PARTITION MECHANISM OF ADSORPTION AND THE ABSENCE OF DISPLACEMENT PHENOMENA IN THE ZONAL ANALYTICAL CHROMATOGRAPHY OF PROTEINS ON BEAD 2-HYDROXY-3-PHENOXYPROPYL-CELLULOSE

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Hydrophobization of bead cellulose is described, carried out by its alkylation with 1,2-epoxy-3--phenoxypropane under the conditions of acid (perchloric acid, borontrifluoride diethyl etherate) and basic (sodium hydroxide) catalysis. Reaction conditions (temperature, reaction time, molar ratio of reactants) have been determined, allowing the hydrophobization of bead cellulose to be carried out to the largest possible extent while maintaining its spherical shape. The nonstoichiometric mechanism suggested for the adsorption of amphiphilic adsorptives on bead 2-hydroxy--3-phenoxypropyl-cellulose (HPP-C) was checked by means of adsorption of six proteins. It was found that the surface of the hydrophobic segment of the adsorbent must be sufficiently large to be able to come in touch with the hydrophobic region of the protein through its multiple residues. In such cases the partitioning of the protein between the hydrophobic segment present as a liquid-like film and the surrounding solution becomes the predominant step of the adsorption. This adsorption mechanism is also reflected in zonal chromatography on bead HPP-C, as no displacement phenomena could be observed in any of the six proteins used. Retention of these proteins has been affected to a decisive extent by the degree of hydrophobization of HPP-C.

The adsorption of amphiphilic solutes on derivatives of bead cellulose has been described in terms of a time-concentration dependence[1,2]. From this dependence, a criterion has been derived, by using which it was possible to distinguish the stoichiometric adsorption from the nonstoichiometric one[3]. The latter type of adsorption became operative in those cases in particular where the adsorbent was either bead 2-hydroxy-3-phenoxypropyl-cellulose[3,4](HPP-C) or 2,3-dihydroxypropyl--(2-hydroxy-3-phenoxy) propyl-cellulose[5]. In such case of adsorption, the partition coefficient remained constant independently of increasing concentration of adsorptive, and adsorption itself proceeded as partitioning of the solute in the liquid–

-liquid system. The hydrophobic substituent, i.e., 2-hydroxy-3-phenoxypropyl group (HPP), should form a binary mixture with the surrounding water, coating the bead cellulose. This binary mixture should possess properties of a liquid-like film and behave as a stationary liquid phase. Amphiphilic adsorptives, e.g., a derivative of phenothiazine (perphenazine) or proteins, would then be adsorbed on bead HPP-C by a mechanism in which partitioning should be the predominant step. Experimental results support this view[6]. Due to the hydrophobic character of the substituent (HPP-group) and amphiphilicity of the adsorptives, adsorption was denoted as hydrophobic. A theoretical investigation of adsorption processes in both analytical and preparative liquid chromatography, including hydrophobic interaction, has been made. Hydrophobic interaction chromatography (HIC) did not follow the stoichiometric displacement model of retention[7].

Up to now, only a single example of application of bead HPP-C in the isolation of proteins has been reported in the literature[8], but the chromatographic separation of calmodulin has not been achieved by using displacing agents typical of HIC (ref.[7]). The objective of this study consisted therefore in an experimental verification of the behaviour of proteins in zonal chromatography on bead HPP-C under conditions where aqueous solutions of solvents (ethyleneglycol) or simple displacing agents (aqueous solutions of salts having various salting-out power), or combined displacing agents, i.e., aqueous solutions of salts containing a competitive macroligand – HPP-poly(oxyethylene) (HPP-POE) were used. In addition, we also intended to elucidate the relations between the parameters of hydrophobic adsorption and hydrophobic zonal chromatography on bead HHP-cellulose of proteins with hydrophobicity defined previously. Further, to check also in HIC the hypothesis of partition mechanism, verified for the case of hydrophobic adsorption[6].

