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ION-EXCHANGE PROPERTIES OF CIBACRON BLUE 3G-A SEPHAROSE (BLUE SEPHAROSE) AND THE INTERACTION OF PROTEINS WITH CIBACRON BLUE 3G-A

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SUMMARY

The affinity for Blue Sepharose of several proteins of known structure showed a pH dependence governed by their isoelectric points; Blue Sepharose behaved like a strong cationic ion exchanger because of the negative charges of its dye ligand, Cibacron Blue. A study of the protein–Cibacron Blue interactions by phase partition and equilibrium dialysis revealed the presence of high-affinity binding sites both in the case of the (di)nucleotide-dependent enzymes that possess the structural domain known as "dinucleotide fold", and in the case of other proteins consisting almost entirely of α-helix (human haemoglobin, cytochrome *c*) or β-sheet (human immunoglobulin G). The presence of additional sites of low affinity, probably situated at the protein surface, was also inferred from the equilibrium dialysis data. In some instances, in contrast with the Sepharose-immobilized dye, the interaction of free Cibacron Blue with proteins was not pH dependent. Steric factors could be responsible for such a differential behaviour. It is suggested that certain nucleotide-dependent enzymes might also bind to Blue Sepharose by ion exchange. Preparative ap-

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plications of these findings are illustrated and discussed in terms of the optimization of affinity chromatography experiments.

INTRODUCTION

Because of the structural analogy between the dye and NADH, the interaction of Cibacron Blue (Cibacron Blue 3G-A, Reactive Blue, C.I. 61211) with (di)nucleotide-dependent enzymes is a standard tool for studying their active sites[1–3]. However, Cibacron Blue binds to dihydrofolate reductase from chicken liver non-competitively with respect to NADPH and competitively with respect to folate, that is, to a site that is not the dinucleotide binding site. The contrary is true for the enzyme from *L. casei*[4]; the spectra of the dye bound to the enzyme from the two sources are different. These two interactions were assumed to be ionic, "non-specific" in the first instance and "specific" for the dinucleotide binding site in the other. The "specific" binding of Cibacron Blue to the "dinucleotide fold" region of liver alcohol dehydrogenase has been directly demonstrated by X-ray diffraction studies[5].

The chromatography on Blue Sepharose (Cibacron Blue 3G-A–Sepharose 4B-CL) is now a well established technique for protein purification[6–10]. According to Gianazza and Arnaud[11], depending on the conditions, the fractionation may be achieved by ion-exchange, diffusion–exclusion, pseudo-ligand affinity or hydrophobic chromatography. With particular emphasis on the first category listed, this paper aims to discuss ways of optimizing Blue Sepharose chromatography experiments. To this end, a parallel investigation of the affinities for Blue Sepharose and free Cibacron Blue of well characterized proteins was undertaken, which outlined significant differences in the interaction of proteins with free and Sepharose-bound dye.

EXPERIMENTAL

Chemicals and materials

The materials were obtained from the following suppliers: cytochrome *c* from horse hearts and bovine serum albumin from Merck (Darmstadt, F.R.G.); Sepharose 6B-CL, Blue Sepharose, DEAE-Sephadex A-25, Sephadex G-25, Sephacryl S200 SF and Dextran 500 from Pharmacia (Uppsala, Sweden); polyethylene glycol 6000 from Loba Chemie (Vienna Fischamend, Austria); and Cibacron Blue 3G-A from Fluka (Buchs, Switzerland). The dye was used without purification. The molar absorption coefficient at 610 nm, in dilute solutions of less than 5 μM, was taken as 13,600[12].

Human haemoglobin

The haemoglobin was purified by chromatography on Blue Sepharose[13].

Ovalbumin

Ovalbumin was prepared from egg whites by ammonium sulphate precipitation at pH 5.0[14].

Human serum immunoglobulin G

This was purified by ammonium sulphate precipitation and negative absorption on DEAE-Sephadex A-25. It was freed from polymeric material by gel filtration through Sepharose 6B-CL.

