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IMMUNOCHEMICAL STUDIES OF THE NON-SPECIFIC INTERACTIONS OF CYANOGEN BROMIDE-ACTIVATED MACROPOROUS AGAROSE-BASED IMMUNOADSORBENTS

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SUMMARY

This paper reports studies of the origin of the undesirable non-specific adsorption in immunoadsorption chromatography. The non-specificity of cyanogen bromide (CNBr)-activated macroporous agarose (Sepharose 4B)-based immunoadsorbents has been assessed from a comparative study of the following parameters: (1) The non-specific adsorption of protein (IgG) on unsubstituted cyanogen bromide-activated Sepharose 4B. (2) The non-specific adsorption of proteins (human serum IgG and sheep IgG) on cyanogen bromide-activated Sepharose 4B–sheep IgG immunoadsorbent. (3) The non-specific adsorption of proteins, immunoglobulin G(IgG) and human serum albumin (HSA) on cyanogen bromide-activated Sepharose 4B–sheep anti-human IgG immunoadsorbent and (4) the non-specific adsorption of peptides and amino acids on cyanogen-bromide activated Sepharose 4B–sheep anti-human IgG immunoadsorbent.

INTRODUCTION

The currently high popularity of solid phase techniques in chemical, biochemical and immunochemical procedures is an index of the intrinsic advantages afforded by immobilized reagents in analytical and preparative applications. Despite the invaluable contribution of immunoadsorption chromatography[1] for isolating antigens and antibodies, its operational efficiency has been severely compromised by various non-biospecific interactions resulting in rather poor small-scale separations and purifications. Furthermore, few successful large-scale separations have been reported in the literature.

The adsorption, onto an adsorbent, of a specific protein from a loaded heterogeneous sample is determined by the optimum conditions favouring complex formation between the immobilized ligand and the compound to be isolated. Such conditions are determined by the following parameters: the solvent[2]; the polarity of the eluent, its ionic strength and pH[3]; temperature[4,5]; charge of the activated matrix and ligand[6,7]; the ratio of the amount of covalently coupled ligand:activated matrix[7,8];

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the ratio of the amount of protein loaded:amount of ligand immobilized[8]; and the accessibility of the reactive groups and the degree of hydrophilicity of the matrix and ligand[5,7,9]. The successful application of immunoadsorption chromatography is largely attributed to the wide use of cyanogen bromide (CNBr)-derivatized macroporous agarose, introduced by Porath and co-workers[10]. The 4% (weight per expanded volume) beaded macroporous agarose available commercially from Pharmacia as Sepharose 4B is the most popular matrix for the synthesis of immunoadsorbents. The popularity of Sepharose has been attributed to its relative inertness[11] (*i.e.* the matrix does not act as an adsorbent), porosity[5], the apparent stability of the derivatized products[12] and their commercial availability.

Eveleigh and Levy[7] identified two types of non-specific interactions: (a) a non-reversible primary reaction that occurs in the first exposure of an immunoadsorbent to a complex biological sample, attributable to inadequate blocking of matrix active sites, remaining from coupling of the ligand to the matrix, with a low-molecular-weight reagent; (b) a reversible non-specific adsorption of certain components that results in a contamination of the eluted fractions and a partial blockage of otherwise specific binding sites. This latter proces presents a tedious problem in terms of obtaining highly pure products by the single step procedure of passing a heterogeneous sample such as serum through an immunoadsorbent column.

Non-specific interactions have been largely attributed to hydrophobic ligands or spacer molecules[13–16], charged groups[6,17–19], steric hindrance arising from the mode of attachment of the ligand[5,11] and occlusion of the ligand by the matrix backbone[5,11]. Although the salt sensitivity of the adsorption of some proteins suggests the possibility of some electrostatic interaction, some types of non-specific binding cannot be reversed by salt, polarity-reducing agents or denaturing agents[5,7,20]. In addition, some proteins such as albumin and ovalbumin[9,20] show irregular adsorption/elution features. O'Carra suggested that when biospecific adsorption and non-biospecific adsorption are balanced, they could act synergistically[5]. The obscure mechanisms of certain non-specific interactions and the severe disparity between the amount of adsorbed and eluted substances[5,7,8] prompted this investigation of the cyanogen bromide-activated matrix, the covalently coupled protein ligand and the loaded protein as possible sources of non-specific interference.

