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SEPARATION OF DEHYDROGENASES ON POLYAMINOMETHYLSTYRENE

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SUMMARY

The binding of dehydrogenases, especially alcohol dehydrogenase, and other proteins to several ion exchangers and hydrophobic polymers was investigated. Quantitative parameters for the stability of the polymer–protein complexes (obtained from double reciprocal plots) indicate a high but different affinity of many proteins for polyaminomethylstyrene. The chromatography of a mixture of five dehydrogenases and human serum albumin on polyaminomethylstyrene is described.

INTRODUCTION

In recent years, the use of affinity chromatography for the purification of enzymes has received considerable attention[1,2]. While many workers have concentrated on adsorbents specific for only one macromolecule, work in other laboratories has been directed towards the development of adsorbents for the purification of groups of enzymes, *e.g.*, dehydrogenases[3]. Recently, purifications of enzymes on hydrocarbon-coated Sepharose, based on the general principle of hydrophobic interactions, have been reported[4–6]. It has been shown previously that the presence of a hydrocarbon extension ("arm") between a ligand and the Sepharose often has a dramatic effect on the binding capacity of a column in affinity chromatography[1,2,7,8]. This phenomenon was often considered to be a disadvantage. Concerning the covalent attachment of enzymes to water-insoluble polymers, some workers have postulated that hydrophobic polymers with hydrophobic groups in the polymer matrix have a destabilizing effect on the bound enzyme[9].

Because hydrophobic interactions are a universal and effective principle of binding, we have investigated the binding of several proteins to some hydrophobic polymers in order to show their applicability in enzyme purification.

MATERIALS AND METHODS

The following enzymes, substrates and polymers are used: yeast alcohol dehydrogenase (lyophilized), isocitrate dehydrogenase, 3-hydroxybutyrate dehydrogenase, ribonuclease, cytochrome *c*, NAD^+ and $NADP^+$ (Boehringer, Mannheim,

G.F.R.); lactate dehydrogenase, malate dehydrogenase and urease (VEB AWD, Dresden, G.D.R.); trypsin and catalase (Reanal, Budapest, Hungary); human serum albumin (Forschungsinstitut für Impfstoffe, Dessau, G.D.R.); polyaminomethylstyrene (VEB Farbenfabrik, Wolfen G.D.R.); polystyrene and polyacrylonitrile (VEB Chemische Werke, Buna G.D.R.); DEAE-, CM-, aminoethyl- and *p*-aminobenzylcellulose, polyamide, Dowex 1-X8, L-malate, D,L-3-hydroxybutyrate and L-lactate (Serva, Heidelberg, G.F.R.).

The activity of the dehydrogenases was measured by following the rate of change in E_{340} with a Beckman DK 2A recording spectrophotometer at 25°. The incubation mixture (total volume 2.0 ml) contained 0.82 m*M* NAD^+ and, according to the enzyme being tested, 0.5 *M* ethanol, 45 m*M* L-lactate, 45 m*M* L-malate or 24 m*M* D,L-3-hydroxybutyrate in 0.067 *M* phosphate buffer, pH 8. The incubation mixture (total volume 2.0 ml) to determine isocitrate dehydrogenase activity contained 0.5 m*M* $NADP^+$, 1 m*M* $MnCl_2$ and 5 m*M* D,L-isocitrate in 0.067 m*M* phosphate buffer, pH 6.5. The activity of the enzymes is expressed in enzyme units (one unit is that amount which will catalyze the transformation of 1 μmole of NAD^+ ($NADP^+$) into NADH (NADPH) per minute under standard conditions).

Protein was determined using the methods of Lowry *et al.*[10] or Webster[11]. The linear gradient from pH 8 to pH 5 was produced with an Ultragrad automatic gradient mixer (LKB, Stockholm, Sweden).

RESULTS AND DISCUSSION

The model studies were carried out with alcohol dehydrogenase. For the binding of substrates to this enzyme, hydrophobic interactions are important[12]. The binding of alcohol dehydrogenase to several ion exchangers and some hydrophobic polymers is shown in Fig. 1. The high affinity of the enzyme for polyaminomethylstyrene (80% of monomers are substituted with amino groups) is remarkable.

The time-dependent binding of alcohol dehydrogenase to polyaminomethylstyrene is shown in Fig. 2. The decrease in enzyme activity with a decrease in protein concentration suggests adsorption and not inactivation of the enzyme.

The behaviour of alcohol dehydrogenase is not characteristic of this enzyme. Some other dehydrogenases and other proteins are bound more strongly to polyaminomethylstyrene than alcohol dehydrogenase (Fig. 3). Some proteins, *e.g.*, ribonuclease, on the other hand, are not bound in our case.

