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Development of an enzyme-linked immunosorbent assay for C.I. Reactive Blue 2 and its application to a comparison of the stability and performance of a perfluorocarbon support with other immobilised C.I. Reactive Blue 2 affinity adsorbents

David J Stewart, Duncan R Purvis, Jennifer M Pitts and Christopher R Lowe

Institute of Biotechnology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QT (UK)

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ABSTRACT

An indirect competitive enzyme-linked immunosorbent assay capable of detecting the reactive triazine dye C I Reactive Blue 2 at concentrations down to 300 p*M* was developed, and representing a 3000-fold higher sensitivity over direct spectrophotometric analysis An investigation of the cross-reactivity of the polyclonal antibody against various compounds structurally related to C I Reactive Blue 2 revealed that the immunoassay appeared to be specific for anthraquinone-containing dyes and was largely unaffected by substitutions at the triazine ring These characteristics suggest that the immunoassay may be exploited to analyse the leaching of the ligand C I Reactive Blue 2 from dye-affinity adsorbents The performance of a novel perfluoropolymer affinity support containing C I Reactive Blue 2 was compared with eight other commercially available dyed affinity adsorbents by three separate criteria pressure–flow-rate characteristics, protein binding capacities and dye leakage under selected conditions All the affinity adsorbents were subjected to six purification cycles of albumin from human plasma prior to comparison In the pressure–flow-rate comparison, the perfluoropolymer support, in contrast to the other adsorbents, showed a non-linear relationship between pressure and flow-rate Human serum albumin dynamic load capacities were determined by frontal analysis and were found to be in the range 10 1–48 5 mg/ml The perfluoropolymer support displayed the lowest capacity, probably because of its lack of porosity and, consequently, low surface area In the dye leakage analysis, the perfluoropolymer support exhibited the lowest leakage of immobilised dye under all conditions tested including exposure to sodium isothiocyanate (5 *M*), hydrochloric acid (1 *M*) and sodium hydroxide (1 *M*) This study suggests that although the novel C I Reactive Blue 2–perfluoropolymer support displays low protein binding capacity, it otherwise compares very favourably with a range of commercially available dye-affinity adsorbents

INTRODUCTION

Immobilised textile dyes are being used increasingly as affinity adsorbents for the purification of biological macromolecules [1-7] The triazine dyes are robust molecules possessing a mixture of aromatic and charged moieties that bind relatively selectively to proteins at sites where natural substrates, inhibitors, cofactors and nucleotides interact The reactive chlorotriazine ring enables facile coupling of the dye to hydroxyl-containing chromatographic matrices, thereby allowing the preparation of versatile affinity adsorbents Thus, not suprisingly, immobilised triazine dyes are increasingly replacing natural biological ligands as adsorbents for affinity chromatography since their low cost and broad applicability coupled with their ease of immobilisation and resistance to chemical and biological degrada-

Correspondence to Dr C R Lowe, Institute of Biotechnology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QT, UK

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tion is particularly advantageous at process scale [7,8] However, the wider acceptance of triazine dyes as affinity ligands, particularly for the purification of high value therapeutic proteins, has been beset by concerns over dye leakage and resultant product contamination either through matrix breakdown or solvolysis of the matrix–ligand bond [1] There still remains a requirement for the development of novel chromatographic matrices and coupling chemistries that are able to withstand not only the high pressures used in high performance affinity chromatography, but also treatment with extremes of acid and alkali used for *in situ* column clean-up, chemical sanitisation and depyrogenation [9]

Perfluorocarbons are chemically and biologically inert, high density (1 8–2 1 g/ml), thermally stable synthetic materials that are totally insoluble in aqueous and organic solvents They are extremely hydrophobic and do not immediately appear to be materials suitable for biochromatography However, effective affinity adsorbents can be synthesised by wetting the surfaces of solid or liquid perfluoropolymers with perfluoroalkylated dyes Thus, the preparation of several bis-perfluoroalkylated dichlorotriazinyl dyes and their subsequent adsorption to a perfluoropolymer matrix and application to the purification of lactate dehydrogenase from a crude rabbit muscle extract by affinity chromatography has been reported [10] These materials, however, were still prone to ligand leakage on exposure to organic solvents or harsh aqueous conditions Since the extent of leakage appeared to depend on the number and length of perfluorocarbon anchoring groups, attachment of the triazine dye to an inert hydrophilic polymer containing a number of pendant perfluorinated anchoring groups should provide a highly stable adsorbed polymeric coating on the perfluoropolymer matrix The synthesis of perfluoroalkylated polyvinyl alcohol, its immobilisation onto a perfluoropolymer matrix and subsequent derivatisation with C I Reactive Blue 2 and its application to the purification of rabbit muscle lactate dehydrogenase and human serum albumin by affinity chromatography has recently been reported [11] The adsorbent exhibited negligible non-specific adsorption and similar capacities and degrees of purification as previously reported for dyed silica [12] Furthermore, a long-term study lasting more than nine months was unable to detect dye leakage spectrophotometrically on exposure of the perfluoropolymer adsorbents to water, sodium hydroxide (1 *M*), hydrochloric acid (1 *M*), urea (1 *M*) or acetone, suggesting that this novel affinity adsorbent might compare very favourably with commercially available dyed affinity adsorbents

Although the textile dyes are intensely coloured, spectrophotometric analysis is limited to a detection limit of 10^{-6} *M* if no prior concentration of the leachate is carried out, in practice, sample turbidity may reduce the sensitivity even further Consequently, the development of a sensitive assay to detect the leakage of ligands, particularly the widely used triazine dye C I Reactive Blue 2, from affinity adsorbents, is now long overdue The development of an enzyme-linked immunosorbent assay (ELISA) [13] for C I Reactive Blue 2 would not only greatly facilitate the assessment of adsorbent stability under operational conditions, but also permit the level of contamination of injectable therapeutic products by leachates to be quantitatively determined

This paper describes the development and application of an enzyme-linked immunoassay for C I Reactive Blue 2 to compare the leakage of dye from the perfluoropolymer support with a number of commercially available C I Reactive Blue 2-containing affinity adsorbents Adsorbent stability was analysed under a variety of conditions and care was taken to treat the materials identically by pre-treating all media with six human serum albumin purification cycles prior to the leakage study In order to offer a comprehensive assessment of the performance of the materials, two further comparisons were undertaken flow-rate was measured as a function of pressure for all the materials, which included inorganic, organic and several agarose-based materials, together with the coated perfluoropolymer adsorbent In addition, the dynamic load capacity of these adsorbents for human serum albumin was measured by frontal analysis to evaluate the performance of the perfluoropolymer support compared to other adsorbents A similar comparative study of commercially available Protein A affinity adsorbents has recently been reported [14]

