Journal of Chromatography, 616 (1993) 197–204
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6867

Behaviour of human immunoglobulin G subclasses on thiophilic gels: comparison with hydrophobic interaction chromatography

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(First received January 12th, 1993; revised manuscript received March 26th, 1993)

ABSTRACT

We have used thiophilic and hydrophobic interaction chromatography in an attempt to obtain enriched human immunoglobulin G (IgG) subclasses from a therapeutic immunoglobulin preparation. Proteins were adsorbed on a thiophilic gel and on Phenyl-, Butyl-, or Octyl-Sepharose in 1 *M* ammonium sulphate. Elution with a decreasing salt gradient produced no marked subclass selectivity, except with Octyl-Sepharose, which yielded a poorly adsorbed fraction somewhat enriched in IgG2, representing *ca.* 20% of the total initial protein. Neither thiophilic nor hydrophobic interaction chromatography appear suitable for an efficient enrichment in subclasses, which all show a broad heterogeneity in their affinity for these columns. The influence of the starting salt concentration was also studied. With thiophilic gels, in the absence of ammonium sulphate, *ca.* 30% of the initial load was not adsorbed, and was found to be enriched in IgG2. At 2.5 and 5% ammonium sulphate, practically no adsorption occurred. At 7.5% ammonium sulphate, the non-adsorbed fraction was enriched in IgG3. With Phenyl-Sepharose, adsorption increased smoothly with the salt concentration. It is concluded that different forces come into play for adsorption on thiophilic gels at low and high salt concentration.

INTRODUCTION

The therapeutic use of immunoglobulin G (IgG) preparations obtained from pools of human sera is well established. In some circumstances, one may wish to use a preparation with as large a specificity range as possible, in which case it is desirable to respect the blood proportions of IgG subclasses during the isolation procedure. In other situations, one may wish to have at hand a preparation enriched in a certain antigenic specificity, because a number of cases have been reported where a particular specificity was carried by a particular IgG subclass [1–3]. It may then be desirable to enrich the proportion of a given subclass in the therapeutic preparation. This is not an easy task, because the plasma IgG present a general similarity and, at the same time, an extreme molecular heterogeneity within each subclass.

A number of attempts to fractionate IgG subclasses from human plasma have been reported. Howard and Virella [4], in an isoelectric focusing study, showed IgG4 to be relatively acidic (p*I* values *ca.* 6) and IgG3 relatively basic (p*I* values between 8 and 9); the IgG2 population migrated at pH values between 6.5 and 8.5, and the IgG1 population between roughly 6.5 and 9.5. In

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agreement with these results, Skvaril and Morell [5], using several anion exchangers, separated a non-adsorbed fraction depleted in IgG4 from an adsorbed fraction enriched in this subclass.

Size fractionation of IgG subclasses could, in principle, separate IgG3 from the rest of the population, because this subclass has a long hinge region compared with other subclasses (62 compared with 12–15 amino acids) [6]. This leads to a molecular mass of 170 000 instead of 150 000 for the other subclasses. Nevertheless, this difference, combined with the subclass proportions in human plasma, is too small to lead to an efficient enrichment on molecular sieve columns [7].

Affinity chromatography of polyclonal IgG or myeloma proteins on subclass-specific monoclonal antibody columns is an efficient separation method [8–10]. Along the same lines, immobilized protein A from *Staphylococcus aureus* has been used to achieve partial separations. For example, IgG3 is known not to bind to protein A [11], a property that was exploited by Hjelm [12] using protein A-Sepharose 4B. This behaviour may, however, not be general, because at least one example of a protein A-binding IgG3 has been reported in the plasma of mongoloid populations [13]. The affinity displayed in this case may be related to the presence of histidine at position 435 of the heavy chain, similar to other subclasses, whereas IgG3 from the plasma of caucasian populations has an Arg-435. Indeed, His-435 was shown to play a role in the affinity between a human Fc domain and fragment B of protein A, as analysed by X-ray diffraction [14]. Chromatography on protein A-Sepharose was also used to separate two fractions enriched in IgG2 on the one hand and IgG1 and IgG4 on the other hand, using a pH gradient [15]. However, none of these affinity chromatographic methods is practical in industrial process.