Quantitative parameters for the stability of the polymer–protein complexes and for the binding capacity of polymers (polyaminomethylstyrene and polystyrene) are obtained from intercepts and slopes of the straight lines obtained by plotting of 1/proteinbound *versus* 1/proteinfree according to Klotz[13]. The comparison of results (Table I) suggests that the amino group of polyaminomethylstyrene participates in the binding process. In particular, the amounts of bound protein are significantly larger in the case of the polyaminomethylstyrene complexes. The stability of the binding between protein and polymer, demonstrated by the dissociation constants, on the other hand, is larger in the case of polystyrene complexes. These differences indicate the involvement of hydrophobic and electrostatic interactions in the case of binding to polyaminomethylstyrene.

The differences between the binding of the proteins investigated to the polymer

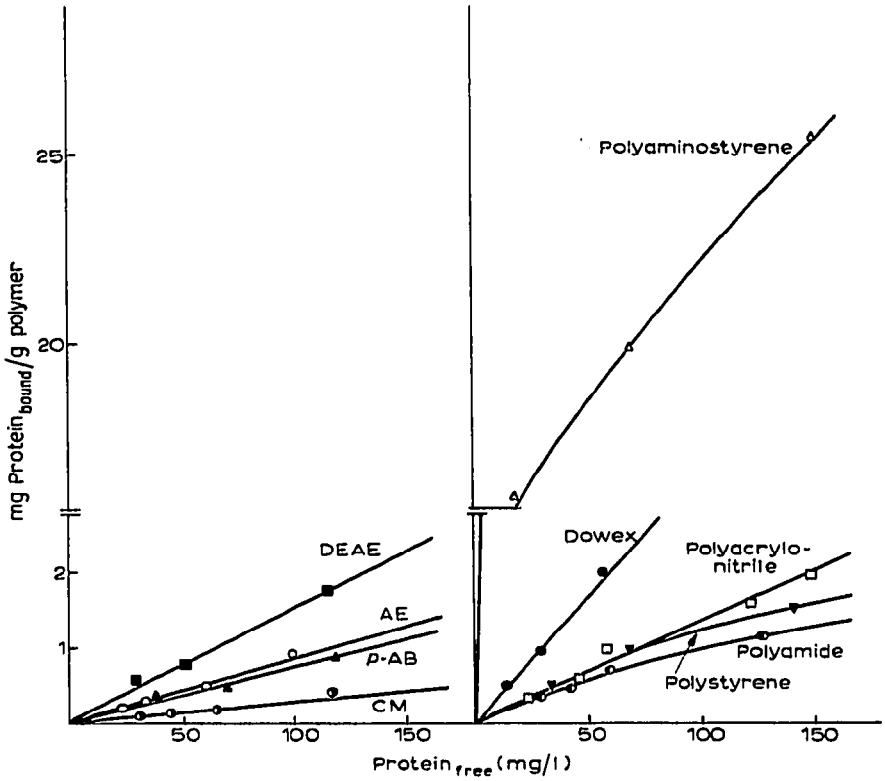

Fig. 1. Binding of alcohol dehydrogenase to ion exchangers and hydrophobic polymers in 0.067 *M* phosphate buffer, pH 8.0, at 25°. The assay mixtures (total volume 2 ml) contained 75–750 μg of alcohol dehydrogenase and a constant amount of the polymer (DEAE-cellulose, 7.2 mg; aminoethyl-cellulose (AE), 57 mg; *p*-aminobenzylcellulose (*p*-AB), 12.4 mg; CM-cellulose, 73 mg; polyamino-methylstyrene, 7.7 mg; Dowex 1-X8, 18 mg; polyacrylonitrile, 20 mg; polystyrene, 100 mg; poly-amide, 40 mg. The mixtures were rapidly stirred for 30 min and then centrifuged. Non-bound protein was determined in the supernatants. Before use in the experiments, all polymers with dissociable groups were activated with 0.1 *N* NaOH or 0.1 *N* HCl and equilibrated with buffer.

suggest that polyaminomethylstyrene may be used in the purification of proteins. The separation of a mixture of five dehydrogenases and human serum albumin on a column of polyaminomethylstyrene is demonstrated in Fig. 4. Malate dehydrogenase and iso-citrate dehydrogenase appear with the void volume or are eluted by buffer without sodium chloride. Using a gradient of pH 8 to 5 and sodium chloride (0.2 *M*), lactate dehydrogenase, alcohol dehydrogenase and albumin are desorbed. 3-Hydroxybutyrate dehydrogenase is bound so tightly to polyaminomethylstyrene that there is no elution of this enzyme by gradients of sodium chloride up to 2.5 *M* and pH 5.0. Using 2.5 *M* sodium chloride in buffer of pH 3, the inactived 3-hydroxybutyrate dehydrogenase can be eluted. In general, however, a recovery of enzymes of 40–70%, based on activity measurements, is obtained. Thus, general inactivation of enzymes by interaction with the polymer is not likely.