MATERIALS

Cyanogen bromide-activated macroporous agarose, CNBr-Sepharose 4B, was purchased from Pharmacia (Uppsala, Sweden). Sheep anti-human IgG (batch Z511G, 24.7 mg/ml), sheep anti-human human serum albumin (HSA) (batch Z464), donkey anti-sheep IgG (batch Z592A) and normal human serum standard (NIRDL, normal human serum standard code No. BR 99) were obtained from Seward Laboratories (London, U.K.). Agarose for immunochemical studies was purchased from Fisons (Loughborough, U.K.). The amino acid standard solution (2.5 μmole/ml) was purchased from Pierce (Rockford, IL, U.S.A.) and the peptides were obtained from Sigma (St. Louis, MO, U.S.A.). Chromatography columns GA 10 × 15 (15 × 1.0 cm) were bought from Wright Scientific (Kenley, U.K.). All materials used for preparing buffers and salt solutions were of AnalaR grade.

METHODS

Treatment of unsubstituted cyanogen bromide-activated Sepharose 4B

Cyanogen bromide-activated Sepharose 4B was swollen in and washed with 10^{-3} *M* hydrochloric acid to remove dextran and lactose stabilisers, filtered, blocked with aqueous ethanolamine solution (1 *M*) by gently stirring magnetically for one hour and then equilibrated in sodium citrate buffer (0.2 *M*, pH 6.5). After degassing, the gel (1 g dry gel, approximately 3.5 ml swollen) was packed in the column, washed with ammonia solution (0.5 *M*, pH 11.5) and then equilibrated in NaCl–Tris buffer [0.5 *M* NaCl, 0.1 *M* tris(hydroxymethyl)aminomethane–HCl, 0.5% (w/v) sodium azide] pH 8.0.

Aliquots (0.1 ml, 0.78 mg IgG) of pooled whole human serum were loaded in three serial adsorption–desorption cycles followed by loadings of 0.2-ml aliquots of the serum (1.56 mg IgG) for twelve cycles and 1-ml aliquots of the serum (7.8 mg IgG) for two subsequent cycles. After each loading the column was washed with NaCl–Tris buffer (pH 8.0) until the $A^{\frac{1}{2}\,cm}_{280\,nm}$ readings corresponded to that of the buffer alone. The column was next eluted with ammonia solution (0.5 *M*, pH 11.5) until the $A^{\frac{1}{2}\,cm}_{280\,nm}$ absorbances of the 4-ml fractions were negligible (≤ 0.005). After elution with ammonia solution the column was re-equilibrated in NaCl–Tris buffer (pH 8.0) before another cycle was commenced.

Preparation of immunoadsorbent columns

Cyanogen bromide-activated Sepharose–sheep anti-human IgG immunoadsorbent columns (1 g dry gel, ≈ 3 ml packed bed volume) for assessment of the non-specific adsorption of IgG and HSA from whole human serum, and of amino acids and peptides from test solutions, were prepared as described previously[19].