Finally, two chromatographic methods have been examined as tools for purifying bulk immunoglobulins. The first one is hydrophobic interaction chromatography (HIC). Doellgast and Plaut [16] separated IgA from IgGs in serum using a column of phenylalanine-Sepharose. On Phenyl-Sepharose, Hrkal and Rejnková [17] found a broad hydrophobicity range for IgG from albumin-depleted human serum. Mouse monoclonal antibodies could also be adsorbed to liquid chromatographic hydrophobic columns and eluted with a decreasing salt gradient [18,19]. The second method is chromatography on thiophilic gels. These derivatized materials were shown to require a lower ammonium sulphate concentration for the adsorption of immunoglobulins than of other proteins, serum albumin in particular [20–22]. Thiophilic gels were therefore proposed for the purification of immunoglobulins (irrespective of class or subclass) from cell culture media [23,24], ascites fluid [23–25] or colostral whey [26]. In neither of these studies with hydrophobic and thiophilic gels was there any attempt at defining a possible selectivity in adsorption or desorption of IgG subclasses.

This paper, as part of a program designed to study the chromatographic behaviour of human IgG subclasses [27], describes the results of an investigation of their behaviour on adsorption on and desorption from three different hydrophobic gels and a thiophilic column.

EXPERIMENTAL

Samples

The starting material for fractionation was the therapeutic intramuscular Allergamma immunoglobulin preparation, made available by the Centre National de Transfusion Sanguine. It is routinely obtained by fractionation with alcohol and caprylic acid of pooled plasma from more than 10 000 donors [28]. Its starting IgG subclass composition was as follows: IgG1 = 52%, IgG2 = 35%, IgG3 = 9%, IgG4 = 4%. The preparation was dialysed against 50 m*M* sodium phosphate buffer (pH 7.0) and adjusted to 30 mg protein per ml.

Analysis of subclass composition was carried out with an enzyme-linked immunosorbent assay (ELISA) test. The monoclonal anti-subclass antibodies were obtained from Oxoid (Bedford, UK) [29] and used at the following dilution in phosphate-buffered saline (PBS) + 0.1% Tween 20 + 1% bovine serum abumin: NL16 (anti-IgG1),

1:5000, HP6014 (anti-IgG2), 1:10 000, ZG4 (anti-IgG3), 1:5000; RJ4 (anti-IgG4), 1:10 000. The reference immunoglobulin (RefIg) used for calibrating the test was Allergamma itself, stored at −80°C; its subclass distribution had been estimated by a radial immunodiffusion test (Miles). The ELISA test was carried out as follows. Ref Ig, or sample (150 μl) in PBS was coated overnight on 96-well microtiter plates (Dynatech Labs.); the wells were washed once with PBS + 0.1% Tween 20 (PBS-T), then saturated for 1 h at 37°C with BSA (1% in 200 μl PBS-T). After one wash with PBS-T, 150 μl of the anti-subclass immunoglobulin dilutions were added, and the plates were incubated for 2 h at 37°C. The wells were then washed three times with 200 μl of PBS-T. This was followed by the addition of 150 μl of a goat anti-mouse IgG, which had been adsorbed on human immunoglobulins prior to conjugation with alkaline phosphatase (Sigma), diluted 1000-fold in PBS-T + 1% BSA. After 1 h at 37°C and three washes with PBS-T, 150 μl of *p*-nitrophenyl phosphate (Sigma; 1 mg/ml in 0.1 *M* diethanolamine buffer, pH 9.3) were added. The reaction was stopped after 45 min at room temperature by adding 50 μl of 3 *M* NaOH and the absorbance at 405 nm was read with a V_{max} Molecular Devices ELISA reader. The calibration curve was determined with six concentrations of the particular subclass in the RefIg sample, ranging from 10 to 250 ng/ml. In this range, the response with respect to concentration was linear for IgG1 and IgG3. The upper limit for linearity was 170 and 90 ng/ml for IgG2 and IgG4, respectively.

