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Chromatography of human immunoglobulin G on immobilized Drimarene Rubine R/K-5BL Study of mild, efficient elution procedures

Sylvie Cochet, M'Hammed Hasnaoui, Martine Debbia, Yolande Kroviarski, Patrick Lambin, Jean Pierre Cartron, Olivier Bertrand*

INSERM U 76 and INTS, 6 Rue Alexandre Cabanel, 75739 Paris, France

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Abstract

A range of substances were screened to find eluents for human immunoglobulin G (IgG) which are retained with a strong affinity by immobilized Drimarene Rubine R/K-5BL. The strong affinity of IgG for the dye is partly due to the presence of copper in the dye. This was suggested by the effect of substances able to make coordination bonds with metals that elute the IgG and also the effect of metal stripping from the immobilized dye. Several mobile phase conditions were found that allowed desorption of retained IgG on immobilized Drimarene Rubine R/K-5BL without using a protein denaturant. A procedure was also devised for separating IgG_2 from other IgG subclasses using chromatography on immobilized Rubine R/K-5BL and column development with an AMP gradient.

1. Introduction

Immobilized dyes have been widely used as chromatographic supports for purifying a variety of proteins, mostly enzymes [1]. Plasma proteins have been also separated by immobilized dye chromatography. Immobilized Cibacron Blue F3GA has been used to purify human serum albumin on an industrial scale [2,3], and Remazol Yellow GGL has been used for the very large-scale purification of transthyretrin [4]. Many publications have described the purification of diverse plasma proteins at least on a laboratory scale [5–12]. Immunoglobulins have been purified and fractionated on immobilized Remazol yellow GGL [13] and on DEAE-Cibacron Blue [14]. Berg and Scouten [15] screened 65 immobilized dyes for their affinity for immunoglobulins and identified two dyes, Drimarene Blue K-R and Rubine R/K-5BL, that had a high affinity for immunoglobulins. Their affinities were so high that no satisfactory procedure was found for eluting proteins that allowed the recovery of immunoglobulins in a native state.

This paper describes the systematic screening of conditions needed to elute immunoglobulins from an immobilized Rubine R/K-5BL column. It was possible to elute immunoglobulins in good yield with mild eluents. This immobilized dye

* Corresponding author.

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was also used to separate immunoglobulin G (IgG) subclasses.

2. Experimental

2.1. Materials

Drimarene Rubine R/K-5BL was a gift from Sandoz Chimie (Rueil Malmaison, France). Sepharose CL-4B was obtained from Pharmacia (Uppsala, Sweden). Placental immunoglobulins were a gift from Pasteur Merieux Serum et Vaccins (Marcy L'Etoile, France). All other chemicals were purchased form Sigma (St. Louis, MO, USA), Merck (Darmstadt, Germany) or Bio-Rad (Richmond, CA, USA).

2.2. Dye immobilization [1]

Sepharose CL-4B was rinsed extensively with water and immersed in 0.2 *M* NaOH containing 2% (w/v) NaCl. The gel was transferred into a vessel containing reactive dye (20 mg/ml gel) (the dye was used as obtained from the manufacturer), then, 0.2 *M* NaOH containing 2% (w/v) NaCl was added so that final reaction volume was twice the gel volume. The gel suspension was tumbled at 60°C for 1 h, then rinsed with 10 volumes of 0.2 *M* NaOH followed by 50% dimethyl sulphoxide in water until the washings were clear. The gel was finally rinsed with water and stored as a suspension in 100 m*M* NaCl containing 0.02% sodium azide.

Dye substitution was measured as follows [1]. Immobilized dye was hydrolysed in 50% acetic acid at 110°C until the gel was completely dissolved and the absorbance was then measured at 531 nm. The substitution level was expressed as milligrams of dye per millilitre of support, using the reactive dye, as obtained from the manufacturer, as a standard.

2.3. Preparation and characterization of immunoglobulins used for screening eluents

We used immunoglobulins prepared from human placenta, subsequently called placental IgGs. The human placental immunoglobulins were manufactured for intravenous administration and hence were treated with dilute plasmin [16]. They were characterized by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) under both reducing and non-reducing conditions followed by Western blotting. Blots were revealed using rabbit antibodies directed against Fab and Fc fragments of immunoglobulins. IgG subclasses were assayed by enzyme-linked immunosorbent assay (ELISA) [17].

In order to obtain, when needed, IgG free of degradation products (subscquently called gel-filtered IgG), placental IgGs (150 mg of IgG in 20 ml of equilibration buffer) were chromatographed on an Ultrogel AcA-54 column (70 cm × 5 cm I.D.) equilibrated in 5 m*M* sodium acetate buffer (pH 6.0) containing 0.1 *M* NaCl operated at a flow-rate of 105 ml/h.