Prior to loading a column with serum it was washed with ammonia solution (0.5 *M*, pH 11.5) and then equilibrated with NaCl–Tris buffer (pH 8.0). Two 3-ml control immunoadsorbent (cyanogen bromide-activated Sepharose 4B–sheep anti-human IgG) columns for the adsorption of aminoacids and peptides contained 2.61 and 9.9 mg, respectively, sheep anti-human IgG per ml of swollen gel. The columns were washed with ammonia solution (0.5 *M*, pH 11.5) and equilibrated in phosphate buffered saline (0.01 *M* Na_2HPO_4/NaH_2PO_4, 0.15 *M* NaCl, pH 7.2), before being loaded with mixed amino acids and mixed peptides. Each column was loaded serially with 250 µl (625 nmole) amino acid standard solution or 0.5 ml (90–123 nmole) mixed peptide solution. The columns were then washed with phosphate buffered saline until the $A^{\frac{1}{2}\,cm}_{250\,nm}$ of the amino acid eluate was negligible (< 0.005) and the absorbance of the peptide eluate was also small (< 0.005). The columns were then eluted with ammonia solution (0.5 *M*, pH 11.5) and the ammonia eluates from the columns loaded with amino acids were monitored for absorbance at 280 nm while the eluates from the columns loaded with peptides were monitored at 220 nm. The pooled ammonia eluates from each adsorption–desorption cycle were analysed on a Locarte automatic amino acid analyser of the University of Birmingham Macromolecular Analysis Service.

Isolation of sheep IgG

Sheep IgG was isolated from 4 ml normal sheep serum (NSS) by precipitation

with 1.3 ml saturated ammonium sulphate (to give a final concentration of 33.3% (w/v) with respect to saturated ammonium sulphate). The precipitate was dissolved in phosphate buffer (0.03 *M*, pH 7.2) and dialysed extensively against saline (0.9% (w/v) sodium chloride). The dialysed fraction was next loaded onto an anion-exchange diethylaminoethyl (DEAE)-cellulose column (10 × 1 cm) equilibrated in phosphate buffer (0.03 *M*, pH 7.2) and subsequently eluted with the same buffer and collected in 4.0 ml fractions on an LKB Uvicord II automatic fraction collector at a flow-rate of 20 ml/h controlled by an LKB Perpex peristaltic pump. The column effluents were monitored at 280 nm and the IgG eluted in the first peak was pooled, dialysed extensively against saline and concentrated by negative-pressure dialysis[21]. The IgG purity was determined by Ouchterlony double immunodiffusion[22] and immunoelectrophoresis[23] using donkey anti-sheep serum. Sheep IgG (1 ml, 30 mg) equilibrated in sodium citrate buffer (0.2 *M*, pH 6.5) was added to cyanogen-activated Sepharose 4B (1 g dry gel) swollen in and washed with 10^{-3} *M* hydrochloric acid and equilibrated in sodium citrate buffer (6.0 ml). A 73-% coupling efficiency was achieved resulting in 27.9 mg IgG/3 ml packed gel volume.

Immunochemical assays

The pooled unadsorbed and pooled desorbed fractions from four 3-ml immunoadsorbent columns (CNBr-Sepharose 4B–sheep anti-human IgG) of identical binding capacity, were quantitated by the radial immunodiffusion method of Mancini[24], using the sheep anti-human albumin and donkey anti-sheep IgG to quantitate the unadsorbed and absorbed fractions, and the purity of the fractions was determined by immunoelectrophoresis[23] and Ouchterlony double immunodiffusion[22]. The potential antigen binding capacity (*C*) of an antibody immunoadsorbent column was derived from the equation: $C = (P - p)T$ where *P* is the amount of antibody added to the actual gel, *p* is the amount of uncoupled antibody and *T* is the titre of the antibody. The titre is defined as the amount of antigen in mg which reacts with a known amount of antiserum in mg at the equivalence point[25]. The equivalence point was determined by precipitin titration followed by manual nephelometric quantitation[26].

RESULTS

Non-specific adsorption of protein (IgG) on ethanolamine-substituted cyanogen bromide-activated Sepharose 4B

The contribution of the ethanolamine-substituted cyanogen bromide-activated Sepharose 4B to the non-specific adsorption of human serum IgG was demonstrated by a plot of the IgG adsorbed by and eluted from the ethanolamine-substituted cyanogen bromide-activated gel as a function of serial adsorption–desorption cycles (Fig. 1). When a 3-ml cyanogen bromide-activated Sepharose 4B column was loaded with whole human serum (0.1 ml, 0.78 mg IgG) for the first four adsorption–desorption cycles followed by a loading of 0.2 ml serum (1.56 mg IgG) for the succeeding ten cycles, there was no detectable adsorption of IgG on the cyanogen bromide-activated Sepharose matrix. However, after the fifteenth cycle when the IgG load (1 ml, 7.8 mg) was increased ten-fold with respect to the initial load, there was a retention of 1.9 mg (24.4% of the loaded IgG) and 1.6 mg (20.5%) in the sixteenth and seventeenth cycles respectively with the corresponding IgG elution of 0.46 mg (24.2% of the IgG retained) and 0.36 mg (22.5%).