EXPERIMENTAL

Materials

Bead porous cellulose "Perloza" (North-Bohemian Chemical Works, Lovosice) had the following parameters: particle size <250 μm, water regain 4·60 (g H_2O/g dry material). Bead 2-hydroxyethyl-cellulose (water regain 3·65) was obtained by the alkylation of bead cellulose with 2-chloroethanol[1]. 1,2-Epoxy-3-phenoxypropane (EPP) was used either as commercial, practical grade (Serva, Heidelberg), or it was prepared by a reaction of 1-chloro-2,3-epoxypropane with phenol followed by the dehydrochlorination with sodium hydroxide[9], the product was redistilled at reduced pressure (b.p. 113–115 °C/1·995 kPa; 120 C 2·66 kPa): UV spectrum (methanol): 272 ($\log \varepsilon = 3{\cdot}24$).

Proteins. Trypsin (EC 3.4.21.4), from bovine pancreas, crystalline, 0·8 TU^{cas}/mg (Spofa, Prague); α-chymotrypsin (EC 3.4.21.1) from bovine pancreas, crystallized three times, freed from salts and lyophilized (Type II), 25– 32 U/mg (Sigma, St. Louis); lysozyme (mucopeptide N-acetalmuramoylhydrolase, EC 3.2.1.17), from chicken egg white, crystallized twice, freed from salts and lyophilized (ICN Pharmaceuticals, Cleveland); bovine serumalbumin, lyophilized

(SEVAC, Prague); immunoglobulin G, human normal ("Norga"), stabilized with glycine (Imuna, Šarišské Michal'any). Ovalbumin was prepared by the precipitation of egg albumin flake (BDH, Poole) with ammonium sulfate[10], followed by dialysis and lyophilization. In some experiments, bovine albumin essentially fatty acid-free was used (Sigma).

Hydrophobization of bead cellulose. Bead 2-hydroxy-3-phenoxypropyl-cellulose (HPP-C) was prepared via alkylation of bead cellulose with 1,2-epoxy-phenoxypropane by both acid (perchloric acid, borontrifluoride diethyl etherate) or base (sodium hydroxide) catalysis. The alkylation catalyzed with perchloric acid was performed by employing a procedure reported for the alkylation with 1-chloro-2,3-epoxypropane[11]. The alkylation of bead cellulose or 2-hydroxyethyl-cellulose catalyzed with borontrifluoride diethyl etherate was conducted either without solvent, or in diethyl ether or 1,4-dioxan[12], each time at room temperature and with an equimolar content of the reagents (related to the glucose unit). In the base catalyzed alkylation bead cellulose (10·0 g) sucked from water by filtration was resuspended in 1,4-dioxan (10 ml), then activated at room temperature for one hour with 10% NaOH (157 μl), an alkylating agent (1·30 g) was added, and the suspension was stirred at 90°C for 5 h. The product (14·1 ml) was washed with ethanol and water.

The degree of substitution (D_s) of modified cellulose was determined spectrophotometrically[4].

2-Hydroxy-3-phenoxypropyl-poly(oxyethylene) (HPP-POE) was prepared similarly to HPP-C, under conditions of catalysis with perchloric acid. Poly(oxyethylene) (POE), a commercial product polyethyleneglycol 4000 made by Lachema, Brno, was alkylated at 60°C for 2 h and at the molar ratio EPP/hydroxylic groups POE 0·26 : 1. The reaction was stopped after twofold extraction with chloroform (1 : 1, v/v), the joined chloroform extracts were dried over anhydrous Na_2SO_4. The filtrate was pre-evaporated in vacuo and then evaporated to dryness after repeated resuspension in acetone. The sirupy residue was dissolved in a minimum volume of acetone, and the product was precipitated by adding diethyl ether. Removing the ether the white sticky residue was dissolved in an acetone – chloroform mixture (8 : 1, v/v). Then again ether was added gradually (three to four times the volume of the acetone – chloroform mixture) until all the product was precipitated. After removal of the ether and drying in vacuo the weight of the product was 70—80% of POE used. The content of HPP-groups in one gram of the dry product (432·3 μmol/g) was determined spectrophotometrically and calculated from the value ε_{270} 1 723 $l\,ml^{-1}\,cm^{-1}$ (methanol, water).

