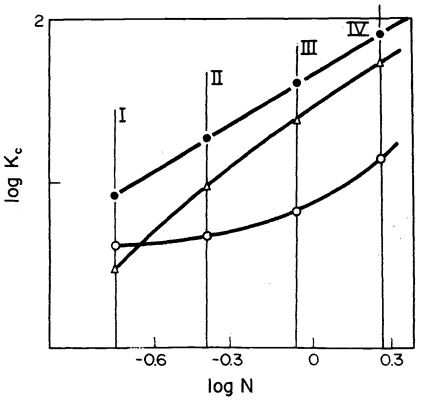

Fig.7. Selectivity coefficient K_c of protein sorption vs N-volume concentration of carboxylic groups in the copolymers of MAA-HPMA containing I, 30; II, 50; III, 75 and IV, 100% MAA. (○) pepsin; (△) chymotrypsinogene, (●) papain.

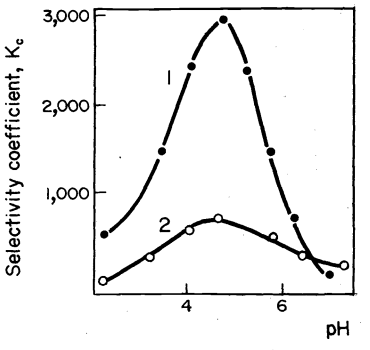

Fig.8. Selectivity coefficient, K_c, of insulin sorption by Biocarb (I) and MK-30 (2) from 0.1 N buffer solutions vs pH.

lecular substrates were achieved for α-amylase and terrilytin protease on the basis of enzyme sorption by Biocarb. The stabilization of Biocarb-sorbed α-amylase (pH 5.5), isolated from the Bac.subtil. culture liquid, was investigated by incubation of the sorption complex in 0.1 N solution of hydrochloric acid at 25 and 37°C (5). After the incubation, α-amylase was desorbed by a 0.4 N borate buffer solution at pH 7.5, in which the activity to soluble starch was determined. A comparison of the degree of acid inactivation of free α-amylase and the polymer sorption complex is shown in Fig.9. α-amylase is completely inactivated in a few minutes, whereas the amylolytic activity in the complex decreases by 50% for about an hour. The inactivation of sorbed amylase depends on several parameters, including the amount of bound enzyme. When an additional amounts of α-amylase is introduced into Biocarb, the degree of stabilization decreases. This phenomenon may be accounted for by the cooperative mechanism of interaction between the enzyme and the sorbent, which is revealed in the S-shaped sorption isotherm. In this case the cooperative interaction leads to destabilization of polyelectrolyte-bound enzyme, like in the case stabilization by soluble polyelectrolytes to be considered below. The acid-resistant polymer complex of α-amylase is dissociated at pH 6.5 (Fig.10) and the active enzyme passes into solution. Acid stabilization is also observed when the terrilytin is sorbed by Biocarb.

The selective interaction of Biocarb with enzymes retains their native

structure, which permits to prepare reversibly dissociating insoluble complexes and to immobilize the enzyme on the sorbent by covalent bonding. This

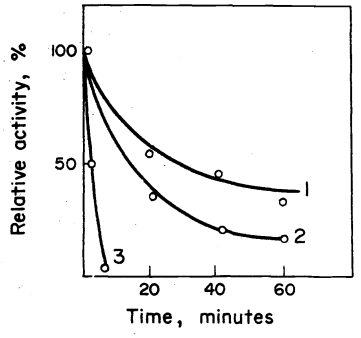

Fig.9. Inactivation of α-amylase (3) and its sorption complexes with Biocarb (I and 2) at pH=2.0
1, 2400 act. units/g, 2, 10000 act. units/g.