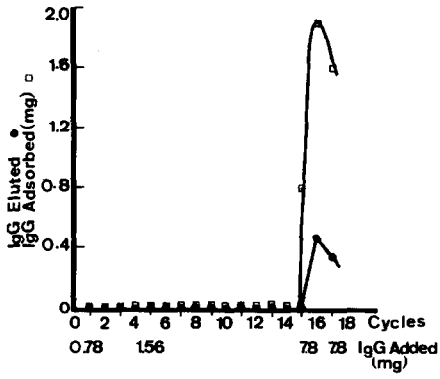

Fig. 1. Non-specific adsorption and elution profiles of human serum IgG on ethanolamine-substituted CNBr-activated Sepharose 4B.

The purity of the eluted IgG was confirmed by immunoelectrophoresis and two-dimensional electrophoresis with the aid of sheep anti-whole human serum. These results demonstrate that although the IgG in serum was not significantly adsorbed on the ethanolamine substituted cyanogen bromide-activated Sepharose 4B matrix after loadings of small volumes of serum in serial adsorption–desorption cycles, there was a pronounced retention of IgG when the volume of serum (1 ml) and hence the amount of IgG (7.8 mg) loaded, was increased ten-fold in the fifteenth cycle. The marked decrease in the amount of IgG retained on the matrix after the peak adsorption in the sixteenth cycle was indicative of the saturation of the column non-specific binding sites. Once a saturation level is attained as a result of specific or non-specific interaction, the amount of material adsorbed decreases asymptotically.

Non-specific adsorption of human serum IgG on a control adsorbent (CNBr-Sepharose 4B–sheep IgG) column

In order to assess the contribution of a macromolecular protein ligand to the

TABLE I

QUANTITATIVE RESULTS OF THE NON-SPECIFIC ADSORPTION OF HUMAN IgG ON A CONTROL ADSORBENT (CNBr-SEPHAROSE 4B–SHEEP IgG) COLUMN

Description	*Cycle*		
	1	*2*	*3*
Application			
IgG applied (mg)	2.32	2.32	2.32
Washing			
IgG unadsorbed (mg)	1.6	1.6	1.92
% IgG unabsorbed	69.0	69.0	83.0
Retention			
IgG adsorbed (mg)	0.72	0.72	0.40
% IgG adsorbed	31.0	31.0	17.2
Elution			
IgG eluted (mg)	Nil	Nil	Nil

TABLE II

QUANTITATIVE RESULTS OF THE NON-SPECIFIC ADSORPTION OF SHEEP SERUM IgG ON A CONTROL ADSORBENT (CNBr-SEPHAROSE 4B–SHEEP IgG) COLUMN

Description	*Cycle*		
	1	*2*	*3*
Application			
Sheep IgG loaded (mg)	2.7	2.7	5.4
Washing			
Sheep IgG unadsorbed (mg)	2.51	2.75	5.16
% sheep IgG unadsorbed	93.0	101.9	95.4
Retention			
Sheep IgG adsorbed (mg)	0.19	0	0.25
% sheep IgG adsorbed	7.0	0.0	4.6
Elution			
Sheep IgG eluted (mg)	Nil	Nil	Nil

phenomenon of non-specific adsorption, sheep IgG was employed as a model non-specific ligand instead of the specific sheep anti-human IgG ligand. When pooled whole human serum (0.2 ml, 2.32 mg IgG) was loaded onto a 3-ml (10.9 mg sheep IgG/ml gel) control adsorbent column (cyanogen bromide-activated Sepharose 4B–sheep IgG) in three successive adsorption–elution cycles and the unadsorbed protein was washed off with NaCl–Tris buffer (pH 8.0) followed by elution with ammonia solution (0.5 *M*, pH 11.5), the results of the column performance were as illustrated in Table I.