Chromatography

Chromatographic gels were bought from Pharmacia (Butyl-Sepharose CL-4B, Octyl-Sepharose CL-4B and Phenyl-Sepharose Fast Flow) or prepared in the laboratory as described previously [20]. Briefly, 200 g of Sepharose 6B (Pharmacia) were washed in 1 l of 0.5 *M* Na_2CO_3, dried by aspiration under vacuum, and resuspended in 200 ml of the same buffer. Divinylsulphone (10 ml) was added, and the mixture was gently stirred overnight at room temperature. The coupled gel was washed with water, dried as above, and resuspended in 200 ml of the starting buffer; after the addition of 20 ml of 2-mercaptoethanol, the suspension was stirred overnight at room temperature. After being washed with water the thiophilic gel was stored in 0.02% NaN_3 at 4°C until use. The capacity of the gels was determined using columns of 1-ml volume, which were loaded with 50 mg of Allergamma in 1 *M* ammonium sulphate and washed with 20 ml of the same solvent. The capacity was defined as the amount of protein eluted with 20 m*M* sodium phosphate buffer (pH 7.0) followed by 60% ethylene glycol in the same buffer. It was found to be 14 mg protein per ml for the thiophilic gel, 9 mg/ml for Butyl-Sepharose and Phenyl-Sepharose, and 5.5 mg/ml for Octyl-Sepharose.

The chromatographic conditions were the following. The gel was packed in an IBF-11 column (11 mm I.D.). Equilibration was carried out in the adsorption buffer at room temperature (20 m*M* sodium phosphate buffer, pH 7.0, with the addition of the indicated ammonium sulphate concentrations). The immunoglobulin sample was adjusted to the required ammonium sulphate concentration by the addition of the solid salt. The solution remained clear up to at least 1 *M* (13.2%), but the samples were filtered through a 0.2-μm filter. The amount of protein applied never exceeded 30–50% of the column capacity, determined as described above. The columns were first washed with 50–80 ml of adsorption buffer, then eluted with a decreasing ammonium sulphate gradient and regenerated as indicated in the figures. For each run, the effluent was pooled so as to have only one non-adsorbed fraction and a limited number of fractions for subclass composition analysis.

RESULTS

Thiophilic gels

Adsorption in 1 M ammonium sulphate on a thiophilic column and elution with a decreasing salt concentration gradient. When the immunoglobulin mixture was adsorbed on the thiophilic gel, *ca.* 4% of the total protein came out immediately but

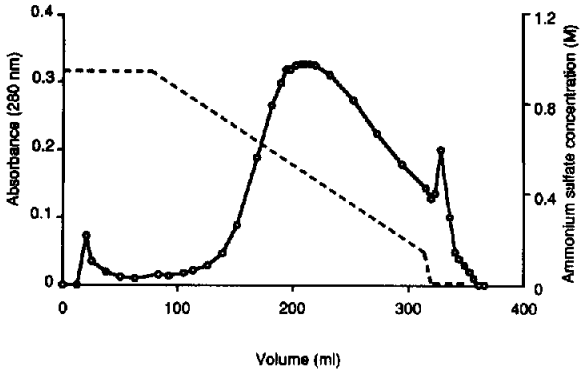

Fig. 1. Elution of immunoglobulins from a thiophilic gel by a gradient of decreasing ammonium sulphate concentration. Gel volume, 17 ml; equilibration buffer, 20 m*M* sodium phosphate buffer and 1 *M* ammonium sulphate (pH 7.0); flow-rate, 1.0 ml/min; gradient volume, 340 ml; protein load, 53 mg. Solid line, absorbance; dashed line, salt molarity.