The change of pH necessary to elute some of the proteins demonstrates the

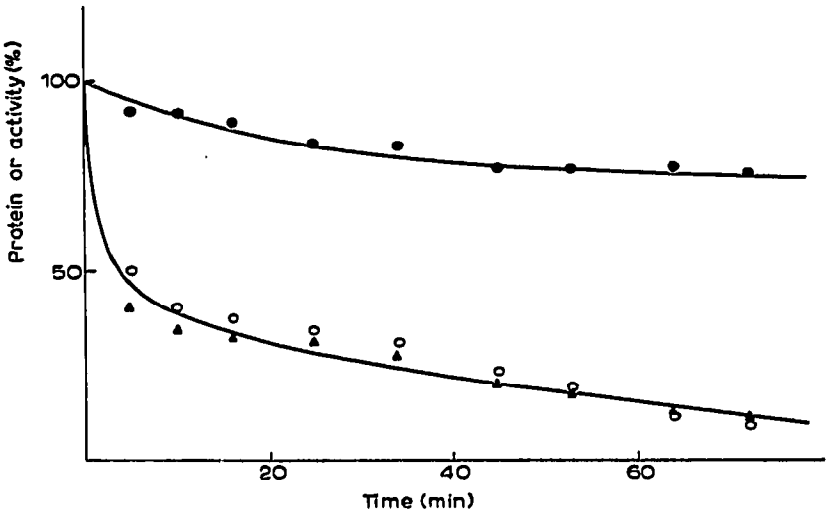

Fig. 2. Rate of binding of alcohol dehydrogenase to polyaminomethylstyrene in 0.067 *M* phosphate buffer, pH 8, at 25°. The incubation mixture contained 30 μg/ml of alcohol dehydrogenase without (●) and with (○; ▲) polyaminomethylstyrene (2.3 mg/ml). Aliquots were removed at the times indicated and, after centrifugation, activities (●; ○) and protein concentrations (▲) were determined.

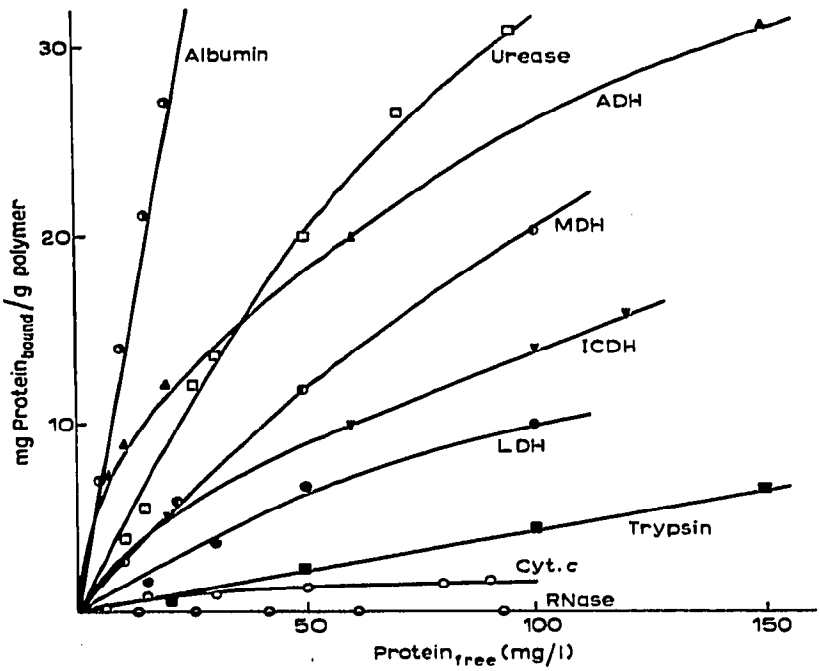

Fig. 3. Binding of proteins to polyaminomethylstyrene in 0.067 *M* phosphate buffer, pH 8.0, at 25°. The assay mixtures (total volume 2 ml) contained the following amounts of polyaminomethylstyrene: 2 mg (human serum albumin); 7.7 mg (alcohol dehydrogenase, malate dehydrogenase, isocitrate dehydrogenase and lactate dehydrogenase); 20 mg (urease); 167 mg (cytochrome *c* and ribonuclease); 176 mg (trypsin). Reaction conditions as in Fig. 1.