MATERIALS AND METHODS

Materials

C I Reactive Blue 2, ovalbumin, bovine serum albumin (fraction V, 96–99%), goat anti-rabbit immunoglobulin G(IgG)–peroxidase conjugate were all purchased from Sigma (Poole, UK) *ortho*-Phenylenediamine was purchased from Dakopatts (High Wycombe, UK) while Falcon 3912 Micro test III flexible microtitre plates were obtained from Beckton Dickinson Labware (Oxnard, CA, USA) Sephadex LH-20, Sephadex G-25 (disposable PD-10 columns) and Mono Q (HR5/5) were obtained from Pharmacia (Milton Keynes, UK) Bromaminic acid was from Sandoz (Basle, Switzerland) and other compounds used in cross-reactivity studies were a kind gift from Dr Steven Burton of the University of Cambridge The following affinity adsorbents were purchased either from manufacturers or suppliers Mimetic Blue 1 A6XL [Affinity Chromatography, (ACL), Isle of Man, UK], Affigel Blue gel (Bio-Rad, UK), Blue-Trisacryl M (IBF, France), Fractogel TSK AF-Blue (Merck, Germany), Blue Sepharose CL-6B (Pharmacia, UK), immobilised Cibacron Blue F3GA (Pierce, UK), Cibacron Blue F3GA = Si500 (Serva, GDR) and C I Reactive Blue 2 Sepharose CL-6B (Sigma, Poole, UK) The particulate perfluoropolymer matrix (6–8m^2/g) was kindly provided by DuPont (Wilmington, DE, USA) Polyvinyl alcohol (average M_r 14 000, 100% hydrolyzed) was purchased from Sigma, perfluorooctanoyl chloride was bought from Fluorochem (Old Glossop, UK) and the triazine dye C I Reactive Blue 2 and acetone (SLR) were from BDH (Poole, UK) Human plasma was obtained from a known donor at the National Blood Transfusion Centre (Nottingham, UK) and tested HIV III$^-$, HBS antigen$^-$ and syphilis$^-$ All other chemicals/biochemicals were of analytical grade and were obtained from Sigma All chromatography was carried out using a Pharmacia FPLC system fitted with two P-500 pumps, twin MV-8 and single MV-7 valves, 50-ml superloop, UV-1 monitor and LCC 500 controller

Preparation of dye-protein conjugates for immunoassay

Conjugates of C I Reactive Blue 2 with ovalbumin and bovine serum albumin were both prepared as follows Crude C I Reactive Blue 2 was purified on Sephadex LH-20 equilibrated with methanol–water (50 50, v/v) according to the method of Pearson *et al* [15] Solid purified dye (200 mg) was added to a solution of protein (100 mg) in 0 2 *M* sodium bicarbonate solution, pH 9 0, (5 ml) and the mixture stirred for 15 h at 36°C, after which unreacted dye was removed by gel filtration on PD-10 columns equilibrated with distilled water The coloured fraction appearing in the void volume was freeze-dried and stored at −20°C The molar ratio of dye to protein in the conjugate was calculated from the absorbance at 620 nm of a solution containing a known weight of conjugate, assuming a molar extinction coefficient of 12 700 M^{-1} cm^{-1} for C I Reactive Blue 2 and molecular masses of 840, 44 000 and 68 800 for C I Reactive Blue 2, ovalbumin and bovine serum albumin, respectively

Preparation of rabbit anti-C I Reactive Blue 2 antibody

Rabbits (New Zealand White, 2 5 kg) were immunised with C I Reactive Blue 2–bovine serum albumin conjugate (11 8 mol dye / mol protein) Primary immunisation was by subcutaneous injections of conjugate made 2 mg/ml in sodium chloride (0 85%, w/v) and emulsified in an equal volume of Freund's Complete adjuvant (100 μg conjugate, total of 2 injections per rabbit) Subcutaneous booster injections of conjugate prepared as before in Freund's Incomplete adjuvant (200 μg conjugate, 2 injections per rabbit) were given six weeks later, followed by subcutaneous booster injections of conjugate made 1 mg/ml in sodium chloride solution (0 85%, w/v)(200 μg conjugate, 2 injections per rabbit) every six weeks Blood was collected from the marginal ear vein two weeks after booster injections and prepared sera were kept frozen at −20°C

Purification of rabbit IgG by ion-exchange chromatography

Buffers A (20 m*M* Tris–HCl, pH 9 0) and B (20 m*M* Tris–HCl, 1 *M* NaCl, pH 7 7) were filtered through 0 2-μm filters and degassed Rabbit sera from two immunised rabbits were transferred into buffer A using disposable PD-10 columns according to the manufacturer's instructions, filtered through 0 2-μm filters and then samples (1 ml) loaded on to a Mono Q anion-exchange column installed in a

comprehensive Pharmacia fast protein liquid chromatography (FPLC) system The salt gradient was applied over a total volume of 18 ml and fractions (1 ml) were collected, assayed for antibody titre using a standard ELISA, aliquotted where appropriate and kept frozen at −20°C

Determination of antibody titre and appropriate antiserum dilution for indirect competitive ELISA

Measurement of antibody titres and determination of appropriate antibody concentration for the indirect competitive enzyme immunoassay were carried out simultaneously In summary, plates precoated with dye–ovalbumin conjugate (4 5 mol dye/mol protein, 10 μg/ml) were incubated with serial dilutions of purified IgG fractions of rabbit antiserum to C I Reactive Blue 2, and following exhaustive washing, with excess anti-rabbit IgG-peroxidase conjugate Maximal binding of anti-dye antibody to the dye hapten was observed for dilutions above 800-fold The antibody dilution for use in the competitive assay was chosen as the dilution at which approximately 70% of maximal antibody binding occurs, thereby providing non-saturated conditions in which added dye competes directly with bound dye for antibody binding

To identify both the fraction containing the highest titre of specific antibody and the appropriate dilution of this fraction for subsequent assays, antigen-coated microtitre plates were prepared by incubating wells with a solution of C I Reactive Blue 2–ovalbumin conjugate (10 μg/ml) in 50 m*M* carbonate, pH 9 6, overnight at 4°C (100 μl/well) Plates were blocked with Marvel (3%, w/v) in phosphate-buffered saline (PBS) containing 0 1% (w/v) Tween 20 (PBST) at 37°C for 2 h (blocking buffer, BB)(200 μl/well) and then thoroughly washed six times (with two 3-min soaks) with 0 9% (w/v) NaCl containing 0 05% (w/v) Tween 20 (washing buffer, WB) Dilutions (1/50) of crude sera and purified fractions from both rabbits were prepared in PBST containing 0 1% (w/v) gelatin (PBST/G) and serially diluted (1 2) across the microtitre plates After 1 h incubation at 37°C, plates were washed as before with WB after which goat anti-rabbit IgG–horse radish peroxidase conjugate diluted 1/1000 in PBST/G was added to the plates (50 μl per well), followed by a further incubation step of 1 h at 37°C *ortho*-Phenylenediamine was made 1 m*M* in 0 1 *M* citric acid–phosphate buffer, pH 5 0 (substrate buffer, SB) and the plates were washed three times with WB followed by distilled water Hydrogen peroxide (30%)(5 μl) was added just prior to addition of the freshly prepared substrate (50 μl/well) After 30 min incubation in the dark at room temperature, 1 *M* H_2SO_4 (50 μl/well) was added to stop the reaction and the absorbances of the wells read at 492 nm using a Titertek ELISA plate reader