Beef muscle lactate dehydrogenase

This was purified as follows: fresh beef muscle was extracted with 3 volumes of 5 m*M* EDTA–1.5 m*M* β-mercaptoethanol (pH 7.0). After filtration, the extract was adjusted to pH 8.0 and Blue Sepharose was added (about 1 l of settled gel for the extract from 2.5 kg of muscle). The suspension was gently stirred until more than 90% of the activity was absorbed. The Blue Sepharose was washed in a column with buffer A [50 m*M* Tris–HCl (pH 8.0)–2 m*M* EDTA–1.5 m*M* β-mercaptoethanol] containing 0.25 *M* sodium chloride. Lactate dehydrogenase was eluted with buffer A containing 2 *M* sodium chloride and precipitated by dialysis against a saturated solution of ammonium sulphate. The enzyme was recovered by centrifugation and equilibrated with buffer A by gel filtration through Sephadex G-25. Finally, it was subjected to re-chromatography on Blue Sepharose, washed with 0.25 *M* sodium chloride in buffer A and eluted with 1 m*M* NADH in buffer A containing 0.25 *M* sodium chloride. The overall yield of the procedure was about 30%.

Pig heart malate dehydrogenase (mitochondrial)

This was purified in parallel with nucleoside diphosphate kinase. The enzymes were absorbed in batch on Blue Sepharose at pH 8.0 from a low ionic strength extract. Washing the gel in a column with buffer A containing 0.3 *M* sodium chloride eluted malate dehydrogenase together with a red protein, possibly cytochrome *c*. Nucleoside diphosphate kinase was eluted with 1.5 m*M* ATP in buffer A containing 0.3 *M* sodium chloride; the further steps in its purification have been described elsewhere[9]. Malate dehydrogenase was precipitated by dialysis against a saturated solution of ammonium sulphate, centrifuged, dissolved in the minimum volume of buffer A and desalted by gel filtration through Sephadex G-25. It was absorbed on a Blue Sepharose column and eluted with a linear gradient of 20–500 m*M* phosphate buffer (pH 6.8), containing 5 m*M* EDTA and 1.5 m*M* β-mercaptoethanol. The enzyme was precipitated as above. It was purified to homogeneity by negative absorption on DEAE-Sephadex A-25 at pH 8.0.

Beef muscle pyruvate kinase

This was purified as in ref. 15 until step 3 of that procedure. The final step was gel filtration through Sepharose 6B-CL.

The purified proteins were stored at 4°C as precipitates in saturated ammonium sulphate solutions. They were equilibrated with the working buffer by gel filtration. All proteins were at least 95% pure as shown by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate. Human immunoglobulin G was found pure by imunoelectrophoretic analysis.

Details of the analytical procedures are given in Table I and the figures.

RESULTS

The dye Cibacron Blue carries three sulphonic groups that are expected to be fully ionized at pH above 3. Indeed, the titration curve (Fig. 1) showed that the molecule did not change its ionization state between pH 5 and 9. This means that any pH dependence of the binding properties of Blue Sepharose is due to changes in the protein ionization.

pH dependence of the binding of proteins to Blue Sepharose

The binding of proteins to Blue Sepharose at different pH values was studied in separate batch experiments. As shown in Table I, three types of behaviour were apparent:

(a) Ovalbumin did not bind to Blue Sepharose; it had a net negative charge within the pH range investigated.

(b) Human haemoglobin, human immunoglobulin G and beef muscle pyruvate kinase bound at acidic pH values, in agreement with their net positive charge, and did not bind at pH values greater than their isoelectric points.

(c) Proteins such as cytochrome *c*, having a net positive charge within the pH range investigated, bound as to a cationic ion exchanger. However, bovine serum albumin, despite having a net negative charge, bound at all pH values, probably through its ligand-binding site(s), which contain clusters of positively charged side-chains and large hydrophobic areas[17]. Finally, beef muscle lactate dehydrogenase and pig heart mitochondrial malate dehydrogenase were bound to Blue Sepharose at all pH values between 5 and 9; these are nucleotide-dependent enzymes and the presence of the "dinucleotide fold" has been demonstrated in the first case[18].

Increased ionic strength reduced the binding of all proteins in a similar fashion, whether at pH 6.3 or 8.0 (Fig. 2).