Non-specific adsorption of sheep serum IgG on a control adsorbent (cyanogen bromide-Sepharose 4B–sheep IgG) column

When a 3-ml control adsorbent column (cyanogen bromide-Sepharose 4B–sheep IgG) was loaded with normal sheep serum (0.1 ml, 2.7 mg IgG) in two successive adsorption elution cycles, followed by a loading of 0.2 ml serum (5.4 mg sheep IgG) in the third cycle and the unadsorbed protein in each cycle was washed off with NaCl–Tris buffer (pH 8.0) followed by elution with ammonia (0.5 *M*, pH 11.5); the results of the column performance were as illustrated in Table II.

Non-specific adsorption of proteins (IgG and HSA) on cyanogen bromide-activated Sepharose 4B–sheep anti-human IgG immunoadsorbent

The non-specific adsorption of protein associated with the interaction of a macromolecular antibody ligand (sheep anti-human IgG), covalently coupled to CNBr-activated Sepharose 4B, with the complementary antigen (human serum IgG) was assessed by loading immunoadsorbent columns of constant binding capacities with varying volumes of whole human serum and monitoring the adsorption and elution patterns of endogenous IgG (Fig. 2) and HSA (Fig. 3). The composite plots show the percentage IgG eluted as a function of the adsorption–desorption cycles resulting from the loading of four immunoadsorbent columns (3 ml) of identical binding capacities (1.6 mg IgG) with pooled whole human serum (0.1–0.7 ml). Although the antigen yield increased with increasing antigen load, the maximum yield

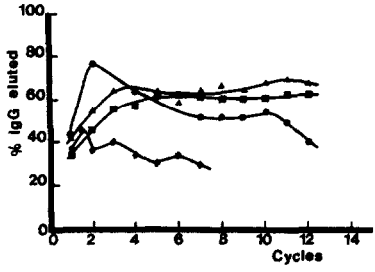

Fig. 2. Composite plots of the percentage human serum IgG eluted on serial adsorption–desorption cycles when increasing amounts of antigen in the form of whole human serum (WHS) were loaded on (CNBr-activated Sepharose 4B–sheep anti-human IgG) immunoadsorbent columns (3 ml gel) of the same potential binding capacity (1.6 mg IgG). Amounts of WHS loaded: ■, 0.1 ml (1.16 mg IgG); ▲, 0.15 ml (1.74 mg); ●, 0.4 ml (4.64 mg); ◆, 0.7 ml (8.12 mg).

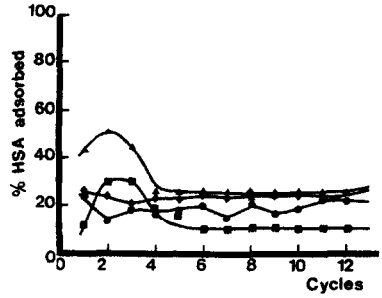

Fig. 3. Composite plots of the percentage HSA adsorbed in serial adsorption–desorption cycles when increasing amounts of antigen in the form of whole human serum was loaded on (CNBr-activated Sepharose 4B–sheep anti-human IgG) immunoadsorbent columns (3 ml) of the same potential binding capacity. Amounts of HSA applied: ■, 2.85 mg; ▲, 4.275 mg; ●, 11.4 mg; ◆, 19.74 mg.

was obtained when the antigen load approximated to three-fold the potential binding capacity of the column. Composite plots of HSA adsorbed against cycles indicated the adsorption of 10–40% of the loaded HSA in the first cycles when the HSA loaded was 2.85 and 4.28 mg respectively. No significant amount of HSA was eluted with 0.5 *M* ammonia. Quantitation of the eluates by Mancini radial immunodiffusion revealed the eluted HSA to be less than 9 μg/ml (0.05–0.35% of the loaded HSA).