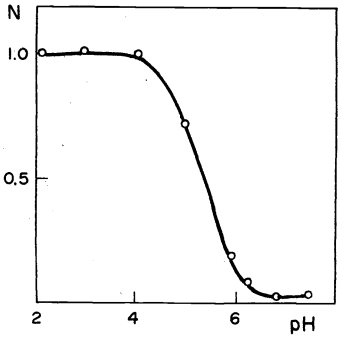

Fig.10. Dissiciation of the sorbed complex α-amylase - Biocarb. N is the portion of enzyme in sorbed state.

approach is similar to the incorporation of enzymes into gels in the cross-linking polymerization in the enzyme solution. However, the sorption method followed by chemical immobilization on highly permeable Biocarb sorbent with very stable structure permits the immobilization of greater amounts of enzyme per unit volume of the swollen copolymer and also favours substrate diffusion to the enzyme. The immobilization of urease on Biocarb was carried out by its sorption from solution and chemical immobilization in the presence of a 0.3% solution of glutaric aldehyde (6). The sorption capacity of Biocarb for urease greatly depends on the pH of the solution and reaches a maximum at the isoelectric point of the enzyme (Fig.11). Urease completely retains its activity on immobilization. The activity of the immobilized urease with respect to urea was studied in a column under dynamic conditions (Fig.12). Stationary

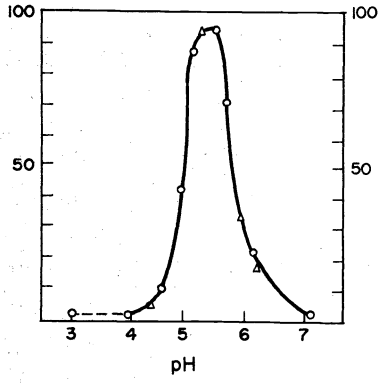

Fig.11. Urease sorption by Biocarb (○) and its activity after chemical immobilization (Δ) vs pH of solution.

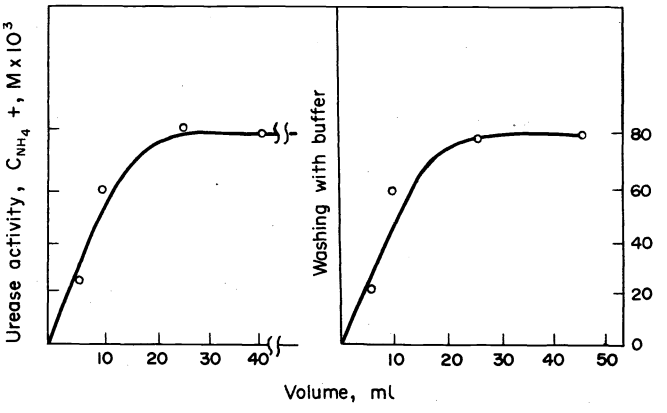

Fig.12. Hydrolytic cycles of 3% urea in column 0.7 x 0.8 cm with immobilized urease, pH 7.0, 25 ml/hr.

conditions were attained in 10-30 min. After the process was finished and the column was washed with a buffer solution, the dynamic cycle of enzymatic activity was made repeatedly, which indicates a long-term use of the immobilized urease in the column process.

The interaction of enzymes with soluble synthetic and natural polymer electrolytes opens up new possibilities to modify the properties of biocatalysts. Enzymes in soluble polymer complexes can be stabilized and even activated (4). It becomes possible also to inhibit the enzymes by soluble polyelectrolytes and to change their enzymatic specificity. Finally, soluble polymeric complexes can interact readily with substrates of high molecular mass. It should be noted that thrombi dissolve faster under the action of trypsin-polyvinylimidazole (PVI) complex (7) than due to trypsin alone. Nucleases are activated or inhibited by dextran-sulphates (DS) and change their specificity. Fig.13 shows that the introduction of DS into the RNase solution can increase or decrease the transferase activity, but the hydrolytic activity is low in all cases. As a rule, the modification of enzymes by synthetic or natural polyelectrolytes is a result of the action of the polymer-carrier on the enzyme macromolecular structure. The stabilization of trypsin is carried on by PVI but not by its analogues of low molecular mass (Fig.14). The polymer effect