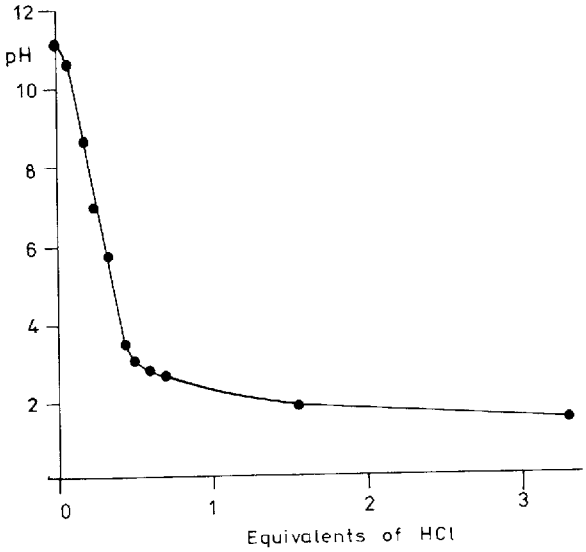

Fig. 1. Titration of Cibacron Blue. 1.15 g of Cibacron Blue was disolved in 80 ml of water, the pH was adjusted to 11.2 with 100 μl of 1 *N* NaOH, then 1 *N* HCl was added in 100-μl increments.

TABLE I

THE pH DEPENDENCE OF THE BINDING OF PROTEINS TO BLUE SEPHAROSE

The binding of proteins to Blue Sepharose under different pH and ionic strength conditions was studied by mixing 1.0 ml of buffered medium containing sodium chloride or free dye as required, 0.2 ml of a 50% suspension of Blue Sepharose in distilled water and 0.1 ml of protein solution to give a final concentration of about 1 mg/ml; 20 m*M* of either sodium succinate, sodium phosphate or Tris–acetate buffers were used in the pH ranges 5–6, 6.5–7 and 7.5–9 respectively. The capped tubes were gently rotated for 20 min. After brief centrifugation, the concentration of unbound protein was determined by reading the absorbance of the supernatant at 280 nm. The symbols + and − signify that over 85% and less than 15%, respectively, of the protein was boound to Blue Sepharose at the pH values indicated. The isoelectric points are taken from ref. 16 or from the specific references quoted in the text.

Protein	*Source*	*pI*	*Binding to Blue Sepharose*	
			pH 5	*pH 9*
Ovalbumin	Hen	4.70	−	−
Oxyhemoglobin	Human blood	6.95	+	−
Immunoglobulin G	Human blood	7	+	−
Pyruvate kinase	Beef muscle	6.75	+	−
Cytochrome *c*	Horse heart	9.04	+	+
Serum albumin	Bovine	4.90	+	+
Lactate dehydrogenase	Beef muscle	8.5	+	+
Malate dehydrogenase (mitochondrial)	Pig heart	9	+	+