Indirect competitive enzyme-linked immunoassay

An indirect competitive assay was used in order to detect free dye In this procedure, free antigen was serially diluted (1 2) across the plate Standard curves were obtained using a series of C I Reactive Blue 2 standards (10 μ*M* to 1 n*M*) serially diluted (50 μl/well) across the plate to which was added a standard aliquot of appropriately diluted antibody (50 μl/well) in 2 × PBST/G Plates were agitated at room temperature for 15 min and then incubated at 37°C for 1 h as before Bound antibody was detected as previously described Unknown samples were similarly diluted and the curves generated compared with standard curves to yield values of the concentration of C I Reactive Blue 2 in the unknown samples Assays were typically carried out in triplicate

The effect on assay sensitivity of antigen coating concentration was studied by incubating microtitre plates with dilutions of antigen ranging from 0 01–10 μg/ml using a lower-substituted dye–ovalbumin conjugate (1 14 mol dye/mol protein) Standard dye solutions were used to assess the sensitivity of the assay depending on the extent of antigen coating

Cross-reactivity of anti-C I Reactive Blue 2 antibody with various analogues and structural fragments of C I Reactive Blue 2

All compounds were made 5 m*M* in water and serial dilutions were prepared as for standard C I Reactive Blue 2 after which the appropriate dilution of antibody was added in 2 × PBST/G The concentration of compound required to yield an absorbance of 1 0 at 492 nm was estimated from the linear region of the antibody binding curves Comparison with the C I Reactive Blue 2 concentration yielded the relative cross-reactivity of the compound for anti-C I Reactive Blue 2 antiserum Assays were performed in triplicate

Synthesis of C I Reactive Blue 2 polyvinyl alcohol-coated perfluoropolymer support Modification of the perfluorocarbon matrix

C I Reactive Blue 2 was immobilised to the polyvinyl alcohol-coated perfluoropolymer support according to the procedure described in ref 11 Immobilised ligand content was estimated according to a previously described procedure [11]

Preparation of affinity adsorbents

Commercial adsorbents were initially prepared as recommended by the manufacturers Where no recommendations were available, gels were mixed/swollen with approximately 50 volumes distilled water, washed with 50 volumes 50 m*M* phosphate buffer, pH 7 0 and then packed in Pharmacia HR5/10 columns (2 ml) Thereafter, experiments were conducted on each 2 ml packed sample in a sequential manner (i) pretreatment, (ii) determination of albumin capacity by frontal analysis, (iii) leakage of dye in buffer, and (iv) leakage of dye in rigorous conditions Flow-rate *versus* pressure analyses were carried out in packed Pharmacia HR5/10 columns using prepared but untreated gels which were discarded after use

Flow-rate versus pressure analysis

Prior to adsorbent packing, all end-frits were sonicated in 1 *M* hydrochloric acid After packing, adsorbent flow-rates were generated using Pharmacia's FPLC system with a safety cut-off at 4 5 MPa Values for flow-rate (ml/min) were made independent of column geometry by dividing by the cross-sectional area of the HR5/10 columns

Pretreatment of affinity adsorbents

Adsorbents were pre-equilibrated in 20 m*M* acetate buffer, pH 5 5 Human plasma was diluted 1/4 in this buffer, filtered through 0 2-μm filter, the pH adjusted to 5 5 with 0 1 *M* hydrochloric acid and 1 ml samples ($\approx$20 mg protein) loaded onto each column Bound protein was eluted with 1 *M* potassium chloride in 20 m*M* phosphate, pH 8 0 in a total volume of 5 ml Each column was then re-equilibrated with 20 m*M* acetate buffer, pH 5 5 and diluted plasma loaded once more In all, each column underwent six plasma load–wash–elute–re-equilibration cycles Columns were run at 1 ml/min, with the exception of Bio-Rad's Affi-Gel Blue matrix which was run at 0 25 ml/min

Dynamic load capacity for human serum albumin

Dynamic load capacities of the adsorbents were determined according to the method described in ref 16 Human serum albumin-binding capacity was measured by pumping 10 mg/ml pure albumin in 20 m*M* acetate buffer, pH 5 5, until the absorbance at 280 nm of the output and input streams were identical Protein loading was halted, the column washed with buffer until the eluent contained negligible protein by absorbance at 280 nm and bound albumin was then eluted with potassium chloride (1 *M*) in 20 m*M* phosphate buffer, pH 8 0, the recovery of bound protein was determined by absorbance at 280 nm Protein concentrations were determined by absorbance at 280 nm assuming an extinction coefficient for pure human serum albumin of 0 53 ml mg^{-1} cm^{-1} [17]

Dye leakage detection by enzyme immunoassay

In order to detect free dye, the indirect competitive ELISA was used A standard curve was obtained using a known solution of C I Reactive Blue 2 (5 μM) serially diluted across the microtitre plate Unknown samples were serially diluted six-fold across identically coated microtitre plates and curves generated were fitted to the standard curve to yield values of the concentration of C I Reactive Blue 2 in each sample For this study conditions were adjusted to detect unknown dye concentrations from the limits of spectrophotometric detection (1 μ*M*) down to 3 n*M* in order to maximise the possible range of detection Where dye was visible in the initial sample, the sample was diluted ten-fold before being serially diluted across the microtitre plate Acidic and basic samples were neutralised with small aliquots of strong base or acid High chaotrope concentrations which interfered with the immunoassay could not be easily removed by techniques such as gel filtration without affecting the dye concentration After thorough investigation, it was concluded that antibody binding in serial dilutions containing below 320 m*M* NaSCN was identical to controls in the absence of chaotrope Therefore, samples containing 5 *M* NaSCN were initially diluted 1 1 with antibody in the first well, yielding a chaotrope concentration of 2 5 *M* NaSCN, and were then serially diluted six-fold across the plate, only the final three wells, containing 313, 157 and 79 m*M* NaSCN, respectively, were used for the analysis of those samples

Dye leakage in buffer

Packed gels (2 ml bed volume) were thoroughly washed with 50 m*M* phosphate buffer, pH 7 0 (50 column volumes) and left to stand in this buffer at room temperature (72 h) Buffer (10 ml) was run through the columns, collected and assayed for leakage of dye by competitive ELISA Leakage data were corrected to give values for the average daily loss of dye from the affinity adsorbents