Adsorption of Proteins on Bead HPP-C

Adsorption was measured by the batch procedure at room temperature. Suspensions containing HPP-C were freed from water by sucking (0·5—100 mg of dry HPP-C) and the corresponding protein at a concentration 100—600 μg/ml in 0·1M-phosphate buffer pH 7·0 with 1M-NaCl were vigorously stirred. An aliquot part of the suspension (c. 1 ml) was filtered in time intervals 5, 15, 30, 60, 120, 180 and 240 min, and the protein concentration was determined in 0·5 ml of the filtrate[13]. Purity of the proteins applied was checked spectrophotometrically, by using values of the absorbance coefficients reported in the literature[14].

Experimental results were treated by employing two procedures.

Method A: Nonlinear regression of the equation[1,2]

$$B = c_0 t/(c_0 t/B'_e + t c_B/B'_e + c_0/v_0 + c_v/v_0)\,, \qquad (1)$$

where B is the amount of the adsorbate at a time t, c_0 is the starting protein (adsorptive) concentration in solution, B_e is the amount of the adsorbate at $t \to \infty$ from a solution of the adsorptive

having $c_0 \to \infty$, c_B and c_v are the concentrations of the adsorptive needed for $B_e = B'_e/2$ or $v_0 = v'_e/2$ respectively, with B_e being the amount of the adsorbate at $t \to \infty$, and v_0, v'_0 respectively are the initial rates of adsorption from solutions of the adsorptive having a real or infinitely high c_0. The equilibrium partition coefficient p_e was calculated from

$$p = p_e t/(t + t_p), \tag{2}$$

where p is B/c_t, c_t being the concentration of the adsorptive in solution at a time t; p_e is defined under equilibrium conditions by the relation $p_e = B_e/(c - c_C B_e)$, with c, c_C being the respective concentrations of the protein and adsorbent[4] in the system (bulk concentrations; $c = c_0$ at $t = 0$); t_p is the time interval, when $p = p_e/2$, defined by $t_p = ct_{0.5}/(c - c_C B_e)$, $t_{0.5}$ being the half-time of adsorption.

Method B: Graphic and linear regression, B_e being determined graphically from the c vs t dependence; the partition coefficient p_e is then calculated by linear regression as the slope of the B_e vs c_e dependence, with c_e being the concentration of the adsorptive in the state of equilibrium.

Zonal Chromatography of Proteins

The experiments were performed in columns 1·2 × 11·3 cm and 2·8 × 13·2 cm packed with bead HPP-C equilibrated by means either of 0·1M phosphate buffer pH 7·0 containing 1M-NaCl (simple displacing agent) or of 0·1M phosphate buffer pH 7·0 containing 1M-NaCl and 1 mM--HPP-POE (combined displacing agent, 2·36 mg polymer/ml). The amount of protein applied in the smaller column was 2 mg/0·5 ml of the simple displacing agent, flow rate 30 ml/h; 1 ml fractions were collected. The protein concentration in the eluate was determined colorimetrically[13]. In larger column, 10 mg of protein dissolved in 5 ml of the simple agent was applied, and 10 ml fractions were collected at the same flow rate. When using the competitive macroligand, i.e. the combined displacing agent for the elution, 2 ml of the obtained eluates were shaken several times with chloroform (4 ml). Thereafter the layers were separated at 4°C, and the protein concentration in the upper layer was determined[13].

Two types of gradient elution were also used, viz., a continuous NaCl gradient ($0 \to 1$ mol l^{-1}) in a 0·1M phosphate buffer pH 7·0, and a discontinuous gradient of ethyleneglycol in water ($0 \to 60$ vol.%), with the concentration step 20%.

The available distribution coefficient K_{av} was determined by the usual procedure, the void volume V_0 (ml) was determined by means of dextran[15] having the relative average molecular weight M_m approx. $2 \cdot 10^6$ (Sigma, St Louis).

RESULTS AND DISCUSSION

Hydrophobization of Bead Cellulose and Adsorption of Proteins

2-Hydroxy-3-phenoxypropyl-cellulose (HPP-C) was obtained by reacting cellulose (the bead type) with 1,2-epoxy-3-phenoxypropane under various reaction conditions. The extent of introduction of the 2-hydroxy-3-phenoxypropyl group (molar degree of substitution, D_s; related to the glucose unit) depends on: the type of catalysis during alkylation, the ratio of the reaction components, the temperature and duration of the reaction (Table I). In both types of catalysis: acid ($HClO_4$, BF_3) and alkali (NaOH), comparable D_s values can be obtained. The reaction catalyzed with BF_3

proceeds satisfactorily already at room temperature. If the reaction is conducted above D_s 0·045, the spherical shape of the sample is altered or even destroyed. With bead 2-hydroxyethyl-cellulose used as the starting material in the reaction, it is assumed that the alkylation will bring about substitution of active hydrogen atoms of the hydroxyl group of the glucose unit and/or of the hydroxyethyl group (products *XI*, *XII*).