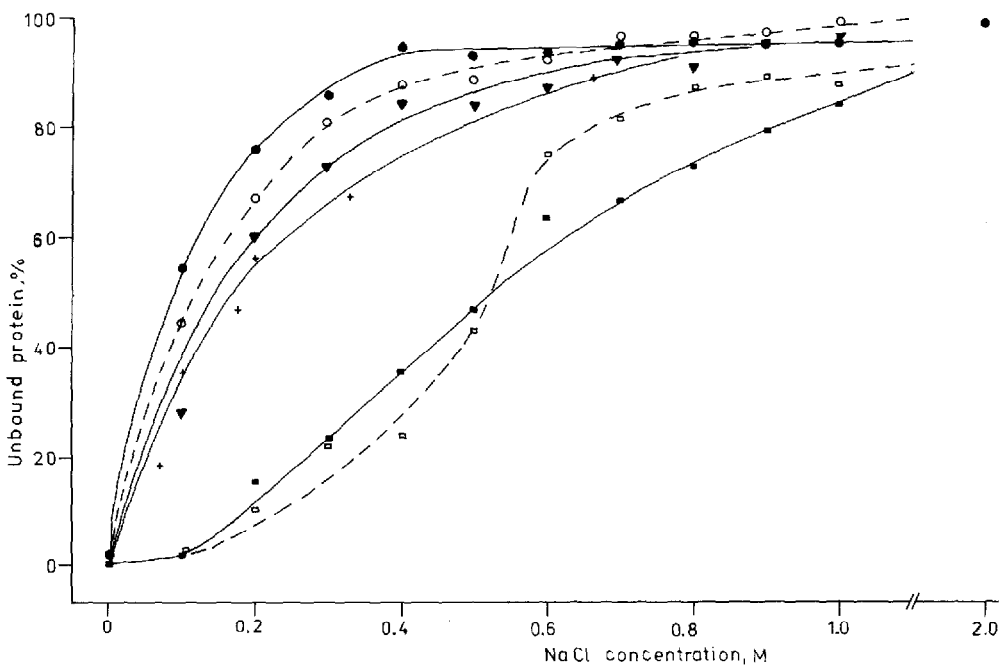

Fig. 2. Percentage of protein eluted from Blue Sepharose by increasing NaCl concentration at pH 6.3 (full line) and pH 8.0 (broken line and open symbols). Pyruvate kinase (+) is at pH 5.0; other symbols refer to cytochrome *c* (○), haemoglobin (▽) and lactate dehydrogenase (□). All curves reach the 100% value at 2.0 *M* NaCl. The experimental conditions are described in Table I.

The dependence on pH is a general feature of the binding to Blue Sepharose, as illustrated with a low ionic strength beef muscle extract (Figs. 3 and 4): the percentage of bound protein was greater in the low pH range, so that with excess of Blue Sepharose at pH 5.0 over 95% of the protein bound to the gel (Fig. 3). Several other crude extracts or partially purified enzymes from various sources showed similar behaviour (results not shown).

Interaction of proteins with Cibacron Blue

The existence of specific dye binding sites in proteins was demonstrated by several approaches, as follows.

(a) The simplest approach was to include free Cibacron Blue in the Blue Sepharose binding assay (Fig. 5). Small concentrations of free dye efficiently prevented the binding to Blue Sepharose of all proteins tested, including immunoglobulin G (not shown), suggesting an affinity of the free dye for these proteins. On addition of the dye, the lactate dehydrogenase–Blue Sepharose complex precipitated.

(b) Qualitative information concerning the binding was obtained by the partition method (Fig. 6). All proteins tested bound the dye despite the unfavourable displacement of the equilibrium due to removal of the unbound dye from the dextran phase. The pH dependence of the dye–protein interaction showed differences com-

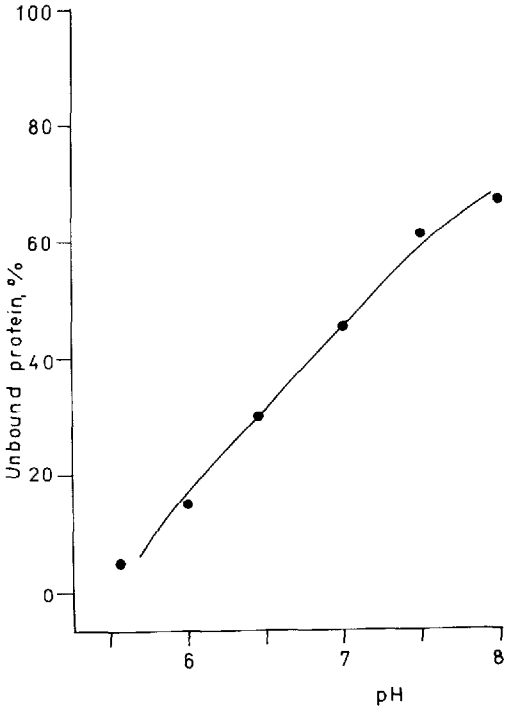

Fig. 3. Percentage of protein from a beef-muscle extract eluted from Blue Sepharose as a function of pH. Minced beef was extracted with 3 volumes of 5 m*M* EDTA–1.5 m*M* β-mercaptoethanol. The supernatant obtained after centrifugation for 10 min at 8000 *g* was desalted by gel filtration through Sephadex G-25, then subjected to the same experimental procedure as described in Table I, except that a 10-fold higher gel to protein ratio was used.