Dye leakage in rigorous conditions

Affinity adsorbents were sequentially exposed to three increasing concentrations of chaotrope (NaSCN), acid and base, after each exposure, the gel was immediately washed with an equal volume of distilled water, and then left while the next adsorbent was challenged Each gel was thus exposed to 10 ml volumes of 0 5 *M* NaSCN, 0 1 *M* HCl, 0 1 *M* NaOH, 1 25 *M* NaSCN, 0 25 *M* HCl, 0 25 *M* NaOH, 5 *M* NaSCN, 1 *M* HCl and 1 *M* NaOH, each exposure followed immediately by a 10 ml water wash and a pause of 140 min before the next challenge All fractions were collected, neutralised where necessary and assayed for C I Reactive Blue 2 by competitive ELISA

RESULTS AND DISCUSSION

ELISA development

Purification and characterisation of rabbit IgG to C I Reactive Blue 2 The fraction containing the highest antibody titre against C I Reactive Blue 2 was purified from the crude antiserum of one New Zealand White rabbit by anion-exchange chromatography and assayed by standard ELISA Fig 1A shows the standard dilution curve for this fraction incubated with bound C I Reactive Blue 2–ovalbumin conjugate (10 μg/ml, 4 5 mol dye/mol protein) and shows that a 1/3200 dilution gives approximately 70% maximal binding of antibody Therefore all competitive assays were carried out using a 1/3200 dilution of the highest titre fraction Fig 1B

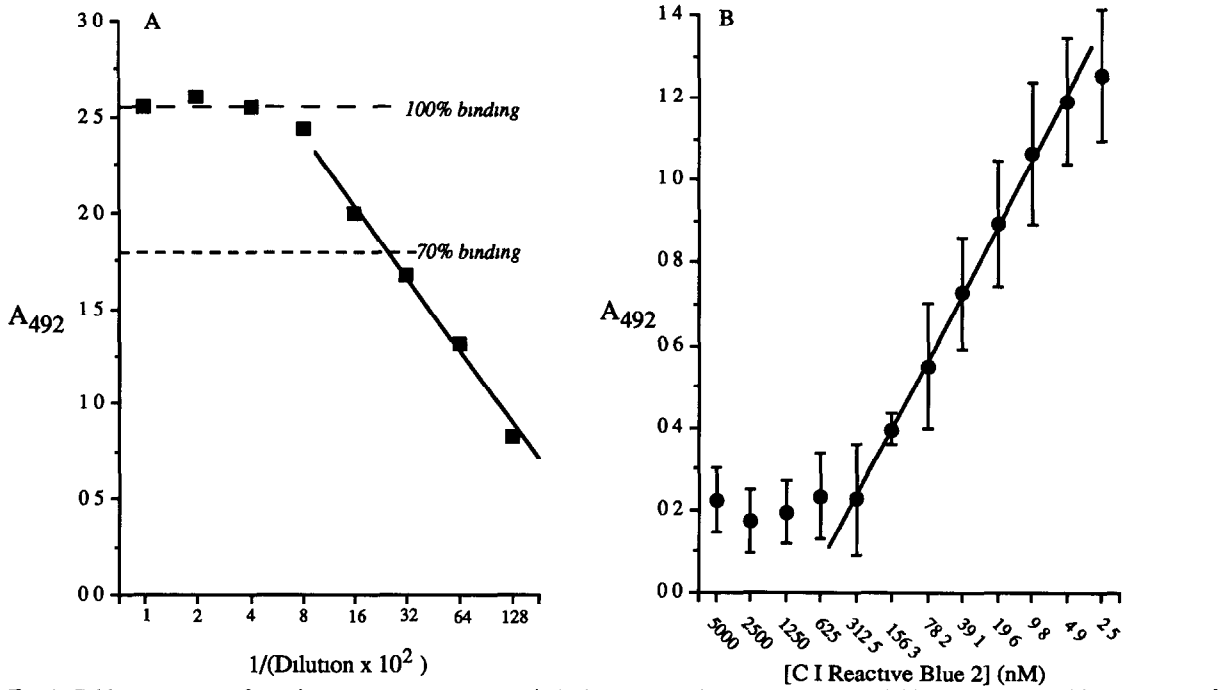

Fig 1 Calibration curves for indirect competitive enzyme-linked immunosorbent assay (A) Serial dilutions of purified IgG fraction of rabbit antiserum to C I Reactive Blue 2 incubated on plates pre-coated with dye–ovalbumin conjugate (4 5 mol dye/mol protein, 10 μg/ml) Values shown represent actual measurements (B) Serial dilutions of C I Reactive Blue 2 incubated together with 1/3200 dilution of purified IgG fraction on identical plates to (A) Values shown represent means and standard deviations of triplicate samples