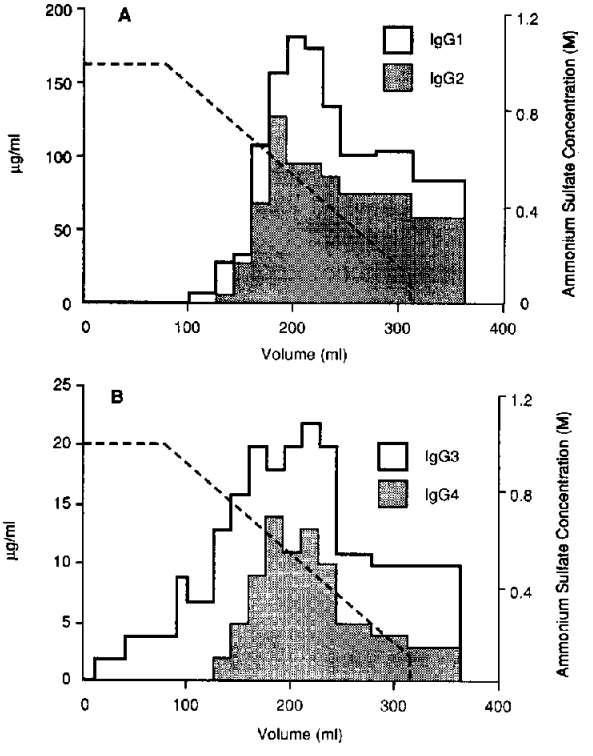

Fig. 2. Proportions of IgG subclasses in the fractions eluted from a thiophilic gel by a gradient of decreasing ammonium sulphate concentration (see Fig. 1): (A) IgG1 and IgG2; (B) IgG3 and IgG4. The starting subclass composition is given in Experimental. The salt molarity is shown by the dashed line.

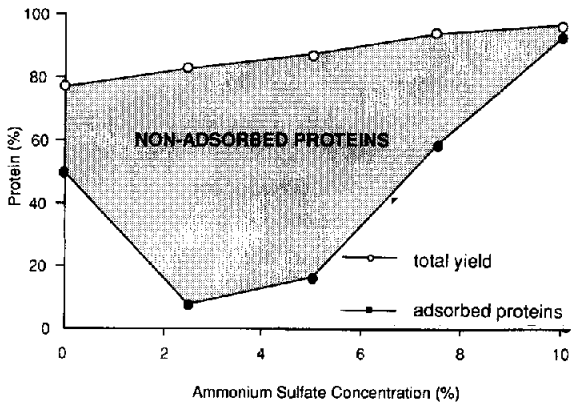

Fig. 3. Influence of the ammonium sulphate concentration on the adsorption of IgG on a thiophilic gel. The protein load was 50 mg on a 10-ml gel bed, equilibrated in 20 m*M* N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulphonic acid) (HEPES) (pH 7.0) containing variable concentrations of ammonium sulphate. The flow-rate was 1.0 ml/min. The non-adsorbed proteins were collected as one fraction, and the adsorbed proteins were eluted as another single fraction using 60% ethylene glycol in 20 m*M* HEPES (1 *M* ammonium sulphate is equivalent to a 13.2% concentration).

most of it was eluted by the decreasing ammonium sulphate concentration gradient (Fig. 1). After regeneration with 60% ethylene glycol in 20 m*M* sodium phosphate buffer, the overall yield was 90–100%. The subclass composition was analysed along the profile as described in Experimental (Fig. 2). The non-adsorbed and early-eluting fractions consisted entirely of IgG3 (*ca.* 10% of total IgG3 in the initial mixture). As for the main broad peak, the IgG subclass distribution along the gradient did not depart dramatically from the starting distribution, but there appeared to be a tendency for IgG2 to elute preferentially at lower ionic strength.