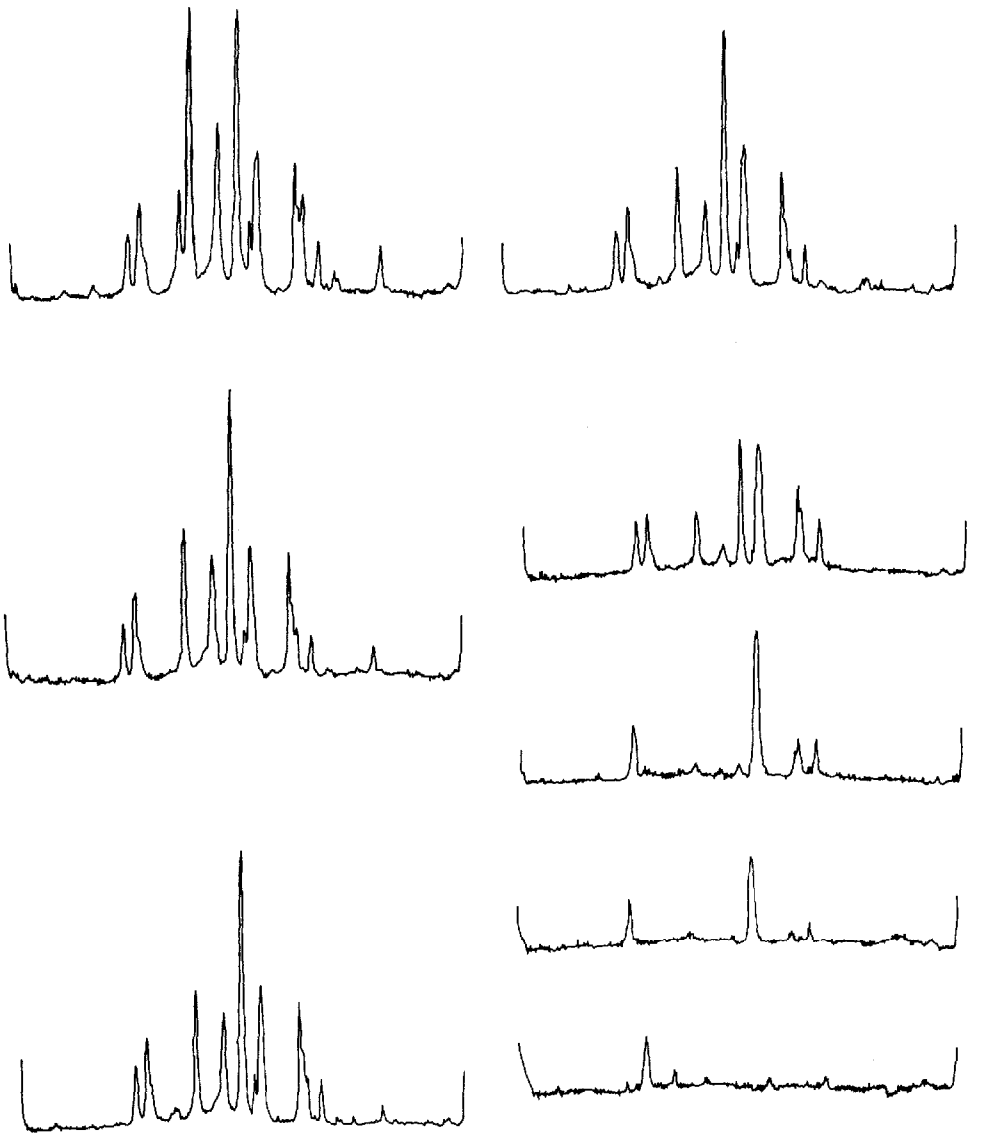

Fig. 4. Sodium dodecylsulphate polyacrylamide gel electrophoresis densitometer traces as a function of pH showing the proteins of the beef muscle extract that did not bind to Blue Sepharose. Details as in Fig. 3. Samples of equal volume were loaded on to each electrophoresis channel. Down the column, from top left, the pH values are: 9.0 (control: total extract), 8.5, 8.0, 7.5, 7.0, 6.5, 6.0, 5.5.

pared with the binding of the same proteins to Blue Sepharose; for example, ovalbumin interacted with free Cibacron Blue where as it was not absorbed by Blue Sepharose.

