POROUS COPOLYMERS OF METHACRYLIC ACID WITH N-(2-HYDROXYPROPYL) METHACRYLAMIDE AND (2-HYDROXYETHYL) METHACRYLATE STUDY OF SORPTION PROPERTIES*

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Porous crosslinked copolymers of methacrylic acid with N-(2-hydroxypropyl) methacrylamide (HPMA) or 2-(hydroxyethyl) methacrylate were prepared, and the relationship between their structure and the sorption of papain, bovine serumalbumin, chymotrypsinogen, pepsin, ovalbumin, insulin, novocain and oleandomycin were investigated. The presence of hydrophilic components in the gel structure makes possible additional interactions between sorbent and the compound sorbed. The occurrence of additional interactions (probably hydrogen bonds) is more pronounced with cation exchangers containing the (2-hydroxyethyl) methacrylate monomeric unit, which favourably affects the sorption of univalent organic cations but at the same time contributes to the denaturation of sorbed proteins. In contrast to univalent organic cations, cation exchangers containing the N-(2-hydroxypropyl) methacrylamide monomeric unit are more advantageous in the sorption of proteins, because due to the lower extent of additional interactions no irreversible denaturation of sorbed labile proteins takes place in this case.

In our earlier paper we reported the synthesis and structure of porous copolymers of methacrylic acid with N-(2-hydroxypropyl) methacrylamide or (2-hydroxyethyl) methacrylate, using 1,3,5-triacryloylhexahydrotriazine as the crosslinking component[1]. The potentiometric properties of these copolymers have been described earlier[2,3]. The copolymers may be used as selective cation exchangers, suitable for the separation of physiologically active compounds. Regularities controlling the sorption of proteins on porous carboxylic cation exchangers have been investigated in detail on the cation exchanger Biokarb (copolymer of methacrylic acid with 1,3,5-triacryloylhexahydrotriazine) prepared by the crosslinking precipitation polymerization[4-7]. The presence of hydrophilic units in the structure of the sorbent affects both the morphology of the resulting copolymer[1] and its sorptional properties by enabling supplementary bonds (*e.g.*, hydrogen bonds) to be formed between sorbent and the compound sorbed.

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This paper reports an investigation of the sorption of papain, bovine serumalbumin, chymotrypsinogen, pepsin, ovalbumin, insulin, novocain and oleandomycin on copolymers of methacrylic acid with N-(2-hydroxypropyl) methacrylamide or (2-hydroxyethyl) methacrylate in order to determine the effect of the content of the hydrophilic component on the sorptional properties of copolymers under study.

EXPERIMENTAL

Methods and Chemicals Used

The copolymers were prepared in the same way as in ref.[1]; their composition is given in Table I. The following chemicals were used throughout the work: papain manufactured by Merck, bovine serumalbumin, chymotrypsinogen manufactured by Reanal, bovine pepsin crystallized three times and crystalline ovalbumin manufactured by Scyuzreachim, insulin (24·3 IU/mg of protein), protease-terrilytin (the proteolytic activity was 50 caseine units/mg protein), oleandomycin phosphate (further called oleandomycin, a macrolide antibiotic having the activity 780 IU/mg), haemoglobin obtained according to Antoni[8], novocain hydrochloride (further called novocain) precipitated three times from methanol, all manufactured in USSR.

The concentrations of insulin and serumalbumin were determined by an evaluation of the optical density of solutions at 280 nm; the other proteins were determined according to Lowry[9], and the concentration of haemoglobin was ascertained by using its optical density at 542 nm. The proteolytic activity of pepsin, papain and terrilytin was determined according to Anson[10], using a 1% solution of casein as the substrate at the optimal pH for the given protease. The concentration of novocain was determined by using the optical density at 262 nm. A method described earlier was employed in the determination of the concentration of oleandomycin by means of the optical density of products of acid hydrolysis at 360 nm[11].