Placental or gel-filtered IgGs were passed through a Sephadex G-25 column equilibrated in buffer A [10 m*M* KOH adjusted to pH 6.0 with solid 2-(N-morpholino)ethanesulphonic acid (MES)] prior to their use for screening eluents.

2.4. Preparation of metal-stripped immobilized dye

Metal was removed from immobilized Rubine R/K-5BL by pumping through the column 0.1 *M* EDTA dissolved in buffer B (10 m*M* KOH adjusted to pH 6.0 with solid MES containing 2 *M* NaCl). The copper content of the EDTA eluate was assayed spectrophotometrically at 632 nm.

2.5. Eluent screening

A column (2.6 cm × 1.1 cm I.D.) of immobilized dye was equilibrated in buffer A at a flow-rate of 20 ml/h. Untreated or metal-stripped immobilized dye was used as required. Two types of experiments were performed, as follows.

(i) A 6-mg amount of placental IgGs dissolved in buffer A was loaded on the immobilized dye column. The column was then developed in

sequence with 20 ml of buffer A, 20 ml of buffer B, 20 ml of buffer B′ (buffer B containing one of the eluents tested), 20 ml of buffer B again and finally with 20 ml of buffer C (buffer B containing 6 *M* urea). Protein-containing fractions eluted with buffers A , B and C and finally with buffer B′ (if the buffer itself did not adsorb at 280 nm) were separately pooled and the absorbance at 280 nm of each pool was evaluated.

(ii) A 6-mg amount of gel-filtered IgGs dissolved in buffer A was loaded on the column. The column was then developed with 20 ml of buffer A′ (buffer A containing one of the putative eluents), 20 ml of buffer A and finally 20 ml of buffer C. The absorbances of pools, made similarly as above, were measured.

The procedures used to calculate the amounts of proteins eluted by buffer B′ and A′ (when these buffers contained UV-absorbing substances) are described in Section 3.

2.6. Separation of IgG subclasses

Gel-filtered IgGs (6 mg dissolved in buffer A) were loaded on an immobilized Rubine R/K-5BL column (2.6 cm × 1.1 cm I.D.) equilibrated in buffer A. The flow-rate was 20 ml/h. After sample loading, the column was rinsed with 20 ml of buffer A and developed with 10 ml of buffer A containing 10 m*M* AMP and then with a linear 90 min gradient from 10 to 100 m*M* AMP (in buffer A). The column was then rinsed with 100 m*M* AMP in buffer A and finally with buffer C. Each fraction (2 ml) was assayed for IgG_2 and for total IgG by ELISA.

2.7. Other techniques

SDS-PAGE [18] was carried out on a Novex (San Diego, CA, USA) apparatus. Protein was assayed by measuring the absorbance at 280 nm or by the Bradford assay [19]. The p*K* values of imidazole and imidazole derivatives were measured using a Tacussel (Villeurbanne, France) TT 60 automatic titrator.

3. Results and discussion

3.1. Characterization of immunoglobulin preparations used as starting material

Placental IgGs had been treated with dilute plasmin to remove aggregates [16], and there was some cleavage of IgGs analogous to that produced by papain generating Fab and Fc fragments (Fig. 1). Western blotting of a non-reduced gel confirmed that fragments with apparent molecular masses of 46 000 and 54 000 reacted with the Fab- and Fc-specific anti-IgG. These degradation fragments will be referred to as Fab and Fc fragments. The band migrating with an apparent molecular mass of 30 000 on the non-reduced gel was identified as a degradation product of Fc origin.

A Coomassie Brilliant Blue-stained smear centred at an apparent molecular mass of 150 000 was also seen on the non-reduced gel. A similar pattern has been already described by others and assumed to be due to proteolytic degradation of IgGs [16]. Gel-filtered IgGs were essentially free of Fab and Fc fragments (Fig. 1) but had some M_r 150 000 smear on the non-reduced gel.

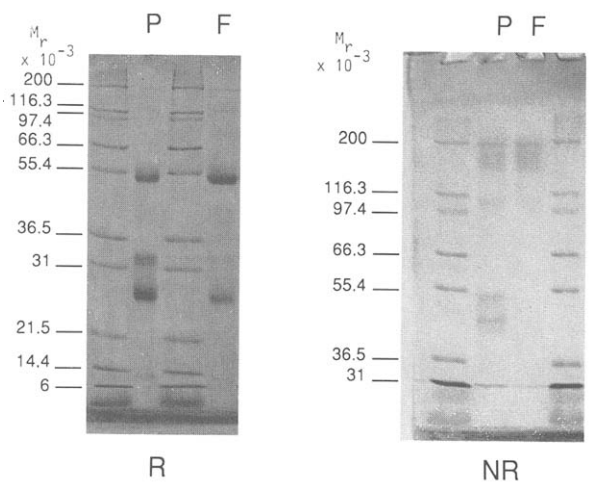

Fig. 1. SDS-PAGE of placental and gel-filtered IgGs. Placental and gel-filtered IgGs were loaded in lanes P and F. Molecular mass standards were loaded in the other lanes. Electrophoreses were run under reducing conditions (R) and non-reducing conditions (NR) on 12.5% and 8% gels.