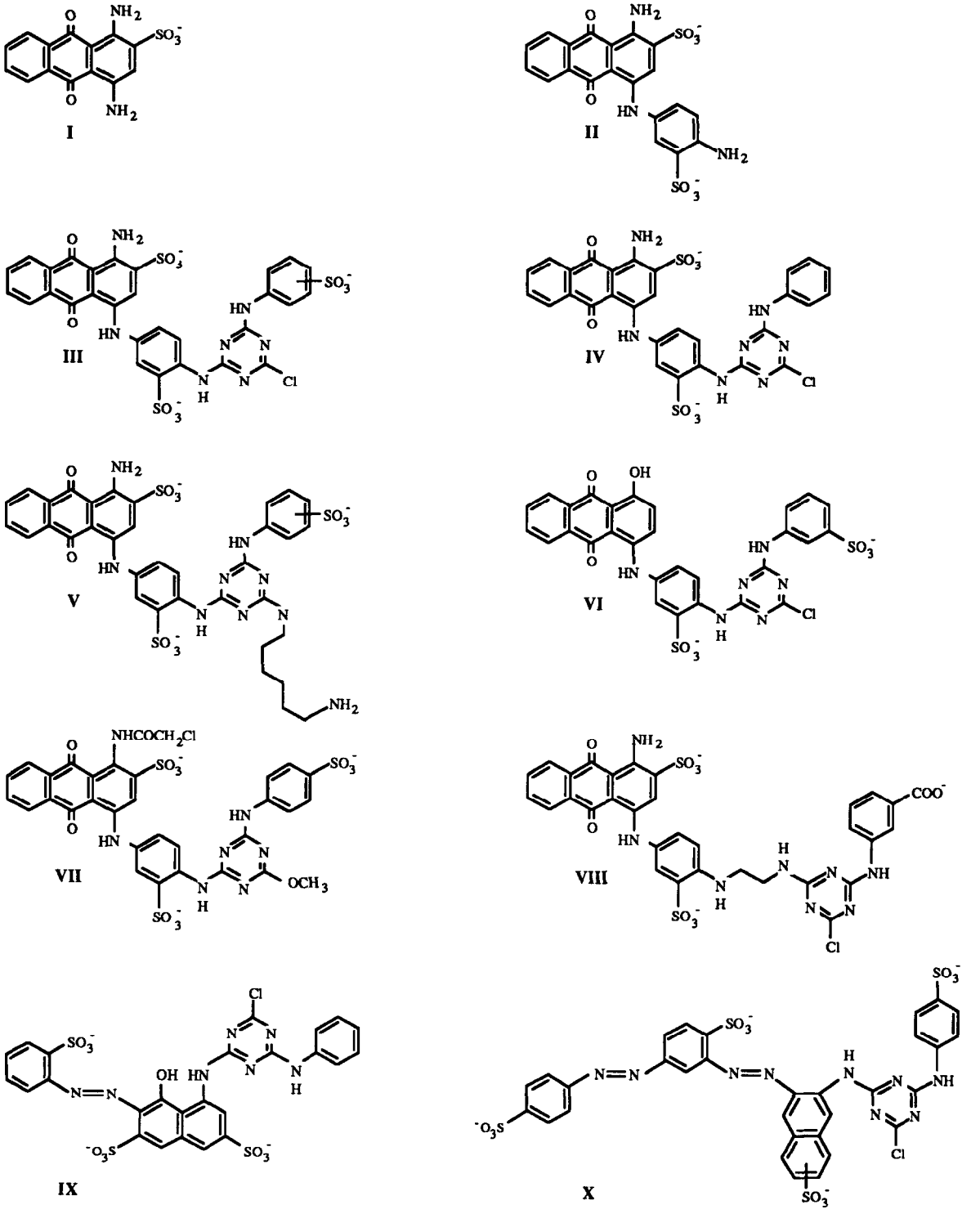

Fig 2 Structures of fragments and analogues of C I Reactive Blue 2 (**I**) = 1,4-diaminoanthraquinone, **II** = Blue base, **III** C I Reactive Blue 2, **IV** = *anilino*-C I Reactive Blue 2, **V** = 1,6- diaminohexane-*m*-C I Reactive Blue 2, **VI** = 1-hydroxy-2-desulpho-*m*-C I Reactive Blue 2, **VII** = acryloyl anthraquinone-methoxy-*p*-C I Reactive Blue 2, **IX** = Procion red H-3B, **VIII** = *m*-benzoic-*s*-triazino-1,2-ethylenediamine-blue base, **X** = Procion brown H-2G All compounds purified by Sepharose LH-20 chromatography according to Lowe and Pearson [2]

shows the mean standard curve and deviation for C I Reactive Blue 2 using the indirect competitive assay across a broad range of dye concentrations (2 4 n*M*–5 μ*M*) The assay is linear between 5 and 300 n*M* C I Reactive Blue 2 under the conditions used

Sensitivity of the competitive ELISA for C I Reactive Blue 2 Analysis of the potential sensitivity of the indirect competitive ELISA was performed using a lighter substituted dye–protein conjugate (1 14 mol dye/mol protein) and a range of coating concentrations down to 10 ng conjugate/ml The sensitivity of the assay increased as the amount of bound antigen decreased and under certain conditions the assay could detect dye concentrations down to 300 p*M* The amount of bound antibody did, however, decrease with lower antigen coating concentrations, producing a lower absorbance value against a fixed background (the plate itself) and thereby more potential for error Amplification systems such as those based on the biotin–avidin system [18] may be expected to increase the sensitivity further This aspect was not investigated since, for our purposes, the assay was required to measure a broad range of dye concentrations up to spectrophotometric detection limits (1 μ*M*) and this necessitated choosing a higher concentration of coating antigen (10 μg/ml) with concomitant loss of sensitivity (>3 n*M*, ≈3 ng/ml)

Specificity of the competitive ELISA for C I Reactive Blue 2 The specificity of the immunoassay was studied by measuring the ability of structural fragments and analogues of C I Reactive Blue 2 and other reactive dyes to block antibody binding of C I Reactive Blue 2-coated microtitre plates Fig 2 shows the structures of some compounds tested for their cross-reactivity with antibody to the parent dye Structures **I** and **II** are intermediates in the synthesis of C I Reactive Blue 2 (**III**), while **IV** is an unsubstituted terminal ring isomer of the dye [19] Compound **V** represents the parent dye substituted at the triazine ring with 1,6-diaminohexane, a bridging or spacer group commonly used in affinity chromatography to separate the affinity ligand from the matrix backbone Compounds **VI** and **VII** each possess substituted anthraquinone moieties, though the latter retains the charged anthraquinone sulphonate group **VIII** is essentially an analogue of C I Reactive Blue 2 possessing an 1,2-ethylenediamine spacer group between the blue base moiety (**II**) and the triazine ring Compounds **IX** and **X** represent two azo triazine dyes, Procion Red H-3B and Procion Brown H-2G respectively, that do not contain the anthraquinone group

TABLE I

CROSS-REACTIVITY OF CERTAIN FRAGMENTS AND STRUCTURAL ANALOGUES OF C I REACTIVE BLUE 2 IN ELISA

Microtitre plates were presented with dye–ovalbumin conjugate (4 5 μmol dye/μmol protein, 10 μg/ml) Plates were incubated with serial dilutions of 5 μ*M* stocks of analogues together with a 1/3200th dilution of purified IgG factor of rabbit antiserum to C I Reactive Blue 2 Values shown represent the means of triplicate samples

Compound	No	Concentration[a] (n*M*)	Relative cross-reactivity (%)
1,4-Diaminoanthraquinone	**I**	360	3 6
Blue base	**II**	32	40 6
C I Reactive Blue 2	**III**	13	100
anilino-C I Reactive Blue 2	**IV**	24	54 2
1,6-Diaminohexane-*m*-C I Reactive Blue	**V**	14	92 9
1-Hydroxy-2-desulpho-*m*-C I Reactive Blue 2	**VI**	180	7 2
Acryloyl anthraquinone-methoxy-*p*-C I Reactive Blue 2	**VII**	180	7 2
m-Benzoic-*s*-triazino-1,2-ethylenediamine-blue base	**VIII**	17	76 5
Procion red H-3B	**IX**	5000	0 3
Procion brown H-2	**X**	3300	0 4

[a] These values represent that concentration of each compound yielding an absorbance at 492 nm of 1 0 in the indirect competitive ELISA