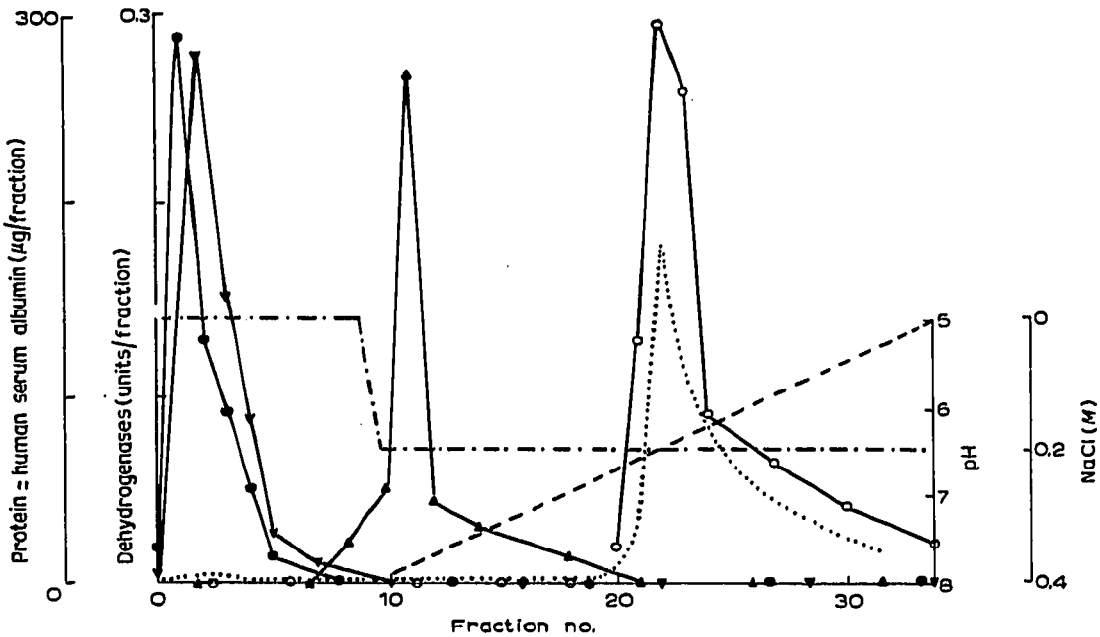

Fig. 4. Chromatography of a mixture of five dehydrogenases and human serum albumin on polyaminomethylstyrene. The polyaminomethylstyrene column (2.5 × 1 cm) contained about 1.4 g of polymer and was equilibrated with 0.067 *M* phosphate buffer, pH 8. A mixture containing 100 μg of malate dehydrogenase, 15 μg of alcohol dehydrogenase, 30 μg of lactate dehydrogenase, 300 μg of isocitrate dehydrogenase, 600 μg of 3-hydroxybutyrate dehydrogenase and 3 mg of human serum albumin in the same buffer (5 ml) was applied. The flow-rate was 0.2 ml/min. Subsequent elution was carried out at 25° with 0.067 *M* phosphate buffer containing 0.3 m*M* EDTA, 0.2 *M* NaCl (–·–·–) and a gradient of pH (— — —). Fractions of 2 ml were collected at a flow-rate of 0.4 ml/min. Enzymic activities were determined as described in the text. ●, Malate dehydrogenase; ▼, isocitrate dehydrogenase; ▲, lactate dehydrogenase; ○, alcohol dehydrogenase. No activity of 3-hydroxybutyrate dehydrogenase was found in any of the fractions. The protein content of the fractions (·····) corresponds approximately to the albumin content.

TABLE I

PARAMETERS OF PROTEIN BINDING TO POLYAMINOMETHYLSTYRENE AND POLYSTYRENE AT 25°

Parameters obtained from double reciprocal plots of $1/\text{protein}_{\text{free}}$ *versus* $1/\text{protein}_{\text{bound}}$.

Protein	*Polymer*	*Dissociation constant of protein–polymer complex (μM)*	*Binding capacity (μmole protein/g polymer)*
Alcohol dehydrogenase	Polyamino-	0.17	0.2
Lactate dehydrogenase	methylstyrene	4.8	0.5
Isocitrate dehydrogenase		2.1	0.6
Malate dehydrogenase		2.4	0.6
Urease		0.4	0.3
Human serum albumin		1.2	2.1
Trypsin		38	2.1
Cytochrome *c*		6.2	0.2
Catalase		>10	?
Ribonuclease		?	~0
Alcohol dehydrogenase	Polystyrene	1.9	0.003
Human serum albumin		0.1	0.001
Ribonuclease		2.4	0.006
Catalase		0.15	0.001

importance of electrostatic interactions. However, the facts that the elution of lactate dehydrogenase (IEP = 4.5) is very simply compared with alcohol dehydrogenase (IEP = 5.4) and that the binding properties are very different in the case of ribonuclease (IEP = 9.7), cytochrome *c* (IEP = 9.8) and trypsin (IEP = 10.8), also illustrate the significance of hydrophobic interactions (see also Hofstee[14]).

We believe that these and similar polymers, characterized by various interactions between protein and polymer, are suitable for protein purifications. Other advantages of their application are their chemical, microbiological and thermal stability.

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