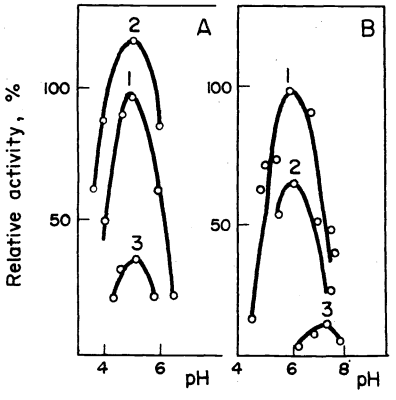

Fig.13. Transferase (A) and hydrolytic (B) activaties of pancreatic RNase and its complexes with DS. 1, RNase; 2, RNase - DS (MM 65000, n^{x}=0.8); 3. RNase - DS (MM 500000, n = 1.4).

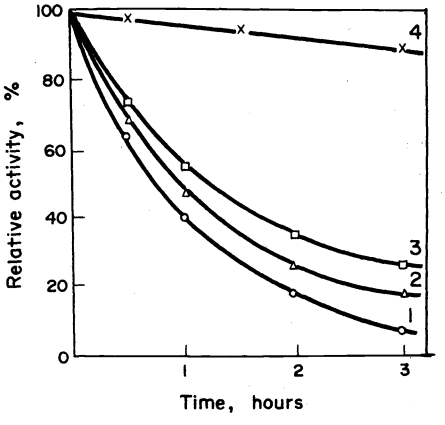

Fig.14. Autolysis of trypsin (1) and its complexes with imidazol (2), poly-N-ethylimidazol (3) and poly-N'-vinylimidazol (4), pH 6.1, 37°.

consists in the polyfunctional interaction of synthetic polyelectrolyte and is revealed in the fixation of the enzyme native conformation in case of stabilization or its disterbance in the case of inhibition. Quantitative methods for the estimation and analysis of thermodynamic and kinetic constants of complexation should be used to determine the mechanism of molecular complexe formation in polymer systems. The equilibrium characteristics of the formation of polymer complexes in solution are usually determined by dynamic methods, such as sedimentation, chromatography (9) along with other physical

[x] n is degree of substitution, number of sulfate groups per monomer unit.

and physico-chemical methods (10). The chromatographic elution analysis of the polymer complex against the background of constant concentration of one of the components, e.g. synthetic polymer electrolyte (11), is a suitable approach to the solution of these problems. The simplest problem is the chromatographic analysis of the system of reversible complexation when one enzyme molecule (E) interacts with synthetic polyelectrolyte (P):

$$E + P = EP \qquad (1)$$

If component concentrations $C_P \gg C_E$, it is possible to introduce the instability constant of the polymer complex in the form:

$$K = C_E / C_{EP} \qquad (2)$$

The analysis of the chromatographic problem has shown that under conditions of equilibrium chromatography the elution curve for the protein is characterized by one maximum, although both the protein and its polymer complex coexist in the solution. This phenomenon is associated with quasi-equilibrium and occurs at low rates of solution flow. The analysis of the chromatographic process of reversibly dissociating system migration under the constancy of coefficients for the distribution of substances between the mobile and the stationary phases (11) leads to the following expression for the constants of the complex instability:

$$K = (V_{EP} - V') / (V' - V_E) \qquad (3)$$

where V' is the elution volume of the complex for the protein at the unimodal shape of the elution curve. V_E is the elution volume of protein obtained in an independent experiment with only one component, and V_{EP} is the elution volume of the stable complex. The value of V_{EP} can be determined, for example, in a gel chromatographic experiment when the complex moves without penetrating the porous gel structure and is eluted with the free volume of the column. The calculation of the instability constant for a polymer complex of pancreatic RNase - DS (MM 500 000, sulphur content 16%) at the DS/RNase weight ratio of 1 : 2 according to eq. (3) yields the value of 5.10^{-2}. The dissociation constants for the complexation of RNase with copolymers of vinyl sulphonate and vinyl pyrrolidone are shown in Fig.15.