The extent of cross-reactivity of a compound with C I Reactive Blue 2 was estimated by comparing the concentration of competing dye analogue necessary to produce an absorbance of 1 0 at 492 nm relative to the required concentration of parent dye The concentration was estimated from the linear region of the mean antibody binding curve for each compound Table I shows that dye analogues (**II, IV, V** and **VIII**) containing the structural motif comprising a 1,4-diaminoanthraquinone ring coupled to a *p*-diaminobenzene sulphonate such as C I Reactive Blue 2 itself (**III**) are potent inhibitors of the immune binding reaction Modification of this structural motif either by removal of the *p*-diaminobenzene sulphonate ring (**I**) or by alteration of the anthraquinone ring itself (**VI** and **VII**) reduce the potency of the competitive binding analogue by at least an order of magnitude Moreover, dyes altogether lacking an anthraquinone group (**IX** and **X**) showed reduced binding by over two orders of magnitude That these dyes cross-react to some extent with antibodies raised to C I Reactive Blue 2 suggests that some weak antigenic determinancy resides in the triazine and terminal sulphonated rings This is supported by the lowered immune response exhibited by compound **IV** that lacks a terminal sulphonate negatively-charged groups are thought to play an important role in antigenic determinancy [20]

The observation that the predominant antigenic determinant of C I Reactive Blue 2 appears to be the structural motif comprising anthraquinone and *p*-diaminobenzene sulphonate rings may depend in large part on the manner of synthesis of the dye–protein conjugate used for immunisation C I Reactive Blue 2 is coupled to the carrier protein by nucleophilic substitution, through, for example, the ε-amino group of a surface lysine residue of bovine serum albumin, at the triazine ring and, therefore, this region of the hapten is least accessible for recognition by the immune system Availability of the anthraquinone and *p*-diaminobenzene sulphonate rings for interaction with antibody has thus conferred the desired specificity on the polyclonal antibody Substitution at the triazine ring, whether by 1,6-diaminohexane or a larger molecule such as degraded fragments of the agarose matrix, should thus have little effect on the recognition of C I Reactive Blue 2 by the antibody Therefore, the rabbit antibody may be fruitfully used to detect low concentrations of both free C I Reactive Blue 2 and derivatives in which the leached dye is attached to matrix fragments via the triazine ring without concentration of the eluent or any other prior treatment of the sample

Evaluation of the adsorbents

Evaluation of the overall performance of an affinity adsorbent for any particular application must include parameters such as mechanical rigidity, capacity for protein binding, and stability under selected conditions These operational parameters determine the maximum permissible flow-rate through the adsorbent, the protein throughput and the degradation of the adsorbent during use and regeneration

Flow-rate versus pressure analysis The two immobilised dye adsorbents based on silica and cross-linked polyvinyl alcohol, Cibacron Blue F3GA Si = 500 and Fractogel TSK AF-Blue, respectively, display a linear relationship between flow-rate and

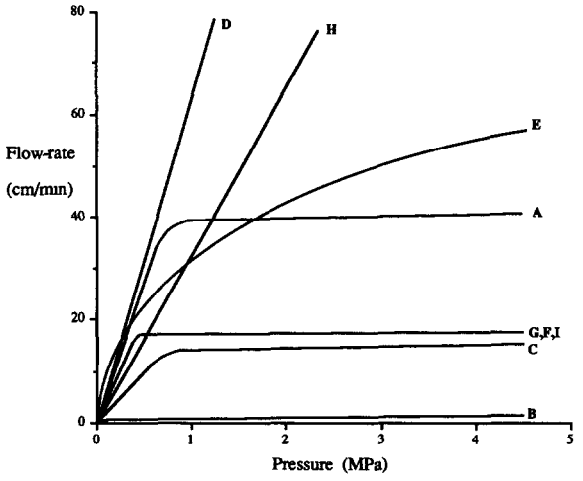

Fig 3 Flow-rate *versus* pressure curves for C I Reactive Blue 2-containing affinity adsorbents Adsorbents were packed in Pharmacia HR 5/10 columns (2 ml) and flow-rates measured as a function of applied pressure and divided by the column cross-sectional area Adsorbents are as follows A = Mimetic Blue A6XL (ACL), B = Affi-Gel Blue (Bio-Rad), C = Blue Trisacryl-M (IBF), D = Fractogel TSK AF-Blue (Merck), E = C I Reactive Blue 2 polyvinyl alcohol-coated perfluorocarbon matrix, F = Blue Sepharose CL-6CB, G = Immobilized Cibacron Blue F3GA (Pierce), H = Cibacron Blue F3GA = Si 500 (Serva), and I = Reactive Blue 2-Sepharose CL-6B (Sigma)

pressure that is characteristic of "hard" chromatography gels and suggests that both materials are suitable for use as high performance media (Fig 3) In contrast, agarose-based and acrylate-based materials only exhibit this proportionality at low pressures, with further increases in pressure beyond a characteristic point producing no further increase in flow-rate, this observation suggests that compression of the gel is occurring, with flow through the column actually decreasing in some cases if further pressure is applied Maximal flow-rates through the agarose-based materials varied from less than 2 cm/min for Affi-Gel Blue to almost 40 cm/min for MIMETIC Blue 1 A6XL and appeared to depend on the extent of cross-linking of the matrix Affi-Gel Blue probably represents native agarose, with Blue Sepharose CL-6B, Immobilised Cibacron Blue F3GA and Reactive Blue 2-Sepharose CL-6B being examples of mildly cross-linked materials, while the rigidity of MIMETIC Blue 1 A6XL must be attributable to a substantial degree of cross-linking In contradistinction to these affinity adsorbents, the coated perfluoropolymer adsorbent exhibits a nonlinear relationship between flow-rate and pressure with an extrapolated working maximum of approximately 80 cm/min This suggests that the perfluoropolymer matrix undergoes slow compression as the pressure is increased Furthermore, it has been observed that the amount of adsorbed polymer coating per unit weight of base matrix also appears to play a part in the pressure-flow-rate performance of the perfluoropolymer adsorbent support Thinner polymeric coatings permit the application of higher flow-rates, although there remains a requirement for sufficient adsorbed polymer to enable immobilisation of the affinity ligand to the matrix and to prevent nonspecific adsorption of protein

Dynamic load capacity for human serum albumin Table II displays the suppliers' figures, where available, for immobilised ligand concentrations per ml of affinity adsorbent together with their measured dynamic load capacities for human serum albumin per ml of each affinity adsorbent Dynamic load capacities do not appear to correlate with ligand densities, since ligand usage (μmol albumin/μmol dye) ranging from 7 8% for the C I Reactive Blue 2 polyvinyl alcohol-coated perfluoropolymer to 31 1% exhibited by Immobilised Cibacron Blue F3GA This difference may result from the fact that while both these gels have comparable ligand densities per unit wet weight (1 9 and 2 0 μmol/g, respectively), the ligand density per unit surface area of the perfluoropolymer adsorbent is much higher because of its low total surface area, and consequently, less protein may be bound per unit volume of adsorbent Use of alternative perfluoropolymer matrices with higher total surface areas would be expected to offer greater protein binding capacities without a requirement for increasing the ligand concentration per unit wet weight of matrix