Of the two parameters which significantly affect the hydrophobic character of bead cellulose, i.e., the degree of substitution (D_s) and the length of the hydrophobic chain[6], this study is concerned with the effect of the former. The hydrophobic character of bead HPP-C was characterized either by using the partition coefficient, p_e, of one of the six proteins present in the system HPP-C – water, or by means of the amount of protein adsorbed at a time $t \to \infty$ (B_e). Being characterized by several

TABLE I

Alkylation of cellulose with 1,2-epoxy-3-phenoxypropane (EPP). Symbols: r_a molar ratio of EPP and glucose units, c_S content of 2-hydroxy-3-phenoxypropyl groups in 1 g of dry product, D_s molar degree of substitution

r_a	Solvent	Temperature °C	Time h	Product	Moisture content mass %	c_S μmol g^{-1}	$D_s \cdot 10^3$
			Reaction catalyzed with $HClO_4$				
0·1	1,4-dioxan	40	2	*I*	86·3	48	7·7
0·25	1,4-dioxan	60	2	*II*	86·6	72	11·7
0·25	1,4-dioxan	70	2	*III*	84·8	93	15·1
1·0	1,4-dioxan	80	2	*IV*	82·9	276	44·8
			Reaction catalyzed with BF_3				
1·0	none	room. temp.	0·5	*V*	75·0	173	28·9
1·0	diethylether	room temp.	0·5	*VI*	76·8	193	31·3
1·0	diethylether	room temp.	1	*VII*	75·5	244	39·6
1·0	diethylether	room temp.	2	*VIII*	76·6	255	41·3
1·0	diethylether	room temp.	2	*IX*	71·5	200	32·4
2·0	1,4-dioxan	room temp.	2	*X*	76·1	182	29·5
1·0[a]	none	room temp.	2	*XI*	64·1	541	87·7
1·0[a]	none	room temp.	2	*XII*	72·3	599	97·1
			Reaction catalyzed with NaOH				
0·15	1,4-dioxan	90	5	*XIII*	79·6	248	40.0

[a] Alkylation of 2-hydroxyethylcellulose.

parameters earlier[16,17], the applied proteins were selected according to their hydrophobicity.

In agreement with preceding results[4,6], there was a considerable influence of D_s on the B_e and p_e values. With decreasing D_s the values of both B_e (Fig. 1) and p_e decreased, too. The extent of influence of D_s depended on the hydrophobicity of the

TABLE II

Partition coefficients p_e of proteins in the system HPP-C IV–water. For computing methods see Experimental (correlation coefficient given in brackets)

Protein	p_e	
	method *A*	method *B*
Trypsin	82·7 ± 4·3	82·8 (0·990)
Chymotrypsin	172·3 ± 3·7	152·1 (0·994)
Lysozyme	188·0 ± 10·5	223·0 (0·963)
Ovalbumin	224·0 ± 11·3	150·4 (0·968)
Serumalbumin	538·0 ± 32·7	614·3 (0·952)
Immunoglobulin G	3 272·3 ± 209·3	3 736·8 (0·998)