Static Arrangement of Sorption

Cation exchangers in the H-form, in equilibrium with the air moisture, grain size 0·1—0·2 mm were used in the experiments. The composition and properties of copolymers under investigation are given in Table I. The proteins were sorbed at room temperature at constant pH (0·01M phosphate buffer) and at constant ionic strength. Weighed amounts of sorbent were 20—25 mg, the volume of protein solution was 10 ml. To suppress the possibility of a cooperative bond between protein and polymer, the sorption properties of the cation exchanger were studied at low concentration of proteins (0·5—1·0 mg/ml). The time needed for the establishment of equilibrium in the static arrangement was 30 h. The sorption capacity per unit weight of the copolymer was determined from the difference between the initial and final protein concentration in solution; the distribution coefficient k_i was calculated using the specific volume.

Dynamical Arrangement of Sorption

The sorption capacity at a constant concentration of the sorbed compound in solution (1 mg/ml) was determined in the column arrangement at the ionic strength of solution 0·05N. A column 1 cm in diameter, was filled with 200—250 mg of the copolymer which was washed with a solution at the rate 30 ml h^{-1} cm^{-2}. The amount of sorbed protein was determined from the concentration difference at the inlet and outlet of the column. After that, the column was washed with a 0·01 *m* buffer solution at the same pH which was used in the sorption. Washing was completed

when the solution leaving the column did not contain any protein. The total amount of protein obtained by washing with the buffer was regarded as molecular sorption of the protein. The protein was desorbed by changing pH of the washing solution: for pepsin, serumalbumin and hemoglobin it was a 0·1M phosphate buffer (pH 7·4), for the other proteins the buffer used was 0·1M-Na_2HPO_4. The amount of desorbed protein was measured; for pepsin, papain and terrilytin, the proteolytic activity was also determined.

Novocain and oleandomycin were desorbed in 0·01M-HCl. The concentration of the organic cation in eluates was determined either photometrically or by means of a Na-electrode after neutralization.

THEORETICAL BACKGROUND

Three physicochemical features should be pointed out in the systems under investigation, namely: *1*) High dispersity of the polymer phase[1]. *2*) Different ratios of the hydrophilic and ionogenic components in the polymers taken for investigation. *3*) Low degree of ionization of carboxylic groups[3]. All these factors appear in the study of the sorption of physiologically active compounds.

The total free energy of a high-disperse system involves the surface energy of the system, which in a general case depends on two radii of the curve of the interface. In the systems studied by us, the interfacial surface area is fixed by covalent crosslinking[1,2], so that the cation exchangers cannot reduce their free energy in further processes by reducing surface areas which separates two phases. On the contrary, in the swelling of microglobules of a heterogeneous polyelectrolyte in water, the specific surface area somewhat increases compared with the dry state. In such systems free energy can be reduced only if the surface tension is reduced too. The decrease in surface tension

TABLE I

Characteristics of Copolymers of Methacrylic Acid (*I*), N-(2-Hydroxypropyl) Methacrylamide[a] (*II*) and (2-Hydroxyethyl) Methacrylate (*III*).

Concentrations of the components of the initial monomeric mixture are given; the other symbols are defined in the Experimental.

Sample No	[*I*]	[*II*]	[*III*]	V_0	V_w	*N*	σ
	mol.%			ml/g		mequiv. ml^{-1}	nm^{-2}
1	10	90	—	1·49	9·7	0·18	0·35
2	30	70	—	1·51	14·8	0·22	0·4
3	50	50	—	4·34	15·2	0·39	1·2
4	75	25	—	3·12	7·5	0·89	1·66
5	10	—	90	4·34	9·6	0·16	0·65
6	30	—	70	4·34	8·3	0·33	1·08
7	50	—	50	5·49	10·7	0·41	1·44
8	75	—	25	4·34	8·7	0·77	1·87
9[b]	100	—	—	2·4	5·5	1·8	2·16

[a] In all copolymers 1,3,5-triacryloylhexahydrotriazine was used as the crosslinking agent (4 mol.% calculated per the monomeric mixture). [b] Biokarb.

may be due to *a*) a change in the ionization on the copolymer surface, *b*) sorption of surface-active compounds. The effect of these two parameters is the cause underlying the higher selectivity of polyelectrolytes towards surface-active compounds containing ionogenic groups.