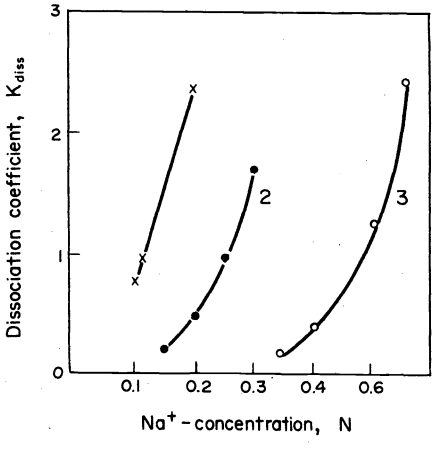

Fig.15. Dissociation constants of the complexes RNase-compolymers of vinyl pyrrolidone and vinyl sulphonate (VS). 1, 18% VS; 2, 27% VS; 3, 50% VS.

The thermodynamic analysis of the interaction of RNase with DS of different molecular masses was carried out for the initial (linear)parts of the isotherms describing the ratio of bound RNase to free RNase. Data in Table 3

show that the inhibition of the enzyme does not at all mean that the interaction between the enzyme and the polymer-carrier is very specific, since the thermodynamic potential of complexation is not very great. However, in this process the enthalpy decreases, i.e., the intermolecular interaction energy is high. In contrast, the complexation with activation is a highly specific process accompanied by a greater decrease in the thermodynamic potential. The increase in entropy is a still more interesting feature of this process, which can be interpreted as the appearance of a set of microstates of the bound enzyme owing to the polyfunctional nature of intermolecular interaction. A system of weak bonds with the energy close to that of the thermal motion leads to the coexistence of a whole set of states differing in the levels of interaction energies.

Table 3

Thermodynamic functions of the formation of polymer complexes of ribonuclease

Polymer		MM.10^{-3}	Degree of substitution, n	Ionic strength	ΔG cal/mole	ΔH cal/mole	ΔS cal/mole degree
Dextran-sulphate	inhibition	500	1.4	0.056	-400	-6000	-20
Dextran-sulphate	inhibition	500	1.4	0.316	-500	-4000	-11
Dextran-sulphate	activation	60	0.8	0.056	-1100	+2500	+13
Sulphoethyl cellulose	activation	150	0.45	0.056	-1500	+2000	+12

We determined the kinetic constants of the formation and dissociation of complexes by using chromatographic analysis of the systems with rapidly established equilibrium between the mobile and the stationary phases for all three interacting components (E, P, EP). In contrast to the chromatographic process described above, here we consider a system in which the equilibrium in the process of complexation itself is absent. This problem is solved (12) for the elution chromatographic process of the movement of a narrow zone of interacting components.

In this case the equation of material balance can be represented by a system of equations for all three components:

$$\left[\alpha + K_{d_i}(1-\alpha)\right]\frac{\partial c_i}{\partial t} = -U\frac{\partial c_i}{\partial x} + \alpha f_i\,(c_E, c_P, c_{EP}, k_1, k_{-1}) \qquad (4)$$

where $f_E = k_1 c_{EP} - k_{-1} c_E c_P$, $f_E = f_P = -f_{EP}$,

x is the coordinate corresponding to the distance from the upper layer of the sorbent, t is the time, c_i are the concentrations of components, k_1 is the rate constant of complexation, k_{-1} is the rate constant of the reverse reaction, α is the relative porosity of the column and K_{d_i} is the coefficient of substance distribution between the phases. The initial and boundary conditions were determined as follows:

$$c_i(x,0) = \begin{cases} \text{const} & (0 < x \leq x_0) \\ 0 & (x > x_0) \end{cases} \qquad c(0,t) = 0 \quad (t > 0) \qquad (5)$$

x_0 is the height of the zone at the column inlet.

The numerical solution of this problem leads to a system of chromatograms shown in Fig.16. It can be seen that as the elution rate of the solution increases, the height and the area of the first maximum corresponding to the complex zone also increase. For any selected rate pair we introduce the function:

$$\varphi = (c^{I}_{U_1} / c^{II}_{U_1}) / (c^{I}_{U_2} / c^{II}_{U_2}) \qquad (6)$$

where $c^{I}_{U_i}$ is the maximum concentration of protein in the first zone (I) and $c^{II}_{U_i}$ is its maximum concentration in the second zone (II) at the elution rates of the solution U_i (U_1 and U_2). The numerical calculation makes it possible to plot a family of curves φ vs.-log K_{-1} (Fig.17) giving the values of k_{-1} and the pairs of elution rates U_1 and U_2.