Influence of ammonium sulphate concentration in the adsorption buffer on the selectivity of subclass retention. Hutchens and Porath [21] showed that the ammonium sulphate concentration influences the adsorption of immunoglobulins on thiophilic gels. Using similar conditions, we investigated the selectivity of retention for the various subclasses. Fig. 3 shows the profile of total retention as a function of ammonium sulphate concentration. It is very similar to that presented by Hutchens and Porath [21]. Fig. 4 gives the

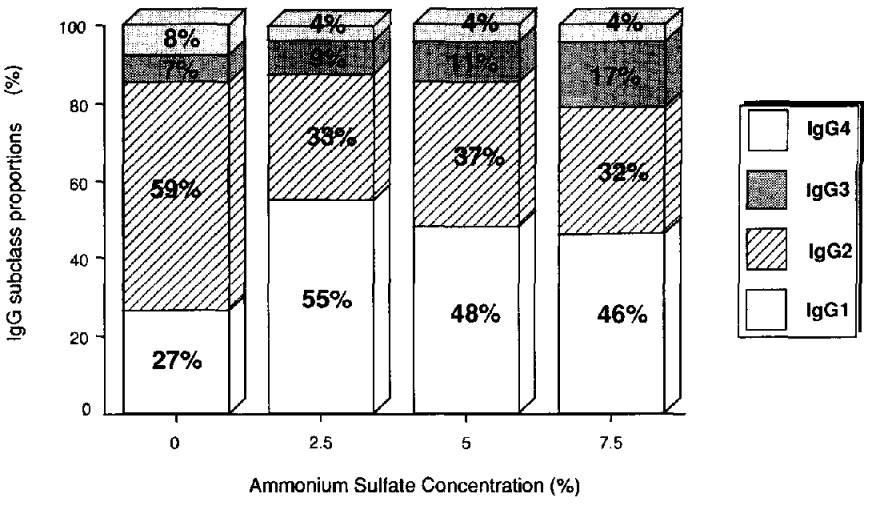

Fig. 4. Distribution of IgG subclasses in the fractions not retained on the thiophilic gel at various ammonium sulphate concentrations (see Fig. 3). The starting subclass composition is given in Experimental.

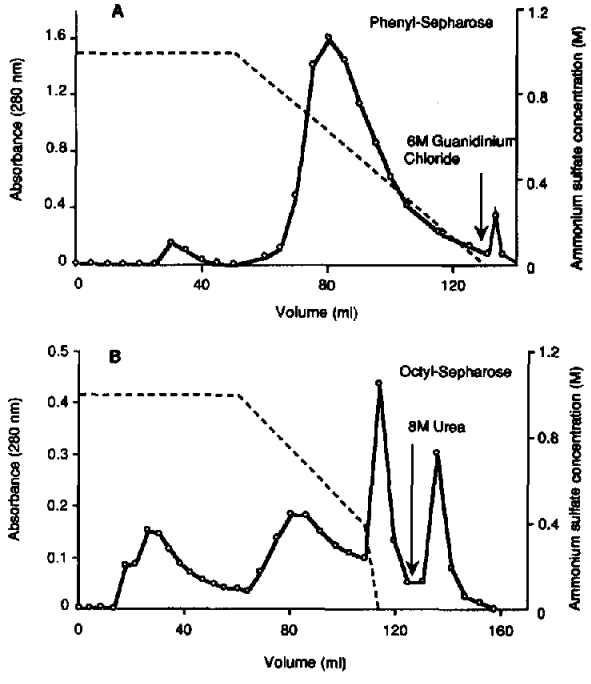

Fig. 5. Elution of immunoglobulins from hydrophobic gels by a gradient of decreasing ammonium sulphate concentration. (A) Phenyl-Sepharose Fast Flow, protein load 40 mg; (B) Octyl-Sepharose CL-4B, protein load 20 mg. General conditions: gel volume, 10 ml; equilibration buffer, 20 m*M* sodium phosphate buffer and 1 *M* ammonium sulphate (pH 7.0); flow-rate, 1.0 ml/min; gradient volume, 100 ml. Solid line, absorbance; dashed line, salt molarity.

subclass proportions in the fractions that were not retained at each ammonium sulphate concentration. In the absence of salt, the non-adsorbed protein (one third of the total load) showed a definite excess of IgG2 (*ca.* 50% of the total IgG2 load), and a deficit in IgG1. But at 7.5% ammonium sulphate, the non-adsorbed protein (also about one third of the load) was enriched in IgG3 and contained 40% of the total IgG3 in the applied sample. Nevertheless, under the conditions where most of the protein was not adsorbed [Fig. 3, 2.5 and 5% $(NH_4)_2SO_4$] or was retained (Fig. 1), the selectivity effects were small (Figs. 2 and 4).