Katchalsky[13] has demonstrated experimentally that the surface activity of polymethacrylic acid in solution varies linearly with the degree of ionization (α). It may be assumed that in cross-linked copolymers of methacrylic acid the surface energy also decreases with increasing α. At the same time, however, it should be borne in mind that carboxylic groups on the surface of microglobules may have a degree of ionization different from those inside them[14].

It has been shown in an earlier paper by Glückauf[15] that the selectivity of ion exchange is predominantly determined by the change in the electrostatic contribution of free energy of the system. Gregor and coworkers[16] calculated the selectivity coefficient for counterions of various sizes assuming that the linear chain forms a cylinder of certain diameter with constant charge density along the axis. They proved experimentally the correctness of their calculations at a low degree of packing of the ion exchanger and demonstrated that the selectivity coefficient increased with the squared radius of the counterion and with charge density along the axis. The selectivity coefficient of ion exchange was calculated from the expression

$$K = \frac{m_1 \cdot c_2}{m_2 \cdot c_1}, \qquad (1)$$

where m_1, m_2 are the macroscopic concentrations of organic counterions and Na^+ in the ion exchanger respectively, c_1, c_2 are the respective concentration of organic and Na^+ ions involved in the exchange in an equilibrium solution.

In the potentiometric titration of carboxylic ion exchangers with triethylbenzylammonium hydroxide we found that the distribution coefficient of this ion increased proportionately to the volume density of carboxylic groups in the polymer[3], which confirms the correctness of Gregor's model for univalent counterions. The model can be used in heterogeneous structures with some restrictions. Such structures are formed in the process of precipitation crosslinking copolymerization, if phase separation occurs in the system during the copolymerization as a result of the fact that the interaction parameter polymer–solvent exceeds the critical limit. This metastable state is fixed by a high number of crosslinks, which prevent the chains from decoiling. For this reason, the model of extended polyelectrolyte chain, similarly to that of the random coil, is not suited for the description of ionization in these systems. Since the sorption of proteins occurs on the surface of microglobules of heterogeneous cation exchangers, we examined the effect of surface density of carboxylic groups on the solution–polymer interface. An approximate estimate of σ (density of ionogenic groups per surface unit) may be made similarly to the calculation of the volume fraction for an arbitrary component in micelles formed in the precipitation polymerization[17], assuming that all microglobules which form the disperse cation exchanger are homogeneous hard globules. The concentration of carboxylic groups in each microglobule is $m \cdot \varrho$, where m is the content of methacrylic acid in one gram of dry polymer (mol/g) and ϱ is the density of homogeneous copolymer in g/cm^3. Assuming that the form and arrangement of microglobules in the cation exchanger do not change on swelling, one may assume that by swelling from V_0 to V_w the size of each microglobule increases V_w/V_0 times. In such case the volume concentration of carboxylic groups in the swollen microglobule is given by

$$M = m \cdot \varrho \cdot V_0/V_w, \qquad (2)$$

where V_0, V_w are the bulk volumes of the dry and swollen cation exchanger respectively (in ml/g).

The average statistical distance between carboxylic groups in the globule is $(M)^{1/3}$ and the respective density of ionogenic groups per surface unit of microglobules is given by

$$\sigma = \left(\frac{N_A}{10^{24}} . N . V_0 \right)^{2/3} , \qquad (3)$$

where N_A is the Avogadro number, N is the average concentration of carboxylic groups in the swollen porous cation exchanger (mequiv./ml), and σ is the number of carboxylic groups per surface unit. In this paper, σ is used as a universal characteristic of the sorbent allowing us to compare the sorption properties of various cation exchangers.