TABLE III

NON-SPECIFIC ADSORPTION OF AMINO ACIDS ON CYANOGEN BROMIDE-ACTIVATED SEPHAROSE 4B–SHEEP ANTI-HUMAN IgG IMMUNOADSORBENT

Amino acid applied	*Percent of applied amino acid adsorbed per cycle*		
	Cycle 1	*Cycle 2*	*Cycle 3*
L-Aspartic acid	55.6	0	0
L-Threonine	6.8	4.6	2.1
L-Serine	71.6	0	0
L-Glutamic acid	42.0	0	0
L-Proline	58.0	0	0
Glycine	8.8	1.9	0
L-Cystine	9.6	5.9	2.6
L-Valine	5.2	0	0
L-Methionine	5.2	1.0	0
L-Isoleucine	5.6	0	0
L-Leucine	9.6	0	0
L-Tyrosine	3.6	0	0
L-Phenylalanine	10.0	2.4	0
L-Histidine	9.0	1.4	0
L-Lysine	10.4	2.8	Not resolved
L-Arginine	20.8	7.3	Not resolved

TABLE IV

NON-SPECIFIC ADSORPTION OF PEPTIDES ON CYANOGEN BROMIDE-ACTIVATED SEPHAROSE 4B–SHEEP ANTI-HUMAN IgG IMMUNOADSORBENT

Peptide applied	*Percent of applied peptide adsorbed per cycle*					
	Cycle 1	*Cycle 2*	*Cycle 3*	*Cycle 4*	*Cycle 5*	*Cycle 6*
Glycyl-L-aspartic acid	0	0	0	0	0	0
Glycyl-L-serine	39.0	20.0	18.3	0	0	0
Glycyl-glycyl-glycine	0	0	0	0	0	0
Glycyl-L-phenylalanine	10	3	0	0	0	0
L-Phenylalanyl-glycine	6.0	0	0	0	0	0

Non-specific adsorption of peptides and amino acids on immunoadsorbent (cyanogen bromide-activated Sepharose 4B–sheep anti-human IgG) column

The adsorption characteristics of amino acids (Table III) and peptides (Table IV) on the test immunoadsorbent (cyanogen bromide-activated Sepharose 4B–sheep anti-human IgG) column proved useful models for exploring the cationic charge distribution on CNBr-activated polysaccharide matrix. A more distinct adsorption profile was apparent for the mixed amino acids loaded than for the loaded mixed peptides.

DISCUSSION

The chromatographic inertness of unsubstituted agarose matrix is commonly assumed by a majority of investigators, but in many cases the controls demonstrating negative adsorption on the matrix were inadequate. Small molecules such as ethanolamine or lysine are commonly employed to block residual active sites in the matrix after ligand substitution of the cyanogen bromide derivatized Sepharose matrix. Furthermore, the contribution of such molecules to non-specific binding has largely been assumed to be negligible although the non-specific adsorption of poly(A) in the nucleic acid field on ethanolamine-substituted Sepharose 2B has been described[27]. In the present work, although only the adsorption and elution of human serum IgG on ethanolamine-substituted cyanogen bromide-activated Sepharose 4B was monitored (Fig. 1), non-specific adsorption was dominant when the serum load was significantly increased. A negligible amount of protein was adsorbed when 0.2 ml of human serum (about 7% of the column volume) was loaded in ten serial adsorption–desorption cycles. The marked retention of IgG in the fifteenth cycle accompanied the ten-fold increase in the initial serum load. The decrease in the amount of IgG adsorbed in the sixteenth cycle indicates the saturation of the adsorptive sites in the column. The results illustrated in Fig. 1 demonstrate that overloading the Sepharose-based immunoadsorbent not only increases the non-specific adsorption of extraneous proteins from a heterogeneous sample but also severely limits the successful re-use of the column.