(c) The binding of Cibacron Blue to human immunoglobulin G and bovine serum albumin was studied by equilibrium dialysis (Figs. 7 and 8). The Scatchard plots showed a common biphasic binding. About one dye molecule bound to one

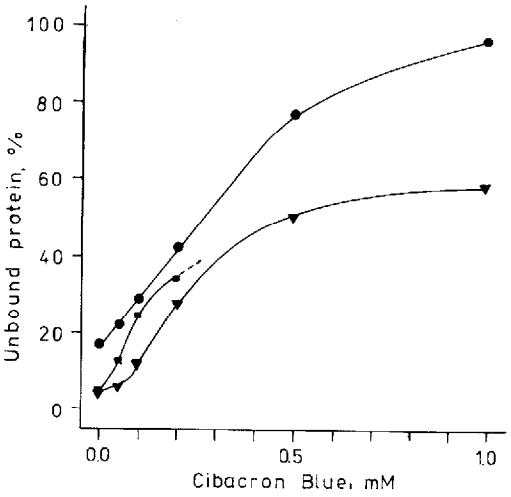

Fig. 5. Percentage of proteir not bound to Blue Sepharose at pH 6.5 in the presence of free Cibacron Blue. Symbols refer to cytochrome *c* (○), lactate dehydrogenase (□), haemoglobin (▼). Experimental details are described in Table I. The free dye from the medium was absorbed prior to measurement on to small columns (30 × 5 mm) of DEAE-Sephadex A-25 equilibrated with 10 m*M* succinate buffer (pH 6.0). By mixing the proteins with unsubstituted Sepharose, no decrease in protein concentration in the supernatant was noted, which thus excluded any interaction with the agarose matrix. Cibacron Blue above 0.2 m*M* caused precipitation of lactate dehydrogenase within the gel.

immunoglobulin G molecule, with a K_d of about 3 μM at pH 6.0 and 6 μM at pH 8.0, in the presence of 0.1 *M* sodium chloride to avoid protein precipitation at low ionic strength. Note that the binding of Cibacron Blue to immunoglobulin G does take place in spite of the lack of affinity of the latter for Blue Sepharose at pH 8.0. Bovine serum albumin bound two dye molecules with a K_d of 7.8 μM at pH 6.8. In both instances, the existence of an indeterminate number of binding sites of low affinity was apparent, but a quantitative analysis was not undertaken because the dye was strongly aggregated in the concentration range where the experimental points were obtained.

DISCUSSION

Our data confirm previous observations and substantiate the idea that ionic forces play an important role in the binding of proteins to Blue Sepharose. As Cibacron Blue, the dye ligand of Blue Sepharose, does not change its ionization state (Fig. 1), the affinity of the proteins for Blue Sepharose is likely to be extremely sensitive to minimal modifications in their electric charge. The fact that Cibacron Blue does not undergo protonation–deprotonation has an additional important preparative consequence: Blue Sepharose behaves like a strong ion exchanger and proteins can be eluted sharply by simple alteration of the pH at low ionic strength. In other words, it has no buffering capacity, a useful feature for high-performance liquid chromatographic applications, because the re-equilibration with the starting buffer would be rapid.