3.2. Characterization of immobilized dye

Dye substitution was 4.3 mg of dye per millilitre of gel. The copper eluted with EDTA from an aliquot of gel (not used for chromatography) was 7.4 μmol per millilitre of gel.

3.3. Identification of molecular species eluted from Rubine column by buffers A, B and C

The elution pattern of placental IgGs with buffers A, B and C is shown at the top of Fig. 2. SDS-PAGE (Fig. 3) and Western blots (not shown) demonstrated that Fab was the only species eluted from the column during sample loading and washing with buffer A. Fab was also eluted by buffer B. The urea-containing buffer C eluted IgGs and the Fc fragment. These results agree well with the data of Berg and Scouten [15]. Gel-filtered IgGs were eluted only with buffer C (not shown).

3.4. Studies with metallized dye: screening of a range of potential eluting species and insight into the molecular mechanism underlying metallized dye affinity for proteins

It has been known since the very early days of immobilized dye chromatography that an enzyme retained on a dye column can often be eluted by adding a natural ligand for the enzyme to the mobile phase [20,21]. It has also been shown that enzymes retained by a dye could be eluted with substances unrelated to one of their natural ligands. For example, chymotrypsinogen can be eluted from an immobilized dye with cytidine monophosphate [22]. Fragments of natural ligands can be more efficient than natural ligands [23]. This leads to the conclusion [23] that "every substance which in some way mimics a dye (*i.e.*, which associates a high density of charged groups with hydrophobic parts) has the potential to elute a protein from a dye column". In keeping with this background, we tried to discover useful substances allowing immunoglobulins to be eluted in a native state from the Rubine column.

Numerical results representative of the eluting power of the various potential eluents dissolved in buffer B were obtained as follows: proteins present in the peak eluted by infusion of buffer C, following buffer B′ infusion onto the column were evaluated by measuring absorbance at 280 nm: the peak eluted by buffer C after the column had been developed with only buffer A and B was fairly constant and corresponded to 43.2% of total loaded protein; if buffer B′ contained a substance that eluted proteins from the column, peak eluted by buffer C was lower (see Fig. 2, centre and bottom). The eluting power of the substance dissolved in buffer B′ was then evaluated from the ratio.

$$\frac{43.2 - \text{protein eluted by buffer C)}}{43.2} \cdot 100$$

Obviously, in each instance of an absorbing and eluting buffer B′ the effective presence of IgGs in the buffer B′-eluted fractions was checked by SDS-PAGE, ELISA and/or Bradford protein assay. When the test substance in buffer B′ did not absorb at 280 nm the proportion of proteins eluted could be evaluated either through direct absorbance measurement of fractions eluted with buffer B′ or through absorbance measurement of fractions eluted with buffer C and calculation as indicated above. The results obtained with the two methods were in satisfactory agreement.

Numerical results for the experiments in which tested substances were dissolved in buffer A were obtained similarly.

Eluting power of phosphate and phosphorylated compounds, nucleosides and nucleobases

Phosphate is known to be a potential eluent of proteins from immobilized dyes [24], but 0.1 *M* phosphate did not elute IgGs from immobilized Rubinol R/K-5BL. Inositol hexaphosphate (IHP), an inexpensive phosphoryl compound that has been used to elute, with a satisfactory selectivity, proteins from a dye column [25], had no effect (0.1 *M* in buffer B) (Fig. 4, top).