Although some of the protein could have been trapped in the interstices of the matrix or coupled to unblocked residual active groups, the simultaneous effects of electrostatic and non-ionic interactions appear to be implicated in the adsorption of

protein on cyanogen bromide-activated gel. As the cationic charges on the isourea derivatives[6,17] became neutralised, other non-covalent interactions would tend to become predominant. Jencks[28] suggested that hydrophobic bonding may be the most single important factor in non-covalent interactions in aqueous solutions where the strengths of electrostatic, charge transfer and hydrogen bonds are reduced by the charge solvating hydrogen bonding ability of water.

The results illustrated in Fig. 1 and in Table I suggest that the sheep IgG ligand contributed positively to non-biospecific adsorption. The 31% and 33% human IgG adsorbed on the adsorbent (cyanogen bromide-activated Sepharose 4B–sheep IgG) column in the first and second adsorption–elution cycles (Table I) respectively cannot be attributed to biospecific adsorption. In addition, comparable loadings of human serum on the ethanolamine-blocked cyanogen bromide-activated Sepharose 4B column (Fig. 1) showed no significant adsorption of human IgG after twelve serial adsorption–elution cycles. The adsorption of human serum IgG must be attributed mainly to the interaction of the loaded antigen with the sheep IgG ligand. The 50% decrease in the column binding capacity in the third adsorption–elution cycle (Table I) is another demonstration of the column saturation effect illustrated in Fig. 1.

In contrast, the loading of sheep serum on the control adsorbent (cyanogen bromide-activated Sepharose 4B–sheep IgG) column resulted in the adsorption of 7% and 0% IgG in the first and second adsorption–elution cycles respectively (Table II). However, a two-fold increase in the amount of sheep serum loaded resulted in only 4.6% adsorption of the loaded IgG. Although some of the IgG adsorbed in cycle 1 was washed off in the unadsorbed fraction in cycle 2, these results would suggest that there was no significant adverse non-specific interaction of sheep serum IgG with the sheep IgG ligand covalently coupled to the Sepharose matrix. This finding would justify the addition of sheep IgG to a coupling mixture in order to minimise the amount of specific ligand substituted. Nevertheless, the cummulative non-specific retention of small amounts of protein on adsorbent columns during serial adsorption–elution cycles would limit the useful re-use of the column by occlusion of active sites and contribute to contamination of eluates with the non-specifically adsorbed proteins.

It is noticeable that in cycles 1 and 3 (Table II) only 7% and 5% respectively of the loaded IgG were retained on the cyanogen bromide-activated Sepharose 4B–sheep IgG adsorbent column loaded with sheep serum. In contrast, 21% and 17.2% of the loaded IgG were retained in cycles 1 and 3 respectively on a similar adsorbent column when human serum was loaded (Table I). In both cases there was no detectable desorption of IgG with ammonia solution (0.5 *M*, pH 11.5). These results demonstrate the retention of protein on the columns, and indicate a stronger interaction of the cyanogen bromide-activated Sepharose 4B–sheep IgG adsorbent with human serum IgG than with sheep serum IgG.

The difference between the amount of protein (from whole human serum) adsorbed on and eluted from an immunoadsorbent (cyanogen bromide-activated Sepharose 4B–sheep anti-human IgG) column (true biospecific) is highlighted in Fig. 2 which shows the percentage of the adsorbed IgG eluted as a function of the adsorption–elution cycles. Although the yield of antigen increased with increasing antigen load, the highest antigen yield resulted when the antigen load approximated to three-fold the potential binding capacity of the column. This corresponded to a

mean percentage IgG elution of 55.0% of the IgG adsorbed in twelve serial adsorption–elution cycles. The non-specific adsorption of contaminated protein is illustrated by the adsorption of HSA from the whole human serum applied to the column as shown in Fig. 3. The HSA loading of 11.4 mg coincided with the IgG load of approximately three-fold the column potential binding capacity and resulted in comparatively low HSA adsorption. Despite the loading of the heterogeneous serum, the immunoadsorbent columns, which had the smallest volumes of serum loaded, showed no significant reduction in biospecific activity after twelve serial cycles (Fig. 2). The column which had the largest volume of serum loaded showed the greatest decrease in biospecific activity after six cycles (Fig. 2).