A further point of interest arising from the capacity data given in Table II is the considerable difference in albumin binding capacities exhibited by the 6% cross-linked agaroses, immobilised Cibacron Blue F3GA, Blue Sepharose CL-6B and Reactive Blue 2-Sepharose CL-6B, these gels exhibited near-identical flow-pressure curves and contained equivalent concentrations of immobilised C I Reactive Blue 2 This observation may reflect the use of an alternative coupling chemistry in the preparation of immobilised Cibacron Blue F3GA Similarly, the substantial albumin-binding capacity of MIMETIC Blue 1 A6XL even over the less cross-linked Affi-Gel Blue may result from the manufacturer's acknowledged use of a spacer-arm to separate the ligand from the matrix and facilitate interaction with the complementary protein [21]

Dye leakage in buffer The dye leakage figures (pmol/day) of the adsorbents shown in Table II were determined after six successive human serum albumin purification cycles, each of which included equilibration, albumin challenge, buffer wash, elution with 1 *M* potassium chloride and re-equilibration These conditions would be expected to displace any unbound dye remaining after implementation of the manufacturers' washing procedures Thus, the leakage of dye from Affi-Gel Blue, Blue Trisacryl-M and Immobilised Cibacron Blue F3GA must represent a realistic daily loss arising either through hydrolysis of the dye–matrix bond or the matrix itself In context, however, this means that even Affi-Gel Blue, the adsorbent displaying the highest rate of ligand loss at 0 0009%/day, would lose less than 0 33% of its immobilised dye per year when standing at room temperature in 50 m*M* phosphate buffer at pH 7 0 No leakage of dye was detectable from the coated perfluoropolymer adsorbent under these conditions

TABLE II

LIGAND CONCENTRATION, HUMAN SERUM ALBUMIN CAPACITY AND STABILITY IN BUFFER OF C I REACTIVE BLUE 2-CONTAINING AFFINITY ADSORBENTS

Adsorbent[a]	Manufacturer/supplier	Ligand content[b] (μmol/ml)	Human serum albumin capacity[e] (mg/ml)	Dye leakage (pmol/day)	Immobilised dye loss (%loss/day)
Mimetic Blue 1 A6XL	ACL	2 5 ± 0 2	48 9	U[f]	–
Affi-Gel Blue	Bio-Rad	>2 3[c]	45 5	43	<0 0009
Immobilised Cibacron Blue F3GA	Pierce	≈2 0	42 3	17	0 0004
Fractogel TSK AF-Blue	Merck	–[d]	40 6	U	–
Cibacron Blue F3GA-silica	Serva	–[d]	33 4	U	–
Blue Trisacryl-M	IBF	≈4 0	29 7	27	0 0003
Blue Sepharose CL-6B	Pharmacia	≈2 0	25 4	U	–
Reactive Blue 2-Sepharose CL-6B	Sigma	≈2 0	23 2	U	–
Blue polyvinyl alcohol-coated perfluorocarbon adsorbent	–	1 9 ± 0 2	10 1	U	–

[a] Packed adsorbents (2 ml) were washed with 50 m*M* phosphate buffer, pH 7 0 (50 column volumes) and then allowed to stand for 72 h in this buffer Further buffer (10 ml) was then added, collected and assayed for C I Reactive Blue 2 by competitive ELISA

[b] Manufacturer/suppliers' figures

[c] Calculated assuming formule weight for C I Reactive Blue 2 = 840

[d] Figures not supplied

[e] Albumin capacities were measured by frontal analysis of 10 mg/ml human serum albumin in 50 m*M* sodium acetate, pH 5 5

[f] Undetectable (< pmol/day)

Dye leakage in rigorous conditions Leakage of immobilised ligand from affinity adsorbents may be attributable to cleavage of the dye–matrix bond, hydrolysis or dissolution of the matrix backbone or displacement of tightly adsorbed but non-covalently bound ligand remaining after adsorbent synthesis Fig 4 displays the results of exposing a number of affinity adsorbents to increasing concentrations of chaotrope, acid and base The effect of high chaotrope concentrations on the immunoassay illustrates how serial dilutions rather than single well samples are required to analyse dye loss under these conditions Leakage of ligand was undetectable from most adsorbents in 0 5 *M* NaSCN and, even in 1 25 *M* NaSCN, the coated perfluoropolymer and MIMETIC Blue 1 A6XL displayed no leakage Under all other conditions, the immunoassay detected loss of dye from all the adsorbents However, the extent of ligand loss varied widely for any given condition for example, in 1 *M* sodium hydroxide solution, loss of only 0 1% of the total dye content of the perfluoropolymer adsorbent was observed, whilst under the same conditions, approximately 13 5% of total ligand content of the silica-based Cibacron Blue F3GA = Si500, assuming an initial dye content of 2 μmol/ml silica, was lost It is well known that silica is labile in alkali and dissolution occurs at pH values above 8 0 [22] In contrast, silica is stable at low pH and, therefore, the leakage exhibited in acidic conditions probably arises from hydrolysis of the siloxane bonds between ligand and the support Pellicular coatings for silica comprising polymers such as dextran have been developed which coat the surface and facilitate ligand immobilisation [23] However, coating imperfections will still allow solvent attack on the base matrix and will inevitably lead to ligand loss

The 1,4-ether bond between the D-galactose and 3,6-anhydro-L-galactose moieties of agarose is known to hydrolyse in acidic conditions, resulting in breakdown of the matrix [24] Furthermore, uncrosslinked polymer chains of agarose are aggregated by hydrogen bonds which may be disrupted by chaotropic agents as well as by acid and base The stabilities of the agarose-based materials follow a heirarchy A ≫ F,G,I > B This order mirrors the results of the flow-rate *versus* pressure analysis and is explicable in terms of the extent of chemical cross-linking introduced within the agarose polymer chains The notable stability of A (MIMETIC