AMP and cyclic AMP, 10 m*M* in buffer B,

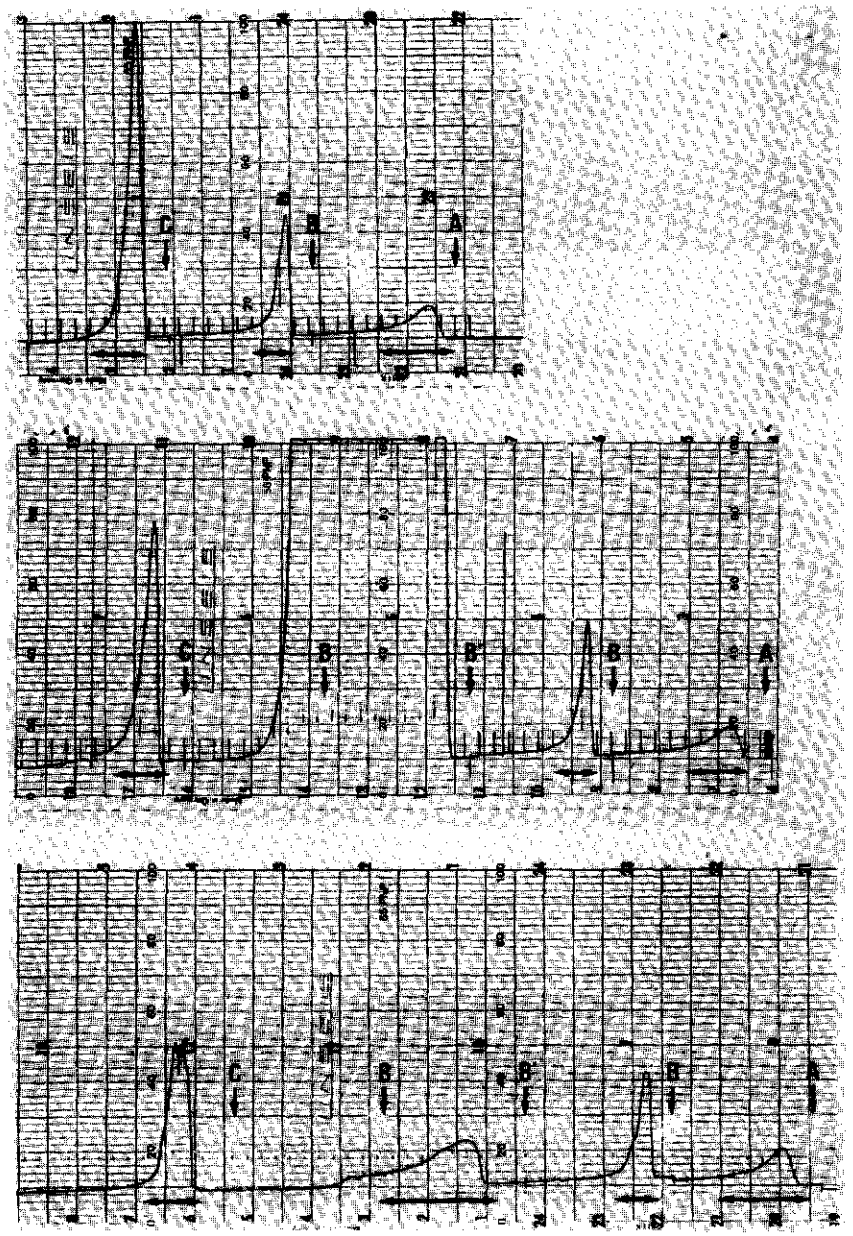

Fig. 2. Chromatography of placental IgGs on immobilized Rubine R/K-5BL. Top, elution with buffers A, B and C; centre, buffer B' contained 10 m*M* ADP; bottom, buffer B' contained 0.5 m*M* imidazole. Vertical arrows indicate buffer changes. The column was 2:6 cm × 1.1 cm I.D. and the flow-rate was 20 ml/h. Fractions were pooled as indicated by the double horizontal arrows and protein content was evaluated by absorbance measurements.

eluted some IgGs from the dye column and at 100 m*M* eluted all the IgGs. Adenosine and adenine (10 m*M*) eluted slightly more IgGs than did AMP or cyclic AMP. NAD and CMP (10 m*M* concentration in buffer B) eluted a significant proportion of IgGs (Fig. 4, top).

The eluting power of AMP and adenosine dissolved in buffer A was also evaluated (see Fig.

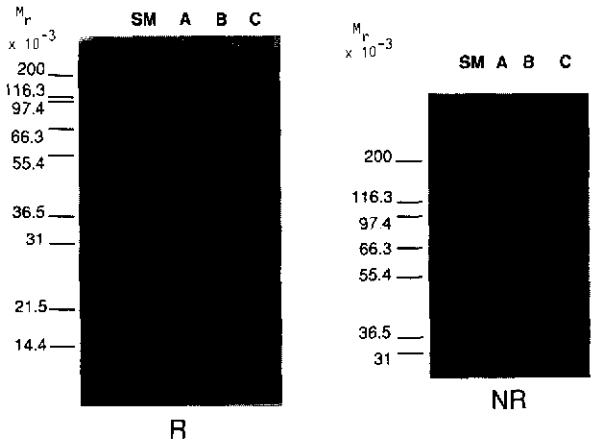

Fig. 3. SDS-PAGE analysis of the chromatogram shown in Fig. 2 (top). An aliquot of starting material for the chromatography (placental IgGs) was loaded in lane SM. Aliquots of the three peaks eluted from the column during sample loading, during buffer B application and during development with buffer C were loaded into lanes A, B and C, respectively. The left lanes of the gels were loaded with molecular mass standards. R and NR indicate reduced and non-reduced conditions, respectively.

4, bottom): 10 m*M* AMP eluted about 20% and 100 m*M* AMP 67% of retained proteins whereas adenosine at the same concentrations had no effect.

Eluting power of amino acids, amino acid derivatives and imidazole compounds

Amino acids were tested because some of them (tryptophan, phenylalanine and tyrosine) are aromatic and bear charges. Some, such as histidine, tryptophan and phenylalanine, have been used in immobilized form to purify immunoglobulins [26–28].