The exploitation of the pH dependence of the binding of proteins to Blue

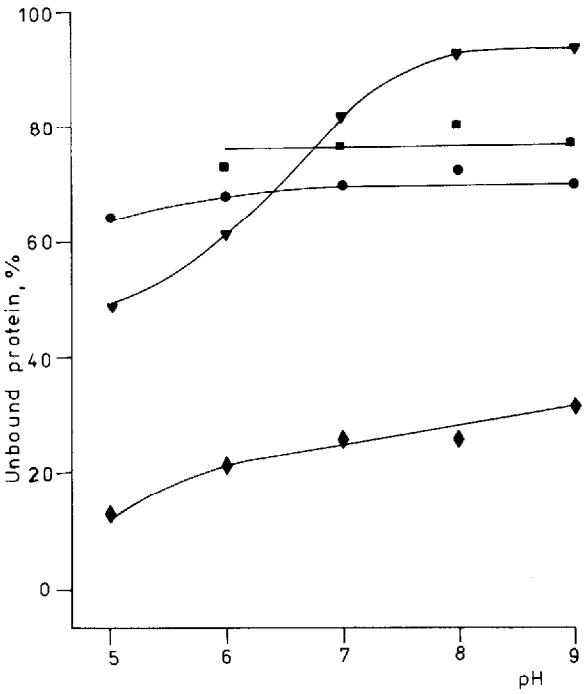

Fig. 6. Percentage of free Cibacron Blue in protein–dye mixtures at various pH values, as reflected by the concentration of dye in the polyethylene glycol phase of a system containing polyethylene glycol 6000 and dextran 500 at a final concentration of 7% each[19]. The free dye (about 20 nmol in a total volume of 0.8 ml) partitioned almost completely into the more hydrophobic polyethylene glycol phase at any pH between 5.0 and 9.0, while the proteins concentrated into the dextran phase. When dye and protein were present together in equimolar ratios, the drop in the dye concentration in the upper, polyethylene glycol phase, monitored at 610 nm, would qualitatively indicate the formation of a dye–protein complex. A thorough quantitative analysis is cumbersome, as one would have to take into account both the affinity constant and the partition coefficient of the dye, which cannot be measured accurately. Symbols refer to haemoglobin (▽), lactate dehydrogenase (□), cytochrome *c* (○) and ovalbumin (◇). At pH 5.0 the lactate dehydrogenase–Cibacron Blue complex precipitated.

Sepharose (Table I and Fig. 3) is an important factor in the optimization of the use of this gel as an affinity material. On the one hand, a protein that binds to Blue Sepharose at any pH between 5 and 9 will be more efficiently in a batch or column experiment performed at as high a pH value as allowed by the protein stability. On the other hand, the use of buffers of relatively high ionic strength (0.2–0.5 *M* sodium chloride) leads to the elution of the weakly bound proteins, including, for example, malate dehydrogenase (0.3 *M* at pH 7.5) or glutamate dehydrogenase (0.5 *M* at pH 7.5; unpublished work). There are relatively few proteins that remain bound to Blue Sepharose under these conditions. Elution with coenzyme, such as in the case of lactate dehydrogenase, nucleotide diphosphate kinase or alanine dehydrogenase[22], delivers proteins of high purity.

Fortuitously, when working at high pH and salt concentrations the interferences due to the non-specific binding of proteins to residual sulphate groups on Sepharose become less important. Moreover, it becomes possible to replace agarose

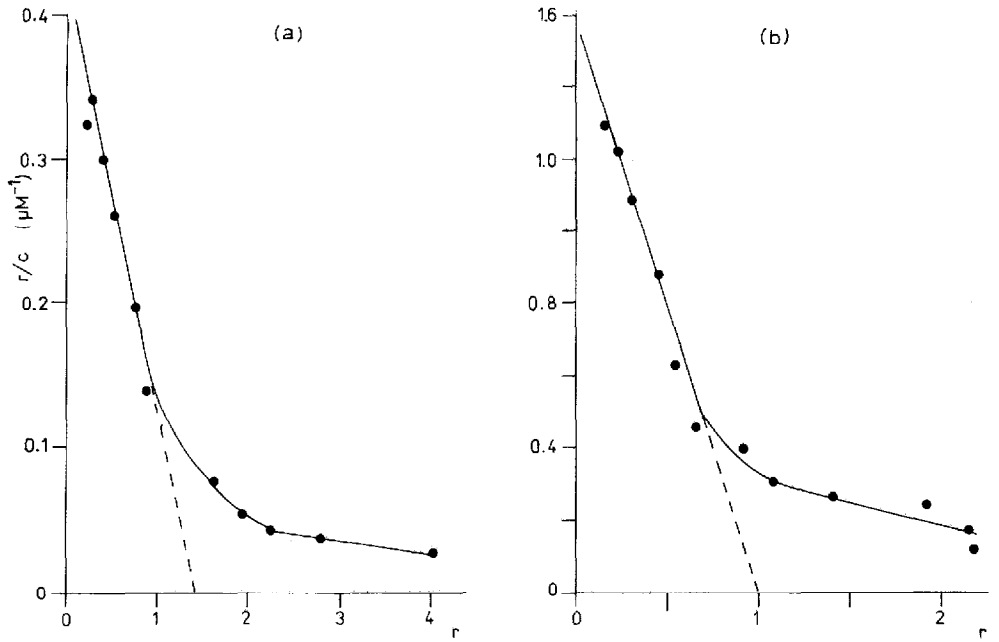

Fig. 7. Scatchard plot based on equilibrium dialysis data obtained with Cibacron Blue–immunoglobulin G complexes: (A) at pH 6.0 in 20 m*M* phosphate buffer 0.1 *M* NaCl; (B) at pH 8.0 in 20 m*M* triethanolamine–acetate buffer–0.1 *M* NaCl. Protein solutions were equilibrated in whisking dialysis bags for 6 days with solutions of Cibacron Blue in an appropriate buffer. The completeness of equilibration was checked by including a blank sample, *viz.*, 4 ml of buffer in a separate bag. Protein was determined by the Gornall *et al.* method[20]. The absorbance of the dye at 540 nm was subtracted after dilution in 0.5 *M* NaOH. Free and free + bound dye were measured at 610 nm, after dilution to an absorbance between 0.1 and 0.15, in a 5-cm cuvette. The data were plotted according to Scatchard[21].

by agar in the synthesis of low-cost affinity materials for large-scale laboratory or industrial applications.