Hydrophobic gels

Adsorption in 1 M ammonium sulphate: influence on adsorption of the ligand nature. On Phenyl-Sepharose FF, only 4% of the proteins was not adsorbed in the presence of 1 *M* ammonium sulphate. The ones that were adsorbed were eluted regularly along the decreasing ammonium sulphate gradient (Fig. 5A). After a final wash with 20 m*M* sodium phosphate buffer (pH 7.0), the total yield was 95%. In agreement with this value, practically no protein was eluted during column regeneration with 6 *M* guanidinium chlo-

ride. With Butyl-Sepharose CL-4B, similar results were obtained (not shown) but with an overall yield of 75% only.

In contrast, on Octyl-Sepharose CL-4B, under the same conditions, but with a lower protein load owing to the lower gel capacity (see Experimental), 21% of the proteins were not retained and the decreasing salt gradient eluted only a further 33% (Fig. 5B). After regeneration with 8 *M* urea, the total yield was only 65%. When the gradient was made between 1 *M* ammonium sulphate and 60% ethylene glycol in the phosphate buffer, it eluted 38% of the loaded protein, and the total yield after 8 *M* urea went up to 82%.

Adsorption in NaCl. The different behaviour of the phenyl and butyl ligands, on the one hand, and the octyl ligand, on the other, was also attested by the results of experiments in which ammonium sulphate was replaced by NaCl. In contrast with the near-total adsorption of the IgGs in 1 *M* $(NH_4)_2SO_4$ on Phenyl-Sepharose, no adsorption occurred on the same gel in 2 *M* NaCl in 20 m*M* sodium phosphate buffer. This is in qualitative agreement with the predictions of Melander and Horváth [30]. However, with Octyl-Sepharose in 1 *M* NaCl, 30% of the protein load was already adsorbed. After 8 *M* urea, the total yield was identical with that obtained with the ammonium sulphate gradient (63%).

Behaviour of IgG subclasses on the hydrophobic columns in 1 M ammonium sulphate. With Phenyl-Sepharose, the non-adsorbed fraction contained 87% IgG3, but represented only 10% of the IgG3 load. Along the gradient, the subclass distribution was independent of the elution molarity, within experimental error (Fig. 6). With Butyl-Sepharose the results were very similar, except that the IgG3 proportion in the non-adsorbed fraction was 54%.

With Octyl-Sepharose, the non-adsorbed fraction represented *ca.* 22% of the IgG2 and 10% of the other subclasses in the initial load (Fig. 7). As a result, the fraction is enriched in IgG2 (52% of the total IgG). When adsorption was carried out in 1 *M* NaCl, the non-adsorbed fraction contained 47% IgG1 and 46% IgG2 (50% of the IgG2 load).

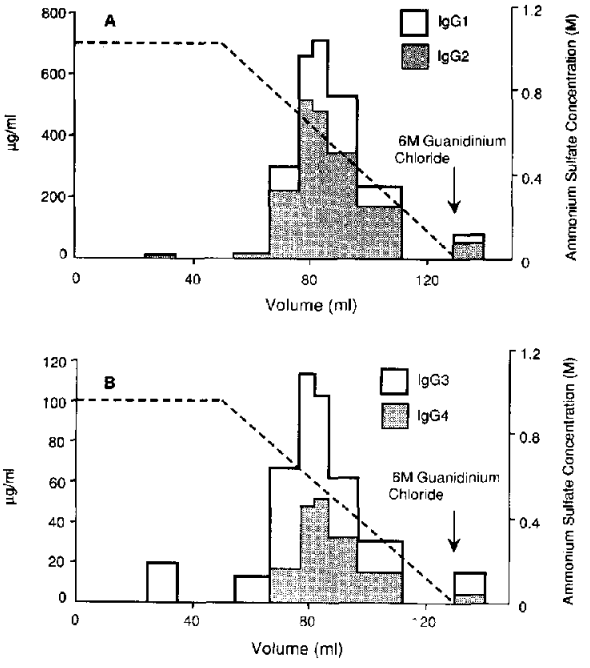

Fig. 6. Distribution of IgG subclasses in the effluent from the Phenyl-Sepharose column (see Fig. 5A): (A) IgG1 and IgG2; (B) IgG3 and IgG4. The starting subclass composition is given in Experimental. The salt molarity is shown by the dashed line.