Tryptophan and phenylalanine (0.1 *M*) eluted some, but not all, of the IgGs (Fig. 5). Phenylalanine ethyl ester and N-acetylphenylalanine were both more efficient than the parent amino acids. Glycine had no effect (0.1 *M* concentration in buffer B′) while glycinamide eluted some of the IgGs. Histidine (0.1 *M*) eluted IgGs quantitatively. The dramatic effect of histidine is clearly due to the imidazole group, as imidazole at 10 m*M* removed all proteins from the Rubine R/K-5BL column. Imidazole and imidazole derivatives were clearly better eluters if the imidazole nitrogen was unprotonated: (i) imidazole is more efficient at pH 9 than at pH 6.0 (see Fig. 5); the p*K* of imidazole is 6.71, but it is well known that immobilized dyes often show a weaker affinity for proteins at alkaline pH; the p*K* values of substituted imidazoles were measured and found to be 7.13 for methylimidazole and 8.63 for nitroimidazole; it is

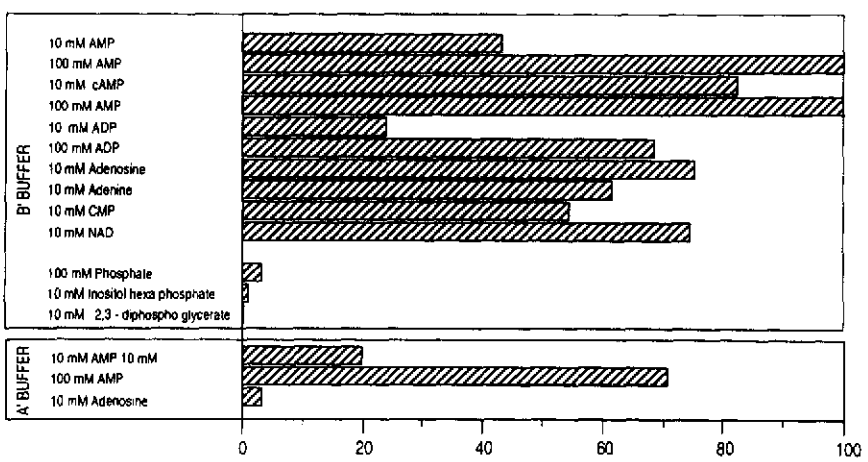

Fig. 4. Amounts of proteins eluted from the metallized Rubine R/K-5BL column with nucleotides, nucleosides nucleobases and some phosphorylated compounds. Numerical values (%) were determined as indicated in the text. Top, test substances were dissolved in buffer B; bottom, test substances were dissolved in buffer A.

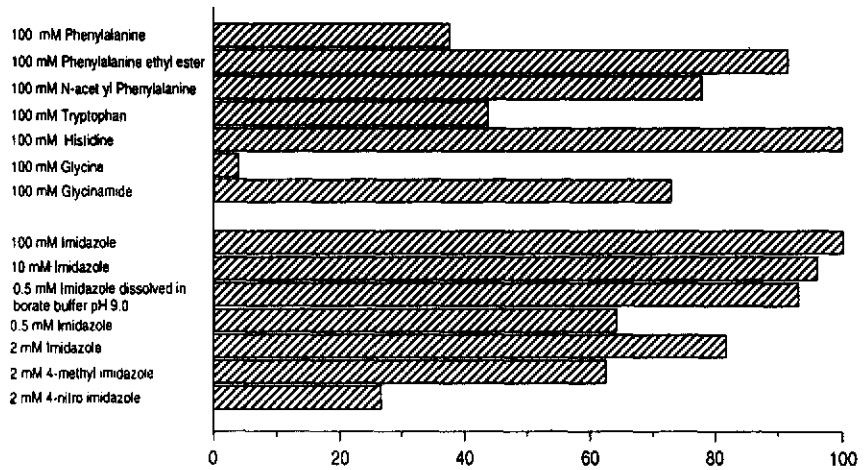

Fig. 5. Amounts of proteins eluted from the metallized Rubine R/K-5BL column by amino acids, derivatives thereof or imidazole compounds. Results shown were obtained by dissolving the tested substances in buffer B. Numerical values (%) were determined as indicated in the text.

obvious that one can rank the eluting power of imidazole and substituted imidazole in order of the nitrogen pK: compounds with higher pK have greater eluting power.

Eluting power of amines and miscellaneous mobile phase conditions

NH_4Cl (100 mM in buffer B) did not elute IgG and Fc fragments retained on the column. Neither diaminohexane nor benzylamine (100 mM) eluted significant amounts of IgGs, but pyridine (10 mM) eluted all the bound IgGs.

Glucosamine (0.1 M) in buffer B eluted more than half of the retained IgGs, but 0.1 M N-acetylglucosamine was much less effective (Fig. 6).