The sorption of proteins depends on the degree of ionization of the polyelectrolyte and on the total charge and its distribution in the protein macromolecule. For this reason, interactions between protein and polymeric matrix depend on pH of the surrounding solution. For each protein there exists an optimal pH of solution at which the capacity and selectivity of sorption are the highest. For proteins having their isoelectric point lower than pK_{char} of the cation exchanger ($pI < pK_{char}$), the optimal pH lies in the region of the isoelectric point of the protein[5,18]. The sorption isotherms of proteins at constant pH in solution usually have the form of Langmuir's isotherm[5]. At small concentrations of protein in the sorbent the sorption capacity is linear-dependent on protein concentration in the equilibrium solution. In such case the selectivity of sorption may be characterized by the distribution coefficient:

$$k_i = m_i / c_i . \qquad (4)$$

The distribution coefficient characterizes the total selectivity of sorption, which includes not only the ion exchange sorption, but also additional interactions between sorbent and sorbate. A special feature of copolymers investigated by us consists in that the ratio of ionogenic to hydrophilic units in the polymeric matrix changes during sorption. The nonionogenic component of the monomeric unit, N-(2-hydroxypropyl) methacrylamide or (2-hydroxyethyl) methacrylate, may form additional hydrogen bonds, which in many cases raises the selectivity of sorption.

RESULTS AND DISCUSSION

The sorption capacities of cation exchangers under investigation for proteins having different molecular weight and electrochemical properties are summarized in Table II. pH of solution from which sorption was carried out is given for each protein. One can see that independently of the character of the hydrophilic comonomer, the sorption capacity of proteins increases with increasing concentration of methacrylic acid in the initial copolymerization mixture (Table I). This dependence allows us to assume that the sorption of proteins on carboxylic cation exchangers is due to interionic or ion-dipole attractive forces. Figs 1 and 2 show the dependence of the distribution coefficients of proteins (k_i) on the volume concentration of carboxylic groups in the copolymer swollen to equilibrium (N).

For all the proteins under investigation, differences were observed in the sorption properties of the copolymer methacrylic acid (I) –N-(2-hydroxypropyl) methacryl-

TABLE II

Sorption Capacities of Copolymers (in mg/g) for Proteins

Sample[a] No	Pepsin pH 3·0	Insulin pH 4·2	Serumalbumin pH 5·6	Chymotrypsinogen pH 6·0	Haemoglobin pH 6·7	Papain pH 7·55
1	—	36·9	13	3	—	—
2	51·3	88·6	22	16	132	90
3	47·7	29·7	16	15	177	148
4	49·4	41·6	123	41	199	187
5	44·6	16·6	21	9	128	80
6	44·6	21·0	24·5	11	104	112
7	93·2	40	38	31	218	189
8	109·4	43·7	45	47	141	193
9	182	435	160	91	212	413

[a] Composition of copolymers *cf.* Table I.

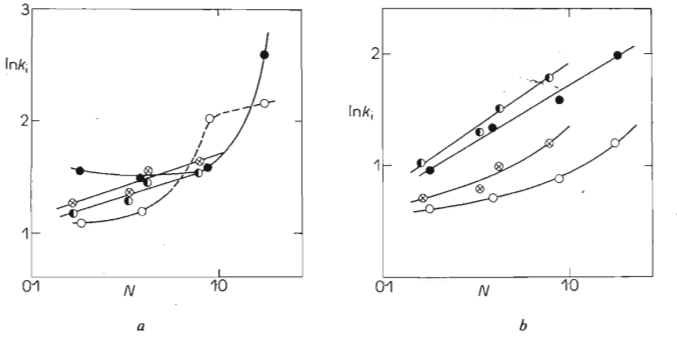

FIG. 1

Dependence of the Distribution Coefficient (k_i) of *a* Insulin (◐, ●) and Serumalbumin (⊗, ○) on the Volume Concentration of Carboxylic Groups (N, mequiv/ml) in the Copolymers of MMA with HEMA (◐, ⊗) and in the Copolymers of MMA with HPMA (●, ○)