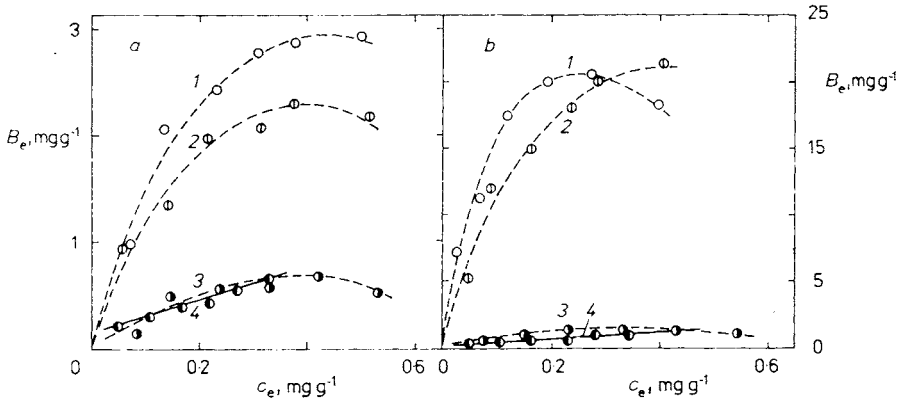

FIG. 1

Adsorption isotherms (broken lines) of proteins on (*a*) mixture of HPP-C I and unsubstituted cellulose (1 : 1, v/v; c_S = 20 μmol g^{-1}) and (*b*) HPP-C *II* (c_S = 72 μmol g^{-1}). Proteins (initial concentrations 100, 200, 300, 400, 500 and 600 mg l^{-1}): *1* immunoglobulin G, *2* serumalbumin, *3* ovalbumin, *4* trypsin. For 4 linear regression: (*a*) $B_e = 1{\cdot}53\, c_e + 0{\cdot}13$ (solid line), $r = 0{\cdot}990$; (*b*) $B_e = 2{\cdot}85\, c_e + 0{\cdot}19$ (solid line), $r = 0{\cdot}985$

protein for which the B_e or p_e values were measured. In Table II, the proteins used are arranged with respect to the p_e values determined by the method *A* in the HPP-C *IV*–water system. Hydrophobicity of the proteins (as characterized by p_e) affected the adsorption process on HPP-C *I*–*IV* so much as to cause a change in the adsorption mechanism. As can be seen in Table II, all the six proteins were adsorbed on most hydrophobic cellulose (HPP-C *IV*) via the partition mechanism. With decreasing c_S (276·1 → 93·4 → 72·4 → 27·0 μmol/g), the adsorption of hydrophobic proteins, especially of immunoglobulin G and serumalbumin, was accompanied by deviations of the partition mechanism, reflected in the nonlinear character of the B_e vs c_e dependence particular at higher concentrations of the adsorptive (Fig. 1). On the contrary, the least hydrophobic protein – trypsin – was adsorbed by the partition mechanism irrespective of the hydrophobicity or D_s of HPP-C. The corresponding dependences were linear, and the values p_e 2·85 and 1·53 determined respectively for the systems water – HPP-C *II* and water mixture of HPP-C *I* and unsubstituted bead cellulose ($c_S = 27\ \mu mol\ g^{-1}$) were statistically significant.

Zonal Chromatography of Proteins on HPP-C

The effect of hydrophobization of bead cellulose on zonal chromatography is illustrated in Fig. 2. The effect of D_s on the retention of protein is particularly evident. While in the case of the least hydrophobic protein – trypsin – the retention on HPP-C *IV* was 75%, the retention of all the other proteins with the exception of chymotrypsin (88%) was quantitative. A decrease in D_s resulted in a complete desorption of trypsin, not only from HPP-C *II* and *I* (Fig. 2*a*), but also from HPP-C *III*. The relation between the retention of trypsin, its hydrophobicity expressed through p_e and the hydrophobicity of HPP-C expressed through D_s could hold also for the other proteins. For instance, p_e ($\leqq 3$) could be a limiting value of the hydrophobicity of protein in the system HPP-C *III* – water, making possible its complete desorption. None of the other proteins could be completely desorbed from HPP-C *III*; immunoglobulin G and serumalbumin almost could not be desorbed at all.

The effect of hydrophobization of bead cellulose on the distribution coefficient, K_{av}, or the elution volume, V_0, of proteins was less important. Differences in the K_{av} values of proteins determined from the elution volumes of proteins on HPP-C with the lowest c_S value (a mixture of HPP-C and unsubstituted bead cellulose 1 : 1 v/v, $c_S \approx 25\ \mu mol\ g^{-1}$) and on unsubstituted bead cellulose were minimal, not exceeding 0·25 (immunoglobulin G). Fig. 2*b* shows the elution profiles of serumalbumin after zonal chromatography on the mixture HPP-C *I* and bead cellulose mentioned above. and of high-molecular weight dextran on bead cellulose. A similar ΔK_{av} values ($c \sim 0{\cdot}1$) was determined from the elution profiles of trypsin after zonal chromatography on bead celluloses just mentioned. This means, of course, that hydrophobization of bead cellulose reduced the separation efficiency of proteins reached by the

size-exclusion chromatography on bead cellulose[18,19]. A positive influence on the separation efficiency of HIC proteins may have the salting out effect of salt[20]. With such salts as sodium chloride, ammonium sulfate, sodium sulfate and sodium citrate arranged in the order of increasing salting out effect, partial precipitation was observed in the case of the most hydrophobic proteins (serumalbumin, immunoglobulin G).