Increasing the pH of mobile phase to 9.0 or lowering it to 5.4 alone eluted only a small fraction of the retained proteins.

Effect of EDTA

The precise structure of Rubine R/K-5BL is not available but it is known to be a copper-containing azo dye [15]. We therefore evaluated the eluting power of EDTA. EDTA eluted a small fraction of the IgGs plus a large amount of copper (corresponding to 5.5 μmol of copper per millilitre of gel). Urea-containing buffer C applied thereafter to the column eluted the remaining retained IgG. The IgG bound to the column may protect the copper from chelation by EDTA because the EDTA eluted only a very small fraction of the bound IgGs and EDTA treatment of the column *after* elution of IgGs removed the copper left on the column after the first EDTA treatment (the amount of copper eluted by the second treatment with EDTA was 1.5 μmol per millilitre of gel). When the column that had been treated twice with EDTA was loaded with another aliquot of the IgG test mixture, most of the absorbance was eluted with buffer B and essentially none with buffer C.

Hence copper plays an important role in the affinity of the immobilized dye for the IgGs. Nevertheless, immobilized Rubine R/K-5BL is not simply some sort of an immobilized metal affinity chromatographic (IMAC) support [29]: lowering the mobile phase pH does not elute retained proteins; raising the ionic strength induces some desorption of retained proteins, as part of the Fab fragment present in placental IgGs and retained on the column is efficiently eluted (see Fig. 2, top), whereas IMAC is normally performed at high ionic strength and EDTA efficiently elutes proteins retained on IMAC supports together with the metal.

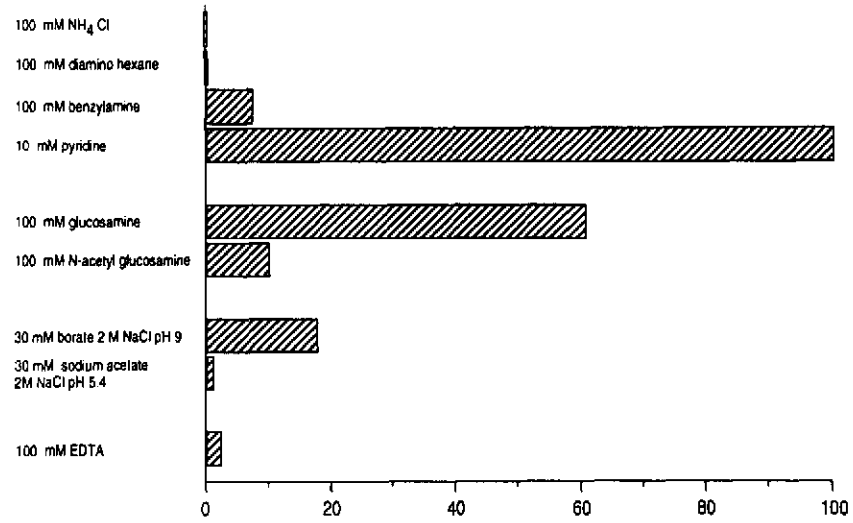

Fig. 6. Amounts of proteins eluted from the metallized Rubine R/K-5BL column by amino-containing substances, miscellaneous additives and mobile phase compositions. Results shown were obtained by dissolving the tested substances in buffer B. Numerical values (%) were determined as indicated in the text.

The copper in azo dyes is often anchored to the organic backbone of the dye by at least three coordination bonds, leaving at best only one bond free to interact with solvent or solutes [30]. The substances that elute significant amounts of IgGs from the metallized immobilized dye (*e.g.*, imidazole or pyridine) can form coordination bonds with metals. Molecules that form bidentate complexes with metals, such EDTA, and unsubstituted amino acids, are not better eluters than substances containing atoms bearing a lone pair of electrons (see results obtained with phenylalanine and its derivatives, with glycine and glycinamide).

Copper in the dye is an important element contributing to the affinity of the dye for the IgGs. Nevertheless, copper does not seem to be the only factor in the affinity of the immobilized dye for the IgGs. The IgGs were retained on the column by probably both coordinative bonding with copper and by electrostatic and/or hydrophobic interactions similar to those usually encountered in immobilized dye chromatography; thus imidazole dissolved in buffer A (a low ionic strength buffer) eluted poorly IgGs from a metallized immobilized dye column (only 2% of the absorbance was eluted with 2 m*M* imidazole dissolved in buffer A). Other experiments were performed to evaluate the eluting power of several substances with a demetallized dye.

3.5. Studies with demetallized dye and design of new elution mixtures for metallized dye

IgGs were quantitatively eluted from the demetallized dye column by raising the ionic strength, as is common with immobilized dyes (Fig. 7); this is usually considered to indicate the importance of electrostatic interactions between proteins and dye.