b) Papain (◐, ●) and Pepsin (⊗, ○) on the Volume Concentration of Carboxylic Groups (N, mequiv/ml) in the Copolymers of MMA with HEMA (◐, ⊗) and in the Copolymers of MMA with HPMA (●, ○)

amide (*II*) and the copolymer methacrylic acid – (2-hydroxyethyl) methacrylate (*III*). In the case of sorption of insulin (Fig. 1), the selectivity for copolymers *I* + *II* increases starting only from 50 mol.% of methacrylic acid in the initial mixture of monomers, while for copolymers *I* + *III* it increases monotonically over the whole range of gel composition. We have shown in an earlier work[1], that in the range of low concentrations of *I* copolymers *I* + *II* possess a lower porosity (heterogeneity) compared with copolymers *I* + *III*. On the other hand, no relationship could be detected between the heterogeneity[1] and size of proteins sorbed. It may be assumed, therefore, that heterogeneity is more important with respect to the sorption surface than with respect to the permeability for proteins. In order to compare the sorption capacities of copolymers having different porous structure expressed through (V_0), we used calculated values of the surface density of carboxylic groups (σ). Fig. 3 shows the dependence of k_i on σ for chymotrypsinogen, papain and pepsin. It is obvious that in the chosen coordinates the binding energy of the protein with the polyelectrolyte is virtually linear dependent on the surface density of carboxylic groups, and that the slope is independent of the character of the hydrophilic comonomer.

In addition to electrostatic interactions occurring in the sorption of biologically active compounds, an important role is also played by additional interactions between

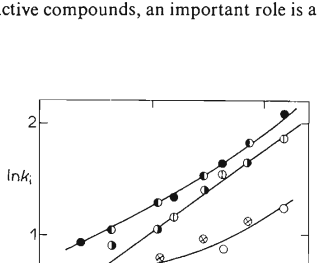

FIG. 2

Dependence of the Distribution Coefficient (k_i) of Papain (◐, ●), Pepsin (◑, ○) and Chymotrypsinogen (◑, ⊗) on the Surface Density of Carboxylic Groups (σ) in the Copolymers of MAA with HEMA (◐, ◑, ◑) and in the Copolymers of MAA with HPMA (●, ○, ⊗)

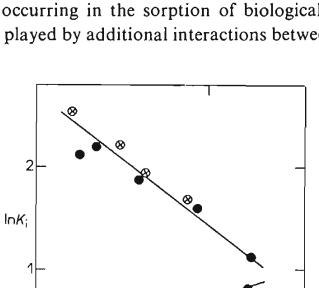

FIG. 3

Dependence of the Selectivity Coefficient of Ion Exchange (k_i) of Novocain (●) and Oleandomycin (⊗) on the Volume Concentration of Carboxylic Groups (N) in the Copolymers of MAA with HEMA (⊗) and in the Copolymers of MMA with HPMA (●)

sorbent and the compound sorbed[19-21]. In this reason, washing of the sorbent with water or diluted buffer without any change in pH may cause desorption of the protein thus sorbed. For papain, pepsin, insulin and chymotrypsinogen, molecular sorption was not higher than 5–10% of the total sorption capacity. For ovalbumin, molecular sorption was distinctly higher. Table III shows a comparison between the total and molecular sorption for ovalbumin and haemoglobin on copolymers *I* + *II*. In these systems molecular sorption is proportional to the content of monomers of the N-(2-hydroxypropyl) methacrylamide unit in the copolymer, but has no important influence on the distribution coefficient.

In the case of the sorption of univalent cations, additional interactions may considerably raise sorption selectivity[21]. Results of the sorption of oleandomycin and novocain summarized in Table IV indicate that the distribution coefficient, k_i, and the coefficient of selectivity of the ion exchange, K_i, of oleandomycin increase with increasing concentration of the hydrophilic monomer in the initial mixture. This influence is stronger with copolymers *I* + *III* than with copolymers *I* + *II*. The molecule of oleandomycin contains several hydroxylic groups able to form hydrogen bonds. As has been shown earlier[11] in the case of the polycondensate based on phenoxyacetic acid, the sorption selectivity of oleandomycin increases depending on the content of phenol or resorcin. As can be seen in Fig. 4, the incorporation of monomeric units of (2-hydroxyethyl) methacrylate or N-(2-hydroxypropyl) methacrylamide into the polymeric structure of these sorbents leads to similar conclusions.