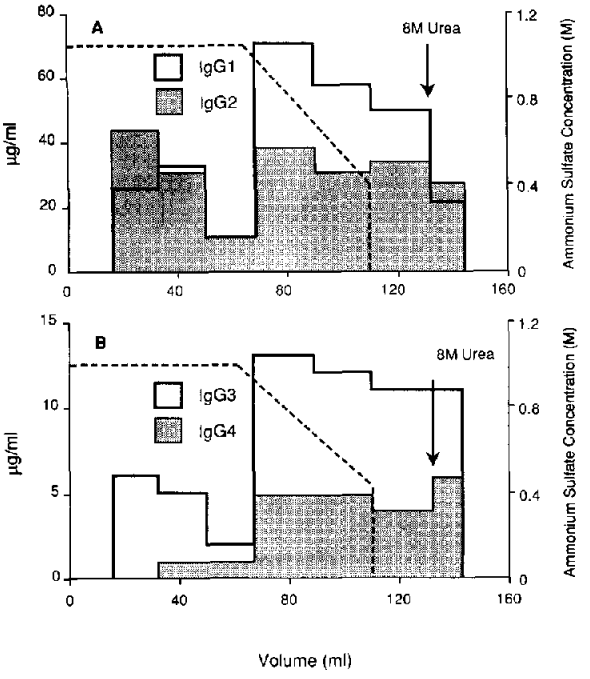

Fig. 7. Distribution of IgG subclasses in the effluent from the Octyl-Sepharose column (see Fig. 5B): (A) IgG1 and IgG2; (B) IgG3 and IgG4. The starting subclass composition is given in Experimental. The salt molarity is shown by the dashed line.

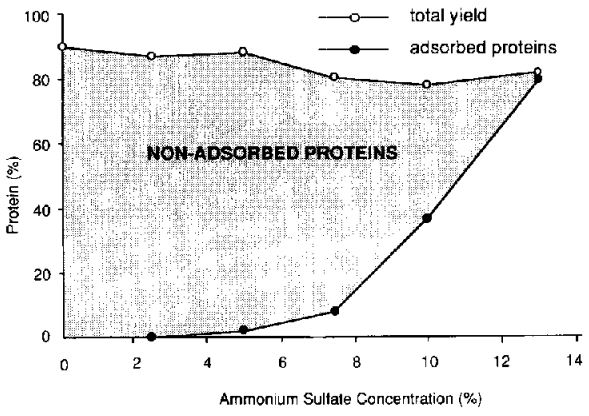

Fig. 8. Influence of ammonium sulphate concentration on the adsorption of IgG on a Phenyl-Sepharose column. The protein load was 40 mg on a 10-ml bed, equilibrated in 20 m*M* HEPES (pH 7.0) containing the indicated concentrations of ammonium sulphate. The flow-rate was 1.0 ml/min. Elution was carried out with 20 m*M* HEPES (pH 7.0).

Influence of ammonium sulphate concentration in the adsorption buffer on the selectivity of the subclass adsorption. In view of the qualitatively similar results obtained with the thiophilic gel and Phenyl- and Butyl-Sepharose when the IgG pool was adsorbed in 1 *M* ammonium sulphate and eluted with a decreasing salt gradient, we tested the influence of the ammonium sulphate concentration on adsorption of the IgG pool on Phenyl-Sepharose. The results, shown in Fig. 8, are clearly different from those of Fig. 3. Adsorption increased as a smooth function of salt concentration and was definitely weaker than on a thiophilic gel at the same ammonium sulphate molarity: at 10% $(NH_4)_2SO_4$, less than half of the IgG was adsorbed on Phenyl-Sepharose, whereas retention was nearly complete on the thiophilic gel. Adsorption was totally non-selective with respect to subclass, because at any point between 0 and 10% salt, the subclass distribution of the eluted fraction was identical with that of the starting material (data not shown). It is only at 1 *M* salt (13.2%) that a small proportion of IgG3 appeared to elute early (see above and Fig. 6B).