Many studies have been reported in the literature describing the interaction of enzymes with substituted cyanogen bromide-activated Sepharose 4B[14,15]. However, no report could be found describing the molecular interaction of amino acids and peptides with immunoadsorbents. The adsorption characteristics of amino acids (Table III) and peptides (Table IV) on the test immunoadsorbent (cyanogen bromide-activated Sepharose 4B–sheep anti-human IgG) gave some insights into the nature of protein–protein interactions with charged absorbents[19]. The significant adsorption of certain amino acids (L-serine, L-proline, L-aspartic acid and L-glutamic acid) and the dipeptide (L-glycyl-L-serine) indicate that contributions from ionic and some non-ionic effects could be concomitantly involved in the adsorption process. This is highlighted by the adsorption of the hydroxymonoamino-monocarboxylic α-amino acids L-serine (72%) and L-threonine (7%) and the cyclic α-amino acid L-proline (58%). The major difference between L-serine and L-threonine is the presence in L-threonine of a methyl group adjacent to the hydroxyl group. The strongly hydrophobic methyl group of threonine could have inhibited adsorption on the hydrophilic Sepharose beads as a result of steric hindrance and possibly hydrogen bonding with the lone pair electrons on the hydroxy group oxygen atom. In contrast, the adsorption of L-proline appears to be determined partly by the hydrophobic methylene groups of the pyrolidine ring. The adsorption of the mono-aminodicarboxylic α-amino acids, L-aspartic acid (56%) and L-glutamic acid (42%), also reflects the contribution of the charged polar groups at pH 6.7 to the adsorption process.

The comparative analysis of the adsorptive characteristics of four dipeptides and one tripeptide (Table IV) showed that only glycyl-L-serine was adsorbed significantly under the experimental conditions. The high adsorption in the first cycle of L-glycyl-L-serine was consistent with the high adsorption of serine (72%) in the first cycle. The high adsorption of aminoacids and peptides in the first cycle could also reflect some binding by unblocked active groups in the adsorbent. In addition, the comparatively small adsorption of peptides could be a reflection of the strong pH dependence observed by Joustra and Axen (1975) for the coupling of glycyl-L-leucine to cyanogen bromide-activated Sepharose 4B[29].

Particularly for immunoadsorption, the attractive features of Sepharose and other forms of macroporous agarose compared to other matrix materials are: their commercial availability; porosity of the beads which allows access to molecules in the million dalton range; their hydrophilic nature; relative chromatographic inertness[11]; and the apparent stability of the derivatized products[12]. Notable limitations of the agarose matrix include: the presence of ester sulphate groups and carboxy groups derived from actual formation with endogenous pyruvic acid; the cationic

charge associated with cyanogen bromide-activated agarose; low mechanical and chemical rigidity; low effective ligand concentration within the gel matrix; solubility in hot water and non-aqueous solvents and the partial retention of biospecifically and non-biospecifically adsorbed molecules resulting in inefficiency and restrictions in re-use[8].

Despite the advantages afforded by cyanogen bromide-activated agarose for synthesizing adsorbents, the inherent nature of the support imposes certain restrictions in its efficient utilization[6–8,19]. The most effective chromatographic systems will best be achieved by the careful control of the matrix activation in order to limit the number of active sites generated and the cross-linking of the matrix[7] and careful attention to coupling and column operation protocols in order to obtain the best balance between the desired biospecific and the adventitious non-biospecific binding resulting from the gross electrostatic, hydrophobic and other non-covalent interactions. For achievement of this optimum an understanding at the planning stage of both the non-specific adsorption likely, as discussed in this paper, and the steps which may be taken (judicious choice of the gel: protein ratio, the composition of eluents and the amount of protein loaded onto the column), as discussed previously[8], are necessary.

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