In order to test the absence of displacement phenomena, in zonal chromatography of hydrophobic proteins on HPP-C the experiments were performed in presence of a competitive ligand, namely, 2-hydroxy-3-phenoxypropyl-poly(oxyethylene) (HPP-POE). It was found, however, that even at its maximum concentration (10 mmol l^{-1}), representing an approximate solubility of HPP-POE in a 50 mM phosphate buffer pH 7·0, the proteins possessing the strongest affinity (immunoglobulin G, serumalbumin) to the low hydrophobized cellulose, i.e. HPP-C *II*, were not desorbed, or their retention did not decrease to any significant extent.

Another test in order to prove the absence of displacement phenomena was gradient elution. Gradient elutions with decreasing NaCl concentration (1 mol $l^{-1} \rightarrow 0$ mol l^{-1}) or increasing ethyleneglycol concentration (0–60 vol.%) were used. No

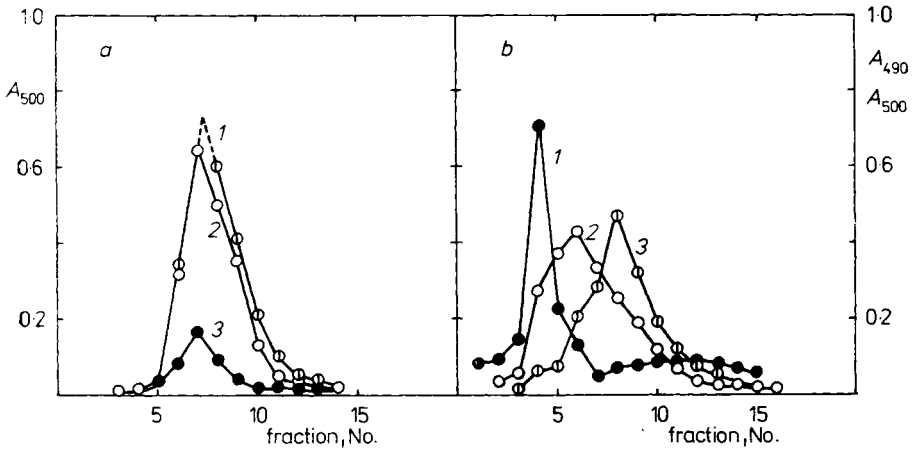

FIG. 2

Zonal chromatography of (*a*) trypsin (fraction 1 ml) and (*b*) serumalbumin and dextran (fraction 10 ml) on bead cellulose and its derivatives (eluent = 0·1M phosphate buffer pH 7·0 containing 1M-NaCl). Adsorbents: (*a*) *1* mixture HPP-C *I* and cellulose (1 : 1, v/v; c_S = 27 μmol g^{-1}), *2* cellulose, *3* HPP-C *IV*; (*b*) *1*, *2* cellulose (*1* in the elution of dextran), *3* mixture HPP-C and cellulose (1 : 1, v/v; c_S 25 μmol g^{-1}). Proteins were determined by using the Folin-phenol reagent[13] (A_{500}), dextran was determined by means of a phenolsulfuric reagent[15] (A_{490})

important influence on retention or on the elution profiles of proteins could be detected in any of them.