In the case of sorption of novocain, no hydrogen bonds between the sorbent and sorbate can be formed; neither are there any conditions permitting the formation of hydrophobic bonds[19]. For this reason, the sorption of novocain can be explained by electrostatic interactions.

TABLE III

Sorption Capacity (*A*, mg/g), Molecular Sorption (*B*, mg/g) and Distribution Coefficient (k_i) of Ovoalbumin and Haemoglobin on Copolymers of N-(2-Hydroxypropyl) Methacrylamide

Sample[a] No	Ovoalbumin			Haemoglobin		
	A	*B*	k_i	*A*	*B*	k_i
1	148	84	8·0	—	—	—
2	233	50	8·9	132	36	35·4
3	179	19	6·9	177	36	55·4
4	98	9	7·9	199	2	48·0
9	277	0	23·2	212	2	33·7

[a] Composition of copolymers *cf.* Table I.

The individual sensitivity of sorbed compound towards the intensity of additional interactions is very important for the preservation of the native conformation of physiologically active compound, especially as regards proteins with labile structure[7]. For all the sorbents investigated in this work, quantitative desorption of pepsin and papain was observed without any loss of enzymatic activity. In the case of terrilytin the preservation of enzymatic activity depended on the type of the hydrophilic comonomer in the sorbent.

TABLE IV

Sorption Capacity (A, mg/g), Distribution Coefficient (k_i) and Ion Selectivity Coefficient (K_i) of Oleandomycin and Novocain on Copolymers of Methacrylic Acid (I), with N-(2-Hydroxypropyl) Methacrylamide (II) or (2-Hydroxyethyl) Methacrylate (III)

C content of nonionogenic monomers in the initial monomeric mixture.

C	Oleandomycin, 0·005 NaCl						Novocain 0·05 NaCl	
mol.%	$II + I$			$III + I$			$II + I$	
	A	k_i	K_i	A	k_i	K_i	A	k_i
0	24	40	14				73	6·4
25	120	97	42	156	120	48	87	5·0
50	174	135	76	198	150	92	84·6	2·4
70	101	84	179	210	120	200	88	2·6
90	90	71	137	261	200	380	52	2·2

TABLE V

Effect of Sorption and Desorption on the Proteolytic Activity of Terrilytin

A sorption capacity (mg/g), C content of nonionogenic monomers in the initial monomeric mixture (mol.%), D proteolytic activity after desorption (%).

	Copolymers $I + II$		Copolymers $I + III$	
C	A	D	A	D
0	206	100		
25	35	89	85	94
50	20	100	41	38
70	9·8	100	73	23
90	6·3	97·7	59	6

The results summarized in Table V show that the incorporation of *III* into the copolymer structure has as a consequence reduction of the proteolytic activity of terrilytin in the process of sorption and desorption. The introduction of units of *II* into the copolymer structure (in spite of decreasing ion capacity) does not exhibit any drop in the proteolytic activity in both processes. It is known that in the neutral pH range protease terrilytin[22,23] in the native form is present by some 45% in the α-helix conformation. With a change in pH of solution, the conformation also changes and the proteolytic activity decreases. Since in the molecule of terrilytin there are no S–S bonds which would stabilize the structure of the macromolecule, the enzyme loses its proteolytic activity in an irreversible manner. It is known that the α-helix conformation of the macromolecule is stabilized by intermolecular hydrogen bonds. A change in this conformation may take place, if the macromolecule participates in other stronger intermolecular bonds. Our results justify us to assume that in the case of copolymer $I + III$, stronger bonds are formed between the polymer and protein than in the case of copolymer $I + II$.

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