Imidazole (in buffer A) eluted only a small proportion of IgGs from demetallized dye. Phosphate (0.1 *M*) which was completely unable to elute IgG from metallized dye eluted more than 65% of the bound IgG from a demetallized dye. IHP (10 m*M*) did not elute significant amounts of IgGs from metallized dye but it eluted nearly 65% of the retained IgGs from the demetallized dye; 25 m*M* IHP eluted 100% of the bound IgGs. It was found that 10 m*M* AMP, cyclic AMP and NAD were all less effective than IHP at removing IgGs from demetallized dye; 100 m*M* AMP in buffer A eluted about 80% of the

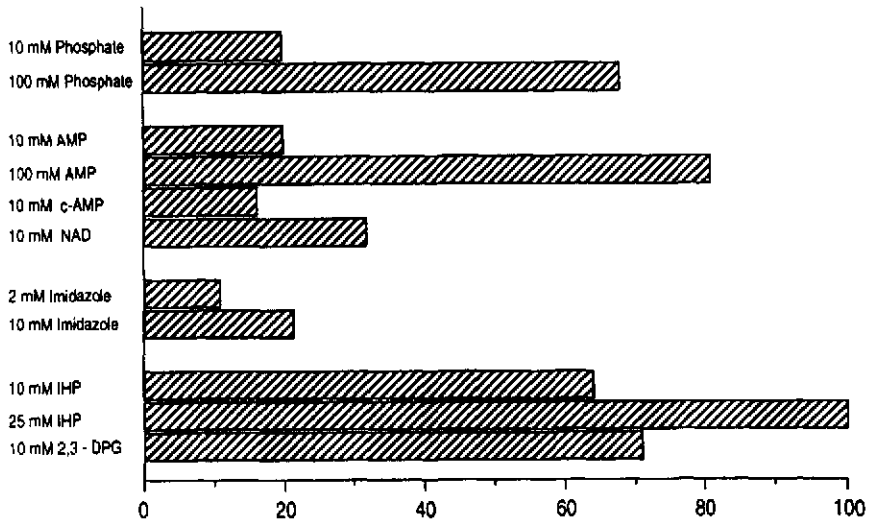

Fig. 7. Amounts of proteins eluted from the metal-stripped Rubine R/K-5BL column by various mobile phase additives (dissolved in buffer A). Numerical values (%) were determined as indicated in the text.

retained proteins from the demetallized dye, as did 100 m*M* AMP in buffer A from a metallized Rubine R/K-5BL column (see above).

We therefore believe that the affinity of Rubine R/K-5BL for IgG is mediated partly by electrostatic (and possibly hydrophobic) interactions, such as those commonly used to explain affinity of dyes for proteins, and partly by coordination to immobilized metal.

Substances such as AMP can probably compete with both types of interactions, as 0.1 *M* AMP also elutes IgGs from an immobilized metallized Rubine R/K-5BL column when dissolved in a low ionic strength buffer (see Fig. 4, bottom). In contrast, other substances probably interfere only with the metal participation in dye bonding to protein (*e.g.*, imidazole), or with the electrostatic components of the interaction (*e.g.*, IHP). We therefore tested new elution mixtures containing substances able to interfere with either component of the Rubine–proteins interactions. The aim was to obtain a non-UV-absorbing, inexpensive eluent that was more efficient than AMP. Buffer A containing 25 m*M* IHP and 10 m*M* imidazole eluted all the IgGs from immobilized (normally metallized) Rubine R/K-5BL. This result is remarkable when it is recalled that at the beginning of this work only urea-containing buffers were known to be able to elute IgGs.

3.6. Use of immobilized Rubine to purify IgGs and separate IgG subclasses

Immobilized Rubine R/K-5BL was not very useful for purifying IgGs from human plasma. Albumin and other proteins were bound to the support along with the IgGs and no eluent has yet been found to elute IgGs with sufficient selectivity to provide a one-step purification procedure (data not shown). In contrast, the dye is promising for separating IgG subclasses. Because the interaction of proteins with metals often involves histidine residues and IgG_2 has one histidine less than IgG_1 (the most abundant IgG subclass) in the constant regions of the γ chain [31], we attempted to separate IgG_2 from the bulk of IgGs on a Rubine R/K-5BL column. There is a need, for therapeutic purposes, for efficient techniques for preparing IgG_2-enriched IgG fractions. An immobilized Rubine R/K-5BL column was loaded with gel-filtered IgGs and developed with a gradient up to 100 m*M* AMP. There are some separation of IgG_2 (Fig. 8). These preliminary results are encouraging. Some