Correlation Analysis

Generally, the protein surface area involved in the adsorption process is no more than a few hundred square angströms. A small area on the surface of biological macromolecules generally determines the chromatographic behaviour in bioaffinity chromatography. In contrast, much larger regions of the surface determine chromatographic behaviour in most of the other modes of chromatography[21]. The surface area of protein in contact with the adsorbent's surface may even be proportionate to the overall surface area of the protein. This ensues from the semilogarithmic dependences $\log p_e$ vs $M_m^{1/3}$ or $\log p_e$ vs $M_m^{2/3}$ (Fig. 3). Exponential terms $M_m^{1/3}$ and $M_m^{2/3}$, (relative molar masses), should vary in parallel with the variation of the diameter or of the surface area of an equivalent hydrodynamic sphere. Assuming that all the proteins under comparison have the same geometrical (globular) shape (specific volume), the same isoelectric point and the net charge[22], the previous relationship will be valid. These assumptions need not be satisfied in the case of lysozyme* which escapes correlation, and partly also with α-chymotrypsin, which reduces the proximity of correlations (r being 0·984 or 0·983). With the other four proteins the correlations are very close ($r = 0{\cdot}9994$ or $r = 0{\cdot}9927$); of these, trypsin is the protein which is adsorbed by the smallest surface. As such, to achieve complete

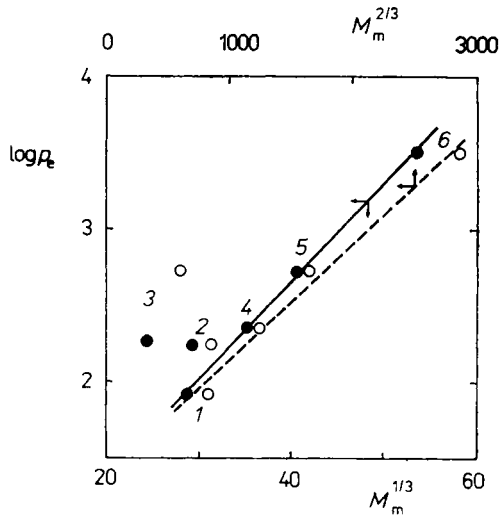

FIG. 3

Correlation of the partition coefficient of proteins ($\log p_e$) and relative molar mass of proteins ($M_m^{1/3}$ ●, $M_m^{2/3}$ ○). Proteins (coupled points): *1* trypsin, *2* α-chymotrypsin, *3* lysozyme, *4* ovalbumin, *5* serumalbumin, *6* immunoglobulin G. Linear regression for *1*, *4*, *5* and *6*: $\log p_e = 0{\cdot}0639\, M_m^{1/3} + 0{\cdot}09985$ (solid line), $r = 0{\cdot}9994$; $\log p_e = 0{\cdot}000755\, M_m^{2/3} + 1{\cdot}379$ (broken line), $r = 0{\cdot}9927$

* For lysozyme it was found that a relatively large portion of the surface determines the chromatographic behaviour[7].

contact it does not require a large surface of the adsorbent, i.e. a liquid-like film represented by the hydrophobic segment. So that all the HPP-C's prepared in this study, including those with a low D_s (of HPP-C *I*, *II*) adsorb trypsin via formally the same mechanism, i.e., the partition mechanism (Fig. 1). On the contrary, the surface area of the liquid-like film formed by the hydrophobic segment of low substituted celluloses (HPP-C *I*, *II*) is obviously too small to allow proteins with a larger surface area, such as ovalbumin, serumalbumin, immunoglobulin G, interact with it through multiple residues[21]. The adsorption of these three proteins on bead HPP-C having the c_S values $\leqq 90$ μmol/g did not proceed by the purely partition mechanism any more (Fig. 1). On the other hand, experiments with zonal chromatography revealed the complete absence of displacement phenomena, independently of both the protein and HPP-C used. A question still remains as to which adsorption and desorption isotherms were reflected by their superposition in the elution profiles obtained by the reported procedures. A model which satisfactorily describes the partition chromatography of proteins is not yet available, also due to the fact that protein can be folding and unfolding during the chromatographic process. Interconversion of conformers in the chromatographic system can also influence the peak shape. Thus, in HIC the tendency is to go to a broad peak in the equilibrium state[21].

It may be said, in conclusion, that in the analytical separation of proteins with different hydrophobicity by zonal chromatography on bead HPP-C, low-substituted HPP-C with $c_S < 50$ μmol/g must be used. The resolution ($\Delta K_{av} \leqq 0{\cdot}2$) and operational loading capacity of such hydrophobic adsorbents is low, however. On HPP-C ($c_S \geqq 90$ μmol/g) complete retention of strongly hydrophobic proteins can be achieved, with the recovery of less hydrophobic proteins being almost quantitative.

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