This method of evaluation of kinetic constants was used to study the rates of the formation and dissociation of the polymer complex in the interaction of pancreatic RNase and DS. The chromatographic process was carried out on a column (1 x 90 cm) packed with Sepharose. A 0.05 M phosphate buffer at pH 7.4

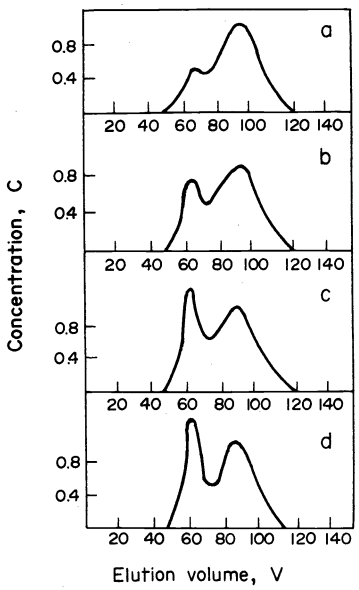

Fig.16. Theoretical elution curves for a two-component reacting system at $k_{-1} = 5.10^{-5}$. (a) U=4.5 ml/hr; (b) U=9 ml/hr; (c) U=18 ml/hr; (d) U=36 ml/hr.

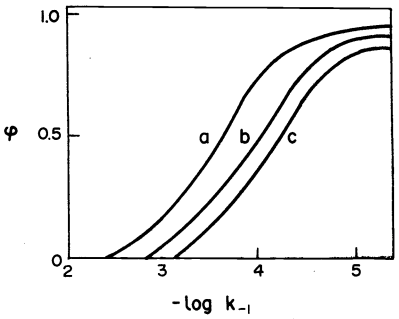

Fig.17. Plots of φ vs.-log k_{-1} (a) U_1= 18 ml/hr, U_2= 36 ml/hr (b) U_1= 9 ml/hr, U_2= 18 ml/hr; (c) U_1= 4.5 ml/hr, U_2= 9 ml/hr.

was used. Each of the components gave a symmetrical elution curve. For RNase V_E = 96 ml, for DS V_p = 57 ml. In the complexation experiment the molar ratio of DS to RNase was 1 : 8. The elution curves are shown in Fig.18. The calculated values of φ for chromatograms (1), (2) and (2), (3) were $\varphi_{1,2}$ =0.77 and $\varphi_{2,3}$ = 0.68. This permitted the determination of the formation and dissociation constants from the theoretical curves in Fig.17. For this system $K_{-1} = (4+2).10^{-5}$ sec^{-1} and $k_1 = (8+4).10^{-4}$ sec^{-1}. These data show that the complexation time of RNase with DS is several hours. Presumably, the probability of the formation and dissociation of the complex is limited by the strict steric complementarity between the polymer components and by the si-

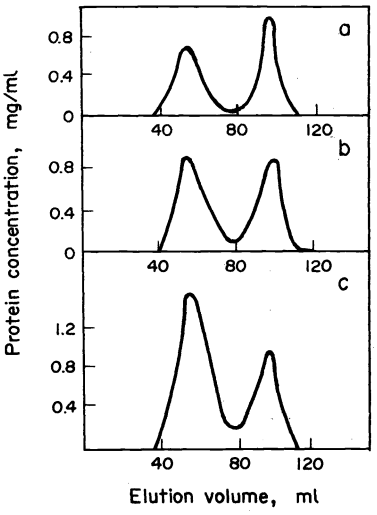

Fig.18. Elution curves for the reacting system RNase-dextran-sulphate (DS) (MM 60000, n = 0.8). (a) U=4.5 ml/hr, (b) U= 9 ml/hr; (c) U = 18 ml/hr.

multaneous break of several intermolecular bonds during dissociation.

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