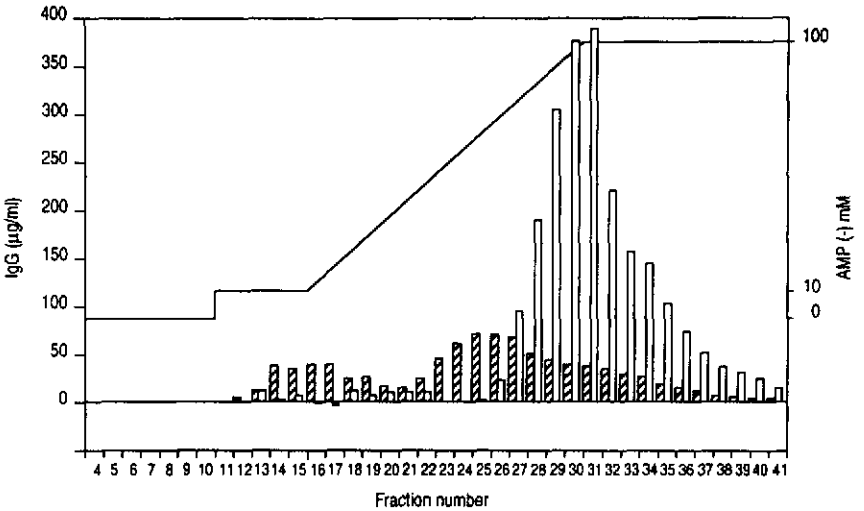

Fig. 8. Separation of IgG_2 from the other subclasses by chromatography on immobilized metallized Rubine R/K-5BL. The column (2.6 cm × 1.1 cm I.D.) was equilibrated and rinsed, after sample loading, with buffer A, then developed with a gradient of AMP dissolved in buffer A (the continuous line shows the gradient profile). The histogram shows the concentrations of IgG_2 (hatched bars) and of IgG of the other subclasses (empty bars) in the collected fractions.

other eluting agents, or mixtures of agents, could well be more efficient and cheaper than AMP.

4. General comments and conclusions

This report is not the first to emphasize the important role of metals in dye–protein interactions. Several papers have shown the dramatic effect of adding metals to mobile phases to promote the binding of proteins to dyes, at least for defined dye–protein pairs [32–35]. A ternary complex between protein, dye and metal seems to be formed in some instances that decreases significantly the dissociation constant of the protein and dye [32]. In other instances the metal could be bound to the dye (through coordination bonds with the azo bridge and sulphonate groups on the dye) [34,35] and this metal-loaded dye may display a greater affinity for the protein of interest. It was suggested that the interaction of metals "with functional groups appended to the dye chromophore stabilizes a particular conformation of the dye which is especially acceptable to the complementary protein" [35].

Proteins could be eluted from immobilized dyes with chelating agents, but all agents are not equally effective (in fact, some of them have little effect [34]). An enzyme retained on a column via a metal-mediated interaction could be eluted with two successive buffers [35]: the first, containing EDTA, presumably interfered with the component of dye–protein interaction due to the metal, and the second (a buffer with higher ionic strength and pH), pumped to the column immediately after the former eluent, presumably interfered with the usual, mostly ionic, component of the dye–protein interactions. This strategy is similar to that which we use to elute IgGs: the eluting mobile phase contained one solute directed against coordination bonds with metals (imidazole) and the other directed against classical dye–protein interactions (inositol hexaphosphate).

In the papers referred to above, the metal was deliberately added to the dye–protein pair. Metals can already be present in the reactive dye as received from the manufacturer: metallization of dyes is used in the dyes and pigments industry to improve light and wet fastness or to produce specific shades [30]. The role of metals in metallized dye–protein interactions have been evaluated by affinity partitioning, chromatography

and spectroscopy [36–39]. It was demonstrated that stripping metal from dyed polyethylene glycol can lead to loss of specificity of the partitioning (partitioning of lactate dehydrogenase) or the selectivity of partitioning can be greater with a metallized dye than after metal stripping (partitioning of glucose-6-phosphate dehydrogenase) [39]. The role of one histidine residue at the active site of alcohol dehydrogenase is crucial for binding of this enzyme to a copper-containing dye [36].

In conclusion, this paper has demonstrated that the systematic screening of eluents can be fruitful: while only denaturing agents were known to elute IgGs from immobilized Rubine R/K-5BL [15], systematic screening allowed the discovery of substances that allowed IgGs to be eluted efficiently in a native state. This screening used, as stated above, aromatic and charged substances, and phosphorylated sugars were also found (in this work and previously [25]) to be potentially useful eluents. Substances able to form coordination bonds with metals have been screened and should probably be systematically tried when a dye structure is unknown. Mixtures of eluents (each being able to compete with one kind of dye–protein interaction) can be very effective, as demonstrated here. Our results and previous work [36–39] demonstrate that interactions of proteins with metals contribute significantly to the affinity of proteins for metallized dyes. Metal stripping can modulate the interactions of proteins with a given immobilized dye. One can guess on the basis of published work [34,35] that changing the metal of a metallized dye could probably alter its properties in interacting with proteins. Rubine R/K-5BL, which is known as a useful reagent for purifying Fab [15], is also suitable for the IgG_2 enrichment of IgG preparations.

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