DISCUSSION

Our results show that, under the usual working conditions (adsorption in 1 *M* ammonium sulphate and elution with a decreasing salt gradient), neither thiophilic gels, nor hydrophobic gels such as Phenyl- or Butyl-Sepharose, display any marked selectivity for human IgG subclasses apart from *ca.* 10% of total IgG3 that was not adsorbed at all on either column. Adsorption on a thiophilic gel in the absence of ammonium sulphate, however, enabled the selective enrichment to 60% of half the total IgG2 load. Furthermore, in the flow-through fraction at 7.5% ammonium sulphate, which encompassed about one third of the load, similar to the situation at 0% ammonium sulphate (Fig. 3), there was enrichment in IgG3 (Fig. 4). These selectivity effects, however, are not sufficiently marked to be of any practical value.

The most interesting aspect of our results lies in a comparison between the properties of the thiophilic gel and Phenyl-Sepharose on the one hand, and of the latter and Octyl-Sepharose on the other. At first sight, in 1 *M* ammonium sulphate, the thiophilic gel does not appear to behave differently from Phenyl-Sepharose. The immunoglobulin preparation appears to show a broad heterogeneity with respect to adsorption properties on both gels. However, variation of the salt adsorption concentration enables a differentiation between the two adsorbents. First, as already noted by Hutchens and Porath [21], we found total adsorption to require a higher ammonium sulphate concentration on the hydrophobic gel. Second, Fig. 8 shows a salt dependence of adsorption, in keeping with that expected for essentially hydrophobic interactions, whereas the situation for the thiophilic gel is different (Fig. 3). The minimum of adsorption at intermediate salt concentration has already been described by Hutchens and Porath [21] for a number of proteins in particular immunoglobulins. We interpreted this behaviour as indicating that different types of interaction are at play at low and high ionic strength: on raising the salt concentration, there would be a shift from electrostatic or polar

interactions to hydrophobic-like interactions. However, this interpretation is certainly too simple and, even if valid in this case, it does not appear to have general value. Indeed, Hutchens and Porath [21] showed that some proteins, such as soybean trypsin inhibitor and lens culinaris lectin, show an adsorption profile similar to that of Fig. 8 rather than that of Fig. 3. Furthermore, the same authors discussed at length the lack of parallelism observed for a number of proteins in their strength of adsorption on hydrophobic and thiophilic gels at neutral pH. Thus our results are in agreement with published ones concerning the specific characteristics of thiophilic chromatography [21].

The different properties of Phenyl- (and Butyl-) Sepharose (Fig. 5A) and Octyl-Sepharose (Fig. 5B) with respect to the immunoglobulin preparations were unexpected. Whereas with the first two gels there was a gradual elution with decreasing ionic strength, irrespective of IgG subclass, discrete populations were eluted from Octyl-Sepharose, with part of IgG2 being the least adsorbed. It is not surprising that some proteins should be more strongly retained by the immobilized octyl chain than by the butyl chain. That there also exists a marked difference between the octyl and the phenyl ligand might be explained by the aromatic character of the phenyl ring, which could give rise in solution to weakly polar interactions such as were observed in crystal structures [31]; these might temper the strength of hydrophobic interactions. But the fact that *ca.* 20% of the immunoglobulins could be eluted from Octyl-Sepharose in 1 *M* ammonium sulphate, usually indicating weak hydrophobic interactions, suggests instead that the octyl arm, because of its bulk, could have a poorer accessibility to some specific sites than the phenyl and butyl arms.

ACKNOWLEDGEMENT

This work was supported by a grant from the Ministère de la Recherche et de la Technologie (programme des Biotechnologies).

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