The weak, pH-dependent binding mode could in itself be used as a purification means. Thus, working with low ionic strength buffer, human haemoglobin bound to Blue Sepharose at pH 7.0 and could be eluted at pH 8.0 with 0.1 *M* triethanolamine–acetate. As catalase (p*I* = 5.4) and carbonic anhydrase (p*I* = 5.3) did not bind at pH 7.0, and as most phosphotransferases and pyridinic dehydrogenases retained their affinity under the conditions used for the elution of haemoglobin, the latter was obtained in a high state of purity[13]. The nucleotide-dependent enzymes could be recovered by elution with 2 *M* sodium chloride solution. Conversely, haemoglobin did not bind to Blue Sepharose if the gel was loaded at pH 8.0 and thus it was eliminated to the extent of 99.99% during the first step of the purification of horse blood nucleoside diphosphate kinase. Combining batch absorption and column elution with ATP, 2.25 kg of protein could be processed using just 800 ml of Blue Sepharose, achieving a purification factor of 6900 in a single step[23].

By studying in parallel the interaction of proteins with free and Sepharose-bound Cibacron Blue, several fundamental differences became apparent. First, the dye binds strongly to ovalbumin (Fig. 6) and to immunoglobulin G (Fig. 7b) at pH values at which these proteins do not bind to Blue Sepharose. This suggests that

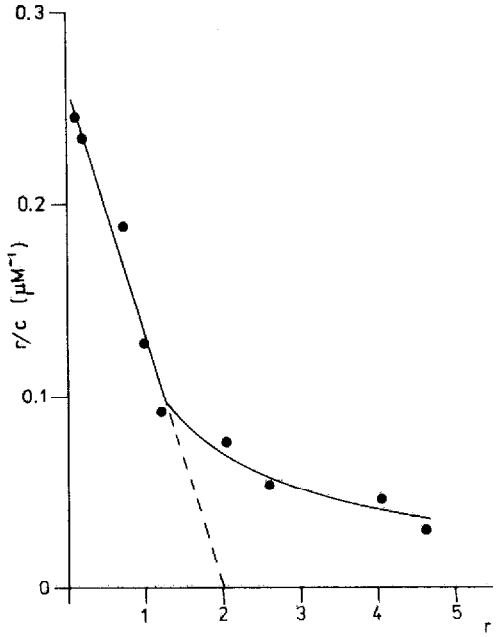

Fig. 8. Scatchard plot based on equilibrium dialysis data obtained with Cibacron Blue–bovine serum albumin complexes in 20 m*M* phosphate buffer (pH 6.8). Experimental procedure as in Fig. 7.

different sites are involved in the interaction with free and immobilized Cibacron Blue. Possibly the sites of (strong) binding of the dye are not sterically accessible to the Sepharose-bound dye. In this way one could explain several discrepancies (unpublished observations): the heart lactate dehydrogenase, with an isoelectric point of 5, which strongly binds Cibacron Blue, has only a very weak affinity for Blue Sepharose and does not bind at all if the protein to gel ratio is high. In contrast, as seen, muscle lactate dehydrogenase (p*I* = 8.5) binds to Blue Sepharose at pH 8.0 even at medium to high ionic strengths. Similarly, the pig heart mitochondrial malate dehydrogenase (p*I* = 9) binds to Blue Sepharose, whereas the cytoplasmic isoenzyme (p*I* = 5) does not.

It is remarkable that the relationship between isoelectric points and Blue Sepharose affinity seems to hold even with at least some nucleotide-dependent enzymes. Provision of the appropriate coenzymes leads to their elution in a manner analogous to classical affinity elution, where the binding of the coenzyme and the interaction with the gel involve different sites on the protein.

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