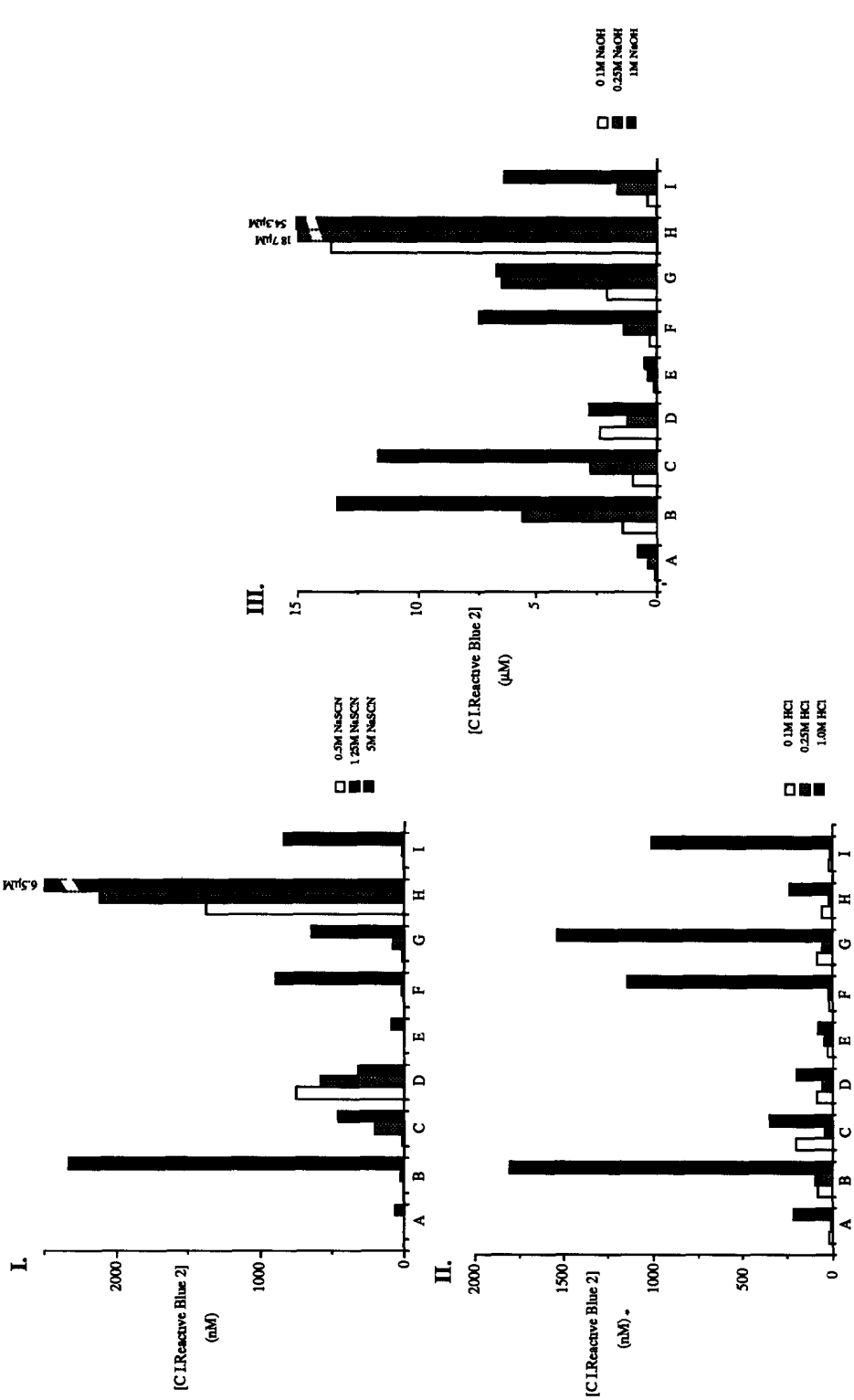

Fig 4 Leakage data for nine C I Reactive Blue 2-containing affinity adsorbents on exposure to conditions of (I) high chaotropic, (II) acidic and (III) basic conditions Packed columns were washed with challenging solution (10 ml) followed by a water wash (10 ml), dye leakage was measured by competitive ELISA and challenging solution and water wash results summed Adsorbents are as follows A = Mimetic Blue A6XL (ACL), B = Affi-Gel Blue (Bio-Rad), C = Blue Trisacryl-M (IBF), D = Fractogel TSK AF-Blue (Merck), E = C I Reactive Blue 2 polyvinyl alcohol-coated perfluorocarbon matrix, F = Blue Sepharose CL-6B, G = Immobilised Cibacron Blue F3GA (Pierce), H = Cibacron Blue F3GA = Si500 (Serva), and I = Reactive Blue 2-Sepharose CL-6B (Sigma)

Blue 1 A6XL) in basic, as well as acidic and chaotropic, conditions may also depend on the unique spacer arm chemistry used to link the dye to the matrix In contrast, direct coupling of the triazine dye to the matrix yields a triazinyl ether bond that may be hydrolysed by extremes of pH [1] The acrylic polymer, Trisacryl Blue-M (C), offers superior stability to the agaroses (F,G,I) in acidic and chaotropic conditions but is less able to withstand alkali Fractogel TSK AF-Blue (D) performs moderately well under all conditions, but it is particularly noticeable that this adsorbent does not withstand lower chaotropic, acidic or basic conditions well, this material may benefit from a pre-treatment that includes exhaustive washing in these conditions prior to use

The C I Reactive Blue 2 polyvinyl alcohol-coated perfluoropolymer displays the lowest leakage under all conditions although slight leakage in both acidic and basic conditions is observed This may be attributable either to hydrolysis of the triazinyl ether bond between the adsorbed polyvinyl alcohol and the triazine dye or to desorption of the entire perfluoroalkylated dyed polymer from the perfluoropolymer surface The use of cross-linking agents to strengthen further the stability of the coating layer may circumvent the latter problem

CONCLUSIONS

The enzyme-linked immunoassay developed for the detection of the triazine dye C I Reactive Blue 2 exhibits a considerable increase in the senstivity of detection over spectrophotometric methods The assay appears to be specific for the anthraquinone moiety of the dye and therefore offers the possibility of monitoring the leakage of immobilised dye from affinity adsorbents irrespective of whether fragments of the matrix remain attached to the dye In applying this immunoassay to a comparison of eight commercial affinity adsorbents and one novel dyed perfluoropolymer support, leakage of ligand from adsorbents in buffer at pH 7 0 was undetectable from most of the adsorbents, although, on exposure to more rigorous conditions that might be applied during the lifetime of a working column, leakage was evident from all the dyed adsorbents The C I Reactive Blue 2 polyvinyl alcohol-coated perfluoropolymer exhibited the lowest loss of ligand in both acidic and basic conditions by a substantial margin, and offered reasonable pressure/flow-rate characteristics with potential maximal flow-rates in the region of 80 cm/min Of the commercial adsorbents, Fractogel TSK-AF Blue would appear to offer advantages for high performance liquid affinity chromatography although careful monitoring of leakage is advisable Overall, MIMETIC Blue 1 offers the best capacity and stability of all commercial adsorbents and its increased rigidity suggests that it is suitable both for low and medium pressure applications Unfortunately, the low binding capacity of the perfluoropolymer for proteins, attributable to its lack of porosity and low surface area, limits its current usefulness Nevertheless, work is underway to remedy these shortcomings by the use of more porous perfluoropolymer matrices The remarkable stability of the perfluoropolymer adsorbents should make them attractive propositions for the purification of therapeutic proteins where harsh conditions could be employed to effect *in situ* depyrogenation, sterilisation and cleaning

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